University of Zagreb

Faculty of Science

Department of Biology

Lucija Kanjer

DIVERSITY OF CYANOBACTERIA IN EPIBIOTIC MICROBIAL COMMUNITIES ASSOCIATED WITH SEA TURTLES

DOCTORAL DISSERTATION

Zagreb, 2025

Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet

Biološki odsjek

Lucija Kanjer

RAZNOLIKOST CIJANOBAKTERIJA U EPIBIOTSKIM MIKROBNIM ZAJEDNICAMA MORSKIH KORNJAČA

DOKTORSKI RAD

Zagreb, 2025

This doctoral thesis was carried out within the Postgraduate Doctoral Programme in Biology at the University of Zagreb, Faculty of Science, Department of Biology, under the supervision of assoc. prof. dr. sc. Sunčica Bosak. The research presented in this doctoral thesis was supported by the Croatian Science Foundation (HRZZ) project “Loggerhead sea turtle (*Caretta caretta*) microbiome: insight into endozoic and epizoic communities – TurtleBIOME” (project number UIP-05- 2017-5635, principal investigator dr. sc. Sunčica Bosak).

Information about Mentor

Sunčica Bosak (07.06.1982.), PhD, Associate Professor

Sunčica Bosak obtained her Diploma in Biology, Ecology in 2006 at the Faculty of Science of the University of Zagreb. She has been employed at the Department of Biology at the Faculty of Science since 2008, first as a junior researcher and a teaching assistant. She obtained her PhD in Interdisciplinary Study of Oceanology at Department of Geology, Faculty of Science in 2013 and then continued to a postdoctoral position at the same institution. In 2016 she became an Assistant Professor and in 2022 an Associate Professor. She has been a teaching assistant at four courses from 2006 to 2008, and since then continues to teach undergraduate and graduate courses of Biological Oceanography, Pelagic Microbiology, Microbial Ecology, Marine Microbial Ecology, and a Field Course in Marine Ecology as a professor. She mentored 19 students in their master and bachelor theses, and two doctoral students.

She was awarded multiple scientific training fellowships that allowed her to conduct research and establish collaborations across Europe (Assemble Plus Transnational Access projects in Italy, Synthesys EU projects in Sweden). During her career so far, she was a part of multiple research projects spanning across the fields of plankton food webs, coastal management and monitoring, impact of antifouling paints on the environment, and microalgae diversity. In 2018 she obtains funding and becomes a principal investigator in an installation research project funded by the Croatian Science Foundation "Loggerhead sea turtle (*Caretta caretta*) microbiome: insight into endozoic and epizoic communities (TurtleBIOME)", that lasts until 2023. Her research is hereafter mostly focused on phytoplankton, primary productivity, diatom taxonomy, and microbiomes of marine vertebrates. She is a member of the User Selection Panel of the Horizon 2020 Research Infrastructure Project AQUACOSM and COST Action Ocean4Biotech Platform, and an associate in two additional research projects (ISLAND, BIOTA).

Currently, she is an author or a co-author of 57 scientific publications, 111 conference abstracts and eight conference papers, including five popular science publications. She was the organizer of the 7th European Phycology Congress in 2019 in Zagreb and has greatly contributed to many science communication and popularization events and workshops (Night of Biology, European Researchers' Night, Festival of Science).

Acknowledgements

I want to express my sincere gratitude to my mentor, Sunčica Bosak, for everything she's done to guide and support me during my doctoral studies. From the lockdown and earthquake aftermath, to relocation, changing plans and juggling work across multiple locations—thank you for always pushing me beyond my comfort zone to achieve exciting scientific goals, while still giving me the space I needed to reflect and grow. Thank you for being my mentor and guiding me to be the scientist I am today.

Thank you to all the members of the Algology Group, who always made me feel welcome and part of a small but wonderful community. To my past and present PhD colleagues, Klara, Antonija M. and Antonija K., thank you for your insightful discussions, lab help, and for always being there to talk or vent. Special thanks to Martina, you were always so kind to me and ready to jump in during lab-related emergencies. I'm also deeply thankful to all the colleagues who offered help in the form of laboratory equipment and space—Prof. Zlatko Liber from the Department of Botany, members of the Plant Physiology Group, and the Animal Physiology Group.

My sincere thanks to Annick Wilmotte for so generously welcoming me into her group at the BCCM Cyanobacteria Collection at the University of Liège, and for guiding me through the world of cyanobacterial taxonomy. Marcelo Vaz, thank you for your hands-on advice and for sparking my true love for cyanobacteria. Dorian and Martina, our time together was far too short, but it left a lasting impact on both my research and me personally. Thank you for all your help and the great moments in the lab!

For their help in providing samples and precious time with sea turtles, I am deeply thankful to Simona Matas, Karen Gobić, and Milena Mičić from Aquarium Pula, as well as Mateja Zekan Lupić and rest of the staff from the Blue World Institute. And to the turtle-diatom expert, Roksana Majewska, thank you for generously sending me your unwanted, cyanobacteria-contaminated cultures.

Finally, to my partner, family and friends: there are no words strong enough to fully express how grateful I am for your love and support. Thank you for always believing in me, especially during the times when I couldn't believe in myself.

Doctoral dissertation

University of Zagreb
Faculty of Science
Department of Biology

DIVERSITY OF CYANOBACTERIA IN EPIBIOTIC MICROBIAL COMMUNITIES ASSOCIATED WITH SEA TURTLES

LUCIJA KANJER

Department of Biology, Faculty of Science, Horvatovac 102a, 10000 Zagreb

Cyanobacteria are key components of marine microbial ecosystems, yet their role in host-associated environments, such as the carapace of sea turtles, remains largely unexplored. The aim of this work is to provide the first detailed characterization of epibiotic cyanobacterial communities associated with sea turtles using 16S rRNA gene amplicon sequencing and cultivation of novel cyanobacterial strains. Cyanobacteria, while not dominant, were found to be consistently present in sea turtle carapace biofilm. The most abundant were filamentous cyanobacteria of the genus *Rhodoploca* and other members of the order Nodosilineales. Turtle age and size were the main drivers of cyanobacterial diversity. The cultivated strains of *Salileptolyngbya* and *Leptothoe* represent potentially newly discovered commensals of sea turtles. Strains belonging to more opportunistic species like *Okeania* and *Spirulina* were also cultivated. The results demonstrate the importance of combining traditional cultivation methods and DNA-metabarcoding to uncover hidden cyanobacterial diversity in the host microbiome, with new insights into marine microbial ecology and sea turtle biology.

(125 pages, 40 figures, 9 tables, 219 references, original in English)

Keywords: Cyanobacteriota, *Caretta caretta*, microbiota, 16S, metabarcoding, cultures

Supervisor: Dr Sunčica Bosak, Associate Professor

Reviewers: Dr Marija Gligora Udovič, Full Professor
Dr Ines Sviličić Petrić, Senior Scientist
Dr Petar Žutinić, Associate Professor

Doktorski rad

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

RAZNOLIKOST CIJANOBAKTERIJA U EPIBIOTSKIM MIKROBNIM ZAJEDICAMA MORSKIH KORNJAČA

LUCIJA KANJER

Biološki odsjek, Prirodoslovno-matematički fakultet, Horvatovac 102a, 10000 Zagreb

Cijanobakterije predstavljaju ključnu komponentu morskih mikrobnih ekosustava, ali njihova uloga u mikrobiomu životinja, kao što su mikrobne zajednice na oklopima morskih kornjača, ostaje uglavnom neistražena. Cilj ovog rada je detaljno karakterizirati zajednice cijanobakterija povezanih s morskim kornjačama pomoću sekvenciranja amplikona gena 16S rRNA i izolacije i uzgoja novih cijanobakterijskih sojeva. Utvrđeno je da su cijanobakterije, iako nisu dominantne, stalno prisutne u biofilmovima oklopa kornjače. Najzastupljenije su bile nitaste cijanobakterije iz roda *Rhodoploca* i ostali pripadnici reda Nodosilineales. Starost i veličina kornjače bili su glavni pokretači raznolikosti cijanobakterijske zajednice. Uzgojeni sojevi *Salileptolyngbya* i *Leptothoe* predstavljaju potencijalno novootkrivene komenzale morskih kornjača. Također su kultivirani sojevi koji pripadaju rodovima kao što su *Okeania* i *Spirulina* koji pripadaju više oportunističkim svojtama, Rezultati pokazuju važnost kombiniranja tradicionalnih metoda uzgoja i DNA-metabarkodiranja za otkrivanje skrivene raznolikosti cijanobakterija u mikrobiomu domaćina, te pridonose novim spoznajama u području morske mikrobne ekologije i biologije morskih kornjača.

(125 stranica, 40 slika, 9 tablica, 219 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: Cyanobacteriota, *Caretta caretta*, mikrobiota, 16S, metabarkodiranje, kulture

Mentor: izv. prof. dr.sc. Sunčica Bosak

Ocjenjivači: prof. dr. sc. Marija Gligora Udovič
dr. sc. Ines Sviličić Petrić, znanstvena savjetnica
izv. prof. dr. sc. Petar Žutinić

TABLE OF CONTENTS

1. INTRODUCTION

Cyanobacteria, previously known as blue-green algae, have played a pivotal role in Earth's evolution (Whitton 2012). These ancient photosynthetic organisms, among the first to utilize sunlight for energy and to produce oxygen, have significantly shaped our planet's atmosphere and continue to impact ecosystems worldwide. When they formed an association with eukaryotic organisms and became the ancestor of chloroplast, they began the second oxygen-revolution. Beyond their historical significance, cyanobacteria play integral roles in modern ecosystems, contributing to nutrient cycling, oxygen production, and sometimes, toxin production. Understanding their diversity and ecological functions is crucial for preserving natural habitats. Today cyanobacteria are also studied for their biotechnological potential in cosmetic industry, as a food supplement and as a resource of novel drugs and other bioactive compounds. Nevertheless, many species are still undescribed and their ecology and systematic is not completely understood.

In nature, microorganisms rarely live on their own, more often they form associations in biofilms or microbial mats. In these structures, every microbial species fulfils a certain function and relies on the interactions with other microbes. In this way they are more resilient to harsh environmental conditions, predators and anthropogenic pressures. In these communities, cyanobacteria are primary producers as they photosynthesize, produce sugars and fix inorganic carbon. Some cyanobacterial species are also able to fix atmospheric nitrogen and make it available for other organisms. Moreover, their secondary metabolites can act as bioactive compounds and deter pathogenic bacteria. The structure and composition of these biofilms and microbial mats depend on various factors, including the type of surface they form on - such as sediments, artificial materials, or even living organisms

Microorganisms found living on animals are part of an animal microbiome. A microbiome is a collection of all microbes, i.e. viruses, bacteria, fungi and microeukaryotes that are associated with a distinct habitat/biome. We often associate the term microbiome with humans or animals as hosts, but we can also recognize other types of microbiomes, such as soil microbiome, water microbiome or microbiome of built-in environment. The most studied microbiome is the human microbiome, and it is followed by microbiomes of animals that provide some value to humans, such as farm animals and pets. The microbiomes of wild animals have just recently gained scientific attention and are still mainly based on answering the question of “who is there?”. However, the knowledge gained from such studies brings the conclusion that every species carries its own unique microbiome often with novel microbial species. In endangered animal species there is an additional research value as the possibility of

extinction is not only applied to animals, but also transferred to its microbial community. This knowledge brings new perspective and alertness of just how important microbiome research is and how it must be implemented in the conservation efforts.

Conservation biologists suggest that studying and maintaining animal microbiomes should be a core component of conservation strategies, as it is essential for addressing challenges like native species reintroduction, captive breeding, and non-native species invasions (Redford et al. 2012, Peixoto et al. 2022). Identifying the microbiota associated with animals has been acknowledged as a supportive measure for sea turtle conservation (Trevelline et al. 2019). Sea turtles, large marine reptiles, include seven extant species (Wyneken et al. 2013). Many of these species are endangered due to a wide range of human activities, including fishing bycatch, ingestion of fishing gear, plastic pollution, and light pollution near nesting sites (Casale et al. 2018). Recently, efforts to characterize and describe the endobiotic microbiota of the gut, cloaca, and oral cavity of sea turtles have increased (Abdelrhman et al. 2016, Biagi et al. 2019, Scheelings et al. 2020, Filek et al. 2021). Nevertheless, the epibiotic microbial community of sea turtles, often overlooked, is just as crucial, as the surface microbiome is the primary interface between an animal and its environment. The composition of epibiotic communities is influenced by factors such as location, diet, and health status, all of which affect the microbiome profile of these animals (Blasi et al. 2022, Kanjer et al. 2022).

The number of known cyanobacterial species is growing daily, following the realization that these species are not cosmopolitan but are highly dependent on their specific habitats (Komárek and Johansen 2015a). Cyanobacteria often form close associations with animals such as sponges and ascidians (Adams et al. 2012). Given that cyanobacteria associated with sea turtles have never been comprehensively studied, it is possible that novel species may be discovered in these unique environments as for instance, numerous novel diatom species have been described from sea turtles worldwide. Furthermore, the search for new cyanobacterial species is not only ecologically significant but also holds potential for discovering species with possible industrial applications, such as in medicine. In summary, cyanobacteria from previously unexplored habitats could reveal surprising new characteristics, emphasizing the importance of basic microbial ecology research.

To address the above-mentioned research gaps, this thesis aims to comprehensively describe the epibiotic cyanobacterial community living in photoautotrophic biofilm growing on the carapaces of sea turtles. To accomplish this goal, dual approach will be used: i) metabarcoding using the 16S rRNA gene amplicons will be applied to describe the composition

and diversity of cyanobacterial community, and ii) cultivation of cyanobacteria will be used to describe and link morphological and genetic characteristics of the cultivated strains and to explore the potential discovery of novel cyanobacterial species.

This dissertation addresses several interlinked objectives, and the following hypotheses are formulated based on these objectives. The first overall objective is to determine the composition, structure and diversity of the cyanobacterial communities of the carapace and skin of sea turtles. The second thesis objective is to analyze the dependence of the composition of cyanobacterial communities on the carapace and skin of sea turtles on the location, size and age of the individuals and it is presumed that the cyanobacterial community of the carapace and skin of sea turtles differs depending on the location, size and age of the turtles. The third objective is to compare the composition of the cyanobacterial community of the microbial biofilm of sea turtle carapaces obtained by the analysis of universal bacterial primers for the V4 region of the 16S rRNA gene and specific cyanobacterial primers for the V6 region of the 16S rDNA gene and it is hypothesized that specific primers for cyanobacteria that amplify the V6 region give better results than universal bacterial primers that amplify the V4 region of 16S rDNA. And lastly, the final objective is to isolate cyanobacterial cultures from the epibiotic biofilm of sea turtle carapace and describe their characteristics using a morphological and molecular approach as it is hypothesized that the carapace habitat of sea turtles represents a source of new cyanobacterial taxa for science.

The structure of this thesis consists of five main chapters: Literature overview, Materials and Methods, Results, Discussion and Conclusion. The literature overview summarizes previous research on cyanobacteria, and epibiotic communities on sea turtles. Results and Material and Methods are each divided into three subchapters, each investigating one research aim. The first part describes the first metabarcoding survey, which aimed to determine how prevalent cyanobacteria are in prokaryotic component of sea turtle biofilms. The second part presents the second metabarcoding survey, which used cyanobacteria-specific primers, and within this survey we were able to investigate diversity and structure of cyanobacterial community on sea turtle carapaces in detail. The third part focuses on cultured cyanobacterial strains, their phylogeny and their association with sea turtle samples. The discussion interprets the findings within the broader context of cyanobacterial ecology, host-microbe interactions, and potential new species of cyanobacteria. Finally, the conclusion summarizes the main contributions of this dissertation and suggests future research directions.

Results presented in this dissertation within the chapter *4.1* were part of the study published as: *Kanjer, L., Filek, K., Mucko, M., Majewska, R., Gračan, R., Trotta, A., Panagopoulou, A., Corrente, M., Di Bello, A., Bosak, S. (2022), Surface microbiota of Mediterranean loggerhead sea turtles unraveled by 16S and 18S amplicon sequencing, Frontiers in Ecology and Evolution 10, doi: 10.3389/fevo.2022.907368* (Kanjer et al. 2022). These results were presented within the thesis, however with additional re-analysis of the dataset using CyanoSeq as reference database, as this database was not published and therefore not available in the period when the first metabarcoding analyses were made. Moreover, results presented within the chapter *4.2* were published as *Kanjer, L., Filek, K., Mucko, M., Zekan Lupić, M., Frleta-Valić, M., Gračan, R. & Bosak, S. (2024), Growing older, growing more diverse: Sea turtles and epibiotic cyanobacteria, Journal of Phycology 60:1390–405* (Kanjer et al. 2024). These results were expanded and a new statistical analysis added to the results.

The scientific contribution of this dissertation is the first comprehensive analysis of epibiotic cyanobacterial communities associated with sea turtles. Specifically, it identifies dominant taxa within these host-associated biofilms and elucidates the principal drivers influencing cyanobacterial diversity. Furthermore, this work characterizes previously undescribed cyanobacterial strains inhabiting sea turtle biofilms, which may possess ecological and biotechnological significance. Through the integration of traditional cultivation methods with culture-independent, DNA-based approaches, the study offers a novel and multifaceted perspective on the role of cyanobacteria in structuring epibiotic microbial communities, as well as their potential functional interactions with the host. The contribution of this work is not only that it advances the field of cyanobacterial research, but also contributes to broader understandings of host–microbiome dynamics and sea turtle biology. This dissertation thus establishes a foundation for future investigations into host-associated cyanobacterial communities and their ecological and evolutionary importance within marine ecosystems.

2. LITERATURE OVERVIEW

2.1 Cyanobacteria

Cyanobacteria are prokaryotic organisms capable of autotrophic photosynthesis. Although today we know that they belong to bacteria, due to their size and the habitat in which they live, they were historically called blue-green algae and were identified and studied together with other eukaryotic phototrophs. Today, they are still studied as part of algal research, and the botanical nomenclature code was being used when describing new species until very recently (Garcia-Pichel et al. 2020). In 2023, the prokaryotic nomenclature code is finally being used to classify cyanobacteria (Oren et al. 2023).

As a group, cyanobacteria are cosmopolitans inhabiting freshwater, terrestrial and marine habitats. They are often pioneers of colonization, and many representatives are extremophiles and can live in nutrient-poor habitats due to the ability of many species to fix nitrogen. Many species of cyanobacteria form symbioses with different groups of eukaryotic organisms such as plants, fungi and animals. Cyanobacteria carry out photosynthesis and fix atmospheric nitrogen, so they often supply their host with assimilated carbon and nitrogen. In return, cyanobacteria receive protection from predators and changing environmental conditions (Adams et al. 2012).

2.1.1 Cell structure and colony morphology

The prokaryotic cell organization hallmarks

The prokaryotic organisms are characterized by the absence of closed membrane systems within the cell, thus lacking nucleus and other cell organelles. Their DNA appeared to be uniformly distributed in the cell, but it is actually arranged in nucleoids that can have different shapes indicative of different taxa (Cepák 1993). They are gram-negative bacteria which means their cell walls contain a thin peptidoglycan layer, placed between the inner cytoplasmic membrane and an outer membrane containing lipopolysaccharides (Stanier and Cohen-Bazire 1977). This basic structure of cell walls is largely uniform in cyanobacteria, but internal cell organization, pigmentation, cell and colony shape all vary greatly between different evolutionary lines and are used for species identification (Komárek and Johansen 2015a).

Thylakoids and pigments – inside and outside

Inside the cell, thylakoids occupy most of the cell's volume. Thylakoids are specialized membranes that house photosynthetic apparatus. The arrangement and pattern of these photosynthetic thylakoids vary among different genera and are useful in identifying higher-order evolutionary and taxonomic relationships (Komárek and Johansen 2015a). Genera in the

order Synechococcales typically have thylakoids located parietally, while more advanced evolutionary lines exhibit parallel or irregular thylakoid positions. For example, in Oscillatoriales, thylakoids are typically arranged in concentric layers, whereas in Nostocales, they often form a radial or irregular network (Komárek and Johansen 2015a). Under light microscopy (LM), cells can appear as pale or bright blue-green, olive green, gray, pink, or violet. These color variations depend on the ratio of photosynthetic pigments, particularly chlorophyll *a* (and sometimes *b* and *d*), as well as phycobilins, allophycocyanin, phycocyanins (blue), and phycoerythrin (red). Phycobilins are localized in phycobilisomes, specialized structures in cyanobacteria that contain these accessory pigments. The proportions of these pigments can either be stable or change in several species and within the same population. This adaptive process is known as photoacclimation, or chromatic adaptation in older literature. In species or strains capable of photoacclimation, green light stimulates the synthesis of phycoerythrin, which captures these wavelengths more efficiently than chlorophyll *a*. Conversely, increased red light stimulates the synthesis of phycocyanin. This process is one of many that allows cyanobacteria to adapt and successfully colonize a wide range of habitats (Komárek and Johansen 2015a, Vidal et al. 2021).

However, the final coloration of cyanobacteria is not solely due to photosynthetic pigments, but also includes pigments found in the external sheath, such as scytonemin, gloeocapsin, and secondary carotenoids. The sheath, which is the outer mucilaginous layer (Komárek and Johansen 2015a), can be present in some cyanobacterial taxa and serves as a distinguishing feature. The structure of sheaths and envelopes around cells is diverse. The mucilage can form a hyaline, amorphous mass, diffuse and marginal envelopes, or structured and layered sheaths. Gelatinous envelopes (fine mucilage) and sheaths (firm or structured external layers) appear to have a protective function. Sheath pigments do not contribute to photosynthesis but act as sunscreen, protecting the cell from UV radiation in environments exposed to intense solar radiation. In addition to UV protection, exopolysaccharides (EPS) in the sheath contribute to biofilm formation, adhesion to surfaces, and protection against desiccation and predation (Garcia-Pichel and Castenholz 1991, Matsui et al. 2012).

Gas vesicles

Gas vesicles, intracellular structures unique to cyanobacteria, are commonly found in planktonic species. With a density of about one-tenth that of water, gas vesicles reduce the overall density of the cells, providing buoyancy and allowing them to float or rise from the

biofilm. Their presence is a characteristic feature of certain genera and species. Cyanobacteria can regulate their buoyancy by synthesizing or collapsing gas vesicles in response to light intensity and nutrient availability, optimizing their position in the water column (Walsby 1994). The accumulation of many gas vesicles forms an aerotope. Aerotopes consist of bundles of cylindrical protein microstructures that make up the gas vesicles. When aerotopes are abundant, they can cause the vegetative cells to appear brownish due to light refraction, which might be mistaken for actual pigmentation (Komárek and Johansen 2015a, Vidal et al. 2021).

Heterocytes – nitrogen capture factories

The cell structure is species-dependent, but there are also specialized cell types for nitrogen fixation (heterocytes) and survival of hard conditions (akinetes) that exhibit a completely different cell organization due to their specialized functions. These types of cells occur only in most evolved cyanobacterial order, Nostocales (Komárek and Johansen 2015b, Vidal et al. 2021).

Heterocytes (historically referred as heterocysts; **Figure 1A**, **B**) are specialized cells that can fix atmospheric nitrogen (N_2) and convert it to ammonium (NH_4^+) which can be used by cyanobacteria, algae and plants for growth. Heterocytes formation occurs in absence of combined-nitrogen source, after vegetative cell division. The cell undergoes a series of cascade metabolic events resulting in a mature heterocysts that express genes related to nitrogen fixation and supply fixed nitrogen to the filament. These cells possess only photosystem I that produces ATP to support nitrogen fixation, but lacks photosystem II that produces oxygen (Zeng and Zhang 2022). Fixation of atmospheric nitrogen is also called diazotrophy and nitrogenase is the enzyme responsible for it. Nitrogenase is damaged by oxygen and needs anoxic conditions for its functioning. Therefore, heterocytes are morphologically different than vegetative cells and can be recognized by thick and air-tight cell walls and no color due to lack of photosynthetic pigments (Vidal et al. 2021). Besides their lack of color, they are usually larger than vegetative cells, have different shapes and light refraction properties. Their position in the trichome is predetermined genetically, meaning that can be an indicative characteristic for identifying different cyanobacterial taxa (Komárek and Anagnostidis 2005). However, their frequency in the population depends on the nitrogen supply in the environment and declines when concentration of NH_4^+ or NO_3^- are higher, they can even lack completely in the ample availability of inorganic nitrogen (Komárek and Johansen 2015b, Vidal et al. 2021).

Akinetes – resting stages for survival

Another type of morphologically distinct cells are akinetes, resting stages (**Figure 1B**). Akinete differentiation is triggered by environmental factors and these forms are meant to survive harsh conditions such as cold temperatures, frost or drought. Like in heterocytes, their cell wall is thickened and refracts light differently that can be easily distinguished from vegetative cells under the microscope. They are often spherical, ellipsoidal or cylindrical in shape, which can differentiate them from heterocytes (Komárek and Johansen 2015b, Vidal et al. 2021). The amount of DNA inside the akinetes increases during their development (Sutherland et al. 1985). There is also a larger accumulation of photosynthetic assimilates, such as glycogen and cyanophycin. Akinetes can remain dormant for long periods and germinate when environmental conditions, such as rising temperatures or increased nutrient availability, become favorable (Kaplan-Levy et al. 2010). The akinete morphology, position in the trichome, cell wall properties and mode of germination are genetically determined and thus important morphological characteristics of species or genera (Komárek and Johansen 2015b, Vidal et al. 2021).

The variable size of cyanobacterial forms

The size of cyanobacteria varies considerably between taxa. Unicellular cyanobacteria have cell diameters ranging from about 0.2 μm to over 40 μm (Vidal et al. 2021). Some filamentous forms can have cell diameters of up to 100 μm, but these cells are generally very short and coin-shaped, so their cell volume is not necessarily much larger than that of other species (Whitton 2012). The length of filaments can reach several millimeters in certain benthic forms. Some colonial forms, such as the species in the *Microcystis* genus, produce massive accumulations in surface blooms that are visible to the naked eye (Paerl and Otten 2013, Komárek and Johansen 2015a). Benthic cyanobacteria such as *Nostoc* can also produce macroscopic colonies in terrestrial or aquatic environments (Dodds et al. 1995).

Trichome, filaments and their branching

The most basic type of cyanobacterial cell is free-living unicellular organism (**Figure 1C**) encapsulated with outer exopolymer sheath. This form of cyanobacteria is called unicellular or coccoid form (Komárek and Johansen 2015a). Cells can also be organized in rows of vegetative cells called a trichome (**Figure 1D, E**). The trichome enclosed within a sheath (**Figure 1D**) is called a filament (Komárek and Johansen 2015b). Filaments can be formed from

one or several trichomes. Depending on the taxa, trichomes can have real (true) or false branching. False branching in filamentous cyanobacteria refers to a situation where new branches appear but the cells within those branches are not directly connected to the main filament cells. It is also called dividing in one plane. Real branching occurs when a trichome divides itself in two planes, and the parent trichome is not discontinued at the spot where branching took place. This kind of branching is typical for more evolutionary complex cyanobacterial groups like family Stigonemataceae inside the order Nostocales (Komárek and Johansen 2015b, Vidal et al. 2021).

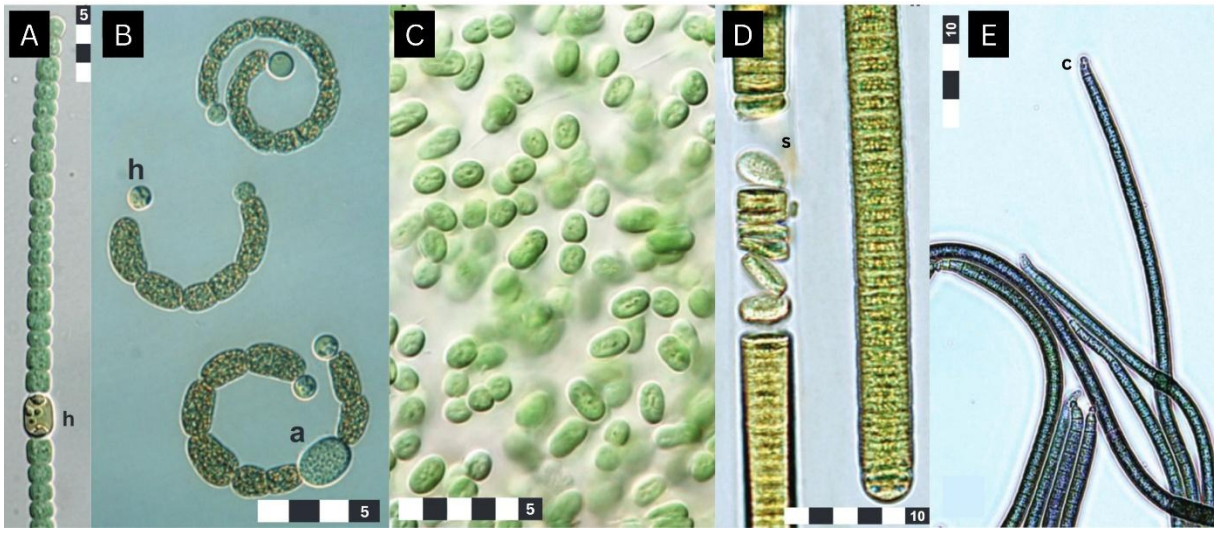


Figure 1. Main cyanobacterial cell and colony shapes and structures: unicellular species *Aphanocapsa* sp. (A); individual cells of *Lyngbya* sp. seen inside of a trichome with sheath (s) (B); purple-colored trichomes without sheaths *of Microcoleus*/*Phormidium* sp. (C); *Anabaena* sp. colony with heterocyte (h) (D); short, coiled trichomes of Anabaenopsis sp. with akinetes (a) and terminal hererocytes (h) (E); photographs are adapted from (Vidal et al. 2021).

Isopolar trichomes contain all the vegetative cells of the same size and shape. Cyanobacteria that possess isopolar trichomes are called filamentous homocytous (or non-heterocytous) cyanobacteria and cyanobacteria that have heteropolar trichomes and have heterocytes are called filamentous heterocytous cyanobacteria (Komárek and Johansen 2015b). However, even in isopolar trichomes, the vegetative cell at the end can have different shape or size. Calyptra, a mucilaginous cap-like structure (**Figure 1E**), can be present in some genera (Komárek and Anagnostidis 2005). Heteropolar trichomes, as e.g. characteristic for the taxa belonging to the family Rivulariaceae, have the last few cells, called apical cells, which can become very thin and elongated, giving the whole colony a hair-like shape (Rippka et al. 1979).

Filamentous cyanobacterial taxa can form hormogonia, short motile filaments meant for reproduction. Hormogonia (sing. hormogonium) are formed by fragmentation of parent trichome. Fragmentation occurs either by creating lamella between cells in one trichome or by sacrificing a part of trichome via necridic cells or necridia, which can be seen by LM in various forms of degradation or empty space inside trichome. Hormogonia are motile fragments, easily transferable from parent biofilm to form new trichomes in new habitat (Meeks and Elhai 2002).

All these features are important in identifying and describing cyanobacterial species. Even today, when we base cyanobacterial phylogeny on the their genetic similarity (Strunecký et al. 2023), it is still important to couple it with morphological descriptions. In history, morphology was the only basis for taxonomical classification systems and even now it is crucial for polyphasic approach in taxa identification (Komárek 2016).

2.1.2 Taxonomy and phylogeny

Cyanobacteria are still commonly known as blue-green algae. Historically classified as algae due to their photosynthetic abilities, large size and ecological roles, these microorganisms were only later recognized as bacteria due to advances in microscopy and molecular biology. This historical misclassification has led to persistent confusion in scientific nomenclature, which continues to affect modern taxonomic frameworks.

Botanical or prokaryotic code?

In botanical nomenclature, cyanobacteria were placed within the International Code of Nomenclature for algae, fungi, and plants (ICN), which further solidified their association with algae. In 1941, cyanobacterial prokaryotic origin was first suspected (Stanier and Van Niel 1941), followed by proposed first name change from blue-green algae to cyanobacteria (Stanier and van Niel 1962). The first major challenge to the algal classification of cyanobacteria arose with the advent of electron microscopy and then molecular studies, including ribosomal RNA sequencing, which provided conclusive evidence that cyanobacteria shared fundamental genetic and biochemical characteristics with prokaryotic bacteria (Woese and Fox 1977, Rippka et al. 1979). Despite their reclassification as bacteria, cyanobacteria remained deeply embedded in botanical and algal ecological studies due to their ecological roles in primary production, nitrogen fixation, and bloom formation, which closely parallel those of true algae.

Because cyanobacterial research goes hand in hand with algal research, newly discovered cyanobacterial taxa did not have all the requirements to be described under

International Code of Nomenclature of Prokaryotes (ICNP). For example, the definition of a species is still ambiguous in cyanobacteria. Classic bacterial methods in determining species like DNA-DNA hybridization cannot be used on many cyanobacterial species due to lack of axenic cultures (Vidal et al. 2021). Under the botanical code, there is no need to deposit axenic cultures, and many species were described using just morphological features. Because of this confusion, there were many unresolved issues like unknown true number of cyanobacterial species, non-existence of type strains, no formally accepted global taxonomy, often using the terms like “groups” or “sections” instead of true names for taxonomic ranks like classes, orders etc.

The long-waited solution to the decades-old problem seems to have finally been proposed. The 2022 revision of the International Code of Nomenclature of Prokaryotes has been published, providing updated guidelines for the naming and classification of prokaryotic organisms, including cyanobacteria (Oren et al. 2023). For it to happen, first, ICNP general considerations and rules regarding cyanobacteria had to be emended (Oren et al. 2021). Next, the name for cyanobacterial phylum had to be resolved and harmonized with prokaryotic code. This was done by validly publishing type genus *Cyanobacterium* after which the cyanobacterial phylum could be validly established and named after type genus and adding suffix *-ota* making the new phylum name Cyanobacteriota (Oren et al. 2022). The revised 2022 version of ICNP now includes all cyanobacterial species previously described within botanical code belonging to phylum Cyanobacteriota under following rules. Cyanobacteria that were previously validly published under the botanical code (ICN) are now also considered validly published under the prokaryotic code (ICNP). This means that previously assigned cyanobacterial names under the botanical code (ICN) will not need to be changed and will be recognized under the prokaryotic code (ICNP) as well. Additionally, newly described cyanobacterial names must now follow the ICNP, but if they were originally published under the ICN, their names can still be considered valid under the ICNP without requiring republication. The decision allows names previously published under the ICN to be recognized under the ICNP, but it does not suggest that new cyanobacterial taxa can continue to be described under the ICN. Cyanobacterial names found in the CyanoDB (Hauer and Komárek 2022) database and website are now officially recognized as approved bacterial names in the ICNP (Oren et al. 2023). This aligns with the goal of bringing cyanobacterial nomenclature fully under the rules of prokaryotic taxonomy while respecting historical names published under the botanical code.

Historical Overview of Cyanobacterial Classification Systems

Cyanobacterial taxonomy has evolved from purely morphology-based systems to classifications integrating molecular and biochemical data. Early efforts, such as Geitler's (1932) classification under the ICN, grouped cyanobacteria based on cell organization and division patterns, later refining this into four primary orders: Chroococcales, Dermocarpales, Pleurocapsales, and Hormogonales (Geitler 1942). Rippka et al. (1979) introduced a bacteriological system, aligning cyanobacteria with prokaryotic lineages by dividing them into five morphological subsections I (Chroococcales), II (Pleurocapsales), III (Oscillatoriales), IV (Nostocales) and V (Stigonematales). Subsequent refinements, including Anagnostidis and Komárek (1985), continued to emphasize morphological traits while acknowledging the need for deeper phylogenetic insights. Their system, adopted in *Bergey's Manual of Systematic Bacteriology* (Castenholz et al. 2001), underscored key features such as heterocyte formation, binary or multiple fission, and true vs. false branching.

With advancements in molecular phylogenetics, polyphasic taxonomy emerged, integrating morphology, cytology, and genetic data (Komárek 2016). Hoffmann et al. (2005) proposed a thylakoid-based classification, revealing polyphyly in traditionally accepted orders like Oscillatoriales and Synechococcales. Komárek et al. (2014) further incorporated 16S rRNA phylogenies, identifying new orders such as Spirulinales and Chroococcidiopsidales, yet challenges remained due to unresolved polyphyletic groups. Polyphasic approaches between 2014 and 2021 led to the discovery of over 270 species in 140 new genera, highlighting inconsistencies between thylakoid structure and phylogenetic relationships (Strunecký et al. 2023).

The most recent advances, such as classification proposed by Strunecký et al. (2023), combined whole-genome cyanobacterial sequence tree with less robust but better sampled 16S rRNA gene phylogeny mapped to the phylogenomic backbone. This refined cyanobacterial taxonomy (**Figure 2** and **Figure 3**), addresses long-standing issues of polyphyly and incorporated reference strains where possible. Their work introduced *Vampirovibriophyceae*, a non-photosynthetic cyanobacterial class, and reorganized problematic taxa. This work provides the desperately needed taxonomic reference point including the whole-genome sequences into polyphasic approach. With current efforts in organizing cyanobacterial taxonomy (Strunecký et al. 2023) and nomenclature under prokaryotic code (Oren et al. 2023), we can finally say that there is hope making order in understanding cyanobacterial phylogeny.

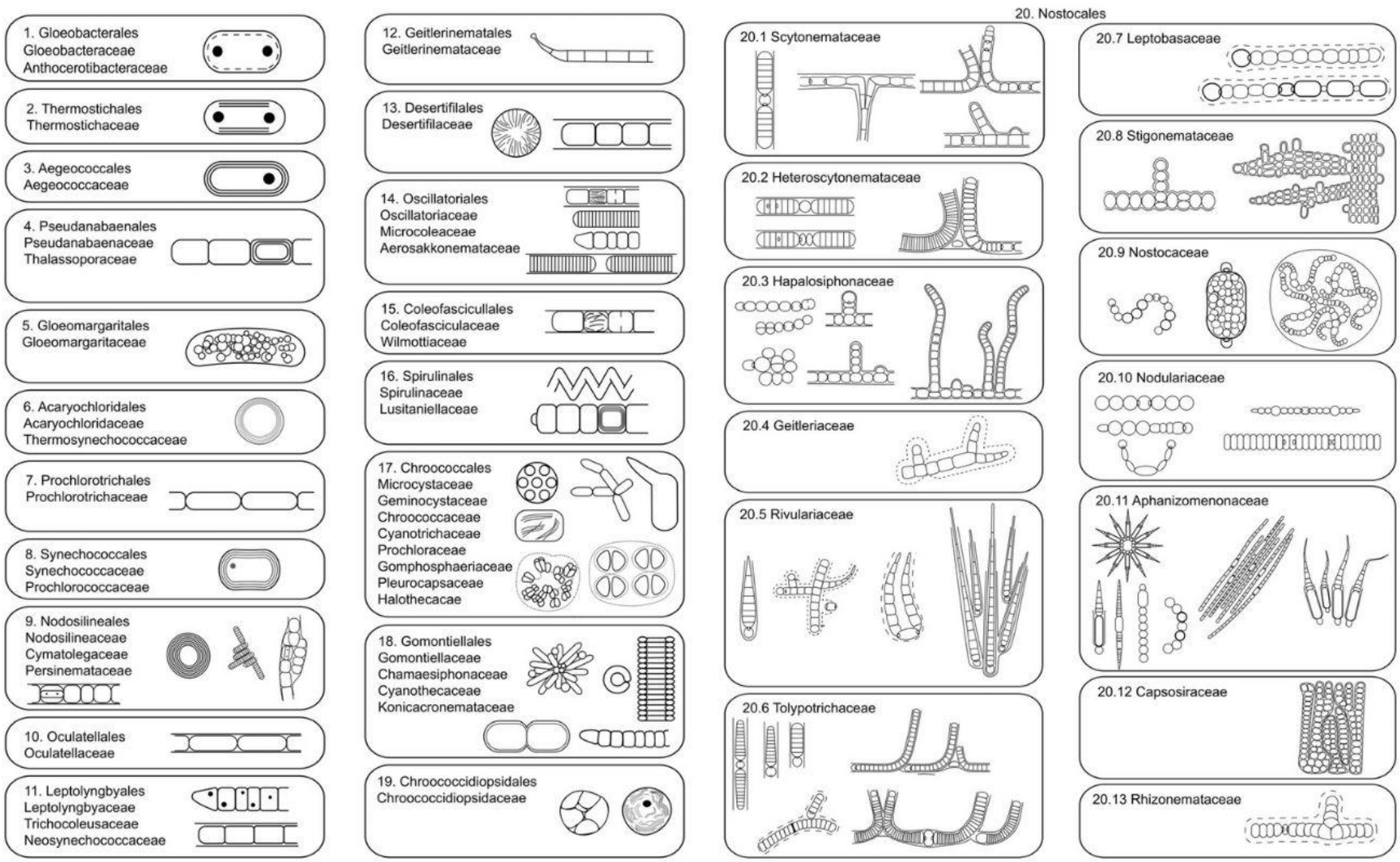


Figure 2. Schematic representations of cyanobacterial orders and families according to the latest updated cyanobacterial classification system (Strunecký et al. 2023).

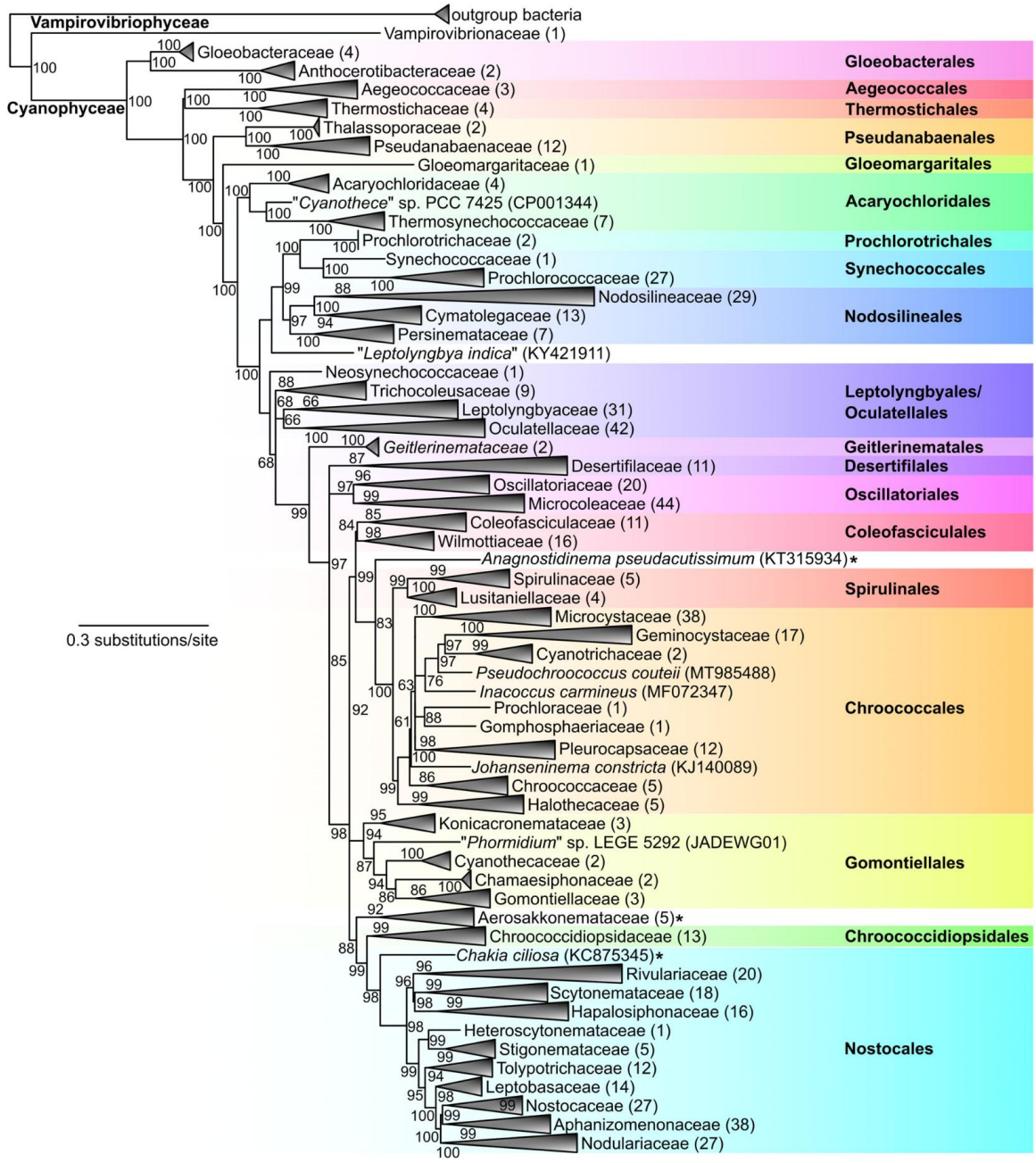


Figure 3. Phylogenetic tree of cyanobacterial orders and families based on 16S r RNA gene according to the latest updated cyanobacterial classification system (Strunecký et al. 2023).

It is important to incorporate current efforts in organizing cyanobacterial taxonomy to create practical tools like CyanoSeq (Lefler et al. 2023) and Cydrasil (Roush et al. 2021) databases that are easy to use with cyanobacterial metabarcoding data and 16S rRNA sequences. And that remains crucial for applied cyanobacterial research in biotechnology, environmental monitoring, and the study of harmful algal blooms (Paerl and Otten 2013).

