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Biofilm formation behaviour of marine filamentous cyanobacterial strains in controlled hydrodynamic conditions

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Summary

Marine biofouling has severe economic impacts and cyanobacteria play a significant role as early surface colonizers. Despite this fact, cyanobacterial biofilm formation studies in controlled hydrodynamic conditions are scarce. In this work, computational fluid dynamics was used to determine the shear rate field on coupons that were placed inside the wells of agitated 12-well microtiter plates. Biofilm formation by three different cyanobacterial strains was assessed at two different shear rates (4 and 40 s⁻¹) which can be found in natural ecosystems and using different surfaces (glass and perspex). Biofilm formation was higher under low shear conditions, and differences obtained between surfaces were not always statistically significant. The hydrodynamic effect was more noticeable during the biofilm maturation phase rather than during initial cell adhesion and optical coherence tomography showed that different shear rates can affect biofilm architecture. This study is particularly relevant given the cosmopolitan distribution of these cyanobacterial strains and the biofouling potential of these organisms.

Introduction

Biofouling is an ongoing concern in aquatic environments and marine applications, leading to material deterioration, surface corrosion, decrease of hydrodynamic performance and significant economic losses (Salta et al., 2013). Moreover, biofilms are recognized as a permanent or temporary refuge for bacterial pathogens in aquaculture facilities (King et al., 2006). Additional problems related to biofouling on marine environments are present in marine underwater support structures, sensors and housings, which are used for on-site monitoring with weekly, monthly or continuous measuring. On these devices, the most obvious problems are related to optical and electrochemical sensors, because even a thin biofilm on the optics can give rise to incorrect measurements (Delauney et al., 2010). Although marine biofouling is a dynamic process encompassing different agents and their interactions, it has been considered that biofilm formation by bacteria and algae is a primordial step that occurs prior to the adhesion of macrofouler organisms such as invertebrate larvae, mussels, seaweeds and barnacles (Mieszkin et al., 2013). Cyanobacteria are a widespread group of photosynthetic prokaryotes which, together with diatoms, constitute the major components of marine biofilms (Salta et al., 2013; Bharti et al., 2017; Di Pippo and Congestri, 2017). Moreover, cyanobacteria produce large amounts of extracellular polymeric substances (EPS), which increase biofilm cohesion (Rossi and De Philippis, 2015). This is particularly important because it has been shown that the main challenge in marine biofouling control is associated with microorganisms which are also responsible for the initiation of biofilm formation and those which are also able to excrete large amounts of EPS (Mieszkin et al., 2013; Salta et al., 2013; Telegdi et al., 2016). Thus, a promising approach to delay macrofouling is to prevent the adhesion and biofilm formation by cyanobacteria.

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In this work, two strains from the Nodosilinea genus, collected from the same site in the Portuguese coast, and a Synechococcaceae strain from a different location were studied. To the best of our knowledge, no studies about the biofilm formation behaviour of these commonly occurring cyanobacterial strains have been published so far.

Amongst the parameters that affect biofilm development, physicochemical factors related with the surface (Crawford et al., 2012) and flow velocity and shear rate (Allen et al., 2018) are some of the most important. There is evidence that shear forces affect not only biofilm structure but also composition, mass transfer, exopolysaccharides production, energy metabolism and can also induce genetic or molecular changes in biofilms (Moreira et al., 2015a). It has been reported that a slow flow of water (0.2 m s⁻¹) promotes the growth of filamentous green algae and cyanobacteria (Minchin and Gollasch, 2003; Flemming et al., 2009) and that biofouling can be favoured at low fluid velocities. These lower flow velocity values can be found in harbours and when underwater devices and sensors are moored or floating in their vicinity (Minchin and Gollasch, 2003). Therefore, it is important to perform biofilm formation studies in controlled hydrodynamic conditions where the shear rate is well defined. Although the effects of hydrodynamic conditions on the architecture of some bacterial biofilms have been described (Teodósio et al., 2011), there is a comparatively low information regarding the effects of hydrodynamics in filamentous cyanobacterial biofilms. Moreover, some filamentous cyanobacteria species produce cyanotoxins, which pose a particular risk to aquatic organisms and to human health (Frazão et al., 2010; Regueiras et al., 2018). Poisoning and additional chronic effects might be caused by the ingestion of contaminated food or even by drinking water during recreational activities (Martins et al., 2005; Buratti et al., 2017).

