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The anoxic stress conditions explored at the nanoscale by atomic force microscopy in highly eutrophic and sulfidic marine lake



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ABSTRACT

Marine Rogoznica lake (RL), an eutrophic and euxinic environment situated at the eastern Adriatic coast was used as a natural laboratory to test the application of atomic force microscopy (AFM) in combination with the electrochemical and high temperature catalytic oxidation (HTCO) measurements for characterization of water column organic matter (OM) and reduced sulfur species (RSS) in relation to seasonal changes of environmental conditions. Water column of RL was explored at the nanoscale by the AFM during the anoxic holomictic event (S1, October 2011) and stratified winter (S2, January 2012) and spring (S3, May 2012) conditions in the oxic and anoxic water samples. Obtained results from the AFM uphold the electrochemical and HTCO measurements, indicating significant difference in the present type of OM during the holomictic, anoxic stress conditions in comparison to the samples collected during the stratification period. Differences in the OM type were discussed in line with the physical and biological processes that occurred in RL during sampling: mixing processes characterized by fast turnover of water layers and biological activity characterized by low (January 2012) and high (May 2012) primary production of diatoms and zooplankton grazing activities.

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1. Introduction

Oxygen deficiency i.e. hypoxia or anoxia occurs throughout the world in the coastal waters and areas of the coastal ocean where oxygen is low or absent in bottom waters. In many modern coastal systems, anthropogenic changes are superimposed on natural variation and increased input of nutrients from anthropogenic sources affect the natural systems, enhancing the algal blooms, and increase in OM flux (Eldridge and Roelke, 2011; Paerl, 2006). Therefore, so-called dead zones are expanding worldwide nowadays. The sinking of OM and subsequent decay leads to high demand in oxygen what can cause periodic or permanent water column hypoxia (0.2 $mg/l < [O_2] < 2mg/l)$, anoxia $([O_2] < 0.2 mg/l)$ or even euxinia ([O₂] < 0.2 mg/l, free [HS⁻]) (Eldridge and Roelke, 2011; Neretin, 2006). Anoxia normally occurs in enclosed basins (including fjordtype estuaries, seawater lakes, and anchialine caves) where physical barriers and density stratification limits the advection of O₂ to the deep waters and remineralization processes enhance deposition of the OM and nutrients in the water column and sediments (Eldridge and Roelke, 2011; Neretin, 2006).

RL, also known as the Dragon Eye, is a unique example of small and shallow (circular shape with an area of 10.276 m², maximum length of 143 m, and a maximum depth of 15 m in the middle of the lake), euxinic and eutrophic marine environment, situated on the Gradina Peninsula at the eastern coast of the Adriatic Sea (43°32'N, 15°58'E middle Dalmatia, Fig. 1a) (Ciglenečki et al., 2015 and references therein). Due to the high phytoplankton activity upper part of the water column is well oxygenated while remineralization processes enhance deposition of organic matter (DOC up to 6 mg/l), and nutrients (NH₄⁺, up to 315 μ M; PO₄³⁻, up to 53 μ M; and SiO₄⁴⁻, up to $680 \,\mu\text{M}$) in hypoxic/anoxic part of the water column where milimolar concentration of sulfide is confirmed (this study and Kršinić et al., 2000; Ciglenečki et al., 2005, 2015; Bura-Nakić et al., 2009; Žic et al., 2013; Pjevac et al., 2015 and references therein). Existence of a strong thermohaline and redox conditions i.e. chemocline can be found at the oxia/anoxia boundary. Around chemocline, a layer with dense population of sulfide oxidizing bacteria (SB) mainly genus Chromatium (purple SB, PSB) and Chlorobi (green SB, GSB) is situated. Population density of GSB is much higher but due to the size of individual bacterial PSB cell, this layer has a purple color (Ciglenčki et al., 1998; Pjevac et al., 2015). There is no direct connection between RL and surrounding sea, however tides are visible on the rocks, what indicates water intrusion through porous carbonate (Žic et al., 2013). The lake is under strong influence of



Fig. 1. (a) Map indicating position of RL and (b) photo of the RL surface water taken during holomictic and stratified conditions. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

atmospheric i.e. meteorological conditions (Ciglenečki et al., 2015) which influence stratification and water column stability, as well position of chemocline. The only input of fresh water to RL is through precipitation.

