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FACULTY OF SCIENCE
DEPARTMENT OF BIOLOGY

Klara Filek

DIVERSITY OF DIATOM AND BACTERIAL COMMUNITIES ASSOCIATED WITH LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*)

DOCTORAL THESIS

Zagreb, 2022

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET
BIOLOŠKI ODSJEK

Klara Filek

RAZNOLIKOST MIKROBNIH ZAJEDNICA DIJATOMEJA I BAKTERIJA GLAVATIH ŽELVI (*CARETTA CARETTA*)

DOKTORSKI RAD

Zagreb, 2022

This doctoral dissertation was carried out within the Postgraduate doctoral programme in Biology at the University of Zagreb, Faculty of Science, Department of Biology (Division of Botany), under the supervision of prof. dr. sc. Sunčica Bosak. The research presented in this doctoral dissertation was supported by the Croatian Science Foundation (CSF) 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), European Union's Horizon 2020 research and innovation programme under grant agreement No 730984 ASSEMBLE Plus project, and FEMS Research and Training Grant FEMS-GO-2019-577. The work of Klara Filek was fully supported by the "Young Researchers' Career Development Project – Training of Doctoral Students" of the CSF funded by the European Union from the European Social Fund. The experimental parts of this thesis were carried out at the University of Zagreb (Croatia) and Ghent University (Belgium).

Mentor Biography

Sunčica Bosak (born 7th June 1982), PhD, Associate professor

Prof. Bosak obtained her Diploma in biology in 2006 at the Faculty of Science of the University of Zagreb. She is 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 Ph.D. in Interdisciplinary Study of Oceanology in 2013 and the 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 for 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 thesis, 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 coauthor of 47 scientific publications, 102 conference abstracts and seven conference papers, including five popular science publications. She was an 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).

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To my family and friends… even though it was often difficult for me to explain what exactly I am doing with turtles, your support was unwavering. Mom and dad thank you for letting me set up random marine Winogradsky columns in your kitchen and taking care of them. Impromptu cakes and fast drives healed some of the scientific woes and celebrated successes. My brother Karlo, thank you for being you – you inspire me to be a better human (except in those special occasions when I am furious with you). To my old and new friends – I admire all of you for the way you decided to explore the world, even if that meant we had to go our separate ways. Thank you for all the adventures, playfulness, and compassion that enriched this PhD journey.

University of Zagreb Doctoral thesis
Faculty of Science
Department of Biology

DIVERSITY OF DIATOM AND BACTERIAL COMMUNITIES ASSOCIATED WITH LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*)

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Faculty of Science, Department of Biology

Loggerhead sea turtles are ancient marine reptiles inhabiting the oceans worldwide. They are colonized by microorganisms both in the gastrointestinal tract and on their carapace and skin. In this thesis that consists of four scientific publications, the loggerhead sea turtle-associated diatom and bacterial communities were investigated by high-throughput sequencing of chloroplast *rbcL* gene and 16S rRNA gene, and cultivation. The culture-independent approach has shown that loggerheads harbor diverse but stable bacterial communities in their cloaca, and that the oral microbiota reflects the turtle's environment. The carapace and skin harbor complex microbial communities often rich in as yet unclassified taxa. Diatoms isolated from carapace and skin were cultivated as xenic monocultures and further characterized using morphology and molecular techniques to elucidate their identity and phylogeny. Novel diatom taxa were discovered, often belonging to the genera *Craspedostauros*, *Fallacia*, or *Amphora* genus. Cultivated diatoms also enabled the characterization of the phycosphere and isolation of diatom-associated bacterial strains.

(112 pages, 3 figures, 146 references, original in English language)
Keywords: microbiome, microbial communities, amplicon sequencing, reptiles, phycosphere
Supervisor: Dr. Sunčica Bosak, Associate Professor
Reviewers: Dr. Tomislav Ivanković, Associate Professor
Dr. Zrinka Ljubešić, Associate Professor
Dr. Wim Vyverman, Professor

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorski rad

RAZNOLIKOST MIKROBNIH ZAJEDNICA DIJATOMEJA I BAKTERIJA GLAVATIH ŽELVI (*CARETTA CARETTA*)

KLARA FILEK

Prirodoslovno-matematički fakultet, Biološki odsjek

Glavate želve su drevni morski gmazovi koji nastanjuju svjetska mora i oceane. Njihov probavni sustav te oklop i koža kolonizirani su mikroorganizmima. U ovoj disertaciji čiji su rezultati predstavljeni u sklopu četiri znanstvene publikacije, mikrobne zajednice dijatomeja i bakterija glavatih želvi su analizirane koristeći molekularne metode sekvenciranja visoke protočnosti kloroplastnog *rbcL* gena i 16S rRNA gena te kultiviranjem. Rezultati metoda neovisnih o kultivaciji su pokazali da su bakterijske zajednice u kloaki glavate želve raznolike ali stabilne te da oralna mikrobiota reflektira okoliš u kojem se kornjača nalazi. Karapaks i koža podloga su složenim mikrobnim zajednicama u kojima su često prisutni još uvijek nepoznati mikroorganizmi. Dijatomeje izolirane s karapaksa i kože kultivirane su kao ksenične monokulture te su korištene morfološke i molekularne metode za njihovu identifikaciju i određivanje filogenije. Otkrivene su nove svojte dijatomeja unutar rodova *Craspedostauros*, *Fallacia* i *Amphora*. Kultivacija dijatomeja također je omogućila analizu fikosfere te izolaciju pripadajućih bakterijskih sojeva.

(112 stranica, 3 slike, 146 literaturnih navoda, jezik izvornika: engleski)
Ključne riječi: mikrobiom, mikrobne zajednice, amplicon sekvenciranje, gmazovi, fikosfera
Mentor: Izv. prof. dr. sc. Sunčica Bosak
Ocjenjivači: Izv. prof. dr. sc. Tomislav Ivanković
Izv. prof. dr. sc. Zrinka Ljubešić
Prof. dr. sc. Wim Vyverman

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List of Publications

This doctoral thesis is based on the following publications. The publications are referred to in the text by their assigned Roman numerals.

I. **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.

II. **Filek K**, Lebbe L, Willems A, Chaerle P, Vyverman W, Žižek M, Bosak S (2022) More than just hitchhikers: a survey of bacterial communities associated with diatoms originating from sea turtles. FEMS Microbiology Ecology 98: fiac104.

III. Ashworth M*, Majewska R*, Frankovich T, Sullivan M, Bosak S, **Filek K**, Van de Vijver B, Arendt M, Schwenter J, Nel R, Robinson N, Gary M, Theriot E, Stacy N, Lam D, Perrault J, Maire C, Manning S (2022) Culturing putatively obligate epizoic diatoms: insights for the evolution and ecology of diatoms and their host. Scientific Reports 12: 15116.

IV. Majewska R, Ashworth MP, Bosak S, Goosen WE, Nolte C, **Filek K**, Van de Vijver B, Taylor JC, Manning SR, Nel R (2021) On sea turtle-associated *Craspedostauros* (Bacillariophyta), with description of three novel species. Journal of Phycology 57: 199-218.

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* these authors contributed equally to this manuscript and should be considered first authors to all academic and professional effects

Extended Summary

Marine microbial communities are often investigated within the scope of biogeochemical cycles, productivity, ecology, and evolution, and were traditionally subject to a point of view that microorganisms are mostly solitary and planktonic. Today, however, it is known that microorganisms seldom act alone and are typically a part of a broad microbial network (Donlan, 2002; Penesyan et al., 2021). A nexus between microbial ecology and clinical microbiology allowed for the recognition of host-microorganism associations that are not necessarily deleterious for the host (as in terms of pathogens) but can also be neutral or even beneficial (McFall-Ngai et al., 2013). Microbial communities in close association to the host are referred to as the microbiota (the assembly of microorganisms) and the microbiome (collection of microorganisms and their genes that form a "theater of activity") (Berg et al., 2020). Both terms are often associated with large unicellular or multicellular hosts, but the terms can also be used to describe the microbial community of any "well-defined habitat" like the Lieberkühn crypts in the small intestine or the phycosphere of microalgae (Berg et al., 2020). In comparison to terrestrial habitats, marine organisms are immersed in the seawater medium and continuously exposed to and colonized by microorganisms. Multicellular hosts, like seaweeds or marine megafauna, that harbor microbial biofilms could be described as "diversity hotspots" as the global oceans are considered, in a microbial sense, "blue deserts" (Polovina et al., 2008). Beyond colonizing available surfaces, microbial communities associated with multicellular marine hosts can strongly impact their host and are therefore increasingly studied (Apprill, 2017). Well established host-microbe associations study systems include bacterial symbiosis with the Hawaiian bobtail squid (*Euprymna scolopes*) and the gutless oligochaete worm (*Ollavius algarvensis*), and microalgal symbiosis with corals (Apprill, 2017). Marine vertebrates are just starting to be investigated as the significance of microbiome extends beyond commonly studied humans or human associated animals in captivity. The importance of studying "wild" microbiomes is widely recognized in evolutionary and conservation biology (Hird, 2017; Trevelline et al., 2019). With climate change and subsequent habitat devastation affecting almost every biome and leading to increased extinction rates (Stork, 2010), it is expected that host-associated microbial communities are also under pressure although at an unknown scale. Loggerhead sea turtles (*Caretta caretta*) are an endangered species inhabiting the oceans globally, and are known to harbor macro- and microepibionts on their skin and shells. Historically, reports on sea turtle epibionts relied on microscopy but today, culture-independent high-throughput sequencing is starting to get widely adapted in the sea turtle microbiome fields, especially the endozoic microbiome. For example, sea turtle endozoic microbial communities have been more extensively studied than the epizoic ones but only in feces, cloaca and distal gut, despite the importance of oral microbiome in vertebrate health. Furthermore, until recently, there were no reports on the microbial communities associated with the skin and carapace of sea turtles elucidated through marker gene sequencing (Rivera et al., 2018; Blasi et al., 2021; Kanjer et al., 2022). Regardless of the attractiveness of large datasets that allow for a fast and relatively easy overview of microbial communities, the efforts are stunned by the lack of reference sequences in respective databases. This emphasizes the need for multiple methodological approaches to understudied microbial habitats, such as the sea turtle, that include the cultivation of microorganisms along with culture-independent approaches.

The aims of this thesis were to: i) analyze the composition and diversity of endozoic bacterial communities of loggerhead sea turtles; ii) characterize epizoic diatom and bacterial communities of the skin and carapace of loggerhead sea turtles, with a focus on diatom-associated bacteria; and iii) isolate and identify diatoms found on the skin and carapace of the loggerhead sea turtles, establish monoculture protocols, and describe newly found diatom taxa.

In this thesis that consists of four scientific publications, the loggerhead sea turtle-associated diatom and bacterial communities were investigated by high-throughput sequencing of chloroplast *rbcL* gene and 16S rRNA gene, and cultivation. The oral and cloacal microbial communities of wild and rehabilitated loggerhead sea turtles revealed that the oral microbiota is reflective of the turtle's environment, while the cloacal microbiota is diverse but stable during short-term rehabilitation (Publication **I**). The epizoic diatom and bacterial communities are diverse and rich in yet unidentified microbial taxa (Publication **II**). Diatoms can be isolated and cultured from multiple sea turtle hosts and manatees and multiple novel species can be found (Publication **II**, **III**, **IV**). Additionally, xenic diatom cultures retain the bacterial environmental signature from their host and enrich specific microbial taxa that are lower in abundance in the total bacterial community of the host turtle, like *Alcanivorax* and *Marinobacter* genera (Publication **II**). Culturing diatom-associated bacteria yielded 127 isolates, out of which 40 were further identified by 16S rRNA sequencing that revealed potential new bacterial genus in the *Flavobacteriaceae* family (Publication **II**). The phylogeny of epizoic diatoms shows that they group based on their host, and that the epizoic habitat preference evolved multiple times in the diatom history (Publication **III**). A novel diatom species *Craspedostauros legouvelloanus* was described form loggerhead sea turtles, and previously described *Craspedostauros alatus* was reported on a loggerhead sea turtle for the first time (Publication **IV**).

The scientific contribution of the thesis is in the multifaceted approach to the loggerhead sea turtle microbiome, encompassing both prokaryotes and microeukaryotes, and culture-independent and cultivation-based methods. Multiple anatomical sites of the host were studied to encompass the endozoic microbiota: cloacal, and for the first time, oral microbial communities; and the epizoic microbiota: the skin and carapace. Culture independent approaches were enriched by cultivation of both diatoms and diatom-associated bacteria. The DNA sequences of the isolated microbial strains supplement the reference databases and will improve microbial identification in future metabarcoding efforts. This thesis also delivers first insights into the diatom-associated bacteria originating from a vertebrate host, which provides a baseline for future vertebrate associated diatom-bacteria interaction studies. Preserving microbial biodiversity in peculiar environments, such as the sea turtle, can potentially support future biotechnological advances. Taken together, the results in this thesis firmly establish loggerhead sea turtles as “hotspots” for macro- and micro-biodiversity, and can be used to steer decision-making in conservation and rehabilitation of endangered marine species.

Prošireni Sažetak

Morske mikrobne zajednice često su istraživane u kontekstu biogeokemijskih ciklusa, primarne produkcije, ekologije i evolucije te su shodno tome bile uključene u tradicionalna promišljanja o mikroorganizmima kao solitarnim i planktonskim stanicama. Danas znamo da mikroorganizmi rijetko djeluju kao individualne stanice već da su češće dio kompleksnih mikrobnih zajednica (Donlan i sur., 2002; Penesyan i sur., 2021). Spoj između mikrobne ekologije i kliničke mikrobiologije omogućio je nova saznanja o odnosu mikroorganizama i njihovih domaćina te utvrdio da mikroorganizmi nisu uvijek pogubni za domaćina već mogu biti neutralni ili pak korisni (McFall-Ngai i sur., 2013). Mikrobne zajednice u bliskoj vezi sa svojim domaćinima se zajednički nazivaju mikrobiotom (skup mikroorganizama) i mikrobiomom (zbir mikroorganizama i njihovih gena koji formiraju „teatar aktivnosti“) (Berg i sur., 2020). Termini mikrobiota i mikrobiom se čestu vežu za veće jednostanične i višestanične domaćine, ali se mogu koristiti i za opisivanje mikrobnih zajednica u staništima koja su fizikalno-kemijski dobro definirana; kao što su Lieberkühnove kripte u malom crijevu ili fikosfera mikroalgi (Berg i sur., 2021). Suprotno od kopnenih staništa, morski domaćini su cijelo vrijeme uronjeni u morski medij te kontinuirano izloženi i kolonizirani mikroorganizmima. Višestanični domaćini, kao što su makroalge ili morska megafauna, koji na svojim površinama nose mikrobne biofilmove bi se mogli smatrati „središtima biodiverziteta“ s obzirom da se globalni oceani često smatraju „plavim pustinjama“ na temelju manje brojnosti mikroorganizama u stupcu vode (Polovina i sur., 2008). Osim što mikroorganizmi nastanjuju slobodne površine morskih domaćina, njihove zajednice tako mogu snažno mogu utjecati i na njihovu fiziologiju (Apprill, 2017). U morskim ekosustavima mikroorganizmi i njihovi domaćini proučavani su u dobro opisanim sustavima kao što su bakterijske simbioze kod havajske lignje *Euprymna scolopes* i mnogočetinaša bez probavnog sustava *Ollavius algarvensis*, te simbioza mikroalgi i koralja (Apprill, 2017). Naime, morski su kralježnjaci tek od nedavno postali važan objekt istraživanja u području mikrobioma. Važnost istraživanja „divljih“ mikrobioma odavno je prepoznata u konzervacijskoj i evolucijskoj biologiji (Hird, 2017; Trevelline i sur., 2019). Sve intenzivnije posljedice klimatskih promjena i posljedična devastacija staništa u gotovo svakom biomu, dovele su do ubrzanih stopa izumiranja vrsta (Stork, 2010), stoga je očekivano da će i mikrobiomi ugroženih domaćina biti pod pritiskom nadolazećih promjena. Glavate želve (*Caretta caretta*) ugrožena su vrsta morskih kornjača koje obitavaju u svjetskim oceanima. Poznato je da su domaćini raznim makroorganizmima (rakovi vitičari i makroalge) i mikroorganizmima na koži i oklopima. Također, istraživanja epibionata na kornjačama su se povijesno uglavnom oslanjala na mikroskopiju ali današnje metode neovisne o kultivaciji koje uključuju i sekvenciranje visoke protočnosti se sve više koriste, posebno u istraživanjima endozojskih mikrobnih zajednica. Tako su endozojske bakterijske zajednice opširno proučavane ali samo u kloaki, distalnom crijevu i fecesu, dok oralne mikrobne zajednice nisu istraživane unatoč poznatoj važnosti u zdravlju kralježnjaka. Do nedavno nije bilo ni saznanja o mikrobnim zajednicama na koži i oklopu morskih kornjača, ali danas je dostupno nekoliko istraživanja na glavatim želvama i zelenim morskim kornjačama (Rivera i sur., 2018; Blasi i sur., 2020; Kanjer i sur., 2022). Usprkos atraktivnosti i pristupačnosti generiranja velike količine podataka kroz metode sekvenciranja visoke protočnosti, posljedično i lakšeg pregleda mikrobnih zajednice, istraživanja u tom smjeru često nailaze na prepreke zbog manjka referentnih DNA sekvenci u relevantnim podatkovnim bazama. Prepreke tog tipa naglašavaju važnost višestrukog pristupa neistraženim staništima kao što su morske kornjače, te obavezno povezivanje kultivacije mikroorganizama sa metodama neovisnim o kultivaciji.

Ciljevi ove disertacije su: i) analiza sastava i raznolikosti endozojskih bakterijskih zajednica glavatih želvi; ii) karakterizacija epizojskih zajednica dijatomeja i bakterija kože i karapaksa glavatih želvi, s fokusom na bakterije usko vezane za dijatomeje; te iii) izolacija i identifikacija dijatomeja s kože i karapaksa, uspostavljanje protokola za monokulture i opisivanje novopronađenih svojti dijatomeja.

U ovoj disertaciji, koja se sastoji od četiri znanstvene publikacije, mikrobne zajednice dijatomeja i bakterija glavatih želvi istražene su kroz sekvenciranje visoke protočnosti kloroplastnih *rbcL* gena i ribosomalnih 16S rRNA gena te kroz kultivaciju. Rezultati istraživanja endozojske mikrobne zajednice divljih i kornjača u rehabilitaciji ukazuju da oralna mikrobiota reflektira okoliš u kojem se kornjača nalazi dok je kloakalna mikrobiota raznolika ali stabilna tokom kratkotrajne rehabilitacije (Publikacija **I**). Epizojske zajednice dijatomeja i bakterija su raznolike te se sastoje od mnoštva još uvijek neidentificiranih mikroba (Publikacija **II**). Dijatomeje s različitih domaćina (morskih kornjača i sirena, tj. morskih krava) se mogu neometano izolirati i kultivirati te su među kultiviranim dijatomejama pronađene i nove svojte (Publikacija **II**, **III** i **IV**). Ksenične kulture dijatomeja također zadržavaju bakterijski „potpis“ domaćina s kojeg su izolirane te omogućavaju rast bakterijama koje se na domaćinu nalaze u niskim brojnostima kao što su rodovi *Alcanivoracax* i *Marinobacter* (Publikacija **II**). Iz kultura dijatomeja uspješno je izolirano i kultivirano 127 sojeva bakterija, od kojih je 40 dalje identificirano pomoću sekvenciranja 16S rRNA gena. Time je otkriveno nekoliko bakterijskih sojeva koji bi mogli biti predstavnici novoga bakterijskog roda unutar porodice *Flavobacteriaceae* (Publikacija **II**). Filogenija epizojskih dijatomeja pokazuje da se grupiraju ovisno o tome s kojeg su domaćina izolirane te da je sklonost epizojskom staništu evoluirala nekoliko puta kroz evolucijsku povijest dijatomeja (Publikacija **III**). Nove vrsta dijatomeje *Craspedostauros legouvelloanus* je opisana s glavate želve, dok je vrsta *Craspedostauros alatus* koja je prethodno opisana s muzejskih uzoraka zelene i Kempijeve želve po prvi put pronađena i na glavatim želvama u Jadranskom moru (Publikacija **IV**).

Znanstveni doprinos ove disertacije nalazi se u višestrukom pristupu proučavanju mikrobnih zajednica glavate želve. Naime, u istraživanjima predstavljenima u sklopu ove disertacije obuhvaćeni su i prokarioti i mikroeukarioti, te su kombinirani kultivacija mikroorganizama i metodološki pristupi neovisni o kultivaciji. Istraživano je nekoliko anatomskih regija glavatih želvi kako bi se obuhvatila endozojska i epizojska mikrobiota: mikrobne zajednice u kloaki i po prvi puta, u usnoj šupljini te mikroorganizmi na koži i karapaksu. Metodološki pristupi neovisni o kultiviranju mikroorganizama su u ovom slučaju obogaćeni upravo kultivacijom dijatomeja i bakterija iz kultura dijatomeja. Sekvencirana DNA kultiviranih dijatomeja i bakterija značajno doprinosi referentnim databazama te na taj način može poboljšati buduće istraživačke napore koji uključuju metabarkodiranje mikroorganizama. Ova disertacija također donosi prve uvide u bakterijske zajednice usko vezane za dijatomeje koje se nalaze na morskim kralježnjacima čime se postavlja osnova za buduća istraživanja interakcija između dijatomeja i bakterija na raznim domaćinima. Također, očuvanje mikrobne bioraznolikosti jedinstvenih staništa, kao što su morske kornjače, može doprinijeti i budućim biotehnološkim inovacijama. Predstavljeni rezultati u ovoj disertaciji snažno podržavaju tezu da su glavate želve središta makro- i mikrobnog biodiverziteta u globalnim oceanima te se dobiveni zaključci mogu iskoristiti pri donošenju odluka o najboljim načinima za očuvanje i rehabilitaciju ugroženih morskih vrsta.

1. Introduction

1.1. Host-microbe associations in marine ecosystems

Marine ecosystems make up to 70% of the Earth's surface and it is estimated that microorganisms comprise 15% of the relative global biomass (~ 81 Gt C), most of which is distributed within marine and deep subsurface environments (Bar-On et al., 2018). Technologies for investigating the scope of microbial phylogenetic and functional diversity have recently become highly accessible, with metagenomic sequencing addressing the problem of ocean under-exploration (Sunagawa et al., 2015, 2020). Approaches including meta-omics, genomics, and culturomics consistently produce new knowledge about the crucial roles of marine microbes in biogeochemical cycles, ecology, and evolution: from primary production to carbon sequestration (Falkowski et al., 2008; Cherabier, Ferrière, 2022), biotechnological and pharmaceutical applications (Paoli et al., 2022), over to pinpointing specific microbial interactions within microalgal blooms, single microbial cells, or metazoan hosts (Amin et al., 2012; Apprill, 2017; Martin et al., 2021; Boscaro et al., 2022).

The majority of microorganisms have traditionally been considered as planktonic and solitary. However, a shift in the microbial paradigm has led to an understanding that microorganisms seldom act alone, and that they are typically a part of a broad microbial network (reviewed by Donlan, 2002; Penesyan et al., 2021). A nexus between microbial ecology and clinical microbiology has facilitated the recognition of microorganisms that do not have a deleterious effect on the host (e.g., infections) but can be neutral or beneficial (McFall-Ngai et al., 2013). Microbial communities in close association with the host are referred to as the microbiota – the assembly of microorganisms; or microbiome – the collection of microorganisms and their genes that form a 'theatre of activity' as defined by Berg et al. (2020). Microbiota and microbiome are mostly associated with larger unicellular or multicellular hosts, but they also describe a microbial community of any 'well-defined habitat with distinct physiochemical properties' (Berg et al., 2020). Those habitats could be different anatomical sites of the metazoan host or the phycosphere, a thin region of diffused nutrients surrounding a microalgal cell.

In comparison to terrestrial habitats, marine organisms are immersed in the seawater medium and continuously exposed to colonization by microorganisms. Yet, based on chlorophyll concentrations, the open oceans are considered to be, in a microbial sense, "blue deserts"; (Polovina et al., 2008). Most microbes (up to 80% of bacteria and archaea) live in biofilms that protect against desiccation, toxins, antibiotics, or predation by grazing organisms (Penesyan et al., 2021). It is well known that bacteria and microalgae colonize submerged surfaces and enable subsequent complex biofilm formation (Dang, Lovell, 2016; Caruso, 2020). Living hosts, such as seaweeds or marine vertebrates, are often colonized by microbes and could be considered "diversity hotspots" in an otherwise resource-limited and microbe-scarce environment (Keller et al., 2021). The marine megafauna (body mass > 45 kg based on Estes et al., 2016) could therefore act as a reservoir of microbes and help microbial dispersal across different geographical locations during long-distance migrations.

The endozoic or epizoic microbiomes are being increasingly studied in marine animals. The research in marine microbiomes today spans from algae, sponges, cnidarians, nematodes, and mollusks, to large vertebrates such as whales and sharks (Apprill, 2017; Apprill et al., 2018; Doane et al., 2020). Some animals seem to not have or need a microbiome in all their life stages (Hammer et al., 2019) while corals, seaweeds and whales harbor consistent and stable microbiomes (Apprill et al., 2017, 2018; Miller et al., 2020; van der Loos et al., 2022). Well established study systems of host-microbe associations in marine ecosystems include bacterial symbiosis with the Hawaiian bobtail squid (*Euprymna scolopes*) and the gutless oligochaete worm (*Ollavius algarvensis*), and microalgal symbiosis with corals (Apprill, 2017). *E. scolopes* lives in a symbiosis with a bioluminescent bacterium *Aliivibrio fischeri* that inhabits the light organ in the squid's mantle. The bacterium helps the squid camouflage by counterillumination (Young, Roper, 1976). However, as Apprill (2017) notes, the rest of the squid's microbiome is understudied except for the female reproductive system - the accessory nidamental glands harbor complex and stable microbial communities (Kerwin, Nyholm, 2018). Another example is the *Ollavius algarvensis* oligochaete that resides in the marine sediment, is 3 cm long, and does not have a mouth or digestive system. Instead of harboring bacteria in the gut, the oligochaete hosts bacterial symbionts just below its cuticle and the bacteria act as a primary food and energy source (Dubilier et al., 2001). Furthermore, various corals live in symbiosis with microalgae in the *Symbodiniaceae* family (endosymbiotic dinoflagellates) that provide oxygen through photosynthesis. Environmental disturbances, such as high temperatures, can lead to coral bleaching i.e., the loss of chloroplasts in endosymbiotic dinoflagellates, and coral death (Suggett, Smith, 2020). Coral-associated microbiomes are now widely studied, including the relationship between the endosymbiotic dinoflagellates and their closely associated bacteria in the '*Symbiodiniaceae* phycosphere' (Peixoto et al., 2017; Garrido et al., 2021).

1.2. The "wild" microbiome: A story in the making

Advances in culture-independent approaches to studying microorganisms have accelerated the field of microbiome and environmental microbiology, leading to an exponential growth in published research over the last 40 years (Figure 1). Most microbiome focus on the bacteria found in the human gut or in animals of importance to humans (companions or food). Nevertheless, such studies have provided a foundation of knowledge on how the microbiome can affect the vertebrate host. The vertebrate microbiome is now known to affect the host's development, immune system maturation and modulation, behavior, reproduction, nutrient acquisition, and metabolism (McFall-Ngai et al., 2013). The brain-gut-microbiome axis is a newly developed concept that connects microbial activity in the gut with neurodegenerative diseases or neurodevelopmental disorders (Martin et al., 2018; Cryan et al., 2019), while recent advances in the skin microbiome revealed that disturbances in the composition of the microbial community of the integument can lead to an impaired skin barrier function and inflammation (Harris-Tryon, Grice, 2022). Yet, most of the host-microbiome research is conducted in a controlled environment or captivity that can alter the natural microbiome of the host (McKenzie et al., 2017). The translation of results obtained in captive animals is challenged, as it seems that rewilding the microbiome of laboratory mice leads to an enhanced immune response that mirrors that of wild mice and humans, emphasizing the need for 'wild' microbiomes in immunology (Rosshart et al., 2019).

Microbial communities associated with vertebrates other than humans or human-associated animals, have been predominantly studied in the gut or feces of captive mammals, fish, and birds with wild hosts initially underrepresented (Colston, Jackson, 2016; Youngblut et al., 2019; Levin et al., 2021). Earlier studies focused on building an inventory of microbes via culturing or short-read sequencing to determine the most dominant bacterial phyla and potential mechanisms in the assembly of the microbial communities (Colston, Jackson, 2016). Later major findings revealed the influence of the host's phylogeny and genetics on the gut microbiome composition in mammals, fish, and reptiles, but in birds the microbial communities reflected diet and geography of the host (Colston, Jackson, 2016; Hird, 2017). Larger-scale studies on wild animal microbiomes had shown that diet modulates the formation of functional microbial guilds while the host's phylogeny can determine the presence or absence of specific bacterial taxa (Youngblut et al., 2019).

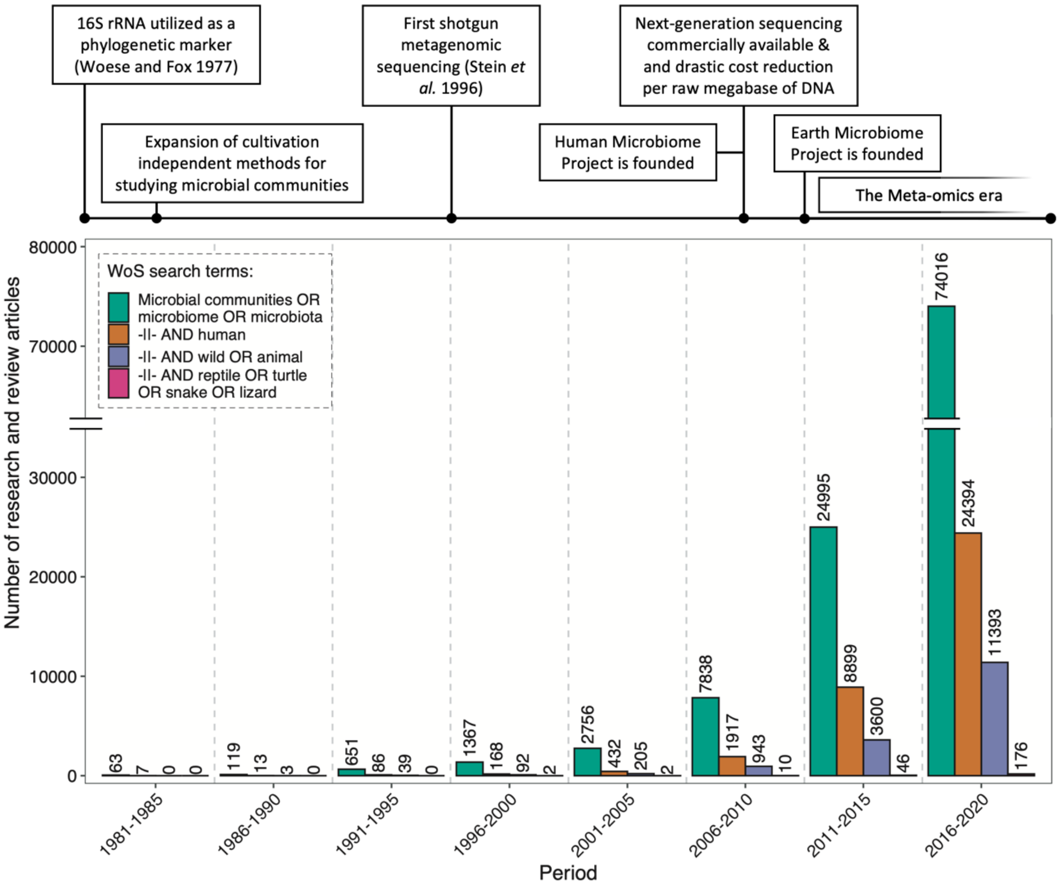

Figure 1. Number of research articles in 5-year periods for different search terms in the Web of Science. The baseline search term is ["microbial communities" OR microbiota OR microbiome] while other terms, like "human" (see legend) are searched for within the initial baseline search findings. The timeline above the figure indicates the most prominent studies and projects relevant for development of the microbiome field (such as Woese, Fox, 1977; Stein et al., 1996).

Regardless of strong phylosymbiosis signals in mammals, in birds and bats the microbiomes converged which could be explained by physiological adaptations needed for powered flight in both groups (Song et al., 2020). Recent *de novo* assembly of bacterial genomes in gut microbiomes of diverse vertebrate hosts has revealed patterns in microbial composition and function based on the host's taxonomy and traits (Levit et al., 2021). Several host classes, including reptiles, host a large reservoir of previously unknown microbes as well

(Levin et al., 2021). The higher proportion of unknown microbes in the reptilian microbiomes could be attributed to the low depth of sampling in the reptilian hosts (Figure 1).

Skin is the largest organ and the first line of defense against hazards in the environment such as injuries, toxins, UV radiation or pathogens. The microbiome is crucial in vertebrate skin barrier maintenance, as it modulates and educates the immune system and protects against pathogens through production of antimicrobial compounds (Ross et al., 2019). Along with humans and their companion animals, amphibians' skin microbiome is one of the most studied due to common infections with a chytrid fungus that led to staggering population decline in frog and salamander populations (Ross et al., 2019; Scheele et al., 2019; Varga et al., 2019). The skin microbiome of the marine megafauna is increasingly investigated in humpback whales, dolphins, killer whales, and sharks. Humpback whales seem to harbor core bacterial communities on their skin (Apprill et al., 2014) that vary in abundance depending on the season and geography (Bierlich et al., 2018). In contrast to other cetaceans, in beluga whales, the skin microbiome seems to be more variable and without a core microbiome (Van Cise et al., 2020). Microbiomes in whales and dolphins seem to be influenced by the host's phylogeny, however the influence of different locations on the microbiome of the same host species was not examined by Apprill et al. (2020). The skin microbiomes of the great white and tiger sharks are strongly influenced by the microorganisms present in the water column (Pratte et al., 2022), while in other shark species, the microbiome is quite distinct from the environment (Storo et al., 2021). In leopard sharks, the abundance of microbial taxa within a microbiome varies over time, while the functionality remains stable across different time points (Doane et al., 2022). Furthermore, Hooper et al. (2019) studied both bacterial and diatom communities from host shotgun sequencing data in killer whales and connected an increase in abundance of diatoms in individuals with poor skin condition. This shows that examining just the bacterial component of the skin microbiome could underestimate the effects of other microbial groups on the host (e.g., fungi, protozoa, or microalgae). Interestingly, recent efforts to characterize the skin microbial communities of sea turtles have shown that bacteria, diatoms, and other microeukaryotes of loggerhead sea turtles differ between the localities of the turtles and whether the microbes grow on the skin or the carapace (Van de Vijver et al., 2020; Kanjer et al., 2022). Initial studies in marine megafauna skin microbiomes have successfully given first insights into the composition of microbial communities, but the impact of the microbial communities on the host or vice versa remains to be determined.

The necessity of 'wild' microbiome research is already recognized in evolutionary and conservation biology (Hird, 2017; Trevelline et al., 2019). Trevelline et al. (2019) go as far as suggesting that the disruption of host-associated microbial diversity should be considered threatening to wildlife populations. With climate change and subsequent habitat devastation affecting almost every biome and leading to increased extinction rates (Stork, 2010), it is expected host-associated microbes are also under pressure although at an unknown scope. Meta-analysis of the endo and epizoic microbiomes of wild hosts in different habitats has shown that internal microbiomes can be explained by host phylogeny and immune complexity, diet, and climate, while the external microbiomes could be explained by climate (temperature and precipitation) (Woodhams et al., 2020). Furthermore, captivity (transient or temporary) is often an element of threatened species management, and it is known to alter the microbiome, but it is unclear how these changes affect the biology and physiology of the host animal (McKenzie et al., 2017). Microbial engineering or microbial stewardship (e.g., prebiotics, probiotics, microbiome transplants) are often suggested as tools to mitigate the potential effects of captivity or to restore a healthy state in vulnerable hosts (West et al., 2019; Peixoto et al., 2022). Integration of the microbial component in traditional conservation efforts is necessary, however, to confidently manipulate host-associated microbial communities and mitigate associated risks, interdisciplinary exchange of knowledge and resources together with targeted implementation is needed (Peixoto et al., 2022).

1.3. The science (biology and ecology) of extant sea turtles

1.3.1. Ancient extant marine reptiles govern the global oceans

There are seven extant species of sea turtles (order Testudines, superfamily Chelonioidea) in the world's oceans: hard-shelled hawksbill (*Eretmochelys imbricata*), Kemp's ridley (*Lepidochelys kempii*), olive ridley (*Lepidochelys olivacea*), green (*Chelonia mydas*), loggerhead (*Caretta caretta*), flatback (*Natator depressus*), and soft-shelled leatherback sea turtle (*Dermochelys coriacea*). The work in this thesis is focused on loggerhead sea turtles of the Mediterranean Sea; consequently, the features specific to them are emphasized throughout this chapter. Loggerhead sea turtles are the largest and most studied hard-shelled sea turtles reaching up to 200 kg in weight and 90 cm in average carapace length, but they come second to the soft-shelled leatherbacks (Lutz et al., 1996). Currently, all species but the flatback sea turtle are considered critically endangered, endangered, or vulnerable according to the IUCN 2020 (https://www.iucnredlist.org) (IUCN-SSC Marine Turtle Specialist Group, 2020). The history of sea turtles dates to 120 million years ago into the Cretaceous era, and the oldest known representative is *Desmatochelys padillai* (Cadena, Parham, 2015). The hallmark of sea turtle anatomy that separates them from their terrestrial or freshwater relatives is the fusiform body shape that allows for less friction and drag during swimming while simultaneously restricting protective head and limb retraction. The hard-shelled sea turtles' carapace (upper shell) and plastron (underside part of the shell) consist of bony plates that fuse with the ribs covered by a layer of keratinous scales (i.e., scutes) whose patterns (number and position) are used for species identification together with head scales and the beak shape. The leatherback sea turtle is soft-shelled and instead of bony plates and scutes, the shell consists of blubber permeated with small bone plates on top of which lays a thick rubbery layer of skin (Lutz et al., 1996).

The flippers are wide, as back flippers are being used for steering and front for propelling with enlarged claws in males used for grasping the female while mating. Sea turtles today inhabit both neritic (near shore) and oceanic (offshore) habitats, and commonly progress from oceanic habitats as hatchlings to neritic foraging habitats as juveniles and adults, depending on resource availability (Wyneken et al., 2012). The beaks of sea turtles reflect their diet: loggerheads have wide and strong jaws that can crush diverse prey with harder shells while the sharp and narrow beak of hawksbills (hence the name) is specialized for reaching the crevices within coral reefs (Wyneken et al., 2012). The globalization of sea turtle research enabled an expansion of observed feeding strategies in many species, revealing surprising patterns and a wider choice of diet items than previously reported (Wyneken et al., 2012). Sea turtles are long-lived, spend 90% of their time submerged (Lutz, Musick, 1996), and are solitary except for synchronized mass nestings of hundreds of Kemp's ridley or olive ridley sea turtles, called arribadas. They are slow to reach sexual maturity (up to 10 years in loggerheads) but once they do, they return to the nesting beach they originated from (natal homing) often migrating over long distances (Wyneken et al., 2012; Hays, Scott, 2013).

Loggerhead sea turtles can be found globally, with nine distinct subpopulations: North Pacific, Mediterranean, Northeast Atlantic, North Indian, South Pacific, Northwest Atlantic, South Atlantic, Southwest Indo-Pacific, Southwest Indian (Wyneken et al., 2012). Loggerhead hatchlings disperse widely from their rookeries, and according to ocean model simulations the largest nesting sites allow easy dispersal to the most productive oceanic habitats (Harrison et al., 2021). There is little evidence for mixing of female populations across basins due to high nesting site fidelity, while males often copulate with females outside of their ancestral nests, therefore connecting regional nesting sites through 'male-mediated gene flow' (Bolten et al., 1998; Bowen, Karl, 2007; Wallace et al., 2010).

The Mediterranean population of loggerheads differs from other populations as adult females are smaller in size (Casale et al., 2018). Although there is an influx of hatchlings from the Atlantic loggerhead population to the Mediterranean Sea, they tend to migrate back to the Atlantic and do not return to foraging sites, while Mediterranean turtles rarely transition to the Atlantic (Casale et al., 2018). Mediterranean loggerheads nest mostly in Greece, Turkey, Libya, and Cyprus, while they forage and winter in the neritic waters of the Adriatic Sea, Tunisia, Greece, Turkey, Egypt, and Spain (Carreras et al., 2006; Casale et al., 2018). Recent estimations show that there could be more than 8000 loggerhead sea turtle nests in the Mediterranean annualy, with estimates of total abundance between 1.2 and 2.4 million individuals (Casale, Heppell, 2016; Casale et al., 2018). A spatial density model of loggerhead abundance based on aerial and shipboard line transect survey data by the Naval Undersea Warfare Center Division (2003-2018) estimates a mean abundance of 994 000 turtles basin wide (Sparks, DiMatteo, 2020). Due to their current high numbers, the Mediterranean loggerhead sea turtle population is considered the least threatened.

1.3.2. Current threats to sea turtle populations

While adult sea turtles often lack natural predators due to body size, they are under great threat of anthropogenic habitat devastation and climate change. Exploitation of sea turtles for food and trade was regulated by Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) and Red List of the IUCN in 1980's (Casale et al., 2018). The main factors affecting sea turtles today are: (1) habitat degradation due to coastal development, tourism, chemical and plastic pollution, especially at nesting sites; (2) hatchling recruitment failure due to light pollution-associated disorientation, predation by mammals, and *Fusarium* spp. infections; (3) incidental catch of turtles by fisheries that can lead to entanglement injuries, internal injuries, and gas embolism, together with injuries caused by speeding boats; and (4) an increase in environmental temperature averages caused by climate change that leads to skewed sex ratios i.e., feminization (Casale et al., 2018; Dimitriadis et al., 2018; Gleason et al., 2020).

Global efforts in sea turtle conservation, which includes rehabilitation in clinics and rescue centers, extensive monitoring, and public outreach, seems to have positive results on global sea turtle abundance estimates (Mazaris et al., 2017). However, rapidly changing environmental conditions and a swift increase in global temperatures could be detrimental to turtles and tortoises despite all the conservation efforts – urging for better research and tools for enhancing conservation strategies (Rilov et al., 2019; Stanford et al., 2020).

1.3.3. Sea turtles as protagonists in scientific studies - from physiology and biologging, to commensals

Innate ability for extended deep dives, long-distance migrating, and natal homing sparked research based on data loggers and satellite telemeters attached to sea turtles. In that way it is possible to track turtle behavior, biogeography, and population distribution remotely. Such studies, along with genetic marker research, enabled description of migratory routes and habitats, and facilitated conservation of important habitats (Godley et al., 2008; Wallace et al., 2010). Besides mapping populations, environmental data loggers attached to sea turtles were recently used in efforts to collect data on the surface and subsurface ocean temperatures *in situ* for tropical cyclone prediction (i.e., biologging) (Bousquet et al., 2020). Moreover, sea turtles are deeply affected by pollution, especially when it comes to debris and microplastic ingestion (Tomas et al., 2001; Lazar, Gračan, 2011; Meaza et al., 2021). Recently, it was discovered that bacteria from loggerheads in the Mediterranean Sea harbor high levels of antimicrobial resistance genes, even when turtles were not treated with antibiotics, thus expanding the role of sea turtles as sentinel species for antibiotic pollution monitoring in marine habitats (Storelli et al., 2005; Pace et al., 2019a; Trotta et al., 2021a, 2021b).

As other marine vertebrates, sea turtles are extensively colonized not only by opportunistic pathogens, but also by symbionts, in the gut as well as on the skin and carapace (Figure 2). The first reports of macro epibionts (> 1 mm) on sea turtles date back to Darwin in the 1850's (Robinson, Pfaller, 2022). Since then, reports on macro epibionts were sporadic with an increase in systematic approach in recent years (Robinson, Pfaller, 2022). It is now known that the macro-epibiont assembly (barnacles, red and green algae, bryozoans, cnidarians etc.) can reflect migratory routes and habitat preferences (Luschi, Casale, 2014).

