Ecology and diversity of cyanobacteria in changing ecosystems in the Eastern Adriatic area

Kolda, Anamarija

Sveučilište u Zagrebu

Faculty of Science
Department of Geology

Anamarija Kolda

ECOLOGY AND DIVERSITY OF CYANOBACTERIA IN CHANGING ECOSYSTEMS IN THE EASTERN ADRIATIC AREA

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Anamarija Kolda

EKOLOGIJA I RAZNOLIKOST CIJANOBAKTERIJA U PROMJENJIVIM EKOSUSTAVIMA U PODRUČJU ISTOČNOG JADRANA

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University of Zagreb
Faculty of Science
Department of Geology

Doctoral thesis

ECOLOGY AND DIVERSITY OF CYANOBACTERIA IN CHANGING ECOSYSTEMS IN THE EASTERN ADRIATIC AREA

ANAMARIJA KOLDA

Faculty of Science, Department of Geology

Cyanobacteria are ubiquitous bacteria that evolved photosynthesis and therefore made life on Earth possible by creating aerobic atmosphere more than 2 billion years ago. As primary colonizers, they have adapted to diverse types of environments, and primordial as well as actual extreme conditions. In that respect, they are considered indicators of the ecological properties of the environment in which they are found. This thesis aims at exploring the vast diversity and ecology of the Cyanobacteria, from ecosystems under different selective pressures: extreme drought and flood conditions as well anthropogenic pressures such as aquaculture, agriculture and urbanization Modern molecular-based methods, with emphasis on the high-throughput sequencing, were the main methods used in this study, thus providing an accurate picture of their diversity and community composition in different ecological niches. In both freshwater and marine ecosystems, habitats such as microbial mats, sediment and water column were explored to give an overview of diversity and adaptation of Cyanobacteria to different environmental conditions. These conditions are ever shifting under the climate change, whose impact is actually observed in the semi-enclosed Adriatic Sea. As a matter of fact, the relevance of the Cyanobacteria is especially highlighted in the vulnerable geographical and geological area of the karstic Adriatic, where the effects of climate change and land-based pollution are acting much faster than in other areas.

(141 pages, 6 figures, 148 references, original in English)

Keywords: Bacteria, microbial mat, intermittent river, seawater, sediment, aquaculture

Supervisors: Dr. Damir Kapetanović, Senior Research Associate
Dr. Zrinka Ljubešić, Associate Professor

Reviewers: Dr. Marija Gligora Udovič, Associate Professor
Dr. Raffaella Casotti, Senior Scientist
Dr. Ines Sviličić Petrić, Senior Research Associate
Dr. Sunčica Bosak, Assistant Professor

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Geološki odsjek

Doktorski rad

EKOLOGIJA I RAZNOLIKOST CIJANOBAKTERIJA U PROMJENJIVIM EKOSUSTAVIMA U PODRUČJU ISTOČNOG JADRANA

ANAMARIJA KOLDA

Prirodoslovno-matematički fakultet, Geološki odsjek

Cijanobakterije su sveprisutne bakterije, koje su razvile oksigenu fotosintezu te omogućile život na Zemlji stvorivši aerobnu atmosferu prije više od 2 milijarde godina. Kao primarni kolonizatori, prilagodili su se različitim vrstama okoliša te ekstremnim uvjetima iz Zemljine prošlosti, kao i današnjim ektremnim uvjetima. Zahvaljujući tim prilagodbama, one su indikatori ekoloških svojstava okoliša u kojem se nalaze. Cilj ovog doktorskog rada je istražiti raznolikost i ekologiju cijanobakterija u ekosustavima koji su pod različitim pritiscima selekcije: ekstremnim sušama i poplavama te antropogenim pritiscima kao što su akvakultura, poljoprivreda i urbanizacija. Molekularne metode, s naglaskom na sekvenciranju druge generacije, su glavne metode korištene u ovom istraživanju. Njihovim implementacijom dobiva se potpunija slika raznolikosti i sastava zajednice cijanobakterija u različitim ekološkim nišama. U slatkovodnim i morskim ekosustavima istraživana su staništa poput mikrobnih obraštaja, sedimenata i vodenog stupca, upravo kako bi se dobio uvid u raznolikost i prilagodbe cijanobakterija različitim uvjetima okoliša. Ti se uvjeti neprestano mijenjaju u skladu s klimatskim promjenama, čiji je utjecaj očit u poluzatvorenom ekosustavu kao što je Jadransko more. Zapravo, važnost istraživanja ekologije cijanobakterija je posebno primjerena u osjetljivom geografskom i geološkom području krškog Jadrana, u kojem su učinci klimatskih promjena i onečišćenja s kopna evidentni mnogo brže nego na drugim područjima.

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Ključne riječi: bakterije, mikrobni obraštaj, povremene rijeke, morska voda, sediment, akvakultura

Mentori: dr.sc. Damir Kapetanović, viši znanstveni suradnik
izv.prof. dr.sc. Zrinka Ljubešić

Ocjenjivači: izv.prof.dr.sc. Marija Gligora Udovič
dr.sc. Raffaella Casotti, znanstveni savjetnik
dr.sc. Ines Sviličić Petrić, viša znanstvena suradnica
doc.dr.sc. Sunčica Bosak

Table of content

LIST OF PUBLICATIONS

I. Žutinić, P., Sviličić Petrić, I., Gottstein, S., Gligora Udovič, M., Kralj Borojević, K., Kamberović, J., **Kolda, A.,** Plenković-Moraj, A., Ternjej, I. (2018). Microbial mats as shelter microhabitat for amphipods in an intermittent karstic spring. *Knowledge and Management of Aquatic Ecosystems* 1, 419.

II. **Kolda, A.**; Petrić, I., Mucko, M., Gottstein, S., Žutinić, P., Goreta, G., Ternjej, I., Rubinić, J., Radišić, M., Gligora Udovič, M. (2019). How environment selects: Resilience and survival of microbial mat community within intermittent karst spring Krčić (Croatia). *Ecohydrology* 12 (2)

III. **Kolda, A.**, Ljubešić, Z., Gavrilović, A., Jug-Dujaković, J., Pikelj, K., Kapetanović, D. (2020). Metabarcoding Cyanobacteria in coastal waters and sediment in central and southern Adriatic Sea. *Acta Botanica Croatica* 79(2)

IV. **Kolda, A.,** Gavrilović, A., Jug-Dujaković, J., Ljubešić, Z. El-Matbouli, M., Lillehaug, A., Lončarević, S., Perić, L., Knežević, D., Vukić Lušić, D., Kapetanović, D. (2020). Profiling of bacterial assemblages in the marine cage farm environment, with implications on fish, human and ecosystem health. *Ecological indicators*, 118 (1 2), 106785

THESIS SUMMARY

The name “cyanobacteria” very concisely describes two most important characteristics of these organisms: firstly, they have specific, diagnostic pigments in their cells that are important link in the process of oxygenic photosynthesis, and secondly, they are prokaryotic organisms. As many other bacteria, cyanobacteria are ubiquitous in many environments, but they have abilities to proliferate in the most extreme ones. Adaptation to high salinity, extremely high and low temperatures, desiccation, high UV radiation, high level of nutrients etc., originate from their long history (3.5 billion years), when the Earth’s environment was utterly extreme (Bellinger and Sigee, 2015). For that reason, they are often considered as pioneers in today’s environments, especially the ones that emulate conditions of the Earth’s past. Through photosynthesis and symbiotic relationships with other prokaryotic and eukaryotic organisms, cyanobacteria have transformed ecosystems and environments in what we observe today. Even though cyanobacteria are crucial part of many ecosystems (especially in the oceans), due to anthropogenically impacted processes of climate change and eutrophication, they are often associated with negative impacts on the environment and health, especially in freshwaters. Their diversity, abundance, positive and negative impact on aquatic and terrestrial ecosystems have been investigated in various research studies. At the same time, for many cyanobacterial genera taxonomical challenges are still present. Transition to molecular-based methods in investigations largely contributed to discovering much greater cyanobacterial diversity and opened up discussion of bridging morphological and genomic data (Komárek et al., 2014). Moreover, molecular-based methods, such as the ones utilizing high-throughput sequencing (HTS) technology, represent fast and relatively low-cost technologies when addressing monitoring issues in the anthropogenically impacted environments. Metabarcoding methods of first (Sanger sequencing) and second generation (HTS) (Santos, 2020) using the 16S rRNA and/or ITS gene markers, are the main methods used in this thesis for the identification of cyanobacteria and other members of bacterial communities in freshwater and marine ecosystem of the Eastern Adriatic Sea.

The aim of this thesis is to describe cyanobacterial community response to environmental pressures derived from climate change and anthropogenic sources, with emphasis on the changes observed at the level of cyanobacterial diversity and community structure. This encompasses studying cyanobacterial community response in both benthic and pelagic systems of different freshwater and marine environments, namely an intermittent river in the Adriatic Sea basin and

coastal waters and sediments of central and southern Adriatic Sea. This aim has been pursued using molecular-based methods, specifically metabarcoding. In doing that, this thesis will address the following hypotheses: i) Cyanobacteria are exceptionally important organisms in extreme environments, as they change/adapt such environments into habitats suitable for growth and proliferation of other organisms; ii) The Adriatic Sea and the Adriatic basin in general are ideal natural models for studying ecosystems influenced by climate change and anthropogenic pressures and iii) contemporary molecular methods using high-throughput sequencing technology, such as metabarcoding, are the most appropriate tools for studying cyanobacteria and for identification of key taxa essential for ecosystems functioning.

In this doctoral thesis, after the introductory chapter, results in the form of four publications are presented together with thorough discussion. The first two publications are concerned to freshwater, benthic cyanobacteria within microbial mats situated in an intermittent river of the Adriatic Sea basin. Intermittent freshwater systems are identified as extremely vulnerable to climate change, but are unfortunately very rarely focus of investigations (Datry et al., 2014). Publication I is concentrated on the description of cyanobacterial microbial mats as food and shelter for the subterranean crustacean *Synurella ambulans* (F. Müller, 1846). Cyanobacteria were investigated using light microscopy and sequencing of the 16S rRNA gene clone libraries (Sanger i.e. first generation sequencing) in the river under intermittent regime. Furthermore, organisms embedded in the microbial mats, for instance diatoms and the amphipod *S. ambulans* were described and enumerated. In that way, cohabitation relationships of cyanobacteria with other eukaryotic organisms were explored. In the Publication II, bacterial component of the cyanobacterial mats were investigated during the course of one year covering eight different time points and all four different seasons (October, November, and December of 2014 and March, April, May, June, and October of 2015), by using both Sanger sequencing of ITS region and HTS of 16S rRNA V1-V3 region, focusing on the hydrological stress as main factor shaping targeted communities. Publications III and IV focus on areas of the central and southern Adriatic Sea impacted by anthropogenic activities (aquaculture), concentrating on the seawater column and surface marine sediments. Bacterial communities, including cyanobacteria, were targeted using HTS of 16S rRNA V1-V3 region. In Publication III, the cyanobacterial component was subsampled from the total Bacteria in order to examine their diversity, ecology and structural changes, as well as to detect the main environmental factors responsible for the observed changes within communities. Finally, Publication IV explores

the impact of aquaculture and other anthropogenic impacts in coastal areas of the Eastern Adriatic Sea on total bacterial community by using HTS and by implementing various bioinformatics tools.

This thesis will, for the first time, provide valuable information on the structure of the cyanobacterial microbial mat community residing in the unexplored intermittent river habitat. Cyanobacteria and other members of bacterial community were described for the first time in this ecosystem by implementing HTS, which is still rarely used in ecological and monitoring studies in Croatia. In the anthropogenically impacted coastal seawater and sediments, situated in the central and southern Adriatic coastal zone, cyanobacteria (as well as other members of prokaryotes) were explored by using the same method. For the first time, benthic cyanobacteria from marine sediment were given consideration in the eastern Adriatic. This thesis also provides insights into sediment bacterial community residing in the in the anthropogenically impacted zones, thematic that is completely overlooked, due to its complexity. Finally, this thesis represents a first step towards the use of total bacterial communities as indicators to assess environmental, animal and human health in sea bass aquaculture system in Croatia. Moreover, this is one of the few studies using HTS metabarcoding of bacterial communities in sea bass farms in the Mediterranean Sea in general.

Climate change and anthropogenic pollution of coastal areas are one of the biggest challenges faced by Mediterranean countries, necessitating investigations such as the ones presented in this thesis. Furthermore, the Eastern Adriatic Sea and the Adriatic basin are even more vulnerable to these changes, due to karstified rocks of semi-enclosed, rugged coastline, offering many locations for various anthropogenic activities and contamination. Therefore, considering climate change and human impact on coastal Adriatic, the results displayed by metabarcoding methods reveal great bacterial diversity, ecological variety and functional potential in the investigated environments. HTS technologies and metabarcoding also show promise as an independent and complementing method of investigation of cyano(bacteria), particularly in the eastern Adriatic.

PROŠIRENI SAŽETAK

Naziv „cijanobakterije“ vrlo sažeto opisuje dvije najvažnije karakteristike ovih organizama: prvo, one imaju specifične dijagnostičke pigmente u svojim stanicama koji su važna karika u procesu fotosinteze kisika, i drugo, one su prokariotski organizmi. Kao i mnoge druge bakterije, cijanobakterije su sveprisutne u mnogim okolišima, no cijanobakterije imaju sposobnost razmnožavanja u onim najekstremnijim. Prilagodbe na uvjete visokog saliniteta, izuzetno visokih i niskih temperatura, isušivanje, visoko UV zračenje, visoka razina hranjivih tvari itd., su prilagodbe koje potječu od postanka cijanobakterija prije 3,5 milijardi godina, kada su uvjeti u okolišima na Zemlji bili isključivo esktremni (Bellinger i Sigee, 2015). Iz tog razloga ih se u današnjim okolišima često naziva pionirima, posebno u onim okolišima koji imaju slične uvjete kao u Zemljinoj prošlosti. Međutim, cijanobakterije su transformirale ekosustave i okoliš kakve ih danas poznajemo postupkom fotosinteze i simbiotskim odnosima s drugim prokariotskim i eukariotskim organizmima. Iako su cijanobakterije još uvijek važan dio mnogih ekosustava, posebno oceana, zbog antropogenih utjecaja (klimatskih promjena i eutrofikacije) mogu imati negativan utjecaj na okoliš i zdravlje. Njihova raznolikost, abundancija te pozitivni i negativni utjecaji na vodene i kopnene ekosustave istražuju znastvenici iz različitih područja. Unatoč tome, mnogi cijanobakterijski rodovi i dalje prestavljaju taksonomske nedoumice zbog dvojne nomenklature. Prelazak na molekularne metode istraživanja cijanobakterija uvelike je pridonio otkrivanju veće cijanobakterijske raznolikosti i otvorio raspravu o premošćivanju taksonomskih razlika dobivenih iz morfoloških i genetskih podataka. Štoviše, molekularne metode, poput onih koje koriste tehnologiju sekvenciranja druge generacije, brže su i sve jeftinije u monitoringu okoliša pod antropogenim utjecajima. Metode metabarkodiranja prve i druge generacije (Santos, 2020) pomoću genskih markera 16S rRNA i/ili ITS, središnja su metoda u ovom doktorskom radu, u identifikaciji cijanobakterija i ostatku bakterijske zajednice u slatkovodnom i morskom ekosustavu istočnog Jadrana.

Ciljevi ovog doktorskog rada su opisati raznolikost, promjene u strukturi i odgovor cijanobakterijskih zajednica na pritiske u okolišu uzrokovane klimatskim promjenama i antropogenim izvorima. To obuhvaća i bentičku i pelagičnu komponentu u slatkovodnom i morskom okolišu: krška rijeka Jadranskog sliva koja ljeti presušuje, te morski stupac i površinski sedimenti središnjeg i južnog Jadranskog mora. Cilj je to postići korištenjem molekularnih metoda, tj. metabarkodiranje. Pri tome će se doktorski rad baviti sljedećom hipotezama: i) Cijanobakterije su iznimno važni organizmi u naseljavanju ekstremnih okoliša i mijenjaju/prilagođavaju takve okoliše

za razvoj drugih organizama; ii) Jadranski sliv i Jadransko more su idealni prirodni modeli za proučavanje ekosustava pod utjecajem klimatskih promjena i antropogenim pritiscima, iii) Suvremene molekularne metode koje koriste tehnologiju sekvenciranja nove generacije, kao što je metabarkodiranje, je odgovarajući alat za proučavanje cijanobakterija i određivanje ključnih vrsta za ekosustave.

U ovom doktorskom radu, nakon uvodnog poglavlja, predstavljeni su rezultati u formi četiriju publikacija te kasnije raspravljeni. Prve dvije publikacije posvećene su slatkovodnim bentičkim cijanobakterijama u mikrobnim obraštajima, smještenim na izvoru povremene tekućice Jadranskog sliva. Povremene tekućice prepoznate su kao izuzetno osjetljive na klimatske promjene, no istovremeno su nedovoljno istražena staništa (Datry i sur., 2014). U Publikaciji I fokus je stavljen na opisivanje cijanobakterijskih mikrobnih obraštaja kao hrane i skloništa za podzemne amfipode *Synurella ambulans* (F. Müller, 1846). Cijanobakterije se istražene pomoću svjetlosne mikroskopije i 16S rRNA sekvenciranja prve generacije u reprezentativnim mjesecima u riječnom ciklusu. Nadalje su opisane vrste i abundancija organizama koji su nalaze u mikrobnim obraštajima, kao što su dijatomeje i *S. ambulans*. Na taj su način istraženi kohabitacijski odnosi cijanobakterija s drugim eukariotskim organizmima. U Publikaciji II, cijanobakterijski mikrobni obraštaji su istraženi tijekom osam mjeseci (listopad, studeni i prosinac 2014 te ožujak, travanj, svibanj, lipanj i listopad 2015). Kako bi se dobio detaljniji uvid u bakterijsku komponentu, napravljeno je sekvenciranje ITS regije sekvenciranjem prve generacije te regije 16S rRNA V1-V3 sekvenciranje druge generacijes fokusom na hidrološki stres koji oblikuje zajednicu. U Publikaciji III i IV provodilo se sezonsko istraživanje uzoraka morske vode i površinskih sedimenata u središnjem i južnom priobalju Jadrana, pod utjecajem antropogenih aktivnosti (akvakultura). Bakterijska zajednica, uključujući cijanobakterije, istraživana je poglavito upotrebom sekvenciranjem druge generacije 16S rRNA V1-V3 regije. U Publikaciji III cijanobakterijska komponenta bila je zasebno analizirana radi detaljnijeg uvida u raznolikost, ekologiju i strukturne promjena, kao i otkrivanja glavnih faktora okoliša koji oblikuju cijanobakterijsku zajednicu u morskoj vodi i sedimentu. Konačno, Publikacija IV detaljno istražuje ukupnu bakterijsku zajednicu pomoću sekvenciranja druge generacije, primjenjujući različite bioinformatičke alate, prvenstveno za procjenu utjecaja akvakulture i drugih antropogenih utjecaja obalnih područja istočnog Jadranskog mora.

Znanstveni doprinos ove teze prvi je uvid u sastav bakterijske zajednice cijanobakterijskih mikrobnih obraštaja, u nedovoljno istraženom okolišu povremenih rijeka. Nadalje, cijanobakterije i ostali članovi bakterijske zajednice prvi put su opisani primjenom sekvenciranja druge generacije,

koji se još uvijek rijetko koristi u ekološkim i monitoring istraživanjima u Hrvatskoj. U središnjem i južnom obalnom području Jadrana cijanobakterije (kao i ostali pripadnici zajednice) istraživane su istom metodom u antropogenim utjecajima obalne morske vode i sedimenata. Dan je detaljan uvid posebno u bakterijsku zajednicu u sedimenatu, staništu koje je gotovo neistraženo u drugim studijama na Jadranu koje koriste sekvenciranje druge generacije. Nadalje, prvi put se na istočnom Jadranu pridala pažnja bentičkim cijanobakterija iz morskog sedimenta. Bitan korak učinjen je upotrebom sekvenciranja druge generacije na bakterijske zajednica iz sustava akvakulture lubina u Hrvatskoj, u procjeni zdravlja okoliša, životinja i ljudi. Štoviše, ovo je jedno od rijetkih istraživanja koje koristi metodu metabarkodiranja sekvenciranjem nove generacije na farmama lubina općenito u Mediteranu.

Klimatske promjene i antopološko onečišćenje su najveći izazovi s kojim se susreću mediteranske zemlje, stoga su istraživanja kao iz ovog doktorata nužna. Posebno jer je područje istočnog Jadrana i Jadranskog mora osjetljivo na takve pritiske, zbog krške geološke podloge i poluzatvorene, vrlo razvedne obale. Stoga, uzimajući u obzir klimatske promjene i utjecaj čovjeka na područje Jadrana, rezultati dobiveni metodom metabarkodiranja pokazali su veliki potencijal kao samostalna metoda te nadopuna drugim metodama istraživanja cijano(bakterija), posebno na istočnom Jadranu.

INTRODUCTION

Cyanobacteria: Prokaryotes essential for life on Earth

Bacterial phyla with link to plant kingdom

Metabolic activity of extremely special prokaryotes – Cyanobacteria – have created probably the most important biogeochemical phenomenon in Earth history, an aerobic atmosphere 2.2-2.4 billion years ago (Ochoa de Alda et al., 2014). Using aerobic photosynthesis cyanobacteria produced oxygen for the first time, raising the oxygen level in the atmosphere and in the ocean, allowing the ozone layer to form so enabling oxygen-using living organisms to thrive and evolve. During a period known as the Great Oxidation Event (GOE), cyanobacteria became one of the main contributors to the biological cycle of carbon and nitrogen (Schirrmeister et al., 2016, Meriluoto et al., 2017), as are still today. Moreover, they synthetize N-(2-Aminoethyl)Glycine, likely the origin molecule for peptide nucleic acids (PNAs), directly impacting evolution of molecules containing genetic information, e.g. RNA (Banack et al. 2012; Schirrmeister et al., 2016). Cyanobacteria incorporate non-photosynthetic line – Melainabacteria – thriving in aphotic habitats using anoxygenic photosynthesis, a predecessor of oxygenic photosynthesis. Photosynthetic Cyanobacteria and Melainabacteria share a common ancestor, which suggests that photosynthesis occurred with strong lateral gene transfer (Soo et al. 2014). Genome of the most recent ancestor has 4.5 Mb, approximately only 4-6% of the genome of modern cyanobacteria. Due to numerous genomic "upgrades", today cyanobacteria have various evolutionary advantages, e.g. ability to create heterocysts and filaments, diazotrophic metabolism and numerous symbiotic adaptations (Larsson et al., 2011; Alvarenga et al., 2017). Furthermore, they have up to two chromosomes (1.4 to 8.2 Mb), but the possibility to make 218 chromosomes in polyploidy events (Griese et al., 2011; Alvarenga et al., 2017). Core genome of cyanobacteria is robust and resistant to horizontal gene transfer and modifications, conserving the essential biochemical paths and complex proteins (Shi and Falkowski 2008; Larsson et al.,2011; Bergman 2011; Alvarenga et al., 2017). Nonetheless, the rest of the genome easily changes and responds quickly to environmental changes (Tenaillon et al. 2016; Alvarenga et al., 2017).

The name "cyanobacteria" originates from Greek word "cyano-" meaning blue, due to pigment phycobilin that in combination with phycoerythrin produces characteristic blue-green colour (Bellinger and Sigee, 2015). Typical prokaryotic structure includes cell size <10 μm in diameter, nucleotide DNA without nuclear membrane structure. Like other Gram negative bacteria,

they have external membrane, plus peptidoglycan cell wall, lipopolysaccharide inside membrane, and certain cyanobacteria also have outer mucous membrane. Specific organelles are located in the protoplasm: carboxysomes (enzymes for CO_2 fixation), cyanophycin (amino acids polymers containing arginine and aspartic acid, used for nitrogen storage), granules of polyphosphate and glycogen, and finally, photosynthetic pigments connected to the thylakoid membranes. On them are located large protein structures such as phycobilisomes, chlorophyll *a* complex, carotenoids, photosynthetic reaction centre and electron transport system (Bellinger and Sigee, 2015). Both oxygenic photosynthesis and cellular respiration occur in thylakoids. Phycobilisomes are arranged in rows and connected to photosystem II situated on the other side of the thylakoid membrane surface, and their role is to collect light for cyanobacterial cell. Their shape can vary from semi-dysmoid to semi-ellipsodial, bicylindrical, in bundles, etc. Phycobilisomes consist of two types of proteins: brightly coloured, polar phycobiliprotein with covalently bound tetrapyrrole chromophores (bilins) and uncoloured, non-polar bound proteins. The association of acidic, polar phycobiliprotein and non-polar, basic binding protein creates the "core-rod" structure, that is very efficient in transporting light energy to the photosynthetic reaction centre. Due to bilins, phycobiliproteins are very fluorescent, and their analysis, along with other non-polar pigments (carotenoids and chlorophylls), can provide insight into cyanobacterial diversity. Moreover, phycobiliproteins can make up to 60% of the protein content of cyanobacteria, and for this reason certain types of cyanobacteria (e.g., *Arthrospira*, marketed as Spirulina) have been used in the human and animal diet (Colyer et al., 2005). Other important processes in which cyanobacteria participate is nitrogen fixation, and further transformation of nitrogen into ammonia ions and amino acids. Nitrogen fixation is usually carried out in heterocysts under aerobic conditions, but there are special strategies of nitrogen fixation for other cyanobacteria without these specialized cells (Whitton and Potts, 2000). In the last decade it was determined that cyanobacteria are the main N_2-fixing organisms in the oceans, even though heterocyst cyanobacteria are scarce in marine environments (Bauer et al., 2008; Whitton and Potts, 2000).

Due to their remarkable features and metabolic adaptations, cyanobacteria reside in all types of habitats; from thermal springs, Antarctic soils and lakes, oceans and seas, river estuaries, freshwater ecosystems, and illuminated rock and soil surfaces (Colyer et al., 2005). They can even inhabit interior of rocks (endoliths), with several adaptations: rock boring (euendolith), colonizing cavities inside porous rocks (cryptoendoliths) and rock's cracks (chasmoendoliths) (Golubic et al., 1981). They are primary colonizers, capable of surviving various extreme conditions such as high

salinity, extreme temperature changes, droughts, large amounts of UV radiation. Cyanobacteria also store nutrients such as phosphorus, nitrogen, carbon, iron, which allows them to grow in conditions where these elements are limited (Meriluoto et al. 2017).

Taxonomic conundrum: Botanists versus Microbiologists

Cyanobacterial taxonomy is a complex subject due to different approaches used. The first classical taxonomy was compiled by Gietler in 1932 according to the instructions of the ICBN (International Code of Botanical Nomenclature), and he calls them blue-green algae or Cyanophyta (Whitton and Potts 2012). Later in the 1970s, Stanier proposed that blue-green algae should be treated as bacteria, change the name to cyanobacteria and adjust their taxonomy according to the rules of the International Code of Nomenclature of Bacteria (Stanier et al. 1978; Whitton and Potts, 2012). Assigning species for cyanobacteria, as well as bacteria in general, is often challenging. Prokaryotes reproduce asexually, so they do not fit into Ernst Mayr's "biological concept of species" - populations that reproduce within their own group and are reproductively isolated from other groups. Molecular definitions of the species are accepted today: if they have over 97% identity of 16S rRNA gene or if they share at least 70% binding in standardized DNA-DNA hybridization, they belong to the same species (Doolittle and Papke, 2006). In the last decade, all nomenclature changes involve molecular research, and the central role of molecular data is particularly emphasized by Komárek et al. (2014). However, the importance of additional inclusion of phenotypic and ecological features for accurate species identification is suggested.

In practice, cyanobacteria are often determined by their morphological features. Morphogenera are often used in the determination literature, i.e. defining genera on the principle of their morphology (Komárek et al., 2014). Morphological characteristics are the basis for species determination, e.g. coccoid or trichal form, branching type, dimensions, presence of akinets and other specialized cells. However, these features often appeared or disappeared during the evolution of species and genera, also due to environmental conditions (Gugger and Hoffmann 2004; Schirrmeister et al., 2011; Komárek et al. 2014; Shih et al. 2013), making this type of characterization unpredictable. Use of electron microscopy and molecular methods have changed drastically our knowledge on cyanobacteria, showing that morphology cannot be the only standard in species taxonomy and determination. A good example of the unreliability of morphology are cyanobacteria grown in culture, which often change their morphology as a response to adverse conditions (Komárek et al., 2014). Moreover, the use of molecular methods has led to the discovery of many

cryptic genera and species, which are morphologically or ecologically almost identical, but stand out when ultrastructural differences, small morphological differences and ecological differences are observed (Komárek et al., 2014).

A compromise can be represented by the "polyphasic approach", in which two main conditions must be met: the taxonomic classification must reflect phylogeny and the species must be monophyletic (Komárek et al., 2014). Knowing the phylogeny, often the data on the morphology and ecology of the species coincide, and it is easier to identify a new genus. The definition of the cyanobacterial genus uses molecular phylogeny based on 16S RNA type species, around which monophyletic groups of strains that connect morphological and other characteristics are collected. According to these characteristics, Komárek et al. (2014) propose a taxonomic revision of Cyanobacteria in 8 orders: Gleobacterales, Synechococcales, Oscillatorales, Chroococcales, Pleurocapsales, Spirulinales, Chrococcidiopsidales and Nostocales (Komárek et al., 2014). It should be emphasized that sometimes 16S RNA analysis is not sufficient for certain genera, therefore it is necessary to sequence additional regions of the genome in order to make a multiple loci analysis. In the discovery of cryptic species and the separation of polyphyletic genera, it is useful to combine phylogeny from most frequently used markers: 16S rRNA and 16S-23S ITS (internal transcribed spacer) region. ITS marker can be observed phylogenetically or through the secondary structure of proteins (Sciuto and Moro 2016). Revision of the genus *Geitlerinema* and description of the new genus *Anagnostidinema* gen. nov. is a successful example of using the secondary structure of the ITS region (Strunecky et al. 2017)

Methodology for cyanobacterial research

Cyanobacteria are a very indicative phyla of the environment they inhabit; hence they are oftentimes investigated using different methods, depending on the scope of the investigation. One of the approaches is water quality monitoring, that uses information on cyanobacterial ecology to assess the trophic state of aquatic environments. Depending on the type of water body, the methods of sampling, detection and monitoring of cyanobacteria vary. Thus, there is a methodology for shallow lakes, deep lakes, reservoirs, rivers, the Baltic Sea and drinking water reservoirs. Ecological monitoring of cyanobacterial blooms is performed by the morphological observation under an inverted light microscope, according to the Utermöhl method (Lund et al., 1958). Sampling of cyanobacteria in aquatic environments can be done in 3 ways: phytoplankton mesh, pump or integrated pump. The collected water samples must be chemically fixed immediately or shortly after

sampling (Bellinger and Sigee, 2015). Results can be further incorporated in ecological theories, such as Reynolds functional groups, e.g. Mantzouki et al. (2016). The authors consider that the key ecological characteristics of cyanobacteria are most important for successful control and management of lakes. Using Reynolds functional groups (Reynolds et al. 2002; Reynolds 2006) they 5 groups of cyanobacteria can be defined: S1 / S2, H1 / H2, LO / LM, SN and R. For each, key ecological properties are defined, related on how species adapt on eutrophication conditions and climate change, then what monitoring and management measures can be applied knowing their strengths and weaknesses.

Bloom-forming cyanobacteria may produce various toxins called cyanotoxins. Traditionally, various chemical methods (e.g. HPLC, LC/MS etc.) have been used to determine cyanobacterial toxins that are classified into families according to chemical structure (Meriluoto et al. 2017). According to the Guidelines for Safe Recreational Water Environments (WHO, 2003), marine blooms are known to cause “marine cyanobacterial dermatitis” that occurs after swimming in such waters. Symptoms including itching, burning, redness, blisters, and peeling of the skin, as reported to be caused by the benthic marine cyanobacterium *Lyngbya majuscula* in Japan, Hawaii, and Australia. Numerous toxic components have been isolated from marine cyanobacteria, such as aplysiatoxin, debromoaplysiatoxin, and lyngbyatoxin A (WHO, 2003; Shimizu 1996). *Nodularia spumenigena*, which produces the toxin nodularin, is considered to be the first cyanobacteria that caused mammal death. Nodularin is a hepatotoxin that enters the body by ingesting contaminated water, and it causes bleeding, liver failure and kidney damage (Eriksson et al. 1988; Sandström et al. 1990). In most cases, the identification of cyanobacterial species is not sufficient to determine whether it is also toxic, as strains with varying degrees of toxicity, but belonging to the same species, may develop (WHO, 2003).

Oceanologists and limnologists use chemical methods such as fluorimetry or spectrophotometry to determine chlorophyll concentrations, which are used as a proxy of phytoplankton biomass (Jeffrey et al., 1997). Wright et al. (1991) introduced the High Performance Liquid Chromatography (HPLC) method which separates more than 50 pigments including chlorophylls (a, b, c), carotenoids and their derivatives by means of 3 solvent ratios (80:20 methanol/ammonium acetate and 0.01% BHT; acetonitrile/water and 0.01% BHT; ethyl acetate). Since then, detailed protocols allow discrimination of phytoplankton photosynthetic and accessory pigments which are used as taxonomic markers (Jeffrey et al., 1997). For cyanobacteria are used β,β-carotene and zeaxanthin – the most important marker pigment for cyanobacteria (shared with

green algae), but also cyanobacteria-specific such as echinenone, canthaxanthin, myxoxanthophyll, etc. (Descy, 2017). However, the carotenoid canthaxanthin can coelute with the cyanobacterial taxonomic marker zeaxanthin, hindering the results. HPLC also allow separation and quantification of divinyl chlorophyll a taxonomical indicator of the cyanobacterium Prochlorococcus, which belongs to marine picoplankton and plays a key role in the global carbon cycle (Ito and Tanaka, 2011). It is one of the few cyanobacteria that does not have phycobilisomes, along with *Prochloron* and *Prochlorothrix* (Biller et al., 2015). It has a unique photosynthetic system of prochlorophyte chlorophyll-binding protein (Pcb) that uses divinyl chlorophyll *a* and divinyl chlorophyll *b* (Biller et al., 2015) instead of monovinil chlorophyll a, and show an incredible photophysiology which allows it to photosynthesize down to the deep layers of the water column in the vast majority of the oceans. Evolutionarily, this is an essential feature that allows these cyanobacteria to efficiently use the blue part of the light spectrum with the help of divinil chlorophyll a (Ito and Tanaka, 2011).

Another method of tracking cyanobacteria is through satellite imaging (optical or satellite remote sensing) which allows to quickly spot blooms and eventually apply countermeasures (Hunter et al., 2017). Cyanobacterial “diagnostic pigments” used in remote sensing are phycocyanin with a characteristic maximum at about 620 nm and 650 nm (Gons et al. 2005; Bresciani et al. 2016) and phycoerythrin with a maximum at 565 and 600 nm (Bresciani et al. 2011, 2016).

Lastly, with development of molecular methods and computational power, molecular methods are used for assessing species richness and abundance, by designing specific oligonucleotide probes with fluorescent labels, which can then be enumerated by epifluorescence microscopy or flow cytometry. Various types of analysis use this basic technology: Fluorescent In Situ Hybridization (FISH), qPCR (quantitative polymerase chain reaction), dot blot hybridization, and whole cell (in situ) hybridization, coupled with epifluorescence microscopy, confocal microscopy and flow cytometry. Especially flow cytometry (FCM) has a wide application as a quantitative method at the single cell level, that can overcome many shortages of traditional microscopy analysis (Dubelaar et al., 2007). Using the lasers as a light source, FCM produces fluorescent and scattered light signals emitted off cell populations and read by detectors, further converted into electronic signals analysed by software (McKinnon, 2018). In that process, can be used to enumerate populations of interest, e.g. DNA binding dyes, viability dyes, ion indicators dyes etc. (McKinnon, 2018). This methodology is very useful for monitoring applications (aquaculture, ballast waters) and ecological applications (population processes, cell processes and functions, abundance), due to its reproducibility and rapidity of analysis (Dubelaar et al., 2007).

Regarding metabarcoding, Sanger sequencing (Sanger and Coulson, 1975), based upon using PCR (Polymerase Chain Reaction) technique, has revolutionized applications in molecular biology. This molecular method allowed bacteria analysis without the need of culturing them, which tremendously updated the field, considering that less than 1% of microbes from the environment are cultured and only a fraction of them is found in culture collections (Solden et al., 2016). This is called first generation sequencing or metabarcoding because it used full length 16S rRNA gene as a marker (approximately 1600bp) in a lengthy procedure: obtaining and cloning of amplicon, adding it into a vector and transform in a host (usually *E. coli*), extracting the plasmid, purification and finally Sanger sequencing of 16S rRNA insert (Santos et al., 2020). 16S rRNA is one of the many marker genes used, but it is considered the golden standard for bacterial taxonomic profiling, due to several advantages: it is present in all prokaryotes including Archaea; it has highly conserved regions targeted by universal primers and by flanking hypervariable regions (V1-V9) and variable regions for identification of specific bacterial groups; high degree of functional conservation and small size of ~1500 bp (Santos et al., 2020). However, even though Sanger sequencing platforms can generate sequences up to 1000 bp with almost 100% accuracy, their number is very limited and time and cost demanding. Besides 16S rRNA gene PCR amplification and subsequent Sanger sequencing, PCR methods as DGGE (Denaturing Gradient Gel Electrophoresis) and T-RFLP (Terminal Restriction Length Polymorphism) were increasingly used in the monitoring of cyanobacteria from environmental samples. For DGGE analysis, the noncoding and variable region ITS (Intergenic Transcribed Spacer), located between 16S-23S rRNA (Luo et al. 2014), is used to obtain more detailed analyses of intraspecies phylogeny (García-Martínez et al. 1996; Boyer et al., 2001).

In the mid-2000s, high throughput sequencing (HTS) technologies became more prevalent due to the ability to process large amounts of biological data, e.g. Illumina platform can generate up to 20 Gb of data, comparing to Sanger sequencing platform output of 1.9-84Kb. Besides the high output and data accuracy, these technologies also removed time-consuming steps such as the cloning of DNA fragments and electrophoretic separation of sequencing products (required for Sanger sequencing) (Santos et al., 2020). HTS includes several sequencing platforms: 454 pyrosequencing (Roche), Ion Torrent (Life Technology) and Illumina (Mandal et al., 2015). This second generation sequencing platforms use PCR amplification of specific regions of the 16S rRNA gene, where out of nine regions V1-V2 and V3-V4 are the most used (Santos et al., 2020). A paired end library is constructed using these regions, with adapters and indexes added to amplicons ends, and finally libraries of ~300 bp are sequenced on the chosen platform. Main advantage over Sanger

sequencing is high number of small gene fragments, which is still sufficient to analyse complete bacterial diversity using bioinformatics tools in downstream analysis and databases (e.g. SILVA, Greengens) in bacterial identification. The field of microbial ecology experienced considerable transformation, accompanied by availability of ever emerging new tools and updates of platforms for bioinformatics analyses (e.g. QIIME/QIIME2, Mothur, Phyloseq). However, biases of the method can be introduced in every step, from using different isolation kits, choosing primers for different regions of 16S rRNA amplification etc., leading to bias in taxonomic assignment, overestimation of certain bacterial groups because of high number of amplification etc.

Third generation sequencing (or long read sequencing) has been developed in the last ten years. It started with Pacific Biosciences (PacBio) single-molecule real-time (SMRT) sequencing in 2011, using Sequel sequencer, and Oxford Nanopore Technologies nanopore sequencing in 2014, using MinION platform. Still, for 16S rRNA metabarcoding studies, there is still a scarcity of bioinformatic tools and protocols designed specifically for the analysis of outputs by these platforms, especially compared to second generation sequencing platforms (Santos et al., 2020).

Symbiotic relationships case in point: Microbial mats

Pronounced potential for symbiotic relationships is characteristic of cyanobacteria, including endosymbiosis. Results by Ochoa de Alda et al. (2014) propose that the origin of plastids happened during diversification of mainly N_2 fixing filamentous cyanobacteria around 1.75–2 Bya ago. In that ecologically important event, cyanobacteria basically started an evolutionary path that led to the origin of plants during the Proterozoic (Schirrmeister et al., 2016). The large metabolic capacity of cyanobacteria has produced important ecological roles and enabled them to achieve mutual relations with various organisms. Their endosymbiosis and episymbiosis with plants, fungi and lichens, diatoms and dinoflagellates, and animals have been documented (Adams 2000; Adams et al., 2013; Alvarenga et al., 2017). Heterotrophic bacteria often form symbiotic relationships with cyanobacteria, bounding to their cells walls or residing in cyanobacterial glycocalyx, profiting from products of photosynthesis, nitrogen fixation and secondary metabolism (Zhubanova et al. 2013; da Silva et al. 2014s).

Microbial mats are the oldest ecosystems on Earth and the most ecologically successful, due to the establishment of a stable but also very plastic community of organisms (Awramik, 1976; Bonilla-Rosso et al., 2012). These are self-sustaining and heterogeneous systems, that are interesting experimental models on which ecosystem response to rapid environmental changes can

be tested (Paerl et al., 2008; Bonilla-Rosso et al., 2012). Additionally, they are remarkable for studying coevolution and ecological interactions such as mutualism, competition, predation, parasitism, commensalism, neutralism (Alvarenga et al., 2014). They are characterized by vertically stratified benthic communities of functional groups of microorganisms, which grow on a solid substrate such as rocks, sand, and other sediments (Bolhuis et al., 2014). Microbial mats are considered analogues of stromatolites, whose fossil remains date 3.5 billion years into the Earth's past (Bolhuis et al., 2014). According to Stal (2012), these are excellent model organisms for the study of Precambrian stromatolites, although nowadays lithification rarely occurs as it did in the Archaic, from which the oldest stromatolites date. In contrast to the high prevalence in geological history, modern microbial mats are limited to several types of freshwater and marine aquatic environments (Bonilla-Rosso et al., 2012). Mats are thought to develop in extreme environments where there is no grazing, which is one of the reasons for the age of stromatolites (Whitton and Potts, 2012). Environments such as open savanna soils, rocks of cold desert of Antarctica, leaf surfaces in wet tropical forests, rocks in the mountains (especially dolomites), are oftentimes covered by cyanobacterial microbial mats. They survive and often thrive in these environments due to adaptations to irradiance, heat and water stress (Lüttge, 1997).

Phototrophic microbial mats develop in an illuminated environment, and are mostly composed of cyanobacteria, and sometimes diatoms, which also participate in photosynthesis. In addition to cyanobacteria, it may contain a vast number of taxonomically different bacterial groups, such as purple sulfur bacteria, green sulfur bacteria etc. Cyanobacteria in a mat can amplify microbial community conditions to perform a particular task (Zhubanova et al., 2013). Good examples of that are microbial mats formed after oil spill where cyanobacteria as pioneers provide the basis for attracting in the mat specifically oil degrading bacteria, helping in bioremediation of the area (Al-Thukair et al., 2007). As primary producers, cyanobacteria produce organic compounds for the growth and production of nonstructural components such as extracellular polymers substances (EPS), which as an adhesive create a stable structure in which organisms grow and which is attached to the substrate surface (Bolhuis et al., 2014). They have a great role in stabilizing sediment, preventing erosion and participating in the terraforming of new environments.

Marine cyanobacteria: Global impact

Marine cyanobacteria play a major role in the global carbon cycle (as primary producers) and in nitrogen fixation, especially in the marine environment that is poor in nitrogen compounds (Brito

et al. 2017). In the marine ecosystems, including coastal zones, the most dominant fraction of cyanobacteria is picocyanobacterial, single cell cyanobacteria under 2-3 μm in diameter dominate (Jasser and Callieri 2017). They account for a very important share in primary production and ocean biomass, exceeding 50% of marine phytoplankton (Paerl, 2012). This is especially true for ultraoligotrophic areas, such as oceans and oligotrophic deep lakes. Picocyanobacteria with other bacteria form the foundations of the microbial food network, especially serving as food for protozooplankton. Heterotrophic nanoflagellates are responsible for the removal of 90% of picocyanobacterial and bacterial biomass, and ciliate for the remaining 10% (Jasser and Callieri 2017). Picocyanobacteria feed to some extent on copepods, rotifers, and mixotrophic algae, forming a trophic link between them and mesozooplankton (Jasser and Callieri 2017; Callieri et al., 2012). Marine picocyanobacteria contain the genera *Synechococcus*, *Chroococcus*, *Prochlorococcus*, and *Synechocystis* (Paerl, 2012). *Synechococcus* and *Prochlorococcus* together contribute about 25% of ocean primary production (Flombaum et al. 2013; Dvořák et al. 2014). These two genera diverged 150 million years ago from a common ancestor and still share many genetic similarities (at least according to 16S rRNA), although they differ significantly in cell size and photosynthetic pigments. Phylogeny made on entire genomes also separate the two genera, although interestingly linking low-light (LL) *Prochlorococcus* to *Synechococcus* rather than high-light *Prochlorococcus*. Their geographical distribution indicates the ecological niche they occupy. *Synechococcus* is ubiquitous in all marine environments, and is particularly dominant in nutrient-rich coastal waters and cold waters of higher latitude. *Prochlorococcus*, in contrast, is found in warm and oligotrophic oceans and seas, such as the Mediterranean Sea (Li, 2000). Due to the phycobilisomes antenna system of *Synechococcus*, it is more adapted to temperature changes and is less sensitive to the toxicity of copper from coastal waters (Biller et al., 2015). Thus, the genus *Synechococcus* is more abundant in marine pelagic picoplankton. Namely, 26 of the currently 43 fully sequenced cyanobacterial genomes belong to this genus (Dvořák et al., 2014). In freshwater environments, picocyanobacteria are divided into 5 genera: *Synechococcus*, *Cyanobium*, *Synechocystis*, *Cyanothece* and *Cyanobacterium* (Jasser and Callieri, 2017). These cyanobacteria can enter the coastal marine zones via freshwater input from the coast, such as estuaries and deltas of rivers. Under special environmental conditions, they can develop mucilage, form colonies and blooms. Examples of genera that do not create blooms are *Aphanocapsa*, *Aphanothece*, *Chroococcus*, *Coelosphaerium*, *Cyanodictyon*, *Merismopedia*, *Snowella*, *Romeria*, etc. (Jasser and Callieri, 2017).

