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# Diatom Polysaccharides: Extracellular Production, Isolation and Molecular Characterization

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Additional information is available at the end of the chapter

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## 1. Introduction

Diatoms (phylum Heterokontophyta, class Bacillariophyceae) are photoautotrophic protists derived from a secondary endosymbiosis involving a heterotrophic eukaryote host and a red alga endosymbiont. The annual primary production of diatoms is estimated to be 20 Pg ( $10^{15}$  g) of carbon, equivalent to 40% of marine and one-fifth of total global primary production and diatoms are responsible for more than half of the organic carbon flux to the deep ocean. DOM (dissolved organic matter) represents the largest pool of organic matter in the sea, with substantial part still uncharacterized [1].

The extracellular polysaccharide production by marine diatoms is a significant route by which photosynthetically produced organic carbon enters the trophic web and may influence the physical environment in the sea as observed for example when massive aggregation events on basin scale occur. Macroscopic dimensions and the massive appearance of gelatinous macroaggregates known as 'mucilage events' occurring episodically in Northern Adriatic are illustrated in Figure 1.

Despite many advances in organic carbon concentration in marine diatoms, the biophysical and biochemical mechanisms of extracellular polysaccharide production remains a significant challenge to resolve at molecular scale in order to proceed towards the understanding of its function at the cellular level and interactions and fate in the ocean. Chemical characterization of diatom extracellular polymer substance (EPS) isolated from laboratory cultures revealed that EPS are predominantly heteropolysaccharides that contain substantial amounts of uronic acid and sulphate residues [4-7]. There are of course other substances derived by disruption or decay of algal cells. Such substances are both of low

and high molecular weight, present mainly as dissolved or particulate matter. Microscopic (transmission electron and atomic force microscopy) and NMR studies reveal that fibrillar polysaccharides formed the bulk of oceanic DOM [8].



**Figure 1.** Macroscopic phenomenon of extracellular polysaccharide gelation in the Northern Adriatic Sea: (a) remote sensing by satellite showing gel phase in red color (adopted from [2]); and (b) at 10 m depth captured by a scuba-diver [3].

Many papers showed that the aldose signatures of marine DOM obtained from different seawater samples around the world is similar to that determined on cultured phytoplankton DOM [9,10] and that the carbohydrate production could be very different among the species selected, growth and environmental conditions [5,7,11-17]. These results are very important in order to understand the role of algal exudation in the aggregation processes observed in all of the seas and in general in carbon cycling in the euphotic zone. Many authors showed that cultured diatoms growth in P-limiting condition determines an increase of polysaccharides exudated by different diatoms species [4,6,7,11,12,15,17,18] both pelagic and benthic.

## 2. Techniques for primary structure characterization

Among the three major classes of biopolymers, the polysaccharides show the greatest chemical and structural variety. The nucleic acids are constructed from a handful of nucleotide bases so that the polymeric structure obtained is invariably linear. The number of amino acid building blocks used to construct the proteins is approximately twenty but, again, the proteins are always linear polymers. On the other hand, polysaccharides display a wide chemical and structural variability that is not found among the polypeptides and polynucleotides mainly due to the multiple hydroxyl functionality of the five- and six-carbon sugars. The replacement of one or more of such sugar hydroxyl functionalities by amine, ester, carboxylate, phosphate or sulfonate groups, leads to the frequent occurrence of tree-like branching and to the huge number of possible polymeric conformations of different solution behavior. For these reasons carbohydrate analysis involves, after isolation and purification, many steps, i.e. the determination of individual monosaccharides, of anomeric linkages, of branching and sequence, of anomeric configuration and, finally, of the chain conformation [19].

## 2.1. Isolation, purification and separation by ultra-filtration or by solvent precipitation

In the last two decades the filter (ultra-)fractionation technology has highly improved the methodologies for isolation and purification of polydisperse biopolymers, consisting of macromolecules, like algal polysaccharides, that present very often a large number of size fractions, going from oligomers of few sugar residues up to several million dalton of molecular weight. The tangential-flow filtration (known also as crossflow filtration) is one of the most useful tools for biopolymer separation and purification, both from seawater and culture medium. The principal advantage of this technique is that the residue which can obstruct the filter is substantially washed away during the filtration process by a tangential flow along the surface of the membrane. Depending on the biopolymer to be retained, membrane cut-off ranges from 1 kD to 1000 kD are used.

For large volumes, as seawater samples, a polysulfone multi-fiber system (hollow fiber tangential flow columns) is useful technique for simultaneous dia-filtration and concentration of samples given the large surface area available (on m<sup>2</sup> scale).

The addition of a non-solvent (or a bad-solvent for polysaccharides) to a given sample containing dissolved polysaccharides (algal cultures or seawater samples) allows the separation of the carbohydrate fraction by precipitation. This is a very common method for many advantages. It is non-destructive, inexpensive and relatively fast allowing also a fractionation in terms of polysaccharide molecular weight. Cold ethanol, isopropanol or acetone are often used and added to the cell-free supernatant of cultures or filtered samples in an appropriate volume to volume ratio (about 4:1). The precipitate is usually re-dissolved in pure water and the solution dialysed exhaustively against EDTA (0.01-0.1 M) and Milli-Q water [6,7,20,21] The precipitation/re-dissolution treatment is commonly performed three-four times depending on the purity to be achieved.

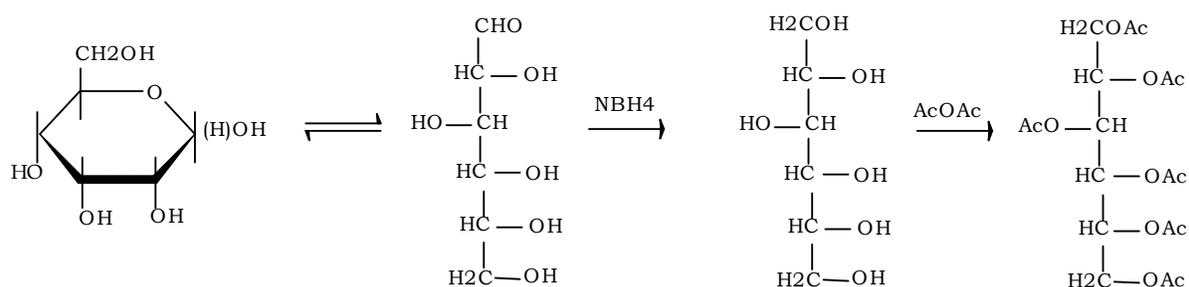
## 2.2. Gas-chromatography of alditol acetates of neutral monosaccharides

Although the excretion of photosynthetic compounds is recognised as the major source of carbohydrates in seawater [10], so far there are not many papers reporting on the molecular composition of carbohydrates in the exudates from diatom cultures [see for examples 6-7,10,22-28]. The molecular-level characterization of dissolved polysaccharides may provide basic information on the origin, the bioreactivity and the fate of these biopolymers. For example, after monosaccharide analysis Hama and Yanagi reported that the turnover rate of dissolved glucose was the highest among dissolved neutral aldoses, while turnover rates of galactose, mannose, xylose, rhamnose and fucose were similar to each other and markedly lower than glucose [29]. This was a significant finding suggesting that the degree of degradability of autotrophic DOM depends mainly on the relative percentage of glucose with respect to other monosaccharides.

Gas-chromatographic (GC) methodologies for neutral monosaccharide analysis used to characterize the primary structure of marine polysaccharides were reported in several

papers [5-7,20,30-39]. In general, the methodology is based on the acid hydrolysis of the polysaccharides and the suitable derivatisation of the saccharidic matter in order to obtain volatile compounds [40]. Thus, hydroxyl groups are subjected to chemical modifications obtaining silylated, acetylated, trifluoroacetylated, methylated or ethylated derivatives. Neutral and amino sugars are commonly analyzed after exhaustive hydrolysis with trifluoroacetic acid and the Neeser acetylation method [41]. The Neeser method provides a simple, rapid, and sensitive analytical method, which has been successfully used on glycoproteins and on plant and microbial cell-wall polysaccharide fractions in order to ensure complete release of amino sugars from glycoproteins, together with minimum losses of neutral sugars with an improved derivatization procedure by treatment with  $\text{CH}_3\text{ONH}_2\cdot\text{HCl}$  in pyridine. In addition the occurrence of uronic acids requires a preliminary reduction process with carbodiimide and  $\text{NaBH}_4$  [42]. The N-acetylated form of amino groups, that often occurs in marine polysaccharides, are removed by hydrolysis.

A very popular method of neutral carbohydrate analysis is the alditol acetate method originally described by Blakeney et al.[43], based on the four-step reaction described as:



**Scheme 1.** Scheme 1

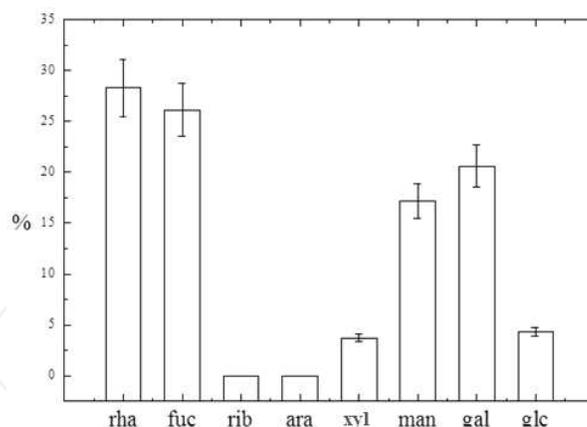
The complete separation of the mixture of neutral and amino sugars is usually obtained on polar capillary column (fused-silica coated with methyl silicone fluid).

As an example, the composition of extracellular polysaccharides produced by marine diatom *Chaetoceros decipiens* at the later exponential growth phase is presented in Figure 2. The exopolysaccharide fraction was isolated and purified by precipitation from bulk solution with isopropanol. The hydrolysis and the gas-chromatographic analysis yielded a suite of six neutral monosaccharides: glucose (glc), galactose (gal), mannose (man), xylose (xyl), rhamnose (rha) and fucose (fuc), present in different amount.

The molar ratio of monosaccharide presented in Table 1 shows rhamnose and fucose as the major components followed by galactose residue. The comparison with the composition of the exopolysaccharides obtained by Myklestad from *Chaetoceros decipiens* culture [5] shows a good agreement even at different growth conditions.

