New insight into colonies of *Microcystis* (Cyanobacteria) as multi-specific floating biofilms

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ABSTRACT

The ability to form biofilms is a functional trait shared by many bacterial species. Biofilms provide bacteria a sheltered environment where the nutrients and oxygen gradients create a heterogeneous matrix and promote cells to differentiate their metabolism and functions according to the position they occupy inside the matrix. Species of the *Microcystis* genus are among the most common bloom-forming cyanobacteria. They are unicellular microorganisms able to form colonies and to reach high biomass during blooms in lakes, reservoirs and estuaries worldwide. Colonial lifestyle provides several advantages under stressing conditions, including adaptation to different light intensities, protection from toxic substances and grazing, while allowing them to grow when the nutrient supply is low. Although the biology, ecology and colony formation have been extensively recognized in *Microcystis* spp., the analysis of the progression from unicellular to multicellular phases in this cyanobacterium have been always addressed as individual phenotypic plasticity and rarely as a multi-specific community of interrelated microorganisms. Here, we re-interpreted the evidence coming from different studies about the *Microcystis* lifestyle and propose a new way to analyze the available information about this cyanobacterial group. We specifically address the characteristics shared by bacterial biofilms and *Microcystis* colonies and suggest that the morphological changes from single cells to colonies are due to a cascade of events leading to the formation of a multi-specific biofilm. Studying the formation of colonies using this framework would help to better understand the life cycle of *Microcystis*, its functional relationship with the associated microbiome and the factors triggering microcystin production, helping to design strategies for prevention and control of the blooms caused by these organisms. Taking into account the biology and the ecological strategies of *Microcystis*, a conceptual model of emergence and decay of these floating multi-specific biofilms is proposed.

Keywords: multi-specific, biofilm, *Microcystis,* colonies, mucilage, EPS, holobiont

INTRODUCTION

Bacterial biofilms are defined as aggregates of microbial cells surrounded by a self-produced polymer matrix that can be composed by a single (mono-specific) or several species (multi-specific) living in a collaborative way (Flemming et al., 2016). Biofilm growth of microorganisms was first defined in medical microbiology, when it was also demonstrated that biofilm-embedded organisms have an increased antimicrobial resistance compared to those growing as planktonic bacteria (Nickel et al., 1985). The classic conceptual model of biofilm formation involves motile planktonic cells that become attached to a surface in response to a variety of environmental signals, such as exposure to subinhibitory concentrations of antibiotics as demonstrated in *P. aeruginosa* and *Escherichia coli* (Hoffman et al., 2005). Attached cells produce a hydrated matrix of extracellular polysaccharides (EPS), extracellular DNA, proteins and lipids (Flemming and Wingender, 2010), changing their structure and functional relationships. There are several key features that distinguish the cells in a biofilm from the planktonic lifestyle. Although biofilm cells encounter stronger gradients of nutrients and waste products than during planktonic life (Stewart and Franklin, 2008), they are embedded in a more controllable environment.

In aquatic cyanobacteria, despite the increasing amount of information regarding their ecology, the biofilm concept is generally associated with benthic species, which form cyanobacterial mats in several aquatic ecosystems (Stal, 2012). Among the planktonic groups we will focus on *Microcystis* spp., a complex of cyanobacteria from the Chroococcales order that live in freshwater and brackish waters. They are Gram-negative bacteria that can be found as single cells or in colonies that float near the surface, reaching colony sizes that can be detected by naked eye. *Microcystis* blooms in eutrophic ecosystems and generally a size spectrum can be found, ranging from ca. 4 µm (single cells) to hundreds of microns (large colonies) (Figure 1) (Reynolds et al., 1981). It has been described that *Microcystis* blooms are composed by populations able to produce secondary metabolites called microcystins, which are toxic to animals and humans (toxins), and by non-toxic populations (Vezie C, et al., 1998). Interestingly, some studies have shown that high temperature (between 25 and 30 °C) promotes the growth of toxic *Microcystis* (Davis et al., 2009), while non-toxic populations seem to have less tolerance to extreme environmental conditions such as nutrient depletion or low light (Van de Waal et al., 2011). Therefore, it is very likely that under current climate warming and worldwide eutrophication scenarios,

a dominance of cyanobacterial blooms containing a higher percentage of toxic *Microcystis* cells will occur. This makes it very relevant to understand the biology and ecology of the whole *Microcystis* community (toxic and non-toxic), with special attention to the toxic component.

Figure 1. *Microcystis* colonies. Large *Microcystis* colonies from Uruguay river (Uruguay-South America) observable at naked eye. A concentrated sample is shown at left.

Current vision of organism´s evolution is increasingly incorporating the concept of holobiont, which recognizes the widespread occurrence of host-associated microbiomes and makes emphasis in the multispecies nature of host–microbiome assemblage (Bordenstein and Theis, 2015). In *Microcystis*, the presence of an external layer of mucilage constituted by extracellular polysaccharides that is heavily populated by other bacterial species has been early discovered and is increasingly studied. The complex microbial structure generated in the mucilaginous colonies and its role in the survival and fitness of the cyanobacterium has started to be studied (Pérez-Carrascal et al., 2021; Schmidt et al., 2020) and points towards a holobiont lifestyle. In this context, studying the role of the heterotrophic counterpart of the

holobiont in *Microcystis* fitness and adaptation to different environmental conditions could provide the key for their global success.

