

# **Physico-Chemical Characterization of Natural and Ex-Situ Reconstructed Sea-Surface Microlayers**

Blaženka Gašparović<sup>1,a</sup>, Zlatica Kozarac<sup>a</sup>, Alain Saliot<sup>b</sup>, Božena Ćosović<sup>a</sup>, Dietmar Möbius<sup>c</sup>

<sup>a</sup>Center for Marine and Environmental Research, Ruđer Bošković Institute, HR-10001 Zagreb, Croatia

<sup>b</sup>Laboratoire de Physique et Chimie Marines de l'Université Pierre et Marie Curie, Observatoire des Sciences de l'Univers, UA CNRS 2076, 4 Place Jussieu, F-75252, Paris Cedex 05, France

<sup>c</sup>Max-Planck-Institut für Biophysikalische Chemie, Postfach 2841, D-37018 Göttingen, Germany

Running title: **Sea-surface microlayers**

Chemical composition and physico-chemical characteristics of natural and *ex-situ* reconstructed sea surface microlayer samples were studied using a complex methodological approach. Surface microlayer samples and the underlying seawater were collected in different seasons and different weather conditions in the northern most part of the Adriatic sea. The techniques used were thin layer chromatography with flame ionization detection for lipid classes analysis, electrochemical characterization of adsorbable organic substances using the ONP probe and monolayer techniques for surface pressure ( $\pi$ ) and surface potential ( $\Delta V$ ) measurements, as well as Brewster angle microscopy.

Our results indicated higher enrichment of organic matter for the microlayer sample collected in the warm period of the year (summer). This was reflected in the high surface activity of the present organic substances, and formation of a more condensed layer than for the other two samples collected in the spring. Of the two spring samples, the one sampled on a sunny day was richer in surface active organic material. We concluded that the most important source of surface active substances is the *in-situ* production of organic substances by the present plankton and bacteria, promoted by sunlight, while anthropogenic input comprises a smaller part of the present organic matter, around 10 % for all samples.

**Key Words:** sea-surface microlayer; lipid analysis; o-nitrophenol; surface active organic substances; voltammetry; monolayer studies; Brewster angle microscopy; northern Adriatic.

## INTRODUCTION

Sea surface microlayer is an important boundary, as an area of exchange of matter and energy, that either affects or is affected by global change (1, 2). Sea surface layer is generally enriched in organic substances, particularly those which are surface active, metal ions, bacteria and other microorganisms, relative to subsurface water (3-5). Organic substances found in the microlayer originate from the *in-situ* production or from different sources and have been brought there by different transport mechanisms from the seawater bulk, atmosphere or land. Adsorbed organic substances change the physical and optical properties, depending on the nature of organics, i.e. the nature of polar groups, architecture of the hydrophobic chain, and ionic strength, pH and temperature.

Although the physical and chemical properties of the sea-surface microlayer have been studied extensively, there is still lack of knowledge about the physico-chemical processes governing the formation and properties of the surface microlayer.

Electrochemical methods have been widely used for the investigation of surface active substances (SAS) in different waters (6 - 9). These methods are based on the principle that organic substances adsorbed on the electrode surface influence the mass and charge transport across such a modified phase boundary. The recently developed electrochemical method using the o-nitrophenol (ONP) probe as a convenient tool for rough characterization of adsorbable organic matter (OM) in the sea surface microlayer and subsurface seawater samples was applied in this work (10, 11). The method is based on the fact that different types of OM, viz. polysaccharides, proteins, lipids and refractory substances such as humic and fulvic acids, cause changes in the electrochemical characteristics of the ONP probe, such as changes in the voltammetric peak and prepeak heights and a shift of the peak potential. The relationship between these parameters serves as a good indicator of the prevailing type of OM in marine samples.

Monolayer studies, mostly measurements of surface pressure-area isotherms and elastic properties of sea-surface films were performed years ago and most of them have been recently reviewed (1, 2). The two most commonly measured properties of monolayers are their surface pressure ( $\pi$ ) and surface potential ( $\Delta V$ ) (12, 13). Surface pressure,  $\pi$ , is the difference between the surface tension of a clean and monolayer coated surface ( $\pi = \gamma_0 - \gamma$ ) and is dependent on lateral pressure in the interface. The surface potential of a monolayer,  $\Delta V$ , is defined as the difference between the measured potential of the

monolayer covered and clean surface, and is primarily dependent on the nature of the polar groups in the interface, and it provides information about the molecular orientation and changes at the interface.

Brewster angle microscopy (BAM) was developed as a powerful method for optical characterization of monolayers at the air/water interface (14, 15). BAM, as a non perturbing technique for the microscopic characterization of the monolayer provides information about the homogeneity of the film, existence and formation of domains, phase transition and adsorption of material from the aqueous phase. Although BAM has been used very successfully to investigate the morphology of lipid and fatty acid monolayers and the liquid crystal phase transition, as well as for studies of protein adsorption and crystallization processes at the air-water interface (15-18), to our knowledge no study of natural sea surface microlayer samples has been reported to date. This methodology for characterization of natural marine samples and samples of phytoplankton culture media has been proposed recently (19).

This study deals with a complex experimental approach for the investigation of natural sea surface microlayers collected from organic-rich and -poor, relatively uncontaminated sea waters. Investigations of representative samples were performed with natural and reconstructed films. Direct measurements in samples without any pretreatment (DOC and electrochemical measurements) provided information about the natural concentration level of OM and the characteristics of these substances. Lipids were estimated from extracted films (4, 20) while, for monolayer studies and BAM measurements, model films ex-situ reconstructed from extracted dissolved lipids of sea-surface microlayer samples were used. The ultimate aim was the physico-chemical characterization of natural sea-surface microlayer samples, comparing the results obtained by different methods.

## **EXPERIMENTAL**

### *1. Study area and collection of samples*

All samples were collected from the semi-enclosed, shallow Gulf of Trieste, the northernmost part of the Adriatic sea (Fig. 1). Stations located off the Bay of Piran were selected and sampled in June 19, 1995 (sample 95-170; 170 being the Julian day) and in March 29, and April 2, 1996 (96-89 and 96-93). Environmental conditions differed considerably: high primary production as inferred from high amount of polar lipids associated with living organisms, 102.04 and 9.85  $\mu\text{g dm}^{-3}$  in sea surface microlayer and underlying water (see Table 1), calm sea, very sunny, sampling in the afternoon- sample 95-170 -

station 1; low primary production (polar lipids concentrations, 5.33 and 1.2  $\mu\text{g dm}^{-3}$ ), calm sea after a strong North wind, sampling in the sunny early afternoon- sample 96-89 - station 1; low primary production (concentrations of polar lipids 2.66 and 0  $\mu\text{g dm}^{-3}$ ), rough sea after heavy rain, sampling in the afternoon- sample 96-93 - station 2.

