

CHARACTERIZATION OF MARINE ORGANIC MATTER IN THE BAY OF BOKA KOTORSKA BY ELECTROCHEMICAL AND ATOMIC FORCE MICROSCOPY IMAGING

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ABSTRACT

In this work is given a brief overview of current application of electrochemical and atomic force microscopy imaging for organic matter characterization in seawater of the bay of Boka Kotorska (south Adriatic sea) in the framework of the bilateral project (Impact assesment and determination of organic pollutants in the waters of the Boka Kotorska). Our particular interest is focused on single and soft organic particles that are not previously studied in this basin. Monitoring of soft particles dynamics in marine system is vital task towards predicting aggregation event, and of importance for tourism and fishery of the region.

Key words: atomic force microscopy, bay of Boka Kotorska, electrochemical imaging, organic matter, organic microparticles

INTRODUCTION

The fate and distribution of organic matter in aquatic system is under influence of different physical and biogeochemical processes. Organic matter of a given chemical composition can occur in a large range of forms

under the influence of the dynamic conditions of particular marine environments: monomer molecules, polymer chains, globules, vesicles and complex networks such as gels (Žutić & Svetličić 2000). Little is known about distribution of environmental particles due to difficulties in sampling, sample handling, insufficient sensitivity and selectivity of analytical methods but also due to the heterogeneity of natural sample (Buffle & Leeuwen 1992). Therefore, seawater sample needs to be unperturbed in order to preserve heterogeneous distribution and primary structure of organic particles. Using traditional oceanographic routine, organic matter in aquatic system had been classified as dissolved and particulate based on filtration through a filter with pore size of 0.5 μm (Golberg et al. 1952). Nowadays, it is known that due to such classification many particle classes remained undetected in spite of their concentration, reactivity and the role in aquatic environment (Koike et al 1990, Wells & Goldberg 1991, 1993, 1994, Alldredge et al. 1993, Long & Azam 1996). About three decades ago, Žutić and coworkers used adhesion-based sensor in discoveries of fluid vesicles in natural waters (Žutić et al. 1984) and of organic film at the freshwater/seawater interface in the Krka estuary (Žutić & Legović 1987). The surface-active particles (SAP) are described as vesicle-like structures formed by self-assembly of polysaccharide, proteinaceous and lipid components of the excretion and decomposition products of phytoplankton (Žutić and Svetličić 2000). Beside of their role as food source, transport of gas, microconstituents, pollutants, nowadays they are considered as precursor particles of mucilage phenomena in the northern Adriatic (more on mucilage phenomena see Gianni et al. 2005) that undergo phase transition to gel phase (Žutić & Svetličić 2000, Svetličić et al. 2005). Here, we will briefly present potentials of surface methods, electrochemical

imaging and atomic force microscopy (AFM) imaging in characterization of marine organic matter. Electrochemical method of amperometry at mercury electrode meets requirements for non-invasive, simple and rapid analysis of aquatic samples suitable for monitoring needs. Such approach enables simultaneous and direct detection of different organic constituents in seawater (e.g. biopolymers, flexible and fluid nonliving and living microparticles) based on their different electrochemical response at the interface, while inorganic particles stay undetected (Ivošević & Žutić 1997, Baldi et al. 1999, Kovač et al. 1999, Svetličić et al. 2006, Ivošević et al. 2007a). Soft fluid-like particles (e.g. organic droplets, living cells, lipid vesicles) in aqueous media can be characterized in terms of concentration, polydispersity, size, reactivity, hydrophobicity and surface charge (Kovač et al. 2000, Svetličić et al. 2001, Svetličić & Hozić 2002, Ivošević et al. 2007b) using amperometry at mercury electrode (electrochemical imaging). Recently, atomic force microscopy (AFM) has been introduced to visualize supramolecular structure of marine gel network and marine biopolymers self-assembly (Mišić Radić et al. 2011). Currently, other marine components such as lipids are under the investigation (Šegota et al. to be submitted). Aim of this study is to combine the two different surface methods (electrochemical imaging and imaging using AFM) covering micro- and nanometric scales to characterize organic matter in the stratified water column of the bay of Boka Kotorska.

MATERIAL AND METHODS

Sampling

Seawater samples were collected in two seasons: spring (April 24, 2013) and late summer (September 19, 2013) at 9 standard stations in the bay of Boka Kotorska (Figure 1).