The presence of an extracellular matrix with a microbiome, the evidence for a quorum sensing signaling system, the different metabolic capacities exhibited by single cells vs. colonies and field observations about *Microcystis* life cycle prompted us to propose that the colonies of these organisms are floating, multispecific biofilms. Here, we present a review of the literature focused on the main characteristics of *Microcystis* colonies resembling those of bacterial biofilms.

MAIN CHARACTERISTICS OF BACTERIAL BIOFILMS

In a biofilm, the cells become embedded within a slimy extracellular matrix that is mainly composed of extracellular polymeric substances and is produced by the cells within the biofilm. This polymeric matrix is composed of EPS, proteins, lipids and DNA (Costerton et al., 1987; Stoodley et al., 2002). When growing as biofilms, bacteria are usually attached to surfaces in high cell density accumulation, where diffusion and physicochemical heterogeneity are linked to large physiological fluctuations (Stewart and Franklin, 2008). Bacterial biofilms can also be considered as hydrogels, which are complex polymers containing many times their dry weight in water.

Biofilms are not just bacterial slime layers but biological ecosystems that work as a functionally coordinated community. The complex network of interactions within the biofilm influences the growth rate and metabolic activity of the participating bacteria, which are affected by the differences in nutrients and oxygen availability within biofilms. The formation and dispersal of biofilms are tightly regulated at the genetic level and triggered by environmental signals (Fazli et al., 2014).

The mechanism involved in biofilm formation and regulation is known to be the quorum sensing (QS), a chemical language used for intercellular communication, which is based on small, self-generated signal molecules called autoinducers that are produced in low concentration by the cells. When enough bacteria are present the concentration of autoinducers reaches a threshold level and bacteria are able to sense their critical mass, repressing or activating target genes (De Kievit and Iglewski, 2000). It has been reported that the genes that are controlled by QS can build up to 10 % of the bacterial genome (Wagner et al., 2003). The functional differences between free-living cells and biofilms have been

extensively demonstrated in pathogenic bacteria, such as *Pseudomonas aeruginosa,* where QS regulates the expression of genes involved in lectins, EPS and exotoxin, among others (Kariminik et al., 2017).

Bacterial biofilms can also exist in the air-liquid interface, forming floating biofilms or pellicles. This interface provides access to oxygen and other gases from the air and nutrients from the liquid phase through opposing gradients (Armitano et al., 2014). In pellicle-forming bacteria, such as *B. subtilis* and *P. aeruginosa*, flagellar motility is required for wild-type pellicle maturation dynamics and is an important trait influencing whether or not cells can form pellicles (Hölscher et al., 2015). This is relevant in the case of heterotrophic bacteria lacking gas vesicles for flotation and need to reach the surface. When growing in culture under static conditions, the cells tend to accumulate at the bottom of the flask, meaning that floating at the surface would be only possible through the generation of positive buoyancy. Some taxa, such as *Gluconacetobacter* spp. are buoyant because they trap CO_2 bubbles generated during the respiration process. Other bacteria can secrete surface-active agents (e.g., surfactants) or synthesize a polysaccharides-rich matrix that avoid the mixing with the liquid medium (Angelini et al., 2009; Koizumi et al., 2008). In the case of *Microcystis*, the position relative to the surface can be achieved thanks to the presence of gas vesicles aggregations or aerotopes in the cytoplasm (Šmarda and Maršálek, 2008), which allow them to regulate their vertical position in the water column and to form the colony in a suitable position receiving the right amount of light, oxygen and CO_2. The EPS matrix of bacteria that are able to form pellicles are usually composed by glucose, galactose, rhamnose, mannose or cellulose (for a review see Armitano et al. 2014), which are also typical components of the extracellular matrix of *Microcystis* (Lei et al., 2007; Li et al., 2009). Thus, *Microcystis* colonies have more than one trait contributing to float near the surface.

CHARACTERISTICS OF *MICROCYSTIS* COLONIES RESEMBLING THOSE OF BACTERIAL BIOFILMS

Table 1 summarizes some of the main biofilm-like characteristics exhibited by *Microcystis* colonies.

Table 1. Traits of bacterial biofilms exhibited by colonies of *Microcystis* spp.

