Review article: Potential application of surface methods for the monitoring of organic matter dynamics in marine systems

Galja Pletikapić[1,2], Nadica Ivošević DeNardis[1]

[1]Division for Marine and Environmental Research, Ruđer Bošković Institute, POB 180, 10000 Zagreb, Croatia

5 [2]FOM Institute AMOLF, POB 41883, 1009 DB Amsterdam, Netherlands *(current address)*

Correspondence to: Nadica Ivošević DeNardis (ivosevic@irb.hr)

Abstract

Marine organic matter has a highly reactive nature; it continuously undergoes biotic and abiotic transformations and consequently exhibits high variation in both time and space, with a tendency to accumulate at the interfaces. To study its role
10 in natural systems, it is of crucial importance to preserve its original state. We present the potential of non-invasive surface methods, namely, the electrochemical method of chronoamperometry at the dropping mercury electrode (DME) applied together with atomic force microscopy imaging (AFM), and their suitability on the basis of field studies undertaken in the northern and southern Adriatic Sea (Boka Kotorska Bay, Montenegro). Electrochemical approach meets the requirements for monitoring purposes due to the simple and fast analysis of a large number of raw seawater samples enabling simultaneous
15 differentiation of organic constituents. On the other hand, atomic force microscopy allows direct visualization of biotic and abiotic particles and provides an insight into structural organization of marine organic matter at micro- and nanometre scales. Application of these methods can contribute to the prediction of mucilage events and to the monitoring of organic pollution in aquatic systems. Moreover, understanding the dynamics of organic matter in seawater is of crucial importance for improvement of national legislation on environmental protection, sustainable development and for management of marine
20 systems.

1 Introduction

Based on the traditional oceanographic routines, organic matter in the marine environment is classified as dissolved (DOM), consisting of truly dissolved and colloidal fractions, and particulate (POM). DOM is manly produced by primary production in surface waters (Carlson, 2002), and 10 % of it can self-assemble to form nano- , micro- or even macrogels (Verdugo et al.,
25 2004; 2012). Because of the great complexity and heterogeneous composition of marine organic matter, its characterization remains a challenge. Characterization of DOM in terms of its composition and molecular structure has advanced

substantially in the last decade, mainly owing to the development of advanced instrumental analytical methods and the corresponding procedures, for example, Fourier-transform ion cyclotron, mass spectrometry, homo- and hetero-correlated multidimensional nuclear magnetic resonance spectroscopy, and excitation emission matrix fluorimetry with parallel factor analysis for UV–fluorescence spectroscopy, along with advances made in sample preparation (i.e., desalting/extraction

5 techniques, Nebbioso and Piccolo, 2013; Roth et al., 2015; Kujawinski et al., 2016; Mopper et al., 2007). In addition, development of particle counters (electronical, electrochemical), histological staining and sophisticated microscopical methods in the last decades helped establish new particle classes, predominantly non-living, which had remained undetected due to their small size, transparency, and susceptibility to degradation (Žutić and Svetličić, 2000). These particle classes have shown large abundance and reactivity, consequently having a crucial role in aquatic environments, particularly in the

10 cycling of nutrients, aggregation of organic matter and food web (Koike et al., 1990; Wells and Goldberg, 1991; 1993; 1994; Alldredge et al., 1993; Long and Azam, 1996). These particles are usually classified as: (i) colloidal particles (5-200 nm) collected by ultracentrifugation and observed under transmission electron microscopy, reaching concentrations up to 10^{12}/L at the thermocline of the North Atlantic and Northwest Pacific (Wells and Goldberg, 1991; 1993); (ii) submicrometre particles (0.4-1µm) detected by Coulter particle counters reaching concentrations up to 10^{10}/L in the top 40 m of the North

15 Pacific of Japan, Northwest Atlantic Schlep (Koike et al., 1990; Longhurst et al., 1992); (iii) surface-active particles (SAP; 1-500 µm), mostly formed from decomposed and excreted phytoplankton products, can reach concentrations up to 10^8/L in the estuarine mixing zones, the sea surface or halocline of the Adriatic Sea (Žutić et al., 1984; Žutić and Legović, 1987; Marty et al., 1988; Svetličić et al., 2005; 2006; Žutić et al., 2004), detected electrochemically. These particles play an important role as precursors of massive aggregate formation; (iv) transparent exopolymeric particles (TEP, 3-100 µm) are

20 flexible, polysaccharide microgels serving as surfaces for bacterial colonization and transfer by adsorption, are detected by Alcian blue staining or spectrophotometrically and may reach concentrations up to 5×10^6/L at the California coast (Alldredge et al., 1993; Passow, 2002; Villacorte et al., 2015); (v) protein containing particles (CSP, 2-500 µm) are visualized by Coomassie blue staining, may reach concentrations up to 10^8/L off the California coast (Long and Azam, 1996), providing a rich nitrogen source for bacteria and other organisms, and (v) giant aggregates (>1m) observed by a scuba

25 diver in the Northern Adriatic Sea (Stachowitch et al., 1990; Leppard et al., 1997; Svetličić et al., 2005; Gianni et al., 2005a). Recently, more attention has been paid to marine gels ranging from nano to macro dimensions (Verdugo et al., 2004; 2012; Mišić Radić et al., 2011). Marine gels are defined as three-dimensional networks of solvated biopolymers embedded in seawater. Their importance in marine ecosystems has been widely recognized among scientists from different fields due to their role in the microbial loop and sedimentation processes, biogeochemical carbon cycling, marine carbohydrate chemistry,

