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Justine Demay

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THÈSE

pour obtenir le grade de

DOCTEUR DU MUSÉUM NATIONAL D'HISTOIRE NATURELLE

Spécialité : Microbiologie

présentée et soutenue publiquement par

Justine DEMAY

le 08/12/2020

Diversité et activités de métabolites de cyanobactéries des boues thermales de Balaruc-les-Bains

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Liste des abréviations

ANTX	Anatoxine
CYN	Cylindrospermopsine
EPS	Exopolysaccharide
HaCaT	Kératinocytes humains
LC-MS	Chromatographie liquide couplée à de la spectrométrie de masse
LC-MS/MS	Chromatographie liquide couplée à la spectrométrie de masse en tandem
LPS	Lipopolysaccharide
MAAs	Mycosporine-like amino acids
PMBC	Cellules mononuclées isolées à partir de sang périphérique humain
PMC	Paris Museum Collection
RAW 264.7	Macrophages murins
RMN	Spectroscopie par résonance magnétique nucléaire

Liste des publications et communications

Publications

Demay, J.; Bernard, C.; Reinhardt, A.; Marie, B. Natural Products from Cyanobacteria: Focus on Beneficial Activities. *Mar. Drugs* 2019, 17, 320.

Conception de la revue, écriture et correction du manuscrit

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Demay, J.; Halary S.; Knittel-Obrecht A.; Villa P.; Duval C.; Hamlaoui S.; Roussel T.; Yéprémian C.; Reinhardt A.; Bernard C.; Marie B. Antioxidant and anti-inflammatory properties of cyanobacteria from thermal mud (Balaruc-les-Bains, France): a multi-approach study. (soumis à *Biomolecules*)

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Communications

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Contexte général de la thèse

Les cyanobactéries sont des microorganismes procaryotes capables de réaliser la photosynthèse oxygénique comme source d'énergie pour leur métabolisme. Elles seraient apparues sur Terre il y a environ trois milliards d'années. Grâce à leur capacité de rejeter du dioxygène (O₂) lors de la photosynthèse, les cyanobactéries sont considérées comme responsables de l'oxygénation de l'atmosphère terrestre primitive, ce qui a conduit à la formation de la couche d'ozone. Les cyanobactéries sont également au cœur de nombreuses théories sur l'évolution de la vie sur Terre puisqu'elles sont à l'origine, par endosymbiose, de l'apparition des chloroplastes, sièges de la photosynthèse des cellules des végétaux supérieurs (Ochoa De Alda et al. 2014). Les cyanobactéries regroupent de nombreuses espèces aux morphologies très diverses, allant des formes unicellulaires aux formes multicellulaires filamenteuses. Elles possèdent des modes de vie et de dispersion très variés. On recense en effet des formes planctoniques, d'autres benthiques, capables de former des efflorescences ou encore de vivre en symbiose avec d'autres organismes. Cette diversité permet aux cyanobactéries de coloniser de nombreux habitats comme les environnements marins, dulcicoles ou terrestres et des environnements extrêmes tels que les sources thermales, les glaciers ou encore les déserts (Mazard et al. 2016; Sciuto and Moro 2015).

Bien que naturels, les phénomènes d'efflorescence de cyanobactéries sont de plus en plus fréquemment observés sur l'ensemble du globe, principalement à cause du réchauffement climatique et de l'eutrophisation des milieux aquatiques, et posent des problèmes notables dans la gestion des ressources en eau. En effet, au-delà des perturbations engendrées sur les réseaux trophiques en terme d'accès aux ressources (nutriments et lumières), les cyanobactéries sont connues pour produire de nombreuses molécules bioactives. Parmi ces composés, les cyanotoxines sont particulièrement étudiées puisqu'elles ont montré des effets néfastes sur la santé humaine (Buratti et al. 2017). On compte par exemple des molécules neurotoxiques (anatoxine-a, saxitoxines), hépatotoxiques (microcystines, nodularines et cylindrospermopsines) et dermatotoxiques (lyngbyatoxines et aplysiatoxines) (Meriluoto et al. 2016). Les cyanotoxines représentent néanmoins une faible proportion de la diversité de molécules produites par les cyanobactéries. Ces molécules présentent d'ailleurs une incroyable variété de structures chimiques (alcaloïdes, terpènes, lipides, polysaccharides, peptides, ...) et peuvent présenter de nombreuses bioactivités (cytotoxiques, antimicrobiennes, anti-inflammatoires, ...) (Ali Shah et al. 2017).

Les métabolites issus des cyanobactéries font, en outre, l'objet de nombreux travaux de recherche dans le but d'identifier de nouveaux composés actifs utilisables par l'Homme. De par leurs bioactivités diverses, les molécules produites par les cyanobactéries sont potentiellement exploitables dans un certain nombre de champs d'application, notamment dans le domaine pharmaceutique (Vijayakumar and Menakha 2015; Tan 2006; William H Gerwick and Fenner 2013), cosmétique (Mourelle et al. 2017), agroalimentaire (Rastogi and Sinha 2009; Singh et al. 2017; Lau et al. 2015; Ciferri and Tiboni 1985) ou encore dans le développement d'énergies renouvelables (Zahra et al. 2020). Des brevets sont régulièrement publiés concernant les molécules produites par les cyanobactéries, leur mode de production et leurs potentielles applications (Pedro N Leao et al. 2016; William H. Gerwick 1990; Dieckmann et al. 2006; W H Gerwick et al. 1995). L'un des meilleurs

exemples de développement d'un médicament à partir d'un métabolite isolé d'une cyanobactérie est celui de la dolastatin 10 (Newman and Cragg 2017). Cette molécule hautement cytotoxique, isolée pour la première fois en 1987, a fait l'objet d'intenses travaux de recherche afin de développer un médicament anticancéreux, le brentuximab vedotin, approuvé par la Food and Drug Administration en 2011 et utilisé depuis dans le traitement du lymphome dans la maladie de Hodgkin (Newman and Cragg 2017).

Malgré les nombreux travaux visant à décrire les molécules produites par les cyanobactéries, peu d'industries utilisent les cyanobactéries ou les microalgues pour produire des molécules pour la pharmaceutique ou la cosmétique. Le marché est globalement dominé par les cultures de *Chlorella vulgaris* et d'*Arthrospira platensis* (« spiruline ») qui sont utilisées « entières » en agroalimentaire et en cosmétique, la biomasse étant produite dans des étangs à ciel ouvert (Apel and Weuster-Botz 2015; Furmaniak et al. 2017). La production industrielle de cyanobactéries présente d'ailleurs de nombreux challenges que ce soit par un procédé « ouvert » ou en bioréacteur « fermé ». En effet, la culture « ouverte » n'est pas toujours adaptée à toutes les espèces de cyanobactéries qui ne sont pas toutes capables d'atteindre de fortes biomasses. De plus les conditions optimales de culture nécessitent souvent une température assez élevée ainsi qu'un taux d'ensoleillement suffisant, ce qui limite l'implantation de telles exploitations dans certaines zones géographiques (Zahra et al. 2020; Furmaniak et al. 2017). La culture en bioréacteurs, quant à elle, est souvent coûteuse car il faut ajouter au réacteur de la lumière, du CO₂ et d'autres nutriments nécessaires à la croissance de la biomasse, ce qui ne peut être utilisé que pour la production de produits à haute valeur ajoutée. Néanmoins, le développement de ce type de procédé permet un contrôle quasi-total de la culture et des conditions (mesure en temps réel du pH, du taux de CO₂ et/ou d'O₂) et présente donc de nombreux intérêts (Apel and Weuster-Botz 2015; R. Singh et al. 2017).

Par leurs propriétés anti-inflammatoires et antioxydantes, certains métabolites produits par les cyanobactéries pourraient d'ailleurs apporter des bénéfices thérapeutiques dans le cadre des cures thermales. Localisée au bord de l'étang de Thau, à cinq kilomètres de Sète, la ville de Balaruc-les-Bains est située à proximité d'une source d'eau thermale exploitée depuis l'Antiquité, notamment pendant l'époque gallo-romaine. Seule station thermale de Méditerranée, la ville est restée un lieu reconnu pour ses traitements en rhumatologie et phlébologie. En 1969, les cures thermales se démocratisent et la ville de Balaruc-les-Bains édifie un bâtiment capable d'accueillir 3 700 curistes par an. Depuis 2015, grâce à la construction d'un nouvel établissement thermal avec de nouveaux aménagements conséquents et soucieux des normes sanitaires modernes, les Thermes de Balaruc-les-Bains sont désormais capables de traiter 54 000 curistes par an, avec une capacité de coordonner 4 200 cures à la journée, faisant des Thermes de Balaruc-les-Bains le premier établissement thermal français.

Si aujourd'hui les curistes peuvent accéder à une large gamme de soins (balnéothérapie, lit de boue, sophrologie, et activités physiques encadrées), historiquement les soins se faisaient par « pélothérapie », c'est-à-dire par application de boues maturées (appelées « péloïdes ») directement en contact avec la peau des patients. Le péloïde est en fait un mélange de boue et d'eau thermale que l'on laisse à l'air libre et exposé à la lumière naturelle pendant plusieurs mois. Durant cette période, appelée « maturation », le mélange eau/boue thermale sera colonisé par de nombreuses espèces de microorganismes (bactéries, microalgues,

cyanobactéries, ...) qui vont constituer la biocénose (ou bioglée) du péloïde ainsi obtenu. Au XIX^{ème} siècle, le péloïde était récolté aux environs immédiats du jaillissement de la source thermale. Ensuite, au début du XX^{ème} siècle, le péloïde était obtenu par maturation des limons récoltés dans l'étang de Thau avec de l'eau thermale prélevée à la source. Dans les années 70, le processus de maturation est devenu semi-industrialisé puisque les sédiments issus de l'étang de Thau et l'eau thermale étaient mis en maturation dans des malaxeurs industriels. Deux études ont d'ailleurs été menées, en mimant le procédé grâce à des systèmes expérimentaux afin d'étudier les différentes étapes de la maturation du péloïde de Balaruc-les-Bains (une thèse menée par Baudinat en 1986 et une étude menée par Dupuis en 1987) (Baudinat 1986; Dupuis 1987). Les deux études ont montré que le développement de microalgues « *sensu lato* »¹ pouvait être plus ou moins rapide et intervenait entre dix jours et sept semaines. En revanche, les deux études s'entendent sur le fait que la communauté microbienne du péloïde était majoritairement dominée par des cyanobactéries et notamment celles du genre *Phormidium*².

Depuis 2015, seul un mélange de boue stérilisée et d'eau thermale est utilisé pour les soins. En effet, les normes et les règles sanitaires drastiques, auxquelles doivent se plier les établissements recevant du public, sont difficiles à mettre en place dans le cadre de l'utilisation de péloïdes « naturels », puisqu'il est difficile de contrôler les communautés microbiennes colonisant naturellement les boues. Néanmoins, au vu des nombreuses molécules produites par les cyanobactéries et les microalgues, les bienfaits de la pélothérapie pourraient être également attribués à la biocénose du péloïde. C'est dans une volonté d'éclaircir ce point qu'est née la collaboration entre les Thermes de Balaruc-les-Bains et l'équipe « Cyanobactéries, Cyanotoxines et Environnement » du Muséum National d'Histoire Naturelle (équipe CCE). Cette collaboration a débuté avec une première convention de recherche et les travaux du Dr. S. Hamlaoui en 2014, plus de 20 ans après les études de Baudinat et Dupuis (détailés Chapitre II). Son travail a eu pour but d'inventorier les communautés de microorganismes peuplant les boues thermales de Balaruc-les-Bains. Dans cet inventaire, ses travaux ont permis la description des cyanobactéries et des microalgues se développant dans un bassin de décantation, dans des conditions mimant la maturation du péloïde pendant une période allant d'avril à octobre (**Figure 1**) (Hamlaoui 2014).

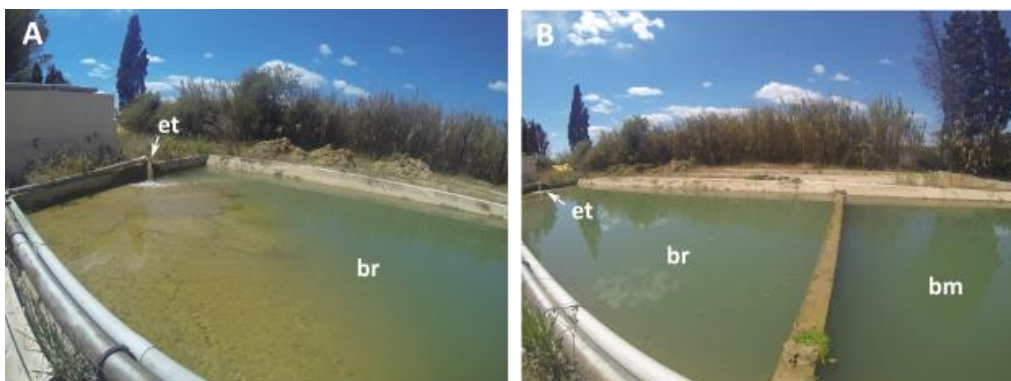


Figure 1. Bassin de maturation des boues. (et) arrivée de l'eau thermale, (br) bassin de rétention, (bm) bassin de maturation des boues.

¹ Microorganismes photosynthétiques eucaryotes et cyanobactéries

² Filamenteuses, filaments non ramifiés, gaine facultative, biofilm en couches fines et lisses.

Neuf espèces de cyanobactéries et quatre espèces de microalgues ont également pu être isolées et conservées dans les collections de cyanobactéries et de microalgues du Muséum National d'Histoire Naturelle (PMC et ALCP, respectivement). Leur innocuité quant à la production de cyanotoxines a également été évaluée. Les cyanobactéries étaient présentes en plus forte proportion que les microalgues, et étaient principalement représentées par les souches clonales appartenant aux espèces : *Planktothricoides raciborskii*, en collection sous le numéro PMC 877.14, *Microcoleus vaginatus* (PMC 879.14) et *Leptolyngbya boryana* (PMC 883.14)³. Ces résultats sont similaires aux deux précédentes études ayant montré une dominance des cyanobactéries possédant le morphotype des *Phormidium*. Par ailleurs, les analyses par ELISA ont permis de déterminer qu'aucune des neuf souches de cyanobactéries n'avait la capacité à produire des cyanotoxines.

Ces résultats ont donc permis de poursuivre la collaboration entre les Thermes et l'équipe CCE. Dans ce contexte, une Convention Industrielle de Formation par la Recherche (CIFRE) a été établie en 2017 entre les Thermes de Balaruc-les-Bains et l'équipe CCE. Les travaux réalisés au cours de cette thèse se sont alors concentrés sur l'étude de la diversité des molécules produites par les neuf cyanobactéries isolées des boues thermales de Balaruc-les-Bains et l'évaluation de leur potentiel thérapeutique en rhumatologie et phlébologie.

Ainsi, les travaux de thèse avaient pour objectifs de répondre aux questions suivantes :

1. Quelle est la relation entre diversité taxonomique, diversité chimique et activité démontrée des métabolites produits par les cyanobactéries ? (**Chap. 1**)
2. Quelle est la taxonomie des souches isolées et le potentiel des souches à produire des métabolites d'intérêt ? (**Chap. 2**)
3. Quelle est la diversité des métabolites des cyanobactéries de Balaruc-les-Bains ? Et quel est leur potentiel d'activités ? (**Chap. 3**)
4. Quelle est la cinétique de production des métabolites des cyanobactéries de Balaruc-les-Bains ? (**Chap. 4**)

Chacun de ces chapitres est organisé sous la forme d'articles publiés, soumis ou en préparation. Enfin, l'ensemble des résultats de ces travaux de thèse sont discutés dans la cinquième et dernière partie pour s'ouvrir ensuite à un certain nombre de perspectives (**Chap. 5**).

Afin de répondre à cette problématique, une revue bibliographique des connaissances sur les métabolites produits par les cyanobactéries et leurs activités référencées a été réalisée. Elle présente l'analyse de la base de données construite à partir de la littérature et questionne l'origine taxonomique des souches productrices de métabolites ainsi que la diversité chimique et des activités démontrées (**Chap. 1**). Les neuf cyanobactéries issues des boues thermales de Balaruc-les-Bains ont été affiliées taxonomiquement en accord avec les dernières données publiées concernant la classification des cyanobactéries et comprend la description de chacune des souches et celle d'un nouveau genre, *Pseudo-chroococcus couteii*. Ensuite, en accord avec l'analyse de la base de données, le potentiel de production de molécules à activités « bénéfiques » a été étudié pour chacune des souches (**Chap. 2**).