Microbial epibionts are studied less extensively, but there are infrequent reports on prokaryotic and eukaryotic microorganisms (meifoauna, diatoms, bacteria) that focus on determining microorganismal population patterns for detecting migratory routes or the health status of the animal (Robinson et al., 2016; Rivera et al., 2018; Ingels et al., 2020; Van de Vijver et al., 2020; Kanjer et al., 2022). Abdelrhman et al., (2016) described the first insights on the gut microbiome of loggerhead sea turtles' feces obtained by 16S rRNA gene amplicon sequencing. Since then, there has been an increasing number of studies that describe the microbial communities of the sea turtle gastrointestinal tract (Ahasan et al., 2017; Price et al., 2017; Campos et al., 2018; Arizza et al., 2019; Biagi et al., 2019; Ahasan et al., 2020; McDermid et al., 2020; Samuelson et al., 2020; Scheelings et al., 2020a; McNally et al., 2021b; Chen et al., 2022), skin lesions (Trotta et al., 2021a), and eggs or nests (Gambino et al., 2020;

Vecchioni et al., 2022) encouraged by clinical microbiology practices in sea turtle rehabilitation and recent advances in the field of wild microbiomes and conservation. Currently, data on gut microbial communities is available for all extant sea turtle species, as each species was sampled for at least one sample type: mucosal, fecal, or cloacal. So far, the oral microbiome has only been investigated in Kemp's ridley sea turtles during rehabilitation (McNally et al., 2021a). Most of the data collected so far has been obtained by opportunistic sampling of turtles during nesting or turtles found as bycatch or injured, which means there are significant gaps in sampling different developmental stages, sexes, and localities.

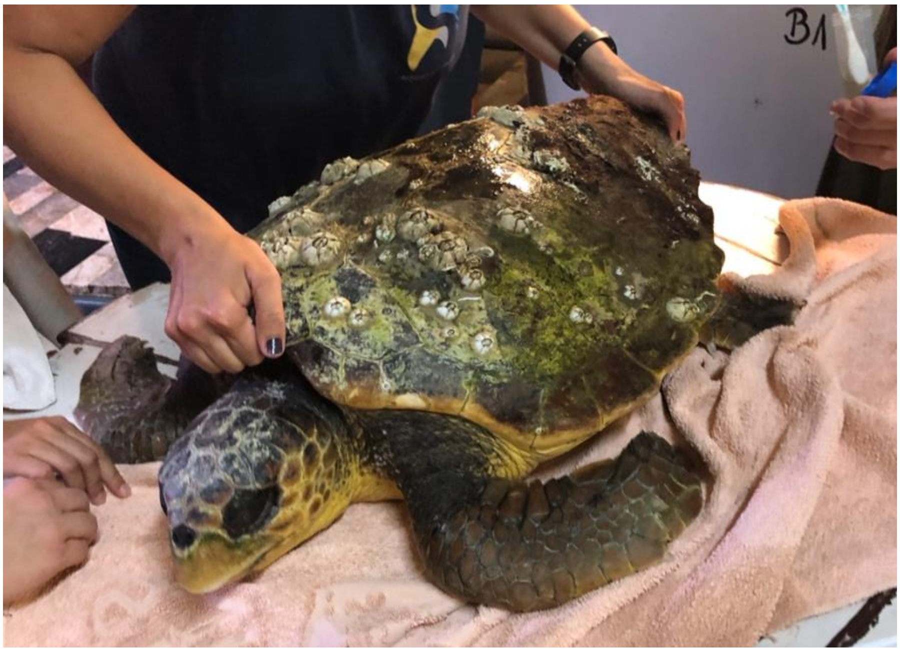

Figure 2. The loggerhead sea turtle named Ella, is undergoing sampling before admission to the sea turtle rehabilitation and rescue center at the Aquarium Pula, Croatia. The carapace and skin are overgrown by barnacles and algae.

1.4. Loggerhead sea turtles as hosts to microorganisms

1.4.1. Endozoic microbial communities of loggerhead sea turtles

Microorganisms in the gastrointestinal tract of loggerhead sea turtles were first investigated in feces and cloacae in turtles undergoing rehabilitation by 16S rRNA gene profiling through amplicon sequencing (Abdelrhman et al., 2016; Arizza et al., 2019; Biagi et al., 2019) or cultivating bacterial isolates (Pace et al., 2019b; Alduina et al., 2020). The main bacterial phyla found in the feces and intestine at the time were reported to be Firmicutes (Bacillota corrig. phyl. nov.), Proteobacteria (Pseudomonadota corrig. phyl. nov.), Fusobacteria

(Fusobacteriota corrig. phyl. nov.), and Bacteroidetes (Bacteroidota corrig. phyl. nov.) with a significant number of Clostridiales indicating dysbiosis (Abdelrhman et al., 2016; Arizza et al., 2019; Oren, Garrity, 2021). Furthermore, microbial community composition was affected by the size of the turtle as measured by CCL (Biagi et al., 2019). Culturing efforts focused mainly on opportunistic bacterial and parasitic pathogens in buccal and cloacal swabs, feces, internal organs, eggs, and nests with detection of antibiotic resistance both in wild and in turtles undergoing rehabilitation (Pace et al., 2019b, 2019a; Alduina et al., 2020; Blasi et al., 2020; Trotta et al., 2021b). Recently, Biagi et al., (2021) investigated the impact of ingested plastic debris on microbial communities in rescued loggerhead sea turtles and found bacterial phylotypes associated with higher proportion of ingested plastic, suggesting that certain bacterial taxa, like *Cetobacterium somerae*, could be used as indicators of plastic ingestion in sea turtles. Wild loggerheads seem to differ in gut microbiota depending on geographically distinct populations, which is thought to be due to differences in environmental conditions and diet (Scheelings et al., 2020b). Contrastingly to previous studies wild loggerhead distal gut microbiota is dominated by Proteobacteria (Pseudomonadota corrig. phyl. nov.) and Bacteroidetes (Bacteroidota corrig. phyl. nov.), with Fusobacteria (Fusobacteriota corrig. phyl. nov.) and Firmicutes (Bacillota corrig. phyl. nov.) less abundant (Scheelings et al., 2020a) albeit the sampled population consisted only of nesting females. It is known that fungal and bacterial infections can affect hatchling success (Alduina et al., 2020; Gambino et al., 2020) with *Fusarium* spp., *Aeromonas* spp., *Citrobacter* spp., and *Pseudomonas* spp. being the main culprits. However, only recently has the microbiota of eggs and nests been investigated in detail as to provide a baseline for further culture independent investigation in microbial factors affecting hatchling mortality and survival (Vecchioni et al., 2022).

1.4.2. Epizoic microbial communities of loggerhead sea turtles

In spite of well-characterized macroepibiont assemblages on sea turtles (Robinson, Pfaller, 2022), the investigations on loggerhead carapace and skin microorganisms remain scarce. Clinically important microorganisms are investigated by culturing bacterial (Trotta et al., 2021b, 2021a) and fungal isolates from skin and carapace lesions, with fungi usually being associated with egg mortality (Cafarchia et al., 2020; Gleason et al., 2020). Recently, communities of microeukaryotes and/or prokaryotes have started to get investigated by microscopy (Ingels et al., 2020; meiofauna), combination of microscopy and metabarcoding (Blasi et al., 2021; microeukaryotes and bacteria), or only metabarcoding (Kanjer et al., 2022; microeukaryotes and bacteria). Notably, diatoms are the exception as they are often studied in sea turtles through morphological approaches leading to new species descriptions. Diatoms are unicellular microalgae (Bacillariophyceae, Stramenopiles) characteristic for their resilient silica frustules that remain stable even after the death of the cell. Diatoms are ubiquitous in aquatic environments and are responsible for 20% of the annual global oxygen production via photosynthesis and carbon cycling (Field et al., 1998; Jin et al., 2006; Tréguer et al., 2018). Their phycosphere often harbors distinct bacterial communities as well (Amin et al., 2012; Figure 3).

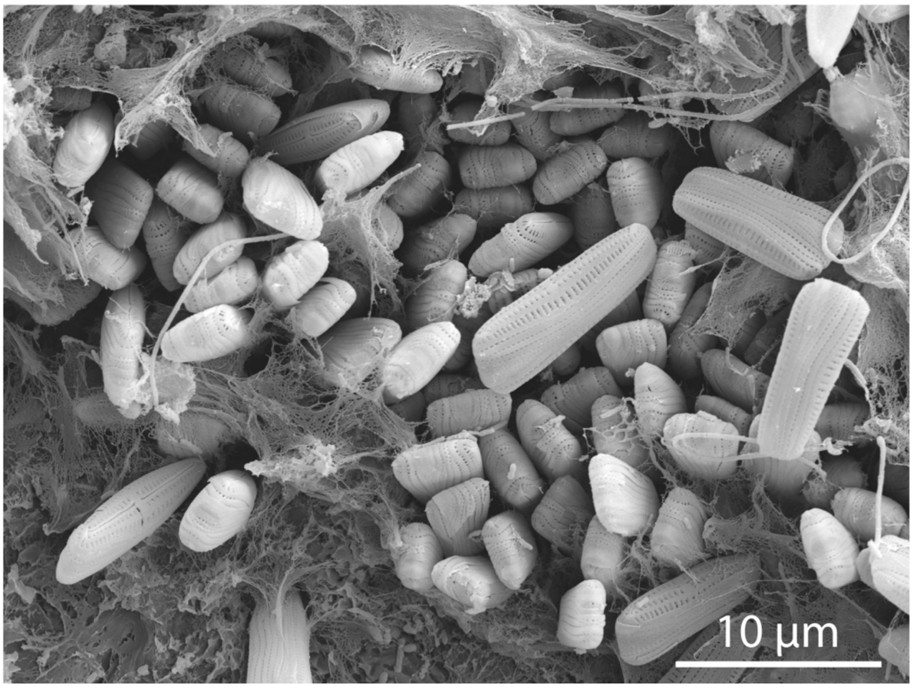

Figure 3. Assemblage of *Poulinea lepidochelicola* diatom cells on the loggerhead sea turtle carapace embedded in extensive mucilage. Bacterial cells can also be observed adhering to the mucilage or directly to the diatom cells.

Morphological analyses of silica frustules to determine the composition of diatom communities is used in marine and freshwater biomonitoring, so the same approach was initially used in studying diatoms associated with sea turtles. After confirmation that all sea turtles harbor diatoms on their surfaces (Robinson et al., 2016), multiple new diatom species were discovered, many of whom are considered epizoic (Majewska et al., 2015, 2017, 2018, 2019). Morphology-based analyses of diatom assemblages in loggerhead sea turtles had shown that epizoic diatoms are abundant and diverse (Kaleli et al., 2020; Kanjer et al., 2020) and differ based on the locality of the turtles (Van de Vijver et al., 2020). Although diatoms were not specifically targeted in the following studies, similar patterns have been observed in culture-independent surveys of bacterial and microeukaryotic communities based on 16S and 18S rRNA gene profiling, respectively (Blasi et al., 2021; Kanjer et al., 2022).

1.5. The importance of culturing host-associated microorganisms

Even though cultivation-independent approaches (e.g., amplicon or metagenomic sequencing) for surveying marine host microbiota or microbiome allow for insights into community dynamics and potential biological diversity, there is a significant gap between sequence-based data and cultivated microbial representatives (Keller et al., 2021). The paradigm that only 1% of microorganisms are culturable was based on high unrecognized diversity detected by 16S rRNA gene sequencing. However, (Martiny, 2019) urges that the 1% paradigm is no longer correct, and many more microbial taxa can indeed be cultured. Information on uncultivated microorganisms has propelled the field of microbial ecology and evolution (Solden et al., 2016), but cultured representatives are still needed for understanding the biology, functions, interactions with the environment (or a host), or for conducting experiments.

In rarely explored habitats such as the marine megafauna, there is a higher incidence of previously unknown DNA sequences attributed to microorganisms (Keller et al., 2021; Levin et al., 2021). Intense culturing efforts in whales and corals yielded 592 microbial isolates (mostly bacterial) that, although biased, still recapitulated the microbial diversity found through sequence-based methods (Keller et al., 2021). In diatoms, the need for cultured representatives is stressed because of historical description of species based solely on morphology of the cell. As described above, diatoms are microalgae with silicate frustules that are predominantly used for their identification, but with the development of molecular methods it was recognized that DNA barcoding is necessary to recognize cryptic or pseudocryptic species (Mann et al., 2010). Metabarcoding of the diatom communities based on chloroplast gene *rbcL* (RuBisCo large subunit gene) on green sea turtles has shown that there is a lack of publicly available reference data and failed in detecting diatom taxa that were otherwise detected by microscopy (Rivera et al., 2018). The discrepancy between sequence-based data and morphological analyses is not unusual and is encountered in freshwater biomonitoring as well (Pérez-Burillo et al., 2020), however, the scope of inconsistencies varies with knowledge about the habitat. For example, benthic diatoms associated with sea turtles are underexplored in comparison to free-living planktonic diatoms, which could lead to a high abundance of misidentified diatom taxa.

Culturing diatoms, along with other microbes, and contributing to reference databases with curated data is therefore unavoidable if high-throughput sequencing approaches are to be used for understanding the complete microbiome of the sea turtle host. Beyond enhancing culture-independent approaches by cultivation, the addition of cultivable microbial strains to sequence-based data can enable focused experimental approaches to reveal host-microbe interactions that span from functional roles of members of the microbiome in health and disease, coevolution, microbiome manipulation ("microbial stewardship"), to exploring the biotechnological potential of previously unexplored niche habitats (Keller et al., 2021; Peixoto et al., 2022).

1.6. Aims and hypotheses

The aims and hypotheses of this doctoral thesis are focused on addressing the gaps in loggerhead sea turtle endozoic and epizoic microbiome research. To that end, this thesis encompasses four scientific publications (**I-IV**).

Aims:

1. Analysis of the composition and diversity of endozoic bacterial communities of loggerhead sea turtles (**Publication I**)

2. Characterization of epizoic diatom and bacterial communities of the skin and carapace of loggerhead sea turtles, with a focus on diatom-associated bacteria (**Publication II**)

3. Isolation and identification of diatoms found on the skin and carapace of the loggerhead sea turtles, establishment of monoculture protocols, and description of newly found diatom taxa (**Publications II, III, and IV**)

Hypotheses:

1. Oral microbiota of loggerhead sea turtles is dynamic and reflective of but also distinct from the environment, while the cloacal community is more stable (**Publication I**)

2. The phycospheres of diatom strains isolated from loggerhead sea turtles maintain the bacterial signature of the host they originated from (**Publication II**)

3. The microbial communities associated with loggerhead sea turtles are a source of novel microbial taxa (**Publications II, III, and IV**)

Publication **I** contributes to the first aim and hypothesis by characterizing oral and cloacal microbial communities of loggerhead sea turtles in the Adriatic Sea. It also contributes to first descriptions of oral microbiota of loggerheads, as previous efforts were based solely on cultivation of clinically relevant strains and did not inform on the whole community. The second aim was explored within Publication **II** that encompassed an amplicon-based survey of diatom and bacterial communities of the skin and carapace. Within Publication **II** diatom cells were isolated, cultivated and identified, directly contributing to the third aim, while the bacterial communities of diatom cultures were investigated by amplicon sequencing and cultivation contributing to the second aim and second and third hypothesis. Publication **III** and **IV** directly contributed to the third aim and hypothesis. Publication **III** describes successful approaches to culturing epizoic diatoms from multiple sea turtle hosts and delivers a library of reference sequences that can enhance future diatom metabarcoding efforts. In Publication **IV** one newly described epizoic diatom species was reported to be found on loggerhead sea turtles in the Adriatic Sea for the first time, and another one was described as a novel species.

Publication I

Filek *et al. anim microbiome* (2021) 3:59
https://doi.org/10.1186/s42523-021-00120-5

Animal Microbiome

RESEARCH ARTICLE **Open Access**

Characterization of oral and cloacal microbial communities of wild and rehabilitated loggerhead sea turtles (*Caretta caretta*)

Klara Filek[1], Adriana Trotta[2], Romana Gračan[1], Antonio Di Bello[2], Marialaura Corrente[2] and Sunčica Bosak[1*]

Abstract

Background: Microbial communities of wild animals are being increasingly investigated to provide information about the hosts' biology and promote conservation. Loggerhead sea turtles (*Caretta caretta*) are a keystone species in marine ecosystems and are considered vulnerable in the IUCN Red List, which led to growing efforts in sea turtle conservation by rescue centers around the world. Understanding the microbial communities of sea turtles in the wild and how affected they are by captivity, is one of the stepping stones in improving the conservation efforts. Describing oral and cloacal microbiota of wild animals could shed light on the previously unknown aspects of sea turtle holobiont biology, ecology, and contribute to best practices for husbandry conditions.

Results: We describe the oral and cloacal microbiota of Mediterranean loggerhead sea turtles by 16S rRNA gene sequencing to compare the microbial communities of wild *versus* turtles in, or after, rehabilitation at the Adriatic Sea rescue centers and clinics. Our results show that the oral microbiota is more sensitive to environmental shifts than the cloacal microbiota, and that it does retain a portion of microbial taxa regardless of the shift from the wild and into rehabilitation. Additionally, Proteobacteria and Bacteroidetes dominated oral and cloacal microbiota, while Kiritimatiellaeota were abundant in cloacal samples. Unclassified reads were abundant in the aforementioned groups, which indicates high incidence of yet undiscovered bacteria of the marine reptile microbial communities.

Conclusions: We provide the first insights into the oral microbial communities of wild and rehabilitated loggerhead sea turtles, and establish a framework for quick and non-invasive sampling of oral and cloacal microbial communities, useful for the expansion of the sample collection in wild loggerhead sea turtles. Finally, our investigation of effects of captivity on the gut-associated microbial community provides a baseline for studying the impact of husbandry conditions on turtles' health and survival upon their return to the wild.

Keywords: Microbiota, Bacterial diversity, Reptile, Rehabilitation, Adriatic Sea, Conservation

Background

Microbial communities associated with vertebrates can influence host's evolution, development, immune system maturation, physiology, nutrient acquisition, health and disease [1, 2]. It is estimated that the host's collection of bacteria could contain at least 100 times the genes as in the host's genome, often adding to the metabolic functions' repertoire, e.g. biochemical pathways in nutrient acquisition [3]. Moreover, we can consider the host and its microbial commensals as a distinct biological entity (holobiont and hologenome) susceptible to the processes of natural selection [4, 5].

Most studies of microbial communities have focused on the distal gut of humans or captive mammals [2, 6] but there are recent growing efforts in investigations of

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free-ranging wild animals. Wild animals are sensitive to environmental perturbations caused by climate change and anthropogenic habitat disruption, therefore investigating wild animal-associated microbial communities contributes to improving existing conservation efforts [7, 8]. Current research covering major vertebrate groups reveal evidence for co-phylogeny of mammals and their microbial communities, microbiome convergence in birds and bats, while microbial assemblages of non-mammalian hosts (e.g. reptiles) are mostly influenced through diet and the environment [9, 10]. Marine animals are permanently immersed in seawater environment, making the microbial dynamics different from those of land-dwelling animals [11]. As expected, marine mammals have been the focus of most vertebrate microbial community studies that undertook a wider sampling effort of body sites other than the distal gut or feces [12–16]. In comparison to other vertebrates, reptiles are still underrepresented in studies of their bacterial communities [6, 17], especially large marine reptiles, such as sea turtles. Sea turtles are large-bodied, long-lived marine top predators, considered as a keystone species, with critical roles in ecosystem processes such as bioturbation, bioaccumulation, energy flow, trophic status and mineral cycling [18]. Loss of foraging and nesting sites, increasing global temperatures, and bycatch are major threats for sea turtles' survival. Currently, there are seven extant sea turtle species listed on the IUCN Red List of Threatened Species [19]: Kemp's ridley (*Lepidochelys kempii*) and hawksbill sea turtles (*Eretmochelys imbricata*) are critically endangered; the green turtle (*Chelonia mydas*) is considered to be endangered; loggerhead (*Caretta caretta*), olive ridley (*Lepidochelys olivacea*) and leatherback (*Dermochelys coriacea*) sea turtles are listed as vulnerable, while data are deficient for the flatback sea turtle (*Natator depressus*). The efforts of sea turtle rescue and rehabilitation initiatives facilitate access for sea turtle-focused research [20] and, consequently, studies on microbial communities of sea turtles are increasing.

To date, microbial assemblages of the sea turtle gut have been described by sequencing the 16S rRNA genes of fecal or cloacal samples in wild, stranded [21, 22], and rehabilitated green sea turtles [23, 24], in juveniles undergoing an ontogenetic shift from pelagic to neritic habitats [25, 26], and mucosa-associated bacterial communities in stranded green turtles [27]. Additionally, there are reports on the gut microbiota of Kemp's ridley turtles undergoing rehabilitation [28] and nesting flatback turtles [29, 30]. Loggerhead sea turtles' fecal and gut microbial communities have been studied mostly in stranded animals or undergoing rehabilitation in the Mediterranean Sea [31–33] with recent reports on nesting females of the USA and Australian populations [30, 34]. Furthermore, Scheelings and colleagues have performed one of the most comprehensive studies on the distal gut microbial communities of all seven species of the sea turtles reporting phylogenetic aspects of sea turtle microbiome evolution [34].

The focus of this study is on the loggerhead sea turtles' oral and distal gut microbiota in both recently caught and turtles undergoing rehabilitation at the Adriatic Sea turtle rescue centers. In addition to distal gut (cloacal) samples, we sampled the buccal (oral) cavity as there are no known reports on 16S rRNA profiling for oral microbial communities in sea turtles to the date of this study. Cultivation-based approaches have shown that oral bacterial communities of loggerhead sea turtles in the Mediterranean harbor antibiotic-resistant bacterial strains and common opportunistic pathogens [35, 36]. NGS amplicon sequencing of resistant bacterial isolates showed that injured Adriatic Sea loggerheads' wounds contain bacteria with multiple antibiotic resistance genes [37]. Aforementioned reports emphasize the idea of sea turtles as sentinel species that can be studied as indicators of marine health and pollution [35]. To fill in the gap in understanding the loggerhead sea turtle microbiota, we provide data on loggerhead oral microbial communities as the oral cavity is the first line in transitioning from external to internal environments of the turtle. The aims of this study were to describe oral and cloacal microbial communities of loggerhead sea turtles and compare them between incidentally caught or stranded and captive animals undergoing rehabilitation. Additionally, we investigated the impact of short-term rehabilitation period on loggerhead microbiota, which could clarify the dynamics of the loggerhead sea turtles' commensal microbes in relation to the turtles' changing environment.

Methods

Target population

We sampled loggerhead sea turtles from the Adriatic Sea that were found floating, stranded on beaches or incidentally caught by fishing boats and then transported to the regional veterinary clinic or rescue center: The Sea Turtle Clinic (STC) of the Department of Veterinary Medicine of University of Bari "Aldo Moro" (Italy) and the Marine Turtle Rescue Center Aquarium Pula (Croatia). Samples collected immediately upon arrival to the treatment facility are considered "wild" as they were taken close to the time of turtle capture and marked as "before" samples in further analyses and text. All turtles were examined for injuries and relevant information were collected during sampling. Healthy individuals were released within 24 h, while others were kept under observation ("short-term rehabilitation") or longer rehabilitation until recovered

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from injuries. List of sampled turtles is presented in Table 1 with an indication of release day.

Sampling of 12 loggerhead turtles (Table 1) was performed by trained personnel during December 2018 and January 2019 in accordance with the 1975 Declaration of Helsinki, as revised in 2013, and the applicable national laws.

Loggerheads' enclosure description

At the STC (Italy) the hospitalized turtles were kept in individual plastic tanks (approximately 1.5 m in diameter and 1 m in depth) with clean artificial saltwater (tap water with added NaCl at least up to 35 ppt salinity). The saltwater was changed every 2–3 days, with routine tank cleaning and disinfecting between saltwater changes. At the Marine Turtle Rescue Center Aquarium Pula (Croatia), the hospitalized turtle was kept in an individual plastic tank (2 m in diameter, 1.5 m in depth) with local seawater pumped and purified through the Aquarium's filtration systems. The tank was occasionally cleaned by scrubbing the algal overgrowth and grime off the tank walls. All turtles in the study were fed with diverse foods ranging from frozen (herring, codfish, mullet) or fresh fish food (squid, pilchard, and mackerel).

Sample collection

The loggerheads' cloacal and oral swab samples were collected either upon arrival of the turtle to the center (further regarded to as cloacal before, CB; oral before, OB) or within the rehabilitation period (after the turtle has spent time in the rescue center, further regarded to as cloacal rehabilitated, CR; oral rehabilitated, OR). When possible, we collected tank water during the rehabilitation period (further regarded to as tank water, W).

Oral swab samples were collected by gentle rotating of sterile dry cotton or synthetic swabs (Aptaca Nuova, Italy) on the tongue and palate mucosa, while cloacal samples were collected by inserting the swabs approximately 10 cm into the cloaca and rotating (Additional file 2: Figure S1). The swabs were collected in triplicate and stored individually in 97% ethanol at − 20 °C until DNA extraction. Samples of the tank water were collected prior to routine tank cleaning or during oral and cloacal sampling, in sterile containers and kept cool until arrival to the lab and filtering. Sampled tank water (250 ml) was vacuum filtered on a 45 mm in diameter, 0.2 µm pore-size sterile Whatman polycarbonate membrane filter (Sigma-Aldrich). Filters were carefully folded with sterile forceps and stored in 96% ethanol at − 20 °C until further processing. In total, 12 loggerhead turtles were sampled: three turtles were sampled twice (upon arrival and during rehabilitation), nine turtles were sampled once (five upon arrival, four during rehabilitation), and tank water samples were collected from three turtle enclosures (Table 1). Cloacal samples were collected from all turtles and sampling periods, while we could not obtain oral samples from three turtles (Table 1; ID010, ID034, and ID040).

DNA extraction and sequencing

Prior to DNA extraction the ethanol was removed from the tubes by pipetting (after centrifugation) and evaporation under laminar flow hood for 24 h. DNA from the filters and swabs was extracted with the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions with several modifications: (1) after transferring the swabs and filters to the PowerBead Tube the samples were incubated for 15 min at 65 °C, (2) instead of beadbeating PowerBead Tubes were vortexed horizontally for 10 min at maximum speed, and (3) all downstream incubation times at 2–8 °C were increased to 15 min. DNA was extracted from each swab and filter individually, and DNA concentrations were measured by NanoDrop ND-1000 V3.8 spectrophotometer (ThermoFisher). For samples with low DNA yield, triplicate DNA isolates were pooled together and concentrated according to the troubleshoot section of the DNeasy Powersoil Kit instructions. Extracted DNA was sent for PCR, library preparation, and 250 × 2 paired-end Illumina MiSeq v2 setup sequencing of the V3-V4 region of 16S rDNA with primers 341F_ill (5′-CCTACGGGNGGCWGCAG-3′) and 805R_ill (5′-GACTACHVGGGTATCTAATCC-3′) [38] to Microsynth (Switzerland).

Sequence analysis

Demultiplexed sequences with removed adapters and linker sequences were obtained from the Microsynth sequencing facility and quality checked with FastQC [39]. Upon inspection, reverse sequences were shown to be of insufficient quality and length in some samples, therefore only forward reads were used in downstream analyses with QIIME 2 2020.2 [40]. Forward demultiplexed reads (Casava 1.8 single-end demultiplexed fastq format) were imported to QIIME 2 and summarized using q2-demux plugin followed by denoising with DADA2 q2-dada2 plugin [41]. Forward sequences were trimmed at 5' end for 10 bp (primer removal) and truncated to 240 bp that produced a final sequence length of 230 bp. DADA2 dereplication produced amplicon sequence variants (ASVs) analogous to 100% operational taxonomic units (OTUs) [42]. ASVs were aligned with mafft [43] (via q2-alignment) and used to construct an unrooted phylogeny tree with fasttree2 [44] (via q2-phylogeny). Taxonomy was assigned to ASVs via q2-feature-classifier [45] classify-sklearn naïve Bayes taxonomy classifier against the SILVA ribosomal RNA sequence database (v. 132)

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Table 1 Information on sampled loggerhead sea turtles and their condition at the time of admission to the rescue centers in the Adriatic Sea

Turtle ID	Origin	Sampling date (YYYY-MM-DD)	Days before sampling	Sampling site			CCL (cm)	Weight (kg)	Sex	Life stage	Reason for admission to rescue center
				Oral	Cloacal	Water					
ID010	CRO, Korčula	2018-12-11	3	–	+	–	NA	40	F	adult	Old head injury; weight was measured prior to release 2019-11-04
		2019-04-08	121	–	+	+	69.7	46.5			
ID019	IT, Barletta	2019-01-09	0	+	+	–	50.7	31	ND	juvenile	Trawling (32 m depth), treated for gas embolism
		2019-01-11	2^R	+	+	–					
ID022	IT, Barletta-Trani	2019-01-10	0^R	+	+	–	72	42	M	adult	Trawling (22 m depth), released during the day
ID023	IT, Trani	2019-01-11	13	+	+	–	64	30.6	ND	juvenile	Trawling (36 m depth) hospitalized at the WWF center in fresh water (7 days leeches removal treatment)
ID026	IT, Molfetta	2019-01-21	6^R	+	+	+	64.5	32.1	M	adult	Found at the beach, not good clinical status
ID028	IT, Barletta	2019-01-17	0	+	+	–	74.5	46	F	adult	Trawling (34 m depth), gas embolism
ID030	IT, Barletta	2019-01-17	0	+	+	–	63	29.5	ND	juvenile	Trawling (34 m depth), gas embolism
		2019-01-21	4^R	+	+	+					
ID034	IT, Bisceglie	2019-01-22	0^R	–	+	–	72	45.6	F	adult	Trawling (40 m depth), good clinical status
ID040	IT, Barletta-Trani	2019-01-28	4^R	–	+	–	66	35.2	ND	juvenile	Trawling (40 m depth), good clinical status
ID041	IT, Barletta-Trani	2019-01-28	4^R	+	+	–	55.5	20	ND	juvenile	Trawling (22 m depth), gas embolism
ID042	IT, Barletta	2019-01-29	0^R	+	+	–	67.5	36.4	ND	juvenile	Trawling (40 m depth), good clinical status
ID044	IT, Bisceglie	2019-01-29	0^R	+	+	–	68	36.3	ND	juvenile	Trawling (30 m depth), good clinical status

Letter "R" in superscript next to the number of day before sampling indicates release into the wild on that day, if not present it means that the turtle was kept in the clinic or rescue center after the sampling

CCL curved carapace length, *ND* not determined

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[46]. Mitochondrial and chloroplast sequences were filtered out via q2-taxa prior to calculating alpha and beta diversity metrics via q2-diversity plugin.

Alpha diversity measurements, including Shannon's diversity index, observed ASVs, and Faith's phylogenetic diversity, were used for inspecting rarefaction curves to determine suitable sampling depth, and the differences between sampling sites were tested by Kruskal–Wallis H test. Beta diversity analyses were performed on rarefied dataset to 3200 sequences per sample to eliminate bias of different sampling depths [47, 48]. Comparisons of microbial communities were performed through Bray–Curtis, unweighted and weighted UniFrac [49, 50] Principal Coordinate Analyses (PCoA) via q2-diversity plugin. Due to intrinsic compositionality of microbial community datasets obtained by sequencing [51] we used an additional beta diversity analysis on non-rarified data through Robust Aitchison Principal Component Analysis (robust PCA; rPCA) via q2-deicode plugin [52]. Robust PCA is based on centered log-transformation and matrix completion, while retaining feature loadings that may discern between potential microbial niches. The analysis was performed after the exclusion of features with less than ten reads across samples. Log-ratios of rPCA feature loadings were inspected through q2-qurro plugin [53]. The permutational multivariate analysis of variance (PERMANOVA) was used to analyze beta diversity statistical differences via q2-diversity plugin, with the Benjamini–Hochberg false discovery rate (FDR) correction for multiple comparisons. Core features and genera (present in 80% or 85% of samples per sampling site) were determined via q2-feature-table plugin. All plots were visualized with ggplot2 [54] in RStudio (v. 1.3.959) and EMPeror [55].

Results

A total of 744 531 high-quality reads were obtained for 15 cloacal, 11 oral, and three tank water samples (29 samples in total). The samples had a mean (±SE) 25 673±3 265 sequences per sample that were clustered to 4476 ASVs (Additional file 1). Predominant phyla of cloacal samples consisted of Proteobacteria, Bacteroidetes, Kiritimatiellaeota, Firmicutes and Spirochaetes (>90% of all cloacal sequences). Oral samples' predominant phyla were Proteobacteria, Bacteroidetes and Planctomycetes (>90% of all oral sequences), while tank water exhibited high prevalence of Proteobacteria, Bacteroidetes and Epsilonbacteraeota (>90% of all tank water sequences). Taxa within phyla varied among individuals, sampling sites, and sampling periods (Additional file 1).

Alpha diversity metrics (Shannon's diversity, observed features, Faith's PD) were calculated for sampling sites; cloacal, oral, and tank water. No significant difference was observed ($p>0.05$, Kruskal–Wallis H test) for different sampling sites in either of alpha diversity metrics tested (Additional file 2: Table S2). Tank water did exhibit higher variation than cloacal and oral samples, possibly due to sample size and differences in origin (artificial saltwater in Italy vs. filtered sea water in Croatia that showed greater diversity) (Fig. 1), but it was not significantly different from other sampling sites (Additional file 2: Table S2).

Bacterial communities of cloacal samples tended to cluster together, regardless of the sampling period, but oral sample communities showed some separation based on sampling before or during rehabilitation according to PCoA plots (Figs. 2a, 3) and rPCA biplot (Fig. 2b). Tank water samples did not show a visible pattern for Bray–Curtis PCoA or Robust Aitchison PCA (Fig. 2), but for UniFrac PCoA the samples tended to cluster near oral samples (Fig. 3). Feature loadings of Robust Aitchison PCA represent highly ranked individual ASVs, mostly uncultured Gammaproteobacteria, *Rhodobacteraceae*, and members of the Kiritimatiellae WCHB1-41 group (Fig. 2b).

Based on PERMANOVA (with 999 permutations) bacterial communities differed significantly ($p<0.05$) between sampling sites and periods (cloacal before, CB; cloacal rehabilitated, CR; oral before, OB; oral rehabilitated, OR; tank water, W) for all used distance metrics tested (Bray–Curtis $p=0.001$, pseudo-F $=2.37$; Robust Aitchison $p=0.002$, pseudo-F $=3.68$; unweighted UniFrac $p=0.001$, pseudo-F $=2.38$; weighted UniFrac $p=0.001$, psuedo-F $=3.59$). Pairwise PERMANOVA testing for sampling site and period groups

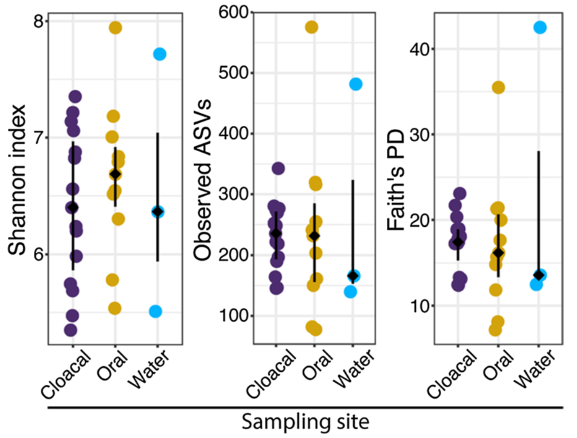

Fig. 1 Alpha diversity (Shannon index, observed ASVs, Faith's Phylogenetic Diversity) of loggerhead cloacal (purple), oral (yellow), and tank water (blue) samples. Filled diamond indicates sample median with lines extending to the upper and lower quartile of sample distribution

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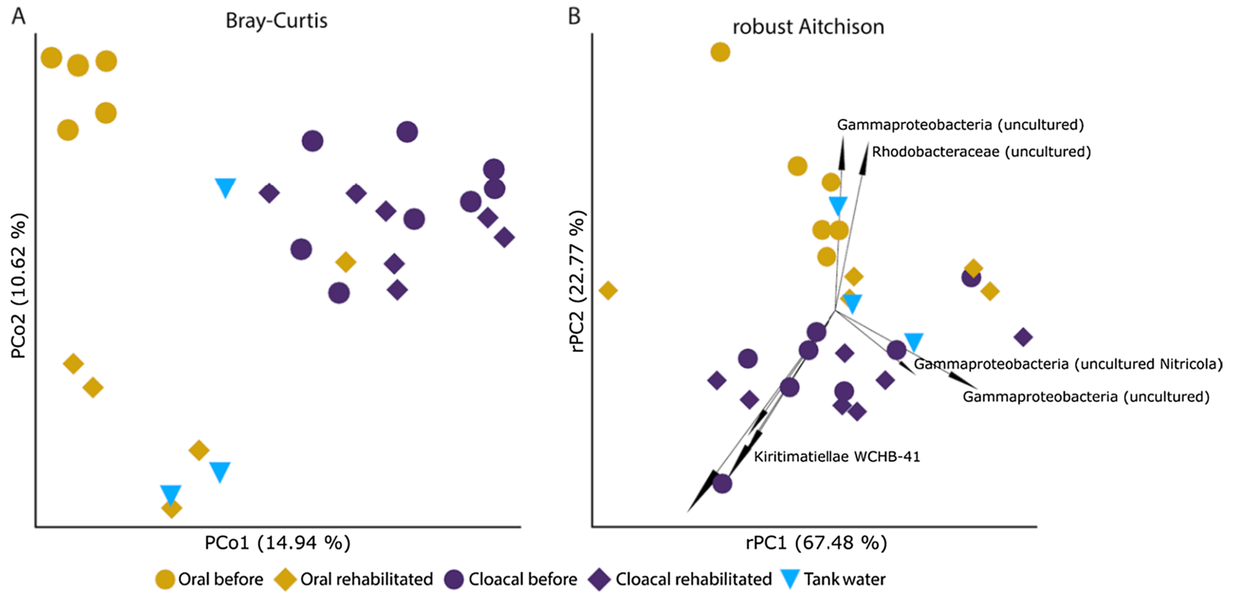

Fig. 2 Comparison of microbial diversity in loggerhead cloacal, oral and tank water samples **a** principal coordinate analysis (PCoA) plot of Bray–Curtis distances and **b** principal component analysis (PCA) biplot of robust Aitchison distances with loadings as individual highly ranked ASVs

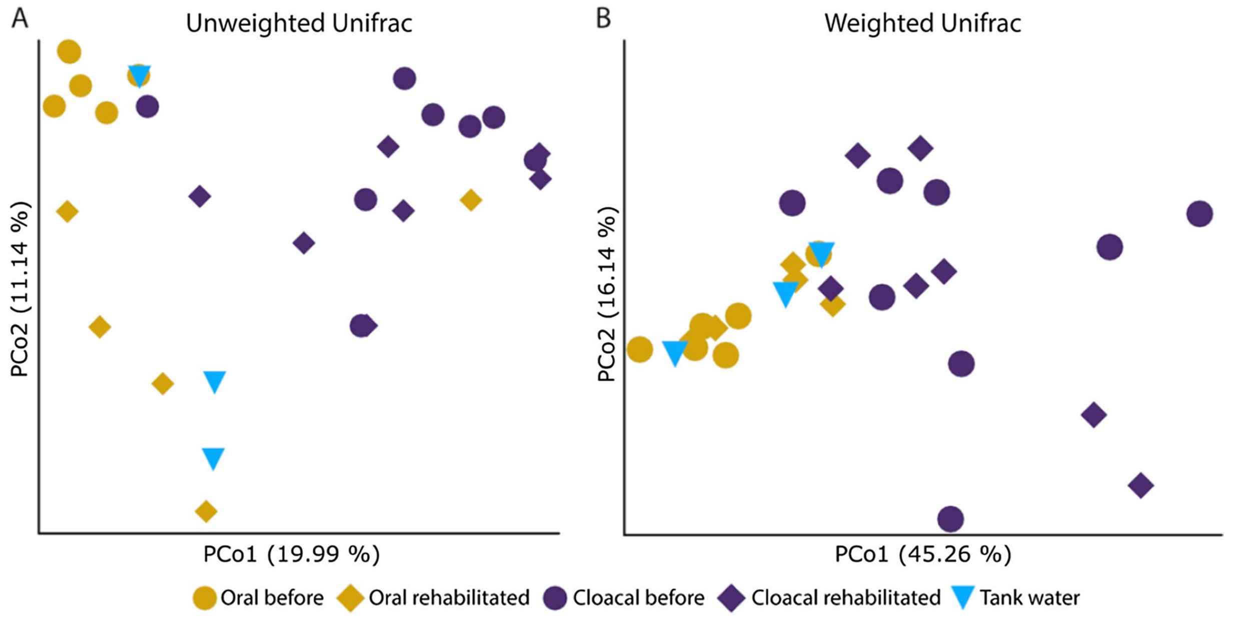

Fig. 3 Comparison of microbial diversity in loggerhead cloacal, oral and tank water samples. Principal component analysis (PCoA) plot of **a** Unweighted Unifrac and **b** Weighted Unifrac

differed between metrics used with the most conservative result obtained from Robust Aitchison distance that detected a significant difference only between CR versus OB ($p = 0.005$, pseudo-F = 12.27) and CB versus OB ($p = 0.005$, pseudo-F = 10.40). Bray–Curtis distance, unweighted and weighted UniFrac distances pairwise test results showed a significant difference for CB versus OR and OR versus OB in addition to the same sampling site

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and period conditions that were observed with Robust Aitchison distance pairwise testing. No significant difference was detected between CB and CR samples. Out of all tested metrics, Bray–Curtis and unweighted UniFrac pairwise test results showed a significant difference ($p < 0.05$) among W versus CB, CR, and OB. We detected no significant difference between W and OR samples, which points to the effects of tank water on the oral microbiota of turtles in rehabilitation. Summary of pairwise PERMANOVA tests per distance metric is shown in Additional file 2: Table S3. Visual inspection of natural log ratios of up to 20% top and bottom feature loadings of the Robust Aitchison PCA biplot (Fig. 2b) shows clear segregation of oral before and oral rehabilitated samples (5%, 10%, and 20% top and bottom features on rPC1), and similar log-ratio values of all cloacal samples to oral rehabilitated samples (20% top and bottom features on rPC2) (Fig. 4).

Bacterial communities were distributed across eleven dominant phyla present at > 1% relative abundance in at least one sampling site (Fig. 3). All sampling sites shared dominant phyla Proteobacteria, Bacteroidetes, and to a lesser extent Epsilonbacteraeota (Table 2). Firmicutes were shared between cloacal and oral samples, while tank water and oral samples shared Actinobacteria. Specific to cloacal samples were Kiritimatiellaeota, Spirochaetes and Lentisphaerae phyla, oral samples harbored *Planctomycetes,* and tank water Patescibacteria and Verrucomicrobia (Fig. 5).