Microlayer was collected from a hand powered small rubber boat with a Garrett-type metallic screen, made of stainless steel (collected film thickness: 0.4 mm) (4, 21). This method permits the collection of large volumes of sea samples even with relatively rough sea conditions, although a dilution of the real sea surface microlayer is occurring. The *in-situ* thickness of the films sampled are not known, so the enrichment of DOC compared to the underlying water could be several orders of magnitude higher than is reported here, applying the concept of a discrete surface film of monomolecular or plurimolecular thickness. Surface underlying water was collected by immersing 20 l glass carboys at a ca. 20 cm depth.

Filtration was performed within one hour, using preheated Whatman GF/F glass fibre filters at 450 °C, with 0.7  $\mu\text{m}$  pore size, to analyze separately the dissolved and particulate phases.

Dissolved lipids were extracted from seawater by doubly distilled chloroform (3 times, third at pH 2) for analysis and the *ex-situ* model experiment. Particulate lipids were extracted from Whatman GF/F glass fibre filters by a modified one phase solvent mixture methanol-water-methylene chloride adapted from Bligh and Dyer (22). After repeated extractions and subsequent phase separation, the lipid extract was recovered in the lower methylene chloride layer, dried by solvent washed magnesium sulfate and evaporated to dryness in a rotary evaporator at a temperature lower than 30 °C. Filters and extracts were kept deep frozen until the analysis.

## 2. Dissolved organic carbon analysis

Determination of the DOC content in the samples was performed using the high temperature catalytic oxidation (HTCO) technique, proposed by Sugimura and Suzuki (23). Model TOC-500 System (Shimadzu) with a highly sensitive Pt catalyst and non-dispersive infrared (NDIR) detector for CO<sub>2</sub> measurements was used. All precautions were in accordance with discussions for measurements of DOC published in the Special issue of Marine Chemistry (24). Organic carbon free Milli-Q water (Millipore, USA) was employed for the preparation of standard solutions (K-hydrogen phthalate)

(Merck, Germany). The blank of the system corresponds to  $0.15 \text{ mg C dm}^{-3}$ , while the precision obtained for the standard concentration of  $1 \text{ mg C dm}^{-3}$  was  $\pm 3.2 \%$  (25).

### 3. Lipid analysis

Total lipid extracts were analyzed for lipid classes by thin layer chromatography with the flame ionization detection (TLC-FID) Iatroscan technique, sensitive and qualitative for each lipid class with internal calibration (20, 26).

Chromarod thin layer chromatography with the flame ionization detection technique was used to measure the dissolved and particulate classes of lipids using a Iatroscan MKV TLC/FID analyzer (26). Lipid extract was analyzed by spotting on SIII chromarods with an automatic syringe and using four consecutive developing solvent systems similar to the multistep procedure described by Parrish (27). Hydrocarbons, wax esters + sterol esters, fatty acid methyl esters and free fatty acids were separated with hexane-diethyl ether-formic acid (98:2:0.5). Triacylglycerols, alcohols, sterols and diacylglycerols were separated with hexane-diethyl ether (87:13), whereas chlorins and monoacylglycerols were separated with diethyl ether-acetone (57:43). Finally, a fourth developing system, chloroform-methanol-water (57:47:1.4) allowed separation of diphosphatidylglycerols and phosphatidylglycerols, and phosphatidylethanolamines.

Separate calibration curves were established for each lipid class, using the following external standards covering the concentration range of the samples (0.1 to 0.5 mg): eicosane for HC (see Table 1 for acronyms), cholesteryl palmitate for WE, methyl behenate for ME, stearic acid for FFA; tripalmitin for TAG, stearic alcohol for ALC,  $\beta$ -sitosterol for ST, 1,3-dipalmitin for DAG, chlorophyll a for PIG, monopalmitoylglycerol for MAG, diphosphatidylglycerol sodium salt for DPG, phosphatidylglycerol ammonium salt for PG, dipalmitylphosphatidylethanolamine for PE. Each sample was analyzed in triplicate. The precision was about 5-10% in absolute weight, and the standard deviation accounted for 1-5% of the relative abundance of any lipid class.

Briefly, polar lipids (DPG + PG) are structural components of biomembranes and reveal the amount of OM associated to living organisms. Triacylglycerols (TG) indicate metabolic reserves. Finally some lipids are derived from the breakdown of glycerides, such as diacylglycerides (DG), monoacylglycerides (MG) and free fatty acids (FFA) and can be thus considered indicators of the state of degradation of biogenic matter.

#### 4. Electrochemical methods

Using ONP as an electrochemical probe, we have investigated SAS in seawater and sea surface microlayer samples by measuring adsorption effects at the mercury electrode/solution interface. Natural samples were measured either directly or after filtration.

Phase sensitive ac voltammetric measurements were performed using the EDT-ECP 110 Modular Research Polarograph (London, England). All experiments were performed in a three electrode system with a hanging mercury drop electrode (HMDE) by Metrohm (Switzerland). Ag/AgCl electrode was used as the reference electrode and a platinum wire as the auxiliary electrode. The ac voltage frequency was 170 Hz, and the p-p amplitude 10 mV. Scan rate was 20 mV s<sup>-1</sup>.

The electrochemical analytical procedure was described previously by Gašparović and Čosović (11). ONP (10<sup>-4</sup> M) was added into the model solution or marine sample just before voltammetric measurement. Model solutions simulated seawater conditions, i.e. 0.55 M NaCl and 2x10<sup>-3</sup> M NaHCO<sub>3</sub>, pH 8.4 were used. OM was accumulated on the mercury electrode at the potential of -0.35 V, prior to the potential scan, by stirring the solution for 1, 3 and 10 min.

Voltammetric measurements for each accumulation time include two scans recorded in the potential range from -0.35 V to -0.9 V. In the first scan, the reduction peak of ONP is recorded, while in the second scan on the same mercury electrode the reduction product gives a new peak at a more positive potential, which is denoted as the ONP prepeak A.

All values of the ONP peak or prepeak heights for a given accumulation time are presented as relative values normalized to the ONP peak or prepeak height in the absence of OM for model system, and normalized to the accumulation time t=0 for seawater samples.

Oleic acid (Fluka, Switzerland) and L- $\alpha$ -phosphatidyl-DL-glycerol (Sigma, USA) were used without further purification. NaCl (heated 450 °C/5 hours) and NaHCO<sub>3</sub> (Kemika, Croatia) were used with prior purification with charcoal. Mercury was purified by double distillation under reduced pressure. All solutions were prepared with deionized water obtained using the Milly-Q Water System (Millipore, Switzerland).

### 5. Monolayer studies

$\pi$ -A and  $\Delta V$ -A isotherms were measured in a rectangular Teflon trough enclosed in a tight box and thermostated. A Wilhelmy balance (15 mm wide filter paper) was used to measure the surface pressure, and the surface potential was measured using a vibrating plate condenser (13).