Exopolysaccharide molar composition	Rha	Fuc	Xyl	Man	Gal	Glc
<i>Chaetoceros decipiens</i>	7	7	1	4	5	1
<i>Chaetoceros decipiens</i> [5]	7	7	0.5	1	3	0.5

**Table 1.** Relative molar composition of exopolysaccharides from *Chaetoceros decipiens* cultures.



**Figure 2.** Monosaccharide pattern of the exopolysaccharides from cultured diatom *Chaetoceros decipiens* [44].

### 2.3. Anionic chromatography for charged and neutral monosaccharides

Carbohydrates in seawater include neutral sugars, aminosugars and acidic sugars, mainly uronic acids, phosphorylated and sulphated sugars [45-47]. By using high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detector (PAD) [38] the simultaneous analysis of mixture of such substituted carbohydrates is possible after acid hydrolysis and neutralization. Chemical derivatization is not required so that time consuming gas-chromatographic methods for uronic residue analysis are avoided.

Monosaccharides are completely separated by an isocratic elution [48-50] or in addition with a gradient course of two mobile eluent phases [51]. Seawater samples and culture media require a desalting step preceding the acid hydrolysis and the use of membrane dialysis (of about 1kDa) instead of resins is strongly recommended [51,52] avoiding the losses in carbohydrate yield.

### 2.4. Methylation analysis and mass spectroscopy

The most widely used method for determining anomeric linkage structure of a polysaccharidic chain is the methylation analysis. The polysaccharide is partially methylated, then hydrolyzed and the resulted partially methylated monosaccharides are acetylated. These methylated alditol acetate sugars allow to establish which carbons are involved in the anomeric linkage. The advent of combined GC and mass spectrometry allows the identification of monosaccharides and provides linkage information on complex polysaccharides. The methylation reaction is commonly performed using Harris' method [53].

Unpublished data (P. Sist) on axenic culture of *Chaetoceros decipiens* are presented in Table 2. The result of the methylation analysis of the exopolysaccharide allowed to identify the linkages among monosaccharides along the polymeric chain. The results showed that fucose and rhamnose were present mainly as terminal residues (t-Rha and t-Fuc) but a lower percentage of rhamnose (8.3%) was linked in the chain backbone (2-Rha) and 5.4% of fucose

Methylation derivative	Linkage	%
2,3,4-Me <sub>3</sub> Rha	t-Rha	21.4
2,3,4-Me <sub>3</sub> Fuc	t-Fuc	10.7
2,3,4,6-Me <sub>4</sub> Man	t-Man <sub>p</sub>	3.2
3,4-Me <sub>2</sub> Rha	2-Rha	8.3
2,3,4,6-Me <sub>4</sub> Glc	t-Glcp	4.0
2,3,4,6-Me <sub>4</sub> Gal	t-Galp	3.2
3,4-Me <sub>2</sub> Fuc	2-Fuc	2.7
3,4,6-Me <sub>3</sub> Man	2-Man <sub>p</sub>	2.3
2,3,4-Me <sub>3</sub> Man	6-Man <sub>p</sub>	3.3
3,5-Me <sub>2</sub> Xyl	2-Xylf	1.0
Fuc	2,3,4-Fuc	5.4
3,4,6-Me <sub>3</sub> Gal	2-Galp	13.4
2,3,6-Me <sub>3</sub> Gal	4-Galp	10.5
2,3,6-Me <sub>3</sub> Glc	4-Glcp	2.4
3-Me Xyl	2,4-Xylp	1.8
3,6-Me <sub>2</sub> Man	2,4-Man <sub>p</sub>	1.9
4,6-Me <sub>2</sub> Man	2,3-Man <sub>p</sub>	1.0

**Table 2.** Methylation derivatives of monosaccharidic units of exopolysaccharide from *Chaetoceros decipiens* culture.

represented branched residues (2,3,4-Fuc). Galactose residues which were linked at carbon 2 and 4 (23.9% of 2-Galp and 4-Galp) resided predominantly in the backbone, while mannose was both a branched residue (2.9% of 2,4-Man and 2,3-Man) and mono-substituted in a linear chain (5.6% of 2-Man and 6-Man).

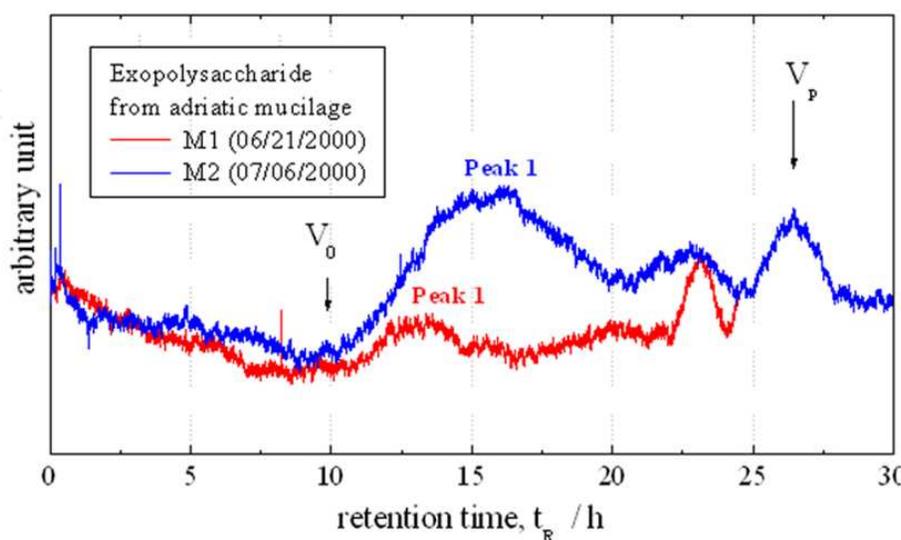
The relatively high percentage of galactose which could be present as  $\alpha$  or as  $\beta$  anomeric configuration in the chain backbone suggested a possible extended and rigid chain conformation of the *D. decipiens* polysaccharide as also found for model polysaccharides in solution [54,55].

## 2.5. Gel permeation chromatography

The heterogeneity and the molecular weight polydispersity of polysaccharide fractions can be analyzed by gel permeation chromatography (GPC) [6,37,56]. This analytical technique is also called size-exclusion chromatography because the fractionation occurs according to the molecular size,  $V$  (not molecular weight!), of the polymer which easily or not permeates pores of a suitable dimension of the gel matrix packed in the column which has no specific, or weak, interactions with eluted polymers.

Unpublished data (R. Urbani) of exopolysaccharides obtained from mucilage marine samples are presented in Figure 3. The purified polysaccharides were dissolved in 0.05M NaCl and solutions were filtered on 0.45  $\mu\text{m}$  filters and injected in a conditioned GPC

column (1.5 m length, 310 mL internal volume), packed with Sepharose CL6B ( $10^4$ - $10^6$  Da). The chromatographic apparatus was pre-equilibrated using 0.05M NaCl at 25°C at a flow rate of 10 mL·h<sup>-1</sup> and the column was calibrated with standard dextran solutions ( $M_w = 2 \cdot 10^4$  Da and  $M_w = 5 \cdot 10^5$  Da). A differential refractive index instrument was used as detector.



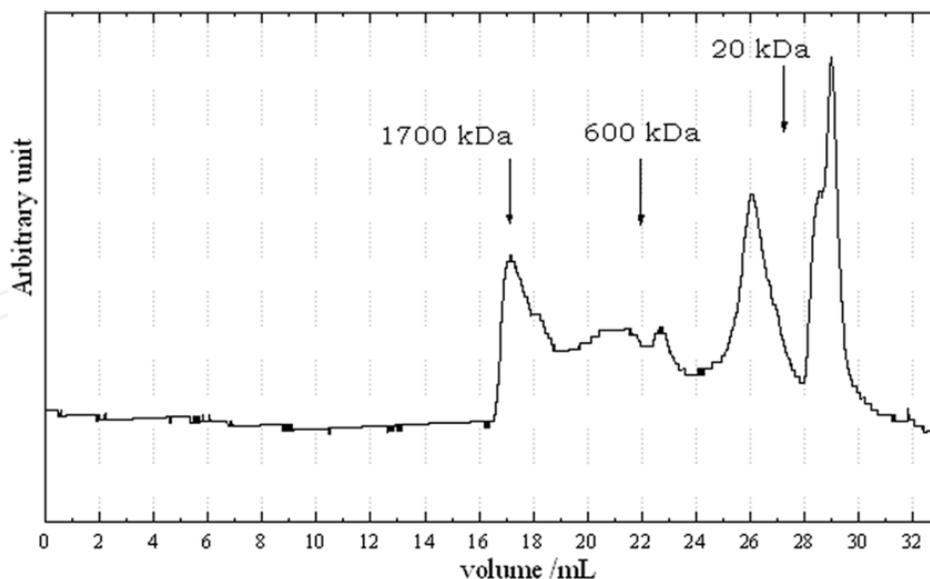
**Figure 3.** GPC of purified exopolysaccharides from Adriatic mucilage.

The small difference in the retention time (volumes) between peak 1 of M1 and M2 samples ( $t_R$  about 15 hours) is related to different stage (or age) of the mucilage aggregates with different degraded state [20]. The other peaks ( $t_R$  about 23 hours) very close to the (total) permeation volume,  $V_p$ , correspond to the low molecular weight fractions, very likely derived from hydrolytic activities of the bacterial pool on the native aggregates. High molecular weight fractions (peak 1 of M1 and M2) were collected, purified and derivatized for gas-chromatographic analysis. Their monosaccharidic patterns shown in Table 3 are quite similar suggesting the same origin of the high molecular weight polysaccharides even for aggregates of different degraded state [20].

Sample	Fraction	Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc
M1	Peak 1	11.8	20.1	--	1.9	8.3	12.2	31.1	14.6
M2	Peak 1	9.3	15.8	--	2.7	13.7	13.3	29.7	15.5

**Table 3.** Relative monosaccharide composition (%) of unfractionated exopolysaccharide and corresponding fraction at high molecular weight.

A rapid and powerful non-preparative technique for the characterization of molecular dimensions of marine dissolved organic matter and exopolymeric material from diatoms cultures is the high performance size-exclusion chromatography (HPSEC) [57]. By using a hydrogel column system (cut-off 50.000-1.000.000 Da) HPSEC experiments were performed on purified exopolysaccharides from *Chaetoceros decipiens* cultures [44] and results are shown in Figure 4.