Further, bacterial taxa classified to genera (or the next available classification level) present at > 2% relative abundance in at least one sampling site and period conditions indicate taxa specificity to habitat and, on the other hand, the possibility of sharing bacterial taxa of the turtle endomicrobiota with the environment (e.g. Bizionia in oral rehabilitated and tank water bacterial communities) (Table 3). WCHB1-41 taxon (phylum Kiritimatiellaeota) was shown to be almost exclusive for cloacal samples (even though turtle ID010 has not had any sequences of that taxon detected), along with *Treponema* 2, *Aeromonas*, unclassified Aeromonadales, *Desulfovibrio*, unclassified *Rikenellaceae*, and *Bacteroides* genus. Oral samples often shared taxa with cloacal and tank water samples with noticeable differences in relative abundance of *Pseudoalteromonas* and unclassified *Helieaceae* that was not found at > 2% in cloacal samples or tank water. Interestingly, only tank water harbored *Bermanella* as it was not detected in cloacal nor oral samples (Table 3). Based on PERMANOVA results (Additional file 2: Table S3), wild oral samples (before) and oral microbiota during rehabilitation differ significantly, which is also reflected in relative abundances of microbial taxa abundance (Table 3). Wild oral samples harbored more Bacteroidales, *Tenacibaculum*, *Rhodobacteraceae*,

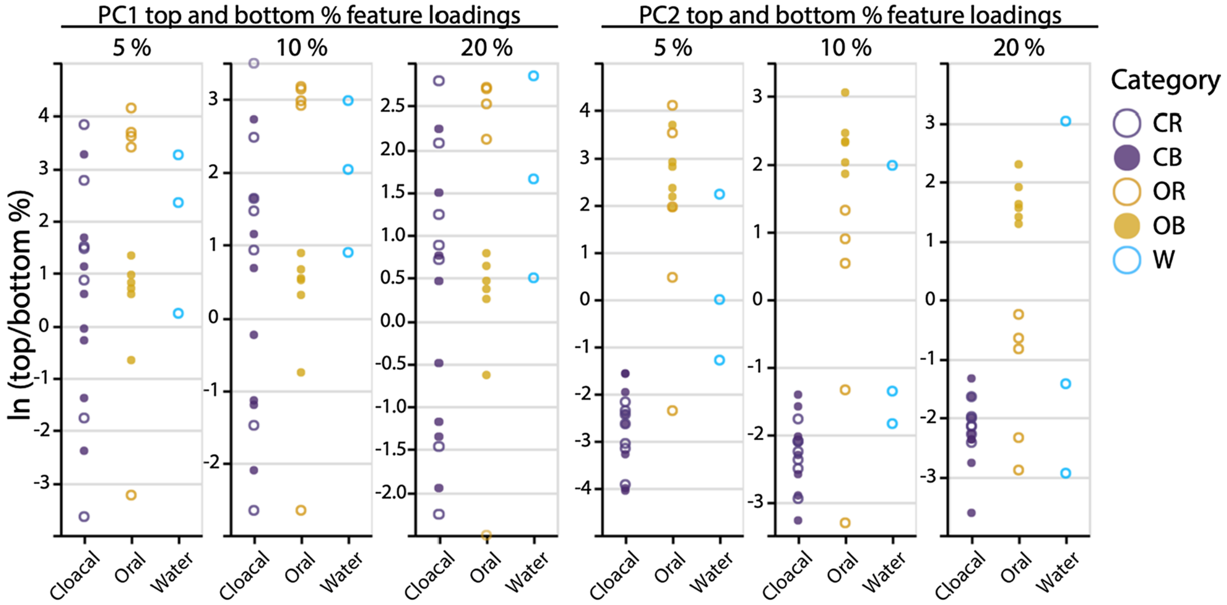

Fig. 4 Natural log-ratios (plotted by QURRO) of loggerhead samples' top and bottom 5%, 10%, and 20% of feature loadings on rPC1 and rPC2 of Robust Aitchison PCA biplot feature loadings by sampling site and period: CB, cloacal before; CR, cloacal rehabilitated; OB, oral before; OR, oral rehabilitated; W, tank water

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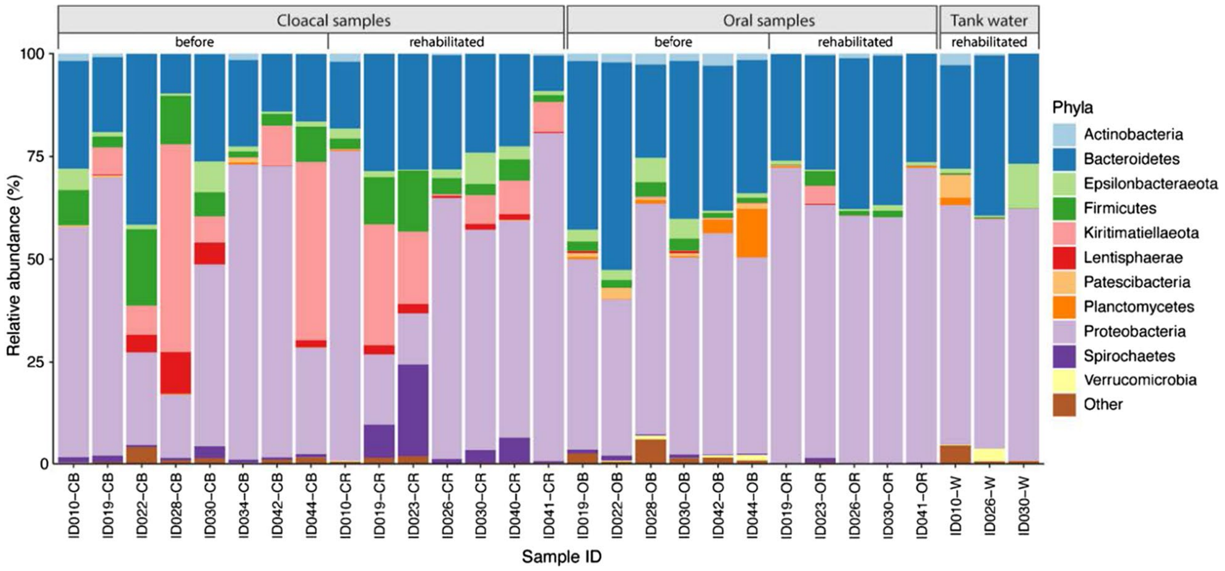

Fig. 5 Relative abundances (%) of bacterial phyla present (> 1% on average per sampling site) in loggerhead cloacal, oral and tank water samples. Turtle ID suffix indicates the sampling site and period (before or during/after rehabilitation) as follows: CB, cloacal before; CR, cloacal rehabilitated; OB, oral before; OR, oral rehabilitated; W, tank water

Table 2 Bacterial phyla of loggerhead sea turtle cloacal and oral samples, and tank water samples from the rescue centers present at > 1% relative abundance on average per sampling site

Bacterial phyla	Cloacal (n = 15)	Oral (n = 11)	Tank water (n = 3)
Actinobacteria	0.52 ± 0.18	**1.37 ± 0.31**	**1.09 ± 0.87**
Bacteroidetes	**21.74 ± 2.16**	**33.88 ± 2.43**	**30.26 ± 4.33**
Epsilonbacteraeota	**2.48 ± 0.63**	**2.02 ± 0.57**	**4.15 ± 3.36**
Firmicutes	**6.74 ± 1.35**	**1.75 ± 0.37**	0.22 ± 0.08
Kiritimatiellaeota	**12.78 ± 4.04**	0.46 ± 0.39	0.03 ± 0.03
Lentisphaerae	**1.99 ± 0.72**	0.14 ± 0.06	0.06 ± 0.03
Patescibacteria	0.17 ± 0.08	0.60 ± 0.24	**1.83 ± 1.83**
Planctomycetes	0.08 ± 0.04	**1.65 ± 1.06**	0.63 ± 0.61
Proteobacteria	**48.60 ± 6.21**	**56.08 ± 3.19**	**58.62 ± 1.56**
Spirochaetes	**3.30 ± 1.47**	0.44 ± 0.14	0.01 ± 0.01
Verrucomicrobia	0.03 ± 0.01	0.30 ± 0.14	**1.14 ± 0.97**

Values represent mean percentage ± SE, with mean values above 1% in bold

Gammaproteobacteria and *Halieaceae*, in comparison to oral samples from turtles in rehabilitation, which showed greater abundance of *Bizionia*, *Pseudoalteromonas*, *Shewanella*, *Pseudomonas*, and *Vibrio*, similar to cloacal and tank water samples (Table 3).

Cloacal samples exhibited two core ASVs (present in more than 85% of samples (12/15)); *Kiritimatiellae* WCHB1-41 and *Treponema* 2. Oral samples did not show any core ASVs at 85% cutoff, but at 80% (8/11 samples) four putative core ASVs were detected, belonging to *Gammaproteobacteria*, *Rhodobacteraceae*, *Pseudoalteromonas*, and *Halieaceae*.

Core taxa collapsed to genus level (present in more than 85% of samples per sampling site) for cloacal samples consisted of uncultured WCHB-41, *Desulfovibrio*, *Bacteroides*, *Shewanella*, *Treponema* 2, *Psychrobacter*, uncultured *Cardiobacteriaceae*, uncultured *Rikenellaceae*, uncultured *Clostridiales* vadin BB60 group, and unassigned *Lachnospiraceae*. Oral samples putative core genera were *Tenacibaculum, Flavobacterium*, and unclassified *Halieaceae*. Genera present both in cloacal and oral samples are *Vibrio*, *Marinifilum*, *Fusibacter* and *Arcobacter* (see Additional file 3).

Discussion

The results of our research on the microbiota of loggerhead sea turtles show that oral and cloacal microbial communities differ, and that oral microbial assemblages are less stable than cloacal in regard to the turtles' changing environment (wild *versus* veterinary clinic enclosures). We provide the first insights into oral bacterial communities of incidentally caught wild loggerhead sea turtles and deliver information on how the oral microbiome might respond to short-term rehabilitation in the recovery rescue centers. While most previous studies from the Mediterranean were based on gut microbiome from sick turtles found stranded or dead [31–33] this paper mostly encompasses loggerheads from the wild,

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Table 3 Bacterial taxa of loggerhead sea turtle cloacal and oral, and tank water samples classified to the genus (or higher taxonomic level) present at > 2% average relative abundance in at least one sampling site and period category (before or wild and during rehabilitation)

Bacterial taxa	**Cloacal samples**		**Oral samples**		**Tank water**
	before (n = 9)	**rehabilitated (n = 6)**	**before (n = 7)**	**rehabilitated (n = 4)**	**rehabilitated (n = 3)**
Phylum Bacteroidetes					
Bacteroidales; unclassified	1.86 ± 0.60	**2.45 ± 1.07**	**2.21 ± 0.28**	0.19 ± 0.09	0.30 ± 0.24
Bacteroides	**2.09 ± 0.61**	1.73 ± 0.76	0.10 ± 0.10	ND	0.13 ± 0.13
Marinifilum	1.56 ± 0.55	**4.23 ± 1.87**	1.37 ± 0.54	0.74 ± 0.32	0.54 ± 0.37
Rikenellaceae; unclassified	**3.03 ± 1.29**	1.10 ± 0.73	0.14 ± 0.13	ND	0.03 ± 0.03
Flavobacteriaceae; unclassified	**2.22 ± 0.88**	1.94 ± 0.33	**13.78 ± 2.93**	**11.54 ± 4.45**	**6.79 ± 1.70**
Bizionia	ND	1.29 ± 0.77	0.03 ± 0.03	**11.51 ± 4.27**	**6.25 ± 6.04**
Flavobacterium	0.10 ± 0.05	0.84 ± 0.49	**2.23 ± 2.12**	**2.16 ± 1.07**	**5.70 ± 5.26**
Tenacibaculum	0.39 ± 0.13	0.50 ± 0.15	**3.44 ± 1.96**	0.81 ± 0.24	**2.77 ± 2.18**
Phylum Kiritimatiellaeota					
WCHB1-41; unclassified	**15.45 ± 6.13**	**8.56 ± 4.26**	0.69 ± 0.61	0.02 ± 0.02	0.03 ± 0.03
Phylum Proteobacteria					
Rhodobacteraceae; unclassified	1.69 ± 0.98	1.08 ± 0.24	**8.18 ± 1.63**	**4.45 ± 1.32**	**3.47 ± 1.41**
Desulfovibrio	**2.76 ± 0.65**	1.54 ± 0.59	0.37 ± 0.15	0.07 ± 0.07	0.08 ± 0.08
Gammaproteobacteria; unclassified	**11.03 ± 5.02**	**22.69 ± 6.90**	**13.83 ± 2.50**	**3.33 ± 2.01**	**2.96 ± 2.10**
Aeromonadales; unclassified	0.19 ± 0.19	**4.74 ± 4.35**	ND	0.01 ± 0.01	ND
Aeromonas	**3.56 ± 3.46**	0.01 ± 0.01	0.44 ± 0.44	ND	0.03 ± 0.03
Colwellia	0.21 ± 0.20	0.04 ± 0.03	0.32 ± 0.18	0.81 ± 0.40	**3.29 ± 2.94**
Pseudoalteromonas	**2.16 ± 1.55**	**2.94 ± 1.12**	1.70 ± 1.04	**19.52 ± 7.69**	**3.13 ± 2.19**
Shewanella	1.54 ± 0.55	**7.15 ± 2.07**	1.76 ± 1.74	**3.86 ± 2.07**	0.77 ± 0.68
Halieaceae; unclassified	0.07 ± 0.06	0.03 ± 0.02	**2.16 ± 0.62**	1.68 ± 0.92	0.02 ± 0.02
Bermanella	ND	ND	ND	ND	**6.20 ± 6.20**
Pseudomonas	0.68 ± 0.33	1.63 ± 0.81	**2.95 ± 2.95**	**5.82 ± 1.99**	**14.10 ± 7.58**
Vibrio	**7.24 ± 3.09**	**3.07 ± 1.01**	1.40 ± 0.50	**8.43 ± 4.17**	1.09 ± 0.44
Phylum Spirochaetes					
Treponema 2	**3.12 ± 2.29**	**2.22 ± 1.02**	0.12 ± 0.11	ND	ND

Values represent mean percentage ± SE, with mean values above 2% in bold

ND not detected

incidentally caught during fishing activities. Thus, we consider microbial communities in samples taken prior to admission to the rescue center or clinic as a close representative of the wild microbiome, comparable to recent studies on wild, nesting, adult loggerhead females intestinal microbiome [30, 34]. Only two turtles in this study had to be hospitalized for longer periods due to head injuries (turtle code ID010) or leeches parasitization (turtle code ID023). On the other hand, oral microbiota of sea turtles has not yet been explored by 16S rRNA gene sequencing, while it has been investigated in the freshwater Krefft's river turtle (*Emydura macquarii krefftii*) and pond slider turtle (*Trachemys s. scripta*) [56, 57].

In our study, alpha diversity metrics did not show significant differences between oral and cloacal body sites or sampling periods (before and during rehabilitation). This could be explained by the size of our target population (relatively small), with juveniles and adults of similar nutritional status, which is insufficient for discovering potential characteristics that could be associated with microbial diversity of samples on this level. In oral microbiomes, ID023 turtle sample is an outlier with much higher microbial diversity, which may be linked with its rehabilitation in the WWF care facility where it was undergoing freshwater treatment for leeches removal prior to admission to the rescue center where it was sampled. Tank water samples from the Aquarium Pula local circulating seawater showed much higher diversity with frequent marine microbial taxa, in comparison to water from the STC in Bari that harbored non-circulating artificial saltwater. Further, aquarium seawater tank exhibited a similar trait to seawater samples in a study by Biagi et al. [33], having a higher diversity of low abundance phyla. Aquarium tank water also had higher abundances

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of phyla Planctomycetes and Patescibacteria which were observed mostly in oral samples before hospitalization. Therefore, the aquarium recirculating tank water could present a more "natural" marine habitat rather than the tanks with artificial seawater.

Beta diversity metrics consistently showed separation of cloacal and oral microbiomes but with different significance detection between sampling period depending on the metric tested by PERMANOVA. Beta diversity measures used in most sea turtle microbiome studies are still Bray–Curtis dissimilarity, unweighted, and weighted Unifrac even though they do not account for the compositionality of microbiome datasets obtained by sequencing [51]. Due to data compositionality, we decided on presenting already widely accepted beta diversity metrics PCoA along with the robust Aitchison distance PCA,argued to be a better choice for compositional data [52, 58]. Our combined results indicate strong differences between wild cloacal microbiota versus both oral sample periods. Moreover, no significant differences were found among tank water and oral rehabilitated microbiota, which emphasizes the impact of the environment on oral microbiota of loggerhead sea turtles.

Reptile oral microbiomes were considered to be influenced by the prey fecal microbiome but Zancolli et al. [57] observed distinct species-specific patterns in snakes and freshwater turtles that undermine the assumption that reptiles' oral cavity is a passive reservoir of microbes. As sea turtles are mostly submerged and in close contact with the water medium (sea), we hypothesized that oral microbiome would resemble the environment. As expected, oral samples clustered based on sampling period with samples before rehabilitation clustered closer to the aquarium free-circulating tank water while oral rehabilitated clustered closer to tank water of enclosures with non-filtered artificial seawater (Bray–Curtis and unweighted Unifrac PCoA). No significant differences were observed between oral and tank water samples, but specific bacterial taxa not found in tank water suggest that the oral microbiome consists, at least partially, of endogenous and transitional microbes from the environment. Proteobacteria and Bacteroidetes, abundant in oral microbiota in our study, were also dominantly present in the oral microbiome of Krefft's river turtle, which was markedly different from the external turtle microbiome and the environment [56]. Comparisons beyond phylum level show that Krefft's river turtle and pond slider turtle share *Burkholderiaceae* and *Weeksellaceae* families not detected in our study [56, 57] while *Flavobacteriaceae* are shared between Kreffts and loggerheads.

In our study, we detected high abundances of ASVs which could not be classified to genera but only to higher taxonomic ranks: Bacteroidales, *Flavobacteriaceae*, *Rhodobacteraceae*, and Gammaproteobacteria. Highly abundant oral ASVs often overlapped in classification with highly abundant taxa in water tanks, but the actual taxonomic diversity between those groups remains to be determined as overly unclassified reads could implicate a high incidence of yet undiscovered bacteria, or insufficient sequence length required for successful taxonomical identification. Interestingly, the genus *Pseudoalteromonas* was more abundant in oral microbiomes of rehabilitated turtles, while unclassified *Halieaceae* were more abundant in oral microbiomes before rehabilitation than in any other sample type. *Halieaceae* are often found in coastal, neritic environment, deep-sea waters, and in demersal animals (e.g. sponges) [59, 60], hence, they could be easily transported from the marine environment and into the oral cavity. *Pseudoalteromonas* spp. are marine bacteria known for production of antimicrobial substances with many of the species found in association with marine eukaryotes [61] which has been proposed as beneficial to its marine hosts [62]. It is possible that the low abundance taxa in wild oral microbiota are enriched by the veterinary clinic's enclosure environment conditions; temperature and nutrient availability are relatively stable in comparison to the turtle's natural habitat. Other taxa that had higher abundances were also notably present in cloacal (*Vibrio* spp., *Shewanella* spp.) or tank water samples (*Pseudomonas* spp., *Bizionia* spp.), which could be transient and non-specific for oral microbiome. At this point, little data are available to compare aquatic turtles' oral microbiomes beyond the superficial taxonomic levels, and according to our results habitat has a significant effect on the sea turtle oral microbiota. Additional sampling across many different groups of turtles and their habitats would be needed to decouple the effects of the habitat from the intrinsic and possibly representative oral microbes. Even though effects of oral microbial communities on the host have been described in humans and other mammals, it is unknown what roles reptile microbiome may have, especially in marine species [15, 63].

Cloacal microbiome samples did not show any significant clustering of different sample traits in our study design, which is consistent with previous reports [31, 32], but there have been reports on effects of the CCL on cloacal microbiota clustering [33]. As sea turtles often exhibit ontogenetic habitat shift and transit from pelagic to neritic prey, the change in the microbiota regarding to the size and age of the individual could be explained by changed preferences in habitat and food. In juvenile green turtles, there is a significant variation in cloacal microbiomes between pelagic and neritic habitats and transition to herbivorous lifestyle [25]. Additionally, green turtles in rescue centers exhibit a microbiome shift

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depending on the type of food they receive during rehabilitation, where the fecal microbiome constitutes of bacterial communities prepped for higher protein content as the recovering turtles are fed mostly seafood, but the community shifts to communities known for metabolization of plant polysaccharide upon introduction of plant food near the end of the recovery [24]. The developmental shift from pelagic to neritic habitats of loggerheads in the central Mediterranean Sea is more relaxed, where juveniles have a short epipelagic stage but later choose the habitat opportunistically, according to food availability and oceanographic features [64]. Consequently, shallow north-central Adriatic Sea enables early recruitment to the neritic habitats where rich and diverse benthic pray is available even to small juveniles (< 30 cm) [64]. Presented microbiome of Adriatic Loggerheads seems to confine with satellite tracking and tagging studies that suggest long-term residence of both adults and juveniles in the shallow neritic Adriatic, with seasonal migrations along the Italian coast to the south during winter [65]. Hence, the differences observed in fecal, cloacal and intestinal microbial communities between loggerheads sampled in the central Mediterranean [31, 32], Australia, or USA [30, 34], may be partially explained by highly opportunistic feeding nature and food availability for sampled turtle populations.

The most comprehensive loggerhead microbiota studies from geographically and genetically distinct healthy nesting females [30, 34], usually linked to neritic feeding grounds, reported that microbial communities of the sea turtle gut are dominated by Proteobacteria, followed by Spirochaetes, Bacteroidetes and Actinobacteria. This coincides with our results on wild and early rehabilitation microbial profiles of cloacal samples. On the other hand, microbial communities dominated by higher proportions of Fusobacteria, Firmicutes, and Bacteroidetes with low abundance of Proteobacteria may be considered atypical and describe fecal microbiota of rehabilitated or stranded turtles, connected with the turtle health status [31–33].

The only study on loggerhead microbiome from the Adriatic Sea [33] on fecal microbial communities of stranded or turtles captured in trawling nets showed high abundance of Firmicutes and Fusobacteria, while Bacteroidetes and Proteobacteria were not pronounced. A significant portion of microbial taxa they reported belonged to *Cetobacterium* and *Clostridium* genera, which were not observed in our study. Since these Adriatic loggerheads shared a similar ecological niche and foraging habitats, described non-Proteobacteria dominated microbiome [33] probably arises from their health status, changes in immunity, rehabilitation treatments, recorded period of starvation, and sampling feces rather than the intestine or cloaca. In our study, we detected two putative core cloacal ASVs belonging to WCHB1-41 and *Treponema* 2; while uncultured Clostridiales and *Lachnospiraceae* were detected as putative core taxa and were not overly abundant. Within phylum Bacteroidetes, major components were *Bacteroides,* which have been observed in loggerhead fecal microbiomes [32, 33] and mammalian microbiome [66], *Marinifilum* spp. (commonly found in seawater), unclassified *Rikenellaceae* (specialized for the digestive tract of different animals) [67] and unclassified *Flavobacteriaceae* found in a wide variety of habitats. Interestingly, a major proportion of reads in cloacal samples belonged to the novel Kiritimatiellaeota phylum (formerly in Verrucomicrobia) and were identified as uncultured eubacterium WCHB1-41 [68]. Uncultured WCHB1-41 have been found in equine vaginal and distal gut microbiome, and rumen of cattle [69–71]. Verrucomicrobia have been found in loggerhead cloacal and fecal samples [33, 34] and it is possible that at least a portion of Verrucomicrobia reads would be classified as Kiritimatiellaeota if SILVA v.132 was used to assign the taxonomy, as in this study and study by Arizza et al. [32].

When discussing the representative microbiome of the turtle gut, it is important to discern between the fecal microbiome that is often affected by food composition [24] and is a better descriptor of gut lumen microbiome, *versus* the microbial communities attached to the mucosa and in direct contact with the host, which might or might not be influenced by the shifts in habitat, environment and food type availability [72]. In our study, we used swabs for both oral and cloacal samples rather than feces, as collecting swab samples is less time-consuming in comparison to collecting fecal samples, relatively non-invasive to the turtle and may be performed during fieldwork or within rescue centers. Our results show that cloacal swabs might be sufficient to describe microbial communities as a proxy to feces and intestinal samples, which would allow for wider and less invasive sampling of loggerheads. Sampling wild microbial communities of loggerheads (among other sea turtles and reptiles in general) is necessary to gain basic insights into reptile microbiomes. A recent study in bacterial communities of wild animals via de-novo metagenome assembly showed that wild microbiomes are a resource for novel bacterial species and biological functions [17]. Furthermore, when identifying unknown bacterial genomes of Reptilia microbiota consisted predominantly of novel microbial members and are under sampled in most meta-microbiome studies [6, 9, 17]. Higher abundances of unclassified members of Proteobacteria, Bacteroidetes and other phyla might then prove to be reservoirs of novel bacterial species with interesting features.

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Microbial community studies should inform conservation efforts and rehabilitation facilities in ways to improve treatments, housing conditions, and preparation for the release of rehabilitated turtles. In this study, we show that the oral microbiota is potentially less stable and more prone to the acquisition of external microbial taxa in comparison to the relatively stable cloacal microbiota. Implications and effects of long-term rehabilitation of turtles in tanks with non-circulating artificial seawater on the turtles are still unknown and should be investigated further. Due to the sensitivity of oral microbiota to external conditions it should be noted that local circulating seawater should be preferred in rescue centers whenever possible, to preserve and enrich bacterial communities.

Conclusions

Our work provided the first insights into oral and cloacal microbiota of incidentally caught and mostly healthy loggerhead sea turtles before admission to the rescue center or clinic and after rehabilitation. Other studies focused on hospitalized, dead, and stranded Mediterranean loggerheads [31–33] while our research provided mostly healthy, wild microbiota information as in recent studies on nesting female loggerheads [30, 34]. We showed that cloacal microbiota remains relatively stable during short-term hospitalization, which has been shown in previous studies. Even though loggerhead oral microbial communities do not completely resemble the microbiota of the turtle's environment, they are dynamic and change swiftly as they accommodate taxa from a new environment. Furthermore, cloacal and oral swabs are sufficient for description of microbial communities of loggerheads and allow quick and non-invasive sampling. As reptile microbial communities are still less investigated, wild sea turtle microbiota characterization provides essential information for the expansion of our knowledge on sea turtle biology and guidelines on how to improve on the conservation efforts for these vulnerable, and highly important keystone species in marine ecosystems.

Abbreviations

ASV: Amplicon sequence variants; CB: Cloacal before; CCL: Curved carapace length; CR: Cloacal rehabilitated; DNA: Deoxyribonucleic acid; IUCN: International Union for Conservation of Nature; OB: Oral before; OR: Oral rehabilitated; PCoA: Principal coordinate analysis; rPCA: Robust Principal component analysis; rRNA: Ribosomal ribonucleic acid; STC: Sea Turtle Clinic; USA: United States of America; W: Tank water; WWF: World Wildlife Fund.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-021-00120-5.

Additional file 1: Table S1. Sequencing results (raw counts) and taxonomy assignments per ASV for all samples in this study.

Additional file 2: Figure S1. Oral (A) and cloacal (B) sampling of loggerhead sea turtle at the Sea Turtle Clinic of the Department of Veterinary Medicine of University of Bari (Italy). Courtesy of Adriana Trotta. **Table S2.** Alpha diversity measures (Shannon's diversity, observed ASVs. Faith's Phylogenetic Diversity) for cloacal, oral and tank water sampling sites with Kruskal–Wallis H test results. Values are represented as mean ± SD, with significance level $\alpha < 0.05$. **Table S3.** A comparison of differences in microbial communities of different sampling sites and periods by pairwise PERMANOVA for Bray–Curtis, Robust Aitchison, unweighted and weighted UniFrac distance metrics. SampSling sites and periods are marked as follows: CB, cloacal before; CR, cloacal rehabilitated; OB, oral before; OR, oral rehabilitated; W, tank water. P-values shown have been FDR corrected. Significance levels are indicated by an asterisk: $p \leq 0.05^*$, $p \leq 0.01^{**}$ with all significant values bolded.

Additional file 3: Table S4. Core taxa of cloacal and oral microbiota at ASV level (85% and 80% cutoff, respectably) and collapsed to the genus level (85% cutoff).

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Authors' contributions

AT, RG, AB, MC, and SB concepted and designed the study; AT and SB collected the samples; AT and KF carried out the laboratory work; KF conducted the bioinformatics, statistical analyses, and data visualization and interpretation; KF wrote the first draft of the manuscript; All authors revised the paper and approved the final version of the manuscript.

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Availability of data and materials

The amplicon sequencing data obtained in this study are available in the European Nucleotide Archive under accession number PRJEB46638. Additional information about data analysis and extended metadata are available in Mendeley Data repository (https://doi.org/10.17632/45skyv5hzy.1).

Declarations

Ethical approval and Consent to participate

Sampling was performed in accordance with the 1975 Declaration of Helsinki, as revised in 2013 and the applicable national laws. The sampling at the Sea Turtle Clinic (Bari, Italy) was conducted with the permission of the Department of Veterinary Medicine Animal Ethic Committee (Authorization # 4/19), while sampling in Croatia was done in accordance with the authorization of the Marine Turtle Rescue Center by the Ministry of Environment and Energy of the Republic of Croatia.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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Additional file 1: Table S1. Sequencing results (raw counts) and taxonomy assignments per ASV for all samples in this study.
Can be accessed at https://doi.org/10.1186/s42523-021-00120-5

Additional file 2: Figure S1. Oral (A) and cloacal (B) sampling of loggerhead sea turtle at the Sea Turtle Clinic of the Department of Veterinary Medicine of University of Bari (Italy). Courtesy of Adriana Trotta. **Table S2.** Alpha diversity measures (Shannon's diversity, observed ASVs. Faith's Phylogenetic Diversity) for cloacal, oral and tank water sampling sites with Kruskal-Wallis H test results. Values are represented as mean ± SD, with significance level $\alpha < 0.05$. **Table 3S.** Comparison of differences in microbial communities of different sampling sites and periods by pairwise PERMANOVA for Bray-Curtis, Robust Aitchison, unweighted and weighted UniFrac distance metrics. Sampling sites and periods are marked as follows: CB, cloacal before; CR, cloacal rehabilitated; OB, oral before; OR, oral rehabilitated; W, tank water. P-values shown have been FDR corrected. Significance levels are indicated by an asterisk: $p \leq 0.05$*, $p \leq 0.01$** with all significant values bolded.

Additional file 3: Table S4. Core taxa of cloacal and oral microbiota at ASV level (85% and 80% cutoff, respectably) and collapsed to the genus level (85% cutoff).
Can be accessed at https://doi.org/10.1186/s42523-021-00120-5

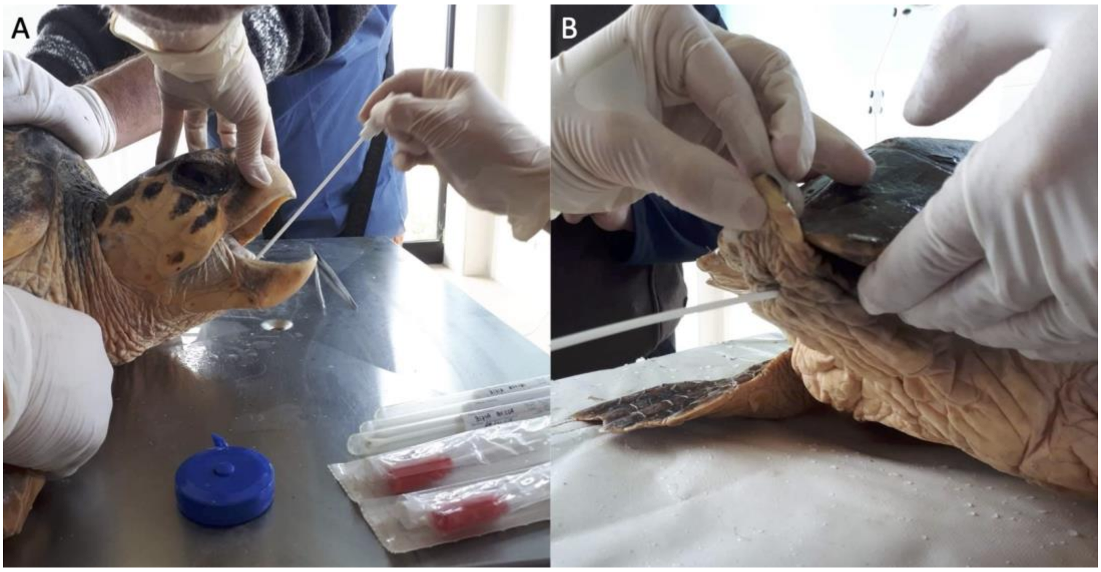

Figure S1.

Table S2.

	Cloacal	Oral	Tank water	p	H
Shannon	6.41 ± 0.66	6.65 ± 0.66	6.53 ± 1.11	0.75	0.59
Observed ASVs	230.93 ± 55.44	237.64 ± 138.24	262.33 ± 189.82	0.89	0.24
Faith's PD	17.30 ± 3.27	17.24 ± 7.72	22.85 ± 17.02	0.89	0.24

Table S3.

		Bray-Curtis		Robust Aitchison		unweighted UniFrac		weighted UniFrac	
Groups	**n**	**pseudo-F**	**p-value**	**pseudo-F**	**p-value**	**pseudo-F**	**p-value**	**pseudo-F**	**p-value**
CB vs. CR	15	1.120	0.284	0.200	0.931	1.094	0.278	0.492	0.804
CB vs. OR	13	2.490	**0.008****	2.280	0.186	2.426	**0.016***	4.227	**0.013***
CB vs. OB	14	3.630	**0.005****	10.400	**0.005****	3.890	**0.003****	6.656	**0.005****
CR vs. OB	13	3.970	**0.008****	12.270	**0.005****	4.020	**0.003****	7.487	**0.005****
CR vs. OR	12	2.400	**0.005****	2.280	0.186	1.908	0.051	4.148	**0.024***
OB vs. OR	11	2.830	**0.008****	3.450	0.153	2.167	**0.003****	3.972	**0.007****
W vs. CR	10	1.870	**0.008****	2.180	0.186	2.156	**0.016***	2.730	0.093
W vs. CB	11	1.840	**0.011***	2.050	0.186	2.233	**0.016***	2.761	0.054
W vs. OR	8	0.940	0.424	0.060	0.971	1.040	0.405	0.691	0.804
W vs. OB	9	2.580	**0.012***	3.600	0.186	2.091	**0.016***	2.324	0.054

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Research Article

More than just hitchhikers: a survey of bacterial communities associated with diatoms originating from sea turtles

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Abstract

Diatoms and bacteria are known for being the first colonizers of submerged surfaces including the skin of marine reptiles. Sea turtle carapace and skin harbor diverse prokaryotic and eukaryotic microbes, including several epizoic diatoms. However, the importance of diatom-bacteria associations is hardly investigated in biofilms associated with animal hosts. This study provides an inventory of diatoms, bacteria and diatom-associated bacteria originating from loggerhead sea turtles using both metabarcoding and culturing approaches. Amplicon sequencing of the carapace and skin samples chloroplast gene *rbcL* and 16S rRNA gene detected, in total, 634 diatom amplicon sequence variants (ASVs) and 3661 bacterial ASVs, indicating high diversity. Cultures of putative epizoic and non-epizoic diatoms contained 458 bacterial ASVs and their bacterial assemblages reflected those of their host. Diatom strains allowed for enrichment and isolation of bacterial families rarely observed on turtles, such as *Marinobacteraceae*, *Alteromonadaceae* and *Alcanivoracaceae*. When accounting for phylogenetic relationships between bacterial ASVs, we observed that related diatom genera might retain similar microbial taxa in culture, regardless of the turtle's skin or carapace source. These data provide deeper insights into the sea turtle-associated microbial communities, and reveal the potential of epizoic biofilms as a source of novel microbes and possibly important diatom-bacteria associations.

Keywords: bacteria diatom interactions, diatoms, epizoic bacteria, epizoic communities, phycosphere

Introduction

Associations of bacteria and microbial eukaryotes (protists) are common across different environments including marine habitats (Husnik et al. 2021). Despite the extent of genomic and metabolic diversity of microbial eukaryotes, and their importance in biogeochemical cycles, most of the information on host-microbe associations has been acquired from studies on animal hosts, particularly the digestive system of mammals (Thompson et al. 2017, Husnik et al. 2021). However, bacterial associations with microbial eukaryotes have been increasingly studied in ciliates, amoeba, dinoflagellates and diatoms, traditionally in terms of endosymbiosis (as plastids or housed within the host cytoplasm, nucleus or mitochondria) and as ectosymbionts (microalgal phycosphere). The range of host-bacteria interactions span from beneficial, commensal, to harmful (e.g. B_{12} vitamin production by bacteria, utilization of host-derived organic matter, competition for resources, antimicrobial compounds production by hosts), sometimes even expanding the host's metabolic "toolbox", but the types of interactions are often overlapping and difficult to decouple (Amin et al. 2012, Seymour et al. 2017, Henry et al. 2021, Husnik et al. 2021, Boscaro et al. 2022).

Diatoms (Bacillariophyceae) are essential and omnipresent primary producers in aquatic environments, responsible for approximately 20% of oxygen production and 40% of primary production and particulate carbon export (Field et al. 1998, Jin et al. 2006, Tréguer et al. 2018). The bulk of research on planktonic diatoms in the water column and benthic diatoms inhabiting sediment biofilms revealed the importance of diatom-bacteria interactions (Amin et al. 2012, Durham et al. 2017, Osuna-Cruz et al. 2020) and bacterial influence on diatoms' community composition and productivity (Koedooder et al. 2019, Majzoub et al. 2019), growth and cell division (Amin et al. 2015, van Tol et al. 2017) and sexual reproduction (Cirri et al. 2018, 2019), while diatoms can directly modulate the bacterial community via secondary metabolite production (Fei et al. 2020, Shibl et al. 2020). Efforts in elucidating the diatom-bacteria associations and interactions are still restricted to somewhat familiar systems of laboratory cultures, plankton or sediment, while studies that expand the diatom-bacteria associations repertoire in other habitats remain scarce.

Marine vertebrates have been reported to be extensively colonized by diatoms along with other macro- and microorganisms as reported by morphology-based approaches and metabarcoding (Frick and Pfaller 2013, Rivera et al. 2018, Hooper et al. 2019, Blasi et al. 2021, Robinson and Pfaller 2022; Kanjer et al. 2022). There are multiple reports on novel diatom taxa associated with sea turtles and their potentially exclusive epizoic lifestyle as they have not yet been found elsewhere (Majewska et al. 2015, 2017, 2020, Riaux-Gobin et al. 2021). Microbial diversity observed on marine vertebrate epidermis suggests marine animals could be "hot spots" for microbial diversity and interactions in often nutrient-

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poor open seas (Keller et al. 2021). In this study we focused on surface microbial communities of loggerhead sea turtles in the Adriatic Sea. Reports on prokaryotic and microeukaryotic data on loggerhead sea turtles show diatoms make up a noticeable portion (up to 25%) of microbial eukaryotes on the carapace and skin of loggerheads, often within complex biofilms dominated by bacterial phyla Proteobacteria (classes Gammaproteobacteria and Alphaproteobacteria), Bacteroidota, Bdellovibrionota, Cyanobacteria and Firmicutes (Blasi et al. 2021; Kanjer et al. 2022). It is still unknown if sea turtle-associated microbial epizoic communities have any effect on their host or *vice versa*, including putative epizoic diatoms. Nonetheless, it is becoming clear that loggerhead sea turtle carapace and skin are dynamic and microbially rich environments with the potential to act as a reservoir of diverse and novel microbial species (Kanjer et al. 2022). Insights into the biodiversity of marine vertebrate host-derived diatoms and diatom-associated bacteria are still lacking, even though they could be crucial for understanding the biology and lifestyle of epizoic diatoms.

The aim of this study was to provide an inventory of diatom, bacterial and diatom-associated bacterial communities originating from several loggerhead sea turtles via marker gene microbial profiling (RuBisCO large subunit gene *rbcL* and 16S rRNA gene) and cultivation. The main objective of this study was to examine the microbial community structure on the surface of loggerhead sea turtles by isolating and cultivating turtle-associated diatom strains, profiling the bacterial communities associated with individual diatom strains in culture, and isolating and cultivating bacteria from several diatom strains. This multilayered approach provides a deeper understanding of sea turtle epizoic biofilm potential as a source of novel microbes, source-to-culture bacterial shifts in diatom strains and potentially important diatom-bacteria associations in epizoic biofilms.

Materials and methods

Loggerhead sea turtle carapace and skin sampling

The samples in this study were collected from four loggerhead sea turtles in the Adriatic Sea during 2019 and are a part of the larger dataset presented in Kanjer et al. (2022). Living samples of carapace (randomized collection across the whole surface) and skin (head, neck and flippers) biofilms used for diatom cell isolations were collected by sterile toothbrushes and resuspended in 50-ml conical sterile tubes containing filtered sea water (Table 1). Individual turtles have a unique identification number prefixed by "ID", while carapace and skin samples identification numbers are prefixed by "TB" (see the columns "Turtle ID" and "Source sample (ID)" in Table 1). Samples intended for microbial metabarcoding were collected as described above and resuspended in 50-ml sterile conical tubes containing 96% ethanol for preservation at −20°C until further processing, as described in the Kanjer et al. 2022. Live samples intended for diatom isolation were diluted in sterile f/2 culture medium (Sigma-Aldrich, Germany) (Guillard's medium for diatoms; Guillard 1975) with salinity matching the sample collection source, either in sterile petri dishes (LLG, Germany) or flat bottom transparent 6- or 24-well plates (Guangzhou Jet Bio-Filtration, China) and incubated at 18–20°C at 7–10 μmol m^2 s^{-1}, 12 : 12 (light: dark) cycle.

Diatom isolation, culturing and identification

Establishing monoclonal diatom cultures

Diatoms were isolated from the diluted carapace and skin source samples within the next 8–10 weeks by weekly screenings using an inverted light microscope (Olympus CKX41, Olympus, Tokyo, Japan) and manual isolation of single diatom cells by micropipetting (Andersen 2005). Monoclonal xenic cultures were established by passaging a single cell through multiple series of sterile f/2 medium that facilitated removal of visible eukaryotic contaminants and preservation of bacteria in the diatom phycosphere (Andersen 2005). For the remainder of the study, diatom strains were grown in 25- or 75-cm^2 cell culture flasks (VWR Avantor, USA), in 17 or 50 ml f/2 medium, respectively, at conditions as described above. Upon reaching higher densities (late exponential or stationary phase) the strains were subcultured. The cultures were subsampled for morphological and molecular analyses upon reaching late exponential phase by pelleting and removing the excess culture medium. Pellets for morphological analyses were stored at 4°C in at least 70% EtOH, while pellets for marker gene analysis were stored at −20°C in 96% EtOH. All diatom strains in this study (Table 1, column "Diatom strain ID") are available at the BCCM/DCG culture collection (https://bccm.belspo.be/about-us/bccm-dcg; see Table S1 for extended metadata and Table S2 for culture collection codes). The diatoms' identification number throughout this manuscript is prefixed by "DM", which stands for "diatom monoculture".

Diatom identification via morphology and rbcL sequencing

Diatom silicate frustules were cleaned of organic matter following Simonsen's cleaning method (Simonsen 1974, Hasle 1978). Diatom samples (5 ml volume in ethanol) were washed with distilled water prior to adding an equal volume of saturated $KMnO_4$ solution and incubating for 24 h at room temperature. After 24 h, an equal volume of concentrated HCl was added to the samples, which were heated over an alcohol burner flame, and then washed with distilled water until neutral pH (approximately five times). Permanent slides were prepared by drying cleaned frustules on 22×22 mm coverslips (Hirschmann, Germany) and mounting with Naphrax (Brunel Microscopes Ltd, Chippenham, UK). Permanent slides were analyzed with Zeiss Axio Imager A2 with DIC and an Axiocam 305 digital camera (Carl Zeiss, Jena, Germany). Stubs for scanning electron microscopy analyses were prepared by drying cleaned frustules onto 3-μm pore size (13 mm in diameter) nucleopore polycarbonate membrane filters (Pleasanton, CA, USA) before sputter-coating. Dried filters were mounted on aluminium stubs with carbon tape and sputter-coated with platinum (10 nm) using a Precision Etching and Coating System, PECS II (Gatan Inc., Pleasanton, CA, USA). The specimens were analyzed with a JEOL JSM-7800F scanning electron microscope (JEOL, Tokyo, Japan).

For molecular identification via the *rbcL* gene, diatom DNA was extracted from the pellets by DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions with an extra pre-processing bead beating step for disrupting the diatom colonies and frustules. The pellets were mixed with 0.5 g of 1.0-mm glass beads (Qiagen) in a sterile 2-ml safe lock microtube and vortexed horizontally at maximum speed for 10 min prior to continuing with the manufacturer's protocol. The extracted DNA was used as a template to amplify the *rbcL* marker as described in Theriot et al. (2015). The initial PCR reactions were performed in 25-μl volume reactions consisting of 1 μl of DNA template, 12.5 μl of Takara EmeraldAmp Master Mix 2x (Takara Bio, Japan), 0.5 μl primers rbcL40+ and rbcL1444- (0.2 μM final concentration) and 10.5 μl of sterile dH_2O, while nested PCR reactions were performed in 50 μl (double the reagents for the 25-μl reaction) with a similar set up as above but different reverse primer rbcL1255- (see Table S3 for the primers list). The thermocycling conditions for the initial reaction were 94°C for 5 min, 30 cycles of 98°C for 10 sec, $T_a^{(initial)}$=50°C or $T_a^{(nested)}$=50°C for 60 sec, 72°C for 2 min and final extension at

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Table 1. Description of diatom strains used in this study. Turtle ID includes carapace and skin source samples taken from a single turtle.

Diatom strain ID	Turtle ID	Source sample (ID)	Scientific name	Host relation**	Time from isolation to harvest (days)	Bacterial isolates
DM0150	ID010	Carapace (TB139)	*Achnanthes squaliformis*	epizoic	388	yes
DM0177	ID010	Carapace (TB139)	*Achnanthes squaliformis*	epizoic	332	yes
DM0178	ID010	Skin (TB140)	*Nitzschia* sp.	non-epizoic	343	no
DM0179	ID010	Skin (TB140)	*Entomoneis* sp.	non-epizoic	332	no
DM0052	ID034	Carapace (TB89)	*Achnanthes elongata*	epizoic	667	no
DM0053	ID034	Carapace (TB89)	*Achnanthes elongata*	epizoic	682	yes
DM0054	ID034	Carapace (TB89)	*Achnanthes elongata*	epizoic	673	no
DM0060	ID034	Carapace (TB89)	*Achnanthes elongata*	epizoic	647	yes
DM0070	ID034	Carapace (TB89)	*Amphora* sp. 1	non-epizoic	649	no
DM0077	ID034	Skin (TB90)	*Poulinea lepidochelicola*	epizoic	644	yes
DM0123	ID047	Carapace (TB115)	*Diploneis* sp.	non-epizoic	503	no
DM0129	ID047	Carapace (TB115)	*Diploneis* sp.	non-epizoic	507	yes
DM0136	ID047	Carapace (TB115*)	*Diploneis* sp.	non-epizoic	512	no
DM0147	ID047	Carapace (TB115*)	*Fallacia* sp.	non-epizoic	495	yes
DM0168	ID073	Carapace (TB155)	*Achnanthes elongata*	epizoic	365	no
DM0170	ID073	Carapace (TB155)	*Achnanthes elongata*	epizoic	367	yes
DM0181	ID073	Carapace (TB155)	*Psammodictyon panduriforme*	non-epizoic	340	yes
DM0182	ID073	Carapace (TB155)	*Amphora* sp. 2	non-epizoic	356	no
DM0183	ID073	Carapace (TB155)	*Psammodictyon* sp.	non-epizoic	333	yes

*In case of ID047 a second carapace sample was obtained a month after the initial one (TB115) and it did not undergo NGS sequencing.
**Host relation status (epizoic or non-epizoic) is based on Ashworth et al. (2022; at https://doi.org/10.21203/rs.3.rs-1041030/v2)

72°C for 15 min. The amplicons were purified by the NucleoSpin Gel and PCR cleanup kit according to the manufacturer's instructions (Macherey-Nagel, Dueren, Germany). Purified products were sent for Sanger sequencing with primers rbcL404+ and rbcL587- to Macrogen (http://dna.macrogen-europe.com).