The mixing between water layers depends on the meteorological conditions which highly influences its intensity and usually can be detected in autumn as a result of termohaline dissolution and stratification breakdown inducing holomictic conditions. If the typical stratification breakdown is accompanied by the fast turnover of the water layers, triggered by extreme weather events (Ciglenečki et al., 2015), fast transport of highly sulfidic bottom water to the surface may cause consumption of all dissolved oxygen and occurrence of anoxic conditions in the whole lake (Bura-Nakić et al., 2012; Ciglenečki et al., 2005). Under these circumstances the presence of different reduced sulfur species (RSS - sulfide, polysulfide and colloidal sulfur formed by oxidation) and relatively high nutrient concentration can be found throughout the water column of RL (Bura-Nakić et al., 2009, 2012; Žic et al., 2013; Ciglenečki et al., 2015 and references therein). Rapid precipitation of colloidal sulfur influence change in the lake's surface color, which in contrast to stratified conditions when the water transparency is high, is non-transparent milky-yellowish (Fig. 1b). If the mixing process is relatively slow, driven only by the termohaline dissolution and vertical diffusion, oxygenated surface waters can diffuse to the bottom anoxic layer, or anoxic bottom waters rich in sulfide and nutrients can diffuse to the surface. In this case the mixing process can last up to 7 days, and may result in oxygenation of the entire water column (Ciglenečki

et al., 2015). Upward, transport of ammonium which is facilitating biologically mediated nitrification is considered to be primarily source of high nitrate concentrations (up to $30 \,\mu$ M) that can be usually detected in the entire water column during winter and spring months. The eutrophication of the lake is strongly influenced by nutrient recycling under anaerobic conditions (Žic et al., 2013; Ciglenečki et al., 2015 and references therein).

Seasonal variation of basic physico-chemical parameters (temperature, salinity, and dissolved oxygen) measured in RL water column from middle of the lake, during 2011 and 2012 are presented in Fig. 2, where stratification breakdown and establishment of holomixis and new stratification cycle are clearly visible.

During the last 20 years of our research activities in RL (1994 – up to day), the anoxic and sulfidic holomictic events were followed twice, in September 1997 and October 2011 (Bura-Nakić et al., 2009, 2012; Ciglenečki et al., 2015). In both occasions high sulfide and ammonium concentrations that were detected through the entire water column, lead to mass mortality of all phytoplankton and benthic organisms (Kršinić et al., 2000; Barić et al., 2003; Ciglenečki et al., 2005). Mass mortality contributed to increased nutrient concentration in RL, even in the surface layer that remained high for several months after holomictic anoxic event due to remineralization processes, decomposition of large amount of particulate organic carbon (POC) and lack of intensive primary production (Kršinić et al., 2000; Barić et al., 2003; Ciglenečki et al., 2005).

Influenced by the work of the Svetličić group who introduced



Fig. 2. Seasonal variation of physico-chemical parameters, temperature, salinity and dissolved oxygen in RL water column in 2011 and 2012.

application of the AFM for the marine ecology studies, including visualization of supramolecular structure of marine gels, and marine biopolimers self-assembly in the North Adriatic (Mišić-Radić et al., 2011; Pletikapić et al., 2012a, 2012b; Svetličić et al., 2005, 2011, 2013; Urbani et al., 2012), in this paper for the first time anoxic stress conditions were explored at the nanoscale in RL water column by use of the AFM. Characteristics of the water column were studied during holomictic anoxic event (S1) in October 2011 and the stratified winter (S2, January 2012) and spring (S3, May 2012) conditions, including highly eutrophic oxic and anoxic lake samples. OM and RSS presence during the selected events were in parallel characterized by standard electrochemical measurements of surface active substances (SAS) and RSS at the Hg electrode (Bura-Nakić et al., 2009; Ciglenečki et al., 1996, 2005; Ćosović and Ciglenečki, 1997; Ćosović and Vojvodić, 1998) while the content of dissolved and particulate organic matter (DOC, POC) was determined by high temperature catalytic oxidation (HTCO) measurements (Dafner and Wangersky, 2002). Obtained results indicate how a combination of selected surface methodology with nano- to microscale imaging, i.e. state-of-the art AFM technique and electroanalytical voltammetric methods could be a good choice for studying different biogeochemical processes in the

marine environment, especially those related with OM and sulfur species cycling, interaction and their distribution between dissolved and colloidal phases.

2. Experimental

2.1. Measurements of physico-chemical conditions in Rogoznica lake

The salinity, temperature, pH, and dissolved oxygen values were recorded by the CTD probe (HQ40d Portable Meter, HACH, USA) in situ during sampling from the middle of RL. Lake samples were collected by 5 L Niskin bottles in two distinct periods: during the holomictic anoxic event in October 2011 (S1), and during the stratified conditions in the water column in January (S2) and May 2012 (S3) at various depths, considering both oxic and anoxic water samples, as already described (Bura-Nakić et al., 2009; Ciglenečki et al., 1996, 2005, 2015; Ćosović et al., 2000; Pjevac et al., 2015).

