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Gram staining reveals diverse bacterial associations in coral cell-associated microbial aggregates in the Pacific Ocean

Chutimon Singhakarn [Corresp., 1, 2], **Robert J Toonen** [1], **Thierry M Work** [2]

[1] Hawai'i Institute of Marine Biology, University of Hawaii at Manoa, Kāne'ohe, Hawai'i, United States

[2] Honolulu Field Station, US Geological Survey, National Wildlife Health Center, Honolulu, Hawai'i, United States

Corresponding Author: Chutimon Singhakarn
Email address: chutimon@hawaii.edu

Cell-Associated Microbial Aggregates (CAMAs) have been observed in 24 coral species from the Pacific Ocean, and studies indicate most contain Gram-negative rods from the genus *Endozoicomonas.* Here, we used histology with Gram staining to evaluate the morphology and distribution of CAMAs in six species of scleractinian corals from Hawaii and Palmyra. Within CAMAs, we observed the coexistence of bacteria with differing morphologies and Gram staining properties both within and among coral species. *Pocillopora* and *Acropora* had mostly Gram-negative rods, whereas Gram-negative cocci dominated in *Porites*. *Acropora* had the highest abundance of Gram-positive CAMAs. The anatomical distribution of CAMAs varied by coral species. CAMAs dominated in the tentacles of *Pocillopora meandrina*, *Pocillopora grandis*, and *Porites evermanni,* were mostly in the coenosarc of *Acropora cytherea*, and were found equally between tentacles and coenosarc in *Porites compressa* and *Porites lobata*. Tissue layer distribution also varied, with CAMAs mainly in the epidermis of *Pocillopora* but in the gastrodermis of *Porites* and *Acropora*. The diversity of bacteria in CAMAs and their anatomic distribution in Pacific corals may be more complex than previously understood. This indicates other bacterial species, in addition to *Endozoicomonas,* are colonizing CAMAs in corals from the Pacific Ocean.

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Chutimon Singhakarn[1,2,3], Robert J. Toonen[1], Thierry M. Work[2]

[1] Hawai‘i Institute of Marine Biology, University of Hawai‘i at Mānoa, Kāne‘ohe, Hawaii, USA
[2] U.S. Geological Survey, National Wildlife Health Center, Honolulu Field Station, Honolulu, Hawaii, USA
[3] Corresponding Author: chutimon@hawaii.edu

Abstract

Cell-Associated Microbial Aggregates (CAMAs) have been observed in 24 coral species from the Pacific Ocean, and studies indicate most contain Gram-negative rods from the genus *Endozoicomonas*. Here, we used histology with Gram staining to evaluate the morphology and distribution of CAMAs in six species of scleractinian corals from Hawaii and Palmyra. Within CAMAs, we observed the coexistence of bacteria with differing morphologies and Gram staining properties both within and among coral species. *Pocillopora* and *Acropora* had mostly Gram-negative rods, whereas Gram-negative cocci dominated in *Porites*. *Acropora* had the highest abundance of Gram-positive CAMAs. The anatomical distribution of CAMAs varied by coral species. CAMAs dominated in the tentacles of *Pocillopora meandrina*, *Pocillopora grandis*, and *Porites evermanni,* were mostly in the coenosarc of *Acropora cytherea*, and were found equally between tentacles and coenosarc in *Porites compressa* and *Porites lobata*. Tissue layer distribution also varied, with CAMAs mainly in the epidermis of *Pocillopora* but in the gastrodermis of *Porites* and *Acropora*. The diversity of bacteria in CAMAs and their anatomic distribution in Pacific corals may be more complex than previously understood. This indicates other bacterial species, in addition to *Endozoicomonas,* are colonizing CAMAs in corals from the Pacific Ocean.

Introduction. Microbes within the surface mucus layer, coral tissue, and skeleton play a vital role in coral holobiont function (Siboni et al., 2008; van Oppen & Blackall, 2019; Tandon et al., 2022; Mohamed et al., 2023). Although substantial knowledge exists on microbial metagenomics of mucus (Lee et al., 2015; Glasl et al., 2016; Hadaidi et al., 2017), relatively less is known about the identity, location, or function of the internal microbiome of corals (Wada et al., 2019; Maire et al., 2023; Maire et al., 2024). As such, interest is growing surrounding bacteria-coral interactions involving Cell-Associated Microbial Aggregates (CAMAs). First observed in the sea anemone *Exaiptasia diaphana* (Palincsar, 1989), CAMAs are now recognized in 24 coral species across the Pacific Ocean, and are particularly prevalent in the genera *Acropora*, *Porites*, and *Pocillopora* (Work & Aeby, 2014). While common among healthy individuals, CAMAs are decreased or absent in *Porites* experiencing tissue loss (Sudek et al., 2012). The high prevalence of CAMAs in some coral tissues not associated with host cell pathology indicates they may play a role in coral health similar to endosymbionts (Work & Aeby, 2014).

Based on molecular and morphological studies of *Stylophora pistillata*, the genus *Endozoicomonas* is the dominant bacterium in CAMAs for that species of coral globally (Neave et al., 2017). *Endozoicomonas* has been confirmed in tissues of *S. pistillata* from the Red Sea (Bayer et al., 2013), the western Pacific (Wada et al., 2022), and Micronesia (Neave et al., 2016) using molecular assays and fluorescence *in situ* hybridization (FISH). However, it is likely that other microbes are involved in CAMA formation and function. For example, in *Acropora*

hyacinthus, five distinct bacterial morphologies were seen in CAMA using fluorescent *in situ* hybridization (FISH) and 16S rRNA probes: rod-shaped, atypical coccus, longer rod morphology, filamentous-like bacteria, and rod-shaped morphology with spore-like structure (Wada et al., 2019). In contrast, bacterial morphology using transmission electron microscopy was similar within and between CAMAs in *Pocillopora acuta* (Maire et al., 2023). There is also evidence of coexistence of multiple bacteria within a single CAMA. For example, *Simkania* and *Endozoicomonas* were identified from CAMA samples using laser microdissection and 16S ribosomal RNA gene metabarcoding (Maire et al., 2023). Application of FISH further demonstrated co-localization within a given aggregate, thereby underscoring CAMA complexity. Three *Endozoicomonas* metagenomes recovered from CAMAs indicate the potential for bacteria synthesizing antioxidants, antimicrobial compounds, and several B vitamins, which may be essential for coral and Symbiodiniaceae health (Maire et al., 2023). However, sorting out how these functions relate to coral health still needs further experimental clarification.

Knowledge of the distribution and diversity of bacteria within CAMA is important to understanding their role in coral health. While molecular methods like metagenomics and *in situ* hybridization are powerful tools, they have limitations. Metagenomics indicates the presence or absence of DNA but gives no information on whether bacteria are intact, their morphology, or anatomic location. *In situ* hybridization localizes organisms to tissues, but requires *a priori* knowledge of the organisms to be identified so that proper probes can be designed. In the absence of such knowledge, when presented with unknown bacteria in tissues, diagnosticians use histological techniques coupled with Gram staining (Gram, 1884; Wilson et al., 2015) to guide laboratory investigations. Gram stains categorize bacteria as Gram-positive (blue to purple) or Gram-negative (red to pink) based on cell wall properties (Smith & Hussey, 2005). Knowing the Gram-status of bacteria in tissues allows for efficient downstream application of appropriate confirmatory diagnostic steps. For example, the presence of Gram-negative bacteria in tissues might lead to the use of culture media selective for growth of that bacteria type for isolation and further characterization (Jung & Hoilat, 2024).

Here, we used Gram stains to evaluate CAMAs in three genera of scleractinian corals (*Acropora*, *Pocillopora*, and *Porites*) that were commonly found to host these microbial aggregates in previous surveys (Work & Aeby, 2014). These coral genera represent three of the most important reef-building families globally (Acroporidae, Pocilloporidae & Poritidae) comprising different evolutionary lineages and life history strategies (Siqueira et al., 2022; Jury et al., 2024). These three families together comprise roughly 95% of coral cover throughout the Hawaiian Archipelago (Franklin et al., 2013). In Hawaii, *Acropora* has a restricted geographic distribution in the Northwestern Hawaiian Islands and is rare from the Main Hawaiian Islands (Walsh et al., 2014; Concepcion et al., 2016). In contrast, *Pocillopora* is a common early colonizer on reefs, whereas *Porites* is a slower growing massive coral most common on more established reefs throughout the Hawaiian Archipelago. Comparing the composition and distribution of CAMAs

across these genera provides a reasonable sampling of scleractinian diversity to assess location and composition of CAMAs among coral taxa.

Materials & Methods.