Cyanobacteria in the Anthropocene

Living with extremes: Cyanobacteria and climate change

Current climate change is evident in ongoing processes of global temperature rise, warmer oceans and ocean acidification, melting ice surfaces and glacial retreat, sea level rise and extreme events – as a result of numerous anthropogenic activities. These processes are greatly impacting the environment and ecosystems' functioning.

The rise in temperature caused by global warming encourages increased growth of cyanobacteria, in contrast to the eukaryotic phytoplankton fraction (Paerl and Huisman, 2008, 2009; Paerl and Paul, 2012). Cyanobacteria have developed several strategies allowing them to adapt to this human-changing environments: resistance to high temperatures, tolerance to reduced light, alterations in floating at different depths within the water column (by pseudovacuole), tolerance to low levels of phosphorus and nitrogen, low pH and low CO_2, symbiotic bonds with aerobic bacteria on the surface of heterocysts (Bellinger and Sigee, 2015). As an example, in freshwater eutrophic lakes of temperate latitudes, where blooms occur in summer, when the temperature is above 20°C and when the lake temperature stratification is established, cyanobacteria e.g., the genus *Microcystis* ascertain dominance over other microalgae because of their ability to float to the desired depth, thus winning the competition for light source and nutrients (Codd et al., 2017). The ecology of e.g. *Microcystis aeruginosa* species is multifaceted, as they occur also in oligotrophic lakes (Bellinger and Sigee, 2015) and may be benthic by forming granular masses at the bottom of the lake (Bellinger and Sigee, 2015). Species of the genus *Microcystis* and *Gloeotrichia* can re-become planktonic by separating from the substrate and becoming free-floating. Ecologically, colonial cyanobacteria are very good bioindicators of a highly eutrophic water body, while unicellular cyanobacteria from the Chrococcales group are indicators of meso- and oligotrophic conditions (Bellinger and Sigee, 2015).

As a consequence of human impacts to these environments, eutrophication of aquatic habitats results in frequent cyanobacterial blooms eventually posing major problems for the environment and human health (Luo et al. 2012; O'Neil et al. 2012). Eutrophication is one of the biggest stressors that can affect freshwater and coastal marine ecosystems. It can result in so-called "dead zones" as a consequence of the lack of the oxygen in the water column, mostly due to the increased frequency, duration, and extent of phytoplankton blooming (McCrackin et al., 2017). In the last 50 years, the dead zone areas within the world's coastal zones has grown to 245 000 km^2 (Diaz and Rosenberg, 2008; McCrackin et al., 2017). The prevalence and blooming of toxic algae has

been likewise on the rise in the last decade, with their occurrence being associated with excessive nutrient supply from coastal zones (Anderson et al. 2008; McCrackin et al., 2017). Lack of fresh water and insufficient wastewater treatment (van Loosdrecht and Brdjanovic, 2014), increased use of fertilizers for intensive agriculture (for human and animal consumption) (Bennett et al., 2001) and global warming, amplifies uncontrolled growth of cyanobacteria and the emergence of toxic strains. Also, high temperatures and insolation favour the growth of cyanobacteria at the expense of phytoplankton in freshwater ecosystems. Intense and extreme rainfall also affects the increased influx of nutrients into aquatic ecosystems and their enhanced eutrophication (Jeppesen et al., 2009).

Intermittent rivers: Future scenario for fluvial waters

Intermittent rivers encompass "all temporary, ephemeral, seasonal, and episodic streams and rivers in defined channels" (Datry et al., 2014). Even though they are present on every continent and constitute more than half of the length of the global rivers network, they are yet not integrated into regional and global analyses (Datry et al., 2018). Due to anthropogenic engineering, many of the large, perennial worlds' rivers are turning into intermittent (e.g. Nile, Colorado, Rio Grande, Yellow River etc.). Several world regions have predominantly intermittent water bodies: Australia, Arctic, Antarctica, Alpine region (Datry et al., 2014). In the Mediterranean region especially, most of the surface water bodies belong to intermittent rivers and ephemeral streams (IRES), and population is heavily dependet on the source of water and sediment they provide (Borg Galea et al., 2019). In the Mediterranean basin, they provide various ecosystem services, e.g. as aquifers, for irrigation, protection from floods, grounds for grazing animals, habitats of medicinal herbs and plants etc. Moreover, due to their complexity (aquatic, semi-aquatic and terrestrial periods), they are hotspots of biodiversity and habitats of endemic species. Finally, during dry periods IRES habitats have important ecological functions of organic matter and nutrient cycling. In the disconnected pools of the river during drying and dry periods, leaf litter and sediment accumulate. Hypoxic conditions cause ammonium and phosphate release from sediments, and dissolved nutrients are converted to evaporites, while aquatic macrophytes and algae are replaced by plants (Datry, 2014). When water flow resumes, solute concentration rises, and progressing water can move many tonnes of organic matter resulting in "pulses" of carbon used by heterotrophic organisms, but also potentially causing hypoxia and mortalities for fish and invertebrates in reservoirs, lakes and coastal areas. This effect of intermittent rivers, as well as greenhouse gas emissions and carbon

sequestrations, are still underexplored (Datry, 2014). Climate is the main driver shaping intermittent rivers, affecting precipitation, wind, temperature fluctuations, which are then mediated by geological characteristics. Other natural and anthropogenic activities are shaping them additionally (Borg Galea et al., 2019). Due to the unpredictability of climate change, the future of these water bodies is uncertain. According to Acuña and Tockner (2010), climate change will prolong the deciduous-tree litter fall by several months in the Mediterranean region, which will result in more organic matter accumulation in dry river channels, increasing the carbon pulses in the wet seasons. At the same time, occurrence, duration and frequency of river drying is prolonged due to agriculture irrigation needs and other anthropogenic uses (Döll and Schmied 2012). In Mediterranean IRES, climate change and anthropogenical activities effect on long-term reduction in number of flow-days and flow decrease is already recorded, which in turn is disturbing ecosystem services they provide (Borg Galea et al., 2019). Principal primary producers in IRES are cyanobacteria and algae, their composition depending on different climatic and geological settings, hydrological patterns, water chemistry, and light irradiance regime (Sabater et al., 2017). In the Mediterranean, intermittent rivers cyanobacterial composition varies from cold and warm arid zones, but they can also acquire likeness to communities in perennial rivers if the flow changes are less intense. During the dry phase, community shifts radically, with desiccation resistant cyanobacteria and green algae as pioneers, followed by diatoms. Duration of dry phase can have lasting impact on their communities, especially if the flowing phase is not long enough for communities to fully recover (Sabater et al., 2017). Cyanobacteria and algae form microbial mats (biofilms), incorporate other bacteria and fungi, while providing habitat for microfauna and meiofauna (Sabater and al, 2016). Cyanobacteria make all this possible by secreting extracellular polymeric substances (EPS), which offers protection from desiccation, UV radiation, biocides etc. by forming microbial mats (biofilms). EPS is retaining extracellular enzymes, supporting utilization of compounds from the environment and transforming them into nutrients for bacteria and algae. It also enables cell to cell communication in close proximity, thus promoting formation of a co-operative community (Sabater and al, 2016).

Aquaculture: Microbial challenges

Aquaculture's primary role is cultivation of aquatic organisms: fish, shellfish, molluscs and crustaceans, macrophytes and algae (FAO, 1988). Human intervention has been involved from the very beginning in the breeding process, and includes juvenile rearing, feeding, protection from diseases and predators, etc., all for the purpose of increasing the production of organisms for human

and animal consumption (FAO, 2014). Aquaculture is one of the fastest growing industries in the world, in just 12 years (2000-2012) production increased from 32.4 to 66.6 million tons globally (FAO, 2014). It can be considered an artificial medium for bacterial development, as nutrients in the form of phosphate and nitrogen metabolites and organic matter are abundant in aquaculture conditions (Martinez-Porchas and Vargas-Albores, 2017). Microorganisms in the aquaculture in the last decade are used as probiotics, for bioremediation of effluent, as food, but also as biomarkers in the environment, which inform about the state of the aquaculture environment (Martinez-Cordova et al., 2015; Martinez-Porchas and Vargas-Albores, 2017). Practice and technology in aquaculture have advanced rapidly, as seen in higher yields, feeding new formulations, new species for cultivation, and genetically modified organisms, recirculation systems, multitrophic systems, and biofloc/biofilm technology (Martinez-Porchas and Vargas-Albores, 2017). The biggest obstacle to aquaculture progress are diseases caused by microorganisms. Financial losses due to diseases in aquaculture reach up to $ 6 billion per year (Assefa and Abbuna, 2018). The health and production of organisms are affected by temperature, pH, dissolved oxygen, salinity, nutrients, and phytoplankton biomass (Martins et al., 2018). Seasonal parameters such as rainfall and sunlight can affect the already mentioned physicochemical and biological parameters.

The bacterial community, residing within its water column as well as in the sediment, is extremely important in aquaculture, providing purification and consequently good quality of water, being responsible for the health of aquatic organisms (Rurangwa and Verdegem, 2015; Martins et al., 2018). Microorganisms in the water column can have a beneficial effect on water quality by mineralizing extensive nutrients loads, reducing the number of pathogenic bacteria and affecting a higher survival rate of larvae. Microbial communities provide a rapid response to changes in the aquatic environment, which can vary from changes in the overall composition of the community and its functionality, to small changes such as (de)activation of certain metabolic pathways (Bentzon-Tilia et al., 2016). In sediments, bacterial communities play a distinct role in biogeochemical cycling of all major nutrients with a crucial role in the decomposition of organic matter. Unfortunately, in the intensive aquaculture systems, high organic production and/or waste accumulation can reduce the capacity of the microbial component to mineralize these compounds. This can eventually result in hypoxia followed by the release of toxic metabolites into the environment (e.g. ammonium, nitrites, hydrogen sulphide), which adversely affects health and survival of aquatic organisms (Robinson et al., 2016). The challenge of making as much profit as possible in aquaculture often means raising large quantities of fish (or other organisms) in a given

area. In such systems, high concentrations of nutrients such as nitrogen and phosphorus can lead to hypereutrophication and eutrophication, often followed by phytoplankton blooms (Granada et al., 2016). Toxins produced by phytoplankton blooms (including cyanobacteria) can cause mortality in aquaculture (McCrackin et al., 2016). In general, eutrophication causes negative economic consequences for the aquaculture (McCrackin et al., 2016). Only 13.9% of nitrogen and 25.4% of phosphorus from fish food is utilized, the rest accumulates in water and sediment (He and Wu, 2003; Zhang et al., 2014). Nitrogen compounds, such as ammonium and nitrite, accumulating in high concentrations, can be toxic to aquatic animals but also to human health (Nora'aini et al. 2005; Zhang et al., 2014), strongly impacting surrounding sites.

Due to their major role in preserving health of the aquaculture, microbial communities are very good indicators of the changes within these ecosystems. Changes in the microbial transport of nitrogen and phosphorus, as well as metabolism, can give indications of the state of water in the farm, as dissolved inorganic nitrogen and phosphorus are one of the most important indicators of self-pollution of the farm and the environment (Bentzon-Tilia et al., 2016). Nevertheless, in light of global warming and climate change, some authors have noticed changes in the structure of microbial communities, with shift from non-toxic to toxic strains following increasing temperatures (Sinden and Sinang, 2016), however, which is a significant cause for concern when it comes to aquaculture management. At the same time, a significant decrease in cyanobacterial blooms was observed at temperatures above 32°C.

Diseases of aquatic organisms are known to be a consequence of the complex interaction between the host, microorganisms, pathogens and environmental conditions (Granada et al., 2016). Unfortunately, environmental conditions persisting in the aquaculture, such as high densities of organisms, increased stress of organisms, excessive fattening and poor water circulation, often promote development and the emergence of diseases, especially in intensive and semi-intensive aquaculture production. Under such degraded environmental conditions, organisms are more susceptible to pathogens, with extremely virulent pathogens known to occur.

Changes in the global climate affect microorganisms in the sediment, water and aquatic organisms themselves. The rise in temperature has also been positively correlated with the growth of aquatic organisms. However, higher temperatures affect susceptibility to disease, spawning time, mortality and impact on the life cycle. All of the above also has socio-economic consequences globally (Woodard, 2015; FAO, 2016). Rising CO_2 and ocean acidification are leading to negative changes for organisms important to aquaculture. These processes cause the physiological changes

in shellfish, from reproduction and growth to shell development. Due to acidification, massive shellfish larvae mortality has been reported in farms. Embryos and larvae of bony fish also show sensitivity (reduced growth and mortality) to sea and ocean acidification (Heuer and Grossel, 2014; FAO, 2016). The impact of climate change on salinity can affect diseases of cultivated organisms. For example, bacteria of the genus *Vibrio* that prefer growth in warm waters (<15 C) of lower salinity (<25 ppm) may, under the influence of climate change, cause disease in farms of temperate and higher latitudes (Rowley et al., 2014; FAO, 2016). Phytoplankton (algae and cyanobacteria) produce oxygen in the water column and can be a source of food, but at the same time in certain unfavourable conditions they can be the cause of death of organisms. Bacteria from the water column and sediment are important for system health and nutrient remineralization, but can be a reservoir of pathogenic bacteria and antimicrobial resistance genes. Therefore, the study of microorganisms in aquaculture must be approached holistically and synergistically, looking at the broader interdisciplinary picture, in order to adequately understand the negative processes and approach their solution.

Finally, the problem of cyanobacterial blooms and cyanotoxins in aquaculture is a common problem that causes large economic damage and has a negative impact on human and animal health, as well as a negative impact on the environment itself (Sinden and Sinang, 2016). Cyanotoxins can cause neurological damage (Metcalf and Codd, 2009) and hepatologic lesions (Eriksson et al., 1988; Sandström et al. 1990). Also, volatile organic compounds (VOCs) such as geosmin and 2-methyl isoborneal (MIB) spread unpleasant odours in drinking water and give a mouldy taste to fish (Sinden and Sinang, 2016). Cyanobacterial blooms cause reduction of oxygen in the water column: cyanobacterial cells floating on the surface impede photosynthesis of phytoplankton, while large numbers of dead cells from the bloom decompose and additionally consume oxygen. Except for fish mortality, blooms and cyanotoxins affect fish appetite and growth, and such fish is often not of sufficient quality for consumption or damaged by toxins (Sinden and Sinang, 2016). Numerous studies have shown that the frequency, intensity and duration of cyanobacterial blooms are globally on the rise, as a result of eutrophication and global warming (Huisman et al., 2018). Therefore, there is a need for new, faster methods detection is large, due to the great economic importance, impact on the diet and health of a large number of people and environmental impact.

Cyanobacteria research in the Eastern Adriatic

Although they are an extremely important part for aquatic ecosystem functioning, especially the marine one, Cyanobacteria research in Croatia was mainly focused on freshwater ecosystems. In them, cyanobacteria were studied as part of phytoplankton community in lakes and rivers, following Water Framework Directive guidelines (Mihaljević et al., 2014; Žutinić et al., 2014; Gligora Udovič et al., 2015) or as bloom-forming phenomena in freshwater ecosystems in continental Croatia (Mihaljević and Stević, 2011). Even though 22% of Croatia is coastal and an additional 35,4% is covering the Adriatic Sea (Ministry of Environment and Energy, Republic of Croatia), investigations on cyanobacteria in eastern Adriatic are scarce. In these few studies investigators have used flow cytometry, epifluorescence microscopy and high throughput sequencing (HTS) in order to study the composition and dynamics of bacterial communities (Šantić et al., 2013; Najdek et al. 2014, Korlević et al., 2014; Paliaga et al., 2017; Babić et al., 2017; Babić et al., 2018; Mucko et al., 2018; Šantić et al., 2018). Of particular interest was spatial and temporal distribution of the *Prochlorococcus* and *Synechococcus* picocyanobacterial community in Adriatic seawater. However, data on the sediment bacterial community, including cyanobacterial fraction, are very scarce (Korlević et al., 2015).

Data produced by flow cytometry in the coastal waters of central and southern Adriatic Sea showed that *Synechococcus* biomass dominates over *Prochlorococcus*, probably due to adaptation to water-column mixing, eurythermal properties and ability to absorb different types of nutrient sources (Šantić et al., 2013). Using epifluorescence microscopy, two ecotypes of *Synechococcus* are detected coexisting, phycocyanin-rich cells (PC-SYN) and phycoerythrin-rich cells (PE-SYN) (Šantić et al., 2018). Two ecotypes were affected by relative ratio of phosphorus availability and total inorganic nitrogen nutrients. PC-SYN cells were dominating during spring, showing a significant positive relationship with temperature and nitrogen, and PE-SYN dominated during winter and autumn positively responding to phosphate availability. In the offshore waters of southern Adriatic, Najdek et al. (2014) investigated picocyanobacteria and bacterial community during convection event, combining methods of flow cytometry and the DGGE analysis. *Prochlorococcus* and *Synechococcus* were more abundant in highly saline and nutrient poor Levantine Intermediate waters then in Southern Adriatic waters, with negative correlations with dissolved inorganic nitrogen, in contrast to results for heterotrophic prokaryotes and picoeukaryotes. Additionally, Korlević et al. (2014) focused its study on changes in the structure and diversity of the total bacterial community during two sampling years (2011/2012) using 454 pyrosequencing and CARD-FISH methods. As observed, cyanobacteria were the second most abundant group within total bacterial community, and

interestingly, their abundance decreased during the convection event (Korlević et al., 2014). Picocyanobacterial community, with emphasis on different *Prochlorococcus* ecotypes, was also investigated in the southern Adriatic during winter convection event in the year 2015 (Babić et al., 2017). By combining flow cytometry with phylogenetic information of the ITS region clone libraries, authors have detected co-occurrence of HLI (MED4) and LLI (NATL2A) Prochlorococcus ecotypes alongside with high diversity of Synechococcus clades. In publication by Babić et al. (2018) and Mucko et al. (2018), Cyanobacteria were further investigated as a part of the total bacterial community by flow cytometry, HPLC and 16S rRNA amplicon high throughput sequencing. In the winter conditions of 2016 when deep convection was absent, *Prochlorococcus* and *Synechococcus* abundances were found to be higher in the coastal areas from 0-30m and offshore surface, euphotic layer, representing 10% of the total bacterial community (Babić et al., 2018). Within the picoplankton community, Cyanobacteria represented 9.25% of the total bacterial community, and their abundances were found to correlate to higher concentrations of pigment: zeaxanthin plus β-carotene for *Synechococcus,* and divinyl chlorophyll *a* for P*rochlorococcus* (Mucko et al., 2018). However, in this study HPLC method was shown to be less sensitive, when compared to flow cytometry and HTS, probably due to very low concentrations of photosynthetic pigments. On the other hand, investigation conducted on the coastal seawater impacted by the wastewaters that underwent only primary treatment (Paliaga et al., 2017), using HTS and epifluorescence microscopy, suggested that cyanobacteria were not major contributor to bacterial community composition. *Synechococcus* prevailed over *Prochlorococcus* abundances, showing preference to eutrophic conditions (Paliaga et al., 2017).

Besides coastal and offshore Adriatic picocyanobacteria, research was also published on cyanobacteria living in the extreme environment of rock pools and splash zones of the karst Adriatic coast (Brandes et al., 2015; Palinska et al., 2017; Vondrášková et al., 2017; Vogt et al., 2019). Brandes et al. (2015), for studying cyanobacteria residing within supratidal rock pools, have used HTS, single-cell and single-filament DNA isolation in addition to microscopy. Cyanobacterial order *Pleurocapsale*s was found as the main contributor in the endolithic community with the highest richness recorded in the most extreme high-salinity samples. Interestingly, these habitats had higher relative community diversity when comparable to the extreme sites of hot and cold deserts (Brandes et al., 2015). In the further investigation conducted by Palinska et al. (2017), total bacterial component was analysed by using HTS, revelling highly diverse community of heterotrophs: *Proteobacteria, Chloroflexi, Actinobacteria, Planctomycetes, Acidobacteria and Spirochaetes.*

However, Cyanobacteria were predominant representing 43% of the community, with a total of 13 morphospecies and 17 genotypes discovered. Proportions of heterotrophic to phototrophic bacteria was found to be dependent on the distance to the sea level, with separation of Cyanobacteria orders: Oscillatoriales in the subtidal zones and Pleurocapsales in the upper wave-spray zone (Palinska et al., 2017). Comparing Croatian sample with tidal flats samples (sediments and water) from 4 other different climate zones (Iceland, Oman, France, Germany), using HTS with specific primers targeting cyanobacteria, showed that Croatian samples were dominantly Pleurocapsales enriched (Vogt et al., 2019). Besides, Croatian sample had high number of location-specific OTUs (72%) but it only shared 5 to 7 OTUs with other locations, which implies very diverse and particular cyanobacterial community, probably derived by characteristics of Adriatic coast limestone (Vogt et al., 2019). In the line with that, Vondrášková et al. (2016) have reported 42 cyanobacterial endolithic and epilithic species in the splash zone of Croatian islands Ugljan and Veruda. Using extensive microscopy efforts (336 samples), they determined vertical gradient as the most important determinant affecting species composition, with *Hyella* spp. and *Gloeocapsopsis crepidinum* as the most dominant (Vondrášková et al., 2016).

THESIS OUTLINE

This doctoral thesis incorporates four scientific publications (I-IV), which are adequately addressing aims and hypotheses of the thesis.

Aims of thesis are:

1. To define the cyanobacterial community response to environmental pressure in the benthic and pelagic systems in freshwater and marine environments. (Publication II, III, IV)
2. To identify changes in the structure of the cyanobacterial communities in an extreme ecosystem that is affected by climate change and anthropogenic pressures (mariculture). (Publication I, II, III, IV)
3. To describe the diversity of cyanobacteria in the intermittent river and aquaculture-influenced marine system in the southern and central Adriatic. (Publication I, II, III, IV)
4. To apply molecular methods and analysis to identify cyanobacteria in the investigated ecosystems. (Publication I, II, III, IV)

Hypothesis of the thesis are:

1. Cyanobacteria are exceptionally important organisms in extreme environments, as they change/adapt such environments into habitats for development of other organisms. (Publication I, II)
2. The Adriatic Sea and the Adriatic basin are ideal natural models for studying ecosystems influenced by climate change and anthropogenic pressures. (Publication I, II, III, IV)
3. Recent innovative molecular methods using high-throughput sequencing technology, such as metabarcoding, are appropriate tools for cyanobacterial research and for the identification of key taxa for ecosystems. (Publication II, III, IV)

First two publications (I, II) answer satisfactory to the first hypothesis; all four publications (I, II, III, IV) pertain to the second hypothesis, and the last three publications (II, III, IV) explore possibilities of the third hypothesis. Aims of the thesis are reached: Publications II, III, IV describe cyanobacterial response to various environmental pressures; publications I, II, III, IV identify changes in the

community and describe diversity of cyanobacteria using observational method such as light microscopy and molecular methods using DNA extraction, PCR amplification and phylogenetic analysis of 16S rRNA and ITS marker genes and HTS of 16S rRNA amplicons and various bioinformatic tools in the analysis microbial mats, seawater and sediment samples in the Eastern Adriatic area (Publications II, III, IV).

INDIVIDUAL PUBLICATIONS

Publication I

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RESEARCH PAPER OPEN ACCESS

Microbial mats as shelter microhabitat for amphipods in an intermittent karstic spring

Petar Žutinić[1,*], Ines Petrić[2], Sanja Gottstein[1], Marija Gligora Udovič[1], Koraljka Kralj Borojević[3], Jasmina Kamberović[4], Anamarija Kolda[2], Anđelka Plenković-Moraj[1] and Ivančica Ternjej[1]

[1] Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia
[2] Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
[3] 3 Minnesota Drive, Great Sankey, WA5 3SY Warrington, UK
[4] Department of Biology, Faculty of Natural Sciences and Mathematics, University of Tuzla, Univerzitetska 4, 75000 Tuzla, Bosnia and Herzegovina

Abstract – Microbial mats represent complex communities where cyanobacteria and diatoms as key organisms provide shelter for diverse assemblages of aquatic invertebrates, like the small stygophilous amphipod *Synurella ambulans*. Studies addressing such communities in the karst springs have rarely examined springheads, and have ignored intermittent springs. During high flow conditions the stygophilic crustaceans are flushed to the surface of a temporary stream Krčić where microbial mats prevent their drift and enables their successful retreat into underground in the periods of drought. The objective of this study was to characterize the microbial mat community of the Krčić Spring as a shelter for *S. ambulans* during strong current and high water level. Representative samples for diatom and cyanobacterial species identification and composition, as well as the fresh mat material for potential animal activity and cyanobacterial phylogenetic analysis were collected. The most dominant diatom was *Achnanthidium minutissimum*, whilst *Fragilaria capucina*, *Meridion circulare*, *Navicula cryptocephala* and *Nitzschia palea* had abundance greater than 0.5%. Morphological observations of cyanobacteria revealed that *Phormidium favosum* was the most dominant, with *Hydrocoleum muscicola* as a subdominant. Cyanobacterial phylogenetic relationship revealed two distinct clusters: (i) "*Phormidium* cluster", confirming morphological observations in both winter and spring samples, and (ii) "*Wilmottia* cluster", a first report for Croatia and found exclusively in the winter sample. Laboratory observations revealed a small stygophilic amphipod *S. ambulans*, hiding and feeding inside the pockets of fresh microbial mat. The intermittent Krčić Spring as a predator-free and competitor-free ecosystem provides a spatiotemporal conformity between microbial mat and stygophilous amphipod.

Keywords: Microbial mat / cyanobacteria / diatoms / amphipods / karstic spring

Résumé – Le tapis microbien comme microhabitat refuge pour les amphipodes dans une source karstique intermittente. Le tapis microbien représente des communautés complexes où les cyanobactéries et les diatomées, en tant qu'organismes clés, abritent divers assemblages d'invertébrés aquatiques, comme les petits amphipodes stygophiles *Synurella ambulans*. Les études portant sur ces communautés dans les sources karstiques ont rarement examiné ces têtes de ruisseaux et ont ignoré les sources intermittentes. Dans les conditions d'écoulement fort, les crustacés stygophiles sont chassés à la surface d'un ruisseau temporaire Krčić où les tapis microbiens empêchent leur dérive et permettent leur retrait dans le sous-sol en période de sécheresse. L'objectif de cette étude était de caractériser la communauté du tapis microbien de la source Krčić comme abri pour *S. ambulans* lors de forts courants et de niveaux d'eau élevés. La diatomée la plus dominante était *Achnanthidium minutissimum*, tandis que *Fragilaria capucina*, *Meridion circulare*, *Navicula cryptocephala* et *Nitzschia palea* avaient une abondance supérieure à 0.5%. Des échantillons représentatifs ont été prélevés pour l'identification et la composition des espèces de diatomées et de cyanobactéries, ainsi que de la matière organique du tapis servant à l'activité animale potentielle et pour l'analyse phylogénétique des cyanobactéries. Les observations morphologiques des cyanobactéries ont

*Corresponding author: petar.zutinic@biol.pmf.hr

révélé que *Phormidium favosum* était le plus dominant, avec *Hydrocoleum muscicola* comme sous-dominant. L'étude phylogénétique cyanobactérienne a révélé deux groupes distincts: (i)"groupe de *Phormidium*", confirmant les observations morphologiques dans des échantillons hivernaux et printaniers, et (ii)"groupe de *Wilmottia*", une première observation pour la Croatie trouvé exclusivement dans l'échantillon hivernal. Les observations en laboratoire ont révélé qu'un petit amphipode stygophile *S. ambulans* se cachait et se nourrissait dans les poches du tapis microbien. La source Krčić intermittente en tant qu'écosystème sans prédateurs et sans concurrents assure une conformité spatio-temporelle entre le tapis microbien et l'amphipode stygophilique.

Mots-Clés : Tapis microbien / cyanobactérie / diatomée / amphipode / source karstique

1 Introduction

Microbial mats are structurally complex communities composed of physiologically and ecologically diverse micro-organisms adapted to gradients of light and nutrient accessibility. They are present in very diverse, often extreme environments with limited or even minimal top-down controls, like ephemeral and intermittent streams and springs (Stal and Caumette, 1994; Stanish *et al.,* 2013). Microbial mats can form dense micrometer-scale communities in which the full plethora of microbial metabolism can be present, usually consisting of filamentous, entangled organisms that produce a macroscopic 'mat-like' structure. These structures exhibit great variety in morphology and composition and may include diverse biofilms of immobilized microorganisms (Bauld, 1984). Cyanobacteria and diatoms are emphasized as the key organisms comprising microbial mats in spring habitats (Esposito *et al.,* 2006; Stal, 2012; Cantonati *et al.,* 2012, 2015, 2016), where these prokaryotic-eukaryotic interactions play an important role in microbial mat development by enabling significant photosynthetic activity (Elster and Komárek, 2003) and nutrient cycling and acquisition (Gooseff *et al.,* 2004; McKnight *et al.,* 2004; Mueller and Vincent, 2006). Their remarkable adaptability, through which they can overcome the extremity of environmental conditions prevailing in many carbonate-rock springs, makes them one of the key components in terms of ecology, management and conservation of spring ecosystems (Cantonati *et al.,* 2015). Taxa populating these habitats must be able to cope with rapid irradiance alterations, high water-level fluctuations and monthslong drought periods. Moreover, a diverse assemblages of aquatic invertebrates, particularly amphipods like the small stygophilous spring dwelling *Synurella ambulans* (F. Müller, 1846), live on and within the microbial mats (Camacho and Thacker, 2006, 2013; Korpinen *et al.,* 2006; Lévesque *et al.,* 2015).

A complex geological history of Croatian Dinaric karst manifests through formation of specific features, like well-developed underground drainage system, strong interactions between circulation of surface and groundwater, presence of unpredictable conduits, fissures and cavities beneath the ground, unexpected connections of water, changes of underground flow path over time, all of which shape a pronouncedly heterogeneous hydrological, hydrogeological, morphological, physicochemical and biological conditions in karst springs (Bonacci, 1993, 2015). The majority of water input into karst spring systems is caused by rainfall, which reaches groundwater *via* infiltration through karst aquifers. Variations in precipitation induce rapid and pronounced oscillations of groundwater, strongly affect spring flow and cause fluctuations in discharge volume, thus making catchment boundaries of springs extremely time-variant (Hao *et al.,* 2012; Bonacci, 2015). Karst spring discharges reflect periods of poor or abundant precipitations due to specific recharge conditions and the locally prevalent climate regime (Bonacci, 2015; Fiorillo *et al.,* 2015). In such conditions streamflow regime is recognized as the principal variable affecting the success and distribution of aquatic biota (Meyer and Meyer, 2000; Meyer *et al.,* 2003; Konrad *et al.,* 2008). Limited knowledge of the effects of temporary seasonal drying of karst springs on stream biota introduces numerous questions when comparing biological integrity of these specific habitats to perennially flowing streams (Reiss and Chifflard, 2015).

There is a paucity of information on the adaptations of various organisms to temporary streamflows and their use of microbial mats during these stressful conditions (McDonough *et al.,* 2011; Robson *et al.,* 2011). Most studies addressing cyanobacterial and diatom communities in karst springs have examined mostly perennial streams, rarely springheads, and have ignored intermittent springs (Cantonati *et al.,* 2012, 2016). Their ecological role as the food source and shelter for freshwater invertebrates is still poorly understood (Camacho and Thacker, 2013; Lévesque *et al.,* 2015). During high flow conditions specimens of the stygophilic amphipod *S. ambulans* are flushed to the surface of a temporary stream Krčić where they can be retained by the microbial mat and moss, thus preventing their drift and facilitating their successful retreat into underground in the periods of drought. The objective of this study was to characterize the microbial mat community in the karstic stream Krčić and its role as a feasible alternative shelter for the subterranean stygophilic crustacean *S. ambulans* during two events: (i) high water level with fast streamflow, and (ii) water level decrease preceding drought.

2 Materials and methods

2.1 Study area

Krčić is a small intermittent stream situated in the middle of Dinaric karst of the southern Croatia, confined mostly to the External Dinarides, which consists predominantly of Triassic, Jurassic and Cretaceous limestones and dolomites (Bonacci *et al.,* 2006; Hajna *et al.,* 2010). The study area falls within a continental climate influenced by the mid-Mediterranean climate with the average annual temperature of 13 °C. The

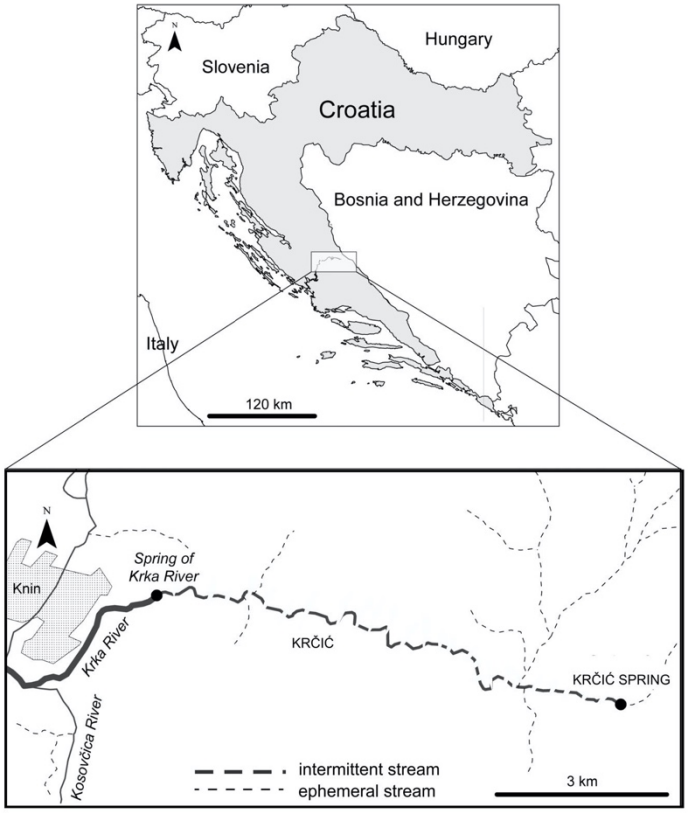

Fig. 1. Map of the investigated area.

summers are hot, with maximum temperature above 30 °C, whilst winters are cold with temperatures as low as −10 °C. The catchment area of Krčić covers 157 km^2 with the surface flow of 10.5 km from the springhead to the waterfall in the Krka River (Bonacci *et al.*, 2006). The spring and the mouth of the river lie between latitudes 44°1′32″N and 44°2′31″N and longitudes 16°19′53″E and 16°14′7″E, respectively (Fig. 1). Strong river bed karstification causes significant water losses along the stream causing alternating surface and subsurface flow (Bonacci, 1987, 1993; Bonacci *et al.*, 2006). Krčić functions as a descending karstic spring (Jukić and Denić-Jukić, 2006) and dries up regularly every year from June to September, usually when springwater discharge of Krka River (below the mouth of Krčić) drops below 4.20 $m^3 s^{-1}$ (Bonacci *et al.*, 2006). However, in 2014 it retained water for the entire year because of exceptionally rainy summer (Fig. 2).

The diverse rheocrene Krčić Spring has multiple spheres of discharge rate over time due to groundwater emerges on the bottom, and along the edges of the springhead as a fast-flowing spring runs. The spring is not shaded by trees and the habitats are well preserved, although arteficial barriers of the 18th century mill building are situated 100 m downstream of the springhead.

2.2 Specimen collection and identification

Representative samples of the microbial mat for diatom and cyanobacterial species identification and composition were collected during two field trips in winter (February) and spring (May) of 2014 from the springhead of the Krčić stream. Months were selected based on their hydrologic conditions. February is characterized by high water level accumulating from precipitation and snowmelt. May is distinguished by a strong decrease in streamflow usually followed by a months-long drought period (Fig. 2). Algae were brushed and scraped from the stone surface (3 × 3 cm^2) and rinsed into a sample jar. Samples were preserved with a buffered 4% formaldehyde solution. Diatom samples were cleaned following Hendey (1964). Acids were neutralised with distilled water until pH reached 7. Cleaned valves were mounted in Naphrax diatom mountant. Light microscope observations were conducted using an Olympus BX51 Microscope (Olympus, Japan). Diatoms were counted under oil immersion at 1000× magnification until a minimum of 400 valves have been counted. Identification of diatoms was performed using Krammer and Lange-Bertalot (1986, 1991b), Lange-Bertalot (2001), and Hofmann *et al.* (2013). For scanning electron microscopy (SEM), part of the clean diatom frustule suspension was filtered

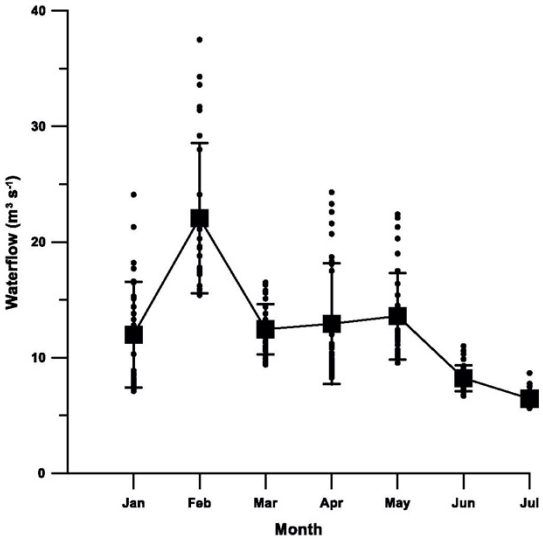

Fig. 2. Waterflow of the lower part of Krčić in the period between January and July 2014 (Meteorological and Hydrological Service, Croatia).

onto Whatman Nucleopore membrane filters (Sigma-Aldrich, USA) with a maximum pore diameter of 0.3 μm, pieces of which were attached to aluminum stubs after air-drying. The stubs were sputter-coated with 30 nm of gold and studied using MIRA3 FEG-SEM microscope (Tescan Orsay Holding, Czech Republic) in the Forensic Science Centre Zagreb.

For the morphological analysis of cyanobacterial samples, length and width of at least 30 cells on each trichome were measured under the 1000× magnification using the BA310 Motic optical microscope (Speed Fair Co. Ltd., China). Identification of cyanobacteria was performed following the most recent literature (Komárek and Anagnostidis, 2005; Palińska *et al.*, 2011; Hašler *et al.*, 2012; Strunecký *et al.*, 2013). Microphotographs were taken with the Optika Pro 3LT camera and processed with Optika Vision Pro Software (Optika Microscopes, Italy).

The fresh mat material, sampled directly at the springhead, was transported to the laboratory for the subtle observations of potential animal activity inside the jelly pockets. Part of fresh mat material was placed into diatom medium (Andersen, 2005) for a few days and sent for DNA extraction.

2.3 DNA extraction from cyanobacterial mats

Approximately 100 mg of the fresh mat material preserved in culture media was used as a starting material for nucleic acid extraction. DNA was extracted from winter and spring samples using NucleoSpin Plant II Kit (Macherey-Nagel, Germany) according to the manufacturer instructions with a few modifications. In order to remove the culture media, mat material was first centrifuged (600 g, 15–30 min), followed by resuspension of the obtained cell in TEN buffer (final concentration 50 mM TrisHCl pH 8; 5 mM EDTA; 50 mM NaCl) with the addition of Sarcosyl (1%). After 1 h incubation at the room temperature, pellets were washed 4 times with TEN buffer solution. Each dry pellet, in which 2–5 small beads were added, was mechanically homogenized to fine powder using FastPrep FP120 Cell Disrupter at 4.5 m s^{-1} for 45 s (Thermo Savant Bio101, Qbiogene, France). The obtained homogenate was then resuspended in 100 μl of TEN buffer solution and incubated at 37 °C (15 min) with lysozyme (final concentration 0.5 mg ml^{-1}). According to the NucleoSpin Plant protocol, Lysis Buffer PL1 and RNase were added and the sample was incubated at 65 °C (30 min). Additional step included the addition of Proteinase K enzyme to the sample and incubation at 55 °C (30 min), followed by a short incubation of the sample at 90 °C (5 min) for Proteinase K inactivation. Samples were then centrifuged (11 000 g, 5 min), after which the extraction continued on the NucleoSpin Filter according to manufacturer's instructions (Macherey-Nagel, Germany). The integrity of the DNA obtained at the end of the protocol was checked by electrophoresis on 1% agarose gel. DNA was quantified at 260 nm using a BioPhotometer (Eppendorf, Germany).

Table 1. Primer sequences used for amplification of cyanobacterial 16S rRNA.

Primer	Sequence (5'–3')
CYA106F	CGC ACG GGT GAG TAA CGC GTG A
CYA359F	GGG GAA TYT TCC GCA ATG GG
CYA781R(a)*	GAC TAC TGG GGT ATC TAA TCC CAT T
CYA781R(b)*	GAC TAC AGG GGT ATC TAA TCC CAT T

* Reverse primer was an equimolar mixture of CYA781R(a) and CYA781R(b).

2.4 PCR amplification and phylogenetic analysis of cyanobacterial 16S rRNA

16S rRNA gene fragments were amplified from the DNA by using two primer sets specifically designed to target cyanobacterial 16S rRNA (Nübel *et al.*, 1997). A product of approximately 700 base pairs (bp) was targeted in the 1st PCR and of approx. 450 bp in the 2nd PCR by using primer set 1 (CYA106F/CYA781R) and primer set 2 (CYA359/CYA781R), respectively. Sequences of the primers used are given in the Table 1. PCR was performed with 100 ng of DNA used as a template under conditions identical to those reported in Srivastava *et al.* (2007). Cycling conditions were as follows: 94 °C for 3 min; followed by 35 cycles each consisting of 1 min denaturation at 94 °C; 1.5 min annealing at 59 °C; 2 min elongation at 72 °C; and a final 5 min elongation at 72 °C. Obtained PCR products were analyzed on agarose gels (2%) before being purified from the reaction using gel extraction kit (GenElute Gel extraction kit, Sigma, USA). Subsequently, purified PCR products were cloned into the plasmid vector pGEM-T Easy according to the manufacturer's instructions (Promega, France). Two libraries were established from winter sample (one from 1st PCR and one from 2nd PCR) and two from those samples collected at the spring time (one from 1st PCR and one from 2nd PCR). Clones were picked from different libraries (approx. 20 from each library) and checked for the correct insert size by vector targeted PCR. Diversity

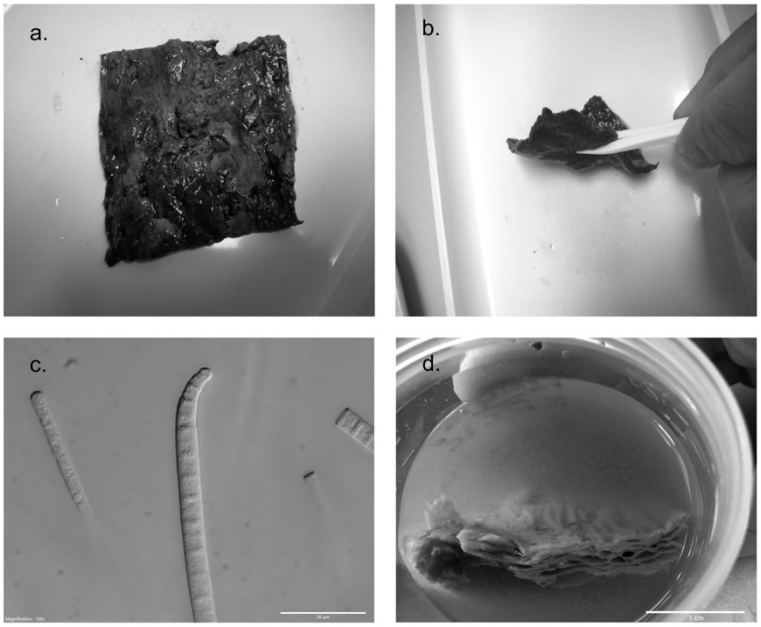

Fig. 3. Components of microbial mats: macroscopic image of microbial mat (a, b); *Phormidium favosum* (c); visible pockets in microbial mat of Krčić Spring (d).

within 16S rRNA sequences was checked by restriction fragment length polymorphism (RFLP) analysis. Amplified 16S rRNA PCR products were digested with restriction endonucleases AluI (Fermentas, Thermo Fisher Scientific, USA) following manufacturer's instructions. Digested fragments were visualized by running electrophoresis on 3% agarose gel. Clones showing different RFLP pattern were selected and further subjected to 16S rRNA gene sequencing (Macrogen, Amsterdam) using universal SP6 primer. Forward and complementary sequences were aligned using the Clustal X2 Multiple Sequence Alignment Program (Larkin *et al.*, 2007). Ambiguities and PCR errors were checked manually and the chromatograms were used to make corrections where it was appropriate. The corrected sequences were aligned with sequences from GenBank. 16S rRNA sequences were compared with known nucleotide sequences using the Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed from multiple alignments drawn by using NJplot software (Perrière and Gouy, 1996) using the neighbour-joining method that included references from GenBank showing highest similarities to our sequences. Nucleotide sequences have been deposited in the Genbank database under the accession numbers KY820663-KY820690.

3 Results

Microbial mat was densely developed in the Krčić Spring during high streamflow in both winter and spring samples (Fig. 3). Total biomass of microbial mat was estimated as chlorophyll *a* concentration. The values of chlorophyll *a* in winter sample were 6.83 $\mu g\,cm^{-2}$, while spring Chl *a* was 68.31 $\mu g\,cm^{-2}$.

3.1 Diatom assemblage

A total of 25 diatom species was recorded from samples at the Krčić Spring. A number of species was rather low and relatively constant (12–15). In every sample one or two species dominated, while other diatoms were poorly represented with only a few frustules. All samples were dominated by *Achnanthidium minutissimum* (Kützing) Czarnecki, with a very high abundance in winter (92%), as well as in spring (35%). During winter only *Fragilaria capucina* Desmazières, *Meridion circulare* (Greville) C.Agardh, *Navicula cryptocephala* Kützing and *Nitzschia palea* (Kützing) W.Smith had abundance greater than 0.5%. The same species were also more abundant in the spring samples, with subdominant *N. palea* (27%) and *M. circulare* (22%).

3.2 Morphological characterization of cyanobacterial taxa

According to microscopic and morphological observations, dominant species in the microbial mat of the Krčić Spring in both winter and spring samples was *Phormidium favosum* Gomont (Fig. 3). The second most dominant species in the community was *Hydrocoleum muscicola* Hansgirg ex Forti.

