



OPEN An exploratory investigation into the microbial and cyanobacterial presence on skin epibiotia and orofacial lesions in estuarine common bottlenose dolphins (*Tursiops truncatus*) through metabarcoding

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Estuarine common bottlenose dolphins (*Tursiops truncatus*) inhabiting the Indian River Lagoon (IRL) have been documented with light brown skin lesions, loosely categorized as “algal sheens”. In recent years, dolphins from the IRL have also exhibited unusual necro-ulcerative orofacial lesions. To date, no metabarcoding investigation has been conducted to determine whether “algal sheens” observed on the dolphins are pathogenic, opportunistic or are indicators of poor health or water quality. The aim of this study was to investigate the etiology of “algal sheens” and orofacial lesions, to assess the pathogenic potential. In this study, 13 skin and “algal sheen” swabs from 11 dolphins were sequenced using metabarcoding techniques. Genus-level identification was achieved and compared. Results from both “algal sheen” and orofacial lesion samples demonstrated higher bacterial diversity than reported in normal bottlenose dolphin skin microbiomes. Previously unreported bacterial genera were identified, genera containing pathogenic species found in both sample types. Species responsible for “algal sheens” were not definitively identified. Numerous cyanobacterial genera typically found in eutrophic conditions were identified.

Keywords Dolphin mortality, Metabarcoding, Pathogenic, *Tursiops truncatus*

Defining the categories and prevalence of estuarine bottlenose dolphin (*Tursiops truncatus* and *Tursiops erebennus*¹) skin lesions have been the focus of recent research, as lesion etiologies have been historically uncharacterized^{2–4}. As with most mammals, dolphin skin is critical to their immune system, acting as a physical barrier and supported by immune cells⁵. Dolphin skin has a high sloughing rate, with the outermost layer of healthy skin shedding once every two hours⁶. Due to this near constant slough rate, exogenous organisms may either colonize and/or penetrate through many cell layers or opportunistically settle on impaired skin that is not sloughing.

Epidermal skin disease, growths, sheens, or mats found on dolphins may be associated with opportunistic colonization of a compromised animal^{7,8}. Dolphin epidermal disease is often associated with freshwater exposure and epidermal growths have been reported originating from fungi, bacteria, and cyanobacteria^{8,9}. Proliferative epidermal growths have also been reported as secondary constituents from animals that have had viruses that cause skin lesions (e.g. poxvirus) or nonspecific physical trauma (e.g. boat strike or entanglement). However, the etiology of these epidermal growths is widely unknown⁷. Skin lesions may persist over many years, potentially indicating chronic decreased immune status or prolonged exposure to poor water quality¹⁰. Epidermal growths

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of photosynthetic organisms on dolphins inhabiting turbid, estuarine water are likely facilitated by compromised animals staying closer to the surface, either through rest behavior^{11,12} or increased respiration.

The epidermal bacterial microbiome of bottlenose dolphins (*T. truncatus*) from both captive and wild animals has been previously characterized through next generation sequencing techniques^{10,13}. Both studies noted a significant degree of differentiation between bacterial microbiomes of individual dolphins. A potential explanation for this variance in wild dolphins is associated with the changing environmental conditions in individuals' habitats¹⁰. Additionally, Russo et al.¹⁰ noted that coastal bottlenose dolphins (those inhabiting areas ~1 km to shoreline) were more susceptible to skin lesions and bacterial skin disease due to the greater variances in environmental disturbance.

The Indian River Lagoon (IRL) is a shallow water, estuarine aggregate of three systems (the Indian and Banana Rivers and Mosquito Lagoon) that extends 250 km along the east coast of Florida. The majority of this microtidal system is enclosed and is subject to low water exchange rates and long water residence times¹⁴. This system has been subject to poor water quality, recurrent harmful algal blooms, seagrass meadow die-offs, fish kills, and multiple dolphin unusual mortality events^{15–17}. Dolphins residing in the IRL are susceptible to a wide range of diseases and anthropogenic pollution, including exposure to *E. coli* and mercury bioaccumulation^{17–19}. IRL dolphins are considered immunocompromised²⁰ and may be more susceptible to opportunistic skin infections as they exhibit high site fidelity in a highly anthropogenically polluted shallow estuarine system^{21,22}. Supporting this, a recent population health evaluation found skin lesions in nearly all free-swimming IRL dolphins²³. A large proportion of animals were observed with light brown epidermal epibiotic growths (loosely described as “algal sheens”), which are currently not known to be pathogenic or benign²³. Additionally, free-swimming animals with extreme orofacial necrosis (often mandible/maxilla grossly exposed) have also been observed (W. Noke Durden, personal observation). To date, the presence of “algal sheens” has not been investigated as a potential health indicator, nor has the etiology or pathogenesis of the orofacial necrosis been determined. A large data gap exists regarding whether “algal sheens” are harmful or could contain species that are opportunistic or pathogenic and lead to infections, such as observed orofacial necrosis.

The purpose of this study was to use metabarcoding techniques to identify microbial assemblages present on stranded IRL dolphins with common epidermal “algal sheens” and orofacial necrosis of unknown origin. Dolphin skin lesions and epidermal growths have previously been investigated through culturing techniques^{8,9}. However, a full characterization of bacterial and cyanobacterial communities found in dolphin lesions or epidermal growths through metabarcoding has not been evaluated. Metabarcoding is beneficial as it allows microbial community comparison without visual identification, as well as the sequencing of species that cannot be cultured. Utilizing this approach, the study aimed to identify the collective community of organisms present on dolphin “algal sheens” and potential causative agents of necrotic orofacial lesions.

Methods

Stranding responses were conducted under a Stranding Agreement between Hubbs Seaworld Research Institute and the National Oceanographic and Atmospheric Administration (NOAA) Fisheries. Skin samples and presumptive “algal sheen” swabs were collected from deceased dolphins between 2010 and 2022 and immediately frozen in either -20°C for “algal sheen” samples and -80°C for necrotic orofacial lesions. The handling of dolphin swabs and tissue samples, along with associated information (e.g. necropsy reports, photographs, GPS coordinates), were collected in accordance with the Marine Mammal Protection Act pursuant to NOAA's Code of Federal Regulations 50CFR216.22. Stranding locations for all dolphins visualized using ArcGIS 10.8.2 (ESRI; Redlands, California).

“Algal sheen” was a presumptive label attributed to unknown orange to beige growths on the animals. Animals selected for analyses were in decomposition categories Code 2 (fresh dead; $n=5$) and code 3 (moderately decomposed; $n=6$)²⁴. Sex was determined by external and internal examination. Age class estimation was based on total length (adult male [≥ 246 cm], adult female [≥ 231 cm], juvenile male [161–245 cm], juvenile female [161–230 cm]²⁵) and reproductive productive assessment was used to refine age class estimates (adult corpora presence or presence of spermatozoa and associated interstitial tissue density²⁶). Body condition was assessed by standard methods²⁷ and animals were systematically evaluated for evidence of human interaction (entanglement, ingestion of marine debris, vessel-strike-propeller wounds^{28,29}). Animals were also thoroughly assessed for epidermal lesion presence and described based on previously published literature^{30–34}. Both “algal sheens” and orofacial lesions were graded based on severity and prevalence. Presumptive “algal sheen” categories were defined to match Bearzi's evaluation of skin lesions³⁵ for consistency. “Algal sheen” prevalence was categorized as mild when confined to one portion of the animal, moderate when it was present along more than one aspect of the animal, but covering less than 50%, or diffuse when the “algal sheen” covered approximately 50% or more of the dolphin. It is important to note that there are many factors that may bias assessment including, overexposed images or observations in shaded areas, may inhibit accurate sheen assessment. Furthermore, carcass transport (stretchered or towed) and/or decomposition may reduce affected areas if sloughed off. The classification severity of orofacial lesions was adapted from Knowlton et al.³⁶, where physical damage from entanglement was graded. Orofacial lesion severity was categorized as follows: Minor necrotic lesions were defined as those that presented necrosis of the skin and/or hypodermis along jaws and/or oral mucosa/palate. Moderate necrotic lesions were defined as those that extended to the bone (maxilla or ramus). Lastly, severe necrotic lesions were defined as those that involved bone erosion of the maxilla, mandible, or tooth loss. For the purpose of this study, samples were classified grossly as those taken from necrotic orofacial lesions or those containing an “algal sheen”. In two cases (Hubbs-2018-Tt and Hubbs-2104-Tt), samples were taken from representative areas where both lesion types were present. A total of 13 samples were sequenced from 11 dolphins (Table 1). Archived samples utilized in this study were limited in scope as they did not have matched healthy tissue or environmental samples for comparison.

