

Characterization of coral mucus for its microbes and antimicrobial properties under healthy and stress conditions

A THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In the School of Biological Sciences & Biotechnology

GOA UNIVERSITY



By

Phartade Vilas Bhagwat

(Reg. no. GU201711823)

CSIR-National Institute of Oceanography,

Dona Paula, Goa India 403004.

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Under the Guidance of

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August 2024

Declaration

As required under the Goa University Ordinance OA-19A, I state that the present thesis entitled “**Characterization of coral mucus for its microbes and antimicrobial properties under healthy and stress conditions**” is my original contribution and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgments have been made, wherever facilities and suggestions have been availed.

Place: Goa University

Phartade Vilas Bhagwat

Date:09.08.2024



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Certificate

This is to certify that the thesis entitled “**Characterization of coral mucus for its microbes and antimicrobial properties under healthy and stress conditions**” submitted by **Mr. Phartade Vilas Bhagwat**, for the award of the degree of Doctor of Philosophy in Biotechnology, is based on the original studies carried out by him under my supervision.

The thesis or any part thereof has not been submitted for any other degree or diploma in any university or institution.

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LIST OF ABBREVIATIONS

1. SML-Surface mucus layer
2. UFM-Unfiltered mucus
3. FM-Filtered mucus
4. AIA-Actinomyces isolation agar
5. DMSO-Dimethyl sulfoxide
6. NBT-Nitro blue tetrazolium chloride
7. PBS-Phosphate buffer saline
8. LB-Luria Bertani
9. rpm-Rotations per minute
10. TE-Tris- Ethylenediaminetetraacetic acid
11. MSA-Mannitol salt agar
12. ZMA-Zobell marine agar
13. EMB-Eosin methylene blue
14. TCBS-Thiosulphate-citrate-bile salts-sucrose agar
15. SDS-PAGE-Sodium dodecyl sulphate polyacrylamide gel electrophoresis
16. EtBr-Ethidium bromide
17. PCR-Polymerase chain reaction
18. PEG-Polyethylene glycol
19. SOD-Superoxide dismutase
20. EDTA-Ethylenediaminetetraacetic acid
21. TEMED-Tetramethyl ethylenediamine
22. NaNO₂-Sodium nitrite
23. AlCl₃-Aluminium chloride
24. NaOH-Sodium hydroxide
25. BHA-Butylated hydroxy anisole
26. BHT-Butylated hydroxytoluene
27. DPPH-2,2-diphenyl-1-picrylhydrazyl
28. HPLC-High performance liquid chromatography
29. EDTA-Ethylenediaminetetraacetic acid
30. TBA-Thiobarbituric acid

31. TCA-Trichloroacetic acid
32. HEPES-Hexacyanoferrate, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
33. HAM-Healthy *Acropora* mucus
34. WPAM-White patch *Acropora* mucus
35. WTAM-White tip *Acropora* mucus
36. ADAM-Algal dominating *Acropora* mucus
37. HPM-Healthy *Porites* mucus
38. PLSM-Pink line syndrome mucus
39. PP-Pink spot *Porites*
40. PB-*Porites* bleached
41. AHL-N-Acyl homoserine lactones
42. CFU-Colony forming unit
43. DNA-Deoxyribonucleic Acid
44. RNA-Ribonucleic Acid
45. FA-Fatty acid
46. MUFA-Monounsaturated fatty acid
47. PUFA-Polyunsaturated fatty acid
48. SAFA-Saturated fatty acid
49. H₂O₂-Hydrogen peroxide

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Abstract

Coral mucus secreted by mucocytes provides a protective physicochemical and physiological barrier between coral tissue and external environmental threats. The biomolecules and nutrients of the secreted mucus are derived from endosymbionts and coral polyps, which support coral functions, such as feeding, sediment clearing, and protection, against numerous biotic and abiotic stressors. The surface mucus layer also houses a diverse community of beneficial microorganisms that defend against pathogens. A coral health survey was conducted over two seasons, analyzing nearby seawater nutrients and physiological conditions. The study also examined both culturable and culture-independent bacterial diversity, alongside nutrients and physiological parameters, in the mucus of healthy and diseased coral species, *Acropora* sp. and *Porites* sp. High nutrients supported the bleached, algal dominance, and diseased state of corals. Metagenomic analysis of the unculturable bacteria supported the culturable diversity results. Besides, variations in the bacterial diversity were observed between *Porites* sp. and *Acropora* sp. mucus. The nutrient rich mucus was mostly reported for their association with bacteria as invaders for causing coral disease among various other associated microbes. In the present study, the ciliates, were also demonstrated for using the coral mucus as feed, proliferation and secondary invaders causing coral disease severity. However, the purpose and the mechanism of mucus release, the effects of mucus components on defense properties, and functional roles in intra- and interspecific interactions were not well described.

The surface mucus layer of corals aids in feeding, silt removal, and defense against pathogens. However, the first line of defense of secreted mucus of corals against tissue damage generated through pathogen or environmental factors is poorly understood. Hence, we used various methods such as a well diffusion assay and tests for quorum quenching, free radical scavenging, antioxidant enzyme expression and phenoloxidase (PO) activity to determine the mucus defense properties using mucus of healthy and diseased *Porites* sp. and *Acropora* sp. Interestingly the coral mucus showed antimicrobial activity against coral pathogens such as bacteria and protozoan ciliates. Besides, mucus inhibition of the *N*-Acyl homoserine lactone (AHL) molecule suggests its quorum quenching defense property. Antioxidant compounds such as phenol and flavonoids. Free radical scavenging of mucus such as hydrogen peroxide, hydroxyl radical, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), was observed significantly more in diseased corals ($p < 0.05$). Antioxidant enzymes superoxide dismutase (SOD), catalase, and peroxidase activity were observed in both the

diseased and healthy coral mucus. The presence of serine and metalloproteases were also detected in coral mucus. Further, phenoloxidase (PO) activity was found maximum in diseased coral mucus affected by pink line syndrome of *Porites* sp. and white patch of *Acropora* sp. than the healthy coral mucus. The above antimicrobial properties, antioxidant enzymes, and quorum quenching properties of coral mucus may aid in understanding the coral's defense and survival against pathogens and any abiotic stress.

Coral mucus contains all essential nutrients such as carbohydrates, amino acids, fatty acids (FA), etc. Although fatty acids, an essential nutrient majorly described from the coral tissue and its endosymbiont, they had not been well described from the coral mucus. Also, there is no clear study on the tissue or endosymbiont as a major contributor to their secretion of fatty acids into the mucus, and their possible role in disease and healthy state. Thus, the present study characterized fatty acid profiles of diseased and healthy *Acropora* sp. and *Porites* sp. mucus. The study reveals that mucus contains health indicators FAs, which are important in metabolism, physiological processes, and stress tolerance. The major mucus fatty acid observed in both the corals was palmitic acid a precursor fatty acid. Besides, mucus FAs were observed for its resemblance with that of polyps and its heterotrophic feeds such as bacteria and planktons than endosymbiont FAs. Thus, the present study aims to understand the mucus components and the complex roles played by mucus in microbial associations, protection and defense responses that influence the coral health.

Chapter one

Introduction and literature review.

1.1 Introduction.

According to recent studies, 20 % of coral reefs have already been irreparably damaged, and additionally, 24 % of the remaining reefs are facing serious threats due to anthropogenic stresses, such as overfishing and eutrophication (Sharma and Ravindran, 2020; Galli et al., 2021). Biotic and abiotic stressors can cause coral bleaching, a process in which the algal symbionts are expelled from the host coral (Hafezi et al., 2020; Ravindran et al., 2022). The absence of the endosymbiont will weaken coral health, and mucus secretion will be affected (Ravindran et al., 2022). Environmental impacts on mucus secretion, including temperature rise, have not been studied in detail (Vaughan et al., 2021). For example, mucus release is enhanced during high sedimentation (Reynaud and Ferrier-Pages, 2019), and when corals are exposed to air at low tide (Wild et al., 2004, 2005, Bhagwat et al., 2024). In addition, mucus aggregations in the water column increase sedimentation and nutrient recycling (Wild et al., 2005; Plass-Johnson et al., 2015), which control microbial activities (Bythell and Wild, 2011). Therefore, a better understanding of the changes in mucus caused by biological and physical stressors may help us to monitor coral health under different environmental conditions.

Mucus is a slimy substance produced by epithelial cells called mucocytes utilizing the organic carbon synthesized by photosynthetic endosymbiotic algae (Brown and Bythell, 2005; Bakshani et al., 2018). Mucus production varies between and within coral species (Coffroth, 1990; Hadaidi et al., 2019). It is mainly composed of proteolytic enzymes, microbes, mucins, proteins, polysaccharides, lipids, and their substructures, such as monosaccharides, amino acids, and mycosporines (Brown and Bythell, 2005; Stabili, 2019). The beneficial microbes within the mucus form a barrier against potential coral pathogens, competing for nutrients and space or producing antibiotics (Ritchie, 2006; Krediet et al., 2013; Raina et al., 2016; Irudayarajan et al., 2023). Coral mucus has several important functions, including protection against pathogens and UV radiation, capturing food, acting as an energy carrier, producing antibiotics, and serving as a nutrient source for coral-associated microbial communities (Bythell and Wild, 2011; Bakshani et al., 2018). Coral mucus contains up to 1000 times more bacteria than seawater (Nakajima et al., 2015). *Vibrio shiloi*, the causative agent of bleaching in the Mediterranean coral, and *Oculina patagonica* bind to the receptors of the surface mucus layer during temperature rise (Thompson et al.,

2014). However, beneficial probiotic bacteria have been demonstrated to enable *O. patagonica* to become resistant to *V. shiloi* infection (Reshef et al., 2006; Mills et al., 2013). Thus, studying the components of coral mucus is of great importance for a better understanding of the entire mucosal system, its interactions with other microorganisms (commensals and pathogens), and their involvement in coral immunity and health (Ravindran et al., 2020; Ravindran et al., 2022 a & b; Ravindran et al., 2023, Irudayarajan et al., 2023; Bhagwat et al., 2023; Bhagwat et al., 2024). Further, although the composition of coral mucus has been described in some studies (Coddeville et al., 2011; Hadaidi et al., 2019), it is limited to a few coral species, and the sources of mucus components in mucus are not clearly understood. Specifically, in India, coral diseases are the least studied, and most of them are not known for their causative agents (Sharma and Ravindran, 2020). The major coral reefs found in India are the Gulf of Kutch, the Gulf of Mannar, Palk Bay, the Andaman and Nicobar Islands, and the Lakshadweep Islands (Fig. 1.1) (Venkataraman, 2006; Bhagwat et al., 2024). Thus, the present study was made in the Gulf of Mannar corals aiming to address the significant role of coral mucus of healthy and diseased corals for their essential components including microbes for influencing coral health and resilience under various biotic and abiotic stress conditions.

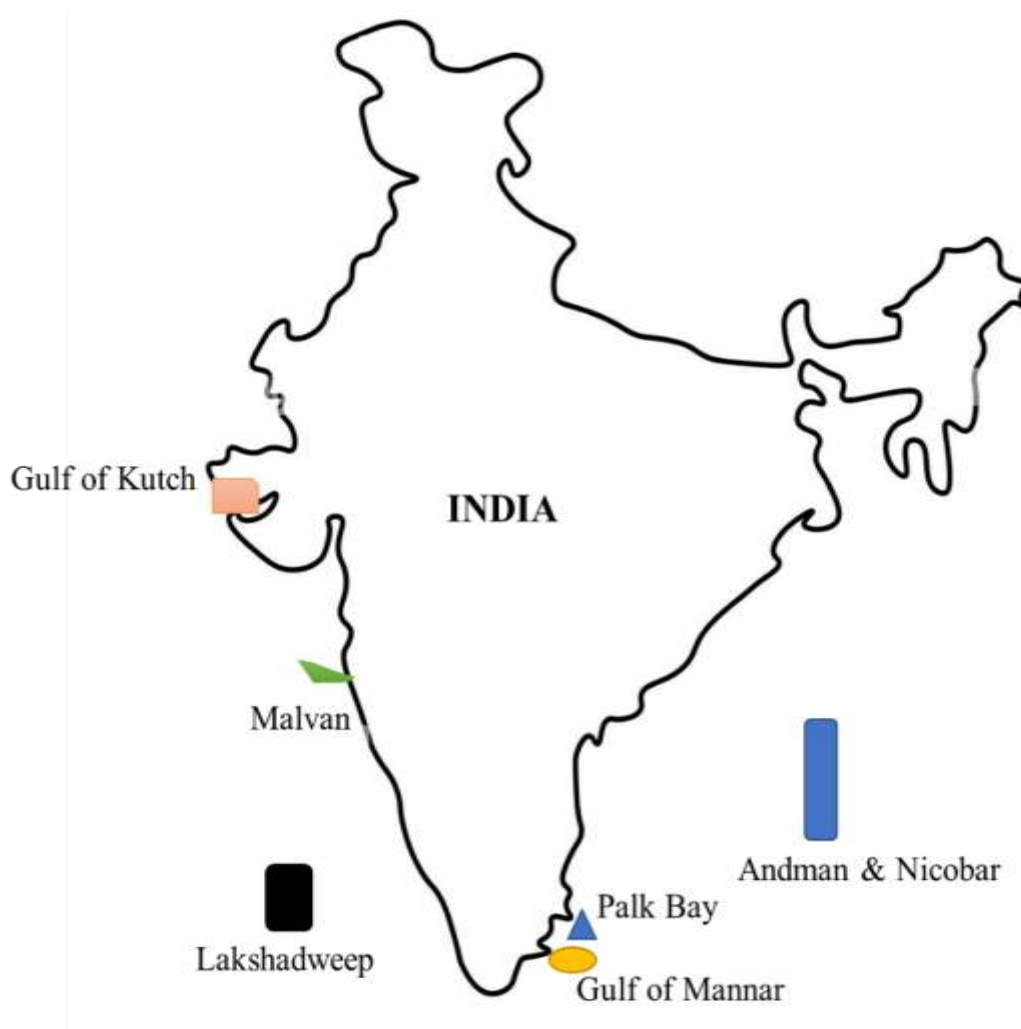


Figure 1.1- Major coral reefs of India. The Gulf of Kutch is characterized by the presence of platform reefs, while the Gulf of Mannar, Palk Bay, and the Andaman and Nicobar Islands feature fringing reefs. Lakshadweep boasts Atolls reef formations and a patchy reef structure is identified in Malvan.

1.2 Coral mucus synthesis and secretion.

The mucocyte cells of the coral polyp synthesize mucus, with the major component being carbon-based carbohydrates, and are then secreted out as a coral surface layer (Brown and Bythell, 2005). The coral-associated endosymbiotic algae are believed to provide the maximum carbon required for mucus synthesis although the source of the entire carbon requirement is not clear (Crossland, 1987; Brown and Bythell, 2005; Fang, 2015). The other compounds required for mucus, such as amino acids, fatty acids, lipids, glycerol and

nicotinamide adenine dinucleotide phosphate (NADPH), come from the coral diet (Hadaidi, 2018). Only mucocytes contribute to mucus synthesis in corals, unlike in humans where multiple cell types are involved (Hadaidi et al., 2019; McShane et al., 2021). Some other gland cells are observed in coral tissues, but their roles are still unclear (Bruckner, 2015). However, in vertebrates, the transformation of similar gland cells to mucus cells has been observed, which may be because these cells are either specialized secretory cells or developing mucocytes (Que, 2015). The distribution of mucocytes varies in both abundance and location, not just within individual colonies but also between coral species (Baker et al., 2022). The production of coral mucus can be triggered by various factors, such as changes in light intensity, water flow, and the presence of predators or food particles (Zetsche et al., 2016). For example, an increase in the sea surface temperature increases the mucocyte density in host tissue; however, it decreases after bleaching (Piggot et al., 2009). In addition, more organic components are found in mucus from stressed than in healthy corals (Lee et al., 2016).

The synthesis of coral mucus is unique because it is derived from a significant portion of the photosynthetically fixed carbon (Brown and Bythell, 2005; Hadaidi et al., 2019), and contains a high concentration of arabinose and xylose, which is not typically found in animal cells. Different coral species have varied rates of mucus production (Crossland et al., 1980a; Kurihara et al., 2018), depending on the endosymbiont contribution of energy-rich glycerol, alanine, and glucose to the host (Burriesci et al. 2012; Gabay 2018) (Fig. 1.2). Excess photosynthetic carbon is eliminated by the coral through mucus release (Bakshani et al. 2018) along with lipids, such as wax esters and triglycerides for mucus synthesis (Krueger et al., 2018). *Acropora* spp. release up to 4.8 L of mucus from a 1m² patch in one day and 56–80 % of the mucus is dissolved in water (Wild et al., 2004), of which 20–40 % of mucus forms strings and filaments (Stabili, 2019). This mucus net filters seawater up to 100 ml/cm² of tissue per hour (Lewis, 1976). Subsequently, heterotrophic coral reef organisms utilize the energy from the materials trapped in the coral mucus and the energy derived from zooxanthellae (Wild et al., 2004). Invertebrate mucus protects, lubricates, and helps in locomotion, mating, homing, and foraging, performing much more diverse functions than in vertebrates (Denny, 1989). In addition, mucus released on the epithelial surface aims to attract particulate matter.

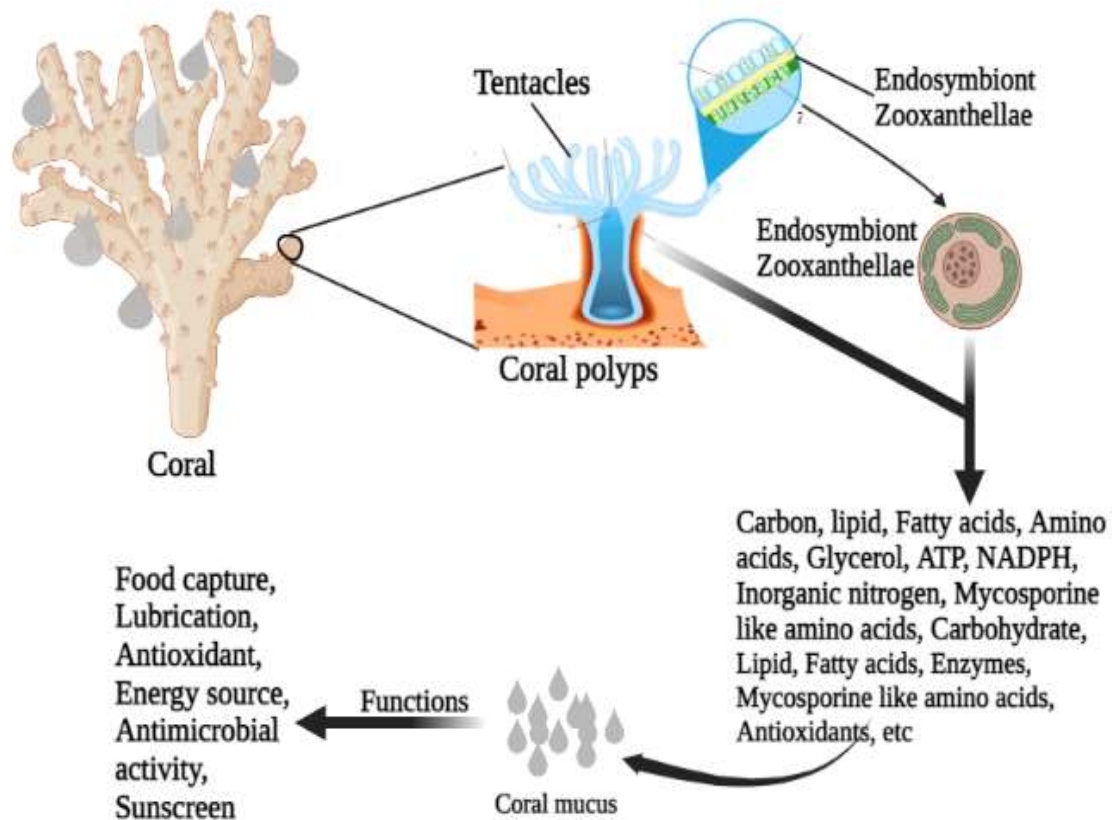


Figure 1.2- Coral mucus and properties. Coral mucus synthesis occurs in coral polyps from mucocyte cells. The mucus has a combination of nutrients provided by both the coral and endosymbiont. The released mucus acts as a first line of defense, with various properties against microbial pathogens, UV radiation, and other stressors that influence the health of the corals.

1.3 Coral mucus and its beneficial role

Coral mucus plays a crucial role in coral health and reef ecosystem. Mucus is composed of various components such as mucins, carbohydrates, fatty acids, lipids, proteins, amino acids, enzymes, and antioxidants (Bhagwat et al., 2024), which were known for their vital roles in improving coral health. These components of the coral mucus not only help in protecting the coral by forming a protective barrier against pathogens and environmental stressors, but also contribute to nutrient cycling within the reef ecosystem (Taniguchi et al.,

2024). Besides, the mucus supports the growth of beneficial microbes, which influence the coral health (Bhagwat et al., 2024). The components of mucus and their beneficial roles are described below:

1.3.1 Mucins and the importance of mucus rheology.

The glycoprotein core, mucin, is made up of 20 % polymers and 80 % sugars and contains cysteine, which undergoes cleavage during mucus dispersion (Bakshani et al., 2018). *O*-glycosylation of mucin occurs on oligosaccharides in the core between serine and threonine and on the *N*-acetylgalactosamine carbohydrate chains, and *N*-glycosylation occurs on asparagine (Corfield, 2015). The side chains of mucins are either branched or linear and up to 20 monosaccharides long and are linked with either $\beta 1 \rightarrow 2$ or $\beta 1 \rightarrow 3$ bonds (Krediet et al., 2009). The structural diversity of mucin is due to its carbohydrate composition (Thornton and Sheehan, 2004). The ability of mucin to form a gel affects mucus function depending on its extensive glycosylation and polymerization ability (Younan et al., 1982). The von Willebrand D (VWD) domain is involved in the polymerization of mucin monomers via intermolecular disulfide bonds (Ambort et al., 2012). The outer layer of the epidermis of fishes continuously produces new goblet cells because they cannot produce mucus more than once. But coral mucus goblet cells can continuously produce mucus from the same cells (Subramanian et al., 2007).

Corals require relatively fluid mucus for their ciliary feeding and to prevent the desiccation of coral polyps by air exposure during low tide (Brown and Bythell, 2005; Bessell-Browne et al., 2017). Based on rheology, coral mucus is distributed as fluid mucus, string, web or flocs, and mucus sheets (Bessell-Browne et al., 2017). Distinct mucin characteristics are present across various layers within the coral surface mucus layer (SML) (Guppy et al. 2019). The mucin concentration and gel-forming properties of mucus are necessary for its protective and lubricating functions (Thornton and Sheehan, 2004). Human mucus becomes hydrated and mucus contents turn it into a gel (Piludu et al., 2003). As polymeric mucins have been detected in coral mucus, similar to vertebrates (Jatkar et al., 2010a), it is possible that coral mucus also contains different types of mucin molecules that convert it from gel to liquid and liquid to gel as needed (Horricks et al., 2019). Because coral mucus is rich in plant-associated sugar arabinose, it may have some carbohydrate chains that

differ from those of mammalian mucins (Wild et al., 2005; Bhagwat et al., 2024). Human mucus changes its rheology from viscoelastic gel to viscous fluid at high stress and returns to a viscous gel at low stress (Jatkar et al., 2010b) making it a lubricant and helping it move under various physiological conditions (Hill et al., 2022). A similar property may also be present in the coral SML, which remains to be investigated. However, corals can produce two types of mucus. The milked mucus has no gel-forming properties but can be converted to a gel by a higher concentration of mucin (Jatkar et al., 2010a), which is very similar to human respiratory mucus (Zetsche et al., 2016). Mucus sheets are sticky mucus layers derived from liquid mucus (Bessell-Browne et al., 2017). These are then transferred into the food web of the reef (Brown and Bythell, 2005).

1.3.2 Carbohydrates

The relative concentration of carbohydrates varies among coral species, yet their composition remains constant, with certain sugars being shared across multiple species, and this variability is intricately linked to host phylogeny, microbial associations, and symbiont composition (Hadaidi et al., 2019). The mucosal sugars glucose, mannose, xylose, and galactose are found in all coral species that have been analyzed, whereas arabinose, *N*-acetylglucosamine, ribose, fucose, and rhamnose are present in only a few species (Table 1.1). Thermal stress has been shown to induce changes in both the enrichment and abundance of various carbohydrate pools, with a notable impact on galactose levels (Hillyer et al., 2017). For example, a significant decrease in glucose, galactose, and mannose was described under rising temperature-stress conditions (Lee et al., 2016). Thus, mannose, glucose, and galactose can serve as markers for temperature stress, while *N*-acetyl glucosamine exhibits anti-inflammatory properties and reduces the production of free radicals (Salvatore et al., 2000). *N*-acetyl galactosamine also helps in the *O*-glycosylation of coral mucin molecules. Xylose and arabinose are types of plant sugars commonly utilized as energy sources, indicating that they may have come from the endosymbiont (Teplitski et al., 2016). Arabinose is widespread in *Acropora* sp., suggesting that it is transferred from algal symbionts to the host (Wild et al., 2005; Wild et al., 2010; Lee et al., 2016).

The carbohydrate composition of mucus differs among the corals *Acropora pharaonis*, *Porites verrucosa*, and *Porites lobata* (Hadaidi et al., 2019). However, *Stylophora*

pistillata and *Pocillopora verrucosa*, two pocilloporid corals, have similar carbohydrates (Neave et al., 2017) and zooxanthellae composition (LaJeunesse et al. 2018). Differences in mucus components could affect microbial community composition in coral reef habitats (Schottner et al., 2009). The most common mucus components observed in corals that occur across a large geographic range (e.g., *A. muricata*, *Pachyseris speciosa*, *Fungia fungites*, *Stylophora* sp., and *Pocillopora* sp.) are fucose, arabinose, glucose, galactose, the amino sugar *N*-acetylglucosamine, xylose, and mannose (Wild et al., 2005; Lee et al., 2016) (Table 1.1). Thus, mucus carbohydrates are essential substrates that support bacterial growth and contribute to the flux of dissolved organic matter (DOM) in seawater (Riemann and Azam, 2002).

Table 1.1 Carbohydrate content of various coral species mucus.

Sr. No.	Carbohydrate	Coral species	Reference
1.	Arabinose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Pachyseris speciose</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>Cespitularia</i> sp., <i>Porites lobata</i> , <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Wild et al., (2005; 2010); Hadaidi et al., (2019).
2.	Fucose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>Cespitularia</i> sp., <i>Galaxea fascicularis</i> , <i>Porites lobata</i> , <i>Ctenactis</i> sp., <i>Pocillopora</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Wild et al., (2005; 2010); Lee et al., (2016); Hadaidi et al., (2019).
3.	Galactose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Acropora formosa</i> , <i>Pachyseris speciose</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>Cespitularia</i> sp., <i>Porites lobata</i> , <i>Galaxea fascicularis</i> , <i>Stylophora pistillata</i> , <i>Pocillopora verrucose</i> , <i>Ctenactis</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Wild et al., (2005; 2010); Lee et al., (2016); Hadaidi et al., (2019).
4.	Glucose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Acropora formosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>Cespitularia</i> sp., <i>P. lobata</i> , <i>G. fascicularis</i> , <i>S. pistillata</i> , <i>P. verrucose</i> , <i>Ctenactis</i> sp., <i>Pocillopora</i> sp., <i>Stylophora</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Wild et al., (2005; 2010); Lee et al., (2016); Hadaidi et al., (2019).
5.	Mannose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Acropora formosa</i> , <i>Pachyseris speciose</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>Cespitularia</i> sp., <i>P. lobata</i> , <i>G. fascicularis</i> , <i>S. pistillata</i> , <i>P. verrucose</i> , <i>Ctenactis</i> sp., <i>Fungia</i> sp., <i>Pocillopora</i> sp., <i>Pachyseris</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Wild et al., (2005; 2010); Lee et al., (2016); Hadaidi et al., (2019).

6.	N-acetyl galactosamine	<i>Acropora formosa</i> , <i>Lemnalia</i> sp., <i>Pachyseris speciose</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Cespitularia</i> sp.	Meikle et al., (1988); Corfield (2015).
7.	N-acetyl glucosamine	<i>Acropora</i> sp., <i>Acropora Formosa</i> , <i>Pachyseris speciose</i> , <i>Fungia fungites</i> , <i>Cespitularia</i> sp. <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>A. pharaonis</i> , <i>P. lobata</i> , <i>G. fascicularis</i> , <i>S. pistillata</i> , <i>Ctenactis</i> sp., <i>Fungia</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Salvatore et al., (2000); Wild et al., (2005; 2010); Hadaidi et al., (2019).
8.	Ribose	<i>A. pharaonis</i> , <i>P. lobate</i>	Wild et al., (2010).
9.	Rhamnose	<i>A. pharaonis</i> , <i>P. lobata</i> , <i>Acropora</i> sp., <i>Madrepora</i> sp., <i>Lophelia</i> sp.	Wild et al., (2010); Hadaidi et al., (2019).
10.	Xylose	<i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemnalia</i> sp., <i>Acropora</i> sp., <i>Fungia</i> sp., <i>Lophelia</i> sp.	Meikle et al., (1988); Wild et al., (2010); Krediet et al., (2013); Hadaidi et al., (2019).

1.3.3 Fatty acids of mucus

The precise understanding of the fatty acid (FA) composition and its role in coral mucus is still inadequate and remains one of the least studied aspects of coral mucus. The most common and abundant FAs observed in the mucus of *Acropora* sp. in Discovery Bay, Jamaica, were palmitic acid and palmitoleic acid (Means and Sigleo, 1986; Wild et al., 2005). These were also found more frequently in corals and zooxanthellae as structural and storage lipids (Patton et al., 1977; Means and Sigleo, 1986). Other FAs, such as myristic acid and linoleic acid, have also been observed in mucus, but in lower amounts (Wild et al., 2005). The most abundant saturated FAs in marine organisms are palmitic acid (C16:0), palmitoleic acid (C16:1), and myristic acid (C14:0), similar to those found in certain unicellular marine algae (Uslu et al., 2021). Even though the presence of FAs in mucus has been reported, their origins have not been documented.

FAs in coral mucus are essential for the health and functioning of coral reefs, providing energy, supporting microbial communities, and aiding in defense mechanisms (Bachok et al., 2006). Polyunsaturated fatty acids (PUFAs), especially $n-3$ and $n-6$ PUFAs, regulate metabolism, including growth, respiration, energy generation, energy reserves, membrane constituents, photosynthesis, planula larvae and egg production, biomass formation and general enzyme activity, survival, and stress resistance (Bachok et al., 2006). C20:5 $n-3$ FAs are essential for sperm maturation and C20:4 $n-6$ is essential for egg maturation, and saturated (SAFA) and monounsaturated FAs (MUFA) are stored as triglycerides and wax esters (Grottoli et al., 2004; Tchernov et al., 2004; Treignier et al., 2008). In the previously studied coral mucus samples, higher concentrations of SAFAs were observed (Sigleo, 1986). In addition, FAs in corals serve as markers for bacterial colonization and diversity, coral health, and food source tracking (Volkman et al., 1980). As the synthesis of FAs in corals and in coral mucus is directly coupled to photosynthesis, there is a significant correlation between dinoflagellate biomarkers and FA concentrations (Oku et al., 2003). Free fatty acids (FFAs) have been found to be involved in maintaining cell membrane stability and cell signaling (Binienda et al., 2013). FFAs are physiologically active as dietary components and participate in cell signaling (Zbigniew et al., 2013). FAs are also required for reproduction and membrane fluidity (Lin et al., 2012). However, the purpose and the function of FAs present in mucus are not well understood and need to be thoroughly

investigated in various genera and species of corals based on their habitat and other environmental parameters.

1.3.4 Mucus lipids

Photosynthetic carbon is stored in the cytoplasm of zooxanthellae, in the form of lipids, which are then incorporated into the coral mucus, where it serves as an important source of energy for a variety of marine organisms (Crossland et al., 1980; Pasaribu et al., 2015). Many galactolipids synthesized by marine diatoms have been shown to induce apoptosis starting at micromolar concentrations in mammalian cells (Andrianasolo et al., 2008), suggesting the function of membrane lipids in regulating the symbiosis between corals and zooxanthellae. Coral mucus is composed of high-energy lipids which are also an important source of energy for fish (Wright et al., 2019). Upon thermal stress, corals may acquire more thermotolerant symbionts (Lajeunesse et al., 2010) having lipids with more SAFAs with a high melting point. This altered membrane lipid composition is observed in bleached corals, indicating differences in heat sensitivity among zooxanthellae lineages (Tchernov et al., 2004).

Zooxanthellae provides precursors and metabolites required for endogenous lipid synthesis in coral hosts mainly through photosynthesis (Oku et al., 2003; Iluz and Dubinsky, 2015), and several studies have demonstrated the rapid conversion of photosynthetically fixed carbon to lipids (Radice et al., 2019). The presence of lipids (e.g., wax esters) in coral mucus has been demonstrated in significant amounts (Crossland, 2021). Corals are rich in lipids, which are the biochemical link between the symbiotic mutualistic association of coral and zooxanthellae (Sikorskaya et al., 2022). Lipids are involved in coral physiological and biochemical processes and are an important source of long-term stored energy as mucus serves as an energy-rich source of nutrition for various organisms (Rodrigues et al., 2008). During bleaching, a change in FA composition and also a reduction in the rate of lipid production may affect the coral's metabolic needs (Grottoli et al., 2004). This reduced energy reserve increases disease susceptibility and mortality (Smith et al., 2020), which may be due to a change in the microbial community in the damaged tissues.

1.3.5 Proteins and/or enzymes of mucus

According to Benavides et al., (2017), a low C: N ratio in coral mucus indicates the presence of a large amount of protein. Previous studies on *Palythoa* sp. and *Fungia* sp. showed that their mucus contains 8–20% organic matter and 1–2% protein (Daumas and Thomassin, 1977; Stabili et al., 2015). Coral mucus from *Acropora*, *Fungia*, and *Sarcophyton* species contains important enzymes, such as peptidases, esterases, and glycosidases (Vacelet and Thomassin, 1991; Bhagwat et al., 2024). Hydroxyproline, found in coral mucus, is crucial for collagen synthesis and maintaining the thermodynamic stability of collagen's triple-helical conformation and associated tissues (Ortega et al., 2018). Fish mucus, on the other hand, is known for its high protein content and enzymes, such as lysozyme, alkaline phosphatase, complement, cathepsin B, transferrin, and C-reactive protein (Subramanian et al., 2007; Zhao et al., 2008; Bhagwat et al., 2024). The mucus of the zoanthid *Palythoa caribaeorum* contains lectins and proteases (Guarnieri et al., 2018). However, phenoloxidase (PO), peroxidases (POD), and antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase, which have been reported from coral tissues (Anithajothi et al., 2014), have not yet been reported in coral mucus. Thus, mucus reported with antimicrobial properties has to be investigated for its potential peptides and proteins.

1.3.6 Amino acids

Corals and zooxanthellae can synthesize 20 different amino acids, with some produced by the coral polyps, others by the zooxanthellae, and yet others by both partners. However, different coral species have different amino acid synthesis abilities (Fitzgerald and Szmant, 1997; Bhagwat et al., 2024). Amino acids can also be taken up via plankton, detritus, and the water column. The acquired amino acids are used by the coral to synthesize proteins for tissue growth, calcification, and other processes (Grover et al., 2008; Shinzato et al., 2014). The production of methionine could be a result of the synergistic action of enzymes from both coral and zooxanthellae, indicating that these symbiotic partners have evolved to mutually benefit each other (Shinzato et al., 2014). Although corals acquire many amino acids, only 16 have been observed in the mucus, including aspartate, glutamine, serine, glycine, threonine, arginine, alanine, tyrosine, valine, phenylalanine, isoleucine, leucine, lysine, histidine and methionine, of several coral species (i.e., *Acropora formosa*, *Pachyseris*

speciosa, *Fungia fungites*, *Sarcophyton* sp., *Lemnalia* sp., *Cespitularia* sp., *Platygyra lamellina*, *Heteroxenia fuscescens*, *Oculina arbuscula* (Meikle et al., 1988; Ducklow and Mitchell, 1979; Means and Sigleo, 1986; Coddeville et al., 2011; Bhagwat et al., 2024).

Analysis of mucus produced by *Palythoa* and *Fungia* species has shown that the amino acids serine, aspartic acid, glutamic acid, and glycine are present in high abundance, and these play important roles in a variety of biological processes, including protein synthesis, cellular signaling, and energy metabolism (Daumas and Thomassin, 1977). Glycine is the most abundant amino acid found in both coral tissues and mucus, particularly in species such as *Montastraea cavernosa* (Daumas and Thomassin, 1977). Glutamic acid, which also acts as an antioxidant in many plants, animals, and bacteria, was more abundant in the mucus of *Fungia* sp., *Acropora* sp., *Platygyra* sp., *Sarcophyton* sp., and *Heteroxenia* sp., compared to serine, and methionine (Ducklow and Mitchell, 1979). Alanine is generally observed in *Porites divaricata*, *Acropora cervicornis*, *Orbicella faveolata*, and the azooxanthellate corals, *Astrangia poculata* and *Tubastraea coccinea* (Fitzgerald and Szmant, 1997; Bhagwat et al., 2024). Similarly, *Fungia scutaria* was reported to utilize tyrosine, lysine, glycine and aspartic acid, from surrounding water (Stephens, 1962; Bhagwat et al., 2024). This suggests that corals acquire some amino acids through heterotrophic feeding from the surrounding water. In addition, both host and zooxanthellae can synthesize amino acids and this varies from species to species.

1.3.7 Mucus antioxidants against oxidative stress.

A decrease in zooxanthellae density is due to oxidative stress causing coral bleaching (Downs et al., 2002; Weis, 2008). Primarily, oxidative stress is generated during photosynthesis (Krieger-Liszkay, 2005). Excessive production of reactive oxygen species (ROS) causes disorganization and rupture of the thylakoid membrane (Tchernov et al., 2004). Free radicals, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($-OH$), degrade mucus glycoproteins at the histidine molecule, so that mucus property, molecular weight, and viscosity are affected (Dailah, 2022). ROS imparts an adverse impact on the mucus defense properties which are then controlled by the mucus antioxidants (Suggett et al., 2008).

The presence of phenoloxidase (PO), an enzyme that activates the invertebrate defense system, was reported in coral secretory mucus (Rivera-Ortega and Thome, 2018).

The mechanism of secretion of this enzyme into coral mucus is not known. However, the expulsion of similar molecules from tissue to the mucus was reported previously (Ritchie, 2006). Thus, phenoloxidase is thought to be an intracellular enzyme, but similar activity was also observed in the mucus of other organisms, such as in lancelets (Zhang and Li, 2000) and some insect cuticles (Arakane et al., 2016). The pro-phenoloxidase (proPO) activates PO, resulting in the synthesis of melanin which in turn activates innate immune responses (Fig. 2.2). The proPO system itself is activated by tyrosine and phenylalanine, which is synthesized by both the coral host as well as zooxanthellae (Fitzgerald and Szmant, 1997). To reduce the impact of toxic by-products, such as reactive oxygen (RO) and other free radicals, antioxidant enzymes are up-regulated to offset self-harm (Gardner et al., 2016). However, the source and the presence of such antioxidant enzymes and non-enzymatic antioxidants in coral mucus are not well studied (Rivera-Ortega and Thome, 2018). Some enzymes, such as superoxide dismutase and glutathione peroxidase, have been reported as stress markers in corals (Gardner et al., 2016). Elevated PO activity triggers melanin production, serving as a signal for corals to react to pathogens, and concurrently functioning as a potent antioxidant against UV radiation (Palmer et al., 2011). The antioxidant capabilities of mucus against oxidative stress may determine the survival of the corals against bleaching and other stressors.

1.3.8 Mucus microbes.

Corals apart from the coral host and endosymbiotic alga, contain a distinct microbial community including fungi, bacteria, archaea, and viruses, as coral mucus is a good source of nutrients for the growth and proliferation of microbes (Bhagwat et al., 2024). The bacterial community provides nutrients, protects from pathogen invasion through antimicrobial production, and also indicates coral health status (described in more detail in 1.3.9) (Ritchie, 2006; Rypien et al., 2010; Roder et al., 2015; Ziegler et al., 2016). Coral mucus and associated beneficial bacteria regulate the microbial population of corals (Ritchie, 2006). The most important function of mucus-associated bacteria is to supply and cycling of nutrients which impacts coral health (Lesser et al., 2004; Wegley et al., 2007). The significance of the surface mucus layer (SML) in coral biology extends beyond its role as a habitat for unique coral-associated microbes. It plays a crucial part in providing nutrition, protection, and cleansing functions (Brown and Bythell, 2005). The SML composition varies depending on

time, species, location, and sloughing (Bhagwat et al., 2024). These structural changes contribute to habitat dynamics, and bacterial communities likely respond to these fluctuations (Nelson et al., 2013). Also, the alterations in the bacterial community structure in coral mucus have an impact on the bioactivities of mucus. For example, healthy mucus showed higher antimicrobial activity than that of bleached coral mucus with different bacterial communities (Cuong et al., 2022).

A diverse number of ciliates associated with many coral diseases have been reported which are opportunistic pathogens and bacterivorous (Sweet and Sere, 2016). There are many coral diseases reported for their association with ciliates such as Skeleton-Eroding Band, which was first described for their ciliate association (Antonius and Lipscomb, 2001), Brown Band Disease associated with ciliate *Philaster guamense* (Lobban et al., 2011), White Syndrome, the most pervasive coral disease (Sweet and Bythell, 2012), White Band Disease, White Plague, Brown Jelly Syndrome and BrB-like syndrome (Randall et al., 2014; Sweet et al., 2014). In addition, some ciliates are also reported for the consumption of endosymbiont *Zooxanthellae* (Qiu et al., 2010). Ciliates are specific to coral species and in vitro, experiments suggest the specificity in the utilization of mucus as substrate efficiently (Ravindran et al., 2020). Although the ciliate association with coral diseases has been reported no study described their role in causing the diseases.

In the mucus of certain *Acropora* and *Porites* species including surface mucus, the presence of fungi (*Thraustochytrium* sp.) has been reported (Raghukumar and Balasubramanian, 1991; Harel et al., 2008). Additionally, various fungal associations have been observed in diseased corals, including a marine fungus linked to black line disease in *Montastrea annularis* star corals (Ramos-Flores, 1983), endolithic fungi in *Porites lobata* hard corals (Le Campion-Alsumard et al., 1995), and a *Scolecobasidium* sp. associated with *Porites lutea*, *Goniopora* sp., and *Montipora tuberculosa* (Raghukumar and Raghukumar, 1991). A well-characterized fungal disease is an aspergillosis in gorgonians (sea fans), caused by *Aspergillus sydowii* (Smith et al. 1996). Apart from this, fungi are reported for their beneficial roles in corals. Several antimicrobial compounds have been identified in fungi associated with coral reefs (Namikoshi et al., 2000; Bugni and Ireland, 2004). Fungi are also reported for their role in nitrogen cycling within the coral ecosystem (Wegley et al., 2007). It is possible that some Mycosporine-like amino acids (MAA) (functions as sun protectant)

can be introduced to the corals from fungi because the similar type of MAAs typically associated with endosymbionts have also been produced by certain associated fungi (Dunlap and Shick, 1998). Certain fungi have been recognized for their participation in the biomineralization of coral skeletons (Raghukumar and Ravindran, 2011). Numerous strains of the fungus *Corallochytrium limacisporum* have been identified in coral mucus. Research has indicated the presence of the key lysine synthesis enzyme alpha aminoadipate reductase (α -AAR) in *C. limacisporum* (Sumathi et al., 2006). Coral mucus, known for its abundance in carbohydrates, proteins, amino acids, and more, has been shown to host *Thraustochytrid* fungi that exhibit robust proteolytic enzyme activity, particularly at alkaline pH (Raghukumar et al., 2008). Consequently, *Thraustochytrids* sp. within coral mucus may contribute to its degradation. Additionally, the presence of *Straminopilan* sp. fungi on coral surfaces and in mucus has been associated with providing nutrition, specifically in the form of polyunsaturated fatty acids, which may aid corals in surviving bleaching events (Harel et al., 2008). Therefore, the role of fungi in the coral holobiont is diverse, with some reports indicating harmful effects while others suggest a beneficial role.

Coral mucus creates an extremely favorable environment for the rapid growth of viruses, fostering the emergence of both temperate and virulent phages (Nguyen-Kim et al., 2015). Therefore, many reports demonstrated the presence of viruses as the most abundant biological entities in mucus (Davy and Patten, 2007; Nguyen-Kim et al., 2015). The majority of viruses found in association with coral are bacteriophages (Nguyen-Kim et al., 2014). Bacteriophages, widely distributed, play a significant role as a key regulator of bacterial populations (Nguyen-Kim et al., 2015). Mucus viruses through the lytic pathway protect corals from bacterial pathogens by cell lysis (Atad et al., 2012; Bourne et al., 2009). Lysogenic phages embedded in mucus serve as a protective shield against other viral infections by integrating into the host's genome (Paul, 2008). In contrast, viruses also can infect the endosymbiont of corals (Correa et al., 2013). In addition, many beneficial bacteria affected by bacteriophages may fail to provide essential nutrition and defense to corals. Elevated temperatures can also trigger latent viruses in *Symbiodinium*, resulting in the substantial mortality of *Symbiodinium*, potentially causing coral bleaching and mortality (Wilson et al., 2005; Correa et al., 2012). Although mucus viruses have been reported to infect all types of coral holobionts, their role is both beneficial as well as harmful for the

corals (Nguyen-Kim et al., 2015). Coral mucus naturally harbors a great number of microorganisms, whose roles are still largely unknown but most probably contribute to mucus balance and biological activities. Microbes especially in mucus are largely understudied, for their specific role in the coral ecosystem and disease causation.

1.3.9 Antimicrobial activity of mucus and mucus-associated microbes

Coral mucus from different habitats is composed of unique components that alter the presence of specific microbes on the surface of different coral species (Guppy and Bythell, 2006; McShane et al., 2021). The natural flora of healthy coral is presumably beneficial and any shift in this bacterial population may be an early indicator of coral stress, such as disease (Nithyanand et al., 2011). Therefore, the SML of coral is a selective medium determined by the host-microbe–environment interactions, where the commensal microbiota act against potential pathogens (Ritchie, 2006; Alagely et al., 2011; Krediet et al., 2013; Teplitski et al., 2016; Irudayarajan et al., 2023). The microbial gene abundance in the coral mucus is known to be involved in stress resistance, virulence, chemotaxis and motility, utilization of FAs and lipids, sulfur and nitrogen metabolism, and secondary metabolism making the mucus-associated bacterium a first line of defense (Bythell and Wild, 2011; Irudayarajan et al., 2023). Thus, some microbial communities in the SML are described as producing defensive antimicrobial chemicals (Orland and Kushmaro, 2009; Irudayarajan et al., 2023). The Caribbean coral *Acropora palmata* demonstrated antibacterial inhibition against the pathogen that causes whitepox disease (Nissimov et al., 2009). Similarly, antibacterial activity was found in many cultivable bacteria of the coral mucus of six scleractinian corals from the Gulf of Eilat (Shnit-Orland and Kushmaro, 2009). Varied bacterial genera of both tissue and mucus of *A. digitifera*, including *Vibrio* sp. and *Bacillus* sp. have been elucidated for their antibacterial activity. *Bacillus* sp. synthesizes a significant peptide group of antibiotics including gramicidin, bacitracin and polymyxin B (Wiese et al., 2009). Thus, the highly specific mucus bacterial community is crucial for the first line of defense of corals against diseases and other environmental stress factors (Kemp et al., 2015). Moreover, specific bacteriophages of the coral SML have been described to treat various bacterial infections as phage therapy (Nguyen et al., 2015; van Oppen, 2019). The SML antimicrobial activity exhibited species specificity, while the mucus-associated beneficial bacteria, such as

Pseudoalteromonas sp. and *Roseobacter* sp., have been described for their antibacterial activity against a number of coral pathogens, including *V. shiloi* (Ortega and Thome, 2018). As a result, beneficial microorganisms of corals (BMCs) are used as probiotic drugs in order to increase coral resistance to environmental stress, and the rotifers, *Brachionus plicatilis*, are employed as carriers to impart BMCs into the coral *Pocillopora damicornis* (Assis et al., 2020).

The biochemicals present in the surface mucus of *Acropora palmata* and *Pseudodiploria strigosa* are responsible for regulating the coral-associated microbial community (Rivera Ortega et al., 2018). Studies have shown that the mucus of *A. palmata* suppresses the growth of various bacterial strains, including *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, and the whitepox disease causative agent, *Serratia marcescens* (Ritchie, 2006). The mucus also exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria (Ritchie, 2006). Similarly, healthy coral mucus obtained from *P. strigosa* has been found to possess antimicrobial activity against *Serratia marcescens* and *Aurantimonas* sp. pathogens (Ortega and Thome, 2018). In addition to its antimicrobial properties, *Galaxea fascicularis* mucus has been found to have potential health benefits for humans, such as apoptotic and anticancer activity, DNase activity, and activity against Topoisomerase I and II enzymes (Ding et al., 1999; Khalesi, 2015). Homarine, an organic compound with antibiotic potential, has been documented in corals for its ability to potentially inhibit the growth of a range of bacteria and fouling diatoms (Maldonado et al., 2016). Fish mucus contains a variety of components, including disulfides, glycoconjugates, antimicrobial peptides (AMPs), lysozyme, cathepsin B, complement, alkaline phosphatase, C-reactive protein, transferrin, and others, which act as effective protection (Meyer et al., 2007; Subramanian et al., 2007; Zhao et al., 2008). AMPs in fish mucus are effective antimicrobial agents against human pathogens, including algae, bacteria, fungi, viruses, or parasites (Komatsu et al., 2009; Fernandes et al., 2010). Although the antimicrobial properties of coral mucus and associated bacteria have been mentioned in numerous papers, the specific components responsible for these properties are not well documented (Wright et al., 2019; Wijayanti et al., 2020), and further research is needed to better understand the immunological properties of coral mucus and the mechanisms by which it provides protection.

1.3.10 Mucus as a sunscreen and effective antioxidant under heat stress

Mycosporine-like amino acids (MAAs) are responsible for shielding corals from the harmful effects of solar ultraviolet (UV) radiation by absorbing approximately 10% of UV radiation. This mechanism is critical for the survival of corals, as exposure to high levels of UV radiation can cause damage to coral DNA, resulting in a range of negative impacts on coral health and the overall health of the surrounding ecosystem. By providing this important protection against UV radiation, MAAs play a key role in maintaining the health and resilience of coral populations (Teai et al., 1998; Kageyama and Waditee-Sirisattha, 2019). Mycosporine-glycine, mycosporine-2glycine, palythine, mycosporine-methylamine-serine, and mycosporine-methylamine-threonine, which were characterized from *Lobophyllia*, *Acropora*, *Fungia*, and *Pocillopora* species, have all been reported from coral mucus (Teai et al., 1998). In addition to their ability to combat oxidative stress, MAAs can protect many marine organisms from the harmful effects of light (Shick and Dunlap, 2002). Similarly, mycosporine-glycine showed an additional antioxidant property in coral mucus along with sunscreen, probably protecting endosymbionts from oxidative damage produced through photosynthesis (Teai et al., 1998; Bhagwat et al., 2024). Biologically harmful UV rays between 310 and 360 nm are absorbed by MAAs. Endosymbionts and the coral hosts are both protected from the harmful effects of UV radiation by mucus MAAs. It was reported that the UV-absorbing molecules in the coral mucus of *Fungia fungites* reduced with depth (Drollet et al., 1997; Bhagwat et al., 2024). The reduction in UV radiation, light, and photosynthesis may be attributed to increased depth, as water absorbs and scatters UV radiation more effectively with increasing depth, leading to a decrease in UV radiation intensity.

Mycosporine-like amino acid concentrations vary depending on coral species and habitat. They have been found in both host tissues as well as in zooxanthellae (Shick and Dunlap, 2002). MAAs are thermally stable, making them useful for scavenging reactive oxygen species under heat stress (Banaszak and Lesser, 2009). MAAs play a role in cellular osmoregulation during salinity stress or in a high-salinity environment (Oren and Gunde-Cimerman, 2007). The combination of mycosporine and glycine, the most common MAAs in coral hosts, protects the coral symbiosis from oxidative damage (Banaszak et al., 2006).

MAA also serves as a nitrogen source within the cell, which is required for growth and proliferation (Oren and Gunde-Cimerman, 2007).

1.3.11 Mucus as a source of nutrients for marine organisms

Mucus produced by coral is a valuable energy source with high glucose content. As glucose is readily available, it is a highly preferred energy source for microbes and other organisms (Ravindran et al., 2023). Thus, coral mucus is an important component of marine ecosystems, providing a rich source of energy for a wide group of organisms (Wild et al., 2010) (Table 1.2). Mucus energy levels are far greater than benthic photosynthetic algae. Further, a few soft corals have also been shown to feed on the mucus of other corals (Coffroth, 1984). Similarly, fishes, such as blue sprat (Eckes et al., 2015), damselfishes (Coles and Strathmann, 1973; McMohan et al., 2016), and other benthic organisms, such as an Acoelomorpha worm (*Waminoa* sp.), bivalves (Shafir and Loya, 1983; Bhagwat et al., 2024), shrimp (*Coralliocaris superba*) (Horka et al., 2016), coral crabs (*Trapezia* sp.) (Shmuel et al., 2022) and some planktonic organisms, such as crown of- thorns starfish larvae (Nakajima et al., 2016), copepods (*Acartia negligence*) (Richman, 1975; Bhagwat et al., 2024), and mysids, (*Mysidium integrum*) (Gottfried and Roman, 1983) also exhibit feeding on coral mucus. Coral mucus was found to be the best nutritional source with a C: N ratio of mucus fluid of 6.9 to 13.7 and mucus sheet of 4.8 to 5.9 (Coffroth, 1990; Bhagwat et al., 2024) (Table 1.2). Since mucus sheets that stay on the coral surface can trap organic materials, their nutritional value is higher than reef benthic algae (~ 1) and reef algal detritus (~ 1) (Wilson et al., 2003). When compared with the other forms of energy sources, reef fish feces contribute to a nutritional value of ~ 10 (Bailey and Robertson, 1982; Bhagwat et al., 2024), phytoplankton ~3.5 (Renaud et al., 1999; Bhagwat et al., 2024), and zooplankton ~5.5 (Ventura, 2006). Furthermore, coral mucus acts as a vehicle for nutrients to reach the mouth (Goldberg 2018). Small reef fish and copepods utilize wax esters and triglycerides as energy sources, both of which are major components of mucus (Ho, 2017). Thus, coral mucus helps in the fulfillment of energy requirements as well as the cycling of nutrients (Wild et al., 2004).

Table 1.2- C/N ratio in fresh coral mucus.

Hard corals	C/N ratio	References
<i>Acropora</i> sp.	8.5	Coles and Strathmann, 1973
<i>Acropora formosagrandis</i>	5.9	Vacelet and Thomassin, 1991
<i>Fungia scutaria</i>	7.5	Coles and Strathmann, 1973
<i>Fungia scutaria</i>	6.9	Krupp, 1982
<i>Fungia echinata</i>	5.6	Vacelet and Thomassin, 1991
<i>Porites compressa</i>	6.0	Coles and Strathmann, 1973
<i>Goniopora lobata</i>	5.9	Coles and Strathmann, 1973
<i>Platygyra lamellina</i>	6.6	Pascal, 1981
<i>P. astreoides</i>	5.9	Coffroth, 1990
<i>P. furcate</i>	4.1	Coffroth, 1990
<i>P. australiensis</i>	5.9	Coffroth, 1990
<i>P. lutea</i>	5.9	Coffroth, 1990
<i>P. lobate</i>	5	Coffroth, 1990
<i>P. murrayensis</i>	5.5	Coffroth, 1990
<i>Porites</i> sp.	4.8	Coffroth, 1990
Soft corals		
<i>Heteroxenia fuscescens</i>	7.3	Pascal, 1981
<i>Sarcophyton</i> cf. <i>glaucom</i>	5.6	Vacelet and Thomassin, 1991
<i>Briarium asbestinum</i>	8.9	Pascal, 1981

1.3.12 Role of mucus in reef ecosystem nutrient cycling

When coral mucus is released into the water column, it is rapidly colonized by microorganisms that break down the organic compounds and release nutrients, such as nitrogen (N₂) and phosphorus (Van Oppen et al., 2019). These nutrients are then available for uptake by other organisms in the ecosystem, such as phytoplankton and zooplankton. Corals obtain N₂ through predation and from the water column and excrete ammonia (Fig. 1.3) (Siboni et al., 2008). Further, Archaea use this ammonia, which plays an important role

in ocean nitrification. During daytime, when oxygen levels are high due to photosynthesis, certain types of Archaea convert ammonia into nitrite. Conversely, at night, when oxygen is low, different types of Archaea carry out denitrification to convert nitrite into nitrogen (Siboni et al., 2008; Siboni et al., 2012). Nitrogen-cycling microbes play a vital role in coral-algae symbiosis, especially under nutrient-rich or -poor conditions for coral productivity (Radecker et al., 2015). Coral mucus contains high concentrations of dissolved organic nitrogen (DON), which can be readily taken up by bacteria for growth and metabolism. Some of these bacteria form symbiotic relationships with coral polyps, where they fix atmospheric nitrogen (N_2) (Diaz, 2017). Other bacteria in the mucus help us to recycle nitrogen within the holobiont by breaking down waste products and releasing ammonium (NH_4^+) and nitrate (NO_3^-) into the surrounding water. These forms of nitrogen can then be taken up by other organisms in the reef ecosystem (Wild et al., 2004).

Up to 80 % of mucus released by corals becomes dissolved organic carbon (DOC) (Wild et al., 2004). Sponges transfer dissolved organic matter (DOM) including DOC and DON to the next trophic level (Rix et al., 2017). Bacteria utilize mucus and break down organic compounds and release carbon dioxide (CO_2) through respiration. This CO_2 can then be taken up by photosynthetic organisms, such as algae and seagrasses, which use it to produce organic carbon through photosynthesis (Haas et al., 2016). This process helps us to maintain the balance of carbon in the reef ecosystem. Coral mucus also contains significant amounts of dissolved organic phosphorus (DOP), which can be taken up by bacteria and recycled within the holobiont (Nakajima et al., 2015). Some of these bacteria are capable of breaking down complex organic phosphorus compounds, such as phospholipids, to release inorganic phosphorus (PO_4^{3-}) into the surrounding water (Bourne et al., 2013). This inorganic phosphorus can then be taken up by algae and other organisms in the reef ecosystem. Mucus functions as nutrient trapping, thus, higher ammonia, nitrate, and phosphate in mucus than in surrounding water suggests its role in nutrient adsorption. In addition, coral mucus transports nutrients into reef sediment (Wild et al., 2005). Overall, coral mucus plays a crucial role in maintaining the health and productivity of reef ecosystems by facilitating nutrient cycling and supporting the diverse array of organisms that inhabit these ecosystems. Recycling these nutrients within the holobiont and the surrounding water helps us to maintain a balance of nutrients and supports the ecosystem's productivity. Additionally, the role of coral mucus in

nutrient cycling highlights the importance of conserving coral reefs and protecting them from human activities that may disrupt this delicate balance.

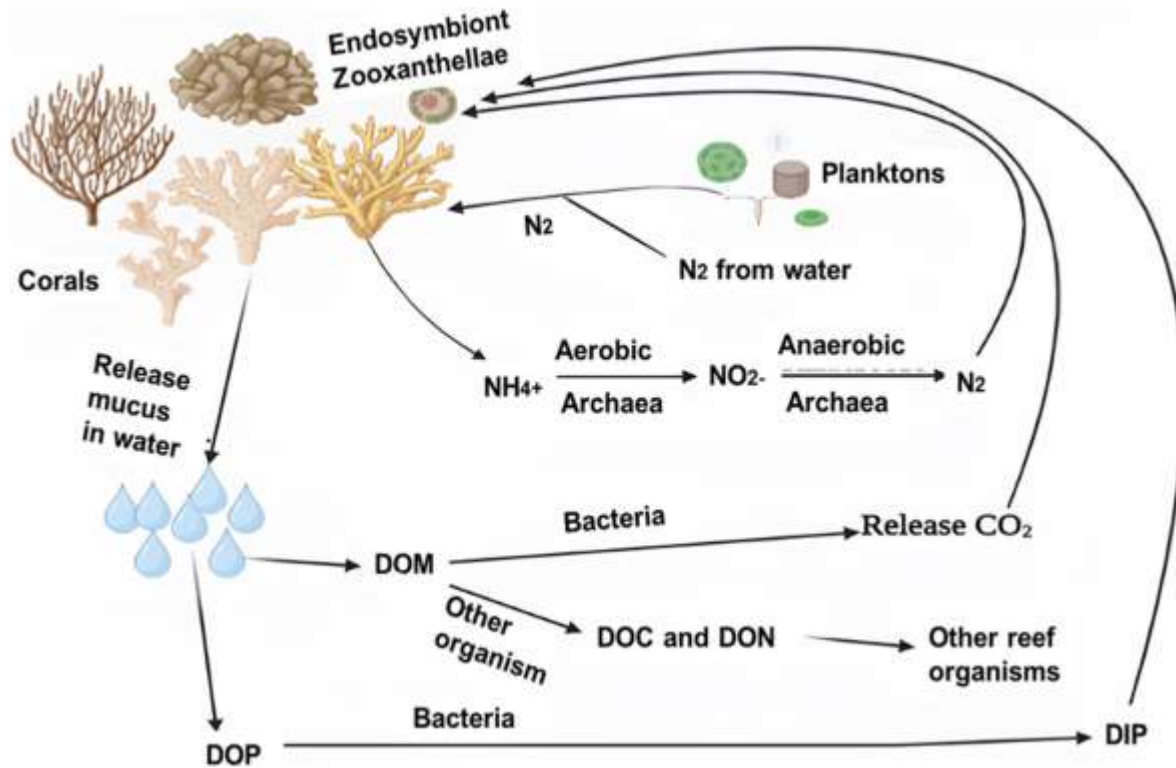


Figure 1.3- Coral mucus in reef nutrient cycling. Corals release mucus rich in DOM to the surrounding water, which is utilized by reef organisms whose respiration returns carbon dioxide and other nutrients required for photosynthesis by the endosymbiont zooxanthellae. Also, endosymbiont-derived organic compounds are released into the mucus and nutrient cycling is activated through various microbes. (DOM-dissolved organic matter, DON-dissolved organic nitrogen, DIP-dissolved inorganic phosphate, DOC-dissolved organic carbon, DOP-dissolved organic phosphate, NH₄-Ammonia, NO₂-nitrite, N₂-nitrogen).

1.4 Objectives

The objectives of this study include:

- To characterize the healthy and diseased coral mucus.
- Evaluation of the beneficial role of coral mucus and associated bacteria for their defense properties.

1.5 Summary

- The coral surface mucus serves as an interface between the coral epithelium and the seawater environment. It provides a primary line of defense and protects coral surfaces from a variety of physio-chemical and microbial invasions.
- As both host and endosymbiont provide mucus constituents, this symbiotic mucus synthesis is different from many other marine organisms.
- The exact mechanism of mucus synthesis, secretion, and sources of other important components present in mucus is unknown, and less information is available about the properties and rheological changes of mucus, which are not fully demonstrated.
- Very less study have been done on coral species for their mucus components, such as carbohydrates, fatty acids, amino acids, proteins, antioxidants, and antimicrobial compounds, and also not all types have been thoroughly explored. Also, mucus defense properties, including antimicrobial peptides, antioxidants, and UV protectants (e.g., Mycosporine-like amino acids), and beneficial microbes, are not well described for their roles in supporting coral health in various stress conditions.
- Coral mucus serves multiple ecological roles with their associated microorganisms and the surrounding environment that need to be studied to understand reef ecosystem functioning.
- The present literature review aids in understanding the role of coral mucus in coral reef ecosystems; however, more detailed studies of coral mucus components and their roles in the coral reef ecosystem by using appropriate biochemical and molecular approaches may provide insights in understanding the possible role of coral mucus in producing coral health.

Chapter two

Physiochemical parameters, isolation of Coral mucus and mucus-associated bacteria from selected study sites of Gulf of Mannar.

2.1 Introduction.

Corals and their surrounding environment play a pivotal role in the health and resilience of the coral reef ecosystems (Bourne et al., 2016). The physical and chemical parameters such as temperature, salinity, pH, and elevated nutrients (for example, phosphate, nitrate, ammonia, and dissolved organic carbon) play a major role in determining the health of the coral and coral reef ecosystem (Bruno et al., 2003). Also, the above various abiotic stress causes the corals to release coral mucus, which protects the health of the corals against biotic and abiotic stress.

Coral holobiont, apart from the coral host and endosymbiotic algae, contains a distinct microbial community including fungi, bacteria, archaea, and viruses, as coral mucus is a good source of nutrients for the growth and proliferation of microbes. The bacterial community provides nutrients, protects from pathogen invasion through antimicrobial production, and also indicates coral health status (Koh et al., 1997; Ritchie, 2006; Rypien et al., 2010; Roder et al., 2015; Ziegler et al., 2016; Roder et al., 2014). Coral-associated microorganisms benefit their host by photosynthesis, nitrogen fixation, etc. The most important function is to supply and cycling of nutrients which impacts coral health (Lesser et al., 2004; Wegley et al., 2007). Coral mucus naturally harbors a great number of microorganisms, whose roles are still not clearly described, which may contribute in nutrient balance and various biological activities that support the healthy coral reef ecosystem. It has been reported that commensal microbiota in mucosal surfaces protect against opportunistic infection (Nan Zhang and QiuShui, 2015). Thus, understanding the intricate dynamics of nutrients, physio-chemical, and biological parameters along with the coral mucus, is an essential requirement for unraveling the impact of environmental stressors on coral health.

Therefore, three different locations have been selected for the coral health survey in the Gulf of Mannar, Tamil Nadu, India. Corals were monitored within a 5-meter radius of each location (Fig. 2.2). They were categorized as healthy, bleached, and diseased and the sample locations were studied for their health conditions and monitored under two times of different months. The physical and chemical parameters were measured and bacteria were isolated. Examination of these parameters, may aid in understanding the responses of corals to changing environmental conditions and provide valuable insights into the mechanisms of coral adaptation and vulnerability.

2.2 Materials and methods.

2.2.1 Study locations and coral health survey.

Sample locations such as Munnaikad Island, Big Manali Island, and T. Nagar Island from the Gulf of Mannar, Tamil Nadu, India have been chosen for the proposed study. Munnaikad Island has been named as sample location 1 (SL1), subsequently Big Manali Island as sample location 2 (SL2), and T. Nagar Island as sample location 3 (SL3) (Fig. 2.1). Corals were monitored within a 5-meter radius of each location. *Porites*, dominant genera in these locations were observed as healthy, bleached, and diseased corals based on their health condition. The same sample locations were studied for their health by recording the longitude and latitude under two different observations. Munnaikad island (SL1) is located on (Latitude: N 09°17' 36.2'', Longitude: E 079°08'12.6''). Big Manali island (SL2) is located at (Latitude: N 09°13'15.6'', Longitude: E 079°08'38.5''). T. Nagar (SL3) is located at (Latitude: N 09°17'45.6'', Longitude: E 79°08'36.8''). The map of the study area is as follows.

2.2.2 Sample collection.

Mucus samples of *Porites* sp. and *Acropora* sp. from different healthy, bleached, and diseased corals (Table 2.1) have been collected from the various islands of GoM (Ravindran et al., 2020) for analysis of associated bacterial communities and mucus components. Diseased mucus samples such as white patch *Acropora* disease and *Porites* pink line syndrome disease were collected along the disease margins (Ravindran et al., 2020). The bleached mucus samples were collected from the bleached fragments of corals. Healthy and pink line syndrome disease *Porites* tissue was scraped with the help of a clean sterile scalpel and immediately transferred to a clean sterile 2 ml microcentrifuge tube. All samples were maintained in cold conditions and brought to the laboratory until further processing. Similarly, water samples near *Porites* sp. corals have been collected.

Table 2.1- Mucus collected from healthy, diseased, and bleached coral species

Sr. No.	Mucus of different corals
1	Healthy <i>Porites</i> sp.
2	Pink line syndrome <i>Porites</i> sp.
3	Bleached <i>Porites</i> sp.
4	Pink spot disease <i>Porites</i> sp.
5	Healthy <i>Acropora</i> sp.
6	White patch disease <i>Acropora</i> sp.
7	Algal dominated <i>Acropora</i> sp.

2.2.3 Isolation of bacterial community from the collected mucus as well as water samples.

After collection of mucus and water samples, 100 µl, 200 µl, and 300 µl of each water sample and in ratios of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} diluted mucus samples were plated on the Zobell Marine Agar (HiMedia) and kept in the incubator for 48 hours at 28°C for proper growth. Dilution 10^{-4} of mucus samples was used for the isolation of pure cultures from individual colonies. Further, each colony was subcultured on a separate Zobell Marine Agar (HiMedia) plate for further bacterial identification using chemical and molecular characters.

2.2.4 Heterotrophic bacterial count in nearby seawater.

The bacterial count (CFU) in nearby healthy, bleached, and diseased coral seawater was conducted. Serial dilutions of each seawater sample (10^1 - 10^4 dilutions) were prepared in sterile seawater to ensure measurable bacterial counts. 100 µl aliquots from each dilution were deposited onto individual Zobell marine agar (HiMedia) plates and evenly spread in duplicate using a glass rod to ensure uniform colony distribution. The plates were then incubated at 28 °C, for 24 hours to allow bacterial colonies to develop. After the incubation period, the dilutions showing isolated colonies were counted. The colony-forming units (CFU/ml) of seawater for each dilution were calculated based on the number of colonies counted and the dilution factor.

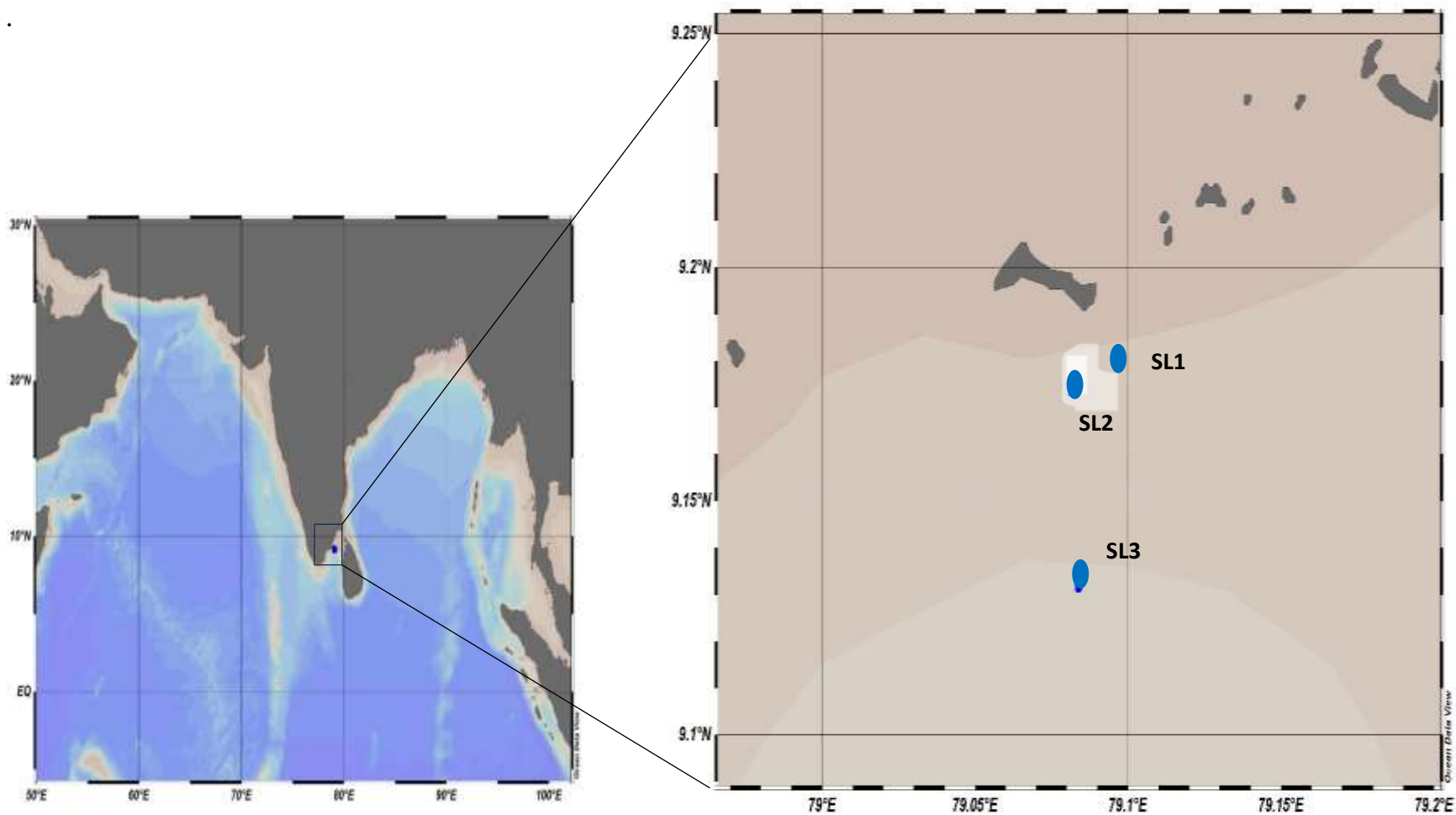


Figure 2.1- Map of sampling locations along the coastline of Mandapam in the Gulf of Mannar.

2.2.5 pH, temperature, and salinity of seawater.

Physiological parameters assessment of the coral surrounding water namely pH, temperature, and salinity were conducted. These assessments were carried out throughout June 2018 and February 2019 at three distinct locations of the Gulf of Mannar labeled as Munnaikad Island (SL1), Big Manali Island (SL2), and T. Nagar (SL3). The physicochemical parameters of the surrounding seawater were measured specifically near the corals. Temperature measurements were acquired using a thermometer, while salinity levels were determined using a refractometer. pH of the water was assessed using pH strips. Data collection and analysis were conducted separately for each collection, and comparisons were made among the three locations.

2.2.6 Nutrient analysis of seawater nearby corals.

The nutrient concentrations (in μM) were measured at the three specified locations in the Gulf of Mannar (GoM), where the field experiments were conducted. Sea water samples were filtered using a $0.22\ \mu\text{m}$ membrane filter in 200 ml syringes, 10 - 25 cm from the bottom of the syringe, and were immediately transferred to acid-washed 200 ml bottles and immediately frozen. Samples were then transported to CSIR-NIO, Goa, and were processed using an automatic analyzer (San Skalar SAN++ Continuous Flow Analyzer). For the nutrient analysis, 10 ml of water near each healthy and diseased coral was taken and utilized. A blank sample containing only 10 ml of MQ water was employed as a control during the analysis. Before processing the autoanalyzer was meticulously calibrated for silica, ammonia, nitrite, nitrate, and phosphate (all chemicals of Merck).

2.2.7 Analysis of carbon from sediment adjacent to healthy and stressed *Porites* sp.

The percentage of carbon was quantified using a CN analyzer (Flash 2000 NC, Thermo Fisher) in sediment samples collected near healthy, bleached, and diseased corals. The sample preparation process began with the even spreading of sediment samples on aluminum trays, followed by drying in an oven at temperatures ranging from $60\ ^\circ\text{C}$ to $70\ ^\circ\text{C}$ until a constant weight was achieved, with the initial weights recorded. After drying, the

sediments were grinded into a homogeneous powder using a mortar and pestle, and the resulting powdered samples were completely transferred to clean, pre-weighed containers. To preserve sample integrity, the containers were stored in a desiccator to prevent moisture absorption. For analysis, 10 mg of the powdered sediment sample was loaded into the CN analyzer and processed for analyzing carbon concentration (Wang and Wang, 2011).

2.2.8 Determination of dissolved oxygen in seawater.

For dissolved oxygen (DO) analysis, water samples were collected according to the health status of the corals. Dissolved oxygen measurements were made using the standard Winkler method (Carvalho et al., 2021). Immediately after collection in BOD bottles, samples were fixed with Winkler's A and B solution (500 µL each). Bottles were sealed tightly with a glass cap to ensure an airtight closure. Samples fixed during sampling with settled ppt were dissolved with 50 % v/v HCl (Merck), and a pipette tip was placed below the sample's surface and released. The solution was mixed and a 50 mL quantity was measured and titrated against a standard 0.1 N Sodium thiosulfate solution (Merck). Samples were collected in duplicates and 2 readings were obtained from each bottle, totaling 4 readings from each sample.

Calculations for DO (mg/L) were as such:

$$\text{DO (mg/L)} = (\text{VNa}_2\text{S}_2\text{O}_3 \times N \times 8) / \text{Vsample}$$

Where: $\text{VNa}_2\text{S}_2\text{O}_3$ = Volume of standardized sodium thiosulfate solution used in the titration (in mL).

N = Normality of the sodium thiosulfate solution

Vsample = Volume of the water sample taken for titration (in mL)

8 = Conversion factor for dissolved oxygen.

2.2.9 Biochemical identification of mucus bacteria.

2.2.9.1 Gram staining

A glass slide with a heat-fixed bacterial smear is first prepared. Then it was air-dried and gently heat-fixed. Then the crystal violet (HiMedia) was added to a smear for a minute,

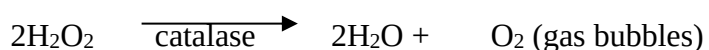
followed by the Gram's iodine (HiMedia). A decolorizing solution (HiMedia) was then used for decolorization. The smear was subsequently counterstained with safranin (HiMedia) for one minute. After gentle rinsing, the slide was air-dried before being examined under a microscope (Tripathi and Sapra 2020). Peptidoglycan and lipopolysaccharide are the major cell wall components in Gram-positive and Gram-negative bacteria, respectively. Gram-positive bacteria remain unaffected when decolorized and retain their purple color throughout the staining procedure. Gram-negative bacteria's lipopolysaccharides are washed out by decolourizer thus forming pores in the cell wall, counter stain enters through these pores and appears pink or red after the staining.

2.2.9.2 Oxidase test

A small amount of bacterial colony was picked from a recently isolated colony using a sterile inoculation loop. Then it was streaked onto an oxidase test strip (HiMedia). The reaction was observed within 10-15 seconds (Shields and Cathcart 2010). Oxidase Discs (HiMedia) were used for the detection of oxidase enzyme production by microorganisms like *Neisseria*, *Alcaligenes*, *Aeromonas*, *Vibrio*'s, *Campylobacter* and *Pseudomonas*, which give positive reactions and for excluding Enterobacteriaceae, which give negative reactions.

2.2.9.3 Catalase test

A small amount of bacterial growth from a recently isolated colony was transferred onto a clean glass slide with the sterile wire loop. Subsequently, a drop of hydrogen peroxide (Merk) solution (3 %) was placed directly onto the bacterial colony on the slide, and the reaction was observed (Reiner 2010). The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.



2.2.9.4 Sugar fermentation test

A small amount of bacterial culture was inoculated into 1.5 ml phenol red fermentation broth (Sigma) in Eppendorf tubes. The medium is then incubated at 28 °C for 24 hours. Durham tubes were placed inverted in each test sample. After the incubation period, the presence of gas bubbles in the Durham tube, along with a change in the color of the medium was observed (Shoaib et al., 2020).

2.2.9.5 Indole test

To perform the indole test, a tryptophan broth (HiMedia) medium was inoculated with the mucus-isolated bacterial culture, followed by incubation at 28 °C for 24 hours. After allowing sufficient growth, Kovac's reagent (HiMedia) was added to the culture. The reaction between the indole gas, produced through the enzymatic conversion of tryptophan, and the reagent was observed (Shoaib et al., 2020).

2.2.9.6 Sulfur test

A bacterial culture was inoculated into a Zobell marine agar (HiMedia) medium and allowed to grow at 28 °C for 24 hours. Following the incubation period, lead acetate solution (HiMedia) was introduced to a portion of the culture. Color change in media was observed (Shoaib et al., 2020).

2.2.9.7 Urease test

A bacterial culture was inoculated into a urea-containing Zobell marine (HiMedia) medium and allowed to incubate at 28 °C for 24 hours. After the incubation period, the medium was examined for a change in pH, typically indicated by a color shift due to the formation of ammonia (Shoaib et al., 2020).

2.2.9.8 Triple sugar iron test

A bacterial culture was inoculated into a TSI agar (HiMedia) slant, and the medium was incubated at 28 °C for 24 hours. After incubation, the TSI agar slant was examined for various reactions. If the bacteria fermented any of the sugars, acid production occurred,

causing a color change in the medium. The color change was observed. The presence of gas production was indicated by the lifting of the agar (Shoaib et al., 2020).

2.2.9.9 Starch hydrolysis test

A few colonies of the test organism were selected using a sterile loop. A starch plate of Mueller Hinton agar (MHA) (HiMedia) was streaked with the selected colonies in the form of a line across the width of the plate. To maximize efficiency, multiple cultures were tested on a single agar plate, with each represented by a distinct line. The plates were then incubated at 28 °C for 24 hours to allow for optimal growth and interaction between the test organism and the starch medium. After incubation, 2-3 drops of 10 % iodine solution were added directly onto the edge of the colonies. It was kept for 10 minutes, during which the iodine solution interacted with the starch substrate. The plates were read immediately after the addition of iodine, as the blue color tended to fade over time (Shoaib et al., 2020).

2.2.9.10 Methyl red test

The bacterial culture was inoculated into a tube containing a peptone glucose broth (HiMedia). The medium was incubated at 28 °C for 24-48 hours under aerobic conditions. To conduct the test, alpha-naphthol (SRL) and potassium hydroxide (KOH) reagents (HiMedia) were added to the broth. The tube was gently mixed and allowed to stand for at 28 °C for 24 hours. The development of a red color indicated a positive VP test result, suggesting the production of acetoin (Shoaib et al., 2020).

2.2.10 Growth of mucus bacteria on selective media.

Different selective media Cetrimide Agar, EMB agar, Mannitol Salt Agar, MacConkey Agar, Thayer Martin Agar, Bismuth Sulfite Agar, and Bile Esculin Agar (all were of HiMedia) were autoclaved at 121 °C for 20 minutes. TCBS Agar plates were made by keeping the TCBS media in a boiling water bath for 15 minutes by proper mixing every 3 minutes interval. Media were allowed to cool and poured into sterile Petri plates in sterile laminar airflow. Plates were kept for solidification. *Porites* and *Acropora* sp. mucus were diluted in 1:10, 1:100, 1:1000, etc. ratios. The diluted mucus was spread-plated on each selective media in duplicates. Plates were kept in an incubator at 28 °C for 24 hours of

incubation. Similarly, mucus was spread plated on common isolation media Zobell Marine Agar. After 24 hours of incubation, colonies were counted and CFU value per mL was calculated.

2.2.11 Bacterial diversity analysis by molecular studies.

2.2.11.1 DNA extraction from healthy and diseased coral *Acropora* and *Porites* mucus for metagenome analysis.

Isolation of DNA from healthy and PLS diseased mucus *Porites* sp. as well as *Acropora* sp. was carried out by using ZR Fungal/Bacterial DNA (MiniPrep™ kit) with slight modification. In 400 µl coral mucus, 500 µl of lysis buffer was added and mixtures were transferred to the ZR BashingBead™ buffer tube. All the samples were vortexed for 3 minutes. Samples were centrifuged in the ZR BashingBead™ lysis tube (0.1 and 0.5 mm) in a microcentrifuge at 10,000 x *g* for 1 minute. From this mixture, up to 400 µl supernatant was transferred to a Zymo-Spin™ III-F filter in a collection tube and centrifuged at 8,000 x *g* for 1 minute. Into this filtrate, 1,200 µl of genomic lysis buffer was added to the collection tube from the above step. The 800 µl of the mixture from this was transferred to a Zymo-Spin™ IIC Column 3 in a Collection Tube and centrifuged at 10,000 x *g* for 1 minute. The flow-through from the Collection Tube was discarded and the above step was repeated. A 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuged at 10,000 x *g* for 1 minute. A 500 µl gDNA Wash Buffer was added to the Zymo-Spin™ IIC Column and again centrifuged at 10,000 x *g* for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl DNA elution buffer was added directly to the column matrix. Again, centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. Concentrations of DNA were measured on a NanoDrop spectrophotometer (Agilent).

2.2.11.2 16s rRNA gene sequencing

Metagenomics study of *Porites* and *Acropora* healthy and diseased mucus was done using V3-V4 region of 16s amplicon, sequencing was done using an Illumina HiSeq2500 with paired-end chemistry of sequencing depth 2 X 250 bp, OTUs generated using QIIME pipeline to identify healthy and diseased coral mucus. Based on this, relative abundance and

diversity matrices using Chao, Shannon, and Reverse Simpson were used to observe phylum, family, class, order, genus, and species.

2.2.11.3 Bioinformatics and Statistics

Amplicon sequences were analyzed in QIIME2 (versions 2019.10) (Bolyen et al., 2019). The demultiplexed reads were imported into QIIME2 and sequences were quality trimmed, chimera removed and filtered with to length >250 bp using DADA2 (Callahan et al., 2016). The resulting feature tables were rarefied for a core diversity analysis including phylogenetic metrics (unifrac) of the alpha and beta diversity with default settings. Representative sequences were aligned and filtered with mafft (Katoh and Standley, 2013) before a phylogenetic tree was calculated and rooted with a fast tree (Price et al., 2010). Kruskal–Wallis tests (Kruskal and Wallis, 1952) were calculated for alpha diversity metrics to define the significance between categorical metadata columns. Community compositions were linked to environmental variables by bioenv tests (Clarke and Ainsworth, 1993). Taxonomic assignments of representative sequences were conducted through a naive-Bayes classifier trained on 16S rDNA V3-V4 primer and the OTUs were clustered at 99 % similarities within the Greengene 13_8 database. The differential abundance of taxa was determined by gneiss (Morton et al., 2017). Longitudinal analysis (Bokulich et al., 2018) was based on changes in Shannon diversity estimates and weighted unifrac distances using linear mixed-effects modeling (Seabold and Perktold, 2010) and non-parametric microbial interdependence tests (Zhang et al., 2017).

2.3 Results.

2.3.1 Coral health survey

The coral health survey (Fig. 2.2) during the first sample collection (June 2018) revealed distinct health conditions of corals across three selected locations (SL1, SL2, and SL3). The Munnaikad Island (SL1) study site was observed dominant with diseased corals (87 %) along with some healthy corals (13 %). There were no instances of bleached corals or corals dominated by algae in this area (Figs. 2.3 and 2.4a). The Big Manali Island (SL2) was identified as a bleached and algal-dominated region. It dominates corals with algae

(52.08 %) and bleached corals (33.33 %) followed by diseased corals (14.58 %). In the Big Manali location, healthy corals were not observed (Figs. 2.3 and 2.4b). The T. Nagar (SL3) location was observed as a healthy location for corals where almost all corals were free from bleaching and disease (97 %). Algal-dominant corals were not observed in this region. However, the percentage of bleached and diseased corals was very low (2 % and 1 % respectively) (Figs. 2.3 and 2.4c). The second collection (February 2019) depicted a significant improvement in coral health in Big Manali Island and T. Nagar Island (Fig. 2.4b and c). On Big Manali Island, the percentage of bleached corals dropped to zero as algal-dominant corals increased by 35.42 % (Fig. 2.4b). The occurrence of diseased corals increased by 2.08 %. In contrast, on Munnaikad Island, the percentage of bleached corals increased by 18 %, while the occurrence of diseased corals decreased by 18 %. Healthy corals on T. Nagar Island saw a modest increase of 1.5 % (Fig. 2.4c). On T. Nagar Island, there was a 2 % decrease in bleached corals, accompanied by a 0.5 % increase in diseased corals.

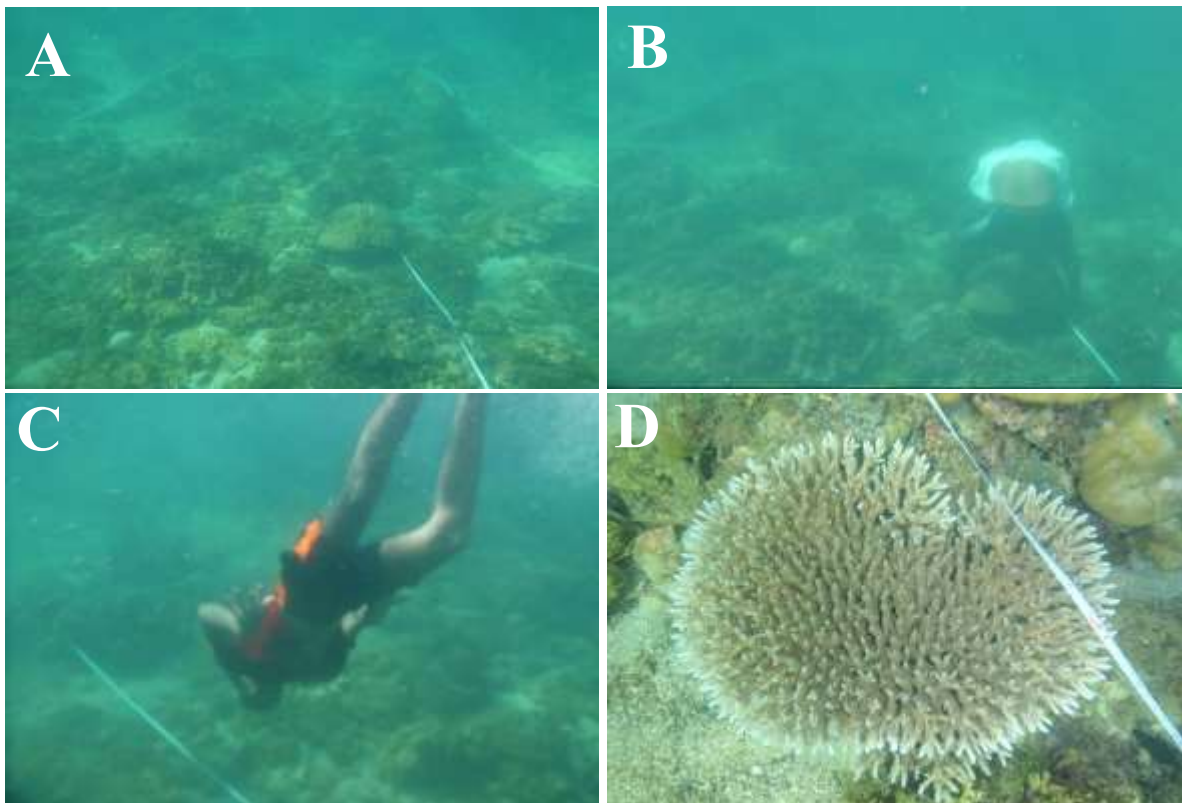


Figure 2.2 The study locations in the Gulf of Mannar regions were marked with 5-meter radius for the observations of disease, healthy and bleached corals. **A.** Munnaikad Island, **B.** Big Manali Island, **C.** T. Nagar Island and **D.** *Acropora* sp. observed in study location.

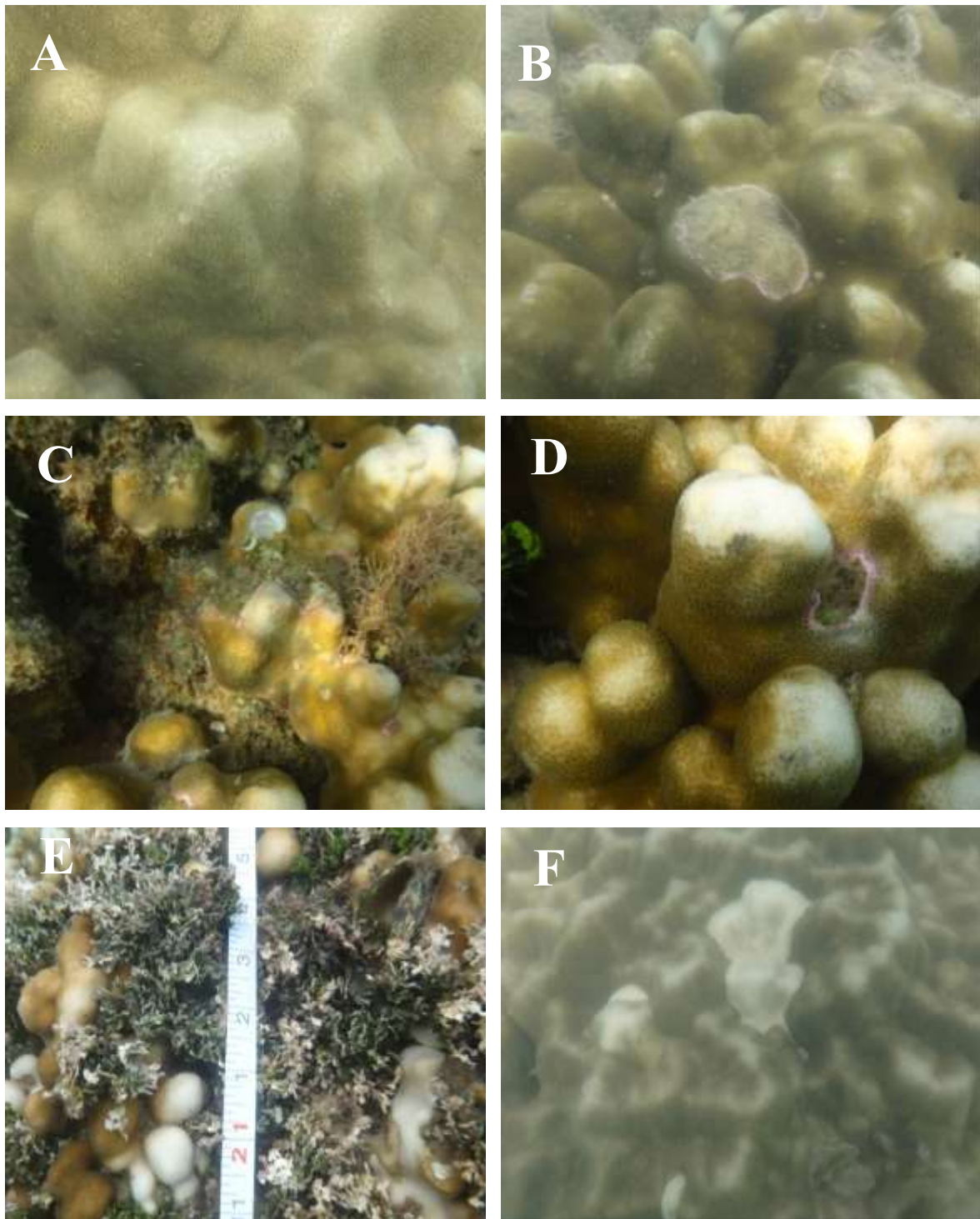


Figure 2.3- Disease and healthy corals of SL1, SL2 and SL3 locations. A. Healthy *Porites* sp., B) Diseased *Porites* sp., C) Algal dominant *Porites* sp., D) Diseased and bleached *Porites* sp., E) Bleached and algal dominated *Porites* sp., F) Bleached *Porites* sp.

