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Bacterial diversity across a highly stratified ecosystem: A salt-wedge Mediterranean estuary

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ABSTRACT

Highly stratified Mediterranean estuaries are unique environments where the tidal range is low and the tidal currents are almost negligible. The main characteristics of these environments are strong salinity gradients and other environmental parameters. In this study, 454 pyrosequencing of the 16S rRNA gene in combination with catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) was used to estimate the bacterial diversity across the Krka estuary in February and July 2013. The comparison of the data derived from these two techniques resulted in a significant but weak positive correlation ($R = 0.28$) indicating a substantial difference in the bacterial community structure, depending on the applied method. The phytoplankton bloom observed in February was identified as one of the main factors shaping the bacterial community structure between the two environmentally contrasting sampling months. *Roseobacter*, *Bacteroidetes* and *Gammaproteobacteria* differed substantially between February and July. Typical freshwater bacterial classes (*Actinobacteria* and *Betaproteobacteria*) showed strong vertical distribution patterns depending on the salinity gradient. *Cyanobacteria* decreased in abundance in February due to competition with phytoplankton, while the SAR11 clade increased its abundance in July as a result of a better adaptation toward more oligotrophic conditions. The results provided the first detailed insight into the bacterial diversity in a highly stratified Mediterranean karstic estuary.

Introduction

Estuaries are dynamic ecosystems where the interaction of freshwater and seawater leads to the formation of specific environments strongly influenced by a combination of physical, chemical and biological drivers. The riverine input of terrigenous sediments and organic matter, together with the mixing processes related to tides, represent some of the main factors that characterize these environments. Salt-wedge estuaries are restricted to the areas of low tides where a sharp halocline differentiates a freshwater layer and a marine layer resulting in a strong vertical contrast ecosystem [18]. Bacterial production and biomass in estuarine ecosystems has been well studied [22,23,47,49]. However, studies describing estuarine communities are not common and have been mainly based on 16S rRNA sequencing [10,20,33,36] or FISH approaches [1,5,70]. In recent years, next-generation sequencing has been used successfully to examine biogeographic patterns in coastal areas and estuaries [10,20]. Studies describing the diversity of bacteria in estuarine ecosystems have identified freshwater-specific and seawater-specific bacterial communities [14,15]. In addition, they have shown the formation of particular estuarine bacterial communities depending on the freshwater residence time [15]. Freshwater communities were commonly characterized by the presence of *Actinobacteria* and *Betaproteobacteria*, while the marine communities were dominated by *Alphaproteobacteria* (mainly the SAR11 clade). On the other hand, *Bacteroidetes* and *Gammaproteobacteria* did not show a clear relationship with the salinity gradient in estuarine ecosystems [1,5,20,36]. The complex dynamics of environmental parameters in estuarine ecosystems represent a

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challenge for the study of microbial communities, but they also provide a unique system for the investigation of the community changes that occur in these steep gradients.

Highly stratified estuaries are characteristic for the areas of the Mediterranean where the tidal range is low and the tidal currents are almost negligible [18]. The karstic Krka River estuary is situated on the eastern coast of the Adriatic Sea (Croatia). The river is characterized by a low terrigenous input due to its karstic drainage area and a series of travertine barriers, lakes and waterfalls that occur before the 23 km long estuary is reached [43]. The presence of a permanent vertical stratification classifies this estuary as a highly stratified type (salt-wedge estuary) where the depth and thickness of the halocline vary depending on the freshwater input. These features make the Krka estuary suitable for studying the effects of environmental forcing on the formation of an organic matter layer at the halocline [71]. Phytoplankton community dynamics [8,13,60,66], bacterial abundance, activity and biomass [22,23], together with the dynamics of physical and chemical parameters [12,28,38–40,60,71], have been largely studied in the Krka River estuary, but no systematic estimate of bacterial diversity or dynamics has been carried out to date. The distribution of *Synechococcus* and *Prochlorococcus* was only recently determined by Šantić et al. [58] using flow cytometry.

Although estuaries are very dynamic and important environments and the microbial communities in many of them have been described, little data is available specifically on bacterial communities in Mediterranean estuaries [64]. Therefore, this current study describes the bacterial diversity along the Krka River estuary in February and July using a combination of 454 pyrosequencing and catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) techniques.

Materials and methods

Sampling and estimation of environmental parameters

Two cruises with the R/V *Hidra* were conducted in the Krka River estuary in winter (25 February) and summer (8 July) of 2013. Samples were collected at three stations located within the Krka River Estuary: E3, E4a and E5, whereas Station AD3, located outside the estuary, was chosen as a reference marine coastal station (Fig. 1). Sampling depths were determined after obtaining the salinity and temperature profile using the Seabird SBE 19 plus CTD probe (SEA-Bird Electronics Inc., USA). The samples for the determination of bacterial community structure, phytoplankton, nutrients and chlorophyll *a* (Chl *a*) were sampled with 5 L Niskin bottles.

