University of Zagreb

Faculty of Science
Department of Geology

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SEDIMENT MICROBIAL COMMUNITIES IN A *Cymodocea nodosa* SEAGRASS MEADOW

DOCTORAL THESIS

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Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet
Geološki odsjek

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MIKROBNE ZAJEDNICE SEDIMENTA U LIVADI MORSKE CVJETNICE *Cymodocea nodosa*

DOKTORSKI RAD

Mentor:
dr. sc. Marino Korlević

Zagreb, 2025.

This doctoral thesis[1] was completed at the Ruđer Bošković Institute, Centre for Marine Research, Laboratory for Marine Microbial Ecology and Laboratory for Benthic Ecology, Rovinj, under the supervision of Marino Korlević, PhD, as part of the Interdisciplinary Doctoral Study in Oceanology at the University of Zagreb Faculty of Science, Department of Geology.

[1] This doctoral thesis was created using the R package bookdown (Xie, 2016, 2025) and is available on GitHub (https://github.com/MicrobesRovinj/Markovski_DoctoralThesis_2025).

SUPERVISOR INFORMATION

Marino Korlević, PhD, graduated in biology from the University of Zagreb Faculty of Science in 2009. In 2015, he completed the Interdisciplinary Doctoral Study in Oceanology at the University of Zagreb Faculty of Science by defending his doctoral thesis “In-depth analysis of the Adriatic Sea bacterial diversity”. He is currently a senior research associate in the Laboratory for Benthic Ecology, Centre for Marine Research, Ruđer Bošković Institute, Rovinj, Croatia.

He has been principal investigator of one project and an associate on 11. He is the first author of three articles, shared co-first author of one, corresponding author of two, first and corresponding author of three, and co-author of 10 scientific articles. Articles published in 2015, 2018, 2020, 2021, and 2022 received the Ruđer Bošković Institute Annual Award. He received the 2016 Annual Award for Young Scientists of the Croatian Microbiological Society, has participated in various international scientific conferences with oral and poster presentations, and is a member of the Croatian Microbiological Society. His main research interest is marine microbial ecology, especially the interactions between microbes and marine macrophytes.

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Doctoral thesis

SEDIMENT MICROBIAL COMMUNITIES IN A *Cymodocea nodosa* SEAGRASS MEADOW

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Abstract

The presence of seagrass in marine environments influences surface sediments and creates specific environmental conditions that support diverse and abundant sediment microbial communities. However, the decline of seagrass meadows of various species has been documented worldwide, including that of *Cymodocea nodosa*, a common seagrass species in the Mediterranean Sea. In the Bay of Saline, located in the northern Adriatic Sea, a decline of a *Cymodocea nodosa* meadow was observed during its monthly status assessment from July 2017 to October 2018. To determine the response of sediment microbes to seagrass meadow decline, surface sediments were collected from two sites during that period, one without vegetation and one with the declining seagrass meadow. The microbial response was assessed by determining community composition and metabolic profile. In addition, the environmental conditions in the sediment were assessed and changes after the onset of meadow decline were detected, such as an accumulation of hydrogen sulphide. Despite these changes, no clear temporal succession of the microbial community in the sediment was observed. Instead, the communities were stratified by sediment depth and exhibited distinct community composition between sites. The bacterial community was mainly comprised of *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota*, and *Campylobacterota*, while the archaeal community was dominated by *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota*, and *Asgardarchaeota*. Functional analysis based on microbial profiles revealed that these communities degrade complex sugars and proteins, producing acetate, formate, and ethanol by fermentation, thereby supporting dissimilatory sulphate reduction. Before the seagrass decline, notable differences in the metabolic profiles were observed between sites. However, during the decline, the profiles converged, with those of the nonvegetated site resembling the ones of the *Cymodocea nodosa* meadow sediment. These results indicate that while the microbial communities in the seagrass meadow sediments remain stable in composition and function during the decline, adjacent communities shift their metabolic profiles, highlighting the broader ecological influence of the decline of seagrass meadows.
(117 pages, 8 figures, 217 references, 3 appendices, original in English)

Keywords: sediment microbial communities, microbial community composition, microbial metabolic profile, *Cymodocea nodosa*, seagrass meadow decline, northern Adriatic Sea

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MIKROBNE ZAJEDNICE SEDIMENTA U LIVADI MORSKE CVJETNICE *Cymodocea nodosa*

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Sažetak

Prisutnost morskih cvjetnica u morskom okolišu utječe na površinske sedimente stvarajući specifične uvjete pogodne za raznolike mikrobne zajednice velikih brojnosti. No, širom svijeta zabilježeno je propadanje livada morskih cvjetnica raznih vrsta, uključujući livade vrste *Cymodocea nodosa*, česte morske cvjetnice u Sredozemnom moru. Propadanje livade ove vrste utvrđeno je i u uvali Saline, smještenoj u sjevernom Jadranskom moru, tijekom redovitog postupka procjene stanja livade morske cvjetnice provedenog od srpnja 2017. do listopada 2018. Tijekom ovog razdoblja uzorkovan je površinski sediment na dvije postaje, jednoj bez vegetacije i jednoj s propadajućom livadom, kako bi se odredio odgovor mikrobnih zajednica sedimenta na propadanje livade morske cvjetnice. Odgovor mikrobnih zajednica utvrđen je određivanjem njihovog sastava i metaboličkog profila. Također, određeni su uvjeti okoliša u sedimentu te su zabilježene promjene uvjeta nakon početka propadanja livade, poput nakupljanja sumporovodika. Unatoč ovim promjenama, nije utvrđena jasna vremenska sukcesija mikrobne zajednice sedimenta. Umjesto toga, uočene su promjene u sastavu zajednica u odnosu na dubinu sedimenta i između postaja. Bakterijsku zajednicu činila su prvenstveno koljena *Desulfobacterota*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota* i *Campylobacterota* te razred *Gammaproteobacteria*, dok su zajednicom arheja prevladavala koljena *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota* i *Asgardarchaeota*. Funkcionalna analiza temeljena na mikrobnim profilima otkrila je da ove zajednice razgrađuju složene šećere i proteine proizvodeći acetat, format i etanol fermentacijom, čime podržavaju disimilatornu redukciju sulfata. Prije propadanja livade uočene su primjetne razlike između postaja u metaboličkim profilima. Međutim, metabolički su profili tijekom propadanja postali vrlo slični, pri čemu su profili nevegetirane postaje počeli nalikovati sedimentu s vrstom *Cymodocea nodosa*. Ovi rezultati ukazuju da, iako sastav i funkcija mikrobnih zajednica u sedimentu livade morske cvjetnice ostaju nepromijenjeni tijekom propadanja, okolne zajednice pokazuju promjene metaboličkog profila naglašavajući širi ekološki utjecaj propadanja livada morskih cvjetnica.
(117 stranica, 8 slika, 217 literaturnih navoda, 3 priloga, jezik izvornika: engleski)

Ključne riječi: mikrobne zajednice sedimenta, sastav mikrobne zajednice, metabolički profil mikroorganizama, *Cymodocea nodosa*, propadanje livade morske cvjetnice, sjeverno Jadransko more

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PROŠIRENI SAŽETAK (EXTENDED ABSTRACT)

Morske cvjetnice nastanjuju i oblikuju plitka obalna sedimentna dna (Duarte, 2002). Njihove livade pohranjuju i time uklanjaju ugljik iz okoliša, čineći ove ekosustave važnim dionicima ublažavanja klimatskih promjena (Duarte, Middelburg i sur., 2005; Duarte i sur., 2013; Kennedy i sur., 2010). Međutim, zbog sve veće izloženosti antropogenim pritiscima uočen je gubitak livada morskih cvjetnica na globalnoj razini (Dunic i sur., 2021; Orth i sur., 2006; Waycott i sur., 2009). Prisutnost ovih biljaka u obalnim sedimentima stvara specifične uvjete okoliša koji podržavaju veliki broj stanica raznolikih i aktivnih mikrobnih zajednica (Duarte, Holmer i sur., 2005). Većina istraživanja mikrobnih zajednica morskih cvjetnica vezana je za epifite i rizosferu (Cúcio i sur., 2016; Korlević i sur., 2021), dok su istraživanja zajednica u sedimentima rijetka. Posljedično su i saznanja o dinamici i utjecaju livade na mikrobne zajednice u sedimentima ograničena. U ovom okolišu, mikrobne zajednice pospješuju oslobađanje i transformaciju nutrijenata tijekom razgradnje organske tvari, podržavajući time rast i primarnu produkciju biljaka. Osim ovakvih povoljnih učinaka, mikrobna aktivnost može imati i negativnih utjecaja na biljke, poput oslobađanja sumporovodika uslijed visokih stopa redukcije sulfata (Duarte, Holmer i sur., 2005). Za rast i razvoj morskih cvjetnica bitno je da povoljni učinci mikrobne aktivnosti, poput oslobađanja i transformacije nutrijenata, nadvladavaju nepovoljne, kao što je proizvodnja sumporovodika (Duarte, Holmer i sur., 2005). Prisutnost livada morskih cvjetnica i mikrobna remineralizacija organske tvari u sedimentu bitni su čimbenici u biogeokemijskom ciklusu ugljika (Duarte, Holmer i sur., 2005; Duarte, Middelburg i sur., 2005). Zbog navedenog, važno je unaprijediti razumijevanje međudjelovanja morskih cvjetnica i svih njihovih mikrobnih zajednica.

Morska cvjetnica *Cymodocea nodosa* rasprostranjena je širom Sredozemnog mora (Green i Short, 2003; Short i sur., 2007). U skladu s podacima o propadanju livada morskih cvjetnica širom svijeta uočeno je i propadanje livada vrste *Cymodocea nodosa* u raznim obalnim područjima (Barsanti i sur., 2007; Boudouresque i sur., 2009; Pérez-Ruzafa i sur., 2006; Shili i sur., 2002), uključujući i sjeverno Jadransko more (Green i Short, 2003; Orlando-Bonaca i sur., 2015, 2019). Zbog gubitka ovih vrijednih staništa te ograničenih saznanja o mikrobnim zajednicama sedimenata morskih cvjetnica, cilj istraživanja bio je utvrditi sastav i raznolikost mikrobnih zajednica u sedimentu livade vrste *Cymodocea nodosa* primjenom sekvenciranja markerskog gena te odrediti funkcionalnu raznolikost i dinamiku zajednica zajedničkom primjenom metoda metagenomike i metaproteomike.

Mjesečno uzorkovanje vrste *Cymodocea nodosa*, vodenog stupca i sedimenta provedeno je u uvali Saline, smještenoj sjeverozapadno od Rovinja, u sjevernom Jadranskom moru, u razdoblju od srpnja 2017. do listopada 2018. Procjena stanja ove morske cvjetnice obavljena je uzorkovanjem unutar livade metodom probnih kvadrata te biometrijskom analizom izdanaka i rizoma s korijenjem. Uzorci jezgara površinskog sedimenta prikupljeni su na dvije postaje, jednoj bez vegetacije i jednoj koja je na početku uzorkovanja sadržavala livadu vrste *Cymodocea nodosa*. Kako bi se utvrdila dinamika uvjeta u okolišu, određen je niz fizikalno-kemijskih parametara

u vodenom stupcu i u prikupljenim jezgrama sedimenta. Prije izdvajanja DNA i proteina, svaka je jezgra sedimenta izrezana u četiri odsječka visine 1 cm. Izdvajanje ukupne DNA provedeno je primjenom smjese organskih otapala prema J. Zhou i sur. (1996), uz modifikacije koje su opisali Pjevac i sur. (2018). Sastav i raznolikost mikrobne zajednice utvrđeni su sekvenciranjem regije V4 gena za 16S rRNA platformom Illumina MiSeq, dok su funkcionalna raznolikost i dinamika zajednica određene iz metaboličkih profila, proizašlih iz zajedničke analize metagenoma i metaproteoma. Metagenomi su sekvencirani analizom ukupne DNA iz odabranih uzoraka platformom Illumina NovaSeq 6000. Ukupni su proteini izdvojeni izravnom lizom mikrobnih stanica inkubacijom sedimenta u lužnatom puferu s visokom koncentracijom natrijevog dodecilsulfata i naknadnim taloženjem s trikloroctenom kiselinom (Chourey i sur., 2010). Sastav metaproteoma, te posljedično i metabolički profil zajednica, određeni su primjenom tandemske masene spektrometrije te identifikacijom proteina usporedbom dobivenih masenih spektara s aminokiselinskim sljedovima proizašlim iz analize metagenoma.

Na početku istraživanja, uvalu Saline naseljavala je morska cvjetnica vrste *Cymodocea nodosa*, čija se prostrana livada protezala od jugozapadnog dijela uvale, dubine 1,5 m, prema središnjem dijelu, dubine 4 m. Na kraju istraživanja, preostalo je svega nekoliko vrlo malih područja naseljenih s vrstom *Cymodocea nodosa*, uz samu obalu u obliku uskih pruga. Biometrijska analiza ukazala je na normalan ciklus rasta izdanaka i rizoma s korijenjem od srpnja 2017. do ožujka 2018. Najviše vrijednosti biometrijskih parametara izdanaka zabilježene su u listopadu 2017., nakon čega je, u studenom 2017., utvrđen snažan pad vrijednosti, u skladu s uobičajenim smanjenim rastom morske cvjetnice zimi (Agostini i sur., 2003; Cancemi i sur., 2002; Najdek i sur., 2020; Terrados i Ros, 1992; Zavodnik i sur., 1998). Međutim, na proljeće 2018., izostao je ponovni rast morske cvjetnice i oporavak livade. Za razliku od izdanaka, biomasa rizoma s korijenjem bila je stabilna do ožujka 2018., kada je uočen njezin pad, koji se nastavio do kraja istraživanja, u skladu s uočenim propadanjem livade. Proces propadanja najvjerojatnije je potaknulo smanjenje dostupnosti svjetla, i posljedično pad fotosintetske aktivnosti, zbog povećane mutnoće mora uzrokovane snažnijim terigenim donosom i resuspenzijom sedimenta. Na snažniji terigeni donos upućivalo je smanjenje saliniteta i povećanje koncentracije partikularne tvari u vodenom stupcu. Pad fotosintetske aktivnosti prouzročio je i pad oksidacijske sposobnosti korijenja, bitnog mehanizama zaštite morske cvjetnice od toksičnog djelovanja sumporovodika. Naime, nakon početka propadanja livade, zabilježeno je povećanje koncentracije i proširenje zone nakupljanja sumporovodika.

Iako su se uvjeti okoliša u sedimentu tijekom istraživanog razdoblja, u kojem je zabilježeno i propadanje livade, mijenjali, analiza raznolikosti zasnovana na sekvenciranju markerskog gena nije otkrila jasnu vremensku sukcesiju mikrobne zajednice sedimenta. Umjesto toga, zabilježene su razlike u zajednicama u odnosu na dubinu sedimenta te između postaje bez vegetacije i postaje koja je na početku istraživanja sadržavala livadu morske cvjetnice. Taksonomska analiza sastava mikrobnih zajednica otkrila je prevladavanje nukleotidnih sljedova domene *Bacteria* nad onima domene *Archaea*. Bakterijsku zajednicu činila su prvenstveno koljena *Desulfobacterota*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota* i *Campylobacterota* te razred *Gammaproteobacteria*, dok su zajednicom arheja prevladavala koljena *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota* i *Asgardarchaeota*. Analizom dinamike taksonomskih skupina utvrđen je pad udjela s dubinom sedimenta domene *Bacteria*, koljena *Bacteroidota* i razreda *Gammaproteobacteria*, dok je za domenu *Archaea* te koljena *Chloroflexi* i *Thermoplasmatota* uočen suprotni trend rasta. Nadalje, udio koljena *Bacteroidota* i *Campylobacterota* bio je veći na postaji koja je na početku istraživanja sadržavala livadu, za razliku od koljena *Crenarchaeota* i razreda *Gammaproteobacteria*, čiji je udio bio veći na postaji bez vegetacije. Uočena vremenska stabilnost zajednica tijekom propadanja livade upućivala je na

potrebu provjere odražavanja ovog obrasca i u funkcionalnoj raznolikosti i dinamici mikrobnih zajednica sedimenta.

Metabolički profili mikrobnih zajednica, proizašli iz zajedničke analize metagenoma i metaproteoma, su za razliku od sastava i raznolikosti zajednica ukazali na promjene tijekom propadanja livade. Prije početka propadanja, uočene su razlike u bogatstvu i indeksu raznolikosti proteina između postaje bez vegetacije i postaje s livadom morske cvjetnice. Ove su razlike bile izraženije u dubljim slojevima sedimenta. Tijekom propadanja, bogatstvo i indeksi raznolikosti proteina u sedimentu bez vegetacije postali su slični onima u sedimentu s propadajućom livadom, dok u samom sedimentu s propadajućom livadom nisu uočene vremenske promjene ovih parametara. U skladu s ovim podacima, vremenske promjene strukture metaboličkog profila uočene su isključivo na postaji bez vegetacije te su, također, bile izraženije u dubljim slojevima sedimenta.

Metaboličkim su profilima mikrobnih zajednica prevladavali proteini vezani uz proizvodnju i pretvorbu energije. Kako bi se utvrdio sastav proteina uključenih u razgradnju organske tvari i njihov odgovor na propadanje livade, praćena je dinamika hidrolitičkih enzima, ABC-transportera (engl. *ATP-binding cassette*), enzima vezanih za razne fermentacijske procese te proteina uključenih u disimilatornu redukciju sulfata. Među hidrolitičkim enzimima najzastupljeniji su bili enzimi za razgradnju ugljikohidrata i proteina. To je bilo u skladu s prisutnošću ABC-transportera, od kojih su najzastupljeniji bili specifični za šećere i aminokiseline, što naglašava važnost ugljikohidrata i proteina za mikrobnu zajednicu u sedimentu. Analiza enzima vezanih za razne fermentacijske procese otkrila je najveću zastupljenost proteina uključenih u fermentaciju acetata, formata i etanola, važnih fermentacijskih produkata u morskim sedimentima. Među proteinima za proizvodnju i pretvorbu energije, jedni od najzastupljenijih bili su enzimi uključeni u disimilatornu redukciju sulfata, odražavajući važnost ovog procesa za mineralizaciju organske tvari u morskim sedimentima. Tijekom propadanja livade, dinamika ABC-transportera, enzima vezanih za fermentacijske procese te proteina uključenih u disimilatornu redukciju sulfata uglavnom je pratila već opisane promjene cjelokupnog metaboličkog profila.

Rezultati istraživanja sastava zajednica u sedimentu morske cvjetnice *Cymodocea nodosa* upotpunjuju opis mikrobnih zajednica povezanih s ovom važnom morskom cvjetnicom Sredozemnog mora. Također, zajednička primjena metoda metagenomike i metaproteomike u određivanju metaboličkog profila zajednica čini iskorak u istraživanju, ne samo mikrobnih zajednica sedimenta morskih cvjetnica, već i općenito zajednica površinskog obalnog sedimenta. Praćenje stanja morske cvjetnice *Cymodocea nodosa* u uvali Saline, tijekom kojeg je zabilježeno propadanje livade, te određivanje sastava i funkcije mikrobnih zajednica u sedimentu pružilo je posebnu priliku za istraživanje odgovora zajednica na propadanje livade morske cvjetnice. Zaključno, rezultati ukazuju na izostanak utjecaja propadanja livade na sastav mikrobnih zajednica sedimenta. Međutim, funkcionalna analiza utvrdila je promjene metaboličkog profila zajednica tijekom propadanja livade, isključivo u okolnom sedimentu bez vegetacije i s izraženijim promjenama u dubljim slojevima sedimenta.

CONTENTS

LIST OF SCIENTIFIC ARTICLES

1. Najdek, M., Korlević, M., Paliaga, P., **Markovski**, **M.**, Ivančić, I., Iveša, Lj., Felja, I., and Herndl, G. J. (2020). Dynamics of environmental conditions during the decline of a *Cymodocea nodosa* meadow. *Biogeosciences*, *17*(12), 3299–3315. https://doi.org/10.5194/bg-17-3299-2020
   Contribution: Investigation and writing—review and editing.

2. **Markovski**, **M.**, Najdek, M., Herndl, G. J., and Korlević, M. (2022). Compositional stability of sediment microbial communities during a seagrass meadow decline. *Frontiers in Marine Science*, *9*, 966070. https://doi.org/10.3389/fmars.2022.966070
   Contribution: Sampling, laboratory and data analyses, writing—original draft, review and editing.

3. **Markovski**, **M.**, Najdek, M., Zhao, Z., Herndl, G. J., and Korlević, M. (2025). Shift in the metabolic profile of sediment microbial communities during seagrass decline. *Environmental Microbiome*, *20*(1), 93. https://doi.org/10.1186/s40793-025-00750-1
   Contribution: Sampling, laboratory and data analyses, writing—original draft, review and editing.

1 INTRODUCTION

Marine sediments cover approximately 70% of the Earth's surface. In these habitats, biomass is primarily constituted by microbes, mainly bacteria and archaea (Nealson, 1997). The estimated number of microbial cells in marine sediments ranges from 2.9×10^{29} to 5.4×10^{29} (Kallmeyer et al., 2012; Parkes et al., 2014). This abundance is approximately equivalent to that found in seawater and soil (Kallmeyer et al., 2012). Microbial cells are ubiquitous in the sediments of all oceans (Kallmeyer et al., 2012; Figure 1), extending from the sediment surface to several kilometres below the seafloor (Inagaki et al., 2015). Generally, the abundance of prokaryotes in the sediment decreases with increasing sediment depth and age (Kallmeyer et al., 2012). The diverse environmental conditions present in different sediments contribute to a great diversity of prokaryotic life in these habitats (Hoshino et al., 2020; Li et al., 2020; Parkes et al., 2014; Tapilatu et al., 2024; C.-J. Zhang et al., 2019). The primary factors influencing the composition of bacterial and archaeal communities in marine sediments are oxygen availability and sediment depth, both of which are influenced by varying sedimentation rates from the water column. These factors affect the formation of specific microbial communities, generally distinguishing deep-sea oxic sediment communities from more anoxic communities in coastal areas (Hoshino et al., 2020; Orsi, 2018). Besides these general trends, other habitat-specific factors further contribute to the differentiation of communities in deep-sea and coastal sediments. For example, environments such as hydrothermal vents (Cerqueira et al., 2018) and cold seeps (Y. Zhang et al., 2012) in the deep sea, or seagrass meadows (Duarte, Holmer, et al., 2005) in coastal sediments, exhibit unique characteristics that shape specific microbial community structures.

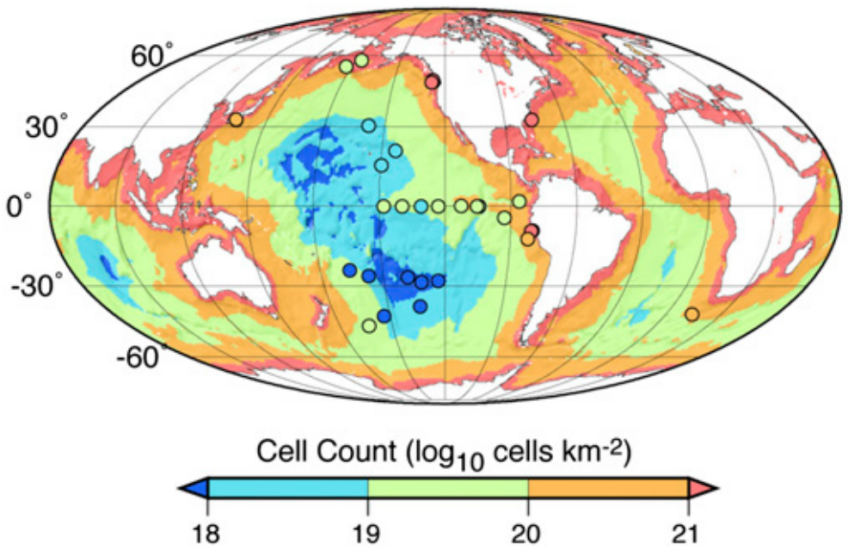

Figure 1. Global distribution of the integrated number of microbial cells in marine sediments. The colours of the dots indicate the number of cells calculated for specific sites. From Kallmeyer et al. (2012).

1.1 Marine sediments: a habitat for microbes

Sedimentation of organic matter from the water column serves as the primary source of electron donors and thus energy for microbes in marine sediments (Jørgensen et al., 2019; Kallmeyer et al., 2012; Orsi, 2018). This process is influenced by several factors, such as the productivity and depth of the overlying water column, as well as terrestrial input (Berger and Wefer, 1990; Jahnke, 1996). High sedimentation rates create anoxic sediments rich in organic matter (Bowles et al., 2014; Jørgensen, 1982), while low rates enable oxygen to penetrate deeper into the sediment (D'Hondt et al., 2009; Røy et al., 2012). The sedimentation rate is closely linked to the productivity of the water column, which tends to be higher on the continental shelf compared to the open ocean (Orsi, 2018). Thus, anoxic conditions in continental shelf sediments develop close to the sediment surface (Bowles et al., 2014), whereas in the open ocean, where both productivity and sedimentation rates are lower, oxygen-rich zones can extend up to 30 m below the sediment surface (Røy et al., 2012).

The composition of the microbial community varies greatly between oxic and anoxic sediments, reflecting the distinct environmental conditions present in these two types of habitats (Hoshino et al., 2020; Orsi, 2018). In oxic sediments, microbial growth is typically limited by the availability of organic matter. In contrast, microbes in anoxic sediments are primarily regulated by the concentrations of terminal electron acceptors, most commonly sulphate. The abundance of microbial cells is markedly greater in anoxic sediments than in their oxic counterparts, with differences reaching up to eight orders of magnitude (Jørgensen and Marshall, 2016; Kallmeyer et al., 2012). For instance, in coastal anoxic mud, microbial cell counts can be as high as 10^{10} cells cm^{-3}, whereas oxic clay found in deep-sea regions, which is poor in organic matter, averages only about 10^{2} cells cm^{-3} (Jørgensen and Marshall, 2016). Global analyses indicate that in oxic sediments, cell abundance drops below 10^{7} cells g^{-1} just 0.1 m beneath the seafloor. In contrast, even at 1 m beneath the seafloor in anoxic sediments, the typical concentration of cells ranges from 10^{7} to 10^{9} cells g^{-1} (Kallmeyer et al., 2012; Orsi, 2018). The highest microbial abundances have been recorded in the shallow, eutrophic regions of the Baltic Sea, while the central areas of the North and South Pacific Ocean gyres exhibit some of the lowest abundances (Jørgensen and Marshall, 2016). Here, at sediment depths greater than 20 m, microbial counts fall below measurable levels (D'Hondt et al., 2009, 2015; Røy et al., 2012). The exceptionally low abundances observed in deep-sea sediments of the great ocean gyres are attributed to extremely low sedimentation rates. These rates allow deep oxygen penetration and extensive mineralisation of organic matter prior to deeper burial (D'Hondt et al., 2009, 2015; Orsi, 2018).

Sediments present a challenging environment for microbial survival due to marked changes in environmental conditions throughout the sediment core. Nevertheless, diverse prokaryotic communities have been identified from the sediment surface down to depths of approximately 2.5 km (Inagaki et al., 2015). The vertical structure of the sediment core reflects the sedimentation of fresh material from the water column to the seabed and its burial over time (Orsi, 2018; Petro et al., 2017). Microbial cells from the water column or the seabed surface are buried alongside this material, gradually becoming separated from the sediment surface while being subjected to changing environmental conditions (Petro et al., 2017). As sediment age and depth increase, microbes encounter an important challenge: the reduction of available organic matter (Jørgensen and Marshall, 2016; Petro et al., 2017). More labile organic compounds are depleted in shallower sediment layers, leading to impoverished organic matter in older and deeper sediments (Jørgensen and Marshall, 2016; Middelburg, 1989; Petro et al., 2017). Consequently, the composition of organic matter shifts towards a higher content of refractory matter, which limits energy sources and results in slower microbial activity in deeper sediment layers (Jørgensen and Marshall, 2016; Orsi, 2018; Petro et al., 2017). Studies have shown that microbial activity decreases by two to three orders of magnitude in sediment layers just a few meters deep compared to surface sediment (Petro et al., 2017; Røy et al., 2012). Additionally, a corresponding decline in cell abundance and an increase in generation time has been observed at the same depth (Kallmeyer et al., 2012; Starnawski et al., 2017; Figure 2). Microbial activity, cell abundance, and generation time can all be linked to sediment age and the declining availability of organic matter (Petro et al., 2017; Starnawski et al., 2017).

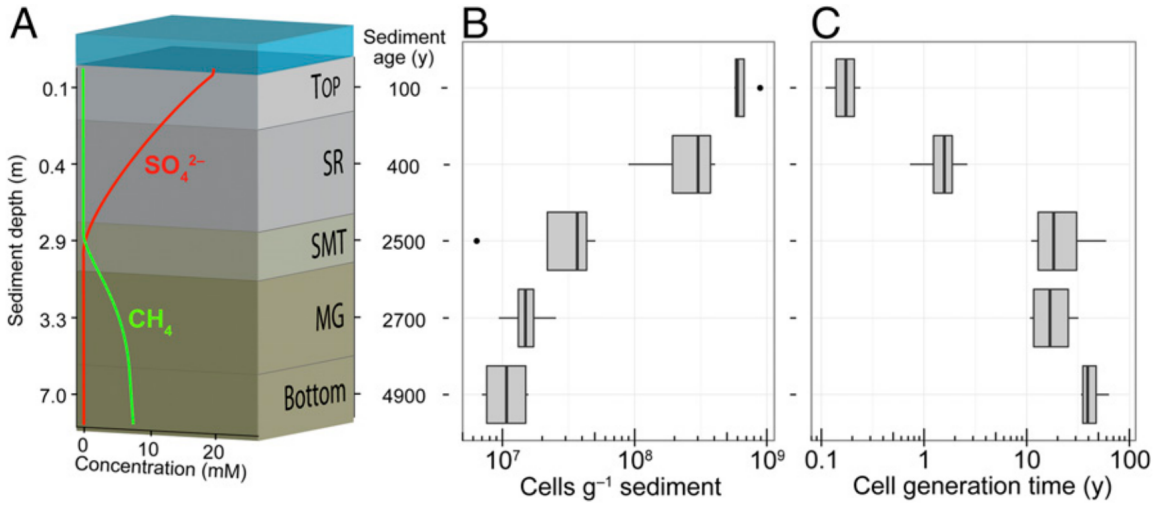

Figure 2. The vertical biogeochemical zonation (A) and the corresponding distribution of microbial cell abundances (B) and generation times (C) in sediments. Biogeochemical zones: Top, surface sediment; SR, upper sulphate-rich sediment; SMT, sulphate–methane transition zone; MG, methanogenic sediment; Bottom, deep methanogenic zone. From Starnawski et al. (2017).

The sediment column is characterised not only by a decline in the availability of organic matter but also by changes in biogeochemical zones as sediment depth increases (Starnawski et al., 2017; Figure 2). These changes are accompanied by variations in specific terminal electron acceptors, with each layer identified by the most energetically favourable and available acceptor of organic matter mineralisation (Canfield et al., 1993; Froelich et al., 1979; Thamdrup et al., 1994). Starting from the top, the biogeochemical zones include an oxic layer, where oxygen (O_2) serves as the terminal electron acceptor; a zone of nitrate (NO_3^-) reduction; zones of manganese [Mn(IV)] and iron [Fe(III)] oxides reduction; a zone of sulphate (SO_4^{2-}) reduction; and the deepest methanogenic layer, where carbon dioxide (CO_2) is converted to methane (CH_4; Orcutt et al., 2011). This biogeochemical stratification can be disrupted in the surface layer due to bioturbation (Kristensen, 2001), typically extending to a depth of about 10 ± 5 cm (Boudreau, 1998; Meysman et al., 2006; Petro et al., 2017). This activity generates a layer of considerable biogeochemical heterogeneity, allowing for localised deeper oxygen penetration (Boudreau, 1998; Meysman et al., 2006). In addition, bioturbation allows for deeper transport of organic matter, which leads to increased microbial activity and dynamics in this layer (Petro et al., 2017). Beneath the bioturbation zone, environmental conditions stabilise, organic matter sources diminish, and diffusion becomes the primary process (Jørgensen and Marshall, 2016; Petro et al., 2017). Here, sulphate reduction is the main process of organic matter mineralisation (Jørgensen, 1982; Petro et al., 2017). This layer is separated from the deeper methanogenic region by a narrow sulphate-methane transition (SMT) zone characterised by sulphate-dependent methane oxidation and by a peak in the sulphate reduction rate (Leloup et al., 2007; Petro et al., 2017; Thomsen et al., 2001). The deeper layers, lacking sulphate, primarily rely on methanogenesis for mineralisation (Petro et al., 2017).

1.2 Molecular techniques for studying sediment microbial communities

Traditionally, microbial communities were primarily described through cultivation techniques (Amann et al., 1995). However, it is now well established that less than 1% of microorganisms can be cultured in laboratory settings (Ferguson et al., 1984; Jannasch and Jones, 1959), a phenomenon preventing the accurate description of microbial populations known as the “great plate count anomaly” (Amann et al., 1995; Staley and Konopka, 1985). This discrepancy is attributed to factors such as the selective nature of culture media, the presence of inactive cells, and the aggregation of bacteria (Jannasch and Jones, 1959). To address the

culture-dependent limitations, Pace et al. (1986) pioneered molecular microbial ecology by cloning DNA extracted from environmental samples and by sequencing the 16S rRNA gene. This innovative approach allowed for the identification of microorganisms without the need for cultivation, enabling studies of microbial ecology based on the molecular phylogeny of the 16S rRNA gene (Rappé and Giovannoni, 2003; Woese et al., 1990; Woese and Fox, 1977). As a result, many novel taxonomic groups were described (Okabe et al., 2010). The assessment of microbial communities in coastal marine sediments began with the study by Gray and Herwig (1996), which reported the phylogenetic composition of a bacterial community within marine nearshore sediments. Nowadays, determining the structure of the microbial community typically involves extracting environmental DNA, amplifying a fragment of the 16S rRNA gene using polymerase chain reaction (PCR), and sequencing with various next-generation sequencing (NGS) technologies, usually the Illumina MiSeq platform (Hoshino et al., 2020; Kozich et al., 2013). Although this methodological approach has revolutionised marine microbial ecology, focusing on a single gene or gene fragment allows for the estimation of only the microbial community composition and dynamics. To characterise the potential and active metabolic processes of microbes, a comprehensive identification and quantification of all present genes and their products is essential (W. Zhang et al., 2010).

A commonly recommended approach to assess the potential metabolic processes carried out by microbes in the environment is metagenomics (Heidelberg et al., 2010). In contrast to traditional genomics, which focuses on the genomic DNA of a single organism, metagenomics explores the collective genomic information of all organisms present in a community (Quince et al., 2017). The typical metagenomic method used in studies is shotgun metagenomics, in which total DNA is isolated from the environmental sample and sequenced in an untargeted (“shotgun”) approach using high-throughput sequencing techniques (Heidelberg et al., 2010; Quince et al., 2017). Usually, total DNA is isolated from the environmental sample and used for metagenomic library preparation and sequencing (Quince et al., 2017). The nucleotide sequences obtained are then assembled into longer sequences, known as contigs, often using bioinformatic tools based on the de Bruijn graph (Quince et al., 2017; Simpson and Pop, 2015). The assembled reads are then compared with existing databases for taxonomic classification and functional annotation (Quince et al., 2017; Simon and Daniel, 2011). An important advantage of this approach is that it eliminates potential biases associated with amplification of specific DNA fragments by PCR, such as limited primer coverage (Apprill et al., 2015; Parada et al., 2016; Simon and Daniel, 2011). Indeed, direct sequencing of total DNA has been shown to be the most accurate method for determining taxonomic composition, even though it requires more resources for

sequencing and computation (Simon and Daniel, 2011; von Mering et al., 2007). Metagenomics is now widely used to characterise microbial diversity and metabolic potential in both surface and deep sediments (Marshall et al., 2018; Moore et al., 2020). However, to comprehensively study the processes carried out by microbial communities, it is essential to integrate other omics approaches that focus on gene products and active processes, such as metaproteomics.

Wilmes and Bond (2004) proposed the term "metaproteomics" to describe the large-scale characterisation of the entire protein composition of a microbial community at a given time. The methods used to identify these proteins are based on approaches originally developed for proteomic analyses (Van Den Bossche et al., 2025). These methods typically involve enzymatic digestion of the isolated proteins, usually with trypsin, separation of the peptides using liquid chromatography, and peptide analysis by tandem mass spectrometry (LC-MS/MS; Van Den Bossche et al., 2025; D.-Z. Wang et al., 2014). A suitable approach for the enzymatic digestion of proteins isolated from environmental samples is filter-aided sample preparation (FASP), which enables the removal of many contaminating compounds that are usually present in environments such as soils and sediments and are often isolated together with proteins (Van Den Bossche et al., 2025; Wiśniewski et al., 2009). In the final bioinformatic analysis step, the peptide spectra are matched with theoretical spectra from protein sequence databases (Saito et al., 2019; Van Den Bossche et al., 2025). This process enables both the identification and quantification of peptides and their corresponding proteins (Van Den Bossche et al., 2025). Determining the full spectrum of proteins produced by microbial communities poses a number of challenges, mainly due to the complexity of metaproteomes (Wilmes et al., 2015). For example, the wide range of abundance of proteins in a metaproteomic sample and the low coverage of protein sequence databases pose limitations to successful protein identification (Saito et al., 2019; D.-Z. Wang et al., 2014; Wilmes et al., 2015). Despite these obstacles, metaproteomics holds great promise in linking genetic potential to phenotype and thus improving our understanding of the processes carried out by microbial communities (Wilmes et al., 2015). In addition, metaproteomic techniques have been successfully applied in various types of marine sediments, e.g. cold seeps (Glass et al., 2014; Stokke et al., 2012), diffuse hydrothermal venting (Urich et al., 2014), mudflat aquaculture (Lin et al., 2015), and chronically petroleum-polluted (Bargiela et al., 2015) sediments.

1.3 Composition of sediment microbial communities

Using the previously described molecular techniques based on 16S rRNA gene sequencing, a comprehensive overview of the taxonomic composition of microbial communities in marine sediments was obtained. Large differences were observed between oxic and anoxic sediments (Hoshino et al., 2020; Figure 3), which could be related to the environmental differences that characterise these types of sediments (Section 1.1). The data showed that the proportion of bacteria in marine sediments is generally higher than that of archaea and that the contribution of archaea is more pronounced in low-oxygen sediments than in those with higher oxygen content (Hoshino et al., 2020; Hoshino and Inagaki, 2019). Within the archaeal community, the phyla *Asgardaeota* and *Crenarchaeota* characterise the anoxic sediments, while the phylum *Thaumarchaeota* dominates in oxic sediments (Durbin and Teske, 2011; Hoshino et al., 2020; Lauer et al., 2016). Furthermore, within the bacterial community, members of *Atribacteria*, *Chloroflexi*, and *Planctomycetes* are predominant in low-oxygen sediments, while *Alphaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes* dominate in sediments with higher oxygen content (Durbin and Teske, 2011; Hoshino et al., 2020).

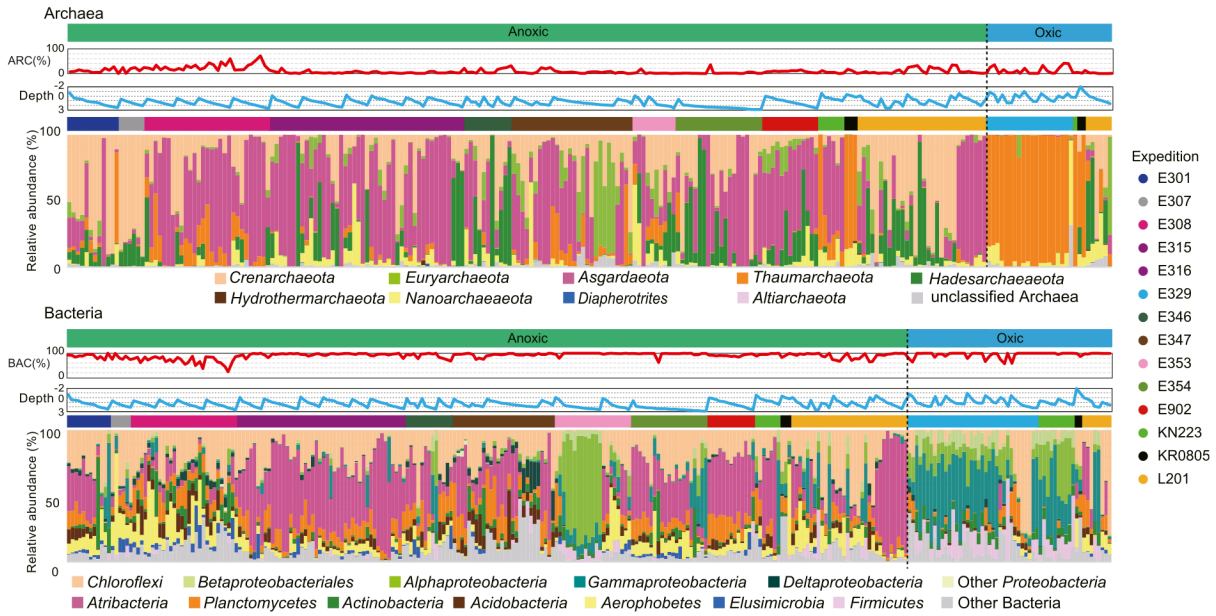

Figure 3. Differences in taxonomic composition between microbial communities in oxic and anoxic marine sediments. The composition was determined using universal primers targeting both archaea and bacteria. The relative abundance of archaeal (ARC) and bacterial (BAC) sequences in each sample is represented by a red line, while sediment depth is shown on a logarithmic scale with a light blue line. Sampling expeditions are colour-coded above the corresponding bar. From Hoshino et al. (2020).

In addition to the differences between oxic and anoxic sediments, major changes in microbial taxonomic composition and richness are generally observed with increasing sediment

depth (Chen et al., 2017; Hoshino et al., 2020; Kirkpatrick et al., 2019; Petro et al., 2017; Walsh et al., 2016). These changes could also be related to shifts in environmental conditions with increasing sediment depth, as in the case of differences between oxic and anoxic sediments (Section 1.1). In general, bacteria dominate over archaea in surface sediments, while their abundance becomes similar with increasing sediment depth (Chen et al., 2017). The higher proportion of archaea in deeper sediments is probably related to their adaptability to limited sources of organic matter (Hoehler and Jørgensen, 2013). Interestingly, differences in the change in archaeal and bacterial richness with increasing sediment depth were observed between oxic and anoxic sediments. In anoxic sediments, both bacterial and archaeal richness generally decrease with increasing sediment depth, whereas in oxic sediments, archaeal richness remains stable and only bacterial richness decreases (Hoshino et al., 2020). Members of *Gammaproteobacteria*, *Deltaproteobacteria* (reclassified as phylum *Desulfobacterota*), *Alphaproteobacteria*, *Acidobacteria*, and *Bacteroidetes* show higher proportions in surface sediments, while the subsurface is characterised by *Atribacteria*, *Chloroflexi*, and various archaeal groups such as *Bathyarchaeota* and *Lokiarchaeota* (Chen et al., 2017; Petro et al., 2017; Waite et al., 2020; Walsh et al., 2016). Interestingly, a clear shift in the taxonomic composition of the bacterial community was observed at a sediment depth of around 1 km (Inagaki et al., 2015). The phyla *Chloroflexi* and *Atribacteria*, which are characteristic of subsurface sediments, become less present and groups known to dominate terrestrial soil habitats such as *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria* begin to dominate. These changes were explained by the preservation of indigenous bacteria in terrigenous sediments over tens of millions of years after burial in the seabed (Inagaki et al., 2015).

1.4 Metabolism of sediment microbial communities

The large differences in environmental conditions between oxic and anoxic sediments as well as throughout the sediment core (Section 1.1) are reflected not only in the composition of the microbial communities (Section 1.3), but also in their metabolism (Orsi, 2018). The sedimentation of organic matter from the water column (Jørgensen et al., 2019; Kallmeyer et al., 2012; Orsi, 2018) and the available terminal electron acceptors (Canfield et al., 1993; Froelich et al., 1979; Thamdrup et al., 1994) are the main factors influencing the metabolism of these communities (Section 1.1). Studies indicate that in oxic sediments, where organic matter is scarce and the oxygen zone can therefore extend very deep into the sediment, chemolithoautotrophic archaea (e.g. ammonia-oxidising members of the phylum *Thaumarchaeota*), the ubiquitous

gammaproteobacterial JTB255 group, and members of the *Nitrospinae* and *Nitrospirae* perform dark carbon fixation (Bienhold et al., 2016; Dyksma et al., 2016; Molari et al., 2013; Orsi, 2018; Tully and Heidelberg, 2016). Furthermore, it appears that ammonia in deep-sea sediments is remineralised from organic compounds by heterotrophic microbes and utilised by ammonia-oxidising *Thaumarchaeota*, highlighting the importance of interactions between different groups of microbes (Orsi, 2018; Tully and Heidelberg, 2016; Wankel et al., 2015). The possibility that some members of *Thaumarchaeota* grow mixotrophically further complicates the understanding of the specific roles that each group plays in these habitats (Qin et al., 2014).