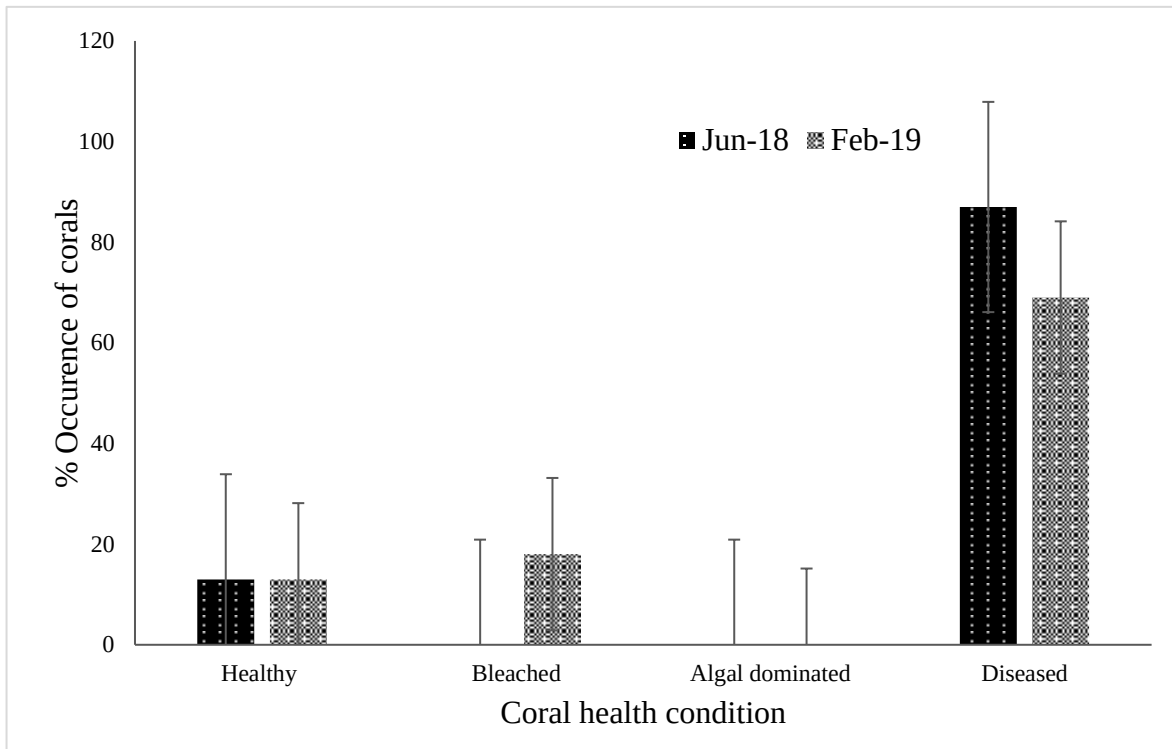


Figure 2.4a- Occurrence of healthy, bleached, algal-dominated, and diseased corals in SL1 location.

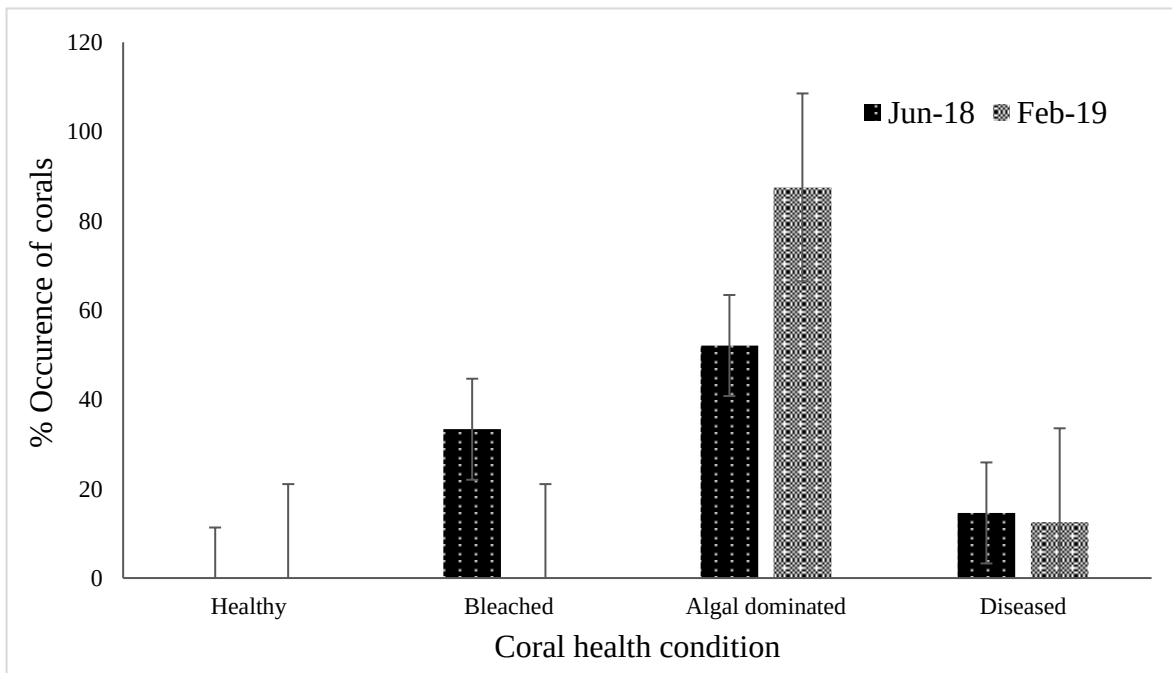


Figure 2.4b- Occurrence of healthy, bleached, algal-dominated, and diseased corals in SL2 location.

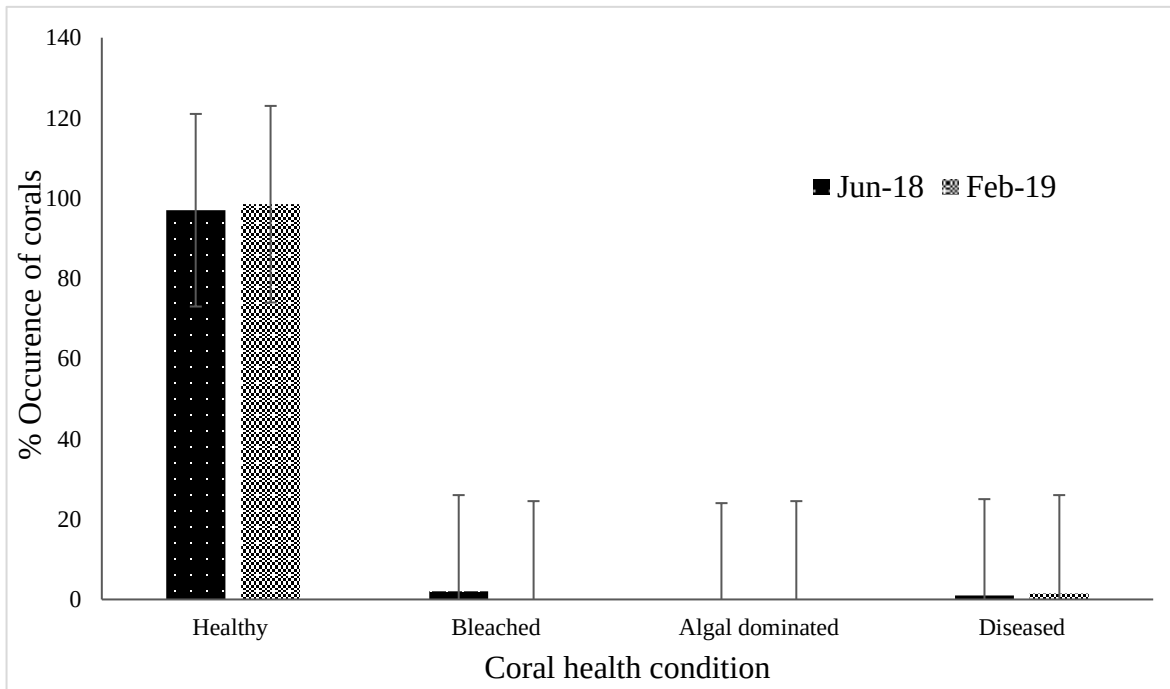


Figure 2.4c- Occurrence of healthy, bleached, algal-dominated, and diseased corals in SL3 location.

2.3.2 pH, temperature, and salinity of the surrounding water.

The parameters such as pH, temperature, and salinity of nearby seawater to coral reefs in the Gulf of Mannar exhibit some variations between June 2018 and February 2019. In June 2018, pH levels remained constant at 7.8 across all locations (Munnaikad Island, Big Manali Island, and T. Nagar Island), while in February 2019, the pH range we observed from 8 to 8.1. In February 2019, at Munnaikad Island, known for its dominance of diseased corals, and Big Manali Island, characterized by bleached and algal-dominant corals, in both locations the pH was observed to be increased by 0.3. Meanwhile, at T. Nagar Island, where healthy corals prevail, the pH increased by 0.2. Temperature fluctuations were noted, with June 2018 recording temperatures ranging between 28 °C and 29 °C, slightly higher than those observed in February 2019, which ranged from 27 °C to 28 °C. Additionally, in February 2019, temperatures were observed to be 1 °C lower across Munnaikad Island, Big Manali Island, and T. Nagar Island. However, salinity levels remained consistent at 3.6 ‰ for both June 2018 and February 2019 collection across all locations, with a slight increase noted during February 2019.

2.3.3 Nutrient levels of seawaters near healthy, diseased, and bleached coral.

Nutrient concentrations of *Porites* sp. nearby water including nitrate, nitrite, ammonia, phosphate, and silicates, were assessed using a nutrient autoanalyzer. In the SL1 location, where bleached, algal dominant, and diseased corals were present, the concentrations of nitrates, nitrite, silica, ammonia, dissolved inorganic nitrogen (DIN), and phosphate were 2.22 $\mu\text{M/L}$, 0.715 $\mu\text{M/L}$, 7.75 $\mu\text{M/L}$, and 1.78 $\mu\text{M/L}$, 5.183 $\mu\text{M/L}$, 0.52 $\mu\text{M/L}$ respectively (Figs. 2.5).

The SL2 location, housing healthy, bleached, and diseased corals was observed. Nutrient concentration was measured. The concentrations of nitrates, nitrite, silica, ammonia, dissolved inorganic nitrogen (DIN), and phosphate were 3.13 $\mu\text{M/L}$, 0.05 $\mu\text{M/L}$, 11.06 $\mu\text{M/L}$, and 1.005 $\mu\text{M/L}$, 6.855 $\mu\text{M/L}$, 1.245 $\mu\text{M/L}$ respectively (Figs. 2.5).

In the SL3 location, healthy, bleached, and diseased corals were observed. A maximum healthy coral percentage was observed in this location. The concentrations of nitrates, nitrite, silica, ammonia, dissolved inorganic nitrogen (DIN), and phosphate near *Porites* sp. water were 2.99 $\mu\text{M/L}$, 0.75 $\mu\text{M/L}$, 12.42 $\mu\text{M/L}$, and 0.91 $\mu\text{M/L}$, 4.655 $\mu\text{M/L}$, 0.225 $\mu\text{M/L}$ respectively (Figs. 2.5).

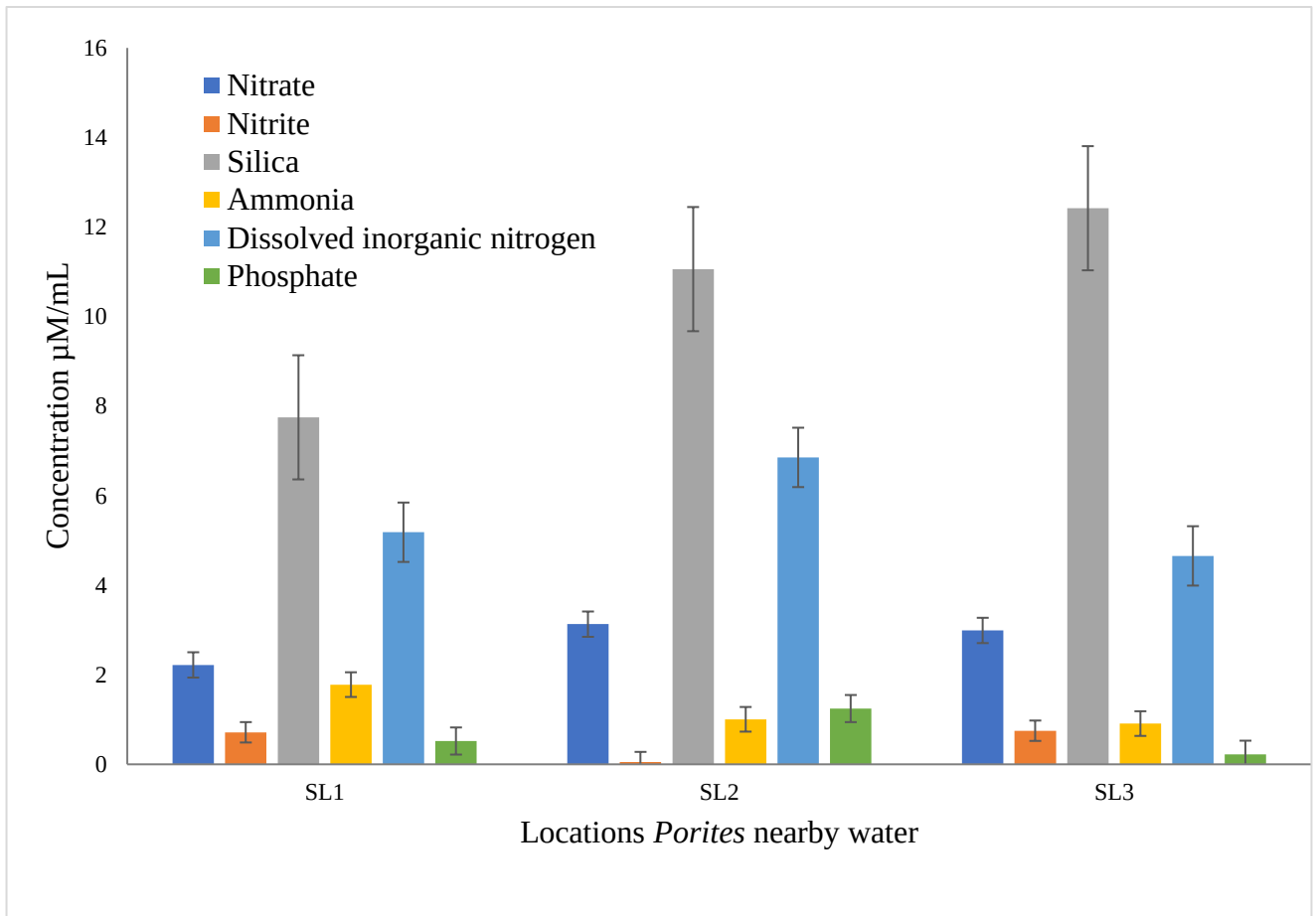


Figure 2.5- Nutrient concentrations in healthy, bleached, and diseased *Porites* nearby water in SL1- Bleached and algal dominant location, SL2- Diseased coral location, and SL3- Healthy coral location.

2.3.4 Total carbon in coral sediment.

In the SL1 location, carbon concentrations in coral-adjacent sediment were measured, revealing percentages of 8.34 % for bleached corals, 8.72 % for diseased corals, and 11.65 % for healthy corals (Fig. 2.6). Moving to the SL2 location, the carbon concentrations were 11.08 % for bleached corals, 11.52 % for diseased corals, and healthy corals were absent in this location. In the SL3 location, healthy corals exhibited a carbon concentration of 8.67 %, bleached corals had 10.18 %, and diseased corals showed 8.57 % (Fig. 2.6).

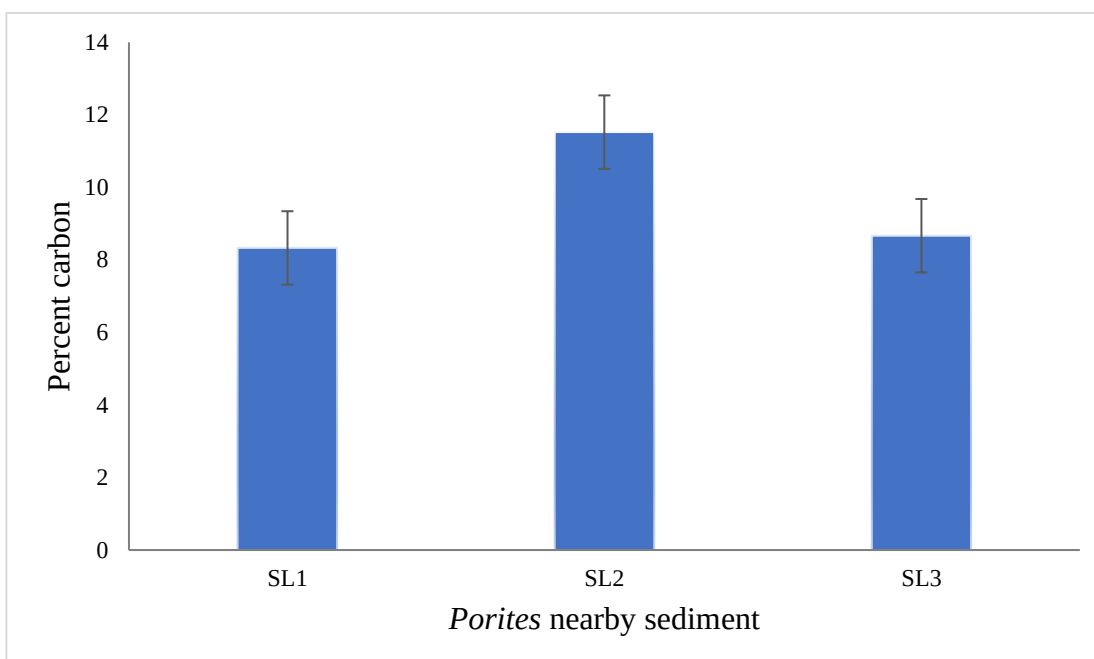


Figure 2.6- Carbon concentration from nearby sediment of healthy, bleached, and diseased *Porites* sp.

2.3.5 Dissolved oxygen, and bacterial count.

In the seawater surrounding coral habitats, the dissolved oxygen (DO) concentration was found to be highest in healthy coral locations i.e., T. Nagar Island followed by bleached and algal dominant location (Big Manali Island) (Fig. 2.7). Diseased coral location (Munnaikad Island) showed comparatively lowest concentration of DO (Fig. 2.7). The bacterial colony forming unit (CFU) values were assessed in different locations to understand the heterotrophic bacterial dynamics in the seawater surrounding coral habitats. Bacterial abundance in nearby coral water of all three locations studied was found to be inversely proportional to the dissolved oxygen concentration (Figs. 2.7). Highest bacterial count was observed in diseased coral surrounding water (Munnaikad Island), followed by bleached-algal dominant location sea water (Big Manali Island). Comparatively lowest bacterial count was observed in healthy coral seawater (T. Nagar Island) (Figs. 2.7).

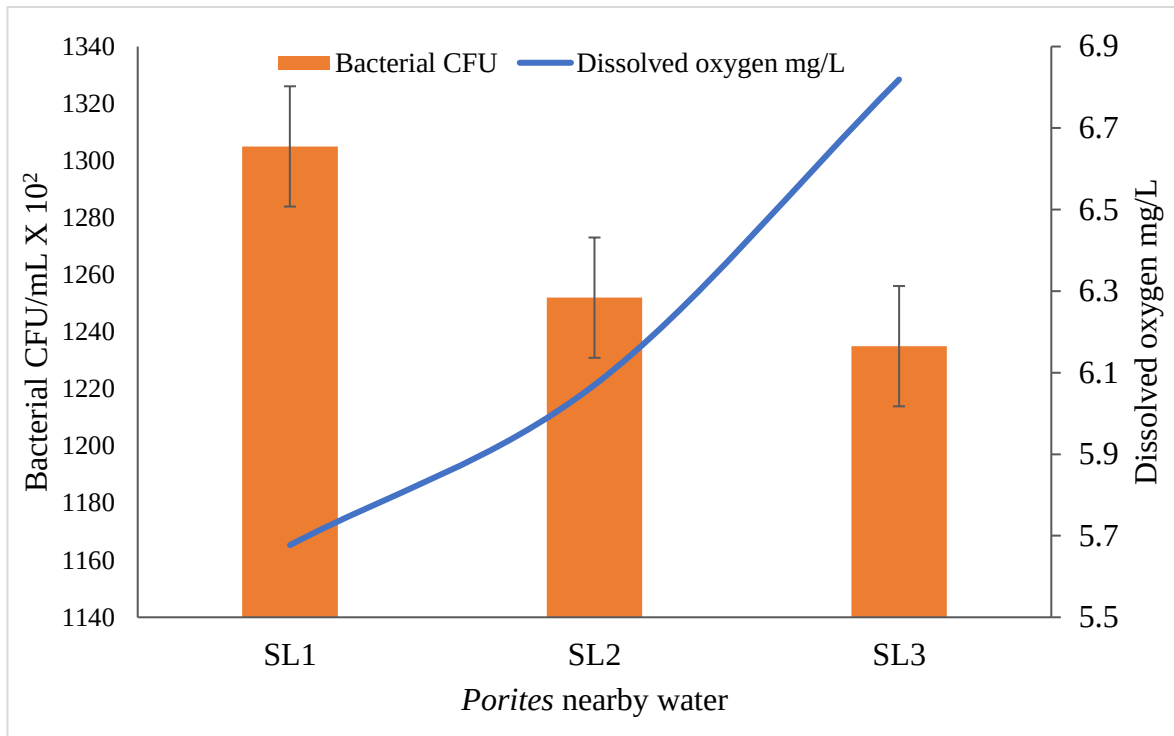


Figure 2.7- Relation between dissolved oxygen concentration and bacterial abundance from nearby healthy, bleached, and diseased *Porites* water.

2.3.6 Influence of nutrients and physical parameters of surrounding water on coral health

Canonical Correspondence Analysis (CCA) was conducted to examine the influence of nutrients such as nitrate, nitrite, ammonia, silica, phosphate, carbon, dissolved inorganic nitrogen, and surface water temperature on coral health. The bleached state of corals exhibits a positive correlation with elevated bacterial Colony Forming Units (CFU), increased temperature, and higher silica and ammonia concentrations, indicating that these factors are linked to coral bleaching. Silica, ammonia, and carbon show a positive association with the dominance of algae on corals, suggesting that higher levels of these components contribute to the prevalence of algae. Conversely, the diseased condition of corals is positively associated with Nitrate, Nitrite, Dissolved Inorganic Nitrogen (DIN), Phosphate, and Dissolved Oxygen, suggesting that elevated levels of these factors are indicative of coral disease. Interestingly, there is also an impact of Nitrate, Nitrite, DIN, Phosphate, and

Dissolved Oxygen on the healthy status of corals, suggesting these factors play a modest role in determining coral health without being the primary drivers (Fig. 2.8).

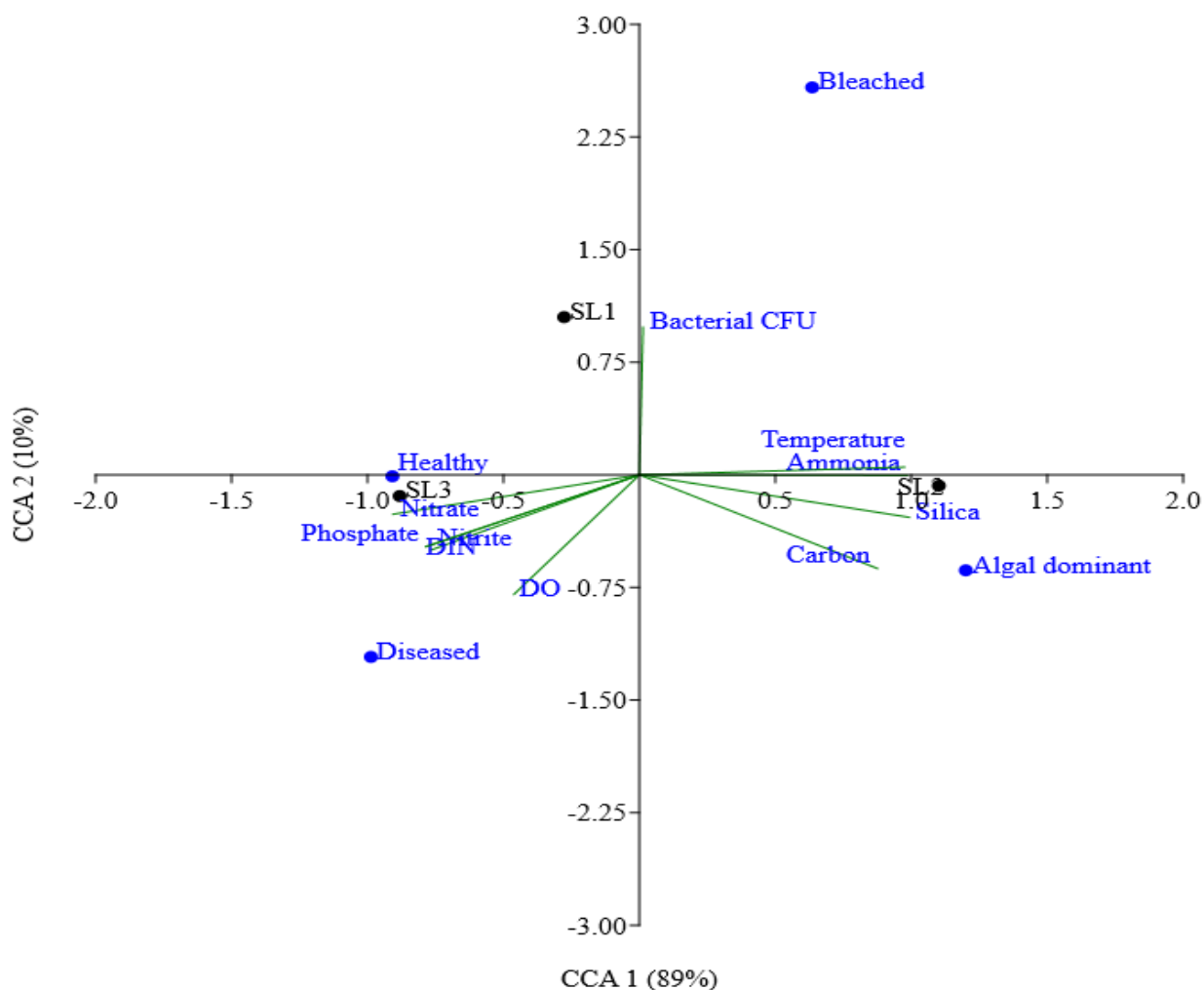


Figure 2.8- Canonical correspondence analysis (CCA) ordination plot of dominant bacterial and fungal genera and environmental variables temperature, salinity, dissolved oxygen (DO), and depth.

2.3.7 Mucus-associated bacteria

The dilution of mucus at $1:10^4$ showed isolated colonies on agar plates (Fig. 2.9). Colonies were counted of each healthy and diseased *Acropora* and *Porites* mucus and CFU/mL was calculated (Fig. 2.10). In both sample collections, algal-dominated *Acropora* mucus showed the highest bacterial counts. The remaining mucus samples showed slightly

similar bacterial counts among each other but as compared to *Acropora* mucus, *Porites* mucus possesses a higher load of bacteria. Based on colony characteristics (morphological and physical) distinct bacterial colonies have been isolated from the mucus of *Acropora* sp. (healthy- 132 colonies June 2018, 98 colonies February 2019, and white patch disease- 141 colonies June 2018, 107 colonies February 2019) and *Porites* sp. bacterial colonies (Healthy- 128 colonies June 2018, 87 colonies February 2019 and pink line syndrome disease-134 colonies June 2018, 93 colonies February 2019) (Fig. 2.11). Primary identification was done by morphology and biochemical characteristics of bacteria by using various biochemical tests and they were characterized in various genera (Figs. 2.12-2.21). The obtained results from these tests were used for segregating the bacteria into different groups based on their biochemical and morphological colony characteristics. Bergen's manual was used for the further characterization of bacteria at the genus level.

In June 2018, various pathogenic bacteria were identified in coral mucus, as detailed in (Table 2.2). *Staphylococcus*, along with *Enterococcus* sp., *Methylococcus* sp., *Stenotrophomonas* sp., and *Moraxella* sp., were exclusively present in both healthy and PLS-diseased *Porites* mucus, except *Staphylococcus* which was observed in white patch *Acropora* disease mucus as well. *Psychrobacter* sp. and *Phenylobacterium* sp. were exclusively associated with healthy and white patch-diseased *Acropora* mucus. *Acinetobacter* sp. was exclusively found in healthy *Porites* mucus. *Thiotrix* sp. was detected in PLS disease *Porites*, healthy *Acropora*, and white patch *Acropora* disease.

In February 2019, a repeat collection revealed that *Staphylococcus* sp. and *Moraxella* sp. were again found exclusively in *Porites* healthy and PLS disease mucus, mirroring the results from June 2018. Additionally, *Pseudomonas* sp. was associated with both healthy and PLS disease *Porites* mucus. The *Cyanobacteria* genus was exclusive to PLS disease, while *Phormidium* was found only in the diseased mucus of both *Acropora* and *Porites*. *Pseudomonas* sp. and *Pseudoalteromonas* sp. were present in all mucus types except for healthy *Porites* mucus. *Psychrobacter* sp. was absent only in healthy *Porites* mucus, while *Phenylobacterium* sp. was exclusively found in *Acropora* mucus (both healthy and PLS disease). *Acinetobacter* sp. was observed exclusively in healthy mucus (both *Acropora* and *Porites*). *Thiothrix* sp. was specifically found in white patch *Acropora* disease (Table 2.3).

However, among two different collections *Methylococcus*, *Enterobacter*, *Brucella*, *Erwinia*, *Serratia*, and *Flavobacteria* diversity was present only in the June 2018 collections mucus *Citrobacter*, *Acrobacter*, *Sulfobacter*, and *Aurantimonas* were present only in the mucus of the February 2019 collection.

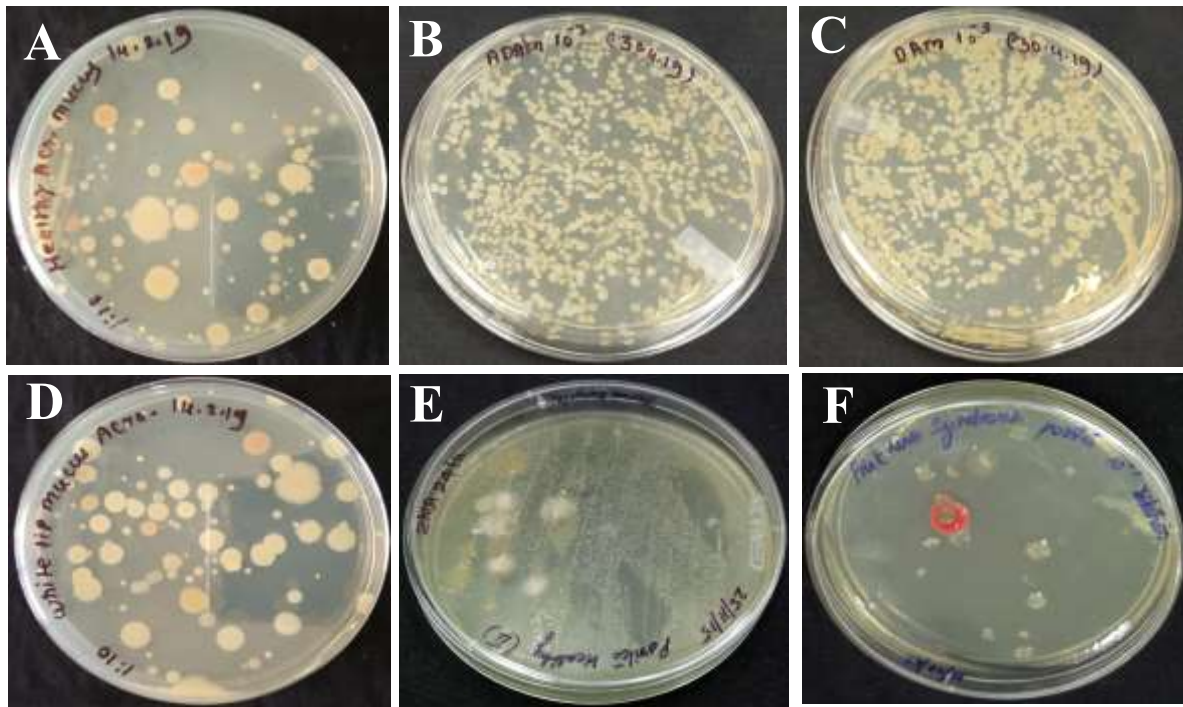


Figure 2.9- Different healthy and diseased coral mucus dilutions were inoculated on Zobell marine agar (ZMA) plates to isolate the bacteria from mucus and incubated at 25 °C temperature. **A.** Healthy *Acropora* mucus showed isolated colonies at 10^{-1} dilution whereas **B.** and **C.** diseased *Acropora* mucus at 10^{-3} , while **D.** White tip *Acropora* mucus **E.** healthy *Porites* mucus and **F.** Pink line syndrome *Porites* mucus showed isolated colonies at dilution 10^{-1} .

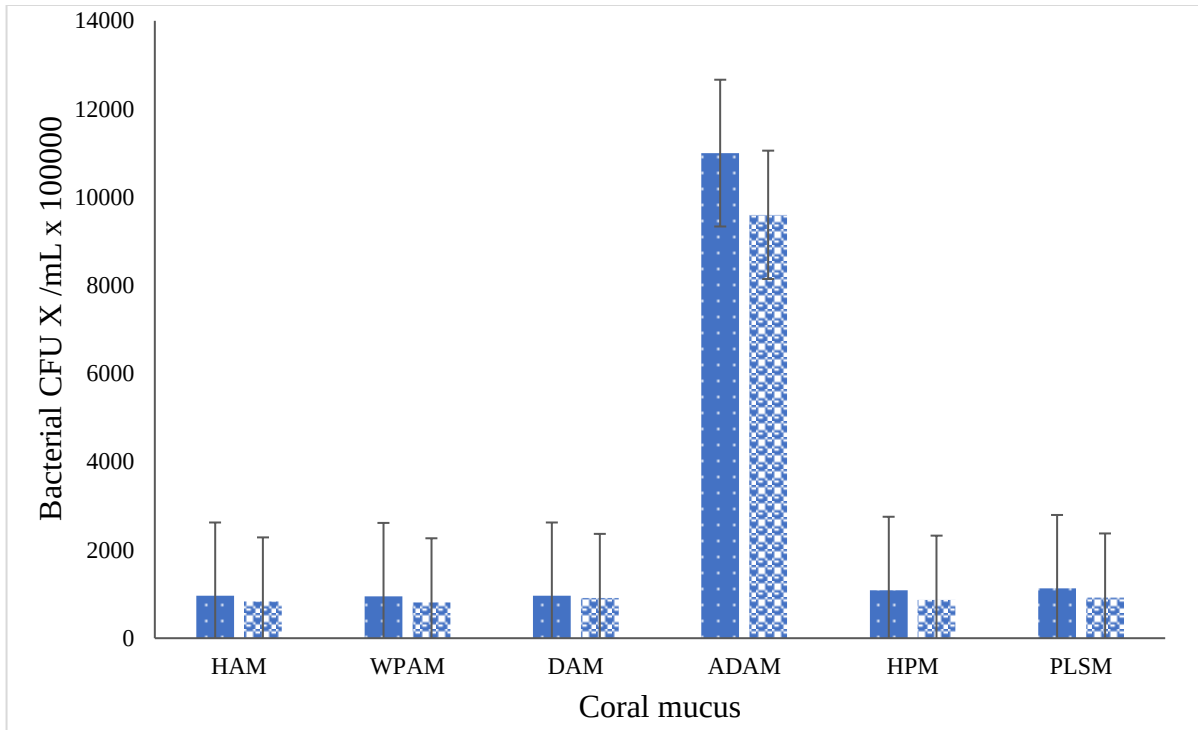


Figure 2.10- Occurrence of heterotrophic bacteria (ZMA agar) in different healthy and diseased mucus samples. HAM-healthy *Acropora* mucus, WPAM-white patch *Acropora* mucus, DAM-dead *Acropora* mucus, ADAM- algal dominated *Acropora* mucus, HPM-healthy *Porites* mucus, PLSPM-Pink line syndrome *Porites* mucus.

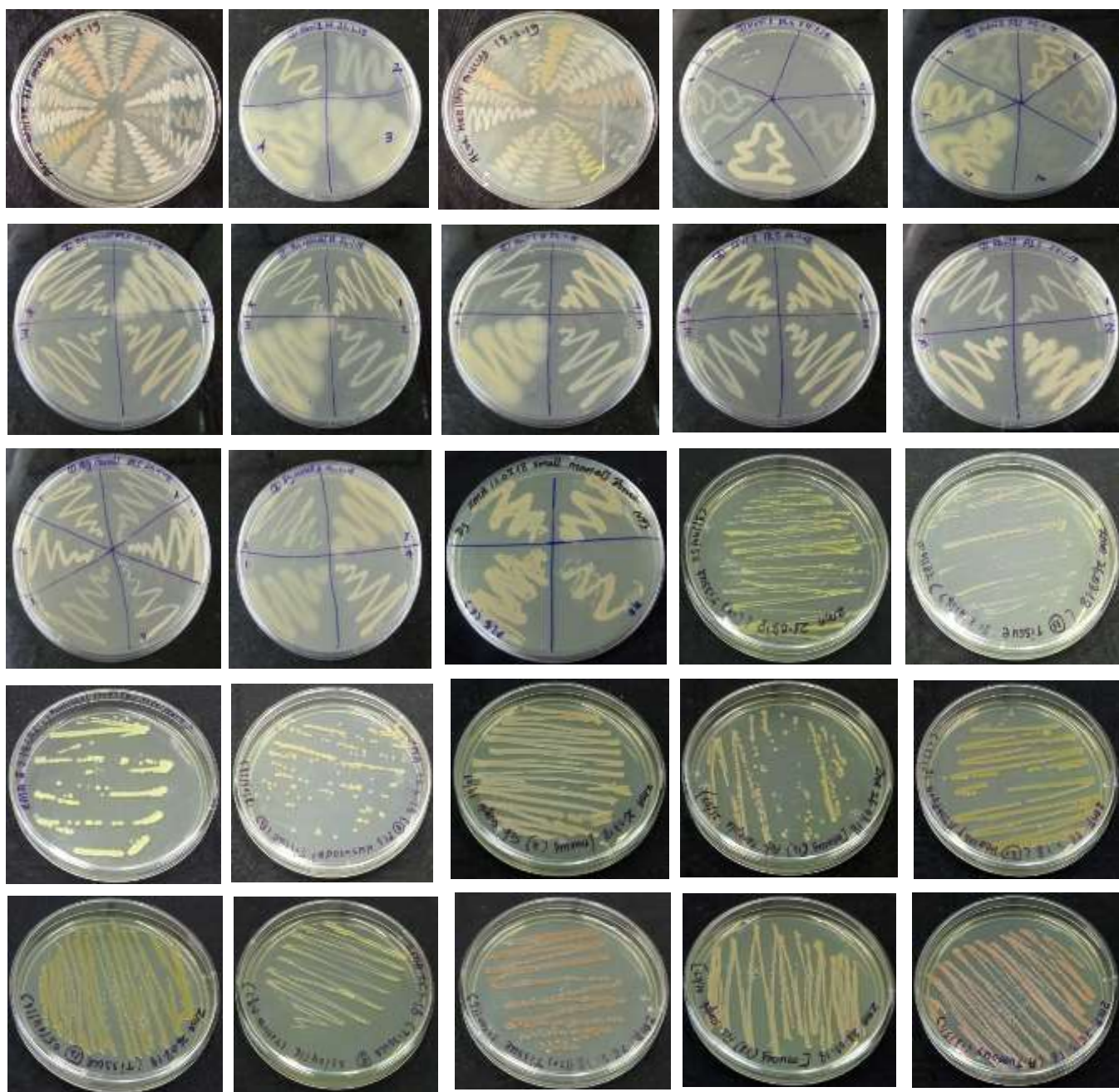


Figure 2.11- Individual separate colonies were picked without contamination and streaked on Zobell marine agar (ZMA) plates and incubated at 25 °C temperature for the bacterial growth.



Figure 2.12- Catalase test: Catalase positive and negative properties of healthy and disease mucus bacterial isolates.



Figure 2.13- Oxidase test: Oxidase positive and negative properties of healthy and Disease mucus bacterial isolates.

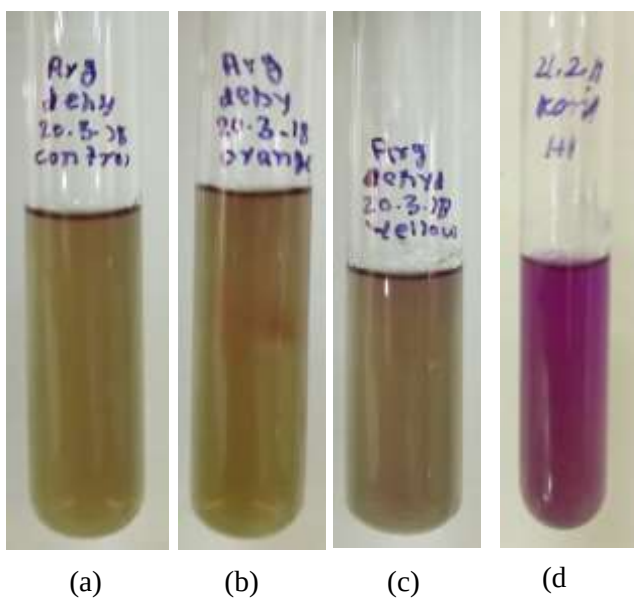


Figure 2.14- Arginine dehydrolase test.

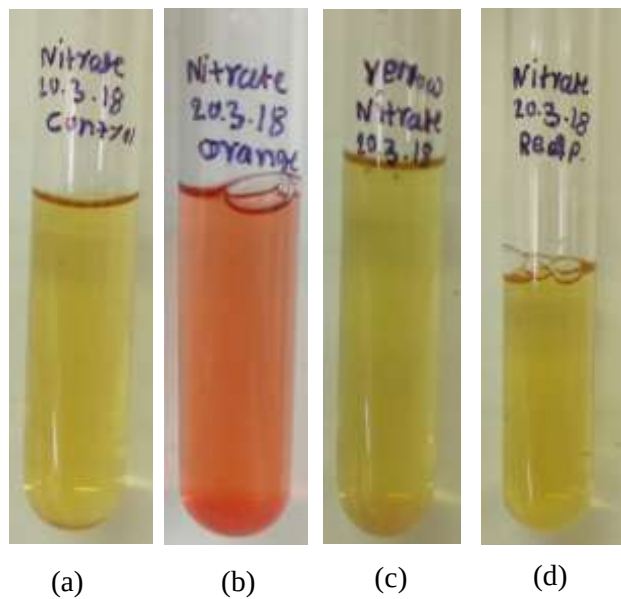


Fig 2.15- Nitrate reduction test.

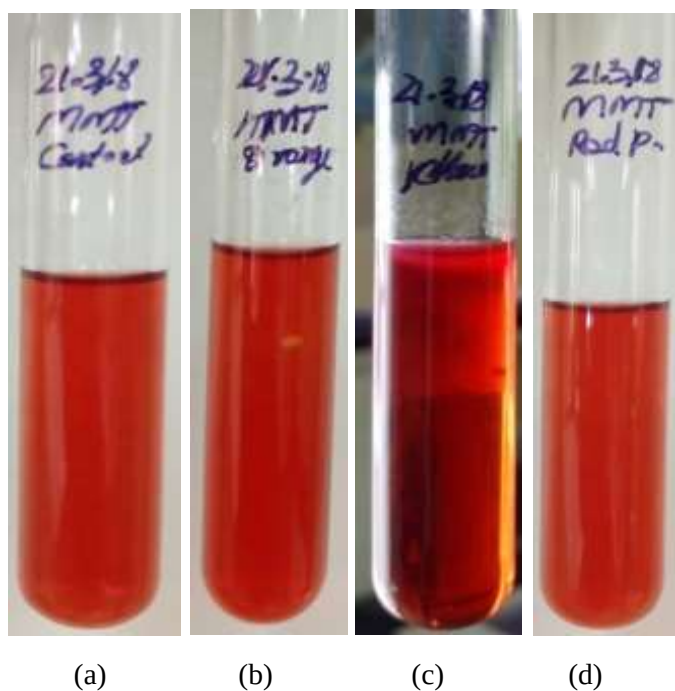


Figure 2.16- Mannitol motility test.

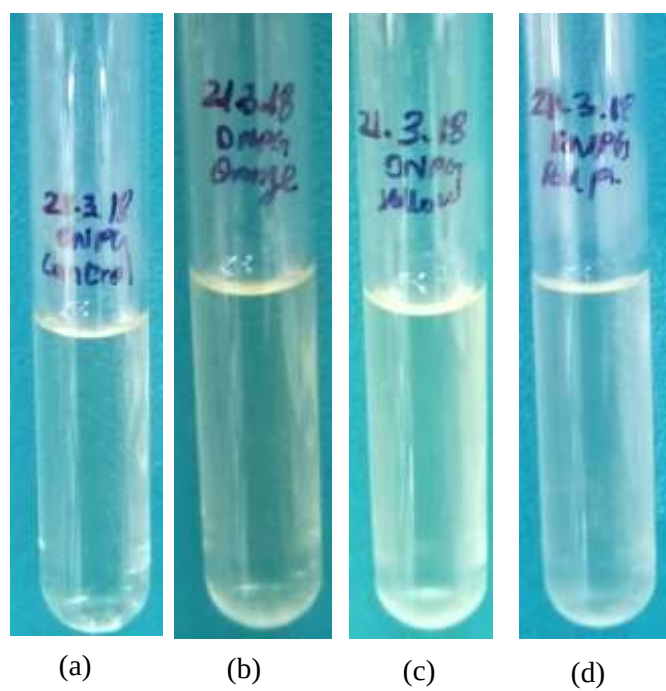


Figure 2.17- ONPG test.

Figures 2.14-2.17 *(a): Control without adding any bacterial strain, (b,c,d): bacteria for positive and negative reactions of the tests.

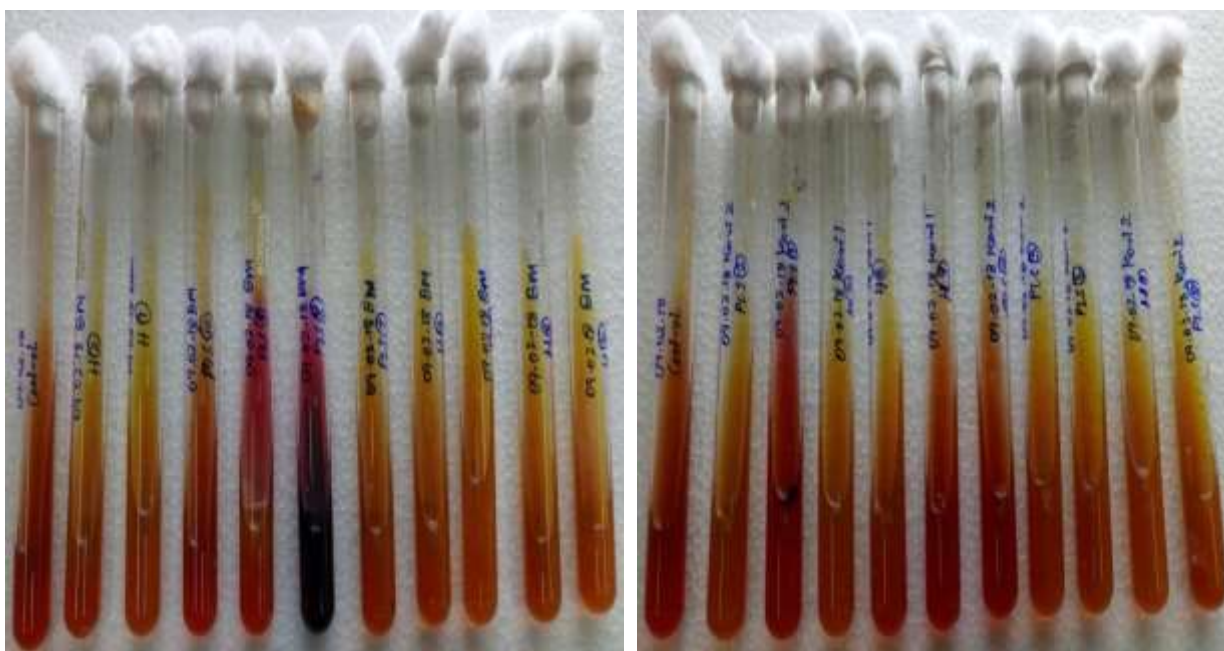


Figure 2.18- Triple Sugar Iron (TSI) fermentation test on *Porites* PLS and healthy mucus.

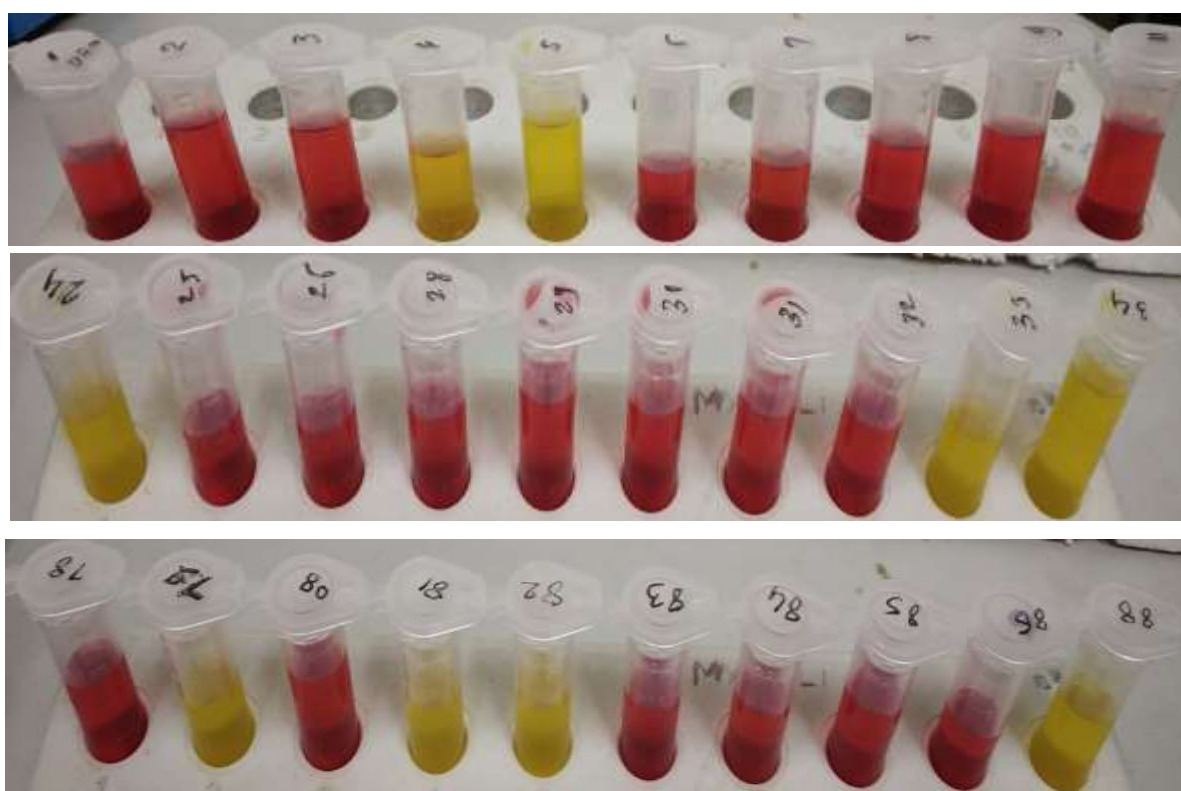


Figure 2.19- Glucose Fermentation test of bacterial isolates of healthy and diseased mucus.

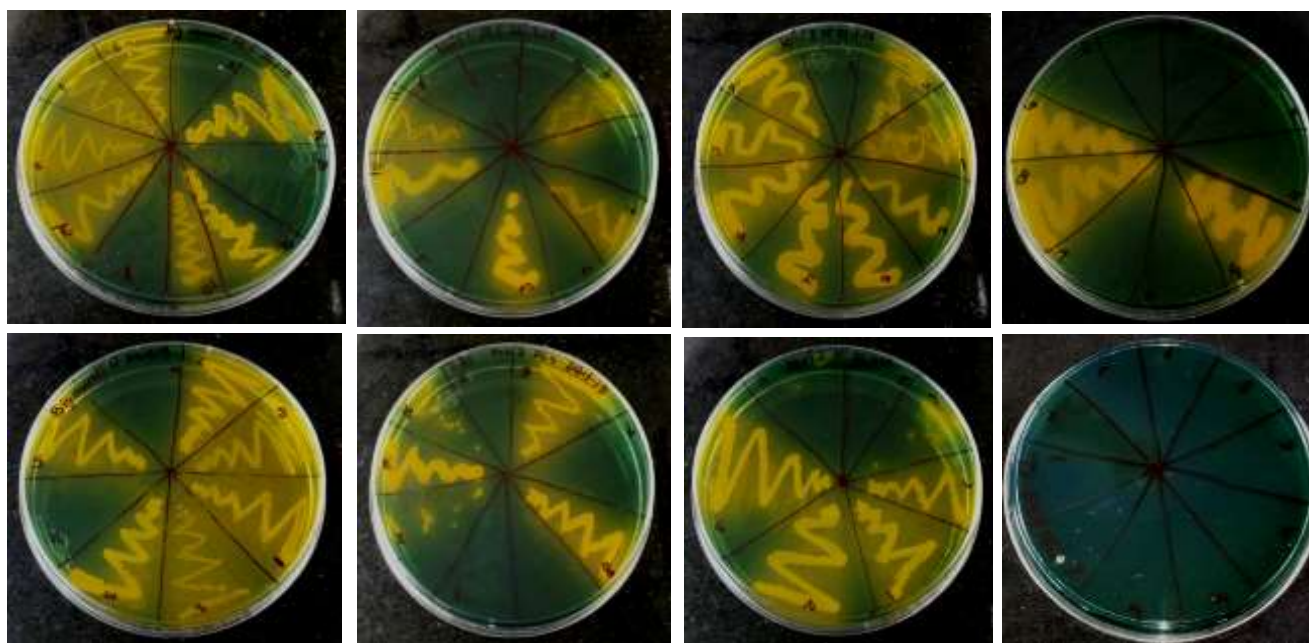


Figure 2.20- *Vibrio* sp. isolation using TCBS- *Vibrio* sp. of diseased, bleached and healthy mucus was isolated using TCBS media.



Figure 2.21- Mannitol salt agar test – Isolation and identification of *Staphylococcus aureus* from the disease and healthy mucus using mannitol salt agar test. (*S. aureus* ferments mannitol and produces yellow-colored colonies surrounded by yellow zones. Coagulase-negative strains of *S. aureus* are usually mannitol non-fermenters and therefore produce pink to red colonies surrounded by red-purple zones).

Table 2.2- Bacterial isolates identified through biochemical characteristics from healthy and diseased, *Porites* and *Acropora* sp. mucus in June 2018.

Healthy <i>Porites</i>	PLS <i>Porites</i>	Healthy <i>Acropora</i>	White patch <i>Acropora</i>
<i>Actinomyces</i>	<i>Actinomyces</i>	<i>Actinomyces</i>	<i>Actinomyces</i>
<i>Vibrio</i>	<i>Vibrio</i>	<i>Vibrio</i>	<i>Vibrio</i>
<i>Micrococcus</i>	<i>Micrococcus</i>	<i>Micrococcus</i>	<i>Micrococcus</i>
<i>Staphylococcus</i>	<i>Staphylococcus</i>	-	<i>Staphylococcus</i>
<i>Neisseria</i>	<i>Neisseria</i>	<i>Neisseria</i>	<i>Neisseria</i>
<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>
<i>Enterococcus</i>	<i>Enterococcus</i>	-	-
<i>Methylococcus</i>	<i>Methylococcus</i>	-	-
<i>Halomonas</i>	<i>Halomonas</i>	<i>Halomonas</i>	<i>Halomonas</i>
<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>
<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas</i>
<i>Enterococcus</i>	<i>Enterococcus</i>	<i>Enterococcus</i>	<i>Enterococcus</i>
<i>Paracoccus</i>	<i>Paracoccus</i>	<i>Paracoccus</i>	<i>Paracoccus</i>
<i>Moraxella</i>	<i>Moraxella</i>	-	-
<i>Yersinia</i>	<i>Yersinia</i>	<i>Yersinia</i>	<i>Yersinia</i>
-	-	<i>Psychrobacter</i>	<i>Psychrobacter</i>
-	-	<i>Phenylobacterium</i>	<i>Phenylobacterium</i>
<i>Corynebacterium</i>	<i>Corynebacterium</i>	<i>Corynebacterium</i>	<i>Corynebacterium</i>
<i>Klebsiella</i>	<i>Klebsiella</i>	<i>Klebsiella</i>	<i>Klebsiella</i>
<i>Enterobacter</i>	<i>Enterobacter</i>	<i>Enterobacter</i>	<i>Enterobacter</i>
<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>
<i>Streptomyces</i>	<i>Streptomyces</i>	<i>Streptomyces</i>	<i>Streptomyces</i>
<i>Roseobacter</i>	<i>Roseobacter</i>	<i>Roseobacter</i>	<i>Roseobacter</i>
<i>Alteromonas</i>	<i>Alteromonas</i>	<i>Alteromonas</i>	<i>Alteromonas</i>
<i>Brucella</i>	<i>Brucella</i>	<i>Brucella</i>	<i>Brucella</i>
<i>Phormidium</i>	<i>Phormidium</i>	<i>Phormidium</i>	<i>Phormidium</i>
<i>Aeromonas</i>	<i>Aeromonas</i>	<i>Aeromonas</i>	<i>Aeromonas</i>

<i>Erwinia</i>	<i>Erwinia</i>	<i>Erwinia</i>	<i>Erwinia</i>
<i>Serratia</i>	<i>Serratia</i>	<i>Serratia</i>	<i>Serratia</i>
<i>Stenotrophomonas</i>	<i>Stenotrophomonas</i>	-	-
<i>Acinetobacter</i>	-	-	-
<i>Cyanobacteria</i>	<i>Cyanobacteria</i>	<i>Cyanobacteria</i>	<i>Cyanobacteria</i>
<i>Flavobacteria</i>	<i>Flavobacteria</i>	<i>Flavobacteria</i>	<i>Flavobacteria</i>
-	<i>Thiothrix</i>	<i>Thiothrix</i>	<i>Thiothrix</i>

Table 2.3- Bacterial isolates identified through biochemical characteristics from healthy and diseased, *Porites* and *Acropora* sp. mucus in February 2019.

Healthy <i>Porites</i>	PLS <i>Porites</i>	Healthy <i>Acropora</i>	White patch <i>Acropora</i>
<i>Actinomycetes</i>	<i>Actinomycetes</i>	<i>Actinomycetes</i>	<i>Actinomycetes</i>
<i>Vibrio</i>	<i>Vibrio</i>	<i>Vibrio</i>	<i>Vibrio</i>
<i>Staphylococcus</i>	<i>Staphylococcus</i>	-	-
<i>Neisseria</i>	<i>Neisseria</i>	<i>Neisseria</i>	<i>Neisseria</i>
<i>Halomonas</i>	<i>Halomonas</i>	<i>Halomonas</i>	<i>Halomonas</i>
<i>Pseudomonas</i>	<i>Pseudomonas</i>	-	<i>Pseudomonas</i>
<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>
<i>Enterococcus</i>	<i>Enterococcus</i>	-	-
<i>Micrococcus</i>	<i>Micrococcus</i>	<i>Micrococcus</i>	<i>Micrococcus</i>
-	<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas</i>	<i>Pseudoalteromonas</i>
<i>Paracoccus</i>	<i>Paracoccus</i>	<i>Paracoccus</i>	<i>Paracoccus</i>
<i>Moraxella</i>	<i>Moraxella</i>	-	-
<i>Yersinia</i>	<i>Yersinia</i>	<i>Yersinia</i>	<i>Yersinia</i>
-	<i>Psychrobacter</i>	<i>Psychrobacter</i>	<i>Psychrobacter</i>
-	-	<i>Phenylobacterium</i>	<i>Phenylobacterium</i>
<i>Corynebacterium</i>	<i>Corynebacterium</i>	<i>Corynebacterium</i>	<i>Corynebacterium</i>
<i>Klebsiella</i>	<i>Klebsiella</i>	<i>Klebsiella</i>	<i>Klebsiella</i>
<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>
<i>Streptomyces</i>	<i>Streptomyces</i>	<i>Streptomyces</i>	<i>Streptomyces</i>

<i>Roseobacter</i>	<i>Roseobacter</i>	<i>Roseobacter</i>	<i>Roseobacter</i>
<i>Alteromonas</i>	<i>Alteromonas</i>	<i>Alteromonas</i>	<i>Alteromonas</i>
-	<i>Phormidium</i>	-	<i>Phormidium</i>
<i>Aeromonas</i>	<i>Aeromonas</i>	<i>Aeromonas</i>	<i>Aeromonas</i>
<i>Citrobacter</i>	<i>Citrobacter</i>	<i>Citrobacter</i>	<i>Citrobacter</i>
<i>Arcobacter</i>	<i>Arcobacter</i>	<i>Arcobacter</i>	<i>Arcobacter</i>
<i>Sulfitobacter</i>	<i>Sulfitobacter</i>	<i>Sulfitobacter</i>	<i>Sulfitobacter</i>
<i>Stenotrophomonas</i>	<i>Stenotrophomonas</i>	-	-
<i>Acinetobacter</i>	-	<i>Acinetobacter</i>	-
-	<i>Cyanobacteria</i>	-	-
<i>Aurantimonas</i>	<i>Aurantimonas</i>	<i>Aurantimonas</i>	<i>Aurantimonas</i>
-	-	-	<i>Thiothrix</i>

2.3.8 Growth of coral mucus bacteria on different selective media.

In the June 2018 dataset, our findings revealed that MacConkey agar ADAM exhibited the highest coliform growth, followed by WPAM and HAM. In the context of *Porites* mucus, HPM demonstrated greater coliform growth compared to PLSM. *Acropora* mucus displayed 0.5-2-fold more *Coliforms* than *Porites* mucus (Fig. 2.22). *Salmonella* sp. exhibited a significantly higher presence in ADAM mucus, surpassing healthy and diseased mucus by 6-8-fold (Fig. 2.23). Thayer Martin agar showed increased *Neisseria* occurrence in healthy mucus and algal-dominant *Acropora* mucus (Fig. 2.24). Similarly, Mannitol salt agar indicated maximum occurrence in healthy and algal-dominant mucus.

All mucus samples on Mannitol salt agar displayed growth, with a more supportive environment for healthy mucus and algal-dominant *Acropora* mucus compared to diseased mucus (Fig. 2.25). Bile esculin agar identified *Enterococcus* exclusively in algal-dominant *Acropora* mucus for *Acropora*, while *Enterococcus* was present in both healthy and diseased *Porites* mucus (Fig. 2.26). *Vibrio* sp. exhibited the highest abundance on TCBS media compared to other specific media, with a higher presence in diseased mucus than in healthy mucus (Fig. 2.27).

By February 2019, the occurrence of bacteria grown on specific media had reduced compared to the June 2018 season. *Neisseria* sp. displayed a slightly increased number in PLSM (Fig. 2.24). The abundance of Mannitol fermenting bacteria remained similar to the earlier collection, with even higher levels in white tip *Acropora* diseased mucus (WTAM) (Fig. 2.25).

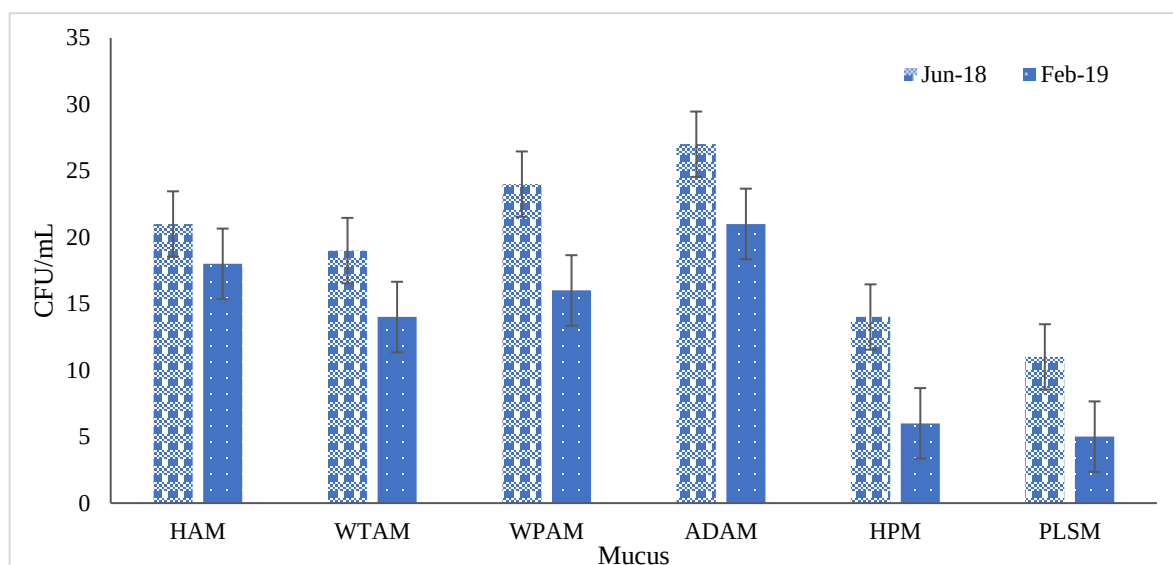


Figure 2.22- Occurrence of *Coliforms* (MacConkey's agar) bacteria in different healthy and diseased mucus samples.

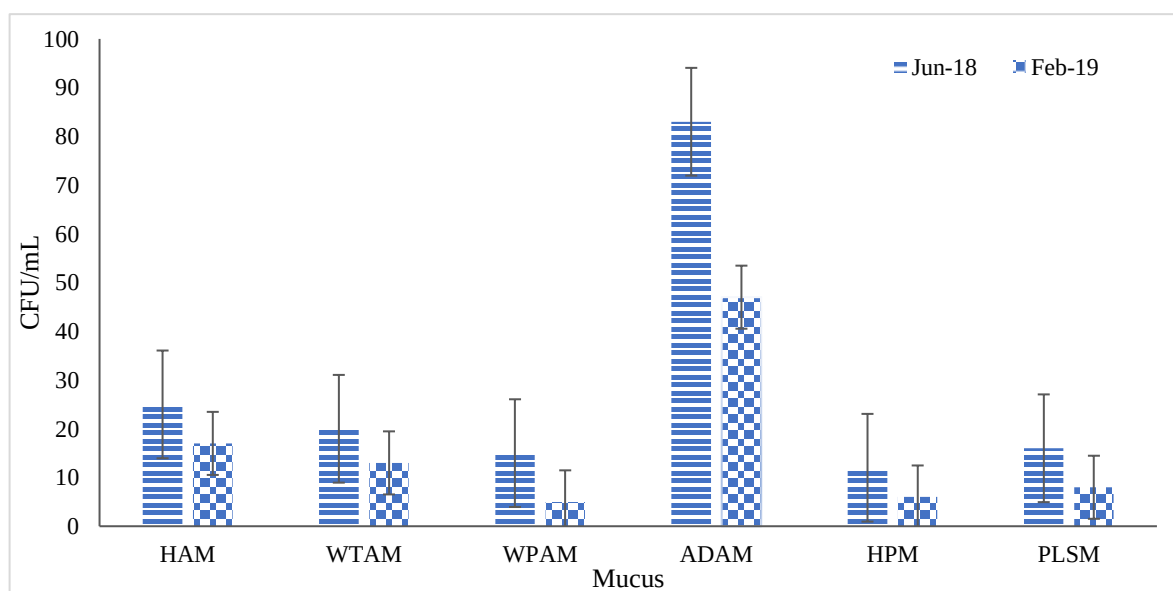


Figure 2.23- Occurrence of *Salmonella* (Bismuth sulfite agar) in different healthy and diseased mucus samples.

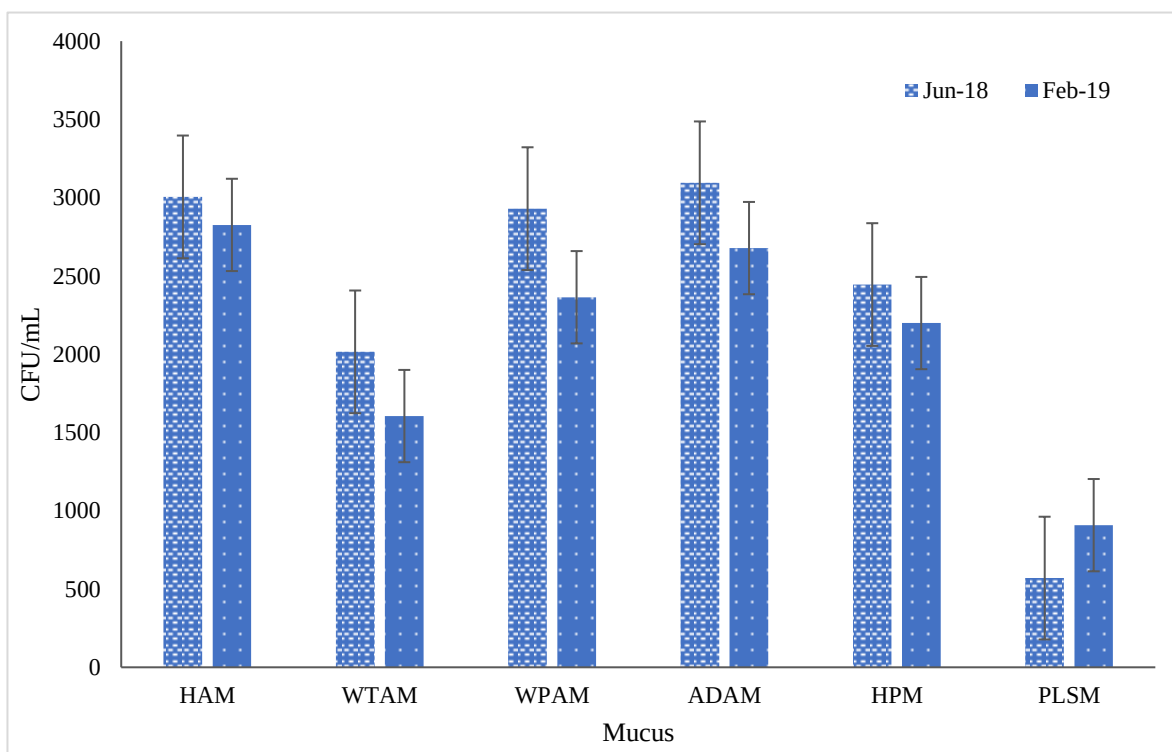


Figure 2.24- Occurrence of *Neisseria* (Thayer martin agar) in different healthy and diseased mucus samples.

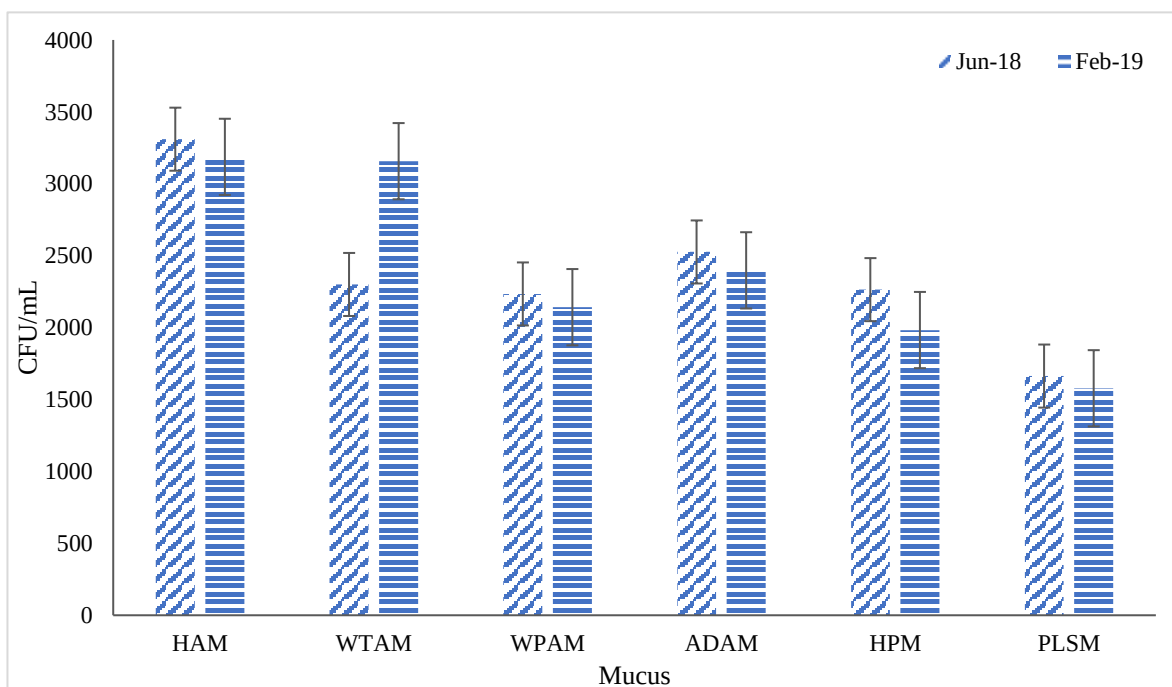


Figure 2.25- Occurrence of gram-positive cocci and mannitol fermenter in different healthy and diseased mucus samples.

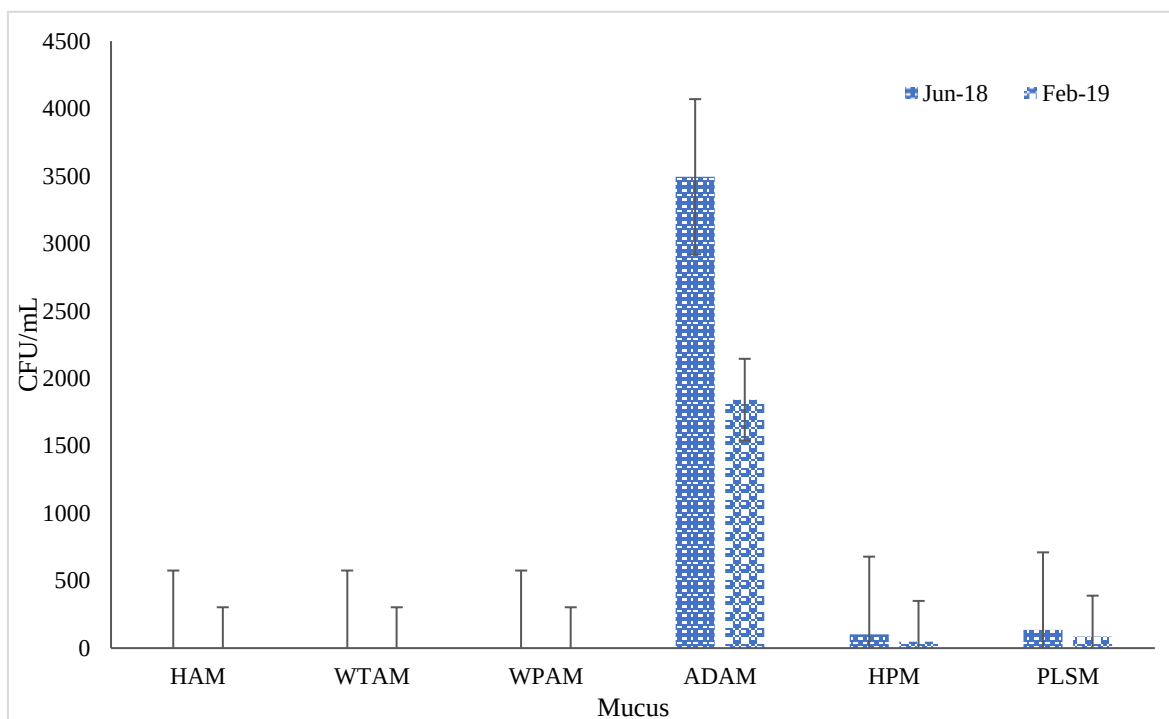


Figure 2.26- Occurrence of *Enterococcus* (Bile esculin agar) in different healthy and diseased mucus samples.

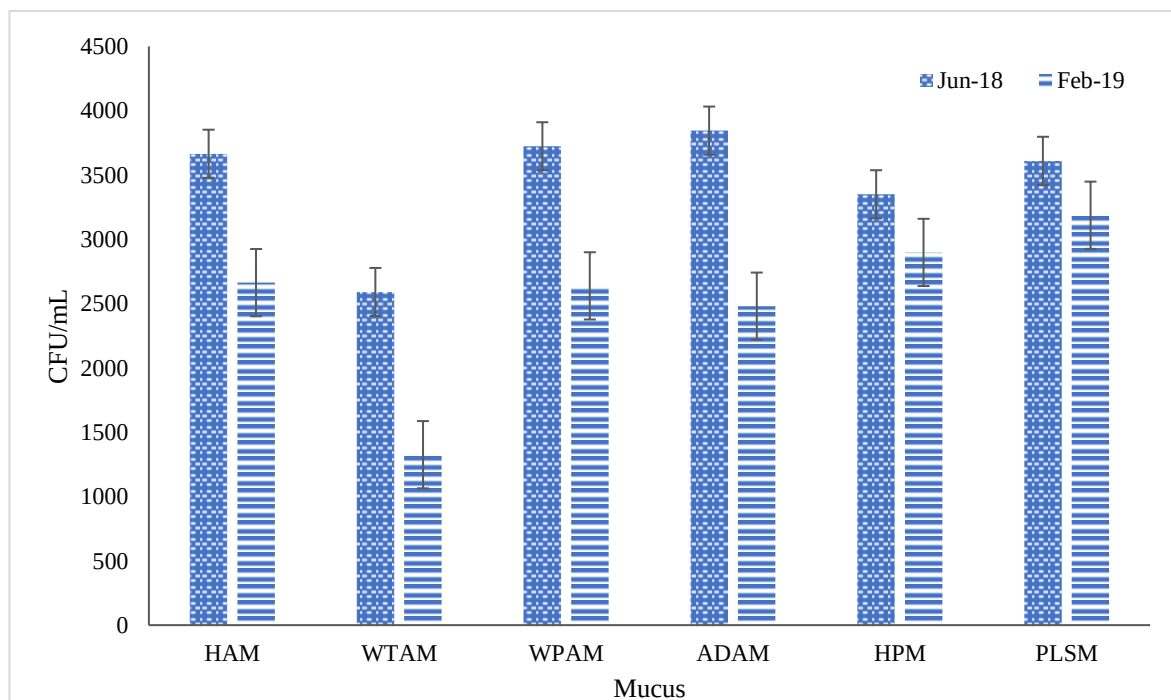


Figure 2.27- Occurrence of *Vibrio* in different healthy and diseased mucus samples.

2.3.9 Metagenome analysis.

2.3.9.1 DNA extraction, Data process, and Diversity index

Coral mucus DNA of the healthy, diseased, pink line syndrome, algal-dominated mucus of *Acropora* sp. and *Porites* sp. was isolated (Fig. 2.28a and b). The isolated different samples of DNA were subjected to metagenome sequence analysis to understand the diversity of the non-culturable microbes.

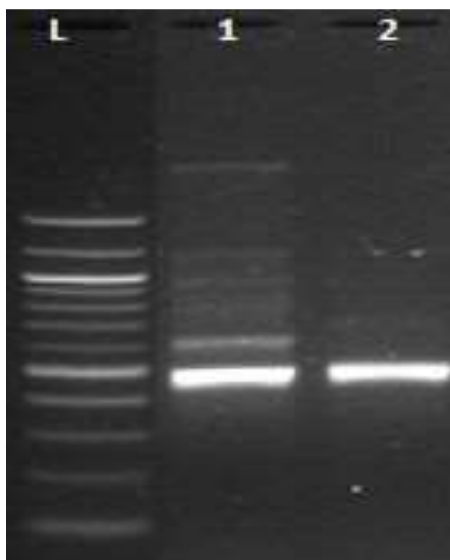


Figure 2.28a- L- ladder, 1. HP1 (Healthy *Porites* sp.) 2. PLS1 (pink line syndrome *Porites* sp.).

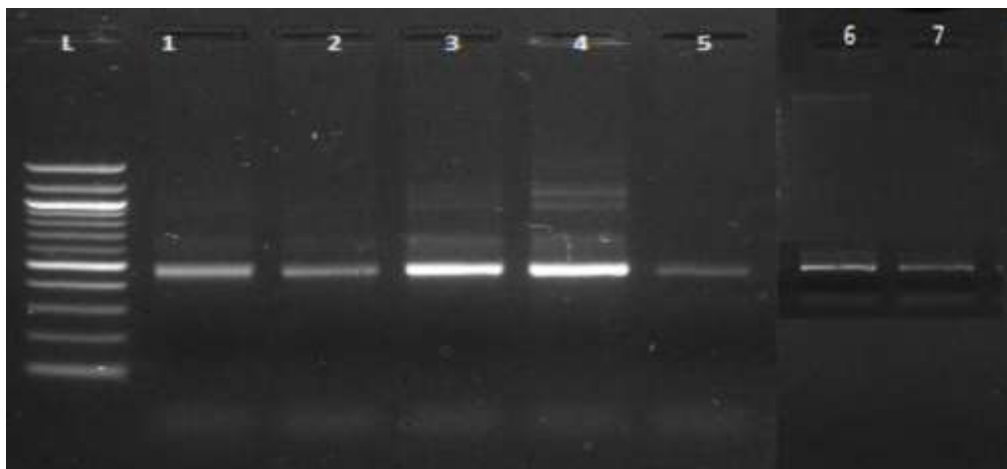


Figure 2.28b- L-ladder, 1-HAM (Healthy *Acropora* sp.), 2-HAM, 3-HPM (Healthy *Porites* sp.), 4- PLSM (pink line syndrome *Porites* sp.), 5- WPAM (White Patch *Acropora* sp.), 6- SL1 algal dominated, 7- SL 2 diseased.

2.3.9.2 Metagenome analysis of coral mucus (non-culturable)

The metagenomic sequence analysis of the V3-V4 regions of coral mucus was done to understand the diversity of the non-culturable microbes. The healthy, diseased, Pink line syndrome, algal-dominated mucus of *Acropora* sp. and *Porites* sp. subjected to metagenome sequence analysis revealed their relative abundance in the phylum, class, family, order, genus, and species level. the

The relative microbial abundance of the healthy and diseased mucus showed the presence of proteobacteria in high OTUs at the phylum level (Fig. 2.19) in diseased mucus. The same corresponds to Gammaproteobacteria at class level and the pathogenic *Vibrio* sp. also belongs to Gammaproteobacteria. The presence of *Vibrio* sp. was observed in the healthy *Porites* mucus and pink line syndrome mucus (PLSM) at phylum, class, order, family, and genus levels (Fig. 2.29). Likewise, the presence of Actinobacteria was viewed in healthy *Acropora* mucus and the algal-dominated and diseased *Porites* sp. mucus. The *Bacillus* sp. that falls under the firmicutes was also seen in all the mucus samples because *Bacillus* and Actinobacteria are known antimicrobial-producing organisms.

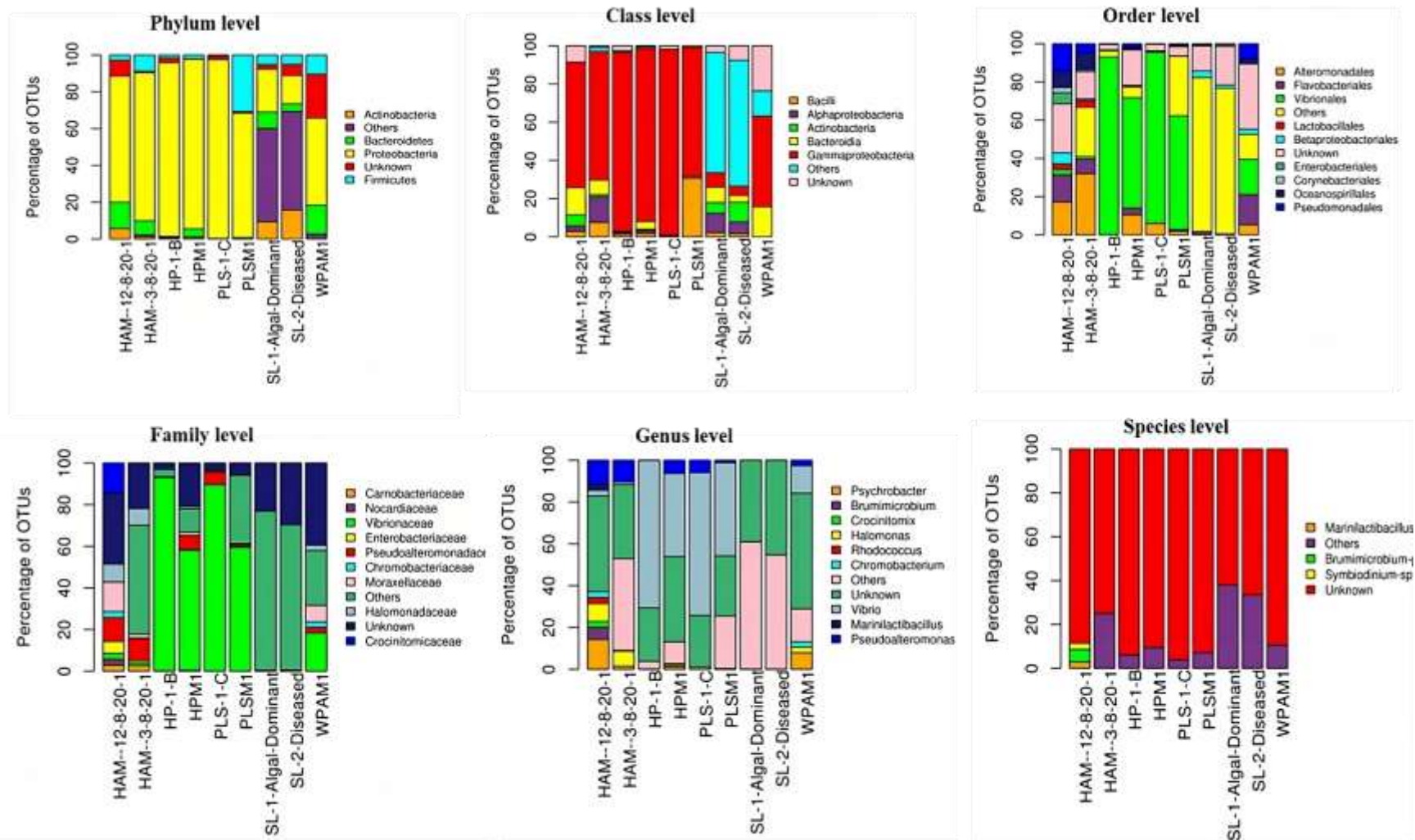


Figure 2.29- Relative abundance of non-culturable mucus microbes in the phylum, class, order, family, genus and species. By metagenome sequence analysis of V3-V4 regions. HAM- Heathy *Acropora* mucus, HP- Healthy *Porites*, HPM- Healthy *Porites* mucus, PLS – Pink line syndrome, WPAM- white patch *Acropora* mucus.

2.3.9.3 Alpha diversity with samples and rarefaction curves

In this section, we analyzed the microbial diversity within the samples by calculating Shannon, Chao1, and observed species metrics. The chao1 metric estimates the species richness while the Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. The observed species metric is the count of unique OTUs identified in the sample. We found significant diversity and richness of species in both healthy and diseased mucus samples of *Acropora* and *Porites* corals. Additionally, the diseases affecting *Acropora* and *Porites* showed considerable diversity. Conversely, healthy *Porites* and *Acropora* exhibited lower levels of diversity and richness. Notably, mucus samples exhibited the highest diversity and richness among all samples tested. The rarefaction curve for each of the metrics is provided in the figures below.

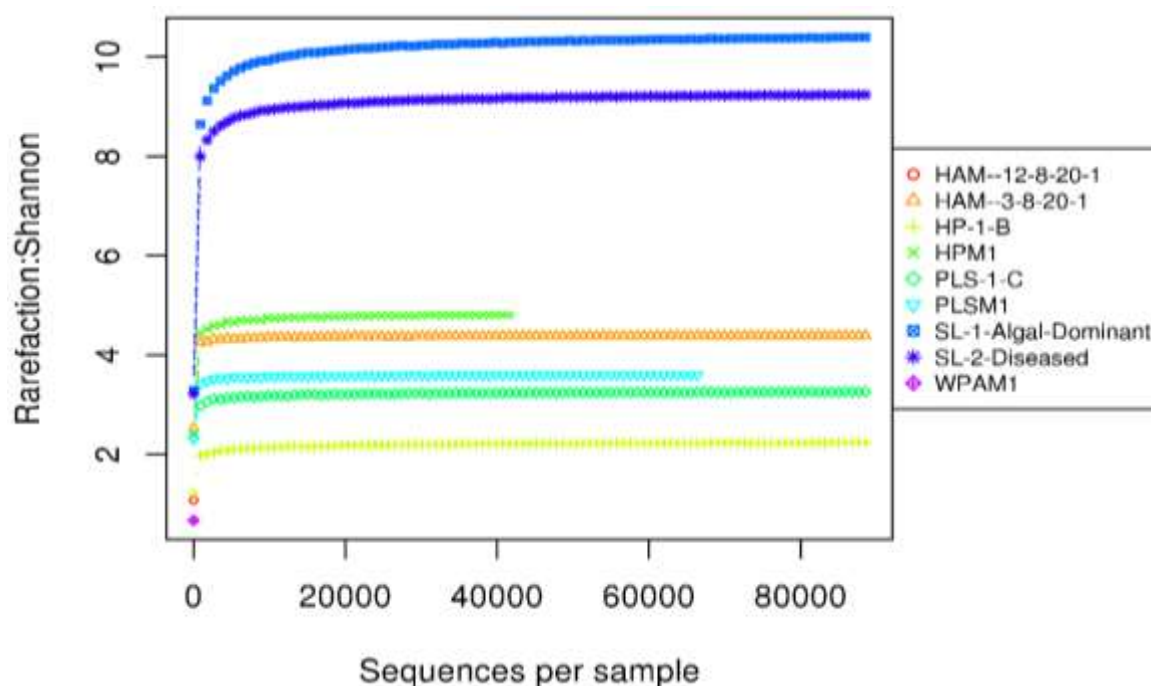


Figure 2.30a- Shannon curve obtained for the samples.

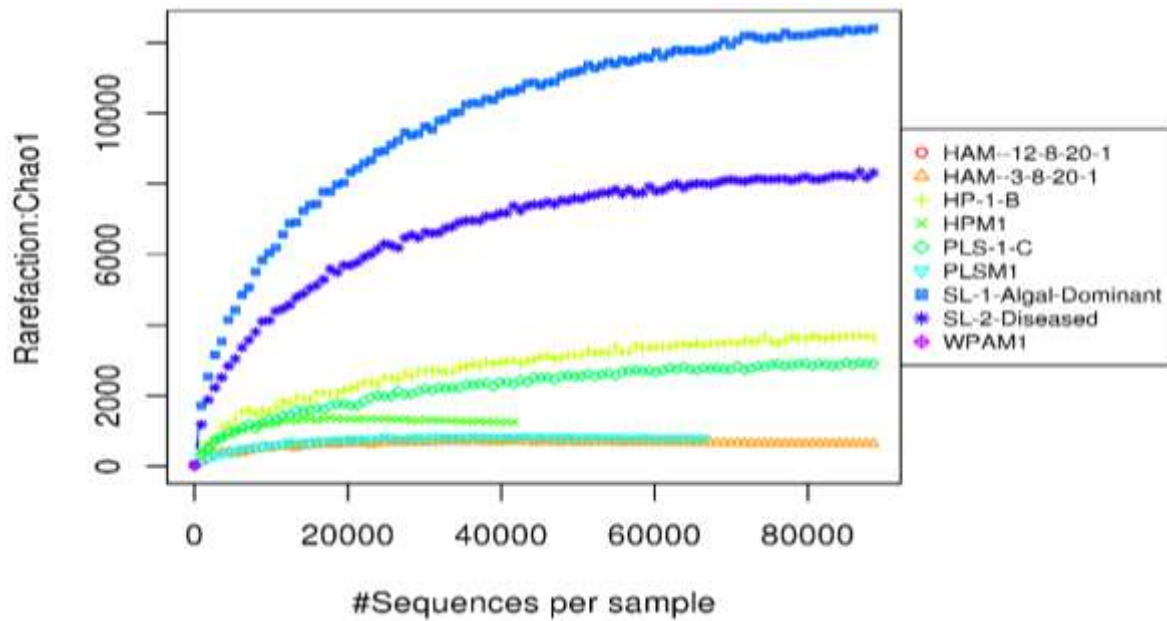


Figure 2.30b- Chao1 curve obtained for the samples.