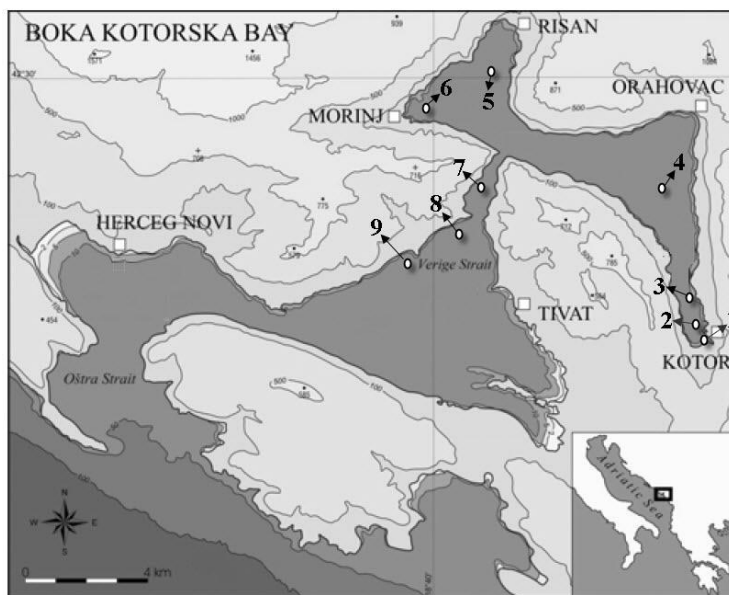


Figure 1. Study area and the sampling stations in the bay of Boka Kotorska (south Adriatic Sea): 1-Gurdić, 2-Škurda, 3-Institute, 4-Kotor, 5-Risan, 6-Morinj, 7-Kamenari, 8-Bijela-fish farms, 9-Bijela shipyard.

The seawater samples were collected with Niskin bottles at 1-5 m depths depending on station. Temperature, salinity, chlorophyll *a* and algae composition were determined by methods commonly used in oceanographic research (Parsons et al. 1985).

Electrochemical method

The chronoamperometry at mercury electrode enables detection of soft organic particle in aquatic samples as described in detail in references (Kovač et al. 2000, Svetličić et al. 2001, Žutić et al. 2004). In principle, a micrometer-sized particle adheres and spread at charged mercury electrode/aqueous electrolyte interface which is registered as well-defined amperometric signal. The signal amplitude reflects a particle size and the signal frequency indicates a particle concentration in seawater sample. The signal frequency is translated into the particle concentration using a corresponding calibration curve with *Dunaliella tertiolecta* cells as standard particles. The polarographic maximum of Hg(II) ions enables determination of adsorbable organic matter (dissolved organic matter and submicron particles) in seawater based on smooth and gradual decrease of reduction current proportional to surfactant concentration in the sample, known as surfactant activity (Zvonarić et al. 1973, Žutić et al. 1977). The surfactant activity of seawater is expressed as the equivalent amount of nonionic synthetic surfactant Triton-X-100 (polyethylene glycol tert-octyl phenyl ether, MW=600) in milligrams per liter. This presents an alternative approach to measure dissolved organic carbon of seawater (Hunter & Liss 1980).

Electrochemical measurements

Electrochemical measurements of seawater samples were performed within 24 hours from the collection. The sample is placed in the electrochemical cell that is opened to air throughout the analysis and thermostated at 20 °C. Dropping mercury electrode had a drop-life of 2.0 seconds, flow-rate of 6.0 mg/s and the maximum surface area of 4.57 mm². All potentials were

referred to an Ag/AgCl (0.1 M NaCl) reference electrode, which was separated from the measured dispersion by a ceramic frit. Its potential was +2 mV vs. calomel electrode (1 M KCl). Electrochemical measurements were performed using a PAR 174A Polarographic Analyzer interfaced to a PC. Analog signals data acquisition was performed with DAQ card-AI-16-XE-50 (National Instruments) input device and the data were analyzed using the application developed in LabView 6.1 software. The current time ($I-t$) curves over 50 mercury drop lives were recorded at the constant potentials of -400 mV. At this potential the dropping mercury electrode is positively charged ($+3.8 \mu\text{C}/\text{cm}^2$) and shows maximum attraction to organic particles adhesion. Surfactant activity of the sample is measured by addition of 500 μL of 0.1 M HgCl_2 in 50 mL of seawater just prior to the measurement.