In contrast to sediments with high oxygen content, anoxic marine sediments are rich in organic matter, which leads to oxygen consumption in the upper centimetres of the sediment (Bowles et al., 2014; Jørgensen et al., 2019; Orsi, 2018; Section 1.1). Below this surface zone, anaerobic microbial metabolism, which relies on the remaining energetically most favourable terminal electron acceptors, is responsible for the mineralisation of organic matter (Canfield et al., 1993; Jørgensen et al., 2019; Middelburg and Levin, 2009; Orsi, 2018; Thamdrup et al., 1994; Figure 4). Aerobic organisms can mineralise the organic matter completely to carbon dioxide via the tricarboxylic acid cycle. In contrast, the organic matter in anoxic sediments is mineralised in an anaerobic food chain (Arndt et al., 2013). Degradation begins with the hydrolytic breakdown of high-molecular-weight organic matter such as carbohydrates and proteins by extracellular enzymes, which can be attached to the cell or released into solution (Arnosti, 2011; Jørgensen et al., 2019). These complex polymers are converted into substrates that are small enough (typically less than ca. 600 Da, possibly higher for polysaccharides) to be transported across cell membranes (Arnosti, 2011; Jørgensen et al., 2019; Reintjes et al., 2017). This initial hydrolysis is often the rate-limiting step in the overall degradation of organic matter, emphasising its crucial role in controlling degradation efficiency (Arndt et al., 2013; Jørgensen et al., 2019). Indeed, research suggests that subsequent mineralisation pathways do not strongly influence degradation rates (Beulig et al., 2018).

The microbial community in the sediment converts small substrates produced by hydrolytic processes such as sugars, amino acids, lipids, organic acids, etc. into a range of products such as short-chain fatty acids (SCFAs; e.g. formate, acetate, propionate, and butyrate), alcohols, hydrogen, and carbon dioxide through multi-step fermentation processes (Jørgensen, 2000; Jørgensen et al., 2019). *Planctomycetes*, *Anaerolineales* (phylum *Chloroflexi*), and *Bathyarchaeota* have been identified as initial fermenters in anoxic sediments, while *Dehalococcoidia* (phylum *Chloroflexi*), *Deltaproteobacteria* (reclassified as phylum *Desulfobacterota*), and *Nanoarchaeota* may be involved in secondary fermentation (Suominen et

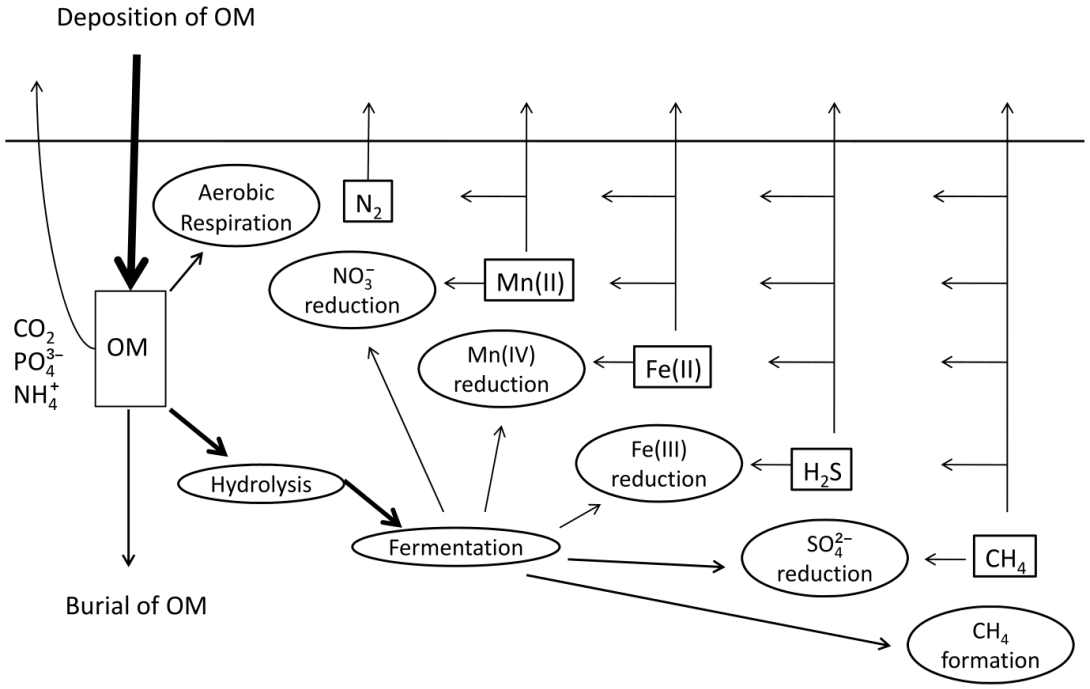

Figure 4. Conceptual model of the degradation of organic matter in marine sediments. High-molecular-weight organic matter is converted into smaller substrates by hydrolysis and fermentation. These products are respired with the most energetically favourable terminal electron acceptors in the following order of utilisation: oxygen (O_2), nitrate (NO_3^-), manganese [Mn(IV)] and iron [Fe(III)] oxides, sulphate (SO_4^{2-}), and carbon dioxide (CO_2). Manganese [Mn(II)], iron [Fe(II)], sulphide (H_2S + HS^- + S^{2-}), and methane (CH_4) are produced and can be oxidised after diffusing upwards. Modified from Middelburg and Levin (2009).

al., 2021; Waite et al., 2020). Since sulphate-reducing microbes are generally unable to utilise the products of hydrolysis of complex organic matter, they take up the substrates produced by the fermenters and respire them to carbon dioxide using sulphate as a terminal electron acceptor (Jørgensen, 2000; Jørgensen et al., 2019). The importance of sulphate reduction is reflected in the estimate that this process in coastal sediments could be responsible for 50% of the mineralisation of organic carbon in the sediment column (Jørgensen, 1982; Jørgensen et al., 2019). The sulphate reducers found in marine sediments belong mainly to uncultured groups within the *Deltaproteobacteria* (reclassified as phylum *Desulfobacterota*), which are only distantly related to cultured sulphate reducers (Jørgensen et al., 2019; Waite et al., 2020). When the sulphate is depleted, the terminal degradation of organic carbon is taken over by methanogenic archaea, whose substrate spectrum is narrower and largely limited to hydrogen, carbon dioxide, and possibly acetate (Jørgensen, 2000; Jørgensen et al., 2019; Petro et al., 2017). Most of the methane produced in the continental shelf and slope sediments diffuses upwards into the SMT zone, where it is oxidised by the anaerobic methanotrophic archaea (ANME), with sulphate serving as a terminal electron acceptor (Egger et al., 2018; Jørgensen et al., 2019).

1.5 Seagrasses

Seagrasses are a polyphyletic group of monocotyledonous angiosperms adapted to a fully submerged lifestyle in marine waters (Les et al., 1997; Orth et al., 2006). It is estimated that they evolved from their terrestrial ancestors around 70 to 100 million years ago (Capó-Bauçà et al., 2022; Orth et al., 2006; Wissler et al., 2011). Colonisation of marine habitats is considered a difficult adaptive obstacle, requiring the acquisition of salinity tolerance, underwater vegetative growth, a sufficient anchoring system to withstand wave action and tidal currents, and hydrophilous (water-mediated) pollination (Arber, 1920). Indeed, seagrasses fulfil all four of these characteristics necessary for plants to thrive in the marine environment (den Hartog, 1970). Moreover, the occurrence of three independent seagrass lineages (Hydrocharitaceae, Cymodoceaceae complex, and Zosteraceae) proves that the acquisition of these traits occurred multiple times and represents an impressive example of convergent evolution in angiosperms (Les et al., 1997). There are approximately 70 species regarded as seagrasses, all of which are classified in the order Alismatales (Short et al., 2011; Unsworth et al., 2022). Within this order, three families consist exclusively of seagrasses, Zosteraceae (three genera), Cymodoceaceae (five genera), and Posidoniaceae (one genus), while the family Hydrocharitaceae contains three genera that are considered seagrasses and 14 genera that are restricted to freshwater habitats (den Hartog and Kuo, 2006). In addition to the species within these genera, many species of the genus *Ruppia* are sometimes considered seagrasses, while species of the genera *Potamogeton* and *Lepilaena* are considered associates of seagrasses or facultative members of the seagrass community (Green and Short, 2003).

Seagrasses occur in shallow marine and estuarine environments of all continents except Antarctica (den Hartog and Kuo, 2006; Green and Short, 2003). The areas containing the highest number of seagrass species are insular South-East Asia, including northern tropical Australia and the Great Barrier Reef, south-eastern India, eastern Africa, southern Japan, and south-western Australia (Short et al., 2007; UNEP-WCMC and Short, 2018; Figure 5). In the Mediterranean Sea, there is a unique temperate–tropical mix of seagrass species (Short et al., 2007). *Zostera marina* Linnaeus and *Nanozostera noltei* (Hornemann) Tomlinson et Posluszny, two species common in temperate regions, occur here alongside the endemic *Posidonia oceanica* (Linnaeus) Delile (Green and Short, 2003; Guiry and Guiry, 2025; Short et al., 2007). In addition to these species, the Mediterranean is also inhabited by *Halophila stipulacea* (Forsskål) Ascherson, an invasive species thought to have been introduced from the Red Sea through the Suez Canal (Lessepsian migration), and by *Cymodocea nodosa* (Ucria) Ascherson, a seagrass whose congeners inhabit

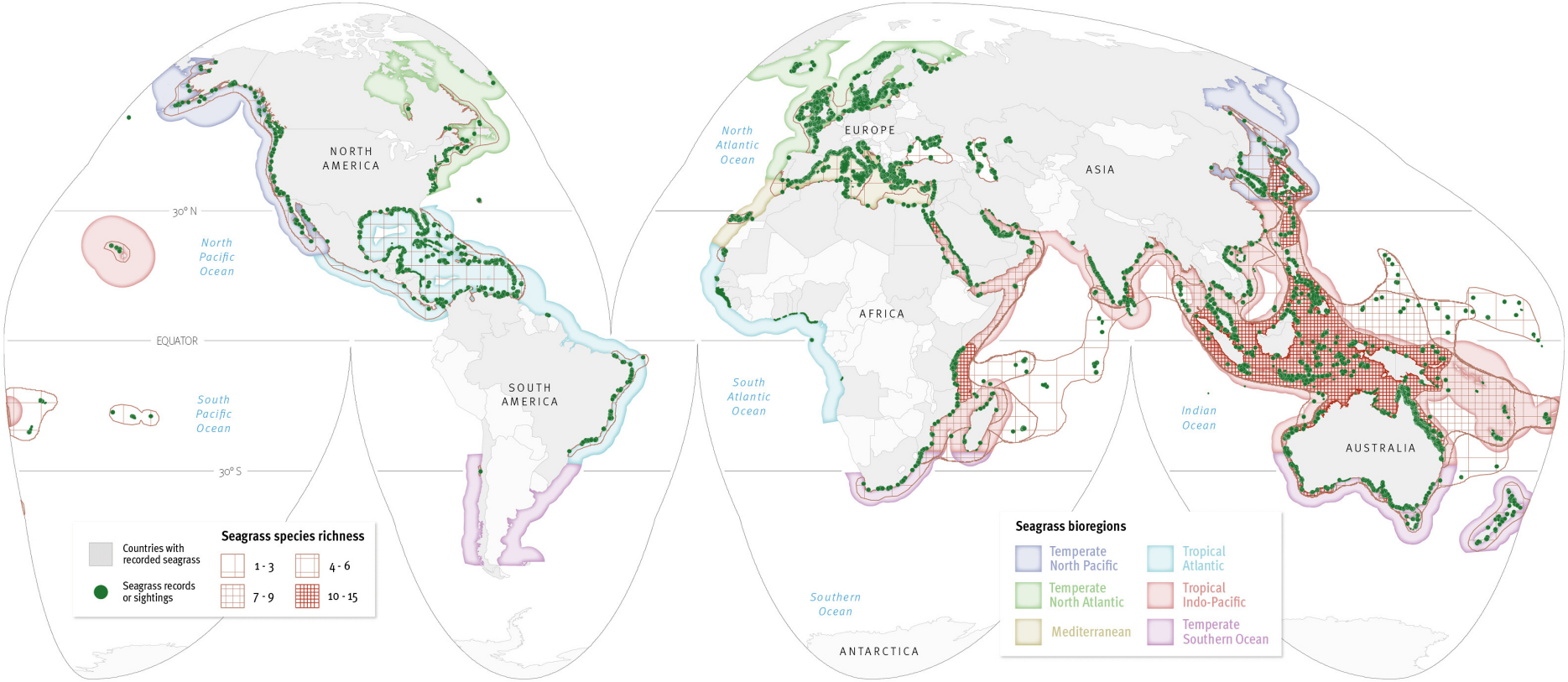

Figure 5. Global distribution of seagrasses, species richness, and bioregions. Data sources: Short et al. (2007) and UNEP-WCMC and Short (2018). Map created by Levi Westerveld/GRID-Arendal (2019) using the Goode homolosine projection (https://grida.no/resources/13590). From UNEP (2020).

the tropical Indo-Pacific (Fritsch, 1895; Green and Short, 2003; Guiry and Guiry, 2025; Short et al., 2007). In addition, *Ruppia* spp. have been found in the Mediterranean Sea, but their distribution is generally restricted to shallow waters characterised by fine sediments and strong salinity fluctuations, such as coastal lagoons and brackish habitats (Green and Short, 2003; Mannino et al., 2015). These seagrass species, which are characteristic of the Mediterranean, have also been found in the Adriatic Sea (Curiel et al., 2021; Green and Short, 2003; Guidetti et al., 2002).

The seagrass *C. nodosa*, whose sediment microbes are the subject of this doctoral thesis, is, as already mentioned, widespread in the Mediterranean (Green and Short, 2003; Short et al., 2007). However, it is not restricted to this area. Its range also includes the Atlantic coast of Africa, including the Canary Islands. Here, the southern limit of distribution is south of the Tropic of Cancer (den Hartog, 1970; Green and Short, 2003; Short et al., 2007). On the European Atlantic coast, it is also found in Portugal, where it is mixed with temperate seagrasses (Short et al., 2007), and in southern Spain (den Hartog, 1970). In the Adriatic Sea, *C. nodosa* is common in both the northern (Agostini et al., 2003; Najdek et al., 2020; Orlando-Bonaca et al., 2015; S. M. Smith et al., 2025; Zavodnik et al., 1998) and southern (den Hartog, 1970; Mačić, 2014) parts of this area of the Mediterranean Sea. This seagrass is generally found in shallow waters, but can sometimes reach a depth of 30 to 40 m. The meadows in shallow and deep waters are typically discontinuous (Green and Short, 2003). *C. nodosa* is usually found in sheltered locations, such as bays and coastal lagoons (Agostini et al., 2003; Green and Short, 2003; Najdek et al., 2020; Orlando-Bonaca et al., 2015; S. M. Smith et al., 2025; Zavodnik et al., 1998). In general, it is considered a pioneer species that often precedes *P. oceanica* in succession, with which it cannot compete (den Hartog, 1970; Green and Short, 2003). The growth pattern is unimodal, with a biomass maximum in summer, a minimum in winter, and a particularly active growth phase in spring (Agostini et al., 2003; Cancemi et al., 2002; Najdek et al., 2020; Terrados and Ros, 1992; Zavodnik et al., 1998). *C. nodosa* is a dioecious species (den Hartog, 1970; Green and Short, 2003). Flowering has been described in spring and summer, while fruits have been collected from spring to late autumn (den Hartog, 1970; Reyes et al., 1995).

Seagrasses are considered ecosystem engineers because they cause physical state changes in biotic and abiotic materials and thus modify, maintain, and create habitats (C. G. Jones et al., 1994; Orth et al., 2006). These environments, known as seagrass meadows, are ecologically important because they provide habitat and food for many animals, serve as nursery areas for the larger ocean, attenuate wave energy, stabilise the sediment, and improve the transparency of the water by trapping sediment particles (Duarte, 2002; Green and Short, 2003; Heck and Valentine,

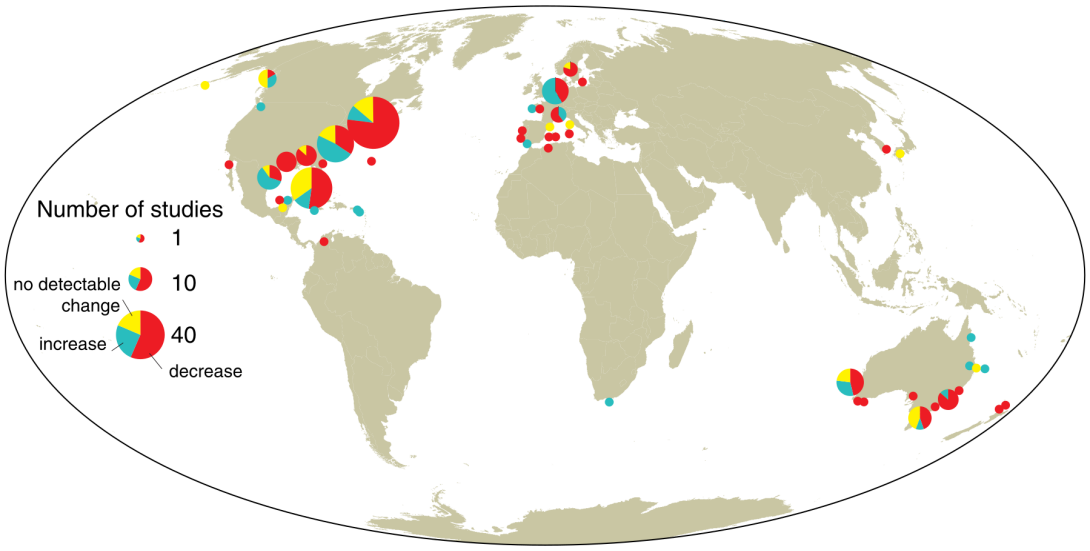

Figure 6. Global assessment of seagrass loss in different coastal areas based on data from 215 sites. An increase or decrease in seagrass area at each site of more than 10% is marked in green and red, respectively. If the final area is within ±10% of the initial area, the change is considered undetectable and marked in yellow. From Waycott et al. (2009).

2006). In addition, the sediments of seagrass meadows are enriched with organic carbon through the trapping of organic particles from the water column, the release of dissolved organic carbon by seagrass roots, and the already mentioned stabilisation of the sediment (Duarte, Holmer, et al., 2005; Terrados and Duarte, 2000; van Katwijk et al., 2010). Moreover, the decomposition of seagrass leaves, roots, and rhizomes increases the availability of organic matter in seagrass meadows (Liu et al., 2017; Peduzzi and Herndl, 1991; Trevathan-Tackett et al., 2020). It is therefore not surprising that the sediments of seagrass meadows are natural hotspots for carbon sequestration. It is estimated that these areas are responsible for up to 20% of global carbon sequestration in marine sediments, although they occupy only 0.1% of the seafloor (Duarte, Middelburg, et al., 2005; Duarte et al., 2013; Kennedy et al., 2010). Due to the importance of seagrass meadows, their observed loss on a global scale is worrying (Dunic et al., 2021; Orth et al., 2006). The area covered by seagrass meadows has declined by 29% worldwide since the end of the 19th century (Waycott et al., 2009; Figure 6). Although more recent estimates have reduced the global loss over the same period to 19%, this is still considerable and the decline continues (Dunic et al., 2021). The loss of seagrass meadows has also been observed in the Mediterranean (de los Santos et al., 2019; Dunic et al., 2021). In fact, together with the Tropical Atlantic, the Mediterranean experienced the greatest loss of seagrass meadows in absolute numbers, with the decline being greatest from the 1940s to the 1980s and the situation remaining stable thereafter (Dunic et al., 2021). A partially similar trend in the loss of seagrass meadows was also observed in the Adriatic Sea. After a sharp decline in the 1970s and 1980s, seagrass meadows experienced a phase of recovery in the 1990s. However, from 2007 to 2013 a

further loss of seagrass meadows was recorded (Danovaro et al., 2020). Although Short et al. (2011) estimated a stable population trend for *C. nodosa*, a more recent study using ecological niche modelling predicts, in the worst-case scenario, a sharp decline in suitable habitats for this species due to climate change (Chefaoui et al., 2018). Indeed, several studies reported the decline of *C. nodosa* meadows in different parts of the Mediterranean (Barsanti et al., 2007; Boudouresque et al., 2009; Pérez-Ruzafa et al., 2006; Shili et al., 2002), including the Adriatic Sea (Green and Short, 2003; Orlando-Bonaca et al., 2015, 2019).

1.6 Microbial communities in sediments of seagrass meadows

Sediments of seagrass meadows are considered hotspots for microbial activity because, as already mentioned, these habitats are enriched with organic matter directly by seagrasses or through various seagrass-mediated processes (Duarte, Holmer, et al., 2005; Section 1.5). Consequently, studies have shown that microbial abundance, activity, and metabolic diversity are higher in seagrass-vegetated than in nonvegetated sediments (Delille et al., 1996; Duarte, Holmer, et al., 2005; Holmer and Nielsen, 1997; Mohapatra et al., 2022; A. Smith et al., 2004). The high organic matter content of seagrass sediments appears to stimulate the proliferation of sulphate-reducing bacteria, which in turn play an important role in the remineralisation of nutrients in these habitats (Blackburn et al., 1994; Duarte, Holmer, et al., 2005; Holmer and Nielsen, 1997; A. Smith et al., 2004). This is consistent with the already recognised importance of sulphate reduction in the mineralisation of organic carbon in coastal sediments (Jørgensen, 1982; Jørgensen et al., 2019). In addition, the facilitated release and transformation of nutrients by microbes during remineralisation supports seagrass growth and photosynthesis (Duarte, Holmer, et al., 2005). However, high rates of sulphate reduction can lead to the accumulation of hydrogen sulphide (H_2S), a potent phytotoxin (Duarte, Holmer, et al., 2005; Holmer and Bondgaard, 2001; Koch and Erskine, 2001), which has been linked to die-off events of seagrass meadows (Borum et al., 2005; Carlson et al., 1994). The diffusion of oxygen from seagrass roots and the reoxidation of hydrogen sulphide back to sulphate has been recognised as a coping mechanism of seagrasses against hydrogen sulphide toxicity (Duarte, Holmer, et al., 2005; Hasler-Sheetal and Holmer, 2015; Holmer et al., 2005; Pedersen et al., 2004). These examples show that seagrasses and sediment microbes live in a state of delicate balance. For the growth and development of seagrasses, it is important that the positive, mutualistic effects of the microbes, such as the remineralisation of nutrients, overcome the negative ones, such as the production of hydrogen sulphide (Duarte, Holmer, et al., 2005).

The composition of microbial communities living in sediments colonised by seagrasses is not as well studied, as research has mainly focused on the rhizosphere and only occasionally sediment communities have been used for comparison. It has been shown that communities in the rhizosphere are not species-specific and differ from those in the sediment (Cúcio et al., 2016; Ettinger et al., 2017; Rabbani et al., 2021; X. Zhang et al., 2020). One of the most important differences observed is the higher proportion of *Deltaproteobacteria* (reclassified as phylum *Desulfobacterota*) in the bulk sediment, a group that contains many sulphate reducers from marine sediments (Ettinger et al., 2017; Waite et al., 2020). In contrast, the rhizosphere is characterised by members of the *Epsilonproteobacteria* (reclassified as phylum *Campylobacterota*; Ettinger et al., 2017; Jensen et al., 2007; Waite et al., 2017, 2018). Microbial communities in sediments colonised by many seagrass species such as *Enhalus acoroides* (Linnaeus f.) Royle, *Thalassia hemprichii* (Ehrenberg) Ascherson (Liu et al., 2018), *Thalassia testudinum* K. D. Koenig (Ugarelli et al., 2024), *Amphibolis antarctica* (Labillardière) Ascherson, *Halodule uninervis* (Forsskål) Ascherson (Fraser et al., 2018), *Halodule wrightii* Ascherson (A. Smith et al., 2004), *Posidonia oceanica* (García-Martínez et al., 2009), *Nanozostera japonica* (Ascherson et Graebner) Tomlinson et Posluszny, and *Zostera marina* (Sun et al., 2020) have been described (Guiry and Guiry, 2025). Studies have often found differences between seagrass-vegetated and nonvegetated areas (Alsaffar et al., 2020; A. Smith et al., 2004; Sun et al., 2020; Zheng et al., 2019). Furthermore, differences in microbial communities have been described even between the periphery and the central region of the seagrass meadow (Ettinger et al., 2017). One of the differences between seagrass-vegetated and nonvegetated areas is the higher abundance of archaea in seagrass-colonised sediments (Zheng et al., 2019). The archaeal community in seagrass sediments generally includes *Crenarchaeota*, *Euryarchaeota*, *Asgardaeota*, *Woesearchaeota*, *Bathyarchaeota*, and *Thaumarchaeota* (Sun et al., 2020; Zheng et al., 2019), while the bacterial community consists predominantly of *Proteobacteria*, *Chloroflexi*, *Planctomycetes*, *Bacteroidetes*, *Acidobacteria*, *Epsilonproteobacteria* (reclassified as phylum *Campylobacterota*), and *Latescibacteria* (Sun et al., 2020; Waite et al., 2017, 2018).

2 OBJECTIVES AND HYPOTHESES

Seagrasses are considered ecosystem engineers because they modify, maintain, and create habitats (C. G. Jones et al., 1994; Orth et al., 2006). The decline and loss of seagrass meadows, which are ecologically important habitats, is worrying as it has been observed on a global scale, including in the Mediterranean and Adriatic Sea (Danovaro et al., 2020; Dunic et al., 2021). Sediments of seagrass meadows are considered hotspots for microbial activity, as these habitats are enriched with organic matter directly by seagrasses or through various seagrass-mediated processes. Several ecological interactions between seagrasses and microbes in the sediment have been described, suggesting that these plants and microbes live in a state of delicate balance (Duarte, Holmer, et al., 2005). Although microbial communities living in the sediments of many seagrass species have been described (Fraser et al., 2018; García-Martínez et al., 2009; Liu et al., 2018; A. Smith et al., 2004; Sun et al., 2020; Ugarelli et al., 2024), little is known about the response of these communities to the decline and loss of seagrasses.

The decline of *Cymodocea nodosa*, whose sediment microbes are the subject of this doctoral thesis, has been observed in various parts of the Mediterranean (Barsanti et al., 2007; Boudouresque et al., 2009; Pérez-Ruzafa et al., 2006; Shili et al., 2002), including the Adriatic Sea (Green and Short, 2003; Orlando-Bonaca et al., 2015, 2019). Although the rhizosphere and epiphytic microbial communities of this seagrass species have been described (Cúcio et al., 2016; Korlević et al., 2021), little is known about the communities living in the sediment of its meadow. Therefore, the objectives of this doctoral thesis were:

1. To assess the composition and diversity of sediment microbial communities in a *C. nodosa* seagrass meadow using a marker gene sequencing approach.
2. To assess the functional diversity and dynamics of sediment microbial communities in a *C. nodosa* seagrass meadow using a metagenomic and metaproteomic approach.

In addition, the following hypotheses were tested:

1. Decline of the *C. nodosa* meadow alters sediment environmental conditions.
2. The sediment microbial community structure differs with sediment depth, between the vegetated and nonvegetated sites, and throughout the study period.
3. The metabolic profile of the sediment microbial community differs with sediment depth, between the vegetated and nonvegetated sites, and throughout the study period.

3 SCIENTIFIC ARTICLES

3.1 Dynamics of environmental conditions during the decline of a *Cymodocea nodosa* meadow

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Dynamics of environmental conditions during the decline of a *Cymodocea nodosa* meadow

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Abstract. The dynamics of the physicochemical and biological parameters were followed during the decline of a *Cymodocea nodosa* meadow in the northern Adriatic Sea from July 2017 to October 2018. During the regular growth of *C. nodosa* from July 2017 to March 2018, the species successfully adapted to the changes in environmental conditions and prevented H_2S accumulation by its reoxidation, supplying the sediment with O_2 from the water column and/or leaf photosynthesis. The *C. nodosa* decline was most likely triggered in April 2018 when light availability to the plant was drastically reduced due to increased seawater turbidity that resulted from increased terrigenous input, indicated by a decrease in salinity accompanied with a substantial increase in particulate matter concentration, combined with resuspension of sediment and elevated autotrophic biomass. Light reduction impaired photosynthesis of *C. nodosa* and the oxidation capability of belowground tissue. Simultaneously, a depletion of oxygen due to intense oxidation of H_2S occurred in the sediment, thus creating anoxic conditions in most of the rooted areas. These linked negative effects on the plant performance caused an accumulation of H_2S in the sediments of the *C. nodosa* meadow. During the decay of aboveground and belowground tissues, culminating in August 2018, high concentrations of H_2S were reached and accumulated in the sediment as well as in bottom waters. The influx of oxygenated waters in September 2018 led to the re-establishment of H_2S oxidation in the sediment and remainder of the belowground tissue. Our results indicate that if disturbances of environmental conditions, particularly those compromising the light availability, take place during the recruitment phase of plant growth when metabolic needs are at a maximum and stored reserves minimal, a sudden and drastic decline of the seagrass meadow occurs.

1 Introduction

Seagrasses are important ecosystem engineers, constructing valuable coastal habitats which play a key role in the preservation of marine biodiversity and carbon sequestration (Duarte et al., 2013; Samper-Villareal et al., 2016). Seagrasses extend their active metabolic surfaces (i.e., leaves, rhizomes and roots) into the water column and in the sediment, where root activity might modify the chemical conditions (Marbà and Duarte, 2001). Their canopies and dense meadows are responsible for trapping substantial amounts of sediment particles and organic matter, enhancing water transparency and sediment stability with the dense network formed by the rhizome (Gacia and Duarte, 2001; Hendriks et al., 2008; Widdows et al., 2008). Seagrass rhizospheres store organic matter (Pedersen et al., 1997), promote sulfate reduc-

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tion (Holmer and Nielsen, 1997), release oxygen (Pedersen et al., 1998) and alter sediment redox potential.

Seagrasses require some of the highest light levels of any plant worldwide to provide oxygen to roots and rhizomes and support a large amount of nonphotosynthetic tissue (Orth et al., 2006). This makes seagrasses sensitive to environmental changes, especially those that deteriorate light availability, such as sediment loading, eutrophication or epiphyte cover on seagrass leaves (Terrados et al., 1998; Halun et al., 2002; Brodersen et al., 2015; Costa et al., 2015). Seagrasses have adapted to a highly variable light environment, providing tolerance to short-term periods of low-light conditions by balancing carbon supply and respiratory requirements. In a healthy growing population this balance is achieved by increasing the photosynthetic activity, reallocating carbohydrate reserves from rhizomes and slowing down growth rates (Collier et al., 2009). Besides metabolic and physiological changes, stress responses under poor light conditions include the shedding of leaves and shoots and production of new, altered tissue. At sublethal light levels, these changes may be permanent. Below these species-specific minimum light requirements seagrass populations are dying off (Collier et al., 2012). Membrane lipids, particularly polyunsaturated fatty acids (PUFAs), as the most responsive constituents have a major role in the adaptation processes of primary producers to fluctuating environmental factors, such as temperature, irradiance or salinity (Viso et al., 1993; Lee et al., 2007; Schmid et al., 2014; Sousa et al., 2017; Beca-Carretero et al., 2018, 2019). The changes in the unsaturation degree (UND) of membrane fatty acids affect the maintenance of membrane functions and membrane resistance to cold stress or poor light conditions. The UND depends mostly on the variation in α-linolenic (C18:3$n-3$; ALA) and linoleic (C18:2$n-6$; LA), the major unsaturated fatty acids in leaves, implicated in the evolution of oxygen during photosynthesis. LA and ALA are derived from oleic acid by desaturation in the chloroplast, and this conversion considerably declines in the dark, being completely inhibited by anaerobiosis (Harris and James, 1965).

Sediments inhabited by seagrasses are usually anoxic, highly reduced and rich in sulfide (H_2S), a strong phytotoxin (Koch and Erskine, 2001) which has been implicated in several die-off events of seagrasses (Carlson et al., 1994; Borum et al., 2005; Krause-Jensen et al., 2011). H_2S is produced by sulfate-reducing bacteria that use sulfate as a terminal electron acceptor for the mineralization of organic matter (Jørgensen, 1977; Capone and Kiene, 1988; Canfield et al., 1993). High H_2S concentrations may occur as a consequence of enhanced mineralization due to increased temperature, organic loading or oxygen depletion (Moeslund et al., 1994; Pérez et al., 2007; Mascaró et al., 2009). Under these conditions, sulfides may intrude into plants. Reoxidation of H_2S in the rhizosphere by the incorporation of S^0 in the belowground tissue has been recognized as a major survival strategy of seagrasses in sulfidic sediments (Pedersen et al., 2004; Holmer et al., 2005; Hasler-Sheetal and Holmer, 2015). Generally, the synergistic effect of oxygen depletion and other stresses, such as sulfide toxicity, may shorten the survival of benthic communities and possibly accelerate mortality events (Vaquer-Sunyer and Duarte, 2010).

The seagrass *Cymodocea nodosa* (Ucria) Ascherson is a widely distributed and common species throughout the Mediterranean (Terrados and Ros, 1992; Pedersen et al., 1997; Cancemi et al., 2002; Agostini et al., 2003). For the northern Adriatic, however, only sparse data are available on the standing crop, seasonal dynamics, or natural and anthropogenic pressures supporting the ecological or conservation status of *C. nodosa* meadows (Zavodnik et al., 1998; Orlando-Bonaca et al., 2015). Although *C. nodosa* shows large phenotypic plasticity, adapting to diverse natural and anthropogenic stressors by physiological and morphological adaptations, a severe decline has been reported during the last few decades in coastal areas (Orth et al., 2006; Short et al., 2011; Tuya et al., 2002, 2014), including the northern Adriatic (Orlando-Bonaca et al., 2015, 2019). One of these declines was documented in our study performed from July 2017 to October 2018 in Saline Bay (northern Adriatic Sea). A series of monthly physicochemical and biological measurements were conducted in *C. nodosa* tissues, sediment underlying the *C. nodosa* meadow, nonvegetated sediments and surrounding water to (i) determine the link between ambient seawater and sediment environmental factors influencing the growth of *C. nodosa*, (ii) document the response of *C. nodosa* to the changes in environmental conditions that led to the meadow decline, and (iii) evaluate the conditions that led to a decline in *C. nodosa*.

2 Materials and methods

2.1 Study site

Saline Bay is located 4 km northwest of Rovinj (Croatia) at the coast of the northern Adriatic Sea (45°7′ 5″ N, 13°37′20″ E; Fig. S1 in the Supplement). The bay represents the terminal shallow part of an 800 m long inlet, open towards the northwest. The southeastern coast of Saline Bay is characterized by relatively pristine conditions, while the northwestern littoral part has been completely modified by the excavation of coastal mud and the addition of large amounts of gravel to create an artificial beach. Large amounts of silty red soil (terra rossa) can be found in the southeastern inner part of the bay in a large muddy flatland which is slowly being eroded by the sea and rain weathering. The main input of freshwater to the bay has been represented by land drainage canals since the year 2017. Even though Saline Bay is protected from the prevailing winds (from the NE and SE), circulations from the northwestern quadrant can occasionally trigger bigger waves, resuspending the surface sediments and giving the waters a muddy appearance. At the

beginning of this study, the seafloor was covered with large *C. nodosa* meadows spreading from the southwestern coastal area (1.5 m depth) towards the central part of the bay (4 m depth), while at the end of the study only a few small patches persisted in tiny stripes along the shoreline.

2.2 Sampling

The sampling was performed for 15 months from July 2017 to October 2018. Seawater for analyses of nutrients, chlorophyll *a* (Chl *a*), particulate matter concentration and prokaryotic abundance was sampled using plastic containers (10 L). *C. nodosa* (3–4 m of depth) was collected together with rhizomes, roots and epiphytic macroalgae by divers using the quadrat sampling method. Three quadrats (20 cm × 20 cm) were randomly scattered in positions of maximum seagrass coverage (e.g., 100 %). Sediment samples were collected inside vegetated and nonvegetated sediment by divers using plastic core samplers (15 cm, 15.9 cm^2). For granulometric composition, organic matter, prokaryotic abundance, total lipid and fatty acid analyses, the cores were cut into 1 cm sections to a depth of 8 cm and lyophilized, except for sections for prokaryotic abundance analysis, which were weighted (approx. 2 g) and fixed with formaldehyde (final conc. 4 % v/v) immediately after slicing the sediment core.

2.3 Temperature (T) and salinity (S) measurements

T was measured continuously (at 30 min intervals) using HOBO Pendant Temperature/Light data loggers (Onset, USA) which were replaced at each sampling. S was measured on sampling dates by a pIONeer 65 probe (Radiometer Analytical, Copenhagen).

2.4 Inorganic nutrients, Chl *a* and particulate matter (PM) analysis

Seawater for all analysis was filtered through combusted Whatman GF/F filters. Nitrate (NO_3), nitrite (NO_2), ammonia (NH_4), phosphate (PO_4) and silicate (SiO_4) were analyzed spectrophotometrically according to Strickland and Parsons (1972). Chl *a* was determined on filters by the fluorometric procedure after extraction in 90 % acetone (Holm-Hansen et al., 1965). PM was determined gravimetrically after filtering up to 5 L of seawater on preweighed filters which were dried (at 60 °C) and reweighed.

2.5 Determining prokaryotic abundance

For determining the prokaryotic abundance in seawater, 2 mL of formaldehyde (final conc. 4 % v/v) fixed samples were stained with 4′,6-diamidino-2-phenylindol (DAPI; 1 µg mL^{-1} final conc.) for 10 min (Porter and Feig, 1980). In sediment samples, prokaryotes were detached from the sediment particles by the addition of Tween 80 (0.05 mL) and ultrasonicated for 15 min (Epstein and Rossel, 1995). After sonication, 1 mL of the supernatant was stained with DAPI (final conc. 5 µg mL^{-1}). DAPI-stained samples were filtered onto black polycarbonate filters (Whatman, Nuclepore, 0.22 µm) and counted under an epifluorescence microscope (Zeiss AxioImager Z1).

2.6 Biometry of *C. nodosa* and epiphytic macroalgae

The material from each quadrat was washed under running seawater to remove sediment. From each quadrat, algae, leaves and rhizomes with roots were separated. The length of the longest leaf on each shoot was measured, and the shoots were counted. Species of macroalgae were determined, and their coverage was estimated according to the Braun-Blanquet scale. Separated samples were washed with filtered and autoclaved seawater, weighed, dried at 60 °C for 48 h, and reweighed. The dry mass was calculated per area (g m^{-2}).

2.7 Granulometric composition of the sediment and its organic matter content

For granulometric analysis of the sediment, each sample was wet sieved through a set of seven standard ASTM sieves (4, 2, 1, 0.5, 0.25, 0.125, 0.063 mm mesh size). The fraction that passed through the 0.063 mm sieve was collected and analyzed following the standard SediGraph procedure (Micromeritics, 2002). The material that was retained on the sieves was dried and weighted. The data obtained by both techniques were merged to obtain a continuous grain size range and analyzed with the statistical package GRADISTAT v 6.0. Sediments were classified according to Folk (1954). The sediment permeability was calculated based on median grain size (d_g) following the empirical relation by Gangi (1985). The organic matter content was determined as ignition loss after heating dried sediment sections at 450 °C for 4 h in a muffle furnace.

2.8 Oxygen (O_2), hydrogen sulfide (H_2S) and redox potential (E_h) profiling

The microprofiles of O_2, H_2S and E_h were measured on intact cores immediately after sampling using a motorized micromanipulator (MMS9083) equipped with microsensors OX-100 and H_2S-200, a redox microelectrode RD-200 coupled with reference electrode REF-RM (Unisense A/S, Denmark). Prior to the measurements, the OX-100 microsensor was calibrated using two-point oxic–anoxic calibration; H_2S-200 was calibrated in fresh Na_2S solutions using eight-point calibration (1–300 µM in a deoxygenated calibration buffer (NaAc–HAc, pH < 4); RD-200 with REF-RM was calibrated using two-point calibration by the simultaneous immersion of electrodes in quinhydrone redox buffers prepared in pH 4 and pH 7 buffers, all according to the manufacturer's recommendations. During measurements, sediment cores were placed in a pool filled with seawater from the sampling site

to maintain in situ temperature. From July to October 2017 H_2S was measured spectrophotometrically in pore waters (Cline, 1969) squeezed out by centrifugation from each section (5 mm) of the sediment cores.

2.9 Total lipids, fatty acid composition and elemental sulfur (S^0)

Lyophilized samples of seagrass tissues, macroalgae, sediment or particulate matter were weighed and extracted into a solvent mixture of dichloromethane / methanol (DCM : MeOH; 2 : 1) in an ultrasonic bath at 35 °C with three solvent mixture changes. The extracts were pooled and separated into layers by the addition of 0.9 % NaCl solution. Lower DCM layers (containing lipids) were released over Na_2SO_4 anhydride, collected in preweighed round bottom flasks and evaporated to dryness using a rotary evaporator. After evaporation, flasks were reweighed, and total lipid concentrations (TLs; mg g^{-1} DW, where DW is dry weight) were calculated from the difference in weight. For fatty acid determination, lipid extracts were saponified (1.2 M NaOH in methanol), acidified (6 M HCl), methylated (14 % BF_3 in methanol) and extracted into DCM.

Fatty acid methyl esters (FAMEs) were analyzed by the Agilent gas–liquid chromatography (GLC) 6890N GC system equipped with a 5973 Network Mass Selective Detector, capillary column (30 m × 0.3 mm × 0.25 µm; cross-linked 5 % phenylmethylsiloxane) and ultrahigh-purity helium as the carrier gas. The GLC settings were as follows: programmed column temperature rise from 145 °C by 4 °C min^{-1} to 215 °C, then by 1 °C min^{-1} to 225 °C and finally by 4 °C min^{-1} to 270 °C at a constant column pressure of 2.17 kPa. Retention times, peak areas and mass spectra were recorded on the ChemStation Software. FAMEs were identified by mass spectral data and family plots of an equivalent chain length (ECL) for GC standards. Applied GC standards were as follows: FAME mix C18–C20, PUFA1 and PUFA3 standards (Supelco, Sigma-Aldrich, Bellefonte, PA, USA) and C4–C24 FAME standard mix, cod liver oil and various individual pure standards (Sigma, Neustadt, Germany).

The following indices of fatty acid profiles were calculated: saturated fatty acids (SATs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and the unsaturation degree (UND). The UND was employed to evaluate the degree of organic matter degradation due to the greater susceptibility of unsaturated, particularly polyunsaturated, components to degradation and calculated according to the formula

$$[1 \cdot (\%\ \text{monoenoic}) + 2 \cdot (\%\ \text{dienoic}) + 3 \cdot (\%\ \text{trienoic}) + 4 \cdot (\%\ \text{tetraenoic}) + 5 \cdot (\%\ \text{pentaenoic}) + 6 \cdot (\%\ \text{hexaenoic})]/\%\ \text{SAT}$$

(Pirini et al., 2007). To evaluate the input of terrestrial organic matter relative to that of marine organic matter in particulate matter, the ratio of terrestrial to aquatic acid (TAR = C24 + C26 + C28/C12 + C14 + C16) was used (Cranwell et al., 1987; Bourbonniere and Meyers, 1996).

In FAMEs, chromatograms elemental sulfur (S^0), eluted as S_8 (m/z 256), was identified by the comparison of retention time with characteristic fragment ions in samples and standard solutions. The concentration of S^0 was estimated on the base of the calibration curve prepared for standard solution of S_8 (Aldrich, Germany) in cyclohexane (2–20 mg L^{-1}). The calibration curve was determined under the same GLC settings as for FAMEs. The limit of detection (LoD) and limit of quantitation (LoQ) were calculated from the parameters of the calibration curve constructed on the basis of the three lowest concentrations in three replicates. The LoD and LoQ (0.92 and 2.80 mg L^{-1}, respectively) were more than twice the values obtained by Rogowska et al. (2016) probably due to the higher injector and column temperature used in this study than the one they proposed as optimal for S determination.

2.10 Data analyses

A multivariate analysis, hierarchical clustering and k-means methods (SYSTAT 12) were applied to group *C. nodosa* aboveground and belowground tissues according to the similarity of their fatty acid profiles and indices, i.e., physiological condition, during the investigated period.

Sediment data were analyzed for two groups of sediment layers, the upper layer (0–4 cm), where most of the rhizomes and roots are located, and the lower layers (5–7 cm). Differences between vegetated and nonvegetated sediment samples in each sediment layer were tested by one-way ANOVA. Correlations among parameters were tested using the Pearson's correlation coefficient (r). The level of statistical significance was $p < 0.05$. A multivariate principal component analysis (PCA; PRIMER 6) was applied to identify the most important variables explaining differences between vegetated and nonvegetated sediments. Correlation matrices were constructed using the following variables: H_2S, E_h, O_2, S^0, prokaryotic abundance (PA), TL and UND. All variables were normalized due to their different scales. Only the principal components with eigenvalues > 1 were considered.