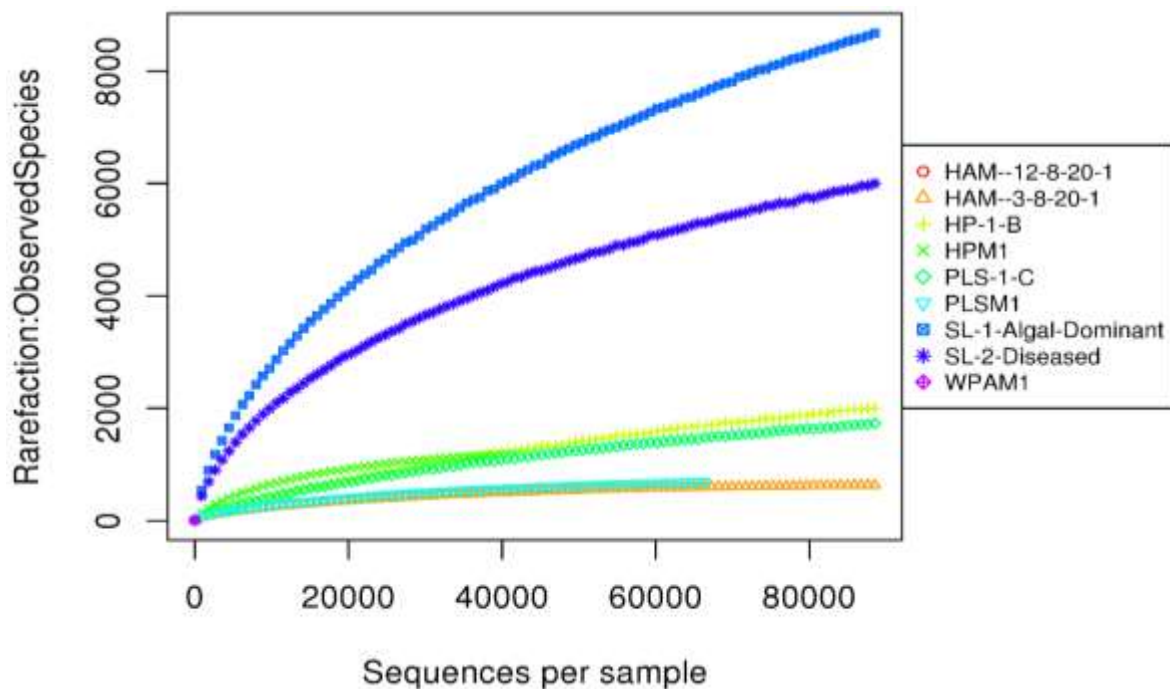


Figure 2.30c- Observed species curve obtained for the samples.

2.3.9.4 Metagenomics (V3-V4) of healthy and diseased *Porites* mucus sample.

Metagenomics study of mucus sample of healthy and diseased *Porites* at phylum level 90-95 % proteobacteria were present in the mucus of healthy *Porites* whereas 95-98 % of proteobacteria are present in disease state. 2-4 % of firmicutes are present in both healthy and diseased states. 3-5 % of phylum were unknown in both healthy and diseased state *Porites* mucus samples (Fig. 2.31A). At the class level, approximately 2-3 % of Bacilli are present in both healthy and disease states. Around 90-95 % of Gammaproteobacteria are present in the mucus of healthy *Porites*. whereas in disease they are more than 95 % which is slightly higher as compared to healthy. More than 3 % of the bacterial class was unknown in both healthy as well as disease states (Fig. 2.31B). At the order level presence of Alteromonadales was seen in the mucus of disease *Porites* sp. which was absent in healthy. Less than 2-3 % Bacillales were present in both healthy and diseased *Porites* sp. mucus however these are abundant in healthy state. Around 90-95 % of Vibrionales were present in both healthy and diseased states. Approximately 3-5 % of bacterial order was unknown (Fig. 2.31C).

At the family level, Pseudoalteromonadaceae was present in the disease state whereas it is absent in the healthy state. Around the same percentage of Vibrionaceae which is 90-95 % is present in both disease and healthy states. Less than 5 % percentage of family present was unknown in both disease and healthy state (Fig. 2.31D). At the genus level bacillus was present in the mucus of healthy *Porites* sp. which was absent in the disease. Approximately 75 % of *Vibrio* is present in both healthy and diseased states of *Porites* sp. mucus. Less than 15 % of the genus belonged to the other group (Fig. 2.31E). At a species level of less than 3 % *Vibrio azureus* was present in mucus samples of both healthy and diseased *Porites* sp. A very small percentage of uncultured *Vibrio* species was present in the mucus of diseased *Porites* sp. (Fig. 2.31F).

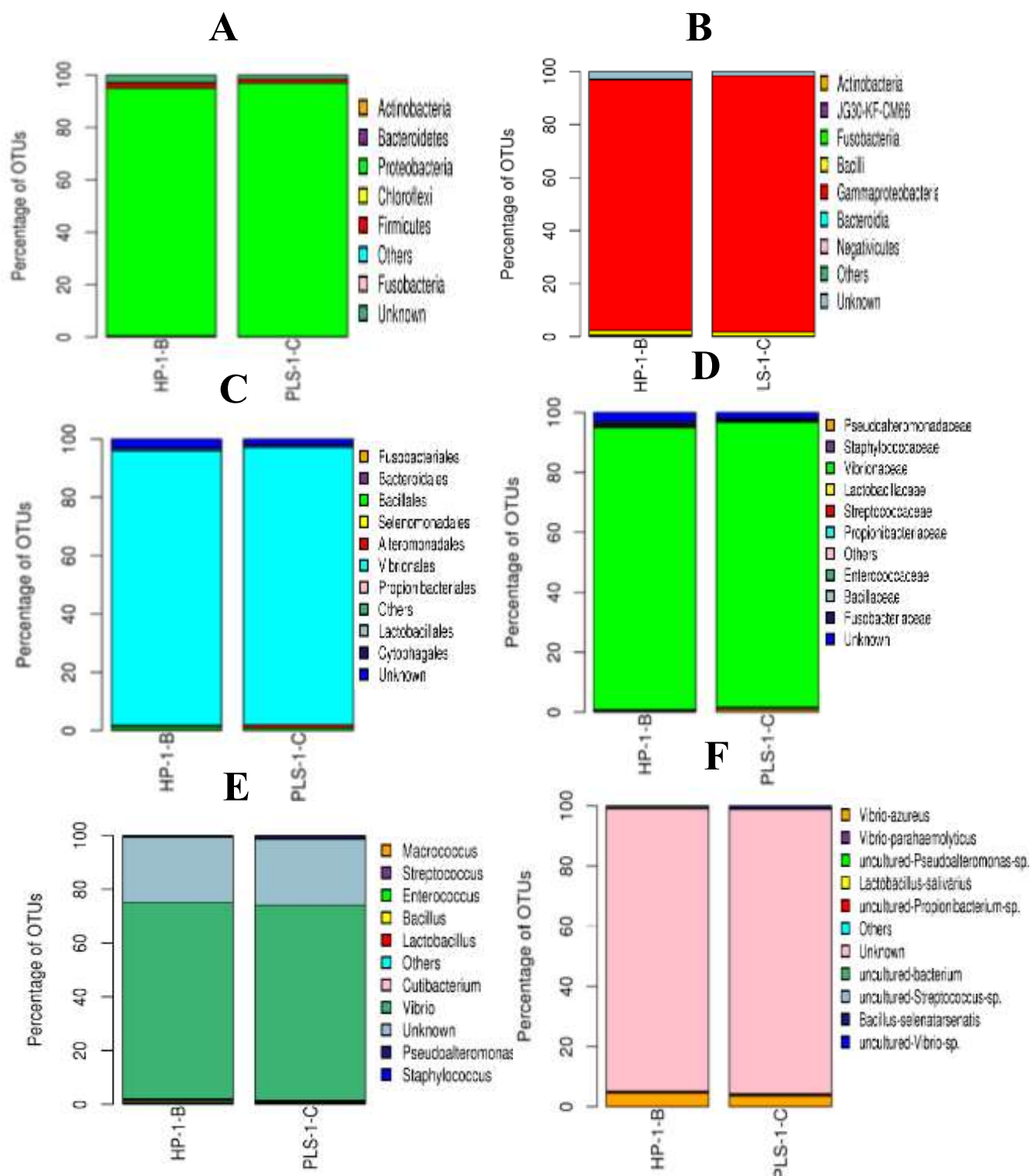


Figure 2.31- Relative abundance of healthy and diseased mucus sample at A) at the phylum level, B) at class level, C) at an order level, D) at the family level, E) at the genus level, F) at the species level.

2.3.9.5 Alpha diversity and rarefaction curves.

In this section, we analyze the microbial diversity within the samples by calculating Shannon, Chao1, and observed species metrics. The chao1 metric estimates the species richness while the Shannon metric is the measure to estimate observed OTU abundances, and accounts for both richness and evenness. The observed species metric is the count of unique OTUs identified in the sample. High diversity was noted in both healthy and diseased *Porites mucus*, although there was comparatively less richness observed in these two sample sets. The rarefaction curve for each of the metrics is provided in the figures below (Fig. 2.32).

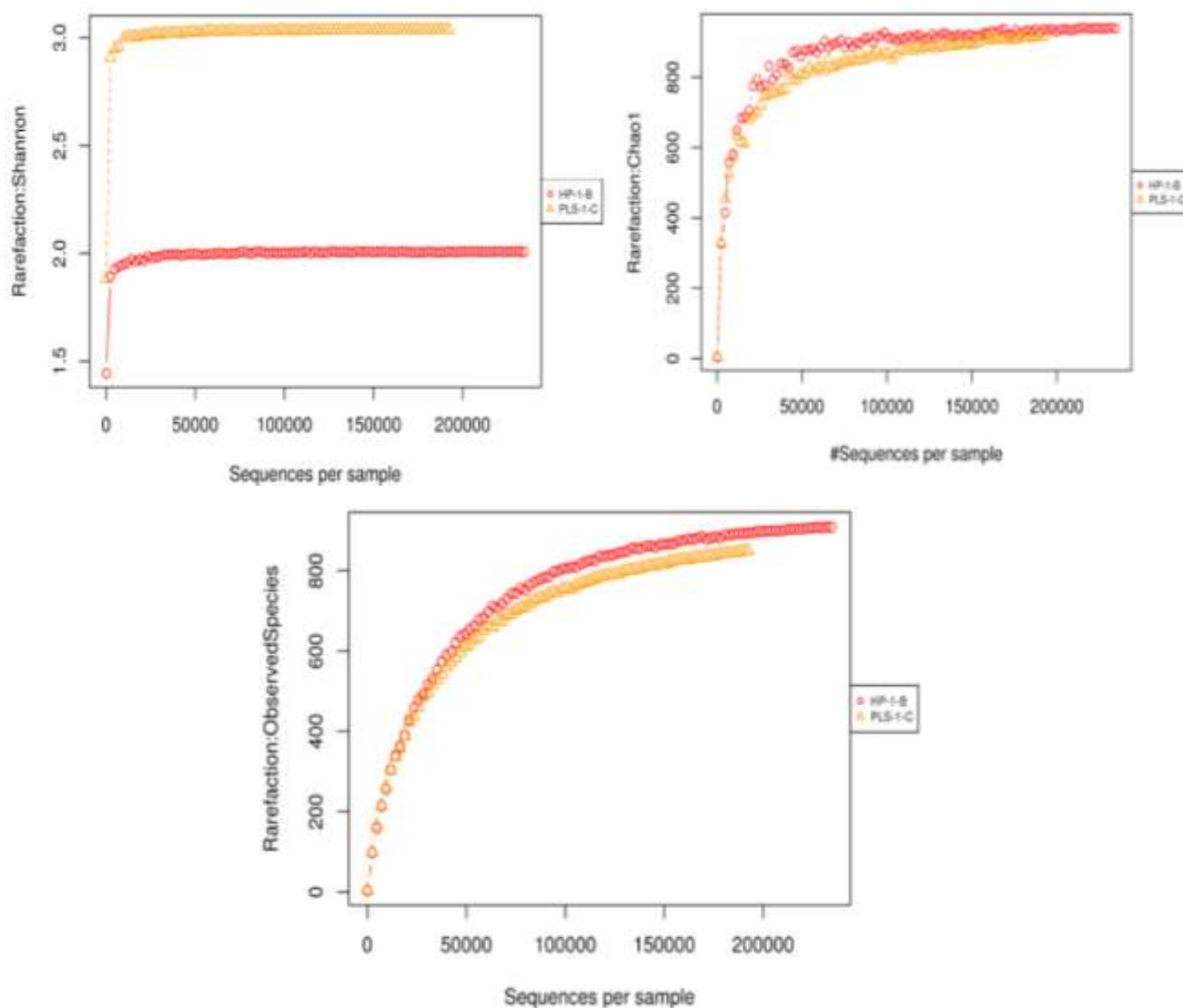


Figure 2.32- Rarefaction curve of healthy and PLS diseased coral mucus with Shannon, Chao1, and observed species.

2.4 Discussion.

During the pre-monsoon period in June 2018, SL1 was dominated by algae and bleached corals. The nutrient concentrations were notably higher at SL1, indicating a potential link between nutrient levels and algal growth (Fig. 2.3). SL2 identified as a diseased location, showed the presence of healthier corals compared to SL1. SL3 represented a healthy coral condition with minimal bleaching and disease. The nutrient concentrations in SL3 exhibited lower than those in SL1 and SL2, supporting the correlation between nutrient levels and coral health (Angelo et al., 2014). February 2019 observations showed significant improvement in SL2 and SL3 (Fig. 2.3), attributed to a temperature and pH drop. However, SL1 did not recover, and algae dominance increased. The impact of rising temperature and pH levels on coral health was observed across all locations, highlighting the critical role of temperature in affecting coral health.

Nitric oxide synthesis linked to nitrite emerged as a factor in coral bleaching, emphasizing the significance of nitrite in influencing the physiological responses of corals (Fernandes et al., 2020; Hawkins et al., 2014). Elevated ammonia levels in bleached and algal-dominant regions indicate potential stress on endosymbiotic algae and polyps (Zhu et al., 2004). High dissolved inorganic nitrogen (DIN) supported the risk of algal overgrowth impacting coral ecosystems (Haas et al., 2009). Also, the earlier study of high nitrogen levels that increased bleaching under heat and light stress (Wiedenmann et al., 2013), supported the present study with the high percentage of bleached corals that have been observed in SL1. The enrichment of nitrates in the presence of algae, as seen in SL1, led to a reduction in measurable nitrate concentrations in the water and an escalation in coral mortality (Fabricius et al. 2013). Elevated nutrient levels have the potential to reduce the heat stress tolerance ability of corals. Tropical reef-building corals commonly flourish in nutrient-poor environments, because of the highly efficient uptake and recycling of nutrients by coral reef organisms (Wiedenmann et al., 2023). The algae account for most of the uptake of dissolved inorganic nitrogen from the environment, mainly in the form of ammonium (NH_4^+) and nitrate (NO_3^-) (Wooldridge, 2013). The coral host can regulate the amount of ammonium and nitrate uptake from the environment by maintaining a tissue-specific gradient and also exercises an internal limitation of ammonium and nitrate availability on *Symbiodinium* (NOAA 2015). Nitrification in corals reduces the nitrogen available for *Symbiodinium*

growth. Denitrification maintains the internal nitrogen limitation of *Symbiodinium*. Endolithic fungi reduce nitrate into ammonium helps to prevent the loss of nitrogen from the holobiont (Bourne et al., 2016). In this way, healthy corals get rid of the harmful effects of excess nitrogenous nutrients by nitrogen cycling. Thus, the healthy symbiotic association of coral requires comparatively less ammonia.

Silica concentrations showed a positive correlation with healthy corals (SL3). Dissolved oxygen concentration was also found slightly higher in healthy association in the healthy coral region (Fig. 2.7). Elevated nitrogen levels promoting zooxanthellae growth align with the observations in SL3, where healthy corals had higher nitrogen levels. Elevated temperature and along with high DOC availability increase nitrogen fixation activity in corals resulting in bleaching and diseases in corals. Increased photosynthesis by zooxanthellae due to excess nitrogen availability may cause CO₂ limitation resulting in coral bleaching (Radecker et al., 2015).

On DO and bacterial counts comparisons, bacterial counts were less in healthy coral water with more DO and more in diseased and algal-dominated coral water with less DO (Fig. 2.7). The opposite trend to that of dissolved oxygen, may be due to the higher the number of bacteria will imply more biological demand for dissolved oxygen for survival. Decreased DO impact on coral physiology (Haas et al., 2014). We observed low DO concentrations in the diseased and bleached coral regions. Comparatively healthy coral *Porites* sp. water has more DO than diseased coral water and diseased *Porites* sp. water has higher DO than algal-dominated water.

In SL1, the observed prevalence of algae and a significant proportion of bleached corals is supported by earlier studies that the temperature rise, light absorption, and elevated algal densities could contribute to bleaching (Fabricius, 2006). Also, enriched nutrients have the potential to boost the productivity of coral reef macroalgae (Fabricius et al., 2012). These increased algal densities have the potential to disrupt corals through various mechanisms, including the release of algal toxins, depletion of oxygen, and the accumulation of decomposing organic material, which may increase the risk of bacterial and fungal infections (Angelo et al., 2013). This correlation was observed in SL1 locations, where high colony-forming unit (CFU) values and algal dominance were identified (Guzman et al. 1990). Enrichment of both nitrogen and phosphate in SL1 and SL2 resulted in destabilizing the

coral–algae symbiosis. Phosphate was found in the optimum requirement range in SL3 which supports coral coloration and metabolic processes and higher in stressed coral regions (Lirman and Fong, 2007). We observed comparatively high carbon concentration in diseased nearby coral sediment (Fig. 2.6). This may be because of the release of stressed coral mucus, which settles in sediment near the coral. In SL2, where coral diseases were observed, there was a notable increase in nutrient levels, which attracts phytoplankton densities that can potentially limit light availability for zooxanthellae, leading to diminished calcification rates. Additionally, the rise in nutrient levels within reef waters may be regarded as a factor contributing to the proliferation of coral diseases (Hallock and Schlager, 1986; Bruno et al., 2003; Voss and Richardson, 2006). Location SL1, where both bleached and diseased corals were present, exhibited higher nutrient concentrations near affected corals. This aligns with existing literature suggesting that increased nutrient levels beyond certain thresholds can have detrimental effects on coral physiology, especially under temperature stress conditions (Angelo et al., 2013).

CCA analysis further supported the statement that higher nutrients were associated with the diseased state of corals. Moderate nutrients were seen associated with healthy corals. Higher ammonia which is detrimental to symbiotic algae (Chen et al., 2017) was seen associated with bleached corals along with silica, carbon, and increased temperature (Fig. 2.8). The possible reason for high nutrients in diseased and algal-dominant coral areas may be high anthropogenic activities as it is situated near the shore.

The total bacteria levels were relatively consistent between healthy and diseased *Acropora* and *Porites* sp. mucus, except in the case of algal-dominant *Acropora* sp. mucus. In healthy *Porites* sp. mucus, concentrations of ammonia and nitrate were higher, as discussed in Chapter 5 (Fig. 5.2). These nutrients were identified as supporting the maximum growth of *Neisseria*, *Enterococcus*, and *Vibrio* (Figs. 2.24, 2.26, and 2.27). Silica and phosphate concentrations were higher in PLSM mucus, coinciding with a notable presence of *Neisseria* and *Vibrio* (Figs. 2.24 and 2.26). Healthy *Acropora* mucus exhibited moderate nutrient concentrations, while WPAM mucus had the lowest levels of the mentioned nutrients, fostering the growth of *Coliforms*, *Salmonella*, *Neisseria*, and *Vibrio* (Figs. 2.22, 2.23, 2.13, and 2.27).

The results of our study, conducted in June 2018 with high temperatures and February 2019 with comparatively low temperatures, revealed interesting patterns. In a previous report on *Acropora* sp. mucus in the Gulf of Mannar, Vibrionaceae members were abundant (Nithyanand and Pandian 2009). Similar findings were observed in our current study, where both *Acropora* and *Porites* mucus exhibited the presence of Vibrionaceae members. Another study highlighted the antimicrobial activity of *Bacillus*, *Planomicrobium*, *Vibrio*, and *Pseudoalteromonas* against coral-associated pathogenic bacteria, aligning with some of our present observations (Shnit-Orland and Kushmaro, 2009).

Furthermore, our study identified bacteria previously reported in coral associations, such as *Staphylococcus* and *Micrococcus* (Table no.2.2). While *Vibrio* sp. have been recognized as pathogens in marine environments (Thompson et al., 2004; Austin et al., 2005), they have specifically been implicated in coral disease in previous research (Banin et al., 2000; Ben-Haim et al., 2003; Cervino et al., 2008; Sussman et al., 2008; Luna et al., 2010). In our study, *Vibrio* sp., *Staphylococcus* sp., and *Pseudoalteromonas* sp. were associated with mucus sampled from both healthy and diseased coral tissues. Notably, *Pseudoalteromonas* has been linked to mortality in marine crustacea (Costa-Ramos and Rowley, 2004) and algae (Lovejoy et al., 1998). However, similar to *Staphylococcus* sp., which is commonly found associated with skin and mucus membranes in warm-blooded organisms, there have been no reports of either causing disease in corals (Wilson et al., 2012).

Previous studies have reported the presence of *Vibrio*, *Staphylococcus*, *Micrococcus*, *Pseudomonas*, and *Microbacterium* in *Acropora* disease (Wilson et al., 2012). The consistent presence of *Staphylococcus* in both healthy and diseased *Porites* mucus suggests a stable association. In *Acropora* mucus, *Staphylococcus* was found only in white patch diseased *Acropora* mucus, particularly in the first collection, indicating a potential correlation with the high temperatures experienced in June 2018.

In a prior investigation of *Porites* sp. mucus in the Persian/Arabian Gulf and the Red Sea, the dominant families identified were Pseudomonadaceae, Dermabacteraceae, and Flavobacteraceae in both locations. Conversely, the seawater near the coral in these locations exhibited a prevalence of Flavobacteriaceae, Halomonadaceae, and Pelagibacteraceae. These findings highlight a distinct difference between the microbial composition of mucus samples and the adjacent seawater in the same coral locations (Hadaidi et al., 2017), which supports

the present study's observations of bacterial isolates that had been screened in culturable and unculturable methods. The study involving diverse coral taxa and their mucus, the dominant microbial species identified included *Herbaspirillum* sp., *Brevibacterium aureum*, *Sphingomonas echinoides*, *Delftia* sp., *Sphingobium yanoikuyae*, and *Staphylococcus epidermidis* (Ainsworth et al., 2015; Hadaidi et al., 2017). Dominant microbial taxa in coral mucus included Gammaproteobacteria, Alphaproteobacteria, Firmicutes, and Cyanobacteria, with variations in their relative abundance observed among coral genera and regions.

The relative microbial abundance of the healthy and diseased mucus showed the presence of proteobacteria in high OTUs at the phylum level in diseased mucus. Distinct coral host-specific communities were identified, such as the prevalence of Clostridiales on *Acropora* sp. in Hoga and the Mexican Caribbean, contrasting with *Porites* sp. and seawater. *Halomonas* sp. dominated with *Porites* sp. and *Acropora* sp. in the Mexican Caribbean, while *Psychrobacter* sp. with *Acropora* and *Porites*. Notably, *Halomonas* sp. dominance was associated with *Acropora* and *Porites* in the Mexican Caribbean, Firmicutes with *Acropora* in the Mexican Caribbean and *Acropora* and *Porites* in Hoga, and Cyanobacteria with *Acropora* and *Porites* in Hoga and *Porites*. Additionally, some studies reported geographically distinct *Psychrobacter* sp. were associated with coral mucus, highlighting the predominance of Clostridiales associated with *Acropora* sp. and reinforcing the evidence for coral host-specific microorganisms (McKew et al., 2012).

Within the healthy mucus of both *Acropora* and *Porites*, Proteobacteria were found to be abundant. In the diseased mucus samples of both *Acropora* and *Porites*, the abundance of Proteobacteria was even higher compared to the healthy mucus samples. As most of the proteobacteria are gram-negative and pathogenic bacteria (Rizzatti et al., 2017). However, many Proteobacteria have been implicated in various functions within coral microbiomes, including nutrient cycling, pathogen defense, and stress tolerance. In the culturable analysis, we found *Vibrio* was abundant in all mucus aligning with unculturable data, with the highest predominance observed in *Porites* diseased mucus, followed by healthy *Porites* mucus (Fig. 2.27). This suggests that *Vibrio* may play a significant role in the microbial community associated with *Porites* corals, particularly during disease states. Conversely, in healthy *Acropora* mucus, we identified *Psychrobacter*, *Pseudoalteromonas*, and *Halomonas* as predominant genera (Fig. 2.29). This indicates a unique bacterial composition associated with

healthy *Acropora* corals, with these genera potentially contributing to the overall health and resilience of the coral holobiont.

2.5 Summary

- A Coral health survey was carried out in a 5-meter radius on the field during sample collection. In the SL1 Algal dominated, SL2 diseased and SL3 healthy corals were predominant.
- Samples (mucus, water, coral tissue, sediment) were collected.
- Physicochemical parameters were analysed in nearby water of healthy, bleached and diseased corals.
- High nutrients support the bleached, algal dominance, and diseased state of corals.
- Total carbon in near diseased coral sediment was more compared to healthy coral. It may be due to photosynthate and carbohydrate-rich mucus released by stressed corals.
- Higher temperature in June 2018 supports more pathogenic bacterial growth compared to lower temperature in February 2019.
- Metagenomic analysis supported the culturable diversity results and also variations in the bacterial diversity were observed in *Porites* and *Acropora* sp. mucus.
- The disease and healthy mucus were collected at different times were found to have varied bacterial occurrence.

Chapter three

Coral-associated ciliates and their
interactions with mucus.

3.1 Introduction

The environmental stress and physical damage on corals have been described for the susceptibility of corals to infectious diseases (Page and Willis., 2008; Katz et al., 2014; Sharma and Ravindran, 2020) induced by pathogenic microbes such as bacteria (Ritchie and Smith, 1995; Richardson, 1898), fungi (Ravindran, 2001), viruses (Rohwer and Thurber, 2009) or different type of microorganisms (Richardson, 1996) including cyanobacteria (Rasoulouniriana et al., 2009; Richardson et al., 2009). The other parasitic flatworms, trematodes have been described to induce infection on *Porites* sp. (Aeby, 2007; Palmer et al., 2009). Protozoans have been reported as direct consumers of coral-associated photosynthetic algae, zooxanthellae (Ulstrup et al., 2007; Qiu, 2010), and coral tissues (Cooper et al., 2007), but only a few of them as possible causative agents of coral diseases (Antonius and Lipscomb, 2001; Rodriguez et al., 2009). Skeleton-Eroding Band was the first coral disease described for the association of a folliculinid ciliate, *Halofolliculina corallasia* (Antonius and Lipscomb, 2001; Winkler et al., 2004). Then various other coral diseases such as brown band (Willis et al., 2004), Caribbean Ciliate Infection disease with ciliates similar to those associated with skeletal eroding band (*Halofolliculina*) (Croquer et al., 2006), white syndrome with the diverse ciliate community (Sweet and Bythell, 2012), white band disease, white plague and brown jelly syndrome have all been described for various ciliates association with the different coral genera (Randall et al., 2014; Sweet et al., 2014; Sweet and Sere, 2015). While previous research has established connections between ciliate species and distinct signs of coral diseases, there is a notable gap in understanding the mechanisms underlying the role of ciliates in disease progression, as well as their patterns of growth, proliferation, and feeding preferences.

This study seeks to address this knowledge gap by investigating the association of ciliates with two specific scleractinian coral disease signs. This study aims to investigate the possible mechanism of ciliates in coral disease lesion association by utilizing nutrient-rich coral mucus, and how mucus impacts their growth stages (adult, dormant, and larvae). The research seeks to uncover the mechanisms that enable ciliates to survive in association with diseased coral lesions (*Acropora* and *Porites* sp. mucus), in the Gulf of Mannar region, India. Protozoans are known for their selective nature of feeding based on the prey size, shape, and

biochemical composition (Dopheide et al., 2014). The chemical composition of coral mucus, derived from photosynthates of symbiotic symbiodiniaceae cells and heterotrophic feeding by corals, is a major nutrient source for various coral-associated organisms (Hadaidi et al., 2019). The composition of coral mucus was analyzed for carbohydrates, amino acids, lipids, and proteins (Brown and Bythell, 2005). Also, no previous studies have been made on growth parameters for the ciliates using nutrient-rich coral mucus as the substrate for understanding the coral-ciliate interactions. Therefore, the ciliates of the present study were investigated for their feeding interactions with two different diseased corals, *Acropora muricata* and *Porites lutea*.

3.2 Material and methods

3.2.1 Protozoan associated with corals

3.2.1.1 Field selection, observation, and sample collection

In June 2018, the ciliates were for the first time observed on the hard coral *A. muricata* and *P. lutea* sp. from the coastal marine National Park of Gulf of Mannar region (GoM) (GPS Locations - N 09°17' 36.2" E 079°08'12.6" and N 09°17'45.6"; E 079°08'36.8") India. The ciliates were monitored during the subsequent months such as February and March 2019. The colonies of shallow barrier reef (2 to 3 m depth) were examined along a 5 m radius from the zone dominated by *A. muricata* and *P. lutea*. A collection samples permit was granted by MoEFCC, New Delhi, India and PCCF, Chennai, Tamil Nadu, India. Small pieces of early/advanced diseased and healthy coral (about ~ 0.5 to 1 cm²) were collected from the field and then transported in aerated small tanks to the laboratory. The surface layers of the diseased and healthy portions of the corals were collected using syringes. Also, *A. muricata* and *P. lutea* mucus were collected using sterile syringes as earlier described by Ducklow and Mitchell. Samples (healthy or diseased) evaluation from *A. muricata* and *P. lutea* was made for lesions, injuries, disease, and presence or absence of ciliates by microscopy (Olympus). Furthermore, the disease severity was examined based on the advancement of lesions and the percentage of tissue loss that occurred in the fragments. The advancement of lesions and the areas of tissue loss were measured as described by (Katz et al., 2014).

3.2.1.2 Ciliate collection and culturing

The ciliate collection was done as described by (Katz et al., 2014). Briefly, the early/advanced diseased and healthy fragments of *Porites* and *Acropora* species were placed in 0.22 μ filter seawater and incubated for 3-5 h at room temperature. After the incubation period, a dense group of ciliates was found moving from the advanced disease fragments to the water surface. The ciliates were then collected and washed with filtered seawater. The washed ciliates were counted under a dissecting microscope and used as an inoculum for the infection experiments.

The harvested ciliates were maintained as pure cultures in the laboratory with wheat grains as a food source to enrich bacteria as described in the earlier methods with minor modifications. Coral-associated ciliate protozoans were grown and cultured *in vitro* using synthetic media namely ASWP – Artificial Seawater for Protozoans prepared according to (Day et.al 2007). Briefly, media preparation was done by adding sea salts to pre-prepared soil extract (106 g of fine crumb, good quality, sieved soil free from pesticide and fertilizers is added to 660 mL of deionized water and autoclaved, allowed to settle for 24 h and again autoclaved. The second time autoclaved soil extract was then decanted and filtered and added to the stock solution (Sodium nitrate (5.625 g), Disodium hydrogen phosphate (0.225 g), Dipotassium hydrogen phosphate (0.188 g) per liter) with sterilized Phosphate buffer saline (PBS) of pH 7.6 – 7.8. To the 300 – 500 μ L of the media, wheat seeds surface sterilized by boiling in water for 4 min was added. For the prepared synthetic media, a bulk suspension of mostly adult ciliate cells is picked and used as an inoculum. Alternatively, a homogenous suspension of cells was prepared and a known number of cells were added as an inoculum. Cells were sub-cultured and re-inoculated into fresh media-containing plates when they attained a high growth density. All cultures are maintained at 30 °C in low light to dark conditions.

3.2.1.3 Microscopic observations and morphological identification of marine ciliates

Collected coral-associated ciliates from the selected diseased portions of *A. muricata* and *P. lutea* surface fragments and behavioral characters were observed under an Olympus binocular microscope. The samples were also wet mounted with a cover slip to restrict the movement of ciliate protozoans. Alternatively, Bouin's Fixative prepared in seawater (30 mL

saturated picric acid, 10 mL formaldehyde, 2 mL glacial acetic acid) was used to immobilize the cells. Higher-magnification images of coral-associated ciliates were observed for morphology and ultrastructure using a Research inverted microscope (Olympus IX51 Inverted Microscope) at 5x, 10x, 40x, and 100x (oil immersion) magnification. Using Cell scope software, images and videos were captured on an Olympus BXI51 microscope. The characteristic features of the ciliates were compared with morphological descriptions from previous studies and the dichotomous key in the 'Marine interstitial Ciliates' was used for ciliate identification. Morphological characteristics, such as cortical and ultrastructural features, provided a means of distinguishing ciliate morphotypes. Features such as kinetosomal make-up and oral infraciliary structures such as the AZM (Adoral Zone of Membranelles) are highly conserved features and together with organelle distribution, size, shape, and color are routinely used for distinguishing genera.

3.2.1.4 Molecular characterization of the ciliates

The molecular characterization protocol of the isolated ciliates started with the washing step, three to five times in sterile seawater, then incubated with an addition of 15 µl of proteinase K (20 mg/ml) in a water bath for 20 minutes at 54 °C. After incubation, they were vortexed for 20 seconds, boiled at 100 °C for 10 minutes, then vortexed for a further 20 seconds, and centrifuged at 16,000 g for 3 minutes. The supernatant of 30 µl was taken off and put into a fresh microcentrifuge tube. This was then stored at -20 °C until further use (Ravindran et al., 2020). Extracted DNA was PCR amplified using universal 18S rRNA gene eukaryotic primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3') (Ravindran et al., 2020). The PCR reactions were performed in a Bio-rad thermal cycler with the following program: initial denaturation at 94 °C for 3 min, followed by 34 cycles of 94 °C for 1 minute, annealing at 50 °C for 1 minute, and 72 °C for 1 minute, with a final extension step of 72 °C for 10 minutes. All PCR products were ethanol-purified and sequenced (Ravindran et al., 2020). Sequences were aligned using the CLUSTAL W sequence alignment program and the Neighbour-joining method was used to reconstruct the phylogenetic tree using the MEGA 7 software program (Ravindran et al., 2020). The topology of the Phylogenetic tree was estimated using the bootstrap analysis

method with 500 replications. The sequences were submitted in NCBI and the GenBank accession numbers were obtained (MT561362- MT561365).

3.2.1.5 Ciliate feeding preferences with coral mucus

The isolated mucus from corals *A. muricata* and *P. lutea* was used in various proportions and combinations to analyze ciliate's feed preference and growth (Ravindran et al., 2020). The substrate composition such as only coral mucus unfiltered (MUF), mucus filter-sterilized (MF), and only seawater and freshwater without any additional media or mucus were used for the experiments. Motile, adult Ciliate cells of about 10 – 15 numbers were added to wells in triplicates per group of the above different substrate combinations. Growth at 28 °C was monitored by counting cells under the microscope at time intervals from 6 hours up to 18 hours period. Means and standard deviations were calculated to enable statistical analysis and understanding of the error rates of each group.

3.3 Results

3.3.1 Field selection, occurrence, and types of ciliates and sample collection

In June 2018 the samples were collected from diseased and healthy *A. muricata* and *P. lutea* and examined under the microscope. The advanced disease lesion of *P. lutea* was found with two stages in the field. One was with dead tissue surrounded with a pink discoloration (Fig. 3.1) and the other with a white mat-like covering on the surface of the coral pink discoloration (Fig. 3.1). Advanced disease lesions of *A. muricata* were observed with disease stage initially with the white syndrome and later turned with brown color (Fig. 3.2). The surface samples were scraped carefully from the healthy and diseased portions of the *A. muricata* and *P. lutea*. Surprisingly the tissue loss of the diseased portion inside the pink line circle and the surface mat region of the *P. lutea* was dominated by a single type of ciliate infection Morpho 1 (Fig. 3.3) along with three other ciliates (Morpho 2, 3 and 4, but no such ciliates were observed in the healthy and early disease lesions of *P. lutea*. *A. muricata* was also observed for a different ciliate (Morpho 2) which is dominated in diseased tissue or surface regions (Fig. 3.4) along with two other ciliates (Morpho 3 and 4), but no such ciliate was found with healthy or early disease regions of the coral tissue and surface. A month later in March 2019, similar observations were found for ciliates occurrence with the healthy, early

and advanced stages of disease condition of the above two different corals. The ciliate dominated (Morpho 1) with that of the *Porites* sp. was not found associated with that of the *A. Muricata* which may be feed specific. Whilst three other ciliates (Morpho 2, 3 and 4) were observed in advanced disease lesions of both the coral genera.

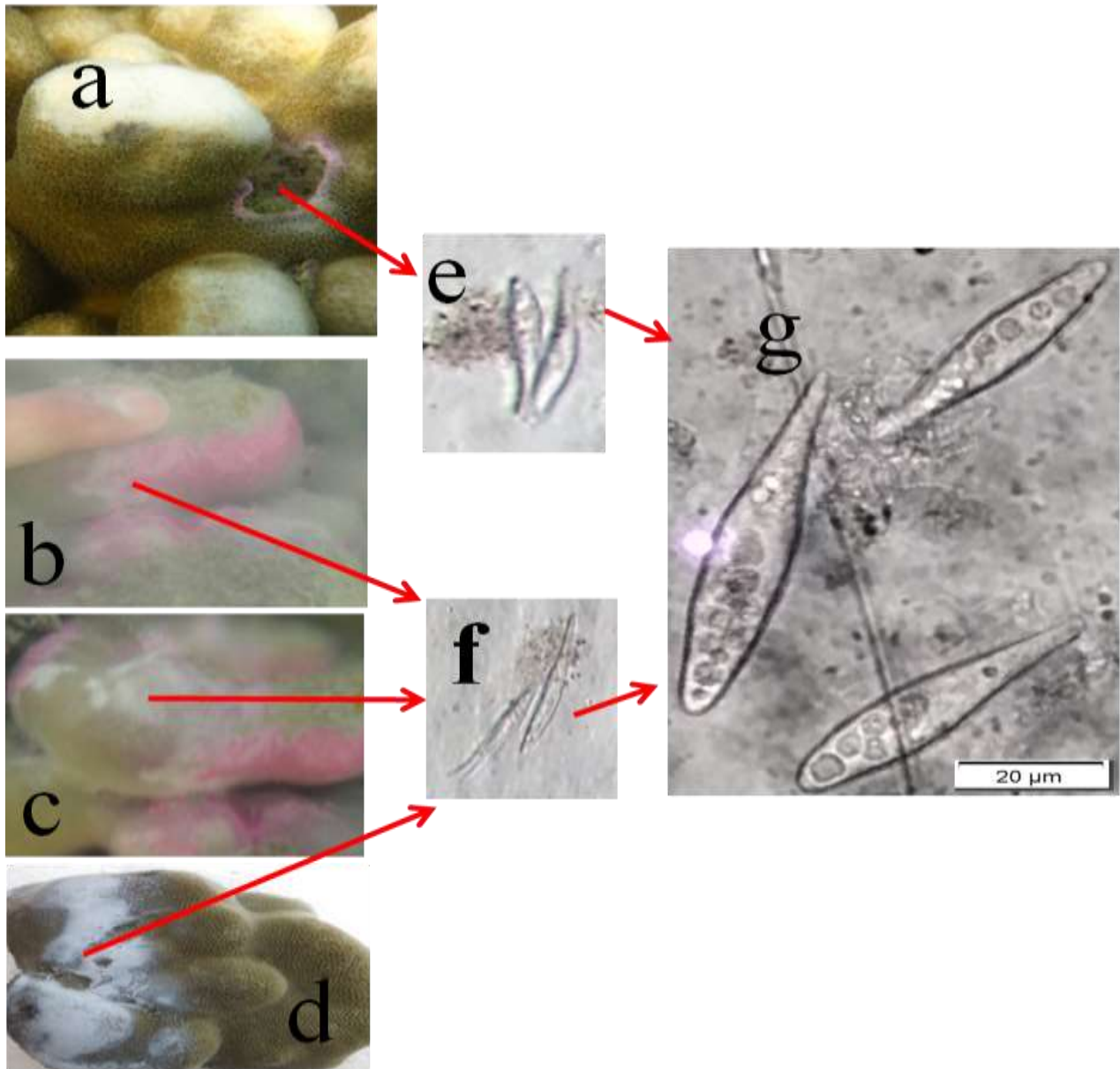


Fig. 3.1- *Porites lutea* disease lesions and the associated ciliate. **a.** tissue with discoloration of pink colour surrounding the dead tissue. **b** and **c.** the tissue with inflammation due to stress and the formation of a white layer. **d.** The tissue with advanced stage with a white mate. **e, f,** and **g** the ciliate isolated from the *Porites lutea* disease lesions.

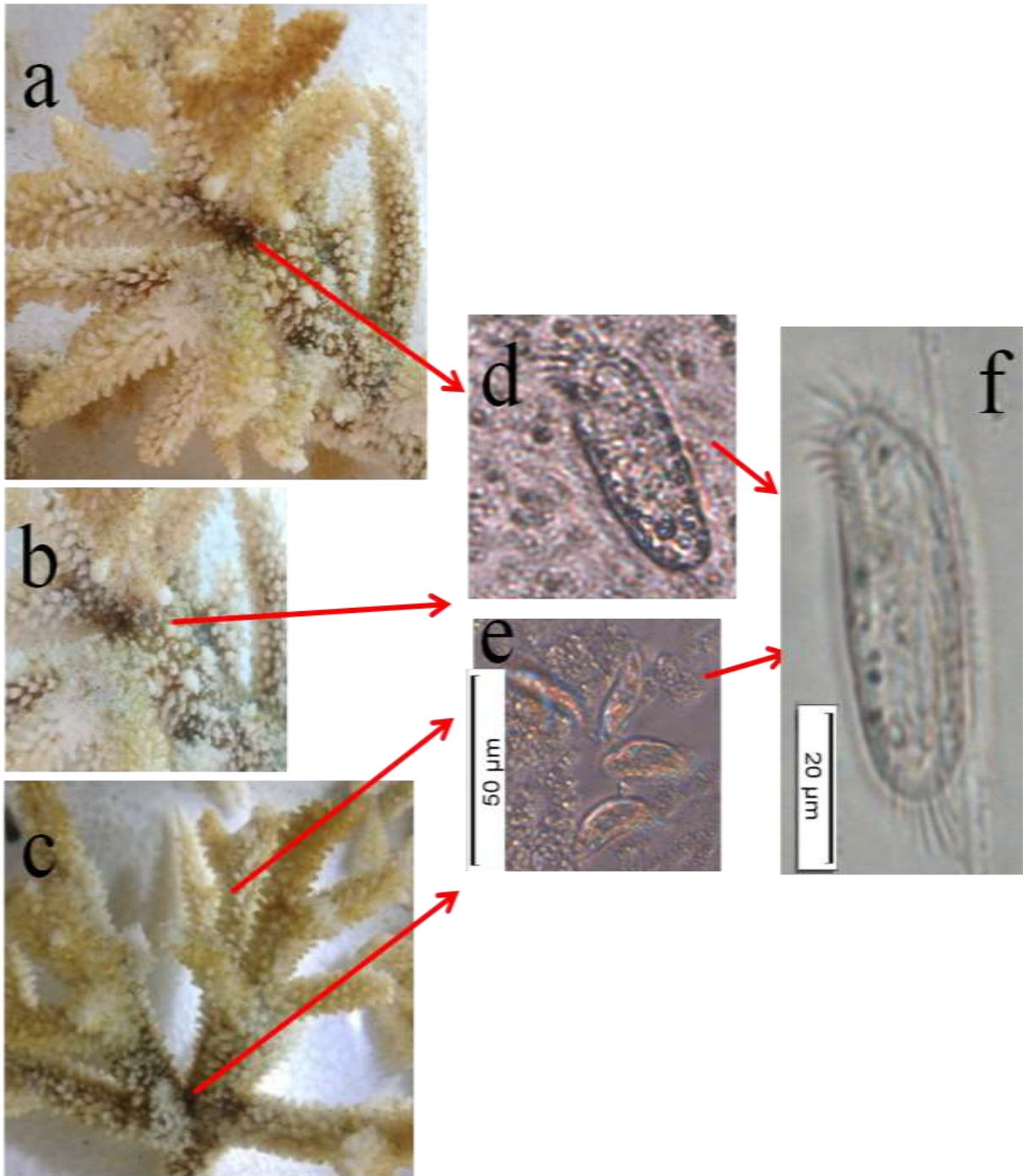


Fig. 3.2- *Acropora muricata* disease lesions and the associated ciliate. **a.** tissue with initial white and later with brown discoloration surrounding dead tissue. **b** and **c.** the tissue with white syndrome due to stress and formation of white and brown discoloration. **d, e,** and **f.** the ciliate isolated from the *Acropora muricata* disease lesions.

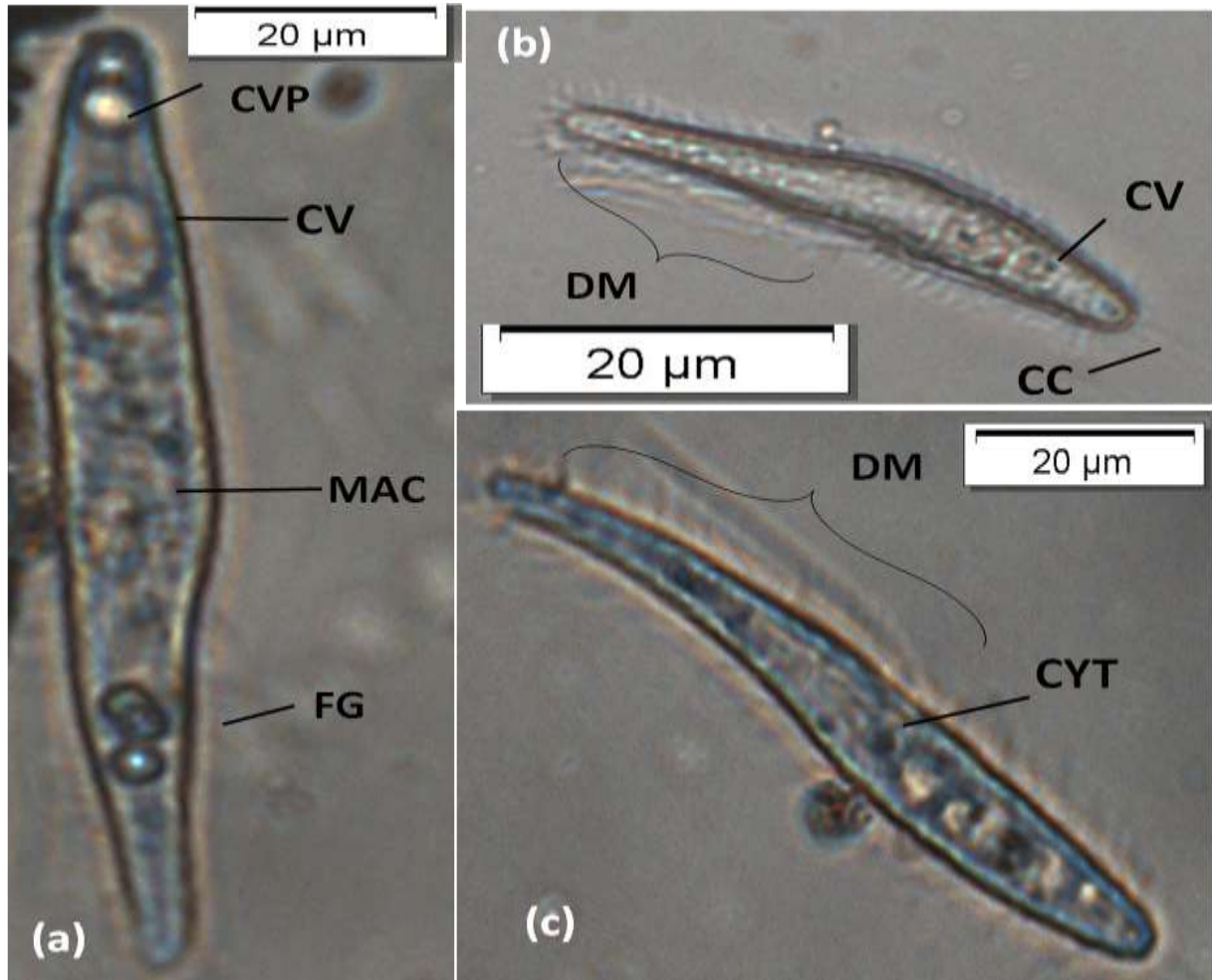


Fig. 3.3- Detailed Image of internal ultrastructure of coral-associated ciliate *Cohnilembus verminus*. **(a)** Showing CVP-Contractile vacuole pore, CV- Contractile vacuole, MAC – Macronuclei, FG- Food granule **(b)** Complete external ciliature of *C.verminus* **(c)** Showing DM Double membranelles, CYT – Cytostome. Scales included are added from microscopy images.

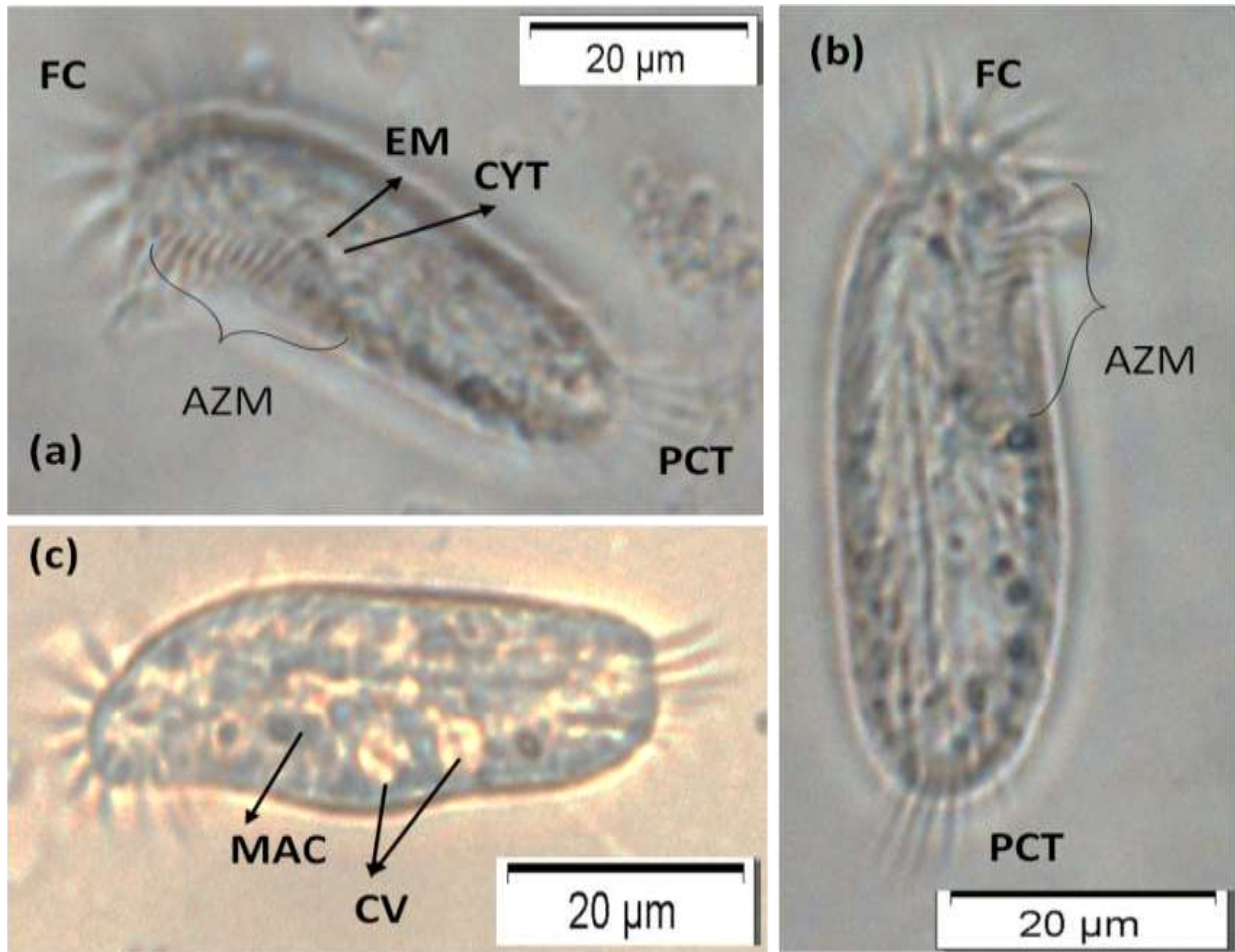


Fig. 3.4- Detailed Image of internal ultrastructure of coral-associated ciliate *Holosticha* sp. (a) Showing sagittal plane view with structures as FC – Frontal cirri, EM- elongated membranelle, CYT – Cytostome, PCT- Posterior cilila tuft and AZM – Adoral zone of membranelles (b) Another view of coral-associated ciliate *Holosticha* sp. (c) Showing MAC – Macronuclear nodules, CV – Contractile vacoules. Scales included are added from microscopy images.

3.3.2 Collection and culturing of coral-associated dominant ciliates

Coral-associated protozoans were successfully grown and cultured *in vitro* using synthetic media namely ASWP – Artificial Seawater for Protozoans. ASWP media along with wheat seeds was seen to support a maximum number of adults of both *Holosticha* and *Cohnilembus* ciliates for a longer period of time as compared to that of the other base media which were used such as *Acropora* sp. coral mucus (filtered and unfiltered) and seawater alone or sea water with mucus.

The isolated ciliates from the diseased corals were placed in the media for their growth. Abundant cells of ciliates, *C. verminus*, and *Holosticha* sp. multiplied and scattered homogeneously on the substrate. Notable differences in the ciliate cell numbers in various media parameters were observed at different time intervals from 6 h to 90 h (Fig. 3.5 and 3.6). Both, *C. verminus* and *Holosticha* sp. ciliates grew well with wheat seeds added media than the media without wheat seeds. This elucidated that the media with wheat seeds generating heterotrophic bacteria was an essential feed for the ciliate's growth. Also, the ciliate, *C. verminus*, and *Holosticha* sp. were observed for its maximum growth of trophont (adult) stage at 8 to 10 h according to the nutrient availability (Fig. 3.5 and 3.6). After consistent nutrient deprivation and feeding it is observed that repeatedly stressed ciliate protozoans transition observed from trophont (adult) to theront (larval) stage during 11 to 14 h which allowed its reproduction and survival (Fig. 3.7 and 3.8). However, repeated deprivation causes the cells to move into a tomont (resting stage) in *C. verminus* (Fig. 3.7) and not in *Holosticha* sp. The theront and tomont stages were found to respond to the last feeding immediately and switch to a trophont (adult stage) (Fig. 3.7 and 3.8). Moreover, the experiments revealed large differences in growth between *C. vermins* and *Holosticha* sp. cells that *C. verminus* cells grew faster/multiplied after 24 h when compared with that of the *H. diademata* cells growth (Fig. 3.5 and 3.6).

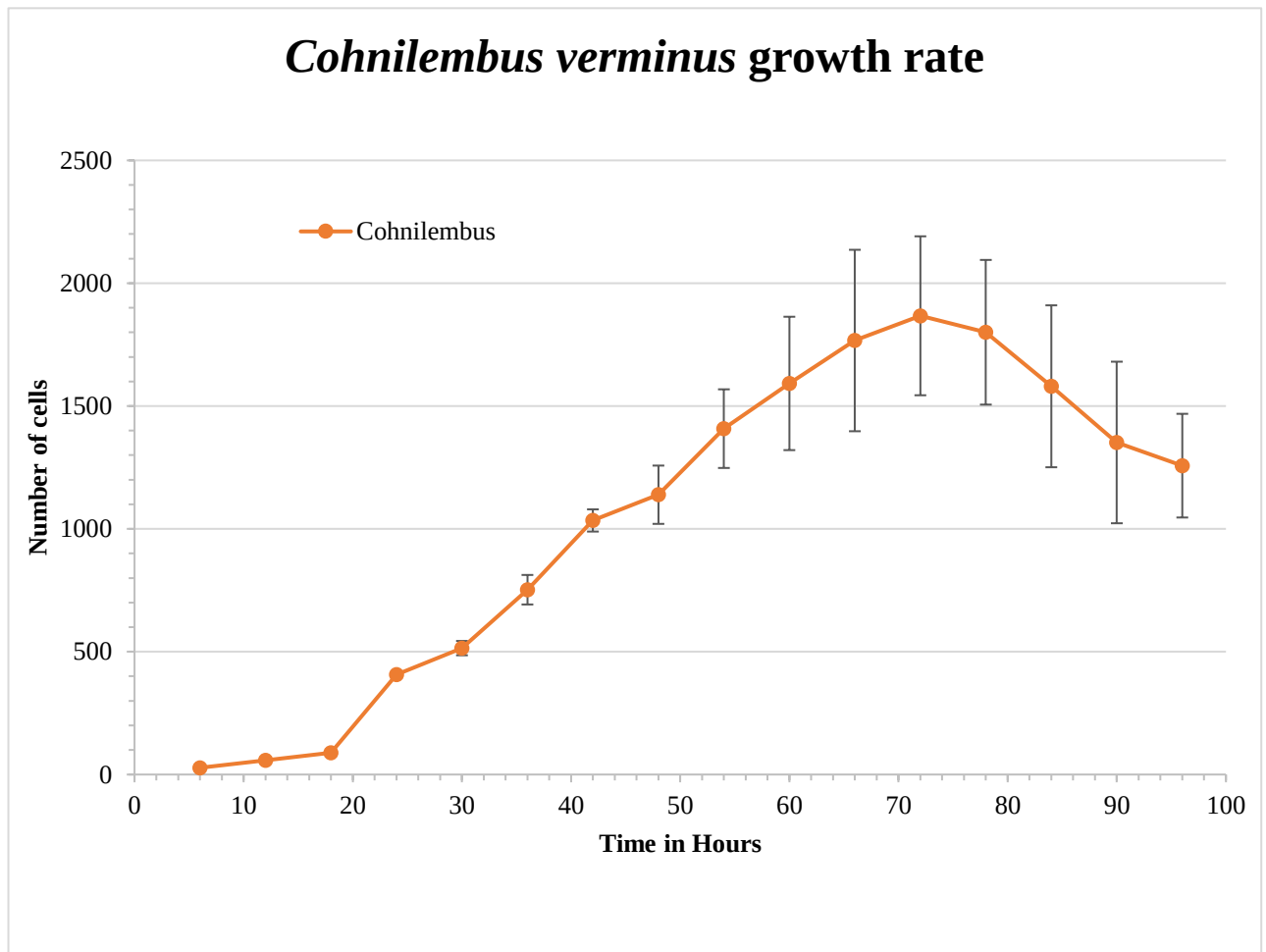


Fig. 3.5- *Cohnilembus* sp. was able to grow rapidly with high cell numbers in the media-added wheat seeds.

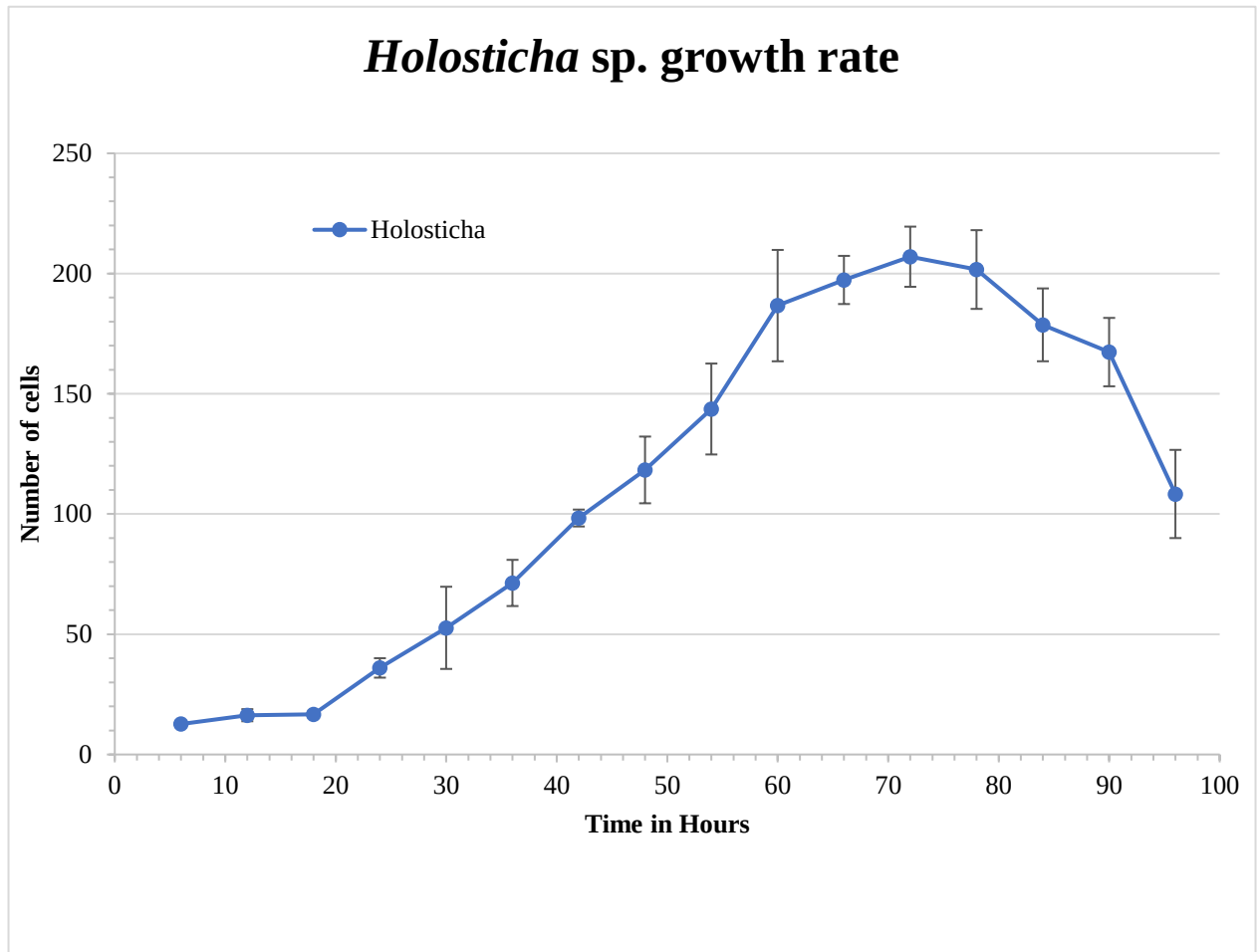


Fig. 3.6- The *Holosticha* sp. was slow in their growth rate with low cell numbers. Both the ciliates showed a rapid increase.

3.3.3- Microscopic identification of ciliates

Morpho 1 – Genus *Cohnilembus* Kahl, 1931; *Cohnilembus verminus* (O. F. Muller, 1786); Syn. *Lembus punctatus* Kahl, 1928; *Lembus reesi* Kahl, 1931.

Description:

Species *Cohnilembus* is a hymenostome ciliate of marine origin and has a tapered elongate shape with the anterior end being narrower and thinner than the posterior end which is slightly ovoid. The outline is very thin, Spindle-shaped or fusiform with the anterior portion considerably narrowed and tapering, size highly variable average length/width - 63 μm (55-77) / 6.05 μm (6-10) μm (Fig. 3.3). The cell is uniformly covered by somatic cilia. Several, relatively long, bent cilia are present at the tip of the narrow anterior end. The anterior half bears a double undulating membrane composed of fused ciliate that leads to the middle of the body. Cilia at the posterior end are slightly longer than those on the cell body with caudal cilia. It often moves forward in a straight line, stops to feed, and then moves again to a new location. Movements are not very active or fast. It was observed for positive thigmotactic. Macronucleus large, elongated to oval in shape, centrally located, which are often irregular-shaped (either two macronuclei or one macronucleus and one micronuclei). One to two micronuclei were closely associated with the macronucleus. The cytosome or mouth was in the center of the body. A contractile vacuole is located at the posterior end.

Morpho 2 - Genus *Holosticha* Wrzesniowski, 1877; Kahl, 1932.

Description:

The body was generally elongated, posteriorly rounded, with size highly variable average length/width 62.5 – 70 μm / 8 - 15 μm (Fig. 3.4). A higher number of marginal and mid-ventral cirri. Seven well-developed frontal cirri at the anterior. Two macronuclei, 2 micronuclei. Contractile vacuole (5-6 μm) in the middle of the body. The adoral zone of membranelles (AZM) extends over about 40% of the body length, having a wide gap at the front end of the body that is caused by the absence of membranelles and separated into two parts with anterior with 7-13 membranelles and the posterior composed of 15-25 membranelles. Active and fast jerky movements in circles were observed. The present study ciliate was similar to that of the *Holosticha diademata*, which is distinguished by the body shape and the presence of contractile vacuole in the post-equatorial position from the other

species. Thus, this species differs by the presence of a contractile vacuole on the post-equatorial position from the very similar *Holosticha pullaster* (in posterior 1/3), with which it is often confused.

3.3.4 The possible life cycle of ciliates

The isolated ciliates were observed for some of the sexual and asexual stages of the life cycle. Sexual reproduction by conjugation of two ciliate cells of a compatible mating type was observed in both the ciliates, *C. vermins* (Fig. 3.7) and *Holosticha cells* (Fig. 3.8). Sexual reproduction or autogamy is always followed by asexual reproduction. In the present study, *Holosticha* sp. and *C. verminus* sp. cells reproduced asexually by transverse division with the anterior half of the ciliate forming a new organism and the other posterior half forming another new cell (Fig. 3.7 and 3.8). *C. verminus* and *Holosticha* sp. were observed for their maximum growth of the trophont (adult) stage at 8 to 10 h (Fig. 3.7 and 3.8). Ciliate protozoans' transition was observed from the trophont (adult) to the front (larval) stage during 11 to 14 h which allowed its reproduction and survival (Fig. 3.5 and 3.6). However, a tomont (resting stage) stage was observed only in *C. verminus* (Fig. 3.7) and not in *Holosticha* sp.

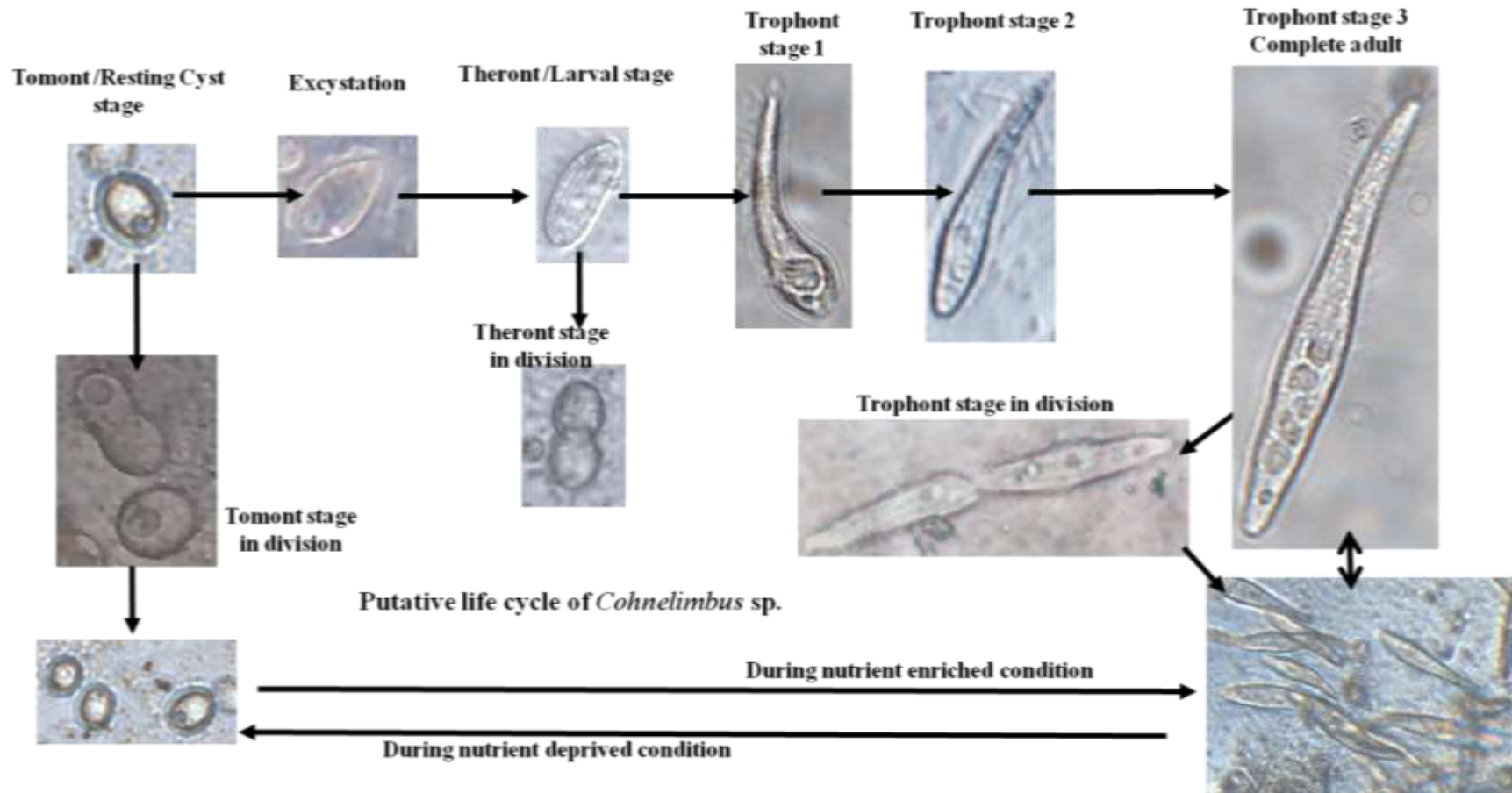


Fig. 3.7- Possible life cycle of *Cohnilembus*: Sexual and asexual reproduction was observed. Conjugation of two ciliate cells of a compatible mating type was with sexual reproduction. Asexual reproduction by transverse division with the anterior half of the ciliate forming a new organism and the other posterior half forming another new cell (Trophont stage in division) as the complete adult stage (Trophont). Tomont stages were found in the division and subsequently developed into theront or larval stages. The theront stages directly develop into Trophont or adult stages during favorable conditions or divide and multiply (Theront stage in division).

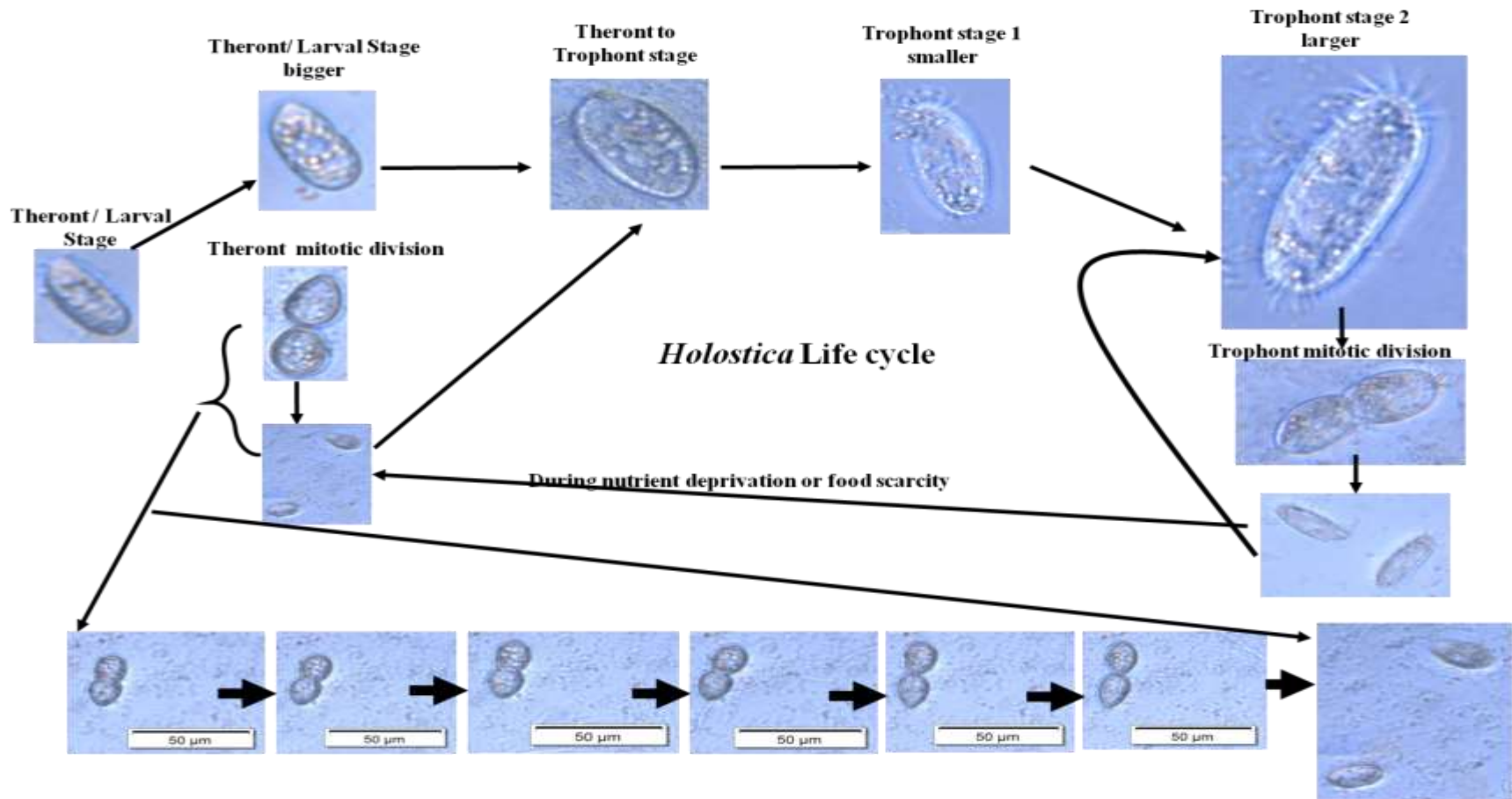


Fig. 3.8- Possible life cycle of *Holosticha*: The sexual and asexual reproduction was observed. Conjugation of two ciliate cells of a compatible mating type was with sexual reproduction. Asexual reproduction by transverse division with the anterior half of the ciliate forming a new organism and the other posterior half forming another new cell (Trophont stage in division) as the complete adult stage (Trophont). No tomont or resting stages were found. The theront stages directly develop into trophont or adult stages during favorable conditions or divide and multiply (Theront stage in division).

3.3.5 Molecular characterization of ciliates

The 18S rRNA gene sequences of the ciliates (GenBank accession numbers: MT561362- MT561365) were compared with sequences from the GenBank database (<http://www.ncbi.nlm.nih.gov>). Ciliate Morpho 1 (MT561362) was closed related to *Cohnilembus verminus* isolate FXP2009051101 (HM236339) with 97.1% sequence homology. Morpho 2 (MT561363) was 100 % similar to the *Uronema marinum* (AY551905). Morpho 3 (MT561364) was 100% similar to the ciliate *Holosticha diademata* isolate JJM2007091003 (KF306396). Morpho 4 (MT561365) was closely related to *Euplotes vannus* strain ME1 (AY361850) with 99.5 % sequence homology. Phylogenetic analysis based on the Neighbor-Joining method clearly showed that all these four ciliates were placed within the clades of respective genera (Fig. 3.9).

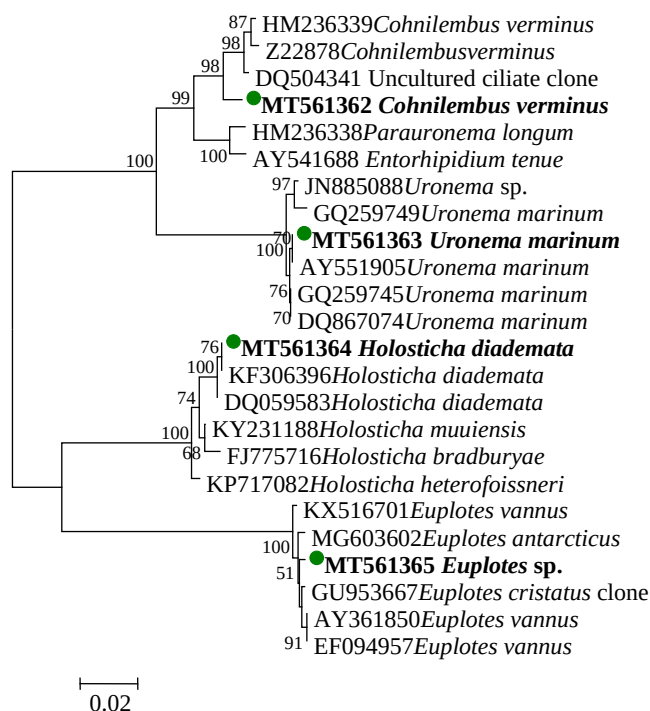


Fig. 3.9- Neighbor-Joining tree based on 18S rRNA gene sequences showing the phylogenetic relationship of Ciliates (in bold letters) with the GenBank closest similar sequences. Bootstrap percentages (500 replicates) are shown next to the branches >50 %. Bar, 0.02 substitutions per nucleotide position.

3.3.6- Ciliate feeding preferences with coral mucus

To analyze the feeding preferences of the ciliates with that of the substrates such as coral mucus (*C. verminus* and *H. diademata*), seawater, and freshwater, the ciliates were subjected to grow under different media components such as only coral mucus (mucus filter-sterilized (MF)) and unfiltered (MUF)), only seawater and freshwater without any additional media or mucus. *C. verminus* cells were placed in the substrate containing *Acropora* and *Porites* mucus for its growth analysis (Fig. 3.11). The ciliate cell numbers (trophont/adult stage) were found high with *Porites* mucus unfiltered (M (PUF)) and *Porites* mucus filtered (M (PF)). In contrast, the poor or negligible increase in ciliate cell numbers was observed in conditions with *Acropora* mucus filtered (M (AF)) and mucus unfiltered (M (AUF)), but the theront or larval (L) and tomont or resting stages (R) are observed more than the *Porites* mucus substrates (Fig. 3.10). While *C. verminus* cells were observed for fewer numbers with only seawater and no growth in freshwater.

Holosticha sp. cells differ from *C. verminus* by slow growth rate (Fig. 3.11) and as well as different feeding preferences. *Holosticha* sp. cells were placed in the substrate containing *Acropora* and *Porites* mucus for its growth analysis (Fig. 3.11). The ciliate cell numbers (trophont/adult stage) were found high with *Acropora* mucus unfiltered (M (AUF)) added with seawater followed by poor or negligible increase in *Acropora* mucus filtered (M (AF)), unfiltered *Porites* mucus (M (PUF)) and filtered *Porites* mucus (M (PF)) added with seawater. The theront or larval (L) stages were observed from the sixth hour and increased during the 18th hour in all the substrates used (Fig. 3.11). No tomont or resting stages were observed in *Holosticha* sp. cells.

Even though, *Holosticha* sp. was able to associate with the disease lesions of both the *A. muricata* and *P. lutea*, its high growth of cell numbers was observed with *Acropora* mucus unfiltered (M (AUF)) added with seawater when compared with that of the other conditions of mucus substrates (Fig. 3.11). Conversely, the good growth of *C. verminus* with PMUF and PMF of *P. lutea* and not in *A. muricata* suggests that *C. verminus* prefers feeding ingredients or components of *P. lutea* mucus than the mucus components of *Acropora* sp. Also, the subsequent field isolation of *C. verminus* from coral, *P. lutea*, and not from *A. muricata* implies that it may be host-specific to *P. lutea*.

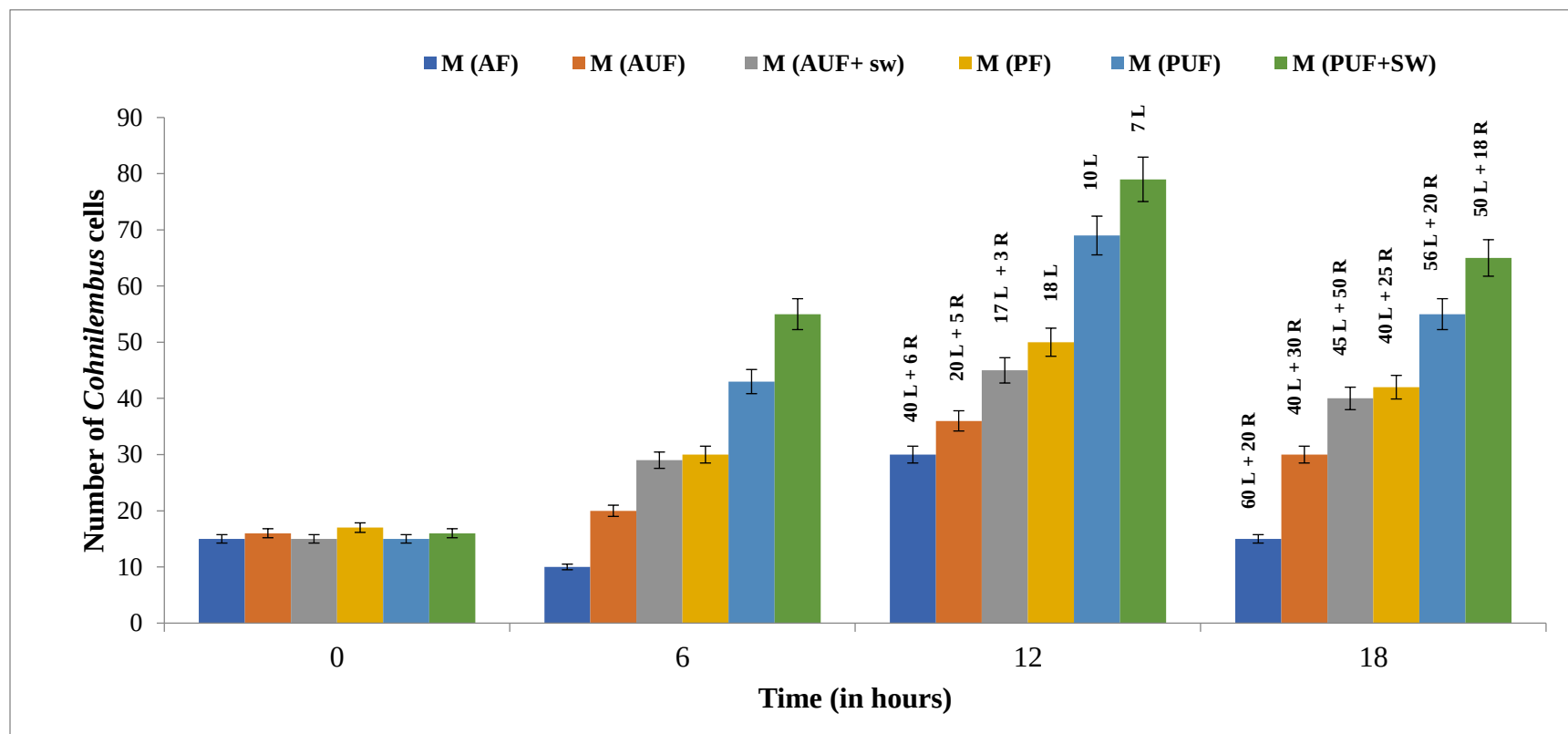


Fig 3.10 *Cohnilembus* sp. growth in coral mucus. *Cohnilembus* sp. cells were placed in the substrate containing *Acropora* and *Porites* mucus for its growth analysis. The ciliate cell numbers (trophont/adult stage) were found high with *Porites* mucus unfiltered (M (PUF)) added with seawater and *Porites* mucus filtered (M (PF)) added with seawater. In contrast, the poor or negligible increase in ciliate cell numbers was observed in conditions with *Acropora* mucus filtered (M (AF)) and mucus unfiltered (M(AUF)), but the theront or larval (L) and tomont or resting stages (R) are observed more than in the *Porites* mucus substrates.

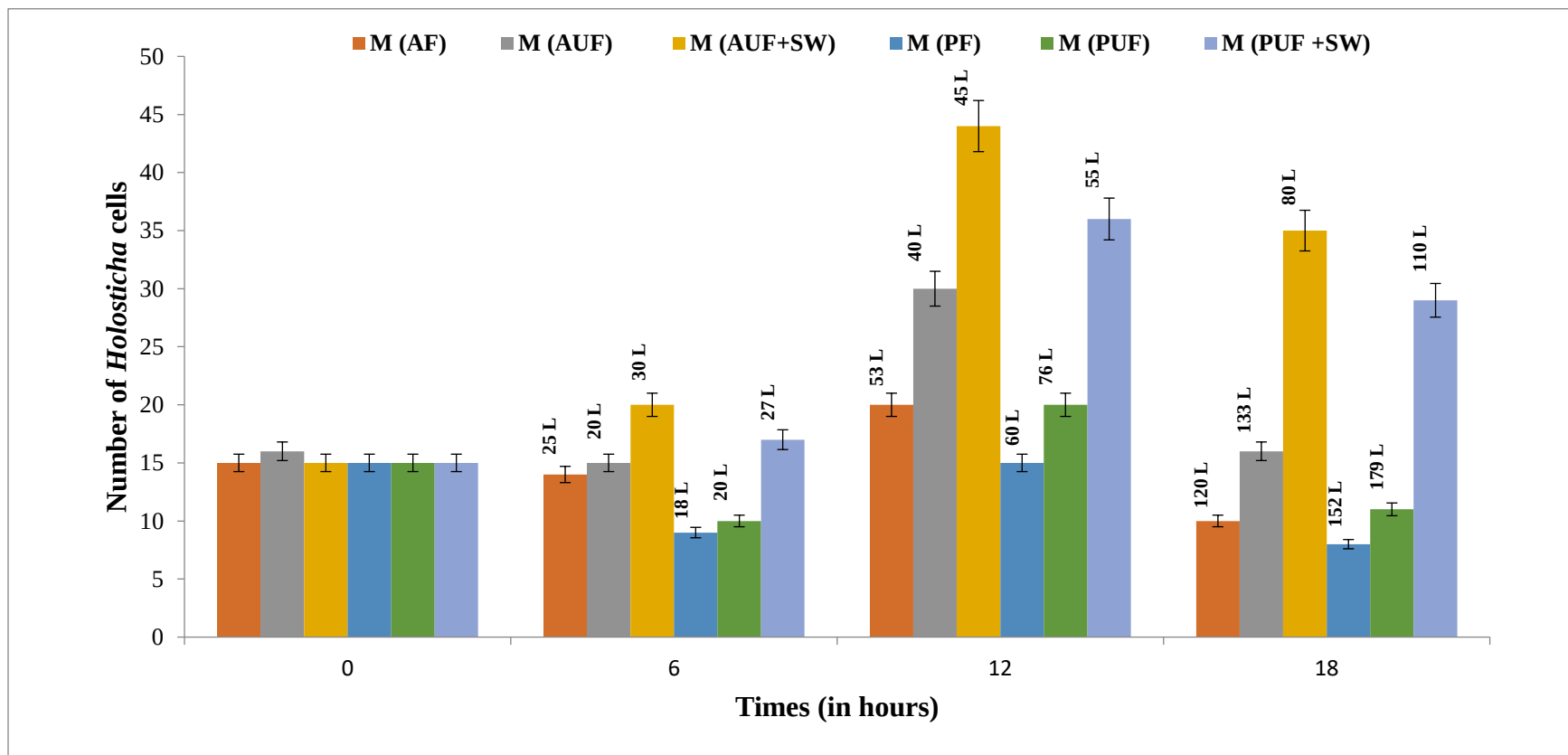


Fig. 3.11- *Holosticha* sp. growth in coral mucus. *Holosticha* sp. cells were placed in the substrate containing *Acropora* and *Porites* mucus for growth analysis. The ciliate cell numbers (trophont/adult stage) were found high with *Acropora* mucus unfiltered (M (PUF)) added with seawater followed by a poor or negligible increase in *Acropora* mucus filtered (M (AF)) and *Porites* mucus (M (PUF)) and M (PF) added with seawater. The theront or larval (L) stages were observed from the sixth hour and increased during the 18th hour in all the substrates used and no *tomont* or resting stages were observed.

3.3 Discussion

The ciliate association with *A. muricata* and *P. lutea* scleractinian corals from coral reef regions of India was investigated for the first time. Other than Indian reefs, only a few studies have been made on ciliates association with corals and all have been described for their occurrence with disease lesions (Sweet and Sere, 2015). Also, the ciliates have been reported for their feeding behavior with bacteria (Dopheide et al., 2011) and bacteria-associated denuded dead tissues (Sweet and Sere, 2015). The ciliates of the present study were also isolated from the diseased lesions of *A. muricata* and *P. lutea* from the Gulf of Mannar Marine National Park coral reef regions (Fig. 3.1 and 3.2; 3.3 and 3.4). Two stages of disease signs were observed for the occurrence of ciliates in *P. lutea*. One with dead tissue surrounded with pink inflammation (Fig. 3.1) and the other was with a white mat-like covering on the surface of the coral (Fig. 3.1). In *A. muricata* ciliates was associated with an initial white syndrome disease sign and with brown to black colour for the later stages (Fig. 3.2). The isolated two ciliates *Holosticha* sp. from both *A. muricata* and *P. lutea*, as well as *C. verminus* from the *P. lutea* (Fig. 3.3 and 3.4) were identified using the taxonomic keys, before morphological descriptions (Carey, 1992; Lee et al., 2000; Weibo-song, 2000; Weibo-song, 2002) and molecular characterization (Fig. 3.9). The similar observation and isolation of ciliates from two different corals with subsequent multiple field trips confirmed the association of ciliates with disease lesions. *C. verminus* belonging to Scutico ciliates often feed on bacteria and the enhanced feeding was observed when attached to a surface compared to free moving ciliate in the water column (Bourne et al., 2008). Therefore, it is possible that during the disease of *P. lutea*, *C. verminus* attached to areas of the dead and decaying tissues (Smith et al., 2015). *C. verminus* earlier has been reported for its association with the white syndrome-affected *Echinopora lamellose* (Smith et al., 2015), brown band disease of *A. muricata* (Bourne et al., 2008), and *A. hyacinthus* (Katz et al., 2014) as a close resemblance. However, the morphological characterization was not done microscopically for critical species-level identification for a *C. verminus* species. Thus, we identified and confirmed for the first time *P. lutea* associated *C. verminus* using microscopic and molecular characterization (Fig. 3.3, Fig. 3.9, and Fig. 3.6) The other dominant ciliate isolated from the diseased *A. muricata* and less found in the *P. lutea* was demonstrated it to be a species within the genus *Holosticha* belonging to hypotrichous ciliate (Fig. 3.9). The present study suggests

that the post-equatorial position contractile vacuole from *Holosticha* sp. had similarity with *Holosticha diademata* (Fig. 3.4). *H. diademata* has been described for its association with various coral disease signs of mostly of *Acropora* spp. and less with that of *P. lutea* from Australia, Solomons, Fiji, Maldives, UK, Venezuela, Columbia, Moyonette, Reunion and S. Africa (Katz et al., 2014; Sweet and Sere, 2015). This prevalent association of *Holosticha* sp. with diseased *Acropora* and *porites* species correlates with the present study and confirmed the occurrence and association of *Holosticha* sp. with the Indian diseased corals.

The coral-associated ciliate species were mostly described based on taxonomic and systematic studies and they were not focused on reproduction dynamics (Long and Zufall, 2010). The life cycle stages such as larvae and resting stages of the ciliate play a major role in their multiplication under favorable feed conditions. We observed some of the possible stages of the life cycle of both *C. verminus* and *Holosticha* sp. which were similar to the characteristic features of Scuticociliates and hypotrichous respectively (Fig. 3.3 and 3.4; Fig. 3.7 and 3.8). The observation suggested that reproductive stages of ciliates (Fig. 5.7 and 5.8) were connected with feeding habits such as trophont, the feeding, and division stage; tomite, swimming stage in search of feed; resting cyst stage (Long and Zufall 2010). Although, many trophonts appeared within 24 hours after inoculation with a known number of ciliate cells in wheat grain medium *C. verminus* cells grew faster/multiplies after 24 h when compared with that of the *Holosticha* sp. cells growth (Fig. 3.5 and 3.6).

No previous studies have been made on growth parameters for both the ciliates *C. vermins* and *Holosticha* sp., especially with coral mucus. To understand the mucus role as a preferred feed for the ciliates we proposed *in vitro* investigations using mucus with (unsterilized) and without (filter sterilized) bacteria (Fig. 3.10 and 3.11). Here, growth rates of cells of *C. vermins* and *Holosticha* sp. varied with different proportions when coral mucus (filtered /unfiltered mucus and mucus diluted with seawater) from *Porites* sp. and *Acropora* sp. was used as a growth medium (Fig. 3.10 and 3.11). Ciliate cells of *C. verminus* and *Holosticha* sp. were also found in moderate numbers with the substrate of seawater supplemented with coral mucus (filtered/unfiltered). This result could be triggered as an effect of active inhibitors from the concentrated mucus which has less effect when mucus was diluted with seawater allowing better microbial growth (Vacelet and Thomassin, 1991). Additionally, the growth of both the ciliates cells observed in the filtered mucus, which has

no bacteria, correlated with the earlier study that ciliates directly use the free sugars of the carbohydrate composition of the mucus (Teixeira et al., 2017). The poor growth of both the ciliates under the concentrated *A. muricata* mucus may be due to the strong inhibitors which had not allowed the growth of prokaryotic and eukaryotic microbes according to the early study (Vacelet and Thomassin, 1991). Secondly, under normal stress conditions, *A. muricata* mucus production was reported for its significant increase in a shorter period as compared to other corals (Thompson et al., 1980). Also, the surface rheology of *Acropora* sp. showed that it more easily and regularly secreted mucus in the form of webs and rings than *Porites* sp. This corroborates with the results showing that the excess supplied mucus in its crude concentrated form was not beneficial for the growth of bacteria and ciliates. However, its diluted form was an efficient food source for bacteria and bacterivorous ciliates majorly due to the absence or diluted effect of inhibitors.

The growth pattern of *C. verminus* and *Holosticha* sp. cells was quite similar when exposed to filtered (PUF) and unfiltered (PUMF) *Porites* sp. mucus (Fig. 3.10 and 3.11). In this case, it was observed that concentrated mucus was a better substrate for growth as compared to diluted mucus (Fig. 3.10 and 3.11). This could be because, firstly, the release of *Porites* sp. mucus took place over a longer period, and hence the net diffusion of nutrients also tends to be slower through the mucus sheet (Johnston and Rohwer, 2007) including the inhibitors. Secondly, the bacterivorous ciliates directly preferred the sugars instead of the bacteria as prey. This result of high cell numbers in both with and without bacteria conditions corroborates (Fig. 3.10 and 3.11) with the earlier study (Teixeira et al., 2017) which demonstrated that the ciliates swiftly ingested free sugars, stored them as the reserve of carbohydrates and outcompeted the bacteria for glucose (Teixeira et al., 2017). Differences in growth patterns of ciliates observed among two different mucus types as substrate reveal that *A. muricata* mucus was a better source of growth for microbes and associated ciliates (Fig. 3.10 and 3.11). This may be attributed to the different mucus carbohydrate compositions between *Porites* and *Acropora*. The levels of basic monosaccharides like glucose, arabinose and mannose are higher in *Acropora* sp. than *Porites* sp. and could be easily consumed by the bacteria and the ciliates (Hadaidi et al., 2019; Teixeira et al., 2017). These observations are corroborated with the present study where both the ciliate preferred as a substrate the *A. muricata* mucus (Fig. 3.10 and 3.11). Conversely, the low levels of basic monosaccharide

and higher levels of low bacterial degradability monosaccharide, fucose, and galactose (Hadaidi et al., 2019) support the present study for growth with lower ciliate cell numbers with *P. lutea* mucus (Fig. 3.10 and 3.11). However, the present study showed that the growth rate of *C. verminus* was more rapid compared to *Holosticha* sp. when growth in various proportions of substrates containing mucus from both the coral genera (Fig. 3.10 and 3.11). This may be due to the selective feeding preferences of the ciliates (Dopheide et al., 2011) such as selective grazing on particular prey types via both mechanosensory and chemosensory cues that have been observed in protozoa (Fenchel, 1980; Ayo et al., 2001).