Coral identified based on growth form, corallites, and verrucae following descriptions from Wells (1998). *Acropora cytherea* colonies are tabulate with fine, upward-projecting branchlets and radial corallites with short open calices and a terminal corallite. *Pocillopora grandis* colonies have stout upright flattened branches with tubercles interspersed with distinct corallites. *Pocillopora meandrina* colonies are similar to *Poc. grandis* but colonies comprise short branches radiating from a central area. *Porites compressa* branches are distinct to fused cylindrical forms with closely apposed small corallites. *Porites lobata* colonies are usually hemispherical or lobed and may be more than 4 meter wide with a smooth surface and closely apposed corallites and yellow to pale brown. *Porites evermanni* colonies are similar but dark brown.

Samples were collected between 2001 and 2021 from the Main and Northwestern Hawaiian Islands, including Island of Hawai‘i, O‘ahu, Kaua‘i, Kānemiloha‘i (French Frigate Shoals), Nalukākala (Maro Reef), and Palmyra Atoll (**Figure 1, Table 1)** under Hawaii Department of Aquatic Resources (Permit: SAP2025-28). During collections, colonies were photographed, and gross lesions were categorized as apparently normal, algae overgrowth, bleaching, discoloration, tissue loss, or growth anomaly (Work & Aeby, 2006, **Figure 2A**). Coral fragments were decalcified using Cal-Ex II (Fisher Scientific, Waltham, Massachusetts, USA), trimmed, and processed following standard histological methods as described (Work & Aeby, 2010). Sections were recut onto glass slides and stained with the modified Brown and Hopps method (Schwartz et al., 1989) (referred to hereafter as Gram stain). Gram stains were performed by Wisconsin Veterinary Diagnostic Laboratory, a laboratory certified by the USDA National Animal Health Laboratory Network (U.S. Food and Drug Administration, 2013). In Gram-positive bacterial cells, the thick peptidoglycan layer in the cell wall prevents the elution of the crystal violet–iodine complex during decolorization, allowing these cells to retain the stain turning them purple-blue (Erkmen, 2021). In contrast, Gram-negative cells have a thinner peptidoglycan layer, which allows the crystal violet–iodine complex to diffuse out during decolorization, leaving the cells visible only after being counterstained with safranin that turns them red.

Some bacteria exhibit Gram-variable staining, meaning Gram-positive bacteria may stain as Gram-negative. This can occur when certain Gram-positive bacteria, such as *Bacillus*, *Butyrivibrio*, and *Clostridium* have a thinner cell wall during exponential growth phase (Beveridge, 2001) or a damaged cell wall (Popescu & Doyle, 1996) thereby not allowing retention of crystal violet-iodine complex. The most common mistake is misidentifying Gram-positive bacteria as Gram-negative due to excessive decolorization, which causes the cells to appear Gram-negative (Popescu & Doyle, 1996). To minimize such errors, we included a known Gram-positive and Gram-negative control in our staining procedure to verify our results. Our

Gram-negative control was kidney tissues from a White-tailed tropicbird (*Phaeton lepturus*) with lesions of Salmonellosis and presence of small Gram-negative rods from which pure cultures of *Salmonella typhimurium* were isolated. Our Gram-positive control was liver tissues from a Laysan duck (*Anas laysanensis*) with lesions of sepsis and presence of large Gram-positive rods from which pure cultures of *Erysipelothrix rhusiopathiae* were isolated.

Two set of coral histological slides were examined by a single observer. The first set was a total of 87 fragments from 76 colonies comprising six coral species including *Acropora cytherea* (n = 4), *Pocillopora grandis* (n = 8), *Pocillopora meandrina* (n = 16), *Porites compressa* (n = 20), *Porites lobata* (n = 20), and *Porites evermanni* (n = 8) **(Supplementary file 1)**. The second set of samples comprised paired normal and lesion fragments from which we enumerated CAMAs per tissue area (cm^2) from paired apparently healthy and lesion fragments of 35 diseased coral colonies. For this, we used a subset of previously examined histology slides (n=52) and added 23 additional slides that were paired with the original slides from the same individual colonies **(Supplementary file 2)**.

Slides were scanned using HP Color LaserJet pro MFP M479fdw (HP Inc., Palo Alto, California, USA). The scanned images were used only to calculate the surface area (cm^2) of tissue from individual fragments using QuPath version 0.4.3 (Bankhead et al., 2017). The assessment of color or Gram stain was done during using BX43 compound microscope (Olympus corporation, Waltham, Massachusetts, USA), and by images of CAMAs from Gram-stained slides that were taken with an INFINITY3 digital microscope camera (Lumenera Corporation, Ottawa, Ontario, Canada). Brightness, contrast, and hue were set at zero. The images were taken at 100X magnification. Distribution of CAMAs was categorized by anatomical location (skeleton, tentacles, mesentery, coenosarc, also known as coenenchyme (Woodley et al., 2016)). Within tissues, CAMA were localized to specific layers including epidermis, mesoglea, gastrodermis, and calicodermis. Bacterial shape within CAMAs was classified as rod and cocci. CAMAs staining red to pink on Gram stain were classified as Gram-negative while those staining purple-to-blue were classed as Gram-positive. CAMAs whose bacteria were densely packed and whose shape could not be clearly categorized were categorized as undetermined and excluded from further analyses (n=150, 11.17% of total observed CAMAs). Number of CAMAs from each individual fragment was normalized by surface tissue area (cm^2) of histology slide to account for different amounts of tissues examined on each slide.

All analyses were done with RStudio version 2023.06.1+524 (Posit team, 2023). Points for box plot/violin plot were jittered to enhance clarity of presentation (Cleveland, 1985). Number of CAMAs per cm^2 did not fit parametric assumptions of normal distribution and equal variance, so they were compared between coral species, gross lesion, and location using Kruskal-Wallis test. Percentage of Gram status and shape were compared between species, anatomy and tissue layer using Pearson's chi-squared test and pairwise proportions tests (post-hoc test). The number of

CAMAs per tissue area (cm²) was compared between apparently normal and lesion fragments from the same colony using the Wilcoxon signed-rank test. All statistics were run using the ggstatplot package version 0.13.0 (Patil, 2021). Shannon's index (Shannon, 1948) was used to compare the diversity of bacterial morphologies observed per coral species with the R package vegan version 2.6-10 (Oksanen et al., 2022).

Results. Of 87 fragments across six species of coral, we identified a total of 1310 CAMAs. The highest median number of CAMAs/cm^2 per fragment was 7.62 in *Por. compressa* (inter quartile range [IQR] = 0.102) followed by *Poc. meandrina* (median = 6.88, IQR = 0.121), *Poc. grandis* (median = 6.84, IQR = 0.192), *Por. evermanni* (median = 5.96, IQR = 0.033), *A. cytherea* (median = 4.17, IQR = 0.036) and *Por. lobata* (median = 2.38, IQR= 0.032). There was no significant difference in the median number of CAMAs/cm^2 between coral species (Kruskal-Wallis test, $p = 0.072$) **(Figure 2B)**. Median number of CAMAs/cm^2 by lesion type ranged from 1.01 for algae overgrowth to 10.45 for bleaching. Number of CAMAs/cm^2 was not significantly different among five gross lesion types (Kruskal-Wallis test, $p = 0.097$) **(Figure 3)**. Median (IQR) of CAMAs/cm^2 per fragment between location were 13.2 (0.106) for the Island of Hawai'i, 6.59 (0.167) for Kaua'i, 4.98 (0) for Nalukākala (Maro Reef), 4.83 (0.051) for Kānemiloha'i (French Frigate Shoals), 4.17 (0.036) for Palmyra, and 3.44 (0.058) for O'ahu. Number of CAMAs/cm^2 differed significantly by location (Kruskal-Wallis test, p-value = 0.0094) with corals from Island of Hawai`i having significantly greater numbers of CAMAs compared to those from O'ahu ($p = 0.0077$) **(Figure 4)**. There was no significant difference (Wilcoxon signed-rank test, $p = 0.10$) in the number of CAMAs/cm^2 between paired normal and lesion fragments from individual diseased colonies (n=35) (**Figure 5**).

Anatomical and tissue layer distributions of CAMAs vary among coral species

The distribution of CAMAs within anatomical compartments and tissue layers varied among coral species. In *Poc. meandrina*, *Poc. grandis*, *and Por. evermanii,* most CAMAs were located in the tentacles, accounting for 96.5%, 95.5%, and 74.6% of anatomical compartments, respectively. *A. cytherea* had CAMAs mainly in the coenosarc. In *Por. compressa* and *Por. lobata*, CAMAs were equally distributed between the tentacles and coenosarc. Three CAMAs were observed in the skeleton, an inanimate portion of the coral **(Figure 6A)**. For tissue layers, CAMAs in *Pocillopora* were mainly in the epidermis (92.7% and 93.9% for *Poc. grandis* and *Poc. meandrina*, respectively; in *Porites* CAMAs were mainly in the gastrodermis. CAMAs in *A. cytherea* were mainly in the gastrodermis and mesoglea with relatively fewer in the calicodermis **(Figure 6B)**.