3 Results

3.1 Water column

Environmental variables

During the summer of 2017 daily means of sea-bottom temperature in the *C. nodosa* meadow ranged between 26 and 28 °C. During autumn seawater temperatures decreased, reaching below 12 °C by the end of December. The coldest period was recorded at the beginning of March lasting only for a few days (min 8.62 °C). From April to mid-July 2018,

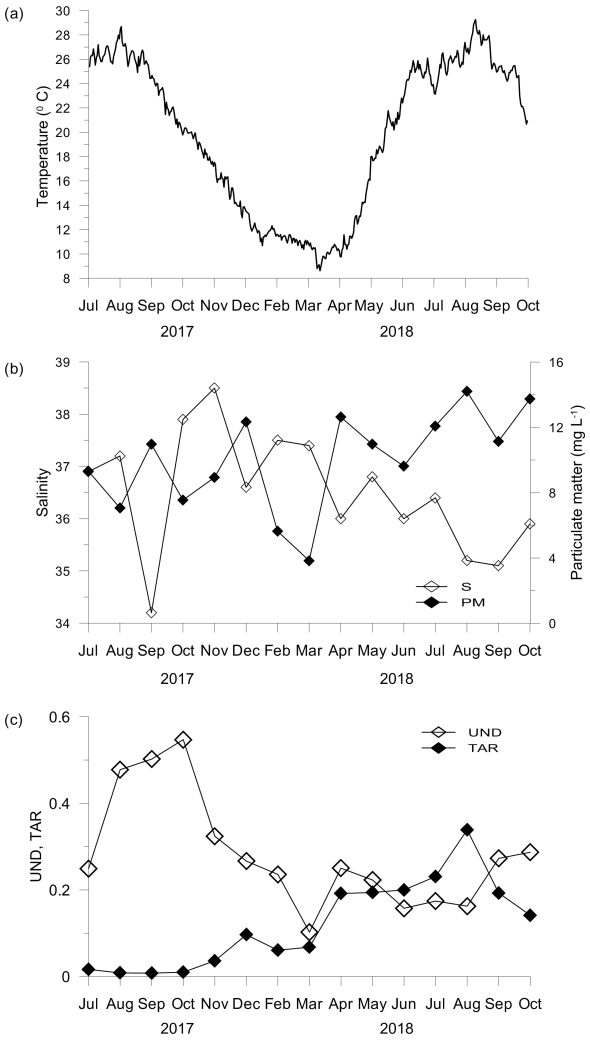

Figure 1. Temperature **(a)**, salinity and particulate matter concentration **(b)**, and unsaturation degree (UND) and terrestrial-to-aquatic ratio (TAR) of the particulate lipid matter **(c)** in seawater.

temperature increased with moderate fluctuations to the maximum of 29.26 °C recorded in August 2018 (Fig. 1a).

Concentrations of inorganic nutrients and Chl *a* were generally low. The highest concentrations (DIN – 8.27 µM; PO_4 – 0.18 µM; SiO_4 – 9.82 µM; Chl *a* – 0.89 µg L^{-1}) associated with the lowest salinity (34.2) were found in September 2017 (Table S1 in the Supplement). The abundance of prokaryotes (2.6–11.3 × 10^5 cell mL^{-1}) varied seasonally and significantly correlated to seawater temperatures ($r = 0.618$; $p < 0.05$). In contrast, salinity (*S*: 34.2–38.5) and concentrations of particulate matter (PM: 3.84–14.21 mg L^{-1}) showed irregular variations (Fig. 1b) and a significant opposite trend ($r = -0.630$; $p < 0.05$).

The particulate lipids exhibited the highest unsaturation degree (UND) during summer and early autumn 2017 and small increases in the UND in April and September and October 2018 (Fig. 1c). The UND was significantly correlated with Chl *a* ($r = 0.603$; $p < 0.05$). In contrast, the terrestrial-to-aquatic ratio (TAR) considerably increased in April and was the highest in August 2018 (Fig. 1c). TAR was correlated negatively to the UND ($r = -0.644$; $p < 0.05$) and positively to particulate matter ($r = 0.641$; $p < 0.05$). Although PUFA with 18 C atoms (C18 PUFA) made the largest contribution to the total PUFA pool, C20 PUFA, mainly of phytoplankton origin, showed a similar trend to that observed for the UND (Fig. S2, Table S2).

3.2 *Cymodocea nodosa* meadow

3.2.1 Biometry

C. nodosa leaves and shoots reached the highest biomass (285.3 ± 57.4 g m^{-2}), length (102.4 ± 26.6 mm) and shoot density (3703 ± 334 shoots m^{-2}) in October 2017 (Fig. 2a). After the appearance of the regular vegetation minimum in November 2017, biometric indices further decreased reflecting the decay of the meadow in summer 2018. In August 2018, only yellow to brownish leaves on sparse shoots were collected (4.5 ± 1.3 g m^{-2}, 5.4 ± 1.3 mm and 30 ± 35 shoots m^{-2}). In September and October 2018, no shoots or leaves were observed (Fig. 2a). The biomass of rhizomes and roots also reached its maximum in October 2017 (599.7 ± 36.8 g m^{-2}). In contrast to leaves and shoots, the belowground biomass was stable until March 2018 when a decline was observed that continued until October 2018 (30.5 ± 6.8 g m^{-2}; Fig. 2a).

3.2.2 Total lipid concentrations (TLs) and fatty acid composition

TL in the *C. nodosa* aboveground tissue (6.7–25.3 ±2.4 mg g^{-1} DW) increased until February 2018, when maximum TLs were measured (Fig. 2b). Thereafter, TLs decreased until August 2018. During this period, the belowground TL (6.3 ± 1.9–15.9 ± 1.1 mg g^{-1} DW) was generally lower than the aboveground TLs and the trend was similar to that of leaves. The minimum TLs were observed in September 2018, while in October 2018, concentrations similar to that measured in October 2017 were observed (Fig. 2b).

The major fatty acid components in *C. nodosa* tissues were palmitic (C16:0) among the saturated fatty acids (SATs) and oleic (C18:1$n-9$) in monounsaturated fatty acids (MUFAs). In the aboveground tissue, the main polyunsaturated fatty acids (PUFAs) were α-linolenic (C18:3$n-3$; ALA) and linoleic (C18:2$n-6$; LA), while in the belowground tissue LA was dominant (Fig. 2b). The dynamics of the UND in the aboveground tissue was principally influenced by changes in

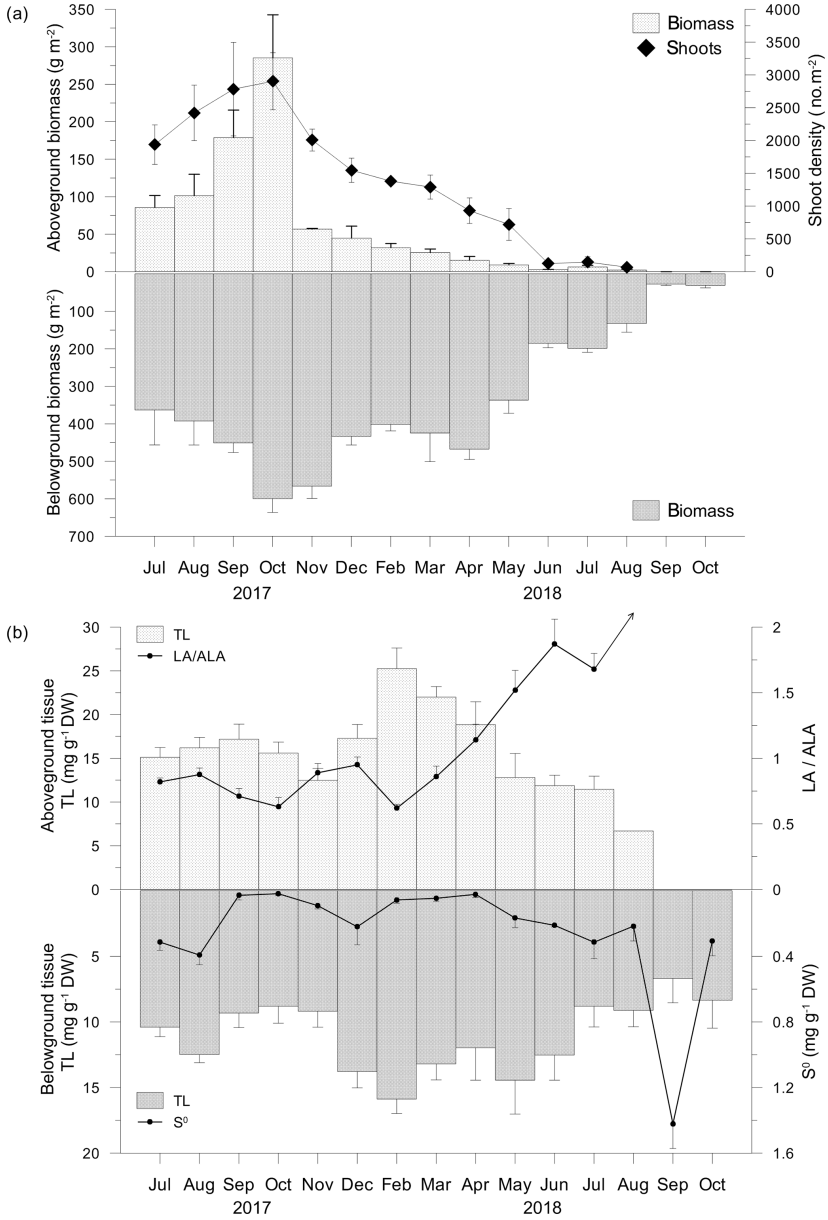

Figure 2. Aboveground and belowground tissue biomasses and shoot density **(a)**, total lipid concentrations (TLs) and linoleic-to-α-linolenic fatty acid ratios (LA / ALA; an arrow indicates an infinite value) in aboveground tissue and TLs and approximated concentrations of elemental sulfur (S^0) in belowground tissue **(b)**.

ALA and LA. LA / ALA ratios were < 1 from July 2017 to March 2018 and > 1 from April to July 2018 (Fig. 2b). In August 2018, the LA / ALA ratio was infinite due to the absence of ALA (Fig. 2b). Elemental sulfur (S^0) was detected only in decaying leaves in August 2018 ($0.21\,mg\,g^{-1}$ DW). In the belowground tissue, S^0 was detected in all samples (Fig. 2b). Higher concentrations were measured during summer 2017 (up to $0.39 \pm 0.06\,mg\,g^{-1}$ DW). S^0 increased from minimum concentrations in April ($0.02 \pm 0.01\,mg\,g^{-1}$ DW) until reaching $1.42\,mg\,g^{-1}$ DW in September 2018 (Fig. 2b).

According to the fatty acid profiles, *C. nodosa* leaves were classified into three groups, except for the leaves collected in August 2018 (Fig. 3). The most distinguishing features specifying physiological differences between

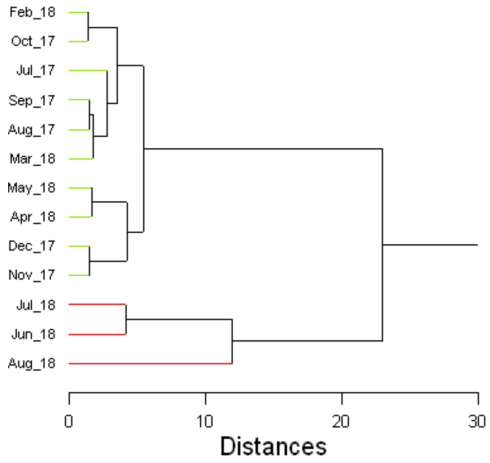

Figure 3. Cluster analysis dendrogram of the fatty acid composition of *C. nodosa* leaves. Summary statistics are given in Table S3.

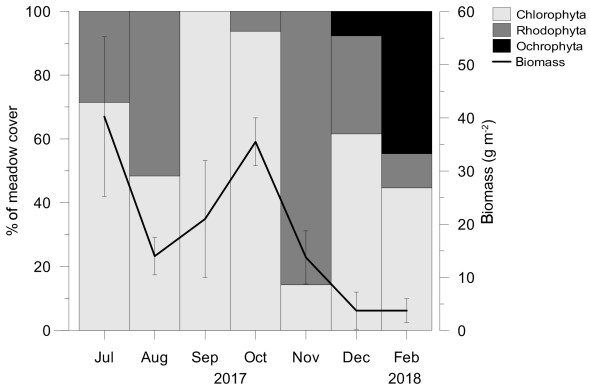

Figure 4. The contribution of macroalgal phyla in a meadow cover and total macroalgal biomass. After February 2018 macroalgae were no longer present in the *C. nodosa* meadow.

Group 1 (July–October 2017 and February–March 2018), Group 2 (November–December 2017 and April–May 2018) and Group 3 (June and July 2018) were decreasing mean values of PUFA, UND, ALA and LA and increasing means of SAT and the proportion of long-chain saturated fatty acids ($C \geq 24$). In the ungrouped leaves from August 2018 ALA was not found and PUFA and UND were at a minimum, while SAT and $C \geq 24$ were at a maximum (Table S3). Three groups of rhizomes and roots (Group 1 – July–October 2017 and February–March 2018; Group 2 – November–December 2017 and April–May 2018; Group 3 – June–October 2018) showed similar characteristics to the groups 1, 2 and 3 of related leaves (Table S4).

3.2.3 Epiphytic macroalgae

From July 2017 to February 2018 different taxa of macroalgae belonging to the three phyla Chlorophyta (*Halimeda tuna*, *Dasycladus vermicularis*, *Cladophora prolifera*, *Udotea petiolata*), Rhodophyta (*Rytiphlaea tinctoria*, *Peyssonnelia* spp., *Gelidium* sp.) and Ochrophyta (*Dictyota dichotoma*) covered the meadow in varying proportions and abundances (Fig. 4). After March 2018, when only few individuals of *Peyssonnelia* sp. were found, macroalgae were no longer present in the *C. nodosa* meadow.

Although the fatty acid profiles of macroalgal communities were highly variable, the contribution of C18 PUFA and C20 PUFA to the total PUFA pool generally depended on the prevailing phyla and their characteristic PUFA pattern. The algae belonging to Rhodophyta and Ochrophyta are richer in C20 PUFA (C20:5$n-3$, C20:4$n-6$), while Chlorophyta generally show a prevalence of C18 PUFA (C18:3$n-3$, C18:2$n-6$; Schmid et al., 2014; Gao et al., 2018). Furthermore, their contribution to biomass varied due to large differences in morphology, which most likely also contributed to the variability in fatty acid profiles. During the dominance of Chlorophyta and Rhodophyta in the macroalgal community, C18 PUFA and C20 PUFA, respectively, showed the highest contribution to the total PUFA pool. In most samples, the lowest contribution to the total PUFA pool was observed for C16 PUFA and C22 PUFA (Fig. S3).

3.3 Sediment

3.3.1 Granulometric composition

According to the granulometric composition, median grain sizes (d_g) and permeability (k), the vegetated and non-vegetated sediments were classified as slightly gravelly sandy mud, fine-grained sediment ($d_g < 165\,\mu m$) and low-permeability sediment to impermeable sediment ($k < 2 \times 10^{-11}\,m^2$). In general, the *C. nodosa* sediment consisted of a significantly higher proportion of sand (Sa) and lower proportion of silt (Si) and clay (C; Sa, $41.11 \pm 4.34\,\%$; Si, $46.44 \pm 2.86\,\%$; C, $9.63 \pm 2.76\,\%$) in comparison to nonvegetated sediment (Sa, $20.53 \pm 10.49\,\%$; Si, $53.24 \pm 6.76\,\%$; C, $23.29 \pm 4.86\,\%$). The median grain size and permeability in *C. nodosa* sediment (d_g, $37.51 \pm 17.97\,\mu m$; k, $1.22 \times 10^{-12} \pm 1.13 \times 10^{-12}\,m^2$) were significantly higher than in nonvegetated sediment (d_g, $10.86 \pm 5.34\,\mu m$; k, $1.04 \times 10^{-13} \pm 1.02 \times 10^{-13}\,m^2$). The upper layers of both cores (0–4 cm) had larger particles, while the lower layers (5–8 cm) showed a uniform distribution of smaller grain sizes (Fig. 5).

3.3.2 O_2, E_h, H_2S and S^0

Oxygen concentrations (O_2) in the bottom water of the *C. nodosa* meadow varied over a wide range (0 µM–$171.4 \pm 17.6\,\mu M$) and generally followed the O_2 saturation trend (Fig. 6a). From May to June 2018, O_2 decreased to below $62.5\,\mu M$, considered as severe hypoxia (Vaquer-Sunyer and Duarte, 2008), and was completely depleted in July 2018

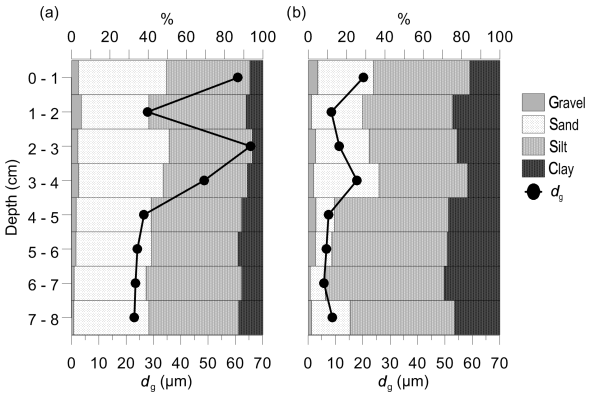

Figure 5. Granulometric composition and median grain size (d_g) of vegetated **(a)** and nonvegetated sediment **(b)**.

(Fig. 6a). From August to October 2018, O_2 increased again. The variations of O_2 in the bottom water of the nonvegetated sediment were similar to those in the *C. nodosa* meadow albeit generally higher (79.4 ± 10.4 µM–212.2 ± 33.4 µM) than in the vegetated sediment except for in September and October 2018 (Fig. 6a).

In general, the O_2 penetration depth in the vegetated and nonvegetated sediment covaried with the O_2 concentration in the bottom layer, penetrating deeper when its concentration in the bottom water was higher (Fig. 6b). In the vegetated sediment, O_2 was mainly depleted down to 1 cm of depth. In the nonvegetated sediment, the oxygen penetration depth was up to 4 times higher than in vegetated sediments, except for the period from August to October 2018 when the penetration depths were similar (Fig. 6b).

The thickness of the oxic ($E_h > 150$ mV) and suboxic (150 mV $> E_h > 0$ mV) layers in the vegetated sediment increased from July 2017 (~ 0.5 cm) to March 2018 (~ 4 cm) and decreased progressively from April (~ 0.8 cm) towards the surface in July 2018, when the entire sediment core was anoxic ($E_h < 0$). From August (~ 1 cm) to October 2018 (~ 2.5 cm) the oxic and suboxic layer thickness increased again (Fig. 7). Oxic conditions ($E_h > 0$) generally reflected O_2 concentrations in the bottom waters. The dynamics of E_h in nonvegetated sediment were similar to those in the vegetated sediment. However, the thickness of the oxic layer was considerably larger than in the vegetated sediment. Reducing conditions ($E_h < 0$) were only recorded in July and August 2017 (Fig. 7).

Concentrations of free H_2S in the pore water of the vegetated sediment generally increased with depth, creating an accumulation zone mainly within the upper sediment layers (1–4 cm; Fig. 7). From July to November 2017, H_2S concentrations increased up to 120 µM (at 4–5 cm). In December 2017, H_2S was low and uniformly distributed throughout the core (< 5 µM). H_2S concentrations increased and the accumulation layer was ascending from March (up to 34.2 ± 12.8 µM; 5–7 cm) to April 2018 (up to 177.2 ± 125.1 µM; 3.5–4.5 cm). During May (up to 107.8 ± 75.9 µM; 2.5–4 cm), June (up to 199.0 ± 6.3 µM; 1.5–6 cm) and July (up to 210.1 ± 138.9 µM; bottom water–6 cm) 2018 a propagation of the accumulation zone was observed in addition to an increase in H_2S (Fig. 7). In August 2018 (up to 1164.1 ± 702.1 µM; bottom water–7 cm) extremely high concentrations over the entire sediment core were recorded. In September and October 2018, H_2S concentrations decreased (down to 140.0 ± 25.3 and 72.7 ± 52.7 µM; bottom water–7 cm and 1–7 cm, respectively). In the nonvegetated sediment, H_2S depth profiles were similar to those in vegetated sediments, but the concentrations were generally lower, except for in the summer of 2017 when the concentrations were comparable but the accumulation zones deeper (Fig. 7).

S^0 mainly occurred in oxic ($E_h > 150$ mV) and suboxic (150 mV $> E_h > 0$ mV) layers of both, vegetated and nonvegetated sediments (Fig. 7). Generally, the ranges of approximated S^0 concentrations in vegetated sediment (8.5×10^{-5}–0.39 mg g^{-1} DW–2.6×10^{-3}–12.1 µmol g^{-1} DW), except for the extreme value in April 2018 (0.99 mg g^{-1} DW–30.8 µmol g^{-1} DW), were similar to those found at the nonvegetated sites (2.9×10^{-4}–0.28 mg g^{-1} DW–9.2×10^{-3}–8.9 µmol g^{-1} DW).

3.3.3 Prokaryotic abundance

Prokaryotic abundance varied largely in vegetated (2.1–39.9×10^7 cells g^{-1} FW, where FW is fresh weight) and nonvegetated (3.7–24.1×10^7 cells g^{-1} FW) sediments. Prokaryotic abundance was significantly higher in the upper layers than in the lower layers of vegetated ($F = 40.553$; $p < 0.05$) and nonvegetated ($F = 52.531$; $p < 0.05$) sediments (Fig. 8). Prokaryotic abundance showed significant monthly changes in the upper ($F = 3.053$; $p < 0.05$) and lower ($F = 5.035$; $p < 0.05$) layer of vegetated sediments, in contrast to both layers of nonvegetated sediments ($p > 0.05$). Prokaryotic abundances were significantly higher in the upper layers ($F = 44.577$; $p < 0.05$) and significantly lower in the lower layers ($F = 5.986$; $p < 0.05$) of vegetated sediments than in the respective layers of nonvegetated sediments (Fig. 8). In the upper sediment layer, prokaryotic abundances were significantly higher in the vegetated than in the nonvegetated sediments from July to October 2017 and from June to August 2018 (Fig. 8). In the lower layers of vegetated sediments, prokaryotic abundance was significantly higher than in the nonvegetated sediments in October 2017 and in August and September 2018 (Fig. 8).

3.3.4 Organic matter, total lipids and fatty acid composition

The concentrations of organic matter (OM) and total lipid concentrations (TLs) were highly correlated in vegetated

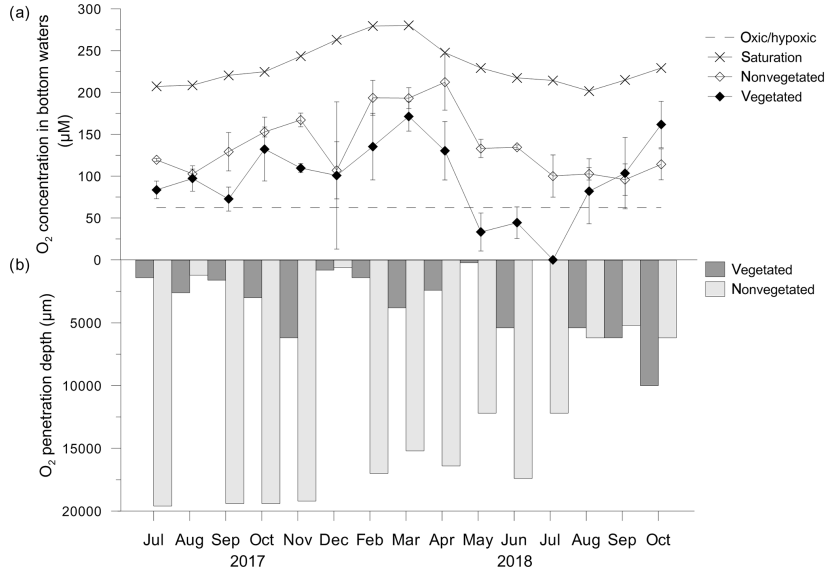

Figure 6. (a) Oxygen (O_2) concentrations in bottom waters in, and **(b)** O_2 penetration depths above, vegetated and nonvegetated sediment. O_2 at the saturation level was calculated according to the temperature and salinity measured in seawater on the sampling dates; O_2 at the hypoxic frontier ($\sim 62.5\,\mu M$) was taken from Vaquer-Sanyer and Duarte (2008).

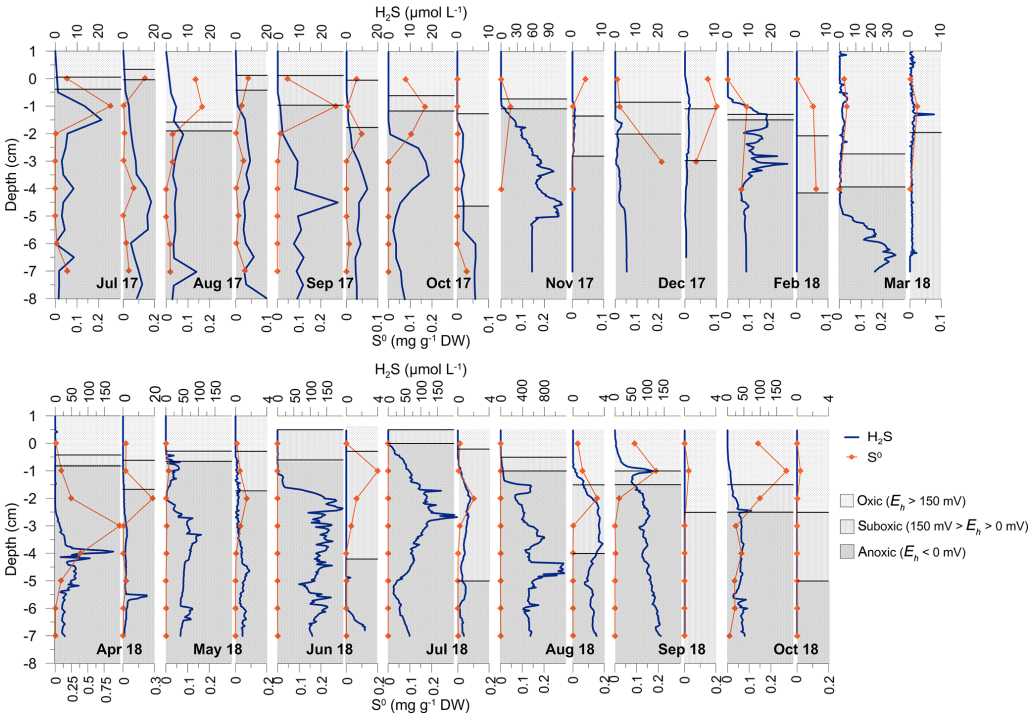

Figure 7. Depth profiles of H_2S and S^0 concentrations in vegetated and nonvegetated sediment (adjacent narrow graphs). The redox potential (E_h) in both sediments is shown as areas corresponding to oxic ($E_h > 150\,mV$), suboxic ($150 > E_h > 0\,mV$) and anoxic ($E_h < 0\,mV$) conditions.

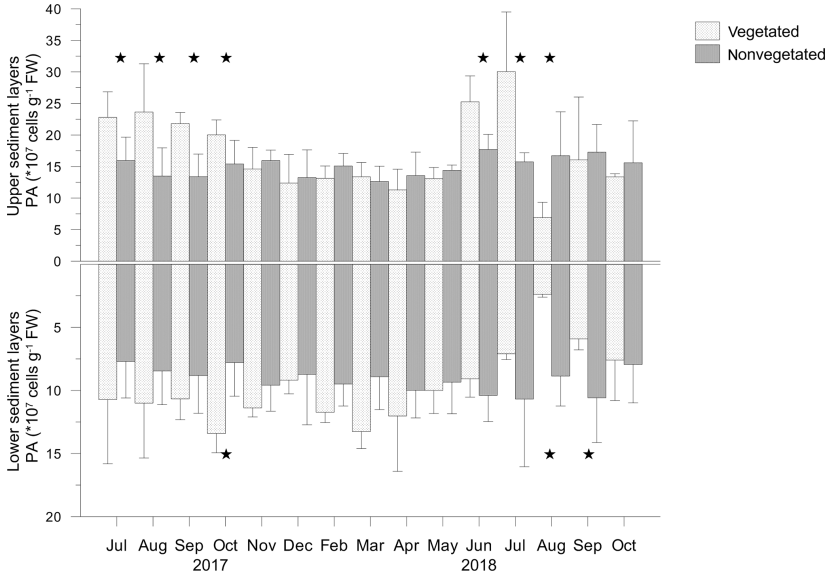

Figure 8. Prokaryotic abundance (PA) in the upper (0–4 cm) and lower (5–8 cm) layers of vegetated and nonvegetated sediments; significant differences in PA between the sediments are indicated by stars.

(OM – 37.6–231.1 mg g^{-1} DW; TL – 0.15–2.75 mg g^{-1} DW; $F = 214.172$; $p < 0.05$) as well as in nonvegetated (OM – 56.7–160.3 mg g^{-1} DW; TL – 0.33–2.39 mg g^{-1} DW; $F = 45.569$; $p < 0.05$) sediments. OM and TL generally decreased with depth and exhibited similar changes throughout the investigated period with significantly higher concentrations in upper sediment layers than in lower sediment layers ($p < 0.05$; Fig. 9).

In the vegetated sediment, the TL showed significant monthly changes in the upper ($F = 11.418$; $p < 0.05$) and lower ($F = 3.186$; $p < 0.05$) sediment layers, in contrast to both layers of nonvegetated sediment ($p > 0.05$). From July to October 2017, in the upper layer of vegetated sediments, TL was significantly higher than in nonvegetated sediments (Fig. 9). From November 2017 onwards, TL decreased slightly until April 2018, reaching similar concentrations to TL in nonvegetated sediments (Fig. 9). TLs decreased markedly in May and continued to decrease until August 2018. During that period, the TL in vegetated sediments was significantly lower than in nonvegetated sediments. In September and October 2018, TLs in vegetated sediments were similar to those in nonvegetated sediments (Fig. 9).

The fatty acid composition of vegetated and nonvegetated sediments was similar and in both layers characterized by the prevalence of SAT (vegetated upper 71.2 %–90.4 %, lower 75.9 %–89.1 %; nonvegetated upper 71.2 %–80.7 %, lower 78.2 %–82.5 %) over MUFA (vegetated upper 7.6 %–22.9 %, lower 9.0 %–19.9%; nonvegetated upper 17.8 %–24.1 %, lower 15.3 %–18.2 %) and PUFA (vegetated upper 1.9 %–6.9 %, lower 1.9 %–5.1 %; nonvegetated upper 1.7 %–4.8 %, lower 1.7 %–3.9 %). The trends of the monthly changes in the UND were similar in both layers of both sediment types. Those variations were less pronounced in the nonvegetated sediment where the UND varied in narrower ranges in both layers (upper 0.26–0.51, lower 0.23–0.33) than it did in vegetated sediment (upper 0.13–0.57, lower 0.14–0.37). From July to October 2017 and in April 2018, the UND was higher in the upper layers of vegetated sediment than in the nonvegetated one, while from November 2017 to March 2018, UNDs of both sediments were lower than in the previous period (Fig. 9). From June to August 2018, the UND decreased considerably in vegetated sediment, being lower than in nonvegetated sediments. During September and October 2018, an increase in the UND was observed in both sediments. In the lower layers, UNDs were similar, except for in July and August 2018 when a considerable decrease in the UND was observed in vegetated sediments (Fig. 9).

The proportions of PUFAs with chain lengths of 16, 18, 20 and 22 C atoms within the PUFA pool were similar between the respective layers of both sediments. Throughout the study period, the highest contribution of C18 PUFA originated from *C. nodosa* detritus and Chlorophyta was observed (Fig. S4, Table S2). From July to October 2017, April to May 2018 and September to October 2018, a contribution of C20 PUFA attributed to phytoplankton and Rhodophyta was also detected. The smallest contribution to the PUFA pool was accounted for by C16 PUFA and C22 PUFA, which were found in seston and macroalgae (Fig. S4, Table S2).

The similarities between the sediments were also observed in the contribution of the main SAT components to the SAT

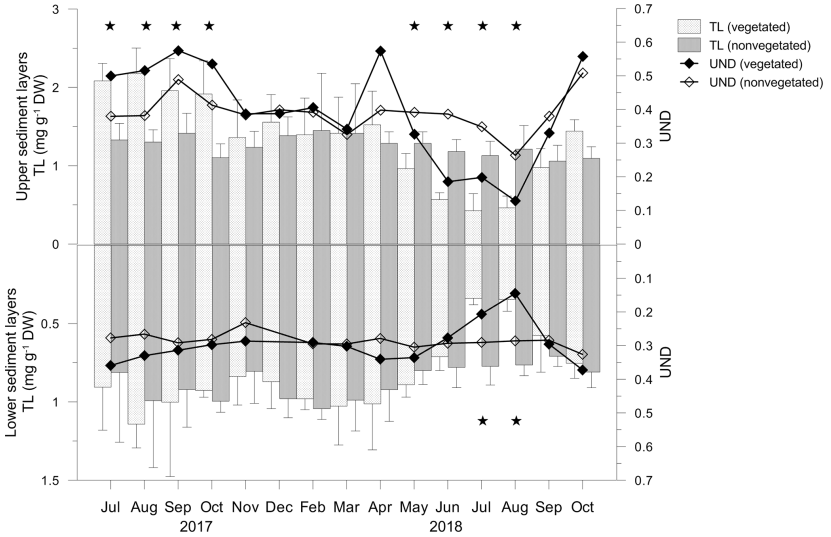

Figure 9. Total lipid concentrations (TLs) and unsaturation degree (UND) in the upper (0–4 cm) and lower (5–8 cm) layers of vegetated and nonvegetated sediments. Significant differences in TLs between the sediments are indicated by stars.

pool from July 2017 to March 2018 and from September to October 2018 (Fig. S4, Table S2). From April to August 2018, an increase in the long-chain ($C \geq 24$) and common (C16:0 + C18:0) fatty acids followed by a decrease in the bacterial fatty acid (BACT) contribution to the SAT pool was observed in both layers of the vegetated sediment. In contrast, the contribution of these components to the SAT pool was fairly invariable in nonvegetated sediments during the same period (Fig. S4, Table S2).

3.3.5 Relationship between different physicochemical parameters

The relationships between H_2S, O_2, TL, S^0, PA, E_h and the UND in vegetated and nonvegetated sediment are shown in the principal component analysis, where PC1 explained 42.5 % and PC2 14.4 % of variability (Fig. 10). The loadings for positive relationships were obtained for H_2S (0.298) on PC1 and E_h (0.541) and O_2 (0.327) on PC2. For the negative relationships, the loadings were for TL (−0.534), the UND (−0.494), S^0 (−0.388), E_h (−0.327), PA (−0.296) and O_2 (−0.191) on PC1 and H_2S (−0.536), S^0 (−0.485), TL (−0.165) and the UND (−0.221) on PC2.

PC1 separated most of the upper sediment layers (July 2017–May 2018, September–October 2018) according to the higher TLs and S^0, a higher UND, and more positive E_h from most of the lower layers and upper layers of vegetated sediments (June–August 2018) with increased H_2S concentrations. On PC2, the vegetated was separated from the nonvegetated sediment due to higher concentrations of H_2S and S^0 and more negative E_h, which characterized vegetated sediments during almost the entire study period. The extreme concentrations of S^0 and H_2S found in the upper layer in April and the lower layer in August 2018, respectively, were responsible for the considerable separation of these layers from all other vegetated layers (Fig. 10).

4 Discussion

Saline Bay is a shallow, highly dynamic coastal area characterized by frequently turbid waters due to the combined effect of land runoff and wind-driven resuspension of fine sediment. Nutrients and Chl *a* (as a proxy for autotrophic biomass) varied in the ranges characteristic for the oligotrophic coastal waters off Rovinj (Ivančić et al., 2018). The dynamics of particulate matter were associated with freshwater input. The higher contribution from autochthonous sources was observed during the increases in autotrophic biomass. However, only in September 2017 was this increase supported by nutrients from the water column, while all other increases were most likely connected to bottom waters where phytoplankton could have been supplied with nutrients through sediment resuspension. The considerable increase in the particulate matter of terrigenous origin from April to August 2018 suggested the enhanced land runoff in that period.

In temperate Mediterranean coastal waters *C. nodosa* meadows show a clear unimodal annual growth cycle, reaching maximum development in summer and a minima during winter, with a particularly active growth phase in spring (Terrados and Ros, 1992; Zavodnik et al., 1998; Agostini et al., 2003). In Saline Bay, the maximum biomass was measured in October 2017. This shift from summer to early autumn was most likely due to intense grazing activities (Cebrian et

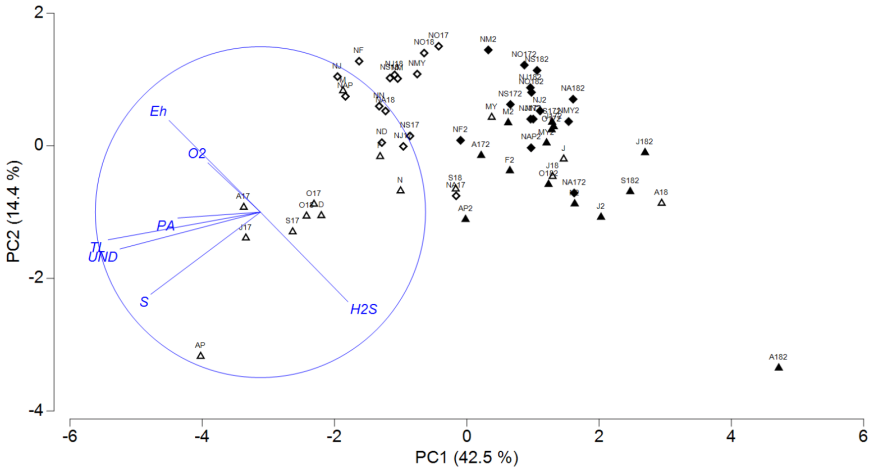

Figure 10. PCA plot of redox potential (E_h), oxygen (O_2), hydrogen sulfide (H_2S), sulfur (S), total lipid concentration (TL), prokaryotic abundance (PA) and unsaturation degree (UND) in the upper (0–4 cm; △, ◇) and lower (5–7 cm; ▲, ◆) layers of vegetated and nonvegetated sediments, respectively. Projections of variables are given in the circle.

al., 1996; Valentine and Duffy, 2006) suggested by a prevalence of visibly grazed leaves during July and August 2017. A growth minimum occurred during late autumn and winter, as commonly observed. However, during the spring of 2018, phenological parameters continued to decrease in spite of established favorable environmental conditions for growth, i.e., increase in water temperature, intensity and period of solar radiation. This decrease continued until the complete extinction of the aboveground tissue in August 2018. The belowground tissue followed a similar trend but with less expressed changes. Still, their recognizable remnants were found after the loss of the aboveground tissues.

Organic matter and closely correlated total lipids in the sediment of *C. nodosa*-rooted area changed significantly throughout the investigated period, in contrast to organic matter in nonvegetated sediment. Nevertheless, considerable similarity in the quality and degradation of lipid matter at both the vegetated and the nonvegetated sites indicates an important contribution of detritus imported from the meadow as a source of organic matter for prokaryotes in nonvegetated sediments. This close coupling could be expected due to site proximity and the lower organic content of the nonvegetated sediment, which should enhance the dependence of prokaryotes on the imports of seagrass detritus from the adjacent meadows (Holmer et al., 2004). The significant enrichment of *C. nodosa* sediment with unsaturated, more labile components only during abundant growth of the meadow could be explained by more efficient entrapment of seston material within the meadow (Gacia and Duarte, 2001). Such easily utilizable organic matter, including dissolved monomeric carbohydrates, leaching out during decomposition of *C. nodosa* leaves stimulated prokaryotic growth as previously observed (Peduzzi and Herndl, 1991).

From July 2017 to March 2018, an adaptation of *C. nodosa* leaves to the decreasing light and temperature occurred. Until October 2017, the temperature of the water column was still optimal for elongation of the leaves and biomass increase, while the ambient light intensities were continuously decreasing. An additional reduction in available light might occur from the self-shading effect due to high canopy biomass and/or shading due to epiphytic macroalgae growth. Desaturation of low and fairly invariable lipids during the most active growth phase suggested an increase in the membrane fluidity to optimize photosynthetic activity under low-light conditions. Such a physiological adaptation was found in seagrasses living along a depth gradient (Beca-Carretero et al., 2019) and in macroalgae in contrasting seasons (Schmid et al., 2014). In late autumn 2017 and 2018, the decrease in desaturation indicated reduced fluidity and activity of photosynthetically active membranes (Quigg et al., 2006; Wacker et al., 2016). This was associated with a decreased abundance of shoots and aboveground biomass. By shedding leaves and shoots the plant further balances metabolic requirements and mobilizes energy from the carbohydrate reserves stored in the belowground tissue (Alcoverro et al., 2001; Lee et al., 2007). During the winter, due to a sharp and continuous decrease in water temperature, rapid desaturation of increasing lipids provided cold resistance, as regularly observed in algae and plants (Terrados and Lopez-Jimenez, 1996; Iveša et al., 2004; Upchurch, 2008).

In a healthy seagrass meadow, the oxygen generated by seagrass photosynthesis is transported to belowground tissues to maintain an oxic microsphere around roots and rhi-

zomes and reoxidize sulfide to nontoxic S^0, thus preventing an invasion of H_2S into the plant (Pedersen et al., 1998; Holmer et al., 2005). S^0 was found in the *C. nodosa* belowground tissue during the entire investigation period, as already observed in seagrasses living in sulfidic sediments (Holmer and Hasler-Sheetal, 2014; Hasler-Sheetal and Holmer, 2015). The relatively low accumulation of H_2S ($< 30\,\mu M$) during the summer and early autumn 2017 indicated that H_2S was apparently rapidly recycled within the rooted area via reoxidation by O_2 to S^0 and/or removal by precipitation with iron compounds. Most of S^0 was found in oxic layers or at suboxic–anoxic boundaries, being in ranges typical for sulfidic coastal sediments (Troelsen and Jørgensen, 1982; Panutrakul et al., 2001; Pjevac et al., 2014). The oxidation of H_2S could occur spontaneously by chemical reaction with free oxygen or mediated by sulfide-oxidizing bacteria surrounding or being attached to seagrass roots (Jørgensen, 1977; Cúcio et al., 2016; Ugarelli et al., 2017; Fahimipour et al., 2017). In November, due to the degradation of organic matter and reduced oxygen production and leakage in the rooted zone caused by *C. nodosa* senescence, the reoxidation capacity of the sediment was greatly decreased. This resulted in considerable accumulation of H_2S ($> 100\,\mu M$) which extended up to the sediment surface. During winter and early spring, H_2S production generally decreased, likely due to the reduced activity of sulfate reducing prokaryotes at lower temperatures, and the sediment gradually shifted towards a more oxidized state. H_2S detected even within the oxic sediment and in the rooted area in February 2018 could be attributed to the sediment heterogeneity and the presence of reducing microniches where anaerobic metabolism could occur regardless of surrounding redox conditions (Jørgensen, 1977; Frederiksen and Glud, 2006).

In April 2018, *C. nodosa* was most probably exposed to increased siltation, due to an intensification of terrigenous input as indicated by a decrease in salinity ($\Delta 1.5$ with respect to March) and a substantial increase in particulate matter concentration (up to 3 times more than in March; Fig. 1b) combined with resuspension of sediment, provoking elevated autotrophic growth. The intensive siltation is associated with the increased light attenuation, both through the direct shading effect of suspended sediments and through the promotion of phytoplankton and epiphyte growth by the associated increase in nutrients (Terrados et al., 1998; Halun et al., 2002; Brodersen et al., 2015). Therefore, the increase in seawater turbidity and considerable sediment redeposition on the leaves might have severely impaired the light availability and slowed down the plant's photosynthetic activity as indicated by LA / ALA being more than 1 in the aboveground tissue, resulting from a decreased conversion of LA to ALA (Harris and James, 1965). When the minimum light requirements ($\sim 14\,\%$ of incidence light) are not met, *C. nodosa* intensely sheds leaves and shoots (Collier et al., 2012). Such light conditions apparently persisted until May 2018 and most likely prevented the re-establishment of photosynthesis, and *C. nodosa* continued to shed shoots and leaves. The reduced photosynthesis and therefore O_2 transport from the leaves to the rhizome–root system probably minimized root respiration. The maintenance of the oxic rhizosphere and the internal O_2 partial pressure in the lacunae further depended mainly on the diffusion of O_2 from the water column. From April to June 2018, O_2 in the bottom water drastically decreased. Although in such conditions of limited light and O_2 the seagrass might be capable of rapidly modulating metabolic pathways and enhance its photosynthetic rate, as shown for *Zostera muelleri* (Kim et al., 2018), it appeared that the O_2 content of the *C. nodosa* belowground tissue was still too low to maintain the internal pressure, and therefore, the plant tissues became potentially accessible to sulfide intrusion (Pedersen et al., 2004).

