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Colonization of diatoms and bacteria on artificial substrates in the northeastern coastal Adriatic Sea

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Abstract – Every surface that is immersed in seawater becomes rapidly covered with an unavoidable biofilm. Such biofilm formation, also known as fouling, is a complex multistage process and not yet thoroughly investigated. In this study, the succession of diatoms and bacteria was investigated during a one month exposure on an artificial substrate of plexiglass (polymer of methyl methacrylate) mounted above the seafloor at a depth of 5 m. For biofilm analyses, the fouling was investigated using selective agar plates, epifluorescence, light and electronic microscopy, as well as high performance liquid chromatography (HPLC) pigment analysis. During biofilm development, the abundance of all biofilm components increased and reached maximum values after a one month exposure. In the bacterial community, heterotrophic marine bacteria were dominant and reached $1.96 \pm 0.79 \times 10^4$ colony forming units (CFU) cm^{-2}. Despite the fact that faecal coliforms and intestinal enterococci were detected in the water column, faecal coliforms were not detected in the biofilm and intestinal enterococci appeared after one month of exposure but in the negligible number of 60 ± 10 CFU cm^{-2}. The phototrophic component of the biofilm was dominated by diatoms and reached a concentration of 6.10×10^5 cells cm^{-2}, which was supported by pigment analysis with fucoxanthin as dominant pigment in a concentration up to 110 ng cm^{-2}. The diatom community was dominated by *Cylindrotheca closterium* and other pennate benthic diatoms. A detailed taxonomic analysis by electronic microscopy revealed 30 different taxa of diatoms. The study confirmed that a plexiglass surface in a marine environment is susceptible to biofouling within 30 days of contact. Furthermore, the colonization process sequence firstly involved bacteria and cyanobacteria, and secondly diatoms, which together formed a primary biofilm in the sea.

Keywords: bacteria, biofilm, biofouling, diatoms, succession

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Introduction

A biofilm is an assemblage of adhered cells and their products on a surface, a 'coating' or 'covering' composed of organisms like bacteria, protozoa, algae and invertebrate animals (O'toole et al., 2000, Stoodley et al., 2002). As a general rule, biofilm growth can be explained in several phases: (i) adsorption – binding of dissolved chemical compounds, macromolecules such as glycoproteins, polysaccharides and proteoglycans, in the first moments of contact with any surface immersed in the seawater, (ii) immobilization – reversible binding of bacterial cells with weak links and interactions on the surface of the substrate, (iii) consolidation – irreversible binding of bacterial cells on the surface of the substrate in which bacterial cells begin to secrete extracellular polymeric substances (EPS), which creates a permanent bond between cells and surfaces, (iv) settling – the final stage in the development of biofilms, when other micro-organisms such as unicellular algae inhabit bacterial colonies and biofilm takes a three-dimensional structure. Colonization of microorganisms on the solid surfaces is a worldwide problem: from offshore oil platforms and bridges that can collapse due to biofouling, through freighters, cruisers and naval vessels to fishing facilities and a variety of fishing gear. The biggest problem of fouling on ships is the possibility of corrosion of the stern, which increases ships fuel consumption up to 30% (De Rincon et al. 2001). Consequently, there is an increased release of greenhouse gases contributing to the major environmental problem of our time. Because of fouling on aquaculture installations for fish, shellfish and other organisms their cultivation is faced with environmental problems such as anoxia, eutrophication, increased turbidity, and all of these can lead to plague organisms and major economic losses (Lewis et al. 1997). Settling allows for the colonization of multicellular organisms such as larvae, invertebrates, multicellular filamentous algae and other macro-invertebrates (Lehaitre and Compère 2007). Periphyton includes all plant and animal organisms attached to various types of substrates that are submerged in water, and that do not penetrate the surface and is divided into 'euperiphyon' (basic part of the periphyton, formed by attached organisms adapted to a sessile lifestyle) and 'pseudoperiphyton' (part that is associated with the periphyton formed by communities of organisms that move freely among attached species, depending on them as a source of food and protection from predators and planktonic organisms caught and retained in a dense network of organic matter).

Investigations of biofilm formation in the marine environment are scarce. It is known that colonization of artificial differs from that of natural substrates (Hamilton and Duthie 1984, Sabater et al. 1998). Periphyton communities in the northern Adriatic consist mainly of diatoms with a distinct seasonal variation. The highest abundance and biomass were observed in the period between February and October (557.156 ± 82.602 cells cm^{-2}), while in the period between January and February abundance and biomass was much lower (365 ± 407 cells cm^{-2}) (Totti et al. 2007). Also, no significant difference in the periphyton community structure and composition were observed in different artificial substrates. Investigation of periphyton development on artificial substrates in the highly stratified estuary of the karstic Zrmanja River (middle Adriatic) (Caput et al. 2008), showed high diatom abundance after a two-week exposure (2.3×10^7 cells cm^{-2}) with species richness 41, while after 4 weeks, abundance doubled and richness increased to 50. Periphyton was composed mostly of *Amphora coffeaeformis* and *Navicula veneta* after 2 weeks; while after 4 weeks, *Melosira moniliformis* was co-dominant. On the other hand, Romagnoli et al. (2007) described the diatom communities associated with *Eudendrium racemosum* as a natural substrate