30 and particle dynamics in the ocean (Žutić and Svetličić, 2000; Azam and Long, 2000; Passow, 2002; Verdugo et al., 2004; 2012). These gels are also bioreactive, since they compartmentalize nutrients and can thus enhance microbial activity (Kepkay, 1994; Azam, 1998). Marine gels can exhibit even macroscopic dimensions when massive gelatinous macroaggregates cover a few square kilometres of the sea surface and form clouds in the water column (Fig.1). This can have harmful consequences for the marine systems and cause substantial damage to the economy of the region. Even though

evidence accumulated from satellite observations to micro-scale studies and a large number of hypotheses on the mechanism of the event have emerged over the last decades (Žutić et al., 2004), what triggers such a large-scale phase transformation of organic matter is still a subject of debate. While relatively broad scientific literature is available on the application of different techniques for characterization of marine organic matter, most of the methods include relatively expensive equipment and/or complex sample preparation procedures that effect the state of the fragile fractions of organic matter. It is now generally agreed that organic matter of a given chemical composition can occur in a large range of forms under the influence of dynamic aquatic conditions: monomer molecules, polymer chains, globules, vesicles and complex networks such as gels (Žutić and Svetličić, 2000). All of these will undoubtedly affect organic matter's persistence, stability, aggregation behaviour and bioavailability. Little is known about the distribution of environmental particles due to the difficulties in sampling, sample handling, insufficient sensitivity and selectivity of analytical methods but also due to the heterogeneity of natural samples (Buffle and Leeuwen, 1992). For example, the seawater sample needs to be unperturbed in order to preserve the heterogeneous distribution and primary structure of organic particles. High heterogeneity makes both the analysis and data interpretation challenging. In this review, we present the potentials of non-invasive surface methods, namely, the electrochemical method of chronoamperometry at DME applied together with atomic force microscopy imaging, and their suitability on the basis of field studies undertaken in the northern and southern Adriatic Sea (Boka Kotorska Bay, Montenegro). We emphasize the importance of no need for sample pretreatment in order to capture the physical structure and organization of organic matter as closely as it exists in the natural system. Every sample pretreatment such as filtration, centrifugation, staining, fixation, reverse osmosis, dialysis introduces artefacts. We particularly focus on marine organic particles in the size range of only a few nanometres to several hundreds of micrometres. This fraction mainly involves soft, hydrophobic, hydrophilic, and reactive particles, susceptible to degradation. Micrometre sized surface-active particles can be measured electrochemically on the single particle level based on their interfacial properties, structural organization and hydrophobic/hydrophilic character. Electrochemical approach is fast compared to other methods, with sample characterization taking only a couple of minutes, making it suitable for analyses of a large number of raw seawater samples applicable for monitoring. On the other hand, structural organization of organic matter on the molecular level and three dimensional images of fine, fragile, and soft structures can be revealed using AFM and correlated to the electrochemical data in order to better understand the biotic as well as abiotic transformation of organic matter at micro and nanoscales as the root of macroscopic giant gel formation in the sea (Ciglenečki and Svetličić, 2015). The advent of AFM opened a possibility to directly explore these processes at the scale that determines the fate of organic matter and its interactions at the interfaces.

2 Surface methods for characterization of marine organic matter

2.1 Electrochemical method

The electrochemical method used here is chronoamperometry of dissolved oxygen at a charged dropping mercury electrode (Barradas and Kimmerle, 1966; Zvonarić et al., 1973). Chronoamperometry at a mercury electrode is an important and

convenient tool for the in situ single particle analysis in an aqueous electrolyte solution. Mercury, as a substrate, has several very unique advantages: it is atomically smooth, fluid and chemically inert with a large set of interfacial data in various aqueous electrolyte solutions. Fluidity of mercury perfectly mimics interactions with other fluid interfaces in contact (e.g. biological membranes, vesicles, bubbles). Electrochemical measurement is performed by immersing the charged DME directly into the electrochemical vessel containing a fresh seawater aliquot of 15 mL. The mercury electrode has a surface area of only $4mm^2$ with the drop life time of 2 seconds. Every 2 seconds, a new and reproducible mercury surface is formed at the end of the glass capillary and the analysis can be repeated many times. This is an important aspect of the method, since the arrival of particles to the interface is a stochastic process and the representative behaviour can be determined only by analysing a larger set of data collected under identical experimental conditions (Kovač et al., 2000). The electrochemical method is based on the sensing of the interfacial properties, hydrophobicity and supramolecular organization of particles, rather than on the chemical composition (Žutić et al., 2004). The main advantage of the electrochemical approach is simultaneous and direct detection of different organic constituents in seawater based on their different electrochemical responses (Fig. 2) while inorganic particles remain undetected (Ivošević and Žutić, 1997; Baldi et al., 1999; Kovač et al., 1999; Svetličić et al., 2006; Ivošević DeNardis et al., 2007a). Organic constituents of seawater can be classified according to their electrochemical responses at the dropping mercury electrode based on the phenomena of molecular adsorption, adhesion and spreading, and particle collision (Žutić et al., 1990; 1993; Tsekov et al., 1999). On the chronoamperometric curve, adsorption of dissolved organic matter and submicron particles is manifested cumulatively as a gradual decrease of the oxygen reduction current (Žutić et al., 1999). On the other hand, adhesion of fluid SAP with hydrophobic properties is detected on the single particle level through spike-shaped signals (Žutić et al. 1993; Svetličić et al., 2001, Ivošević et al., 2007b). Collision of gel microparticles with the flexible three-dimensional network and thus possessing a hydrophilic character is detected also on the single particle level through specific dip-shaped signals (Svetličić et al., 2006; Ivošević DeNardis et al., 2007a). The dropping mercury electrode as an in situ sensor, enables characterization of fluid SAP in the aqueous environment (Žutić et al., 1984; Žutić and Legović, 1987; Žutić et al., 2004) in terms of concentration, size, reactivity and hydrophobicity (Žutić et al., 1993; Kovač et al., 2000; Ivošević et al., 2007b). Fig. 3 shows the attractive interaction between an oil droplet and the charged electrode/seawater interface. Oil droplet, as a subclass of surface-active particles, adheres and spreads at the charged interface, causing double layer charge displacement and establishment of the flow of the compensating current known as adhesion signal. Signal amplitude reflects the particle size, signal shape reflects the physicochemical properties of the particle and signal frequency indicates particle concentration in the seawater sample. Signal frequency is translated into particle concentration using the corresponding calibration curve with *Dunaliella tertiolecta* cells as standard particles (Kovač et al., 2000). Chronoamperometric measurements meet the requirements for monitoring based on the following advantages (i) simple and fast analysis (100 seconds per sample) of soft micrometre particles in the size and concentration ranges of 1-500 µm and 10^5-10^8 particles/L, respectively, and (ii) no sample pretreatment. In order to access more quantitative information from the amperometric signal of an organic particle, the measurement has to be performed in a previously deaerated solution under nitrogen purging for a few minutes so as to