³ Anciennement identifiées comme *Phormidium nigrum*, *P. autumnale* et *Leptolyngbya foveolarum*

L'analyse des neuf souches de cyanobactérie a été conduite en utilisant plusieurs méthodes globales à haut débit type « omique ». Plusieurs approches ont donc été utilisées conjointement dans le but de caractériser la richesse de production en métabolites des neuf cyanobactéries. Un premier volet d'analyse génomique a été mené via le séquençage, l'assemblage et l'étude des génomes des neuf cyanobactéries ainsi qu'un second volet d'analyse de métabolomique non-ciblée en spectrométrie de masse des extraits de ces cyanobactéries. Ces deux approches complémentaires permettent, d'une part, l'identification du potentiel de production de métabolites en identifiant les clusters de gènes de biosynthèse et, d'autre part, l'observation globale des métabolites produits par les souches dans des conditions définies. Un troisième volet permettant la caractérisation des bioactivités exhibées par les mêmes extraits bruts a également été abordé comme une première étape de futurs travaux de développement. Ces différentes approches, indépendantes de prime abord, ont finalement montré une parfaite complémentarité et ont permis d'obtenir une vision globale du potentiel des cyanobactéries de Balaruc-les-Bains dans des perspectives de développement thérapeutique (**Chap. 3**). La quatrième partie présente l'analyse cinétique des métabolites produits par *Aliinostoc* sp. PMC 882.14 dans différentes conditions de culture (obtenus lors du stage de M2 conduit par Damien Le Moigne) (**Chap. 4**). Les différentes questions et objectifs de la thèse ainsi que leur articulation sont résumés en **Figure 2**.

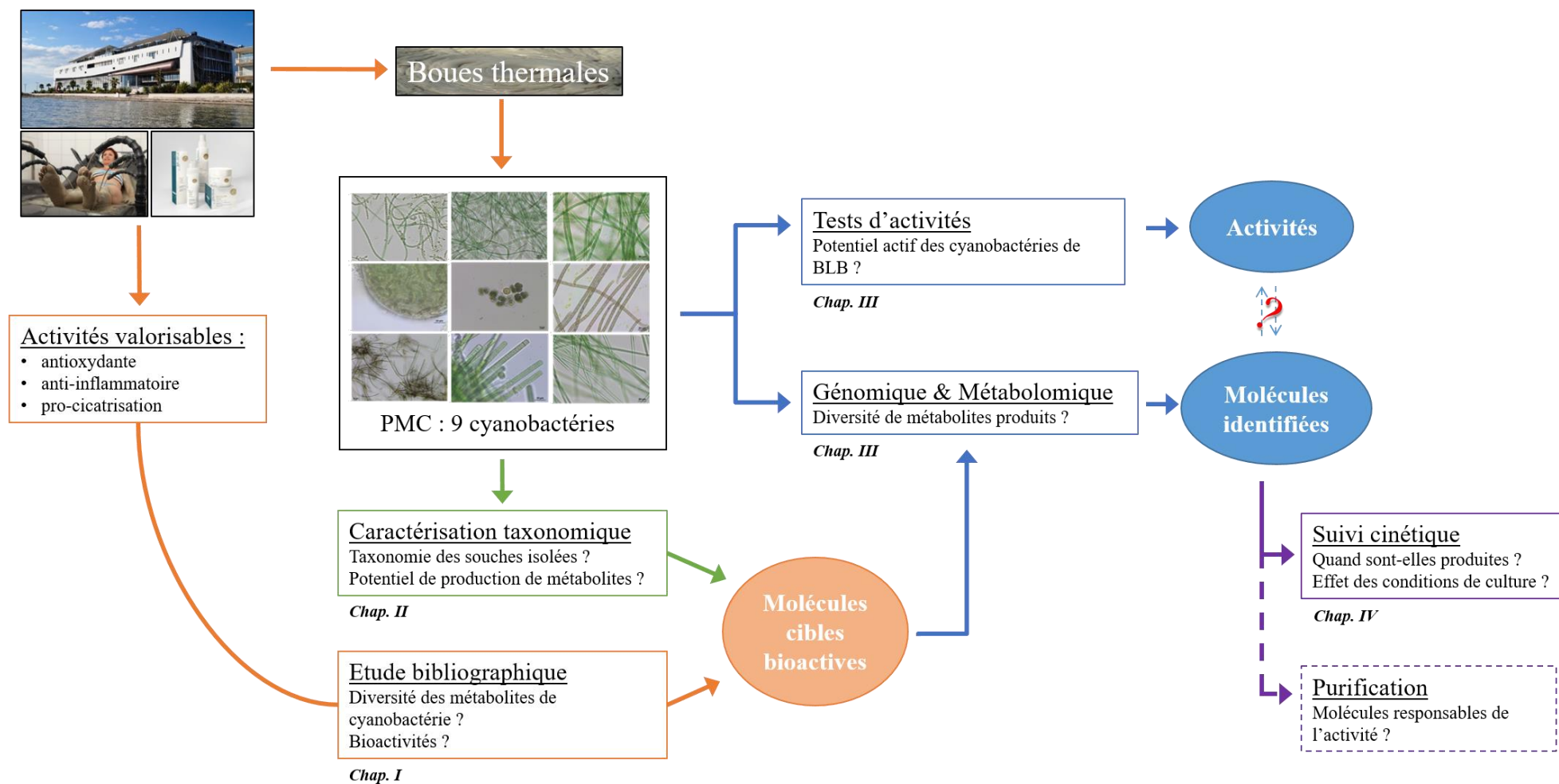


Figure 2. Schéma général des objectifs de la thèse

Chapitre I

Introduction :

Diversité des métabolites produits par les cyanobactéries et leurs activités

Chapitre I – Introduction

DIVERSITE DES METABOLITES PRODUITS PAR LES CYANOBACTERIES ET LEURS ACTIVITES

I.1. Contexte de l'étude

Depuis des millénaires, l'Homme a utilisé son environnement, et notamment les plantes, pour guérir maladies, plaies et infections en tout genre (Petrovska 2012). Ce savoir s'est transmis de génération en génération à travers les différentes communautés. Avec l'avancée des techniques scientifiques et les débuts de l'ethnopharmacologie au XIX^{ème} siècle, les propriétés curatives de ces différents remèdes ont pu être décryptées et les molécules bioactives identifiées. On peut par exemple citer la quinine, une molécule antipaludéenne issue du quinquina, ou bien l'acide salicylique, le principe actif de l'aspirine, issu du saule. Plus tard, de nouvelles molécules actives ont été identifiées à partir de microorganismes comme les bactéries et les champignons, notamment des molécules antibiotiques telle que la pénicilline découverte par A. Fleming en 1928 à partir de moisissures du genre *Penicillium* (G. D. Wright 2019).

Dans les années 80s-90s, la découverte et la caractérisation de molécules naturelles sont peu à peu abandonnées, perçues comme un processus long, coûteux et difficile (difficulté de purification du métabolite actif et difficulté d'obtenir des quantités suffisantes de matériel biologique). Les industries pharmaceutiques investissent alors dans le criblage haut débit de molécules de synthèse afin de développer de nouveaux principes actifs (Beutler 2019). Paradoxalement, cette baisse d'intérêt des entreprises pharmaceutiques intervient alors que le développement des techniques d'étude des produits naturels est de plus en plus pointu, avec une meilleure compréhension des voies de biosynthèse des molécules d'intérêt. En effet, les molécules naturelles présentent de nombreux avantages pour le développement de nouveaux médicaments. Au-delà de leur immense diversité structurale, ces molécules ont été conservées au fil de l'évolution pour le gain compétitif qu'elles représentent. Elles sont donc adaptées à leur rôle biologique (défense, allélopathie, ...) et possèdent les qualités chimiques et physiques nécessaires pour être bioactives (affinité avec les récepteurs chez la cellule cible, capacité à traverser les membranes biologiques, ...) (G. D. Wright 2019).

C'est le développement des techniques omiques telle que la génomique, c'est-à-dire l'étude du génome complet d'un organisme à partir du séquençage de son matériel génétique, ou la métabolomique (l'étude de l'ensemble des métabolites produits à un temps t par un individu) qui ont permis un nouvel essor de la recherche sur les molécules naturelles, en particulier celles produites par les microorganismes (Niu and Li 2019; Machado et al. 2017). En effet, ces avancées technologiques ont permis la généralisation du séquençage du génome des bactéries, permettant la caractérisation des voies de biosynthèse de métabolites secondaires par la découverte des voies non ribosomiques telles que les NRPS (non-ribosomal peptide synthase) et les PKS (polyketide synthase) ainsi que leur organisation en îlots de gènes de biosynthèse (Machado et al. 2017; G. D. Wright 2019).

Les cyanobactéries, bien que sous-représentées dans les bases de données publiques en termes de nombre de génomes disponibles (Alvarenga et al. 2017), ont été, elles aussi, particulièrement étudiées ces 20 dernières années pour la production de métabolites secondaires, avec la découverte de molécules très actives telles que les cyanotoxines ou encore les dolastatines, potentiellement exploitables dans plusieurs champs d'application (Ali Shah et al. 2017; Janssen 2019; Martins and Vasconcelos 2015; Chlipala et al. 2011). De nombreuses revues ont d'ailleurs été publiées concernant les métabolites isolés des cyanobactéries, se concentrant sur différents aspects tels que les voies de biosynthèse démontrées (Dittmann et al. 2015), la diversité des structures chimiques (Ali Shah et al. 2017) ou encore les activités spécifiques (Vijayakumar and Menakha 2015; Mourelle et al. 2017). Néanmoins, peu de revues apportent des éléments de réponse quant à la diversité des cyanobactéries productrices (Tidgewell et al. 2010) ou permettent de lier diversité taxonomique, classe chimique des molécules et bioactivité.

Dans ce cadre, et pour répondre à la première question de la thèse : *Quelle est la relation entre diversité taxonomique, diversité chimique et activité démontrée des métabolites produits par les cyanobactéries ?*, une revue exhaustive de la littérature a été réalisée (1970-2018) concernant les métabolites produits par les cyanobactéries et leurs bioactivités dans le but de comparer (i) les différentes origines taxonomiques des molécules bioactives, (ii) leurs diversités structurales, (iii) les différentes activités décrites et (iv) d'identifier les molécules les plus valorisables en termes d'activités bénéfiques. En parallèle, cette revue a également permis (v) de faire un inventaire des

molécules décrites pour les cyanobactéries appartenant aux genres des souches étudiées en relation avec les activités d'intérêt pour les Thermes de Balaruc-les-Bains.

I.2. Résumé

Une base de données regroupant les 260 familles de métabolites (regroupées selon leurs voies de biosynthèse et leurs structures) produits par les cyanobactéries a été créée, construite à partir de 670 articles scientifiques. La base de données contient les différentes caractéristiques des molécules telles que leur structure, leur classe chimique, leur voie de biosynthèse, la ou les souche(s) à partir desquelles le métabolite a été décrit (espèce, genre, famille, ordre), l'écosystème dont est issue la souche productrice et le site d'échantillonnage. Les différents tests d'activités et leurs résultats ont également été relevés pour chacun des métabolites. Une attention particulière a été accordée aux 47 molécules issues des cyanobactéries possédant des activités « bénéfiques », à fort potentiel de valorisation dans les domaines pharmaceutique, cosmétique, biotechnologique ou agricole.



Review



Natural Products from Cyanobacteria: Focus on Beneficial Activities

Justine Demay ^{1,2}, Cécile Bernard ^{1,*}, Anita Reinhardt ² and Benjamin Marie ¹

Parmi la diversité des molécules référencées, il apparaît que, sur les 300 genres de cyanobactéries décrits par Komárek et al. 2014 (Komarek et al. 2014), seulement 90 ont été décrits comme produisant des métabolites bioactifs. Aux différents échelons taxonomiques, les résultats ont montré une diversité assez faible. A l'échelle des genres, il existe de fortes disparités en terme de nombre de composés décrits, avec, par exemple, le genre des *Lyngbya-Moorea* produisant à lui seul 85 familles de métabolites, soit plus de la moitié des molécules référencées pour les souches appartenant aux Oscillatoriales.

La revue a également montré que les métabolites issus des cyanobactéries sont très divers en terme de structure chimique avec une dizaine de classes chimiques différentes (alcaloïdes,

depsipeptides, lipides, lipopeptides, macrolides, peptides, polysaccharides, terpènes, polycétides, ...). Parmi les activités recherchées, on retrouve principalement 14 familles d'activités avec les activités toxiques (léthalité contre un organisme/embryon, cytotoxicité, hépatotoxicité, neurotoxicité, dermatotoxicité), les activités antimicrobiennes (antibactérienne, antivirale, antifongique, antialgale, antiparasitaire) et les activités sur les processus cellulaires (anti-inflammatoire, antioxydant, inhibition d'enzymes).

Aucune relation directe n'a d'ailleurs été montrée entre le type d'activité révélée et la classe chimique des molécules produites par les cyanobactéries, même si au sein d'une même famille de métabolites (par exemple les microcystines) l'activité est bien souvent la même avec des modulations de l'effet (plus ou moins fort) en fonction des résidus incorporés par chaque variant.

Ces résultats, au regard de la littérature entourant la découverte de nouvelles molécules bioactives, ont permis de mettre en lumière plusieurs points. Le premier est que ce type d'étude bibliographique est dépendant de l'identification des taxa étudiés ; en effet, de nombreux genres étant polyphylétiques (*Lyngbya*, *Phormidium*, *Nostoc* ...), il est difficile de faire des corrélations entre les cyanobactéries qui produisent une famille de métabolites. Le second est le fait que de nombreuses voies de biosynthèse demeurent inconnues pour la plupart des familles de métabolites de cyanobactéries. La proportion de génomes de cyanobactéries disponibles dans les bases de données publiques est faible par rapport au nombre de génomes disponibles. De ce fait, de nombreuses découvertes sont encore à faire sur la caractérisation des îlots de gènes de biosynthèse et leurs mécanismes de régulation.

Enfin, cette revue a permis de réaliser un inventaire des molécules potentiellement produites par les neuf souches étudiées au cours de cette thèse (voir **Tableau 1, Chap. II, part. 3**).

I.3. Article 1

*marine drugs*

Review

Natural Products from Cyanobacteria: Focus on Beneficial Activities

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Abstract: Cyanobacteria are photosynthetic microorganisms that colonize diverse environments worldwide, ranging from ocean to freshwaters, soils, and extreme environments. Their adaptation capacities and the diversity of natural products that they synthesize, support cyanobacterial success in colonization of their respective ecological niches. Although cyanobacteria are well-known for their toxin production and their relative deleterious consequences, they also produce a large variety of molecules that exhibit beneficial properties with high potential in various fields (e.g., a synthetic analog of dolastatin 10 is used against Hodgkin's lymphoma). The present review focuses on the beneficial activities of cyanobacterial molecules described so far. Based on an analysis of 670 papers, it appears that more than 90 genera of cyanobacteria have been observed to produce compounds with potentially beneficial activities in which most of them belong to the orders Oscillatoriales, Nostocales, Chroococcales, and Synechococcales. The rest of the cyanobacterial orders (i.e., Pleurocapsales, Chroococcidiopsales, and Gloeobacterales) remain poorly explored in terms of their molecular diversity and relative bioactivity. The diverse cyanobacterial metabolites possessing beneficial bioactivities belong to 10 different chemical classes (alkaloids, depsipeptides, lipopeptides, macrolides/lactones, peptides, terpenes, polysaccharides, lipids, polyketides, and others) that exhibit 14 major kinds of bioactivity. However, no direct relationship between the chemical class and the respective bioactivity of these molecules has been demonstrated. We further selected and specifically described 47 molecule families according to their respective bioactivities and their potential uses in pharmacology, cosmetology, agriculture, or other specific fields of interest. With this up-to-date review, we attempt to present new perspectives for the rational discovery of novel cyanobacterial metabolites with beneficial bioactivity.

Keywords: cyanobacteria; natural products; metabolites; biological activities; producers; chemical classes

1. Introduction

Cyanobacteria belong to an ancient group of photosynthetic prokaryotes that present a very wide range of cellular strategies, physiological capacities, and adaptations that support their colonization of very diverse microenvironments worldwide. As a consequence, cyanobacteria occur in varied and often extreme habitats and are then able to settle in diverse biotopes (e.g., marine,

terrestrial, freshwater, thermal springs) (Whitton and Potts 2012; Kurmayer et al. 2016; Mazard et al. 2016). They are also well known for the production of a wide variety of bioactive natural products, including some potent toxins (e.g., microcystins, anatoxins, and saxitoxins) (Kurmayer et al. 2016; Mazard et al. 2016). Due to the remarkable capability of cyanobacteria to proliferate and form toxic blooms that induce potential human health consequences (Buratti et al. 2017), numerous studies have been conducted to develop tools for the monitoring of such blooms (Humbert and Törökne 2017; Salmaso et al. 2017) or effective strategies for the mitigation of their overgrowth (Paerl and Otten 2013). On the contrary, certain cyanotoxins could also constitute a promising opportunity for drug development such as certain cancer therapies (Zanchett and Oliveira-Filho 2013).

