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Chemically mediated interactions between *Microcystis* and *Planktothrix*: impact on their growth, morphology and metabolic profiles

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Running title: *Microcystis* and *Planktothrix* chemical interaction

Originality Significance Statement

The authors confirm that all the reported work is original, and to our knowledge this is the first report showing that physiological responses to co-culturing *Microcystis* and *Planktothrix* strains are reflected in a change in the production of the metabolites by both interacting species. These results are strong indications that potential exchange of info-chemicals in co-culture chambers occurred and is sufficient for an allelopathic activity.

Summary

Freshwater cyanobacteria are known for their ability to produce bioactive compounds, some of which have been described as allelochemicals. Using a combined approach of co-cultures and analyses of metabolic profiles, we investigated chemically mediated interactions between two cyanobacterial strains, *Microcystis aeruginosa* PCC7806 and *Planktothrix agardhii* PCC7805. More precisely, we evaluated changes in growth, morphology and metabolite production and release by both interacting species. Co-culture of *Microcystis* with *Planktothrix* resulted in a reduction of the growth of *Planktothrix* together with a decrease of its trichome size and alterations in the morphology of its cells. The production of intracellular compounds by *Planktothrix* showed a slight decrease between mono and co-culture conditions. Concerning *Microcystis*, the number of intracellular compounds was higher under co-culture condition than under monoculture. Overall, *Microcystis* produced a lower number of intracellular compounds under monoculture than *Planktothrix*, and a higher number of intracellular compounds than *Planktothrix* under co-culture condition. Our investigation did not allow us to identify specifically the compounds causing the observed physiological and morphological changes of *Planktothrix* cells. However, altogether, these results suggest that co-culture induces specific compounds as a response by *Microcystis* to the presence of *Planktothrix*. Further studies should be undertaken for identification of such potential allelochemicals.

Introduction

Phytoplankton succession is strongly constrained by resources and abiotic parameters (exploitative competition) and predation, as well as interacting phytoplankton species (interference competition) (Reynolds, 1984; Gross *et al.*, 2012). The latter includes an active process involving synthesis and release of organic bioactive compounds that directly interfere

with competitors, and may be classified as allelochemicals (Molisch, 1937; Rice, 1984). The mechanisms of allelochemical action described for phytoplankton are photosynthesis inhibition, enzyme inhibition, cell lysis, inhibition of nucleic acid synthesis and reactive oxygen species production (Legrand *et al.*, 2003; Leflaive and Ten-Hage, 2007).

Cyanobacteria are well known for their ability to produce a wide variety of secondary metabolites, some of which have been described as allelochemicals (Berry, 2008; Leão *et al.*, 2009a; Leflaive and Ten-Hage, 2007). As secondary metabolites, cyanotoxins have also been identified and studied on the base of their allelopathic properties conferring to the cyanobacteria a competitive advantage over other phytoplanktonic species. Microcystins (MCs), are the largest and most structurally diverse group of cyanotoxins that may have a significant negative impact on the aquatic ecosystem and present a hazard to animal and human health (Zanchett and Oliveira-Filho, 2013). Previous studies have reported that MCs reduce or inhibit growth and photosynthetic processes in aquatic plants (Pflugmacher, 2002; Jang *et al.*, 2007), green algae (*e.g.* *Chlamydomonas* (Kearns and Hunter, 2000), and cyanobacteria, including *Nostoc*, *Anabaena*, *Synechococcus* and *Aphanizomenon* species (Singh *et al.*, 2001; Hu *et al.*, 2004, 2005, 2008). In addition to MCs, several other cyanobacterial metabolites have also been shown to have inhibitory activity against photoautotrophs, specifically including kasumigamide (Ishida and Murakami, 2000), microcin SF608 (Wiegand *et al.*, 2002) and fatty acids (Ikawa *et al.*, 1996, 1997).

In temperate eutrophic freshwaters, *Microcystis* and *Planktothrix* may co-occur, with *Planktothrix* being an early colonizer and *Microcystis* appearing subsequently (Engelke *et al.*, 2003; Jang *et al.*, 2006; Pitois *et al.*, 2014; Reichswaldt *et al.*, 2015; Guellati *et al.*, 2017). In a previous laboratory study, Engelke *et al.* (2003) reported an increase of MC concentrations in *M. aeruginosa* PCC 7820 cells in response to the presence of the non-MC-producing *P. agardhii* CYA 29 or its spent medium, and that this interaction led to an inhibition of

P. agardhii growth. Given the evidence showing the likely role of allelopathic compounds in this inter-specific interaction, we investigated chemically mediated interactions between both cyanobacteria, the MC-producing *M. aeruginosa* PCC 7806 strain and the non-MC-producing *P. agardhii* 7805 strain, using an approach of co-cultures (strains separated by a membrane) and metabolic profiling in order to relate the presence or absence of specific markers to the allelopathic action of the strains. Our purpose-built co-culture chambers allowed to study species interactions mediated by diffusible signals that can influence the growth of the organisms and the production of metabolites. The metabolic profiling consisted first to detect the overall intra- and extracellular compounds produced by the cyanobacterial strains through implementing a non-targeted analytical strategy. Second, as both strains have their genomes sequenced and their potential to synthesize different bioactive compounds described (Frangeul *et al.*, 2008; Humbert *et al.*, 2013; Pancrace *et al.*, 2017), we further focused on four distinct classes of cyanobacterial peptides, namely the microcystins, the cyanopeptolins, the cyanobactins and the aeruginosins. These widely distributed peptides have been studied for potential pharmaceutical applications and, in many cases, protease inhibitory activity has been found (Namikoshi and Rinehart, 1996; Welker and von Döhren, 2006) and except for microcystins, only little is known about their allelopathic activity. This combined approach has recently been used to examine the impact of intra-species interactions between MC-producing and non-MC-producing *Microcystis* strains on the nature of the bioactive compounds produced and provided new insights into the factors that regulate the production of MCs and other cyanopeptides (Briand *et al.*, 2016). Hence, this approach has been applied in the present study to provide a better understanding of the allelopathic role of the cyanobacterial bioactive metabolites in interactions between species. More precisely, we evaluated changes in growth, morphology and in metabolites production and release by both interacting strains.

Results

Growth of M. aeruginosa and P. agardhii in mono- and co-culture

The growth of both strains under both conditions was monitored until the cultures reached the end of the exponential phase or even the stationary phase. Given that we used a high nutrient BG11 medium (240 mg L⁻¹ of N and 7.2 mg L⁻¹ of P) and that the concentration of cells were not higher than 2x10⁷ cells mL⁻¹ (data not shown), it is unlikely that the stationary phase was due to nutrient limitation but most likely due to light, pH or CO₂.

The biovolume of the *M. aeruginosa* strain in co-culture showed a smaller increase than in monoculture during the exponential growth phase until day 16. The final biovolume reached at the end of the co-culture experiment was however similar to that in monoculture after a sudden increase observed on day 18 concomitant with the decrease in the biovolume of *Planktothrix* (Fig. 1A). The growth rates of *Microcystis* were different between the culture conditions, respectively $\mu = 0.10 \pm 0.01 \text{ day}^{-1}$ under monoculture and $\mu = 0.07 \pm 0.003 \text{ day}^{-1}$ under co-culture (Fig. 1B). In co-culture conditions, the growth of *P. agardhii* was significantly reduced from day 9 when compared with the monocultures (Fig. 1A). At the end of the experiments (on day 21), the *P. agardhii* biovolumes were significantly three-fold lower under co-culture condition than under monoculture condition. The *P. agardhii* growth rates under co-culture condition was $0.08 \pm 0.02 \text{ day}^{-1}$, which represent a reduction of 28 % compared with those in the monoculture ($\mu = 0.11 \pm 0.02 \text{ day}^{-1}$; Fig. 1B).

Morphological alterations of the P. agardhii cells in co-culture

Culturing *P. agardhii* cells with *M. aeruginosa* cells resulted in a decrease of trichome size of *P. agardhii*, but also in alterations and deformations in the morphology of the cells (Fig. 2). While the monoculture condition was characterized by a population with a trichome length

superior to 100 μm (65 – 85 %; Fig. 2 A and B-i), under co-culture condition, the *Planktothrix* population was dominated (60 – 85 %; Fig. 2A and B-ii) by smaller trichomes (< 100 μm). Similarly, the cell volume of *P. agardhii* was lower than under monoculture condition from day 9 after inoculation with *Microcystis* population (Fig. 2C). No significant change in the cell volume and morphology was observed in the *Microcystis* population between mono- and co-culture conditions (data not shown).