Field identifier	Sample no	Date	Decomposition code	Sex	Age class	Type of lesion	Prevalence of lesion	Location of algae	Additional skin lesions present	Body condition	Genbank accession number
Hubbs-1319-Tt	Sample 1	3/10/2013	2	M	Adult	Algal sheen	Moderate on head, left lateral and flukes	Rostrum, gape and lateral sides	No	Thin	SAMN47938985
Hubbs-2018-Tt	Sample 2 (algae) Sample 3 (jaw necrosis)	3/23/2020	2 (alive prior)	M	Juvenile	Algal sheen and necrotic orofacial lesions	Algal sheen: diffuse when observed free swimming, mild when recovered Orofacial lesion: Severe	Face and dorsal aspect of the head	Ovoid, tattoo-like lesions; extensive orofacial necrosis with bone erosion, oral mucosa, tongue, and palate present extensive necrotic tissue	Emaciated	SAMN47938986, SAMN47938987
Hubbs-2226-Tt	Sample 4	7/24/2022	2	M	Juvenile	Necrotic orofacial	Severe	NA	Deep, necrotic orofacial lesions with bone involvement; raised skin lesions on the leading edge of the right pectoral flipper	Emaciated	SAMN47938988
Hubbs-1037-Tt	Sample 5	3/4/2010	2 (alive prior)	M	Adult	Algal sheen; freshwater exposure	Diffuse	Lateral sides, ventrum, dorsum	Freshwater exposure lesions: raised, ulcerated and lesions with red mottling	Ideal	SAMN47938989
Hubbs-2004-Tt	Sample 6	2/9/2020	3 (early)	F	Adult	Algal sheen	Moderate on face and peduncle	Dorsal and lateral aspects	Pox-like and ulcerated	Emaciated	SAMN47938990
Hubbs-2015-Tt	Sample 7	4/22/2020	3 (early)	F	Adult	Algal sheen; consistent with freshwater exposure	Mild overall coverage, large percentage on face	Lateral sides and face	Ovoid "rust" colored lesions consistent with freshwater exposure	Emaciated; pregnant	SAMN47938991
Hubbs-2046-Tt	Sample 8	12/12/2020	3	M	Adult	Algal sheen	Moderate; diffuse on dorsal fin, flippers and face	Diffusely over body	Pox like lesions, ulcerated lesions	Thin	SAMN47938992
Hubbs-1249-Tt	Sample 9	8/20/2012	2	M	Juvenile	Necrotic orofacial lesions	Moderate; tongue is truncated	NA	Jaw and palate necrosis	Emaciated	SAMN47938993
Hubbs-2021-Tt	Sample 10	6/28/2020	3 (late)	F	Adult	Algal sheen	Mild	Surrounding dorsal fin	CBD	CBD	SAMN47938994
Hubbs-2104-Tt	Sample 11 (algae) Sample 12 (jaw necrosis)	1/31/2021	3	M	Juvenile	Algal sheen and necrotic orofacial lesions	Algal sheen: moderate (lateral face, Orofacial lesion: severe)	Peduncle and face	Pox-like lesion; extensive orofacial necrosis and bone erosion and oral mucosa, tongue and palate necrosis	Emaciated	SAMN47938995, SAMN47938996
Hubbs-2206-Tt	Sample 13	4/3/2022	3 (early)	F	Juvenile	Algal sheen	Mild (rostrum, gape)	Rostrum and gape	No	Ideal	SAMN47938997

Table 1. Sample information and life history characteristics associated for each sampled Indian River Lagoon dolphin. Decomposition codes included code 2 (early decomposition) and code 3 (moderate decomposition).

Samples were extracted using a DNeasy PowerSoil Extraction Kit (Qiagen, Hilden, Germany) with a negative control (*i.e.* kit buffer blank) for each subset of extractions and for each primer (Nanopure H₂O). The kit buffer blank was subjected to the same procedures as all samples and later used in the sequence analysis to account for laboratory contamination. Samples were prepared using a two-step PCR approach for Illumina amplicon sequencing, targeting the V4-V5 region of the 16S rRNA gene. The initial PCR was completed to amplify the three barcode markers in individual reactions using specific primers with the attached Illumina adapter (Supp Table 1). The primary PCR amplification was completed in 25 µl reactions using 12.5 µl of Q5 High-Fidelity 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 1.0 µl of each primer (1 µM), 9.5 µl RNase-free H₂O, and 1 µl eDNA. Thermocycler parameters were tailored for the individual primer sets used to amplify the 16S regions (Supp Table 1). Following initial PCR amplification, samples were sent to the University of Tennessee, Knoxville (UTK) to be cleaned using Agencourt AmPure XP beads (Beckman Coulter Inc., Indianapolis, IN, USA) and quantified using a Qubit Fluorometer (v.2.0; ThermoFisher Scientific, Waltham, MA, USA). Samples were then normalized, and a second PCR reaction was performed at UTK to apply the indexing primers following the

Illumina 16S protocol³⁷. A second PCR clean-up was performed, and samples were quantified using a Qubit Fluorometer. Libraries were loaded with 25% PhiX clustering control on the Illumina MiSeq platform for 300 b.p. × 2 paired end reads using the V3 kit.

Demultiplexing and adapter removal was performed by the genomics facility at UTK, which provided a single fastq file for forward and reverse reads for each sample at each primer. Primers were trimmed using cutadapt version 4.2³⁸. Reads were quality filtered based on Q30 scores and trimmed to remove low-quality reads using the DADA2 pipeline³⁹. R1 and R2 sequences were truncated and filtered to remove any low-quality reads ($\text{truncLen} = c(240, 180)$, $\text{maxEE} = c(2, 2)$, $\text{truncQ} = 2$) for a total retained length (42), ensuring a sufficient read overlap for the merging step in DADA2. Filtered reads were denoised and dereplicated using DADA2 to produce Amplicon Sequence Variants (ASVs). Singletons (sequences with only one read assigned) and chimeras were removed from the dataset. For both the universal 16 s rRNA dataset and the cyanobacterial specific 16 s rRNA dataset, the SILVA database (release 138.1)⁴⁰ appended with CyanoSeq⁴¹ was employed to assign taxonomy. ASVs identified as chloroplast or mitochondria in the 16S dataset were removed, as these are not prokaryotic sequences (Supp Material).

Alpha and beta diversity assessments were conducted to determine mean species diversity across samples (*i.e.* within-sample diversity) and between samples (*i.e.* between sample diversity). Alpha diversity was assessed in R.Studio (ver 4.5.2) using Chao1, Shannon and Inverse Simpson methods and were compared between groups with different body conditions as well as lesion types. For Beta Diversity, an exploratory Bray Curtis analysis was conducted and visualized using Principal Coordinates Analysis (PCoA) and significance was assessed using an exploratory Permutational multivariate analysis of variance (PERMANOVA) was conducted to analyze significance. In these analyses only lesion groups could be assessed due to limited sample sizes in body condition groups.