**Figure 4.** HPSEC chromatogram of purified exopolysaccharide from cultured *Chaetoceros decipiens*.

Two distinct peaks were observed: at lower ( $V=16-20$  mL) and at higher retention volume ( $V=25-30$  mL) which corresponded to about 1700 kDa and 20 kDa molecular weight substances, respectively. The low molecular size peaks were near the permeation volume ( $V_p$ ) very likely due to oligosaccharides with an average number of monosaccharidic residues of about 110 units. The authors (R. Urbani, P. Sist) hypothesized that *Chaetoceros decipiens* produces a high molecular weight exopolysaccharide and some oligomers at low molecular weight probably derived from the release of storage carbohydrates by cultured diatom in given culture condition [44].

### 3. Solution and aggregation properties

In the macromolecular science it is well documented that going from flexible polysaccharides toward more rigid and extended chains, as well as with the presence of an ordered sequence in the primary structure, there is a general propensity of polymer chains to form chain associations/aggregations or multiple helical structures. Even if that sequence specificity were present, the absence of pronounced amphiphilic character in the sugar building blocks is not favorable for the formation of some kind of globular folding that occurs in many proteins and some nucleic acids [58]. In other words such globular structures are unknown in polysaccharides. Nonetheless, ordered conformations were proposed to represent the structure of many polysaccharides, both in solid state and in solution. In some cases the functionality is most closely associated with the occurrence of a randomly coiling polymeric character, that is, a propensity for the chain to move continuously through a vast range of nearly equally energetic conformations.

The presence of charged groups on the polysaccharidic chain confers peculiar properties to the macromolecules favoring, for example, the solubility or the association/dissociation processes in solution. On the polymer side the presence of the charged groups influences

strongly all the conformational properties by enhancing the chain dimensions and increasing the hydrodynamic volume. Knowledge of polysaccharidic chain structures, from single chain up to the three-dimensional molecular shape and chain association, is essential to understand their capability to form supra-molecular structures including physical gels [54].

### 3.1. Viscometry and light scattering characterization

Physico-chemical techniques are widely used in order to estimate the biopolymer features such as chain stiffness and chain dimensions which are strictly related to the polymer propensity to give aggregation and highly structured systems like gels. The Smidsrød-Haug parameter B [59] measured by using capillary viscosity techniques as a function of ionic strength is related to chain stiffness: the more flexible the chain, the higher the response of the intrinsic viscosity on the ionic strength variation and the higher the B value.

Intrinsic viscosities,  $[\eta]$ , of exopolysaccharides from Adriatic mucilage (M1 and M2) were measured in Cannon-Ubbelohde suspended-level capillary viscometers at different ionic strength and obtained by linear regression of reduced specific viscosities,  $\eta_{sp}/C$  ( $dL \cdot g^{-1}$ ), as a function of polymer concentrations. By plotting the intrinsic viscosity (the polymer hydrodynamic volume) as a function of the inverse of the root of ionic strength (Figure 5), both exopolysaccharides show a linear reduction of  $[\eta]$  at increasing of ionic strength due to screening effect of the salt on polymer charges. From the Smidsrød-Haug equations [59]:

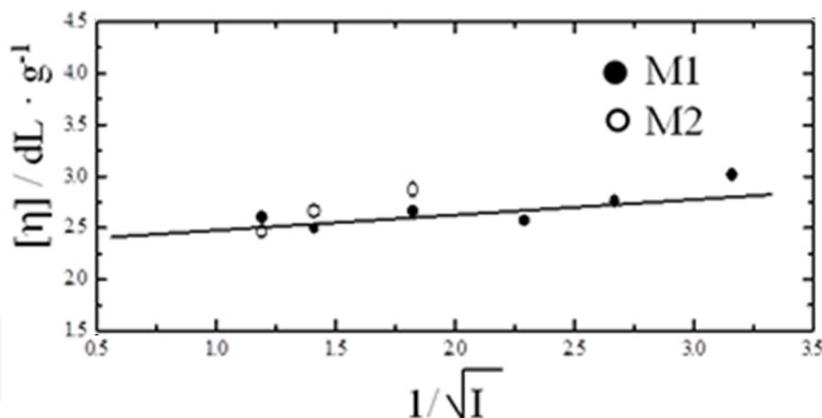
$$[\eta] = A + S' k^{-1} \approx A + S(\sqrt{I})^{-1}$$

the stiffness parameter B is obtained where  $[\eta]_{0.1}$  is the intrinsic viscosity at 0.1 M ionic strength:

$$B = \frac{S}{([\eta]_{0.1})^{1.3}}$$

It is interesting to compare the B stiffness parameter of exopolysaccharides with those reported in the literature for other polysaccharides of similar molecular weight. This is shown in Table 4 where dextran sulphate and polyphosphate represent rather flexible polymers with a high B value and, on the other hand, DNA and xanthan represent typical stiff polymers exhibiting a low B parameter. The M1/M2 exopolysaccharides exhibited a value similar to that of alginate considered as a semiflexible polymer.

Static and dynamic laser light scattering (SLLS and DLLS, respectively) are techniques widely used for polymer characterization, measuring average chain properties and thermodynamic quantities in solution. These properties are related to the propensity of the polymer system to give elongated and stiff chain: the radius of gyration ( $R_G$ ), the weight-average molecular weight ( $M_w$ ), the second virial coefficient ( $A_2$ ) and the hydrodynamic diameter ( $d_h$ ).



**Figure 5.** Plot of intrinsic viscosity *vs.* inverse of the square root of ionic strength for M1 and M2 polysaccharides.

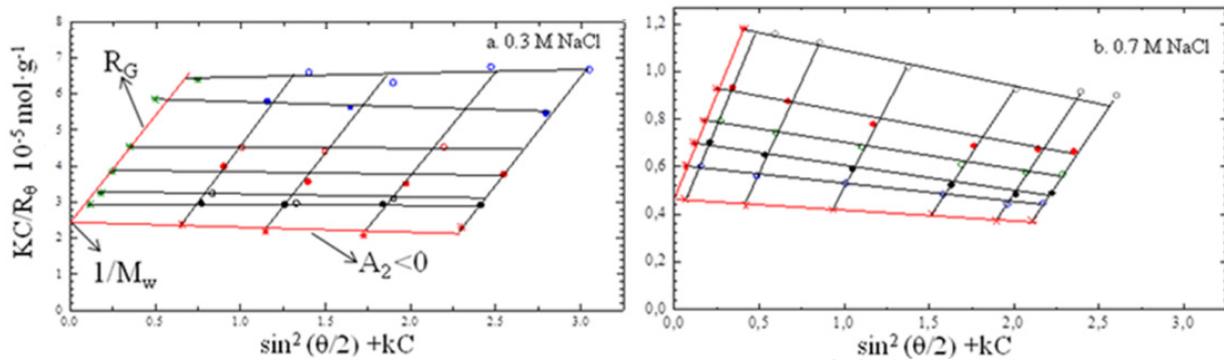
Polymer	B
Polyphosphate	0.44
Dextran sulphate	0.23
Hyaluronic acid	0.065
Alginate (mannuronic rich)	0.040
M1, M2	0.036
Alginate (guluronic rich)	0.031
DNA	0.0055
Xanthan	0.0053

**Table 4.** Stiffness parameters (B) of different model polymers.

Measurement of the scattering intensity at many angles allows the evaluation of the radius of gyration  $R_G$ , while by measuring the scattering intensity for many samples of various concentrations, the coefficient  $A_2$  is obtained. Simultaneous linear least squares fits to both the angular and concentration dependence of scattering intensities are employed for the properties determination.

In Figures 6a and 6b the two Zimm plots of the M2 exopolysaccharide in 0.3M and 0.7M NaCl solutions, respectively, are presented. The results show a constancy of  $R_G$  (and  $M_w$ ) at different salt concentrations, while  $A_2$  becomes more negative for higher salt concentration as a consequence of the screening effect on polymer charges which may lead to an extensive degree of aggregation. In general, with respect to the thermodynamic stability of the polymer solution, negative  $A_2$  values are a clear indication of the tendency of polysaccharide solution to undergo a phase separation to form an amorphous carbohydrate solid phase or a gel-like structure.

With comparison to model polysaccharides of different stiffness having the same  $M_w$  (Table 5), M2 polysaccharide possess higher  $R_G$  value than the flexible and coiled pullulan but also than the semi-rigid chain of the wellan and alginate biopolymers.



**Figure 6.** Zimm plot of M2 at two ionic strength (a) 0.3M and (b) 0.7M NaCl.

Sample	$R_G$ /nm
Pullulan	24
Wellan	68
Alginate	73
M2	155 <sup>a</sup>

<sup>a</sup> in 0.3M NaCl

**Table 5.** Radius of gyration of purified polysaccharides M1 and M2 compared to model polysaccharides with  $M_w=220,000$  dalton in 0.1M NaCl.

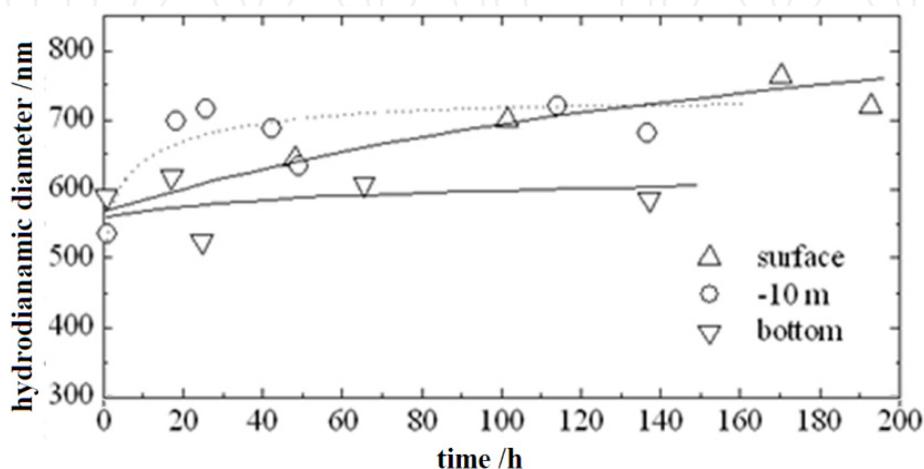
Finally, taking into account all these results one may say that the M2 polysaccharide shows a marked polyelectrolytic behavior and an intrinsic chain stiffness, favoring at higher salt concentrations chain-to-chain association and/or gel formation. Thus, coupled to other favorable environmental conditions, the salinity gradient of halocline in the seawater column could play an important role in the first stage of aggregation of dissolved biopolymers.