Untargeted metabolomic profiling

In order to better understand the physiological and morphological responses to interactions between species, changes in the production of the overall compounds produced by both strains under both conditions were also analyzed. Based on untargeted metabolomic profiling, a total of 2730 different putative compounds were found in the entire experiment (Fig. 3). Out of the 2730 putative compounds, 822 (421+34+367) were specific to *Microcystis*, 506 (133+12+361) were only found in *Planktothrix*, whereas 1402 putative compounds were shared by both strains independently of the culture condition and the fraction. Out of the 1402 putative shared compounds, 344 were found in all samples, 352 were specific to extracellular fractions and 453 were only found in intracellular fractions.

We further evaluated the number of putative metabolites in intracellular fraction produced by both strains (*e.g.* 1400 and 1354 putative intracellular compounds respectively for *Microcystis* and *Planktothrix*; Fig. 3) to disentangle those produced under co-culture condition from those produced under monoculture condition for each strain. While 75% (197 out of the 263) of the putative compounds produced under mono-culture were also present in co-culture conditions (Fig. 4A), *Microcystis* produced five times more putative intracellular metabolites under co-culture condition (1334=1137+197) than under monoculture one (263=66+197; Fig. 4A). Out of the 1400 putative intracellular compounds, 390 were

produced by only *Microcystis* under co-culture and 24 under monoculture. Concerning *Planktothrix*, a decrease of 15% of the number of putative intracellular compounds produced under co-culture was observed compared to the number of putative metabolites recovered under monoculture (respectively $926=266+660$ and $1088=428+660$; Fig. 4A). Out of the 1354 putative intracellular compounds produced by *Planktothrix*, 124 were exclusively produced by this species under co-culture and 174 under monoculture. Forty-nine percent (660 out of the 1354) of putative intracellular compounds were produced under both mono- and co-culture conditions (Fig. 4A). Interestingly, *Microcystis* produced a lower number of putative intracellular compounds under monoculture than *Planktothrix* (respectively 263 and 1088; Fig. 4A). Inversely, *Microcystis* produced a higher number of putative compounds under co-culture condition than *Planktothrix* (respectively 1334 and 926; Fig. 4A).

We also attempted to disentangle extracellular compounds released under co-culture condition from those released under monoculture condition for each strain (Fig. 4B). The total number of released putative compounds by both strains under co-culture condition is the same as they shared the same medium (746). Out of the 746 released putative compounds under co-culture condition, 370 and 369 metabolites were also retrieved respectively in *Microcystis* and *Planktothrix* media under monoculture. A lower number of putative compounds released by *Microcystis* under co-culture condition than under monoculture one was observed (respectively 746 and 954, among which 367 were exclusively found in the medium extract of *Microcystis*). In the *Planktothrix* extracellular fraction, 629 and 746 putative compounds were found in mono- and co-culture conditions respectively. Out of the 629 putative extracellular compounds detected under monoculture, 133 were *Planktothrix*-specific substances. Interestingly, *Microcystis* released a higher number of putative extracellular compounds (954) under monoculture than *Planktothrix* (629).

Targeted metabolomic profiling

We further focused on some identified peptides produced by both strains in order to evaluate changes in relative concentrations in intra- and extracellular fractions under mono- and co-culture conditions as a response to interspecific interactions. For *M. aeruginosa*, we focused on nine peptides (two microcystins, three cyanopeptolins and four cyanobactins; Table 1), described in a previous study as major peptides produced by this strain in monoculture under the same growth condition (Briand *et al.*, 2016). For *P. agardhii*, nine different compounds were identified (Table 1). Two cyanobactins were identified as previously described compounds (prenylagaramide B and C; Murakami *et al.*, 1999; Donia and Schmidt, 2011; Fig. S1). The other seven peptides were potentially novel analogues of a known peptide class: two chlorinated aeruginosins (Fig. S2) and five cyanopeptolins (Fig. S3), based on their fragmentation patterns. According to a recent review by Kurmayer *et al.* (2014) cyanopeptolins are among the most reported secondary metabolites produced by *Planktothrix*.

Changes in cyanopeptide relative concentrations in each strain were studied (i) as a function of the physiological status of the strains under mono- and co-culture conditions and (ii) in response to interspecific interaction. Both strains produced different peptides. Hence, for each strain, the peptide quantifications were normalized against their respective quantifications under monoculture at day 7. Figures 5 and 6 show the relative concentrations of the identified intracellular (Fig. 5A and 6A) and extracellular (Fig. 5B and 6B) compounds produced by *Microcystis* (Fig. 5) and *Planktothrix* (Fig. 6) under mono- and co-culture conditions during the exponential growth phase (day 7) and the stationary phase (day 21).

Changes in the relative concentrations of identified cyanopeptides in M. aeruginosa under mono- and co-culture conditions. In mono-culture, the relative concentrations of Aer A, B,

C and D compounds in *Microcystis* were significantly lower at the end of the stationary phase than those measured at the beginning of the growth phase (Mono T7 versus Mono T21, Fig. 5A and Table 2). Those compounds were retrieved in significantly higher relative concentrations in the medium at day 21 than at day 7 (Mono T7 versus Mono T21, Fig. 5B and Table 2), suggesting a release of these compounds into the medium over the course of the experiment. The amounts of the other cyanopeptides (MCs and Cya) did not show significant differences as a function of the growth phase of the cells, neither in the cell fraction nor in the medium. In the co-culture, no general trend was observed in any of the fractions, except for Aer B, for which the decrease in the intracellular fraction and increase in the medium was significant between day 7 and day 21 (Co-culture T7 versus Co-culture T21, Fig. 5A, B and Table 2).

After 7 days of co-culture, the intracellular relative concentrations of cyanopeptides in *Microcystis* were not affected by the presence of *Planktothrix* (Mono T7 versus Co-culture T7, Fig. 5A and Table 2). However, their relative concentrations in the medium were lower than in monoculture (Mono T7 versus Co-culture T7, Fig. 5B and Table 2). After 21 days of co-culture, the presence of *Planktothrix* led to an increase of the intracellular content of seven of the nine compounds produced by *Microcystis* (des-MCLR, Cya A, B, C, Aer A, C and D, Fig. 5A and Table 2). The difference was significant for 5 compounds: Cya A, B, C and Aer A and D (Mono T21 versus Co-culture T21, Fig. 5A and Table 2). When comparing the relative extracellular concentrations of *Microcystis* compounds between the mono- and the co-culture conditions at T21, we found that all relative concentrations decreased in the presence of *Planktothrix* (Mono T21 versus Co-culture T21, Fig. 5B and Table 2).

Changes in the relative concentrations of identified cyanopeptides in P. agardhii under mono- and co-culture conditions. In the *Planktothrix* monoculture, the intracellular content

of the peptides were significantly lower at day 21 than at day 7 (Mono T7 versus Mono T21, Fig. 6A and Table 2). Those compounds were detected in the medium and their relative concentrations were not significantly different between day 7 and day 21 (Mono T7 versus Mono T21, Fig. 6B and Table 2), with the exception of the mono-chlorinated aeruginosin compound (Arg Cl), exclusively detected after 21 days in both fractions and in both conditions. Under co-culture condition, Arg Cl₂, Cya 1105 and Pre B exhibited a very similar pattern with a decrease of their relative content in the cell fraction between day 7 and day 21, whereas the amount of the other compounds did not change between both sampling days (Co-culture T7 versus Co-culture T21, Fig. 6A and Table 2). Relative concentrations of the cyanopeptides in the medium did not change between day 7 and day 21 (Co-culture T7 versus Co-culture T21, Fig. 6B and Table 2).

The relative concentrations of all investigated cyanopeptides produced by *Planktothrix* were not affected by the presence of *Microcystis*, neither in the cyanobacterial cell fraction nor in the shared medium at day 7 and day 21 (Mono T7 versus Co-culture T7 and Mono T21 versus Co-culture T21, Fig. 6A, B and Table 2).

Discussion

Although secondary metabolites are generally accepted to play an important role in mediating interspecific communication among cyanobacteria (Kaplan *et al.*, 2012, 2016), their exact functional roles are still to be elucidated. Combining physiological and untargeted metabolomic approaches on co-culturing cyanobacterial strains allowed to better understand underlying mechanisms due to interspecific interactions among cyanobacteria. In this study, we demonstrated that the presence of *Microcystis aeruginosa* affected the growth and the morphology of *Planktothrix agardhii* cells and that these physiological responses were reflected in a change in the production of the metabolites by both interacting species. These

results are strong indications that potential exchange of info-chemicals in co-culture chambers occurred between both strains and is sufficient for an allelopathic activity.

