Acta Bot. Croat. 79 (2), 157–169, 2020
DOI: 10.37427/botcro-2020-021

CODEN: ABCRA 25
ISSN 0365-0588
eISSN 1847-8476

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 a, b). 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-

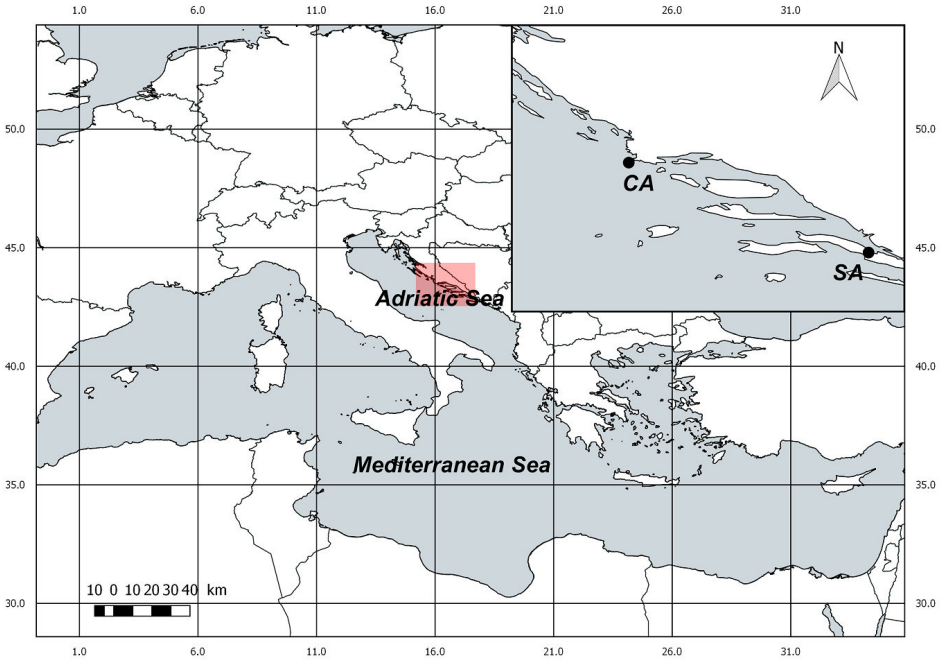

Fig. 1. Study sites in the central and southern East Adriatic Sea (CA – central Adriatic, SA – southern Adriatic).

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

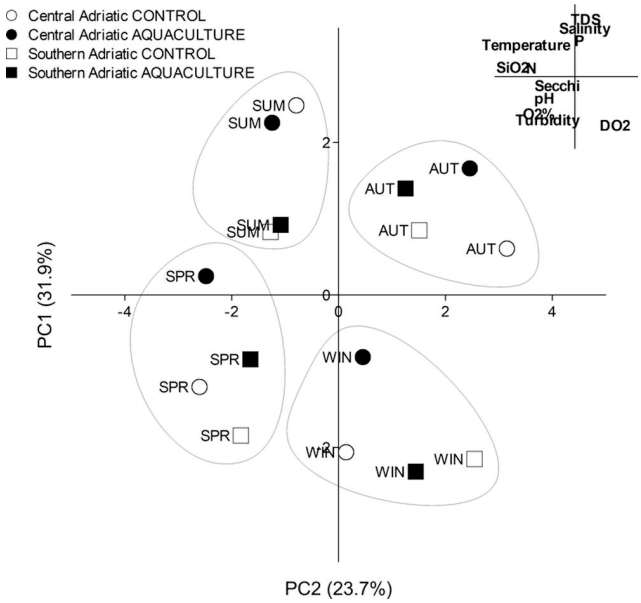

Fig. 2. Principal component analysis ordination graph of physicochemical parameters of seawater column (T – temperature, SiO_2 – silicon dioxide, N – total nitrogen, P – total phosphorous, Secchi – Secchi disc, Turb – turbidity, Sal – salinity, TDS – total dissolved solids, DO_2 – dissolved oxygen, O_2% – 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