Bacterial morphology and Gram staining

Of 1310 CAMAs, we were able to confidently determine bacteria shape and Gram status for 1193. CAMAs had four bacteria morphologies: Gram-negative rods **(Figure 7A)** Gram-negative cocci **(Figure 7B-C)**, Gram-positive rods **(Figure 7D)**, and Gram-positive cocci **(Figure 7E)**. Avian tissues infected with *Salmonella typhimurium* showed small Gram-negative rods **(Figure 7F)** whilst avian tissues infected with *Erysipelothrix rhusiopathiae* showed larger Gram-positive rods **(Figure 7G)**. In corals, CAMAs were dominated by Gram-negative bacteria except for *A. cytherea* where 36% of CAMAs were Gram-positive (Pearson's chi-squared, $p = 0.1$). Bacteria within CAMAs in *A. cytherea, Poc. meandrina* and *Poc. grandis* were rod-shaped whereas those in CAMAs from *Por. compressa, Por. evermanni*, and *Por. lobata* were mostly coccoid with a minority being rod-shaped bacteria (13%, 8% and 7%, respectively) **(Figure 8A)**. We observed coexistence of bacteria differing in both bacterial shape and Gram staining characteristics within the same CAMAs for 34% of fragments where morphology of CAMAs could be reliably identified. The diversity of bacterial morphologies was highest in *A. cytherea* with a Shannon index of 0.768, followed by *Por. compressa* (0.471), *Por. meandrina* (0.402), *Por. lobata* (0.397), *Por. evermanni* (0.267), and *Poc. grandis* (0.241) **(Figure 8B)**.

Discussion.

In contrast to Sudek et al. (2012), who found a 74% reduction of CAMAs in *Porites* bleaching with tissue loss versus healthy colonies, we observed no significant difference in the number of CAMAs per cm² between paired visually normal and lesioned fragments from the same individual diseased colonies. This discrepancy could be due to differences in sample type, as our study focused solely on fragments from diseased colonies, a recognized limitation of our study. Also, we did not examine lesions of bleaching with tissue loss, so perhaps there are particular responses of CAMAs with certain lesion types. For instance, we saw that corals with algal overgrowth exhibited fewer CAMAs compared to corals with other types of gross lesions. Algal overgrowth on corals associated with phase shifts driven by nutrient overload is a well-recognized issue in coral reef ecosystems (Bell & Elmetri, 1995; Done, 1992; McCook, 1999). Molecular studies have shown that nutrient pollution and increased algal cover can shift bacterial communities, with an increase in opportunistic Proteobacteria and a decrease in Actinobacteria within the surface mucus layer of corals (Haas et al., 2016; Zaneveld et al., 2016). Although the relationship between nutrient levels, algal overgrowth and CAMA abundance remains unclear, it may warrant further investigation. Temporal studies of CAMAs abundance could help determine whether declines in CAMAs precede or follow algal overgrowth, and sampling only normal colonies would be useful to assess the status of CAMA in clinically normal *Pocillopora, Porites* and *Acropora.*

Most CAMAs in *Pocillopora* were found in the epidermis of the tentacles, while in *Porites evermanni*, *Porites lobata*, and *Porites compressa*, they were primarily located in the gastrodermis of the tentacles and coenosarc. This distribution may reflect the anatomy of these corals, as more complex perforate corals like *Acropora* and *Porites* (Okubo, 2016) may provide a greater variety of habitats and cell types available for bacterial colonization. Similarly, in *Acropora*, CAMAs were predominantly found in the coenosarc, which is abundant between widely spaced corallites (Fenner, 2005). These contrast with non-perforate Pocillopora where CAMA were almost exclusively in epidermis of tentacles. In other invertebrates, the location of microbial aggregations is often linked to specific physiological functions of the host. For example, *Aliivibrio fischeri* in squid aggregate in the cilia of its light organ behind the gut-ink sac, thereby facilitating bioluminescence during nighttime activity (Nyholm & McFall-Ngai, 2021). Similarly, *Wolbachia* bacteria in mosquitoes preferentially localize in specific regions of the oocyte during oogenesis, enabling them to infect the female germline and induce parthenogenesis, turning unfertilized eggs into diploid females (Correa & Ballard, 2016). In corals, the specific localization of CAMAs could similarly influence physiological functions. For example, CAMAs in the gastrodermis may play a role in nutrient absorption and digestion, while those in the epidermis might affect interactions with the external environment or contribute to host defense. Investigating how the anatomical location of CAMAs relates to coral function could provide valuable insights into their role in coral health.

Although DNA sequencing is commonly used to identify microbial communities, Gram staining remains a valuable tool for initial surveys and has a long history of use in diagnostic pathology. As such, our study complements existing studies on CAMA by providing important information of localizing organism to anatomical location. For example, most CAMAs comprised Gram-negative rods, morphologically consistent with the genus *Endozoicomonas*, which would accord with other studies that show this bacterium to be widely abundant across various marine invertebrates and fish (Neave et al. 2016; Pogoreutz & Ziegler 2024). *Endozoicomonas* has been identified from CAMAs in *Stylophora pistillata*, *Pocillopora verrucosa*, and *Pocillopora acuta* (Bayer et al., 2013; Neave et al., 2017; Maire et al., 2023). However, the presence of Gram-positive rods and cocci and Gram-negative cocci indicates additional species of bacteria exist in at least five species of Pacific corals. Gram-positive bacteria in coral tissues have been extensively documented using both culture-dependent (Sweet et al., 2021) and culture-independent (Ainsworth et al., 2015) methods. For example, *A. cytherea* hosts a small proportion of Gram-positive cocci bacteria (<1%) identified as *Candidatus actinomarina* (Qin et al., 2022), whereas *Por. compressa* and *Por. lobata* harbor Actinobacteria (6% and 23%, respectively) (Ritchie, 2005). In addition to filamentous forms, Actinobacteria exhibit a wide range of morphologies including coccoid and rod-coccoid (Ventura et al., 2007), morphologies similar to what was observed here.

A. cytherea exhibited the greatest diversity of CAMAs, with multiple morphologies and Gram staining. Other authors have also noted varying morphologies of bacteria in CAMAs from *Acropora*. For example, Wada et al. 2019 found different morphologies of CAMA in *A. hyacinthus* from Australia. This variability of bacteria within a CAMA could indicate organisms that share metabolic byproducts, a phenomenon known as bacterial cross-feeding reviewed by Smith et al., (2019). Bacterial diversity among and within CAMA could also potentially enhance host resilience akin to algal endosymbionts where different genera can vary spatially and temporally within individual coral colonies (Rouzé et al., 2019; Rocha de Souza et al., 2023) where this diversity enhances resilience during environmental stress (Cunning et al., 2018). Analogously, microbial diversity within CAMAs may also be a strategy that helps corals adapt to environmental perturbations. We have anecdotally observed that disease outbreaks in Hawaii often involve *Por. evermanni,* a species with particularly low diversity of CAMAs (as assessed by Gram staining). Understanding the specific bacterial species within these CAMAs and exactly how they interact with the host would be crucial to understand their role in coral immunity and health. Future research could focus on methods to grow these CAMA in culture (Sweet et al., 2021), laser capture microdissection to identify targeted CAMAs by molecular means (Maire et al., 2021; Maire et al., 2023; Maire et al., 2024), eliminating or introducing CAMAs into corals experimentally and monitoring host fitness (Palincsar et al., 1989; Schuett et al., 2007), or assessing viability of bacteria using methods like propidium monoazide (Nocker et al. 2007).

Extracellular Gram-negative rods were observed in all tissue layers of the tentacles of *Poc. grandis*, as well as in the skeletons of *A. cytherea* and *Por. lobata*. This could indicate the rupture of the double-layer membrane surrounding bacterial aggregates (Palincsar et al., 1989). These CAMAs might infect neighboring cells through intercellular spread, similar to obligate intracellular bacteria like *Rickettsia* spp. (van Schaik et al., 2013), or they may persist outside host cells as facultative bacteria (Maire et al., 2023). Some bacteria in CAMAs may be facultative intracellular organisms. For example, Gram-negative rods, such as *Endozoicomonas* recovered from CAMAs, possess relatively large genomes ranging from 5.6 to 6.9 million base pairs (Mbp) (Maire et al., 2023) indicating it to be a facultative symbiont, because obligate endosymbionts typically have much smaller genomes (<1.5 Mbp) (Darby et al., 2007). The evidence of *Endozoicomonas* exhibiting diverse aggregation patterns, ranging from contained aggregates to irregular shapes lacking clear boundaries, also supports the hypothesis that some bacteria within CAMAs may be facultative intracellular organisms (Gotze et al., 2024). The difficulty in culturing bacteria from CAMAs and their cnidarian host cells presents a substantial challenge in understanding their role in coral health, their colonization processes, and regulatory mechanisms. Historically, large advances in our understanding of host-microbe interactions have stemmed from the ability to manipulate both bacteria and their hosts. For example, studies on *Wolbachia* in insects have illuminated how this symbiont influences reproduction and population dynamics (Fallon, 2021), whereas research on the symbiosis between squid and *Aliivibrio fischeri* has provided insights into bacterial colonization and bioluminescence (Nyholm &

McFall-Ngai, 2021). Future research could focus on developing new methods for culturing CAMAs, such as culturomics (Vanstokstraeten et al., 2022) and in vitro cultivation of bacteria from CAMAs with coral primary cells (Nowotny et al., 2021).