Allelopathy is usually tested by experimental approaches as culturing with cell-free filtrates or using high concentrations of isolated compounds to induce biological effects (Babica *et al.*, 2006; Leflaive and Ten-Hage, 2007). These approaches are artificial and not representative of the natural ecosystem. Direct cultivation of strains/species allowing cell-to-cell interaction is a more realistic approach to mimic interspecific interaction. Recent studies have shown stronger growth inhibition in green algae and diatoms in direct co-cultivation with *M. aeruginosa* than in membrane-separated co-culture or with cyanobacteria exudates (Dunker *et al.*, 2013, 2017; Wang *et al.*, 2017). However direct co-cultivation does not as easily allow for monitoring the respective physiological and metabolic responses of both interacting organisms. Here we used co-culture chambers allowing cells to grow without cell-to-cell contact while sharing the same media and being exposed to dissolved chemicals from the other species. Thus, membrane-separated co-culture is a good way to disentangle the metabolic response of the interacting organisms. In previous studies, the use of co-culture chambers without physical contact has facilitated the investigation of chemical communication between interacting planktonic organisms (Schatz *et al.*, 2005; Paul *et al.*, 2013; Briand *et al.*, 2016; Dunker *et al.*, 2017).

Results from both strains time-course monoculture experiments revealed a general response in the targeted metabolome with respect to time. The relative cellular concentrations of almost all identified compounds decreased between the exponential and stationary phases, suggesting that their production is linked to cellular division. This decrease in the intracellular fraction was accompanied by a concomitant decrease in the extracellular fraction of *Planktothrix* but by a significant increase in the extracellular fraction of *Microcystis* suggesting a release through cells lysis and/or an active transport. However, given the smaller

proportion of extracellular form of each compounds (maximum 25% of the total, data not shown), the total amount of these compounds produced by *Microcystis* also decreased between the exponential and stationary phases. This interesting result is in accordance with the well-established observations that production of MC is generally directly related to growth cycle (Orr and Jones, 1998; Briand *et al.*, 2012), and to our knowledge has never been reported for all other peptides presented in this study.

Both cyanobacterial strains grew exponentially in monoculture whereas their growth was impaired in co-culture with a stronger growth inhibition of *P. agardhii* leading to the dominance of *M. aeruginosa*. As temperature, light and nutrient availability were similar between treatments, these abiotic factors presumably did not play a role in the observed differences between mono- and co-culture treatments. Specific care was taken to work with axenic strains and to avoid bacterial contamination of the cultures, therefore an influence of compounds that bacteria might have released in the culture medium can also be excluded.

Possible explanation for the decrease of both cell counts in co-cultures is that the production of metabolites is biochemically and energetically costly, and that these requirements compete with nutrients and energy investments in cell structure and growth. The untargeted metabolomic approach used in this study, enables the observation of a large array of metabolites involved in the specialized metabolism of the organism and can better estimate the overall cost associated with the production of metabolites rather than focusing on specific compounds. For *Microcystis*, the number of intracellular compounds is five-fold higher in co-culture suggesting a potential cost of metabolite production on its growth. However, negative impact on *Microcystis* growth by allelochemicals from *Planktothrix* may not be ruled out. In contrast, a slight decrease of the total number of intracellular metabolites produced by *Planktothrix* was observed in co-culture compared to monoculture condition. Hence the growth response of *Planktothrix* to co-culture is not entirely associated with the

cost of metabolites production but may come from impact of specific allelopathic compounds produced by *Microcystis* and released in the media. Disentangle when the observed growth responses are caused by allelochemicals or by the costs associated to the production of compounds is an important and critical question but would require further investigations on the nature of released compounds. Our experiment is consistent with other studies showing that exudates of *Microcystis* can influence the physiology of other cyanobacteria (Bartova *et al.*, 2011; Maria *et al.*, 2015; Yang *et al.*, 2014) or green algae (Bittencourt-Oliveira *et al.*, 2015; Ma *et al.*, 2015; Dunker *et al.*, 2017; Song *et al.*, 2017; Wang *et al.*, 2017). Exposure of *Microcystis* strains may result in various effects on the targeted organism ranging from alteration of growth and cellular morphology, photosynthesis, changes in pigment, protein, increased formation of reactive oxygen species, activation of gene expression or cell lysis. However, the involved mechanism remains to be elucidated. Most of the studies link the negative impact to the production of toxic compounds, *i.e.* microcystin (Leão *et al.*, 2009) and suggest MC as a potential allelochemical allowing MC-producing species to outcompete. Whereas, other studies showed allelopathic inhibition by compounds other than microcystins (Legrand *et al.*, 2003; Schatz *et al.*, 2005; Oberhaus *et al.*, 2008; Yang *et al.*, 2014). Our study cannot conclude on a direct role of MC on the growth and morphology of *Planktothrix* because we did not test the addition of MC extract to *Planktothrix* culture.

Co-culturing *Microcystis* and *Planktothrix* resulted in significant variations on the production of metabolic compounds. First of all, *Microcystis* contained five times more putative intracellular compounds in response to the presence of *Planktothrix*. Among those compounds, 390 compounds were *Microcystis*-specific substances, exclusively produced in response to the interaction. From the targeted analysis, five major peptides (Cya A, B, C and Aer A, D) were found at higher concentrations at the end of the co-culture experiment. Although the overall production of intracellular compounds produced by *Planktothrix* was

slightly weaker under co-culture than monoculture condition (both overall and for selected cyanopeptides), 124 *Planktothrix*-specific compounds were exclusively produced in response to the interaction. An increase in the production of intracellular metabolites in response to interactions between species was also observed by Engelke *et al.* (2003) who reported, in contrast with our study, higher MC concentrations when *P. agardhii* or spent medium was added to cultures of *M. aeruginosa*. In a previous study investigating interactions within species under the same experimental condition (co-culture setup and temperature, light and nutrient), Briand *et al.* (2016) observed higher relative concentrations of MC and the other major cyanopeptides produced by *M. aeruginosa* PCC 7806 in co-culture with the non-MC-mutant (MT) strain. However, the addition of MC on MT did not induce the increase of overall compounds suggesting that MC was not the major inducing peptide. The fact that MC is not upregulated here, in the presence of *Planktothrix*, suggests that under the experimental conditions this metabolite does not play a role neither in the growth inhibition nor in the initiation of the production of additional compounds. By contrast, other compounds which are upregulated or induced (*e.g.* Cya A, B, C and Aer A, D and among the 390 *Microcystis*-specific compounds) may have played a major role in chemical communication, influencing *Planktothrix* morphology and growth and would be the most likely candidates to act as allelochemicals. The widely distributed cyanopeptolins act as serine protease inhibitors (Martin *et al.*, 1993; Gademann and Portmann, 2008), while aerucyclamides were found to have cytotoxic activities with regard to the freshwater crustacean *Thamnocephalus platyurus* (Portmann *et al.*, 2008a and b). More recently, Aer A, B, D and Cya B produced by *M. aeruginosa* PCC7806 were found to be putative inducible defense molecules against predation by *Daphnia* (Sadler and von Elert, 2014). Whether these compounds are produced as a signal of stress or serve as a competitive advantage is still unknown. Most likely, these cyanopeptides have several functions, thus allowing cyanobacteria to efficiently colonize and

dominate highly variable environment. The variations in production of specific compounds with physiological status and in response to intra- and interspecific cyanobacterial interactions reinforce the need to analyze the entire metabolome of the interacting organisms to assess the production of potential active compounds. Simultaneously, transcriptome-based characterization of interactions between co-occurring organisms would contribute to the current knowledge about the molecular mechanisms involved in the intra- and interspecies cyanobacterial interactions. Regulatory molecular mechanisms, such as activity of transposable elements, enabling cells to face various environmental conditions, like N availability (Steffen *et al.*, 2014) and grazing (Harke *et al.*, 2017), may facilitate adaptability through the regulation of the production of metabolites.

Despite being conducted under laboratory conditions and on two species belonging to two genera, our experiments provide evidence that chemical cue(s) specific to each strain induced the stimulated production of intracellular compounds and the negative impact on *Planktothrix* growth, supporting the idea that cyanobacteria have the potential to interact with each other via released compounds. Hence, we further examined the exometabolome involving dissolved chemical signals likely implied in allelopathic interactions. Interestingly, a lower number of specific compounds released by *Microcystis* under co-culture condition than under monoculture condition was observed. Moreover, species-specific compounds induced in co-culture were not all retrieved in the shared media, as well as in targeted analysis, a significant decrease of the extracellular form of most peptides was observed. Likewise, in a recent study investigating interactions within species between *M. aeruginosa* PCC 7806 and the non-MC-mutant (MT) strain, compounds detected at higher relative concentrations in cells were not necessarily found at higher relative concentrations in the media and some of them were not detected at all (Briand *et al.*, 2016). Inhibition of excretion or transformation of those compounds might cause the observed effect. Active uptake of

compounds by the other co-cultured strain is also a possibility in the same line that Bar-Yosef *et al.* (2010). They showed that under P limitation, the presence of cylindrospermopsin-producing *Aphanizomenon* promotes P supply by inducing alkaline phosphatase secretion by other phytoplankton. At this stage of our study, we are not able to discriminate specific compounds released by each strain. As the metabolome of *Planktothrix* was more stable than that of *Microcystis*, an approach with spikes of *Planktothrix* exudates into *Microcystis* cultures to induce changes in metabolite expression may provide further insights. Furthermore, untargeted metabolic footprint associated with stable isotope labelling may be an alternative way to characterize co-exposed cultures of micro-organisms and could help explore the chemical nature of the solutes that mediate biological interactions (Baran *et al.*, 2010; 2011; Weber *et al.*, 2013). Moreover, as different strains of the same species may exhibit diversity in their production of allelopathic compounds (Oberhaus *et al.*, 2008) and as the results come from a single experiment, where all treatments were conducted in duplicates, further repeated in depth mechanistic co-culture experiments using different strains are needed to firmly establish specificity of these interactions.