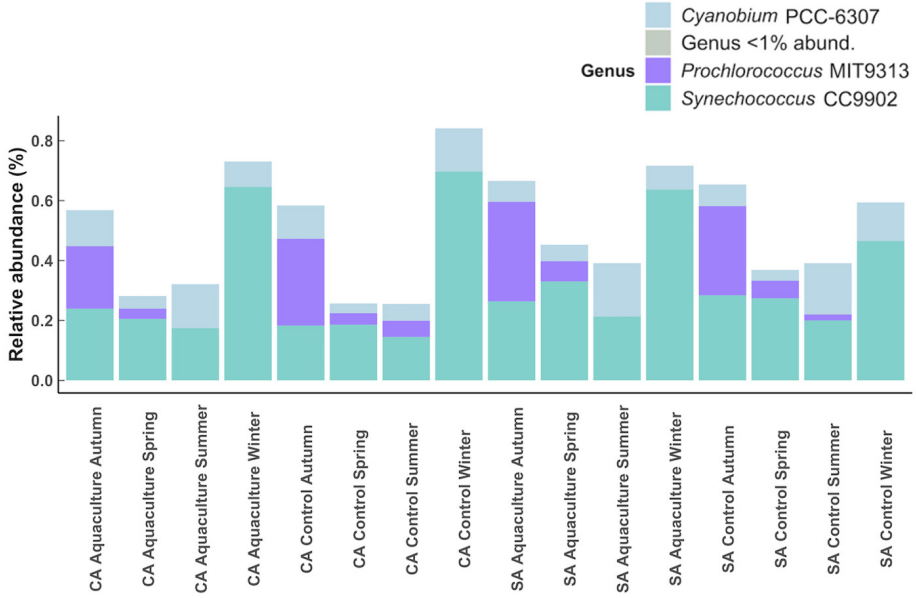

Fig. 3. Relative abundances (% of total sequence number) of cyanobacterial genera in all sampling points of coastal seawater (CA – central Adriatic, SA – southern Adriatic, Aquaculture – location under the influence of fish farms, Control – control location).

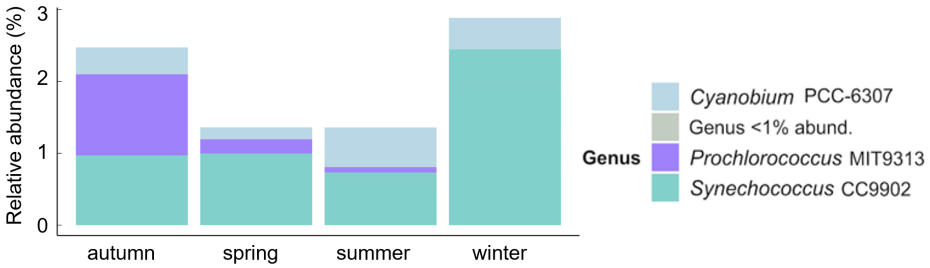

Fig. 4. Relative abundances (% of total sequence number) of cyanobacterial genera in seawater during four seasons, including combined central and southern Adriatic samples.