throughout an annual cycle in the Ligurian Sea. Diatom abundance values ranged from 46.752 ± 24.684 cells mm^{-2} in February 2003 to 917 ± 331 cells mm^{-2} in October 2003, while biomass ranged from 1.94 ± 1.94 to 0.013 ± 0.003 µg C mm^{-2} for the same periods. In contrast, filamentous cyanobacteria appeared with high densities between late spring and summer with maximum abundance of 29.872 ± 4.482 cells mm^{-2} and biomass of 0.32 ± 0.18 µg C mm^{-2}. On average, motile diatoms represented the most abundant fraction of diatom communities (73%), followed by adnate (17%), erect (7%) and tube-dwelling growth forms (3%). Considering biomass, motile diatoms represented 48% of the total biomass value, followed by erect (25%), adnate (18%) and tube-dwelling diatoms (9%) (Romagnoli et al. 2007).

The aim of this study was to investigate the succession and settling of benthic microalgae in the process of formation of primary biofilm in the northeast Adriatic Sea. An additional importance of this research is the interdisciplinary approach in which various methods were combined to obtain a better insight into the biofilm formation. Thus, the objectives of this study were to (i) determine the quantitative and qualitative composition of diatoms and bacteria on artificial plexiglass plates, (ii) to demonstrate their distribution through the investigated time of 30 days and (iii) to provide insight in their probable mutual influence on the growth and development of the biofilm.

Materials and methods

Sampling

Periphyton sampling was carried out in the bay Val de Lesso in the city of Rovinj (45.060N, 13.3748E) in the period from September 9th to October 7th 2013. The bay is under moderate anthropogenic influence due to the surrounding inhabited area. It is shallow (maximum depth up to 10 m) and the seabed is covered by seagrass *Cymodocea nodosa*. The late summer/early autumn was chosen for the exposure period according to Hillebrand and Sommer (1997) due to the appropriate temperature for the endorsement of speed of settlement on artificial substrates. Continuous weather measurements were conducted at a meteorological station located at Sv. Ivan na Pučini, Rovinj, western Istrian coast (45.04746N, 13.62167E). The data were supplied by the Croatian Meteorological and Hydrological Service (Tab. 1). All data regarding abundance of bacteria and diatoms were statistically analyzed by software STATISTICA 7.0 and 2007 Microsoft Office Suite Service Pack 3.

Plexiglass was used as a substrate for biofilm formation following the recommendation of Slàdečkovà (1962a) because of its convenience compared to a natural substrate. Dimensions of plates were 70 × 20 × 2 mm (l/w/h) for bacteriological sampling and 100 × 70 × 2 mm (l/w/h) for algological sampling. The plates were set in a steel structure with plastic rails (Fig. 1a, 1b). In an effort to refine the terminology Slàdečkovà (1962a) discovered periphyton exclusive of epiphytic and epizoic forms. Periphyton was subdivided into 'euperiphyon', immobile organisms attached to the substrate by means of rhizoides, gelatinous stalks, or other holdfast mechanisms, and 'pseudoperiphyton' forms that are free-living, mobile, or creeping among or within the euperiphyton. The plates were set vertically in order to reduce the accumulation of sludge and biomass of 'pseudoperiphytic' diatoms (Slàdečkovà 1962a). The construction was set in the sea at a depth of 5 m in the meadow of seagrass *Cymodocea nodosa* and raised 30 cm above the sediment. The depth of five

Tab. 1. Hydrographic parameters of sampling events at Bay Val de Lesso, Rovinj in the period from September 9th to October 7th 2013, Avg. – average value, wind strength is shown in values according to Beaufort scale (0–12).

Parameters / Date	9-Sept	10-Sept	11-Sept	13-Sept	16-Sept	7-Oct
Avg. daily temperature (°C)	24.2	23.1	18.3	17.3	18.6	14.2
Avg. sea temperature (°C)	22.20	22.00	22.00	22.20	22.00	19.90
Daily pressure (hPa)	1014.4	1013.3	1011.3	1016.9	1003.7	1020.9
Precipitation (mm)	0.3	0.0	0.2	0.5	48.9	20.8
Wind direction	SSE	SE	SE	ESE	NE	ENE
Wind strength	4	1	1	1	3	1

Fig. 1. Construction with plexiglass plates placed in meadow of *Cymodocea nodosa* seagrass (a), plexiglass plates placed in steel structure with plastic rails (b).

meters ensures enough light for biofilm development, while benthic impacts of waves and tides do not interfere with the succession. Two large and two small plexiglass plates were placed into the construction for each sample with a total of 24. Plexiglas plates were previously disinfected with 70% ethanol and stored in sterile containers. The collected material for diatom analyses was preserved in 4% formaldehyde.