Experimental procedures

Cyanobacterial strains and culture conditions

Two axenic cyanobacterial strains were used: the unicellular MC-producing *M. aeruginosa* PCC 7806 and the filamentous non-MC-producing *Planktothrix agardhii* PCC 7805. Both strains have their genomes sequenced and their potential to synthesize different bioactive compounds has been described (Frangeul *et al.*, 2008; Humbert *et al.*, 2013; Pancrace *et al.*, 2017). The cultures were cultivated in BG11 medium (Stanier *et al.*, 1971) and grown under a 12:12 h light:dark regime using daylight white fluorescent tubes (Toshiba, 15 W, FL15D) with $35 \mu\text{mol m}^{-2} \text{s}^{-1}$ illumination at a constant temperature of 22°C. The cultures were

maintained in exponential growth phase by repeated dilution in fresh culture medium, whereas the axenicity was regularly evaluated as in (Briand *et al.*, 2012). Both strains are available from the Pasteur Culture collection of Cyanobacteria (<https://webext.pasteur.fr/cyanobacteria/>).

Mono- and co-culture experiments

Mono- and co-culture experiments were performed in the purpose-built growth chamber used in Briand *et al.* (2016). The device consists of two chambers with a 90 mm flat edge opening on their median part and a 25 mm round neck on the top. Both parts are separated by a 0.45 μm cellulose nitrate membrane filter (Whatman, Buckinghamshire, UK) that enabled the passage of fluids and dissolved substances between the two chambers, but not cells. A total of six devices (12 chambers) were used in order to have the following conditions in duplicate: (1) *Microcystis* monocultures (M/M) were initiated by inoculation of *M. aeruginosa* PCC7806 cells in both chambers, (2) *Planktothrix* monocultures (P/P) consisted of both chambers inoculated with *P. agardhii* PCC7805 cells and (3) Co-culturing experiments (M/P) consisted of one chamber inoculated with *M. aeruginosa* and the other with *P. agardhii*. Inoculates of cultures were centrifuged (10 min at 4000 g and 22°C) and washed with sterile BG11 medium twice before being added at the same biovolume (120 mm³ L⁻¹) to the respective chamber containing 425 mL of BG11 medium. The devices were gently shaken at 60 rpm. Monocultures and co-cultures experiments were performed once, at the same time under 12:12 h light:dark regime, 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination and 22°C. Samples were taken every two or three days for growth kinetics and physiological cell characteristics. Samples were harvested at the beginning of the exponential growth phase (day 7) and at the stationary phase (day 21) to study the intra- and extracellular secondary metabolic profiles by UHPLC-QTOF-MS/MS.

Growth kinetics and physiological cell characteristics

Aliquots of culture (4 mL) were taken every two or three days to estimate cell concentration and cell volume. Cyanobacterial growth was estimated by converting the optical density (OD) at 750 nm, measured using an UVIKON-XS double-beam spectrophotometer (Bio-Tek Instruments Inc, Winooski, USA), into cell concentration (cells mL⁻¹) based on the highly significant positive correlations between these two parameters for each strain ($R^2 = 0.993$, $n = 30$, $p < 0.01$ for *M. aeruginosa* and $R^2 = 0.996$, $n = 30$, $p < 0.01$ for *P. agardhii*, data not shown). Initially, cell concentration was measured using a Nageotte counting chamber with an Olympus BX50 microscope at 400× magnification (Olympus Optical Co, Tokyo, Japan).

The cell volumes were also measured using an Olympus BX50 microscope at 400× magnification (Olympus Optical Co, Tokyo, Japan). At least 30 images of single cells were taken from each replicate of each experiment with a Power HAD DXC-950P camera (Sony Corporation, Tokyo, Japan) and measured using PegasePro®Full version 4.0 software (2I System, Paris, France). The precision was $\pm 0.1 \mu\text{m}$. This equipment was calibrated using a stage micrometer, and the cell area was computed by the analyzer from the number of pixels forming the image. The cell volume was calculated using the formula for a spherical shape for *M. aeruginosa* cells and a cylindrical shape for *P. agardhii* taken from Sun and Liu (2003).

The specific growth rates, μ (day⁻¹) were calculated during the exponential growth phase (between day 4 and day 16) according to the following equation: $(\ln N_2 - \ln N_1) / (t_2 - t_1)$, where N_1 and N_2 are biovolume (mm³ L⁻¹) at time t_1 and t_2 , respectively. Day 4 was chosen to represent the beginning of the complete interspecific interaction after the metabolites had time to diffuse across the membrane as tested in Briand *et al.* (2016).

Sampling, extraction and internal standard addition

Samples were harvested at the beginning of the exponential growth phase (day 7; 60 ml) and at the stationary phase (day 21; 200 ml) to study the intra- and extracellular secondary metabolic profiles of each strain under mono- and co-culture conditions. The volume of each chamber was harvested separately and centrifuged (for 10 min at 4000 g and 22°C) to separate supernatants from biomass. Freeze-dried biomass was extracted up to three times in CH₂Cl₂/MeOH (2:1, v/v). After removal of the solvent under nitrogen, the intracellular extracts were dissolved in 200 µL CH₃CN/H₂O (1:1, v/v), using sonication (2 min). Each intracellular extract was purified using a disposable 100 mg reversed-phase C₁₈ cartridge (SupelcleanTM LC-18 SPE, Supelco Analytical, Bellefonte, PA, USA) by loading 60 µL and rinsing with 3 mL CH₃CN to remove compounds that would not elute from reversed-phase column material. Supernatants containing extracellular compounds were extracted onto SPE-C₁₈ cartridges (Discovery[®] DSC-18, 1000 or 5000 mg depending on the volume of supernatant, Supelco Analytical, Bellefonte, PA, USA). Compounds were recovered from these cartridges by elution with 18 or 60 mL CH₃CN. Intracellular and extracellular extracts were evaporated to dryness under nitrogen and kept frozen until analysis.

Dry intra- and extracellular extracts were dissolved with CH₃CN and internal standard (BOC-L-protected Ornithine, 0.25 mg mL⁻¹ in CH₃CN, Sigma-Aldrich, St Louis, MO, USA) were added before UPLC-QTOF-MS analysis.

UHPLC-QTOF-MS/MS analysis, metabolomic profiling and data processing

As microbiological samples may comprise up to tens of thousands of metabolites of high diversity an untargeted metabolomics approach with ultra-high performance technology was used to allow for profiling of a large number of compounds. We coupled ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry

(UHPLC-QTOF-MS), previously developed by (Mondeguer *et al.*, 2012), and subsequently applied multivariate statistical analysis to highlight differences in *Microcystis aeruginosa* monoculture, *Planktothrix agardhii* monoculture and their co-cultures and so examine if this unbiased approach without preconceptions confirms the biological hypotheses posed.

The samples were analyzed by high-resolution tandem mass spectrometry (HRMS/MS), through implementing a non-targeted analytical strategy to detect the overall small intra- and extracellular compounds produced by the cyanobacterial strains recovered from the biomass and the culture medium. We further focused on the production of some targeted compounds produced specifically by *Microcystis aeruginosa* or *Planktothrix agardhii*.

Instrumentation

An Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6550 iFunnel QTOF hybrid mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with dual Jetstream® electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution to calibrate continuously detected m/z ratios was used. The Agilent MassHunter Workstation software (version B.07) was used to process the raw MS data, including extraction of molecular features (MFs). Mass Profiler Professional software (version 13.1.1) was used for generation of molecular formula, library searching and database searching.

LC-QTOF-MS/MS analysis

Without additional preparation, aliquots (5 μ l) of each extract were separated on a Kinetex 2.6 μ m XB C₁₈ 100Å (Phenomenex, Le Pecq, France) column (100 \times 2.1 mm) maintained at 40°C, with water (A) and 95% acetonitrile / water (B), both containing 2 mM ammonium

formiate and 50 mM formic acid. The flow rate was set at 0.3 ml min⁻¹. The gradient was as follow: starting at 5% B during 2 min then rising from 5% B to 50% B in 6 min then raise to 75% B in 3.50 min to finally raise to 100% B and maintain at 100% B during 2 min, subsequently returned to initial condition in 0.50 min and re-equilibrate at 5% B during 4 min.

