J Appl Phycol
DOI 10.1007/s10811-013-0028-1

Characterization of exopolysaccharides produced by seven biofilm-forming cyanobacterial strains for biotechnological applications

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Received: 21 December 2012 / Revised and accepted: 25 March 2013

Abstract The molecular identification of seven biofilm-forming cyanobacteria and the characterization of their exopolysaccharides were made and considered in terms of potential biotechnological applications. The studied strains were isolated from phototrophic biofilms taken from various Italian sites including a wastewater treatment plant, an eroded soil, and a brackish lagoon. The polysaccharides were characterized by use of ion exchange chromatography, circular dichroism, and cytochemical stains. All strains produced exopolysaccharides with differing ratios of hydrophobic and hydrophilic moieties depending on the species, the polysaccharide fraction (i.e., whether capsular or released), and the ambient conditions. It was shown that the anionic nature of the exopolysaccharides was due to the presence of carboxylic and sulfated groups and is likely the main characteristic with industrial applicability. Potential biotechnological applications are discussed.

Keywords Cyanobacteria · 16S rRNA gene · EPS · Released polysaccharides · Capsular polysaccharides · Biotechnological applications

This study is dedicated to the memory of Prof. Patrizia B. Albertano (1952–2012)

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Introduction

Biofilms are complex and dynamic microbial communities that can have substantial phenotypic diversity allowing adaptation to widely different and variable environments (Battin et al. 2003). Biofilm microorganisms live embedded in a self-produced matrix of hydrated exopolymeric substances (EPS) that are the key to their ecological success (Battin et al. 2007; Flemming and Wingender 2010). EPS mainly consist of exopolysaccharides, proteins, nucleic acids and lipids that form a three-dimensional polymer network attaching the biofilm to surfaces and holding cells in close proximity resulting in a synergistic community (Flemming and Wingender 2010). High water retention capacity of the EPS from strong binding of water molecules with exopolysaccharides helps protect biofilms from desiccation (Pereira et al. 2009; Di Pippo et al. 2011). Exopolysaccharides also have many other roles such as protection from UV radiation and predation, biofilm attachment, detachment and mechanical strength, as well as

Published online: 21 April 2013

antibiotic resistance, and exo-enzymatic degradation activity (Wingender et al. 1999; Sutherland 2001; Pereira et al. 2009).

There is a good deal of interest in EPS from the industrial sector including the food, pharmaceutical, biomedical, and bioremediation fields (Rehm 2010). This is due to the wide compositional diversity of EPS and their physical, rheological, and other distinctive properties. Therefore, investigations into EPS components can have an economic as well as a scientific relevance.

As is often the case, exopolysaccharides can represent the dominant fraction of the EPS and potential applications are many (De Philippis and Vincenzini 1998). With this in mind, cyanobacteria are of interest as they have long been known to produce large amounts of exopolysaccharides (Pereira et al. 2009), which can be attached to the cell surface (capsular/bound polysaccharides, CPS) or be released (released polysaccharides, RPS) into the environment (De Philippis and Vincenzini 2003). It is also known that cyanobacterial exopolysaccharides are more complex than those of other microbes, with many potentially useful physicochemical properties (Li et al. 2001; Pereira et al. 2009). Yet, it was not until relatively recently that this large and diverse production received significant consideration resulting in their use as industrial gums, bioflocculants, emulsifiers, viscosifiers, medicines, soil conditioners, and biosorbants (Li et al. 2001). The sugar compositions of CPSs can be markedly different to those of the RPSs suggesting different biosynthesis mechanisms, that are, in turn, dependent on environmental conditions (Li et al. 2001; Micheletti et al. 2008). Responses of cyanobacteria to different experimental conditions, however, seem to be species- and/or strain-dependent making more difficult the optimization of exopolysaccharide production (Pereira et al. 2009). In this context, the identification and cataloging of cyanobacterial strains and their exopolysaccharide composition appears to be fundamental.

The focus in this study is on cyanobacteria isolated from a range of environments that would require distinctly different protective properties of their EPS. Two strains were taken from a wastewater treatment plant of the airport of Fiumicino, Rome, Italy. Wastewaters are known to contain a wide number of life-threatening contaminants of varied concentrations (e.g., pathogens, toxins, heavy metals, pharmaceuticals, etc.) and variable pH and temperature. Four strains were isolated from an Italian shallow lagoon (Cabras Lagoon, Sardinia), in which, there are large shifts in salinity, and polluted waters (Magni et al. 2005). The seventh strain was isolated from an eroded soil (Valle dell'Esaro, Cosenza, Italy). It has been reported that cyanobacterial exopolysaccharides play a significant role in reclaiming desert soils, aiding water retention and stabilization (Mager and Thomas 2011). In addition, the selection of biofilm-forming strains was made for ease of harvest at industrial scales, as it has long been recognized that the recovery of microplankton represents a major cost in mass cultivation (Kumar et al. 2010; Christenson and Sims 2012).

Molecular identification was carried out and strains were grown in batch cultures and, at the stationary phase, biomass production was evaluated and physicochemical characterization of their CPS and RPS fractions were made. This study also reports on the first attempt to characterize exopolysaccharides extracted from single-species biofilms. Three of the seven strains were, in fact, also grown in a microcosm appositely made for biofilm development. Strains were tested for forming single-species biofilms and for producing exopolysaccharides. Results are also discussed with respect to future experimental endeavors to unravel the possible optimization strategies for the application of these cyanobacterial exopolysaccharides in biotechnology.

Materials and methods

Identification of the cyanobacterial strains

Table 1 lists the names and the origins of the seven biofilm-forming cyanobacteria used in this study. Strains were isolated from biofilms grown in eroded soils (VRUC162), in the sedimentation tank of an Italian wastewater treatment plant (VRUC164, VRUC165), and from microphytobenthos growing on sediments of the shallow Cabras Lagoon, Sardinia, Italy (VRUC163, VRUC166, VRUC167, and VRUC 168).