Atomic force microscopy

AFM connects the nanometer and micrometer length scales utilizing a sharp probe tip that senses interatomic forces acting between the surface of a sample and the atoms at the apex of the tip. The physical basis behind AFM and its ability to „feel“ the surface, make AFM a versatile tool in biophysics allowing high resolution imaging, nanomechanical characterization and measurements of inter and intramolecular forces in living and non-living structures. Thanks to the simple principle on which it is based, the AFM is a surprisingly small and compact instrument. Its use includes electronic control unit, computer and usually two monitors for simultaneous checking of image and imaging parameters. The probe which scans the sample surface consists of a cantilever and the tip located at the free end of a cantilever. The deflection of the cantilever is measured by an

optical detection system. Registered values of cantilever deflection are electronically converted into pseudo 3D image of a sample. AFM is a non-destructive method which gives real 3D images of the sample with a vertical resolution of 0.1 nm and lateral resolution of 1 nm. Measured forces range from 10^{-6} N to 10^{-11} N.

Sample preparation for AFM imaging

The samples were prepared following procedure developed for seawater samples (Mišić Radić et al. 2011). Direct deposition of a whole seawater on freshly cleaved mica and rinsing is the procedure that causes the least impact on the original structures of organic assemblies in seawater. A 5 μ L aliquot of seawater was deposited on freshly cleaved mica sheet mounted on the sample holder, dried for 20 min and then dipped for 30 s in ultrapure water. This procedure was repeated three times and the sample was allowed to dry in an enclosed Petri dish before imaging.

AFM measurements

AFM imaging of seawater sample was performed using MultiMode Scanning Probe Microscope with a Nanoscope IIIa controller (Bruker, Billerica USA) with a vertical engagement (JV) 125 μ m scanner. To minimize the forces of interaction between the tip and the surface during the tapping mode imaging, the ratio of the set point amplitude to the free amplitude (A/A_0) was maintained at 0.8-0.9 (soft tapping). Processing and analysis of images was carried out using the NanoScopeTM software (Digital Instruments, Version V5.31r1). All images are presented as raw data except for the first-order two-dimensional flattening. Imaging was performed in air at 25 °C and humidity of 50–60 % (ambient conditions).

The tapping mode imaging was applied using silicon tips (TESP, Bruker, nom. freq. 320 kHz, nom. spring constant of 42 Nm^{-1}). The linear scanning rate was optimized around 1.0 Hz for the tapping mode with scan resolution of 512 samples per line.

RESULTS AND DISCUSSION

We selected Kotor station as our case study since it is situated in the center of the biggest Kotor bay characterized by karstic rivers, underground springs and diminished human activity. Figure 2 shows depth distributions of temperature and salinity measured in two seasons.

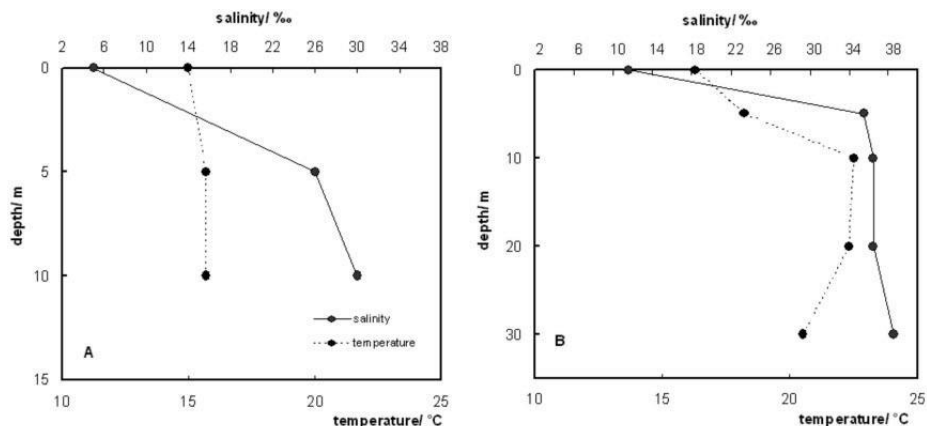


Figure 2. Vertical profiles of temperature and salinity at the station Kotor taken on April 24 (A) and on September 19 2013 (B).