Diatom strains identified as *Achnanthes elongata*, *Achnanthes squaliformis* and *Poulinea lepidochelicola* were considered to be exclusively epizoic diatoms, while other diatoms were categorized as non-epizoic for the purposes of this study based on Ashworth et al. (2022).

Microbial community profiling in source samples and diatom strains

Source samples processing and amplicon sequencing

Carapace and skin samples (preserved in ethanol) were collected and processed within the Kanjer et al. study (2022). Briefly, total DNA was extracted using the DNeasy PowerSoil kit (Qiagen) following the manufacturer's guidelines with several modifications and sent for amplicon sequencing of the V4 region of the 16S rRNA gene by 515F and 806R primers (Apprill et al. 2015, Parada et al. 2016). Within this study, to gain information on diatom community composition, a portion of the same DNA was used to sequence a 312-bp barcode of the *rbcL* gene by combining the forward (DiatrbcL1F_708F_1, DiatrbcL2F_708F_1 and DiatrbcL3F_708F_1) and reverse (DiatrbcL1R_708F_1 and DiatrbcL2R_708F_1) primers from the Vasselon et al. (2017) study into one forward primer (5′-A GGTGAAYWAAAGGTTCWTAYTTAAA-3′) and one reverse primer (5′-CCTCTAATTTACCWACNACWG-3′), as listed in Table S3. The sequencing was performed on the Illumina platform with MiSeq 250×2 bp paired-end chemistry at MrDNA (TX, USA).

Diatom monoclonal cultures total DNA extraction and amplicon sequencing

Diatom strains were maintained in culture for at least a year and passed several rounds of subculturing before harvesting the pellet for bacterial profiling (Tables 1 and S1). The strains were then grown in duplicates in 75-cm^2 cell culture flasks as described above and were harvested upon reaching the late exponential phase. The cells were collected and pelleted in 50-ml conical sterile tubes by centrifuging at 5000 g for 10 min prior to removing the supernatant. The pellet was transferred to a sterile 2 ml microtube, pelleted again by centrifuging at 16 000 g for 10 min with supernatant removed and then stored at −80°C until DNA extraction. The DNA was extracted by DNeasy PowerLyzer Microbial kit (Qiagen) following the manufacturer's instructions with several modifications: the cultures were disrupted by bead-beating with a mixture of sharp carbon and glass beads at 30 Hz, SL buffer was heated to 60°C before use and the elution buffer was incubated on the filter for 5 min before centrifugation and final elution of DNA. The quality and quantity of DNA were checked with Nanodrop and Qubit prior to sending each replicate's DNA for sequencing of the V4 region of 16S rRNA gene with the 505F and 806R primers (Apprill et al. 2015, Parada et al. 2016) at Microsynth (Switzerland).

Bacterial cultivation from diatom monoclonal cultures

To survey the culturable bacteria within the diatom cultures, 10 diatom cultures (Table 1) were harvested in the late exponential phase and used to culture bacteria. The diatom cultures were grown as described above in 25-cm^2 cell culture flasks (15–20 ml f/2) in duplicate and, upon reaching sufficient density, harvested in 15-ml conical sterile tubes for further processing.

To increase the chances for successful bacterial isolation, two approaches were used in diatom pellet pre-treatment before culturing bacteria: crushing and washing the pellets.

Crushed pellets: The 15-ml tube was centrifuged at 8000 g for 10 min before removing the supernatant up to 1 ml of residual pellet and media. The 1 ml of material was transferred to a 1.5-ml sterile microtube and centrifuged at 16 000 g for 5 min before removing the residual supernatant. The pellet was then crushed by a sterile plastic pestle attached to an electric screwdriver for 5 sec. After crushing the pellet, the material was resuspended in 200 μl

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of sterile 0.9% NaCl solution and serially diluted. One hundred μl of each serial dilution (from 10^0 to 10^{-6}) was plated on Marine Agar (MA; Difco, Detroit, USA) plates and incubated at 20°C for 48 h, and then if growth was visible the plates were incubated at 15°C for 96 more hours, or if growth was not visible the plates were incubated at 20°C for 96 more hours.

Washing pellets: The pellet was transferred to a round bottom sterile tube prior to washing several times with 10 ml sterile 0.9% NaCl solution (8000 g 10 min, three times), after which the pellet was resuspended in 1 ml of 0.9% NaCl and serially diluted before plating on the MA. The incubation and growth conditions were as described above.

Single bacterial colonies were inspected under a stereo microscope, replated and incubated for 2–6 days (until growth was visible) at 20°C. Once pure, bacterial strains were collected from plates into Microbank$^{\text{TM}}$ vials (Fischer Scientific) for cryopreservation. Some colonies of the pure strains were collected using a pipette tip and resuspended in an alkaline lysis buffer for DNA extraction (Niemann et al. 1997).

For identification of the bacterial isolates 16S rRNA genes were amplified by PCR using pA (8f) and pH (20r) (Edwards et al. 1989) primers in 25-μl volume reactions. The PCR reactions contained 2 μl of alkaline lysis DNA template, 2.5 μl of dNTPs, 2.5 μl of Qiagen PCR buffer 10x, 0.25 μl of 10 μM primers, 0.5 μl of Qiagen Taq DNA polymerase and 17 μl Milli-Q water. The thermocycling conditions were: intial step 95°C for 5 min, three cycles of 95°C for 1 min, 55°C for 2 min 15 sec, 72°C for 1 : 15 min, 30 cycles of 95°C for 35 sec, 55°C for 2 min 15 sec, 72°C for 1 min 25 sec and a final extension of 72°C for 7 min. The products were inspected on agarose gel and purified using Nucleofast PCR purification plates (Macherey-Nagel, Dueren, Germa) according to the manufacturer's instructions. For initial identification, the V1-V3 region was sequenced with BLK1 primer (Cleenwerck et al. 2007) at Eurofins Genomics (https://eurofinsgenomics.eu/). In a second round the amplicons were completely sequenced with additional primers (Coenye et al. 1999). All primers used in this study are listed in Table S3.

Bioinformatic and data analyses

Diatom and bacteria marker gene sequences processing

Sequences for *rbcL* gene obtained by Sanger sequencing were inspected for quality and assembled into a contig in Geneious Prime v. 2022.0.2. 16S rRNA sequences were assembled and checked for quality using BioNumerics 7.6.3 (Applied Maths) and identified using EZBioCloud (Yoon et al. 2017; https://www.ezbiocloud.net). The sequences were aligned in AliView v. 1.28 (Larsson 2014) using MUSCLE (Edgar 2004). To be able to compare the diatom and bacterial cultures marker genes with amplicon-based next generation sequencing (NGS) data, the full length *rbcL* and 16S rRNA gene sequences were trimmed to their corresponding NGS regions in AliView. Maximum likelihood phylogenetic trees for full length marker gene sequences were generated by IQ-TREE (using ModelFinder and UFBoot2=1000) and visualized by Interactive Tree of Life (iTOL) (Nguyen et al. 2015, Kalyaanamoorthy et al. 2017, Hoang et al. 2018, Letunic and Bork 2021). GenBank accession codes for all diatom and bacterial strains can be found in Table S2.

Amplicon sequencing bioinformatic and statistical analyses

Source sample (carapace and skin) sequences obtained from MrDNA were pre-processed by FASTqProcessor (MrDNA) to remove all non-biological sequences (primers, linkers, adapters) prior to importing the data to QIIME 2 in "EMP protocol" multiplexed paired end fastq format. The carapace and skin 16S rRNA gene amplicon sequencing data obtained from Kanjer et al. (2022) were processed independently within this study to be able to compare them with the amplicon sequencing data of diatom cultures. Diatom cultures sequences obtained from Microsynth were already trimmed and were imported to QIIME 2 in the Cassava 1.8 paired end demultiplexed format. Both *rbcL* and 16S rRNA gene sequencing data were processed with QIIME 2 v. 2021.4 (Bolyen et al. 2019), with the same tools but with specific parameters for each sequencing batch described in detail in resources provided in the Data and code availability section. The imported sequences were demultiplexed by q2-demux and denoised by q2-dada2 (DADA2; Callahan et al. 2016), which produced amplicon sequence variants (ASVs, 100% operating taxonomic units). Up to this point each sequencing batch was processed separately to reduce denoising errors and were merged accordingly after the DADA2 output; diatom source samples (carapace and skin) *rbcL* amplicon sequencing in one group; 16S rRNA gene diatom monoclonal culture samples in the second group and source samples (carapace and skin) 16S rRNA gene amplicon sequencing data in the third group (see Data and code availability). Further, analyses of 16S rRNA gene amplicon sequencing data were performed separately for diatom culture replicates, merged diatom replicates data, source samples data and merged diatom and source samples data. Sequences were aligned by MAFFT (Katoh 2002) and FastTree2 in q2-phylogeny plugin was used to construct a phylogenetic tree (Price et al. 2010). Taxonomy was assigned to ASVs through q2-feature-classifier (Bokulich et al. 2018, Robeson et al. 2021) classify-sklearn naive Bayes taxonomy classifier in SILVA v. 138 99% 505F-806R nb classifier (Pruesse et al. 2007) for 16S rRNA gene reads and Diat.barcode v. 10 for diatom *rbcL* reads (Rimet et al. 2019). Reads assigned to chloroplasts and mitochondria were removed from the 16S rRNA amplicon sequencing data before further processing.

To investigate alpha and beta diversity the whole 16S rRNA amplicon sequencing dataset was rarefied to 32 660 reads per sample based on inspection of rarefaction curves via q2-diversity plugin. Alpha diversity indices (Shannon's entropy, Pielou's evenness, Faith's phylogenetic diversity, observed ASVs) were calculated via q2-diversity plugin. Beta diversity was explored via q2-diversity plugin on rarefied data with Bray-Curtis, Jaccard, unweighted UniFrac, weighted UniFrac (Lozupone and Knight 2005, Lozupone et al. 2011) and generalized UniFrac (Chen et al. 2012) distances. Unrarefied data were analyzed through robust Aitchison distance via q2-deicode plugin to cater for the compositionality of amplicon sequencing data (Gloor et al. 2017, Martino et al. 2019). Principal coordinates analyses (PCoA) for Bray-Curtis, Jaccard, all UniFrac distances and principal components analysis (PCA) for robust Aitchison (rPCA) were performed by q2-diversity and q2-deicode, respectively. Along with robust Aitchison distance, multiple conventional beta diversity indices were used to best represent and visualize data as Bray-Curtis dissimilarity and weighted UniFrac distance are affected by the most abundant members of the bacterial community, while the effects of low abundance or rare microbial taxa can be observed with Jaccard and unweighted UniFrac distances. Multi-way permutational multivariate analysis of variance (i.e. Adonis PERMANOVA) (Anderson 2001) was used to estimate the relative impact of factors (turtle host ID and diatom genus) on the bacterial communities in diatom cultures (permutations=9999, {vegan} v. 2.5–7, Oksanen et al. 2020; {pairwiseAdonis} v. 0.4, Arbizu 2017). Data exploration and visualizations were performed with R v. 4.1.1 in RStudio (R Core Team 2021, {qiime2R} v. 0.99, Bisanz 2018; {tidyverse}, Wick-

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ham et al. 2019; {ggplot2}, Wickham 2016; see Data and code availability). To investigate the cultured diatom and bacterial strains presence in the amplicon sequencing data, trimmed marker gene sequences were merged with ASVs, aligned in AliView, processed with IQ-TREE and visualized by iTOL, as described above.

Data and code availability

Diatom and bacterial strains used in this study are available at the BCCM/DCG and LMG culture collections, respectively (see the culture codes in Table S2). Raw amplicon sequences (with removed non-biological sequences) are available at the European Nucleotide Archive under the accession numbers PRJEB47668 (diatom monoclonal culture 16S rRNA sequences), PRJEB51458 (total sea turtle surface 16S rRNA sequences; sample accession numbers ERS10917111, ERS10917104, ERS10917103, ERS10917093, ERS10917093, ERS10917091) and PRJEB51297 (total sea turtle surface *rbcL* sequences). Full *rbcL* sequences per diatom strain and 16S rRNA gene sequences per bacterial isolate are available in GenBank (*rbcL* OM686876-OM686892; 16S OM959184-OM959220 and ON040652-ON040654; accession numbers per strain are listed in Table S2). All other data supporting the conclusions in this manuscript are available in the supplementary materials.

Data and code used for bioinformatic analyses, statistical analyses and data visualizations are available at GitHub (https://github.com/kl-fil/Filek_et_al._2022-diatom_microbiota) and Mendeley Data (DOI: 10.17632/4r6568xcpw.1).

Results

Loggerhead-associated diatom monoclonal cultures and source sample diatom community composition

Within this study we isolated and cultivated diatom strains of diverse diatom taxa and established xenic monoclonal cultures. Only cultures without detected eukaryotic contaminants were chosen for this study (Table 1). Isolated diatoms were identified as belonging to eight different genera and 11 species (Fig. 1B-L), including the putative epizoic diatoms *Achnanthes elongata*, *Achnanthes squaliformis* and *Poulinea lepidochelicola* (Fig. 1B, C, H, M). *Achnanthes* and *Poulinea* in cultures exhibited high polysaccharide secretion in the form of stalks or mucus sheaths enabling cells to connect and form chains and/or colonies (Fig. 1M) and attach to the cell culture flask surfaces. Other genera did not show such behavior under the conditions in this study except for colony formation of *Amphora* sp. 2 (DM0182), whose cells tended to cluster together in the water column and rarely attached to the cell flask surfaces. *Diploneis*, *Amphora*, *Nitzschia*, *Fallacia* and *Psammodictyon* strains readily attached to surfaces, but formation of chains or stalks was not detected. Relative relationships between diatom strains in this study (except *Nitzschia* sp. DM0178 and *Diploneis* sp. DM0136, for which the *rbcL* sequences could not be obtained) based on *rbcL* marker gene are shown within the maximum likelihood phylogenetic tree in Fig. S1A.

NGS of the *rbcL* marker gene amplicons in carapace and skin source samples yielded 458 069 high quality *rbcL* sequences (median=37 756.5) across 619 ASVs. The samples showed high proportions of *Nitzschia*, *Amphora*, *Halamphora* and *Navicula* genera along with unclassified ASVs based on Diat.barcode taxonomy classifications of the *rbcL* marker region (Fig. S1B). However, when the NGS *rbcL* amplicon marker was extracted from full size *rbcL* sequences obtained for diatom strains, and compared with the NGS results sequence annotations, we observed discrepancies in Diat.barcode assigned taxonomy for barcodes associated with newly described epizoic taxa *A. elongata*, *A. squaliformis* and *P. lepidochelicola* (Fig. S2). Positioning of diatom strain extracted *rbcL* barcodes in the phylogenetic tree (Fig. S2) coincided with NGS sequences annotated as *Nitzschia* spp. (DM0052, DM0053, DM0054; *A. elongata*), *Bacillariaceae* (DM0060, DM0168, DM0170; *A. elongata*), *Amphora* spp. (DM0077; *P. lepidochelicola* and DM0150, DM0177 *A. squaliformis*) with low confidence values (Table S4). Alignment of NGS *rbcL* ASVs and *rbcL* barcode sequences of diatom strains found exact matches for most diatoms except *Fallacia* sp. (DM0147), *Psammodictyon panduriforme* (DM0181), *Amphora* sp. 2 (DM0182) and *Psammodictyon* sp. (DM0183). Matched *rbcL* ASVs were present in source samples at mostly around 1% relative abundance. Interestingly, *Amphora* sp. 1 (DM0070) matched ASV was present at 48% relative abundance in its source sample, while *P. lepidochelicola* (DM0077) was present at 32% in its source sample (Table S4), thus forming a significant portion of the diatom assemblage of the turtle-associated microbial biofilm. Epizoic diatoms *A. elongata* and *A. squaliformis* were present in their corresponding source samples at less than 1% and at 3% relative abundance, respectively. For other strains that could not be matched to an *rbcL* ASV we examined the closest neighbors in the phylogenetic tree (Fig. S2) and their presence was also around 1% in at least one source sample (Table S4).

Bacterial communities of source samples (carapace and skin) and diatom monoclonal cultures

Source samples yielded 856 010 16S rRNA gene sequences (median=159 904.5) across 4275 ASVs. Diatom cultures (19 strains in two replicates, $n_{(total)}=38$) yielded 2 297 642 high quality 16S rRNA gene sequences (median=63 314.5) across 485 ASVs. Chloroplast reads encompassed 497 842 sequencing reads in diatom cultures NGS data; on average, 21% of reads across all samples were associated with chloroplasts (ranging from 1% to 75% of relative abundance). After filtering chloroplast and mitochondria sequences, source samples retained 3661 ASV and diatom monocultures 458 ASVs; with 216 ASVs in common. Shared ASVs comprised an average 40% relative abundance (SD±0.2) of diatom-associated bacteria, and average 8% relative abundance (SD±0.6) of source sample bacterial community (Table S5).

Within sample bacterial community diversity

Source samples exhibited high alpha diversity (Fig. S3) with 917 ASVs on average. Diatom cultures contained 52 ASVs on average (spanning from 18 to 101) with Shannon's entropy, observed ASVs and Faith's phylogenetic diversity index several times lower than in source samples (Fig. S3). Pielou's evenness and Faith's phylogenetic diversity index showed some diatom cultures are dominated by specific bacterial ASVs and lack phylogenetic diversity, while others have a more equal prevalence of ASVs and higher diversity. No metadata categories were found to be responsible for such observations.

Bacterial community composition and structure

Relative abundance of microbial taxa (Fig. 2) shows general reduction in the number of bacterial phyla in diatom cultures versus source samples (Fig. 2B). Diatom monocultures contained 17 phyla in total, reduced in comparison with source samples, which contained 36 (Table S5). Source samples completely lacked Elusimicrobiota phylum, which was found in only one diatom culture (*A. elongata*, DM0052) and contained only one ASV (1606) belonging to

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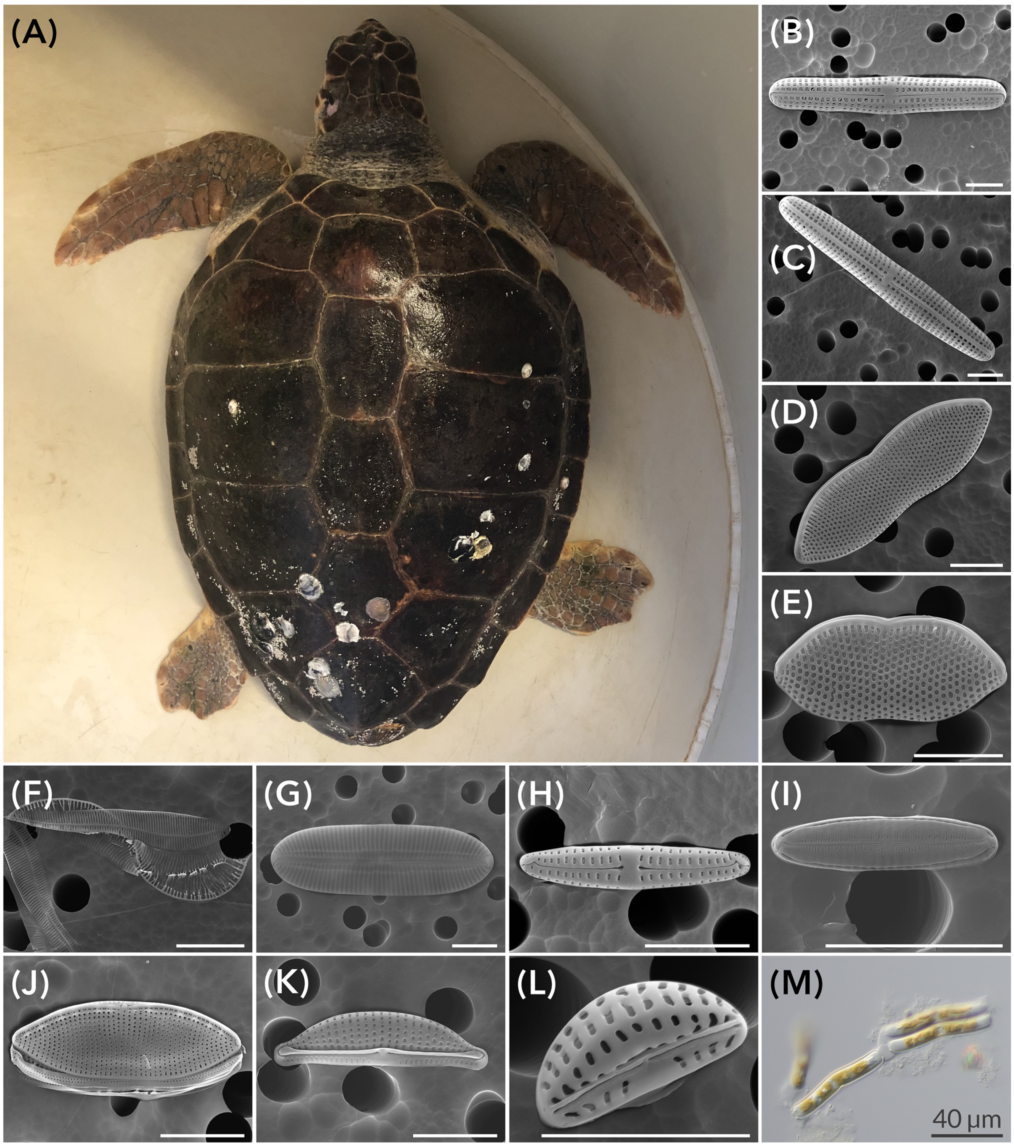

Figure 1. Loggerhead sea turtle and isolated diatoms. Loggerhead sea turtle Merry Fisher ID010 (**A**), and scanning electron micrographs of diatom taxa (**B**-**L**): *Achnanthes elongata* (B), *Achnanthes squaliformis* (C), *Psammodictyon* sp. (D), *Psammodictyon panduriforme* (E), *Entomoneis* sp. (F), *Diploneis* sp. (G), *Poulinea lepidochelicola* (H), *Fallacia* sp. (I), *Nitszchia* sp. (J), *Amphora* sp. 1 (K), *Amphora* sp. 2 (L), light micrograph of *A. elongata* cells in monoculture (**M**). All scales are 5 μm unless marked differently.

Elusimicrobium genus at the relative abundance of 0.2% (159 out of 78 562 reads). *Rhodobacteraceae* were abundant both in diatom and source samples, while *Thalassospiraceae* and *Stappiaceae* seem to be enriched in monocultures while being part of the rare biosphere at less than 0.1% of relative abundance (Pascoal et al. 2021) in source samples (Fig. 2C). In class Gammaprotebacteria the effect of enriched taxa is more pronounced as families *Alteromonadaceae*, *Cellwelliaceae*, *Marinobacteaceae*, *Alcanivoracaceae* and *Nitricolaceae* are more abundant in monocultures (Fig. 2D). Several taxa within Bacteroidota phylum also show the enrichment pattern (*Crocinitomicaceae*, Sphingobacteriales NS11.12) (Fig. 2E), but *Phycispheraceae* within Planctomycetota show higher abundance in only a subgroup of epizoic diatom strains originating from TB89, TB90 and TB139 source samples, even though they are barely present in TB89 and TB90 (Fig. 2F).

Shared bacterial taxa and individual ASVs

Source and monoculture samples shared bacterial families *Rhodobacteriaceae* and *Flavobacteriaceae*, while 85% (21/25 samples) shared additional *Hyphomonadaceae* and *Stappiaceae*. At the genus level, 80% of samples (20/25) shared unclassified members of *Rhodobacteraceae*, *Alcanivorax* and *Labrenzia*. Source samples shared 50 ASVs, of which 41 seem to be part of the rare biosphere (at less than 0.01% on average across samples) and rarely present in diatom monocultures. Only ASV 1206 (uncultured *Oligoflexaceae*) reached a relative abundance of 10% and 43% in source samples. Source samples exhibited much higher diversity than diatom cultures and consistently harbored members of Proteobacteria, Bacteriodota, Bdellovibrionota, Actinobacteriota, Myxococcota, Planctomycetota, Verrucomicrobiota, Cyanobacteria, Deinococcota, Desulfobactaerota, Chloroflexota, Firmicutes,

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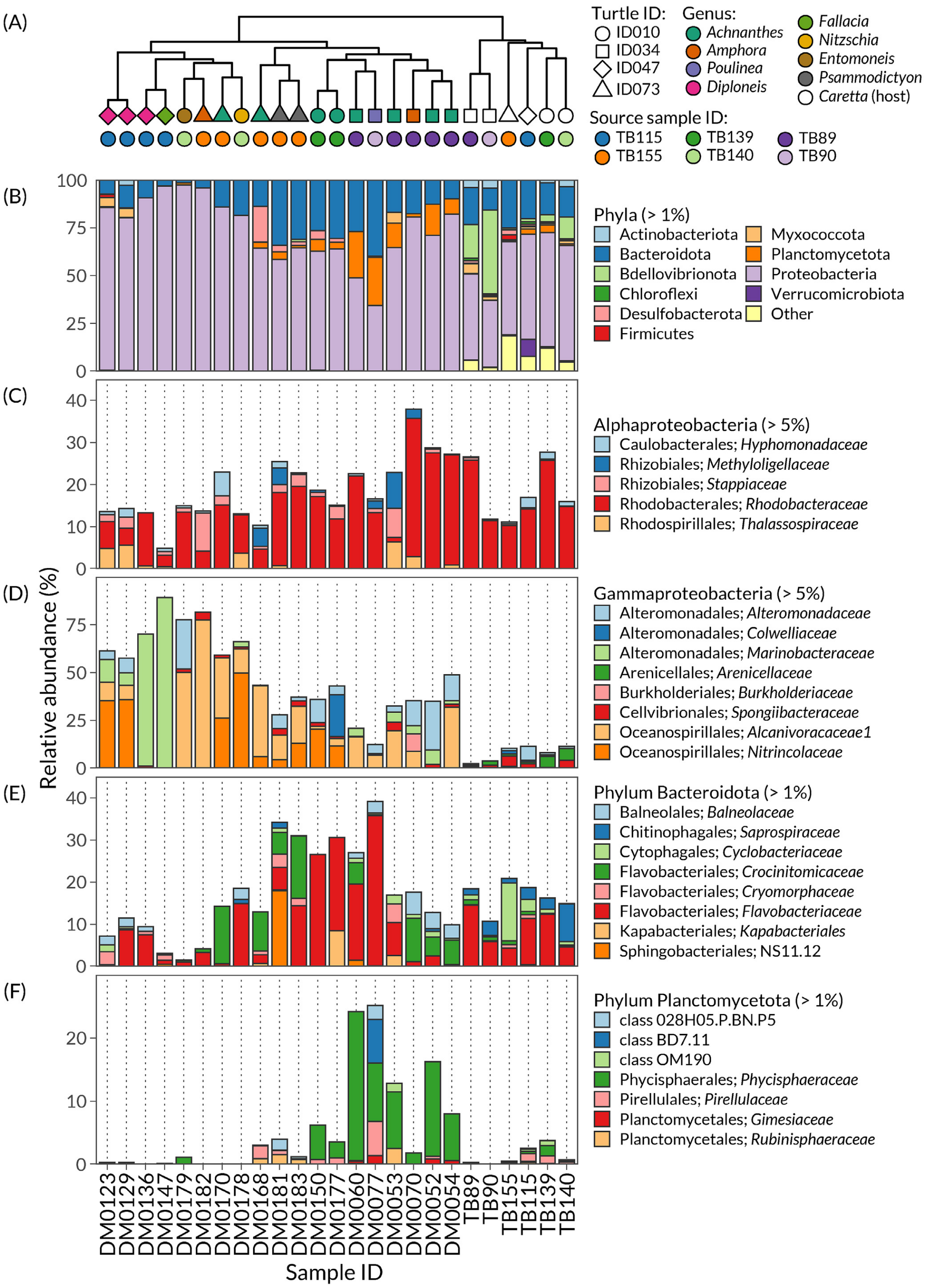

Figure 2. Relationships between samples and relative abundance of bacterial taxa in cultivated diatom strains and source samples. Generalized UniFrac dendrogram shows relationships between samples (**A**) with indication of diatom genus (color) and turtle ID (shape) at the tips, while specific source samples (colors) are indicated in symbols beneath. Relative abundances of bacterial taxa are presented at the levels of bacterial phyla above 1% (**B**), classes Alphaproteobacteria (**C**) and Gammaproteobacteria (**D**) above 5%, phyla Bacteroidota (**E**) and Planctomycetota (**F**) above 1% relative abundance in at least one sample. Most common orders and families or closest taxonomic identification are shown.

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Campilobacterota, Spirochaetota and SAR324 Marine group B phyla (Table S5).

All diatom strains shared Proteobacteria and Bacteroidota, while only 80% of monocultures (15/19) shared Planctomycetota phylum. *Alcanivorax* spp., unclassified *Rhodobacteraceae*, *Labrenzia* spp., *Marinobacter* spp., *Methylophaga* spp. and uncultured Parvibaculales are commonly found in monocultures (75% of samples). ASV 2509, classified as uncultured Parvibaculales, was found in 15/19 diatom cultures and in 2/6 source samples below 1% relative abundance. Interestingly, diatom cultures originating from the same source shared 3–5 ASVs, except in the TB139 source group where the two cultures were *A. squaliformis* as they shared 14 ASVs. Similarly, *Achnanthes* strains originating from the source sample TB155 shared 13 ASVs (out of 45 and 43 total observed features), but the *Achnanthes* strains originating from TB89 shared only five ASVs. The difference between these three groups of *Achnanthes* is that source samples TB139 and TB155 had up to two to three times higher ASVs to begin with, in comparison with TB89. All *Achnanthes* strains shared just the previously mentioned ASV 2509, while *Achnanthes* from TB139 and TB155 shared four ASVs (belonging to *Alcanivorax* spp., *Porticoccus* spp., unclassified Parvibaculales and *Labrenzia* spp.) (Table 2). Notably, *Diploneis* sp. strains (DM123, DM129) originating from TB115 shared 71 ASV (out of 100 and 101 total observed ASVs per strain), while *Psammodictyon* strains (DM0181, DM0183) from TB155 shared 34 ASVs (out of 71 and 58 total observed ASVs per strain) (Table 2). On the other hand, *Psammodyction* and *Achnanthes* strains from TB155 shared only seven ASVs.

Beta diversity analyses of bacterial communities

Taxonomic composition and individual bacterial ASV sharing between diatom strains is reflected in beta diversity metrics (Figs 3, S4 and S5). Compositional data analysis through rPCA shows a general pattern of samples separating based on diatom genus (Fig. 3A) and carapace or skin source sample (Fig. 3C). Highly ranked feature loadings of the rPCA overlap with previously observed taxa often found in diatom strains (genera *Alcanivorax*, *Neptuniibacter*, *Marinobacter*, *Alteromonas*, *Phycisphaera*). Generalized UniFrac considers the phylogenetic distances between ASVs and their abundance in each sample, balancing between the "weight" of highly abundant taxa (weighted UniFrac) and rare taxa (unweighted UniFrac), so the PCoA accordingly shows similarities between diatom strain samples with closely related bacterial taxa (Fig. 3B and D) and reiterates the source sample groupings observed with robust Aitchison distance.

Dominant bacterial taxa tend to drive groupings between samples based on the source sample ID (Figs S4A, S4B, S5A and S5B), while low abundance taxa tend to affect groupings in such a way that samples start reflecting the diatom genus groups (Figs S4D and S5D). Regardless, presence-absence metrics seem to separate diatom bacterial communities based on origin, revealing the environmental signature (Figs S4C and S5C). When source samples and diatom monocultures were investigated together, despite their extreme differences in microbial richness and diversity, the above mentioned patterns recurred (Figs S6 and S7).

Adonis PERMANOVA showed significant differences when diatom monoculture samples are grouped by their individual turtle host of origin (combined carapace and skin samples; Turtle ID), and an effect of genus grouping was observed (Table 3). With generalized UniFrac 34% of variation is explained by turtle ID (F-model=3.309, Pr(>F)=0.0001) and 35% by diatom genus (F-model=1.698, Pr(>F)=0.0007), while using the robust Aitchison 69% of variation is attributed to Turtle ID (F-model=13.897, Pr(>F)=0.0001) and 15% to diatom genus (albeit genus being not significantly different in this case Pr(>F)=0.1). Pairwise ADONIS showed differences between individual turtle hosts grouped by Turtle ID ID034 vs. ID047 (generalized UniFrac F-model=4.14, R^2=0.34, Pr(>F)=0.042; robust Aitchison F-model=13.8, R^2=0.63, Pr(>F)=0.03) and ID034 vs. ID074 (generalized UniFrac F-model=3.1, R^2=0.25, Pr(>F)=0.024; robust Aitchison F-model=23, R^2=0.72, Pr(>F)=0.24).

Bacterial isolates from diatom monoclonal cultures

A total of 125 bacterial isolates were obtained from 10 diatom cultures (Tables 1 and S2). Partial sequencing of the 16S rRNA gene was used to identify the strains of interest (possibly unique) and 40 strains were chosen for full 16S rRNA sequencing (Table S2). Based on the V4 region of the full 16S rRNA sequence, 39 out of 40 bacterial strains were matched with an ASV in diatom and source sample sequencing data (Fig. 4). Notably, ASVs of five bacterial isolates were not detected in the diatom strain they originated from but were detected in other diatom strains; the only bacterial strain not matched with an ASV was classified as belonging to the genus *Actibacterium* (Fig. 4).

Based on full 16S rRNA sequence we managed to obtain identification for ASVs that were classified differently by SILVA or had assigned taxonomy only above genus level: several ASVs that were assigned to *Rhodobacteraceae* by SILVA were identified as belonging to genera *Leisingera*, *Jindonia*, *Tritonibacter*, *Celeribacter* and *Antarctobacter.* ASV 2615 and ASV 2918 were assigned to genus *Labrenzia* and *Sedimentitalea* by SILVA (confidence at 0.91 and 0.72), but we identified them as belonging to *Roseibium* and *Sulfitobacter* genus, respectively. Additionally, with SILVA two ASVs (824 and 974) were assigned to genera *Aquibacter* and *Winogradskyella*, while full 16S rRNA sequences indicate they could potentially belong to a new genus in the *Flavobacteriaceae* family. Multiple bacterial strains matched with one ASV even though they differed in full length 16S rRNA sequences, such as *Tritonibacter scottomolliaceae* and *Tritonibacter mobilis*, *Alteromonas* spp. and *Alcanivorax* spp. (Fig. 4, Tables S5 and S6).

ASVs matched to bacterial isolates were detected in at least one diatom monoculture (except ASV 3821); however, only 14 out of 30 matched ASVs were present in source samples and at less than 1% relative abundance (Fig. 4, Table S6). Interestingly, based on the ASVs, *Alcanivorax* spp. were enriched in most diatom cultures, while in source samples they were detected at less than 1% relative abundance (2–15 reads in four out of six source samples) and ASV 3821 (matching to *Acinetobacter lwoffii*) was detected only in source samples and not in monocultures (Fig. 4, Table S6).

Discussion

Investigations of diatom-bacteria associations, although crucial for understanding global ecological processes, are still limited mostly to habitats such as sediment biofilms or planktonic communities. In this study we provide a multi-level inventory of diatoms and bacteria associated with loggerhead sea turtles. Our approach combined PCR-based surveys of microbial communities (*rbcL* and 16S rRNA gene amplicon sequencing) in source samples as well as isolating and culturing non-model and novel diatom taxa, thus showing that marine reptiles are valuable "hot spots" of diatom and bacterial diversity (Hooper et al. 2019, Keller et al. 2021). As diatoms are hosts to bacteria within their phycosphere we further surveyed the bacterial community retained in diatom

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Table 2. Shared ASVs within diatom genera originating from the same source sample with closest ASV identification for ASVs present above 1% average relative abundance.

Scientific name (source sample)	Diatom strain ID	Shared ASVs	Total ASVs	Relative abundance of shared ASV within sample	Closest ASV identification (average relative abundance across selected samples)
Achnanthes elongata (TB89)	DM0052 DM0053 DM0054 DM0060	5	68 38 50 53	24% 15% 28% 27%	*Phycisphaera* (11%), *Pseudophaeobacter* (7%), *Marinobacter* (4%), Parvibaculales (1%); *Maritalea* (<1%)
Achnanthes squaliformis (TB139)	DM0150 DM0177	14	46 43	20% 21%	*Algisphaera* (3%), *Rhodobacteraceae* (4%), PB19 (3%), *Sulfitobacter* (2%), *Alcanivorax* (2%), *Spongiibacter* (1%), *Porticoccuks* (1%)
Achnanthes elongata (TB155)	DM0168 DM0170	13	56 23	42% 45%	*Alcanivorax* (34%), *Maricaulis* (2%), *Rhodobacteraceae* (1%), *Labrenzia* (1%), *Porticoccus* (1%)
Diploneis sp. (TB115)	DM0123 DM0129	71	100 101	91.0% 90%	*Neptuniibacter* (31%), *Marinobacter* (9%), *Alcanivorax* (8%), *Alteromonas* (5%), *Nannocystaceae* (5%), *Thalassospira* (4%), *Amphritea* (2%), NRL-2 (2%), *Nitrincolaceae* (2%), *Labrenzia* (2%), *Rhodobacteraceae* (2%), *Maricalus* (1%)
Psammodictyon pandurifome (TB155) *Psammodictyon* sp. (TB155)	DM0181 DM0183	34	71 58	50% 63%	*Alcanivorax* (16%), *Crocinitomix* (9%), *Alteromonas* (7%), *Spongiibacter* (2%), *Labrenzia* (2%), *Rhodobacteraceae* (6%), *Tenacibaculum* (2%), *Porticoccus* (1%), *Methyloligellaceae* (2%), 028H05-P-BN-P5 (1%), *Cryomorphaceae* (1%)

Table 3. Adonis (PERMANOVA) results based on generalized Unifrac and robust Aitchison distances for diatom strain microbial communities with two categorizations: Turtle ID as individual turtle host and diatom genus. F statistic *P*-values significance level is Pr(>F)<0.05 (in bold).

	Generalized Unifrac			Robust Aitchison		
	F-model	R^2	Pr(>F)	F-model	R^2	Pr(>F)
Turtle ID	3.309	0.34	**0.0001**	13.897	0.69	**0.0001**
Genus	1.698	0.35	**0.0007**	1.579	0.16	0.1

monoclonal cultures. Bacterial communities of diatom phycospheres revealed instances of bacterial taxa enrichment and potentially important diatom-bacteria associations.

Sea turtle carapace and skin harbor diverse microbial communities

Sea turtles harbor diverse macro- and microorganisms on their carapace and skin (Frick and Pfaller 2013, Rivera et al. 2018, Van de Vijver et al. 2020, Kanjer et al. 2022). Bacterial communities associated with sea turtle skin and carapace are highly diverse and reflect the sampling locality of the turtle (expanded in Kanjer et al. 2022). The carapace and skin samples in this study were obtained from turtles that were sampled before admission to rehabilitation, during rehabilitation and post-rehabilitation after spending time in an open pool with recirculating sea water (see Table S1), which could affect the epizoic biofilm composition. Although our study focused on a limited number of turtles, our isolation and cultivation efforts led to establishing cultures of several newly described diatom species *A. elongata*, *A. squaliformis* and *P. lepidochelicola* and potential new species such as *Diploneis* sp., *Fallacia* sp., *Amphora* spp. and *Psammodictyon* sp. (Majewska et al. 2015, 2017).

Phycosphere bacterial community composition reflects diatoms' source environment and genus

Similar to other benthic diatom-bacteria biofilms, diatoms on sea turtles can be observed in dense assemblages, surrounded by extracellular polysaccharides and bacteria either on the diatom cells or surrounding organic matter (Bosak, unpublished data). Because diatom cells in this study were washed through several series of sterile growth medium during isolation, we assume that the bacteria transferred with the diatoms are the ones found in close association with the diatom cells' phycosphere in their natural habi-

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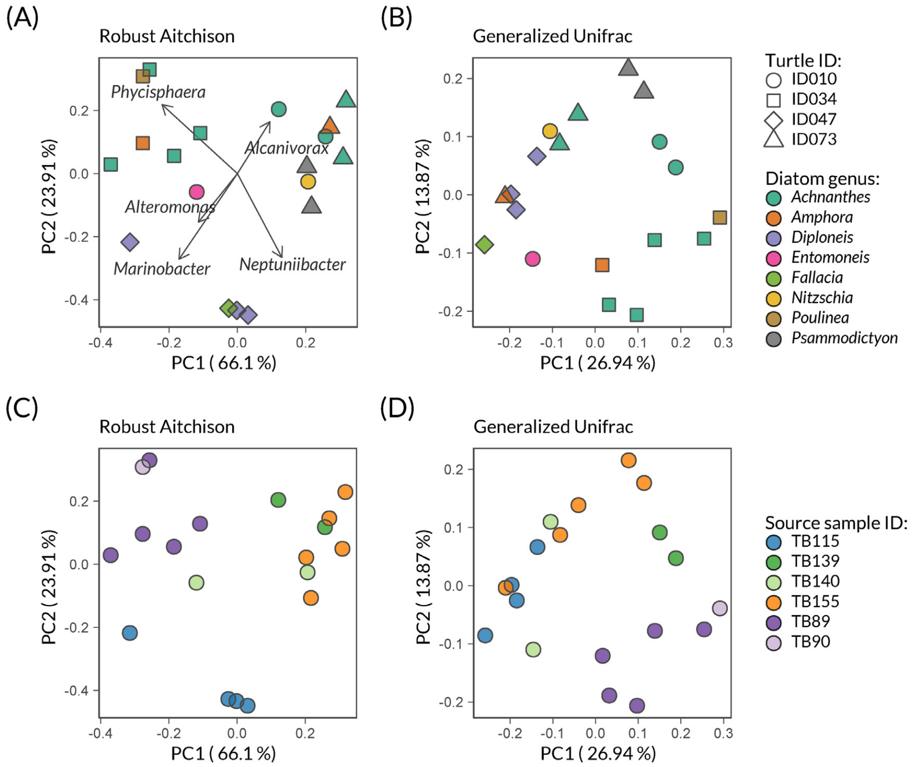

Figure 3. Bacterial community structure in cultivated diatom strains. Principal component analysis (PCA) of robust Aitchison distance (**A, C**) and principal coordinate analysis (PCoA) of generalized UniFrac (**B, D**) show sample clustering by turtle ID and diatom genus (A, B) or by specific source sample (C, D).

tat therefore allowing us to reveal potentially relevant diatom-bacteria associations. Specific ASVs detected both in source samples and in diatom cultures comprised only a small proportion of the total microbial community in source samples, while they made up almost half of the community in diatom cultures. Bacterial ASVs that were not detected in source samples but are present in diatom cultures could have been a part of rare taxa in source samples and possibly only became detectable once enriched in diatom cultures. Beta diversity analyses that do not consider the bacterial ASVs' phylogenetic relationships (Jaccard or Bray-Curtis) consistently grouped the diatom strain bacterial communities based on the source sample. However, once phylogenetic distance between bacterial ASVs was considered (unweighted, weighted and generalized UniFrac) the strength of the source sample effect lessened, and possible effects of the diatom host "selection" were accentuated. Our results support the findings that closely related diatom species recruit bacteria from their immediate environment and retain the environmental signature in culture while also selecting for related bacterial taxa, depending on their lifestyle and functions provided by the bacterial community (Amin et al. 2012, Seymour et al. 2017, Behringer et al. 2018, Crenn et al. 2018, Majzoub et al. 2019, Stock et al. 2019, 2022, Mönnich et al. 2020, Barreto Filho et al. 2021).