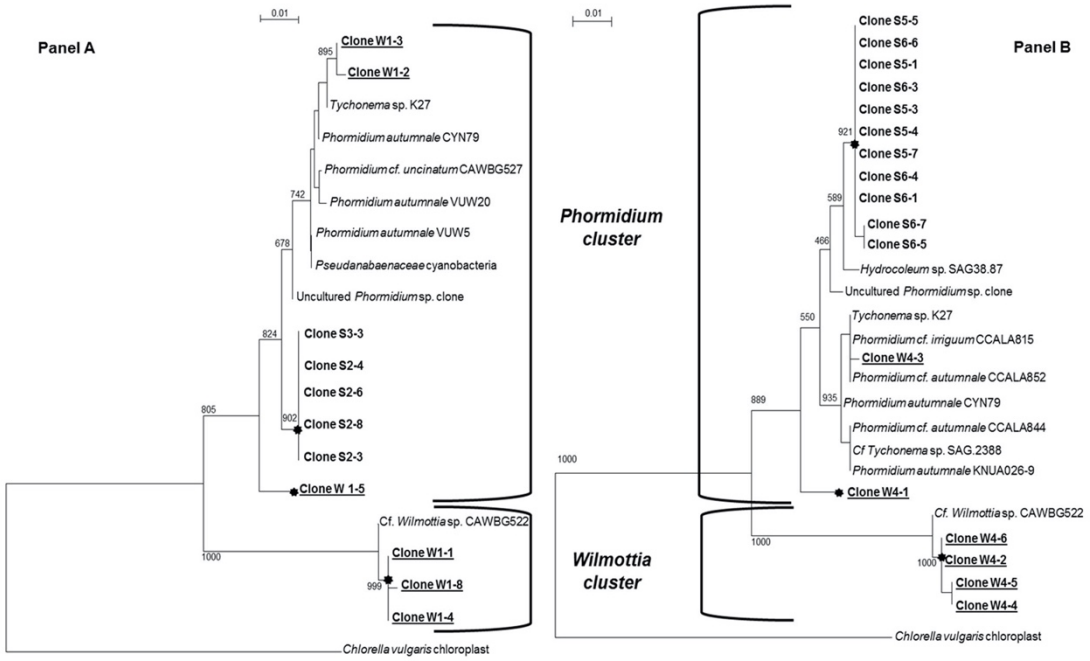

Fig. 4. Phylogenetic relationship of 16S rRNA larger fragment (A) and smaller fragment amplicon (B) amplified in clones originating from the Krčić Spring mat to the most related strains selected from GenBank database. Sequences obtained from the winter sample clone libraries are underlined and marked with "Clone W", while those from spring clone libraries with "Clone S". Sub-clusters are marked with asterisk. Molecular phylogenetic analyses were conducted in Clustal X2. The bootstrap consensus tree was inferred from 1000 replicates.

3.3 *Phormidium favosum* Gomont

Thallus blue-green, layered and soft. Trichomes blue-green, narrow, curved at both ends, unconstricted at the cross walls. Apical cells well developed, capitate, with well-formed wall thickenings (calyptrae). Cells with distinct granules agglomerated at the cross walls. Cell width from 3.1 to 5.6 μm (avg. 4.7 μm), length between 2.9 and 4.5 μm (avg. 3.7 μm); cells only slightly wider than long (length/width ratio 1.3). Atypical morphotypes also present, with capitate and widely rounded apical cells.

3.4 *Hydrocoleum muscicola* Hansgirg ex Forti

Thallus light brown, soft, placed underneath blue-green layers of *Phormidium*. Sheaths thin, colourless, usually enclosing one or up to several trichomes. Trichomes pale green, 2.9–4.5 μm (avg. 3.4 μm) wide. Cells 2.2–4.1 μm (avg. 3.2 μm) long, on average almost isodiametric (length/width ratio 1.1). Apical cells widely rounded, without calyptrae.

3.5 Molecular phylogeny of cyanobacterial mats

By using modified extraction protocol 24–113 ng μl^{-1} of high-quality DNA was successfully obtained from cyanobacterial mats collected in the Krčić Spring at both winter and spring sampling times. PCR amplification of the cyanobacterial 16S rRNA gene gave amplicons of the expected sizes: (i) in 1st PCR larger fragment of 650 bp, and (ii) in 2nd PCR smaller fragment of 450 bp. After cloning into appropriate vector four separate clone libraries were established: (i) winter sample/larger fragment, (ii) winter sample/smaller fragment, (iii) spring sample/larger fragment, and (iv) spring sample/smaller fragment. Clones were further subjected to RFLP analysis in order to check the diversity of cyanobacterial communities. Clones showing different restriction patterns were assembled into different RFLP families (results not presented). For further sequencing, at least one representative member of each of the RFLP family (altogether 28 clones) was chosen. Based on the sequences pairwise alignment phylogenetic tree was built (Fig. 4). Majority of sequences shared high homology (98–99%) with the 16S rRNA gene of the Oscillatoriales genera *Tychonema K. Anagnostidis* and *J. Komárek*, *Pseudanabaena Lauterborn* and *Hydrocoleum Kützing* ex Gomont but mainly matched to *Phormidium autumnale* Gomont. Similarities between 16S rRNA sequences of these genera did not allow more clear identification of the species. A smaller number of sequences were identified as *Wilmottia* O. Strunecký, J. Elster and J. Komárek.

Analogous phylogenies were obtained by analysing both smaller and larger 16S rRNA gene fragments sequences (Fig. 4). Sequences clearly grouped into 2 separate clusters: (i) "Phormidium cluster", and (ii) "Wilmottia cluster", with the

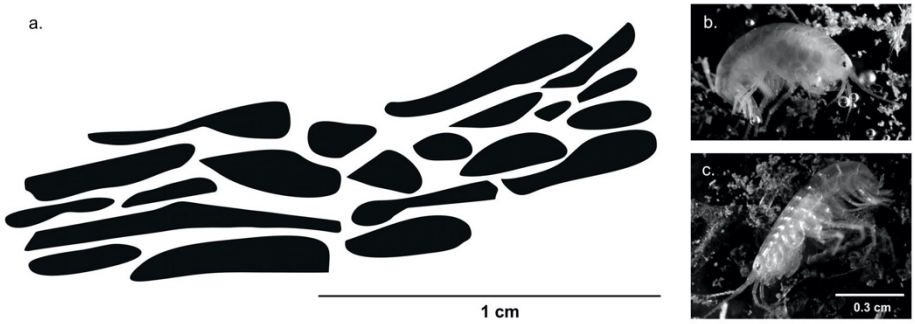

Fig. 5. Pockets of microbial mat, detailed scheme (a); *Synurella ambulans* (b) female specimen; (c) male specimen.

majority of sequences falling into "Phormidium cluster". Sequence comparison showed 7–10% difference in 16S rRNA among the clusters, thus indicating a distinct phylogenetic separation. Cyanobacteria selected from the GenBank were shown to originate from geographically distinct regions, such as German, Spanish and Swedish streams, but also streams from New Zealand and Polar Regions. Phylogenetic tree revealed that the representatives of the "Phormidium cluster" could be found in the Krčić Spring in both spring (Clones S3-3, S2-4, S2-6, S2-8, S2-3, S5-5, S6-6, S5-1, S6-3, S5-3, S5-4, S5-7, S6-4, S6-1, S 6-7, S6-5) and winter (Clones W1-5, W1-3, W1-2, W4-1, W4-3) seasons. However, clones forming "Wilmottia cluster" originated exclusively from the sample collected at the winter time. These winter clones (clones W1-1, W1-8, W1-4, W4-6, W4-5 and W4-4) showed 99% homology to *Wilmottia* sp. CAWBG522 isolated from benthic freshwater habitats in New Zealand. In addition to main clusters two sequences W1-5 (larger 16S rRNA fragment) and W4-1 (smaller 16S rRNA fragment, based on the 97% similarity cut-off value, formed distinct operational taxonomic units (OTUs) inside "*Phormidium* cluster".

3.6 Microbial mat as a shelter for crustaceans

Laboratory observations on fresh microbial mat revealed a small stygophilic amphipod species *S. ambulans*. The adult specimens were less than 7 mm in size, hiding and feeding on fine particulate organic matter (FPOM) inside the pockets of thin cyanobacteria-diatom jelly coating in size of cca 1–8 mm (Fig. 5). The average densities of crustaceans inside the pockets were presented in Table 2.

4 Discussion

Recent studies on cyanobacterial representatives of lotic aquatic systems emphasize the problems of distinguishing cyanobacterial species based on morphological features and ecological preferences (Komárek, 2016), specifically referring to the *Phormidium sensu lato* group (Palińska and Marquardt, 2007; Strunecký *et al.,* 2013). The classification of *P. sensu lato* by Komárek and Anagnostidis (2005) has recently been supplemented by several new genera based on the combination of both morphological and genetic features, namely the 16S rRNA gene sequencing (Komárek, 2010; Strunecký *et al.,* 2010; Chatchawan *et al.,* 2012). *P. autumnale* Gomont, a key species within the complex, encompasses a cluster denominated as a group VII (Komárek and Anagnostidis, 2005), comprising species characterized by very similar morphological traits and widely diverse, ubiquitous morphotypes adapted to various biotopes (Strunecký *et al.,* 2013). Subtle differences in colony formation, trichome organization and exploitation of distinct habitats can be noted amid the cluster.

Table 2. Number of specimens of *Synurella ambulans* per square 10 × 10 cm in mucilage microbial mat at the Krčić Spring.

Month	Avg ± SD	Min	Max
Winter (February 2014)	6.75 ± 4.50	3	12
Spring (May 2014)	4.50 ± 2.38	2	7

Microbial mats predominately composed of *P. autumnale* complex prevail and dominate in fast flowing streams with rapid water current and stony bottom (Komárek *et al.,* 2012), as was the case in the Krčić Spring. *P. favosum*, which is included into group VII, is attributed with isodiametric cells, trichomes shortly attenuated towards the trichome ends, and the presence of well-developed calyptrate apical cells. The ecotype-based differences between *P. favosum* and *P. autumnale*, where the former is a stenothermal species inhabiting cold oligotrophic streams and rivers (Strunecký *et al.,* 2012), while the other is typical for meso- to eutrophic waters (Izaguirre *et al.,* 2001; Palińska and Marquardt 2007; Strunecký *et al.,* 2010; Hašler *et al.,* 2012; Du, 2013; Loza *et al.,* 2013) corresponded to morphological observations of species forming mats, as well as habitat characteristics of the Krčić Spring.

P. autumnale and other species within the group VII share similar features with the *Microcoleus sensu stricto* complex, *i. e*. homogeneous cell division and thylakoid structure, as noted by Boyer *et al.* (2002) and Komárek and Anagnostidis (2005). Consequent research on *Microcoleus vaginatus* and *P. autumnale* samples from different aquatic systems yielded a taxonomic revision of *P. autumnale* into *Microcoleus autumnalis* (Trevisan former Gomont) Strunecký, Komárek and Johansen comb. nov., and *P. favosum* into *Microcoleus favosus* (Gomont) Strunecký, Komárek and Johansen comb.

nov. (Strunecký *et al.,* 2013). Still, the taxonomic uncertainty of identifying morphospecies could produce spurious phylogenetic inferences (Bagley *et al.,* 2015), specially when RNA sequencing of samples classified as *P. autumnale* indicates the presence of several different genotypes (Strunecký *et al.,* 2013). Furthermore, when compared with the results of a partial 16S rRNA phylogeny, ecotype preferences used to separate *M. vaginatus* from *P. autumnale* proved fairly inaccurate. In addition, all subsequent taxonomic revisions of *Microcoleus* complex should include more genetic markers due to high similarity of the 16S rRNA genes. Due to aforementioned reasons the authors have decided to use the nomenclature following the sequence names in the NCBI database.

As in the previous case, genera *Microcoleus* and *Hydrocoleum* are also morphologically poorly delineated. Although Komárek and Anagnostidis (2005) made distinctions based on the sheath structure, as being hyaline in *Microcoleus* opposed to lengthwise striated in *Hydrocoleum*, they stated that a phenotype with both features may appear, thus recommending a detailed molecular review in both genera.

H. muscicola is a freshwater species characterized by olive green to reddish, often warty thallus with trichomes 3–4 μm wide. According to Komárek and Anagnostidis (2005), it was reported from the hilly and mountainous streams in Austria (Carinthia, Tyrol) and Croatia (Dalmatia), and from tufa barrier waterfalls in Greece (Edessa, Macedonia). From the metaphyton samples collected in Nahuel Huapi National Park (Patagonia, Argentina) Wenzel and Díaz (2008) described following morphological characteristics of *H. muscicola*: trichomes unconstricted, roughly parallel, 3–4 μm in width, gradually attenuated at the ends; sheath hyaline, unbranched, narrow; cells 1.5–3 μm in length, with granulations near the transverse walls; apical cells conical or rounded, sometimes capitated. This was in accordance with morphological observations on *H. muscicola* described herein, specifically the width of thalli, the size and structure of individual cells, and the shape of apical cells.

Results of the phylogenetic analysis confirmed presence of the Oscillatoriales cyanobacteria in the microbial mats of the Krčić Spring. Even though two different primers were used in order to elucidate cyanobacteria to the species level, cyanobacterial genera *Phormidium, Hydrocoleum* and *Tychonema* were not readily distinguished by their 16S rRNA gene sequences. These species showed high sequence similarity form a single clade with 80–90% bootstrap support, as was previously shown by Harland *et al.* (2014). Nevertheless, clear separation between two phylogenetically distinct clusters implied that two cyanobacterial populations dominate microbial mats in the Krčić Spring with clear seasonal differentiation. The *Phormidium* population, found in both winter and spring samples, supports characterization of dominant species appertaining to P. *autumnale* complex. Other population comprised cyanobacteria identified as *Wilmottia* species. This finding was surprising since the cyanobacterial ribotypes corresponding to *Wilmottia* sp., to our knowledge, have not yet been reported in Croatia. Interestingly, this population was found exclusively in the winter sample. Ecology of *Wilmottia* species is considered to be very distinct, with low temperatures and long frozen periods. However, cyanobacteria isolated from cold environments have temperature optima growth rates in the range 15–20 °C, suggesting that they likely had their evolutionary origins within temperate latitudes (Jungblut *et al.,* 2009), and only subsequently colonized perennial cold habitats. Considering this, *Wilmottia* in the Krčić Spring could be endemic for this area, although a broader investigation on the topic is needed for further conclusions. In addition, subclustering inside *Phormidium* population (clones W4-1, W1-5) indicated a possibile existence of cyanobacteria genotypes specific for Croatian region. Moreover, those strains were found to be exclusively related to winter season period.

M. circulare is a pennate araphid diatom with thickened silica ribs (costae) oriented transversely across the valve (Kociolek *et al.,* 2011). It is a widely distributed member of benthic and periphytic (epilithic and epiphytic) communities of various freshwater ecosystems including brooks (Krejci and Lowe, 1987), streams (B-Béres *et al.,* 2016), karstic springs and spring-fed streams (Cantonati and Ortler, 1998; Delgado *et al.,* 2013) and alpine springs (Cantonati and Lange-Bertalot, 2010; Mogna *et al.,* 2015). It forms fan-shaped colonies with cells enclosed within a mucilaginous polysaccharide matrix (Lock *et al.,* 1984). Ecologically, *M. circulare* is classified as a crenophilic (Krammer and Lange-Bertalot, 1991a), alkaliphilous and oligo-eutraphentic species (Van Dam *et al.,* 1994; Cantonati and Ortler, 1998), commonly present in calcareous medium-to-high-altitude (500–1500 m a.s.1.) springs and streams (Margalef, 1949; Symoens, 1957). It is a microthermal species growing abundantly in the cold water within a temperature range of 7–15 °C (Whitford, 1960; Krejci and Lowe, 1987), with phosphorus-poor, high-light conditions (Cox, 1993; Hill *et al.,* 2011), all of which conformed to the Krčić Spring.

As one of the most widely recognized freshwater diatoms, *A. minutissimum*, a species corresponding to a still largely unresolved species complex, is frequently dominating benthic algal communities in the upper courses of streams and rivers (Virtanen *et al.,* 2011; Noga, 2012; Novais *et al.,* 2015; Cyr, 2016). Despite its omnipresence and importance in water quality assessments, *A. minutissimum* is attributed to a rather difficult identification due to small cell size, inter- and intraspecific varieties and insufficiently studied ecology (Potapova and Ponader, 2004; Ponader and Potapova, 2007; Novais *et al.,* 2015). It is an early colonizing species (Falasco *et al.,* 2012) reported to successfully recolonize and develop dense populations in the fast water substrata after repeated annual dry phases (Della Bella *et al.*, 2017). The properties of Krčić Spring corresponded to water associated with high abundance and frequency of *A. minutissimum*, like streamflow variability and slighty alkaline conditions (Noga *et al.,* 2014; Della Bella *et al.*, 2017; Jakovljević *et al.,* 2016). *A. minutissimum* is reported as a pioneering species during the initial processes of biofilm formation (Sekar *et al.,* 2004; Leinweber and Kroth, 2015). It is often the most numerous diatom occuring in *P. autumnale* and *P. favosum*-dominated microbial mats (Kelly, 2012; Brasell *et al.,* 2015), as was the case in Krčić Spring, where it could efficiently utilise potency by forming pads and stalks, thus additionally anchoring the biofilm.

N. palea is a pennate diatom with the nitzschioid eccentically positioned raphe within a keel supported by fibulae (Spaulding and Edlund, 2008), lanceolate valves, barely visible striae with parallel sides and poles terminating with subcapitate apices (Kociolek *et al.,* 2011). It is a benthic diatom with wide geographical distribution in freshwater lentic

and lotic habitats (Trobajo *et al.,* 2010). *N. palea* is a taxonomically problematic species encompassing a multitude of cryptic and pseudocryptic species into a *N. palea* complex (Trobajo *et al.,* 2009). It is capable of surviving extreme environmental conditions prevailing in the Krčić Spring, like high streamflow variability and annual periods of drought, as was also noted in Australian dryland river systems (McGregor *et al.,* 2006). *N. palea* was also found in tufa-forming microbial mats from the Harz Mountains karst streams in Germany (Arp *et al.,* 2010). Due to its exceptional tolerance to organic pollution and heavy metals (Palmer, 1969), biomass increase of *N. palea* is an indication of anthropogenic pollution (Tornés *et al.,* 2007; Bere *et al.,* 2014). The low abundance of *N. palea* recorded in Krčić Spring during the study period suggests minimum anthropogenic pressure on the stream. In that regard, close monitoring of *N. palea* could provide valuable information of a possible increase of human-induced impacts on the extremely vulnerable karst environments (Trobajo *et al.,* 2009; Delgado *et al.,* 2012).

N. cryptocephala is a benthic solitary pennate diatom with symmetrical biraphid morphological features (Potapova, 2011). Although common in a multitude of freshwater habitats, this polymorphic diatom is comprised of a complex of pseudo-cryptic species with presumably restricted distributions (Poulíčková *et al.,* 2010). It is a common member of metaphytic communities in well illuminated, alkaliphilous, lotic habitats with relatively low nutrient concentrations (Blinn and Bailey, 2001; Cox *et al.,* 2011).

F. capucina is an araphid pennate diatom frequently noted in benthic assemblages (Tuji and Williams, 2006; Boulêtreau *et al.,* 2010) with many recognized varieties. Although tolerant to increase in concentrations of heavy metals, it usually favours rheophilic, alkaliphilic habitats with lower water temperature, and low to moderate nutrient conditions (Kelly *et al.,* 2005). The high abundance of *F. capucina* was recorded in several Croatian karst aquatic systems, like the karst stream Jankovac (Špoljar *et al.,* 2012) and karst submountain river Lika (Mejdandžić *et al.,* 2015). The most dominant epilithic taxa recorded in the Su Gologone karst spring in Italy were alkaliphilous, oligotrophic species, including *F. capucina* and *N. cryptocephala* (Lai *et al.,* 2016). Delgado *et al.* (2012) have split diatom taxa from 60 Mediterranean temporary karst streams into two groups based on their sensitivity or tolerance. *F. capucina* and *N. cryptocephala* were placed into sensitive taxa indicating reference sites, while *N. palea* was grouped into tolerant taxa, in which case the abundance of species was referring to disturbance. Moreover, a study on the 371 streams from Portugal on both siliceous and carbonate (karst) bedrock (Feio *et al.,* 2012) indicated that all three aforementioned diatoms preferred streams on non-siliceous substrate, with mean water temperature of 15 °C, increased hardness and alkalinity, and higher degree of temporality.

4.1 Interspecies cohabitation on the edge

Pockets of microbial mat in Krčić Spring serve as an important shelter for stygophilic amphipod *S. ambulans* when moss as the primary substratum is not fully developed. The cyanobacteria-diatom mat protects amphipods from drift, usually during the flow activation in autumn or from the Dinara mountain snowmelt in spring. Therefore, the amphipods are presumably well adapted to: (1) changing currents caused by seasonal drying of the spring, and (2) changing of living substrates.

The low dispersal ability of the stygophilous amphipod *S. ambulans* causes specific selection of offered substrates, such as microbial mat, within the relatively small scale of spring patches. Since the inhabitants of subterranean ecosystems might be poor competitors in more ecologically complex surface environments (Fišer *et al.,* 2014), surface-dwelling species may limit the extant of the distribution of *S. ambulans* in pockets of the microbial mat in photic habitats. Moreover, the intermittent Krčić Spring as a predator-free (fishes) and competitor-free (epigean amphipods) ecosystem provides a spatiotemporal conformity between microbial mat and stygophilous amphipod. Furthermore, microbial mat provides a large surface for small biota in a relatively stable habitat transition of aquatic-terrestrial ecotones (Cantonati *et al.,* 2016) and can serve as a capturer of organic matter between the stony substrate of the Krčić Spring. On the other hand, flow rate variability and successive community dynamic and competition between the microbial mat and moss community in intermittent spring could cause changes in the choice of neighbouring shelters by amphipods, choosing moss cushions at the top of the stony substrate as a more dominant organic substrate in the Krčić Spring.

5 Conclusion

Succession of cyanobacteria and diatoms causes shift in food web structure of intermittent springs, not only when the spring dry up or during the flow oscillation, but moreover as a consequence of evolutionary internal clock in species adapted to temporary environment. Microbial mat most likely uses chemical signals in intra- and inter- species communication about physico-chemical conditions of the environment, but the information flow could also come from animals such as crustaceans and aquatic plants, playing a significant role in the organization and function of superorganism structure such as microbial mat in intermittent springs.

A huge negative impact of global warming on temporary karstic streams may be critical for the survival of some isolated and rare species of cyanobacteria and diatoms, as well as some sophistically adapted aquatic invertebrates living on the ecotone between groundwater and surface water. Moreover, any disturbance across a wide range of spatiotemporal scale of the aquifer-spring-terrestrial system can change the community structure or even induce removal of species.

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RESEARCH ARTICLE

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How environment selects: Resilience and survival of microbial mat community within intermittent karst spring Krčić (Croatia)

Anamarija Kolda[1] | Ines Petrić[2] | Maja Mucko[3] | Sanja Gottstein[3] | Petar Žutinić[3] | Gordana Goreta[4] | Ivančica Ternjej[3] | Josip Rubinić[5] | Maja Radišić[5] | Marija Gligora Udovič[3]

[1] Laboratory for Aquaculture and Pathology of Aquatic Organisms, Division for Marine and Environmental Research, Ruđer Bošković Institute, Zagreb, Croatia

[2] Laboratory for Environmental Microbiology and Biotechnology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Zagreb, Croatia

[3] Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia

[4] Public Institution "National Park Krka", Šibenik, Croatia

[5] Department of Hydrotechnics, Faculty of Civil Engineering, University of Rijeka, Rijeka, Croatia

Correspondence
Ines Petrić, Laboratory for Environmental Microbiology and Biotechnology, Division for Marine and Environmental Research, Ruđer Bošković Institute, Zagreb, Croatia.
Email: ipetric@irb.hr

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Abstract

We conducted an on-site monitoring study involving seasonal collection of microbial mats samples from the Krčić spring located in the Dinaric karst in Croatia. This intermittent karst spring is characterized by oligotrophic conditions and extreme water regime fluctuations. Extreme conditions at the Krčić included summer drought followed by strong rains in the autumn as well as freezing ambient temperature during winter. By using two phylogenetic markers, 16S rRNA (total Bacteria) and internal transcribed spacer (ITS) region (Cyanobacteria), we aimed to detect the impact of intense seasonal fluctuations of environmental parameters in shaping (and/or selecting) the microbial mat community of the Krčić spring. Microbial mat community was found to harbour bacteria belonging to 11 different phyla with Cyanobacteria making the community core (>50%), followed by Alphaproteobacteria. The most abundant cyanobacterial genera included *Microcoleus*, *Phormidium*, and uncultured Antarctic cyanobacterium. In two mat samples, collected during conditions of low temperatures and strong bora wind (winter 2014) and during drought period (summer 2015), Cyanobacteria were diminished within the community. Under the extreme cold populations found to proliferate included Planctomycetes and candidate phylum TM6, found specifically in this sample. Members of the phyla Firmicutes were strictly found during the drought summer period followed by *Cytophagia-Fibrella*, *Polymorphobacter*, *Polaromonas*, and *Massilia*. During the event of high water inflow following the drought, Cyanobacteria represented 90% of the community in which specific desiccant-tolerant *Chroococcidiopsis*, *Calothrix*, and *Pleurocapsa* species appeared having mechanisms for quick recolonization of environments.

KEYWORDS

cyanobacteria, extreme conditions, intermittent river, ITS sequencing, microbial mat community, NGS

1 | INTRODUCTION

Microbial mats represent horizontally stratified benthic communities growing on solid substrate. In this complex network of microenvironments, various functional groups of organisms coexist, each carrying specific biological role (Bolhuis, Cretoiu, & Stal, 2014; van Gemerden, 1993; Stal, 2012). Our knowledge on microbial mat mostly comes from eukaryotic macroscopic communities while relationships, interactions, and functioning of bacterial and archaeal communities are largely unknown (Prieto-Barajas, Valencia-Cantero, & Santoyo, 2018). However, by playing a pivotal role in mediating biogeochemical processes, microbial component of this community is crucial for the mat sustainability. Different studies have shown that, depending on the environment, microbial mats differ from each other with

cyanobacteria and microalgae (diatoms, green algae etc.) usually making a phototrophic backbone, being followed by anoxygenic photosynthetic bacteria, green sulfur bacteria and purple bacteria, aerobic heterotrophs and anaerobes, sulfate-reducing bacteria, sulfur oxidizing bacteria, and methanogenic archaea (Prieto-Barajas et al., 2018). Even though studied for several decades, only recent introduction of molecular biological techniques involving metagenomic analyses using high-throughput DNA and RNA sequencing allowed full assessment of diversity within these communities (Bolhuis et al., 2014; Schneider, Arp, Reimer, Reitner, & Rolf, 2013).

As a consequence of changes exerted on the freshwater environment coming from both natural and anthropogenic causes, services provided by the microbial component of the mat community might be altered. This could eventually lead to the domino effect influencing higher-level organisms and ultimately the whole ecosystem by impairing its capacity to provide food, protect livelihoods, maintain clean water, and recover from stress. Disturbances in freshwater environments have traditionally been measured by using benthic macroinvertebrate and diatom assemblages in the ecological status assessments. Microbial communities quickly respond to perturbations in their environment (Shade et al., 2012) with changes manifesting on the level of diversity, structure, and/or function. Still, they are largely neglected in freshwater studies, excluded in ecosystem/local and global biogeochemical process models and disregarded as monitoring parameters. As it has been highlighted by Rousk and Bengtson (2014), development of new models of biogeochemical cycles that factor in microbial physiology, ecology, and biogeochemistry is greatly needed. This is even more evident for intermittent river systems that constitute more than half of the length of the global rivers network, yet are not integrated into regional and global analyses (Datry et al., 2018). Microbial mats in such ecosystems can be considered natural laboratories in which adaptation to the extremes could be studied.

The karst spring Krčić, located in the Outer Dinarids in Croatia, forms a unique hydrographic system together with the Krčić River and Krka River (Bonacci, Jukić, & Ljubenkov, 2006). It represents a typical intermittent ecosystem showing seasonal extremes in environmental conditions with alternations in dry (summer) and wet (autumn/spring) period (Figure 1). Variations in rainfall induce rapid and pronounced oscillations of groundwater, which strongly affect spring flow and cause fluctuations in the discharge volume (Bonacci, 2014; Fiorillo et al., 2015; Hao et al., 2012). The drying of Krčić begins downstream towards the spring, usually starting in the middle of July and ending during first heavy autumn rains (over 80–100 mm of precipitation within few days on the Dinara Mountain), usually in the middle or towards the end of September (Bonacci et al., 2006). Different studies have shown that biota is rarely resilient to these types of disturbances with negative consequences seen on the level of virtually all biotic communities and biogeochemical processes (Datry, Larned, & Tockner, 2014).

In this study, we conducted an on-site monitoring of the Krčić spring that involved seasonal collection of microbial mat samples over 12-month period. Krčić was chosen as an ideal model for exploring dynamics of microbial mat communities in intermittent ecosystems. With its almost routine seasonal shifts in water regime, the spring provides excellent opportunity to study the response of a semiclosed mat community to environmental disturbance. Even though molecular tools are widely accessible, study of cyanobacterial diversity still largely includes use of microscopic techniques. These techniques, besides being labour intensive, often overlook species richness. Therefore, to define the structure and dynamics of microbial mat community, our study relied solely on molecular techniques. We hypothesized that due to seasonal extremes, microbial mat community will be altered and will experience several cycles of growth, with its members showing differences in resilience and survival strength as a consequence of extreme cold, extreme drought, and high-water inflow.

2 | METHODS

2.1 | Study site

Krčić is an intermittent river in the Dinaric karst area of central Dalmatia in the southern Croatia, geographically located between latitudes 44° 1′ 32′′N, 44° 2′ 31′′N and longitudes 16° 19′ 53″E and 16° 14′ 7″E (Žutinić et al., 2018). Krčić springs at the foot of the Dinara Mountain at 370 m a.s.l., runs for 11.5 km and ends as a 20-m long waterfall called Topoljski buk into the Krka River (Bonacci et al., 2006; Jukić, 2006). Geology of the Krčić catchment includes impermeable Triassic dolomites, semipermeable and permeable Jurassic dolomites and

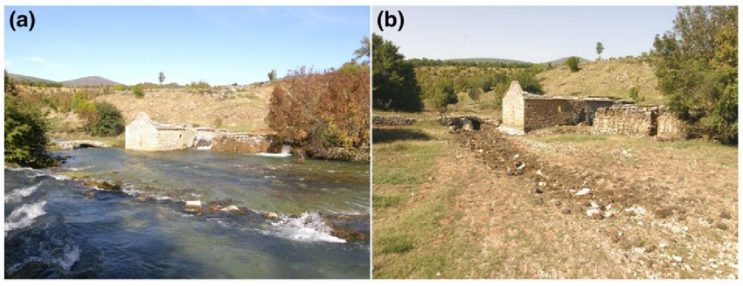

FIGURE 1 Intermittent River Krčić during high water flow condition (Panel A, October 21, 2015) and drought period (Panel B, September 19, 2015; photo. S. Gottstein)

limestone, as well as Quaternary alluvial deposits (Bonacci, 1985; Jukić, 2006) in Kninsko polje area, which are remnants of glacial Mindel and Riss age (Marjanac & Marjanac, 2004). The formation of tufa barriers is also a heritage from the interglacial periods during Pleistocene, which is interspersing with glacial times and is forming even nowadays (Friganović, 1961; Perica, Orešić, & Trajbar, 2005). The geomorphology of the Krčić area is firmly shaped by strong water and bora wind erosion, coupled with anthropogenic and zoogenic influences that have led to destruction of tufa barriers whose purpose was detention of water. The extensive livestock grazing of sheep and goats intensified hydrological processes, consequently stronger droughts and flooding periods and shaped barren landscape (Perica et al., 2005).

Today's climate is continental with mid-Mediterranean influences, causing high-temperature contrast between summer and winter seasons (max. >30°C, min. <−10°C; Žutinić et al., 2018). The annual precipitation for the area is high, but with very irregular distribution. The periodicity of karst spring Krčić mostly depends on the amount of rainfall, which reaches groundwater via infiltration through heterogeneous karst aquifers inducing oscillations of the spring flow (Bonacci, 2014; Fiorillo et al., 2015; Hao et al., 2012). During summer months, high evaporation leads to pronounced droughts, whereas during winter, high-water flows induce flooding in the Knin Valley and the downstream parts of the Krka River valley. A 14-year-long monitoring investigation of hydrological conditions at five stations along the Krčić River showed a pattern of drought on different stations: from 49 days on the spring of Krčić, 19–143 days in the middle of the river up to 90 days on the outlet (waterfall) of Krčić (Bonacci, 1985).

2.2 | Hydrology of the study area

Since Krčić Spring is not used by the Croatian Meteorological and Hydrological service (DHMZ) in regular monitoring of local hydrological conditions, we decided to use data collected on other stations in order to determine the hydrological conditions of the Krčić Spring during sampling years 2014 and 2015. Results of the hydrological surveys that covered the available monitoring data were previously published in Bonacci (1983, 1985), Bonacci and Ljubenkov (2005), Bonacci et al. (2006), and Jukić (2006). Machine learning and data mining methods, designed to develop models using high-dimensional predictors, were used to determine needed flow rates for the Krčić Spring. There are several river basins adjacent with Krčić (Cetina, Krka, Zrmanja, and Čikola) that were active during the selected hydrological conditions monitoring period. Based on the previous correlation analyses, two stations were selected: station Vinalić 1, a perennial station on the spring section of the Cetina River, and station Ružić 1 on the Čikola River, characterized by regular annual monthly drying, like Krčić. For constructing the model, series of the daily flow data as well as series of the estimated flow values for the previous 15, 30, and 60 days from the selected stations Krčić, Ružić 1, and Vinalić 1 were analysed by using the M5P induction regression induction algorithm (Quinlan, 1986) and the Waikato Environment for Knowledge Analysis (WEKA) program package (Hall et al., 2009; Witten, Frank, & Hall, 2005). Data from the periods 1950–1979 were used to train the model, that is, for its generation and validation, and the data from the periods 1980–1990 were used for its testing. The testing was performed using correlation coefficient (r), root mean square error, mean absolute error, and relative absolute error.

The model used in the study generates the interdependence of the dependent variables (daily flows on Krčić) in the function of independent variables (daily flows and from them derived average flow rates on Vinalić 1 and Ružić 1). In a way that their set linear functional link determines the weights based on the training of the model, it generates different branches of the regression decision tree in dependence of the model with the separated characteristic thresholds of the analysed indicators.

2.3 | Sampling of microbial mat and environmental parameters at the site

Samples of fresh microbial mats were collected from the Krčić Spring from October 2014 to October 2015, covering eight different time points and all four different seasons (October, November, and December of 2014 and March, April, May, June, and October of 2015). Symbols of different sampling time points are shown in Table 1. Environmental parameters (pH, conductivity, oxygen concentration, and saturation) were measured in situ by WTW Multi 3430 Set F 2FD47F (Sentix 940/Tetracon 925/FDO 925), whereas water samples were taken for water chemistry and measured accordingly: chemical oxygen demand (COD; potassium permanganate oxidation by Standard Methods—APHA 2005) and alkalinity (titration with 0.1 M HCl with methyl orange used as an indicator in titration). The water flow values were measured with SonTek FlowTracker. All values are shown in the Table 1. Collected samples of the microbial mats were cut in squares (3 × 3 cm), and subsamples were taken for molecular analysis, frozen at −20°C and transported to the laboratory for further DNA extraction.

2.4 | DNA extraction from samples

Total DNA was extracted from samples by using NucleoSpin Plant II kit (Macherey-Nagel, Germany) following manufacturer's instructions with slight modifications. Subsamples of 200–300 mg of the centrifuged microbial mat (600 *g*, 15 min) were resuspended in a TEN buffer solution (50 mM TrisHCl pH = 8; 5 mM EDTA; 50 mM NaCl, final concentrations) and incubated for 1 hr at room temperature with N-lauroylsarcosine sodium salt (1% final concentration; Sigma, USA). For removal of possible Polymerase chain reaction (PCR) inhibitors, pellets were washed and recentrifuged four times with TEN buffer solution (600 *g*, 15 min). Mechanical disruption of the cells was done by addition of three small beads to dry pellets and shaking on a Vortex-Genie 2 (MoBio, USA) for 5 min at max. speed. Homogenate was then resuspended in 100 µl of TEN buffer solution and incubated at 37°C (30 min) with lysozyme (0.5 mg/ml final concentration) to achieve chemical lysis of the cells. Samples were further incubated at 65°C (30 min) after addition of Buffer PL1 (Nucleospin Plant kit, Macherey-Nagel, Germany) and Rnase (Qiagen,

TABLE 1 Environmental and hydrological parameters of the sampling site

Symbol	Date	T (°C). air	T (°C). water	O_2 saturation (%)	Dissolved O_2 (mg O_2/L)	Conductivity (µs/cm)	pH	Alkalinity (HCl/ml)	$CaCO_3$ (mg/L)	COD (O_2 mg/L)	Flow max. (m/s)	Flow min. (m/s)	Flow avg. (m/s)
Oct-14	20/10/2014	16.5	9.4	109.9	12.8	411	7.47	4.1	205	1.18	1.25	0.87	0.53
Nov-14	30/11/2014	14.1	9.1	98.9	10.85	395	7.42	4.0	200	1.49	1.27	0.19	0.74
Dec-14	29/12/2014	2.1	8.9	107.5	12.08	406	7.47	3.9	195	1.49	10.4	0.44	0.82
Mar-15	07/03/2015	6.3	9.0	99.8	11.08	356	7.58	4.25	212.5	0.86	0.95	0.47	0.70
Apr-15	29/04/2015	12.5	9.0	99.5	11.00	355	7.56	3.8	200	0.80	1.00	1.44	0.67
May-15	05/05/2015	14.00	9.0	98.4	10.95	352	7.55	3.5	185	0.71	1.04	0.42	0.64
Jun-15	02/06/2015	24.9	9.1	103.0	11.5	366	7.59	3.2	170	0.79	0.69	0.00	0.21
Oct-15	21/10/2015	13.50	10.8	100.3	10.63	345	7.99	4.0	170	0.94	1.02	0.40	0.67

Germany). Modificational step included the additional incubation at 55°C (30 min) with Proteinase K (Promega, USA). Further steps were carried out following NucleoSpin Plant protocol. Nucleic acid quantification was done on BioSpec-nano (Shimadzu, Japan) while its integrity was checked by electrophoresis on 1% agarose gel.

2.5 | Amplicon sequencing and bioinformatics

Total extracted DNA was sent for 16S rRNA gene library preparation and Illumina MiSeq sequencing to Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) using inhouse 27Fmod (5′-AGRGTTTGATCMTGGCTCAG-3′) as forward and 519Rmod (GTNTTACNGCGGCKGCTG) as a reverse primer, covering both bacteria and archaea. The PCR program included a 28-cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Sequencing was performed by Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the Illumina Inc. (San Diego, CA, USA) guidelines. Obtained raw joined reads were quality checked with FastQC ver. 0.11.5. (Andrews, 2010). The 16S rRNA gene sequences were joined and depleted of barcodes in QIIME1 1.9.1 using extract_barcodes.py and join_paired_ends.py (Caporaso et al., 2010). Sequences with read length of more than 250 BP, q value of more than 20 with no ambiguity and homopolymer length of less than 6 BP were selected using split_libraries.py command. From a total of 691,446 raw sequences, further analyses included 300,362 sequences (length = min. 348 BP, max. 598 BP, and avg. 484 BP) ranging between 24,983 and 44,686 sequences per sample (Table 2). After demultiplexing, sequences were checked for chimeras and were sorted in operational taxonomic units (OTUs) by USEARCH (version 6.1) and UCHIME (version 4.2) using the default settings, generating a total of 1,780 OTUs (Edgar, 2010; Edgar, Haas, Clemente, Quince, & Knight, 2011). Taxonomic assignments were carried out using SILVA 123 QIIME1 compatible database under 97% of identity. Raw sequences are deposited in European Nucleotide Archive under project number PRJEB26287.

2.6 | Data analysis

Alpha diversity indices Chao1, observed OTUs, Simpson and Shannon, were calculated using QIIME with alpha_diversity.py command. To visualize taxonomic composition, bar plots and pie charts indicating percentage of detected OTUs and cluster dendrograms with sample separation based on calculated Bray–Curtis dissimilarity distance matrix were generated using R software (version 3.0.1, R Development Core Team, 2008) using the VEGAN (version 2.5–3) and ggplot2 (version 3.1.0) packages (Oksanen et al., 2007; Wickham, 2011).

TABLE 2 Illumina MiSeq sequencing quality and species richness and diversity among 16S rRNA gene libraries

Sample	No. of good quality sequences	No. of observed OTUs[a]	PD whole tree	Chao1	Shannon	Simpson
Oct-14	35,021	883	33.398	817.561	5.420 (226)	0.923 (13)
Nov-14	39,768	702	30.905	699.748	4.547 (94)	0.852 (7)
Dec-14	39,837	1105	46.829	994.189	6.522 (680)	0.956 (23)
Mar-15	44,686	598	25.518	585.164	3.837 (46)	0.861 (7)
Apr-15	42,231	509	24.632	506.053	3.407 (30)	0.775 (4)
May-15	30,947	593	28.459	575.115	4.927 (138)	0.904 (10)
Jun-15	24,983	522	29.874	614.669	5.661 (287)	0.953 (21)
Oct-15	42,889	641	25.415	492.811	3.093 (22)	0.660 (3)
Average	37,545	522	30.629	660.664	4.677	0.861

Note. Numbers in brackets = calculated "Effective numbers of species."

[a] Operational taxonomic units (OTUs) were defined at a 97% sequence identity threshold.

2.7 | PCR amplification and cloning of cyanobacterial ITS region

PCR amplification of the 16S rRNA plus the internal transcribed spacer (ITS) region was performed in 25-μl (final volume) reaction mixture containing 12.5 μl of the EmeraldAmp Max PCR Master Mix premix (TaKaRa Bio, Otsu, Japan) provided with polymerase, optimized buffer, MgCl2, and dNTP mix (composition is proprietary), 1 μl of each of the primer (20 μM), 2 μl of the template DNA and ddH2O. Specific primers CYA106F (5′-CGG ACG GGT GAG TAA CGC GTG A-3′) and 23S0R (5′-CTT CGC CTC TGT GTG CCT AGG T-3′) amplifying cyanobacterial 16S rRNA plus adjacent ITS were used in the study (Amarouche-Yala, Benouadah, El Ouahab Bentabet, & López-García, 2014). PCR conditions were as follows: 94°C for 2 min; 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min; final extension at 72°C for 7 min (Amarouche-Yala et al., 2014). PCR amplicons (cca 2,000 BP) were excised from the gel and cleaned with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). 16 rRNA/ITS gene libraries were constructed using the pGEM®-T Vector Systems (Promega, USA) following manufacturer's instructions. Clone inserts were checked by PCR using flanking vector primers sp6 and T7. Inserts of the correct size were further submitted to Macrogen (Amsterdam, The Netherlands) for partial sequencing with the specific cyanobacterial reverse primer CYA-1380R (5′-TAA CGA CTT CGG GCG TGA CC-3′; Amarouche-Yala et al., 2014). Retrieved sequences were edited, checked manually, and compared with the National Center for Biotechnology Information (NCBI) Genbank database using Blast tool.

Our ITS sequences, along with those selected from the NCBI database, were aligned using ClustalX 2.1 software (http://www.clustal.org/clustal2/). MEGA 7.0.26 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) was used to perform further phylogenetic analysis, both maximum likelihood and neighbour joining. The Kimura two-parameter model and a discrete Gamma distribution for non-uniformity of evolutionary rates among sites (K2 + G) were selected according to lowest Bayesian information criterion (BIC) scores (Tamura et al., 2013). The reliabilities of phylogenetic relationships were evaluated using a nonparametric bootstrap analysis with 1,000 replicates. Phylogenetic tree was visualized by using Interactive Tree of Life software (https://itol.embl.de/; Letunic & Bork, 2007). Based on the 97% threshold value, ITS sequences were grouped into different clusters (i.e., Operational Taxonomic Unit, OTUs). Obtained ITS sequences have been deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/) under accession numbers MK275309–MK275583.

3 | RESULTS

3.1 | Dynamics of environmental and hydrological parameters at Krčić

Year 2014 was characterized by a rainy summer, without a typical drought period, followed by exceptionally rainy autumn months (Žutinić et al., 2018). In December 2014, after a strong bora wind storm, environment around Krčić Spring has frozen. In the following spring of 2015, Krčić was characterized by high water resulting from the intensive snow melt. As usually, the water flow of Krčić decreased in May 2015 and was followed by a summer drought period from June 26 to October 10 in 2015. In October 12, 2015, a new rain season was established. Measured environmental parameters (Table 1) indicated that both ambient temperatures were altered according to the season (min. in Dec-14, 2.1°C; max. in June 15, 24.9°C). Saturation, measured during the day, was at 100% through the sampling period. COD values were constantly low, however, slightly higher between Oct-14 and Dec-14 (autumn/winter "wet period"), as compared with period Mar-15 to Jun-15 (spring/summer "drying period"; 1.4 and 0.82, respectively). Other parameters did not change significantly. Values of all measured nutrients (P and N) were constantly measured below the detection limit throughout the studied period (data not shown).

The hydrological assessment of the conditions under which sampling was carried out during 2014 and 2015 had to be done without any available hydrological monitoring results. Only data on the relative water level at the sampling point and on the recorded events of Krčić flow during sampling series were available. This challenge was overcome by a model. Figure S1, provided in the supplementary materials, shows the result of testing generated and measured flow models for the hydrological years 1982–1983, during which the largest range of

fluxes were recorded on the Krčić river. From this result, it was apparent that the generated model has a very good forecasting potential, if the previous and current flows at stations Vinalić 1 and Ružić 1 are known. This was also confirmed by the analysis showing correlation between measured and modelled flow for the entire model-testing period (1980–1990; Figure S2). Based on the tested models, modelling of the Krčić flow was conducted for the sampling period of 2014–2015. Results of the model-based discharge and water level readings during the sampling period suggest very good correlation between the fluctuation of the measured relative water levels and generated flow values (Figure 2), therefore indicating very good predictions for low water levels record, as well as for drying periods. Model therefore confirmed periodicity of the high water levels observed during spring and autumn periods and low water levels during summer periods. It confirmed strong drought during summer 2015 followed by high water in the following autumn period.