Two main aspects known as the chemical diversity and the related bioactivity have to be considered when considering the application potential of natural products produced by cyanobacteria. The chemical diversity of metabolites produced by these organisms has been well described and about 15 reviews have been already published in the past 20 years, dealing with their structural and chemical diversity (Tidgewell et al. 2010; Ali Shah et al. 2017; Mi et al. 2017; Wang et al. 2017; Chlipala et al. 2011; Sivonen et al. 2010) or their respective biosynthetic pathways (Dittmann et al. 2015; Pattanaik and Lindberg 2015). Beyond the notorious harmful effects of cyanotoxins, other cyanobacterial natural products show a wide range of bioactivities that could be potentially useful for diverse applications (R. Singh et al. 2017; J. S. Singh et al. 2016; William H Gerwick and Fenner 2013; Tânia K Shishido et al. 2015; Vijayakumar and Menakha 2015). So far, among the existing reviews related to the diversity of cyanobacterial metabolites, only one has addressed the relative taxonomical positions of the different producing strains (Tidgewell et al. 2010). A few taxa appear to be especially prolific producers of a large set of metabolites, while others still remain to be investigated. Recent genomics approaches and genome sequencing have been important steps in the elucidation of the pathways implicated in the biosynthesis of natural products. Their wide structural diversity has been described as a consequence of the numerous biosynthetic pathways developed by cyanobacteria in order to produce these metabolites (Dittmann et al. 2015). Most of the active cyanobacterial molecules are considered as being produced either through the non-ribosomal peptide (NRP) or the hybrid polyketide-NRP biosynthetic pathways (Ali Shah et al. 2017), or by the ribosomal synthesis of pro-peptides that are post-translationally modified (RiPP). Previous genome analysis demonstrated that the diversity of the known metabolites is merely a fraction of the true metabolic potential of cyanobacteria (Dittmann et al. 2015). Concerning bioactivity, cyanobacteria have long been a source of molecules with a potent nutritional property (J. S. Singh et al. 2016). Aztec civilizations consumed cyanobacteria (*Spirulina*) in their routine diet (Ciferri and Tiboni 1985), and Chadian populations still use them as one of their substantial food sources (Abdulqader et al. 2000). Besides nutritional and probiotic purposes (Chlipala et al. 2011; Vijayakumar and Menakha 2015), cyanobacteria are well-known as an important source of metabolites with technological applications in the biotechnical or pharmaceutical fields, which lead to an increase in interest in these research realms (Ali Shah et al. 2017). Most bioactivities described to date are the antibacterial, antifungal, anti-cancerous, immunosuppressive, anti-inflammatory, and anti-tuberculosis activities that have the potential to be used in fields such as pharmacology, cosmetology, agriculture, the food industry, or as biofuel (R. Singh et al. 2017). Cyanobacteria cells represent a sustainable resource for biotechnology due to their photosynthetic, N-fixation, and autotrophic capacities (R. Singh et al. 2017; J. S. Singh et al. 2016; Lau et al. 2015). Due to the current increase in their pharmaceutical value and in their application prospects for use in medicine or biotechnology, the exploration of uncovered cyanobacterial taxa constitutes a promising strategy to efficiently explore the chemical diversity of their bioactive compounds.

The present review globally and systematically describes current knowledge on the biological activities described for cyanobacterial natural products, and, thanks to the construction of a specific and freely available molecular database, regroups all information described so far concerning the chemical structures, the producing organisms, and the various bioactivities of all the different cyanobacterial metabolite families. This original material allows us to depict, from data based on

exhaustive literature, which kinds of bioactive metabolite are potentially produced by the different cyanobacterial taxa. In this case, the producer organisms were considered at different taxonomic levels (family, order, and genus) and are referenced according to their original habitats (freshwater, marine, and others). The chemical diversity is described with respect to the different kinds of bioactivity and the potential links between them are questioned, according to their potential or effective molecular mechanisms of action. A specific focus on 47 cyanobacterial compounds presenting beneficial bioactivities is detailed and discussed regarding their potential in pharmaceutical, cosmetical, biotechnical, and agricultural applications, which opens new perspectives on the discovery of novel and potent bioactive cyanobacterial molecules.

2. Methods for Dataset Construction

A database was constructed using different search engines, notably PubMed and Google Scholar. The keywords used were “cyanobacteria,” “metabolite” or “natural product,” “beneficial” and “activity,” or “biological properties.” The database was first based on reviews and further completed with recent publications dealing with the isolation of new compounds from cyanobacteria.

The main entries into the database were the names of the metabolites. To avoid bias in the counting of metabolites, we stored all the data for each molecule and its variants as a “family.” In fact, there are still no molecular classification references for a natural product description. As discussed by Janssen (Janssen 2019), there is no standardized naming system along cyanobacterial metabolites, as in natural product discovery in general, that could induce an underestimation of the real diversity of natural products and to hide the potential link between their chemical structures, biosynthetic pathways, and evolution routes. Thus, such a valuable classification of cyanobacterial metabolites is still needed, notably in the current context of genomic and metabolomic development.

In our database, metabolites were grouped and classified based on different criterion, initially selected by different authors (Dittmann et al. 2015; Janssen 2019; Chlipala et al. 2011). First, they were classified according to their biosynthetic pathways based on the genomic data reviewed by Dittmann et al. (e.g. microcystins, cryptophycins, and aeruginosins) (Dittmann et al. 2015). Secondly, when biosynthetic information was not available, metabolites were classified, according to their structural homology, as proposed by Boudreau et al., (Boudreau et al. 2012), Janssen (Janssen 2019), and Chlipala et al. (Chlipala et al. 2011), supposing that they might be sharing at least a similar, if not the same, biosynthetic route (e.g. kulolide-family, aerucylamides, and cyanopeptolins). In most cases, metabolite variants have a few differences occurring on a few residues and have conserved the specific structure of their metabolite family. For example, the cyanopeptolin-like family, which contains, so far described, 139 variants, is comprised of a core structure of six amino acid residues and a variable side chain containing between 1 and 3 residues. The sequence of the amino acids in the core structure is usually composed with: Thr-[Leu|Arg|Tyr]-Ahp-[Ile|Phe|Thr|Leu|Val]-N-Me[Tyr|Phe]-[Val|Ile] (see Supplementary data S1). Some amino acids are variable (in brackets) and some others are identical in the large majority of the variants, notably the 3-amino-6-hydroxy-2-piperidone (Ahp), and the threonine (Thr) that support the side chain and close the cycle with an ester bond linkage (S1) (Chlipala et al. 2011).

The data collected were then classified depending on the chemical class of the compound, the chemical structure, and the strain producing the metabolites with all the taxonomic information (species, genus, family, and order), in accordance with Komarek et al. (2014) (Komarek et al. 2014). In addition, we compiled the demonstrated activities for the purified compounds. Fourteen classes of activity were mostly tested through the literature: lethality (against brine shrimp, and other small invertebrates), neurotoxicity, hepatotoxicity, dermal toxicity, cytotoxicity, anti-inflammatory activity, antioxidant activity, antiviral, antibacterial, antifungal, antialgal, antiprotozoal, serine protease inhibition, and other types of enzyme inhibition.

Additionally, 670 publications were analyzed, dating from the 1970s until today (April 2019). Around 1630 unique molecules have been reported so far and were grouped in 260 families of metabolites (see Supplementary data S2). To validate the knowledge depth of our work, a rarefaction

curve of the number of molecule families was constructed using the number of analyzed publications (Figure 1).

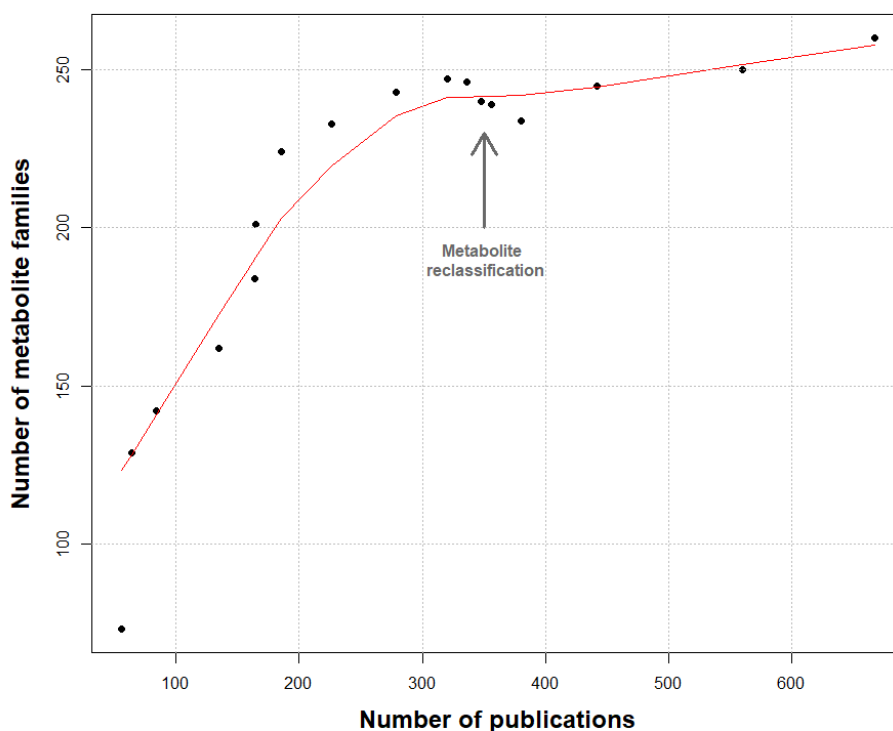


Figure 1. Evolution of the cumulative number of metabolite families according to the number of analyzed publications used for the construction of the database. The arrow indicates a reclassification event of all the structural variants of one molecule in a unique entry of “family” (Boudreau et al. 2012; Janssen 2019; Chlipala et al. 2011). We observed a progressive stabilization of the number of compound families in the database that supports the postulation of the exhaustiveness of the present database.

3. Taxonomy of the Producing Strains

The 260 families of molecules were attributed to cyanobacteria at their different taxonomic levels (order, family, and genus) (Figure 2). Some families of compounds can be produced by different strains and, thus, occur at different taxonomical levels. For example, microcystins are produced by various strains belonging to seven different genera, five families, and three orders.

The Oscillatoriales produces the largest number of metabolite families (153 families, 46.5%). The strains belonging to the Nostocales are also considerable producers of metabolites with 98 families (29.7%). The other main producers are the strains belonging to Chroococcales and Synechococcales, which exhibit, respectively, 34 and 31 described molecule families (10.3% and 9.4%). It is interesting that, except for these four orders, the others (i.e., Pleurocapsales, Chroococciopsales, Gloeobacterales, and Spirulinales) remain weakly represented in the database: less than five families of metabolite have been reported so far for all of them.

Some metabolites have been isolated from cyanobacterial assemblages without accurate identification of the producer organisms. For these cases, the authors identified the genera of the two dominant cyanobacteria of the assemblage but could not accurately determine which one of them produces which molecule (Soares et al. 2014; Vining et al. 2015; L M Nogle et al. 2001; Iwasaki et al. 2014; Harrigan, Yoshida, et al. 1998; A. Pereira et al. 2009; Graber and Gerwick 1998; A. R. Pereira et

al. 2010; 2011; Lisa M. Nogle and Gerwick 2002b; Williamson et al. 2004; Harrigan, Luesch, Yoshida, Moore, Nagle, Biggs, et al. 1999; Namthip Sitachitta et al. 2000). Tidgewell et al. (2010) (Tidgewell et al. 2010) also identified the prevalence of marine cyanobacterial products within Oscillatoriales and Nostocales with 58% and 24% of the isolated molecules, respectively. Within Oscillatoriales, members of the genus *Lyngbya*, and, notably, *Lyngbya majuscula* produce the highest number of metabolites. This benthic genus is widely spread through the tropical marine ecosystem and has been widely studied because of its toxicity and implication in many dermatitis cases around the world (N. J. Osborne et al. 2007; Nicholas J Osborne and Shaw 2008). A number of studies have been conducted on the *Lyngbya* genus, and a high number of new metabolites have been described. In fact, *Lyngbya* is, to date, the most productive genus of bioactive cyanobacterial compounds (Figure 2B). Recent studies showed that *Lyngbya* is polyphyletic (Engene et al. 2010; Komarek et al. 2014) and using polyphasic approaches, *Lyngbya* has been split into four new genera: *Moorea* (Engene et al. 2012), *Okeania* (Engene et al. 2013), *Limnoraphis* (Komarek et al. 2013), and *Microseira* (Mcgregor and Sendall 2015). Some marine strains previously identified morphologically as *Lyngbya majuscula* and *Lyngbya sordida* were, therefore, renamed as *Moorea producens*, and some strains of *Lyngbya bouillonii* were renamed to *Moorea bouillonii* on the basis of molecular and phylogenetic analyses (Engene et al. 2012). In the same way, some freshwater strains morphologically identified as *Lyngbya wollei* were separate from the *Lyngbya* genus and described as *Microseira wollei* after analysis of their phylogenetic position (Mcgregor and Sendall 2015).

According to this information, we decided to present the number of metabolite families produced by the *Lyngbya* and the *Moorea* genera together (reported as *Lyngbya-Moorea* in Figure 2B), given that the majority of families isolated from *Lyngbya* species were reported to be from *Lyngbya majuscula* (46 of 78 described from all the *Lyngbya*) or from *Lyngbya* spp. strains sampled from tropical marine environments (22 of 78), as described for the *Moorea* genus and were possibly misidentified with regard to this newly described genus (Engene et al. 2012).

At the family level, the main producers of known bioactive compounds belong to Oscillatoriaceae (30.3%, producing 122 families of compounds), followed by Nostocaceae and Microcoleaceae (17.2% and 10.9% for 69 and 48 molecule families, respectively) (Figure 2A). At the genus level (Figure 2B), *Lyngbya-Moorea* exhibits the highest number of isolated compounds (85 families of metabolites representing 20.6%), in accordance with the perceived richness of production for the *Lyngbya* genus due to its polyphyletic status (Engene et al. 2011). *Nostoc* is the second most prolific genus of bioactive compound families with 50 isolated families so far (12.1% of the total number of families of metabolites). The other most important genera are *Anabaena*, *Oscillatoria*, and *Microcystis* (with 32, 31, and 27 families of molecules, respectively, representing 7.8%, 7.5%, and 6.6%) (Figure 2B).

When looking at the habitats of these cyanobacteria, a large number of compounds were isolated from marine environments (148 families of metabolite in the database, which means 53% of the families of metabolites) in comparison to the number of strains isolated from freshwater environments (77 families of metabolites, 27.6%) (Figure 2B). However, this difference might be at least partly due to the high number of compounds isolated from the marine species *Lyngbya majuscula-Moorea producens* (49 families of molecules, 18.8% of the families in the database) and to the existence of various research programs focused on marine species (e.g., the Panama International Cooperative Biodiversity Group, ICBG).

Overall, we observed that diversity at the genus level is important, as illustrated by the 90 different genera present in the database. Moreover, 65 different genera have been reported to produce less than four molecules (Figure 2B). We also noticed that five molecules were isolated from *Lyngbya/Schizothrix* assemblages and five others from unidentified strains of cyanobacteria (Figure 2B). Thus, at the genus level, the diversity of producers is large with a high number of genera studied (90 different genera). Nevertheless, these genera generally belong to the same orders (e.g. Oscillatoriales, Nostocales, Synechococcales, and Chroococcales) while some orders were not studied. For example, among the Pleurocapsales order, only four genera have been reported to

produce metabolites. As a result, the covered diversity appears not to be exhaustive and can still be increased.

According to Shih et al. (2013) (Shih et al. 2013), the genomic potential of cyanobacteria to produce secondary metabolites is high with more than 70% of the studied strains presenting non-ribosomal peptide synthase (NRPS) or polyketide synthase (PKS) gene clusters in their genomes. In particular, they identified one strain belonging to the *Fischerella* genus (*Fischerella* sp. PCC 9339) that exhibits 22 NRPS/PKS clusters in its genome. On the contrary, only five compound families have been isolated from the genus *Fischerella* so far and are listed on the present database. Moreover, it is interesting to note that, among the 126 strains analyzed by Shih et al. (2013) (Shih et al. 2013), only 14 were formally reported to produce characterized metabolites.