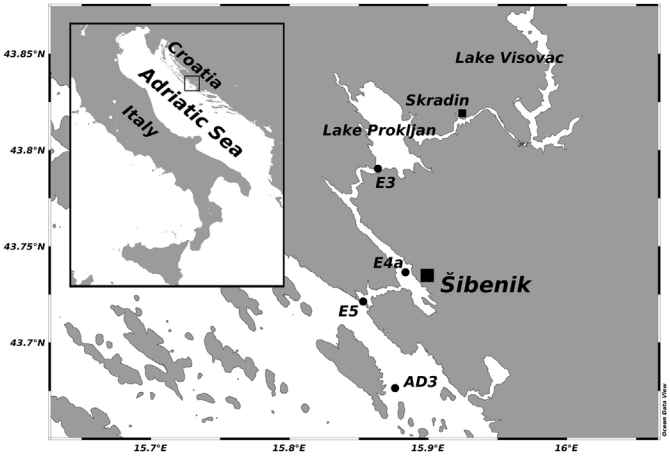

Fig. 1. Study area and sampling stations inside (E3, E4a and E5) and outside (AD3) the Krka estuary.

The concentrations of nitrate (NO_3^-), nitrite (NO_2^-), orthophosphate (PO_4^{3-}) and orthosilicate (SiO_4^{4-}) were determined according to Strickland and Parsons [57], while the concentration of ammonium (NH_4^+) was determined according to Ivančić and Degobbis [32]. Dissolved inorganic nitrogen (DIN) was expressed as the sum of the nitrate, nitrite and ammonia concentrations. The concentrations of particulate (POC; >0.7 μm) and dissolved organic carbon (DOC; <0.7 μm) were determined by a high-temperature catalytic oxidation (HTCO) method [16] with a TOC-VCPH-5000 solid-sample total organic carbon (TOC) analyzer (Shimadzu, Japan) coupled to an SSM-5000A solid-sample combustion unit (Shimadzu, Japan), according to previously described protocols [17,45]. For Chl *a* determination, 1 L of seawater was filtered onto 25 mm GF/F filters (Whatman, UK), stored immediately in liquid nitrogen and afterwards at -80 °C, and the Chl *a* concentrations were determined by reversed-phase high-performance liquid chromatography (HPLC; Spectra System, Model UV 2000) [4]. The samples for phytoplankton analyses were fixed with formaldehyde (2% v/v final concentration) immediately after collection.

Phytoplankton determination

Sub-samples of 50 mL were analyzed by a Zeiss Axiovert 200 inverted microscope (Zeiss, Germany) after sedimentation for 24 h [65]. Cells larger than 20 μm were designated as microphytoplankton, and cells between 2–20 μm as nanophytoplankton.

454 pyrosequencing

Seawater aliquots of 1 L were vacuum-filtered through 0.2 μm Nuclepore™ polycarbonate membrane filters (Whatman, UK) with a peristaltic pump. Filters were stored in 1 mL sucrose buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose), frozen in liquid nitrogen and subsequently stored at -80 °C. The DNA was extracted according to Massana et al. [42]. The bacterial V1-V2 16S rRNA region was amplified using bacterial primers 27Fmod (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519Rmodbio (5′-GTNTTACNGCGGCKGCTG-3′) in four parallel reactions. The primers were modified for 454 pyrosequencing, so that each forward primer contained a gene specific sequence (27Fmod) extended at the 5′-end with a 10 bp barcode sequence (specific for each sample) and an adapter sequence A, while the reverse primer contained a gene specific sequence (519Rmodbio) extended at the 5′-end with an adapter sequence B. Each 25 μL PCR reaction contained: 1× Green GoTaq® Flexi Buffer (Promega, USA), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.15 mg BSA, 0.2 μM of forward and reverse primers, 0.625 U of GoTaq® Flexi DNA Polymerase (Promega, USA) and 20 ng of DNA template. The PCR amplification conditions were: 5 min initial denaturation at 95 °C, 30 cycles of 40 s denaturation at 94 °C, 40 s annealing at 53 °C and 1 min elongation at 70 °C, finalized by 10 min at 70 °C. After pooling of the replicate reactions, PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and sent for GS FLX Titanium and GS FLX+ 454 pyrosequencing at Eurofins (Ebersberg, Germany).

Sequence analysis

Obtained standard flowgram format (SFF) files were extracted using an sff_extract script (available at http://bioinf.comav.upv.es/sff_extract/index.html) applying the sff_extract -c command that allows sequence quality checking. Fasta files were split according to the barcode sequence using mothur [52]. Sequences containing any differences in the barcode or primer sequence were removed in the