Isolates VRUC162, VRUC163, and VRUC164 were previously identified as *Trichormus variabilis*, *Anabaena augstumalis*, and *Phormidium autumnale*, respectively (Bruno et al. 2012). The other four isolates (VRUC 165, VRUC 166, VRUC 167, and VRUC 168) were identified here by means of molecular taxonomic methods. After centrifugation, pellets of each strain were repeatedly frozen and thawed and then DNA was extracted using the GeneMATRIX Plant and fungi DNA purification kit (EURx). The 16S rRNA gene was amplified using the cyanobacterial specific primers CYA359 and the prokaryotic universal primer C (5′-ACGGGCGGTGTGTAC-3′) (Nübel et al. 1997). Amplifications were run in a GeneAmp PCR system 2700 (Applied Biosystems) using the conditions previously tested (Bruno et al. 2009). PCR products (≈1,000 bp) were cloned into pGEM-T Easy vector (Promega) and sequenced using primers CYA359 and C (Bruno et al. 2012). The 16S rRNA gene sequences were analyzed using the BLAST function of GenBank at the National Center NCBI electronic site (http://www.ncbi.nlm.nih.gov/). Sequences were compared with those of the other strains under study previously obtained (Bruno et al. 2012) and those from GenBank. Multiple sequence alignments were conducted with the CLUSTAL W

Table 1 Designation, origin, and medium of the tested strains

Strain designation	Origin	Medium
Trichormus variabilis VRUC162	Eroded soils Valle dell'Esaro (CZ)	$BG11_0$*
Anabaena augstmalis VRUC163	Cabras lagoon sediments (OR)	$BG11_0$
Phormidium autumnale VRUC164	Wastewater Treatment Plant of Fiumicino Airport (Rome)	modified BG11**
Synechocystis aquatilis VRUC165	Wastewater Treatment Plant of Fiumicino Airport (Rome)	modified BG11
Calothrix sp. VRUC166	Cabras lagoon sediments (OR)	$BG11_0$
Nostoc sp. VRUC167	Cabras lagoon sediments (OR)	$BG11_0$
Trichormus variabilis VRUC168	Cabras lagoon sediments (OR)	$BG11_0$

*Rippka et al. 1979

**BG11 modified using only nitrates as nitrogen source and increasing the concentration of PO_4^{3-} in order to obtain a final N:P ratio of 15 in the medium (K_2HPO_4 16 mg L^{-1}) (Guzzon et al. 2005)

program (http://www.ebi.ac.uk/). PAUP* 4.0 b10 software package (Swofford 2002; Posada 2008) was used to generate maximum-parsimony and distance trees. The robustness of the inferred phylogenies was determined by bootstrap analysis based on 500 re-samplings of data. The 16S rRNA gene sequence of *Gloeobacter violaceus* PCC7421 was used as an out-group for the construction of trees. The 16S rRNA gene sequences obtained in this study were deposited in the GenBank database under accession numbers JX456610, JX456611, JX456612, and JX456613 for VRUC165, VRUC166, VRUC167, and VRUC168, respectively.

Cultures

Each of the seven isolates (300 mL) was grown in batch culture (500-mL flasks) with a specific liquid medium for each strain (Table 1) in a controlled chamber at 18 °C, 60 % relative humidity (RH) and a photosynthetic photon flux density of 18 μmol photons m^{-2} s^{-1} with a light:dark regime of 14:10 h. Subsamples (1 mL) were taken in triplicate every 48 h and their growth estimated by measuring the optical density (Cary Varian 50 Bio UV visible spectrophotometer) at 730 nm. When the stationary phase was reached, the obtained biomass was used for biomass and EPS analyses.

Three of the seven strains of cyanobacteria (VRUC 162, VRUC 163, and VRUC 164) were also cultivated in a semicontinuous culture system (Fig. 1). The system is a flow-lane incubator placed in a controlled chamber at 25 °C and 60 % RH. It consists of four separate light chambers (see Fig. 1), illuminated at 90 μmol photons m^{-2} s^{-1} from above following a light:dark regime of 16:8 h. Medium was pumped over the growth substrate (42 polycarbonate slides) at a flow rate of 25 L h^{-1} (current speed of 5 cm s^{-1}). The flow-lane system had four light sensors per lane: one for the control of incident light, located under the lamp, and the other three, positioned directly under three selected slides, to monitor the transmittance. A decrease of transmitted light was given as an estimate of biomass accumulation. This system allowed continuous tracking of biofilm development and the construction of growth curves. Development was based on the absorption of light by the biofilm and different phases of the growth cycle were given as initial stage (10 %), active stage (50 %), and mature stage (90 %) (Zippel et al. 2007). Samples for biomass and EPS analysis were collected at the mature stage.

Biomass

The daily biomass productivity was calculated by dividing the difference between the dry weights (T=105 °C, overnight) at the end and at the start of the experiment by its duration (days) (Mata et al. 2010). All samples were taken in triplicate.

Exopolysaccharides: extraction and analysis

The biomass obtained from strains grown in batch culture was used to extract two fractions of exopolysaccharides: CPS and RPS following the procedure described in Barranguet et al. (2004, 2005). Biomass harvested from

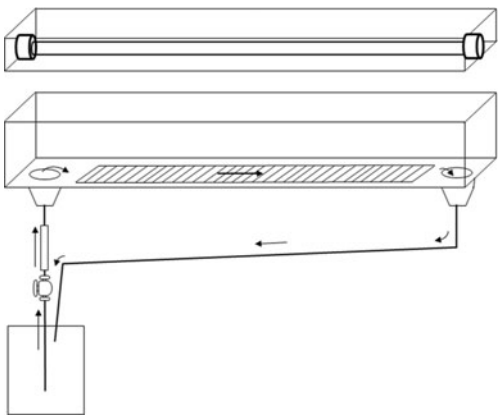

Fig. 1 Scheme of a chamber of the continuous flow-lane incubator prototype used to culture biofilm-forming cyanobacteria strains. A flow channel through medium circulated over a surface covered with polycarbonate slides. Each chamber contained the light source and the circulation of culture medium could be regulated. See text for details