Bacteriological analysis

For bacteriological analysis, plates were removed from the structure after 1 h, 12 h, 24 h, one week and one month of contact. Plates were gently washed with 100 mL of sterile saline (0.8% NaCl) and immersed in Schott bottles containing 150 mL of sterile saline solution so that the entire surface of the plate was immersed in liquid. Biofilm was dispersed using an ultrasonic probe (40 W, 120 one second cycles). After treatment with ultrasound

supernatant, samples were collected and inoculated on the appropriate culture medium in duplicates. Dispersed biofilm for marine heterotrophic bacteria analysis was decimally diluted, while for coliform and enterococci analysis the samples were filtered through a nitrocellulose filter (0.2 μm) and then inoculated onto selective agar plates in duplicates. The number of heterotrophic marine bacteria was determined on Marine agar, DIFCO, USA, (25 °C, 72 h). The number of faecal coliforms was determined on m-FC agar, Biolife, Italy (44.5 °C, 24 h). The number of intestinal enterococci was determined on Slanetz-Bartley agar, Biolife, Italy (35 °C, 72 h).

Diatom and phytoplankton analysis

For algological analysis, plates were sampled after 1 h, 12 h, 24 h, 48 h, 96 h, one week and one month and placed in sterile bags with care that the biofilm was not damaged. Fouling was scraped off the inner and outer side of the plates using a brush and dispersed in a known volume of 2% formaldehyde solution. Cell counts were obtained by the inverted microscope Zeiss Axiovert 200 using a previously described method (Utermöhl 1958). Subsamples of 10 ml were analysed microscopically after sedimentation for 24 h. Microplankton (MICRO) cells (longer than 20 μm) were counted under a magnification of 400× (1–2 transects), as well as 200× and 100× (transects along the rest of the counting chamber base plate). The samples were acid cleaned (HCl/H_2SO_4) prior to qualitative analysis using scanning electron microscope (SEM) Philips 515. For direct plate analyses plexiglass plates were pre-dried at room temperature. Fouling on such prepared plates was observed by SEM Philips 515 and, after being dyed with DAPI, by epifluorescence microscopy (Zeiss Axio Imager Z1).

To get insight into the natural periphytic community, leaves of seagrass *Cymodocea nodosa* were sampled and phytoplankton samples also, to establish stabile surrounding phytoplankton assemblages. Leaves of seagrass *C. nodosa* were sampled on the first day of the experiment and the periphytic community was acid cleaned (HCl/H_2SO_4) and examined by SEM Philips 515. For analysis of the surrounding phytoplankton community, samples were taken at a depth of 5 m by 5 L Niskin bottle after 1 h, 24 h, 48 h, 96 h, one week and one month. Samples of 50 mL were sedimented for 24 h and analysed following the Utermöhl method (1958) with an inverted microscope (Zeiss Axiovert 200).

Pigment analysis

In order to gather information about the chemotaxonomic composition of the periphytic community, pigments were analyzed using high performance liquid chromatography (HPLC). Re-suspended samples were filtered on 0.7 μm pore size Whatman Glass Fibre Filters (GF/F) and preserved in liquid nitrogen until the analysis. The extraction in 4 mL of cold 90% acetone was performed by sonication, and the extract was clarified by centrifugation. Pigments were separated by reversed phase HPLC following the protocol of Barlow et al. (1997). Extracts were mixed 1:1 (v/v) with 1 M ammonium acetate and injected into an HPLC system equipped with 3 mm Thermo Hypersil column MOS2 (C-8, 120 A pore size, 150 × 4.6 mm) (Thermo Hypersil-Keystone). Pigments were separated at a flow rate of 1 mL min^{-1} using a linear gradient program with a duration of 40 min. Solvent A consisted of 70:30 (v/v) methanol: 1 M ammonium acetate and solvent B was 100% methanol. Chlorophyll and carotenoids were detected by absorbance at 440 nm (Spectra System, Model UV

2000). Qualitative and quantitative analyses of individual pigments were performed by external standard calibration using authentic pigment standards (VKI, Denmark).

Results

During sampling, weather conditions varied from day to day, which was reflected in the structure of the water column and consequently the structure of the periphytic community and biofilm formation. After 30 days of exposure, a slimy mucous biofilm was present on plexiglass plates. The main living constituents of the biofilm were attached cells of bacteria, cyanobacteria, diatoms, dinoflagellates, green (Ulvophyceae) and brown (Phaeophyceae) algae.