The surface layer temperature was $14.9 \text{ }^{\circ}\text{C}$ and salinity was $4.9 \text{ }_{\text{‰}}$. Below the surface layer, at the 5 m depth, higher salinity was measured, i.e. $26 \text{ }_{\text{‰}}$ (Figure 2A). Seawater was sampled in April only at the surface. Based on long-term monitoring program of the northern Adriatic sea, the highest abundance of reactive organic microparticles is found at the surface layer up to 5 m depth (Project Jadran). The electrochemical measurement

is performed by immersing working electrode (dropping mercury electrode) directly into the electrochemical vessel containing seawater aliquot of 15 mL with salinity adjustment to 35 ‰. Electrochemical responses recorded in seawater samples taken in spring and late summer at the station Kotor are shown on Figure 3.

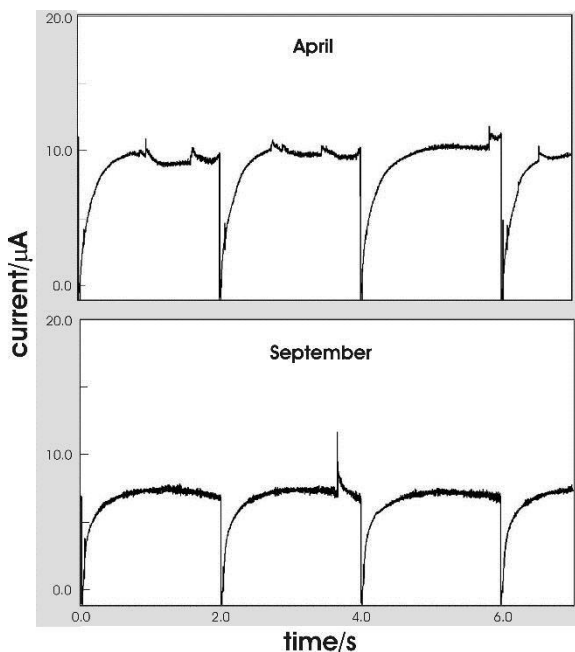


Figure 3. Amperometric signals of microparticles (over three consecutive current-time curves) in the seawater taken at the station Kotor, depth 0 m, on April and on September, are recorded at the potential of -400 mV.

Amperometric signals of SAP are superimposed on the oxygen reduction current. Each amperometric signal corresponds to the detection of single SAP while amplitude variation reflects their size distribution. The irregular appearance of amperometric signals is due to the spatial heterogeneity inherent to dispersed system and stochastic nature of the particles encounter with the electrode. In April, signal amplitude and signal

duration of SAP detected at the station Kotor did not vary significantly and could be related to signals of dinoflagellates and marine vesicles. Electrochemical measurement takes about 2 minutes per sample to detect soft particles in the size range from 1-500 μm and concentration range 10^5 - 10^8 particles/L. Maximum of SAP concentration was determined at the station Kotor ($1.1 \times 10^7/\text{L}$) which was close to the critical microparticle concentration ($\sim 2 \times 10^7/\text{L}$) where large-scale phase transition from dispersed to gel state takes place in the northern Adriatic sea (Svetličić *et al.* 2005). At stations of Risan and Bijela shipyard, concentrations of SAP were about $6.5 \times 10^6/\text{L}$ due to the influence of fish farms and shipyard. Minimum of SAP concentration was determined to be $0.2 \times 10^6/\text{L}$ at the station Gurdić where salinity was minimal (2.8 ‰) due to the freshwater inputs. The number of SAP appears to correlate with chlorophyll *a* data as showed during mesocosm bloom experiment (Žutić *et al.* 2004). Concentration of chlorophyll *a* was located within the limits, typical for spring season, i.e. 2.0 $\mu\text{g}/\text{L}$ at the station Morinj, while increased concentration of 5.0 $\mu\text{g}/\text{L}$ were measured at the station Bijela shipyard (Krivokapić *et al.* 2011). Concentrations of chlorophyll *a* in the northern Adriatic in the spring are ranged from 10-100 ng/L with maximal value up to 2 $\mu\text{g}/\text{L}$ (Mikac 2013). Nutrients (nitrates, nitrites, phosphates, silicates) were within the limits typical for the bay of Boka Kotorska (Campanelli *et al.* 2009). Microphytoplankton abundance was from 10^4 to 10^6 cells/L. Diatom cells (Bacillariophyceae) prevailed in populations microphytoplankton at all stations, with the highest density of 3.42×10^5 cells/L. The highest dinoflagellate abundance was 2.2×10^6 cells/L, while other cell types Coccolithophorids reach value of 10^5 cells/L. The maximum abundance of the silicoflagellate was 560 cells/L (Drakulović