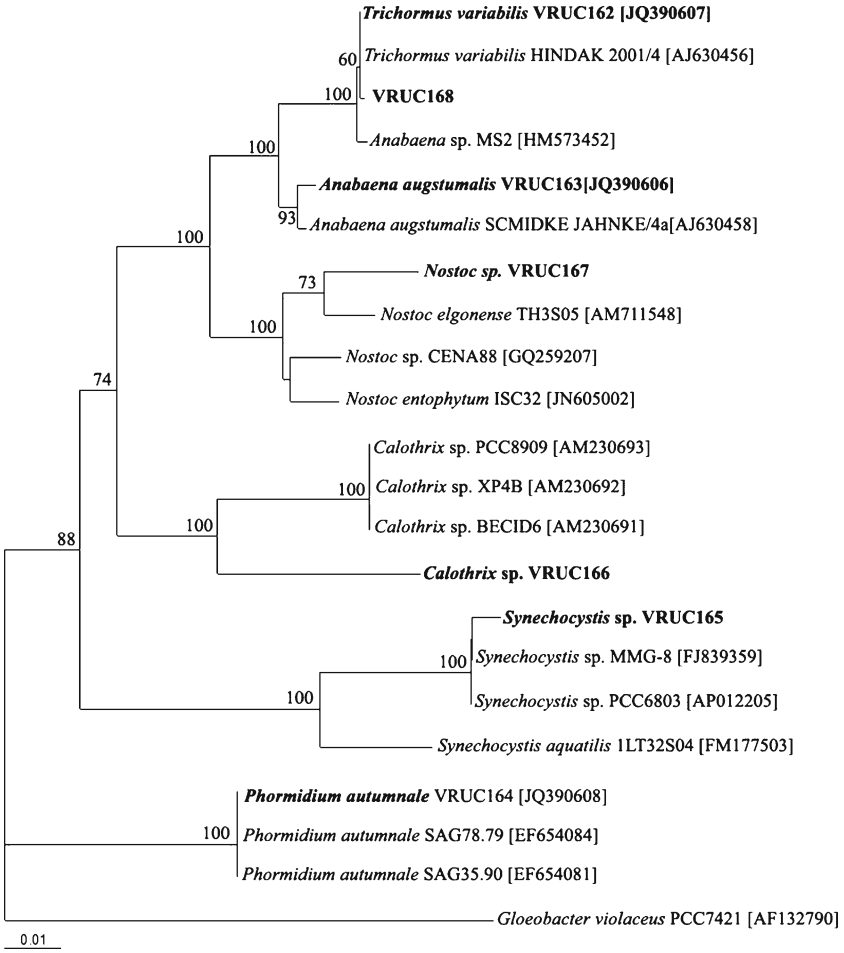

Fig. 2 Phylogenetic relationships of the seven cyanobacterial strains investigated (*in bold*) and related cyanobacteria based on 16S rRNA gene sequences. *Numbers near nodes* indicate bootstrap values >50 % for distance joining analyses. *G. violaceus* PCC7421 is the out-group taxon. GenBank accession numbers in *square brackets*

the flasks was centrifuged (5,000 rpm, 10 min) and the supernatant containing the RPS was separate from the pellet, containing the CPS and the cells. Exopolysaccharides in the supernatant were quantified (see below), while the pellet was incubated in 0.1 M sulfuric acid at 95 °C for 1 h. After the incubation, samples were centrifuged (3,500 rpm, 5 min) and the resulting supernatant (bound/capsular extracellular carbohydrates) was separated from the pellet (cells)

Table 2 Biomass productivity, CPS, and RPS amount obtained for the seven strains cultivated in this study

Strain label	Volumetric biomass productivity (gDW L^{-1} day^{-1})	mg CPS gDW^{-1}	CPS %	mg RPS gDW^{-1}	RPS %
Batch culture					
Trichormus variabilis VRUC162	0.04 (0)	76.49 (17.59)	7.65 (1.76)	28.39 (6.3)	2.84 (0.63)
Anabaena augstmalis VRUC163	0.02 (0.001)	50.46 (16.42)	5.05 (1.64)	30.41 (9)	3.04 (0.9)
Phormidium autumnale VRUC164	0.06 (0.004)	1.74 (0.75)	0.17 (0.07)	18.50 (4.8)	1.85 (0.48)
Synechocystis aquatilis VRUC165	0.09 (0.006)	0.37 (0.55)	0.04 (0.06)	9.00 (5.1)	0.9 (0.51)
Calothrix sp. VRUC166	0.04 (0.001)	17.59 (18.24)	1.76 (1.82)	55.01 (35)	5.5 (3.5)
Nostoc sp. VRUC167	0.04 (0.002)	2.96 (2.38)	0.30 (0.24)	73.80 (33.4)	7.38 (3.34)
Trichormus variabilis VRUC168	0.04 (0)	10.03 (4.53)	1.01 (0.45)	86.70 (11.9)	8.67 (1.19)
Incubator biofilm	Areal biomass productivity (gDW m^{-2} day^{-1})				
Trichormus variabilis VRUC162	1.00 (0.19)	4.50 (2.26)	0.45 (0.23)		
Anabaena augstmalis VRUC163	2.98 (0.33)	14.73 (4.38)	1.47 (0.44)		
Phormidium autumnale VRUC164	3.05 (0.05)	12.47 (2.31)	1.25 (0.23)		

Standard deviation in brackets

Table 3 Monosaccharide composition of CPS and RPS fractions extracted from strains grown in the two culture systems

Strain	Monosaccharides (% mol)												
	Fuc	Rha	GalN	Man	GlcN	Gal	Glc	Xyl	Fru	Rib	Ara	GalAc	GlucAc
CPS (batch)													
Trichormus variabilis VRUC 162	6.71	0.34	1.57	–	0.58	7.81	74.22	4.38	–	1.21	3.19	–	–
Anabaena augstmalis VRUC 163	1.03	0.41	–	4.54	0.50	6.51	68.78	2.85	–	–	0.05	15.34	–
Phormidium autumnale VRUC 164													
Synechocystis aquatilis VRUC 165	3.79	2.84	–	–	9.16	–	81.99	0.95	–	–	–	–	1.26
Calothrix sp. VRUC 166	3.13	–	0.47	6.73	7.49	11.28	52.98	8.80	1.69	–	4.54	1.42	3.17
Nostoc sp. VRUC 167	3.13	–	0.90	11.55	3.42	12.80	52.70	8.56	–	–	3.99	–	2.95
Trichormus variabilis VRUC 168	0.81	–	–	15.79	3.46	12.02	51.44	14.29	–	–	–	1.58	0.62
CPS (Incubator)													
Trichormus variabilis VRUC 162	10.53	0.43	0.20	0.76	–	12.49	59.52	14.80	–	–	0.37	–	0.91
Anabaena augstmalis VRUC 163	5.16	–	–	7.19	6.08	12.13	56.91	10.63	–	–	–	–	1.90
Phormidium autumnale VRUC 164	9.01	5.90	–	11.24	–	10.15	44.79	–	0.64	4.18	9.11	3.34	1.64
RPS (batch)													
Trichormus variabilis VRUC 162	8.10	0.39	1.15	5.51	2.30	8.48	55.36	10.96	–	6.76	0.99	–	–
Anabaena augstmalis VRUC 163	2.80	0.09	2.37	6.31	–	12.17	47.89	–	–	–	–	28.37	–
Phormidium autumnale VRUC 164	0.17	65.69	–	2.93	–	0.55	3.31	0.35	2.55	21.87	–	2.59	–
Synechocystis aquatilis VRUC 165	16.40	8.00	–	2.73	18.72	3.14	26.01	8.81	–	–	–	8.41	7.79
Calothrix sp. VRUC 166	2.20	–	–	6.27	5.04	12.49	56.26	6.35	–	–	9.46	1.02	0.91
Nostoc sp. VRUC 167	5.50	2.08	–	6.86	1.89	9.93	26.44	25.31	–	–	8.19	8.60	5.20
Trichormus variabilis VRUC 168	4.35	1.30	–	7.54	1.24	12.55	35.96	20.68	–	–	3.86	8.46	4.05

