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Investigation of Sea-surface Microlayer and Phytoplankton Culture Samples by Monolayer Techniques and Brewster Angle Microscopy*

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Natural samples of sea-surface microlayer and phytoplankton culture samples have been studied by monolayer techniques and by Brewster angle microscopy (BAM). Surface pressure-area (π-A) and surface potential-area (ΔV-A) isotherms have been measured. Simultaneously BAM video images have been recorded.

The π-A isotherms, as well as BAM images of monolayers of dipalmitoyl phosphatidylcholine (DPPC), dimyristoylphosphatidic acid (DMPA) and dioctadecyldimethyl ammonium bromide (DOMA) spread on an aqueous subphase containing a sample of phytoplankton culture show that surface active substances released by phytoplankton influence molecular organization as well as domain morphology of the lipid monolayers.

The sea-surface microlayer sample spread at the air/water interface exhibits the characteristics of a liquid expanded phase without undergoing a phase transition. The BAM images taken from this film depend on surface pressure, showing at low surface pressures liquid condensed domains surrounded by a liquid expanded phase, and at higher surface densities only a liquid condensed phase.

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INTRODUCTION

The sea-surface microlayer has been defined as the top 1 to 1000 μm of the ocean surface. It is derived from multiple sources and is composed of different natural and anthrophogenic hydrophobic compounds, *i.e.* of dissolved and insoluble surface active material such as free fatty acids, alcohols, hydrocarbons, lipids, proteins, amino acids and also of more oxygenated molecules of higher molecular weight, such as glycopeptido-lipido-oligosaccharide complexes.[1,2] The main source of the natural (non-pollutant) substances is biota of the water column below the surface. Surface active substances are formed as a product of phytoplankton metabolism, as well as a product of degradation and decomposition processes of dead organisms.[3,4] Air bubbles, rising through the water column, scavenge these organic materials and bring them to the surface. In the surface microlayer in coastal environments high concentrations of toxic chemicals are also often found. The hydrophobic fraction of surface active material prevails in the surface microlayer but, depending on the primary production and season, the hydrophilic fraction can be suprisingly high.[5]

Although the physical and chemical properties of sea-surface microlayers have been studied extensively,[1,2] knowledge is still lacking about the physico-chemical processes governing the formation and properties of the surface microlayers which represent the interfacial region where many important bio-physico-chemical processes and the flux of gases take place. There is increasing evidence for the importance of studies of the nature, properties and role of the sea-surface microlayer. Sea-surface microlayers are sites of adverse biological effects and a medium for the transfer of energy and material between the sea and the atmosphere, thus playing an important role in the marine environmental protection and global changes.

The recently developed Brewster angle microscopy (BAM) has been shown to be a very powerful method for the optical characterization of monolayers at the air/water interface.[6,7] In the past, fluorescence microscopic techniques were extensively used to investigate various aspects of the dynamics and molecular organization in monolayers.[8,9] The coexistence of solid/liquid and/or liquid/gas domains was visualized under the microscope. However, the use of fluorescent probes has several disadvantages by comparison with the Brewster angle microscopy as a non-perturbing technique for the microscopic characterization of the monolayer. The principle, design and applications of BAM have been described in the literature.[6,7,10–17] Here, it is sufficient to say that 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° 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 on the monolayer morphology, which can be visualized.

The method provides information about the homogeneity of the film, existence and formation of domains, phase transitions and adsorption of material from the aqueous subphase.

The aim of this work was to apply the monolayer techniques (surface pressure and surface potential measurements) and Brewster angle microscopy to the investigation of natural samples, such as phytoplankton culture and sea-surface microlayer samples.

EXPERIMENTAL

Procedure

Surface pressure-molecular area (π-A) and surface potential-molecular area (ΔV-A) isotherms have been 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.

The morphology of monolayers was investigated using a commercial Brewster angle microscope BAM 1 manufactured by NFT (Nanofilm Technologie GmbH), Göttingen, Germany, and a closed homemade trough. The principle and design of the Brewster angle microscope have been described elsewhere.[6,7,11]

Chemicals and Samples

Dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidic acid (DMPA) and dioctadecyl dimethyl ammonium bromide (DOMA) were purchased from Sigma Chemical Co. and used as received.

Chloroform (HPLC), *p.a.* grade used as spreading solvent was obtained from Baker Chemicals, Holland.

Deionized water from a Milli-Q system (Millipore Corp.) was used to prepare the subphase.

The phytoplankton culture sample and the sea-surface microlayer sample were taken during the field experiment which was conducted in Piran, Slovenia, in 1995. The procedure and results of the whole experiment will be published elsewhere.[18] Suffice it to say that the natural sea-water sample was enriched with nutrients as well as micro- and macroelements necessary for plankton growth. Under these conditions cultures present in the sample show enhanced and faster growth. The sample that we analyzed was taken in the stationary growing phase.

The sample of sea-surface microlayer was taken with Garrett's sampler, 16-mesh stainless-steel screen (80 x 70 cm), which collects the top 100–150 μm of water surface.[19] The samples were extracted with an organic solvent[20] and after evaporation of the solvent kept in deep freeze. The sample for monolayer analysis was prepared by dissolving the material in chloroform and spreading it at the air/water interface.

All measurements were performed at room temperatures.