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-

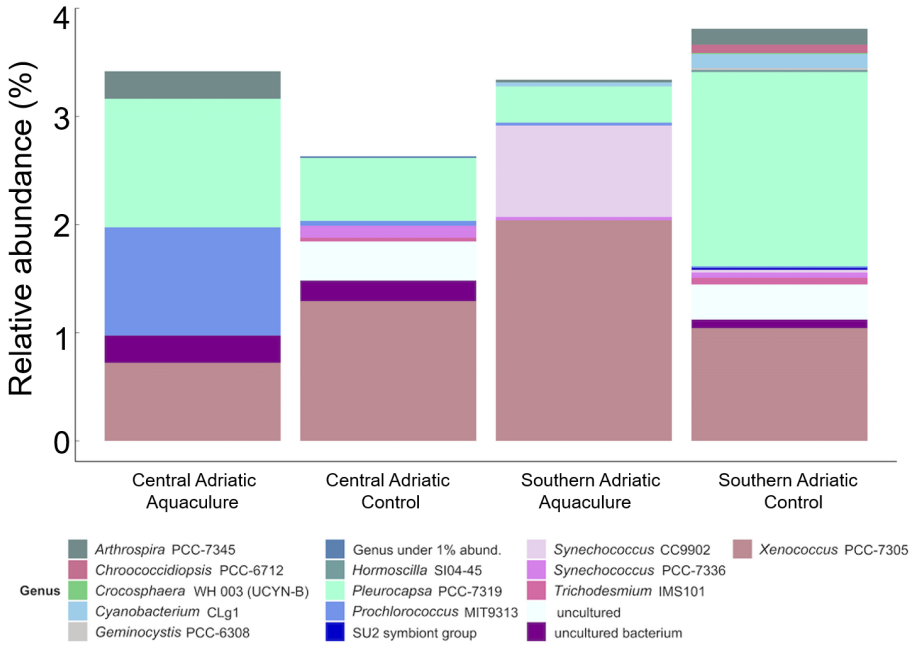

Fig. 5. Relative abundances (% of total sequence number) of cyanobacterial genera in sediment, relating to locality and site type (combined central Adriatic aquaculture and control sites, and southern Adriatic aquaculture affected and control sites).

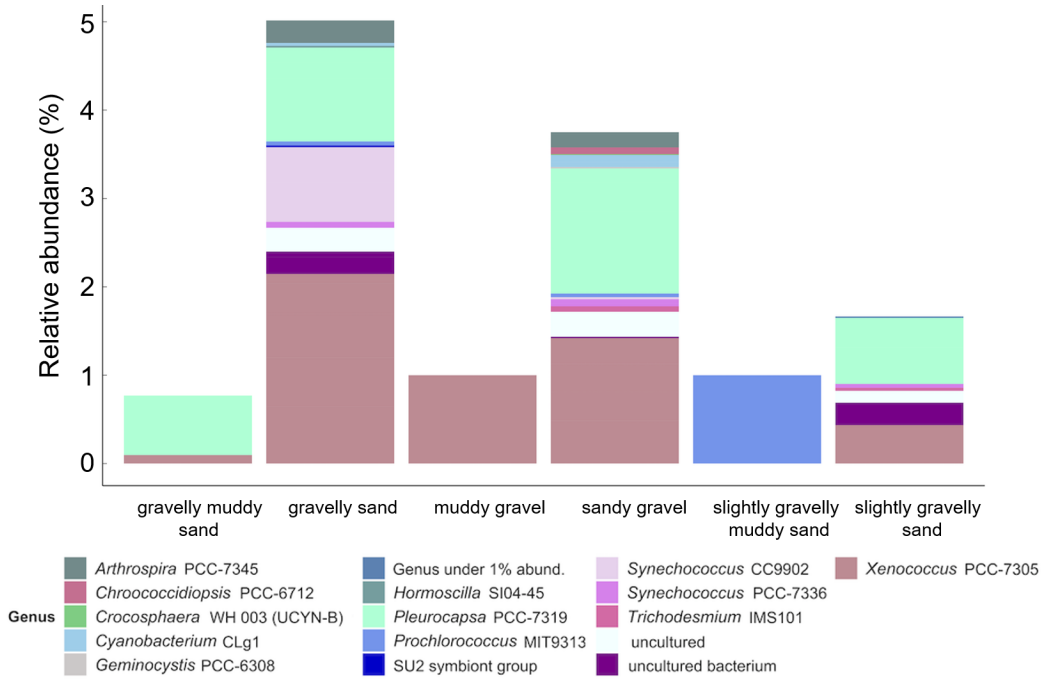

Fig. 6. Relative abundances (% of total sequence number) of cyanobacterial genera in sediment, in relation to sediment type (combined central Adriatic aquaculture and control sites, and southern Adriatic aquaculture affected and control sites).

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*, P*hormidiacaeae* 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 physico-chemical 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 et al. 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.