remove redox reaction of dissolved oxygen. In this way, it is possible to retrieve information about: organic particle diameter, particle surface area at the interface, number of molecules in the monolayer, surface charge of the particle, critical interfacial tensions of adhesion, and kinetic parameters of the adhesion process through analysis of the amperometric signal using a reaction kinetics model of the first order and the corresponding methodology (Ivošević DeNardis et al., 2012;

5   Ivošević DeNardis et al., 2015). On the other hand, determination of adsorbable organic matter (dissolved organic matter and submicron particles) in seawater is based on a 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). Surfactant activity of seawater is expressed as the equivalent amount of the nonionic synthetic surfactant Triton-X-100 (polyethylene glycol tert-octyl phenyl ether, MW=600) in milligrams per litre. The polarographic maximum of Hg(II) ions offers an alternative

10   approach to measuring dissolved organic carbon of seawater (Hunter and Liss, 1981).

2.2 Atomic force microscopy (AFM)

Atomic force microscopy was recently introduced as a tool in marine ecology (Svetličić et al., 2005; Malfatti et al., 2009;

15   2010; Urbani et al., 2012; Bosak et al., 2012; Svetličić et al., 2013 and references therein). The advantages of AFM over conventional light or electron microscopy are: (i) 3D high resolution imaging of different seawater constituents (biotic and abiotic) connecting micrometric and nanometric dimensions, (ii) samples can be non-destructively imaged in the air and under near natural aqueous conditions, (iii) nanomechanical measurements (Young's modulus as a measure of elasticity), deformation, hydrophobicity, and (iv) determination of intra- and inter-molecular forces in heterogeneous molecular

20   assemblies (Pletikapić et al., 2014). All these unique advantages make AFM a powerful tool in marine environmental studies, well suited either for investigating the mechanism of marine mucilage formation or for closer morphological description of marine cells (Pletikapić et al., 2012a; Francius et al., 2008). AFM is based on a relatively simple principle; it involves raster scanning of a sharp and hard tip (probe) located at the free end of a flexible cantilever. The tip is scanned over the surface of a sample, sensing the interaction forces between the tip and sample (Fig. 5). The sample is mounted on a

25   piezoelectric scanner, which allows three-dimensional positioning with subnanometre accuracy. Interaction between the tip and the surface of the sample leads to cantilever bending, which is measured by laser light reflected from the cantilever to a position sensitive photodetector. As changes in cantilever deflection result in variation of the distance between the tip and sample, a constant distance is re-established with a feedback loop between the sample/tip positioning system and a computer controlled piezoelectric scanner. Registered values of cantilever deflection are electronically converted into a pseudo 3D

30   image of the sample. As a result, AFM gives real 3D images of the sample with a vertical resolution of 0.1 nm and lateral resolution of 1 nm.

3 Field study in the Northern Adriatic Sea

3.1 Monitoring of marine organic matter distribution by electrochemical based detection

A field-oriented study in the Northern Adriatic was carried out in the framework of the long-term National Monitoring
5   Programme (Systematic Study of the Adriatic Sea as a Base for Sustainable Development of the Republic of Croatia, 1998-
2012). The study refers to the monitoring of organic microparticle distribution as an early warning sign for mucilage
formation. We introduced a direct electrochemical method to probe the state of marine organic matter without perturbing its
original heterogeneous distribution. Data were collected over a period of 10 years and those referring to station SJ 105 are
shown in Fig. 4. This particular sampling station was selected because it is distant from the coastline and thus the influence
10   of direct input of inland freshwater is avoided. SAP concentrations ranged from $5x10^5$ to $6.x10^6$/L, showing spatiotemporal
variability with special patterns depending on the sampling depth in the water column and/or season. For example,
significantly higher SAP concentrations (about two times) occurred in the region under the direct influence of nutrient rich
freshwater inputs of the Po River (SJ 101, 103, 108). In the first three year period (2002, 2003, 2004), a mucilage event
appeared. In the period just before the mucilage event, SAP concentration rose to 1×10^7/L in the subsurface layer (0-10 m,
15   SJ 101, 103, 108), indicating the forthcoming event. In contrast, low concentration of SAP in the subsurface layer at station
SJ105 in 2007 is in line with the absence of mucilage events. Hence, SAP was identified as a reactive precursor for mucilage
formation in seawater. Concentrations of GeP were usually around 10^5/L in the years without mucilage events (0.2×10^5/L is
lower electrochemical detection limit). Elevated concentration of GeP, by about one order of magnitude (15×10^6/L), was
determined in all sampling stations at the time of mucilage events (2002-2004). Abundance of GeP was on average 10 % of
20   SAP values (Project Jadran). Surfactant activity reflecting the concentration of submicron and dissolved organic fractions
varied in the range from 0.5 to 2 mg/L in the years without mucilage events and decreased with the depth profile. Elevated
surfactant activity up to 3.4 mg/L was determined during the mucilage event. In addition, a net increase of surfactant activity
in the whole water column was observed at the end of years without mucilage events as a consequence of prolonged
accumulation of organic matter in the water column. It was therefore concluded that the likelihood of mucilage formation
25   depends mainly on new primary production, specific structural organization of the newly produced organic matter and,
above all, hydrographic and meteorological conditions in the seawater (Project Jadran).