Bacteria

Heterotrophic marine bacteria reached the number of $1.96 \pm 0.79 \times 10^4$ CFU cm^{-2} during one month of exposure (Fig. 2A). Despite the fact that faecal coliforms and intestinal enterococci were present in the water column at the beginning of the experiment (faecal

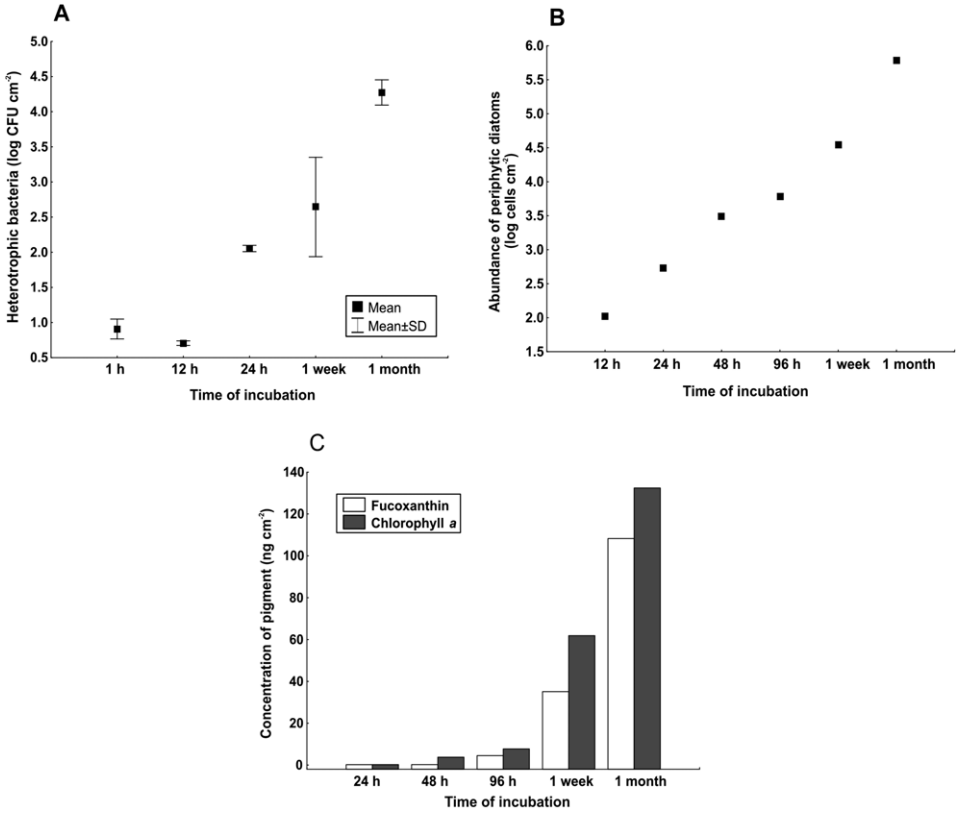

Fig. 2. Abundance (expressed as logarithmic value) of heterotrophic marine bacteria through exposure time from 1 h to one month (a); Abundance (expressed as logarithmic value) of periphytic diatoms through exposition time from 12 h to one month (b); Concentration of pigments fucoxanthin and Chl *a* through exposure time from 24 h to one month (c).

coliforms 255 ± 78 CFU L^{-1}, intestinal enterococci 180 ± 28 CFU L^{-1}) no coliforms were detected in the biofilm during the entire experiment. Enterococci were present after one month of exposure in a negligible number (60 ± 10 CFU cm^{-2}).

Diatoms

Periphytic diatoms also showed a steady increase in abundance during the month of exposure of the plexiglass plates (Fig. 2B). The absolute maximum value of abundance of periphytic diatoms was 6.10×10^5 cells cm^{-2} at 30 days of exposure.

The pioneer species *Cylindroteca closterium* and *Nitzschia longissima* were determined and they re-occurred with planktonic species *Dactyliosolen fragilissimus*, *Diploneis bombus*, *Proboscia alata* and group *Pseudo-nitzschia pseudodelicatissima* »sensu lato« (Tab. 2). These were followed by *Thalassionema nitzschioides*, *Leptocylindrus danicus*, *Microtabella interrupta*, *Pleurosigma angulatum*, *Licmophora* sp. and *Melosira nummuloides*. The species with the greatest abundance was *Cylindroteca closterium* (5.5×10^4 cells cm^{-2}).

A total of 30 diatom taxa were determined (Tab. 3) in the periphyton assemblage from the plexiglass plates and the dominant taxa were: *Navicula* sp. (71%), *Thalassiosira* sp. (71%), *Amphora ovalis* (71%), *Amphora* sp. (43%), *Licmophora* sp. (43%), *Mastogloia* sp. (43%), *Proschkinia bulnheimii* (43%), *Thalassionema nitzschioides* (43%), *Cyclotella* sp. (43%). The qualitative analysis of stable diatom community on *Cymodocea nodosa* revealed a total of 10 species of which *Amphora ovalis*, *Cyclophora* sp., *Navicula* sp. and *Proschkinia bulnheimii* were most frequent. Three taxa were recorded only by analyzing leaves of *Cymodocea nodosa*: *Achnanthes* sp., *Cyclophora* sp. and *Microtabella interrupta.*

Tab. 2. List of all diatom taxa present in biofilm at different exposure times during research period as well as their abundance.