3.2 | Diversity indices

Illumina MiSeq amplicon sequencing of the 16S rRNA gene libraries generated from all eight microbial mat samples resulted in a total of 300,363 good quality sequences obtained, with an average of 37,545 sequences obtained for each of the samples (Table 2). A similarity threshold of 97% was used to cluster these reads resulting in an average of 522 OTUs obtained for each of the sample. Lowest OTUs were obtained for a sample collected in April 2015 (509), while the highest number of OTUs for the sample collected in December 2015 (1105). Average Chao1 index for all eight samples was 660.664, with lowest value found for Oct-15 and highest for samples Oct-14 and Dec-14, indicating generally higher number of singletons (rare species). The average values of Simpson and Shannon indices, responsible for determining overall species richness and diversity, were 0.860 and 4.677 respectively, with visible variation between different samples. In order to give a particular value, both indices were converted to true diversity, that is, effective numbers of species by using formula *exp (Shannon index value)* or 1/(1-Simpson value; Jost, 2006) as presented in the Table 2. In this way, biodiversity among communities was more easily compared. This further showed that community from the Dec-14 had 680 equally common species being 23 and 30 times more diverse than those from Apr-15 and Oct-15, which had only 30 and 22 equally common species, respectively. Communities from Oct-14, Nov-14, May-15, and Jun-15 had, in average, 187 equally common species. The similar result was observed when comparing effective numbers of species obtained based on the Simpson index. Low Chao1 and low indices in samples from Apr-15 and Oct-15 indicated low number of rare species and low overall species richness. Sample from Dec-14 had high Chao1 in combination with high Shannon and Simpson values, indicating higher number of rare species along with high overall species richness. Interestingly, sample from Jun-15 had lower number of rare species, while keeping relatively high overall species richness.

3.3 | Changes in the structure of total microbial mat community based on 16S rRNA amplicon sequencing

Cluster dendrogram of eight samples collected at the Krčić Spring at different time points based on the Bray–Curtis dissimilarity distance matrix is presented in Figure 3. Results suggested a strong separation of the sample collected in June -15 from the rest of the samples. In addition, samples Oct-15 and Dec-14 were found to group together and separate from the cluster including samples from Nov-14, Mar-15, Apr-15, Oct-14, May-15.

Sequencing results, presented in the Figure 4, clearly showed that the structure and diversity of microbial mat communities varied in different samples collected throughout the year. Over 50% of sequences in most of the samples corresponded to phylum Cyanobacteria reaching highest numbers (>85%) in samples collected in March 2015 (Mar-15) and October 2015 (Oct-15). Exceptions were samples Dec-14 and Jun-15 in which Cyanobacteria represented 24.2 and 11.5% of the total community, respectively, and in which >50% of sequences belonged to phyla Proteobacteria and Planctomycetes (Dec-14) or only to Proteobacteria (Jun-15). On the phylum level, after Cyanobacteria, Proteobacteria accounted for the second largest cluster in all samples, representing in average 28% of the total microbial mat community. Alphaproteobacteria represented dominant class of

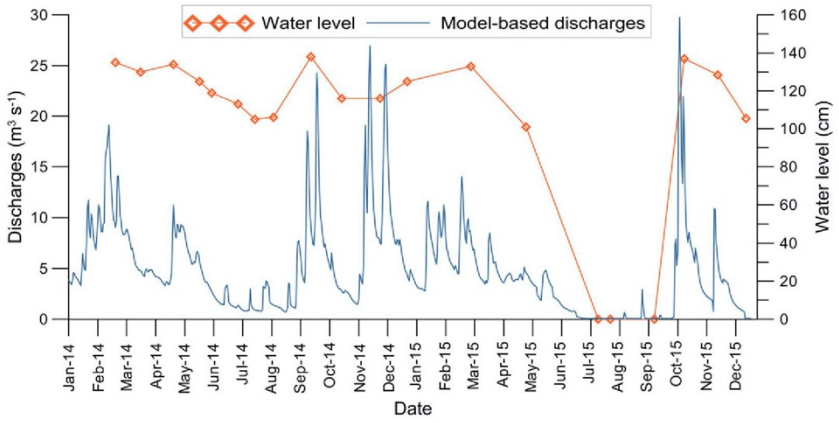

FIGURE 2 The dynamics of water flow generated by model and measured values during the sampling period at Krčić (2014–2015)

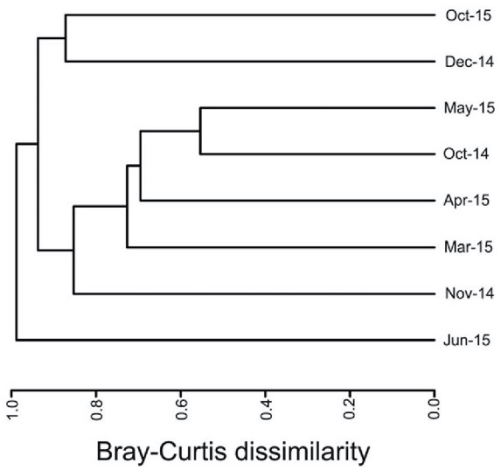

FIGURE 3 Hierarchical cluster dendrogram of microbial mat samples collected at Krčić Spring at eight different time points based on the pairwise Bray–Curtis dissimilarity

Proteobacteria (average 18% of the community), followed by Betaproteobacteria and Gammaproteobacteria each accounting for 4% of the total microbial mat community. Other phyla, accounting in average <10% of total sequences, included Bacteroidetes, Firmicutes, Planctomycetes, Verrucomicrobia, uncultivated candidate phylum TM6 and SM2F11, Chloroflexi, Acidobacteria, and Actinobacteria cluster. However, number of sequences retrieved for these phyla, as well as for three different Proteobacteria classes were shown to greatly fluctuate depending on the sample studied. For each of these clusters, highest number of sequences were found as follows: (a) for Bacteroidetes in sample May-15 (13%), (b) for Firmicutes in sample Jun-15 (12%), (c) for Planctomycetes in sample Dec-14 (28%), (d) for Verrucomicrobia in sample Oct-14 (6%), (e) for Chloroflexi and Acidobacteria in sample Oct-15 (2 and 1% respectively),(f) for Actinobacteria and Gammaproteobacteria in Jun-15 and Oct-15 (1,5 and 15% respectively), (g) for Alphaproteobacteria in Oct-14, Nov-14, and Jun-15 (average 34%), and (h) for Betaproteobacteria in Apr-15 and Jun-15 (11%). Sequences of the uncultivated candidate phylum TM6, representing 11% of all sequences, were retrieved only from the sample collected in December 2014.

Changes in the bacterial community structure on the level of different bacterial genera, when dominant cyanobacterial cluster is omitted from the analysis, are presented in Figure 5. Only those samples in which cyanobacteria did not represent majority of the sequences (>50%) are presented in the figure. High genus diversity was observed in samples Oct-14 (Panel A, 16 genera), Dec-14 (Panel C, 12 genera), and Jun-15 (Panel D, 16 genera), but with clear differences in the community composition. Genera that appeared in June included *Bacillus* and *Planomicrobium* (Firmicutes), *Pseudomonas* (Gammaproteobacteria) and *Polymorphobacter*, *Polaromoas*, and *Massilia* within Alphaproteobacteria class. Samples from Oct-14 and Nov-14 were dominated with Alphaproteobacteria, including specific including different genera from Rhizobiales, Rhodobacterales, and Rhodospirillales. Winter sample collected in the Dec-14 was characterized by the appearance of uncultivated TM6 bacterium, but included specific genera such as *Pirellula*, *Planctomyces*, *Shewanela*, and *Luteolibacter*. In summer sample, collected during drought in 2015, Firmicutesx, Actinobacteria, and *Pseudomonas* appeared.

3.4 | Changes in cyanobacterial community structure based on 16S rRNA amplicon sequencing

Results of the 16S rRNA gene amplicon sequencing identified that six different known cyanobacterial genera and one population of

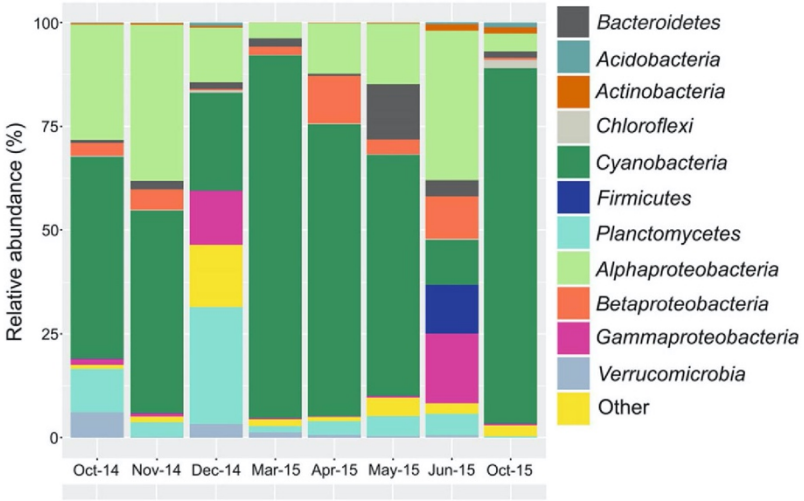

FIGURE 4 Changes in structure of the total microbial mat community (major phyla with >1% relative sequence contribution), as revealed by high throughput amplicon sequencing, in 8 samples collected throughout the year at the Krčić Spring

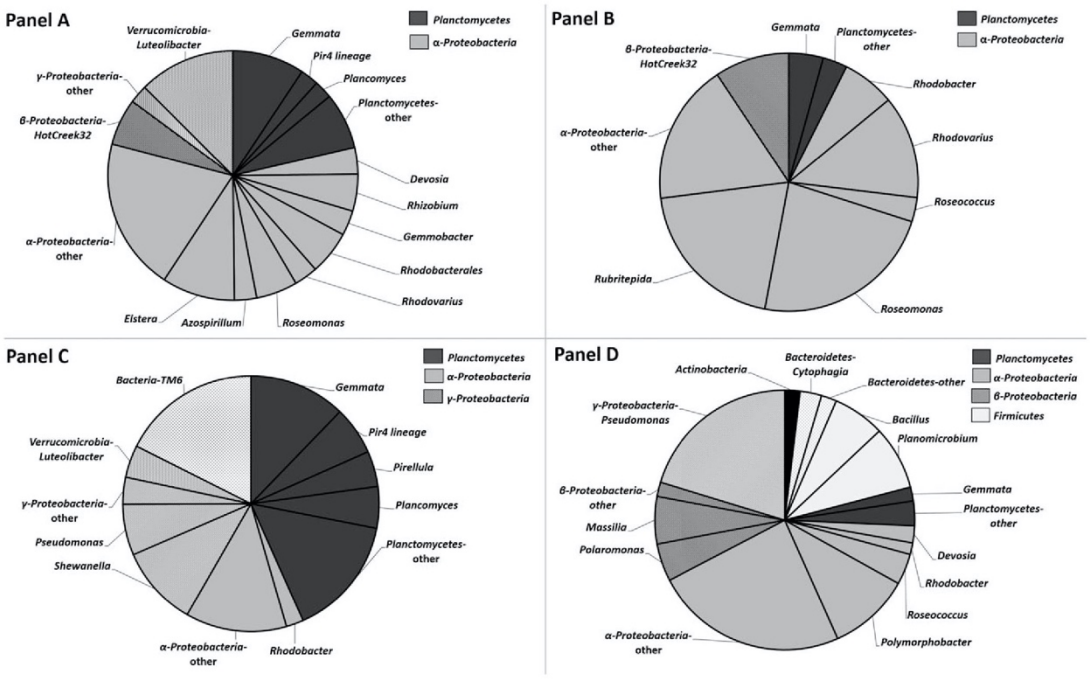

FIGURE 5 Changes in bacterial community structure, on the level of different bacterial genera, observed within microbial mat community for samples collected in October 2014 (Panel A), November 2014 (Panel B), December 2014 (Panel C), and June 2015 (Panel D) at the Krčić Spring

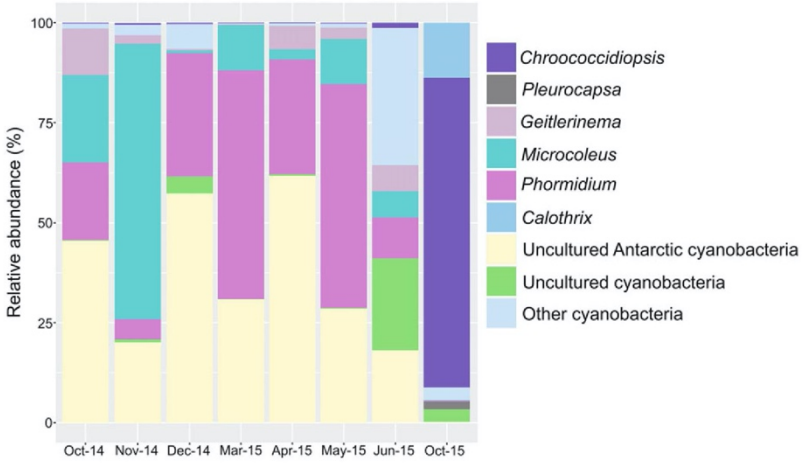

FIGURE 6 Changes in relative abundances of different cyanobacterial populations within cyanobacterial community in eight different samples collected throughout the year at the Krčić Spring

cyanobacteria taxonomically similar to the uncultured Antarctic cyanobacteria can be identified within the microbial mat of the Krčić Spring (Figure 6). Even though found in slightly different percentages in different sampling periods four populations, including *Microcoleus*, *Phormidium*, *Getlerinema*, and uncultured Antarctic cyanobacteria species, were found to represent cyanobacterial core in all samples in which cyanobacteria dominated (>50% of total sequences) total mat community. The only strongly differing sample was Oct-15, in which 70% of all sequences belonged to *Chroococcidiopsis*, 12% to *Calothrix*, and 2% to *Pleurocapsa*. These genera did not appear in other samples. In samples where cyanobacteria were poorly represented (Dec-14 and Jun-15), majority of sequences were identified as uncultured cyanobacteria.

3.5 | Analysis of cyanobacterial community structure and diversity by sequencing of the specific ITS region

Cladogram of genotypic diversity of the cyanobacterial 16 rRNA/ITS region obtained by Sanger sequencing from all samples is presented in the Figure 7. Results suggested that Cyanobacteria can be grouped into eight different clusters (named Cluster 1–Cluster 8) based on the 3% dissimilarities observed between sequences (97% threshold value). Majority of the sequences grouped within Cluster 1 belong to genera *Phormidium/Microcoleus/Tychonema/Oscillatoria/Stanieria*, all six being phylogenetically closely related. This cluster contained sequences retrieved from all eight collected time points. Clusters 2, 3, 4, and 5 were closely related. Cluster 2 contained sequences found solely in the sample collected in October 2015 during flooding. These sequences showed highest similarity to *Chroococcidiopsis* (i.e., *C. muralis*) strains from the hot and cold desert regions, with clear separation into sub-Cluster 2b (group of two sequences) closely related to the above mentioned strains, and sub-Cluster 2a (group of 28 sequences) showing to be phylogenetically more distant from *Chroococcidiopsis* strains and closely related to uncultured cyanobacterium clone from quartz hypoliths. Cluster 3 contained sequences found in several samples including Dec-14, Mar-15, Apr-15, and May-15 and was most closely related to strains isolated from different freshwater systems (*Phormidium/Wilmotia*-related). Sequences originating from sample collected during strong winter (Dec-14) grouped into Cluster 5 that were closely related to uncultured cyanobacteria isolated from extreme environments (glacial lakes, dry Antartic valleys, ice cores). Specific group of sequences forming Cluster 6 showed 99% similarity to uncultured cyanobacterium isolated from glacier, correlated to genus *Anagnostidinema gen. nov.* (Strunecký et al., 2017) formerly classified as genus *Geitlerinema* (not shown on dendogram). Clusters 4, 7, and 8, each included only several sequences found exclusively in the sample Jun-15. Cluster 4 contained two sequences from drought summer sample (Jun-15) correlated to one *Microcoleus* strain, Cluster 7 showed closest relationship to the *Leptolyngbya* genus, while sequences grouping into Cluster 8 had no clear phylogenetic relation to known cyanobacteria with 99% similarity to the uncultured cyanobacterium isolated from karstwater rivers.

4 | DISCUSSION

4.1 | Diversity and role of the microbial mat community

Our study conducted on the intermittent Krčić Spring indicated that microbial mat community developing seasonally within this ecosystem is facing strong alternations in its structure and diversity. Complexity and dynamics of mat communities are known to be shaped by primary production and/or by the alternation in the nutrient regimes within the ecosystem (Zeglin, 2015), but are likewise strongly affected by the level of disturbance in the environment that can include diel, seasonal, or intermittent extreme weather-driven fluctuations in abiotic factors (Bonilla-Rosso et al., 2012; Grime, 1973; Tilman, 1990). Microbial mats are situated in the shallow water as a periphyton on mesolital and macrolital zone. They may come on the edges of the Krčić Spring where the water strongly oscillates depending on the flow, additionally getting more exposed to the sharp seasonal variations in air temperatures.

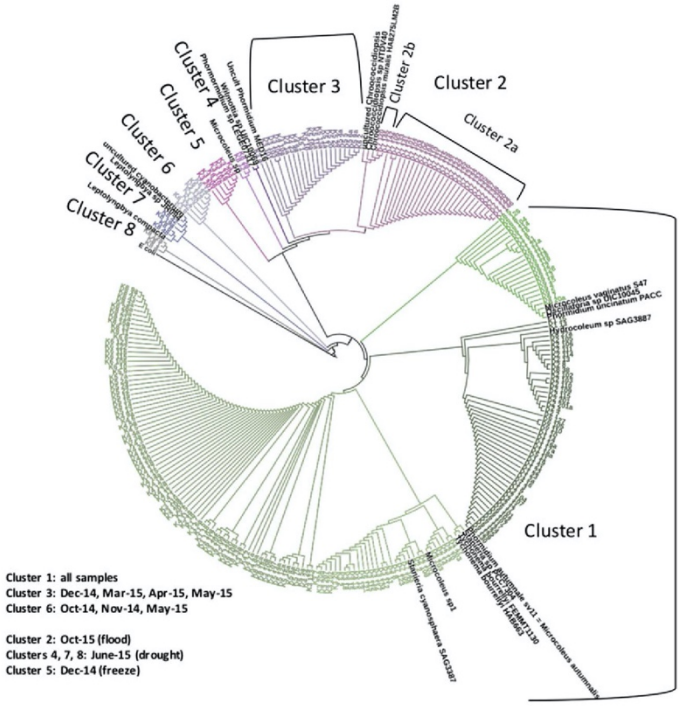

FIGURE 7 Cladogram of genotypic diversity of ITS region in Cyanobacteria found in eight different samples collected throughout the year at the Krčić SpringCluster 1 = *Phormidium*-related; Cluster 2 = *Chroococcidiopsis*-related; Cluster 3 = *Wilmottia*-related; Cluster 4 = *Microcoleus*-related; Cluster 5 = uncultured cyanobacteria from extreme environments; Cluster 6 = *Anagnostidinema (Geitlerinema)*-related; Cluster 7 = *Leptolyngbya*-related; Cluster 8 = uncultured cyanobacterium

In these situations, the ability of ecosystems to adapt to the exerted changes depends on the duration and the intensity of the change, as well as on its biological diversity. In the study conducted by Preisner, Fichot, and Norman (2016), significant changes in diversity and shifts in biogeochemical cycling potential were recorded within microbial mats as a consequence of changes exerted to the ecosystem. Although nutrient level and physicochemical measurements (low COD, constant high oxygen saturation) confirmed Krčić as a pristine ecosystem, with year-long oligotrophic nutrient status, at the same time, this river showed intense seasonal fluctuations in its water regime. Long-term hydrological measurements on several gauging stations already affirmed the cyclic periodicity of the Krčić river brought on by summer droughts (Bonacci et al., 2006) and erratic but substantial rainfall periods (850–1750 mm along Krka River; Penzar & Penzar, 1990; Perica et al., 2005). Based on measured hydrological conditions and observed disturbances exerted onto the microbial mat community, periodicity of the Krčić river can be divided into (a) drought period generated during summer season, (b) strong rains following in the autumn months, (c) freezing ambient temperature during winter period, and (d) higher water levels reached in the spring time as a consequence of snow melt. Such alternations in dry and wet conditions are known to affect virtually all biotic communities and biogeochemical processes, however, studies on the changes exerted onto microbial component of the intermittent rivers are still rare.

Although clearly seasonally impacted, microbial mat community in the Krčić spring was preserved throughout the year, indicating its tolerance to low temperature, high water level, prolonged desiccation, and high solar irradiances. This diverse microbial community consisted of an array of different functional groups of bacteria coming from 11 different phyla (including two candidate phyla TM6 and SM2F11) presumably highly adapted to the oligotrophic conditions. In almost all seasons, filamentous Cyanobacteria belonging to the Oscillatoriales were predominant bacterial microorganisms, thus confirming their key role within the community. Oscillatoriales, that included populations of *Microcoleus* and *Phormidum* species, is widely distributed lineage, preferentially in colder environments (Casamatta, Johansen, Vis, & Broadwater, 2005; Jungblut, Lovejoy, & Vincent, 2010; Tang, Tremblay, & Vincent, 1997), designated as most abundant Cyanobacteria in calcifying freshwater microbial mats (Cadel-Six et al., 2007; Bissett, Reimer, de Beer, Shiraishi, & Arp, 2008; Beraldi-Campesi et al., 2012; Perri, Manzo, & Tucker, 2012; Wood, Smith, et al., 2012; Schneider, Reimer, Hahlbrock, Arp, & Rolf, 2015). Interestingly, mat community harboured one abundant population of cyanobacteria (in average 20%) related to the uncultured Antarctic cyanobacterium, giving a clue to the specific extreme environmental condition defining Krčić. During autumn and spring season, we noted an occasional contribution (4.1–5.7%) of the genus *Geitlerinema* (*Anagnostidinema gen. nov.*), previously reported in different environments, but with clear preference for carbonate-rich waters and unpolluted freshwater environments (Komárek, 1992; Margheri, Piccardi, Ventura, Viti & Giovannetti, 2003; Tavera, Novelo, & Lopez, 2013; Brinkmann et al., 2015). The dominance of cyanobacteria in this karst ecosystem concurred with similar research conducted on German karsts (Arp et al., 2010; Schneider et al., 2015). By producing biomass, extracellular polymers and other organic matter cyanobacteria provide nutrition source for other heterotrophic and lithotrophic microorganisms running a variety of mineralisation processes and conversions (Bolhuis et al., 2014; Schneider et al., 2015; Ward, Ferris, Nold, & Bateson, 1998). This is especially important in the low-nutrient environment such as Krčić, in which both production and recycling of nitrogen and sulfur compounds are required for the sustainability of this system. In nutrient-depleted environments, cyanobacteria are generally also considered as the principal diazotrophs. However, the magnitude and importance of this fixation in lotic ecosystems are poorly understood (Berrendero et al., 2016).

After photosynthetic cyanobacteria, members of the class Alphaproteobacteria represented not only the second most abundant but also the most divergent group at the site. This was not surprising since Cyanobacteria and Proteobacteria often coexist as dominant groups in these types of mats (Beraldi-Campesi et al., 2012; Heath, Wood, & Ryan, 2010; Ng et al., 2006). With photosynthetic cyanobacteria constantly providing aerobic milieu, aerobic heterotrophic Proteobacteria are expected to play a major role in organic carbon mineralisation. Planctomycetes appeared as the third most abundant group. As suggested, they catalyse important transformations in carbon (sugar) and nitrogen (anammox) cycles in ecosystems (Fuerst & Sagulenko, 2013). Alphaproteobacteria were represented by an array of bacteria belonging to Rhodobacterales, Rhodospirillales, and Rhizobiales, identified previously as members of surface mat layers (Schneider et al., 2013). Rhodobacterales are also designated as the most common and dominant primary surface-colonizing bacterial group with the ability to react to a low level of nutrient faster than other bacteria (Dang, Li, Chen, & Huang, 2008). Genus *Rhodobacter* represents a versatile photoheterotrophic group of organisms that can adapt to different trophic strategies and can, therefore, occupy various niches in the mats (Garrity, Bell, & Lilburn, 2005). Some of the identified genera represent anaerobic phototrophic purple nonsulfur bacteria living photoheterotrophic during the day while in dark they grow chemotrophically. This suggests existence of anoxic niches within the surface mat or occurrence of species with characteristics differing from known species. In addition, members of *Rhizobiales* are also known as diazotrophs. Interestingly, no genera that could be clearly associated to the ecologically important processes of nitrification or sulfate-reduction were identified within the mat community.

4.2 | Extreme events shifting mat community structure

Cyanobacteria/Alphaproteobacteria/Planctomycetes group were, based on phylogenetic analysis, designate as microbial mat community core, associated to production and organic carbon mineralisation. However, as it was clear, extremes in hydrological conditions have strongly impacted these populations, altering their abundance and diversity, thus potentially affecting biogeochemical cycling. These changes were recognized at both higher and lower taxonomic levels within the community. The extremes included time point characterized by low ambient temperatures (reaching almost 0°C) and strong bora wind in winter 2014 and drought period in summer 2015. During these extremes, a general increase in overall species richness and diversity was recorded. As already well documented, if disturbances are intermediate in frequency and intensity, they can create opportunities for development of microniches that add to

diversity (Bonilla-Rosso et al., 2012; Connell, 1978; Grime, 1973; Rainey & Travisano, 1998). An interesting parallel can be made between our microbial mats and two oligotrophic microbial mats from the investigation conducted by Bonilla-Rosso et al. (2012). In the study of microbial mats in oligotrophic ponds, Bonilla-Rosso et al. (2012) speculated that the microbial mat from the oligotrophic desiccation pond would have higher diversity than expected for such environment, because of the intermediate disturbance. It was evident that, in the conditions of extreme cold and extreme drought at the Krčić Spring, depending on the resilience and survival of different community members to these disturbances, microbial mat community has evolved in two different directions. Cyanobacteria, known to be able to survive in unfavourable and extreme conditions (Bolhuis et al., 2014), were interestingly diminished within the mat community during both extreme time events, representing in avg. 17% of the community. At the same time, it was obvious that specific environmental conditions favouredenrichment of specific phyla and classes of bacteria within the community.

Microbial mat developed during the winter extreme event (winter 2014) seems to harbour populations of bacteria highly adapted to the cold and freeze, which was also supported by high Chao values indicating a high number of rare species. As opposed to mat communities developed in similar extreme polar regions, in which Cyanobacteria represent a dominant group (Prieto-Barajas et al., 2018), community from the Krčić included enrichment with bacteria belonging to Gammaproteobacteria and Planctomycetes. In addition, candidate phyla TM6 and SM2F11, as well as bacteria from the genera *Shewanella* (Gammaproteobacteria) and *Pirellula* (Planctomycetes) were exclusively enriched in this extreme winter conditions. Species of SM2F11, Shewanella and Pirellula, were previously identified in different extreme cold environments for which they evolved a number of adaptive strategies with the aim to maintain vital cellular functions in conditions of cold, desiccation, radiation, excessive UV radiation and temperature, and low nutrient availability (De Maayer, Anderson, Cary, & Cowan, 2014; Glöckner et al., 2003; Ntougias et al., 2016). Recent studies also identified some members of phylum Planctomycetes as colonizers of extreme acidic environments (Fuerst & Sagulenko, 2013). Current knowledge on the ecology of the candidate phylum TM6 is still limited and therefore its appearance in high numbers in the condition recorded during winter 2014 is difficult to explain. Gene sequencing suggests it has a parasitic lifestyle with eukaryotic host. Likewise, this phylum is affiliated with the *Patescibacteria superphylum* found in extreme environments of hypersaline microbial mats, sulfur springs, arsenic-rich sediments, and biofilms collected from showerheads (Yeoh et al., 2015). *Pseudomonas* (Gammaproteobacteria), found in higher numbers during this winter condition, are known to be able to survive in cold environments with adaptation including activation of specific metabolic pathways (Tribelli & López, 2018).

Mat community shaped by the second extreme event, that is, drought recorded in the summer 2015, was highly diverse and had a unique structure, clustering away from all other samples. Proteobacteria, that included both Alpha, Beta, and Gamma classes, again outnumbered Cyanobacteria, making 2/3 of the total mat community. Under these extreme drought, Firmicutes (genera *Bacillus* and *Planomicrobium*), Actinobacteria (*Arthrobacter*), and *Cytophagia-Fibrella* (Bacteroidetes) emerged as new community members. In addition, new genera were enriched within Proteobacteria: *Polymorphobacter* genera (Alphaproteobacteria), *Polaromonas* and *Massilia* (Betaproteobacteria), and *Pseudomonas* (Gammaproteobacteria). Interestingly, some of these bacteria were found as members of soil microbial community of the Antarctica ice-free regions (Tahon & Willems, 2017). As stated in this research, in the conditions of oligotrophy and strong solar radiation, aerobic anoxygenic phototrophic bacterial groups are those adopted to use sunlight and convert it into chemical energy in order to support life. Drought phenomenon is also known to lead to alternations in environmental factors including pronounced oligotrophy, increased solute concentration and oxygen content, reduced substrate diffusion, and osmolyte production (Naylor & Coleman-Derr, 2018). In this type of stress, an increase in the ratio of Gram-positive phyla Firmicutes and Actinobacteria was observed (Chodak, Gołębiewski, Morawska-Płoskonka, Kuduk, & Niklińska, 2015; Naylor & Coleman-Derr, 2018), concurring with our results. Many Gram-positive bacteria, characterized metabolically as "oligotrophs," can survive better in these conditions by adaptations including thicker cell walls, sporulation, and production of osmolytes (Naylor, DeGraaf, Purdom, & Coleman-Derr, 2017). As also seen for Krčić, drought might not alter the only abundance but composition within Proteobacteria, as suggested in recent studies on soil and peatland (Naylor & Coleman-Derr, 2018; Potter et al., 2017). In the Krčić Spring, switch was observed on the level of Beta, Gamma, and Alpha classes, later being interestingly represented by unidentified populations of bacteria. This suggested the potential presence of novel species and metabolic traits in this habitat. Due to conditions similar to those in higher altitude (dryness, strong UV radiation, and low concentration of organic materials), it was not surprising to find *Polaromonas* and *Polymorphobacter* genera, found in unvegetated periglacial soils in high-elevated environments and ice-free polar regions (Darcy, Lynch, King, Robeson & Schmidt, 2016; Tahon & Willems, 2017). *Polaromonas* is a metabolically diverse "opportunitroph" capable of taking advantage of transient periods of higher temperatures and substrate availability for development (Darcy et al., 2016). *Bacillus* strains, with the ability to produce of heat-resistant endospores and sphingolipids in the membrane, and *Massila*, are likewise able to tolerate abiotic stressors including drought. *Pseudomonas* (making 16% of the community) species, found to proliferate during both cold and drought extremes in Krčić, obviously possess metabolic features allowing them to survive in different extreme environmental conditions. Many exopolysaccharide-producing drought tolerant bacteria are *Pseudomonas* species (Ali, Vardharajula, & Venkateswar, 2013). Due to the strong drought during which this ecosystem is turning to terrestrial, it is not surprising that many identified bacterial strains are common soil isolates.

The third type of disturbance was exerted onto the mat community in spring 2014 and autumn 2015, when a previously dormant river was again reactivated after a period of cold and drought by high water inflow. This rewetting event conditioned new possibilities for substrate colonization resulting in the formation of a newly colonized mats (de Nijs, Hicks, Leizeaga, Tietema & Rousk, 2018; Schimel, Balser & Wallenstein, 2007). During these water inflow periods, mat community had a low overall diversity and species richness with mat being almost solely represented by cyanobacteria (around 90% of the total community). After a winter decline cyanobacterial genera

Phormidium, uncultured Antarctic cyanobacteria and *Microcoleus* were the first colonizers of the mat. This is presumably due to the filamentous structure of the trichomes allowing these cyanobacteria to quickly cover different kind of substrates (e.g., wet rocks). Both genera, belonging to the family Oscillatoriaceae, are known as the most numerous dwellers in karst freshwater microbial mats (Beraldi-Campesi et al., 2012; Bissett, de Beer, et al., 2008; Cadel-Six et al., 2007; Perri et al., 2012; Schneider et al., 2015; Wood, Kuhajek, de Winton, & Phillips, 2012). These organisms, by synthetize extracellular polymers serving as a glue for other microorganisms, provide stabile foundation between mat and the substrate (Bolhuis et al., 2014; De Philipps & Vincenzini, 1998; Grant & Gust, 1987). Different colonizers were found to dominate mat community during second water inflow occurring after summer drought. In this period, we found exclusive populations of cyanobacteria identified as *Chroococcidiopsis* (family Chroococcidiopsidaceae), *Calothrix* (family Rivulariaceae), and *Pleurocapsa* (family Hyellaceae), with almost sole domination by the *Chroococcidiopsis* species. All three genera are notable for their resistance to desiccation and later rehydration, which enables them to inhabit extremely arid hot and cold deserts (Potts, 1999a, 1999b). In addition, *Chroococcidiopsis* and *Pleurocapsa* have a specific mode of reproduction (multiple fission) and reproductive cells (baeocysts) that facilitate fast colonization of the extreme environments. *Calothrix* has similar adaptations called akinets, dormant cells that help conserving genetic material in adverse conditions (Adams & Duggan, 1999). Moreover, they have specialized "survival cells" and DNA reparation pathways (Fewer, Friendl & Büdel, 2002; Potts, 1999a, 1999b; Yasui & McReady, 1998) that help them endure periods of desiccation and nitrogen limitation, such as those occurring in summer period in Krčić Spring. As a pioneer genus of cyanobacteria in extreme environments, *Chroococcidiopsis* was considered a candidate for Mars terraforming more than 20 years ago (Friedmann & Ocampo-Friedmann, 1995). These cyanobacterial populations were not identified in other sampling periods and were probably present only in very low numbers. Such cosmopolitan organisms with ability to live in the most extreme environments on Earth are usually not able to compete well with specialized organisms in species-rich communities in more moderate conditions (Friedmann & Ocampo-Friedmann, 1995). This is recognized as characteristic of primitive organisms whose survival depended on the endurance of extreme conditions, where they are dominant organism forming monospecific populations (Billi, Friedmann, Hofer, Caiola, & Ocampo-Friedmann, 2000; Friedmann & Ocampo-Friedmann, 1995; Lacap-Bugler et al., 2017). Even though other bacterial groups were very lowly represented in the mat community in this second water inflow period, we found samples to be enriched with specific populations of bacteria including *Chloroflexi* (nonsulfur green bacteria usually responsible for anoxygenic photosynthesis) and Acidobacteria. Acidobacteria is an understudied but one of the most widespread soil bacterial phyla and can represent up to 52% of the community (Kielak, Barreto, Kowalchuk, van Veen, & Kuramae, 2016). Interestingly, members of Acidobacteria were found to have a role in recovering soils as beneficial to soil nutrient cycling and plant growth after drastic disturbance (Huang et al., 2015), which could explain their appearance in microbial mat community after this autumn rewetting event.

4.3 | Cyanobacterial diversity

Molecular techniques for identification of Cyanobacteria based on the 16S rRNA analysis are widely employed. However, due to high similarities of cyanobacterial 16S rRNA genes, analyses based on this marker can be insufficient to fully characterize cyanobacterial diversity within the ecosystem. To gain full insights into the cyanobacterial diversity of Krčić Spring, we used second ITS gene marker. Taxonomic information confirmed *Phormidum/Microcoleus* as the core of the cyanobacterial community, containing highly related sequences found at all-time points during our investigation, as well as specific appearance of cyanobacteria in connection to the extreme events of freezing ambient temperature, drought and flood. Interestingly, when compared with 16S rRNA, many of these sequences could be, based on ITS, identified also as *Tychonema*, found in tufa-forming biofilms in the German karstwater river (Arp et al., 2010), *Hydrocoleum* or *Stanieria cyanosphaera* (syn. *Chrococcidiopsis stanieria*), found in mineral springs and pools (Komárek & Hindák, 1975). This example highlights high genetic similarity between these cyanobacterial genera as well as potential discrepancies that came with morphological identification of the submitted sequences. Furthermore, we have to be careful with the identification of some BLAST sequences, for example, *Tychonema bourellyi*, which is a planktonic species characteristic for producing toxic algae blooms in glacial lakes (Salmaso, Cerasino, Boscaini, & Capelli, 2016). In general, when compared with 16S rRNA analysis, ITS marker led to slightly better resolution with identification of several new cyanobacterial populations. Sequences clustering with *Chroococcidiopsis* species, appearing in the period after-summer flood, suggested existence of two evolutionary closely related but diverse cyanobacterial populations, one being related to known *Chroococcidiopsis* and other to uncultured cyanobacteria found in quartz hypoliths. In the period after-winter flood, we found cyanobacterial population closely related to *Wilmottia*, genera already recorded in the Krčić Spring during winter time (Žutinić et al., 2018). One new cyanobacterial population, correlated to species found in extreme environments of glacial lakes, dry Antartic valleys and ice cores, was found characteristically during winter period. This population of cyanobacteria probably reflects group named "uncultured Antarctic cyanobacteria" representing 14% of the sequences identified by the 16S rRNA analysis. Interestingly, the ITS analysis of the sample collected in summer drought revealed presence of three groups of cyanobacteria, probably representing very low abundant populations. In agreement to 16S rRNA analysis, the first group contained species of the genus *Leptolyngbya*. The second one separated into *Microcoleus*-related group, phylogenetically very distant from the core *Microcoleus/Phormidum* cluster. The third group contained specific cyanobacteria related to the uncultured cyanobacterium from karst water rivers. Finally, we concur that combination of two molecular markers allowed more precise identification of the members of cyanobacterial community within this intermittent karst spring.

5 | CONCLUSION

Investigations on the disturbances that may affect dynamics within microbial mat communities are important ecological tools aiming to recognize potentially negative effects of the observed changes on

the biogeochemical processes that form the foundation of the whole ecosystem. In this study, we used Krčić Spring as a representative of intermittent Dinaric spring to study resilience and survival of different community members to seasonal disturbances in environmental conditions that included extremes in air temperature and hydrological regime (water flow, velocity, and quantity). These disturbances have clearly impacted microbial mat community with different community members showing ability to survive and proliferate in such extreme conditions. During stable water regime, autotrophs and heterotrophs coexisted with less overall richness within the community. Extreme events of freezing ambient temperatures and drought affected mat community by allowing heterotrophic bacteria to emerge in larger numbers and diversity, while in the conditions of dormant river reactivation, Cyanobacteria emerged as first colonizers of the newly established mat community. Even though this study represents only a snapshot of the investigated mat community, it gives a good indication of possible adaptations of the community to this extreme environment. Several annual research cycles on the site will be necessary in order to confirm ability of the Krčić mat community to recover from the exerted disturbances. In addition, further investigations will aim at recognizing potential alternations in biogeochemical cycling within the ecosystem, much needed to clearly define impacts of intermittent disturbances to this fragile Krčić river ecosystem.

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ORCID

Anamarija Kolda https://orcid.org/0000-0001-5537-5414
Ines Petrić https://orcid.org/0000-0003-3875-9373

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Metabarcoding Cyanobacteria in coastal waters and sediment in central and southern Adriatic Sea

Anamarija Kolda[1]*, Zrinka Ljubešić[2], Ana Gavrilović[3], Jurica Jug-Dujaković[4], Kristina Pikelj[5], Damir Kapetanović[1]

[1] Ruđer Bošković Institute, Zagreb, Croatia
[2] University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6, Zagreb, Croatia
[3] University of Zagreb, Faculty of Agriculture, Department of Fisheries, Apiculture, Wildlife Management and Special Zoology, Zagreb, Croatia
[4] Sustainable Aquaculture Systems, Inc., Frenchtown, New Jersey, USA
[5] University of Zagreb, Faculty of Science, Department of Geology, Zagreb, Croatia

Abstract – Seasonal sampling of the seawater column and sediment in Adriatic coastal areas affected by various anthropogenic activities, primarily aquaculture, was conducted during 2017. In total, 32 samples from two sites (central and southern Adriatic) were analysed by 16S rRNA amplicon sequencing. This approach was selected to test the possibilities of using metabarcoding in studying marine cyanobacteria, exploring their ecology and potential as an indicator group in anthropologically stressed coastal environments. Additionally, physico-chemical water column parameters, sediment granulometry and composition were assessed. Water column revealed a seasonal variation of amplicon sequencing variants (ASVs) closely related to *Cyanobium* PCC-6307, *Prochlorococcus* MIT9313 and *Synechococcus* CC9902, as well as seasonal grouping of physico-chemical parameters in PCA analysis. Sediment analysis uncovered greater community richness of 13 cyanobacterial genera and two uncultured groups. The most abundant in sandy gravels and gravelly sand type of sediments were ASVs closely related to *Pleurocapsa* PCC-7319 and *Xenococcus* PCC-7305. Furthermore, identified cyanobacterial ASVs predominantly displayed similarity to isolates from tropical areas (e.g. *Neolyngbya*, *Chroococcidiopsis*, *Trichodesmium*, etc.), which could indicate the tropicalization process already ongoing in the fish fauna of the Adriatic Sea.

Keywords: Adriatic Sea, ecology, marine cyanobacteria, metabarcoding, sediment, water column

Introduction

Researching cyanobacteria brings several powerful facts into focus: they are (i) remarkably old organisms – as old as 3.5 billion years (Bellinger and Sigee 2015), (ii) the makers of the aerobic atmosphere in which life, as we know it, exists (Meriluoto et al. 2017), (iii) the main atmospheric nitrogen fixators in global oceans (Whitton and Potts 2012), (iv) one of the main primary producers in the oceans (Paerl 2012), (v) evolutionarily important for chloroplast origin through endosymbiosis (Margulis 1970), and finally, (vi) the creators of the oldest ecosystems – microbial mats (Green and Jahnke 2010). Although cyanobacteria are more commonly investigated in freshwater environments due to intensifying problems of eutrophication and production of cyanotoxins, cyanobacteria are an ecologically extremely important group in marine environments, both planktonic and benthic cyanobacteria. Their role in nutrient cycling, especially as primary producers and nitrogen fixators is of the essence (Whitton and Potts 2012).

Ecological monitoring of cyanobacteria includes many different methods such as the classical morphological counting method using light microscopy (Lund et al. 1958) and chemical methods e.g. HPLC (Colyer et al. 2005), flow cytometry (Casotti et al. 2000) and satellite remote sensing (Gons et al. 2005). However, in the last decade we have entered the era of "omics", thanks to large advances in molecular methodology as well as in computational power and various bioinformatic tools (Heilderberg et al. 2010). The inability of standard culture techniques to isolate more than 99% of bacteria in the environment (Handelsman 2004) encourages the use of community sequencing approaches or

*Corresponding author e-mail: Anamarija.Kolda@irb.hr

metagenomics, which started to unveil a veritable black box of microbial diversity in marine science (Hugenholtz and Tysen 2008). Metagenomics requires only environmental samples of soil, water, etc., from which environmental DNA (eDNA) is isolated (Mandal et al. 2015). Therefore, cyanobacterial taxonomy has transitioned from dependence on morphological features/data to sequencing data. Although their taxonomic relationships are often confusing, and their nomenclature has been established by both botanists and microbiologists, there are efforts to overcome these issues through a polyphasic approach (Komárek 2016). The most popular phylogenetic marker in prokaryotic metabarcoding is 16S rRNA gene, due to its presence in all prokaryotes. 16S rRNA contains many variable but also highly conserved regions, more specific phylogenetic markers that can provide higher genetic resolution are widely used for Cyanobacteria, e.g. ITS, internal transcribed spacer region of 16S-23S rRNA (Huo et al. 2018). The combination of two markers markers, 16S rRNA and ITS, has been successfully applied in the identification of freshwater cyanobacteria in Croatia (Kolda et al. 2019). However, for metabarcoding studies, 16S rRNA is selected due to comprehensive public databases (i.e. SILVA, Greengenes, etc.) that do not exist for other (cyano-) bacterial markers.

The present study is conducted in the Adriatic Sea, a semi-enclosed basin in the northernmost part of the Mediterranean Sea, and distinctively subdivided into northern, central and southern Adriatic Sea. The eastern coast of the Adriatic Sea is marked by a high, rocky, and rugged coastline offering many habitats ideal for fisheries and aquaculture (Dragičević et al. 2017). Aquaculture is one of the fastest-growing industries in the world with 60 million tonnes of exported farmed aquatic organisms annually, which is a 245% increase in the last 40 years (FAO 2018). In the conditions of fish farming, nutrients, excretions of organisms, and food residues can cause eutrophication in the environment in which aquaculture is practised (Bentzon-Tilia et al. 2016). Only 13.9% of the nitrogen and 25.4% of the phosphorus from the fish feed is utilized, and the rest accumulates in the water and sediment (Zhang et al. 2014). In addition to these compounds leading to eutrophication, nitrogenous compounds such as ammonium and nitrite at high concentrations can be toxic to aquatic animals as well as damaging to human health (Zhang et al. 2014).

Investigations of marine cyanobacteria and other prokaryotes in the Adriatic Sea focused on modern molecular methods (the study of composition and dynamics of bacterial communities) are scarce. To our knowledge, these methods have not been employed in the investigation of aquaculture-impacted sites in the eastern Adriatic Sea. They have mainly addressed cyanobacteria as part of bacterioplankton in offshore waters in the southern Adriatic (Najdek et al. 2014, Babić et al. 2018, Mucko et al. 2018), and in wastewater-impacted coastal zones of the northern Adriatic (Paliaga et al. 2017). Bacterial communities of surface sediments are less researched, except sediments impacted by industry and tourism in the northern Adriatic (Korlević et al. 2015). Coastal areas are interesting to investigate, not just for the obvious anthropogenic influences, i.e. aquaculture, but for others that may be concealed (untreated wastewater) or seasonally impacted (effluents from agriculture or tourism pressures). Although influences are evident or assumed, it is difficult to discern whether there is a main stressor, and if so which, or whether they are working in sync at different times of the year. However, their influence evidently exists in the structure of a microbial community.

We hypothesize that the composition, diversity, and ecology of cyanobacteria can be changed rapidly in anthropogenically impacted coastal marine ecosystems. Recognizing these changes on this level could contribute to determining the ecological state of these human-impacted environments. In order to determine that, firstly we need to establish "what is there" using metabarcoding techniques and bioinformatics tools. Cyanobacteria are already widely used as eutrophication indicators in freshwater ecosystems, and highly eutrophicated marine ecosystems (e.g. Baltic sea) (Vahtera et al. 2007). Likewise, their importance is noted in the Marine Strategy Framework Directive under Descriptor 5: Eutrophication (Criteria: undesirable changes in algal community structure) (MSFD, 2008/56/EC). Therefore, we wanted to test the possibility of using specific marine Cyanobacteria as potential indicators of marine ecosystem ecological status in the highly impacted coastal zone, as they are in the freshwater environment. Lastly, our objective is to test the viability of metabarcoding as a standard monitoring method in investigating anthropogenically impacted coastal waters and sediments.