In conclusion, our study investigated the association of ciliates with diseased lesions of *Acropora* and *Porites* sp. corals. Ciliates' feeding preference on mucus suggests that mucus plays an important role in the growth, proliferation, and adult active stages of the ciliates. Mucus ciliate interaction further confirms that the ciliate *Cohnilembus verminus* was specific to *Porites* sp. and *Holosticha* sp. was more specific to *Acropora* sp. corals.

3.4 Summary

- Coral mucus serves as a good source of nutrients and supports the ciliate's growth which may help ciliates survival and association with corals diseased lesions.
- The association between *Cohnilembus* and diseased lesions is exclusive to coral *Porites*, while *Holosticha* is observed in both *Acropora* and *Porites* corals.
- Both ciliates exhibit both sexual and asexual reproduction, with the resting cyst observed only in *Cohnilembus*. However, *Holosticha* was observed for higher growth rate.
- *Cohnilembus* displays faster growth compared to *Holosticha*. *Acropora* mucus serves as a nutrient source for both ciliates, but *Holosticha* more predominantly utilizes it.

Chapter four

Beneficial properties of coral mucus.

4.1 Introduction

Coral reefs are unique, diverse, economically important, and productive ecosystems, hence their description as the tropical rainforests of the ocean (Rosenberg et al., 2007). However, corals that form reefs are under threat due to various biotic and abiotic stressors such as disease, UV-radiation and temperature rise (Sharma and Ravindran, 2020). These stressors also influence the dissociation of the symbiont from the host coral which leads to coral bleaching, a response against oxidative stress (Downs et al., 2002). The generation of such oxidative stress takes place when the production and accumulation of reactive oxygen species (ROS) become uncontrollable by the host. These ROS damage the cell's macromolecules such as lipids, DNA, and proteins (Schieber and Chandel, 2014), and act as damaging or signaling molecules (Sharma et al., 2012). Besides, due to the expelled endosymbiont, the host coral becomes weak and immunocompromised. This allows primary and secondary pathogens to infect the coral tissue causing disease that leads to coral mortality.

Several studies have described the role of bacteria and ciliates as primary and secondary pathogens influencing the disease progression in immunocompromised corals (Sweet and Sere, 2016; Ravindran et al., 2020; Ravindran et al., 2021). Therefore, understanding the various responses of coral reefs to disease and bleaching events is critical for their natural resilience. Furthermore, the possible evolutionary adaptation of corals to various stress conditions enhanced the stress tolerance level of individual corals, implying that corals could remember and respond to their previous environmental experiences (Hackerott et al., 2021). This supports the past reports of experimental data showing that some Scleractinian corals have adaptive-like immune responses in addition to innate immunity (Rosenberg et al., 2007). This phenomenon is known as experience-mediated tolerance, and it occurs when corals that were previously susceptible to bleaching caused by a specific bacterial disease may develop immunity to it. For example, it has been established that the coral *Oculina patagonica* can become resistant to reinfection by *Vibrio shiloi* after being severely impacted by bleaching events caused by this bacterium (Reshef et al., 2006).

Coral mucus is a complex mixture of protein, carbohydrates, and lipids produced by mucocytes in the epidermal layer and utilized by the coral as an adaptation mechanism against various stress conditions (Brown and Bythell, 2005). As a result, coral surface tissue

that secretes the surface mucus layer (SML) serves as a first line of defense as well as a physiochemical and physiological barrier between animal tissue and external environment stresses (Brown and Bythell, 2005, Geffen et al., 2009, Bakshani et al., 2018). The bioactive compounds in coral mucus have been experimentally proven to have antimicrobial activity in the defense against infectious disease by inhibiting *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Vibrio* sp. and *Serratia marcescens* (Brown and Bythell, 2005, Ritchie, 2006, Bakshani et al., 2018). Besides, Mycosporine-like amino acids (MAA), which act as a sunscreen that absorbs UV radiation (Teai et al., 1998), and phenoloxidase (PO) and melanin activity in mucus of healthy and diseased *Pseudodiploria strigosa* (Rivera-Ortega and Thome, 2018) have also been described. Prophenoloxidase (ProPO) is a major enzyme of coral innate immunity (Cerenius et al., 2008) which gets upregulated due to oxidative stress. During proteolysis, ProPO gets converted into phenoloxidase (PO) by serine proteinase activity which finally stimulates the melanin synthesis pathway (Mydlarz et al., 2009), an indicator for corals to respond against pathogens (Palmer et al., 2011). The antioxidants, phenols, and flavonoids found in coral mucus that play an important role in free radical scavenging activity and the quorum quenching characteristic that inhibits infections and promotes coral health have not been studied or reported. It is believed that such defensive substances in mucus are derived as secretes from coral tissue to mucus (Fleming, 1922; Ritchie, 2006). However, no study was described for the presence of antioxidants and other defense substances reported from coral mucus.

Coral disease outbreaks due to bacterial infections have increased significantly. Especially, various *Vibrio* sp. involved in causing numerous coral diseases, namely *Vibrio rotiferanus*, *V. harveyi*, *V. alginolyticus*, *V. proteolyticus* together have been described as a consortium that causes Yellow blotch/band disease (YBD) (Cervino et al., 2008); *V. parahaemolyticus*, as the causative agent of *Porites* ulcerative white spot disease (PUWS) (Arboleda and Reichardt, 2010), a proline-rich P-toxin of *V. shiloi* induced bleaching of *Oculina patagonica* (Banin et al., 2001); *V. coralliilyticus*, the causative pathogen of white syndrome (WS) and bleaching in the coral *Pocillopora damicornis* (Ben-Haim et al., 2003a; Ben-Haim et al., 2003b). *V. carchariae*, *V. harveyi* causing white band in *Acropora* sp. (Ritchie and Smith, 1998). Also, the role of lysogenic prophages in the conversion of non-pathogenic to virulent strains has been described in a wide range of species, including *Vibrio*

cholerae, *Staphylococcus aureus*, and *Escherichia coli* (Fortier and Sekulovic, 2013) and especially in the coral pathogen, *V. coralliilyticus* (Weynberg et al., 2015).

The coral pathogen, *V. parahaemolyticus*, and *S. aureus* as control pathogens have been taken in the present study for their inhibition and suppression of virulence if any by coral mucus. Besides, the coral surface mucus layer that represents the primary defense against pathogens has been characterized for their defense properties such as the antimicrobial capabilities of mucus extend to a range of well-known pathogens associated with coral, including previously identified strains such as *Psychobacter alimentarius*, *P. pulmonis*, *Bacillus paramycoides*, *Pseudoalteromonas carrageenovora*, *P. espiana*, *P. neustonica*, and various *Vibrio* species. Additionally, beneficial bacterial properties and nitrogen cycling by mucus microbes by eliminating harmful excess ammonia were also analyzed. The mucus quorum quenching, antioxidants (for DPPH, hydroxyl radical and hydrogen peroxide scavenging, and metal chelating and reducing power activities and antioxidant enzymes (Superoxide dismutase, catalase, peroxidase, phenol oxidase) which may aid in understanding coral survival and resilience under various biotic and abiotic stress conditions.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Ammonium sulphate, Phosphate buffer saline, SDS, Coomassie blue stain, methanol, silver stain, developing solution, terminating solution, Muller-Hinton agar, Zobell Marine Broth (Himedia), Tris-EDTA buffer, Actinomycetes isolation agar (AIA), ISP2 media, destain solution, staining buffer (potassium phosphate buffer, EDTA, TEMED, riboflavin, and NBT), hydrogen peroxide, potassium ferric cyanide(Himedia), ferric chloride (Himedia), ammonium chloride, streptomycin, nitrite, sulphanilamide solution, N (1-naphthyl)-ethylene diamine dihydrochloride solution, Luria-Bertani (LB) agar (Himedia), nutrient soft agar (Himedia), Penicillin G (Himedia), starch, Iodine, Tryptic Soy broth, Dimethyl sulfoxide (DMSO), Sodium bicarbonate, Folin-Ciocalteu reagent, gallic acid, sodium nitrite (NaNO_2), aluminium chloride (AlCl_3), sodium hydroxide (NaOH), quercetin, ethanol, xylenol orange, butylated hydroxy anisole (BHA), and butylated hydroxytoluene (BHT) were obtained from Merck (Mumbai, India). DPPH was purchased from Sigma-Aldrich (Steinheim, Germany).

H₂O₂, HPLC grade methanol, deoxyribose, ferric chloride, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA), potassium hexacyanoferrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ferrous sulphate, ferrozine was of HiMedia (Mumbai, India). FOX reagent, Mannitol, Riboflavin, potassium ferric cyanide, guaiacol (Sigma-Aldrich). All other reagents were analytical grade.

4.2.2.1 Mucus protein extraction

Coral mucus 20 ml was centrifuged for 10 minutes, at 10,000 rpm (Thermo Scientific) to allow all the cell mass and impurities to settle down. Into the mucus supernatant, the desired amount of Ammonium sulphate i.e. 9.55 gm to get desired saturation, for protein precipitation at 25 °C (HiMedia, Mumbai, India) was dissolved by continuous stirring on a magnetic stirrer for 3 h till the Ammonium sulphate crystal formation, followed by 15 min of centrifugation at 10,000 rpm to pellet out the protein. Pellets were dissolved in phosphate-buffered saline (PBS) to analyze proteins. Protein samples were dialyzed out of the Ammonium sulphate with phosphate buffer saline pH 7.4.

4.2.2.2 Protein profile of diseased and healthy mucus

The above isolated protein samples were checked for their protein profile loading in a 10 % gel SDS-PAGE. After electrophoresis gel was kept with Coomassie blue stain for overnight incubation and then with the de-stain solution to remove the stain from the gel to visualize the protein bands on the gel. Further silver staining has been performed for better clarity of the protein separation (Hempelmann et al., 2017). Gel kept in fixation solution (containing 50 % methanol, 12 % acetic acid, and 0.05 % formalin) overnight. Fixation prevents protein movement from the gel matrix and removes interfering ions and detergent from the gel. Gel washed with 20 % methanol for 20 minutes by changing the solution 3 times in between. The washing solution (50 % methanol) was discarded and the gel was treated with a sensitizing solution (20 mg sodium thiosulphate / 100 ml quantity) for 1 minute with rotation, which increases sensitivity and contrast of staining. The sensitizing solution was discarded and the gel was washed twice with distilled water for 1 minute. Then gel was kept in a silver staining solution (200 mg silver nitrate, 75 µl formalin / 100 ml distilled water) in the dark with shaking for 20 minutes to allow silver ions to bind to the protein. After

washing with distilled water twice for 30 seconds to remove unbound silver ions. Rinsed with developing solution (6 % sodium carbonate, 50 µl formalin, and 0.8 mg sodium thiosulphate) and then incubated with the more developing solution with rotation until bands appear. As soon as bands developed reaction was stopped by adding a terminating solution (50 % methanol and 12 % acetic acid).

4.2.3 Mucus antimicrobial activity against pathogens

The disc diffusion method was performed to determine the antimicrobial activity of mucus against coral pathogens *Vibrio parahaemolyticus* and *Staphylococcus aureus* isolated from diseased corals. Different concentrations of *Acropora* sp. and *Porites* sp. mucus were used (20 µl, 30 µl, 50 µl, 70 µl, and 100 µl). The *S. aureus* and *V. parahaemolyticus* were spread-plated on Muller-Hinton agar plates and varied concentrations of mucus were placed in discs aseptically over the pathogen spread agar plates and incubated at 28 °C for 24 h.

4.2.4 Quorum quenching analysis

N-Acyl homoserine lactone (AHL) molecule quorum quenching by coral mucus was carried out by well diffusion plate assay (Tait et al., 2010). Quorum sensing (QS) regulates the violacein pigment production of *Chromobacterium violaceum* and is exploited for inhibitors of QS (McLean et al., 2004). Briefly, *C. violaceum* culture 100 µl was spread plated on Luria-Bertani (LB) agar plates. Wells were pricked in the spread agar plates and 100 µl of mucus was added to the well. The Plates were incubated for 24 h at 30 °C. After incubation, 0.7 % of nutrient-soft agar containing 200 µl of *C. violaceum* per 10 ml soft agar was poured over the colonies. All the plates were further incubated at 30 °C for 24 h and plates were monitored.

Violacein pigment production was tested based on the earlier method (Choo et al., 2006). Briefly, in 1.8 ml Tryptic soy (TS) broth, 0.1 ml *C. violaceum* culture and 0.1 ml mucus were added. *C. violaceum* was grown on TS broth at 30 °C on the shaker. After incubation, to precipitate the violacein pigment, 1 ml culture was centrifuged at 13,000 rpm for 10 min. Then 1 ml of DMSO was added to the pellet and resuspended well. Optical density was checked at 585 nm. The control was made without the addition of mucus. All tests were

performed in triplicates. Percentages of violacein pigment production are calculated by the following formula:

$$(\text{Absorbance of tested concentration at 585 nm} / \text{absorbance of the control at 585 nm}) \times 100.$$

The AHL lactonase enzyme activity on Penicillin G was done by incubating the reaction i.e., 200 μ l Penicillin G 20 mg/ml, 200 μ l 0.1 % starch, 200 μ l mucus sample, and 200 μ l water at 40 °C for 15 min (Mukherji and Prabhune, 2013). The lactonase enzyme is of the beta-lactamase superfamily that acts on the beta-lactam ring of Penicillin. Because of the high temperature used during the assay, some amount of Penicillin gets degraded. So, to avoid false readings, results were observed immediately after the addition of iodine. Controls were made without penicillin and enzyme, and only without enzyme. The exact mechanism behind the assay was that the AHL lactonase hydrolyzes penicillin into penicilloic acid. After the production of penicilloic acid, iodine is not free to interact with the 0.1 % starch and has no production of the blue colour, which confirms the presence of quorum quenching molecule in coral mucus. Thus, in control, without coral mucus, there is no formation of penicilloic acid and the iodine reacts with the starch and forms blue colour. Thus, the formation of the blue color depends on the interaction of free iodine with 0.1 % starch. The color formation was noted within 10 seconds of the 10 % Iodine reagent addition.

4.2.5 Antioxidants scavenging assays

4.2.5.1 Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging assay was determined using the method of Ravindran and Naveenan (2011). Mucus (25 μ l, 50 μ l, and 75 μ l) were taken and 50 mM of hydrogen peroxide (HiMedia Mumbai, India) was mixed in a ratio of 1: 1 (v / v). The reaction mixture was incubated for 30 min, at room temperature. After 30 min of incubation, 10 μ l of HPLC grade methanol, and 0.9 ml FOX reagent (ferrous oxidation-xylenol orange reagent) were added to the 90 μ l hydrogen peroxide solution (Sigma-Aldrich). The mixture was thoroughly mixed and placed at room temperature for 30 min. Blank was made by adding all the reagents except the sample. The ferric-xylenol orange complex formation was measured at 560 nm. The scavenging activity was measured based on inhibition of the complex

formation of ferric-xylene orange. To calculate scavenging activity, the following formula has been used:

$$\% \text{ H}_2\text{O}_2 \text{ scavenging} = [(\text{Control absorbance} - \text{sample absorbance}) / \text{control absorbance}] \times 100$$

4.2.5.2 Hydroxyl radical scavenging

The hydroxyl radical scavenging assay of coral mucus was carried out using the earlier method (Ravindran and Naveenan 2011; Ravindran et al., 2012a). The activity is based on spectrometric estimation of the breakdown product of 2-deoxyribose by condensation with Thio-barbituric acid (TBA). The hydroxyl radicals were generated by the Fe^{3+} ascorbate EDTA H_2O_2 system i.e. Fenton reaction. The reaction mixture consisted of 1 ml of 20 mM phosphate buffer (pH-7.4) containing 2.8 mM deoxyribose, 100 μM ferric chloride, 1 mM H_2O_2 , 100 μM EDTA, and different concentrations of *Porites* sp., *Acropora* sp. mucus (25 μl , 50 μl , and 75 μl). Ascorbic acid (100 μM) was added at last followed by incubation for 1 h at 37 °C. Then, 1 ml of 1 % Thio-barbituric acid and 2.8 % Trichloroacetic acid were added and the reaction was incubated for 15 min at 90 °C for color development. After cooling the tubes, optical density was measured at 532 nm. Mannitol was used as a control. The following formula measured the scavenging activity:

$$\% \text{ Hydroxyl radical scavenging} = [1 - (\text{sample absorbance} / \text{blank absorbance})] \times 100.$$

4.2.5.3 DPPH scavenging assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was performed as per earlier demonstrated methods (Ravindran and Naveenan 2011; Ravindran et al., 2012a) by adding 800 μl of 1 mM DPPH (Sigma- Aldrich Steinheim, Germany) solution prepared in 95 % ethanol with mucus (25 μl , 50 μl , and 75 μl). The reactions were vortexed well and kept at room temperature for 30 min in the dark. Control was made by using 1 ml of ethanol. Samples were centrifuged at 13,500 rpm for 5 min at RT. Optical density was measured at 517 nm. Butylated hydroxyanisole (BHA) (Merk Mumbai, India) was used as a reference compound.

% DPPH scavenging activity = [(Absorbance of the blank - Absorbance of the sample) / Absorbance of the blank] X 100.

4.2.5.4 Metal chelating assay

The metal ion chelating capacity of the coral mucus was quantified by the modified protocol (Dinis et al., 1994; Ravindran and Naveenan 2011; Ravindran et al., 2012a). The experiment was performed in HEPES buffer (pH 7.2, 20 mM). Different concentrations of mucus (25 µl, 50 µl, and 75 µl) were mixed with 12.5 µM ferrous sulphate solution. Into the reaction, 75 µM ferrozine solution was added and shaken vigorously. The mixture was kept for 20 mins and incubated at room temperature. After 20 min of incubation, the optical density was measured at 562 nm. EDTA was used as a positive control. The chelating capacity percentage was calculated by using the formula:

% Chelating activity = [(Absorbance of the blank - Absorbance of the sample) / Absorbance of the blank] X 100.

4.2.5.5 Reducing power determination

The reducing power of the mucus samples was determined by the method of (Ravindran et al., 2012a) with slight modification. Various concentrations of mucus (25 µl, 50 µl, and 75 µl) extracts (25 µl, 50 µl, and 75 µl) 0.5 ml were mixed with an equal volume of phosphate buffer (0.2 M pH-6.6) and 0.5 ml of 0.1 % potassium hexacyanoferrate. This reaction mixture was incubated at 50 °C for 20 min in a boiling water bath. After incubation 0.5 ml of 10 % Trichloroacetic acid was added to stop the reaction and 1 ml of the upper portion of the reaction mixture was mixed with 1 ml of distilled water, 0.1 ml of 0.01 % Ferric chloride (FeCl₃) followed by incubation at room temperature for 10 min. Optical density was measured at 700 nm. An increase in absorbance indicates increased reducing power.

4.2.6 Coral mucus antioxidant enzyme spectrophotometric assays

4.2.6.1 Superoxide dismutase activity

An assay of superoxide dismutase activity was done using the earlier method of our laboratory (Ravindran et al., 2012b). Coral mucus extract was diluted with 0.3 mM sodium

cyanide, 0.2 ml EDTA (0.1 M) solution, and 0.1 ml NBT (1.5 mM). Immediately 0.05 ml riboflavin (0.12 mM) was added and incubated for 12 min in light. Blank was made with distilled water in the place of coral samples. Optical density was measured at 560 nm. SOD activity was expressed as U/mg protein. The enzyme causing half the maximum inhibition reduction in NBT is known as one unit of SOD.

4.2.6.2 Catalase activity

Catalase activity was determined by using our earlier lab protocol (Ravindran et al., 2012b). 20 µl of healthy and diseased coral *Acropora* sp. and *Porites* sp. mucus extract was mixed separately into the reaction mixture of 30 µl phosphate buffer (50 mM) (pH 7.0) and 50 µl H₂O₂ (50 mM). The activity was measured at an optical density of 240 nm. Control was made without coral mucus and tissue samples. Catalase activity was calculated using the molar extinction coefficient for H₂O₂ at 240 nm (Anderson et al., 1995).

4.2.6.3 Peroxidase assay

Peroxidase activity assay was done by using the protocol developed by (Kiran et al., 2012). Mucus extract, 200 µl was mixed with 3.5 ml phosphate buffer pH 6.5 (100 mM) and added to 100 µl guaiacol (20 mM) (Sigma-Aldrich) in 10 mM phosphate buffer (pH 6.5) in the cuvette. The reaction was initiated by adding 200 µl of 200 mM H₂O₂ (Sigma-Aldrich), which was prepared in 10 mM phosphate buffer (pH 6.5). Spectrophotometric reading was measured at 430 nm. The change in absorbance per minute was measured from the linear phase. The blank was prepared with water. Peroxidase enzyme activity was expressed in units per mg protein.

4.2.6.4 Phenol oxidase assay

Phenol oxidase activity was measured by the procedure of Mydlarz and Palmer (2011) with slight modification. Dilutions were made by adding 30 µl of the coral mucus extract with 40 µl of 50 mM phosphate buffer, pH 7.5. To this reaction mixture, 25 µl of 0.1 mg/mL trypsin (HiMedia Mumbai, India) was added and kept for 10 mins incubation. After incubation, 30 µl of the guaiacol (10 mM stock concentration, 2.6 mM final concentration,

Sigma-Aldrich) was added and the developed coloured product was measured at an optical density of 470 nm for 15 min. Values are represented as units per mg of protein.

4.2.7 Electrophoretic assay

4.2.7.1 Superoxide dismutase activity

Superoxide dismutase (SOD) enzyme activity of healthy and diseased coral mucus of *Acropora* sp. and *Porites* sp. was checked in native-PAGE gel. SOD activity was visualized on the 10 % gel by the activity staining procedure (Ravindran et al., 2012b). After electrophoresis, the gel was washed with distilled water 2–3 times. Then it was treated with 0.1 % NBT (Sigma-Aldrich) on the shaker for 15 min in the dark. The gel was washed 2–3 times with distilled water. Riboflavin (Sigma-Aldrich) solution was added to the gel in the dark with shaking for 15 min. Again, the gel was washed 2–3 times with distilled water. Immediately the gel was exposed to light until the appearance of SOD activity bands on the gel.

4.2.7.2 Catalase activity assay

Catalase activity staining was performed on 6 % native-PAGE gel by the earlier method (Ravindran et al., 2012b). After electrophoresis, the gel was washed two times with distilled water. The gel was then rinsed with 50 mM phosphate buffer containing H₂O₂ 0.01 M, and for 20 min gentle rocking was given. Further, the gel was kept in 30 ml of 2 % potassium ferric cyanide (Sigma-Aldrich) and 30 ml of 2 % ferric chloride (HiMedia Mumbai, India) and mixed well until the appearance of yellow-colored bands over the lightbox. Mucus catalase activity was also demonstrated on a clean glass slide against 3 % hydrogen peroxide as a substrate. 10 µl mucus was added to the substrate and the production of air bubbles was observed to confirm the presence of catalase enzymes.

4.2.8. Screening of mucus-associated bacteria for their beneficial role

4.2.8.1 Antimicrobial activity of beneficial bacteria (BB) by well diffusion method

Coral-associated bacteria of BB 1-7 (seven strains) were isolated and identified. After 24 hours of the bacterial culture incubation at 120 rpm at 28 °C on a shaker in the media Zobell Marine Broth (Himedia), the 50 ml culture was centrifuged at 10,000 rpm for 10 min

and the supernatant was used for extracellular protein extraction by ammonium sulphate precipitation method. In 50 ml of supernatant, 23.89 gm ammonium sulphate had been dissolved at 25 °C to get 70 % saturation. This mixture was kept on a magnetic stirrer with continuous stirring for 3 hours. After incubation mixture was centrifuged at 10,000 rpm for 15 min and the pellet was suspended in 1 ml of TE buffer and stored at -20 °C until further use. For intracellular 10 ml of PBS have been added to the cell pellet and incubated for 10 min. After centrifuging at 8000 rpm for 10 min and the supernatant was discarded. The washing step was repeated. Then into the pellet ice cold 10 ml acetone was added and kept on ice for 30 min. After incubation, the samples were centrifuged at 8000 rpm for 10 min and the supernatant was discarded. Into pellet 1 ml of 1 %, SDS have been added to the pellet and incubated for 5 min. After incubation samples were centrifuged at 8000 rpm for 10 min and the supernatant was discarded. The pellets have been dissolved in 1 ml of PBS and stored at -20 °C. Protein concentration was measured by using nanodrop (Thermofisher) and also analyzed on 10 % SDS-PAGE gel.

4.2.8.2 Coral beneficial bacteria and pathogens used for antibacterial analysis

The extracted extracellular and intracellular proteins of the above BB strains (BB1- 2 strains and BB2- 5 strains) and coral mucus protein were used for their antimicrobial activity potential against various coral-associated pathogens such as *Serratia nematophila*, *Serratia marcescens*, *Vibrio vulnificus* (and 3 other species of *Vibrio*) including *MRSA*, *S. aureus*, which have been procured and maintained in the laboratory on Muller Hinton Agar (Himedia) plate by well diffusion method. 100 µl of a freshly grown active broth culture of coral pathogen spread plated on MHA plates and 100 µl intracellular and extracellular protein samples which were extracted from BB added into the well and kept in the incubator for 16-24 hours with PBS as control at an appropriate optimum temperature depending upon pathogen optimum growth conditions. After incubation, the zone of inhibition was observed and measured in mm, and plates were photographed.

4.2.8.3 Antimicrobial activity of Actinomycetes by cross streak plate method against known pathogens

Actinomycetes isolated from coral mucus grown on specific media such as Actinomycetes isolation agar (AIA) checked for their antimicrobial activity against known pathogens which were *S. marcescens*, *V. cholera*, *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi*, MRSA, *P. pulmonis* and *S. aureus* (which have been procured and maintained in the laboratory) on ISP2 media. Actinomycetes cultures were streaked on the middle of the ISP2 media plate and coral pathogens were streaked perpendicular to the actinomycetes culture. Plates were kept for 24 hours of incubation at 28 °C temperature.

4.2.8.4 In-vitro nitrite production by mucus-associated archaea and bacteria

Acropora sp. and *Porites* sp. HAM (healthy *Acropora* mucus), DAM (diseased *Acropora* mucus), HPM (healthy *Porites* mucus), and PLSM (*Porites* pink line syndrome mucus). mucus from each coral was collected. The collected 2 ml mucus was filtered through a 0.2 µm filter paper. The filter paper containing retained microbes was then transferred into 10 ml of sterile seawater, supplemented with 1 mM ammonium chloride, and maintained at a temperature range of 22-25 °C. This reaction mixture was then incubated for up to 28 days. To eliminate nitrified bacterial activity, two flasks were incubated with 500 mg/L streptomycin along with mentioned mucus microbial samples. Control was used as 8 ml filtered mucus in 2 ml sterile seawater and 1 mM ammonium chloride. Nitrite concentration was measured after 0,2,15,28 days intervals (Siboni et al., 2008).

Nitrite production estimation was carried out by diluting nitrite stock solution by 2 µm/L. 2 ml of samples were aliquoted in triplicates similarly blanks were made with 2 ml D/W. To each tube 40 µl sulphanilamide solution was added and followed by 40 µl N (1-naphthyl)-ethylene diamine dihydrochloride solution was added and mixed well after each solution addition. Incubate the mixture for 10 minutes. Absorbance was measured at 543 nm against D/W as a reference.

The factor (FNO₂) was calculated:

$$FNO_2 = 2 / \text{mean absorbance of standard NO}_2 - \text{mean abs. of the blank.}$$

4.2.9 Statistical analysis

All the experiments were carried out in triplicates. One-way ANOVA was used to compare means of the antioxidant assays and enzyme activities of healthy and diseased coral mucus (healthy *Acropora* sp. mucus, white patch *Acropora* sp. mucus, healthy *Porites* sp. mucus, and pink line syndrome *Porites* sp. mucus) samples by using SPSS software. The coefficient of determination r^2 was calculated using Microsoft Excel.

4.3 Results

4.3.1 Mucus protein profile

The study involved the extraction of mucus protein to conduct various enzyme assays. To analyze the protein composition, the protein profiles of different mucus samples were examined, including mucus from healthy *Porites* and various disease states such as pink line syndrome, white patch 1, white patch 2, and the black mat. This analysis was carried out using SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), (Fig. 4.1). When comparing healthy *Porites* mucus to diseased *Porites* mucus, it was observed that the expression of proteins in the diseased state was relatively higher (as depicted in Fig. 4.2). Notably, the protein bands in healthy *Porites* mucus appeared lighter in comparison to those in diseased *Porites* mucus. SDS-PAGE analysis of *Acropora* mucus (healthy vs. diseased) also revealed distinctive band patterns (Figure 4.3). The banding differences indicate variations in protein expression and composition between the two states. The observed difference in band intensity indicates potential variations in protein composition between healthy and diseased states, reflecting an alteration in the mucus protein profile under stressful conditions. This response is likely associated with stress and serves a defensive function in the coral.



Fig. 4.1- SDS page gel protein profile. Well, 1. Protein ladder 2. Healthy *Acropora* mucus 3. Pink line syndrome *Porites* mucus 4. Healthy *Acropora* mucus 5. White patch *Acropora* mucus 6. Healthy *Porites* mucus 7. Healthy *Porites* mucus 8. Black matt disease mucus.

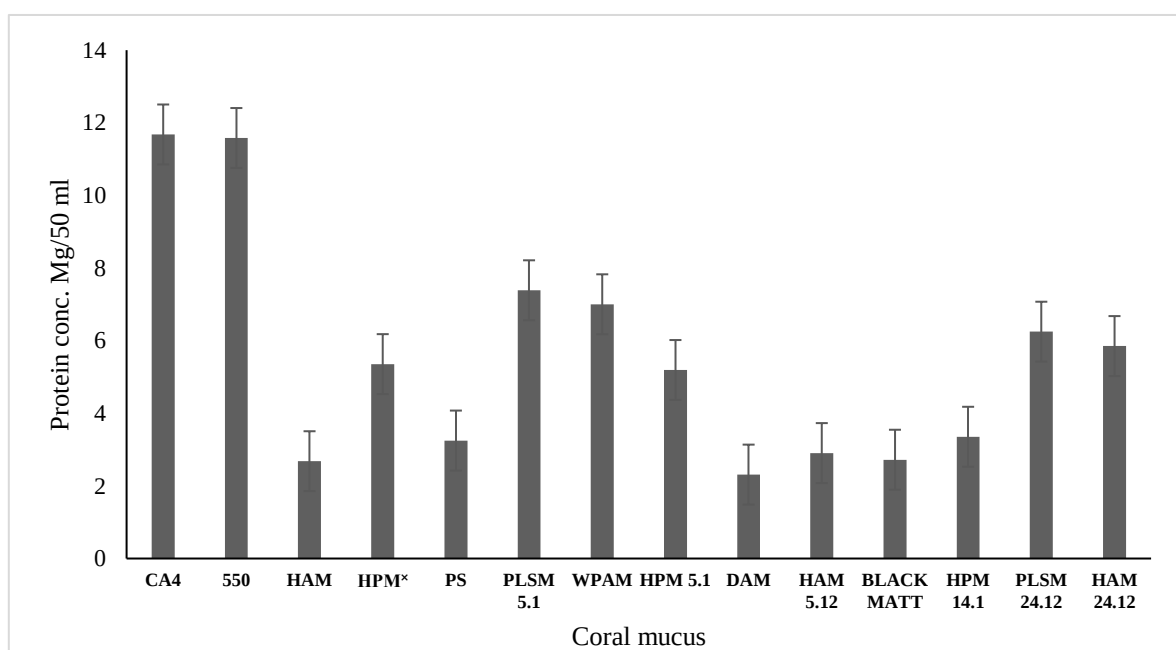


Fig. 4.2- Protein concentration found in coral mucus by Lowry method. CA- Mucus beneficial bacteria, HAM- Healthy *Acropora* mucus, WPAM- White patch *Acropora* mucus, HPM- Healthy *Porites* mucus, PLSM- pink line syndrome *Porites* mucus, PS- pink spot *Porites*, 550- *Bacillus*.

4.3.2 Coral mucus antimicrobial activity against bacteria

The healthy and diseased *Acropora* sp. and *Porites* sp. (Fig. 4.3A) mucus was observed for significant activity by inhibiting the coral pathogens of *S. aureus* and *V. parahaemolyticus* (Fig. 4.3B). The filtered mucus of *Porites* sp. and *Acropora* sp. were observed for potential antimicrobial activity with an increased concentration against *S. aureus* and *V. parahaemolyticus* (Fig. 4.3B). Thus, the zone of inhibition was increased with the increased concentration of mucus, suggesting the antagonistic ability of the coral mucus against the coral bacterial pathogens was concentration-dependent.

4.3.3 Quorum quenching property of coral mucus

A zone of clearance was observed in a *C. violaceum* spread plate assay around the well-containing diseased and healthy mucus (Fig. 4.4A). This reveals that mucus can interfere with QS produced by coral host pathogens. The Violet colour of the violacein pigment with quorum sensing property was seen in the negative control wells. The inhibition of quorum sensing by mucus was investigated by the use of violacein pigment of *C. violaceum* that was estimated by spectrophotometric assay. Violacein pigment, a quorum-sensing molecule of *C. violaceum* production was inhibited by healthy and diseased coral mucus (Fig. 4.4B). The healthy *Acropora* sp. mucus, healthy *Porites* sp. mucus, and *V. harveyi* showed maximum inhibition of violacein pigment production due to the abundance of quorum quencher molecules. Whereas less inhibition was seen in PLSM (pink line syndrome *Porites* sp. mucus) and white patch *Acropora* sp. diseased coral mucus. Control only with *C. violaceum* showed maximum production of violacein. Therefore, a fall in the violacein concentration suggests the inhibition of quorum sensing. The healthy and diseased *Acropora* sp. and *Porites* sp. mucus were observed to be positive for lactonase enzyme presence (Fig. 4.4C). The AHL lactonase of the coral mucus hydrolyzes penicillin into penicilloic acid, confirming the presence of quorum quenching molecule in coral mucus (Fig. 4.4C).

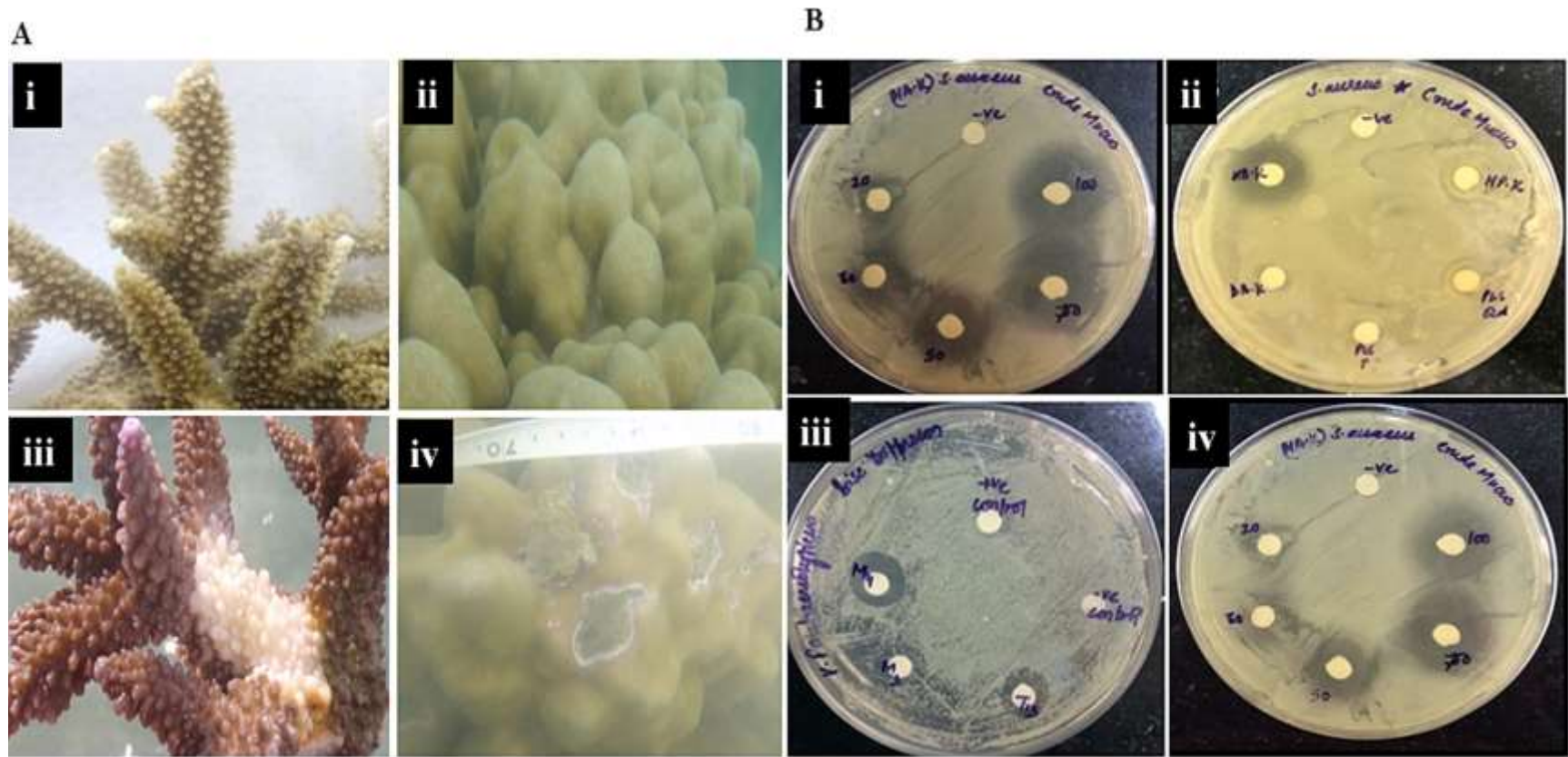


Figure 4.3- A. Healthy and diseased *Acropora* sp. and *Porites* sp. **i.** *Acropora* sp. healthy, **ii.** *Porites* healthy, **iii.** *Acropora* sp. white patch disease, **iv.** *Porites* sp. pink line syndrome disease. **B.** Antimicrobial activity of mucus against coral pathogens- **i.** *Acropora* and *Porites* mucus activity against *S. aureus* **ii.** *Porites* mucus activity against *S. aureus*, **iii.** *Acropora* and *Porites* mucus activity against *Vibrio parahaemolyticus*, **iv.** Filtered *Acropora* and *Porites* mucus against *S. aureus*.

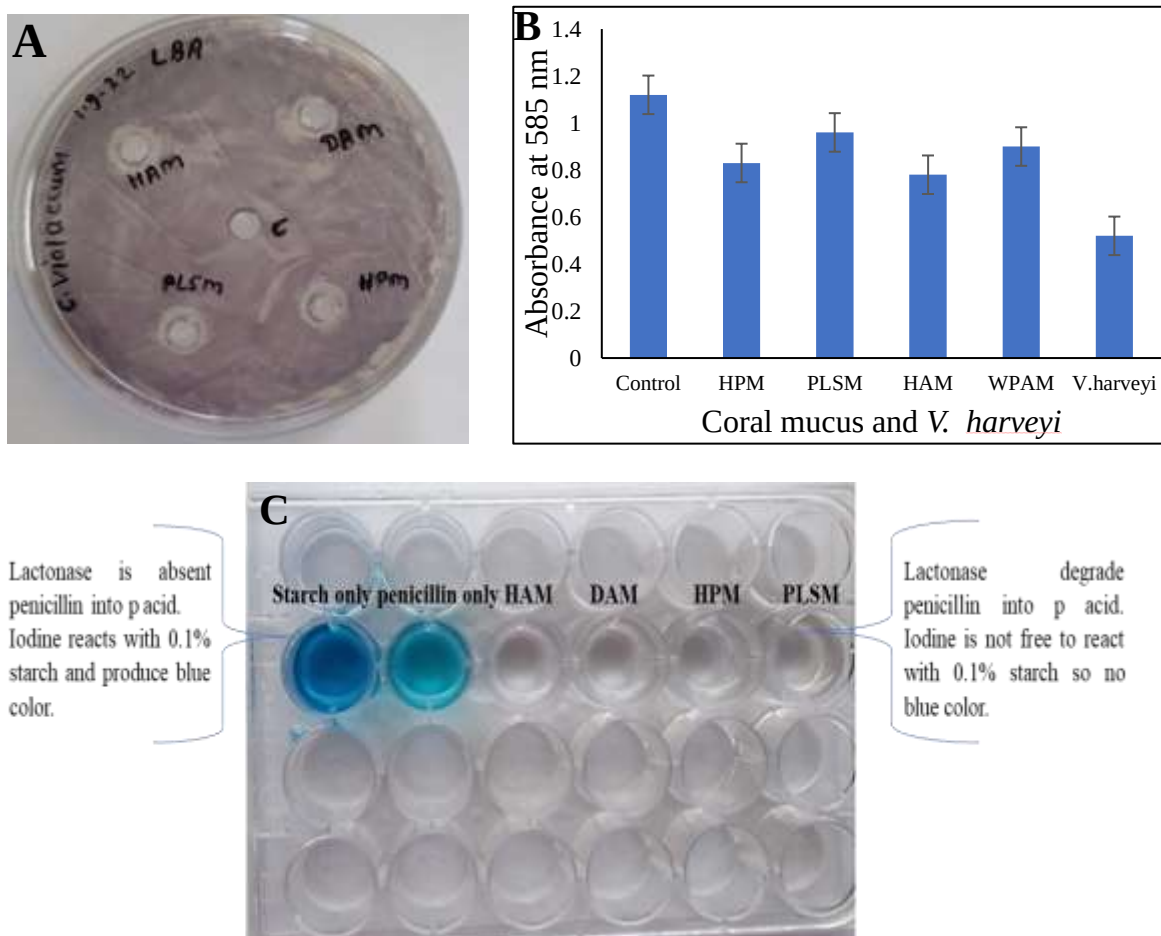


Figure 4.4- AHL quorum sensing molecule inhibition assay. **A.** (i) Positive control *V. harveyi* inhibiting violet color by degrading AHL molecule of *C. violaceum*. (ii) In wells CA4- *C. amphilecti* inhibited the AHL molecule and produced a clear zone of inhibition. (iii) Healthy and diseased *Acropora* and *Porites* mucus showing zone of AHL inhibition. **B.** Iodometric assay to determine the hydrolyzing activity of mucus AHL lactonase. Wells containing positive control penicillin without test samples showing blue color formation. Healthy *Acropora*, white patch *Acropora*, healthy *Porites* and pink line syndrome disease *Porites* mucus shows no color formation. **C.** Inhibition of violacein production by coral mucus. Violacein production was measured spectrophotometrically as described in the materials and methods. Data are presented as absorbance at 585 nm. (HAM- Healthy *Acropora* sp. mucus, DAM/WPAM-White patch *Acropora* sp. mucus, HPM- Healthy *Porites* sp. mucus, PLSM- Pink line syndrome *Porites* sp. mucus).

4.3.4 Coral's antioxidant scavenging activity

4.3.4.1 Hydrogen peroxide scavenging activity

Diseased mucus actively scavenged hydrogen peroxide and showed maximum activity (Fig. 4.5). The healthy coral mucus of both *Acropora* sp. and *Porites* sp. coral species exhibited poor hydrogen peroxide scavenging properties than the diseased coral mucus. One-way analysis of variance (ANOVA) determined a significant increase in (more 12.75 % ($p < 0.05$)) hydrogen peroxide scavenging activity in the diseased *Porites* sp. mucus than in healthy mucus (8.91 %). *Porites* sp. mucus displayed approximately 2-fold more hydrogen peroxide scavenging than healthy *Acropora* sp. 7.65 % and diseased *Acropora* sp. 8.91 % mucus at 50 μ l concentration. While the diseased *Porites* sp. mucus exhibited a maximum activity of 14.57 % at a 75 μ l concentration.

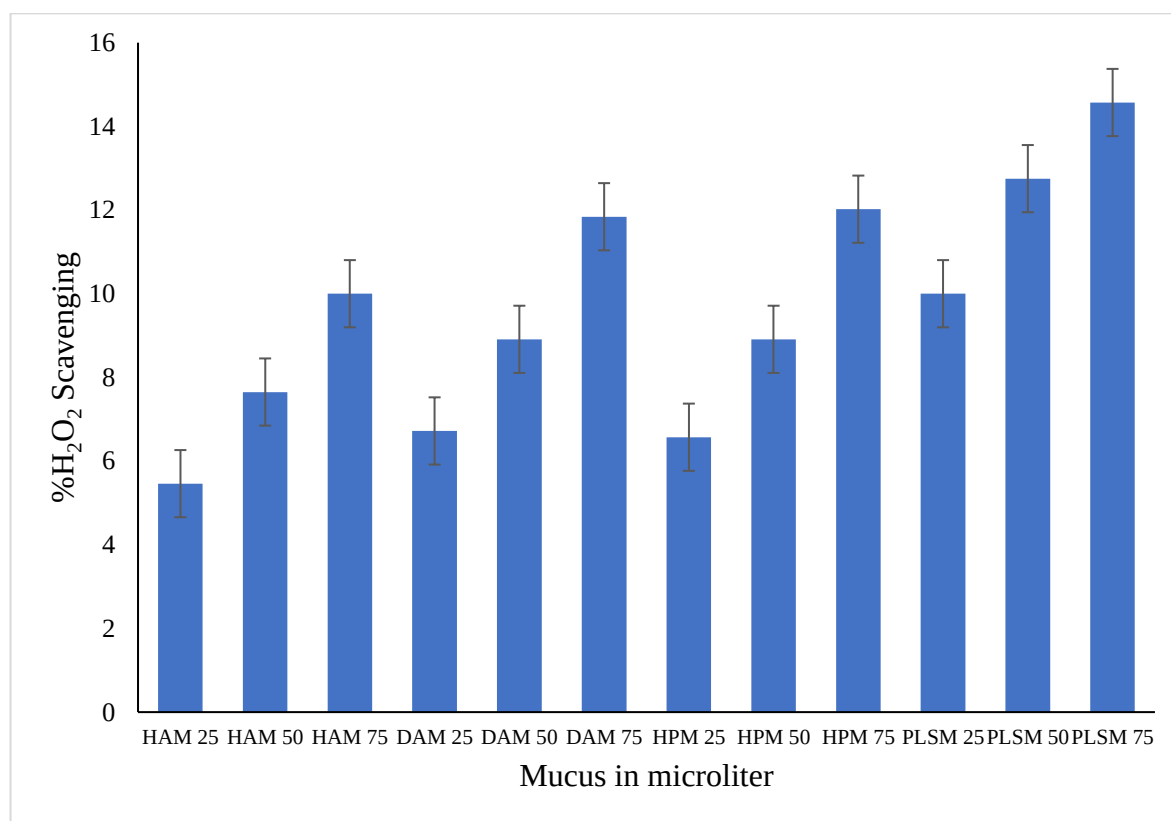


Figure 4.5- Hydrogen peroxide free radical scavenging activity at increasing concentrations of mucus. Values are mean $\pm$ SD ($n = 3$). All the experiments were carried out in triplicates. HAM- Healthy *Acropora* mucus ($r^2 = 0.9987$, p -value = 0.02), DAM-White patch *Acropora* mucus ($r^2 = 0.9997$, p -value = 0.009), HPM- Healthy *Porites* mucus ($r^2 = 0.9926$, p -value = 0.54), PLSM- Pink line syndrome *Porites* mucus ($r^2 = 0.9849$, p -value = 0.7).

4.3.4.2 Hydroxyl radical scavenging activity

Coral mucus hydroxyl radical scavenging capacity was analyzed and compared with standard mannitol (Fig. 4.6). The hydroxyl radical scavenging activity of *Acropora* sp. mucus and *Porites* sp. mucus was subsequently found high in diseased corals. Hydroxyl radical scavenging of *Acropora* sp. diseased mucus at 75 μ l concentration was statistically significant ($p < 0.03$) in diseased mucus (5.40 %) than in healthy mucus (3.85 %). Similarly, *Porites* sp. mucus also revealed statistically significant ($p < 0.05$) scavenging activity in diseased mucus compared to healthy mucus at all three concentrations (25 μ l, 50 μ l, and 75 μ l). The scavenging activity at 25 μ l concentration, varies from 0.98 % in healthy *Acropora* sp. mucus to 5 % in pink line syndrome-affected *Porites* sp. mucus. As compared to *Acropora* sp. mucus, *Porites* sp. mucus displayed 2–2.5-fold more activity in healthy and up to 3.5-fold more activity in diseased mucus to scavenge the hydroxyl radicals.

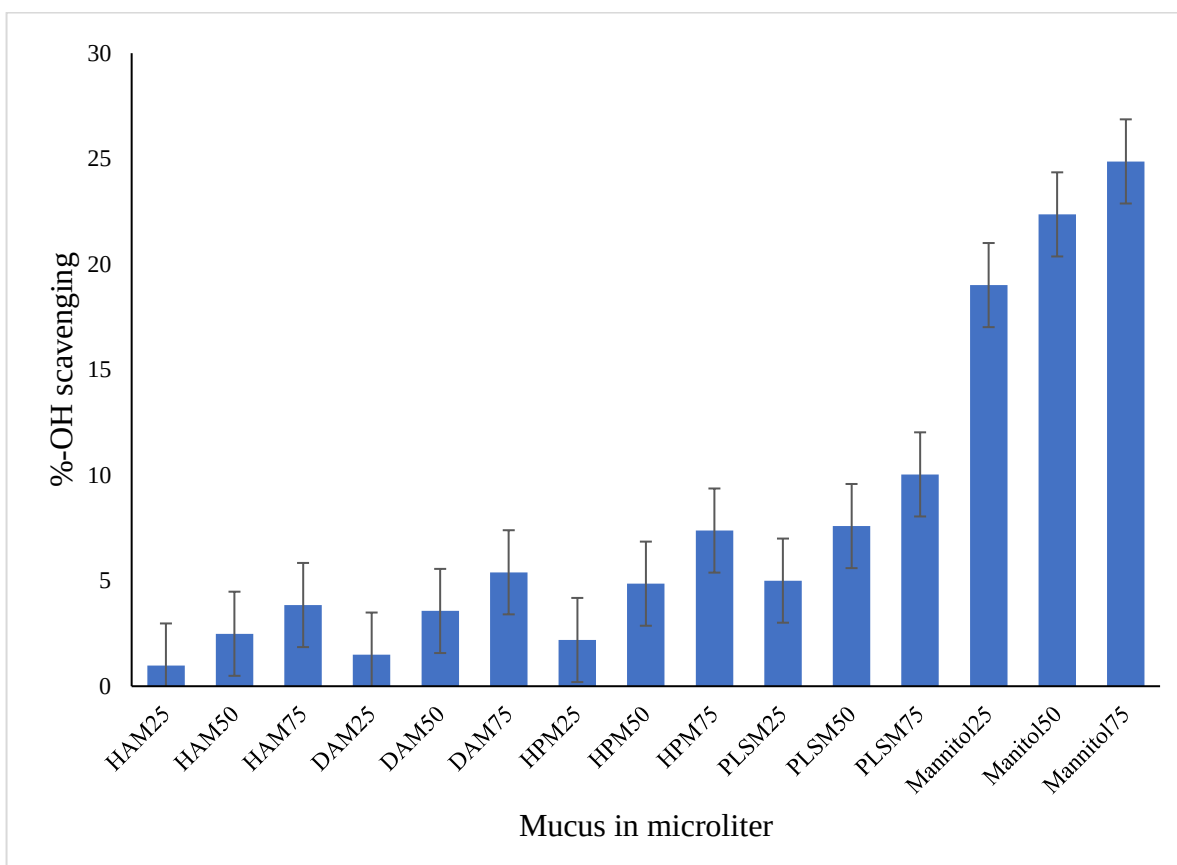


Figure 4.6- Hydroxyl radicals scavenging capacity observed in corals. Mannitol is used as a reference compound.

4.3.4.3 DPPH scavenging activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was demonstrated based on the ability of DPPH to react with proton donors such as phenols (Fig. 4.7). The DPPH scavenging activity was checked for the *Porites* sp. and *Acropora* sp. mucus and the butylated hydroxy anisole (BHA) was used as the reference compound. Wherein, the reaction mixture changed color from purple to yellow. At 25 μ l concentration of mucus, healthy *Acropora* sp. showed (19.49 %) DPPH scavenging, while *Porites* sp. mucus displayed significance ($p < 0.03$) in diseased mucus (23.13 %) than healthy mucus (20.94 %). Similarly, at 75 μ l concentration, DPPH scavenging was found more ($p < 0.01$) in diseased *Porites* sp. mucus (27.69 %) than in healthy *Porites* sp. mucus (25.68 %). Thus, the ability of coral mucus to scavenge the DPPH radicals was observed predominantly more in diseased coral mucus.

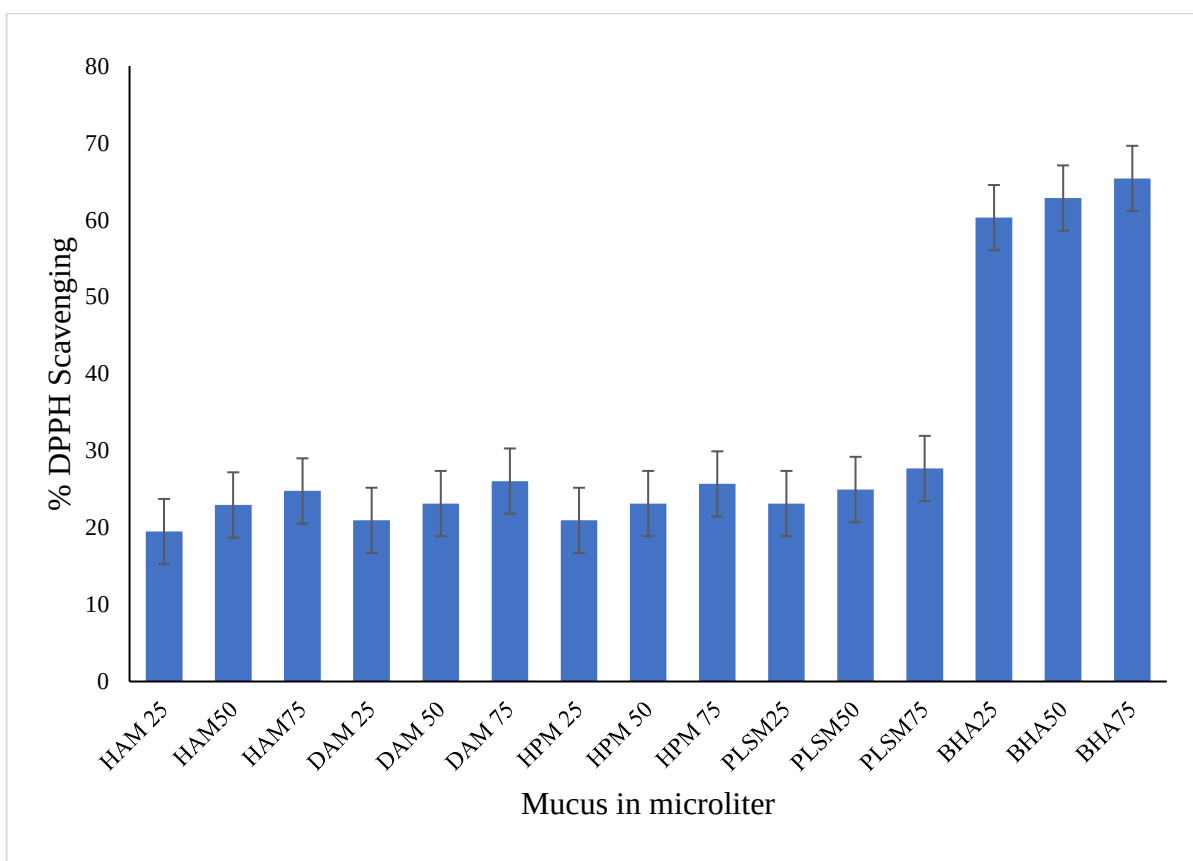


Figure 4.7- The 2,2-diphenylpicrylhydrazyl (DPPH) scavenging activity present in coral mucus. BHA- Butylated hydroxy anisole is used as standard.

4.3.4.4 Metal chelating assay

The metal ion chelation capacity of coral *Acropora* sp. and *Porites* sp. mucus was analyzed (Fig. 4.8). EDTA was used as a reference compound and the metal ion chelation was observed in all the coral mucus. Diseased *Acropora* sp. mucus at 75 μ l concentration showed statistically significant ($p < 0.05$) metal chelation than *Acropora* sp. healthy mucus. *Porites* sp. diseased mucus at 25 μ l ($p < 0.02$) and at 75 μ l concentration ($p < 0.01$) showed significantly more activity (8.47 % and 10.12 %) than healthy *Porites* sp. mucus (7.4 % and 8.96 %). The capacity of mucus to capture and bind reactive metal ions was found high in *Porites* sp. diseased mucus. Thus, coral mucus interferes with the formation of the Fe²⁺ - ferrozine complex and captures the ferrous ion before ferrozine.

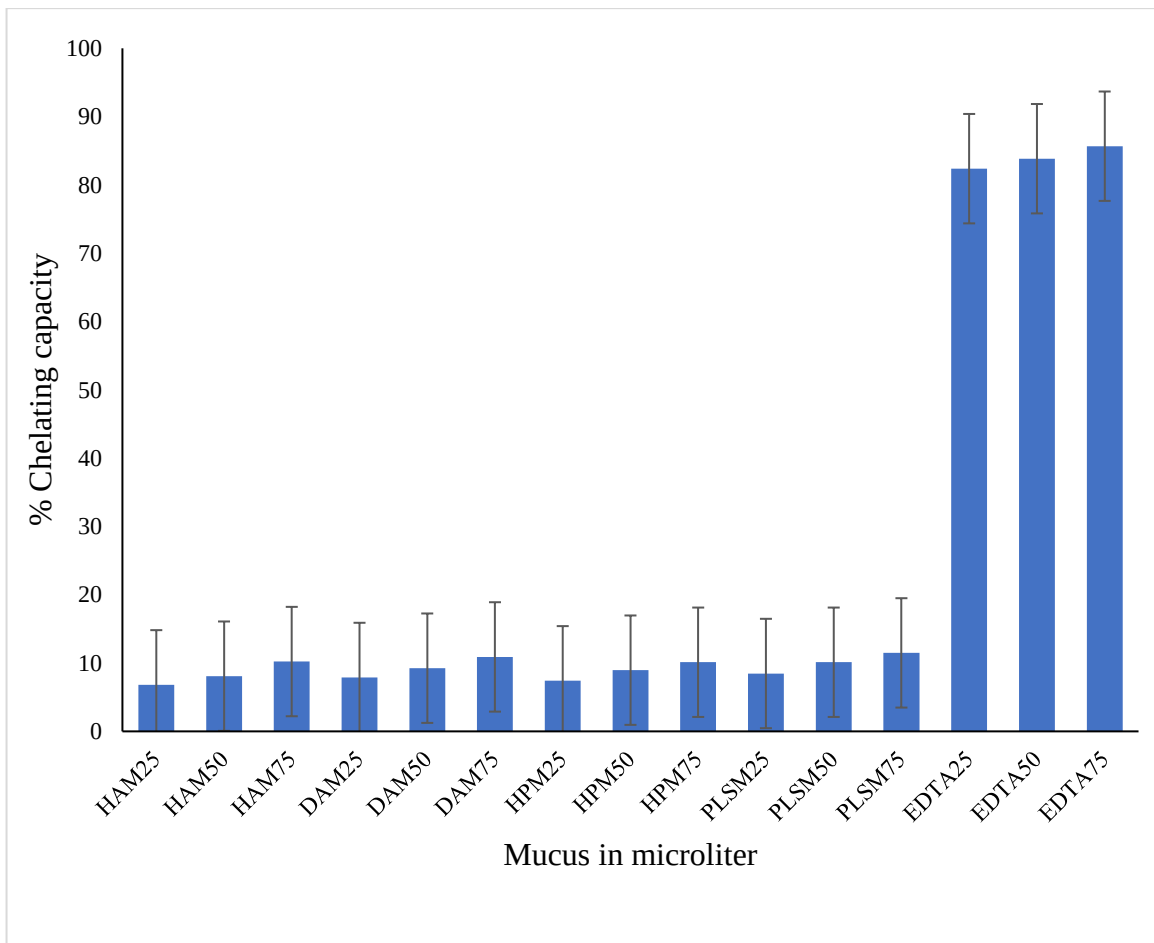


Figure 4.8- Metal ion chelation capacity of corals, EDTA-Ethylenediamine tetra-acetic acid.

4.3.4.5 Reducing power assay

There were significant linear correlations ($r^2 = p < 0.05$) between total antioxidants and the reducing ability of coral mucus (Fig. 4.9). The potential of mucus to convert harmful reactive oxygen species into less harmful substances was analyzed at various concentrations of mucus. In both *Porites* sp. and *Acropora* sp., the reducing power of mucus was observed high in diseased corals than in healthy ones. There was a significant difference ($p < 0.05$) between the reducing power of healthy and diseased *Acropora* sp. mucus and *Porites* sp. mucus. In diseased *Acropora* sp. mucus reducing power was 0.11 unit and whereas in *Porites* sp. diseased mucus it was found 0.12 unit which was recorded maximum in mucus.

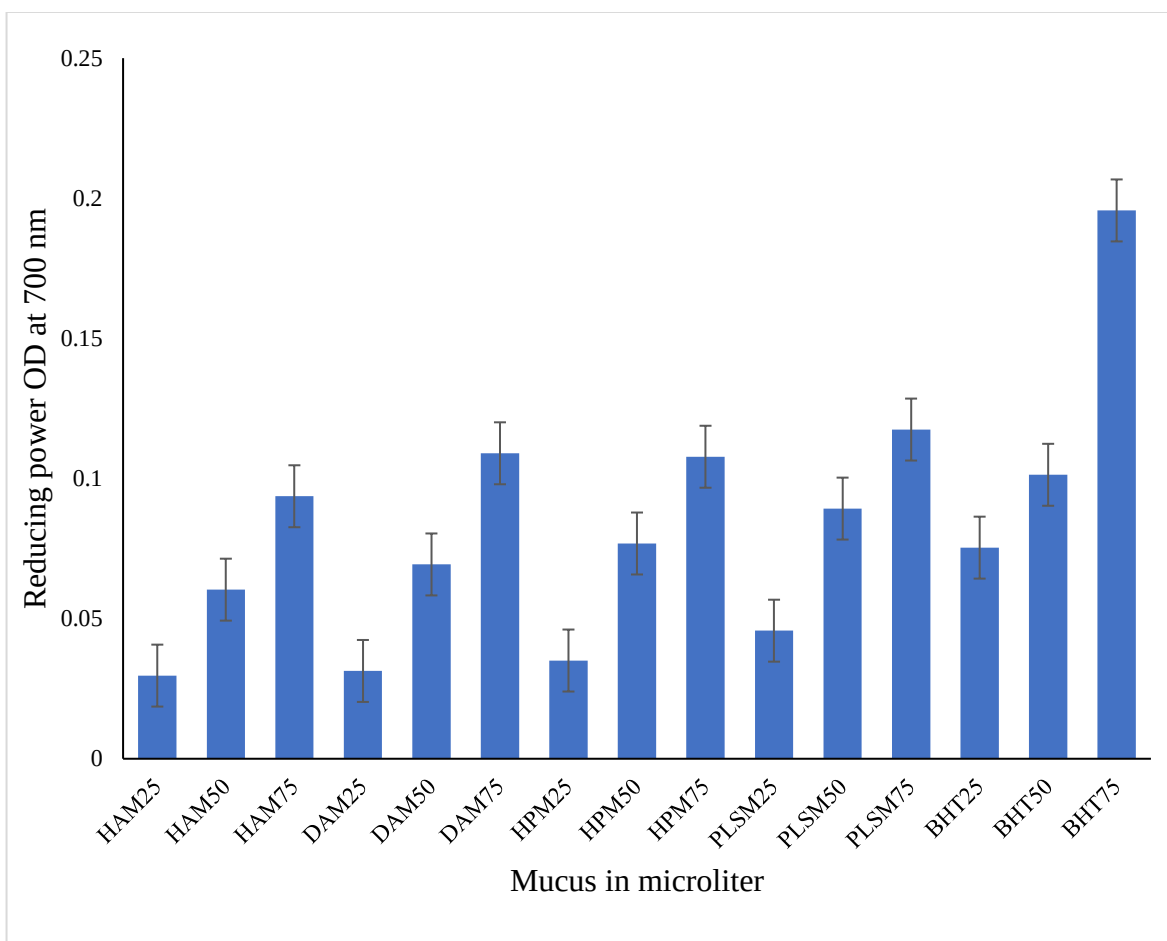


Figure 4.9- Relationship between reducing the power of coral mucus and total antioxidant. BHT -Butylated hydroxytoluene is used as a reference compound.

4.3.5 Coral mucus antioxidant enzymes

4.3.5.1 Superoxide dismutase activity

Acropora sp. and *Porites* sp. healthy and diseased mucus exhibited high SOD activity which was expressed in U/mg of proteins (Fig. 4.10). Maximum activity was observed in *Porites* sp. pink line syndrome mucus (1.43 U/mg of proteins) which was significantly more ($p < 0.05$) than the activity found in healthy *Porites* sp. mucus (0.95 U/mg of proteins). Superoxide dismutase enzyme activity was less when compared with that of healthy *Acropora* sp. mucus (0.80 U/mg of protein) than in diseased *Porites* sp. mucus (1.37 U/mg of proteins) which was significant ($p < 0.05$). The superoxide dismutase enzyme activity of mucus was confirmed by developing a zymogram on electrophoresis by specific staining. SOD reacted with the reaction mixture's substrate nitro-blue tetrazolium (NBT). White-colored SOD bands had been detected on a blue background in a native-PAGE gel (Fig. 4.11A).

4.3.5.2 Catalase activity

Catalase activity of *Acropora* sp. and *Porites* sp. mucus was performed by spectrophotometric assay for quantitative analysis. The activity was 2-fold more in diseased mucus compared to healthy (Fig. 4.10). Catalase activity was higher in diseased *Porites* sp. mucus (1.35 U/mg of protein) than in healthy *Porites* sp. mucus (1.12 U/mg of protein). Similarly, less activity was noticed in healthy *Acropora* sp. mucus (1.07 U/mg of protein) than in diseased *Acropora* sp. mucus (1.15 U/mg of protein). Maximum catalase activity was observed in pink line syndrome of *Porites* sp. Further, the catalase enzyme activity of mucus was analyzed qualitatively on native page gel by specific staining, by providing hydrogen peroxide as a substrate. Yellow-coloured catalase enzyme activity bands (Fig. 4.11B) had formed against a blue-green background and Ferricyanide precipitation caused negative staining of the catalase enzyme. Besides, the presence of catalase in mucus was reconfirmed on the slide with the cleavage of hydrogen peroxide into water and oxygen bubbles (Fig. 4.11C).

4.3.5.3 Peroxidase activity

In vitro peroxidase activity of coral mucus was demonstrated spectrophotometrically and expressed as U/mg of protein (Fig. 4.10). The peroxidase activity was significantly higher ($p < 0.05$) in pink line syndrome-affected *Porites* sp. mucus (2.12 U/mg of protein) than in healthy *Porites* sp. (1.11 U/mg of protein). *Acropora* sp. mucus also showed significantly ($p < 0.05$) more activity in white patch diseased mucus which was higher (1.62 U/mg of protein) than healthy mucus (0.52 U/mg of protein). Among different mucus, peroxidase activity was observed more in pink line syndrome-affected *Porites* sp. Maximum activity was seen in pink line syndrome *Porites* sp. mucus as far as mucus peroxidase activity is concerned.

4.3.5.4 Phenol oxidase

The presence of phenol oxidase enzyme has been checked in healthy and diseased mucus (Fig. 4.10). One-way analysis of variance (ANOVA) demonstrated that pink line syndrome affected *Porites* sp. mucus (1.03 U/mg of protein) was statistically significantly different ($p < 0.05$) and more from healthy *Porites* sp. mucus (0.34 U/mg of protein) as far as phenol oxidase activity was concerned. Phenol oxidase activity was also observed significantly more ($p < 0.05$) in diseased *Acropora* sp. mucus (0.85 U/mg of protein) than in healthy *Acropora* sp. mucus (0.27 U/mg of protein). PO activity observed in *Porites* sp. mucus was 2-fold more than in *Acropora* sp. mucus. Phenol oxidase activity noticed was 2–3-fold higher in diseased mucus than in healthy mucus of both the coral species.

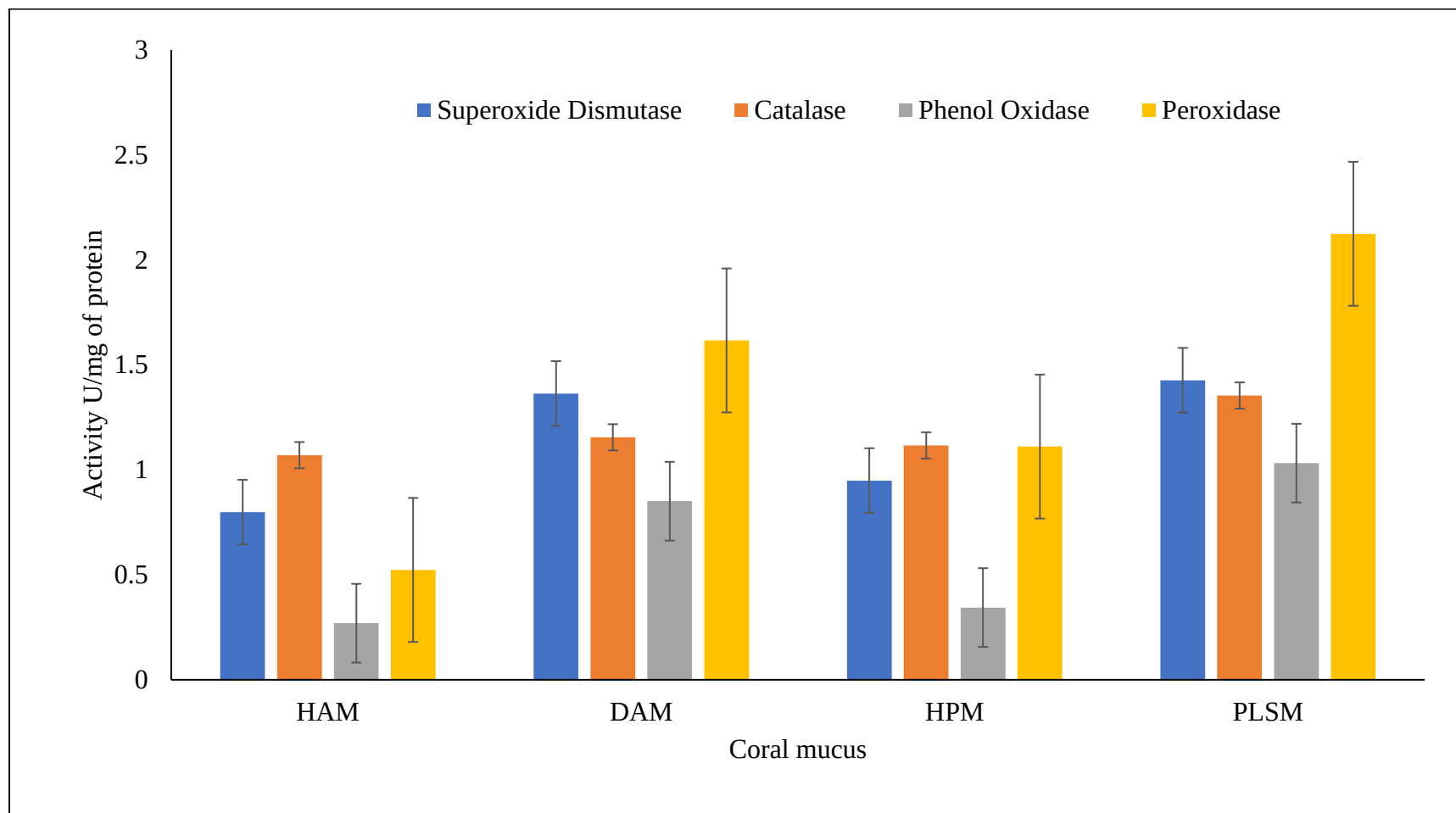


Figure 4.10- Antioxidant enzyme assay of coral mucus - Superoxide dismutase, catalase, phenoloxidase, and peroxidase enzyme activity assay of coral mucus of healthy and diseased *Acropora* sp. and *Porites* sp. Data represented with three technical replicates mean $\pm$ SD ($n = 3$). HAM- Healthy *Acropora* sp. mucus, DAM- Diseased *Acropora* sp. mucus, HPM- Healthy *Porites* sp. mucus, PLSM- pink line syndrome disease *Porites* mucus.

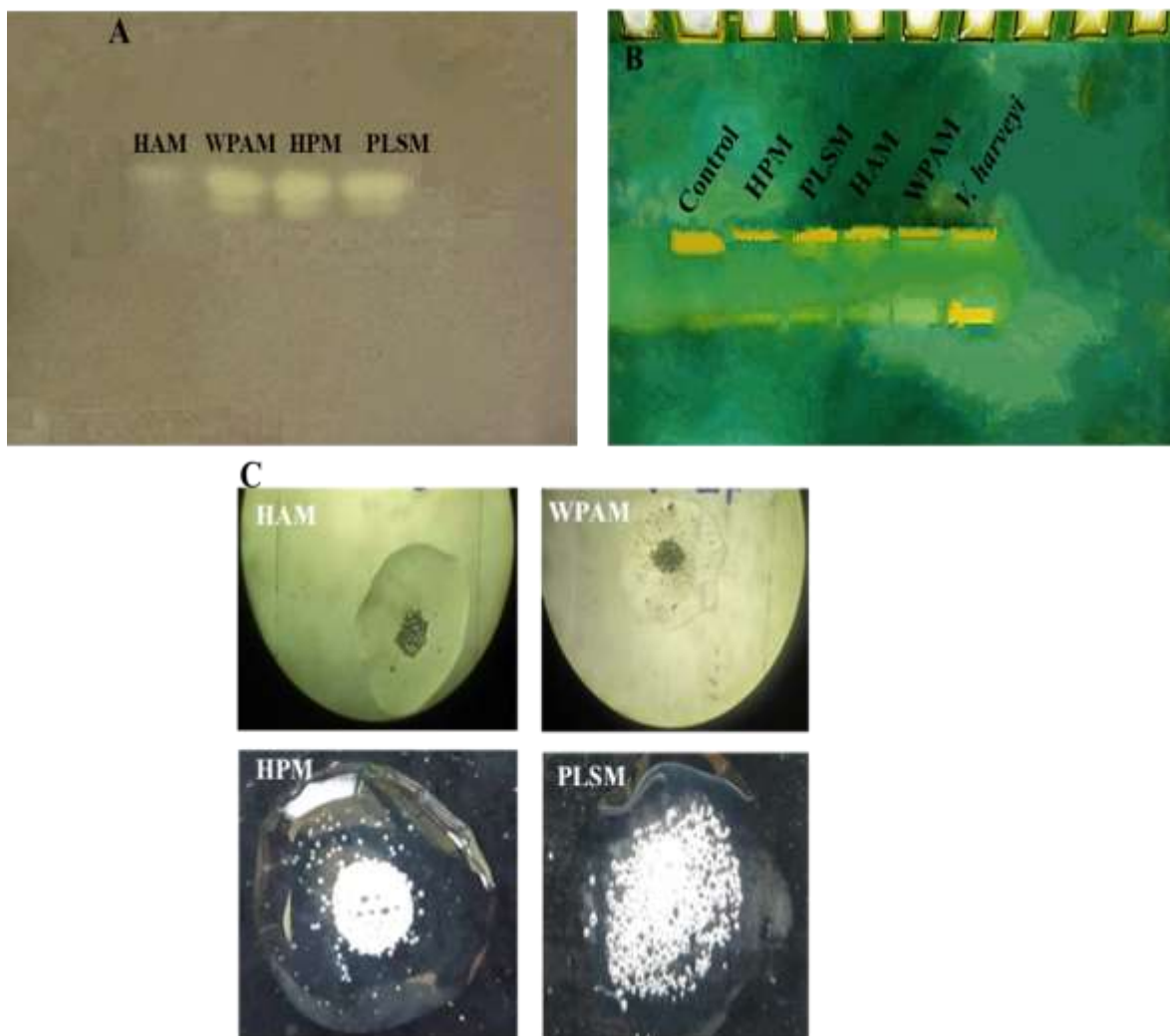


Figure 4.11- Native Page gel examination of SOD and CAT – **A.** Superoxide dismutase enzyme activity on 10 % native-PAGE gel with an equal concentration of protein 50 μg (well 1 to 4) HAM- healthy *Acropora* mucus, WPAM- white patch *Acropora* mucus protein, HPM- healthy *Porites* mucus, PLSM- Pink line syndrome *Porites* mucus. **B.** Catalase enzyme activity on 6 % native PAGE gel in well 1. control, 2. healthy *Porites* mucus, 3. pink line syndrome *Porites* mucus, 4. healthy *Acropora* mucus, 5. white patch *Acropora* mucus protein, and 6. *V. harveyi*. **C.** Catalase test of mucus against 3 % hydrogen peroxide. All corals mucus i.e. HAM-healthy *Acropora* mucus, WPAM-white patch disease *Acropora* mucus, HPM-healthy *Porites* mucus, and PLSM-pink line syndrome disease *Porites* mucus showing positive catalase test.

4.3.6 Mucus-associated potential beneficial bacteria

4.3.6.1 Beneficial bacteria antimicrobial activity

Potential antimicrobial bacterial species were isolated, identified and screened for their antimicrobial activity against coral pathogens. Two species of BB have been studied BB 1 and 2 (two strains) and BB 3-7 (five strains) for intracellular as well as extracellular protein for their antimicrobial activity against various coral-associated known previously reported pathogens (Fig. 4.12) such as *Psychobacter alimentarius*, *P. pulmonis*, *Bascillus paramycoids*, *Pseudoalteromonas carragenevora*, *P. espiana*, *P. neustonica*, *Vibrios*, (which has been previously isolated and maintained from our laboratory) on Muller Hinton Agar (Himedia) plate by well diffusion method. The antimicrobial potential of BB strains to act against the above-mentioned pathogens is shown in (Table 4.1).

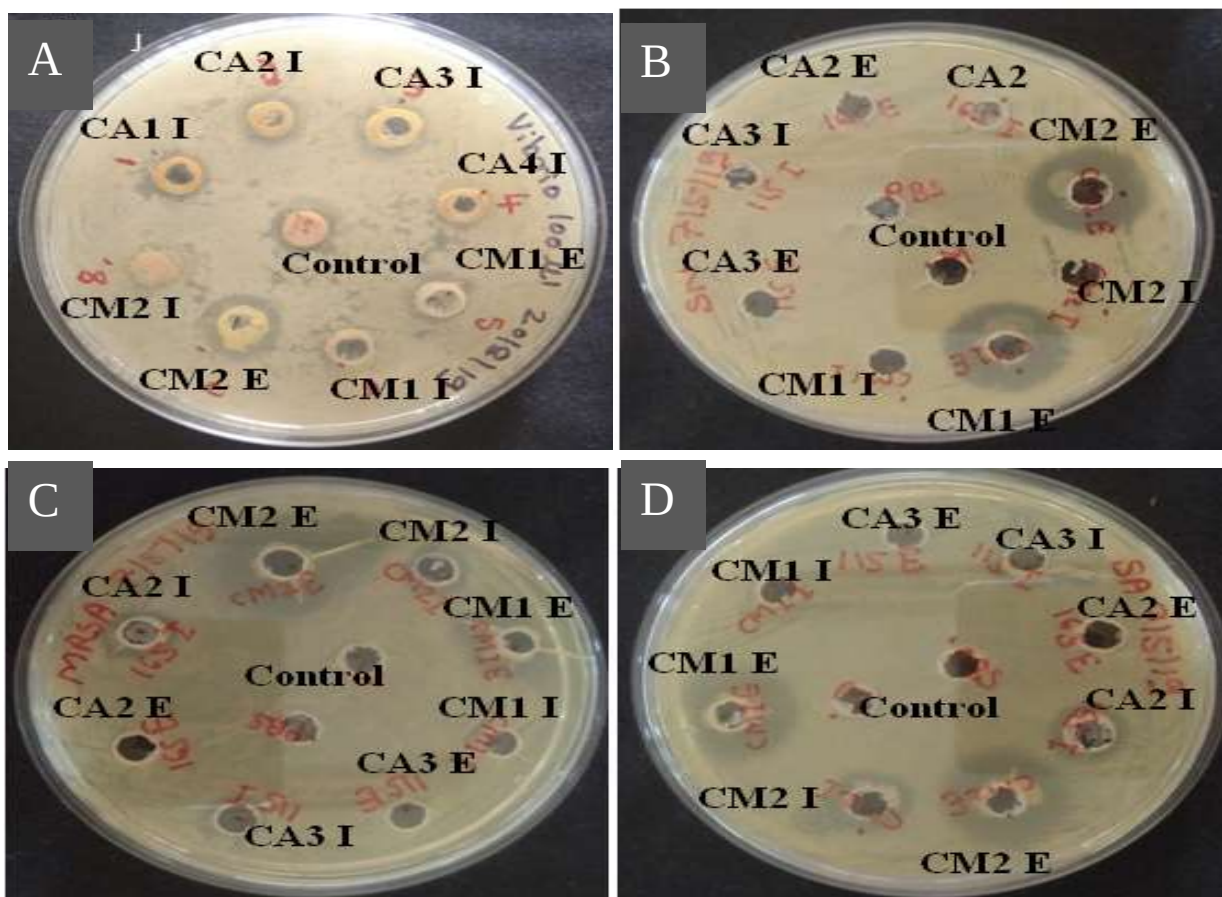


Figure 4.12- Antimicrobial activity of BB1 (CA1 and CA2) and BB2 (CM1, CM2, CM3, CM4 and CM5) bacterial protein against coral pathogens. A.- *Vibrio* sp., B.- *Serretia nematophila*, C.- MRSA, D.- *S. aureus*, G.- *Psychrobacter pulmonis* and H.- *Vibrio*.

Table 4.1- Antimicrobial activity of beneficial bacteria (BB) BB1 (2 strains) and BB2 (5 strains) intracellular and extracellular protein against coral pathogens (zone of inhibition in mm).

BB → Pathogens ↓	BB1E	BB1I	BB2E	BB2I	BB1E	BB1I	BB2E	BB2I	BB3E	BB3I	BB4E	BB4I	BB5E	BB5I
<i>Serratia nematophila</i>	19	-	18	-	-	-	-	-	-	-	-	-	-	-
<i>Serratia marscencens</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Vibrio vulnificus</i>	23	-	24	-	-	14	-	-	-	-	10	12	-	15
<i>MRSA</i>	16	12	20	11	-	-	9	-	-	9	-	-	-	
<i>S.aures</i>	19	-	18	12	-	13	10	-	-	10	-	13	-	11
<i>Vibrio</i> Yellow	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Vibrio</i> Green	-	-	-	-	-	13	-	-	-	-	-	13	-	12

4.3.6.2 Antimicrobial activity of Actinomycetes against coral pathogens

Actinomycetes cultures isolated from coral mucus by using Actinomycetes-specific media showed antimicrobial activity against coral pathogens. Actinomycetes B2SD1 showed antimicrobial activity against *MRSA*, Actinomycetes B1WH1 strain showed inhibition against *V. parahaemolyticus*, and *V. harveyi* (Fig. 4.13).

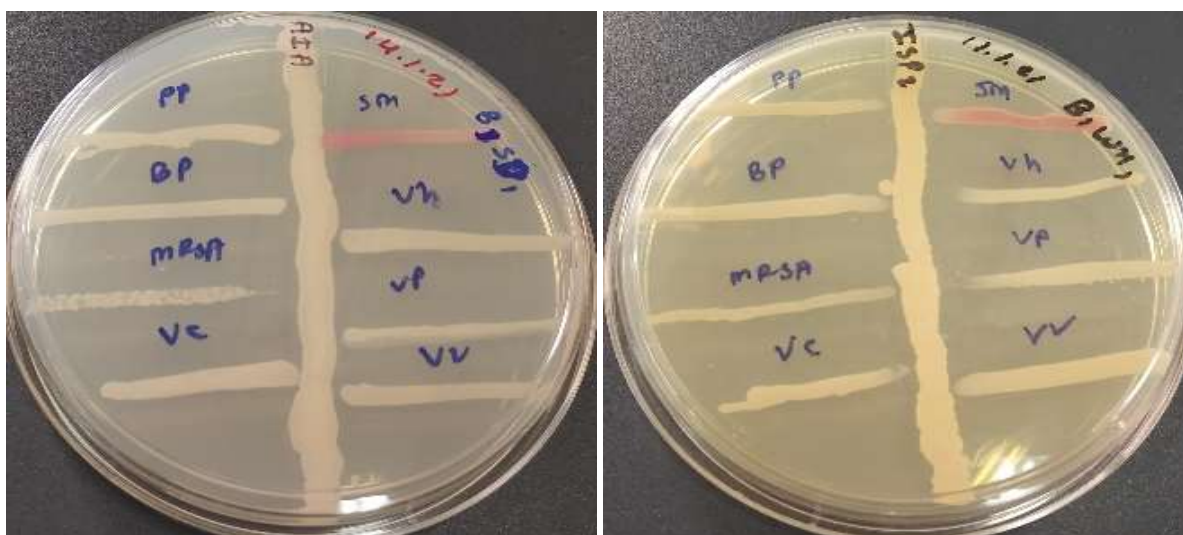


Figure 4.13- Actinomycetes activity against coral pathogens. Actinomycetes (a) B2SD1 showed against *MRSA* and (b) B1WH1 against *V. parahaemolyticus* and *V. harveyi*.

4.3.6.3 Protein profile of mucus and BB strains

The study identified potential antimicrobial beneficial bacterial strains in coral mucus, including two strains of BB1 and five strains of BB2. To differentiate these strains at the primary level, we conducted SDS-PAGE analysis of their differential protein profiles (Fig. 4.14). The results revealed distinctive expression patterns in the protein bands of BB1 in comparison to BB2. Among the five strains of BB2, the qualitative analysis indicated similar expression of protein bands, with well numbers four and five less clearly visible. Conversely, in BB2, well numbers six and seven exhibited similar expression patterns but differed when compared to BB1. The extracellular protein profiles associated with potential antimicrobial activity were visualized on a 10% SDS-PAGE gel.

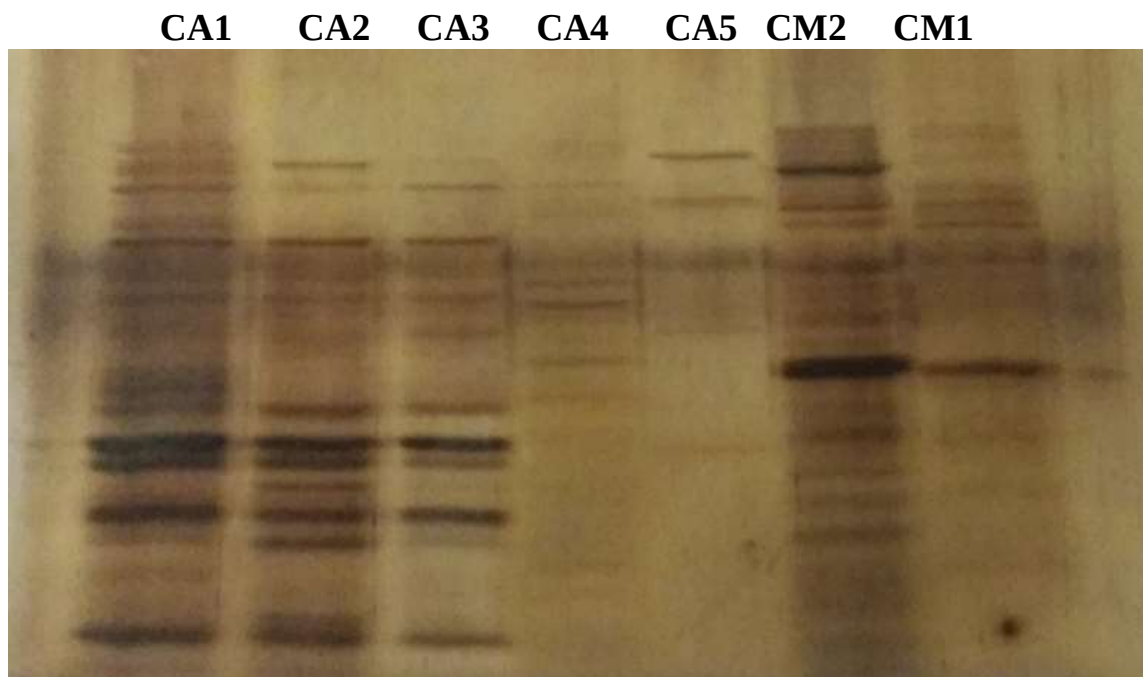


Fig. 4.14- Extracellular protein bands of BB1 and BB2 on 10 % SDS-PAGE gel. Well CA1, CA2, CA3, CA4 and CA5 extracellular proteins of five strains of BB1 while CM1 and CM2 two strains of BB2.

4.3.7 Nitrite production of healthy and diseased mucus microbes

Coral mucus HAM-healthy *Acropora* mucus, DAM-dead *Acropora* mucus, HPM-healthy *Porites* mucus, and PLSM-pink line syndrome mucus bacteria were checked for nitrite production for 28 days. In the first 15 days, there was no production, or its negligible. After 28 days of incubation, we found maximum nitrite production by the *Porites* pink line syndrome disease mucus bacteria which was 14.04 micromole/Litre (Fig. 4.15), and then by healthy *Acropora* mucus, diseased *Acropora* mucus, and healthy *Porites* mucus bacteria almost similar. It was also seen that; streptomycin had eliminated nitrifying bacterial activity in all mucus bacterial test flasks containing streptomycin and showed less nitrite production than samples without streptomycin.

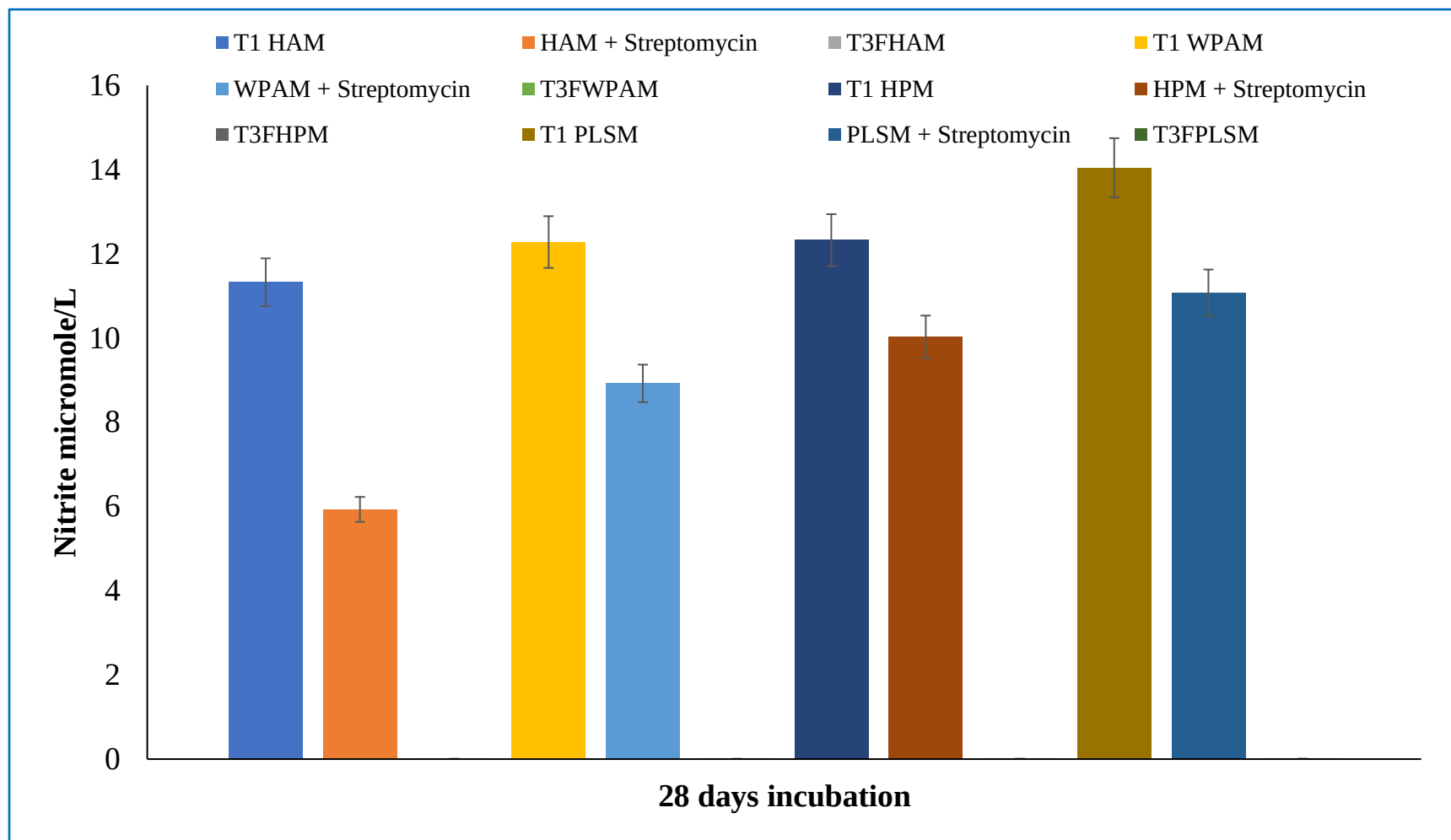


Figure 4.15- Production of nitrite by coral mucus bacteria/archaea under 28 days incubation. Streptomycin is used as an inhibitor for nitrifying bacteria for control.

4.4 Discussion

Acropora sp. and *Porites* sp. mucus exhibited antimicrobial properties against various known coral pathogens such as *S. aureus* and *V. parahaemolyticus* (Fig. 4.3B). Thus, the antimicrobial activity suggests the possible mucus role in the first line of defense that favours the corals health and resilience. This preliminary defense property of mucus prompted us to screen the various mucus defense components that have been much studied in other organisms such as fish mucus but not in coral mucus. The results revealed the antioxidants and antioxidant enzymes of diseased coral mucus for maximum activity of free radical scavenging than the healthy mucus, which may be due to signals/factors involved in the activation of the corals natural defense signals against any biotic or abiotic stress.