Although investigations of natural samples often suffer from irreproducibility, these measurements were reproducible, probably due to the fact that the phytoplankton culture sample was already in the stationary growing phase and the monolayer of the sea-surface microlayer was *ex-situ* reconstructed from the material of the natural sample previously extracted with chloroform.

RESULTS AND DISCUSSION

Phytoplankton Culture Sample

Surface Pressure-Area (π-A) Isotherms

Measurements of the surface pressure of the phytoplankton culture sample during a period of 2 hours did not show any considerable change in surface pressure, indicating that no appreciable accumulation of surface active substances released by this phytoplankton sample occurs on the free air/solution interface. If lipid monolayers were present on top of the solution containing the phytoplankton sample, an increase of surface pressure in time was observed. The same was recently recorded for other phytoplankton samples taken in the same area during 1996 (unpublished data).

Monolayers of DPPC, DMPA and DOMA were spread on the phytoplankton culture sample and *π-A* isotherms were recorded. The zwitterionic lipid DPPC and the negatively charged amphiphilic lipid DMPA belong to the group of phospholipids which constitute a major lipid material in biomembranes. Monolayers of DPPC, as well as monolayers of natural lecithins are probably the most extensively used models in studies of the phenomena observed in biomembranes.[21] Although DPPC is not a perfect model for cell membrane, it is very often used in monolayer studies since it is insoluble in water and forms a very stable monolayer at the air/water interface, as compared to natural lecithins. In natural aquatic systems, artificial cationic detergents (quarternary ammonium salts) are predominant in the group of positively charged lipids. Protamines can be found among substances of biological origin carrying a positive charge, especially in marine waters. They represent a group of simple proteins which occur combined with nucleic acids in fish sperm. Since protamines are water soluble, they do not form a stable monolayer. Therefore, we used DOMA instead, which is a cationic amphiphilic lipid forming very stable and well defined monolayers at the air/water interface.

The morphology and molecular organization characteristics of the lipid monolayers depend strongly on the nature and concentrations of other ions and molecules in the subphase.[22–25]

Surface pressure-area (*π-A*) isotherms for DPPC, DMPA and DOMA monolayers spread on water (curve 1) and on the subphase containing the plankton culture sample (curves 2 and 3) are shown on Figures 1–3. *π-A* iso-

therms were recorded immediately after the spreading (curves 1 and 2) and 1 hour after the spreading (curves 3), respectively. The accumulation time of 1 hour was chosen because preliminary results showed that in longer accumulation times no appreciable change of π-A isotherms was observed, indicating that equilibrium was reached in the adsorption process. Arrows on the isotherms correspond to BAM pictures.

DPPC spread on water exhibits a rather condensed film with a phase transition around 5 mN/m (Figure 1, curve 1). The isotherm of the DPPC monolayer recorded immediately after its spreading on the plankton culture subphase shows expansion of the area/monolayer molecule and a phase transition shifts from A = 0.8 to A = 1.0 nm^2. Upon further compression the isotherm curve 2 coincides with the isotherm of DPPC on water at an area/lipid molecule of $A \approx 0.43$ nm^2. The π-A isotherm of the DPPC monolayer on the plankton subphase recorded 1 hour after spreading shows expansion of the area/monolayer molecule in the whole area region and even in the condensed phase, at $\pi \approx 35$ mN/m the area/lipid molecule is increased by approximatelly 0.07 nm^2. The phase transition almost dissapears.

The surface pressure-area (π-A) isotherm for DMPA monolayer spread on water is of a condensed type without phase transition (Figure 2, curve 1). The π-A isotherm for DMPA monolayer spread on the plankton culture sample is not different from the isotherm for DMPA on water if recorded immediately (curve 2) but is more expanded if recorded after an accumulation time of 1 hour (curve 3). The isotherm shows expansion in the whole range of surface pressure up to a value around 30 mN/m. By further compression,

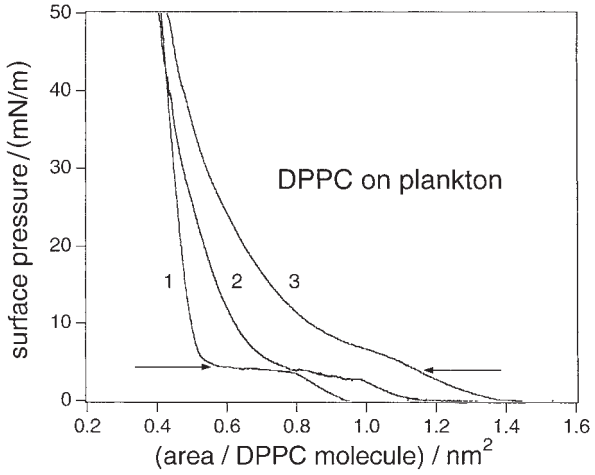


Figure 1. Surface pressure-area (π-A) isotherms for the DPPC monolayers spread on water (1) and on the phytoplankton sample (2, 3). Accumulation time 0 (1, 2) and 1 hour (3).