Detection was carried out by operating in full scan and auto MS/MS in positive ion mode. The capillary voltage, nozzle voltage and fragmentor voltage were set to 4500, 500 and 365 V, respectively. The sheath gas was at 250°C (11 mL min⁻¹) and the drying gas at 160°C (11 mL min⁻¹) and nebulizer 45 psi. Nitrogen was used as collision gas.

Mass spectra were acquired in full scan analysis over an m/z range of 100–1700 using a range of 2 spectra s⁻¹ and storage in both profile and centroid mode. MS/MS spectra were acquired in auto MS/MS mode over an m/z range 50 to 1700 with a MS and MS/MS scan rate of 1 spectra s⁻¹. The collision energy was applied with a slope of 0.5 and an offset of 30. A maximum of 3 precursors per cycle was selected with an active exclusion of 5 spectra after 30 s.

Our instrument gave a resolution of 15,000 Full Width at Half Maximum (FWHM) at m/z 118.0862 and 30,000 FWHM at m/z 922.0098. To assure the desired mass resolution, continuous internal calibration (Tuning Mix) was performed during analyses by using the signals at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonated hexakis (1H,1H,3Htetrafluoropropoxy) phosphazine (HP-921)] in the positive ion mode. All samples analyzed (28), were injected twice in the same batch and methanol injections were included every five samples as a blank run to assess carry-over.

Data processing and statistical analysis

Several pre-processing steps (feature findings, alignment, filtration and normalization) were used to reduce a large number of data to an operational size and to obtain an adequate set of compounds before applying multivariate analysis.

To process all data obtained by LC–QTOF in full scan MS mode, treatment of raw data files was started by extraction of potential molecular features (MFs) with the suited algorithm included in the software. The Molecular Feature Extraction (MFE) algorithm is optimized to improve the extraction of metabolite information. Peak filters were set to centroid height and minimum threshold absolute abundance of ions was set at 100 counts for positive ionization mode with all charge states allowed for the obtained ions. This rather low cut-off value was established taking into account the low intensity of the molecular microcystin-LR toxin ($[M+H]^+$ at m/z 995.588 @ RT 8.049 min) in intra- or extracellular *Microcystis aeruginosa* monoculture. Additionally, the isotopic distribution to consider MFs as valid should be defined by two or more ions (with a peak spacing tolerance of 0.002 m/z , plus 5.0 ppm in mass accuracy). Ion and adduct formation in the positive mode ($[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$), as well as neutral loss by dehydration, were included to identify features corresponding to the same potential metabolite. Thus, ion clusters with identical elution profiles and related m/z values (representing different adducts or isotopes of the same compound) were extracted as molecular features characterized by their retention time (RT), intensity in the apex of the chromatographic peaks and accurate mass. In this way, raw data files were created in compound exchange format files (cef files) for each sample and exported into the Mass Profiler Professional software package (version 13.1.1, Agilent Technologies, Santa Clara, CA, USA) for further processing. The parameters of Mass Profiler Professional were set as follows: Created next experiment type: Combined (Identified + Unidentified); Organism: None. Workflow type: Data Import Wizard. Minimum absolute abundance: 100 counts; Compound alignment: RT window = 0.5% + 0.5 min, Mass window = 15.00 ppm

+2.0 mDa; Baseline Option: None. To better represent the overall production of compounds expressed by both strains under mono- and co-culture conditions, data from the duplicated injections and the duplicated cultures at each time point (day 7 and day 21) were grouped to generate eight replicates. Only peaks present in both injections, and in the two culture duplicates and at both time points were selected before applying statistical analysis (only compounds that were present in 8 of 8 replicates were accepted to be taken further). In the software this filtration is referred to as a frequency-filter.

Identification of metabolites and their relative quantification

To identify cyanobacterial secondary metabolites, mass spectra were manually verified in MassHunter Qual (version B 7.0) for molecular ion masses of known peptides and their respective adducts (*e.g.* sodium adducts with $\Delta m = 22$ Da compared to the singly protonated molecular mass), and loss of water peaks ($\Delta m = 18$ Da). Further, the mass differences between and the relative intensity of isotopic peaks were analyzed to gain information on the presence of possible halogen atoms (*e.g.* chlorine, bromine). MS/MS data were then analyzed for indicative fragments or series of fragments by comparison to fragment spectra of known peptides according to Welker *et al.* (2006).

In order to compare relative concentrations of specific metabolites between different treatments, the peak area was determined with the software MassHunter Qual (version B 7.0). The quantification was based on the ratio of the peak area of metabolites and the added internal standard, BOC-L-protected ornithine. Data were then normalized to the dry weight for intracellular metabolites and to the volume for extracellular metabolites as described in Winnikoff *et al.* (2014). We only quantified the compounds relatively, therefore we cannot draw conclusions about absolute contents of respective compounds in any of the experiments.

Statistical analyses

Differences in growth and normalized relative cyanopeptide concentrations were evaluated using unpaired *t*-test or one-way ANOVA with Tukey's post test. The analyses were conducted with the software GRAPHPAD PRISM 4.00 (San Diego, CA, USA). In all cases, differences were accepted as significant when $P < 0.05$. Values are given as mean \pm standard deviation.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Table and Figures

Table 1. Peptide classes previously known to be produced by the two species, also identified in the strains of the present study.

<i>Microcystis aeruginosa</i> PCC 7806			
Peptide class	<i>m/z</i> [M+H]⁺	Retention time (min)	Assignment (Reference)
Microcystin	981.5417	8.098	Des-MCLR (Mayumi <i>et al.</i> , 2006)
	995.5585	8.098	MC-LR (Mayumi <i>et al.</i> , 2006)
Cyanopeptolin	929.5336	8.554	Cyanopeptolin B (Martin <i>et al.</i> , 1993)
	943.5512	8.63	Cyanopeptolin C (Martin <i>et al.</i> , 1993)
	957.5586	8.63	Cyanopeptolin A (Martin <i>et al.</i> , 1993)
Cyanobactin	517.2241	10.526	Aerucyclamide C (Portmann <i>et al.</i> , 2008b)
	533.2014	10.754	Aerucyclamide B (Portmann <i>et al.</i> , 2008a)
	535.2171	10.374	Aerucyclamide A (Portmann <i>et al.</i> , 2008a)
	603.1525 [M+OH] ⁺	7.34	Aerucyclamide D (Portmann <i>et al.</i> , 2008b)

<i>Planktothrix agardhii</i> PCC 7805				
Peptide class	<i>m/z</i> [M+H]⁺	Retention time (min)	Assignment (Reference)	Comment
Aeruginosin	835.3323	6.581	Aeruginosin 834	chlorinated
	869.2929	6.809	Aeruginosin 868	dichlorinated
Cyanopeptolin	1106.5439	8.368	Cyanopeptolin 1105	
	1120.5596	8.427	Cyanopeptolin 1119	
	1134.4815	8.443	Cyanopeptolin 1133	
	1145.5436	8.899	Cyanopeptolin 1144	
	1091.5324	9.446	Cyanopeptolin 1090	
Cyanobactin	929.5150	10.374	Prenylagaramide B (Murakami <i>et al.</i> , 1999; Donia and Schmidt, 2011)	
	1021.6108	10.602	Prenylagaramide C (Donia and Schmidt, 2011)	

Table 2. Statistical results relative to figure 5 and 6. ANOVA/Tukey's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$, ns: non-significant, nd: not detected, Mono: monoculture.

	<i>Microcystis</i>	des-MCLR	MC-LR	Cya A	Cya B	Cya C	Aer A	Aer B	Aer C	Aer D
Intracellular	Mono. T7 vs. Mono. T21	ns	ns	ns	ns	ns	**	***	*	*
	Co-culture T7 vs. Co-culture T21	*	ns	ns	ns	ns	ns	**	ns	ns
	Mono. T7 vs. Co-culture T7	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Mono. T21 vs. Co-culture T21	ns	ns	*	*	*	**	ns	ns	**
Extracellular	Mono. T7 vs. Mono. T21	ns	ns	ns	nd	ns	****	****	***	***
	Co-culture T7 vs. Co-culture T21	ns	ns	ns	nd	*	ns	**	ns	ns
	Mono. T7 vs. Co-culture T7	ns	ns	ns	nd	ns	***	****	*	***
	Mono. T21 vs. Co-culture T21	ns	*	*	nd	*	****	****	***	***
	<i>Planktothrix</i>	Arg Cl	Arg Cl ₂	Cya 1105	Cya 1119	Cya 1133	Cya 1144	Cya 1090	Pre B	Pre C
Intracellular	Mono. T7 vs. Mono. T21	**	****	**	**	**	*	**	***	*
	Co-culture T7 vs. Co-culture T21	***	****	***	ns	ns	ns	ns	**	ns
	Mono. T7 vs. Co-culture T7	ns	ns	*	ns	ns	ns	ns	ns	ns
	Mono. T21 vs. Co-culture T21	ns	ns	ns	ns	ns	ns	ns	ns	ns
Extracellular	Mono. T7 vs. Mono. T21	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Co-culture T7 vs. Co-culture T21	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Mono. T7 vs. Co-culture T7	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Mono. T21 vs. Co-culture T21	ns	ns	ns	ns	ns	ns	ns	ns	ns

Figure 1. The biovolumes (A) and growth rates (B) of both *M. aeruginosa* and *P. agardhii* under mono- and co-culture conditions. The asterisks indicate the biovolumes of *P. agardhii* in the co-culture that were significantly different from those in monoculture with $P < 0.05$ in unpaired t-test.

