

Presence of dispersed diesel fuel in the water column of the Boka Kotorska Bay: a case study

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ABSTRACT

We detected, characterized and visualized the presence of dispersed oil in the seawater due to the recent accidental sinking of the ship and oil spill in the Boka Kotorska bay (south Adriatic sea). Diesel fuel was dispersed in the form of micro and nano droplets in the whole water column as determined by conventional light microscopy and electrochemical and atomic force microscopy imaging. Revealed presence of dispersed diesel fuel poses a serious threat to plankton community dynamics and could enter the food web in this semiclosed bay. Consequently, monitoring of dispersed fuel micro- and nanodroplets is recommended due to the intensive boat trafficking and shipyard activity in the Boka Kotorska bay.

Keywords: Atomic force microscopy, Boka Kotorska bay, diesel fuel droplets, electrochemical particle detection, oil pollution

INTRODUCTION

In the framework of the ongoing bilateral project, we have recently characterized marine organic matter in the Boka Kotorska bay (Ivošević DeNardis *et al.* 2013, Dautović *et al.* 2012, Campanelli *et al.* 2009). The Boka Kotorska bay is a semiclosed basin of Montenegro characterized with

karstic rivers and underground springs. Our particular interest was to understand seasonal distribution of microscopic and fluid surface-active particles (SAP) that are important as food source and for transport of gas, microconstituents and pollutants. The surface-active particles were also identified as reactive precursor for macroaggregate formation in the Northern Adriatic (Žutić & Svetličić 2000, Gianni *et al.* 2005). The combination of complementary surface methods (electrochemical and atomic force microscopy, AFM) allowed extending the characterization of organic constituents down to nanometric scale providing important insight into their fate in aquatic system.

This study is triggered by the recent accidental sinking of the ship and spilling of diesel fuel in the Boka Kotorska bay aiming to detect and characterize the form of the remaining diesel fuel material after cleaning action. We postulate the presence of remaining fuel in the form of dispersed droplets based on our experience in studying diesel fuel degradation by bacteria in the Venice Lagoon (Baldi *et al.* 1999). Oil droplets are one of subclass of SAP that have been studied extensively in terms of adhesion and spreading behavior at model charged interface (Žutić *et al.* 1993, Ivošević *et al.* 1994, 1999, Ivošević & Žutić 1997, 1998, 2002) which offered rationale for development of an adhesion-based electrochemical sensor applicable for *in situ* particle analysis (Kovač *et al.* 2000, Žutić *et al.* 2004) including marine environment. Here, we will use light microscopy after staining, electrochemical detection and atomic force microscopy imaging in order visualize and characterize the presence of dispersed oil in the sampled seawater after diesel oil spill accident.

MATERIAL AND METHODS

Sampling

Seawater samples were collected at the station Rt Murva in the bay of Boka Kotorska, at four depths on November 14, 2013 (Figure 1), 34 days after the sinking accident. The seawater samples were collected with Niskin bottles at 0, 5, 10 and 15 m depths.

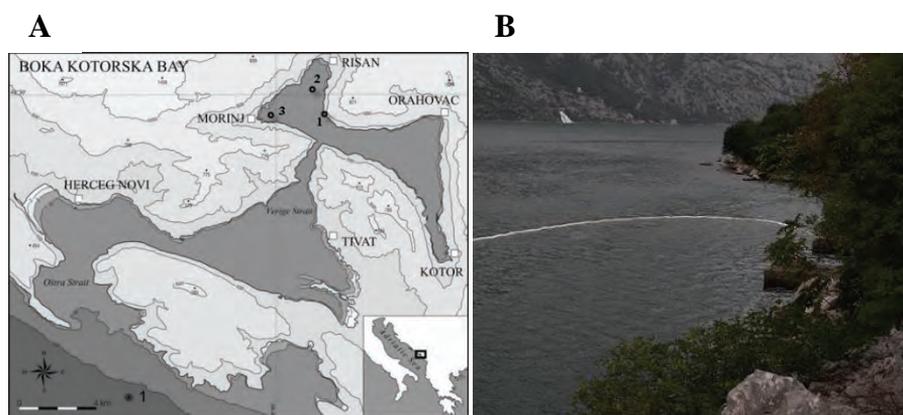


Figure 1. Study area and the sampling stations in the bay of Boka Kotorska (south Adriatic sea): 1-Rt Murva, 2-Risan 3-Morinj (A). Photograph of the polluted area surrounded by floating fence at the Rt Murva (B).