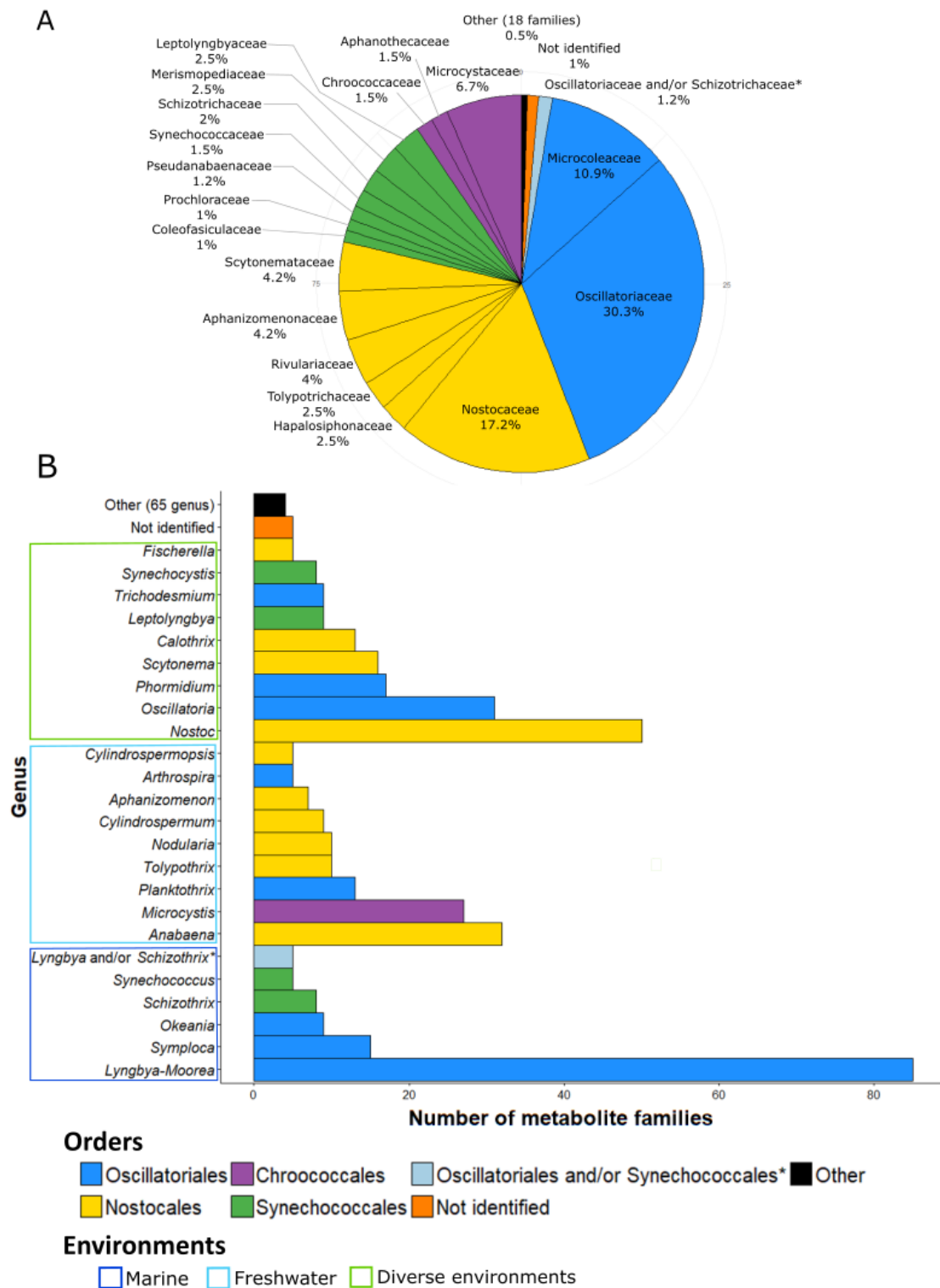


Figure 2. Proportion of families of compound by taxonomical level. **A/** The pie chart represents the percentage of compound families for each taxonomical family. Note that some compound families can be produced by several cyanobacterial families. The “Other” category concerns other taxonomical families that produce less than two compound families. **B/** The histogram shows the number of compound families for each genus. The “Other” category corresponds to genera producing less than four compound families. * indicates cyanobacterial assemblages whom the real metabolite producer

is still undetermined. The boxes indicate the environmental origins for the corresponding genera. For both charts, the colors correspond to the taxonomical order of each genus or family.

In addition, the best producer genus, *Lyngbya-Moorea*, remains rarely studied at the genomic level: four genomes are available in the Genbank database and another three are available on the Microscope platform (Médigue et al. 2018). Considering the number of compounds isolated from the *Lyngbya-Moorea* genus (85 compound families), most of the links between the identified molecules and the responsible biosynthetic gene clusters remain to be characterized. We also compared our collected data with those reported by Dittman et al. (2015) (Dittmann et al. 2015) in order to determine when the isolated molecule families are linked with a specific and identified biosynthetic gene cluster. This review showed that less than 20% of the molecule families from the database are associated with specific identified biosynthetic gene clusters. Thus, the biosynthetic pathways of a large majority of compounds is still unknown as well as the regulation mechanisms controlling their production. Therefore, these observations highlight part of the remaining possibilities for the discovery of new molecules, gene production, and biosynthetic pathways.

4. Chemical Diversity and Bioactivity of Natural Products from Cyanobacteria

Each of the 260 families of compounds was classified by chemical classes and bioactivity (Figures 3 and 4). The 260 families of compounds were classified by their chemical classes, and 10 different classes were listed: alkaloids, depsipeptides, lipopeptides, macrolides/lactones, peptides, terpenes, polysaccharides, lipids, polyketides, and others (Figure 3). Of the 260 metabolite families, 66 belong to the peptide class. Together with the depsipeptide and lipopeptide classes, they represent 133 families of compounds (51%) derived from peptides. This is not surprising, regarding the diversity of biosynthetic pathways described in cyanobacteria: NRPS (non-ribosomal peptide synthase), PKS (polyketide synthase) and RiPPs (ribosomally synthesized and post-translationally modified peptides) with the ability to produce a wide range of metabolites and notable peptides (Dittmann et al. 2015) (Figure 3).

Fourteen major activities have been listed from the literature (lethality, neurotoxicity, hepatotoxicity, dermaltoxicity and cytotoxicity, anti-inflammatory, antioxidant, antiviral, anti-microbial, antibacterial, antifungal, and antiprotozoal activities as well as protease and enzyme inhibition activities). Cytotoxic activity against various cell lines is the most frequently detected type of bioactivity with up to 110 families of the 260 listed. On the other hand, lethality and the antibacterial activities have been detected for 54 and 43 compound families, respectively (Figure 4).

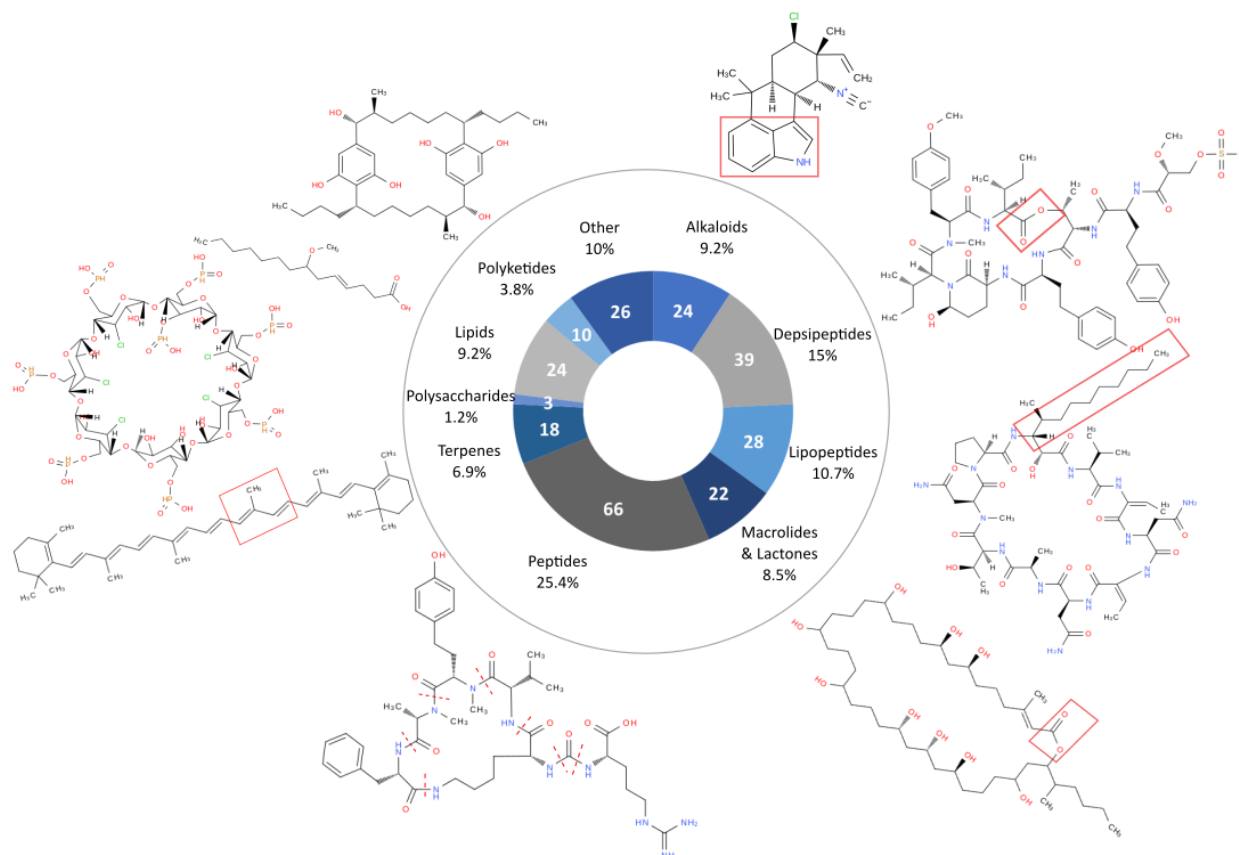


Figure 3. Classification of the 260 cyanobacterial metabolite families according to their respective chemical classes. All the molecules have been classified into these different classes according to their respective structural characteristics. For example, the depsipeptides are a class of peptides containing an ester bond and macrolides are molecules exhibiting a macrocycle and one or more lactone functions. Some examples of cyanobacterial molecules belonging to these classes are illustrated. Hapalindole A (alkaloids), Oscillapeptin A (depsipeptides), Minutissamide A (lipopeptides), Caylobolide B (macrolides/lactones), Anabaenopeptin E (peptides), β -carotene (terpenes), Cyclodextrin phosphate (polysaccharides), Lyngbic acid (lipids), and Cyliindrocyclophane A (polyketides). The main characteristics of each chemical class are highlighted in red. All the structures were obtained from the ChEMBL Database (<https://www.ebi.ac.uk/chembl/>).

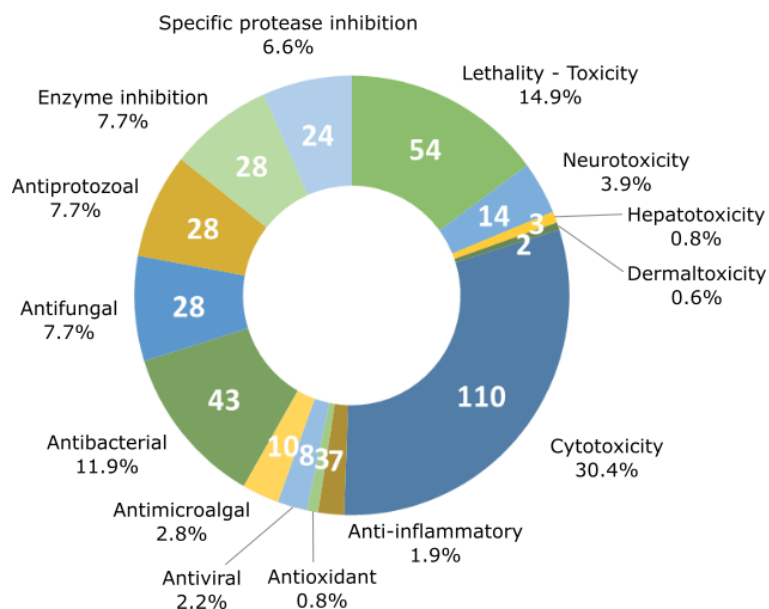


Figure 4. Number of metabolite families observed for each type of activity. The percentage represents the proportion of one activity compared to the whole occurrence of activities detected ($n = 362$). Some compounds present various activities and are considered several times.

The number of compounds displaying each tested activity is shown in Figure 5. The activities of molecules have been tested against different targets ranging from a specific cellular mechanism to an entire organism. For example, the inhibitory activity of proteases and other enzymes was shown to target enzymatic processes when the lethality and antimicrobial activity were tested against whole organisms. The lethality tests were generally realized against small invertebrates such as the brine shrimp crustacean *Artemia salina*, the gastropod mollusk *Biomphalaria glabrata*, and the crustacean *Thamnocephalus platyurus*. The present analysis confirms preceding observations (i.e., that cytotoxicity is the most commonly detected activity, followed by lethality and antibacterial activity). Some activities were detected only for a restricted number of compounds: dermaltoxicity concerned only two families of metabolites (aplysiatoxins and lyngbyatoxins) (R. E. Moore, Blackman, et al. 1984; Cardellina et al. 1979), hepatotoxicity was observed for three families (cylindrospermopsins, microcystins, and nodularins) (Ohtani et al. 1992; MacKintosh et al. 1990; Jokela et al. 2017), antioxidant and anti-inflammatory activities were observed for four (carotenoids, chlorophylls, mycosporine-like amino acids, and phycocyanins) (Stahl and Sies 2003; Lanfer-Marquez et al. 2005; Jain et al. 2017; C. Romay et al. 1998), and seven metabolite families (coibacins, honaucins, aeruginosins, malyngamides, phycocyanin, scytonemin, and tolypodiol) (Balunas et al. 2012; Choi, Mascuch, et al. 2012; Kapuścik et al. 2013; Shaala et al. 2013; Ch. Romay et al. 2003; Rajesh Prasad Rastogi et al. 2015; Michèle R. Prinsep et al. 1996), respectively. Nevertheless, there are only a few examples of these activities being tested by authors in comparison with cytotoxicity and lethality, which have been investigated far more regularly. In terms of anti-inflammatory activity, all seven tested molecules cited above were positive for this type of activity, and 53% of the studied molecule families have been tested for cytotoxic activity, while only 2.7% have been tested for anti-inflammatory activity. In parallel, some of these metabolite families can exhibit more than one activity. In fact, a total of 362 activities have been detected for the whole of the 260 metabolite families.

Focusing on the chemical classes, it appears that there is no specific indication that one chemical class exhibits specific activities with regard to other classes. The results from the review showed that the polysaccharide class presents only two tested activities (enzyme inhibition and antiviral activity), but only three types of polysaccharides isolated from cyanobacteria have been observed so far (calcium spirulan, cyclodextrins, and iminotetrasaccharide) (T. Hayashi et al. 1996; Entzeroth et al. 1986; Thammana et al. 2006). Five chemical classes (the alkaloids, the depsipeptides, the lipopeptides,

the macrolides, and the peptides) seem to present a remarkably large set of activities. When comparing the number of detected activities with the number of molecules belonging to each chemical class, the most bioactive molecules were shown to be the alkaloids, the lipopeptides, and the polyketides, which exhibit respectively 2.2, 1.9, and 1.8 activities per molecule on average.

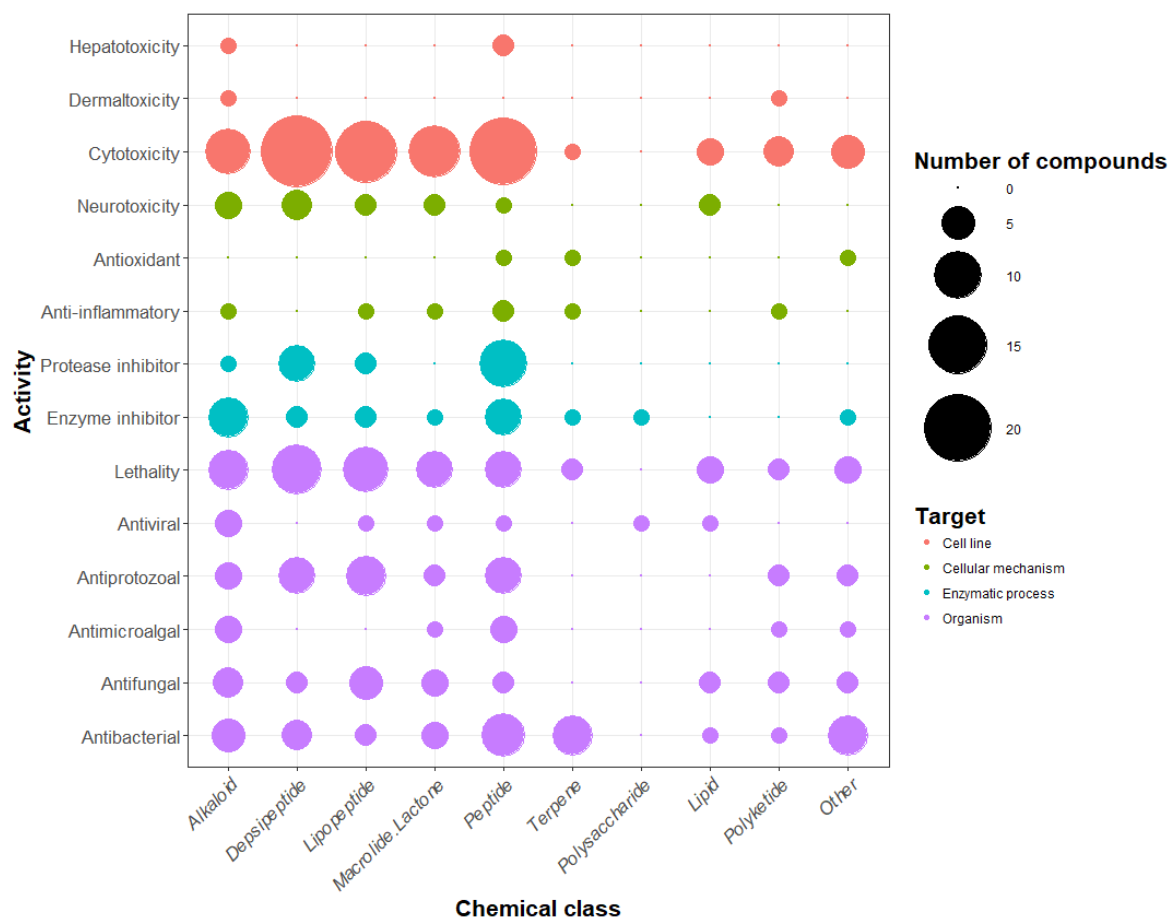


Figure 5. Classification of the 260 metabolite families according to their respective activities and chemical classes. The number of metabolite families is symbolized by the disc diameters, for each activity and each chemical class. For example, the first circle represents the number of alkaloids that exhibit a hepatotoxic activity (in this case, one family of metabolites). Colors correspond to the different categories of activity targets. For example, cytotoxicity and hepatotoxicity are tested *in vitro* against cell lines while neurotoxicity, antioxidant, and anti-inflammatory activities can be biochemically tested for specific cellular mechanisms (such as the sodium influx, the scavenging of ROS (reactive oxygen species), and the inhibition of cytokines).