Diatoms enrich bacterial taxa that are otherwise scarce

Studies so far have shown diatom phycosphere is usually dominated by Proteobacteria (mainly Alphaproteobacteria) and Bacteroidota phyla members: *Sulfitobacter*, *Roseobacter*, *Ruegeria*, *Marinobacter*, *Alteromonas* and *Flavobacterium* (Amin et al. 2012, Goecke et al. 2013, Seymour et al. 2017, Majzoub et al. 2019), and that bacterial consortia are stable over time in xenic diatom monoclonal cultures (Behringer et al. 2018, Barreto Filho et al. 2021). While we consistently observed Alphaproteobacteria in diatom cultures, we also detected a strong enrichment of members of Gammaproteobacteria, namely *Nitrincolaceae* that are usually detected in diatom blooms (Liu et al. 2020) and *Alcanivoracaceae* that are predominant in oil-contaminated sea water (Kasai et al. 2002, Bookstaver et al. 2015). Historically, the genus *Alcanivorax* has been associated with hydrocarbon degradation, while recent studies show *A. borkumensis* is common in the plastisphere in the Mediterranean Sea with the ability to degrade low density polyethylene (Delacuvellerie et al. 2019). *Alcanivorax venustensis* and *A. borkumensis* were readily isolated from most diatom cultures in this study as it is possible that the diatom hosts produce organic nutrients beneficial to *Alcanivorax*. To the authors' knowledge, *Alcanivorax* genus has not yet been reported in other diatoms in culture. Even so, *Alcanivorax* has been reported in the phycosphere of dinoflagellates (Denaro et al. 2021), it was isolated from commercial *Nannochloropsis* cultures grown in plastic bags of ProviAPT reactors (Giraldo et al. 2019) and was found to be a major constituent of tidal biofilms where it could consume diatom-produced hydrocarbons (Coulon et al. 2012).

On the other hand, Planctomycetota phylum *Phycisphaeraceae* members were enriched in only a subset of diatom strains (genera *Achnanthes* and *Poulinea*). Diatom *P. lepidochelicola* harbored uncultured Planctomycetota 028H05-P-BN-P5 and BD7-11, uncultured *Phycisphaera* sp. and genera *Blastopirellula* and *Gimesia*, which have been described previously as intimately associated with macroalgal surfaces (Lage and Bondoso 2014, Bondoso et al. 2017, Wiegand

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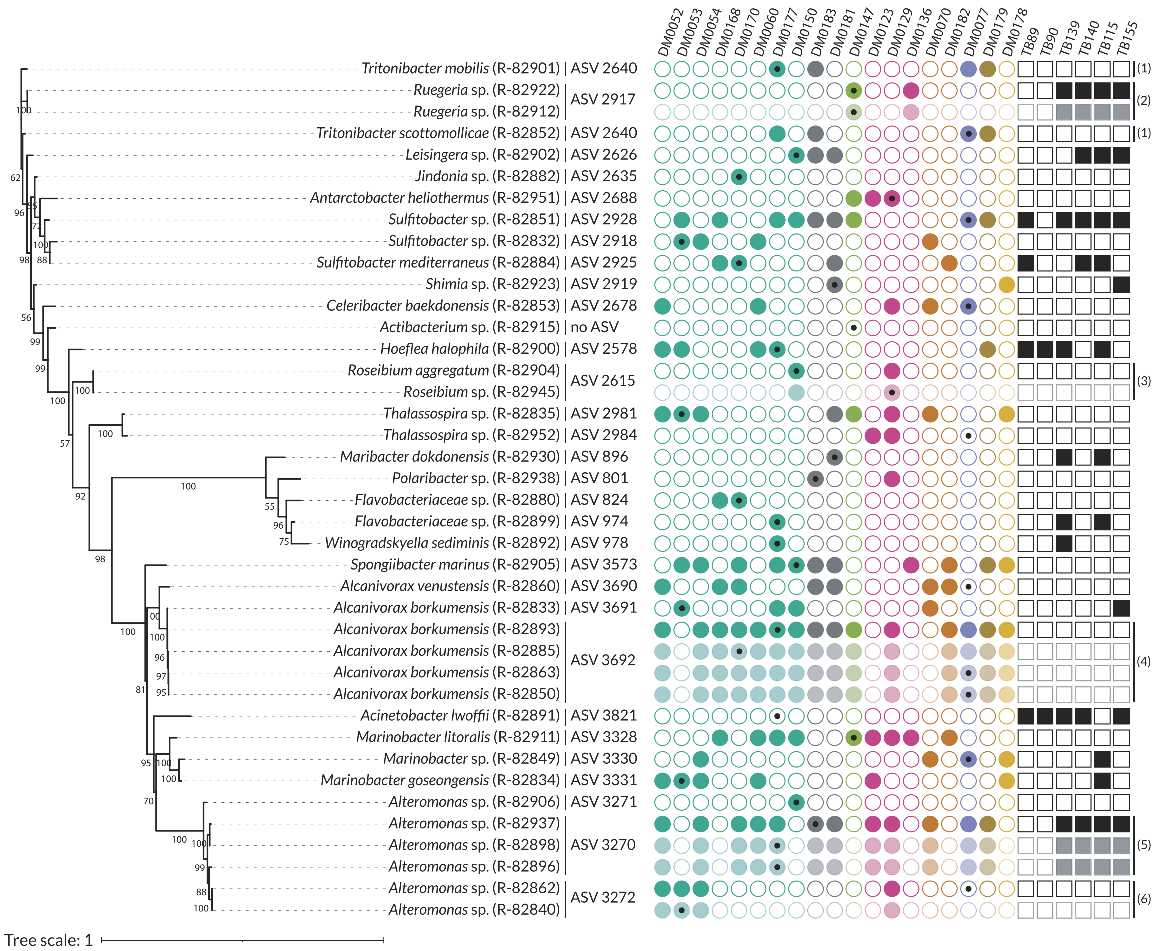

Figure 4. Relative relationships of bacterial isolates from diatom cultures and their matching ASV presence in diatom or source samples. Maximum likelihood phylogenetic tree of full 16S rRNA gene sequences of bacterial isolates was constructed in IQ-Tree and modified in iTOL (bootstrap values shown). ASVs matched with bacterial isolates were identified based on the V4 region of the full 16S rDNA of bacterial isolates. Their presence or absence in the diatom and source NGS data is shown for each bacterial strain by filled or empty circles, respectively. Diatom strain of origin for each bacterial isolate is indicated by a black dot in the presence/absence matrix. As several bacterial isolates were matched with an identical ASV there is repetition in rows of presence/absence data indicated by faded colors and numbers on the side of the matrix as follows: (1) ASV 2640, (2) ASV 2917, (3) ASV 2615, (4) ASV 3692, (5) ASV 3270, (6) ASV 3272.

et al. 2020). Members of phylum Planctomycetota are known for their uncommon traits: endomembranes, anammoxomes, reproduction by budding and good attachment abilities (Lage and Bondoso 2014 and the references therein) that might have facilitated colonization or even just close association with diatoms exhibiting high polysaccharide production during colony, chain and stalk formation (as observed in *Achnanthes* and *Poulinea* cultures).

Bacterial isolates from diatom cultures revealed that several bacterial taxa detected by NGS were readily cultured on MA, even demonstrating the potential for new species discovery as several bacterial isolates were recognized as potential novel genera within the *Flavobacteriaceae* family. These results complement previous efforts in characterizing bacteria associated with diatom cultures (Goecke et al. 2013, Stock et al. 2019, 2022). However, our culturing efforts were limited to 10 out of 19 diatom strains used in this study and biased both by diatom strain selection and growth media selection. Ideally, expanding culturing efforts to other diatoms from sea turtle surface could provide greater diversity of culturable bacteria as observed in a recently published inventory of bacterial isolates from corals and skin of cetaceans (Keller et al. 2021).

Potential factors responsible for shaping diatom associated bacterial communities

Investigations focused on laboratory cultures cannot grasp the complexities of microbial networks found in nature as bacterial communities of cultures often differ strongly from those of single cells (Crenn et al. 2018, Boscaro et al. 2022). It is rarely expected that the epizoic benthic diatoms live as single cells during most of their lifetime as they are found in biofilms. Also, we cannot assume diatom biofilms on sea turtles are limited to a single species but are often mixed. Consequently, there will be several layers and modes of interactions between diatoms, bacteria and even other microorganisms inhabiting biofilms on sea turtles.

The importance of host anatomy and spatial structure of microbial communities is recognized in vertebrate microbiome, emphasizing the effects of spatiotemporal microbiome variability from skin or gastrointestinal tract down to individual skin pores or even crypts of Lieberkühn (Conwill et al. 2022 and the references therein). Similar efforts to investigate anatomical sites and their specific microbial communities are often undertaken in echinoderms (Jackson et al. 2018), marine sponges (Hentschel et al. 2012,

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Verhoeven et al. 2017) and corals (van Oppen and Blackall 2019, Keller et al. 2021). In this study there are several levels of host anatomy: sea turtle carapace and skin, and diatoms either as members of complex biofilms on the turtle or as laboratory cultures. The loggerhead sea turtle carapace is a hard bone shell covered by a living epidermis with a thick outer layer of keratin scutes that are shed periodically. The macro- (position on the carapace) and microanatomy (carapace scutes' morphology) could affect both diatom colonization, localization and their associated bacteria composition through light and nutrient availability, probability of mechanical removal via sea currents or turtle behavior and colonization by other turtle epibionts (Blasi et al. 2021). Here, we collected the total microbial community from the carapace surface or skin and as a result could not test macro- and microanatomy effects on associated diatoms and bacteria. Additionally, at the phycosphere level, diatom colony or cell morphology could potentially affect the microbes that are in the near vicinity or attached to diatom cells directly. Naturally, large diatoms have a greater cell surface that could facilitate bacterial attachment in comparison with small diatoms that could prove difficult to colonize individually (Amin et al. 2012, Seymour et al. 2017), which could provide a possible explanation for the lowest number of observed ASVs in *Amphora* sp. 2 and *Nitzschia* sp. (around 5 and 10 micrometers long, respectively), but not in DM0170 *A. elongata* strain (around 35–40 micrometers long). Growth in colonies could further enable diatom-bacterial associations through extensive diatom polysaccharide production, cell-to-cell attachment, branching with mucilage pad junctions and stalk formation that could fortuitously provide extra surface and different "microanatomical" niches for closely associated bacteria.

Exact mechanisms of microbial community assembly in diatom hosts were not the focus of this study, but it seems diatom strains investigated here are open to diverse colonization rather than being restricted to a small number of bacterial symbiont taxa. Hosting a repertoire of diverse bacteria (and in turn harboring their metabolic potential) could possibly prove beneficial to complex microbial communities in dynamic environments (Henry et al. 2021, Pfister et al. 2022). However, it is difficult to infer the functions and potential benefits, or lack thereof, in microbial assemblages solely through marker gene amplicon sequencing data as marker gene sequences do not reflect the ecotypes and genomic diversity in microbes (Sjöqvist et al. 2021). Similarly, even though in our study most diatom strain *rbcL* sequences were matched to *rbcL* ASVs in source samples, taxonomic assignment of matched *rbcL* ASVs in source samples via Diat.barcode database did not parallel our morphology-based identification of novel diatom species (e.g. genera *Achnanthes*, *Poulinea* and *Fallacia*; see Fig. S1). Current *rbcL* databases still lack sequenced representatives of diatom groups such as benthic pennate diatoms, which leads to inconsistencies between sequencing and morphology data (Rivera et al. 2018). To investigate lesser known diatom taxa (as in this study) and their communities, expanding diatom isolation, cultivation and marker gene sequencing efforts is necessary. Hence, comprehensive diatom and bacteria community, and subsequently strain characterization, is needed to gain insight into functions and metabolic exchanges within epizoic diatom-bacteria communities. Even though studying complex diatom biofilms remains challenging, especially on marine vertebrate hosts, a combination of culture-based and culture-independent approaches focusing on individual diatom taxa and their associated bacteria can provide a baseline for discovering diatom-bacteria association patterns and hypothesis generation.

Ethics declarations

Sampling was performed in accordance with the 1975 Declaration of Helsinki, as revised in 2013 and the applicable national laws. The sampling at the Sea Turtle Clinic (Bari, Italy) was conducted with the permission of the Department of Veterinary Medicine Animal Ethic Committee (Authorization # 4/19), while sampling in Croatia was done in accordance with the authorization of the Marine Turtle Rescue Center by the Ministry of Environment and Energy of the Republic of Croatia.

Author notes

KF, SB, PC, WV and AW conceptualized the study. KF, LL and MŽ performed the laboratory work. Data curation, formal analysis, data visualization and writing of the original draft was performed by KF. SB, KF and AW provided funding for the research. All the authors were involved in the revision and editing of the manuscript.

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Supplementary data

Supplementary data are available at FEMSEC online.

Conflict of interest statement. The authors declare no conflict of interest.

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Publication III

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Cultivating epizoic diatoms provides insights into the evolution and ecology of both epibionts and hosts

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Our understanding of the importance of microbiomes on large aquatic animals—such as whales, sea turtles and manatees—has advanced considerably in recent years. The latest observations indicate that epibiotic diatom communities constitute diverse, polyphyletic, and compositionally stable assemblages that include both putatively obligate epizoic and generalist species. Here, we outline a successful approach to culture putatively obligate epizoic diatoms without their hosts. That some taxa can be cultured independently from their epizoic habitat raises several questions about the nature of the interaction between these animals and their epibionts. This insight allows us to propose further applications and research avenues in this growing area of study. Analyzing the DNA sequences of these cultured strains, we found that several unique diatom taxa have evolved independently to occupy epibiotic habitats. We created a library of reference sequence data for use in metabarcoding surveys of sea turtle and manatee microbiomes that will further facilitate the use of environmental DNA for studying host specificity in epizoic diatoms and the utility of diatoms as indicators of host ecology and health. We encourage the interdisciplinary community working with marine megafauna to consider including diatom sampling and diatom analysis into their routine practices.

Abbreviations

POE	Putatively obligate epizoic
SEM	Scanning electron microscope
bs	Bootstrap support
pp	Posterior probability

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Common health indicators currently used to monitor cetaceans, sirenians and sea turtles include mortality rates, demographics, disease prevalence and frequency of stranding events. Since animal-associated microbiota may affect and be affected by their host, both internal and external microbiome composition at any given time could also reflect mid- and longer-term effects of disturbances or stressors experienced by the animal[1]. New health and fitness indices based on compositional changes in the native microbiomes could be a valuable addition to comprehensive health assessments for aquatic vertebrates[2].

Studies on the external microbiome of large aquatic vertebrates have typically focused on the bacterial and/or viral components. In contrast, epizoic microeukaryotes remain poorly explored despite the observation of diatoms on whales over a century ago[3,4]. Diatoms (Bacillariophyta) are a diverse group of largely photosynthetic microalgae characterized by their uniquely shaped siliceous thecae (frustules) and are commonly found in the plankton and benthos of many different aquatic habitats. Recent studies have expanded the known diversity of epizoic diatoms through increased sampling of hosts to include sea turtles[5–22], sea snakes[23] and manatees[24,25].

Competition for limited resources among diatoms has led to niche partitioning and significant habitat specificity in some taxa. The epizoic diatom communities growing on aquatic vertebrates appear to be formed by a combination of opportunistic surface-attached taxa and putatively obligate epizoic (POE) taxa. While the opportunistic taxa are shared across the benthic habitats of the local environment, the POE taxa thus far have only been observed in the epizoic microbiome[7,21,26,27]. This mixture of opportunistic and POE taxa is an intriguing assemblage, as it is potentially influenced by the host's biology (e.g. physiology, anatomy and host-specific prokaryotic microbiome) and behavior (e.g. long-distance migrations, diving, basking, and terrestrial nesting which expose epibionts to extremes in temperature, pressure, irradiance, nutrient concentration and desiccation) as well as the environment (e.g. mean temperature, salinity, nutrient load, local biocenoses). Moreover, the unique and highly specific diatom flora composition can be documented long past the death of the diatom cells by the weathering-resistant inorganic frustules. This has resulted in diatoms being utilized extensively for paleoecological reconstructions and bioindication in freshwater environments; for multiple reviews, see[28]. Similar diatom-based health indices may be developed for the marine animals and their habitats.

However, before this can happen, at least two issues must be addressed:

1) We must expand upon our knowledge of the specific molecular, genomic and ecological nature of the interactions between POE diatoms and their host and environment.

2) We need to simplify the identification of epizoic diatoms, which currently requires specialized equipment (such as electron microscopy) and literature that can be highly fragmented and incomplete, particularly in the case of marine diatoms.

Both of these issues could be addressed by metagenomic and metabarcoding techniques, respectively. Currently, however, the dearth of reference data—both in annotated genome and transcriptomes as well as vouchered DNA barcodes for diatoms—would limit the effectiveness of either effort. For example, a metabarcoding attempt on sea turtle epiflora[29] failed to recover some of the diatom taxa identified in microscopical surveys, including the dominant POE taxon *Labellicula lecohuiana* Majewska, De Stefano & Van de Vijver. The authors acknowledged that this failure was likely due to the lack of any relevant reference sequences for the genus *Labellicula*. Further, the position of *Labellicula* in the molecular phylogeny of diatoms is unknown. This uncertainty significantly hinders any bioinformatic efforts to find sequence data even closely related to *Labellicula* among both the metabarcoding reads and the reference databases. Many other POE taxa have uncertain phylogenetic affinities within the raphid diatoms, including *Tursiocola* Holmes, Nagasawa & Takano, *Epiphalaina* Holmes, Nagasawa & Takano and the "*Tripterion* complex". This latter assemblage of diatom genera (*Tripterion* Holmes, Nagasawa & Takano, *Chelonicola* Majewska, De Stefano & Van de Vijver, *Poulinea* Majewska, De Stefano & Van de Vijver and *Medlinella* Frankovich, Ashworth & M.J.Sullivan) is of particular taxonomic interest as they represent a radiation of exclusively epizoic diatom taxa. Their current taxonomy is not universally accepted[15], and distinguishing the genera can be difficult without the use of electron microscopy due to a similar overall frustule morphology (heteropolar, stalked and septate or pseudoseptate) and relatively small size (< 20 um).

To address the aforementioned issues, we have cultured and sequenced DNA data from POE diatom taxa. These were isolated from sea turtles and manatees from the wild, rehabilitation and rescue centers as well as aquaria from the United States of America, The Bahamas, Croatia, Italy and South Africa. While DNA sequence data from vouchered specimens alone would be useful for molecular identification, the ability to maintain these diatoms away from their hosts facilitates the formulation of hypotheses and laboratory experiments to test the molecular nature of the relationship between the diatom and host.

Results

Culture success.

We successfully cultured > 600 strains, both POE and opportunistic diatoms on the epizoic habitat. This manuscript focuses on 76 of these sequenced strains (Table 1) and the sequences from the single-cell DNA extractions of the non-photosynthetic *Tursiocola* spp. (Figs. 1, 2). Sequence data from 21 additional diatoms are included (Figs. S1, S2). While these additional sequenced diatom taxa were isolated from epizoic collections, they are known opportunistic taxa, occur in non-epizoic habitats, or their habitat preferences are unclear.

Target POE taxa.

POE taxa were identified based on the available literature and included diatom species that have only ever been observed in association with the epizoic habit being found on multiple animal specimens[6,8,10,11,14–16,24,25,30]. Among these were epizoic taxa typically reaching high relative abundances (> 25%)—*Achnanthes elongata* Majewska & Van de Vijver, *Chelonicola costaricensis* Majewska, De Stefano & Van de Vijver, *C. caribeana* Riaux-Gobin, Witkowski, Ector & Chevallier, *Craspedostauros danayanus* Majewska & Ashworth, *Medlinella amphoroidea* Frankovich, Ashworth & M.J.Sullivan, *Poulinea lepidochelicola* Majewska,

Host species	Location	Host status	POE Diatoms cultured (# of strains) [total cultures]
Chelonia mydas (Green Sea Turtle)	Bahamas	Wild animal: "turtle1"	Cca (2), Td (2), Pv (1) [9]
Chelonia mydas (Green Sea Turtle)	Durban, South Africa	Aquarium resident: "Calypso"	Cco (1), Cm (3), Pl (2) [14]
Chelonia mydas (Green Sea Turtle)	Durban, South Africa	Aquarium resident: "Wasabi"	Ma (1), Pl (4) [12]
Chelonia mydas (Green Sea Turtle)	Florida, USA	Wild animal: "FL noname"	Ae (2), Tg (1) [6]
Chelonia mydas (Green Sea Turtle)	Florida, USA	Rehabilitation animal: "Fleming"	Ae (5), Pl (3), Pv (3) [22]
Eretmochelys imbricata (Hawksbill Sea Turtle)	Texas, USA	Aquarium resident: "Einstein"	Ca (2) [4]
Eretmochelys imbricata (Hawksbill Sea Turtle)	Durban, South Africa	Aquarium resident: "Tripod"	Ma (3) [11]
Lepidochelys kempii (Kemp's Ridley Sea Turtle)	Georgia, USA	Wild animal: "Z6"	Ae (3) [12]
Dermochelys coriacea (Leatherback Sea Turtle)	Kosi Bay, South Africa	Wild animal: "ZA0019A/ZA1824E"	Cd (1) [7]
Caretta caretta (Loggerhead Sea Turtle)	Durban, South Africa	Aquarium resident: "Shiv"	Pl (3) [6]
Caretta caretta (Loggerhead Sea Turtle)	Kosi Bay, South Africa	Wild animal: "ZA00940/ZA10860"	Ma (1) [4]
Caretta caretta (Loggerhead Sea Turtle)	Kosi Bay, South Africa	Wild animal: "ZA1595E/ZA1826E"	Pl (1) [10]
Caretta caretta (Loggerhead Sea Turtle)	Kosi Bay, South Africa	Wild animal	Csp (2) [10]
Caretta caretta (Loggerhead Sea Turtle)	Florida, USA	Wild animal: "A2"	Ae (7) [8]
Caretta caretta (Loggerhead Sea Turtle)	Florida, USA	Wild animal: "CC032217a"	Cca (2), Pv (2) [19]
Caretta caretta (Loggerhead Sea Turtle)	Florida, USA	Wild animal: "FL Christine"	Cca (2) [6]
Caretta caretta (Loggerhead Sea Turtle)	Brijuni Islands, Croatia	Aquarium resident: "Lunga"	Ps (1) [3]
Caretta caretta (Loggerhead Sea Turtle)	Bisceglie, Italy	Rehabilitation animal: "Iracus"	Pl (3) [38]
Lepidochelys olivacea (Olive Ridley Sea Turtle)	Long Beach, California	Aquarium resident: "LoMain"	Pl (1) [14]
Lepidochelys olivacea (Olive Ridley Sea Turtle)	Florida, USA	Rehabilitation animal: "Harry"	Ae (2), Pl (3) [10]
Trichechus manatus latirostris (West Indian Manatee)	Florida, USA	Wild animal: "FLMan40"	Ae (2) [11]
Trichechus manatus latirostris (West Indian Manatee)	Georgia, USA	Wild animal: "CGA1605"	Ae (1) [23]

Table 1. POE diatoms cultured in this study, sorted by host species. POE diatoms are abbreviated and followed by the number of strains cultured from the indicated host: Ae = *Achnanthes elongata*, Ca = *Craspedostauros alatus*, Cd = *Craspedostauros danayanus*, Cm = *Craspedostauros macewanii*, Cca = *Chelonicola caribeana*, Cco = *Chelonicola costaricensis*, Csp = *Chelonicola sp*., Ma = *Medlinella amphoroidea*, Pl = *Poulinea lepidochelicola*, Ps = *Proschkinia sulcata*, Pv = *Proschkinia vergostriata*, Td = *Tursiocola denysii*, Tg = *Tursiocola guyanensis*.

De Stefano & Van de Vijver, *Tursiocola* spp., as well as species often present on animals but never exceeding 10% of the diatom relative abundance—*Craspedostauros alatus* Majewska & Ashworth, *C. macewanii* Majewska & Ashworth, *Proschkinia sulcata* Majewska, Van de Vijver & Bosak and *P. vergostriata* Frankovich, Ashworth & M.J Sullivan. SEM images of some of these taxa sampled for DNA can be found in Fig. 3. This list of POE taxa is not exhaustive as the full diversity of POE diatoms remains to be documented. Moreover, it does not include several probable POE species (e.g. *Achnanthes squaliformis* Majewska & Van de Vijver, *Navicula dermochelycola* Riaux-Gobin, Witkowski, Kociolek & Chevallier), which have not yet been isolated and cultured.

Molecular phylogenetic results. The currently recognized POE strains were predominantly located in two clades in the molecular phylogeny—*Achnanthes* sensu stricto + *Craspedostauros* (Fig. 1) and the clade containing the *Tripterion* complex, *Tursiocola* and *Proschkinia* (Fig. 2). With regards to *Achnanthes*, most of the sampled diversity comes from three species of sea turtles (green, Kemp's ridley and loggerhead) and West Indian manatees sampled in the southeastern US. These strains formed a well-supported clade (ML bootstrap support [bs] = 100%, BI posterior probability [pp] = 1.0) sister to the rest of the sequenced *Achnanthes* spp. The

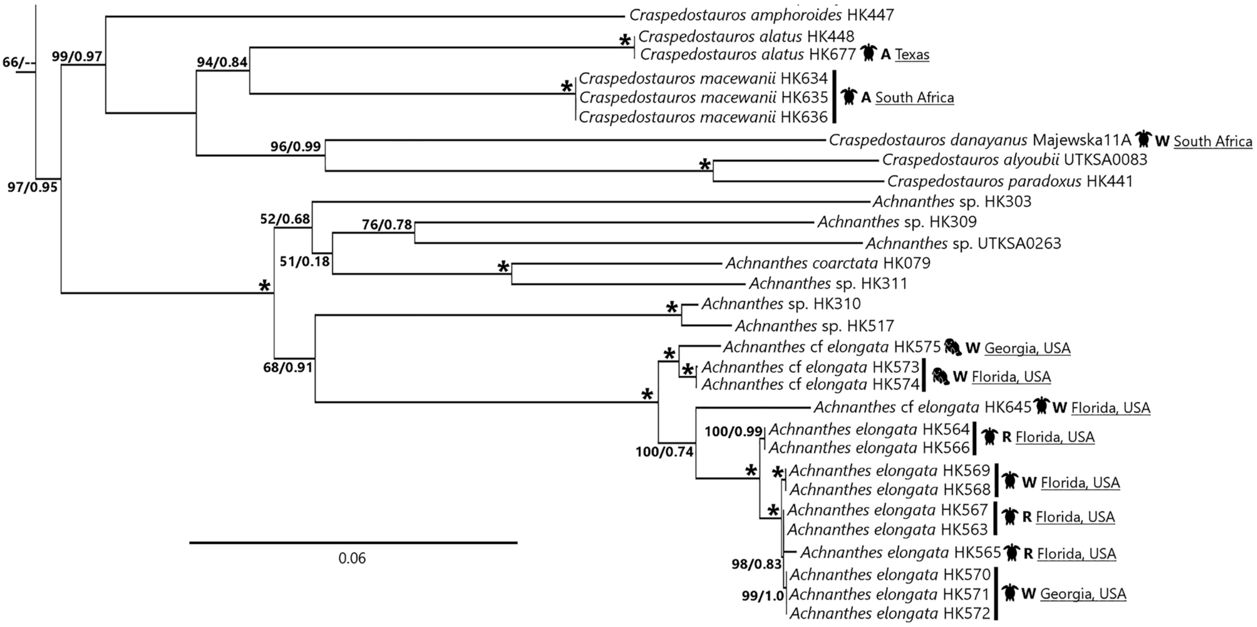
Craspedostauros amphoroides HK447
Craspedostauros alatus HK448
Craspedostauros alatus HK677 A Texas
Craspedostauros macewanii HK634
Craspedostauros macewanii HK635
Craspedostauros macewanii HK636
A South Africa
Craspedostauros danayanus Majewska11A W South Africa
Craspedostauros alyoubii UTKSA0083
Craspedostauros paradoxus HK441
Achnanthes sp. HK303
Achnanthes sp. HK309
Achnanthes sp. UTKSA0263
Achnanthes coarctata HK079
Achnanthes sp. HK311
Achnanthes sp. HK310
Achnanthes sp. HK517
Achnanthes cf elongata HK575 W Georgia, USA
Achnanthes cf elongata HK573
Achnanthes cf elongata HK574
W Florida, USA
Achnanthes cf elongata HK645 W Florida, USA
Achnanthes elongata HK564
Achnanthes elongata HK566
R Florida, USA
Achnanthes elongata HK569
Achnanthes elongata HK568
W Florida, USA
Achnanthes elongata HK567
Achnanthes elongata HK563
R Florida, USA
Achnanthes elongata HK565 R Florida, USA
Achnanthes elongata HK570
Achnanthes elongata HK571
Achnanthes elongata HK572
W Georgia, USA
66/--
99/0.97
97/0.95
94/0.84
96/0.99
52/0.68
76/0.78
51/0.18
68/0.91
100/0.99
100/0.74
98/0.83
99/1.0
*
0.06

Figure 1. Maximum likelihood phylogenetic tree derived from a concatenated 3-gene DNA sequence dataset, representing the *Achnanthes*, *Craspedostauros* and *Staurotropis* clades (complete tree shown in Fig. S1). Support values (ML bootstrap support/BI posterior probability) shown above nodes; "*" = nodes with 100%/1.0 values. Taxon name followed by DNA extraction voucher number or strain ID. Taxa isolated from epizoic habitats followed by a diagrammatic representation of the host from which the strain was isolated, and metadata on the location and setting in which the host was sampled (A = aquarium, R = rehabilitation facility, W = wild).

POE *Achnanthes* clade also sorted by host, with strains collected from manatee (100%/1.0 bs/pp) and sea turtle (100%/0.74 bs/pp) hosts in their own clades. The POE *Craspedostauros* taxa showed a different pattern to the rest of the POE diatoms. Their clade included both POE and non-POE species, with POE taxon *C. danayanus* sister to *C. alyoubii* and *C. paradoxus* (96%/0.99 bs/pp) rather than to the POE *C. macewanii* and *C. alatus*.

The "*Tripterion* complex + " clade (strains illustrated in Fig. 3a–d) was resolved with strong support (100%/1.0 bs/pp). While we were able to sample taxa from the *Chelonicola*, *Poulinea* and *Medlinella* genera in this complex, we were unable to observe any taxa within *Tripterion* sensu stricto in our collections. The "*Tripterion* complex + " clade also contained the POE genus *Tursiocola* and *Proschkinia* Karayeva, which has both POE and non-POE species, as well as the non-epizoic genera *Stauroneis* Ehrenberg, *Craticula* Grunow, *Parlibellus* E.J.Cox, *Fistulifera* Lange-Bertalot and some monoraphid genera such as *Schizostauron* Grunow and *Astartiella* Witkowski, Lange-Bertalot & Metzeltin. The molecular data suggested no common origin for the POE clades; *Tursiocola* and the *Tripterion* complex are sister to non-POE taxa rather than each other, and the POE *Proschkinia* (*P. vergostriata* and *P. sulcata*) formed a clade sister (100%/1.0 bs/pp) to the rest of the *Proschkinia* spp.

Within *Tursiocola*, both nutritional types appear monophyletic, with the non-photosynthetic manatee-associated taxa (*T. alata*, *T. bondei*, *T. varicopulifera* and *T. ziemanii*) and the photosynthetic sea turtle-associated taxa (*T. denysii* and *T. guyanensis*) in their own clades (100%/1.0 bs/pp for both clades). It should be noted, however, that there were only two photosynthetic *Tursiocola* taxa sampled. Tree topology in the *Tripterion* complex remained the same regardless of analysis, with *Chelonicola costaricensis* "Majewska21C" + *Poulinea lepidochelicola* (100%/1.0 bs/pp) sister to *Medlinella amphoroidea* + *Chelonicola* sp. "Majewska39A/40A" + *C. caribeana* (92%/0.74 bs/pp).

Only two clades in the *Tripterion* complex had any geographic variation: the *Poulinea* clade and *Chelonicola caribeana* clade. For *Poulinea*, strains collected in South Africa were not monophyletic, with "Majewska 17C" sister to the rest of the clade, which included strains isolated from the Adriatic, Florida, California and South Africa. It should be noted that the Florida clade represented strains collected from a single location—a rehabilitation facility—while the South African strains were isolated from collections of both wild and captive host animals. The *C. caribeana* clade, on the other hand, contained strains isolated exclusively from wild host animals in South Africa, Florida and the Bahamas, with the South African strains ("Majewska39A/40A") sister to the rest.

Discussion

Based on our molecular phylogeny, it appears that the epizoic habit has evolved several times and in several different raphid diatom morphotypes: elongate biraphid (*Tursiocola* and *Proschkinia* , Fig. 3f,g, respectively) and monoraphid frustules (*Achnanthes*, Fig. 3e), asymmetric, clavate biraphid frustules (*Tripterion* complex, Fig. 3a) and thin oval monoraphid frustules (*Bennettella, Epipellis*[31]). These independent gains of the epizoic habit could be driven by the host biology and evolution. The various epizoic diatom lineages, if eventually resolved to be

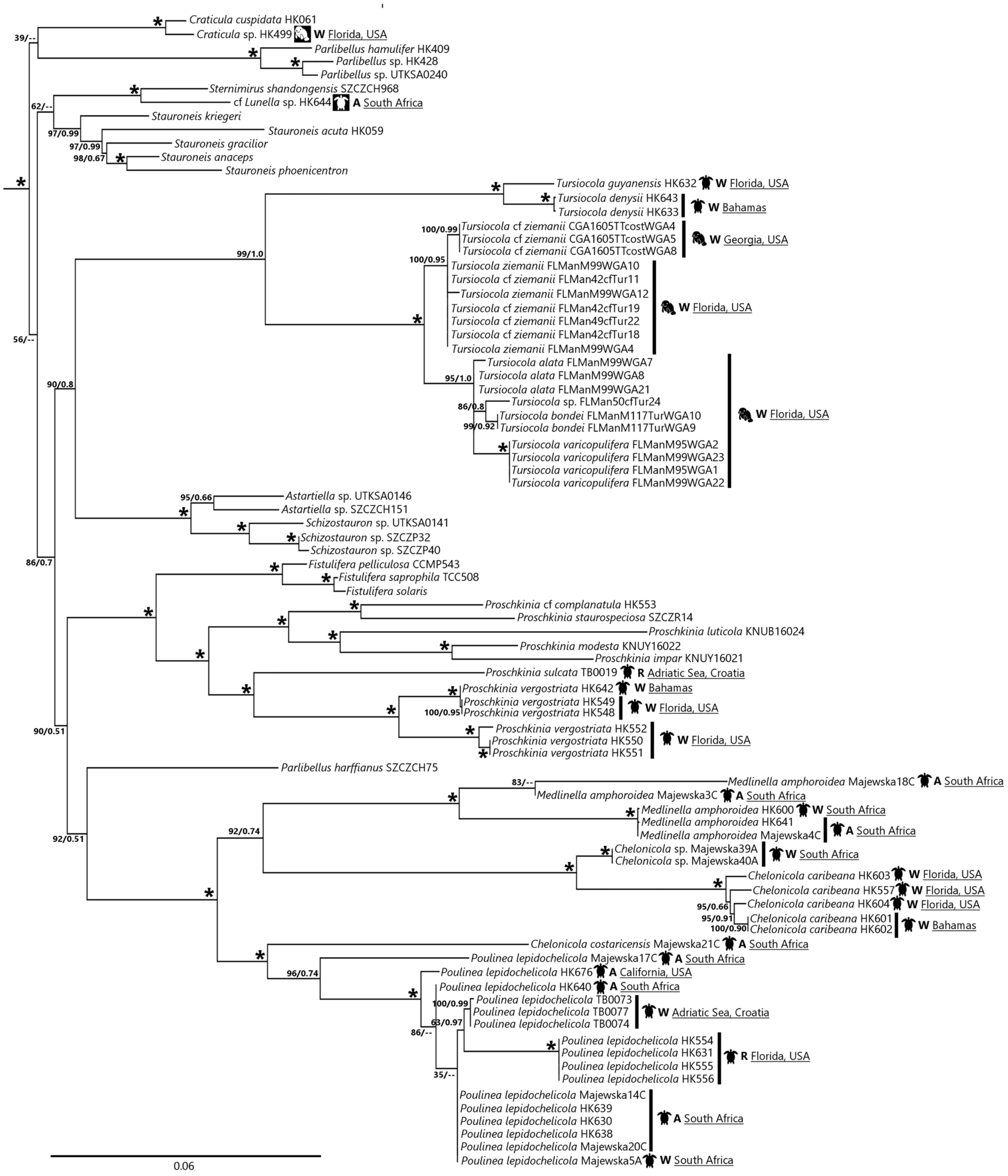
Craticula cuspidata HK061
Craticula sp. HK499 W Florida, USA
Parlibellus hamulifer HK409
Parlibellus sp. HK428
Parlibellus sp. UTKSA0240
Sternimirus shandongensis SZCZCH968
cf Lunella sp. HK644 A South Africa
Stauroneis kriegeri
Stauroneis acuta HK059
Stauroneis gracilior
Stauroneis anaceps
Stauroneis phoenicentron
39/--
62/--
97/0.99
97/0.99
98/0.67
56/--
*
Tursiocola guyanensis HK632 W Florida, USA
Tursiocola denysii HK643
Tursiocola denysii HK633
W Bahamas
Tursiocola cf ziemanii CGA1605TTcostWGA4
Tursiocola cf ziemanii CGA1605TTcostWGA5
Tursiocola cf ziemanii CGA1605TTcostWGA8
W Georgia, USA
Tursiocola ziemanii FLManM99WGA10
Tursiocola cf ziemanii FLMan42cfTur11
Tursiocola ziemanii FLManM99WGA12
Tursiocola cf ziemanii FLMan42cfTur19
Tursiocola cf ziemanii FLMan49cfTur22
Tursiocola cf ziemanii FLMan42cfTur18
Tursiocola ziemanii FLManM99WGA4
W Florida, USA
Tursiocola alata FLManM99WGA7
Tursiocola alata FLManM99WGA8
Tursiocola alata FLManM99WGA21
Tursiocola sp. FLMan50cfTur24
Tursiocola bondei FLManM117TurWGA10
Tursiocola bondei FLManM117TurWGA9
Tursiocola varicopulifera FLManM95WGA2
Tursiocola varicopulifera FLManM99WGA23
Tursiocola varicopulifera FLManM95WGA1
Tursiocola varicopulifera FLManM99WGA22
W Florida, USA
100/0.99
100/0.95
99/1.0
95/1.0
86/0.8
99/0.92
90/0.8
Astartiella sp. UTKSA0146
Astartiella sp. SZCZCH151
Schizostauron sp. UTKSA0141
Schizostauron sp. SZCZP32
Schizostauron sp. SZCZP40
95/0.66
86/0.7
Fistulifera pelliculosa CCMP543
Fistulifera saprophila TCC508
Fistulifera solaris
Proschkinia cf complanatula HK553
Proschkinia staurospeciosa SZCZR14
Proschkinia luticola KNUB16024
Proschkinia modesta KNUY16022
Proschkinia impar KNUY16021
Proschkinia sulcata TB0019 R Adriatic Sea, Croatia
Proschkinia vergostriata HK642 W Bahamas
Proschkinia vergostriata HK549
Proschkinia vergostriata HK548
W Florida, USA
100/0.95
Proschkinia vergostriata HK552
Proschkinia vergostriata HK550
Proschkinia vergostriata HK551
W Florida, USA
90/0.51
Parlibellus harffianus SZCZCH75
Medlinella amphoroidea Majewska18C A South Africa
Medlinella amphoroidea Majewska3C A South Africa
83/--
Medlinella amphoroidea HK600 W South Africa
Medlinella amphoroidea HK641
Medlinella amphoroidea Majewska4C
A South Africa
Chelonicola sp. Majewska39A
Chelonicola sp. Majewska40A
W South Africa
Chelonicola caribeana HK603 W Florida, USA
Chelonicola caribeana HK557 W Florida, USA
Chelonicola caribeana HK604 W Florida, USA
Chelonicola caribeana HK601
Chelonicola caribeana HK602
W Bahamas
95/0.66
95/0.91
100/0.90
92/0.74
92/0.51
Chelonicola costaricensis Majewska21C A South Africa
Poulinea lepidochelicola Majewska17C A South Africa
Poulinea lepidochelicola HK676 A California, USA
Poulinea lepidochelicola HK640 A South Africa
Poulinea lepidochelicola TB0073
Poulinea lepidochelicola TB0077
Poulinea lepidochelicola TB0074
W Adriatic Sea, Croatia
100/0.99
63/0.97
86/--
96/0.74
Poulinea lepidochelicola HK554
Poulinea lepidochelicola HK631
Poulinea lepidochelicola HK555
Poulinea lepidochelicola HK556
R Florida, USA
35/--
Poulinea lepidochelicola Majewska14C
Poulinea lepidochelicola HK639
Poulinea lepidochelicola HK630
Poulinea lepidochelicola HK638
Poulinea lepidochelicola Majewska20C
A South Africa
Poulinea lepidochelicola Majewska5A W South Africa
0.06

Figure 2. Maximum likelihood phylogenetic tree derived from a concatenated 3-gene DNA sequence dataset, representing the clade containing the *Tripterion* complex, *Tursiocola* and *Proschkinia* clades (complete tree shown in Fig. S1). Support values (ML bootstrap support/BI posterior probability) shown above nodes; "*" = nodes with 100%/1.0 values. Taxon name followed by DNA extraction voucher number or strain ID. Taxa isolated from epizoic habitats followed by a diagrammatic representation of the host from which the strain was isolated, and metadata on the location and setting in which the host was sampled (A = aquarium, R = rehabilitation facility, W = wild). Black host icon = POE taxon; white host icon = unclear habitat preference.

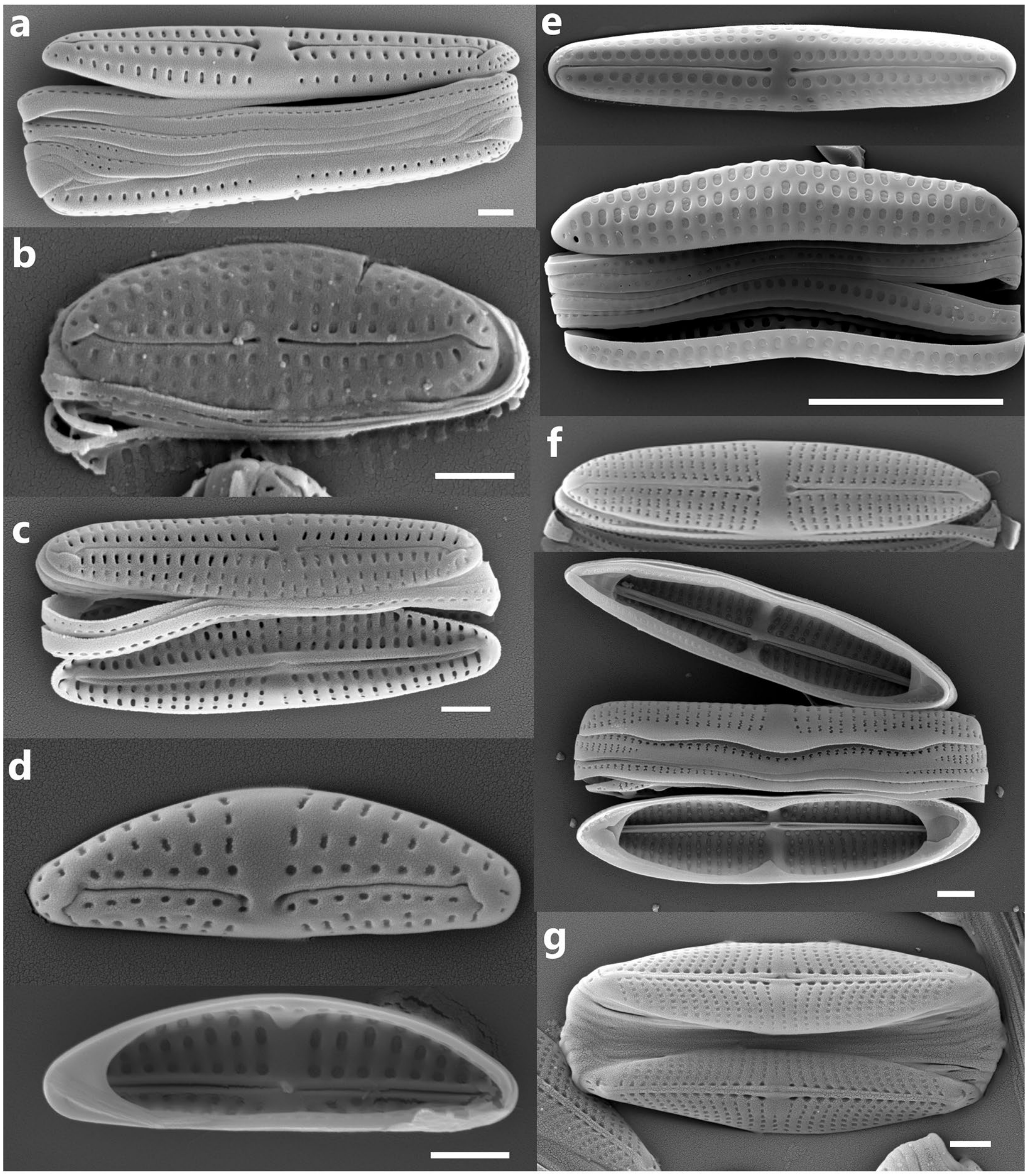

Figure 3. Scanning electron micrographs of some of the POE diatom taxa successfully cultured and sampled for DNA. a = *Poulinea lepidochelicola* HK630, complete frustule. b = *Chelonicola* cf *costaricensis* Majewska 21C, valve exterior. c = *Chelonicola* sp. Majewska 40A, complete frustule. d = *Medlinella amphoroidea* HK600 (valve exterior above, interior below). e = *Achnanthes elongata* HK563 (valve exterior above, complete frustule below). f = *Tursiocola denysii* HK633 (valve exterior above, complete frustule below). g = *Proschkinia vergostriata* HK552, complete frustule. All scale bars = 1 µm.

closely linked to a specific type of host animal, might have diverged from non-epizoic taxa under different ecological and evolutionary constraints and at different times corresponding to the emergence of various groups of marine megafauna.