Dynamic laser light scattering (DLS), also called photon correlation spectroscopy, is a technique for the determination of hydrodynamic diameter of macromolecules and particulate matter in solution. In DLS the fluctuation of the scattered light due to the Brownian motion of the molecules is detected by a photon counting detector. This technique is used nowadays to measure particle sizes at the nanometer scale and to follow the kinetics of particle formation from dissolved EPS material. As reported by Verdugo [60,61] marine aggregates may self-assemble from free DOM biopolymers and this process was easily followed by DLS.

Following the same procedure the authors (R. Urbani, P. Sist) measured hydrodynamic diameters using Adriatic seawater samples collected during a three-years monitoring activity (1999-2001). Polymer assembly was monitored for 8 days by analyzing the scattering fluctuations detected at a 45° scattering angle. The autocorrelation function of the scattering intensity fluctuations was averaged over a 1-min sampling time and the particle size distribution calculated by the CONTIN method. Figure 7 shows an example of time

dependence of hydrodynamic diameters in three seawater samples taken at different depths.

Although the aggregation kinetics was the same (asymptotic shaped curve in Figure 7) the highest value (1  $\mu\text{m}$ ) of hydrodynamic diameter was significantly lower than values reported by Verdugo group (several micrometers) for oceanic samples [60,61] and cultured diatoms, as well [62].



**Figure 7.** Hydrodynamic diameter in dependence on time for Adriatic seawater samples.

#### 4. The supramolecular organization of polysaccharide fibrils: from cell release to giant gel networks

Diatoms excrete large quantities of extracellular polymeric substances, both as a function of their motility system and as a response to environmental conditions [63,64]. The natural occurrence of EPS is closely linked to diatom biomass, a pattern consistent over both macro (km) and micro ( $\mu\text{m}$ ) scales. We will focus on diatom exopolysaccharides produced in cell culture and from large aggregates they form in marine environment [3]. Those polysaccharides stand out by their supramolecular organization and gelling capacity due to hydrogen bonding and electrostatic interaction resulting in formation of reversible physical gel. Unlike chemical gels that are formed by chemical reaction using a crosslinking agent, the characteristics of polysaccharide electrolytes is to form gels by physical bonds through intermolecular forces among polymer chains [65,66].

Intermolecular interactions are the basis of life, and an extremely important part of biological research, so an enormous range of techniques have been applied to their study. Among the new methodologies (experimental and theoretical) developed and applied to polysaccharide conformation and dynamics, solution properties, chain aggregation and gelation, the results obtained using atomic force microscopy (AFM) have been pointed out among those giving the most striking results [67]. AFM connects the nanometer and micrometer length scales utilizing a sharp probe tip that senses interatomic forces acting

between the surface of a sample and the atoms at the apex of the tip. The physical basis behind AFM and its ability to „feel“ the surface, make AFM a versatile tool in biophysics allowing high resolution imaging, nanomechanical characterization and measurements of inter and intramolecular forces in living and non-living structures [68]. Thanks to the simple principle on which it is based, the AFM is a surprisingly small and compact instrument. Its use includes electronic control unit, computer and usually two monitors for simultaneous checking of image and imaging parameters. The probe which scans the sample surface consists of a cantilever and the tip located at the free end of a cantilever. The deflection of the cantilever is measured by an optical detection system. Registered values of cantilever deflection are electronically converted into pseudo 3D image of a sample. AFM is a non-destructive method which gives real 3D images of the sample with a vertical resolution of 0.1 nm and lateral resolution of 1 nm. Measured forces range from  $10^{-6}$  N to  $10^{-11}$  N.

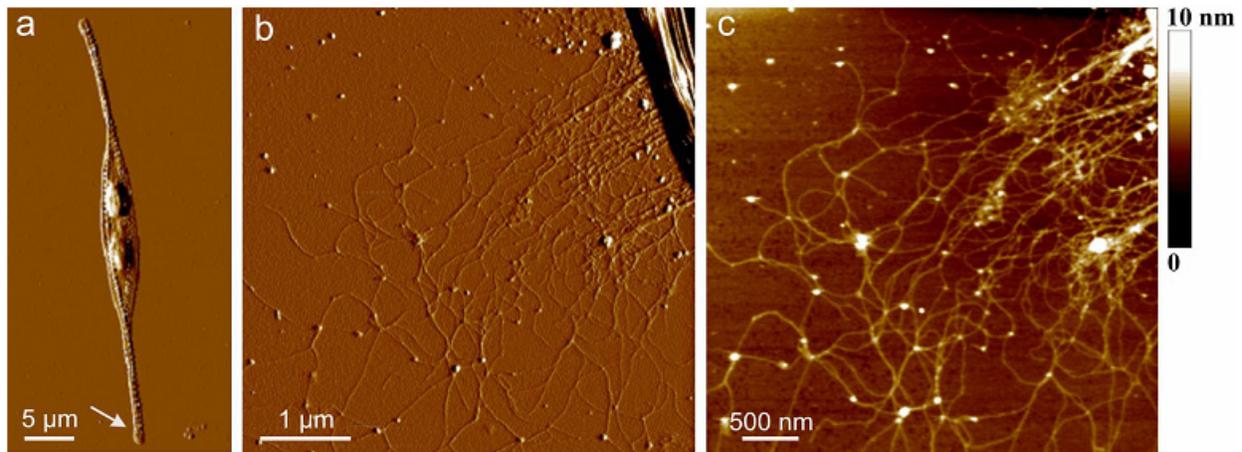
Here we will cover the recent achievements using AFM as the principal method in revealing the supramolecular organization of diatom exopolysaccharide fibrils beyond the chemical composition.

Polysaccharide samples for AFM imaging [69-72] and polysaccharide gels [70,73-77] are usually spread on freshly cleaved mica surface. The imaging of hydrated samples is preferably conducted in air to inhibit the unfavourable motion of polysaccharides in liquid medium. Such AFM studies have been validated against data obtained directly under buffers, transmission electron microscopy (TEM) studies and cryo-AFM. Balnois and Wilkinson [78] showed that when AFM is operated under ambient conditions, the thin water layer both sorbed to the biopolymers and present on the mica surface maintains molecular structure during AFM imaging.

Protocols for marine sample AFM imaging have been developed only recently: single diatom cells and released polymers [79]; isolated polysaccharides from diatom cultures [3,79]; marine gel polymers and networks [80]. The samples were prepared using the drop deposition method (5-10  $\mu$ L aliquots) and mica as a substrate. Mica sheets were placed in enclosed Petri dishes for approximately 30–45 min to allow biopolymers and cells to settle and attach to the surface. Samples were then rinsed in ultrapure water (three times for 30 s) and placed in enclosed Petri dish to evaporate the excess of water on the mica. The rinsing step was necessary to remove the salt crystals that would hamper imaging. AFM imaging was performed in air at room temperature and 50–60% relative humidity. In contact mode the force was kept at the lowest possible value in order to minimize the forces of interaction between the tip and the surface. For imaging in tapping mode the ratio of the set point amplitude was maintained to the free amplitude ( $A/A_0$ ) at 0.9 (light tapping).

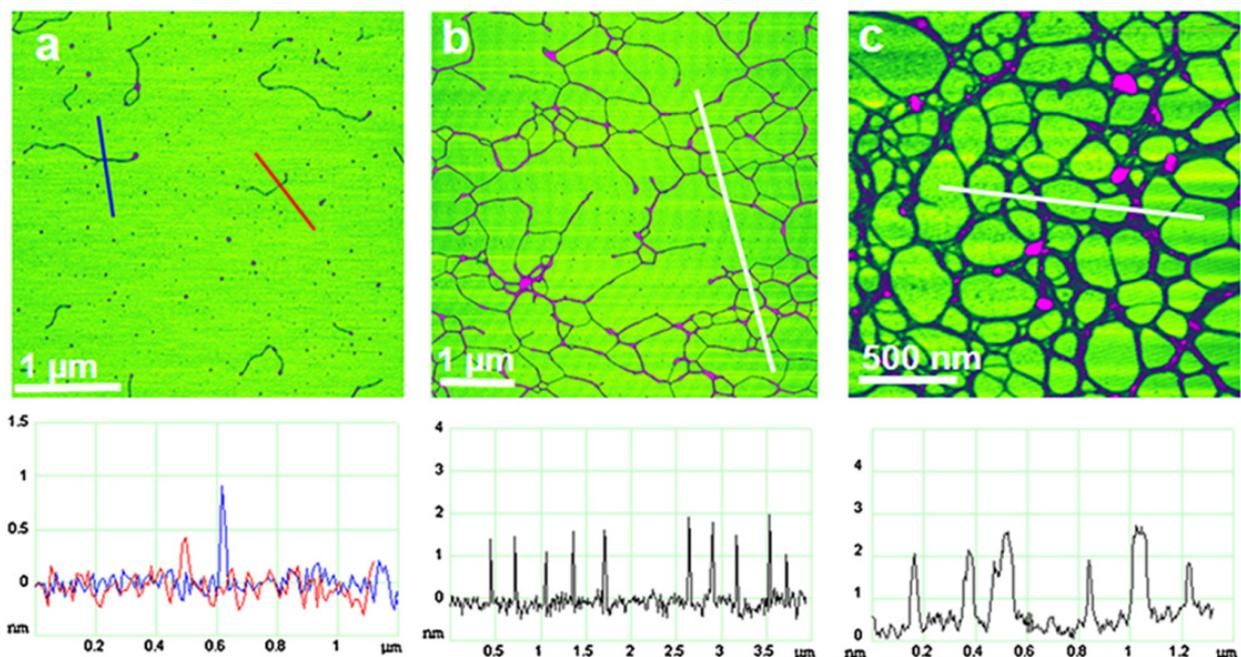
#### **4.1. Exopolysaccharide production at a single cell level**

The ubiquitous marine diatom *Cylindrotheca closterium*, isolated from the northern Adriatic Sea, was used in AFM studies of exopolysaccharide production at a single cell level.