2.2. Electrochemical measurements

The concentration of reduced sulfur species based on

acidification step (Ciglenečki and Ćosović, 1997; Bura-Nakić et al., 2009) was determined as total RSS (RSS_t) including volatile (RSS_v, mainly composed of sulfide) and non-volatile (RSS_{nv}, mainly composed of elemental sulfur and non-purgeable organosulfur compounds) fraction in non-filtered samples by use of the cathodic stripping voltammetry. An Autolab PGSTAT 128N instrument connected to 663VA Stand (Methrom, Switzerland) were used. Measurements were performed in three electrode cell purged with N₂. The Hg electrode was used as working electrode, and Ag/AgCl (3 M KCl) and carbon rod was used as a reference and auxiliary electrode, respectively.

AC voltammetric measurements (out of phase mode) for determination of surface active substances (SAS) in filtered and nonfiltered samples were performed with BAS 100A (BASi, USA) connected to manually controlled hanging mercury drop electrode (HMDE) as a working electrode. The change of the capacitive current due to SAS adsorption on the Hg electrode was recorded at a -0.6 V (vs. Ag/AgCl) after a certain accumulation time, following the work of Ćosović and Vojvodić (1998) and Ćosović and Ciglenečki (1997). The decrease in the capacitive current was related to the concentration of SAS in the samples as an equivalent to nonionic surfactant Triton-X-100 (mg/l).

2.3. Dissolved and particulate organic matter measurements (DOC, POC)

The DOC concentrations were analyzed in duplicate using the sensitive high temperature catalytic oxidation (HTCO) method (Dafner and Wangersky, 2002) on a Model TOC-VCPH (Shimadzu) with silica catalyst (Elemental Microanalysis, UK) and non-dispersive infrared (NDIR) detector for CO_2 as already described in Dautović et al. (2012). POC was analyzed with a solid sample module SSM-5000A associated with a TOC-VCPH carbon analyzer, calibrated with glucose. Prior to analysis, filtration of the samples were performed on Whatman GF/F glass fiber filters, pore size 0.7 μ m, previously combusted for 4 h at 450 °C to separate POC and DOC fraction.

2.4. Microscopic measurements

The samples of RL water for AFM imaging were taken at different events (S1–S3), filtered through the 0.2 μ m Whatman GF/F filter and stored at 4 °C until the imaging was performed. Prior to the imaging the filter was rinsed with a small volume of the MQ water to resuspend the material from the filter. The drop deposition method was used to transfer the 5 μ L of resuspended sample on freshly flame annealed Au(111) electrode which was left to dry under laboratory atmosphere conditions. The samples prepared for the AFM after the imaging were used for the SEM imaging and the EDS analysis.

AFM (Nanoscope III, Digital Instruments, USA) was used for visualization of the RL samples on the Au(111) surface. Imaging was performed ex-situ under laboratory atmosphere conditions, with operating the AFM in the "tapping" mode using standard silicon probes (TESP-tips) provided by Digital Instruments (Batina et al., 2003). Tips were 125 µm long and had typical resonance frequencies between 294 and 375 kHz. Typically, scanning started at 40 μ m \times 40 μ m areas, and gradually the image size was reduced to $1 \,\mu\text{m} \times 1 \,\mu\text{m}$. All AFM images were recorded during the slow scan rate (1 Hz), to avoid sample damaging. The images have been recorded in the height mode, which is related to the sample surface topography. Images presented in the paper are shown in the top-view mode. Quantitative evaluation of particle dimensions was performed using the Nanoscope software. Scanning electron micrographs were obtained by JEOL7600F scanning electron microscope (SEM) (Jeol, USA) equipped with Energy dispersive X-ray spectroscopy (EDS) detector (Oxford, UK) for elemental analysis.