In order to analyse and predict flow conditions in an easy to handle biofilm reactor assay like a 12-well microtiter plate, flow simulation and modelling are highly useful. Computational fluid dynamics (CFD) is a numerical simulation technique that involves the solution of fluid flow and/or heat/mass transfer problems and provides a detailed understanding of flow phenomena that are difficult to analyse experimentally. Although CFD applications in biotechnological and biological processes are common (Dean et al., 2016), this technique is not commonly used to predict the shear rate occurring inside reactors that are used in marine biofilm research. A detailed hydrodynamic analysis of these biofilm reactors is useful, and CFD is a powerful tool used to model flow situations to assess if the hydrodynamic conditions in vitro are similar to those prevailing in some marine systems in situ. In order to define the operational conditions that can be used in vitro to simulate the hydrodynamics prevailing in natural aquatic environments, the first goal of this research was to predict the shear rate occurring in a marine biofilm reactor (agitated 12-well microtiter plates). The second goal was to evaluate the effects of different shear rates and surface properties on the biofilm formation behaviour of different marine cyanobacteria. The chosen surfaces, glass and perspex, represent commonly submerged artificial surfaces found on different equipment in aquatic and marine environments such as underwater windows of boats, aquaculture equipment, flotation spheres, moored buoys, underwater cameras, measuring devices or sensors (Roy, 1983; Taylor, 1996; Blain et al., 2004; King et al., 2006).

**Results and discussion**

The first goal of this study was the determination of the shear rate field in the bottom of an agitated 12-well microtiter plate and to assess its similarity to published shear rate values occurring in some marine systems. Secondly, the effects of shear rate and surface properties on the biofilm formation behaviour of different cyanobacterial strains were also evaluated.

Flow simulation on the 12-well microtiter plates was performed by CFD. Figure 1 shows the free liquid surface at a given instant upon agitation of the microtiter plate at two shaking frequencies. The liquid surface is not flat in either condition, and the surface inclination is higher at the highest shaking frequency. The velocity profiles were also determined for both shaking frequencies (Supporting Information, S1—Velocity profiles). With a higher fluid velocity (obtained at the higher shaking frequency), a decreased liquid depth (associated to the surface inclination) was obtained. As previously suggested, the increased fluid velocity may facilitate mass transfer of
nutrients from the bulk liquid to the biofilm and also access to oxygen and light may be promoted in this condition (Gomes et al., 2014).

The shear rate distribution on the surface at different degrees of a full rotation is shown in Fig. 2, where it can be seen that higher shear rate values were obtained at the highest shaking frequency. In a full rotation (360°) of the orbital platform, the instant maximum and minimum wall shear rate values at any given point on the coupon surface are compensated over time. Therefore, the representative wall shear rate was obtained by averaging the results throughout a complete rotation cycle over the coupon surface.

Figure 3 shows the shear strain rate variation over time. An average shear rate value of 40 s$^{-1}$ was obtained for 185 rpm, reaching a maximum value of 120 s$^{-1}$, whereas an average value of 4 s$^{-1}$ was calculated for 40 rpm, reaching a maximum value of 11 s$^{-1}$. At the shaking frequency of 185 rpm, the range of shear rates that can be reached includes different values that can be found in marine environments. Although a shear rate of 50 s$^{-1}$ has been reported for a ship in a harbour (Bakker et al., 2003), equipment and devices which are in similar environments (partially submerged or even moored equipment such as underwater cameras, flotation spheres and sensors) and even transparent hulls of boats or their windows are likely to be subjected to similar shear values. Moreover, this marine reactor also allows the biofilm development at lower shear rate conditions (at 4 s$^{-1}$), which is important because lower fluid velocities promote marine biofouling (Minchin and Gollasch, 2003; Flemming et al., 2009).