Materials and methods

Sampling

Samples of the water column and surface sediments were collected in the scope of the AQUAHEALTH project: from two sampling locations at the first site in central Adriatic (CA) and two sampling locations at the second site in the southern Adriatic Sea (SA). Both sites are in the coastal area affected by various anthropogenic influences (overpopulation, wastewaters, tourism, agriculture etc), but predominantly by aquaculture – European seabass cage farms. The site in the central Adriatic is characterized by more oligotrophic conditions and is under the effect of the open sea, while the southern Adriatic site is a moderately eutrophic enclosed bay with the strong freshwater influence of the Neretva River (Fig. 1). At both sites, two sampling locations were selected, first in the cage farm area (CA – Movar Cove N 43.509141, E 15.96268; SA – Mali Ston Bay N 42.922510, E 17.474728, respectively) and second, as a control point away from the farm (CA control N 43.504971, E 15.952208; SA control N 42.93022, E 17.49925, respectively). Sampling was conducted in all four seasons during 2017 (February, June, September, November).

Niskin sampler was used to collect water column composite samples (maximum depth 20 m) for molecular anal-

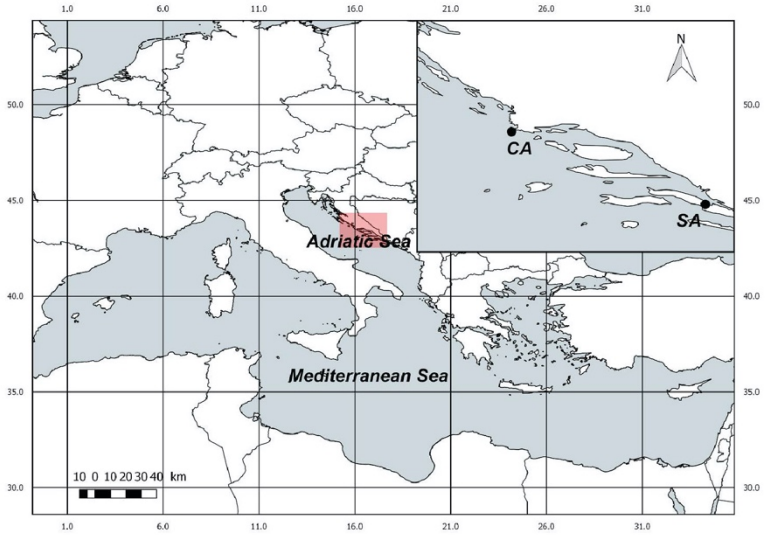

Fig. 1. Study sites in the central and southern East Adriatic Sea (CA – central Adriatic, SA – southern Adriatic).

ysis in 1 L bottles from four depths (250 mL from surface, 5 m, 10 m and bottom layer). Seawater was collected in 250 mL bottles for water chemistry analysis from each depth. Physico-chemical parameters (salinity, dissolved oxygen and oxygen saturation, temperature, turbidity, pH, total dissolved solids) were measured *in situ* by probes: SevenGo pro/OptiOx, SevenGo pro pH/Ion (Mettler Toledo, Ohido, US). The water column transparency was determined by a Secchi disc. Immediately after sampling, samples for molecular analysis were filtered through 0.2 µm pore filters (Whatman, Sigma Aldrich, UK) in triplicate (300 mL per filter), frozen in liquid nitrogen until transported to the laboratory, where they were stored at −20 °C. Surface sediment samples were collected by a diver, stored on ice and transported to the laboratory, where they were stored at −20 °C.

Water column nutrients and granulometric analysis of sediment

Total nitrogen was determined by oxidative digestion with peroxydisulfate (ISO 11905-1: 1997); total phosphorus was determined with ammonium molybdate using Hach spectrophotometer DR/6000 (ISO 6878:2004); and the amount of silicon dioxide was determined by the Hach method 8186 – heteropoly blue using a DR/6000 Hach spectrophotometer. All values were expressed in mg L^{-1}.

To determine grain size, 100 g of dried sediment was weighed from each sample and sieved through 7 standard stainless sieves to separate coarse-grained (> 0.063 mm) and fine-grained (< 0.063 mm) fractions. The suspension with fraction < 0.063 mm was analysed using Micromeritics Sedigraph 5100. Sediment particles found in coarse-grained sediment (> 0.063 mm) were randomly separated from each fraction and microscopically examined under a binocular microscope for qualitative bulk identification. The sediment texture for the whole sediment fractions range (0.005-2.00 mm) was determined according to the Folk (1954) classification scheme.

DNA extraction and amplicon sequencing

Total DNA was extracted from filters and sediment samples by using DNeasy PowerSoil kit (Qiagen, Germany), following the manufacturer's instructions with minor changes. Modifications involved mechanical disruption on Vortex-Genie 2 (MoBio, USA) for 15 min at maximum speed and incubation at 37 °C for 30 min with the addition of 2 µL of lysozyme (0.5 mg mL^{-1} solution). Extracted DNA yield and quality were measured by spectrophotometry (BioSpec Nano, Shimadzu, Japan), while the integrity of DNA was checked on 1% agarose gel. Samples of total extracted DNA were sent for 16S rRNA gene library preparation and amplicon next-generation sequencing to Molecular Research LP (Shallowater, Texas, USA). Sequencing was performed on the Illumina MiSeq (Illumina, Chesterfold, UK) platform following the manufacturer's guidelines (MR DNA; www.mrdnalab.com, Shallowater, Texas, USA). The 16S rRNA gene V1-V3 variable region was targeted by PCR primers 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′), with a barcode on the forward primer. The PCR program included a 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, with a final elongation step at 72 °C for 5 minutes. PCR products were visualized on 2% agarose gel to check the success of amplification and the relative intensity of bands.

Bioinformatics and statistical analysis

Reads were processed using QIIME 2 2019.4 (Bolyen et al. 2019). Pipeline included several steps: importing and demultiplexing of raw sequence data, quality filtering and denoising using DADA2 plugin (Challahan et al. 2016) and taxonomy assignment of the resulting amplicon sequencing variants (ASVs) using Naïve Bayes classifier pre-trained on the SILVA 132 database with 99% OTU identity threshold. From the total bacterial community, taxa filtering was performed to include only cyanobacterial ASVs and excluding chloroplast and mitochondrial sequences from the data. The cladogram was constructed using plugin q2-phylogeny: the MAFFT program was used to perform multiple sequence alignment, masking ambiguously aligned regions and applying FastTree for creating a cladogram from the masked alignment. The generated tree (Online Suppl. Fig. 1) was visualized in iTOL 4.4.2. (Letunic and Bork 2019). Sequences that were poorly identified or defined as "uncultured" were searched in the NCBI GenBank database using the BLAST search tool, and those with low identity threshold were pruned from the tree. Sample frequency was added using FeatureTable[Frequency] (Online Suppl. Fig. 1). Generated phylogenetic tree was visualized in iTOL using FeatureData[AlignedSequence] file generated from QIIME2. Leaf labels were automatically assigned by adding FeatureData[Taxonomy] file, and multivalue bar chart with sample frequencies was created with FeatureTable[Frequency] file. Downstream analysis and taxa bar plot visualizations were performed in RStudio version 1.2.1335, using qiime2R (Bisanz 2018), phyloseq (McMurdie and Holmes 2013) and ggplot2 (Wickham 2016) packages. Statistical analysis of physico-chemical parameters of seawater was conducted using Primer 5.2.9 and visualization further executed in Grapher™ version 8.2.460 (Golden Software, LLC, Colorado, USA). Raw sequences reads are deposited in European Nucleotide Archive (ENA) under project number PRJEB34935.

Results

Physico-chemical parameters of water column and sediment granulometry

The principal component analysis includes physico-chemical parameters of seawater (Tab. 1) in all sampling locations during all seasons in 2017 (Fig. 2). PC1 axis explains 31.9% of the variance in physico-chemical data (eigenvalue 3.50), while PC2 axis explains 23.7% (eigenvalue 2.61) (Online Suppl. Tab. 1). By using PCA it was not possible to identify a clear pattern of grouping or separation of sampling sites. However, the seasonal pattern is easily identified for all sampling sites and locations. Winter samples have been grouped mostly in the negative part of the PC2 axis, positively correlated with dissolved oxygen. Most of the spring samples are grouped in the negative part of the PC1 and PC2 axis, correlating with pH, turbidity, transparency and percentage of O_2 in the water column. Summer samples are described by temperature, SiO_2 and total nitrogen in the positive area of the both the PC axis. Samples from the autumn were characterized by TDS, salinity and total phosphorus, and thereby grouped in the positive part of the PC1 and the PC2 axis.

Analysed sediments were predominantly classified as gravelly sands with various and generally low proportions of gravel and mud (Tab. 2) Generally, CA sediments are mostly gravelly sands, with a muddy component present in aquaculture sites in autumn and summer. Sediment in the SA con-

Tab. 1. Median values for physico-chemical parameters of seawater in sampling sites during seasons in 2017. Site: CA – central Adriatic, SA – southern Adriatic, Aq – site type under the influence of fish farms, Co – control site type, S.disc – Secchi disc, Turb – turbidity, Sal – salinity, TDS – total dissolved solids, T – temperature, DO_2 – dissolved oxygen, O_2% – oxygen saturation, N – total nitrogen, P – total phosphorus, SiO_2 – silicon dioxide, NA – not measured). Values for physico-chemical data

Season	Site	S.disc (m)	Turb	Sal	TDS (mg L^{-1})	T (°C)	pH	DO_2 (mg L^{-1})	O_2 (%)	N (mg L^{-1})	P (mg L^{-1})	SiO_2 (mg L^{-1})
Winter	CA Co	19	2.63	33.10	25.47	13.83	7.89	10.54	102.02	NA	NA	NA
	CA Aq	15	1.10	33.48	25.78	14.13	8.05	10.13	98.73	NA	NA	NA
	SA Co	14	4.98	34.28	26.48	12.25	8.09	10.95	101.63	0.30	0.008	0.12
	SA Aq	12.5	5.88	33.75	26.05	12.01	7.83	10.63	101.63	0.60	0.01	0.26
Spring	CA Co	15	27.53	33.30	25.60	23.45	7.88	8.95	103.73	0.90	0.02	1.46
	CA Aq	12	44.63	34.30	26.03	22.18	7.85	9.12	102.65	1.13	0.06	2.38
	SA Co	9.5	54.33	33.40	25.50	22.25	7.83	9.29	105.83	0.60	0.02	1.08
	SA Aq	10	23.90	33.68	25.70	21.30	7.92	9.33	104.65	0.68	0.05	1.19
Summer	CA Co	17	1.98	36.45	27.63	21.8	7.95	9.01	102.85	0.53	0.03	1.29
	CA Aq	12	1.78	33.48	27.25	21.95	8.04	8.46	96.98	0.83	0.05	1.50
	SA Co	15	0.78	34.85	26.48	21.53	7.91	9.12	102.9	1.00	0.02	0.80
	SA Aq	14	0.83	34.65	26.33	21.35	7.93	8.97	100.85	0.83	0.02	1.02
Autumn	CA Co	7	NA	34.5	26.43	16.63	7.04	9.72	99.68	0.23	0.06	0.14
	CA Aq	15	0.25	34.63	26.33	16.80	7.29	9.43	97.1	0.50	0.06	0.13
	SA Co	5	1.00	34.18	26.20	15.70	7.95	9.70	97.53	0.58	0.06	0.40
	SA Aq	9	0.50	34.53	26.58	15.25	7.79	9.78	98.03	0.68	0.07	0.61

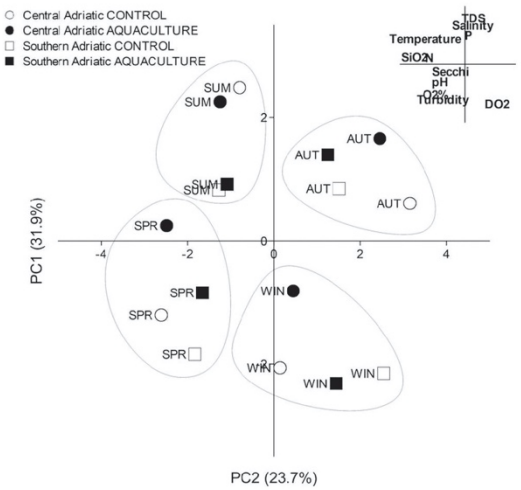

Fig. 2. Principal component analysis ordination graph of physicochemical parameters of seawater column (T – temperature, SiO_2 – silicon dioxide, N – total nitrogen, P – total phosphorous, Secchi – Secchi disc, Turb – turbidity, Sal – salinity, TDS – total dissolved solids, DO_2 – dissolved oxygen, O_2% – oxygen saturation; WIN – winter, SPR – spring, SUM – summer, AUT – autumn). Number of samples = 16.

trol location is, for the most part, sandy gravel, with more variation in the aquaculture location – gravelly sands with muddy gravel, with one sample of sandy gravel that was taken only in the vicinity of the fish cage due to inaccessibility. Most of the sediment samples were composed of biogenic carbonate clasts, generally, shell debris containing molluscan fragments with less present foraminifera tests, echinoid fragments, worm tubes and bryozoans. Textural characteristics did not show any regularity attributable to the sampling location or season.

Tab. 2. Textural characteristics of surface sediment samples after Folk (1954). CA – central Adriatic, SA – southern Adriatic, Aquaculture – site type under the influence of fish farms, Control – control site type.

Locality / Site type	Season	Classification after Folk (1954)
CA Aquaculture	Winter	Slightly gravelly sand – (g)S
	Spring	Gravelly sand – gS
	Summer	Gravelly muddy sand – gmS
	Autumn	Slightly gravelly muddy sand – (g)mS
CA Control	Winter	Slightly gravelly sand – (g)S
	Spring	Gravelly sand – gS
	Summer	Gravelly sand – gS
	Autumn	Gravelly sand – gS
SA Aquaculture	Winter	Gravelly sand – gS
	Spring	Gravelly sand – gS
	Summer	Muddy gravel – mG
	Autumn	Sandy gravel – sG
SA Control	Winter	Sandy gravel – sG
	Spring	Sandy gravel – sG
	Summer	Gravelly sand – gS
	Autumn	Sandy gravel – sG

Cyanobacteria community relative abundance and diversity

Using the metabarcoding molecular approach, 32 samples were analysed with 10102 ASV assigned at 99% similarity threshold. Out of that number, 437 ASV were defined as "Cyanobacteria". Additional filtering of sequences identified as "Chloroplast" was applied, resulting in the identification of a total of three cyanobacterial genera from the water column, and 13 genera and two uncultured groups in the surface sediments. Planktonic picocyanobacteria *Cyanobium* PCC-6307, *Prochlorococcus* MIT9313 and *Synechococcus* CC9902 were detected in the water column, as shown in Figs. 3 and 4. The difference in the community of marine picocyanobacteria was not due to sites (CA/SA) or location type (aquaculture/control point), but a seasonal pattern was observed. Although *Prochlorococcus* was absent from winter

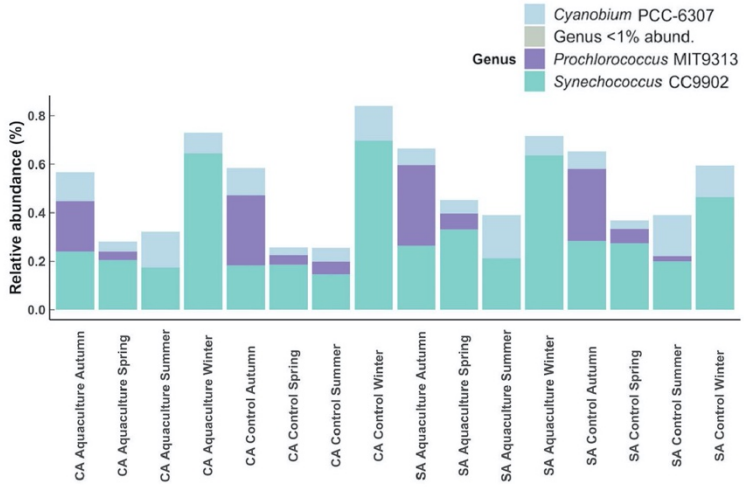

Fig. 3. Relative abundances (% of total sequence number) of cyanobacterial genera in all sampling points of coastal seawater (CA – central Adriatic, SA – southern Adriatic, Aquaculture – location under the influence of fish farms, Control – control location).

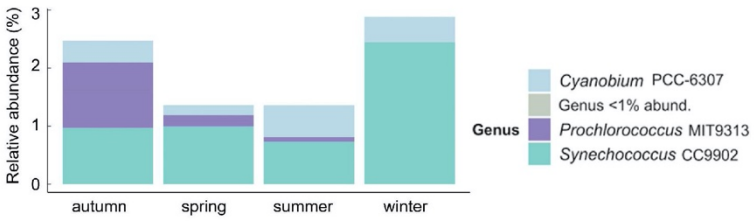

Fig. 4. Relative abundances (% of total sequence number) of cyanobacterial genera in seawater during four seasons, including combined central and southern Adriatic samples.

samples, the relative abundance of *Synechococcus* CC9902 can reach even 70%. However, *Prochlorococcus* MIT9313 had the highest abundance in autumn samples (up to 29%). Remarkably, the freshwater genus *Cyanobium* was represented in all samples, especially in summer samples (17%).

Sediment samples showed location and site type differentiation (Fig. 5). At the same time, there seems to be an indication of community structure connected to the type of sediment (Fig. 6). In total, 13 cyanobacterial genera closely related to strains (*Arthrospira* PCC-7345, *Chroococcidiopsis* PCC-6712, *Crocosphaera* WH0.03, *Cyanobacterium* CLG1, *Geminocystis* PCC-6308, *Hormoscilla* SI04-45, *Pleurocapsa* PCC-7319, *Prochlorococcus* MIT9313, *Synechococcus* CC9902 and *Synechococcus* PCC-7336, *Trichodesmium* IMS10, *Xenococcus* PCC-7305 and SU2 symbiont group) and 2 uncultured groups were detected. Samples from the SA showed a higher diversity of genera over CA samples (13 + 2 uncultured and 6 + 2 uncultured, respectively). Sediment characterized as sandy gravel contains the highest number of genera (11 + 2 uncultured), and it is most represented in SA. In general, control locations on both sites have higher richness (total of 12 + 2 uncultured cyanobacterial genera) than the sites near fish cages (7 + 1 uncultured).

Genera *Xenococcus* and *Pleurocapsa* (order Pseudocapsales) were represented and dominant in most samples. Planktonic cyanobacteria were also represented in sediment samples, e.g. *Prochlorococcus* and *Synechococcus*, but mainly in aquaculture locations. Interestingly, in the water column only *Synechococcus* CC9902 was detected, and not *Synechococcus* PCC-7336. Some genera, e.g., *Crocosphaera*, *Cyanobacterium*, *Geminocystis* (order Chroococcales) and *Chroococcidiopsis* PCC-6712 (order Chroococcidiopsidales) were only detected in the SA control location. *Hormoscilla* SI04-45 (*Hormoscilla spongeliae* (Gomont) Anagnostidis et Komárek) belonging to the order Oscillatoriales, was identified only in the summer sample in the SA control location, along with the unicellular SU2 symbiont group. *Arthrospira* PCC-7345 (Osciallatoriales, *Phormidiaceae*) was detected at both sites with 25% max. relative abundance in the CA aquaculture location. Only *Prochlorococcus* showed seasonal occurrence in the sediment. It was detected in autumn samples, in which it was the most abundant in the water column. The group "uncultured" contained ASVs of fami-

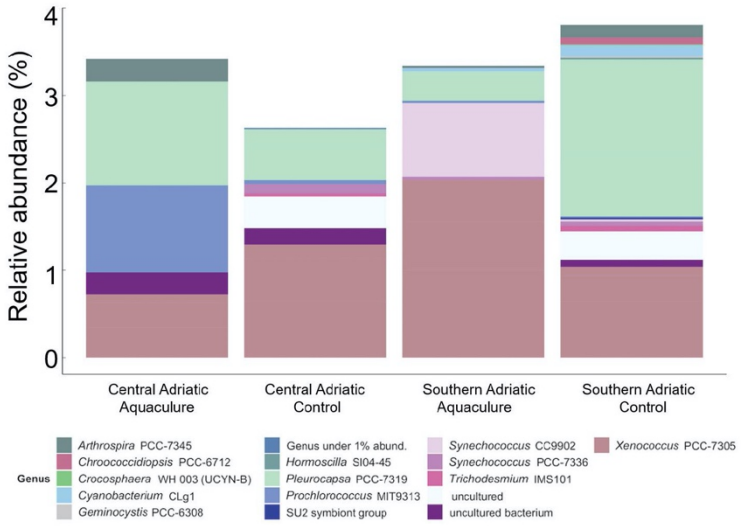

Fig. 5. Relative abundances (% of total sequence number) of cyanobacterial genera in sediment, relating to locality and site type (combined central Adriatic aquaculture and control sites, and southern Adriatic aquaculture affected and control sites).

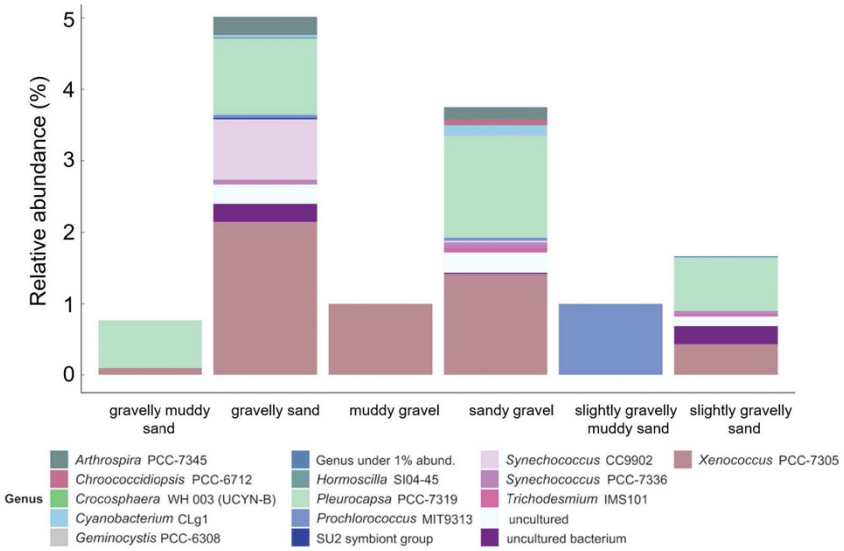

Fig. 6. Relative abundances (% of total sequence number) of cyanobacterial genera in sediment, in relation to sediment type (combined central Adriatic aquaculture and control sites, and southern Adriatic aquaculture affected and control sites).

lies *Leptolyngbyanceae* and *Xenococcaceae*, while the group "uncultured bacterium" comprised *Cyanobacteriaceae* and *Melainabacteria*.

Cladogram (On-line Suppl. Fig. 1) shows the genotypic diversity of cyanobacterial 16S rRNA gene sequences constructed from a total of 100 identified taxa or sequences, after removal of chloroplast, uncultured and poorly identified sequences. SILVA taxonomy is based on Bergey's Taxonomic Outlines or, in cases of rapid taxonomy changes, on the "List of Prokaryotic Names with Standing in Nomenclature". Topological differences between the SILVA Ref (NR 99) trees and other resources are expected, since SILVA taxonomy employs a phylogeny-based process using guide trees (Yilmaz et al., 2014).

Sample frequency bar plots visually demonstrate the separation of taxa found in the water column and in the sediment – planktonic and (mostly) benthic genera. Water column samples show lower number of taxa, but much higher sample frequency then ASVs from the sediment. Water column samples are mainly represented by the family *Cyanobiaceae*, consisting of the genera *Synechococcus* CC9902, *Prochlorococcus* MIT9313 and *Cyanobium* PCC-6307. Using BLAST, several unidentified sequences were re-assigned and showed a similarity to various coastal cyanobacterial strains. For instance, ASV found in autumn in SA is identified as *Cyanobium* sp. CSZ that was isolated in a eutrophic coastal lagoon in the Baltic coast. ASV detected in winter and spring on both sites was uncultured *Synechococcus* sp. clone KOT-S4UC, isolated from the coastal Arabian Sea. A sequence detected in water and sediment at both sites shows a relation to *Synechococcus* Minos12 isolated from the Mediterranean Sea, which appears to be non-motile (clade III). Sequences similar to Atlantic strains were found in winter and spring waters (uncultured *Synechococcus* sp. clone DWH – surface water of the Gulf of Mexico and *Synechococcus* sp. WH 8020 – New England coastal strain).

Sediment samples show high diversity incorporating cyanobacterial families *Xenococcaceae*, *Microcysteaceae*, *Cyanobacteriaceae*, *Phormidiacaeae* and *Rivulariaceae*. Some of the ASVs determined as the "uncultured" strains, closely related to the non-photosynthetic cyanobacteria of the Melainabacteria group, are found in sediments in CA. Many other sequences similar to strains in tropical and subtropical regions (e.g. uncultured bacterium clone bac98c and uncultured bacterium clone bac129c) share similarities with bacteria isolated in oolitic sands of Highborne Cay (Bahamas). Sediments from autumn control samples in SA contain ASVs similar to *Neolyngbya irregularis* ALCB 114389 and *Neolyngbya arenicola* ALCB 114386, newly described filamentous benthic cyanobacteria from Brazilian coast. Sequences also showed similarities with uncultured cyanobacterium clone RII-OX103 isolated from subtidal surface sediments of Cíes Islands (NW coast of Spain). Some ASVs seems to belong to plankton, e.g. *Chroococcidiopsis* sp. CC-MP2 that is classified as a saltwater strain isolated from Micronesia and similar habitats (Pavilion Beach, Sand Island, Midway Atoll, Midway Islands).

There are also ASVs pointing to nutrient cycling roles (nitrogen and carbon cycles), such as uncultured Chroococcales cyanobacterium clone D10, diazotrophic cyanobacteria isolated from salt marshes and uncultured bacterium clone OS02-CYA-1 from intertidal marine sediments with different organic substrate utilization. Another potential diazotrophic ASV found in both CA and SA (winter) is similar

to the strain *Trichodesmium erythraeum* SERB 14, isolated from Great Nicobar Biosphere Reserve.

Some ASVs are hinting at biofilm and microbial mat formation in the sediments, e.g. *Aphanocapsa* sp. HBC6 and uncultured bacterium clone CI5cm.45 that have similarities with isolates from stromatolites of Highborne Cay in the Bahamas. ASVs from control location in SA during summer and autumn are similar to uncultured cyanobacterium clone AO26 found in anoxic and suboxic layers of permeable sediments from the South Atlantic Bight (Hunter et al., 2006), a shallow submarine hydrothermal system (Hirayama et al. 2007), a coral reef sediment (Sørensen et al. 2007, Gao et al. 2011). A sequence detected in sandy gravels of the SA control location during summer and autumn may be *Romeria* sp. (Synechococcales cyanobacterium LEGE 06003), isolated from Buarcos Beach in Portugal.

Lastly, the cyanobacterial propensity for symbiotic relationships is also shown, in ASVs from CA similar to Uncultured *Calothrix* sp. clone 10010_AA1_t7 and Uncultured cyanobacterium clone STX_22 isolated from the coral host in the Caribbean.

Discussion

The present study focused on discovering the composition, diversity, and ecology of cyanobacteria from the water column and sediment in rapidly changing and anthropogenically impacted coastal marine ecosystems. Using metabarcoding techniques and bioinformatics tools, we wanted to establish not only cyanobacterial taxa present in these ecosystems, but also whether they can have indicator value, as in freshwater ecosystems. Water column samples in this study display a seasonal variance, but do not show any difference between locations influenced by aquaculture activates and control locations, in contrast to sediment samples. The cyanobacterial community of the sediment seems to be affected by a muddy component, and to have a preference for a sandy gravel type of sediment away from aquaculture impacted locations. It is evident from the cyanobacterial composition that sediment samples have overall larger community richness than water samples, although the sampling frequency of ASVs is higher in seawater. Overall, detected Cyanobacteria in water column and sediment were not exclusively marine genera, and evidence of freshwater and coastal eutrophication was found from the cyanobacterial composition.

In the water column, although we expected to find a distinction between cyanobacterial assemblages in aquaculture impacted sites vs. control and variation between southern and central Adriatic locations, no significant difference was observed. This could be due to the similar physico-chemical parameters, as measured at both sites and locations. Additionally, it could indicate that these two marine aquacultures have well-managed systems which did not provoke triggers for dramatically different assemblages in the water column. However,a seasonal pattern is observed, both in physicochemical parameters groupings in PCA (Fig. 2) and in the picocyanobacterial taxa from metabarcoding results (Figs. 2, 4). The ecological importance of picocyanobacteria in the world's oceans cannot be stressed enough since they are one of the most important primary producers. They constitute over 50% of marine phytoplankton (Paerl 2012) and out of that percentage, *Synechococcus* and *Prochlorococcus* account for approximately half of primary production in the ocean (Flombaum et al. 2013, Dvořák et al. 2014). On a global ocean scale, the prevalence of *Prochlorococcus* or *Synechococcus* depends on their environmental preferences – for *Prochlorococcus* ecotypes and *Synechococcus* clades (Zwirglmaier et al. 2008). Investigations of picocyanobacteria in the eastern Adriatic Sea showed dominance in the abundance of *Synechococcus* over *Prochlorococcus* (Šantić et al. 2013, Paliaga 2017, Mucko et al. 2018), which was also confirmed in this study (Fig. 4). *Prochlorococcus* MIT9313 strain belongs to an ecotype of low-light adapted *Prochlorococcus*, which could indicate occasional decreased light availability in the water column. This strain belongs to subclade IV and has one of the largest genomes, which indicates a higher ability to respond to environmental stress (Gómez-Baena et al. 2009). Moreover, it is shown that this particular strain has an important role in carbon cycling due to its carbon-concentration mechanism (Scott et al. 2007), and can utilize organic nitrogen compounds such as urea and amino acids (Zubkov et al. 2003, Scott et al. 2007) excreted by the fish in aquaculture facilities (Lazzari and Baldisserotto 2008). Investigating offshore oligotrophic southern Adriatic waters, Babić et al. (2018) also discovered low-light ecotype of *Prochlorococcus*, however, they were OTUs closely related to the *Prochlorococcus* NATL2A strain. This could demonstrate that *Prochlorococcus* MIT9313 is more adapted to the coastal, anthropogenically impacted water environment. Regarding its absence from the winter samples (Fig. 4), the explanation could be a combination of high light transparency (SA – 12.5 m and 14 m; CA – 19 m and 15 m) in the water column and lower temperature (SA 12.01-12.25 °C; CA 13.84-14.13 °C), presented in Tab. 1. This is in concordance with the reports by Zinser et al. (2007) from experimental data that involved growth rates depending on temperature and light, and Rocap et al. (2003) analysis of the *Prochlorococcus* MIT9313 genome, which established the loss of many genes encoding phycobilisome structural proteins and enzymes that are involved in phycobilin biosynthesis. With respect to salinity, values are lower in all sampling sites than the Adriatic Sea mean values, which clearly points to freshwater influence. As reported by Russo et al. (2012), depending on the season, salinity varies between 37.84 and 38.89, but in our sampling points, they range from 33.10 (min.) to 36.45 (max.). The proliferation of several freshwater genera, e.g. *Cyanobium*, *Geminocystis*, *Cyanobium* and *Chrococcidiopsis*, could signify that input throughout the year in both sites. In the SA site, this is definitely the freshwaters of the Neretva River coming into the Mali Ston Bay, while in CA it could indicate occasional submarine springs that are common for the karstic coast of the eastern Adriatic Sea (Pikelj and Juračić 2013). In agreement with this, *Chroococcidiopsis cyanosphaera* Komárek et Hindák (sub SAG 33.87), origi-

nating from mineral springs and pools was detected (Online Suppl. Fig. 1). *Cyanobium* in coastal waters could not only signify freshwater influence but additional eutrophic conditions according to Pulina et al. (2011). In our samples, their highest abundances were found during the summer at aquaculture locations on both sites (SA – 17.81%, CA – 14.67%). Eutrophication generated or aided by aquaculture can have a negative impact on the productivity of the industry. It can be destructive to less tolerant species in the phytoplankton community and also lead to an increase of the cyanobacteria fraction (Pulina et al. 2011). Cyanobacterial blooms, most challenging in freshwater ecosystems, are also well documented in Mediterranean lagoons (Chomérat et al. 2007). They are reported in Ca'Pisani lagoons in the western coast of the Adriatic Sea (Sorokin et al. 2006), in conditions of intensive aquaculture in which a cyanobacterial bloom followed and surpassed the bloom of the potentially toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech. Therefore, the questions arise: in the face of global climatic perturbations, is there a possibility of picocyanobacterial blooms becoming a regular occurrence in the coastal bay areas (not just more secluded lagoons), especially areas affected by the additional pressure of aquaculture? In that sense, the advantages of having a long memory of sediment sample could be very informative. Some of the planktonic genera detected in sediments could be troublesome in the future, e.g. *Trichodesmium erythraeum* Ehrenberg ex Gomont. Specifically, this generally innocuous nitrogen fixator from tropical waters is forming potentially toxic blooms. Their decomposing blooms can affect aquaculture sites by creating anoxic conditions leading to mortalities (Negri et al. 2004). Furthermore, a large percentage of water column ASVs showed similarity to the eutrophic strain *Synechococcus* CC9902. OTUs similar to this strain were recorded in Croatia for the first time in the active bacterial community of the naturally eutrophic, marine meromictic Rogoznica Lake, situated in the coastal area of the central Adriatic Sea (Čanković et al. 2019). Furthermore, *Synechococcus* CC9902 (clade IV) was found to survive even in anoxic and dark conditions, and showed the highest abundances during the winter, as in this study (Fig. 4). The potential aquaculture-related concern could be that this strain was firstly isolated from coastal waters off California, where it can form extensive blooms (Hamilton et al. 2014). Experiments performed by Hamilton et al. (2014) on the native fish under the bloom concentration of the *Synechococcus* CC9902, showed a negative effect on the behaviour of the fish. This suggested the possibility of sublethal effects of *Synechococcus* blooms on coastal fish populations if climate change predictions come true since fish (regardless of the type of diet) absorb water through drinking, gills, eyes and skin (Flombaum et al. 2013, Hamilton et al. 2014).

Considering sediment, almost all samples contained the genera *Xenococcus* and *Pleurocapsa*, making them core genera in the cyanobacterial community. Unsurprisingly, they are microbial mat-forming cyanobacteria and first colonizers in marine sediments. *Xenococcus* forms colonies attached to any substrate, e.g. stones, alga etc., while *Pleurocapsa* is a unicellular, pseudofilamentous genus that can grow layers of cells on limestone substrate, and some species are endolithic (Goh et al. 2009). Results of the grain size analysis support the proliferation of these two genera – analysed sediments fit into the average coarse-grained carbonate biogenic sediment typical for the eastern part of the Adriatic Sea. While being composed mainly of biogenic shell debris, the grain size of the sampled sediment usually varies due to the presence of dominant organisms and the degree of biogenous skeletal detritus decomposition (Pikelj et al. 2016). The role of cyanobacteria in sediment is of importance, since they produce extracellular polymeric substances (EPS) (Golubic et al. 2000) that stabilize loose sediments, prevent erosion, and protect them from various biotic and abiotic stressors (Costa et al. 2018). These processes are active today, as they were in the ancient stromatolites (Bolhuis et al. 2014). EPS are produced by both filamentous cyanobacteria moving through sediment particles (Golubic et al. 2000) and unicellular non-motile cyanobacteria (Rossi and De Philippis 2015), many of whom are present in the sediment samples (Fig. 5). The sediment analysis of this study suggests that gravel and sand components create a higher number of niches for a large percentage of genera, while samples with muddy component contain 1-2 genera. This is close to the finding of Stal (2010) on cyanobacteria in intertidal coasts, where they appeared frequently in sandy sites, but did not "proliferate on muddy or wave-exposed sites" (Andersson et al. 2014). Moreover, cyanobacterial community richness in sediment samples from this study seems to be largely affected in aquaculture locations, even if they are determined as sandy gravel or gravelly sands (Figs. 5, 6). With the exception of the sandy gravel sample in SA aquaculture location in autumn, they all have an extremely low number of genera. This could indicate a continuing disturbance produced by the aquaculture activities on the community, but, in addition, the fish rearing probably generates a muddy component in the sediment (Tamminen et al. 2011). Aquaculture locations with muddy components also have higher abundances of planktonic *Prochlorococcus* and *Synechococcus*, which implies fish ingestion of picocyanoplankton and accumulation in sediment via fish excretion. In sediment, some members in the cyanobacteria community seem to indicate a light deprivation and anoxic condition that is at least intermittently occurring. Although cyanobacteria are oxygenic phototrophic organisms, Miyatake et al. (2013) confirmed that cyanobacteria and diatoms can survive in dark and anoxic conditions by glucose utilization, proposing a mixotrophic way of living for these organisms usually known as primary producers, e.g. *Cyanobacterium* CLG1 is known to synthesize both glycogen and starch (Kadouche et al. 2016). *Geminocystis* strain PCC-6308 can accumulate a large amount of phycoerythrin (Hirose et al. 2015), which could help it in light acclimation, and detection of a non-photosynthesizing group of cyanobacteria Melainabacteria (Di Rienzi et al. 2013) supports this claim. Additionally, sediment harbours ASVs similar to the benthic strain *Synechococcus* PCC-7336, clustering differently from

other *Synechococcus* representatives (On-line Suppl. Fig. 1). *Synechococcus* PCC-7336 has an unusually large genome that contains type V polymerase proteins rarely found in other cyanobacteria, but common in plants. Additionally, Li et al. (2015) have found 107 kinases and regulators stimulating gene expression to environmental stress, making it highly adaptable to light/oxygen deficiency.

Finally, results markedly present a number of cyanobacterial ASVs related to the various strains from tropical areas (On-line Suppl. Fig. 1). They are present mostly in sediment samples (although some of them are planktonic), e.g. *Neolyngbya*, *Chrococcidiopsis*, *Trichodesmium*, *Aphanocapsa*, *Cyanobacterium*, *Crocospaera, Xenococcus* and many "Uncultured" cyanobacteria strains. Additionally, in seawater, there are ASVs closely related to *Synechococcus* strains from warm seas (the Gulf of Mexico, Arabian Sea). This tropical affinity or "tropicalization" is a trend most evident in wild fish composition in the Adriatic Sea within the last two decades, starting with the arrival of Lessepsian fish species from the Indo-Pacific (Dragičević et al. 2017). According to Ibarbalz et al. (2019), investigations in the temperate zone confirm the trend of tropicalization in marine plankton. It is not surprising that microbiota in our research, *Cyanobacteria* specifically, are mirroring a trend that is well underway throughout the food web.

Conclusion

This study was conducted to test the viability of marine cyanobacteria in human-impacted coastal zones as valuable indicators of ecological states, in the same way that they are used in freshwater ecosystems and the Marine Strategy Framework Directive. By using a metabarcoding approach, we wanted to circumvent the shortcomings of other methods, e.g. the light microscopy counting method. Although there are biases in metabarcoding method, especially if only a resident community is being investigated (DNA), it can deliver valuable information about "what was there". By linking that knowledge with physico-chemical parameters in the water column and granulometric analysis of sediment, it allowed us the opportunity to hypothesise the ecological preferences of taxa found. Therefore, this study provides a starting point in the investigation of the cyanobacterial community in coastal waters and sediments in the Adriatic Sea impacted by aquaculture and proposes the metabarcoding method as a suitable monitoring tool.

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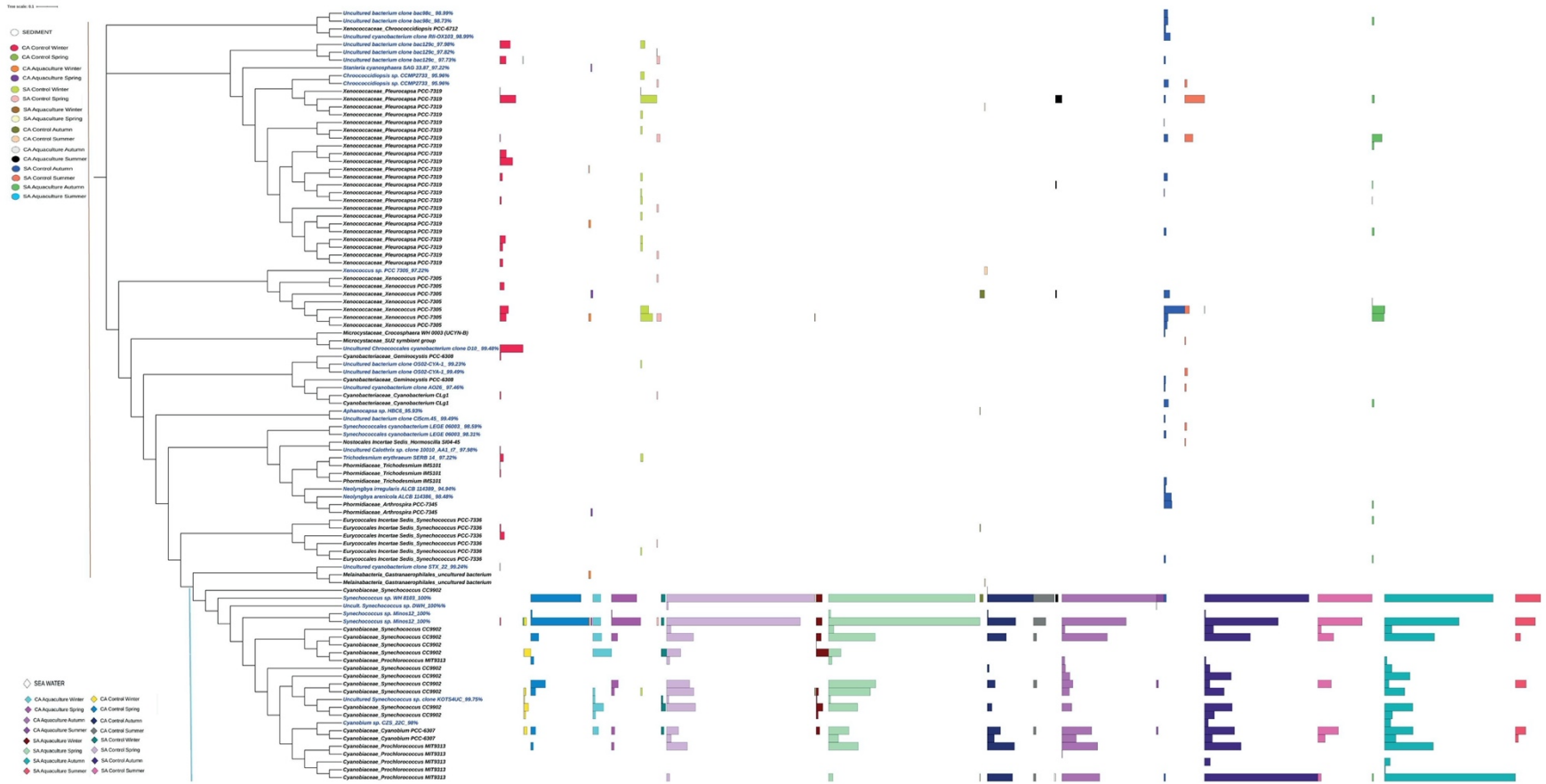
On-line Suppl. Tab. 1. Results of principal component analysis.

	PC1	PC2	PC3
Eigenvalues	3.50	2.61	1.75
% variance explained	31.9	23.7	15.9
Cumulative % explained	31.9	55.6	71.5
Eigenvectors			
Secchi Depth	−0.153	−0.085	−0.527
Turbidity	−0.238	−0.332	0.347
Salinity	0.088	0.403	−0.261
TDS	0.095	0.477	−0.269
Temperature	−0.425	0.265	0.178
pH	−0.267	−0.130	−0.423
DO_2	0.351	−0.406	−0.155
O_2 %	−0.314	−0.328	−0.014
N	−0.428	0.071	−0.112
P	0.040	0.350	0.445
SiO_2	−0.495	0.077	−0.001



On-line Suppl. Fig. 1. Cladogram displaying grouping of 100 cyanobacterial ASVs, with addition of multi value bar chart of sample frequencies in different ASVs (CA – central Adriatic, SA – southern Adriatic, Aquaculture – site type under the influence of fish farms, Control – control site type; à - sea water sample type, **O** – sediment sample type). Leaf labels are automatically assigned by SILVA database (black letters), or NCBI GenBank database BLAST search hit results (blue letters).

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Profiling of bacterial assemblages in the marine cage farm environment, with implications on fish, human and ecosystem health

Anamarija Kolda[a,*], Ana Gavrilović[b], Jurica Jug-Dujaković[c], Zrinka Ljubešić[d], Mansour El-Matbouli[e], Atle Lillehaug[f], Semir Lončarević[f], Lorena Perić[a], Dražen Knežević[g], Darija Vukić Lušić[h], Damir Kapetanović[a]

[a] Ruđer Bošković Institute, Division for Marine and Environmental Research, Zagreb, Croatia
[b] University of Zagreb, Faculty of Agriculture, Department of Fisheries, Apiculture, Wildlife Management and Special Zoology, Zagreb, Croatia
[c] Sustainable Aquaculture Systems, Inc., Frenchtown, NJ, USA
[d] University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia
[e] Department For Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine, Wien, Austria
[f] Norwegian Veterinary Institute, Department of Fish Health and Biosecurity, Oslo, Norway
[g] Croatian Agency for Agriculture and Food, Osijek, Croatia
[h] Department of Environmental Health, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

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ABSTRACT

This research presents a comprehensive study of bacterial assemblages within the water column and in the surface sediments in the zone of two European sea bass cage farms. By the application of the high-throughput amplicon sequencing of 16S rRNA gene, and further implementing microbial ecology tools, a bacterial segment from cage culturing systems and their respective controls were analyzed, with special reference to potential impact on animal, human and environmental health. Samples of seawater and sediments were collected seasonally, at locations situated in the central and southern Adriatic Sea. Bacterial composition was significantly different in the seawater vs. sediment. No significant differences in alpha diversity in sediments were indicated between aquaculture and control sites, and it appears that it is not affected by farming practices. Control sediments have higher relative abundance of aerobic and facultative anaerobic bacteria, while aquaculture sediments are markedly anaerobic. Sediments largely contain functional groups for respiration of sulfate and sulfur compounds, though doubly more in aquaculture sites. Seasonal groupings of bacterial assemblages were confirmed in the seawater, with higher relative abundance of known aquaculture pathogens (except *Photobacterium* in the winter samples) detected in the winter and summer, opposed to other two seasons. Rare taxa were analyzed in the sediment and in the water column in the search for known fish pathogens, with five genera detected: *Vibrio*, *Pseudomonas*, *Photobacterium*, *Tenacibaculum* and *Mycobacterium*. Biomarkers important for the impact of aquaculture on the environment were identified, e.g. *Blastopirullela*, Sva0081, *Suflurovum*, *Spirochaeta* 2, etc., as well as human and fish potential pathogens: *Vibrio ichtyoentery*, *V. harvey*, *Acinetobacter lwoffi*, *A. johnsonii*, *Clostridium perfringens*, etc. Chemoheterotrophy has emerged as the dominant functional group in both environments. Regarding priorities for aquaculture microbial management, seawater seems to contain a higher percentage of taxa connected to health-related functional groups.