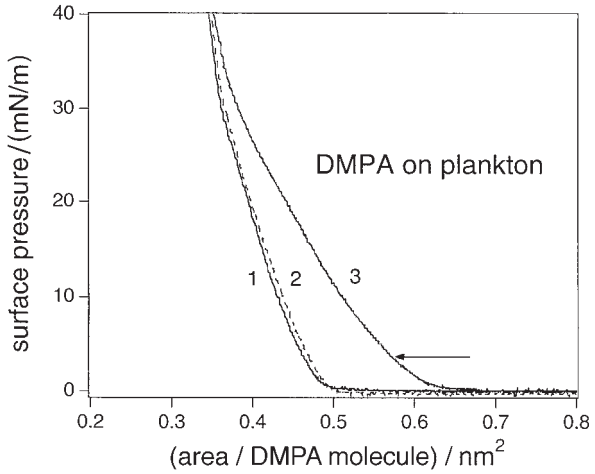


Figure 2. Surface pressure-area (π-A) isotherms for the DMPA monolayers spread on water (1) and on the phytoplankton sample (2, 3). Accumulation time 0 (1, 2) and 1 hour (3).

the area reached almost the same value as that for DMPA monolayer spread on water.

The isotherm of the DOMA monolayer spread on water is of the liquid expanded type with a phase transition around 13 mN/m (Figure 3, curve 1). The isotherms of DOMA monolayers spread on the plankton culture sub-

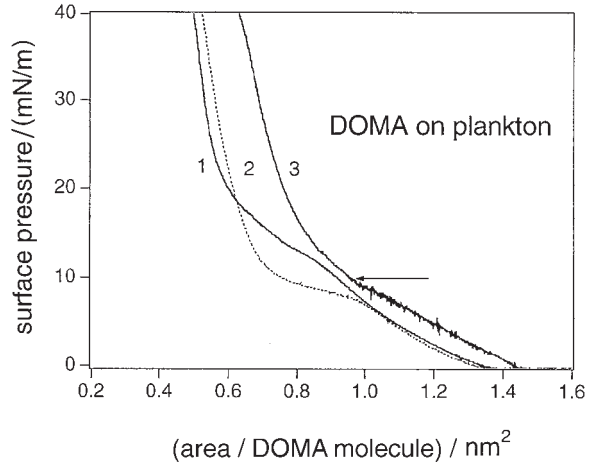


Figure 3. Surface pressure-area (π-A) isotherms for the DOMA monolayers spread on water (1) and on the phytoplankton sample (2, 3). Accumulation time 0 (1, 2) and 1 hour (3).

phase change depending on the accumulation time (Figure 3, curves 2 and 3). The isotherm recorded immediately after the spreading of the monolayer (Figure 3, curve 2) is different in the phase transition region (area between $A = 0.62$ and $A = 1.0$ nm^2). It shows contraction with respect to DOMA on water at larger areas $A > 1.0$ nm^2 ($\pi \approx 7.5$ mN/m). The area/DOMA molecule for the monolayer in the condensed phase ($A < 0.6$ nm^2, $\pi > 20$ mN/m) reached almost the same value as that for the DOMA monolayer spread on water (it is larger only by approximately 0.025 nm^2). The condensation of the DOMA monolayer in the range of 8 mN/m $\leq \pi \leq$ 18 mN/m can be attributed to the decrease of repulsing forces between the head groups of DOMA due to the electrostatic interaction with negatively charged ions and/or groups present in the subphase. In recent studies of cospread and adsorbed DOMA/Na-pyrene sulphonate monolayers, condensation of the DOMA monolayer at low surface pressures (till $\pi = 16.5$ mN/m) was observed as well.[26,27] The adsorption of counterions at the monolayer/subphase interface may be specific or nonspecific and leads to significant changes in intermolecular electrostatic interactions at the interface. Regarding the DOMA monolayers, it is known that counterions have an important and specific influence on the double layer forces.[22,25] The isotherm of the DOMA monolayer spread on plankton and recorded after 1 hour of accumulation (Figure 3, curve 3) is expanded in the whole area range and shows no phase transition. In the densely packed phase ($A > 20$ mN/m) the area increase is 0.17 nm^2/DOMA molecule.

An expansion of the area/monolayer molecule was observed for all three investigated monolayers, depending on accumulation time. The phase transitions of DPPC and DOMA monolayers observed on water dissapeared when isotherms were recorded after a longer accumulation time.

Adsorption of molecules dissolved in the bulk solution on to the air/solution interface usually causes changes of surface characteristics like surface tension and surface potential. If lipid monolayers are present on top of the solution, both the electrostatic and hydrophobic interactions between the lipid and solute molecules influence the monolayer characteristics. The expansion of π-A isotherms and the disappearance of phase transitions are usually attributed to the penetration of solute molecules into the lipid film, *i.e.* the solution solutes may be partially or completely incorporated into the hydrophobic region of a lipid monolayer. The thermodynamics of penetration was established years ago by Ter-Minassian Saraga.[28] However, the expansion of isotherms can be also interpreted in terms of adsorption underneath the lipid monolayer and formation of a sublayer.[24,26,27,29]

In adsorption at interfaces, both hydrophobic and electrostatic interactions determine the surface excess of solute species at natural phase boundaries. In the case of charged polyelectrolytes present in solution, an interaction with functional groups of lipid films can be expected as well.