Light microscopy after staining

Oil droplets and their colonization by bacteria were visualized by epifluorescence microscope (Olympus, BX51 equipped with DP70 Digital Camera System) after staining. Fluorescent molecular probe, Nile red (9-(Diethylamino)-5H-benzo[a]phenoxazin-5-one, Sigma) is used to stain oil droplets in seawater (Greenspan *et al.* 1985). Stock solutions of Nile red (1 and 10 mg/mL) were prepared in acetone and stored in dark at 4°C. Staining was carried on fresh seawater samples. Nile red solution (0.5 mg/mL in acetone) was added to seawater samples (1:100 v/v) and incubated for 5-10 min. The bacterial communities were observed by epifluorescence

microscopy after staining with DAPI (4,6-diamino-2-phenylindole; Porter & Feig 1980).

Electrochemical method

The chronoamperometry at mercury electrode enables detection of soft organic particles in aquatic samples as described in detail (Žutić *et al.* 1993, Kovač *et al.* 2000, Svetličić *et al.* 2001, Žutić *et al.* 2004). In principle, a micrometer-sized particle adheres and spreads 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 being 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 mg/L. This presents an alternative approach to measure dissolved organic carbon (DOC) of seawater (Hunter & Liss 1980).

Electrochemical measurements

The 15 mL of seawater sample was placed in the electrochemical cell that was 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 was positively charged (+3.8 μC/cm²) and shows maximum attraction to organic particles adhesion. Surfactant activity of the sample was measured by addition of 500 μL of 0.1 M HgCl₂ in 50 mL of seawater just prior to the measurement. Electrochemical measurement enables detection of soft particles in the size range 1-500 μm and concentration range 10⁵-10⁸ particles/L.

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. Unlike traditional microscopes, AFMs do not use lenses, so the size of the tip rather than diffraction effects generally limits their resolution. Soon after its invention (in 1986) AFM has emerged as a bio-imaging technique capable of measurements on unstained and unfixed biological molecules and assemblies in air and under fluid. Besides imaging at multiple spatial

scales, it can also serve as a mechanical probe of these types of systems. 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, a 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 the 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 the 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). 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 rinsed with 50 μ L ultrapure water. The rinsing procedure was repeated three times and the sample was allowed to dry in an enclosed Petri dish before imaging. The rinsing of samples with ultrapure water was necessary to remove the salt crystals that would hamper AFM imaging under ambient conditions. All measurements were performed in air at room temperature and 50–60 % relative humidity.

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 μm scanner. The tapping mode imaging was applied using silicon tips (TESP, Veeco, nom. freq. 320 kHz, nom. spring constant of 42 N/m). To minimize the forces of interaction between the tip and the surface, 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 V614r1). The linear scanning rate was optimized around 1.0 Hz with scan resolution of 512 samples per line.

RESULTS AND DISCUSSION

The ship in question (PR-38 „Tunj“) had overall length of 32.7 meters and weight of 325 tons, holding oil and diesel oil (D2). The ship sank on the October 11, 2013 at the Rt Murva. Immediate cleaning action of polluted region of about 1000 m^2 was undertaken using the floating fence and oil spill adsorbent pads. Seawater samples were taken at the station Rt Murva, approximately four meters from the floating fence. Table 1 summarized temperature, pH and salinity data in respect of depths at the sampling site. Maximum depth at the corresponding sampling site was 18.2 m.

The surface layer temperature was 16.0 $^{\circ}\text{C}$ and salinity was 16.7 ‰. At the 5 m depth, temperature was 19.5 $^{\circ}\text{C}$ and measured salinity was higher i.e. 32.8 ‰ indicating existence of density gradient.

Table 1. Temperature, pH and salinity of seawater measured at the station of Rt Murva in the Boka Kotorska bay.