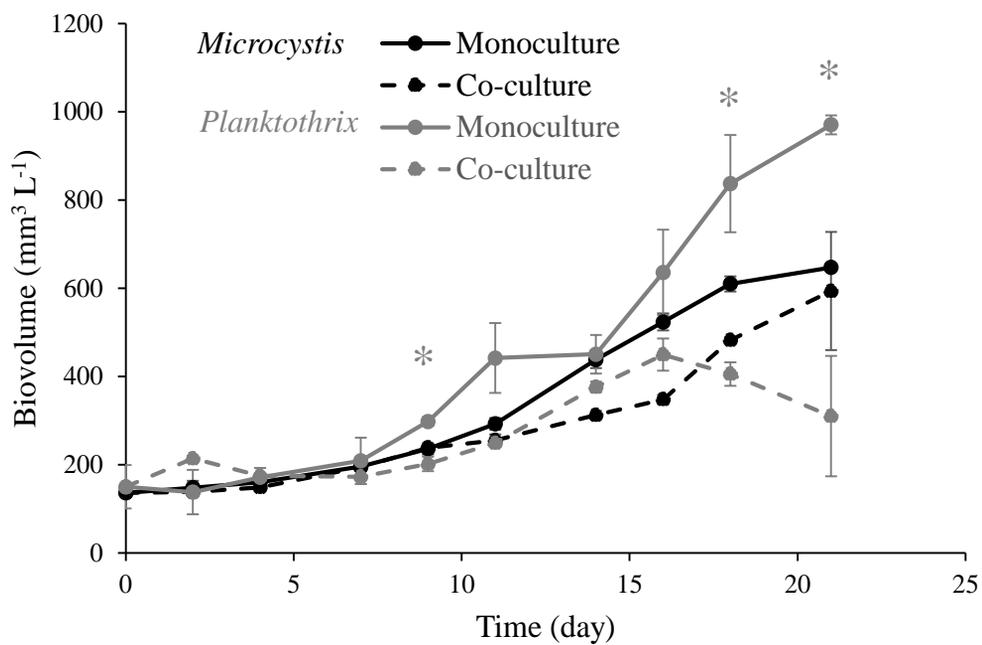
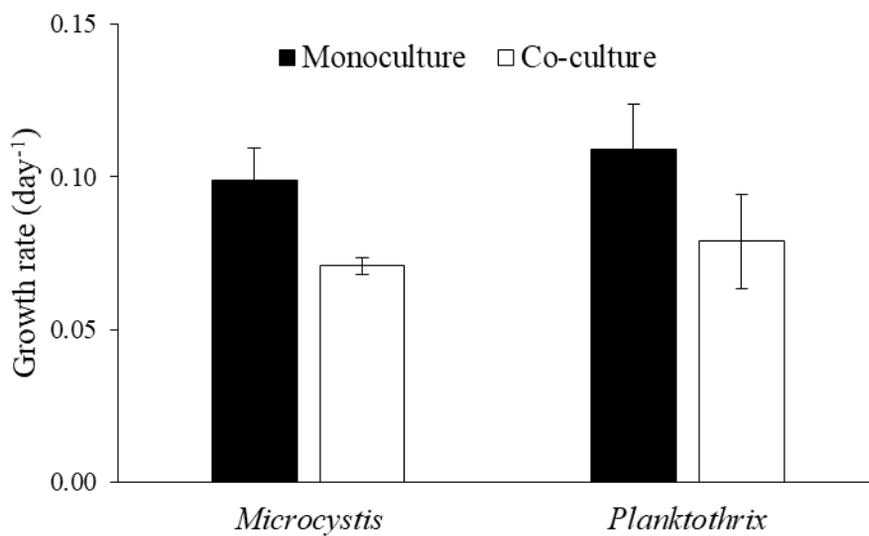
Figure 2. Percentage of trichomes and morphology of *P. agardhii* cells grown under mono- and co-culture conditions. **A** Percentage of trichomes lengths higher (solid bars) and lower (hatched bars) than 100 μm under mono- (black bars) and co-culture (grey bars) conditions. **B** Representative microphotographs highlighting the morphology of *P. agardhii* cells grown under mono- (i) and co-culture (ii) conditions. **C** Volume of *P. agardhii* cells under mono- (solid black line) and co-culture (grey dashed line) conditions.

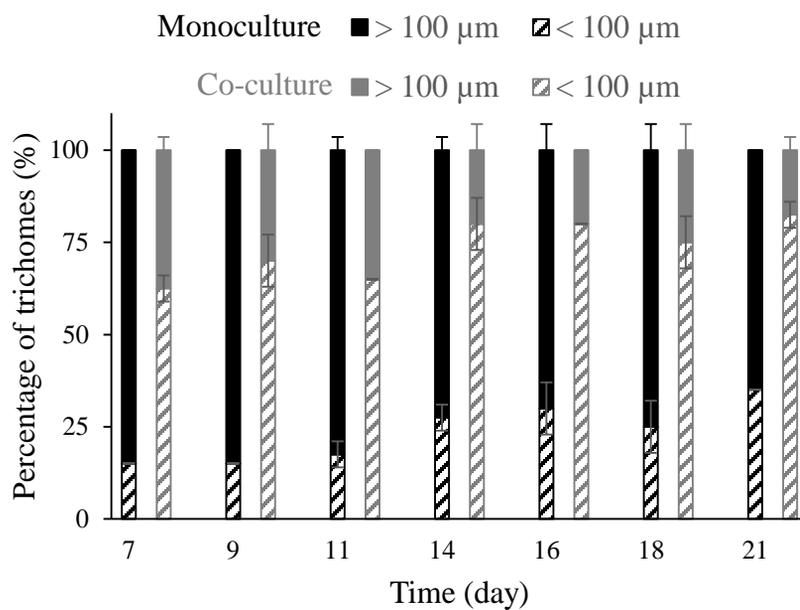
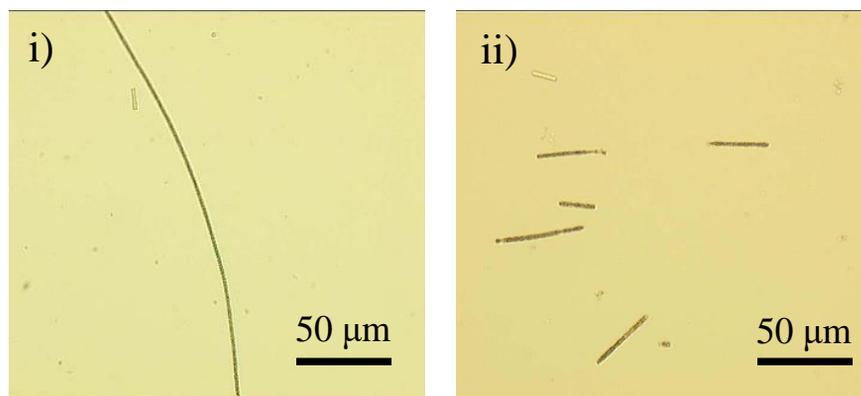
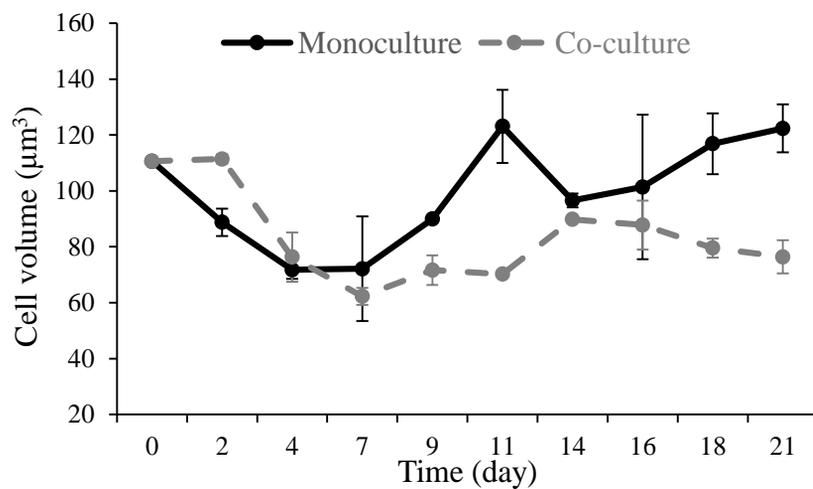
Figure 3. Venn diagram representing the number of specific and shared compounds recovered from both monoculture and co-culture conditions by *Microcystis* and *Planktothrix* strains in intra- and extracellular fractions during the experiment (data from day 7 and day 21 were grouped).