From the values of surface active substances and dissolved organic carbon (DOC) reported elsewhere,[5] it was concluded that the phytoplankton culture was in the stationary growing phase. Fractionation of dissolved organic solutes was done by sorption on XAD-8 resin,[5,30] and the content of surface active substances and dissolved organic carbon in fractions were also determined.[31] Regarding the surface active material, the hydrophobic neutral fraction was dominant (42.3%) if compared with hydrophobic acid (28.6%) and hydrophilic (29.1%) fractions. However, DOC values showed the predominance of the hydrophilic fraction (56.1%), while in hydrophobic neutral and hydrophobic acid fractions the values of DOC were 17.1 and 26.8%, respectively. This discrepancy can be attributed to the fact that the sample was taken during the bloom of diatoms.[32] It is known that diatoms excrete a large quantity of hydrophilic carbohydrates[33] which are part of the DOC content but their contribution to the amount of surface active substances is negligible.

In the work reported here, both the hydrophobic and partial electrostatic binding contribute to the interaction of plankton exudates with the lipid monolayers.

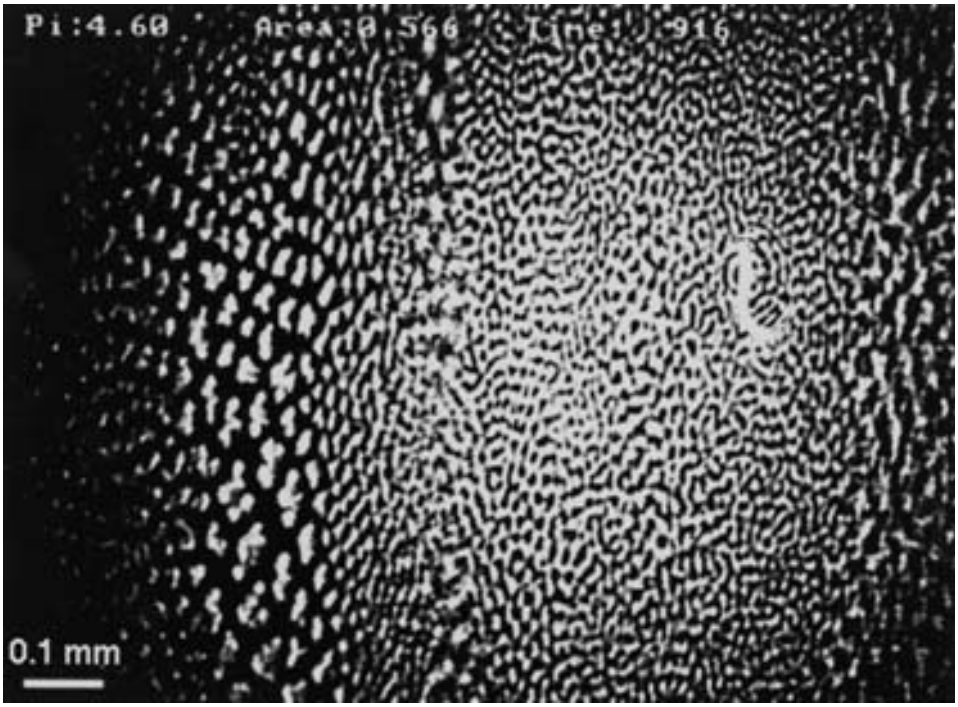


Figure 4a. BAM video image of a DPPC monolayer spread on water. Surface pressure π = 4.6 mN/m.

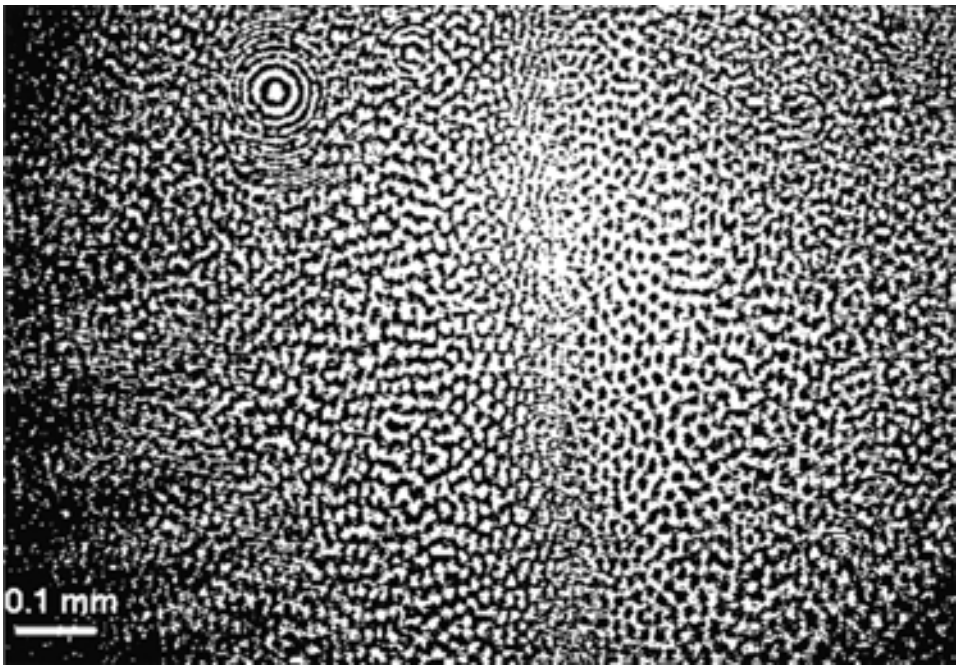


Figure 4b. BAM video image of DPPC spread on the phytoplankton subphase. Surface pressure π = 3.80 mN/m.