Depth/m	t/°C	pH	S / ‰
0	16.0	8.29	16.7
5	19.5	8.18	32.8
10	20.1	8.12	36.0
15	20.7	8.01	36.5

Seawater samples were completely clear with a slight scent of diesel fuel. To test the presence of diesel fuel oil, we performed light microscopy experiments using Nile red, which indicated presence of oil droplets (Figure 2). Dispersed droplets were in the range from 1 μm up to 30 μm in measured samples (0m, 5m and 10m) with the small ones being the most abundant.

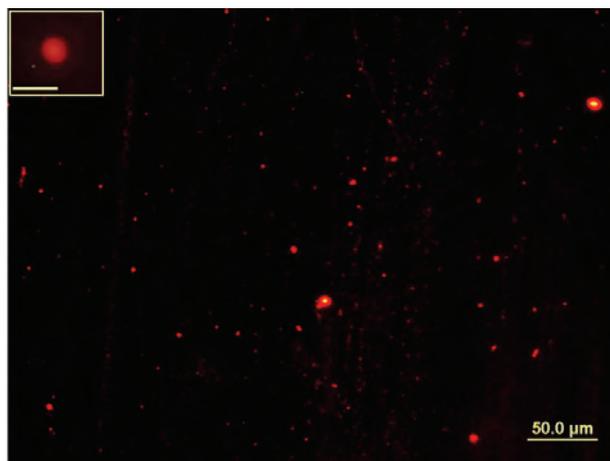


Figure 2. Dispersed diesel fuel droplets stained by Nile red and visualized by epifluorescence microscopy. Inset shows oil droplet at higher magnification (scale bar denotes 2 μm).

Most of the particles were spherical with regular shape, uniformly stained as shown for the oil droplet of $\sim 1 \mu\text{m}$ diameter presented in the inset of the Figure 2.

Electrochemical characterization

Surface-active particles shows tendency to accumulate at the density (salinity and temperature) gradients (Žutić & Legović 1987). We expected to detect most of electrochemical adhesion signals in sample taken at depth of 5 meters. The electrochemical measurement was performed by immersing working electrode (dropping mercury electrode) directly into the electrochemical vessel containing seawater sample with salinity adjustment to 35 ‰. Signals appeared stochastically, therefore, we followed adhesion signals continuously over 100 seconds to determine droplet concentration in the seawater sample. The highest droplet concentration of $2.0 \times 10^5/L$ was determined at the 5 m depth, while at the other depths concentration of droplet was bellow detection limit for electrochemical detection. Characteristic electrochemical signals of fluid droplets recorded in the seawater sample taken at the station Rt Murva, depth 5 m were shown in Figure 3.

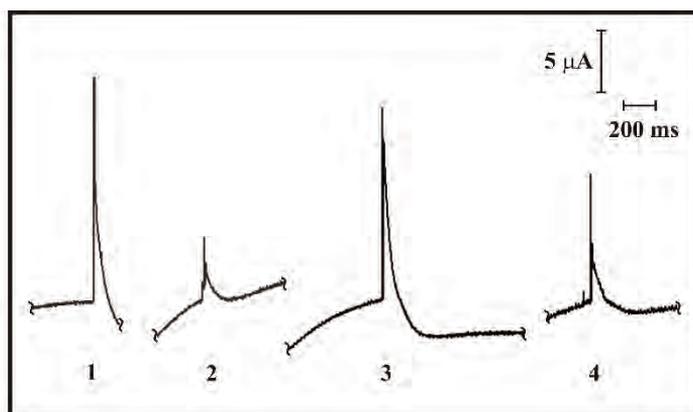


Figure 3. Four segments of the oxygen reduction current at the dropping mercury electrode with superimposed signals of oil droplets in seawater sample taken at the station Rt Murva, depth 5 m, recorded at the potential of -400 mV.

The signals amplitude and duration were in the range of 5 to 18 μA and 200 to 600 ms, respectively. The shape and duration of recorded signals corresponded to signals of fuel oil droplets (Baldi *et al.* 1999) and of model hydrocarbon droplets (Ivošević and Žutić 1997) recorded under similar experimental conditions. In the case of oil droplet recorded as signal 4 (Figure 2), adhesion, deformation and spreading to monolayer thickness over the mercury electrode/seawater interface was completed within time of 280 ms. In contrast, signal duration of phospholipid vesicles for the same signal amplitude was significantly shorter, namely between 10 and 40 ms (Ivošević *et al.* 2007).