Taxa / Exposure time	Abundance (cells cm^{-2})						
	1 h	12 h	24 h	48 h	96 h	1 week	1 month
Dactyliosolen fragilissimus (Bergon) Hasle	2						
Cylindroteca closterium (Ehrenberg) Reimann & J. C. Lewin	16		61	114	151	7234	55764
Diploneis bombus Ehrenberg	2						
Leptocylindrus danicus Cleve				26			
Licmophora sp.						1426	37176
Melosira nummuloides C. Agardh							703
Microtabella interrupta (Ehrenberg) Round					201		88
Nitzschia longissima (Brébisson) Ralfs	16				25	23	
Pleurosigma angulatum (Quekett) W. Smith				2			
Proboscia alata (Brightwell) Sundström	65			23	25		
Pseudo-nitzschia pseudodelicatissima »sensu lato«	194		163	137		182	
Thalassionema nitzschioides (Grunow) Mereschkowsky		25	82	524	780	251	
Unindentified pennate diatoms	307	53	225	2234	4909	25722	515814

Tab. 3. List of all diatom taxa analyzed with SEM at different exposure times, C – diatom taxa found on leaves of seagrass *Cymodocea nodosa*, Fr. – occurrence frequency of taxa (diatom taxa found on *C. nodosa* were excluded), 0* – zero frequency for taxa found only on leaves of *C. nodosa*.

Taxa (30 in total)	Exposure time							C	Fr. (%)
	1 h	12 h	24 h	48 h	96 h	1 week	1 month		
Achnanthes sp.								+	0*
Amphora coffeaeformis Cleve	+				+	+	+	+	58
Amphora ovalis (Kützing) Kützing			+	+	+	+	+	+	71
Amphora sp.					+	+	+	+	43
Bacteriastrum sp.							+		14
Cocconeis sp.					+	+		+	29
Cyclophora sp.								+	0*
Cyclophora tenuis Castracane						+			14
Cyclotella sp.	+			+			+		43
Cylindrotheca closterium (Ehrenberg) Reimann & J. C. Lewin						+	+		29
Diploneis bombus Ehrenberg							+		14
Diploneis sp.					+	+	+		43
Fallacia sp.							+		14
Gomphonema sp.							+		14
Grammatophora marina (Lyngbye) Kützing							+		14
Licmophora flabellata (Grev.) C. Agardh							+		14
Licmophora sp.					+	+	+		43
Mastogloia sp.	+				+		+	+	43
Mastogloia undulata Grunow							+		14
Microtabella interrupta (Ehrenberg) Round								+	0*
Navicula sp.	+			+	+	+	+		71
Nitzchia sp.			+			+		+	29
Paralia sulcata (Ehrenberg) Cleve	+								14
Pleurosigma angulatum (Quekett) W. Smith							+		14
Proschkinia bulnheimii (Grunow) Karayeva					+	+	+	+	43
Psammodictyon mediterraneum (Hustedt) D. G. Mann						+	+	+	29
Rhizosolenia sp.	+								14
Thalassionema nitzschioides (Grunow) Mereschkowsky			+		+		+		43
Thalassiosira sp.	+	+	+		+		+		71
Triceratium sp.	+								14

The surrounding phytoplankton assemblage was composed of 24 dominant taxa of Bacillariophyceae, six dominant taxa in the group Dinophyceae and the groups Cryptophyceae and Chlorophyceae. The composition of phytoplankton throughout the exposure period was relatively consistent with the dominant group *Pseudo-nitzschia pseudodelicatissima* »sensu lato« whose greatest abundance was 10.2×10^4 cells L^{-1}.

Biofilm pigments

HPLC pigment analysis revealed 10 different pigments belonging to different groups: peridinin, diadinoxanthin, butinin, chlorophyll *a* (Chl *a*), chlorophyll *c* (Chl *c*), fucoxanthin, hexanoiloxifucoxanthin, zeaxanthin, β-carotene and prasinoxanthin, fucoxanthin (concentration up to 110 ng cm^{-2}) and Chl *a* (concentration up to 135 ng cm^{-2}) being the dominant pigments (Fig. 2C). During this study Chl *c*, prasinoxanthin and zeaxanthin were detected after 96 h of exposure. Chl *a*, fucoxanthin and peridinin were dominant from beginning. Also, the concentration of fucoxanthin was significantly lower than the concentration of Chl *a* in the first four days of exposure, and then increasingly reached similar concentrations (fucoxanthin 108.272 ng cm^{-2}, Chl *a* 132.424 ng cm^{-2}) (Fig. 2C).

Direct biofilm visualisation

During the colonization of the plexiglass plates and biofilm generation some parts of the surface remained uninhabited, while the others developed a comprehensive three-dimensional biofilm (Figs. 3, 4). Biofilm thickness was not homogeneous in all parts of the substrate and varied from a few μm to a few cm depending on the state of succession on the plates.