2.1.3 Ecology and diversity

Cyanobacteria's ecological roles extend beyond primary production and nitrogen fixation. They are among the earliest oxygenic photosynthesizers and have played a major role in shaping Earth's atmosphere and biosphere (Schopf 2000). They are distributed worldwide in various habitats, ranging from deserts to open oceans, freshwater lakes, polar regions, and even extreme environments such as hot springs and hypersaline lakes (Whitton and Potts 2007).

Aquatic forms

Aquatic forms are the most well-known and researched cyanobacteria. In lakes and large water bodies, they are almost always present. Their presence increases with eutrophication, which is why they are often associated with toxic blooms. In some cases, up to 50% of the total biomass in a bloom can be produced by a single species, such as *Microcystis* (Komárek and Johansen 2015b). The most common aquatic genera include *Microcystis, Dolichospermum, Aphanizomenon, Planktothrix*, and *Synechococcus*. Marine planktonic picocyanobacteria, such as *Prochlorococcus*, play a crucial role in global biogeochemical cycles, contributing to approximately 20% of total primary production (Partensky et al. 1999, Flombaum et al. 2013). *Trichodesmium*, another marine cyanobacterium, is a key nitrogen fixer in oligotrophic tropical oceans (Capone et al. 1997).

Terrestrial forms

Terrestrial cyanobacteria, such as *Nostoc*, *Scytonema*, and *Chroococcidiopsis*, are found in almost all soil types, where they contribute to nitrogen fixation and soil stabilization (Belnap 2003). During periods of heavy rainfall or in excessively moist soils, cyanobacteria can form characteristic dark green or blue-green layers visible to the naked eye. In desert environments, *Chroococcidiopsis* survives extreme desiccation and UV radiation by living within rock surfaces as an endolithic organism (Billi and Potts 2002).

Benthic forms

Benthic cyanobacteria often form microbial mats in aquatic environments, where genera such as *Phormidium*, *Oscillatoria*, and *Lyngbya* dominate. These mats can be characteristic of the type of substrate they colonize, ranging from sediments to rocky surfaces (Stal 2012). Although planktonic cyanobacteria are more commonly associated with cyanotoxins, benthic species are also capable of toxin production, but their toxicity is less studied. For example, *Aetokthonos hydrillicola* grows epiphytically on the aquatic plant

Hydrilla verticillata in stagnant waters in the United States and has been implicated in the deaths of waterfowl and eagles due to its neurotoxic effects (Wilde et al. 2005).

Cyanobacterial research in the Adriatic Sea

Studies in the Adriatic Sea have primarily focused on the fluctuating abundance of planktonic picocyanobacteria in relation to the physiochemical conditions of the water column of Adriatic sea (Babić et al. 2017, Mucko et al. 2018). However, cyanobacteria are far more abundant and taxonomically diverse in benthic and phototrophic fouling communities, yet their ecological significance in these habitats remains largely understudied, particularly in the Adriatic. Research on cyanobacterial communities in coastal waters and sediments affected by human activity suggests that DNA metabarcoding is an effective tool for tracking shifts in cyanobacterial community (Kolda 2018, Kolda et al. 2020). In these altered ecosystems, changes in the cyanobacterial community have been linked to processes such as coastal eutrophication and tropicalization (Kolda 2020). A comparative study on cyanobacterial diversity across tidal flats in different latitudinal regions found that Croatian cyanobacterial communities are distinct, with only a small fraction of species being accurately classified at the genus level (Vogt et al. 2019).

Cyanobacterial symbioses

Besides free-living life forms, cyanobacteria also engage in various symbiotic relationships. Many species form symbioses with diverse eukaryotic groups, including such as plants, fungi and animals (Mutalipassi et al. 2021). Due to their ability to perform photosynthesis and fix atmospheric nitrogen, cyanobacteria often supply their hosts with essential carbon and nitrogen while receiving protection from predators and environmental fluctuations in return (Adams et al. 2012). Lichenized cyanobacteria, such as *Nostoc*, associate with fungi to form lichens, providing photosynthetic products to their fungal partners (Rikkinen 2015).

Among cyanobacterial symbioses with animals, those involving sponges have drawn considerable scientific interest. These associations are notable for the production of biologically active secondary metabolites by the sponges and their cyanosymbionts (Wilkinson 1980). In certain sponge species, cyanobacterial symbionts can constitute up to 40% of the organism's total biomass. While this relationship is generally considered a facultative mutualism (where cyanobacteria promote sponge growth without being essential for their survival) studies on the

Mediterranean sponge *Ircinia fasciculata* suggest an obligate mutualistic relationship, as the loss of cyanosymbionts led to the sponge's rapid decline (Cebrian et al. 2011). Beyond sponges, cyanobacteria also form symbiotic relationships with other marine organisms, including tunicates (Ascidia), echiurans, isopods (Isopoda), and hydras (Adams et al. 2012). Simpler organisms like sponges and corals typically harbor cyanobacteria within their tissues, whereas vertebrates tend to have more superficial associations, with cyanobacteria residing on their external surfaces (Mutalipassi et al. 2021). Symbiotic relationships between vertebrates and algae are relatively uncommon, with one notable exception being the coevolution of sloths and their epizoic photosynthetic community, which includes cyanobacteria (Kaup et al. 2021).

While cyanobacteria frequently form mutualistic and commensal associations, they can also negatively impact aquatic animals. Studies on commercially significant fish species in aquaculture highlight the harmful effects of cyanobacterial presence on fish health (Drobac et al. 2016, Kolda 2020). Additionally, cyanobacteria can indirectly influence animal well-being by altering their gut microbiome composition (Duperron et al. 2019, Sehnal et al. 2021). A microcosm study by Duperron et al. (2019) observed an increase in potentially pathogenic bacteria in the gut microbiome of fish *Oryzias latipes* when exposed to toxic *Microcystis aeruginosa* cyanobacteria.

The ecological roles of benthic cyanobacteria, particularly in marine environments, remain poorly understood, as noted by Wood et al. (2020). Certain marine cyanobacteria deter herbivores and coral reef fish by producing toxic compounds (Capper et al. 2016). In Egyptian coastal waters, blooms of the benthic cyanobacterium *Oscillatoria acutissima* have been linked to substantial fish mortality (Ismael 2012). Additionally, cyanobacterial toxins have been detected in dolphins, raising concerns about their potential health effects (Brown et al. 2018, Davis et al. 2019). Cyanobacterial toxins may also impact sea turtles. For instance, *Lyngbya majuscula* (now reclassified as *Moorena producens*; Engene et al. 2012, Tronholm and Engene 2019), which grows epiphytically on seagrass consumed by green turtles, produces lyngbyatoxin. This toxin has been associated with an increase in the prevalence and severity of diseases such as fibropapillomatosis (Arthur et al. 2008). Such impacts can extend throughout the marine food web, as cyanotoxins consumed by small fish and shellfish can bioaccumulate and affect larger predators, including sea lions, dolphins, birds, and turtles.

2.2 Epibiosis on sea turtles

Underwater surfaces, whether living or non-living, are inevitably colonized by organisms. Initially, a biofilm of microorganisms forms, subsequently followed by the colonization of larger organisms, or macroorganisms. When the colonized surface is inanimate, such as rocks, boats, or plastic, the resulting community is referred to as fouling. However, when organisms grow on a living host, such as plants or animals, the relationship is termed epibiosis. In this context, the host organism is the entity upon which other organisms grow, and these attached organisms are known as epibionts (**Figure 4A**).

2.2.1 Sea turtles as host organisms

Sea turtles are large marine reptiles belonging to the order Testudines. There are seven currently living species, they all belong to the family Cheloniidae or hard-shelled turtles, except leatherback sea turtle which belongs to the family Dermochelyidae and whose shell is covered with skin instead of keratin scutes (Spotila 2004).

Sea turtles are widely distributed across the world's oceans, inhabiting tropical and subtropical waters (Wyneken et al. 2013), with some species ranging into temperate zones, like Mediterranean Sea (Casale 2010). They are commonly found in coastal areas, including sandy beaches, coral reefs, estuaries, and seagrass beds, where they forage and nest (Spotila 2004). Different species have distinct habitat preferences; for instance, the leatherback turtle (*Dermochelys coriacea* Vandelli, 1761) is known for its deep-sea pelagic lifestyle, while green turtles (*Chelonia mydas* Linnaeus, 1758) are more associated with seagrass meadows (Lutz et al. 2002). Nesting occurs on sandy beaches, primarily in tropical and subtropical regions, with major nesting sites located in Australia, Costa Rica, Florida, and Southeast Asia (Miller 1997). For the Mediterranean Sea, nesting sites are along southeastern coast, mainly that of Greece, Cyprus, Turkey and Cyprus (Margaritoulis and Rees 2011, Casale et al. 2018). Nesting in the southern Adriatic Sea is only sporadically reported, in Italy (Bentivegna et al. 2010) and Albania (Piroli and Haxhiu 2020). The distribution of sea turtles is influenced by ocean currents, temperature, and food availability, and their migratory behavior allows them to travel vast distances between feeding and nesting sites (Bolten 2002).

The life cycle of sea turtles is complex and involves several distinct stages, including egg, hatchling, juvenile, subadult, and adult phases (**Figure 4B**), with a strong emphasis on long-distance migration. Female sea turtles lay their eggs on sandy beaches, burying them in

nests where incubation lasts around 45–70 days, depending on temperature (Miller 1997). Hatchlings emerge at night and make their way to the ocean, guided by natural light cues, but many fall prey to predators such as birds, crabs, and fish (Witherington and Salmon 1992). The surviving hatchlings enter an oceanic phase, often referred to as the "lost years," during which they drift with ocean currents and feed on plankton and small organisms (Musick and Limpus 1997). As they grow, juveniles shift to coastal habitats, where they adopt species-specific diets, such as herbivory in green turtles (*Chelonia mydas*) and omnivory in loggerheads (*Caretta caretta* Linnaeus, 1758) (Bjorndal 1997). After reaching sexual maturity, which can take decades, adult sea turtles undertake long migrations between foraging and nesting grounds, often returning to their natal beaches to reproduce (Bowen and Karl 2007). This migratory cycle repeats over multiple reproductive seasons, with females typically nesting every 2–4 years.

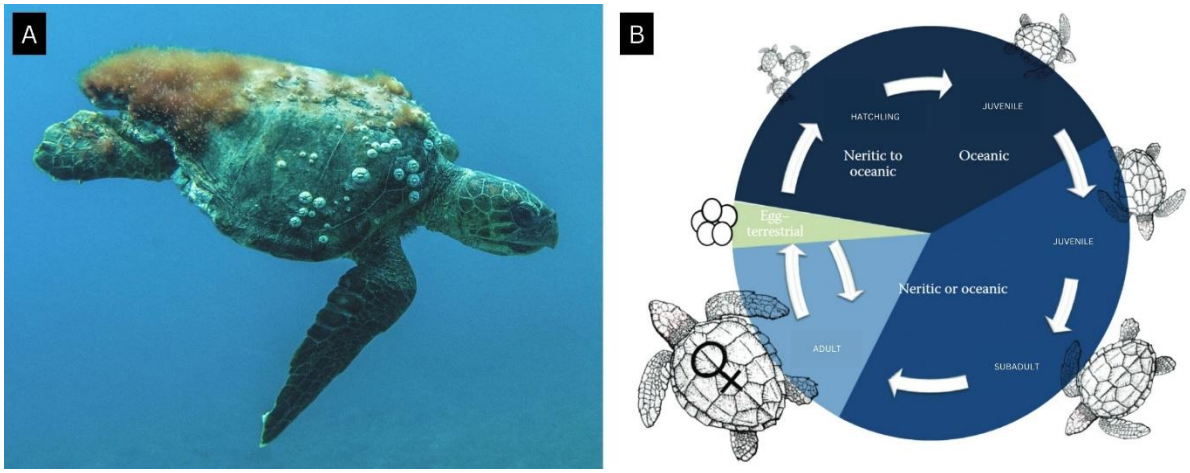


Figure 4. Loggerhead sea turtle with visible epibionts: barnacles and algae; photo by Marinko Babić (A); Conceptual model of loggerhead life stages and habitat type (B), Dark blue represents the oceanic phase occupied by hatchlings and younger juveniles, medium blue represents transition between oceanic and neritic phase in older juveniles and subadult turtles. Pale blue represents complete transition to neritic phase by adult turtles. Graphic is adapted from TEWG (2009) and Wyneken et al. (2013).

Sea turtles play a crucial role in marine ecosystems, contributing to the health of seagrass beds, coral reefs, and oceanic food webs. Green turtles (*Chelonia mydas*) help maintain seagrass ecosystems by grazing, while hawksbill turtles (*Eretmochelys imbricata* Linnaeus, 1822) regulate sponge populations on coral reefs, promoting biodiversity (Bjorndal and Jackson 2002). However, sea turtle populations face significant threats, including habitat destruction, climate change, fisheries bycatch, pollution, and illegal poaching (Wallace et al. 2011). Conservation efforts focus on protecting nesting beaches, reducing bycatch through turtle-

excluder devices (TEDs), and mitigating climate change impacts on nesting sites (Lutcavage et al. 1997). International agreements such as the Convention on International Trade in Endangered Species (CITES) and the Inter-American Convention for the Protection and Conservation of Sea Turtles aim to safeguard these species (Mast et al. 2006). Public awareness campaigns and community-based conservation programs also play a vital role in ensuring the survival of sea turtles for future generations.

2.2.2 Macro-epibionts

As with many phenomena in biology, the study of sea turtle epibiosis began with Charles Darwin (Darwin 1851, 1854). Darwin's anecdotal observations documented barnacles (families *Lepadidae* and *Balanidae*) inhabiting the surfaces of sea turtles. Since then, research on sea turtle epibionts has grown significantly, shedding light on the complex ecological relationships that occur on the shells of these marine reptiles.

Macro-epibionts on sea turtles have been extensively studied, offering valuable insights into various aspects of sea turtle biology that would otherwise remain unknown (Frick and Pfaller 2013). However, meta-analyses suggest that many macro-epibiont species remain undiscovered, indicating significant potential for further study in this field.

The relationships between sea turtles and their epibionts can be classified as opportunistic, commensal, or parasitic. Opportunistic epibionts are the most common and are typically free-living organisms that colonize sea turtles as they would any other suitable surface. Commensal epibionts benefit from their association with sea turtles in specific ways, without harming the host. In contrast, parasitic epibionts harm the sea turtle. To date, no mutualistic relationships between sea turtles and individual epibiont species have been documented. Epibionts on sea turtles can also be classified based on their mode of attachment. Sessile or sedentary epibionts permanently attach themselves to the host's surface, while motile epibionts remain unattached, moving freely over the turtle's body. The sedentary form of epibiosis, where organisms affix themselves permanently to the host, is the most commonly observed type on sea turtles (Frick and Pfaller 2013).

Barnacles had been found as loyal epibionts of sea turtles for more than 30 million years (Collareta et al. 2023). Some species, such as *Chelonibia testudinaria* Linnaeus, 1758 and *Chelonibia caretta* Spengler 1790, are considered obligate commensals of sea turtles, relying on the turtle for habitat and dispersal without causing harm. An interesting characteristic of *Chelonibia* species is their ability to produce plastic phenotypes depending on their host. For

instance, *C. testudinaria* (sea turtles), *C. manati* (manatees), and *C. patula* are phenotypically different, yet genetically identical (Cheang et al. 2013). Other barnacle species, like *Platylepas hexastylos* and *Platylepas decorata*, can cause minor injuries to turtles by embedding into their soft tissues. These barnacles often act as pioneering colonizers, establishing themselves first on the turtle's shell, thereby creating conditions suitable for other epibionts, such as amphipods, to attach later (Frick and Pfaller 2013). Barnacles provide a stable surface, facilitating the accumulation of a diverse community of organisms. Their presence can also serve as an indicator of how long a turtle has been in a particular environment (Frick and Pfaller 2013).

The genus *Hyachelia* consists of amphipods that have developed an obligate commensal relationship with sea turtles, co-evolving alongside them (Iwasa-Arai et al. 2023). For example, *Hyachelia lowryi* Serejo & Sittrop, 2009 has been recorded on hawksbill turtles, while other species in the genus have been found on green and loggerhead turtles. These amphipods typically inhabit the carapace and skin folds of their hosts (Iwasa-Arai et al. 2023).

Polysiphonia carettia is a species of red algae first described in 1971 from loggerhead sea turtles in California (Hollenberg 1971). *P. carettia* was first recorded in the Mediterranean Sea in 2001 (Baez et al. 2001) and, in 2016, in the Adriatic Sea, on Savudrija peninsula (Battelli and Rindi 2016). This alga exclusively colonizes the carapaces of cheloniid sea turtles, such as the loggerhead (*Caretta caretta*), and has not been found on other substrates, while other species of the same genus can also be found on abiotic substrata. The association between *P. carettia* and sea turtles is a notable example of obligate commensalism, where the alga benefits from the mobile habitat provided by the turtle's carapace, allowing it to access diverse marine environments. This relationship also highlights the role of sea turtles in facilitating the distribution of certain epibiotic species across different marine habitats (Battelli and Rindi 2016).

Living on a sea turtle offers several advantages for epibionts, such as an expansive and relatively predator-free habitat. Barnacles, in particular, benefit from increased water flow as the turtle swims, enhancing their ability to filter feed and obtain nutrients (Pfaller et al. 2008). Additionally, algae that grow on the turtle's carapace are exposed to greater sunlight, promoting photosynthesis and growth (Shine et al. 2010). These favorable conditions make turtles an ideal substrate for various epibiotic organisms. Despite the benefits, epibionts also face challenges when living on turtles, such as the risk of being physically removed. Turtles frequently groom themselves, scraping their shells against rocks to remove growing carapace pieces and

consequently dislodging epibionts (Wahl 1989). Additionally, during mating, friction and contact with other turtles may lead to the accidental removal of epibionts. This constant risk of dislodgment forces epibionts to develop strong attachment mechanisms (Frick and Pfaller 2013 and references within).

Epibionts can provide a significant advantage to turtles by enhancing their camouflage in their natural habitat. The accumulation of barnacles, algae, and other organisms helps turtles blend into their surroundings, making them less visible to predators such as sharks. This natural form of camouflage can be particularly beneficial in both oceanic and coastal environments, offering an additional layer of protection (Frick and Pfaller 2013). While some epibionts are harmless or beneficial, others can pose health risks to turtles. Parasitic leeches, for example, are known to transmit the fibropapilloma virus, which causes tumor-like growth that can impair the turtle's health (Greenblatt et al. 2004). Additionally, certain barnacle species can embed deeply into the turtle's skin, creating open wounds that may become infected with pathogenic bacteria (George 1996). While epibionts are generally considers harmless, these health issues can impact the turtle's overall fitness and reduce survival of already sick animals (Frick and Pfaller 2013).

Studying epibiotic communities on sea turtles provides valuable insights into their biology, including migration patterns, habitat use, and health status. The composition of epibiotic communities on sea turtles varies depending on whether they inhabit oceanic or neritic environments. Oceanic communities are typically characterized by species adapted to open-water conditions, while neritic communities include organisms found in coastal and shallower waters. By analyzing the types of epibionts present on a turtle, we can infer its movement patterns and determine whether it has spent more time in offshore or nearshore waters. This information is valuable for understanding migration routes and habitat preferences. By analyzing epibiont diversity and distribution, we can also gain a better understanding of the turtle's life history and movement across different environments. This information contributes to conservation efforts and helps in developing strategies for protecting these endangered species (Frick and Pfaller 2013). More specifically, epibiont research has led to significant discoveries in sea turtle conservation, including the identification of previously unknown nesting beaches (Caine 1986). By studying the epibionts on nesting females, researchers were able to track their movements and identify key breeding grounds. These findings were later validated using molecular tools (Bowen et al. 1993, Encalada et al. 1998), confirming the importance of integrating epibiont studies with genetic analysis for conservation purposes.

Moreover, the presence and composition of epibionts can serve as an effective tool for identifying habitat shifts in juvenile turtles. As turtles transition from the open ocean (pelagic) to coastal (neritic) foraging grounds, the types of epibionts attached to them change accordingly. By analyzing these shifts, researchers can gain valuable insights into developmental stages and habitat preferences of juvenile turtles. Another successful application of epibiont research in conservation is utilizing the known growth rates of barnacles to estimate arrival of turtles at their nesting grounds (Eckert and Eckert 1988). By measuring the size of barnacles attached to a turtle's shell, researchers can approximate the duration the turtle has spent in a specific area. This method provides useful data for tracking migration timelines and improving conservation strategies for nesting populations.

After over a century of research on sea turtle epibionts, Robinson and Pfaller (2022) conducted an extensive review of existing studies. They identified loggerhead sea turtles as hosting the highest richness and diversity of macro-epibionts. However, they noted that, based on sampling efforts, not all species living on loggerheads have been documented. The current body of literature remains incomplete, highlighting the need for further research. They also stated that while numerous factors contribute to the diversity of epibiont taxa, a wide geographic range is not one of them. Instead, the diversity of habitats visited by an individual turtle throughout its life and the physical characteristics of the substrate, such as its structure and tissue type, are far more influential. For instance, loggerheads, with their thick keratin scutes, provide a favorable surface for epibiont attachment. In contrast, leatherbacks, which have skin covering their shells, are less suitable for colonization by epibionts. The review also pointed out a sampling bias toward nesting female turtles and emphasized the importance of studying male and juvenile turtles in both oceanic and neritic habitats to gain a more comprehensive understanding of epibiont communities across different sexes and development ages.

2.2.3 Micro-epibionts

Despite growing research efforts, significant knowledge gaps remain in understanding the relationship between sea turtles and their epibionts. One such gap involves microbial communities inhabiting the surface of sea turtles, extending beyond what is visible to the naked eye. Among microbial epibionts, diatoms were the first group to receive attention. Studies utilizing microscopy, cultivation, molecular phylogeny, and metabarcoding have revealed putatively obligate epizoic diatom species (Rivera et al. 2018, Majewska et al. 2021, Ashworth et al. 2022) with new evidence emerging each year. These findings complement the broader

narrative of obligate macro-commensals coexisting with (still putative) diatom micro-commensals.

Bacteria are an indispensable part of host-associated microbiomes. Initial studies on bacteria on the surfaces of sea turtles focused on bacterial infections in sea turtle wounds and the anthropogenic impact of drug excrement pollution, which led to the detection of antibiotic-resistant bacteria (Alduina et al. 2020). Alarmingly, antibiotic-resistant strains found on sea turtles originate entirely from their environment, as turtles are not systematically treated with antibiotics (Trotta et al. 2021b, 2021a). This discovery raised numerous questions, including how can we know if human activity influences the sea turtle microbiome when baseline microbial communities remain largely unknown? The need to explore the baseline microbiome of sea turtles, its constituents and main drivers began to arise.

The advancement of culture-independent, DNA-based methods has made it possible to start answering those questions and shift research from a pathogen-focused perspective to a microbiome-focused approach. The first study describing the baseline surface microbiota of sea turtles examined three juvenile loggerheads in the Tyrrhenian Sea (Blasi et al. 2022). Subsequent studies have expanded this research to other sea turtle species, including hawksbill and green turtles from Iran (Loghmannia et al. 2023), loggerhead and green turtles from Italy and Israel (Bachmann et al. 2024; preprint) and leatherback sea turtles from Florida (Kuschke et al. 2024). Blasi et al. (2022) combined macro- and micro-epibiont analyses on anterior vs. posterior carapace scutes, reporting Firmicutes (dominated by Staphylococcaceae) and Proteobacteria (mainly genus *Kiloniella*) as the most abundant bacterial phyla. Loghmannia et al. (2023) found that Proteobacteria dominate both green and hawksbill turtles, followed by Bacteroidetes and Cyanobacteria. Their findings suggest that turtle species, rather than location, primarily drive microbiota differences. Bachmann et al. (2024) reported classes Gammaproteobacteria and Bacteroidia as being the most abundant on both loggerhead and green turtles, making up to 60-80% of total bacterial abundance. The investigated drivers of change were species and location (tested only in loggerheads). On leatherback sea turtles the most dominant phyla were Proteobacteria and Bacteroidota. They also investigated the influence of age and body part (carapace vs. flipper) on the difference of microbiota and found out that age has more influence, older turtles carrying more diverse communities and flippers carrying more diversity than carapace. Overall, these studies show dominance of Proteobacteria on the surface of sea turtles but also that both internal (age, body part) and external factors

(location) govern the difference in microbiota of sea turtles while maintaining similar patterns within single sea turtle species. While it is debated if every animal even have the innate microbiome (Hammer et al. 2019), current studies suggest each species may carry a unique core microbiota, which is in some part also susceptible to environmental influences. Yet, insufficient evidence exists to determine whether sea turtles are truly microbiome-dependent or if observed patterns result from study design limitations.

Current research represents only a preliminary glimpse into sea turtle microbiome diversity, mainly reporting bacterial composition at the phylum level. At such a broad taxonomic resolution, it is difficult to draw definitive ecological or functional conclusions, as metabolic and ecological diversity within bacterial phyla is vast. Additionally, each of the studies mentioned contains unidentified sequences, potentially representing undiscovered bacterial taxa that could reshape our understanding of sea turtle microbiomes. While diatom research suggests the presence of putatively obligate epizoic species, bacterial studies have yet to reveal or disprove a similar pattern. Further research from multiple perspectives is necessary to draw more concrete conclusions. Investigating specific bacterial groups in greater detail may uncover patterns similar to those observed in diatoms.

Reports on cyanobacteria within sea turtle microbiomes are scarce, but existing studies indicate their presence among at least top ten most abundant phyla across different species. On hawksbill and green turtles from Iran, cyanobacteria had been reported as second or third most abundant phyla, depending on the sample. “Unidentified cyanobacterium” being the most abundant genera and driving 8% dissimilarity between green and hawksbill species and 6% dissimilarity between green turtles and stone biofilm sample (Loghmannia et al. 2023). Other abundant cyanobacterial genera from that study included *Lyngbya*, *Leptolyngbya*, *Cyanobacterium*, *Phormidium* and *Chrococcidiopsis*. In the study by Blasi et al. (2022) Cyanobacteria comprised only 1% of total bacterial community, though higher abundances of Pseudanabaenaceae and Rivulariaceae were observed microscopically on anterior scutes. Bachmann et al. (2024) detected cyanobacterial sequences only on green turtles while loggerheads carried no cyanobacteria. Kuschke et al. (2024) reported Cyanobacteria in the top 10 bacterial phyla with 1.4% cyanobacteria in nesting leatherback turtles and a greater dominance of cyanobacteria on flippers (2.2%) than on carapace (0.48%). Although these studies consistently detect cyanobacteria among the most abundant phyla, a deeper understanding of their role within the sea turtle microbiome is needed.

The taxonomic complexity of cyanobacteria poses a challenge, as commonly used bacterial reference databases contain numerous misclassifications (Lefler et al. 2023). This underscores the need to integrate metabarcoding with other methods like cultivation and microscopy to refine taxonomic resolution and ecological interpretation.

Metabarcoding, or amplicon sequencing, has become one of the most widely used techniques today, largely due to its affordability, well-established analytical pipelines, and the lack of a need for specialized taxonomists (Kolda 2018). Ongoing efforts to refine reference databases (Roush et al. 2021, Lefler et al. 2023) present a promising avenue for advancing ecological research. As a result, it is unsurprising that metabarcoding is frequently employed in ecological studies to assess cyanobacterial diversity across various environments.

Notably, no attempts have been made to cultivate these epizoic cyanobacteria. This is a consequence of the general difficulties with cyanobacterial isolation, especially in low abundance environments. Cyanobacterial cultivation is very different from heterotrophic bacteria cultivation (Andersen 2005, Haande et al. 2017). Their slow growth rates, faster-growing algal contamination, problems with obtaining axenic strains makes cyanobacterial cultivation time-consuming and tedious (Schmelling and Bross 2024). When combined with the fact that not all bacteria can be cultivated, according to the "great plate anomaly" theory (Razumov 1932, Staley and Konopka 1985), culturing cyanobacteria as a way of capturing true diversity has long been abandoned. However, cultures play a crucial role in the comprehensive characterization of cyanobacterial taxa, integrating molecular, microscopic, and eco-physiological data (Komárek 2016). Furthermore, isolating and culturing microbial strains can provide insights that cannot be obtained through metabarcoding alone, such as physiological traits, growth dynamics, chemical and antibiotic susceptibility, and cyanotoxin production. Additionally, cultivated strains serve as a foundation for future taxonomic research (Fountain-Jones et al. 2024).

Future research should focus on identifying the presence of host-specific cyanobacterial associations, exploring their ecological roles, and investigating environmental and physiological drivers of their abundance. A deeper understanding of cyanobacteria will not only aid sea turtle conservation, but also advance broader cyanobacterial taxonomy and ecology.

3. MATERIALS AND METHODS

This research included analyses of cyanobacterial communities using a dual approach: a cultivation-independent approach (DNA metabarcoding/16S rRNA amplicon sequencing) and cultivation of sea turtle-associated cyanobacterial strains. The metabarcoding was performed in two rounds, the first metabarcoding survey was performed using universal bacterial primers to gain information of the contribution of cyanobacteria among all bacteria. The second metabarcoding survey was performed with specific cyanobacterial primers to obtain enough sequences for detailed insight into epibiotic cyanobacterial community and valid statistical results. Cultivation of cyanobacterial strains was implemented as a complimentary method to describe the composition of epibiotic cyanobacterial community and to get insight into the morphology and phylogeny of culturable taxa.

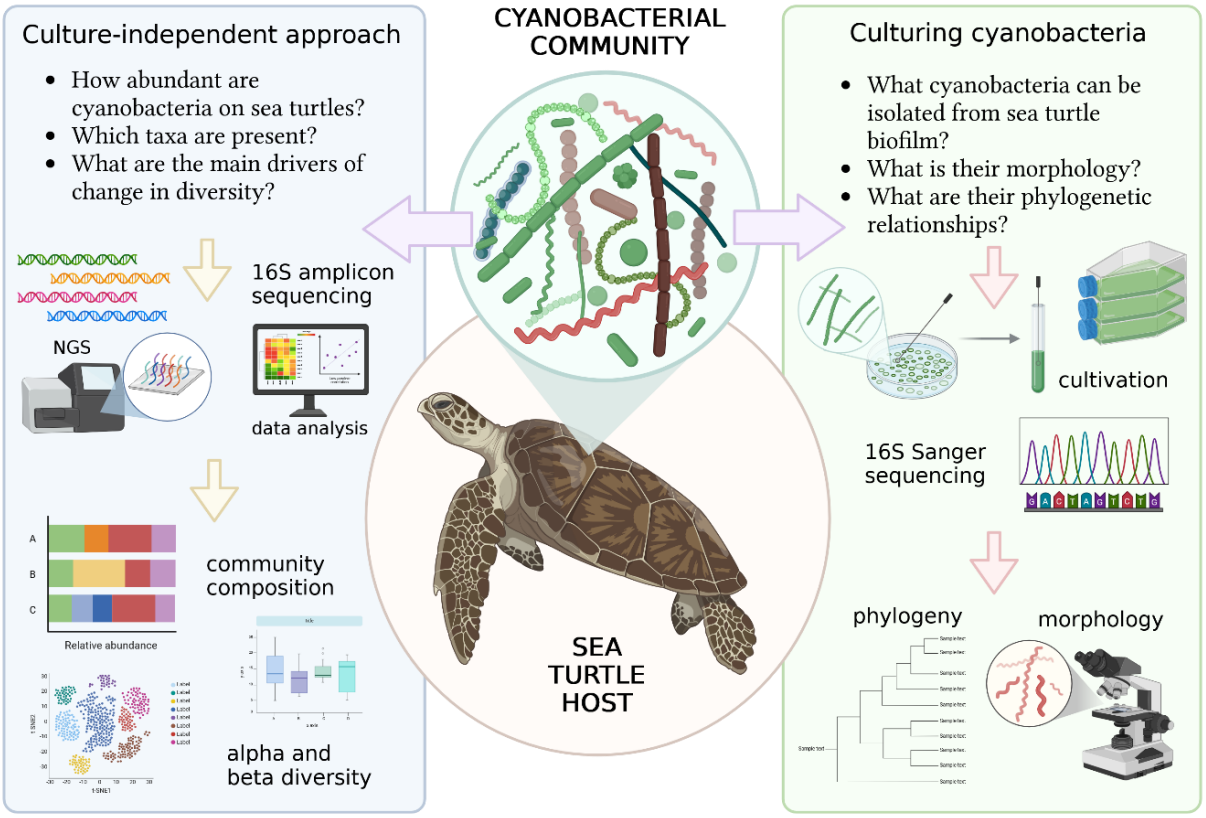


Figure 5. Graphical overview of the main research approaches, research questions and methods used in the dissertation.

3.1 Sampling of loggerhead sea turtles

Loggerhead sea turtles (*Caretta caretta*) were sampled both in rehabilitation centers and in the wild during tagging activities, as part of the project TurtleBIOME. Loggerheads included healthy and injured animals from different areas of the Mediterranean Sea: the Adriatic, Ionian, Tyrrhenian and Aegean seas. The turtles were sampled in three centers for rehabilitation of sea turtles (Sea Turtle Rescue Center Aquarium Pula and Blue World institute Lošinj in Croatia and The Archelon Sea Turtle Protection Society in Greece) and one veterinary clinic (The Sea Turtle Clinic, STC, Department of Veterinary Medicine, University of Bari "Aldo Moro" in Bari, Italy). In Greece, loggerheads foraging in Amvrakikos Bay were sampled by volunteers from the Archelon association during their regular monitoring activities, and on Rethymnos (Crete) during nesting and monitoring on beaches. The sampling was performed in accordance with the Helsinki Declaration of 1975, revised in 2013, and applicable national laws. Sampling at the Sea Turtle Clinic (Bari, Italy) was carried out with the permission of the Committee for Animal Ethics of the Institute of Veterinary Medicine (Authorization #4/19), while sampling in Croatia was carried out under the authorization of the Sea Turtle Rescue Center of Aquarium Pula by the Ministry of Protection environment and energy of the Republic of Croatia. Sampling activities in Greece were carried out with the permission of the Greek Ministry of Agriculture and Environment. A leatherback and a hawksbill sea turtle were sampled by Dr. Roksana Majewska in South Africa, cultures initially isolated by her and clone of this cultures provided for the research within this thesis. Details about sea turtles sampled for each experiment can be found in the following chapters.

3.2 Cultivation-independent DNA-based approach

3.2.1 First metabarcoding survey - universal prokaryotic amplicons

Sampling sea turtles for first metabarcoding survey

For the first metabarcoding survey a total of 26 loggerhead sea turtles were sampled from four regions within the Mediterranean: the Adriatic Sea (n = 14), the Ionian Sea (n = 9), the Tyrrhenian Sea (n = 1), and the Aegean Sea (n = 2), following the guidelines outlined by Pinou et al. (2019). The locations where turtles were originally found are shown in **Figure 6**.

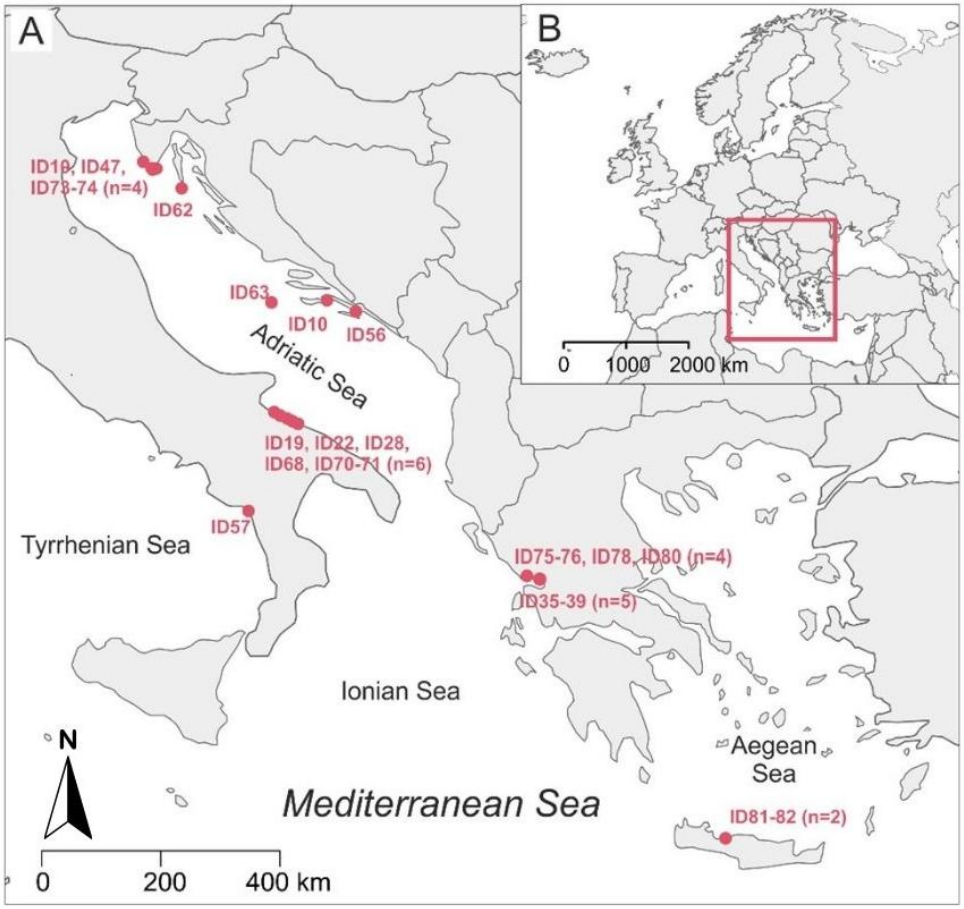


Figure 6. Map of the origin localities of sampled loggerhead turtles for first metabarcoding with universal bacterial primers; Turtle ID code is indicated with red circles (A); position of our study area in map of Europe (B). The map was made using R packages maps (Becker et al., 2021) and mapdata (Becker et al., 2018).

One individual (ID010) was sampled twice, once upon arrival at the rescue center Aquarium Pula and again after approximately a year of rehabilitation. In total, 54 samples were obtained between August 2018 and November 2019 (**Table 1**) from Marine Turtle Rescue Center Aquarium Pula and Blue World Institute Lošinj in Croatia, The Archelon Sea Turtle Protection Society in Greece and The Sea Turtle Clinic, STC, Department of Veterinary Medicine, University of Bari “Aldo Moro” in Bari, Italy. Turtles were designated either as "wild" if sampled immediately upon capture before being placed in a rehabilitation pool, or as "admitted" if immersed in a rehabilitation pool before sampling. The time interval between admission and sampling varied between 1 and 10 days, except for ID010.

Table 1.Sea turtle and sample information for first metabarcoding survey with universal bacterial primers for 16S V4 region. Sampling date is provided in format DD.MM.YYY., curved carapace length (CCL) is expressed in centimeters.

Turtle ID	Turtle name	Carapace ID	Skin ID	Origin sea	Sampling date	CCL
ID010	Merry Fisher	TB31	TB32	Adriatic Sea	11.12.2018.	n.a.
		TB139	TB140	Adriatic Sea	04.11.2019.	69.7
ID019	Tarcontes	TB49	TB50	Adriatic Sea	09.01.2019.	50.7
ID022	Reti	TB55	TB56	Adriatic Sea	10.01.2019.	72
ID028	Murrana	TB73	TB74	Adriatic Sea	17.01.2019.	74.5
ID034	Iracus	TB89	TB90	Adriatic Sea	22.01.2019.	72
ID035	GTB1	GTB11	GTB12	Ionian Sea	01.08.2018.	78.6
ID036	GTB2	GTB21	GTB22	Ionian Sea	01.08.2018.	51
ID037	GTB3	GTB31	GTB32	Ionian Sea	01.08.2018.	69.6
ID038	GTB4	GTB41	GTB42	Ionian Sea	01.08.2018.	58.5
ID039	GTB5	GTB51	GTB52	Ionian Sea	01.08.2018.	53.2
ID047	Žal	TB115	TB116	Adriatic Sea	08.05.2019.	53.5
ID056	Samba	TB117	TB118	Adriatic Sea	09.06.2019.	74.0
ID057	Angelo	TB119	TB120	Tyrrhenian Sea	24.06.2019.	77
ID062	Zoki	TB129	TB130	Adriatic Sea	30.07.2019.	54
ID063	CC Vis 1901	TB131	TB132	Adriatic Sea	10.06.2019.	24
ID068	Kanooh	TB145	TB146	Adriatic Sea	25.07.2019.	46.5
ID070	Futon	TB149	TB150	Adriatic Sea	24.07.2019.	43
ID071	Cosmyn	TB151	TB152	Adriatic Sea	23.10.2019.	65.2
ID073	Marvin	TB155	TB156	Adriatic Sea	20.11.2019.	32.2.
ID074	Ryan	TB157	TB158	Adriatic Sea	20.11.2019.	n.a.
ID075	AMV No1 GTB6	GTB61	GTB62	Ionian Sea	02.07.2019.	64.5
ID076	AMV No2 GTB7	GTB71	GTB72	Ionian Sea	01.07.2019.	62.9
ID078	AMV No4 GTB9	GTB91	GTB92	Ionian Sea	01.07.2019.	55.3
ID080	AMV No6 GTB11	GTB111	GTB112	Ionian Sea	01.07.2019.	66.5
ID081	RETH No1 GTB12	GTB121	GTB122	Aegean Sea	10.07.2019.	n.a.
ID082	RETH No1 GTB13	GTB131	GTB132	Aegean Sea	14.07.2019.	n.a.

Two separate samples were obtained from each turtle – one from the carapace and another one from the skin (**Figure 7**). Biofilm samples were collected using either sterile scalpels or clean toothbrushes, then immediately suspended in 96% ethanol (Marotz et al. 2021) within sterile 50 ml conical tubes. While carapace samples were randomly taken from the entire shell, skin samples were specifically collected from the upper part of head, neck, and flippers. All samples were stored at -20°C until further analysis.

Figure 7. Handling and sampling of loggerhead sea turtle skin (left image) and carapace (right image) by trained and authorized personnel in Sea Turtle Clinic in Bari, Italy.

Sequence data processing for first metabarcoding survey

DNA extraction and sequencing were conducted in two separate rounds: in 2019 (20 samples from ID10, ID19-39) and 2020 (34 samples from ID10, ID47-82). For both rounds, before DNA extraction, samples were centrifuged in 15 ml tubes (2500 rpm for 10 min, Centurion Scientific K3 Series, UK), and the ethanol supernatant was removed, leaving only the pelleted material for further analysis. DNA was extracted in duplicates from 0.25 g of ethanol-free samples using the DNeasy PowerSoil kit, following the manufacturer's protocol with some modifications. The modifications were made in both rounds using trial and error methods because the original protocol did not provide enough DNA material or we did not have access to the bead-beater. The samples were placed into PowerBead tubes and incubated at 50°C for 15 minutes. Incubation times for solutions C1, C2, and C3 were adjusted: 30 minutes at 65°C for C1, and 15 minutes at 4°C for the others. Instead of bead-beating, a horizontal vortexing method was used with an IKA VXR basic Vibrax shaker for 10 minutes at a maximum speed of 2200 rpm. The DNA was then eluted in 50 ml of DNase-free molecular-grade water after incubating at room temperature for 2 minutes. DNA purity and concentration were assessed using a NanoDrop ND-1000 V3.8 spectrophotometer (Thermo Fisher).

Extracted DNA was sequenced using the Illumina MiSeq System, generating 2×250 bp paired-end reads. The 16S rRNA gene V4 region was amplified using universal prokaryotic primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al. 2015, Parada et al. 2016). The sequencing was conducted at Molecular Research MrDNA (Shallowater, TX, United States).

Sequences obtained from MrDNA were processed using FASTqProcessor (MrDNA) to remove non-biological sequences before exporting them in a QIIME2-compatible format ("EMP protocol" multiplexed paired-end fastq format). These sequences were then imported into QIIME2 version 2020.6 (Bolyen et al. 2019). Demultiplexing was conducted via the q2-demux plugin, and sequence denoising was performed using the q2-dada2 plugin (Callahan et al. 2016). Taxonomic assignment of amplicon sequence variants (ASVs) was conducted using the q2-feature-classifier plugin with a naïve Bayes classifier trained on SILVA v.138 (99% 505F-806R nb classifier) (Quast et al. 2013) and re-analysis was made with taxonomic assignment using CyanoSeq reference database integrated into SILVA v.138 (Lefler et al. 2023). Prior to further analysis, mitochondrial and chloroplast sequences were removed.

Data visualization of taxa bar plots was generated in R (R Core Team 2024) within R Studio (R Project for Statistical Computing) using packages dplyr (Wickham et al. 2023), ggplot2 (Wickham 2016), RColorBrewer (Neuwirth 2022) and patchwork (Pedersen 2024). The relative abundance of various sample groups was calculated by summing the ASV counts for each taxon and dividing them by the total sequence count for that group.

3.2.2 Second metabarcoding survey - specific cyanobacterial amplicons

Sampling sea turtles for second metabarcoding survey

For the second metabarcoding survey, the samples were collected only from carapaces of loggerhead sea turtles from the Adriatic Sea (**Figure 8**). This study included samples from total of 28 turtles: 19 juveniles, 5 sub-adults, and 4 adults (**Table 2**). Developmental stage (age) classifications were based on carapace length: juveniles had a curved carapace length (CCL) of ≤59.9 cm, sub-adults ranged from 60 to 69.9 cm, and adults had a CCL of ≥70 cm, following Mariani et al. (2023) (**Figure 9**). Most of the turtles in this study were either stranded, injured, or accidentally captured by fishermen along the eastern Adriatic coast between 2020 and 2021 (Figure 7) before being transported to two Croatian Sea Turtle Rescue Centers: the Blue World Institute in Mali Lošinj and Aquarium Pula in Pula. Additionally, three turtles (TB181, TB183, and TB185) were sampled as part of research conducted by the Blue World Institute in the Vis archipelago, Croatia, and were immediately released after sampling (**Table 2**).