Next, we compared concentrations of SAP and surfactant activity determined in September 19, 2013, at stations Risan and Morinj (Figure 1), with those determined at the Rt Murva in November. The concentrations of SAP determined at Risan and Morinj in September are in the same order of magnitude as determined at the station of Rt Murva. The most pronounced difference was reflected in two times lower surfactant activity (i.e. 1.2 mg/L) at the station of Rt Murva than at stations Risan and Morinj. The elevated values of surfactant activities determined at stations Risan and Morinj could be ascribed to the very dry season with no freshwater input and nearby fish farms.

AFM characterization

Submicrometer sized population of fuel oil droplets were visualized by AFM and typical examples are shown in Figures 4 and 5.

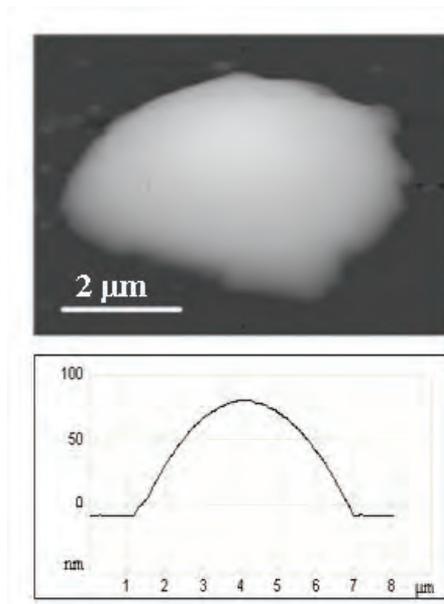


Figure 4. Topographic 3D view with vertical profile of a single droplet in seawater sample taken at station Rt Murva, depth 0 m.

Hydrophobic droplets adopted a spherical cap shape due to adhesion and spreading over the mica surface and are in line with reported AFM results for hydrocarbon oil droplets deposited at mica (Pompe *et al.* 1998) and other the solid interfaces (Zhang & Druker 2008, Tongcher *et al.* 2006). On the other hand, we visualized the lens-like shape of hexadecane macroscopic droplet deposited at uncharged mercury interface (Ivošević & Žutić 1998). The lens-like shape of oil deposited droplet was controlled by changing the applied potential at the electrode/solution interface (Ivošević & Žutić 2002). The height at the droplet apex presented in Figure 4 is only 90 nm while it's width is 6 μm , as evident from the vertical profile across the center of the droplet. The imaged droplet diameter in seawater would be 520 nm. An example of smaller oil droplet spread over mica forming spherical cap is shown in Figure 5.

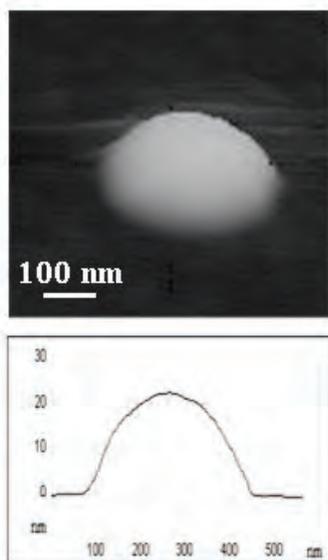


Figure 5. Topographic 3D view with vertical profile of a single oil nanodroplet in seawater sample taken at station Rt Murva, depth 5 m.

The maximum height of the droplet deposited on the mica substrate is 22 nm with the diameter of 380 nm. The imaged droplet diameter in seawater would be only 80 nm. It is worth noting that similar class of submicron particles were imaged at the station Škudra (cruiser port) in April 2013.

Biodegradation

To verify whether biodegradation process occurred, seawater sample was stained with DAPI to detect presence of bacteria (Figure 6).

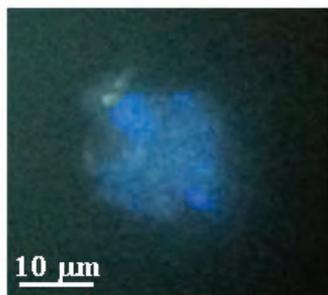


Figure 6. Colonized oil droplet by bacterial communities stained with DAPI.