**Figure 8.** (a) AFM image of the whole *C. closterium* cell presented as deflection data. Arrow indicates the position of the polymer excretion site; (b) The released polymers still attached to the apex of the cell rostrum, deflection data, scan size  $5\ \mu\text{m} \times 5\ \mu\text{m}$ ; (c) Released polymers presented as height data, scan size  $4\ \mu\text{m} \times 4\ \mu\text{m}$  and vertical scale shown as the color bar [3].

Figure 8a revealed the general features of a live *C. closterium* cell with the two chloroplasts and its drawn-out flexible rostra. Arrow indicates the position of polymer release shown in Figures 8b and 8c. The bundles of polymer fibrils extended up to  $10\ \mu\text{m}$  from cell surface. Their heights are  $5\text{--}7\ \text{nm}$  at the position close to the site of excretion. At a distance of  $1\ \mu\text{m}$  the dense network is observed with fibril heights of  $2\text{--}3\ \text{nm}$ . At even larger distances the network is less dense with the fibril heights in the range of  $0.4$  to  $1.2\ \text{nm}$ . The lower value of fibril height corresponds to the single monomolecular polysaccharide chains [71]. At this



**Figure 9.** AFM images (tapping mode) of polysaccharides isolated from the *C. closterium* culture medium and dissolved in ultrapure water: (a) single fibrils (concentration  $5\ \text{mg/L}$ ) vertical scale  $2.5\ \text{nm}$ ; (b,c) fibril networks (concentration  $10\ \text{mg/L}$ ). Vertical scales:  $5\ \text{nm}$  (b) and  $10\ \text{nm}$  (c) [79].

larger distance, the network appeared with incorporated spherical nanoparticles–globules. The globules are found to interconnect two or more fibrils. The globules may represent positively charged proteins whose function before the release is efficient intracellular packing of negatively charged polysaccharide fibrils, in line with molecular crowding in living cells [81].

#### 4.2. Extracted and purified polysaccharides of *C. closterium*

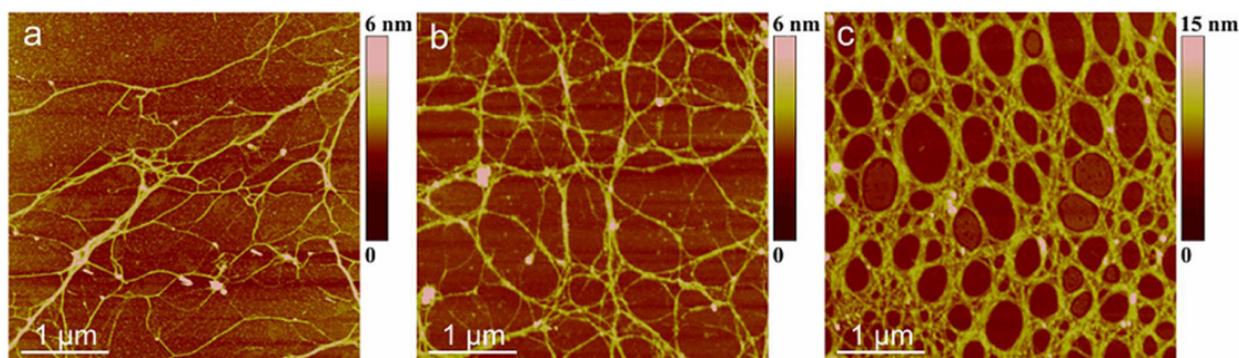
The polysaccharide fraction isolated from the axenic *C. closterium* culture medium [7] was used to test the capacity of photosynthetically produced polymers to self-assemble into gel phase.

The isolated polysaccharides for AFM imaging were prepared in two concentrations (5 and 10 mg/L). Single fibrils prevail in samples prepared with polysaccharide concentration of 5 mg/L. Dissolved polysaccharides resumed a flexible fibrillar structure (Figure 9a) with fibril heights of 0.4 and 0.9 nm. The value of 0.4 nm corresponds to polysaccharide single molecular chain. AFM imaging of samples prepared from the solution containing 10 mg/L revealed fibrillar networks varying in the degree of fibril associations (Figures 9b,c). The fibril heights in the networks span over the same range (0.9–2.6 nm) and the mode of fibril association follows the same pattern. However, the segments forming the network shown in Figure 9c are significantly wider (50 vs. 140 nm), suggesting side-by-side associations once the maximum height of individual fibrils is reached. If we take the value of 2.6 nm observed using AFM analysis as the maximum fibril height, then up to six monomolecular fibrils can constitute a single polysaccharide fibril [79]. The absence of globules in the AFM images of isolated polysaccharides also indicates that the globules, which appeared as a constitutive component in the EPS of single cells (Figures 9b,c), are not polysaccharides.

#### 4.3. Evolution of marine gel polymer networks

The fact that the isolated polysaccharide fraction has the capacity to self-assemble into a gel network in pure water is an important finding with implications on the mechanism of the macroscopic gel phase formation in marine systems. The marine gel is characterized as a thermoreversible physical gel and the dominant mode of gelation as crosslinking of polysaccharide fibrils by hydrogen bonding which results in helical structures and their associations [80]. This mechanism contrasts a more generally established view [82,83] that marine gel phase formation proceeds via cross-linking of negatively charged biopolymers (namely polysaccharides) by  $\text{Ca}^{2+}$  ions. Only recently, Ding *et al.* [62] reported that diatom EPS can spontaneously self-assemble in calcium-free artificial sea water, forming microscopic gels of 3–4  $\mu\text{m}$ . They pointed out an overlooked issue of crosslinkers other than calcium ions in the formation of marine polymer networks.

Figure 10 represents the evolution of polymer networks of the macroscopic gel phase in the northern Adriatic Sea [84,85]. Samples were prepared from the macroaggregates with different residence time in the water column, from early stage of gel phase formation to the



**Figure 10.** Evolution of polymer networks in the macroscopic gel phase from (a) to (c): early stage of gel phase formation to condensed gel network of older macroaggregate. AFM images are acquired in contact mode and presented as height data, scan size  $4\ \mu\text{m}\times 4\ \mu\text{m}$  [3].

condensed (mature) gel network of an older macroaggregate. The long polymer strands with small patches of initial fibril associations (Figure 10a) coexisted with the continuous gel network shown in Figure 10b. With the prolonged residence time (one month) the more condensed network is formed as presented in Figure 10c. The analysis of fibril heights for early and mature gel state is given in Table 6. The fibril heights for the early stage of gel network correspond to the fibril heights produced by Adriatic *C. closterium*.

Polysaccharide fibrils	Number of fibrils analyzed	Fibril height /nm	
		Mean value	Range
Attached to the diatom cell	120	$0.85 \pm 0.32$	0.4 - 1.8
Marine gel network: early stage	189	$0.92 \pm 0.40$	0.4 - 2.0
Marine gel network: mature gel	178	$3.58 \pm 0.76$	1.6 - 5.0

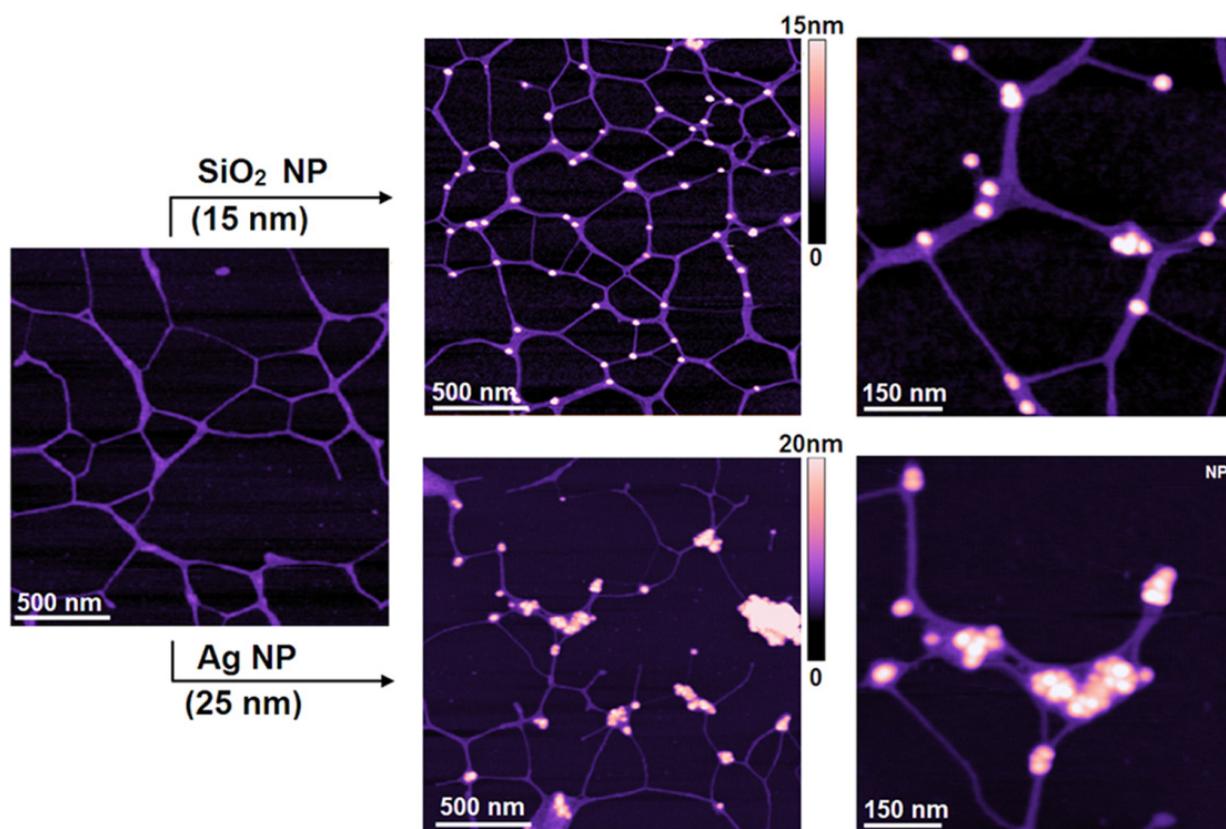
**Table 6.** Comparison of polysaccharide fibril heights [3].