The presence of CAMAs in apparently normal corals reported by others (Wada et al. 2019, 2022) and the presence of CAMAs in visually normal fragments in our study indicates they may play an important role in coral physiology, akin to mutualistic intracellular bacteria in insects that influence nutrition, immunity, and evolution (Eleftherianos et al. 2013; Coolen et al. 2022). Bacterial endosymbionts in insects are vital for maintaining host health, particularly in the face of emerging diseases, environmental stress, and climate change (Vásquez et al., 2023). For example, *Buchnera* bacteria provide essential amino acids to aphids, allowing them to subsist on nutrient-poor diets like plant sap (Gündüz & Douglas, 2009). Given these parallels, it seems reasonable to hypothesize that CAMAs may play analogous roles in corals. The genomic functional characterization of *Endozoicomonas* in marine hosts indicates *Endozoicomonas* within CAMAs may have wide range symbiotic spectrum from mutualism and commensalism to opportunism and parasitism (Pogoreutz & Ziegler, 2024). *Endozoicomonas* exhibit aggregative behavior in the gill and digestive epithelium and have been associated with parasitic and pathogenic relationships with fish and clams (Katharios et al., 2015; Bennion et al., 2021). In contrast to CAMAs in corals, we observed no associated host cell pathology. The squid-Vibrio model (McFall-Ngai, 1999) might serve as an informative procedural analogue towards better understanding how CAMA interact and beneficially or adversely affect the coral host.

Conclusions. A study of CAMAs in diseased corals using morphology and Gram staining revealed morphological differences among individual CAMAs, ranging from coccoid to rod-shape and from Gram-negative to Gram-positive, highlighting complexity of CAMAs. Corals affected by algal overgrowth had fewer CAMAs compared to those with other types of lesions, which indicates that CAMAs may be involved in the microbial community shifts associated with nutrient pollution and increased algae cover. Further, geographic variations in CAMA abundance were found in corals from the Island of Hawai‘i having significantly higher numbers compared to O‘ahu, potentially reflecting anthropogenic effects that are much greater on the densely populated island of O‘ahu. Future research could focus on identifying the specific microbial species within CAMAs. Confirming CAMAs abundance and complexity between healthy and diseased coral, especially algae overgrowth could highlight the potential role and dynamic of CAMAs.

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Table 1(on next page)

Number of fragments sampled for histology partitioned by location and species.

Depths at the sampled sites are 3-10 meters.

1

Species	Kānemilohaʻi (French Frigate Shoals)	Island of Hawaiʻi	Oʻahu	Kauaʻi	Nalukākala (Maro Reef)	Palmyra Atoll	Total
Pocillopora grandis	1			12			13
Pocillopora meandrina		8	1	12			21
Porites compressa	3	4	13				20
Porites evermanni	5		3				8
Porites lobata	1	3	15		1		20
Acropora cytherea						5	5
Total	10	15	32	24	1	5	87

2

Figure 1

A map showing the location of coral collection sites in the Pacific Island region including Island of Hawai‘i, O‘ahu, Kaua‘i, Kānemiloha‘i (French Frigate Shoals-FFS), Nalukākala (Maro Reef) and Palmyra Atoll.

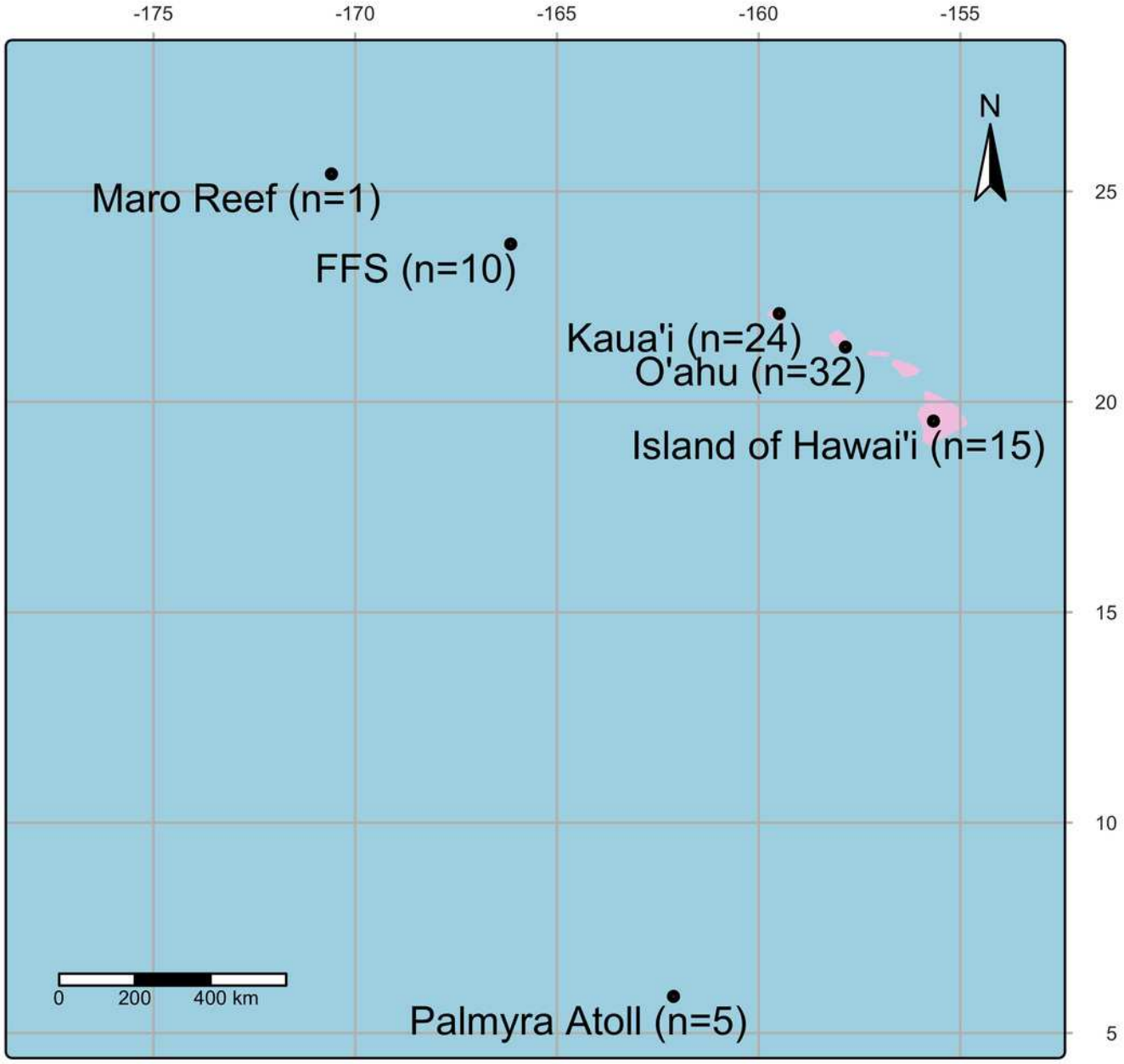


Figure 2

Number of Cell-Associated Microbial Aggregates (CAMAs) among coral species.

(A) Percentage of gross lesion types within coral species. (B) Number of CAMAs per cm^2 in individual fragments by corals species. The colors of point indicate gross lesion in each fragment. The box extent shows the middle 50% of number of CAMA/cm^2 in individual coral specie. The upper and lower whiskers show upper 25% and lower 25% of Number of CAMAs/cm^2 ,excluding outliner in individual coral specie. Middle line and red number indicates median CAMA/cm^2 of each coral specie. *Poc*.= *Pocillopora* , *Por* .= *Porites* , and *A* .= *Acropora.*