In this study, the quorum quenching property of the healthy and diseased *Acropora* sp. and *Porites* sp. mucus (Fig. 4.4A, B, C) suggests that it may contain enzymes such as lactonase, which competitively inhibits the AHL molecule by binding with AHL receptors. Thus, the AHL lactonase enzyme in the coral mucus, which inactivates penicillin's beta-lactam ring, a member of the beta-lactamase superfamily (Huma et al., 2011), that converts penicillin into penicilloic acid was elucidated. This coral mucus was also confirmed for its inhibition of violacein pigment production, a quorum sensing molecule produced by *C. violaceum* (Fig. 4.4A, B), suggesting the vital role of the mucus components in inhibiting pathogens that cause infections in corals.

Acropora sp. and *Porites* sp. mucus exhibited the presence of antioxidants, which have free radical scavenging properties, reducing power, and metal ion chelating capacity. This antioxidant activity was expressed more in diseased mucus than in healthy. However, the activity levels differed in *Acropora* sp. and *Porites* sp. mucus. This variation may be due to the *Symbiodinium* type that has been associated (McGinty et al., 2012; Krueger et al., 2014; Ravindran et al., 2022). Also, excess hydrogen peroxide (H_2O_2) radicals cause the dissociation of zooxanthellae from the coral host by damaging holobionts cells or triggering cell signaling pathways of the symbiont (Weis, 2008). Hence H_2O_2 scavenging antioxidants and catalase enzymes were analysed and found to be maximum in diseased coral mucus suggesting that diseased corals express effective defense capability to fight against H_2O_2 which helps in preventing bleaching (Fig. 4.5). Hydroxyl and DPPH radicals are the major free radicals that cause lipid peroxidation and severe cell damage (Aurand et al., 1977) which

affects the thylakoid membrane, phospholipids (Lesser, 1996; Tchernov et al., 2004) and the photosynthesis ability of endosymbionts. The mucus of the present study showed effective scavenging activity against DPPH and diseased mucus of *Porites* sp. was observed with maximum activity (Fig. 4.7). Activity of the chelating agent was also observed in mucus (Fig. 4.8). These sequestering agents can prevent corals from oxidative damage by reacting with free unstable metal ions and reduce their harm by forming a stable compound. A significant amount of reducing agents in mucus reveals that it can effectively reduce the harmful oxidants by the addition or removal of hydrogen (Fig. 4.9).

The presence of free radical-scavenging antioxidant enzymes in the coral mucus was also tested. Catalase inactivates this H_2O_2 into oxygen and water. The SOD and catalase enzyme expression was observed more in diseased coral mucus as compared to healthy mucus, which might be due to the high oxidative stress in diseased mucus (Figs. 4.10 and 4.11). Further, peroxidase enzymes protect the coral from the fungal pathogen, which has been reported in coral disease aspergillosis caused by *Aspergillus sydowii* (Mydlarz and Harvell, 2007). Peroxidase activity of the coral mucus was observed to be the highest in the *Porites* sp. diseased mucus (Fig. 4.10). Earlier findings on antioxidant enzyme cascade of the coral tissues have also reported the upregulation against ROS generation due to thermal and salt stress (Almeida and Mascio, 2011). This supports our present results on the upregulation of SOD, catalase, and peroxidase enzymes in diseased mucus (Figs. 4.10 and 4.11). The presence of phenoloxidase activity in coral mucus of the present study (Fig. 4.10) was also reported in some cnidarian mucus such as healthy and black band disease of *Pseudodiploria strigosa*, healthy and bleached *Cassiopea xamachana* and *Exaiptasia pallida* mucus (Rivera-Ortega and Thome, 2018; Palmer et al., 2011). Coral mucus (Fig. 4.3), along with beneficial bacteria BB1 and BB2 (7 strains) (Fig. 4.12) and *Actinomyces* sp. (Fig. 4.13), possess antimicrobial properties that can effectively combat various pathogens, including those that affect coral (Table 4.1). The combination of mucus and beneficial bacteria creates a potent antimicrobial cascade. Through the SDS page there was a primary differentiation observed between two BB strains (Fig. 4.14).

Corals obtain N_2 through predation and from water and excrete ammonia (Fig. 4.15) (Siboni et al. 2008). Further, archaea use this ammonia, which plays an important role in ocean nitrification. During the daytime when oxygen levels are high due to photosynthesis,

certain types of archaea convert ammonia into nitrite. Conversely, at night when oxygen is low, different types of archaea carry out denitrification to convert nitrite into nitrogen (Siboni et al. 2008). Marine archaea play a main role in oceanic nitrification (Wuchter et al. 2006). Archaea observed in coral mucus may also be central to nitrogen recycling, by process of nitrification and denitrification results in regulating the concentration of excess ammonium (Siboni et al. 2012). Nitrogen is a limiting factor for endosymbiont growth and density. Nitrogen cycling is important in maintaining the symbiosis and functioning of coral holobionts (Radecker et al. 2015). Coral mucus contains high concentrations of dissolved organic nitrogen (DON), which bacteria can readily take up for growth and metabolism. Some of these bacteria form symbiotic relationships with coral polyps, where they fix atmospheric nitrogen (N_2) into forms that can be used by the corals (Diaz 2017). Other mucus bacteria may help in recycling nitrogen within the holobiont by breaking down waste products and releasing ammonium (NH_4^+) and nitrate (NO_3^-) into the surrounding water.

4.5 Summary

- Coral mucus demonstrates potent antimicrobial properties against diverse pathogens. Beneficial bacteria associated with coral mucus, such as BB1, BB2 and *Actinomycetes*, have also been identified and exhibit effective antimicrobial activity against various coral pathogens.
- The mucus exhibits quorum quenching (QQ) properties, suggesting a potential role in preventing pathogenicity.
- The inhibition of AHL molecules in mucus implies QQ against quorum-sensing (QS) bacteria, potentially involving enzymes like lactonase, which was then confirmed by lactonase enzyme assays.
- Additionally, mucus displays ROS scavenging properties, with relatively higher levels observed in diseased mucus.

Chapter five
Characterization of coral mucus for its
components.

5.1 Introduction

Coral mucus has several important functions, including protection against pathogens and UV radiation, acting as an energy carrier, and serving as a nutrient source for coral-associated microbial communities (Bakshani et al., 2018; Bythell and Wild, 2011; Bhagwat et al., 2024). The mucus produced by coral is a valuable energy source with high glucose content. As glucose is readily available, mucus is a highly preferred energy source for microbes and other organisms (Ravindran et al., 2023). Thus, mucus is an important component of marine ecosystems, providing a rich energy source for a wide group of organisms (Wild et al., 2010). Mucus energy levels are far greater than benthic photosynthetic algae (Bhagwat et al., 2024). Thus, many organisms have also been described for their feed on mucus (Eckes et al., 2015; McMohan et al., 2016; Horka et al., 2016; Shmuel et al., 2022). Coral mucus is the best nutritional source with a C: N ratio of mucus fluid of 6.9 to 13.7 and mucus sheet of 4.8 to 5.9 (Coffroth, 1990). Mucus helps in the fulfillment of energy requirements as well as the cycling of nutrients (Wild et al., 2004). Coral mucus plays a crucial role in maintaining the health and productivity of reef ecosystems by facilitating nutrient cycling and supporting the diverse array of organisms that inhabit these ecosystems. Recycling these nutrients within the holobiont and the surrounding water helps to maintain a balance of nutrients and supports the ecosystem's productivity.

Coral mucus has been also demonstrated for its antimicrobial activity against coral disease-causing pathogens (Nissimov et al., 2009). In addition, antioxidants rich in total phenols and flavonoids have been studied in coral tissues (Sadarun et al., 2022). These antioxidants are vital for countering harmful free radicals and oxidative stress in organisms. Suggesting that they could serve a protective function for the corals against various biotic and abiotic stressors. The presence of these antioxidants in coral mucus also suggests protective functions against environmental stress for corals (Hawkins et al., 2015). Thus, investigating healthy and diseased coral mucus for antimicrobial, antioxidant, and nutrient properties may provide insights into their possible role in influencing coral health.

Fatty acids are important biomolecules for every organism. Corals acquire essential fatty acids (EFAs) through their symbiotic association with algae zooxanthellae (Kellogg and Patton, 1983; Patton and Burris, 1983; Patton et al., 1983; Harland et al., 1993) or through *de novo* synthesis by host cells using glucose from zooxanthellae as the main carbon source

(Oku et al., 2003). Corals cannot produce essential fatty acids (EFAs), because they cannot synthesize precursors such as linolenic (C18:3n-3) and linoleic (C18:2n-6) acids (Latyshev et al., 1991; Bell et al., 2007). However, they can acquire it from heterotrophic feeding on plankton or dissolved organic matter (Sebens, 1987; McAuley, 1994; Ferrier-Page and Gattuso, 1998). So, EFAs act as markers for dietary sources in coral. FAs C16:0 name and C18:0 name are key precursors for PUFAs C18:2n-6 and C18:3n-3 biosynthesis. Coral FA synthesis is directly linked to photosynthesis (Oku et al., 2003). Since corals derive most of their PUFAs from zooxanthellae (Papina et al., 2003), algae loss is directly related to PUFA levels (Graeve et al., 1994; Zhukova and Aizdaicher, 1995). Thus, during bleaching, a response to heat stress provides the importance of fatty acids requirement for coral survival (Grottoli et al., 2004; Rodrigues et al., 2008). Corals are physiologically active due to the FA's presence as food ingredients and molecules, that are involved in cell signaling and membrane stabilization through palmitoylation and myristoylation (Binienda et al., 2013). Fatty acids are also major components of lipids, essential components of cell membrane lipids, and precursors of bioactive metabolites (Sargent et al., 1990).

Coral mucus secreted by mucocytes provides a protective physicochemical and physiological barrier between coral tissue and external environmental threats. The biomolecules and nutrients of the secreted mucus are derived from endosymbionts, and coral polyps and support coral functions, such as feeding, sediment clearing, and protection, against numerous biotic and abiotic stressors. The surface mucus layer also houses a diverse community of beneficial microorganisms that defend against pathogens. Enzymes including peptidases, esterases, and glycosidases were observed and described in mucus. We have seen the presence of phenoloxidase, an intracellular enzyme in secretory mucus (in Chapter 4), generally triggers melanin synthesis, providing fast-acting components of invertebrate immunity in disease resistance. However, the purpose and the mechanism of mucus release, the effects of mucus components on defense properties, and functional roles in intra- and interspecific interactions are not well described. Thus, in the present study, healthy and diseased coral mucus has been analyzed for their nutrients, antioxidants, and fatty acids to understand their role in influencing coral health under various biotic and abiotic stress.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Sodium bicarbonate, Folin-Ciocalteu reagent (HiMedia), Sodium nitrite (HiMedia), Aluminum chloride (Sigma-Aldrich), Sodium hydroxide (Merck), methanol (SRL), chloroform (SRL), potassium hydroxide (Merck), hexane (SRL), sulfuric acid (SRL), sodium cyanide, EDTA (Merck), NBT (HiMedia), riboflavin (HiMedia), hydrogen peroxide, trypsin (HiMedia), guaiacol (HiMedia), phosphate buffer saline, potassium ferric cyanide (Sigma-Aldrich) and ferric chloride (Sigma-Aldrich).

5.2.2 Mucus nutrient analysis

Mucus samples from healthy and diseased coral *Acropora* and *Porites* sp. were promptly transported to the laboratory under cold conditions to maintain their integrity. Upon arrival, the samples underwent processing within 24 hours to ensure the precision of the subsequent results. The mucus samples were subjected to nutrient analysis, including the evaluation of silica, ammonia, nitrite, nitrate, and phosphate contents through an autoanalyzer (San Skalar SAN⁺⁺ Continuous Flow Analyzer). For the nutrient analysis, 10 ml of each healthy and diseased mucus sample, diluted in a 1:9 ratio with MQ water (mucus: MQ water), was utilized. A blank sample containing only 10 ml of MQ water was employed as a control during the analysis. Before processing the autoanalyzer was meticulously calibrated for silica, ammonia, nitrite, nitrate, and phosphate (all of Merck), and the instrument settings were optimized.

5.2.3 Total soluble phenol content assay

The total soluble phenolic content of coral mucus and tissue was estimated by our earlier lab protocol (Ravindran and Naveenan 2011). To estimate total soluble phenol, the positive control used was gallic acid. A hundred μ l of the mucus and tissue extract sample was mixed with 2 ml of sodium bicarbonate (2 %) and kept for 2 minutes incubation at room temperature. After incubation, 100 μ l of Folin-Ciocalteu reagent was added to the mixture and placed in the dark for 30 minutes. The samples were vortexed. Absorption was measured

at 725 nm spectrophotometrically. All the chemicals used for assay were of Merck Mumbai, India.

5.2.4 Total flavonoids content assay

To quantify the total flavonoid content of coral mucus and tissue, quercetin was used as the standard (Ravindran and Naveenan 2011). The 100 µl of the coral mucus and tissue extract was mixed in 300 µl of D/W, then 30 µl of sodium nitrite (5 % NaNO₂). Tubes were incubated for five minutes. To this mixture, 30 µl of Aluminum chloride (10 % AlCl₃) was added and mixed well and the reaction mixture was placed for 5 minutes at room temperature. 200 µl Sodium hydroxide (1 mM NaOH) was added to the reaction and the final volume was made up of 1 ml by adding D/W and mixed well. All chemicals used were from Merck Mumbai, India. Optical density was observed at 510 nm.

5.2.5 Fatty acid composition of mucus

5.2.5.1 Lipid Extraction.

Lipid Extraction from various healthy and diseased samples of *Porites* sp. and *Acropora* sp. mucus was carried out according to the Folch method (Folch et al., 1956). Coral mucus of about 0.2 ml was added to 5 ml of methanol: chloroform 1:2 and kept for ultrasonication for up to 30 minutes at 40 °C. Then the samples were transferred to the separating funnel. 2 ml of potassium hydroxide (KOH) was added (5 grams in 50 ml of methanol) to the sample for saponification and mixed well and allowed for the separation till the separate layer formed. The upper layer was removed using a Pasteur pipette and transferred to the pear-shaped flask.

5.2.5.2 Fatty acid analysis of healthy, bleached, and diseased *Porites* sp. and *Acropora* sp. corals mucus by GC-MS

Methylation was done by adding 2 ml of sulphuric acid in the separated supernatant layer and allowing for 2 hours in a dry bath with shaking in between. After methylation, 2 ml hexane was added to each sample and vortexed. The upper layer was separated using a Pasteur pipette and samples were allowed to dry after nitrogen gas evaporation. Hexane (gas chromatography grade) 100 µl was added to each dried sample and vortexed. The samples

were again subjected to methylation as above. 2 ml of sulfuric acid was added to the hexane fraction and non-hexane fraction (sulfuric acid methylated) as well. After sulfuric acid methylation, the samples were transferred to the 30 ml glass stopper vials and incubated in the water bath for 2 hours at 80 °C. Samples were mixed properly in between. After methylation, samples were kept at room temperature, and 2 ml of hexane was added, mixed well, and the supernatant layer was separated, containing the fatty acid (FA). The extracted fatty acids were analyzed by using a GC/MS Gas Chromatograph/Mass Spectrometer system.

5.2.6 Statistical analysis

All the experiments were carried out in triplicates. One-way ANOVA was used to compare means of the healthy and diseased coral mucus (healthy *Acropora* mucus, white patch *Acropora* mucus, healthy *Porites* mucus, and pink line syndrome *Porites* mucus) and tissue samples (healthy *Porites* and pink line syndrome *Porites*) antioxidant assays and enzyme activity. The coefficient of determination r^2 was calculated using Microsoft Excel 2019.

5.3 Results

5.3.1 Mucus nutrients

The analysis of healthy and diseased mucus from *Acropora* and *Porites* sp. (Fig. 5.1) involved assessing their nutrient content, including silica, ammonia, nitrite, nitrate, and phosphate, using an autoanalyzer (Fig. 5.2). Maximum phosphate concentration was observed in *Porites* pink line syndrome disease, while the minimum was recorded in white patch *Acropora* mucus. Ammonia concentration was found to be higher in healthy mucus, with comparatively elevated levels in *Porites* species mucus. Similarly, silicate levels were 5-10 times higher in *Porites* mucus compared to other mucus samples. Nitrite concentrations were consistently low across all studied mucus, ranging from 0.25 to 0.55 $\mu\text{M/L}$. Nitrate concentrations were higher in healthy mucus, reaching a maximum in healthy *Porites* mucus. In comparison to the water surrounding nearby coral (as illustrated in Chapter 2, Figure 2.4), mucus exhibited notably elevated levels of ammonia and phosphate. Nitrate also demonstrated a slightly increased concentration within the mucus. Conversely, nitrite and

silica were substantially more abundant in the water than in the mucus. Silica was observed to be higher in concentration in healthy water compared to healthy mucus, while in diseased conditions, it was more prevalent in the mucus than in the water.

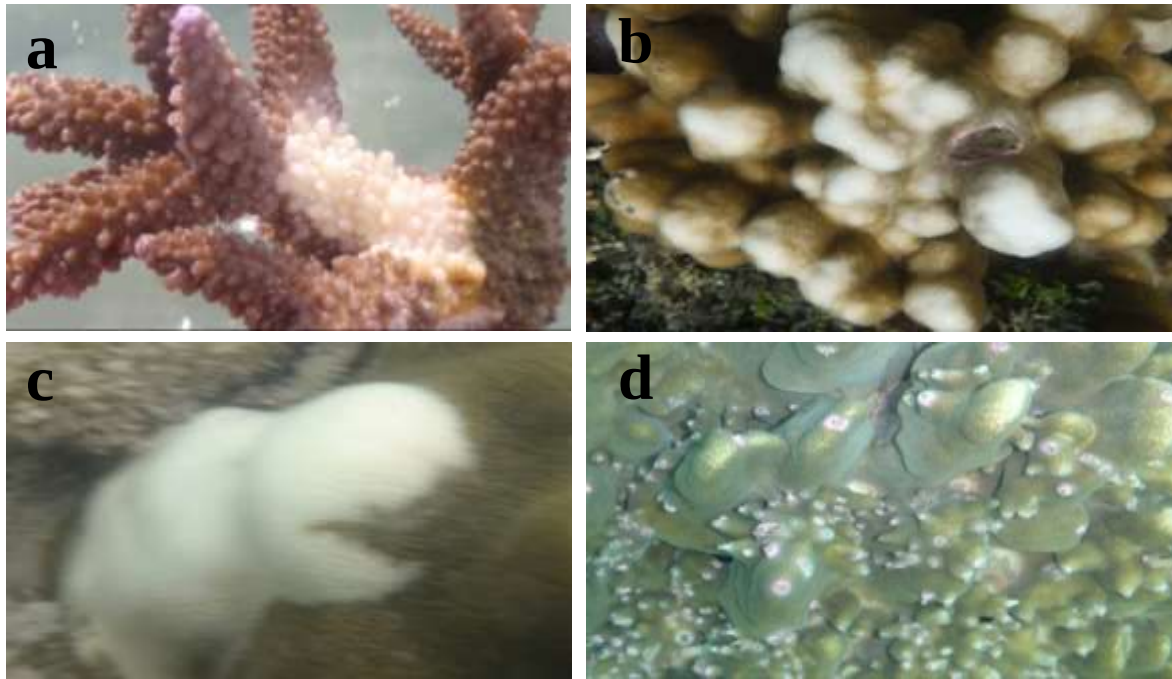


Figure 5.1- Diseased mucus sample collected from the *Porites* and *Acropora* corals located at the Gulf of Mannar island. **a.** White patch diseased *Acropora*, **b.** pink line syndrome diseased *Porites*, **c.** bleached *Porites*, **d.** pink spot disease *Porites*.

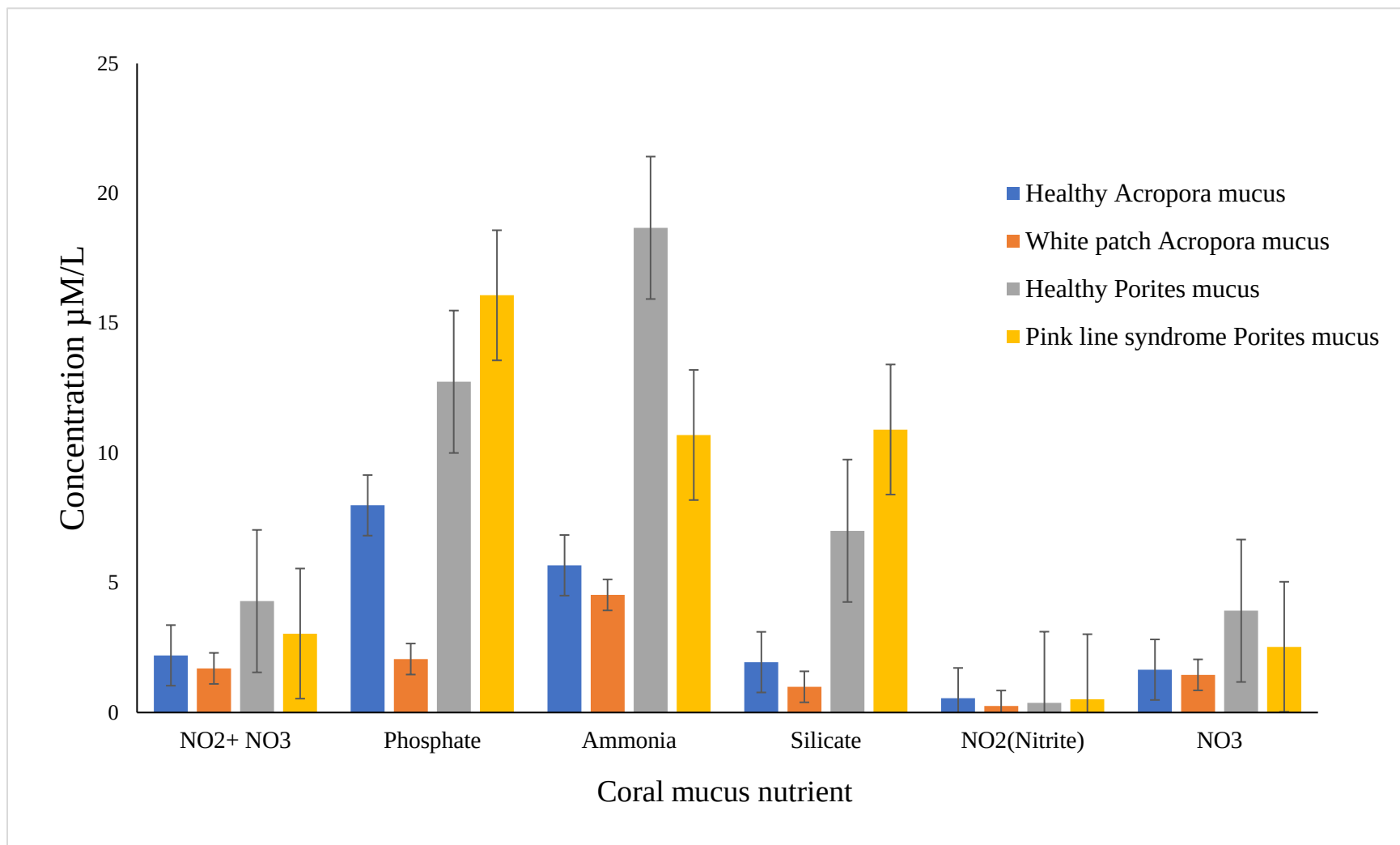


Figure 5.2- Nutrient analysis of healthy *Acropora*, white patch *Acropora*, healthy *Porites* and pink line syndrome disease *Porites* mucus.

5.3.2 Total antioxidant contents of coral mucus

The quantitative determination of total phenolic was carried out using the Folin–Ciocalteu reagent. Gallic acid was used as a reference compound for phenol content, the quercetin standard curve was plotted as concentration against absorbance, and slope values were determined for flavonoid determination. Diseased *Acropora* mucus showed significantly ($p > 0.0004$) more concentration of 3.67 $\mu\text{g/ml}$ of total phenol than healthy *Acropora* mucus which was 2.30 $\mu\text{g/ml}$. The determined total soluble phenol (Fig. 5.2) concentration was found to be significantly higher ($p > 0.00002$) in diseased *Porites* mucus 14.54 $\mu\text{g/ml}$ compared to the healthy *Porites* mucus 9.86 $\mu\text{g/ml}$. However, only diseased coral mucus was observed to significantly produce a larger repertoire of non-enzymatic antioxidant compounds as compared to healthy coral mucus as far as the levels of total soluble phenol were concerned. The determined flavonoid (Fig. 5.3) concentration of *Acropora* mucus was significantly ($p > 0.0002$) more in the diseased mucus 18.3 $\mu\text{g/ml}$ than in healthy *Acropora* mucus 7.9 $\mu\text{g/ml}$. The flavonoid concentration of diseased *Porites* mucus was 16.09 $\mu\text{g/ml}$ which was significantly ($p > 0.0001$) more than healthy *Porites* mucus at 9.55 $\mu\text{g/ml}$.

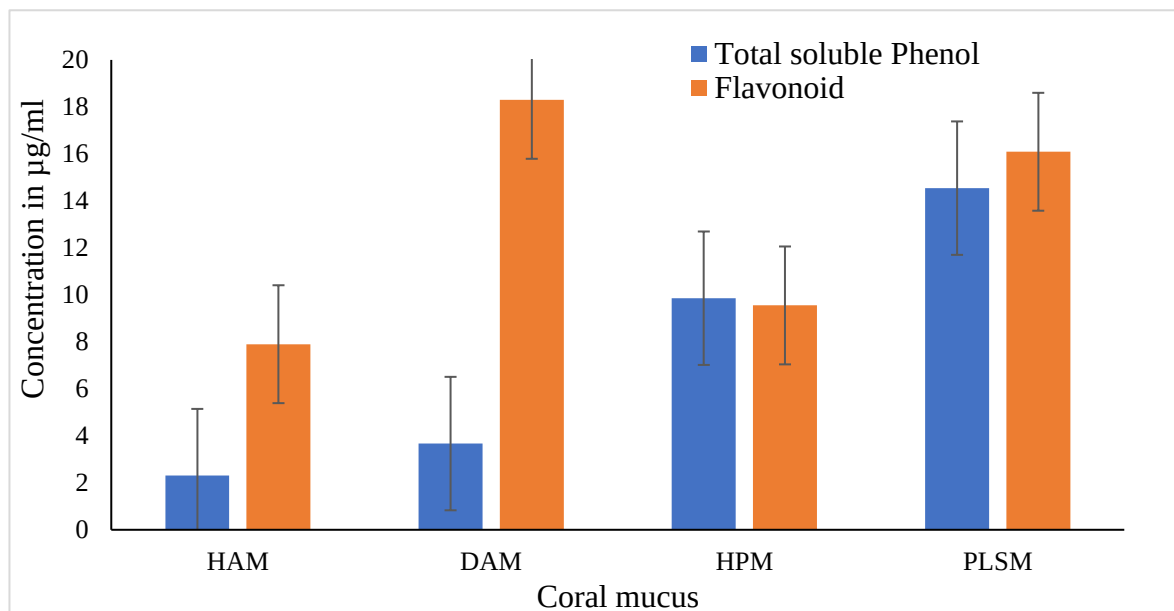


Figure 5.3- Total soluble phenol and flavonoid concentration of coral mucus and tissue. Values are mean $\pm$ SD ($n = 3$). HAM- Healthy *Acropora* mucus, DAM-White patch *Acropora* mucus, HPM- Healthy *Porites* mucus, PLSM- Pink line syndrome *Porites* mucus, HPT- Healthy *Porites* tissue, PLSPT- Pink line syndrome *Porites* tissue.

5.3.3 Fatty acid profile of mucus

In the present study, healthy *Acropora* mucus (17 FAs) possesses more diversity and abundance of fatty acids than white patch disease *Acropora* mucus (6 FAs). While in *Porites* healthy mucus (11 FAs), bleached mucus (10 FAs), pink spot mucus (14 FAs), and PLS disease mucus (12 FAs), FAs diversity was more but abundance was similar (Table 5.1 and 5.2). The relative percentage of FAs in healthy and diseased *Acropora* and *Porites* sp. were varied. In healthy *Acropora* mucus, saturated and monounsaturated fatty acids (MUFAs) were more diverse and their relative percentage was high in white patch *Acropora* disease mucus. Polyunsaturated fatty acids (PUFAs) were absent in diseased *Acropora* mucus. The relative percentage of saturated fatty acids (SAFAs) in healthy *Porites* mucus was much higher than in diseased and bleached *Porites* mucus. MUFAs were very low in diseased mucus compared to bleached and diseased *Porites* mucus and their diversity was also slightly more than healthy and bleached *Porites* mucus. PUFAs were more in bleached *Porites* mucus than healthy and diseased mucus. Long-chain PUFAs were abundant in bleached and diseased *Porites* mucus.

5.3.4 Biomarker FAs in mucus

Fatty acid constituents of *Acropora* (Fig 5.4) and *Porites* (Fig 5.5) coral mucus were analyzed for their biomarkers, saturated (SAFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). The *Symbiodiniaceae* markers 18:2n-6, C22:5n-3, and C22:6n-3 (Graeve et al., 1994, Zhukova and Aizdaicher, 1995), polyp tissue markers 20: 4n-6, 22: 4n-6 and 22: 5n-3, bacterial biomarker FAs C15:0, C16:1n-9, C16:1n-7, C17:0 (Volkman et al., 1980, Jeffries, 1972, Bachok et al., 2006) were present in mucus. Polyps and *Symbiodinium* FAs predominated in *Acropora* mucus. In addition, C16:1n-7 and C17:0 sulfate-reducing bacterial markers were also seen in mucus (Findlay and Dobbs, 2018). The essential FA docosahexaenoic acid (DHA, C22:6n-3) was present in the *Acropora* host, *Symbiodinium*, and *Porites* mucus but absent in *Acropora* mucus. Arachidonic acid (ARA, C20:4n-6) was present in healthy, bleached, and diseased *Porites* mucus as well as healthy *Acropora* mucus and was predominant in all hosts other than *Symbiodinium*. Health indicator FAs and tropic marker FAs ratio from mucus FAs were calculated to know the sources of FAs in mucus, coral's feeding pattern, and health condition (Table. 5.1 and 5.2).

Table 5.1- Fatty acid content of *Acropora* host, *Symbiodinium* and mucus with relative percentage.

	<i>Acropora samoensis</i> (Kim et al., 2021)		<i>Acropora intermedia</i> (Imbs et al., 2014)		<i>Acropora muricata</i> (Imbs et al., 2014)		<i>Acropora muricata</i> mucus (Present study)	
Fatty acid	<i>Symbiodinium</i>	Host	<i>Symbiodinium</i>	Host	<i>Symbiodinium</i>	Host	Healthy	Diseased
C12:00	-	-	-	-	-	-	-	2.69
C14:00	3.37	2.66	2.7	1.9	3.5	3.1	1.42	-
C15:00	-	-	-	-	-	-	0.38	-
C16:00	22.25	31.06	18.6	20.1	16.3	30.1	29.36	60.43
C17:00	-	-	-	-	-	-	0.33	-
C18:00	5.73	14.34	5.4	13.6	4.7	10.1	35.19	28.26
C20:00	0.13	0.59	0.3	2.2	0.2	1.0	0.88	-
C22:00	0.17	0.15	-	-	-	-	-	-
Σ SFA	31.65	48.8	27	37.8	24.7	44.3	67.56	91.38
C16:1n-9	2.31	2.29	1.5	1.2	0.5	1.1	0.51	0.9
C16:1n-7	0	0	1.9	1.1	1.1	2.6	0.65	2.8
C18:1n-9	2.35	2.66	2.9	3	3.3	5.1	3.04	4.92
C18:1n-7			0.6	0.3	0.2	0.4	-	-
C20:1n-9(CGA)	0.48	2.88	1.3	2.8	0.6	1.7	1.47	-
C22:1n-9	0.13	0.22	-	-	-	-	-	-
Σ MUFA	5.27	8.05	8.2	8.4	5.7	10.9	5.67	8.62
C18:3n-3 (ALA)	2.35	11.98	-	-	-	-	3.08	-
C18:4n-3 (SDA)	10.39	1.26	13	0.9	13.1	0.9	-	-
C18:5n-3	-	-	2.8	0.2	3.9	0.2	-	-
C20:5n-3 (EPA)	13.72	5.69	14.7	8.7	7.7	4.4	3.67	-
C22:5n-3 (DPA)	0.4	0.96	2.1	4.3	1.9	2.4	-	-

C22:6n-3 (DHA)	19.59	6.66	8.4	5.0	9.2	3.6	-	-
Σ n-3 PUFA	46.45	26.55	42	19.1	35.8	11.5	6.75	-
C18:2n-6 (LA)	0.93	0.81	1	0.6	0.8	1.1	2.38	-
C18:3n-6 (GLA)	13.55	5.47	8.2	2.7	8.2	2.8	-	-
C20:2n-6	0	0	2	0.4	1.2	0.5	0.6	-
C20:3n-6 (DGLA)	0.35	1.04	0.4	1	-	1.8	0.62	-
C20:4n-6 (ARA)	1.28	7.17	5.3	17.2	8.0	10.2	9.74	-
C22:4n-6 (ADA)	0.48	2	2.3	8.1	3.4	10.7	6.68	-
C22:5n-6	-	-	-	-	-	0.1	-	-
Σ n-6 PUFA	16.59	16.49	11.676	30	21.6	27.2	20.02	-
C16:2n-7	-	-	-	0.2	1.6	0.2	-	-
C18:2n-7	-	-	0.1	-	0.3	0.1	-	-
Σ PUFA	63.04	43.04	53.776	49.3	59.3	39	26.77	-
PUFA: SFA	1.99	0.9	1.99	1.3	2.4	0.9	0.4	-
EPA: ARA	10.8	0.8	2.8	0.5	0.96	0.43	0.4	-
EPA: DHA	0.7	0.8	1.75	1.74	0.84	1.22	-	-
n-3: n-6	2.8	1.6	3.6	0.64	1.66	0.42	0.3	-

Table 5.2- Fatty acid content of *Porites* host, *Symbiodinium* and mucus with relative percentage.

Fatty acid	<i>Porites cylindrica</i> (Imbs et al., 2014)		Healthy <i>Porites</i> mucus	Bleached <i>Porites</i> mucus	Pink line syndrome <i>Porites</i> mucus	Pink spot disease <i>Porites</i> mucus
	<i>Symbiodinium</i>	Host				
C12:00	-	-	3.66	-	-	-
C14:00	2.2	1.8	5.48	-	3.76	6.89
C15:00	-	-	1.31	0.9	-	1.77
C16:00	16	17.9	40.8	-	2.01	1.92
C17:00	-	-	-	9.52	5.795	6.87
C18:00	3.5	18.2	28.06	-	3.66	2.7
C20:00	0.4	0.9	-	6.68	-	-
C22:00	-	-	-	-	-	-
Σ SFA	22.1	38.8	79.31	17.1	15.225	20.15
C16:1n-9	0.8	1.3	4.67	39.175	45.33	39.4
C16:1n-7	2.7	1.9	1.48	-	1.255	1.25
C18:1n-9	2.7	3.3	-	23.91	26.25	24.19
C18:1n-7	0.3	0.4	-	-	-	-
C20:1n-9(CGA)	0.9	3.6	-	-	-	0.77
C22:1n-9	-	-	-	-	-	-
Σ MUFA	7.4	10.5	6.15	63.085	72.835	65.61
C18:3n-3 (ALA)	-	-	-	-	-	-
C18:4n-3 (SDA)	14.1	1.3	-	-	-	-
C18:5n-3	2.6	0.2				
C20:5n-3 (EPA)	15.8	10.2	-	-	-	-
C22:5n-3 (DPA)	1.2	4	4.3	1.2	1.3	1.4
C22:6n-3 (DHA)	11.4	3.6	1.7	0.9	1.1	0.5

Σ n-3 PUFA	45.1	19.3	6	2.1	2.4	1.9
C18:2n-6 (LA)	1.1	0.9	-	7.72	7.61	6.75
C18:3n-6 (GLA)	14.4	2.7	14	11.82	4.285	3.85
C20:2n-6	2.6	0.4	-	-	-	-
C20:3n-6 (DGLA)	0.4	0.8	-	-	-	-
C20:4n-6 (ARA)	2.9	13.7	3.4	0.9	1.3	1
C22:4n-6 (ADA)	1.2	8.1	-	-	-	-
C22:5n-6	1.2	8.1	-	-	-	-
Σ n-6 PUFA	23.8	34.7	17.4	20.44	13.195	11.6
C16:2n-7	0.9	0.1	-	-	-	-
C18:2n-7	0.1	-	-	-	-	-
Σ PUFA	69.9	54.1	14	19.54	11.895	13.5
PUFA: SFA	3.2	1.4	0.18	1.14	0.78	0.69
EPA: ARA	5.45	0.74	-	-	-	-
EPA: DHA	1.39	2.83	-	-	-	-
n-3: n-6	1.89	0.56	0.3	0.1	0.2	0.2

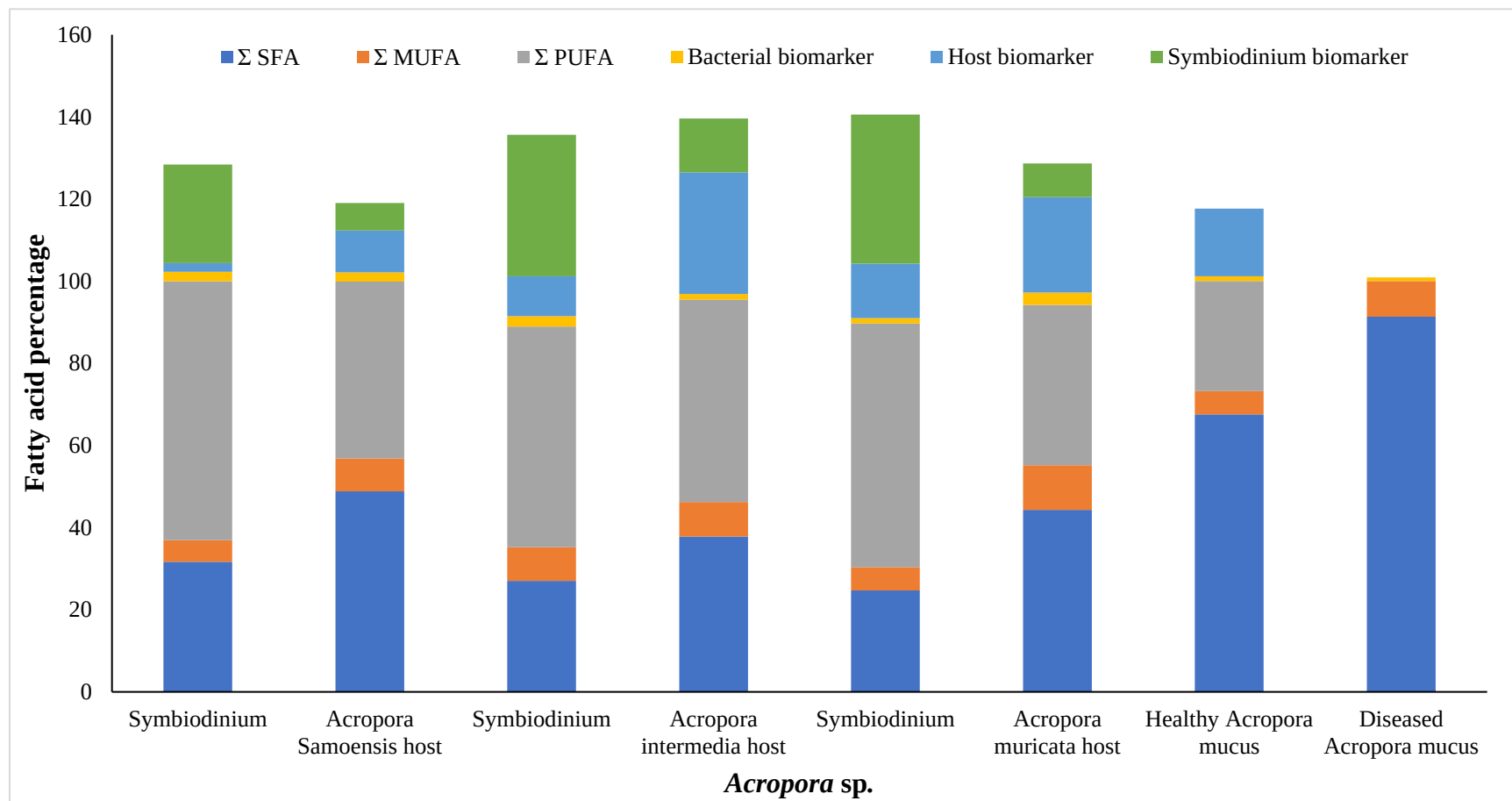


Figure 5.4- Comparison of the relative concentrations of fatty acid classes (% of total FAs) among healthy *Acropora* mucus and diseased *Acropora* mucus with *Acropora samoensis*, *Acropora intermedia*, *Acropora muricata* host FAs and its *Symbiodinium*s. SAFAs-saturated fatty acids, MUFAs-monounsaturated fatty acids, PUFAs-polyunsaturated fatty acids, *Symbiodinium* biomarker FAs, coral host biomarker FAs, and bacterial biomarker FAs.

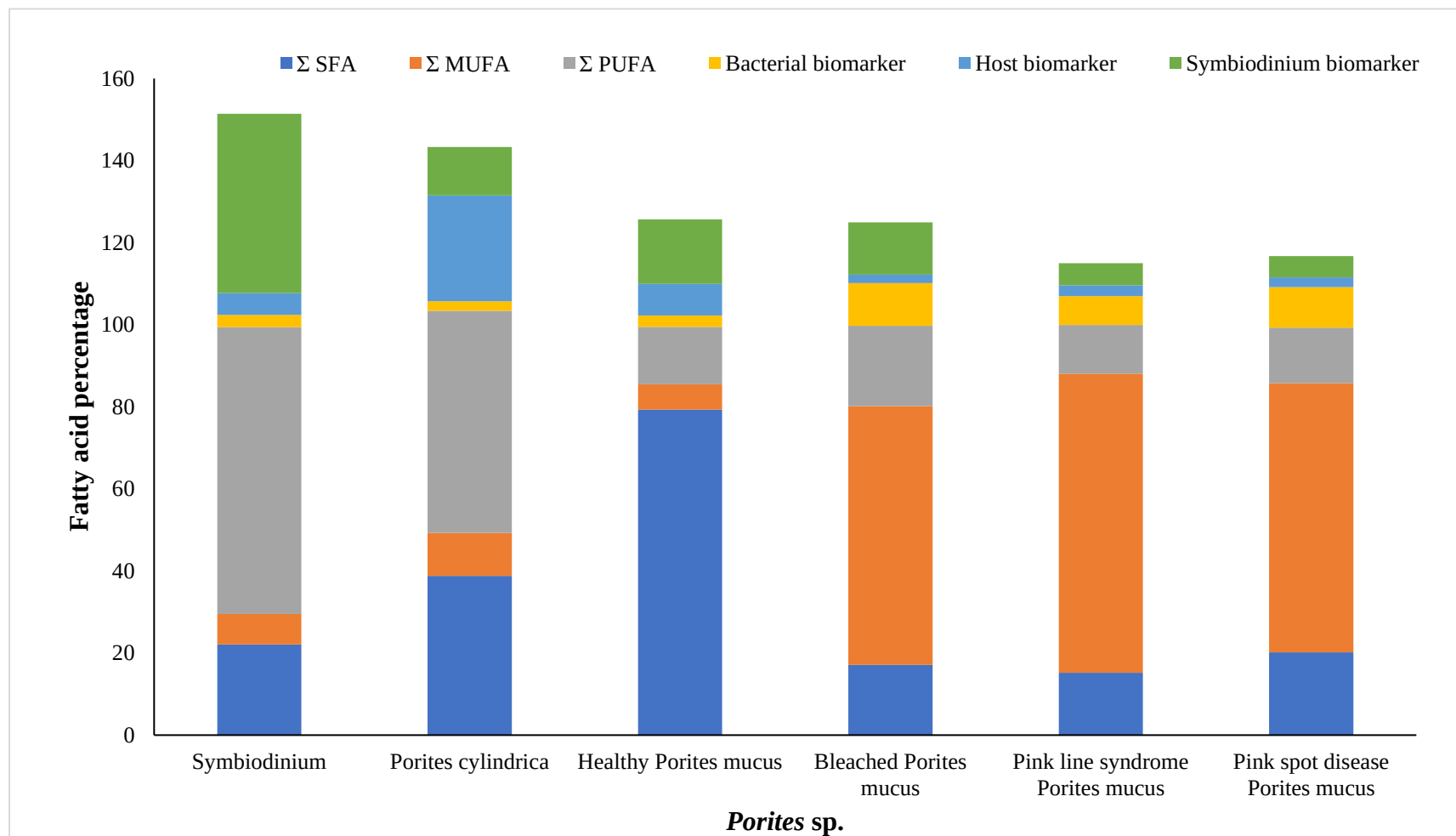


Figure 5.5- Comparison of the relative concentrations of fatty acid classes (% of total FAs) among healthy, bleached, pink line syndrome, and pink spot diseased *Porites* mucus with its *Symbiodinium* and host. SAFAs-saturated fatty acids, MUFAs-monounsaturated fatty acids, PUFAs-polyunsaturated fatty acids, *Symbiodinium* biomarker FAs, coral host biomarker FAs, and bacterial biomarker FAs.

5.4 Discussion

The nutrient analysis findings indicated differences in the nutrient concentration between healthy and diseased mucus (Fig. 5.2). Phosphate is an essential nutrient for coral growth, but excessive levels can contribute to nutrient pollution and coral stress (Dunn et al., 2012). *Porites* mucus affected by pink line syndrome disease showed a high concentration of phosphate (Fig. 5.2). Ammonia was observed very high in all mucus as reported mucus acts as a sink for excess ammonia and protects corals from detrimental effects of ammonia. Ammonia is a nitrogen compound that can arise from various sources, including the breakdown of organic matter. Silica is also an important nutrient for coral growth (Anjum et al., 2020). Interestingly, significantly higher levels of silicate were observed in the *Porites* mucus compared to *Acropora* (Fig. 5.2). Higher silica was found in healthy coral surrounding water than mucus, it may be due to high anthropogenic activities. The silicate in *Porites* mucus was reported to be 5-10 times higher than that in *Acropora* mucus. This difference indicates variations in nutrient uptake and utilization between these two coral types.

As illustrated in Chapter 4, (Figure 4.15), there was a higher abundance of nitrite-producing microbes, including archaea, in diseased mucus, capable of oxidizing ammonia into nitrite. These transformations can influence the concentrations of ammonia and nitrite within the mucus compared to the water. Research suggests a positive correlation between archaeal concentration and nitrite and ammonia levels (Siboni et al., 2008). This implies that mucus rich in ammonium and other metabolic by-products of corals offers a nutrient-rich environment for these microorganisms. Nitrite concentrations were found to be very low across all studied mucus. Nitrate concentrations were generally higher in healthy mucus compared to diseased mucus. The maximum nitrate concentration was detected in the mucus of healthy *Porites*. Nitrate levels can reflect the overall health of the coral reef ecosystem, as elevated levels may indicate excess nutrient input (D'Angelo and Wiedenmann, 2014). High ammonia levels result from increased microbial activity, such as the decomposition of organic matter in mucus. High levels of ammonia coupled with low nitrite levels suggest a disruption in the nitrification process. This could occur due to low oxygen, inhibited microbial activity, or imbalances in nutrient availability. Factors such as pollution, habitat degradation, temperature fluctuations, and ocean acidification can disrupt nutrient cycling processes and compromise coral health and resilience. Groundwater discharge, degradation

of diatoms, and other siliceous microorganisms contribute to higher levels of silica in seawater. Coral and its species type influence the silica concentration which actively utilizes silica from the seawater for skeletal growth.

In Chapter 4, we analyzed the scavenging activity of mucus (Figs. 4.5-4.9). To identify the molecules responsible for the antioxidant activity we characterized coral mucus for its antioxidants. *Acropora* sp. and *Porites* sp. mucus exhibited the presence of antioxidants such as soluble phenol and flavonoids (Fig. 5.3). Total phenol and flavonoids were expressed more in diseased mucus than in healthy. This supports our earlier results which showed high ROS scavenging activity in diseased mucus (as explained in Chapter 4). These sequestering agents prevent corals from oxidative damage by reacting with free radicals and reduce their harm by forming a stable compound. Especially flavonoids are recognized for their capacity to absorb ultraviolet (UV) radiation (Salian et al., 2021). The GoM corals are in shallow habitats where corals may face high sunlight exposure hence flavonoids in mucus may act as a protective shield for corals, protecting them against UV-induced harm such as reduced photosynthesis. Additionally, Phenolic compounds have antimicrobial and allelopathic properties that aid corals in defending against harmful bacteria, fungi, and other pathogens (Gross, 2003; Couttolenc et al., 2022).

FAs in mucus constrain studying their role and whether Symbiont or host secretes them into mucus. Only (Ravindran et al., 2020) reported coral mucus for their fatty acid profile but there was no clear study describing the fatty acid role and source in the mucus. As photosynthetic by-products cannot fulfil the coral's need for nitrogen and phosphorus and other factors required for growth and reproduction which need to be acquired by heterotrophy (Battey and Patton, 1986; Ferrier-Pages et al., 2003). Some FA was also recorded as derived from the host (Dunn et al., 2012). Interestingly some reports showed the transfer of PUFAs from corals to symbionts (Imbs et al., 2014). In addition, FAs play various important roles in corals, n3 and n6 PUFAs regulate growth, respiration, metabolism, energy, photosynthesis, larvae, and egg production (Rinkevich, 1989; Harland et al., 1992; Tchernov et al., 2004; Imbs et al., 2007; Treignier et al., 2008). FAs n3 provides survival and resistance to biotic and abiotic stress (Romdhane et al., 1995). Some fatty acids only get synthesized by *Symbiodinium* and act as markers of symbiosis (Imbs et al., 2010). Some biomarker FAs of

Symbiodinium in the host lipid indicate the transfer of fatty acids (Papina et al., 2003; Treignier et al., 2008).

Non-photosynthetic corals, which heavily rely on heterotrophic feeding, exhibited higher levels of C22:5n-3 FA (Imbs et al., 2010; Figueiredo et al., 2017). This suggests that these corals primarily obtain this FA through their diet rather than synthesizing it internally. The higher abundance of C22:5n-3 in healthy *Porites* mucus and host tissue (Table 5.2) indicates a substantial contribution of heterotrophic feeding to the coral's nutritional intake. This is supported by the elevated levels of C18:1n-9 fatty acids in bleached and diseased *Porites* mucus (Table 5.2), indicative of increased heterotrophic feeding under stress conditions (Radice et al., 2019). Similarly, both healthy and diseased *Acropora* specimens showed higher levels of C18:1n-9, emphasizing the importance of heterotrophic feeding in these corals (Table 5.1). Furthermore, the presence of various PUFAs, including C18:2n-6, C20:3n-6, C20:4n-6 (ARA), and C22:4n-6, reflects the reliance on heterotrophic nutrient sources (Armstrong et al., 1994), particularly evident in bleached *Porites*, diseased *Porites*, and healthy *Acropora* mucus. The ratio of EPA to DHA (C20:5n-3 to C22:6n-3) serves as an indicator of autotrophic versus heterotrophic feeding (Rocker et al., 2019). Higher ratios suggest autotrophy, while lower ratios indicate heterotrophy. The significantly higher EPA:DHA ratio observed in *Acropora* and *Porites* hosts, as well as their *Symbiodinium*, and only in healthy *Acropora* mucus, suggests predominant autotrophic feeding. Conversely, higher levels of DHA in coral hosts and only in *Porites* mucus imply heterotrophic feeding, likely originating from the host. Moreover, the ratio of PUFA to SFA serves as a proxy for dietary preferences, with higher ratios indicating a carnivorous feeding preference (Rocker et al., 2019). The elevated PUFA:SFA ratio in all *Symbiodinium* compared to the host, as well as in bleached and diseased *Porites* mucus, suggests a shift towards carnivorous heterotrophic feeding, particularly under stress conditions. Thus, the presence and abundance of heterotrophic FAs in coral mucus highlight the transfer of these FAs in mucus by the host.

Desaturases D12 and D15, identified in certain coral species (Kabeya et al., 2018), play a crucial role in synthesizing long-chain fatty acids (FAs). Additionally, *Symbiodinium*, the symbiotic algae within coral tissues, has been documented to produce essential PUFAs such as C18:3n-3 and C18:2n-6, which can be transferred to the coral host (Bachok et al., 2006; Teece et al., 2011). Furthermore, the host coral itself is capable of synthesizing n-3

PUFAs (Kabeya et al., 2018), indicating multiple sources of essential FAs within the coral symbiosis. In addition, corals acquire FAs through heterotrophic feeding, as demonstrated by studies showing uptake of n-3, n-6, and n-9 FAs (Hinrichs et al., 2013; Seemann et al., 2013). The heterotrophic feeding behavior of corals is influenced by various factors, including coral morphology, polyp abundance, *Symbiodinium* density, and nematocyte types (Teece et al., 2011), as well as environmental factors such as light, depth, and temperature (Oku et al., 2003; Harland et al., 1992). These factors collectively influence the availability and composition of FAs in coral tissues and mucus. Determining the specific sources of FAs in coral mucus poses challenges due to the dual capabilities of the coral host and its symbiotic algae. Both the host coral and *Symbiodinium* can synthesize FAs via the *de novo* pathway and acquire them through heterotrophic feeding. Consequently, the FA content in coral mucus likely results from a complex interplay between endogenous synthesis and external uptake, influenced by environmental conditions and symbiotic dynamics.

The n-3 PUFA ratio was higher in *Acropora Symbiodinium* and healthy *Acropora* mucus than in diseased *Acropora* mucus (Table 5.1). Similarly, it was higher in *Porites Symbiodinium* and healthy *Porites* mucus than in bleached and diseased *Porites* mucus (Table 5.2). The higher the ratio healthier the coral. With the help of desaturase enzymes photosynthesising invertebrates converts 16:0 (palmitic acid; PA), into long-chain PUFAs, C18:3n-3 and C18:2n-6 (Kellogg and Patton, 1983; Dalsgaard et al., 2003). EPA: ARA ratio was also higher in *Symbiodinium* and healthy *Acropora* mucus. EPA was absent in diseased *Acropora* mucus, healthy *Porites* mucus, bleached *Porites* mucus, and diseased *Porites* mucus. SAFAs and MUFAs are easily utilized energy sources (Tocher, 2003). Whereas, PUFAs are used for membrane fluidity, reproduction, cellular functioning, and the immune mechanism (Tocher, 2003; Funk, 2001). Lipids act as long-term energy in the form of triglycerides and wax esters and are a part of structural phospholipids and sterols which maintain structure and integrity (Saunders et al., 2005; Imbs et al., 2010). To maintain this, a proper balance of long-chain fatty acids is required (Russo, 2009; Richier et al., 2010). Long-chain PUFAs perform an important function in corals in particular eicosapentaenoic acid (C20:5n-3, EPA), anti-inflammatory and docosahexaenoic acid (C22:6n-3, DHA), membrane fluidity (Dalsgaard et al., 2003; Guil-Guerrero, 2007; Russo, 2009). Arachidonic acid (C20:4n-6, ARA), helps in the synthesis of important immune response hormones

(Tocher, 2003; Russo, 2009). The n-3 and n-6 PUFA ratio indicates disease conditions and provides nutrients (Clarke and Jump, 1993; Nomura et al., 2013). FA suggests the health condition of the coral, as C18:2n-6 and C18:3n-6 are essential for proper physiological functioning, metabolism, and stress resilience. More n-3 LC PUFA helps in the cell membrane and electron flow in chloroplast under reduced light conditions (Hazel and Williams, 1990; Mock and Kroon, 2002). The n-6 PUFA can help under increased thermal stress to maintain membrane fluidity (Nettleton, 1995). Higher concentrations of long-chain n-3 PUFA suggest corals increased ability to grow, survive, and stress resistance (Bachok et al., 2006). The lower proportion of n-3 LC: n-6 LC was due to more n-6 LC PUFA acquired by heterotrophic feeding (Simopoulos, 2008).

FAs biomarker confirms the FA contribution of *Symbiodiniaceae* and polyps to the mucus. PUFAs particularly C20:4n-6 and C22:4n-6 in mucus suggest a healthy association of *Symbiodinium* with the polyps (Treignier et al., 2008). Bacterial biomarker FAs may come in mucus from the degradation of bacteria by the mucus. The thermal stress FA markers may help in monitoring past temperature encounters on corals. A low level of PUFAs in diseased *Acropora* mucus and pink spot *Porites* mucus indicates the possible loss of *Symbiodinium*. Similarly, in bleached *Porites* mucus low levels of the dinoflagellate biomarkers C22:5n-3, and C22:6n-3 indicate the loss of *Symbiodinium*. EPA, DHA and ARA maintain *Symbiodinium* population, immune response, metabolism and respiration (Yamashiro et al., 1999; Hulbert, 2003). Autotrophy marker stearidonic acid (SDA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA) (Table 5.1) was absent in *Acropora* mucus but present excess in *Acropora Symbiodinium* followed by host (Table 5.1 and 5.2). DHA and DPA were present in all *Porites* mucus and more in healthy *Porites* mucus which suggest autotrophic feeding preference of healthy corals. SDA was absent in *Porites* mucus. Heterotrophy marker cis-gondoic acid (CGA) was abundantly present in healthy *Acropora* mucus and absent in diseased *Acropora* mucus. Whereas in *Porites* it was present in pink spot disease mucus. Also, CGA was more in hosts than their *Symbiodinium* which suggests they have come in mucus by heterotrophic feed and from host (Mies et al., 2018). Increased temperatures are associated with higher levels of unsaturated and long-chain fatty acids, as observed in bleached *Porites* mucus (Mies et al., 2018).

5.5 Summary

- Coral mucus exhibits varying nutrient levels, including silica, ammonia, nitrite, nitrate, and phosphate, serving as a sink for excess ammonia through nitrogen cycling which is important in nutrient cycling and as a feed for various organisms.
- Diseased mucus displays higher levels of antioxidants (phenol, flavonoids).
- Fatty acid composition in coral mucus resembles heterotrophic feed and polyp fatty acids. The presence of fatty acid markers suggests the mutual transfer of fatty acids among *Symbiodinium*, polyps, and also from the bacteria.
- Mucus fatty acids serve as indicators of health and symbiosis, playing vital roles in metabolism, physiological processes, and stress tolerance.
- Healthy, diseased and bleached mucus were observed for varied fatty acids profiles. However, bleached coral mucus was observed for less fatty acids, which may be due to the loss of endosymbionts.
- The diversity in fatty acids indicates stress on corals, influencing mucus fatty acid composition.

BIBLIOGRAPHY

- Aeby GS (2007). Spatial and temporal patterns of *Porites strematodiasis* on the reefs of Kaneohe Bay, Oahu, Hawaii. *Bulletin of Marine Science* 80:209–218.
- Almeida EA, Mascio P (2011). Hypometabolism and antioxidative defense systems in marine invertebrates hypometabolism. In Anna, N., and Michal, C., (eds) *Hypometabolism: Strategies of Survival in Vertebrates and Invertebrates*. Research Signpost Kerala, India, 39–55.
- Ambort D, Johansson ME, Gustafsson JK, Ermund A, Hansson GC (2012). Perspectives on mucus properties and formation-lessons from the biochemical world. *Cold Spring Harbor Perspectives in Medicine* 2(11):014159.
- Andrianasolo EH, Haramaty L, Vardi A, White E, Lutz R, Falkowski P (2008). Apoptosis-inducing galactolipids from a cultured marine diatom, *Phaeodactylum tricornutum*. *Journal of Natural Products* 71(7):1197–1201.
- Anithajothi R, Duraikannu K, Umagowsalya G, Ramakritinan CM (2014). The presence of biomarker enzymes of selected scleractinian corals of Palk Bay, southeast coast of India. *BioMed Research International* 2014, 684874.
- Anjum AS, Sun KC, Ali M, Riaz R, Jeong SH (2020). Fabrication of coral-reef structured nano silica for self-cleaning and super-hydrophobic textile applications. *Chemical Engineering Journal* 401:125859.
- Antonius AA, Lipscomb D (2001). First protozoan coral-killer identified in the Indo-Pacific. *Atoll Research Bulletin* 481:1-21.
- Arakane Y, Noh MY, Asano T, Kramer KJ (2016). Tyrosine metabolism for insect cuticle pigmentation and sclerotization. *Extracellular composite matrices in arthropods*, pp. 165–220.
- Arboleda MDM, Reichardt WT (2010). *Vibrio* sp. causing *Porites* ulcerative white spot disease. *Diseases of Aquatic Organisms* 90 (2):93–104.
- Atad I, Zvuloni A, Loya Y, Rosenberg E (2012). Phage therapy of the white plague-like disease of *Favia fava* in the Red Sea. *Coral Reefs* 31:665–670.
- Aurand LW, Boone NH, Giddings CG (1977). Superoxide and singlet oxygen in milk lipid peroxidation. *Journal of Dairy Science* 60(3):363-369.

- Ayo B, Santamaría E, Latatu A, Artolozaga I, Azúa I, Iriberrí J (2001). Grazing rates of diverse morphotypes of bacterivorous ciliates feeding on four allochthonous bacteria. *Letters in Applied Microbiology* 33:455–460.
- Bachok Z, Mfilinge P, Tsuchiya M (2006). Characterization of fatty acid composition in healthy and bleached corals from Okinawa, Japan. *Coral Reefs* 25(4):545–554.
- Bailey TG, Robertson DR (1982). Organic and caloric levels of fish feces relative to its consumption by coprophagous reef fishes. *Marine Biology* 69(1):45–50.
- Baker LJ, Reich HG, Kitchen SA, Grace Klings J, Koch HR, Baums IB, ..., Thurber RV (2022). The coral symbiont *Candidatus Aquarickettsia* is variably abundant in threatened Caribbean acroporids and transmitted horizontally. *The ISME Journal* 16(2):400–411.
- Bakshani CR, Morales-Garcia AL, Althaus M, Wilcox MD, Pearson JP, Bythell JC, Burgess JG (2018). Evolutionary conservation of the antimicrobial function of mucus: A first defense against infection. *npj Biofilms and Microbiomes* 4(1):14.
- Banaszak AT, Barba Santos MG, LaJeunesse TC, Lesser MP (2006). The distribution of mycosporine-like amino acids (MAAs) and the phylogenetic identity of symbiotic dinoflagellates in cnidarian hosts from the Mexican Caribbean. *Journal of Experimental Marine Biology and Ecology* 337(2):131–146.
- Banaszak AT, Lesser MP (2009). Effects of solar ultraviolet radiation on coral reef organisms. *Photochemical and Photobiological Sciences* 8(9):1276–1294.
- Banin ES, Khare SK, Naider F, Rosenberg E (2001). Proline-rich peptide from coral pathogen *Vibrio shiloi* that inhibits photosynthesis of zooxanthellae. *Applied Environmental Microbiology* 67:1536–1541.
- Bell MV, Dick JR, Anderson TR, Pond DW (2007). Application of liposome and stable isotope tracer techniques to study polyunsaturated fatty acid biosynthesis in marine zooplankton. *Journal of Plankton Research* 29:417–422.
- Benavides M, Bednarz VN, Ferrier-Pagès C (2017). Diazotrophs: Overlooked key players within the coral symbiosis and tropical reef ecosystems? *Frontiers in Marine Science* 4:10.
- Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, Rosenberg E, (2003a). *Vibrio coralliilyticus* sp. nov., a temperature-dependent

- pathogen of the coral *Pocillopora damicornis*. The International Journal of Systematic and Evolutionary Microbiology 53:309- 315.
- Ben-Haim Y, Zicherman-Keren M, Rosenberg E (2003b). Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. Applied Environmental Microbiology 69 (7), 4236–4242.
- Berdy J (2005). Bioactive microbial metabolites. Journal of Antibiotics 58(1):1–26.
- Bessell-Browne P, Fisher R, Duckworth, A, Jones R (2017). Mucous sheet production in Porites: An effective bioindicator of sediment related pressures. Ecological Indicators 77:276–285.
- Bhagwat PV, Ravindran C, Irudayarajan L (2023). Characterization of healthy and diseased invertebrate coral mucus for their defense properties. Journal of Invertebrate Pathology 201:108001.
- Bhagwat PV, Ravindran C, Irudayarajan L (2024). Beneficial properties of mucus in coral adaptations and ecological interactions. Marine Biology 171(2):46.
- Binienda ZK, Sarkar S, Silva-Ramirez S, Gonzalez C (2013). Role of free fatty acids in physiological conditions and mitochondrial dysfunction. Food and Nutrition Sciences 04(9):6–15.
- Birchenough GM, Johansson ME, Gustafsson JK, Bergström JH, Hansson G (2015). New developments in goblet cell mucus secretion and function. Mucosal immunology 8(4):712-719.
- Bishop DG, Kenrick JR (1980). Fatty acid composition of symbiotic *Zooxanthellae* in relation to their hosts. Lipids 15(10):799.
- Boechat IG, Adrian R (2005). Biochemical composition of algivorous freshwater ciliates: you are not what you eat. FEMS microbiology ecology 53:393-400.
- Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, ..., Gregory Caporaso J (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:1-17.
- Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, ..., Caporaso JG (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37(8):852-857.

- Bourne D, Boyett H, Henderson M, Muirhead A, Willis B (2008). Identification of a Ciliate (Oligohymenophorea: Scuticociliatia) associated with Brown Band Disease on corals of the Great Barrier Reef. *Applied Environmental Microbiology* 74:883-888.
- Bourne DG, Dennis PG, Uthicke S, Soo RM, Tyson GW, Webster N (2013). Coral reef invertebrate microbiomes correlate with the presence of photosymbionts. *The ISME journal* 7(7):1452-1458.
- Bourne DG, Garren M, Work TM, Rosenberg E, Smith GW, Harvell CD (2009). Microbial disease and the coral holobiont. *Trends in Microbiology* 17:554–562.
- Bourne DG, Morrow KM, Webster NS (2016). Insights into the coral microbiome: underpinning the health and resilience of reef ecosystems. *Annual review of microbiology* 70:317-340.
- Brown B, Bythell J (2005). Perspectives on mucus secretion in reef corals. *Marine Ecology Progress Series* 296:291–309.
- Brown BE, Bythell JC (2005). Perspectives on mucus secretion in reef corals. *Marine Ecology Progress Series* 296:291–309.
- Bruckner AW (2015). White syndromes of western Atlantic reef-building corals. *Diseases of Coral* 316–332.
- Bruno JF, Petes LE, Harvell CD, Hettinger A (2003). Nutrient enrichment can increase the severity of coral diseases. *Ecology Letters* 6:1056–1061
- Bugni TS, Ireland CM (2004). Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Natural Product Reports* 21:143–163.
- Burriesci MS, Raab TK, Pringle JR (2012). Evidence that glucose is the major transferred metabolite in dinoflagellate–cnidarian symbiosis. *Journal of Experimental Biology* 215(19):3467-3477.
- Bythell JC, Wild C (2011). Biology and ecology of coral mucus release. *Journal of Experimental Marine Biology and Ecology* 408(1–2):88–93.
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13(7):581–583.
- Carey PG (1992). Marine interstitial ciliates. An illustrated key. *Chapman & Hall, London*. 368.

- Carreau JP, Dubacq JP (1978). Adaptation of a macro-scale method to the micro-scale for fatty acid methyl transesterification of biological lipid extracts. *Journal of Chromatography A* 151(3):384–390.
- Cerenius L, Lee BL, Soderhall K (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in immunology* 29(6):263-271.
- Cervino JM, Thompson FL, Gomez-Gil B, Lorence EA, Goreau TJ, Hayes RL, Winiarski-Cervino KB, Smith GW, Huguen K, Bartels E (2008). The *Vibrio* core group induces yellow band disease in Caribbean and Indo-Pacific reef-building corals. *Journal of Applied Microbiology* 105:1658–167.
- Chalfie M, Kain S (2006). *Green fluorescent protein: Properties, applications, and protocols*. Wiley Interscience.
- Chen HK, Wang LH, Chen WNU, Mayfield AB, Levy O, Lin CS, Chen CS (2017). Coral lipid bodies as the relay center interconnecting diel-dependent lipidomic changes in different cellular compartments. *Scientific Reports* 7(1):1–13.
- Chen Y, Ma J, Huang H, Chen P, Chao F, Luo W (2017). Effects of Temperature, pH, Ammonia Nitrogen Content and Salinity on Coral (*Acropora millepora* & *Palythoa* sp.) Bleaching. *Agricultural Biotechnology* 6(6):52-59.
- Choo JH, Rukayadi Y, Hwang JK (2006). Inhibition of bacterial quorum sensing by vanilla extract. *Letters in applied microbiology* 42(6):637-641.
- Clarke KR, Ainsworth M (1993). A method of linking multivariate community structure to environmental variables. *Marine Ecology-Progress Series* 92:205-205.
- Coddeville B, Maes E, Ferrier-Pages C, Guerardel Y (2011). Glycan profiling of gel forming mucus layer from the scleractinian symbiotic coral *Oculina arbuscula*. *Biomacromolecules* 12(6):2064–2073.
- Coffroth MA (1984). Ingestion and incorporation of coral mucus aggregates by a gorgonian soft coral. *Marine Ecology Progress Series* 17(2):193–199.
- Coffroth MA (1990). Mucous sheet formation on poritid corals: An evaluation of coral mucus as a nutrient source on reefs. *Marine Biology* 105(1):39–49.
- Coles SL, Strathmann R (1973). Observations on coral mucus “flocs” and their potential trophic significance 1. *Limnology and Oceanography* 18(4):673–678.

- Cooper W, Lirman O, Schmale M, Lipscomb D (2007). Consumption of coral spat by histophagic ciliates. *Coral Reefs* 26, 249-250.
- Corfield AP (2015). Mucins: A biologically relevant glycan barrier in mucosal protection. *Biochimica et Biophysica Acta* 1850(1):236–252.
- Cornick S (2018). SNARE-mediated exocytosis of MUC2 from intestinal goblet cells (Doctoral thesis, University of Calgary, Calgary, Canada).
- Correa AM, Welsh RM, Thurber RLV (2013). Unique nucleocytoplasmic dsDNA and +ssRNA viruses are associated with the dinoflagellate endosymbionts of corals. *ISME Journal* 7:13–27.
- Couttolenc A, Medina ME, Trigos Á, Espinoza C (2022). Antioxidant capacity of fungi associated with corals and sponges of the reef system of Veracruz, Mexico. *Electronic Journal of Biotechnology* 55:40-46.
- Croquer A, Bastidas C, Lipscomb D (2006). Folliculinid ciliates: a new threat to Caribbean corals? *Disease of Aquatic Organisms* 69:75–78.
- Crossland CJ (1987). In situ release of mucus and DOC-lipid from the corals *Acropora variabilis* and *Stylophora pistillata* in different light regimes. *Coral Reefs* 6(1):35–42.
- Crossland CJ (2021) HERMATYPIC CORAL, ACROPORA cf. ACUMINATA. In *Endosymbiosis and cell biology: A synthesis of recent research*. Proceedings of the International Colloquium on Endosymbiosis and Cell Research, Tübingen, April 1980 (Vol. 1, p. 163). Walter de Gruyter GmbH & Co KG.
- Crossland CJ, Barnes DJ, Borowitzka MA (1980). Diurnal lipid and mucus production in the staghorn coral *Acropora acuminata*. *Marine Biology*, 60(2–3):81–90.
- Cuong DM, Hien PTT, Van Ngoc B (2022). Changes in microbial community composition affect bioactivities of mucus isolated from coral *Acropora millepora*. *Vietnam Journal of Biotechnology* 20(2):387-392.
- D Ainsworth T, Krause L, Bridge T, Torda G, Raina JB, Zakrzewski M, ..., Leggat W. (2015). The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. *The ISME journal* 9(10):2261-2274.

- D'Angelo C, Wiedenmann J (2014). Impacts of nutrient enrichment on coral reefs: new perspectives and implications for coastal management and reef survival. *Current Opinion in Environmental Sustainability* 7:82-93.
- Dailah HG (2022). Therapeutic potential of small molecules targeting oxidative stress in the treatment of chronic obstructive pulmonary disease (COPD): A comprehensive review. *Molecules* 27(17):5542.
- Dalsgaard J, St John M, Kattner G, Muller-Navarra DC, Hagen W (2003). Fatty acid trophic markers in the pelagic marine environment. *Advances in Marine Biology* 46:225-340.
- Daumas R, Thomassin BA (1977). Protein fractions in coral and zoantharian mucus, possible evolution in coral reef environment. In *Proceedings of the (3rd) Int. Coral Reef Symp. (Miami)* 1 (pp. 517–525).
- Davy JE, Patten NL (2007). Morphological diversity of virus-like particles within the surface microlayer of scleractinian corals. *Aquatic Microbial Ecology* 47:37–44.
- De Carvalho CC, Caramujo MJ (2018). The various roles of fatty acids. *Molecules* 23(10):2583.
- Denny MW (1989). Invertebrate mucous secretions – Functional alternatives to vertebrate paradigms. *Symposia of the Society for Experimental Biology* 43:337–366.
- Desbois AP (2013). Antimicrobial properties of eicosapentaenoic acid (C20: 5n–3). *Marine Microbiology* 351-367.
- Diaz L (2017) Response of the coral associated nitrogen fixing bacteria toward elevated water temperature. *Journal of Water Resources and Ocean Science* 6(6):98-109.
- Ding JL, Fung FMY, Ng GWS, Chou LM (1999). Novel Bioactivities from a Coral, *Galaxea fascicularis*: DNase-like Activity and Apoptotic Activity Against a Multiple-Drug-Resistant Leukemia Cell Line. *Marine Biotechnology* 1(4):328–336.
- Dopheide A, Lear G, Stott R, Lewis G (2011). Preferential Feeding by the Ciliates *Chilodonella* and *Tetrahymena* spp. and Effects of These Protozoa on Bacterial Biofilm Structure and Composition. *Applied Environmental Microbiology* 77:4564-4572.
- Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM (2002). Oxidative stress and seasonal coral bleaching. *Free Radical Biology and Medicine* 33(4):533–543.

- Drollet JH, Teai T, Faucon M, Martin PMV (1997). Field study of compensatory changes in UV-absorbing compounds in the mucus of the solitary coral *Fungia repanda* (Scleractinia: Fungiidae) in relation to solar UV radiation, sea-water temperature, and other coincident physico-chemical parameters. *Marine and Freshwater Research* 48(4):329–333.
- Ducklow HW, Mitchell R (1979). Bacterial populations and adaptations in the mucus layers on living corals 1. *Limnology and Oceanography*, 24(4):715–725.
- Dunlap WC, Shick JM (1998). Ultraviolet radiation-absorbing mycosporine-like amino acids in coral reef organisms: a biochemical and environmental perspective. *Journal of Phycology* 34:418–430.
- Dunn JG, Sammarco PW, LaFleur Jr G (2012). Effects of phosphate on growth and skeletal density in the scleractinian coral *Acropora muricata*: A controlled experimental approach. *Journal of Experimental Marine Biology and Ecology* 411:34-44.
- Eckes M, Dove S, Siebeck UE, Grutter AS (2015). Fish mucus versus parasitic gnathiid isopods as sources of energy and sunscreens for a cleaner fish. *Coral Reefs* 34(3):823–833.
- Fabricius KE (2006). Effects of irradiance, flow, and colony pigmentation on the temperature microenvironment around corals: Implications for coral bleaching?. *Limnology and oceanography* 51(1):30-37.
- Fabricius KE, Crossman K, Jonker M, Mongin M, Thompson A (2023). Macroalgal cover on coral reefs: Spatial and environmental predictors, and decadal trends in the Great Barrier Reef. *PLoS One* 18(1):0279699.
- Fabricius KE, De'ath G, Humphrey C, Zagorskis I, Schaffelke B (2013). Intra-annual variation in turbidity in response to terrestrial runoff on near-shore coral reefs of the Great Barrier Reef. *Estuarine, Coastal and Shelf Science* 116:57-65.
- Falkowski PG, Dubinsky Z, Muscatine L, Porter JW (1984). Light and the bioenergetics of a symbiotic coral. *BioScience* 34(11):705–709.
- Fang JHS (2015). Community analysis of coral mucus-associated bacteria and impact of temperature and CO₂ changes on them (Doctoral dissertation. Swinburne University of Technology (Sarawak campus)).

- Fenchel T (1980). Suspension feeding in ciliated protozoa: functional response and particle size selection. *Microbial Ecology* 6:1–11.
- Fernandes de Barros Marangoni L, Ferrier-Pagès C, Rottier C, Bianchini A, Grover R (2020). Unravelling the different causes of nitrate and ammonium effects on coral bleaching. *Scientific Reports* 10(1):11975.
- Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu J, Datta P, McCormick M, Huang L, McDermott E, Eisenlohr L, Landel CP, Alnemri ES (2010). The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. *Nature Immunology* 11(5):385–393.
- Figueiredo C, Baptista M, Rosa IC, Lopes AR, Dionisio G, Rocha RJ, Cruz IC, Kikuchi RK, Simoes N, Leal MC, Tojeira I (2017). 3D chemoecology and chemotaxonomy of corals using fatty acid biomarkers: Latitude, longitude and depth. *Biochemical systematics and ecology* 70:35–42.
- Findlay RH, Dobbs FC (2018). Quantitative description of microbial communities using lipid analysis. *Handbook of methods in aquatic microbial ecology* 271–284.
- Fitzgerald LM, Szmant AM (1997). Biosynthesis of “essential” amino acids by scleractinian corals. *Biochemical Journal* 322(1):213–221.
- Fleming A (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of The Royal Society of London B* 93 (653):306–317.
- Folch J, Lees M, Sloane-Stanley GH (1956). A simple method for the isolation purification of total lipids from animal tissues. *Journal of Biological Chemistry* 226:497–509.
- Fortier LC, Sekulovic O (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence* 4 (5):354–365.
- Funk CD (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294(5548):1871–1875.
- Gabay Y (2018). The cellular and physiological basis of host-symbiont specificity in a model cnidarian-dinoflagellate symbiosis (Doctoral dissertation, Open Access Te Herenga Waka-Victoria University of Wellington).

- Galli P, Montano S, Seveso D, Maggioni D (2021). Coral reef biodiversity of the Maldives. Atolls of the Maldives. Nissology and Geography. Rowman and Littlefield, Lanham, 196-212.
- Gardner SG, Nielsen DA, Laczka O, Shimmon R, Beltran VH, Ralph PJ, Petrou K (2016). Dimethylsulfoniopropionate, superoxide dismutase and glutathione as stress response indicators in three corals under short-term hyposalinity stress. *Proceedings. Biological Sciences* 283(1824):20152418.
- Glasl B, Herndl GJ, Frade PR (2016). The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance. *ISME Journal* 10(9):2280–2292.
- Goldberg WM (2018). Coral food, feeding, nutrition, and secretion: A review. In *Marine organisms as model systems in biology and medicine* (pp. 377–421).
- Gottfried M, Roman MR (1983). Ingestion and incorporation of coral-mucus detritus by reef zooplankton. *Marine Biology* 72(3):211–218.
- Graeve M, Kattner G, Hagen W (1994). Diet induced changes in the fatty acid composition of Arctic herbivorous copepods: experimental evidence of trophic markers. *Journal of Experimental Marine Biology and Ecology* 182:97–110.
- Gross EM (2003). Allelopathy of aquatic autotrophs. *Critical reviews in plant sciences* 22(3-4):313-339.
- Grottoli AG, Rodrigues LJ, Juarez C (2004). Lipids and stable carbon isotopes in two species of Hawaiian corals. *Porites compressa* and *Montipora verucosa*, following a bleaching event. *Marine Biology* 145:621–631.
- Guarnieri MC, de Albuquerque Modesto JC, Pérez CD, Ottaiano TF, Ferreira RDS, Batista FP, de Brito MV, Campos IHMP, Oliva MLV (2018). Zoanthid mucus as new source of useful biologically active proteins. *Toxicon* 143:96–107.
- Guil-Guerrero JL (2007). Stearidonic acid (18: 4n-3): Metabolism, nutritional importance, medical uses and natural sources. *European Journal of Lipid Science and Technology* 109(12):1226–1236.
- Guppy R, Brown B, Bythell JC (2019). Preserving the viscous coral surface mucus layer using low-acid glycol methacrylate (GMA) resin. *Coral Reefs* 38(3):521-526.

- Guppy R, Bythell JC (2006). Environmental effects on bacterial diversity in the surface mucus layer of the reef coral *Montastraea faveolata*. *Marine Ecology Progress Series* 328:133-142.
- Guzman P, Ecker JR (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *The Plant Cell* 2(6):513-523.
- Haas A, Al-Zibdah M, Wild C (2009). Effect of inorganic and organic nutrient addition on coral–algae assemblages from the Northern Red Sea. *Journal of Experimental Marine Biology and Ecology* 380(1-2):99-105.
- Haas AF, Fairoz MFM, Kelly LW, Nelson CE, Dinsdale EA, Edwards RA et al (2016). Global microbialization of coral reefs. *Nature Microbiology* 1(7):16042.
- Haas, A. F., Smith, J. E., Thompson, M., & Deheyn, D. D. (2014). Effects of reduced dissolved oxygen concentrations on physiology and fluorescence of hermatypic corals and benthic algae. *Peer Journal* 2:235.
- Hackerott S, Martell HA, Eirin-Lopez JM (2021). Coral environmental memory: Causes, mechanisms, and consequences for future reefs. *Trends in Ecology and Evolution* 36 (11):1011-1023.
- Hadaidi G, Gegner HM, Ziegler M, Voolstra CR (2019). Carbohydrate composition of mucus from scleractinian corals from the central Red Sea. *Coral Reefs* 38(1):21–27.
- Hadaidi G, Röthig T, Yum LK, Ziegler M, Arif C, Roder C, ..., Voolstra CR (2017). Stable mucus-associated bacterial communities in bleached and healthy corals of *Porites lobata* from the Arabian Seas. *Scientific Reports* 7(1):45362.
- Hadaidi GA (2018). Coral-associated bacterial community dynamics in healthy, bleached, and disease states [Doctoral Dissertation].
- Hafezi M, Giffin AL, Alipour M, Sahin O, Stewart RA (2020). Mapping long-term coral reef ecosystems regime shifts: A small island developing state case study. *Science of the Total Environment* 716:137024.
- Hallock P, Schlager W (1986). Nutrient excess and the demise of coral reefs and carbonate platforms. *Palaios* 389-398.
- Harel M, Ben-Dov E, Rasoulouniriana D, Siboni N, Kramarsky-Winter E, Loya Y,... Kushmaro A (2008). A new Thraustochytrid, strain Fng1, isolated from surface

- mucus of the hermatypic coral *Fungia granulosa*. FEMS Microbiology Ecology 64(3):378-387.
- Harland AD, Navarro JC, Spencer-Davies P, Fixter LM (1993). Lipids of some Caribbean and Red Sea corals: total lipid, wax esters, triglycerides and fatty acids. Marine Biology 117:113–117.
- Harland AD, Spencer-Davies P, Fixter LM (1992). Lipid content of some Caribbean corals in relation to depth and light. Marine Biology 113:357–361.
- Hawkins TD, Krueger T, Becker S, Fisher PL, Davy SK (2014). Differential nitric oxide synthesis and host apoptotic events correlate with bleaching susceptibility in reef corals. Coral Reefs 33, 141-153.
- Hawkins TD, Krueger T, Wilkinson SP, Fisher PL, Davy SK (2015). Antioxidant responses to heat and light stress differ with habitat in a common reef coral. *Coral Reefs*, 34, 1229-1241.
- Hazel JR, Williams EE (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Progress in Lipid Research 29(3):167–227.
- Hempelmann Ernst, Krafts K (2017). The mechanism of silver staining of proteins separated by SDS polyacrylamide gel electrophoresis. Biotechnic & Histochemistry 92:1-7.
- Hill DB, Button B, Rubinstein M, Boucher RC (2022). Physiology and pathophysiology of human airway mucus. Physiological Reviews 102(4):1757–1836.
- Hillyer KE, Dias DA, Lutz A, Wilkinson SP, Roessner U, Davy SK (2017). Metabolite profiling of symbiont and host during thermal stress and bleaching in the coral *Acropora aspera*. Coral Reefs 36:105-118.
- Hinrichs S, Patten NL, Allcock RJN, Saunders SM, Strickland D, Waite AM (2013). Seasonal variations in energy levels and metabolic processes of two dominant *Acropora* species (*A. spicifera* and *A. digitifera*) at Ningaloo Reef. Coral Reefs 32(3):623–635.
- Ho TK (2017). Dredging land reclamation causing mucus development in massive spherical corals in the Spratly Islands, South China sea: The effects on China's. Journal of Fisheries International Industry.

- Hoogenboom M, Rottier C, Sikorski S, Ferrier-Pages C (2015). Among-species variation in the energy budgets of reef-building corals: Scaling from coral polyps to communities. *Journal of Experimental Biology* 218(24):3866–3877.
- Horka I, De Grave S, Franssen CH, Petrusek A, Ďuriš Z (2016). Multiple host switching events shape the evolution of symbiotic palaemonid shrimps (Crustacea: Decapoda). *Scientific Reports* 6(1):26486.
- Horricks RA, Herbinger CM, Lillie BN, Taylor P, Lumsden JS (2019). Differential protein abundance during the first month of regeneration of the Caribbean star coral *Montastraea cavernosa*. *Coral Reefs* 38(1):45–61.
- Huang YS, Huang WC, Li CW, Chuang LT (2011). Eicosadienoic acid differentially modulates production of pro inflammatory modulators in murine macrophages. *Molecular and Cellular Biochemistry* 358:85–94.
- Hulbert AJ (2003). Life, death and membrane bilayers. *Journal of Experimental Biology* 206:2303-2311.
- Huma N, Shankar P, Kushwah J, Bhushan A, Joshi J, Mukherjee T, Raju SC, Purohit HJ, Kalia VC (2011). Diversity and polymorphism in AHL-lactonase gene (aiiA) of *Bacillus*. *Journal of Microbiology and Biotechnology* 21(10):1001-1011.
- Iluz D, Dubinsky Z (2015). Coral photobiology: New light on old views. *Zoology* 118(2):71–78.
- Imbs AB, Latyshev NA, Dautova TN, Latypov YY (2010). Distribution of lipids and fatty acids in corals by their taxonomic position and presence of zooxanthellae. *Marine Ecology Progress Series* 409:65–75.
- Imbs AB, Latyshev NA, Zhukova NV, Dautova TN (2007). Comparison of fatty acid compositions of azooxanthellate *Dendronephthya* and zooxanthellate soft coral species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 148(3):314-321.
- Imbs AB, Yakovleva IM (2012). Dynamics of lipid and fatty acid composition of shallow water corals under thermal stress: an experimental approach. *Coral Reefs* 31:41-53.

- Imbs AB, Yakovleva IM, Dautova TN, Bui LH, Jones P (2014). Diversity of fatty acid composition of symbiotic dinoflagellates in corals: evidence for the transfer of host PUFAs to the symbionts. *Phytochemistry* 101:76-82.
- Irudayarajan L, Ravindran C, Raveendran HP, (2023). Antimicrobial activity of coral-associated beneficial bacteria against coral disease causing microbial pathogens. *Journal of Basic Microbiology* 64(1):81-93.
- Ivanova EP, Nguyen SH, Guo Y, Baulin VA, Webb HK, Truong VK, Wandiyanto JV, GarveyCJ, Mahon PJ, Mainwaring DE, Crawford RJ (2017). Bactericidal activity of self-assembled palmitic and stearic fatty acid crystals on highly ordered pyrolytic graphite. *Acta Biomaterialia* 59:148–157.
- Jeffries PH (1972). Fatty acid ecology of tidal marsh. *Limnology and Oceanography* 17:433-440.
- Johnston I, Rohwer F (2007). Microbial landscapes on the outer tissue surfaces of the reef building coral *Porites compressa*. *Coral Reefs* 26:375-383.
- Jr Thompson JH, Shinn EA, Bright TJ (1980). Effects of drilling mud on seven species of reef building corals as measured in the field and laboratory. In: Geyer RA (ed) *Marine environmental pollution, vol 1: Hydrocarbons Elsevier, New York Amsterdam*. 433-454.
- Jump DB, Clarke SD, MacDougald O, Thelen A (1993). Polyunsaturated fatty acids inhibit S14 gene transcription in rat liver and cultured hepatocytes. *Proceedings of the National Academy of Sciences* 90(18):8454–8458.
- Kabeya N, Fonseca MM, Ferrier DEK, Navarro JC, Bay LK, Francis DS, Tocher DR, Castro LFC, Monroig O (2018). Genes for de novo biosynthesis of omega-3 polyunsaturated fatty acids are widespread in animals. *Science Advances* 4:6849.
- Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution* 30(4): 772-780.
- Katz S, Pollock F, Bourne D, Willis B (2014). Crown-of-thorns starfish predation and physical injuries promote brown band disease on corals. *Coral Reefs* 33:705-716.

- Kawashima H, Toyoda-Ono Y, Suwa Y, Kiso Y (2009). Subchronic (13-week) oral toxicity study of dihomono- γ -linolenic acid (DGLA) oil in rats. *Food and chemical toxicology* 47(6):1280-1286.
- Kellogg RB, Patton JS (1983). Lipid droplets, medium of energy exchange in the symbiotic anemone *Condylactis gigantea*: a model coral polyp. *Marine Biology* 75:137–149.
- Kiran CR, Rao DB, Sirisha N, Rao TR (2012). Impact of germination on biochemical and antioxidant enzymes of *Ceiba pentandra* (Kapok) seeds. *American Journal of Plant Sciences* 3(09):1187.
- Koh EGL (1997). Do Scleractinian Corals Engage in Chemical Warfare Against Microbes? *Journal of Chemical Ecology* 23:379–398.
- Krueger T, Becker S, Pontasch S, Dove S, Hoegh-Guldberg O, Leggat W, Fisher PL, Davy SK (2014). Antioxidant plasticity and thermal sensitivity in four types of *Symbiodinium* sp. *Journal of Phycology* 50(6):1035-1047.
- Krupp DA (1982). The composition of the mucus from the mushroom coral *Fungia scutaria*. In: *Proceedings of the 4th Int. Coral Reef Symposium* 2:9–73.
- Kruskal WH, Wallis WA (1952). Use of ranks in one-criterion variance analysis. *Journal of the American statistical Association* 47(260):583-621.
- LaJeunesse TC, Smith R, Walther M, Pinzón J, Pettay DT, McGinley M, Aschaffenburg M, Medina-Rosas P, Cupul-Magaña AL, Pérez AL, Reyes-Bonilla H, Warner (2010). Host–symbiont recombination versus natural selection in the response of coral dinoflagellate symbioses to environmental disturbance. *Proceedings of the Royal Society B: Biological Sciences* 277(1696):2925–2934.
- Latyshev NA, Naumenko NV, Svetashev VI, Latypov YY (1991). Fatty acids of reef building corals. *Marine Ecology Progress Series* 76:295–301.
- Le Campion-Alsumard T, Golubic S, Hutchings P (1995a). Microbial endoliths in skeletons of live and dead corals: *Porites lobata* (Moorea, French Polynesia). *Marine Ecology Progress Series* 117:149–157.
- Lee JJ, Leedale GF, Bradbury PC (2000). *An Illustrated Guide to the Protozoa: Organisms Traditionally Referred to as Protozoa, or Newly Discovered Groups*. 2nd edn. II. Massachusetts: *Blackwell Scientific*. 690–1432.

- Lee, S. T., Davy, S. K., Tang, S. L., & Kench, P. S. (2016). Mucus sugar content shapes the bacterial community structure in thermally stressed *Acropora muricata*. *Frontiers in microbiology* 7:371.
- Lesser MP (1996). Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnology and Oceanography* 41(2):271-283.
- Lesser MP, Mazel CH, Gorbunov MY, Falkowski PG (2004). Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. *Science* 305:997–1000.
- Lirman D, Fong P (2007). Is proximity to land-based sources of coral stressors an appropriate measure of risk to coral reefs? An example from the Florida Reef Tract. *Marine Pollution Bulletin* 54(6):779-791.
- Lobban CS, Raymundo LM, Montagnes DJ (2011). *Porpostoma guamensis* n. sp., a Philasterine Scuticociliate associated with Brown-Band Disease of corals. *Journal of Eukaryotic Microbiology* 58(2):103-113.
- Long H, Zufall R (2010). Diverse modes of reproduction in the marine free-living ciliate *Glaucanema trihymene*. *BMC Microbiology* 10:108.
- Mascarelli PE, Bunkley-Williams L (1999). An experimental field evaluation of healing in damaged, unbleached and artificially bleached star coral, *Montastraea annularis*. *Bulletin of Marine Science* 65(2):577–586.
- McAuley PJ (1994). Interactions between host and symbionts in algal invertebrate intracellular symbioses. *Botanical Journal of Scotland* 47:97–112.
- McGinty ES, Pieczonka J, Mydlarz LD (2012). Variations in reactive oxygen release and antioxidant activity in multiple *Symbiodinium* types in response to elevated temperature. *Microbial Ecology* 64(4):1000-1007.
- McKew BA, Dumbrell AJ, Daud SD, Hepburn L, Thorpe E, Mogensen L, Whitby C (2012). Characterization of geographically distinct bacterial communities associated with coral mucus produced by *Acropora* spp. and *Porites* spp. *Applied Environmental Microbiology* 78(15):5229-5237.
- McLean RJ, Pierson III LS, Fuqua C (2004). A simple screening protocol for the identification of quorum signal antagonists. *Journal of microbiological methods* 58(3):351-360.