At the same time, the sediment was enriched with fresh organic matter derived from increased autotrophic biomass in bottom waters. In addition to the induction of the bloom, strong sediment resuspension, most likely by aeration, stimulated the intense oxidation of H_2S that started to be produced in the rooted zone (up to $180\,\mu M$), due to increased activity of sulfate-reducing prokaryotes possibly triggered by the increase in temperature. An increase in S^0 concentration that reached its maximum in the same layer suggests a simultaneous oxidation of the produced H_2S. The sulfide oxidation probably caused oxygen depletion in the rooted zone and anoxic zone extension up to the sediment subsurface. In May 2018, the excess of organic matter accumulated in April 2018 was degraded. The concentrations of S^0, detected only in the suboxic layer, considerably decreased possibly by disproportionation or respiration by members of the sulfate reducing bacteria (Pjevac et al., 2014).

During June and July 2018, a sudden and significant deterioration in *C. nodosa* physiological conditions was indicated by the further increase in the LA / ALA ratio in the leaves and by the overall saturation of decreasing lipids in aboveground and belowground tissues. Additionally, the loss of leaf tissue negatively impacted the photosynthetic carbon fixation and therefore oxygen production, including the transport of oxygen to belowground tissue (Lee and Dunton, 1997; Lee et al., 2007). The belowground tissue that was not supported by photosynthetically derived oxygen became anoxic. Thus induced anaerobiosis most likely caused a complete inhibition of the fatty acid desaturation chain (Harris and James, 1965) and a permanent breakdown of photosynthesis leading to the final decay of the aboveground biomass and considerable loss of belowground biomass. As the bottom waters were completely depleted in O_2 the whole plant was exposed to sulfides. H_2S inhibits cytochrome c oxidase by binding to regulatory sites on the enzyme, reducing the rate of cellular respiration and leading to the chemical asphyxiation (Nicholls et al., 2013).

From June to August 2018, the decomposition of organic matter, encompassing the entire sediment core, was intensified and accompanied by a large increase in H_2S concentra-

tions (up to 1200 µM). The degradation process involved rhizomes and roots, as suggested by the apparent loss of belowground biomass. Such loss typically occurs in the first stage of plant decay, the leaching phase (Trevathan-Tackett et al., 2017). Readily available, soluble carbohydrates that largely contribute to the leachate mass (Vichkovitten and Holmer, 2004) most probably supported the increase in prokaryotic abundance observed in June and July 2018. However, the significant decrease in prokaryotic abundance that coincided with a maximum degradation of organic matter and H_2S production in August 2018 might indicate that remaining compounds were not degradable by the sulfate reduction pathway (Arndt et al., 2013) and needed the presence of prokaryotes specialized in the anaerobic degradation of refractory compounds, including cellulose and lignin.

During September and October 2018, H_2S concentrations drastically decreased, and the sediment was gradually enriched in fresh organic matter. Due to the combined effect of freshened oxygenated water inflow and resuspension which gradually deepened the oxic layer, reoxidation of H_2S increased. Biogeochemical studies suggest that most sulfides (80 %–90 %) are eventually reoxidized; 10 %–20 % are ultimately buried as complexes with iron (i.e., FeS, FeS_2) or with organic matter after sulfurization (Jørgensen, 1977, 1982). H_2S scavenging with iron and formation of iron sulfides might be more important in Saline Bay, since terrestrial waters are washing out terra rossa, rich in Fe oxides and oxyhydroxides (Durn, 2003). For this reason, sediment cores were most likely always black with sulfuric odor, irrespective of H_2S concentrations or the presence of vegetation.

5 Conclusions

Our results provide insights into the interaction of multiple stressors that have led to the meadow decay, triggered in the sensitive recruitment phase of meadow growth. Even after the improvement of the sediment conditions by the end of the summer of 2018, *C. nodosa* was not able to recolonize its previously occupied areas. This finding combined with a visible alteration of the water column and sediment indicates a considerable loss of the *C. nodosa* habitat. Further research is needed to examine the fate of Saline Bay meadows and an eventual recolonization of the area.

Beyond seagrass itself, this loss had extensive consequences as it has endangered many species that depend on seagrass for food, shelter and nursery. Given the lack of data on the ecological and conservation status of the still numerous seagrass meadows along the northern Adriatic coast, the identification and monitoring of the main pressures acting on them are needed to protect such valuable habitats from degradation and extinction.

Data availability. The underlying data sets presented as figures and tables within this paper and the Supplement are available upon request by contacting the corresponding author.

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/bg-17-3299-2020-supplement.

Author contributions. MN, MK and GJH conceptualized the study; the investigation was carried out by MK, PP, MM, II, LI, IF and MN; MN conducted formal analysis and wrote the original draft, which was reviewed and edited by MK, GJH, PP, LI, II, IF and MM.

Competing interests. The authors declare that they have no conflict of interest.

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3.2 Compositional stability of sediment microbial communities during a seagrass meadow decline

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Compositional stability of sediment microbial communities during a seagrass meadow decline

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The presence of seagrass shapes surface sediments and forms a specific environment for diverse and abundant microbial communities. A severe decline of *Cymodocea nodosa*, a widespread seagrass species in the Mediterranean Sea, has been documented. To characterise and assess the changes in microbial community composition during the decline of a *Cymodocea nodosa* meadow, Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene was performed. Samples of surface sediments from two sites, one without any vegetation and one with a declining *Cymodocea nodosa* meadow, were collected at monthly intervals from July 2017 to October 2018. Microbial communities were stratified by sediment depth and differed between the vegetated and the nonvegetated site. Although the *Cymodocea nodosa* meadow declined to a point where almost no leaves were present, no clear temporal succession in the community was observed. Taxonomic analysis revealed a dominance of bacterial over archaeal sequences, with most archaeal reads classified as *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota*, and *Asgardarchaeota*. The bacterial community was mainly composed of *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota*, and *Campylobacterota*. Our results show that sediment microbial communities are remarkably stable and may resist major disturbances such as seagrass meadow decline.

KEYWORDS

sediment microbial communities, *Cymodocea nodosa*, seagrass meadow decline, northern Adriatic Sea, Illumina 16S rRNA sequencing

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Introduction

Shallow coastal sediments are often colonized by seagrasses, which cover approximately 0.1 to 0.2% of the global ocean (Duarte, 2002). Seagrasses penetrate the sediment with their roots and rhizomes forming extensive meadows. The presence of seagrass meadows shapes surface sediments and provides a specific environment for diverse and abundant microbial communities (Duarte et al., 2005). Sediments colonized by seagrasses are considered hotspots for microbial activity as seagrass meadows enrich the underlying sediment with organic matter (Duarte et al., 2005). High organic matter content is mainly achieved by releasing dissolved organic carbon from seagrass roots and by trapping organic particles from the water column (Duarte, 2002). Moreover, seagrasses stabilize the underlying sediment, promoting the accumulation of organic matter and sediment particles (Fonseca and Kenworthy, 1987; Terrados and Duarte, 2000; van Katwijk et al., 2010). In addition, seagrass beds can also increase the availability of organic matter through the decomposition of detached leaves, roots and rhizomes (Jensen et al., 2007; Liu et al., 2017).

Studies of marine sediment microbial communities primarily focus on changes in microbial abundance and activity with sediment depth (Jørgensen and Marshall, 2016; Petro et al., 2017; Starnawski et al., 2017; Orsi, 2018). Depth-dependent changes in taxonomic composition have been well described differentiating surface sediment communities dominated by *Bacteria*, especially *Proteobacteria*, from deeper communities characterized by *Archaea* (Orcutt et al., 2011; Chen et al., 2017; Petro et al., 2017). Coastal surface sediments colonized by seagrass are not as well investigated due to studies focusing primarily on rhizosphere communities and only occasionally including sediment communities for comparison (Cúcio et al., 2016; Rabbani et al., 2021). Communities in the rhizosphere are not species-specific and differ from those in the sediment (Cúcio et al., 2016; Ettinger et al., 2017; Zhang et al., 2020). One of the main differences is the higher relative abundance of *Desulfobacterota*, one of the most abundant sulphate reducing bacteria in seagrass sediments, in contrast to the rhizosphere, which is characterized by *Epsilon proteobacteria* (Ettinger et al., 2017). When sediment microbial communities were described, the main focus was on the differences between vegetated and nonvegetated sites (Zheng et al., 2019; Sun et al., 2020). In addition, these studies showed that communities differ even with respect to the meadow edge (Ettinger et al., 2017). However, little is known about the response of these communities to seagrass decline. As only limited information is available on the succession of microbial communities in seagrass sediments it is hard to predict how and if seagrass decline influences the underlying sediment communities. It was reported that the sulphate-reducing community in seagrass sediments changes over time (Smith et al., 2004) and that seagrass sediment microbial communities change according to nutrient availability (Guevara et al., 2014). Furthermore, seagrass restoration was also found to alter the sediment microbial community (Bourque et al., 2015). These studies suggest that a temporal community pattern may be observed in sediment communities of seagrass meadows and that these communities could also change as a result of seagrass decline.

In the Mediterranean Sea, *Cymodocea nodosa* is a widespread seagrass species declining in coastal areas (Ruiz Fernandez et al., 2009; Tuya et al., 2014; Orlando-Bonaca et al., 2015). The rhizosphere and epiphytic communities of *C. nodosa* have been described (Cúcio et al., 2016; Korlević et al., 2021a), however, little is known about sediment communities underlying *C. nodosa* meadows. The aim of the present study was to characterize the taxonomic composition of sediment communities of a *C. nodosa* meadow and to assess the temporal dynamics of these communities. As the studied meadow experienced a major decline (Najdek et al., 2020), we investigated whether this event affected the sediment microbial community structure.

Materials and methods

Sampling

Sediment cores were sampled in a declining *C. nodosa* meadow (vegetated site) and at an adjacent area without any vegetation (nonvegetated site) both located in the Bay of Saline, east coast of the northern Adriatic Sea (45°7′5″ N, 13°37′20″ E). (Figure 1). One sediment core from each site was collected monthly from July 2017 to October 2018 (Supplementary Table S1) by diving using 15 cm long plastic core samplers. Sediment samples were immediately transported on ice to the laboratory and stored at −80°C until further processing. A detailed description of the study site, the decline of the *C. nodosa* meadow and the dynamics of environmental conditions during the decline are provided in Najdek et al. (2020). Briefly, at the beginning of the study the seagrass *C. nodosa* formed a large and dense meadow at the vegetated site. Seagrass roots and rhizomes penetrated into slightly gravelly sandy mud, while shoots and leaves were present from the southwestern coastal area up to the central part of the bay which was without any vegetation. Following the regular vegetation minimum in November 2017, shoots and leaves started to decline, while roots and rhizomes persisted longer. At the end of the study,

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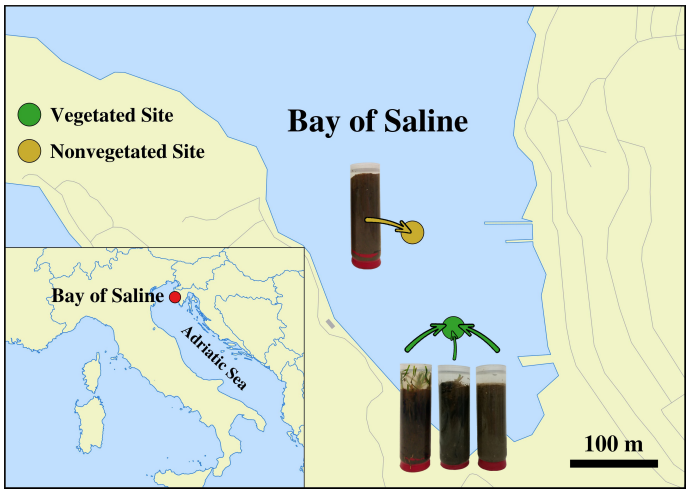

FIGURE 1
Location of the vegetated (declining *Cymodocea nodosa* meadow) and nonvegetated site in the Bay of Saline, northern Adriatic Sea, together with visual representations of vegetated and nonvegetated sediment cores (© OpenStreetMap contributors, www.openstreetmap.org/copyright).

after a severe meadow decline at the vegetated site only very small patches persisted along the shoreline.

DNA isolation

Total DNA from sediment samples was extracted following a modified (Pjevac et al., 2018) isolation protocol of Zhou et al. (1996). Prior to DNA isolation, cores were cut into four different 1 cm sections: top (0 – 1 cm), bottom (7 – 8 cm), and two middle sections: upper middle (1 – 3 cm) and lower middle (3 – 6 cm) section. Sediment samples were weighted (2 g) avoiding roots and rhizomes from vegetated cores, mixed with 5.4 ml of extraction buffer (100 mM Tris [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM Na_3PO_4 [pH 8.0], 1.5 M NaCl, 1% CTAB) and 10 µl of proteinase K (20 mg ml^{-1}) and incubated by horizontal shaking at 225 rpm at 37°C for 30 min. Thereafter 1.2 ml of 10% SDS was added and the mixture incubated again by horizontal shaking at 225 rpm at 65°C for 60 min. The supernatant was collected after centrifugation at 3220 × g at room temperature for 10 min and mixed with an equal volume of chloroform:isoamyl alcohol (1:1). The aqueous phase was retrieved after centrifugation at 3220 × g at room temperature for 10 min. The extraction procedure with the organic solvent mixture was repeated twice. After the final extraction 0.6 volumes of isopropanol were added to precipitate the DNA. The mixture was incubated at 22°C for 60 min and centrifuged at 3220 × g at room temperature for 45 min. The obtained pellet was washed twice with 10 ml of chilled 70% ethanol, centrifuged at 3220 × g at room temperature for 10 min after each washing, and finally resuspended in 100 µl of deionized water.

Illumina 16S rRNA sequencing

The V4 region of the 16S rRNA gene was sequenced using a two-step PCR approach described previously (Korlević et al., 2021b). Briefly, the V4 region was amplified using the 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) primers from the Earth microbiome project (https://earthmicrobiome.org/protocols-and-standards/16s/), which contained a sequence tag on the 5′ end (Caporaso et al., 2011; Caporaso et al., 2012; Apprill et al., 2015; Parada et al., 2016). Purified samples were sent for Illumina MiSeq sequencing (2 × 250 bp) at IMGM Laboratories (Martinsried, Germany) where the second PCR of the two-step PCR approach was performed using primers targeting the tag region incorporated in the first PCR. These primers also contained adapter and sample-specific index sequences. For each sequencing batch, a positive and a negative control were also sequenced. The positive control consisted of a mock community composed of uniformly mixed DNA from 20 different bacterial strains (ATCC MSA-1002, ATCC, USA), while PCR reactions without DNA template served as the negative control. Sequences obtained in this study have been deposited in the European Nucleotide Archive at EMBL-EBI under the accession numbers SAMEA11293274 – SAMEA11293412 and SAMEA6648825.

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Sequence and data analysis

Sequences were analysed on the computer cluster Isabella (University Computing Centre, University of Zagreb) using version 1.45.2 of mothur (Schloss et al., 2009) according to the MiSeq Standard Operating Procedure (MiSeq SOP; https://mothur.org/wiki/miseq_sop) (Kozich et al., 2013) and recommendations given by the Riffomonas project (https://riffomonas.org) to foster data reproducibility. Alignment and classification were performed using the 138.1 release of the SILVA SSU Ref NR 99 database (https://www.arb-silva.de) (Quast et al., 2013; Yilmaz et al., 2014). A cut-off of 97% was used to cluster sequences into operational taxonomic units (OTUs).

Pipeline data processing and visualization were done using R (version 3.6.3) (R Core Team, 2020) combined with packages vegan (version 2.5.7) (Oksanen et al., 2020) tidyverse (version 1.3.1) (Wickham et al., 2019) and multiple other packages (Neuwirth, 2014; Xie, 2014; Xie, 2015; Xie et al., 2018; Xie, 2019; Edwards, 2020; Wilke, 2020; Xie et al., 2020; Xie, 2021a; Xie, 2021b; Allaire et al., 2021; Zhu, 2021). Observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index and Inverse Simpson diversity index were calculated after normalization to the minimum number of reads per sample to account for different sequencing depths using vegan's function `rrarefy` (Oksanen et al., 2020). Chao1 and ACE estimators were calculated using vegan's function `estimateR`, while Shannon and Inverse Simpson diversity indices were obtained using vegan's function `diversity` (Oksanen et al., 2020). To express both diversity indices in terms of effective number of OTUs the exponential of the Shannon diversity index was retrieved (Jost, 2006). The proportions of shared community members between different sediment layers and the two sites were expressed as the Bray-Curtis similarity coefficient calculated on the OTU data table using vegan's function `vegdist` and transformed from dissimilarities to similarities (Legendre and Legendre, 2012; Borcard et al., 2018; Oksanen et al., 2020). The Principal Coordinate Analysis (PCoA) was performed on Bray-Curtis dissimilarities based on OTU abundances using the function `wcmdscale` (Legendre and Legendre, 2012; Oksanen et al., 2020). Differences between communities of different layers, sites, years, and decay periods were tested by performing the Analysis of Similarities (ANOSIM) using vegan's function `anosim` and 1000 permutations (Oksanen et al., 2020). When differences between years or decay periods were tested samples were grouped based on sampling year (2017 and 2018) and decay of roots and rhizomes (before and after decay). The period prior to the decay included samples retrieved from the begining of the study until and including February 2018, while the period after the decay included samples taken after February 2018. To calculate the proportion of OTU community variation explained by environmental variables (redox potential [E_h], oxygen [O_2], hydrogen sulfide [H_2S], sulfur [S^0], organic matter content, and prokaryotic abundance) reported in Najdek et al. (2020) vegan's function `RsquareAdj` was applied on the results of the distance-based Redundancy Analysis (db-RDA) (Borcard et al., 2018; Oksanen et al., 2020). To calculate the db-RDA vegan's function `capscale` on OTU data and explanatory environmental variables was performed. The analysis was computed using the Bray-Curtis dissimilarity index and the Lingoes correction for negative eigenvalues (Legendre and Legendre, 2012; Borcard et al., 2018; Oksanen et al., 2020). In addition, differences between richness estimators, diversity indices, and relative sequence abundances were tested by performing the Mann-Whitney *U* test (function `wilcox.test`), when two groups were compared, or the Kruskal-Wallis *H* test (function `kruskal.test`) followed by a pairwise comparison using the Mann-Whitney *U* test (function `pairwise.wilcox.test`), when more than two groups were compared. Bonferroni correction was applied to address the problem of multiple comparisons.

In total 3.3 million sequences were obtained after quality curation and exclusion of sequences without known relatives (no relative sequences), and eukaryotic, chloroplast, and mitochondrial sequences. Altogether, 68 samples from the vegetated site and 68 from the nonvegetated site were analysed. The number of reads per sample ranged from 9,722 to 55,381 (Supplementary Table S1). Even with the highest sequencing effort the rarefaction curves did not level off as commonly observed in high-throughput 16S rRNA amplicon sequencing approaches (Supplementary Figures S1, S2). After quality curation and exclusion of sequences as mentioned above, reads were clustered into 89,488 different OTUs. Normalization to the minimum number of sequences (9,722) described earlier resulted in 64,335 distinct OTUs ranging from 1,774 to 3,576 OTUs per sample (Supplementary Figure S3). Based on the positive control, a sequencing error rate of 0.01% was calculated which is in line with previously reported values for high-throughput sequencing data (Kozich et al., 2013; Schloss et al., 2016). Following quality curation, the negative controls yielded on average 34.2 ± 62.6 sequences. The detailed analysis procedure is available in a Github repository (https://github.com/MicrobesRovinj/Markovski_SalineSediment16S_FrontMarSci_2022).

Results

To assess the richness and diversity of microbial communities in sediments of the Bay of Saline the observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index, and Inverse Simpson diversity index were calculated (Figure 2). The observed number of OTUs was similar between the vegetated (2,746.7 ± 398.4 OTUs) and the nonvegetated site (2,883.0 ± 353.1 OTUs) and showed no statistical difference (p = 0.06). Interestingly, both the highest

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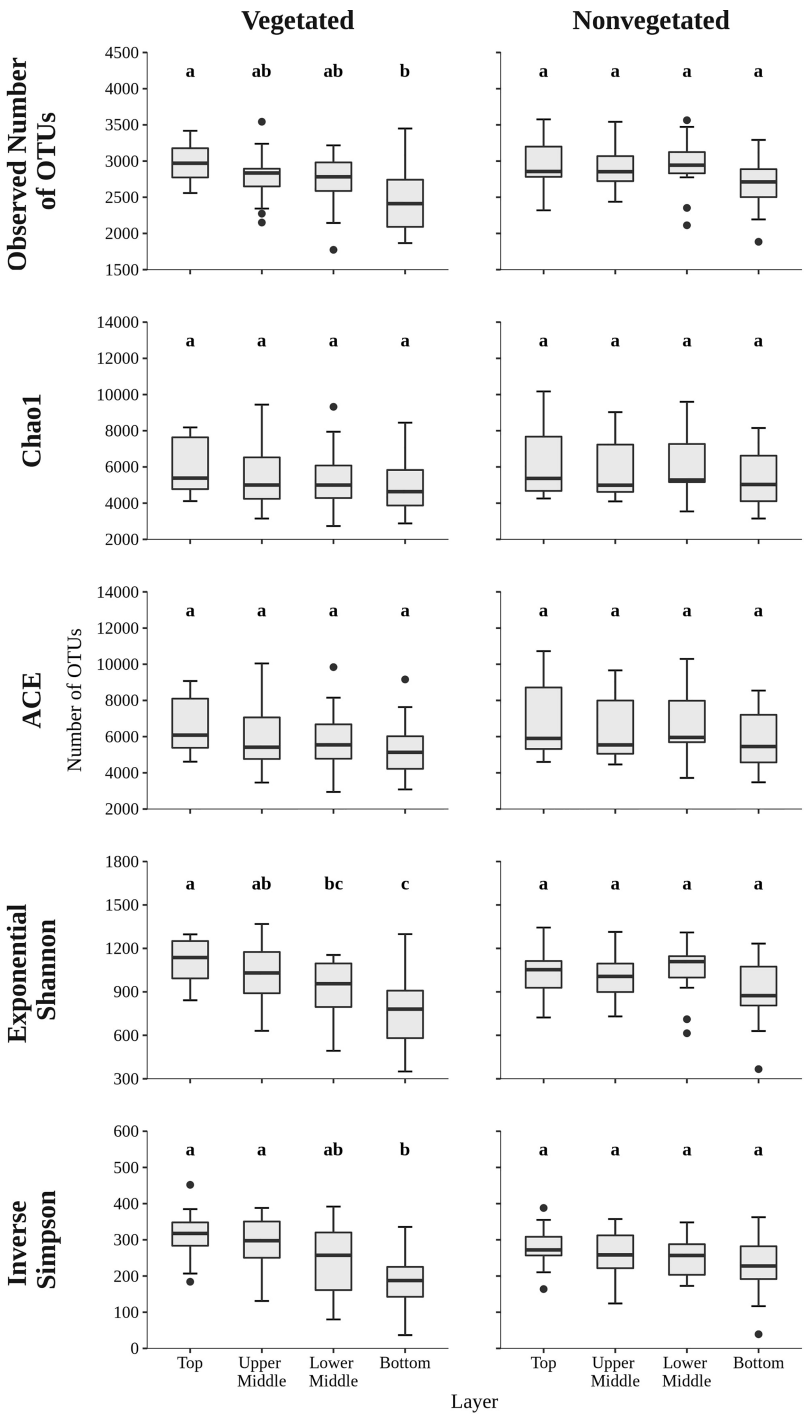

FIGURE 2
The observed number of OTUs, Chao1, ACE, exponential of the Shannon diversity index, and Inverse Simpson diversity index of sediment microbial communities sampled in different sediment layers of the vegetated and nonvegetated site in the Bay of Saline. Different letters correspond to statistically significant differences.

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and lowest number of OTUs were observed at the vegetated site, more specifically the highest number was found in the top layer (2,976.1 ± 262.0 OTUs) and lowest in the bottom layer (2,500.4 ± 462.7 OTUs). These layers were also the only ones showing statistical difference at the vegetated site (Figure 2 and Supplementary Table S2). In contrast, the observed number of OTUs at the nonvegetated site was similar across sediment layers and did not show significant differences (Figure 2 and Supplementary Table S3), although the lowest value was also observed in the bottom layer (2,700.8 ± 378.8 OTUs). During the study period, the observed number of OTUs was variable, with no clear temporal trend observed (Supplementary Figure S3). Chao1, ACE, exponential of the Shannon diversity index and the Inverse Simpson diversity index of sediment communities at the site with and without vegetation were very similar, with no estimate or index showing a statistically significant difference (all $p > 0.1$). In addition, the Chao1 and ACE richness estimators also showed no significant differences between sediment layers (Figure 2 and Supplementary Tables S2, S3). In contrast, diversity indices at the vegetated site showed a difference between the top and bottom layer and between the upper middle and bottom layer, with exponential of the Shannon diversity index also showing a significant difference between the top and lower middle layer (Figure 2 and Supplementary Table S2). At the nonvegetated site, the different sediment layers showed no statistical difference in either richness or diversity (Figure 2 and Supplementary Table S3). Temporal variability in richness estimates and diversity indices was high at both sites, with no clear trend (Supplementary Figures S3, S4).

To evaluate the dynamics of sediment microbial communities Principal Coordinate Analyses (PCoA) of Bray-Curtis distances based on OTU community data were performed. PCoA of all samples differentiated communities based on sediment depth along the first axis, whereas samples from the vegetated and nonvegetated site were separated along the second axis (Figure 3). ANOSIM confirmed that sediment communities in the Bay of Saline differed between sediment layers with some overlap ($R = 0.48$, $p < 0.001$), while the communities of the vegetated and nonvegetated site showed a higher degree of overlap ($R = 0.27$, $p < 0.001$). When communities of different sediment layers were analysed separately, a clearer differentiation between communities of the vegetated and nonvegetated site was observed ($R = 0.45 – 0.49$, all $p < 0.001$). Interestingly, when samples from the same layer of the vegetated and nonvegetated site were compared, the top layers of the sediment showed the highest degree of similarity (Bray-Curtis, 0.64), while the lowest degree of similarity was observed in samples from the upper middle and bottom layers (Bray-Curtis, 0.59) (Figure 3 and Supplementary Figure S5). When samples from each site were analysed

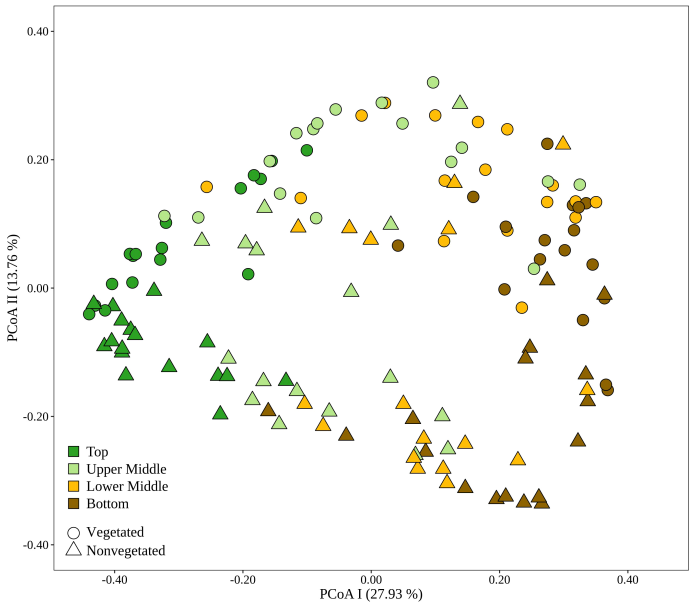

FIGURE 3
Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarities based on OTU abundances of sediment microbial communities sampled in the Bay of Saline. Samples from different sites are labelled with different symbols while samples from different sediment layers are indicated by colour. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.

separately, the previously observed differentiation of samples based on sediment depth was noted (Figure 4) (ANOSIM; vegetated, R = 0.50, $p < 0.001$ and nonvegetated R = 0.49, $p < 0.001$) with the highest degree of similarity observed between samples from middle layers (Bray-Curtis; vegetated, 0.71 and nonvegetated, 0.71) and between lower middle and bottom layers (Bray-Curtis; vegetated, 0.69 and nonvegetated, 0.71) (Supplementary Figure S5). Also, to determine the main environmental parameters governing community changes OTU data were linked to a set of environmental variables reported by Najdek et al. (2020) using db-RDA. Only a small proportion (R_a^2 = 18.3%) of the observed community variation could be explained by the environmental variables. To determine whether there is a temporal succession in the community pattern, samples from each layer and site were analysed separately to exclude the effects of sediment depth and vegetation, which have been shown to primarily influence sediment community structure (Figure 4). No grouping of samples by month was observed in any of the layers and sites analysed. Although Najdek et al. (2020) described a sharp decline in above ground biomass in the same meadow since the beginning of 2018, we did not detect a clearly defined grouping of samples based on sampling year in all the analysed layers (ANOSIM; vegetated, R = 0.06 – 0.26, p = 0.05 – 0.18 and nonvegetated, R = 0.03 – 0.18 p = 0.05 – 0.29). In addition, we also analysed the samples according to the reported decline of roots and rhizomes, as belowground biomass showed a later onset of decline than the aboveground biomass (Najdek et al., 2020). However, this analysis also did not reveal a grouping in any of the tested layers (ANOSIM; vegetated, R = 0.07 – 0.19, p = 0.05 – 0.18 and nonvegetated, R = 0.16 – 0.20, p = 0.05 – 0.06). Furthermore, as with the community analysis, taxonomic classification of all samples also did not indicate a temporal succession but a fairly stable community composition was detected in all layers both at the vegetated and nonvegetated site (Supplementary Figure S6).

Archaeal sequences comprised 9.5 ± 4.7% of all reads. Sequences classified as *Archaea* increased in relative abundance from the top (4.5 ± 1.6%) to the bottom sediment layer (14.1 ± 4.0%). The archaeal community was comprised of *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota*, and *Asgardarchaeota* (Figure 5). *Nanoarchaeota* comprised 3.6 ± 1.3% of all sequences and were evenly distributed across the different sediment layers, whereas all other archaeal phyla showed a depth-related pattern. All *Nanoarchaeota* related sequences were classified as *Woesearchaeales*, with 28.2 ± 13.5% of sequences further classified as SCGC AAA011-D5. A particularly pronounced depth-related pattern was found in *Thermoplasmatota*. Sequences classified as *Thermoplasmatota* comprised 4.1 ± 1.2% of all sequences in the bottom sediment layer and only 0.7 ± 0.6% in the top layer. The majority of sequences related to this group was further classified as Marine Benthic Group D and DHVEG-1. *Crenarchaeota* comprised 1.8 ± 2.3% of all reads. This group had a higher relative sequence abundance at the nonvegetated (2.7 ± 2.8%) than at the vegetated site (1.0 ± 0.9%) ($p < 0.0001$). The vast majority of *Crenarchaeota* related sequences were classified as *Bathyarcheia*. Out of all reads, *Asgardarchaeota* comprised 0.9 ± 0.7% of sequences that could all be further classified as *Lokiarchaeia*.

Overall, bacterial sequences (90.5 ± 4.7%) dominated over archaeal ones and were mainly comprised of *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota*, and *Campylobacterota* (Figure 5). Of all reads, *Desulfobacterota* was the most abundant taxon in the middle (upper middle, 19.4 ± 2.0% and lower middle, 20.2 ± 3.2%) and bottom layers (18.3 ± 3.1%) (Figures 5, 6). *Desulfobacterota* consisted mainly of *Desulfosarcinaceae*, *Desulfatiglandaceae*, *Desulfocapsaceae*, *Desulfobulbaceae*, and uncultured members of the order *Syntrophobacterales* (Figure 6). Sequences classified as *Desulfocapsaceae* showed affinity for the top sediment layer, where they comprised 26.3 ± 8.2% of *Desulfobacterota* reads compared to the bottom layer where they constituted only 3.8 ± 3.3% of *Desulfobacterota* reads. *Desulfosarcinaceae* and *Desulfobulbaceae* varied depending on the site. In the whole microbial community, *Desulfosarcinaceae* reads were more abundant at the vegetated (8.6 ± 2.7%) than nonvegetated site (6.1 ± 2.7%) ($p < 0.0001$), while sequences classified as *Desulfobulbaceae* were less represented at the vegetated (0.8 ± 0.7%) than at the nonvegetated site (1.3 ± 0.7%) ($p < 0.0001$).

Gammaproteobacteria comprised most of the *Proteobacteria* sequences (87.6 ± 4.1%) and made up the majority of all reads in the top sediment layer (23.2 ± 6.2%) (Figures 5, 6). This group was represented with more sequences at the nonvegetated (14.8 ± 8.9%) than at the vegetated site (9.5 ± 7.4%) ($p < 0.001$). Out of all gammaproteobacterial sequences, 25.2 ± 8.3% of reads could not be further classified than to the class *Gammaproteobacteria* (Figure 6). Sequences that could be further classified were mainly assigned to *Thiotrichaceae*, B2M28, *Woeseiaceae*, *Halieaceae* and *Thioalkalispiraceae* (Figure 6). The observed difference between the relative abundance in *Gammaproteobacteria* at the two sites was particularly pronounced for *Thioalkalispiraceae*. Sequences of this group were more abundant at the nonvegetated (1.1 ± 0.8%) than at the vegetated site (0.3 ± 0.3%) ($p < 0.0001$).

Sequences classified as *Bacteroidota* were more abundant in the top sediment layer (16.9 ± 2.7%) with their relative abundance decreasing with sediment depth and reaching a minimum in the bottom layer (6.7 ± 2.2%) (Figures 5, 6). A higher relative abundance of *Bacteroidota* sequences was observed at the vegetated site (12.0 ± 4.5%) than at the nonvegetated site (9.8 ± 4.6%) ($p < 0.01$). *Bacteroidota* were mainly composed of sequences without known relatives within *Bacteroidales*, Bacteroidetes BD2-2, *Cyclobacteriaceae*, *Flavobacteriaceae*, *Prolixibacteraceae* and *Saprospiraceae* (Figure 6). In contrast to *Bacteroidota*, sequences classified as *Chloroflexi* increased with sediment depth (top layer, 4.8 ± 2.0% and bottom layer, 13.8 ± 2.7%) (Figures 5, 7). *Chloroflexi* were

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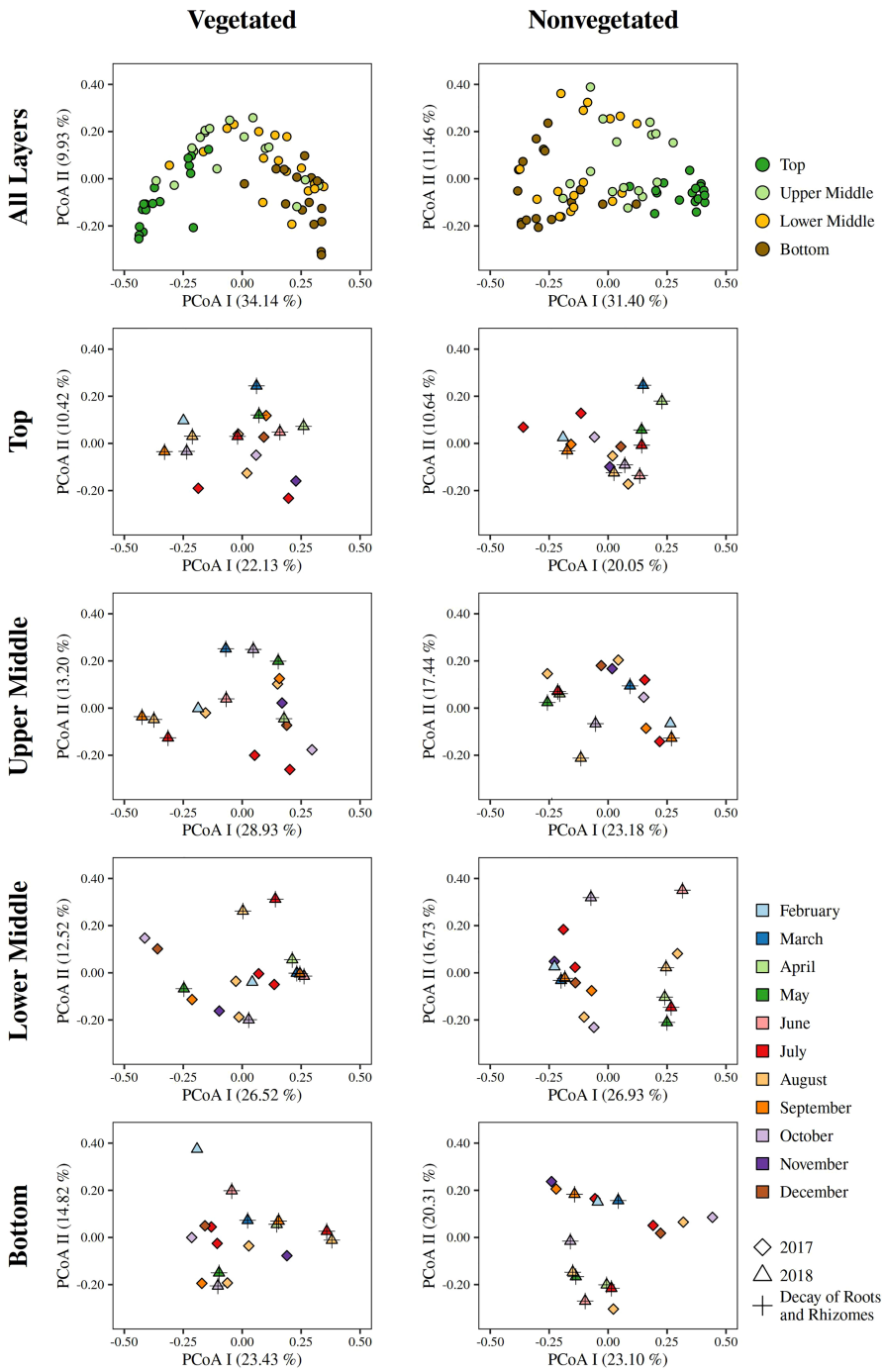

FIGURE 4
Principal Coordinates Analyses (PCoA) of Bray-Curtis dissimilarities based on OTU abundances of sediment microbial communities, of all and individual sediment layers, sampled at the vegetated and nonvegetated site in the Bay of Saline. The proportion of explained variation by each axis is shown on the corresponding axis in parentheses.

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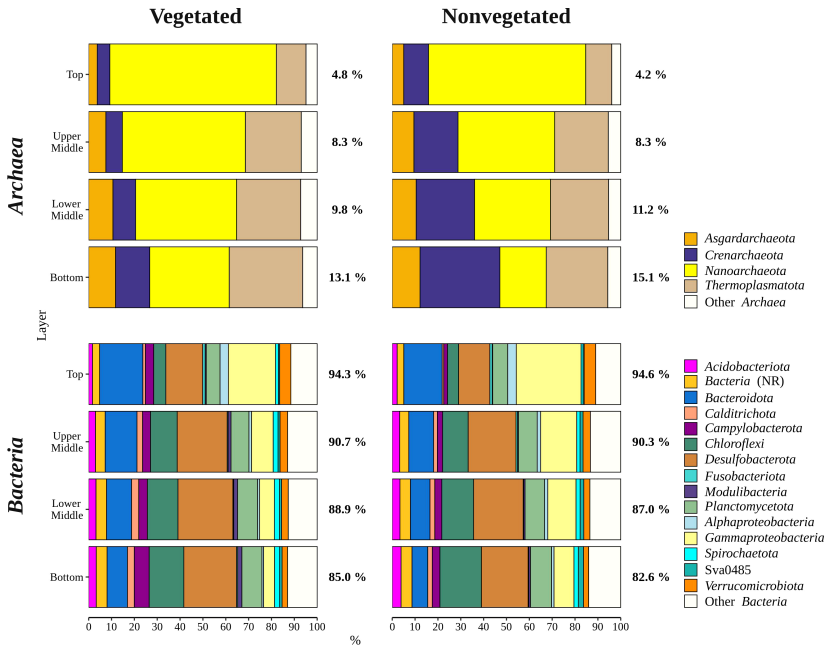

FIGURE 5
Taxonomic classification and relative contribution of the most abundant bacterial and archaeal (≥ 3%) sequences in sediment communities sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known relatives.

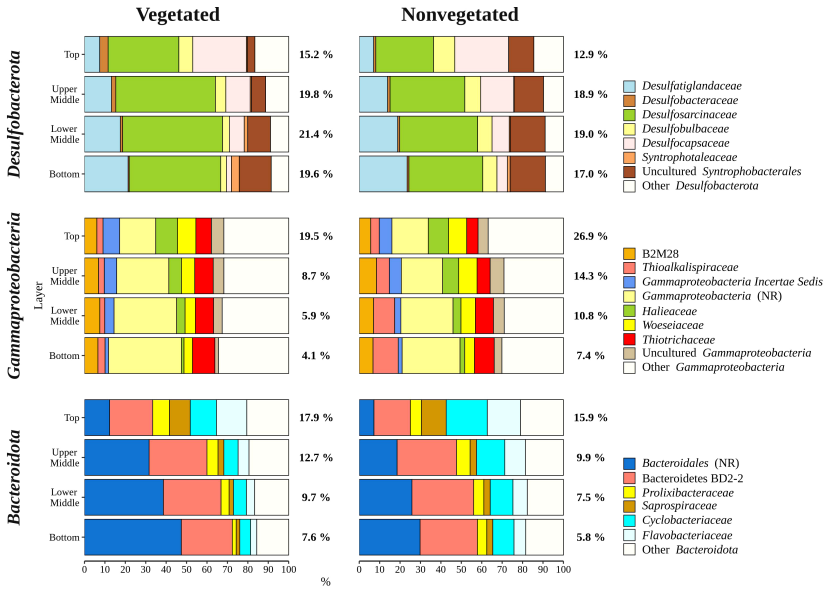

FIGURE 6
Taxonomic classification and relative contribution of the most abundant (≥ 2%) taxonomic groups within *Desulfobacterota*, *Gammaproteobacteria*, and *Bacteroidota* in sediment communities sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known relatives.

mainly composed of *Anaerolineaceae*, while SBR1031, uncultured *Anaerolineae*, sequences without known relatives within *Anaerolineae* and *Dehalococcoidia*, AB-539-J10, and *Ktedonobacteraceae* made up the remainder of the *Chloroflexi* community (Figure 7).

Planctomycetota were evenly represented in the middle (upper middle, 7.3 ± 0.9% and lower middle, 7.6 ± 0.9%) and bottom layers (7.5 ± 1.0%), and less abundant in the top layer (6.0 ± 0.7%), showing no difference between the sites (vegetated, 7.0 ± 1.2% and nonvegetated, 7.1 ± 1.0%) (Figures 5, 7). The *Planctomycetota* community consisted mainly of SG8-4, *Pirellulaceae*, 4572-13, and sequences that could not be further classified (no relative *Planctomycetota*) (Figure 7). A high proportion of *Planctomycetota* reads (39.1 ± 5.1%) were assigned to other *Planctomycetota*, indicating a high diversity within this group. *Campylobacterota* comprised on average 3.1 ± 3.0% of all sequences (Figures 5, 7). Overall, no pattern related to sediment depth was observed for this group. Slightly higher values were characteristic for the vegetated (3.9 ± 3.5%) than the nonvegetated site (2.3 ± 2.3%) ($p < 0.001$). When differences between sites were tested for all sediment layers, only the difference in the top layer between the two sites (vegetated, 3.4 ± 1.8% and nonvegetated, 1.5 ± 1.6%) was significant ($p < 0.01$). Reads related to *Campylobacterota* could be further classified into two families, *Sulfurimonadaceae* and *Sulfurovaceae* (Figure 7). Of these two families, *Sulfurimonadaceae* showed an area-related difference in relative abundance. Higher values were found at the vegetated (2.3 ± 3.4%) than at the nonvegetated site (0.7 ± 1.8%) ($p < 0.0001$). *Sulfurimonadaceae* consisted of the genus *Sulfurimonas*, while *Sulfurovaceae* consisted of the genus *Sulfurovum*.

