The *Microcystis*-microbiome interactions: origins of the colonial lifestyle

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Abstract

Species of the *Microcystis* genus are the most common bloom-forming toxic cyanobacteria worldwide. They belong to a clade of unicellular cyanobacteria whose ability to reach high biomasses during blooms is linked to the formation of colonies. Colonial lifestyle provides several advantages under stressing conditions of light intensity, ultraviolet light, toxic substances and grazing. The progression from a single-celled organism to multicellularity in *Microcystis* has usually been interpreted as individual phenotypic responses of the cyanobacterial cells to the environment. Here, we synthesize current knowledge about *Microcystis* colonial lifestyle and its role in the organism ecology. We then briefly review the available information on *Microcystis* microbiome and propose that changes leading from single cells to colonies are the consequence of specific and tightly regulated signals between the cyanobacterium and its microbiome through a biofilm-like mechanism. The resulting colony is a multi-specific community of interdependent microorganisms.

Keywords: *Microcystis,* microbiome, colonies, mucilage, EPS, holobiont

Introduction

It has been since a long time ago that microbiologists have noticed that bacteria do not always live as single cells. Many of the known bacterial species have the ability to grow in a multicellular and coordinate way, the biofilms. 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. 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 (Sauer *et al.* 2022). 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. After a while, sessile cells arranged in microcolonies from where some cells can escape to return to the planktonic lifestyle and subsequently colonize a new surface (Petrova and Sauer 2016). Although biofilm cells encounter stronger gradients of nutrients and waste products than during planktonic life, they are embedded in a more controllable environment (Stewart and Franklin 2008).

In the case of aquatic cyanobacteria, despite the increasing amount of information regarding their ecology, the biofilm concept is generally associated with benthic species, which form 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 form dense blooms in eutrophic ecosystems (Paerl 1988; Huisman *et al.* 2018) and can be found as single cells or in colonies floating near the surface, with a size spectrum ranging from ca. 4 µm (single cells) to hundreds of microns (large colonies) (Reynolds *et al.* 1981) that can be detected by naked eye.

Interestingly, *Microcystis* belongs to a phylogenetic group of unicellular cyanobacteria and its ability to form colonies is usually considered as an ecological aggregation strategy to avoid predation or protect from ultraviolet radiation, among others. In this context, colony formation by these organisms has been explained either by cell division (the usual bacterial process to multiply) or cell adhesion (Yang

et al. 2008). However, recent genomic evidence suggests that colonies in *Microcystis* result from clonal expansion rather than cell aggregation (Carrascal *et al.* 2021).

In spite of the amount of information regarding *Microcystis* ecology, colony formation and toxicity, little is known about the biological interactions taking place inside the colony and their role in *Microcystis* biology and evolution. Here, we focus on i) the characteristics shared by bacterial biofilms and *Microcystis* colonies; ii) the current knowledge about colony formation process in *Microcystis*; iii) the evidence on the existence of quorum sensing (QS) in *Microcystis* and; iv) the information about community composition and function of the colony-associated microbiota. Based on this, we propose that the morphological, functional and microbiome compositional changes occurring from single cells to colonies are consequence of biological and ecological interactions between the cyanobacterium and the heterotrophic bacteria. These specific and carefully regulated interactions are bi-directional and induce the development of a mucilaginous envelope that will host the heterotrophic community through a biofilm-like mechanism. Taking this into account a conceptual model of emergence and decay of these floating multi-specific biofilms of *Microcystis* is presented.

Microcystis blooms and microcystins production

Microcystis blooms are composed by a mixture of populations able to produce secondary metabolites called microcystins that are toxic to animals and humans, and by non-toxic populations. It has been shown that high water temperature (between 25 and 30 °C) promotes the growth of *Microcystis* populations able to produce microcystin (toxic), while non-toxic populations seem to have less tolerance to variable environmental conditions (Davis *et al.* 2009; Van de Waal *et al.* 2011). Therefore, it is very likely that under the current climate warming and worldwide eutrophication scenario a dominance of cyanobacterial blooms containing a higher percentage of toxic *Microcystis* will occur (Paerl and Huisman 2008; Kruk *et al.* 2023), making it very relevant to understand the biology and ecology of these organisms.