Sampling occurred as soon as possible after the turtles' arrival at the rescue centers. For this part of the study only biofilm samples obtained from carapaces were used. The biofilm was

randomly sampled from the carapace surface using a clean toothbrush (Dentalux) and resuspended in 96% ethanol within 50 ml collection tubes. Additionally, microbial biofilm samples were collected from the inner plastic surfaces of the rehabilitation pools where turtles TB217 (sample ID: TB217P) and TB219 (sample ID: TB219P) were housed. Furthermore, a separate sample (sample ID: VRS) was obtained by scraping submerged rocks from a beach near Aquarium Pula (44.833372, 13.833015). All samples were preserved in 96% ethanol at -20°C until DNA extraction.

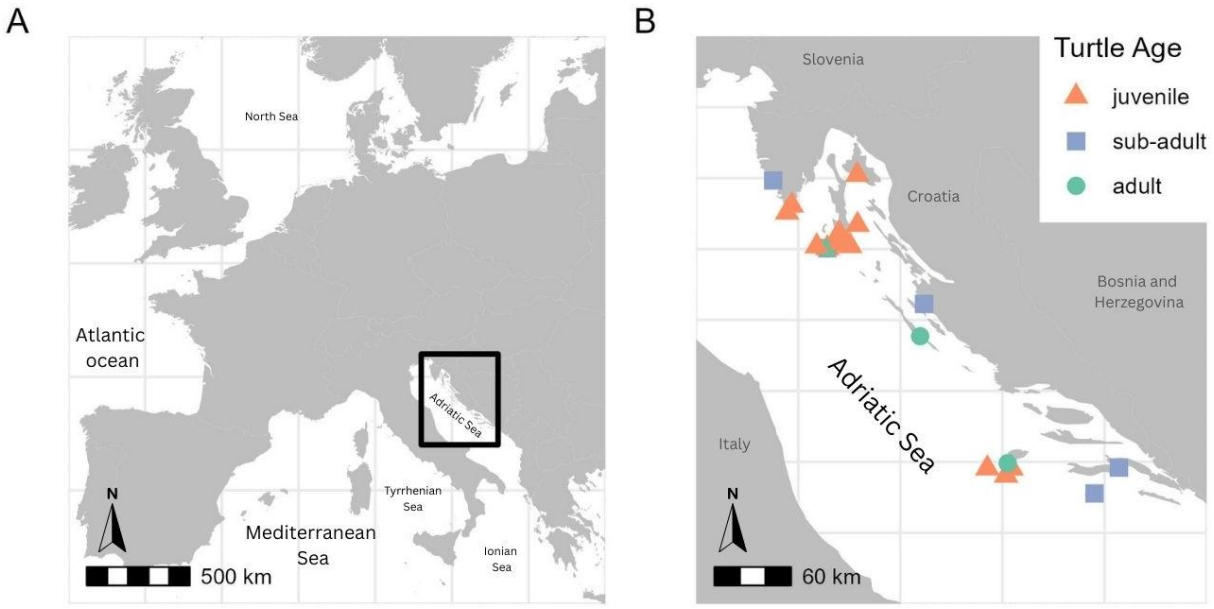


Figure 8. Maps of locations of sampled loggerhead sea turtles in the second metabarcoding survey; A – map of Europe with indicated study area in black rectangle; B – map of Adriatic Sea with indicated locations of each sampled animal, triangle shapes represent juvenile animals, squares represent sub-adult animals and circles represent adult animals.

Morphological examination of cyanobacteria in epibiotic biofilms

To observe cyanobacteria within the epibiotic microbial biofilm, scanning electron microscopy (SEM) was conducted on shed scutes from turtle TB235 (named Kolumbo, CCL = 65 cm), which was sampled by the Sea Turtle Rescue Centre at Aquarium Pula on 13.10.2023. This sample was used solely for morphological analysis and was not included in the amplicon sequencing. The scutes were chemically dried using HMDS (Oshel 1997), then mounted on aluminum stubs with carbon tape, and sputter-coated with a 15 nm platinum layer using the Precision Etching and Coating System (PECS II, Gatan Inc., Pleasanton, CA, USA). Imaging was performed with a JEOL JSM-7800F scanning electron microscope (JEOL, Tokyo, Japan)

at the Department of Physics, Centre for Micro and Nano Sciences and Technologies, University of Rijeka, Croatia.

Figure 9. Examples of loggerhead sea turtles with indicated curved carapace length (CCL) measurements according to Mariani et al. (2023); A – juvenile sea turtle (TB189); sub-adult sea turtle (TB191); adult sea turtle (TB199).

Sequence data processing and statistical analysis for second metabarcoding survey

Before DNA extraction, samples were centrifuged in 15 ml tubes (2500 rpm for 10 min, Centurion Scientific K3 Series, UK), and the ethanol supernatant was removed, leaving only the pelleted material for further analysis. DNA was extracted from 250 mg of each sample using the DNeasy PowerLyzer PowerSoil kit (Qiagen, Germany), following the manufacturer's protocol with slight modifications. After adding the C1 solution, samples were incubated at 70°C for 10 min. Bead beating was performed using a Qiagen TissueLyser (Retsch, Germany) set to 30 Hz for 1 min. The final DNA elution step was done using 50 µl of Solution C6, with a 5-minute incubation at room temperature before the last centrifugation step. A negative control (nuclease-free water, W4502 Sigma-Aldrich) was included and processed alongside the samples using the same DNA extraction kit. DNA concentration and purity were assessed using a Biospec-Nano spectrophotometer (Shimadzu, Japan).

Extracted DNA samples were sent to Microsynth AG (Balgach, Switzerland) for library preparation and sequencing. Sequencing was carried out on an Illumina MiSeq System, generating 2×300 bp paired-end reads with cyanobacteria-specific custom primers targeting the V6 region: 1328F (5'-GCTAACGCGTTAAGTATCCCGCCTGG-3') and 1664R (5'-GTCTCTCTAGAGTGCCCAACTTAATG-3') (Lee et al. 2017).

Table 2. Information about sampled loggerhead sea turtles from the second metabarcoding survey; sampling date is given in format: DD.MM.YYYY.; CCL = curved carapace length; CCW = curved carapace width. Turtle age groups were determined as follows: juveniles ≤59.9 cm CCL, sub-adults 60-69.9 cm CCL, adults ≥70 cm CCL, CCL and CCW are expressed in centimetres.

Sample ID	Turtle name	Sampling date	Sampling season	Age	CCL	CCW	Rescue centre	Longitude	Latitude	Coast	Condition
TB159	Ella	30.06.2020	summer	Sub-adult	62.0	58.0	Aquarium Pula	15.23235	44.11306	south	injured
TB163	Huanita	20.03.2020	spring	Sub-adult	68.0	66.0	Aquarium Pula	16.90186	42.77756	south	injured
TB167	Maro	21.07.2020	summer	Sub-adult	67.0	63.2	Aquarium Pula	17.14156	42.95932	south	injured
TB175	Maksimus	28.07.2020	summer	Juvenile	45.2	44.2	Aquarium Pula	13.89056	44.75832	north	good
TB177	Špela	3.8.2020	summer	Sub-adult	68.0	67.0	Aquarium Pula	13.75431	44.98378	north	poor
TB181	CC_VIS_2001	29.06.2020	summer	Juvenile	42.0	37.0	Blue World Institute	16.03579	42.90401	south	good
TB183	CC_VIS_2002	11.08.2020	summer	Juvenile	31.0	29.0	Blue World Institute	15.85258	42.9546	south	good
TB185	CC_VIS_2003	20.08.2020	summer	Juvenile	31.0	28.0	Blue World Institute	16.09005	42.9536	south	good
TB189	Valbiska	5.11.2020	autumn	Juvenile	30.0	27.5	Blue World Institute	14.57858	45.02314	north	injured
TB191	FS94	30.11.2020	autumn	Sub-adult	67.5	66.0	Blue World Institute	14.28518	44.50634	north	good
TB195	FS35	10.12.2020	autumn	Juvenile	45.0	41.0	Blue World Institute	14.28518	44.50634	north	good
TB197	FS25	19.12.2020	autumn	Adult	70.0	67.0	Blue World Institute	14.28518	44.50634	north	good
TB199	FS60	19.12.2020	autumn	Adult	73.0	68.0	Blue World Institute	14.28518	44.50634	north	good
TB201	Apox	14.1.2021	winter	Juvenile	36.0	34.0	Blue World Institute	14.44744	44.54678	north	injured
TB203	CC_Lošinj_2102	25.01.2021	winter	Juvenile	55.0	53.5	Blue World Institute	14.40861	44.57298	north	injured
TB205	Zlata	03.02.2021	winter	Juvenile	35.5	33.0	Blue World Institute	14.4418	44.54294	north	poor
TB207	Noemi	16.02.2021	winter	Juvenile	40.5	38.0	Blue World Institute	14.50811	44.52218	north	injured
TB209	Sanjin	16.02.2021	winter	Juvenile	32.0	29.0	Blue World Institute	14.44744	44.54678	north	injured
TB211	CC_Lošinj_2106	08.03.2021	winter	Juvenile	40.0	38.0	Blue World Institute	14.40082	44.61342	north	injured
TB213	Natanael	10.03.2021	winter	Juvenile	33.0	29.0	Blue World Institute	n.a.	n.a.	n.a.	poor
TB215	Karlo Albano	25.03.2021	spring	Adult	73.0	73.0	Aquarium Pula	15.19374	43.88625	south	poor
TB217	Oliver Raul	23.04.2021	spring	Juvenile	26.0	24.2	Aquarium Pula	13.93734	44.80744	north	poor
TB219	Martin	23.04.2021	spring	Juvenile	37.8	34.8	Aquarium Pula	14.47877	44.53431	north	poor
TB221	CC_Lošinj_2112	25.05.2021	spring	Juvenile	31.0	28.0	Blue World Institute	14.42382	44.56873	north	poor
TB223	Bova	08.06.2021	spring	Adult	70.0	64.0	Blue World Institute	16.051	42.991	south	injured
TB227	Calimero	06.04.2021	spring	Juvenile	52.0	52.0	Blue World Institute	14.18147	44.51976	north	injured
TB229	Marijana	06.04.2021	spring	Juvenile	26.0	23.0	Blue World Institute	14.58064	44.67156	north	good
TB231	CC_Lošinj_2111	21.04.2021.	spring	Juvenile	27.0	25.5	Blue World Institute	14.43973	44.54774	north	poor

Primer and adapter sequences were trimmed, and demultiplexing was performed by the sequencing service provider. The demultiplexed sequences were then imported into the QIIME2 environment (Bolyen et al. 2019) using Casava 1.8 demultiplexed paired-end format. Sequence denoising was conducted using DADA2 (q2-dada2 plugin), generating amplicon sequence variants (ASVs) (Callahan et al. 2016). The V6 sequences were truncated at 258 bp for forward reads and 162 bp for reverse reads. A second truncation was applied at 240 bp for forward reads and 200 bp for reverse reads.

Taxonomic classification of ASVs was performed using the q2-feature-classifier plugin (Bokulich et al. 2018), with the CyanoSeq reference database integrated into SILVA v.138 (Lefler et al. 2023). Prior to further analysis, chloroplast sequences were filtered out, retaining only sequences belonging to the Cyanobacteriota phylum. QIIME2 artifacts, including the feature table, taxonomy assignments, rooted phylogenetic tree, and metadata table, were imported into the R environment (R version 4.2.2, R Studio version 2023.09.1) using the qiime2R package (Bisanz 2018).

Compositional taxa bar plots were used for illustrating cyanobacterial community composition on Class, Order, Family and Genus level using dplyr (Wickham et al. 2023), ggplot2 (Wickham 2016) and RColorBrewer (Neuwirth 2022) packages for R. Venn diagram was used to show shared and unique cyanobacterial ASVs across juvenile, sub-adult and adult turtle groups as well as number of core cyanobacterial ASVs and it was made using ggvenn (Yan 2023) package for R and Canva editor. For alpha and beta diversity analyses, dataset was rarefied to the read depth of 10,098. Due to low number of reads (less than 10,098) samples TB211, TB213, TB167, TB217P, TB219P and negative control were excluded from diversity analyses.

Alpha diversity was inferred using 3 indices: Observed ASV richness (measuring number of ASVs present in a sample), Shannon's diversity index and Faith's Phylogenetic index. For determining which factors influence the cyanobacterial community the most, I examined categorical and numerical factors separately. Categorical factors were Age (**Table 2**), Recovery Centre (**Table 2**), Condition (good, poor or injured), Coast (north and south) and Season (spring, summer, autumn and winter; according to the sampling date in **Table 2**). Kruskall-Wallis test was used in assessing the influence of categorical factors on alpha diversity indices and differences were visualised using boxplots. Post-hoc Dunn test was used for assessing within-group difference of significant factors. Pearson correlation was used to assess the association

between numerical factors (CCL, CCW, Latitude and Longitude) and alpha diversity metrices. Those analyses were performed in R using packages: phyloseq (McMurdie and Holmes 2013), picante (Kembel et al. 2010), dunn.test (Alexis Dinno 2014), writexl (Ooms 2017) and patchwork (Pedersen 2024).

For each alpha diversity metric, a model fitting was performed to see how well the collected turtle information explain the cyanobacterial diversity and to see which factors contributed to it the most. Three separate models were performed for three response variables: Observed ASV richness, Shannon diversity index and Faith's phylogenetic diversity index. Predictor variables were CCL, longitude, recovery centre and turtle condition. Variables Age and Coast were excluded because they were originally derived from CCL and Longitude variables, respectively, which were already included in the models. Only Longitude (and not Latitude) was included in the models as location effect test because of the position of Adriatic Sea that spans in the north-south direction. Variable Season was excluded from the models because it showed collinearity with other variables. Negative binomial generalized linear model (GLM) was used of Observed ASV richness due to the discrete nature of the data, while linear model (LM) was used for Shannon's diversity index and Faith's phylogenetic diversity. Forest plot of the estimated effects of predictors was used to visualize the effect of each predictor variable. Additional R packages used for this analysis were: MASS (Ripley and Venables 2009), car (Fox et al. 2001), AER (Kleiber and Zeileis 2008), pscl (Jackman 2005), and sjPlot (Lüdecke 2013).

To reduce dimensionality of multivariate data and to visualise the most influential cyanobacterial genera, I used principal components analysis (PCA) on center-log transformed data merged at genus level. Top 10 different genera that had the most influence on PC1 and PC2 were visualised as arrows in PCA visualisation. To determine which factors contribute the most to variation in the beta diversity of the cyanobacterial community on sea turtle carapaces (calculated as Bray-Curtis distance), I used permutational multivariate analysis of variance (PERMANOVA). Pairwise PERMANOVA with Bonferroni correction of p-value was performed for the factors that showed to be significant in global PERMANOVA. Additional R packages used for this analysis were: microbiome (Lahti and Sudarshan 2012), ggfortify (Horikoshi et al. 2016), ggpubr (Kassambara 2023), vegan (Oksanen et al. 2020) and pairwiseAdonis (Arbizu 2017). Code for data analysis for this thesis is available on GitHuB link: https://github.com/lucijakanjer/PhD-disertation.

3.3 Cultivation of sea turtle-associated cyanobacteria

3.3.1 Sampling and isolation of cyanobacterial strains

Live biofilm samples were collected from sea turtles of three species (loggerhead, hawksbill, and leatherback) between 2019 and 2022 (**Table 3**; **Supplement 1**). Individuals originated from the Adriatic Sea (Croatia) and South Africa. Sampling was performed by scraping the carapace or skin with a clean toothbrush, from random locations. The collected biofilm was resuspended in 0.2 µm syringe-filtered seawater and stored at room temperature in sterile 50 mL conical tubes for transport.

Upon arrival in the laboratory, samples were transferred to sterile 100×15 mm plastic Petri dishes and supplemented with sterile growth medium to initiate enrichment cultures. Four cyanobacteria-specific media: BG-11 marine, BG-11_0 marine, MN, and ASN-III (recipes in **Table 4**; Rippka et al., 1979) were used in isolation to maximize the likelihood of cultivating different cyanobacterial species. Media were sterilized by autoclaving prior to use. Each enrichment culture and later isolated strains were kept in the culture room, under a 16:8 h light:dark cycle at 22°C with white, fluorescent light.

Table 3. List of sea turtles from which cyanobacteria cultures were obtained.

Turtle ID	species	age	body part	collection date	location	strains
RM1	leatherback	adult	skin	14.12.2021.	Kosi Bay, SA	CY006
RM2	hawksbill	juvenile	skin	02.12.2020.	Bayworld, Port Elizabeth, SA	CY007
RM4	loggerhead	adult	carapace	06.07.2019.	uShaka Sea World, Durban, SA	CY009
TB215 Karlo Albano	loggerhead	adult	carapace	25.03.2021.	Telašćica, Croatia	CY002, CY004
TB235 Kolumbo	loggerhead	juvenile	carapace	13.10.2022.	Kornati, Croatia	CY011

From enrichment cultures, isolation of monocultures was performed using two techniques: direct micropipette isolation from liquid media and needle isolation from streaked solid media. Growth of enrichment cultures was regularly monitored (at least once a week) by inverted light microscopy (Olympus CX41). Once macroscopical growth of cyanobacteria had developed, typically after 1–4 weeks it was transferred to new sterile liquid medium and single trichomes were isolated using micropipette with mouthpiece into new Petri dish. Second

method of isolation, needle isolation, was performed as follows: macroscopic growth from initial enrichment cultures was scooped with microbiological inoculation loop and streaked on sterile solid media agar (1.5%) plate. After 1-4 week incubation, agar plates were observed under microscope and single trichomes were picked up with inoculation needle and transferred to sterile liquid media in 24-well plate. In cases where diatoms or other eukaryotic contaminants were present (in cultures from either technique), cycloheximide was added at concentrations between 100–500 µg/mL to suppress their growth. After another 1-4 week incubation period, in Petri dishes or wells of 24-well plate where only single cyanobacterial species grew without contaminants, we considered it as successfully established strain.

Table 4. Growth media recipes, adjusted from Rippka et al. (1979).

	BG-11 marine	**BG-11$_0$ marine**	**MN**	**ASN-III**
Aquarium Salts	35 g	35 g		25 g
$NaNO_3$	1.5 g		0.75 g	0.75 g
$K_2HPO_4 \cdot 3H_2O$	0.04 g	0.04 g	0.02 g	0.02 g
$MgSO_4 \cdot 7H_2O$	0.075 g	0.075 g	0.04 g	3.5 g
$CaCl_2 \cdot 2H_2O$	0.036 g	0.036 g	0.02 g	0.5 g
EDTA - $Na_2 \cdot 2H_2O$	0.001 g	0.001 g	0.0005 g	0.0005 g
Citric acid monohydrate	0.006 g	0.006 g	0.003 g	0.003 g
Ammonium ferric citrate	0.006 g	0.006 g	0.003 g	0.003 g
$NaHCO_3$	0.15 g	0.15 g		
$Na2CO_3 \cdot 10H2O$	0.054 g	0.054 g	0.02 g	0.054 g
$MgCl_2 \cdot 6H_2O$				2 g
KCl				0.05 g
A5 trace metals*	1 mL	1 mL	1 mL	1 mL
MilliQ water	1L	1L	250 mL	1L
Filtered seawater			750 mL	
pH	7	7	7	8.5

*Trace metal mix A5 contains (g/L): H_3BO_3, 2.86; $MnCI_2 \cdot 4H_20$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $NaMoO_4 \cdot 2H_20$, 0.390; $CuSO_4 \cdot 5H_20$, 0.079; $Co(N0_3)_2 \cdot 6H_20$, 0-0494.

All established strains, either by micropipette or needle isolation technique, were transferred and kept in sterile Falcon tubes of 15 mL in sterile BG-11 marine medium. They were transferred to a new Falcon tube with sterile BG-11 marine medium once every 1-3 months to keep strains actively growing. From this point on they were morphologically, molecularly and phylogenetically analyzed in the same way.

3.3.2 Morphological characterization of cyanobacterial strains

Morphological characterization of isolated strains was conducted using light microscopy on Olympus BX43 with SC100 camera and on Zeiss Axio Imager.A2 equipped with an Axiocam 305. Microphotographs were taken and morphometric measurements were made on at least 30 cells from 5–6 trichomes per strain. Cell dimensions (length and width) were recorded, and qualitative morphological features, such as pigmentation, presence of heterocysts or akinetes, and cell arrangement, were described.

3.3.3 Molecular identification of cyanobacterial strains

Molecular identification of cyanobacterial strains was based on 16S rRNA marker gene sequences which have been amplified and sequenced with adjacent internal transcribed spacer (ITS) as 16S-ITS region. Only 16S rRNA gene was used for phylogenetic tree construction in this thesis.

DNA isolation, PCR amplification and sequencing of strains

Genomic DNA was isolated from strains CY002 and CY004 using DNeasy PowerSoil PowerLyzer (Qiagen, Hilden, Germany) extraction kit according to the manufacturer's protocol. From strains CY006, CY007, CY009 and CY011 DNA was isolated using the NucleoSpin Soil kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions with one modification. During the sample lysis step, instead of vortexing for 5 minutes, this step was prolonged to 10 minutes. Digital Vortex Mixer (VWR) with Horizontal-(24) multitube holder (Scientific Industries, Inc.) was used at the maximum speed of the instrument, 2500 rpm. After extraction of DNA, the concentration of DNA in the samples was measured on NanoVue Plus Spectrophotometer (VWR).

After successful DNA extraction, 16S-ITS region of the rRNA gene was amplified by polymerase chain reaction (PCR) on PCR thermocycler Techne Prime Thermo Cycler T100 for strains CY002 and CY004 and on Thermal Cycler (Bio-Rad, USA) for strains CY006, CY007, CY009 and CY011. Universal bacterial primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and cyanobacteria-specific 23S30R primer (5'-CTTCGCCTCTGTGTGCCTAGGT-3') were used (Taton et al. 2003). The PCR reactions were performed in 50 µL reactions containing 31.3 µL of sterile water (Sigma Aldrich, USA), 5 µL of PCR TransTaq buffer (TransGene Biotech, China), 5 µL of BSA buffer (TransGene Biotech, China), 1 µL of dNTP (New England Biolabs, USA), 1.25 µL of each primer, 0.2 µL of the polymerase Staq+ (TransGene Biotech, China) and

1-8 µL volume of DNA, depending on the DNA concentation. The thermocycling conditions were 94°C for 10 min, 35 cycles of 94°C for 45 sec, 57°C for 45 sec and 68°C for 2 min. The protocol ends with final extension for 7 minutes at 68°C and cools down to 16°C.

The quality of the amplification was verified by running the electrophoresis. The gel was made with 1,5% of agarose in the buffer TAE (1×), the solution was heated to dissolve the agarose and once it cooled down to 40°C, 2 µL of GelRed (Biotium, USA) was added to the gel. The wells of the gel were charged with a mixture of 2 µL of loading dye (Thermo Fisher Scientific, USA) and 8 µL of PCR products. A DNA ladder, Lambda DNA/Hind III Marker (Thermo Fisher Scientific, USA), was also loaded on the gel. The electrophoresis ran for 1 hour with a current of 100 V and was visualized on the UV transilluminator.

The PCR products were purified with NucleoSpin Gel and PCR cleanup kit according to the manufacturer's instructions (Macherey-Nagel, Dueren, Germany). Quantity of DNA in the purified PCR product was measured on NanoVue before sequencing preparation in order to calculate correct volumes to prepare. For sequencing the entire 16S and ITS region, along with above mentioned 27F and 23S30R, four more primers were employed: 359F (5'-GGGGAATYTTCCGCAATGGG-3'), 979F (5'-CGATGCAACGCGAAGAAC-3'), 1092R (5'-GCGCTCGTTGCGGGACTT-3') and 1492R (5'-TACCTTGTTACGACTT-3') for each sample (following the method from Taton et al. 2003). For sequencing, more primers were used than for PCR amplification because the 16S-ITS region is long and combining sequences from multiple primers makes the sequencing result more accurate and reliable. Each sample sent to sequencing measured 10 µL with 2-8 ng/µL sample DNA concentration and 5 µM primer concentration. Purified PCR products from strains CY002 and CY004 were sent for Sanger sequencing to the Macrogen sequencing center (Amsterdam, Netherlands). Purified PCR products from strains CY006, CY007, CY009 and CY011 were sent to the GIGA sequencing center (University of Liège, Belgium) for Sanger sequencing.

Phylogenetic analyses of cyanobacterial strains

Obtained sequences from cyanobacterial strains were visualized and edited in Geneious Prime 2024.0 Software (https://www.geneious.com). From 16S-ITS region, only 16S amplicon was used to assemble the final phylogenetic tree and infer phylogeny. Low quality sites were removed and contigs were assembled to contain full length 16S amplicon. Resulting sequences were searched via BLAST tool against GenBank NCBI database. Along with those sequences, I added 10 best BLAST hit sequences and representative sequences of the selected genera,

families and orders. For more broader context, reference sequences (order Nodosilineales, Oscillatoriales and Spirulinales) from CyanoSeq database obtained via their Zenodo repository were added. All of those sequences were used to perform multiple sequence alignment using Clustal Omega (Sievers et al. 2011) plugin within Geneious software. The resulting alignment was used to make phylogenetic tree. Maximum likelihood (ML) phylogenetic tree was calculated using IQ-TREE 2 program (Minh et al. 2020). The substitution model was estimated using ModelFinder tool. Tree was calculated using TIM3e+R5 substitution model and 1000 bootstrap permutations using ultrafast bootstrap method (Hoang et al. 2018). To infer the best global tree, 300 runs were performed. Tree was visualized in iTOL tree viewer and edited in Canva editor.

Construction of ASV-strains phylogenetic tree

To combine and compare results obtained from metabarcoding and from strains together, the 16S rRNA sequences obtained from cultivated strains were cut to their V6 region to match the V6 region of the cyanobacterial ASVs from the metabarcoding survey. The ASVs and V6 region of cultivated strains were aligned using Clustal Omega (Sievers et al. 2011) plugin within Geneious software and the phylogenetic tree was calculated using IQ-TREE 2. The substitution model was estimated using ModelFinder tool. Tree was calculated using K3P+I+G4 substitution model and 1000 bootstrap permutations using ultrafast bootstrap method (Hoang et al. 2018). Tree was visualized using iTOL and bubble plot of ASV presence/absence data was added on the plot using iTOL editor. Only areas of interest were cut and shown using Canva editor.

4. RESULTS

4.1 Contribution of cyanobacteria to the total prokaryotic community of the sea turtles' carapace and skin

Results of the first metabarcoding survey, revealing relative abundances and contribution of cyanobacteria on the sea turtle carapace and skin samples are shown on **Figure 10**. Cyanobacteria were identified in all samples with average abundance of 3% of total bacterial community. Within-sample cyanobacteria abundance varied from 0.01% in sample TB157 to 19.77% in sample GTB131. Most abundant cyanobacterial genera are shown on **Figure 10A** with taxonomy assigned by SILVA database, where *Phormidium*_MBIC10003, unidentified Cyanobacteria and *Acrophorium*_PCC-1375 were three most abundant genera. Genera like *Pleurocapsa*, *Limnothrix*, *Geitlerinema*, *Chroococcidiopsis*, *Phormidesmis* and *Arthrospira* were also present in high abundances.

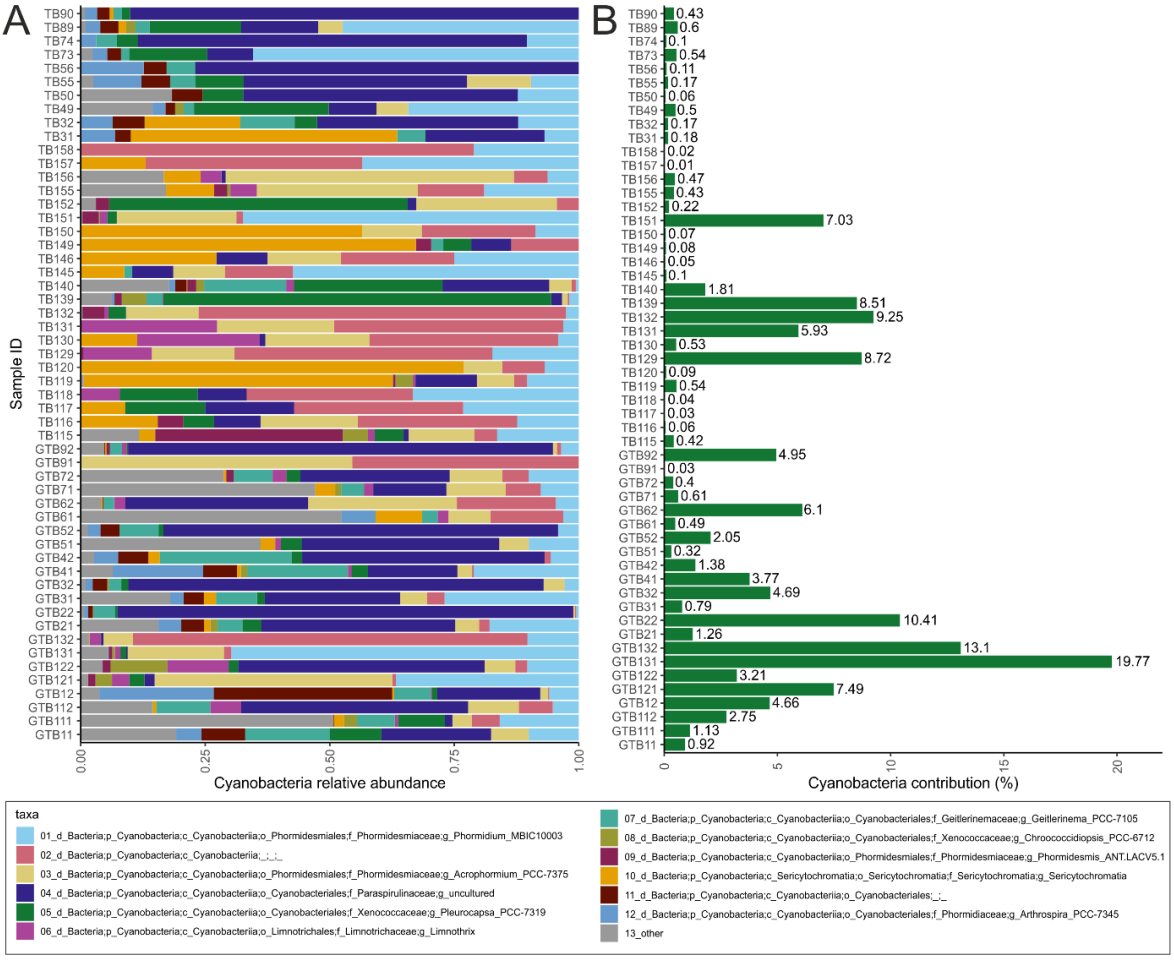


Figure 10. Relative abundances of cyanobacterial genera within the Cyanobacteriota phylum with SILVA 138 database for assigning taxonomy:. (A); contribution of Cyanobacteriota to total bacterial community within a sample based on relative abundance (B).

These results are part of a comprehensive analysis of bacterial and micro-eukaryotic communities on mediterranean loggerhead turtles as reported in Kanjer et al. 2022. In this dissertation, the dataset was re-analyzed with taxonomy assigned by CyanoSeq database (**Figure 11**) to be comparable with the second metabarcoding survey as it is better in assigning cyanobacterial taxonomy.

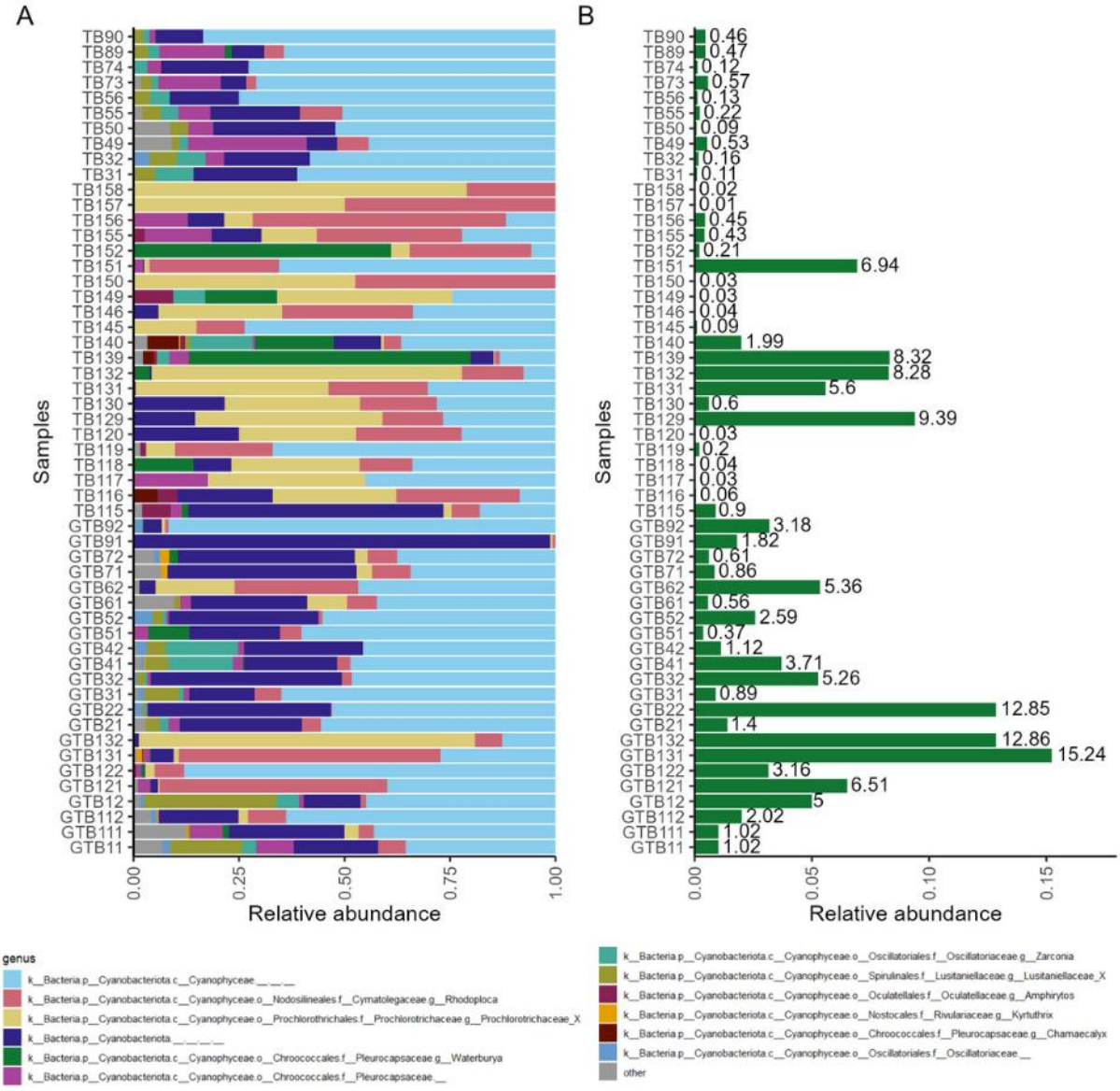


Figure 11. Relative abundances of cyanobacterial genera within the Cyanobacteriota phylum with CyanoSeq database used for assigning taxonomy: (A); contribution of Cyanobacteriota to total bacterial community within a sample based on relative abundance (B).

The re-analyzed results showed an average of 2.48% cyanobacterial abundance with a maximum of 15.24% of cyanobacteria in a single sample and minimum of 0.01% (**Figure 11B**). The most abundant genera assigned using CyanoSeq database (**Figure 11A**) were unidentified Cyanophyceae, *Rhodoploca* and Prochlorotrichaceae_X. The assigned genera that were also abundant were *Waterburya*, *Zarconia*, *Amphirytos*, *Kyruthrix*, and *Chamaecalyx*.

4.2 Detailed characterization of cyanobacterial community associated with sea turtles from Adriatic sea

Results of the second metabarcoding included detailed characterization of the epibiotic cyanobacterial community: (i) composition (chapter 4.2.2), (ii) alpha diversity (chapter 4.2.3) and beta diversity (chapter 4.2.4). Part of this results was published in Kanjer et al. (2024) where the turtle age and size were reported there as most important factor driving cyanobacterial diversity. This dissertation expands this previous research with graphical representations of all cyanobacterial taxon categories and statistics on influence of other factors on alpha diversity. Multivariate analyses using linear and generalized linear models further revealed how these factors interact to shape community composition. Finally, the integration of ASV data with strains in a combined phylogenetic tree (Chapter 4.4) provided a broader context, linking sequence-based community data with morphologically and genetically characterized strains.

4.2.1 Microscopic observations in epibiotic microbial community

Scanning electronic microscopy analyses of the biofilm formed on loggerhead scutes of the one individual turtle TB235 showed presence of homocytous filamentous cyanobacterial trichomes (**Figure 12**) embedded with organic matter and other prokaryotic and microeukaryotic cells. They most likely belong to orders like Oscillatoriales (**Figure 12A**, **B**), Spirulinales (**Figure 12C**, **D**), Nodosilineales, Leptolyngbyales, Oculatellales (**Figure 12E**), Pseudanabenales or Prochlorotrichales (**Figure 12F**), however this identification was based solely on morphological characteristics, and therefore exact taxonomical identification was not possible.

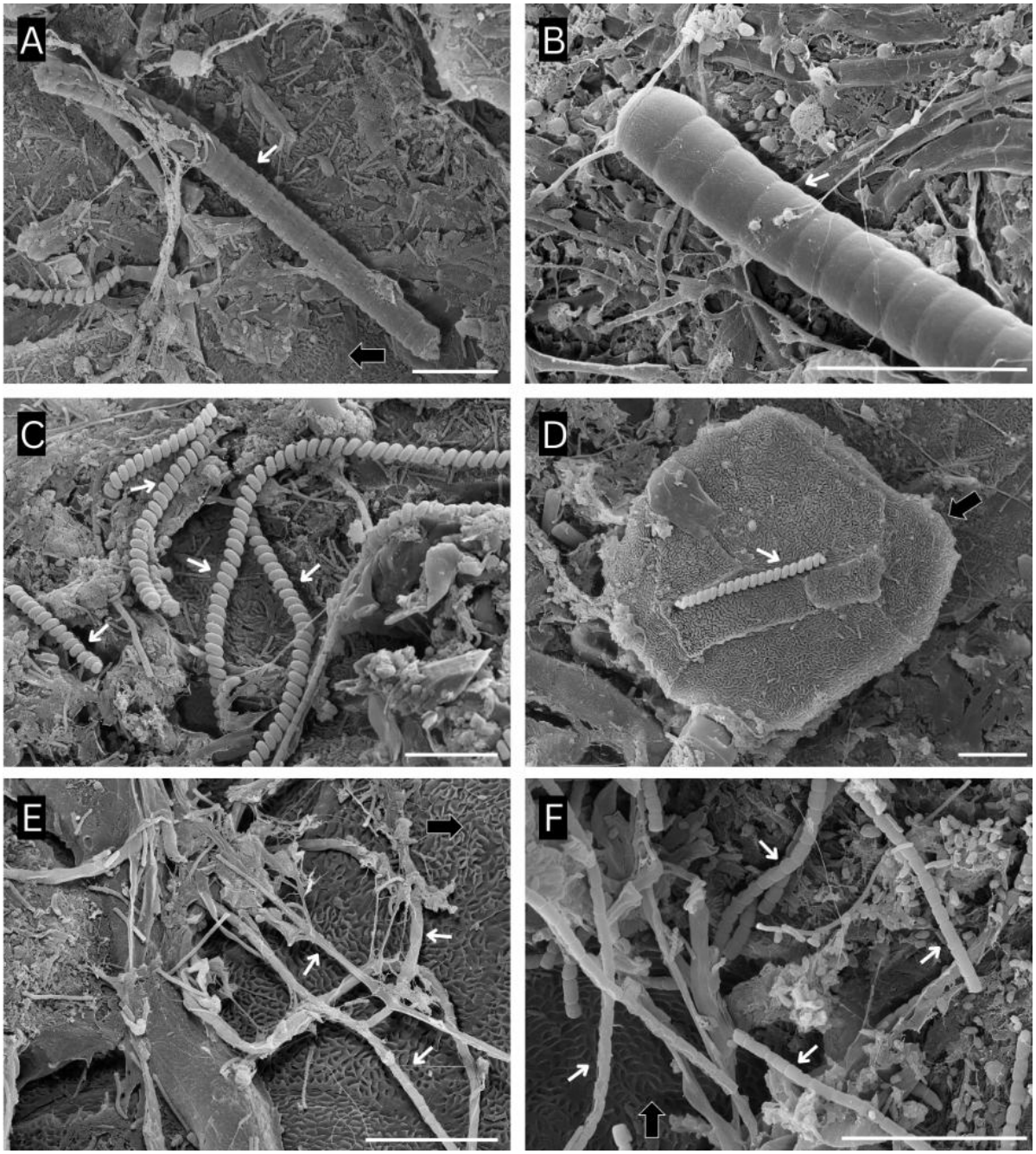


Figure 12. Scanning electron microscopy (SEM) images of cyanobacteria (white arrows) in the epibiotic microbial community growing on the sub-adult loggerhead sea turtle carapace surface (black arrows) sampled from sea turtle TB235; Oscillatoriales (A, B); Spirulinales (C, D); Nodosilineales, Leptolyngbyales or Oculatellales (E); Pseudanabenales or Prochlorotrichales (F); Scale bar represents length of 10 μm.

4.2.2 Cyanobacterial composition in sea turtles from different ages

High throughput sequencing in second metabarcoding survey yielded 2,359,741 high quality cyanobacterial sequences arranged in 551 ASVs and in 32 samples. Median sequence frequency per sample is 20,069.5 (maximum 412,998.0, minimum 107.0). In the samples TB209 and TB211 no cyanobacterial sequences were identified and therefore these samples were excluded from further analyses leaving a total of 30 samples. In the samples obtained from adult sea turtles, altogether 102 cyanobacterial ASVs were recorded; for subadult sea turtles, there were 104 ASVs, and for juvenile sea turtles, there were 67 ASVs (Figure 3). All groups shared 63 cyanobacterial ASVs; adults and subadults shared 35 ASVs, and subadults and juveniles also shared 35 ASVs, while adults and juveniles shared 17 ASVs (**Figure 13**).

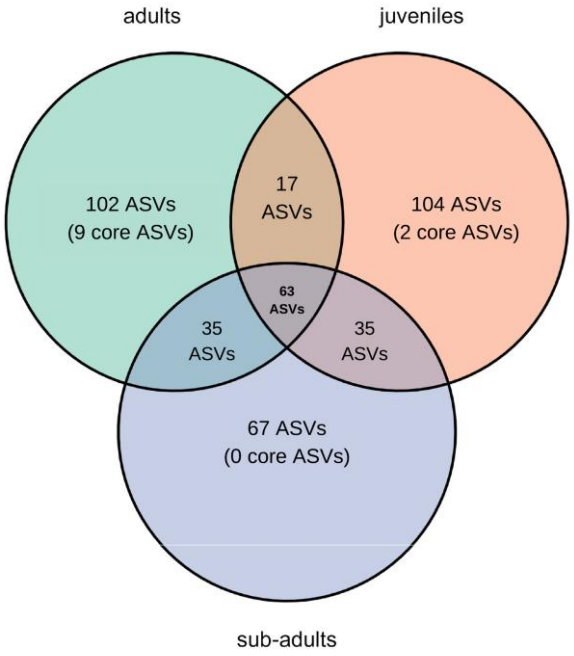


Figure 13. Venn diagram showing the number of detected cyanobacterial ASVs and number of core ASVs for each sea turtle group (adult, subadult, and juvenile) and number of ASVs shared among the groups.

Analysis of cyanobacterial relative abundances across samples revealed a consistent dominance of class Cyanophyceae across juvenile, sub-adult, and adult samples (**Figure 14**). Sericytochromatia and Vampirivibrionia were present in lower proportions across all developmental stages, and sub-adult sample TB191 had notably higher proportion of Sericytochromatia but was still dominated by Cyanophyceae. The juvenile, sub-adult, and adult

samples exhibit a similar dominance of Cyanophyceae, with some variability in other classes. The rock biofilm (R) and pool biofilm (P) samples also show exclusive prevalence of Cyanophyceae and classes Sericytochromatia and Vampirivibrionia were not recorded in those samples. Unclassified sequences were present in all sea turtle samples and in rock biofilm sample.