Discussion

Sediments of seagrass meadows harbour diverse, abundant, and active microbial communities (Smith et al., 2004; Duarte et al., 2005; Sun et al., 2015). Although research on microbial communities of seagrass meadows mainly focused on rhizosphere communities, some studies also included the underlying and surrounding sediment (Jensen et al., 2007; Cúcio et al., 2016; Zhang et al., 2020). As with most sediments, a vertical structuring has been found in the microbial communities of seagrass meadow sediments (Sun et al., 2020). Furthermore, a difference between prokaryotic communities of seagrass meadow sediments and nonvegetated sediments has been observed (Ettinger et al., 2017; Zheng et al., 2019). Temporal studies of these communities are generally rare,

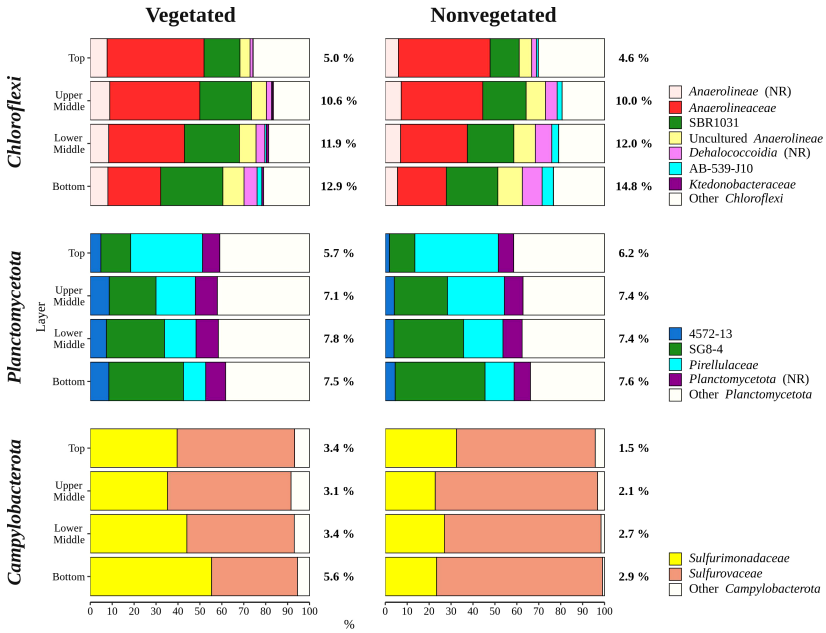

FIGURE 7
Taxonomic classification and relative contribution of the most abundant (≥ 1%) taxonomic groups within *Chloroflexi*, *Planctomycetota*, and *Campylobacterota* in sediment communities sampled at the vegetated and nonvegetated site in the Bay of Saline. NR – sequences without known relatives.

and little is known about how microbial communities in seagrass meadow sediments change with meadow decline and loss. In this study, we assessed the microbial communities in the sediment of a declining *C. nodosa* meadow to gain further insights into the taxonomic composition, vertical structuring, and dynamics of microbial communities in seagrass meadow sediments while at the same time comparing them with bare sediments of a nearby site.

Shannon and Simpson indices account for both richness and evenness and are less sensitive to rare taxa than richness estimators such as ACE and Chao1 (Bent and Forney, 2008). We found no difference in richness (Chao1 and ACE) between sediment layers, suggesting that the observed rare taxa did not play a key role in the vertical structuring of the sediment community in the Bay of Saline (Figure 2). In contrast, diversity indices at the vegetated site showed a depth related pattern (Figure 2). Diversity was highest in the first centimetre of the sediment and differed from the deepest layer (7 – 8 cm). This is consistent with previous studies of marine sediments that describe a decrease in community diversity from the surface to deeper sediment layers, even at small scales within the first few meters (Petro et al., 2017; Hoshino et al., 2020). Seagrasses are known to stabilize the sediment and reduce sediment resuspension (Terrados and Duarte, 2000; van Katwijk et al., 2010). It is possible that the presence of the seagrass, especially roots and rhizomes, increase diversity differences between the top and bottom layer by stabilizing the sediment. In contrast, mechanical mixing may homogenise the sediment together with microbial cells causing more similar microbial diversity in different layers. In addition, seagrass meadows increase the organic matter content of the sediment through the decay of dead tissue (Jensen et al., 2007; Liu et al., 2017), which may have further contributed to the observed differences between sediment layers. Vertical structuring of sediment communities is typically achieved through burial, which is accompanied by selection based on successive changes in environmental conditions (Petro et al., 2017; Kirkpatrick et al., 2019; Marshall et al., 2019). Specific environmental conditions surrounding roots and rhizomes may act as a filter during burial, separating the top from the bottom layer. In contrast, the sediment of the nonvegetated site remained vertically more stable in terms of richness and diversity.

Another component known to differentiate communities in marine sediments besides depth stratification is site-specificity (Polymenakou et al., 2005; Hamdan et al., 2013), which is even more pronounced in seagrass meadows where sediment microbial communities differ not only between the vegetated and nonvegetated area, but also towards the edge of the seagrass patch (Ettinger et al., 2017). In this study, we also observed a grouping of samples according to the two sites (Figure 3), while the microbial communities of both the vegetated and nonvegetated site were stratified according to sediment depth. This is in line with Sun et al. (2020) who noted that the seagrass *Zostera marina* and *Zostera japonica* influence the vertical organisation of microbial communities in the sediment. Although the microbial communities at the vegetated site were distinct from the ones at the nonvegetated site, a high degree of overlap was present. Given that the two sampling sites were in close proximity to each other, a high degree of similarity is not surprising. The microbial communities in the Bay of Saline most likely originate from the same source and only through burial undergo a specific selection characteristic for each site. This type of community structuring (Hamdan et al., 2013; Walsh et al., 2016; Petro et al., 2019) is further supported by the highest degree of similarity between the vegetated and nonvegetated site observed in the top sediment layer. Also, such a high similarity of the top sediment layer may be attributed to imports of seagrass detritus to the nonvegetated site. As one of the main carbon sources in *C. nodosa* meadows (Holmer et al., 2004), seagrass detritus may easily be transported to the adjacent nonvegetated site forming similar communities in the top sediment layer. To assess the temporal dynamics of the microbial community, we analysed each sediment layer and site separately to exclude the influence of sediment depth and site-specificity. Because microbial communities of surface sediments have shorter generation times and higher biomass than communities at deeper sediment strata, and seagrass meadow sediments are hotspots for microbial activity (Duarte et al., 2005; Starnawski et al., 2017), successional changes during the decline of a seagrass meadow could be expected. Surprisingly, the decline of the *C. nodosa* meadow in the Bay of Saline appeared to have little or no effect on the microbial community, as we did not observe any grouping of communities according to month, year, or meadow condition (Figure 4). In addition, no temporal patterns were observed in the taxonomic composition, richness, or diversity of the microbial community. Such a stable community structure and low proportion of community variation explained by the available environmental variables (Najdek et al., 2020) could be caused by a greater proportion of dormant or dead microbial cells remaining in the sediment, leading to a perceived taxonomic stability (Luna et al., 2002; Jones and Lennon, 2010; Cangelosi and Meschke, 2014; Carini et al., 2016; Torti et al., 2018; Bradley et al., 2019). Taxonomic identification by molecular methods such as sequencing of the 16S rRNA gene cannot distinguish between active and dormant cells, nor whether the cell is alive or dead (Cangelosi and Meschke, 2014). Indeed, it has been reported that in coastal marine sediments dead cells account for 70% of all bacterial cells, while among living bacterial cells only 4% grow actively (Luna et al., 2002). Furthermore, it is possible that the change in community composition may be delayed given that microbial communities in marine sediments often have very long generation times (Jørgensen and Marshall, 2016; Starnawski et al., 2017) and that some recognizable remnants of roots and rhizomes were still observed at the end of the study (Najdek et al., 2020). High metabolic versatility of microbial

community members which allows functional continuity to be maintained despite changes in composition (Louca et al., 2018), may also allow for some degree of compositional stability despite changing environmental conditions. Indeed, a decoupling of microbial composition and biogeochemical processes has been observed in sediments. Bowen et al. (2011) have shown that microbial communities in sediments are able to resist compositional changes despite significant variations in external nutrient supply, while Marshall et al. (2021) found that the composition of the nitrogen cycling community might change but these compositional changes are not reflected in functional changes.

The archaeal community of both sites was comprised of *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota*, and *Asgardarchaeota* which are all typical sediment members (Zheng et al., 2019; Sun et al., 2020). We found a nearly threefold increase in the relative abundance of *Archaea* in the deepest sediment layer compared to the top layer (Figure 5). This is not surprising as it has been well documented that *Bacteria* dominate the upper sediment layers while at deeper layers the distribution between *Bacteria* and *Archaea* is more uniform (Chen et al., 2017). A particularly pronounced increase in relative abundance with depth was observed for *Thermoplasmatota* at both sites. It is possible that oxygen penetration in the uppermost sediment layer caused such a pronounced change as representatives of the Marine Benthic Group D and DHVEG-1, accounting for the majority of sequences within the phylum *Thermoplasmatota* (Rinke et al., 2019), are known to be restricted to anoxic environments (Lloyd et al., 2013).

The main difference between the archaeal community of the vegetated and nonvegetated site was the increased presence of *Crenarchaeota* in the nonvegetated sediment (Figure 5). This difference resulted from a much greater increase in the relative abundance of *Bathyarcheia* with increasing depth at the nonvegetated site (Figure 5). In a study comparing archaeal communities in the sediment of a *Zostera marina* meadow with those of bare sediment, a higher presence of *Bathyarchaeota* was found in the vegetated sediment, which is not consistent with our results (Zheng et al., 2019). This discrepancy could have been caused by patchiness and different sampling strategies. In contrast to the three samples per vegetated and nonvegetated sediment in the study of Zheng et al. (2019), we analysed sixty-eight samples from each site. *Bathyarcheia*, formerly known as the Miscellaneous Crenarchaeotal Group (MCG), are typically present in deeper sediment layers as they are well adapted to energy limitation (Kubo et al., 2012). Since seagrasses are known to directly and indirectly enrich the underlying sediment with organic matter (Terrados and Duarte, 2000; Duarte, 2002; Duarte et al., 2005; Jensen et al., 2007; van Katwijk et al., 2010; Liu et al., 2017), it is possible that the presence of *C. nodosa* caused the observed lower relative abundance of this group in the sediment at the vegetated site.

The sediment bacterial community of both sites consisted of taxonomic groups commonly found in marine sediments such as *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*, *Chloroflexi*, and *Planctomycetota* (Walsh et al., 2016; Hoshino et al., 2020), along with *Campylobacterota*, characteristic of seagrass meadows (Jensen et al., 2007). These major groups showed different patterns in relative abundance depending on sediment depth (Figures 6, 7). The proportion of *Gammaproteobacteria* and *Bacteroidota* decreased with sediment depth, while the relative abundance of *Chloroflexi* increased (Figure 6). Although the proportion of *Desulfobacterota* remained similar in all sediment layers, *Desulfocapsaceae*, a major constituent of the *Desulfobacterota* community, decreased with sediment depth (Figure 6). *Gammaproteobacteria* and *Desulfobacterota* (formerly known as *Deltaproteobacteria*), were reported to decrease with sediment depth, while *Chloroflexi* increased (Petro et al., 2017). Also, Smith et al. (2004) documented no vertical trend in sulphate-reducing prokaryotes (*Desulfobacterota*) over a similarly small depth range. Reduction of sulphate is one of many processes that affects pH in sediments, while oxygen penetration controls the depth of pH minima (Silburn et al., 2017). Because *Desulfocapsaceae* are neutrophilic (Galushko and Kuever, 2021) it is possible that depletion of oxygen below the first centimetre and an increase in hydrogen sulphide with sediment depth (Najdek et al., 2020) contributed to the observed vertical trend of this group. The pronounced decline in *Gammaproteobacteria* after the top centimetre could also be attributed to the oxygen penetration depth observed in the Bay of Saline (Najdek et al., 2020) coinciding with the abrupt change in the relative abundance of this class. Oxygen availability could also influence the vertical distribution of *Chloroflexi* and *Planctomycetota* (Figure 7), as these phyla are known to be prevalent in anoxic sediments (Hoshino et al., 2020). In addition to oxygen availability, the decline of *Gammaproteobacteria* and *Bacteroidota* with sediment depth may also be related to the lower availability of fresh organic matter in deeper layers (Middelburg, 1989), as both of these groups are known to break down and assimilate fresh detritus in coastal sediments (Gihring et al., 2009).

The differences in taxonomic composition of microbial communities from the vegetated and nonvegetated site were not as pronounced as those influenced by sediment depth. *Gammaproteobacteria* made up a large proportion of the microbial community at the nonvegetated site, and as with vertical structuring, their higher presence at this site could be explained by oxygen availability. This class contains representatives with a wide range of metabolisms, including aerobic species (Gutierrez, 2019), which could benefit from the higher oxygen availability at the nonvegetated site (Najdek et al., 2020). Indeed, a study by Ettinger et al. (2017) also found a higher presence of *Gammaproteobacteria* in the sediment outside a seagrass meadow. The most pronounced difference in the taxonomic composition of this class between the vegetated and nonvegetated site is the higher relative abundance of *Thioalkalispiraceae* in the nonvegetated sediment (Figure 6). This higher relative abundance could be due to differences in organic matter content. In fact, *Thioalkalispiraceae* are known to

be chemolithoautotrophs (Mori et al., 2011; Mori and Suzuki, 2014) and thus may rely on inorganic compounds rather than organic matter supplied by the seagrass. Slight differences were also observed in the *Desulfobacterota* community between the vegetated and nonvegetated site. *Desulfosarcinaceae* were more pronounced at the vegetated site, while *Desulfobulbaceae* were more pronounced at the nonvegetated site (Figure 6). Although both families have been associated with the rhizosphere of seagrasses (Cúcio et al., 2016), our results are consistent with previous studies that reported a high presence of *Desulfosarcinaceae* in vegetated sediments and higher relative abundances of *Desulfobulbaceae* in the nonvegetated sediment (Smith et al., 2004; García-Martínez et al., 2009). The most abundant *Desulfobacterota* familiy at both the vegetated and nonvegetated site was *Desulfosarcinaceae*. The high metabolic versatility of this group (Watanabe et al., 2020) may have lead to its even greater proliferation at the vegetated site (Figure 6) where high concentrations of different carbon substrates may become available during decomposition of organic matter. In contrast to *Gammaproteobacteria* and *Desulfobacterota*, a higher relative abundance of *Bacteroidota* at the vegetated site may be influenced by the presence of the plant itself. Seagrass cell walls contain polysaccharides like cellulose (Pfeifer and Classen, 2020) and *Bacteroidota* have been identified as decomposers of macromolecules such as cellulose (Thomas et al., 2011). The differences between the vegetated and nonvegetated sediment communities were also reflected in the higher proportion of *Campylobacterota* related sequences at the vegetated site. *Campylobacterota*, formerly known as *Epsilonproteobacteria*, are known to be closely associated with roots and rhizomes of seagrasses, particularly *Sulfurimonadaceae* (Jensen et al., 2007). In this study, the family *Sulfurimonadaceae* also contributed highly to *Campylobacterota* at the vegetated site (Figure 7). This high contribution may be caused by close proximity of the sampled sediment to roots and rhizomes. The seagrass selects the rhizosphere microbial community from the surrounding bulk sediment by enrichment of certain taxa and depletion of others (Cúcio et al., 2016; Zhang et al., 2020; Zhang et al., 2022). It may be possible that roots and rhizomes to some degree also alter the composition of the community in the surrounding sediment in their close proximity forming the observed structure of *Campylobacterota* in our samples.

Taken together, sediment microbial communities in the Bay of Saline were depth stratified, and differed between the vegetated and nonvegetated site, however, remained temporally stable. Although the *C. nodosa* meadow experienced a sharp decline during the investigation period, no pronounced change in the microbial community was observed. The characterization of the sediment microbial community of the declining *C. nodosa* meadow in the Bay of Saline forms the basis for further studies based on methods that can differentiate active communities or methods that can provide insight into the prevailing metabolic processes during the period of seagrass decline.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repositories andaccession numbers can be found in the article.

Author contributions

MN, GH and MK designed the study. MM, MN and MK performed sampling and laboratory analyses. MM and MK analyzed the data. MM prepared the manuscript with editorial help from MN, GH and MK. All authors contributed to the article and approved the final submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.966070/full#supplementary-material

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3.3 Shift in the metabolic profile of sediment microbial communities during seagrass decline

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Shift in the metabolic profile of sediment microbial communities during seagrass decline

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Abstract

Background Seagrass meadows are highly productive ecosystems that are considered hotspots for carbon sequestration and microbial activity. In seagrass sediments, microbial communities break down organic matter, facilitating the release and transformation of nutrients that support plant growth and primary production. The decline of seagrass meadows of various species has been documented worldwide, including that of *Cymodocea nodosa* (Ucria) Ascherson, a widespread seagrass in the Mediterranean Sea. To assess the influence of seagrass decline on the metabolic profile of sediment microbial communities, metaproteomes from two sites, one without vegetation and one with a declining *Cymodocea nodosa* meadow, were characterised at monthly intervals from July 2017 to October 2018.

Results Prior to seagrass decline, differences in the metabolic profiles between the vegetated and nonvegetated sediment were found, particularly in the deeper sediment layers. During the decline, these differences diminished as microbial communities in nonvegetated sediments exhibited increased protein richness and diversity, aligning more closely with those at the vegetated site. Notably, temporal variations in the structure of the metabolic profile were only observable in the nonvegetated sediment and were also more pronounced at greater sediment depths. Finally, the assessment of proteins involved in organic matter degradation such as ABC transporters, fermentation-mediating enzymes, and proteins involved in dissimilatory sulphate reduction mirrored these shifts.

Conclusions Overall, the main results of this study suggest that the presence of seagrass meadows influences the metabolic profile of microbial communities in sediments, highlighting the distinctions between nonvegetated and seagrass-colonised sediments. In particular, the loss of seagrass leads to a shift in the metabolic profile of sediment communities in the surrounding area, while the metabolic profiles of previously colonised sediments appear to be more resilient to seagrass loss.

Keywords Sediment microbial communities, *Cymodocea nodosa*, Seagrass meadow decline, Northern Adriatic Sea, Metaproteomics, Microbial metabolic profile

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Background

The biomass in marine sediments consists mainly of prokaryotes, whose richness and abundance are comparable to that in the water column [1–3]. The main factor determining the abundance and activity of these microorganisms is the availability of organic matter [3–5]. The complete mineralisation of organic compounds in anoxic environments such as most coastal sediments requires complex microbial interactions [6–9]. The stepwise degradation of organic matter begins with the breakdown of complex organic polymers such as carbohydrates or proteins by extracellular enzymes that can be released into solution or remain associated with the cell. These enzymes convert high-molecular-weight organic matter to substrates that are small enough to be transported into the cell [10]. Part of these hydrolytic products are fermented to short-chain fatty acids (SCFAs) and alcohols that facilitate anaerobic microbial respiration, e.g. by sulphate-reducing bacteria or methanogens [7, 9].

Shallow coastal sediments colonised by seagrasses are considered a special type of habitat where microbes break down organic matter, facilitating the release and transformation of nutrients that support plant growth and primary production [11]. Such areas are hotspots for microbial activity, as seagrasses enrich the sediment with organic matter by excreting organic carbon, trapping organic particles from the water column, and stabilising the sediment. In addition, the decomposition of seagrass leaves, roots, and rhizomes contributes to the enrichment of the sediment with organic matter [11–14]. Consequently, microbial communities in seagrass sediments are metabolically more diverse and active than those inhabiting bare sediments [15]. Taxonomic analyses showed differences between communities at vegetated and nonvegetated sites [16–21] and indicated that microbial communities even differ with respect to the meadow edge [22].

In order to obtain a comprehensive overview of the microbial communities living in sediments colonised by seagrasses, methods that allow functional characterisation, such as metaproteomics, must be applied. This high-throughput "meta-omics" approach is emerging as an important tool for deciphering the key components that determine the function of microbial ecosystems [23]. In addition, this approach has the potential to provide insights into the biogeochemical cycling in marine sediments and to assess the response of microbes to environmental change [24]. Metaproteomics is closely linked to metagenomics, as genome information in combination with data on expressed proteins not only provides information on the functional potential of microbial populations, but also on which metabolism is active in an ecosystem [25]. Metaproteomics has already been used to analyse microbial metabolic processes in cold seeps [26, 27], diffuse hydrothermal venting [28], mudflat aquaculture [29], and chronically petroleum-polluted [30] sediments, but to our knowledge there are no metaproteomic studies on microbial communities in seagrass meadow sediments.

About 19% of seagrass meadows worldwide have been lost since 1880 [31]. A decline of *Cymodocea nodosa* (Ucria) Ascherson, a widespread and common seagrass species throughout the Mediterranean Sea [32], has been observed [33–36], including in the northern Adriatic Sea [37–39]. However, there is little information on microbial dynamics during seagrass decline, making it difficult to predict how the loss of seagrass influences the microbial community in the sediment. The few available studies on microbial community succession in seagrass sediments suggest that changes could be expected. For instance, changes in sulphate-reducing bacteria in seagrass bed sediments over time were reported [40], as well as community changes in response to nutrient availability [41] and seagrass restoration [42]. However, in a previous study, we investigated the diversity and dynamics of sediment microbial communities during the decline of the seagrass species *C. nodosa* and found a notable compositional stability in response to such a major disturbance [21]. The aim of the present study was to characterise the metabolic profile of prokaryotic communities in *C. nodosa* meadow sediments using a metaproteomic approach, with the hypotheses that: (i) there are differences in metabolic profiles between vegetated and nonvegetated sediments that vary with sediment depth, and (ii) the decline of seagrass meadows leads to a shift in the microbial metabolic profile that is not uniform across sediment layers.

Methods

Sampling

Sampling for DNA and protein isolation was performed as described in Markovski et al. (2022) [21]. Briefly, sediment samples were collected in a declining *C. nodosa* meadow (vegetated site) in the Bay of Saline, a shallow and dynamic coastal area 4 km north-west of Rovinj, Croatia, on the east coast of the northern Adriatic Sea (45°7′ 5″ N, 13°37′ 20″ E). An adjacent area without seagrass (nonvegetated site) was also sampled in the same bay. From July 2017 to October 2018, a sediment core was taken monthly at each site by diving with plastic core samplers. As seagrass surface sediments show vertical patterns of environmental conditions [39] and microbial community structures [19], the sediment cores were cut into four sections of 1 cm length each: the top (0–1 cm), the bottom (7–8 cm), and two middle sections: upper middle (2–3 cm) and lower middle (3–6

cm; Supplementary Table S1). A detailed description of the sampling site, the environmental conditions, and the decline of the *C. nodosa* meadow can be found in Najdek et al. (2020) [39]. In brief, at the beginning of the study, part of the bay was covered with a large and dense seagrass meadow extending from the south-western coastal area towards the central part of the bay. The seagrass showed a regular growth minimum in November 2017. After that, the shoots and leaves began to decline, while roots and rhizomes persisted longer, until March 2018. At the end of the study, only small patches of the meadow were still present in the form of tiny strips along the shoreline [39]. The decline of the meadow was attributed to the reduced light availability caused by the increased turbidity of the seawater due to the increased terrigenous input [39].

DNA isolation

Total DNA from each sediment section was isolated using a modified isolation protocol [43] based on Zhou et al. (1996) [44] as described in Markovski et al. (2022) [21]. In brief, 2 g of sediment were weighed, avoiding roots and rhizomes in vegetated cores, mixed with the extraction buffer and proteinase K, and incubated by horizontal shaking at 37 °C for 30 min. After the addition of SDS, the mixture was incubated again by horizontal shaking at 65 °C for 60 min. The sediment particles were removed by centrifugation and the supernatant was extracted three times with an equal volume of chloroform:isoamyl alcohol (1:1). DNA precipitation was performed by adding isopropanol and incubating the mixture at 22 °C for 60 min. The DNA pellet obtained after the centrifugation step was washed twice with cold (−20 °C) 70% ethanol, air-dried, and resuspended in 100 µl of deionised water.

Metagenomics

Due to the limited number of sediment metagenomes that could have been sequenced, we selected four DNA samples from Markovski et al. (2022) [21] collected in August 2018 from the top (0–1 cm) and lower middle (4–5 cm) layers of both the vegetated and nonvegetated sites (Supplementary Table S2). These selected DNA samples were sent on dry ice to IMGM Laboratories (Martinsried, Germany) for metagenomic sequencing. The genomic DNA was purified using AMPure XP Beads (Beckman Coulter, USA) at a bead:DNA ratio of 1:1 (v/v) and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). The integrity of the DNA was checked on a 1% agarose gel. Metagenomic sequencing libraries were prepared from 100 or 300 ng of genomic DNA using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs, USA) according to the manufacturer's protocol. Fragments of 500–700 bp were selected using the AMPure XP Beads, enriched by PCR for 5 or 6 cycles, and quality controlled. The individual libraries generated from different DNA input samples were pooled and sequenced on an Illumina NovaSeq 6000 sequencing system (2 × 250 bp).

The sequences obtained were analysed on the Life Science Compute Cluster (LiSC; CUBE—Computational Systems Biology, University of Vienna). MEGAHIT (version 1.2.9) [45], with default settings, was used to assemble individual metagenomic libraries and putative genes were predicted from contigs longer than 200 bp using Prodigal (version 2.6.3) [46] in metagenome mode (-p meta). Predicted genes were functionally annotated using the eggNOG mapper (version 2.1.9) [47] with the eggNOG database (version 5.0.2) [48]. Taxonomic classification was performed using the lowest common ancestor algorithm from DIAMOND (version 2.0.15) [49] against the non-redundant NCBI database (NR). Phylogeny was determined using the top 10% of hits with an e-value < 1×10^{-5} (--top 10). Sequence renaming and the calculation of metagenomic statistics were performed using the tools from BBTools (https://jgi.doe.gov/data-and-tools/bbtools). In total, metagenomic sequencing generated between 205,085,833 and 216,556,629 sequence pairs (Supplementary Table S2). After the removal of low-quality reads, sequences were assembled into 21,634,340 to 33,248,196 contigs, with L50 ranging from 590 to 601 bp. Coding sequence (CDS) prediction generated between 27,526,969 and 42,249,295 CDSs, while functional annotation resulted in 19,599,377 to 29,892,039 annotated CDSs.

Protein isolation

The proteins were isolated from the same sediment sections that were used for DNA isolation [21]. For each of the 15 sampling dates, one protein sample was isolated for each of the four sections collected from the vegetated and nonvegetated site, resulting in a total of 120 protein samples (Supplementary Table S1). The SDS-based lysis method with trichloroacetic acid (TCA) precipitation described in Chourey et al. (2010) [50] and modified by Hultman et al. (2015) [51] was used. To 5 g of sediment, 10% (w/w) polyvinylpolypyrolidone (PVPP) was added. The mixture was suspended in 5 ml protein extraction buffer (4% SDS; 100 mM Tris–HCl, pH 8.0) and vortexed. After incubation in boiling water for 5 min, the samples were sonicated and incubated again in boiling water for 5 min. Sonication was performed using the Sonopuls HD 4100 probe sonicator (Bandelin, Germany) equipped with an UW 100 ultrasonic transducer and a TS 103 probe. The solution was sonicated at 75% of

the maximum amplitude (245 µm) for 2 min at an interval of 10 s on and 10 s off. The sediment particles were removed by centrifugation for 20 min at 4 °C and 4,500 × *g*. The supernatant was transferred to a clean tube and mixed with 1 M dithiothreitol (DTT; final concentration 24 mM). The proteins were precipitated with cold (4 °C) 100% TCA (final concentration 20%) overnight at −20 °C. The protein pellet was obtained by centrifugation for 40 min at 4 °C and 10,000 × *g*. The obtained pellet was washed three times with cold (−20 °C) acetone and centrifuged after each washing step for 5 min at 4 °C and 20,000 × *g*. The pellet was transferred to a clean 1.5 mL tube during the first washing step. The dried pellet was stored at −80 °C until further processing.

Metaproteomics

The filter-aided sample preparation (FASP) [52] procedure was used to perform trypsin digestion. Isolated proteins were processed using the FASP Protein Digestion Kit (Expedeon, UK) according to the manufacturer's instructions, with minor modifications [53]. Briefly, the protein pellet was solubilised in the urea sample buffer included in the kit, amended with DTT, and centrifuged to remove larger particles. The trypsin digestion was performed on the column filter overnight at 37 °C for 18 h. The resulting filtrate containing peptides was acidified to a final concentration of 1% trifluoroacetic acid (TFA). Digested peptides were desalted using the Pierce C18 Tips (Thermo Fisher Scientific, USA) according to the manufacturer's instructions and sent to the Proteomics Facilities of the University of Vienna for mass spectrometry analysis.

MS/MS spectra were obtained using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) and searched against a protein database containing amino acid sequences of predicted CDSs from combined metagenomes that were sequenced and analysed as described above. MS/MS spectra were successfully generated for 118 samples (Supplementary Table S1). Before the protein database search, the predicted CDSs were clustered at 90% similarity using CD-HIT (version 4.6.8). Peptides were identified using the SEQUEST-HT engine and validated with Percolator, all within Proteome Discoverer (version 2.1; Thermo Fisher Scientific, USA). The probability of false peptide identification was reduced by applying the target-decoy approach. Only peptides with a false discovery rate < 1% were retained. Protein identification required at least two peptides, including one unique peptide. Quantification of the relative abundance of proteins was conducted using a chromatographic peak area-based label-free quantitative method [54, 55], where the peak areas of unique peptides were summed and normalised to the normalised area abundance factor (NAAF). In total, 67,947 different proteins were identified from the obtained MS/MS spectra. Of all identified proteins, 94.6% were annotated by the eggNOG database. To focus exclusively on microbial communities, only proteins classified as *Archaea* and *Bacteria* using the NCBI database were retained. As a precautionary measure, proteins that were not taxonomically classified as *Archaea* or *Bacteria* by the eggNOG database were also removed, leaving a total of 57,305 proteins in the dataset.

Data analysis

Data processing and visualisation were performed using R (version 4.5.0) [56] combined with the tidyverse package (version 2.0.0) [57, 58] and several other packages [59–78]. The Shannon diversity index was calculated using the function `diversity` from the vegan package (version 2.6.10) [71]. To express the diversity index in terms of the effective number of proteins, the exponential of the Shannon diversity index was calculated [79]. Differences between the number of observed proteins, the exponential of the Shannon diversity index, and the NAAFs between sites, sediment layers, i.e. sections of sediment cores, and the period before and during the decline of the *C. nodosa* meadow were tested by applying the Mann–Whitney *U* test using the function `wilcox.test` [56]. The Bonferroni correction was applied to solve the problem of multiple comparisons using the function `p.adjust` [56]. Differences in the structure of the microbial metabolic profiles between sites, sediment layers, and the period before and during the meadow decline were tested on Bray–Curtis dissimilarities based on protein NAAFs by performing the Analysis of Similarities (ANOSIM) using the function `anosim` from the vegan package and 999 permutations [71]. The grouping of samples into the period before and during meadow decline was based on the status assessment of the *C. nodosa* meadow reported by Najdek et al. (2020) [39], focusing on the belowground biomass. The sampling period from the beginning of the study until February 2018 was labelled the period before decline, while the period after this month was referred to as the period of meadow decline. Principal Coordinate Analysis (PCoA) was performed on Bray–Curtis dissimilarities based on protein NAAFs using the function `wcmdscale` from the vegan package. If necessary, the Lingoes correction method was applied to account for negative eigenvalues [71, 80, 81].

Results

To assess the richness and diversity of isolated proteins from the sediment microbial communities in the Bay of Saline, the number of observed proteins and the

exponential of the Shannon diversity index were calculated. Samples from each layer were grouped based on the sampling site and the period before and during the meadow decline. Comparisons were made both between sampling sites within each period and between different periods at each individual site (Fig. 1). In all layers, significantly ($p < 0.05$) higher numbers of observed proteins were found during the period before meadow decline at the vegetated (top, 35,626–37,937 proteins; upper middle, 32,494–39,996 proteins; lower middle, 35,220–39,713 proteins; and bottom, 32,183–37,440 proteins) compared to the nonvegetated (top, 29,217–36,284 proteins; upper middle, 29,312–36,755 proteins; lower middle, 25,752–33,630 proteins; and bottom, 27,672–33,922 proteins) site. In contrast, no significant changes between sites were observed in all layers during the period of decline. In addition, no significant changes were found at each site between the two periods. When analysing the exponential of the Shannon diversity index, significant changes were observed only in the lower middle and bottom layer (Fig. 1). Here, in agreement with the number of observed proteins, higher values were found during the period before meadow decline at the vegetated (lower middle, 7,594.2–11,300.0 proteins and bottom, 3,927.3–10,300.9 proteins) compared to the nonvegetated site (lower middle, 1,497.2–3,070.7 proteins and bottom, 586.6–2,696.5 proteins). Also, in agreement with the number of observed proteins, no significant changes were observed between the sites during meadow decline. Additionally, the Shannon diversity index showed significant changes in these layers between the two periods. In the lower middle layer of the vegetated site, significantly higher values were observed before (7,594.2–11,300.0 proteins) than during (4,254.3–9,227.5 proteins) meadow decline. However, in the bottom layer of the nonvegetated site significantly higher values were found during (1,815.8–6,775.9 proteins) than before the meadow decline (586.6–2,696.5 proteins).

ANOSIM testing of Bray–Curtis dissimilarities was applied to determine the changes in the structure of the metabolic profile of the sediment microbial communities. When all proteins from all samples were analysed

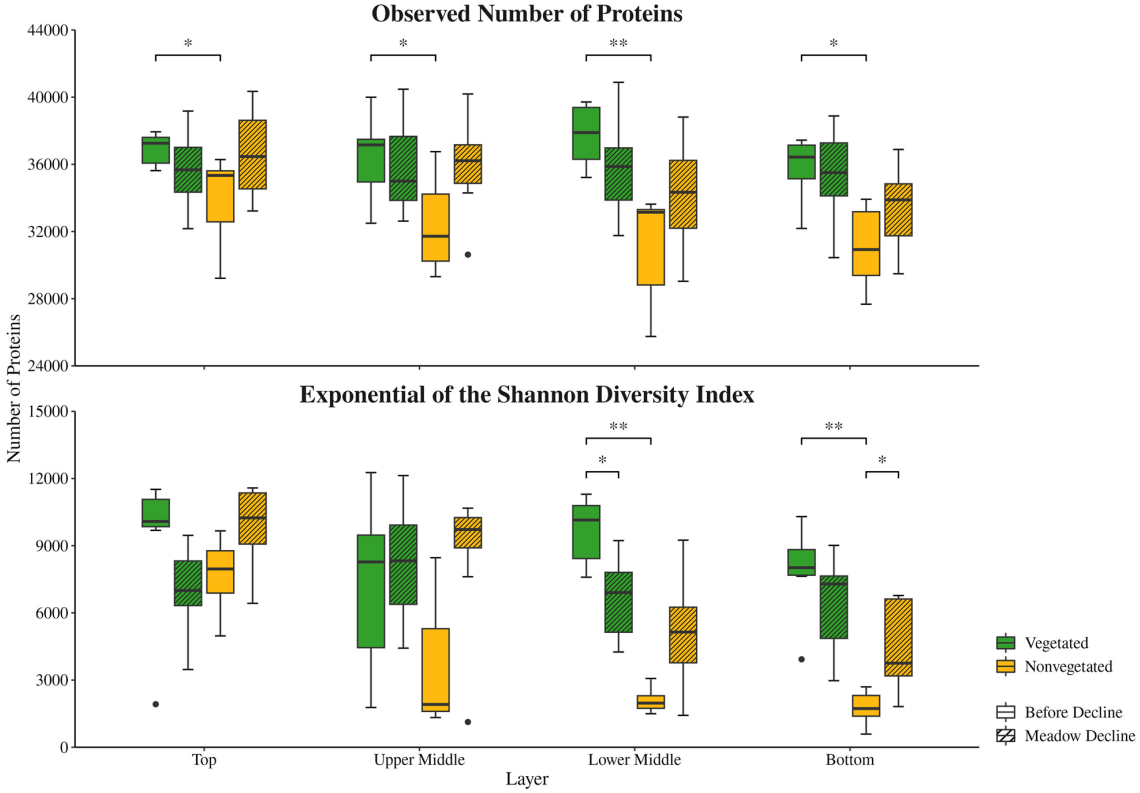

Fig. 1 The observed number of proteins and the exponential of the Shannon diversity index of sediment microbial communities in the Bay of Saline. Samples were collected in different sediment layers at the vegetated and nonvegetated site before and during the decline of the *C. nodosa* meadow. The asterisks indicate the level of statistical significance: ***p* < 0.05 and ****p* < 0.01, while the dots represent outliers

together, no strong differentiation was observed between sites, layers, or the period before and during meadow decline (ANOSIM, R = 0.14–0.24, all $p < 0.01$). To determine whether only a part of the metabolic network showed any differentiation, proteins classified in the Cluster of Orthologous Genes (COG) category C (energy production and conversion), the most abundant category in our samples (see below, Supplementary Table S3), were analysed separately. However, no strong differentiation was observed between sites, layers, or decline periods when only these proteins were considered (ANOSIM, R = 0.15–0.21, all $p < 0.01$). In addition, the separate analysis of samples from the vegetated and nonvegetated site of all and COG C categorised proteins did also not reveal a strong differentiation between layers or decline periods (ANOSIM, R = 0.09–0.26, all $p < 0.01$), with the exception of a more pronounced separation observed at the nonvegetated site between the periods before and during meadow decline. This separation could be observed when all proteins (ANOSIM, R = 0.33, $p < 0.01$) and, especially, when proteins from the functional COG category C (ANOSIM, R = 0.51, $p < 0.01$) were considered. To gain a clearer overview of this separation, samples from the nonvegetated site were analysed using PCoA (Fig. 2). A distinction of samples from the lower middle and bottom layer retrieved during the period before meadow decline from all other samples was noticed. This distinction could be observed when all proteins were analysed together, but especially when proteins from the functional COG category C were considered (Fig. 2). Furthermore, to gain a better insight in the change of the structure of the metabolic profile between the period before and during meadow decline, samples from each site and layer were analysed separately. Sediment layers of the vegetated site did not show any strong differentiation between these two periods when either all (ANOSIM, R = 0.05–0.31, $p = 0.01–0.24$) or only COG C categorised proteins (ANOSIM, R = 0.12–0.30, $p = 0.01–0.07$) were considered. In contrast, a pronounced separation between the two periods was observed in different layers of the nonvegetated site (Fig. 3). When comparing the layers of this site, the lowest distinction between the two periods was observed in the top layer (ANOSIM; all proteins, R = 0.35, $p < 0.01$; COG C proteins, R = 0.38, $p < 0.01$), middle in the upper and lower middle layer (ANOSIM; all proteins, R = 0.29–0.45, all $p < 0.01$; COG C proteins, R = 0.53–0.62, all $p < 0.01$), and the highest in the bottom layer (ANOSIM; all proteins, R = 0.53, $p < 0.01$; COG C proteins, R = 0.95, $p < 0.01$).

A total of 52,270 different proteins were assigned to a COG functional category. The most abundant COG category in terms of the number of proteins it contained (8,224 proteins) and their NAAFs (15.2%) was the functional COG category C, which comprises proteins for energy production and conversion (Supplementary Table S3). To detect how the decline of the meadow affected the energy production and conversion of sediment microbial communities in the Bay of Saline, we assessed the NAAF dynamics of the functional COG category C in each sediment layer. When comparing the sites before the meadow decline, significant ($p < 0.05$) differences were only observed in the bottom layer where the proteins of this functional category comprised a larger proportion at the nonvegetated (19.5–31.7%) than at the vegetated (13.8–26.2%) site. No significant difference was found between the sites during the decline.

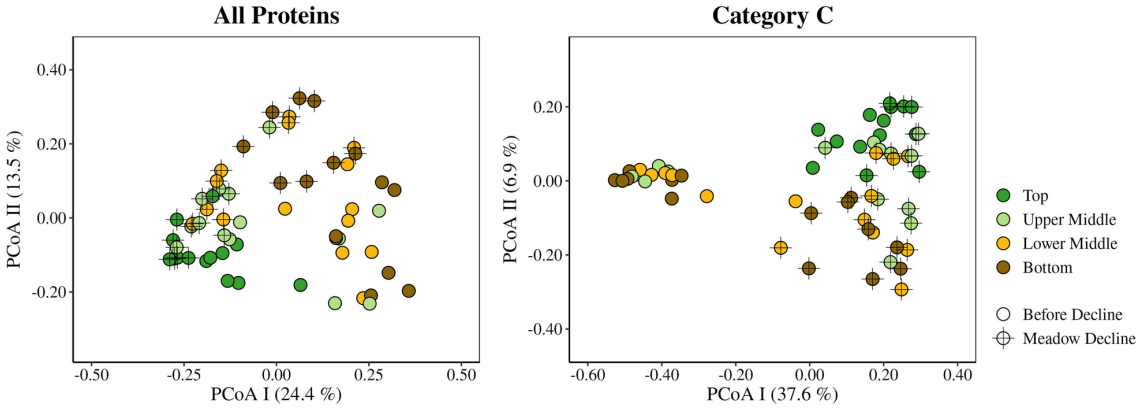

Fig. 2 PCoA of Bray–Curtis dissimilarities of microbial proteins sampled in the sediment of the Bay of Saline. All proteins and proteins classified only into COG category C (energy production and conversion) were analysed. Only samples collected in sediment layers at the nonvegetated site before and during the decline of the *C. nodosa* meadow are shown. The proportion of variation explained by each axis is indicated in parentheses on the corresponding axis

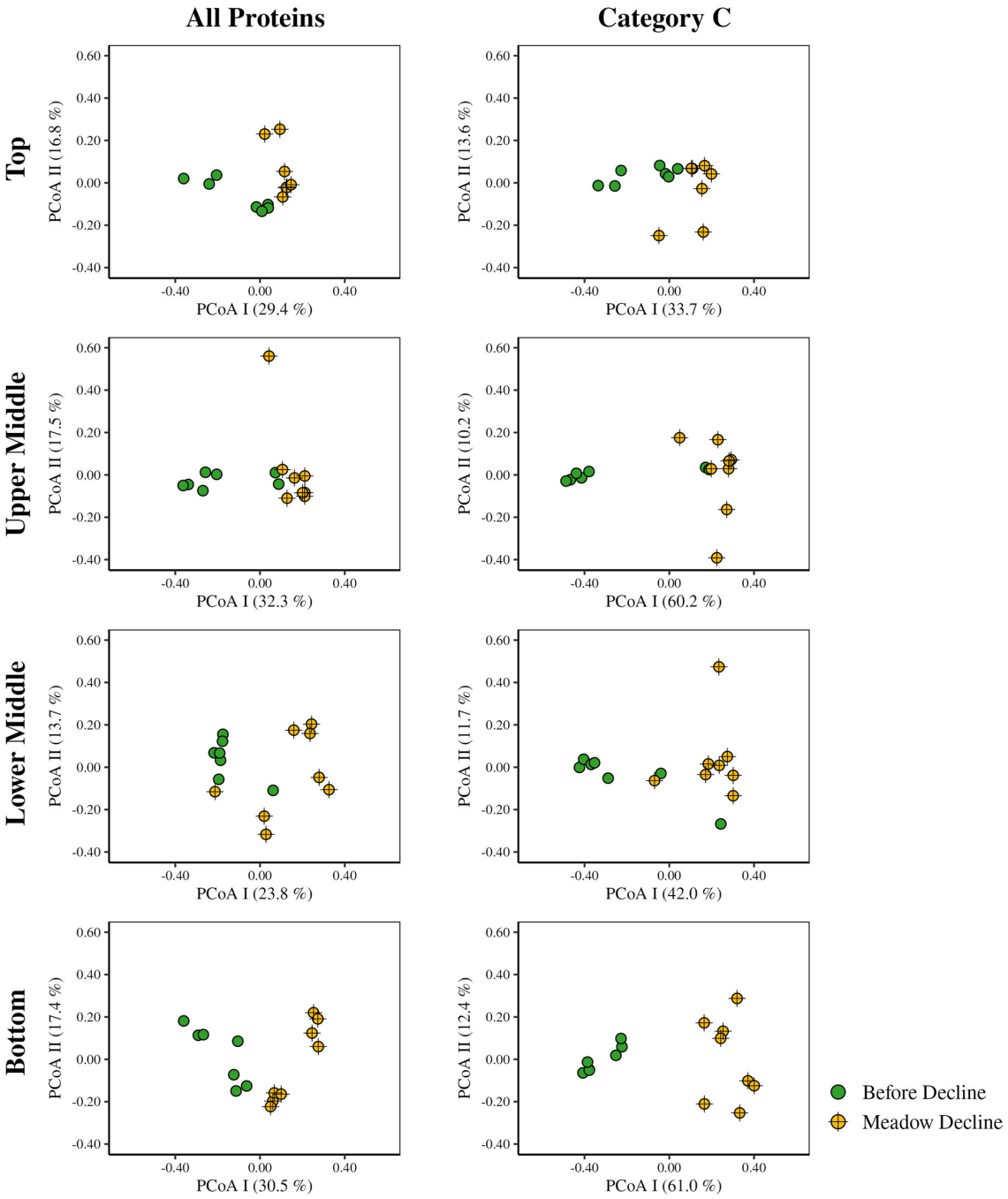

Fig. 3 PCoA of Bray–Curtis dissimilarities of microbial proteins sampled in each layer in the sediment of the Bay of Saline. All proteins and proteins classified only into COG category C (energy production and conversion) were analysed. Only samples collected at the nonvegetated site before and during the decline of the *C. nodosa* meadow are shown. The proportion of variation explained by each axis is indicated in parentheses on the corresponding axis

When comparing the layers of the individual sites before and during the decline, we detected a significant change in the proportion of the functional COG category C only in the bottom layer of the nonvegetated site. Here, a significant decrease in the proportion of this functional category was observed between the period before (19.5–31.7%) and during (8.2–13.9%) meadow decline.