Until now, studies on the ecology of *Microcystis* have focused on the determinants of its growth, potential toxicity and diversity (Dick *et al.* 2021). More recently, the structure and function of its microbiome and its role in the survival and fitness of the cyanobacterium have started to be included (Jankowiak and Gobler

2020; Schmidt *et al.* 2020; Carrascal *et al.* 2021). However, there is no consensus on the mechanisms that determine the production of microcystins, the density and persistence of blooms or the microbiome community structure. In this sense, the evidence from different studies is frequently contradictory, since some works are based on axenic cultures of unicellular forms (hard to find in nature), others on environmental samples and others analyse and compare sequences obtained either from isolates, environmental DNA or enrichments from blooms, making generalizations difficult (Pimentel and Giani 2014; Martin *et al.* 2020; Zhou *et al.* 2020; Yang *et al.* 2022; Dai *et al.* 2023; Yin *et al.* 2024). Another possible explanation for the contradictions is that the factors driving bloom formation may be uncoupled from those driving toxicity, perhaps due to complex regulation pathways associated not only to the cyanobacterium, but also to its heterotrophic partners.

Similarities between *Microcystis* colonies and biofilms

In biofilms, attached cells produce a hydrated matrix of extracellular polysaccharides (EPS), extracellular DNA, proteins and lipids, changing their structure and functional relationships (Stoodley *et al.* 2002). But bacterial biofilms can also exist in the air-liquid interface forming floating biofilms or pellicles. This interface provides access to oxygen and other gasses from the air, as well as nutrients from the liquid phase through opposing gradients (Armitano, Méjean and Jourlin-Castelli 2014).

Microcystis colonies are extremely buoyant, commonly forming wind-blown scums. Their position relative to the surface can be achieved thanks to the presence of gas vesicles aggregations 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 to receive the right amount of light, oxygen, CO_2 and nitrogen, which is necessary to build the protein vesicles (Wu *et al.* 2023). The EPS matrix contributes to buoyancy and has the same composition that has been described for pellicle-forming bacteria (Armitano, Méjean and Jourlin-Castelli 2014), such as glucose, galactose, rhamnose, mannose or cellulose (Lei *et al.* 2007; Li *et al.* 2009). This matrix creates a microenvironment called the phycosphere, where complex ecological interactions between phytoplankton and bacteria occur (Seymour *et al.* 2017).

Colony formation in *Microcystis* can be induced by abiotic factors causing stress, such as low temperature (15 °C) and low light intensity (10 μmol photons m^{-2} s^{-1}) (Yang *et al.* 2012; Li *et al.* 2013; Xu *et al.* 2016). In the presence of high concentration of calcium (Wang *et al.* 2011; Sato *et al.* 2017) and lead, the formation of colonies reaching more than 100 μm diameter can be induced and its EPS acts trapping the metal ions (Bi *et al.* 2013). As for bacterial biofilms, the ability of *Microcystis* to form colonies has also been linked to antibiotic resistance, since low concentrations of aminoglycoside antibiotics induced cell aggregation, suggesting a protective role for the EPS (Tan *et al.* 2018). Another characteristic shared by biofilms and *Microcystis* colonies is cellular motility. Genes encoding for type IV pili (e.g., *pilT*) have been found in *Microcystis aeruginosa* PCC 7806 (Nakasugi and Neilan 2005), which may indicate that cells can move by means of twitching motility during the initial arrangement of the cells inside the growing colony (Maier and Wong 2015). 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.