Hydrocarbon-degrading bacteria could present up to 10% of the total bacterial population (Hedlund *et al.* 1999), thus biodegradation process is rather slow. We studied bacterial efficiency in degradation of diesel fuel droplets using electrochemical based detection and molecular probes with confocal laser-scanning microscopy (Baldi *et al.* 1999, Žutić *et al.* 1999, Svetličić *et al.* 1997). Bacterial biomass usually increases during oil biodegradation. In contrast to the increase of bacterial biomass, based on report of, it was found that phytoplankton primary production could decrease up to 40% and 3 years period was required for algal community recovery after heavy fuel oil spill in the Marmara sea (Turkey, Tas & Okus 2007). Further, presence of oil has serious impact on survival of fish eggs and larvae, shellfish, hatching percentage, swimming ability, fertility in copepods as reported for the Baltic sea (Alvinge *et al.* 2001). In spite of the risk on the sensitive marine ecosystem, up to now no actions were taken for withdrawal of the ship PR-38 „Tunj“ from the the Boka Kotorska bay.

CONCLUSION

One month after the mechanical removal of the oil spill, due to the accidental sinking of the ship, we have detected presence of dispersed oil

droplets in the whole water column. The presence of fuel oil in the form of dispersed microdroplets were confirmed using conventional light microscopy and electrochemical adhesion imaging while population of nanodroplets were identified and characterized using AFM and developed protocol for marine sample analysis. The characterized oil droplets were in the continuum size range from micro to nano. Electrochemical detection allowed fast, simple and direct measurement, gaining information about microdroplet concentration, size and polydispersity level. On the other hand, atomic force microscopy allowed visualization of oil droplets present in the seawater sample on the nanometer level not easily assessed by other techniques. The complementary surface techniques applied within this case study, showed potential to be used for testing and optimizing cleanup performance after accidental events but also for monitoring of the water quality in areas exposed to the intensive boat trafficking, anchoring and shipyard activity in the semiclosed bay as Boka Kotorska.

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REFERENCES

- Alvinge, E., D. Stoica, K. Iversen (2001): Oil Pollution in the Baltic Sea and the Effects on Fish and Fisheries. Environmental Studies, Aarhus University
- Baldi, F., N. Ivošević, A. Minacci, M. Pepi, R. Fani, V. Svetličić, V. Žutić (1999): Adhesion of *Acinetobacter venetianus* to diesel fuel droplets studied by in situ electrochemical and molecular probes. Appl. Environ. Microbiol., 65: 2041-2048.
- Campanelli, A., A. Bulatović, M. Cabrini, F. Grilli, Z. Kljajić, R. Mosetti, E. Paschini, P. Pierluigi, M. Marini (2009): Spatial distribution of physical, chemical and biological oceanographic properties, Phytoplankton, nutrients and Coloured Dissolved Organic Matter (CDOM) in the Boka Kotorska Bay (Adriatic Sea). Geofizika, 26: 1–14.
- Dautović, J., S. Strmečki, B. Pestorić, V. Vojvodić, M. Plavšić, S. Krivokapić, B. Čosović (2012): Organic matter in the karstic enclosed bay (Boka Kotorska Bay, south Adriatic Sea). Influence of freshwater input. Fresen. Environ. Bull., 21: 995-1006.
- Giani, M., D. Degobbi, A. Rinaldi (2005): Mucilages in the Adriatic and Tyrrhenian seas. Sci. Total. Environ., 353: 1-380.
- Greenspan, P., E. P. Mayer, S. D. Fowler (1985): Nile red: a selective fluorescent stain for intracellular lipid droplets. J Cell Biol. 100: 965-73.
- Hedlund, B.P., A.D. Geiselbrecht, T.J. Bair, J.T. Staley (1999): Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. Appl. Environ. Microbiol., 65: 251-259.
- Hunter, K.A. & P.S. Liss (1980): Polarographic measurement of surface-active material in natural waters. Wat. Res., 15 203-215.
- Ivošević, N., J. Tomaić, V. Žutić (1994): Organic droplets at an electrified interface: critical potentials of wetting measured by polarography. Langmuir, 10:2415–2418.
- Ivošević, N., & V. Žutić (1997): Polarography of marine particles: A model study. Croat. Chem. Acta, 70: 167–178.
- Ivošević, N., J. Tomaić, V. Žutić (1998): Spreading and detachment of oil droplets at an electrified interface. Langmuir, 14:231-234.