Results

Dolphin life history

Dolphins in this study stranded throughout the Indian River Lagoon, though animals with orofacial necrosis were localized to the Banana and Indian Rivers (Fig. 1). Eight of the nine marked dolphins could be identified as IRL residents from an existing photo ID catalog¹². Some sampled dolphins ($n=2$; 18.2%) had evidence of entanglement, both active (Hubbs-2018-Tt) and distant (*e.g.*, years prior; Hubbs-2206-Tt). Most dolphins (72.7%) presented in a compromised nutritional state (thin, $n=2$; emaciated, $n=6$), with two animals in ideal body condition and one animal where body condition could not be determined (Table 1). Lesion prevalence for the two aforementioned categories was slightly sex skewed ($n=7$ males [63.6%], $n=4$ [36.4%] female dolphins), as commonly reported in prior IRL dolphin studies^{12,42}. Most dolphins ($n=7$, 63.6%) presented other lesions (*e.g.*, ulcerated, pox-like, lesions consistent with freshwater exposure) in addition to epidermal “algal sheen”. “Algal sheen” was found predominately on the rostrum/head and lateral sides of the body (Table 1) and was not documented on the ventrum. “Algal sheen” was observed in adults ($n=6$) and juveniles ($n=3$) (total 9/11, 81.8% of tested samples), whereas orofacial necrosis was only observed in juveniles ($n=4$) (Table 1; Fig. 2). Histology reports were available for seven animals, most of which demonstrated lung disease (*e.g.*, pulmonary fibrosis or pneumonia) ($n=6$, 54.5% of sampled animals) and presented grossly in diminished body condition (thin or emaciated). All animals with orofacial lesions were emaciated ($n=4$ individuals, 100%), while animals with “algal sheens” ($n=9$ individuals) ranged in body condition, including ideal ($n=2/9$; 22.2%), thin ($n=2/9$; 22.2%) and emaciated ($n=4/9$; 44.4%). The body condition of the remaining individual was not reported.

Collective results

Data from the 16S rRNA gene were the only sequences that passed quality checks. There were 18,628 total ASV counts across all samples after quality control filtering (Supp Table 2), 1,899 of which were unique ASVs (Genbank BioSample accession numbers SAMN47938985-SAMN47938997). No single ASV was shared across all dolphin samples, demonstrating bacterial differences between areas of “algal sheens” and “orofacial necrosis” (*e.g.*, Hubbs-2018-Tt and Hubbs-2104-Tt). The reported chimera rate was 17.8%.

Alpha diversity measures revealed that numbers of ASVs differed between lesion and body condition categories. From an individual sample perspective, Chao1 species richness (S) ranged from 33 to 434 (mean = 197.9), Simpson Index (D') ranged from 4.13 to 18.63 (mean = 10.92) Shannon diversity (H') ranged from 2.06 to 3.84 (mean = 3.01). The average S, H' and D' differed between emaciated ($n=8$; mean S = 212.5, mean H' = 3.05, mean D' = 10.71), thin ($n=2$; mean S = 179, mean H' = 2.94, mean D' = 10.87) and ideal ($n=2$; mean S = 200, mean H' = 3.4, mean D' = 15.24), body types respectively (Fig. 3). Low sample sizes prevented assessment of significance, however, at a high level, individuals in ideal body condition may have higher skin microbial diversity when “algal sheens” are present as compared to emaciated groups (Fig. 3). When comparing S, H' and D' between lesion type, diversity indices indicated that “algal sheens” were more diverse in two of the three alpha diversity matrixes (“Algal sheen” $n=9$; mean S = 216.13 mean H' = 3.05, mean D' = 10.76), (Orofacial lesion $n=4$; mean S = 156.5, mean H' = 2.94, mean D' = 11.24) (Fig. 3). This may indicate that “algal sheens” contain more bacterial constituents compared to necrotic orofacial lesions.

Beta diversity only analyzed comparisons between lesion groups, as opposed to body condition, due to limited sample sizes ($n=2$) for both thin and ideal body condition categories. The PERMANOVA demonstrated significant difference ($\alpha=0.012$) between lesion groups. The first axis of the PCoA accounted for 17.6% the total variance, while the second axis accounted for 10.9% of the total variance between bacterial communities (Fig. 4). Preliminary analysis also portrayed clustering based on lesion type, particularly those with orofacial lesions (Fig. 4).

The most abundant phyla present in all samples were Bacteroida followed by Firmicutes and Proteobacteria (Fig. 5). These results contrasted those of the skin microbiota in a previous study that evaluated estuarine

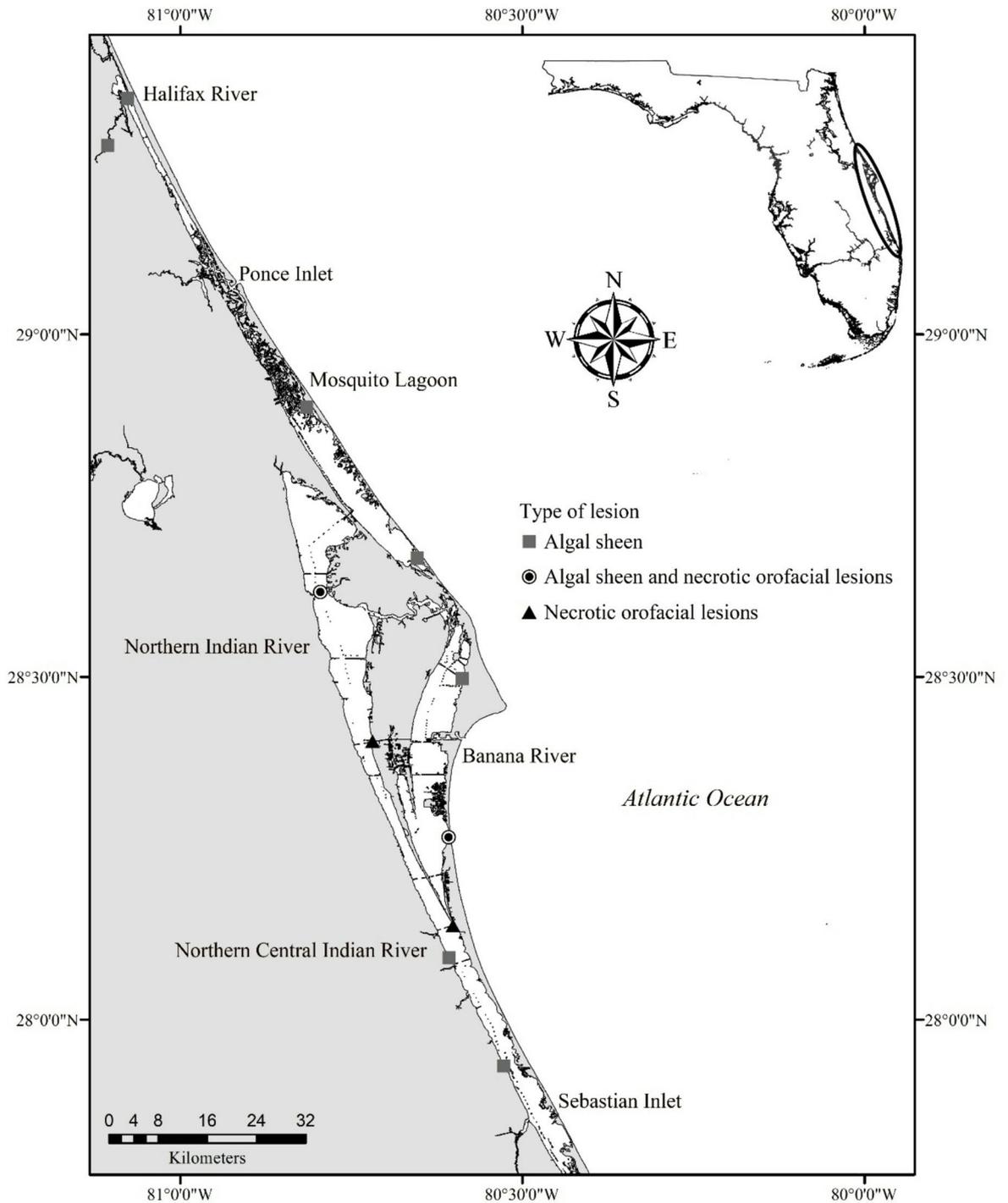


Fig. 1. A map of the Indian River Lagoon along the east coast of Florida (Ponce Inlet to Jupiter Inlet, inset). The study site included the following sub-basins: Halifax River, Mosquito Lagoon, Northern Indian River, Banana River, and the North-Central Indian River. Stranding locations of bottlenose dolphins sampled with necrotic orofacial lesions and “algal sheens” are depicted.

dolphins in Sarasota Bay, where Proteobacteria was the most abundant bacterial phylum in healthy skin samples⁴³. Furthermore, the sequenced microbial diversity for lesion samples in this study was greater than those reported from skin samples in Robles-Malagamba et al.⁴³.