3.2 Structural organization of marine organic matter at the nanometric level, visualized by AFM

30   In one of the first AFM studies of marine organic matter, Santschi and coworkers (1998) used AFM to image individual
fibrillar polysaccharides in marine macromolecular organic matter. From there on, potential AFM has been recognized to
study both the networks secreted by marine bacteria (Malfatti et al., 2009) and marine fibrils forming giant gel
macroaggregates formed in the Northern Adriatic (Volleinder et al., 1999; Giani et al., 2005b), their self-assembly and
molecular structure at different levels of association (Mišić Radić et al., 2011). However, application of AFM imaging for

monitoring by itself is often not suitable. This is due to the large number of samples required in the monitoring studies, making AFM analysis, if used alone, a time consuming process. It is therefore recommended to analyse seawater samples first with some more rapid and "bulk" technique (i.e. chronoamperometry) in order to identify samples of interest. For AFM imaging, the drop deposition method modified for marine samples and imaging in air were found to be most adequate for

5   visualization of macromolecular organic matter organization (Pletikapić et al., 2011; Mišić Radić et al., 2011 for more detail). Typically, AFM measurements of marine organic samples are performed at room temperature and 50-60 % of relative humidity, which leaves samples with a small hydration layer adhering to the substrate, helping maintain the native structure (Balnois and Wilkinson, 2002). It was found that direct deposition of a drop of seawater (5μL) on freshly cleaved mica, followed by the rinsing of sea salts and evaporation of water excess, was the procedure that caused the least impact on

10 the original structures of biopolymers and other assemblies in seawater (Mišić Radić, 2011; Svetličić et al., 2013). Such an approach could offer an alternative way to visualize fragile organic structures when dealing with a large number of seawater samples. While an aqueous environment would be preferable, direct AFM imaging of marine polymers, vesicles and organic droplets as very soft and deformable structures is often hampered by their weak interaction with the interface. While interpreting the data, one should consider possible surface and/or dehydration artefacts. Representative images of marine

15 fibril forming networks with different levels of biopolymer entanglements and with different pore sizes are shown in Fig. 6. It is interesting to note that the appearance of marine gel networks at the nanometric scale, as shown in the corresponding Figure, coincided with the occurrence of gel microparticles detected electrochemically, as shown in Fig. 4, at the same sampling time. Image analysis revealed that biopolymer heights were in the 0.5- 2.5 nm range, while pore sizes ranged from ~ 40 nm to more than 1μm along the longest axis. Svetličić et al., 2011, found that these parameters (pore size, fibril height)

20 can be related to the aging of gel aggregates in the water column, i.e. older aggregates contained smaller pores and thicker fibrils, while "young" aggregates had a looser structure with smaller fibrils and bigger pore sizes. In addition, gel network formation was visualized at station SJ 107 in August 2010, when concentrations of micrometre sized GeP and SAP were low (0.2×10^6/L and 2.3×10^6/L, respectively). Significantly higher concentration of submicron organic content (surfactant activity, 2.88 mg/L) was detected 2 months prior to the mucilage event at the depth of 10 m, in 2010, which could act as a pool for

25 biopolymer self-organization towards gel network formation. One of the most comprehensive chemical characterization studies of Northern Adriatic gel macroaggregates showed that carbohydrates are the main organic fraction and that the average ratio of polysaccharides to total organic carbon is 41.5 % (Gianni et al., 2005b). Exopolysaccharides released by the diatom *Cylindrotheca closterium* under the conditions of increased production comprised a simpler system, which had the same chemical composition of polysaccharides as marine gels, indicating that the diatom exopolymeric substance was the

30 most likely origin of marine gels (Urbani et al., 2005). In addition, striking similarity was found in both marine gels and reconstituted polysaccharide networks (obtained from the same diatom) in terms of their mechanical properties as well as morphological features (Pletikapić et al., 2014). Marine gels were characterized in the non-imaging mode of AFM, the so called "fishing", where single polymers and/or their more complex associations are "picked up from the surface (fished) by the AFM probe and extended and/or unfolded/unzipped by increasing the distance of the AFM tip from the surface. This

approach enabled relating mechanical properties to different biopolymer morphologies (from single fibril, helical structures to more complex junction zones in networks with a higher degree of crosslinking), revealing new information about network architecture and connectivity. Networks were characterized as physical gels in line with DCS measurements (Mišić et al., 2011), where the level of association can be controlled by dilution, adjusting the ionic strength and stirring (Pletikapić et al.,

5   2014). Due to their dense 3D network structure and "sticky" nature, marine gels show a potential to scavenge nanoparticles that accumulate in the junction zones of the network (Svetličić et al., 2011; Pletikapić et al., 2012b). In this way, marine gels might stabilize nanoparticles and prevent aggregation and export from the water column. Recent review highlights potential of AFM in revealing new information about the interaction of inorganic nanoparticles with living and non living organic matter (Ciglenečki and Svetličić 2015). As native marine gels are constantly exposed to mechanical perturbations caused by

10   local hydrodynamics (turbulence and shear) as well as large-scale physical processes (strong winds and currents), the application of hundreds of piconewtons of force is highly biologically relevant. It is expected that AFM will reveal new classes of submicron particles in the future, and contribute to the understanding of the dynamic process of transformation and biogeochemical cycling of organic matter in marine systems. Vesicular structures are another structural form of organic matter in seawater (Fig 6). Existence of vesicle-like assemblies has already been inferred by Nagata and Kirchman (1997)

15   but vesicles were for the first time electrochemically detected in seawater samples (Žutić et al., 1984). Recently, it was reported that membrane vesicles of 100 nm in diameter can be released by marine cyanobacteria and are frequently found in marine ecosystems in the concentration range 10^5 to 10^6 vesicles/mL (Soler et al., 2015). Such vesicles may entrap nutrients, toxins and a variety of other organic molecular species present in seawater. These components may be concentrated and preserved from degradation in the inner cavity of the vesicle and/or attached to the membrane of the vesicle. This ability of