- Means JC, Sigleo AC (1986). Contribution of coral reef mucus to the colloidal organic pool in the vicinity of Discovery Bay, Jamaica, WI. *Bulletin of Marine Science* 39(1):110–118.
- Meesters EH, Bak RP (1993). Effects of coral bleaching on tissue regeneration potential and colony survival. *Marine Ecology Progress Series* 189–198.
- Meikle P, Richards GN, Yellowlees D (1987). Structural determination of the oligosaccharide side chains from a glycoprotein isolated from the mucus of the coral *Acropora formosa*. *Journal of Biological Chemistry* 262(35):16941-16947.
- Meikle P, Richards GN, Yellowlees D (1988). Structural investigations on the mucus from six species of coral. *Marine Biology* 99:187-193.
- Mies M, Guth AZ, Tenorio AA, Banha TNS, Waters LG, Polito PS, Taniguchi S, Bicego MC, Sumida PYG (2018). In situ shifts of predominance between autotrophic and heterotrophic feeding in the reef-building coral *Mussismilia hispida*: an approach using fatty acid trophic markers. *Coral Reefs* 37(3):677–689.
- Mills E, Shechtman K, Loya Y, Rosenberg E (2013). Bacteria appear to play important roles in both causing and preventing the bleaching of the coral *Oculina patagonica*. *Marine Ecology Progress Series* 489:155-162.
- Mock T, Kroon BM (2002). Photosynthetic energy conversion under extreme conditions II: the significance of lipids under light limited growth in Antarctic sea ice diatoms. *Phytochemistry* 61(1):53–60.
- Morrison-Gardiner S (2002). Dominant fungi from Australian coral reefs. *Fungal Divers* 9:105-121.
- Morton JT, Sanders J, Quinn RA, McDonald D, Gonzalez A, Vázquez-Baeza Y, ..., Knight R (2017). Balance trees reveal microbial niche differentiation. *MSystems* 2(1):162-16.
- Mutha RE, Tatiya AU, Surana SJ (2021). Flavonoids as natural phenolic compounds and their role in therapeutics: An overview. *Future journal of pharmaceutical sciences* 7:1-13.
- Mydlarz LD, Couch CS, Weil E, Smith G, Harvell CD, (2009). Immune defenses of healthy, bleached and diseased *Montastraea faveolata* during a natural bleaching event. *Diseases of aquatic organisms* 87(1-2):67-78.

- Mydlarz LD, Harvell CD (2007). Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. *Comparative Biochemistry and Physiology A Molecular and Integral Physiology* 146(1):54–62.
- Mydlarz LD, Palmer CV (2011). The presence of multiple phenoloxidases in Caribbean reef-building corals. *Comparative Biochemistry and Physiology A Molecular and Integral Physiology* 159(4):372–378.
- Namikoshi M, Kobayashi H, Yoshimoto T, Meguro S, Akano K (2000). Isolation and characterization of bioactive metabolites from marine-derived filamentous fungi collected from tropical and sub-tropical coral reefs. *Chemical and Pharmaceutical Bulletin* 48:1452–1457.
- Nelson CE, Goldberg SJ, Kelly LW, Haas AF, Smith JE, Rohwer F *et al* (2013). Coral and macroalgal exudates vary in neutral sugar composition and differentially enrich reef bacterioplankton lineages. *ISME Journal* 7:962–979.
- Nettleton JA (1995). Omega-3 fatty acids and health. In *Omega-3 fatty acids and health* (pp. 64–76). Springer, Boston, MA.
- Nguyen-Kim H, Bettarel Y, Bouvier T, Bouvier C, Doan-Nhu H, Nguyen-Ngoc L, ... Brune J (2015). Coral mucus is a hot spot for viral infections. *Applied and Environmental Microbiology* 81(17):5773–5783.
- Nguyen-Kim H, Bouvier T, Bouvier C, Doan NH, Nguyen NL, Rochelle-Newall E, Desnues C, Reynaud S, Ferrier-Pages C, Bettarel Y (2014). High occurrence of viruses in the mucus layer of scleractinian corals. *Environmental Microbiology* 6:675–682.
- Nissimov, J., Rosenberg, E., & Munn, C. B. (2009). Antimicrobial properties of resident coral mucus bacteria of *Oculina patagonica*. *FEMS Microbiology Letters* 292(2):210–215.
- Nomura M, Kamogawa H, Susanto E, Kawagoe C, Yasui H, Saga N, Hosokawa M, Miyashita K (2013). Seasonal variations of total lipids, fatty acid composition, and fucoxanthin contents of *Sargassum horneri* (Turner) and *Cystoseira hakodatensis* (Yendo) from the northern seashore of Japan. *Journal of Applied Phycology* 25:1159–1169.
- Oku H, Yamashiro H, Onaga K (2003). Lipid biosynthesis from [¹⁴C]-glucose in the coral *Montipora digitata*. *Fisheries Science* 69(3):625–631.

- Page CA, Willis BL (2008). Epidemiology of skeletal eroding band on the Great 1 Barrier Reef and the role of injury in the initiation of this widespread coral disease. *Coral Reefs* 27:257–272.
- Palmer C, Roth M, Gates R (2009). Red fluorescent protein responsible for pigmentation in Trematode-Infected *Porites compressa* tissues. *Biology Bulletin* 21:68-74.
- Palmer CV, Traylor-Knowles NG, Willis BL, Bythell JC (2011). Corals use similar immune cells and wound-healing processes as those of higher organisms. *PloS one* 6(8):23992.
- Papina M, Meziane T van, Woesik R (2003). Symbiotic zooxanthellae provide the host-coral *Montipora digitata* with polyunsaturated fatty acids. *Comparative Biochemistry and Physiology B* 135:533–537.
- Pasaribu B, Weng LC, Lin IP, Camargo E, Tzen JT, Tsai CH, Ho SL, Lin MR, Wang LH, Chen CS, Jiang PL (2015). Morphological variability and distinct protein profiles of cultured and endosymbiotic Zooxanthellae cells isolated from *Exaiptasia Pulchella*. *Scientific Report* 5(1):15353.
- Pascal H (1981). Bacterial utilization of mucus on the coral reef of Aqaba (Red Sea). In *Proc. 4th int. Coral Reef Symp., Univ. Philippines, Manila* 1:669–677.
- Patton JS, Battery JF, Rigler MW, Porter JW, Black CC, Burris JE (1983). A comparison of metabolism of bicarbonate ¹⁴C and acetate 1-¹⁴C and the variability of species lipid components in reef corals. *Marine Biology* 75:121-130.
- Patton JS, Burris JE (1983). Lipid synthesis and extrusion by freshly isolated *zooxanthellae* (symbiotic algae). *Marine Biology* 75:131–136.
- Paul JH (2008). Prophages in marine bacteria: dangerous molecular time bombs or the key to survival in the seas?. *The ISME journal* 2(6):579-589.
- Piludu M, Rayment SA, Liu B, Offner GD, Oppenheim FG, Troxler RF, Hand AR (2003). Electron microscopic immunogold localization of salivary mucins MG1 and MG2 in human submandibular and sublingual glands. *Journal of Histochemistry and Cytochemistry* 51(1):69–79
- Qiu D, Huang L, Huang H, Yang J, Lin S (2010). Two functionally distinct ciliates dwelling in *Acropora* corals in the South China Sea near Sanya, Hainan Province, China, *Applied and Environmental Microbiology* 76:5639-5643.

- Radecker N, Pogoreutz C, Voolstra CR, Wiedenmann J, Wild C (2015). Nitrogen cycling in corals: The key to understanding holobiont functioning? Trends in Microbiology 23(8):490–497.
- Radice VZ, Brett MT, Fry B, Fox MD, Hoegh-Guldberg O, Dove SG (2019). Evaluating coral trophic strategies using fatty acid composition and indices. PLoS ONE 14(9):0222327.
- Raghukumar C, Raghukumar S (1991). Fungal invasion of massive corals. PSZNI Marine Ecology 12:251–260.
- Raghukumar S, Balasubramanian R (1991). Occurrence of thraustochytrid fungi in corals and coral mucus, Indian Journal of Marine Sciences 20:176–181.
- Ramos-Flores T (1983). Lower marine fungus associated with black line disease in star corals (*Montasrea annularis* E & S). Biology Bulletin 165:429–435.
- Randall C, Jordan-Garza J, vanWoesik R (2014). Ciliates associated with signs of disease on two Caribbean corals. Coral Reefs 34:243-247.
- Randall CJ, Jordán-Garza AG, Muller EM, Van Woesik R (2014). Relationships between the history of thermal stress and the relative risk of diseases of Caribbean corals. Ecology 95(7):1981-1994.
- Rasoulouniriana D, Siboni N, Ben-Dov E, Kramarsky-Winter E, Loya Y, Kushmaro A (2009). *Pseudoscillatoria coralii* gen. nov., sp. nov., a cyanobacterium associated with coral black band disease (BBD). Disease of Aquatic Organisms 87:91-96.
- Ravindran C, Bhagwat PV, Silveira KA, Shivaramu MS, Lele UP (2020). Characterization of coral associated ciliates and their interactions with disease lesion progression of Indian Scleractinian corals. Microbial Pathogenesis 149:104472.
- Ravindran C, Bhagwat PV, Silveirza KA, Shivaramu MS, Upendra PL (2020). Characterization of coral associated ciliates and their interactions with disease lesion progression of Indian Scleractinian corals. Microbial Pathogenesis 149:104472.
- Ravindran C, Irudayarajan L, Raveendran HP (2023). Possible beneficial interactions of ciliated protozoans with coral health and resilience". Applied and Environmental Microbiology 0121723.

- Ravindran C, Lawrance I, Vasanth AJ (2022). Endosymbiotic *Symbiodinium* clades occurrence and influence on coral growth and resilience during stress. *Symbiosis* 86:261–272.
- Ravindran C, Lawrance I, Vasanth AJ (2022b). Endosymbiotic *Symbiodinium* clades occurrence and influence on coral growth and resilience during stress. *Symbiosis* 86: 261-272.
- Ravindran C, Naveenan T, Varatharajan GR, Rajasabapathy R, Meena RM (2012a). Antioxidants in mangrove plants and endophytic fungal associations. *Botanica Marina* 55(3):269–279.
- Ravindran C, Raveendran HP, Irudayarajan L (2021). Ciliated protozoan occurrence and association in the pathogenesis of coral disease. *Microbial Pathogenesis* 162:105211.
- Ravindran C, Varatharajan GR, Rajasabapathy R, Vijayakanth S, Kumar AH, Meena RM (2012b). A role for antioxidants in acclimation of marine derived pathogenic fungus (NIOCC 1) to salt stress. *Microbial Pathogenesis* 53 (3-4):68–79.
- Ravindran J, Raghukumar C, Raghukumar S (2001). Fungi in *Porites lutea*: association with healthy and diseased corals. *Diseases of Aquatic Organisms* 47:219–228.
- Reiner K (2010). Catalase test protocol. American society for microbiology 1-6.
- Renaud SM, Thinh LV, Parry DL (1999). The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. *Aquaculture* 170(2):147–159.
- Reshef L, Korez O, Loya Y, Zilber-Rosenberg I, Rosenberg E (2006). The coral probiotic hypothesis. *Environmental Microbiology* 8 (12):2068–2073.
- Reynaud S, Ferrier-Pagès C (2019). Biology and ecophysiology of Mediterranean cold–water corals. In *Mediterranean cold-water corals: Past, present and future: Understanding the deep-sea realms of coral*, 391–404.
- Richardson L (1996). Horizontal and vertical migration patterns of *Phorridium corallyticum* and *Beggiatoa* spp. associated with black-band disease of corals. *Microbial Ecology* 32:323-335.
- Richardson L, Miller A, Broderick E, Kaczmarzky L, Gantar M, Stanic D, Sekar R (2009). Sulfide, microcystin, and the etiology of black band disease. *Diseases of Aquatic Organisms* 87:79- 90.

- Richardson LL (1998). Coral diseases: what is really known? *Trends in Ecology and Evolution* 13:438-443.
- Richman S, Loya Y, Slobocckin LB (1975). The rate of mucus production by corals and its assimilation by the coral reef copepod *Acartia negligens* 1. *Limnology and Oceanography* 20(6):918–923.
- Rinkevich B (1989). The contribution of photosynthetic products to coral reproduction. *Marine Biology* 101:259–263.
- Ritchie (2006). Regulation of microbial populations by coral surface mucus and mucus associated bacteria. *Marine Ecology Progress Series* 322:1–14.
- Ritchie KB, Smith GW (1995). Preferential carbon utilization by surface bacterial communities from water mass, normal, and white-band diseased *Acropora cervicornis*. *Molecular Marine Biology and Biotechnology* 4:345-352.
- Ritchie KB, Smith GW (1998). Type II white-band disease. *Revista the Biologia Tropical* 46(5):199-203.
- Rivera-Ortega J, Thome PE (2018). Contrasting antibacterial capabilities of the surface mucus layer from three symbiotic Cnidarians. *Frontiers in Marine Science* 5:392.
- Rix L, de Goeij JM, van Oevelen D, Struck U, Al-Horani FA, Wild C, Naumann MS (2017). Differential recycling of coral and algal dissolved organic matter via the sponge loop. *Functional Ecology* 31(3):778-789.
- Rizzatti G, Lopetuso LR, Gibiino G, Binda C, Gasbarrini A (2017). Proteobacteria: a common factor in human diseases. *Biomedical Research International* 2017(1):9351507.
- Rocker MM, Francis DS, Fabricius KE, Willis BL, Bay LK (2019). Temporal and spatial variation in fatty acid composition in *Acropora tenuis* corals along water quality gradients on the Great Barrier Reef, Australia. *Coral Reefs* 38(2):215–228.
- Roder C, Bayer T, Aranda M, Kruse M, Voolstra CR (2015). Microbiome structure of fungid coral *Ctenactis echinata* aligns with environmental differences. *Molecular Ecology* 24:3501–3511.
- Roder C, et al. (2014). Bacterial profiling of White Plague Disease in a comparative coral species framework. *ISME Journal* 8:31–39.

- Rodrigues LJ, Grottoli AG, Pease TK (2008). Lipid class composition of bleached and recovering *Porites compressa* Dana, 1846 and *Montipora capitata* Dana, 1846 corals from Hawaii. *Journal of Experimental Marine Biology and Ecology* 358:136–143.
- Rohwer F, Thurber R (2009). Viruses manipulate the marine environment. *Nature* 459:207-212.
- Romdhane MS, Devresse B, Lege'r PH, Sorgeloos P (1995). Effects of feeding ((-3) HUFA enriched Artemia during a progressively increasing period on the larviculture of freshwater prawns. *Aquaculture International* 3:236 242.
- Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I (2007). The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* 5(5):355-362.
- Rosenberg, E (2021). Microbiomes. *Current Knowledge and Unanswered Questions*. Springerbook.
- Russo GL (2009). Dietary n-6 and n-3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. *Biochemical Pharmacology* 77:937–946.
- Rypien KL, Ward JR, Azam F (2010). Antagonistic interactions among coral-associated bacteria. *Environmental Microbiology* 12:28–39.
- Sadarun B, Rahmatika NS, Yodha AWM, Fristiohady A, Sundowo A, Baharum SN (2022). Antioxidant and Cytotoxic Properties of Soft Coral *Nepthea* sp. *Ilmu Kelautan: Indonesian Journal of Marine Sciences* 27(1).
- Sahidin I, Sadrun B, Rahmatika NS, Yodha AWM, Fristiohady A, Sundowo A, Fajriah S (2023). Phytochemical screening and antioxidant and cytotoxic activities of ethyl acetate subfractions of soft coral *Nepthea* sp. growing in Southeast Sulawesi. *Journal of Applied Pharmaceutical Science* 13(2):099-105.
- Salian A, Dutta S, Mandal S (2021). A roadmap to UV-protective natural resources: Classification, characteristics, and applications. *Materials Chemistry Frontiers* 5(21):7696-7723.
- Salvatore S, Heuschkel R, Tomlin S, Davies SE, Edwards S, Walker-Smith J, ..., Murch SH (2000). A pilot study of N-acetyl glucosamine, a nutritional substrate for

- glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Alimentary Pharmacology and Therapeutics* 14(12):1567-1579.
- Sargent JR, Bell MV, Henderson RJ, Tocher DR (1990). Polyunsaturated fatty acids in marine and terrestrial food webs. In: Mellinger J, editor. *Nutrition in Wild and Domestic Animals*. Basel: Karger 11–23.
- Schieber M, Chandel NS (2014). ROS function in redox signaling and oxidative stress. *Current Biology* 24(10):453-462.
- Schottner S, Hoffmann F, Wild C, Rapp HT, Boetius A, Ramette A (2009). Inter-and intra-habitat bacterial diversity associated with cold-water corals. *The ISME journal* 3(6):756-759.
- Seabold S, Perktold J (2010). Statsmodels: econometric and statistical modeling with python. *SciPy* 7:1.
- Sebens KP (1987). Coelenterata. In: Pandian TJ, Vernberg FJ (eds) *Animals energetics*. Academic, San Diego 55–120.
- Seemann J, Sawall Y, Auel H, Richter C (2013). The use of lipids and fatty acids to measure the trophic plasticity of the coral *Stylophora subseriata*. *Lipids* 48(3):275–286.
- Shafir A, Loya A (1983). Consumption and assimilation of coral mucus by the burrowing mussel *Lithophaga lessepsiana*. In *Proceedings of the International Conference in Marine Science Red Sea* 9:135–140.
- Sharma D, Ravindran C (2020). Diseases and pathogens of marine invertebrate corals in Indian reefs. *Journal of Invertebrate Pathology* 173:107373.
- Sharma P, Jha AB, Dubey RS, Pessarakli M (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany* 2012:217037.
- Shick JM, Dunlap WC, Pearse JS, Pearse VB (2002). Mycosporine-like amino acid content in four species of sea anemones in the genus *Anthopleura* reflects phylogenetic but not environmental or symbiotic relationships. *Biological Bulletin* 203(3):315–330.
- Shields P, Cathcart L (2010). Oxidase test protocol. *American Society for Microbiology* 1-9.
- Shmuel Y, Ziv Y, Rinkevich B (2022). Coral-inhabiting *Trapezia* crabs forage on demersal plankton. *Frontiers in Marine Science* 9:1652.

- Shnit-Orland M, Kushmaro A (2009). Coral mucus-associated bacteria: A possible first line of defense. *FEMS Microbiology Ecology* 67(3):371–380.
- Shoaib M, Muzammil I, Hammad M, Bhutta ZA, Yaseen I (2020). A mini-review on commonly used biochemical tests for identification of bacteria. *International Journal of Research Publications* 54(1):1-7.
- Shukla MR, Yadav R, Desai A (2009). Catalase and superoxide dismutase double staining zymogram technique for *Deinococcus* and *Kocuria* species exposed to multiple stresses. *Journal of basic microbiology* 49(6):593-597.
- Siboni N, Ben-Dov E, Sivan A, Kushmaro A (2008). Coral-associated ammonium oxidizing Crenarchaeota and their role in the coral holobiont nitrogen cycle. In *Proceedings of the 11th International Coral Reef Symposium* 252-256.
- Siboni N, Ben-Dov E, Sivan A, Kushmaro A (2008). Global distribution and diversity of coral associated Archaea and their possible role in the coral holobiont nitrogen cycle. *Environmental Microbiology* 10(11):2979-2990.
- Siboni N, Ben-Dov E, Sivan A, Kushmaro A (2012). Geographic specific coral-associated ammonia-oxidizing archaea in the northern Gulf of Eilat (Red Sea). *Microbial Ecology* 64:18-24.
- Sikorskaya TV, Ermolenko EV, Efimova KV (2022). Lipids of Indo-Pacific gorgonian corals are modified under the influence of microbial associations. *Coral Reefs* 41(2):277–291.
- Simopoulos AP (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine* 233:674–688.
- Smith D, Leary P, Craggs J, Bythell J, Sweet M (2015). Microbial Communities associated with healthy and white syndrome-affected *Echinoporala mellosa* in aquaria and experimental treatment with the antibiotic ampicillin. *PLOS ONE* 10:0121780.
- Smith GW, Ives LD, Nagelkerken IA, Ritchie KB (1996). Caribbean sea-fan mortalities. *Nature* 383:487.
- Smith HA, Conlan JA, Pollock FJ, Wada N, Shore A, Hung JYH, Aeby GS, Willis BL, Francis DS, Bourne DG (2020). Energy depletion and opportunistic microbial

- colonisation in white syndrome lesions from corals across the Indo-Pacific. *Scientific Reports* 10(1):19990.
- Stabili L (2019). The mucus of marine invertebrates: Cnidarians, polychaetes, and echinoderms as case studies. In *Enzymatic technologies for marine polysaccharides* (pp. 151–162). CRC Press.
- Stabili L, Schirosi R, Parisi MG, Piraino S, Cammarata M (2015). The mucus of *Actinia equina* (Anthozoa, Cnidaria): An unexplored resource for potential applicative purposes. *Marine Drugs* 13(8):5276–5296
- Stephens GC (1962). Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. *Biological Bulletin* 123(3):648–659.
- Subramanian S, MacKinnon SL, Ross NW (2007). A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* 148(3):256–263.
- Suggett DJ, Warner ME, Smith DJ, Davey P, Hennige S, Baker NR (2008). Photosynthesis and production of hydrogen peroxide by Zooxanthellae (pyrrhophyta) phylotypes with different thermal tolerances 1. *Journal of Phycology* 44(4):948–956.
- Sweet M, Bythell J (2012). Ciliate and bacterial communities associated with White Syndrome and Brown Band Disease in reef-building corals. *Environmental microbiology* 14(8):2184-2199.
- Sweet M, Sere M (2015). Ciliate communities consistently associated with coral Diseases. *Journal of Sea Research* 113:119-131.
- Sweet MJ, Croquer A, Bythell JC (2014). Experimental antibiotic treatment identifies potential pathogens of white band disease in the endangered Caribbean coral *Acropora cervicornis*. *Proceedings of the Royal Society B* 281:20140094.
- Sweet MJ, Sere MG (2016). Ciliate communities consistently associated with coral diseases. *Journal of Sea Research* 113:119–131.
- Tait K, Hutchison Z, Thompson FL, Munn CB (2010). Quorum sensing signal production and inhibition by coral-associated *Vibrios*. *Environmental Microbiology Reports* 2(1):145-150.

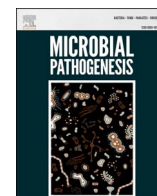
- Taniguchi A, Kuroyanagi Y, Aoki R, Eguchi M (2023). Community Structure and Predicted Functions of Actively Growing Bacteria Responsive to Released Coral Mucus in Surrounding Seawater. *Microbes and Environments* 38(3):23024.
- Taniguchi A, Nishimura S, Eguchi M (2024). Coral mucus effects on bacterial growth, respiration, and grazing mortality in reef systems. *Journal of Experimental Marine Biology and Ecology* 573:152003.
- Tchernov D, Gorbunov MY, De Vargas C, Narayan Yadav SN, Milligan AJ, Häggblom M, Falkowski PG (2004). Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proceedings of the National Academy of Sciences of the United States of America* 101(37):13531–13535.
- Teai T, Drollet JH, Bianchini JP, Cambon A, Martin PMV (1998). Occurrence of ultraviolet radiation-absorbing mycosporine-like amino acids in coral mucus and whole corals of French Polynesia. *Marine and Freshwater Research* 49:127–132.
- Teece MA, Estes B, Gelsleichter E, Lirman D (2011). Heterotrophic and autotrophic assimilation of fatty acids by two scleractinian corals, *Montastraea faveolata* and *Porites astreoides*. *Limnology and Oceanography* 56(4):1285–1296.
- Teixeira C, Lana R, Tao J, Hackmann T (2017). Comparing the responses of rumen ciliate protozoa and bacteria to excess carbohydrate. *FEMS Microbiology and Ecology* 93:1-12.
- Teplitski M, Krediet CJ, Meyer JL, Ritchie KB (2016). Microbial interactions on coral surfaces and within the coral holobiont. *The Cnidaria, Past, Present and Future: The World of Medusa and Her Sisters* 331-346.
- Thompson JR, Rivera HE, Closek CJ, Medina M (2014). Microbes in the coral holobiont: Partners through evolution, development, and ecological interactions. *Frontiers in Cellular and Infection Microbiology* 4:176.
- Thornton DJ, Sheehan JK (2004). From mucins to mucus: Toward a more coherent understanding of this essential barrier. *Proceedings of the American Thoracic Society* 1(1):54–61
- Tocher DR (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science* 11(2):107–184.

- Tranter PRG, Nicholson DN, Kinchington D (1982). A description of spawning and post-gastrula development of the cool temperate coral, *Caryophyllia smithi*. Journal of the Marine Biological Association of the United Kingdom 62(4):845–854.
- Treignier C, Grover R, Ferrier-Pages C, Tolosa I (2008). Effect of light and feeding on the fatty acid and sterol composition of zooxanthellae and host tissue isolated from the scleractinian coral *Turbinaria reniformis*. Limnology and Oceanography 53(6):2702–2710.
- Tripathi N, Sapra A (2020). Gram staining.
- Ulstrup K, Kuhl M, Bourne D (2007). Zooxanthellae harvested by ciliates associated with Brown Band Syndrome of corals remain photosynthetically competent. Applied Environmental Microbiology 73:1968–1975.
- Urich K (1994). Comparative Animal Biochemistry. Springer Verlag, 782 pp. ISBN 3-540-57420 4. Journal of Evolutionary Biology 9(6):1029–1030.
- Uslu L, Sayin S, Naz M, Taskin E, Soyler O, Saygili I, Cetin Z, Dinler ZM, Isik O (2021). Proximate analysis and fatty acid profile of some brown macroalgae collected from the Northeastern Mediterranean coast. Fresenius Environment Bulletin 30:9433–9437.
- Vacelet E, Thomassin BA (1991). Microbial utilization of coral mucus in long term in situ incubation over a coral reef. Hydrobiologia 211(1):19–32.
- Van Oppen MJ, Blackall LL (2019). Coral microbiome dynamics, functions and design in a changing world. Nature Reviews Microbiology 17(9):557–567.
- Vaughan EJ, Wilson SK, Howlett SJ, Parravicini V, Williams GJ, Graham NAJ (2021) Nitrogen enrichment in macroalgae following mass coral mortality. Coral Reefs 40(3):767–776.
- Venkataraman, K. (2006). *Coral reefs in India*. Chennai: National Biodiversity Authority.
- Ventura M (2006) Linking biochemical and elemental composition in freshwater and marine crustacean zooplankton. Marine Ecology Progress Series 327:233–246.
- Volkman JK, Johns RB, Gillan FT, Perry GJ, Bavor Jr HJ (1980). Microbial lipids of an intertidal sediment—I. Fatty acids and hydrocarbons. Geochimica et Cosmochimica Acta 44(8):1133–1143.

- Voss JD, Richardson LL (2006). Nutrient enrichment enhances black band disease progression in corals. *Coral Reefs* 25:569-576.
- Wegley L, Edwards R, Rodriguez-Brito B, Liu H, Rohwer F (2007). Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*. *Environmental Microbiology* 9:2707–2719.
- Weis VM (2008). Cellular mechanisms of Cnidarian bleaching: Stress causes the collapse of symbiosis. *Journal of Experimental Biology* 211(19):3059–3066.
- Weynberg KD, Voolstra CR, Neave MJ, Buerger P, Van Oppen MJ (2015). From cholera to corals: Viruses as drivers of virulence in a major coral bacterial pathogen. *Scientific Reports* 5(1):1–9.
- Wiebo-Song (2000). Morphological and taxonomical studies on some marine Scuticociliates from China Sea, with description of two new species, *Philaster idesarmatalis* sp. n. and *Cyclidium varibonneti* sp. n. (Protozoa: Ciliophora: Scuticociliatida). *Acta Protozoologica* 39:95-322.
- Wiebo-Song, Wilbert N (2002). Faunistic studies on marine ciliates from the Antarctic Benthic area, including descriptions of one epizoic form, 6 new species and, 2 new genera (Protozoa: Ciliophora) *Acta Protozoologica* 41:23-61.
- Wiedenmann, J., D'angelo, C., Mardones, M. L., Moore, S., Benkwitt, C. E., Graham, N. A., ... & Genin, A. (2023). Reef-building corals farm and feed on their photosynthetic symbionts. *Nature* 620(7976):1018-1024.
- Wiese J, Thiel V, Nagel K, Staufenberger T, Imhoff JF (2009). Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria Saccharina* from the Baltic Sea. *Marine Biotechnology* 11(2):287–300.
- Wijayanti DP, Sabdono A, Dirgantara D, Widyananto PA, Sibero MT, Bhagooli R, Hidaka M (2020). Antibacterial activity of acroporid bacterial symbionts against White Patch Disease in Karimunjawa Archipelago, Indonesia. *The Egyptian Journal of Aquatic Research* 46(2):187-193.
- Wild C, Huettel M, Klueter A, Kremb SG, Rasheed MY, Jorgensen BB (2004). Coral mucus functions as an energy carrier and particle trap in the reef ecosystem. *Nature* 428(6978):66-77.

- Wild C, Naumann M, Niggel W, Haas A (2010). Carbohydrate composition of mucus released by scleractinian warm- and cold-water reef corals. *Aquatic Biology* 10(1):41–45.
- Wild C, Woyt H, Huettel M (2005) Influence of coral mucus on nutrient fluxes in carbonate sands. *Marine Ecology Progress Series* 287:87–98.
- Willis B, Page C, Dinsdale E (2004). Coral disease on the Great Barrier Reef. In: E Rosenberg, Y Loya, editors. *Coral Disease and Health*. Berlin, Germany: Springer Verlag 69–104.
- Wilson B, Aeby GS, Work TM, Bourne DG (2012). Bacterial communities associated with healthy and *Acropora* white syndrome-affected corals from American Samoa. *FEMS microbiology ecology* 80(2):509-520.
- Wilson SK, Bellwood DR, Choat JH, Furnas MJ (2003). Detritus in the epilithic algal matrix and its use by coral reef fishes. *Oceanography and Marine Biology*, 41:279–310.
- Winkler R, Antonius A, Renegar DA (2004). The skeleton eroding band disease on coral reefs of Aqaba, Red Sea. *Marine Ecology—Pubblicazioni Della Stazione Zoologica Di Napoli I* 25:129–144.
- Wooldridge, S.A., 2013. Breakdown of the coral-algae symbiosis: towards formalizing a linkage between warm-water bleaching thresholds and the growth rate of the intracellular zooxanthellae. *Bio Geosciences* 10(3):1647-1658.
- Wright RM, Strader ME, Genuise HM, Matz M (2019). Effects of thermal stress on amount, composition, and antibacterial properties of coral mucus. *Peer Journal* 7:6849.
- Wuchter C, Abbas B, Coolen MJ, Herfort L, van Bleijswijk J, Timmers P et al (2006). Archaeal nitrification in the ocean. *Proceedings of the National Academy of Sciences* 103(33):12317-12322.
- Xie D, Jackson EN, Zhu Q (2015). Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered *Yarrowia lipolytica*: from fundamental research to commercial production. *Applied Microbiology and Biotechnology* 99:1599–1610.
- Yamashiro H, Oku H, Higa H, Chinen I, Sakai K (1999). Composition of lipids, fatty acids and sterols in Okinawan corals. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 122(4):397–407.

- Younan F, Pearson J, Allen A, Venables C (1982). Changes in the structure of the mucous gel on the mucosal surface of the stomach in association with peptic ulcer disease. *Gastroenterology* 82(5 Pt 1):827–831.
- Zainudin Bachok AE, Prosper Mfilinge AE, Makoto Tsuchiya (2006). Characterization of fatty acid composition in healthy and bleached corals from Okinawa, Japan. *Coral Reefs* 25:545–554.
- Zetsche EM, Baussant T, Meysman FJ, Van Oevelen D (2016). Direct visualization of mucus production by the cold-water coral *Lophelia pertusa* with digital holographic microscopy. *PLOS ONE* 11(2):0146766.
- Zhang N, He QS (2015). Commensal microbiome promotes resistance to local and systemic infections. *Chinese Medical Journal* 128(16):2250-2255.
- Zhang S, Li G (2000). Presence of phenoloxidase and prophenoloxidase in the epidermal cells and the epidermis mucus of the lancelet *Branchiostoma belcheritsingtauense*. *Ophelia* 52(3):207–212.
- Zhang Y, Han SW, Cox LM, Li H (2017). A multivariate distance-based analytic framework for microbial interdependence association test in longitudinal study. *Genetic epidemiology* 41(8):769-778.
- Zhao X, Findly RC, Dickerson HW (2008). Cutaneous antibody-secreting cells and B cells in a teleost fish. *Developmental and Comparative Immunology* 32(5):500-508.
- Zhu B, Wang G, Huang B, Tseng CK (2004). Effects of temperature, hypoxia ammonia and nitrate on the bleaching among three coral species. *Chinese Science Bulletin* 49:1923-1928.
- Zhukova NV, Aizdaicher NA (1995). Fatty acid composition of 15 species of marine microalgae. *Phytochemistry* 39(2):351–356.
- Ziegler M, et al (2016). Coral microbial community dynamics in response to anthropogenic near a major city in the central Red Sea. *Marine Pollution Bulletin* 105:629–640.



Characterization of coral associated ciliates and their interactions with disease lesion progression of Indian Scleractinian corals

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ABSTRACT

Ciliates associated with advanced diseased lesions of *Acropora* sp. and *Porites* sp. in the field were isolated and characterised using microscopic and molecular analysis. The identified dominant coral-associated ciliates as *Holosticha* sp. and *Cohnilembus verminus* was propagated *in vitro* and taken for further study. Ciliates high cell numbers with substrate containing bacteria-free mucus confirms the feeding preference for nutrients in mucus instead of bacteria. Therefore, fatty acid composition of the coral mucus was analysed and noted for the different composition levels of SAFA, MUFA and PUFA in both the genera. This suggests the possible feed specific interactions of ciliates with coral mucus and tissues. Conversely, *Holosticha* sp. was observed for invading the host cells for its voracious ingestion of Symbiodiniaceae cells and tissues. Moreover, the aquarium based investigation revealed that the ciliates migrate to the injured and early disease signs of corals enhancing the tissue loss and disease lesion progression. Thus, our results indicate that the ciliates interact with the immunocompromised disease corals and play a major role in progression of disease lesions leading to rapid coral mortality.

1. Introduction

The environmental stress and physical damages on corals have been described for the susceptibility of corals to infectious diseases [1–3] induced by pathogenic microbes such as bacteria [4–6], fungi [7–9], viruses [10–12] or different type of microorganisms [13,14] including cyanobacteria [15–21]. The other parasitic flatworms, trematodes have been described to induce infection on *Porites* sp [22–25]. Protozoans have been reported as direct consumers of coral-associated photosynthetic algae, zooxanthellae [26,27] and coral tissues [28], but only a few of them as possible causative agents of coral diseases [29–32]. Skeleton-Eroding Band was the first coral disease described for the association of a foliicolinid ciliate, *Halofolliculina corallasia* [31,33]. Then various other coral disease such as brown band [34,35], Caribbean Ciliate Infection disease with ciliates similar to those associated with skeletal eroding band (*Halofolliculina*) [36,37], white syndrome with diverse ciliate community [38], white band disease, white plague and brown jelly syndrome have all been described for various ciliates association with the different coral genera [39–41]. Although the above studies had been reported the association of ciliate species with specific

coral disease signs, there are no published reports describing on ciliate – Indian coral interactions. Thus, in this study, we aimed to describe the association of ciliates with two different scleractinian corals disease signs from the coral reefs of the Gulf of Mannar regions, India. Protozoan are known for their selective nature of feeding based on the prey size, shape and biochemical composition [42]. The chemical composition of coral mucus, derived from photosynthates symbiotic symbiodiniaceae cells and heterotrophic feeding by corals, is a major nutrient source for various coral associated organisms [43]. The composition of coral mucus was analysed for carbohydrates, amino acids, lipids and proteins [44]. However, no study has been reported for the fatty acid profile of the mucus; fatty acids, are essential for energy use and storage, cell membrane structure and gene regulation [45]. Also, no previous studies have been made on growth parameters for the ciliates using nutrient rich coral mucus as substrate for understanding the coral-ciliate interactions. Therefore, the ciliates of the present study were investigated for their feeding interactions with two different diseased corals, *Acropora muricata* and *Porites lutea*. Firstly, it was analysed the feeding preferences of ciliates for coral tissues or the symbiont algae. Secondly, it was studied the dissolved chemical components such as fatty acids, carbohydrates

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and composition of bacteria in the mucus of two different coral genera and their distinct role, if any, in ciliate feeding preferences.

2. Materials and methods

2.1. Field selection, observation, and sample collection by

In June 2018, the ciliates were for the first time observed on the hard coral *A. muricata* and *P. lutea* sp. from the coastal marine National Park of Gulf of Mannar region (GoM) (GPS Locations - N 09°17' 36.2"E 079°08'12.6" and N 09°17'45.6"; E 079°08'36.8") India. The ciliates were monitored during the subsequent months such as February and March 2019. The colonies of shallow barrier reef (2–3 m depth) were examined along a 5 m radius from the zone dominated by *A. muricata* and *P. lutea*. Collection samples permit was granted by MoEFCC, New Delhi, India and PCCF, Chennai, Tamil Nadu, India. Small pieces of early/advanced diseased and healthy coral (about ~0.5–1 cm²) were collected from the field then transported in aerated small tanks to the laboratory. The surface layers of the diseased and healthy portion of the corals were collected using syringes. Also, *A. muricata* and *P. lutea* mucus were collected using sterile syringes as earlier described by Ducklow and Mitchell [13]. Samples (healthy or diseased) evaluation from *A. muricata* and *P. lutea* was made for lesions, injuries, disease and presence or absence of ciliates by microscopy (Olympus). Furthermore, the disease severity was examined based on the advancement of lesions and percentage of tissue loss that occurred in the fragments. The advancement of lesions and the areas of tissue loss were measured as described by Katz et al., [2].

2.2. Ciliate collection and culturing

Ciliate collection was done as described in Katz et al., [2]. Briefly, the early/advanced diseased and healthy fragments of *Porites* and *Acropora* species were placed in 0.22 µ filter sea water and incubated for 3–5 h at room temperature. After the incubation period a dense group of ciliates were found moving from the advanced disease fragments to the water surface. The ciliates were then collected and washed with filtered sea water. The washed ciliates were counted under a dissecting microscope and used as an inoculum for the infection experiments.

The harvested ciliates were maintained as pure cultures in the laboratory with wheat grains as a food source to enrich bacteria as described in earlier method [46] with minor modifications. Briefly, media preparation was done by adding sea salts to pre-prepared soil extract (106 g of fine crumb, good quality, sieved soil free from pesticide and fertilizers is added to 660 mL of deionized water and autoclaved, allowed to settle for 24 h and again autoclaved. The second time autoclaved soil extract was then decanted and filtered and added to the stock solution (Sodium nitrate (5.625 g), Disodium hydrogen phosphate (0.225 g), Dipotassium hydrogen phosphate (0.188 g) per liter) with sterilized Phosphate buffer saline (PBS) of pH 7.6–7.8. To the 300–500 µL of the media, wheat seeds surface sterilized by boiling in water for 4 min was added. To the prepared synthetic media a bulk suspension of mostly adult ciliate cells is picked and used as an inoculum. Alternatively, homogenous suspension of cells was prepared and a known number of cells were added as an inoculum. Cells were sub-cultured and re-inoculated into fresh media containing plates when it attains a high density of growth. All cultures are maintained at 30 °C in low light to dark conditions.

2.3. Microscopic and molecular characterization of the ciliates

Collected coral associated ciliates from the selected diseased portions of *A. muricata* and *P. lutea* surface fragments and behavioural characters were observed under an Olympus binocular microscope. The samples were also wet mounted with a cover slip to restrict the movement of ciliate protozoans. Alternatively, Bouin's Fixative prepared in seawater

(30 mL saturated picric acid, 10 mL formaldehyde, 2 mL glacial acetic acid) was used to immobilize the cells. Higher-magnification images of coral-associated ciliates were observed for morphology and ultrastructure using a Research inverted microscope (Olympus IX51 Inverted Microscope) at 5x, 10x, 40x and 100x (oil immersion) magnification. Images and videos were captured on an Olympus BXI51 microscope using Cell scope software. The characteristic features of the ciliates were compared with morphological descriptions from previous studies [47–50] and the dichotomous key in the 'Marine interstitial Ciliates' [47] were used for ciliate identification. Morphological characteristics, such as cortical and ultrastructural features, provided a means of distinguishing ciliate morphotypes. Features such as kinetosomal make-up and oral infraciliary structures such as the AZM (Adoral Zone of Membranelles) are highly conserved features and together with organelle distribution, size, shape and color are routinely used for distinguishing genera [47–50].

Molecular characterization protocol of the isolated ciliates started with washing step, three to five times in sterile seawater, then incubated with an addition of 15 µl of proteinase K (20 mg/ml) in a water bath for 20 min at 54 °C. After incubation, they were vortexed for 20 s, boiled at 100 °C for 10 min, then vortexed for a further 20 s and centrifuged at 16,000 g for 3min. Supernatant of 30 µl was taken off and put into a fresh micro-centrifuge tube. This was then stored at –20 °C until further use. Extracted DNA was PCR amplified using universal 18S rRNA gene eukaryotic primers NS1 (5'-GTAGTCATATGCTTGCTC-3') and NS8 (5'-TCCGCAGGTTACCTACGGA-3'). The PCR reactions were performed in a Bio-rad thermal cycler with the following program: an initial denaturation 94 °C for 3 min, followed by 34 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min, and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. All PCR products were ethanol-purified and sequenced. Sequences were aligned using the CLUSTAL W sequence alignment program [51] and Neighbor-joining [52] method was used to reconstruct phylogenetic tree using the MEGA 7 software program [53]. The topology of Phylogenetic tree was estimated using the bootstrap analysis method [54] with 500 replications. The sequences were submitted in NCBI and the GenBank accession numbers obtained (MT561362- MT561365).

2.4. Ciliate feeding preferences with coral mucus

The isolated mucus from corals *A. muricata* and *P. lutea* was used in various proportions and combinations to analyse ciliates feed preference and growth. The substrate composition such as only coral mucus unfiltered (MUF), mucus filter-sterilized (MF), only seawater and freshwater without any additional media or mucus were used for the experiments. Motile, adult Ciliate cells of about 10–15 numbers were added to wells in triplicates per group of the above different substrate combinations. Growth at 28 °C was monitored by counting cells under the microscope at time intervals from 6 h up to 18 h period. Means and standard deviations were calculated to enable statistical analysis and understand the error rates of each group.

2.5. Biochemical analysis of coral mucus

Lipid Extraction from mucus samples was carried out according to Folch et al. (1957) method [55]. Samples were added to 5 ml of methanol: chloroform 1:2 and kept for ultra-sonication up to 30 min at 40 °C. Then the samples have been transferred to the separating funnel. 2 ml of 10% KOH in methanol was added to the sample for the saponification and mixed well and allowed for the separation until a separate layer formation. The upper layer (supernatant) was transferred to Pear shape flask. Transesterification of the fatty acid components of the total lipid was carried out following the method described by Carreau and Dubacq [56]. Fatty acid methyl esters were analysed on Gas Chromatography mass spectrometer (GCMS) system (QP-2010, Shimadzu, Japan) equipped with a DB-23 capillary column (60 m × 0.25 µm ID, 0.25 µm film

thickness) as described in Trivedi et al., [57].

2.6. Ciliate ingestion of symbiodiniaceae cells

The multiwell plate containing the mucus with seawater was used for analyzing the ingestion of Symbiodiniaceae cells by the ciliates. Symbiodiniaceae cells associated coral tissues were placed in the multiwell plate containing mucus with seawater. Ciliates isolated from both the corals of about 10–15 cells were added to the above media and observed the ciliates ingestion of Symbiodiniaceae cells. The above ciliate ingestion experiments are performed at 28 °C.

2.7. Ciliate infection and reisolation from coral fragments

The early disease and healthy fragments were used for the ciliate infection studies as described by Katz et al., [2]. The coral fragments were acclimatized for 10 days in an aquaria where the temperature, pH and salinity were maintained and checked regularly. Oxygen was provided through an air pump. A cycle of 12 h of light was provided. The ciliates free healthy, early disease sign and artificially injured corals were examined and confirmed using a stereo and compound microscope. The ciliates were inoculated with healthy, early disease sign and injured corals fragments and controls were placed for each of the three conditions with no ciliates treatment. Coral fragments were monitored daily basis and photographed. The rates of tissue loss are measured using time series photographs as earlier described by Randall et al., [39]. All treatment was made in triplicates.

2.8. Statistical analysis

All data are given as the mean $\pm$ SD of triplicates ($n = 3$). Analysis of variance (ANOVA) test ($P < 0.05$) was performed using Microsoft Excel 2010. Analysis of variance was performed for all the assays with different media as groups to calculate the significance of variance and Turkey's Post hoc test for Honestly Significant Differences also performed. Statistical differences at $P < 0.05$ were considered to be a significant difference.

3. Results

3.1. Occurrence and type of ciliates

In June 2018 the samples were collected from diseased and healthy *A. muricata* and *P. lutea* and examined under the microscope. The advanced disease lesion of *P. lutea* was found with two stages in the field. One was with dead tissue surrounded with a pink discoloration (Fig. 1a) and the other was with the white mat-like covering on the surface of the coral pink discoloration (Fig. 1a). Advanced disease lesions of *A. muricata* was observed with disease stage initially with white syndrome and later turned with brown colour (Fig. 1b). The surface samples were scraped carefully from the healthy and diseased portions of the *A. muricata* and *P. lutea*. Surprisingly the tissue loss of the diseased portion inside the pink line circle and the surface mat region of the *P. lutea* was dominated with a single type of ciliate infections Morpho 1 (Fig. 1a) along with three other ciliates (Morpho 2, 3 and 4, but no such

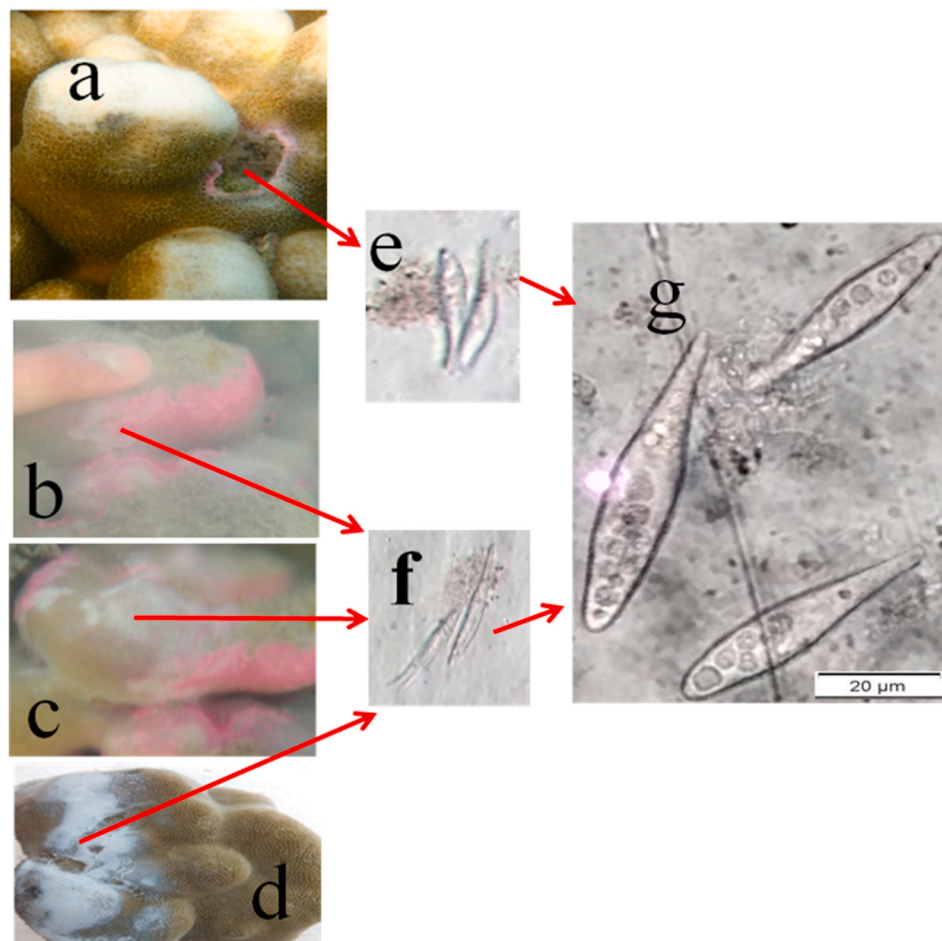


Fig. 1a. *Porites lutea* disease lesions and the associated ciliate. a. tissue with discoloration of pink colour surrounding the dead tissue. b and c. the tissue with inflammation due to stress and formation of white layer. d. The tissue with advanced stage with white mate. e, f and g the ciliate isolated from the *Porites lutea* disease lesions.

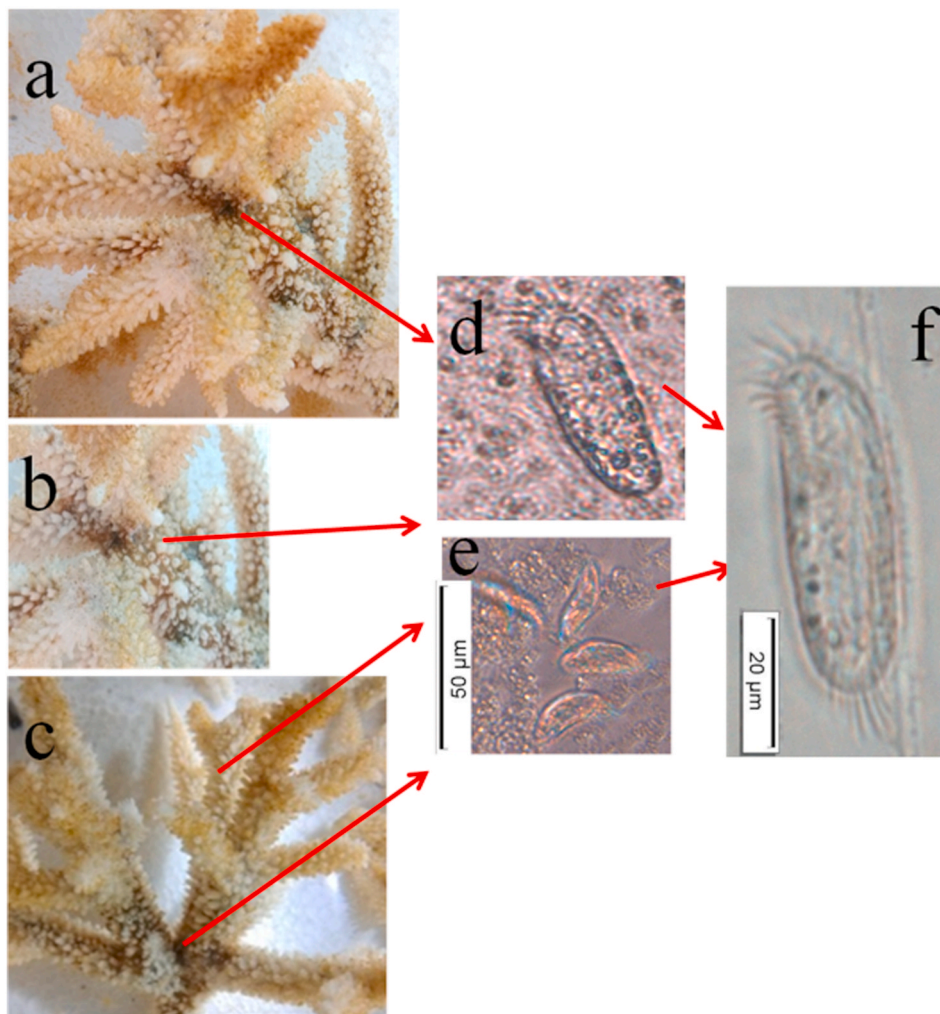


Fig. 1b. *Acropora muricata* disease lesions and the associated ciliate. a. tissue with initial white and later with brown discoloration surrounding dead tissue. b and c. the tissue with white syndrome due to stress and formation of white and brown discoloration. d, e and f. the ciliate isolated from the *Acropora muricata* disease lesions.

ciliates were observed in the healthy and early disease lesions of *P. lutea*. *A. muricata* also observed for a different ciliate (Morpho 2) which is dominated in diseased tissue or surface regions (Fig. 1b) along with two other ciliates (Morpho 3 and 4), but no such ciliate was found with healthy or early disease regions of the coral tissue and surface. A month later in March 2019, the similar observations were found for ciliates occurrence with the healthy, early and advanced stages of disease condition of the above two different corals. The ciliate dominated (Morpho 1) with that of the *Porites* sp. was not found associated with that of the *A. muricata* which may be of feed specific. Whilst three other ciliates (Morpho 2, 3 and 4) were observed in advanced disease lesions of both the coral genera.

3.2. Morphology

Morpho 1 – Genus *Cohnilembus* Kahl, 1931; *Cohnilembus verminus* (O. F. Müller, 1786); Syn. *Lembus punctatus* Kahl, 1928; *Lembus reesi* Kahl, 1931.

Description (Fig. 2a):

The outline is very thin, Spindle-shaped or fusiform with anterior portion considerably narrowed and tapering, size highly variable average length/width - 63 µm (55–77)/6.05 µm (6–10) µm. The cell is uniformly covered by somatic cilia. Several, relatively long, bent cilia are present at the tip of the narrow anterior end. Anterior half bears a

double undulating membrane composed of fused ciliate that leads to the middle of the body. Cilia at the posterior end are slightly longer than those on the cell body with caudal cilia. It often moves forward in a straight line, stops to feed and then moves again to a new location. Movements are not very active or fast. It was observed for positively thigmotactic. Macronucleus large, elongate to oval in shape, centrally located, which are often irregular-shaped. One to two micronuclei closely associated with the macronucleus. Cytosome or mouth was in the center of the body. A contractile vacuole is located at the posterior end.

Morpho 2 - Genus *Holosticha* Wrzesniewski, 1877; Kahl, 1932.

3.3. Description (Fig. 2b)

The body generally elongate, posterior rounded, size highly variable average length/width 62.5–70 µm/8–15 µm. Higher number of marginal and mid-ventral cirri. Seven well developed frontal cirri at anterior. Two macronuclei, 2 micronuclei. Contractile vacuole (5–6 µm) in middle of the body. Adoral zone of membranelles (AZM) extending over about 40% of body length, having a wide gap at the front end of the body that is caused by the absence of membranelles and separated into two parts with anterior with 7–13 membranelles and the posterior composed of 15–25 membranelles. Active and fast jerky movements in circles were observed. The present study ciliate was similar to that of the *Holosticha diademata*, which is distinguished by the body shape and the presence of

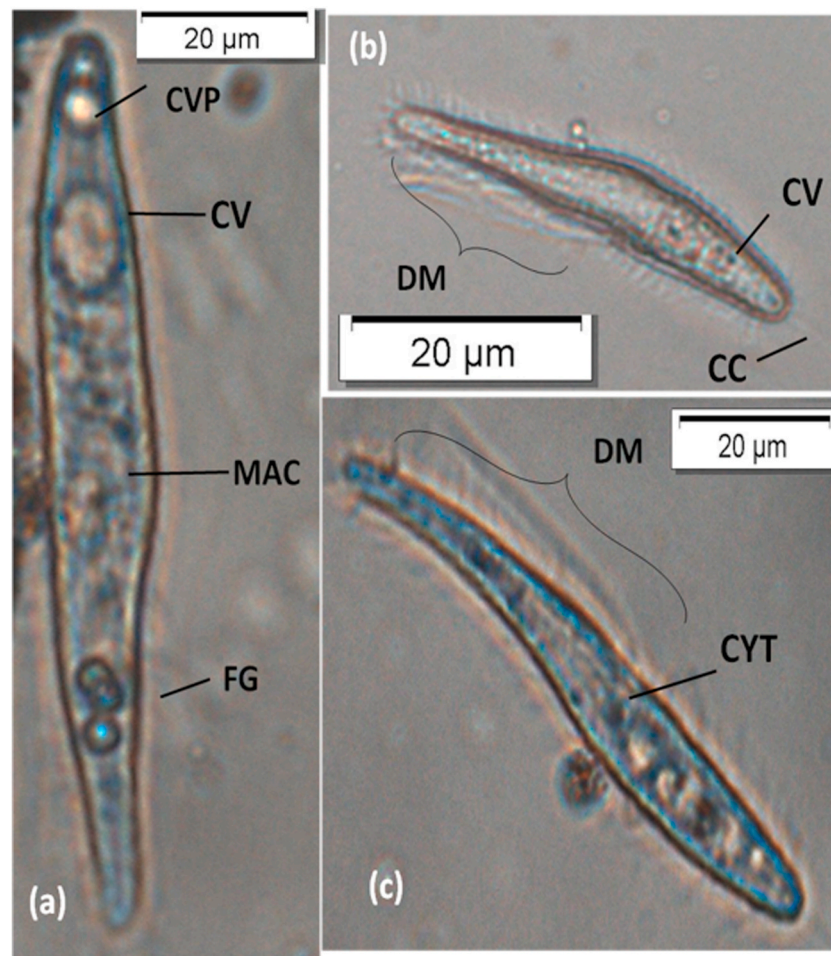


Fig. 2a. Detailed Image of internal ultrastructure of coral associated ciliate *Cohnilembus verminus*. (a) Showing CVP-Contractile vacuole pore, CV- Contractile vacuole, MAC – Macronuclei, FG- Food granule (b) Complete external ciliature of *C. verminus* (c) Showing DM Double membranelles, CYT – Cytostome. Scales included are added from microscopy images.

contractile vacuole in the post-equatorial position from the other species. Thus, this species differs by the presence of a contractile vacuole in the post-equatorial position from the very similar *Holosticha pullaster* (in posterior 1/3), with which it is often confused.

Morpho 3 - Genus *Uronema* Dujardin, 1841.

Description:

The body is generally elongate, ovoid to pyriform, size highly variable average length/width 30–60 µm/8–15 µm. The anterior end is truncated with a small apical plate, and posterior end is narrowly rounded. Macronucleus is spherical near the mouth or towards anterior end. Single contractile vacuole caudally positioned. Cilia are densely arranged and it has a single caudal cilium. The ciliate moves swiftly and also remains motionless when undisturbed.

Morpho 4 - Genus *Euplotes* Ehrenberg, 1830.

3.4. Description

The body is Dorso-ventrally flattened, ovoid in shape. The length/width highly variable 100–140 µm/10–25 µm. Large AZM (adoral zone of membranelles) usually extending at least two-thirds the body length. Marginal rows of cirri absent usually 1 or 2 weak cirri on the left margin. In addition, there are ten to nineteen well developed frontoventrals, five or six transverse and four to five rigid caudal cirri. The macronucleus is C- shaped macronucleus and with the spherical micronucleus.

3.5. Molecular characterization of ciliates

The 18S rRNA gene sequences of the ciliates (GenBank accession numbers: MT561362- MT561365) were compared with sequences from the GenBank database (<http://www.ncbi.nlm.nih.gov>). Ciliate Morpho 1 (MT561362) was closely related to *Cohnilembus verminus* isolate FXP2009051101 (HM236339) with 97.1% sequence homology. Morpho 2 (MT561363) was 100% similar with the *Uronema marinum* (AY551905). Morpho 3 (MT561364) was 100% similar to the ciliate *Holosticha diademata* isolate JJM2007091003 (KF306396). Morpho 4 (MT561365) was closely related to *Euplotes vannus* strain ME1 (AY361850) with 99.5% sequence homology. Phylogenetic analysis based on Neighbor-Joining method clearly showed that all these four ciliates placed within the clades of respective genera (Fig. 3).

3.6. The possible life cycle of ciliates

The isolated ciliates were observed for some of the sexual and asexual stages of the life cycle. Sexual reproduction by conjugation of two ciliate cells of a compatible mating type was observed in both the ciliates, *C. verminus* (Fig. 4a) and *Holosticha* cells (Fig. 4b). Sexual reproduction or autogamy is always followed by asexual reproduction. In the present study, *Holosticha* sp. and *C. verminus* cells reproduced asexually by transverse division with the anterior half of the ciliate forming new organism and other posterior half forming another new cell (Fig. 4a and b). *C. verminus* and *Holosticha* sp. was observed for its

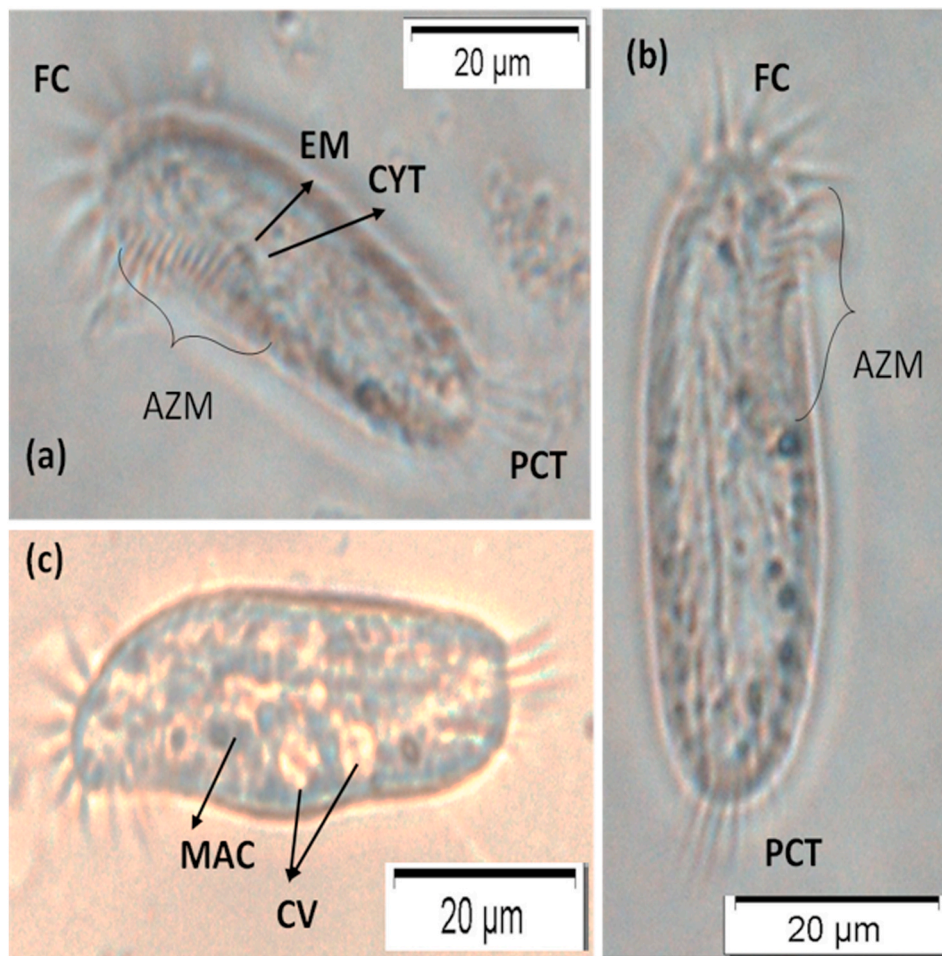


Fig. 2b. Detailed Image of internal ultrastructure of coral associated ciliate *Holosticha* sp. (a) Showing sagittal plane view with structures as FC – Frontal cirri, EM – elongated membranelle, CYT – Cytostome, PCT – Posterior cilia tuft and AZM – Adoral zone of membranelles (b) Another view of coral associated ciliate *Holosticha* sp. (c) Showing MAC – Macronuclear nodules, CV – Contractile vacuules. Scales included are added from microscopy images.

maximum growth of trophont (adult) stage at 8–10 h (Fig. 4a and b). Ciliate protozoans transition observed from trophont (adult) to theront (larval) stage during 11–14 h which allowed its reproduction and survival (Fig. 5 a and b). However, a tomont (resting stage) stage was observed only in *C. verminus* (Fig. 4a) and not in *Holosticha* sp.

3.7. Growth experiments

3.7.1. In vitro propagation of ciliates

The isolated ciliates from the diseased corals were placed in the media for its growth. Abundant cells of ciliates, *C. verminus*, and *Holosticha* sp. multiplied and scattered homogeneously on the substrate. Notable differences in the ciliate cell numbers in various media parameters were observed at different time intervals from 6 h to 90 h (Fig. 5 a and b). Both, *C. verminus* and *Holosticha* sp. ciliates grew well with wheat seeds added media than the media without wheat seeds. This elucidated that the media with wheat seeds generating heterotrophic bacteria was an essential feed for the ciliates growth. Also the ciliate, *C. verminus* and *Holosticha* sp. was observed for its maximum growth of trophont (adult) stage at 8–10 h according the nutrient availability (Fig. 5a and b). After consistent nutrient deprivation and feeding it is observed that repeatedly stressed ciliate protozoans transition observed from trophont (adult) to theront (larval) stage during 11–14 h which allowed its reproduction and survival (Fig. 4a and b). However, repeated deprivation causes the cells to move into a tomont (resting stage) in *C. verminus* (Fig. 4 a) and not in *Holosticha* sp. The theront and tomont

stages were found responded to the last feeding immediately switch to a trophont (adult stage) (Fig. 4a and b). Moreover, the experiments revealed large differences in growth between *C. verminus* and *Holosticha* sp. cells that *C. verminus* cells grew faster/multiply after 24 h when compared with that of the *H. diademata* cells growth (Fig. 5 a and b).

3.8. Ciliate feeding preferences with coral mucus

To analyse the feeding preferences of the ciliates with that of the substrates such as coral mucus (*C. verminus* and *H. diademata*), seawater, freshwater, the ciliates were subjected to grow under different media components such as only coral mucus (mucus filter-sterilized (MF)) and unfiltered (MUF)), only seawater and freshwater without any additional media or mucus (Fig. 8a and b). *C. verminus* cells were placed in the substrate containing *Acropora* and *Porites* mucus for its growth analysis (Fig. 6a). The ciliate cell numbers (trophont/adult stage) were found high with *Porites* mucus unfiltered (M (PUF)) and *Porites* mucus filtered (M (PF)). In contrast, the poor or negligible increase in ciliate cell numbers was observed in conditions with *Acropora* mucus filtered (M (AF)) and mucus unfiltered (M (AUF)), but the theront or larval (L) and tomont or resting stages (R) are observed more than the *Porites* mucus substrates (Fig. 6a). While, *C. verminus* cells was observed for fewer numbers with only sea water and no growth in freshwater.

Holosticha sp. cells differ with *C. verminus* by slow in growth rate (Fig. 6b) and as well as different feeding preference. *Holosticha* sp. cells were placed in the substrate containing *Acropora* and *Porites* mucus for

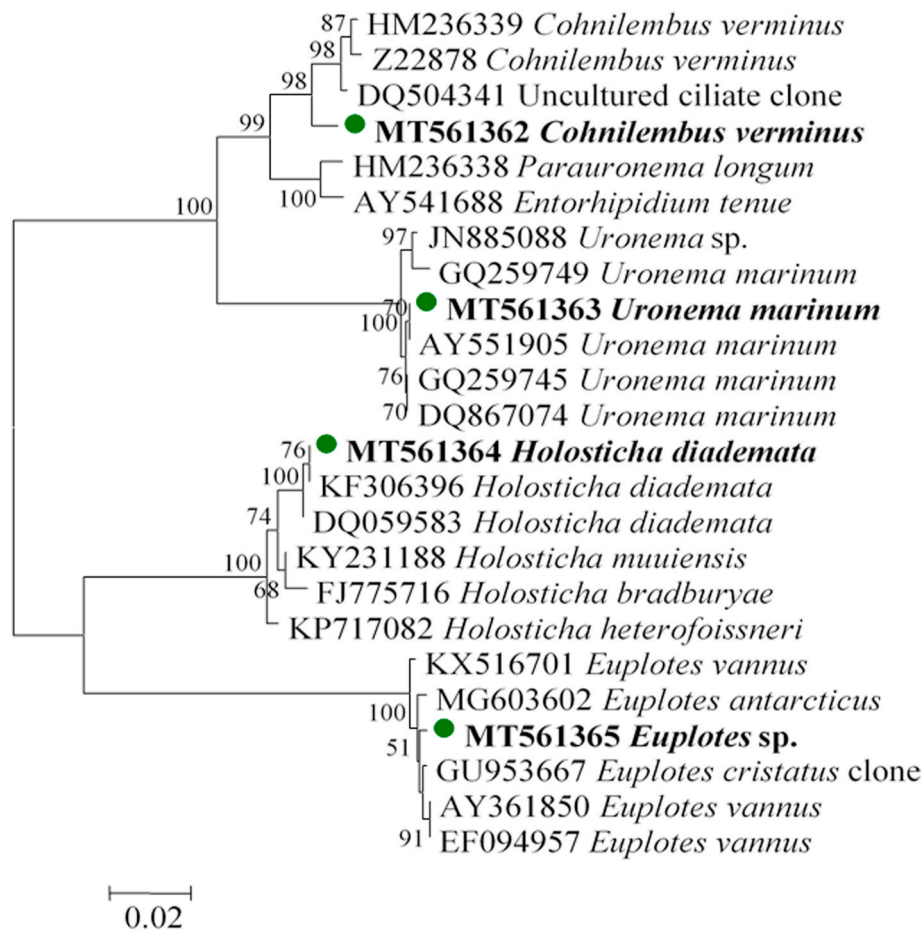


Fig. 3. Neighbor-Joining tree based on 18S rRNA gene sequences showing the phylogenetic relationship of Ciliates (in bold letters) with the GenBank closest similar sequences. Bootstrap percentages (500 replicates) are shown next to the branches >50%. Bar, 0.02 substitutions per nucleotide position.

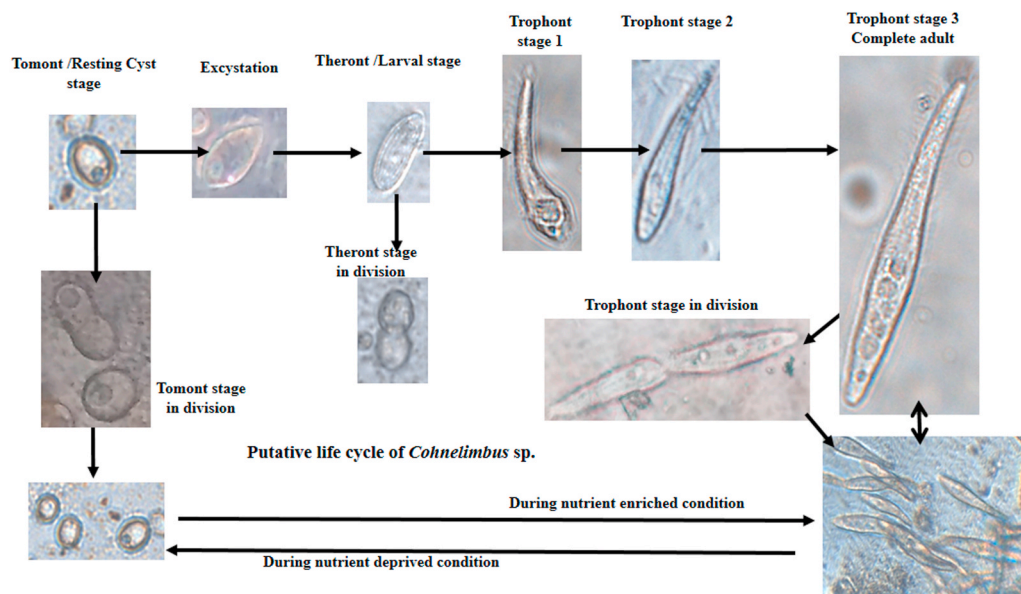


Fig. 4a. Possible life cycle of *Cohnilembus*: The sexual and asexual reproduction was observed. Conjugation of two ciliate cells of a compatible mating type was with sexual reproduction. Asexual reproduction by transverse division with the anterior half of the ciliate forming new organism and other posterior half forming another new cell (Theront stage in division) as complete adult stage (Trophont). Tomont stages were found in division and subsequently developed into theront or larval stages. The theront stages directly developed into Trophont or adult stages during favourable condition or divide and multiply (Theront stage in division).

its growth analysis (Fig. 6b). The ciliate cell numbers (trophont/adult stage) was found high with *Acropora* mucus unfiltered (M (AUF)) added with sea water followed by poor or negligible increase in *Acropora* mucus filtered (M (AF)), unfiltered *Porites* mucus (M (PUF)) and filtered

Porites mucus (M (PF)) added with sea water. The theront or larval (L) stages were observed from sixth hour and increased during the 18th hour in all the substrates used (Fig. 6b). No tomont or resting stages were observed in *Holosticha* sp. cells.

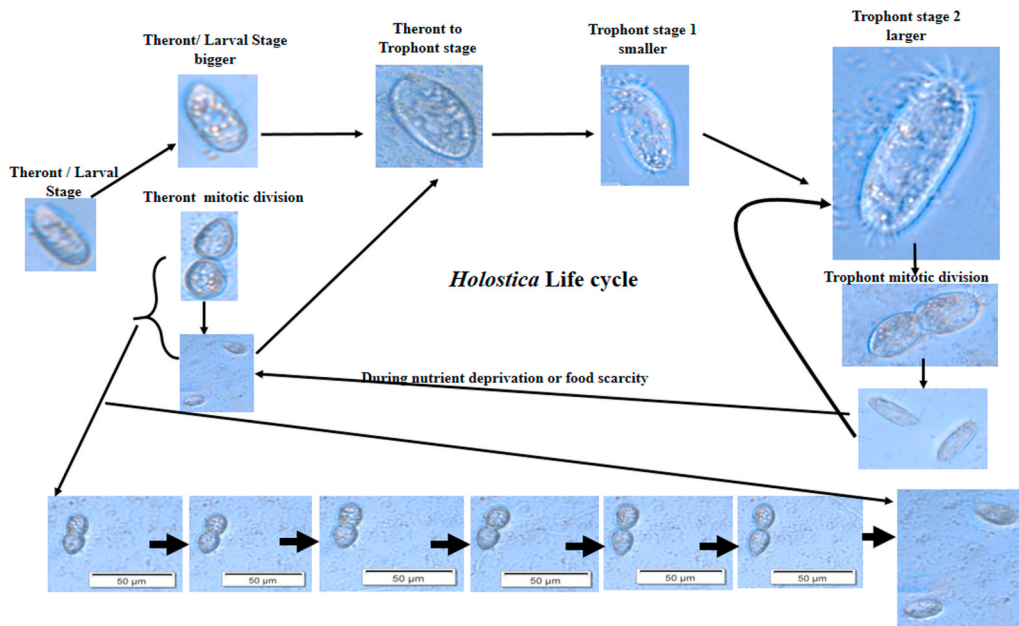


Fig. 4b. Possible life cycle of *Holosticha*: The sexual and asexual reproduction was observed. Conjugation of two ciliate cells of a compatible mating type was with sexual reproduction. Asexual reproduction by transverse division with the anterior half of the ciliate forming new organism and other posterior half forming another new cell (Trophont stage in division) as complete adult stage (Trophont). No tomont or resting stages was found. The theront stages directly developed into trophont or adult stages during favourable condition or divide and multiply (Theront stage in division).

Even though, *Holosticha* sp. was able to associate with the disease lesions of both the *A. muricata* and *P. lutea*, its high growth of cell numbers was observed with *Acropora* mucus unfiltered (M (AUF)) added with sea water when compared with that of the other conditions of mucus substrates (Fig. 6b). Conversely, the good growth of *C. verminus* with PMUF and PMF of *P. lutea* and not in *A. muricata* suggests that *C. verminus* prefer feeding ingredients or components of *P. lutea* mucus than the mucus components of *Acropora* sp. Also, the subsequent field isolation of *C. verminus* from coral, *P. lutea* and not from *A. muricata* implies that it may be of host-specific to *P. lutea*.

3.9. Fatty acid composition of mucus

The compositions of all fatty acid classes were similar in both the *Porites* sp. and *Acropora* sp. coral mucus (Table 1). However, *Porites* sp. mucus had higher carbon-specific concentrations of saturated (SAFA) and monounsaturated (MUFA) fatty acids, whilst *Acropora* sp. had higher polyunsaturated fatty acids (PUFA) (Table 1). The main saturated fatty acids (16: 0 and 18: 0) was observed in both the coral mucus but 16: 0 acid was high (53.8%) in *Porites* sp. and 18: 0 acid was high (35.19%) in *Acropora* sp. mucus. The essential MUFA 18: 1 (n-7) was 8.5% in *Porites* sp. mucus and 3.04% in *Acropora* sp. mucus. The PUFAs of symbiodiniaceae cells (18:2 (n-6), 18: 3 (n-3), 20: 5 (n-3), and 22: 6 (n-3)) and polyp tissues (20: 4 (n-6), 22: 4 (n-6) and 22: 5 (n-3)) were predominated in *Acropora* sp. mucus, except the 18: 2 (n-6), 18: 3 (n-3) (Table 1).

3.10. Ciliate ingestion of symbiodiniaceae cells

Microscopic observation revealed two dominant types of ciliates association with the diseased *P. lutea* (*C. verminus*) and *A. muricata* (*H. diademata*). The ciliate, *C. verminus* associated with the *P. lutea* showed no consumption of Symbiodiniaceae instead it was observed for the rapid feeding of bacterial cells and dead coral tissues. Conversely, the other ciliate, *H. diademata* associated with that of the diseased *A. muricata* was observed for its voracious ingestion of Symbiodiniaceae cells (Fig. 7) and as well as feeding of the dead coral tissues. The Symbiodiniaceae cells ingestion varied among the ciliate cells from one to >25 cells (Fig. 7). *Holosticha* sp. activity of invading the host cells and ingesting the Symbiodiniaceae cells of diseased coral confirms, *H. diademata*, a significant pathogen of a diseased coral and causes tissue

loss which leads to coral mortality.

3.11. Ciliate infection and re-isolation from coral fragments

The infection and re-isolation of *Holosticha* sp. and *C. verminus* from diseased and artificially injured *A. muricata* and *P. lutea* was conducted and also the tissue loss was determined. Ciliate free early disease sign, artificially injured and healthy coral fragments of *A. muricata* and *P. lutea* were placed in the aquaria. The mixed adult ciliate cells of *Holosticha* sp. and *C. verminus* each about 250 were inoculated by placing the ciliates on the surface of healthy, early disease and injured coral fragments. Controls were placed with uninoculated ciliates for all the healthy, early disease sign and artificially injured conditions. Every 12 h the disease progression was monitored with tissue loss and ciliates counts. After 12 h the ciliates were reisolated from the infected corals and the disease progression with tissue loss were observed in both the ciliates inoculated early disease and injured corals (Fig. 8a and b). *Holosticha* sp. was reisolated from the early disease and artificially injured *A. muricata* and *P. lutea* but not from the healthy fragments and as well as from the ciliates uninoculated fragments. As expected, the *C. verminus* was isolated only from the injured and diseased tissues of *P. lutea* and not from the *A. muricata*. However, both the ciliates were very few on re-isolation from aquaria experiments. Total ciliate numbers and the average percentage area of tissue loss were increased in 12 h and also subsequent hours up to the observed period of 72 h in both the ciliates treated corals (Fig. 8a and b). Healthy fragments and the uninoculated controls were not observed for any ciliates or tissue loss (Fig. 8a and b).

4. Discussion

The ciliate association with *A. muricata* and *P. lutea* scleractinian corals from coral reef regions of India was investigated for the first time. Other than Indian reefs, only a few studies have been made on ciliates association with corals and all have been described for their occurrence with disease lesions [41]. Also, the ciliates have been reported for their feeding behaviour with bacteria [42] and bacteria associated denuded dead tissues [41]. The ciliates of the present study were also isolated from the diseased lesions of *A. muricata* and *P. lutea* from the Gulf of Mannar marine national park coral reef regions (Fig. 1a and b; 2 a and b). Two stages of disease signs were observed for the occurrence of

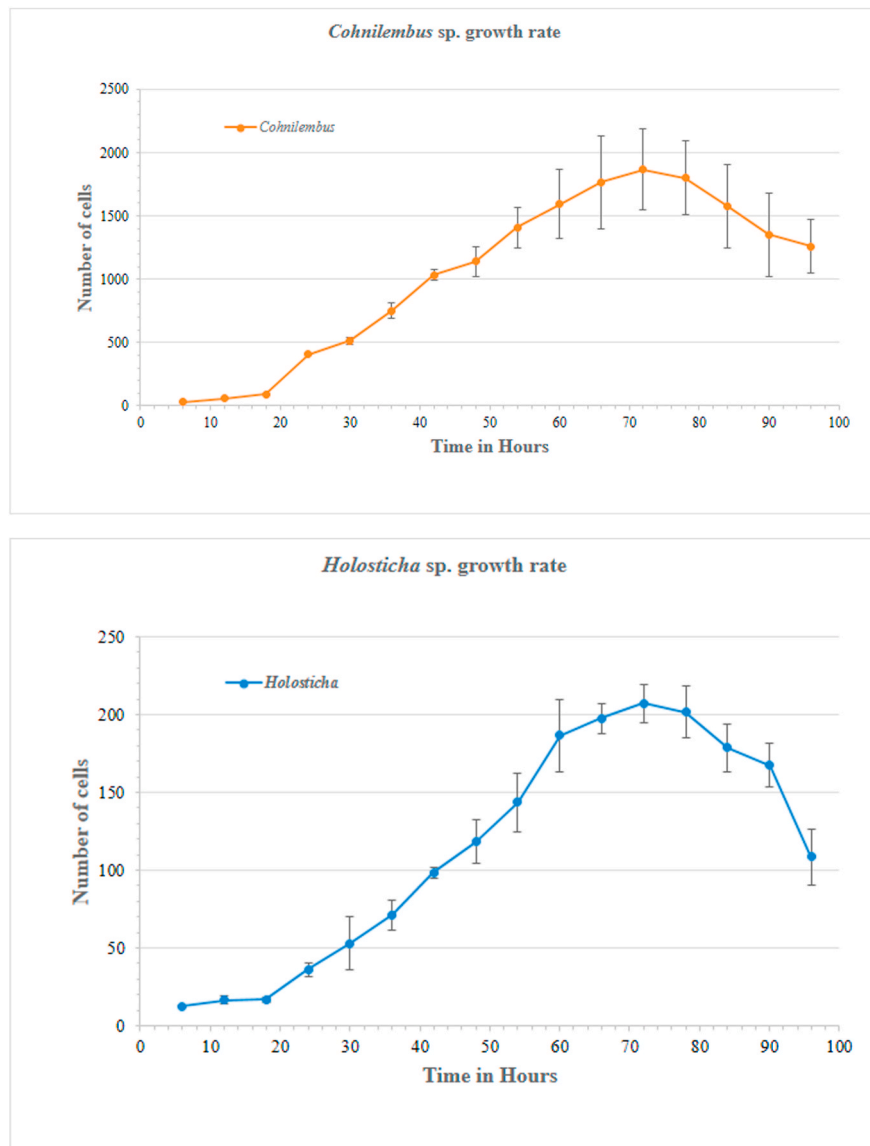


Fig. 5. (a and b). a).The growth of ciliates. a). ciliate, *Cohnilembus* sp. was able to grow rapidly with high cell numbers in the media added wheat seeds. b). The *Holosticha* sp. was slow in their growth rate with low in cell numbers. Both the ciliates showed a rapid increase.

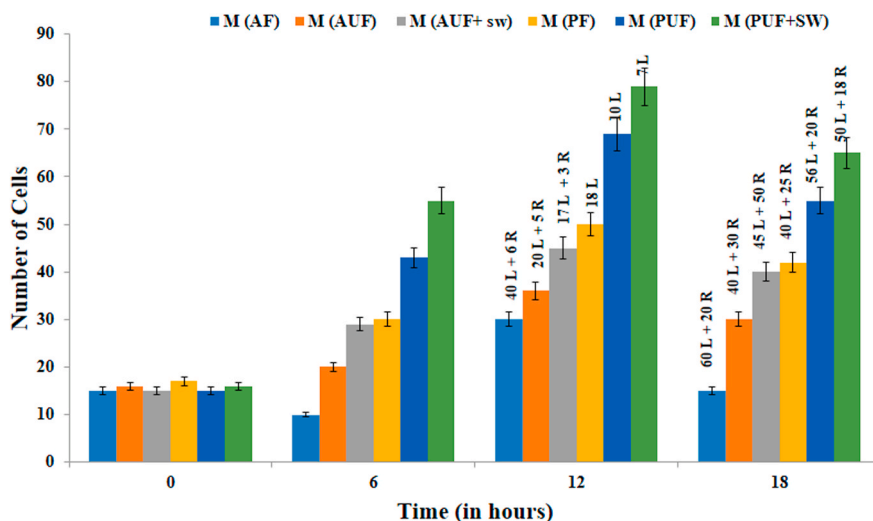


Fig. 6a. *Cohnilembus* sp. growth in coral mucus. *Cohnilembus* sp. cells were placed in the substrate containing *Acropora* and *Porites* mucus for its growth analysis. The ciliate cell numbers (trophont/adult stage) were found high with *Porites* mucus unfiltered (M (PUF) added with sea water and *Porites* mucus filtered (M (PF) added with sea water. In contrast, the poor or negligible increase in ciliate cell numbers was observed in conditions with *Acropora* mucus filtered (M (AF) and mucus unfiltered (M (AUF), but the theront or larval (L) and tomont or resting stages (R) are observed more than in the *Porites* mucus substrates.

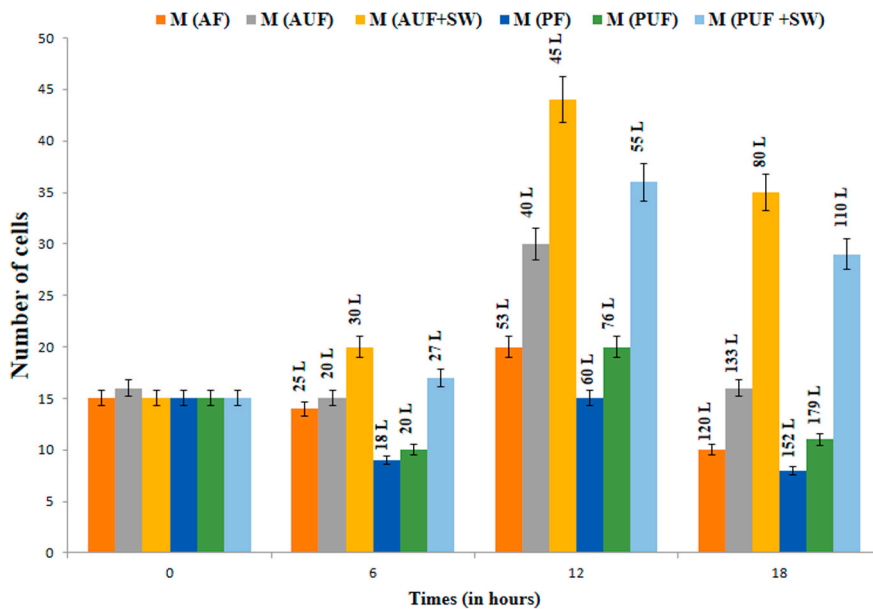


Fig. 6b. *Holosticha* sp. growth in coral mucus. *Holosticha* sp. cells were placed in the substrate containing *Acropora* and *Porites* mucus for its growth analysis. The ciliate cell numbers (trophont/adult stage) were found high with *Acropora* mucus unfiltered (M (PUF) added with sea water followed by poor or negligible increase in *Acropora* mucus filtered (M (AF) and *Porites* mucus (M (PUF) and M (PF) added with sea water. The theront or larval (L) stages were observed from sixth hour and increased during the 18th hour in all the substrates used and no tomont or resting stages were observed.

Table 1

Fatty acid composition of total lipids of *Acropora* sp. and *Porites* sp. mucus sample (% of total fatty acids, values more than 0.2% are given).

Fatty acid		<i>Acropora</i> sp.	<i>Porites</i> sp.
C12:0	Dodecanoic acid	1.93	3.66
C14:0	Tetradecanoic acid	1.42	5.48
C15:0	Pentadecanoic acid	0.38	1.31
C16:0	Hexadecanoic acid	29.36	53.8
C16:1(n-9)	<i>cis</i> -7-hexadecenoic acid	0.51	2.67
C16:1(n-7)	<i>cis</i> -9-hexadecenoic acid	0.65	1.48
C17:0	Heptadecanoic acid	0.33	2.6
C18:0	Octadecanoic acid	35.19	23.46
C18:1(n-9)	<i>cis</i> -9-octadecenoic acid	3.04	8.5
C18:2(n-6)	<i>cis</i> -9,12-octadecadienoic acid (z,z)	2.38	6.1
C18:3(n-3)	<i>cis</i> -9,12,15-octadecatrienoic acid	3.08	4.5
C20:0	Eicosanoic acid	0.88	1.0
C20:1(n-9)	<i>cis</i> -11-eicosenoic acid	1.47	1.0
C20:2(n-6)	<i>cis</i> -11,14-eicosadienoic acid	0.6	1.7
C20:3(n-6)	<i>cis</i> -8,11,14-eicosatrienoic acid, (z,z,z)	0.62	0.84
C20:4(n-6)	<i>cis</i> -5,8,11,14-eicosatetraenoic acid	9.74	3.3
C20:5(n-3)	<i>cis</i> -5,8,11,14,17-eicosapentaenoic acid	3.67	4.1
C22:4(n-6)	<i>cis</i> -7,10,13,16-docosatetraenoic acid	6.68	5.6
C22:5(n-3)	<i>cis</i> -7,10,13,16,19-docosapentaenoic acid	1.2	0.9
C22:6(n-3)	<i>cis</i> -4,7,10,13,16,19-docosahexaenoic acid	7.4	4.54
SAFA		69.49	91.31
MUFA		5.67	13.65
PUFA		35.37	32.58

ciliates in *P. lutea*. One with dead tissue surrounded with pink inflammation (Fig. 1a) and the other was with the white mat-like covering on the surface of the coral (Fig. 1a). The pink coloration of a coral has been described as a response to an infected tissue either for photoprotection or radical oxygen species quenching and also cytotoxic defence during the stressed condition [25,58]. In this study, after the pink coloration in *P. lutea* a thick white mat layer on the surface by the coral mucus was observed (Fig. 1a). This immune response had been observed earlier especially for the mucus from *Porites* species forming a layer of coat or mat in defending against sediment or temperature variations [59] and also entrapping the microbial pathogens such as bacteria, protozoa [60]. In *A. muricata* ciliates was associated with an initial white syndrome

disease sign and with brown to black colour for the later stages (Fig. 1b). The isolated two ciliates *Holosticha* sp. from both *A. muricata* and *P. lutea*, as well as *C. verminus* from the *P. lutea* (Fig. 2a and b) were identified using the taxonomic keys, before morphological descriptions [47–50] and molecular characterization (Fig. 3). The similar observation and isolation of ciliates from two different corals with subsequent multiple field trips confirmed the association of ciliates with disease lesions. *C. verminus* belonging to Scuticociliates often feed on bacteria and the enhanced feeding was observed when attached to a surface compared to free moving ciliate in the water column [61]. Therefore, it is possible that during the disease of *P. lutea*, *C. verminus* attached to areas of the dead and decaying tissues [62]. *C. verminus* earlier has been reported for its association with the white syndrome affected *Echinopora lamellosa* [62], brown band disease of *A. muricata* [61] and *A. hyacinthus* [2] as a close resemblance. However, the morphological characterization were not done microscopically for critical species level identification for a *C. verminus* species. Thus, we identified and confirmed for the first time *P. lutea* associated *C. verminus* using microscopic and molecular characterization (Fig. 2a., 3 and 4a) The other dominant ciliate isolated from the diseased *A. muricata* and less found in the *P. lutea* was demonstrated it to be a species within the genus *Holosticha* belonging to hypotrichous ciliate (Fig. 3). The present study suggests that the post-equatorial position contractile vacuole from *Holosticha* sp. had similarity with *Holosticha diademata* (Fig. 2b). *H. diademata* has been described for its association with various coral disease signs of mostly of *Acropora* spp. and less with that of *P. lutea* from Australia, Solomons, Fiji, Maldives, UK, Venezuela, Columbia, Moyonette, Reunion and S. Africa [2,41]. This prevalent association of *Holosticha* sp. with diseased *Acropora* and *porites* species correlates with the present study and confirmed the occurrence and association of *Holosticha* sp. with the Indian diseased corals.

The coral associated ciliate species were mostly described based on the taxonomic and systematic studies and there were not focused on reproduction dynamics [63]. The life cycle stages such as larvae and resting stages of the ciliate play a major role for their multiplication under favourable feed conditions. We observed some of the possible stages of life cycle of both *C. verminus* and *Holosticha* sp. which were similar to the characteristic features of Scuticociliates and hypotrichous respectively (Fig. 2a and b; Fig. 4a and b). The observation suggested that reproductive stages of ciliates (4 a and b) were connected with feeding habits such as trophont, the feeding, and division stage; tomite, swimming stage in search of feed; resting cyst stage [63]. Although,

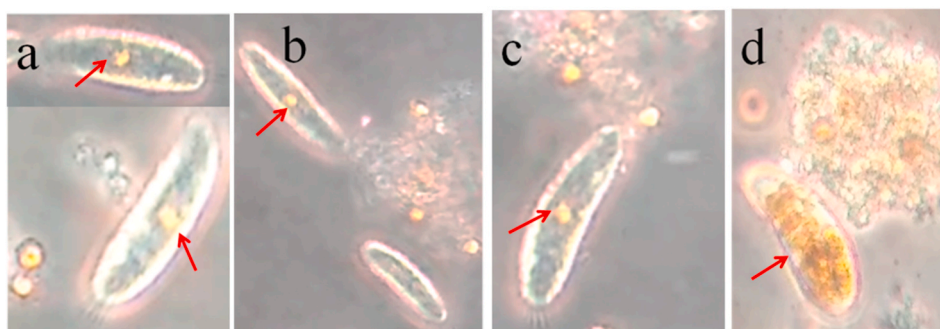


Fig. 7. Ciliate ingestion of Symbiodiniaceae cells. *Holosticha* sp. ciliate cells with Symbiodiniaceae cells. Arrows denote Symbiodiniaceae cells.

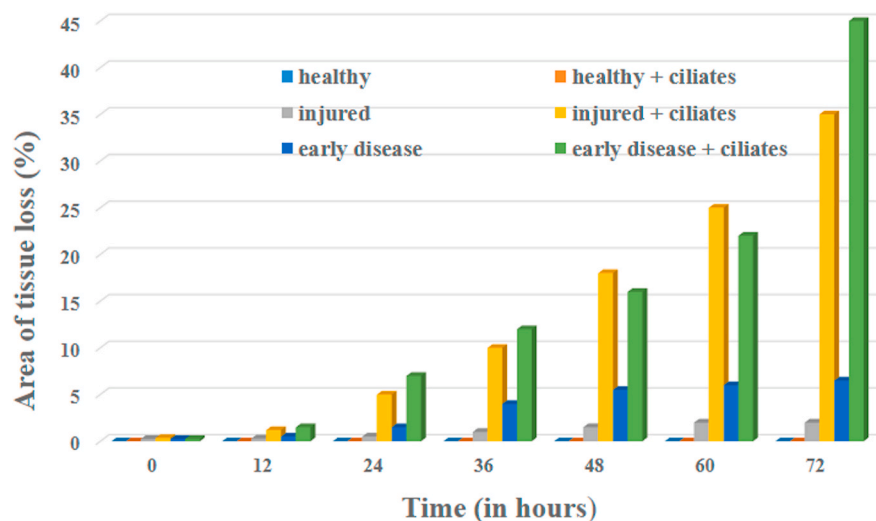


Fig. 8a. Tissue loss of *Porties* sp. inoculated with and without ciliates. Artificially injured and early disease sign *Porties* without ciliates was observed for minimal level of tissue loss. The ciliates inoculated early disease sign and injured *Porties* were observed for the high percentage of tissue loss under increased time series. No tissue loss were observed in only healthy and healthy inoculated with ciliates.