#### 4.4. Exopolysaccharides interactions with nanoparticles

Diatom EPS production increases as a feedback response to the presence of NPs and may thus contribute to detoxification mechanisms [86,87]. Specifically, in the study of Ag NPs toxicity to the marine diatoms *C. closterium* and *C. fusiformis* [88] an increase of EPS production was documented and the incorporation of Ag NPs was clearly demonstrated. Although Ag NPs integrated in EPS–gel network are beneficial to the diatom cell (detoxification), their accumulation and persistence in microenvironments prolong their presence in the water column and make NPs available to higher organisms. Residing in a gel environment, particles are prevented from aggregation and export from the water column.

The diatom EPS component responsible for the NPs interaction was identified by bringing in direct contact polysaccharide fraction isolated from *C. closterium* EPS with Ag and SiO<sub>2</sub> NPs.

The polysaccharide fibril network was prepared by dissolving the polysaccharide fraction isolated from the *C. closterium* culture medium in ultrapure water at concentration of 20  $\mu\text{g}/\text{mL}$  and stirred for 45 minutes before adding NPs, 10  $\mu\text{g}/\text{mL}$  Ag-citrate coated NP, nominal size  $\sim 25$  and SiO<sub>2</sub> NP (LUDOX® HS-40, Sigma), nominal size 15nm were used.



**Figure 11.** Interaction of polysaccharide network and nanoparticles visualized by AFM. The polysaccharide network was preformed in 20 mg/L of isolated polysaccharides in ultrapure water before addition of 10 mg/L NPs.

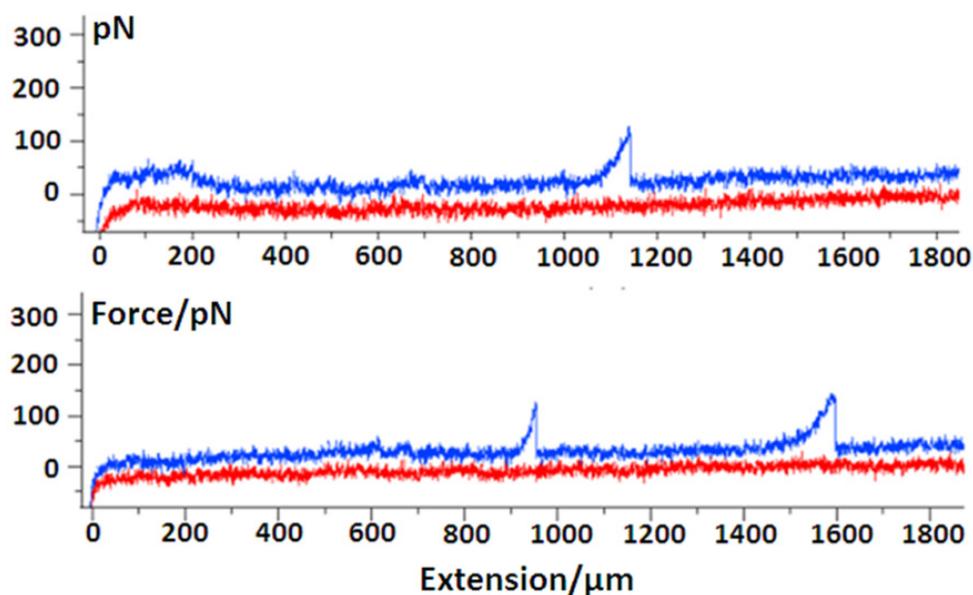
The Ag and SiO<sub>2</sub> NPs were detected exclusively on polysaccharide fibrils as single spherical particles or their agglomerates as shown by AFM images (Figure 11). The NPs did not induce cross-linking of fibrils nor change the fibril heights. Rather, particles are imbedded into the preexisting polysaccharide network. Besides the significance for the environment, such interaction of NPs and diatom polysaccharides can be applied for the design of composite materials, such as biocompatible gels with new properties [89]. Diatom extracellular polysaccharides could be also used as a capping agent giving rise to the stability of NPs in liquid environments over a broad range of ionic strength and pH.

#### 4.5. Force spectroscopy as future prospective

In AFM force spectroscopy mode a single molecule or fiber is stretched between the AFM flexible cantilever tip and a flat substrate mounted on a highly accurate piezoelectric positioner. Polysaccharide molecule, protein or other biopolymer, is either adsorbed to the substrate or linked to it through the formation of covalent bonds. When the tip and substrate are brought together and then withdrawn, one or more molecules can attach to the tip by adsorption. As the distance between the tip and substrate increases, extension of the molecule generates a restoring force that causes the cantilever to bend. The deflection of the

cantilever measures the force on the polymer with an accuracy of  $\sim 5$  pN, while the piezoelectric positioner records the changes in the molecule's end-to-end length with an accuracy of 0.1nm.

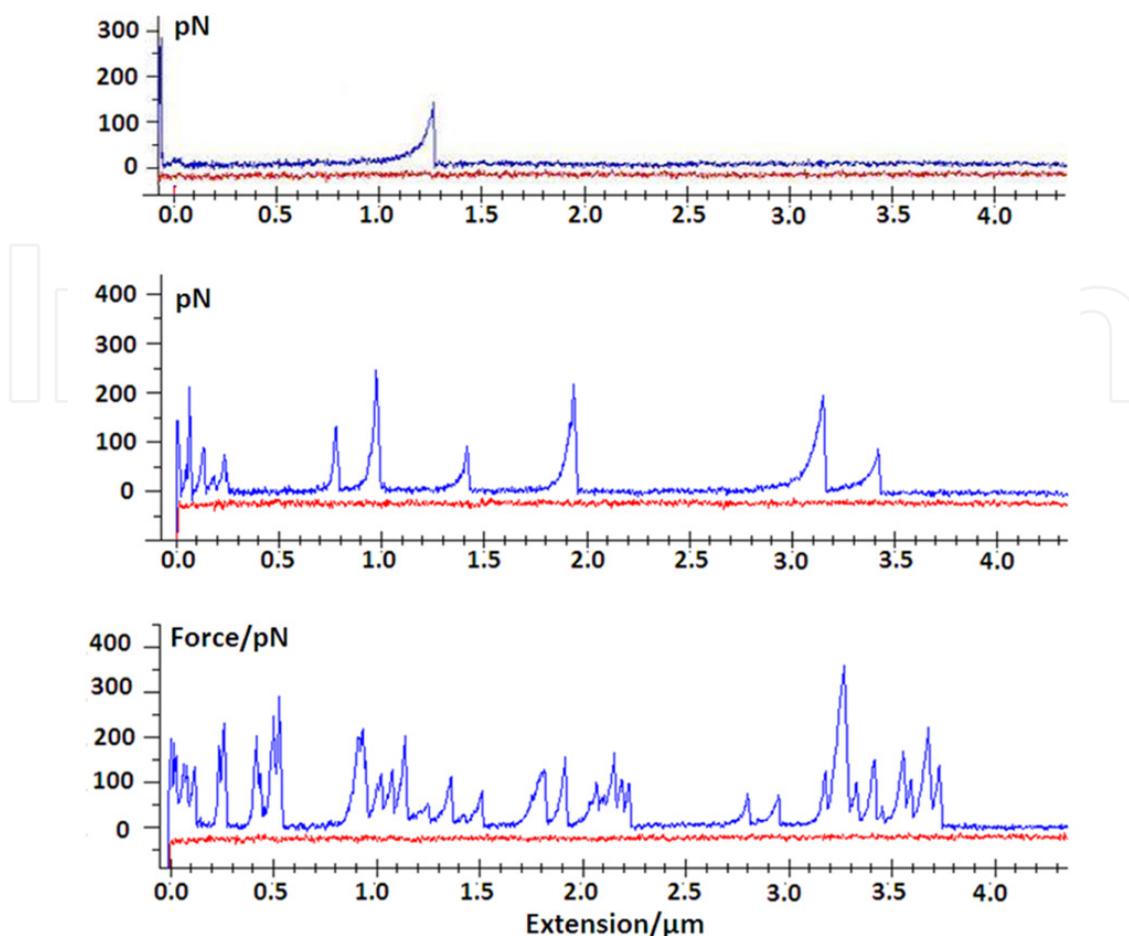
AFM force spectroscopy is widely used method in polymer biophysics allowing measuring mechanical properties of single molecules, and with a possibility to directly quantify the forces involved in both intra- and inter-molecular polymer interactions [90-93]. It is also adopted in advancing diatom research into the nanotechnology era [94]. Most of the work done so far on measuring forces with AFM has distinguished non adhesive and adhesive EPS components and discovered the adhesive properties and designs that give explanation as to why diatoms have the great tendency to attach to surfaces. However, force spectroscopy has not yet been performed on single diatom polysaccharide fibrils or their networks. The data that follows are the results of exploratory experiments conducted on diatom polysaccharide molecules (single fibrils, Figure 12) and on marine gel polysaccharide network (Figure 13) in filtered seawater.



**Figure 12.** Force approach (in red) and extension (in blue) curves acquired for polysaccharide single fibrils in filtered seawater. Two typical curves are shown.

The extension curve with the two rupture events in Figure 12 could result from the two individual fibrils of different length (0.9 and 1.5  $\mu\text{m}$ ) simultaneously attached to the tip. The force spectrum signature for polysaccharide fibrils assembled in marine gel network is by far more complex (Figure 13) [95]. Assigning the underlying disentanglement events is in progress.

The force spectra can provide the critical piece of information that will allow us to characterize and quantify physical forces in polysaccharide network assemblies. Further developments will contribute to the new field of nanoecology and open the possibilities for rational design of polysaccharide gels with desired properties.



**Figure 13.** Force approach (in red) and extension (in blue) curves of polysaccharide fibrils assembled in marine gel network acquired in filtered seawater.