These observations highlight a bias in the bioactivities searched from the isolated molecules. First, reported activities were those that researchers decided to test. Thus, the metabolite bioactivity profile could be underestimated because of the number of tests realized and remains the main limitation for the description of the potential applications of the bioactive molecules. In addition, there is still no consensus concerning the dose and dilution threshold that should be considered for each individual bioactivity test. In some cases, the concentration difference, used to determine if two distinct molecules are active, is important. For example, odoamide (Sueyoshi et al. 2016), which is a cyclic depsipeptide member of the aurilides family, and scytoscalarol (Mo, Kronic, Pegan, et al. 2009), a sesterterpene, have both been described as being “cytotoxic.” However, their respective IC_{50} values appear to be very different: 26.3 nM against HeLa S3 human cervical cancer cells for odoamide and 135 μ M against Vero cells for scytoscalarol, which represents a concentration difference of 500 times between their respective inhibition potentials. Furthermore, tests can be realized against several cell

lines and strains presenting different sensitivity responses, which limit the comparison between results.

With 10 chemical classes and 14 types of bioactivity, the cyanobacterial metabolites are diverse and highly active. However, half of the families of metabolites listed in the database are peptides or peptide derivatives. This could be due to the importance of the peptide biosynthetic pathway (NRPS, PKS, and RiPPs) or the extraction methods used, which might favor peptide extraction. We did not observe a link between chemical classes and activities, but this observation must be considered carefully with regard to the low number of investigated molecules in some classes (i.e., polyketides, polysaccharides, and terpenes). The most frequently detected activity for cyanobacterial metabolites is cytotoxicity (42% of the metabolite families), whereas antioxidant or anti-inflammatory activities were detected for only 1.5% and 2.7% of the families. This imbalance is due to the frequency at which tests were carried out. In fact, cytotoxicity was tested for 53% of the molecules, while anti-inflammatory activity was only tested in 2.7%. This observation may reflect the research inclination to find new pharmaceutical compounds, notably cytotoxic compounds that are usable in cancer therapy, and suggests the potential for the discovery of new activities for application in other fields.

5. Beneficial Activities of Natural Products Produced by Cyanobacteria

In this review, we further considered and developed 47 examples of molecules that are considered as exhibiting potential beneficial activities for several purposes. The 260 families of compounds could have a wide range of applications, e.g., agriculture, pharmacology, cosmetology, or in the food industry. For potential applications in agriculture, cyanobacterial compounds could be useful for alternative soil fertilization methods and as chemical pesticides (J. S. Singh et al. 2016). The potential pharmaceutical applications of cyanobacterial metabolites include the development of new antibiotics, antibacterial drugs, or antiviral drugs (Vijayakumar and Menakha 2015). Metabolite families were selected because of their specific features described below in each bioactivity-related section.

5.1. Antimicrobial Activity

Antimicrobial compounds that do not present toxic effects are particularly of interest for applications in the food industry in order to clean processing equipment or for food preservation (Sung et al. 2013; Abushelaibi et al. 2012). Cyanobacteria produce 85 families of metabolites isolated from various strains, which display potent antimicrobial activity (representing a third of the 260 molecule families listed in the database) (J. S. Singh et al. 2016). Below, we summarize the different antimicrobial metabolites (organized by type of antimicrobial activity) that have been isolated from cyanobacteria so far and the corresponding relevant information (see Tables 1–5). We also detail some examples of specific molecules that exhibit interesting bioactivity profiles such as the selective profile for their activity, which present broad-spectrum action together with the absence of associated cytotoxicity.

5.1.1. Antibacterial Activity

Among the metabolite families listed, 43 molecules exhibit antibacterial activity, which represents 17% of the families. These components were, in general, tested against different types of bacteria: GRAM-negative, GRAM-positive mycobacteria, and cyanobacteria.

Among the 43 molecules, 22 are also cytotoxic and 16 present lethal activity against small invertebrates. Only three of them—eucapsitrione, kulolide-like molecules, and abietic acid—may have specific antimicrobial activity and no activity against other microorganisms.

Eucapsitrione and kulolide-like molecules (Table 1, details available in Supplementary data S3) show antibacterial activity (against *Mycobacterium tuberculosis*) without inhibitory activity against the yeast *Candida albicans* (Sturdy et al. 2010; Lisa M. Nogle and Gerwick 2002a). Eucapsitrione is an anthraquinone derivative molecule isolated from the cyanobacterium *Eucapsis* sp. (UTEX 1519)

(Sturdy et al. 2010). This phenolic compound family is well-known in plants and some microorganisms, and has demonstrated a large range of bioactivities, including antimicrobial, antioxidant, anti-inflammatory, and potent anticancer properties (Kosalec et al. 2013; E. J. Yang et al. 2018; J. G. Park et al. 2016; Yen et al. 2000). This opens up other perspectives and applications for these anthraquinone derivatives isolated from cyanobacteria, such as eucapsitrione. However, so far, its other potential bioactivities have not been tested.

Table 1. Antibacterial molecules extracted from the database and discussed in this review.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Eucapsitrione	Anthraquinone derivative	-Antibacterial -No antifungal -Cytotoxic	<i>Eucapsis</i> sp. UTEX 1519	(Sturdy et al. 2010)
Kulolide-like analogs	Depsipeptide	-Antibacterial -No antifungal -Antiprotozoal -Lethal -Cytotoxic -VGSC (Voltage Gate Sodium Channel) activation	<i>Lyngbya majuscula</i> , <i>Rivularia</i> sp., <i>Moorea producens</i> , <i>Okeania</i> sp., <i>Symploca hydnooides</i> , <i>Oscillatoria margaritifera</i>	(Reese et al. 1996; Lisa M. Nogle and Gerwick 2002a; Bunyajetpong et al. 2006; Tripathi, Puddick, Prinsep, Lee, et al. 2010; Karla Lynn Malloy 2011; Mevers et al. 2011; Montaser et al. 2011; Salvador et al. 2011; Boudreau et al. 2012; Almaliti et al. 2017; Iwasaki et al. 2017; H. Luesch, Pangilinan, et al. 2001; Medina 2009)
Abietic acids	Terpene	-Antibacterial -No lethality -No antialgal	<i>Plectonema radiosum</i> LEGE 06105, <i>Nostoc</i> sp. LEGE 06077 and LEGE 07365, <i>Chroococcidiopsis</i> sp. LEGE 06174, <i>Synechocystis</i> sp. LEGE 06079, <i>Synechocystis salina</i> LEGE 06099, <i>Leptolyngbya ectocarpi</i> LEGE 11425, <i>Nodosilinea</i> sp. LEGE 13457, <i>Nodosilinea nodulosa</i> LEGE 07084	(Costa et al. 2016)
Hapalindole-like	Alkaloid	-Antibacterial -Antifungal -Antialgal -Cytotoxic -Insecticidal -Lethal activity	<i>Hapalosiphon fontinalis</i> , <i>Westiellopsis</i> sp., <i>Fischerella musicola</i> , <i>Hapalosiphon welwitschii</i> , <i>Westiella intricata</i> , <i>Fischerella ambigua</i> ,	(Acuña et al. 2015; Becher and Jüttner 2005; Becher et al. 2007; Cagide et al. 2014; Etchegaray et al. 2004; Hillwig, Zhu, et al. 2014; Hillwig, Fuhrman, et al.

-Reverse multidrug resistance (MDR)	<i>Hapalosiphon delicatulus</i> , <i>Hapalosiphon hibernicus</i> ,	2014; Huber et al. 1998; H. Kim, Lantvit, et al. 2012; H. Kim, Krunic, et al. 2012;
-VGSC modulator	<i>Westiellopsis prolifica</i> , <i>Fischerella</i> sp., <i>Hapalosiphon laingii</i>	Klein et al. 1995; Koodkaew et al. 2012; Micallef et al. 2014; 2015; Mo, Krunic, Chlipala, et al. 2009; Mo et al. 2010; R. E. Moore, Cheuk, et al. 1984; R. E. Moore, Yang, et al. 1987; R. E. Moore, Cheuk, et al. 1987; R. E. Moore et al. 1989; A. Park et al. 1992; Raveh and Carmeli 2007; Schwartz et al. 1987; Smitka et al. 1992; Stratmann, Moore, et al. 1994; Walton et al. 2014)

More details about compound activities are available in Supplementary data S3.

The kulolide-like family includes 44 related molecules. Kulolide, which is the first molecule of the family to be discovered, was isolated from a cephalaspidean mollusk *Philinopsis speciosa* (Reese et al. 1996). Luesch and co-workers (2001) isolated the first cyanobacterial analogues of this family, naming them the pitipeptolides, and proposed a cyanobacterial origin for kulolide itself (H. Luesch, Pangilinan, et al. 2001). All members of the kulolide-like family share chemical similarities and can be categorized into two subgroups: those containing 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya) and those containing 3-hydroxy-2-methyl-7-octynoid acid (Hmoya) (Boudreau et al. 2012). The same activities were not tested for all analogues, but some of them have shown antibacterial, antiprotozoal, cytotoxic, and even lethal activities (Table 1).

The third example of a family of molecule presenting a specific anti-bacterial activity is that of abietic acids (Table 1). Abietic acid is a terpene that is generally found in resin and used by conifers as a defense metabolite (Costa et al. 2016). It demonstrates anti-cyanobacterial activity against *Synechococcus nidulans*, and seems to be non-toxic to *Chlorella vulgaris* and the brine shrimp *Artemia salina* (Table 1). It has been suggested that its activity and defense mechanisms could be equivalent to those of coniferous plants, i.e., trapping microorganisms or acting as allelochemical compounds. These non-toxic properties are compelling for the development of specific anti-cyanobacterial products.

The hapalindole-like group is a family of alkaloids, which contains around 80 related molecules (Acuña et al. 2015; Becher and Jüttner 2005; Becher et al. 2007; Cagide et al. 2014; Etchegaray et al. 2004; Hillwig, Zhu, et al. 2014; Hillwig, Fuhrman, et al. 2014; Huber et al. 1998; H. Kim, Lantvit, et al. 2012; H. Kim, Krunic, et al. 2012; Klein et al. 1995; Koodkaew et al. 2012; Micallef et al. 2014; 2015; Mo, Krunic, Chlipala, et al. 2009; Mo et al. 2010; R. E. Moore, Cheuk, et al. 1984; R. E. Moore, Yang, et al. 1987; R. E. Moore, Cheuk, et al. 1987; R. E. Moore et al. 1989; A. Park et al. 1992; Raveh and Carmeli 2007; Schwartz et al. 1987; Smitka et al. 1992; Stratmann, Moore, et al. 1994; Walton et al. 2014) (Table 1). These metabolites were previously isolated from *Hapalosiphon*, *Fischerella*, *Westiellopsis*, and *Westiella* genera. They show a wide range of activity, most notably, antibacterial activity against 27 various bacterial strains, together with antifungal and anti-algal activities. They are also cytotoxic and exhibit additional insecticidal activity. Some of them were even able to reverse drug resistance in cancer cell lines (Hillwig, Zhu, et al. 2014; Stratmann, Moore, et al. 1994) (Table 1). They putatively exhibit modulatory activity on sodium channels (Cagide et al. 2014), which could explain their diverse bio-activities.

Table 2. Antialgal molecules extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Cyanobacterin	Lactone derivative	-Antialgal -Anti-cyanobacterial -Growth inhibition	<i>Scytonema hofmanni</i> UTEX 2349, <i>Nostoc linckia</i> CALU 892	(Mason et al. 1982; Gromov et al. 1991; Gleason and Case 1986; F. Ishibashi et al. 2005)
Fischerellins	Polyketide	-Antialgal -Anti-cyanobacterial -Antifungal -Lethal -Growth inhibition	<i>Fischerella musicola</i> , <i>Fischerella</i> sp., <i>Fischerella ambigua</i> , <i>Fischerella tesserantii</i>	(Gross et al. 1991; Hagmann and Jüttner 1996; Papke et al. 1997; Srivastava et al. 1999)
Westiellamide-like analogs	Peptide	-Antialgal -Anti-cyanobacterial -No antifungal -Lethal activity -Cytotoxic	<i>Westiellopsis prolifica</i> EN-3-1, <i>Nostoc</i> sp. 31, <i>Stigonema dendroideum</i> IA-45-3, <i>Oscillatoria raoi</i> TAU IL-76-1-2, <i>Nostoc spongiaeforme</i> var. <i>tenue</i> str. Carmeli	(Ogino et al. 1996; Todorova et al. 1995; Michele R. Prinsep et al. 1992; Admi et al. 1996; Banker and Carmeli 1998; Jüttner et al. 2001)
Ambigols	Alkaloid	-Antialgal -Antibacterial -Antifungal -Antiprotozoal -Lethal activity -Cytotoxicity	<i>Fischerella ambigua</i> 108b	(Falch et al. 1993; A. D. Wright et al. 2005)

		-Enzyme inhibition	
Schizotrin-like analogs	Peptide	-Antialgal -Antibacterial -Antifungal -Antiprotozoal -Lethal activity -Cytotoxicity	<i>Schizothrix</i> sp. TAU IL-82-2, <i>Lyngbya</i> sp. 36.91, <i>Phormidium</i> sp. LEGE 05292, <i>Tychonema</i> sp. CCAP 1462/13 (An et al. 2007; Berry et al. 2004; Dias et al. 2017; P. N. Leao et al. 2010; Mehner, Müller, Krick, et al. 2008; Pergament and Carmeli 1994; Ribeiro et al. 2017; Zainuddin et al. 2009)

5.1.2. Antialgal Activity

Antialgal activity was tested generally against microalgae, and 10 families of metabolites were shown to present such activity. Among these 10 families, four also exhibited anti-cyanobacterial activity, and it can be supposed that these molecules may be acting against general photosynthesis mechanisms. For example, cyanobacterins isolated from two strains, *Scytonema hofmanni* UTEX 2349 and *Nostoc linckia* CALU 892 (Mason et al. 1982; Gromov et al. 1991), were shown to present significant antimicrobial activity directed against a large panel of microalgal and cyanobacterial strains (Table 2). These compounds also inhibit the growth of eight angiosperm plants, such as duckweed (*Lemna* genus), pea, corn, sorrel, black bindweed, wild oat, and green foxtail (Gleason and Case 1986) (Table 2). Gleason and Case (1986) showed that this activity is due to the inhibition of the Hill reaction in photosystem II without inhibition of photosystem I (Gleason and Case 1986).

Another example is the fischerellin family. These compounds were observed in four strains belonging to the *Fischerella* genus. They show a wide range of activities comprising growth inhibition of *Lemna minor*, antifungal and lethal activities, and antialgal and anti-cyanobacterial activities. Hagmann & Jüttner (1996) showed that fischerellins A is an effective inhibitor of photosystem II (Hagmann and Jüttner 1996) (Table 2).

The westiellamide-like analogs family comprise 12 related cyclic peptides isolated from five strains belonging to four different genera (Table 2). The related molecules, known as the bistratamides, were previously isolated from the ascidian *Lissoclinum bistratum* (Foster et al. 1992), and authors hypothesized a cyanobacterial symbiont origin for these molecules (Michele R. Prinsep et al. 1992). This family of compounds have been shown to have anti-algal and anti-cyanobacterial activities (Table 2), but they did not show any antifungal activity against the yeast *Saccharomyces cerevisiae* (Ogino et al. 1996; Todorova et al. 1995; Michele R. Prinsep et al. 1992; Admi et al. 1996; Banker and Carmeli 1998; Jüttner et al. 2001). Moreover, one of them, dendroamide A, has shown the ability to reverse the multi-drug resistance of a human breast carcinoma cell line (MCF-7/ADR) (Ogino et al. 1996). The MCF-7/ADR cell line overexpresses the P-glycoprotein pump, which transports drugs outside of the cell, providing higher resistance to chemical treatment. Dendroamide A is able to specifically inhibit the action of the P-glycoprotein pump, which allows the drug to penetrate and lyse the cells, so it has potential anticancer applications.