Discussion

Research of periphytic algae on natural and artificial substrates is faced with an array of challenges: quantifying the substrate surface as well as the diversity and inaccuracies of the techniques of removing fouling makes it difficult to compare published results (Barbiero 2000). The best practice for studying processes of migration, colonization and growth is: (i) carefully to choose the artificial substrate, its material surface, texture and size (Cattaneo and Amireault 1992), (ii) to adjust for easy handling (Weitzel et al. 1979), (iii) to determine the appropriate location and duration of exposure of the substrate (iv) and to adjust material for the selective attachment of benthic flora (Snoeijs 1991). In our study plexiglass was selected as artificial substrate which facilitated removal and the measurement of surface fouling, allowed easier handling and reduced the possibility of settling of organisms that are not of interest for the development of primary biofilms. Moreover, we confirmed that the plexiglass surface in a marine environment is susceptible to biofouling within 30 days of contact. However, the dominating factor involved in the initial attachment of a bacterial cell to a surface has remained elusive.Today it is thought that a multitude of factors are involved in processes of settling, including surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness and surface micro-topography (Palmer et al. 2007). Our finding that faecal coliforms and intestinal enterococci were detected in the water column, but not in a biofilm may be explained with chemical composition, surface roughness and micro-topography. Coliforms are known to inhabit mixed-population bio-

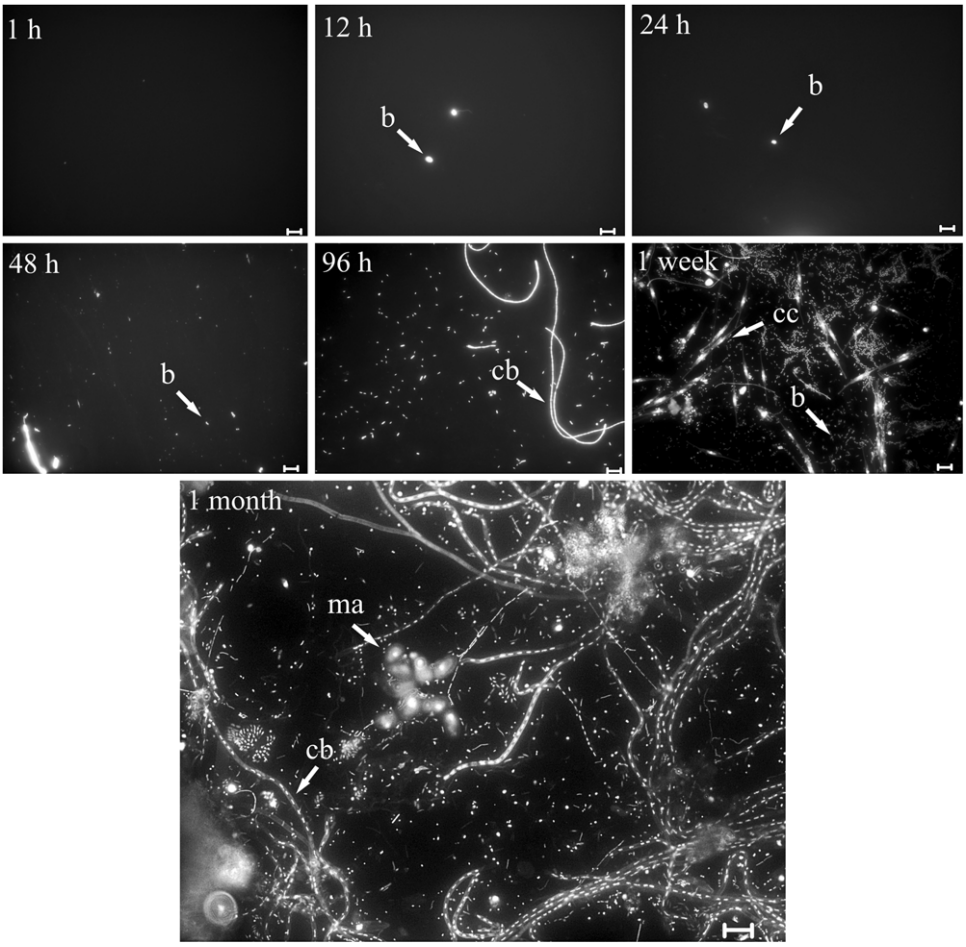

Fig. 3. Direct plate analysis under epifluorescence microscope through the exposure time from 1 h to 1 month; b – bacterial cells; cb – cyanobacterial chains; cc – cells of *Cylindrotheca closterium*; ma – macro-aggregate. Bars = 10 μm.

films in water distribution systems (Camper et al. 1996). In laboratory conditions the persistence of coliforms in mixed biofilms on polycarbonate surfaces was highly dependent on the growth rate of the inoculum and type of substratum. Significantly higher numbers of both heterotrophs and coliforms were found on steel (reactive surface) than on polycarbonate (inert surface) (Camper et al. 1996). Bacteria (including coliforms) that initially successfully colonized the surface can readily acclimatize to low-nutrient conditions (seawater) (Novitsy and Morita 1978, Kurath and Morita 1983, Camper et al. 1996). Thus, the absence of faecal coliforms and intestinal enterococci in the biofilm in our experiment was likely due to the choice of substratum; the mentioned species were unable to initially attach to inert surface of plexiglass and continue their growth in the biofilm.