No single genus was identified across all sequenced dolphins, but members of the genus *Burkholderia* were found in 12 of the 13 (92.3%) dolphin samples, in varying abundance. Seven genera were reported in 76.9% (10/13) of sequenced samples, including *Bacteroides*, *Psychrobacter*, *Clostridium*, *Porphyromonas*, *Tenacibaculum*, *Fusobacterium* and *Fusibacter* (Fig. 3). *Bacteroides* was in high abundance when sequenced (counts ranging



Fig. 2. Epidermal abnormalities evaluated in bottlenose dolphins: (A) and (B): lesions suspected to be “algal sheens”, (C) and (D): necrotic orofacial lesions.

from 176 to 30,268). Of these seven genera, five were also reported in the top 20 most abundant genera (i.e. all but *Fusobacterium* and *Fusibacter*). Interestingly, *Bacteroides* was reported as one of the top 20 most abundant bacterial skin genera from offshore, but not coastal dolphins in Russo et al.¹⁰. *Porphyromonas* spp. was reported across 69.2% (9/13) of all samples and is known to be a part of mammalian anaerobic biomes associated with skin, the oral cavity, and urogenital locations⁴⁴. Further, it was also reported as a microbial skin constituent from dolphins residing in both coastal and offshore environments¹⁰. *Tenacibaculum* spp. was reported in 76.9% of samples (10/13) and has also reported in high abundance in dolphin skin samples in Sarasota Bay⁴³, the upper respiratory tract of IRL dolphins⁴⁵, as well as wounds of other marine mammals⁴⁶. Although the species was not identified, many members of this genus are opportunistic fish pathogens⁴⁷. Lesions range from necrosis, erosion and ulceration of the mouth syndrome⁴⁷. As these correspond with clinical features observed in sequenced dolphins ($n = 5$, 45.5%), it is pertinent to further investigate of its role on dolphin morbidity (Fig. 6).

Necrotic orofacial lesions versus “algal sheens”

When comparing sequence data abundances between the two skin lesion types, the majority of dolphins with necrotic orofacial lesions had top 20 genera abundances of *Amnipylo* spp. (4/4, 100%), *Aureispira* spp. (3/4, 75%), *Flavobacterium* spp. (3/4, 75%), *Nautella* spp. (3/4, 75%) *Bacteroides* spp. (3/4, 75%), *Treponema* spp. (3/4, 75%) and *Hathewayia* spp. (4/4, 100%) (Fig. 7). While *Treponema* spp. has been reported from dolphin genital samples in Sarasota Bay⁴³, this is the first report from any other bodily location.

Animals with “algal sheens” shared more top 20 bacterial genera when compared to those with necrotic orofacial lesions (Fig. 6). For example, all animals with sheens had *Clostridium* spp., *Tenacibaculum* spp., and *Vibrio* spp. in their top 20 abundances (Fig. 8). These genera are species rich, with both pathogenic and species belonging to normal flora, found in saltwater environments^{43,48}, and their presence may be a consequence of environmental conditions. Further research should be conducted to determine the species present, as most of these animals also presented “pox-like” or “ulcerated” lesions with no defined etiology (Table 1). *Flavobacterium* spp. were also found across many “algal sheen” animals ($n = 7/9$, 78%), warranting further investigation as this phylogenetically diverse genus has species that are apart of normal microbial flora as well as opportunistically pathogenic members⁴⁹. The presence of *Aureispira* spp., *Epibacterium* spp., *Hathewayia* spp., *Nautella* spp., and *Thalassotalea* spp. were found across at least six samples (66–89%). All members of the genus *Aueispira* have been sequenced from marine environments^{50,51}, though never from a marine mammal.

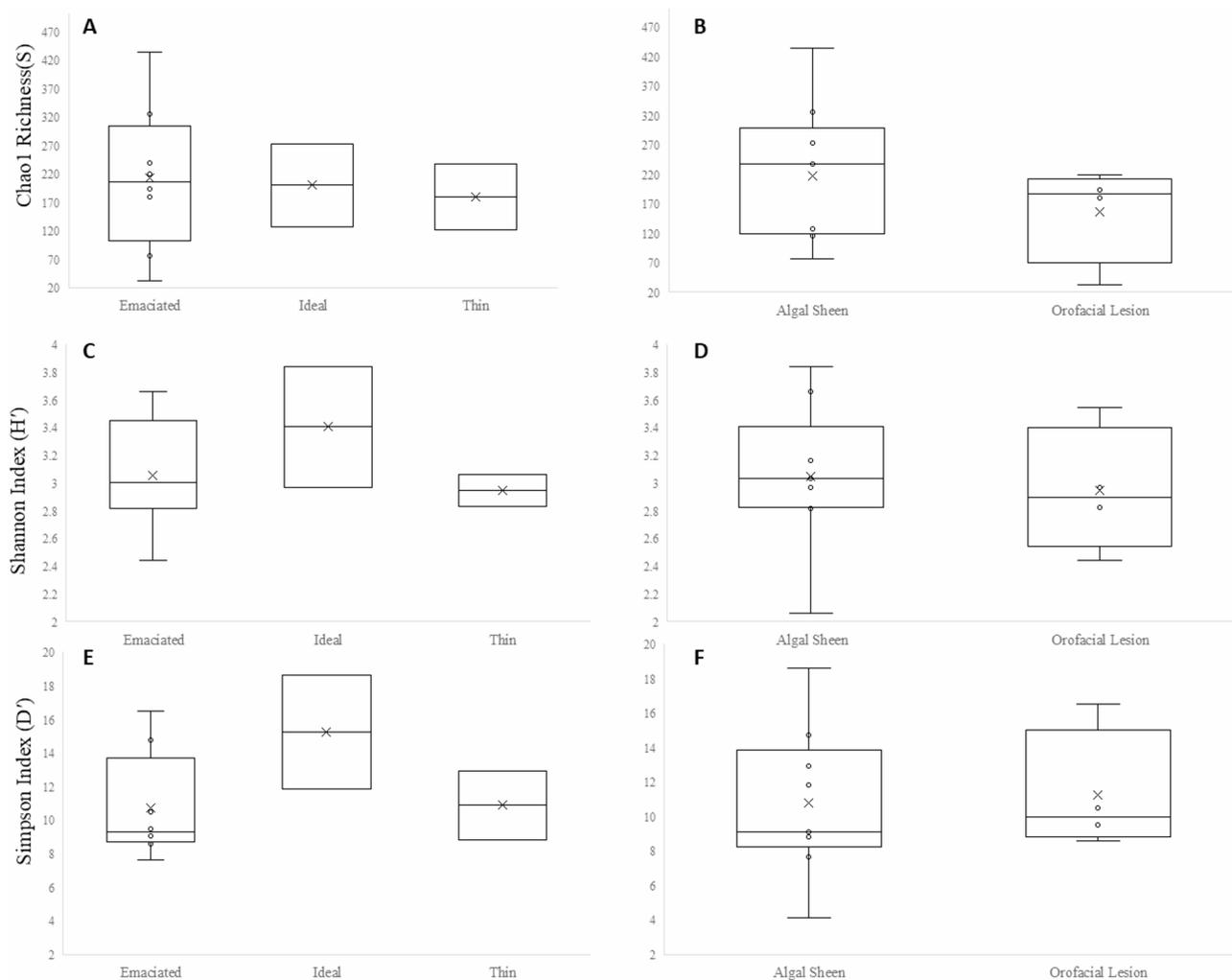


Fig. 3. Alpha diversity measures as box-and-whisker plots between dolphin body condition (A, C, E) and lesion categories (B, D, F).