Among the antialgal compounds, two have a remarkably broad spectrum of antimicrobial activities: the ambigols and the schizotrin-like analogs families, both show antialgal, antibacterial, antifungal, and antiprotozoal activities (Table 2). Three ambigol variants were isolated from *Fischerella ambigua* strain 108b, while the schizotrin-like family includes 13 structurally related molecules isolated from four different strains (Table 2). In addition to these antimicrobial activities, the ambigols also present enzyme inhibition activity against cyclooxygenases and HIV-1 reverse transcriptase. The members of the schizotrin-like family, the portoamides (isolated from *Phormidium* sp. LEGE 05292), have also shown mitochondrial metabolism inhibition activity, which induces a further decrease in the cellular ATP content in cells exposed to portoamides (Ribeiro et al. 2017). This property is also promising for the development of drugs acting against tumors and cancers (Ramsay et al. 2011).

Via their main antialgal action (i.e., photosynthesis inhibition), the molecules have been shown to present other potential uses and could be used as alternatives to chemical herbicides based on PSII inhibition (e.g., 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU). These families of compounds could be used to develop new algaecides and herbicides and/or to develop new pharmaceutical drugs.

5.1.3. Antifungal Activity

Twenty-eight families of compounds showed antifungal activities. Toxicity tests were carried out against diverse fungal species, which are mostly pathogenic ones. Quite common ones include *Candida albicans*, *Saccharomyces cerevisiae*, *Penicillium notatum*, and *Aspergillus oryzae*, and less common

ones include *Trichophyton mentagrophytes* and *Ustilago violacea*. Among these compounds, 11 showed several other types of antimicrobial activity in addition to antifungal activity. Only two metabolite families, hassallidins and lyngbyabellins, demonstrated specific antifungal activity without presenting any antibacterial activity. The hassallidins are cyclic glycolipopeptides isolated from three strains belonging to the Nostocales (Table 3). Four variants have been characterized so far (T. H. Bui et al. 2014; Neuhof et al. 2006; 2005; Pancrace et al. 2017; Vestola et al. 2014), and the non-ribosomal peptide gene cluster responsible for hassallidin biosynthesis has been identified. Thus, the hassallidins cluster was detected by bioinformatics analysis of the genomes of four heterocytous cyanobacteria, *Aphanizomenon gracile*, *Cylindrospermopsis raciborskii*, *Nostoc* sp., and *Tolypothrix* sp., and hassallidins production was confirmed by LC/MS analysis (Table 3). Recently, Pancrace et al. (2017) identified the hassallidins gene cluster and characterized a new hassallidins variant from *Planktothrixserta* (PCC 8927), which is a nitrogen-fixing, non-heterocytous forming strain (Pancrace et al. 2017). They concluded that the strain gain of the cluster occurred by horizontal transfer and, therefore, questioned the natural product distribution and diversity among cyanobacteria.

Table 3. Antifungal molecules extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Hassallidins	Glycolipopeptide	-Antifungal -No antibacterial activity	<i>Hassalia</i> sp. B02-07, <i>Anabaena</i> sp. (SYKE 748A, 90y1998, 90M3, 299B, 258, SYKE763A, 0TU33S16, 0TU43S8, 1TU33S8, 1TU35S12, 1TU44S9, 1TU44S16, SYKE971/6, NIVA-CYA269/2, NIVA- CYA269/6, XPORK5C, XSPORK7B, XSPORK36B, XSPORK14D, BECID19), <i>Anabaena cylindrica</i> Bio33 <i>Cylindrospermopsis raciborskii</i> (ATC-9502 & CS-505), <i>Aphanizomenon gracile</i> Heaney/Camb 1986 140 1/1, <i>Nostoc</i> sp. (159 & 113.5), <i>Tolypothrix</i> sp. PCC 9009 <i>Planktothrix sarta</i> PCC 8927	(T. H. Bui et al. 2014; Neuhof et al. 2005; 2006; Vestola et al. 2014; Pancrace et al. 2017)
Lyngbyabellins	Depsipeptide	-Antifungal -No antibacterial activity -Lethal activity -Cytotoxic	<i>Lyngbya majuscula</i> <i>Lyngbya</i> sp., <i>Lyngbya bouillonii</i> <i>Moorea bouillonii</i>	(Choi, Mevers, et al. 2012; B. Han et al. 2005; Hendrik Luesch et al. 2000; Marquez et al. 2002; K. E. Milligan et al. 2000; Ramaswamy et al. 2007; P. G. Williams, Luesch, et al. 2003; Matthew et al. 2010)

Micro-guanidines	Guanidine derivative	-Antifungal -No cytotoxicity -No protease inhibition	<i>Microcystis</i> sp. TAU IL-306, <i>Microcystis aeruginosa</i> TAU IL-374	(Adiv and Carmeli 2013; Gesner-Apter and Carmeli 2008; Lifshits and Carmeli 2012)
Majusculamides	Lipopeptide	-Antifungal -Cytotoxic -Immunosuppressive activity -Actin filaments disrupting -Anti-settlement activity	<i>Lyngbya majuscula</i> , <i>Lyngbya polychroa</i>	(Marner et al. 1977; Carter et al. 1984; R. E. Moore and Entzeroth 1988; Koehn et al. 1992; Meickle et al. 2009; Simmons et al. 2009; Tan et al. 2010)

The lyngbyabellins are cyclic depsipeptides. They were isolated from *Lyngbya* and *Moorea* species (Table 3). Hectochlorin is the only member of the family that was tested for antibacterial and antifungal activity, which showed no antibacterial activity but displayed antifungal activity against *Candida albicans* (Marquez et al. 2002). The distinctive feature of the lyngbyabellins is that they can also disrupt actin filaments. Luesch et al. (2000) (Hendrik Luesch et al. 2000) and Han et al. (2005) (B. Han et al. 2005) showed that cells exposed to Lyngbyabellin A and E lost their microfilament network, which caused cell cycle arrest at the cytokinesis phase. Marquez et al. (2002) (Marquez et al. 2002) showed that the same process appears with cells exposed to hectochlorin. They also demonstrated that the molecule stimulates actin polymerization and then induces cell cycle disorders.

Microguanidines are guanidine derivatives isolated from two strains of *Microcystis* (Table 3). These molecules showed antifungal activity against *Saccharomyces cerevisiae* E4orf4 without cytotoxic activity. This specificity could be of interest for the development of new antifungal products (Gesner-Apter and Carmeli 2008).

Majusculamides are lipopeptides produced by *Lyngbya majuscula* and *Lyngbya polychroa*. These metabolites combine antifungal and cytotoxic activities with immunosuppressive and anti-settlement properties (Carter et al. 1984; Koehn et al. 1992; Marner et al. 1977; Meickle et al. 2009; R. E. Moore and Entzeroth 1988; Simmons et al. 2009; Tan et al. 2010). Simmons et al. (2009) (Simmons et al. 2009) also demonstrated the ability of majusculamides to disrupt actin filaments that may explain these specific properties (Table 3).

5.1.4. Antiviral Activity

Viral diseases are one of the main health concerns around the world. According to the World Health Organization (WHO), HIV and AIDS caused around one million deaths in 2017 (World Health Organisation 2018b). We noted that eight families of cyanobacterial compounds have shown antiviral activity. Antiviral activity was generally determined by testing against the human immunodeficiency virus (HIV-1 or HIV-2) or the *Herpes simplex virus* (HSV-1 or HSV-2). The aplysiatoxins showed activity against Chikungunya's virus (CHIKV) (Gupta et al. 2014) (Table 4), but are also very active dermatotoxins (R. E. Moore, Blackman, et al. 1984; Chlipala, Sturdy, et al. 2010) and tumor-promoting molecules due to their capacity to activate protein kinase C (PKC), which is an enzyme that plays roles in cell proliferation, differentiation, and apoptosis (Gupta et al. 2014) (Table 4). Recently, Han et al., demonstrated that two aplysiatoxin analogues showed the capability to inhibit the potassium channels (B. N. Han et al. 2018), which opens interesting perspectives for the study and use of these molecules for drug development.

Table 4. Antiviral molecules extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Aplysiatoxins	Alkaloid	-Antiviral -Dermatitis and swimmer's itch agents -Cytotoxic	<i>Lyngbya majuscula</i> , <i>Schizothrix calcicola</i> , <i>Oscillatoria nigro-viridis</i> , <i>Trichodesmium erythaeum</i>	(Rajesh P Rastogi et al. 2015; Gupta et al. 2014; Chlipala, Tri, et al. 2010; B. N. Han et al. 2018; Mynderse et al. 1977; R. E. Moore,

				Blackman, et al. 1984)
				(Boyd et al. 1997; B. Dey et al. 2000; Matei et al. 2016)
Cyanovirin-N	Protein	-Antiviral -No cytotoxicity -Stop fusion and transmission of HIV-1 virus	<i>Nostoc ellipsosporum</i> <i>Cyanothece</i> sp.	
				(T. Hayashi et al. 1996; K. Hayashi et al. 1996; Mader et al. 2016)
Calcium spirulan	Polysaccharide	-Antiviral -No cytotoxicity -Low anticoagulant activity	<i>Arthrospira platensis</i>	

Two other families of molecules have shown antiviral activity against a large panel of viruses. The first one, cyanovirin-N analogs, have been isolated from *Nostoc ellipsosporum* (Boyd et al. 1997) and *Cyanothece* sp. (Matei et al. 2016) (Table 4). These molecules are proteins belonging to the lectins class because of their ability to bind glycans. Cyanovirins show inhibitory activity against HIV-1, HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus, HHV-6, and measles virus (Boyd et al. 1997; B. Dey et al. 2000). Also, they inhibit Ebola and influenza viruses (Matei et al. 2016). Nevertheless, cyanovirins are not active against some viruses, such as human herpesvirus A (HHV-1), cytomegalovirus, and adenovirus type 5 (B. Dey et al. 2000). Cyanovirins are also non-cytotoxic for non-infected cells (at concentrations required for antiviral activity) (Boyd et al. 1997; B. Dey et al. 2000) (Table 4). In fact, cyanovirin-N binds gp120, which is a glycoprotein component of the HIV envelope. As a result, the molecule inhibits membrane fusion into target cells and stops virus transmission. Calcium spirulan has been isolated from *Arthrospira platensis* (anc. *Spirulina platensis*) and is a sulphated polysaccharide. It shows antiviral activity against a wide range of viruses including HIV-1, HSV-1, the human cytomegalovirus (HCMV), measles virus, mumps virus, and influenza virus, in addition to a low cytotoxicity against several cell lines (ID50 values between 2900 and 7900 µg/mL) (Table 4) (T. Hayashi et al. 1996; Mader et al. 2016). Furthermore, calcium spirulan seems inactive against *Poliovirus* and *Coxsackievirus*, two non-enveloped viruses, which means that it likely has selective activity for enveloped viruses. Hayashi et al. (1996) (T. Hayashi et al. 1996) also showed that this molecule inhibits virus penetration in targeted cells. Other sulphated polysaccharides are known for their anticoagulant and antiviral activity, such as heparin or dextran sulphate (Wijesekara et al. 2011; Baba et al. 1988). In comparison to these molecules, calcium spirulan showed a lower anticoagulant activity and a longer half-life in blood (K. Hayashi et al. 1996), which confirms its promising potential for the development of new specific antiviral drugs.

5.1.5. Antiprotozoal Activity (Against Malaria, Leishmaniosis, Chagas Disease)

The last kind of antimicrobial properties tabulated is antiprotozoal activity. Protozoans are eukaryotic microorganisms, some of them have parasitic lifestyles and are well-known for their involvement in human diseases such as Malaria, Leishmaniosis, Chagas' disease, and Trypanosomiasis. These diseases represent a huge problem in tropical countries where the parasite is transmitted by mosquitoes. The WHO identified more than 210 million Malaria cases in 2016 (World Health Organisation 2019). Therein, molecules with antiprotozoal activity are actively being sought in order to develop new drugs against these diseases.

Table 5. Antiprotozoal molecules extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
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Companeramides	Depsipeptide	-Antiprotozoal -No significant cytotoxicity	<i>Leptolyngbya</i> sp. or « <i>Hyalidium</i> »	(Vining et al. 2015)
Hoshinolactam	Lactam	-Antiprotozoal -No cytotoxicity	<i>Oscillatoria</i> sp.	(Ogawa et al. 2017)
Dolastatins	Peptide	-Antiprotozoal -Lethal -Cytotoxic	<i>Lyngbya majuscula</i> , <i>Symploca hydroides</i> , <i>Lyngbya</i> sp., <i>Symploca</i> sp. VP642, <i>Lyngbya-Schizothrix</i> assemblage	(Davies-Coleman et al. 2003; Fennell et al. 2003; Harrigan, Luesch, et al. 1998; Harrigan, Luesch, Yoshida, Moore, Nagle, and Paul 1999; Harrigan, Yoshida, et al. 1998; H. Luesch, Moore, et al. 2001; Hendrik Luesch et al. 2002; Mitchell et al. 2000; L M Nogle et al. 2001; Lisa M. Nogle and Gerwick 2002a)

From the review, 28 cyanobacterial metabolites showed antiprotozoal activities. Tests have been conducted against several strains of *Plasmodium falciparum* (causative agent of Malaria), *Leishmania donovani* (Leishmaniosis), *Trypanosoma cruzi* (Chagas' disease), and *Trypanosoma brucei* (Sleeping sickness). Among the 28 concerned families of molecules, 19 showed antiprotozoal activity against drug-resistant strains, especially against chloroquine-resistant strains of *Plasmodium falciparum* (see Table 5). Nevertheless, most of them are less active than the antibiotics currently used. For example, companeramides are cyclic depsipeptides produced by a cyanobacterium previously identified as *Leptolyngbya* sp. (now *Hyalidium*) (Vining et al. 2015) (Table 5). Companeramides showed antimalarial activity against three strains of chloroquine-resistant *Plasmodium falciparum*. They also showed no significant cytotoxicity against the cell lines used in the test, which constitutes a unique property for the development of specific but non-toxic antimalarial drugs. Unfortunately, the activity of companeramides against the parasite is 100-fold lower than that of chloroquine (a commonly used drug), which reduces their potential utilization.

However, some molecules show promise as substitutes for antibiotic treatment because of their strong activity against the parasite. This is the case for hoshinolactam and dolastatins. Hoshinolactam is an aromatic molecule belonging to the lactam chemical class (Ogawa et al. 2017). It was isolated

from an environmental sample rich in *Oscillatoria* sp. and has shown antiprotozoal activity against *Trypanosoma brucei* (IC₅₀ = 3.9 nM) with no cytotoxicity against MDR-5 (the host cell, IC₅₀ > 25 µM) (Table 5). Furthermore, the IC₅₀ of pentamidine (another commonly used drug) against *Trypanosoma* species is 4.7 nM. Thus, the activity of hoshinolactam is equivalent to that of the antibiotics, and hoshinolactam represents a promising alternative to pentamidine for Trypanosomiasis treatment (Ogawa et al. 2017).

Dolastatins are a well-studied family of peptides. The first members of this family were isolated in 1977 from the sea hare *Dolabella auricularia* (Pettit 1997). In 1998, other molecules belonging to the dolastatins family were isolated from the cyanobacteria *Lyngbya majuscula* and *Symploca hydroides*, which leads to the hypothesis that dolastatins isolated from the mollusc have a cyanobacterial dietary origin (Flahive and Srirangam 2005). Dolastin 10, which is one of the dolastatin-related molecules, is the most potent antiprotozoal metabolite discovered so far from cyanobacteria. This exhibits an IC₅₀ of 0.1 nM (the IC₅₀ of chloroquine is, on average, 5 nM for the chloroquine-sensitive strain of *P. falciparum*) (Fennell et al. 2003). Dolastatins are also strongly cytotoxic molecules (Table 5). They are able to inhibit tubulin polymerization, which induces cellular cycle arrest and apoptosis (Bai et al. 1990). Antiprotozoal and cytotoxic activities are both the result of this property. Therefore, there is no apparent specificity for this molecule to act directly against the parasite itself, where the cellular host is likely the most potent target of dolastatins. For this reason, Fennell et al. (2003) (Fennell et al. 2003) concluded that dolastatins do not constitute a promising antiprotozoal drug despite their strong activity.

5.2. Potential Anticancer Activity

Currently, cancers constitute the most important non-transmittable diseases worldwide. According to the WHO, cancer was the cause of one in six deaths (9.6 million) in 2018 (World Health Organisation 2018a). The annual cost of cancer in 2010 was estimated to be \$1.16 trillion USD (B. W. Stewart and Wild 2014). That is why numerous studies have been conducted to understand the physiology of different cancers and to find new efficient anticancer drugs. For this purpose, researchers are looking for molecules, and, notably, natural products that are able to kill cells or inhibit cell proliferation. In this section, we selected some particular cyanobacterial metabolites for which the mode of action is known as significant examples. We feature metabolites acting on the microtubule or the microfilament network, histone deacetylase and proteases inhibitors, and molecules with the ability to reverse multi-drug resistance.