Biofilm trait	In *Microcystis* colonies	References
Presence and composition of EPS matrix	Yes (glucose, xylose, galactose, fucose, rhamnose, arabinose)	Otsuka *et al.*, 2000; Lei *et al.*, 2007; Li *et al.*, 2009; Bi *et al.*, 2013; Li *et al.*, 2013b; Wang *et al.*, 2013; Liu, Huang, and Qin, 2018
Heterogeneity of the matrix	Yes	Sampognaro *et al.*, 2020
Buoyancy by EPS formation	Yes	Wang *et al.*, 2011; Xiao *et al.*, 2018; Chen *et al.*, 2019
Buoyancy by other mechanisms (gas vesicles)	Yes	Thomas and Walsby, 1985; Deacon and Walsby, 1990; Mlouka *et al.*, 2004
Physiological changes between single cells and multicellular stage	Yes	Gan *et al.*, 2012; Deus *et al.*, 2020; Harke and Gobler, 2013
Presence of a quorum sensing mechanism	Yes	Zhai *et al.*, 2012
Increased resistance to antimicrobial or toxic compounds	Yes	Bi *et al.*, 2013; Tan *et al.*, 2018

Presence and composition of extracellular polysaccharides in colonies: its effect on *Microcystis* morphology and ecology

Cyanobacteria of the *Microcystis* genus exhibit high phenotypic plasticity. They can exist as single or paired cells when growing fast under axenic conditions in laboratory cultures, but in nature are usually found as colonies (Xiao et al., 2017) displaying different morphologies, including irregular, sponge-like, spherical and elongated (Komárek and Komárková, 2002). These colonies consist of groups of cells stick to each other by a mucus envelope mainly composed of EPS (Hall-Stoodley et al., 2004)

192 known as mucilage. This region that surrounds the cells creates a microenvironment known as the
193 phycosphere (Bell and Mitchell, 1972), where complex ecological interactions between phytoplankton
194 and bacteria occur (Jiang et al., 2007; Seymour et al., 2017).

195 Colony formation in *Microcystis* has been attributed to i) cell division: after binary fission cells
196 remain attached and daughter cells become enveloped in a layer of mucilage that prevents their
197 separation; or ii) cell adhesion: single cells aggregate via the secretion of sticky mucilage (Kessel and
198 Eloff, 1975; Yang et al., 2012). In any case, colonies are extremely buoyant, commonly forming wind-
199 blown scums (Znachor et al., 2006). As mentioned above, their buoyancy is achieved mainly by the
200 presence of aerotopes (Šmarda and Maršálek, 2008) and helped by the presence of the thick matrix of
201 EPS produced by the cells and typically composed by glucose, xylose, galactose, fucose, arabinose and
202 rhamnose (Lei *et al.*, 2007; Li *et al.*, 2009) (Table 1) (Figure 2). It has been demonstrated that the
203 morphology of *Microcystis* colonies in culture could change and the solubilization of the mucilage could
204 induce changes in colonial morphology (Li et al., 2009; Otsuka et al., 2000; Wang et al., 2013).

205 The presence of a mucilaginous envelope also provides *Microcystis* with an advantage to withstand
206 alterations of the physical environment, such as osmotic stress (Kehr and Dittmann, 2015). Moreover,
207 it has been shown that after strong mixing events large mucilaginous colonies have a faster recovery of
208 the near to surface position than single cells and small-sized colonies (Kruk et al., 2017). Floating rates
209 of medium size *Microcystis* colonies (ca. 100 μm) rarely exceeded ± 30 μm s^{-1}, whereas colonies
210 considerably larger than this are reported to achieve flotation rates of 300 μm s^{-1} (Ganf, 1974;
211 Humphries and Imberger, 1983; Reynolds, 2007). This buoyancy would favor the access to sunlight by
212 the cells located at the center of the colony. In addition, it has been shown that estuarine to marine
213 salinity levels promoted an increase in the thickness of the mucilage and a decrease of cell-free space
214 resulting in higher cell density, which serve as a defense mechanism to cope with salinity stress
215 (Sampognaro et al., 2020). Since *Microcystis* colonies contain many times its dry weight in water they
216 would match the criteria for being classified as hydrogels.