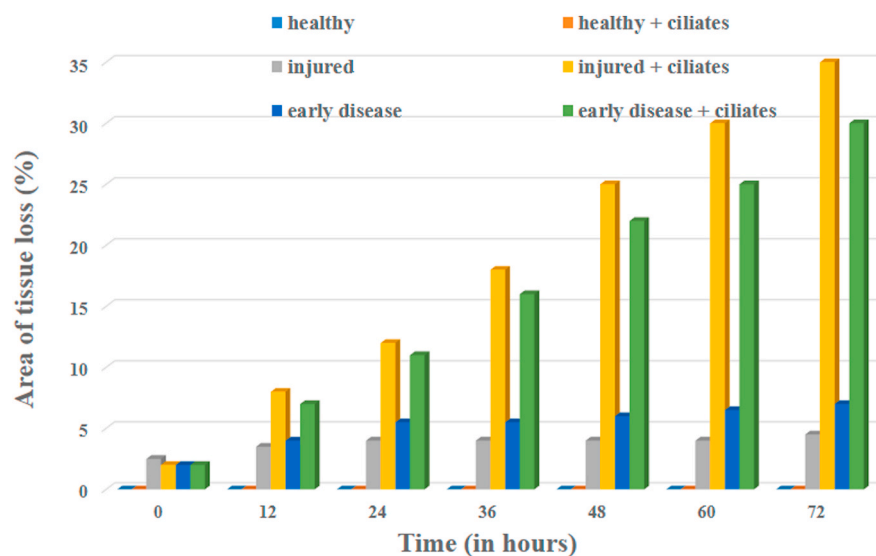


Fig. 8b. Tissue loss of *Acropora* sp. inoculated with and without ciliates. Artificially injured and early disease sign *Acropora* without ciliates was observed for minimal level of tissue loss. The ciliates inoculated early disease sign and injured *Acropora* were observed for the high percentage of tissue loss under increased time series. No tissue loss were observed in only healthy and healthy inoculated with ciliates.

many trophonts appeared within 24 h after inoculation with known number of ciliate cells in wheat grain medium *C. verminus* cells grew faster/multiplies after 24 h when compared with that of the *Holosticha* sp. cells growth (Fig. 5a and b).

No previous studies have been made on growth parameters for both the ciliates *C. verminus* and *Holosticha* sp. especially with coral mucus. To understand the mucus role as a preferred feed for the ciliates we proposed *in vitro* investigations using mucus with (un sterilized) and without (filter sterilized) bacteria (Fig. 6a and b). Here, growth rates of cells of *C. verminus* and *Holosticha* sp. varied with different proportions when coral mucus (filtered/unfiltered mucus and mucus diluted with seawater) from *Porites* sp. and *Acropora* sp. was used as a growth medium (Fig. 6a and b). Ciliate cells of *C. verminus* and *Holosticha* sp. were also found in moderate numbers with the substrate of seawater was supplemented with coral mucus (filtered/unfiltered). This result could be triggered as an effect of active inhibitors from the concentrated mucus which has less effect when mucus was diluted with seawater allowing better microbial growth [64]. Additionally, the growth of both the ciliates cells observed in the filtered mucus, which has no bacteria, correlated with the earlier study that ciliates directly use the free sugars of the carbohydrate composition of the mucus [65]. The poor growth of both the ciliates under the concentrated *A. muricata* mucus may be due to the strong inhibitors which had not allowed the growth of prokaryotic and eukaryotic microbes according to the early study [64]. Secondly, under normal stress conditions, *A. muricata* mucus production was reported for its significant increase in a shorter period as compared to other corals [66]. Also, the surface rheology of *Acropora* sp. showed that more easily and regularly secreted mucus in the form of webs and rings than *Porites* sp. This corroborates with the results showing that the excess supplied mucus in its crude concentrated form was not beneficial for the growth of bacteria and ciliates. However, its diluted form was an efficient food source for bacteria and bacterivorous ciliates majorly due to the absence or diluted effect of inhibitors.

The growth pattern of *C. verminus* and *Holosticha* sp. Cells was quite similar when exposed to filtered (PUF) and unfiltered (PUMF) *Porites* sp. mucus (Fig. 6a and b). In this case, it was observed that concentrated mucus was a better substrate for growth as compared to diluted mucus (Fig. 6a and b). This could be due to the fact that, firstly, release of *Porites* sp. mucus took place over a longer period of time and hence the net diffusion of nutrients also tends to be slower through the mucus sheet [67] including the inhibitors. Secondly, the bacterivorous ciliates directly preferred the sugars instead of the bacteria as prey. This result of high cell numbers in both with and without bacteria conditions corroborates (Fig. 6a and b) with the earlier study [65] which demonstrated that the ciliates swiftly ingested free sugars, stored them as reserve of carbohydrates and outcompeted the bacteria for glucose [65]. Differences in growth patterns of ciliates observed among two different mucus types as substrate reveal that *A. muricata* mucus was a better source of growth for microbes and associated ciliates (Fig. 6a and b). This may be attributed to a different mucus carbohydrate compositions between *Porites* and *Acropora*. The levels of basic monosaccharide like glucose, arabinose and mannose are higher in *Acropora* sp. than *Porites* sp. and could be easily consumed by the bacteria and the ciliates [43,65]. These observations are corroborated with the present study where both the ciliate preferred as a substrate the *A. muricata* mucus (Fig. 6a and b). Conversely, the low levels of basic monosaccharide and higher levels of low bacterial degradability monosaccharide, fucose and the galactose [43] supports the present study for growth with lower ciliate cell numbers with *P. lutea* mucus (Fig. 6a and b). However, the present study showed that the growth rate of *C. verminus* was more rapid compared to *Holosticha* sp. when growth in various proportions of substrates containing mucus from both the coral genera (Fig. 6a and b). This may be due to the selective feeding preferences of the ciliates [42] such as selective grazing on particular prey types via both mechano sensory and chemosensory cues that have been observed in protozoa [68,69]. Also earlier report described species-specific differences in ciliates

biochemical composition fed with same diet [70]. Thus, for the first time, we analysed the fatty acid composition of the mucus, an essential nutrient used for energy storage and gene regulation [45]. The results revealed the composition of all fatty acid classes were similar in both the *Porites* sp. and *Acropora* sp. coral mucus (Table 1). The obtained fatty acid composition of the mucus was found similar with that of the tissues of *Porites* sp. and *Acropora* sp. [71]. However, the fatty acids levels had different proportions in both genera of the mucus (Table 1) and tissues [71], which supports the *C. verminus* high growth due to a preferred feed on PMUF, PMF (Fig. 6a) or on the diseased tissues (Fig. 8a) of *P. lutea*. Moreover, the PUFAs synthesized by Symbiodiniaceae cells (18:2 (n-6), 18:3 (n-3), 20:5 (n-3), and 22:6 (n-3)) and host polyp tissues (20:4 (n-6), 22:4 (n-6) and 22:5 (n-3)) (Imbs et al., 2014) were predominated in *Acropora* sp. mucus, except the 18:2 (n-6), 18:3 (n-3) which were observed in *Porites* mucus (Table 1). The 18-carbon chain n-6 and n-3 fatty acids are linoleic acid (18:2 (n-6)) and α -linolenic acid (18:3 (n-3)) are produced only by the plants (here, Symbiodiniaceae cells) [72]. These fatty acids on further desaturation and elongation process in the tissues (corals and ciliates) give rise to essential fatty acids such as Arachidonic acid (AA) 20:4 (n-6), Adrenic acid (AdA) 22:4 (n-6) and Docosapentaenoic acid (DPA) 22:5 (n-3), Eicosapentaenoic acid (EPA) 20:5 (n-3), and Docosahexaenoic acid (DHA) 22:6 (n-3) [72]. Thus the high levels of these acids in *Porites* sp. mucus along with the other nutrients such as amino acids and carbohydrates may change the *C. verminus* feeding habits to specific host.

The two types of ciliates were also observed for their different relationships with the diseased and healthy coral host. *C. verminus* which has been isolated only from the coral *P. lutea* moved slowly in the culture and rapidly grew in various substrates *in vitro* as well as feeding on detritus and bacteria of the coral tissue. However, *C. verminus* didn't ingest Symbiodiniaceae cells, although it has been earlier observed for ingestion of Symbiodiniaceae cells [2]. In contrast, the other *Holosticha* sp. moved rapidly and voraciously ingested Symbiodiniaceae (Fig. 7) cells, in accord with the previous study for engulfing symbiodiniaceae cells [2].

Therefore, the ciliates isolated from the diseased coral lesions may act as a secondary pathogens causing further tissue loss in the host. This was in agreement with the previous report of six species for the ingestion of Symbiodiniaceae cells among the 28 species of ciliates associated with the various coral diseases studied [41]. The photosynthetically competent ingested Symbiodiniaceae cells inside the ciliate, *Holosticha* sp. with deep color pigments (Fig. 7) has to be further analyses of its autotrophy as previously described [26]. However, the ingestion and digestion of Symbiodiniaceae cells by the ciliate affects the coral health.

The ciliate identified in this study such as *Holosticha* sp. and *C. verminus* have already been previously described for their association with diseased corals [2,6,41,61,62]. Hence they are likely colonizers of the vulnerable coral sand feeders of bacteria associated with the dead and decaying tissues of denuded skeleton left by the coral disease lesions. Ciliates have also been shown to invade and devour the tissues [28, 73] of vulnerable corals suggested that ciliate infections are a serious threat to coral reef health [40]. However, Koch's postulates have not yet been fulfilled for any of the ciliates associated with coral lesions [40]. In the present study, inoculation of ciliates into the aquaria containing the healthy, early disease sign and artificially injured fragments elucidated the rapid development of disease lesions followed by tissue loss in the diseased and injured fragments (Fig. 8a and b). Also, intriguingly, *C. verminus*, one among the two ciliates of the present study was isolated only from the *P. lutea* from the three different collections from the field with the same disease lesions. Based on this observation, we inoculated *in vitro* *C. verminus* and *Holosticha* sp. to both the early disease and artificially injured coral genera of *A. muricata* and *P. lutea*. Interestingly, it was observed that *C. verminus* was able to infect only the diseased or injured tissues of *P. lutea* (Fig. 8 and b), while, *Holosticha* sp. was found in both the coral genera. However, the association of these ciliates with coral disease and their role in coral disease

interaction has to be further investigated. This may also aid to understand the biochemical feed specific *C. verminus* with *P. lutea*.

5. Conclusion

The ciliate association with the diseased corals, *A. muricata* and *P. lutea* from India was reported for the first time and also elucidated for its influence in the disease lesion progression. Ciliates were isolated from the advanced diseased corals from the field and identified using microscopic and molecular characteristics. *C. verminus* was isolated only from the advanced diseased lesions of *P. lutea*, while the *Holosticha* sp. was observed for its association with both the *A. muricata* and *P. lutea*. Also, *C. verminus* high growth with the *Porites* mucus as substrate and its reisolation from early disease sign and artificial injured *P. lutea* and not from *A. muricata* tissues suggests for its possible feed specific interactions. Thus, the fatty acid composition of *A. muricata* and *P. lutea* mucus was analysed and noted for the different levels of SAFA, MUFA and PUFA in two of the genera. Conversely, *Holosticha* sp. was found for its voracious ingestion of Symbiodiniaceae cells and other dead tissues contributing to the tissue loss. Further, the ciliates inoculated under aquaria conditions demonstrated that both the ciliates were found migrating towards the artificially injured and early disease sign corals influencing the tissue loss and disease lesion progression leading to coral mortality. The further experimental analysis on the ciliate feed specific interactions may aid in understanding the influence of ciliate in coral disease lesion progression. This work provides the first demonstration of two different genera of ciliate association with diseased corals from Indian reefs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2020.104472>.

References

- [1] C.A. Page, B.L. Willis, Epidemiology of skeletal eroding band on the Great Barrier Reef and the role of injury in the initiation of this widespread coral disease, *Coral Reefs* 27 (2008) 257–272.
- [2] S. Katz, F. Pollock, D. Bourne, B. Willis, Crown-of-thorns starfish predation and physical injuries promote brown band disease on corals, *Coral Reefs* 33 (2014) 705–716.
- [3] D. Sharma, C. Ravindran, Diseases and pathogens of marine invertebrate corals in Indian reefs, *J. Invertebr. Pathol.* 173 (2020) 1–11.
- [4] P. Garrett, H. Ducklow, Coral diseases in Bermuda, *Nature* 253 (1975) 349–350.
- [5] K.B. Ritchie, G.W. Smith, Preferential carbon utilization by surface bacterial communities from water mass, normal, and white-band diseased *Acropora cervicornis*, *Mol. Mar. Biol. Biotechnol.* 4 (1995) 345–352.
- [6] L.L. Richardson, Coral diseases: what is really known? *Trends Ecol. Evol.* 13 (1998) 438–443.
- [7] T. Le Campion-Alsumard, S. Golubic, K. Priess, Fungi in corals – symbiosis or disease – interaction between polyps and fungi causes pearl-like skeleton biomineralization, *Mar. Ecol. Prog. Ser.* 117 (1995) 137–147.
- [8] S. Morrison-Gardiner, Studies on the morphology and ecology of fungi associated with the Australian marine environment. PhD Thesis, James Cook University, Townsville., 2001, p. 246.
- [9] J. Ravindran, C. Raghukumar, S. Raghukumar, Fungi in *Porites lutea*: association with healthy and diseased corals, *Dis. Aquat. Org.* 47 (2001) 219–228.
- [10] W. Wilson, A. Dale, J. Davy, S. Davy, An enemy within? Observations of virus-like particles in reef corals, *Coral Reefs* 24 (2005) 145–148.
- [11] S. Davy, S. Burchett, A. Dale, P. Davies, J. Davy, C. Muncke, et al., Viruses: agents of coral disease? *Dis Aquat Organ* 69 (2006) 101–110.
- [12] F. Rohwer, R. Thurber, Viruses manipulate the marine environment, *Nature* 459 (2009) 207–212.
- [13] H. Ducklow, R. Mitchell, Bacterial populations and adaptations in the mucus layers on living corals, *Limnol. Oceanogr.* 24 (1979) 715–725.
- [14] L. Richardson, Horizontal and vertical migration patterns of *Phormidium coralyticum* and *Beggiatoa* spp. associated with black-band disease of corals, *Microb. Ecol.* 32 (1996) 323–335.
- [15] R. Carlson, L. Richardson, Oxygen and sulfide dynamics in a horizontally migrating cyanobacterial mat: black band disease of corals, *FEMS Microbiol. Ecol.* 18 (1995) 155–162.
- [16] L. Richardson, K. Kuta, Ecological physiology of the black band disease cyanobacterium *Phormidium coralyticum*, *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 43 (2003) 287–298.
- [17] I. Kuffner, V. Paul, Effects of the benthic cyanobacterium *Lyngbya majuscula* on larval recruitment of the reef corals *Acropora surculosa* and *Pocillopora damicornis*, *Coral Reefs* 23 (2004) 455–458.
- [18] M. Sussman, D. Bourne, B. Willis, A single cyanobacterial ribotype is associated with both red and black bands on diseased corals from Palau, *Dis Aquat Organ* 69 (2006) 111–118.
- [19] D. Rasoulouniriana, N. Siboni, E. Ben-Dov, E. Kramarsky-Winter, Y. Loya, A. Kushmaro, *Pseudoscillatoria coralii* gen. nov., sp. nov., a cyanobacterium associated with coral black band disease (BBD), *Dis Aquat Organ* 87 (2009) 91–96.
- [20] L. Richardson, A. Miller, E. Broderick, L. Kaczmarek, M. Gantar, D. Stanic, R. Sekar, Sulfide, microcystin, and the etiology of black band disease, *Dis Aquat Organ* 87 (2009) 79–90.
- [21] D. Stanic, S. Oehle, M. Gantar, L. Richardson, Microcystin production and ecological physiology of Caribbean black band disease cyanobacteria, *Environ. Microbiol.* 13 (2011) 900–910.
- [22] G.S. Aebly, Trade-offs for the butterfly fish, *Chaetodon multicinctus*, when feeding on coral prey infected with trematode metacercariae, *Behav. Ecol. Sociobiol.* 52 (2002) 158–165.
- [23] G.S. Aebly, Corals in the genus *Porites* are susceptible to infection by a larval trematode, *Coral Reefs* 22 (2003), 216–216.
- [24] G.S. Aebly, Spatial and temporal patterns of *Porite strematodiasis* on the reefs of Kaneohe Bay, Oahu, Hawaii, *Bull. Mar. Sci.* 80 (2007) 209–218.
- [25] C. Palmer, M. Roth, R. Gates, Red fluorescent protein responsible for pigmentation in Trematode-Infected *Porites compressa* tissues, *Biol Bull* 21 (2009) 68–74.
- [26] K. Ulstrup, M. Kuhl, D. Bourne, Zooxanthellae harvested by ciliates associated with Brown Band Syndrome of corals remain photosynthetically competent, *Appl. Environ. Microbiol.* 73 (2007) 1968–1975.
- [27] D.J. Qiu, L.M. Huang, H. Huang, J.H. Yang, S.J. Lin, Two functionally distinct ciliates dwelling in *Acropora* corals in the South China Sea near Sanya, Hainan Province, China, *Appl. Environ. Microbiol.* 76 (2010) 5639–5643.
- [28] W. Cooper, O. Lirman, M. Schmale, D. Lipscomb, Consumption of coral spat by histophagic ciliates, *Coral Reefs* 26 (2007) 249–250.
- [29] E.C. Peters, Diseases of coral-reef organisms, in: C. Birkeland (Ed.), *Life and Death of Coral Reefs*, Chapman & Hall, New York, 1997, pp. 114–136.
- [30] C. Cerrano, G. Bavestrello, C.N. Bianchi, R. Cattaneo-vietti, S. Bava, C. Morganti, C. Morri, P. Picco, G. Sara, S. Schiaparelli, A. Sicardi, F. Sponga, A catastrophic mass-mortality episode of gorgonians and other organisms in the Ligurian Sea (North-western Mediterranean), summer, *Ecol. Lett.* 3 (1999) 284–293.
- [31] A.A. Antonius, D. Lipscomb, First protozoan coral-killer identified in the Indo-Pacific, *Atoll Res. Bull.* 481 (2001) 1–21.
- [32] S. Rodríguez, A. Cróquer, H. Guzmán, C. Bastidas, A mechanism of transmission and factors affecting coral susceptibility to *Halofolliculina* sp. infection, *Coral Reefs* 28 (2009) 67–77.
- [33] R. Winkler, A. Antonius, D.A. Renegar, The skeleton eroding band disease on coral reefs of Aqaba, Red Sea, *Marine Ecol.—Pubblicazioni Della Stazione Zoologica Di Napoli I* 25 (2004) 129–144.
- [34] C.S. Lobban, L. Raymundo, D.J.S. Montagnes, *Porpostoma guamensis* n. sp., a Philasterine Scuticociliate associated with brown-band disease of corals, *J. Eukaryot. Microbiol.* 58 (2011) 103–113.
- [35] B. Willis, C. Page, E. Dinsdale, Coral disease on the great barrier reef, in: E. Rosenberg, Y. Loya (Eds.), *Coral Disease and Health*, Springer-Verlag, Berlin, Germany, 2004, pp. 69–104.
- [36] A. Croquer, C. Bastidas, D. Lipscomb, a. Folliculinid ciliates, A new threat to Caribbean corals? *Dis Aquat Organ* 69 (2006) 75–78.
- [37] A. Croquer, C. Bastidas, D. Lipscomb, R.E. Rodríguez-Martínez, E. Jordan-Dahlgren, H.M. Guzmán, First report of folliculinid ciliates affecting Caribbean scleractinian corals, *Coral Reefs* 25 (2006) 187–191.

- [38] M. Sweet, J. Bythell, Ciliate and bacterial communities associated with White Syndrome and Brown Band Disease in reef-building corals, *Environ. Microbiol.* 14 (2012) 2184–2199.
- [39] C. Randall, J. Jordan-Garza, R. vanWoesik, Ciliates associated with signs of disease on two Caribbean corals, *Coral Reefs* 34 (2014) 243–247.
- [40] M.J. Sweet, A. Croquer, J.C. Bythell, Experimental antibiotic treatment identifies potential pathogens of white band disease in the endangered Caribbean coral *Acropora cervicornis*, *Proc. R. Soc. B.* 281 (2014) 20140094.
- [41] M. Sweet, M. Séré, Ciliate communities consistently associated with coral Diseases, *J. Sea Res.* 113 (2015) 119–131.
- [42] A. Dopheide, G. Lear, R. Stott, G. Lewis, Preferential feeding by the ciliates *Chilodonella* and *Tetrahymena* spp. and effects of these Protozoa on bacterial biofilm structure and composition, *Appl. Environ. Microbiol.* 77 (2011) 4564–4572.
- [43] G. Hadadi, H. Gegner, M. Ziegler, C.R. Voolstra, Carbohydrate composition of mucus from scleractinian corals from the central Red Sea, *Coral Reefs* 38 (2019) 21–27, 2019.
- [44] B. Brown, J. Bythell, Perspectives on mucus secretion in reef corals, *Mar. Ecol. Prog. Ser.* 296 (2005) 291–309.
- [45] V.Z. Radice, M.T. Brett, B. Fry, M.D. Fox, O. Hoegh-Guldberg, S.G. Dove, Evaluating coral trophic strategies using fatty acid composition and indices, *PLoS One* 14 (2019) 9.
- [46] J.G. Day, U.E.M. Achilles-Day, S. Brown, A. Warren, Cultivation of algae and protozoa, in: C.J. Hurst, G.R. Kuds, M.J. McInerney, L.D. Stezenbach, M. V. Walter (Eds.), *Manual of Environmental Microbiology*, ASM Press, Washington DC, 2007, pp. 79–92.
- [47] P.G. Carey, *Marine Interstitial Ciliates. An Illustrated Key*, Chapman & Hall, London, 1992, 368.
- [48] J.J. Lee, G.F. Leedale, P.C. Bradbury, *An Illustrated Guide to the Protozoa: Organisms Traditionally Referred to as Protozoa, or Newly Discovered Groups*, second ed. II, Blackwell Scientific, Massachusetts, 2000, pp. 690–1432.
- [49] Wiebo-Song, Morphological and taxonomical studies on some marine Scuticociliates from China Sea, with description of two new species, *Philaster idesarmatalis* sp. n. and *Cyclidiumvaribonneti* sp. n. (Protozoa: Ciliophora: Scuticociliatida), *Acta Protozool.* 39 (2000) 95–322.
- [50] Wiebo-Song, N. Wilbert, Faunistic studies on marine ciliates from the Antarctic Benthic area, including descriptions of one epizoid form, 6 new species and, 2 new genera (Protozoa: Ciliophora), *Acta Protozool.* 41 (2002) 23–61.
- [51] J.D. Thompson, D.G. Higgins, T.J. Gibson, Clustal W, Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* 22 (1994) 4673–4680.
- [52] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol. Biol. Evol.* 4 (1987) 406–425.
- [53] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, *Mol. Biol. Evol.* 33 (2016) 1870–1874.
- [54] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, *Evolution* 39 (1985) 783–791.
- [55] Jordi Folch, M. Lees, G.H. Sloane, A. Stanley, A simple method for the isolation and purification of total lipid from animal tissues, *J. Biol. Chem.* 226 (1) (1957) 497–509.
- [56] J.P. Carreau, J.P. Dubacq, Adaptation of a macroscale method to the micro-scale for fatty acid methyl transesterification on biological lipid extracts, *J. Chromatogr.* 151 (1978) 384–390.
- [57] T. Trivedi, D. Jain, N.S.S. Mulla, N.S.S. Mamatha, R.S. Damare, R.A. Sreepada, S. Kumar, V. Gupta, Improvement in biomass, lipid production and biodiesel properties of a euryhaline *Chlorella vulgaris* NIOCCV on mixotrophic cultivation in wastewater from a fish processing plant, *Renew. Energy* 139 (2019) 326–335.
- [58] C. Palmer, L. Mydlarz, B. Willis, Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals, *Proc. Biol. Sci.* 275 (2008) 2687–2693.
- [59] J.E. Duerden, The role of mucus in corals, *J. Cell Sci.* 2 (1906) 591–614.
- [60] C.R. Bakshani, A.L. Morales-Garcia, M. Althaus, M.D. Wilcox, J.P. Pearson, J. C. Bythell, J.G. Burgess, Evolutionary conservation of the antimicrobial function of mucus: a first defence against infection, *NPJ Biofilms Microbiomes* 4 (2018) 14.
- [61] D. Bourne, H. Boyett, M. Henderson, A. Muirhead, B. Willis, Identification of a ciliate (Oligohymenophorea: Scuticociliatia) associated with Brown band disease on corals of the great barrier reef, *Appl. Environ. Microbiol.* 74 (2008) 883–888.
- [62] D. Smith, P. Leary, J. Craggs, J. Bythell, M. Sweet, Microbial Communities associated with healthy and white syndrome-affected *Echinopora mellosa* in aquaria and experimental treatment with the antibiotic ampicillin, *PLoS One* 10 (2015), e0121780.
- [63] H. Long, R. Zufall, Diverse modes of reproduction in the marine free-living ciliate *Glaucanema trihymene*, *BMC Microbiol.* 10 (2010) 108.
- [64] E. Vacelet, B. Thomassin, Microbial utilization of coral mucus in long term *in situ* incubation over a coral reef, *Hydrobiologia* 211 (1991) 19–32.
- [65] C. Teixeira, R. Lana, J. Tao, T. Hackmann, Comparing the responses of rumen ciliate protozoa and bacteria to excess carbohydrate, *FEMS Microbiol. Ecol.* 93 (2017) 1–12.
- [66] J.H. Jr Thompson, E.A. Shinn, T.J. Bright, Effects of drilling mud on seven species of reef-building corals as measured in the field and laboratory, in: R.A. Geyer (Ed.), *Marine environmental pollution 1, Hydrocarbons* Elsevier, New York Amsterdam, 1980, pp. 433–454.
- [67] I. Johnston, F. Rohwer, Microbial landscapes on the outer tissue surfaces of the reef-building coral *Porites compressa*, *Coral Reefs* 26 (2007) 375–383.
- [68] T. Fenchel, Suspension feeding in ciliated protozoa: functional response and particle size selection, *Microb. Ecol.* 6 (1980) 1–11.
- [69] B. Ayo, E. Santamaría, A. Latatu, I. Artolozaga, I. Azúa, J. Iriberrí, Grazing rates of diverse morphotypes of bacterivorous ciliates feeding on four allochthonous bacteria, *Lett. Appl. Microbiol.* 33 (2001) 455–460.
- [70] I.G. Boechat, R. Adrian, Biochemical composition of algivorous freshwater ciliates: you are not what you eat, *FEMS Microbiol. Ecol.* 53 (2005) 393–400.
- [71] N.A. Latyshev, N.V. Naumenko, V.I. Svetashev, Y. Ya Latypov, Fatty acids of reef-building corals, *Marine Ecol. Prog. Series* 76 (1991) 295–301.
- [72] B. Imbs, B. Andrey, Ly PT. Dang, Viacheslav G. Rybin, Vasily I. Svetashev, Fatty acid, lipid class, and phospholipid molecular species composition of the soft coral *Xenia* sp. (Nha Trang Bay, the South China Sea, Vietnam), *Lipids* 50 (2015) 575–589.
- [73] M. Nuges, R. Bak, Brown-band syndrome on feeding scars of the crown-of-thorn starfish *Acanthaster planci*, *Coral Reefs* 28 (2009) 507–510.



Characterization of the defense properties of healthy and diseased coral mucus

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ABSTRACT

The surface mucus layer of corals aids in feeding, silt removal, and defense against pathogens. However, first line of defense of secreted mucus of corals against tissue damage generated through pathogen or environmental factors is poorly understood. Hence, we used various methods such as a well diffusion assay and tests for quorum quenching, free radical scavenging, antioxidant enzyme expression and phenoloxidase (PO) activity to determine the mucus defense properties using mucus of healthy and diseased *Porites* sp. and *Acropora* sp. Interestingly the coral mucus showed antimicrobial activity against coral pathogens such as bacteria and protozoan ciliates. Inhibition of the *N*-Acyl homoserine lactone (AHL) molecule suggests quorum quenching. Free radical scavenging of mucus was screened using hydrogen peroxide, hydroxyl radical, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methods, which was found significantly more in diseased corals ($p < 0.05$). Antioxidant enzymes superoxide dismutase (SOD), catalase, and peroxidase activity were observed in both the diseased and healthy coral mucus. The presence of serine and metalloproteases was also detected in coral mucus. Further, phenoloxidase (PO) activity was highest in diseased coral mucus affected by pink line syndrome and white patch *Acropora* sp. disease than the healthy coral mucus. Thus, the present study of antimicrobial properties, antioxidant enzymes, and quorum quenching properties in coral mucus may aid in understanding the corals defense and survival against pathogens and any abiotic stress.

1. Introduction

Coral reefs are unique, diverse, economically important, and productive ecosystems, hence their description as the “rainforests” of the ocean (Rosenberg et al., 2007). However, corals that form reefs are under threat due to various biotic and abiotic stressors such as disease, UV-radiation and temperature rise (Sharma and Ravindran, 2020). These stressors also influence the dissociation of the symbiont from the host coral which leads to coral bleaching, a response against oxidative stress (Downs et al., 2002). The generation of such oxidative stress takes place when the production and accumulation of reactive oxygen species (ROS) become uncontrollable by the host. These ROS damage the cells macromolecules such as lipids, DNA, and proteins (Schieber and Chan-del, 2014) and act as damaging or signalling molecules (Sharma et al., 2012). Besides, due to the expelled endosymbiont, the host coral becomes weak and immunocompromised. This allows primary and secondary pathogens to infect the coral tissue causing disease that leads to

coral mortality.

Several studies have described the role of bacteria and ciliates as primary and secondary pathogens influencing the disease progression in immunocompromised corals (Sweet and Sere, 2016; Ravindran et al., 2020; Ravindran et al., 2021). Therefore, understanding the various responses of coral reefs to disease and bleaching events is critical for their natural resilience. Furthermore, the possible evolutionary adaptation of corals to various stress conditions enhanced the stress tolerance level of individual corals, implying that corals could remember and respond to their previous environmental experiences (Hackerott et al., 2021). This supports the past reports of experimental data showing that some Scleractinian corals have adaptive-like immune responses in addition to innate immunity (Rosenberg et al., 2007). This phenomenon is known as experience-mediated tolerance, and it occurs when corals that were previously susceptible to bleaching caused by a specific bacterial disease may develop immunity to it. For example, it has been established that the coral *Oculina patagonica* can become resistant to

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reinfection by *Vibrio shiloi* after being severely impacted by bleaching events caused by this bacterium (Reshef et al., 2006).

Coral mucus is a complex mixture of protein, carbohydrates, and lipids produced by mucocytes in the epidermal layer and utilised by the coral as an adaptation mechanism against various stress conditions (Brown and Bythell, 2005). As a result, coral surface tissue that secretes the surface mucus layer (SML) serves as a first line of defense as well as a physiochemical and physiological barrier between animal tissue and external environment stresses (Brown and Bythell, 2005, Geffen et al., 2009, Bakshani et al., 2018). The bioactive compounds in coral mucus have been experimentally proven to have antimicrobial activity in the defense against infectious disease by inhibiting *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Vibrio* species and *Serratia marcescens* (Brown and Bythell, 2005, Ritchie, 2006, Bakshani et al., 2018). Besides, Mycosporine-like amino acids (MAA), which act as a sunscreen that absorbs UV radiation (Teai et al., 1998), and phenoloxidase (PO) and melanin activity in mucus of healthy and diseased *Pseudodiploria strigosa* (Rivera-Ortega and Thome, 2018) have also been described. Prophenoloxidase (ProPO) is a major enzyme of coral innate immunity (Cerenius et al., 2008) which gets upregulated due to oxidative stress. During proteolysis, ProPO gets converted into phenoloxidase (PO) by serine proteinase activity which finally stimulates the melanin synthesis pathway (Mydlarz et al., 2009), an indicator for corals to respond against pathogens (Palmer et al., 2011). The antioxidants, phenols, and flavonoids found in coral mucus that play an important role in free radical scavenging activity and the quorum quenching characteristic that inhibits infections and promotes coral health have not been studied or reported. It is believed that such defensive substances in mucus are derived as secreted from coral tissue to mucus (Fleming, 1922; Ritchie, 2006). However, no study was described for the presence of antioxidants and other defense substances reported from coral mucus.

Coral disease outbreaks due to bacterial infections have increased significantly. Especially, various *Vibrio* sp. involved in causing numerous coral diseases, namely *Vibrio rotiferanus*, *V. harveyi*, *V. alginolyticus*, *V. proteolyticus* together have been described as a consortium that causes Yellow blotch/band disease (YBD) (Cervino et al., 2008); *V. parahaemolyticus*, as the causative agent of Porites ulcerative white spot disease (PUWS) (Arboleda and Reichardt, 2010), a proline-rich P-toxin of *V. shiloi* induced bleaching of *Oculina patagonica* (Banin et al., 2001); *V. corallilyticus*, the causative pathogen of white syndrome (WS) and bleaching in the coral *Pocillopora damicornis* (Ben-Haim et al., 2003a; Ben-Haim et al., 2003b). *V. carchariae*, *V. harveyi* causing white band in *Acropora* spp. (Ritchie and Smith, 1998). Also, the role of lyso-genic prophages in the conversion of non-pathogenic to virulent strains has been described in a wide range of species, including *Vibrio cholerae*, *Staphylococcus aureus*, and *Escherichia coli* (Fortier and Sekulovic, 2013) and especially in the coral pathogen, *V. corallilyticus* (Weynberg et al., 2015).

The coral pathogen, *V. parahaemolyticus* and *S. aureus* as control pathogen has been taken in the present study for their inhibition and suppression of virulence if any by coral mucus. Besides, the coral surface mucus layer that represents the primary defense against pathogens has been characterized for their defense properties such as antimicrobial, quorum quenching, antioxidants (for DPPH, hydroxyl radical and hydrogen peroxide scavenging, and metal chelating and reducing power activities) and antioxidant enzymes (Superoxide dismutase, catalase, peroxidase, phenol oxidase) which may aid in understanding coral survival and resilience under various biotic and abiotic stress conditions.

2. Materials and methods

2.1. Sample collection and preparation of extract

Coral mucus samples were collected from the Big Manali island within a 5-meter radius transect, habitats at N 09°13'15.6" E

079°08'38.5", at depth 1–1.5 m, Temperature 29 °C, Gulf of Mannar, India. Healthy coral mucus was collected *in situ* with a sterile syringe from the surface of the individual healthy coral colonies as earlier collected by Ravindran et al., (2020). Similarly, diseased *Acropora* sp. (white patch) and *Porites* sp. (pink line syndrome) mucus were collected along the disease margins from every single individual colony. The collected fresh mucus samples were stored below 4 °C and examined for all the experimental analysis that was done in triplicates.

2.2. Mucus antimicrobial activity against pathogens

The disc diffusion method was performed to determine the antimicrobial activity of mucus against coral pathogens *Vibrio parahaemolyticus* and *Staphylococcus aureus* isolated from diseased corals. Different concentrations of *Acropora* sp. and *Porites* sp. mucus were used (20 µl, 30 µl, 50 µl, 70 µl, and 100 µl). The *S. aureus* and *V. parahaemolyticus* were spread-plated on Muller-Hinton agar plates and varied concentrations of mucus were placed in discs aseptically over the pathogen spread agar plates and incubated at 28 °C for 24 h.

2.3. Coral mucus inhibition of ciliates growth

Adult ciliates with an equal and known number i.e., 15 or more ciliates of *Holosticha diademata* and *Cohnelimbus verminus* isolated from diseased corals (Ravindran et al., 2020) were used in mucus inhibition assay. Four treatments were tested with the above ciliates such as ciliates with only seawater, only coral mucus (with bacteria), only coral mucus 0.22 µm filtered (without bacteria) and mucus with seawater. Plates were incubated at 28 °C. The number of ciliates was counted and observed every 8 h, 24 h, and 48-hour time intervals under a microscope. All the experiments were carried out in triplicates.

2.4. Quorum quenching analysis

N-Acyl homoserine lactone (AHL) -molecule quorum quenching by coral mucus was carried out by well diffusion plate assay (Tait et al., 2010). Quorum sensing (QS) regulates the violacein pigment production of *Chromobacterium violaceum* and is exploited for inhibitors of QS (McLean et al., 2004). Briefly, *C. violaceum* culture 100 µl was spread plated on Luria-Bertani (LB) agar plates. Wells were pricked in the spread agar plates and 100 µl of mucus was added to the well. The Plates were incubated for 24 h at 30 °C. After incubation, 0.7 % of nutrient soft agar containing 200 µl of *C. violaceum* per 10 ml soft agar was poured over the colonies. All the plates were further incubated at 30 °C for 24 h and plates were monitored.

Violacein pigment production was tested based on the earlier method (Choo et al., 2006). Briefly, in 1.8 ml Tryptic soy (TS) broth, 0.1 ml *C. violaceum* culture and 0.1 ml mucus were added. *C. violaceum* was grown on TS broth at 30 °C on the shaker. After incubation, to precipitate the violacein pigment, 1 ml culture was centrifuged at 13,000 rpm for 10 min. Then 1 ml of DMSO was added to the pellet and resuspended well. Optical density was checked at 585 nm. The control was made without the addition of mucus. All tests were performed in triplicates. Percentages of violacein pigment production are calculated by the following formula:

$$\left(\frac{\text{Absorbance of tested concentration at 585 nm}}{\text{Absorbance of the control at 585 nm}} \right) \times 100.$$

The AHL lactonase enzyme activity on Penicillin G was done by incubating the reaction i.e., 200 µl Penicillin G 20 mg/ml, 200 µl 0.1 % starch, 200 µl mucus sample, and 200 µl water at 40 °C for 15 min (Mukherji and Prabhune, 2013). The lactonase enzyme is of the beta-lactamase superfamily that acts on the beta-lactam ring of Penicillin. Because of the high temperature used during the assay, some amount of Penicillin gets degraded. So, to avoid false readings, results were

observed immediately after the addition of iodine. Controls were made without penicillin and enzyme, and only without enzyme. The exact mechanism behind the assay was that the AHL lactonase hydrolyzes penicillin into penicilloic acid. After the production of penicilloic acid, iodine is not free to interact with the 0.1 % starch and has no production of the blue colour, which confirms the presence of quorum quenching molecule in coral mucus. Thus in control, without coral mucus there is no formation of penicilloic acid and the iodine reacts with the starch and forms blue colour. Thus, the formation of the blue color depends on the interaction of free iodine with 0.1 % starch. The color formation was noted within 10 s of the 10 % Iodine reagent addition.

2.5. Characterization of antioxidant properties

2.5.1. Total soluble phenol content assay

The total soluble phenolic content of coral mucus was estimated by using our earlier lab protocol (Ravindran and Naveenan 2011). To estimate total soluble phenol, the positive control used was gallic acid. A 100 µl extract of the mucus sample was mixed with 2 ml of sodium bicarbonate (2 %) and kept for 2 min of incubation at room temperature. After incubation, 100 µl of Folin-Ciocalteu reagent was added to the mixture and placed in the dark for 30 min. The samples were vortexed and absorbance was measured at 725 nm spectrophotometrically.

2.5.2. Total flavonoids content assay

To quantify the total flavonoid content of coral mucus, quercetin was used as the standard (Ravindran and Naveenan 2011). The 100 µl of the coral mucus was mixed in 300 µl of distilled water and 30 µl of sodium nitrite (5 % NaNO₂). Tubes were incubated for five minutes. To this mixture, 30 µl of Aluminium chloride (10 % AlCl₃) was added, and mixed well and the reaction mixture was placed for 5 min at room temperature. Then 200 µl Sodium hydroxide (1 mM NaOH) was added to the reaction and the final volume was made up to 1 ml by adding distilled water and mixed well. Optical density was observed at 510 nm.

2.6. Antioxidants scavenging assays

2.6.1. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging assay was determined using the method of Ravindran and Naveenan (2011). Mucus (25 µl, 50 µl, and 75 µl) were taken and 50 mM of hydrogen peroxide (HiMedia Mumbai, India) was mixed in a ratio of 1:1 (v/v). The reaction mixture was incubated for 30 min, at room temperature. After 30 min of incubation, 10 µl of HPLC grade methanol, and 0.9 ml FOX reagent (ferrous oxidation – xylenol orange reagent) were added to the 90 µl hydrogen peroxide solution (Sigma-Aldrich). The mixture was thoroughly mixed and placed at room temperature for 30 min. Blank was made by adding all the reagents except the sample. The ferric-xylenol orange complex formation was measured at 560 nm. The scavenging activity was measured based on inhibition of the complex formation of ferric-xylenol orange. To calculate scavenging activity, the following formula has been used:

$$\% \text{H}_2\text{O}_2 \text{ scavenging} = \left[\frac{\text{Control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right] \times 100.$$

2.6.2. Hydroxyl radical scavenging

The hydroxyl radical scavenging assay of coral mucus was carried out using the earlier method (Ravindran and Naveenan 2011; Ravindran et al., 2012a). The activity is based on spectrometric estimation of the breakdown product of 2-deoxyribose by condensation with Thio-barbituric acid (TBA). The hydroxyl radicals were generated by the Fe³⁺ ascorbate EDTA H₂O₂ system i.e. Fenton reaction. The reaction mixture consisted of 1 ml of 20 mM phosphate buffer (pH-7.4) containing 2.8 mM deoxyribose, 100 µM ferric chloride, 1 mM H₂O₂, 100 µM

EDTA, and different concentrations of *Porites* sp., *Acropora* sp. mucus (25 µl, 50 µl, and 75 µl). Ascorbic acid (100 µM) was added at last followed by incubation for 1 h at 37 °C. Then, 1 ml of 1 % Thio-barbituric acid and 2.8 % Trichloroacetic acid were added and the reaction was incubated for 15 min at 90 °C for color development. After cooling the tubes, optical density was measured at 532 nm. Mannitol was used as a control. The scavenging activity was measured by the following formula:

$$\% \text{Hydroxyl radical scavenging} = \left[1 - \frac{\text{sample absorbance}}{\text{blank absorbance}} \right] \times 100.$$

2.6.3. DPPH scavenging assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was performed as per earlier demonstrated methods (Ravindran and Naveenan 2011; Ravindran et al., 2012a) by adding 800 µl of 1 mM DPPH (Sigma-Aldrich Steinheim, Germany) solution prepared in 95 % ethanol with mucus (25 µl, 50 µl, and 75 µl). The reactions were vortexed well and kept at room temperature for 30 min in the dark. Control was made by using 1 ml ethanol. Samples were centrifuged at 13,500 rpm for 5 min at RT. Optical density was measured at 517 nm. Butylated hydroxyanisole (BHA) (Merk Mumbai, India) was used as a reference compound.

$$\% \text{DPPH scavenging activity} = \left[\frac{\text{Absorbance of the blank} - \text{Absorbance of the sample}}{\text{Absorbance of the blank}} \right] \times 100$$

2.6.4. Metal chelating assay

The metal ion chelating capacity of the coral mucus was quantified by the modified protocol (Dinis et al., 1994; Ravindran and Naveenan 2011; Ravindran et al., 2012a). The experiment was performed in HEPES buffer (pH 7.2, 20 mM). Different concentrations of mucus (25 µl, 50 µl, and 75 µl) were mixed with 12.5 µM ferrous sulphate solution. Into the reaction, 75 µM ferrozine solution was added and shaken vigorously. The mixture was kept for 20 mins and incubated at room temperature. After 20 min of incubation, the optical density was measured at 562 nm. EDTA was used as a positive control. The chelating capacity percentage was calculated by using the formula: % Chelating activity = $\left[\frac{\text{Absorbance of the blank} - \text{Absorbance of the sample}}{\text{Absorbance of the blank}} \right] \times 100$.

2.6.5. Reducing power determination

The reducing power of the mucus samples was determined by the method of (Ravindran et al., 2012a) with slight modification. Various concentrations of mucus (25 µl, 50 µl, and 75 µl) extracts (25 µl, 50 µl, and 75 µl) 0.5 ml were mixed with an equal volume of phosphate buffer (0.2 M pH-6.6) and 0.5 ml of 0.1 % potassium hexacyanoferrate. This reaction mixture was incubated at 50 °C for 20 min in a boiling water bath. After incubation 0.5 ml of 10 % Trichloroacetic acid was added to stop the reaction and 1 ml of the upper portion of the reaction mixture was mixed with 1 ml of distilled water, 0.1 ml of 0.01 % Ferric chloride (FeCl₃) followed by incubation at room temperature for 10 min. Optical density was measured at 700 nm. An increase in absorbance indicates increased reducing power.

2.7. Coral mucus antioxidant enzyme spectrophotometric assays

2.7.1. Superoxide dismutase activity.

An assay of superoxide dismutase activity was done using the earlier method of our laboratory (Ravindran et al., 2012b). Coral mucus extract was diluted with 0.3 mM sodium cyanide, 0.2 ml EDTA (0.1 M) solution, and 0.1 ml NBT (1.5 mM). Immediately 0.05 ml riboflavin (0.12 mM) was added and incubated for 12 min in light. Blank was made with distilled water in the place of coral samples. Optical density was measured at 560 nm. SOD activity was expressed as U/mg protein. The enzyme causing half the maximum inhibition reduction in NBT is known as one unit of SOD.

2.7.2. Catalase activity

Catalase activity was determined by using our earlier lab protocol (Ravindran et al., 2012b). 20 µl of healthy and diseased coral *Acropora* sp. and *Porites* sp. mucus extract was mixed separately into the reaction mixture of 30 µl phosphate buffer (50 mM) (pH 7.0) and 50 µl H₂O₂ (50 mM). The activity was measured at an optical density of 240 nm. Control was made without coral mucus and tissue samples. Catalase activity was calculated using the molar extinction coefficient for H₂O₂ at 240 nm (Anderson et al., 1995).

2.7.3. Peroxidase assay

Peroxidase activity assay was done by using the protocol developed by (Kiran et al., 2012). Mucus extract, 200 µl was mixed with 3.5 ml phosphate buffer pH 6.5 (100 mM) and added to 100 µl guaiacol (20 mM) (Sigma-Aldrich) in 10 mM phosphate buffer (pH 6.5) in the cuvette. The reaction was initiated by adding 200 µl of 200 mM H₂O₂ (Sigma-Aldrich), which was prepared in 10 mM phosphate buffer (pH 6.5). Spectrophotometric reading was measured at 430 nm. The change in absorbance per min was measured from the linear phase. The blank was prepared with water. Peroxidase enzyme activity was expressed in units per mg protein.

2.7.4. Phenol oxidase assay

Phenol oxidase activity was measured by the procedure of Mydlarz and Palmer (2011) with slight modification. Dilutions were made by adding 30 µl of the coral mucus extract with 40 µl of 50 mM phosphate buffer, pH 7.5. To this reaction mixture, 25 µl of 0.1 mg/mL trypsin (HiMedia Mumbai, India) was added and kept for 10 mins incubation. After incubation, 30 µl of the guaiacol (10 mM stock concentration, 2.6 mM final concentration, Sigma-Aldrich) was added and the developed coloured product was measured at an optical density of 470 nm for 15 min. Values are represented as units per mg of protein.

2.8. Electrophoretic assay

2.8.1. Mucus protein extraction

Coral mucus 20 ml was centrifuged for 10 min, at 10,000 rpm (Thermo scientific) to allow all the cell mass and impurities to settle down. Into the mucus supernatant, the desired amount of Ammonium sulphate i.e. 9.55 gm to get desired saturation, for protein precipitation at 25 °C (HiMedia, Mumbai, India) was dissolved by continuous stirring on a magnetic stirrer for 3 h till the Ammonium sulphate crystal formation, followed by 15 min of centrifugation at 10,000 rpm to pellet out the protein. Pellets were dissolved in phosphate buffered saline (PBS) to analyze proteins. Protein samples were dialyzed out of the Ammonium sulphate with phosphate buffer saline pH 7.4.

2.8.2. Superoxide dismutase activity

Superoxide dismutase (SOD) enzyme activity of healthy and diseased coral mucus of *Acropora* sp. and *Porites* sp. was checked in native-PAGE gel. SOD activity was visualized on the 10 % gel by the activity staining procedure (Ravindran et al., 2012b). After electrophoresis, the gel was washed with distilled water 2–3 times. Then it was treated with 0.1 % NBT (Sigma-Aldrich) on the shaker for 15 min in the dark. The gel was washed 2–3 times with distilled water. Riboflavin (Sigma-Aldrich) solution was added to the gel in the dark with shaking for 15 min. Again, the gel was washed 2–3 times with distilled water. Immediately the gel was exposed to light until the appearance of SOD activity bands on the gel.

2.8.3. Catalase activity assay

Catalase activity staining was performed on 6 % native-PAGE gel by the earlier method (Ravindran et al., 2012b). After electrophoresis, the gel was washed two times with distilled water. The gel was then rinsed with 50 mM phosphate buffer containing H₂O₂ 0.01 M, and for 20 min gentle rocking was given. Further, the gel was kept in 30 ml of 2 %

potassium ferric cyanide (Sigma-Aldrich) and 30 ml of 2 % ferric chloride (HiMedia Mumbai, India) and mixed well until the appearance of yellow-colored bands over the light box. Mucus catalase activity was also demonstrated on a clean glass slide against 3 % hydrogen peroxide as a substrate. 10 µl mucus was added to the substrate and the production of air bubbles was observed to confirm the presence of catalase enzymes.

2.8.4. Gelatin zymography for protease detection in coral mucus

The gelatin zymography was done to characterize and profile the proteases on a 12 % resolving polyacrylamide gel with 0.1 % gelatin and 0.15 % SDS following the protocol of Subramanian et al. (2007). The classes of protease present in the coral mucus were determined using specific inhibitors such as phenylmethylsulphonyl fluoride (5 mM), benzamidinium hydrochloride (5 mM), EDTA (5 mM), 1 mg/ml *N*-ethylmaleimide, 10 µg/ml pepstatin and *o*-phenanthroline (5 mM). All chemicals were used of make by HiMedia Mumbai, India except BHT which was of make by Merk Mumbai, India.

2.9. Statistical analysis

All the experiments were carried out in triplicates. One-way ANOVA was used to compare means of the antioxidant assays and enzyme activities of healthy and diseased coral mucus (healthy *Acropora* sp. mucus, white patch *Acropora* sp. mucus, healthy *Porites* sp. mucus, and pink line syndrome *Porites* sp. mucus) samples by using SPSS software. The coefficient of determination r^2 was calculated using Microsoft Excel.

3. Results

3.1. Coral mucus antimicrobial activity against bacteria

The healthy and diseased *Acropora* sp. and *Porites* sp. mucus was observed for significant activity by inhibiting the coral pathogens of *S. aureus* and *V. parahaemolyticus* (Fig. 1A). The filtered mucus of *Porites* sp. and *Acropora* sp. were observed for potential antimicrobial activity with an increased concentration against *S. aureus* and *V. parahaemolyticus* (Fig. 1B). Thus, the zone of inhibition was increased with the increased concentration of mucus, suggesting the antagonistic ability of the coral mucus against the coral bacterial pathogens are concentration dependant.

3.2. Antimicrobial activity of coral mucus against ciliates

Growth and proliferation of both the ciliate such as *Cohnelimbus* sp. (Fig. 2) and *Holosticha* sp. (Fig. 2) were observed to be inhibited by *Acropora* sp. mucus. Wherein, *Cohnelimbus* sp. exhibited more sensitivity to *Acropora* sp. mucus compared to *Holosticha* sp. While seawater alone showed support for both the ciliate growth. Seawater mixed with mucus showed intermediate growth. The ciliate inhibition rates were measured by an increase in incubation time is directly proportional to a decrease in ciliate numbers.

3.3. Quorum quenching property of coral mucus

A zone of clearance was observed in a *C. violaceum* spread plate assay around the well-containing diseased and healthy mucus (Fig. 3A). This reveals that mucus can to interfere with QS produced by coral host pathogens. The Violet colour of the violacein pigment with quorum sensing property was seen in the negative control wells. The inhibition of quorum sensing by mucus was investigated by the use of violacein pigment of *C. violaceum* that was estimated by spectrophotometric assay. Violacein pigment, a quorum sensing molecule of *C. violaceum* production was inhibited by healthy and diseased coral mucus (Fig. 3B). The healthy *Acropora* sp. mucus, healthy *Porites* sp. mucus, and *V. harveyi* showed maximum inhibition of violacein pigment production due to the

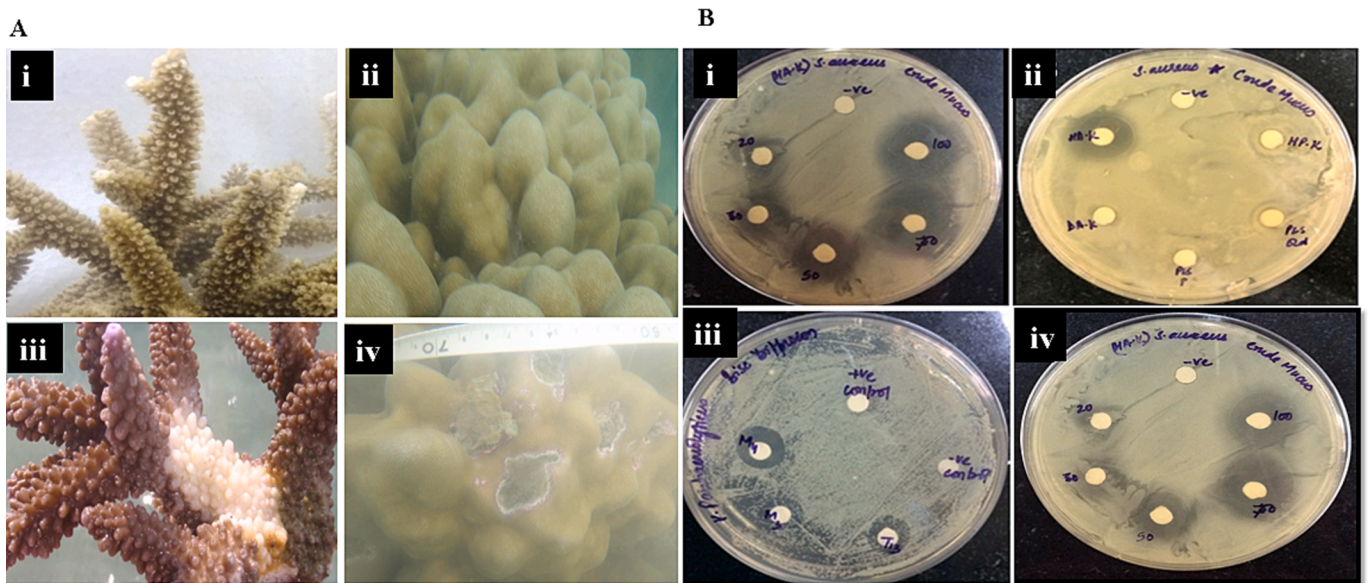


Fig. 1. A. Healthy and diseased *Acropora* sp. and *Porites* sp. i. *Acropora* sp. healthy, ii. *Porites* healthy, iii. *Acropora* sp. white patch disease, iv. *Porites* sp. pink line syndrome disease. B. Antimicrobial activity of mucus against coral pathogens- i. *Acropora* and *Porites* mucus activity against *S. aureus* ii. *Porites* mucus activity against *S. aureus*, iii. *Acropora* and *Porites* mucus activity against *Vibrio parahaemolyticus*, iv. Filtered *Acropora* and *Porites* mucus against *S. aureus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

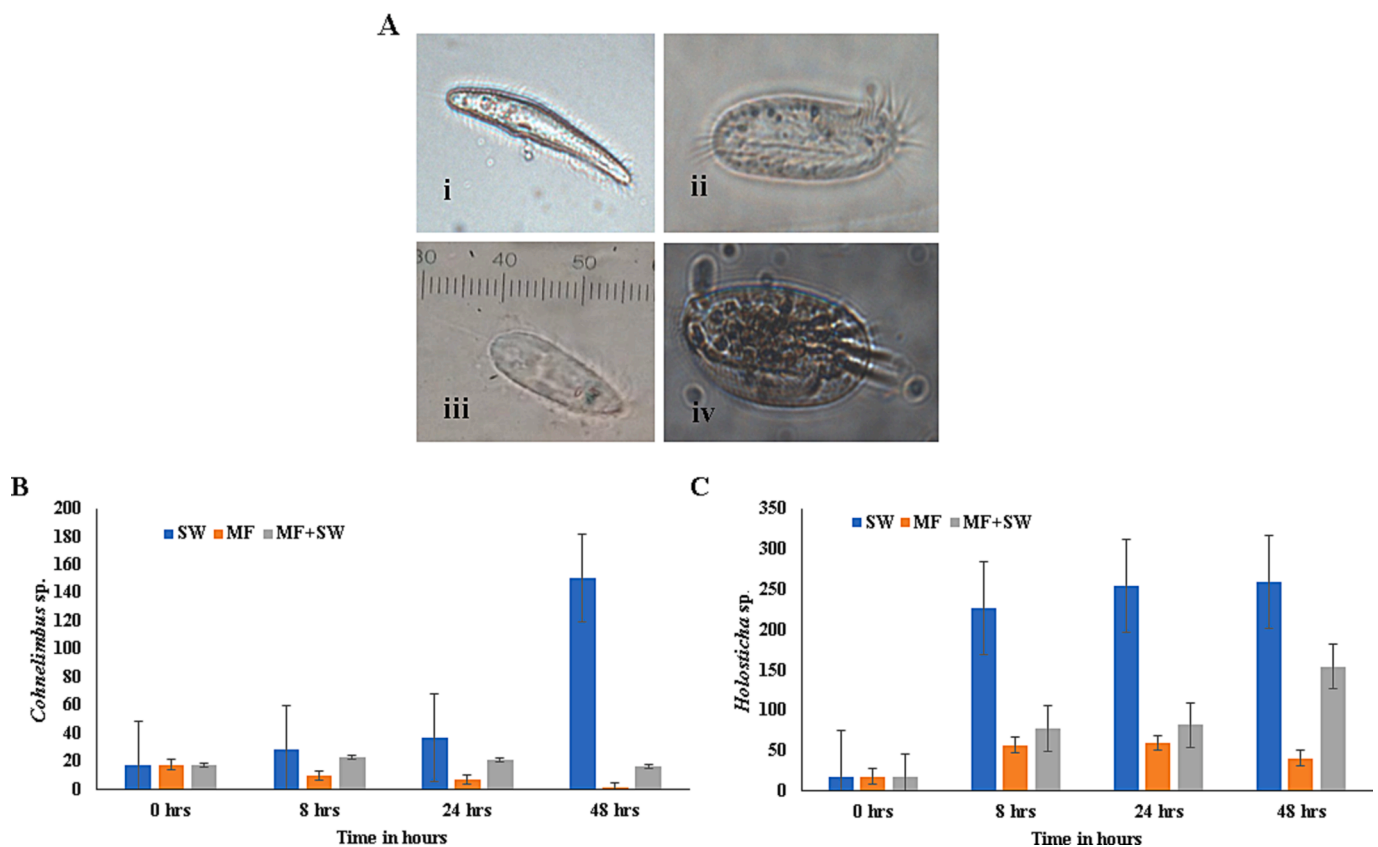


Fig. 2. Antimicrobial activity of mucus against eukaryotic protozoan ciliates. A. Ciliates associated with diseased corals i. *Cohnelimbos* sp. ii. *Holosticha* sp. iii. *Uronema* sp. iv. *Euplotes* sp. B. *Acropora* sp. mucus inhibits the growth of *Cohnelimbos* sp. C. *Acropora* sp. mucus inhibits the growth of *Holosticha* sp. (SW- Seawater, MF- mucus filter and MF + SW- mucus filter and seawater).

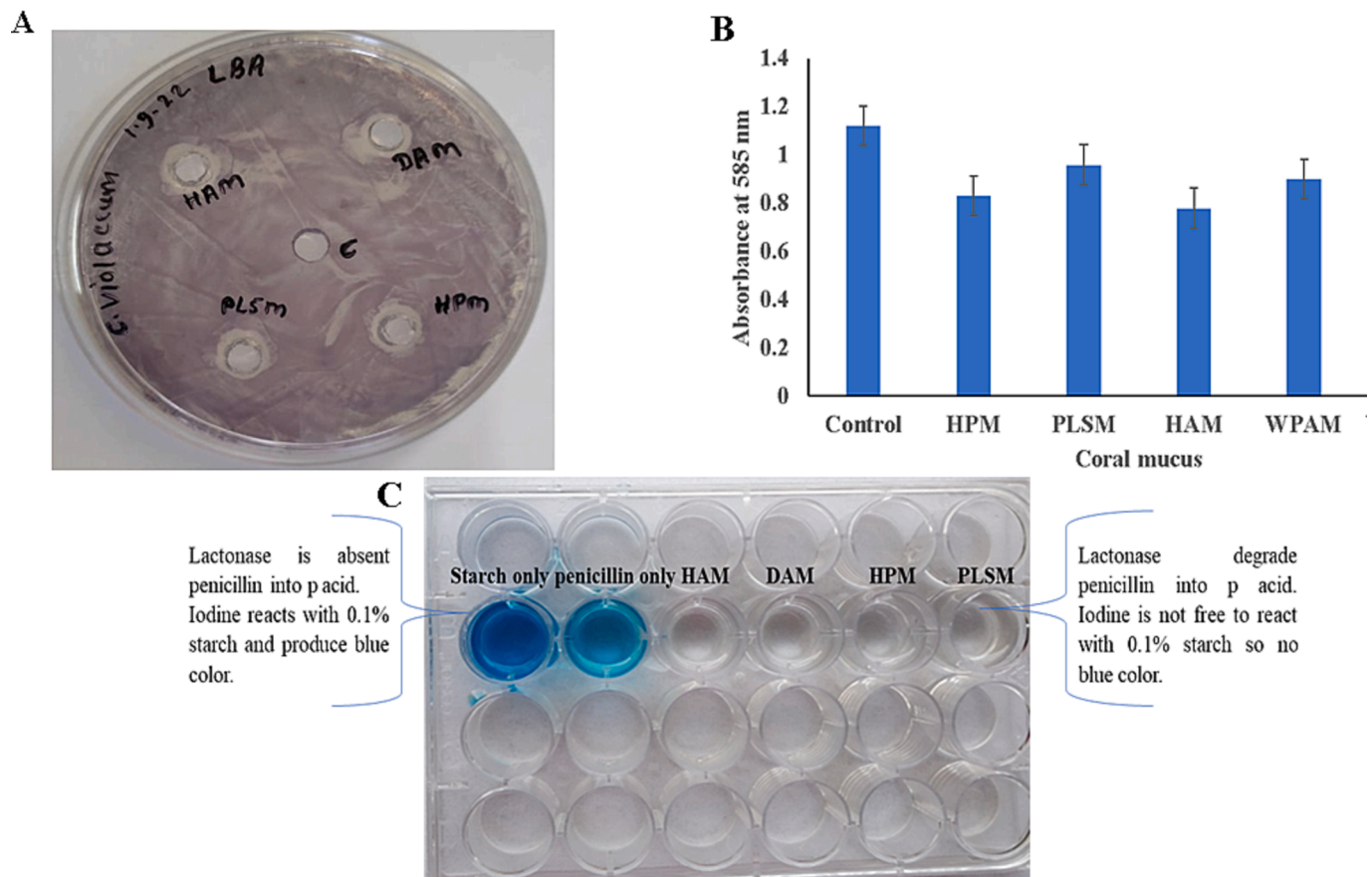


Fig. 3. AHL quorum sensing molecule inhibition assay. **a.** (i) Positive control *V. harveyi* inhibiting violet color by degrading AHL molecule of *C. violaceum*. (ii) In wells CA4- *C. amphilecti* inhibited the AHL molecule and produced a clear zone of inhibition. (iii) Healthy and diseased *Acropora* and *Porites* mucus showing zone of AHL inhibition. **b.** Iodometric assay to determine the hydrolyzing activity of mucus AHL lactonase. Wells containing positive control penicillin without test samples showing blue color formation. Healthy *Acropora*, white patch *Acropora*, healthy *Porites* and pink line syndrome disease *Porites* mucus shows no color formation. **c.** Inhibition of violacein production by coral mucus. Violacein production was measured spectrophotometrically as described in the materials and methods. Data are presented as absorbance at 585 nm. (HAM- Healthy *Acropora* sp. mucus, DAM/WPAM-White patch *Acropora* sp. mucus, HPM- Healthy *Porites* sp. mucus, PLSM- Pink line syndrome *Porites* sp. mucus). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

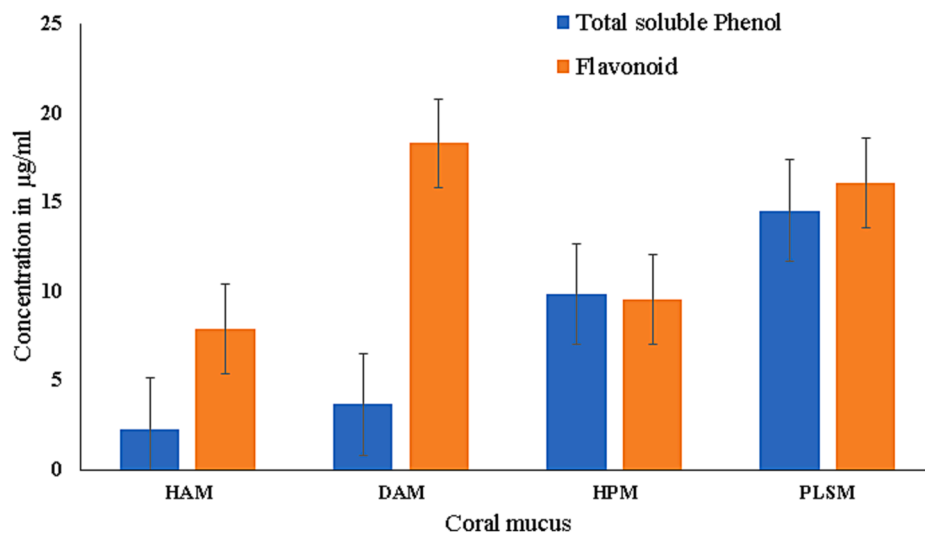


Fig. 4. Total antioxidants in coral mucus- Total soluble phenol and flavonoid concentration of coral mucus. Values are mean $\pm$ SD (n = 3). HAM- Healthy *Acropora* mucus, DAM-White patch *Acropora* sp. mucus, HPM - Healthy *Porites* mucus, PLSM- Pink line syndrome *Porites* sp. mucus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

abundance of quorum quencher molecules. Whereas less inhibition was seen in PLSM (pink line syndrome *Porites* sp. mucus) and white patch *Acropora* sp. diseased coral mucus. Control only with *C. violaceum* showed maximum production of violacein. Therefore, a fall in the violacein concentration suggests the inhibition of quorum sensing.

The healthy and diseased *Acropora* sp. and *Porites* sp. mucus were observed to be positive for lactonase enzyme presence (Fig C). The AHL lactonase of the coral mucus hydrolyzes penicillin into penicilloic acid, confirming the presence of quorum quenching molecule in coral mucus (Fig C).

3.4. Total antioxidant contents of coral mucus

The quantitative determination of total phenolic content was determined by the use of Folin-Ciocalteu reagent. Gallic acid was used as a reference compound for phenol content while the quercetin standard curve was plotted as concentration against absorbance and slope values were determined for flavonoid determination. Diseased *Acropora* sp. mucus showed significantly ($p < 0.04$) more concentration of $3.67 \mu\text{g}/\text{ml}$ of total phenol than healthy *Acropora* sp. mucus which was $2.30 \mu\text{g}/\text{ml}$. The determined total soluble phenol (Fig. 4) concentration was found to be significantly more ($p < 0.02$) in diseased *Porites* sp. mucus $14.54 \mu\text{g}/\text{ml}$ compared to the healthy *Porites* sp. mucus $9.86 \mu\text{g}/\text{ml}$. However, only diseased coral mucus was observed to significantly produce a larger repertoire of non-enzymatic antioxidant compounds as compared to healthy coral mucus as far as the levels of total soluble phenol were concerned. The determined flavonoid (Fig. 4) concentration of *Acropora* sp. mucus was significantly ($p < 0.02$) more in the

diseased mucus $18.3 \mu\text{g}/\text{ml}$ than in healthy *Acropora* sp. mucus $7.9 \mu\text{g}/\text{ml}$. The flavonoid concentration of diseased *Porites* sp. mucus was $16.09 \mu\text{g}/\text{ml}$ which was significantly ($p < 0.01$) more than healthy *Porites* sp. mucus $9.55 \mu\text{g}/\text{ml}$.

3.5. Coral's antioxidant scavenging activity

3.5.1. Hydrogen peroxide scavenging activity

Diseased mucus actively scavenged hydrogen peroxide and showed maximum activity (Fig. 5A). The healthy coral mucus of both *Acropora* sp. and *Porites* sp. coral species exhibited poor hydrogen peroxide scavenging properties than the diseased coral mucus. One-way analysis of variance (ANOVA) determined a significant increase in (more 12.75 % ($p < 0.05$)) hydrogen peroxide scavenging activity in the diseased *Porites* sp. mucus than in healthy mucus (8.91 %). *Porites* sp. mucus displayed approximately 2-fold more hydrogen peroxide scavenging than healthy *Acropora* sp. 7.65 % and diseased *Acropora* sp. 8.91 % mucus at $50 \mu\text{l}$ concentration. While the diseased *Porites* sp. mucus exhibited a maximum activity of 14.57 % at a $75 \mu\text{l}$ concentration.

3.5.2. Hydroxyl radical scavenging activity

Coral mucus hydroxyl radical scavenging capacity was analyzed and compared with standard mannitol (Fig. 5B). The hydroxyl radical scavenging activity of *Acropora* sp. mucus and *Porites* sp. mucus was subsequently found high in diseased corals. Hydroxyl radical scavenging of *Acropora* sp. diseased mucus at $75 \mu\text{l}$ concentration was statistically significant ($p < 0.03$) in diseased mucus (5.40 %) than in healthy mucus (3.85 %). Similarly, *Porites* sp. mucus also revealed statistically

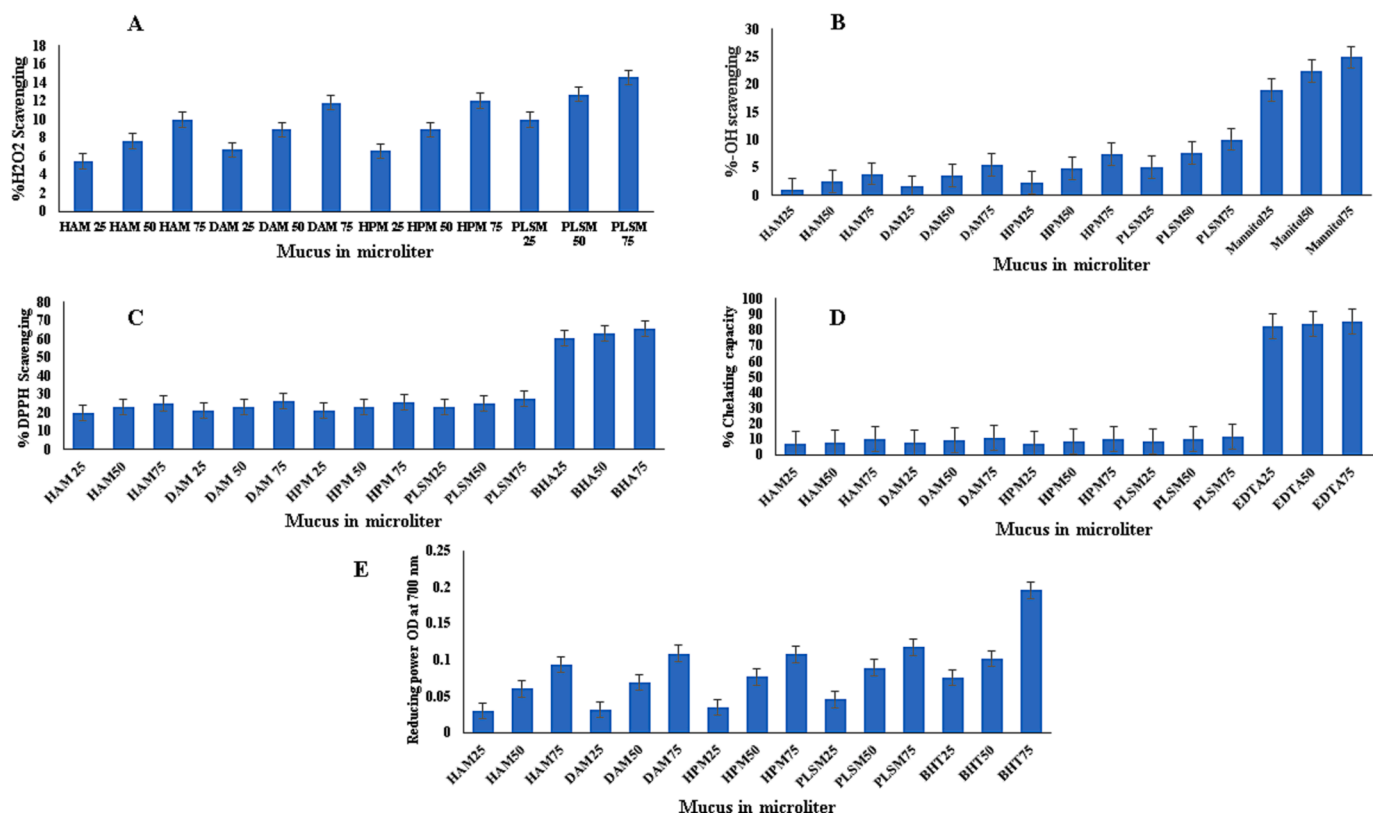


Fig. 5. Antioxidant assay of coral mucus- Antioxidant properties of diseased and healthy coral mucus. **a.** Hydrogen peroxide free radical scavenging activity at increasing concentrations of mucus. **b.** Hydroxyl radicals scavenging capacity observed in corals. Mannitol is used as a reference compound. **c.** 2,2-diphenylpicrylhydrazyl (DPPH) scavenging activity present in coral mucus. BHA- Butylated hydroxy anisole is used as standard. **d.** Metal ion chelation capacity of corals, EDTA-Ethylenediamine tetra-acetic acid. **e.** Relationship between reducing the power of coral mucus and total antioxidant. BHT -Butylated hydroxytoluene is used as a reference compound. Values are mean $\pm$ SD ($n = 3$). All the experiments were carried out in triplicates. HAM- Healthy *Acropora* mucus ($r^2 = 0.9987$, p -value = 0.02), DAM-White patch *Acropora* mucus ($r^2 = 0.9997$, p -value = 0.009), HPM- Healthy *Porites* mucus ($r^2 = 0.9926$, p -value = 0.54), PLSM- Pink line syndrome *Porites* mucus ($r^2 = 0.9849$, p -value = 0.7). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significant ($p < 0.05$) scavenging activity in diseased mucus compared to healthy mucus at all three concentrations (25 μ l, 50 μ l, and 75 μ l). The scavenging activity at 25 μ l concentration, varies from 0.98 % in healthy *Acropora* sp. mucus to 5 % in pink line syndrome affected *Porites* sp. mucus. As compared to *Acropora* sp. mucus, *Porites* sp. mucus displayed 2–2.5-fold more activity in healthy and up to 3.5-fold more activity in diseased mucus to scavenge the hydroxyl radicals.

3.5.3. DPPH scavenging activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was demonstrated based on the ability of DPPH to react with proton donors such as phenols (Fig. 5C). The DPPH scavenging activity was checked for the *Porites* sp. and *Acropora* sp. mucus and the butylated hydroxy anisole (BHA) was used as the reference compound. Wherein, the reaction mixture changed colour from purple to yellow. At 25 μ l concentration of mucus, healthy *Acropora* sp. showed (19.49 %) DPPH scavenging, while *Porites* sp. mucus displayed significance ($p < 0.03$) in diseased mucus (23.13 %) than healthy mucus (20.94 %). Similarly, at 75 μ l concentration, DPPH scavenging was found more ($p < 0.01$) in diseased *Porites* sp. mucus (27.69 %) than in healthy *Porites* sp. mucus (25.68 %). Thus, the ability of coral mucus to scavenge the DPPH radicals was observed predominantly more in diseased coral mucus.

3.5.4. Metal chelating assay

The metal ion chelation capacity of coral *Acropora* sp. and *Porites* sp. mucus was analyzed (Fig. 5D). EDTA was used as a reference compound and the metal ion chelation was observed in all the coral mucus. Diseased *Acropora* sp. mucus at 75 μ l concentration showed statistically significant ($p < 0.05$) metal chelation than *Acropora* sp. healthy mucus. *Porites* sp. diseased mucus at 25 μ l ($p < 0.02$) and at 75 μ l concentration ($p < 0.01$) showed significantly more activity (8.47 % and 10.12 %) than healthy *Porites* sp. mucus (7.4 % and 8.96 %). The capacity of mucus to capture and bind reactive metal ions was found high in *Porites* sp. diseased mucus. Thus, coral mucus interferes with the formation of the Fe 2^{+} -ferrozine complex and captures the ferrous ion before ferrozine.

3.5.5. Reducing power assay

There were significant linear correlations ($r^2 = p < 0.05$) between total antioxidants and the reducing ability of coral mucus (Fig. 5E). The potential of mucus to convert harmful reactive oxygen species into less harmful substances was analyzed at various concentrations of mucus. In

both *Porites* sp. and *Acropora* sp., the reducing power of mucus was observed high in diseased corals than in healthy ones. There was a significant difference ($p < 0.05$) between the reducing power of healthy and diseased *Acropora* sp. mucus and *Porites* sp. mucus. In diseased *Acropora* sp. mucus reducing power was 0.11 unit and whereas in *Porites* sp. diseased mucus it was found 0.12 unit which was recorded maximum in mucus.

3.6. Coral mucus antioxidant enzymes

3.6.1. Peroxidase activity

In vitro peroxidase activity of coral mucus was demonstrated spectrophotometrically and expressed as U/mg of protein (Fig. 6). The peroxidase activity was significantly higher ($p < 0.05$) in pink line syndrome-affected *Porites* sp. mucus (2.12 U/mg of protein) than in healthy *Porites* sp. (1.11 U/mg of protein). *Acropora* sp. mucus also showed significantly ($p < 0.05$) more activity in white patch diseased mucus which was higher (1.62 U/mg of protein) than healthy mucus (0.52 U/mg of protein). Among different mucus, peroxidase activity was observed more in pink line syndrome-affected *Porites* sp. Maximum activity was seen in pink line syndrome *Porites* sp. mucus as far as mucus peroxidase activity is concerned.

3.6.2. Phenol oxidase

The presence of phenol oxidase enzyme has been checked in healthy and diseased mucus (Fig. 6). One-way analysis of variance (ANOVA) demonstrated that pink line syndrome affected *Porites* sp. mucus (1.03 U/mg of protein) was statistically significantly different ($p < 0.05$) and more from healthy *Porites* sp. mucus (0.34 U/mg of protein) as far as phenol oxidase activity was concerned. Phenol oxidase activity was also observed significantly more ($p < 0.05$) in diseased *Acropora* sp. mucus (0.85 U/mg of protein) than in healthy *Acropora* sp. mucus (0.27 U/mg of protein). PO activity observed in *Porites* sp. mucus was 2-fold more than in *Acropora* sp. mucus. Phenol oxidase activity noticed was 2–3-fold higher in diseased mucus than in healthy mucus of both the coral species.

3.6.3. Superoxide dismutase activity

Acropora sp. and *Porites* sp. healthy and diseased mucus exhibited high SOD activity which was expressed in U/mg of proteins (Fig. 6). Maximum activity was observed in *Porites* sp. pink line syndrome mucus

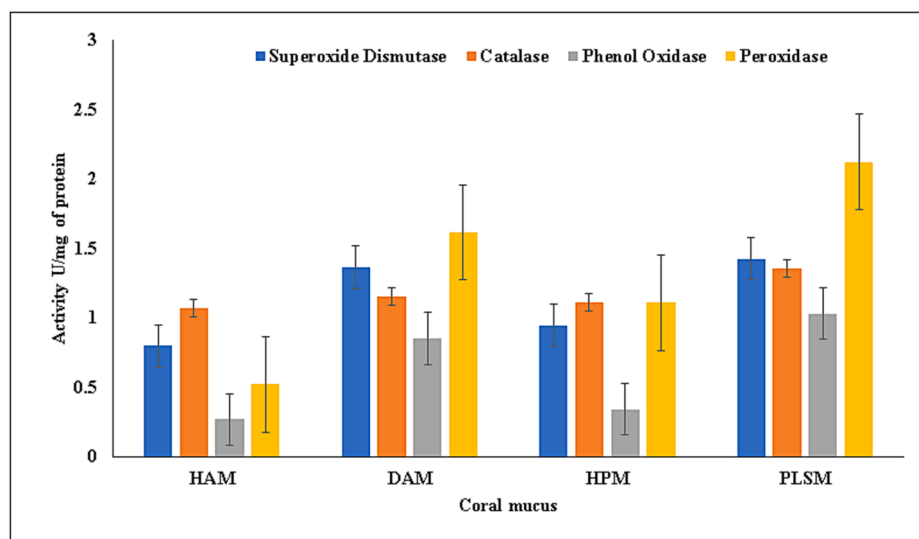


Fig. 6. Antioxidant enzyme assay of coral mucus - Superoxide dismutase, catalase, phenoloxidase, and peroxidase enzyme activity assay of coral mucus of healthy and diseased *Acropora* sp. and *Porites* sp. Data represented with three technical replicates mean $\pm$ SD ($n = 3$). HAM- Healthy *Acropora* sp. mucus, DAM- Diseased *Acropora* sp. mucus, HPM- Healthy *Porites* sp. mucus, PLSM- pink line syndrome disease *Porites* mucus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(1.43 U/mg of proteins) which was significantly more ($p < 0.05$) than the activity found in healthy *Porites* sp. mucus (0.95 U/mg of proteins). Superoxide dismutase enzyme activity levels were less when compared with that of healthy *Acropora* sp. mucus (0.80 U/mg of protein) than in diseased *Porites* sp. mucus (1.37 U/mg of proteins) which was significant ($p < 0.05$).

The superoxide dismutase enzyme activity of mucus was confirmed by developing a zymogram on electrophoresis by specific staining. SOD reacted with the reaction mixture's substrate nitro-blue tetrazolium (NBT). White-colored SOD bands had been detected on a blue background in a native-PAGE gel (Fig. 7A).

3.6.4. Catalase activity

Catalase activity of *Acropora* sp. and *Porites* sp. mucus was performed by spectrophotometric assay for quantitative analysis. The activity was 2-fold more in diseased mucus compared to healthy (Fig. 6). Catalase activity was high in diseased *Porites* sp. mucus (1.35 U/mg of protein) than in healthy *Porites* sp. mucus (1.12 U/mg of protein). Similarly, less activity was noticed in healthy *Acropora* sp. mucus (1.07 U/mg of protein) than in diseased *Acropora* sp. mucus (1.15 U/mg of protein). Maximum catalase activity was observed in pink line syndrome of *Porites* sp.

Further, the catalase enzyme activity of mucus was analysed qualitatively on native page gel by specific staining, by providing hydrogen peroxide as a substrate. Yellow coloured catalase enzyme activity bands (Fig. 7B) had formed against a blue-green background and Ferricyanide precipitation causes negative staining of the catalase enzyme. Besides, the presence of catalase in mucus was reconfirmed on the slide with the cleavage of hydrogen peroxide into water and oxygen bubbles (Fig. 7C).

3.6.5. Detection of protease in coral mucus

In this study the metalloproteases were observed in healthy and diseased mucus of *Acropora* sp., while the *Porites* sp. mucus exhibited both serine and metalloprotease (Fig. 8). The cysteine and aspartic protease were not observed in both the coral mucus (Fig. 8). The healthy and diseased mucus of *Porites* sp. exhibited the presence of metalloprotease by the inhibition activity of EDTA and O-phenanthroline, compared with the control or no inhibitor treatment. The serine protease inhibition was achieved in the presence of PMSF (Fig. 8 A & B) as an inhibitor in both the diseased and healthy mucus of *Porites* sp. Unlikely, the diseased mucus of *Acropora* sp. displayed a lower level of inhibition of metalloprotease by O-phenanthroline alone. However, the healthy mucus of *Acropora* sp. exhibited the presence of metalloprotease by both the inhibitors such as EDTA and O-phenanthroline. The presence of other proteases such as cysteine and aspartic protease inhibition was not achieved in both the coral mucus during the treatment with *N*-ethylmaleimide and Pepstatin respectively.

4. Discussion

Acropora sp. and *Porites* sp. mucus exhibited antimicrobial properties against various known coral pathogens such as *S. aureus* and *V. parahaemolyticus* (Fig. 1B). The present study shows that the coral mucus has activity not only against pathogenic bacteria but also against coral diseased lesions associated protozoan ciliates such as *Cohnelimbis* (Fig. 2B) and *Holosticha* (Fig. 2C). Thus, the antimicrobial activity suggests the possible mucus role in the first line of defense that favours the corals health and resilience. This preliminary defense property of mucus prompted us to screen the various mucus defense components that have been much studied in other organisms such as fish mucus but not in coral

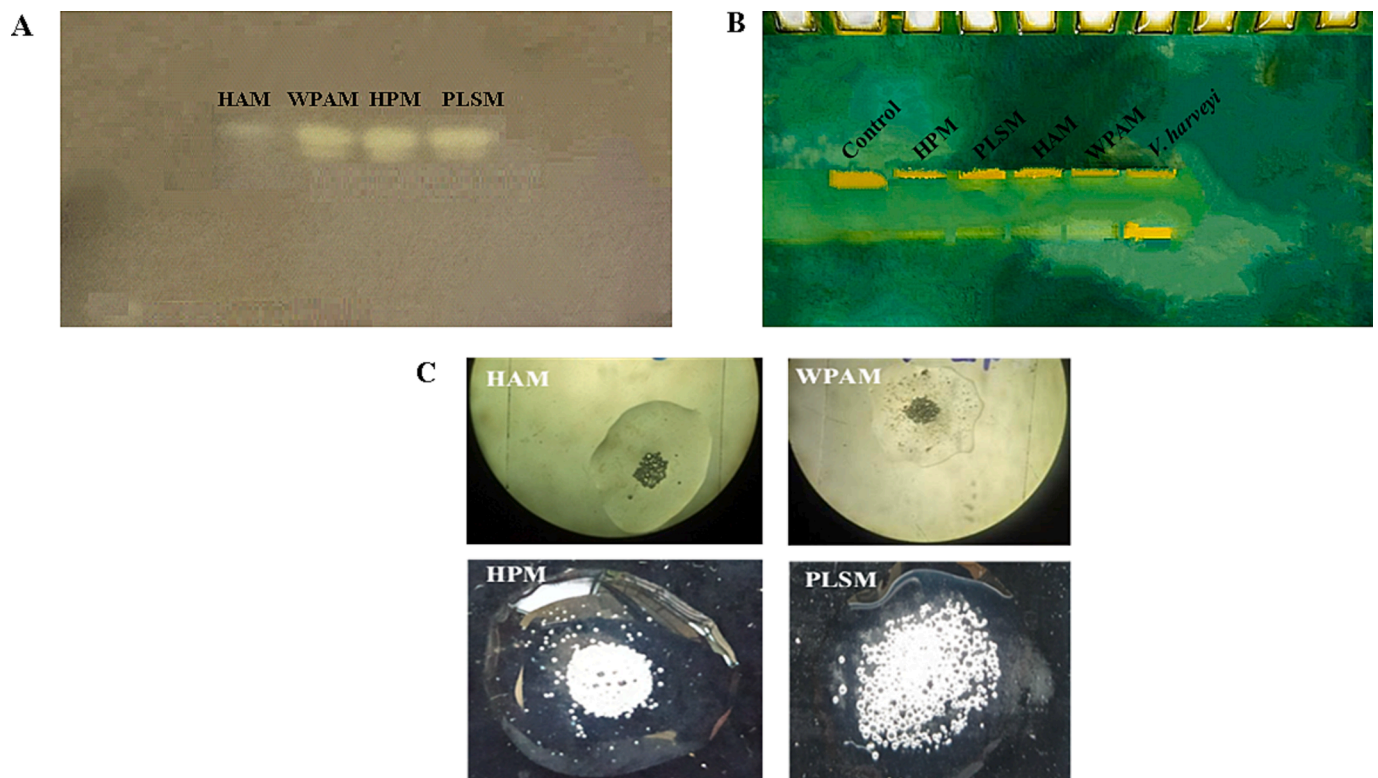


Fig. 7. Native Page gel examination of SOD and CAT – A. Superoxide dismutase enzyme activity on 10 % native-PAGE gel with an equal concentration of protein 50 μ g (well 1 to 4) HAM- healthy *Acropora* mucus, WPAM- white patch *Acropora* mucus protein, HPM- healthy *Porites* mucus, PLSM- Pink line syndrome *Porites* mucus. B. Catalase enzyme activity on 6 % native PAGE gel in well 1. control, 2. healthy *Porites* mucus, 3. pink line syndrome *Porites* mucus, 4. healthy *Acropora* mucus, 5. white patch *Acropora* mucus protein, and 6. *V. harveyi*. C. Catalase test of mucus against 3 % hydrogen peroxide. All corals mucus i.e. HAM-healthy *Acropora* mucus, WPAM-white patch disease *Acropora* mucus, HPM-healthy *Porites* mucus, and PLSM-pink line syndrome disease *Porites* mucus showing positive catalase test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

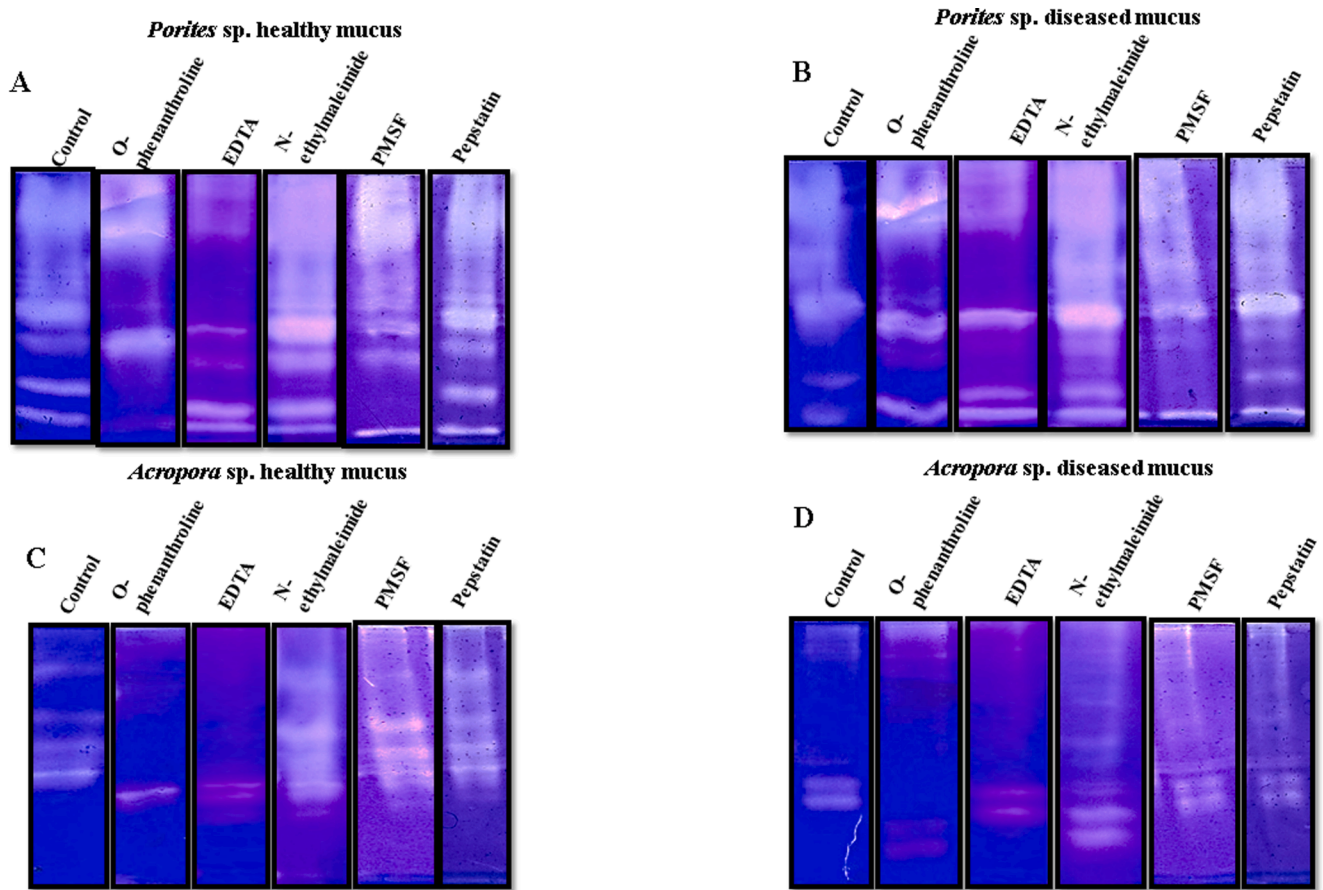


Fig. 8. Zymography to determine the presence of protease types in the mucus of *Acropora* sp. and *Porites* sp. **A-** *Porites* sp. healthy mucus with the presence of serine and metalloproteases. **B-** *Porites* sp. diseased mucus with the presence of serine and metalloproteases. **C-** *Acropora* sp. healthy mucus with the presence of metalloprotease. **D-** *Acropora* sp. diseased mucus with the presence of metalloprotease. Whereas the other proteases such as cysteine and aspartic proteases were not observed in both the healthy and diseased coral mucus. (EDTA- ethylenediamine tetra-acetic acid, PMSF- phenylmethylsulfonyl fluoride).

mucus. The results revealed the antioxidants and antioxidant enzymes of diseased coral mucus for maximum activity of free radical scavenging than the healthy mucus, which may be due to signals/factors involved in the activation of the corals natural defense signals against any biotic or abiotic stress.

In this study, the quorum quenching property of the healthy and diseased *Acropora* sp. and *Porites* sp. mucus (Fig. 3A, B, C) suggests that it may contain enzymes such as lactonase, which competitively inhibits the AHL molecule by binding with AHL receptors. Thus, the AHL lactonase enzyme in the coral mucus, which inactivates penicillin's beta-lactam ring, a member of the beta-lactamase superfamily (Huma et al., 2011), that converts penicillin into penicilloic acid was elucidated. This coral mucus was also confirmed for its inhibition of violacein pigment production, a quorum sensing molecule produced by *C. violaceum* (Fig. 3A, B), suggesting the vital role of the mucus components in inhibiting pathogens that cause infections in corals.