Cyanobacterial genera

In the Robles-Malagamba et al.⁴³ study, the presence of epidermal cyanobacteria was reported for three dolphins. In this study, cyanobacterial species were observed on 9 of the 11 (81.8%) sampled dolphins, with multiple species being sequenced from five animals (Fig. 9). It is possible that cyanobacteria may opportunistically colonize immunocompromised or debilitated IRL dolphins. In the Robles-Malagamba et al.⁴³ study, dolphins were randomly selected, whereas our study targeted animals with similar ailments (e.g. orofacial necrosis) or visual lesions (e.g. “algal sheen”). A total of 39 cyanobacterial sequences were amplified, 38 were identifiable to the genus level. The only other reported species of cyanobacteria known to aggregate on the epidermis of compromised dolphins, *Komarekiella delphini-convector*⁹, was not present in any samples. Members of the genera *Pseudosynechococcus* and *Vulcanococcus* were present in highest abundance across three of the nine (33%) dolphins. The majority of dolphins with epidermal “algal sheens” ($n = 5/9$, 55.5%) had singular genera present in high abundance, with the highest cyanobacterial diversity retrieved from Sample 8 (Fig. 6). Interestingly, cyanobacteria were found present on the four animals with necrotic orofacial lesions, two of which also had the genus *Vulcanococcus*.

Discussion

The aim of this study was to investigate the microbial diversity present on dolphins with epidermal “algal sheens” and unusual necrotic orofacial lesions. This high-level investigation could not nor elucidate the species responsible for the “algal sheens”. It is possible that these sheens represent a mixed microbial community (e.g., diatoms, oomycetes, etc.), but the 18S region was not amplified for confirmation. This study used archived frozen samples collected between 2012 and 2022, which may be confounded by storage temperature (all “algal sheen” samples stored at $-20\text{ }^{\circ}\text{C}$ while all mouth lesions stored at $-80\text{ }^{\circ}\text{C}$), matrix type (swab versus tissue) and anatomical site collection. Although direct comparisons cannot be made, high level assessment indicated differences in individual microbial communities were influenced by body condition and lesion type. At the time of collection, matched healthy tissue samples and environmental samples were not taken, limiting direct

PCoA - Bray–Curtis Dissimilarity (Dolphin Lesions)

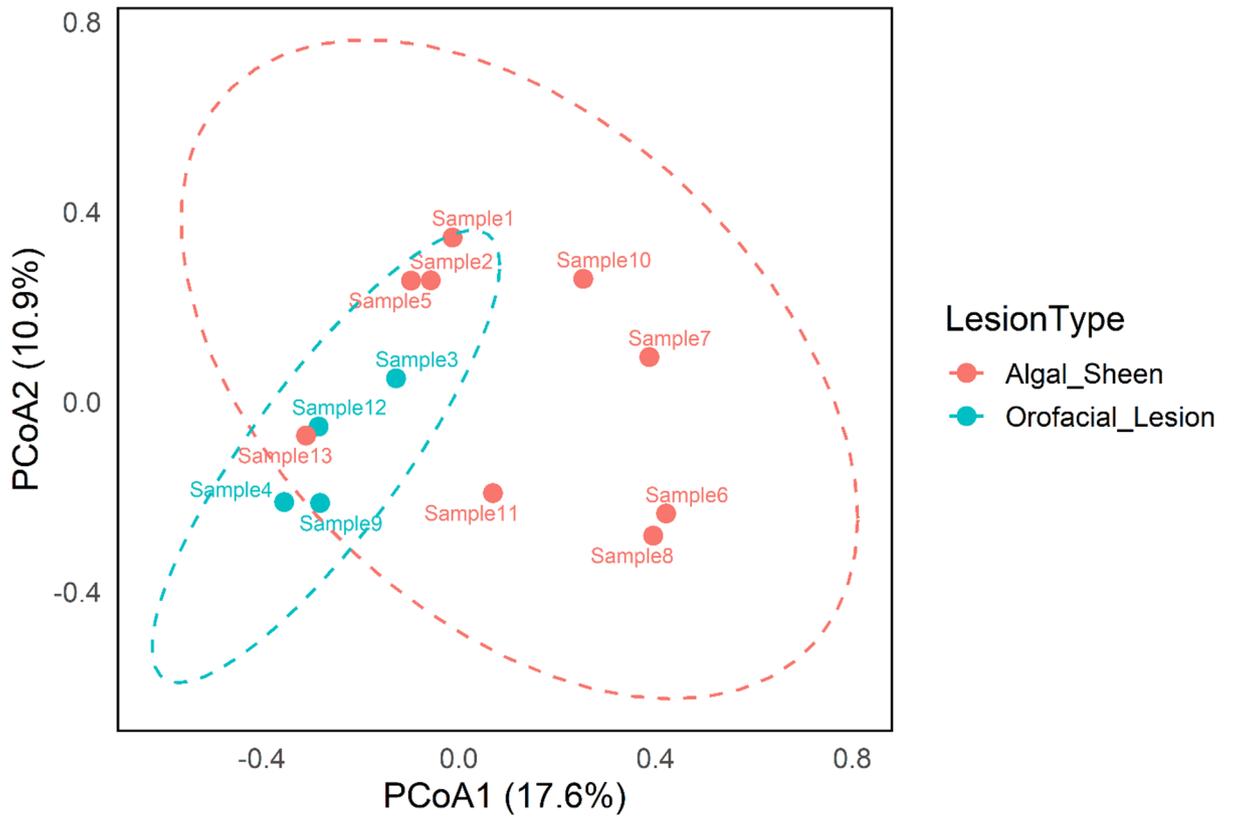


Fig. 4. PCoA demonstrating results of Beta diversity analysis between lesion groups (“algal sheen” and orofacial lesions). Each axis demonstrates a percentage of variation and dashed lines represent 95% confidence ellipses.

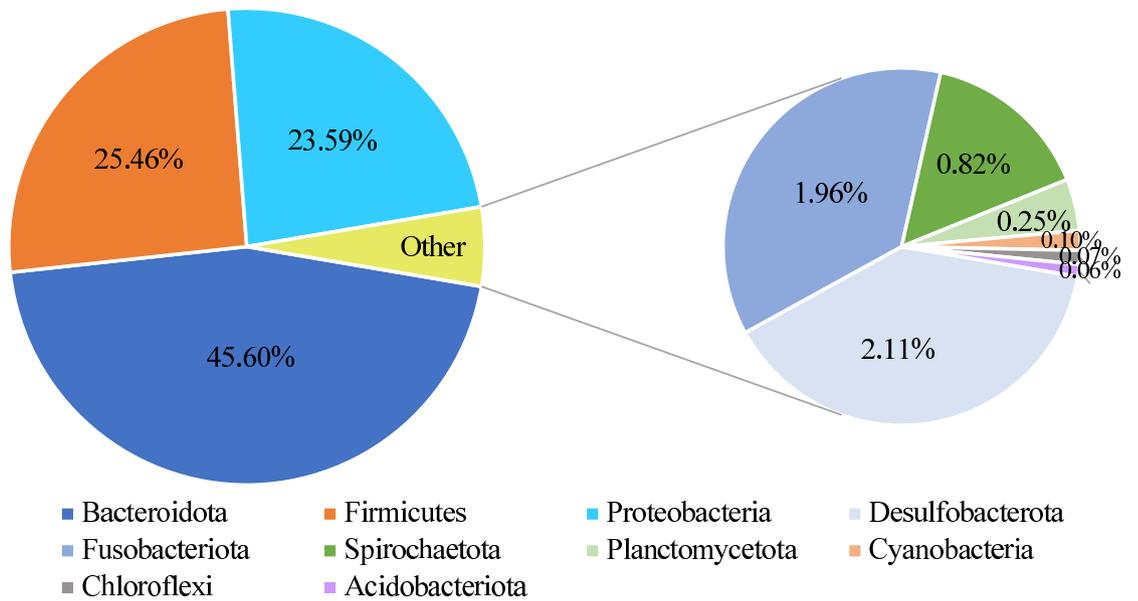


Fig. 5. Top 10 phyla based on mean percent presence across all samples from Indian River Lagoon dolphins.