20   vesicles to transport and deliver diverse compounds in discrete packages adds another layer of complexity to the flow of information, energy and biomolecules in marine microbial communities (Biller et al., 2014). For identification of vesicle like structures, AFM can be a very suitable tool due to the high resolution imaging and easy sample preparation. Such vesicles (Fig. 7) were also periodically encountered during the diatom bloom experiment and during the Northern Adriatic field studies by AFM (Svetličić et al., 2008). It is likely that the abundance of such structures is highly underestimated. Due to the

25   small size and relatively soft and fragile character of these structures, during AFM imaging with relatively higher forces, particularly when contact mode is used, vesicles are swept away by the AFM tip and remain undetermined. Proper care while setting imaging parameters, scanning at low force (i.e. Peak Force Tapping mode) and coupling AFM with other bulk methods such as electrochemistry to identify the sample of interest with higher concentrations of vesicles may resolve the issue. Such an approach was used to study vesicle like particles in the Boka Kotorska Bay (Ivošević DeNardis et al., 2013;

30   2014). The most important findings show that both surface methods, electrochemical and AFM, provide comparable results on different scales. To summarize, application of AFM opens a possibility to directly explore the main organic assemblies found in seawater, their molecular organization and stability, providing new data and enhancing our understanding of the gel formation mechanism and the role of marine gels in ocean biogeochemical cycles. This information is difficult to access by conventional analytical methods. However, despite all, AFM is not yet exploited up to its full potential. Significant advances

in the field are expected by integrating AFM into versatile hybrid devices that would combine two or three complementary techniques in one instrument, allowing a more detailed and comprehensive analysis of marine samples. While simultaneous AFM imaging and mechanical mapping (stiffness, friction, dissipation and/or adhesion) is already showing its potential by increasing the number of studies conducted on biological samples (Dufrene et al., 2013, and references therein), including also marine samples (Francius et al., 2008; Pletikapić et al., 2012), of particular interest for investigation of marine organic matter is coupling AFM with different optical, spectroscopic and/or interfacial techniques (Flores and Toca-Herreta, 2009). In line with the topic covered in this review, integration of mercury as a substrate in the AFM setup is drawing particular attention (Schon et al., 2013). An AFM cantilever has been developed using a mercury fountain pen probe allowing simultaneous probing of mechanical and electrical properties, for instance in biological membrane research. In addition, a hybrid AFM- optical (fluorescence) microscope (Kassies et al., 2005; Geisse et al., 2009) would be extremely useful for deepening the research on different classes of organic particles traditionally detected by staining and microscopic analysis. An additional step forward in the field is expected by combining AFM and IR to provide simultaneous correlation of topographical (organizational) and chemical data (Dazzi et al., 2012; Amenabar et al., 2013; Kulik et al., 2014). Alternatively, one could use chemically functionalized tips, and by simultaneously performing imaging and force curve acquisition, generate chemical maps (Blanchette et al., 2008). Such studies would allow relating molecular organization of marine organic matter with chemical information on the µm and nm scales, which would undoubtedly widen the spectrum of information about samples of interest.

3.3 A field case study in the Southern Adriatic Sea (Boka Kotorska Bay, Montenegro): Oil spill incident

The field case study was undertaken because of the recent accidental sinking of a ship and spilling of diesel fuel in the bay of Boka Kotorska (Fig. 8, Ivošević DeNardis et al., 2014). The ship had an overall length of 32.7 metres and weighed 325 tons, carrying oil and diesel oil (D2). The ship sank on October 11, 2013 at Rt Murva (15 m depth). One month after the mechanical removal of oil spill, dispersed oil droplets were still present in the whole water column. Characterized oil droplets were in the continuum size range from micro to nano. Smaller oil droplets tend to accumulate at the halocline, while larger rise to the surface where they may coalesce. It was electrochemically detected that the highest concentration of micrometre droplets was 2.0×10^5/L at the 5 m depth (i.e., at the density gradient), while droplet concentrations were below detection limit at other depths. Dispersed droplets were in the size range from 1 µm up to 30 µm in measured samples (determined by microscopy and Nile red staining). Bacterial colonization of micrometre sized fuel droplets indicated that a biodegradation process occurred, as revealed by epifluorescence microscopy and DAPI staining (Fig. 9a). Hydrocarbon-degrading bacteria could account for up to 10 % of the total bacterial population (Hassanshahian and Cappello, 2013), thus the biodegradation process is rather slow. Hydrocarbon-degrading bacteria utilize oil droplets as a carbon and energy source. Bacterial efficiency in the degradation of diesel fuel droplets can be measured as a change in droplet concentration, distribution, size, bacterial film formation, bacterial oxygen consumption and through chemical changes of oil composition

(Baldi et. al., 1997; 1999). AFM could be of particular importance in imaging interactions between bacteria and oil droplets on the nanometeric scale, which would enable better understanding of the mechanism of biofilm formation and therefore provide an insight into the efficiency of the biodegradation process. In addition, force spectroscopy can be applied to probe the adhesion force between the bacterial cell and the droplet and to measure nanomechanical properties of both the bacterial

5   cell and oil droplets. Moreover, AFM can provide an insight into the nanoscale surface morphology of biofilms or aggregates. AFM images of larger and smaller. nanometre sized oil droplets spread over the mica substrate forming spherical caps, as shown in Figs. 9b and c. The imaged droplet diameter in seawater would correspond to 520 and 80 nm, respectively (Ivošević DeNardis et. al., 2014). Nanometre sized droplets were detected in the whole water column as reactive and stable fractions that persist longer and can therefore cause a significant decrease of phytoplankton primary production. Recovery of