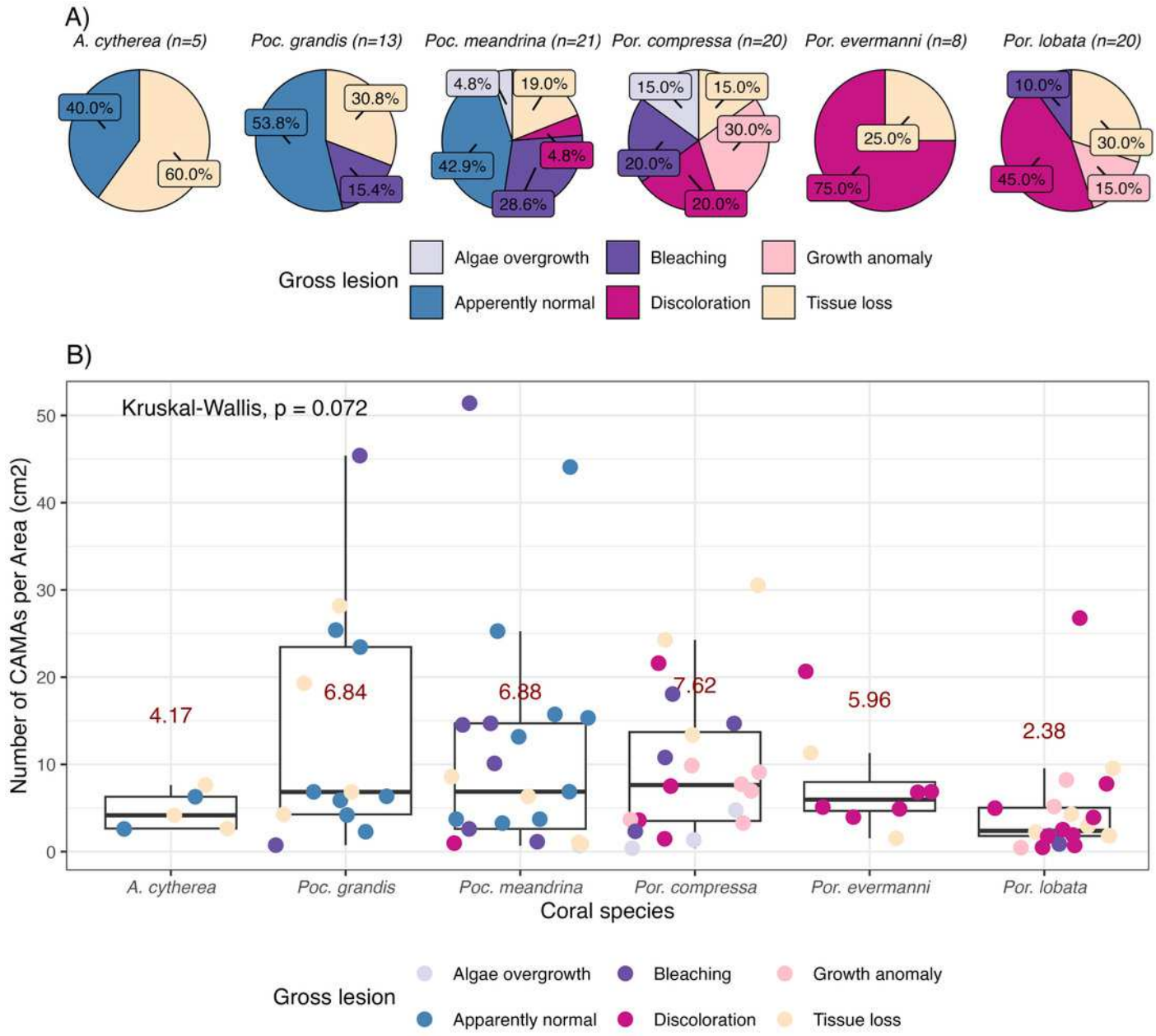

A)
A. cytherea (n=5)
Poc. grandis (n=13)
Poc. meandrina (n=21)
Por. compressa (n=20)
Por. evermanni (n=8)
Por. lobata (n=20)
40.0%
60.0%
53.8%
30.8%
15.4%
4.8%
19.0%
42.9%
4.8%
28.6%
15.0%
15.0%
30.0%
20.0%
20.0%
25.0%
75.0%
10.0%
30.0%
45.0%
15.0%
Gross lesion
Algae overgrowth
Apperently normal
Bleaching
Discoloration
Growth anomaly
Tissue loss
B)
Kruskal-Wallis, p = 0.072
Number of CAMAs per Area (cm2)
0
10
20
30
40
50
4.17
6.84
6.88
7.62
5.96
2.38
A. cytherea
Poc. grandis
Poc. meandrina
Por. compressa
Por. evermanni
Por. lobata
Coral species
Gross lesion
Algae overgrowth
Apparently normal
Bleaching
Discoloration
Growth anomaly
Tissue loss

Figure 3

Boxplot for number of Cell-Associated Microbial Aggregates (CAMAs) per area (cm 2) by gross lesions.

The box extent shows the middle 50% of number of CAMA/cm 2 by gross lesion. The upper and lower whiskers show upper 25% and lower 25% of Number of CAMAs/cm^2 ,excluding outliner in individual gross lesion. Middle line and red number indicates median number of CAMA/cm^2 across gross lesion. Points indicate outlier data. Algae overgrowth has the lowest median number 1.01 of CAMAs/cm^2 . Bleaching has the highest median number 10.45 of CAMAs/cm^2 . Example of gross lesion (margin indicated by arrowhead) (A) algae overgrowth in *Pocillopora meandrina* (B) bleaching in *Porites lobata* (C) discoloration in *Porites evermanni* (D) growth anomaly in *Porites compressa;* note corals exhibiting excessive growth of skeleton in relation to adjacent polyps on the same colony (E&F) tissue loss in *Poc. meandrina* and *Acropora cytherea*, respectively.

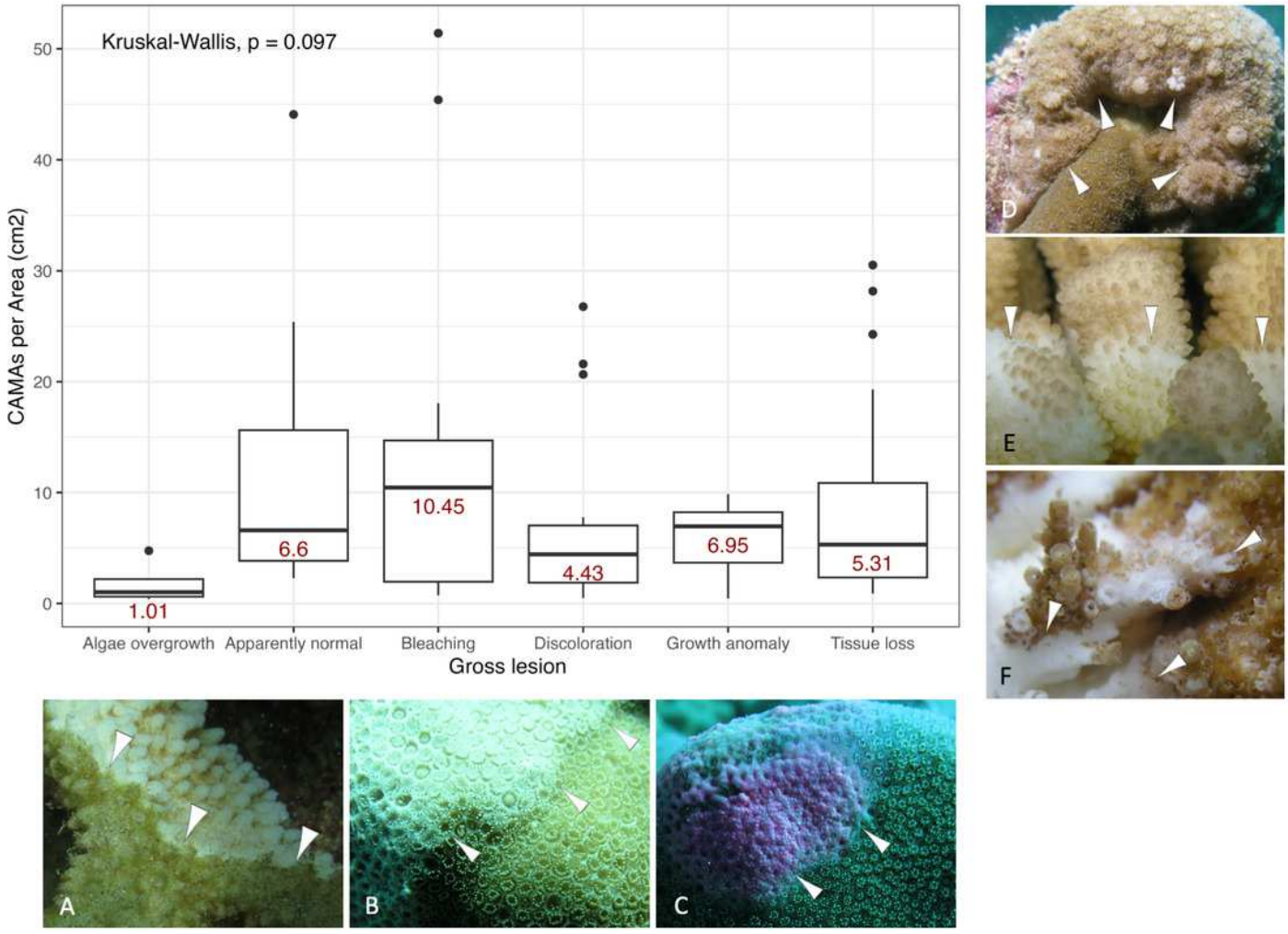

Kruskal-Wallis, p = 0.097
CAMAs per Area (cm2)
50
40
30
20
10
0
1.01
6.6
10.45
4.43
6.95
5.31
Algae overgrowth
Apparently normal
Bleaching
Discoloration
Growth anomaly
Tissue loss
Gross lesion
A
B
C
D
E
F

Figure 4

Boxplot/violin plots showing the number of Cell-Associated Microbial Aggregates (CAMAs) per area (cm^2) across different locations.