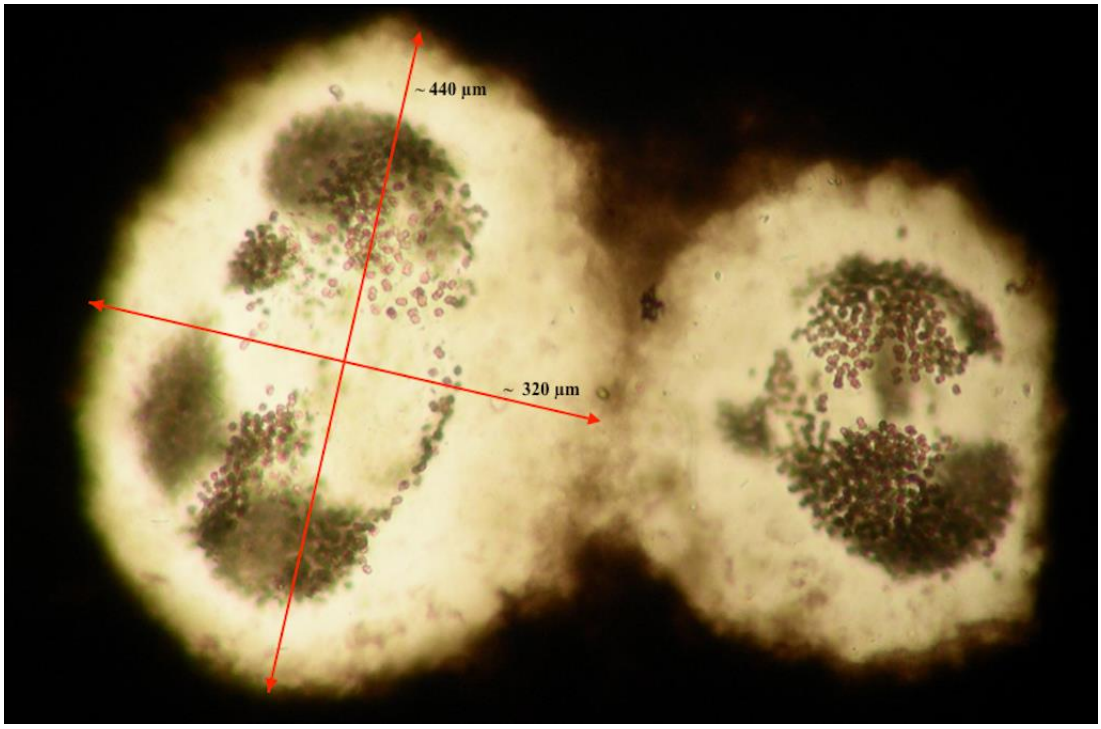

Figure 2. ***Microcystis*** **mucilage.** Chinese ink staining of *Microcystis* spp. colonies reveals the thick layer of mucilage. Brightfield, 40x magnification.

Colony formation in *Microcystis* can be induced by abiotic factors, such as low temperature (15 °C) and low light intensity (10 µmol photons m^{-2} s^{-1}), which enable them to develop colonies up to 100 µm diameter (Li et al., 2013; Xu et al., 2016; Yang et al., 2012). On the opposite hand, an increased growth rate is observed at higher light intensities, with a concomitant increase of intracellular polysaccharides consumption that provokes a decreased propensity to form colonies (Xiao et al., 2018). It has been described that in presence of high concentration of calcium (Sato et al., 2017; Wang et al., 2011) and lead (Bi et al., 2013), the formation of colonies reaching more than 100 µm diameter can be induced. In addition, the exposure to metals also showed to induce the secretion of EPS that helps to precipitate the metal ions, acting as a mechanism to avoid metal poisoning (Bi et al., 2013). The ability to form colonies was also linked to antibiotic resistance. In this sense, low concentrations of aminoglycoside antibiotics induced the aggregation of *Microcystis* cells, suggesting a protective role for the EPS (Zhang et al., 2018).

Nutrients can also affect colony formation, as previously demonstrated by Ma et al. (2014), who found that addition of nitrogen and phosphorus provoked disaggregation of colonies in culture; while (Zhu et al., 2016) found a general decrease in colony size at increasing nutrient concentrations in the field, potentially resulting from increased growth rate. Interestingly, the EPS amounts produced by single-celled laboratory strains have shown to be intensely reduced compared to freshly isolated colonies, which can have up to 10-fold higher quantities of EPS (Wang et al., 2011). Thus, the production of an EPS-rich mucilaginous envelope seems to be a response to adverse environmental conditions.

Evidence for a quorum sensing (QS) mechanism in *Microcystis*

Early studies suggested that microcystins could act as a signaling or QS molecule (Dittmann et al., 2001). The MrpA protein (a microcystin-related protein) was found to be strongly expressed in wild-type *Microcystis* PCC 7806, but became undetectable in a mutant lacking a gene involved in microcystin synthesis, *mcyB*. This protein showed similarity to the RhiA protein from *Rhizobium leguminosarum*, which is encoded by the *rhiABC* operon and it is controlled by quorum-sensing mediators (Supplementary Table 1). This finding led the authors to suggest a QS role of microcystins (Dittmann et al., 2001).

Zhai et al. (2012) reported evidence indicating the presence of QS-related signal molecules, the acylated homoserine lactones (AHLs) in cultures of *M. aeruginosa* PCC-7820. Electron microscope photographs of *M. aeruginosa* supplemented with AHLs showed a shift from single free-living cells to a biofilm-like membrane, which led to a stronger aggregation of the cells compared to controls without AHLs. This suggests that QS might play an important role in the environmentally-driven morphological changes of *M. aeruginosa*, providing strong evidence that it regulates colony formation (Zhai *et al.*, 2012) through a coordinated multicellular behavior, as described for biofilms. More recently, Herrera and Echeverry (2021) applied several AHLs known to be involved in QS in Gram negative bacteria to cultures of *Microcystis* and found a correlation with colony-forming activity for most of them. This finding is very interesting, since it also points to a QS-based mechanism associated with the growth of the colonies. Moreover, using ELISA assays, they found increased microcystin levels with some AHLs. They propose that the source of the AHLs could be *Microcystis* or members of the microbiome present

in the phycosphere, meaning that the QS would be an ability conferred by the cyanobacterium and its microbiome, acting cooperatively as a holobiont. Further studies analyzing the presence of the genes encoding for the AHLs synthesis should be performed to confirm this.