10   phytoplankton variability and abundance after a heavy fuel oil spill in the Marmara Sea, Turkey, required a time period of three years (Tas and Erdogan, 2007). The consequences of anthropogenically caused oil spills are known to provoke serious ecosystem disturbances influencing the variability in planktonic dynamics, survival of fish eggs, larvae, shellfish, hatching, swimming activity, fertility in copepods, and overall tropic interactions. For example, dispersed diesel oil had a harmful effect on the cardiac activity of Mediterranean mussels (Bakhmet et al., 2009; Martinović et al., 2015) important for

15   adequate protection of shellfish farms, breeding control and preventing food poisoning of humans. Monitoring of water quality should be recommended due to the intensive boat trafficking and shipyard activity in the Boka Kotorska Bay in order to decrease the risk to the sensitive marine ecosystem. Boka Kotorska Bay is a semiclosed basin of Montenegro characterized by Karst rivers and underground springs, which affect the temperature, density and salinity of seawater (Campanelli et al., 2009). To understand the fate and behaviour of dispersed diesel fuel under the complex hydrodynamic

20   conditions requires a comprehensive and interdisciplinary approach. Supplementary modelling data of the corresponding geographical area would be of benefit in order to assess the scale of oil spreading and trajectory, important for emergency responses and decision matters. Such approach can help understand the scale of the problem and efficient coordination between local authorities, research and industry in order to enable environmental protection as well as sustainable development.

25   **4 Conclusions**

Organic matter in the water column has a highly reactive nature; it continuously undergoes biotic and abiotic transformations and consequently exhibits high variation in time and space, with a tendency to accumulate at the interfaces. Biogeochemical transformation of organic matter is rather complex process, where chemical composition of the seawater plays an important role. The fate of marine organic matter depends on the inorganic constituents but as well on biota, influencing on its physico-

30   chemical properties, distribution, stability and bioavailability of metal-organic complexes. To study the role that organic matter plays in marine systems, it is of crucial importance to characterize it in such a way as to preserve its original state. Hence, the electrochemical method of chronoamperometry at the dropping mercury electrode meets the requirements of non-

invasive, simple and fast analysis of raw aquatic samples, enabling simultaneous differentiation of organic constituents. On the other hand, atomic force microscopy allows direct visualization of biotic and abiotic particles in seawater at micro- and nanometre scales without the need for vacuum and complex sample preparation. A combination of complementary methods enables us to set a continuous scale description of marine organic matter from the micrometre to nanometre scale. The first

5   field oriented study showed how distribution of organic microparticles in the Northern Adriatic can be related to mucilage formation. A few months prior to the mucilage event, the concentration of accumulated surface-active particles in the halocline was 1×10^{7}/L, supported by certain meteorological and hydrodynamics conditions, and a large scale sol to gel organic matter phase transformation occurred. Such microparticles were therefore identified as reactive precursors of macroaggregate formation in seawater. Formation of a gel network at the nanometric scale coincided with elevated

10   concentrations of flexible gel microparticles or surfactant activity prior or after the macroaggregation event. The second field oriented study showed the potential of surface methods for testing the cleanup performance after oils spill events during accidental sinking of the ship in the Boka Kotorska Bay (Montenegro). In spite of the mechanical removal of the main oil spill content, the remaining micro and nanodroplets detected in the collected sample represent a threat to the nearby mussel farms, fisheries and tourism. Possible applications of these surface methods include prediction of mucilage events and

15   monitoring of organic pollution. Interdisciplinary approach and long-term measurements of relevant parameters are required in order to better understand the spatiotemporal distribution of organic matter in aquatic systems and consequently its role in the biogeochemical cycles. In the future, it is expected that integration of experimental data (on nanometre and micrometre scales) with mathematical models (on the kilometre scale), (taking into account meteorological data, oceanographic conditions, circulation pattern, satellite data) could contribute to a better understanding of organic matter dynamics in aquatic

20   systems as well as to evaluation of the ecological status of the corresponding system. Potential application of surface methods to the monitoring of organic matter dynamics in marine systems would be of great benefit to environmental protection, development and management of the coastal area, especially for tourism, fishery and cruiser trafficking.

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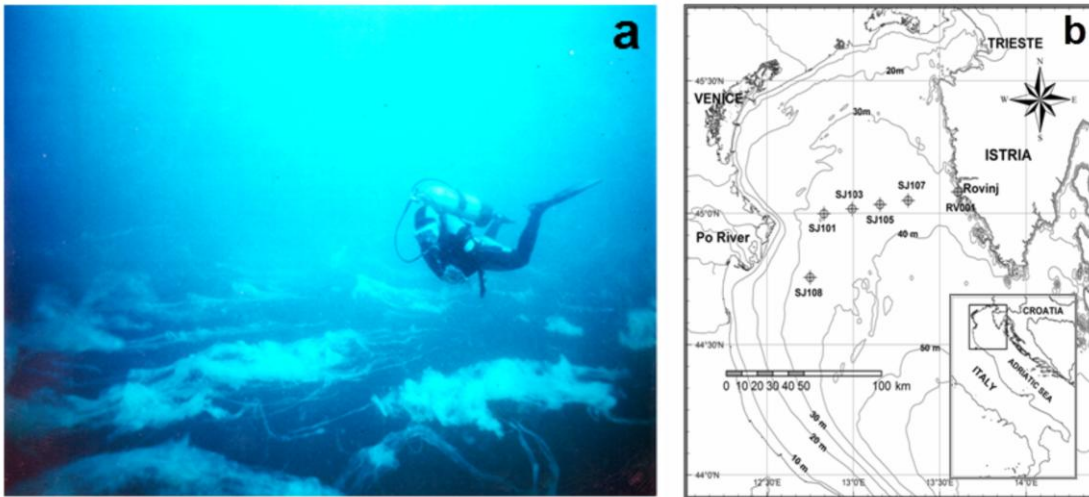


Figure 1: Macroaggregation phenomena in the Northern Adriatic (a) giant gel aggregate at 10 m depth as captured by a scuba-diver in August 1997 (courtesy of Gerald Müler-Niklas), and (b) map of the study area (inset) and sampling stations in the Northern Adriatic.