Among others, the eco-physiological constraints shaping epizoic diatom speciation through adaptive radiation would include the nature and character of the animal substrate. Variations of the dermal layer of sirenians and sea turtles including the ultrastructure, topology, physiology (e.g. shedding patterns), and biochemistry (e.g. enzymatic activity) would require different attachment and colonization (and re-colonization) strategies, thus encouraging the development of specific adaptations. Such a specific adaptation is evidenced by *Melanothamnus maniticola* Woodworth, Frankovich & Freshwater, an epizoic red alga on manatees that has unique skin penetrating rhizoids that anchor the thallus to the deeper epidermis and permit the alga to persist as the host surface skin cells are shed[32]. In marine reptiles, the carapace scutes are often shed periodically, while the skin scales are either shed continuously (sea turtles) or the epidermis is renewed completely in a process called ecdysis (sea snakes[33]). These patterns differ from those observed in marine mammals in which skin shedding may be regulated by external factors such as temperature[34]. Similarly, animals with different diving regimes may host diatoms with different physiological and metabolic adaptations as various stages of photosynthesis will be differently affected by changes in hydrostatic pressure related to the depth, duration, and frequency of dives[35].

Moreover, the diversification dynamics in POE diatoms may be linked to the host animal behavior and lifestyle. The niche heterogeneity, biodiversity, productivity, and nutrient concentrations typical of shallow-water habitats occupied by sirenians and some sea turtles may increase colonization rates by new species and favor benthic diatom immigration to the epizoic community, thus spurring the observed diversity of diatom forms associated with manatees[24,25] or sea turtles using neritic foraging habitats (e.g. loggerheads;[21]). The opposite phenomenon could explain low epizoic diatom diversity on leatherback sea turtles[5,30], and pelagic sea snakes[23] that spend significant time feeding in the pelagic zone rather than on benthic organisms[36]. This follows the general pattern of low macro-epibiotic diversity on leatherbacks[37]. Epizoic diatom diversity might also be driven by intrinsic biotic factors, such as gregariousness and range of the host species as both factors may affect the new species encounter and colonization rates. However, in these systems in which epizoic diatom species richness is driven mainly by speciation rates as opposed to benthic species immigration, the total epizoic diatom diversity may remain low. The higher number of diatom taxa observed on neritic megafauna species as compared to open-water animals seem to support this hypothesis[20].

Currently, taxon sampling is still scattered, and while strains were isolated from multiple geographic localities, much of the strain diversity in species-level clades come from a single collection. The Florida *Poulinea lepidochelicola* clade, for example, represents strains isolated exclusively from the Turtle Hospital rehabilitation facility in Marathon, Florida. Among the South African *P. lepidochelicola* strains, six strains (Majewksa 14C, Majewska 20C, HK630, HK638, HK639 and HK640) came from collections from three turtles at the uShaka Sea World facility in Durban, and likely represent one population. However, a morphological difference does exist between the sequenced *Medlinella amphoroidea* strains from South Africa and the type population of Florida Bay. The valve areolae of the former appear to be occluded by hymenes (Fig. 3d) as opposed to the volae of the type population[14]. Whether this corresponds to a genetic, and perhaps species differentiation remains to be seen, once the Florida Bay population is sequenced.

While we do not yet have enough information to assign any sort of host specificity to certain POE diatom taxa, we have enough DNA sequence data to suggest that some genetic differentiation among POE diatoms is occurring. While we do not know if the genetic distance between the Florida, Mediterranean and South African *Poulinea* strains is driven by speciation or intraspecific biogeography, they are genetically distinct. Data collected from loggerheads suggests little mixing between sea turtle individuals across ocean basins[38], with the Mediterranean population being distinct from the northeast Atlantic one, which is then distinct from northwest Atlantic (including the Gulf of Mexico) population. Even within closer geographic boundaries, such as the western Atlantic, there is demonstrated genetic distance between POE strains (*C. caribeana* of Florida and the Bahamas; *Achnanthes elongata* of Florida and Georgia) in DNA sequence markers which are generally considered too conserved to show intraspecific variation in diatoms[39,40].

The collection of molecular information from a larger number of POE diatom strains may reveal whether genetic diversity in epizoic diatoms reflects biogeographic, ecological, and behavioral patterns observed in the host animal populations. For example, it was demonstrated that sea turtle phylogeography is shaped by the sea turtle species thermal regime and habitat preference[41]. Provided the close relationship between epizoic diatoms and sea turtles holds up under the scrutiny of increased data sampling, it may be expected that POE diatoms associated with the cold-tolerant leatherbacks, which are able to use the southwestern corridors to migrate across the oceans, will be characterized by lower genetic diversity than diatom taxa growing on tropical species such as green turtles, hawksbills, and olive ridley sea turtles, whose Atlantic and Indo-Pacific populations appear to be genetically distinct[42]. This knowledge may significantly advance our understanding about evolutionary relationships between diatoms and their animal hosts as well as shed more light on the mechanistic processes of divergence and adaptive evolution of diatoms and other marine microbes.

This study lays the groundwork for biodiversity and biogeographical work in marine epibioses by starting the development of a database of DNA sequence data from 16 of the known POE diatom species for sea turtles and manatees. These sequences will also be useful in not only identifying more POE taxa, but searching for potential refugia of these taxa in non-epizoic habitats. Large areas of the world's marine shallow benthic environment are poorly studied for diatoms, and therefore we cannot exclude the possibility that the POE taxa do exist outside of epizoic habitats. Even in localities that are relatively well-studied for benthic diatoms, variation in the composition and relative abundance in an assemblage due to substrate specificity and seasonality make the assembly of an exhaustive diatom flora extremely difficult. Environmental DNA surveys, such as metabarcoding, have an advantage over microscope-based surveys with regards to relatively small-sized taxa. Based on the molecular phylogeny of the *Tripterion* complex, it is easy to see how these taxa might have remained undetected in a bioinformatic summary of OTUs by sequence similarity, as there is significant genetic difference between the *Tripterion* complex and the only other sequenced representatives of the Rhoicospheniaceae—the freshwater taxon *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot. In fact, there are no morphological characters exclusive to the taxa in the molecular clade containing *Tursiocola* and the *Tripterion* complex that would cause a diatomist to expect a close match in sequence identity to the POE taxa. With curated sequence data now available for the most common POE taxa, we may find evidence for their occurrence in non-epizoic habitats through eDNA studies.

One of the stated goals of this study was to generate additional DNA sequence data from POE diatom taxa on sea turtles and sirenians. This goal was greatly aided by our ability to culture many of these POE diatoms away from their hosts, which raises several questions about the ecological requirements and adaptations of epizoic diatoms. The isolated strains of POE diatoms, which can be maintained in artificial conditions and without the animal hosts, provide opportunities to further study the molecular, genomic and physiological nature of the unique relationship between the diatoms and marine megafauna in a laboratory setting. For example, we can examine how different species may be affected by different conditions or possess specific adaptations to epizoic lifestyle. It is possible that some trade-off in obtaining those adaptations makes the POE taxa less competitive in non-epizoic benthic environments. We know little about the extent to which the microbes associated with the diatom ("phycosphere") might affect the competitive ability of diatoms, and/or whether the phycosphere may itself manufacture some critical compound only in an epizoic community. Since all cultured POE diatoms were maintained as non-axenic cultures, it is yet unclear what role the bacterial strains played in the development and survival of the targeted diatom species and whether the long-term maintenance of axenic POE strains would be feasible. Future studies may also determine the number of evolutionary leaps to the epizoic habitat and the number of host switches, shedding more light on the co-evolution of diatom-animal relationships.

Methods

Cultures and microscopy. Diatoms were collected from the skin of West Indian manatees and the skin and carapace of six species of sea turtles (see Table 1 for details). These collections were made following the protocol outlined by Pinou et al.[43]. Wild sea turtles were either sampled on nesting beaches after oviposition (as to not disturb the nesting process) or from turtles captured in water via a rodeo method[44]. The seven sea turtles resident at the uShaka Sea World in Durban (South Africa) were sampled during feeding. The Adriatic Sea turtles were sampled upon arrival to the rescue center after being caught accidentally during trawling (Iracus) or during rehabilitation in an outdoor pool with freely circulating seawater (Lunga). Manatees were sampled during annual health assessments conducted by the USGS Sirenia Project.

Individual diatom cells were isolated by micropipette into sterile f/2 culture medium[45] with a salinity matching that of the collection area. Strains isolated from the Bahamas, and the US were maintained under natural light in a north-facing window at UT Austin at room temperature (between 20 and 24 °C). South African strains were lit by natural light from a south-facing window and maintained at a temperature of 20–24 °C at the Unit of Environmental Sciences and Management in Potchefstroom. The strains isolated from the Adriatic were grown at 18–20 °C at 7–10 µmol m^2 s^{-1}, 12:12 (light:dark) cycle .In the case of non-photosynthetic taxa (like some *Tursiocola* species), individual cells were documented by light micrograph ("photovouchered") and isolated into WGA whole-genome amplification cocktail[25].

Cultures were harvested into separate pellets for microscopy preparation and DNA sequencing. Pellets for microscopy were cleaned with hydrogen peroxide and nitric acid, rinsed to neutral pH and dried onto 22 × 22 mm and 12 mm coverslips for light microscopy (LM) and scanning electron microscopy (SEM), respectively. Permanent mounts for the LM slides were made with Naphrax® mounting medium (Brunel Microscopes, www.brunelmicroscopessecure.co.uk) and micrographs were taken with a Zeiss Axioskop. Coverslips for SEM were coated with iridium by a Cressington 208 Bench Top Sputter Coater (Cressington Scientific Instruments, Watford, UK) and micrographs taken with a Zeiss SUPRA 40 VP scanning electron microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Additional micrographs of the strains are available from the authors.

DNA isolation, amplification and sequencing. Pellets for DNA sequencing were extracted using the DNeasy Plant Minikit, with an extra 45 s incubation in a Beadbeater (Biospec Products, Bartlesville, OK, USA) with 1.0 mm glass pellets for colony and frustule disruption. The nuclear-encoded ribosomal SSU and chloroplast-encoded *rbc*L and *psb*C markers were amplified by PCR using the primers outlined in Theriot et al.[46] in 25 µL reactions with 1–3 µL of template DNA, 0.5 µL of each primer, 0.25 µL of Taq polymerase, 12.5 µL of pre-mixed FailSafe Buffer E (Lucigen Corporation) and 8.25–10.25 µL of sterile water. PCR conditions were identical for *rbc*L and *psb*C: 94 °C for 3.5 min., 35 cycles of (94 °C for 30 s, 48 °C for 60 s., 72 °C for 2 min.), and a final extension at 72 °C for 15 min. PCR conditions for SSU were: 94 °C for 3.5 min., 35 cycles of (94 °C for 30 s., 51 °C for 60 s., 72 °C for 3 min.), and a final extension at 72 °C for 15 min. The amplicons were purified using an EXO-SAP protocol: a 3 µL of an EXO-SAP solution containing 0.5 µL of shrimp alkaline phosphatase, 0.25 µL of exonuclease I and 2.25 µL of sterile water were added to the PCR products and incubated at 37 °C for 30 min. followed by 80 °C for 15 min. Purified products were then sequenced on an ABI 3730 DNA Analyzers using BigDye Terminator v3.1 chemistry.

Sequence data were added to a dataset of raphid and araphid pennate diatoms, with *Asterionellopsis glacialis* used as an outgroup (see Table S1 for GenBank accession numbers). SSU data were aligned by the SSUalign program, using the covariance model outlined in Lobban et al.[47]. Data were initially partitioned by gene, by paired and unpaired sites in SSU secondary structure and codon position in *rbc*L and *psb*C. Model testing and grouping of partitions were performed by PartitionFinder 2[48] using all nucleotide substitution models, linked branches, and rcluster search[49] settings for trees inferred by RAxML 8[50]. The best model was chosen using the corrected Akaike information criterion (AICc). Maximum Likelihood and Bayesian Inference based phylogenies were inferred using IQ-TREE version 1.6.12 for Linux[51] with partitioned models[52] and multi-threaded MPI hybrid variant of ExaBayes version 1.5[53], respectively. Nodal support for the maximum likelihood phylogeny was assessed using 1000 bootstrap replicates via IQ-TREE. ExaBayes analyses included four independent runs with two coupled chains where branch lengths were linked. Convergence parameters included an average deviation of split frequencies (ASDSF) of less than or equal to 5% with a minimum of 10,000,000 generations. Bayesian nodal support was assessed using posterior probabilities, with the first 25% of the trees removed as "burn-in".

Data availability

DNA sequence data generated for this study are published on the NCBI GenBank online sequence depository under the accession numbers listed in Table S1. Additional micrographs and cleaned voucher material from the sequenced cultures are available from lead author MPA.

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Author contributions

M.P.A. and R.M. contributed equally to the study design, culture isolation and manuscript writing and editing. M.P.A. also extracted and sequenced DNA and constructed the phylogenetic datasets, while R.M. collected samples in South Africa and the Bahamas and managed South African collections. T.F. contributed to managing collections in the US and study design and interpretation, as well as manuscript editing. M.S. contributed to data interpretation and manuscript editing. S.B. and K.F. contributed to collecting samples in Croatia, extracting and sequencing DNA as well as study design, interpretation and manuscript editing. B.V. contributed to study design, data interpretation and manuscript editing. M.A., J.S., N.I.S., J.R.P. and C.A.M. collected samples in the US and contributed to data interpretation and manuscript editing. R.N. obtained permits for sample collection in South Africa and contributed to manuscript editing. N.J.R. and M.G. obtained permits and coordinated sample collection in the Bahamas and contributed to data interpretation and manuscript editing. E.C.T. and D.W.L. contributed to the phylogenetic analysis of DNA data, data interpretation and manuscript editing. S.R.M. contributed to culture maintenance, DNA work in the US and manuscript editing.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Information

Supplemental Figure S1. Complete Maximum Likelihood phylogenetic tree derived from a concatenated 3-gene DNA sequence dataset. Support values (ML bootstrap support) shown above nodes. Taxa isolated from epizoic habitats followed by a diagrammatic representation of the host from which the strain was isolated, and metadata on the location and setting in which the host was sampled (A = aquarium, R = rehabilitation facility, W = wild). Black host icon = POE taxon; white host icon = unclear habitat preference.

Supplemental Figure S2. Complete Bayesian Inference phylogenetic tree derived from a concatenated 3-gene DNA sequence dataset. Support values (BI posterior probability) shown above nodes. Taxa isolated from epizoic habitats followed by a diagrammatic representation of the host from which the strain was isolated, and metadata on the location and setting in which the host was sampled (A = aquarium, R = rehabilitation facility, W = wild). Black host icon = POE taxon; white host icon = unclear habitat preference.

Supplemental Table S1. Taxa, strain voucher ID and GenBank accession numbers for strains used in the DNA sequence data phylogenetic analysis. Collection site for sample of original strain isolation, or culture collection strain number, is also included (where known). Ingroup taxa (raphid pennates) provided first in the table; outgroup taxa (araphid pennates) follow after table break. Taxa are listed alphabetically. If species unknown, authority for genus is listed.

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Publication IV

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ON SEA TURTLE-ASSOCIATED *CRASPEDOSTAUROS* (BACILLARIOPHYTA), WITH DESCRIPTION OF THREE NOVEL SPECIES[1]

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The current study focuses on four species from the primarily marine diatom genus *Craspedostauros* that were observed growing attached to numerous sea turtles and sea turtle-associated barnacles from Croatia and South Africa. Three of the examined taxa, *C. danayanus* sp. nov., *C. legouvelloanus* sp. nov., and *C. macewanii* sp. nov., are described based on morphological and, whenever possible, molecular characteristics. The new taxa exhibit characters not previously observed in other members of the genus, such as the presence of more than two rows of cribrate areolae on the girdle bands, shallow perforated septa, and a complete reduction of the stauros. The fourth species, *C. alatus*, itself recently described from museum sea turtle specimens, is reported for the first time from loggerhead sea turtles rescued in Europe. A 3-gene phylogenetic analysis including DNA sequence data for three sea turtle-associated *Craspedostauros* species and other marine and epizoic diatom taxa indicated that *Craspedostauros* is monophyletic and sister to *Achnanthes*. This study, being based on a large number of samples and

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animal specimens analyzed and using different preservation and processing methods, provides new insights into the ecology and biogeography of the genus and sheds light on the level of intimacy and permanency in the host–epibiont interaction within the epizoic *Craspedostauros* species.

Key index words: **barnacle; *Chelonibia*; *Craspedostauros*; epizoic diatom; leatherback; loggerhead; phylogeny; *Platylepas*; sea turtle**

Abbreviations**: BS, bootstrap support; CRW, comparative RNA Web; ML, maximum likelihood**

Diatom communities inhabiting both the skin and the carapace of marine turtles are composed largely of species not reported from other biotic or abiotic substrata (Frankovich et al. 2015, 2016, Majewska et al. 2015a,b, 2017a,b, Robinson et al. 2016, Azari et al. 2020, Majewska 2020, Riaux-Gobin et al. 2020). These observations suggest a certain level of host-specific evolutionary adaptations used by sea turtle-associated diatoms. Although intimate relationships between animals and microbes are common and extensively studied, reports of truly epizoic microalgae are generally rare (Ezenwa et al. 2012, Redford et al. 2012, Apprill 2017). Perhaps due to the fact that ubiquitous photosynthetic organisms, such as diatoms, are not immediately perceived as an essential element of any vertebrate microbiome, these new findings are particularly noteworthy. Based on their high frequency of occurrence and the high relative abundances recorded from various sea turtle species and geographic regions, as well as a lack of records from other types of substrata, several of the newly described sea turtle-associated diatom taxa are currently believed to be strictly epizoic or even sea turtle-specific. While this may be the case, many other diatoms present in samples from sea turtles are likely to be opportunistic species that attached to biofilm in the later stages of its development (Majewska et al. 2015b, 2017b, 2019, 2020, Kaleli et al. 2020, Van de Vijver et al. 2020). Although opportunistic taxa often dominate specific epizoic habitats in terms of diversity, they rarely reach high relative abundance, which may suggest they lack some key functional adaptations to the epizoic lifestyle.

The present study focuses on the sea turtle-associated diatom species belonging to the genus *Craspedostauros.* At present, the genus comprises ten validly described species including one, *Craspedostauros alatus,* described from museum specimens of sea turtles (Cox 1999, Sabbe et al. 2003, Van de Vijver et al. 2012, Ashworth et al. 2017, Majewska et al. 2018). *Craspedostauros* is a predominantly marine genus, although *C. laevissimus* is described as "a widespread endemic species restricted to the Antarctic Continent" and may be of brackish or freshwater origin (Sabbe et al. 2003, Van de Vijver et al. 2012). The typical morphological characters of the genus include cribrate areolae, numerous doubly perforated girdle bands, two fore and aft chloroplasts, and a usually narrow stauros. The latter is reduced or strongly reduced in two species, *C. alyoubii* and *C. paradoxus**. Molecular phylogenetic analysis indicated that the genus is closely related to *Achnanthes* and *Staurotropis* (Ashworth et al. 2017). Both taxa, as well as another marine genus *Druehlago*, which has yet to be characterized using molecular approaches, share several morphological similarities with *Craspedostauros* (Cox 1999, Ashworth et al. 2017). For example, all the above-mentioned taxa possess valves and girdle bands perforated by cribrate areolae. *Craspedostauros* and *Druehlago* share the general frustule morphology, including frustules with central constriction (Ashworth et al. 2017), while the fore and aft arrangement of chloroplasts, typical of *Craspedostauros,* can be observed in several *Achnanthes* species (Cox 1999).

Three previously undescribed species, *Craspedostauros danayanus* Majewska et Ashworth sp. nov., *C. legouvelloanus* Majewska et Bosak sp. nov., and *C. macewanii* Majewska et Ashworth sp. nov., were found in the course of an ongoing survey on sea turtle-associated diatoms and are described in the current paper. Moreover, a population of *C. alatus* is for the first time reported from Europe. The large number of samples analyzed along with the different preservation and processing techniques applied allowed us to document the ultrastructure of the frustule and, whenever possible, the morphology of the plastids as well as the colony type and attachment mode of the cells. These observations were supplemented by a 3-gene phylogenetic analysis including DNA sequence data for three sea turtle-associated *Craspedostauros* species and other marine and epizoic diatom taxa.

*The specific epithet in *Craspedostauros paradoxa* should be changed to "*paradoxus*" following the recommendations of the International Code of Nomenclature for algae, fungi, and plants (Articles 23.5 & 62; Turland et al. 2018).

MATERIALS AND METHODS

Material collection and preservation. Diatom samples were collected from captive and wild sea turtles from Croatia and South Africa (Fig. 1). All biofilm samples from carapace and skin were taken using single-use sterile toothbrushes following the sampling protocols suitable for diatom culturing and standard morphology-based diatom analysis of Pinou et al. (2019). In Croatia, single skin and carapace samples were collected from each of 38 loggerhead sea turtles, *Caretta caretta*, rescued and rehabilitated at the Marine Turtle Rescue Centre in Aquarium Pula between 2016 and 2019, on the day of or shortly after their arrival at the facility. In South Africa, single skin and carapace biofilm samples were collected from each of 78 loggerheads and 20 leatherbacks, *Dermochelys coriacea*, nesting in Kosi Bay (Indian Ocean) over two nesting seasons, in 2017/2018 and 2018/2019. In addition, 6-mm skin biopsy punches were taken from either front or rear flippers of 30 loggerheads and six leatherbacks and preserved in 4% formaldehyde solution in seawater immediately after collection. Samples of the sea turtle-associated barnacles *Chelonibia testudinaria* from 100 + loggerheads and *Platylepas coriacea* from 15 leatherbacks were taken using a plastic paint scraper or a blunt knife over four nesting seasons, from 2015/2016 to 2018/2019. Barnacle samples comprised of more than one specimen were divided into two parts and either frozen (−20°C) or fixed with 4% formaldehyde solution in seawater. Single-specimen barnacle samples were frozen (−20°C). Finally, skin and carapace samples were collected from three loggerheads, three green turtles, *Chelonia mydas*, and one hawksbill, *Eretmochelys imbricata*, resident at the uShaka Sea World in Durban on June 28, 2019.

Material collection was performed by, or under close supervision of, qualified field researchers, and the applied techniques and procedures respected ethical principles of the Declaration of Helsinki (World Medical Association 2013) as well as all applicable national laws.

Type slides and unmounted diatom material used in this study are deposited in the following herbaria: South African National Diatom Collection (SANDC), Croatian National Diatom Collection (HRNDC), and Meise Botanic Garden Herbarium (BR; Index Herbariorum, http://sweetgum.nybg.org/science/ih/).

Material processing and microscopy. Diatoms were detached from the frozen barnacles using a Transsonic T310 (Elma, Singen, Germany) ultrasound bath as described by Majewska et al. (2020). Diatom biofilm from the sea turtle skin, carapace, and barnacles was cleaned from organic matter using either a rapid digestion with a mixture of concentrated HNO_3 and H_2SO_4 (at a ratio of 2:1) following von Stosch's method (South African and Croatian samples; Hasle and Syvertsen 1997) or heated 37% H_2O_2 with addition of $KMnO_4$ (Croatian culture strain; van der Werff 1953). Cleaned material was mounted on slides using Naphrax (Brunel Microscopes Ltd, Chippenham, UK; Croatian samples) and Pleurax prepared following von Stosch (1974; South African samples). The slides were examined using a Nikon Eclipse 80i light microscope with Differential Interference Contrast (DIC) and a Nikon DS-Fi1 5MP digital camera (Nikon Instruments Inc., Melville, NY; South African samples) as well as a Zeiss Axio Imager A2 with DIC and an Axiocam 305 digital camera (Carl Zeiss, Jena, Germany; Croatian samples). In addition, fresh material containing living diatoms attached to the sea turtle scutes and skin flakes was stained with blue writing ink (Scheaffer ®) to reveal the colonies of the diatom-associated bacteria.

For scanning electron microscopy (SEM), the oxidized suspension was filtered through 1-μm or 1.2-μm Isopore™ (Merck Millipore, Darmstadt, Germany) or 3-μm Nucleopore (Nucleopore, Pleasanton, CA, USA) polycarbonate membrane filters. Formalin-preserved skin and barnacle samples were

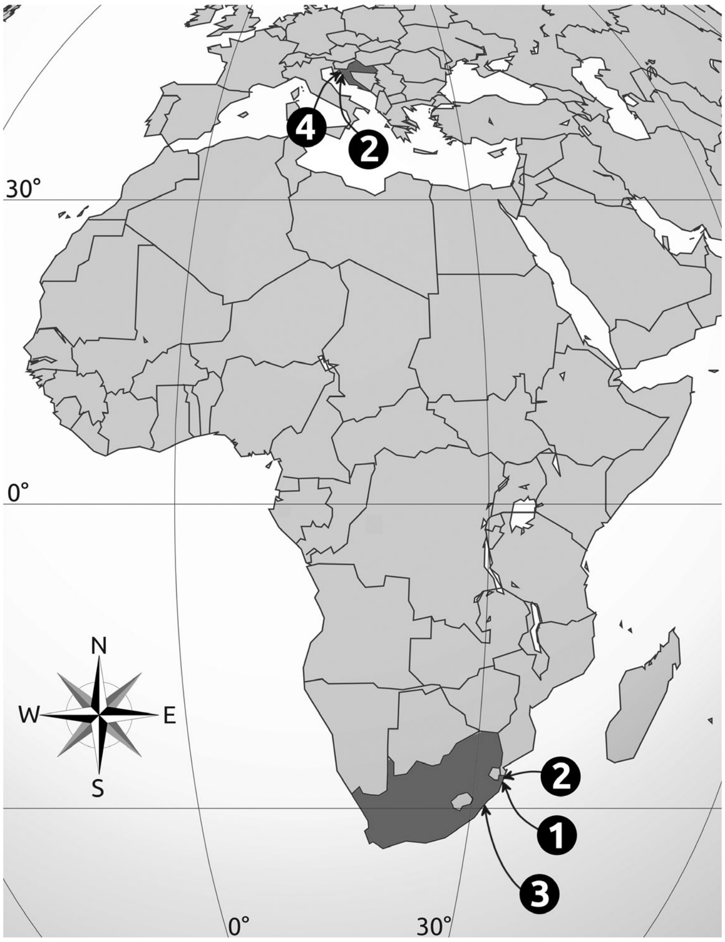


FIG. 1. Sampling locations for *Craspedostauros danayanus* (1), *C. legouvelloanus* (2), *C. macewanii* (3), and *C. alatus* (4).

dehydrated in an alcohol series (30%, 50%, 60%, 70%, 80%, 90%, 95%, 99.9%) followed by critical point-drying in an E3100 Critical Point Dryer (Microscience Division, Watford, UK). Subsequently, the samples were mounted on aluminum stubs with carbon tape and sputter-coated with either gold-palladium using Cressington 108Auto and Cressington 208HR sputter-coaters (Cressington Scientific Instruments Ltd., Watford, UK), palladium using a Precision Etching and Coating System, PECS II (Gatan Inc., Pleasanton, CA, USA), or iridium using Emitech K575X (Emitech Ltd., Ashford, Kent, UK) and Cressington 208 Bench Top sputter-coaters. Diatom specimens were analyzed with JEOL JSM-7800F, JEOL JSM-7001F (JEOL, Tokyo, Japan), FEI Quanta Feg 250 (FEI Corporate, Hillsboro, OR, USA), Zeiss Ultra Plus (Carl Zeiss, Oberkochen, Germany), and Zeiss SUPRA 40 VP (Carl Zeiss Microscopy, Thornwood, NY, USA) scanning electron microscopes at 3–10 kV. To determine the relative abundance of the new species, 400 diatom valves were counted and identified in each sample along arbitrarily chosen transects using SEM. The morphology and frustule ultrastructure of the new taxa was compared with those of all known *Craspedostauros* species worldwide. Taxonomic terminology follows that used in the previous studies focused on this genus (Cox 1999, Sabbe et al. 2003, Van de Vijver et al. 2012, Ashworth et al. 2017, Majewska et al. 2018). The term "rectelevatum" (Van de Vijver et al. 2010) is used to describe the internal central thickening formed by partially fused central helictoglossae, which was present in all the novel taxa, either with or without an additional central knob-like structure.

Culturing. Living diatoms from the fresh material (unpreserved samples containing sea turtle biofilm and filtered seawater; Pinou et al. 2019) were isolated using a glass pipette with a tip pulled and thinned over a flame into 16 × 100 mm glass culture tubes (South African strains) or plastic culture flasks (Croatian strains) filled with 34 PSU (South African strains) or 38 PSU (Croatian strains) f/2 growth medium (Guillard 1975). Strains were lit by natural light from a south-facing window (South African strains) or white fluorescent light with a photoperiod of 12 h (Croatian strain) and maintained at a temperature of 20–24°C. The well-grown cultures were divided into two parts, one of which was used for DNA extraction. The remaining part was cleaned with a mixture of 30% H_2O_2 and 70% HNO_3 and rinsed with distilled water until near-neutral pH was reached in the fluid phase. The Croatian strain (PMFTB0003) was cleaned using saturated $KMnO_4$ solution and ca. 30% HCl following a protocol modified slightly from Simonsen (1974). Permanent microscopy slides and SEM stubs were prepared as described above.

DNA preparation and phylogenetic analysis. The cultures were harvested as cell pellets using an Eppendorf 5415C centrifuge (Eppendorf North America, Hauppauge, NY, USA) for 10 min at 8,000 rpm. The QIAGEN DNeasy Plant Mini Kit (QIAGEN Sciences, Valencia, CA, USA) was used for DNA extraction following the manufacturer's protocol, with the addition of an initial cell disruption by 1.0 mm glass beads in a Mini-Beadbeater (Biospec Products, Inc, Bartlesville, OK, USA) for 45 s. PCR-based DNA amplification and di-deoxy Sanger sequencing of small-subunit nuclear rRNA and the chloroplast-encoded rbcL and psbC markers followed Theriot et al. (2010).

Phylogenetic analysis of the DNA sequence data was conducted using a three-gene dataset: nuclear-encoded small-subunit (SSU) rRNA, and plastid-encoded *rbc*L and *psb*C. Alignment of the SSU sequences, accounting for secondary structure, was carried out using the SSUalign program (Nawrocki et al. 2009), with the covariance model based on the 10 diatoms included with the program download, plus 23 additional diatoms from the CRW website (Cannone et al. 2002). Post alignment, SSU sequences were concatenated with the chloroplast sequences into a single matrix (Table S1 in the Supporting Information). Eight separate partitions were created for the data (SSU paired and unpaired sites, plus the first, second, and third codon positions of each of *rbc*L and *psb*C). This dataset and partitioning scheme were run under maximum likelihood (ML) using RAxML ver. 8.2.7 (Stamatakis 2014) compiled as the pthread-AVX version on an Intel i7-based processor, using the GTR + G model. Twenty-five replicates, each with 500 rapid BS replicates, were run with ML optimizations. Bootstrap support was assessed using the BS replicates from the run with the optimal ML score.

RESULTS

The analyzed biofilm samples contained three previously undescribed species of *Craspedostauros* as well as *C. alatus.* These species and populations are described below.

Craspedostauros danayanus Majewska & Ashworth sp. nov. (Figs. 2 and 3)

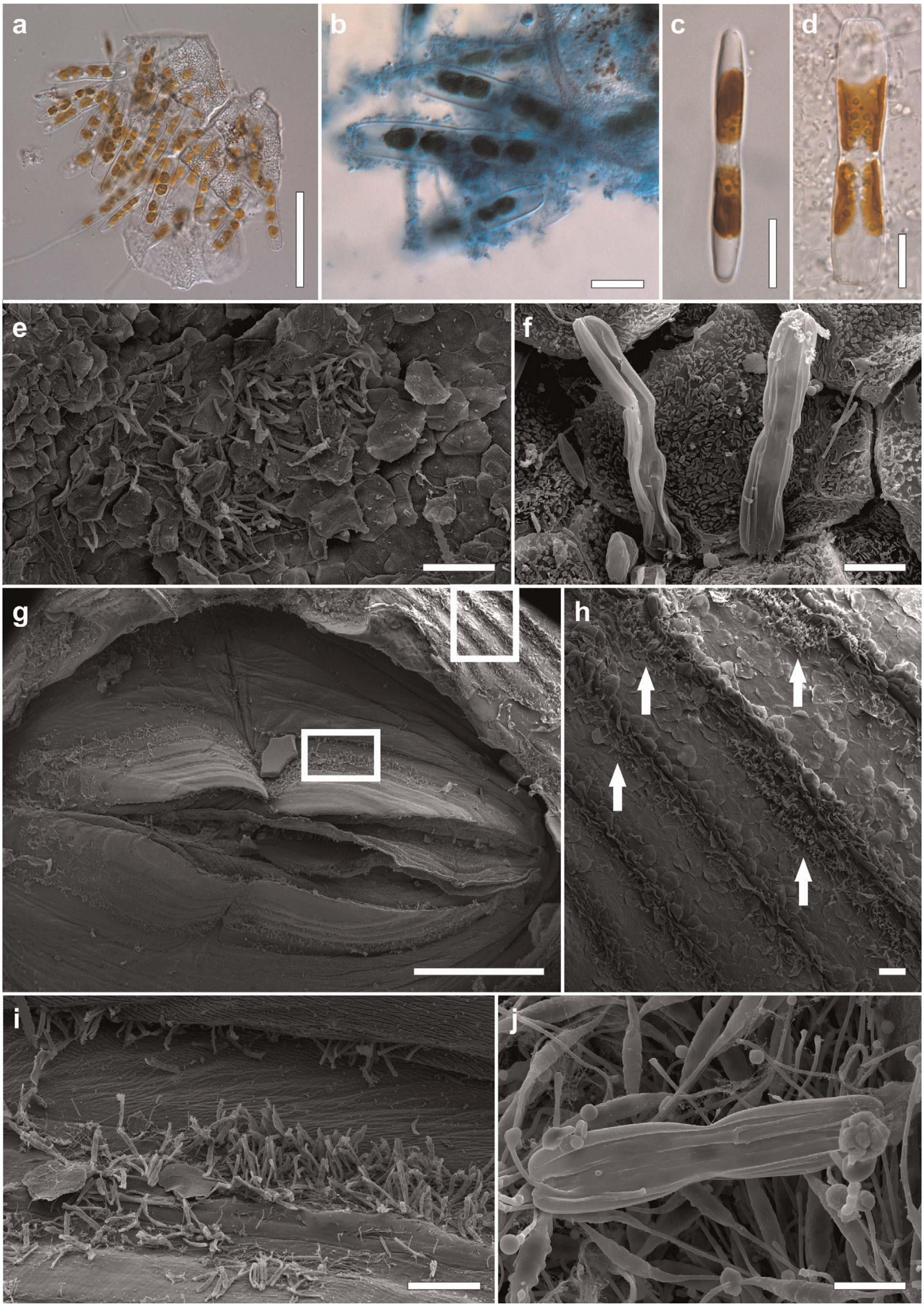
Diagnosis. Valves linear and narrow; stria density high (≥49 in 10 µm); stauros, internal central knob, and external central lip-like silica flaps absent. Accession numbers of sequence data representative of this taxon (but **not** the holotype): MT432485 (*rbc*L) and MT432505 (*psb*C).

Holotype. Permanent slide SANDC ST012 (prepared from sample ZA0019A/ZA1824E).

Type locality. Mabibi Beach, Elephant Coast, South Africa (27°21′30″ S, 32°44′20″ E). Collected from the barnacle *Platylepas coriacea* growing on an egg-laying leatherback sea turtle (tag numbers: ZA0019A, ZA1824E) by R. Majewska, December 7, 2018.

Etymology. The epithet honors Danay A. Stoppel (North-West University, Potchefstroom, South Africa), who made the first observations of the new taxon, in recognition of her contribution to the sea turtle diatom project in South Africa.

FIG. 2. *Craspedostauros danayanus.* (a) Living cells of *C. danayanus* and *Cylindrotheca* sp. attached to the leatherback skin scales (light microscopy). (b) Stained colony of *C. danayanus* and associated bacteria on the leatherback skin scales. (c) Valve view of a living cell (cultured strain). (d) Girdle view of a living cell (cultured strain). (e–j) Scanning electron micrographs of *C. danayanus* attached to its original substratum. (e) Monospecific colony growing among the flaking skin of leatherback (dorsal side of the hind flipper). (f) Extremely delicate and fragile cells of *C. danayanus* attached to the leatherback skin (dorsal side of the hind flipper). (g) An overview of the leatherback-associated barnacle, *Platylepas coriacea*, colonized by *C. danayanus.* (h) A detail of the external part of the barnacle with a sheath of host sea turtle tissue overgrown with *C. danayanus.* Arrows indicate some of the monospecific clumps of *C. danayanus* colonies. (i) A detail of the moveable plates of the barnacle overgrown with *C. danayanus.* (j) A single cell of *C. danayanus* among dense colony of *Cylindrotheca* sp. attached to the folds in the moveable plates of *P. coriacea.* Scale bars: 10 µm = panels b–d, f and j; 50 µm = panel a; 100 µm = panels e, h and i; 1 mm = panel g.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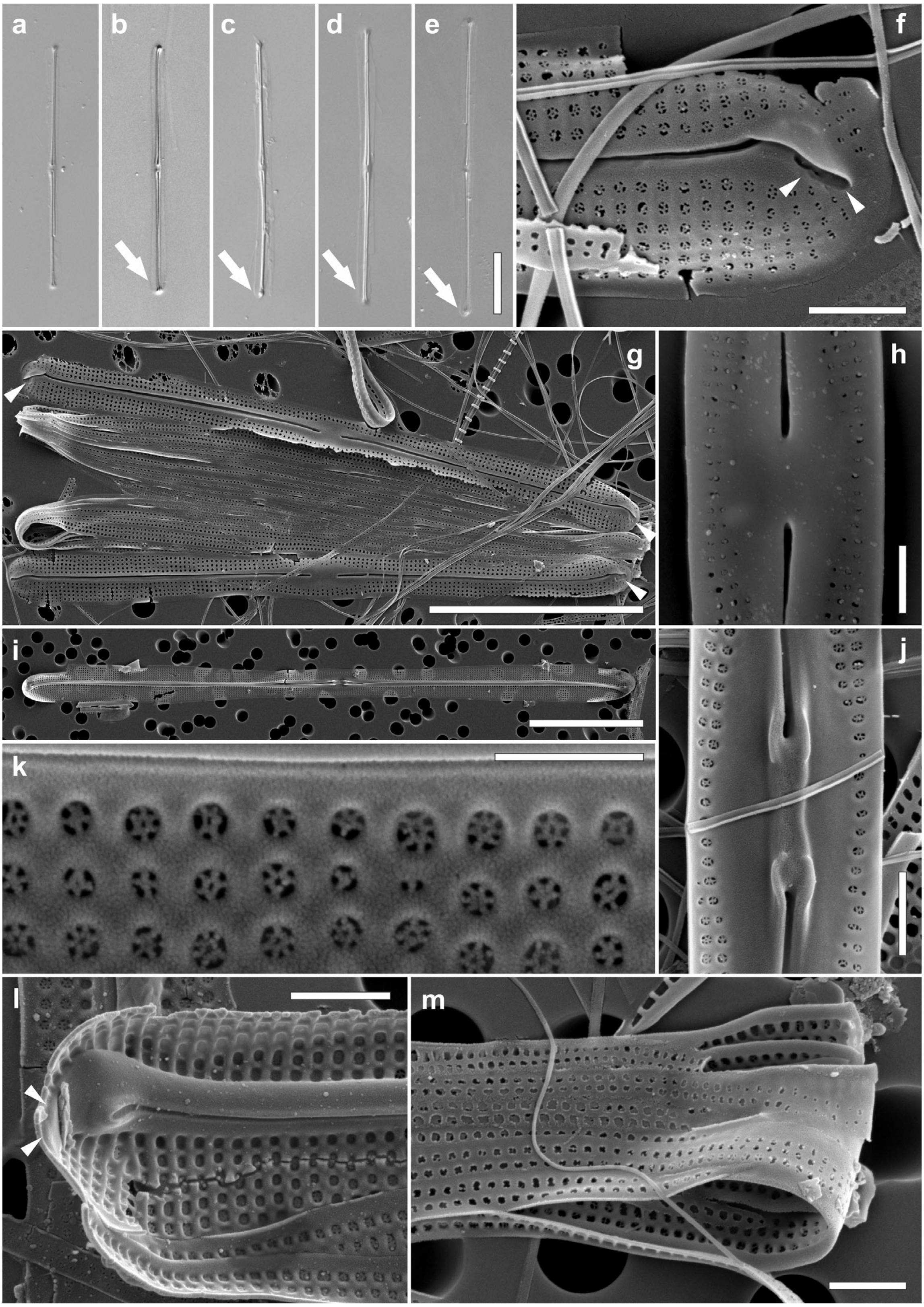
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Fig. 3. *Craspedostauros danayanus.* (a–e) Valve view (light micrographs). Arrows indicate the barely noticeable valve margins. (f–m) Scanning electron micrographs. (f) Detail of the apical part of the valve (external view). Arrowheads indicate the large irregular depression at the fold of the apical silica flap. (g) Frustule with partially detached girdle bands (external view). Arrowheads indicate the large irregular depression at the fold of the apical silica flap. (h) Detail of the central part of the valve (external view). (i) Internal valve view. (j) Detail of the central part of the valve (internal view). (k) Cribrate areolae (internal view). (l) Detail of the apical part of the valve (internal view). Arrowheads indicate the asymmetrical thickening extending from the apical part of the raphe-sternum toward the valve margin. (m) Detail of the girdle bands. Scale bars: 10 µm = panels a–e, g, i; 1 µm = panels f, h, j–m.

Description. Cells with two fore and aft H-shaped chloroplasts (Fig. 2, a–d). Frustules extremely delicate and very lightly silicified (Figs. 2, e–j; 3, a–e). In girdle view, frustules rectangular, moderately constricted at the center (Fig. 2, d, f, and j). Valves narrow, linear, very slightly constricted in the valve middle, with bluntly rounded apices (Figs. 2c and 3, a–e).

Light microscopy (Fig. 3, a–e): Valve dimensions ($n = 30$): length 28–61 µm, width 2–2.5 µm, length/width ratio: 14–30.5. In cleaned (acid-digested) material, partially dissolved valve margins barely noticeable (Fig. 3, a–e, arrows), intact frustules absent. Striae indiscernible (Fig. 3, a–e). Raphe-sternum thickened, clearly visible (Fig. 3, a–e). Thickenings at both central and terminal raphe endings (Fig. 3, a–e).

Scanning electron microscopy (Fig. 3, f–m): Externally: In cleaned material, valve face appearing flat, with very shallow mantle and straight margin (Fig. 3, f and g). Striae uniseriate, 49–51 in 10 µm, parallel, becoming radiate toward the apices, alternate or opposite, composed of up to eight areolae (Fig. 3, f and g). Areolae largely similar in size, becoming somewhat smaller around the central area, squarish to roundish, externally occluded by cribra (Fig. 3, f–h). Each cribrum perforated by 2–8 pores (Fig. 3f). Axial area narrow (Fig. 3, f and g). Raphe-sternum not raised (Fig. 3, f–h). Raphe branches straight (Fig. 3g). Central area large, symmetrical, fusiform (Fig. 3, g and h). Central raphe endings straight, elongated, slightly expanded (Figs. 3, g and h). Terminal raphe endings disappearing under somewhat triangular silica flaps extending from the raphe-sternum, giving the impression of unilaterally bent terminal raphe fissures (Fig. 3, f and g). A large, irregular depression present at the apical flap fold (Fig. 3, f and g, arrowheads). Shortened striae composed of cribrate areolae radiating around the apices beyond the apical silica flaps (Fig. 3f). Asymmetrical pore-free area present beyond the terminal raphe endings in the immediate vicinity of the apical flap fold (Fig. 3f).