3. Results and discussion

3.1. Holomictic event

In October 2011 drastic change in the atmospheric conditions influence rapid cooling of the surface water. Sudden drop of air temperature (for 15 °C in less than 12 h) accompanied by strong bora wind provoked stratification breakdown and fast turnover of the water layers (Ciglenečki et al., 2015). Mixing of the water layers ended by formation of holomictic conditions with appearance of anoxia in the entire water column. During holomictic event (S1) uniform distribution of the different physico-chemical parameters were measured in the water column of the lake: salinity reaching 37, temperature of 21 °C, and oxygen ranging 0–0.86 mg/l resulting in small variations in observed concentrations of RSS, DOC and POC with depth (Fig. 3a and b). In such conditions, concentrations of RSSt were same order of magnitude as RSSnv and ranged between 6×10^{-8} and 1.7×10^{-7} M (Fig. 3a). During the holomictic event, difference between RSS_t and RSS_{nv} diminished compared to the stratified water column conditions. Small differences between usually high concentration of volatile fraction of reduced sulfur species (RSS_v), mainly attributed to sulfide (Bura-Nakić et al., 2009; Ciglenečki et al., 2005), and RSS_{nv} is a consequence of rapid chemical oxidation of sulfide and precipitation of the colloidal elemental sulfur (S°) throughout the water column (Bura-Nakić et al., 2009, 2012; Ciglenečki et al., 2005). In stratified conditions hydrogen sulfide can be oxidized to zero-valent sulfur and other intermediate sulfur compounds by phototrophic SB (Overmann, 2006; Canfield et al., 2005; Kamyshny et al., 2011). Presence of SB was confirmed in the RL chemocline layer during stratification period, interestly during the holomictic event the GSB and PSB were not detected in the water column (Ciglenečki et al., 1998; Pjevac et al., 2015). These organisms (primarily GSB and PSB) can also reoxidize S° and number of other reduced sulfur compounds (including thiosulfate) to sulfate. However, the most dominant oxidized form is S°, especally in rapid chemical oxidation, similar as recorded in RL in September 1997 and October 2011 (Fig. 1b).

The distribution of DOC and POC in S1 (Fig. 3b) was relatively uniform and with higher concentration than usually recorded in this period of year (Ciglenečki et al., 2015), average values of 2.092 mg C/l and of 0.73 mg/l, respectively were recorded. The contribution of POC to the total organic carbon (TOC) varied between 23.4% and 31.2%. Increased concentration of DOC - POC, and SAS as well, recorded in the entire water column could be consequence of two processes: (1) upwelling of anoxic water enriched with suspended OM containing detritus, sediment particles, dissolved OM, nutrients and RSS from the bottom to the oxic part of the lake and (2) release of the huge amount of dissolved and particulate OM due to anoxic stress and euxinic conditions that provoked phytoplankton cell lysis, similar as reported for benthic diatoms incubated in the sulfidic conditions (Ciglenečki et al., 2003). As a consequence of upwelling, surface water enrichment with_OM and nutrients lead to a pronounced primary production of selected and rare phytoplankton species that occurred in later months in RL, followed by the pronounced grazing activities, as recorded in January 2012 (S2) (Ciglenečki et al., 2015).

Despite of the uniform DOC and POC distribution, SAS measurements and AFM imaging show different concentration and type of the OM present in the samples collected at the 2 m and 11 m depth during the S1 event (Fig. 3c–f). The images of 2 m depth sample (Fig. 3c, d) revealed aggregates with the branched structure at the larger scale (data not shown) with decreasing the







Fig. 3. (a, b) Physico-chemical parameters of the water column in RL measured during the holomictic, S1 event (October, 2011); (c, d) AFM images of 2 m depth sample, recorded with magnification of (c) $6.2 \ \mu m \times 6.2 \ \mu m$ and (d) $2.1 \ \mu m \times 2.1 \ \mu m$; (e, f) AFM images of 9 m depth sample, recorded with magnification of (e) $10.0 \ \mu m \times 10.0 \ \mu m$ and (f) $3.6 \ \mu m \times 3.6 \ \mu m$.

scanned surface during imaging, aggregates of 250–300 nm width and 70–80 nm height, mainly following the edges of the Au(111) grains are reveled. Around aggregates a flat Au(111) terraces are covered with small nanoparticles (NPs), 10–30 nm width and 10– 20 nm height. In deeper part of the lake, at 11 m depth, the material separated in the spherical aggregates with diameters from 130 to 140 nm was dominant feature on the images (Fig. 3e–f). However, formation of the aggregates is not preferably observed only at the edges of the Au(111) grain as previously detected at 2 m depth. The aggregates form some kind of growth along the edges of Au(111) terraces but most of the surface is covered with spherical NPs, 4–49 nm diameter and 6–50 nm high. The imaged



Fig. 4. (a, b) Physico-chemical parameters of the water column in RL measured during the stratified, S2 event (January, 2012); (c, d) AFM images of 5 m depth sample, recorded with magnification of (c) 16.7 μ m × 16.7 μ m and (d) 2.1 μ m × 2.1 μ m; (e, f) AFM images of 11 m sample, recorded with magnification of (e) 9.9 μ m × 9.9 μ m and (f) 1.1 μ m × 1.1 μ m; squares – represents NaCl, and ovals – represents inorganic NPs or HS NPs.