1. Introduction

Aquaculture and fisheries have significant importance as a source of food and employment for the earth's growing population, which is predicted to reach around nine billion by 2050 (FAO, 2018). In the 2016, fish production reached 171 million tones, mostly directed for human consumption (FAO, 2018). Due to rapid growth worldwide, aquaculture is afflicted by disease outbreaks as well as raising environmental concerns (Assefa and Abunna, 2018). It is apparent that there has been a progressive increase in the number of new bacterial taxa associated with fish diseases that are cause of severe economic losses to aquaculture (Austin and Austin, 2016). Human infections caused by food borne pathogens transmitted from raw or undercooked aquatic organisms are quite common and therefore, they are cause for

* Corresponding author at: Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia.
E-mail address: Anamarija.Kolda@irb.hr (A. Kolda).

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concern (Novoslavskij et al., 2016). Although the strong-evidence of food borne diseases outbreaks caused by fish consumption are well documented worldwide (EFSA and ECDC, 2019), the number of peer-reviewed articles published is disproportioned to the industry's worldwide importance – particularly regarding environmental, human and animal health impact. Besides productivity optimization (in terms of quality nutrition of farmed animals, water quality monitoring and effluent control), aquaculture management requires a strict control of pathogen and disease outbreak in order to preserve the health of farmed animals and end-consumers' safety (Moriarty, 1997). Incorporating tools of microbial ecology may have an important role in aquaculture management, especially in the study of the animal disease transmission and control. Strong evidence of using microbial ecology tools in monitoring coastal waters and sediments quality have been published lately (Wang et al., 2020; Moncada et al., 2019; Hattenrath-Lehmann et al., 2019; Martins et al., 2018; Seo et al., 2017).

Potential fish bacterial pathogens are usually heterotrophs from the water column and sediment, which in the case of deteriorating farming practice (e.g. high organism density, increase of organic matter, high ammonia and hydrogen sulfide concentrations, low oxygen levels) can turn pathogenic (Moriarty, 1997). The benthic areas under and in the close vicinity of the fish cages are covered with organic matter mostly comprised of decaying feed pellets and fish waste (Verhoeven et al., 2018). In the water column, eutrophication processes can lead to microbial imbalances that could cause potentially toxic algal blooms and oxygen depletion (Moncada et al., 2019). Degradation of organic matter by heterotrophic bacteria can lead to hypoxic and anoxic conditions (Tamminen et al., 2011). Important role of bacteria in such environments lay in their efficient use of high nutrient concentrations (Moncada et al., 2019), as in e.g., nitrate removal. Bacteria in the environment also greatly affect farmed organism's health, either by protecting the organisms from the disease or being the source of often fatal diseases. That prompted more studies that have focused on the application of the 16S rRNA amplicon sequencing of bacterial communities in the aquaculture industry (Bentzon-Tilia et al., 2016). Microbiota from the water environment directly impacts fish microbiota (Legrand et al., 2018), while in turn sediment resuspension in the water column can affect water microbial flora (Holmer et al., 2005).

This research presents a comprehensive study of bacterial assemblages within the water column and surface sediments in the aquaculture of the European sea bass (*Dicentrarchus labrax*). European sea bass is farmed almost exclusively in the Mediterranean, and Croatia is one of the main producers (Commission and General, 2012). In order to recognize the bacterial indicators of water quality and benthic impact, as well as potential fish and human pathogenic bacteria, the objective of this study is to understand all aspects of microbial ecology of the seawater and sediment. The first aim was to analyze diversity, i.e. richness and taxonomic composition, of microbial assemblages from aquaculture systems and their respective controls. The second aim involves gaining more insight into sediment/water ecosystem functioning and characteristics of present bacteria, using phenotyping tools and functional groups annotation. Implementation of functional groups is recommended to further complement the taxonomic data, and grouping of the biologically interpretable phenotypes within bacterial community is preferred to the "bag-of-genes" approach in analysis of environmental microbiomes (Ward et al., 2017). A third aim was to investigate aspects of the bacterial assemblages that highlight the potential impact on health: animal, human and environmental, which are inseparable if we want to approach this subject in a holistic, in-depth manner.

2. Materials and methods

2.1. Sampling

Samples of seawater and sediments were collected during 2017 at two aquaculture locations (i.e. near the fish cage) and two control locations, situated in the central and southern Adriatic Sea, marked as Site 1 and Site 2 (Suppl. Fig. S1). Site 1 located in the Movar cove more under the effect of open sea, with no apparent anthropogenic influence besides fish farming. Site 2 is situated in the Mali Ston Bay, under the freshwater influence of Neretva river, coastal residential areas, touristic and fish and bivalve farming activities. Sampling was conducted seasonally (in February for winter, early June for spring, early September for summer, and in November for autumn). A total of 16 seawater samples and 16 surface sediment samples were collected. Surface sediment are shallowly spread on the rocky bottom, and samples were collected by diver (depth 20 m). Granulometric analysis of surface sediments characterized sediments of these locations as predominantly biogenic carbonate clasts. Textural characteristics were determined according to Folk (1954): mostly gravelly sand at Site 1, and sandy gravel at Site 2, with muddy component present at the aquaculture locations (Kolda et al., 2020). Water samples consisted of 1L composite samples that were prepared from water collected from four depths (0 m, 5 m, 10 m, bottom) by a Niskin sampler. Physico-chemical characteristics measured *in situ* were: transparency, salinity, dissolved oxygen, oxygen saturation, temperature, turbidity, pH, total dissolved solids. Total nitrogen, total phosphorus and silicon dioxide were measured in the laboratory (Kolda et al., 2020). Seawater was filtrated through 0.2 μm pore filters (Whatman, Sigma Aldrich, UK), frozen in liquid nitrogen during transportation and stored at −20 °C in laboratory. Surface sediment samples were stored at −20 °C.

2.2. DNA extraction and amplicon sequencing

Total DNA was extracted from filters and sediment samples by using DNeasy PowerSoil kit (Qiagen, Germany) on both sample types, following the manufacturer's instructions with minor changes: mechanical disruption on Vortex-Genie 2 (MoBio, USA) for 15 min at maximum speed and incubation at 37 °C for 30 min with the addition of 2 μL of lysozyme (0.5 mg mL^{-1} solution). Extracted DNA yield and quality were measured by spectrophotometry (BioSpec Nano, Shimadzu, Japan), while integrity of DNA was checked on 1% agarose gel. Samples of total extracted DNA were sent for 16S rRNA gene library preparation and 16S rRNA high-throughput amplicon sequencing to Molecular Research LP (Shallowater, Texas, USA). Sequencing was performed on the Illumina MiSeq (Illumina, Chesterfold, UK) platform using pair-end approach (2 × 250 bp), following the manufacturer's guidelines (MR DNA; www.mrdnalab.com, Shallowater, Texas, USA). The 16S rRNA gene V1-V3 variable region was targeted by PCR primers 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′), with barcode on forward primer. The PCR program included a 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. PCR products were visualized on 2% agarose gel to check the success of amplification and a relative intensity of bands.

2.3. Sequence analysis

Before processing, raw sequences were checked using FastQC tool (Andrews, 2010). Reads were processed using QIIME 2 2019.10, in pipeline that included several steps: importing and demultiplexing of raw sequence data, quality filtering and denoising using DADA2 plugin (Challahan et al., 2016) designed to correct Illumina-sequenced amplicon errors. Taxonomy assignment of resulting amplicon sequencing variants (ASVs) was obtained by using Naïve Bayes classifier pretrained on the SILVA 132 database 99% OTUs identity threshold. From the total bacterial community, taxa filtering was performed to include only bacterial ASVs, excluding Archaea, chloroplast, mitochondrial and unassigned sequences from the data set. Phylogenetic tree is generated

as a support for phylogenetic diversity metrics used in q2-diversity plugin. Raw sequences reads are deposited in European Nucleotide Archive (ENA) under project number PRJEB34935.

3. Statistical analysis

3.1. Diversity indices and composition

Alpha and beta diversity of bacterial community was analyzed in QIIME2 (version 2019.10) using q2-diversity plugin, including: Pielou evenness, Abundance-based Coverage Estimator (ACE), Chao1, Margalef, Faith PD, Simpson index and alpha rarefaction curve, and plotted in Excel 2013. Community richness – Shannon (alpha diversity), Unweighted UniFrac Principal Coordinate Analysis – PCoA (beta diversity) and taxonomic composition were furthermore analyzed in RStudio environment (version 3.6.2), using qiime2R (Bisanz, 2018), phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2016) packages. Box and Whisker plots of potential fish pathogens in the rare taxa relative abundance were performed in Grapher™ version 8.2.460 (Golden Software, LLC, Colorado, USA). Statistical outputs of diversity indices defined strategy for downstream analysis, i.e., for seawater samples parameter "season" was applied, and for the sediments samples the spatial parameter (aquaculture vs. control location).

3.2. Organism-level microbiome phenotypes analysis

Proportion of 9 categories of microbial phenotypes within microbiomes was determined: oxygen utilization (anaerobic, anaerobic and facultatively anaerobic bacteria), Gram staining, biofilm formation, oxidative stress tolerance, mobile elements and pathogenic potential. OTU table was made in QIIME2 using VSEARCH (Rognes et al., 2016) to cluster the DADA2-denoised reads into OTUs, and picked with Greengenes database version 13_8 at 99% OTUs identity threshold. QIIME2 artefact was transformed into BIOM format file version 1.0, JSON (McDonald et al., 2012). Generated OTU table with the mapping file was uploaded into web-based version of BugBase tool (Ward et al., 2017) using default threshold. Samples were plotted according to the season (seawater samples) and the location (sediment samples). Phenotype determination plots were generated using the results of Kruskal–Wallis test and pairwise Mann-Whitney-Wilcoxon test with False Discovery Rate (FDR) p-values.

3.3. Functional annotation of prokaryotic taxa

Ecologically relevant function prediction was made using FAPROTAX database (Louca et al., 2016). Python script collapse_table.py was combined with OTU table (Greengenes 99% identity threshold) made in BIOM JSON format version 2.1.4. Generated functional table was further analyzed in RStudio according to the season (seawater) and the site location (sediment), using packages reshape2 (Wickham, 2007), ggpubr (Kassambara, 2014) and ggplot2 (Wickham, 2016).

4. Results

4.1. Alpha and beta diversity of bacterial community in seawater and sediment

High-throughput sequencing of 32 samples yielded 784,258 quality sequences, featuring 8,836 ASVs at the 99% similarity level. Mean frequency per sample was 24,508 (min. 12,259; max. 39,639). Alpha diversity analysis of Shannon index values was plotted to make a comparison of seawater and sediment samples, according to the location and the site through four seasons (Fig. 1). Species richness was higher in sediments vs. seawater samples (Kruskal-Wallis group p-value = 0.000002). Comparisons of alpha diversity for control and aquaculture locations, both for seawater and sediment, were not statistically different (data not shown). In sediments, richness was not affected by the season, although the lowest value for community richness was 7.14 in summer at the Site 2 aquaculture location, and the highest in the winter 9.09 at the Site 1 aquaculture location. The seawater column displayed seasonal variation in community richness, with the highest values in autumn (7.77), and the lowest in the winter (4.01) (Fig. 1). Kruskal-Wallis pairwise values for summer-winter, autumn-spring and spring-summer groups were p-value = 0.15; spring-winter group p-value = 0.04, for autumn-winter group p-value = 0.02. Other alpha diversity indices showed similar differences comparing water column and sediment samples (S2). The rarefaction curves showed sufficient saturation of richness (Shannon) for all samples (S3).

Beta diversity plot (Unweighted UniFrac) however, showed differentiation of sediment samples according to the location (control and aquaculture), but did not separate central and southern Adriatic (Site 1 and Site 2, respectively). The seawater column again displayed tendency for seasonal clustering, with closer groupings of spring and autumn samples, and winter and summer samples (Fig. 2).

4.2. Taxonomic composition of bacterial communities in surface sediments

The taxonomic analysis, where the non-rarefied data according to McMurdie and Holmes (2014) were used, revealed 53 genera, in addition to uncultured bacteria groups (Fig. 3A). Different taxa formed bacterial assemblages at aquaculture and control location at both investigated sites. Out of 10 most abundant taxa at aquaculture location, *Sufurovum* was the most abundant (2.54–41.29% relative abundance), followed by *Eudoraea* (1.26–11.54%), *Maribacter* (1.05–7.43%), Sva0081 sediment group (1.31–6.11%), *Spirochaeta* 2 (1.58–4.29%), *Lutimonas* (1.96–4.88%), *Arcticiflavibacter* (1.06–2.78%) and *Aquibacter* (1.30–2.87%). Uncultured bacteria were also highly represented with families Anaerolineaceae, Desulfobulbaceae, Marinifilaceae, Saprospiraceae, Sandaracinaceae, Syntrophobacteraceae, Microtrichaceae, Thiotrichaceae, Flavobacteriaceae and Ruminococcaceae (1.03–6.97%), uncultured gamma proteobacterium – Bacteroidetes BD2-2 (1.22–2.86%) and uncultured Bacteroidetes – Bac22 (1.27–2.50%).

Control surface sediments contained the highest relative abundance of genus *Eudoraea* (1.17–17.23%), followed by *Zeaxanthinibacter* (2.76–9.86%), *Woeseia* (1.36–5.67%), *Rubripirellula* (1.48–5.01%), Sva0096 marine group (1.26–3.48%), *Bythopirellula* (1.09–1.98%), *Arcobacter* (1.56–4.04%), *Anderseniella* (1.00–2.55%) and *Actibacter* (1.14–4.44%). Additionally, very high percentage contained genera under 1% or the rare taxa (min. 29.57%, max. 48.82%).

4.3. Taxonomic composition of bacterial communities in seawater

Distinctive bacterial communities were formed seasonally in seawater column samples, with a total of 68 genera and several uncultured groups (Fig. 3B). Samples from winter and summer group shared several most abundant taxa. They were represented with high relative abundance of *Alcanivorax* (2.16–38.59%; 1.70–16.16%), *Halomonas* (6.07–7.20%; 2.53–23.39%), *Pelagibacterium* (2.20–5.14%; 1.35–10.50%), *Phenylobacterium* (11.71–26.52%; 2.27–15.92%), *Pseudomonas* (3.86–11.20%; 5.63–13.73%) and *Vibrio* (1.54–3.63%; 1.55–6.76%). Other representative genera in winter were *Psychrobacter* (4.96–23.40%), *Cobertia* (1.92–13.81%), *Altererythrobacter* (1.30–3.83%) and *Pseudoalteromonas* (1.23–2.64%). Summer-enriched genera were *Muricauda* (8.87%), *Erythrobacter* (1.04–4.58%), *Dokdonia* (1.56–9.12%) and *Bacillus* (1.28–21.09%).

Likewise, spring and autumn samples shared SAR11-Clade Ia (5.61–14.85%; 10.70–24.27%), NS4 marine group (4.35–10.22; 2.33–4.98%), NS5 marine group (2.33–5.88; 4.77–10.63%), *Saggitula* (23.62%; 13.63%) and strain of *Synechococcus* CC9902 (1.25–5.93%; 2.68–3.78%). Other genera characterizing spring samples were OM60(NOR5) (1.55–3.48%), *Litoricola* (4.78–10.66%), *Coraliomargarita*

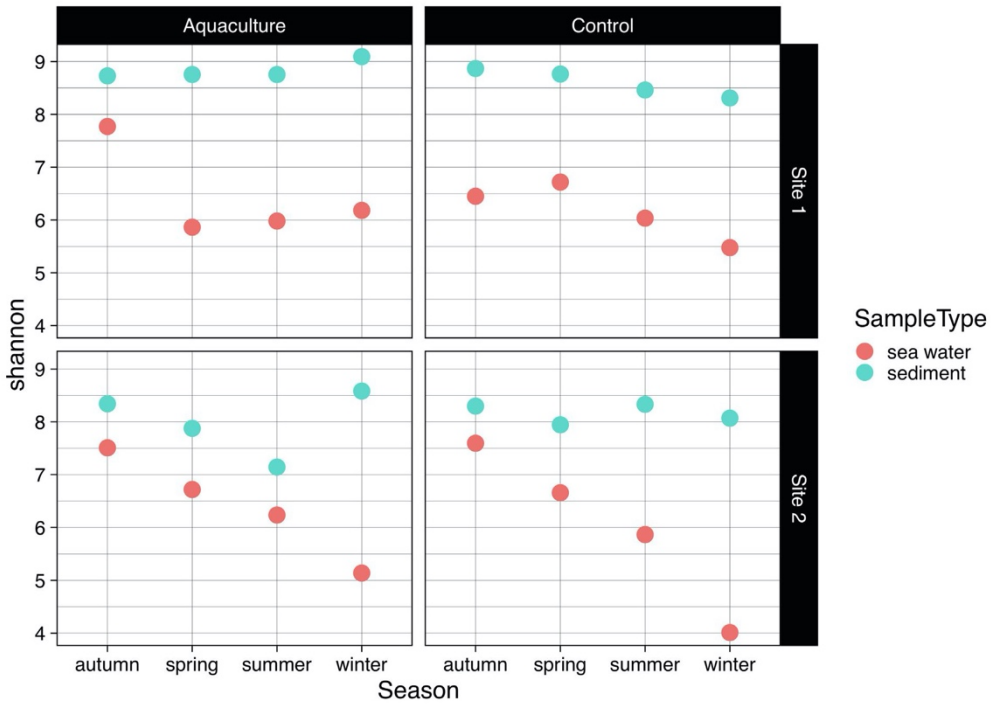

Fig. 1. Alpha diversity analysis measured using Shannon index of surface sediment and seawater samples according to the location types and the sites through seasons.

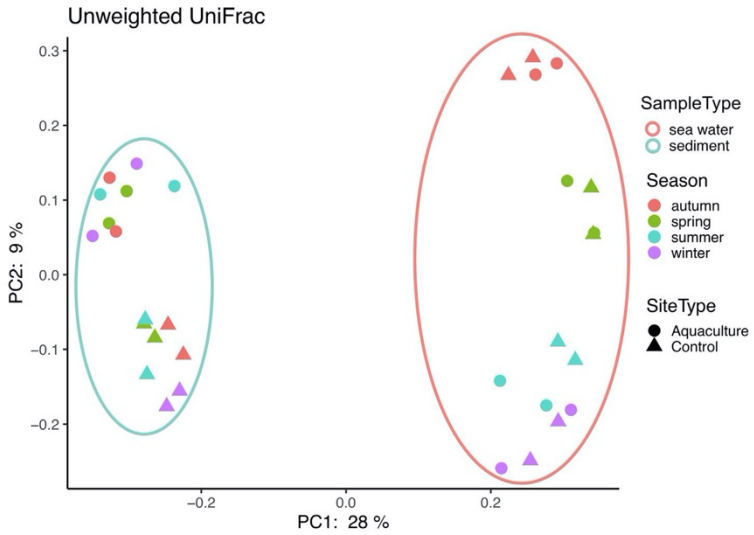

Fig. 2. Principal coordinate analysis (PCoA) of beta diversity analysis based on unweighted Unifrac index, of surface sediment and seawater samples according to the location and the sites through seasons.

(1.15–5.51%), candidatus Puniceispirillum (1.31–2.85%) and uncultured group containing SAR116, Cryptomorphaceae and Flavobacteriaceae families (1.03–2.36%). Additional genera abundant in autumn samples were strain of *Prochlorococcus* MIT9313 (1.46–4.59%), *Glaciecola* (14.85%), SAR11-Clade Ib (2.42–6.15%), Candidatus Actinomarina (2.57–2.92%) and uncultured genera of Cryomorphaceae and Rhodobacteriaceae familes (1.23–8.85%). Seawater samples also contained a high percentage of rare taxa (min.

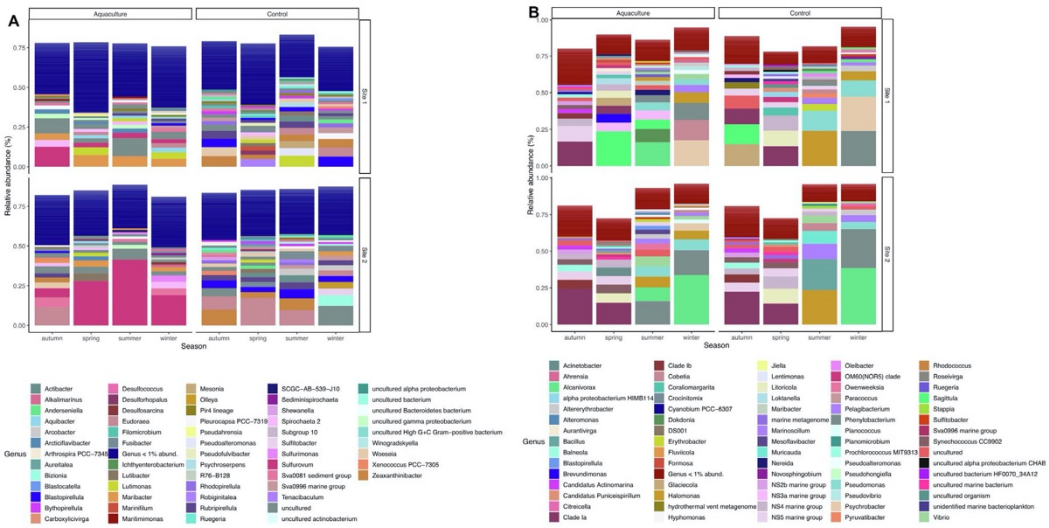

Fig. 3. Taxonomical structure of bacterial assemblages over 1% relative abundance, V1-V3 hypervariable region of 16S rRNA at genus level in: (A) surface sediment; (B) in seawater column.

9.38%, max. 27.69%).

4.4. Rare taxa and conditionally rare taxa harboring potential fish pathogens

Rare taxa were analyzed in the search for known fish pathogens in the sediment and in the water column. Out of 7 genera selected by Martins et al. (2018), 5 genera were detected in analyzed samples: *Vibrio*, *Pseudomonas*, *Photobacterium*, *Tenacibaculum* and *Mycobacterium*. Sediment samples were separated according to aquaculture and control sites, as per diversity outputs, in order to discern whether the farming process was main pathogen generator. A relative abundance of pathogens from sediment are presented in Fig. 4(A). Potential pathogen genera are below 1% abundance in samples from the aquaculture sites (max. values: *Vibrio* = 0.30%; *Pseudomonas* = 0.05%; *Tenacibaculum* = 0.09%), with *Mycobacterium* and *Photobacterium* absent from the aquaculture samples. Control samples harbored representatives from all pathogen genera observed in the study, with *Tenacibaculum* up to 4.25% relative abundance, crossing into conditionally rare taxa category. Other genera are still below 1% (max. values: *Vibrio* = 0.37%; *Pseudomonas* = 0.09%; *Photobacterium* = 0.08%, *Mycobacterium* = 0.05%). The relative abundance of pathogenic genera in seawater was presented according to the season (as per diversity outputs) in order to detect season/seasons were pathogens are the most abundant, and are showed in Fig. 4(B). Vibrio and *Pseudomonas* transition into conditionally rare taxa during winter and summer, as shown in Fig. 3(B), but during the spring and autumn *Vibrio* was absent from the samples. Pseudomonas maximum relative abundance during these months was 0.04% in spring and 0.27% in autumn, respectively. *Photobacterium* was recorded in the summer (0.02%),

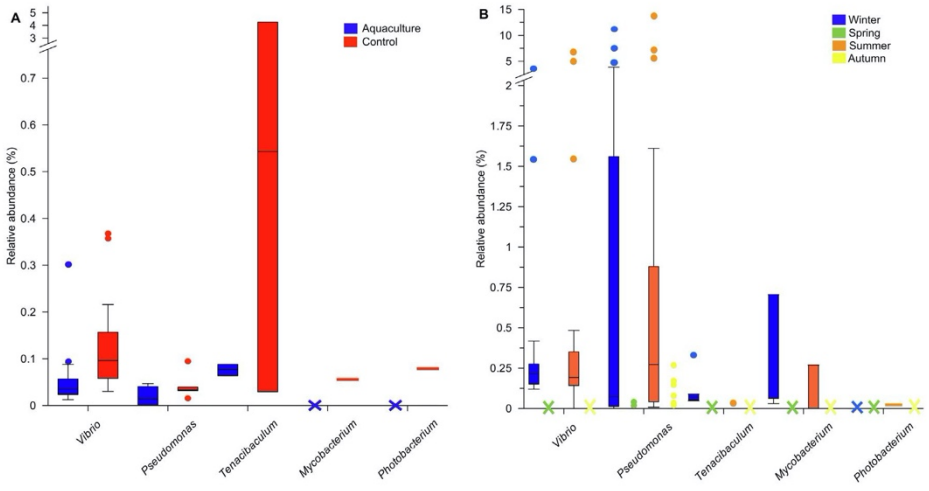

Fig. 4. Boxplots illustrating potential aquaculture pathogens in rare taxa from: (A) sediment and (B) seawater column.

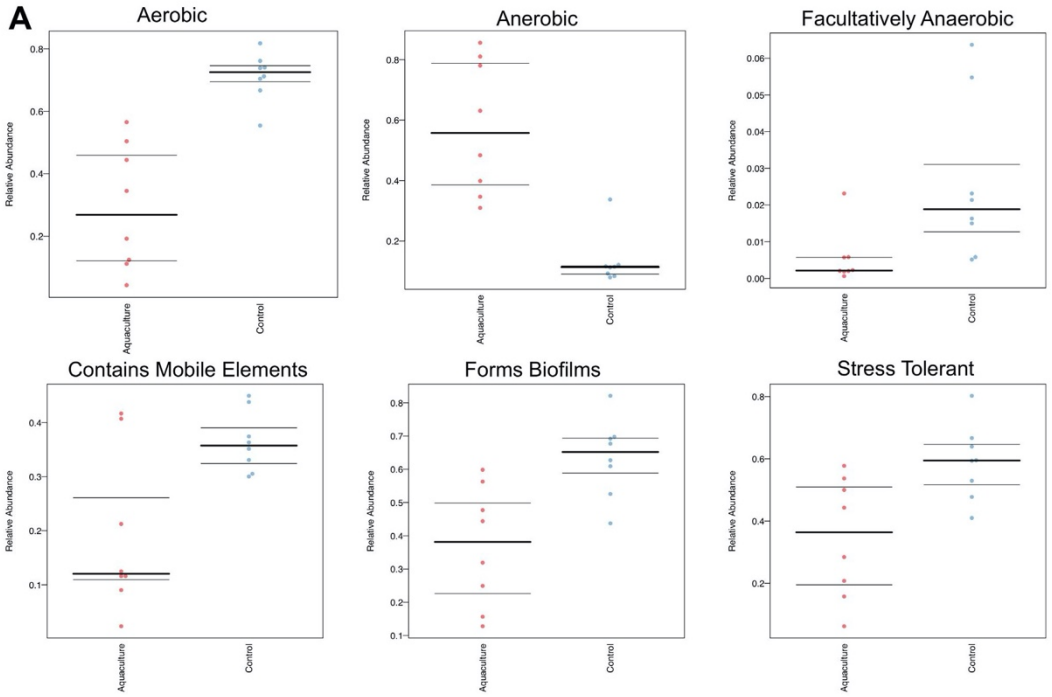

Fig. 5. Relative abundance (%) at organism-level of statistically significant microbial phenotypes: (A) from sediment according to the location and (B) from seawater according to the season.

while *Tenacibaculum* and *Mycobacterium* were present in the summer (max. 0.03%; 0.27%) and winter (max. 0.33%; 0.71%).

4.5. Phenotypic characterization of bacterial community in seawater and sediment

The results of microbial phenotypes i.e. organism metabolic characteristics analyses are presented on Fig. 5, while the statistical results are presented in Suppl. Tab. (S4, S5, S6). For sediment, diversity outputs were followed in order to analyze phenotypic differences between aquaculture and control bacterial communities. However, other parameters were checked, but phenotypes from sediment did not significantly vary regarding seasons and sites (Site 1 and Site 2 – central and southern Adriatic). Statistically significant differences were found at aquaculture vs. control locations at both investigated sites, in regard to oxygen utilization in the sediment samples. Specifically, higher relative abundance of anaerobic taxa was recorded in samples from aquaculture in comparison with control samples (median value 55.7% and 11.3%, respectively). The control had a significantly higher abundance of stress tolerant (med. 59.4%), biofilm forming (med. 65.2%) and mobile elements containing taxa (med. 35.7%). Relative abundance of taxa for Gram staining and potential pathogens phenotypes were not statistically different at aquaculture vs. control location (S4).

In the sea water samples, special variations in phenotypes regarding sites and locations were not determined. However, seasonal differences in phenotypes were observed (S4, Suppl. Tab. 2), as per diversity outputs. Spring and autumn samples showed statistically higher relative abundance of anaerobic taxa (med. value 10.0% and 5.3%, respectively). Winter and summer have significantly higher relative abundance of stress tolerant (med. 46.25%; 54.45%) and potentially pathogenic taxa (med. 36.25%; 39.7%). Significant difference between seasons regarding Gram staining phenotype was not found.

4.6. Ecological functional profiling of bacterial communities in sediment and water

Functional profiling of bacterial communities in water column and sediment samples is presented in Fig. 6. Out of 90 functional groups, 59 groups were represented with at least one record. Out of 1441 records, 44.62% were assigned to at least one group, and remaining 55.38% records could not be assigned to any group. The most represented functional groups in both, water column and sediment, were chemoheterotrophs and aerobic chemoheterotrophs (Fig. 6), although they were slightly more dominant in the water column (median 23.48%;23.52%) than in surface sediments (median 20.42%;22.91%).

Ecological functional groups from all sediment samples are shown in Fig. 6(A). Functional distinction was evident between aquaculture and control. In the aquaculture locations, dominant functional groups were related to sulfate respiration (min. 11.68%, max. 35.28%) and respiration of sulfur compounds (min.12.81%, max.35.28%) versus control (1.59–14.69%; 1.98–14.69%).

Water column samples clustered functionally into 2 groups: spring and autumn, and summer and winter in Fig. 6B. Spring and autumn group had dominant groups representing phototrophy, photoautorotphy, oxygenic photoautotrophy (up to 12.70%) and cyanobacteria (up to 11.51%). Additional high abundance ecological group was “intracellular parasites” with up to 34,14% relative abundance. Summer-winter group is characterized by respiration, reduction and denitrification of nitrogen compounds (nitrate, nitrite) (min. 0.10%, max. 4.05%). Other significant processes involve hydrocarbon degradation (min. 1.78%, max. 11.9%) and fermentation (min. 0.24%, max. 2.33%). During these seasons, human pathogens and animal

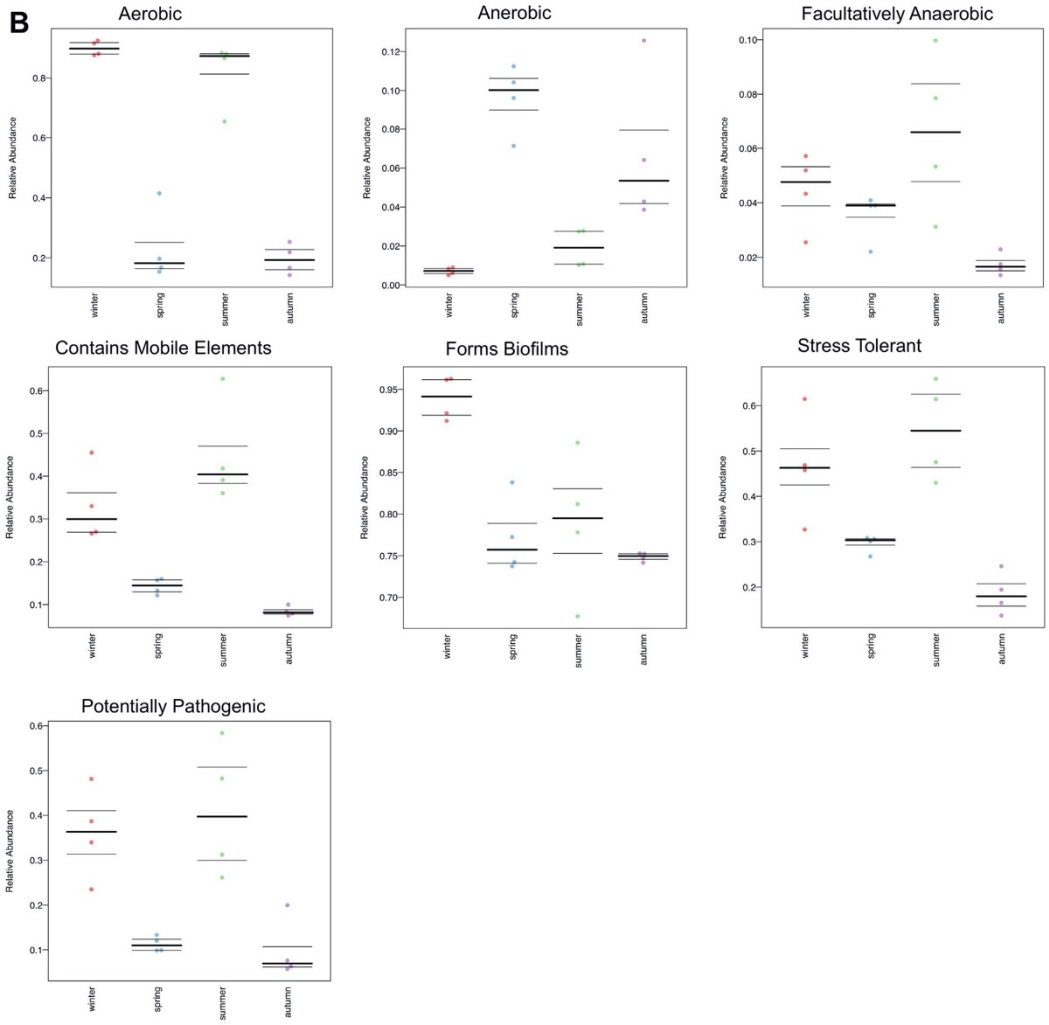

Fig. 5. (*continued*)

parasites/symbionts were detected in higher abundances (min. 0.08%, max. 3.77% and min. 0.23%, max. 3.77%, respectively).

Detected taxa from health related functional groups are shown in Table 1. They are categorized into: human pathogens (pneumonia, nosocomia, all), fish parasites, human and mammal gut, animal parasites/symbionts, plant pathogens, predatory or exoparasitic and intracellular parasites. Taxa relating to human health were pathogenic bacteria *Acinetobacter lwoffi*, *A. johnsonii*, *Clostridium perfringens*, *Pseudomonas mendocina* and *P. stutzeri*. Fish pathogens *Vibrio ichthyoentery* and *V. harveyi* were detected (Table 1).

5. Discussion

The majority of studies in aquatic microbial ecology cover partially the aquaculture ecosystem, either the water column (Martins et al., 2018; Duarte et al., 2019) or the benthic sediment (Tamminen et al., 2011; Verhoevern et al., 2019), while the studies using a holistic approach are even more scarce (Sun et al., 2019). Apart from investigations of sea bass farms in the Atlantic (Pimentel et al., 2017; Martins et al., 2018; Rosado et al., 2019; Duarte et al., 2019), publications regarding European sea bass aquaculture microbiome are scarce. So far, to our knowledge, benthic sediments associated with sea bass cage aquaculture in the Mediterranean have been covered only by Maldonado et al. (2005) and Ape et al. (2019). By analyzing a wide range of data derived from 16S rRNA high-throughput amplicon sequencing and implementing microbial ecology tools, the goal of this study was to characterize microbial assemblages using comprehensive approach.

Diversity statistics outputs defined how to manage other analysis of bacterial assemblages. Specifically, diversity results of the seawater analysis showed seasonality, while the results of sediment samples showed spatial differences (aquaculture vs. control locations). In contrast to current protocols in aquaculture management to analyze physico-chemical parameters, the approach used in this study was to define "who is there", i.e. who are the members of the present bacterial community, in order to predict "what they do" (Nemergut et al., 2013).

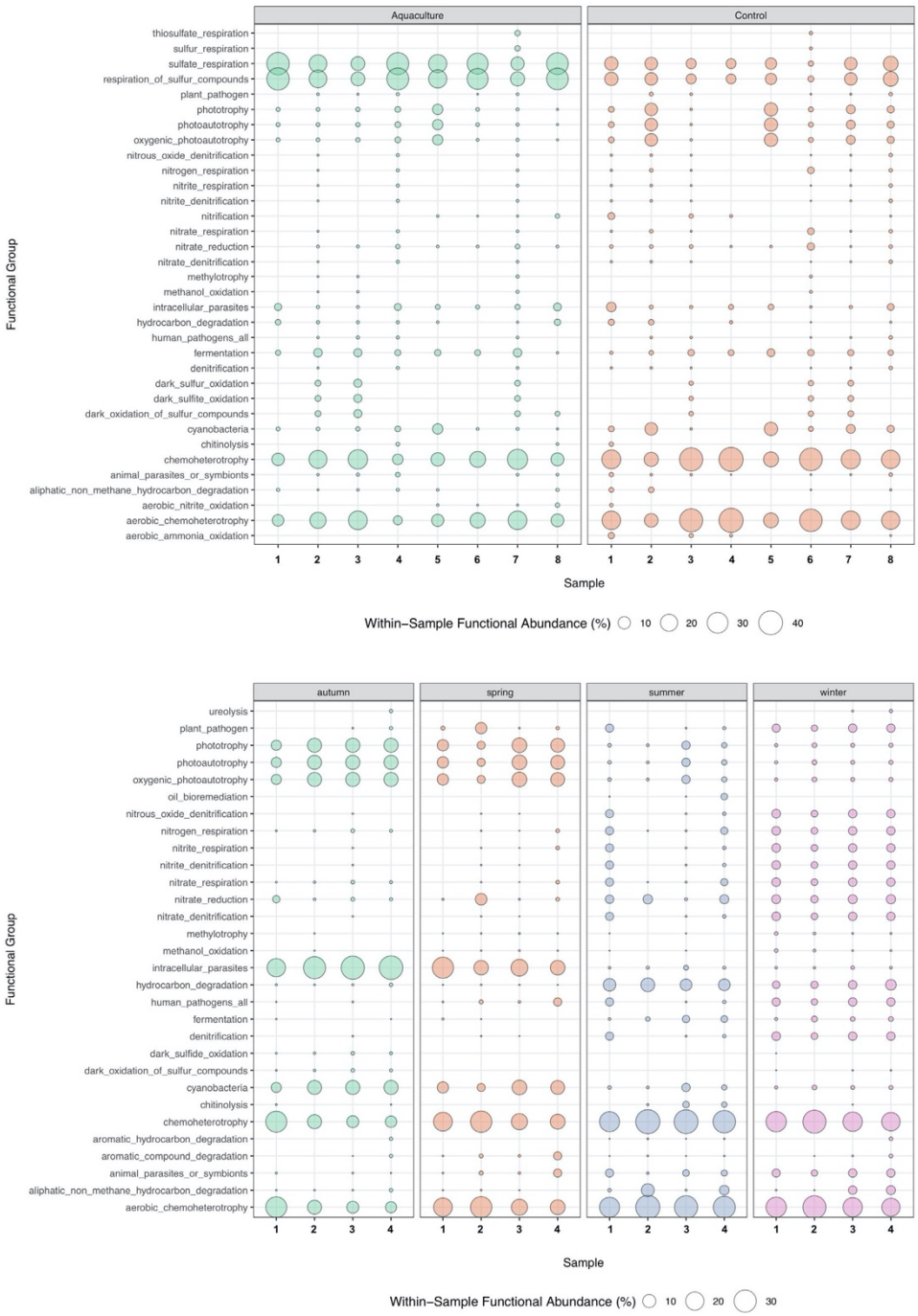

Fig. 6. Bubble plot representing bacterial functional groups from: (A) sediments, according to the location (Aquaculture/Control: 1 – Site 1 autumn, 2 – Site 1 winter, 3 – Site 1 spring, 4 – Site 1 summer, 5 - Site 2 autumn, 6 – Site 2 winter, 7 – Site 2 spring, 8 – Site 2 summer) and (B), according to the season (Autumn/Spring/Summer/Winter: 1 – Site 1 control, 2 – Site 1 aquaculture, 3 – Site 2 control, 4 – Site 2 aquaculture).

Table 1
OTUs identified in health related functional groups.

Health related functional groups	Identified OTUs
human_pathogens_pneumonia	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter lwoffii
human_pathogens_nosocomia	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter; lwoffii
human_pathogens_all	Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium; perfringens
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter; johnsonii
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter; lwoffii
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; mendocina
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; stutzeri
fish_parasites	Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio; ichthyoenteri
human_gut	Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium; perfringens
mammal_gut	Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium; perfringens
animal_parasites_or_symbionts	Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium; perfringens
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter; johnsonii
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter; lwoffii
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; mendocina
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; stutzeri
	Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio; ichthyoenteri
	Proteobacteria; Gammaproteobacteria; Vibrionales; Pseudoalteromonadaceae;Vibrio; harveyi
plant_pathogen	Actinobacteria; Actinobacteria; Actinomycetales; Nocardiaceae; Rhodococcus; fascians
	Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas; diminuta
	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas; stutzeri
predatory_or_exoparasitic	Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bacteriovoracaceae; Bacteriovorax
intracellular_parasites	Proteobacteria; Alphaproteobacteria; Rickettsiales
	Proteobacteria; Alphaproteobacteria; Rickettsiales; AEGEAN_112
	Proteobacteria; Alphaproteobacteria; Rickettsiales; Pelagibacteraceae
	Proteobacteria; Gammaproteobacteria; Legionellales; Coxiellaceae

This could be useful in identifying potential aquaculture bioindicators (Moncada et al., 2019), as these bacterial genera would be informative when establishing aquaculture management strategies. Therefore, the comprehensive research at the genus level of both abundant and rare taxa was performed. Researchers are usually concentrated on the abundant taxa since they are considered “environmental engineers”, directly impacting processes in the environment (Nyirabuhoro et al., 2019). Rare taxa are usually discarded from the analysis, even though this group seems to be more responsive to potential environmental disturbance, activating and increasing their abundance accordingly (Shade et al., 2014; Mo et al., 2018; Nyirabuhoro et al., 2019). Taxonomic profiling alone, although informative, is unable to fully explain differences between the seawater and sediment environment. Moreover, it cannot fully explain variations in the same environment. Nonetheless, in combination with phenotypical and functional profiling, it can contribute to the characterization of aquaculture system. Functional groups can give a more comprehensive information and clearer representation of the environmental conditions that are shaping metabolic niches in bacterial communities (Louca et al., 2016). The importance of considering ecologically functional groups in aquaculture management and microbial ecology has been recognized for some time (Moriarty, 1997).

5.1. Surface sediments: Effect of location on diversity, phenotype and putative function in the bacterial assemblages

Sediment bacterial assemblages were defined by location, forming distinct “aquaculture communities” and “control communities”. Structurally, control communities contain more phototrophic, photoautotrophic and cyanobacterial taxa, pointing to more oxidized sediment. They also have slightly more abundant taxa connected to health-related functional groups. This condition was also supported by rare taxa analysis: control had higher relative abundances of pathogenic genera than aquaculture locations, in juxtaposition to results of other investigations (Vezzulli et al., 2002; Tamminen et al., 2011). In the control sediments, *Tanacibaculum* was occasionally enriched. That is alarming since tenacibaculosis, beside vibriosis, is the most recorded sea bass infection in the Central Mediterranean (Muniesa et al., 2020). Moreover, controls demonstrate a more robust phenotypic profile, recording more taxa that are tolerant to oxidative stress, form biofilms and contain mobile elements. This finding poses important question: is aquaculture production the main source of pathogens in the given area? It appears that other anthropogenic sources, potentially untreated wastewaters (especially sewage wastewaters), could be the source of pathogens. Eastern Adriatic coastal urban areas, like the rest of Mediterranean, release sewage waters untreated or only primary treated (Paliaga et al., 2017). Even though primary treated wastewaters showed limited influence on seawater bacterial assemblages due to dilution and spreading by currents (Paliaga et al., 2017), it may be that they accumulate in sediments and consequently present the potential reservoir of pathogens.

Regarding environmental health, control sediments have higher relative abundance of aerobic and facultatively anaerobic bacteria, while aquaculture sediments are distinctly anaerobic. Sediments largely contain functional groups for respiration of sulfate and sulfur compounds, though doubly more in aquaculture sites. That is worth noting, as it indicates anoxic sediments. Sulfate respiration shift is occurring through the remineralization of decaying organic matter, where sulfate reducing bacteria are reducing sulfate to sulfide, resulting in toxic hydrogen sulfide accumulation (Wasmund et al., 2017; Moncada et al., 2019). In the aquaculture sediments, *Sulfurovum* (Choi et al., 2018) and Sva0081 are significant sulfur-oxidizing chemolitoautotrophs (Liu et al., 2014; Mino et al., 2014). Moreover, *Sufurovum* and *Spirochaeta* 2 are genera identified as bacterial bioindicators connected to various degrees of aquaculture-impacted sediments: *Sufurovum* from low impacted to recently disturbed sites and *Spirochaeta* 2 from a highly impacted sediments in salmon aquaculture (Verhoeven et al., 2018). Organic matter degradation processes are evident in bacterial assemblage: *Eudorea* and *Zeaxanthinibacter* belong to family Flavobacteriaceae, known carbon degraders (Kahng et al., 2010). *Zeaxanthinibacter* is specifically recognized for degrading decaying phytoplankton, and *Maribacter* is effective in organic compounds removal (COD). Enrichment with organic matter in the environment stimulates chemoheterotrophs (Cifuentes and Lindemann, 1993), which are one of the dominant functional groups in both sample types, sediment and sea water column. This indicates specific organic matter loading in the entire coastal environment.