Acknowledgements

This research was supported by the project AQUA-HEALTH (Aquatic microbial ecology as an indicator of the health status of the environment) funded by the Croatian Science Foundation (code: 3494). Assistance and advice from Lorena Perić, PhD in the statistical analysis is greatly appreciated, as well as help from Ines Sviličić Petrić, PhD for advice in DNA extraction and valuable suggestions for improvements on the manuscript. Likewise, we are thankful to the reviewers for their insightful comments, which helped us to improve our work. We appreciate Jakov Žunić for sampling effort and Zvjezdana Šoštarić Vulić for chemical analysis of seawater. We are grateful to AWS Educate for providing computational resources for analysis.

References

Andersson, B., Sundback, K., Hellman, M., Hallin, S., Alsterberg, C., 2014: Nitrogen fixation in shallow-water sediments: Spatial distribution and controlling factors. Limnology and Oceanography 59, 1932–1944.

Babić, I., Mucko, M., Petrić, I., Bosak, S., Mihanović, H., Vilibić, I., Dupčić Radić, I., Cetinić, I., Balestra, C., Casotti, R., Ljubešić, Z., 2018: Multilayer approach for characterization of bacterial diversity in a marginal sea: From surface to seabed. Journal of Marine Systems 184, 15–27.

Bellinger, E.G., Sigee D.C., 2015: Freshwater algae: Identification, enumeration and use as bioindicators. John Wiley and Sons, Ltd. Chichester.

Bentzon-Tilia, M., Sonnenschein, E.C., Gram, L., 2016: Monitoring and managing microbes in aquaculture – Towards a sustainable industry. Microbial Biotechnology 9, 576–584.

Bisanz, J.E., 2018: qiime2R: Importing QIIME2 artifacts and associated data into R sessions. Retrieved September 11, 2019 from https://github.com/jbisanz/qiime2R.

Bolhuis, H., Cretoiu, M.S., Stal, L.J., 2014: Molecular ecology of microbial mats. FEMS Microbial Ecology 90, 335–350.

Bolyen, E., Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S. 2nd, Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. 2019: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37, 852–857.

Casotti, R., Brunet, C., Aronne, B., Ribera d'Alcala, M., 2000: Mesoscale features of phytoplankton and planktonic bacteria in

a coastal area as induced by external water masses. Marine Ecology Progress Series 195, 15–27.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016: DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13, 581–583.

Chomérat, N., Garnier, R., Bertrand, C., Cazaubon, A., 2007: Seasonal succession of cyanoprokaryotes in a hyperetrophic oligo-mesohaline lagoon from the South of France. Estuarine, Coastal and Shelf Science 72, 591-602.

Colyer, C.L., Kinkade, C.S., Viskari, P.J., Landers, J.P., 2005: Analysis of cyanobacterial pigments and proteins by electrophoretic and chromatographic methods. Analytical and Bioanalytical Chemistry 382, 559–569.

Costa, J.A.V., Moreira, J.B., Lucas, B.F., da Silva Braga, V., Cassuriaga, A.P.A., Greque de Morais, M., 2018: Recent Advances and Future Perspectives of PHB Production by Cyanobacteria. Industrial Biotechnology 14, 249–256.

Čanković, M., Žučko, J., Dupčić Radić, I., Janeković, I., Sviličić Petrić, I., Ciglenečki-Jušić, I., Collins, G., 2019: Microbial diversity and long-term geochemical trends in the euxinic zone of a marine, meromictic lake. Systematic and Applied Microbiology 42, 126016.

Di Rienzi, S.C., Sharon, I., Wrighton, K. C., Koren, O., Hug, L. A., Thomas, B.C., Goodrich, J.K., Bell, J.T., Spector, T.D., Banfield, J.F., Ley, R.E., 2013: The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. eLife 2:e01102.

Dragičević, B., Matić-Skoko, S., Dulčić, J., 2017: Trends in Fisheries and Aquatic Animal Health, In Berillis, P. (ed.)., Fish and fisheries of the eastern Adriatic Sea in the light of climate change, 1–22. Bentham e-books, Sharjah

Dvořák, P., Casamatta, D.A., Poulíčková, A., Hašler, P., Ondřej, V., Sanges, R., 2014: *Synechococcus*: 3 Billion Years of Global Dominance. Molecular Ecology 23, 5538–5551.