et al. 2012). Dissolved organic matter was characterized as surfactant activity. The surfactant activity of seawater samples varied from 0.84 to 1.58 mg/L in April, with the highest value measured at stations of Škudra and Bijela shipyard. Surface coverage of electrode (Θ) by surface-active dissolved organic matter was 0.25 for sample collected in April (Ivošević & Žutić 1997, Kovač et al. 1999, Žutić et al. 1999).

In late summer, salinity was higher on the surface than in previous sampling period, due to very dry season (Figure 2B). Figure 3 shows typical electrochemical response recorded in seawater sample taken on September, where signal count significantly decreases and oxygen reduction current decreases almost to the diffusion controlled value. The reason for this is increased content of adsorbable organic matter present in the sample. Surface coverage of electrode (Θ) by surface-active dissolved organic matter was 0.88 for sample collected in September. Concentration of SAP at the station Kotor was one order of magnitude lower (1.0×10^6 /L), while surfactant activity was two times higher than in the spring season (i.e. 2.28 mg/L). Maximum of SAP concentration and surfactant activity was measured at the station Škudra at 10 m depth, i.e. 2.3×10^6 /L and 3.29 mg/L, respectively. We noticed that surfactant activity was about 20 % higher on the western part of the bay (from station Risan to Bijela) mainly because of fish farms location, shipyard and regular ferry line. On the eastern part of the bay, at the station Škudra, surfactant activity was high in both seasons probably due to the cruiser anchoring and the partial discharge of sewage with streams flood. According to the long-term data of the northern Adriatic, elevated value of surfactant activity was observed usually towards the end of the summer if aggregation event did not appear and large amount of organic matter persists in the aquatic system. Dautović et al. 2012

reported that concentrations of dissolved organic carbon (DOC) in the bay of Boka Kotorska increase from spring to autumn months, with the values ranging from 0.64 to 1.43 mg C L⁻¹ as measured during 2008 and 2009.

The samples of particular interest, after being characterized electrochemically, were imaged with AFM. Figure 4 (A) represents the topographic view of seawater sample collected from the station Kotor in spring and imaged in air.

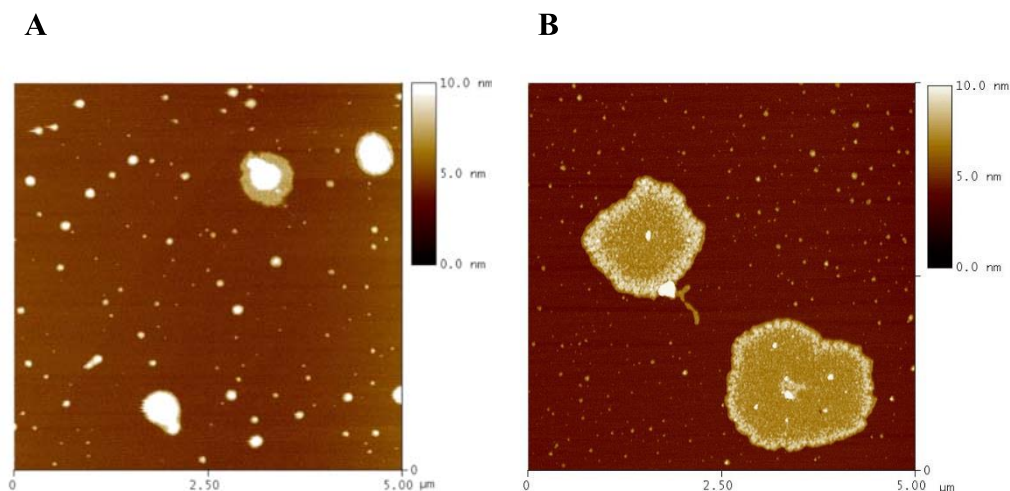


Figure 4. AFM images of seawater sample taken at station Kotor, depth 0 m, in spring season. Images are presented as height data with vertical scales of 10 nm.