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## 5. References

- [1] David L Kirchman (2008) *Microbial Ecology of the Oceans*. New York: John Wiley & Sons. 2<sup>nd</sup> edition. 512 p.
- [2] Zambianchi E, Calvitti C, Cecamore P, D’Amico F, Ferulano E, Lanciano P (1992) The mucilage phenomenon in the Northern Adriatic sea summer 1989: a study carried out with remote sensing techniques. *Sci. Total Environ. (Suppl.)* 581–598.
- [3] Svetličić V, Žutić V, Mišić Radić T, Pletikapić G, Hozić Zimmerman A, Urbani R (2011) Polymer Networks Produced by Marine Diatoms in the Northern Adriatic Sea. *Mar. Drugs* 9: 666-679.
- [4] Myklestad SM, Haug A (1972) Production of carbohydrate by the marine diatom *Chaetoceros affinis*: I. Effect of the conc. of nutrients in the culture medium. *J. Exp. Mar. Biol. Ecol.* 9: 125-136.
- [5] Myklestad SM (1995) Release of extracellular products by phytoplankton with special emphasis on polysaccharides. *Sci. Tot. Environ.* 165: 155-164.
- [6] Magaletti E, Urbani R, Sist P, Ferrari CR, Cicero AM (2004) Abundance and Chemical Characterization of Extracellular Carbohydrates Produced by the Marine Diatom *Cylindrotheca fusiformis*. *Eur. J. Phycol.* 39: 133-142.
- [7] Urbani R, Magaletti E, Sist P, Cicero AM (2005) Extracellular Carbohydrates Released by the Marine Diatoms *Cylindrotheca closterium*, *Thalassiosira pseudonana* and *Skeletonema costatum* : effect of P-depletion and growth status. *Sci. Tot. Environ.* 353: 300-306.
- [8] Santschi PH, Balnois E, Wilkinson KJ, Zhang J, Buffle J (1998) Fibrillar polysaccharides in marine macromolecular organic matter as imaged by atomic force microscopy and transmission electron microscopy. *Limnol. Oceanogr.* 43: 896–908.
- [9] Biersmith A, Benner R (1998) Carbohydrates in phytoplankton and freshly produced dissolved organic matter. *Mar. Chem.* 63: 131-144.
- [10] Aluwihare L, Repeta DJ (1999) A comparison of the chemical characteristics of oceanic DOM and extracellular DOM produced by marine algae. *Mar. Ecol. Prog. Ser.* 186: 105-117.
- [11] Myklestad S (1974) Production of carbohydrates by marine planktonic diatoms I: Comparison of nine different species in culture. *J. Exp. Mar. Biol. Ecol.* 15: 261–74.
- [12] Myklestad S (1977) Production of carbohydrates by marine planktonic diatoms II: Influence of the N/P ratio in the growth medium on the assimilation ratio, growth rate, and production of cellular and extracellular carbohydrates by *Chaetoceros affinis* var *willei* (Gran) Hustedt and *Skeletonema costatum* (Grev) Cleve. *J. Exp. Mar. Biol. Ecol.* 29: 161–79.
- [13] Chen W, Wangersky PJ (1996) Rates of microbial degradation of dissolved organic carbon from phytoplankton cultures. *J Plankton Res.* 18: 1521–33.

- [14] Alldredge AL (1999) The potential role of particulate diatom exudates in forming nuisance mucilaginous scums. *Ann. Ist. Super Sanita`* 35: 397– 400.
- [15] Alcoverro T, Conte E, Mazzella L (2000) Production of mucilage by the Adriatic epipellic diatom *Cylindrotheca closterium* (Bacillariophyceae) under nutrient limitation. *J. Phycol.* 36: 1087– 1095.
- [16] Staats N, Stal LJ, Mur LR (2000) Exopolysaccharide production by the epipellic diatom *Cylindrotheca closterium*: effects of nutrient conditions. *J. Exp. Mar. Biol. Ecol.* 249: 13– 27.
- [17] Granum E, Kirkvold S, Mykkestad SM (2002) Cellular and extracellular production of carbohydrates and aminoacids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion. *Mar. Ecol. Prog. Ser.* 242: 83– 94.
- [18] Guerrini F, Cangini M, Boni L, Trost P, Pistocchi R (2000) Metabolic responses of the diatom *Achnanthes brevipes* (Bacillariophyceae) to nutrient limitation. *J. Phycol.* 36: 882– 890.
- [19] Gerald O Aspinall (1982) *The Polysaccharides*. New York: Academic Press. 248 p.
- [20] Giani M, Berto D, Zangrando V, Castelli S, Sist P, Urbani R (2005) Chemical Characterisation of Different Typologies of Mucilaginous Aggregates in the Northern Adriatic Sea. *Sci. Tot. Environ.* 353: 232-246.
- [21] Urbani R, Brant DA (1992) Kelco microbial polysaccharides S-130 (Welan) and S-657 display similar dilute aqueous solution behaviour. *Carbohydr. Polym.* 11: 169–191.
- [22] Khodse VB, Fernandes L, Gopalkrishna VV, Bhosle NB, Fernandes V, Matondkar SGP, Bhushan R (2007) Distribution and seasonal variation of concentrations of particulate carbohydrates and uronic acids in the northern Indian Ocean. *Mar. Chem.* 103: 327–346.
- [23] Yang GP, Zhang YP, Lu XL, Ding HB (2010) Distributions and seasonal variations of dissolved carbohydrates in the Jiaozhou Bay, China. *Estuar. Coastal Shelf Sci.* 88: 12-20.
- [24] Khodse VB, Fernandes L, Bhosle NB, Sardesai S (2008) Carbohydrates, uronic acids and alkali extractable carbohydrates in contrasting marine and estuarine sediments: Distribution, size fractionation and partial chemical characterization. *Org. Geochem.* 39: 265-283.
- [25] Lai J, Yu Z, Song X, Cao X, Han X (2011) Responses of the growth and biochemical composition of *Prorocentrum donghaiense* to different nitrogen and phosphorus concentrations. *J. Exp. Mar. Biol. Ecol.* 405: 6-17.
- [26] Meon B, Kirchman DL (2001) Dynamics and molecular composition of dissolved organic material during experimental phytoplankton blooms. *Mar. Chem.* 75: 185-199.
- [27] Hama T, Yanagi K, Hama Y (2004) Decrease in molecular weight of photosynthetic products of marine phytoplankton during early diagenesis. *Limnol. Oceanogr.* 49: 471-481.
- [28] McCarthy M, Hedges J, Benner R (1996) Major biochemical composition of dissolved high molecular weight organic matter in seawater. *Mar. Chem.* 55: 281-297.
- [29] Hama T, Yanagi K (2001) Production and neutral aldose composition of dissolved carbohydrates excreted by natural marine phytoplankton populations. *Limnol. Oceanogr.* 46: 1945 – 1955.

- [30] Allan GG, Lewin J, Johnson PG (1972) Marine polymers. IV Diatoms polysaccharides. *Bot. Mar.* 15: 102-108.
- [31] Aluwihare LI, Repeta DJ, Chen RF (1997) A major biopolymeric component to dissolved organic carbon in surface seawater. *Nature* 387: 166-169.
- [32] Skoog A, Benner R (1997) Aldose in various size fractions of marine organic matter: Implications for carbon cycling. *Limn. Oceanogr.* 42: 1803-1813.
- [33] Biersmith A, Benner R (1998) Carbohydrates in phytoplankton and freshly produced dissolved organic matter. *Mar. Chem.* 63: 131-144.
- [34] Benner R, Opsahl S (2001) Molecular indicators of the sources and transformation of dissolved organic matter in the Mississippi river plume. *Org. Geochem.* 32: 597-611.
- [35] Cotrim da Cunha L, Serve L, Blazi JL (2002) Neutral sugar as biomarkers in the particulate organic matter of a French Mediterranean river. *Organic Geochem.* 33: 953-964.
- [36] Repeta D, Quan TM, Aluwihare L, Accardi AM (2002) Chemical characterization of high molecular weight dissolved organic matter in fresh and marine waters. *Geochim. Cosmochim. Acta* 66: 955-962.
- [37] Sartoni G, Urbani R, Sist P, Nuccio C, Berto D, Giani M (2008) Benthic mucilaginous aggregates in the Mediterranean Sea: Origin, chemical composition and polysaccharides characterization. *Mar. Chem.* 111: 184-198.
- [38] Kaiser K, Benner R (2009) Biochemical composition and size distribution of organic matter at the Pacific and Atlantic time-series stations. *Mar. Chem.* 113: 63-77.
- [39] Giani M, Sist P, Berto D, Serrazanetti G, Ventrella V, Urbani R (2012) The organic matrix of pelagic mucilaginous aggregates in the Tyrrhenian Sea (Mediterranean Sea). *Mar. Chem.* 132-133: 83-94.
- [40] Christophers J, Biermann, Gary D McGinnis (1988) *Analysis of Carbohydrates by GLC and MS*. Boca Raton: CRC Press. 304 p.
- [41] Neeser JR (1985) G.l.c. of O-methylxime and alditol acetate derivatives of neutral sugars, hexosamines, and sialic acids: "one-pot" quantitative determination of the carbohydrate constituents of glycoproteins and a study of the selectivity of alkaline borohydride reductions. *Carbohydr. Res.* 138: 189-198.
- [42] Taylor RL, Conrad HE (1972) Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl group. *Biochemistry* 11: 1383-1388.
- [43] Blakeney AB, Harris PJ, Henry RJ, Stone BA (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* 113: 291-299.
- [44] Sist P (2005) *Ciclo biogeochimico della sostanza organica in ambiente marino costiero: caratterizzazione e ruolo dei biopolimeri marini*. PhD Thesis, University of Trieste, Italy.
- [45] Mopper K (1977) Sugars and uronic acids in sediment and waters from the Black Sea and North Sea with emphasis on analytical techniques. *Mar. Chem.* 5: 585-603.