Chloroform (HPLC), p.a. grade used as spreading solvent was obtained from Baker Chemicals, Holland. Deionized water from a Milli-Q system (Millipore, Switzerland) was used to prepare the subphase.

### 6. Brewster angle microscopy

The morphology of monolayers was investigated using a commercial Brewster angle microscope BAM 1 manufactured by NFT (Nanofilm Technologie GmbH), Göttingen, Germany (14) and a closed homemade trough. The principle and design of the Brewster angle microscope have been described elsewhere (14 - 16). BAM is based on lateral changes of the refractive index and/or thickness of the film. P-polarized light is focussed on the clean air/water interface at the Brewster angle, which is about  $53^\circ$  when using visible light, and no reflection occurs. If the angle is kept constant, formation of a monolayer changes the optical situation and reflection is observed, containing information about the homogeneity of the film, existence and formation of domains, phase transitions and adsorption of material from the aqueous subphase.

## RESULTS AND DISCUSSION

### 1. Lipid analysis

Lipids can be used as source indicators through investigation of various marker series (28) or by considering the relative abundance of lipid classes (20, 27, 29). Lipid classes reveal contributions from living organisms (PL), metabolic reserves (TG) and breakdown products from freshly synthesized OM (MG, DG and FFA).

Table 1 shows the lipid class composition of the particulate and dissolved fractions of seawater samples 95-170, 96-89 and 96-93 measured with Iatroscan, expressed in  $\mu\text{g dm}^{-3}$  and in percent (%).



In sample 95-170, lipids were present in both the dissolved and particulate phases. Comparing dissolved lipids in the microlayer sample with subsurface water it is visible that there was enrichment in the microlayer of 1.96, which coincides with that found for the DOC value, whereas a higher enrichment factor value was found for particulate lipids, i.e. 2.86. Since the concentration of total dissolved lipids in the microlayer sample 95-170 was  $124 \mu\text{g dm}^{-3}$ , which corresponds to approximately 60  $\mu\text{g}$  of organic carbon, using the ratio from Bunt (30), lipids constituted a small part of dissolved organic material relative to other microlayers collected in other areas and seasons (31) and constituted only 2 % of the DOC concentration ( $3.24 \text{ mg dm}^{-3}$ ), suggesting that predominant SAS were lipid modified organic substances, which is in agreement with previous findings of D'Arrigo (32).

The microlayer was characterized by a high content of phospholipids (PL) with 44 % and 42 % of total lipids in the particulate and dissolved phases, respectively. This indicated a significant contribution of the OM from the living organisms such as phytoplankton and/or microorganisms having a low content of substances associated with energy reserves, as shown by the low content of triacylglycerols (TG), 5 %. The dissolved phase is also characterized by a low TG content, 1 %. Well-preserved recently-biosynthesized exudates can also participate in this highly PL-rich material in the dissolved phase. Noteworthy is the high contribution to the dissolved phase of monoacylglycerols (MG) (30%) and free fatty acids (FFA) (21 %). As these compounds mainly derive from the degradation of other lipid classes, such as TG and PL, as compared to the particulate phase, their abundance might be explained by intense bacterial degradation and possibly by photooxidation processes that occurred at the sea surface. Such degradation processes might also explain the low TG content along with the flight of TG-rich organisms, such as zooplankton, from the extreme surface due to high light and ultra-violet intensity.

Composition of the underlying water significantly differs from that of the microlayer, characterized by a lower content of diphosphatidylglycerols and phosphatidylglycerols (DPG + PG) (13 and 22 % in particulate and dissolved phases, respectively), and a high content of TG (37 %) in the particles as compared with 10 % in the dissolved phase. There was also a decoupling between the composition of the dissolved and particulate phases with regard to FFA, abundant in the particulate phase (30%), lower in the dissolved phase (17 %) and more pronounced for MG, predominant in the dissolved phase (38 %) and very low in the particulate phase (4 %).

Total lipid concentrations were much lower in spring 1996 than those encountered in June 1995, as it could be expected from the lower biological activity in the period of cold waters in the northern Adriatic.

Enrichment factors for sample 96-89 were as follows: 1.7 for particles; 29.1 for dissolved lipids (only DPG + PG were identified in the microlayer!). Apart from this microlayer sample for dissolved lipids, FFA predominated in all samples, especially in the particles in the underlying water (63%) and in the microlayer (39%). This strongly suggests that the OM was mainly composed of degraded material. Noteworthy is the high enrichment factor found for HC, 17.3, which suggested some contamination of the area due to the harbour activities and potential ship traffic.

Sample 96-93 showed different characteristics. A depletion of total lipids was observed for particles (6.5 against 19.5  $\mu\text{g dm}^{-3}$ ); but the sea was rough, and intense rains might have enhanced a scavenging effect. Enrichment was recorded for dissolved lipids, with a ratio of 4. DPG + PG prevailing in the microlayer for both the particulate (33%) and dissolved (60.5%) phases. FFA prevail in the underlying water (82.4%) but not in the microlayer (14.7%). FFA are very depleted in the microlayer (0.96 against 16.1  $\mu\text{g/l}$ ), but also in the dissolved phase (0.66 - 1.64).

## *2. Electrochemical study of natural sea surface microlayers*

The basic principle of the ONP, as an electrochemical probe, is that electrochemical characteristics like the ONP peak and prepeak heights, as well as the peak potential, change in comparison with the solution without the presence of surface active organic material. With an increasing concentration of OM, i.e. with the increasing surface coverage of the electrode by the adsorbed layer (longer accumulation time), the ac voltammetric peak of the ONP is generally decreased and shifted towards more negative potentials (10). Briefly, the ONP prepeak height is sensitive to the acidity of the organic substances adsorbed on the mercury electrode, and it increases in the presence of acidic organic substances, or decreases in the presence of neutral or positively charged OM in comparison with that in the absence of OM. The shift of the ONP peak potential ( $\Delta E_p$ ) is very sensitive to the hydrophobicity of the present organic substances. Roughly, in the presence of adsorbed hydrophilic organic substances, the ONP peak potential is shifted by no more than 100 mV, of conditionally hydrophobic ones between 100-170 mV, while in the presence of very hydrophobic organic substances, such as fatty acids, a strong shift of the ONP peak potential, 170 mV or more, occurs.

Since the sea surface microlayer is generally known as an area of enrichment of many different organic substances, especially hydrophobic ones, we have chosen two model substances, a long chain unsaturated fatty acid-oleic acid and a polar lipid L- $\alpha$ -phosphatidil-DL-glycerol, to illustrate how such lipid substances change the electrochemical characteristics of the ONP probe. As shown in Fig. 2., very hydrophobic substances such as oleic acid, starting from very low concentrations, change the ONP electrochemical characteristics in a very specific way. Disappearance of the prepeak at the plateau of the adsorption isotherm of oleic acid as well as an increase of the ONP peak height after an initial decrease are seen. In contrast, polar lipids showed almost negligible effects on the electrochemical characteristics of the ONP probe for concentrations that might be found in real samples.