Although different experimental setups have been used to study the effect of hydrodynamics particularly at later stages of marine biofilm development, including larval settlement (Qian et al., 1999, 2000), the use of agitated microtiter plates in the laboratory can be very convenient because it is an affordable and inexpensive platform. Microtiter plates present several advantages such as low volume requirements (reduced operational costs) are easy to handle and enable the control of different parameters like temperature and shear rate. Also, in the real scenario of a ship in a harbour, the shear rate field will oscillate due to tidal currents and because of the wakes.

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caused by passing ships. Since the agitated microtiter plate induces a wave-like shear, it generates a flow topology that is more similar to marine systems than most flow systems.

Although CFD studies have already been performed on our group for 96-well plates (Gomes et al., 2014), the present study focuses on 12-well microtiter plates, which enable a higher shear rate range (as can be found in marine environments) and the use of coupons to test different surfaces. A previous study using micro- and macro-flow systems to test Escherichia coli adhesion (Moreira et al., 2015b) has shown that when the shear forces are kept constant between these platforms, the obtained results were similar despite the large differences in size. Edge effects were also not shown to be important given the relative dimensions of the system and of the bacteria. Since in the present study, the shear rate range achieved (Fig. 3) comprises the values found on real aquatic environments (50 s⁻¹; Bakker et al., 2003), 12-well microtiter plates may be used as a marine biofilm reactor to mimic these environments.

Surface hydrophobicity was assessed by contact angle measurement with water (θw). Contact angle determination provides information about the interaction of the fluid with a solid material through the determination of the wettability and hydrophobicity of the surface. While wettability refers to the tendency of fluid spreading on a surface, hydrophobicity describes the tendency of non-polar molecular aggregation and consequently water molecule repulsion (Yuan and Lee, 2013). By definition, surfaces can be classified into hydrophilic or hydrophobic if the contact angle of water with the surfaces is lower or higher than 90° respectively (Sedlmeier et al., 2008). Thus, low contact angle values indicate liquid spreading along the surface, which is associated with high wettability and hydrophilic properties. In turn, low wettability is associated with hydrophobicity. Since the contact angle of water on glass was θw = 48.55 ± 2.41 and in perspex θw = 73.05 ± 3.42, perspex is relatively hydrophobic compared to glass, exhibiting a lower wettability. Although lower values for glass have been reported, some studies performed with glass surfaces, also showed values in the 40° range (Sener et al., 2013; Thukkaram et al., 2014; Gomes et al., 2015; Moreira et al., 2015a). This slightly increased value may be related to the composition of this specific glass although other glass surfaces that can be used to construct submerged devices (underwater cameras, measuring devices or sensors) can have different hydrophobicities.

Cyanobacterial biofilm formation was monitored for 49 days, and the evolution of wet weight and chlorophyll a content is shown in Figs 4–6. For all cyanobacterial strains, biofilm mass was in general higher at the lowest shear rate on both surfaces although statistically significant differences were mostly found on glass. Biofilm mass obtained on glass under low agitation was on average 22.4% higher for Nodosilinea sp. LEGE 06020 (Fig. 4A), 58% higher for the unidentified filamentous Synechococcales LEGE 07185 (Fig. 5A) and 32% higher for Nodosilinea sp. LEGE 06022 (Fig. 6A) when compared to those obtained at higher shear conditions. Likewise, chlorophyll a was 29.5%, 93.9% and 62.7% higher in these conditions for each of the strains respectively (Fig. 4B, 5B, 6B). On perspex, the biofilm wet weight was on average 17.6%, 37.2%, 26.9% higher at 4 s⁻¹ (Fig. 4C, 5C, 6C), whereas chlorophyll a was 31.5%, 91.9%, 70.8% higher at this shear rate (Fig. 4D, 5D, 6D).