Morphology of DPPC, DMPA and DOMA Monolayers Spread on Water and the Subphase Containing the Phytoplankton Culture Sample

BAM video images were recorded simultaneously with the measuring of the surface pressure-area isotherms.

Brewster angle microscopy enables one to investigate artificial membrane systems such as monolayers of DPPC using a method most suitable for detecting domain formation or defect structures with a lateral size of several μm.[34] BAM, a non-perturbing technique, is very promising for direct observation of DPPC monolayers and their ordering phenomena on the microscale. The domains formed by compression of DPPC monolayers exhibit a wide variety of shapes depending on the details of experimental conditions.[34,35]

BAM images of DPPC spread on water and on phytoplankton culture subphase are shown in Figures 4a and b. As the DPPC solution is spread at the air/water or air/plankton interface, a homogeneous image is obtained. By compressing the monolayer, bright domains of liquid condensed phase showing optical anisotropy appear at surface pressures at which the phase transition in the surface pressure-area isotherms starts. By further com-

pression to higher surface pressures, the domains dissapear and a homogeneous film is formed. Such behavior is observed on both subphases but the domains of DPPC on the plankton culture subphase are smaller and the homogeneous film is formed around 20 mN/m *i.e.* at a lower surface pressure, as compared to the DPPC spread on water.

When the DMPA monolayer is spread on water, a homogeneous film is observed during the whole compression process. The BAM image of the DMPA monolayer spread on the plankton subphase (at π = 3.6 mN/m) is shown in Figure 5. Coexistence of the liquid and liquid condensed phase of the DMPA monolayer with oblong domains can be seen. In a part of the image the domains are in contact, forming small stripes.

DOMA at the air/water interface shows a homogeneous and featureless morphology, which is in agreement with previously reported data.[25] In the presence of plankton exudates, the morphology of the DOMA monolayer is different. BAM image of the DOMA monolayer spread on the plankton subphase is shown in Figure 6. Dendritic structures appear in the liquid/liquid condensed coexistence region (π = 10.5 mN/m). Upon further compression to a densely packed phase, the monolayer shows characteristics of a homoge-

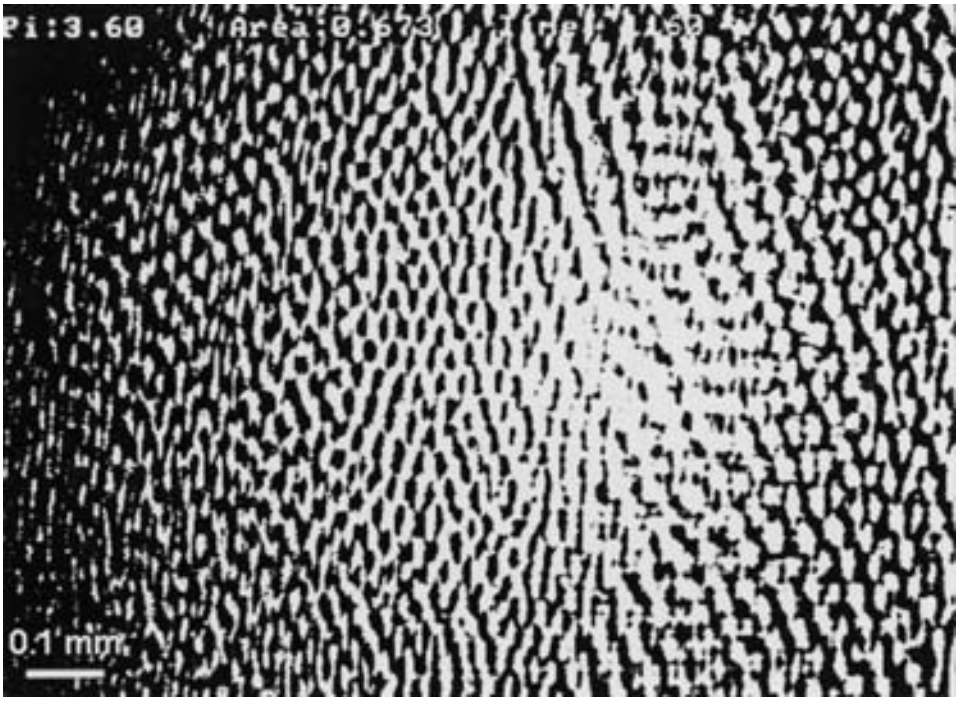


Figure 5. BAM video image of DMPA spread on the phytoplankton subphase. Surface pressure π = 3.60 mN/m.