Fuc fucose, *Rha* rhamnose, *GalN* galactosamine, *Man* mannose, *GlcN* glucosamine, *Gal* galactose, *Glc* glucose, *Xyl* xylose, *Fru* fructose, *Rib* ribose, *Ara* arabinose, *GalA* galacturonic acid, *GlcA* glucuronic acid

and precipitated in 96 % cold ethanol. Both the RPS and CPS fractions were quantified spectrophotometrically using the phenol-sulfuric acid method (Dubois et al. 1956). A standard curve was made using glucose standards. At the end of the extraction, the pellet containing the cells was stained with 0.1 % [*w*/*v*] Alcian Blue in 0.5 M acetic acid to check the extraction efficiency. Observations of the stained samples were made using a light microscope to determine the intactness of the cells and whether any CPS remained after the extraction (Barranguet et al. 2004). CPS produced by strains grown in the incubator was extracted following the same protocol (Barranguet et al. 2004). Analyses were performed on three replicates for each sample.

Subsequently, lyophilized subfractions of RPS and CPS were hydrolyzed with 2 N trifluoroacetic acid for 120 min at 120 °C and then filtered using ultracentrifuge filters (Amicon Ultra-4) at 7,000 rpm for 20 min, dried in a rotary evaporator, and analyzed by using a Dionex ICS-2500 ion exchange chromatograph (IEC, USA) equipped with a gold working electrode and a 250-mm×4.6 mm i.d. Carbopac PA1 column (Dionex, USA). Eluents used were MilliQ-grade water (A), 185 mM sodium hydroxide (B), and 488 mM sodium acetate (C). In the first stage of the analysis (from injection time 0 to 7 min), the eluent was composed of 84 % A, 15 % B, and 1 % C; in the second stage (from 7 to 15 min), the eluent was 50 % B and 50 % C; a finale stage (from 15 to 30 min) was carried out with the same eluent as in the first stage. Peaks for each sugar were identified on the basis of the retention time of known standards. The flow rate was kept at 1 mL min^{-1}.

All the RPS and CPS fractions were also analyzed by means of circular dichroism (CD). Measurements were performed using a Jasco Spectropolarimeter J600, equipped with quartz cells of 0.5 cm optical length, using original Jasco software (20 nm min^{-1} scanning velocity). The trends of CD spectra were analyzed as a function of changing pH. The extract pH was adjusted using either 0.02 M $HClO_4$ or 0.02 M NaOH. The spectra were processed by standardizing the results for the dilution and the optical path of the cell.

Cytochemical characterization of exopolysaccharides by light and confocal microscopy

The seven strains were also observed at light microscope after staining for 10 min with Alcian Blue (AB) 1 % in HCl 0.5 N (pH 0.5) specific for sulfated polysaccharides or in 3 % acetic acid (pH 2.5) specific for carboxylic polysaccharides (Bellezza et al. 2006).

Moreover, fresh samples were stained with 100 μg mL^{-1} concanavalin A-Alexa Fluor 488- conjugate, for 20 min at room temperature (Zippel and Neu 2005), to visualize glucopyranose and mannopyranose in the envelopes of the microorganisms (Bellezza et al. 2005) and with 3 μg mL^{-1} acridine orange, for 5 min (Bellezza et al. 2005) for labeling nucleic acids and polysaccharides. Stained samples were carefully washed three times to remove unbound dyes and observed using a confocal laser scanning microscope (CLSM) Olympus FV1000, IX81 inverse microscope at×60 objective. Excitation was set to 488 and 635 nm, and two channels for detecting chlorophyll *a* (650–750 nm emission range) and neutral monosaccharidic groups labeled by the fluorochromes (500–590 nm emission range) were used. Three-dimensional images were constructed from series of 2-D cross-sectional images (*x*-*y* plane) that were captured at 0.5-μm intervals along the *z*-axis using IMARIS 6.2.0 software (Bitplane AG, Switzerland).

Statistical analyses

Statistical analyses were performed using SPSS, version 13. Kruskal–Wallis nonparametric tests were used to assess for any significant differences in biomass production and the production rates of CPS and RPS among the diverse species. Post hoc pair-wise comparisons were made using Mann–Whitney *U* tests to identify significant differences between species and culture methods.