Analysis of the biofilm wet weight and chlorophyll a suggests that, despite the tenfold difference on average shear rate, biofilm development was similar in both hydrodynamic conditions for the first two weeks. This suggests that shear rate had a lower impact on initial cell adhesion and a more noticeable effect during biofilm maturation, which in turn may be the result of two opposing effects caused by higher flow velocities (Supporting Information, S1 – Velocity profiles). Although nutrient mass transfer from the bulk liquid and oxygen transfer are facilitated at higher flow velocities, which may promote the biofilm development, the increased shear rate may hamper cell adhesion and may be more challenging for biofilm cohesion (erosion and sloughing events may be promoted). It is likely that the relative importance of these effects varies along the biofilm development stages. Since the effect of fluid velocity was more noticeable during biofilm maturation rather than during cell adhesion, it seems that the increased shear stress was not a significant obstacle to attachment whereas biofilm erosion may be the cause for a lower biofilm accumulation at the higher shear rate (Duddu et al., 2009). It has been suggested that monitoring the chlorophyll a production can be a good way to follow cyanobacterial growth (Lawton et al., 1999; Chorus and Cavalieri, 2000). A correlation between chlorophyll a production and biofilm wet weight was observed in some cases (Supporting Information, S2 – Correlation between wet weight and chlorophyll a). This correlation was more noticeable at the lowest shear rate (4 s⁻¹) for all cyanobacterial strains (Supporting Information, S2 – Correlation between wet weight and chlorophyll a; Fig. S2.1 A,C; S2.2 A,C; S2.3 A,C).

In this study, the effect of surface hydrophobicity was probably less important than the hydrodynamic effect, because similar values of wet weight and chlorophyll a were obtained for biofilms developed under different surfaces at the same hydrodynamic conditions (Supporting Information, S3 – Evaluation of cyanobacterial biofilm development under the same hydrodynamic condition on different surfaces; Fig. S3.1; Fig. 3.2). The fact that the
hydrodynamic conditions may be more important than surface properties during initial biofilm development has already been shown during *E. coli* adhesion to glass and PDMS (Moreira *et al.*, 2014) where the effects of the surface properties were modulated by the shear forces. However, biofilm development of *Nodosilinea* sp. LEGE 06022 has also shown significant differences between glass and perspex surfaces (Supporting Information, S3 – Evaluation of cyanobacterial biofilm development under the same hydrodynamic condition on different surfaces; Fig. S3.3), with greater biofilm development on perspex, the most hydrophobic surface.

Since most commonly applied imaging methods to analyse biofilms are ex situ and destructive (Azeredo *et al.*, 2017), optical coherence tomography (OCT) is an interesting technique enabling in situ, non-invasive and real-time imaging without affecting biofilm structure (Wagner and Horn, 2017). Moreover, as biofilm thickness has a strong effect on the performance of underwater devices (Delauney *et al.*, 2010), the knowledge of this biofouling parameter is essential for the design and maintenance of marine equipment. Therefore, biofilm thickness, roughness and structure analyses were performed by OCT from day 28, because the shear rate effect became more pronounced from day 21 (Figs 4–6). Figure 7 shows the biofilm thickness and roughness coefficient evolution and Figs 8–10 shows representative images obtained at the end of the experiment.

The biofilm thickness values obtained for the highest shear rate (40 s\(^{-1}\)) were lower than for the lowest shear rate (4 s\(^{-1}\); Fig. 7A–C). This was more noticeable for biofilm development of the unidentified filamentous Synchococcales LEGE 07185 (Fig. 7B) and *Nodosilinea* sp. LEGE 06022 (Fig. 7C) strains. For all cases except for *Nodosilinea* sp. LEGE 06020 at 4 s\(^{-1}\) and *Nodosilinea* sp. LEGE 06022 at 40 s\(^{-1}\), (in which similar values were achieved, Fig. 7A and C respectively), biofilm thickness increased from day 28 to the end of the experiment. The highest values of biofilm thickness were observed for *Nodosilinea* sp. LEGE 06020 at 4 s\(^{-1}\) on glass (around 446 \(\mu\)m; Fig. 7A, 49 days) and the lowest for *Nodosilinea* sp. LEGE 06022 at 40 s\(^{-1}\) on glass (around 16 \(\mu\)m; Fig. 7C, 42 days). Differences on biofilm thickness between the surfaces were more evident on *Nodosilinea* sp. LEGE 06022 (Fig. 7C), with higher biofilm development on perspex, as previously reported for wet weight and chlorophyll \(a\) quantification (Supporting Information, S3 – Evaluation of cyanobacterial biofilm...