Interestingly, no significant differences in species richness between

aquaculture and control sites was found, as was in the sediments of sea bass farm and control investigated by Ape et al. (2019). Although it seems intuitive that the aquaculture-impacted site will have decreased richness, it appears that it is not affected by farming practices. This is in concordance with the findings of Tamminen et al. (2011) and Moncada et al. (2019). One explanation could be the nature of sediment's environmental compartment, which is characterized by high alpha diversity in general (Lozupone and Knight, 2008; Nemergut et al., 2013).

5.2. Seawater column: Seasonal impact on diversity, phenotype and putative function in the bacterial assemblage

In the seawater column, seasonality had crucial impact on bacterial assemblages, also observed at sea bass farms in Spain (Maldonado et al., 2005) and Portugal (Martins et al., 2018). Seasonal pattern of the physico-chemical parameters in the water column has been observed in previously published study (Kolda et al., 2020). In terms of microbial ecology, there is no regional scale difference between same habitat type, as suggested by Martiny et al. (2011). Alpha diversity was higher in spring and autumn assemblages, while significantly lower in summer and especially winter. Taxonomic profiling revealed that winter and summer, and spring and autumn bacterial assemblages shared about half of the most abundant taxa. Winter and summer assemblages included functional groups responsible for oil bioremediation, nitrogen/nitrate/nitrite respiration, nitrite/nitrate denitrification and hydrocarbon degradation. That is established in the taxonomic composition with enrichment of *Alcanivorax* and *Halomonas* genera (Ibacache-Quiroga et al., 2018). Spring and autumn held more anaerobic and facultatively anaerobic bacterial assemblages and a higher functional abundance of phototrophic/photoautotrophic metabolism and cyanobacterial taxa. In the community structure, taxa connected to algal derived organic molecules and dissolved organic matter (DOM) is detected. Even though phytoplankton blooms were not observed during sampling, indication of increased phytoplankton concentration is present in the bacterial composition: SAR11 (Eiler et al., 2009), NS4 and NS5 marine group (Díez-Vives et al., 2019; Seo et al., 2017), *Caraliomargarita* (Hattenrath-Lehmann et al., 2019) and *Litoricola* (Wang et al., 2020).

Known pathogenic genera in sea bass aquaculture are found mostly in the rare taxa. However, in summer and winter samples, enrichment with genera *Vibrio* and *Pseudomonas* was observed. Phenotype characterization also demonstrated that winter and summer bacterial assemblages had a more robust phenotypic profile: taxa that are tolerant to oxidative stress, form biofilms and have mobile elements. Moreover, higher relative abundance of known aquaculture pathogens (except *Photobacterium* in the winter samples) were detected in the winter and summer, opposed to other two seasons. Winter and summer communities also have a high functional abundance of intracellular parasites, even when pathogens were detected in very low abundances. In spring of 2017 at Site 2, an outbreak of vibriosis caused by *Vibrio* (*Listonella*) *anguillarum* was reported (Kapetanović et al., 2019). By sampling conducted in June, *Vibrio* was not detected in the bacterial community in the water or in the sediment, but high *Vibrio* abundances were detected in the previous, winter sampling (February), referring to the possible outbreak of disease. This time lag between *Vibrio* detected in the winter sampling and the outbreak of the disease in the springtime, was an essential period during which precautions and additional care for fish could have been administrated (e.g. probiotics and other immunostimulants, avoidance of stressful conditions, etc.), especially in period when the water temperature starts to rise. On the other hand, summer samples (September 2017) contained a large abundance of *Vibrio*, *Pseudomonas* and other potential pathogens (Fig. 4b), but there was no disease outbreak. Possible explanation may be the high percentage of the genus *Bacillus* (1.28–21.09%, Fig. 3), which is reported to have probiotic proprieties (Chu et al., 2015; Bentzon-Tilia et al., 2016). This was also confirmed in a recent study by Sun et al. (2019).

5.3. Sediment and seawater microbial ecology: Point of reference for microbial health management in aquaculture

Bacterial composition was significantly different in the seawater vs. sediment, probably due to selection driven by the specific environmental conditions in those habitats (Nyirabuhoro et al. 2019). That finding corresponds with the results of previous investigations (Moncada et al., 2019; Sun et al., 2019, 2020). The seawater column had lower number of genera under 1%, which points to high diversity and bacterial function in sediment (Fig. 1, S2, Fig. 3). This condition was probably caused by a higher concentration of nutrients and more substrate present, especially at aquaculture locations (Verhoeven et al., 2018). Another hypothesis could be that many of the rare taxa recorded are dormant species, and as such invisible to selection of abiotic and biotic pressure (Nyirabuhoro et al., 2019). Dormancy could be a major cause for high abundances of rare taxa in the sediment, while in seawater it could be due to passive dispersal via air and wind (Nemergut et al., 2013). Additionally, rare taxa in coastal waters are less responsive to environmental conditions than abundant taxa, which makes them sensitive to stochastic processes, namely to drift (Nemergut et al., 2013; Mo et al., 2018; Liu et al., 2019). It is uncertain how much of bacterial community members are actually active at the time of the sampling, and according to some findings (Nemergut et al., 2013), it could be less than 10%. However, pathogens from rare taxa have increased reacting to changes in the environment, as shown for *Tenacibaculum* in sediment and *Vibrio* and *Pseudomonas* in seawater column. In that regard, even though some of the rare taxa could be dormant at the time, they represent a "reservoir of genetic diversity" (Nemergut et al., 2013) and consequently, significant functional potential for the ecosystem. Therefore, the role of 16S rRNA amplicon data giving us insight in the functional potential of the entire bacterial assemblage should not be disregarded.

Returning to the priorities for aquaculture microbial management, water habitat seems to contain a higher percentage of taxa connected to health-related functional groups. Known human and animal pathogens are presented in the analysis of the rare taxa, phenotyping and functional group analyses. Furthermore, potential pathogens could be more prevalent outside of the actual fish farm, as shown in sediment results. Control locations therefore suggest strong surrounding impact on the aquaculture environment. An investigation by Martins et al. (2018) demonstrated a similar discovery: a month before the actual disease outbreaking at the sea bass farm, they evidenced a rise of *Vibrio* OTU-s and a decrease of bacteria antagonistic to pathogens in the estuarine waters outside the fish farm.

In this study, a several aquaculture biomarkers important for environmental impact were identified: *Blastopirullela*, Sva0081, *Suflurovum*, Bacteroidetes BD2-2, *Spirochaeta* 2, Anaerolineaceae, Marinimicrobia (SAR406), as well as human and fish potential pathogens: *Vibrio ichtyoentery*, *V. harvey*, *Acinetobacter lwoffi*, *A. johnsonii*, *Clostridium perfringens*, *Photobacterium*, *Tanacibaculum* etc. Chemoheterotrophy has emerged as the dominant functional group in both environments (sediment and seawater). That may seem irrelevant, as aerobic chemoheterotrophs are reported to be the most diverse group on the OTU and genus level in the study of global oceans (Louca et al., 2016). However, in the aquaculture systems, chemoheterotrophic bacteria are evidence of nutrient rich environment, as they are essential for degradation of organic compounds, and in the bioremediation of water and sedimentary processes (Kämpfer et al., 1993; Konhauser and Gingras, 2007).

6. Conclusion

Bacterial composition associated with sea bass cage aquaculture was significantly different in the seawater vs. sediment, probably due to selection driven by the specific environmental conditions in those habitats. The seawater column had lower number of rare taxa, which

points to high diversity of bacterial functions in the sediment. Diversity of the microbial community in seawater showed seasonality, while the results of sediment samples showed spatial differences (aquaculture vs. control). Regarding oxygen utilization, control sediments have higher relative abundance of aerobic and facultative anaerobic bacteria, while aquaculture sediments are distinctly anaerobic. In sediments, no significant differences in species richness were indicated between aquaculture and control, and it appears that it is not affected by farming practices. Regarding priorities for aquaculture microbial management, analyses showed that potential pathogens could be more prevalent outside of the actual fish farm. Control locations therefore suggest strong surrounding's impact on the aquaculture environment. Water habitat appears to contain a higher percentage of potential health-impacting taxa. Composition and functional group analysis revealed important biomarkers for the aquaculture environment: *Blastopirullela*, Sva0081, *Suflurovum*, Bacteroidetes BD2-2, *Spirochaeta* 2, Anaerolineaceae, Marinimicrobia (SAR406), as well as human and fish potential pathogens: *Vibrio ichtyoentery*, *V. harvey*, *Acinetobacter lwoffi*, *A. johnsonii*, *Clostridium perfringens*, *Photobacterium* and *Tanacibaculum*. Furthermore, five genera of rare taxa known as fish pathogens (*Vibrio*, *Pseudomonas*, *Photobacterium*, *Tenacibaculum* and *Mycobacterium*) were detected in the sediment and in the water column. Even though these taxa could be dormant at the time, they stand as reservoir of potential risk for the aquaculture system.

Conclusively, monitoring bacterial assemblages associated with sea bass cage aquaculture in benthic sediments and sea water column can alert to deteriorating environmental health, which in turn impacts farmed animals and human consumers. Using microbial ecology tools, adequately adjusted aquaculture management could manipulate diversity, taxonomic structure and functions, to promote optimal water quality and improve sediment organic load.

CRediT authorship contribution statement

Anamarija Kolda: Investigation, Formal analysis, Writing - original draft. **Ana Gavrilović:** Investigation, Writing - original draft. **Jurica Jug-Dujaković:** Investigation, Writing - original draft. **Zrinka Ljubešić:** Supervision, Writing - review & editing. **Mansour El-Matbouli:** Writing - review & editing, Validation. **Atle Lillehaug:** Writing - review & editing, Validation. **Semir Lončarević:** Writing - review & editing, Validation. **Lorena Perić:** Writing - review & editing, Data curation. **Dražen Kněžević:** Writing - review & editing. **Darija Vukić Lušić:** Writing - review & editing. **Damir Kapetanović:** Funding acquisition, Project administration, Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106785.

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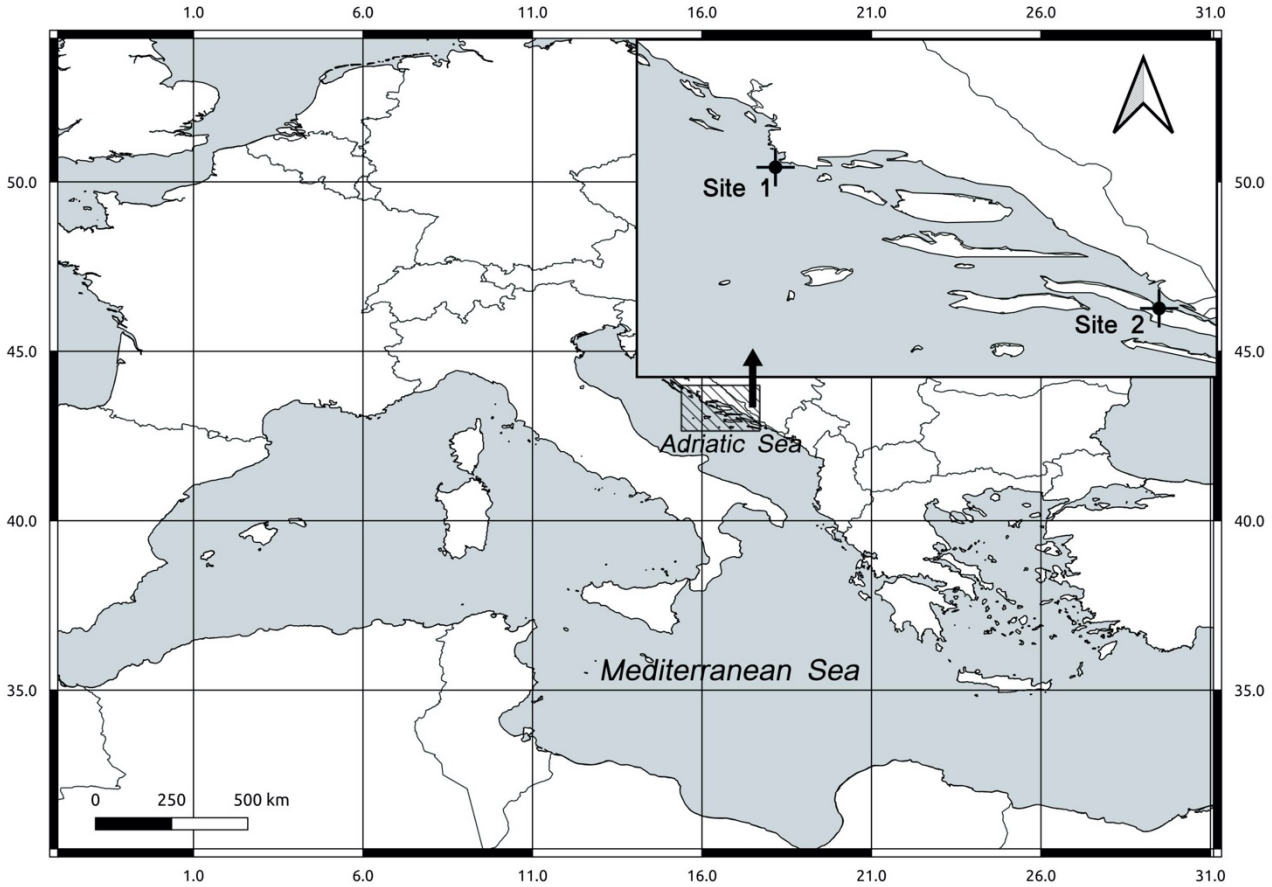
Supplementary Material

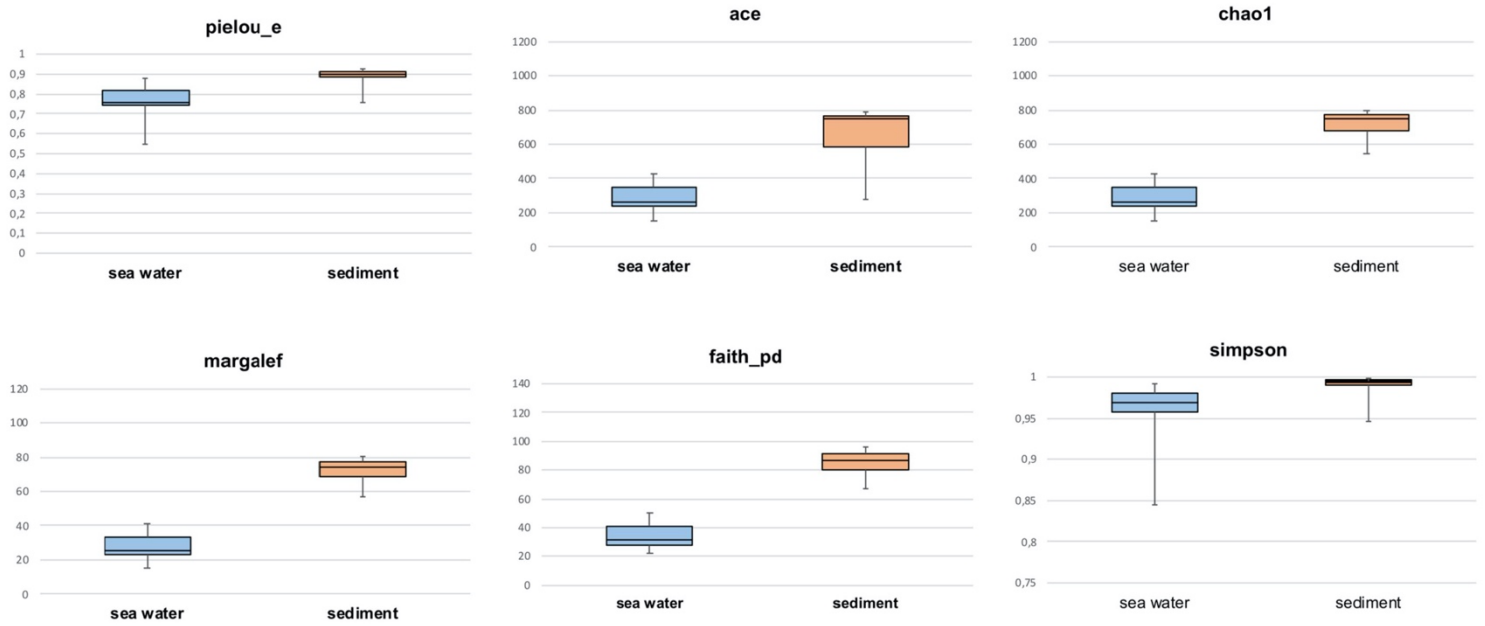
Diversity, phenotype and functional profiling of bacterial assemblages in the marine cage farm environment, with implications on health

Anamarija Kolda*, Ana Gavrilović, Jura Jug-Dujaković, Zrinka Ljubešić, Mansour El-Matbouli, Atle Lillehaug, Semir Lončarević, Lorena Perić, Dražen Knežević, Darija Vukić-Lušić, Damir Kapetanović

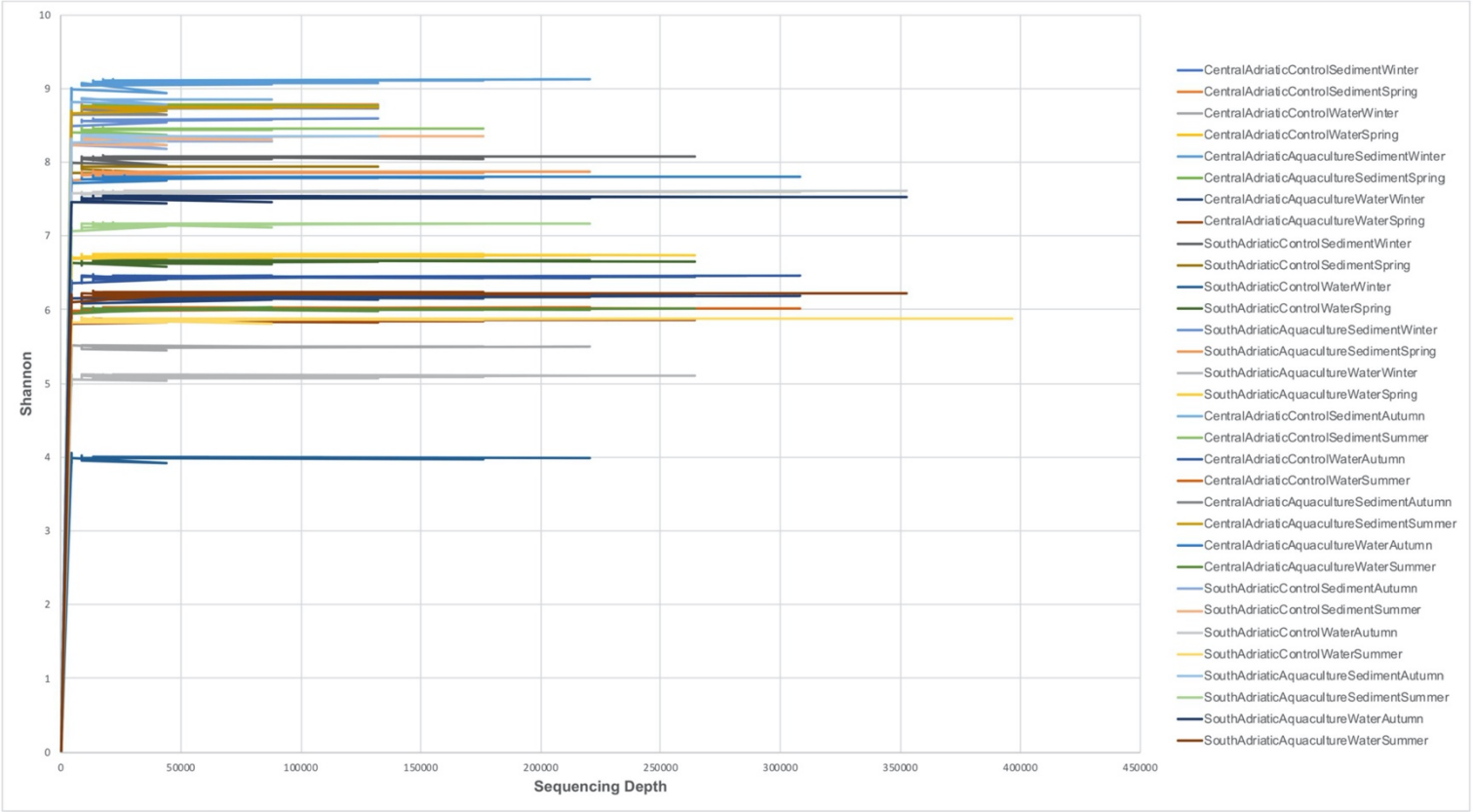
*Corresponding author: Anamarija.Kolda@irb.hr, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia



Supplementary Figure S1.
Map of investigated sites (Site 1: farm location N 43.509141, E 15.96268 and control location N 43.504971, E 15.952208; Site 2: farm location N 42.922510, E 17.474728 and control location N 42.93022, E 17.49925)



Supplementary Figure S2.
Boxplots illustrating alpha diversity indices (Pielou evenness, Abundance-based Coverage Estimator (ACE), Chao1, Margalef, Faith PD and Simpson index) in bacterial assemblages of seawater and sediment samples, at 99% similarity level of ASVs.
Data for the diversity indices were rarefied according to QIIME2 tools, to 392,288 sequences and total of 8,653 features. Sampling depth in core metric analysis (–p-sampling-depth) was 12,259, the minimum number of remaining read counts among all samples.



Supplementary Figure S3
Alpha rarefaction curve illustrating total of 32 samples, constructed on Shannon index
Data for the diversity indices were rarefied according to QIIME2 tools, to 392,288 sequences and total of 8,653 features. Sampling depth in core metric analysis (–p-sampling-depth) was 12,259, the minimum number of remaining read counts among all samples.

Supplementary Table 1. S4 Surface sediment bacterial assemblage phenotype statistics

		Relative abundance with phenotype				
Phenotype	Season	Mean	Median	Standard deviation	Mann-Whitney-Wilcoxon Test p value	FDR-corrected p-value
Aerobic	Aquaculture	0.29	0.27	0.22	0.0003	0.0003
	Control	0.71	0.72	0.08		
Anaerobic	Aquaculture	0.57	0.55	0.00	0.0003	0.0003
	Control	0.13	0.11	0.02		
Facultatively Anaerobic	Aquaculture	0.005	0.002	0.007	0.010	0.010
	Control	0.03	0.02	0.02		
Contains Mobile Elements	Aquaculture	0.18	0.12	0.15	0.038	0.038
	Control	0.36	0.35	0.05		
Forms Biofilms	Aquaculture	0.36	0.38	0.18	0.005	0.005
	Control	0.63	0.65	0.11		
Stress Tolerant	Aquaculture	0.34	0.36	0.19	0.014	0.014
	Control	0.58	0.59	0.12		
Gram Negative	Aquaculture	0.94	0.95	0.05	0.959	0.959
	Control	0.95	0.95	0.02		
Gram Positive	Aquaculture	0.06	0.04	0.05	0.959	0.959
	Control	0.05	0.04	0.02		
Potentially Pathogenic	Aquaculture	0.30	0.29	0.09	0.505	0.505
	Control	0.27	0.25	0.07		

Supplementary Table 2.A. S5 Sea water column bacterial assemblage phenotype statistics seasonally

Proportion with phenotype					
Phenotype	Season	Mean	Median	Standard deviation	Kruskal-Wallis Test group p value
Aerobic	winter	0.90	0.90	0.02	0.008
	spring	0.23	0.18	0.12	
	summer	0.82	0.87	0.11	
	autumn	0.20	0.19	0.05	
Anaerobic	winter	0.01	0.01	0.00	0.005
	spring	0.10	0.10	0.02	
	summer	0.02	0.02	0.01	
	autumn	0.07	0.05	0.04	
Facultatively Anaerobic	winter	0.04	0.05	0.01	0.018
	spring	0.04	0.04	0.01	
	summer	0.07	0.07	0.03	
	autumn	0.02	0.02	0.00	
Contains Mobile Elements	winter	0.33	0.30	0.09	0.004
	spring	0.14	0.14	0.02	
	summer	0.45	0.40	0.12	
	autumn	0.08	0.08	0.01	
Forms Biofilms	winter	0.94	0.94	0.03	0.027
	spring	0.77	0.76	0.05	
	summer	0.79	0.79	0.09	
	autumn	0.75	0.75	0.01	
Stress Tolerant	winter	0.47	0.46	0.12	0.005
	spring	0.30	0.30	0.02	

	summer	0.54	0.54	0.11	
	autumn	0.19	0.18	0.05	
Gram Negative	winter	0.98	0.98	0.01	0.309
	spring	0.98	0.98	0.01	
	summer	0.96	0.97	0.04	
	autumn	0.98	0.98	0.01	
Gram Positive	winter	0.02	0.02	0.01	0.309
	spring	0.02	0.02	0.01	
	summer	0.04	0.03	0.04	
	autumn	0.02	0.02	0.01	
Potentially Pathogenic	winter	0.36	0.36	0.10	0.008
	spring	0.11	0.11	0.02	
	summer	0.41	0.40	0.15	
	autumn	0.10	0.07	0.07	

Supplementary Table 2.B. S6 Sea water column bacterial assemblage phenotype statistics of season groups

Phenotype	Season groups	Pairwise Mann-Whitney-Wilcoxon Test p-values	FDR-corrected pairwise p-values
Aerobic	winter_vs_spring	*	*
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	*	*
	spring_vs_summer	*	*
	spring_vs_autumn	n.s.	n.s.
	summer_vs_autumn	*	*
Anerobic	winter_vs_spring	*	*
	winter_vs_summer	*	*
	winter_vs_autumn	*	*
	spring_vs_summer	*	*
	spring_vs_autumn	n.s.	n.s.
	summer_vs_autumn	*	*
Facultatively Anerobic	winter_vs_spring	n.s.	n.s.
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	*	n.s.
	spring_vs_summer	n.s.	n.s.
	spring_vs_autumn	*	n.s.
	summer_vs_autumn	*	n.s.
Contains Mobile Elements	winter_vs_spring	*	*
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	*	*
	spring_vs_summer	*	*
	spring_vs_autumn	*	*
	summer_vs_autumn	*	*
Forms Biofilms	winter_vs_spring	*	*

	winter_vs_summer	*	*
	winter_vs_autumn	*	*
	spring_vs_summer	n.s.	n.s.
	spring_vs_autumn	n.s.	n.s.
	summer_vs_autumn	n.s.	n.s.
Stress Tolerant	winter_vs_spring	*	*
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	*	*
	spring_vs_summer	*	*
	spring_vs_autumn	*	*
	summer_vs_autumn	*	*
Gram Negative	winter_vs_spring	n.s.	n.s.
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	n.s.	n.s.
	spring_vs_summer	n.s.	n.s.
	spring_vs_autumn	n.s.	n.s.
	summer_vs_autumn	n.s.	n.s.
Gram Positive	winter_vs_spring	n.s.	n.s.
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	n.s.	n.s.
	spring_vs_summer	n.s.	n.s.
	spring_vs_autumn	n.s.	n.s.
	summer_vs_autumn	n.s.	n.s.
Potentially Pathogenic	winter_vs_spring	*	*
	winter_vs_summer	n.s.	n.s.
	winter_vs_autumn	*	*
	spring_vs_summer	*	*
	spring_vs_autumn	n.s.	n.s.
	summer_vs_autumn	*	*

DISCUSSION

Cyanobacterial microbial mats in the intermittent river: Habitat and natural laboratory for studying the extremes

First two publications have answered most of the thesis aims (second, third and fourth): (i) they have identified changes in the structure of cyanobacterial community in the extreme ecosystem affected by the climate change, (ii) described diversity of cyanobacteria in the intermittent river situated in the Adriatic basin, and finally, (iii) applied both molecular (Publication I, II) and observational method (light microscopy, Publication I) for the identification of cyanobacteria. This type of research is additionally significant in the light of the expected rise in numbers of intermittent water bodies worldwide, due to the effects of climate change and anthropogenic interference.

First hypothesis: "C*yanobacteria are exceptionally important organisms in extreme environments, as they change/adapt such environments into habitats for development of other organisms"*, have been confirmed, and first two publication have addressed this in depth – Publication I by explaining the importance of interactions between different trophic groups, including prokaryotes (bacterial community with emphasis on cyanobacteria), eukaryotic component (diatom assemblage) and macroinvertebrates (*Synurella ambulans)* for the ecosystem functioning, while in the Publication II insights are given on the resilience and survival of microbial mat community in this extreme ecosystem with strong seasonal disturbances. Furthermore, the second hypothesis: "*The Adriatic Sea and the Adriatic basin are ideal natural models for studying ecosystems influenced by climate change and anthropogenic pressures*." has also been confirmed, as the study site was the intermittent river Krčić, as part of the Adriatic basin (Krka river sub-basin). The effects on the microbial communities have been reported, as well as the hydrological cycle of the Krčić during 2014 and 2015 (Publication II). Year 2014 was characterized by a rainy summer, without a typical drought period, which was established again in the summer of 2015. These conditions produced distinct bacterial communities in the microbial mat, giving opportunity to witness changes in the environment. Finally, the third hypothesis: "*Contemporary molecular methods using high-throughput sequencing technology, such as metabarcoding, are appropriate tools for cyanobacterial research and for the identification of key taxa for ecosystems*", has been confirmed, as with the combination of 16S rRNA cyanobacteria-specific primers (Publication I), 16S rRNA amplicon sequencing and internal transcribed spacer (ITS) region (Publication II), intense

hydrological changes were detected in shaping (and/or selecting) the microbial mat community of the Krčić.

Cyanobacteria displaying environmental selection in the intermittent river ecosystem

Publication I has approached the importance of microbial mats as a shelter microhabitat during two environmental extremes - high flow and drought periods, and as a feeding place of the amphipod *S. ambulans*. In this publication, for the first time, identification of cyanobacteria forming microbial mats was conducted on one Croatian intermittent karstic spring/river. The Krčić river is specifically selected as a representative of IRES, due to extreme conditions, mostly as a result of intense water regime fluctuations, but also due to low temperature, prolonged desiccation, and high solar irradiance. These extreme conditions encompass: a drought period generated during summer season, strong rains following in the autumn months, freezing ambient temperature during winter period, and higher water levels reached in the spring time as a consequence of snow melt. Two months that have distinct hydrological conditions - February (winter), representing month with high water extreme, and May (spring), representing starting point of drought period, were selected for sampling and consequent analyses. Morphological analysis was conducted on diatoms and cyanobacteria, with additional molecular identification of cyanobacteria using 16S rRNA marker with specific primers designed to target only cyanobacteria (DNA extraction, construction of clone libraries and consequently phylogenetic analysis) and crustacean identification and enumeration. Morphological observations defined Oscillatoriales cyanobacteria as dominant members of the microbial mat, with *Phormidium favosum* found in both winter (high water) and spring (beginning of drought) samples and *Hydrocoleum muscicola* identified as subdominant species. Molecular phylogeny analysis further confirmed Oscillatoriales predominance in microbial mat, but provided additional data. Using these tools *Phormidium autumnale* and genera *Tychonema*, *Pseudoanabena* and *Hydrocoleum* arise as dominant cyanobacteria within microbial mats. Interestingly, in addition to these species *Wilmottia* was identified within the mats, found exclusively in the winter sample characterized by cold and high water. This is considered as a first report of the presence of *Wilmottia* in Croatia. Considering this, *Wilmottia* in the Krčić Spring could be endemic for this area, although a broader investigation on the topic is needed for further conclusions. Phylogenetic tree further suggested clear separation of these two cyanobacterial clusters (7-10% difference): "*Phormidium*" cluster occurring both in winter and spring, and exclusively winter "*Wilmottia*" cluster. Study has clearly showed that pockets of microbial mat, formed in Krčić Spring, serve as an important shelter

for the stygophilic amphipod *S. ambulans* when moss as the primary substratum is not fully developed. Cyanobacteria of order Oscillatoriales seem to be the key for development of microbial mat: they synthesize extracellular polymers serving as a glue for other microorganisms (e.g. diatoms), providing stabile foundation between mat and the substrate and organic source for other heterotrophic and lithotrophic bacteria. The cyanobacteria-diatom mat protects amphipods from drift, usually during the flow activation in autumn or from the Dinara mountain snowmelt in spring.

Results from the Publication I were used as a starting point for the more detailed analysis presented in Publication II. Microbial mats, forming within this extreme ecosystem, were this time studied by sampling conducted throughout the whole year period and by combining two gene regions analysis - 16S rRNA (V1-V3 region) as a universal bacterial marker gene and ITS as a specific cyanobacterial marker gene. This information gave much needed insights into adaptation capability of the microbial mats thriving in this extreme ecosystem. 16S rRNA analysis suggested complex microbial mat community consisted of 11 different bacterial phyla with Cyanobacteria making the community core (>50%), and Proteobacteria (Alphaproteobacteria) emerging as second most dominant phylum. Most abundant cyanobacterial genera included *Microcoleus*, *Phormidium* and uncultured Antarctic cyanobacterium. Other dominant phylum was Proteobacteria (Alphaproteobacteria). ITS marker results grouped majority of cyanobacteria within Cluster 1, encompassing genera *Phormidium*/*Microcoleus*/*Tychonema*/*Oscillatoria*/*Stanieria*, all six being phylogenetically closely related. However, during conditions of low temperatures and strong bora wind (winter 2014) and during drought period (summer 2015), Cyanobacteria were reduced within the community. Under the extreme cold, Planctomycetes and candidate phylum TM6 proliferated. Members of the phyla Firmicutes were strictly found during the drought summer period followed by genera *Cytophagia-Fibrella*, *Polymorphobacter*, *Polaromonas*, and *Massilia*. Nonetheless, during high water inflow following the drought, Cyanobacteria represented 90% of the community in which specific desiccant-tolerant genera (*Chroococcidiopsis*, *Calothrix*, and *Pleurocapsa*) appeared to have mechanisms for quick recolonization.

In order to identify key cyanobacteria in the ecosystem, two markers were used with sometimes opposite results, but giving better insight into the community when combined. For example, potentially endemic genus *Wilmottia* was only detected with ITS marker. ITS analysis suggested the presence of a small population of "*Wilmottia*" cyanobacteria present in both winter and spring months. This genus was previously detected in a winter sample by cyanobacteria-specific 16S rRNA clone libraries (Publication I). To resolve this issue, and with aim to remove other possible

discrepancies, data of the 16S rRNA amplicon sequencing from the Publication II was re-analysed. New pipeline established for Publication III and IV was applied, using QIIME2 platform and new version of SILVA database v. 132 taxonomy assignment at 99% OTU identity threshold (Fig. 1.). Interestingly, the re-analysis suggested that genus *Wilmottia* was abundantly present in all months except pre-drought (Jun-15) and after-drought (Oct-15), when its relative abundance was under 1%. In other months, *Wilmottia* relative abundance ranged between 1.20% in during winter freeze to immense 28.45% during spring snow melt flow. Sequences from the *Wilmottia* genus were found to be closely related to strain *Wilmottia* Ant-Ph58, isolated from pond in Antarctica (Comte et al., 2007). Thus, *Wilmottia* could potentially belong to the puzzling "Uncultured Antarctic cyanobacteria" group from 16S rRNA amplicon analysis in Publication II. Example of *Wilmottia* shows that ITS was a good phylogenetic marker in detecting cyanobacterial members of the community. At the same time, due to limited scope of the SILVA database at the time of 16S rRNA analysis (Publication II), *Wilmottia* sequences could not be more precisely assigned then as "Uncultured Antarctic cyanobacteria". Ribosomal RNA databases such as SILVA are continually quality checking and updating datasets of bacterial rRNA sequences. In that manner, every subsequent analysis is giving new understanding of the data. Obtained results demonstrate rapid change in bioinformatics tools and databases, becoming more sensitive and advanced in helping researchers in better characterization of bacterial communities.

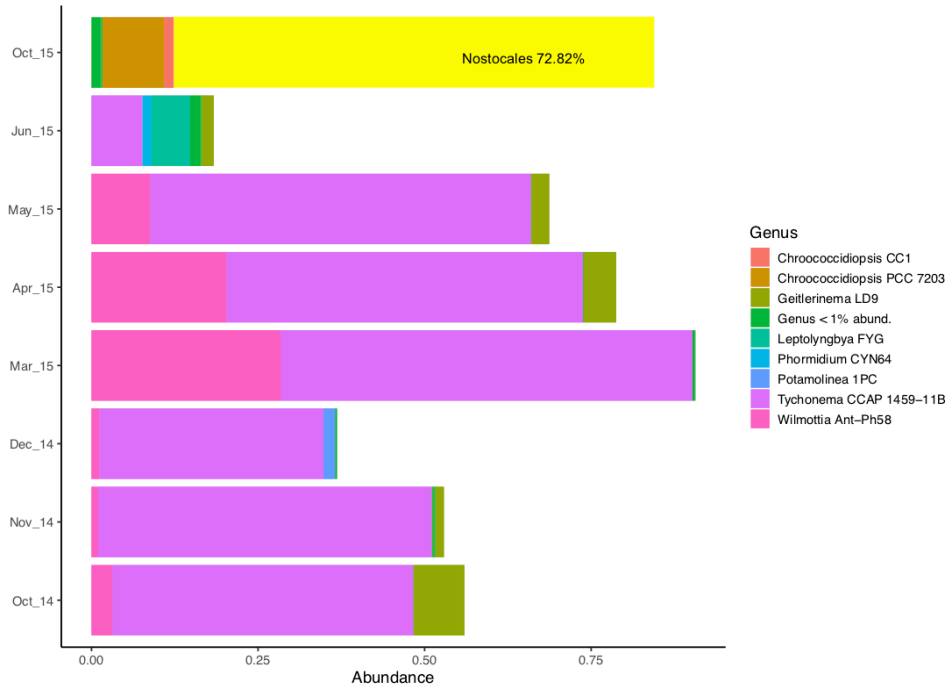


Fig. 1. Relative abundances within cyanobacterial community in Krčić Spring on genus level, SILVA database v. 132 at 99% OTU identity threshold

Re-analysis also detected two new cyanobacterial genera, that were probably sorted as "uncultured" and "other" group (Publication II), due to limitations of the database at the time: *Leptolyngbya* (similar to strain *Leptolyngbya* FYG) and *Potamolinea* (strain *Potamolinea* 1PC). Additionally, group of cyanobacterial genera that had relative abundance under 1% (rare taxa) (Fig.1), was found to be extremely diverse and comprised of: *Aliterella* CENA595, *Calothrix* PCC−6303, *Chalicogloea* CCALA 975, *Chamaesiphon* PCC−7430, *Leptolyngbya* ANT.L52.2, *Loriellopsis* LF−B5, *Microseira* Carmichael−Alabama, *Phormidesmis* ANT.LACV5.1, *Phormidium* SAG 37.90, *Pleurocapsa* PCC−7319, and two uncultured cyanobacterial populations. Interestingly, rare cyanobacterial taxa were present only in pre-drought and post-drought months. This could indicate special functions these cyanobacteria perform in the most extreme conditions.

The main issue arising during molecular analysis was the assignation of sequences to genus *Tychonema*. Previously determined as a genus *Phormidium* (Publication II), these sequences were identified as *Tychonema* CCAP 1459-11B in the re-analysis. However, results of ITS marker pointed out *Phormidium*-related population as a largest cluster comprising both *Phormidium autumnale* and *Tychonema bourrellyi* species (Publication II). Due to ecology of *T. bourrellyi* as a planktonic bloom-forming species (Suda et al. 2002), this was considered as misidentification on the part of NCBI GenBank database. However, study by the Brinkmann et al. (2015) conducted in German karstic streams also found in biofilms sequences closely related to *Tychonema bourrellyi Anagnostidis and Komárek* strain CCAP 1459 /11B. That information could confirm the wider distribution of this strain, not only as planktonic, but as benthic species specific for growing on karstic substrate. Still, to double-check this taxonomic assignment, data from new analysis were assigned using Greengenes database (13_8 at 99% OTUs identity threshold), which assigned these sequences again as *Phormidium* genus. This result affirms already proposed complex questions on the phylogeny of the genus *Phormidium*, whose phenotypical and phylogenetic heterogeneity requires revision of the genus, on which results more precise conclusions could be drawn. However, their function as the main builders of the microbial mat in the conditions of established water flow still stands.

Furthermore, re-analysis elucidated results on genus *Chroococcidiopsis* and perplexing population of quartz hypoliths, important for recolonizing the ecosystem after drought. In the Publication II in the post-drought period (Oct-15), was observed that cyanobacteria predominate microbial mats of which 70% belonged to the species *Chroococcidiopsis* (Publication II). Interestingly, the updated SILVA database re-assigned these large numbers of sequences only to order level Nostocales (Fig.1). Besides this conundrum, similarity to two different strains of

Chroococcidiopsis were detected by new analysis: Chroococcidiopsis CC1 (1.40%) and Chroococcidiopsis PCC 7203 (9.25%). This finding converges to the results of the ITS marker, which has detected two sub-clusters related to *Chroococcidiopsis*: sub-Cluster 2a and sub-Cluster 2b. It should be noted that classification in SILVA is based on Bergey's Taxonomic Outlines, meaning that Nostocales encompasses 15 cyanobacterial families (e.g. Chroococcidiopsaceae, Nostocaceae, Oscillatoriaceae, Phormidiaceae, Xenococcaceae etc.). Better result is obtained with Greengenes database 13_8 (99% OTUs identity threshold), that managed to assign these sequences to family level (*Xenococcaeae*). Nonetheless, assignation to genus, species or strain was unsuccessful when checking these sequences against NCBI GenBank database using BLAST tool. Still, BLAST search revealed 96% identity to uncultured bacteria from the environmental samples of worldwide genomic diversity of "quartz hypoliths". Hypoliths are defined as extremophile microorganisms from hot and cold deserts, living under rocks that provide them refugium or at the rock-soil interface (Cameron and Blank 1965; Schlesinger et al. 2003). Sequences belonging to "quartz hypotlits" were also detected in ITS clone libraries. Hypothetically, these undetermined sequences could belong to *Chroococcidiopsis*, as it is a well-known hypolithic genus from hot and cold deserts. However, since they seem to be very specific, they could present novel species of *Chroococcidiopsis* for Adriatic area or similar type of environment.

Generally, updated analysis has shown to match better to the results of the ITS phylogenetic analysis, which was, up till now, regarded as a better phylogenetic marker in Publication II. This revision upgraded and advanced data on the structure of the microbial mat community within this extreme environment. It is clear that the microbial mat community, especially its cyanobacterial fraction, is still highly underinvestigated and unknown in these extreme types of ecosystems. Their proper identification is of utmost importance in order to recognize and understand the key primary production players in the intermittent rivers.

Bacterial community providing insight into putative ecosystem function

As shown in Publication II, besides cyanobacteria, other members of the microbial mat community also responded to the imposed environmental stressors, especially observed during the drought and after drought period, when apparent changes in their composition and abundances took place. It was noticeable that specific environmental conditions favoured enrichment of specific phyla and classes of bacteria within the microbial mat community. There are four extreme events

shaping specific communities: summer drought period, winter bora and freeze, spring high flows and autumn rewetting after drought period.

Bacterial community during winter freeze showed high alpha diversity and moreover, high rare species richness (Publication II). However, although in extreme polar regions Cyanobacteria represent a dominant group in microbial mats, community from Krčić had diminished cyanobacteria number and enrichment with other phyla, most notably Planctomycetes. Species of SM2F11, *Shewanella* and *Pirellula*, were previously identified in different extreme cold environments, possessing a number of adaptive strategies in conditions of cold, desiccation, radiation, excessive UV radiation and temperature, and low nutrient availability. Planctomycetes are phyla characteristic of many types of microbial mats, colonizing even most extreme acidic environments (Publication II).

During spring flood conditions, community richness was lower and communities are represented mostly by Cyanobacteria (order Oscillatoriales), which build thick microbial mats. However, novel phylum was revealed in new taxonomic analysis - Patescibacteria (Fig. 2). These oligotrophic bacteria are characteristic for groundwater habitat (Herrmann et al., 2019). Patescibacteria was also detected in aquaculture sediments in central Adriatic location, which confirmed the assumption that freshwater input in this area has a source in underground submarine karstic springs (Publication III). Patescibacteria are most abundand during spring flooding, suggesting that they are flushed from the underground into Krčić spring. However, this phylum is present in all sampling points, although under 1% of relative abundance.

Summer drought, interestingly, selected for a community that had overall high community richness. Firmicutes are missing in the results of taxonomic re-analysis (Fig. 2), which is also confirmed by phenotype analysis (Gram-positive phenotype is absent, Fig. 4). This is another example of using updated databases for added insight. With Firmicutes absent from the community, re-analysis endorsed Krčić river as an oligotrophic environment. Drought community also has highest abundance of Actinobacteria, known as metabolic oligotrophs, which thrive in drought conditions of increased solute concentration and oxygen content, reduced substrate diffusion, and osmolyte production (Publication II). Furthermore, several new phyla typical for extreme environments are detected by updated taxonomic analysis: Armatimonadetes (present only during drought) and Deinococcus−Thermus. Adaptations of these two phyla fit drought conditions. Armatimonadetes representatives have been found in typically extreme environments: hot springs (Kanakratana et al. 2004), geothermal soils (Stott et al. 2008), hypersaline microbial mats (Ley et al. 2006) and variety of soils (Chow et al. 2002; Lesaulnier et al. 2008). Deinococcus−Thermus is known

for resistance to extreme radiation, desiccation and thermophilic characteristics (Pavkov-Keller et al., 2011). This phylum is also present in autumn community after the drought.