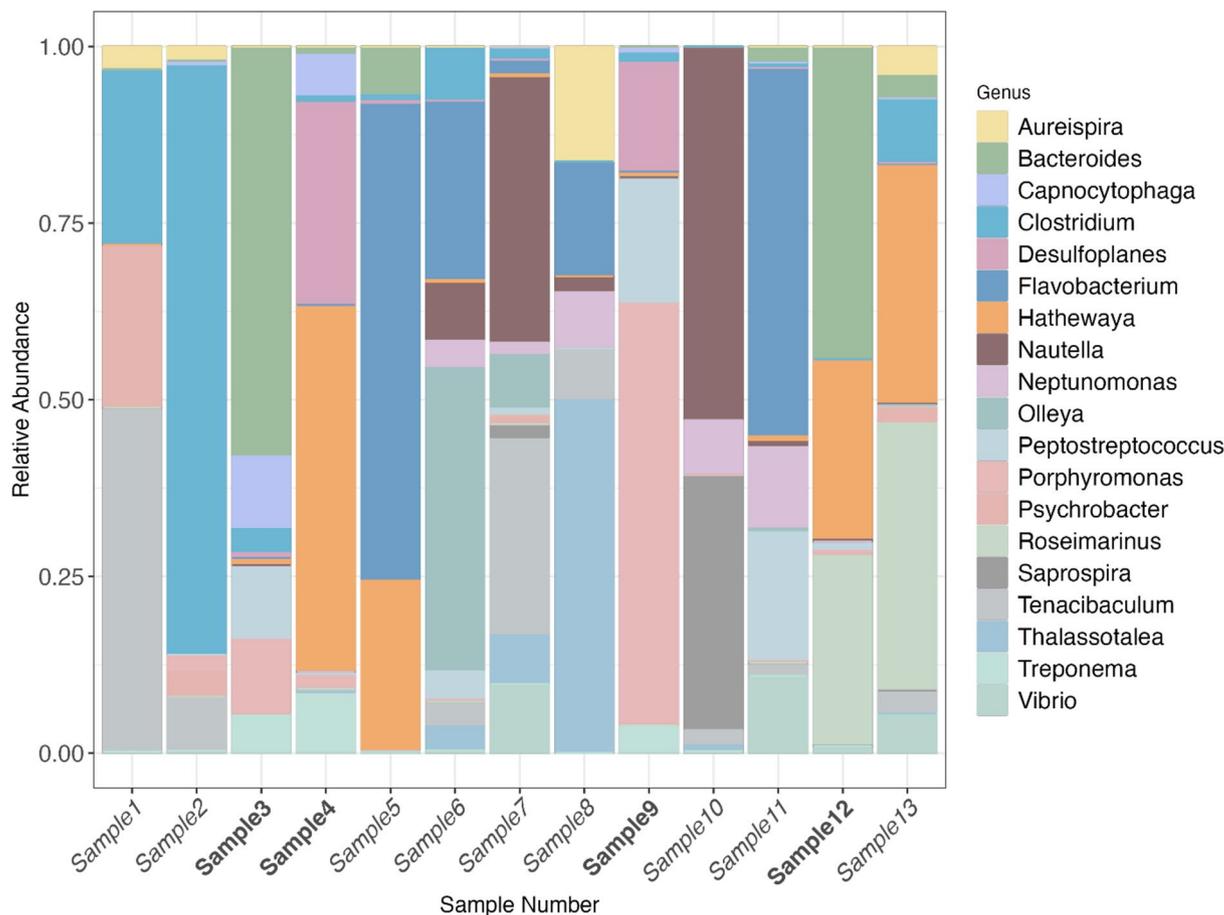


Fig. 6. Relative abundance of the top 20 sequenced genera (representing 49% of total bacterial abundance) across all Indian River Lagoon dolphin samples. Sample type is differentiated with “algal sheens” in italics and necrotic orofacial lesions in bold.

comparative analysis between grossly healthy skin microbiomes and potential environmental constituents. Further research is required for a more in-depth comparisons, such as competitive exclusion and identification of opportunistic or pathogenic colonizers, between healthy and lesioned skin microbiomes. Additionally, future samples should be taken for histopathology, as this may provide more insight into whether bacterial isolates were disseminated into tissues and help elucidate pathogenic potentials.

The results from this study contrasted published results of epidermal skin microbiomes from grossly healthy bottlenose dolphins^{10,43}. The microbial diversity in samples from both epidermal “algal sheens” and necrotic orofacial lesions were greater than those reported from skin samples of healthy individuals. The observed differences in these studies may reflect variation in sample types (e.g. archived frozen samples from deceased animals versus swabs and skin biopsies from live individuals), tissue condition (necrotic versus samples grossly healthy), age class differences between studies (i.e., Robles-Malagamba et al.⁴³ predominantly sampled calves and lactating females, contrasting adult males and juveniles sampled in this study), differences in amplicon region or underlying differences in ecosystem microbiotas. PERMANOVA analysis demonstrated significant differences in diversity between samples taken from orofacial lesion and “algal sheens”, though caveated with small sample sizes.

Sequencing results demonstrated multiple genera with pathogenic members across all dolphins, as well as other unique genera that have not been previously reported as apart of healthy dolphin skin microbiota. This may be due to a potential biofilm being formed on “algal sheen” individuals or opportunistic colonization of the epidermis from changed environmental microbiota from eutrophic or polluted environments. The *Burkholderia/Caballeronia* genus complex is ecologically diverse, being reported from soils, plants, and from human and animal tissues⁵². Members of these genera are known plant and mammal pathogens, though most species have non-pathogenic relationships with plants or unknown ecological niches in the soil⁵². Some species of *Burkholderia* cause zoonotic infections, including Glander’s Disease, which is characterized through ulcerated lesions in mucosal membranes and skin⁵³. This disease is most prevalent in solipeds and is rare in the United States. However, as the etiology of the necrotic orofacial lesions is still unknown, further investigation is warranted. More recently, *Caballeronia* spp. have been noted as promoting plant growth in nutrient limited environments⁵⁴, although impacts on dolphin epidermis are currently unknown. *Psychrobacter* spp. are known to inhabit both cold and warm saline habitats⁵⁴, and more recently described as a part of skin microbiota for