An important factor during the first week of succession is the strength and direction of the wind because it contributes to the mixing of the water column. The occurrence of plank-

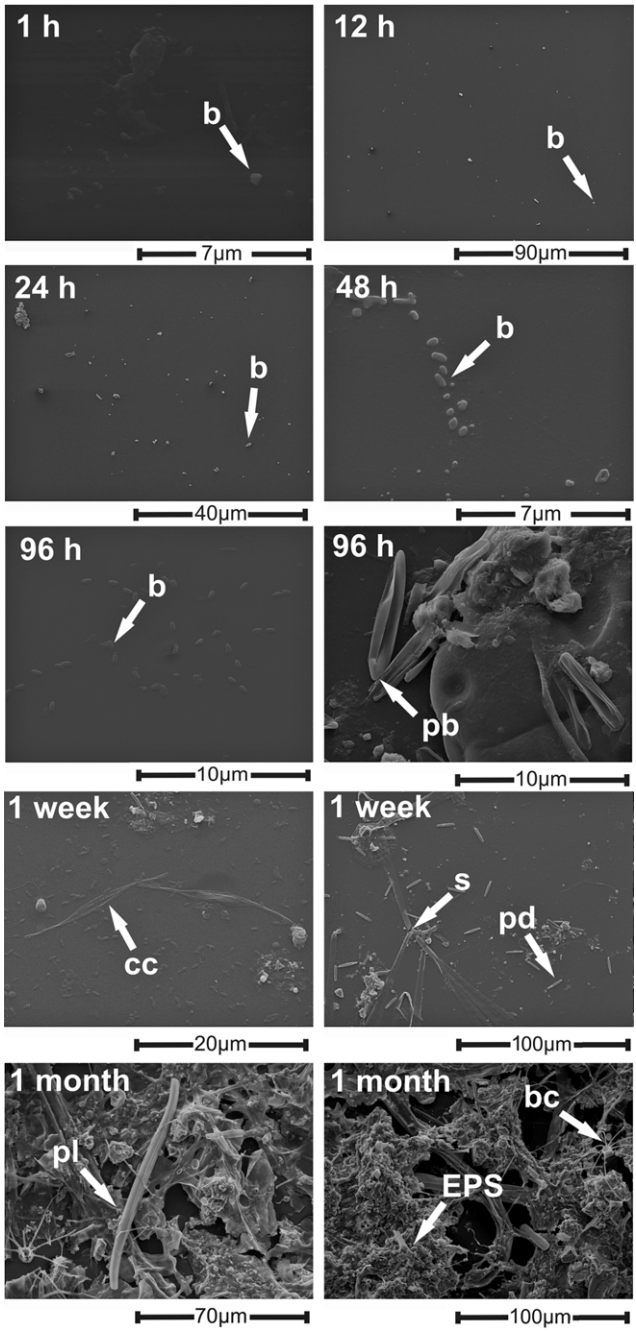

Fig. 4. Direct plate analysis under scanning electron microscope through the exposure time from 1 h to 1 month; b – bacterial cells; bc – cells of *Bacteriastrum* sp.; cc – cells of *Cylindrotheca closterium*; EPS – extracellular polymer substances produced by bacterial and diatom cells; pb – cells of *Proschkinia bulnheimii*; pd – unidentified pennate diatoms; pl – cells of *Pleurosigma* sp.

tonic diatoms among the pioneer species on the plates might be explained by these environmental conditions. In this study we recorded *Dactyliosolen fragilissimus*, *Proboscia alata*, *Thalassionema nitzschioides*, and *Leptocylindrus danicus*, all common planktonic diatoms of the north-eastern Adriatic Sea (Godrijan et al. 2013). Records of frustules of planktonic taxa found in a dense biofilm can be explained by their natural settling and entanglement after death. Frustules were caught and retained in the dense organic network of the biofilm.

The composition of phytoplankton throughout the exposure period was relatively consistent with the dominant group being *Pseudo-nitzschia pseudodelicatissima* »sensu lato« whose greatest abundance was observed after a month of exposure (10.2×10^4 cells L^{-1}). The group *P. pseudodelicatissima* »sensu lato« is positively correlated with temperature in the northern Adriatic, as was the case in the western Mediterranean, where higher abundances were recorded in the warmer part of the year, for example, in the late summer and autumn (Ljubešić et al. 2011, Marić et al. 2012).