There is growing evidence relating colony size with the amount of microcystin they produce. For example, it has been shown that colonies in the size range from 60 to150 μm diameter are those producing higher amounts of microcystins compared to single cells or smaller colonies (Gan *et al.* 2012; Deus Álvarez *et al.* 2020). On the other hand, depletion of extracellular microcystin concentrations showed a decrease in colony size (Gan *et al.* 2012). Thus, the evidence suggests that released microcystins could act as an infochemical-related mechanism involved in the biofilm maintenance. However, if microcystins are involved in a QS-like mechanism remains uncertain.

Regarding QS, acylated homoserine lactones (AHLs) have been found in cultures of *M. aeruginosa* PCC-7820 (Zhai *et al.* 2012). Electron microscope photographs of *M. aeruginosa* supplemented with AHLs showed a shift from single free-living cells to a biofilm-like membrane. 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 through a coordinated multicellular behaviour as that described for biofilms. This was confirmed more recently, when addition of several AHLs from Gram negative bacteria to cultures of

Microcystis induced colony formation (Herrera and Echeverri 2021). The fact that AHLs belonging to several species were able to induce a response in *Microcystis* implies that the QS behaviour leading to colony formation could be triggered by members of the microbiome. Moreover, (Shi *et al.* 2022) showed that several transcripts for pathways involved in biofilm formation were enriched in the *Microcystis* colonial form compared to single cells. These transcripts belonged mainly to heterotrophic bacteria from the microbiota, meaning that QS in *Microcystis* is an ability conferred by the cyanobacterium and its microbiome acting cooperatively. This kind of multi-species, multicellular behaviour may have ecosystem-level effects on several processes, e.g., nutrient cycling, toxin biosynthesis, bloom stability, etc. (Van Le *et al.* 2022).

The *Microcystis* holobiont

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 on the multispecies nature of host–microbiome assemblage (Bordenstein and Theis 2015). In the case of *Microcystis*, the colonial organism is in fact composed of a myriad of different bacterial species interacting and exchanging common goods (nutrients, gasses, carbon, genes) inside the mucilaginous envelope of the cyanobacterium, which confers it an extremely high ability to survive in different environmental conditions (Cook *et al.* 2020). Thus, it seems sound to conclude that the colonial organism we call *Microcystis* is in fact a holobiont. But, how is this prokaryotic holobiont formed?

It has been reported that the highly diverse microbiome of *Microcystis* colonies differs markedly from that present in single cells (Wu *et al.* 2019). Co-cultivation of axenic, single-celled cultures of *Microcystis* with heterotrophic bacteria isolated from *Microcystis* colonies stimulated cyanobacterial growth and induced the production of EPS, allowing to reconstitute colony formation (Reynolds 2007; Shen *et al.* 2011; Wang *et al.* 2016). Moreover, the existence of a metabolic interdependence between *Microcystis* and its microbiome has been proposed (Jackrel *et al.* 2019; Cook *et al.* 2020), suggesting that the ability to compete with other phytoplankton groups would not be determined by the toxin production but by genes from its microbiome (Schmidt *et al.* 2020). Therefore, there is evidence of a clear and strong relationship between the presence of an extracellular matrix and the recruitment of heterotrophic bacteria,

which stimulate colonial growth through QS to form a three-dimensional structure where the exchange of common goods occurs. This constitutes a complex holobiont organism whose formation must have involved the establishment of a symbiotic relationship early in the evolution of the cyanobacterium. As a unicellular cyanobacteria, *Microcystis* can only achieve a multicellular stage through its relationship with the symbiotic partners. This hypothesis would also explain the reversion from colonies to single cells observed when isolating *Microcystis* from environmental samples (Wang *et al.* 2015), probably due to the several dilutions and washing steps that remove the attached bacteria.