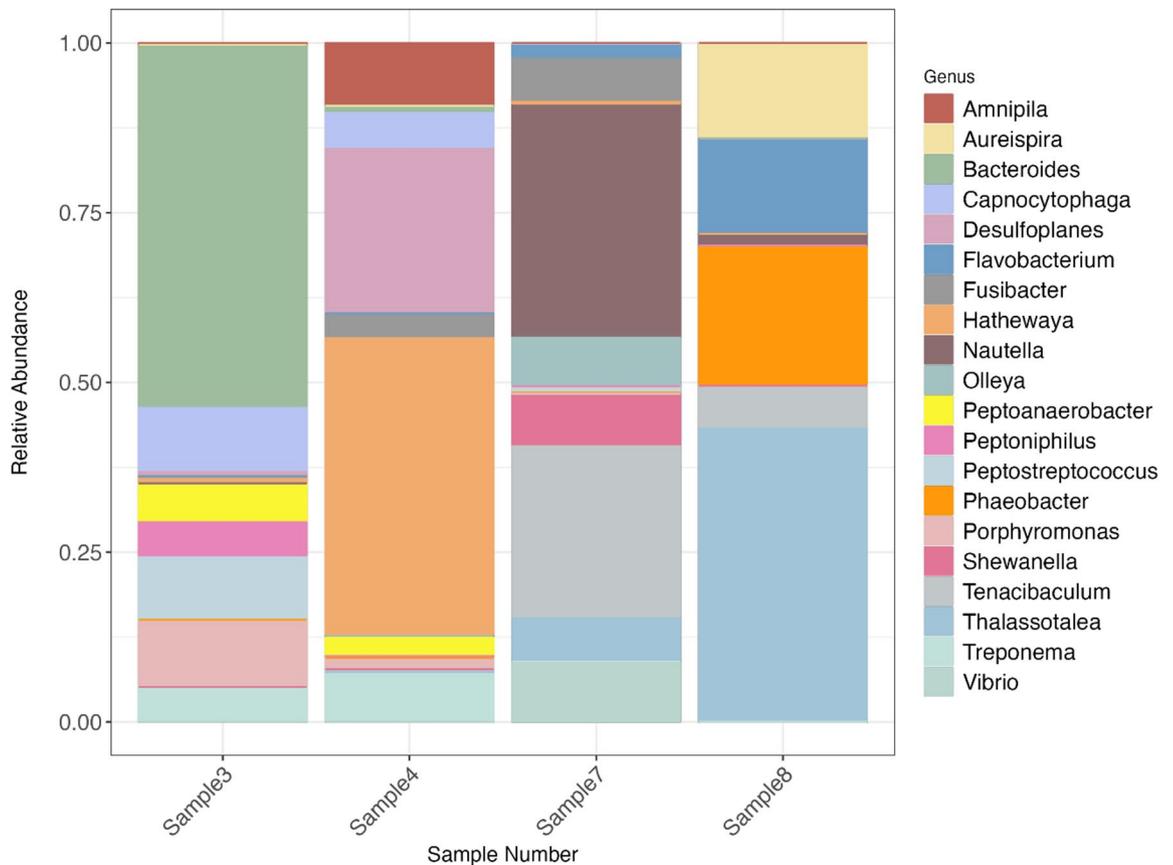


Fig. 7. Relative abundance of the top 20 sequenced genera (representing 66% of total bacterial abundance) across Indian River Lagoon dolphins with necrotic orofacial lesions.

bowhead (*Balaena mysticetus*) and killer whales (*Orcinus orca*)⁵⁵. Members of this genus can be pathogenic to mammals and fish, with ailments ranging from bacteremia, keratoconjunctivitis and endocarditis and opportunistic agents, though the mechanism of transmission is unknown⁵⁶. *Clostridium* is an ecologically broad genus often found inhabiting soils and is a part of normal intestinal biota⁴⁴. However, there are pathogenic species, known to cause botulism, cellulitis, and gas gangrene⁴⁴. Currently, it is unknown whether *Porphyromonas* spp. are a benign member of the oral and skin microbiota⁵⁷, or an opportunistic pathogen when the host animal has compromised homeostasis⁴⁵. Additionally, members of the genus *Treponema* were sequenced. Members of this genus can be highly pathogenic in humans, being responsible for syphilis, bejel, and yaws^{58,59}, yielding ulcerative skin lesions⁶⁰. As the etiology of *Treponema* spp. infection is consistent with the gross clinical signs of the animals sequenced, further investigation is required. Members from the genus *Tenacibaculum* also contain marine pathogens that can produce ulcers (including mouth erosion) in fish^{49,61}. *Hathewayia* is also a prominent genus containing pathogenic species known to cause gas gangrene and rarely, necrotizing disease, through the action of collagenases and lecithinases^{48,62,63}. Dolphins with “algal sheens” may be more susceptible to genera with pathogenic species, especially when growing near or on the rostrum or any other area that may facilitate an opportunistic infection.

Epizotic growth on marine vertebrates, and dolphins in particular, has long been documented^{64–66}. Some of these epizotic microbes are new to science (e.g., *Chelonicola* spp., *Craspedostauros* spp, and *Poulinea* spp⁶⁷), and may have developed specialized features for attachment (e.g., the “fastigium” of *Tursiocolasp*⁶⁸). Some epizotic algae have been associated with deceased dolphins, such as the cyanobacterium *Komarekiella delphiniconvector*⁹. This is the first report of the presence of multiple taxa of cyanobacteria on the epidermis of bottlenose dolphins. No single cyanobacterium was present across all sampled animals. Further assessment, including culturing, is required to determine whether the “algal sheens” are cyanobacterial, algal, or other eukaryotic microbes. BLAST matches indicated that the cyanobacteria found in swab samples were present in other intertidal or coastal habitats, including members of the genus *Synechocystis* and *Pseudosynechococcus*, and from the family *Prochlorococcaceae*⁶⁹. Additional cyanobacterial genera were found only in hypereutrophic, anoxic, and oil polluted water bodies or sediment, such as members of the genera *Vulcanococcus*^{70,71}, *Anathece*⁷², and *Inmanicoccus*⁷³, and from the family *Microcystaceae*⁷⁴. Other instances of cyanobacteria did not have BLAST matches above 95%. This may indicate that undescribed taxa of cyanobacteria are able to colonize dolphin epidermis, but this requires further investigation. The cyanobacterial genera found on “algal sheen” and animals with orofacial lesions may be indicators of poor water quality (e.g., eutrophication) as well as immunocompromised status of the animal (e.g., lowered sloughing state), as a majority of the identified genera

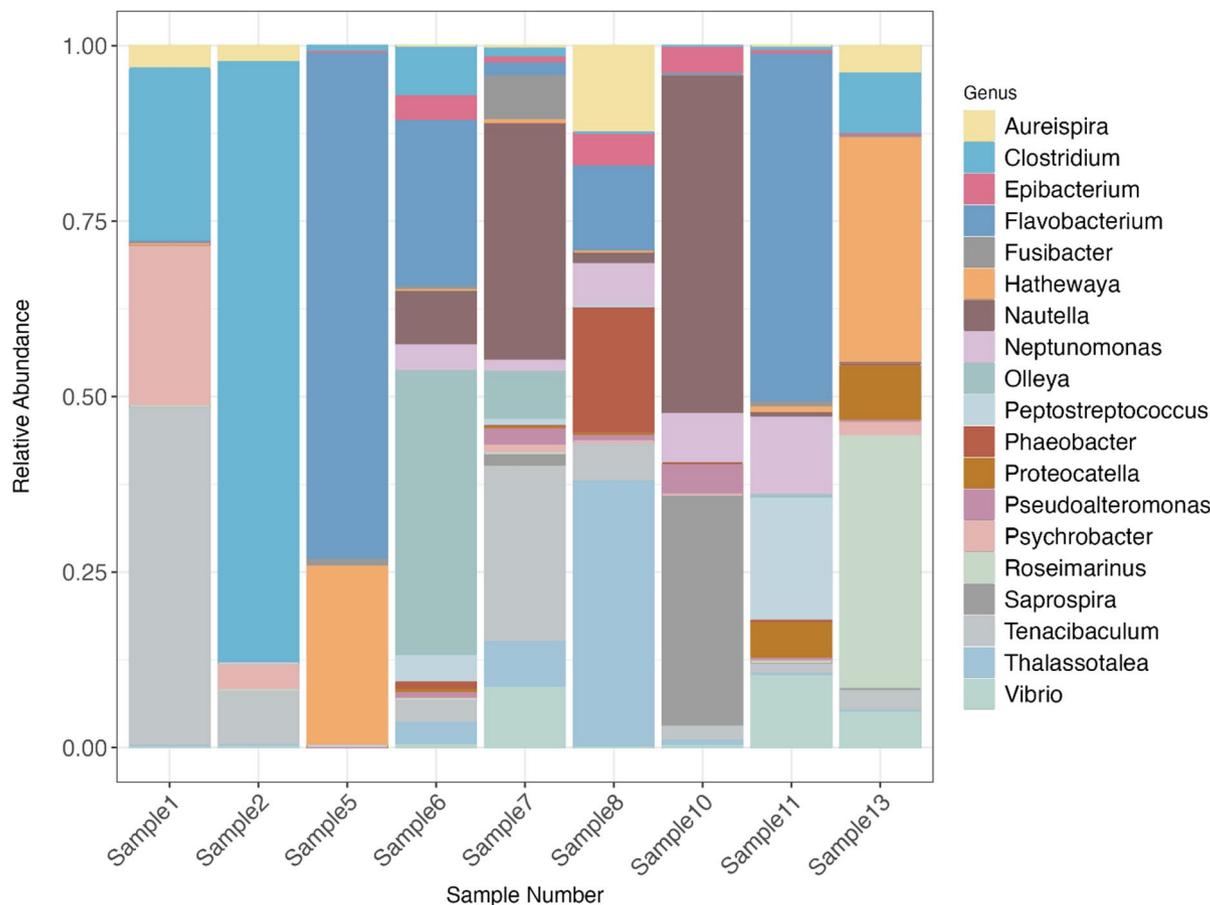


Fig. 8. Relative abundance of the top 20 sequenced genera (representing 61% of total bacterial abundance) across Indian River Lagoon dolphins with “algal sheens”.