As succession progressed, planktonic species were replaced by benthic diatoms the abundance of which increased exponentially. The taxonomic composition and abundance of periphytic diatoms in this study confirms previous research done in the northern Adriatic (Totti et al. 2007, Caput et al. 2008). According to Denicola and McIntyre (1990), diatoms of the genus *Licmophora*, *Cocconeis* and *Achnantes* are among the most prominent colonizers after the first week of exposure. They are accompanied by other motile pennate diatoms which are competitors for light and nutrients such as those from the genus *Amphora*. As confirmed by a quantitative analysis of the biofilm, the qualitative analysis shows that in the initial formation of biofilms, from the first to the fourth day of immigration, the most important factor is planktonic species (Stevenson 1986), while later (from the 7th to the 30th day) there is a significant development of benthic diatoms, which are attached with the entire surface of the valve (e. g. genera *Cocconeis* and *Amphora*) and EPS production in the form of stands (*Licmophora* sp.), apical plate, mucilage plates and cell membranes (Hoagland et al. 1993). Additionally Munda (2005) in her study near Piran (Gulf of Trieste) describes primary colonizers as *Achnanthes* and *Licmophora* species which form dense epilithic populations due to their attachment by stalks. Also noteworthy is the centric diatom *Paralia sulcata,* which is regarded as an indicator species of coastal upwelling situations and is common in plankton and the benthos under low light conditions, which are an advantage to it (Munda 2005). In our study *P. sulcata* was recorded at first sampling event when the water column was mostly under wind pressure resulting in mixed conditions. Our study correlates with previous findings (Caput et al. 2008) and the abundances of *Amphora coffeaeformis* and *Navicula veneta* that dominated ($f \geq 83\%$, abundance $> 10^6$ cells cm^{-2}) in the Zrmanja estuary. In our study, in addition to these two species, *Nitzschia longissima* ($f = 80\%$, abundance 1.5×10^6 cells cm^{-2}) was co-dominant.

The genera *Cyclophora*, *Achnanthe*s and species *Microtabella interrupta* were not recorded in the biofilm on the plates at any sampling event. This could be explained by hypothesizing that they settle later during the biofilm formation or rather that they prefer natural substrates such as seagrass. According to Romagnoli et al. (2007) who compared microalgae grown on artificial substrata and those that settled on *E. racemosum*, on average diatom abundance values were significantly lower on artificial substrata (2.521 ± 1.784 cells mm^{-2}) than on the hydroid (4.737 ± 3.919 cells mm^{-2}, $p < 0.01$). The same pattern was observed for diatom biomass, which showed significantly lower values on mimic substrata

(0.033 ± 0.027 µg C mm^{-2}) than on *E. racemosum* (0.093 ± 0.088 µg C mm^{-2}, $p < 0.005$). Many studies have shown that macrophytes generally serve as a source of nutrients for periphyton, which makes plexiglass a relatively poor substrate (Cattaneo and Kalff 1979, Carignan and Kalff 1982, Burkholder and Wetzel 1990, Cattaneo and Amireault 1992).

The chemotaxonomic analysis of the biofilm provided an excellent confirmation method for our microscopic results. Chl *c*, prasinoxanthin and zeaxanthin were detected after 96 h of exposition which nicely correlated with the appearance of cyanobacteria on the plates after the first week. The presence of Chl *a* and fucoxanthin from the beginning revealed diatoms as primary colonizers.

Descriptive methods of direct plate visualisation with epifluorescence and electron microscopy in this study gave a better insight into the sequence of colonization on artificial substrates and into the three-dimensional structure of this early biofouling stages. So far, these methods have not been used in research and the data obtained in this study can serve as starting point for the development of such methods in the future. During the first hours of contact with seawater we could observe the deposition of a thin film on the substrate. Subsequently, we confirmed that the bacteria are the first colonizers in the process of immobilization (reversible binding), where they first examine the substrate and check for the availability of nutrients, which is followed by the process of consolidation (irreversible binding) when they begin to exude EPS (Lehaitre and Compère 2007). On this sticky mixture of proteins, proteoglycans and carbohydrate diatoms start to attach, and they tend to form permanently adhesive complex structures such as pads, stalks, capsules and tubes. Even though we observed a distinct succession pattern in the biofilm formation, the rather two-dimensional structure of the observed biofilm seems to indicate, that colonization is a rather individual process of direct interaction between the cells and the substrate. However the maturation of the supposedly organic but abiotic thin film covering the substrate surface might explain the observed succession pattern. Also, differences in a presumed time lag of growth induction after attachment to a surface might contribute to the succession of species observed during the biofilm formation.

In conclusion, this study confirmed that plexiglass surfaces in a marine environment are susceptible to biofouling within 30 days of contact. Abundance of bacterial cells and diatom cells increased through the investigated period and they interacted, creating an optimized micro-environment. With HPLC analysis this study confirmed a temporal distribution pattern during the process of biofilm formation: first colonizers are bacteria and cyanobacteria, and second are diatoms, which together form a primary biofilm in the sea. In natural biofilms, niche formation might, in addition to other factors, explain the success and distribution pattern of certain diatoms and associated bacteria. They can support each other by equilibrium of the cross-feeding, possibly optimized by exchange of chemical factors. These associations can be specific or random. It is likely that cross-feeding partners may change due to various factors such as light, temperature, water currents etc., or presence of other microorganisms and their secretions. Further, these interactions appear to initialize the formation of diatom biofilms and aggregates, as has been shown with marine microbial communities. However, we were able to demonstrate a certain spatial independence between bacterial and diatom early settlers, which indicates the importance of abiotic components in the early biofilm formation.

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