Conceptual model for colony formation in *Microcystis* holobiont

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. *Microcystis* can switch from single cells to colonies organized into a coordinated functional community that is embedded in an EPS matrix teemed with a diversity of heterotrophic bacteria living mainly in a cooperative manner with the cyanobacterial cells (Figure 1). The change from single cells to multicellular organization would be triggered by autoinducers molecules (e.g., AHLs) synthesized either by the cyanobacterium, by the microbiome, or both, in response to environmental cues (e.g., resource-rich conditions). As the population grows, the resources become less available and the AHLs upregulate a number of functional genes allowing the organisms to thrive under conditions that would not be favourable, such as nutrients or light shortage, oxidative stress, etc. The main components of the biofilm mucilage are EPS, DNA from lysed cells, proteins, lipids and heterotrophic bacteria that live embedded in this matrix. This bacterial community has a very constant structure, its functional relationships with the cyanobacterium are closely intertwined and involves the trade of different goods, allowing the holobiont to survive. The resulting 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 that thrives in a highly diverse array of environmental conditions.

Future directions

Understanding the mechanism underlying the multispecific biofilm (colony) formation in *Microcystis* holobiont would help to unveil the role of the microbiome in the evolution and environmental performance of these organisms. This will be useful to determine not only the biotic or abiotic conditions triggering microcystin production, but also to uncover the role of microcystin in the holobiont ecology and, therefore, in blooms development. We expect that this kind of knowledge would improve current (and sometimes contradictory) models of growth, fitness, dispersal and decay of these cyanobacteria, contributing to water management and risk assessment.

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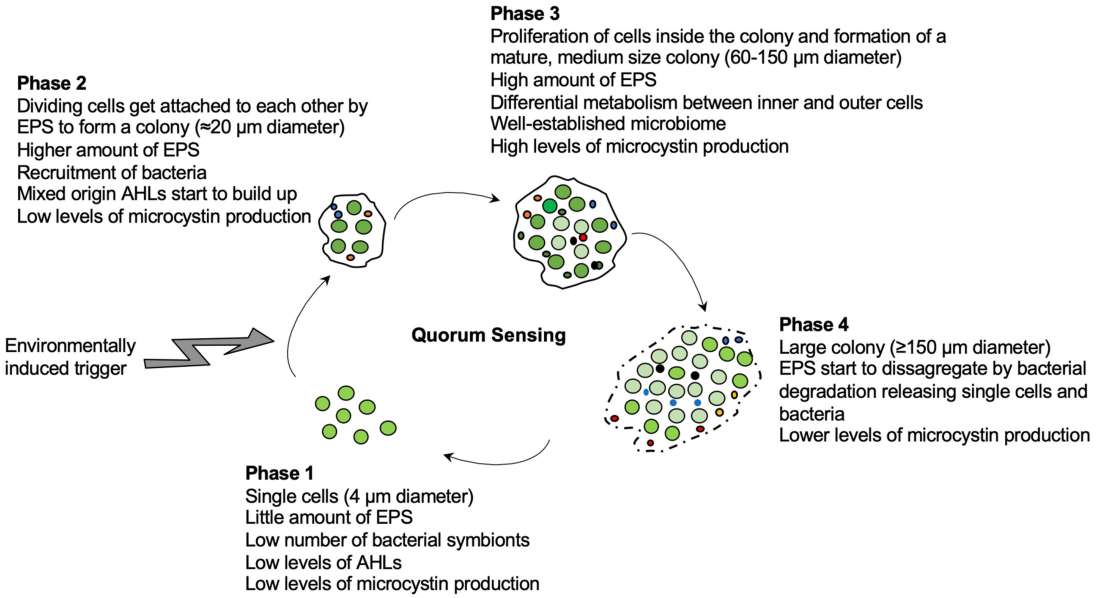

Figure 1. **Proposal for the floating biofilm formation of *Microcystis***. Four phases can be distinguished during the development of a *Microcystis* biofilm according to its lifestyle (single celled vs. attached), EPS and microcystin production, presence of an established microbiome and autoinducers 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 and their interactions with cyanobacterial cells allow 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).