aggregates accumulated near the edges of the Au(111) grain at 2 m depth sample are in accordance with SAS data, probably reflecting more hydrophilic character of the OM. The distinctive difference in the shape of the imaged aggregates at various depths indicates two types of OM present during the S1 holomictic event. At 2 m depth sample the shape and size of smaller particles which are forming the aggregate were clearly distinguishable, while

aggregates imaged at 11 m depth sample resemble gel phase. Most of the gel forming biopolymers are macromolecules which form stable self-assembling networks in which NPs can be imbedded (Buffle et al., 1998; Lead et al., 2005; Lead and Wilkinson, 2006; Pletikapić et al., 2012a). It was expected that the colloidal S° formed during the S1 would react with the OM and be imbedded in the aggregates. However, the EDS analysis of the samples



Fig. 5. (a, b) Physico-chemical parameters of the water column in RL measured during the stratified, S3 event (May, 2012); (c–f) AFM images of samples collected at (c) 2 m (magnification 15.8 μ m × 15.8 μ m), (d) 5 m (magnification 17.2 μ m × 17.2 μ m) and (e, f) 9 m depths (magnification 13.2 μ m × 13.2 μ m and 2.5 μ m × 2.5 μ m); squares – represents NaCl, and ovals – represents inorganic or HS NPs.

imaged by AFM did not reveal the sulfur presence. Also the EDS analysis of the remained filters after sample preparation for AFM imaging revealed very low concentration of the S° in their porous structure, less than 1% w/w. We assume that formed colloidal S° could have different fate in RL water column. It can be stabilized by solubilization through interaction with the hydrophobic OM as already obtained in the seawater mixture containing elemental sulfur and humic acid (Bura-Nakić et al., 2009); interaction with nucleophil groups of OM could transform it to cross-linked sulfopolysulfides (Amrani and Aizenshtat, 2004; Bura-Nakić et al., 2009) or it can undergo fast aggregation and sedimentation due to high ionic strength conditions. Recorded physico-chemical conditions in the lake together with AFM images, supports an idea that the solubilization and formation of sulfopolysulfides are more feasible processes.

Some small features in AFM images presented in Fig. 3c-f in accordance with EDS data, probably reflects presence of NaCl crystals, humic substances (HS), and inorganic NPs resuspended from the sediment during mixing process. Clearly, two different types of NPs were imaged, some of cubic shape (probably NaCl) and other more spherical (probably HS and inorganic NPs). Sample collected at the deeper part of the water column shows noticeable increase (3-4 times) in the NPs concentration on the Au terraces (Fig. 3d and f), indicating stronger interaction and already assumed transfer of the inorganic NPs or HS resuspended from the sediment during the mixing event. In the literature the reported diameter of the single HS NPs is between 2.5 and 13 nm in diameter and the highest range is from 1 to 10 nm (Baalousha and Lead, 2013; Chena et al., 2007; Wilkinson et al., 1999). It is interesting that in the most of the checked literature where the HS was explored with the microscopic techniques, formation of the HS aggregates was observed but the 3D growth was absent (Baalousha and Lead, 2013; Chena et al., 2007; Wilkinson et al., 1999).

3.2. Stratified conditions

Sampling events S2 (Fig. 4a, b) and S3 (Fig. 5a, b), show distinctive differences in the physico-chemical parameters measured within the RL's water column, with evident strong thermohaline stratification accompanied by chemocline both situated at 5 m depth during S2 and at 8 m depth during S3. The chemocline was situated unusually high, especially in S2 event if compared with previously reported data (Bura-Nakić et al., 2009; Ciglenečki et al., 2005). Chemocline usually coincides with thermohaline position in RL which usually is placed around 8–9 m depth, with a tendency to move deeper during the summer–autumn period. Sun heating, evaporation and absence of the rainfall precipitation influence weakening of the water column stratification and in the same time suppress the anoxic water layer during summer (Ciglenečki et al., 2005, 2015). However, similar situation was not recorded in 2012 (Fig. 2).