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**Fig. 4.** Evaluation of *Nodosilinea* sp. LEGE 06020 biofilm development. The parameters analysed refer to wet weight (A, C), chlorophyll \(a\) (B, D). Biofilms were formed in glass (A, B) or perspex (C, D), at two average shear rates, 4 s\(^{-1}\) (closed circles) and 40 s\(^{-1}\) (open circles) during 49 days. Standard deviations from three replicates are represented. Symbols * and ** indicate statistically different values for \(P < 0.1\) and \(P < 0.05\) (respectively) at each incubation time.
development under the same hydrodynamic condition on different surfaces; Fig. S3.3). Large variations on biofilm roughness under different shear rate were only observed for the unidentified filamentous Synechococcales LEGE 07185 (Fig. 7E), in which lower values of the roughness coefficient were obtained for the lowest shear rate. In general, no significant differences between glass and perspex were observed for biofilm roughness coefficient ($P = 0.20$ for Nodosilinea sp. LEGE 06020, Fig. 7D; $P = 0.66$ for the unidentified filamentous Synechococcales LEGE 07185, Fig. 7E and $P = 0.07$ for Nodosilinea sp. LEGE 06022; Fig. 7F). The highest (about 1.14) and the lowest (0.14) values of biofilm roughness coefficient were obtained for the unidentified filamentous Synechococcales LEGE 07185 at $40 \text{ s}^{-1}$ on perspex (Fig. 7E, 49 days) and Nodosilinea sp. LEGE 06020 at $4 \text{ s}^{-1}$ on perspex (Fig. 7D, 35 days) respectively. Biofilm of Nodosilinea sp. LEGE 06020 obtained at a higher shear rate (Figs 8B,D) seemed more compact than those obtained at low shear (Fig 8A,C). This effect was not observed for the unidentified filamentous Synechococcales LEGE 07185 and Nodosilinea sp. LEGE 06022 possibly because a lower biofilm biomass was obtained at a higher shear rate ($40 \text{ s}^{-1}$) for both strains.

A compact architecture probably increases biofilm cohesion and may therefore be an adaptation to the environmental shear forces (Teodósio et al., 2011; Graba et al., 2013). The formation of a less compact structure at lower shear may enhance mass transfer of nutrients to the inner layers of the biofilm (Teodósio et al., 2011).

For most cases, except for biofilms obtained at $40 \text{ s}^{-1}$ on glass (Figs 8, 9 and 10B,F) and for Nodosilinea sp. LEGE 06020 on perspex at $4 \text{ s}^{-1}$ (Fig. 8C,G), the presence of three-dimensional streamers on the biofilm surface could be observed (Figs 8A,E,D,H; 9A,E,C,G,D,H; 10A,E,C,G,D,H). However, these structures were more noticeable for Nodosilinea sp. LEGE 06022 on perspex at lower shear rate ($4 \text{ s}^{-1}$; Fig. 10C,G). The formation of these streamers may contribute to the formation of ‘sieve-like’ networks that can capture cells and other biomass (Drescher et al., 2013), therefore, contributing to the biofilm growth.

Concluding remarks

The CFD analysis in this work shows that the agitated 12-well microtiter plates can be used to attain shear rate values that can mimic aquatic environments. It was
demonstrated that OCT analysis can be a valuable tool to analyse the architecture of marine biofilms and to further understand the biofilm formation dynamics. It was shown that hydrodynamics play a crucial role in the development of these biofilms and that these effects are likely to be more important during biofilm maturation rather than during the initial adhesion phase. Cyanobacterial biofilm formation studies performed in controlled hydrodynamic conditions are scarce, but the importance of studying filamentous cyanobacterial strains with such a cosmopolitan distribution is even more relevant given the biofouling potential of these organisms in different ecosystems around the globe.