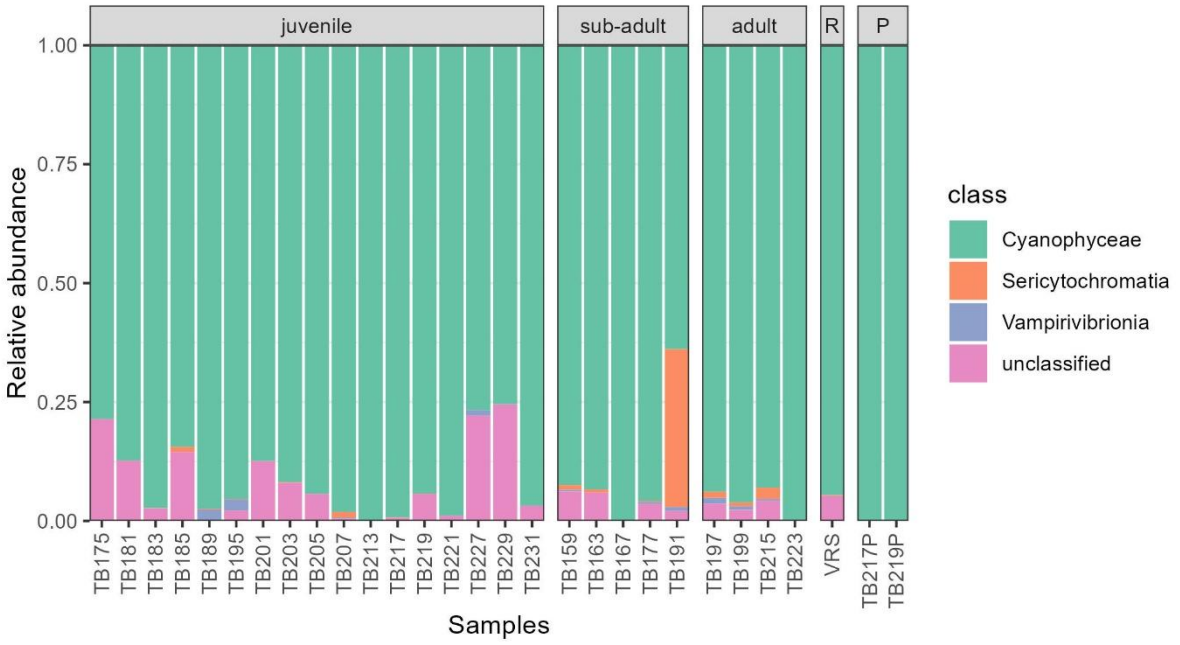


Figure 14. Relative abundance of cyanobacterial classes (kingdom Cyanobacteriota) in samples of sea turtle carapace biofilm, "unclassified" category represents sequences from kingdom Cyanobacteriota that cannot be classified further. Each bar represents one sample, sea turtle samples are divided according to their developmental stages into juvenile, sub-adult and adult groups, "R" represents rock biofilm sample and "P" represents pool samples.

At the order level, Nodosilineales were consistently dominant across all sample groups (**Figure 15**). Along with prevalence of Nodosilineales, juvenile samples also exhibited a higher proportion of Prochlorotrichales, whereas sub-adult and adult samples contained a more even distribution of Chroococcales. The pool biofilm (P) samples also exhibited a high abundance of Nodosiilineales. The rock biofilm sample showed no prevalence of a single order but rather a mix of Nodosilineales, Chroococcales, unclassified Cyanophyceae and Acaryochloridales. Unclassified sequences were present in all types of samples, but mostly in juvenile turtle samples.

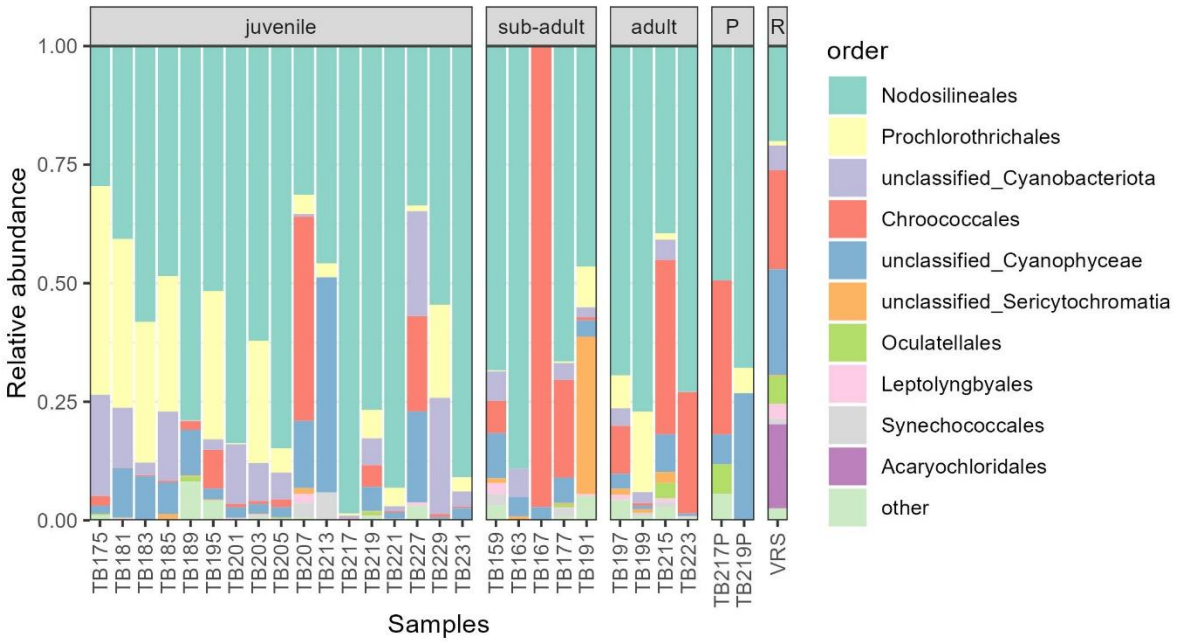


Figure 15. Relative abundance of ten most abundant cyanobacterial orders (kingdom Cyanobacteriota) in samples of sea turtle carapace biofilm, "other" category represents sum of abundances of all other orders present in a sample. Each bar represents one sample, sea turtle samples are divided according to their developmental stages into juvenile, sub-adult and adult groups, "R" represents rock biofilm sample and "P" represents pool samples.

At the family level, families Cymatolegaceae and Nodosilineaceae from the order Nodosilineales were the most abundant in whole dataset (**Figure 16**). Prochlorotrichaceae appeared in greater proportions in juvenile samples, while sub-adult, adult, rock and pool biofilm exhibited lower proportions of that family. Sub-adult samples were also dominated by Nodosilineaceae and Cymatolegaceae, except for sample TB167 which was dominated by Pleurocapsaceae. Adult turtle samples were also dominated by Nodosilineaceae and Cymatolegaceae, but display more diverse family compositions as well, for example, a notable proportion of Geminocystaceae was noted in the sample TB215. Pool biofilm (P) samples also showed dominance of Nodosilineaceae with contribution of Cymatolegaceae, Pleurocapsaceae, Geminocystaceae and several unclassified families. Rock biofilm sample was not dominated by a single family, but rather a mix of Cymatolegaceae, Nodosilineaceae, Pleurocapsaceae, Geminocystaceae, Oculatellaceae and several unclassified families.

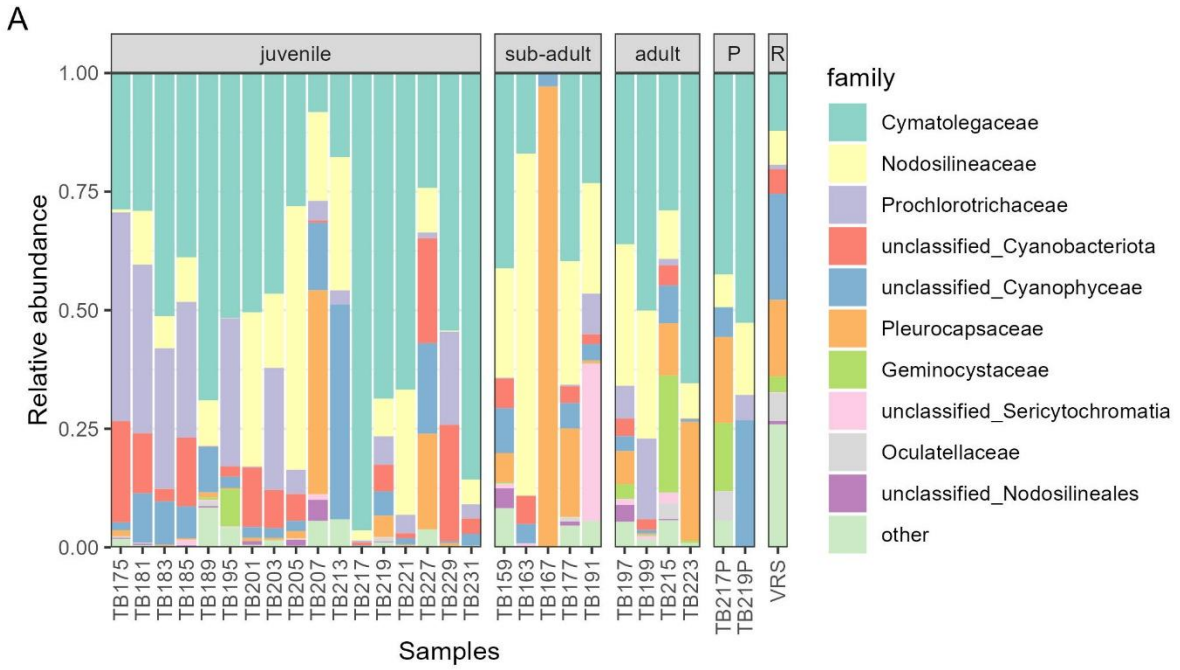


Figure 16. Relative abundance of ten most abundant cyanobacterial families (kingdom Cyanobacteriota) in samples of sea turtle carapace biofilm, "other" category represents sum of abundances of all other families present in a sample. Each bar represents one sample, sea turtle samples are divided according to their developmental stages into juvenile, sub-adult and adult groups, "R" represents rock biofilm sample and "P" represents pool samples.

At the genus level, the most dominant genera include *Rhodoploca*, Prochlorotrichaceae_X, and unclassified Nodosilineaceae (**Figure 17**). Juvenile samples are largely dominated by *Rhodoploca* and Prochlorotrichaceae_X, with contribution of other genera such as *Leptothoe*, *Cymatolege* and *Salileptolyngybya*. Also, a high proportion of unclassified sequences is also reported in juvenile samples. Sub-adult turtle samples display a more diverse genera composition with constant occurrence of *Rhodoploca*, *Cymatolege*, *Leptothoe* and *Salileptolyngybya*. Genus *Odorella* was also present in sub-adult turtle samples, while sample TB167 was completely dominated by that genus. Additionally, sub-adult turtle samples also exhibit high proportions of unclassified sequences. Adult turtle samples were dominated by *Rhodoploca* with the occurrence of Prochlorotrichaceae_X, *Leptothoe*, *Salileptolyngbya*, *Picosynechococcus* and *Odorella*. The pool biofilm (P) samples contained a diverse mix of genera, with *Rhodoploca* being dominant and *Leptothoe*, *Cymatolege*, *Picosynechococcus*, and *Odorella* being notable. The rock biofilm sample contained the highest number of "other" genera with notable presence of *Leptothoe* and unclassified sequences.

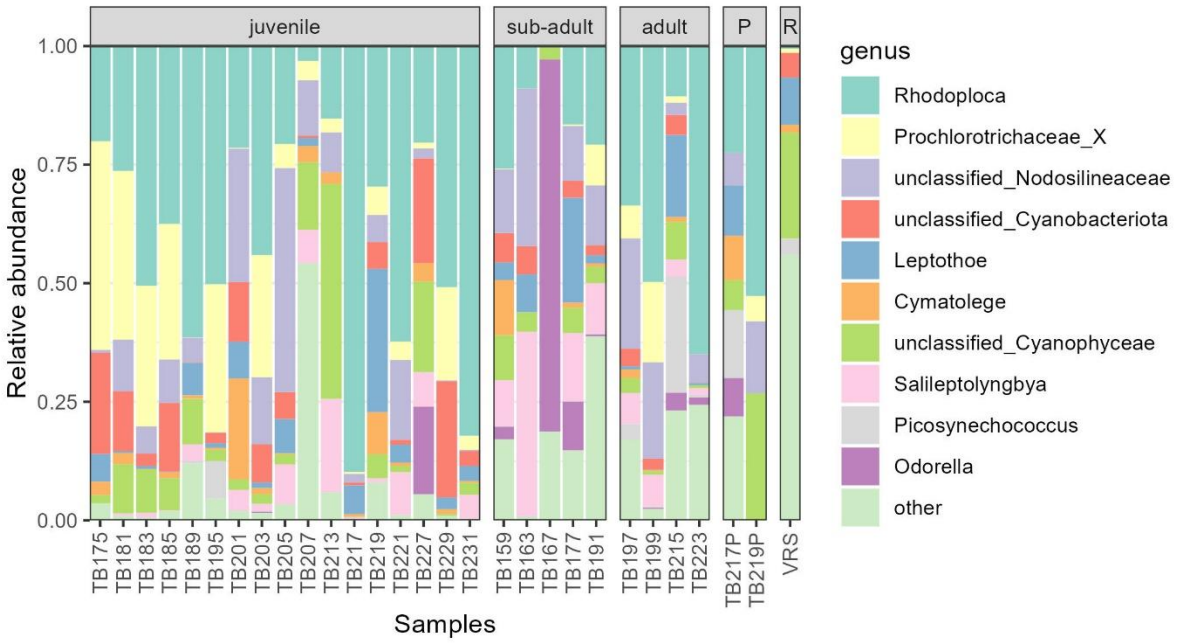


Figure 17. Relative abundance of ten most abundant cyanobacterial genera (kingdom Cyanobacteriota) in samples of sea turtle carapace biofilm, "other" category represents sum of abundances of all other genera present in a sample. Each bar represents one sample, sea turtle samples are divided according to their developmental stages into juvenile, sub-adult and adult groups, "R" represents rock biofilm sample and "P" represents pool samples.

Identifying cyanobacterial core ASVs in epibiotic communities

There were nine core cyanobacteria features (ASVs) identified in 100% of samples in adult sea turtle group (**Supplement 2**) and they were identified as belonging to genera *Rhodoploca*, *Salileptolyngbya*, *Picosynechococcus* and *Odorella*. There were also four core ASVs that were identified only to the family level *Nodosilineaceae* (BLAST showed 98.45% identity with *Leptolyngbya* sp. PCC 7124), phylum *Cyanobacteriota* (BLAST showed 100% identity with *Cyanobacterium* sp. BBD-AO-Red), order *Caenarcaniphilales* (BLAST showed 99.61% identity with Uncultured bacterium clone QSW25) and class *Cyanophyceae* (BLAST showed 98.06% identity with *Copelandiella yellowstonensis* YNP83A-MA6 and *Geitleribactron purpureum* Tovel-1). Two core ASVs were present in 100% samples in the juvenile turtle group (**Supplement 3**), one belonged to the genus *Rhodoploca*, and the other ASV as belonging to the family *Nodosilineaceae* (BLAST showed 98.45% identity with *Leptolyngbya* sp. PCC 7124). In sub-adult class there were no core features identified in 100% of samples, but there were five features identified in 80% of samples (**Supplement 4**). Those cyanobacteria belong to the genera *Rhodoploca*, *Salileptolyngbya*, *Odorella*, one ASV belonging to the family *Nodosilineaceae* (BLAST showed 98.45% identity with *Leptolyngbya*

sp. PCC 7124), one belonging to class *Cyanophyceae* (BLAST showed 98.06% identity with *Copelandiella yellowstonensis* YNP83A-MA6 and *Geitleribactron purpureum* Tovel-1) and one ASV belonging to phylum *Cyanobacteriota* (BLAST showed 100% identity with *Cyanobacterium* sp. BBD-AO-Red).

4.2.3 Alpha diversity of the cyanobacterial communities

Influence of categorical variables on cyanobacterial alpha diversity

Alpha diversity analysis (**Table 5**) indicated that the observed ASV richness (median = 47, range = 27–196), Shannon diversity (median = 1.97, range = 0.62–3.82) and Faith's phylogenetic (median = 1.58, range = 1.06-4.04) varied across samples.

Table 5. Descriptive statistics (minimum, 1st quartile, median, mean, 3rd quartile and maximum) of alpha diversity measures (observed ASV richness, Shannon diversity and Faith's phylogenetic diversity) of cyanobacterial community on sea turtle carapaces.

	Observed ASV richness	Shannon diversity	Faith's Phylogenetic diversity
Minimum	27.00	0.619	1.061
1st quartile	37.00	1.844	1.301
Median	47.00	1.970	1.576
Mean	62.21	2.162	1.825
3rd quartile	68.00	2.536	2.046
Maximum	196.00	3.824	4.037

The analysis of influence of categorical variables alpha diversity revealed significant differences based on factors Age and Recovery Centre, while other factors such as Condition, Coast, and Season did not show statistically significant effects (**Table 6**). Specifically, Shannon diversity varied significantly across age groups ($p = 0.0142$). However, Observed ASVs and Faith's PD did not differ significantly by age. Recovery Centre significantly influenced Observed ASVs ($p = 0.02826$) and Faith's PD ($p = 0.0353$), suggesting location-based microbial diversity differences, though Shannon diversity was not significantly affected. Condition, coast, and season had no significant impact on any alpha diversity metric.

Table 6. Kruskal-Wallis test results for different categorical factors and alpha diversity metrics (Observed ASV richness, Shannon diversity and Faith's Phylogenetic diversity). Reported results contains chi-squared ($\chi 2$) test statistic, degrees of freedom (df), and significance of the test based on $\alpha = 0.05$.

Factor	Index	$\chi 2$	df	p-value
Age (juvenile, sub-adult, adult)	Observed ASVs	1.7149	2	0.4243
	Shannon	8.5085	2	0.0142*
	Faith's PD	4.4955	2	0.1056
Recovery Centre (Aquarium Pula, Blue World Inst.)	Observed ASVs	4.8119	1	0.02826*
	Shannon	2.4923	1	0.1144
	Faith's PD	4.4308	1	0.0353*
Condition (good, poor, injured)	Observed ASVs	2.6111	2	0.271
	Shannon	1.9272	2	0.3815
	Faith's PD	2.5658	2	0.2772
Coast (north, south)	Observed ASVs	2.0611	1	0.1511
	Shannon	0.54857	1	0.4589
	Faith's PD	0.21429	1	0.6434
Season (spring, summer, autumn, winter)	Observed ASVs	3.1172	3	0.3739
	Shannon	0.65684	3	0.8833
	Faith's PD	0.87158	3	0.8323

Post-hoc Dunn's test (**Table 7**) was performed on factors for indices that showed significant differences in Kruskal-Wallis test to observe between what groups lays the difference. The post-hoc test showed that for Shannon diversity in Age factor, juveniles had significantly lower diversity than sub-adults ($p = 0.0208$) and adults, but no significant difference was observed between sub-adult and adult Shannon diversity.

Table 7. Dunn's Post-Hoc test results for Shannon diversity for juvenile, sub-adult and adult turtles in the Age category. Reported results contain mean Rank Difference, adjusted p-value and significance indicated with * for $\alpha < 0.1$ and ** for $\alpha < 0.05$.

Comparison	Mean Rank Difference	Adjusted p-value
juvenile - adult	1.999	0.0684*
juvenile – sub-adult	-2.461	0.0208**
sub-adult - adult	-0.3627	1.0000

Detailed visualization of alpha diversity indices across different age categories (juvenile, sub-adult, and adult; **Figure 18**) showed that sub-adult sea turtles had highest median values of observed richness, Shannon diversity and Faith's PD. Juvenile sea turtles had the lowest value for all alpha diversity indices, but also showed the smallest spread of the data points. Adult sea turtles showed the largest spread of data points. No significant differences were observed in Observed ASV richness ($p = 0.4243$) or Faith's phylogenetic diversity ($p = 0.1056$) as shown **Table 6**. However, Shannon diversity differed significantly among age groups ($p = 0.0142$), with post-hoc Dunn's test indicating a significant difference between juveniles and sub-adults ($p = 0.0208$) as shown in **Table 7**.

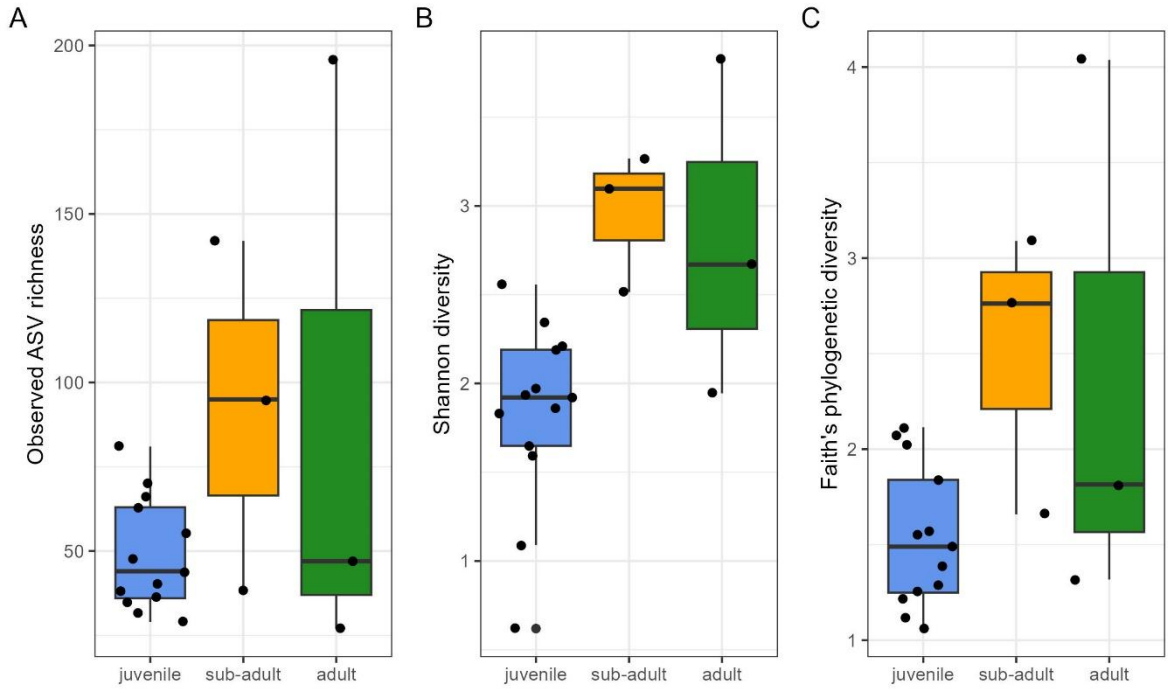


Figure 18. Alpha diversity metrics visualizations by Age category, observed ASV richness (A), Shannon diversity (B) and Faith's Phylogenetic diversity. Boxes are colored by category, juvenile are blue, sub-adult are orange and adult are green. Number of samples in each category: juvenile n = 13, sub-adult = 3, adult = 3.

Differences in alpha diversity indices in turtles from different Recovery Centers (Blue World Institute – BWI and Aquarium Pula) are shown on **Figure 19**. Cyanobacterial community on sea turtles from Aquarium Pula showed higher median values of observed ASV richness, Shannon diversity and Faith's PD. Sea turtles from BWI expressed lower alpha diversity indices, but also the smaller spread of the data. Observed ASV richness and Faith's phylogenetic

diversity showed significant differences between recovery centres (p = 0.0283 and p = 0.0353, respectively), suggesting differences in cyanobacterial richness and phylogenetic diversity based on recovery centre. However, Shannon diversity did not show a significant difference (p = 0.1144) as shown in **Table 6**.

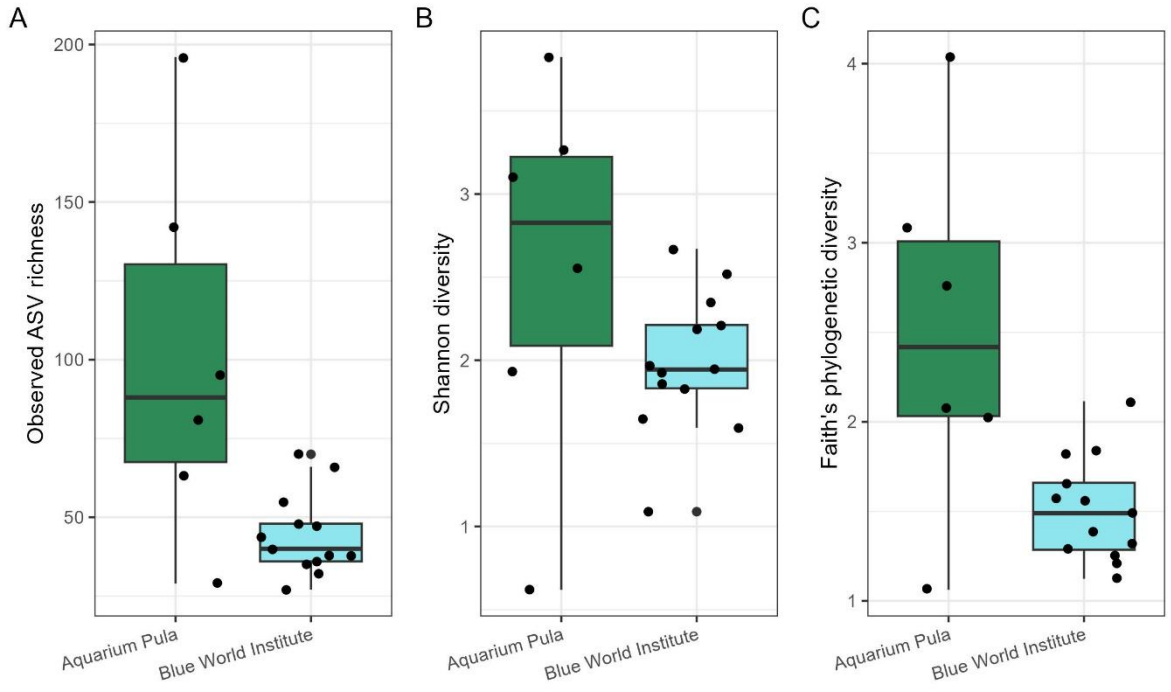


Figure 19. Alpha diversity metrics visualizations by Recovery Centre category, observed ASV richness (A), Shannon diversity (B) and Faith's Phylogenetic diversity (C). Boxes are colored by category, Aquarium Pula is green, sand Blue World Institute is blue. Number of samples in each category: Aquarium Pula n = 6, Blue World Institute n = 13.

The visualization of differences between alpha diversity indices based on turtle condition (good, poor or injured) is shown on **Figure 20**. Although the values are really close to one another, the highest observed ASV richness, Shannon diversity and Faith's PD is observed in injured turtles. The lowest cyanobacterial richness, diversity and phylogenetic diversity is found in turtles that had good condition. Turtles that had poor condition had the largest spread of data of all alpha diversity metrices. None of diversity metrics (Observed ASV richness, p = 0.271; Shannon diversity, p = 0.3815; Faith's phylogenetic diversity, p = 0.2772) showed significant differences between health conditions as shown in **Table 6**.

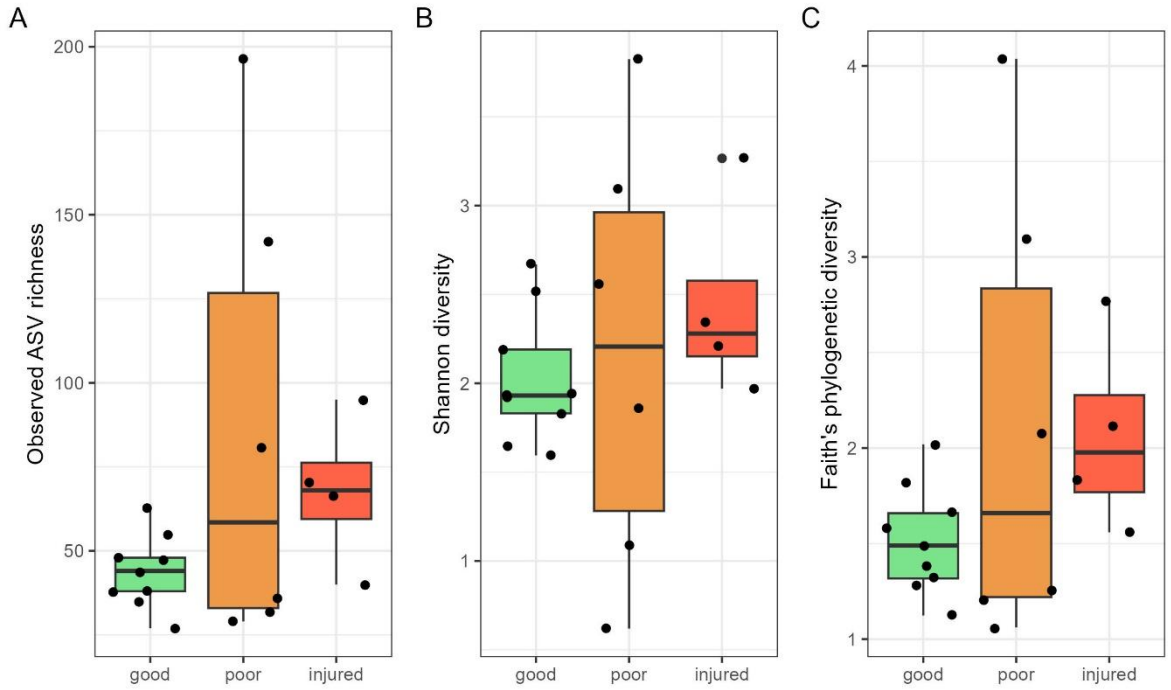


Figure 20. Alpha diversity metrics visualizations by Condition category, observed ASV richness (A), Shannon diversity (B) and Faith's Phylogenetic diversity (C). Boxes are colored by category, good is green, poor is orange and injured is red. Number of samples in each category: good n = 9, poor = 6, injured = 4.

Alpha diversity indices for individuals from different coastal regions of Adriatic (north and south Adriatic) showed similar median values for cyanobacterial community of sea turtles from north and south coast (**Figure 21**). Turtles from the south Adriatic had larger spread of data in the upper part of the distribution. They also had higher maximum values for all three indices. However, no significant differences were detected for Observed ASV richness ($p = 0.1511$), Shannon diversity ($p = 0.4589$) or Faith's phylogenetic diversity ($p = 0.6434$), as shown in **Table 6**.

Visualization of difference in cyanobacterial alpha diversity on sea turtles sampled in different seasons is shown on **Figure 22**. No clear seasonal pattern is observed, besides larger spread of data in turtles sampled in the spring and summer and higher maximum values of warmer seasons (spring and summer). However, none of the three metrics, Observed ASV richness ($p = 0.3739$), Shannon diversity ($p = 0.8833$) or Faith's phylogenetic diversity ($p = 0.8323$), showed significant seasonal differences, as shown in **Table 6**.

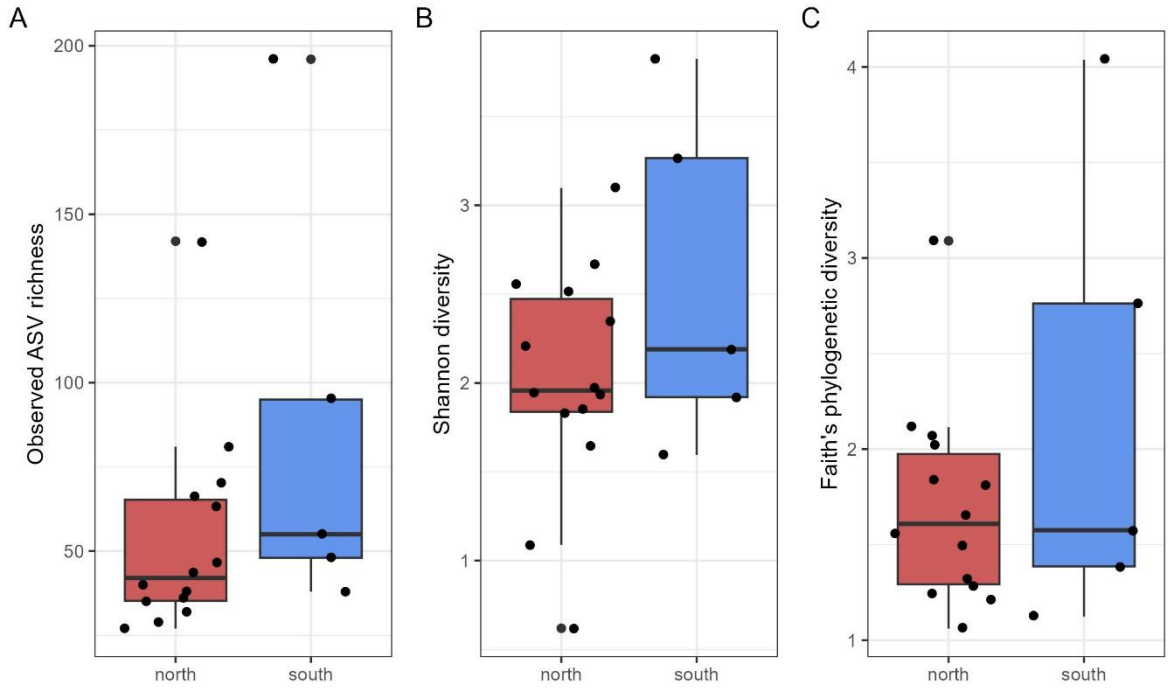


Figure 21. Alpha diversity metrics visualizations by Coast category, observed ASV richness (A), Shannon diversity (B) and Faith's Phylogenetic diversity (C). Boxes are colored by category, north is red, and south is blue. Number of samples in each category: north n = 14, south n = 5.

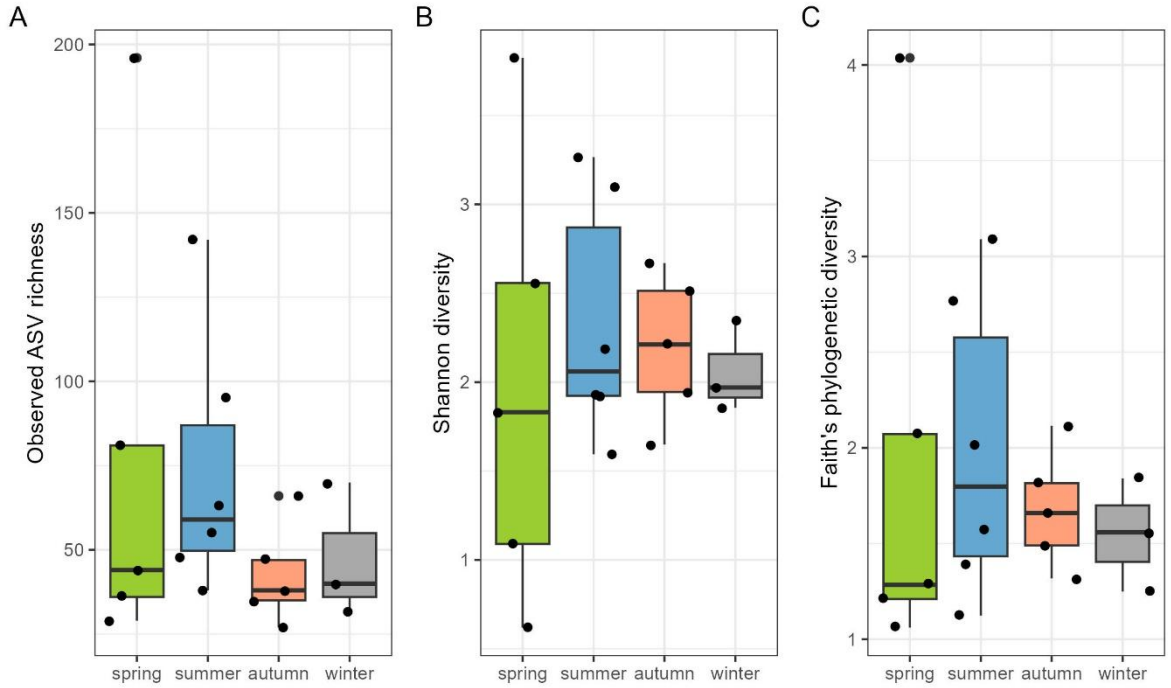


Figure 22. Alpha diversity metrics visualizations by Season category, observed ASV richness (A), Shannon diversity (B) and Faith's Phylogenetic diversity (C). Boxes are colored by category, spring is green, summer is blue, autumn is pink and winter is gray. Number of samples in each category: spring n = 5, summer n = 6, autumn n = 5, winter n = 3.

Influence of numerical variables on cyanobacterial diversity

The analysis of influence of numerical variables (carapace length, CCL; carapace width CCW, longitude and latitude) was performed using Pearson moment-correlation test (**Table 8**).

Carapace length (CCL) was significantly positively correlated with Shannon diversity (**Figure 23**B) and Faith's Phylogenetic diversity (**Figure 23**C). Observed ASV richness also increased with CCL (**Figure 23**A) but was only borderline significant (**Table 8**). Correlation of alpha diversity indices and carapace width (CCW) followed a similar pattern but showed all indices to be significantly positively correlated with CCW (**Table 8**): observed ASV richness (**Figure 24**A), Shannon (**Figure 24**B), Faith's Phylogenetic diversity (**Figure 24**C).

Latitude and longitude did not exhibit a significant correlation with microbial diversity indices (**Table 8**). Observed ASV richness (**Figure 25**A and **Figure 26**A), Shannon diversity index (**Figure 25**B and **Figure 26**B), and Faith's PD (**Figure 25**C and **Figure 26**C) all show weak and non-significant relationships with latitude and longitude where microbial diversity appears to be highly variable without a clear latitudinal and longitudinal pattern.

Table 8. Pearson correlation results between variables (CCL, CCW, Latitude, Longitude) and diversity indices (Observed ASV richness, Shannon diversity index and Faith's Phylogenetic Diversity index); correlation coefficient (r), t-value, p-value (* indicated significance for $\alpha = 0.05$); df = 17.

Variable	Index	Correlation (r)	t-value	p-value
CCL	Observed ASV richness	0.4496	2.0756	0.0534
	Shannon diversity index	0.6989	4.0301	0.0009*
	Faith's PD	0.5793	2.9305	0.0093*
CCW	Observed ASV richness	0.4815	2.2651	0.0368*
	Shannon diversity index	0.7056	4.1058	0.0007*
	Faith's PD	0.6060	3.1414	0.0059*
Latitude	Observed ASV richness	-0.0005	-0.00208	0.9984
	Shannon diversity index	-0.0459	-0.18979	0.8517
	Faith's PD	0.0947	0.39211	0.6998
Longitude	Observed ASV richness	0.0642	0.26511	0.7941
	Shannon	0.1282	0.53292	0.6010
	Faith's PD	-0.0159	-0.06594	0.9482

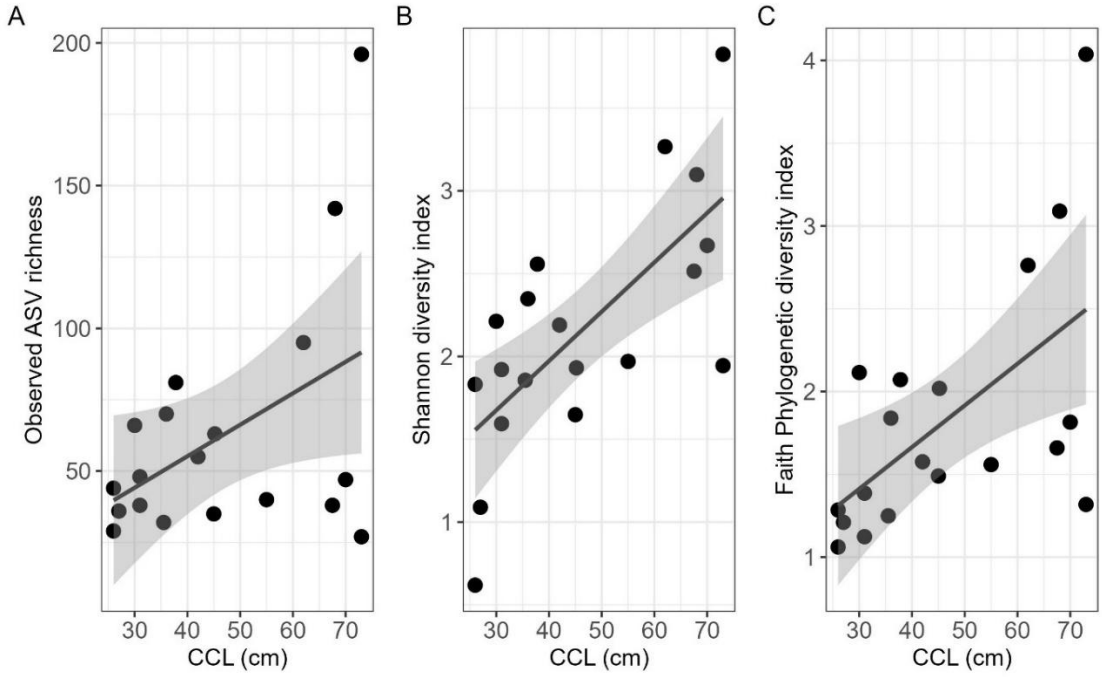


Figure 23. Carapace length (CCL) and alpha diversity metrics' relationship. (A) Observed ASV richness, (B) Shannon diversity index, and (C) Faith's Phylogenetic Diversity index as a function of curved carapace length (CCL) in cm. Black dots represent individual samples, black lines indicate fitted regression models, and the shaded gray areas denote 95% confidence intervals.

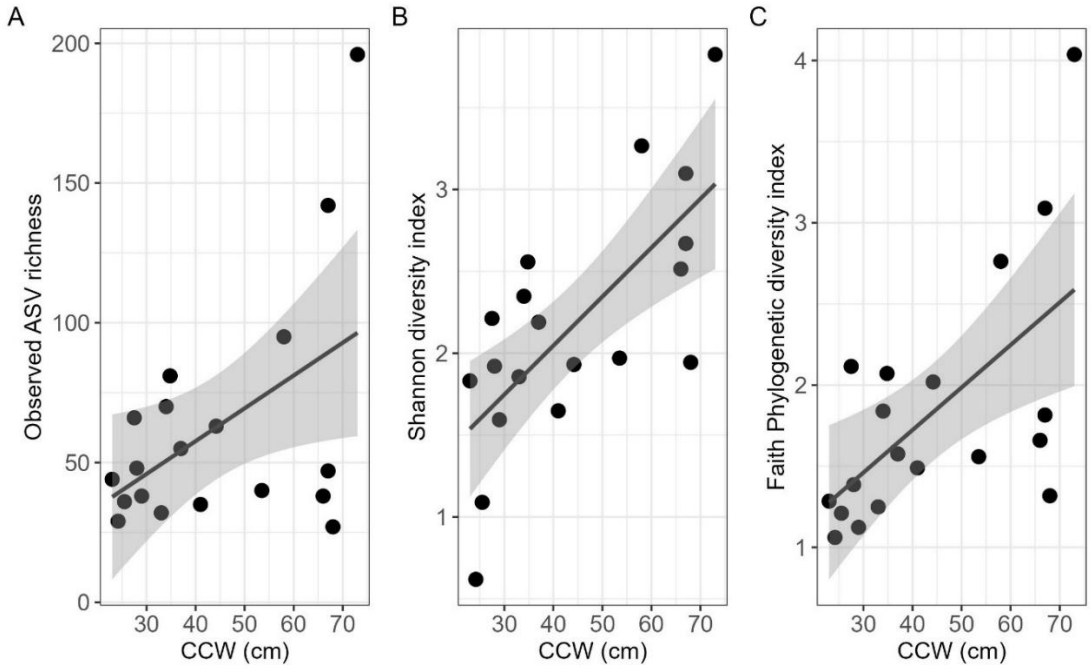


Figure 24. Carapace width (CCW) and alpha diversity metrics' relationship. (A) Observed ASV richness, (B) Shannon diversity index, and (C) Faith's Phylogenetic Diversity index as a function of CCW (cm). Black dots represent individual samples, black lines indicate fitted regression models, and the shaded gray areas denote 95% confidence intervals.

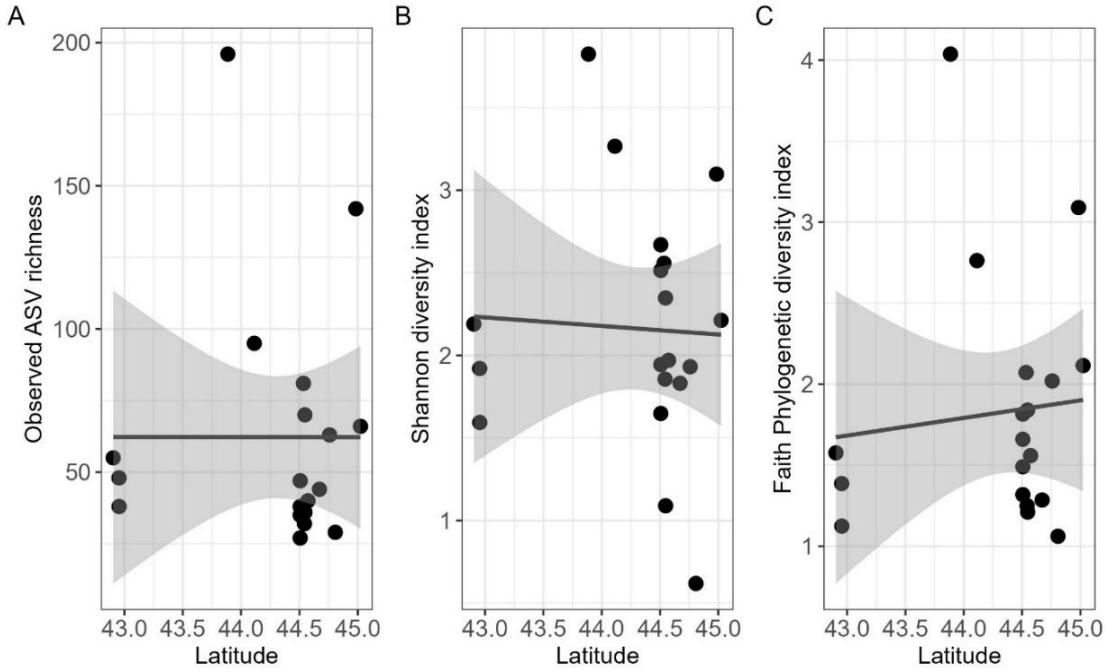


Figure 25. Latitude and alpha diversity metrics' relationship. (A) Observed ASV richness, (B) Shannon diversity index, and (C) Faith's Phylogenetic Diversity index as a function of latitude. Black dots represent individual samples, black lines indicate fitted regression models, and the shaded gray areas denote 95% confidence intervals.