Phenotypic and functional differences between single cells and colonies

The cells growing in a *Microcystis* colony are physiologically distinct from single cells (Table 1), for example in terms of toxin production. In this sense, there is a growing body of evidence relating the colony size to toxicity and identifying that colonies in the size range from 60 to150 µm (diameter or maximum linear dimension) are those producing higher amount of microcystins compared to single cells or colonies smaller than 20 µm diameter (Deus Álvarez et al., 2020; Gan et al., 2012). Cultures of *M. wesenbergii* DC-M1, *M. ichthyoblabe* TH-M1 and *Microcystis* sp. FACHB1027 treated with microcystin-RR developed colonies significantly larger than the control and provoked the upregulation of genes related to the synthesis of polysaccharides: *capD*, *csaB*, *tagH* and *epsL*, resulting in a significant increase of EPS (Supplementary Table 1). This is especially relevant in the case of the non-toxic species *M. wesenbergii*, since the interaction with exogenous microcystin affected growth and colony size and suggests that during a bloom the toxin produced by toxic species would also promote the growth of non-toxic ones. On the other hand, depletion of extracellular microcystin concentrations caused a decrease in colony size, indicating that released microcystins may be involved in maintaining the colony size of *Microcystis* (Gan et al., 2012), regardless of their toxin-production ability.

It has been also shown that when subjected to intense grazing *Microcystis* cells increase the abundances of transcripts encoding extracellular polysaccharides and gas vesicles (Harke and Gobler, 2013). This is in agreement with early findings by Reynolds et al. (1981), who reported that *Daphnia* grazing pressure is stronger on small colonies having less amount of mucilage, indicating that the increase of EPS production could be a mechanism to avoid predation (Reynolds et al., 1981).

Interestingly, genes encoding for type IV pili (e.g., *pilT*) have been found in *Microcystis aeruginosa* PCC 7806 (Nakasugi and Neilan, 2005) (Supplementary Table 1). These pili are present in many gram-negative bacteria systems and are involved in several functions such as cell adhesion, twitching motility, and natural transformation (Mattick, 2002). In several bacterial species, they are also related to biofilm formation via bacterial migration (Barken et al., 2008). For example, in the case of *P. aeruginosa* are

necessary for cap formation of the mushroom-shaped structured biofilm (Klausen et al., 2003), while in *Clostridium difficile* promote early biofilm formation (Maldarelli et al., 2016). We hypothesize that the presence of *pilT* in *Microcystis* cells (see Nakasugi and Neilan, 2005 for images reference) reveals their ability to move and could have a role during the initial arrangement of the cells inside the growing colony. When individual cells initiate a biofilm, they are surrounded by small amounts of EPS and are probably capable of independent movement by means of twitching motility mediated by these pili. As the colony grows and the biofilm starts to mature, water channels develop and a differentiation in physiological processes among cells start to establish in response to conditions in their particular environments (Stoodley et al., 2002).

In a previous study addressing the individual activity of cells in the colony using the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), we found that cells located at the inner part of the colonies exhibited a lower respiration activity than those in the peripheric, suggesting a less active metabolic state (Kruk et al., 2017). This kind of differential activity can be also found in mature biofilms of several bacterial species. In *P. putida* and *E. coli* the cellular activity in the center of cell clusters diminished as the clusters grew larger. This activity can be restored after carbon sources addition, suggesting that cell activity in the inner part of the clusters would be controlled by resources availability (Sternberg et al., 1999).

When growing under laboratory conditions, *M. aeruginosa* colonies disaggregate and resulting single cells have significantly lower chlorophyll a, phycocyanin and total carbohydrate than cells in colonies (Reynolds et al., 1981; Wang et al., 2015). This induction of a unicellular lifestyle has not been explored enough and might be the consequence of dilution and selection in a favorable milieu. This change from colonies to single cells that occurs in cultures might reflect the different ecological requirements between both morphological states and call for caution when analyzing the ecology and environmental preferences of these organisms by using isolates from the laboratory.

Potential role of the *Microcystis* microbiome in colony formation

As it can be seen in Figure 3, the mucilage of *Microcystis* is populated by a high number of heterotrophic bacteria growing at expense of the EPS carbon, as well as other cyanobacteria and sometimes even protists. The interactions occurring between individual organisms within this

phycosphere have an ecosystem-level effect on several processes, e.g., nutrient cycling, toxin biosynthesis, etc. (Bell and Mitchell, 1972; Seymour et al., 2017). The incorporation of bacteria into the phycosphere likely occurs through chemotaxis, random contacts and vertical transmission (Seymour et al., 2017).

In the case of *Microcystis*, it has been shown that heterotrophic bacteria stimulated cyanobacterial growth and induced the production of EPS (Wang et al., 2016). In fact, their presence was early documented by Reynolds et al. (1981), who found that *Microcystis* mucilage was crowded with rod-shaped bacteria that were frequently observed on the periphery of planktonic colonies in their stationary or decline phases (Table 2).