Internally: Raphe slit opening laterally onto the more or less uniformly thickened and distinctly raised raphe-sternum (Fig. 3i). Stauros absent (Fig. 3, i and j). Central area mirroring the external structure in size and shape (Fig. 3, i and j). Central raphe endings elongated, very slightly unilaterally bent, terminating onto weakly constricted rectelevatum (Fig. 3, i and j). Terminal raphe endings positioned somewhat laterally on a large and rounded apical part of the raphe-sternum, terminating in helictoglossae (Fig. 3, i and l). Asymmetrical thickening extending from the apical part of the raphe-sternum toward the valve margin, corresponding to the external apical silica flaps (Fig. 3l, arrowheads). Areolae externally occluded with cribra (Fig. 3, j–l).

Cingulum composed of numerous (14+) open copulae, bearing two rows of typically squarish, roundish, or elongated areolae, ca. 50–60 in 10 µm (Fig. 3, g, l, and m). Areolae occluded externally by cribra (Fig. 3, l and m).

Taxonomic notes and comparison to other Craspedostauros *species. Craspedostauros danayanus* is most similar to *C. paradoxus*, sharing the general valve outline and lacking the stauros. However, *C. danayanus* differs from the latter in being distinctly smaller (28–61 µm vs. 80–85 µm) and more slender (2–2.5 µm vs. 6.5–9 µm), possessing a higher stria density (49–51 vs. 36–40 in 10 µm), and lacking the lip-like silica flaps (externally) and the central knob (internally) present in *C. paradoxus* (Table 1).

Ecology: Epizoic on carapaces of adult leatherback sea turtles and on leatherback-associated barnacles *Platylepas coriacea* growing on adult leatherbacks from Kosi Bay (South Africa). Attaching to the animal surface through one end of the valve, motile in culture.

The taxon was found in 12 leatherback skin samples (out of 20 examined) and in all *Platylepas coriacea* samples examined ($n = 15$) reaching relative abundances of 35% (skin samples) and 79% (barnacle samples). It was found in neither loggerhead nor loggerhead-associated barnacle samples from the same location (Kosi Bay, South Africa). Leatherback skin samples containing *Craspedostauros danayanus* were dominated by *Navicula dermochelycola*, *Tursiocola neliana*, and *Poulinea* spp. The new taxon was dominant in most of the *P. coriacea* samples along with *Cylindrotheca* sp. Both taxa colonized various anatomical parts of the barnacle showing preference for rough surfaces and cavities. The extremely lightly silicified frustules may be an adaptation to the pelagic lifestyle of the host, as the open ocean waters contain significantly lower concentrations of dissolved silica than coastal habitats (Tréguer et al. 1995).

TABLE 1. Comparison of *Craspedostauros alatus*, *C. danayanus*, *C. legouvelloanus*, and *C. macewanii* with several morphologically similar *Craspedostauros* taxa (after Cox 1999, Ashworth et al. 2017, Majewska et al. 2018)

Character	*C. paradoxus*	*C. capensis*	*C. britannicus*	*C. australis*	*C. alyoubii*	*C. alatus*	*C. danayanus*	*C. legouvelloanus*	*C. macewanii*
Valve length (µm)	80–85	25–35	14–60	35–78	83–105	20–37 (26–34)[a]	28–61	18–34 (23–39)[a]	26–51
Valve width (µm)	6.5–9	4.5–5.5	5–6	4–6	6–10	3–5 (3–5)[a]	2–2.5	3–5 (–6)[a]	4.5–5.5
Stria density (in 10 µm)	36–40	19	~24	35	~40	26–28 (24–27)[a]	49–51	46–49 (40–44)[a]	28–31
Valve outline	Linear, slightly constricted	Lanceolate, constricted	Linear to narrow lanceolate	Linear	Linear, slightly constricted	Linear to linear-lanceolate, slightly constricted	Linear, very slightly constricted	Linear to linear-lanceolate, slightly constricted	Linear to linear-lanceolate, slightly constricted
Valve margin at center	Straight	Straight	Slightly expanded	Straight	Straight	Very slightly expanded	Straight	Clearly expanded	Straight
Valve face-mantle junction	Indistinct	Indistinct	Indistinct	Indistinct	Indistinct	Distinct	Indistinct	Indistinct	Distinct
External central area	Roundish, irregular	Bow tie-shaped fascia	Bow tie-shaped fascia	Bow tie-shaped fascia	Rectangular fascia	Rectangular fascia	Fusiform	Rectangular fascia	Bow tie-shaped fascia
Central lip-like silica flaps	Prominent	Rudimentary	Rudimentary	Rudimentary	Prominent	Rudimentary	Absent	Well-developed	Rudimentary
Areola size	Largely similar	Variable	Similar	Similar	Similar	Variable	Similar	Similar	Similar
Number of pores per cribrum	4–5	5–13	5(+)	4	4–5	highly variable	6–8	4	Highly variable
Internal central area	Rectelevatum + knob	Rectelevatum + knob	Helictoglossae	Rectelevatum + knob	Rectelevatum + knob	Rectelevatum	Rectelevatum	Rectelevatum + knob with central cavity	Rectelevatum + knob
Stauros	Strongly reduced/absent	Present	Present	Present	Somewhat reduced	Present	Absent	Present	Present
Reference	Ashworth et al. 2017	Cox 1999	Cox 1999	Cox 1999	Ashworth et al. 2016	Majewska et al. 2018	this article	this article	this article

[a] Values and descriptions given in brackets refer to the Adriatic populations

Craspedostauros legouvelloanus Majewska & Bosak sp. nov. (Figs. 4 and 5)

Diagnosis. Valves linear to linear-lanceolate; valve margin centrally expanded; stria density high (≥40 in 10 μm); valve areolae uniform, with four pores per cribrum; external central lip-like silica flaps well-developed; stauros narrow; internal central knob present; girdle bands with shallow septa.

Holotype. Permanent slide SANDC ST003 (prepared from sample ZA0762D/ZA0763D).

Paratype. Permanent slide HRNDC 000150 (prepared from sample TB13).

Isotypes. Permanent slides BR 4601 and BR 4602.

Type locality. Kosi Bay, South Africa (26°59′39″ S, 32°51′60″ E). Collected from the carapace of an egg-laying loggerhead sea turtle (tag numbers: ZA0762D, ZA0763D) by R. Majewska, December 15, 2017 (holotype).

Marine Turtle Rescue Centre, Pula, Croatia (44°50′07″ N, 13°49 ′58″ E). Collected from a semi-adult female loggerhead named “Mimi” by K. Gobić Medica, May 28, 2019 (paratype).

Etymology. The epithet honors Dr Diane Z. M. Le Gouvello du Timat (Nelson Mandela University, Port Elizabeth, South Africa), who assisted during the type material collection, in recognition of her invaluable help and ongoing support and contribution to the sea turtle diatom project and sea turtle research in South Africa.

Description. Light microscopy (Fig. 4, a–f): Intact frustules lying almost always in girdle view (due to large cell depth/valve width ratio), slightly constricted in the middle (Fig. 4, a, b, and d–f), with several girdle bands (Fig. 4, b, d, and f). Valve margin expanded at the center (Fig. 4, a, d, and f). Frustules lightly silicified and delicate. Valves narrow, linear to linear-lanceolate, slightly constricted at the central area, with bluntly rounded apices (Fig. 4c). Valve dimensions ($n = 30$): length 18–34 μm, width 3–5 μm, length/width ratio: 5.6–9.4. Striae indiscernible (Fig. 4, a–f). Stauros narrow (Fig. 4, a, c–f), widening toward the biarcuate valve margins (Fig. 4f, arrows). Raphe-sternum clearly visible (Fig. 4, a–f). Raphe straight, biarcuate in girdle view (Fig. 4, a, b, d, and f).

Scanning electron microscopy (Fig. 4, g–p): *Externally*: Valves somewhat convex, with no clear valve face-mantle junction (Fig. 4, g–i). Valve margin clearly expanded at the center beyond the stauros (Fig. 4i). Striae uniseriate, 46–49 in 10 μm, parallel throughout the valve center, becoming convergent near the apices, alternate or opposite, composed of up to 13 areolae (Fig. 4, g, h and n). Areolae similar in size throughout the entire valve, squarish, externally occluded by cribra (Fig. 4, g–i and n). Each cribrum perforated by 4 pores (Fig. 4, g–i and n). Axial area very narrow (Fig. 4, g and h). Raphe-sternum very slightly raised (Fig. 4, g–i). Raphe branches more or less straight (Fig. 4g). Central area forming a narrow rectangular fascia (Fig. 4, g and n). Central raphe endings covered entirely by rimmed lip-like silica flaps extending from one side of the axial area (Fig. 4, g and n). At the apices, axial area expanding into somewhat triangular silica flaps covering the terminal raphe endings giving the impression of unilaterally bent terminal raphe fissures (Fig. 4, g–i). An oval or irregular depression present at the apical flap fold (Fig. 4g, arrows). Shortened stria composed of regular areolae and simple puncta radiating around the apices beyond the terminal raphe endings (Fig. 4, g–i).

Internally: Raphe slit opening laterally onto the uniformly thick and clearly raised raphe-sternum (Fig. 4, k and l). Stauros raised, very narrow, broadening abruptly at the mantle expansion and merging with the pore-free area at the valve margin (Fig. 4, l and o), slightly more expanded on the side corresponding to the external lip-like silica flaps (Fig. 4l, arrowheads, o and p). Central raphe endings straight or slightly unilaterally bent, terminating onto rectelevatum (Fig. 4, k, l, o, and p). A blunt cylindrical knob with a small central cavity present between the raphe endings (Fig. 4, k, l, o, and p). Areolae externally occluded by cribra, appearing sunken, especially close to the stauros (Fig. 4, o and p). Stauros-adjacent virgae appearing hollow, suggesting a more complex valve structure in that area (Fig. 4o, arrowheads). Terminal raphe endings positioned somewhat laterally on the raphe-sternum, terminating onto prominent helictoglossae. At the apices, raphe-sternum expanded laterally toward the valve margin, merged with pore-free area corresponding to the external apical silica flaps (Fig. 4, l and m).

Cingulum composed of numerous (12+) open copulae, bearing two rows of typically squarish or elongated areolae, ca. 50–60 in 10 μm (Fig. 4, h–k). Areolae occluded externally by cribra with 4–12 pores per cribrum (Fig. 4, h–k). Valvocopula curved, distinctly narrower, and pore-free beside the stauros (Fig. 4i, arrowheads). An internal ridge (septum) perforated by puncta present in each copula except for valvocopula (Fig. 4, i–k, arrowheads).

Adriatic population (Fig. 5, a–g). Specimens resembling *Craspedostauros leguovelloanus* were found on the carapace of six loggerhead sea turtles sampled on the Croatian coast of the Adriatic Sea. Most of the morphological features observed in the Adriatic population (Fig. 5, a–g) agreed well with those found in *C. legouvelloanus*. The cells possessed two fore and aft H-shaped chloroplasts (Fig. 5a, arrows) as observed previously in other *Craspedostauros* species (Cox 1999, Ashworth et al. 2017, Majewska et al. 2018). The specimens were slightly longer (23–39 μm) and wider (3.5–6 μm, length/width ratio: 5.2–7.8, $n = 25$) than those from the South African population, and their stria density was lower (40–44 in 10 μm vs. 46–49 in 10 μm; Table 1). In general, the frustules showed a relatively high

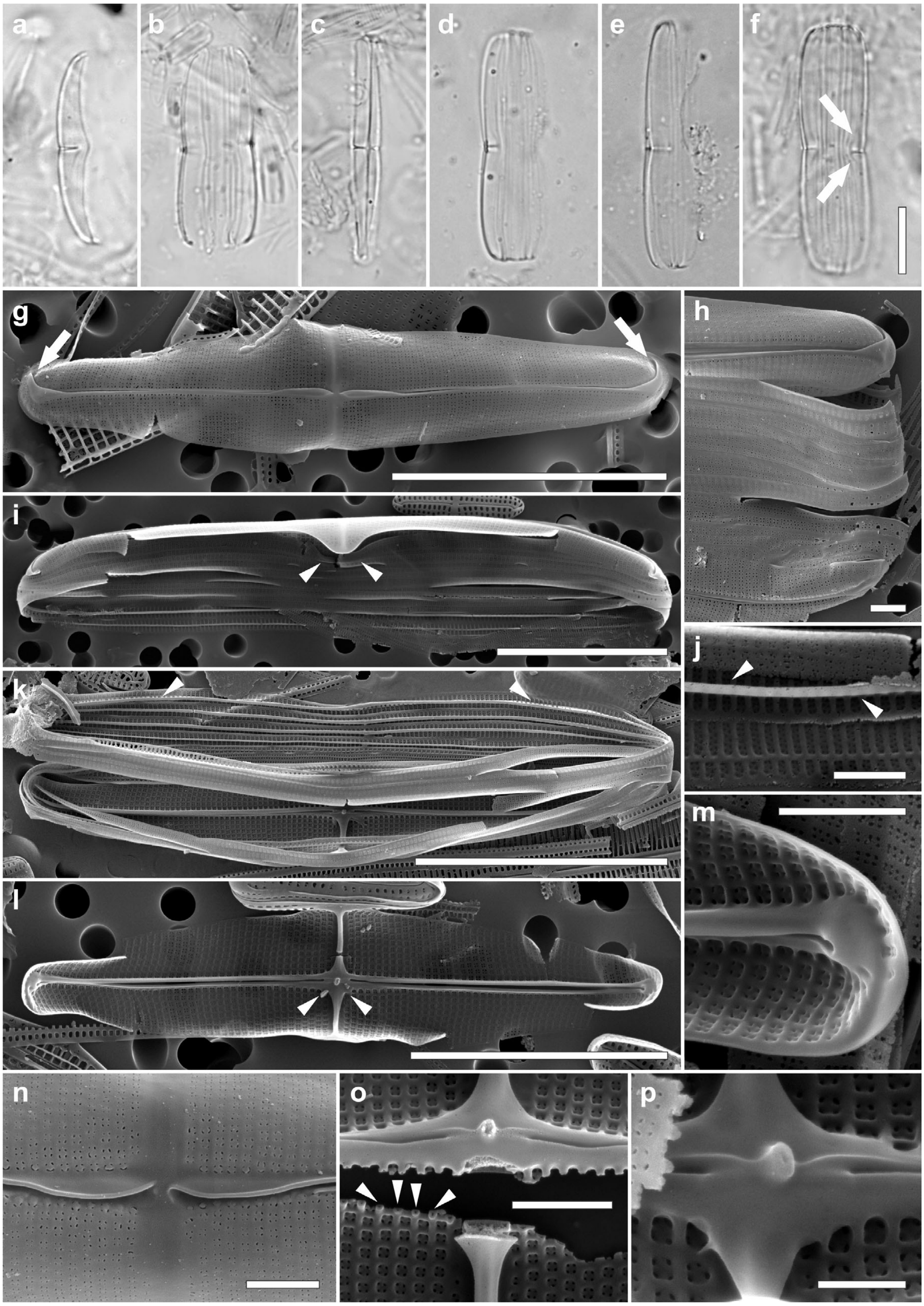
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FIG. 4. *Craspedostauros legouvelloanus*. (a–f) Light micrographs. (a, b, d–f) Girdle view. (a) Valve with two girdle bands attached. (d and e) Frustules with detached valves. (b and f) Complete frustules. Arrows indicate the biarcuate valve margin. (c) Valve view. (g–p) Scanning electron micrographs. (g) External valve view. Arrows indicate depressions at the apical flap-fold. (h) Detail of the apical part of the frustule (external view). (i) Valve with attached girdle bands (girdle view). (j) Detail of the girdle bands (internal view). Arrowheads indicate the internal thickening (septum). (k) Valve with partially detached girdle bands (internal view). (l) Internal valve view. Arrowheads indicate the slight expansion of the stauros on the side corresponding to the external lip-like silica flaps. (m) Detail of the apical part of the valve (internal view). (n) Detail of the central part of the valve (external view). (o and p) Detail of the central part of the valve (internal view). Arrowheads indicate the hollows in the stauros-adjacent virgae. Scale bars: 10 μm = panels a–g, i, k and l; 1 μm = panels h, j and m–o; 500 nm = panel p.

degree of irregularity in the areolae structure and the size and shape of stauros, axial area, and facia (Fig. 5, b–e).

Taxonomic notes and comparison to other Craspedostauros *species*. Currently, *Craspedostauros legouvelloanus* is the only known *Craspedostauros* species with septate girdle bands, although this may be an artifact of incomplete morphological descriptions that rarely mention or present the internal girdle band structure. Valves of *C. legouvelloanus* differ from those of all known stauros-bearing *Craspedostauros* species in possessing a very high stria density (above 40 in 10 μm). Although a similarly high or higher stria density was observed in *C. alyoubii* (~40 in 10 μm) and *C. danayanus* (49–51 in 10 μm), the two species are larger (83–105 μm and 28–61 μm) than *C. legouvelloanus* (18–34 [39] μm) and their general morphology differs considerably from that of the current taxon in, for example, possessing a reduced or strongly reduced stauros (Table 1). Several of the characters of *C. legouvelloanus*, such as largely uniform valve areolae with four pores per cribrum and internal central knob, agree with the description of *C. australis* (Cox 1999). However, the new species

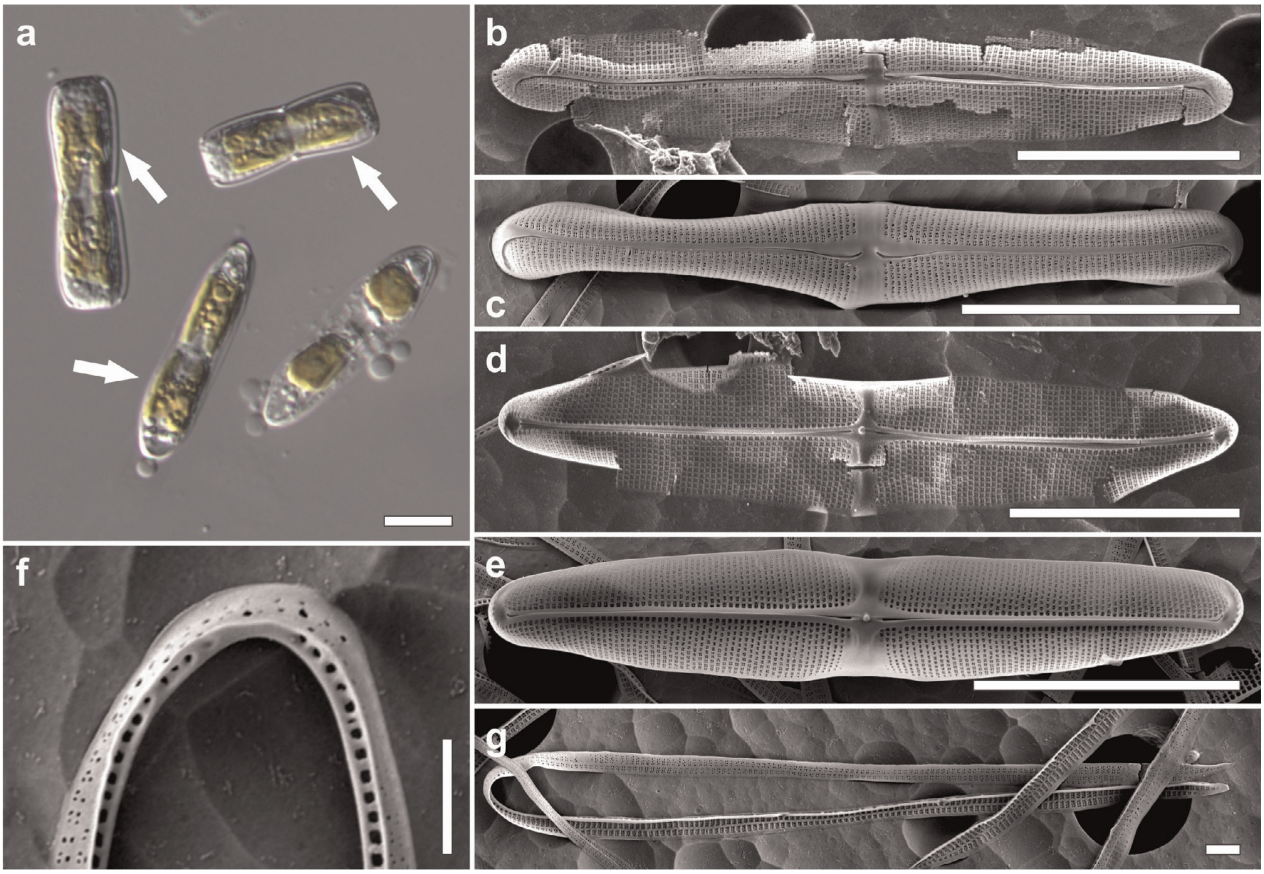


FIG. 5. *Craspedostauros legouvelloanus*. (a) Living cells in culture (light microscopy). Arrows indicate the H-shaped chloroplasts with one lobe pressed against each valve, a feature characteristic of the genus. (b) External valve view (wild population). (c) External valve view (cultured strain). (d) Internal valve view (wild population). (e) Internal valve view (cultured strain). (f) Detail of a girdle band showing internal thickening (septum) with perforations. (g) A single girdle band (external and internal view). Scale bars: 10 μm = panels a–e; 1 μm = panels f and g.

can be easily distinguished from the latter by its clearly centrally expanded valve margin and well-developed lip-like silica flaps externally covering the central raphe endings, features that are absent in *C. australis* (Table 1).

The specimens from the Adriatic population of *Craspedostauros legouvelloanus* exhibited numerous irregularities in the shape and size of taxonomically important characters such as areolae, striae, stauros, and central area. However, overall, they did not differ significantly from the type population. High morphological plasticity and polymorphy in diatoms have been reported from both epizoic and non-epizoic habitats (Cox 2011, De Martino et al. 2011, Riaux-Gobin et al. 2014, 2017, Urbánková et al. 2016, Edlund and Burge 2019), and it is conceivable that the morphological differences observed between the two populations could be induced by environmental triggers, such as differences in salinity or nutrient concentrations (Schultz 1971, Czarnecki 1994, De Martino et al. 2011, Cox 2014). Unfortunately, the Croatian strain PMFTB0003 (Fig. 5, a, c, e, and f) isolated from sample TB13 did not survive and its DNA could not be obtained at the time of this study. Therefore, in the light of the current lack of any additional information about the phylogenetic relationships between the two populations, they should be considered conspecific until otherwise proven.

Ecology: Epizoic on carapaces and skin of adult loggerhead sea turtles and on loggerhead-associated barnacles *Chelonibia testudinaria* growing on adult loggerheads from Kosi Bay (South Africa) and the Adriatic Sea (Croatia). Attaching to the animal surface through one end of the valve, motile in culture.

Although the taxon was present in numerous samples, its relative abundance rarely exceeded 4% of the total diatom number. Samples with *Craspedostauros legouvelloanus* from both locations were each dominated by *Poulinea* spp., *Berkeleya* spp., *Halamphora* spp., and *Nitzschia* spp., with addition of *Achnanthes elongata*, *Cyclophora tenuis*, *Proschkinia* spp., *Navicula* spp., *Licmophora* spp., and *Haslea* spp.

***Craspedostauros macewanii* Majewska & Ashworth sp. nov. (Fig. 6)**

Diagnosis. Valves linear to linear-lanceolate; valve margin straight, not expanded centrally; stria density 28–31 in 10 μm; valve face-mantle junction distinct; external central lip-like silica flaps rudimentary; apical hyaline zone extended; stauros and internal central knob present; girdle bands with up to five rows of squarish areolae. Accession numbers of sequence data representative of this taxon (but **not** the holotype): MT432486-MT432488 (*rbc*L) and MT432506 (*psb*C).

Holotype. Permanent slide SANDC ST242 (prepared from sample ST242/USW).

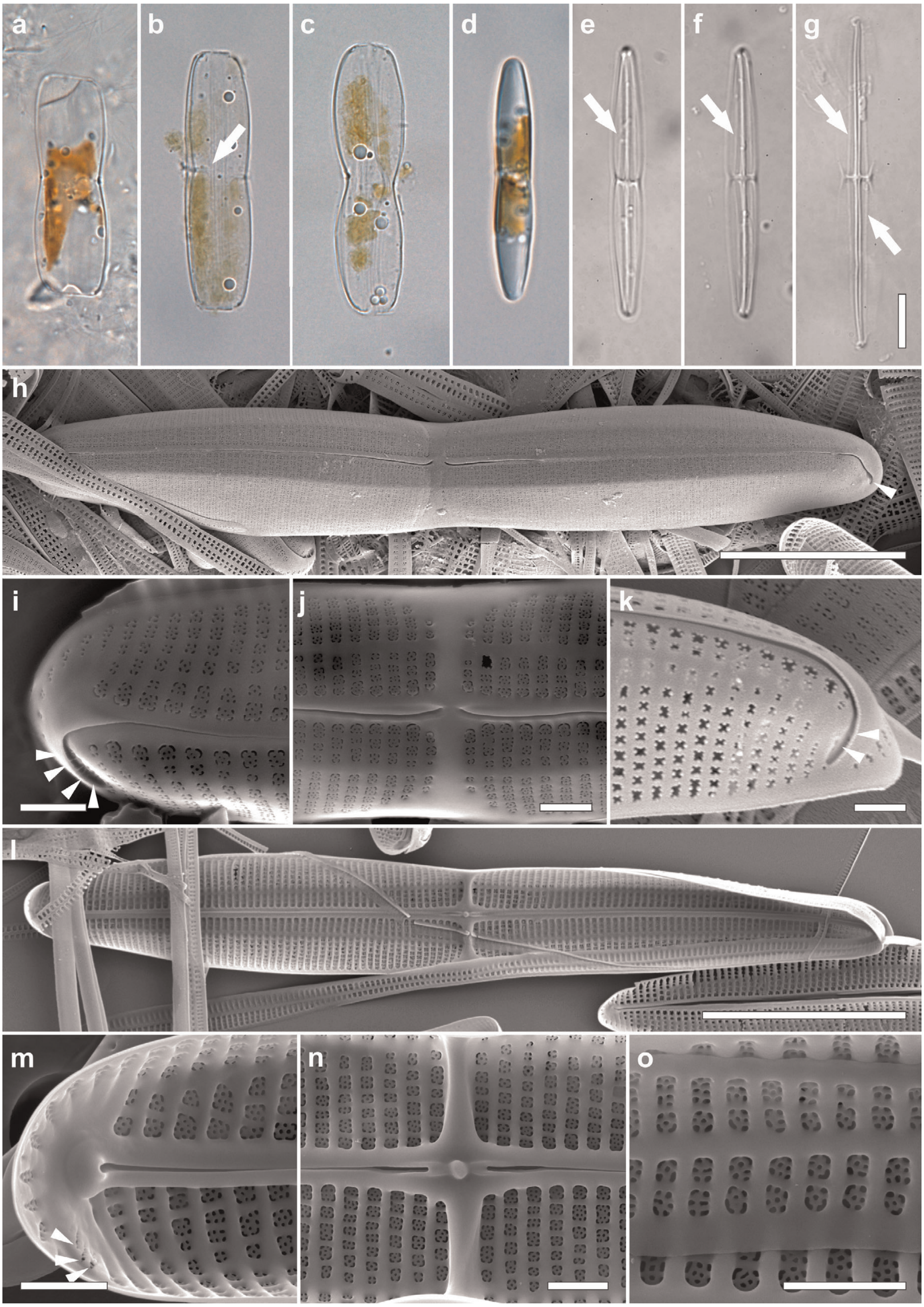
Type locality. uShaka Sea World, Durban, South Africa (29°52′02.79″ S, 31°02′45.29″ E). Collected from the carapace of a captive juvenile loggerhead named “Bubbles” by R. Majewska, June 28, 2019.

Etymology. The epithet honors Tony McEwan, the uShaka Sea World director, whose scientific enthusiasm and support to the sea turtle diatom project are highly appreciated and acknowledged.

Description. Light microscopy (Fig. 6, a–g): Cells with two fore and aft H-shaped chloroplasts (Fig. 6, a and d). Frustules delicate and lightly silicified (Fig. 6, a–g). In girdle view, frustules rectangular, moderately to strongly constricted at the center (Fig. 6, a–c). Cingulum composed of several girdle bands (Fig. 6, b, and c). Valves narrow, linear to linear-lanceolate, slightly constricted at the central area, with bluntly rounded apices (Fig. 6, d–g). Valve margin straight (Fig. 6b, arrow). Valve dimensions ($n = 20$): length 26–51 μm (up to 65 μm in culture), width 4.5–5.5 μm (up to 6 μm in culture), length/width ratio: 5.4–11.3. Valve face-mantle junction visible on each side of the raphe (Fig. 6, e–g, arrows). Striae barely discernible, 28–31 in 10 μm (Fig. 6, e–g). Central area narrow, bow tie-shaped (Fig. 6, e–g). Raphe-sternum thickened (Fig. 6, e–g). Raphe straight (Fig. 6g) with thickenings at the terminal raphe endings (Fig. 6, e–g).

Scanning electron microscopy (Fig. 6, h–o). Externally: Valves slightly concave at the center, with distinct valve face-mantle junction marked by a narrow pore-free area (Fig. 6h and j). Valve face flat (Fig. 6h). Mantle very deep (Fig. 6h). Valve margin straight, with narrow pore-free area at the mantle edge (Fig. 6, i and j). Striae uniseriate, parallel through most of the valve, becoming convergent near the apices, alternate or opposite, composed of up to 21 areolae (2–8 on the valve face and up to 13 on the mantle; Fig. 6, h–k). Areolae similar in size, squarish, externally occluded by cribra (Fig. 6, i–k). Areolae bordering the narrow axial area usually only slightly larger and somewhat irregular in shape

Fig. 6. *Craspedostauros macewanii.* (a–g) Light micrographs. (a–d) Fresh (unpreserved) material. (a and d) Living cells. (a) Girdle view. (d) Valve view. (b and c) Damaged cells in girdle view with the cell content (including plastids) spilling beyond the cell wall. (b) Arrow indicates the straight valve margin. (e–g) Cleaned material. Detached valves in valve view. Arrows indicate the distinct valve face-mantle junction. (h–o) Scanning electron micrographs. (h) External valve view. (i) Detail of the apical part (external valve view). (j) Detail of the central area (external valve view). (k) Detail of the apical part (external girdle view). (l) Internal valve view and partially detached valvocopula. (m) Detail of the apical part (internal valve view). Arrowheads indicate several small areolae present at the end of the curved thickening. (n) Detail of the central area (internal valve view). (o) Detail of the valvocopula (internal view). Scale bars: 10 μm = panels a–h and l; 1 μm = panels i–k and m–o.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(Fig. 6, i–k). Each cribrum perforated by highly variable number of pores (up to 13+; Fig. 6, i–k). Raphe branches more or less straight (Fig. 6h). Central area in the form of a narrow bow tie-shaped fascia (Fig. 6, h and j). Central raphe endings covered by small lip-like silica flaps extending from one side of the axial area (Fig. 6, h and j). Apices pore-free (Fig. 6, h, i, and k). Terminal raphe endings covered by triangular silica flaps giving the impression of unilaterally bent terminal raphe fissures (Fig. 6, h, i, and k). An oval or irregular depression (Fig. 6, h, arrowhead, i and k) with several small areolae (Fig. 6, i and k, arrowheads) present at the apical flap fold. Shortened striae composed of a single areola (occasionally with additional puncta) radiating around the apices beyond the terminal raphe endings (Fig. 6, i and k).

Internally: Raphe slit opening more or less centrally onto the uniformly thick raphe-sternum (Fig. 6, l–n). Stauros raised, narrow, tapering toward the valve face-mantle junction and widening significantly on the valve mantle toward the mantle edge (Fig. 6, l and n). Central raphe endings straight, elongated, terminating onto rectelevatum (Fig. 6, l and n). A flatly ended cylindrical knob present at the central nodule (Fig. 6, l and n). Areolae externally occluded by cribra, appearing sunken, especially close to the raphe-sternum (Fig. 6, m and n). Terminal raphe endings terminating onto prominent helictoglossae within an expanded and thickened pore-free area corresponding to the curvature of the external silica flaps (Fig. 6, m). Several small areolae present at the end of the curved thickening (Fig. 6m arrowheads).

Cingulum composed of numerous open copulae bearing up to five (6?) rows of cribrate squarish or elongated areolae divided by a central pore-free area, ca. 38–45 in 10 μm (Fig. 6, h, l, and o). Advalvar part of valvocopula pore-free beside the stauros (Fig. 6l).

Taxonomic notes and comparison to other Craspedostauros *species.* The morphological character pattern in *Craspedostauros macewanii* is most similar to *C. australis* and *C. capensis.* The three species share several features such as the presence of a bow tie-shaped fascia, rudimentary lip-like silica flaps extending from the raphe-sternum and partially covering the external central raphe endings, valve margin straight at the center, and internally, a single knob at the central nodule (Table 1). Moreover, valve dimensions of *C. macewanii* (26–51 μm long, 4.5–5.5 μm wide) overlap with those reported for *C. australis* (35–78 μm long, 4–6 μm wide) and *C. capensis* (25–35 μm long, 4.5–5.5 μm wide). In *C. macewanii,* however, the stria density (28–31 in 10 μm) is significantly higher than in *C. capensis* (~19 in 10 μm) and lower than in *C. australis* (35 in 10 μm). In addition, *C. macewanii* can be distinguished from both *C. australis* and *C. capensis* by the presence of a distinct valve face-mantle junction running as a narrow, though clearly visible, pore-free ridge from apex to apex. *Craspedostauros macewanii* differs further from *C. capensis* in possessing areolae of a similar size throughout the entire valve (variable in *C. capensis*), and from *C. australis* in having convergent stria at the apices (parallel in *C. australis*) and extended apical hyaline zone (Cox 1999). The new taxon is also the only *Craspedostauros* species with girdle bands perforated by up to five rows of squarish areolae instead of the two rows of usually transapically elongated areolae observed in other species. This, however, may again be an artifact of the lack of detailed information regarding the girdle structure and ornamentation in several other *Craspedostauros* species (Cox 1999).

Ecology: Epizoic on skin and carapaces of captive loggerheads and green turtles. Attaching to the animal surface through one end of the valve, motile in culture.

The taxon was found on two captive loggerheads (a juvenile named "Bubbles" and an adult female named "DJ") and two captive green turtles (a subadult named "Calypso" and an adult male named "Napoleon") each time reaching relative abundance of 0.5%–1%. All carapace samples containing *C. macewanii* were dominated by the so-called "marine gomphonemoids": *Poulinea* spp. and *Chelonicola* spp., accompanied by *Amphora* spp., *Nitzschia* spp., *Achnanthes elongata,* and *A. squaliformis,* whereas the most abundant taxa in the four skin samples were *Tursiocola* spp., *Medlinella* sp., and the two previously mentioned *Achnanthes* species.

Craspedostauros alatus Majewska & Ashworth (Fig. 7)

Craspedostauros alatus was found on the carapaces of several loggerhead sea turtles sampled at the Marine Turtle Rescue Centre in Pula, Croatia. The taxon co-occurred with *C. legouvelloanus.* As in the case of the latter, relative abundance of *C. alatus* was low (ca. 1–3% of the total diatom number). The observed morphological features of the Adriatic population agreed with the original description of the species (Majewska et al. 2018; Fig. 7, Table 1). The examined specimens were 26–34 μm long and 3–5 μm wide (length/width ratio: 6.3–8.8), with stria density 24–27 in 10 μm ($n = 20$), and possessed all species-specific features, including a very distinct valve face-mantle junction and deep mantle (Fig. 7f, arrows, g–i), wing-like silica flaps at the apices (Fig. 7h), and rectelevatum with central cavity (Fig. 7, k and l).

DNA-BASED PHYLOGENY

The genus *Craspedostauros* is monophyletic based on DNA sequence data generated from cultured material to date (Fig. 8), though not with strong bootstrap support (bs < 50%). Regarding the taxa

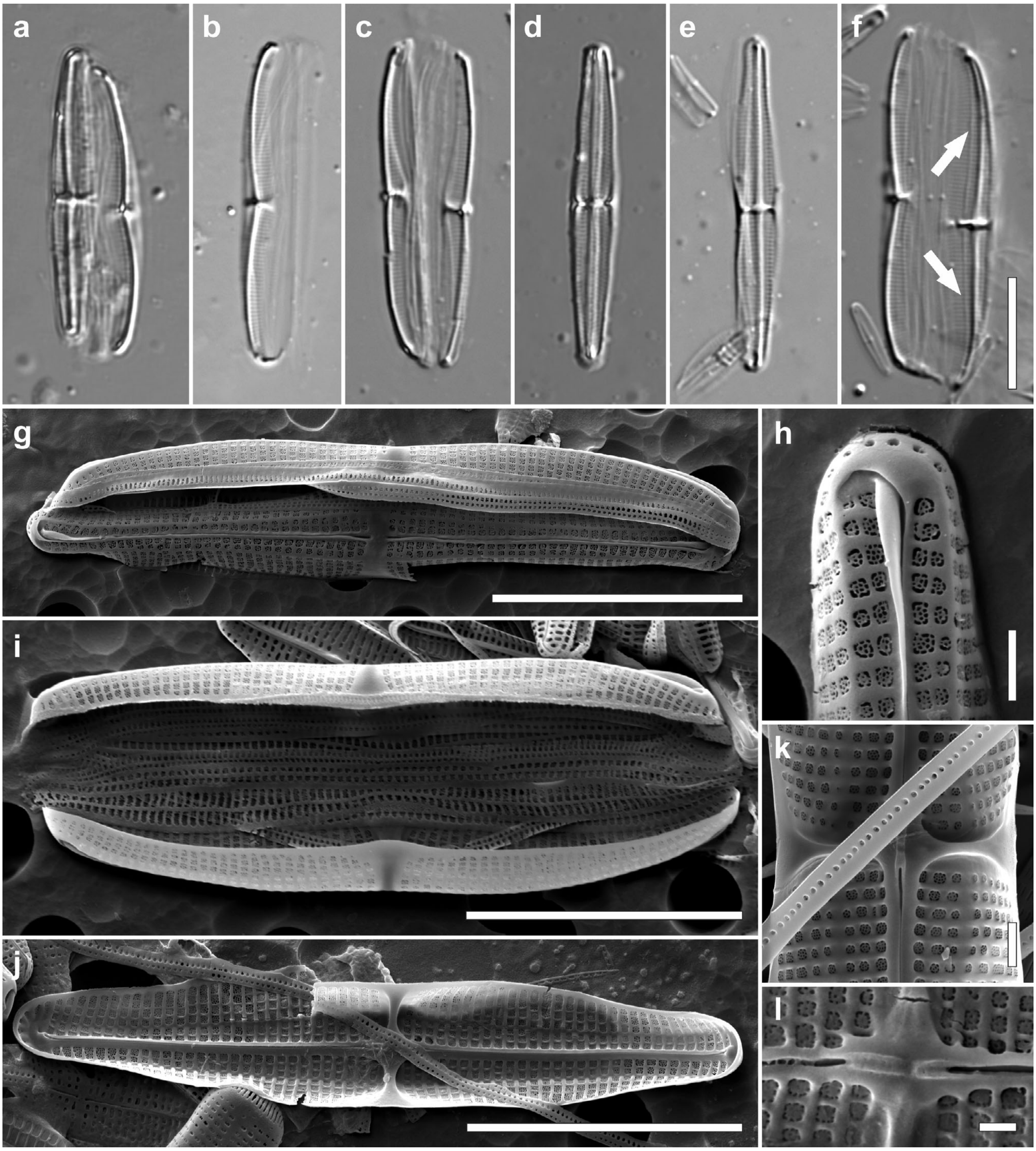

FIG. 7. *Craspedostauros alatus* (Adriatic population). (a–f) Light micrographs. (a, d and e) Valve view. (a) Broken frustule with both valves lying in valve view. (b) Single valve with attached girdle bands. (c and f) Girdle view. Arrows indicate the clear valve face-mantle junction. (g–l) Scanning electron micrographs. (g) Frustule with partially detached girdles bands (external view). (h) Detail of the apical part of the frustule with the winged-liked silica flaps, a feature typical of the species (external view). (i) Frustule with partially detached girdles bands (external girdle view). (j) Internal valve view. (k and l) Detail of the central part of the valve (internal view). Scale bars: 10 µm = panels a–g, i and j; 1 µm = panels h and k; 500 nm = panel l.

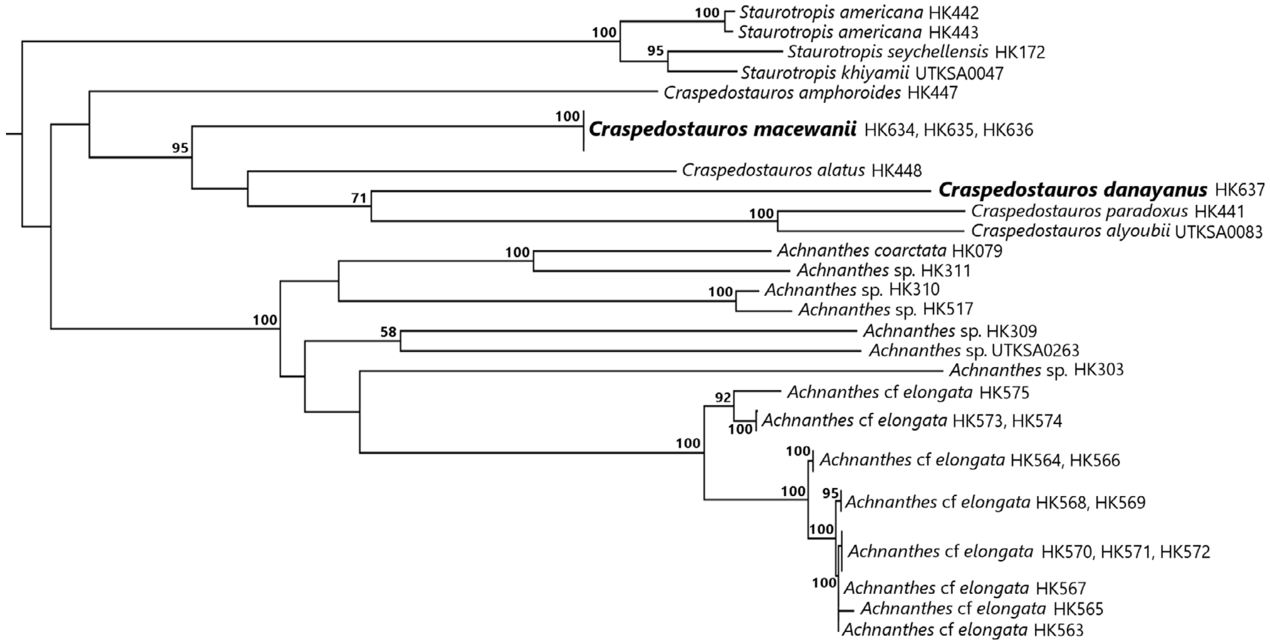
100 Staurotropis americana HK442
Staurotropis americana HK443
100
95 Staurotropis seychellensis HK172
Staurotropis khiyamii UTKSA0047
Craspedostauros amphoroides HK447
100 Craspedostauros macewanii HK634, HK635, HK636
95
Craspedostauros alatus HK448
71 Craspedostauros danayanus HK637
100 Craspedostauros paradoxus HK441
Craspedostauros alyoubii UTKSA0083
100 Achnanthes coarctata HK079
Achnanthes sp. HK311
100 Achnanthes sp. HK310
Achnanthes sp. HK517
100
58 Achnanthes sp. HK309
Achnanthes sp. UTKSA0263
Achnanthes sp. HK303
92 Achnanthes cf elongata HK575
100 Achnanthes cf elongata HK573, HK574
100
100 Achnanthes cf elongata HK564, HK566
100 95 Achnanthes cf elongata HK568, HK569
100
Achnanthes cf elongata HK570, HK571, HK572
100 Achnanthes cf elongata HK567
Achnanthes cf elongata HK565
Achnanthes cf elongata HK563

FIG. 8. Maximum likelihood (ML) phylogram based on the 3-gene dataset (nuclear-encoded ribosomal SSU, chloroplast-encoded rbcL, psbC markers). For clarity, only the clade of raphid diatoms containing *Staurotropis*, *Craspedostauros*, and *Achnanthes* is presented in the figure. The ML tree presenting the complete taxon sampling can be viewed in Figure S1.

described here, *Craspedostauros macewanii* is sister to the reminder of the clade (except *C. amphoroides*) with high support (bs = 96%), while *C. danayanus* is sister to *C. alyoubii* and *C. paradoxus* (bs = 71%).