In the Figure 4A, the dome-shaped particles adhered to mica surface with diameter ranging from 600 nm to 750 nm with the maximum height from 15 nm to 20 nm. They most likely represent vesicle-like structures spreaded over hydrophilic surface (Svetličić *et al.* 2008). The measured heights of the spreaded layer surrounding the particle were between 2.9 nm and 3.2 nm, corresponding to the thickness of the lipid bilayer captured in air (Kanno *et al.* 2002). The fully spread particles were also encountered as shown in Figure 4B. They appears as flat patches from 1 μm to 2 μm in diameter with pronounced rim and the average height of 2.5 nm that again

could correspond to the thickness of the lipid bilayer. Seawater sample collected at the station Kotor in late summer is imaged by AFM (Figure 5).

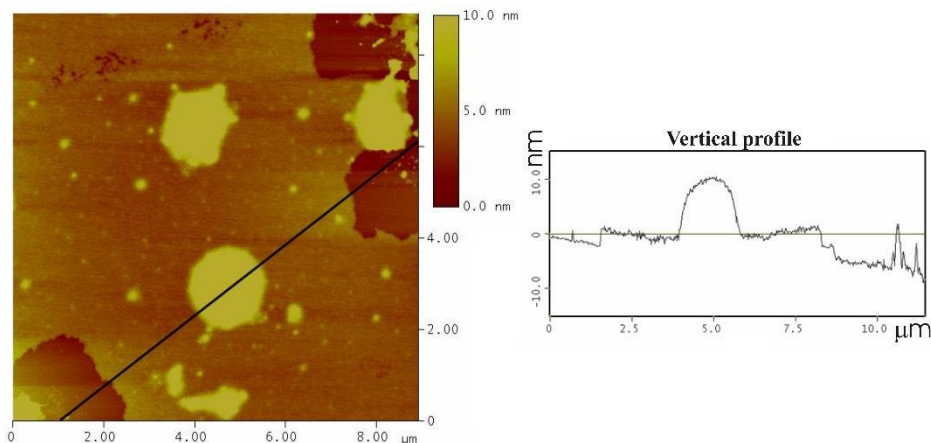


Figure 5. AFM image of seawater sample taken at the station Kotor, depth 0 m, in late summer. Vertical profile is shown along indicating line.

In this picture the dome-shaped particles are within continuous layer with thickness of 2.3 nm. Diameter of the dome-shaped particle is 2 μm and height of 10 nm. This finding is in agreement with electrochemical analysis in terms of particle size and dissolved organic content.

CONCLUSION

The combination of complementary surface methods extends the characterization of organic constituents down to nanometric scale providing important insight into their fate in aquatic system. The current data most likely reflect seasonal dynamics of marine organic matter in the bay. In spring, large input of freshwater and consequently stratification of water column were present. We detected high concentration of surface-active particles in surface layer which is in agreements with chlorophyll *a* data. In contrast, in late summer season, input of freshwater was minimal. The

concentration of surface-active particles was one order of magnitude lower while surfactant activity was two times higher than in the spring season. Atomic force microscopy imaging offers direct visualization of submicron-sized surface-active particles in the near natural environments. Due to highly reactive nature of organic matter to interact at many interfaces in water column, a long-term measurement of relevant parameters are needed in order to better understand spatio-temporal distribution of organic matter in aquatic system towards forecasting of aggregation event. Such study would be of great benefits towards environmental protection, development and management of the bay of Boka Kotorska, especially for tourism, fishery and cruiser trafficking.

ACKNOWLEDGMENT

This work is primarily supported by the Croatian–Montenegro bilateral program of cooperation in Science and Technology through the bilateral projects (0411B016 and 01321), with additional funding through the projects 098-0982934-2744 by the Croatian Ministry of Science, Education and Sports, and National Scientific Project (01-588) provided by the Montenegro Ministry of Science. Authors thank Dragana Drakulović and Branka Pestorić for providing phytoplankton and chlorophyll *a* data. Thanks to Captain of R/V *Nemirna2* and to Milena Mitrić, Sandra Marković and Rajko Martinović for their help during field work.

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