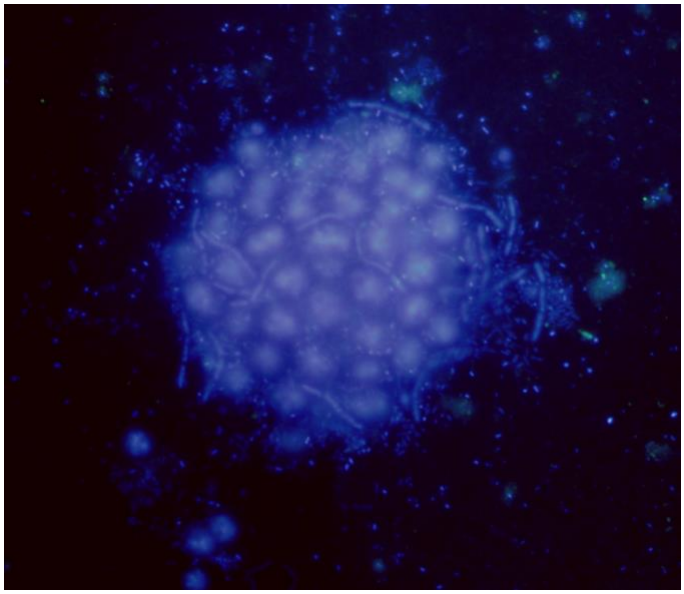

Figure 3. ***Microcystis*** **microbiome**. DAPI-stained *Microcystis* spp. colony showing the bacteria attached to the mucilage. 1000x magnification.

Table 2. Bacterial groups that have been described as associated to *Microcystis* colonies through different methodological approaches (bacterial cultures, 16S rDNA and shotgun metagenomics, metagenome-assembled genomes).

Phylum	Class	Order/Family/Genus	Found in colony or free-living	References
Proteobacteria	Alphaproteobacteria	Rhizobiales, Sphingomonadales, Rhodospirillales, Caulobacterales	Colony-attached	Li et al., 2018; Jankowiak and Gobler, 2020; Cook et al., 2019; Wu et al., 2019; Pérez-Carrascal et al., 2021; Jackrel et al., 2019; Li et al., 2018;
		Rhodocyclaceae	Colony-attached	
		Pleomorphomonas	Colony-attached	
		Candidatus Phycosocius bacilliformis	*M. aeruginosa* phycosphere	
		Rhodobacter	Colony-attached	
		Methylobacterium	Colony-attached	
		Roseomonas	Colony-attached	
		Pseudanabaena	Colony-attached	
	Betaproteobacteria	*Burkholderia*	Colony-attached	
		Alcaligenaceae		
	Gammaproteobacteria		Colony-attached	
	Epsilonproteobacteria		Colony-attached	
Bacteroidetes	Cytophagia, Sphingobacteriia, Chitinophagia	Chitinophagaceae, Cytophagales	Found in free-living and colony-attached fractions	Wu et al., 2019; Pérez-Carrascal et al., 2021
Firmicutes				Wang et al., 2016
Gemmatimonadetes		*Gemmatimonas*	Colony-attached	Yang et al., 2017; Pérez-Carrascal et al., 2021
Verrucomicrobia			Colony-attached	Cai et al., 2014

The bacterial community inhabiting the mucilage has shown to differ markedly from that present in free-living *Microcystis* (Wu et al., 2019). This highly diverse microbiome surrounding *Microcystis* colonies and living in close cooperative way allows the cyanobacteria the access to specific compounds, such as vitamins and some components of the outer membrane lipopolysaccharide, while providing bacteria with highly bioavailable carbon. The bacterial community in the phycosphere has shown to be

highly structured and related to the size of the colonies, suggesting highly specific conditions within the *Microcystis* (Cai et al., 2014). Interestingly, it has been shown that although *Microcystis* microbiomes diverged in taxonomy along a phosphorus concentration gradient, they converged in function, indicating a metabolic interdependence between the host and its microbiome (Jackrel et al., 2019). Cook et al. (2020) found that *Microcystis* microbiome is highly similar across global blooms regardless of the environmental differences, pointing out the existence of a stable associated microbiome that might be involved in colony formation (Cook et al., 2020). Moreover, it has been recently found by single-colony metagenomic sequencing that *Microcystis* microbiome is genotype-specific, and that closely related genotypes have similar microbiomes (Pérez-Carrascal et al., 2021). Similarly, Tu et al. (2019) found that colonies of the same *Microcystis* species have very similar community composition. Indeed, the non-toxic *M. wesenbergii* microbiome harbored a very different composition compared to that from toxic *M. aeruginosa* and *M. panniformis,* which implies that microcystin could play a structuring role in the community.