3.2.1. Winter time

In S2 event (Fig. 4) the oxic water layer was characterized with lower temperature, 6–10 °C, and salinity, 30–31, while in the anoxic layer uniform temperature of 17 °C and salinity around 38 were measured. The oxic part (0–3 m) was slightly saturated with oxygen (71–123%) and RSS_t, dominantly in RSS_{nv} form, did not exceed 1×10^{-8} M. In the anoxic part (5–13 m) the oxygen concentrations dropped to 0 mg/l and accordingly, the RSS_t mostly present in the form of free sulfide increased up to 8.1×10^{-5} M. The concentration of DOC and POC for the S2 event had an average value of 1.44 mg C/l and 1.061 mg C/l, respectively, with POC reaching up to the 57% of TOC. Maximum POC value of 1.4 mg C/l were measured around the chemocline which was situated at 5 m depth. In accordance with previous reports POC, DOC and SAS maximum values were always recorded at the chemocline in stratified conditions (Ciglenečki et al., 2005; Ćosović et al., 2000; Penezić et al., 2010: Pievac et al., 2015: Šestanović et al., 2005). DOC concentrations were higher than typically measured during winter months (Ciglenečki et al., 2005; Ćosović et al., 2000) and displayed no depth variations. Such relatively high DOC could be explained as a consequence of increased herbivore grazing activity that was recorded in the lake water column in January 2012 (Ciglenečki et al., 2015). With grazing, significant fraction of OM can be released in the water column through fecal material, excretion by metazoan zooplankton and release of organic substrates of phytoplanktonic origin during 'sloppy feeding' (Richardot et al., 2001), which have been already discussed as important sources of OM in RL (Ciglenečki et al., 2005). On the other side increased POC concentrations could be an indicator of the relatively high bacterial activity (Pjevac et al., 2015; Šestanović et al., 2005) as well as pronounced remineralization of the freshly produced OM in the surface water after mixing event in S1.

AFM images of samples collected during S2 event at 5 m depth show presence of the aggregates on the edges and the big spherical features which had length of several microns, $1-7 \mu m$, a width of 500 nm, and height of 200-300 nm (Fig. 4c). These structures were not observed in the samples collected during the holomictic S1 event. Sample taken at 5 m depth with maximum accumulation of POC is closely related to intense microbial and chemical activity at the chemocline, which is an ideal surrounding for release of extracellular polymeric substances (EPS). Previous RL's studies showed that bacterial community has a high abundance at the chemocline but with rather low productivity per cell, while in the surface layer there is a low abundance of bacteria with a high productivity (Pjevac et al., 2015; Šestanović et al., 2005). In the literature, dimensions of the bacteria captured under AFM have dimensions exceeding the 1 µm (Obst and Dittrich, 2005; Schilardi et al., 2010), for the Chlorobi bacterial cell the reported size range is 1–3 µm (Pjevac et al., 2015; Overmann, 2006). From the revealed AFM images taken at the larger magnification $(35 \,\mu m \times 35 \,\mu m)$ it is possible that some of the larger features represent bacteria. With higher magnification only smaller features were visible, spreading all over the Au grains with the densely packed surface (Fig. 4d). Spherical features have a diameter of 24 nm, while rectangular elongated features about 80-83 nm length and 43 nm width, with heights ranging from 10 to 20 nm for both shapes. It is interesting that the edges of the grain which usually attracts the hydrophilic features are free of the material. In the more enlarged image, it is revealed that the NPs are formed at the terraces of the Au, what reflects probable presence of NaCl, inorganic and HS NPs. The SEM image (Fig. 6) indicates exactly the same as AFM, huge aggregates distributed over the Au grain surface. EDS analysis of the samples during SEM imaging showed that the big aggregates are composed of: 23.82% of C, 10.76% N, 8.64% of O, 8.52% of Na, 6.79% Cl, 3.16% Ni, and 38.30% Au (Ni and Au are present due to the characteristics of the substrate used for imaging, Fig. 5a).

There is a distinctive difference between the images obtained from the 5 m (oxic) (Fig. 4c, d) and 9 m (anoxic) (Fig. 4e, f) depth in the S2 event. The images taken form 9 m sample does not show aggregates of micron sizes. The large features mostly have a width of 500–515 nm and height of 270–290 nm, and mostly are developed close to the edge of the grain. Elemental analysis of 9 m sample compared to 5 m sample showed higher percentage of C (53.90%), O (14.84%), Na (8.54%), Cl (7.89%), and Au (14.83%, Au is from substrate used during imaging) in larger aggregates (Fig. 4b). Increased content of carbon can be explained as a consequence of very fast transport of OM through the relative shallow water column of RL and its accumulation in the anoxic water where reminaralization is dominating (Bura-Nakić et al., 2009; Ćosović et al.,



Fig. 6. SEM images of the samples collected during S2 event (January, 2012) at (a) 5 m and (b) 9 m depths in the water column of RL and corresponding EDS spectar.