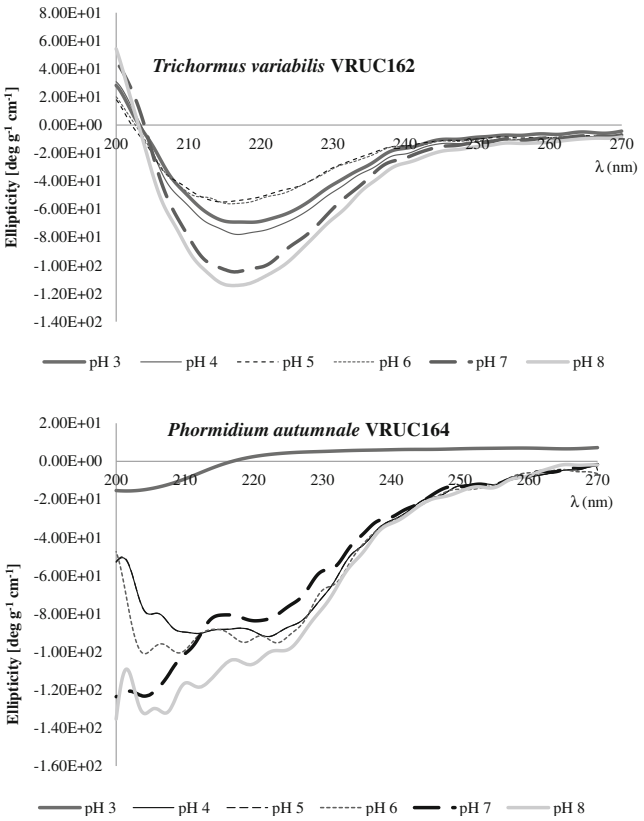

Fig. 3 Dichroic spectra of aqueous solution of capsular exopolysaccharides (CPS) obtained from the two strains **a** *T. variabilis* VRUC162 and **b** *P. autumnale* VRUC164 analyzed at variable pH

Results

Strain identification

Isolates VRUC162, VRUC163, and VRUC164 were previously identified as *T. variabilis*, *A. augstumalis*, and *P. autumnale*, respectively (Bruno et al. 2012). The other four isolates (VRUC 165, VRUC 166, VRUC 167, and VRUC 168) were successfully identified here with partial 16S rRNA gene sequences (≈900 bp). The maximum-parsimony, the maximum-likelihood (not shown), and the distance trees (Fig. 2) had the same topology. VRUC165 clustered with two strains of *Synechocystis* sp. sharing 99.4 % of sequence identity supported by 100 % of bootstrap values. VRUC166, compared in BLAST, was most closely related to three strains of *Calothrix* sp. although they had low sequence identity (94.4 %). The isolate VRUC167 clustered with strains of *Nostoc elgonense*, *Nostoc entophytum*, and *Nostoc* sp. sharing 96.7–97.5 % of sequence identity. VRUC168 formed a cluster with strains of *T. variabilis* with a sequence identity 99.8 % with the isolate VRUC162 and 99.9 % with *T. variabilis* HINDAK 2001/4.

Biomass and exopolysaccharide analysis

Strains grown in batch culture created flocs that were visually of similar dimensions (except for *Synechocystis* sp.) and reached the stationary phase at around 25–34 days (at 25 days for VRUC 165; 32 days for VRUC 162, VRUC 163; and VRUC 164, and 34 days for VRUC 166, VRUC 167, and VRUC 168). The three strains grown in the incubator prototype showed an initial uneven adhesion to the substratum after which the spaces eventually filled in as the biofilm developed forming a homogenous covering of the entire growing surface. Table 2 reports the biomass productivity and the amount of CPS and RPS attained by the strains grown in culture. In batch culture, no significant differences between biomass values were obtained ($P>0.05$). In the incubator, *A. augstumalis* VRUC163 and *P. autumnale* VRUC164 produced the most biomass, 2.98±0.33 and 3.05±0.05 g DW m^{-2} day^{-1}, respectively. The heterocystous strains *T. variabilis* VRUC162 and *A. augstumalis* VRUC163 grown in batch culture produced the most CPS ($P<0.05$), while *Calothrix* sp. VRUC 166 and *Nostoc* sp. 167 produced the most RPS ($P<0.05$). However, when grown as biofilms in the incubator, *T. variabilis* VRUC