It has been reported that high temperatures (32 °C) provoke changes in the composition of heterotrophic bacterial communities embedded into the mucilage of *Microcystis* (Dziallas and Grossart, 2011). These associated heterotrophic bacteria can stimulate cyanobacterial growth and induce the production of EPS that is relevant for the optimum development of *Microcystis* (Reynolds, 2007). Besides, interspecies interactions could promote EPS production (Yang et al., 2008), and co-cultivation of axenic, single-celled cultures of *Microcystis* with heterotrophic bacteria isolated from *Microcystis* colonies stimulated the production of EPS, allowing to reconstitute colony formation (Shen et al., 2011). Moreover, removing the EPS had a detrimental effect on the auto-aggregation abilities of heterotrophic bacteria isolated from *Microcystis* colonies, which suggest that EPS plays a relevant role in the recruitment of bacteria by promoting their attachment (Zhang et al., 2018). More recently, Schmidt et al. (2020) have found that during invasion experiments the ability of *M. aeruginosa* to compete with other phytoplankton species is not determined by the ability to produce the toxin, but by genes from its microbiome. This points to an important role of host-associated bacteria in mediating phytoplankton interspecies interactions.

The resulting cooperative microbial network, which strongly agrees with the holobiont concept as the ecological and evolutionary unit, might be the key for *Microcystis* success under changing environments. The role of the *Microcystis* microbiome is just starting to be discovered and further research is needed to shed light on the role of specific heterotrophic organisms in colony formation and in the persistence of *Microcystis*.

Further evidence on the similarity between the colonial lifestyle and biofilms

Sigee et al. (2007) reported the presence of programmed cell death (PCD) during a late summer bloom of *Microcystis*. More recently, Hu and Rzymski (2019) proposed that under certain stressing conditions (excessive salt concentration, exogenous oxidants, ultraviolet radiation, herbicides, among others) a PCD-like mechanism would cause apoptosis and significant release of microcystin in *Microcystis.* The released microcystin would have an extracellular function, not yet described, which benefits the rest of the population inside the colony. These authors also speculate on the similarities between *Microcystis* colonies and bacterial biofilms, emphasizing the central role of the PCD and microcystin release in bloom development. They also propose that non-microcystin producing *Microcystis* would benefit from the microcystin released by the toxic populations (Hu and Rzymski, 2019).

These antecedents constitute additional evidence that point towards a kind of multicellular behavior within the colonies, such as that existing in the bacterial biofilms. In multicellular organisms, PCD removes cells having molecular errors in order to keep homeostasis and a healthy organism development, prioritizing the benefit of the organism over the survival of the cell. In the case of bacteria, this behavior would not provide any benefit to an individual cell but would confer an advantage to the remaining cells (Allocati et al., 2015; Bayles, 2014; Lewis, 2000). In *Microcystis*, colony formation provides a highly efficient survival strategy under adverse environmental conditions (low nutrient levels, high grazing pressure, high ultraviolet radiation).

PROPOSAL OF A CONCEPTUAL MODEL FOR BIOFILM FORMATION IN *MICROCYSTIS* SPP.

The colonies of *Microcystis* spp. share several characteristics with bacterial biofilms. They can switch from single planktonic cells to aggregates (colonies) organized into a coordinated functional community that is embedded in an EPS matrix, which composition highly resembles that found in bacterial biofilms and that is teemed with a diversity of heterotrophic bacteria living in a cooperative manner with the cyanobacterial cells (Figure 4). The change from single or few cells to multicellular organization would be triggered by autoinducers molecules, such as AHLs, which could be synthesized either by the cyanobacterium or by the microbiome, and that build up during exponential growth under resource-rich conditions. As the population grows, the resources become less available and the AHLs upregulate a number of functional genes (e.g., the microcystin biosynthesis cluster *mcy*) that allow the organisms to thrive under conditions that would not be favorable, such as nutrients and light shortage. This kind of multi-specific biofilm is not built from the attachment of the cells to an abiotic or biotic surface, but on the attachment of cells to each other to form a floating biofilm and allowing *Microcystis* spp. to thrive in a highly diverse array of environmental conditions.