The box extent shows the middle 50% of number of CAMA/cm^2 by locations. The upper and lower whiskers show upper 25% and lower 25% of Number of CAMAs/cm^2 ,excluding outliner in individual locations. Middle line and red dot indicates median number of CAMA/cm^2 across locations. The violin width shows frequency of the value, the wider sections indicating higher frequently of the value of CAMAs/cm^2 . Corals from Island of Hawai ‘ i had significantly more CAMAs/cm^2 than those from O ‘ ahu (Kruskal-Wallis test, $p = 0.0094$; Dunn's post-hoc test with Holm correction, $p = 0.0077$).

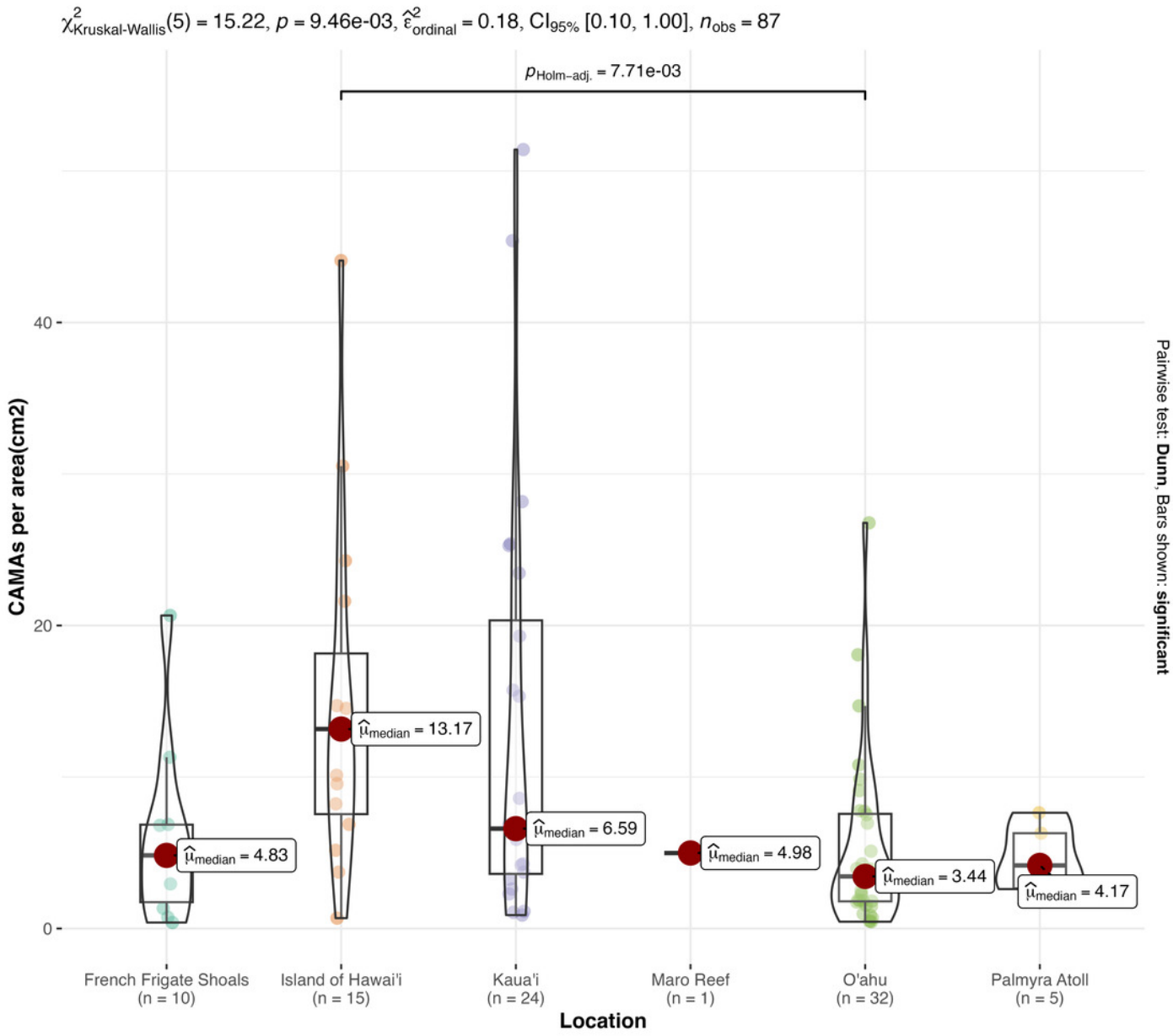

$\chi^2_{Kruskal\text{-}Wallis}(5) = 15.22$, $p = 9.46e\text{-}03$, $\hat{\varepsilon}^2_{ordinal} = 0.18$, $CI_{95\%}$ [0.10, 1.00], $n_{obs} = 87$
$p_{Holm\text{-}adj.} = 7.71e\text{-}03$
CAMAs per area(cm2)
40
20
0
$\hat{\mu}_{median} = 4.83$
$\hat{\mu}_{median} = 13.17$
$\hat{\mu}_{median} = 6.59$
$\hat{\mu}_{median} = 4.98$
$\hat{\mu}_{median} = 3.44$
$\hat{\mu}_{median} = 4.17$
French Frigate Shoals (n = 10)
Island of Hawai'i (n = 15)
Kaua'i (n = 24)
Maro Reef (n = 1)
O'ahu (n = 32)
Palmyra Atoll (n = 5)
Location
Pairwise test: Dunn, Bars shown: significant

Figure 5

Boxplot/violin plots showing Cell-Associated Microbial Aggregates (CAMAs) per area (cm^2) in paired apparently normal and lesion fragments from diseased coral colonies (n = 35).

The box extent shows the middle 50% of number of CAMA/cm^2 by status of fragments. The upper and lower whiskers show upper 25% and lower 25% of Number of CAMAs/cm^2 ,excluding outliner in individual status. Middle line indicates median number of CAMA/cm^2 across status. The violin width shows frequency of the value, the wider sections indicating higher frequently of the value of CAMAs/cm^2 . There was no significant difference in CAMAs per area between normal and lesion fragments (Wilcoxon signed-rank test, p = 0.10).