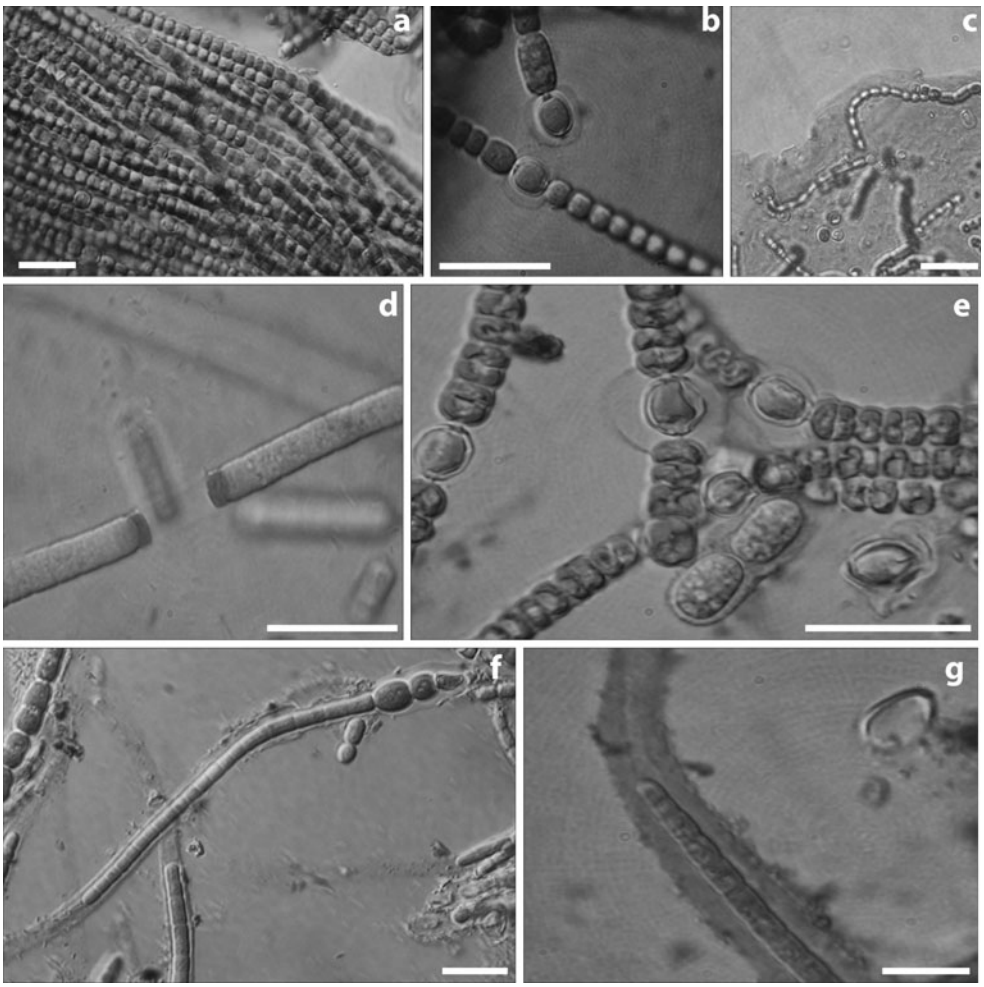

Fig. 4 Light micrographs of cyanobacterial strains after staining with Alcian Blue (AB) dyes. **a**, **b** Reaction of *A. augstumalis* VRUC163 with AB pH 2.5; **c** sulfated polysaccharides (AB pH 0.5) in the RPS produced by *Nostoc* sp. VRUC167; **d** partial reaction to AB pH 0.5 of *P. autumnale* VRUC164; **e** positive reaction to AB pH 0.5 of *T. variabilis* VRUC162 particularly evident around the heterocysts and akinetes; **f**, **g** positive reaction to AB pH 2.5 of *Calothrix* sp. VRUC166. *Scale bars*: 20 μm

162, *A. augstumalis* VRUC163, and *P. autumnale* VRUC164 in general produced significantly much lower CPS than in batch culture ($P<0.01$).

IEC analyses of the CPS polysaccharide identified compositions of between seven (*A. augstmalis* VRUC 163) and 11 (*Calothrix* sp. VRUC 166) different monomers. The RPS were composed of between seven (*A. augstmalis* VRUC 163) and ten (*T. variabilis* VRUC 162, *Nostoc* sp. VRUC 167, strain VRUC 168) different monomers (Table 3). Glucose was the dominant component of both fractions comprising on average around 60 % of the CPS fractions when strains were grown in batch culture, around 50 % in the CPS fractions when strains were grown in the incubator prototype and 36 % in the RPS fractions. At least one uronic acid (galacturonic or glucuronic) was also detected in most samples, except for the RPS and the CPS produced by *T. variabilis* VRUC162 grown in batch culture. The RPS released in batch cultures by *A. augstumalis* VRUC163 showed the highest uronic acid proportion (28.4 %), followed by RPS secreted by *Synechocystis* sp. VRUC165 (16.2 %) and CPS of *A. augstumalis* VRUC163 (15.3 %) when grown in batch culture. Total proportion of uronic acids was on average of around 8 % of the CPS fractions when strains were grown in batch, ca. 5 % in the CPS fractions when strains were grown in the incubator prototype and 14 % in the RPS fractions. Amino sugars, namely *N*-acetyl glucosamine and *N*-acetyl galactosamine, were also detected in most of the analyzed fractions.

CD analyses showed in the CPS and RPS fractions of all strains an ellipticity typical for carboxylic moieties between 200 and 250 nm (Fig. 3). The variation of pH caused changes in the ellipticity, most likely due to protonation–deprotonation processes. No appreciable differences in trend could be seen in the spectra, and no symptoms of spectral perturbations are traceable except that of *P. autumnale* VRUC164 (Fig. 3b). There was absence of ellipticity in the region between 270 and 300 nm (data not shown) typically indicative of the presence of tryptophan and phenylalanine amino acid residues.

Cytochemical characterizations of exopolysaccharides by light and confocal microscopy are shown in Figs. 4 and 5. Staining with AB pH 2.5 revealed the presence of carboxylic groups in the envelopes of *A. augstumalis* and the strain VRUC168, particularly evident around the heterocysts (Fig. 4b, e), and in that of *Synechocystis* sp. No staining was