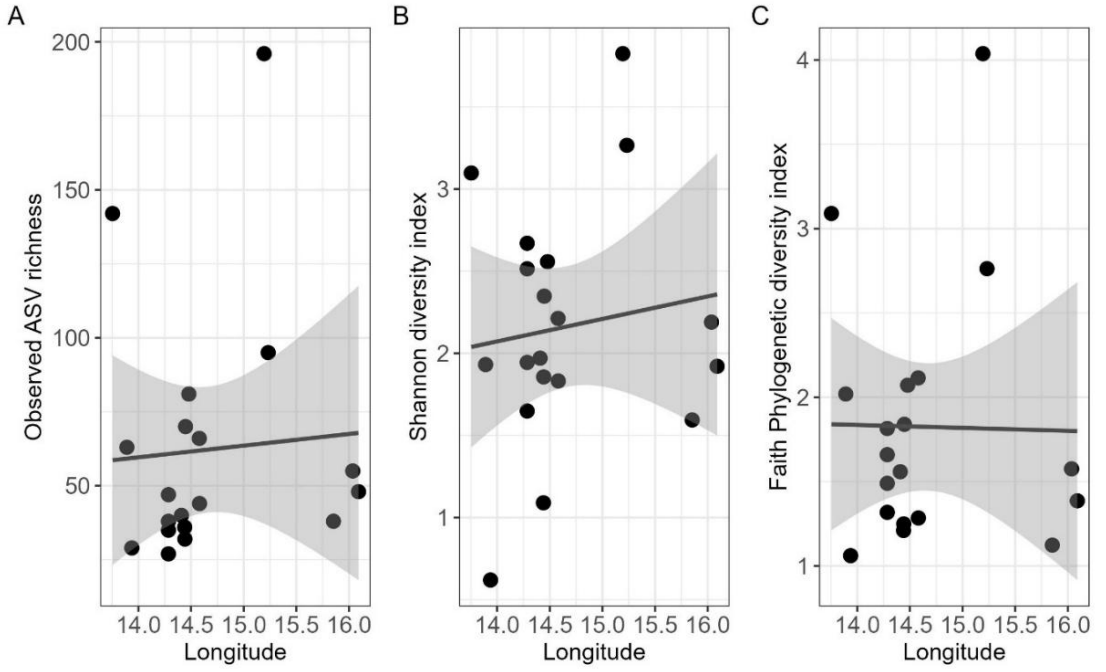


Figure 26. Longitude and alpha diversity metrics' relationships. (A) Observed ASV richness, (B) Shannon diversity index, and (C) Faith's Phylogenetic Diversity index as a function of latitude. Black dots represent individual samples, black lines indicate fitted regression models, and the shaded gray areas denote 95% confidence intervals.

Results on the cyanobacterial alpha diversity model fit

Poisson GLM was used to test the effect of turtle size (CCL), longitude, recovery centre and condition. The used model showed signs of overdispersion (dispersion = 5.766486, z = 3.4837, p-value = 0.0002), meaning that variance of the data is greater than the mean and that violates the assumption of Poisson distribution. Because of that, a negative binomial GLM was performed on the same data which added extra dispersion parameter and allowed a better fit of the model, with the AIC value of 175.68 and the deviance of 18.845. The model explained 69.3% of the variance (Nagelkerke $R^2 = 0.693$). Larger turtles ($\beta = 0.010$, $p = 0.022$) and those at higher longitudes ($\beta = 0.252$, $p = 0.027$) had significantly greater ASV richness. Turtles from the Blue World Institute had significantly lower richness ($\beta = -0.627$, $p = 0.001$). Injured turtles had higher richness ($\beta = 0.428$, $p = 0.027$), but turtles in poor condition showed no significant difference ($p = 0.163$). Visualization is provided in **Table 9** and **Figure 27A**.

A linear model (LM) was used to assess the effect of turtle size (CCL), longitude, recovery centre, and condition on Shannon diversity. The model explained 70.4% of the variance in Shannon diversity ($R^2 = 0.704$, adjusted $R^2 = 0.591$, F-statistics (on 5 predictors, 13 residual degrees of freedom) = 6.197, $p = 0.0038$). Larger turtles had significantly higher Shannon diversity ($\beta = 0.031$, $p < 0.001$). Longitude also had a significant positive effect on Shannon diversity ($\beta = 0.393$, $p = 0.042$). However, the recovery centre did not show a significant effect ($p = 0.238$). Similarly, injured turtles tended to have higher diversity, but this effect was not statistically significant ($p = 0.105$), and there was no significant difference for turtles in poor condition ($p = 0.467$). Visualization is provided in **Table 9** and **Figure 27B**.

A linear model (LM) was used to examine the effect of turtle size (CCL), longitude, recovery centre, and condition on phylogenetic diversity (PD). The model explained 72.8% of the variance in PD ($R^2 = 0.728$, adjusted $R^2 = 0.623$, F-statistics (on 5 predictors, 13 residual degrees of freedom) = 6.951, $p = 0.0023$). Larger turtles had significantly higher PD ($\beta = 0.024$, $p = 0.0036$). Longitude showed a positive effect on PD, but this was not statistically significant ($p = 0.101$). Turtles from the Blue World Institute had significantly lower PD ($\beta = -0.727$, $p = 0.024$). Injured turtles tended to have higher PD, but this effect was only marginally significant ($p = 0.075$), and there was no significant difference for turtles in poor condition ($p = 0.197$). Visualization is provided in **Table 9** and **Figure 27C**.

Table 9. Models' summary of key results from three models: a Negative Binomial Generalized Linear Model (GLM) for Observed ASV richness, and two Linear Models (LM) for Shannon Diversity Index and Faith's Phylogenetic Diversity Index. The table includes coefficient estimates, standard errors, z/t-values, p-values, and the level of statistical significance. Significant variables are marked with asterisks: $p < 0.05$ (*), $p < 0.001$ (**), and $p < 0.0001$ (***).

Variable	Estimate	Std. Error	z/t value	p-value
GLM: Observed ASV richness (Negative Binomial)				
CCL	0.0105	0.0046	2.282	0.02251*
Longitude	0.2528	0.1146	2.206	0.02742*
RecoveryCentre (Blue World Institute)	-0.6274	0.1919	-3.269	0.00108**
Condition (injured)	0.4284	0.1932	2.217	0.02663*
Condition (poor)	0.2914	0.2089	1.395	0.16305
LM: Shannon Diversity Index				
CCL	0.0313	0.007	4.501	0.00060***
Longitude	0.3925	0.1737	2.259	0.04167*
RecoveryCentre (Blue World Institute)	-0.36	0.291	-1.237	0.23796
Condition (injured)	0.5073	0.2914	1.741	0.10536
Condition (poor)	0.2345	0.3134	0.749	0.46748
LM Faith's Phylogenetic Diversity Index				
CCL	0.0241	0.0068	3.541	0.00362**
Longitude	0.3004	0.1701	1.766	0.1008
RecoveryCentre (Blue World Institute)	-0.7272	0.2849	-2.553	0.02407*
Condition (injured)	0.5526	0.2853	1.937	0.07481
Condition (poor)	0.4174	0.3068	1.361	0.19677

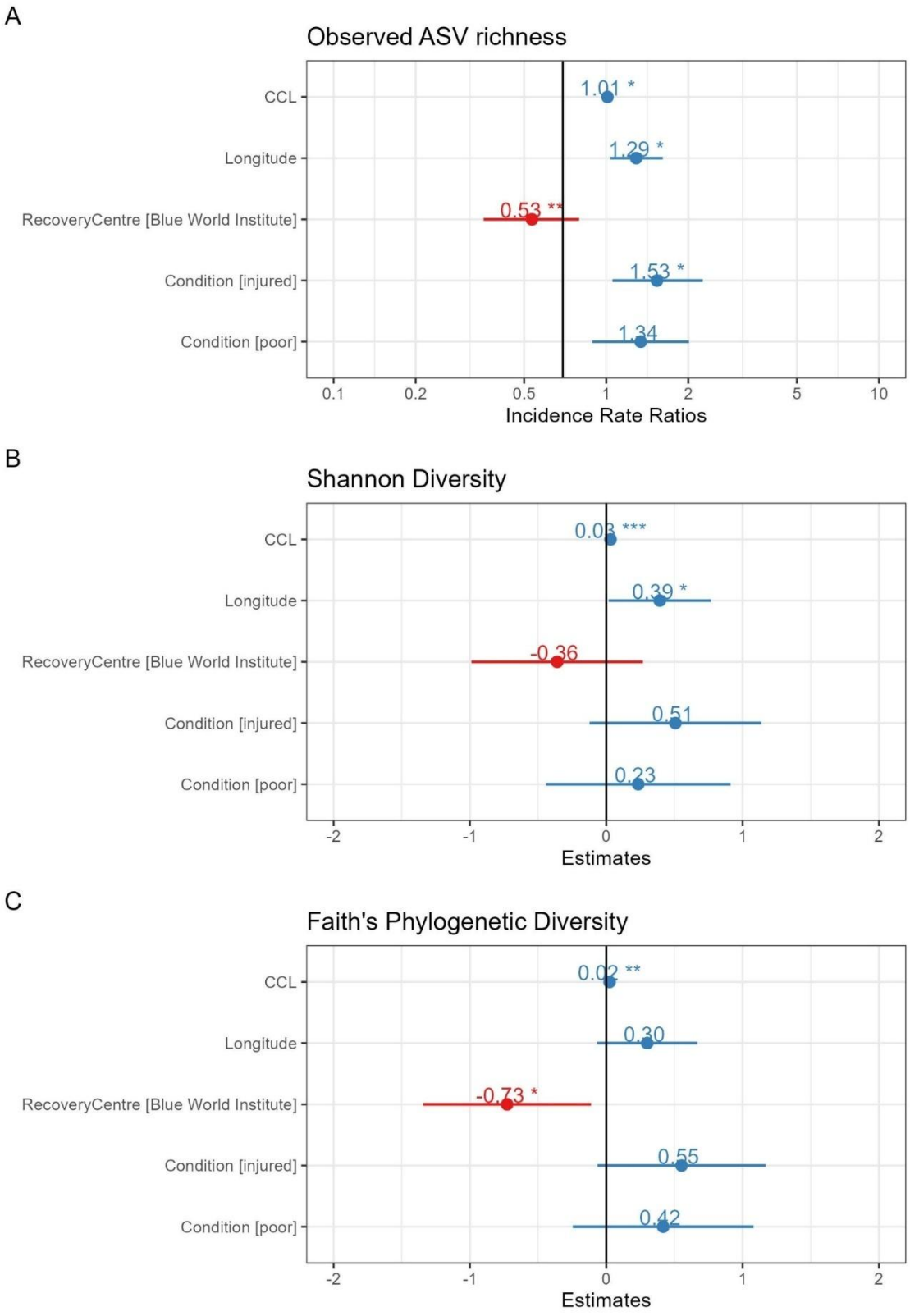


Figure 27. Forest plot of the estimated effects of predictors in GLM for Observed ASV richness (A), LM for Shannon diversity (B) and LM for Faith's Phylogenetic Diversity (C). Dots and values represent intercepts (β) for each predictor, horizontal bars represent 95% confidence intervals. Blue represents the positive effect of a predictor, and red represents negative effect of a predictor. Asterisks represent significance: $p \leq 0.001$ (***), $p \leq 0.01$ (**) and $p \leq 0.05$ (*).

4.2.4 Beta diversity of cyanobacterial communities

To reduce dimensionality of multivariate data and to visualise the most influential cyanobacterial genera, I used principal components analysis (PCA) on center-log transformed data merged at genus level. PCA of cyanobacterial community (**Figure 28**) showed PC1 explained 18.21% variance in the data and PC2 14.05% of variance, together 32.26% variance. The direction and length of the arrows indicate how strongly individual genera correlate with PC1 and PC2. Genus *Odorella* was strongly associated with variation along PC1, while Nodosilineaceae_XXX, *Spirulina*, *Metis*, *Solentia*, *Parasynechococcus*, *Cymatolege* and Prochlorotrichaceae_X contribute to variance along PC2. Orientation of genera arrow suggests higher numbers of *Odorella*, *Hyella* and *Heteroleiblenia* in sub-adult and adult groups, while juvenile groups were characterized by higher numbers of Prochlorotrichaceae_X, *Cymatolege* and *Parasynechococcus*.

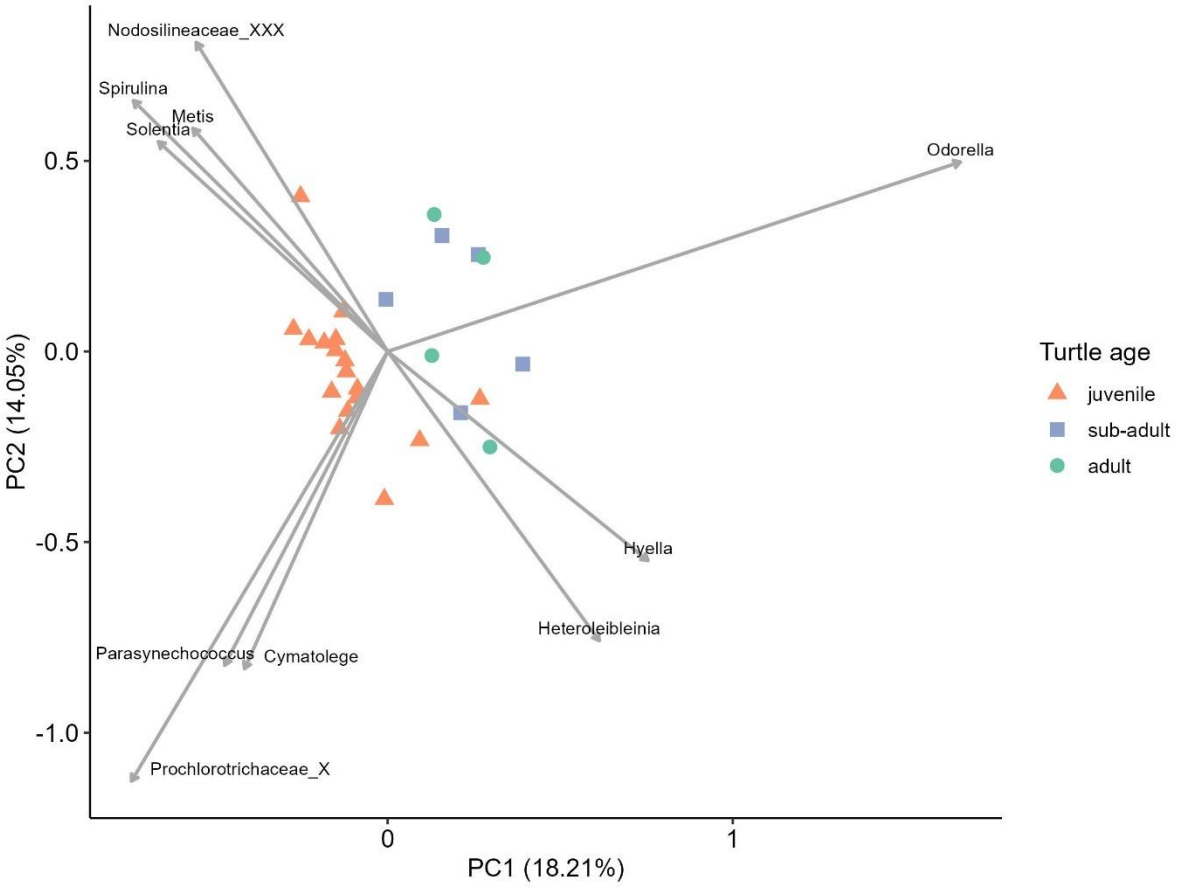


Figure 28. Principal component analysis (PCA) of center-log transformed genera counts distance matrix for cyanobacterial community on sea turtle carapace (n = 26). Data points represent each turtle sample colored by different age categories (juvenile: orange triangles, sub-adults: purple squares and adults: green circles). Arrows represent the top 10 most influential cyanobacterial genera.

To determine which factors contribute the most to variation in the beta diversity of the cyanobacterial community on sea turtle carapaces (calculated as Bray-Curtis distance), I used permutational multivariate analysis of variance (PERMANOVA). The global PERMANOVA test (**Table 10**) assesses whether a given variable (e.g., turtle condition, age, season) has a significant overall effect on community composition. The results showed that the tested variables explained 49.1% of the total variance, while 50.9% remained unexplained. Among the variables, turtle length (CCL, Pseudo-F = 3.8, p = 0.001) and turtle condition (Pseudo-F = 1.7, p = 0.025) were significant predictors, contributing 12.1% and 10.9% of the explained variance, respectively. Longitude, recovery center, and season did not show significant effects.

Table 10. Permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance matrix for cyanobacterial community on sea turtle carapace (n = 26). Variables included in the model were turtle length (CCL), location (longitude), recovery centre, turtle condition and sampling season. Reported results include degrees of freedom (df), proportion of explained variance by model (R^2), Pseudo-F test statistics and p-value. Asterisks represent significance: $p \leq 0.001$ (***), $p \leq 0.01$ (**) and $p \leq 0.05$ (*).

Variable	df	R^2	Pseudo-F	p-value
CCL	1	0.12099	3.8021	0.001 ***
Longitude	1	0.03440	1.0811	0.313
Recovery Centre	1	0.02826	0.8881	0.548
Condition	2	0.10905	1.7134	0.025 *
Season	3	0.12422	1.3012	0.109
Residual	16	0.50913		
Total	24	1.00000		

Because turtle condition was a significant factor in the global PERMANOVA, a pairwise PERMANOVA was conducted to examine which specific condition groups differed (**Table 11**). However, none of the pairwise comparisons remained significant after Bonferroni correction, despite an unadjusted p-value of 0.041 for the *Good vs. Injured* group. This suggests that while turtle condition influences community composition overall, the differences between individual condition groups are not strong enough to remain significant after adjusting for multiple comparisons.

Similarly, pairwise comparisons were performed for turtle age categories (as an indirect measure of turtle size, since CCL is a continuous variable, **Table 11**). Significant differences were found between juvenile and sub-adult turtles (Pseudo-F = 2.7, p = 0.003, adjusted p =

0.009) and between juvenile and adult turtles (Pseudo-F = 3.1, p = 0.001, adjusted p = 0.003). No significant differences were observed between sub-adults and adults (Pseudo-F = 0.82, p = 0.701, adjusted p = 1.0). The non-metric multidimensional scaling (NMDS) plots (**Figure 29**) visualize these patterns, illustrating the distribution of samples based on turtle age and condition.

Table 11. Pairwise permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis distance matrix for cyanobacterial community on sea turtle carapace (n = 26). Variables tested are turtle age and turtle condition. Reported results include degrees of freedom (df), proportion of explained variance by model (R^2), Pseudo-F test statistics p-value and adjusted p-value using Bonferroni method. Asterisks represent significance: $p \leq 0.001$ (***), $p \leq 0.01$ (**) and $p \leq 0.05$ (*).

Variable	Comparison	df	R^2	Pseudo-F	p-value	Adjusted p-value
Age	Sub-adult vs. Juvenile	1	0.12525	2.7204	0.003***	0.009***
	Sub-adult vs. Adult	1	0.10513	0.8224	0.701	1.000
	Juvenile vs. Adult	1	0.14837	3.136	0.001***	0.003***
Condition	Injured vs. Good	1	0.11186	2.0152	0.041*	0.123
	Injured vs. Poor	1	0.08068	1.2286	0.215	0.645
	Good vs. Poor	1	0.09369	1.4472	0.141	0.423

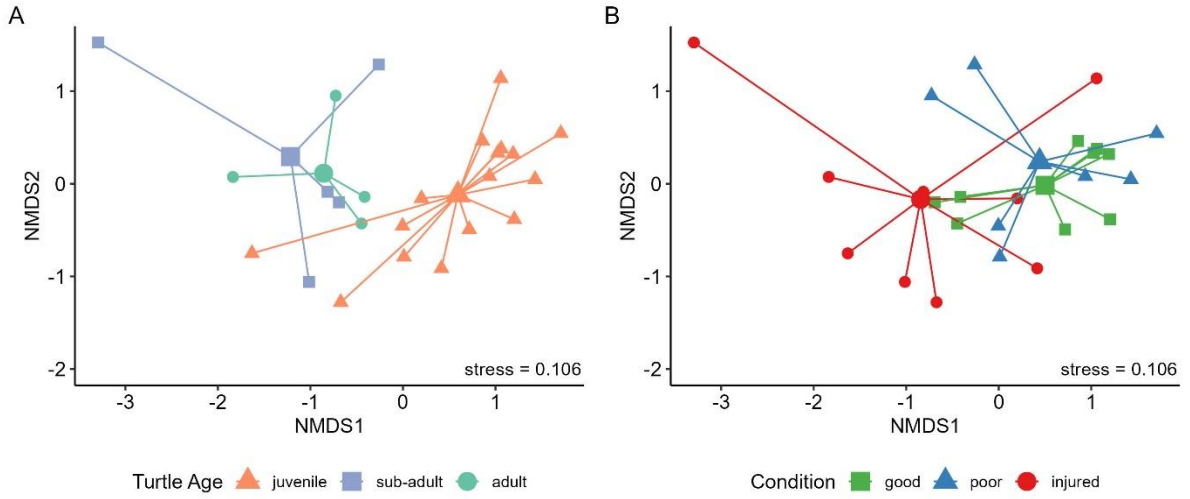


Figure 29. Non-metric multidimensional scaling visualizations (NMDS) based on Bray-Curtis distance matrix for cyanobacterial community on sea turtle carapace (n = 26), grouped by turtle age (A) and turtle condition (B). Larger points represent centroids of each group with lines connecting them to each sample point.

4.3 Characterization and identification of cultivated cyanobacterial strains

Cyanobacterial isolation from epizoic biofilms with no visible cyanobacterial growth proved to be a slow and labor-intensive process, with a low yield of viable strains despite repeated efforts and long incubation periods. Nevertheless, six uni-cyanobacterial strains were successfully established during this study (CY002, CY004, CY006, CY007, CY009, and CY011). An additional six strains, isolated and preliminarily characterized by master student Ela Pahor (Pahor 2024) were included in the molecular and phylogenetic analyses. All strains belonged to filamentous, homocytous cyanobacteria, with CY011 assigned to the order Spirulinales, CY002 and CY004 to Oscillatoriales, and the remaining nine strains to the order Nodosilineales. Morphological and taxonomic details of each strain are described below. The full phylogenetic tree is provided in **Supplement 5**, with selected clades shown in figures throughout the section.

4.3.1 Characterization of *Okeania* sp. CY002 and CY004

Morphology and culture growth of* Okeania *strains CY002 and CY004

Strain CY002 exhibits a dark green to blackish coloration in culture, forming floating clumps and adhering to the flask wall (**Figure 30**). It is a filamentous, homocytous cyanobacterium with large, long, isopolar, and non-coiled trichomes that cluster into mats. Sheaths, necridic cells, and calyptra are present. The trichomes are cylindrical, usually not attenuated at the ends, with rounded apical cells, sometimes bearing calyptra. Cells are not constricted at cross walls, with a mean width of 11.11 µm and a mean length of 2.19 µm. Aerotopes are observed but infrequent, and hormogonia formation is present.

Similar to CY002, strain CY004 is dark green to blackish in liquid medium, forming floating clumps and attaching to the flask wall (**Figure 31**). It is a filamentous, homocytous strain with large, long, isopolar, and non-coiled trichomes that form clusters and mats. Sheaths, necridic cells, and calyptra are present. The trichomes are cylindrical, usually not attenuated at the ends, with rounded apical cells that may or may not bear calyptra. Cells are not constricted at the cross walls, with a mean width of 8.63 µm and a mean length of 2.29 µm. Aerotopes are infrequent, and hormogonia formation is observed.

Figure 30. Microphotographs of strain CY002 under 200× magnification (upper left image), 400× magnification (upper right and lower left image) and 1000× magnification (lower middle and lower left image).

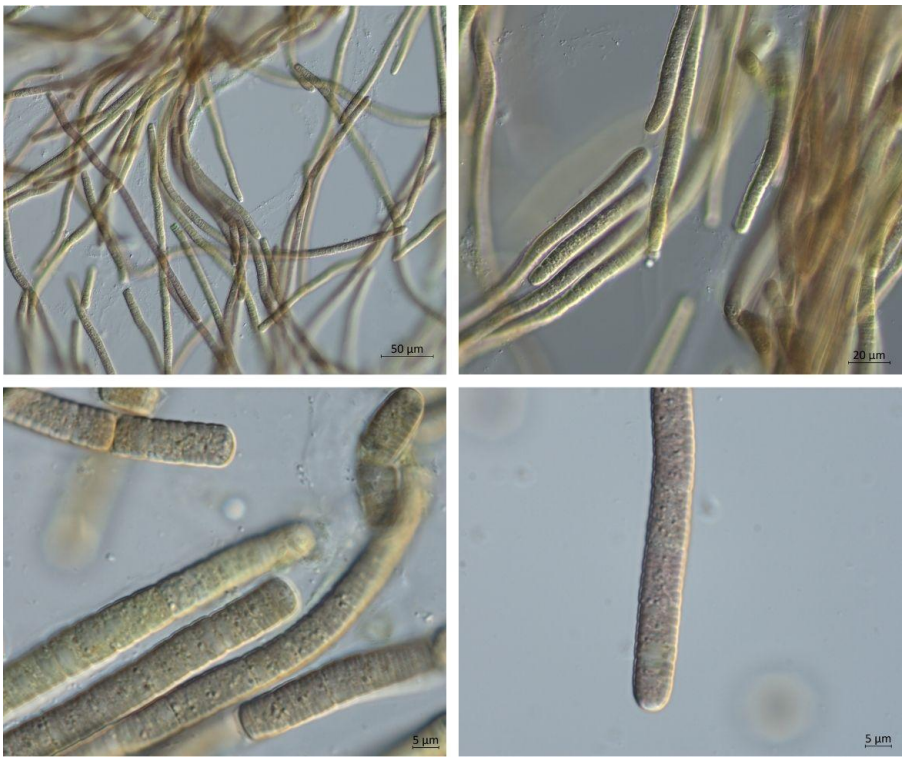


Figure 31. Microphotographs of strain CY004 under 200× magnification (upper left image), 400× magnification (upper right image) and 1000× magnification (lower left and lower right image).

Phylogenetic identity of Okeania *strains CY002 and CY004*

A phylogenetic analysis was conducted to determine the evolutionary placement of *Microcoleaceae* strains CY002 and CY004 based on 16S rRNA gene sequences. The maximum likelihood phylogenetic tree (**Figure 32**A) includes a broad range of cyanobacterial taxa, with a focus on the *Microcoleaceae* clade, where the strains of interest are positioned. In the detailed phylogenetic tree (**Figure 32**B), CY002 and CY004 cluster closely together within the *Microcoleaceae* family. Both strains are positioned within a subclade that includes one *Blennothrix* and several *Okeania* species. The phylogenetic placement suggests that CY002 and CY004 are more closely related to *Okeania* sp. (KiyG1), *Okeania erythroflocculosa* (FFP12-1) and *Okeania comitata* 3L-OSC than to other members of the family. Bootstrap values at major nodes support the stability of this clade, indicating a strong evolutionary relationship among these. These results suggest that CY002 and CY004 form a distinct lineage within *Microcoleaceae*, exhibiting close phylogenetic affinities with *Okeania* and *Blennothrix* species.

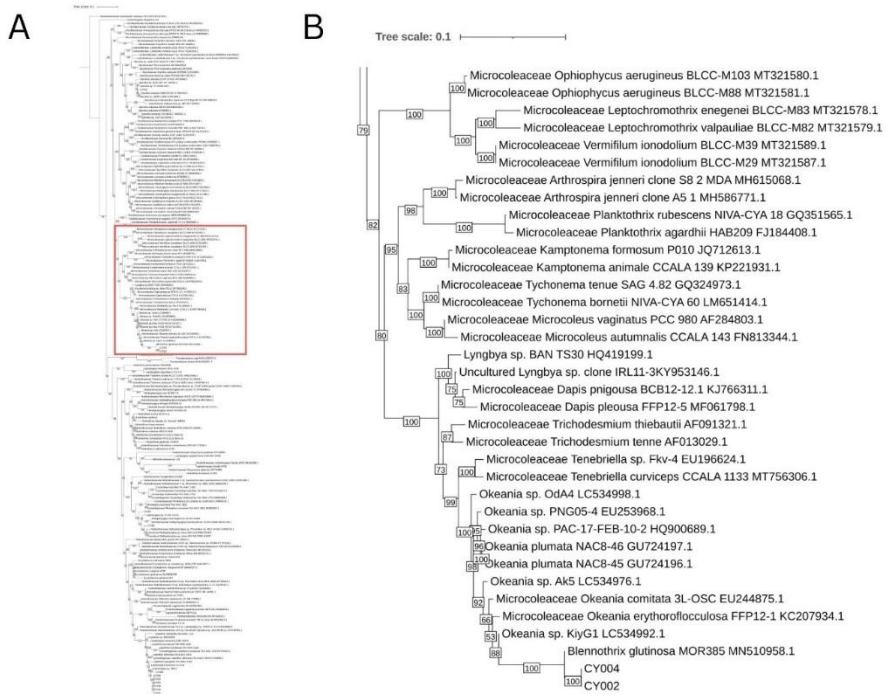


Figure 32. Phylogenetic tree of Microcoleaceae strains, including strains CY002 and CY004 based on 16S rRNA gene. (**A**) A comprehensive phylogenetic tree showing the evolutionary relationships among various cyanobacterial strains, with the relevant section highlighted. (**B**) A magnified view of the Microcoleaceae clade, highlighting the placement of CY002 and CY004. The tree was constructed using the maximum likelihood method, with bootstrap values provided at major nodes. The scale bar represents 0.1 substitutions per nucleotide position.

4.3.2 Characterization of *Spirulina* sp. CY011

Morphology and culture growth of Spirulina sp. strain CY011

Strain CY011 was optimally growing in BG-11 marine medium, displaying pink coloration and forming mats attached to the flask wall or floating on the water surface (**Figure 33**). It is a filamentous, homocytous strain with thin, long, isopolar, and highly coiled trichomes, exhibiting a visible screw-like movement. Neither necridic cells nor calyptra are present. The trichomes are cylindrical and typically not attenuated at the ends. Due to the highly coiled nature of the trichomes, individual cells are not clearly distinguishable.

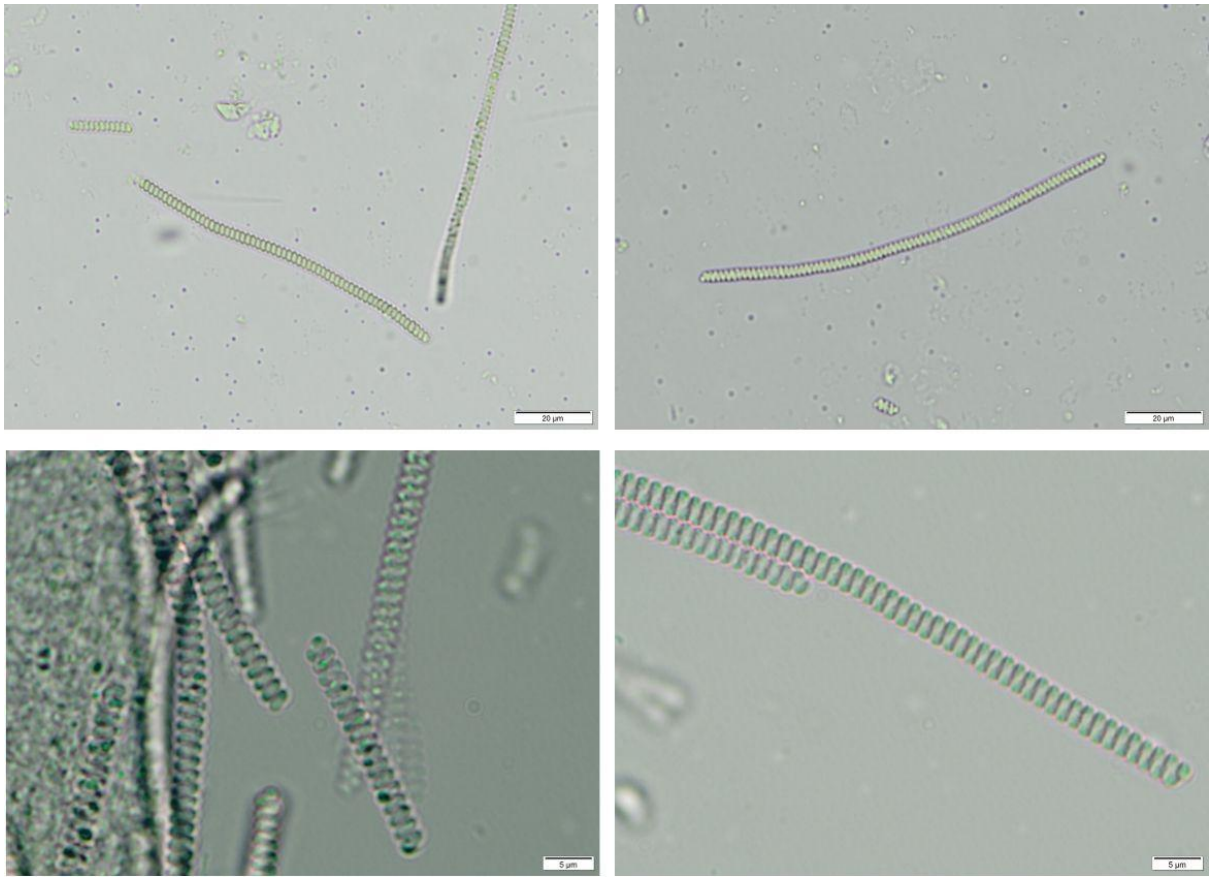


Figure 33. Microphotographs of strain CY011 under 400× magnification (upper right and upper left image) and 1000× magnification (lower left and lower right image).

Phylogenetic identity of Spirulina* sp. *strain CY011

To determine the evolutionary position of the cultured *Spirulina* strain CY011, a phylogenetic analysis was conducted based on 16S rRNA gene sequences. The constructed

maximum likelihood tree (**Figure 34**A) reveals that CY011 clusters within the *Spirulina* clade, sharing a close phylogenetic relationship with *Spirulina subsalsa* and related taxa.

In the detailed view of the *Spirulina* clade (**Figure 34**B), CY011 is positioned in a subclade alongside *Spirulina subsalsa* strains, including strain SAB05015501 and LEGE 11439. The relatively high bootstrap support at this node suggests a well-supported evolutionary relationship. This clustering indicates that CY011 is closely related to *S. subsalsa* rather than other *Spirulina* species, such as *S. major* or *Halospirulina tapetecola*.

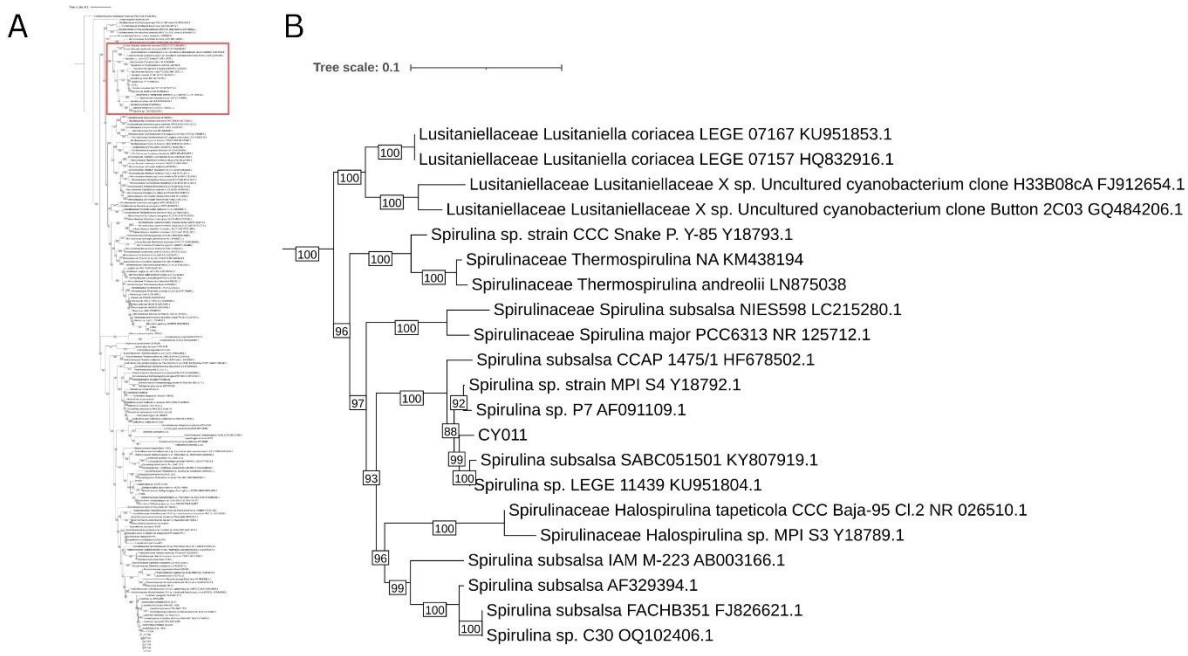


Figure 34. Phylogenetic tree of *Spirulina* strains, including strain CY011 based on 16S rRNA gene. (**A**) A comprehensive phylogenetic tree showing the evolutionary relationships among various cyanobacterial strains, with the relevant section highlighted. (**B**) A magnified view of the *Spirulina* clade, highlighting the placement of CY011. The tree was constructed using the maximum likelihood method, with bootstrap values provided at major nodes. The scale bar represents 0.1 substitutions per nucleotide position.

4.3.3 Characterization of *Salileptolyngbya* sp. CY006 and CY007

Morphology and culture growth of Salileptolyngbya sp. strains CY006 and CY007

Strain CY006 growed in BG-11 marine medium, displaying a pink coloration and growing attached to the flask wall (**Figure 35**). It is a filamentous, homocytous strain with thin, long, isopolar, and non-coiled trichomes. Sheaths and necridic cells are absent. The trichomes are cylindrical and usually not attenuated at the ends, with rounded apical cells lacking calyptra.

Cells are constricted at cross walls, with a mean length of 1.46 µm and a mean width of 1.04 µm. Older trichomes often contain significantly elongated cells.

CY007 is best maintained in BG-11 marine medium, exhibiting a brown or purple coloration and growing attached to the flask wall (**Figure 36**). This filamentous, homocytous strain has thin, long, isopolar, and non-coiled trichomes. Sheaths and necridic cells are absent. The trichomes are cylindrical, typically not attenuated at the ends, with rounded apical cells lacking calyptra. Cells are constricted at cross walls, with a mean length of 1.47 µm and a mean width of 1.08 µm. Older trichomes display notably longer cells.

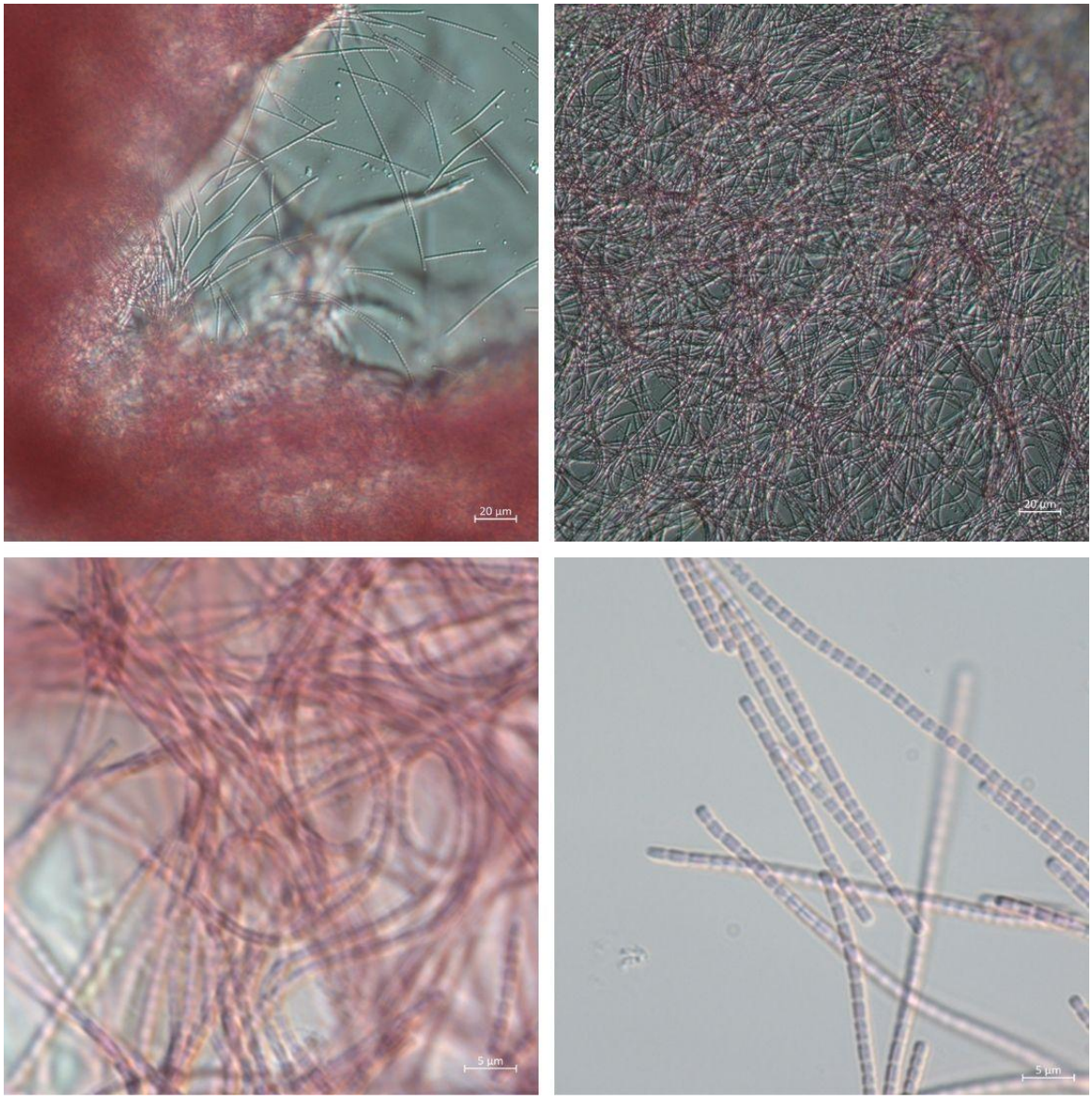


Figure 35. Microphotographs of strain CY006 under 400× magnification (upper right and left image) and 1000× magnification (lower left and lower right image).

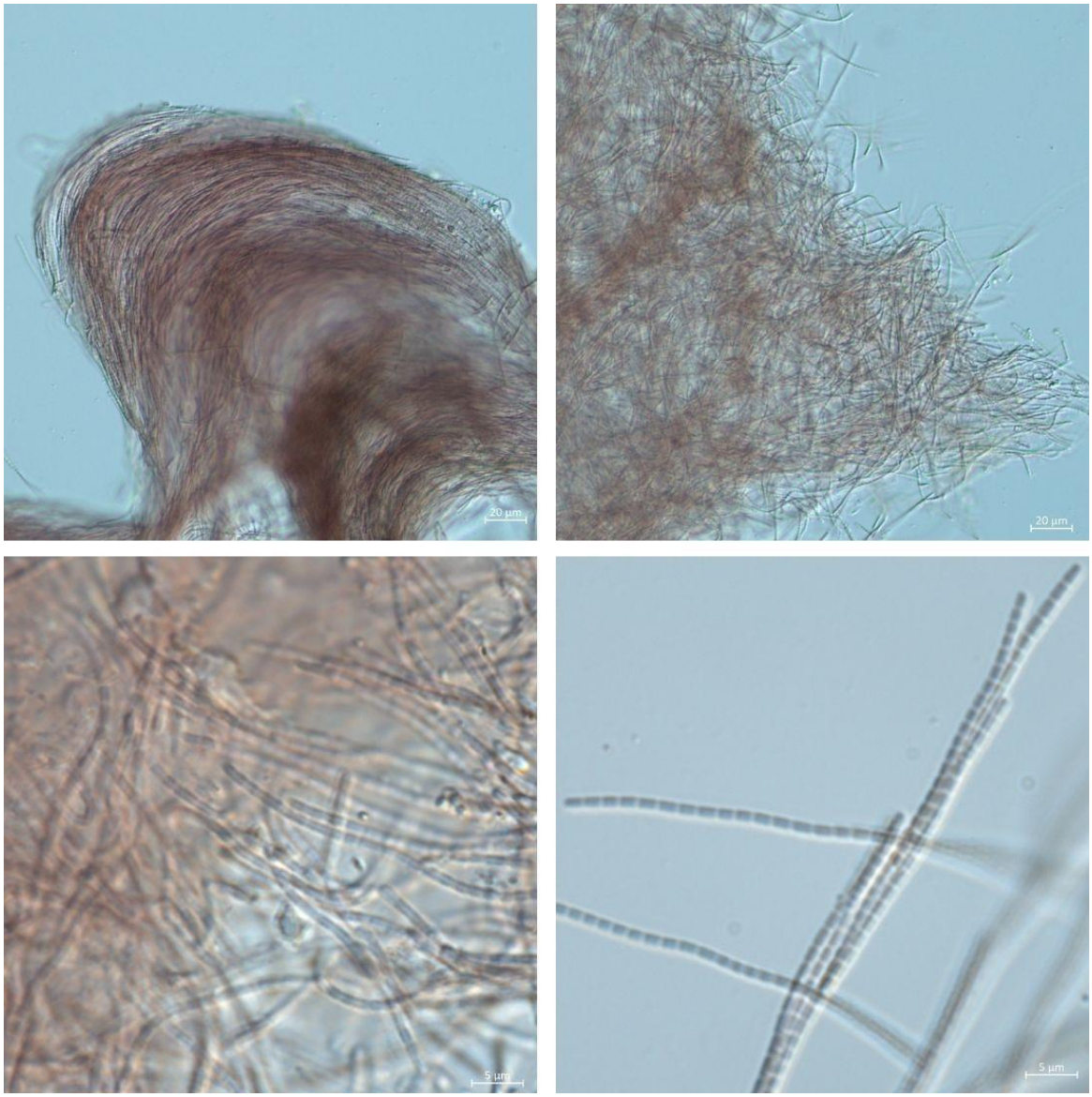


Figure 36. Microphotographs of strain CY007 under 400× magnification (upper right and left image) and 1000× magnification (lower left and lower right image).