Figure 4. Number of compounds recovered under both mono- and co-culture conditions by *Microcystis* and *Planktothrix* strains (**A**: intracellular compounds, **B**: extracellular compounds). Common compounds are compounds that are recovered in both mono- and co-culture conditions. Number in brackets refers to specific compounds produced and released either by *Microcystis* or *Planktothrix*.

Figure 5. Relative concentrations given as a fold change over *M. aeruginosa* under monoculture condition at day 7 of selected intracellular (**A**) and extracellular (**B**) compounds for *M. aeruginosa* under mono- and co-culture conditions at day 7 and 21. Aer: aerucyclamide, Cya: cyanopeptolin, MC: microcystin.

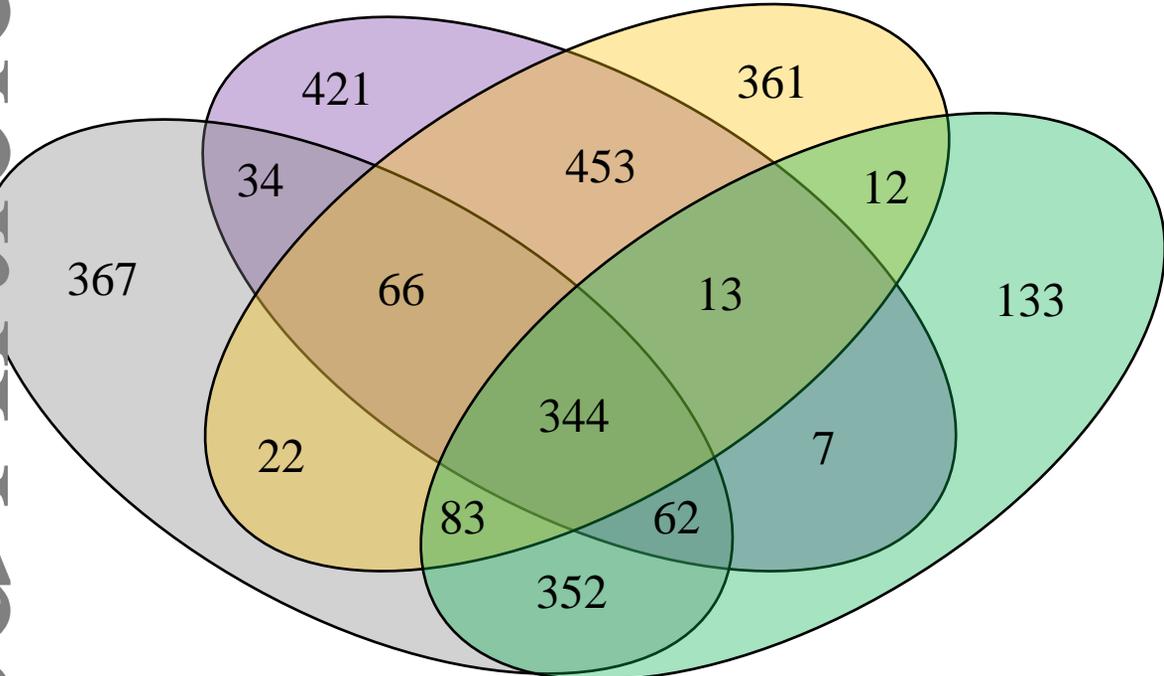
Figure 6. Relative concentrations given as a fold change over *P. agardhii* under monoculture condition at day 7 of selected intracellular (**A**) and extracellular (**B**) compounds for *M. aeruginosa* under mono- and co-culture conditions at day 7 and 21. Arg: aeruginosin, Cya: cyanopeptolin, Pre: prenylagaramide.

A**B**

A**B****C**

Microcystis:
Intracellular compounds
(1400)

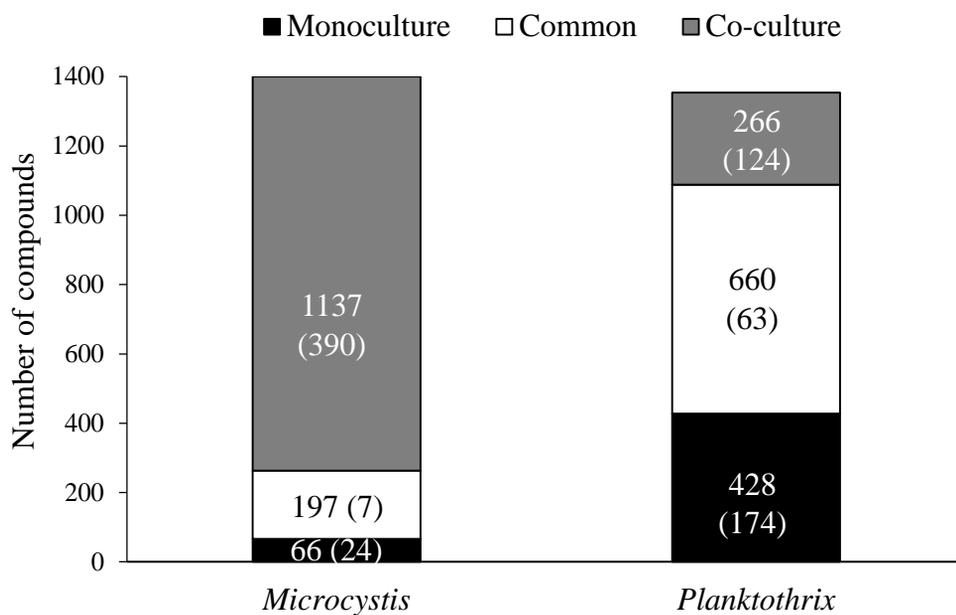
Planktothrix:
Intracellular compounds
(1354)



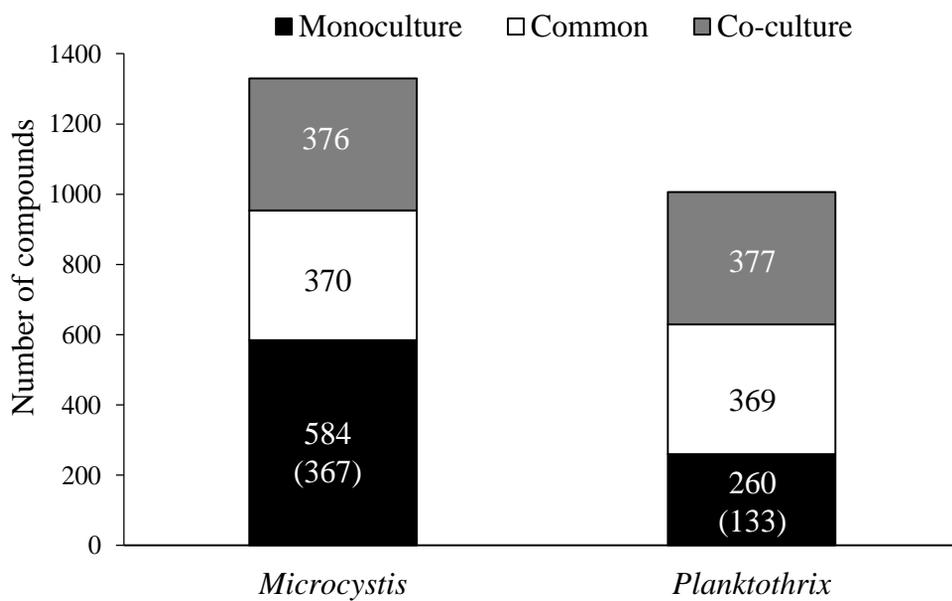
Microcystis:
Extracellular compounds
(1006)