As the mercury electrode is very hydrophobic, selective adsorption of surface active organic constituents of the sea surface microlayer, is occurring, so that by using electrochemical measurements characteristics of that OM can be followed.

Table 2 and Fig. 3 present the electrochemical characteristics of the ONP voltammetric peak and prepeak for the sea surface microlayer sample and subsurface seawater (95-170) which were collected and analyzed in June 1995 in the northern Adriatic sea. The corresponding DOC values were 3.24 and 1.62 mg dm<sup>-3</sup> which gave an enrichment factor of 2 for the dissolved OM content in the microlayer. A strong shift of the ONP peak potential for short and long accumulation times and disappearance of the prepeak for longer adsorption time, as well as the decrease at shorter time, followed by an increase of the ONP peak height at longer adsorption time, indicated a dominant influence of SAS of strongly hydrophobic character, as compared with the model substance - oleic acid shown in Fig. 2. These effects were, however, more pronounced for the nonfiltered microlayer sample than for the filtered one. From the calibration curve for oleic acid, it was evaluated that the present OM exhibited same influence on the ONP probe, viz. 0.8 mg dm<sup>-3</sup> oleic acid for the nonfiltered sample and 0.6 mg dm<sup>-3</sup> oleic acid for the filtered sample. No presence of other organic substances was observed due to the masking effect of the very hydrophobic substances adsorbed on the mercury electrode.

Considering the changes of the ONP electrochemical characteristics for increasing adsorption time, it can be assumed that, when a complete coverage of the electrode is reached and no peak potential shift occurs the peak and prepeak heights still change, probably because the present adsorbed layer changes as well, maybe in terms of forming more condensed packed layer(s) or a reorientation of the molecules adsorbed on the electrode.

In the subsurface seawater sample, the prevailing SAS showed a different adsorption behaviour at the mercury electrode/solution interface, as compared to the microlayer sample, i.e. a smaller shift of the ONP peak potential, a decrease of the main peak height, and a pronounced increase of the prepeak height, the latter being indicative of the presence of organic molecules bearing negative charges (11). The changes of the ONP voltammograms indicate predominance of more hydrophilic acidic organic substances.

Table 2 presents also the electrochemical characteristics of the ONP voltammetric peak for two sea surface microlayer samples and their respective subsurface seawater samples, which were collected in March and April 1996.

From the data obtained using the ONP probe in sample 96-89, a strong influence of the adsorbed OM on the voltammetric characteristics of the ONP probe in both nonfiltered and filtered samples was observed, allowing the conclusion that hydrophobic SAS were the predominant fraction in the sea surface microlayer sample 96-89. From the big shift of the ONP peak potential, for the longer accumulation time, an increase of the ONP peak height for the 10 min accumulation time as well as the disappearance of the prepeak for the same accumulation time are evident. These changes of the ONP electrochemical characteristics were qualitatively the same for the filtered sample, but shifted to the longer accumulation time, indicating that hydrophobic substances were partly removed during filtration. From the calibration curves for oleic acid, it was evaluated that the present OM exhibited the same influence on the ONP probe as 0.2-0.3 mg dm<sup>-3</sup> oleic acid for the nonfiltered sample, and 0.1-0.2 mg dm<sup>-3</sup> oleic acid for the filtered sample. Results of the lipid analysis showed that only polar lipids were present in the dissolved fraction of the surface microlayer sample 96-89. Since polar lipids, as illustrated in Fig. 2., do not influence the characteristics of the ONP probe, the most probable explanation for the changes of the ONP probe observed in the microlayer sample 96-89 could be the presence of other hydrophobic substances, not determined as lipid classes.

Despite the similar DOC values for the sea surface microlayer sample and the subsurface seawater 96-89 (Table 2), the influence of the present organic substances on the electrochemical characteristics of the ONP probe is different. In the subsurface seawater the dominant OM were very probably polysaccharides in combination with fulvic type substances. The presence of that type of organic substances was assumed from the small shift of the ONP peak potential, even for the 10 min accumulation, and parallel to that of an intense decrease of the ONP main peak height. From the calibration curves for polysaccharide dextran T-4 ( $M_w=4\ 000$ ) and fulvic acid isolated from lagoon

sediment (11), the presence of  $0.4 \text{ mg dm}^{-3}$  fulvic acid and  $0.5\text{-}0.6 \text{ mg dm}^{-3}$  polysaccharide was evaluated. However, this is only a rough approximation.

Regarding the microlayer sample 96-93 from Table 2 one might conclude, mostly from the small potential shift (from  $9\text{-}57 \text{ mV}$  and  $53 \text{ mV}$ ), that the present SAS were more hydrophilic. The increased ONP prepeak height indicated the presence of acidic substances, most probably humic type substances, which mostly contributed to the acidity of the seawater samples (33). Comparison of the electrochemical characteristics of the ONP probe obtained in the nonfiltered and filtered samples, as well as in the underlying seawater, suggested that the prevailing SAS were similar in all three samples, differing only in their concentrations. Evaluated concentrations of SAS in equivalents of humic acid and polysaccharide of dextran T-4 type were  $0.3\text{-}0.4 \text{ mg dm}^{-3}$  and  $0.2\text{-}0.3 \text{ mg dm}^{-3}$ , for all three samples, respectively.

### 3. Monolayer study of reconstructed sea surface microlayers

*Ex-situ* reconstructed sea-surface microlayers from the dissolved lipid extract have been studied by monolayer techniques and by Brewster angle microscopy (BAM). Surface pressure-area ( $\pi\text{-A}$ ) and surface potential-area ( $\Delta V\text{-A}$ ) isotherms have been measured. Simultaneously, BAM video images have been recorded. It should be pointed out that A in this case means the area occupied by the film in  $\text{cm}^2$ . Since surface concentrations and molecular weights cannot be specified, it is not possible to use a specific area expressed in area/molecule. This is the reason why areas in  $\pi\text{-A}$  isotherms of natural films are usually scaled as a trough area in  $\text{cm}^2$  and not as area/molecule in  $\text{\AA}^2$  or  $\text{nm}^2$ . However, Frew and Nelson (34, 35) recently developed a method for isolating marine microlayer slick surfactants by solid phase adsorption using reversed (C18) cartridges. Lipid content was examined and the  $\pi\text{-A}$  isotherms of microlayer films could be for the first time expressed on a specific area basis. Such approach is very helpful for improvement the characterization of SAS in the surface microlayer by using monolayer techniques.