Phylogenetic identity of Salileptolyngbya strains CY006 and CY007

The constructed maximum likelihood tree (**Figure 37**A) of cyanobacterial sequences shows placement of strains CY006 and CY007 into order Nodosilineales, within family Nososilineaceae and into the *Salileptolyngbya* genus clade.

Strain CY006 falls into the *Salileptolygbya* clade showing close relationship with sequences *Salileptolyngbya* sp. (BDU 141041) and two unculuted *Salileptolyngbya* sp. clones (ASV-1113 and ASV-512). The relationship is supported by high bootstrap values. Strain CY007 is sister clade to all analyzed *Salileptolyngba* sequences showing a highly bootstrap

supported relationship with *Salileptolyngbya* genus, but not closely related sequences to the exact strain sequences.

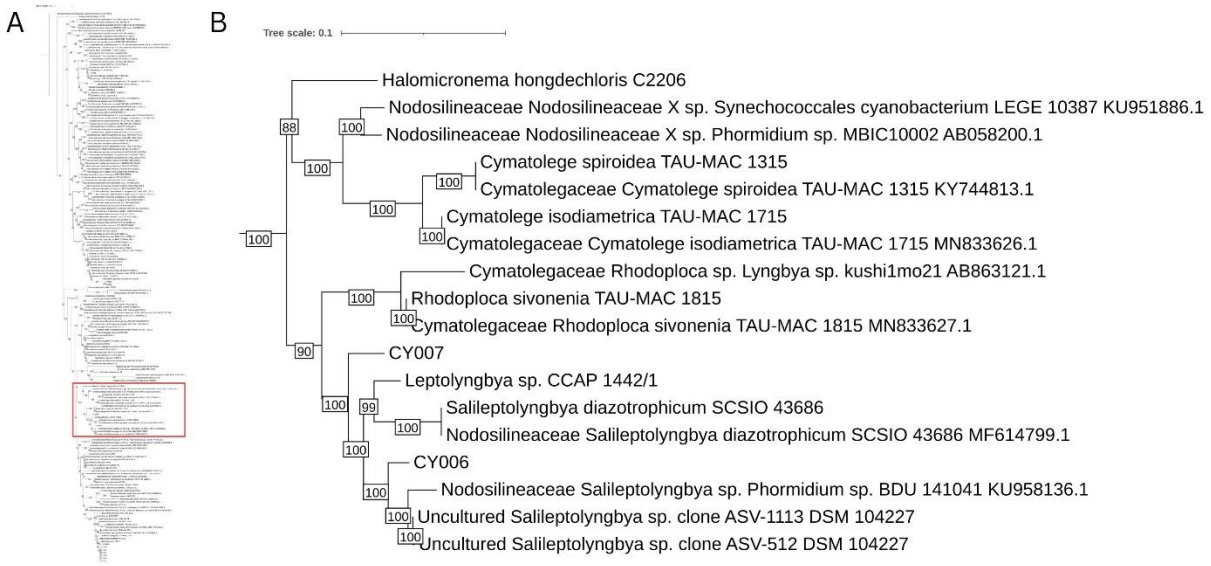


Figure 37. Phylogenetic tree of *Salileptolyngbya* strains, including strains CY006 and CY007 based on 16S rRNA gene. (**A**) A comprehensive phylogenetic tree showing the evolutionary relationships among various cyanobacterial strains, with the relevant section highlighted. (**B**) A magnified view of the *Salileptolyngbya* clade, highlighting the placement of CY006 and CY007. The tree was constructed using the maximum likelihood method, with bootstrap values provided at major nodes. The scale bar represents 0.1 substitutions per nucleotide position.

4.3.4 Characterization of *Leptothoe* sp. CY009 and CY047-CY052

Morphology and culture growth of Leptothoe sp. strains CY009 and CY047-CY052

Strain CY009 thrives in BG-11 marine medium, appearing brown in color and forming floating clumps (**Figure 38**). It is a filamentous, homocytous strain with thin, long, isopolar trichomes that are sometimes coiled. Sheaths are present, while necridic cells and calyptra are absent. The trichomes are cylindrical, usually not attenuated at the ends, with rounded apical cells. Cells are constricted at cross walls, with a mean length of 2.21 µm and a mean width of 1.39 µm. Aerotopes are present.

The morphology of strains CY047-CY052 were described in detail in work by Pahor (2024) and are included here only as part of the phylogenetic analyses since all of these strains are isolated under the same TurtleBIOME project and come from host animals from the same rescue centers and same locations.

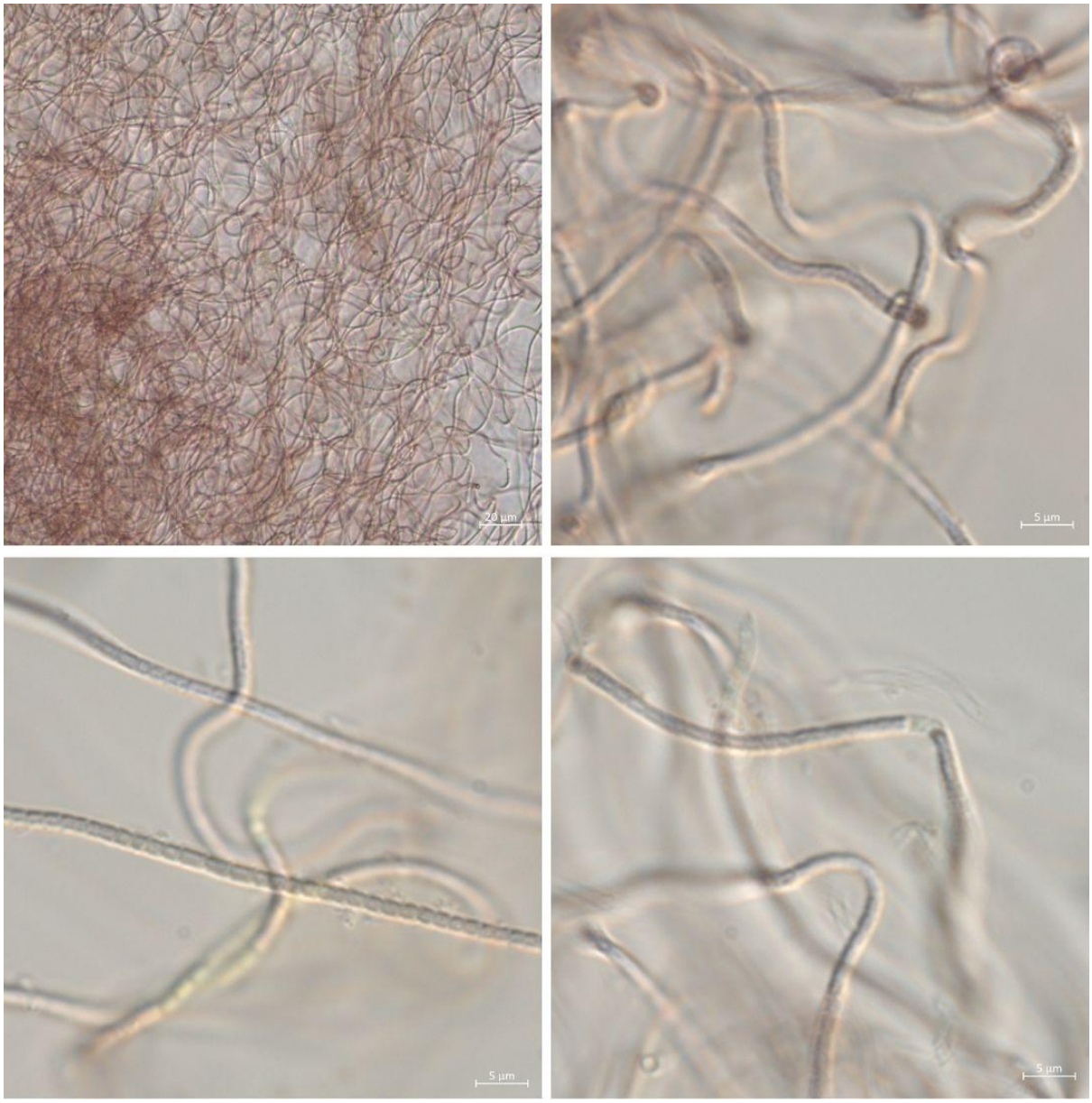


Figure 38. Microphotographs of strain CY009 under 400× magnification (upper right and left image) and 1000× magnification (lower left and lower right image).

Phylogenetic identity of Leptothoe strains CY009 and CY047-CY052

Cyanobacterial 16S maximum likelihood tree reveals placement of strains CY009 and CY047-CY052 into order Nodosilineales, family Cymatolegaceae and into the genus *Leptothoe*. Strain CY009 shows close relationship to sequence *Leptolyngbya* sp. HBC2 with high bootstrap value. Strains CY047-CY052 show also close relationship with sequence *Leptolyngbya* sp. HBC2, but also *Leptolyngbya ectocarpi* ULC424 and *Leptothoe spongobia* TAU-MAC 1015.

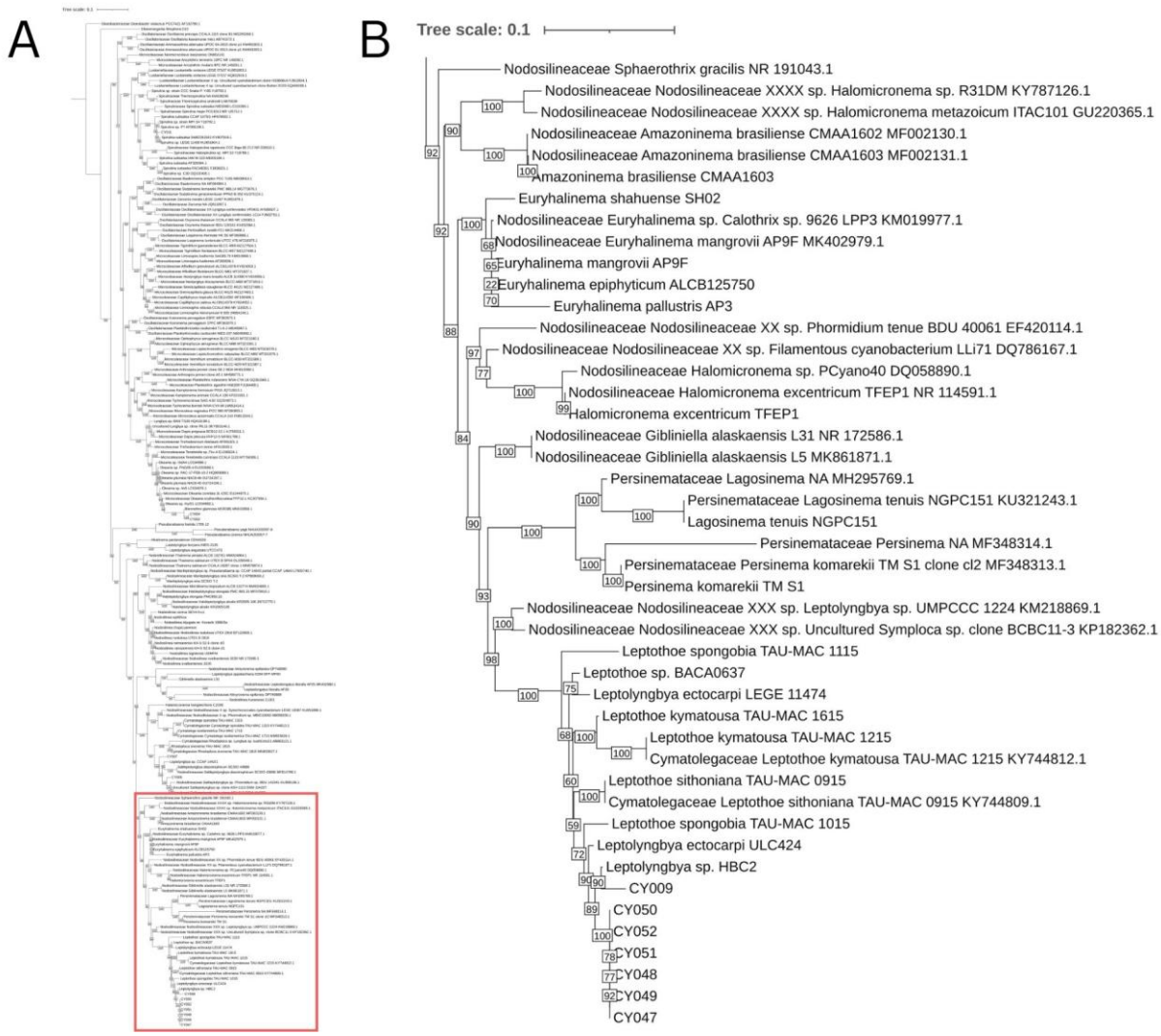


Figure 39. Phylogenetic tree of *Leptothoe* strains, including strains CY009 and CY047-CY052 based on 16S rRNA gene. (**A**) A comprehensive phylogenetic tree showing the evolutionary relationships among various cyanobacterial strains, with the relevant section highlighted. (**B**) A magnified view of the *Leptothoe* clade, highlighting the placement of CY009 and CY047-CY052. The tree was constructed using the maximum likelihood method, with bootstrap values provided at major nodes. The scale bar represents 0.1 substitutions per nucleotide position.

4.4 Connecting culture efforts with DNA-based work

The phylogenetic tree constructed from the 16S rRNA V6 region sequences includes both cultured strains and amplicon sequence variants (ASVs) from the metabarcoding survey, revealing phylogenetic relationships among the sequences. Full tree image is available on the link https://github.com/lucijakanjer/PhD-disertation and could not be included in printed version due to resolution. Most important clades related to cultured strains are shown in **Figure 40**. The presence of ASVs in different sample types, including juvenile, sub-adult, and adult sea turtle samples, as well as reference samples (samples obtained from the environment, not from

sea turtles), where only three clades are shown, are the ones with ASVs closely related to the cultured strains. Strains CY002 and CY004 were not included in this analysis because of a short sequence that did not contain region V6 of 16S rRNA.

ASVs clustering with cultured strain CY011 were identified as order Chroococcales, mainly genera *Xenococcus* and *Chlorogloea*. The *Chlorogloea* ASV most closely related to strain CY011 is present in 4 samples of juvenile turtles (TB175, TB181, TB185, TB201 and TB203) and one sample of sub-adult turtle (TB159), but not in adult turtles or reference samples. From the same clade, ASVs identified as *Xenococcus*, *Chlorogloea,* unidentified Pleurocapsaceae ASV and unidenfied Chroococcales ASV were present in adult turtle sample TB215. From the same clade, ASVs identified as *Geminocystis* and *Xenococcus* and were present in VRS sample, but not in turtle samples. Strain CY006 is closely related to ASVs from Nodosilineales order, four sequences identified as *Salileptolyngbya* and one as *Rhodoploca*. Together, *Salileptolyngbya* ASVs are present in most juvenile turtle samples (except TB175, TB195, TB221 and TB227), most sub-adult turtle samples (except TB167) and most adult turtle samples (except TB223). *Rhodoploca* ASV was present only in sub-adult turtle sample TB167. None of the ASVs from this clade closely related to strain CY006 were present in reference samples. Another clade was found further down in the tree with strain CY007 grouped with three *Salileptolyngbya* ASVs and on unidentified Nodosilineales ASV. Three *Salileptolyngbya* sequences were found in juvenile, sub-adult and adult samples (except in samples TB175, TB185, TB195, TB203, TB205, TB213, TB227, TB229, TB163, TB191 and TB199) and one of those three *Salileptolyngbya* ASVs was also recorded in reference VRS sample. Unidentified Nodosilineales ASV from the same clade is found only in sub-adult turtle sample TB177. Strains CY009 and CY047-CY052 were grouped in the polyphyletic clade with *Leptothoe* ASVs. There were 15 ASVs classified as genus *Leptothoe* from order Nodosilineales. Together, they were present in juvenile turtle samples (TB175, TB181, TB189, TB201. TB203, TB207, TB217, TB219 and TB229) sub-adult turtle samples (TB159 and TB 177) and in adult turtle sample TB215. Four of those *Leptothoe* ASVs were also present in reference sample VRS. Other reference samples from pools TB217P, TB219P and negative control contained none of the sequences that clustered together with cultured strains.

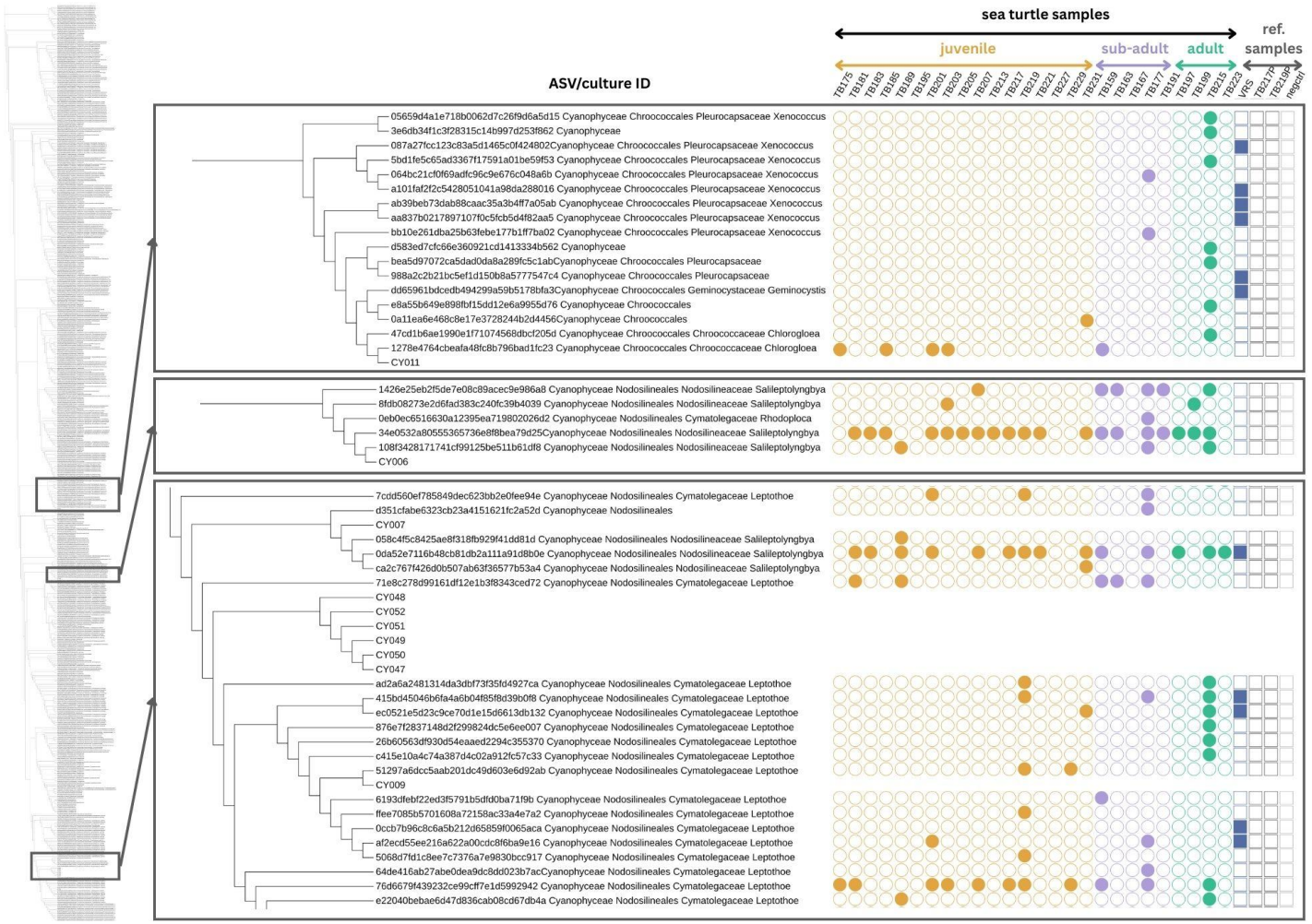


Figure 40. Phylogenetic tree of 16S rRNA V6 region sequences from cyanobacterial strains and amplicon sequence variants (ASVs) obtained from a metabarcoding survey of sea turtle-associated samples. The tree includes sequences from juvenile (yellow), sub-adult (purple), and adult (green) sea turtles, as well as reference samples. The presence of ASVs in different sample types is visualized using a bubble matrix, where the size of each circle represents the relative abundance of a given ASV in a specific sample. Cultured strains are labeled with their respective IDs (CYXXX), while ASVs are identified by unique sequence codes and identified taxonomic ranks.

5. DISCUSSION

This dissertation provides the results of analysis of cyanobacterial communities in sea turtle epibiotic biofilms using combined efforts of culture-dependent and culture-independent approaches. This is the first work that used this combined approach to study host-related cyanobacteria associated with sea turtles. The first metabarcoding survey, using universal bacterial primers, revealed that cyanobacteria are a consistent, although not dominant part of Mediterranean loggerhead sea turtle epibiotic microbial communities in carapace and skin samples. It also gave an overview of cyanobacterial genera present in those communities.

The second metabarcoding survey went into more detailed analysis of cyanobacterial community composition of Adriatic loggerhead sea turtles. Cyanobacteria from order Nodosilineales were found to be dominant, with genus *Rhodoploca* from family Cymatolegaceae being the most abundant. Other genera from family Cymatolegaceae, like *Leptothoe* and *Cymatolege*, were also found to be abundant in sea turtle associated biofilms. Cyanobacterial diversity in sea turtle epibiotic biofilm was influenced by several factors. Turtle size and, consequently, their age is a factor that was proven significant for both alpha and beta diversity. Moreover, alongside turtle size, factors like recovery centre, longitude and turtle condition are found to influence observed richness, longitude are found to influence Shannon diversity and recovery centre to influence Faith's diversity. Beta diversity, along with turtle size and age, was also influenced by turtle condition.

Culturing efforts resulted in six isolated cyanobacterial strains which were morphologically characterized and phylogenetically assigned to genera *Okeania*, *Spirulina*, *Salileptolyngbya* and *Leptothoe*. All cultured strains belong to filamentous homocytous cyanobacteria, as the most common type of cyanobacteria found in biofilms. From the metabarcoding results it can be inferred that *Okeania* and *Spirulina* most likely represent opportunistic commuters on sea turtle carapaces, while *Salileptolyngbya* and *Leptothoe* represent cyanobacterial commensals of sea turtles and potentially new species for science to describe.

The following discussion will cover every aspect of the results in detail, beginning with the first metabarcoding survey and the importance of selecting an appropriate, regularly updated reference database tailored to the microbial group of interest. It will then examine the cultured diversity of cyanobacteria and highlight the unique insights that can only be obtained from strains and how it connects with the information gained from metabarcoding studies. This will show how both approaches complement each other in describing cyanobacterial diversity.

5.1 Who are the main cyanobacterial inhabitants on sea turtle backs?

Studies investigating animal microbiome should not start with the assumption that every animal species has a core microbiome, but according to Hammer et al. (2019) this needs to be proven. The same authors emphasize that sequencing results should be carefully studied to test not only which microbes are there, but where they could come from, are they constant inhabitants of that animal and what function could they perform in/on that animal? The signs that animals do not have core-microbiomes are: 1) that variations between microbial communities associated with different individuals are too large (because those microbes come from host food, environment and laboratory contamination); 2) they have small number of total microbial species comparing to similar species that are known to host true microbiome; 3) they have no evident places on/in body to store beneficial microbes; and lastly, 4) there is no clear signal of microbiome change between hosts with different life-styles, nutrition and host fitness.

Although this study does not deal with gut or other host associated microbiomes, but rather only a cyanobacterial community in surface biofilms of sea turtles, the same principle signs could be discussed. Applying the same perspective on the results presented in this thesis, I can draw several conclusions. First, the variation in microbiota composition between individuals is notable, with individuals lacking cyanobacteria completely, which could support the fact that sea turtles do not host innate cyanobacterial microbiota. However, the presence of Cymatolegaceae and *Rhodoploca* can be observed regularly on sea turtles. This could mean that they are the core taxa and others are more opportunistic. Secondly, the number of species on each individual varies, but since not a lot is known about reptile surface microbiomes, especially the cyanobacterial component, direct comparisons cannot be made and the question whether this number is low or high is still ambiguous. Further on, designated spaces on the carapace for storing beneficial microbes appear to be missing. However, sea turtle carapace is known to harbor macro-epibiont commensals, which can provide necessary three-dimensional structure for biofilm-forming cyanobacteria colonization. Lastly, if we look at the change in microbiome signal from different lifestyles, we can see a signal in increasing diversity as the sea turtle grows, as well as other factors like turtle condition affecting cyanobacterial diversity. This does not necessarily confirm that sea turtles have core cyanobacterial community, since the swimming, diving and foraging habits, as well as the diet change from juvenile to adult turtles. Therefore, the increased diversity could also be the consequence of changing environment and

the transient species are only temporarily included within the community. All of this underscores the need for studies like the present one, bringing baseline information for estimation of the true importance of cyanobacteria on sea turtles.

The results obtained within this dissertation indicate that filamentous cyanobacteria dominate sea turtle biofilms, as confirmed by metabarcoding and SEM imaging. Filamentous homocytous cyanobacterial genera are the most common type recorded in this study and most commonly associated with marine benthic habitats, often found in biofilms and forming microbial mats (Charpy et al. 2010, Cardoso et al. 2017, Brocke et al. 2018). Filamentous taxa, such as *Oscillatoria*, *Hydrocoleum*, *Phormidium*, *Lyngbya* and *Leptolyngbya*, are well adapted to biofilm formation (Charpy et al. 2010), offering structural advantages over unicellular and heterocytous groups. In marine habitats, filamentous cyanobacteria contribute to biofilm formation by providing a stable substrate for other microorganisms, act as primary producers by fixing carbon, adhere to various surfaces such as rocks and macroalgae and produce secondary metabolites that inhibit the growth of competing microorganisms (Stal 2012). Sea turtles seem to provide additional surfaces for cyanobacterial colonization and could act as a highly mobile reservoir with unknown implications for the host's health and effects on the environment.

In the first metabarcoding survey, filamentous cyanobacterial genera such as *Phormidium, Limnothrix, Geitlerinema, Phormidesmis* and *Arthrospira*, appeared to be the most abundant genera using SILVA database. The presence of genera that live solitary or form irregular clusters, such as *Pleurocapsa* and *Chroococcidiopsis*, were also observed. After re-analysis using CyanoSeq database, none of the filamentous genera were found to be among dominant, but instead were replaced by filamentous *Rhodoploca* and Prochlorotrichaceae_X similarly to the results obtained from the second metabarcoding survey. Genera that form pseudofilaments or irregular clusters, such as *Waterburya* and *Chamaecalyx*, (Pleurocapsaceae) were also observed, but were not the same as the ones identified with SILVA. This is probably a result of reassignment of species to their correct genera provided by new molecular evidence. The inaccurate taxonomic assignment of cyanobacteria by the SILVA database is a known issue (Lefler et al. 2023). This is evident in the results of the first metabarcoding survey and its subsequent re-analysis using CyanoSeq. For instance, SILVA assigns ASVs to taxa such as "Acrophorium," "Phormidesmiaceae," and "Paraspirulinaceae" which are names that do not correspond to any recognized cyanobacterial taxa. As a result, no reliable information exists for

these groups, and they are absent from the most up-to-date CyanoDB and CyanoSeq databases. The strong differences between the two sets of results are most likely due to SILVA's outdated and inaccurate cyanobacterial taxonomy, which has not kept pace with recent taxonomic revisions and newly described taxa.

In the first metabarcoding survey published in Kanjer et al. (2022), order Nodosilineales was not yet established and couldn't be recognised as dominant. Although the dominant genus *Rhodoploca* was described in 2021 (Konstantinou et al. 2021), it was not included in SILVA taxonomy, like many more cyanobacterial species until the integration with CyanoSeq. Nodosilineales is a recently defined group within the updated cyanobacterial classification (Strunecký et al. 2023). Members of this order are primarily filamentous, characterized by a cell width of less than 2 μm and parietal thylakoids. They are widely distributed across diverse ecological habitats, spanning marine and freshwater environments (Strunecký et al. 2023). Within the order Nodosilineales, ASVs affiliated with the family Cymatolegaceae, particularly the genera *Rhodoploca*, *Cymatolege*, and *Leptothoe*, were notably abundant in our turtle-associated biofilms. Among them, *Rhodoploca* emerged as both the most prevalent genus and a core taxon across juvenile, sub-adult, and adult turtles. Species belonging to Cymatolegaceae predominantly inhabit marine or brackish environments and exhibit morphological diversity, with pink-colored trichomes serving as a distinctive shared characteristic. Interestingly, all three genera were originally isolated and described from marine sponges in the Aegean Sea (Caroppo et al. 2012, Konstantinou et al. 2019, 2021). This group also demonstrates variability in its photosynthetic apparatus, with some species reportedly lacking chromatic adaptation or utilizing far-red light for photosynthesis (Strunecký et al. 2023).

Cyanobacteria capable of utilizing far-red light typically thrive in low-light environments, where this adaptation provides a survival advantage (Gan et al. 2014, Antonaru et al. 2020). The dominant cyanobacterial orders identified in this study, Nodosilineales and Prochlorotrichales, are known to exhibit far-red light photoacclimation (FaRLiP), allowing for rapid adaptation to changing light conditions, an advantageous trait for organisms inhabiting a mobile, diving host (Gisriel et al. 2023). However, the ability to utilize far-red light is not universally present across all members of these orders and cannot be reliably inferred from 16S rRNA gene sequences alone (Antonaru et al. 2020). While this study does not specifically investigate the presence or abundance of FaRLiP cyanobacteria, it is important to recognize that this capability could enhance survival on sea turtle carapaces, where light availability fluctuates

due to diving behavior. This effect may be particularly significant during winter months when turtles exhibit overwintering behavior, often resting on the seafloor for extended periods with only occasional surfacing (Hochscheid et al. 2005).

The second most abundant order, Prochlorotrichales, currently represented by a single genus *Prochlorothrix* (Strunecký et al. 2023), was particularly prominent in juvenile turtles but largely absent in sub-adults and adults, except in individuals TB191 and TB199. Unlike Nodosilineales, which possess motile trichomes encased in sheaths, Prochlorotrichales form non-motile trichomes, potentially placing them at a disadvantage when colonizing the turtle carapace. This could explain their greater prevalence in the less diverse microbial communities of juveniles before Nodosilineales establishes its ecological dominance.

Unicellular cyanobacteria, such as *Synechococcus* and *Prochlorococcus*, dominate planktonic environments, thriving as free-living cells. These taxa are less common in stable biofilms due to their limited surface adhesion capabilities, but are still recorded in sea turtle carapace biofilms. Heterocytous cyanobacteria, including *Nostoc* and *Calothrix*, possess heterocytes that allow them to fix atmospheric nitrogen. While they form biofilms in freshwater and terrestrial habitats, they are less prevalent in marine epibiotic communities. No heterocyte-forming cyanobacteria were observed in high abundances in this study.

One of the core ASVs, identified as *Cyanobacterium* sp. BBD-AO-Red, is linked to black band disease in corals worldwide (Buerger et al. 2016). Another core ASV was classified as either *Copelandiella yellostonensis* (Kaštovský et al. 2023), a thermophilic mat-forming cyanobacterium related to *Leptolyngbya*, or *Geitleribactron purpureum*, a unicellular, heteropolar cyanobacterium known for forming purple-hued biofilms on rocks (Cantonati et al. 2014). Both taxa belong to the family Leptolyngbyaceae (Mareš and Cantonati 2016).

5.2 What drives cyanobacterial diversity change?

The first metabarcoding survey did not yield enough cyanobacterial sequences using universal bacterial primers (V4) and detailed statistical analysis of factors influencing cyanobacterial diversity could not be performed. Therefore, the second metabarcoding survey using specific cyanobacterial (V6) primers were used and the results confirmed the second hypothesis set in this thesis work, that *to investigate the diversity of cyanobacteria from the*

microbial biofilm of sea turtle carapaces, specific primers for cyanobacteria that amplify the V6 region give better results than universal bacterial primers that amplify the V4 region of 16S rDNA.

The results of the second metabarcoding survey indicate a significant increase in epibiotic cyanobacterial community diversity as sea turtles transition from juvenile to adult stages and confirm the first thesis hypothesis that the cyanobacterial community of the carapace and skin of sea turtles differs depending on the location, size and age of the turtles. This shift likely coincides with expanded migratory routes to breeding and nesting areas in adulthood, highlighting the dynamic nature of loggerhead sea turtle microbiota throughout their life cycle. The loggerhead's development progresses through four distinct phases: hatchling, juvenile, sub-adult, and adult. These stages are marked by an ontogenetic habitat shift from the oceanic phase to the neritic phase (Wyneken et al. 2013). Hatchlings and juveniles predominantly occupy oceanic waters, where they swim and forage near the surface due to their underdeveloped lungs, which limit deep diving. As they mature, their lung capacity increases, allowing for deeper dives and benthic foraging, a defining trait of the sub-adult phase that marks their transition to neritic habitats. Once they reach sexual maturity, adult turtles primarily reside in neritic environments, where they feed on benthic organisms. As omnivores and bioturbators of marine sediments, adult loggerheads actively disturb the substrate during foraging, which may facilitate the colonization of new cyanobacteria on their carapace (Lazar et al. 2011). Recent research (Baldi et al. 2023, Mariani et al. 2023) suggests that in the Mediterranean loggerhead population, the boundary between the oceanic and neritic phases is less distinct, largely due to the availability of shallow foraging areas, particularly in the Adriatic Sea (Casale et al. 2008). Despite this, our results indicate clear differences in cyanobacterial composition between juvenile and adult turtles, reflecting key aspects of their biology and ecology. During the juvenile phase, rapid carapace growth results in frequent shedding of old biofilms along with scutes. Since biofilm formation on surfaces typically begins with bacterial and cyanobacterial colonization and can occur within (Rubio 2002, Mejdandžić et al. 2015, Dang and Lovell 2016, Grzegorczyk et al. 2018), this rapid turnover could create new microhabitats for cyanobacteria to establish. The observation that juvenile turtles harbor less complex and diverse cyanobacterial communities may stem from their reduced interaction with benthic habitats and the continuous formation of primary biofilms, compared to adults, whose carapace supports more established biofilm communities at different successional stages. While juvenile and adult turtles in the Mediterranean may forage in overlapping locations, differences in the time spent

in these areas could influence their exposure to cyanobacteria. Additionally, biofilm ecology itself may play a role, as older biofilms tend to support more structurally complex and taxonomically diverse microbial communities (Fenchel and Kühl 2000, Mejdandžić et al. 2015).

Turtle health condition was among the most influential factors affecting cyanobacterial diversity. The three categories used to classify turtle conditions may have been too broad, potentially masking stronger patterns of association. Previous studies have documented that sick turtles, especially those unable to swim properly, tend to accumulate epibiotic macro-organisms, such as macroalgae and barnacles, due to prolonged surface dwelling or inactivity on the seafloor (Frick and Pfaller 2013). This biofouling process is often preceded by microbial biofilm formation, suggesting that microbial and cyanobacterial communities may serve as early indicators of deteriorating health.

The site of turtle rehabilitation, whether at the Blue World Institute (BWI) or Aquarium Pula, had a notable impact on cyanobacterial community composition. Observed ASV richness and phylogenetic diversity were significantly affected, with turtles sampled from BWI exhibiting lower richness and phylogenetic diversity than those from Aquarium Pula. Differences in water circulation and filtration systems between facilities could contribute to variations in microbial assemblages. Similar studies have demonstrated that captivity conditions can indeed influence the gut microbiota of other reptiles (Keenan et al. 2013, Sandri et al. 2020). However, in this study, the turtles were sampled as quickly as possible upon their arrival and did not remain in rehabilitation facilities long enough to undergo a complete cyanobacterial community shift. An additional factor influencing these differences is the role of Aquarium Pula as a medical center, where more seriously injured or sick turtles are housed. Since severely injured turtles experience prolonged immobility, they may be more prone to cyanobacterial colonization, particularly by opportunistic taxa. Reduced mobility can facilitate biofilm formation, as stationary conditions and prolonged exposure to light enhance cyanobacterial proliferation (Frick and Pfaller 2013).

Longitude was used as a proxy to examine whether the north-south placement of the Adriatic Sea influenced the cyanobacterial communities on sampled turtles. No direct correlation was found between longitude and alpha diversity metrics. However, in statistical models, longitude was a significant contributing factor to observed ASV richness, with turtles from higher longitudes exhibiting richer and more diverse cyanobacterial communities (as

measured by Shannon diversity) compared to turtles from lower longitudes. This indicates that, although longitude alone does not directly drive cyanobacterial diversity, it still influences community composition in conjunction with other factors and that aligns well with the hypothesis that *the cyanobacterial community of the carapace and skin of sea turtles differs depending on the location, size and age of the turtles.* Previous studies on sea turtle microbiota have highlighted location as a key determinant of epibiotic microbial community structure, often due to regional differences in environmental parameters and host behavior (Van de Vijver et al. 2020, Kanjer et al. 2022). The relatively weak signal of longitude in this study may be attributed to the fact that all sampled turtles belong to the same mediterranean population, which is highly migratory (Casale et al. 2008). The Adriatic Sea is a relatively small basin (Cognetti et al. 2000), allowing turtles to travel between locations rapidly, potentially preventing the establishment of regionally distinct cyanobacterial communities. However, seasonal migrations and prolonged residence in specific feeding grounds might still play a role in shaping microbial assemblages, warranting further investigation.

Seasonal variations in water temperature are generally among the strongest drivers of cyanobacterial growth in aquatic and marine environments (Paerl and Otten 2013). However, in this study, seasonality did not significantly contribute to cyanobacterial community differences. Additionally, season was excluded from LM and GLM analyses due to collinearity with other variables. One possible explanation is that seasonal effects were masked by differences in turtle health condition. Turtles sampled in spring and summer were generally in better condition than those sampled in autumn and winter, which may have confounded any potential seasonality-driven changes in cyanobacterial communities. To more accurately assess seasonal influences, future studies should focus exclusively on wild turtles in good health from the same geographic region, an approach that was beyond the scope of this study.

Outside of scope of this study was also the influence of anthropogenic pressure on epibiotic cyanobacterial community, but represent important avenues for future research. Planktonic cyanobacterial taxa are known to thrive in warm temperatures, eutrophic waters, and human-impacted environments whereas for the benthic taxa there is not enough information on their bloom drivers (Ahern et al. 2007, Paerl and Otten 2013). Additionally, sea turtles have been documented as reservoirs for pathogenic and antibiotic-resistant bacteria (Trotta et al. 2021a, 2021b), which could further influence the structure of their cyanobacterial biofilms. With increasing anthropogenic pressures on coastal waters in the Adriatic and Mediterranean

Seas, there is a growing need for studies that examine the impacts of pollution, climate change, and antibiotic resistance on host-associated microbial communities. Understanding how these factors shape cyanobacterial biofilms on sea turtles could provide valuable insights into ecosystem health and host-microbe interactions in marine environments.

The eastern Adriatic coastline is predominantly characterized by limestone rocky shores, with only a limited number of sandy beaches. Consequently, past research has largely focused on endolithic and supratidal cyanobacteria (Brandes et al. 2015, Palinska et al. 2017, Vondrášková et al. 2017, Vogt et al. 2019). These studies have found that the most abundant cyanobacteria belong to the former order Pleurocapsales, a morphologically diverse group with randomly distributed baeocytes (internal spores) within young pseudofilaments (Komárek et al. 2014), now reclassified as the family Pleurocapsaceae within the order Chroococcales (Strunecký et al. 2023). This group of cyanobacteria is commonly found in epilithic and endolithic habitats worldwide. A study by Kolda et al. (2020) investigating cyanobacteria in sediments and coastal waters under anthropogenic influence in Croatia found that Pleurocapsaceae dominated, particularly the genera *Xenococcus* and *Pleurocapsa*. In contrast, while Chroococcales and Pleurocapsaceae were present in our turtle epibiotic samples, they did not exhibit dominance. Among the identified genera, *Odorella*, recently described as a cryptic genus within *Pleurocapsa* (Shalygin et al. 2019), was highly represented. It is possible that Kolda et al. (2020) classified *Odorella* under *Pleurocapsa*, as taxonomic distinctions were only clarified later. Notably, *Odorella* is the first genus within Pleurocapsaceae confirmed to produce microcystins and other cyanotoxins, with a total of 38 identified bioactive molecules. Both *Odorella* and unidentified Pleurocapsaceae taxa were more frequently detected in sub-adult and adult turtle samples. Regarding diversity and taxonomic richness, our study found higher Shannon diversity in turtle-associated cyanobacterial communities compared to those in Croatian tidal flats (ASV richness: 53; Shannon diversity: 0.8) (Vogt et al. 2019). However, our samples exhibited lower ASV richness (median = 47) than those from intertidal environments. Similarly, a study on supratidal endolithic cyanobacteria (Brandes et al. 2015) reported lower OTU richness (ranging from 2 to 25) but greater overall diversity (average Shannon diversity of 4.13) than observed in our dataset.

Our results show both similarities and differences compared to previous studies on cyanobacteria associated with sea turtles, although none have provided such detailed analysis. In hawksbill and green turtles from Iran, cyanobacteria were reported as the second or third

most dominant phylum depending on the sample (Loghmannia et al. 2023). This aligns with my findings, where cyanobacteria were consistently present but not dominant within the epibiotic microbial community. The commonly detected genera in that study included *Lyngbya*, *Leptolyngbya*, *Cyanobacterium*, *Phormidium*, and *Chroococcidiopsis*. While these specific genera were not identified in our study, they share similar ecological requirements with the cyanobacterial taxa we observed. The discrepancy may again reflect differences in reference databases used for taxonomic assignment. Blasi et al. (2022) reported cyanobacteria as comprising only 1% of the total bacterial community, though higher abundances of Pseudanabaenaceae and Rivulariaceae were observed microscopically on anterior scutes. Pseudanabaenaceae were not detected in our study, but this may be due to their morphological similarity to members of the Nodosilineales. In contrast to our results, Bachmann et al. (2024) detected cyanobacterial sequences exclusively on green turtles, with none found on loggerheads. Meanwhile, Kuschke et al. (2024) reported cyanobacteria among the top ten phyla in nesting leatherback turtles, comprising 1.4% of the total community and showing higher abundance on flippers (2.2%) compared to the carapace (0.48%). This is similar to our finding that older turtles, which have had longer environmental exposure, harbor greater cyanobacterial diversity.

To better understand the ecological interactions between cyanobacteria and sea turtles, it is useful to draw comparisons with well-documented microbial associations. Research on epibiotic diatoms has revealed a notable degree of host specificity, as they are consistently present across all sampled sea turtles, with the community largely dominated by putatively epizoic species (Robinson et al. 2016, Ashworth et al. 2021). In contrast, cyanobacteria do not exhibit such uniformity in distribution – some turtles lack detectable cyanobacterial sequences altogether (Kanjer et al. 2022). While a significant proportion of cyanobacterial sequences in our study remained unidentified, the results suggest that these microbes are not strictly host-specific. Instead, the turtle's carapace supports a variety of common benthic taxa, including members of the family Cymatolegaceae, which are also found in marine sponges. These cyanobacteria are known for their ability to tolerate diverse light conditions and produce a range of bioactive compounds, including cyanotoxins.

Recent research suggests that the production of microcystins may be more widespread among marine cyanobacteria than previously recognized (Konstantinou et al. 2019). These cyanotoxins, which are produced by specific cyanobacterial taxa, have been shown to possess

antibiotic properties, raising the possibility of their application in the development of new antimicrobial agents. This potential medical relevance warrants further exploration. However, microcystin production within sea turtle biofilms presents dual concern. On one hand, it may serve as a natural deterrent against pathogenic bacterial colonization (Ramos et al. 2015), thereby benefiting the turtle's microbiome. On the other hand, it poses a potential risk to vertebrates (Zhou et al. 2021), including the host turtle itself. Given the close relationship between toxin-producing cyanobacteria and sea turtles, there is a possibility that exposure to these compounds could negatively impact turtle health, particularly for species already classified as endangered. Juvenile turtles in particular may be more vulnerable to cyanobacterial dominance by a single species, increasing the likelihood of toxic species becoming established on their carapaces. Since cyanotoxins have been identified in taxa residing on sea turtles, their presence could potentially contribute to health issues that lead to turtle rehabilitation and hospitalization. Notably, the role of cyanobacteria in sea turtle health has not been thoroughly examined, nor has their presence been considered as a factor in the admission criteria for turtles in rescue centers. Beyond their potential impact on sea turtles, the spread of toxic cyanobacteria in benthic habitats can disrupt broader ecological dynamics. When toxin-producing species become dominant, they may outcompete other microorganisms, reduce biodiversity and have cascading effects on benthic food webs, potentially affecting invertebrates and fish that rely on these habitats (Zhang et al. 2022).