- Ivošević, N., J. Tomaić, V. Žutić (1999): Wetting equilibria of hydrocarbon droplets at an electrified interface. *Langmuir*, 15:7063–7068.
- Ivošević, N. & V. Žutić (2002): Effect of electrical potential on adhesion, spreading and detachment of organic droplets at aqueous metallic interface. *In*: Mittal K. (Ed.): Contact Angle, Wettability and Adhesion, AH Zeist, The Netherlands, VSP, pp. 549–561.
- Ivošević DeNardis, N., V. Žutić, V. Svetličić, R. Frkanec, J. Tomašić (2007): *In situ* amperometric characterization of liposome suspensions with concomitant oxygen reduction. *Electroanal.*, 19: 2444-2450.
- Ivošević DeNardis, S. Šegota, V. Svetličić, A. Castelli, Z. Kljajić (2013): Characterization of marine organic matter in the bay of Boka Kotorska by electrochemical and atomic force microscopy imaging. *Studia Marina*, 26: 5-22.
- Kovač, S., R. Kraus, S. Geček, V. Žutić (2000): Cell suspension as a model system for electrochemical analysis. *Croat. Chem. Acta*, 73: 279-291.
- Mišić Radić, T., V. Svetličić, V. Žutić, B. Boulgaropoulos (2011): Seawater at the nanoscale: marine gel imaged by atomic force microscopy. *J. Mol. Recognit.*, 243: 397-405.
- Pompe, T., A. Fery, S. Herminghaus (1998): Imaging liquid structures on inhomogeneous surfaces by scanning force microscopy. *Langmuir*, 14: 2585-2589.
- Porter, K.G., & Y.S. Feig (1980): The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr.*, 25: 943-948.
- Svetličić, V., N. Ivošević, V. Žutić, D. Fuks (1997): Polarography of marine bacteria: *Croat. Chem. Acta*, 70: 141–150.
- Svetličić, V., N. Ivošević, S. Kovač, V. Žutić (2001): Charge displacement by adhesion and spreading of a cell. *Bioelectrochem.*, 53:79-86.
- Tas, S., & O. Erdogan (2007): Effects of oil pollution on the phytoplankton community in the Kucukcekmece bay (north-east sea of Marmara, Turkey). *Rapp. Comm. int. Mer Medit.*, 38: 320-320.
- Tongcher, O., R. Sigel, K. Landfester (2006): Liquid Crystal Nanoparticles Prepared as Miniemulsions. *Langmuir*, 22: 4504-4511.
- Zhang X. H. & W. Ducker (2008): Interfacial Oil Droplets. *Langmuir* 24: 110-115.
- Zvonarić, T., V. Žutić, M. Branica (1973): Determination of surfactant activity by polarography. *Thalassia Jugosl.*, 9: 65-75.

- Žutić, V., B. Ćosović, Z. Kozarac (1977): Electrochemical determination of surface active substances in natural waters. *J. Electroanal. Chem.*, 78: 113-121.
- Žutić, V. & T. Legović (1987): A film of organic matter at the fresh-water/sea-water interface of an estuary. *Nature*, 328: 612–614.
- Žutić, V., S. Kovač, J. Tomaić, V. Svetličić (1993): Heterocoalescence between dispersed organic microdroplets and a charged conductive interface. *J. Electroanal. Chem.*, 349:173-186.
- Žutić, V., N. Ivošević, V. Svetličić, R. A. Long, F. Azam (1999): Film formation by marine bacteria at a model fluid interface. *Aquat. Microb. Ecol.* 17:231-238.
- Žutić, V. & V. Svetličić. (2000): Interfacial Processes. *In*: Wangersky, P. (Ed.): *The Handbook of Environmental Chemistry. Marine Chemistry, Vol. 5, Part D*, Springer-Verlag, Berlin-Heidelberg, pp. 150–165.
- Žutić, V., V. Svetličić, N. Ivošević, A. Hozić, O. Pečar (2004): Northern Adriatic mesocosm experiment Rovinj 2003: Dynamics of organic microparticles studied by the electrochemical technique. *Period. Biolog.*, 106: 67-74.