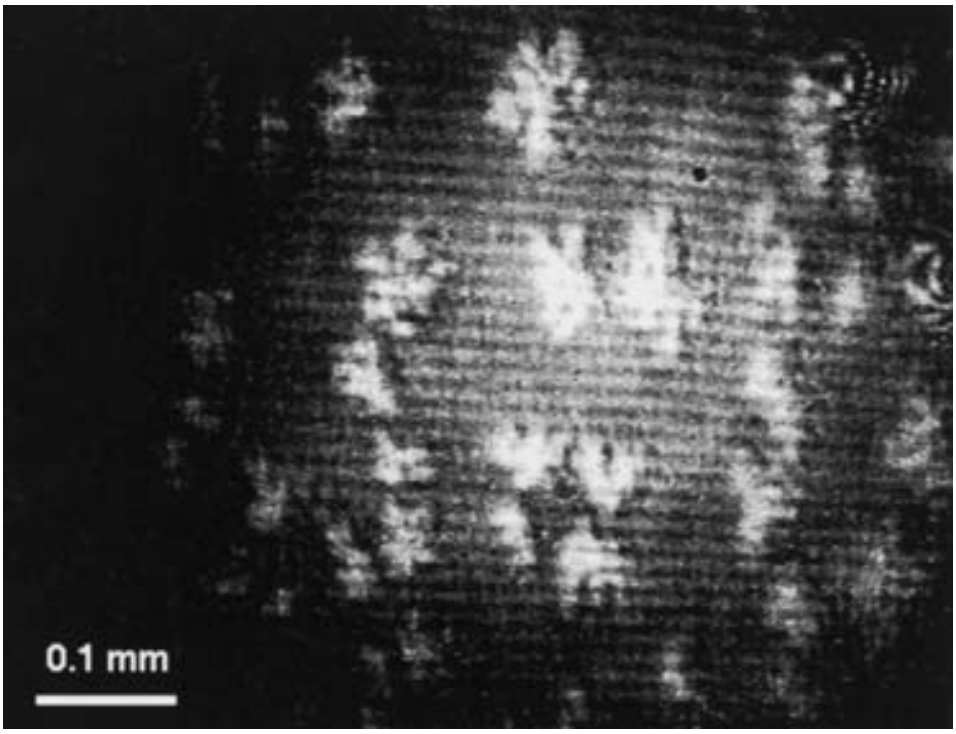


Figure 6. BAM video image of DOMA spread on the phytoplankton subphase. Surface pressure π = 10.5 mN/m.

neous film. The horizontal regular lines are interference fringes. It was recently found that the halogen counterions present in solution, specially Br^-, influence strongly the morphology of DOMA monolayers, wich is reflected in the formation of dendritic structures in the liquid/solid coexistence region.[25]

It is obvious that surface active substances released by phytoplankton influence the molecular organization characteristics and domain morphology of lipid monolayers spread on the plankton sample. Since the characteristics of the plankton exudates are dependent on the predominant species, such studies using different plankton culture samples taken in various seasons can be very helpful in characterization of organic material formed in natural aquatic system.

Sea-surface Microlayer

Monolayer studies, mostly measurements of surface pressure-area isotherms and elastic properties of sea-surface films were performed years ago.[36–40] Surface pressure as the difference between the surface tension of

the clean and monolayer coated surface gives us characteristics of the interface lateral pressure. The greatest problem in examining natural films by monolayer studies is that some very important parameters, such as the number of molecules and their molecular weight, *i*.e. the real composition of the sample, are unknown. Some assumptions about the chemical composition have been made on the basis of comparing the data for natural films with those for a series of model substances.[38] This is the reason why the area in π-*A* isotherms of natural films is usually scaled in the area of trough in cm^2 and not in area/molecule in nm^2. In this work, area is also given in cm^2 of trough at which film compression started after spreading. Recently, N. M. Frew and R. K. Nelson[41,42] developed a method for isolating marine microlayer slick surfactants and presented the π-*A* isotherms for marine microlayer films scaled for the first time according to specific area using measured chemical properties of the constituents of natural films.

Here, we present the use of recently developed Brewster angle microscopy for visualization of the sea-surface microlayer.

Simultaneously with the BAM video images, the surface pressure-area (π-*A*) and surface potential-area (ΔV-*A*) isotherms of the sea-surface microlayer were recorded to correlate the morphology and the monolayer phase state. The isotherms are shown in Figure 7. The film exhibits 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 mN/m. Positive values of the surface potential were observed in the whole region of the isotherm. Surface potential does not show fluctuations and the magnitude of ΔV increases as the film area is reduced, so the surface dipole mo-

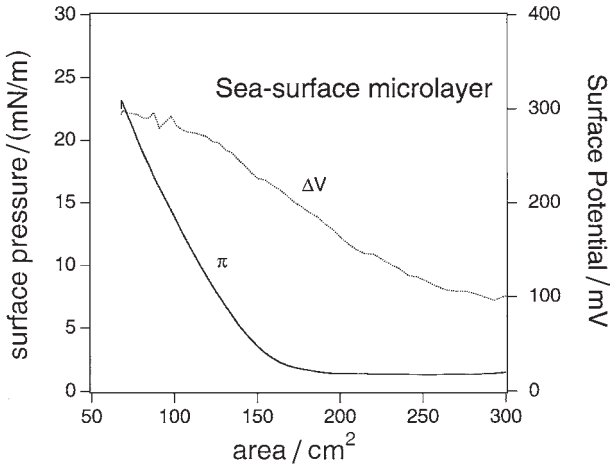


Figure 7. Surface pressure-area (π-*A*) and surface potential-area (ΔV-*A*) isotherms of the sea-surface microlayer sample.