European Commission, 2008: Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for Community actions in the field of marine environmental policy (Marine Strategy Framework Directive), Official Journal of the European Communities, L164/19.

FAO, 2018: The State of Food and Agriculture 2018. Migration, agriculture and rural development. Rome.

Flombaum, P., Gallegos, J.L., Gordillo, J.F., Rincón, J., Zabala, L.L., Jiao, N., Karl, D.M., Li, W.K.W., Lomas, M.W., Veneziano, D., Vera, C.S., Vrugt, J.A., Martiny, A.C., 2013: Present and future global distributions of the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. Proceedings of the National Academy of Sciences of the United States of America 110, 9824–29.

Folk, R. L., 1954: The distinction between grain size and mineral composition in sedimentary rock nomenclature. Journal Geology 62, 344–356.

Gao, Z., Wang, X., Hannides, A.K., Sansone, F.J., Wang, G., 2011: Impact of redox-stratification on the diversity and distribution of bacterial communities in sandy reef sediments in a microcosm. Chinese Journal of Oceanology and Limnology 29,1209–1223.

Goh, F., Allen, M.A., Leuko, S., Kawaguchi, T., Decho, A.W., Burns, B.P., Neilan, B.A., 2009: Determining the specific microbial populations and their spatial distribution within the stromatolite ecosystem of Shark Bay. Multidisciplinary Journal of Microbial Ecology 3, 383–396.

Golubic S., Seong-Joo L., Browne K.M., 2000: Microbial Sediments, In: Riding R.E., Awramik S.M. (eds.), Cyanobacteria: Architects of Sedimentary Structures, 57–67. Springer, Berlin, Germany.

Gons, H.J., Hakvoort, H., Peters, S.W.M., Simis, S.G.H., 2005: Harmful Cyanobacteria, In: Huisman, J., Matthijs, H.C.P., Visser, P. M. (Eds.), Optical detection of cyanobacterial blooms, 177-199. Springer-Verlag, Berlin/Heidelberg.

Gómez-Baena, G., Rangel, O.A., López-Lozano, A., García-Fernández, J.M., Diez, J. 2009: Stress responses in *Prochlorococcus* MIT9313 vs. SS120 involve differential expression of genes encoding proteases ClpP, FtsH and Lon. Research in Microbiology 160, 567–575

Green, S.J., Jahnke, L.L., 2010: Molecular investigations and experimental manipulations of microbial mats: a view to paleomicrobial ecosystems. In: Seckbach, J., Oren, A Microbial Mats: Modern and Ancient Microorganisms in Stratified Systems, 183–206. Springer, Dordrecht.

Hach, 1997: Water Analysis Handbook, 3rd ed. Hach Company, Loveland, CO.

Hamilton, T.J., Paz-Yepes, J., Morrison, R. A., Palenik, B., Tresguerres, M., 2014: Exposure to bloom-like concentrations of two marine *Synechococcus* cyanobacteria (strains CC9311 and CC9902) differentially alters fish behaviour. Conservation Physiology 2, cou020.

Handelsman, J., 2004: Metagenomics: Application of genomics to uncultured microorganisms. Microbiology and Molecular Biology Reviews 68, 669–685.

Heidelberg, K.B., Gilbert, J., Joint, I., 2010: Marine genomics: At the interface of marine microbial ecology and biodiscovery, Microbial Biotechnology 3, 531–43.

Hirayama, H., Sunamura, M., Takai, K., Nunoura, T., Noguchi, T., Oida, H., Furushima, Y., Yamamoto, H., Oomori, T., Horikoshi, K, 2007: Culture-dependent and -independent characterization of microbial communities associated with a shallow submarine hydrothermal system occurring within a coral reef off Taketomi Island, Japan. Applied Environmental Microbiology 73,7642–7656.