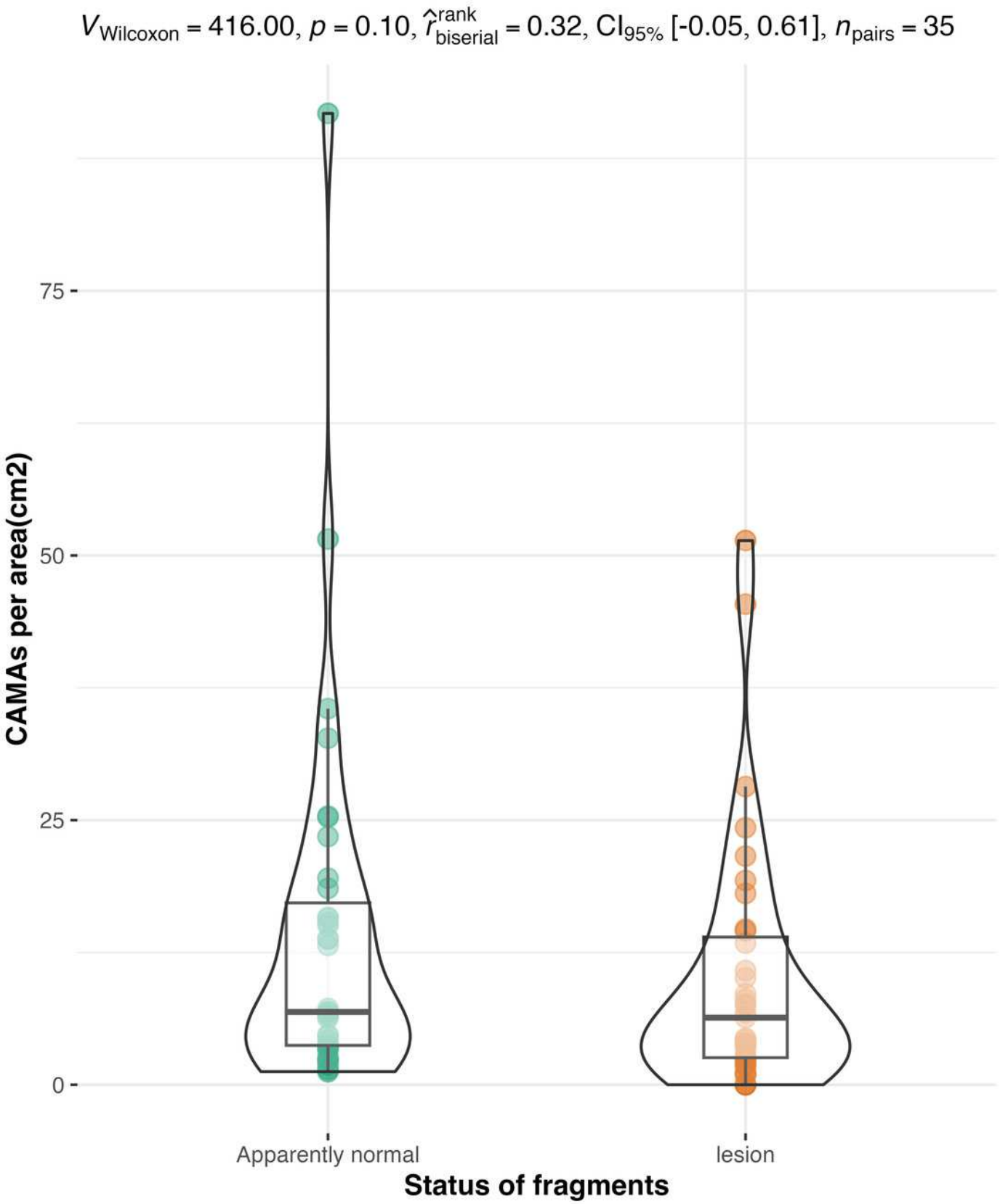

$V_{\text{Wilcoxon}} = 416.00$, $p = 0.10$, $\hat{r}^{\text{rank}}_{\text{biserial}} = 0.32$, $\text{CI}_{95\%}$ [-0.05, 0.61], $n_{\text{pairs}} = 35$
75
50
25
0
CAMAs per area(cm2)
Apparently normal
lesion
Status of fragments

Figure 6

Distribution of CAMAs by (A) anatomical location (B) tissue layers.

In A, contrast preponderance of bacteria in coenosarc of *Acropora cytherea*, *Porites compressa*, and *Porites lobata* compared to tentacles for *Pocillopora* spp. and *Porites evermanni*. In B, note dominance of epidermal bacterial colonization in *Pocillopora* spp. in contrast to gastrodermis for *Porites* spp. and *A. cytherea*. (n = total CAMA count). *Poc*.=*Pocillopora*, *Por*.=*Porites*, and *A*.=*Acropora.*

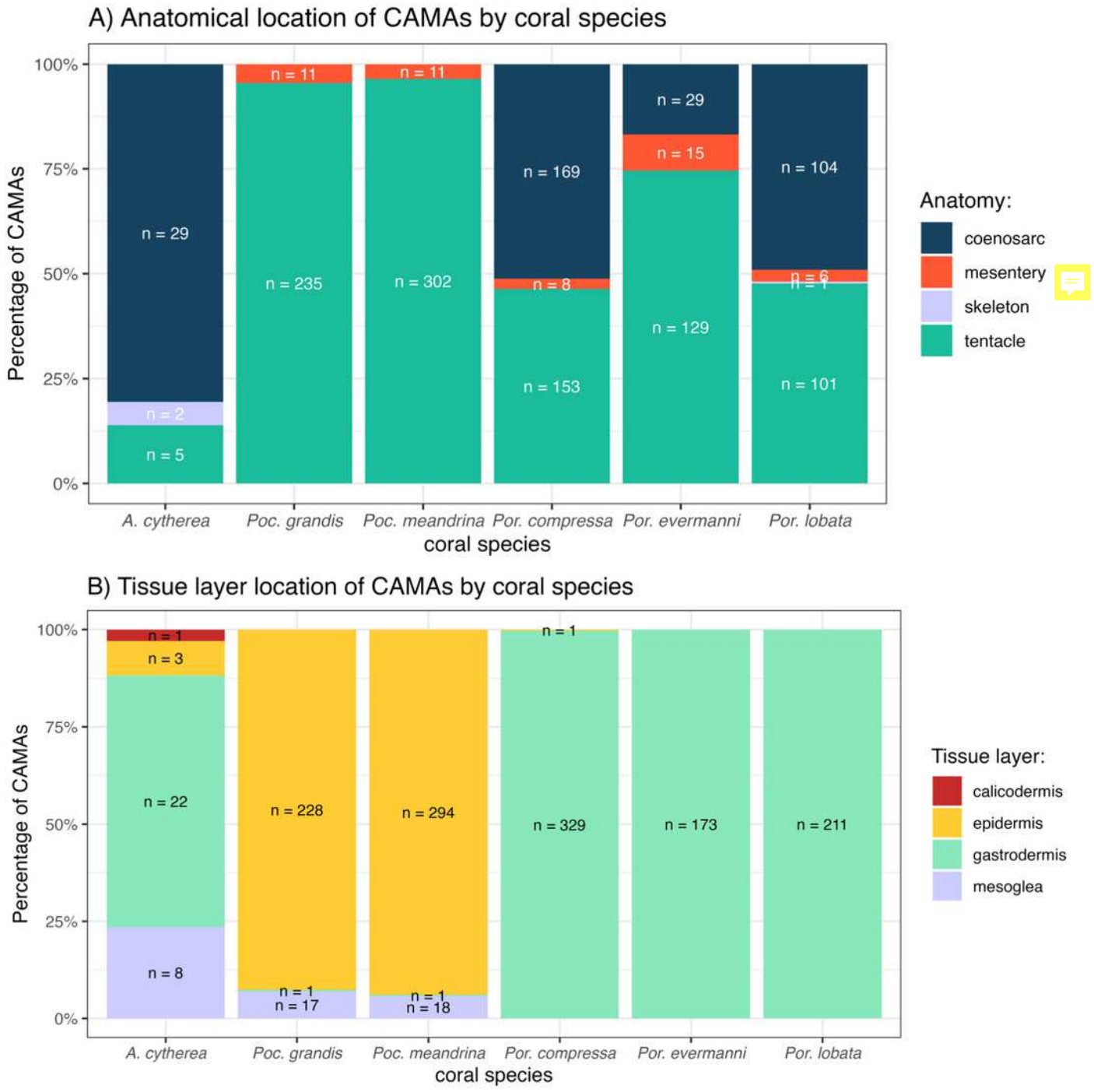

A) Anatomical location of CAMAs by coral species
Percentage of CAMAs
100%
75%
50%
25%
0%
n = 29
n = 2
n = 5
n = 11
n = 235
n = 11
n = 302
n = 169
n = 8
n = 153
n = 29
n = 15
n = 129
n = 104
n = 6
n = 101
A. cytherea
Poc. grandis
Poc. meandrina
Por. compressa
Por. evermanni
Por. lobata
coral species
Anatomy:
coenosarc
mesentery
skeleton
tentacle
B) Tissue layer location of CAMAs by coral species
Percentage of CAMAs
100%
75%
50%
25%
0%
n = 1
n = 3
n = 22
n = 8
n = 228
n = 1
n = 17
n = 294
n = 1
n = 18
n = 1
n = 329
n = 173
n = 211
A. cytherea
Poc. grandis
Poc. meandrina
Por. compressa
Por. evermanni
Por. lobata
coral species
Tissue layer:
calicodermis
epidermis
gastrodermis
mesoglea

Figure 7

Gram stain images of Cell-Associated Microbial Aggregates (CAMAs) from various Pacific Ocean corals and reference Gram-stain images of confirmed using culture and molecular techniques.

(A) Gram-negative rods clustered (black arrow) and diffusely distributed (white arrowhead) in the epidermis and gastrodermis of the tentacle of *Poc illopora grandis* from Kaua‘i. (B) Gram-negative cocci (arrow) in the coenosarc skeleton of *Porites lobata* from O‘ahu. (C) Gram-negative cocci (arrow) in the gastrodermis of *Porites compressa* from O‘ahu. (D) Gram-positive rod (arrow) in the basal body wall gastrodermis of *Por. compressa* from the Island of Hawai‘i. (E) Gram-positive cocci CAMAs (arrow) in the gastrodermis of *Porites lobata* from the Island of Hawai‘i. Abbreviations: g = gastrodermis, e = epidermis, sk = skeleton, z = symbiodiniaceae, black arrowhead = calicodermis, arrow = CAMAs. (F) clustered of *Salmonella typhimurium,* Gram-negative rods (black arrow) in the kidney of White-tailed tropicbird (*Phaeton lepturus*). (G) *Erysipelothrix rhusiopathiae,* Gram-positive rods focally distributed (black arrow) in the kidney of a Laysan duck (*Anas laysanensis*). Scale bar = 10 µm (A&D) and 30 µm for all other plates.