ment is relatively insensitive to compression, wich means that only the molecular density and not the molecular arrangement changes by compression. In order to measure a π-*A* isotherm, we had to spread a very large volume (500 μl) of solution and compress the film very fast (compression velocity was 170 cm^2/min). For comparison, the usual spreading volumes for insoluble lipid monolayers are 30–50 μl at a concentration of 1 mM and a 10 times slower compression velocity is usually applied. These results indicate that the film contains a high fraction of water soluble hydrophilic material, which is bound to the insoluble hydrophobic part. This is in agreement with the results obtained by thin layer chromatography with the flame ionization detection (TLC-FID) Iatroscan technique, which was applied to the estimation of total lipids and the lipid class composition of the microlayer. Accordingly, polar lipids (diphosphatidylglycerols and phosphatidylglycerols) as well as monoacylglycerols and free fatty acids prevailed among the lipids.[43]

In BAM experiments we tested the sea-surface microlayer sample under compression. The images derived from the film were dependent on the surface pressures (Figures 8a-c). At a low surface pressure ($\pi \approx 0.25$ mN/m), the liquid condensed domains can be seen as small bright areas in the liquid ex-

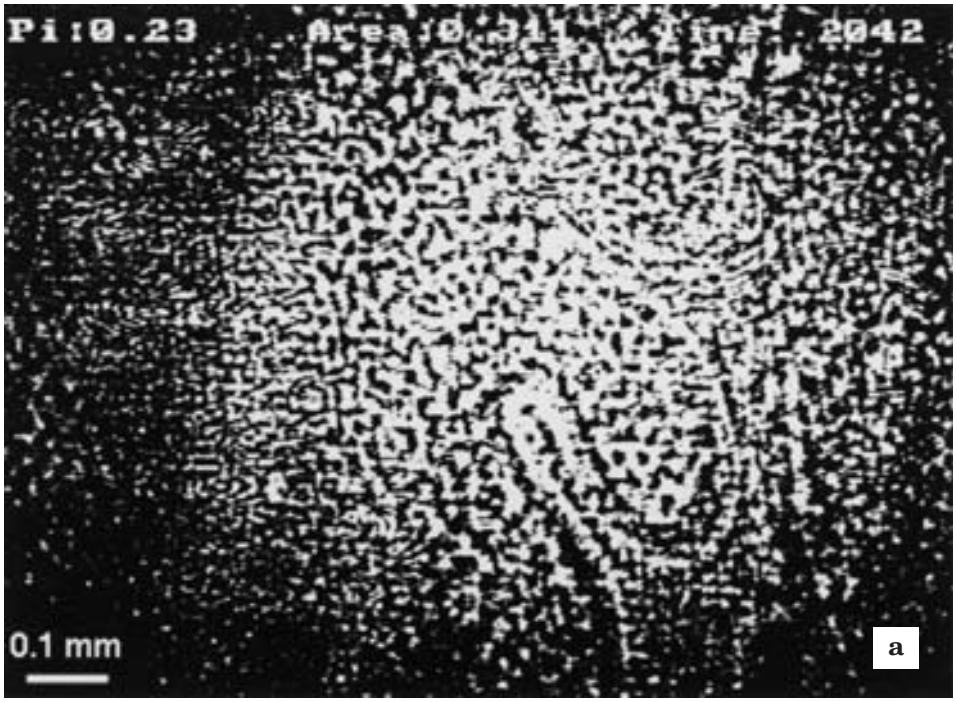
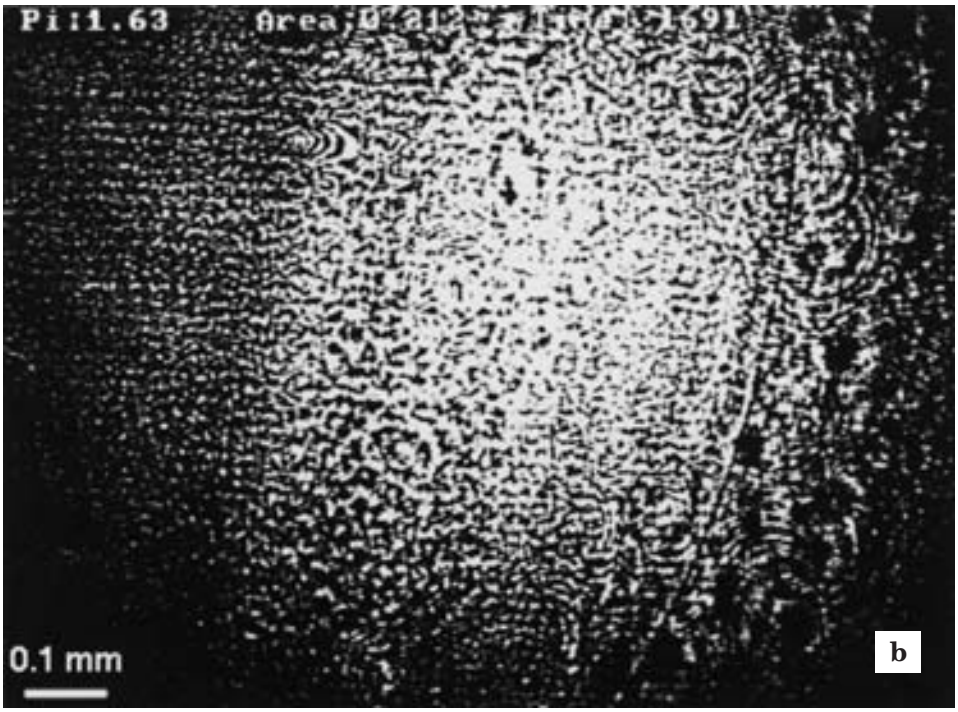
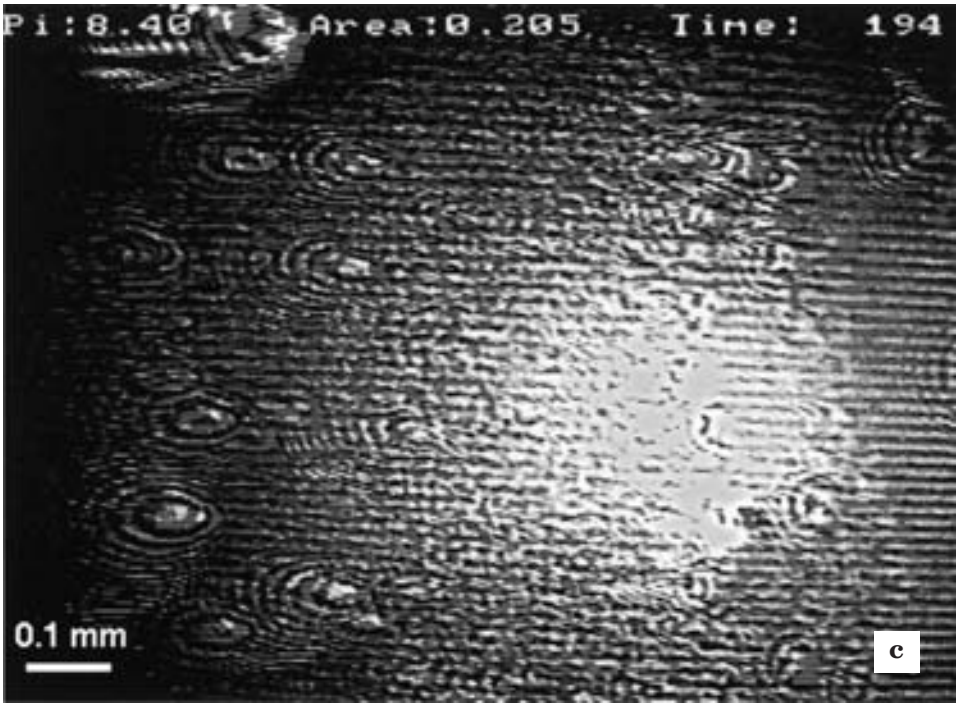