Hirose, Y., Katayama, M., Ohtsubo, Y., Misawa, N., Iioka, E., Suda, W., Oshima, K., Hanaoka, M., Tanaka, K., Eki, T., Ikeuchi, M., Kikuchi, Y., Ishida, M., Hattori, M., 2015: Complete Genome Sequence of Cyanobacterium *Geminocystis* sp. Strain NIES-3709, Which Harbors a Phycoerythrin-Rich Phycobilisome. Genome Announcements 3, e00385-15.

Hugenholtz, P., Tysen, G.W., 2008: Metagenomics. Nature 455, 481–483.

Hunter, E. M., Mills, H. J., Kostka, J. E., 2006: Microbial community diversity associated with carbon and nitrogen cycling in permeable shelf sediments. Applied and environmental microbiology 72, 5689–5701.

Huo, D., Chen, Y., Zheng, T., Liu, X., Zhang, X., Yu, G., Qiao, Z., Li, R., 2018: Characterization of *Microcystis* (Cyanobacteria) genotypes based on the internal transcribed spacer region of rRNA by next-generation sequencing. Frontiers in Microbiology 9, 971.

Ibarbalz, F.M., Henry, N., Brandão, M.C., Martini, S., Busseni, G., Byrne, H., Coelho, L.P., Endo, H., Gasol, J.P., Gregory, A.C., Mahé, F., Rigonato, J., Royo-Llonch, M., Salazar, G., Sanz-Sáez, I., Scalco, E., Soviadan, D., Zayed, A.A., Zingone, A., Labadie, K., Ferland, J., Marec, C., Kandels, S., Picheral, M., Dimier, C., Poulain, J., Pisarev, S., Carmichael, M., Pesant, S., Acinas, S.G., Babin, M., Bork, P., Boss, E., Bowler, C., Cochrane, G., de Vargas, C., Follows, M., Gorsky, G., Grimsley, N., Guidi, L., Hingamp, P., Iudicone, D., Jaillon, O., Kandels, S., Karp-Boss, L., Karsenti, E., Not, F., Ogata, H., Pesant, S., Poulton, N., Raes, J., Sardet, C., Speich, S., Stemmann, L., Sullivan, M.B., Sunagawa, S., Wincker, P., Marcel Babin, Emmanuel Boss, Daniele Iudicone, Olivier Jaillon, Silvia G. Acinas, Ogata, H., Pelletier, E., Stemmann, L., Sullivan, M.B., Sunagawa, S., Bopp, L., de Vargas, C., Karp-Boss, L., Wincker, P.,

Lombard, F., Bowler, C., Zinger, L., 2019: Global trends in marine plankton diversity across kingdoms of life, Cell, e21, 1084–1097.

ISO 11905-1:1997: Water quality – Determination of nitrogen – Part 1: Method using oxidative digestion with peroxodisulfate. Retrieved April 8, 2019 from https://www.iso.org/standard/2155.html

ISO 6878:2004: Water quality – Determination of phosphorus – Ammonium molybdate spectrometric method. Retrieved April 8, 2019 from https://www.iso.org/standard/36917.html

Kadouche, D., Ducatez, M., Cenci, U., Tirtiaux, C., Suzuki, E., Nakamura, Y., Putaux, J.L., Terrasson, A.D., Diaz-Troya, S., Florencio, F.J., Arias, M C., Striebeck, A., Palcic, M., Ball, S.G., Colleoni, C., 2016: Characterization of Function of the GlgA2 Glycogen/Starch Synthase in *Cyanobacterium* sp. Clg1 Highlights convergent evolution of glycogen metabolism into starch granule aggregation. Plant Physiology 171, 1879–1892.

Kolda, A., Petrić, I., Mucko, M., Gottstein, S., Žutinić, P., Goreta, G., Ternjej, I., Rubinić, J., Radišić, M., Gligora Udović, M., 2019: How environment selects: Resilience and survival of microbial mat community within intermittent karst spring Krčić (Croatia). Ecohydrology 12, e2063.

Komárek, J., 2016: A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. European Journal of Phycology 51, 346–353.

Korlević, M., Ristova, P.P., Garić, R., Amann, R., Orlić, S., 2015a: Bacterial diversity in the South Adriatic Sea during a strong, deep winter convection year. Applied Environmental Microbiology 81, 1715–1726.