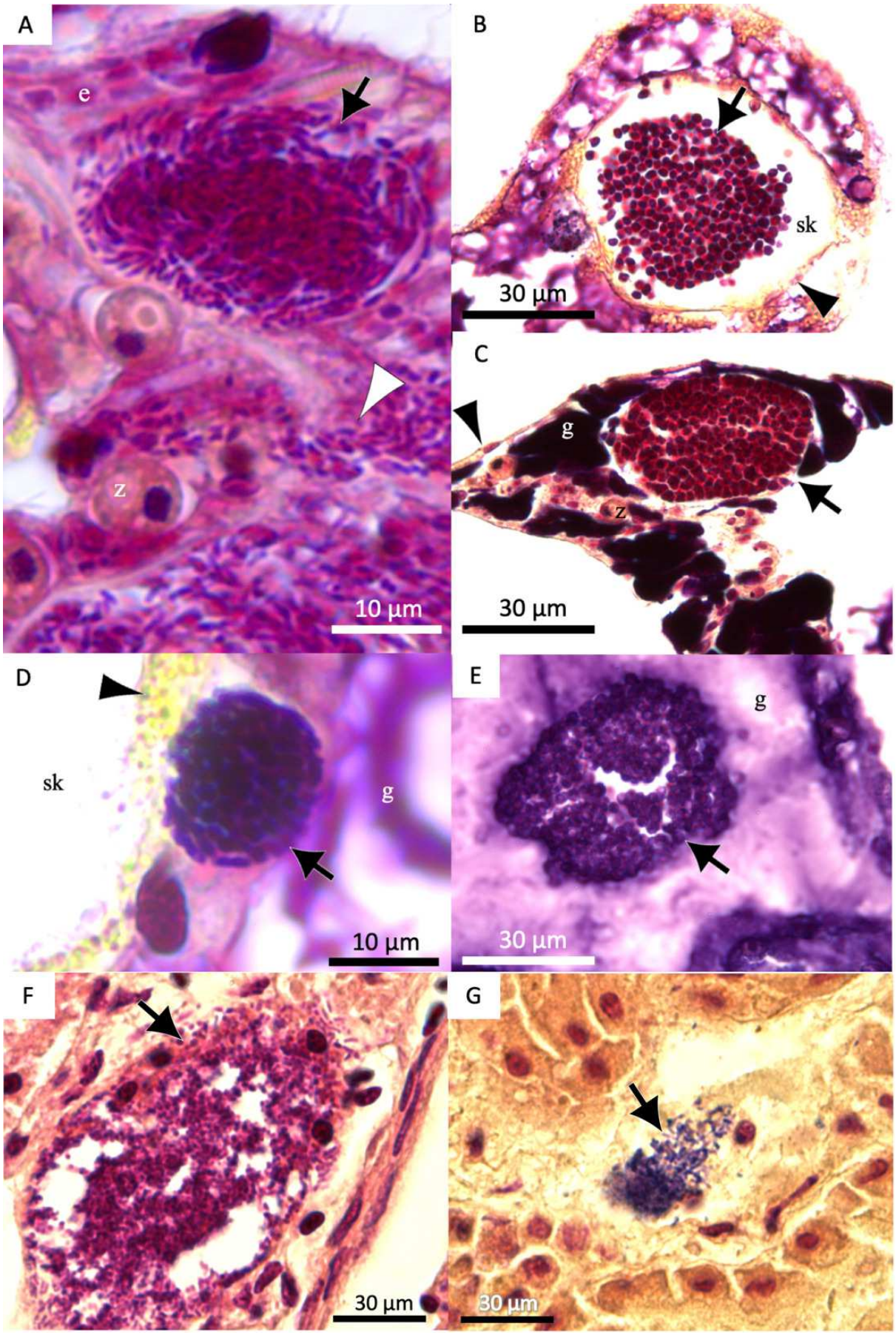
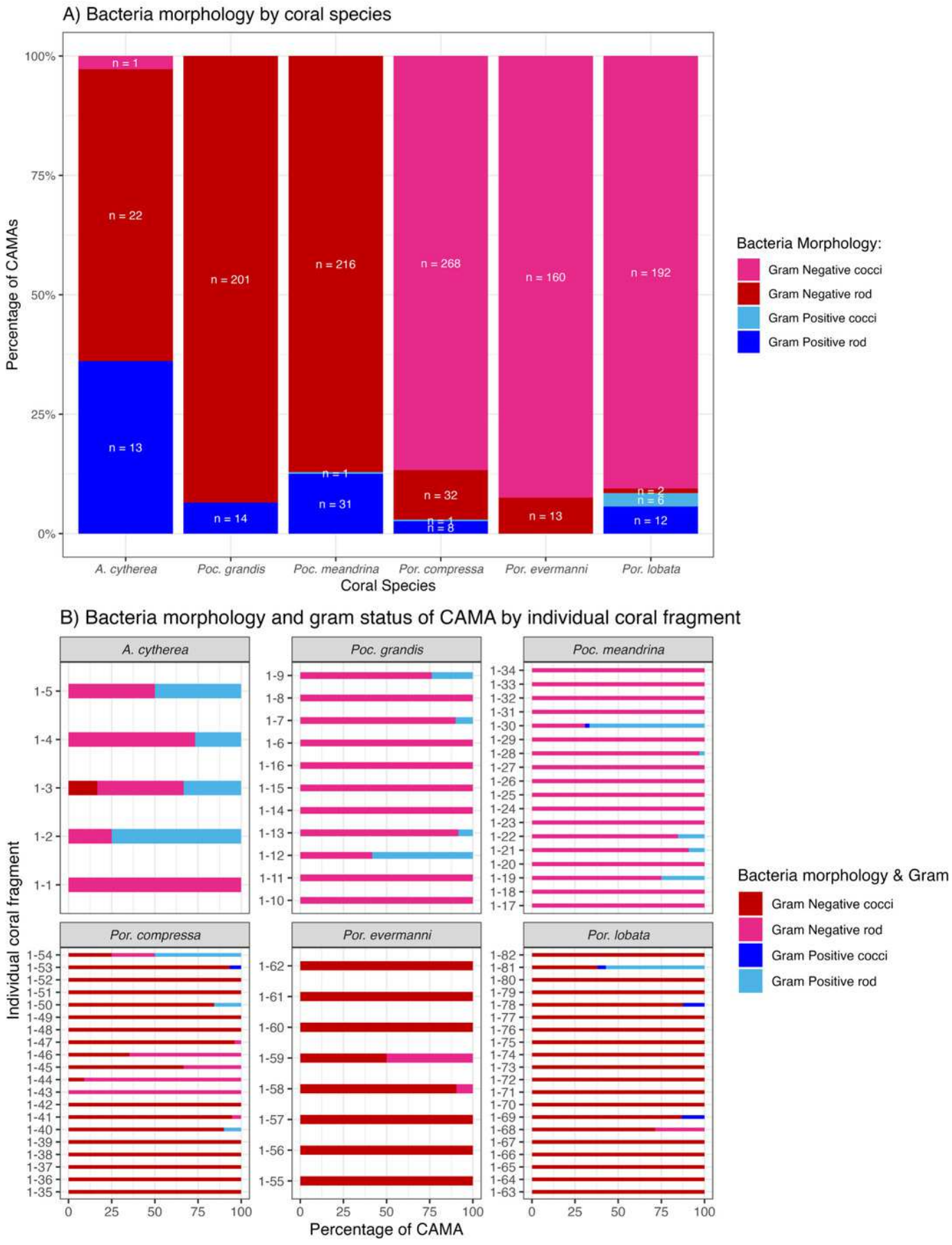

A
e
z
10 µm
B
sk
30 µm
C
g
z
30 µm
D
sk
g
10 µm
E
g
30 µm
F
30 µm
G
30 µm

Figure 8

Morphology and gram status of Cell-Associated Microbial Aggregates (CAMAs) by (A) coral species and (B) individual coral fragment.

(A) Note that Gram-negative rods dominate for *Porites* (*Por.*), Gram-negative coccoid dominate for *Pocillopora* (*Poc.*) and *Acropora* (*A.*) are distinguished by relatively high abundance of Gram-positive bacteria. (n = total CAMA count). (B) Note low diversity of bacteria morphologies in *Por. evermmani.*


A) Bacteria morphology by coral species
Percentage of CAMAs
100%
75%
50%
25%
0%
n = 1
n = 22
n = 13
n = 201
n = 14
n = 216
n = 1
n = 31
n = 268
n = 32
n = 1
n = 8
n = 160
n = 13
n = 192
n = 2
n = 6
n = 12
A. cytherea
Poc. grandis
Poc. meandrina
Por. compressa
Por. evermanni
Por. lobata
Coral Species
Bacteria Morphology:
Gram Negative cocci
Gram Negative rod
Gram Positive cocci
Gram Positive rod
B) Bacteria morphology and gram status of CAMA by individual coral fragment
Individual coral fragment
Percentage of CAMA
Bacteria morphology & Gram
Gram Negative cocci
Gram Negative rod
Gram Positive cocci
Gram Positive rod