Figure 8. BAM video images of the sea-surface microlayer. π = 0.23 mN/m (a); π = 1.63 mN/m (b); π = 8.40 mN/m (c).


Pi:1.63
0.1 mm
b


Pi:8.40
Area:0.205
Time: 194
0.1 mm
c

panded phase which is visualized as dark background (Figure 8a). Upon compression, these domains are pushed together (Figure 8b) and, at higher surface pressures (> 5 mN/m), only the liquid condensed phase can be seen (Figure 8c). (The nearly horizontal stripes are interference fringes and the elliptical features are attributed to dust particles.)

CONCLUSION

There is increasing evidence for the importance of the studies of the nature, properties and role of the sea-surface microlayers and films formed in an estuary at the fresh water/sea water interface.

Monolayer techniques and Brewster angle microscopy have been proved to be very efficient analytical tools for a physico-chemical characterization of natural films and phytoplankton culture samples. They can be used for the investigations of interfacial layers in real aquatic systems in order to improve our understanding of the mechanisms and physicochemical processes at natural phase boundaries, which play an important role in the marine environmental protection and global change. The experimental approach presented in this work will be applied to the studies of natural films taken at different locations and in different seasons. Special attention will be paid to the studies in the sea area with a high primary production characterized by intensive phytoplankton blooms and the formation of high densities of large gelatinous mucous macroaggregates.

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SAŽETAK

Ispitivanje uzoraka morskog površinskog mikrosloja i fitoplanktonske kulture monoslojnim tehnikama i mikroskopijom pod Brewsterovim kutem

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Prirodni uzorci morskog površinskog mikrosloja i fitoplanktonske kulture ispitivani su monoslojnim tehnikama (mjerenje površinskog tlaka π i površinskog potencijala ΔV) i refleksijskom mikroskopijom pod Brewsterovim kutem.

Rezultati ispitivanja pokazali su da površinski aktivne tvari koje nastaju bilo metaboličkim djelovanjem fitoplanktona ili njegovim raspadom mijenjaju molekularnu organizaciju i morfologiju lipidnih monoslojeva, kako neutralnih tako i nabijenih. Ispitivani su slijedeći lipidni monoslojevi: dipalmitoilfosfatidilkolin (DPPC), dimiristoilfosforna kiselina (DMPA) i dioktadecildimetilamonijev bromid (DOMA).

Uzorak morskog površinskog mikrosloja pokazao je karakteristike tekuće ekspandirane faze bez faznog prijelaza. Mikrografije snimljene pod Brewsterovim kutem pokazale su ovisnost strukture filma o površinskom tlaku. Kod niskih površinskih tlakova dobivene su tekuće kondenzirane nakupine okružene tekućom ekspandiranom fazom dok je kod viših površinskih gustoća filma prisutna samo kondenzirana faza.