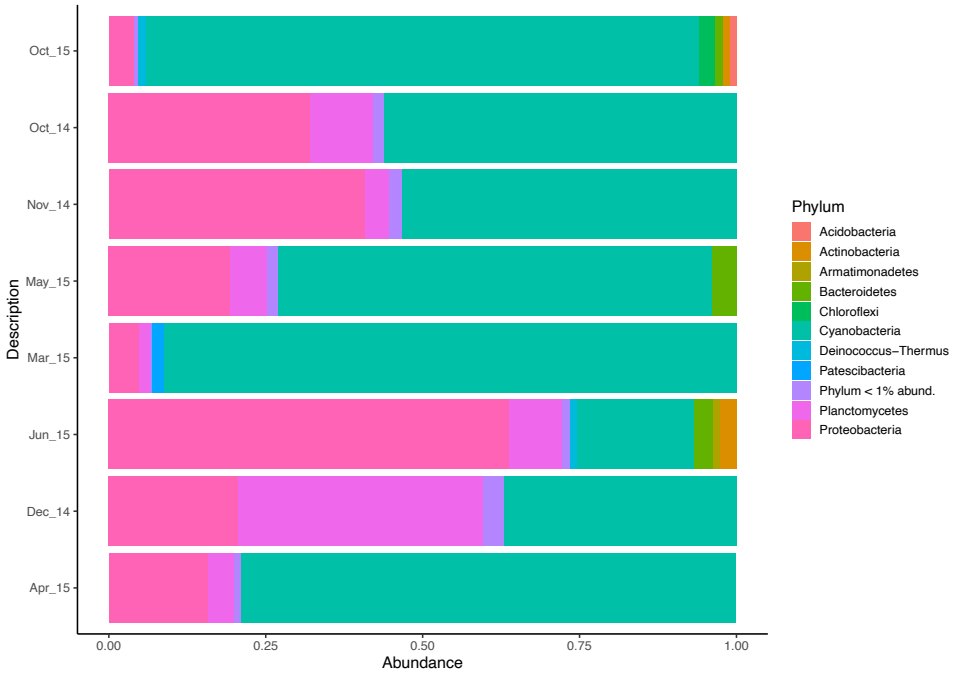


Fig. 2. Relative abundances within bacterial community in Krčić Spring on phyla level, SILVA database v. 132 at 99% OTU identity threshold

Bioinformatics tools for phenotype profiling and functional groups used in Publication IV were applied on data from Publication II. Functional groups analysis of microbial mat according to Louca et al. (2016), derived much simpler characterization and is congruent with conclusions from Publication II: main groups are: (oxygenic) photoautotrophy, phototrophy and cyanobacteria (Fig. 3). This once more supports the claim of the first hypothesis, cyanobacteria are indeed the backbone of microbial mats, gathering the specific microbial consortia according to extreme environmental conditions (Al-Thukair et al., 2007).

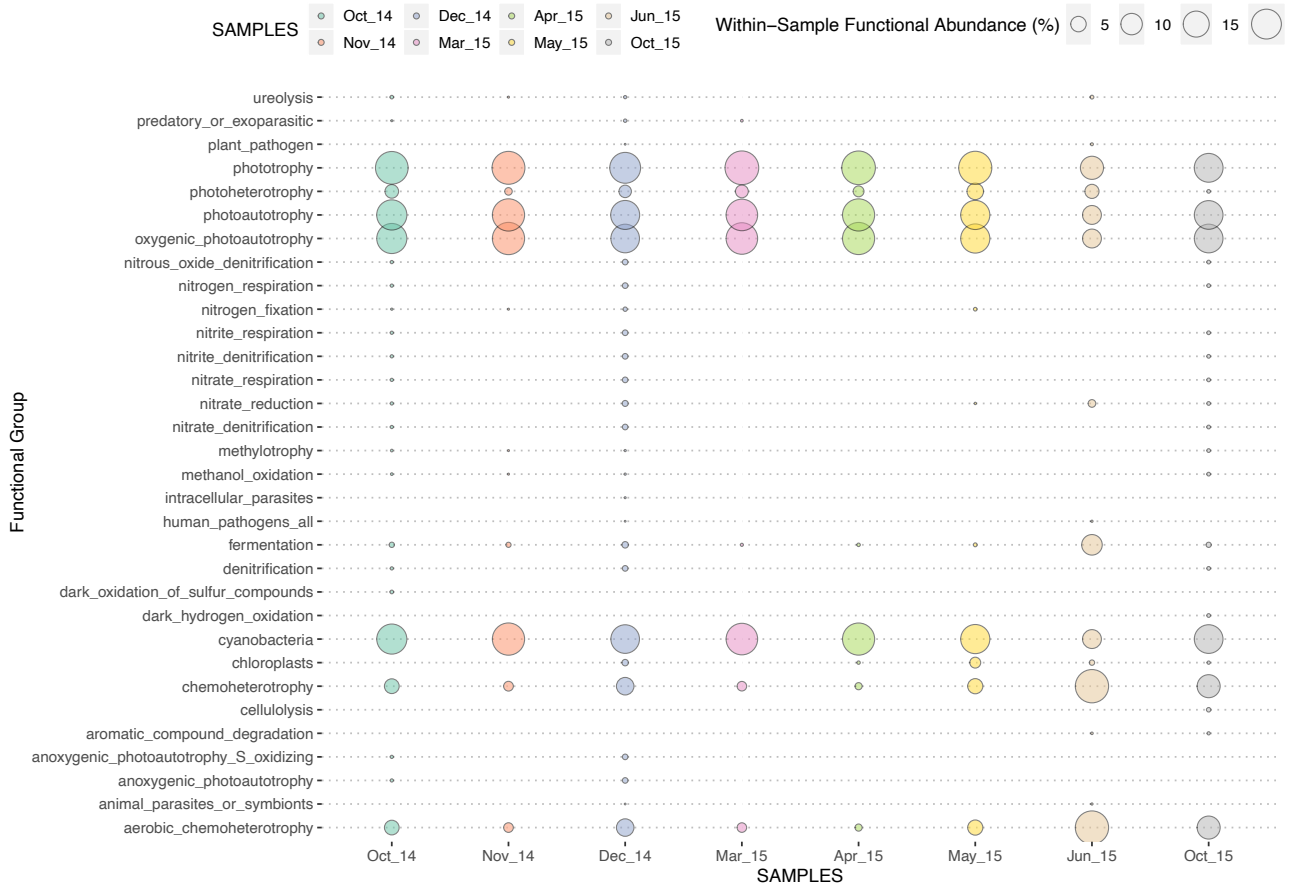


Fig. 3. Functional groups within bacterial community in Krčić Spring

Community from the drought period is an exception, when (aerobic) chemoheterotrophy and fermentation groups have higher relative abundance, presumably degrading cyanobacterial and algal biomass and participating in a sedimentary process. This is also supported by abundance of soil bacteria, e.g. *Polaromonas* and *Polymorphobacter* (Publication II).

Phenotype profiling Ward et al., in Fig. 4., confirmed dominant aerobic lifestyle inside the microbial mat: only aerobic and facultatively anaerobic phenotypes are detected. This was not surprising since Cyanobacteria and Proteobacteria often exist as co-dominant groups in microbial mats, with photosynthetic cyanobacteria constantly providing aerobic environment, and aerobic heterotrophic Proteobacteria playing a major role in organic carbon mineralisation (Publication II).

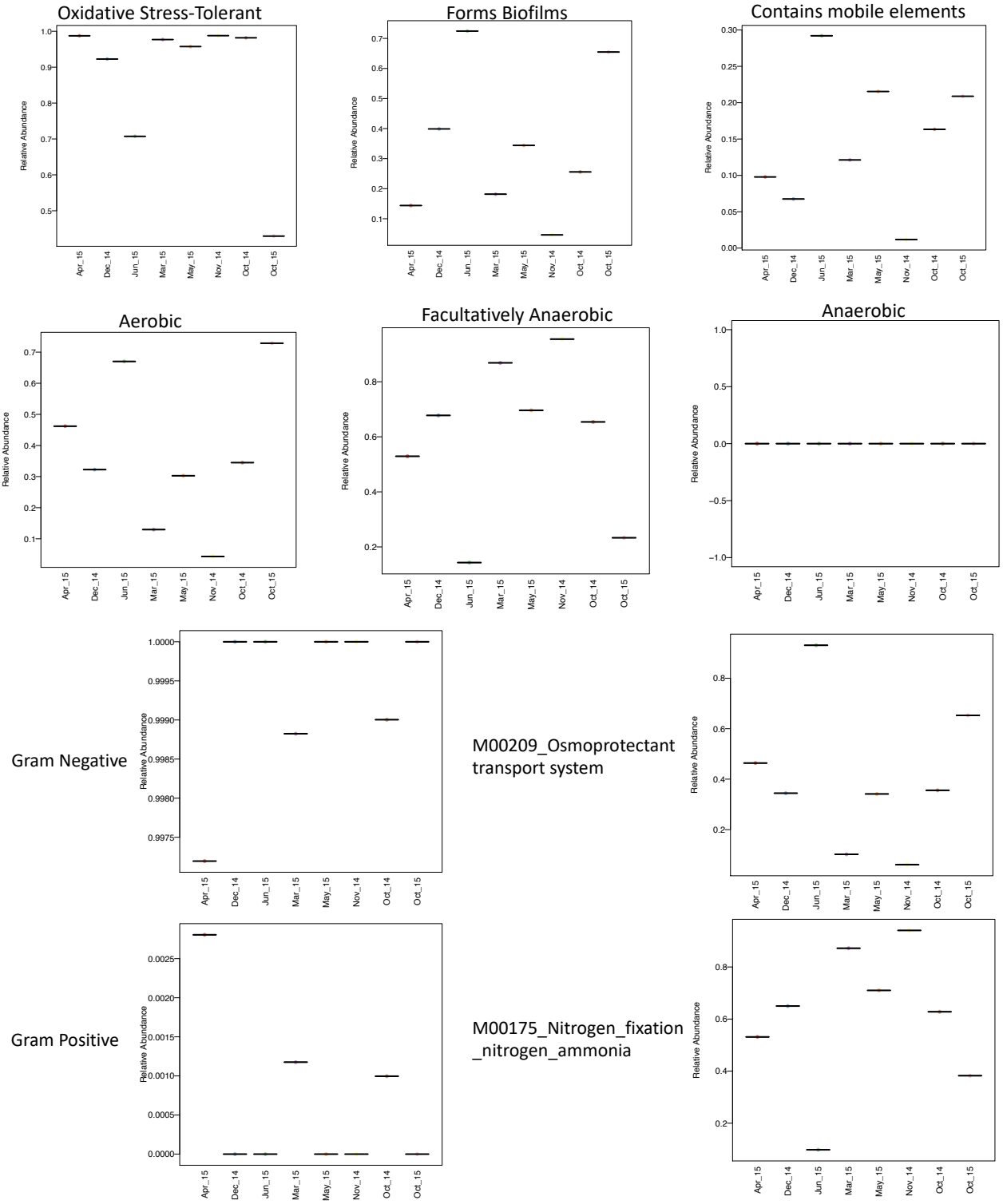


Fig. 4. Relative abundance (%) at organism-level of ten categories of microbial phenotypes from Krčić Spring microbial mat

In general, phenotype analysis suggests that the microbial mat community is mainly composed of Gram-negative members (over 99% for all samples). That is anticipated, as Cyanobacteria and Proteobacteria are both Gram-negative, and represent the majority of the microbial mat community. Regarding the potential of the microbial mats to cope with negative environmental

conditions, results showed that oxidative stress tolerant phenotypes are present in over 90% relative abundance in all samples, except during drought (70.7%) and post-drought (42.9%). This could be connected to lower abundance of Cyanobacteria in the drought and predominance of coccoid Cyanobacteria after drought, while microbial mat was still establishing. Presumably, oxidative stress is a result from the hydrogen peroxide – a toxic by-product of aerobic respiration, present in the developed microbial mat with high cyanobacterial fraction. Furthermore, samples from pre-drought and post-drought possess high relative abundance of "biofilm forming phenotype" (72.4% and 65.5%, respectively) and "mobile elements containing phenotype" (29.2% and 20.8%, respectively). This suggests colonization in the stressful environment, when thick microbial mats (characteristic during high water flow) are not established.

Possibilities of phenotype profiling tools are numerous by using KEEG microbial pathway database, which offers various ways of exploring potential functionality of microbiome. For example, the osmoprotectant transport system pathway, which protects bacterial cells from osmotic stress during drought, has the highest relative abundance in drought and post-drought samples (93.05% in Jun-15 and 65.28% in Oct_15). Interestingly, the drought sample has the lowest values for nitrogen fixation pathway (9.7%), which could be connected to low abundance of Cyanobacteria, generally considered as the principal diazotrophs in nutrient-depleted environments. These are one of the many possible pathways coverage predictions that can be used in exploration of microbiomes. Although, without the mRNA analysis (showing metabolic activity of bacterial community), we can only presume that enriched taxa are actually active in the time of sampling and performing functions. The results of the analysis of 16S rRNA and RNA transcripts can differ greatly, since transcriptomics provide insight into the actual amount of transcribed genes (Gierga et al., 2012; Bolhuis et al., 2014).

In conclusion, recent research on cyanobacterial mats has been turned from traditional morphological approach to molecular based HTS research (Schneider et al., 2015, Bolhuis et al., 2014; Bonilla-Rosso et al., 2012). DNA data can provide valuable insights into diversity and functional potential of the microbial community. Bioinformatics tools using HTS 16S rRNA amplicon data can offer good starting points in investigations of environmental samples, and results can be directed further in experimental design for metatranscriptomics and/or metabolomics studies. It is an important attempt to elucidate conditions in the habitat that is cross between aquatic and terrestrial, an oligotrophic system under hydrological stress, additionally increased by an unpredictable pattern of climate change. Study of the intermittent river Krčić showed that high

water flow is a stressor to which cyanobacteria are responding well, while their richness dropped when environmental conditions turned to cold and/or low water levels. These findings are not surprising considering similar behaviour of cyanobacteria in other extreme environments. In the Brandes et al. (2015) research of rock pools of Croatian Adriatic (another extreme environment), the highest cyanobacteria richness and diversity were measured in the high-salinity samples and the lowest in low-salinity samples. Why is this so? As other extremophiles, cyanobacteria, when compared to other bacteria, easily proliferate in their particular extreme niche. Drought is definitely the most important stressor on many organisms, including cyanobacteria, but due to their remarkable adaptations, they can survive desiccation and thrive again in the rewetting process (Lüttge, 1997), as shown in Publication II. However, how will cyanobacteria from intermittent rivers be affected in the light of extreme events caused by climate change? Total unpredictably is a characteristic of extreme events. The drought periods could be prolonged indefinitely, not giving microbial mat communities opportunity to restore. Or in case of continual flows by excessive rain and snow melt, cyanobacterial mat growth could be prolonged. However, cold periods could also be on the rise, characterized by strong bora winds and freezing ambient temperature, during which Cyanobacteria abundance and diversity could fall again. In the events of prolonged drought and cold periods, certain cyanobacterial genera could be replaced by other, more adapted genera or other bacterial phyla. Monitoring of cyano(bacterial) components in these areas could potentially give indications of climate shifts ahead. As a final point, cyanobacteria are essential for microbial mat formation, giving living substrate to diatoms and shelter and food for macroinvertebrates. Any positive or negative effects of climate change on this ecosystem could disrupt the delicate balance of intermittent river biological segments, affecting the survival of these organisms.

Coastal anthropogenic pressures: Characteristics of Cyanobacteria from water column and sediment

Publication III and IV are covering research conducted within the marine coastal ecosystem, describing possible anthropogenic impact on the bacterial communities residing within seawater and sediment. These two publications are studying eutrophic ecosystem being under strong anthropogenic influence, in contrast to highly oligotrophic freshwater system under intermittent water regime (Publications I and II). This juxtaposition between two ecosystems is however bridged by the general importance of cyanobacteria in these types of environments, changing as a consequence of anthropogenic pressures and/or climate change. In them, for the most part,

cyanobacteria present a principal and crucial segment of the microbial communities. In addition, all studies are conducted within the geographical and geological area of the karstic Adriatic, considered as a fragile region, in which effects of climate change and land-based pollution are observed much faster than in other areas. Samples of water column and surface sediments were seasonally collected at the locations situated in the central and southern Adriatic Sea coast. These locations are affected by various anthropogenic influences (urbanization, wastewaters, tourism, agriculture etc.), but predominantly by the aquaculture, i.e. European seabass cage farms. Results of the Publication III and IV are following established aims of the doctoral thesis: (i) defining both cyanobacterial and total bacterial community in pelagic and benthic system of marine environment, (ii) identifying changes in their structure and diversity as a consequence of anthropogenic pressures; (iii) applying molecular methods, namely metabarcoding, for the identification of targeted communities within the aquaculture system. Following proposed aims, both the second and third hypothesis of the thesis have been confirmed.

While in the Publication III cyanobacterial structure and diversity has been in the focus of the study, in the Publication IV cyanobacteria are analysed with the rest of the bacterial assemblage. Publication III aimed at testing the possibility of using metabarcoding specifically to investigate marine cyanobacteria, explore their ecology and potential as an indicator group in these anthropologically stressed coastal environments, while Publication IV uses a more holistic approach, combining sequencing data and microbial ecology tools, to determine the potential impacts of the aquaculture on animal, human and environmental health. In Publication III, the possibility of using specific marine Cyanobacteria as potential indicators of marine ecosystem ecological status in the highly impacted coastal zone is tested. Cyanobacteria are already widely used as eutrophication indicators in freshwater ecosystems, and highly impacted marine ecosystem. Simultaneously, the metabarcoding method by using HTS was tested as a standard monitoring method in investigating anthropogenically impacted coastal waters and sediments. Both aims were met, since detected Cyanobacteria in the water column and sediment were not exclusively marine genera. Evidence of freshwater influence, coastal eutrophication, as well as tropicalization process aided by climate change effects were found in the cyanobacterial composition.

Research presented in Publication IV resulted in a comprehensive and holistic study of bacterial assemblages' dynamics in the zone of two European sea bass cage farms. Results indicated clear a difference between water column and sediment habitats, harbouring distinct microbial communities. They are formed probably due to selection driven by the specific environmental

conditions in those habitats, requiring specific microorganisms to perform distinctive functions for the habitat. Importantly for aquaculture microbial management, analyses showed that potential pathogens could be more prevalent outside of the actual fish farm. Furthermore, seawater contained a higher percentage of potential health-impacting taxa. For both habitats, important biomarkers for the aquaculture environment were detected, as well as human and fish potential pathogens.

Cyanobacteria and other members of bacterial community in seawater

Cyanobacteria from seawater did not show any difference between locations under aquaculture influence and control locations. Cyanobacterial community from seawater showed lower diversity, composed of only 3 genera: *Prochlorococcus*, *Syenchococcus* and *Cyanobium*. Even so, these genera are more abundant when compared to cyanobacteria found in sediment. Although this was surprising, no impact of the aquaculture activity was detected on the cyanobacterial assemblages from the water column. Two explanations are proposed, (1) either this is the consequence of similar physico-chemical parameters measured on both sites, or (2) fish farms have well-managed systems which do not provoke the triggers for dramatical change in the water column community. Seawater bacterial community (Publication IV), as well as cyanobacterial fraction (Publication III) were found to differ depending on seasonal environmental factors. Cyanobacteria were more abundant in spring and autumn, also confirmed by higher relative abundance of functional groups for phototrophy/oxygenic photoautrotrophy/cyanobacteria. Simultaneously, these two seasons generated a bacterial community that harboured more facultative anaerobic bacteria and anaerobic bacteria, and less oxidative stress tolerant community, potentially from degradation of organic matter. On the positive side, spring and autumn also had lower relative abundance of potential pathogens. However, in summer and winter samples genera *Vibrio* and *Pseudomonas* (Gammaproteobacteria) are enriched. As a consequence, an outbreak of vibriosis caused by *Vibrio* (*Listonella*) *anguillarum* was reported before spring sampling. In the total bacterial community at the phyla level (Fig. 5), Cyanobacteria were found in higher relative abundance in seawater, especially during spring (min. 2.69%, max. 17.93%) and autumn (min. 5.04%, max. 13.82%) (Fig. 5). Relative uniformity of seawater samples is clearly evident on the phyla level (Fig. 5), mostly consisting of Proteobacteria (Alpha- and Gammaproteobacteria), Bacteroidetes and Cyanobacteria. Gammaproteobacteria phyla, besides containing many pathogens (*Vibrio*,

Pseudomonas, *Photobacterium*), integrate many genera responsible for dissolving organic matter, being important in the eutrophic coastal marine areas (genera *Alcanivorax, Halomonas, Litoricola*). This function is extremely important in marine ecosystems, with other phyla being abundantly represented for the same function in our samples: Alphaproteobacteria (SAR11) and Bacteroidetes (NS4 and NS5 marine group) (Publication IV).

Cyanobacteria in seawater related to strains *Cyanobium* PCC-6307, *Prochlorococcus* MIT9313 and *Synechococcus* CC9902 represented abundant taxa in seawater samples, with abundance and ratio found to depend on the collection season (Publication III and IV). Unfortunately, assignation at the genus level for Cyanobacteria in about half of samples was under 50% of ASVs (Amplicon Sequence Variants), which displays potential biases of using universal primer sets targeting V1-V3 region for studying Cyanobacteria (Publication III). New evidence presented in the study by Huber et al. (2019) indicates that the hypervariable regions V5, V6, and V7 are those most informative for studying genera *Synechococcus*, *Prochlorococcus* and *Cyanobium*, with successful assignment of 97% of reads at the genera level. This information is valuable by giving indication on the effectiveness of utilizing 16S rRNA. However, for identification of picocyanobacteria, targeting some specific gene regions should also be incorporated in the future experimental design.

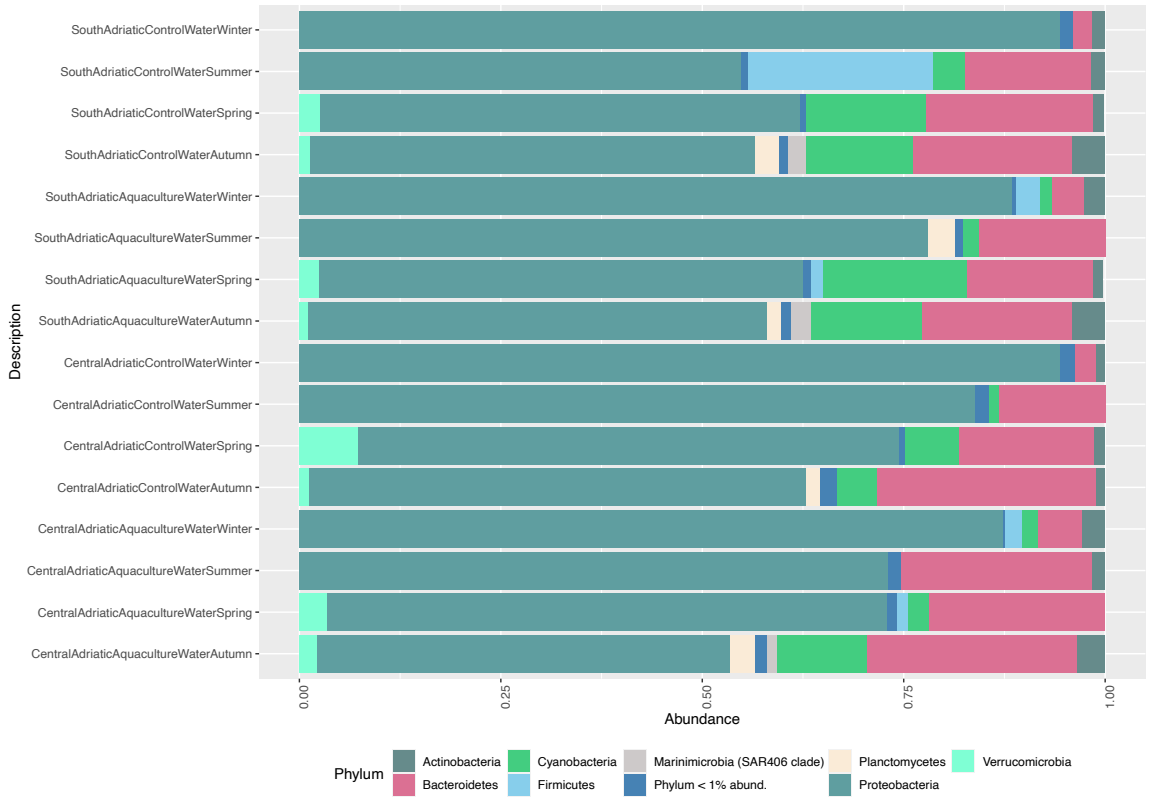


Fig. 5. Relative abundances of bacterial community in seawater column on phyla level

Investigation on the structure of the picocyanobacterial community suggested that, similar to their general distribution patterns in the eastern Adriatic Sea (Šantić et al. 2013, Paliaga 2017, Mucko et al. 2018), *Synechococcus* dominate over *Prochlorococcus* also in the impacted coastal ecosystem. Presumably *Synechococcus* related to the strain CC9902 found in the water column (Publication III) could represent a phycocyanin rich ecotype (Šantić et al., 2018), according to data from Haverkamp et al. (2008) on the gene cpcBA (green PC pigment). The potential aquaculture-related concern arises from the information on this strain being firstly isolated from coastal waters off California, where it formed extensive blooms and affected fish behaviour (Hamilton et al. 2014). Even though in the report by Kim et al. (2018) *Synechococcus* CC9902 was detected only in summer samples at 24°C, results in Publication III showed it was present in the water column throughout the year (12.01 – 23.34°C). That could indicate broader ecological valence of this ecotype then presumed before, and/or successful adaptation to both lower and higher water temperatures.

Interestingly, when compared to the study of Babić et al. (2017), which pointed out HLI ecotype MED4 and LLI ecotype NATL2A as typical *Prochlorococcus* residing within the southern Adriatic offshore waters, in samples collected in seawater of the fish farms only LL adapted *Prochlorococcus* (MIT9313) was detected. It can be presumed that *Prochlorococcus* MIT9313 is more adapted to the coastal, anthropogenically impacted environment, due to its abilities to respond to the environment stress and lower light availability, which are characteristic for more eutroficated coastal zones characterized by high primary production and presence of various size particles (Cloern et al, 2014). This strain is also successful in utilizing organic nitrogen compounds and could have function in transformation of organic matter (fish food) as well as fish excreted urea and amino acids, found in this environment in extensive amounts, as a by-product of fish farming. This implies ecological importance of Prochlorococcus MIT9313 in the functioning of this aquaculture ecosystem. Among detected cyanobacteria not all could be assigned as being exclusively marine genera:e.g. *Cyanobium*, *Geminocystis* and *Chrococcidiopsis*, giving evidence of the freshwater and coastal eutrophication. In coastal water of the Eastern Adriatic *Cyanobium* was not recorded before, and the closest findings were north-west Adriatic of Italian lagoons (Sorokin et al., 2006), Mediterranean lagoons (Pulina et al., 2011) and lagoons of coastal southern Baltic Sea (Albrecht et al., 2017). Detection of the *Cyanobium* genus brought concerns of the eutrophication processes in these waters (Publication III) with clear indication that the aquaculture environment is changing with potential negative impact on the productivity of the industry. Consequently, this genera can be

destructive to less tolerant species in the phytoplankton community, and lead to an increase of cyanobacteria fraction (Pulina et al. 2011) with potential cyanobacterial blooms.

Concerning standard physico-chemical parameters measured in seawater, as shown in the Publications III (and especially the Publication II), environmental parameters seem to play a big role in triggering cyanobacteria proliferation (Mella-Flores et al., 2011). But some of unmeasured parameters in Publications, such as concentration of Fe from iron particles from Saharan dust, precipitation, high solar irradiation and air temperature, calm weather, land runoffs etc., may have great impact on formation of e.g. benthic cyanobacterial mats in surface sediments (Brocke et al., 2015; Brocke et al., 2018). Even registering low nutrient levels (total N and P in Publication II and III) may be misleading, due to nutrients probably being quickly converted to biomass (Brocke et al., 2015). Furthermore, measured physico-chemical parameters and using correlation methods in explaining bacterial distribution and dynamics in seasonal samplings, could be insufficient describers of factors that shape bacterial community, as seen in Korlević et al. (2014). However, in this study some correlations on the co-existence of certain bacterial groups were observed (Publication IV). That implies on the important role of microbial ecology, i.e. bacterial networking and different trophic interactions (with each other and other (micro)organisms), and/or interactions with other environmental parameters, on shaping microbial communities abundance and dynamics.

The Adriatic Sea coastal area is shallow, as is generally the whole Adriatic Sea (Artegiani et al., 1997). Similarly, to the rest of Mediterranean, the coast is densely populated largely without municipal wastewater treatment (Paliaga et al., 2017) with settlements being built mainly on porous limestone bedrock. Additionally, coastal karstic rivers are a source of agricultural and other runoffs to the Adriatic basin. These conditions are a “fertile ground” on which climate change driven events could lead to proliferation of benthic cyanobacteria in sediments as well as potentially toxic cyanobacteria in water column. Investigation of Paliaga et al. (2017) on the effect of wastewaters on the bacterial community in the northern Adriatic also support the findings in Publication IV. Specifically, seawater samples from control location showed higher anthropogenic impact, especially regarding pathogenic bacteria, but generally, seawater communities seem to be shaped by seasonal conditions, as shown in Paliaga et al. (2017).

In conclusion, it is clear that specific cyanobacteria have good potential to be used as early indicators of the changes in the marine environments as a consequence of human impacts. Coastal environments are being constantly subjected to multiple anthropogenic pressures, including

pollution and climate change. Studies in the last several decades pointed out particular vulnerability of the Mediterranean Sea, with many local variations (Mella-Flores et al., 2011). At the same time, unique hydrogeological features of Eastern Mediterranean, and its northernmost corner – Adriatic Sea – makes it an ideal location to observe early changes in the marine environment. Mella-Flores et al. (2011) already proposed the use of *Prochlorococcus* HLII (eMIT9312) and *Synechococcus* clade II (CC9605) as bioindicators of changes in the marine environment. Results produced from Publication III, could propose adding *Synechococcus* (CC9902) and *Prochlorococcus* (MIT9313) as potential bioindicators of changes in the coastal Adriatic area. Moreover, cyanobacterial composition in samples suggested a tropicalization process that cannot be ignored. This process was evident from genera mostly found in sediment, although some of them are planktonic, e.g. *Neolyngbya*, *Chrococcidiopsis*, *Trichodesmium*, *Aphanocapsa*, *Cyanobacterium*, *Crocosphaera*, *Xenococcus*. In seawater, many sequences were closely related to *Synechococcus* strains found up till now exclusively in the warm seas, e.g. Gulf of Mexico and Arabian Sea. Furthermore, results from Publication IV suggests that some strains could be used as important biomarkers for the aquaculture environment including Marinimicrobia (SAR406) and human and fish potential pathogens: *Vibrio ichtyoentery*, *V. harvey*, *Acinetobacter lwoffi*, *A. johnsonii*, *Clostridium perfringens*, *Photobacterium* and *Tanacibaculum*. Accordingly, this thesis should be considered as a strong starting point for possible usage of metabarcoding method as a suitable monitoring tool for ecosystem health.

Cyanobacteria and other members of bacterial community in sediment

Impact of the aquaculture on this ecosystem was more clearly seen within the sediment, as the bacterial community collected underneath the cages noticeably differed from the one at the control site. Interestingly, besides benthic cyanobacteria, a “record” of planktonic cyanobacteria was detected within surface sediments that were not present in seawater samples (Publication III). Furthermore, total bacterial community in sediments was more stable, contrary to seawater samples which provide momentary “snapshot” of the community.

As confirmed by other studies (Vogt et al., 2019), sediment grain size and type seems to be one of the most important factors influencing composition, abundances and distribution of cyanobacteria. Indeed, when compared to the overall microbial community, grain size seemed to be crucial for cyanobacterial diversity in the sediment samples analysed. Muddy component in aquaculture sediments seems to be antagonistic to cyanobacteria. However, cyanobacterial diversity has increased in the sandy gravel type of sediment, located mainly on the control locations.

Benthic cyanobacteria were part of the abundant taxa mostly on the sandy gravels at the control sites, while in the aquaculture impacted sites they were part of rare taxa and had considerably lower diversity. Coarse-grained sediment was shown to be unfavourable for development of mats which require certain abundance of filamentous cyanobacteria, but at the same time have high abundances of unicellular cyanobacteria which act as pioneers and part of "early stage communities" (Vogt et al., 2019). That finding was also observed in aquaculture impacted sites (Publication III), and goes in line with the first hypothesis. Sandy sediments harboured diverse cyanobacterial families, predominantly Pleurocapsales, but including Oscillatoriales, Chroccocales, Chroccocidiopsales and Synechoccales (Publication III), which is congruent with Vogt et al. (2019). As reported for epilitic and endolitic taxa from Adriatic coast (Brandes et al., 2015; Palinska et al., 2017; Vondrášková et al., 2017; Vogt et al., 2019), cyanobacteria from order Pleurocapsales are most often dominant community members, as it is in the sediment samples in Publication III (genera *Pleurocapsa* and *Xenococcus*). Quite possibly, cyanobacteria discovered in the marine sediments could be epiliths and endoliths, adapted to the same extreme lifestyle in sediment as their littoral counterparts. ASVs similar to *Pleurocapsa* PCC-7319 were not recorded in eastern Adriatic sediments before. Intriguingly, they were detected also in the rare taxa of microbial mat in Krčić in Nov-14, Jun-16 and Oct-15 (re-analysis). This strain seems to be representative of species *Pleurocapsa minor* Hansgirg, found in both marine and freshwater habitats (Loza et al., 2013; Dvořák et al., 2017). Nearly all samples contained genera *Pleurocapsa and Xenococcus*, making them core genera in the cyanobacterial community. These genera are microbial mat-forming cyanobacteria and first colonizers in marine sediments. Cyanobacteria play important role in stabilization of surface sediments, from low level (loose sediment covered by thin biofilms), medium (sediment surface stabilized by EPS) and high (well stabilized and laminated microbial mats) (Vogt et la., 2019). Findings of *Pleurocapsa* and *Xenococcus* could indicate beginning of low to medium level microbial mat formation, and emphasize cyanobacterial function as sediment stabilizers. Additionally, *Chroococcidiopsis* found in control sediments (Publication III) is well known representative of endolithic and hypolithic communities (Brandes et al., 2015). Since cyanobacterial communies in sediments of Eastern Adriatic have not been investigated by HTS metabarcoding method before Publication III, there are no comparable results. However, they share high similarity to the extremophiles from the splash zones and tidal pools described by Brandes et al. (2015), Palinska et al. (2017), Vondrášková et al. (2017) and Vogt et al. (2019).

Regarding total bacterial community, there was no significant difference in species richness in aquaculture vs. control sediment, as shown by the diversity metrics (Publication IV). Even so, control sediments contained more aerobic and facultatively anaerobic bacteria, which is concurrent with cyanobacteria found to be enriched at control location (Publication III). Moreover, control sediments had higher relative abundance in functional groups: phototrophy/oxygenic photoautrotrophy/cyanobacteria then aquaculture impacted ones (Publication IV). At the same time, aquaculture sediments were markedly anaerobic, containing functional groups for respiration of sulfate and sulfur compounds. These obvious functional differences clearly pointed out control locations as a more balanced and healthier benthic ecosystem. However, regarding presence of pathogens – important threats for animal/human health, were more abundant in control site sediments (e.g. *Tenacibaculum*). Moreover, it seems that control sediments are more anthropogenically impacted, but presumably by sources other than aquaculture, possibly wastewaters (Publication IV). On the phyla level (Fig. 6.), that can be seen by the enrichment of the bacteria belonging to organic-degrading phyla Planctomycetes and Bacteroidetes. Additionally, Firmicutes were found in higher abundances during summer when, due to tourism and overpopulation, pressures on the coastal ecosystems are higher – suggesting enrichment with nutrients. In sediments under fish cages however, Epsilonbacteraeota (genera *Sulfurovum*) and Spirochaetes (Spirochaeta 2) are dominant within the total community. These bacteria have already been connected to aquaculture impacted sediments being labelled as important sulfur-oxidizing chemolitoautotrophs in these ecosystems. This clear indication of anoxic nature of sediments under fish cages represents environmental health concern, even if the level of detected pathogens is low – therefore giving deceptive low health risk.

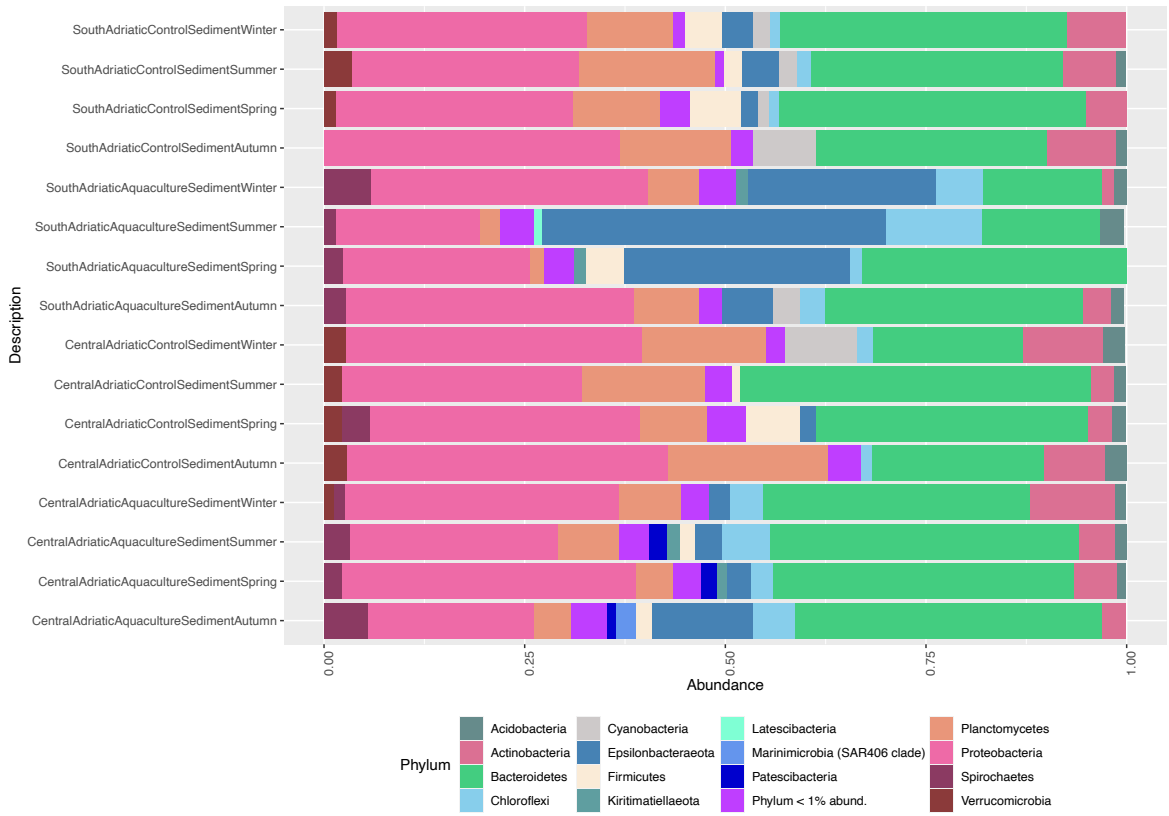


Fig. 6. Relative abundances of bacterial community in surface sediments on phyla level

In research by Al-Thukair et al. (2007) and Brocke et al. (2015), anthropogenically impacted sites are defined as extreme habitats, and consequently, are characterized by the presence of benthic cyanobacterial mats. At such locations, cyanobacterial communities are considered to be vital for conducting natural biodegradation processes (Al-Thukair et al., 2007). However, even though it seems logical that aquaculture sediments should have established benthic mats performing biodegrading function, control sediments were found to harbour more abundant and diverse cyanobacterial communities. Could it be that cyanobacteria are performing biodegradation in more aerobic, sandy control sediments, while aquaculture samples need more specialized bacteria for anoxic, muddy sediments (e.g. *Sulfurovum*)?

In conclusion, investigations on the sediment microbial communities in the eastern Adriatic using HTS metabarcoding method are rare (Korlević et al., 2015). Furthermore, bacterial communities on the aquaculture-impacted sediments, using the HTS metabarcoding method were not described before. Benthic cyanobacterial mats presence, composition and diversity in the eastern Adriatic are certainly fascinating novel area for future investigations, especially since they are connected to higher organic content in the sediments (Brocke et al., 2015), implicating anthropogenic impact. Even though Cyanobacteria are central in this thesis, in sediments they were

found to be less abundant. As shown on phyla level (Fig. 6), in almost half of the studied samples (43%) cyanobacteria relative abundances are mostly under 1%. Even though cyanobacteria fall into the rare taxa category, they have considerably greater diversity than in the seawater and are indicated as community members with high ecological importance. Their importance lies in the fact that rare taxa in ecosystems seem to be more responsive to disturbances in the environment. Other bacterial taxa in this "rare" category, identified as being likewise important, include fish and human pathogens of the genera *Vibrio*, *Pseudomonas*, *Photobacterium* and *Tenacibaculum*. This thesis correspondingly proposes important biomarkers for aquaculture system, whose presence can reflect the state of environmental health within the sediments: *Blastopirullela*, Sva0081, *Suflurovum*, Bacteroidetes BD2-2, Spirochaeta 2, *Anaerolineaceae* and Marinimicrobia (SAR406), as well as mentioned pathogens. On that basis, methods and results of this thesis should be considered as a first step towards recognizing the importance of (cyano)bacterial players as health-state indicators of the coastal marine environments, as well as of economically significant food industry such as aquaculture.

CONCLUSIONS

Results presented in publications encompassing this doctoral thesis, along with new data discussed, offer the opportunity to highlight several important closing remarks:

1. Freshwater microbial mats are described for the first time in the intermittent karstic environment in Croatia, representing extreme habitat that is even more amplified by climate change.
2. Cyanobacteria are essential for microbial mat formation, giving living substrate to diatoms and serving as a shelter and food for stygophilic crustacean *S. ambulans*. Cyanobacterial relative abundance, composition and seasonal dynamic, along with the rest of the bacterial community, is described for the first time using high throughput sequencing in the intermittent karstic environment.
3. Cyanobacteria are the main constituents of microbial mats and pioneers in ecosystem rebuilding after drought stress. They strongly respond to other imposed environmental stressors in this extreme ecosystem. Monitoring of cyano(bacterial) components in intermittent ecosystems could potentially give indications of climate shifts.
4. Pelagic and benthic cyanobacteria, along with the rest of bacterial assemblage, have been described for the first time by high throughput sequencing in the aquaculture system in Croatia. Furthermore, analysis of cyanobacteria from the marine sediments are novel for the eastern Adriatic.
5. This thesis correspondingly proposes important biomarkers for the aquaculture system, whose presence can reflect the state of environmental health within the aquaculture sediments. However, control sediments and seawater bacterial community was a better reference concerning human and animal health.
6. ITS and 16S rRNA genetic markers potential in assessing the bacterial community was compared. Updated version of SILVA database brought disparate results closer together, and detected new cyanobacterial genera and bacterial phyla. Expending possibilities of characterizing bacterial community using emerging bioinformatics tools (e.g. functional groups, phenotype profiling) were explored.
7. High throughput sequencing of 16S rRNA (V1-V3 region) gene marker has displayed promising results in describing total bacterial community, including cyanobacterial fraction.

Due to relatively low price and fast results, it shows potential in describing and monitoring rapidly changing environments focusing on bacterial components.

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CURRICULUM VITAE

Anamarija Kolda was born in Slavonski Brod, Croatia, 8th of April 1988. Completing the elementary and high school in Zagreb, she continued her higher education at the University of Zagreb, Faculty of Croatian Studies. After attainment of Bachelor's degree in Communication Studies and Sociology in 2010, she enrolled in the undergraduate programme of Environmental Science at Faculty of Science, Department of Biology (Zagreb). She volunteered for several years at the Laboratory for the freshwater algae, under the supervision of Marija Gligora Udovič, PhD, and on several algology and biospeleology field trips. After completing the Master studies in Environmental Science in 2016, she was employed at Ruđer Bošković Institute (RBI) in the Laboratory for Aquaculture and Pathology of Aquatic Organisms, as a research assistant on the project "AQUAHEALTH - Microbial Ecology of Water as an Indicator of the Environmental Health", principal investigator Damir Kapetanović, PhD (Croatian Foundation for Science). She enrolled in PhD studies (Oceanology) at the Department of Geology, Faculty of Science (Zagreb) in the same year, under the mentorship of Damir Kapetanović, PhD (RBI) and Zrinka Ljubešić, PhD (Department of Biology). During her employment, she participated in several educational workshops and training abroad and in Croatia, concerning marine conservation, *Cyanobacteria* and microbial ecology, "omics" technologies and bioinformatics. She was also an active participant on three bilateral projects: with Montenegro, Germany and Serbia. In the scope of a bilateral project with Germany, she was a guest at Helmholtz Centre for Environmental Research GmbH – UFZ, Leipzig for the training on specific isolation and description of lytic viruses (Antonis Chatzinotas, PhD). For two consecutive years (2017, 2018), she was a member of teaching field trips for students of Queen Mary University of London, UK, in the organisation of Croatian association of freshwater ecologists. She participated in international scientific conferences with five oral presentations and seven poster presentations. She is a first author in four published scientific papers and one professional paper, co-author in two other published scientific papers, 23 conference abstracts, two conference proceedings papers and four monitoring reports. She is a current member of Croatian Botanical Society and Croatian Microbiological Society.

SCIENTIFIC ACTIVITY AND PUBLICATIONS

CROSBI PROFILE: Anamarija Kolda (CROSBI Profile: 34065, MZO: 360296)

ORIGINAL SCIENTIFIC PAPERS:

Kapetanović, Damir; Gavrilović, Ana; Jug-Dujaković, Jurica; Vardić Smrzlić, Irena; Kazazić, Snježana; Bojanić-Rašović, Mirjana; **Kolda, Anamarija**; Pešić, Ana; Perić, Lorena; Žunić, Jakov et al. Assessment of microbial sea water quality and health status of farmed European seabass (Dicentrarchus labrax) in Eastern Adriatic Sea (Montenegro and Croatia) // ***Studia Marina***, 32 (2019), 2; 52-64 doi:10.5281/zenodo.3584222

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MULTISEK (Multimetric evaluation of crenobiocoenosis) funded by the Environmental Protection and Energy Efficiency Fund (code: 351-01/13-01/108)

Bilateral project Croatia-Montenegro: "Influence of ecological factors on health of sea bass (Dicentrarchus labrax), with special reference to the occurrence of vibriosis in Croatia and Montenegro"

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WORKSHOPS AND SCIENTIFIC TRAINING:

Integrating 'Omics' Technologies into Aquatic Ecology, Fondazione Edmund Mach, San Michele All'Adige (Italy)

Determination Course of Freshwater and Terrestrial Cyanobacteria, University of South Bohemia, České Budějovice (Czech Republic)

International School of Marine Conservation Science, University of Primorska, Oregon State University, Koper (Slovenia)

Introduction to R syntax and of language R and its application in basic statistical and graphical data analysis, University Computational Centre - SRCE, Zagreb (Croatia)

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