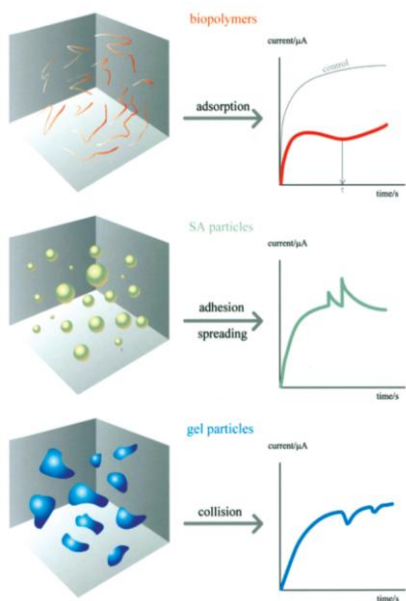



Figure 2: Illustration of different classes of organic constituents in seawater and their electrochemical signals at the electrode: biopolymer and small colloids, surface-active particles and gel particles (Svetličić et al., 2006).

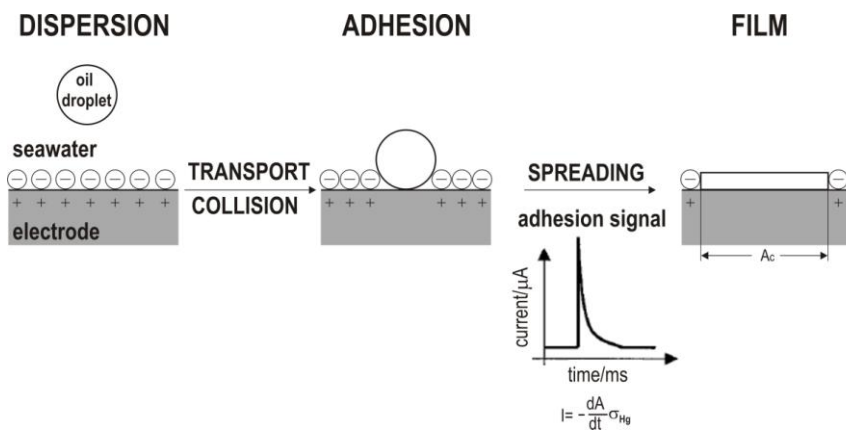



Figure 3: Basic principle of electrochemical adhesion based detection: Interaction of oil droplets with the charged electrode interface.

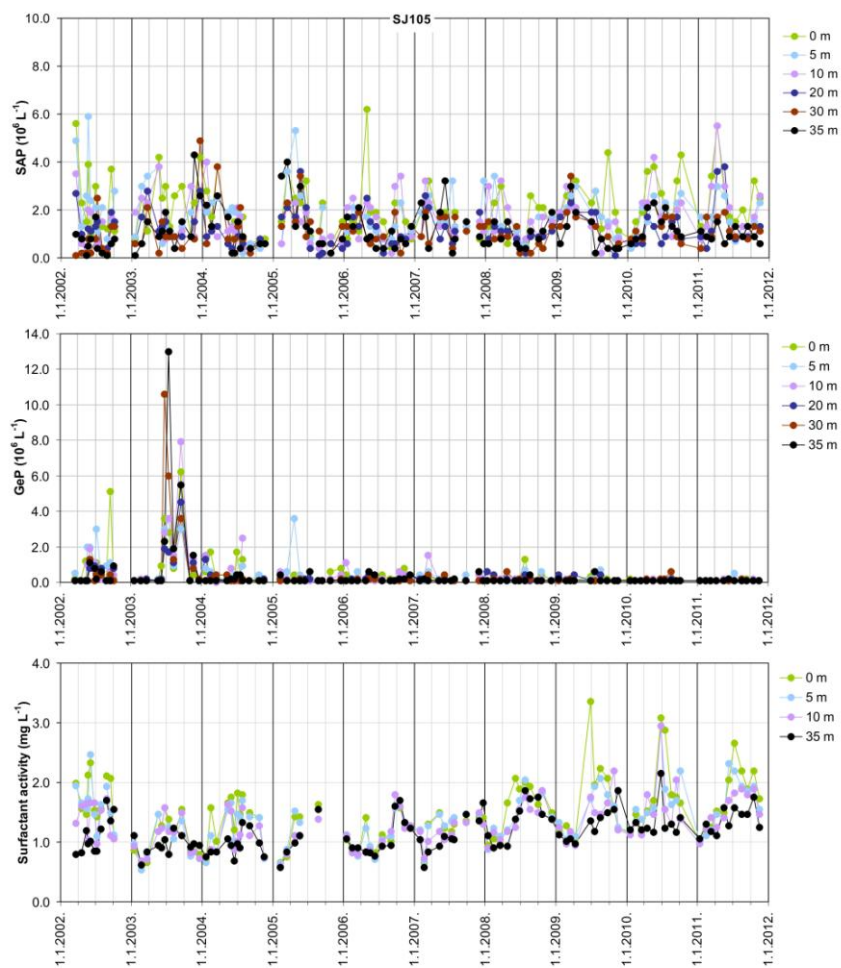



Figure 4: Annual variation of concentrations of: surface-active particles (SAP), gel microparticles (GeP) and surfactant activity determined by electrochemical analysis of raw seawater samples taken at sampling station SJ 105 in the Northern Adriatic (Project Jadran).