The  $\pi\text{-A}$  and  $\Delta V\text{-A}$  isotherms of the sea-surface microlayer 95-170 are shown in Fig. 4. The film exhibited the characteristics of a liquid expanded phase without undergoing a phase transition. Collapse of the film occurred at a rather low surface pressure of  $23 \text{ mN/m}$ . Positive values of surface potential have been observed in the whole region of the isotherm.

The  $\pi$ -A and  $\Delta V$  -A isotherms for the subphase and sea-surface microlayer of samples 96-89 and 96-93 taken in 1996, are shown in Figs. 5 and 6. Both results for sample 96-89 showed that the surface microlayer was enriched in surface active material by comparison with the content of SAS in the subphase. Such behaviour was not observed in sample 96-93 where the difference between the surface active content of the subphase and that in the microlayer was almost negligible. In both cases, the isotherms of expanded type without phase transition were observed.

Surface potential values were positive in the whole area region for all microlayer samples. Surface potential did not show any fluctuation and the magnitude of  $\Delta V$  increased as the molecular area was reduced so that the surface dipole moment was relatively insensitive to compression, indicating that only the molecular density and not the molecular arrangement changed due to the compression of these monolayers.

Values for  $\Delta V$  in the compressed state of monolayers for samples 95-170, 96-89 and 96-93, were 300, 270, and 290 mv, respectively. Years ago Jarvis et al. (36) estimated the  $\Delta V$  values for 17 seawater microlayer samples from coastal waters as well as from the open ocean. The values were in the same range as ours, i.e. 240-375 mV . It is known that the  $\Delta V$  values for free fatty acids are low, for example for stearic acid - 2 mV and for oleic acid 46 mV, while they are much higher for esters, alcohols, and amines (400-650 mV). The magnitude of the observed surface potentials for the seawater microlayer samples presented in this work indicates that mixed films were formed where the high potential compounds such as alcohols, esters, and amines were coadsorbed with low potential free fatty acids. Comparison of these results with the lipid class in dissolved fractions (Table 1) points out to the conclusion that for samples 95-170 and 96-93 such interpretation is a reasonable one.

From the  $\pi$ -A isotherms, thermodynamic parameters describing the states of monolayers, namely compressional modulus  $C_s^{-1}$ , limiting surface area  $A_o$ , and compressibility could be calculated (12). The limiting specific area could be obtained by extrapolating the linear part of the surface pressure-area curve to zero surface pressure. The compressional modulus  $C_s^{-1}$  was calculated from the relation:

$$C_s^{-1} = -A (d\pi/dA)$$

where  $A$  and  $\pi$  are area and surface pressure, respectively. The inverse value of the compressional modulus  $C_s^{-1}$  is compressibility, and the higher  $C_s^{-1}$  means a more condensed (less compressible) monolayer. Values for the limiting area  $A_o$ , compressional modulus  $C_s^{-1}$ , and compressibilities of the sea-surface microlayers studied in this work are given in Table 3. For comparison, it is known that the

compressibilities of the monolayers of cholesterol and stearic acid are 0.0012 and 0.0019 m/mN, of some proteins between 0.015 and 0.03 m/mN, of sedimentary humic acid in different molecular weight range between 0.024 and 0.036 (37), of nonionic surfactant polyoxyethylene(6)dodecanol 0.056 m/mN, of chlorophyll 0.021 m/mN, of vitamin A 0.019 m/mN and of sodiumdodecylbenzenesulfonate 0.017 m/mN (38). Compressibility values for a series of natural films were found to be in a range between 0.0402 and 0.0805 m/mN (38). Our results are in very good agreement with these values.

However, when the compressibility data of pure compounds are compared to our data for natural OM from *ex-situ* reconstructed films, the general results indicate that natural films are not composed primarily of simple compounds. More complex mixtures containing polar compounds bound to the hydrophobic core of insoluble species were indicated.

This is also in good agreement with the recently obtained data (39) for a microlayer sample taken in the middle Adriatic, extracted with chlorophorm and then *ex-situ* reconstructed on the water surface. First, the original sample was put in the trough and the DPPC monolayer was spread over it. The  $\pi$ -A isotherms for DPPC were recorded without accumulation and after 1 hour of accumulation. The compressibility values calculated from these isotherms were 0.023 and 0.063 m/mN, respectively. For comparison, the compressibility value for the DPPC monolayer on deionized water is 0.010 m/mN. The compressibility value of the sample extracted with chlorophorm and spread on water surface was found to be 0.064 m/mN, which corresponds to the value for the DPPC monolayer spread on the original sample and measured after 1 hour of accumulation.

#### 4. Brewster angle microscopy

In BAM experiments, we tested the sea-surface microlayer sample under compression. The images derived from film 95-170 were dependent on the surface pressure. At low surface pressure ( $\pi \approx 0.25$  mN/m) liquid condensed domains could be seen as small bright areas in the liquid expanded phase, visualized as a dark background (Fig. 7). It should be mentioned that this figure has been already published in the paper where BAM is recommended as a method for characterization of natural films (19). Upon compression, these domains were pushed together and at higher surface pressures ( $>5$  mN/m) only the liquid condensed phase could be seen.

Similar behaviour was observed with sample 96-93. The BAM image for this sample, taken at a low surface pressure ( $\pi = 0.4$  mN/m), is shown in Fig. 8. The picture shows the familiar circular patches that

the condensed phase of the microlayer forms in the expanded phase. By compressing, the domains become smaller and densely packed. The homogeneous BAM image in the whole surface pressure region was observed with the microlayer sample 96-89 (not presented here). It means that only one monolayer phase was present and by compressing the film only the brightness of the image increased without formation of any structures. This is in good agreement with the results for the lipid class composition of dissolved fraction of microlayer sample 96-89, presented in Table 1, showing that only fraction DPG+PG was identified in the sample.

## CONCLUSIONS

According to the results obtained by different approaches for investigation of sea surface microlayer samples, very contrasted situations have been encountered underlying the extreme variability of this boundary layer. Sample 95-170, taken during a very sunny day in June, is the richest one in OM and also in OM possessing very hydrophobic properties. This is visible from the obtained DOC value ( $3.24 \text{ mg C dm}^{-3}$ ) and concentration of total lipids ( $0.36 \text{ mg dm}^{-3}$ ), as well as the biggest changes of the electrochemical probe. This is clearly reflected in the highest compressional modulus ( $\text{Cs}^{-1}$ ) value of 21.632 indicating a more condensed layer than for the other two investigated samples.

In the second microlayer sample 96-89, taken during a sunny day in early spring, the presence of hydrophobic SAS was noticed with the electrochemical probe, but in a lower concentration, as confirmed by lipid analysis ( $41.8 \text{ } \mu\text{g dm}^{-3}$ ).