Consistent with other molecular phylogenetic studies which include the genus (Ashworth et al. 2017), the position of the *Craspedostauros* clade can be found in a poorly supported (bs < 50%) assemblage containing the *Staurotropis* clade and a clade of marine *Achnanthes* species. This assemblage, in turn, forms a clade with the Bacillariales (Fig. S1 in the Supporting Informations), though the relationship between the *Staurotropis* + *Achnanthes*+ *Craspedostauros* clade and the three Bacillariales clades is poorly resolved. For taxa, strain voucher ID and GenBank accession numbers for strains used in the analysis see Table S1.

DISCUSSION

The three new species described in the current study share most of the morphological characters typical of the genus *Craspedostauros*, such as squarish or rectangular areolae occluded by cribra on the valve and girdle bands, multiple copulae with at least two rows of perforations, and two fore and aft chloroplasts. Their linear or linear-lanceolate valve outline and the central constriction of the cell seen in girdle view resemble previously described species. Two of the new species, *C. macewanii* and *C. legouvelloanus*, present features not previously observed in any other member of the genus. The former possesses more than two rows of cribrate areolae on the girdle bands, while the latter shows shallow perforated septa. The leatherback-associated *C. danayanus*, in turn, presents a complete reduction of the stauros being the second, after *C. paradoxus*, member of the genus lacking this character.

It is interesting to note that as the number of character states, such as the reduction/loss of the stauros (*Craspedostauros paradoxus* and *C. danayanus*) or addition of septate copulae (C. *legouvelloanus*), within *Craspedostauros* changes, the molecular data remain constant in their support (however tenuous) of monophyly for the genus. Cox (1999) ascribed the constricted girdle view to the presence of the stauros. However, the frustules of the two species lacking the latter still show the central constriction, which may indicate that the lack of stauros is a secondary loss. One of the morphological features of the genus which has been maintained, regardless of newly described diversity, is the cribrate areolar covering. While the degree of cribrum poration might change among species, the overall gestalt ultrastructure remains unchanged. This cribrum ultrastructure is also seen in *Staurotropis* and the *Achnanthes* species, which are typically found (again, somewhat tenuously) sister to the *Craspedostauros* clade in molecular phylogenies. While there are general morphological similarities between the three genera, such as the stauros (though missing in some species of *Craspedostauros* and *Achnanthes*) and the fore and aft H-shaped or plate-like chloroplasts (missing in *Staurotropis* and some species of *Achnanthes*), the cribrate areolae ultrastructure remains constant. In this context, the phylogenetic position of the genus *Druehlago*, which shares the same cribrum ultrastructure and the same chloroplast morphology as *Staurotropis* and *Achnanthes longipes*, but thus far lacks a stauros-bearing taxon, is all the more intriguing (Ashworth et al. 2017).

Microscopical analyses of the fresh and critical point-dried sea turtle skin pieces and barnacles revealed the mode of attachment and growth form of *Craspedostauros danayanus* that attaches to the animal substratum through one pole of the cell. A similar mode of attachment to the natural substratum was observed in several members of the genus (R. Majewska, pers. obs.) suggesting that these taxa can either develop as firmly attached, sessile colonies, or remain motile in less favorable conditions (e.g., in culture tubes).

In the course of the ongoing surveys on sea turtle-associated diatoms, a recently described taxon, *Craspedostauros alatus*, was observed growing on the carapaces of several loggerhead sea turtles rescued in Croatia. *Craspedostauros alatus* was originally described from museum specimens of juvenile Kemp's ridleys (*Lepidochelys kempii*) and a juvenile green turtle found cold-stunned and beyond recovery on a New York (USA) beach during various seasons between 2012 and 2014 (Majewska et al. 2018). Although the relative abundance of *C. alatus* did not exceed 5.5% (current study, Majewska et al. 2018), observations of this taxon on a sea turtle from the Adriatic Sea may indicate that a) *C. alatus* is not an uncommon element of the sea turtle diatom flora, and b) being associated with highly migratory animals such as sea turtles its geographical range is likely linked to that of its hosts.

A similar conclusion can be drawn based on the records of *Craspedostauros legouvelloanus*. The species occurred on several of the Adriatic loggerheads as well as on dozens of loggerheads and their associated barnacles sampled on the eastern coast of South Africa. Even though the diatom was found in two different ocean basins, it cannot be excluded that the sea turtles acted as vectors that facilitated its dispersal among the various seas and oceans. There is strong observational and molecular evidence that the Indian Ocean loggerheads interact and mate with the Atlantic members of the species (Bowen et al. 1994, Bowen and Karl 2007, Le Gouvello et al. 2020). Thus, it is conceivable that any diatom able to endure the changing conditions during the migrations of their hosts and survive in competition with native flora would inoculate all appropriate and available media and substrata encountered. With the exception of *C. danayanus*, the sea turtle-associated *Craspedostauros* species, although common on sea turtles, were never among the dominant taxa, and it is still unclear whether the animal body surface is their preferred or an alternative habitat as yet they have not been recorded from non-sea turtle substrata. It is possible that the occurrence of these species in the sea turtle biofilm samples is linked to the presence of some other sea turtle epibionts (e.g., barnacles, sponges, and bryozoans).

Craspedostauros danayanus, in turn, dominated most of the leatherback skin and barnacle samples that were analyzed, and it is likely that this taxon is highly adapted to the conditions provided by the smooth body of the largest of the sea turtles. Being associated with both the skin and the leatherback-specific barnacle species, *Platylepas coriacea,* its relationship with the host may be obligatory. Leatherbacks, contrary to other extant sea turtles, show a fully oceanic developmental pattern spending most of their lives in highly homogenous open-water environments devoid of refugia (Bolten 2003). They are unique among modern reptiles in being endothermal (Frair et al. 1972). This ability allows them to survive in both tropical and near-freezing waters (James et al. 2006). They are also significantly faster swimmers and deeper divers than other sea turtles (Eckert 2002, Doyle et al. 2008). Therefore, microhabitats provided by these animals, and thus diatom communities contributing to their microbiomes, would differ substantially from those present on other sea turtles. Under such unique conditions, far from the diverse, species-rich shallow-water ecosystems, specific eco-physiological adaptations may be required to survive, and fewer diatom species would manage to thrive on the demanding substratum. An analogous phenomenon is reported from marine cetaceans that seem to be colonized by only a few, highly specialized, diatom taxa (e.g., Nemoto 1956, Holmes et al. 1993, Ferrario et al. 2018). Future studies will likely reveal a more complete picture of the diversity and biogeography of sea turtle-associated *Craspedostauros* and other diatom taxa.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Maximum likelihood tree based on the 3-gene dataset (nuclear-encoded ribosomal SSU, chloroplast-encoded rbcL, psbC markers) with bootstrap values from 1,000 pseudoreplicates over the corresponding nodes. The araphid pennate taxon *Asterionellopsis socialis* was used as the outgroup.

Table S1. Taxa, strain voucher ID and GenBank accession numbers for strains used in the DNA sequence data phylogenetic analysis.

3. Discussion

3.1. Loggerhead sea turtles harbor diverse microbial communities

Aims and hypotheses of this doctoral thesis were successfully addressed by the included publications. The first aim "Analysis of the composition and diversity of endozoic bacterial communities of loggerhead sea turtles", was contributed to by the Publication **I** that encompassed characterization of oral and cloacal microbiota of wild and rehabilitated loggerhead sea turtles in the Adriatic Sea by 16S rRNA gene amplicon sequencing. It expands on the previous studies of loggerhead gut microbiome in the Mediterranean Sea (Abdelrhman et al., 2016; Arizza et al., 2019; Biagi et al., 2019) by combining data from wild and rehabilitated sea turtles. The immediate novelty of Publication **I** is in the description of the oral microbiota of loggerheads by amplicon sequencing for the first time. At the time of Publication **I** oral microbial communities of sea turtles were described only in cold stunned Kemp's ridley sea turtles, and in wild Kemps and green sea turtles (McNally et al., 2021a, 2021b). The second aim "Characterization of epizoic diatom and bacterial communities of the skin and carapace of loggerhead sea turtles, with a focus on diatom-associated bacteria" was directly contributed to by Publication **II**, as it included an amplicon-based survey of loggerhead carapace and skin diatom communities by sequencing the chloroplast *rbcL* gene, and bacterial communities by sequencing the 16S rRNA gene. The analysis of bacterial communities of carapace and skin in Publication **II** is performed on several turtles from the Kanjer et al. (2022) study. Additionally, it contributes to the third aim "Isolation and identification of diatoms found on the skin and carapace of the loggerhead sea turtle, establishment of monoculture protocols, and description of newly found diatom taxa" as 19 diatom strains were isolated and cultured, as well (Publication **II**). Diatom-associated bacteria in individual strains were examined by 16S rRNA gene amplicon sequencing, contributing to the second aim, and by culturing bacterial isolates thus contributing directly to the third aim. This is the first time that the bacteria associated with epizoic diatoms were investigated and cultured, contributing to the efforts in describing the phycosphere of planktonic and benthic diatoms (Amin et al., 2015; Barreto Filho et al., 2021; Stock et al., 2022). Publications **III** and **IV** directly contributed to the third aim, by establishing culturing protocols for epizoic diatoms across sea turtle hosts, including the loggerheads in Publication **II**, and describing new species in the potentially epizoic diatom genus *Craspedostauros*: *Craspedostauros alatus* Majewska & Ashworth was reported to be found on loggerhead sea turtles in the Adriatic Sea, and *Craspedostauros legouvelloanus* Majewska & Bosak was described as a novel species in Publication **IV**.

3.1.1. Oral and cloacal microbiome of loggerhead sea turtles

Investigations in the endozoic microbiome of vertebrates hold promises of detangling healthy states from dysbiosis, improved treatment and conservation as it is known that the microbiome can greatly impact the physiology of the host (McFall-Ngai et al., 2013). Pioneering studies focused on characterizing the gut microbial assemblages presented data on microbial community composition but the clear connections between the gut microbiome and sea turtle physiology are still lacking (Abdelrhman et al., 2016; Ahasan et al., 2017; Samuelson et al., 2020). Even though oral microbiomes have elsewhere been recognized as crucial in host's physiology (Bik et al., 2016), in reptiles they just recently started garnering the attention of microbiome researchers (Zancolli et al., 2015; McNally et al., 2021b, 2021a).

The microbial community composition of the buccal cavity of loggerheads, first described in Publication **I**, strongly reflects the environment of the turtle. As several of the loggerhead sea turtles in Publication **I** were sampled before admission to the rescue center, their microbiomes were considered "wild" or at least a close representative. Once the turtles were admitted to the rescue center, they were housed in large plastic tanks and immersed in filtered recirculating seawater or non-circulating artificial saltwater. Conditions in the tanks differ from those in the wild (e.g., constant temperatures, variation in macro- and micronutrients, fecal contamination) which also affects the tank water (environmental) microbial community composition and functions. The oral bacterial communities differed between "wild" and turtles in rehabilitation as indicated by beta diversity metrics (Bray Curtis and unweighted and weighted UniFrac) and pairwise PERMANOVA results, while there were no differences detected between the oral microbiota of turtles in rehabilitation and their tank water (Publication **I**). The turtles that were sampled for oral microbiota stayed in rehabilitation up to 13 days which is considered short-term (Biagi et al., 2019; Publication **I**), so the results represent the shift from "wild" to "in rehabilitation" microbiota in a very short time. The recirculating seawater tank bacterial community was often grouping closer to the "wild" oral bacterial communities in the PCoA results. Members of the Planctomycetota phyla and Patescibacteria group were highly abundant in recirculating tank seawater and in oral samples before admission to the center, indicating that the recirculating tank water could present a more natural marine habitat in comparison to the tanks with artificial seawater. The composition of microbes shows that genus *Pseudoalteromonas* was more abundant in oral samples in turtles undergoing rehabilitation, and other potentially transient taxa like *Vibrio* and *Shewanella* (from cloaca), and *Pseudomonas* and *Bizionia* (from tank water) were often found. Members of the *Halieaceae* genus, however, were more abundant in all oral samples than in any other sample type which indicates they could be specific for buccal mucosa. The only other studies characterizing oral microbiome of sea turtles include cold-stunned juvenile Kemp's ridley sea turtles (McNally et al., 2021a) and wild juvenile Kemp's ridley and green sea turtles (McNally et al., 2021b). Both studies were published at the time Publication **I** was undergoing review, so they were not included in the initial discussion. The oral microbiota of loggerheads overlaps with the Kemp's ridley sea turtles only in relative abundances of *Flavobacteriaceae* and *Rhodobacteraceae* families, while there are no similarities with the oral microbiota of green sea turtles (McNally et al., 2021a, 2021b; Publication **I**). McNally et al. (2021a) included samples of the boat surface where the turtles were sampled and the surrounding seawater, but contrastingly to Publication **I** they did not detect significant overlaps in environmental and oral microbes. In the case of *Halieaceae* spp., McNally et al. (2021a) detected the genus in sea water at lower relative abundance but have not mentioned it in the rest of their results. In loggerhead sea turtles, however, this group was consistent in oral samples but rare or non-existent in cloacal and tank water samples (Publication **I**). This indicates there could be some members of the *Halieaceae* genus recruited to the mucosa of loggerheads but no other sea turtles. In cold-stunned Kemp's ridley sea turtles undergoing rehabilitation the oral microbiota upon intake resembled the "wild" microbiota, and there was a significant shift in microbial community composition after four weeks of rehabilitation in tanks with filtered saltwater, regardless of the antibiotic treatment (McNally et al., 2021a). In that study the authors did not obtain tank water samples to track the potential exchange of microbes between the turtle and the environment, but their results emphasize the impact of captivity on oral microbiomes, similar to findings in Publication **I**.

The gut microbiota of loggerheads was studied in feces (Abdelrhman et al., 2016; Arizza et al., 2019; Biagi et al., 2019), and in distal colon and cloaca (Scheelings et al., 2020; Publication **I**). Phyla Firmicutes (Bacillota corrig. phyl. nov.), Proteobacteria (Pseudomonadota corrig. phyl. nov.) and Bacteroidota were observed as dominating the fecal microbiota along with Clostridia in stranded turtles (Abdelrhman et al., 2016; Arizza et al., 2019). In a wide range of short- and long-term rehabilitated turtles the feces consisted mostly of Firmicutes (Bacillota corrig. phyl. nov.) and Fusobacteria (Fusobacteriota corrig. phyl. nov.), rich in *Cetobacterium* spp. (associated with plastic ingestion) and *Clostridium* spp., and with a low exchange of microbes with the surrounding tank water (Biagi et al., 2019, 2021). In Publication **I** the exchange of microbes with the environment seemed one directional, meaning the cloacal microbes were expectedly found in the tank water probably due to fecal contamination. Cloacal microbiota of loggerheads in the Publication **I** mainly consisted of Proteobacteria (Pseudomonadota corrig. phyl. nov.), Bacteroidetes (Bacteroidota corrig. phyl. nov.), Kiritimatiellaeota (Kiritimatiellota corrig. phyl. nov.), Spirochaetes (Spirochaetota corrig. phyl. nov.), and Firmicutes (Bacillota corrig. phyl. nov.) which coincides with other studies on loggerheads that sampled the distal colon rather than feces (Scheelings et al., 2020b, 2020a). It is known that the gut consists of anatomical niches with differing microbial communities, specifically the fecal microbiome that is a better representative of gut lumen microbiome and is affected by food composition (Bloodgood et al., 2020) while the mucosal microbial communities consist of microbes intimately attached to the host with possible greater impacts on the physiology than the lumen microbes (Ingala et al., 2018). The differences between loggerhead fecal and cloacal or distal gut microbial communities could be explained by differences in anatomical sites sampled, but also the turtle population properties as there could be differences between wild healthy and stranded turtles, females and males, juveniles or adults. Since the Adriatic loggerheads share a similar ecological niche and foraging habitats, the non-Proteobacteria (Pseudomonadota corrig. phyl. nov.) dominated microbiome in initial studies is probably connected to the turtle's health status, changes in immune response, rehabilitation treatments, diet, and sampling feces rather than the intestine or cloaca (Biagi et al., 2019; Publication **I**). Compared to the other sea turtle species, the loggerhead sea turtle nesting females' distal gut microbiome clusters with those of Hawksbill, flatback and olive ridley sea turtles on the interspecies level (Scheelings et al., 2020a), but on the intraspecies scale exhibits differences between geographically distinct populations (Scheelings et al., 2020b). The interspecies patterns could be explained by similarities in physiology, habitats, evolution and diet preferences across several species, while interspecies differences might reflect the immediate availability of food in a certain habitat (oceanic vs. neritic). Taken together, the results presented in Publication **I** show that the oral microbiota consists of transient microbial taxa, and taxa potentially specific for mucosa and rarely found in other sources, while the cloacal microbiota is stable during short-term rehabilitation confirming the first hypothesis stating that “oral microbiota of loggerhead sea turtles is dynamic and reflective of but also distinct from the environment, while the cloacal community is more stable”.

In studies on other sea turtle species, feces or sections across the gut were usually the preferred sample type for microbial analyses. However, in recent studies on Kemp’s ridley and green sea turtles the results obtained from cloacal swabs were sufficient to detect shifts in microbial communities of turtles in rehabilitation, and to discern between wild Kemp’s ridley and green sea turtles (McNally et al. 2021a). Along with Publication **I**, this work shows that cloacal swabs might be sufficient to describe microbial communities as a proxy to feces and intestinal samples, which would allow for wider and less invasive sampling of loggerheads and other sea turtle species. Cloacal sampling could therefore mitigate difficulties connected with the unpredictability of sampling feces in wild animals.

3.1.2. Carapace and skin microbiome of loggerhead sea turtles

As mentioned in the introduction, the loggerhead skin bacterial microbiome was mostly investigated through traditional culturing methods associated with intact skin and wounds that identified bacteria well known for being opportunistic pathogens like *Escherichia*, *Pseudomonas*, and *Klebsiella* rich in genes conferring antibiotic resistance (Alduina et al., 2020; Trotta et al., 2021b). Recently, Blasi et al. (2021) reported on crustaceans and algal growth in carapaces of ten loggerhead sea turtles in the Mediterranean Sea, along with characterization of bacterial communities in three out of ten turtles. There was an increase in algal coverage from anterior to posterior scutes that is probably related to drag and resistance generated during the turtle’s swimming (Blasi et al., 2021). The three turtles were sampled on their anterior and posterior carapace scutes and the dominant phyla were Firmicutes (Bacillota corrig. phyl. nov.) and Proteobacteria (Pseudomonadota corrig. phyl. nov.) while other taxa were present at less than 1% relative abundance (Blasi et al., 2021). Contrastingly, a recent study including 16S and 18S rRNA gene sequencing of the carapace and skin samples of 26 loggerheads in the Adriatic, Ionian, Tyrrhenian and Aegean Seas revealed highly diverse prokaryotic and microeukaryotic communities (Kanjer et al., 2022) that reflected the turtle’s locality and anatomy. Kanjer et al. (2022) showed that Proteobacteria (Pseudomonadota corrig. phyl. nov.), Bacteroidota, Bdellovibrionota and Cyanobacteria are the most abundant groups on the turtle skin and carapace. The total microeukaryotic community consisted mostly of Alveolata and Stramenopiles, with dominant classes being Oligohymenophorea, Bacillariophyta and Labyrinthulomucetes, Phyllopharingea, among whom the core taxa were ciliate Zoothamnium and diatom *Nitzschia communis* according to 18S rRNA gene sequences (Kanjer et al., 2022). Loggerhead sea turtles investigated in Publication **II** were a part of the Adriatic Sea cohort studied in Kanjer et al. (2022) and their carapace and skin contained 36 bacterial phyla and exhibited high alpha diversity. Opportunistic pathogens were usually not found or were below 1% relative abundance in skin and carapace samples (Kanjer et al., 2022; Publication **II**) implicating such bacteria could be localized to breaches in the skin barrier and diseased areas (Trotta et al., 2021b). Sampling the whole carapace instead of being limited to two sublocations encompassed all the micro niches available at the carapace, rather than localized patches of microorganisms. The total community approach showed to be beneficial in capturing the biodiversity available on the carapace and skin, but it does lack insight into specific niches that can be found within carapace scutes or around crustacean or algae growth. Areas rich in macroepibionts could be facilitating the formation of their own microbial consortia that are supported by the crustacean or algal byproducts (Amin et al., 2012; Durán et al., 2022; van der Loos et al., 2022; Publication **IV**).

Furthermore, the carapace and skin samples from loggerhead sea turtles in Publication **II** were analyzed for diatom communities by *rbcL* gene amplicon sequencing that provided first insights into diatom communities on loggerheads via metabarcoding. Van de Vijver et al. (2020) described diatom communities in the Mediterranean loggerheads solely based on morphology and reported clustering of samples based on turtle geography. Based on morphology, the highest number of taxa was found in genera *Mastogloia*, *Nitzschia*, *Amphora*, and *Navicula* that were present in all samples, with most frequently occurring species including *Nitzschia* CRO sp. 2, *Amphora crenulata*, *Nitzschia* cf. *inconspicua*, and *Cocconeis lineata*. The metabarcoding data (Publication **II**) showed that, based on *rbcL* sequences, the highly abundant genera were *Nitzschia*, *Amphora*, *Halamphora*, *Navicula, and* unclassified Bacillariophyceae which coincides with the morphology data by Van de Vijver et al. (2020). However, by using the *rbcL* DNA marker of 19 isolated diatom strains it seems that the epizoic diatoms (genera *Achnanthes* and *Poulinea*) were often misassigned to *Amphora* or *Nitzschia* genera, or to the *Bacillariaceae* family. Therefore, a proportion of sequences attributed to the most common diatom taxa mentioned above could easily belong to other diatom taxa without representative sequences available in the databases. Most of the diatoms isolated from carapace or skin in Publication **II** were present in amplicon sequencing data but at lower relative abundance (< 1%), with the exception of *Amphora* sp. 1 in one carapace sample that reached up to 50% relative abundance and *Poulinea lepidochelicola* on one skin and one carapace sample that reached 32% and 6% relative abundance, respectively. A study that was conducted on green sea turtles of the Mayotte Island north of Madagascar combined the morphology-based methods and sequencing to show that the diatom assembly was different based on the approach (Rivera et al., 2018). Nevertheless, the loggerhead sea turtles harbor diverse assemblages of diatoms, with the community consisting of presumably non-epizoic generalist diatom taxa and putative epizoic diatoms (Publication **II**). The skin seems to get colonized by epizoic diatoms in higher abundance than the carapace which means skin could be a preferred habitat for some of the putative epizoics. However, as Van de Vijver et al. (2020) notes, the true breadth of epizoic diatom taxa diversity remains to be determined.

3.1.3. Cultivation of epizoic microorganisms

Diatoms inhabiting marine megafauna, like the sea turtles or manatees, can be successfully isolated and grown in culture (Publications **II**, **III**, and **IV**). As mentioned before, 19 diatom strains were isolated and cultivated within the scope of Publication **II**, out of which two were determined as potential new species (*Fallacia* sp. and *Amphora* sp. 2). Several other diatom strains require deeper analysis before determining if they belong to an already described species or could represent novel taxa. Since the diatoms in Publication **II** were isolated from the total carapace or skin sample by manual pipetting, and washed through several rounds of sterile medium, it is considered that only intimately associated bacteria from the phycosphere continued to grow together with the diatom in culture. In that way an inventory of diatom-associated bacterial taxa could be described for each diatom culture (Publication **II**). Bacteria-diatom interactions are important and often complex but were so far described mostly in planktonic diatoms or tidal mudflats (Koedooder et al., 2019; Stock et al., 2019, 2022; Fei et al., 2020; Shibl et al., 2020). Diatom-bacteria associations or interactions have not yet been described in habitats related to vertebrate hosts. Publication **II** provides first insights into bacterial communities intimately associated with diatoms from the sea turtle enabling further focused investigations in diatom and bacterial taxa of interest. Importantly, the phycosphere habitat enriched bacterial taxa rarely observed or even not detected in initial samples (carapace and skin) like *Alcanivorax* spp., *Marinobacter* spp., and *Phycisphaera* spp. (Publication **II**). Still, diatoms isolated from the same host retained the bacterial signature of their host-of-origin in cultures that were maintained for more than one year, and subcultivated several times prior to harvest for DNA isolation (Publication **II**). These findings support the second hypothesis stating that "the phycospheres of diatom strains isolated from loggerhead sea turtles maintain the bacterial signature of the host they originated from". Based on research in mechanisms of diatom phycosphere bacterial community assembly (Stock et al., 2022) it could be possible that turtle-associated benthic diatoms follow similar patterns and recruit microbes in their immediate environment based on net positive (or neutral) interactions resulting in varying abundances of diatom specialist, generalist or transient taxa over time (personal communication with Amaranta Focardi at ISME18).

Additionally, 125 bacterial strains were cultivated from 10 out of 19 diatom cultures, out of which 40 unique bacterial strains were characterized by 16S rRNA gene sequencing and cryopreserved for future inquiries into their physiology, metabolism, and biotechnological potential (Publication **II**). Among sequenced bacterial isolates, several bacterial strains were identified as potential novel genera in the *Flavobacteriaceae* family (Publication **II**). Since the culturing was performed only on Marine Agar, it seems that the expansion of culturing efforts on multiple nutritional and environmental conditions (incubation temperature and time) could yield even more diverse and interesting microbial groups (Keller et al., 2021).

Diatom cultivation from sea turtles and manatees yielded representative sequences for several putative epizoic diatoms (*Achnanthes* spp., *Poulinea lepidochelicola*, *Proschkinia* spp. *Tursiocola* spp., *Medlinella amphoroidea*, *Chelonicola* spp.) (Publication **III**). Phylogenetic analyses had shown that the *Achnanthes* spp. separate into two clades depending on the host they were isolated from (Publication **III**). Also, epizoic diatoms are polyphyletic which means that the epizoic habitat evolved several times in diatom evolution and in different diatom morphotypes (Publication **III**). It seems possible that epizoic diatoms emerged from generalist diatom taxa and evolved to endure a dynamic host habitat. Multiple diatom strains identified only to the genus level (with several species suggestions) in Publication **III** could, upon detailed inspection, be determined as novel species (similar to Publication **II**). In Publication **IV**, three novel diatom species in the *Craspedostauros* genus were described from sea turtles: *Craspedostauros danayanus* Majewska & Ashworth from barnacles *Platylepas coriacea* growing on a leatherback sea turtle, *Craspedostauros macewanii* Majewska & Ashworth from loggerhead sea turtle carapace, and *Craspedostauros legouvelloanus* Majewska & Bosak also from loggerhead sea turtles. *C. danayanus* and *C. macewanii* were successfully isolated and cultured from their respective source samples so their DNA was sequenced and used for phylogenetic analyses. Unfortunately, the *C. legouvelloanus* culture that was isolated from the Adriatic loggerhead sea turtle was maintained in culture only for a short time before death, making it impossible to sequence the DNA. The death of diatom cells in culture might have been due to sub-optimal culturing conditions for that specific diatom, rather than the impossibility of growing in culture as the related species can indeed be cultured (Publication **III** and **IV**). Furthermore, *C. legouvelloanus* was found together with previously described

Craspedostauros alatus (Majewska et al., 2018; Publication **IV**) on loggerhead sea turtles in the Adriatic Sea. *C. alatus* was initially described from the museum specimens of juvenile green and Kemp's ridley sea turtles (Majewska et al., 2018), and Publication **IV** was the first to report it on a loggerhead sea turtle from the Adriatic Sea. Interestingly, *C. alatus* and *C. legouvelloanus* are found in lower abundance on the sea turtle carapace (up to 3% and 4%, respectively) and are often found within diatom assemblages dominated by other epizoic like *Achnanthes elongata* and *Poulinea* spp., along with diatoms expected to be generalists like *Nitzschia*, *Navicula* and *Halamphora* genera (Majewska et al., 2018; Publication **IV**). The *Craspedostauros* clade is monophyletic and sister to *Achnanthes* (Publications **III** and **IV**) and similarly to *Achnanthes elongata*, *Craspedostauros* spp. attach to the sea turtle surface by one end of the cell and are motile in culture. However, it is not yet clear if the turtle habitat is preferred or if these diatoms can be found in other habitats in future studies. Taken together, potential new diatom and bacteria species (Publication **II** and **III**), and the newly described diatom taxa (Publication **IV**) support the third hypothesis stating that "the microbial communities associated with loggerhead sea turtles are a source of novel microbial taxa".

3.2. Integrating the findings on loggerheads' microbiota

The approach to the turtle microbiome within this thesis was not limited to a single microbial group but is multifaceted and included both prokaryotes and microeukaryotes. Multiple anatomical sites of the host were included to encompass the gut (oral and cloacal microbiota) and the skin and carapace as they are the organs or anatomical regions first to interact with the turtle's environment. Culture independent approaches were enriched by cultivation of both diatoms and diatom-associated bacteria. The sea turtle, abundant both in diatoms and bacteria, was deemed as a good host to obtain first insights about vertebrate associated diatoms and their bacteria.

From the turtle-centric perspective, the sea turtle harbors diverse and rich microbial communities both on its surfaces and in the gastrointestinal tract. The microbial assemblages of the loggerhead sea turtle holobiont are strongly affected by the environment and most probably the turtle's behavior and physiology. The sea turtle host has, therefore, a reservoir of microbes out of which some could be proven to be neutral or beneficial, with potential detrimental effects if the turtle host is impaired as that can allow for the progression of opportunistic pathogens. The "hotspot" of microbial diversity on the loggerhead sea turtle could also harbor microbes capable of producing various compounds that facilitate multiple levels of host-microbe or microbe-microbe interactions. As sea turtles are reptiles, they have developed immune systems with innate and adaptive responses with subtle differences from other vertebrate groups (Zimmerman, 2020). In mammals the microbiota of the skin and mucosa is crucial in establishing the barrier from potential infections and immune system education and maturation however, knowledge on mucosal immunity in reptiles is lacking. Surface biofilms, oral and cloacal microbiota, and their products could be important factors in future studies on reptilian immune responses. A novel study on loggerhead sea turtle nests and associated microorganisms has established a framework to start examining the effects of microorganisms on hatchling health, microbiome seeding sources, and the transmission of microorganisms from the mother to the hatchlings (Vecchioni et al., 2022). If the microbial community on the sea turtle mother is in a dysbiosis state with higher incidences of pathogens, the opportunistic pathogens could possibly be transferred to the eggs and presumably non-contaminated nests thus affecting the hatchlings and their development.

To better understand the sea turtle holobiont, studies focusing on one specific anatomical site or trait should be combined and analyzed all together. Meta-analyses could then elucidate the dynamics of microbial colonization of the host, potential exchange of microorganisms between the environment and the external or internal microbiome, and pinpoint the importance of specific microbial taxa in host biology. For example, Planctomycetota phylum members are known for extensively colonizing the surfaces of macroalgae (van der Loos et al., 2022) and have been described in microalgae phycosphere (Lage, Bondoso, 2014; Publication **II**). Planctomycetota have also been observed in the bacterial communities of the oral mucosa where similar lifestyle strategies (attaching to living surfaces) could be useful, however the source of Planctomycetota can still be from food, the environment, or the sea turtle surface microbiome. Deeper investigation in the specific members of Planctomycetota and potential other microbial taxa shared between the epizoic and endozoic microbiome could deliver new insights into the biology of the host and the importance of microbial diversity associated with marine megafauna. To address specific host-microbe-environment interactions, the findings of existing studies need to be integrated to coordinate focused sampling efforts of host and the environment in future studies.

The barrier to investigating the sea turtle epizoic and endozoic microbiomes by culture-independent approaches that yield vast amounts of data is often in the lack of reference sequences in databases for both prokaryotes and eukaryotes. Lack of reference sequences can impair best efforts in either amplicon-based surveys or metagenomic genome assemblies. High abundance of unassigned Gammaproteobacteria in oral and cloacal microbiota (McNally et al., 2021a, 2021b; Publication **II**) indicate high unrecognized diversity, already observed in several in other reptilian microbiomes (Levin et al., 2021). The unassigned sequences overwhelmed the epizoic microbiome dataset as well, both at the 16S rRNA and 18S rRNA gene level, or in the *rbcL* gene sequences. The culture-independent approach can give a broad overview of the community and its dynamics but cannot necessarily inform on the lifestyle of the microorganisms without proper sequence assignment and knowledge on the biology of detected microbes. While in bacteria the unassigned sequences often represent yet undiscovered or uncultured taxa, in diatoms, a large proportion of unassigned sequences reflects the gaps in obtaining representative sequences of already described taxa, potential cryptic species, and general undiscovered diversity of benthic diatoms.

Based on cell morphology, however, diatom communities can still be characterized, but without insights into (semi-)cryptic diversity. Scanning electron micrographs (SEM) of microbial biofilms can visually inform on the lifestyle of diatoms and bacteria directly on the carapace rather than inferring traits from sequencing data. In SEM images like Figure 3 and in Publication **IV** it is visible that common epizoic diatoms are attached to the turtle by one pole of the cell and that they produce mucilage appendages and stalks, sometimes in dense assemblages surrounded with bacteria, under smaller pieces of keratinous scutes that can allow them to avoid drag in water and resist abrasion. Moreover, if cultured, some of the lifestyle strategies of epizoic diatoms can be examined over time in a controlled environment. For example, *Poulinea lepidochelicola* and *Achnanthes* spp. cells form colonies and branch out on stalks, or by connected cells, which lifts them above the surface they are attached to, both in culture and as observed in SEM images of the loggerhead sea turtle carapace. The more generalist or opportunist taxa like *Amphora* spp. or *Navicula* spp. will often stay close to the surface and not branch out which shows different strategies in colonizing host surfaces. The issue with morphology-based approach of analyzing diatom communities is that silica frustules can stay present in the sample even after cell death. Consequently, the diatom assemblage data obtained through morphology alone would then not represent the actual living community present in that habitat. Furthermore, morphology-based methods can be difficult and time consuming, requiring highly skilled diatom taxonomists that might not be always available. The best approach to studying peculiar and uncharacterized habitats such as the sea turtle is, when possible, to complement traditional methods, based on morphology and microscopy, with culturing and high-throughput culture-independent approaches.

4. Conclusions

The main findings of this thesis can be summarized in the following conclusions:

- Oral microbial communities of loggerhead sea turtles reflect the environment but also contain microbial taxa specific for buccal mucosa, which indicates that the enclosure environment during rehabilitation affects the oral microbiome and should be considered when deciding on rehabilitation protocols. On the other hand, the cloacal microbiota is confirmed to be stable during short term rehabilitation as described in previous studies on loggerhead fecal and cloacal microbiota.
- Samples obtained by swabbing the cloaca or oral mucosa can detect shifts in microbial community with high efficacy. This can be utilized in fieldwork for fast and relatively noninvasive sampling as opposed to distal gut swabs or feces collection that can be difficult and unpredictable in wild animals.
- Epizoic microbial communities of sea turtles harbor diverse bacterial and diatom communities with a high potential for discovery of novel microbial taxa.
- Isolation and cultivation of diatom strains from the epizoic habitat is possible and can provide DNA reference data for future metabarcoding studies. Along with better linking of metabarcoding- and morphology-based diatom community studies, additional reference sequences can lessen the need for highly skilled and trained taxonomists and expand the use of DNA-based approaches for diatom community surveys associated with marine vertebrates.
- Diatom strains isolated from a specific turtle host seem to recapitulate their host of origin both in phylogeny and in phycosphere bacterial community composition.
- Culturing diatoms in xenic cultures also enables isolation of bacterial taxa that are otherwise rare in the total turtle-associated biofilm community; possibly by providing more suitable conditions or nutrients needed by those specific bacteria.

5. Literature

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Curriculum Vitae

Klara Filek was born in Zadar (Croatia) on November 16th, 1992. She attended multiple elementary schools in Rijeka and Zadar and has spent one year in The Woodlands (Texas, USA) attending high school before coming back to Zadar and continuing her education at the Franjo Petrić Gymnasium. In 2011 she enrolled in a bachelor programme in biology at the Faculty of Science, University of Zagreb where she attained a title of bachelor in biology (univ. bacc. biol.). In 2016 she continued her education at the Uppsala University in Sweden and graduated as a Master of Science in biology (specialization in microbiology and immunology). She started working as a research assistant and has enrolled in a PhD programme in biology at the Faculty of Science, University of Zagreb in 2018. During her PhD she worked within the Croatian Science Foundation project "Loggerhead sea turtle (*Caretta caretta*) microbiome: insight into endozoic and epizoic communities (TurtleBIOME)" led by Sunčica Bosak. She published 6 scientific publications and has, in total, 25 conference proceedings with 10 active participations. She is the recipient of two research and training grants (FEMS Research and Training Grant, Assemble Plus TA), two poster awards (MLD5 and IDS2021), and two scientific photography awards (Croatian Science Foundation and the NCCR Microbiomes in Switzerland). Additionally, she was a teaching assistant in undergraduate and graduate courses of Histology and Histochemistry, Microbial Ecology, and Pelagic Microbiology. She also contributed to scientific popularization and communication events through writing several blog posts (TurtleBIOME web and International Day of Microorganisms) and organizing workshops for the Night of Biology, European Researchers' Night, and Festival of Science.

Scientific Publications

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Kanjer L, **Filek K**, Bosak S (2021) Cyanobacteria associated with sea turtles: a diversity study using metabarcoding approach. Simpozij studenata doktorskih studija PMF-a

Filek K, Kanjer L, Chaerle P, Vyverman W, Bosak S (2021) Diatom co-cultures: close encounters of *Achnanthes elongata* and *Psammodictyon* sp. Simpozij studenata doktorskih studija PMF-a

Filek K, Kanjer L, Matek A, Trotta A, Majewska R, Ashworth MP, Van de Vijver B, Bosak S (2020) A polyphasic approach to identification and characterization of epibiotic diatoms of loggerhead sea turtles in the Adriatic Sea. Simpozij studenata doktorskih studija PMF-a

Kanjer L, **Filek K**, Matek A, Majewska R, Van de Vijver B, Ashworth MP, Gračan R, Lazar B, Bosak S (2019) Diatom genus Poulinea as epibiont on Adriatic loggerhead sea turtles. 6th Croatian Botanical Symposium

Bosak S, Majewska R, **Filek K**, Van de Vijver B (2019) New observations on some sea turtle associated *Craspedostauros* species. 12th Central European Diatom Meeting

Filek K, Majewska R, Van de Vijver B, Robert K, Mucko M, Frankovich TA, Ashworth MP, Sullivan M, Bosak S (2019) Comparative analysis of the epibiotic diatom assemblage on loggerhead sea turtles in pre- and post-hospitalization period. 7th European Phycological Congress Programme

Višić H, **Filek K**, Mucko M, Trotta A, Panagopoulou A, Lukač M, Corrente M, Di Bello A, Gračan R, Bosak S (2019) Metagenomic characterization of the surface biofilm on Mediterranean loggerhead sea turtles. 7th European Phycological Congress

Van de Vijver B, **Filek K**, Gračan R, Mucko M, Višić H, Robert K, Majewska R, Frankovich TA, Ashworth MP, Bosak S (2019) The TurtleBIOME project: insight into epizoic diatom communities on loggerhead sea turtles. 24th Nordic Diatomist Meeting

Filek K, Kanjer Lucija, Matek A, Trotta A, Majewska R, Ashworth MP, Van de Vijver B, Bosak S (2019) A polyphasic approach for identification of epibiotic diatoms associated with loggerhead sea turtles in Adriatic Sea. The Molecular Life of Diatoms 5

Bosak S, Višić H, **Filek K**, Robert K, Van de Vijver, B, Trotta A, Panagopoulou Aliki, Majewska R (2019) DNA metabarcoding and morphological analyses of the diatom biofilm associated with loggerhead sea turtles in the Mediterranean Sea. The Molecular Life of Diatoms 5

Bosak S, Gračan R, Mucko M, Višić H, **Filek K**, Gobić Medica K, Mičić M, Lukač M, Basu S, Orlić S, Majewska R, Frankovich TA, Ashworth MP, Van de Vijver B (2018) Loggerhead sea turtle microbiome – TURTLEBIOME project: insight into endozoic and epizoic communities. 6th Mediterranean Conference on Marine Turtles

Workshops & Trainings

2022 – "Leadership in science" hosted by Penkala Association in Zagreb

2022 – "Unix and Shell scripting for bioinformatics" course at Physalia Courses, Berlin, Germany

2021 – „Data visualization in R with ggplot2" course at Physalia Courses, Berlin, Germany

2021 – "ISB Virtual Microbiome Series" – Course and Symposium with the Institute for Systems Biology

2021 - "Data visualization with Python" course at Physalia Courses, Berlin, Germany

2021 – "Data analysis with the Tidyverse" course at Physalia Courses, Berlin, Germany

2020 – FEMS Research and Training project: Unveiling microbial relationships within the sea turtle epizoic biofilm – diatom-bacteria interactions; Ghent University, Belgium

2020 – Assemble Plus TA project: Diatom interactions in the sea turtle epizoic biofilm – EpiDiaInter; Ghent University, Belgium

2020 – „Metagenomics, metatranscriptomics, and "Meta'omics for microbial community studies" course at Physalia Courses, Berlin, Germany

2020 – „Programming in Python “ (D460) at SRCE

2019 – „Basics of programming (Python)“ (D450) at SRCE

2019 – Assemble+ TA project: Characterization of the epizoic microbiota of the Mediterranean Sea turtles – MICROTURTLES; Stazione Zoologica Antun Dohrn, Naples, Italy

2019 May – Trainers' Training – within framework of the "Techno-Past Techno Future: European Researchers' Night" project (Projectus Group)

2018 – Scientific training at Belgian Coordinated Collections of Microorganisms Diatoms collection (BCCM/DCG) in diatom single cell isolation, culture maintenance and growth, and cryopreservation, Ghent University, Belgium

2018 Nov – "R syntax and using R in basic statistical and graphic data analysis " (S720) at SRCE

2017 – Research training at the Koskiniemi lab at the Uppsala University

Activities

2022 – EU TalentON participant: Creating solutions for the EU Mission „Restore our Oceans and Waters“ in Leiden, Netherlands

2022 – Educational workshop organizer at the science popularization event "Day and Night at the Faculty of Science"

2021 – International Day of Microorganisms Volunteer: social media and blog: "What can we learn from microbial jungles carried by sea turtles?"

2021 – Educational workshop organizer for "Science festival Zagreb 2021"

2019 – Educational Workshop organizer at European Researchers' Night in Zagreb

2019 – Technical team member at the 7th European Phycological Congress (EPC7)

2019 – Educational workshop and exhibition organizer at the science popularization event "Day and Night at the Faculty of Science" with Academy of Fine Arts

2017 – Course Information Day presenter for Master Programme in Biology; specialization: Microbiology and Immunology at Uppsala University

2014-2015 – Microbiology interest section co-leader at Biology Students Association (BIUS)

2015 – Research-educational project „Papuk 2015“ as a Microbiology section co-leader

2015 – Microbiology workshop organizer and presenter at science popularization event „Misija IRB-a: Potraga za jezgrom“ (with BIUS)

2015 – Animal Physiology Workshop organizer and presenter at the event „Night of Biology“ at the Faculty of Science, University of Zagreb

2014 – Research-educational project „Grabovača 2014“ as a member of Photography interest section

2014 – Microbiology Workshop Presenter at the science popularization event „Night of Biology“ at the Faculty of Science, University of Zagreb

Awards and Grants

2022 – EU TalentOn 2nd prize winner in the Mission Arena „Restore our Oceans and Waters“ (European Commission and Leiden2022)

2022 – Best scientific photo award in the category of "Fieldwork", chosen by the Croatian Science Foundation (CSF)

2021 – Travel award – 9th Conference of Mikrobiokosmos travel grant sponsored by International Society for Microbial Ecology

2021 – Poster award – 1st prize at the International Diatom Symposium 2021 for: Filek et al. (2021) "Loggerhead sea-turtle-associated epizoic vs. non-epizoic diatoms: isolation, identification, and co-cultivation experiments"

2020 – ASSEMBLE Plus TA Grant – European Union's Horizon 2020 research and innovation programme under grant agreement No 730984, ASSEMBLE Plus project: "Diatom interactions in the sea turtle epizoic biofilm – EpiDiaInter"

2020 – FEMS Research and Training Grant „Unveiling microbial relationships within the sea turtle epizoic biofilm – diatom-bacteria interactions“

2019 – Poster Award - 2nd prize at EMBO conference MLD5 for: Filek et al. (2019) „A polyphasic approach for identification of epibiotic diatoms associated with loggerhead sea turtles in Adriatic Sea “

2019 – Travel grant - EMBO conference 2019 The Molecular Life of Diatoms (MLD5)

2015 – Special Rector's Award for Research-educational project "Grabovača 2014."