- [46] Kaiser K, Benner R (2000) Determination of amino sugars in environmental samples with high salt content by high-performance anion-exchange chromatography and pulsed amperometric detection. *Anal. Chem.* 72: 2566–2572.
- [47] Leppard GG (1995) The characterization of algal and microbial mucilage's and their aggregates in aquatic ecosystems. *Sci. Tot. Environ.* 165: 103–131.
- [48] Metaxatos A, Panagiotopoulos C, Ignatiades L (2003) Monosaccharide and aminoacid composition of mucilage material produced from a mixture of four phytoplanktonic taxa, *J. Exp. Mar. Biol. Ecol.* 294: 203–217.
- [49] Goldberg SJ, Carlson CA, Hansell DA, Nelson NB, Siegel DA (2009) Temporal dynamics of dissolved combined neutral sugars and the quality of dissolved organic matter in the northwestern Sargasso Sea. *Deep-Sea Res.* 56: 672–685.
- [50] Goldberg SJ, Carlson CA, Brock B, Nelson NB, Siegel DA (2010) Meridional Variability in Dissolved Organic Matter Stocks and Diagenetic State Within the Euphotic and Mesopelagic Zone of the North Atlantic Subtropical Gyre. *Mar. Chem.* 119: 9-21.
- [51] Engel A, Händel N (2011) A novel protocol for determining the concentration and composition of sugars in particulate and in high molecular weight dissolved organic matter (HMW-DOM) in seawater. *Mar. Chem.* 127: 180-191.
- [52] Girollo D, Ortolano PIC, Vieira AAH (2007) Bacteria–algae association in batch cultures of phytoplankton from a tropical reservoir: the significance of algal carbohydrates. *Freshwater Biol.* 52: 1281–1289.
- [53] Harris PJ, Henry RJ, Blakeney AB, Stone BA (1984) An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.* 127: 59-73.
- [54] Urbani R, Cesàro A (2001) Chain Conformation of Polysaccharides in Different Solvents. In: Wypych G, editor. *Handbook of Solvents*. Toronto: Chem Tech Publishing. pp. 706-735.
- [55] Ruggiero JR, Urbani R, Cesàro A (1995) Conformational Features of Galacturonans: II. Configurational Statistics of Pectic Polymers. *Int. J. Biol. Macromol.* 17: 213-218.
- [56] Mandal SK, Singh RP, Patel V (2011) Isolation and Characterization of Exopolysaccharide Secreted by a Toxic Dinoflagellate, *Amphidinium carterae* Hulburt 1957 and Its Probable Role in Harmful Algal Blooms (HABs). *Microb. Ecol.* 62: 518–527.
- [57] Minor EC, Simjouw JP, Boon JJ, Kerkhoff AE, Van der Horst J (2002) Estuarine/marine UDOM as characterized by size-exclusion chromatography and organic mass spectrometry, *Mar. Chem.* 78: 75–102.
- [58] Gerald O Aspinall (1982) *The Polysaccharides*. New York: Academic Press. 248 p.
- [59] Smidsrød O, Haug A (1971) Estimation of the relative stiffness of the molecular chain in polyelectrolytes from measurements of viscosity of different ionic strengths. *Biopolymers* 1: 1213-1227.
- [60] Chin WC, Orellana MV, Verdugo P (1998) Spontaneous assembly of marine dissolved organic matter into polymer gels. *Nature* 391: 568–72.

- [61] Verdugo P, Santschi PH (2010) Polymer dynamics of DOC networks and gel formation in seawater. *Deep-Sea Res. II* 57: 1486-1493.
- [62] Ding YX, Hung CC, Santschi PH, Verdugo P, Chin WC (2009) Spontaneous Assembly of Exopolymers from Phytoplankton. *Terr. Atmos. Ocean. Sci.* 20: 741-747.
- [63] Underwood GJC, Paterson DM (2003) The importance of extracellular carbohydrate production by marine epipelagic diatoms. *Adv. Bot. Res.* 40: 184-240.
- [64] Underwood GJC, Boulcott M, Raines CA, Waldron K (2004) Environmental effects on exopolymer production by marine benthic diatoms: dynamics, changes in composition, and pathways of production. *J. Phycol.* 40: 293-304.
- [65] Rinaudo M (2005) Advances in characterization of polysaccharides in aqueous solution and gel state. In: Dumitriu S, editor. *Polysaccharides-Structural Diversity and Functional Versatility*. New York: Marcel Dekker. pp. 237-252.
- [66] Jacob N Israelachvili (2010) *Intermolecular and surface forces*. London: Academic Press. 3<sup>rd</sup> edition. 700 p.
- [67] Cesàro A, Bellich B, Borgogna M (2012) Biophysical functionality in polysaccharides: from Lego-blocks to nano-particles. *Eur. Biophys. J.* 41(4): 379-95.
- [68] Peter Eaton, Paul West (2010) *Atomic Force Microscopy*. New York: Oxford University Press. 248 p.
- [69] Kirby AR, Gunning AP, Morris VJ (1996) Imaging polysaccharides by atomic force microscopy. *Biopolymers* 38: 355-366.
- [70] Victor J Morris, Andrew R Kirby, A Patrick Gunning (1999) *Atomic Force Microscopy for Biologists*. London: Imperial College Press. 332 p.
- [71] Abu-Lail NI, Camesano TA (2003) Polysaccharide properties probed with atomic force microscopy. *J. Microsc.* 212: 217-238.
- [72] Abu-Lail N.I, Camesano T.A (2004) Atomic force microscope and single-molecule force microscopy studies of biopolymers. In: Schwarz J, Contescu C, Putyera K, editors. *Dekker Encyclopedia of Nanoscience and Nanotechnology*. London: Taylor & Francis. pp. 119-131.
- [73] McIntire TM, Brant DA (1997) Imaging of individual biopolymers and supramolecular assemblies using noncontact atomic force microscopy. *Biopolymers* 42: 133-146.
- [74] Ikeda S, Morris VJ, Nishinari K (2001) Microstructure of aggregated and nonaggregated kappa-carrageenan helices visualized by atomic force microscopy. *Biomacromolecules* 2: 1331-1337.
- [75] Keresztes Z, Rigó T, Telegdi J, Kálmán E (2001) Investigation of biopolymer networks by means of AFM. *Appl. Phys. A.* 72: 113-116.
- [76] Funami T, Hiroe M, Noda S, Asai I, Ikeda S, Nishinari K (2007) Influence of molecular structure imaged with atomic force microscopy on the rheological behavior of carrageenan aqueous systems in the presence or absence of cations. *Food Hydrocolloid.* 21: 617-629.
- [77] Noda S, Funami T, Nakauma M, Asai I, Takahashi R, Al-Assaf, S, Ikeda S (2008) Molecular structures of gellan gum imaged with atomic force microscopy in relation to

- the rheological behavior in aqueous systems. 1. Gellan gum with various acyl contents in the presence and absence of potassium. *Food Hydrocolloid*. 22: 1148–1159.
- [78] Balnois E, Wilkinson KJ (2002) Sample preparation techniques for the observation of environmental biopolymers by atomic force microscopy. *Colloid. Surf. A* 207: 229–242.
- [79] Pletikapić G, Mišić Radić T, Hozić Zimmermann A, Svetličić V, Pfannkuchen M, Marić D, Godrijan J, Žutić V (2011) Extracellular polymer release AFM imaging of extracellular polymer release by marine diatom *Cylindrotheca closterium* (Ehrenberg) Reiman & J.C. Lewin. *J. Mol. Recognit.* 24: 436–445.
- [80] Mišić Radić T, Svetličić V, Žutić V, Boulgaropoulos B (2011) Seawater at the nanoscale: Marine gel imaged by atomic force microscopy. *J. Mol. Recognit.* 24: 397–405.
- [81] Falciatore A, Ribera d'Alcala M, Croot P, Bowler C (2000) Perception of environmental signals by a marine diatom. *Science* 288: 363–2365.
- [82] Verdugo P, Alldredge AL, Azam F, Kirchmand DL, Passow U, Santschi PH (2004) The oceanic gel phase: a bridge in the DOM–POM continuum. *Mar. Chem.* 92: 67–85.
- [83] Verdugo P, Orellana MV, Chin WC, Petersen TW, van den Eng G, Benner R, Hedges J (2008) Marine biopolymer self-assembly: implications for carbon cycling in the ocean. *Faraday Discuss.* 139: 393–398.
- [84] Stachowitsch M, Fanuko N, Richter M (1990) Mucous aggregates in the Adriatic Sea: an overview of stages and occurrence. *Mar. Ecol.* 11: 327–350.
- [85] Precali R, Giani M, Marini M, Grilli F, Ferrari CR, Pećar O, Paschini E (2005) Mucilaginous aggregates in the northern Adriatic in the period 1999–2002: typology and distribution. *Sci Total Environ.* 353: 10–23.
- [86] Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao AJ, Quigg A, Santschi P, Sigg L (2008) Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* 17: 372–386.
- [87] Miao AJ, Schwehr KA, Xu C, Zhang SJ, Luo ZP, Quigg A, Santschi P (2009) The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. *Environ. Pollut.* 157: 3034–3041.
- [88] Pletikapić G, Vinković Vrček I, Žutić V, Svetličić V (2012) Atomic force microscopy characterization of silver nanoparticles interactions with marine diatom cells and extracellular polymeric substance. *J. Mol. Recognit.* 25: 309–317.
- [89] Boissière M, Tourrette A, Devoisselle JM, Di Renzo F, Quignard F (2006) Pillaring effects in macroporous carrageenan-silica composite microspheres. *J Colloid Interface Sci.* 294: 109–16.
- [90] Bustamante C, Macosko JC, Wuite GJ (2000) Grabbing the cat by the tail: manipulating molecules one by one. *Nat. Rev. Mol. Cell Biol.* 1: 130–6.
- [91] Bhanu P. Jena, J. K. Heinrich Hörber (2006) *Force Microscopy: Applications in Biology and Medicine*. New Jersey: John Wiley & Sons. 300 p.
- [92] Yves Dufrene (2011) *Life at the Nanoscale: Atomic Force Microscopy of Live Cells*. Singapore: Pan Stanford Publishing. 460 p.

- [93] Alexander Noy (2007) Handbook of Molecular Force Spectroscopy. Livermore: Springer. 300 p.
- [94] Higgins M.J, Wetherbee R (2011) The Role of Atomic Force Microscopy in Advancing Diatom Research into the Nanotechnology Era. In: Dufrene Y, editor. Life at the Nanoscale: Atomic Force Microscopy of Live Cells. Singapore: Pan Stanford Publishing. pp. 405-420.
- [95] Pletikapić G, Mišić Radić T, Berquand A, Murvai U, Kellermayer MSZ, Vinković Vrček I, Žutić V, Svetličić V (2012) Marine diatom cells and extracellular polymers: nanostructure, nanomechanics and interactions with nanoparticles. International Conference: From Solid State to BioPhysics VI: From Physics to Life Sciences, 9<sup>th</sup>-16<sup>th</sup> June 2012, Dubrovnik, Croatia.