As the COG categories provide only a broad overview, the predicted CDSs were also classified using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthology (KO) database to gain better insight into the metabolic profile. A total of 1,408 different KO entries were present in the dataset, while 37,243 proteins were assigned to one or more of these KO entries. As the functional COG category C was the most abundant in our dataset (Supplementary Table S3), we aimed to further explore the dynamics of the most pronounced KO entries within this category. The F-type H^+-transporting ATPase subunit c (ATPF0C, atpE; 20.0%), the K^+-stimulated pyrophosphate-energised sodium pump (hppA; 7.9%), and the adenylylsulphate reductase subunits A (aprA; 6.2%) and B (aprB; 6.1%) represented the highest proportion (NAAF) within the functional COG category C (Fig. 4). As the samples from the nonvegetated site showed a clear separation based on the decline periods, especially when the COG category C dataset was considered (Fig. 2), we compared the proportion of these three proteins at the nonvegetated site before and during meadow decline (Fig. 4). We observed a significant ($p < 0.05$) decrease in the proportion of the F-type

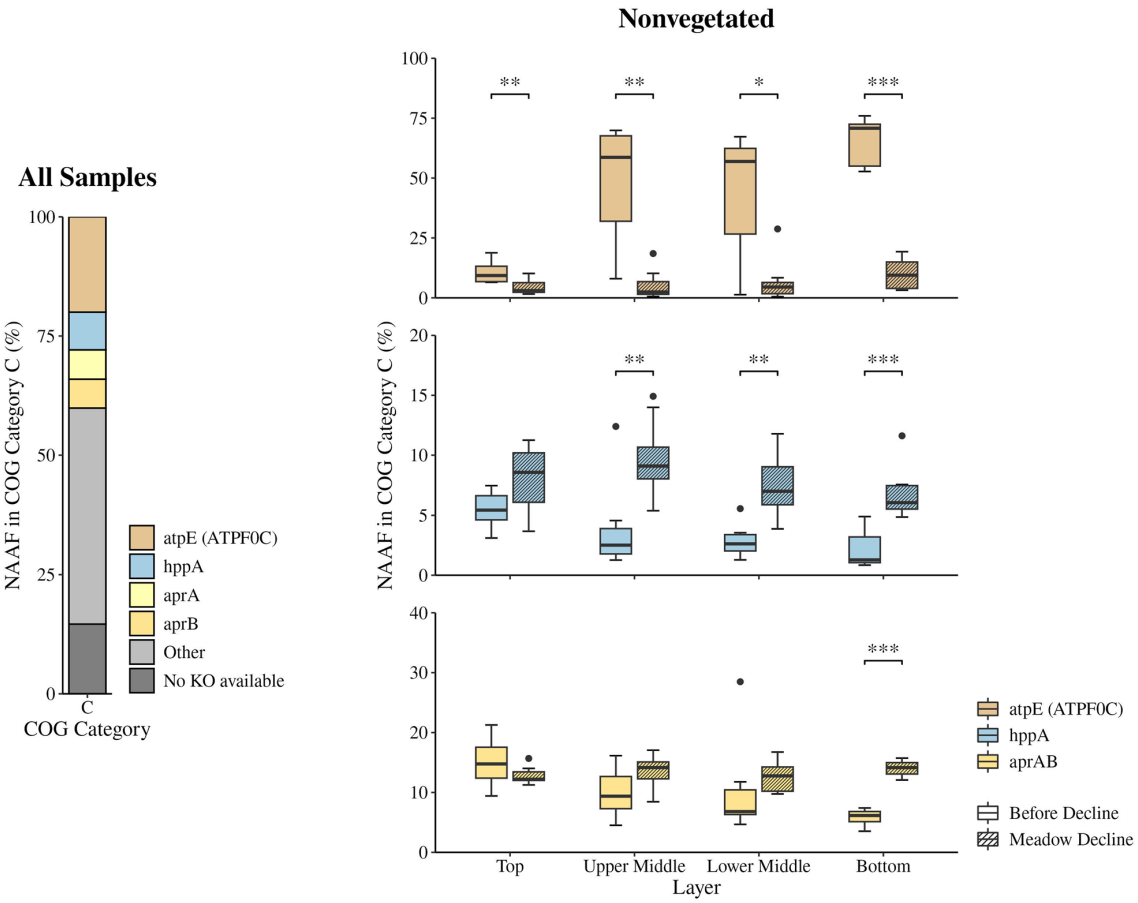

Fig. 4 Proportion of the most abundant (> 3%) KEGG KO entries within the functional COG category C (energy production and conversion) in all samples and changes in the proportion of the same entries in each layer at the nonvegetated site before and during the decline of the *C. nodosa* meadow in the Bay of Saline. The proportion was calculated using the NAAF. The asterisks indicate the level of statistical significance: ***p* < 0.05, ****p* < 0.01, and *****p* < 0.001, while the dots represent outliers

H^+-transporting ATPase subunit c during the decline in all layers. This decrease was particularly pronounced in the bottom layer, where this protein constituted between 52.7 and 76.0% of all COG C categorised proteins before the decline. During meadow decline, its proportion dropped to between 3.2 and 19.3%. Although not as pronounced as the change in the F-type H^+-transporting ATPase subunit c, the K^+-stimulated pyrophosphate-energised sodium pump also showed a significant shift between the two periods in all layers, except the top layer. The most significant shift was observed in the bottom layer, where this protein increased from between 0.8 and 4.9% of all COG categorised proteins before the decline to between 4.9 and 11.6% during meadow decline ($p < 0.001$). The proportion of the adenylylsulphate reductase subunits A and B also increased during the decline in all layers, with the exception of the top layer. However, this shift was only significant in the bottom layer, where this protein increased from 3.5 to 7.4% of all COG categorised proteins before the decline to 12.1 to 15.7% during meadow decline.

The degradation of complex organic matter by the sediment microbial community in the Bay of Saline was evaluated by assessing the dynamics of the carbohydrate, protein, and lipid hydrolytic enzymes (Fig. 5). The dynamics of carbohydrate hydrolytic enzymes was determined using Carbohydrate-Active enZymes (CAZymes), whose proportion did not significantly ($p < 0.05$) change between sites or decline periods, with the exception of the top sediment layer at the nonvegetated site. Here, a significant decrease in the proportion of CAZymes was observed from the period before decline (0.28–0.43%) to the period of meadow decline (0.22–0.28%; Fig. 5). Proteins assigned to the glycoside hydrolase families GH5 and GH9 were the most abundant of all CAZymes (47.2%). To assess protein degradation, we focused on proteins assigned as peptidases in KEGG. The proportion of these enzymes significantly increased in the upper middle layer of the vegetated site from the period before decline (0.15–0.45%) to the period of meadow decline (0.34–0.61%; Fig. 5). Peptidases were almost exclusively comprised of metalloendopeptidases and serine endopeptidases (93.3%). Compared to CAZymes and peptidases, lipases were the least represented in our data (Fig. 5).

We assessed the dynamics of ATP-binding cassette (ABC) transporters to evaluate the uptake of hydrolytic products by prokaryotic cells. Substrate-binding proteins classified as ABC transporters in the KEGG Pathway (map02010) were selected and further manually classified into the following categories based on the molecules they transport: sugar, peptide, amino acid, urea, lipid, polyol, phosphate, and mineral and organic ion (Fig. 5). Sugar (38.0%) and amino acid (31.5%) transporters were the most abundant among all selected ABC transporters. In the lower middle and bottom layer, a significantly ($p < 0.05$) higher proportion of sugar ABC transporters was observed at the vegetated (lower middle, 2.90–4.57%; bottom, 3.90–5.52%) than at the nonvegetated (lower middle, 2.25–3.20%; bottom, 1.75–2.81%) site during the period before meadow decline (Fig. 5). In contrast, no significant differences in the proportion of ABC transporters targeting sugars between sites were observed during the period of meadow decline. These transporters also showed a significant increase in the bottom layer of the nonvegetated site from the period before decline (1.75–2.81%) to the period of meadow decline (2.16–6.79%). The proportion of ABC transporters targeting amino acids only showed a significant increase in the bottom layer of the nonvegetated site from the period before decline (2.05–3.02%) to the period of meadow decline (2.76–4.62%).

To evaluate the role of fermentation processes at these two sites, we selected enzymes from the KEGG database that are thought to be involved in mediating various fermentation products such as carbon dioxide, formate, acetate, acetone, ethanol, lactate, acetoin, propionate, and butyrate (Supplementary Table S4). Of these selected enzymes, our dataset contained pyruvate:ferredoxin oxidoreductase, pyruvate formate-lyase, acetyl-CoA hydrolase, acetate kinase, and alcohol, formate, and lactate dehydrogenase (Fig. 6). Formate dehydrogenase (45.2%), pyruvate:ferredoxin oxidoreductase (31.4%), and alcohol dehydrogenase (17.0%) were the most prominent of all fermentation-mediating enzymes detected. Significantly ($p < 0.05$) higher proportions of formate dehydrogenase were detected before meadow decline in the lower middle layer of the vegetated (0.28–0.45%) compared to the nonvegetated (0.20–0.30%) site. In contrast, no significant differences were observed during the decline. A similar trend was observed for pyruvate:ferredoxin oxidoreductase in the bottom layer, which had a higher proportion of this enzyme before the decline at the vegetated (0.14–0.30%) compared to the nonvegetated (0.09–0.22%) site. However, no significant differences between sites were observed during the decline (Fig. 6). Alcohol dehydrogenase showed significant differences between sites in both the lower middle and bottom layer. In the lower middle layer, a higher proportion of this enzyme was observed at the vegetated than at the nonvegetated site before (vegetated, 0.08–0.16%; nonvegetated, 0.04–0.07%) and during meadow decline (vegetated, 0.07–0.43%; nonvegetated, 0.04–0.11%). The same pattern of higher proportions of this enzyme at the vegetated site before (vegetated, 0.08–0.26%; nonvegetated, 0.05–0.07%) and during meadow

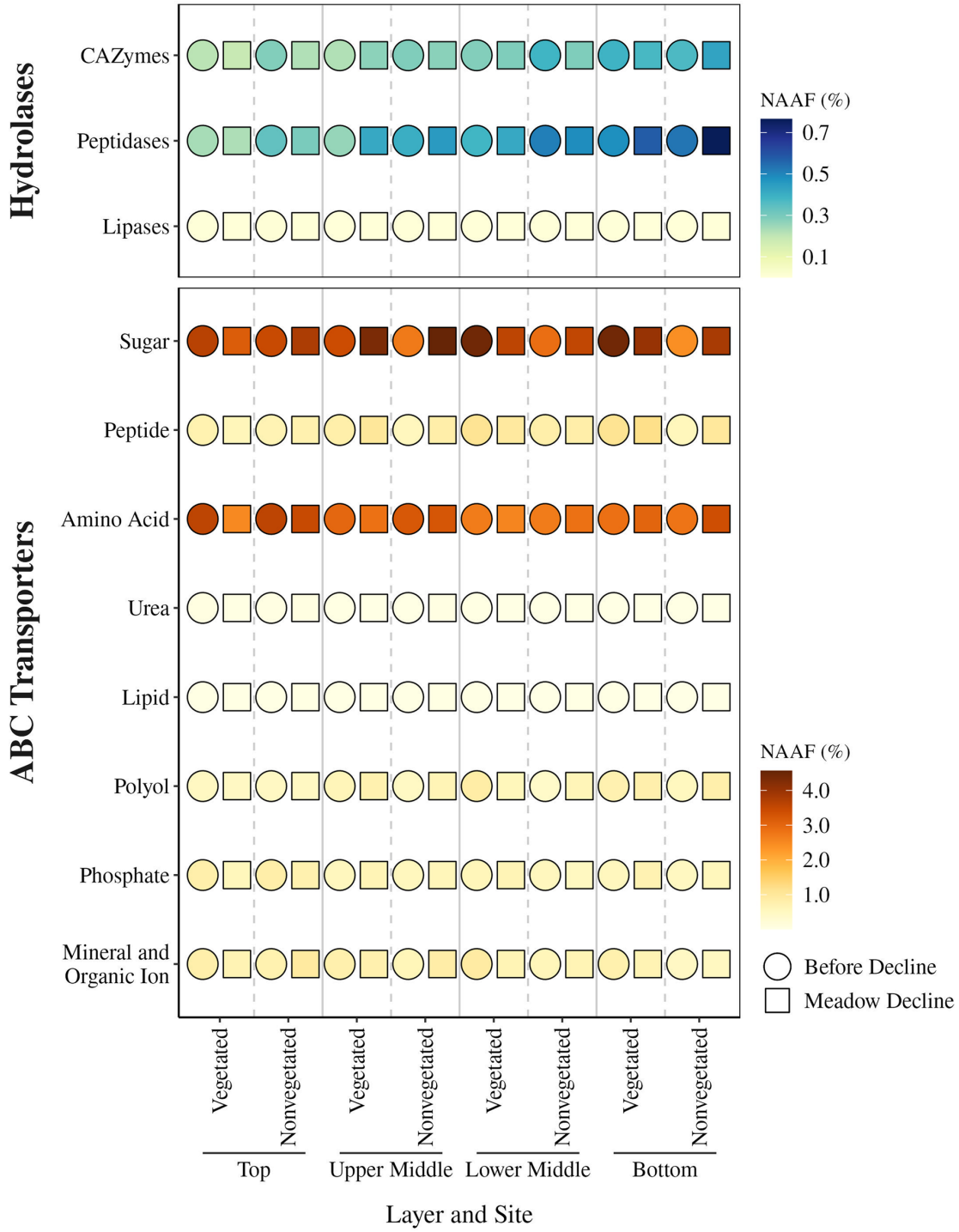

Fig. 5 Median proportion of groups of hydrolases and ABC transporters in sediment layers at the vegetated and nonvegetated site before and during the decline of the *C. nodosa* meadow in the Bay of Saline. The proportion was calculated using the NAAF

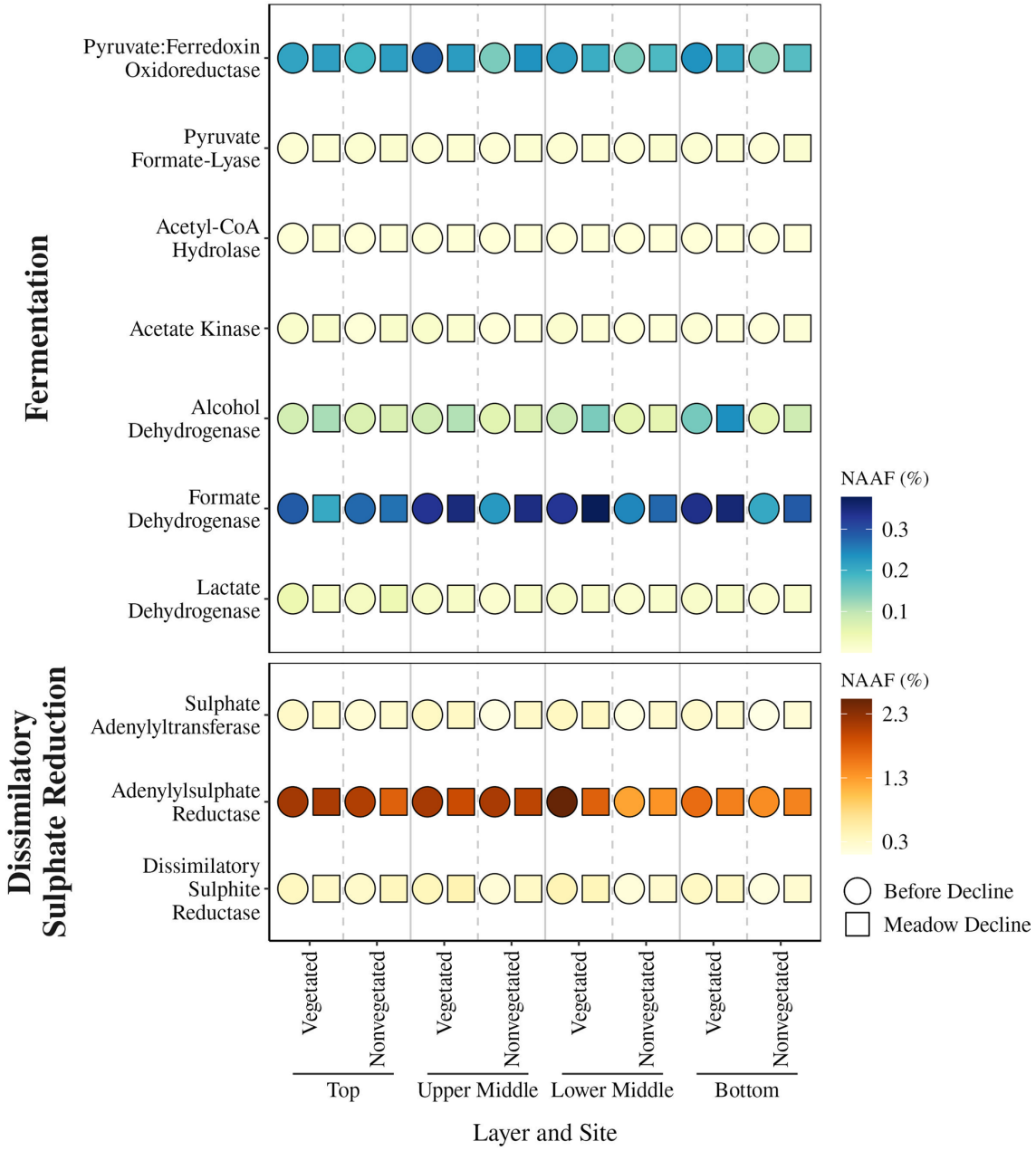

Fig. 6 Median proportion of enzymes involved in mediating various fermentation products and dissimilatory sulphate reduction in sediment layers at the vegetated and nonvegetated site before and during the decline of the *C. nodosa* meadow in the Bay of Saline. The proportion was calculated using the NAAF

decline (vegetated, 0.13–0.88%; nonvegetated, 0.06–0.14%) was also detected in the bottom layer.

To obtain an overview of the different microbial metabolic processes occurring in the sediment of the Bay of Saline, we selected KEGG modules describing methane-, nitrogen-, and sulphur-related processes (Supplementary Table S5). Among all tested modules we found proteins involved in the following processes: methanogenesis,

nitrogen fixation, dissimilatory nitrate reduction, denitrification, assimilatory and dissimilatory sulphate reduction, and thiosulphate oxidation by the SOX complex. Since one of the most prominent proteins in the functional COG category C (energy production and conversion) was the adenylylsulphate reductase (Fig. 4), which is involved in dissimilatory sulphate reduction, the dynamics of the enzymes involved in this process were investigated in more detail (Supplementary Table S6). Of the enzymes involved in dissimilatory sulphate reduction, our dataset contained sulphate adenylyltransferase, adenylylsulphate reductase, and dissimilatory sulphite reductase (Fig. 6). The proportion of adenylylsulphate reductase was much higher (75.0%) than that of sulphate adenylyltransferase (11.1%) and dissimilatory sulphite reductase (13.9%). Significantly ($p < 0.05$) higher proportions of sulphate adenylyltransferase were observed before meadow decline at the vegetated than at the nonvegetated site in the upper middle (vegetated, 0.18–0.55%; nonvegetated, 0.10–0.28%), lower middle (vegetated, 0.27–0.61%; nonvegetated, 0.06–0.19%), and bottom (vegetated, 0.17–0.32%; nonvegetated, 0.06–0.20%) layer. In addition, significantly higher proportions were also found in the bottom layer of the nonvegetated site during decline (0.14–0.35%) compared to the period before meadow decline (0.06–0.20%). Proportions of the adenylylsulphate reductase showed significant changes in the lower middle layer, where higher values were observed before meadow decline at the vegetated (1.99–3.05%) compared to the nonvegetated (1.22–2.53%) site. Also, in the same layer of the vegetated site significantly higher proportions were detected before decline (1.99–3.05%) compared to the period of meadow decline (1.33–2.40%). In the lower middle and bottom layer, dissimilatory sulphite reductase showed higher proportions before meadow decline at the vegetated (lower middle, 0.37–0.78%; bottom, 0.25–0.42%) than at the novegetated (lower middle, 0.16–0.40%; bottom, 0.11–0.21%) site. In addition, significantly higher proportions were detected in the bottom layer of the nonvegetated site during decline (0.17–0.39%) compared to the period before meadow decline (0.11–0.21%). In contrast, no significant differences between sites were observed during the meadow decline for either of these enzymes (Fig. 6).

Discussion

Seagrass meadow habitats are highly productive ecosystems [82] that support high biodiversity [83]. In the present study, before the decline of the *C. nodosa* meadow, higher values of the number of observed proteins and of the exponential of the Shannon diversity index were found in the vegetated compared to the nonvegetated sediment. These differences were more pronounced in the deeper parts of the sediment (i.e. in the lower middle and bottom layer). During meadow decline, the differences began to disappear and the values of the number of observed proteins and of the Shannon diversity index were similar to those observed in the sediment previously inhabited by the meadow. In addition, the structure of the metabolic profile of the communities inhabiting the nonvegetated sediment showed a separation between the period before and during meadow decline, especially in the deeper parts of the sediment and when only proteins for energy production and conversion were considered. This pattern was not observed for the communities at the vegetated site. The difference between the microbial communities inhabiting the vegetated and nonvegetated sediment in the period before meadow decline is not surprising, as several studies have found that the presence of seagrass leads to the formation of sediment communities that differ in composition [16–22] and function [15] from communities inhabiting nonvegetated sediments. In addition, the higher values of the number of observed proteins and of the Shannon diversity index at the vegetated site before the decline are consistent with other studies reporting higher metabolic diversity and microbial community activity in seagrass sediments compared to nonvegetated areas and with higher organic matter content in seagrass-inhabited sediments [11, 15]. In addition, the greater differentiation observed in the deeper layers in terms of protein richness and diversity is consistent with a previous study on the same sediment communities, which found a greater community separation between sites in the deeper parts of the sediment [21]. In contrast, the less pronounced differentiation observed for the same parameters and for the structure of the metabolic profile in the top and upper middle layer could be explained by the input of organic matter derived from the vegetated site, making the communities in the upper part of the sediment more similar to each other. Indeed, organic matter imported from the seagrass meadow has been shown to be an important source for prokaryotes in nonvegetated sediments [84].

The lack of differences between sites during the meadow decline in the number of observed proteins and in the exponential of the Shannon diversity index indicates the presence of a more uniform microbial metabolic profile during this period, similar to the metabolic profile observed at the vegetated site prior to decline. This observation is supported by the greater similarity in the structure of the metabolic profile of the communities at the nonvegetated site during decline with the metabolic profile of the communities in the upper sediment prior to decline. Because seagrass meadows fix the sediment by reducing resuspension rates and sediment mixing [85], we hypothesise that resuspension, mixing, and

transport between sites are enhanced when the meadow is no longer present, allowing greater input of fresh organic matter to the nonvegetated sediment. Indeed, Najdek et al. (2020) [39] reported higher levels of total lipids and organic matter at the nonvegetated site during the *C. nodosa* decline from May to August 2018. The uniformity of the microbial profile observed at the vegetated site during the study could be the result of maintaining the source of organic matter during the decline of the seagrass through the decay of leaves, roots, and rhizomes [11–14].

The analysis of the functional COG categories showed that category C, which includes proteins for energy production and conversion, was the most abundant. This is consistent with the metagenomic study of Habibi et al. (2023) [86], who reported that energy production and conversion was also one of the most abundant functional COG categories in coastal sediments. Among these proteins, F-type H^+-transporting ATPase subunit c, K^+-stimulated pyrophosphate-energised sodium pump, and adenylylsulphate reductase subunits A and B exhibited the highest proportion. The pronounced presence of the F-type H^+-transporting ATPase subunit c in the deeper parts of the nonvegetated sediment prior to decline could be explained by the involvement of this enzyme in the generation of membrane potential. The F-type ATPase can work in both directions, utilising the proton gradient to generate ATP or hydrolysing the ATP to generate the membrane potential [87]. The high proportion of the K^+-stimulated pyrophosphate-energised sodium pump in our dataset indicates the coupling of the energy released by pyrophosphate hydrolysis with the active transport of cations across membranes [88, 89]. The proportion of this protein was increased in the deeper parts of the nonvegetated sediment during meadow decline, reflecting the need of microbial communities for more active cross-membrane transport probably due to the increased input of fresh organic matter from the vegetated site. The high proportion of adenylylsulphate reductase subunits A and B among the proteins for energy production and conversion is not surprising, as this enzyme is part of dissimilatory sulphate reduction to sulphide, a predominant terminal pathway of organic matter mineralisation in anoxic seabeds, where it reduces adenosine-5′-phosphosulphate to sulphite [90]. Furthermore, its significantly higher proportion in the nonvegetated sediment during decline could also be explained by the enhancement of this terminal pathway as a result of increased input of fresh organic matter from the vegetated site.

High molecular weight organic matter in marine sediments must be converted into low molecular weight molecules by various hydrolytic enzymes so that it can be taken up by cells [10]. Important components of organic matter in coastal marine sediments are carbohydrates, proteins, and lipids [91]. In our dataset, CAZymes and peptidases were more abundant than lipases, which may indicate the importance of carbohydrates and proteins as sources of organic matter for the microbial community. Among the CAZymes, the glycoside hydrolase families GH5 and GH9 were the most abundant. These families contain members capable of hydrolysing plant organic matter such as cellulose [92–94]. The presence of enzymes acting on cellulose is not surprising as cellulose is a major component of seagrass cell walls and contributes between 20 and 77% to the dry plant material [95–97]. Metalloendopeptidases and serine endopeptidases made up the vast majority of peptidases in our dataset. A high proportion of these enzymes among the extracellular proteases has already been reported for coastal sediments [98–100]. CAZymes and peptidases showed no dynamics from pre-decline to meadow decline, except that CAZymes decreased in the top layer of the nonvegetated sediment and peptidases increased in the upper middle layer of the vegetated sediment. Studies have shown that the molar carbon:nitrogen content in seagrass litter decreases during the decomposition process, which could be explained by increased microbial colonisation of detrital matter and microbial utilisation of exogenous nitrogen which could be related to the observed decrease in CAZymes and increase in peptidases [12, 14].

Once complex organic compounds have been broken down, the hydrolytic products can be transported into the cells. Prokaryotes utilise various transport proteins, including ABC transporters, to import substrates. Our metaproteomic dataset contained a large amount of ABC transporters for sugars and amino acids. Previous studies of sediment metagenomes also reported a high proportion of ABC transporters [86, 101]. The dynamics of ABC transporters reflected the overall dynamics of the metabolic profile. ABC transporters for sugars showed a higher proportion in the deeper parts of the vegetated compared to the nonvegetated sediment before meadow decline and showed no differences between sites during the decline, reflecting the higher organic matter content and corresponding demand for ABC transporters in seagrass-inhabited sediments [11, 15]. In addition, ABC transporters for sugars and amino acids showed an increase in their proportion in the bottom layer of the nonvegetated sediment from the period before decline to the period of meadow decline, which could be attributed to changes in organic matter content during the decline of the meadow.

The hydrolytic products of the degradation of complex organic matter, such as simple sugars and amino acids, can be imported and consumed by fermenting

microbes. We have identified several enzymes involved in mediating various fermentation products, of which formate dehydrogenase, pyruvate:ferredoxin oxidoreductase, and alcohol dehydrogenase are the most abundant. Microcosm studies of the anaerobic degradation of organic matter in marine sediments revealed that acetate, formate, and ethanol are the most common fermentation products [102, 103]. In addition, direct measurements of sediment pore water have revealed the presence of methanol and ethanol [104]. It is therefore not surprising that the most common fermentation-mediating enzymes we found are putatively involved in the metabolism of acetate, formate, and ethanol. In addition, pyruvate:ferredoxin oxidoreductase and alcohol dehydrogenase have also been reported to be important fermentation-mediating enzymes in Baltic Sea sediments [105]. Similar to the dynamics of the ABC transporters, the fermentation-mediating enzymes also reflected the overall dynamics of the metabolic profile. Formate dehydrogenase, pyruvate:ferredoxin oxidoreductase, and alcohol dehydrogenase showed increased proportions in the deeper parts of the vegetated compared to the nonvegetated sediment before meadow decline, reflecting the higher organic matter content and possibly a higher fermentation rate in seagrass-inhabited sediments [11, 15].

The final step in the anaerobic degradation of organic matter involves the utilisation of simpler compounds such as SCFAs, including acetate and formate, and alcohols by the sulphate-reducing bacteria or methanogens [7, 9]. One of the most prominent proteins in functional COG category C was adenylylsulphate reductase, which is involved in dissimilatory sulphate reduction (Fig. 6). Dissimilatory sulphate reduction is known to be a predominant terminal pathway of organic matter mineralisation in anoxic seabeds [90]. Sulphate adenylyltransferase (Sat), adenylylsulphate reductase (Apr), and dissimilatory sulphite reductase (Dsr), enzymes involved in the dissimilatory sulphate reduction pathway shared by known sulphate-reducing microorganisms [90], were also detected in our metaproteomic dataset. The dynamics of these enzymes showed some common patterns that were comparable to the overall dynamics of the metabolic profile. Overall, higher proportions of these enzymes were found in the deeper parts of the vegetated compared to the nonvegetated site before the meadow decline, while such differences were not present during decline. This pattern as well as the overall dynamics of the metabolic profile could be explained by the higher organic matter content in the seagrass-inhabited sediments before decline [11, 15] and by the increased input of fresh organic matter into the nonvegetated area during meadow decline, probably as a result of increased resuspension rates and sediment mixing [85].

Metaproteomics has great potential to provide insights into the microbial response to environmental change in marine systems [24]. In the present study, metaproteomic analysis of MS/MS spectra using sequenced metagenomes from a subset of the same samples led to the identification of 57,305 proteins, more than in other metaproteomic studies of marine sediments [26, 28–30, 106]. Using this approach, it was possible to assess the dynamics of the metabolic profile of microbial communities in the sediment of a declining *C. nodosa* meadow and the surrounding nonvegetated sediment. Consequently, it was possible to assess the impact of seagrass decline on the metabolic profile of these communities.

Conclusions

Seagrass sediments are considered natural hotspots for carbon sequestration, as some estimates suggest that up to 20% of global carbon sequestration in marine sediments occurs in these carbon-rich sediments, although they occupy only 0.1% of the seafloor [107–109]. Due to the role of seagrasses in carbon sequestration and their decline observed worldwide [31, 33–39], it is important to gain knowledge about the influence of this phenomenon on the processes carried out by the microbial community in the sediment.

The results of the present study show that the differences in the metabolic profile between the microbial communities inhabiting the vegetated and nonvegetated sediment, observed in the period before the meadow decline, disappeared during the decline. In addition, the metabolic profile of the nonvegetated communities approached that of the vegetated communities during the decline of the meadow. This phenomenon was more pronounced in the deeper parts of the nonvegetated sediment, indicating a stronger influence of the seagrass decline on the metabolic profile of the communities in this sediment layer. The differentiation between the vegetated and nonvegetated sediment when the meadow was present and the stronger shift in the profile observed in the deeper parts of the nonvegetated sediment during the decline could be explained by the intensity of the input of organic matter derived from the seagrass [11, 15]. The presence of the seagrass meadow reduces the intensity of resuspension, mixing, and transport between sites [85], thereby reducing the input of organic matter to the areas with bare sediment. However, with the decline of the seagrass, these processes are likely to be more intense, allowing organic matter to spread more easily throughout the area.

While these results provide a valuable foundation for future research, the relatively short sampling duration may not be sufficient to observe the full extent of microbial community response to seagrass decline,

emphasising the need for longer-term studies. Furthermore, additional studies on microbial communities inhabiting sediments influenced by other seagrass species are needed to confirm the generalisability of the results reported in this study.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00750-1.

Additional file

Supplementary file 1 (pdf 55 KB)

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Author Contributions
MN, GJH, and MK designed the study. MM, MN, ZZ, and MK performed sampling and laboratory analyses. MM, ZZ, and MK analysed the data. MM prepared the manuscript with editorial help from MN, ZZ, GJH, and MK. All authors reviewed the manuscript and approved the final submitted version.

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Data Availability
The raw metagenomic sequences obtained in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under the accession number PRJEB75905 (https://www.ebi.ac.uk/ena/browser/view/PRJEB75905). The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [110] partner repository with the dataset identifier PXD054602. Following the recommendations given from the Riffomonas project to enhance data reproducibility (http://www.riffomonas.org), the detailed analysis procedure, including the R Markdown file for this article, is available as a GitHub repository (https://github.com/MicrobesRovinj/Markovski_SalineSedimentMetap_EnvironMicrobiome_2025).

Declarations

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no Conflict of interest.

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4 DISCUSSION

Studies on the temporal dynamics of microbial communities in seagrass sediments are rare (Bourque et al., 2015; García-Martínez et al., 2009; A. Smith et al., 2004), so there is a lack of knowledge on the response of these communities to ecological events, such as the decline of seagrass meadows. Consequently, there were no previously available data on the response of sediment microbes to the decline of seagrass meadows of the species *Cymodocea nodosa*, nor a comprehensive characterisation of the sediment microbial community associated with this seagrass species living outside the rhizosphere (Cúcio et al., 2016). The present doctoral thesis, composed by studying sediment microbes during the decline and loss of a *C. nodosa* meadow in the Bay of Saline, on the eastern coast of the northern Adriatic Sea from July 2017 to October 2018, represents a unique contribution to the understanding of the response of these microbes to such an ecological event. The assessment of the status of the *C. nodosa* seagrass meadow and the environmental conditions in the sediment together with the determination of the community composition and the metabolic profile of the microbial communities in the sediment allowed a comprehensive assessment of the influence of seagrass decline on the microbes in the sediment. In addition, the evaluation of the same parameters in the upper 8 cm of the surface sediment at the site with and without seagrass vegetation enabled the assessment of the changes associated with sediment depth and the influence of seagrass cover on the microbes in the sediment.

4.1 Loss of the seagrass meadow

Part of the seafloor in the Bay of Saline was originally covered with a large *Cymodocea nodosa* meadow extending from the south-western coastal area at a depth of 1.5 m to the central part of the bay at a depth of 4 m. However, by the end of 2018 only a few patches were still present in the form of tiny strips along the shoreline. The biometric analysis of the seagrass showed an increase in shoot biomass and density from July until October 2017, when maximum values were reached. In November 2017, a sharp decline in these biometric indices was observed, followed by a further decline that continued in spring and summer 2018. In September and October 2018, no more seagrass leaves or shoots were found. The biomass of roots and rhizomes also peaked in October 2017, but unlike shoots, it lasted longer and remained stable until March 2018, when it began to decline until the end of the study. The maximum biometric values observed in October 2018 could be related to the unimodal growth pattern of this seagrass species. Indeed, *C. nodosa* shows a biomass maximum in summer, a minimum in winter, and a particularly active growth phase in spring (Agostini et al., 2003; Cancemi et al., 2002; Najdek et al., 2020; Terrados and Ros, 1992; Zavodnik et al., 1998). The shift of the biomass maximum from summer to early autumn could be due to the increased activity of grazers in July and August

2017, as visibly grazed leaves were observed during this period (Cebrián et al., 1996; Heck and Valentine, 2006; Valentine and Duffy, 2006). The sharp decline in the biometric indices of the shoots in November 2017 is consistent with the growth pattern of this seagrass species and its reduced growth in winter (Agostini et al., 2003; Cancemi et al., 2002; Najdek et al., 2020; Terrados and Ros, 1992; Zavodnik et al., 1998). However, the active growth and recovery of the seagrass in spring 2018 did not take place, which initially led to the loss of seagrass shoots and leaves and subsequently of roots and rhizomes.

The loss of the seagrass meadow was most likely triggered in spring 2018 by the increased turbidity of the seawater, which was a result of the intensified terrigenous input and sediment resuspension. The increased terrigenous input was, in turn, indicated by a decrease in salinity and an increase in the concentration of particulate matter in the seawater. Consequently, the increased turbidity reduced the availability of light to the seagrass and impaired its photosynthesis. Reduced photosynthetic activity can be detrimental to seagrass as it impairs the plant's ability to cope with the high concentration of hydrogen sulphide (H_2S) in the sediment (Duarte, Holmer, et al., 2005). Hydrogen sulphide inhibits cytochrome *c* oxidase (Nicholls et al., 2013) and has therefore been identified as a potent phytotoxin (Holmer and Bondgaard, 2001; Koch and Erskine, 2001). The reoxidation of hydrogen sulphide back to sulphate using oxygen diffusing from seagrass roots has been recognised as a coping mechanism of seagrasses against hydrogen sulphide toxicity (Duarte, Holmer, et al., 2005; Hasler-Sheetal and Holmer, 2015; Holmer et al., 2005; Pedersen et al., 2004). Internal oxygen stress and hydrogen sulphide toxicity have indeed been linked to die-off events of seagrass meadows (Borum et al., 2005; Carlson et al., 1994). The importance of hydrogen sulphide is also emphasised by the increase in its concentration and the expansion of its accumulation zone observed during the decline of the meadow in spring and summer 2018. This trend ended in August 2018, when extremely high concentrations of hydrogen sulphide were detected throughout the sediment core. It is therefore not surprising that seagrasses, which are often found in sediments with high sulphide concentrations, have one of the highest light requirements among plants, in order to supply their roots and rhizomes with oxygen and to maintain a considerable amount of non-photosynthetic tissue (Dennison et al., 1993; Duarte, Holmer, et al., 2005; Orth et al., 2006). Therefore, it is once again needed to emphasise that seagrasses are very sensitive to environmental changes, especially those that disrupt light availability, such as sediment load, eutrophication, and epiphyte cover of seagrass leaves (Brodersen et al., 2015; Costa et al., 2015; Halun et al., 2002; Terrados et al., 1998).

4.2 Changes associated with sediment depth

In order to study the sediment microbial communities in the Bay of Saline, their composition and metabolism were evaluated. The microbial community composition was determined by next-generation sequencing (NGS) of the V4 region of the 16S rRNA gene, while the communities metabolism was assessed by determining their metabolic profile using metaproteomics. The microbial community structure, based on bioinformatic analysis of the sequences of the V4 region, revealed that the microbial communities in the sediment were primarily stratified by sediment depth, as suggested by the visual appearance of some sediment cores (Figure 7), and secondarily differed between the seagrass-vegetated and nonvegetated site. Although the community richness estimators (Chao1 and ACE) showed no differences in relation to sediment depth at both sites, the diversity indices (exponential of the Shannon diversity index and Inverse Simpson) revealed a pattern in relation to sediment depth at the vegetated site, with the highest values observed in the first centimetre of sediment and the lowest in the deepest layer (7–8 cm). Diversity indices account for both richness and evenness and are less sensitive to rare taxa than richness estimators, suggesting that rare taxa did not play a key role in the observed changes in alpha diversity associated with sediment depth (Bent and Forney, 2008). This observed change in diversity indices is consistent with previous studies that have described a decrease in alpha diversity from the sediment surface to deeper sediment layers, even at small scales such as within the first few metres (Hoshino et al., 2020; Petro et al., 2017). The cause of the observed changes in alpha diversity associated with sediment depth in the seagrass-vegetated sediment could be the stabilisation of the sediment by the meadow (Terrados and Duarte, 2000; van Katwijk et al., 2010), the increase in organic matter content in the sediment by the seagrass due to the decay of dead tissue (Duarte, Holmer, et al., 2005; Liu et al., 2017; Peduzzi and Herndl, 1991; Trevathan-Tackett et al., 2020), and the creation of specific environmental conditions around roots and rhizomes that can act as a filter when microbial cells are buried, promoting the separation of different layers (Kirkpatrick et al., 2019; Marshall et al., 2019; Petro et al., 2017). In contrast, the stability of alpha diversity throughout the nonvegetated sediment core may be due to the absence or reduced influence of these processes.

The taxonomic classification of the obtained partial 16S rRNA sequences showed the dominance of the domain *Bacteria* over the domain *Archaea*. The bacterial community consisted mainly of *Desulfobacterota*, *Gammaproteobacteria*, *Bacteroidota*, *Chloroflexi*, *Planctomycetota*, and *Campylobacterota*, while the archaeal community mainly comprised *Nanoarchaeota*, *Thermoplasmatota*, *Crenarchaeota*, and *Asgardarchaeota*. All of these taxa are typical of marine

Figure 7. Sediment cores sampled on 14 September 2017 in the Bay of Saline in the *Cymodocea nodosa* seagrass meadow (vegetated site; left) and in the adjacent area without seagrass vegetation (nonvegetated site; right). The black zone, which is indicative of the formation of iron sulphides, is visible in the sediment core sampled from the vegetated site (left). The length of the plastic core sampler is 15 cm.

sediments (Hoshino et al., 2020; Sun et al., 2020; Walsh et al., 2016; Zheng et al., 2019), with *Campylobacterota* being characteristic of the seagrass rhizosphere (Ettinger et al., 2017; Jensen et al., 2007). The observed trend of the proportion of archaea increasing with sediment depth and the proportion of bacteria decreasing is similar to trends observed for deeper sediment cores, which show a dominance of bacteria in the upper sediment parts and a similar proportion of bacteria and archaea in the deeper sediment (Chen et al., 2017). In addition to this general trend, the main taxa whose proportion changed with sediment depth were *Thermoplasmatota*, *Chloroflexi*, *Gammaproteobacteria*, and *Bacteroidota*. The increase in the proportion of *Thermoplasmatota* and *Chloroflexi* with sediment depth and the simultaneous decrease in the proportion of *Gammaproteobacteria* could be related to the measured general oxygen depletion after the first centimetre of sediment. Studies have associated Marine Benthic Group D and DHVEG-1, which make up the majority of *Thermoplasmatota* sequences in the sediment of the Bay of Saline, and *Chloroflexi* with anoxic sediments, while *Gammaproteobacteria* are more characteristic of oxic sediments (Durbin and Teske, 2011; Hoshino et al., 2020; Lloyd et al., 2013). In addition to oxygen depletion, the availability of fresh organic matter could also influence the changes in the proportion of microbial taxa with sediment depth. The ability

of *Gammaproteobacteria* and *Bacteroidota* to degrade and assimilate fresh detritus in coastal sediments may have influenced the decline in their relative abundance with sediment depth, as it is known that fresh organic matter content is less available in deeper sediment layers (Gihring et al., 2009; Middelburg, 1989).

In contrast to the analysis of the V4 region of the 16S rRNA gene, which showed that the microbial communities in the sediment were primarily stratified by sediment depth and secondarily differed between the vegetated and nonvegetated site, the main factor influencing the microbial metabolic profile was the decline of the *C. nodosa* seagrass meadow. The influence of decline was observed in protein richness and diversity, the structure of the metabolic profile, and the assessed proteins involved in organic matter degradation. The influences of sediment depth or site were secondary; that is, the effects of seagrass meadow decline were not observed or were not of the same magnitude at each site and in every layer. Due to the primary influence of seagrass decline, the effect of sediment depth on the microbial metabolic profile is discussed in detail below in Section 4.4, where the effects of seagrass decline are considered.

4.3 Site differences: influence of seagrass cover

Sampling sediment cores from two sites, one with a declining *Cymodocea nodosa* meadow (vegetated site) and the other without seagrass vegetation (nonvegetated site), made it possible to assess the influence of seagrass cover on the microbes in the sediment. As previously mentioned, the analysis of the V4 region of the 16S rRNA gene revealed that the microbial communities in the sediment showed differences between the vegetated and nonvegetated site. These differences were also suggested on some sampling dates by the distinct visual appearance of the sediment cores from the vegetated and nonvegetated site as shown in Figure 7. Changes in alpha diversity with sediment depth were only observed in the vegetated sediment. As these differences between sites are related to sediment depth, they are discussed in detail in Section 4.2 when the changes with sediment depth are considered. The observed differences in microbial communities between sites are not surprising, as it is known that communities in marine sediments are site-specific (Hamdan et al., 2013; Polymenakou et al., 2005), which may only be more pronounced if one of the sites is influenced by seagrass cover. Indeed, data have shown that microbial communities in the sediment differ not only between areas with and without vegetation (Alsaffar et al., 2020; A. Smith et al., 2004; Sun et al., 2020; Zheng et al., 2019), but also between the periphery and the central region of the seagrass meadow (Ettinger et al., 2017). Although the communities from the vegetated and nonvegetated sediment showed differences,

there was still a high degree of overlap in community composition. The highest degree of overlap was observed in the top sediment layer. These observations are not surprising as the microbes living in these sediments likely originate from the same source and only undergo site-specific selection through burial (Hamdan et al., 2013; Petro et al., 2019; Walsh et al., 2016). In addition, the observed highest degree of overlap in community composition in the top sediment layer could be due not only to the same source of origin of the microbes, but also to the same carbon source, as seagrass detritus is one of the main carbon sources in *C. nodosa* meadows and can be imported into the nonvegetated site (Holmer et al., 2004).