Experimental procedures

CFD analysis

For the flow simulation inside a 12-well microtiter plate, a cylindrical well (diameter of 22 mm and height of 18 mm) containing liquid (3 ml of water) and gas (air, filling the remaining volume), at 25°C, was simulated using Ansys Fluent CFD package (version 14.5). The domain was built in Design Modeller 14.5 and discretized into a grid of 40,441 hexahedral cells by Meshing 14.5. The two-phase flow in the vessel was simulated by the VOF methodology (Hirt and Nichols, 1981). The air/water interface was determined by the Geo-Reconstruct method (Youngs, 1982). The PISO algorithm was used to solve the velocity–pressure coupled equations, the QUICK scheme for the discretization of the momentum equations and the PRESTO! scheme for pressure discretization. The surface tension effects were modelled by the continuum surface force (Brackbill et al., 1992), which was introduced through a source term in the momentum equation. An accelerating reference frame was adopted, and the effect of the circular orbital motion on the fluid was modelled by a source term. The no-slip boundary condition and a contact angle of 83° were considered for all the walls (Gomes et al., 2014).

Simulations were made for rotation frequencies of 40 and 185 rpm and an orbital diameter of 25 mm. For each case, 8 s of physical time were simulated with a fixed time step of $2.5 \times 10^{-4}$ s. The primary numerical results obtained, for each time step, were the velocity components, the pressure and the liquid or gas phase volume fractions. For each simulation, after the stabilization of the flow, the average instantaneous shear rate was obtained by integrating an instantaneous solution over the bottom surface of the well (corresponding to the
coupon surface). The time-averaged shear rate was calculated by averaging the instantaneous shear rate throughout a complete rotation cycle.

**Organisms and inocula preparation**

In order to evaluate the behaviour of different filamentous cyanobacteria from different marine environments, three cyanobacterial strains were used in this study. All strains were obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) located at CIIMAR, Portugal (Ramos et al., 2018). *Nodosilinea* sp. LEGE 06020 and *Nodosilinea* sp. LEGE 06022 (order Synechococcales) were isolated from a seawater sample collected in the surf zone and from a sample scraped from a wave-sheltered rock in the intertidal zone respectively (Ramos et al., 2018). Both strains were originally collected at Coxos beach, Portugal (39°00′16.4″N 9°25′33.0″W). The unidentified filamentous Synechococcales LEGE 07185 was isolated from a submerged stone collected in a tide pool, at Lavadores beach, Portugal (41°07′45.1″N 8°40′06.9″W; Ramos et al., 2018). Cells were grown in 750 ml culture in Z8 medium (Kotai, 1972) supplemented with 25 g L⁻¹ of synthetic sea salts (Tropic Marin) and B₁₂ vitamin. Cultures were performed under 14 h light (10–30 μmol photons m⁻² s⁻¹)/10 h dark cycles, at 25°C. Cyanobacterial strains from

**Fig. 7.** Evaluation of cyanobacterial biofilm thickness (left; A–C) and roughness coefficient (right; D–F) in both surfaces and different shear rates (A, D – *Nodosilinea* sp. LEGE 06020; B, E – unidentified filamentous Synechococcales LEGE 07185; C, F – *Nodosilinea* sp. LEGE 06022). Mean values ± SD from three replicates for each sampling day are represented. For each condition, different lowercase letters indicate significant differences between incubation times (28 days - very light grey bars; 35 days - light grey bars; 42 days – dark grey bars; 49 days – black bars; P ≤ 0.05; unpaired t-test).
unidentified filamentous Synechococcales and *Nodosilinea* genus are present in dissimilar geographies, including different continents (Europe, Africa, America and Antarctica). Specimens can be found in different environments and ecologies such as brackish water, freshwater, hypersaline, marine and even terrestrial (Ramos et al., 2018). This cosmopolitan distribution highlights the importance of studying biofilm formation by these organisms.