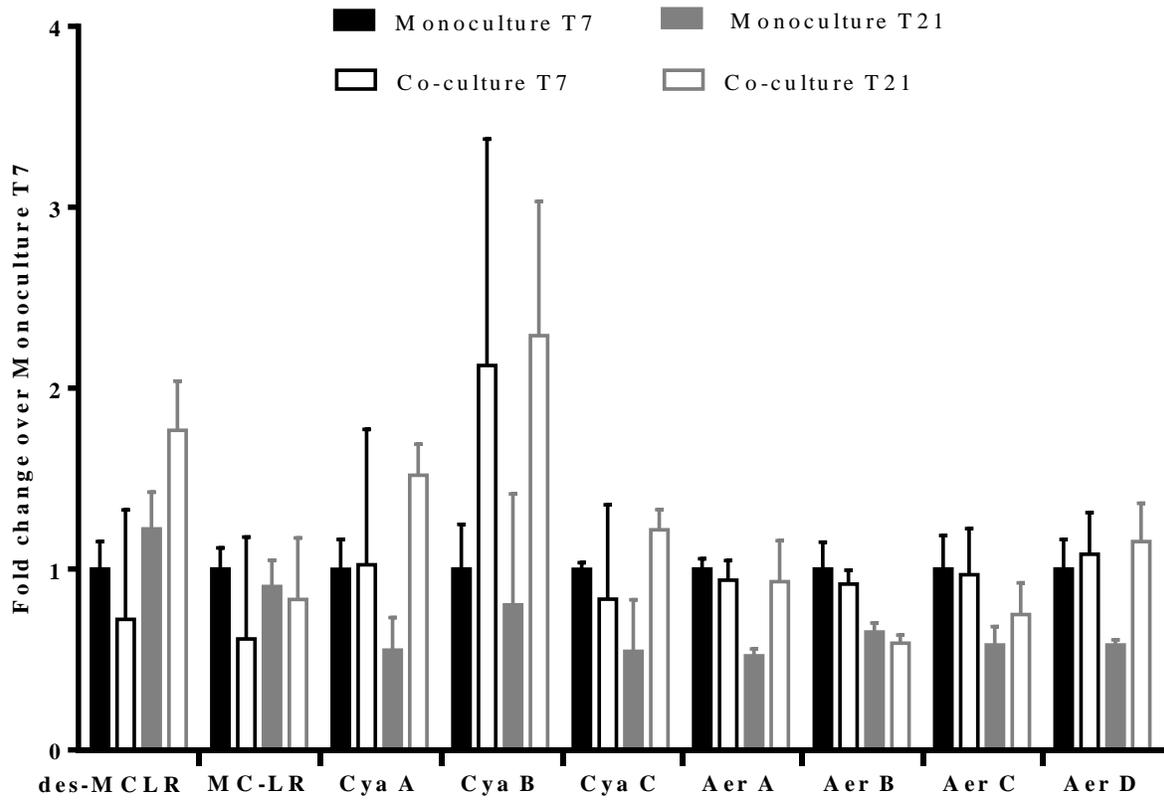
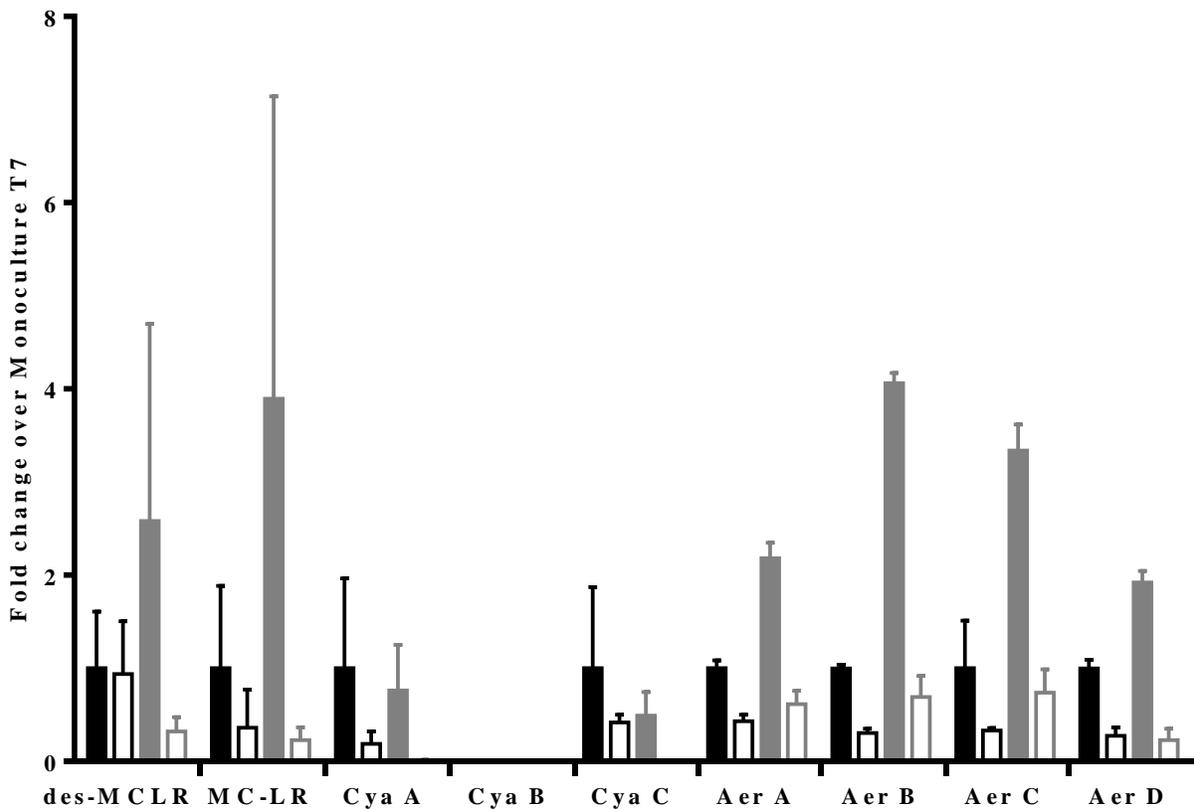
Planktothrix:
Extracellular compounds
(1330)

A: Intracellular

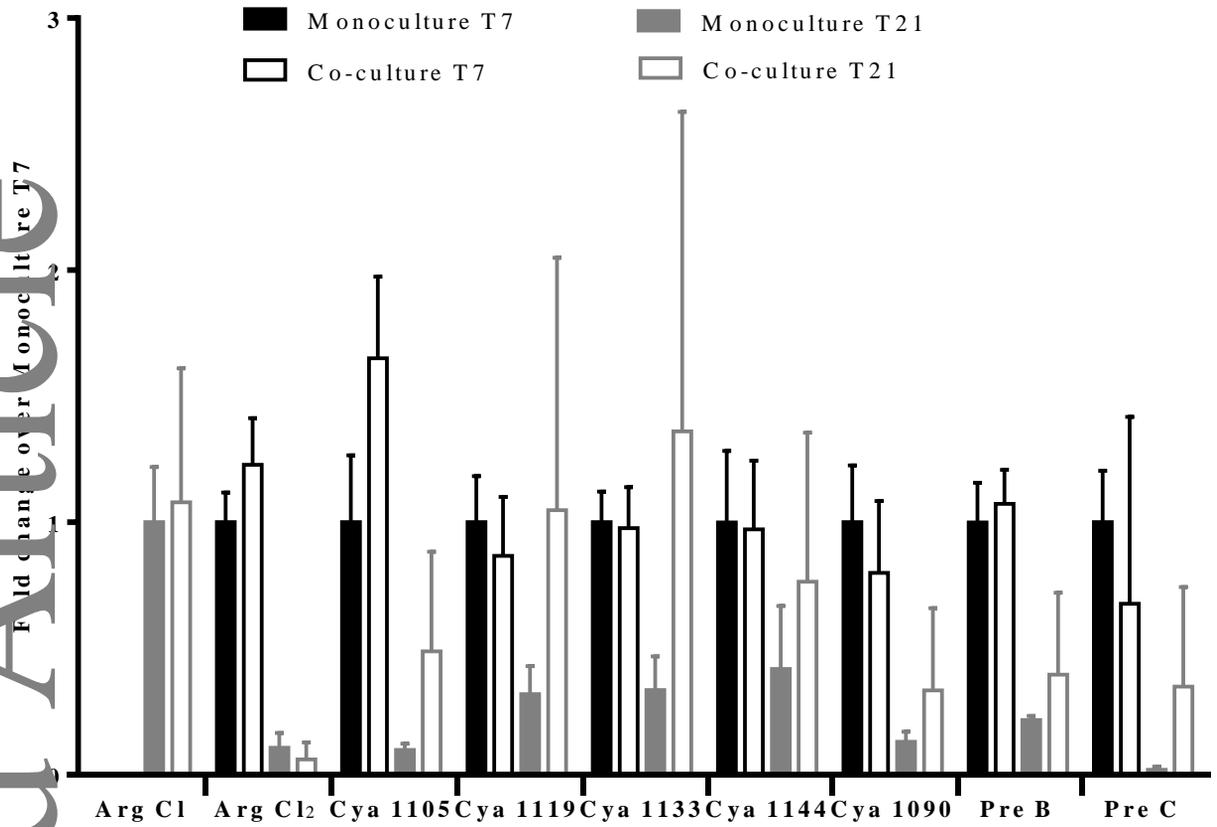


B: Extracellular



A: Intracellular**B: Extracellular**

A: Intracellular



B: Extracellular