Korlević, M., Žučko, J., Najdek Dragić, M., Blažina, M., Pustijanac, E., Vojvoda Zeljko, T., Gačeša, R., Baranašić, D., Starčević, A., Diminić, J., Long, P., Cullum, J., Hranueli, D., Orlić, S., 2015b: Bacterial diversity of polluted surface sediments in northern Adriatic Sea determined by pyrosequencing. Systematic and Applied Microbiology 38, 189–197.

Lazzari, R., Baldisserotto, B., 2008: Nitrogen and phosphorus waste in fish farming. Boletim do Instituto de Pesca Sao Paulo 34, 591–600.

Letunic, I., Bork, P., 2019: Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Research 47, W256–W259.

Li, Y., Rao, N.N., Yang, Y., Zhang, Y., Gu, Y.N., 2015: Gene annotation and functional analysis of a newly sequenced *Synechococcus* strain. Genetics and Molecular Research14, 12416–12426

Lund, J. W. G., Kipling, C., Le Cren, E. D., 1958: The Inverted Microscope Method of Estimating Algal Numbers and the Statistical Basis of Estimations by Counting. Hydrobiologia 11, 143–170.

Mandal, S.D., Panda, A.K., Bisht, S.S., Kumar, N.S., 2015: Microbial Ecology in the Era of Next Generation Sequencing. Journal of Next Generation Sequencing & Applications S1, 1–6

Margulis, L., 1970: Origin of eukaryotic cells; evidence and research implications for a theory of the origin and evolution of microbial, plant, and animal cells on the Precambrian earth. Yale University Press, New Haven-London.

McMurdie, P.J., Holmes, S., 2013: phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE. 8, e61217.

Meriluoto, J., Spoof, L., Codd, G.A., 2017: Handbook of cyanobacterial monitoring and cyanotoxin analysis. European Cooperation in the Field of Scientific and Technical Research (Organization), John Wiley & Sons, Chichester.

Miyatake, T., MacGregor, B.J., Boschker, H.T.S., 2013: Depth-Related Differences in Organic Substrate Utilization by Major Microbial Groups in Intertidal Marine Sediment. Microbial Ecology 79, 389–392.

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

Najdek, M., Paliaga, P., Šilović, T., Batistić, M., Garić, R,. Supić, N., Ivančić, I., Ljubimir, S., Korlević, M., Jasprica, N., Hrustić, E., Dupčić-Radić, I., Blažina, M., Orlić, S., 2014: Picoplankton community structure before, during and after convection event in the offshore waters of the southern Adriatic Sea. Biogeosciences 11, 2645–2659.

Negri, A.P, Bunter, O., Jones, B., Llewellyn, L., 2004: Effects of the bloom-forming alga *Trichodesmium erythraeum* on the pearl oyster *Pinctada maxima*. Aquaculture 232, 91–102.

Paerl, H.W., 2012: Ecology of Cyanobacteria II, In Whitton, Brian A. (Ed.), Marine Plankton, 127-153. Springer, Dordrecht.

Paliaga, P., Korlević, M., Ivančić, I., Najdek, M., 2017: Limited influence of primary treated sewage waters on bacterial abundance, production and community composition in coastal seawaters. Marine Environmental Research. 131, 215–226.

Pikelj, K., Jakšić, L., Aščić, Š., Juračić, M., 2016: Characterization of the fine-grained fraction in the surface sediment of the eastern Adriatic channel areas. Acta Adriatica 57, 195–208.

Pikelj, K., Juračić, M., 2013: Eastern Adriatic Coast (EAC): Geomorphology and coastal vulnerability of a karstic coast. Journal of Coastal Research 29, 944–957.

Pulina, S., Padedda, B.M., Sechi, N., Luglie, A., 2011: The dominance of cyanobacteria in Mediterranean hypereutrophic lagoons: a case study of Cabras Lagoon (Sardinia, Italy). Scientia Marina 75, 111–120.