**Surface analysis**

In order to assess the biofilm behaviour of the cyanobacterial strains, two artificial surfaces commonly placed in aquatic environments, glass and perspex, were tested. Surface analysis was performed by measuring the contact angles with water ($\theta_w$) as reference liquid. This determination was performed in three independent experiments at 25°C ± 2°C, by the sessile drop method using a contact angle meter (Dataphysics OCA 15 Plus, Filderstadt, Germany), as described in Gomes et al., 2015. On each experiment, at least 25 determinations for each material were made.

**Biofilm formation**

Glass (Vidraria Lousada, Lda, Portugal) and perspex (Neves & Neves, Lda, Portugal) coupons (1 cm²) were immersed in a solution of 2% (vol/vol) TEGO 2000®...
industrial detergent (Johnson Diversey, Northampton, United Kingdom; Meireles et al., 2017) and sterile distilled water for 20 min under agitation (150 rpm). In order to remove any remaining disinfectant, coupons were rinsed in sterile distilled water and air-dried. Additionally, glass coupons were autoclaved (121°C, 15 min; Azevedo et al., 2006). After drying, all coupons were aseptically pre-weighted. Biofilm formation was assessed on 12-well microtiter plates (VWR International, Carnaxide, Portugal) under optimized conditions for cyanobacterial growth. Transparent double-sided adhesive tape was used to fix the coupons, and once the tape was placed in the wells, all coupons and the plates were subjected to UV sterilization for 30 min after which the sterile coupons were fixed. A volume of 3 ml of each cyanobacterial suspension in Z8 medium (prepared as described above) supplemented with nutritional factors, which support the growth and cyanobacteria maintenance (Kotai, 1972), was inoculated in each well. For control, Z8 medium without cyanobacterial cells was introduced on the wells. Observations demonstrated that the applied methodology was efficient for surface sterilization because no bacterial growth was observed. Microtiter plates were incubated at 25°C in an orbital shaker with a 25 mm orbital radius (Agitorb 200ICP, Norconcessus, Portugal) at 40 and 185 rpm. Biofilm development of the three cyanobacterial strains was followed for 7 weeks (49 days), because it is

Fig. 9. Representative images obtained by OCT of unidentified filamentous Synechococcales LEGE 07185 biofilms formed after 49 days on glass and perspex. A–D. 2D cross-sectional OCT images (scale bar = 200 μm). E–H. 3D OCT images, top view projection (the colour scale shows the range of biofilm thickness). Biofilms were formed at two different shear rates, 4 s⁻¹ (A, C, E, G) and 40 s⁻¹ (B, D, F, H). [Color figure can be viewed at wileyonlinelibrary.com]
accepted that a two-month interval for maintenance is the minimum duration for economically viable underwater monitoring systems (Blain et al., 2004). During this incubation time, the medium was replaced twice a week. In order to mimic real light exposure periods, a photoperiod of 14 h light (8–10 μmol photons m$^{-2}$ s$^{-1}$/10 h dark cycles) was applied.