5.2.1. Cytotoxic Activity

The first type of activity test was performed to determine the potential of molecules as anticancer agents due to cytotoxicity. Different cell lines derived from tumor cells, like the HeLa cell line (derived from cervical cancer), KB (HeLa derivative), LoVo (human colon tumor), H-460 (human lung cancer), and MCF-7 (human breast cancer) have been used to assess this activity. Most of the time, the molecules investigated were tested against two or more cell lines to detect a potent specificity and to evaluate their potential for drug development. According to this review, 110 families of metabolites isolated from cyanobacteria showed cytotoxicity, which represents 43% of the molecule families listed in the database.

The best example of potent anticancer molecules derived from cyanobacteria is the dolastatin family (Flahive and Srirangam 2005). One synthetic analogue of dolastatin 10, monomethyl auristatin E, is actually used to treat Hodgkin's lymphoma in the drug Brentuximab vedotin (Flahive and Srirangam 2005). Luesch et al. (2001) (H. Luesch, Moore, et al. 2001) showed that dolastatin 10 and symplostatin 1 are 100-fold more efficient than vinblastine (anticancer drug extracted originally from the Madagascar periwinkle) against the same cell line due to their ability to depolymerize microtubules. Unfortunately, dolastatins also have strong cytotoxicity (H. Luesch, Moore, et al. 2001; Mirsalis et al. 1999). Researchers found a way to reduce this toxicity by coupling monomethyl

auristatin E with a chimeric antibody against CD30 (tumor necrosis factor receptor, highly expressed in Hodgkin's lymphoma) in order to target only tumor cells (Francisco et al. 2003). Since then, other antibody drugs linked (ADC) with monomethyl auristatin E have been developed. For example, glembatumumab vedotin is currently under clinical trial. This drug targets GPNMB (glycoprotein non-metastatic melanoma protein B), which is a glycoprotein expressed in melanoma and breast tumors (Rose et al. 2017). In addition to the dolastatins, other cyanobacterial metabolites destabilize the microtubule network. Notably, one such metabolite is tubercidin, which is a nucleoside produced by *Tolypothrix byssoidea*, *Tolypothrix distorta*, *Plectonema radiosum*, and *Scytonema saleyeriense* var. *indica* (Barchi et al. 1983; J. B. Stewart et al. 1988). This molecule was previously isolated from the bacterium *Streptomyces tubercidicus*. Tubercidin has shown inhibition of cell proliferation with an IC₅₀ of 248 nM (Table 6). Tubercidin acts against dolastatins showing a microtubule stabilizing activity comparable to taxol bioactivity (Mooberry et al. 1995). Its cytotoxicity is due to its stabilizing property, which causes mitotic arrest at G2/M transition and stops growth (Altmann 2001).

Table 6. Cytotoxic metabolites extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Tubercidin	Nucleoside	-Cytotoxic -Microtubule stabilizer	<i>Tolypothrix byssoidea</i> H-6-2, <i>Scytonema saleyeriense</i> var. <i>indica</i> CV-14-1, <i>Plectonema radiosum</i> DF-6-1, <i>Tolypothrix distorta</i> BL-11-2	(Barchi et al. 1983; Mooberry et al. 1995; J. B. Stewart et al. 1988)
Aurilides	Depsipeptide	-Cytotoxic -Lethal activity -Anti-swarmling -Antiprotozoal -Induce loss of microfilament network	<i>Lyngbya majuscula</i> , <i>Okeania</i> sp., <i>Lyngbya</i> sp.	(B. Han et al. 2006; Sueyoshi et al. 2016; Tripathi, Puddick, Prinsep, Rottmann, et al. 2010; Tripathi et al. 2011; P. G. Williams, Yoshida, Quon, et al. 2003)
Swinholide-type	Macrolide	-Cytotoxic -Actin microfilament disruption	<i>Symploca</i> sp., <i>Geitlerinema</i> sp., <i>Nostoc</i> sp. UHCC0451, <i>Phormidium</i> sp.	(Andrianasolo et al. 2005; Humisto et al. 2018; Tao et al. 2018)
Anabaenolysins	Lipopeptide	-Cytotoxic -Antifungal -Hemolytic activity -Ability to permeabilize cell membranes	<i>Anabaena</i> sp. XPORK 15F, <i>Anabaena</i> sp. XSPORK 27C	(Ofteidal et al. 2012; Jokela et al. 2012)

Another mechanism of cytotoxicity noted from cyanobacterial metabolites is the destabilization of actin microfilaments. As tubulin microtubules, actin microfilaments are key cytoskeleton components of cells. Microfilaments are involved in several mechanisms: cell division (cytokinesis), cell motility, cell adhesion, exocytosis, and endocytosis (Rao and Li 2004). Thus, molecules with actin-modulating activity are sought in order to develop anticancer drugs because of their ability to induce apoptosis (Rao and Li 2004). Four cyanobacterial metabolite families have shown disrupting activity of the actin microfilament network: the lyngbyabellins, the majusculamides, the aurilides, and the swinholide-like molecules (Table 6).

Lyngbyabellins and majusculamides, as mentioned above, have shown antifungal activity that likely corresponds to their ability to modulate actin polymerization (Hendrik Luesch et al. 2000; B. Han et al. 2005; Marquez et al. 2002; Simmons et al. 2009). Aurilides are cyclic depsipeptides, and the first member of this family was isolated from the sea hare *Dolabella auricularia* (Suenaga et al. 2004). Since then, seven other related molecules have been isolated from two cyanobacterial genera: *Lyngbya* and *Okeania* (B. Han et al. 2006; Sueyoshi et al. 2016; Tripathi, Puddick, Prinsep, Rottmann, et al. 2010; Tripathi et al. 2011; P. G. Williams, Yoshida, Quon, et al. 2003), and one from *Philinopsis speciosa* (cephalaspidean mollusc) (Nakao et al. 2004). Aurilides showed nanomolar cytotoxic activity associated with a moderate toxicity to *Artemia salina*. Two analogues, lagunamides A and B, have also shown antimalarial activity and anti-swarming activity against *Pseudomonas aeruginosa* (Tripathi, Puddick, Prinsep, Rottmann, et al. 2010) (Table 6). Han et al. (2006) (B. Han et al. 2006) showed that aurilides induce microfilament disruption at the micromolar level. They concluded that this disrupting activity is likely related to their toxic and antimicrobial activities.

Swinholide-type molecules were macrolides, originally isolated from the sponge *Theonella swinhoei* (Carmeli and Kashman 1985). In 2005, Andrianasolo et al. (2005) (Andrianasolo et al. 2005) succeeded in isolating swinholide A and two new related molecules (ankaraholides A and B) from two cyanobacteria (*Symploca* sp. and *Geitlerinema* sp., respectively), which leads to the hypothesis of a symbiotic origin of the compounds isolated from the sponge (Andrianasolo et al. 2005) (Table 6). More recently, Humisto et al. (2018) identified the swinholide biosynthetic cluster in *Nostoc* sp. (Table 6) (Humisto et al. 2018), and Tao et al. (2018) isolated nine swinholide-related metabolites from a marine *Phormidium* sp. (Tao et al. 2018). Swinholide A, isolated from the marine sponge, showed microfilament-disrupting activity by stabilizing actin dimers (Bubb et al. 1995). In addition to their cytotoxic activity, cyanobacterial swinholides also showed the same actin-disrupting activity, which is of interest for the development of related anticancer drugs (Andrianasolo et al. 2005).

Other metabolites with noticeable cytotoxicity are anabaenolysins, which are lipopeptides isolated from two strains of the *Anabaena* genus (Jokela et al. 2012) (Table 6). Anabaenolysins showed cytotoxicity against all of the 10 cell lines tested, with LC₅₀ values between 4 and 20 μ M depending on the cell lines and the anabaenolysin variants (Jokela et al. 2012). In addition, using a trypan dye exclusion assay, these authors showed that anabaenolysins have a unique profile. Instead of excluding the dye, cells showed an influx of trypan dye, which means that anabaenolysins permeabilize cell membranes until necrotic death (Jokela et al. 2012). Anabaenolysins are able to solubilize the lipid component of the cell membrane, and likely acts with the same mechanism as the detergent digitonin. Anabaenolysins particularly target cholesterol-containing membranes and do not induce permeabilization of mitochondria membranes. As detergents, anabaenolysins also show hemolytic activity, but at lower concentrations than digitonin and surfactin (Oftedal et al. 2012). In addition, Oftedal et al. (2012) showed that the permeabilization ability of anabaenolysins also allows the internalization of nodularin (Oftedal et al. 2012). This property is of interest for the development of a drug administration strategy involving anabaenolysins as a synergistic compound and other bioactive molecules that cannot be passed through the membrane within the targeted cells alone.

Six cyanobacterial families of compounds showed the ability to reverse multi-drug resistance (MDR) in addition to their cytotoxic properties. These include the cryptophycins (Golakoti et al. 1994; 1995; Subbaraju et al. 1997; Smith et al. 1994), the hapalindole-like metabolites (Stratmann, Moore, et al. 1994), hapalosin (Stratmann, Burgoyne, et al. 1994), the patellamides (Schmidt et al. 2005; A. B.

Williams and Jacobs 1993), the tolyporphins (Michèle R. Prinsep et al. 1992; 1995), and the westiellamide-like (Ogino et al. 1996) molecules (see Supplementary data S3). Among them, five families displayed MDR reversal activity by acting on the P-glycoprotein pumps (except for cryptophycins and patellamides for which the MDR reversal mechanisms have still not been described). P-glycoprotein is a glycosylated transmembrane protein that transports drugs and toxins out of the cell. This protein is often overexpressed in cancer cells and leads to resistance against standard chemotherapeutics because of its lower accumulation in targeted cells (Nanayakkara et al. 2018). Thus, metabolites with the ability to inhibit this efflux pump are of interest for developing anticancer drugs or to supplement current chemotherapeutic strategies in order to increase their efficiency on resistant cancer cells.

5.2.2. Protease Inhibitory Activity

Proteases are a widespread family of enzymes found in most, if not all, organisms. They are involved in a large number of pathways including coagulation, inflammation, digestion, hemostasis, and blood pressure regulation (Ilies et al. 2002; Drag and Salvesen 2010). There are several types of proteases that are classified by their specific hydrolysis mechanisms. The major groups are the metalloproteinases, the serine proteases, the cysteine proteases, the threonine proteases, and the aspartic acid proteases (Drag and Salvesen 2010). Because of their ubiquity, these enzymes are attractive targets for the development of new drugs against diverse diseases (Ilies et al. 2002). Some proteases have also shown the potential to act against thrombotic diseases (Ilies et al. 2002), hypertension (Drag and Salvesen 2010), pulmonary diseases (T. Dey et al. 2018), asthma (Guay et al. 2006), pathogenic microorganisms (M. R. Williams et al. 2017; Lee et al. 2017), and even cancers (Drag and Salvesen 2010; Adams 2004). According to our investigation, 24 families of metabolites presenting diverse protease inhibitor activities have been isolated from cyanobacteria to date. These compounds have shown inhibitory activity against a wide range of proteases, including enzymes belonging to the cathepsin family or the well-known serine proteases trypsin, chymotrypsin, and thrombin. Only three metabolite families have shown an inhibitory activity against cathepsins. Cathepsins are frequently overexpressed in cancer cells and are involved in tumorigenesis, cell invasion, and metastasis (Koblinski et al. 2000; Kos et al. 2014; Bian et al. 2016; Garcia et al. 1996; Lankelma et al. 2010; Leto et al. 2010). One of these three families, the spumigins, isolated from *Nodularia spumigena* and *Anabaena compacta* (Fujii et al. 1997; Anas et al. 2012; Fewer et al. 2009), is a set of linear peptides that are structurally similar to the aeruginosins (Table 7). They showed inhibitory activity against several proteases including trypsin, thrombin, plasmin, and cathepsin B (Anas et al. 2012). All of these proteases are potentially involved in cancer cell processes, and, notably, cathepsin B has been proposed to be a promising target for anticancer drug development (Gondi and Rao 2013; Kos et al. 2014).

Table 7. Serine protease inhibitor metabolites extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Spumigins	Peptide	-Proteases inhibitory activity	<i>Nodularia spumigena</i> AV1 & CCY 9414, <i>Anabaena compacta</i> NIES-835	(Anas et al. 2012; Fewer et al. 2009; Fujii et al. 1997)
		-Protease inhibitory activity	<i>Microcystis</i> sp., <i>Microcystis aeruginosa</i> ,	(Adiv et al. 2012; Al-Awadhi,
Cyanopeptolin-like	Depsipeptide	-Other enzyme inhibition	<i>Aphanocapsa</i> sp.; <i>Microchaete laktahensis</i> ,	Salvador, et al. 2017; Banker and Carmeli 1999;
		-Cytotoxic	<i>Planktothrix agardhii</i> ,	Beresovsky et al.
		-Lethal	<i>Scytonema hofmanni</i> ,	2006; Blom et al.
		-Antibacterial	<i>Lyngbya</i> sp.,	2003;
		-Antifungal	<i>Lyngbya confervoides</i> ,	

-Antiprotozoal	<p><i>Lyngbya</i> spp., <i>Lyngbya semiplena</i>, <i>Microcystis viridis</i>, <i>Diclothrix utahensis</i>, <i>Nostoc</i> sp., <i>Nostoc minutum</i>, <i>Planktothrix rubescens</i>, <i>Lyngbya majuscula</i>-<i>Schizothrix</i> sp. (Assemblage), <i>Stigonema</i> sp., <i>Symploca</i> sp., <i>Symploca hydnooides</i>, <i>Nostoc insulare</i></p>	<p>Bonjouklian et al. 1996; Choi et al. 2008; Elkobi-Peer and Carmeli 2015; Fujii et al. 2000; Gallegos et al. 2018; Grach- Pogrebinsky et al. 2003; Gunasekera et al. 2010; Harada et al. 2001; Harrigan, Luesch, et al. 1998; Harrigan, Luesch, Yoshida, Moore, Nagle, and Paul 1999; Ishida et al. 1997; 1998; 1995; Itou et al. 1999; Iwasaki et al. 2014; Jakobi et al. 1995; Kang, Kronic, et al. 2012; Kaya et al. 1996; Kodani et al. 1999; Kwan, Taori, et al. 2009; Lifshits et al. 2011; Lifshits and Carmeli 2012; Linnington et al. 2008; Lodin- Friedman and Carmeli 2013; Hendrik Luesch et al. 2002; Martin et al. 1993; Matern et al. 2001; 2003; Matthew et al. 2008; Taori et al. 2007; Mehner, Müller, Kehraus, et al. 2008; Murakami et al. 1997; L M Nogle et al. 2001; Okano et al. 1999; Okino, Murakami, et al. 1993; Okino et al.</p>
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				1997; Okumura et al. 2009; Ploutno and Carmeli 2002; Ploutno et al. 2002; Reshef and Carmeli 2001; Sano and Kaya 1995; 1996; Shin et al. 1995; Stolze et al. 2012; Taori et al. 2008b; Thorskov Bladt et al. 2014; Tsukamoto et al. 1993; Vegman and Carmeli 2013; Von Elert et al. 2005; Zafirir-Ilan and Carmeli 2010; Fujii et al. 2002)
Carmaphycins	Peptide	-Protease inhibition -Cytotoxic -Antiprotozoal	<i>Symploca</i> sp. WHG NAC15/Dec/08–5	(A. R. Pereira et al. 2012; LaMonte et al. 2017)

Another example of metabolites with interesting activity is the cyanopeptolin-like family. This family is the second in terms of the number of structural analogues isolated, after the microcystins (respectively 140 and 246 molecular variants described so far). Currently, more than 50 papers have reported on the isolation and activities of these metabolites. They are cyclic depsipeptides isolated from 12 different cyanobacterial genera (Table 7). Among the large number of analogs, a wide range of activity has been reported for these cyanobacterial metabolites including protease activity and other types of enzyme inhibition, cytotoxicity, lethal activity, and antimicrobial activity, which open various possibilities for developing therapies targeting cancer cells or microorganisms or those that fight some diseases like emphysema (Okino et al. 1997), pancreatitis (Hirota et al. 2006), or thrombosis (Hilpert et al. 1994). Nevertheless, this large number of activities can also represent a problem such as how to develop a therapeutic drug exhibiting a specific activity. It would be interesting to study some analogs in more depth or to conduct a structure–activity relationship study in order to increase the specificity of synthetic variants.