Rocap, G., Larimer, F.W., Lamerdin, J., Malfatti, S. Chain, P., Ahlgren, N.A., Arellano, A., Coleman, M., Hauser, L., Hess, W.R., Johnson, Z.I., Land, M., Lindell, D., Post, A.F., Regala, W., Shah, M., Shaw, S.L., Steglich, C., Sullivan, M.B., Ting, C.S., Tolonen, A., Webb, E.A., Zinser, E.R., Chisholm, S.W., 2003: Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature 424, 1042–1047.

Rossi, F., De Philippis, R., 2015: Role of Cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. Life 5, 1218–1238.

Russo, A., Carniel, S., Sclavo, M., Krzelj, M., 2012: Modern Climatology, In: Wang, S.-Y. S., Gillies, R.R. (Eds.) Climatology of the Northern-Central Adriatic Sea, IntechOpen

Scott, K.M., Henn-Sax, M., Harmer, T.L., Longo, D.L., Frame, C.H., Cavanaugh, C.M., 2007: Kinetic isotope effect and biochemical characterization of form IA RubisCO from the marine cyanobacterium *Prochlorococcus marinus* MIT9313. Limnology and Oceanography 52, 2199–2204.

Sorokin, Y.I., Sorokin, P.Y., Ravagnan, G., 2006: Hypereutrophication events in the Ca'Pisani lagoons associated with intensive aquaculture. Hydrobiologia, 571, 1–15.

Sørensen, K., Glazer, B., Hannides, A., Gaidos, E., 2007: Spatial structure of the microbial community in sandy carbonate sediment. Marine Ecology Progress Series 346, 61–74.

Stal, L.J., 2010: Microphytobenthos as a biogeomorphological force in intertidal sediment stabilization. Ecological Engineering 36, 236–245

Šantić, D., Krstulović, N., Šolić, M., Ordulj, M., Kušpilić, G., 2013: Dynamics of prokaryotic picoplankton community in the central and southern Adriatic Sea (Croatia). Helgoland Marine Research 67, 471–481.

Tamminen, M., Karkman, A., Corander, J., Paulin, L., Virta, M., 2011: Differences in bacterial community composition in Baltic Sea sediment in response to fish farming. Aquaculture 313, 15–23.

Vahtera, E., Conley, D.J., Gustafsson, B., Kouse, H., Pitkänen, H., Savchuk, O.P., Tamminen, T., Viitasalo, M., Voss, M., Wasmund, N., Wulf, F., 2007: Internal ecosystem feedbacks enhance nitrogen-fixing Cyanobacteria blooms and complicate

management in the Baltic Sea. AMBIO: A journal of the Human Environment 36, 186–194.

Whitton, B.A., Potts, M., 2012: Ecology of Cyanobacteria II. In: Whitton, B.A. (Ed.), Introduction to the Cyanobacteria, 1–13. Springer, Dordrecht.

Wickham, H., 2016: ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014: The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Research 42, D643–D648.

Zhang, X., Shu, M., Wang, Y., Fu, L., Li, W., Deng, B., Liang, Q., Shen, W. (2014). Effect of photosynthetic bacteria on water quality and microbiota in grass carp culture. World Journal of Microbiology & Biotechnology 30, 2523–2531.

Zinser, E.R., Johnson, Z.I., Coe, A., Karaca, E., Veneziano, D., Chisholm, S. W., 2007: Influence of light and temperature on *Prochlorococcus* ecotype distributions in the Atlantic Ocean, Limnology and Oceanography 52, 2205–2220.

Zubkov, M. V., Fuchs, B. M., Tarran, G.A, Burkill, P.H., Amann, R., 2003: High rate of uptake of organic nitrogen compounds by *Prochlorococcus* cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Applied and Environmental Microbiology 69, 1299–1304.

Zwirglmaier, K., Jardillier, L., Ostrowski, M., Mazard, S., Garczarek, L., Vaulot, D., Not, F., Massana, R., Ulloa, O., Scanlan, D.J., 2008: Global phylogeography of marine *Synechococcus* and *Prochlorococcus* reveals a distinct partitioning of lineages among oceanic biomes. Environmental Microbiology 10, 147–161.