**Biofilm analysis**

*Optical coherence tomography.* Biofilm development was primarily assessed by determination of biofilm structure, thickness and roughness through OCT (Thorlabs Ganymede Spectral Domain Optical Coherence Tomography system with a central wavelength of 930 nm, Thorlabs GmbH, Dachau, Germany). Sampling was performed every 7 days, and at each sampling day, 3 coupons for each surface and hydrodynamic condition for all strains were analysed. Culture medium was carefully removed, and the wells were filled with 3 ml of sterile sodium chloride solution (8.5 g L$^{-1}$). The solution was carefully removed to eliminate loosely attached cyanobacteria. Subsequently, the wells were filled again with 3 ml of sterile sodium chloride solution and imaged. The captured volume was ~ 4 × 4 × 3 mm$^3$ (509 × 313 × 1024 pixels$^3$). Since biofilms are mainly composed
(90%) of water (Bakke et al., 2001), the refractive index was set to 1.40, close to the refractive index of water (1.33). 2D and 3D imaging were performed, and for each coupon, a minimum of three fields of view were analysed to ensure accuracy and reliability of the results obtained. Image analysis was performed as previously described (Gusnaniar et al., 2017). First, the bottom of the biofilm was determined as the best fitting parabole (or hyperboloid when imaged in 3D) that connects the white pixels resulting from light reflection on the substratum surface. Subsequently, a grey-value threshold that separates the biofilm from the background was calculated on the basis of the grey-value histogram of the entire image (Otsu, 1979). The upper contour line of the biofilm was defined as those pixels in the image that have a grey value just higher than the grey-value threshold and are connected to the bottom of the biofilm by pixels with grey values higher than the grey-value threshold. The image was then converted to a binary black and white image, and objects not connected to the bottom were rejected from the biofilm structure. Finally, the mean biofilm thickness was calculated based on the number of pixels between the bottom of the biofilm and the upper contour line for each vertical line in the image. Biofilm roughness was analysed by determination of the roughness coefficient, because this parameter allows comparing the structure of different biofilms, across different studies as well as across scales, once this value is normalized to mean biofilm thickness. The relative roughness coefficient $R^*_{\text{fi}}$ was calculated using the following Eq. 1, according Wagner and Horn, 2017:

$$R^*_{\text{fi}} = \frac{1}{N} \sum_{i=1}^{N} \frac{|L_{F, i} - \overline{L}_{F}|}{\overline{L}_{F}},$$

where $L_{F, i}$ is a local biofilm thickness measurements at location $i$ and $N$ equals the number of thickness measurements and $\overline{L}_{F}$ is the mean biofilm thickness.

**Wet weight determination and chlorophyll a quantification.** In order to complement the characterization of cyanobacterial biofilms, the determination of their wet weight and chlorophyll a content was performed. To determine the wet weight, coupons were detached from the wells with the aid of sterile tweezers and weighted. Biofilm wet weight was obtained by the difference to the initial coupon weight (determined prior to inoculation).

Chlorophyll a is the predominant and the unique pigment present in all groups of alga and cyanobacteria, and it is commonly used as biomass indicator in aquatic environments. Cyanobacterial cells were detached from the coupons by immersing each coupon in 2 ml of 8.5 g L$^{-1}$ sodium chloride solution and vortexing (VV3, VWR) for 2 min at maximum rotational speed (2500 rpm). Coupons were then removed from the liquid and observed under the microscope to confirm complete cell detachment. Detached cells were harvested by centrifugation (3202g, for 5 min at room temperature) and the supernatant discarded. Since chlorophyll pigments are light sensitive, the following chlorophyll extraction procedures were performed in the dark using a protocol adapted from Henriques et al., 2007. A volume of 2 ml of 99.8% methanol (Methanol ACS Basic, Scharlab Basic, Barcelona) was added for chlorophyll extraction, and suspensions were incubated at 4°C for a period of 24 h for maximal chlorophyll a extraction. Samples were then centrifuged at 3202g, for 5 min at room temperature, and the supernatant was transferred to a glass cuvette. The absorbance at 750 nm (turbidity), 665 nm (chlorophyll a) and 652 nm (chlorophyll b) was determined using a V-1200 spectrophotometer (VWR International China Co., Ltd, Shanghai, China). The chlorophyll a concentration ($\mu$g cm$^{-2}$) was calculated through the following Eq. 2 (Porra et al., 1989):

$$\text{Chl a (}\mu\text{g mL}^{-1}) = 16.29 \times A_{665} - 8.54 \times A_{652}.$$  (2)

**Statistical analysis**

All experiments were performed in triplicate. Data analysis was performed using the statistical program GraphPad Prism® for Windows, version 6.01 (GraphPad Software, Inc, San Diego, CA, USA), and results were compared using unpaired $t$-tests with a confidence level of 95% (**$P < 0.05$) and 90% (*$P < 0.1$).

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**References**


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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Appendix S1. Supporting Information.