Despite similar DOC values (1.35 and 1.37) for the microlayer sample 96-89 and subsurface water, selective enrichment of SAS in the microlayer sample is confirmed by the four times enrichment of total lipids and a different electrochemical behaviour of these two samples using the ONP probe, as well as by the recorded  $\pi$ -A and  $\Delta V$  -A isotherms.

All measurements have shown that the third microlayer sample (96-93) is not enriched with SAS, as indicated by similar electrochemical behaviour of the microlayer and the underlying water samples. As slightly higher concentration of total lipids is observed in the subsurface layer. It is probably due to heavy rain dilution and subsequent scavenging in the microlayer as well as mixing of surfacial and deeper water layers by wind.



Comparison of the obtained results shows that changes in the chemical composition are reflected in changed chemical and physical properties, which was recorded by the chemical and physical methods applied. Namely, different acidic-hydrophobic-hydrophilic properties of the present organic matter changed the ONP electrochemical characteristics in different ways, also reflected in different surface pressures and surface potentials.

The OM in the seasurface microlayer originate from the atmosphere, from the land or from the main seawater body or they can be produced *in-situ* by the present organisms. Our experiments showed that the most important source is the *in-situ* production of organic substances by the present plankton and bacteria, promoted by sunlight. At the same time, anthropogenic input of OM, indicated by aliphatic hydrocarbons which constitute a small part of total lipids, was found to be around 10 % for all samples.

Here, we have illustrated how a multitechnical approach can contribute to a better understanding of the origin of OM, as well as the connection between the chemical nature and physical behaviour of organics in natural marine samples.

#### **ACKNOWLEDGEMENT**

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## FIGURE CAPTIONS

**FIG. 1.** Map of the sampling stations in the northern most Adriatic sea.

**FIG. 2.** (a) Dependence of the normalized current of ONP peak on the concentration of oleic acid (1) and L- $\alpha$ -phosphatidyl-DL-glycerol (5). Adsorption isotherm for oleic acid (2). (b) Dependence of the normalized current of ONP prepeak on the concentration of oleic acid (3) and L- $\alpha$ -phosphatidyl-DL-glycerol (6). Dependence of the shift of ONP peak potential on the concentration of oleic acid (4) and L- $\alpha$ -phosphatidyl-DL-glycerol (7). Accumulation time 1 min.

**FIG. 3.** A.c. voltammograms of ONP for sample 95-170: (a) microlayer sample and (b) underlying seawater. Accumulation times (1 and 4) 0 min, (2 and 5) 1 min, and (3 and 6) 3 min. Curves (1-6)-first scan, curves (A)-second scan at the same mercury drop, recorded immediately.

**FIG. 4.** Surface pressure-area ( $\pi$ -A) and surface potential-area ( $\Delta V$ -A) isotherms of the sea surface microlayer sample 95-170.

**FIG. 5.** Surface pressure-area ( $\pi$ -A) and surface potential-area ( $\Delta V$ -A) isotherms of the sea surface microlayer sample 96-89 and of the subsurface seawater sample.

**FIG. 6.** Surface pressure-area ( $\pi$ -A) and surface potential-area ( $\Delta V$ -A) isotherms of the sea surface microlayer sample 96-93. (1, 1a) surface microlayer; (2, 2a) subsurface.

**FIG. 7.** BAM video image of the seawater microlayer sample 95-170. Published in *Croatica Chemica Acta* (19).

**FIG. 8.** BAM video image of the seawater microlayer sample 96-93.

## TABLE CAPTIONS

### TABLE 1

Lipid class composition of the particulate and dissolved fractions of seawater samples 95-170, 96-89 and 96-93 measured with Iatroscan, expressed in  $\mu\text{g dm}^{-3}$  and in percent (%). HC: aliphatic hydrocarbons; SE: wax and sterol esters; ME: methyl esters; FFA: free fatty esters; TG: triacylglycerols; ALC: free alcohols; ST: free sterols; DG: diacylglycerols; MG: monoacylglycerols; DPG+PG: diphosphatidylglycerols + phosphatidylglycerols; PE: phosphatidylethanolamines; PC: phosphatidylcholine; PIG: pigments.

**TABLE 2**

Electrochemical characteristics of ONP voltammetric peak for seawater microlayer samples and subsurface waters for different accumulation times (1, 3 and 10 minutes)

<sup>a</sup>shift of peak potential of ONP voltammetric peak,  $\Delta E = E_p - E_{p0}$ ,  $E_p$  is ONP peak potential for selected adsorption time,  $E_{p0}$  is the peak potential at  $t=0$

<sup>b</sup>normalized peak current of ONP;  $i_p$  is the peak current for selected adsorption time,  $i_{p0}$  is the peak current at  $t=0$

<sup>c</sup>normalized prepeak current of ONP (second scan);  $i_A$  is the prepeak A current for the selected adsorption time,  $i_{A0}$  is the prepeak current at  $t=0$

<sup>d</sup>prepeak disappears at the completely covered electrode

<sup>e</sup>although electrochemical data are given for nonfiltered sample, DOC value is given for comparison

**TABLE 3**

Limiting specific area, compressional modulus and compressibilities of the sea-surface microlayers.

	MICROLAYER				UNDERLYING WATER			
	Particulate $\mu\text{g dm}^{-3}$	%	Dissolved $\mu\text{g dm}^{-3}$	%	Particulate $\mu\text{g dm}^{-3}$	%	Dissolved $\mu\text{g dm}^{-3}$	%
Sample 95-170								
<b>HC</b>	22.34	10.0	3.18	3.0	7.28	9.0	1.71	3.0
<b>SE</b>	4.60	2.0	0.00	0.0	0.55	1.0	0.00	0.0
<b>ME</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.78	1.0