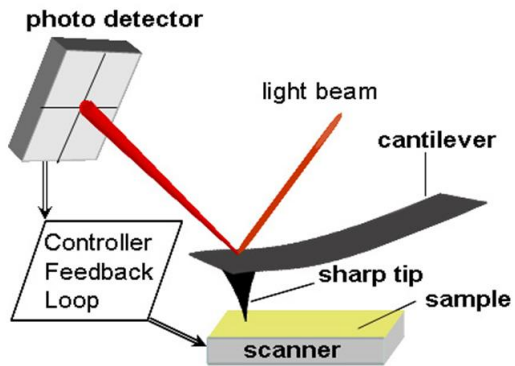
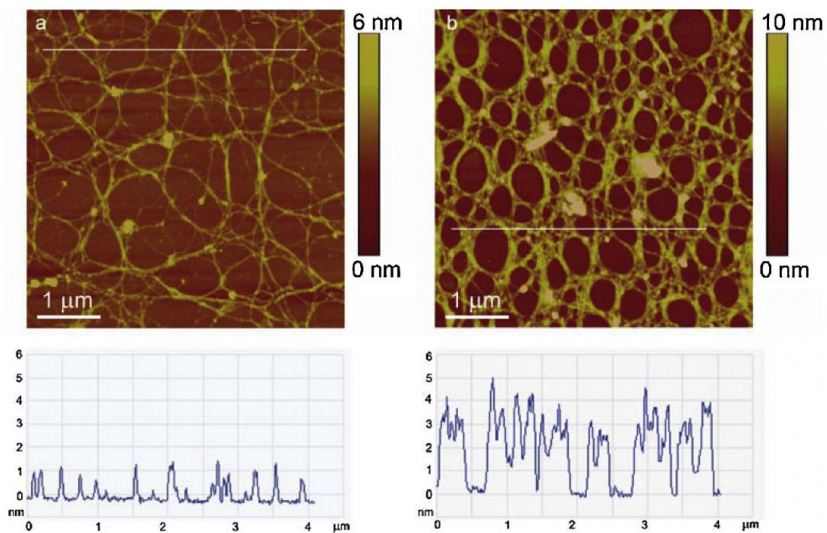



Figure 5: Basic principle of atomic force microscopy.

10   **Figure 6: AFM topographic images of marine gel representing networks with (a) a low degree of fibril crosslinking and with (b) a high degree of crosslinking with the corresponding cross-sections along the indicated lines. Marine gel samples were collected in the Northern Adriatic (SJ 105) in June 23, 2003, at a depth of 10-13 m (Mišić Radić et al., 2011).**

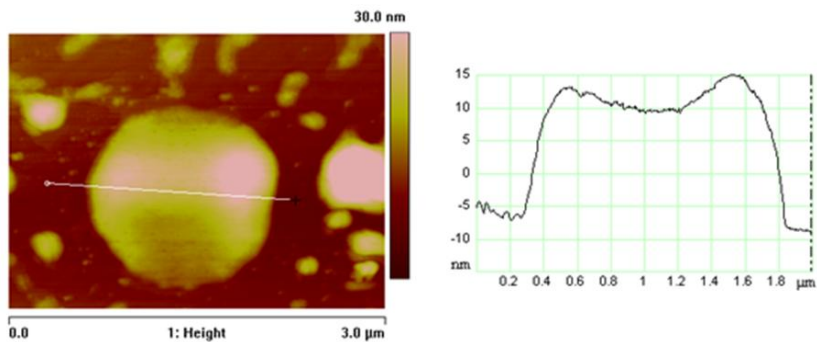



Figure 7: Large marine vesicle of 1.5 μm diameter imaged by AFM with the corresponding cross-section along the indicated line. Samples were collected in the Northern Adriatic (SJ 101) in May 18, 2010, at a depth of 0 m.

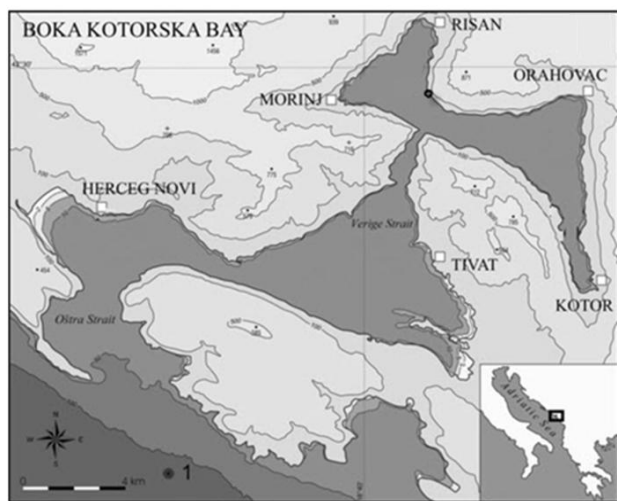



Figure 8: Sampling station of Rt Murva (●) in the bay of Boka Kotorska (Montenegro, inset: study area, southern Adriatic Sea, adopted from Ivošević DeNardis et al., 2014).

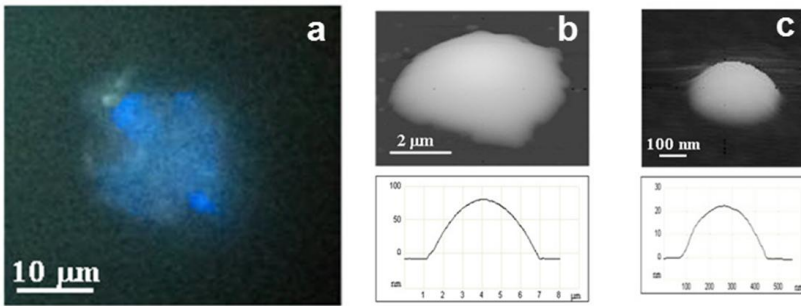



Figure 9: Dispersed fuel oil droplets observed (a) under an optical microscope with DAPI staining, (b) three-dimensional views of typical nanometre sized fuel oil droplets in the seawater sample taken at the Rt Murva depth of 0 m and (c) depth of 5 m visualized by AFM with the corresponding vertical profiles. Vertical profile enables to calculate oil droplet diameter in seawater (adopted from Ivošević DeNardis et al., 2014).