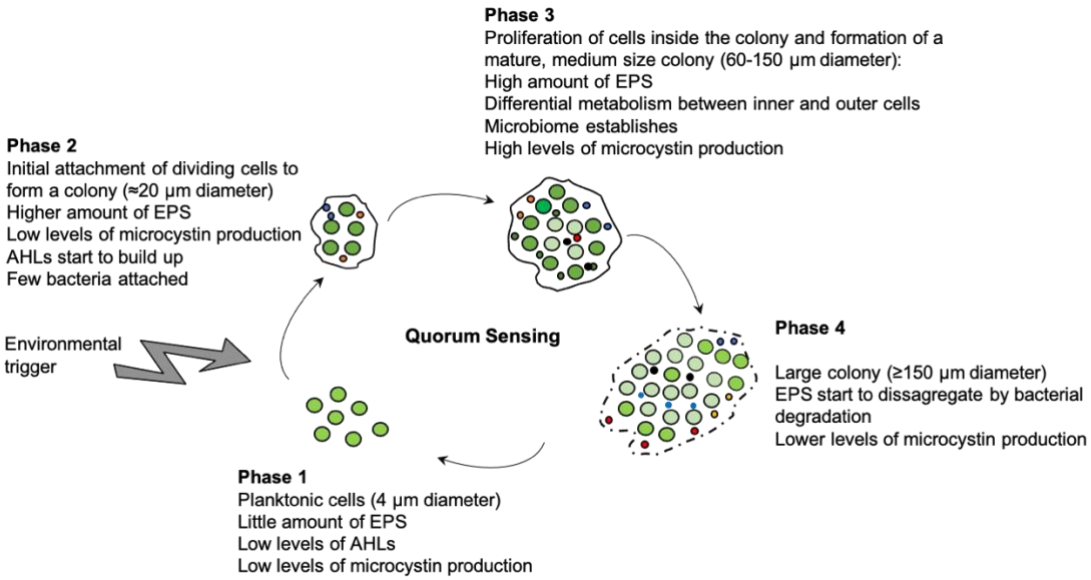

Figure 4. **Proposal for the floating biofilm formation in colonies of *Microcystis***. Four phases can be distinguished during the development of a *Microcystis* community according to lifestyle (single celled vs attached), EPS and microcystin production, presence of an established microbiome and autoinducers concentration (AHLs). Phase 1 is composed of single cells (4 µm diameter, green circles) having little amount of EPS mucilage, low

levels of microcystin production and low levels of AHLs. Phase 2 starts with the initial attachment of dividing cells to each other to form a colony surrounded by a higher amount of EPS mucilage, cells probably mobilize inside the colony and they have low levels of microcystin synthesis while AHLs start to build up and other bacteria (smaller red, blue and black circles) start to attach to the EPS. In Phase 3, the proliferation of cells inside the colony allows the formation of a mature biofilm, with elevated amounts of EPS, high levels of microcystin production and clearly different metabolism between inner and outer cells. A microbiome is well established. The Phase 4 is characterized by large, amorphous colonies, low levels of microcystin production and disaggregation of the mucilage by bacterial degradation of the EPS (typically at the end of a bloom). We propose that the onset of a bloom will depend on abiotic and biotic conditions and on the phase of the *Microcystis* community, being more likely to develop a high biomass in a short time period during phase 3 (active cells, with high microcystin production rates).

It must be noticed that, as it has been described for temperate lakes, the sediment can be the source of *Microcystis* colonies that will trigger the water column colonization when environmental conditions are favorable, especially in temperate lakes where this kind of annual cycle has been described (Reynolds et al., 1981; Yang et al., 2020). The phase at which these colonies will be recruited after winter would depend on the lake temperature and the physiological state of the overwinter organisms.

More data and information gathered from studies specifically addressing the biofilm formation in *Microcystis* are needed in order to develop mathematical models describing the growth and dispersal of the colonies. This, together with morphological, gene expression and functional studies of different *Microcystis* species would bring new insight into the life cycle of this relevant group of organisms.

Understanding the mechanism underlying biofilm formation in *Microcystis* spp. and the role of heterotrophic bacterial community in toxin synthesis and environmental performance will improve the current models of growth, fitness and dispersal of these cyanobacteria.

CONCLUSIONS

1) The information gathered so far about colony formation in *Microcystis* spp. suggests that the mechanisms involved in this process are the same as those defined for biofilm formation in a number of bacterial species. Single-celled *Microcystis* are able to multiply while producing a mucilaginous

envelope that contributes to the differentiation into a colony. Colonies are described to form either by cell division or by cell aggregation; in any case, the presence of an extracellular matrix ensures the confinement of the cells into a tridimensional, secluded structure. The triggers to switch between both lifestyles would involve environmental cues that induce cellular stress such as salinity, oxidative damage due to ROS (inducing microcystin production and export), predation and low nutrient availability.

2) The colonial or biofilm stage, while provokes a reduced specific growth rate, provides *Microcystis* with a sheltered milieu. As a consequence, several gradients of resources (oxygen, light, nutrients) are generated, with the concomitant creation of different micro-environments inside the colony. Thus, cells located in these different micro-environments will have different metabolic rates (e.g., respiration, photosynthesis, microcystin production, etc.).

3) The main components of the biofilm mucilage are EPS, DNA from lysed cells, proteins, lipids and heterotrophic bacteria that live embedded in this mucus. The bacterial community associated with *Microcystis* colonies is starting to be explored and recent findings suggest that there is a quite constant microbiome, in which functional relationships with the cyanobacterium are closely intertwined. This relationship involves the trade of different goods that allows the survival and increases the fitness of the colony as a multispecies biofilm community.

4) The concentration of microcystin has been shown to be linked to the production of EPS by the cells, suggesting that the toxicity potential of *Microcystis* could be the consequence of cells adopting a biofilm lifestyle.

5) The presence of AHLs, the autoinducers molecules involved in bacterial QS behavior, has been detected in *M. aeruginosa* and experimental evidence suggests that the increase of AHLs provokes a shift from single-cell to a biofilm-like status. This strongly suggests that the QS system regulates the coordinated cellular behavior leading to biofilm formation.

6) As stated by Xiao et al. in 2018, we still do not have a clear explanation for the observed differences in colonial morphology among different *Microcystis* species. This gap in knowledge could be shortened if colonies start to be studied using the biofilm approach, since morphology differences (differences in biofilm architecture) could be attributed to different phases of biofilm maturation and would account for the environmental conditions to which the organisms were subjected.

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