<b>FFA</b>	24.20	10.0	25.71	21.0	23.21	30.0	10.60	17.0
<b>TG</b>	11.86	5.0	1.00	1.0	29.41	37.0	6.09	10.0
<b>ALC</b>	9.23	4.0	2.69	2.0	0.43	1.0	2.37	4.0
<b>ST</b>	10.45	4.0	1.77	1.0	3.11	4.0	1.26	2.0
<b>DG</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
<b>MG</b>	42.19	18.0	37.48	30.0	3.22	4.0	23.58	38.0
<b>DPG + PG</b>	102.04	44.0	51.60	42.0	9.85	13.0	13.93	22.0
<b>PE</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
<b>PC</b>	5.36	2.0	0.88	1.0	0.00	0.0	1.52	2.0
<b>PIG</b>	0.00	0.0	0.00	0.0	1.39	2.0	1.02	2.0
<b>Total</b>	232.3	100	124.3	100	78.5	100	62.9	100
Sample 96-89								
<b>HC</b>	2.08	13.4	0.00	0.0	0.12	1.3	0.17	18.1
<b>SE</b>	0.50	3.2	0.00	0.0	0.00	0.0	0.07	7.3
<b>ME</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
<b>FFA</b>	6.12	39.4	0.00	0.0	5.76	63.0	0.33	36.4
<b>TG</b>	0.67	4.3	0.00	0.0	0.58	6.3	0.15	16.5
<b>ALC</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
<b>ST</b>	0.86	5.5	0.00	0.0	0.47	5.1	0.20	21.6
<b>DG</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
<b>MG</b>	0.00	0.0	0.00	0.0	0.79	8.6	0.00	0.0
<b>DPG + PG</b>	5.33	34.3	26.16	100.0	1.20	13.1	0.00	0.0
<b>PE</b>	0.00	0.0	0.00	0.0	0.13	1.5	0.00	0.0
<b>PC</b>	5.36	0.0	0.00	0.0	0.00	0.0	0.00	0.0
<b>PIG</b>	0.00	0.0	0.00	0.0	0.10	1.1	0.00	0.0
<b>Total</b>	15.6	100	26.2	100	9.1	100	0.9	100
Sample 96-93								
<b>HC</b>	0.81	12.4	1.08	8.6	0.36	1.9	0.20	6.5
<b>SE</b>	0.19	2.9	0.65	5.2	0.17	0.9	0.06	1.9
<b>ME</b>	0.00	0.0	0.00	0.0	0.00	0.0	0.06	1.8
<b>FFA</b>	0.96	14.7	0.66	5.3	16.10	82.4	1.64	53.1
<b>TG</b>	0.91	13.9	1.16	9.2	0.91	4.6	0.17	5.5
<b>ALC</b>	0.00	0.0	0.31	2.5	0.0	0.0	0.00	0.0
<b>ST</b>	0.22	3.3	0.39	3.1	0.0	0.0	0.15	4.9
<b>DG</b>	0.00	0.0	0.00	0.0	0.0	0.0	0.00	0.0
<b>MG</b>	1.07	16.4	0.00	0.0	2.01	10.3	0.00	0.0
<b>DPG + PG</b>	2.16	33.0	7.57	60.5	0.0	0.0	0.00	0.0
<b>PE</b>	0.00	0.0	0.70	5.6	0.0	0.0	0.81	26.3
<b>PC</b>	0.00	0.0	0.00	0.0	0.0	0.0	0.00	0.0
<b>PIG</b>	0.22	3.3	0.00	0.0	0.0	0.0	0.00	0.0
<b>Total</b>	6.5	100	12.5	100	19.5	100	3.1	100

Sample	DOC mg dm <sup>-3</sup>	$\Delta E/mV^a$			$i_p/i_{p0}^b$			$i_A/i_{A0}^c$		
		1	3	10	1	3	10	1	3	10
95-170										
Microlayer NF		142	142	147	1.26	1.93	0.82	0.3	0 <sup>d</sup>	0
Microlayer F	3.24	130	144	144	0.83	1.18	1.34	0.7	0	0
Subsurface NF	1.62-F <sup>e</sup>	42	65	-	0.67	0.61	-	2.9	3.6	-

96-89										
Microlayer NF		64	108	163	0.64	0.40	0.68	2.0	2.4	0
Microlayer F	1.35	30	84	148	0.72	0.53	0.55	1.7	2.5	0
Subsurface NF	1.37-F <sup>e</sup>	11	45	69	0.86	0.68	0.35	1.4	2.3	1.3
96-93										
Microlayer NF		9	41	57	0.79	0.64	0.44	1.8	3.1	1.8
Microlayer F	1.39	12	39	53	0.82	0.66	0.54	1.6	2.3	1.8
Subsurface NF		6	36	50	0.83	0.66	0.49	1.5	2.9	2.8

Sample	$A_0$ (cm <sup>2</sup> )	$C_s^{-1}$ (mN/m)	compressibility (m/mN)
95-170	100	21.632	0.046
96-89	154	12.996	0.077
96-93	157	13.138	0.076

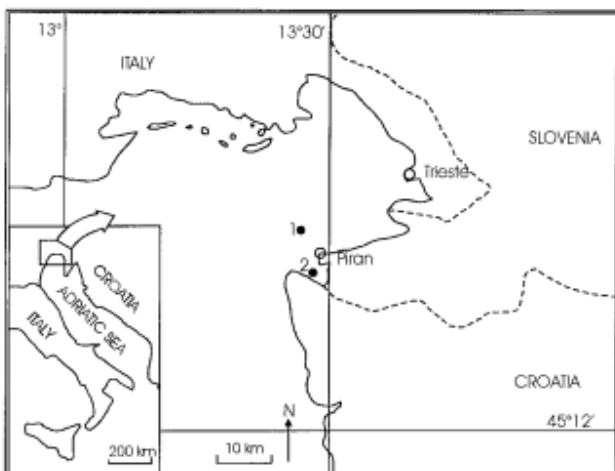


FIG. 1. Map of the sampling stations in the northernmost Adriatic sea.