The impact of climate change on cyanobacterial dynamics is a growing concern, with rising global temperatures and increasing oceanic instability predicted to favor the proliferation of opportunistic and potentially harmful cyanobacteria (Urrutia-Cordero et al. 2020). Cyanobacterial blooms have been widely documented in freshwater ecosystems, where they contribute to eutrophication and produce harmful toxins (O'Neil et al. 2012). However, the long-term effects of climate change on marine cyanobacteria, particularly those colonizing epibiotic surfaces such as sea turtle carapaces, remain largely unexplored. One of the key knowledge gaps in this field is understanding how increasing ocean temperatures influence cyanobacterial community composition on sea turtles. Higher temperatures may promote the growth of thermophilic or opportunistic cyanobacteria, potentially shifting community structure and favoring toxin-producing taxa (Walls et al. 2018). The possibility that warmer conditions could enhance toxin production raises significant concerns for turtle health and broader marine ecosystem stability (Konstantinou et al. 2019). Furthermore, climate change is expected to drive shifts in ocean circulation patterns and nutrient availability (Lynch-Stieglitz et al. 2024, Sun et

al. 2024), which could influence cyanobacterial colonization on sea turtles. Increased nutrient runoff from coastal areas due to extreme weather events may lead to higher cyanobacterial loads in nearshore environments (Duperron et al. 2019, Jaegge et al. 2023, Li et al. 2023), potentially increasing the likelihood of colonization on turtles inhabiting these regions. Additionally, ocean acidification may select for more resilient, potentially harmful cyanobacterial taxa (Wu et al. 2021), further altering the microbial landscape of turtle epibiota. Another unexplored aspect is the potential influence of climate change on turtle behavior and how this might indirectly impact cyanobacterial dynamics. For example, rising sea temperatures have already been linked to changes in sea turtle nesting behavior (Sousa-Guedes et al. 2025). If turtles shift their habitats in response to climate change, they may encounter new microbial communities, leading to previously undocumented cyanobacterial associations.

5.3 Culturable diversity of cyanobacteria on sea turtles

Although only six cyanobacterial strains were successfully obtained from epibiotic samples associated with sea turtles, this outcome reflects the difficulty and time-consuming nature of traditional cyanobacterial isolation techniques. Cultivating filamentous benthic cyanobacteria from complex biofilms requires repeated transfers, long incubation times, and careful monitoring to eliminate contaminants, making even a small number of stable monocultures a significant achievement. These results underscore the critical importance of cultivation, despite its challenges, for advancing our understanding of cyanobacterial diversity. Many key taxa observed on sea turtle carapaces, such as *Salileptolyngbya* and *Leptothoe*, remain poorly characterized in environmental sequencing datasets due to limited reference genomes and unresolved taxonomy. Cultivation enables detailed morphological, ecological, and phylogenetic analyses, which are essential for distinguishing cryptic diversity, identifying potential novel species, and exploring bioactive compound production. Without cultivation, the ecological roles and potential applications of these cyanobacteria would remain largely hidden. The cultivation results confirmed the third thesis hypothesis that the *caparapace habitat of sea turtles represents a source of new cyanobacterial taxa for science*.

The general morphology of species belonging to the order Oscillatoriales is characterized by non-heterocytous trichomes that are either unconstricted or only slightly constricted, typically composed of cells that are shorter than they are wide. Cell diameter within Oscillatoriales varies considerably, ranging from 2 to 115 µm. This order includes two

phylogenetically distinct and well-supported families: Oscillatoriaceae and Microcoleaceae. Strains CY002 and CY004 conform to the morphological characteristics of Oscillatoriales and are phylogenetically assigned to the family Microcoleaceae. Morphologically, members of the Microcoleaceae family, like other Oscillatoriales, often possess large filaments with discoid cells. They may also produce calyptra and commonly exhibit mucilaginous sheaths (Strunecký et al. 2013). This family encompasses a diverse range of filamentous genera, including taxa ecologically important in soils (e.g., *Microcoleus*), freshwater benthic environments (e.g., *Kamptonema*, *Tenebriella*), and planktonic habitats in both freshwater and saline waters (e.g., *Planktothrix*, *Limnospira*, *Limnoraphis*, *Trichodesmium*). A distinct lineage within Microcoleaceae includes species forming extensive benthic mats in marine, tidal, or brackish environments. These were previously classified under the genus *Lyngbya*, which has since been revealed to be polyphyletic and redefined as a morpho-genus (Engene et al. 2010, Strunecký et al. 2023). Traditionally, *Lyngbya* encompassed large, unbranched filamentous cyanobacteria with discoid cells enclosed in sheaths (Geitler 1932, Castenholz et al. 2001, Komárek and Anagnostidis 2005). However, several new genera have since been described from *Lyngbya*, including *Neolyngbya*, *Dapis*, *Capilliphycus*, *Okeania*, and *Affixifilum*. These filamentous taxa generally exhibit cell widths between 8 and 50 µm, often contain gas vesicles, and possess the ability to fix nitrogen. This functional trait links them to *Trichodesmium*, a well-known pelagic nitrogen-fixing cyanobacterium that is phylogenetically grouped with this clade. Strains CY002 and CY004 exhibit several defining features of this group, including sheath-bearing filaments, occasional presence of calyptra and aerotopes. Their phylogenetic placement, confirmed through phylogeny analysis, supports their inclusion in this lineage.

The genus *Okeania* was first described by Engene et al. (2013) and includes cyanobacteria found in tropical and subtropical marine environments, often forming benthic mats. *Okeania* species are among the most common, abundant, and widely distributed cyanobacteria in warm marine habitats, though they may also appear in temperate regions during more warmer periods or extreme events (Engene et al. 2013). Strains CY002 and CY004 are phylogenetically closest to *Blennothrix glutinosa*, but are placed within the *Okeania* clade. This suggests that strains indeed belong to several *Okeania* species, but further morphological and genetic comparisons are warranted. Of the five currently recognized *Okeania* species, strains CY002 and CY004 most closely resemble *Okeania comitata*, particularly in terms of its dark coloration and cell size. *O. comitata* has a cell width of 10–12 µm, compared to 11.11 µm (CY002) and 8.63 µm (CY004). Similarly, *O. comitata* has a cell length of 1.5–2.5 µm, while

CY002 and CY004 measure 2.19 µm and 2.29 µm, respectively. Other morphological characteristics are also congruent with *O. comitata* description. *O. comitata*, previously known as *Oscillatoria nigro-viridis*, is known to produce the bioactive compounds viridamides A and B, which exhibit potent activity against three parasitic protozoa: *Trypanosoma cruzi*, *Leishmania mexicana*, and *Plasmodium falciparum* (Simmons et al. 2008). Further phylogenetic analysis using additional gene markers is necessary to confirm the placement of strains CY002 and CY004 within *Okeania* and to assess their potential as new species. Regarding their presence on sea turtle carapaces, representatives of the order Oscillatoriales, family Microcoleaceae, and genus *Okeania* were not abundant in the analyzed biofilm samples. Strains CY002 and CY004 were isolated from Karlo Albano (TB215), an adult male sea turtle whose carapace was predominantly colonized by Nodosilineales and Chroococcales. The successful isolation of *Okeania* strains may reflect their opportunistic growth under culture conditions, rather than high in situ abundance. These strains may be capable of rapid growth in warmer environments, suggesting they could become more prevalent under future climate warming scenarios. Although this study did not explore the presence of nitrogen-fixing genes or bioactive compound production in CY002 and CY004, previous research (Simmons et al. 2008, Engene et al. 2013) has shown that *Okeania* species possess nitrogenase genes and can produce antiprotozoal compounds. In the epibiotic biofilm community on sea turtle carapaces, *Okeania* may play important ecological roles as primary producers, nitrogen fixers, and potential deterrents of protozoan parasites.

Strain CY011, with its highly coiled trichomes, is a typical representative of the genus *Spirulina* in the order Spirulinales, a placement that is also confirmed by phylogenetic analysis. Historically, members of the Spirulinales were defined morphologically as thin, motile, and narrowly spiraled trichomes lacking sheaths, typically inhabiting halophilic environments (Strunecký et al. 2023). Today, the order Spirulinales comprises two families: Spirulinaceae, which includes taxa with characteristically spiraled trichomes, and Lusitaniellaceae, which currently contains a single species, *Lusitanella coriacea* (Brito et al. 2017). The latter is morphologically cryptic, closely resembling other thin filamentous cyanobacteria with longer cells, such as members of Prochlorotrichaceae, Nodosilineaceae, and some Leptolyngbyaceae. The family Spirulinaceae currently includes two monophyletic, euryhaline genera: *Spirulina* and *Halospirulina*. Both genera are characterized by thin, tightly coiled trichomes that lack sheaths and are typically found in benthic environments or habitats with high salinity or conductivity (Strunecký et al. 2023). The genus *Spirulina* is defined by filamentous,

unbranched trichomes that never possess sheaths. These filaments are rarely solitary (i.e., free-floating) and are more commonly found in clusters or as fine mats (Hauer and Komárek 2022). They also can reach impressive sizes that may be macroscopically visible, regularly covering substrates and providing biofilm structure for other organisms like diatoms and nematodes (Włodarska-Kowalczuk et al. 2014). The trichomes are screw-like coiled along their entire length, with a constant coil width. The coiling pattern is species-specific. *Spirulina* species are typically present in the benthos of freshwater, brackish, or marine environments. Among all known species, *Spirulina subsalsa* exhibits the most tightly coiled trichomes (Komárek and Anagnostidis 2005), a feature also observed in strain CY011. The filament coloration is highly variable, ranging from blue-green, emerald-green, olive green, and blackish to yellowish, pale violet, or pink-reddish hues (Hauer and Komárek 2022) observed in strain CY011. Based on morphological and phylogenetic data, strain CY011 can be confidently assigned to the genus *Spirulina*, and most likely to the species *S. subsalsa*. However, a more detailed multigene phylogenetic analysis would be required to confirm its species-level classification. Although "Spirulina" is a widely recognized name in the context of human nutritional supplements, the commercial product actually originates from *Arthrospira platensis*. This species was formerly classified within *Spirulina* but was later reassigned to the genus *Arthrospira* following sufficient taxonomic and molecular evidence (Papapanagiotou and and Gkelis 2019). Nonetheless, the true *Spirulina* species, particularly *S. subsalsa*, are known producers of several bioactive compounds, including cytotoxic and anti-cancer agents (Szubert 2018), as well as anti-inflammatory and antithrombotic compounds (Shiels 2022). Moreover, *S. subsalsa* has been shown to exhibit toxic effects on the blue shrimp *Penaeus stylirostris*, causing tissue necrosis (Lightner 1978). Strain CY011 was isolated from the carapace of a juvenile loggerhead sea turtle named Kolumbo (sample ID TB235). While the carapace sample from TB235 was not included in the metabarcoding analysis, it was examined using scanning electron microscopy (SEM). SEM micrographs clearly show the presence of *Spirulina*-like filaments on the carapace scutes. In the metabarcoding dataset, representatives of the genus *Spirulina*, family Spirulinaceae, or order Spirulinales were not found in high abundance. This suggests that *Spirulina* may be an opportunistic colonizer on the sea turtle host, as observed in the TB235 sample, rather than a dominant or consistent community member. Within the sea turtle carapace biofilm, *Spirulina* could serve as a primary producer, contributing to overall biofilm productivity. Additionally, the genus's ability to secrete bioactive compounds may influence the structure of the epibiotic community. However, due to the known toxic effects of *S. subsalsa*

on invertebrates, its presence, especially in high abundance, should be approached with caution. It may have harmful impacts on co-occurring animal epibionts or potentially even on the turtle host itself.

Strains CY006 and CY007 were identified as belonging to the genus *Salileptolyngbya* (family Nodosilineaceae), while strains CY009 and CY047–CY052 were assigned to the genus *Leptothoe* (family Cymatolegaceae). All strains are part of the order Nodosilineales. The order Nodosilineales is ecologically diverse and is primarily defined by its monophyletic clustering in multigene and 16S rRNA gene phylogenies**,** as revised by Strunecký et al. (2023). Most members are filamentous, with cell widths below 2 μm and parietal thylakoids. Species with isodiametric cells up to twice as long before division can be cryptic to Leptolyngbyaceae, while those with cylindrical cells several times longer than wide resemble but are slightly thinner than members of Prochlorotrichales (Strunecký et al. 2023). The family Nodosilineaceae is typified by the genus *Nodosilinea* (Perkerson III et al. 2011), but encompasses strains with diverse trichome morphologies and ecological requirements. Trichomes can be straight, flexuous, bent, or spirally coiled—solitary or entangled, and sometimes forming nodules. They can reproduce by disintegration into motile hormogonia without forming necridic cells. Cell size is variable depending on growth conditions but typically ranges from 1–3.5 μm in width and 5–10 μm in length, showing slight constriction at cross walls. Most members are halotolerant, and some are capable of nitrogen fixation. There are currently nine genera in the family: *Nodosilinea*, *Amazoninema*, *Leptoelongatus*, *Marileptolyngbya*, *Salileptolyngbya*, *Euryhalinema*, *Gibliniella*, *Haloleptolyngbya* and *Halomicronema*. Most of them are described recently as cryptic to *Leptolyngbya* (fine filaments with sheaths) and *Psuedanabaena* (fine filaments without sheaths). The genus *Salileptolyngbya* was first described in 2018 with the type species *S. diazotrophicum*, isolated from a planktonic sample at a depth of 200 m (Zhou et al. 2018). It forms fine mats at the bottom of culture flasks and is capable of nitrogen fixation. A second species, *S. insularis*, was recently described from marine benthic habitats in Brazil (Loureiro de Araújo et al. 2023). Additional seventeen *Salileptolyngbya* strains without species-level classification have been cultured and examined in Morais et al. (2025), suggesting that the genus harbors unrecognized species diversity. Strains CY006 and CY007 likely represent undescribed species of *Salileptolyngbya*, based on their phylogenetic placement and morphological traits. They are morphologically similar to *Salileptolyngbya diazotrophicum* and *Salileptolyngbya insularis*, showing comparable filament dimensions and constricted cell walls. However, unlike *S. diazotrophicum*, which has firm multilayered sheaths, CY006 and CY007

lack sheaths, similar to *S. insularis*. The strains also differ in pigmentation: CY006 appears pink, CY007 brown, compared to the blue-green of *S. diazotrophicum* and moss-green of *S. insularis*. Ecologically, CY006 and CY007 were isolated from turtle carapace biofilms, more similar to the benthic origin of *S. insularis* than the planktonic origin of *S. diazotrophicum*. Notably, two red-pigmented environmental strains of *Salileptolyngbya* from the Japanese benthos were found to produce bioactive compounds, kinenzoline (Kurisawa et al. 2021) and bromoiesol sulfates (Ebihara et al. 2021), with proven inhibitory effects against *Trypanosoma brucei rhodesiense*, the causative agent of African sleeping sickness. In this study, *Salileptolyngbya* was frequently detected in loggerhead turtle carapace biofilms across all life stages, with the highest abundances observed in sub-adults. It was not found in high abundance in sample from rocky coast or rehabilitation pools. ASVs closely clustering with CY006 were exclusive to turtle samples, while only one of five ASVs clustering with CY007 was also present in a rocky substrate reference sample. This suggests that *Salileptolyngbya* colonizes turtles from surrounding benthic habitats and that some members of the genus may be constant, possibly commensal, residents of the turtle carapace. In this host-associated biofilm, they likely contribute as primary producers, nitrogen fixers, and potential bioactive compound producers that may deter parasitic protozoa.

The family Cymatolegaceae, within the order Nodosilineales, exhibits considerable morphological variability, including both filamentous and unicellular (pseudofilamentous) cyanobacteria. Filamentous forms are characterized by relatively thin, elongated, or isodiametric cells measuring 0.9–2.2 μm in width and 0.8–3.0 μm in length. Genera such as *Cymatolege*, *Rhodoploca*, and *Leptothoe* were all recently isolated from marine sponges (Caroppo et al. 2012, Konstantinou et al. 2019, 2021). A common feature among them is a pinkish trichome color. They thrive in marine or brackish environments and may either lack chromatic adaptation (Rippka et al. 1979) or utilize far-red light for photosynthesis (Strunecký et al. 2023). *Leptothoe* is a recently established genus from marine sponges in the Aegean Sea and encompasses *Leptolyngbya*-like cyanobacteria. Currently, three species are recognized: *L. kymatousa*, *L. spongobia*, and *L. sithoniana*. While all three species exhibit similar morphological traits, they differ slightly in filament and cell shapes. *L. sithoniana* features the most isodiametric cells, while the others are more elongated. *L. kymatousa* is distinguished by its curvy, wavy filaments that form irregular entangled clusters, whereas the other species tend to have straighter trichomes. The fine, curvy, brown filaments of strains CY009 and CY047–CY052 match the morphological description of *L. kymatousa* most closely. However, many

Leptothoe and *Leptolyngbya* sequences of unknown species designation show high similarity to these strains in phylogenetic analyses. Additionally, Morais et al. (2025) report five newly cultured *Leptothoe* strains without species-level classification. These findings highlight the potential for CY009 and CY047–CY052 to represent novel, undescribed *Leptothoe* species. The genus *Leptothoe* is notable for its bioactive potential. It has been shown to produce anticancer compounds (Ferreira et al. 2022), antimicrobial substances effective against *Staphylococcus aureus* (Konstantinou et al. 2020), and compounds useful in the cosmetic industry for preserving dermal matrix components (Morone et al. 2022). Other studies report its ability to detoxify copper and exhibit cytotoxicity against cancer cell lines (Avalon et al. 2024). In sea turtle-associated biofilms, *Leptothoe* likely plays multiple ecological roles beyond primary production and nitrogen fixation. Given its ability to produce bioactive metabolites and its strong biofouling potential (Romeu et al. 2023), *Leptothoe* may significantly influence the structure and composition of the surrounding microbial community. Its frequent presence on sea turtles suggests it is a core cyanobacterial genus and a likely commensal partner of these marine megafauna.

6. CONCLUSION

This research presents the first comprehensive characterization of epibiotic cyanobacterial communities associated with loggerhead sea turtles, combining both 16S rRNA metabarcoding and cultivation-based approaches. All main aims were accomplished and the hypotheses confirmed: (i) sea turtle age and size were identified as key factors driving the cyanobacterial diversity with location statistically significant but a secondary factor in influencing cyanobacterial community; (ii) universal bacterial V4 primers did not provide adequate number of sequences for detailed cyanobacterial analysis from the microbial biofilm of sea turtle carapaces, while specific cyanobacterial primers that amplify the V6 region of 16S rDNA solved that problem and enabled full cyanobacterial diversity analysis; (iii) phylogenetic analysis of cultured strains belonging to genera *Salileptolyngbya* and *Leptothoe* show potential to be described as new species and this confirms the final hypothesis that the carapace habitat of sea turtles represents a source of new cyanobacterial taxa for science

The main findings of the thesis can be synthesized as follows:

- This study provides initial evidence that loggerhead turtles harbor core cyanobacterial taxa as part of their epibiotic microbial community. While not dominant, cyanobacteria represent a consistent and ecologically relevant component of the microbial community on the carapace.
- Filamentous, non-heterocytous cyanobacteria were the most frequently observed morphotype, as confirmed by both metabarcoding and microscopy. This aligns with previous reports of these cyanobacteria in benthic biofilms and microbial mats, which provide structural support for diverse microbial assemblages.
- The choice of reference database was shown to critically affect cyanobacterial taxonomic resolution. The CyanoSeq database outperformed the more general reference database such as SILVA, allowing for more accurate diversity estimates and improved classification.
- Members of the order Nodosilineales, particularly the family Cymatolegaceae and the genus *Rhodoploca*, were dominant across majority of samples. Other notable groups included Prochlorotrichales, which were more prevalent in juveniles, and Chroococcales, which were more abundant in sub-adult and adult turtles.
- Turtle age, inferred from body size, was identified as the primary factor shaping cyanobacterial diversity. It was the only factor significantly correlated with both alpha and beta diversity, with larger (and older) turtles hosting more diverse communities.

- Additional influencing factors included turtle condition, sampling longitude, and the rehabilitation center of origin. Seasonality, however, did not significantly affect cyanobacterial composition.
- Cultivation efforts revealed a diverse range of cyanobacteria, including potentially commensal genera such as *Salileptolyngbya* and *Leptothoe*, as well as opportunistic taxa like *Okeania* and *Spirulina*. Morphological and phylogenetic analyses suggest that several of these strains may represent yet uncultured and undescribed, novel taxa.
- Phylogenetic attribution of cyanobacterial strains with genera that are known for producing bioactive compounds with antiparasitic or anticancer properties underlines both their ecological significance in host-associated microbiomes and their biotechnological potential.

This research contributes to turtle conservation by establishing baseline data on the epibiotic cyanobacterial communities associated with sea turtles. Monitoring microbial diversity, especially in relation to turtle age, health status, and rehabilitation history, may offer a non-invasive indicator of host well-being and environmental exposure. Incorporating cyanotoxin screening can also be proposed as part of diagnostic protocols in turtles with no clear cause of illness. Incorporating microbial data into rehabilitation and rewilding programs has the potential to enhance long-term health outcomes for sea turtles.

Future research should explore the functional roles of dominant cyanobacteria in the turtle epibiotic microbial community, particularly regarding host interactions, biofilm dynamics, and nutrient cycling. Following 16S metabarcoding, the next step is to implement multi-omics approaches (such as metagenomics, metatranscriptomics, and metabolomics) to achieve higher taxonomic resolution and to uncover the genetic and metabolic functions of these microbial communities. This would help clarify the specific ecological roles played by cyanobacteria in turtle-associated biofilms. Finally, further cultivation, genome sequencing, and chemical screening of novel cyanobacterial strains may lead to the discovery of new bioactive compounds with ecological and pharmacological relevance.

7. REFERENCES

Abdelrhman, K.F.A., Bacci, G., Mancusi, C., Mengoni, A., Serena, F. & Ugolini, A. 2016. A First Insight into the Gut Microbiota of the Sea Turtle Caretta caretta. *Frontiers in Microbiology*. 0:1060.

Adams, D.G., Duggan, P.S. & Jackson, O. 2012. Cyanobacterial Symbioses. *In* Whitton, B. A. [Ed.] *Ecology of Cyanobacteria II: Their Diversity in Space and Time*. Springer Netherlands, Dordrecht, pp. 593–647.

Ahern, K.S., Ahern, C.R. & Udy, J.W. 2007. Nutrient additions generate prolific growth of *Lyngbya majuscula* (cyanobacteria) in field and bioassay experiments. *Harmful Algae*. 6:134–51.

Alduina, R., Gambino, D., Presentato, A., Gentile, A., Sucato, A., Savoca, D., Filippello, S. et al. 2020. Is *Caretta caretta* a Carrier of Antibiotic Resistance in the Mediterranean Sea? *Antibiotics*. 9:116.

Alexis Dinno 2014. dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums.

Anagnostidis, K. & Komárek, J. 1985. Modern approach to the classification system of cyanophytes. 1 - Introduction. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 291–302.

Andersen, R.A. 2005. Algal Culturing Techniques - 1st Edition. Elsevier Academic Press. 592 pp.

Antonaru, L.A., Cardona, T., Larkum, A.W.D. & Nürnberg, D.J. 2020. Global distribution of a chlorophyll f cyanobacterial marker. *ISME J*. 14:2275–87.

Apprill, A., Mcnally, S., Parsons, R. & Weber, L. 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*. 75:129–37.

Arbizu, P.M. 2017. pairwiseAdonis: Pairwise Multilevel Comparison using Adonis.

Arthur, K., Limpus, C., Balazs, G., Capper, A., Udy, J., Shaw, G., Keuper-Bennett, U. et al. 2008. The exposure of green turtles (Chelonia mydas) to tumour promoting compounds produced by the cyanobacterium Lyngbya majuscula and their potential role in the aetiology of fibropapillomatosis. *Harmful Algae*. 7:114–25.

Ashworth, M., Majewska, R., Frankovich, T.A., Sullivan, M., Bosak, S., Filek, K., de Vijver, B.V. et al. 2021. Culturing Putatively Obligate Epizoic Diatoms: Insights for the Evolution and Ecology of Diatoms and Their Host. *Research Square*.

Ashworth, M.P., Majewska, R., Frankovich, T.A., Sullivan, M., Bosak, S., Filek, K., Van de Vijver, B. et al. 2022. Cultivating epizoic diatoms provides insights into the evolution and ecology of both epibionts and hosts. *Scientific Reports*. 12:15116.

Avalon, N.E., Reis, M.A., Thornburg, C.C., Williamson, R.T., Petras, D., Aron, A.T., Neuhaus, G.F. et al. 2024. Leptochelins A–C, Cytotoxic Metallophores Produced by Geographically Dispersed *Leptothoe* Strains of Marine Cyanobacteria. *Journal of the American Chemical Society*. 146:18626–38.

Babić, I., Petrić, I., Bosak, S., Mihanović, H., Dupčić Radić, I. & Ljubešić, Z. 2017. Distribution and diversity of marine picocyanobacteria community: Targeting of Prochlorococcus ecotypes in winter conditions (southern Adriatic Sea). *Marine Genomics*. 36:3–11.

Bachmann, V.M.B.M., Hochscheid, S., Affuso, A., Pace, A., Lalzar, M., Levy, Y., Kan, G. et al. 2024. Characterization of Respiratory, Skin, and Cloaca Microbiota in Mediterranean Loggerhead and Green Sea Turtle Populations. Research Square.

Baez, J.C., Camiñas, J.A., Valeiras, J., Conde, F. & Flores-Moya, A. 2001. First record of the epizoic red seaweed *Polysiphonia carettia* Hollenberg in the Mediterranean Sea. *Acta Botanica Malacitana*. 26:197–201.

Baldi, G., Miglianti, M., Salvemini, P. & Casale, P. 2023. Diet of loggerhead turtles in the Gulf of Manfredonia, South Adriatic Sea: evidence of winter feeding and anthropogenic impacts. *Marine Biology*. 170:169.

Battelli, C. & Rindi, F. 2016. First report of the epizoic red alga *Polysiphonia carettia* (Hollenberg, 1971) on the loggerhead turtle Caretta caretta in the Adriatic Sea. *Acta Adriatica*. 57:173–8.

Bentivegna, F., Rasotto, M.B., De Lucia, G.A., Secci, E., Massaro, G., Panzera, S., Caputo, C. et al. 2010. Loggerhead Turtle (Caretta caretta) Nests at High Latitudes in Italy: A Call for Vigilance in the Western Mediterranean. *Chelonian Conservation and Biology*. 9:283–9.

Biagi, E., D'Amico, F., Soverini, M., Angelini, V., Barone, M., Turroni, S., Rampelli, S. et al. 2019. Faecal bacterial communities from Mediterranean loggerhead sea turtles (Caretta caretta). *Environmental microbiology reports*. 11:361–71.

Billi, D. & Potts, M. 2002. Life and death of dried prokaryotes. *Research in Microbiology*. 153:7–12.

Bisanz, J.E. 2018. qiime2R: Importing QIIME2 artifacts and associated data into R sessions. v0.99.
Bjorndal, K.A. 1997. Foraging ecology and nutrition of sea turtles. *In The Biology of Sea Turtles, Volume I*. pp. 199–231.
Bjorndal, K.A. & Jackson, J. (Eds.) 2002. Roles of Sea Turtles in Marine Ecosystems: Reconstructing the Past. *In The Biology of Sea Turtles, Volume II*. CRC Press.
Blasi, M.F., Rotini, A., Bacci, T., Targusi, M., Ferraro, G.B., Vecchioni, L., Alduina, R. et al. 2022. On *Caretta caretta*'s shell: first spatial analysis of micro- and macro-epibionts on the Mediterranean loggerhead sea turtle carapace. *Marine Biology Research*. 1–13.
Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A. et al. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome 2018 6:1*. 6:1–17.
Bolten, A.B. (Ed.) 2002. Variation in Sea Turtle Life History Patterns: Neritic vs. Oceanic Developmental Stages. *In The Biology of Sea Turtles, Volume II*. 0 ed. CRC Press, pp. 295–310.
Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H. et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology 2019 37:8*. 37:852–7.
Bowen, B., Avise, J.C., Richardson, J.I., Meylan, A.B., Margaritoulis, D. & Hopkins-Murphy, S.R. 1993. Population Structure of Loggerhead Turtles (*Caretta caretta*) in the Northwestern Atlantic Ocean and Mediterranean Sea. *Conservation Biology*. 7:834–44.
Bowen, B.W. & Karl, S.A. 2007. Population genetics and phylogeography of sea turtles. *Molecular Ecology*. 16:4886–907.
Brandes, M., Albach, D.C., Vogt, J.C., Mayland-Quellhorst, E., Mendieta-Leiva, G., Golubic, S. & Palinska, K.A. 2015. Supratidal Extremophiles—Cyanobacterial Diversity in the Rock Pools of the Croatian Adria. *Microbial Ecology*. 70:876–88.
Brito, Â., Ramos, V., Mota, R., Lima, S., Santos, A., Vieira, J., Vieira, C.P. et al. 2017. Description of new genera and species of marine cyanobacteria from the Portuguese Atlantic coast. *Molecular Phylogenetics and Evolution*. 111:18–34.
Brocke, H.J., Piltz, B., Herz, N., Abed, R.M.M., Palińska, K., John, U., den Haan, J. et al. 2018. Nitrogen fixation and diversity of benthic cyanobacterial mats on coral reefs in Curaçao. *Coral Reefs*. 37.
Brown, A., Foss, A., Miller, M.A. & Gibson, Q. 2018. Detection of cyanotoxins (microcystins/nodularins) in livers from estuarine and coastal bottlenose dolphins (Tursiops truncatus) from Northeast Florida. *Harmful Algae*. 76:22–34.
Buerger, P., Alvarez-Roa, C., Weynberg, K.D., Baekelandt, S. & Oppen, M.J.H. van 2016. Genetic, morphological and growth characterisation of a new Roseofilum strain (Oscillatoriales, Cyanobacteria) associated with coral black band disease. *PeerJ*. 4:e2110.
Caine, E.A. 1986. Carapace epibionts of nesting loggerhead sea turtles: Atlantic coast of U.S.A. *Journal of Experimental Marine Biology and Ecology*. 95:15–26.
Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J. & Holmes, S.P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*. 13:581–3.
Cantonati, M., Komárek, J., Hernández-Mariné, M. & Angeli, N. 2014. New and poorly known coccoid species (Cyanoprokaryota) from the mid-depth and deep epilithon of a carbonate mountain lake. *Freshwater Science*.
Capone, D.G., Zehr, J.P., Paerl, H.W., Bergman, B. & Carpenter, E.J. 1997. Trichodesmium, a Globally Significant Marine Cyanobacterium. *Science*. 276:1221–9.
Capper, A., Erickson, A.A., Ritson-Williams, R., Becerro, M.A., Arthur, K.A. & Paul, V.J. 2016. Palatability and chemical defences of benthic cyanobacteria to a suite of herbivores. *Journal of Experimental Marine Biology and Ecology*. 474:100–8.
Cardoso, D.C., Sandionigi, A., Cretoiu, M.S., Casiraghi, M., Stal, L. & Bolhuis, H. 2017. Comparison of the active and resident community of a coastal microbial mat. *Sci Rep*. 7:2969.
Caroppo, C., Albertano, P., Bruno, L., Montinari, M., Rizzi, M., Vigliotta, G. & Pagliara, P. 2012. Identification and characterization of a new Halomicronema species (Cyanobacteria) isolated from the Mediterranean marine sponge Petrosia ficiformis (Porifera). *Fottea*. 12:315–26.
Casale, P. 2010. Sea turtles in the Mediterranean : Distribution, threats and conservation priorities. IUCN. 304 pp.

Casale, P., Abbate, G., Freggi, D., Conte, N., Oliverio, M. & Argano, R. 2008. Foraging ecology of loggerhead sea turtles Caretta caretta in the central Mediterranean Sea: evidence for a relaxed life history model. *Marine Ecology Progress Series*. 372:265–76.

Casale, P., Broderick, A.C., Camiñas, J.A., Cardona, L., Carreras, C., Demetropoulos, A., Fuller, W.J. et al. 2018. Mediterranean sea turtles: Current knowledge and priorities for conservation and research. *Endangered Species Research*. 36:229–67.

Castenholz, R.W., Wilmotte, A., Herdman, M., Rippka, R., Waterbury, J.B., Iteman, I. & Hoffmann, L. 2001. Phylum BX. Cyanobacteria. *In* Boone, D. R., Castenholz, R. W. & Garrity, G. M. [Eds.] *Bergey's Manual of Systematic Bacteriology: Volume One : The Archaea and the Deeply Branching and Phototrophic Bacteria*. Springer, New York, NY, pp. 473–599.

Cebrian, E., Uriz, M.J., Garrabou, J. & Ballesteros, E. 2011. Sponge Mass Mortalities in a Warming Mediterranean Sea: Are Cyanobacteria-Harboring Species Worse Off? *PLOS ONE*. 6:e20211.

Cepák, V. 1993. Morphology of DNA-Containing Structures (nucleoids) as a Prospective Character in Cyanophyte Taxonomy. *Journal of Phycology*. 29:844–52.

Charpy, L., Palinska, K.A., Casareto, B., Langlade, M.J., Suzuki, Y., Abed, R.M.M. & Golubic, S. 2010. Dinitrogen-Fixing Cyanobacteria in Microbial Mats of Two Shallow Coral Reef Ecosystems. *Microb Ecol*. 59:174–86.

Cheang, C.C., Tsang, L.M., Chu, K.H., Cheng, I.-J. & Chan, B.K.K. 2013. Host-Specific Phenotypic Plasticity of the Turtle Barnacle *Chelonibia testudinaria*: A Widespread Generalist Rather than a Specialist. *PLoS One*. 8:e57592.

Cognetti, G., Lardicci, C., Abbiati, M. & Castelli, A. 2000. The Adriatic Sea and the Tyrrhenian Sea. *Seas at the millennium - an environmental evaluation - Volume 1*. 267–84.

Collareta, A., Rasser, M.W., Frey, E. & Harzhauser, M. 2023. Turtle barnacles have been turtle riders for more than 30 million years. *PalZ*. 97:353–63.

Dang, H. & Lovell, C.R. 2016. Microbial Surface Colonization and Biofilm Development in Marine Environments. *Microbiology and molecular biology reviews: MMBR*. 80:91–138.

Darwin, C. 1851. A monograph on the sub-class Cirripedia, with figures of all the species. Ray Society, London, United Kingdom. 400 pp.

Darwin, C. 1854. A monograph on the sub-class Cirripedia, with figures of all the species. The Balanidæ, (or sessile cirripedes); the Verrucidæ, etc. etc. etc. London: The Ray Society. Volume 2. Ray Society, London, United Kingdom. 684 pp.

Davis, D.A., Mondo, K., Stern, E., Annor, A.K., Murch, S.J., Coyne, T.M., Brand, L.E. et al. 2019. Cyanobacterial neurotoxin BMAA and brain pathology in stranded dolphins. *PLOS ONE*. 14:e0213346.

Dodds, W.K., Gudder, D.A. & Mollenhauer, D. 1995. The Ecology of Nostoc. *Journal of Phycology*. 31:2–18.

Drobac, D., Tokodi, N., Lujić, J., Marinović, Z., Subakov-Simić, G., Dulić, T., Važić, T. et al. 2016. Cyanobacteria and cyanotoxins in fishponds and their effects on fish tissue. *Harmful Algae*. 55:66–76.

Duperron, S., Halary, S., Habiballah, M., Gallet, A., Huet, H., Duval, C., Bernard, C. et al. 2019. Response of Fish Gut Microbiota to Toxin-Containing Cyanobacterial Extracts: A Microcosm Study on the Medaka (Oryzias latipes). *Environmental Science & Technology Letters*. 6:341–7.

Ebihara, A., Iwasaki, A., Miura, Y., Jeelani, G., Nozaki, T. & Suenaga, K. 2021. Isolation and Total Synthesis of Bromoiesol sulfates, Antitrypanosomal arylethers from a Salileptolyngbya sp. Marine Cyanobacterium. *The Journal of Organic Chemistry*. 86:11763–70.

Eckert, K.L. & Eckert, S.A. 1988. Pre-Reproductive Movements of Leatherback Sea Turtles (*Dermochelys coriacea*) Nesting in the Caribbean. *Copeia*. 1988:400–6.

Encalada, S.E., Bjorndal, K.A., Bolten, A.B., Zurita, J.C., Schroeder, B., Possardt, E., Sears, C.J. et al. 1998. Population structure of loggerhead turtle (*Caretta caretta*) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA control region sequences. *Marine Biology*. 130:567–75.

Engene, N., Cameron Coates, R. & Gerwick, W.H. 2010. 16S rRNA gene heterogeneity in the filamentous marine cyanobacterial genus *Lyngbya*. *Journal of Phycology*. 46:591–601.

Engene, N., Paul, V.J., Byrum, T., Gerwick, W.H., Thor, A. & Ellisman, M.H. 2013. Five chemically rich species of tropical marine cyanobacteria of the genus Okeania gen. nov. (Oscillatoriales, Cyanoprokaryota). *Journal of Phycology*. 49:1095–106.

Engene, N., Rottacker, E.C., Kaštovský, J., Byrum, T., Choi, H., Ellisman, M.H., Komárek, J. et al. 2012. *Moorea producens* gen. nov., sp. nov. and *Moorea bouillonii* comb. nov., tropical marine cyanobacteria rich in bioactive secondary metabolites. *International Journal of Systematic and Evolutionary Microbiology*. 62:1171–8.

Fenchel, T. & Kühl, M. 2000. Artificial Cyanobacterial Mats: Growth, Structure, and Vertical Zonation Patterns. *Microbial Ecology*. 40:85–93.

Ferreira, L., Sousa, M.L., Ribeiro, T., Morais, J., Forero, A., Castelo-Branco, R., Leão, P.N. et al. 2022. Uncovering the Cyanobacterial Chemical Diversity: The Search for Novel Anticancer Compounds. *Biology and Life Sciences Forum*. 14:49.

Filek, K., Trotta, A., Gračan, R., Di Bello, A., Corrente, M. & Bosak, S. 2021. Characterization of oral and cloacal microbial communities of wild and rehabilitated loggerhead sea turtles (Caretta caretta). *Animal Microbiome*. 3:59.

Flombaum, P., Gallegos, J.L., Gordillo, R.A., Rincón, J., Zabala, L.L., Jiao, N., Karl, D.M. et al. 2013. Present and future global distributions of the marine Cyanobacteria Prochlorococcus and Synechococcus. *Proceedings of the National Academy of Sciences of the United States of America*. 110:9824–9.

Fountain-Jones, N.M., Giraud, T., Zinger, L., Bik, H., Creer, S. & Videvall, E. 2024. Molecular ecology of microbiomes in the wild: Common pitfalls, methodological advances and future directions. *Molecular Ecology*. 33:e17223.

Fox, J., Weisberg, S. & Price, B. 2001. car: Companion to Applied Regression.

Frick, M. & Pfaller, J.B. 2013. Sea Turtle Epibiosis. *In* Wyneken, J., Lohmann, K. J. & Musick, J. A. [Eds.] *The Biology of Sea Turtles, Volume III*. CRC Press, Boca Raton, pp. 304–47.

Gan, F., Zhang, S., Rockwell, N.C., Martin, S.S., Lagarias, J.C. & Bryant, D.A. 2014. Extensive remodeling of a cyanobacterial photosynthetic apparatus in far-red light. *Science*. 345:1312–7.

Garcia-Pichel, F. & Castenholz, R.W. 1991. Characterization and Biological Implications of Scytonemin, a Cyanobacterial Sheath Pigment1. *Journal of Phycology*. 27:395–409.

Garcia-Pichel, F., Zehr, J.P., Bhattacharya, D. & Pakrasi, H.B. 2020. What's in a name? The case of cyanobacteria. *Journal of Phycology*. 56:1–5.

Geitler, L. 1932. Cyanophyceae. *In Kryptogamen-Flora von Deutschland, Österreich Und Der Schweiz*. 2nd ed. Akademische Verlagsgesellschaft, Leipzig, pp. 673–1196.

Geitler, L. 1942. Schizophyta: Klasse Schizophyceae. Ducker & Hulmblot, Berlin. 232 pp.

George, R.H. 1996. Health Problems and Diseases of Sea Turtles. *In The Biology of Sea Turtles, Volume I*. CRC Press.

Gisriel, C.J., Bryant, D.A., Brudvig, G.W. & Cardona, T. 2023. Molecular diversity and evolution of far-red light-acclimated photosystem I. *Frontiers in Plant Science*. 14.

Greenblatt, R.J., Work, T.M., Balazs, G.H., Sutton, C.A., Casey, R.N. & Casey, J.W. 2004. The Ozobranchus leech is a candidate mechanical vector for the fibropapilloma-associated turtle herpesvirus found latently infecting skin tumors on Hawaiian green turtles (Chelonia mydas). *Virology*. 321:101–10.

Grzegorczyk, M., Pogorzelski, S.J., Pospiech, A. & Boniewicz-Szmyt, K. 2018. Monitoring of Marine Biofilm Formation Dynamics at Submerged Solid Surfaces With Multitechnique Sensors. *Frontiers in Marine Science*. 5.

Haande, S., Jasser, I., Gugger, M., Hagman, C., Wilmotte, A. & Ballot, A. 2017. Isolation, Purification, and Cultivation of Toxigenic Cyanobacteria. *In Molecular Tools for the Detection and Quantification of Toxigenic Cyanobacteria*. John Wiley and sons LTD, pp. 43–78.

Hammer, T.J., Sanders, J.G. & Fierer, N. 2019. Not all animals need a microbiome. *FEMS Microbiology Letters*. 366:fnz117.

Hauer, T. & Komárek, J. 2022. CyanoDB 2.0 - On-line database of cyanobacterial genera. Univ. of South Bohemia & Inst. of Botany AS CR. Available At: http://www.cyanodb.cz/ (last accessed February 20, 2025).

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q. & Vinh, L.S. 2018. UFBoot2: Improving the Ultrafast Bootstrap Approximation. *Molecular Biology and Evolution*. 35:518–22.

Hochscheid, S., Bentivegna, F. & Hays, G.C. 2005. First records of dive durations for a hibernating sea turtle. *Biology Letters*. 1:82–6.
Hoffmann, L., Komárek, J. & Kaštovský, J. 2005. System of cyanoprokaryotes (cyanobacteria) state in 2004. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 95–115.
Hollenberg, G.J. 1971. Phycological notes. V. New species of marine algae from California. *Phycologia*. 10:11–6.
Horikoshi, M., Tang, Y., Dickey, A., Grenié, M., Thompson, R., Selzer, L., Strbenac, D. et al. 2016. ggfortify: Data Visualization Tools for Statistical Analysis Results.
Ismael, A.A. 2012. Benthic bloom of cyanobacteria associated with fish mortality in Alexandria waters. *The Egyptian Journal of Aquatic Research*. 38:241–7.
Iwasa-Arai, T., Andrade, S., Miguel, C., Siqueira, S., Werneck, M., Leite, F., Moraes, L. et al. 2023. Coevolutionary Implications of Obligate Commensalism in Sea Turtles: the Case of the Genus *Hyachelia* Barnard, 1967 (Crustacea, Amphipoda). *Zoological Studies*. 62:1–12.
Jackman, S. 2005. pscl: Political Science Computational Laboratory.
Jaegge, A.C., Raabe, J.M., Phillips, Z.B., Bernard, T.L. & Stauffer, B.A. 2023. Flood-driven increases in phytoplankton biomass and cyanobacteria abundance in the western Atchafalaya-Vermilion Bay System, Louisiana. *Hydrobiologia*. 850:4413–41.
Kanjer, L., Filek, K., Mucko, M., Majewska, R., Gračan, R., Trotta, A., Panagopoulou, A. et al. 2022. Surface microbiota of Mediterranean loggerhead sea turtles unraveled by 16S and 18S amplicon sequencing. *Frontiers in Ecology and Evolution*. 10.
Kanjer, L., Filek, K., Mucko, M., Zakan Lupić, M., Frleta-Valić, M., Gračan, R. & Bosak, S. 2024. Growing older, growing more diverse: Sea turtles and epibiotic cyanobacteria. *Journal of Phycology*. 60:1390–405.
Kaplan-Levy, R., Hadas, O., Summers, M., Rücker, J. & Sukenik, A. 2010. Akinetes: Dormant Cells of Cyanobacteria. *In Dormancy and Resistance in Harsh Environments, Topics in Current Genetics*. pp. 5–27.
Kassambara, A. 2023. ggpubr: "ggplot2" Based Publication Ready Plots. 0.6.0.
Kaštovský, J., Johansen, J.R., Hauerová, R. & Akagha, M.U. 2023. Hot Is Rich—An Enormous Diversity of Simple Trichal Cyanobacteria from Yellowstone Hot Springs. *Diversity*. 15:975.
Kaup, M., Trull, S. & Hom, E.F.Y. 2021. On the move: sloths and their epibionts as model mobile ecosystems. *Biological Reviews*. 96:2638–60.
Keenan, S.W., Engel, A.S. & Elsey, R.M. 2013. The alligator gut microbiome and implications for archosaur symbioses. *Scientific Reports*. 3:2877.
Kembel, S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., Blomberg, S.P. et al. 2010. Picante: R tools for integrating phylogenies and ecology. *Bioinformatics*. 26:1463–4.
Kleiber, C. & Zeileis, A. 2008. AER: Applied Econometrics with R.
Kolda, A. 2018. Metode istraživanja cijanobakterija u vodenim ekosustavima. *Hrvatske vode*. 26:271–80.
Kolda, A. 2020. Ecology and diversity of Cyanobacteria in changing ecosystems in the Eastern Adriatic area; Doctoral Thesis; University of Zagreb, Faculty of Scinece, Department of Geology. Doctoral Thesis, University of Zagreb, Zagreb, 141 pp.
Kolda, A., Ljubešić, Z., Gavrilović, A., Jug-Dujaković, J., Pikelj, K. & Kapetanović, D. 2020. Metabarcoding Cyanobacteria in coastal waters and sediment in central and southern Adriatic Sea. *Acta Botanica Croatica*. 79:157–69.
Komárek, J. 2016. A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. *European Journal of Phycology*. 51:346–53.
Komárek, J. & Anagnostidis, K. 2005. Süßwasserflora von Mitteleuropa, Bd 19/2: Cyanoprokaryota, Part 2: Oscillatoriales [English].
Komárek, J. & Johansen, J.R. 2015a. Coccoid Cyanobacteria. *In* Wehr, J. D., Sheath, R. G. & Kociolek, J. P. [Eds.] *Freshwater Algae of North America (Second Edition)*. Academic Press, Boston, pp. 75–133.
Komárek, J. & Johansen, J.R. 2015b. Filamentous Cyanobacteria. *In* Wehr, J. D., Sheath, R. G. & Kociolek, J. P. [Eds.] *Freshwater Algae of North America (Second Edition)*. Academic Press, Boston, pp. 135–235.