Taxonomic analysis of the partial 16S rRNA sequences obtained revealed differences between the vegetated and nonvegetated sites for some groups. *Crenarchaeota* and *Gammaproteobacteria* showed a higher proportion in the sediment of the nonvegetated site, while the proportion of *Bacteroidota* and *Campylobacterota* was higher in the vegetated sediment. Interestingly, different groups within the *Desulfobacterota* showed different affinities, with *Desulfosarcinaceae* showing a higher proportion in the vegetated sediment and *Desulfobulbaceae* in the nonvegetated sediment. The differences in proportions observed for *Crenarchaeota* between sites were the result of the much greater increase in *Bathyarcheia*, the major component of *Crenarchaeota* in the Bay of Saline, with increasing sediment depth at the nonvegetated site. *Bathyarcheia*, formerly known as the Miscellaneous Crenarchaeotal Group (MCG), are well adapted to energy limitation and are usually found in deeper sediment layers (Kubo et al., 2012). It is possible that the presence of *C. nodosa* has caused the observed lower proportion of this group in the vegetated sediment, as seagrasses enrich the sediment with organic matter directly or through various seagrass-mediated processes (Duarte, Holmer, et al., 2005; Liu et al., 2017; Peduzzi and Herndl, 1991; Terrados and Duarte, 2000; Trevathan-Tackett et al., 2020; van Katwijk et al., 2010). The higher proportion of *Gammaproteobacteria* in the nonvegetated sediment could, like its higher proportion in the top sediment layer (Durbin and Teske, 2011; Hoshino et al., 2020), be related to oxygen availability, as oxygen penetration depth was generally higher in the nonvegetated sediment. Indeed, Ettinger et al. (2017) also found a higher proportion of *Gammaproteobacteria* in the sediment outside the seagrass meadow than inside it. *Thioalkalispiraceae*, one of the main groups within the *Gammaproteobacteria*, showed a strong difference in proportion between sites. The higher proportion of this group in the nonvegetated sediment could be related to the lower organic matter content in this sediment, as *Thioalkalispiraceae* are known to be chemolithoautotrophic (Mori et al., 2011; Mori and Suzuki, 2014) and may rely on inorganic compounds rather than the organic matter provided by the seagrass.

In contrast to the proportion of *Crenarchaeota* and *Gammaproteobacteria*, the proportion of *Bacteroidota* and *Campylobacterota* was higher in the vegetated sediment. The higher proportion of *Bacteroidota* in the sediment of the vegetated site could be related to the higher content of plant material in this sediment. Members of *Bacteroidota* have been identified as decomposers of macromolecules such as cellulose (Thomas et al., 2011), and similar to angiosperm land plants, cellulose is the main component of seagrass cell walls (Pfeifer and Classen, 2020; Syed et al., 2016; Torbatinejad and Sabin, 2001). The higher proportion of *Campylobacterota* in the vegetated sediment is not surprising, as this group and especially members of its family *Sulfurimonadaceae* are known to be closely associated with seagrass roots and rhizomes (Ettinger et al., 2017; Jensen et al., 2007). In the Bay of Saline, the *Sulfurimonadaceae* also accounted for a high proportion of the sequences classified as *Campylobacterota* in the vegetated sediment. The proximity of the sampled sediment to seagrass roots and rhizomes probably influenced the higher proportion of *Campylobacterota* and consequently of *Sulfurimonadaceae* in the vegetated sediment. Since seagrasses are known to select the microbial community in the rhizosphere from the bulk sediment by enriching some taxa and depleting others (Cúcio et al., 2016; X. Zhang et al., 2020, 2022), it is possible that seagrass roots and rhizomes alter the microbial community composition in the nearby sediment to some extent in a similar manner.

In general, *Desulfobacterota* was the most abundant high-taxonomic rank in the sediment microbial community in the Bay of Saline. This is consistent with other studies that have identified *Desulfobacterota* as one of the major high-taxonomic ranks in the sediment colonised by seagrass (Cúcio et al., 2016; Ettinger et al., 2017; García-Martínez et al., 2009). Although the proportion of *Desulfobacterota* did not show strong differences between sites or with increasing sediment depth, some groups within this phylum showed a different affinity for vegetated and nonvegetated sediment, as previously mentioned. The observed higher proportion of *Desulfosarcinaceae* in the vegetated sediment and *Desulfobulbaceae* in the nonvegetated sediment is consistent with previous studies that reported a high proportion of *Desulfosarcina*-related sequences in seagrass-vegetated sediments and a higher number of sequences related to *Desulfobulbaceae* in the nonvegetated sediment (García-Martínez et al., 2009; A. Smith et al., 2004). In addition, the high metabolic versatility of *Desulfosarcinaceae* (Watanabe et al., 2020) may have led to their greater proliferation in vegetated sediment, where high concentrations of various carbon substrates may become available during organic matter decomposition (Duarte, Holmer, et al., 2005; Liu et al., 2017; Peduzzi and Herndl, 1991; Trevathan-Tackett et al., 2020).

As already mentioned (Section 4.2), the main factor influencing the metabolic profile of the microbial communities in the sediment was the decline of the *C. nodosa* meadow, while the influence of sediment depth and site were secondary. The influence of site and thus of seagrass cover was particularly evident before the decline of seagrass, especially in the deeper sediment layers. This effect was observed in protein richness and diversity, the structure of the metabolic profile, and the assessed proteins involved in organic matter degradation. Due to the primary influence of seagrass decline, the effect of the sampling site on the metabolic profile of the microbial communities in the sediment is discussed in detail in Section 4.4, where the effects of seagrass decline are considered.

4.4 Temporal differences: influence of seagrass decline

The analysis of a comprehensive set of 118 metaproteomes, whose proteins were identified by searching a database composed of the sequenced metagenomes, made it possible to assess the dynamics of the metabolic profile of the microbial sediment communities in the Bay of Saline. In addition, the sampling of sediment cores in the period before and during the decline of a *Cymodocea nodosa* meadow allowed the assessment of the temporal dynamics and thus the influence of seagrass decline on the same metabolic profile. The influence of the decline of the meadow was observed in protein richness and diversity, the structure of the metabolic profile, and the assessed proteins involved in organic matter degradation. Prior to the decline, higher values of the number of observed proteins and the exponential of the Shannon diversity index were found in the vegetated sediment compared to the nonvegetated sediment. Moreover, these differences were more pronounced in the deeper parts of the sediments, i.e. in the lower middle and bottom layer (Figure 8). The differences between the sites began to disappear during the decline of the meadow and the values of the number of observed proteins and the exponential of the Shannon diversity index were similar to those found in the sediment previously inhabited by the meadow. In addition to these differences, the structure of the metabolic profile of the sediment communities of the nonvegetated site also showed temporal differences. A separation was found between the period before and during the decline of the meadow, especially in the deeper parts of the sediment and when only proteins belonging to the Cluster of Orthologous Genes (COG) category C, which includes proteins for energy production and conversion, were considered. In contrast, no temporal pattern was found for the communities of the vegetated site (Figure 8).

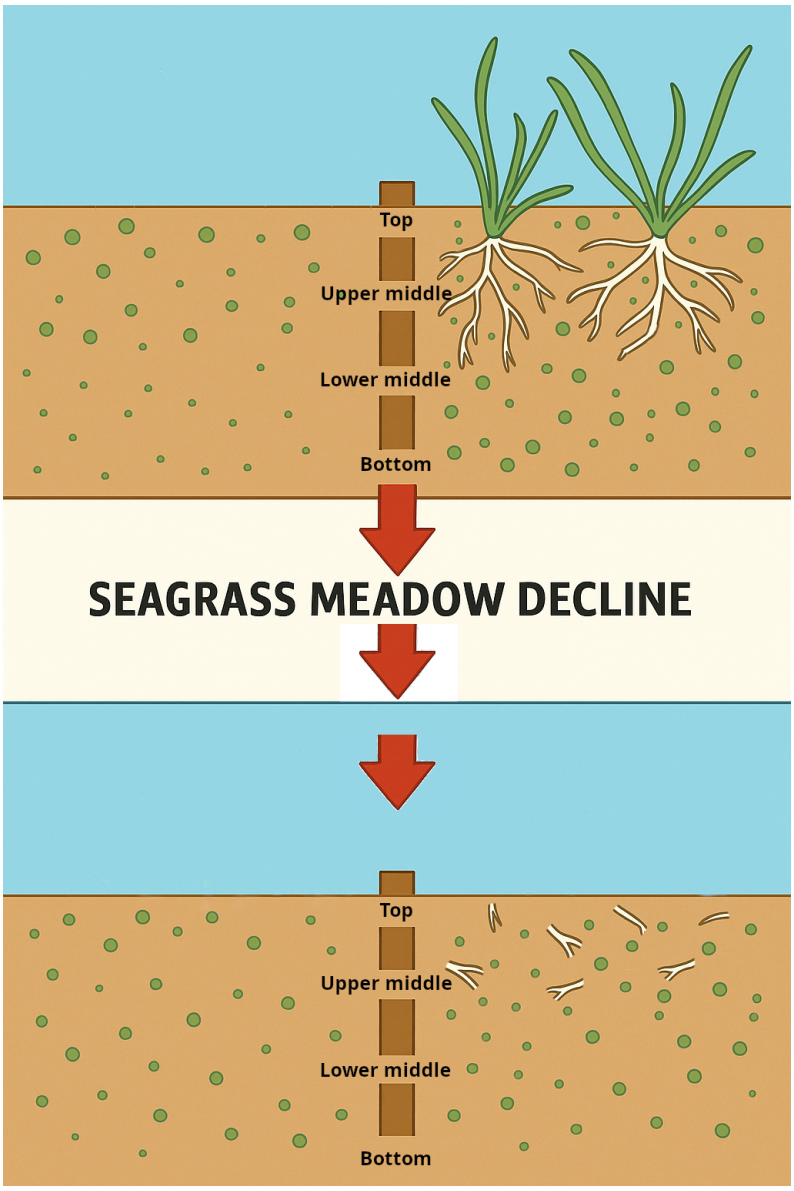

Figure 8. A conceptual diagram illustrating the shift in the metabolic profile (green dots) of sediment microbial communities during the decline of a *Cymodocea nodosa* meadow in the Bay of Saline. Prior to the decline (upper panel), differences in metabolic profiles were observed between the vegetated and nonvegetated site, particularly in the deeper sediment layers. During the decline (lower panel), these differences diminished and the metabolic profile of the nonvegetated site began to resemble that of the vegetated site. The analysis was based on sediment cores cut into four sections of 1 cm length each: the top (0–1 cm), the bottom (7–8 cm), and two middle sections: upper middle (2–3 cm) and lower middle (3–6 cm).

Differences between sites discussed in Section 4.3 were accompanied by differences in metabolic profiles between sites observed prior to decline of the meadow. This is not surprising, as data have shown that microbial communities in vegetated sediments differ in composition (Alsaffar et al., 2020; Ettinger et al., 2017; Sun et al., 2020; Zheng et al., 2019) and function (Mohapatra et al., 2022) from communities in nonvegetated sediments. The higher protein richness and diversity observed at the vegetated site prior to meadow decline is consistent with

other studies reporting higher metabolic diversity and microbial activity in sediments colonised by seagrasses, which may be related to the higher organic matter content in these sediments (Duarte, Holmer, et al., 2005; Holmer and Nielsen, 1997; Mohapatra et al., 2022; A. Smith et al., 2004). Furthermore, the greater differentiation in protein richness and diversity between sites over the same time period in the deeper parts of the sediment is consistent with the previously discussed results showing greater separation in community composition between sites in these parts of the sediment. In contrast, the lack of such differentiation of the same parameters in the upper parts of the sediment, i.e. in the top and upper middle layer, could be explained by the input of seagrass-derived organic matter into the nonvegetated site, as organic matter from the *C. nodosa* meadow can be imported into the nonvegetated site and has been shown to be an important source for the prokaryotes living in this sediment (Holmer et al., 2004). These observations are also supported by the dynamics of the structure of the metabolic profile observed at the nonvegetated site, which showed that samples originating from the upper parts of the sediment were grouped together regardless of the period of sampling.

The lack of differences in protein richness and diversity between sites during the decline of the meadow suggests a more uniform metabolic profile of the microbial communities in the sediment during this period, similar to the metabolic profile of the communities of the vegetated site prior to the decline. Furthermore, this observation is supported by the dynamics of the structure of the metabolic profile observed at the nonvegetated site. Samples collected during the decline grouped with samples collected in the upper parts of the sediment before the decline. As seagrass meadows are known to stabilise the sediment by reducing its resuspension and mixing (Duarte, Holmer, et al., 2005; Terrados and Duarte, 2000; van Katwijk et al., 2010), the lack of differentiation between sites during the decline of the meadow could be the result of a greater input of fresh organic matter into the nonvegetated sediment due to increased resuspension, mixing, and transport between sites when the meadow is no longer present. Indeed, during the decline of the meadow from May to August 2018, higher concentrations of total lipids and organic matter were detected at the nonvegetated than at the vegetated site. In contrast, the uniformity of the microbial profile observed at the vegetated site throughout the study period could be the result of maintaining the source of organic matter during the decline due to decay of leaves, roots, and rhizomes (Duarte, Holmer, et al., 2005; Liu et al., 2017; Peduzzi and Herndl, 1991; Trevathan-Tackett et al., 2020).

Given the great importance of sediments colonised by seagrasses for carbon sequestration and the role of microbial communities in these habitats in the mineralisation of organic matter (Duarte, Middelburg, et al., 2005; Duarte et al., 2013; Kennedy et al., 2010), it is interesting

to evaluate the dynamics of microbial proteins involved in the degradation of organic matter during such an ecological event as the decline of a seagrass meadow. In addition, analysis of the functional COG categories showed that category C, which includes proteins for energy production and conversion, is the most abundant. This is consistent with metagenomic studies, which also found that energy production and conversion is one of the most abundant functional COG categories in coastal sediments (Habibi et al., 2023). Proteins involved in organic matter degradation were assessed by analysing the dynamics of hydrolytic enzymes, ATP-binding cassette (ABC) transporters, fermentation-mediating enzymes, and proteins involved in dissimilatory sulphate reduction. The analysis of hydrolytic enzymes showed that Carbohydrate-Active enZymes (CAZymes) and peptidases were more abundant than lipases, indicating the importance of carbohydrates and proteins as sources of organic matter for the microbes in the sediment of the Bay of Saline. Among the CAZymes, the glycoside hydrolase families GH5 and GH9, which contain members that can hydrolyse plant organic matter such as cellulose, were the most abundant (Aspeborg et al., 2012; Berlemont and Martiny, 2016; Drula et al., 2022). This is not surprising since, as already mentioned, cellulose is the main component of the cell walls of seagrasses (Pfeifer and Classen, 2020; Syed et al., 2016; Torbatinejad and Sabin, 2001). The peptidases consisted almost exclusively of metalloendopeptidases and serine endopeptidases, similar to other coastal sediments where high proportions of these enzymes were found among the extracellular proteases (Z. Liu et al., 2023; X.-Y. Zhang et al., 2015; M.-Y. Zhou et al., 2013). In general, CAZymes and peptidases showed no response to meadow decline, except that the proportion of CAZymes in the top sediment layer of the nonvegetated site decreased from pre-decline to meadow decline, while the proportion of peptidases in the upper middle sediment layer of the vegetated site increased during the same period. These patterns could be explained by the increased microbial colonisation of detritus and microbial utilisation of exogenous nitrogen during seagrass tissue decomposition, as studies have shown that the molar carbon:nitrogen content in seagrass litter decreases during this process (Liu et al., 2017; Peduzzi and Herndl, 1991).

Prokaryotes utilise various transport proteins, including ABC transporters (Davidson and Chen, 2004), to import hydrolytic products into the cell. The proportion of ABC transporters found in the sediment of the Bay of Saline is consistent with other studies that have also found a high abundance of these transporters in marine sediments (Habibi et al., 2023; S. Wang et al., 2020). Among the ABC transporters, those for sugars and amino acids were the most abundant and their dynamics reflected the overall dynamics of the metabolic profile. Before the decline of the meadow, ABC transporters for sugars showed a higher proportion in the deeper parts of the

vegetated sediment than in the nonvegetated sediment. This distribution pattern reflects the higher organic matter content and thus the greater demand for ABC transporters in vegetated sediments (Duarte, Holmer, et al., 2005; Mohapatra et al., 2022). In contrast, these ABC transporters did not show different proportions between sites during the decline of the meadow. In addition, the ABC transporters for sugars and amino acids showed an increase in their proportion in the bottom sediment layer at the nonvegetated site from before the decline to the decline of the meadow, which could be the result of a greater input of fresh organic matter into the nonvegetated sediment during the decline (Duarte, Holmer, et al., 2005; Terrados and Duarte, 2000; van Katwijk et al., 2010), as previously mentioned.

The organic matter in anoxic sediments is mineralised in an anaerobic food chain in which fermentation processes carried out by microbes are an essential component (Arndt et al., 2013). Among the fermentation-mediating enzymes analysed, formate dehydrogenase, pyruvate:ferredoxin oxidoreductase, and alcohol dehydrogenase were the most abundant. This is not surprising, as microcosm studies of the anaerobic degradation of organic matter in marine sediments have found acetate, formate, and ethanol to be the most common fermentation products (Graue et al., 2012; Pelikan et al., 2021), while direct measurements of sediment pore water have demonstrated the presence of methanol and ethanol (Zhuang et al., 2014). In addition, pyruvate:ferredoxin oxidoreductase and alcohol dehydrogenase have been reported to be important fermentation-mediating enzymes in Baltic Sea sediments (Zinke et al., 2019). Similar to the dynamics of the ABC transporters, the dynamics of the fermentation-mediating enzymes also reflected the overall changes of the metabolic profile. Prior to the decline of the meadow, the proportion of formate dehydrogenase, pyruvate:ferredoxin oxidoreductase, and alcohol dehydrogenase was found to be higher in the deeper parts of the sediment at the vegetated site than at the nonvegetated site, reflecting the higher organic matter content and possibly a higher fermentation rate in the seagrass-colonised sediments (Duarte, Holmer, et al., 2005; Mohapatra et al., 2022).

Given that dissimilatory sulphate reduction is recognised as the predominant terminal pathway of organic matter mineralisation in anoxic seabeds (Jørgensen, 1982; Jørgensen et al., 2019), that *Desulfobacterota*, a group known to contain sulphate reducers found in marine sediments, was the most abundant high-taxonomic rank in the sediment microbial community in the Bay of Saline, and that one of the most abundant proteins in the functional COG category C was adenylylsulphate reductase, a protein known to be involved in dissimilatory sulphate reduction, it is interesting to assess the dynamics of the enzymes involved in this process in more detail. The metaproteomes of the microbial community in the sediment of the Bay of Saline

contained sulphate adenylyltransferase (Sat), adenylylsulphate reductase (Apr), and dissimilatory sulphite reductase (Dsr), enzymes involved in the dissimilatory sulphate reduction pathway and shared by known sulphate-reducing microbes (Jørgensen et al., 2019). These enzymes showed changes comparable to the dynamics of the overall metabolic profile. In general, the proportion of these enzymes was higher in the deeper parts of the sediment at the vegetated site than at the nonvegetated site before the decline of the meadow, while such differences were not observed during the decline. Similar to the dynamics of the overall metabolic profile, these observations could be explained by the higher organic matter content in the seagrass-colonised sediments before the decline (Duarte, Holmer, et al., 2005; Mohapatra et al., 2022) and by a greater input of fresh organic matter into the nonvegetated sediment during the decline (Duarte, Holmer, et al., 2005; Terrados and Duarte, 2000; van Katwijk et al., 2010), as previously mentioned.

In contrast to all these observations of the dynamics of the microbial metabolic profile, the community composition determined by analysing the V4 region of the 16S rRNA gene showed no temporal dynamics and thus no influence of the decline of the seagrass meadow. Even when the composition was analysed in a way that excludes the previously established influence of sediment depth and sampling site, no grouping of communities by month, year, or the period before and during seagrass meadow decline could be observed. Such a stable community composition and, in contrast, a dynamic metabolic profile could be the result of a large amount of extracellular DNA or a large proportion of dormant or dead microbial cells in marine sediments (Bradley et al., 2019; Cangelosi and Meschke, 2014; Carini et al., 2016; S. E. Jones and Lennon, 2010; Luna et al., 2002; Torti et al., 2015, 2018). It is estimated that over 80% of DNA in marine sediments is extracellular (Dell'Anno et al., 2002; Dell'Anno and Danovaro, 2005). Furthermore, dead cells can account for up to 70% of all bacterial cells in coastal marine sediments, while only 4% of living cells are actively growing (Luna et al., 2002). Molecular techniques such as 16S rRNA gene sequencing or metagenomics are usually based on the isolation of total DNA and therefore cannot distinguish whether the DNA originates from living, dead, active, or dormant cells or the extracellular DNA pool (Cangelosi and Meschke, 2014; Torti et al., 2015). In addition, the metabolic versatility of the microbes may contribute to some extent to the observed community stability, as the same community members may start to perform different metabolic functions during the decline of the seagrass meadow (Bowen et al., 2011; Louca et al., 2018). Also, a longer sampling period after the loss of the seagrass meadow may be required to observe the changes in community composition, as the microbes living in marine sediments often have very long generation times (Jørgensen and Marshall, 2016; Starnawski et al., 2017). The inability of the 16S rRNA gene sequencing approach to detect temporal changes in community composition

highlights the importance of molecular techniques that enable functional characterisation, such as metaproteomics, in determining microbial response to environmental change in marine systems (Saito et al., 2019).

5 CONCLUSIONS

Seagrass decline is a globally recognised issue with far-reaching consequences. While the effects of this decline on coastal ecosystems have been extensively documented, a critical aspect has been systematically overlooked, the sediment microbial community. Given the gap in previous research, this doctoral thesis addresses these knowledge gaps by providing a comprehensive analysis of sediment microbial communities throughout the decline of a seagrass meadow. Moreover, it offers the first detailed description of sediment microbial communities underlying a *Cymodocea nodosa* seagrass meadow, for which only rhizosphere and epiphytic communities had previously been studied. In addition, it is among the first to use a combined metagenomic and metaproteomic approach to study microbial community metabolism, not only in seagrass sediments but also in coastal surface sediments, providing valuable data for further research.

This thesis consists of three scientific articles that represent a stepwise progression of research: the first (**Dynamics of environmental conditions during the decline of a *Cymodocea nodosa* meadow**) documents the decline of the meadow alongside environmental conditions in the sediment; the second (**Compositional stability of sediment microbial communities during a seagrass meadow decline**) assesses the structure and dynamics of the sediment microbial communities; and the third (**Shift in the metabolic profile of sediment microbial communities during seagrass decline**) examines the metabolism of these microbial communities. The data presented in these scientific articles (Section 3) and discussed in Section 4 confirm the hypotheses:

1. Decline of the *C. nodosa* meadow alters sediment environmental conditions;

It is clear that the decline of the seagrass meadow changed the environmental conditions in the sediment. In particular, an increase in hydrogen sulphide concentration and the expansion of its accumulation zone was observed during the decline of the meadow.

2. The metabolic profile of the sediment microbial community differs with sediment depth, between the vegetated and nonvegetated sites, and throughout the study period;

The metabolic profile of the sediment microbial communities was primarily influenced by the decline of the seagrass meadow. Before the seagrass decline, differences in the metabolic profile of the microbial communities in the sediment were observed between the seagrass-vegetated and the nonvegetated site, especially differing in the deeper sediment parts and not differing closer to the sediment surface. During the decline, these differences in the deeper sediment diminished and the metabolic profile of the microbial communities in the sediment at the nonvegetated site began to resemble that of the vegetated site.

Interestingly, the microbial community composition did not follow the same trend, allowing only for a partial confirmation of the hypothesis:

3. The sediment microbial community structure differs with sediment depth, between the vegetated and nonvegetated sites, and throughout the study period;

Analysis of the V4 region of the 16S rRNA gene clearly showed that the microbial communities in the sediment were primarily stratified by sediment depth and secondarily differed between the seagrass-vegetated and nonvegetated site. In contrast to the metabolic profile of the communities, no temporal changes and thus no response to the decline of the seagrass could be observed in the structure of the microbial communities.

Taken together the collected data once again confirm the previously established notion that vegetated sediments harbour distinct microbial communities from the ones found in the nonvegetated sediments, but also highlight the great influence of the seagrass meadow on the adjacent nonvegetated sediment, reflected in a highly similar microbial community in upper sediment parts. Throughout meadow decline the microbial communities underlying the seagrass meadow exhibit great stability, while the communities of the deeper layers of the adjacent sediment remain compositionally the same and adapt their metabolic profile as a response to greater availability of fresh organic matter. These communities become highly similar to those found in the seagrass sediment throughout the whole sediment core, indicating the intensified spread of seagrass derived organic matter throughout the whole area, in turn fuelling organic matter decomposition accompanied by high concentrations of hydrogen sulphide. In conclusion, the clear influence of seagrass on a wide sediment area has been demonstrated through assessment of environmental parameters and the use of molecular methods. This influence was only more emphasised with meadow decline, highlighting the broader ecological influence of the decline of seagrass meadows. The comprehensive temporal dataset generated, including data of sediment environmental conditions, microbial community composition, and metabolic functions, provides a strong foundation for further studies on sediment microbial communities of seagrass meadows and coastal surface sediment in general.

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7 BIOGRAPHY

Marsej Markovski was born on 6 January 1992 in Pula, Croatia. He completed his primary and secondary education in Rovinj, Croatia, and earned his bachelor's degree in aquaculture in 2013 and his master's degree in mariculture in 2016 from the University of Dubrovnik. Since 2017 he has been a professional associate at the Centre for Marine Research, Ruđer Bošković Institute, Rovinj. In 2019, he enrolled in the Interdisciplinary Doctoral Study in Oceanology at the University of Zagreb Faculty of Science. He is the first author of two scientific articles and co-author of six. Articles published in 2018, 2020, 2021, and 2022 received the Ruđer Bošković Institute Annual Award.

Publications

1. **Markovski**, **M.**, Najdek, M., Zhao, Z., Herndl, G. J., and Korlević, M. (2025). Shift in the metabolic profile of sediment microbial communities during seagrass decline. *Environmental Microbiome*, *20*(1), 93. https://doi.org/10.1186/s40793-025-00750-1
2. Korlević, M., **Markovski**, **M.**, Herndl, G. J., and Najdek, M. (2022). Temporal variation in the prokaryotic community of a nearshore marine environment. *Scientific Reports*, *12*(1), 16859. https://doi.org/10.1038/s41598-022-20954-6
3. **Markovski**, **M.**, Najdek, M., Herndl, G. J., and Korlević, M. (2022). Compositional stability of sediment microbial communities during a seagrass meadow decline. *Frontiers in Marine Science*, *9*, 966070. https://doi.org/10.3389/fmars.2022.966070
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APPENDICES

Appendix A

Supplement of Biogeosciences, 17, 3299–3315, 2020
https://doi.org/10.5194/bg-17-3299-2020-supplement

Biogeosciences Open Access EGU

Supplement of

Dynamics of environmental conditions during the decline of a *Cymodocea nodosa* meadow

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Table S1. Concentrations of nutrients (orthophosphates − PO_4, nitrates − NO_3, nitrites − NO_2, ammonia − NH_4 and silicates − SiO_4), chlorophyll *a* (Chl *a*) and prokaryotic abundances (PA) in the water column of Saline Bay

Sampling date	PO_4 µM	NO_3 µM	NO_2 µM	NH_4 µM	SiO_4 µM	Chl*a* μgL^{-1}	PA $\cdot 10^5$cells mL^{-1}
July 2017	0.015	1.132	0.046	0.480	2.046	0.30	6.7
August 2017	0.045	1.374	0.077	1.195	1.583	0.31	7.8
September 2017	0.176	6.242	0.142	1.885	9.824	0.89	7.9
October 2017	0.030	0.743	0.063	0.505	2.470	0.63	4.7
November 2017	0.020	1.120	0.060	0.820	2.640	0.61	2.6
December 2017	0.020	0.570	0.050	0.460	2.350	0.40	4.3
February 2018	0.080	2.150	0.110	1.140	2.273	0.31	4.0
March 2018	0.000	1.667	0.136	0.542	3.199	0.30	3.9
April 2018	0.000	1.004	0.029	0.616	3.805	0.55	6.6
May 2018	0.000	0.855	0.010	0.314	1.341	0.47	8.3
June 2018	0.000	1.650	0.037	0.702	1.901	0.31	9.8
July 2018	0.010	1.162	0.025	1.152	1.254	0.43	11.3
August 2018	0.025	0.652	0.043	0.906	1.853	0.39	6.7
September 2018	0.000	0.487	0.048	0.487	3.522	0.39	5.7
October 2018	0.195	0.855	0.043	0.721	0.769	0.55	4.4

Table S2. Fatty acid markers assigned to organic sources and fatty acid pools: SAT – saturated fatty acids, PUFA – polyunsaturated fatty acids

Fatty acids	Source	Pool
C15:0i, ai; C15:0; C16:0i; C17:0i, ai; C17:0Δ; C17:0; C18:1(n-7)	Bacteria / Detritus	SAT
C20:5(n-3), C20:4(n-3)	Phytoplankton / Seston Macroalgae; Rhodophyta, Ochrophyta	PUFA
C18:3(n-3); C18:2(n-6)	Macroalgae; Chlorophyta *C. nodosa*	PUFA
C ≥ 24 (C24:0, C26:0, C28:0)	Vascular plants; terrestrial / seston Detritus (*C. nodosa*)	SAT
C16:0, C18:0	Common to all sources	SAT

Table S3. F-ratios, ranges (min and max), means and standard deviations (sd) of fatty acids proportions and ratios for groups of *C. nodosa* leaves obtained by K-means procedure. SAT – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, UND – unsaturation degree, BACT – bacterial fatty acids.

		Group 1				Group 2				Group 3				August
Fatty acid	F- ratio	min	mean	max	sd	min	mean	max	sd	min	mean	max	sd	2018
C14:0	8,431	0,68	0,93	1,25	0,21	0,62	0,90	1,17	0,23	0,85	1,57	2,92	1,02	2,92
C16:1(n-7)	0,507	1,14	2,15	4,79	1,47	0,68	1,36	2,08	0,61	0,47	2,29	4,10	2,57	3,10
C16:0	6,014	15,11	19,87	25,18	3,53	19,87	21,07	22,94	1,44	26,51	27,99	29,46	2,09	28,63
C18:2(n-6)	9,921	21,27	23,36	27,00	2,79	21,75	27,11	32,92	5,22	16,20	17,56	18,92	1,92	5,96
C18:3(n-3)	33,576	24,75	31,41	34,96	3,72	21,67	24,34	26,28	1,95	8,67	12,22	15,77	5,02	0,00
C18:1(n-9)	1,214	3,93	4,94	5,43	0,60	3,56	6,11	8,04	2,22	4,65	6,21	7,76	2,20	7,52
C18:0	5,571	2,13	2,56	3,29	0,43	2,33	2,83	3,61	0,61	2,33	2,95	3,56	0,87	5,02
C20:0	0,713	0,43	0,70	1,26	0,31	0,54	0,70	0,86	0,18	0,41	0,42	0,42	0,01	0,69
C22:0	0,570	1,54	2,43	4,40	1,09	2,47	2,90	3,40	0,43	1,69	2,02	2,34	0,46	2,88
≥ C24	147,894	0,79	2,22	3,15	0,83	3,07	3,92	4,80	0,89	13,26	14,82	16,37	2,20	22,35
SAT	103,457	29,14	31,79	33,99	2,02	33,02	36,01	40,11	3,43	53,89	57,24	60,59	4,74	78,26
MUFA	2,717	8,65	9,83	11,75	1,30	9,76	10,46	10,96	0,50	10,57	11,87	13,16	1,83	12,74
PUFA	123,611	55,11	58,08	59,47	1,71	49,22	53,23	56,70	3,36	26,17	30,43	34,68	6,02	5,96
UND	89,096	4,85	5,16	5,37	0,23	3,49	4,14	4,58	0,51	1,42	1,61	1,79	0,26	0,34
BACT	2,362	1,76	3,16	5,15	1,20	1,72	3,07	4,63	1,19	2,96	4,81	6,66	2,62	13,14

Group 1: July-October 2017, February-March 2018

Group 2: November-December 2017, April-May 2018

Group 3: June-July 2018

Table S4. F-ratios, ranges (min and max), means and standard deviations (sd) of fatty acids proportions and ratios for groups of *C. nodosa* rhizomes and roots obtained by K-means procedure. SAT – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, UND – unsaturation degree, BACT – bacterial fatty acids.

		Group 1				Group 2				Group 3			
Fatty acid	F-ratio	min	mean	max	sd	min	mean	max	sd	min	mean	max	sd
C14:0	2,243	0,50	1,09	1,71	0,51	1,12	1,35	1,74	0,27	1,77	1,99	2,38	0,24
C16:1(n-7)	1,894	1,12	2,12	4,24	1,12	1,40	2,21	2,52	0,54	1,79	4,11	8,86	2,82
C16:0	2,517	17,72	23,11	30,15	5,05	18,35	22,82	25,60	3,39	18,96	24,69	27,42	3,33
C18:2(n-6)	36,160	21,06	27,91	33,29	4,62	21,28	23,26	26,28	2,22	13,34	15,53	17,30	1,75
C18:3(n-3)	3,504	3,12	4,60	8,95	2,96	4,02	4,53	5,59	0,72	3,17	4,26	5,15	0,74
C18:1(n-9)	11,067	12,15	13,39	14,65	1,09	11,73	12,55	12,91	0,56	6,41	7,48	8,89	0,92
C18:0	2,640	3,42	4,76	7,15	1,40	4,10	4,65	5,27	0,50	3,40	4,28	5,49	0,96
C20:0	0,618	0,85	0,99	1,59	0,32	0,62	0,82	1,02	0,23	0,42	0,70	1,04	0,27
C22:0	0,328	1,37	2,55	6,00	1,73	1,71	2,10	2,49	0,44	1,26	2,40	4,65	1,41
≥C24	9,814	4,75	7,35	13,08	3,08	9,90	13,61	20,45	4,78	9,20	22,54	34,46	9,15
SAT	59,790	36,90	45,14	56,75	7,46	49,44	51,49	54,00	2,36	59,83	65,20	67,96	3,44
MUFA	4,420	17,19	20,78	28,56	4,23	17,10	18,59	20,78	1,58	11,35	13,68	18,50	2,82
PUFA	39,164	24,15	33,92	43,66	7,36	27,33	29,59	33,46	2,76	19,85	21,12	22,62	1,11
UND	18,765	1,21	2,06	2,77	0,61	1,38	1,55	1,76	0,20	0,78	0,90	1,17	0,16
BACT	1,625	5,10	6,24	8,35	1,17	5,49	7,34	8,71	1,34	6,72	9,09	10,91	2,45

Group 1: July - October 2017, February - March 2018

Group 2: November-December 2017, April -May 2018

Group 3: June -October 2018

Figure S1. Map of the sampling area

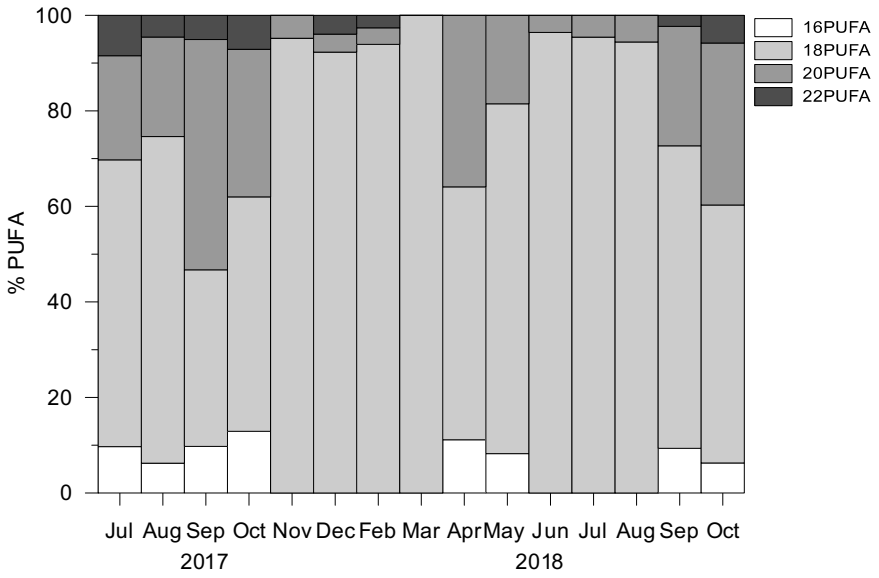

Figure S2. Contributions of PUFA with 16, 18, 20 and 22C atoms to PUFA pool of particulate matter in seawater

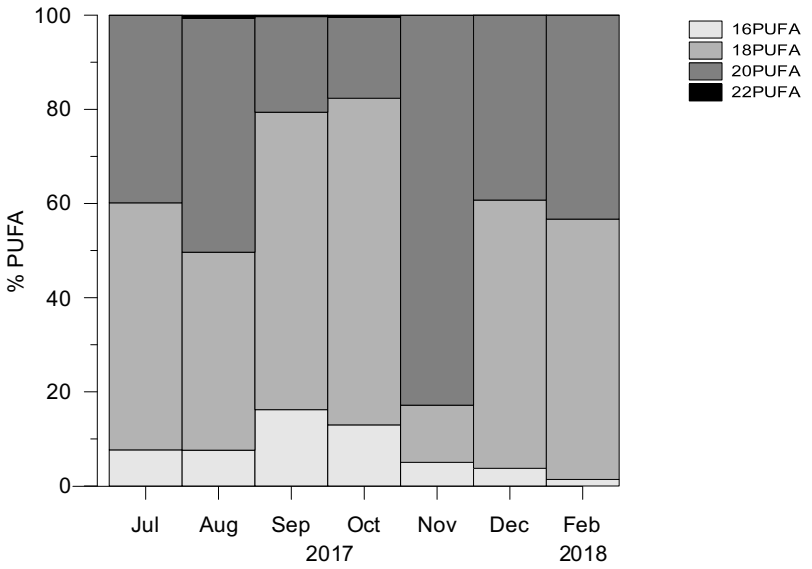

Figure S3. Contributions of PUFA with 16, 18, 20 and 22C atoms to PUFA pool of epiphytic macroalgae mix during their notable presence in a *C. nodosa* meadow

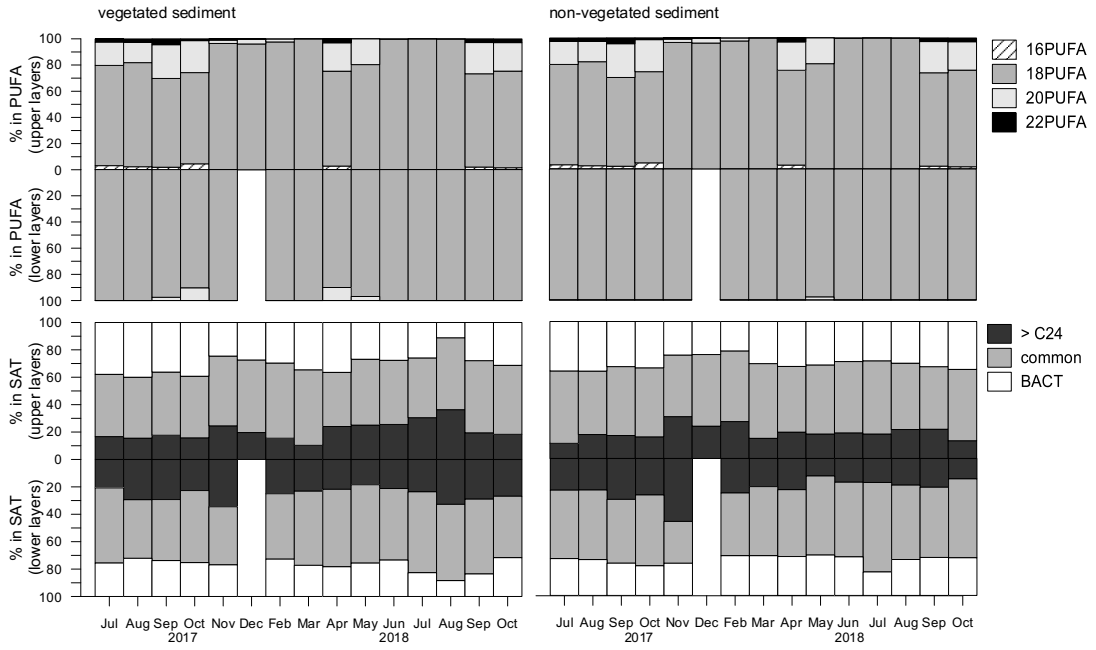

Figure S4. Contributions of PUFA with 16, 18, 20 and 22C atoms to PUFA pool and contributions of bacterial (BACT), common and long chain fatty acids (C ≥ 24) to SAT pool in the upper (0 - 4 cm) and lower (5 - 8 cm) layers of vegetated and non-vegetated sediments.