2000; Penezić et al., 2010; Svensen et al., 2008). It is interesting that on 9 m filtered N was not detected, implying that N in the 5 m sample was probably related to the freshly produced OM by grazing activities that could be accumulated at the chemocline and further decomposed while sinking in the anoxic water at 9 m depth. Increased concentration of small spherical nanofeatures that covered the substrate have a 25–35 nm diameter with a height of 8–12 nm probably are NaCl crystals, HS and inorganic NPs. An increase in the concentration of the smallest NPs can be related to the higher salinity. In this case too, the SEM supports the AFM results.

3.2.2. Spring time

Usually in spring high primary production can be noticed in RL, which is mainly related to diatom blooms (Ciglenečki et al., 2005; Ćosović et al., 2000; Kršinić et al., 2013). Phyisico-chemical conditions in the lake during the S3 event (Fig. 5a, b) reflected meteorological and atmospheric spring conditions and were characterized with the higher temperature, 24–26.2 °C, and salinity, 35 in oxic and 20.2–22.5 °C and 37–38, respectively in anoxic water layer. The position of the chemocline was shifted to the deeper part of the water column (8 m). In the oxic part oxygen ranged from 8.4 to 5 mg/l and RSS_t concentrations were below 1×10^{-8} , while in the anoxia the RSS_t concentrations were between 3.3×10^{-5} and 3.75×10^{-4} M (Fig. 5a). The higher RSS_t concentrations in anoxic part in comparison to S2 event can be attributed to higher bacterial activity that is recorded at higher temperatures (Pjevac et al., 2015).

The DOC and POC concentrations for S3 had an average values of 1.91 mg C/l and 1,59 mg C/l, respectively (Fig. 5b), indicating the increase in phytoplankton abundance and productivity in the lake. The POC vertical profile displayed increased concentration around the chemocline. Maximum POC concentration was 3.5 mg/l

representing more than 60% of TOC. Such high POC accumulation can be again explained by high bacterial and phytoplankton activities. Maximum of total bacteria cell counts, up to 6.4×10^6 cell/ml was detected around the chemocline (Pjevac et al., 2015) while the maximum phytoplankton cell density, up to 3×10^6 cell/l was recorded between 2 and 5 m. The high contribution of POC to organic carbon is already detected during diatom blooms in RL (Penezić et al., 2010) and North Adriatic samples (Malej et al., 2003). Increased SAS concentration and its vertical distribution within the water column, especially of filtered fraction which follows the best POC profile (Fig. 5b), additionally reflects intensive diatom blooms (ćosović et al., 2000), mainly attributed to *Cyclotella choctawhatcheeana* and *Dactyliosolen fragilissimus* (Malešević et al., 2015; Ciglenečki et al., 2015).

The AFM images of oxic samples taken at the 2 m and 5 m and anoxic sample from 9 m depth, during the S3 event (Fig. 5c-f) revealed different type of material in the water column in comparison to the S1 and the S2 events. Images most probably indicate polysaccharide type OM which is known to form a biopolymer gel matrix after being produced by diatoms (Pletikapić et al., 2012a, 2012b; Svetličić et al., 2011, 2013). According to AFM, most intense production of such organic material was in the surface layer. In the AFM image of sample collected at 2 m, the edges of grains are not observed because the surface was covered with thick OM film. At 5 m AFM image this effect is still visible, but it looks that the amount of freshly produced OM was lower; the step edges are visible and presence of NPs is revealed. Less OM production at 5 m depth is also indicated by DOC, POC and SAS profiles (Fig. 5b). The image, taken at 9 m resembles to the images obtained from the oxic water layer but with evidently less OM. According to EDS analysis, 9 m filter sample contained up to 45% of C and 22% of N what goes in line with already discussed higher phytoplankton activity and higher POC and PON production near the chemocline (Svensenet al., 2008).

3.3. Normalized surfactant activity: OM with SAS properties

A large part of dissolved organic matter in the sea represents organic compounds that possess surface active properties. In natural waters SAS is either dissolved, particulate or colloidal and filtration through 0.45 μ m glass filter is commonly used to separate particulate fraction from the dissolved and colloidal. It is important to know that POC retained on the filter may consist of surface active components which contribution to the total surfactant activity may range from several percent to about 15% in seawater, and up to 70% in estuarine samples (Ćosović and Vojvodić, 1998). During filtration, part of the SAS fraction can be retained on the filter and surfactant activity would be reduced in the filtered samples. However, in some samples where predominantly hydorphilic macromolecules are present, conformation changes that may occur during filtration on SAS can increase surfactant activity.