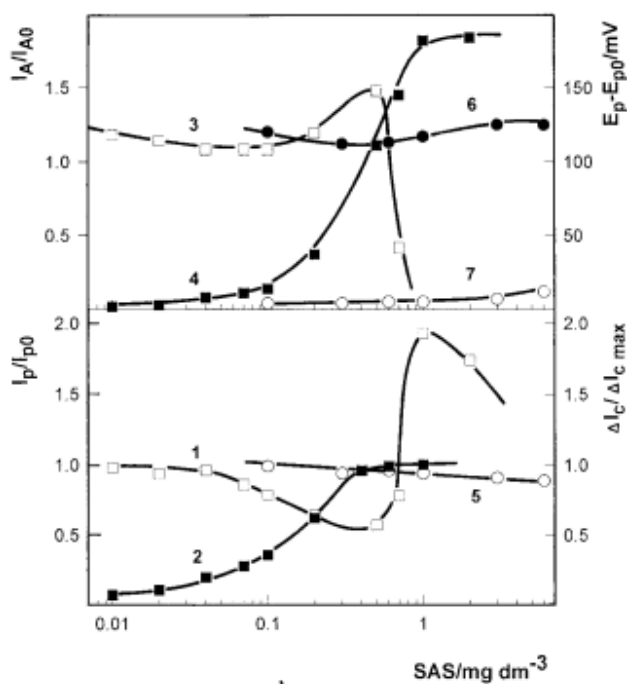
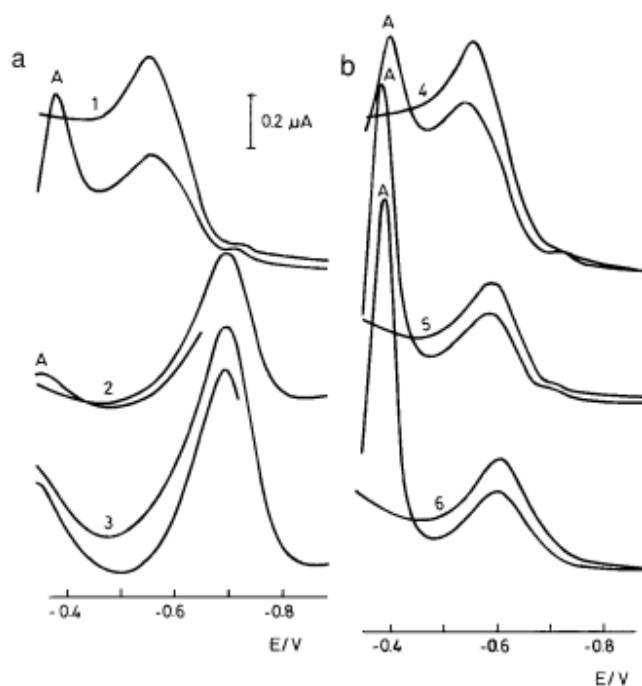
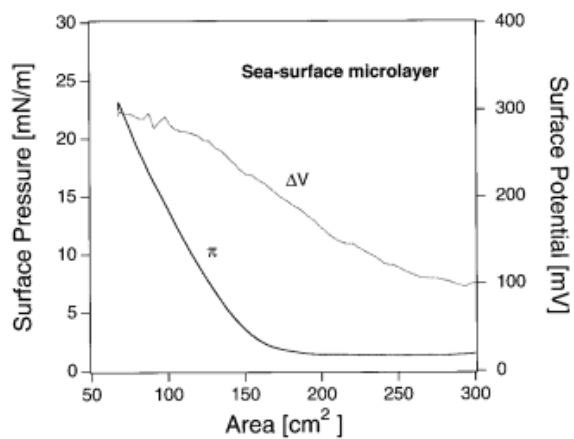


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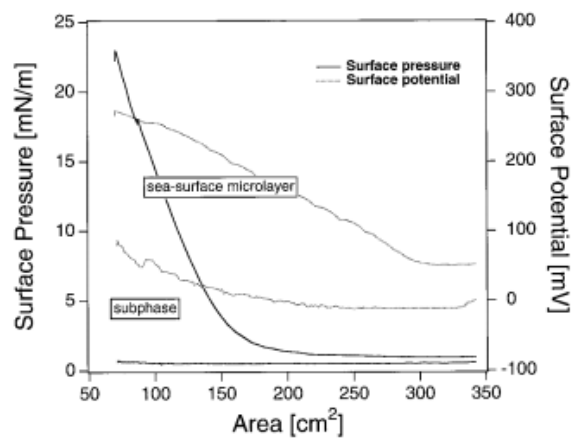




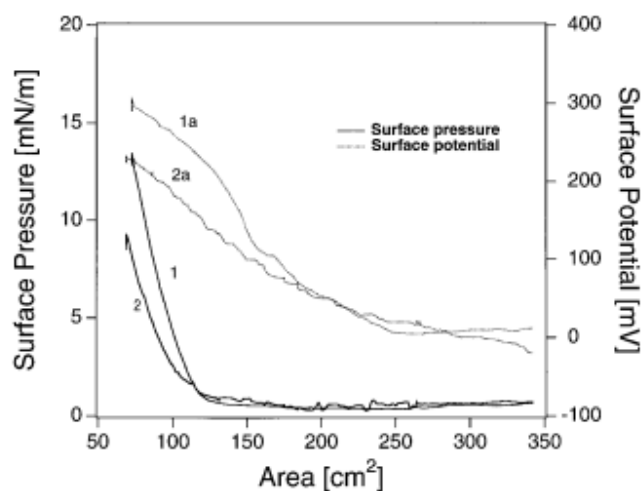
**FIG. 3.** AC voltammograms of ONP for sample 95-170: (a) microlayer sample and (b) underlying seawater. Accumulation times: (1 and 4) 0 min, (2 and 5) 1 min, and (3 and 6) 3 min. Curves 1–6, first scan, curve A, second scan at the same mercury drop, recorded immediately.



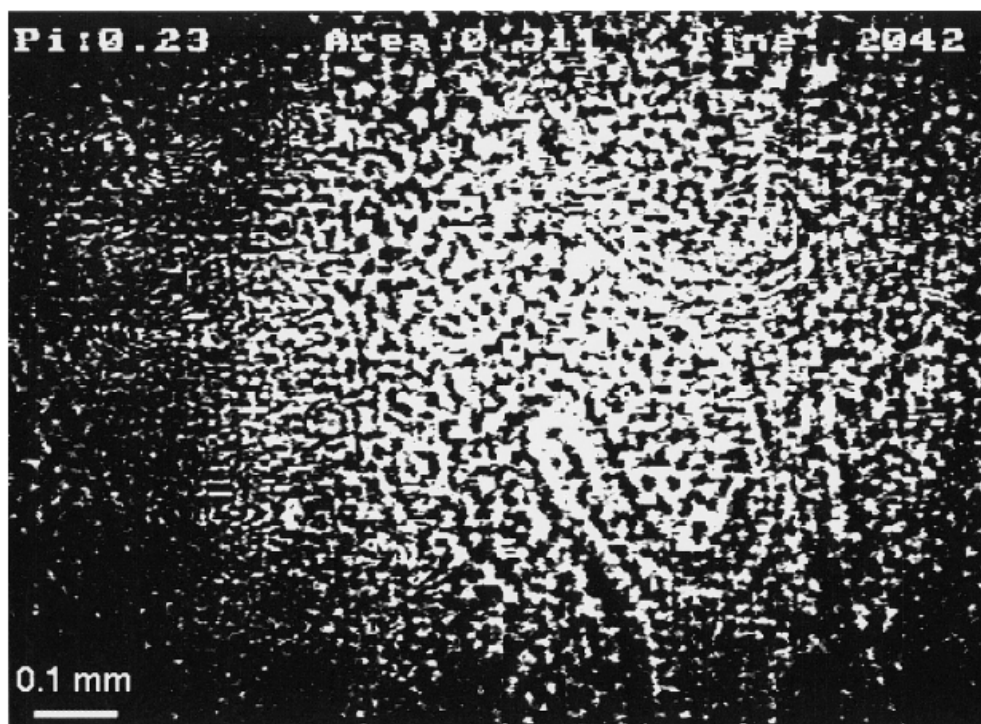
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**FIG. 6.** Surface pressure–area ( $\pi$ – $A$ ) and surface potential–area ( $\Delta V$ – $A$ ) isotherms of the sea-surface microlayer sample 96-93: (1, 1a) surface microlayer; (2, 2a) subsurface.



**FIG. 7.** BAM video image of the seawater microlayer sample 95-170. Published in *Croatia Chemica Acta* (19).