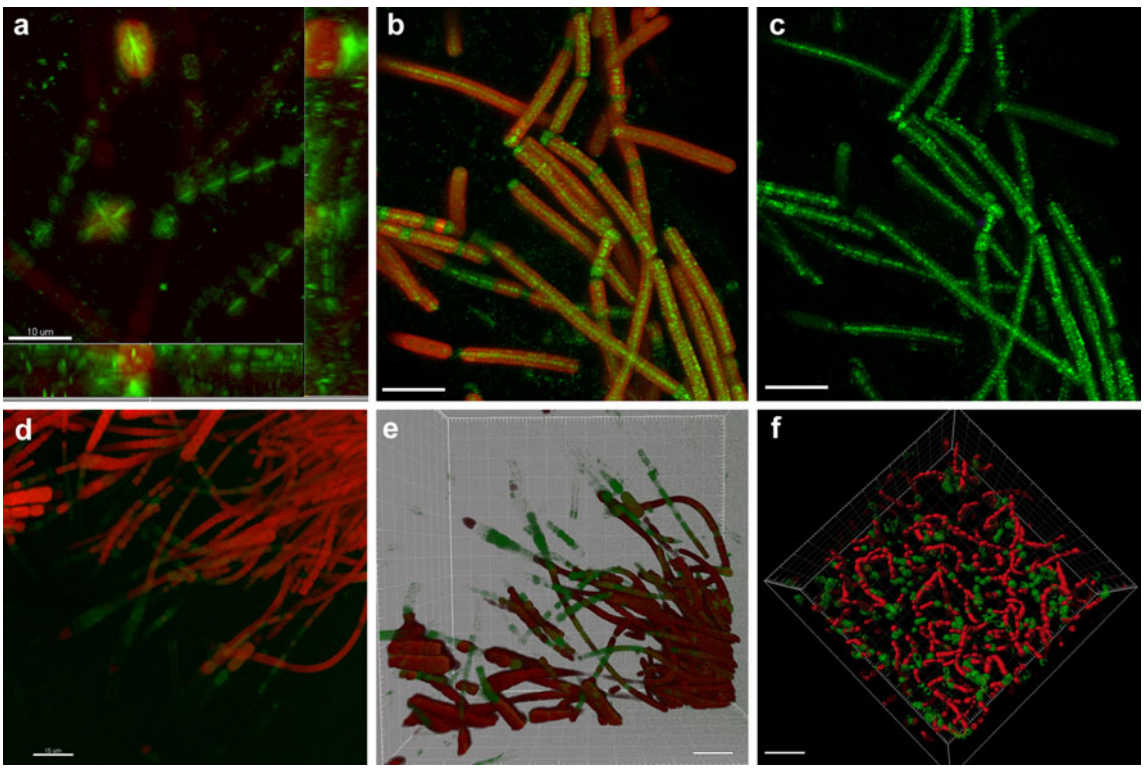

Fig. 5 CLSM images after staining with fluorochromes. **a** Reaction of *A. augstumalis* VRUC163 to concanavalin A-Alexa Fluor 488conjugate showing neutral polysaccaridic material (*green signal*) deposited around the vegetative cells and akinetes, where filaments attached to the substratum; **b**, **c** fine neutral polysaccharidic envelope surrounding the filament of *P. autumnale* VRUC164; **d**, **e** *Calothrix* sp. VRUC166 after staining with concanavalin A-Alexa Fluor 488-conjugate showing the positive reaction of the basal part of the filament and the 3D reconstruction image of the filaments of *Calothrix* sp. and the envelopes around the basal part; **f** 3D reconstruction of the reaction of *Nostoc* sp. with concanavalin A-Alexa Fluor 488-conjugate showing the presence of an envelope around the heterocysts (*green*). The *red signal* was due to the Chl *a* autofluorescence in vegetative cells and akinetes. See text for details. *Scale bars*: 10 μm in **a**–**c** and 15 μm in **d**–**f**

found in *P. autumnale*, *T. variabilis*, and *Nostoc* sp. In *Calothrix* sp. VRUC166, only the terminal part of the sheath stained, whilst the envelope surrounding the basal part did not react. Staining with AB at pH 0.5 indicated the presence of sulfated residues in the exopolysaccharides of the heterocystous cyanobacteria studied, except for that of *Nostoc* sp. VRUC167. No sulfated residues were detected in *Synechocystis* sp., while *P. autumnale* only stained in the apical part of the filament (Fig. 4d). *Nostoc* sp. VRUC167 showed anionic polysaccharides only in its RPS (Fig. 4c).

The CLSM observations using the two fluorochromes showed neutral polysaccharidic envelopes surrounding the cells of all strains (Fig. 5). In *A. augstumalis* VRUC163, there was a nonhomogenous distribution of neutral sugars along the filament and around the akinetes (Fig. 5a). Figure 5a also shows a thickening, where neutral polysaccharides seemed to be accumulated, in the external part of the vegetative cells and akinetes adhering to the substratum. LM observations showed trichomes of *P. autumnale* VRUC164 without clearly defined sheaths, but CLSM observations after the addition of acridine orange and the separation of the two channels, revealed a fine neutral polysaccharidic envelope surrounding the entire trichome (Fig. 5b, c). *Calothrix* sp. showed a clearly defined neutral polysaccharidic envelope surrounding only the basal part of the filament (Fig. 5d, e, green). In *Calothrix* sp. VRUC166, only the vegetative cells in the basal part of the filament reacted with the fluorochrome (Fig. 5d, e, green), with no reaction at the heterocysts, clearly evident in the 3D reconstruction image (Fig. 5e) that also shows that the apical hyaline cells did not contain neutral sugars. LM observations of *Nostoc* sp. VRUC167 did not reveal any envelope surrounding the filament. However, confocal observations using concanavalin A-Alexa Fluor 488 conjugate showed the presence of an envelope surrounding the heterocysts only (Fig. 5f, green signal).

Discussion

The seven strains used in this study produced exopolysaccharide fractions (CPS and RPS) with complex monosaccharide compositions, similar to those synthesized by a variety of previously studied cyanobacteria grown in batch cultures (De Philippis and Vincenzini 1998; Bellezza et al. 2006). The exopolysaccharides had both hydrophobic and hydrophilic properties that render these polymers potentially useful for different biotechnological applications, i.e., as emulsifiers or bioflocculants (Pereira et al. 2009). The deoxy-sugars rhamnose (0.09-65.69 mol %) and fucose (0.17–16.40 mol %) are known to confer hydrophobic properties to polysaccharides (Bellezza et al. 2006), and the uronic acids (up to 28.37 mol %) and sulfated groups, detected by means of LM staining, are known to contribute to the anionic nature and the sticky character of polysaccharides (Decho et al. 2005; Mancuso Nichols et al. 2005). This was particularly evident in the RPS extracted from strains isolated from microphytobenthos growing in sediments of a shallow Italian lagoon (Cabras Lagoon, Sardinia) and in particular for *A. augstumalis* VRUC 163 producing both RPS and CPS fractions with high glucuronic acid content (28.37 and 15.34 mol %, respectively). High uronic content of RPS (16.20 mol %) was also found in *Synechocystis* sp. VRUC 165 which was isolated from a wastewater treatment plant. Since the hydrophilic nature of sugars can promote the formation of ionic bonds with charged molecules such as metal cations, it signifies the potential efficacy of the studied species, and above all *A. augstumalis* VRUC 163 and *Synechocystis* sp. VRUC 165, in wastewater remediation (Pereira et al., 2009; De Philippis and Micheletti 2009; Mancuso Nichols et al. 2005; De Philippis et al. 2011).

The hydrophobic and hydrophilic features of the CPS and RPS have also been implicated in the adhesion of cells to surfaces and to survive desiccation to some degree (Rossi et al. 2012). This being so, *T. variabilis* VRUC162, isolated from eroded and desiccated soils, had an unexpectedly low uronic acid content. However, it was found to be surrounded by an envelope with sulfated residues that can also significantly contribute to the binding of water molecules (Rees 1972). It has to be stressed that these data are still insufficient for envisaging the industrial use of the polysaccharides produced by these isolated cyanobacteria. Indeed, apart from performing other tests on the physicochemical properties of the polymers, it is also necessary to understand the dynamics of polysaccharide productivity of the strains in order to be able to produce the quantity of polysaccharides desired.

Among all the tested strains, *T. variabilis* VRUC 162, *A. augstumalis* VRUC 163, and *Calothrix* sp. VRUC 166 grown in batch culture showed the highest CPS values, comparable with those obtained from other strains of cyanobacteria grown in static culture (Huang et al. 1998; Nicolaus et al. 1999; Bellezza et al. 2006). The exopolysaccharide proportion of the cultured biomass (%) was found to be higher when strains were grown as plankton rather than a biofilm in the incubator.