are found grow in poor water quality environments. This study could not determine whether cyanobacterial presence was representative of passive colonizers post-infection or mortality, or were collected as a result of deposition from carcass recovery. Additional biopsy testing of live individuals would be valuable in confirming whether cyanobacteria are colonizing on live, free-swimming individuals.

It bears noting that all microbiome studies may suffer from two limitations. First, primer biases may impact which sequences are preferentially amplified⁷⁵. Second, community assessments are only as robust as their reference libraries. CyanoSeq⁴¹, while the most robust, curated cyanobacterial database, is still only as reliable as the deposited reference sequences. Future re-assessment of sequences should be conducted as databases are updated for increased clarity.

The causative agent of orofacial necrosis could not be determined. Interestingly, orofacial necrosis was only observed in male, juvenile dolphins, though this may be an artifact of sample collection due to small sample size. Juvenile males have high prevalence of “raking” as a result of scraping their teeth on the skin of other juveniles during social interactions and competitive behavior⁷⁵. This may make them more susceptible to bacterial or viral infections that ultimately cause orofacial necrosis. Furthermore, juvenile dolphins are known to spend more time engaging in play behavior⁷⁶ and male dolphins have been found to become entangled more frequently than females⁷⁷. Orofacial lesions may also be a secondary or opportunistic bacterial infections following entanglement. Two of the reported individuals (Hubbs-1249-Tt, Hubbs-2018-Tt) were either recovered with fishing gear present in mouth (Hubbs-2018-Tt) or had scars consistent with prior human interaction (Hubbs-1249-Tt). Previous reports have documented bottlenose dolphin maxilla damage resulting from entanglement^{78,79} as well as the potential that entanglements perpetuate mouth infections, as speculated in some entangled North Atlantic right whales⁸⁰. Orofacial lesions clearly impacted body condition, as all dolphins with this ailment were emaciated. When comparing body conditions, high level assessment of microbial diversity differed, but further investigation with larger sample sizes is warranted. It is likely that the animals in the IRL are exposed to pathogenic bacteria as the estuary is susceptible to an influx of terrestrial pollutants¹⁴ and is subject to eutrophication and recurrent harmful algal blooms^{81,82} due to the shallow, microtidal nature of the system^{14,83}. Additional molecular approaches (e.g., use of 18S primers) may help characterize additional members of the eukaryotic microbial community.

This research sets the foundation for the utilization of skin microbiota to investigate dolphin and ecosystem health. Further research is required to identify the causative agents of “algal sheens” and necrotic orofacial

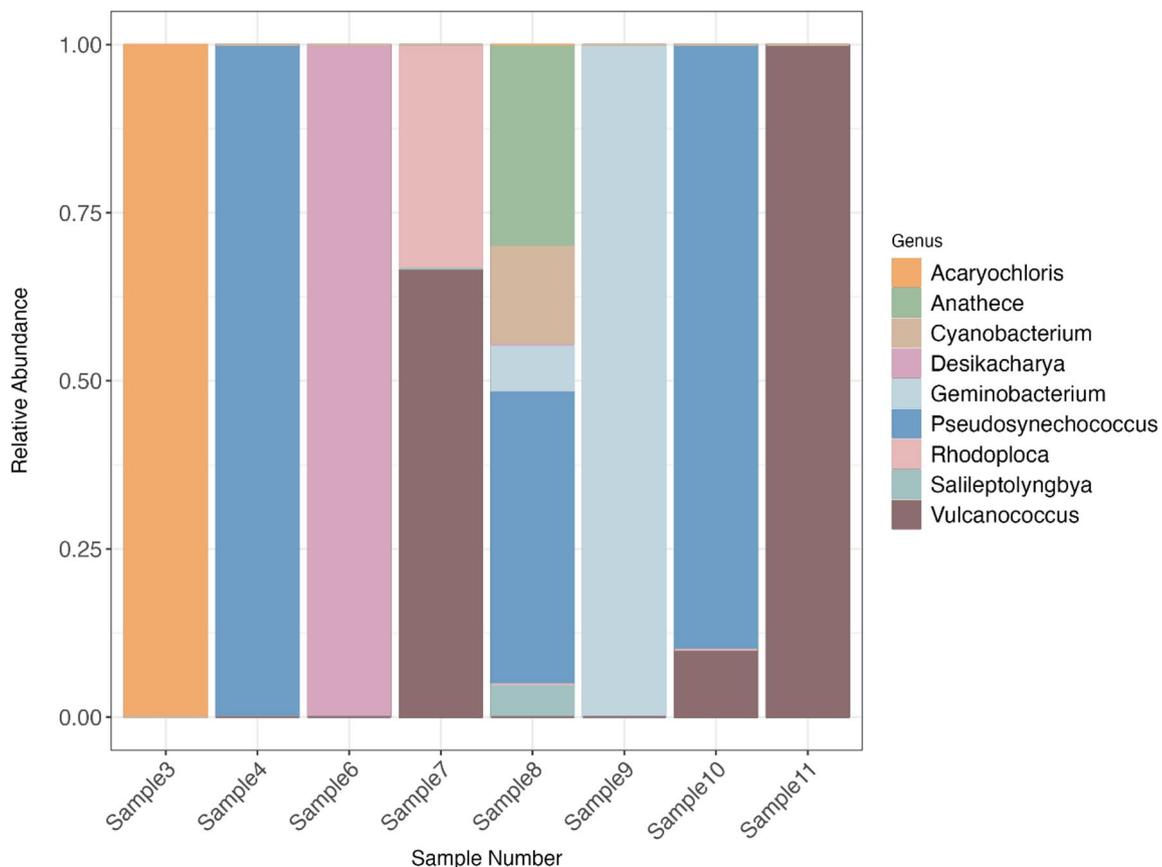


Fig. 9. Relative abundance of cyanobacterial species sequenced from epidermal “algal sheens” sampled from Indian River Lagoon dolphins.

lesions. As the IRL is responsible for 50% of east Florida’s fish catch⁸³ and is valued at \$9.9 billion annually for economic contributions that center around the viability of the ecosystem (e.g. recreational use, fishing, ecotourism, real estate)^{81,82}, further investigation into these species is critical to understanding ecosystem and human health. The ailments identified in this research are also of human health concern given the high rates of fish consumption and the extensive recreational use of the lagoon for recreation, particularly as the IRL has been the site of hundreds of human vibriosis cases⁸⁴. Further investigation is warranted to assess human health risks for pathogenic bacterial infections, from species common to the area.

Data availability

This data has been submitted to Genbank Biosample (accession numbers SAMN47938985-SAMN47938997) that will become publicly available after publication.

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Education at the University of North Florida.

Author contributions

AB wrote the main manuscript text and DAC extracted and amplified all samples and provided funding. WND collected all life history information for all sampled dolphins. AB and WND prepared Table 1, WND prepared Fig. 1, AB and WND prepared Figs. 2, AB prepared Figs 3, 4, and 5 and 3. CM prepared Figs 6, 7, 8 and 9. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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