In S1 and S3 similar concentration and distribution of DOC and SAS (both filtered and non-filtered fraction) were recorded in the water column of the lake (Figs. 3b and 5b). In S3 the largest fraction of DOC was SAS material that was removed by filtration. It was most likely surface-active particles as vesicle-like structure formed by self-assembly of polysaccharides, protein, and lipid components of the excretion and decomposition products of phytoplankton (Žutić and Svetličić, 2000). In S1 also one part of the SAS was removed by filtration, but according to electrochemical measurements, SAS material was present in lower amounts (Fig. 3b). Transport of the more refractory fraction of OM from the sediment and bottom waters (mainly HS, lipids) due to intensive water layer mixing and in the same time, excretion of OM by cell lysis due to anoxic stress conditions are main processes that contributed to the release and distribution of SAS and DOC in S1. In S3 intensive diatom and bacterial activities, detected in the oxic water column and at the chemocline was the main source of OM which DOC fraction was uniformly distributed from the surface to the bottom.

To get more insight to the seasonal variability of predominant SAS material in RL samples taken at S1-S3 events, obtained SAS concentrations (expressed in equivalent amounts of model surfactant T-X-100, mg/l) were normalized to the sample DOC content and correlated with normalized surfactant activities obtained for different model organic compounds as representative constituents of OM in natural waters (Ćosović and Vojvodić, 1998; Ćosović et al., 2000, 2007) (Fig. 7). The comparison of normalized surfactant activity of S1-S3 samples in accordance with AFM, suggests significant changes of dissolved OM regarding to content of DOC and its SAS fraction. Samples with higher content of DOC and SAS were present more in S1, then S3 and S2 events (Fig. 7). The SAS/ DOC ratio for all sampling events, shows the dominant presence of a more hydrophilic material (in S2 more than in S3 and S1 events) which can be attributed to preferably polysaccharide and the HS type of OM. Recently published papers on RL reported that diatoms which are known as producer of more hydrophilic and the polysaccharide type of the surface active OM, took over the domination of phytoplankton assemblages, contributing on average up to 94% (Kršinić et al., 2013). In accordance, comparison of normalized surfactant activity of the RL samples from earlier investigated period, 1994-2000, (Ćosović et al., 2000; Ciglenečki et al., 2005) and this study suggest significant changes, from more hydrophobic to the more hydrophilic character of DOM. Likewise, observed higher concentration of SAS in some filtered samples, especially these sampled in S1 event, indicate possible conformational changes of OM during filtration and can be related to prevalence of more hydrophilic macromolecules (Ćosović and



Fig. 7. Correlation of SAS (equivalent to Triton-X-100) and DOC values measured in the samples of RL during S1–S3 events. Symbols correspond to RL samples and lines correspond to different model substances: (1) triton (T-X-100), (2) polysaccharide dextran (T-500), (3) fulvic acid (FA), (4) humic acid and (5) polysaccharide xanthane.

(Data for model substances taken from Cosovic et al. (2000, 2007))

Vojvodić, 1998).

4. Conclusion

Results from this paper indicate how combination of selected surface methodology covering nano- to micro-scales imaging accompanied with the electrochemical and HTCO measurements could be a good choice for studying different biogeochemical processes in the oxic and anoxic marine environment related with OM and sulfur species cycling, interaction and their distribution between dissolved and colloidal phases.

Production of diverse type of OM during different seasons (autumn, winter, and spring) and physico-chemical conditions (holomixis and during stratification in oxic and anoxic water column conditions) in the small, eutrophic and euxinic marine lake system was confirmed by AFM imaging and were supported by the electrochemical and the HTCO measurements. Obtained differences are in agreement with biochemical and physical processes that occurred in the lake during selected sampling events (S1–S3). Intensive mixing processes governed by fast turnover of the water layers marked out S1, while the biological activity of zoo- and phyto-plankton populations influenced S2 and S3 events. In accordance, significant changes of dissolved OM regarding to content of DOC and its SAS fraction were confirmed. DOC with higher content of the SAS was dominating in S1 event, while SAS/DOC ratio for all sampling events shows the dominant presence of preferably hydrophilic material which can be attributed to the polysaccharide and HS type of OM. AFM additionally offered visualization of OM material that was present in the water column and demonstrated the significant difference in the OM regarding the physico-chemical conditions and biology in the lake. Further work on interpretation of data is in a process to obtain a better correlation between such complex natural processes and distribution of nano-sized material seen by AFM.

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