Appendix B

Supplementary material

Compositional stability of sediment microbial communities during a seagrass meadow decline

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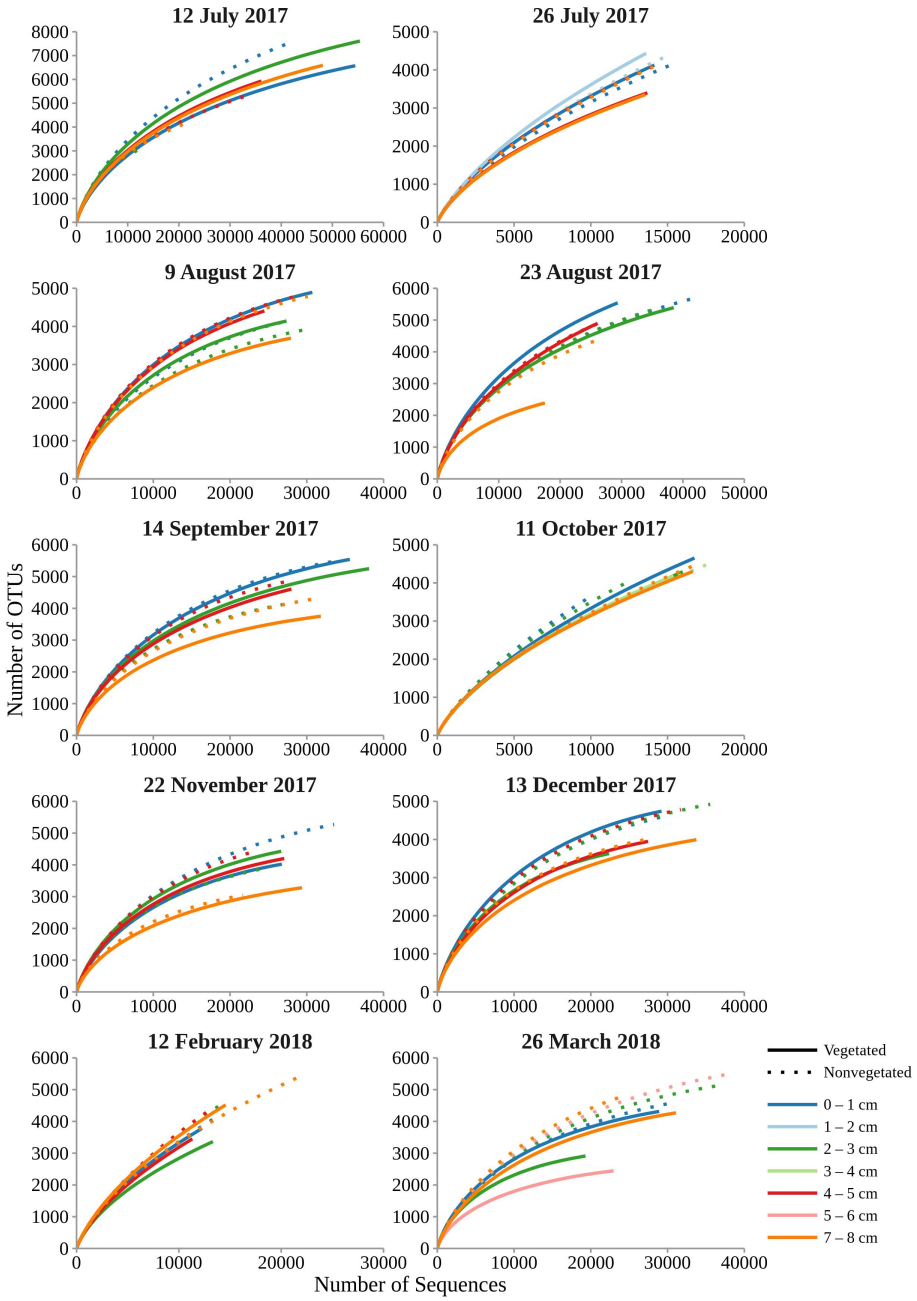
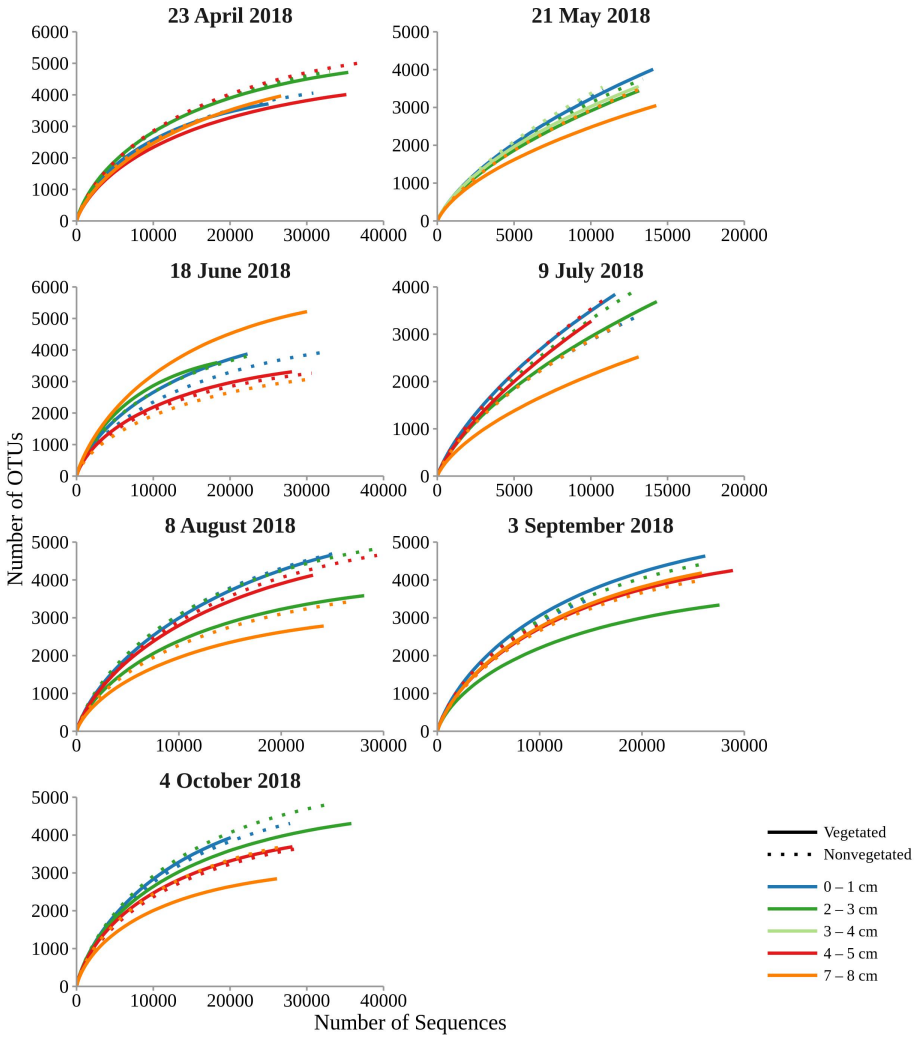
Running title: Compositional stability of sediment communities

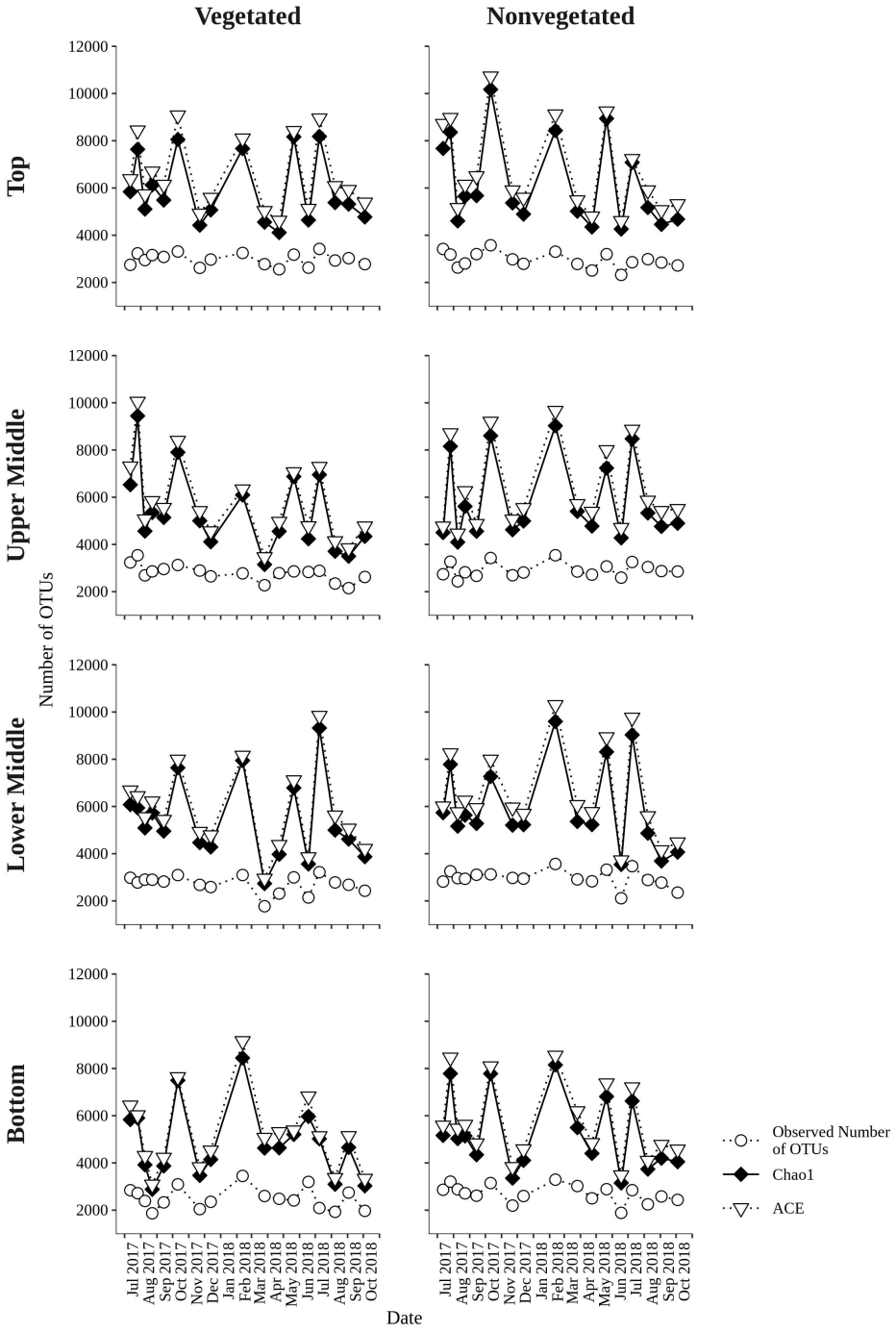
Supplementary figures



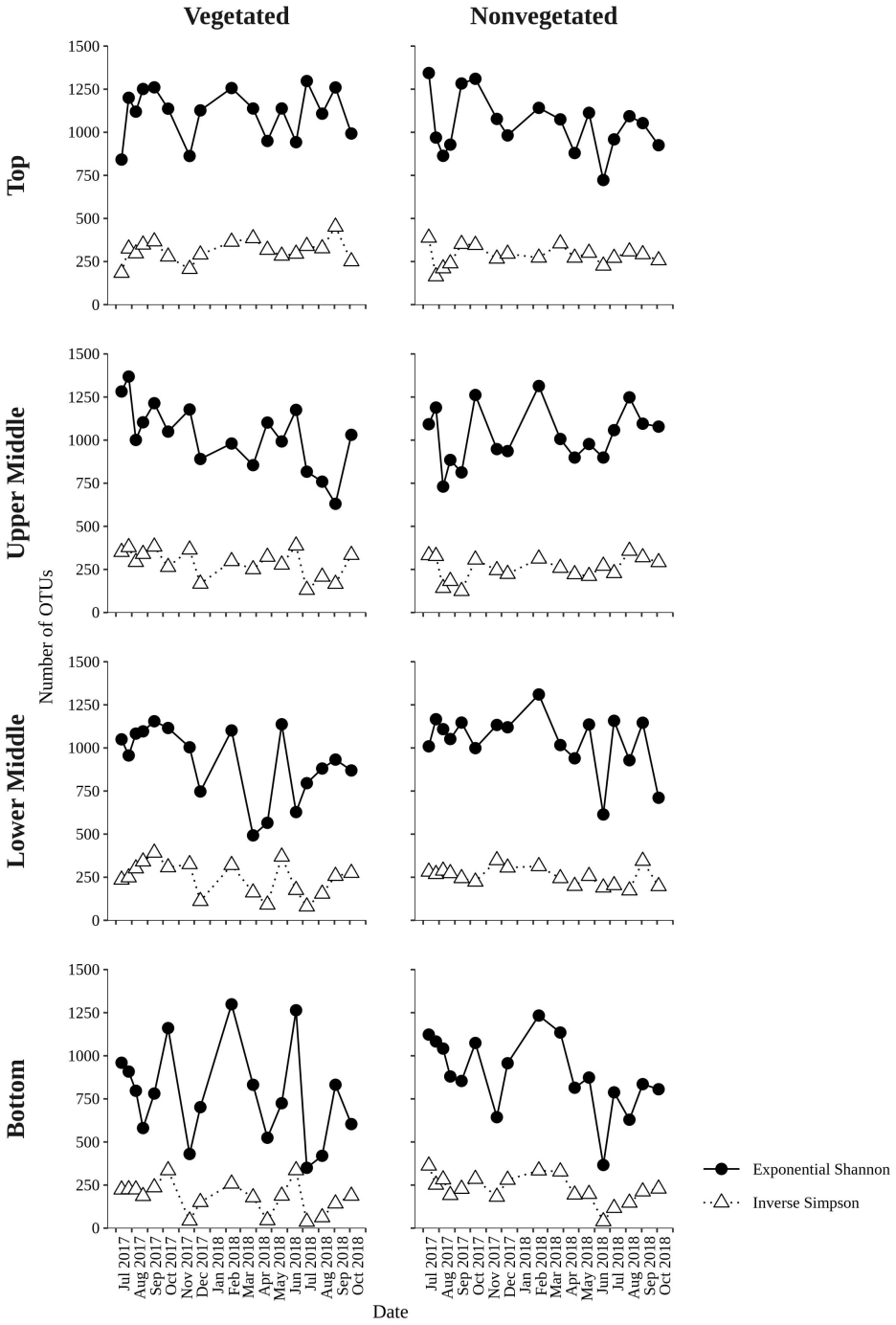
Supplementary Figure S1. Rarefaction curves of sediment microbial communities sampled at the vegetated and nonvegetated site in the Bay of Saline from 12 July 2017 to 26 March 2018.



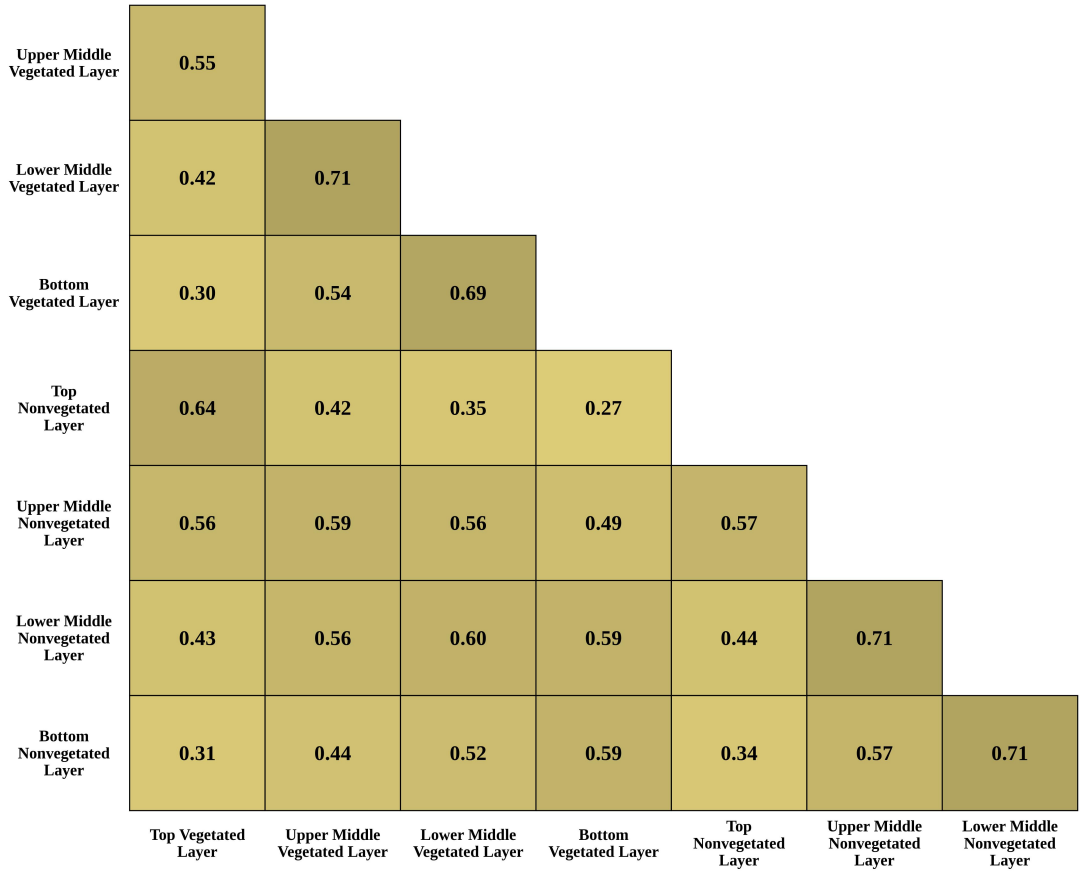
Supplementary Figure S2. Rarefaction curves of sediment microbial communities sampled at the vegetated and nonvegetated site in the Bay of Saline from 23 April 2018 to 4 October 2018.



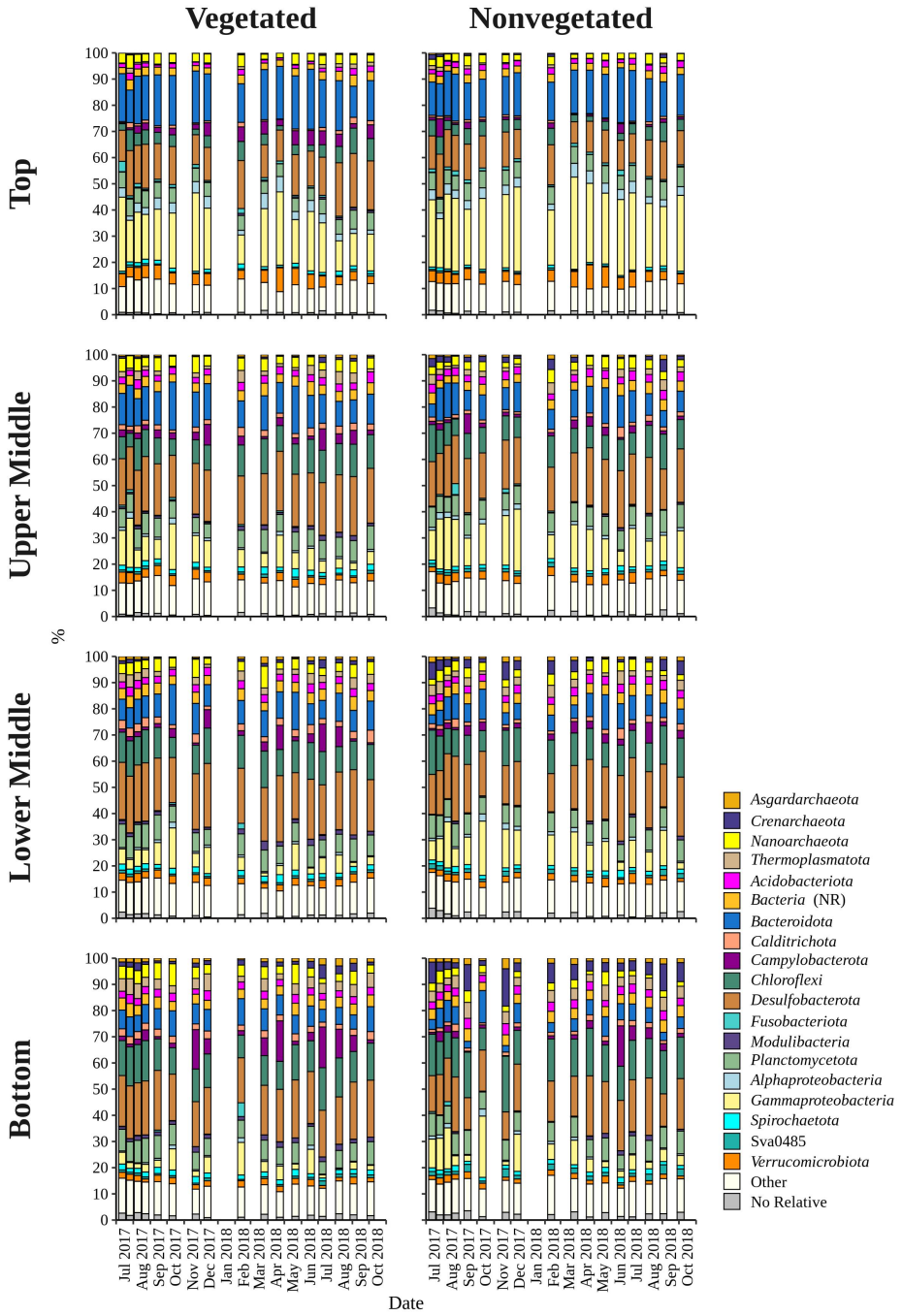
Supplementary Figure S3. Temporal dynamics of the observed number of OTUs, Chao1, and ACE of sediment microbial communities sampled in different sediment layers of the vegetated and nonvegetated site in the Bay of Saline.



Supplementary Figure S4. Temporal dynamics of the exponential Shannon diversity index and Inverse Simpson diversity index of sediment microbial communities sampled in different sediment layers of the vegetated and nonvegetated site in the Bay of Saline.



Supplementary Figure S5. Shared sediment microbial communities (Bray-Curtis similarity coefficient) between different sediment layers and sites in the Bay of Saline.



Supplementary Figure S6. Taxonomic classification and relative contribution of the most abundant bacterial and archaeal sequences (≥ 3 %) of each sample taken in different sediment layers from the vegetated and nonvegetated site in the Bay of Saline. No Relative (NR) – sequences without known relatives.

Supplementary tables

Supplementary Table S1. Sample ID, sampling date and site, sediment depth, no. of sequences and no. of OTUs of each sample. The number of sequences and OTUs was calculated after exclusion of sequences without known relatives (no relative sequences) and eukaryotic, chloroplast and mitochondrial sequences.

Sample ID	Date	Site	Sediment Depth	No. of Sequences	No. of OTUs
76	12 July 2017	Vegetated	0 – 1 cm	54,476	6,574
77	12 July 2017	Vegetated	2 – 3 cm	55,381	7,608
78	12 July 2017	Vegetated	4 – 5 cm	36,129	5,929
79	12 July 2017	Vegetated	7 – 8 cm	48,135	6,590
80	12 July 2017	Nonvegetated	0 – 1 cm	42,466	7,601
81	12 July 2017	Nonvegetated	2 – 3 cm	13,568	3,210
82	12 July 2017	Nonvegetated	4 – 5 cm	34,483	5,387
83	12 July 2017	Nonvegetated	7 – 8 cm	21,281	4,147
96	26 July 2017	Vegetated	0 – 1 cm	14,141	4,126
97	26 July 2017	Vegetated	1 – 2 cm	13,607	4,434
98	26 July 2017	Vegetated	4 – 5 cm	13,686	3,396
99	26 July 2017	Vegetated	7 – 8 cm	13,558	3,352
100	26 July 2017	Nonvegetated	0 – 1 cm	15,116	4,113
101	26 July 2017	Nonvegetated	1 – 2 cm	14,701	4,318
102	26 July 2017	Nonvegetated	4 – 5 cm	14,012	4,080
103	26 July 2017	Nonvegetated	7 – 8 cm	14,494	4,158
116	9 August 2017	Vegetated	0 – 1 cm	30,722	4,894
117	9 August 2017	Vegetated	2 – 3 cm	27,363	4,141
118	9 August 2017	Vegetated	4 – 5 cm	24,476	4,405
119	9 August 2017	Vegetated	7 – 8 cm	27,941	3,691
120	9 August 2017	Nonvegetated	0 – 1 cm	27,645	4,152
121	9 August 2017	Nonvegetated	2 – 3 cm	30,154	3,929
122	9 August 2017	Nonvegetated	4 – 5 cm	29,084	4,816
123	9 August 2017	Nonvegetated	7 – 8 cm	30,128	4,787
136	23 August 2017	Vegetated	0 – 1 cm	29,381	5,541
137	23 August 2017	Vegetated	2 – 3 cm	38,507	5,391
138	23 August 2017	Vegetated	4 – 5 cm	26,101	4,896
139	23 August 2017	Vegetated	7 – 8 cm	17,524	2,388

Supplementary Table S1. Sample ID, sampling date and site, sediment depth, no. of sequences and no. of OTUs of each sample. The number of sequences and OTUs was calculated after exclusion of sequences without known relatives (no relative sequences) and eukaryotic, chloroplast and mitochondrial sequences. *(continued)*

Sample ID	Date	Site	Sediment Depth	No. of Sequences	No. of OTUs
140	23 August 2017	Nonvegetated	0 – 1 cm	41,344	5,663
141	23 August 2017	Nonvegetated	2 – 3 cm	35,724	5,361
142	23 August 2017	Nonvegetated	4 – 5 cm	26,572	4,919
143	23 August 2017	Nonvegetated	7 – 8 cm	26,310	4,385
156	14 September 2017	Vegetated	0 – 1 cm	35,609	5,541
157	14 September 2017	Vegetated	2 – 3 cm	38,113	5,249
158	14 September 2017	Vegetated	4 – 5 cm	27,996	4,606
159	14 September 2017	Vegetated	7 – 8 cm	31,834	3,750
160	14 September 2017	Nonvegetated	0 – 1 cm	33,666	5,487
161	14 September 2017	Nonvegetated	2 – 3 cm	28,251	4,174
162	14 September 2017	Nonvegetated	4 – 5 cm	27,073	4,830
163	14 September 2017	Nonvegetated	7 – 8 cm	31,422	4,316
176	11 October 2017	Vegetated	0 – 1 cm	16,751	4,653
177	11 October 2017	Vegetated	2 – 3 cm	15,991	4,292
178	11 October 2017	Vegetated	3 – 4 cm	15,385	4,191
179	11 October 2017	Vegetated	7 – 8 cm	16,641	4,300
180	11 October 2017	Nonvegetated	0 – 1 cm	9,722	3,576
181	11 October 2017	Nonvegetated	2 – 3 cm	12,470	4,038
182	11 October 2017	Nonvegetated	3 – 4 cm	17,612	4,486
183	11 October 2017	Nonvegetated	7 – 8 cm	17,080	4,514
196	22 November 2017	Vegetated	0 – 1 cm	26,747	4,023
197	22 November 2017	Vegetated	2 – 3 cm	26,663	4,429
198	22 November 2017	Vegetated	4 – 5 cm	27,062	4,200
199	22 November 2017	Vegetated	7 – 8 cm	29,368	3,280
200	22 November 2017	Nonvegetated	0 – 1 cm	33,555	5,271
201	22 November 2017	Nonvegetated	2 – 3 cm	24,602	3,898
202	22 November 2017	Nonvegetated	4 – 5 cm	22,820	4,398
203	22 November 2017	Nonvegetated	7 – 8 cm	21,685	3,046
216	13 December 2017	Vegetated	0 – 1 cm	29,217	4,742
217	13 December 2017	Vegetated	2 – 3 cm	22,379	3,630

Supplementary Table S1. Sample ID, sampling date and site, sediment depth, no. of sequences and no. of OTUs of each sample. The number of sequences and OTUs was calculated after exclusion of sequences without known relatives (no relative sequences) and eukaryotic, chloroplast and mitochondrial sequences. *(continued)*

Sample ID	Date	Site	Sediment Depth	No. of Sequences	No. of OTUs
218	13 December 2017	Vegetated	4 – 5 cm	27,460	3,948
219	13 December 2017	Vegetated	7 – 8 cm	33,757	3,994
220	13 December 2017	Nonvegetated	0 – 1 cm	30,110	4,634
221	13 December 2017	Nonvegetated	2 – 3 cm	35,557	4,921
222	13 December 2017	Nonvegetated	4 – 5 cm	31,732	4,784
223	13 December 2017	Nonvegetated	7 – 8 cm	26,860	3,988
236	12 February 2018	Vegetated	0 – 1 cm	12,249	3,778
237	12 February 2018	Vegetated	2 – 3 cm	13,328	3,364
238	12 February 2018	Vegetated	4 – 5 cm	11,317	3,449
239	12 February 2018	Vegetated	7 – 8 cm	14,574	4,518
240	12 February 2018	Nonvegetated	0 – 1 cm	13,730	4,146
241	12 February 2018	Nonvegetated	2 – 3 cm	14,416	4,590
242	12 February 2018	Nonvegetated	4 – 5 cm	13,317	4,423
243	12 February 2018	Nonvegetated	7 – 8 cm	21,480	5,363
256	26 March 2018	Vegetated	0 – 1 cm	28,906	4,313
257	26 March 2018	Vegetated	2 – 3 cm	19,307	2,911
258	26 March 2018	Vegetated	5 – 6 cm	22,957	2,444
259	26 March 2018	Vegetated	7 – 8 cm	31,090	4,270
260	26 March 2018	Nonvegetated	0 – 1 cm	30,528	4,579
261	26 March 2018	Nonvegetated	2 – 3 cm	36,972	5,142
262	26 March 2018	Nonvegetated	5 – 6 cm	38,650	5,522
263	26 March 2018	Nonvegetated	7 – 8 cm	24,660	4,834
276	23 April 2018	Vegetated	0 – 1 cm	25,010	3,714
277	23 April 2018	Vegetated	2 – 3 cm	35,406	4,712
278	23 April 2018	Vegetated	4 – 5 cm	35,154	4,008
279	23 April 2018	Vegetated	7 – 8 cm	26,658	3,965
280	23 April 2018	Nonvegetated	0 – 1 cm	30,854	4,055
281	23 April 2018	Nonvegetated	2 – 3 cm	33,005	4,742
282	23 April 2018	Nonvegetated	4 – 5 cm	37,048	5,017
283	23 April 2018	Nonvegetated	7 – 8 cm	16,686	3,271

Supplementary Table S1. Sample ID, sampling date and site, sediment depth, no. of sequences and no. of OTUs of each sample. The number of sequences and OTUs was calculated after exclusion of sequences without known relatives (no relative sequences) and eukaryotic, chloroplast and mitochondrial sequences. *(continued)*

Sample ID	Date	Site	Sediment Depth	No. of Sequences	No. of OTUs
296	21 May 2018	Vegetated	0 – 1 cm	14,063	4,009
297	21 May 2018	Vegetated	2 – 3 cm	13,148	3,441
298	21 May 2018	Vegetated	3 – 4 cm	13,120	3,553
299	21 May 2018	Vegetated	7 – 8 cm	14,266	3,050
300	21 May 2018	Nonvegetated	0 – 1 cm	10,825	3,462
301	21 May 2018	Nonvegetated	2 – 3 cm	13,392	3,750
302	21 May 2018	Nonvegetated	3 – 4 cm	10,768	3,541
303	21 May 2018	Nonvegetated	7 – 8 cm	13,103	3,471
316	18 June 2018	Vegetated	0 – 1 cm	22,280	3,874
317	18 June 2018	Vegetated	2 – 3 cm	18,356	3,602
318	18 June 2018	Vegetated	4 – 5 cm	28,066	3,309
319	18 June 2018	Vegetated	7 – 8 cm	30,028	5,217
320	18 June 2018	Nonvegetated	0 – 1 cm	32,190	3,929
321	18 June 2018	Nonvegetated	2 – 3 cm	22,167	3,797
322	18 June 2018	Nonvegetated	4 – 5 cm	30,626	3,264
323	18 June 2018	Nonvegetated	7 – 8 cm	30,259	3,071
336	9 July 2018	Vegetated	0 – 1 cm	11,589	3,844
337	9 July 2018	Vegetated	2 – 3 cm	14,299	3,690
338	9 July 2018	Vegetated	4 – 5 cm	10,031	3,276
339	9 July 2018	Vegetated	7 – 8 cm	13,117	2,521
340	9 July 2018	Nonvegetated	0 – 1 cm	13,328	3,425
341	9 July 2018	Nonvegetated	2 – 3 cm	12,897	3,926
342	9 July 2018	Nonvegetated	4 – 5 cm	11,252	3,822
343	9 July 2018	Nonvegetated	7 – 8 cm	11,902	3,211
356	8 August 2018	Vegetated	0 – 1 cm	24,862	4,654
357	8 August 2018	Vegetated	2 – 3 cm	28,104	3,584
358	8 August 2018	Vegetated	4 – 5 cm	23,108	4,125
359	8 August 2018	Vegetated	7 – 8 cm	24,151	2,782
360	8 August 2018	Nonvegetated	0 – 1 cm	25,781	4,741
361	8 August 2018	Nonvegetated	2 – 3 cm	29,308	4,822

Supplementary Table S1. Sample ID, sampling date and site, sediment depth, no. of sequences and no. of OTUs of each sample. The number of sequences and OTUs was calculated after exclusion of sequences without known relatives (no relative sequences) and eukaryotic, chloroplast and mitochondrial sequences. *(continued)*

Sample ID	Date	Site	Sediment Depth	No. of Sequences	No. of OTUs
362	8 August 2018	Nonvegetated	4 – 5 cm	29,364	4,649
363	8 August 2018	Nonvegetated	7 – 8 cm	26,580	3,422
376	3 September 2018	Vegetated	0 – 1 cm	26,186	4,632
377	3 September 2018	Vegetated	2 – 3 cm	27,575	3,337
378	3 September 2018	Vegetated	4 – 5 cm	28,887	4,249
379	3 September 2018	Vegetated	7 – 8 cm	25,855	4,184
380	3 September 2018	Nonvegetated	0 – 1 cm	14,868	3,481
381	3 September 2018	Nonvegetated	2 – 3 cm	26,415	4,443
382	3 September 2018	Nonvegetated	4 – 5 cm	11,945	3,010
383	3 September 2018	Nonvegetated	7 – 8 cm	25,845	3,993
396	4 October 2018	Vegetated	0 – 1 cm	20,085	3,933
397	4 October 2018	Vegetated	2 – 3 cm	35,809	4,306
398	4 October 2018	Vegetated	4 – 5 cm	28,130	3,692
399	4 October 2018	Vegetated	7 – 8 cm	26,114	2,845
400	4 October 2018	Nonvegetated	0 – 1 cm	27,823	4,307
401	4 October 2018	Nonvegetated	2 – 3 cm	32,430	4,796
402	4 October 2018	Nonvegetated	4 – 5 cm	28,865	3,645
403	4 October 2018	Nonvegetated	7 – 8 cm	26,764	3,695

Supplementary Table S2. Statistic of richness estimator and diversity index parameters of sediment microbial communities sampled in different sediment layers of the vegetated site in the Bay of Saline. Parameters were tested by applying the Kruskal-Wallis *H* test followed by a pairwise comparison using the Mann-Whitney *U* test. Bonferroni correction was used to address the problem of multiple comparisons.

	Kruskal-Wallis *H* test			**Mann-Whitney *U* test**	
Parameter	***H***	***df***	***p***	**Comparisons Between Sediment Layers**	***p***
Observed No. of OTUs	11.5	3	< 0.01	Top Layer – Upper Middle Layer	0.78
				Top Layer – Lower Middle Layer	0.38
				Top Layer – Bottom Layer	< 0.05
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	0.21
				Lower Middle Layer – Bottom Layer	0.63
Chao1	4.7	3	0.20	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.23
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	1.00
				Lower Middle Layer – Bottom Layer	1.00
ACE	5.7	3	0.13	Top Layer – Upper Middle Layer	0.78
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.15
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	1.00
				Lower Middle Layer – Bottom Layer	1.00
Exponential Shannon	16.4	3	< 0.001	Top Layer – Upper Middle Layer	0.95
				Top Layer – Lower Middle Layer	< 0.05
				Top Layer – Bottom Layer	< 0.01
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	< 0.05
				Lower Middle Layer – Bottom Layer	0.63

Supplementary Table S2. Statistic of richness estimator and diversity index parameters of sediment microbial communities sampled in different sediment layers of the vegetated site in the Bay of Saline. Parameters were tested by applying the Kruskal-Wallis *H* test followed by a pairwise comparison using the Mann-Whitney *U* test. Bonferroni correction was used to address the problem of multiple comparisons. *(continued)*

	Kruskal-Wallis *H* test			**Mann-Whitney *U* test**	
Parameter	***H***	***df***	***p***	**Comparisons Between Sediment Layers**	***p***
Inverse Simpson	16.4	3	< 0.001	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	0.32
				Top Layer – Bottom Layer	< 0.01
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	< 0.05
				Lower Middle Layer – Bottom Layer	0.44

Supplementary Table S3. Statistic of richness estimator and diversity index parameters of sediment microbial communities sampled in different sediment layers of the nonvegetated site in the Bay of Saline. Parameters were tested by applying the Kruskal-Wallis *H* test followed by a pairwise comparison using the Mann-Whitney *U* test. Bonferroni correction was used to address the problem of multiple comparisons.

	Kruskal-Wallis *H* test			**Mann-Whitney *U* test**	
Parameter	***H***	***df***	***p***	**Comparisons Between Sediment Layers**	***p***
Observed No. of OTUs	5.1	3	0.16	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.59
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	0.89
				Lower Middle Layer – Bottom Layer	0.27
Chao1	3.7	3	0.30	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.51
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	1.00
				Lower Middle Layer – Bottom Layer	0.95
ACE	4.1	3	0.26	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.44
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	1.00
				Lower Middle Layer – Bottom Layer	0.95
Exponential Shannon	7.1	3	0.07	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.21
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	0.35
				Lower Middle Layer – Bottom Layer	0.16

Supplementary Table S3. Statistic of richness estimator and diversity index parameters of sediment microbial communities sampled in different sediment layers of the nonvegetated site in the Bay of Saline. Parameters were tested by applying the Kruskal-Wallis *H* test followed by a pairwise comparison using the Mann-Whitney *U* test. Bonferroni correction was used to address the problem of multiple comparisons. *(continued)*

	Kruskal-Wallis *H* test			**Mann-Whitney *U* test**	
Parameter	***H***	***df***	***p***	**Comparisons Between Sediment Layers**	***p***
Inverse Simpson	4.7	3	0.20	Top Layer – Upper Middle Layer	1.00
				Top Layer – Lower Middle Layer	1.00
				Top Layer – Bottom Layer	0.25
				Upper Middle Layer – Lower Middle Layer	1.00
				Upper Middle Layer – Bottom Layer	1.00
				Lower Middle Layer – Bottom Layer	1.00

Appendix C

Supplementary material

Shift in the metabolic profile of sediment microbial communities during seagrass decline

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Supplementary tables

Supplementary Table S1 Sample ID, sampling date and site, and sediment depth and layer for each protein sample. Samples for which no MS/MS spectra were obtained are indicated with an asterisk.

Sample ID	Date	Site	Sediment Depth (cm)	Sediment Layer
MM_1	12 July 2017	Nonvegetated	0 – 1	Top
MM_2			2 – 3	Upper Middle
MM_3			4 – 5	Lower Middle
MM_4			7 – 8	Bottom
MM_5	12 July 2017	Vegetated	0 – 1	Top
MM_6			2 – 3	Upper Middle
MM_7			4 – 5	Lower Middle
MM_8			7 – 8	Bottom
MM_9	9 August 2017	Nonvegetated	0 – 1	Top
MM_10			2 – 3	Upper Middle
MM_11			4 – 5	Lower Middle
MM_12			7 – 8	Bottom
MM_13	9 August 2017	Vegetated	0 – 1	Top
MM_14			2 – 3	Upper Middle
MM_15			4 – 5	Lower Middle
MM_16			7 – 8	Bottom
MM_17	14 September 2017	Nonvegetated	0 – 1	Top
MM_18			2 – 3	Upper Middle
MM_19			4 – 5	Lower Middle
MM_20			7 – 8	Bottom
MM_21	14 September 2017	Vegetated	0 – 1	Top
MM_22			2 – 3	Upper Middle
MM_23			4 – 5	Lower Middle
MM_24			7 – 8	Bottom
MM_25	11 October 2017	Nonvegetated	0 – 1	Top
MM_26			2 – 3	Upper Middle
MM_27			3 – 4	Lower Middle
MM_28			7 – 8	Bottom
MM_29	11 October 2017	Vegetated	0 – 1	Top
MM_30			2 – 3	Upper Middle
MM_31			3 – 4	Lower Middle
MM_32			7 – 8	Bottom
MM_33	22 November 2017	Nonvegetated	0 – 1	Top
MM_34			2 – 3	Upper Middle
MM_35			4 – 5	Lower Middle
MM_36			7 – 8	Bottom
MM_37	22 November 2017	Vegetated	0 – 1	Top
MM_38			2 – 3	Upper Middle
MM_39			4 – 5	Lower Middle
MM_40			7 – 8	Bottom

Supplementary Table S1 Sample ID, sampling date and site, and sediment depth and layer for each protein sample. Samples for which no MS/MS spectra were obtained are indicated with an asterisk. *(continued)*

Sample ID	Date	Site	Sediment Depth (cm)	Sediment Layer
MM_41	13 December 2017	Nonvegetated	0 – 1	Top
MM_42			2 – 3	Upper Middle
MM_43			4 – 5	Lower Middle
MM_44			7 – 8	Bottom
MM_45	13 December 2017	Vegetated	0 – 1	Top
MM_46			2 – 3	Upper Middle
MM_47			4 – 5	Lower Middle
MM_48			7 – 8	Bottom
MM_49	12 February 2018	Nonvegetated	0 – 1	Top
MM_50			2 – 3	Upper Middle
MM_51			4 – 5	Lower Middle
MM_52			7 – 8	Bottom
MM_53	12 February 2018	Vegetated	0 – 1	Top
MM_54			2 – 3	Upper Middle
MM_55			4 – 5	Lower Middle
MM_56			7 – 8	Bottom
MM_57*	26 March 2018	Nonvegetated	0 – 1	Top
MM_58			2 – 3	Upper Middle
MM_59			5 – 6	Lower Middle
MM_60			7 – 8	Bottom
MM_61	26 March 2018	Vegetated	0 – 1	Top
MM_62			2 – 3	Upper Middle
MM_63*			5 – 6	Lower Middle
MM_64			7 – 8	Bottom
MM_65	23 April 2018	Nonvegetated	0 – 1	Top
MM_66			2 – 3	Upper Middle
MM_67			4 – 5	Lower Middle
MM_68			7 – 8	Bottom
MM_69	23 April 2018	Vegetated	0 – 1	Top
MM_70			2 – 3	Upper Middle
MM_71			4 – 5	Lower Middle
MM_72			7 – 8	Bottom
MM_73	21 May 2018	Nonvegetated	0 – 1	Top
MM_74			2 – 3	Upper Middle
MM_75			3 – 4	Lower Middle
MM_76			7 – 8	Bottom
MM_77	21 May 2018	Vegetated	0 – 1	Top
MM_78			2 – 3	Upper Middle
MM_79			3 – 4	Lower Middle
MM_80			7 – 8	Bottom

Supplementary Table S1 Sample ID, sampling date and site, and sediment depth and layer for each protein sample. Samples for which no MS/MS spectra were obtained are indicated with an asterisk. *(continued)*

Sample ID	Date	Site	Sediment Depth (cm)	Sediment Layer
MM_81	18 June 2018	Nonvegetated	0 – 1	Top
MM_82			2 – 3	Upper Middle
MM_83			4 – 5	Lower Middle
MM_84			7 – 8	Bottom
MM_85	18 June 2018	Vegetated	0 – 1	Top
MM_86			2 – 3	Upper Middle
MM_87			4 – 5	Lower Middle
MM_88			7 – 8	Bottom
MM_89	9 July 2018	Nonvegetated	0 – 1	Top
MM_90			2 – 3	Upper Middle
MM_91			4 – 5	Lower Middle
MM_92			7 – 8	Bottom
MM_93	9 July 2018	Vegetated	0 – 1	Top
MM_94			2 – 3	Upper Middle
MM_95			4 – 5	Lower Middle
MM_96			7 – 8	Bottom
MM_97	8 August 2018	Nonvegetated	0 – 1	Top
MM_98			2 – 3	Upper Middle
MM_99			4 – 5	Lower Middle
MM_100			7 – 8	Bottom
MM_101	8 August 2018	Vegetated	0 – 1	Top
MM_102			2 – 3	Upper Middle
MM_103			4 – 5	Lower Middle
MM_104			7 – 8	Bottom
MM_105	3 September 2018	Nonvegetated	0 – 1	Top
MM_106			2 – 3	Upper Middle
MM_107			4 – 5	Lower Middle
MM_108			7 – 8	Bottom
MM_109	3 September 2018	Vegetated	0 – 1	Top
MM_110			2 – 3	Upper Middle
MM_111			4 – 5	Lower Middle
MM_112			7 – 8	Bottom
MM_113	4 October 2018	Nonvegetated	0 – 1	Top
MM_114			2 – 3	Upper Middle
MM_115			4 – 5	Lower Middle
MM_116			7 – 8	Bottom
MM_117	4 October 2018	Vegetated	0 – 1	Top
MM_118			2 – 3	Upper Middle
MM_119			4 – 5	Lower Middle
MM_120			7 – 8	Bottom

Supplementary Table S2 Sample ID, sampling site, sediment layer and depth, sampling date, number of raw sequence pairs, number of assembled contigs by MEGAHIT, N50 and L50 assembly statistics, number of predicted CDSs by Prodigal, and number of eggNOG-mapper annotated CDSs.

Sample ID	Site	Layer (Depth)	Date	No. of Raw Sequence Pairs	No. of Contigs	N50*	L50 (bp)*	No. of Predicted CDSs	No. of Annotated CDSs
356	Vegetated	Top (0 – 1 cm)	8 August 2018	205,085,833	32,026,408	8,760,379	601	40,693,178	29,364,186
358		Lower Middle (4 – 5 cm)		209,632,803	33,248,196	9,111,820	590	42,249,295	29,892,039
360	Nonvegetated	Top (0 – 1 cm)	8 August 2018	213,766,540	21,634,340	6,073,512	595	27,526,969	19,599,377
362		Lower Middle (4 – 5 cm)		216,556,629	27,534,653	8,174,204	592	34,788,216	24,307,842

* The notation was preserved from the original output of BBTools statswrapper.sh.

Supplementary Table S3 The proportion of each COG functional category (NAAF) and the number of proteins assigned to each category. The proportion and the number of proteins assigned to category C (energy production and conversion) are highlighted.

COG Category	NAAF (%)	Number of Proteins
C – Energy production and conversion	**15.18**	**8,224**
S – Function unknown	12.62	6,299
G – Carbohydrate transport and metabolism	11.45	6,823
E – Amino acid transport and metabolism	9.25	6,893
M – Cell wall/membrane/envelope biogenesis	8.89	2,999
P – Inorganic ion transport and metabolism	8.43	4,441
Multiple functional categories	7.65	2,603
O – Posttranslational modification, protein turnover, chaperones	7.02	2,901
J – Translation, ribosomal structure and biogenesis	5.28	1,507
H – Coenzyme transport and metabolism	2.29	1,494
Q – Secondary metabolites biosynthesis, transport and catabolism	2.26	1,356
F – Nucleotide transport and metabolism	1.78	1,475
I – Lipid transport and metabolism	1.72	1,421
N – Cell motility	1.38	599
K – Transcription	1.35	1,169
U – Intracellular trafficking, secretion, and vesicular transport	1.27	731
L – Replication, recombination and repair	0.98	397
T – Signal transduction mechanisms	0.79	574
V – Defense mechanisms	0.23	217
D – Cell cycle control, cell division, chromosome partitioning	0.18	147
Total	100.00	52,270

Supplementary Table S4 Overview of selected enzymes, their enzymatic products, and corresponding KO entries used to evaluate mediation processes of various fermentation products.

Name	Product	KO Entry
Pyruvate:ferredoxin oxidoreductase	Acetyl-CoA, carbon dioxide	K00169 K00170 K00171 K00172 K03737
Pyruvate formate-lyase	Acetyl-CoA, formate	K00656
Acetyl-CoA hydrolase	Acetate	K01067
Acetate kinase	Acetate	K00925
Acetoacetate decarboxylase	Acetone, carbon dioxide	K01574
Alcohol dehydrogenase	Ethanol	K00001 K04022 K13951 K13952 K13954 K13980 K18857 K00002 K13979 K00114
Formate dehydrogenase	Carbon dioxide	K00122 K00123 K00124 K00126 K00127 K22515 K05299 K15022 K00125 K22516
Lactate dehydrogenase	Lactate	K00016
Acetolactate decarboxylase	Acetoin, carbon dioxide	K01575
Methylmalonyl-CoA decarboxylase	Propionyl-CoA, carbon dioxide	K11264 K01604
Lactoyl-CoA dehydratase	Acryloyl-CoA	K20626 K20627
Propionaldehyde dehydrogenase	Propionyl-CoA	K13922
Butyrate kinase	Butyrate	K00929
Butyryl-CoA:acetate CoA transferase	Butyrate, acetyl-CoA	K01034 K01035

Supplementary Table S5 Overview of KEGG modules used for assessing various types of microbial metabolism.

Type of Metabolism	KEGG Module
Methanogenesis	M00567 M00357 M00356 M00563
Methane oxidation	M00174
Nitrogen fixation	M00175
Assimilatory nitrate reduction	M00531
Dissimilatory nitrate reduction	M00530
Denitrification	M00529
Nitrification	M00528
Complete nitrification, comammox	M00804
Anammox	M00973
Assimilatory sulphate reduction	M00176
Dissimilatory sulphate reduction	M00596
Thiosulfate oxidation by SOX complex	M00595

Supplementary Table S6 Enzymes involved in dissimilatory sulphate reduction and their KO entries.

Name	KO Entry
Sulphate adenylyltransferase	K00958
Adenylylsulphate reductase	K00394 K00395
Dissimilatory sulphite reductase	K11180 K11181 K27196