Komárek, J., Kaštovský, J., Mareš, J. & Johansen, J.R. 2014. Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia*. 86:295–335.

Konstantinou, D., Mavrogonatou, E., Zervou, S.-K., Giannogonas, P. & Gkelis, S. 2020. Bioprospecting Sponge-Associated Marine Cyanobacteria to Produce Bioactive Compounds. *Toxins*. 12:73.

Konstantinou, D., Voultsiadou, E., Panteris, E. & Gkelis, S. 2021. Revealing new sponge-associated cyanobacterial diversity: Novel genera and species. *Molecular Phylogenetics and Evolution*. 155:106991.

Konstantinou, D., Voultsiadou, E., Panteris, E., Zervou, S.-K., Hiskia, A. & Gkelis, S. 2019. *Leptothoe*, a new genus of marine cyanobacteria (Synechococcales) and three new species associated with sponges from the Aegean Sea. *Journal of Phycology*. 55:882–97.

Kurisawa, N., Otomo, K., Iwasaki, A., Jeelani, G., Nozaki, T. & Suenaga, K. 2021. Isolation and Total Synthesis of Kinenzoline, an Antitrypanosomal Linear Depsipeptide Isolated from a Marine Salileptolyngbya sp. Cyanobacterium. *The Journal of Organic Chemistry*. 86:12528–36.

Kuschke, S.G., Wyneken, J. & Miller, D. 2024. Baseline Skin Microbiota of the Leatherback Sea Turtle. *Microorganisms*. 12:925.

Lahti, L. & Sudarshan, S. 2012. microbiome R package.

Lazar, B., Gračan, R., Katić, J., Zavodnik, D., Jaklin, A. & Tvrtković, N. 2011. Loggerhead sea turtles (Caretta caretta) as bioturbators in neritic habitats: an insight through the analysis of benthic molluscs in the diet. *Marine Ecology*. 32:65–74.

Lee, E., Khurana, M.S., Whiteley, A.S., Monis, P.T., Bath, A., Gordon, C., Ryan, U.M. et al. 2017. Novel Primer Sets for Next Generation Sequencing-Based Analyses of Water Quality. *PLOS ONE*. 12:e0170008.

Lefler, F.W., Berthold, D.E. & Dail Laughinghouse IV, H. 2023. CyanoSeq: a database of cyanobacterial 16S rRNA sequences with curated taxonomy. *Journal of Phycology*. n/a.

Li, N., Zhang, Yunlin, Zhang, Yibo, Shi, K., Qian, H., Yang, H., Niu, Y. et al. 2023. The unprecedented 2022 extreme summer heatwaves increased harmful cyanobacteria blooms. *The Science of the Total Environment*. 896:165312.

Loghmannia, J., Nasrolahi, A. & Dobretsov, S. 2023. Epibiotic bacteria on the carapace of hawksbill and green sea turtles. *Biofouling*. 39:385–98.

Loureiro de Araújo, V., Ferreira dos Santos ,Márcio, Selbach Schnadelbach ,Alessandra, de Castro Nunes ,José Marcos & and Aguiar Caires, T. 2023. New genera of thin homocyted cyanobacteria from Brazilian tropical and subtropical marine islands. *European Journal of Phycology*. 58:457–68.

Lüdecke, D. 2013. sjPlot: Data Visualization for Statistics in Social Science.

Lutcavage, M., Plotkin, P., Witherington, B.E. & Lutz, P.L. 1997. Human Impacts on Sea Turtle Survival.

Lutz, P.L., Musick, J.A. & Wyneken, J. (Eds.) 2002. The Biology of Sea Turtles, Volume II. CRC Press, Boca Raton. 472 pp.

Lynch-Stieglitz, J., Vollmer, T.D., Valley, S.G., Blackmon, E., Gu, S. & Marchitto, T.M. 2024. A diminished North Atlantic nutrient stream during Younger Dryas climate reversal. *Science*. 384:693–6.

Majewska, R., Ashworth, M.P., Bosak, S., Goosen, W.E., Nolte, C., Filek, K., Van de Vijver, B. et al. 2021. On Sea Turtle-associated Craspedostauros (Bacillariophyta), with Description of Three Novel Species. *Journal of Phycology*. 57:199–218.

Mareš, J. & Cantonati, M. 2016. Phylogenetic position of Geitleribactron purpureum (Synechococcales, Cyanobacteria / Cyanophyceae) and its implications for the taxonomy of Chamaesiphonaceae and Leptolyngbyaceae. *Fottea*. 16:104–11.

Margaritoulis, D. & Rees, A.F. 2011. Loggerhead Turtles Nesting at Rethymno, Greece, Prefer the Aegean Sea as Their Main Foraging Area. *Marine Turtle Newsletter*. 131:12–4.

Mariani, G., Bellucci, F., Cocumelli, C., Raso, C., Hochscheid, S., Roncari, C., Nerone, E. et al. 2023. Dietary Preferences of Loggerhead Sea Turtles (Caretta caretta) in Two Mediterranean Feeding Grounds: Does Prey Selection Change with Habitat Use throughout Their Life Cycle? *Animals*. 13:654.

Marotz, C., Cavagnero, K.J., Song, J., McDonald, D., Wandro, S., Humphrey, G., Bryant, M. et al. 2021. Evaluation of the Effect of Storage Methods on Fecal, Saliva, and Skin Microbiome Composition. *mSystems*. 6:e01329–e01320.

Mast, R.B., Pilcher, N.B., Hutchinson, B.J. & Hutchinson, A. 2006. Marine Turtle Specialist Group Hosts Third Burning Issues Workshop.

Matsui, K., Nazifi, E., Hirai, Y., Wada, N., Matsugo, S. & Sakamoto, T. 2012. The cyanobacterial UV-absorbing pigment scytonemin displays radical-scavenging activity. *The Journal of General and Applied Microbiology*. 58:137–44.

McMurdie, P.J. & Holmes, S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*. 8:e61217.

Meeks, J.C. & Elhai, J. 2002. Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiology and molecular biology reviews: MMBR*. 66:94–121; table of contents.

Mejdandžić, M., Ivanković, T., Pfannkuchen, M., Godrijan, J., Marić Pfannkuchen, D., Hrenović, J. & Ljubešić, Z. 2015. Colonization of diatoms and bacteria on artificial substrates in the northeastern coastal Adriatic Sea. *Acta Botanica Croatica*. 74:407–22.

Miller, J.D. 1997. Reproduction In Sea Turtles. *In The Biology of Sea Turtles, Volume I*. CRC Press.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A. & Lanfear, R. 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*. 37:1530–4.

Morais, J., Cruz, P., Hentschke, G.S., Silva, B., Oliveira, F., Neves, J., Silva, R. et al. 2025. Diversity of Cyanobacterial Genera Present in Cabo Verde Marine Environments and the Description of *Gibliniella gelatinosa* sp. nov. *Plants*. 14:299.

Morone, J., Lopes, G., Morais, J., Neves, J., Vasconcelos, V. & Martins, R. 2022. Cosmetic Application of Cyanobacteria Extracts with a Sustainable Vision to Skincare: Role in the Antioxidant and Antiaging Process. *Marine Drugs*. 20:761.

Mucko, M., Bosak, S., Casotti, R., Balestra, C. & Ljubešić, Z. 2018. Winter picoplankton diversity in an oligotrophic marginal sea. *Marine Genomics*. 42:14–24.

Musick, J.A. & Limpus, C.J. 1997. Habitat Utilization and Migration in Juvenile Sea Turtles. *In The Biology of Sea Turtles, Volume I*. CRC Press.

Mutalipassi, M., Riccio, G., Mazzella, V., Galasso, C., Somma, E., Chiarore, A., de Pascale, D. et al. 2021. Symbioses of Cyanobacteria in Marine Environments: Ecological Insights and Biotechnological Perspectives. *Marine Drugs*. 19:227.

Neuwirth, E. 2022. RColorBrewer: ColorBrewer Palettes. 1.1-3.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Lagendre, P., McGlinn, D., Minchin, P.R. et al. 2020. vegan community ecology package version 2.5-7.

O'Neil, J.M., Davis, T.W., Burford, M.A. & Gobler, C.J. 2012. The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae*. 14:313–34.

Ooms, J. 2017. writexl: Export Data Frames to Excel "xlsx" Format.

Oren, A., Arahal, D.R., Göker, M., Moore, E.R.B., Rossello-Mora, R. & Sutcliffe, I.C. 2023. International Code of Nomenclature of Prokaryotes. Prokaryotic Code (2022 Revision). *International Journal of Systematic and Evolutionary Microbiology*. 73:005585.

Oren, A., Arahal, D.R., Rosselló-Móra, R., Sutcliffe, I.C. & Moore, E.R.B. 2021. Emendation of General Consideration 5 and Rules 18a, 24a and 30 of the International Code of Nomenclature of Prokaryotes to resolve the status of the Cyanobacteria in the prokaryotic nomenclature. *International Journal of Systematic and Evolutionary Microbiology*. 71:004939.

Oren, A., Mareš, J. & Rippka†, R. 2022. Validation of the names Cyanobacterium and Cyanobacterium stanieri, and proposal of Cyanobacteriota phyl. nov. *International Journal of Systematic and Evolutionary Microbiology*. 72:005528.

Oshel, P. 1997. HMDS and Specimen Drying for SEM: *Microscopy Today*. 5:16.

Paerl, H.W. & Otten, T.G. 2013. Harmful cyanobacterial blooms: causes, consequences, and controls. *Microb Ecol*. 65:995–1010.

Pahor, E. 2024. Polifazna karakterizacija sojeva cijanobakterija iz bentičkih i epibiotskih staništa istočnog Jadranskog mora. Master thesis, University of Zagreb, Faculty of Science, Zagreb, 95 pp.

Palinska, K.A., Abed, R.M.M., Vogt, J.C., Radtke, G. & Golubic, S. 2017. Microbial Endoliths on East Adriatic Limestone Coast: Morphological vs. Molecular Diversity. *Geomicrobiology Journal*. 34:903–15.

Papapanagiotou, G. & and Gkelis, S. 2019. Taxonomic revision of commercially used Arthrospira (Cyanobacteria) strains: a polyphasic approach. *European Journal of Phycology*. 54:595–608.

Parada, A.E., Needham, D.M. & Fuhrman, J.A. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental microbiology*. 18:1403–14.

Partensky, F., Hess, W.R. & Vaulot, D. 1999. Prochlorococcus, a marine photosynthetic prokaryote of global significance. *Microbiology and molecular biology reviews: MMBR*. 63:106–27.

Pedersen, T.L. 2024. patchwork: The Composer of Plots. 1.3.0.

Peixoto, R.S., Voolstra, C.R., Sweet, M., Duarte, C.M., Carvalho, S., Villela, H., Lunshof, J.E. et al. 2022. Harnessing the microbiome to prevent global biodiversity loss. *Nature Microbiology*. 7:1726–35.

Perkerson III, R.B., Johansen, J.R., Kovácik, L., Brand, J., Kaštovský, J. & Casamatta, D.A. 2011. A Unique Pseudanabaenalean (cyanobacteria) Genus *Nodosilinea* Gen. Nov. Based on Morphological and Molecular Data. *Journal of Phycology*. 47:1397–412.

Pfaller, J.B., Bjorndal, K.A., Reich, K.J., Williams, K.L. & Frick, M.G. 2008. Distribution patterns of epibionts on the carapace of loggerhead turtles, *Caretta caretta*. *Mar. Biodivers. Rec.* 1:e36.

Pinou, T., Domènech, F., Lazo-Wasem, E., Majewska, R., Pfaller, J., Zardus, J. & Robinson, N. 2019. Standardizing Sea Turtle Epibiont Sampling: Outcomes of the Epibiont Workshop at the 37 th International Sea Turtle Symposium.

Piroli, V. & Haxhiu, I. 2020. Nesting of the loggerhead turtle (Caretta caretta) in the southeast Adriatic confirmed. *Natura Croatica : Periodicum Musei Historiae Naturalis Croatici*. 29:23–30.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. et al. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*. 41:D590–6.

R Core Team 2024. R: The R Project for Statistical Computing. R Foundation for Statistical Computing, Vienna. Austria.

Ramos, D.F., Matthiensen, A., Colvara, W., de Votto, A.P.S., Trindade, G.S., da Silva, P.E.A. & Yunes, J.S. 2015. Antimycobacterial and cytotoxicity activity of microcystins. *The Journal of Venomous Animals and Toxins Including Tropical Diseases*. 21:9.

Razumov, A. 1932. The direct method of calculation of bacteria in water: Comparison with the Koch method. *Mikrobiologija*. 1:131–46.

Redford, K.H., Segre, J.A., Salafsky, N., del Rio, C.M. & McAloose, D. 2012. Conservation and the Microbiome. *Conservation Biology*. 26:195–7.

Rikkinen, J. 2015. Cyanolichens. *Biodiversity and Conservation*. 24.

Ripley, B. & Venables, B. 2009. MASS: Support Functions and Datasets for Venables and Ripley's MASS.

Rippka, E., Deruelles, J. & Waterbury, N.B. 1979. Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Journal of General Microbiology*. 111:1–61.

Rivera, S.F., Vasselon, V., Ballorain, K., Carpentier, A., Wetzel, C.E., Ector, L., Bouchez, A. et al. 2018. DNA metabarcoding and microscopic analyses of sea turtles biofilms: Complementary to understand turtle behavior. *PLoS ONE*. 13:1–20.

Robinson, N.J., Majewska, R., Lazo-Wasem, E.A., Nel, R., Paladino, V., Rojas, L., Zardus, J.D. et al. 2016. Epibiotic Diatoms Are Universally Present on All Sea Turtle Species. *PLoS ONE*. 11:e0157011.

Robinson, N.J. & Pfaller, J.B. 2022. Sea Turtle Epibiosis: Global Patterns and Knowledge Gaps. *Frontiers in Ecology and Evolution*. 10.

Romeu, M.J., Morais, J., Gomes, L.C., Silva, R., Vasconcelos, V. & Mergulhão, F.J.M. 2023. Characterization and biofouling potential analysis of two cyanobacterial strains isolated from Cape Verde and Morocco. *FEMS Microbiology Ecology*. 99:fiad004.

Roush, D., Giraldo-Silva, A. & Garcia-Pichel, F. 2021. Cydrasil 3, a curated 16S rRNA gene reference package and web app for cyanobacterial phylogenetic placement. *Scientific Data*. 8:230.

Rubio, C. 2002. Compréhension des mécanismes d'adhésion des biofilms en milieu marin en vue de la conception de nouveaux moyens de prévention. Université Paris 6.
Sandri, C., Correa, F., Spiezio, C., Trevisi, P., Luise, D., Modesto, M., Remy, S. et al. 2020. Fecal Microbiota Characterization of Seychelles Giant Tortoises (Aldabrachelys gigantea) Living in Both Wild and Controlled Environments. *Frontiers in Microbiology*. 11.
Scheelings, T.F., Moore, R.J., Van, T.T.H., Klaassen, M. & Reina, R.D. 2020. The gut bacterial microbiota of sea turtles differs between geographically distinct populations. *Endangered Species Research*. 42:95–108.
Schmelling, N.M. & Bross, M. 2024. What is holding back cyanobacterial research and applications? A survey of the cyanobacterial research community. *Nature Communications*. 15:6758.
Schopf, J. 2000. The Fossil Record: Tracing the Roots of the Cyanobacterial Lineage. *The ecology of cyanobacteria*.
Sehnal, L., Brammer-Robbins, E., Wormington, A.M., Blaha, L., Bisesi, J., Larkin, I., Martyniuk, C.J. et al. 2021. Microbiome Composition and Function in Aquatic Vertebrates: Small Organisms Making Big Impacts on Aquatic Animal Health. *Frontiers in Microbiology*. 12:567408.
Shalygin, S., Huang, I.-S., Allen, E.H., Burkholder, J.M. & Zimba, P.V. 2019. Odorella benthonica gen. & sp. nov. (Pleurocapsales, Cyanobacteria): an odor and prolific toxin producer isolated from a California aqueduct. *Journal of Phycology*. 55:509–20.
Shine, R., Brischoux, F. & Pile, A.J. 2010. A seasnake's colour affects its susceptibility to algal fouling. *Proceedings of the Royal Society B*. 277:2459–64.
Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R. et al. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*. 7:539.
Simmons, T.L., Engene, N., Ureña, L.D., Romero, L.I., Ortega-Barría, E., Gerwick, L. & Gerwick, W.H. 2008. Viridamides A and B, Lipodepsipeptides with Anti-Protozoal Activity from the Marine Cyanobacterium Oscillatoria nigro-viridis. *J Nat Prod*. 71:1544–50.
Sousa-Guedes, D., Campos, J.C., Bessa, F., Mexico, F.F. y C. de, Lasala, J.A., Marco, A. & Sillero, N. 2025. The effects of warming on loggerhead turtle nesting counts. *Journal of Animal Ecology*. 94:566–81.
Spotila, J.R. 2004. Sea Turtles: A Complete Guide to Their Biology, Behavior, and Conservation. JHU Press. 254 pp.
Stal, L.J. 2012. Cyanobacterial Mats and Stromatolites. *In* Whitton, B. A. [Ed.] *Ecology of Cyanobacteria II: Their Diversity in Space and Time*. Springer Netherlands, Dordrecht, pp. 65–125.
Staley, J.T. & Konopka, A. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Review of Microbiology*. 39:321–46.
Stanier, R.Y. & Cohen-Bazire, G. 1977. Phototrophic Prokaryotes: The Cyanobacteria. *Annual Review of Microbiology*. 31:225–74.
Stanier, R.Y. & Van Niel, C.B. 1941. The Main Outlines of Bacterial Classification. *Journal of Bacteriology*. 42:437–66.
Stanier, R.Y. & van Niel, C.B. 1962. The concept of a bacterium. *Archiv. Mikrobiol.* 42:17–35.
Strunecký, O., Ivanova, A.P. & Mareš, J. 2023. An updated classification of cyanobacterial orders and families based on phylogenomic and polyphasic analysis. *Journal of Phycology*. 59:12–51.
Strunecký, O., Komárek, J., Johansen, J., Lukešová, A. & Elster, J. 2013. Molecular and morphological criteria for revision of the genus *Microcoleus* (Oscillatoriales, Cyanobacteria). *Journal of Phycology*. 49:1167–80.
Sun, S., Thompson, A.F., Yu, J. & Wu, L. 2024. Transient overturning changes cause an upper-ocean nutrient decline in a warming climate. *Nature Communications*. 15:7727.
Sutherland, J.M., Reaston, J., Stewart, W.D.P. & Herdman, M. 1985. Akinetes of the Cyanobacterium Nostoc PCC 7524: Macromolecular and Biochemical Changes during Synchronous Germination. *Microbiology*. 131:2855–63.
Taton, A., Grubisic, S., Brambilla, E., De Wit, R. & Wilmotte, A. 2003. Cyanobacterial Diversity in Natural and Artificial Microbial Mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a Morphological and Molecular Approach. *Applied and Environmental Microbiology*. 69:5157–69.

TEWG 2009. An assessment of the loggerhead turtle population in the western northern Atlantic Ocean, a report of the Turtle Expert Working Group. Technical Memorandum No. NMFS-SEFSC-575.
Trevelline, B.K., Fontaine, S.S., Hartup, B.K. & Kohl, K.D. 2019. Conservation biology needs a microbial renaissance: a call for the consideration of host-associated microbiota in wildlife management practices. *Proc Biol Sci*. 286:20182448.
Tronholm, A. & Engene, N. 2019. *Moorena* gen. nov., a valid name for "*Moorea* Engene & al." nom. inval. (Oscillatoriaceae, Cyanobacteria). *Notulae algarum*. 122.
Trotta, A., Cirilli, M., Marinaro, M., Bosak, S., Diakoudi, G., Ciccarelli, S., Paci, S. et al. 2021a. Detection of multi-drug resistance and AmpC β-lactamase/extended-spectrum β-lactamase genes in bacterial isolates of loggerhead sea turtles (*Caretta caretta*) from the Mediterranean Sea. *Marine Pollution Bulletin*. 164:112015.
Trotta, A., Marinaro, M., Sposato, A., Galgano, M., Ciccarelli, S., Paci, S. & Corrente, M. 2021b. Antimicrobial Resistance in Loggerhead Sea Turtles (*Caretta caretta*): A Comparison between Clinical and Commensal Bacterial Isolates. *Animals*. 11:2435.
Urrutia-Cordero, P., Zhang, H., Chaguaceda, F., Geng, H. & Hansson, L.-A. 2020. Climate warming and heat waves alter harmful cyanobacterial blooms along the benthic-pelagic interface. *Ecology*. 101:e03025.
Van de Vijver, B., Robert, K., Majewska, R., Frankovich, T.A., Panagopoulou, A. & Bosak, S. 2020. Geographical variation in the diatom communities associated with loggerhead sea turtles (Caretta caretta). *PLoS ONE*. 15.
Vidal, L., Ballot, A., Azevedo, S.M.F.O., Padisák, J. & Welker, M. 2021. Introduction to cyanobacteria. *In Toxic Cyanobacteria in Water*. 2nd ed. CRC Press.
Vogt, J.C., Abed, R.M.M., Albach, D.C. & Palinska, K.A. 2019. Latitudinal gradient of cyanobacterial diversity in tidal flats. *PLOS ONE*. 14:e0224444.
Vondrášková, A., Fibich, P., Lepš, J. & Kaštovský, J. 2017. Determinants of cyanobacterial species composition in the splash zone of two Croatian islands. *European Journal of Phycology*. 52:179–87.
Wahl, M. 1989. Marine epibiosis. I. Fouling and antifouling: some basic aspects. *Marine Ecology Progress Series*. 58:175–89.
Wallace, B.P., DiMatteo, A.D., Bolten, A.B., Chaloupka, M.Y., Hutchinson, B.J., Abreu-Grobois, F.A., Mortimer, J.A. et al. 2011. Global conservation priorities for marine turtles. *PLoS One*. 6:e24510.
Walls, J.T., Wyatt, K.H., Doll, J.C., Rubenstein, E.M. & Rober, A.R. 2018. Hot and toxic: Temperature regulates microcystin release from cyanobacteria. *The Science of the Total Environment*. 610–611:786–95.
Walsby, A.E. 1994. Gas vesicles. *Microbiological Reviews*. 58:94–144.
Whitton, B. & Potts, M. 2007. Ecology of Cyanobacteria II. pp. 1–11.
Whitton, B.A. 2012. The Ecology of Cyanobacteria: Their Diversity in Time and Space. Springer, Dordrecht Heidelberg NewYork London. 759 pp.
Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
Wickham, H., François, R., Henry, L., Müller, K., Vaughan, D., Software, P. & PBC 2023. dplyr: A Grammar of Data Manipulation. 1.1.4.
Wilde, S.B., Murphy, T.M., Hope, C.P., Habrun, S.K., Kempton, J., Birrenkott, A., Wiley, F. et al. 2005. Avian vacuolar myelinopathy linked to exotic aquatic plants and a novel cyanobacterial species. *Environmental Toxicology*. 20:348–53.
Wilkinson, C.R. 1980. Cyanobacteria symbiotic in marine sponges. *In* Schenk, H. E. A. & Schwemmler, W. [Eds.] *Endosymbiosis and cell biology: A synthesis of recent research. Proceedings of the International Colloquium on Endosymbiosis and Cell Research, Tübingen, April 1980*. De Gruyter, pp. 553–64.
Witherington, B.E. & Salmon, M. 1992. Predation on Loggerhead Turtle Hatchlings after Entering the Sea. *Journal of Herpetology*. 26:226–8.
Włodarska-Kowalczuk, M., Balazy, P., Kobos, J., Wiktor, J., Zajączkowski, M. & Moskal, W. 2014. Large red cyanobacterial mats (*Spirulina subsalsa* Oersted ex Gomont) in the shallow sublittoral of the southern Baltic. *Oceanologia*. 56:661–6.

Woese, C.R. & Fox, G.E. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences*. 74:5088–90.

Wood, S.A., Kelly, L.T., Bouma-Gregson, K., Humbert, J.-F., Laughinghouse IV, H.D., Lazorchak, J., McAllister, T.G. et al. 2020. Toxic benthic freshwater cyanobacterial proliferations: Challenges and solutions for enhancing knowledge and improving monitoring and mitigation. *Freshwater Biology*. 65:1824–42.

Wu, S., Mi, T., Zhen, Y., Yu, K., Wang, F. & Yu, Z. 2021. A Rise in ROS and EPS Production: New Insights into the Trichodesmium erythraeum Response to Ocean Acidification. *Journal of Phycology*. 57:172–82.

Wyneken, J., Lohmann, K.J. & Musick, J.A. 2013. The Biology of Sea Turtles. CRC Press, Taylor & Francis Group, Boca Raton, London, New York. 446 pp.

Yan, L. 2023. ggvenn: Draw Venn Diagram by "ggplot2." 0.1.10.

Zeng, X. & Zhang, C.-C. 2022. The Making of a Heterocyst in Cyanobacteria. *Annual Review of Microbiology*. 76:597–618.

Zhang, W., Liu, J., Xiao, Y., Zhang, Y., Yu, Y., Zheng, Z., Liu, Y. et al. 2022. The Impact of Cyanobacteria Blooms on the Aquatic Environment and Human Health. *Toxins*. 14:658.

Zhou, C., Chen, H., Zhao, H. & Wang, Q. 2021. Microcystin biosynthesis and toxic effects. *Algal Research*. 55:102277.

Zhou, W.-G., Ding, D.-W., Yang, Q.-S., Ahmad, M., Zhang, Y.-Z., Lin, X.-C., Zhang, Y.-Y. et al. 2018. *Marileptolyngbya sina* gen. nov., sp. nov. and *Salileptolyngbya diazotrophicum* gen. nov., sp. nov. (Synechococcales, Cyanobacteria), species of cyanobacteria isolated from a marine ecosystem. *Phytotaxa*. 383:75–92.

8. SUPPLEMENTS

Supplement 1. Health status and detailed information about sea turtles from which the cyanobacterial strains were obtained.

Sea turtle ID	Health status	Sampling person	Comment
RM1	healthy (nesting)	Roksana Majewska	Tag numbers: ZA0122C (right); ZA0123C (left)
RM2	cold-stunned, fully recovered	Roksana Majewska	Stranded on 14 Feb 2020 at Cannon Rocks, Eastern Cape
RM4	healthy	Roksana Majewska	In captivity since 4 January 2013 (shows typically sea turtle diatom flora with ca. 50% of POE taxa on the carapace and ca. 70% of POE on the skin)
TB215	Floating, problems with diving	Lucija Kanjer, Klara Filek	Male turtle, CCL = 74 cm, CCW = 71 cm, weight = 80 kg. Present stamp from Albanian Center for Sea Turtle Recovery.
TB235	Floating, problems with diving	Simona Matas	Unknown gender, CCL = 65 cm, weight= 23 kg. Spotted from a boat floating near the island of Kornati. After reporting to number 112, the employees of NP Kornati were alerted, who transported her to Murter, where she was taken over from the veterinary service in Šibenik. Upon arrival at the Center, it was placed in the intensive care unit. The examination revealed a disturbance of buoyancy, a high degree of malnutrition and the presence of a large number of leeches (*Ozobranchus* sp.).

Supplement 2. List of core ASV features found on 100% sampled adult sea turtles.

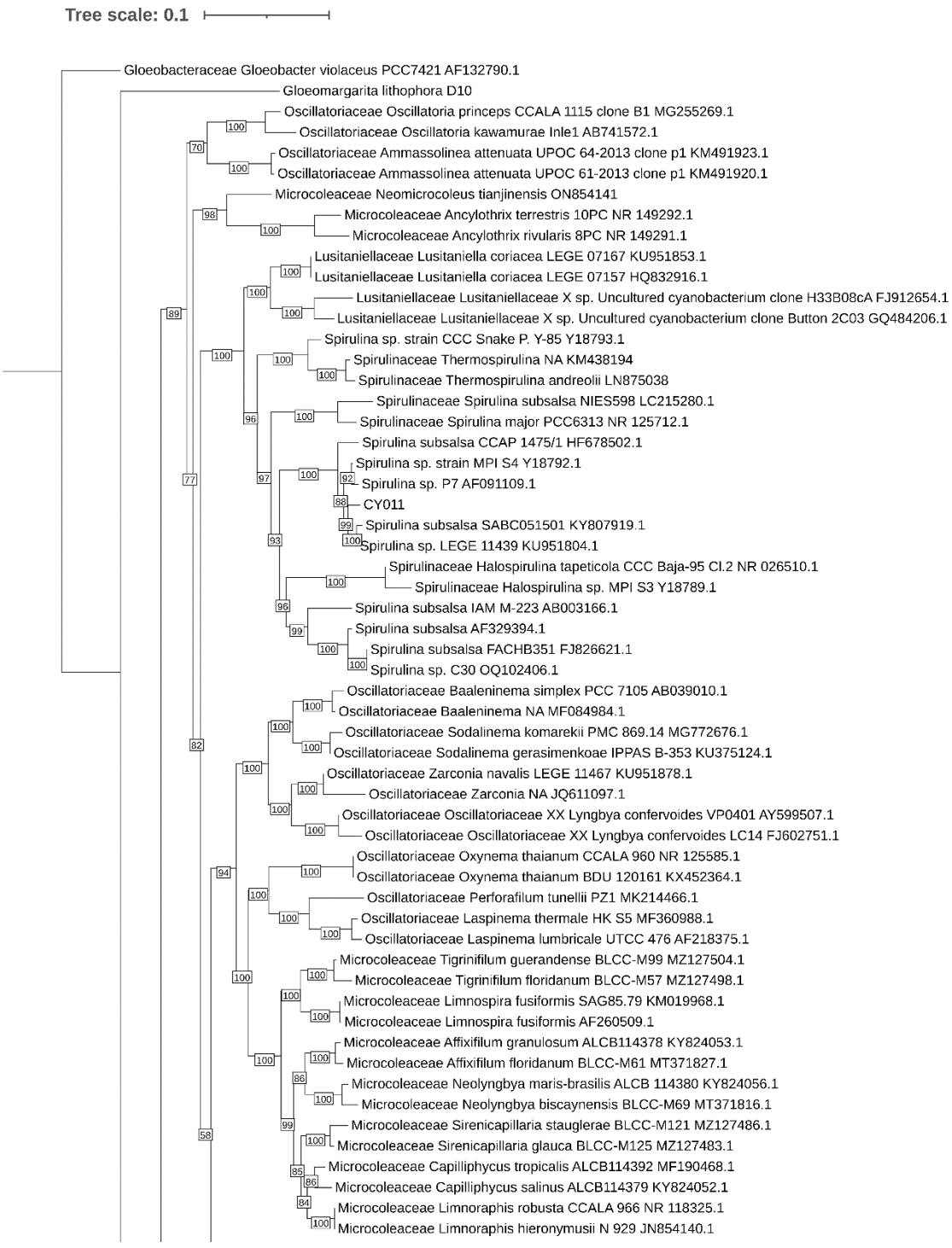
Feature ID	Taxonomy	2%	9%	25%	50%	75%	91%	98%
aff17db4bacf1a70202722947efa9597	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Cymatolegaceae; g__Rhodoploca	2471.58	2753.61	3398.25	3876.5	5210.5	7498.18	8499.04
53c43c446d6bebfe5e1fed3849851f0e	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Cymatolegaceae; g__Rhodoploca	131.68	505.06	1358.5	1876.5	2058.75	2267.55	2358.9
87657d89bb52bc67a9f526c287bfebab	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae	345.96	496.32	840	1534.5	2676.75	3880.11	4406.58
242d31fbc0813a1d46e832063be078e0	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Chroococcales; f__Geminocystaceae; g__Picosynechococcus	23.42	24.89	28.25	338	5305.5	14251.7	18165.7
feae23eace96b6a91c4092e3d6cca7c7	k__Bacteria; p__Cyanobacteriota	13.8	41.1	103.5	274.5	595.75	946.63	1100.14
063ab3be2eca0352fffa01cd7f26a2b8	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae; g__Salileptolyngbya	63.58	83.11	127.75	158.5	271.5	474.06	562.68
4ea64199b3e627481695cbbbb9d46cae	k__Bacteria; p__Cyanobacteriota; c__Vampirivibrionia; o__Caenarcaniphilales; f__; g__NA	9.26	24.17	58.25	117.5	180.25	221.05	238.9
a93c71f8ea3d5695d7e6348a1dbeba3d	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Chroococcales; f__Pleurocapsaceae; g__Odorella	17.54	19.43	23.75	60	1145.25	3163.65	4046.7
a2fbe9fa0a81488838ea66ba6f4bf6fd	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae	5.54	7.43	11.75	30	72.25	122.65	144.7

Supplement 3. List of core ASV features found on 100% sampled juvenile sea turtles.

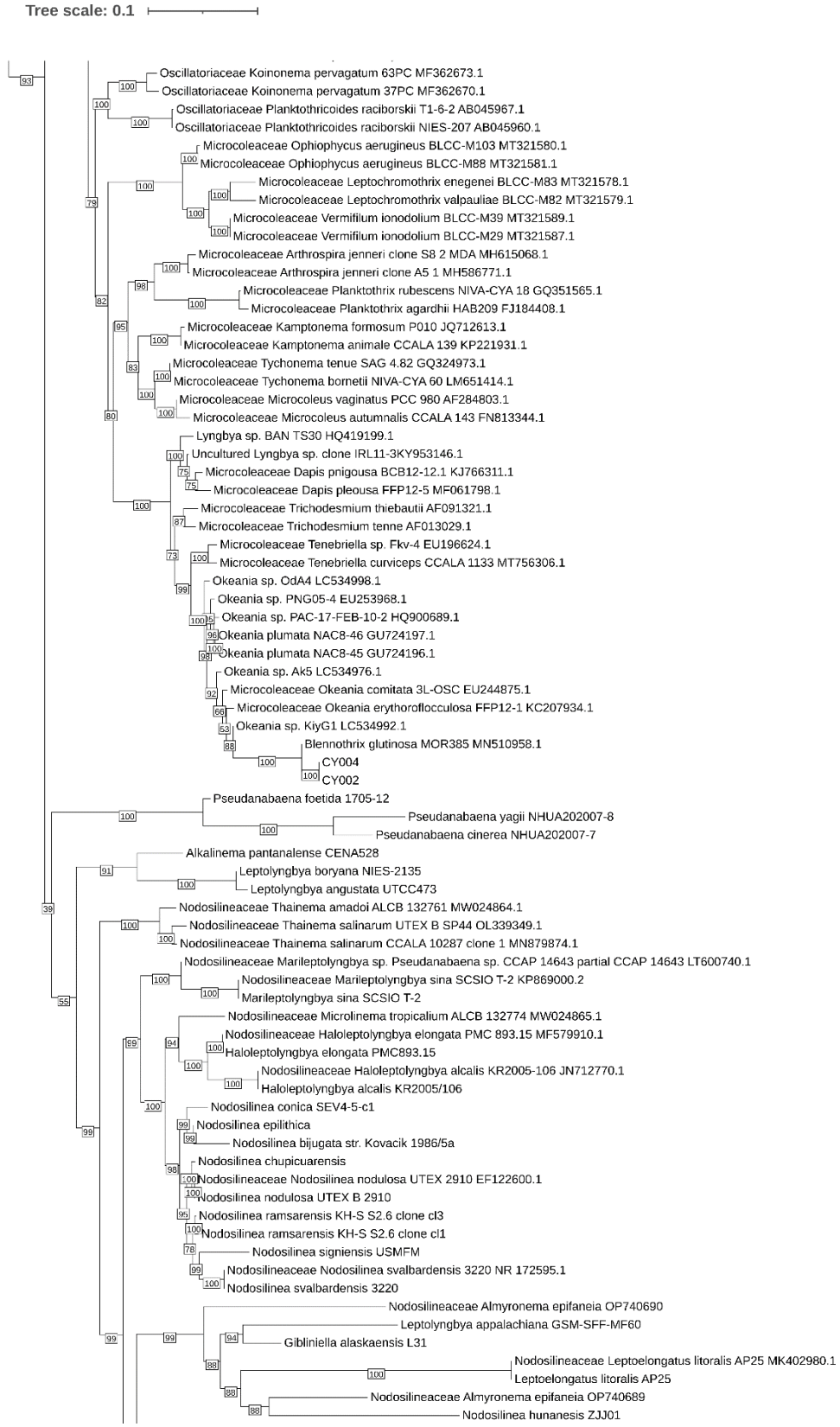
Feature ID	Taxonomy	2%	9%	25%	50%	75%	91%	98%
53c43c446d6bebfe5e1fed3849851f0e	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Cymatolegaceae; g__Rhodoploca	103.32	389	5792	20847	57653	111726.3	240106.8
87657d89bb52bc67a9f526c287bfebab	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae	17	37.24	703	1627	8939	17835.72	85469.44

Supplement 4. List of core ASV features found on 80% sampled sub-adult sea turtles.

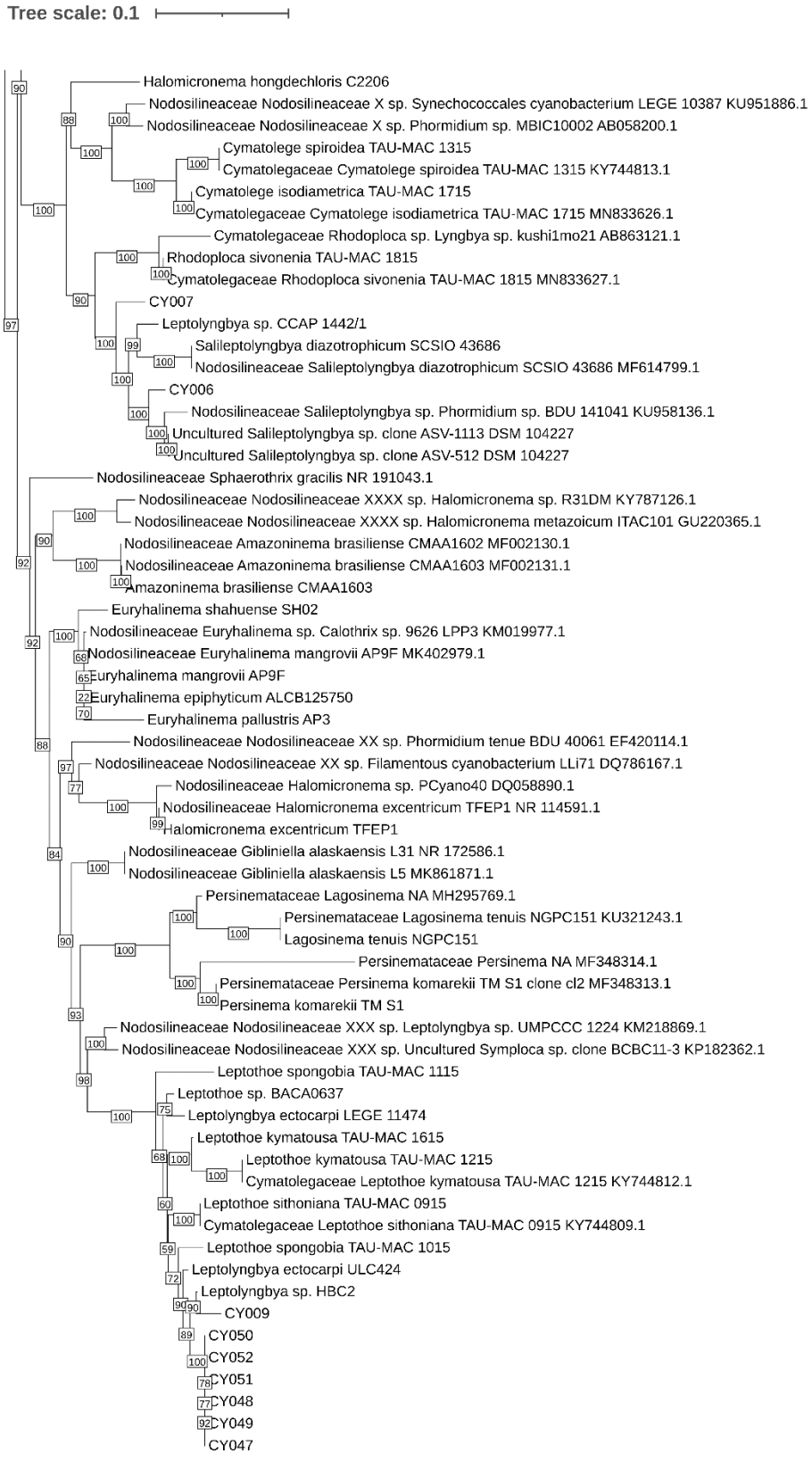
Feature ID	Taxonomy	2%	9%	25%	50%	75%	91%	98%
87657d89bb52bc67a9f526c287bfebab	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae	96.4	433.8	1205	2265	2382	8330.16	10932.48
aff17db4bacf1a70202722947efa9597	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Cymatolegaceae; g__Rhodoploca	26.08	117.36	326	2222	4119	19396.44	26080.32
34e0532a80555365736e8f8bea79d25c	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae; g__Salileptolyngbya	25.28	113.76	316	726	1979	11013.88	14966.64
063ab3be2eca0352fffa01cd7f26a2b8	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae; g__Salileptolyngbya	4.24	19.08	53	706	766	4952.24	6783.72
feae23eace96b6a91c4092e3d6cca7c7	k__Bacteria; p__Cyanobacteriota	2.4	10.8	30	209	991	4341.4	5807.2
53c43c446d6bebfe5e1fed3849851f0e	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Cymatolegaceae; g__Rhodoploca	10.8	48.6	135	146	931	989.24	1014.72
1426e25eb2f5de7e08c89acec9e986a8	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Nodosilineales; f__Nodosilineaceae; g__Salileptolyngbya	1.6	7.2	20	127	249	459.56	551.68
a93c71f8ea3d5695d7e6348a1dbeba3d	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae; o__Chroococcales; f__Pleurocapsaceae; g__Odorella	6.72	30.24	84	84	529	12124.52	17197.56
a2fbe9fa0a81488838ea66ba6f4bf6fd	k__Bacteria; p__Cyanobacteriota; c__Cyanophyceae	2.8	12.6	35	80	216	651.84	842.52



Supplement 5. Maximum likelihood phylogenetic tree of cyanobacterial strains based on 16S rRNA gene with bootstrap values on nodes. Tree includes cultured strains from this survey and reference strains mainly from orders Oscilatoriales, Spirulinales and Nodosilineales with *Gloeobacter violaceus* PCC 7421 strain as root (tree continues on the next page).



Supplement 5. (continued from the previous page).



Supplement 5. (continued from the previous page).

9. CURRICULUM VITAE

Lucija Kanjer was born on 7th July 1995 in Pula (Croatia) where she attended elementary and high school. During high school she showed keen interest in natural sciences and attended various extracurricular activities and competitions. In 2014 she enrolled in Undergraduate study of Biology at the Faculty of Science, University of Zagreb and obtained her bachelor diploma in 2017. The same year she enrolled in graduate study of Ecology and Nature Preservation at the Faculty of Science, University of Zagreb and obtained her master diploma in 2020 with the thesis named "Epizoic diatom genus *Poulinea* on loggerhead sea turtles from the Adriatic Sea". After her diploma she worked on freshwater diatom research and on curation of Croatian National Diatom Collection. She enrolled in PhD program in October 2020 on the research project "Loggerhead sea turtle (*Caretta caretta*) microbiome: insight into endozoic and epizoic communities (TurtleBIOME)". She published 5 scientific publications, of which 4 where she was the first author, 27 conference proceedings with 9 active participation (8 poster and 1 oral presentation). She was awarded with FEMS Research and Training Grant which enabled her to spend scientific training at BCCM Cyanobacteria Collection at University of Liège in Belgium. She participated as a teaching assistant on graduate and undergraduate courses Analysis of Biological Data, Pelagic Microbiology, and Field Course. She co-mentored one Master thesis. She contributed to the popularization of science via several workshops on events "Festival of Science and Day" and "Night at the Faculty of Science". She is a member of Croatian Microbiology Society (HMD) and Croatian Botanical Society (HBoD).