Given the different nutrient, temperature, and light regimes in the two culture systems, any comparisons are to be taken tenuously. It is highly likely that the differences observed in polysaccharide production are more probably to be due to different stresses enforced by the two culture techniques rather than the different life-forms. In the batch cultures, the medium was not renewed during incubation, and likely led to potential nutrient, and light limitation during community development. In the incubator, a replete nutrient supply was maintained by frequent medium replacement and advective transport to the biofilm by the flowing medium. Any hindrance of molecular diffusion into the biofilm caused by the compact biofilm morphology is likely offset by diffusion hindrance of nutrients

out of the biofilm combined with rapid recycling of autochthonous organics within the biofilm by the accumulated hydrolytic enzyme suite in the EPS (Wetzel 2001; Battin et al. 2003; Ellwood et al. 2012).

Since the temperature and light environment was more intense in the incubator system compared to batch culture, an extraction of more EPS was expected from biomass grown in the incubator. It is known that higher temperature and light intensities favor the increase of EPS production (Di Pippo et al. 2012). This has been previously reported on EPS production by diatoms (Staats et al. 2000; Wolfstein and Stal 2002) and cyanobacteria (Vincenzini et al. 1990; Moreno et al. 1998; Otero and Vincenzini 2003) demonstrating a clear and direct relationship between the production of EPS and oxygenic photosynthesis; it would therefore be quite reasonable to expect increasing EPS production with an increase in temperature and light. It is possible that the biofilm thickness and its multi-stratified structure caused strong attenuation of light in the upper biofilm layers. But since the transmitted light (light passing through the full biofilm thickness) was at minimum 10 % of the incident light, it is likely that light limitation was negligible given that cyanobacteria are generally adapted to low light environments (Kühl et al. 1996). Low medium nutrient concentration is also known to induce an increase in exopolysaccharide production (Pereira et al. 2009). Therefore, the results showing that batch cultures produced more exopolysaccharide per unit biomass were to be expected. Based on the current experimental setup, higher biomass production in the incubator system would be expected, but the differences between the systems do not allow comparisons to be made. That said, all the three strains grown in the incubator produced biomass with areal productivity values within the ranges obtained for other marine and freshwater microalgae grown in large-scale systems such as open ponds (Mata et al. 2010). The fact that stress results in enhanced exopolysaccharide production but also reduced biomass yield, means there is an industrial dilemma for the growth of cyanobacteria for the production of polysaccharides. Solutions to this dilemma can be derived from both engineering design and knowledge of the biology of the organism involved. To obtain enough biomass, it would be simple to scale up the operation in terms of area or volume to arrive at the required biomass/polysaccharide production. However, if scaling up is not economically or spatially feasible, it might be astute to have a two-phase process, where the first phase involves growth in optimized growth conditions followed by a second phase where a stress is induced to augment polysaccharide production.

Another important biotechnological aspect related to the use of these polymers concerns the possibility to obtain exopolysaccharides with a stable conformation at varied conditions. CD results here showed that polysaccharide conformation of all but one strain were stable; the small differences observed in the intensity were most likely due to the sum of the contributions from the protonated and unprotonated carboxyl chromophores (Cesaro et al. 1990). This stability derives from a random arrangement of polysaccharides in solution, whose chains have a disordered sequence of saccharide units (Cesaro et al. 1990). *P. autumnale* VRUC 164 was a good EPS producer, but the polysaccharide fraction was shown to be unstable, and therefore of little use in most applications. Both CD and IEC confirmed the presence of uronic carboxylic groups, embedded in the chiral saccharide moieties that have also been shown in other natural and cultured phototrophic biofilms (Bellezza et al. 2005; Congestri et al. 2006; Di Pippo et al. 2009). These carboxylic groups and the presence of sulfated groups found in most CPS fractions of the isolates studied here makes them potentially useful in pharmacological applications (Schaeler and Krylov 2000; Zheng et al. 2006). The location of anionic and neutral residues in the CPS and in the solubilized RPS found in the culture medium was observed in samples stained with Alcian Blue dyes and with fluorochromes. The diverse distribution of neutral and charged exopolysaccharides along the trichomes of most of the strains becomes important information for the biotechnological application of these cyanobacteria strains. Sulfated and carboxylic polysaccharides surrounded the entire filaments of most of the strains, with an intensification at the heterocysts.

Neutral exopolysaccharides were also found to be specifically deposited (i.e., *Calothrix* sp.) within the envelope around the vegetative cells and akinetes, especially where filaments attached to the substratum. Diverse compositions of polysaccharides surrounding vegetative cells and heterocysts likely reflects the different roles of polymers at these positions (Helm & Potts 2012; Wolk et al. 1994). It is known that the neutral fraction, in particular, if reach in deoxy-sugars, is more suitable for the adhesion to solid substrates (Rossi et al. 2012) while the negatively charged fractions are known to bind metal cations (Pereira et al. 2009). The realization of potential biotechnological uses for cyanobacterial EPS is increasing; however, with these advancements, new biological and engineering questions arise that indicate a need for further research to be able to refine viable production processes.

Conclusions and future perspectives

- Most of the species analyzed here showed interesting exopolysaccharide characteristics that could be exploited for biotechnological application(s). A particularly interesting application is the heavy metal removal by the strain *A. augstumalis* VRUC 163 grown in the incubator system. High biomass production was coupled with exopolysaccharides of stable conformation and high

proportion of carboxylic residues, principally located in the envelope surrounding the heterocysts. It would be interesting to grow this strain in a biofilm-based culture system under optimized conditions to obtain biomass and then to induce nutrient stress to determine if there is an increase in carboxylated polysaccharide production.

- Although direct comparisons of biomass and EPS productivity in the two culture systems used here cannot be accurately evaluated, the use of biofilm reactors could represent a more economically viable option for biomass (and therefore polysaccharide) production at a pilot and subsequently at an industrial scale, considering the high costs associated with the harvesting of microplankton (Zhang et al. 2010).
- Biomass leftover from EPS production can also represent a valuable commodity that could be used for the extraction of other valuable by-products and the production of biofuels (Quintana et al. 2011).

Acknowledgments The authors acknowledge Dr. Elena Romano from the Centre of Advanced Microscopy (CAM), Department of Biology, University of Rome "Tor Vergata," for her assistance in the use of the facility, Roberto Targa for his assistance in the composition of figures, and the anonymous reviewers for their constructive comments. This research was partly supported by CNR-IAMC, National Research Council, Institute for Coastal Marine Environment UO Oristano, Italy.

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