Acropora sp. and *Porites* sp. mucus exhibited the presence of antioxidants, which have free radical scavenging properties, reducing power, and metal ion chelating capacity. This antioxidant activity was expressed more in diseased mucus than in healthy. However, the activity levels differed in *Acropora* sp. and *Porites* sp. mucus. This variation may be due to the *Symbiodinium* type that has been associated (McGinty et al., 2012; Krueger et al., 2014; Ravindran et al., 2022). Also, excess hydrogen peroxide (H_2O_2) radicals cause the dissociation of zooxanthellae from the coral host by damaging holobionts cells or triggering cell signaling pathways of the symbiont (Weis, 2008). Hence H_2O_2 scavenging antioxidants and catalase enzymes were analysed and found to be maximum in diseased coral mucus suggesting that diseased corals

express effective defense capability to fight against H_2O_2 which helps in preventing bleaching (Fig. 5A). Hydroxyl and DPPH radicals are the major free radicals that cause lipid peroxidation and severe cell damage (Aurand et al., 1977) which affects the thylakoid membrane, phospholipids (Lesser, 1996; Tchernov et al., 2004) and the photosynthesis ability of endosymbionts. The mucus of the present study showed effective scavenging activity against DPPH and diseased mucus of *Porites* sp. was observed with maximum activity (Fig. 5C). A significant amount of reducing agents in mucus reveals that it can effectively reduce the harmful oxidants by the addition or removal of hydrogen (Fig. 5D). Activity of the chelating agent was also observed in mucus (Fig. 5E). These sequestering agents can prevent corals from oxidative damage by reacting with free unstable metal ions and reduce their harm by forming a stable compound.

The presence of free radical-scavenging antioxidant enzymes in the coral mucus was also tested. Catalase inactivates this H_2O_2 into oxygen and water. The SOD and catalase enzyme expression was observed more in diseased coral mucus as compared to healthy mucus, which might be due to the high oxidative stress in diseased mucus (Figs. 6, 7). Further, peroxidase enzymes protect the coral from the fungal pathogen, which has been reported in coral disease aspergillosis caused by *Aspergillus sydowii* (Mydlarz and Harvell, 2007). Peroxidase activity of the coral mucus was observed to be the highest in the *Porites* sp. diseased mucus (Fig. 6). Earlier findings on antioxidant enzyme cascade of the coral tissues have also reported the upregulation against ROS generation due to thermal and salt stress (Almeida and Mascio, 2011). This supports our present results on the upregulation of SOD, catalase, and peroxidase enzymes in diseased mucus (Figs. 6, 7).

The presence of phenoloxidase activity in coral mucus of the present study (Fig. 6) was also reported in some cnidarian mucus such as healthy and black band disease of *Pseudodiploria strigosa*, healthy and bleached *Cassiopea xamachana* and *Exaptasia pallida* mucus (Rivera-Ortega and Thome, 2018; Palmer et al., 2011). Further, the involvement of proteases in coral mucus is least explored and in the case of fish mucus, these proteases play a vital role in innate immune functions (Ingram, 1980). Based on the nature of the chemical catalysis group they are classified into cysteine, serine, aspartic and metalloproteases (Hartley, 1960). They are known for pathogen damage through pathogenic bacterial protein cleavage and function in indirect production and activation of antimicrobial peptides, complement and immunoglobulins (Cho et al., 2002a; Cho et al., 2002b). In this study, the coral mucus as well displayed the presence of proteases such as serine and metalloproteases in *Porites* sp. and metalloproteases in *Acropora* sp. (Fig. 8). Thus, this result was observed to be the first report in the case of coral mucus and the specific function of these proteases needs further detailed investigations.

5. Conclusion

The presence of antioxidants including phenoloxidase and other enzymes such as peroxidase, superoxide dismutase, metalloprotease, serine protease, and catalase in healthy and diseased *Acropora* sp. / *Porites* sp. mucus is first documented in this work. Quorum quenching and antibacterial activity of the coral mucus supports its potential as an antimicrobial agent against coral-associated microbial infections. The differential expression of antioxidants in healthy and diseased mucus explores the signals/factors involved in the activation of the immune signals against the invasion of pathogenic stress. Thus, the present study supports the presence of biomolecules in coral mucus involved in coral defense against various biotic and abiotic stresses. In addition, these biomolecules of the coral mucus act as biomarkers to determine the corals health status. Therefore, further study on the mechanism of synthesis, source, and involvement of these enzymes and other components in coral mucus may help in understanding the corals natural defenses against pathogenic infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Almeida, E.A., Mascio, P., 2011. Hypometabolism and antioxidant defense systems in marine invertebrates hypometabolism. In: Anna, N., Michal, C. (Eds.), *Hypometabolism: Strategies of Survival in Vertebrates and Invertebrates*. Research Signpost Kerala, India, pp. 39–55.

Anderson, M.D., Prasad, T.K., Stewart, C.R., 1995. Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. *Plant Physiol.* 109 (4), 1247–1257.

Arboleda, MDM, Reichardt, W.T., 2010. *Vibrio* sp. causing *Porites* ulcerative white spot disease. *Dis Aquat Org.* 90 (2), 93–104.

Aurand, L.W., Boone, N.H., Giddings, C.G., 1977. Superoxide and singlet oxygen in milk lipid peroxidation. *J. Dairy Sci.* 60 (3), 363–369.

Bakshani, C.R., Morales-Garcia, A.L., Althaus, M., Wilcox, M.D., Pearson, J.P., Bythell, J.C., Burgess, J.G., 2018. Evolutionary conservation of the antimicrobial function of mucus: A first defence against infection. *npj Biofilms Microbiomes* 4, 14.

Banin, E.S., Khare, S.K., Naider, F., Rosenberg, E., 2001. Proline-rich peptide from coral pathogen *Vibrio shiloi* that inhibits photosynthesis of zooxanthellae. *Appl Environ Microbiol* 67, 1536–1541.

Ben-Haim, Y., Thompson, F.L., Thompson, C.C., Cnockaert, M.C., Hoste, B., Swings, J., Rosenberg, E., 2003a. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. *Int J Syst Evol Microbiol* 53, 309–315.

Ben-Haim, Y., Zicherman-Keren, M., Rosenberg, E., 2003b. Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. *Appl. Environ. Microbiol.* 69 (7), 4236–4242.

Brown, B.E., Bythell, J.C., 2005. Perspectives on mucus secretion in reef corals. *Mar. Ecol. Prog. Ser.* 296, 291–309.

Cerenius, L., Lee, B.L., Soderhall, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol.* 29 (6), 263–271.

Cervino, J.M., Thompson, F.L., Gomez-Gil, B., Lorence, E.A., Goreau, T.J., Hayes, R.L., Winiarski-Cervino, K.B., Smith, G.W., Hughen, K., Bartels, E., 2008. The *Vibrio* core group induces yellow band disease in Caribbean and Indo-Pacific reef-building corals. *J Appl Microbiol* 105, 1658–167.

Cho, J.H., Park, I.Y., Kim, H.S., Lee, W.T., Kim, M.S., Kim, S.C., 2002a. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *FASEB J.* 16 (3), 429–431.

Cho, J.H., Park, I.Y., Kim, M.S., Kim, S.C., 2002b. Matrix metalloproteinase 2 is involved in the regulation of the antimicrobial peptide parasin I production in catfish skin mucosa. *FEBS Lett.* 531 (3), 459–463.

Choo, J.H., Rukayadi, Y., Hwang, J.K., 2006. Inhibition of bacterial quorum sensing by vanilla extract. *Lett. Appl. Microbiol.* 42 (6), 637–641.

Dinis, T.C., Madeira, V.M., Almeida, L.M., 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* 315 (1), 161–169.

Downs, C.A., Fauth, J.E., Halas, J.C., Dustan, P., Bemiss, J., Woodley, C.M., 2002. Oxidative stress and seasonal coral bleaching. *Free Radic. Biol. Med.* 33 (4), 533–543.

Fleming, A., 1922. On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc. Lond. B* 93 (653), 306–317.

Fortier, L.-C., Sekulovic, O., 2013. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence* 4 (5), 354–365.

Geffen, Y., Ron, E.Z., Rosenberg, E., 2009. Regulation of release of antibacterials from stressed scleractinian corals. *FEMS Microbiol. Lett.* 295 (1), 103–109.

Hackerott, S., Martell, H.A., Eirin-Lopez, J.M., 2021. Coral environmental memory: Causes, mechanisms, and consequences for future reefs. *Trends Ecol. Evol.* 36 (11), 1011–1023.

Hartley, B.S., 1960. Proteolytic enzymes. *Annu. Rev. Biochem.* 29 (1), 45–72.

Huma, N., Shankar, P., Kushwah, J., Bhushan, A., Joshi, J., Mukherjee, T., Raju, S., Purohit, H.J., Kalia, V.C., 2011. Diversity and polymorphism in AHL-lactonase gene (aIIA) of *Bacillus*. *Journal of Microbiol Biotechnol* 21, 1001–1011.

Ingram, G.A., 1980. Substances involved in the natural resistance of fish to infection—a review. *J. Fish Biol.* 16 (1), 23–60.

Kiran, C.R., Rao, D.B., Sirisha, N., Rao, T.R., 2012. Impact of germination on biochemical and antioxidant enzymes of *Ceiba pentandra* (Kapok) seeds. *Am. J. Plant Sci.* 03 (09), 1187–1192.

Krueger, T., Becker, S., Pontasch, S., Dove, S., Hoegh-Guldberg, O., Leggat, W., Fisher, P. L., Davy, S.K., 2014. Antioxidant plasticity and thermal sensitivity in four types of *Symbiodinium* sp. *J. Phycol.* 50 (6), 1035–1047.

Lesser, M.P., 1996. Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnol. Oceanogr.* 41 (2), 271–283.

McGinty, E.S., Pieczonka, J., Mydlarz, L.D., 2012. Variations in reactive oxygen release and antioxidant activity in multiple *Symbiodinium* types in response to elevated temperature. *Microbial Ecology* 64 (4), 1000–1007.

McLean, R.J., Pierson III, L.S., Fuqua, C., 2004. A simple screening protocol for the identification of quorum signal antagonists. *J. Microbiol. Methods* 58 (3), 351–360.

Mukherji, R., Prabhune, A., 2013. A rapid iodometric method for qualitative detection of AHL lactonases. *World J. Pharm. Res.* 3 (1), 1126–1132.

Mydlarz, L.D., Couch, C.S., Weil, E., Smith, G., Harvell, C.D., 2009. Immune defenses of healthy, bleached and diseased *Montastraea faveolata* during a natural bleaching event. *Dis. Aquat. Organ.* 87 (1–2), 67–78.

Mydlarz, L.D., Harvell, C.D., 2007. Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 146 (1), 54–62.

Mydlarz, L.D., Palmer, C.V., 2011. The presence of multiple phenoloxidases in Caribbean reef-building corals. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 159 (4), 372–378.

Palmer, C.V., Traylor-Knowles, N.G., Willis, B.L., Bythell, J.C., Roberts, J.M., 2011. Corals use similar immune cells and wound-healing processes as those of higher organisms. *PLoS One* 6 (8), e23992.

Ravindran, C., Bhagwat, P.V., Silveira, K.A., Shivaramu, M.S., Upendra, P.L., 2020. Characterization of coral associated ciliates and their interactions with disease lesion progression of Indian Scleractinian corals. *Microb. Pathog.* 149, 104472.

Ravindran, C., Lawrence, I., Vasanth, A.J., 2022. Endosymbiotic *Symbiodinium* clades occurrence and influence on coral growth and resilience during stress. *Symbiosis* 86, 261–272.

- Ravindran, C., Naveenan, T., 2011. Adaptation of marine derived fungus *Chaetomium globosum* (NIOCC 36) to alkaline stress using antioxidant properties. *Process Biochem.* 46 (4), 847–857.
- Ravindran, C., Naveenan, T., Varatharajan, G.R., Rajasabapathy, R., Meena, R.M., 2012a. Antioxidants in mangrove plants and endophytic fungal associations. *Bot. Mar.* 55 (3), 269–279.
- Ravindran, C., Raveendran, H.P., Irudayarajan, L., 2021. Ciliated protozoan occurrence and association in the pathogenesis of coral disease. *Microbial Pathogenesis* 162, 105211.
- Ravindran, C., Varatharajan, G.R., Rajasabapathy, R., Vijayakanth, S., Kumar, A.H., Meena, R.M., 2012b. A role for antioxidants in acclimation of marine derived pathogenic fungus (NIOCC 1) to salt stress. *Microb Pathog.* 53 (3–4), 68–79.
- Reshef, L., Koren, O., Loya, Y., Zilber-Rosenberg, I., Rosenberg, E., 2006. The coral probiotic hypothesis. *Environ Microbiol.* 8 (12), 2068–2073.
- Ritchie, K.B., 2006. Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar. Ecol. Prog. Ser.* 322, 1–14.
- Ritchie, K.B., Smith, G.W., 1998. Type II white-band disease. *Rev Biol Trop.* 46 (5), 199–203.
- Rivera-Ortega, J., Thome, P.E., 2018. Contrasting antibacterial capabilities of the surface mucus layer from three symbiotic Cnidarians. *Front. Mar. Sci.* 5, 92.
- Rosenberg, E., Koren, O., Reshef, L., Efrony, R., Zilber-Rosenberg, I., 2007. The role of microorganisms in coral health, disease and evolution. *Nat. Rev. Microbiol.* 5 (5), 355–362.
- Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. *Curr. Biol.* 24 (10), 453–462.
- Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot.* 2012, 217037.
- Sharma, D., Ravindran, C., 2020. Diseases and pathogens of marine invertebrate corals in Indian reefs. *J. Invertebr. Pathol.* 173, 107373.
- Subramanian, S., MacKinnon, S.L., Ross, N.W., 2007. A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Comparative Biochemistry and Physiology Part b: Biochemistry and Molecular Biology* 148 (3), 256–263.
- Sweet, M.J., Sere, M.G., 2016. Ciliate communities consistently associated with coral diseases. *J. Sea Res.* 113, 119–131.
- Tait, K., Hutchison, Z., Thompson, F.L., Munn, C.B., 2010. Quorum sensing signal production and inhibition by coral-associated *Vibrios*. *Environ. Microbiol. Rep.* 2 (1), 145–150.
- Tchernov, D., Gorbunov, M.Y., De Vargas, C., Yadav, S.N., Milligan, A.J., Häggblom, M., Falkowski, P.G., 2004. Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proc. Natl. Acad. Sci.* 101 (37), 13531–13535.
- Teai, T., Drollet, J.H., Bianchini, J.P., Cambon, A., Martin, P.M.V., 1998. Occurrence of ultraviolet radiation-absorbing mycosporine-like amino acids in coral mucus and whole corals of French Polynesia. *Mar. Freshw. Res.* 49, 127–132.
- Weis, V.M., 2008. Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. *J. Exp. Biol.* 211 (19), 3059–3066.
- Weynberg, K.D., Voolstra, C.R., Neave, M.J., Buerger, P., Van Oppen, M.J., 2015. From cholera to corals: Viruses as drivers of virulence in a major coral bacterial pathogen. *Sci. Rep.* 5 (1), 1–9.



Beneficial properties of mucus in coral adaptations and ecological interactions

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Abstract

Coral mucus secreted by mucocytes provides a protective physicochemical and physiological barrier between coral tissue and external environmental threats. The biomolecules and nutrients of the secreted mucus are derived from endosymbionts, coral polyps and support coral functions, such as feeding, sediment clearing and protection, against numerous biotic and abiotic stressors. The surface mucus layer also houses a diverse community of beneficial microorganisms that defend against pathogens. Enzymes including peptidases, esterases, and glycosidases were observed and described in mucus. Most importantly, the presence of phenoloxidase, an intracellular enzyme in secretory mucus, generally triggers melanin synthesis, providing fast-acting components of invertebrate immunity in disease resistance. However, the purpose and the mechanism of mucus release, effects of mucus components on defense properties, and functional roles in intra- and interspecific interactions are not well described. Thus, the present review aims to understand the mucus components and the complex roles played by mucus in microbial associations, feeding, and resilience that influence coral health.

Keywords Coral · Mucus · Nutrients · Environment · Pathogens · Innate immunity

Introduction

According to recent studies, 20% of coral reefs have already been irreparably damaged, and additionally 24% of the remaining reefs are facing serious threats due to anthropogenic stresses, such as overfishing and eutrophication (Sharma and Ravindran 2020; Galli et al. 2021). Biotic and abiotic stressor can cause coral bleaching, a process in which the algal symbionts are expelled from the host coral (Hafezi et al. 2020; Ravindran et al. 2022). The absence of the endosymbiont will weaken coral health, and mucus secretion will be affected (Ravindran et al. 2022). Environmental impacts, including temperature rise, on the secretion

of mucus have not been studied in detail (Vaughan et al. 2021). For example, mucus release is enhanced during high sedimentation (Reynaud and Ferrier-Pages 2019), and when corals are exposed to air at low tide (Wild et al. 2004, 2005). In addition, mucus aggregations in the water column increase sedimentation and nutrient recycling (Wild et al. 2005; Plass-Johnson et al. 2015), which control microbial activities (Bythell and Wild 2011). Therefore, a better understanding of the changes in mucus caused by biological and physical stressors may help us to monitor coral health under different environmental conditions.

Mucus is a slimy substance produced by epithelial cells called mucocytes utilizing the organic carbon synthesized by photosynthetic endosymbiotic algae (Brown and Bythell 2005; Bakshani et al. 2018). Mucus production varies between and within coral species (Coffroth 1990; Hadaidi et al. 2019). It is mainly composed of proteolytic enzymes, microbes, mucins, proteins, polysaccharides, lipids and their substructures, such as monosaccharides, amino acids and mycosporines (Brown and Bythell 2005; Stabili 2019; Vilas Bhagwat et al. 2023). The beneficial microbes within the mucus form a barrier against potential coral pathogens, competing for nutrients and space or producing antibiotics (Ritchie 2006; Krediet et al. 2013; Raina et al. 2016;

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Irudayarajan et al. 2023). Coral mucus has several important functions, including protection against pathogens and UV radiation, capturing food, acting as an energy carrier, producing antibiotics, and serving as a nutrient source for coral-associated microbial communities (Bakshani et al. 2018; Bythell and Wild 2011; Vilas Bhagwat et al. 2023). Coral mucus contains up to 1000 times more bacteria than seawater (Nakajima et al. 2015). *Vibrio shiloi*, the causative agent of bleaching in the Mediterranean coral, and *Oculina patagonica* bind to the receptors of the surface mucus layer during temperature rise (Thompson et al. 2014). However, it has been demonstrated that beneficial probiotic bacteria enable *O. patagonica* to become resistant to *V. shiloi* infection (Reshef et al. 2006). Thus, studying the components of coral mucus is of great importance for a better understanding of the entire mucosal system, its interactions with other microorganisms (commensals and pathogens), and their involvement in coral immunity and health. Further, although the composition of coral mucus has been described in some studies (Coddeville et al. 2011; Hadaidi et al. 2019), it is limited to a few coral species and the sources of mucus components in mucus are not clearly understood. Therefore, the present study aims to describe the significant role of coral mucus in maintaining the health and functionality of coral reef ecosystems, especially the synthesis and constituents of coral mucus and their critical roles in protecting coral health.

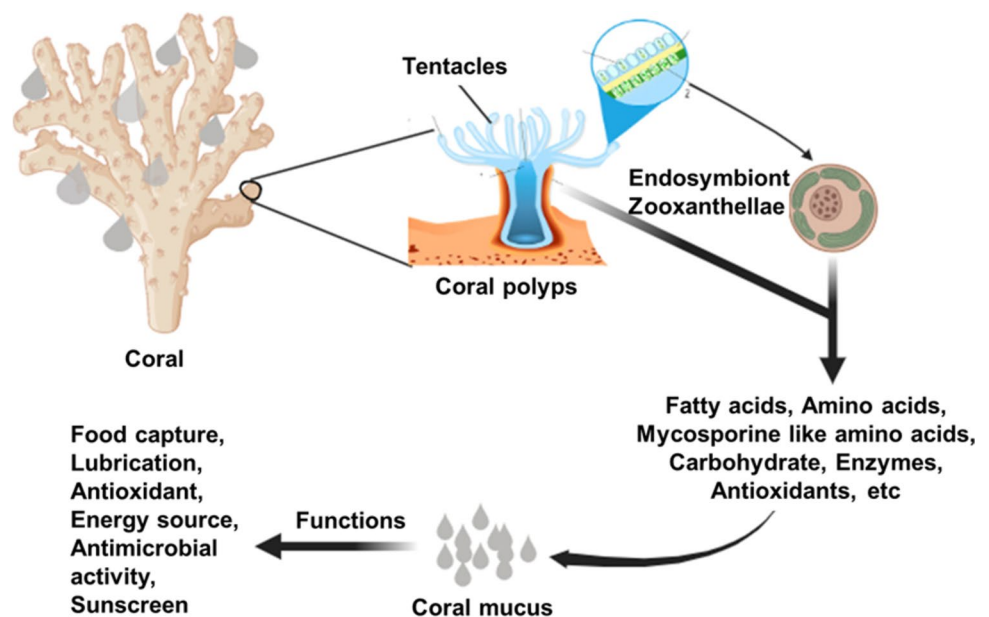
Coral mucus synthesis and secretion

The mucocyte cells of the coral polyp synthesize mucus, with the major component being carbon-based carbohydrates, and are then secreted out as a coral surface layer

(Brown and Bythell 2005). The coral-associated endosymbiotic algae are believed to provide the maximum carbon required for mucus synthesis although the source of the entire carbon requirement is not clear (Crossland 1987; Brown and Bythell 2005; Fang 2015) (Fig. 1). The other compounds required for mucus, such as amino acids, fatty acids, lipids, glycerol and nicotinamide adenine dinucleotide phosphate (NADPH), come from the coral diet (Hadaidi 2018). Only mucocytes contribute to mucus synthesis in corals, unlike in humans where multiple cell types are involved (Hadaidi et al. 2019; McShane et al. 2021). Some other gland cells are observed in coral tissues, but their roles are still unclear (Bruckner 2015). However, in vertebrates, the transformation of similar gland cells to mucus cells has been observed, which may be because these cells are either specialized secretory cells or developing mucocytes (Que 2015). The distribution of mucocytes varies in both abundance and location, not just within individual colonies but also between coral species (Baker et al. 2022). The production of coral mucus can be triggered by various factors, such as changes in light intensity, water flow, and the presence of predators or food particles (Zetsche et al. 2016). For example, an increase in the sea surface temperature increases the mucocyte density in host tissue; however, it decreases after bleaching (Piggot et al. 2009). In addition, more organic components are found in mucus from stressed than in healthy corals (Lee et al. 2016).

The synthesis of coral mucus is unique because it is derived from a significant portion of the photosynthetically fixed carbon (Hadaidi et al. 2019; Brown and Bythell 2005), and contains a high concentration of arabinose and xylose, which is not typically found in animal cells. Different coral species have varied rates of mucus production

Fig. 1 Coral mucus and properties. Coral mucus synthesis occurs in coral polyps from mucocyte cells. The mucus has a combination of nutrients provided by both the coral and endosymbiont. The released mucus acts as a first line of defense, with various properties against microbial pathogens, UV radiation and other stressors that influence the health of the corals



(Crossland et al. 1980; Kurihara et al. 2018), depending on the endosymbiont contribution of energy-rich glycerol, alanine, and glucose to the host (Burriesci et al. 2012; Gabay 2018) (Fig. 1). Excess photosynthetic carbon is eliminated by the coral through mucus release (Bakshani et al. 2018) along with lipids, such as wax esters and triglycerides for mucus synthesis (Krueger et al. 2018). *Acropora* spp. release up to 4.8 L of mucus from a 1 m² patch in one day and 56–80% of the mucus is dissolved in water (Wild et al. 2004), of which 20–40% of mucus forms strings and filaments (Stabili 2019). This mucus net filters seawater up to 100 ml cm² of tissue per hour (Lewis 1976). Subsequently, heterotrophic coral reef organisms utilize the energy from the materials trapped in the coral mucus and the energy derived from zooxanthellae (Wild et al. 2004). Invertebrate mucus protects, lubricates, and helps in locomotion, mating, homing, and foraging, performing much more diverse functions than in vertebrates (Denny 1989). In addition, mucus released on the epithelial surface aims to attract particulate matter.

Mucus composition

Mucins and the importance of mucus rheology

The glycoprotein core, mucin, is made up of 20% polymers and 80% sugars and contains cysteine, which undergoes cleavage during mucus dispersion (Bakshani et al. 2018). *O*-glycosylation of mucin occurs on oligosaccharides in the core between serine and threonine and on the *N*-acetylgalactosamine carbohydrate chains, and *N*-glycosylation occurs on asparagine (Corfield 2015). The side chains of mucins are either branched or linear and up to 20 monosaccharides long and are linked with either $\beta 1 \rightarrow 2$ or $\beta 1 \rightarrow 3$ bonds (Krediet et al. 2009). The structural diversity of mucin is due to its carbohydrate composition (Thornton and Sheehan 2004). The ability of mucin to form a gel affects mucus function depending on its extensive glycosylation and polymerization ability (Younan et al. 1982). The von Willebrand D (VWD) domain is involved in the polymerization of mucin monomers via intermolecular disulfide bonds (Ambort et al. 2012). The outer layer of the epidermis of fishes continuously produces new goblet cells because they cannot produce mucus more than once. But coral mucus goblet cells can continuously produce mucus from the same cells (Subramanian et al. 2007).

Corals require relatively fluid mucus for their ciliary feeding and to prevent the desiccation of coral polyps by air exposure during low tide (Brown and Bythell 2005; Bessell-Browne et al. 2017). Based on rheology, coral mucus is distributed as fluid mucus, string, web or flocs, and mucus sheets (Bessell-Browne et al. 2017). Distinct mucin

characteristics are present across various layers within the coral surface mucus layer (SML) (Guppy et al. 2019). The mucin concentration and gel-forming properties of mucus are necessary for its protective and lubricating functions (Thornton and Sheehan 2004). Human mucus becomes hydrated and mucus contents turn it into a gel (Piludu et al. 2003). As polymeric mucins have been detected in coral mucus, similar to vertebrates (Jatkar et al. 2010a), it is possible that coral mucus also contains different types of mucin molecules that convert it from gel to liquid and liquid to gel as needed (Horricks et al. 2019). Because coral mucus is rich in plant-associated sugar arabinose, it may have some carbohydrate chains that differ from those of mammalian mucins (Wild et al. 2005). Human mucus changes its rheology from viscoelastic gel to viscous fluid at high stress and returns to a viscous gel at low stress (Jatkar et al. 2010b) making it a lubricant and helping it move under various physiological conditions (Hill et al. 2022). A similar property may also be present in the coral SML, which remains to be investigated. However, corals can produce two types of mucus. The milked mucus has no gel-forming properties but can be converted to a gel by a higher concentration of mucin (Jatkar et al. 2010a), which is very similar to human respiratory mucus (Zetsche et al. 2016). Mucus sheets are sticky mucus layers derived from liquid mucus (Bessell-Browne et al. 2017). These are then transferred into the food web of the reef (Brown and Bythell 2005).

Carbohydrates

The relative concentration of carbohydrates varies among coral species, yet their composition remains constant, with certain sugars being shared across multiple species, and this variability is intricately linked to host phylogeny, microbial associations, and symbiont composition (Hadaidi et al. 2019). The mucosal sugars glucose, mannose, xylose, and galactose are found in all coral species that have been analyzed, whereas arabinose, *N*-acetylglucosamine, ribose, fucose, and rhamnose are present in only a few species (Table 1). Thermal stress has been shown to induce changes in both the enrichment and abundance of various carbohydrate pools, with a notable impact on galactose levels (Hillyer et al. 2017). For example, a significant decrease in glucose, galactose, and mannose was described under rising temperature-stress conditions (Lee et al. 2016). Thus, mannose, glucose, and galactose can serve as markers for temperature stress, while *N*-acetyl glucosamine exhibits anti-inflammatory properties and reduces the production of free radicals (Salvatore et al. 2000). *N*-acetyl galactosamine also helps in the *O*-glycosylation of coral mucin molecules. Xylose and arabinose are types of plant sugars commonly utilized as energy sources, indicating that they may have come from the endosymbiont (Teplitski et al. 2016).

Table 1 Carbohydrate content of various coral species mucus

S. no	Carbohydrate	Coral species	References
1	Arabinose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>Cespitulatia</i> sp., <i>Porites lobata</i> , <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Wild et al. (2005, 2010), Hadaidi et al. (2019)
2	Fucose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>Cespitulatia</i> sp., <i>Galaxea fascicularis</i> , <i>Porites lobata</i> , <i>Ctenactis</i> sp., <i>Pocillopora</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Wild et al. (2005, 2010), Lee et al. (2016), Hadaidi et al. (2019)
3	Galactose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Acropora formosa</i> , <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>Cespitulatia</i> sp., <i>Porites lobata</i> , <i>Galaxea fascicularis</i> , <i>Stylophora pistillata</i> , <i>Pocillopora verrucosa</i> , <i>Ctenactis</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Wild et al. (2005, 2010), Lee et al. (2016), Hadaidi et al. (2019)
4	Glucose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Acropora formosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>Cespitulatia</i> sp., <i>P. lobata</i> , <i>G. fascicularis</i> , <i>S. pistillata</i> , <i>P. verrucosa</i> , <i>Ctenactis</i> sp., <i>Pocillopora</i> sp., <i>Stylophora</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Wild et al. (2005, 2010), Lee et al. (2016), Hadaidi et al. (2019)
5	Mannose	<i>Acropora</i> sp., <i>Fungia</i> sp., <i>Acropora formosa</i> , <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>Cespitulatia</i> sp., <i>P. lobata</i> , <i>G. fascicularis</i> , <i>S. pistillata</i> , <i>P. verrucosa</i> , <i>Ctenactis</i> sp., <i>Pocillopora</i> sp., <i>Stylophora</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Wild et al. (2005, 2010), Lee et al. (2016), Hadaidi et al. (2019)
6	N-acetyl galactosamine	<i>Acropora formosa</i> , <i>Lemmalia</i> sp., <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Cespitulatia</i> sp.	Meikle et al. (1988), Corfield (2015), Shane et al. (2021)
7	N-acetyl glucosamine	<i>Acropora</i> sp., <i>Acropora Formosa</i> , <i>Pachyseris speciosa</i> , <i>Fungia fungites</i> , <i>Cespitulatia</i> sp., <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>A. pharaonis</i> , <i>P. lobata</i> , <i>G. fascicularis</i> , <i>S. pistillata</i> , <i>Ctenactis</i> sp., <i>Fungia</i> sp., <i>Pachyseris</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Salvatore et al. (2000), Wild et al. (2005, 2010), Hadaidi et al. (2019)
8	Ribose	<i>A. pharaonis</i> , <i>P. lobate</i>	Wild et al. (2010)
9	Rhamnose	<i>A. pharaonis</i> , <i>P. lobata</i> , <i>Acropora</i> sp., <i>Madrepora</i> sp., <i>Lophelia</i> sp.	Wild et al. (2010), Hadaidi et al. (2019)
10	Xylose	<i>Fungia fungites</i> , <i>Sarcophyton</i> sp., <i>Lemmalia</i> sp., <i>Acropora</i> sp., <i>Fungia</i> sp., <i>Lophelia</i> sp.	Meikle et al. (1988), Wild et al. (2010), Krediet et al. (2013), Hadaidi et al. (2019)

Arabinose is widespread in *Acropora* spp., suggesting that it is transferred from algal symbionts to the host (Wild et al. 2005, 2010; Lee et al. 2016).

The carbohydrate composition of mucus differs among the corals *Acropora pharaonis*, *Porites verrucosa*, and *Porites lobata* (Hadaidi et al. 2019). However, *Stylophora pistillata* and *Pocillopora verrucosa*, two pocilloporid corals, have similar carbohydrate (Neave et al. 2017) and zooxanthellae composition (LaJeunesse et al. 2018). Differences in mucus components could affect microbial community composition in coral reef habitats (Schottner et al. 2009). The most common mucus components observed in corals that occur across a large geographic range (e.g., *A. muricata*, *Pachyseris speciosa*, *Fungia fungites*, *Stylophora* sp., and *Pocillopora* sp.) are fucose, arabinose, glucose, galactose, the amino sugar *N*-acetylglucosamine, xylose, and mannose (Wild et al. 2005; Lee et al. 2016) (Table 1). Thus, mucus carbohydrates are essential substrates that support bacterial growth and contribute to the flux of dissolved organic matter (DOM) in seawater (Riemann and Azam 2002).

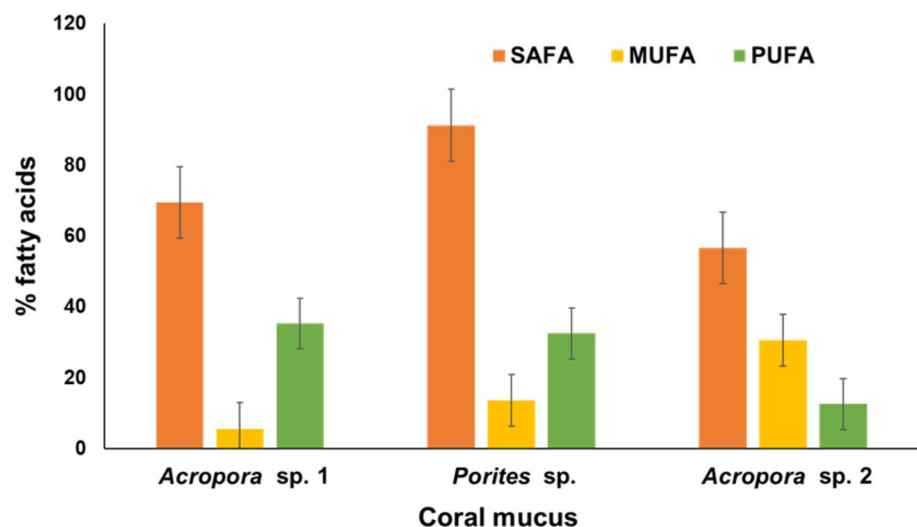
Fatty acids of mucus

The precise understanding of the fatty acid (FA) composition and its role in coral mucus is still inadequate and remains one of the least studied aspects of coral mucus. It was reported that the FA contents of *Acropora* and *Porites* species were almost the same, but the proportions were different not only in the mucus but also in comparison with the tissues (Ravindran et al. 2020). The most common and abundant FAs observed in the mucus of *Acropora* sp. in Discovery Bay, Jamaica, were palmitic acid and palmitoleic acid (Means and Sigleo 1986; Wild et al. 2005). These were also found more frequently in corals and zooxanthellae as structural and storage lipids (Patton et al. 1977; Means and

Sigleo 1986). Other FAs, such as myristic acid and linoleic acid, have also been observed in mucus, but in lower amounts (Wild et al. 2005). The most abundant saturated FAs in marine organisms are palmitic acid (C16:0), palmitoleic acid (C16:1), and myristic acid (C14:0), similar to those found in certain unicellular marine algae (Uslu et al. 2021). Even though the presence of FAs in mucus has been reported, their origins have not been documented.

FAs in coral mucus are essential for the health and functioning of coral reefs, providing energy, supporting microbial communities, and aiding in defense mechanisms (Bachok et al. 2006). Polyunsaturated fatty acids (PUFAs), especially *n*-3 and *n*-6 PUFAs, regulate metabolism, including growth, respiration, energy generation, energy reserves, membrane constituents, photosynthesis, planula larvae and egg production, biomass formation and general enzyme activity, survival, and stress resistance (Bachok et al. 2006). C20:5 n -3 FAs are essential for sperm maturation and C20:4 n -6 is essential for egg maturation, and saturated (SAFA) and monounsaturated FAs (MUFA) are stored as triglycerides and wax esters (Grottoli et al. 2004; Tchernov et al. 2004; Treignier et al. 2008). In all of the previously studied coral mucus samples, higher concentrations of SAFAs were observed (Fig. 2). In addition, FAs in corals serve as markers for bacterial colonization and diversity, coral health, and food source tracking (Volkman et al. 1980). As the synthesis of FAs in corals and in coral mucus is directly coupled to photosynthesis, there is a significant correlation between dinoflagellate biomarkers and FA concentrations (Oku et al. 2003). Free fatty acids (FFAs) have been found to be involved in maintaining cell membrane stability and cell signaling (Binienda et al. 2013). FFAs are physiologically active as dietary components and participate in cell signaling (Zbigniew et al. 2013). FAs are also required for reproduction and membrane fluidity (Lin et al.

Fig. 2 Fatty acid contents of mucus. Percent occurrence of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), and saturated fatty acids (SAFA) in coral mucus (data calculated from the means of fatty acids reported in Sigleo (1986) and Ravindran et al. (2020))



2012). However, the purpose and the function of FAs present in mucus are not well understood and need to be thoroughly investigated in various genera and species of corals based on their habitat and other environmental parameters.

Mucus lipids

Photosynthetic carbon is stored in the cytoplasm of zooxanthellae, in the form of lipids, which are then incorporated into the coral mucus, where it serves as an important source of energy for a variety of marine organisms (Crossland et al. 1980; Pasaribu et al. 2015). Many galactolipids synthesized by marine diatoms have been shown to induce apoptosis starting at micromolar concentrations in mammalian cells (Andrianasolo et al. 2008), suggesting the function of membrane lipids in regulating the symbiosis between corals and zooxanthellae. Coral mucus is composed of high-energy lipids which are also an important source of energy for fish (Wright et al. 2019). Upon thermal stress, corals may acquire more thermotolerant symbionts (Lajeunesse et al. 2010) having lipids with more SAFAs with a high melting point. This altered membrane lipid composition is observed in bleached corals, indicating differences in heat sensitivity among zooxanthellae lineages (Tchernov et al. 2004).

Zooxanthellae provides precursors and metabolites required for endogenous lipid synthesis in coral hosts mainly through photosynthesis (Oku et al. 2003; Iluz and Dubinsky 2015), and several studies have demonstrated the rapid conversion of photosynthetically fixed carbon to lipids (Radice et al. 2019). The presence of lipids (e.g., wax esters) in coral mucus has been demonstrated in significant amounts (Crossland 2021). Corals are rich in lipids, which are the biochemical link between the symbiotic mutualistic association of coral and zooxanthellae (Sikorskaya et al. 2022). Lipids are involved in coral physiological and biochemical processes and are an important source of long-term stored energy as mucus serves as an energy-rich source of nutrition for various organisms (Rodrigues et al. 2008). During bleaching, a change in FA composition and also a reduction in the rate of lipid production may affect the coral's metabolic needs (Grottoli et al. 2004). This reduced energy reserve increases disease susceptibility and mortality (Smith et al. 2020), which may be due to a change in the microbial community in the damaged tissues.

Proteins/peptides of mucus

According to Benavides et al. (2017), a low C:N ratio in coral mucus indicates the presence of a large amount of protein. Previous studies on *Palythoa* sp. and *Fungia* sp. showed that their mucus contains 8–20% organic matter and 1–2% protein (Daumas and Thomassin 1977; Stabili et al. 2015). Coral mucus from *Acropora*, *Fungia*, and *Sarcophyton*

species contains important enzymes, such as peptidases, esterases, and glycosidases (Vacelet and Thomassin 1991; Vilas Bhagwat et al. 2023). Hydroxyproline, found in coral mucus, is crucial for collagen synthesis and maintaining the thermodynamic stability of collagen's triple-helical conformation and associated tissues (Ortega et al. 2018). Fish mucus, on the other hand, is known for its high protein content and enzymes, such as lysozyme, alkaline phosphatase, complement, cathepsin B, transferrin, and C-reactive protein (Subramanian et al. 2007; Zhao et al. 2008). The mucus of the zoanthid *Palythoa caribaeorum* contains lectins and proteases (Guarnieri et al. 2018). However, phenoloxidase (PO), peroxidases (POD), and antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase, which have been reported from coral tissues (Anithajothi et al. 2014), have not yet been reported in coral mucus. Thus, mucus reported with antimicrobial properties has to be investigated for its potential peptides and proteins.

Amino acids

Corals and zooxanthellae can synthesize 20 different amino acids, with some produced by the coral polyps, others by the zooxanthellae, and yet others by both partners. However, different coral species have different amino acid synthesis abilities (Fitzgerald and Szmant 1997). Amino acids can also be taken up via plankton, detritus, and the water column. The acquired amino acids are used by the coral to synthesize proteins for tissue growth, calcification, and other processes (Grover et al. 2008; Shinzato et al. 2014). The production of methionine could be a result of the synergistic action of enzymes from both coral and zooxanthellae, indicating that these symbiotic partners have evolved to mutually benefit each other (Shinzato et al. 2014). Although corals acquire many amino acids, only 16 have been observed in the mucus, including aspartate, glutamine, serine, glycine, threonine, arginine, alanine, tyrosine, valine, phenylalanine, isoleucine, leucine, lysine, histidine and methionine, of several coral species (i.e., *Acropora formosa*, *Pachyseris speciosa*, *Fungia fungites*, *Sarcophyton* sp., *Lemnalia* sp., *Cespitularia* sp., *Platygyra lamellina*, *Heteroxenia fuscensens*, *Oculina arbuscula* (Meikle et al. 1988; Ducklow and Mitchell 1979; Means and Sigleo 1986; Coddeville et al. 2011).

Analysis of mucus produced by *Palythoa* and *Fungia* species has shown that the amino acids serine, aspartic acid, glutamic acid, and glycine are present in high abundance, and these play important roles in a variety of biological processes, including protein synthesis, cellular signaling, and energy metabolism (Daumas and Thomassin 1977). Glycine is the most abundant amino acid found in both coral tissues and mucus, particularly in species such as *Montastraea cavernosa* (Daumas and Thomassin 1977). Glutamic acid, which also acts as an antioxidant in many plants, animals,

and bacteria, was more abundant in the mucus of *Fungia* sp., *Acropora* sp., *Platygyra* sp., *Sarcophyton* sp., and *Heteroxenia* sp., compared to serine, and methionine (Ducklow and Mitchell 1979). Alanine is generally observed in *Porites divaricata*, *Acropora cervicornis*, *Orbicella faveolata*, and the azooxanthellate corals, *Astrangia poculata* and *Tubastrea coccinea* (Fitzgerald and Szmant 1997). Similarly, *Fungia scutaria* was reported to utilize tyrosine, lysine, glycine and aspartic acid, from surrounding water (Stephens 1962). This suggests that corals acquire some amino acids through heterotrophic feeding from the surrounding water. In addition, both host and zooxanthellae can synthesize amino acids and this varies from species to species.

Mucus antioxidants against oxidative stress

A decrease in zooxanthellae density is due to oxidative stress causing coral bleaching (Downs et al. 2002; Weis 2008). Primarily, oxidative stress is generated during photosynthesis (Krieger-Liszka 2005). Excessive production of reactive oxygen species (ROS) causes disorganization and rupture of the thylakoid membrane (Tchernov et al. 2004). Free radicals, such as hydrogen peroxide (H₂O₂) and hydroxyl radicals (–OH), degrade mucus glycoproteins at the histidine molecule, so that mucus property, molecular weight, and viscosity is affected (Dailah 2022). ROS imparts an adverse impact on the mucus defense properties which are then controlled by the mucus antioxidants (Suggett et al. 2008).

The presence of phenoloxidase (PO), an enzyme that activates the invertebrate defense system, was reported in coral secretory mucus (Rivera-Ortega and Thome 2018; Vilas Bhagwat et al. 2023). The mechanism of secretion of this enzyme into coral mucus is not known. However, the expulsion of similar molecules from tissue to the mucus was reported previously (Ritchie 2006). Thus, phenoloxidase is thought to be an intracellular enzyme, but similar activity was also observed in the mucus of other organisms, such as in lancelets (Zhang and Li 2000) and some insect cuticles (Arakane et al. 2016). The pro-phenoloxidase (proPO) activates PO, resulting in the synthesis of melanin which in turn activates innate immune responses. The proPO system itself is activated by tyrosine and phenylalanine, which is synthesized by both the coral host as well as zooxanthellae (Fitzgerald and Szmant 1997). To reduce the impact of toxic by-products, such as reactive oxygen (RO) and other free radicals, antioxidant enzymes are up-regulated to offset self-harm (Gardner et al. 2016). However, the source and the presence of such antioxidant enzymes and non-enzymatic antioxidants in coral mucus are not well studied (Vilas Bhagwat et al. 2023). Some enzymes, such as superoxide dismutase and glutathione peroxidase, have been reported as stress markers in corals (Gardner et al. 2016). Elevated PO activity triggers melanin production, serving as a signal for

corals to react to pathogens, and concurrently functioning as a potent antioxidant against UV radiation (Palmer et al. 2011). The antioxidant capabilities of mucus against oxidative stress may determine the survival of the corals against bleaching and other stressors.

Antimicrobial activity of mucus and mucus-associated microbes

Coral mucus from different habitats is composed of unique components that alter the presence of specific microbes on the surface of different coral species (Guppy and Bythell 2006; McShane et al. 2021). The natural flora of healthy coral is presumably beneficial and any shift in this bacterial population may be an early indicator of coral stress, such as disease (Nithyanand et al. 2011). Therefore, the SML of coral is a selective medium determined by host–microbe–environment interactions, where the commensal microbiota act against potential pathogens (Ritchie 2006; Alagely et al. 2011; Krediet et al. 2013; Teplitski et al. 2016; Irudayarajan et al. 2023). The microbial gene abundance in the coral mucus is known to be involved in stress resistance, virulence, chemotaxis and motility, utilization of FAs and lipids, sulfur and nitrogen metabolism, and secondary metabolism making the mucus-associated bacterium a first line of defense (Bythell and Wild 2011; Irudayarajan et al. 2023). Thus, some microbial communities in the SML are described as producing defensive antimicrobial chemicals (Orland and Kushmaro 2009; Irudayarajan et al. 2023). The Caribbean coral *Acropora palmata* demonstrated with antibacterial inhibition against the pathogen that causes white pox disease (Nissimov et al. 2009). Similarly, antibacterial activity was found in many cultivable bacteria of the coral mucus of six scleractinian corals from the Gulf of Eilat (Orland and Kushmaro 2009). Varied bacterial genera of both tissue and mucus of *A. digitifera*, including *Vibrio* sp. and *Bacillus* sp. have been elucidated for their antibacterial activity. *Bacillus* sp. synthesizes a significant peptide group of antibiotics including gramicidin, bacitracin and polymyxin B (Wiese et al. 2009). Thus, the highly specific mucus bacterial community is crucial for first line of defense of corals against diseases and other environmental stress factors (Kemp et al. 2015). Moreover, specific bacteriophages of the coral SML have been described to treat various bacterial infections as phage therapy (Nguyen et al. 2015; van Oppen 2019). The SML antimicrobial activity exhibited species specificity, while the mucus-associated beneficial bacteria, such as *Pseudoalteromonas* sp. and *Roseobacter* sp., have been described for their antibacterial activity against a number of coral pathogens, including *V. shiloi* (Ortega and Thome 2018). As a result, beneficial microorganisms of corals (BMCs) are used as probiotic drugs in order to

increase coral resistance to environmental stress, and the rotifers, *Brachionus plicatilis*, are employed as carriers to impart BMCs into the coral *Pocillopora damicornis* (Assis et al. 2020).

The biochemicals present in the surface mucus of *Acropora palmata* and *Pseudodiploria strigosa* are responsible for regulating the coral-associated microbial community (Rivera-Ortega et al. 2018). Studies have shown that the mucus of *A. palmata* suppresses the growth of various bacterial strains, including *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, and the white pox disease causative agent, *Serratia marcescens* (Ritchie 2006; Vilas Bhagwat et al. 2023). The mucus also exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria (Ritchie 2006; Vilas Bhagwat et al. 2023). Similarly, healthy coral mucus obtained from *P. strigosa* has been found to possess antimicrobial activity against *Serratia marcescens* and *Aurantimonas* sp. pathogens (Ortega and Thome 2018). In addition to its antimicrobial properties, *Galaxea fascicularis* mucus has been found to have potential health benefits for humans, such as apoptotic and anti-cancer activity, DNase activity, and activity against Topoisomerase I and II enzymes (Ding et al. 1999; Khalesi 2015). Homarine, an organic compound with antibiotic potential, has been documented in corals for its ability to potentially inhibit the growth of a range of bacteria and fouling diatoms (Maldonado et al. 2016). Fish mucus contains a variety of components, including disulfides, glycoconjugates, antimicrobial peptides (AMPs), lysozyme, cathepsin B, complement, alkaline phosphatase, C-reactive protein, transferrin, and others, which act as effective protection (Meyer et al. 2007; Subramanian et al. 2007; Zhao et al. 2008). AMPs in fish mucus are effective antimicrobial agents against human pathogens, including algae, bacteria, fungi, viruses, or parasites (Komatsu et al. 2009; Fernandes et al. 2010). Although the antimicrobial properties of coral mucus and associated bacteria have been mentioned in numerous papers, the specific components responsible for these properties are not well documented (Wright et al. 2019; Wijayanti et al. 2020), and further research is needed to better understand the immunological properties of coral mucus and the mechanisms by which it provides protection.

Mucus as a sunscreen and effective antioxidant under heat stress

Mycosporine-like amino acids (MAAs) are responsible for shielding corals from the harmful effects of solar ultraviolet (UV) radiation by absorbing approximately 10% of UV radiation. This mechanism is critical for the survival of corals, as exposure to high levels of UV radiation can cause damage to coral DNA, resulting in a range of negative impacts

on coral health and the overall health of the surrounding ecosystem. By providing this important protection against UV radiation, MAAs play a key role in maintaining the health and resilience of coral populations (Teai et al. 1998; Kageyama and Waditee-Sirisattha 2019). Mycosporine-glycine, mycosporine-2glycine, palythine, mycosporine-methylamine-serine, and mycosporine-methylamine-threonine, which were characterized from *Lobophyllia*, *Acropora*, *Fungia*, and *Pocillopora* species, have all been reported from coral mucus (Teai et al. 1998). In addition to their ability to combat oxidative stress, MAAs can protect many marine organisms from the harmful effects of light (Shick and Dunlap 2002). Similarly, mycosporine-glycine showed an additional antioxidant property in coral mucus along with sunscreen, probably protecting endosymbionts from oxidative damage produced through photosynthesis (Teai et al. 1998). Biologically harmful UV rays between 310 and 360 nm are absorbed by MAAs. Endosymbionts and the coral hosts are both protected from the harmful effects of UV radiation by mucus MAAs. It was reported that the UV-absorbing molecules in the coral mucus of *Fungia fungites* reduced with depth (Drollet et al. 1997). The reduction in UV radiation, light, and photosynthesis may be attributed to increased depth, as water absorbs and scatters UV radiation more effectively with increasing depth, leading to a decrease in UV radiation intensity.

Mycosporine-like amino acid concentrations vary depending on coral species and habitat. They have been found in both host tissues as well as in zooxanthellae (Shick and Dunlap 2002). MAAs are thermally stable, making them useful for scavenging reactive oxygen species under heat stress (Banaszak and Lesser 2009). MAAs play a role in cellular osmoregulation during salinity stress or in a high-salinity environment (Oren and Gunde-Cimerman 2007). The combination of mycosporine and glycine, the most common MAAs in coral hosts, protects the coral symbiosis from oxidative damage (Banaszak et al. 2006). MAA also serves as a nitrogen source within the cell, which is required for growth and proliferation (Oren and Gunde-Cimerman 2007).

Mucus as a source of nutrients for marine organisms

Mucus produced by coral is a valuable energy source with high glucose content. As glucose is readily available, it is a highly preferred energy source for microbes and other organisms (Ravindran et al. 2023). Thus, coral mucus is an important component of marine ecosystems, providing a rich source of energy for a wide group of organisms (Wild et al. 2010) (Table 2). Mucus energy levels are far greater than benthic photosynthetic algae. Further, a few soft corals have also been shown to feed on the mucus of other

Table 2 C/N ratio in fresh coral mucus

Hard corals	C/N ratio	References
<i>Acropora</i> sp.	8.5	Coles and Strathmann 1973
<i>Acropora formosagrandis</i>	5.9	Vacelet and Thomassin 1991
<i>Fungia scutaria</i>	7.5	Coles and Strathmann 1973
<i>Fungia scutaria</i>	6.9	Krupp 1982
<i>Fungia echinata</i>	5.6	Vacelet and Thomassin 1991
<i>Porites compressa</i>	6.0	Coles and Strathmann 1973
<i>Goniopora lobata</i>	5.9	Coles and Strathmann 1973
<i>Platygyra lamellina</i>	6.6	Pascal 1981
<i>P. astreoides</i>	5.9	Coffroth 1990
<i>P. furcata</i>	4.1	Coffroth 1990
<i>P. australiensis</i>	5.9	Coffroth 1990
<i>P. lutea</i>	5.9	Coffroth 1990
<i>P. lobata</i>	5	Coffroth 1990
<i>P. murrayensis</i>	5.5	Coffroth 1990
<i>Porites</i> sp.	4.8	Coffroth 1990
Soft corals		
<i>Heteroxenia fuscescens</i>	7.3	Pascal 1981
<i>Sarcophyton</i> cf. <i>glaucum</i>	5.6	Vacelet and Thomassin 1991
<i>Briarium asbestinum</i>	8.9	Pascal 1981

corals (Coffroth 1984). Similarly, fishes, such as blue sprat (Eckes et al. 2015), damselfishes (Coles and Strathmann 1973; McMohan et al. 2016), and other benthic organisms, such as an Acoelomorpha worm (*Waminoa* sp.), bivalves (Shafir and Loya 1983), shrimp (*Coralliocaris superba*) (Horka et al. 2016), coral crabs (*Trapezia* sp.) (Shmuel et al. 2022) and some planktonic organisms, such as crown-of-thorns starfish larvae (Nakajima et al. 2016), copepods (*Acartia negligence*) (Richman 1975), and mysids, (*Mysidium integrum*) (Gottfried and Roman 1983) also exhibit feeding on coral mucus. Coral mucus was found to be the best nutritional source with an C:N ratio of mucus fluid of 6.9 to 13.7 and mucus sheet of 4.8 to 5.9 (Coffroth 1990) (Table 2). Since mucus sheets that stay on the coral surface can trap organic materials, their nutritional value is higher than reef benthic algae (~ 1) and reef algal detritus (~ 1) (Wilson et al. 2003). When compared with the other forms of energy sources, reef fish feces contribute to a nutritional value of ~ 10 (Bailey and Robertson 1982), phytoplankton ~ 3.5 (Renaud et al. 1999), and zooplankton ~ 5.5 (Ventura 2006). Furthermore, coral mucus acts as a vehicle for nutrients to reach the mouth (Goldberg 2018). Small reef fish and copepods utilize wax esters and triglycerides as energy sources, both of which are major components of mucus (Ho 2017). Thus, coral mucus helps in the fulfillment of energy requirements as well as the cycling of nutrients (Wild et al. 2004).

Role of mucus in reef ecosystem nutrient cycling

When coral mucus is released into the water column, it is rapidly colonized by microorganisms that break down the organic compounds and release nutrients, such as nitrogen (N_2) and phosphorus (Van Oppen et al. 2019). These nutrients are then available for uptake by other organisms in the ecosystem, such as phytoplankton and zooplankton. Corals obtain N_2 through predation and from the water column and excrete ammonia (Fig. 3) (Siboni et al. 2008). Further, Archaea use this ammonia, which plays an important role in ocean nitrification. During daytime, when oxygen levels are high due to photosynthesis, certain types of Archaea convert ammonia into nitrite. Conversely, at night, when oxygen is low, different types of Archaea carry out denitrification to convert nitrite into nitrogen (Siboni et al. 2008, 2012). Nitrogen-cycling microbes play a vital role in coral–algae symbiosis, especially under nutrient-rich or -poor conditions for coral productivity (Radecker et al. 2015). Coral mucus contains high concentrations of dissolved organic nitrogen (DON), which can be readily taken up by bacteria for growth and metabolism. Some of these bacteria form symbiotic relationships with coral polyps, where they fix atmospheric nitrogen (N_2) (Diaz 2017). Other bacteria in the mucus help us to recycle nitrogen within the holobiont by breaking down waste products and releasing ammonium (NH_4^+) and nitrate (NO_3^-) into the surrounding water. These forms of nitrogen can then be taken up by other organisms in the reef ecosystem (Wild et al. 2004).

Up to 80% of mucus released by corals becomes dissolved organic carbon (DOC) (Wild et al. 2004). Sponges transfer dissolved organic matter (DOM) including DOC and DON to the next trophic level (Rix et al. 2017). Bacteria utilize mucus and break down organic compounds and release carbon dioxide (CO_2) through respiration. This CO_2 can then be taken up by photosynthetic organisms, such as algae and seagrasses, which use it to produce organic carbon through photosynthesis (Haas et al. 2016). This process helps us to maintain the balance of carbon in the reef ecosystem. Coral mucus also contains significant amounts of dissolved organic phosphorus (DOP), which can be taken up by bacteria and recycled within the holobiont (Nakajima et al. 2015). Some of these bacteria are capable of breaking down complex organic phosphorus compounds, such as phospholipids, to release inorganic phosphorus (PO_4^{3-}) into the surrounding water (Bourne et al. 2013). This inorganic phosphorus can then be taken up by algae and other organisms in the reef ecosystem. Mucus functions as nutrient trapping, thus, higher ammonia, nitrate, and phosphate in mucus than in surrounding water suggest its role in nutrient adsorption. In addition, coral mucus transports nutrients

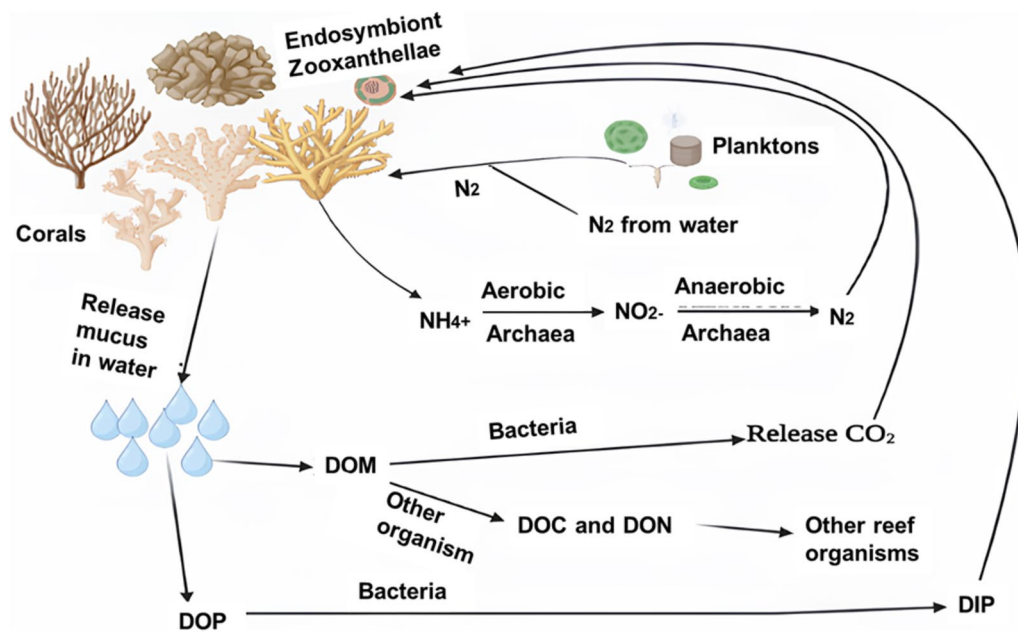


Fig. 3 Coral mucus in reef nutrient cycling. Corals release mucus rich in dissolved organic matter (DOM) to the surrounding water, which is utilized by reef organisms whose respiration returns carbon dioxide and other nutrients required for photosynthesis by the endosymbiont zooxanthellae. Also, endosymbiont derived organic com-

pounds are released into the mucus and nutrient cycling is activated through various microbes. (DOM—dissolved organic matter, DON—dissolved organic nitrogen, DIP—dissolved inorganic phosphate, DOC—dissolved organic carbon, DOP—dissolved organic phosphate, NH₄—Ammonia, NO₂—nitrite, N₂—nitrogen)

into reef sediment (Wild et al. 2005). Overall, coral mucus plays a crucial role in maintaining the health and productivity of reef ecosystems by facilitating nutrient cycling and supporting the diverse array of organisms that inhabit these ecosystems. Recycling these nutrients within the holobiont and the surrounding water helps us to maintain a balance of nutrients and supports the ecosystem's productivity. Additionally, the role of coral mucus in nutrient cycling highlights the importance of conserving coral reefs and protecting them from human activities that may disrupt this delicate balance.

Conclusion

The coral surface mucus serves as an interface between the coral epithelium and the seawater environment. It provides a primary line of defense and protects coral surfaces from a variety of physio-chemical and microbial invasions. It acts as a high-energy substrate for bacterial heterotrophic activity. As both host and endosymbiont provide mucus constituents, this symbiotic mucus synthesis is different from many other marine organisms. The exact mechanism of mucus synthesis, secretion, and sources of other important components present in mucus is unknown, and less information is available about the properties and rheological changes of

mucus, which are not fully demonstrated. Only certain coral species have been studied for their mucus components, such as carbohydrates, fatty acids, amino acids, proteins, antioxidants, and antimicrobial compounds, but even among those, not all types have been thoroughly explored. Also, mucus defense properties, including antimicrobial peptides, antioxidants, and UV protectants (e.g., Mycosporine-like amino acids), and beneficial microbes, are not well described for their roles in supporting coral health in various stress conditions. Coral mucus serves multiple ecological roles with their associated microorganisms and the surrounding environment that need to be studied to understand reef ecosystem functioning. Thus, the present review aids in understanding the role of coral mucus in coral reef ecosystems; however, more detailed studies of coral mucus components and their roles in the coral reef ecosystem by developing appropriate biochemical and molecular approaches may provide insights into protecting and restoring these valuable ecosystems.

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Author contributions PVB wrote the original draft, performed data analysis. CR Conceptualization, fund acquisition, writing and editing the original draft, performed data analysis. LI wrote the original draft, performed data analysis. All authors revised and approved the final manuscript.

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Declarations

Conflict of interest The authors have no conflict of interests.

Ethical standards Not applicable.

References

- Alagely A, Krediet CJ, Ritchie KB, Teplitski M (2011) Signaling-mediated cross-talk modulates swarming and biofilm formation in a coral pathogen *Serratia marcescens*. ISME J 5(10):1609–1620
- Ambort D, Johansson ME, Gustafsson JK, Ermund A, Hansson GC (2012) Perspectives on mucus properties and formation—lessons from the biochemical world. Cold Spring Harb Perspect Med 2(11):014159
- Andrianasolo EH, Haramaty L, Vardi A, White E, Lutz R, Falkowski P (2008) Apoptosis-inducing galactolipids from a cultured marine diatom, *Phaeodactylum tricornutum*. J Nat Prod 71(7):1197–1201
- Anithajothi R, Duraikannu K, Umagowsalya G, Ramakritinan CM (2014) The presence of biomarker enzymes of selected scleractinian corals of Palk Bay, southeast coast of India. Biomed Res Int 2014:684874
- Arakane Y, Noh MY, Asano T, Kramer KJ (2016) Tyrosine metabolism for insect cuticle pigmentation and sclerotization. In: Cohen E, Moussian B (eds) Extracellular composite matrices in arthropods. Springer, Berlin, pp 165–220
- Assis JM, Abreu F, Villela HM, Barno A, Valle RF, Vieira R, Taveira I, Duarte G, Bourne DG, Høj L, Peixoto RS (2020) Delivering beneficial microorganisms for corals: rotifers as carriers of probiotic bacteria. Front Microbiol 15(11):608506
- Bachok Z, Mfilinge P, Tsuchiya M (2006) Characterization of fatty acid composition in healthy and bleached corals from Okinawa. Japan Coral Reefs 25(4):545–554
- Bailey TG, Robertson DR (1982) Organic and caloric levels of fish feces relative to its consumption by coprophagous reef fishes. Mar Biol 69(1):45–50
- Baker LJ, Reich HG, Kitchen SA, Grace Klings J, Koch HR, Baums IB, Muller EM, Thurber RV (2022) The coral symbiont *Candidatus Aquarickettsia* is variably abundant in threatened Caribbean acroporids and transmitted horizontally. ISME J 16(2):400–411
- Bakshani CR, Morales-Garcia AL, Althaus M, Wilcox MD, Pearson JP, Bythell JC, Burgess JG (2018) Evolutionary conservation of the antimicrobial function of mucus: a first defense against infection. NPJ Biofilms Microbiomes 4(1):14
- Banaszak AT, Barba Santos MG, LaJeunesse TC, Lesser MP (2006) The distribution of mycosporine-like amino acids (MAAs) and the phylogenetic identity of symbiotic dinoflagellates in cnidarian hosts from the Mexican Caribbean. J Exp Mar Biol Ecol 337(2):131–146
- Banaszak AT, Lesser MP (2009) Effects of solar ultraviolet radiation on coral reef organisms. Photochem Photobiol Sci 8(9):1276–1294
- Benavides M, Bednarz VN, Ferrier-Pagès C (2017) Diazotrophs: overlooked key players within the coral symbiosis and tropical reef ecosystems? Front Mar Sci 4:10
- Bessell-Browne P, Fisher R, Duckworth A, Jones R (2017) Mucous sheet production in Porites: an effective bioindicator of sediment related pressures. Ecol Ind 77:276–285
- Binienda ZK, Sarkar S, Silva-Ramirez S, Gonzalez C (2013) Role of free fatty acids in physiological conditions and mitochondrial dysfunction. Food Nutr Sci 04(9):6–15
- Bourne DG, Dennis PG, Uthicke S, Soo RM, Tyson GW, Webster N (2013) Coral reef invertebrate microbiomes correlate with the presence of photosymbionts. ISME J 7(7):14521458
- Brown BE, Bythell JC (2005) Perspectives on mucus secretion in reef corals. Mar Ecol Prog Ser 296:291–309
- Bruckner AW (2015) White syndromes of western Atlantic reef-building corals. In: Woodley CM, Downs CA, Bruckner AW, Porter JW, Galloway SB (eds) Diseases of coral. Wiley, New York, pp 316–332
- Burriesci MS, Raab TK, Pringle JR (2012) Evidence that glucose is the major transferred metabolite in dinoflagellate–cnidarian symbiosis. J Exp Biol 215(19):3467–3477
- Bythell JC, Wild C (2011) Biology and ecology of coral mucus release. J Exp Mar Biol Ecol 408(1–2):88–93
- Coddeville B, Maes E, Ferrier-Pages C, Guerardel Y (2011) Glycan profiling of gel forming mucus layer from the scleractinian symbiotic coral *Oculina arbuscula*. Biomacromol 12(6):2064–2073
- Coffroth MA (1984) Ingestion and incorporation of coral mucus aggregates by a gorgonian soft coral. Mar Ecol Prog Ser 17(2):193–199
- Coffroth MA (1990) Mucous sheet formation on poritid corals: an evaluation of coral mucus as a nutrient source on reefs. Mar Biol 105(1):39–49
- Coles SL, Strathmann R (1973) Observations on coral mucus “flocs” and their potential trophic significance 1. Limnol Oceanogr 18(4):673–678
- Corfield AP (2015) Mucins: a biologically relevant glycan barrier in mucosal protection. Biochem Biophys Acta 1850(1):236–252
- Crossland CJ (1987) In situ release of mucus and DOC-lipid from the corals *Acropora variabilis* and *Stylophora pistillata* in different light regimes. Coral Reefs 6(1):35–42
- Crossland CJ (2021) Hermatypic coral, *Acropora* cf. *acuminata*. In: Endosymbiosis and cell biology: a synthesis of recent research. Proceedings of the International Colloquium on Endosymbiosis and Cell Research, Tübingen, April 1980, vol 1, pp 163. Walter de Gruyter GmbH & Co KG
- Crossland CJ, Barnes DJ, Borowitzka MA (1980) Diurnal lipid and mucus production in the staghorn coral *Acropora acuminata*. Mar Biol 60(2–3):81–90
- Dailah HG (2022) Therapeutic potential of small molecules targeting oxidative stress in the treatment of chronic obstructive pulmonary disease (COPD): a comprehensive review. Molecules 27(17):5542
- Daumas R, Thomassin BA (1977) Protein fractions in coral and zoantharian mucus, possible evolution in coral reef environment. In: Proceedings of the (3rd) Int. Coral Reef Symp. (Miami), vol 1, pp 517–525
- Denny MW (1989) Invertebrate mucous secretions—functional alternatives to vertebrate paradigms. Symp Soc Exp Biol 43:337–366
- Diaz L (2017) Response of the coral associated nitrogen fixing bacteria toward elevated water temperature. J Water Resour Ocean Sci 6(6):98–109

- Ding JL, Fung FMY, Ng GWS, Chou LM (1999) Novel bioactivities from a coral, *Galaxea fascicularis*: DNase-like activity and apoptotic activity against a multiple-drug-resistant leukemia cell line. *Mar Biotechnol* 1(4):328–336
- Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM (2002) Oxidative stress and seasonal coral bleaching. *Free Radical Biol Med* 33(4):533–543
- Drollet JH, Teai T, Faucon M, Martin PMV (1997) Field study of compensatory changes in UV-absorbing compounds in the mucus of the solitary coral *Fungia repanda* (Scleractinia: Fungiidae) in relation to solar UV radiation, sea-water temperature, and other coincident physico-chemical parameters. *Mar Freshw Res* 48(4):329–333
- Ducklow HW, Mitchell R (1979) Bacterial populations and adaptations in the mucus layers on living corals 1. *Limnol Oceanogr* 24(4):715–725
- Eckes M, Dove S, Siebeck UE, Grutter AS (2015) Fish mucus versus parasitic gnathiid isopods as sources of energy and sunscreens for a cleaner fish. *Coral Reefs* 34(3):823–833
- Fang JHS (2015) Community analysis of coral mucus-associated bacteria and impact of temperature and CO₂ changes on them (Doctoral dissertation, Swinburne University of Technology (Sarawak campus))
- Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu J, Datta P, McCormick M, Huang L, McDermott E, Eisenlohr L, Landel CP, Alnemri ES (2010) The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. *Nat Immunol* 11(5):385–393
- Fitzgerald LM, Szmant AM (1997) Biosynthesis of “essential” amino acids by scleractinian corals. *Biochem J* 322(1):213–221
- Gabay Y (2018) The cellular and physiological basis of host-symbiont specificity in a model cnidarian-dinoflagellate symbiosis (Doctoral dissertation, Open Access Te Herenga Waka-Victoria University of Wellington)
- Galli P, Montano S, Seveso D, Maggioni D (2021) Coral reef biodiversity of the Maldives. *Atolls of the Maldives. Nissology and geography*. Rowman and Littlefield, Lanham, pp 196–212
- Gardner SG, Nielsen DA, Laczka O, Shimmon R, Beltran VH, Ralph PJ, Petrou K (2016) Dimethylsulfoniopropionate, superoxide dismutase and glutathione as stress response indicators in three corals under short-term hyposalinity stress. *Proc Biol Sci* 283(1824):20152418
- Goldberg WM (2018) Coral food, feeding, nutrition, and secretion: a review. In: Kloc M, Kubiak JZ (eds) *Marine organisms as model systems in biology and medicine*. Springer, Berlin, pp 377–421
- Gottfried M, Roman MR (1983) Ingestion and incorporation of coral-mucus detritus by reef zooplankton. *Mar Biol* 72(3):211–218
- Grottoli AG, Rodrigues LJ, Juarez C (2004) Lipids and stable carbon isotopes in two species of Hawaiian corals. *Porites compressa* and *Montipora verucosa*, following a bleaching event. *Mar Biol* 145:621–631
- Grover R, Maguer J-F, Allemand D, Ferrier-Pagès C (2008) Uptake of dissolved free amino acids (DFAA) by the scleractinian coral *Stylophora pistillata*. *J Exp Biol* 211:860–865
- Guarnieri MC, de Albuquerque Modesto JC, Pérez CD, Ottaiano TF, Ferreira RDS, Batista FP, de Brito MV, Campos IHMP, Oliva MLV (2018) Zoanthid mucus as new source of useful biologically active proteins. *Toxicon* 143:96–107
- Guppy R, Bythell JC (2006) Environmental effects on bacterial diversity in the surface mucus layer of the reef coral *Montastrea faveolata*. *Mar Ecol Prog Ser* 328:133–142
- Guppy R, Brown B, Bythell JC (2019) Preserving the viscous coral surface mucus layer using low-acid glycol methacrylate (GMA) resin. *Coral Reefs* 38(3):521–526
- Haas AF, Fairoz MFM, Kelly LW, Nelson CE, Dinsdale EA, Edwards RA et al (2016) Global microbialization of coral reefs. *Nat Microbiol* 1(7):16042
- Hadaidi G, Gegner HM, Ziegler M, Voolstra CR (2019) Carbohydrate composition of mucus from scleractinian corals from the central Red Sea. *Coral Reefs* 38(1):21–27
- Hadaidi GA (2018) Coral-associated bacterial community dynamics in healthy, bleached, and disease states. (Doctoral Dissertation), KAUST Research Repository. Doi: <https://doi.org/10.25781/KAUST-0UN42>
- Hafezi M, Giffin AL, Alipour M, Sahin O, Stewart RA (2020) Mapping long-term coral reef ecosystems regime shifts: a small island developing state case study. *Sci Total Environ* 716:137024
- Hill DB, Button B, Rubinstein M, Boucher RC (2022) Physiology and pathophysiology of human airway mucus. *Physiol Rev* 102(4):1757–1836
- Hillyer KE, Dias DA, Lutz A, Wilkinson SP, Roessner U, Davy SK (2017) Metabolite profiling of symbiont and host during thermal stress and bleaching in the coral *Acropora aspera*. *Coral Reefs* 36:105–118
- Ho TK (2017) Dredging land reclamation causing mucus development in massive spherical corals in the Spratly Islands, South China Sea: The effects on China's fishing industry. Poster session presented at Virginia Commonwealth University Poster Symposium for Undergraduate Research and Creativity, Richmond, VA
- Horka I, De Grave S, Franssen CH, Petrusek A, Đuriš Z (2016) Multiple host switching events shape the evolution of symbiotic palaemonid shrimps (Crustacea: Decapoda). *Sci Rep* 6(1):26486
- Horricks RA, Herbinger CM, Lillie BN, Taylor P, Lumsden JS (2019) Differential protein abundance during the first month of regeneration of the Caribbean star coral *Montastraea cavernosa*. *Coral Reefs* 38(1):45–61
- Iluz D, Dubinsky Z (2015) Coral photobiology: new light on old views. *Zoology* 118(2):71–78
- Irudayarajan L, Ravindran C, Raveendran HP (2023) Antimicrobial activity of coral-associated beneficial bacteria against coral disease-causing microbial pathogens. *J Basic Microbiol*. <https://doi.org/10.1002/jobm.202300338>
- Jatkar AA, Brown BE, Bythell JC, Guppy R, Morris NJ, Pearson JP (2010a) Coral mucus: the properties of its constituent mucins. *Biomacromol* 11(4):883–888
- Jatkar AA, Brown BE, Bythell JC, Guppy R, Morris NJ, Pearson JP (2010b) Measuring mucus thickness in reef corals using a technique devised for vertebrate applications. *Mar Biol* 157:261–267
- Kageyama H, Waditee-Sirisattha R (2019) Antioxidative, anti-inflammatory, and anti-aging properties of mycosporine-like amino acids: molecular and cellular mechanisms in the protection of skin-aging. *Mar Drugs* 17(4):222
- Kemp DW, Rivers AR, Kemp KM, Lipp EK, Porter JW, Wares JP (2015) Spatial homogeneity of bacterial communities associated with the surface mucus layer of the reef-building coral *Acropora palmata*. *PLoS ONE* 10(12):0143790
- Khalesi MK (2015) Corals. *Springer handbook of marine biotechnology*. Springer, Berlin, pp 179–217
- Komatsu K, Tsutsui S, Hino K, Araki K, Yoshiura Y, Yamamoto A, Nakamura O, Watanabe T (2009) Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. *Dev Comp Immunol* 33(4):499–506
- Krediet CJ, Ritchie KB, Cohen M, Lipp EK, Sutherland KP, Teplitski M (2009) Utilization of mucus from the coral *Acropora palmata* by the pathogen *Serratia marcescens* and by environmental and coral commensal bacteria. *Appl Environ Microbiol* 75(12):3851–3858

- Krediet CJ, Ritchie KB, Alagely A, Teplitski M (2013) Members of native coral microbiota inhibit glycosidases and thwart colonization of coral mucus by an opportunistic pathogen. *ISME J* 7(5):980–990
- Krieger-Liszskay A (2005) Singlet oxygen production in photosynthesis. *J Exp Bot* 56(411):337–346
- Krueger T, Bodin J, Horwitz N, Loussert-Fonta C, Sakr A, Escriu S, Fine M, Meibom A (2018) Temperature and feeding induce tissue level changes in autotrophic and heterotrophic nutrient allocation in the coral symbiosis—a NanoSIMS study. *Sci Rep* 8(1):12710
- Krupp DA (1982) The composition of the mucus from the mushroom coral *Fungia scutaria*. In: Proceedings of the 4th Int. Coral Reef Symp., vol 2, pp 9–73
- Kurihara H, Ikeda N, Umezawa Y (2018) Diurnal and seasonal variation of particle and dissolved organic matter release by the coral *Acropora tenuis*. *PeerJ* 6:5728
- LaJeunesse TC, Smith R, Walther M, Pinzón J, Pettay DT, McGinley M, Aschaffenburg M, Medina-Rosas P, Cupul-Magaña AL, Pérez AL, Reyes-Bonilla H, Warner ME (2010) Host–symbiont recombination versus natural selection in the response of coral–dinoflagellate symbioses to environmental disturbance. *Proc R Soc B* 277(1696):2925–2934
- LaJeunesse TC, Parkinson JE, Gabrielson PW, Jeong HJ, Reimer JD, Voolstra CR, Santos SR (2018) Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Curr Biol* 28(16):2570–2580.e6. <https://doi.org/10.1016/j.cub.2018.07.008>
- Lee ST, Dav SK, Tang SL, Kench PS (2016) Mucus sugar content shapes the bacterial community structure in thermally stressed *Acropora muricata*. *Front Microbiol* 7:371
- Lewis RW (1976) Mucus globule membrane: an hypothesis concerning its role in determining the viscosity of mucus. *J Theor Biol* 61(1):21–25
- Lin C, Wang LH, Fan TY, Kuo FW (2012) Lipid content and composition during the oocyte development of two gorgonian coral species in relation to low temperature preservation. *PLoS ONE* 7(7):38689
- Maldonado A, Johnson A, Gochfeld D, Slattery M, Ostrander GK, Bingham JP, Schlenk D (2016) Hard coral (*Porites lobata*) extracts and homarine on cytochrome P450 expression in Hawaiian butterflyfishes with different feeding strategies. *Comp Biochem Physiol c: Toxicol Pharmacol* 179:57–63
- McMahon KW, Thorrold SR, Houghton LA, Berumen ML (2016) Tracing carbon flow through coral reef food webs using a compound-specific stable isotope approach. *Oecologia* 180(3):809–821
- McShane A, Bath J, Jaramillo AM, Ridley C, Walsh AA, Evans CM, Thornton DJ, Ribbeck K (2021) Mucus. *Curr Biol* 31(15):938–945
- Means JC, Sigleo AC (1986) Contribution of coral reef mucus to the colloidal organic pool in the vicinity of Discovery Bay, Jamaica. *WI Bull Mar Sci* 39(1):110–118
- Meikle P, Richards GN, Yellowlees D (1988) Structural investigations on the mucus from six species of coral. *Mar Biol* 99(2):187–193
- Meyer W, Seegers U, Schnapper A, Neuhaus H, Himstedt W, Toepfer-Petersen E (2007) Possible antimicrobial defense by free sugars on the epidermal surface of aquatic vertebrates. *Aquat Biol* 1(2):167–175
- Nakajima R, Nakatomi N, Kurihara H, Fox MD, Smith JE, Okaji K (2016) Crown-of-thorns starfish larvae can feed on organic matter released from corals. *Diversity* 8(4):18
- Nakajima R, Tanaka Y, Yoshida T, Fujisawa T, Nakayama A, Fuchinoue Y, Othman BHR, Toda T (2015) High inorganic phosphate concentration in coral mucus and its utilization by heterotrophic bacteria in a Malaysian coral reef. *Mar Ecol* 36(3):835–841
- Neave MJ, Rachmawati R, Xun L, Michell CT, Bourne DG, Apprill A, Voolstra CR (2017) Differential specificity between closely related corals and abundant *Endozoicomonas* endosymbionts across global scales. *ISME J* 11(1):186–200
- Nguyen-Kim H, Bettarel Y, Bouvier T, Bouvier C, Doan-Nhu H, Nguyen-Ngoc L, Nguyen-Thanh T, Tran-Quang H, Brune J (2015) Coral mucus is a hot spot for viral infections. *Appl Environ Microbiol* 81(17):5773–5783
- Nissimov J, Rosenberg E, Munn CB (2009) Antimicrobial properties of resident coral mucus bacteria of *Oculina Patagonica*. *FEMS Microbiol Lett* 292(2):210–215
- Nithyanand P, Indhumathi T, Ravi AV, Pandian SK (2011) Culture independent characterization of bacteria associated with the mucus of the coral *Acropora digitifera* from the Gulf of Mannar. *World J Microbiol Biotechnol* 27(6):1399–1406
- Oku H, Yamashiro H, Onaga K (2003) Lipid biosynthesis from [¹⁴C]-glucose in the coral *Montipora digitata*. *Fish Sci* 69(3):625–631
- Oren A, Gunde-Cimerman N (2007) Mycosporines and mycosporine-like amino acids: UV protectants or multipurpose secondary metabolites? *FEMS Microbiol Lett* 269(1):1–10
- Palmer CV, Traylor-Knowles NG, Willis BL, Bythell JC (2011) Corals use similar immune cells and wound-healing processes as those of higher organisms. *PLoS ONE* 6(8):23992
- Pasaribu B, Weng LC, Lin IP, Camargo E, Tzen JT, Tsai CH, Ho SL, Lin MR, Wang LH, Chen CS, Jiang PL (2015) Morphological variability and distinct protein profiles of cultured and endosymbiotic Zooxanthellae cells isolated from *Exaiptasia Pulchella*. *Sci Rep* 5(1):15353
- Patton JS, Abraham S, Benson AA (1977) Lipogenesis in the intact coral *Pocillopora capitata* and its isolated Zooxanthellae: evidence for a light-driven carbon cycle between symbiont and host. *Mar Biol* 44(3):235–247
- Piggot AM, Fouke BW, Sivaguru M, Sanford RA, Gaskins HR (2009) Change in Zooxanthellae and mucocyte tissue density as an adaptive response to environmental stress by the coral. *Montastraea Annularis Mar Biol* 156(11):2379–2389
- Piludu M, Rayment SA, Liu B, Offner GD, Oppenheim FG, Troxler RF, Hand AR (2003) Electron microscopic immunogold localization of salivary mucins MG1 and MG2 in human submandibular and sublingual glands. *J Histochem Cytochem* 51(1):69–79
- Plass-Johnson JG, Cardini U, Van Hoytema N, Bayraktarov E, Burghardt I, Naumann MS, Wild C (2015) Coral bleaching. In: Armon RH, Hänninen O (eds) Environmental indicators. Springer, Dordrecht, pp 117–146
- Que J (2015) The initial establishment and epithelial morphogenesis of the esophagus: a new model of tracheal–esophageal separation and transition of simple columnar into stratified squamous epithelium in the developing esophagus. *Wiley Interdiscip Rev Dev Biol* 4(4):419–430
- Radecker N, Pogoreutz C, Voolstra CR, Wiedenmann J, Wild C (2015) Nitrogen cycling in corals: the key to understanding holobiont functioning? *Trends Microbiol* 23(8):490–497
- Radice VZ, Brett MT, Fry B, Fox MD, Hoegh-Guldberg O, Dove SG (2019) Evaluating coral trophic strategies using fatty acid composition and indices. *PLoS ONE* 14(9):0222327
- Raina JB, Tapiolas D, Motti CA, Foret S, Seemann T, Tebben J, Willis BL, Bourne DG (2016) Isolation of an antimicrobial compound produced by bacteria associated with reef-building corals. *PeerJ* 4:2275
- Ravindran C, Lawrance I, Vasanth AJ (2022) Endosymbiotic *Symbiodinium* clades occurrence and influence on coral growth and resilience during stress. *Symbiosis* 86:261–272
- Ravindran C, Bhagwat PV, Silveira KA, Shivaramu MS, Lele UP (2020) Characterization of coral associated ciliates and their interactions with disease lesion progression of Indian scleractinian corals. *Microb Pathog* 149:104472

- Ravindran C, Irudayarajan L, Raveendran HP (2023) Possible beneficial interactions of ciliated protozoans with coral health and resilience. *Appl Environ Microbiol* 89:e0121723
- Renaud SM, Thinh LV, Parry DL (1999) The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. *Aquaculture* 170(2):147–159
- Reshef L, Kore O, Loya Y, Zilber-Rosenberg I, Rosenberg E (2006) The coral probiotic hypothesis. *Environ Microbiol* 8:2068–2073
- Reynaud S, Ferrier-Pagès C (2019) Biology and ecophysiology of Mediterranean cold-water corals. In: Orejas C, Jiménez C (eds) *Mediterranean cold-water corals: past, present and future: understanding the deep-sea realms of coral*. Springer, Cham, pp 391–404
- Ritchie KB (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar Ecol Prog Ser* 322:1–14
- Rivera-Ortega J, Thomé PE (2018) Contrasting antibacterial capabilities of the surface mucus layer from three symbiotic cnidarians. *Front Mar Sci* 5:392
- Rix L, de Goeij JM, van Oevelen D, Struck U, Al-Horani FA, Wild C, Naumann MS (2017) Differential recycling of coral and algal dissolved organic matter via the sponge loop. *Funct Ecol* 31(3):778–789
- Rodrigues LJ, Grottoli AG, Pease TK (2008) Lipid class composition of bleached and recovering *Porites compressa* Dana 1846 and *Montipora capitata* Dana 1846 corals from Hawaii. *J Exp Mar Biol Ecol* 358(2):136–143. <https://doi.org/10.1016/j.jembe.2008.02.004>
- Salvatore S, Heuschkel R, Tomlin S, Davies SE, Edwards S, Walker-Smith J, Murch SH (2000) A pilot study of *N*-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment Pharmacol Ther* 14(12):1567–1579
- Schottner S, Hoffmann F, Wild C, Rapp HT, Boetius A, Ramette A (2009) Inter- and intra-habitat bacterial diversity associated with cold-water corals. *ISME J* 3(6):756–759
- Shafir A, Loya A (1983) Consumption and assimilation of coral mucus by the burrowing mussel *Lithophaga lessepsiana*. *Int Conf Mar Sci Red Sea* 9:135–140
- Sharma D, Ravindran C (2020) Diseases and pathogens of marine invertebrate corals in Indian reefs. *J Invertebr Pathol* 173:107373
- Shick JM, Dunlap WC, Pearse JS, Pearse VB (2002) Mycosporine-like amino acid content in four species of sea anemones in the genus *Anthopleura* reflects phylogenetic but not environmental or symbiotic relationships. *Biol Bull* 203(3):315–330
- Shinzato C, Inoue M, Kusakabe M (2014) A snapshot of a coral “holobiont”: a transcriptome assembly of the scleractinian coral, *Porites*, captures a wide variety of genes from both the host and symbiotic zooxanthellae. *PLoS ONE* 9(1):e85182
- Shmuel Y, Ziv Y, Rinkevich B (2022) Coral-inhabiting *Trapezia* crabs forage on demersal plankton. *Front Mar Sci* 9:1652
- Shnit-Orland M, Kushmaro A (2009) Coral mucus-associated bacteria: a possible first line of defense. *FEMS Microbiol Ecol* 67(3):371–380
- Siboni N, Ben-Dov E, Sivan A, Kushmaro A (2008) Global distribution and diversity of coral associated Archaea and their possible role in the coral holobiont nitrogen cycle. *Environ Microbiol* 10(11):2979–2990
- Siboni N, Ben-Dov E, Sivan A, Kushmaro A (2012) Geographic specific coral-associated ammonia-oxidizing archaea in the northern Gulf of Eilat (Red Sea). *Microb Ecol* 64:18–24
- Sikorskaya TV, Ermolenko EV, Efimova KV (2022) Lipids of Indo-Pacific gorgonian corals are modified under the influence of microbial associations. *Coral Reefs* 41(2):277–291
- Smith HA, Conlan JA, Pollock FJ, Wada N, Shore A, Hung JYH, Aebys GS, Willis BL, Francis DS, Bourne DG (2020) Energy depletion and opportunistic microbial colonisation in white syndrome lesions from corals across the Indo-Pacific. *Sci Rep* 10(1):19990
- Stabili L (2019) The mucus of marine invertebrates: Cnidarians, polychaetes, and echinoderms as case studies. In: Trincone A (ed) *Enzymatic technologies for marine polysaccharides*. CRC Press, Boca Raton, pp 151–162
- Stabili L, Schirosi R, Parisi MG, Piraino S, Cammarata M (2015) The mucus of *Actinia equina* (Anthozoa, Cnidaria): an unexplored resource for potential applicative purposes. *Mar Drugs* 13(8):5276–5296
- Stephens GC (1962) Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. *Biol Bull* 123(3):648–659
- Subramanian S, MacKinnon SL, Ross NW (2007) A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Comp Biochem Physiol Part B Biochem Mol Biol* 148(3):256–263
- Suggett DJ, Warner ME, Smith DJ, Davey P, Hennige S, Baker NR (2008) Photosynthesis and production of hydrogen peroxide by Zooxanthellae (pyrrhophyta) phylotypes with different thermal tolerances I. *J Phycol* 44(4):948–956
- Tchernov D, Gorbunov MY, De Vargas C, Narayan Yadav SN, Milligan AJ, Häggblom M, Falkowski PG (2004) Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proc Natl Acad Sci USA* 101(37):13531–13535
- Teai T, Drollet JH, Bianchini JP, Cambon A, Martin PMV (1998) Occurrence of ultraviolet radiation-absorbing mycosporine-like amino acids in coral mucus and whole corals of French Polynesia. *Mar Freshw Res* 49(2):127–132
- Teplitski M, Krediet CJ, Meyer JL, Ritchie KB (2016) Microbial interactions on coral surfaces and within the coral holobiont. The Cnidaria, past, present and future: the world of medusa and her sisters, pp 331–346
- Thompson JR, Rivera HE, Closek CJ, Medina M (2014) Microbes in the coral holobiont: partners through evolution, development, and ecological interactions. *Front Cell Infect Microbiol* 4:176
- Thornton DJ, Sheehan JK (2004) From mucins to mucus: toward a more coherent understanding of this essential barrier. *Proc Am Thorac Soc* 1(1):54–61
- Treignier C, Grover R, Ferrier-Pagès C, Tolosa I (2008) Effect of light and feeding on the fatty acid and sterol composition of *Zooxanthellae* and host tissue isolated from the scleractinian coral *Turbinaria reniformis*. *Limnol Oceanogr* 53(6):2702–2710
- Uslu L, Sayin S, Naz M, Taskin E, Soyler O, Saygili I, Cetin Z, Dinler ZM, Isik O (2021) Proximate analysis and fatty acid profile of some brown macroalgae collected from the Northeastern Mediterranean coast. *Fresenius Environ Bull* 30:9433–9437
- Vacelet E, Thomassin BA (1991) Microbial utilization of coral mucus in long term in situ incubation over a coral reef. *Hydrobiologia* 211(1):19–32
- Van Oppen MJ, Blackall LL (2019) Coral microbiome dynamics, functions and design in a changing world. *Nat Rev Microbiol* 17(9):557–567
- Vaughan EJ, Wilson SK, Howlett SJ, Parravicini V, Williams GJ, Graham NAJ (2021) Nitrogen enrichment in macroalgae following mass coral mortality. *Coral Reefs* 40(3):767–776
- Ventura M (2006) Linking biochemical and elemental composition in freshwater and marine crustacean zooplankton. *Mar Ecol Prog Ser* 327:233–246

- Vilas Bhagwat P, Ravindran C, Irudayarajan L (2023) Characterization of the defense properties of healthy and diseased coral mucus. *J Invertebr Pathol* 201:108001
- Volkman JK, Johns RB, Gillan FT, Perry GJ, Bavor HJ Jr (1980) Microbial lipids of an intertidal sediment—I. Fatty acids and hydrocarbons. *Geochemica Et Cosmochimica Acta* 44(8):1133–1143
- Weis VM (2008) Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. *J Exp Biol* 211(19):3059–3066
- Wiese J, Thiel V, Nagel K, Staufenberg T, Imhoff JF (2009) Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. *Mar Biotechnol* 11(2):287–300
- Wijayanti DP, Sabdono A, Dirgantara D, Widyananto PA, Sibero MT, Bhagooli R, Hidaka M (2020) Antibacterial activity of acroporid bacterial symbionts against White Patch Disease in Karimunjawa Archipelago, Indonesia. *Egypt J Aquat Res* 46(2):187–193
- Wild C, Huettel M, Klueter A, Kremb SG, Rasheed MY, Jorgensen BB (2004) Coral mucus functions as an energy carrier and particle trap in the reef ecosystem. *Nature* 428(6978):66–77
- Wild C, Naumann M, Niggel W, Haas A (2010) Carbohydrate composition of mucus released by scleractinian warm- and cold-water reef corals. *Aquat Biol* 10(1):41–45
- Wild C, Woyt H, Huettel M (2005) Influence of coral mucus on nutrient fluxes in carbonate sands. *Mar Ecol Prog Ser* 287:87–98
- Wilson SK, Bellwood DR, Choat JH, Furnas MJ (2003) Detritus in the epilithic algal matrix and its use by coral reef fishes. *Oceanogr Mar Biol* 41:279–310
- Wright RM, Strader ME, Genuise HM, Matz M (2019) Effects of thermal stress on amount, composition, and antibacterial properties of coral mucus. *Peer J* 7:6849
- Younan F, Pearson J, Allen A, Venables C (1982) Changes in the structure of the mucous gel on the mucosal surface of the stomach in association with peptic ulcer disease. *Gastroenterology* 82(5 Pt 1):827–831
- Zetsche EM, Baussant T, Meysman FJ, Van Oevelen D (2016) Direct visualization of mucus production by the cold-water coral *Lophelia pertusa* with digital holographic microscopy. *PLoS ONE* 11(2):0146766
- Zhang S, Li G (2000) Presence of phenoloxidase and prophenoloxidase in the epidermal cells and the epidermis mucus of the lancelet *Branchiostoma belcheritsingtauense*. *Ophelia* 52(3):207–212
- Zhao X, Findly RC, Dickerson HW (2008) Cutaneous antibody-secreting cells and B cells in a teleost fish. *Dev Comp Immunol* 32(5):500–508

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