Lastly, another class of inhibitors that would be of interest for the development of new therapeutics against tumors is the proteasome inhibitors. Proteasome or ubiquitin-proteasome is a multi-enzymatic complex of eukaryotes. It is involved in protein degradation in a different way than the lysosomes (Hilpert et al. 1994). Because proteasome catalysis is involved in a wide variety of essential pathways, including cell-cycle progression and the regulation of apoptosis, it is a potent target for cancer therapy. Moreover, malignant cells have been shown to be more affected by proteasome inhibitors than normal cells, which reduce the potentially deleterious side effects of these molecules (Adams 2004). Four cyanobacterial families of metabolites were described to inhibit the 20S core of proteasome: the carmaphycins (A. R. Pereira et al. 2012), the cylindrocyclophanes (H. T. N. Bui et al. 2007; Chen et al. 1991; Chlipala, Sturdy, et al. 2010; Kang, Santarsiero, et al. 2012; Luo et al. 2014; B. S. Moore et al. 1992; Nakamura et al. 2012; Preisitsch et al. 2015), the nostocyclopeptides (Golakoti et al. 2001; Krunic et al. 2010), and nostodione (Shim et al. 2008; Kobayashi et al. 1994) (see

Supplementary data S3). Among them, the most efficient 20S proteasome inhibitors are the carmaphycins, which exhibit an IC₅₀ of around 2.5 nM (A. R. Pereira et al. 2012), whereas the other compounds present a micromolar range of action (Krunic et al. 2010; Shim et al. 2008; Chlipala, Sturdy, et al. 2010) (Table 7). Only two carmaphycin variants (A and B) have been isolated from *Symploca* sp., so far. These molecules are linear peptides with cytotoxic and antiprotozoal activities. They show the additional ability to inhibit the 20S proteasome activity in yeast and *Plasmodium* by interacting with the $\beta 5$ subunit (A. R. Pereira et al. 2012; LaMonte et al. 2017). These bioactivities are interesting for the use of carmaphycins as anticancer or antimalarial therapeutics. Two studies were conducted to enhance the specificity of carmaphycins for either applications. To develop a specific antimalarial drug, LaMonte et al. (2017) synthesized synthetic analogues of carmaphycin B and identified one analog with a selectivity index of 380 for antiprotozoal activity over cytotoxic activity (LaMonte et al. 2017). On the other hand, Almaliti et al. (2018) studied the potential of carmaphycins as anticancer drugs and as an antibody–drug conjugate (ADC) in order to enhance the selectivity of the molecules for cancer cells and to reduce the potential side effects (Almaliti et al. 2018).

Therefore, cyanobacterial metabolites with protease inhibition activities were shown to be less specific for further use, but the synthesis of synthetic analogs increased the selectivity of some of these molecules.

5.2.3. Histone Deacetylase Inhibitors

Histone deacetylases (HDACs) are enzymes involved in re-modeling the chromatin and the acetylation/deacetylation of histone and non-histone proteins. Furthermore, histone deacetylases play a key role in histone–DNA interactions and in the binding to transcription factors. HDACs have also been identified as potent regulators of gene expression (Salvador-Reyes and Luesch 2015; Bolden et al. 2006). Because cancer generally emerges from genetic mutations inducing hyperactivation of oncogenes or loss of tumor-suppressor genes, targeting mechanisms that are involved in the epigenetic regulation of genes is a promising strategy for the development of anti-tumor drugs (Bolden et al. 2006).

Table 8. HDACs inhibitor metabolites extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Largazole	Depsipeptide	-Histone deacetylases inhibitor -Cytotoxic -Other enzyme inhibition -Pro-drug	<i>Symploca</i> sp.	(Taori et al. 2008a; Y. Liu et al. 2010; Wu et al. 2013; Zhou et al. 2014; Poli et al. 2017; Ungermannova et al. 2012)
Santacruzamate A	Carboxylic acid derived	-Histone deacetylases inhibitor -Cytotoxic	<i>Symploca</i> sp. PAC-19-FEB-10-1	(Pavlik et al. 2013)

Two molecules showing histone deacetylase inhibitory activity have been isolated from cyanobacteria so far, which are known as largazole and santacruzamate A. Both of these molecules come from *Symploca* sp. strains (Table 8). Largazole has shown inhibition against 12 class I HDACs in addition to inhibition of the ubiquitin-activating enzyme (E1). It has also shown cytotoxicity to several cell lines (Table 8). Largazole acts as a pro-drug—the molecule needed to be activated by hydrolysis to release its active form, the largazole thiol (Salvador-Reyes and Luesch 2015). Santacruzamate A has also shown histone deacetylase inhibition and cytotoxicity. It shares some structural features with suberoylanilide hydroxamic acid (SAHA), which is a clinically approved

HDAC inhibitor that is used to treat refractory cutaneous T-cell lymphoma (Pavlik et al. 2013). Salvador-Reyes and Luesch (2015) performed an in-depth review of the activities and mechanisms of action of these two metabolites (Salvador-Reyes and Luesch 2015). They highlighted the high potency of largazole in anticancer drug development, while the potency of santacruzamate seems to remain more limited.

5.3. Anti-Inflammatory and Antioxidant Activity

In this section, we specifically describe cyanobacterial metabolites that showed no cytotoxicity associated with their anti-inflammatory or antioxidant properties.

5.3.1. Anti-Inflammatory Activity

According to our review, seven metabolite families isolated from cyanobacteria were found to have anti-inflammatory activity (aeruginosins, coibacins, honaucins, malyngamides, phycocyanin, scytonemin, and tolypodiol).

Currently, anti-inflammatory molecules have been widely studied in order to develop new therapeutics directed against chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, chronic obstructive pulmonary disease, multiple sclerosis, and inflammatory bowel disease (Hanke et al. 2016). Anti-inflammatory compounds can also be useful against cardiovascular diseases, such as atherosclerosis (Ridker and Lüscher 2014), and neurodegenerative diseases like Parkinson's disease (Rocha et al. 2015).

Table 9. Anti-inflammatory metabolites extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Aeruginosins	Peptide	-Anti-inflammatory activity -Protease inhibitor -No cytotoxicity	<i>Microcystis aeruginosa</i> NIES-98, NIES-298, NIES-101, NIES-89. <i>Microcystis viridis</i> NIES-102, <i>Planktothrix agardhii</i> CYA 126/8. <i>Nodularia spumigena</i> CCY9414. <i>Nostoc</i> sp. Lukesova 30/93	(Ishida et al. 1999; 2007; Kapuścik et al. 2013; Matsuda et al. 1996)
Phycocyanin	Peptide	-Anti-inflammatory -Antioxidant -Specific inhibitor of COX-2 -No lethality	All	(Benedetti et al. 2004; Kuddus et al. 2013; C. Romay et al. 1998; Ch. Romay et al. 2003; A. Patel et al. 2006)
Scytonemin	Alkaloid	-Anti-inflammatory -Enzyme inhibition -No cytotoxicity	<i>Stigonema</i> sp., <i>Nostoc punctiforme</i> , <i>Anabaena variabilis</i> , <i>Anabaena ambigua</i> , <i>Aphanocapsa/Synechocystis</i> sp. (assembly), <i>Aulosira fertilissima</i> , <i>Calothrix</i> sp., <i>Calothrix parietina</i> , <i>Calothrix crustacea</i> , <i>Chlorogloeopsis</i> sp., <i>Chroococcidiopsis</i> sp., <i>Chroococcus</i> sp.; <i>Cylindrospermum</i> sp., <i>Diplocolon</i> sp., <i>Entophysalis granulosa</i> , <i>Gloeocapsa</i> sp., <i>Hapalosiphon</i> sp., <i>Hapalosiphon fontinalis</i> ; <i>Lyngbya</i> sp., <i>Lyngbya aestuarii</i> , <i>Nostoc parmelioides</i> , <i>Nostoc commune</i> , <i>Nostoc microscopium</i> , <i>Nostoc pruniforme</i> , <i>Phormidium</i> sp., <i>Pleurocapsa</i> sp., <i>Rivularia atra</i> , <i>Rivularia</i> sp., <i>Schizothrix</i> sp., <i>Scytonema</i> sp., <i>Tolyothrix</i> sp., <i>Tolypothrix tenni</i> , <i>Westiellopsis prolifica</i> , <i>Scytonema hoffmani</i>	(Ferran Garcia-Pichel and Castenholz 1991; Proteau et al. 1993; Rajesh Prasad Rastogi et al. 2015; Soule et al. 2007; C. S. Stevenson et al. 2002; Christopher S Stevenson et al. 2002)

Anti-inflammatory tests have been performed *in vitro* or *in vivo* in mice. For example, malyngamides have been shown to inhibit superoxide production generated by inflammation-promoting agents (Karla L. Malloy et al. 2011), and honaucins inhibit pro-inflammatory cytokine expression (Choi, Mascuch, et al. 2012) in the murine macrophage cell line RAW264.7. The mouse ear edema assay has been performed *in vivo* by observing the resorption of ear edema in the presence of anti-inflammatory compounds, such as phycocyanin (Ch. Romay et al. 2003), scytonemin (C. S. Stevenson et al. 2002), and tolypodiol (Michèle R. Prinsep et al. 1996), which have shown noteworthy activities by using this assay.

Three metabolites seem to be particularly interesting according to their specific bioactivity profiles: the aeruginosins, phycocyanin, and scytonemin, which have not shown any toxicity when tested *in vitro* or *in vivo*. Aeruginosins have shown anti-inflammatory properties using the AlphaLISA assay. They are able to down-regulate the level of pro-inflammatory mediators (IL-8 and ICAM-1) in stimulated endothelial cells (Kapuścik et al. 2013) without affecting the viability of two different cell lines (Kapuścik et al. 2013) (Table 9). Aeruginosins have also shown serine protease inhibitory activity against trypsin, thrombin, and plasmin (Ishida et al. 1999), and their corresponding biosynthetic gene cluster was first identified in *Planktothrix agardhii* and *Nodularia spumigena* (Table 9) (Ishida et al. 2007; Fewer et al. 2009). Currently, no correlation between serine protease inhibition and the anti-inflammatory activity of aeruginosins were shown. However, on neutrophils, it has been shown that some serine proteases (elastase, cathepsin G, and proteinase 3) are responsible for the conversion and activation of proinflammatory chemokines (and notably, interleukine-8 (IL-8)) and are able to conserve or enhance the inflammation response (Padrines et al. 1994; Wiedow and Meyer-Hoffert 2005; Pham 2006). In this regard, it will be compelling to further test whether aeruginosins are capable of inhibiting other serine proteases, notably elastase, cathepsin G, and proteinase 3, in order to determine whether the down-regulation of IL-8 induced by the aeruginosins is mediated through serine protease inhibition processes.

Phycocyanin is a phycobiliprotein, constituting one of the major cyanobacterial pigments, together with the chlorophylls and phycoerythrin. It is involved in light-harvesting and the energy transfer of phycobilisomes within the outer membrane of thylakoids. In addition, phycocyanin has shown a wide variety of beneficial properties including antioxidant, anti-inflammatory, neuroprotective, and hepatoprotective activities (Ch. Romay et al. 2003) (Table 9). Authors of phycocyanin studies have reviewed the main features of phycocyanin anti-inflammatory mechanisms. Phycocyanin is able to scavenge ROS, has anti-lipoperoxidative effects, and inhibits cyclooxygenases (specifically COX-2) as well as TNF- α release. All of these properties are unique from the perspective of new therapeutics development targeting neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, or Huntington's disorder, or as an anti-inflammatory agent (Ch. Romay et al. 2003).

Scytonemin is an alkaloid pigment found in the sheath of some cyanobacteria and, particularly, on some organisms living in extreme environments (Rajesh Prasad Rastogi et al. 2015). Scytonemin synthesis is mainly induced by UV-A exposure in order to reduce heating and the oxidative stress (Rajesh Prasad Rastogi et al. 2015). Scytonemin is mainly involved in photoprotection by UV-absorption (Rajesh Prasad Rastogi et al. 2015). It has also been shown to have anti-inflammatory activity with no cytotoxicity against non-proliferating cells (C. S. Stevenson et al. 2002; Rajesh Prasad Rastogi et al. 2015; Christopher S Stevenson et al. 2002). In addition, scytonemin has been shown to inhibit polo-like kinase 1 (PLK1), which is an enzyme involved in the phosphorylation and activation of proteins such as cdc25C, which is involved in cell cycle progression and the G2/M transition in the cell cycle (Table 9). As a consequence, scytonemin can repress cell proliferation (C. S. Stevenson et al. 2002; Rajesh Prasad Rastogi et al. 2015; Christopher S Stevenson et al. 2002). Therefore, scytonemin could be a promising compound for use in the development of anticancer therapeutics, sunscreen agents, or anti-inflammatory drugs.

Last but not least, as mentioned above, ambigol have been shown to inhibit cyclooxygenases. Cyclooxygenases are enzymes belonging to the oxidoreductase enzymatic class. Two related

isoforms, COX-1 and COX-2 (Dubois et al. 1998), have been discovered so far and are involved in inflammation processes through the synthesis of prostaglandins from arachidonic acid. Some classical anti-inflammatory molecules are known to target COX. For example, aspirin, which is the most famous COX inhibitor discovered so far, is a nonsteroidal anti-inflammatory drug (NSAID) (Patrignani and Patrono 2015). For these reasons, ambigol is a promising cyanobacterial anti-inflammatory compound. Nevertheless, further studies are still needed in order to describe its activities and potential unexpected side effects in-depth (Vane and Botting 1998).

5.3.1. Antioxidant Activity

Oxidative stress is widely recognized to be implicated in neurodegenerative diseases (Uttara et al. 2009; G. H. Kim et al. 2015), metabolic disorders (Rani et al. 2016), hypertension (Baradaran et al. 2014), liver diseases (Li et al. 2015), and cardiovascular diseases (Siti et al. 2015). Thus, antioxidant molecules are required to develop or supplement therapy for reducing the harmful effects of oxidative stress.

According to our review, only four compounds isolated from cyanobacteria show antioxidant properties. As mentioned above, this low number in comparison to cytotoxic or antimicrobial compounds might be due to the fact that this activity has been poorly tested in secondary metabolites and its testing has generally been limited to pigments or molecules implicated in light-harvesting or UV protection. Antioxidant activity has been characterized for the carotenoids, chlorophyll, the mycosporine-like amino acids (MAAs), and the phycobiliproteins such as phycocyanin (Table 10).

Table 10. Antioxidant metabolites extracted from the database.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Carotenoids	Terpenoid	-Antioxidant -Sunscreen	All	(Wada et al. 2013; Stahl and Sies 2003; Hirschberg and Chamovitz 1994)
Chlorophylls	Chlorin	-Photosynthesis -Antioxidant -Pro-oxidant (sensitizer for singlet oxygen production)	All	(Wada et al. 2013; Lanfer-Marquez et al. 2005)
MAAs	Cyclohexenone linked with an amino acid	-Antioxidant -Sunscreens	<i>Synechocystis</i> sp. PCC 6803, <i>Gloeocapsa</i> sp. CU-2556, <i>Aphanothece halophytica</i> , <i>Gloeocapsa</i> sp., <i>Euhalothece</i> sp., <i>Microcystis aeruginosa</i> , <i>Arthrospira</i> sp. CU2556, <i>Lyngbya</i> sp. CU2555, <i>Leptolyngbya</i> sp., <i>Phormidium</i> sp., <i>Lyngbya</i> cf. <i>aestuarii</i> , <i>Microcoleus chthonoplastes</i> , <i>Microcoleus</i> sp., <i>Oscillatoria spongelidae</i> , <i>Trichodesmium</i> spp., <i>Anabaena</i> sp., <i>Anabaena</i> <i>doliolum</i> , <i>Anabaena variabilis</i> PCC 7937, <i>Nostoc</i> sp., <i>Nostoc commune</i> var. <i>Vaucher</i> , <i>Nostoc commune</i> , <i>Scytonema</i> sp., <i>Nostoc punctiforme</i> ATCC 29133, <i>Nostoc</i> sp. HKAR-2 and HKAR-6, <i>Nodularia baltica</i> , <i>Nodularia harveyana</i> , <i>Nodularia spumigena</i> , <i>Aphanizomenon flos-aquae</i> , <i>Chlorogloeopsis</i> PCC 6912	(Jain et al. 2017; Wada et al. 2013; Balskus and Walsh 2010; Waditee- Sirisattha et al. 2014)
Phycocyanin	Peptide	- Anti- inflammatory - Antioxidant - Specific inhibitor of COX-2 - No lethality	All	(Benedetti et al. 2004; Kuddus et al. 2013; A. Patel et al. 2006; C. Romay et al. 1998; Ch. Romay et al. 2003)

Carotenoids are orange pigments that are localized in the thylakoid membrane. They absorb light between 400 and 500 nm and are involved in photosynthesis by transferring energy to chlorophyll through a single-singlet energy transfer mechanism (Kosourov et al. 2016; Hirschberg and Chamovitz 1994). Five carotenoids are found in the majority of cyanobacteria: β -carotene, zeaxanthin, nostoxanthin, echinenone, and canthaxanthin (Wada et al. 2013). In addition to their role in light harvesting, carotenoids act as potent photoprotectant molecules and show antioxidant activity through ROS scavenging (Wada et al. 2013; Kosourov et al. 2016) (Table 10).

Chlorophylls are the ubiquitous pigments of photosynthetic organisms. Chlorophyll *a* is the major isoform used by cyanobacteria with most absorbing light at 660 nm (Wada et al. 2013). Chlorophylls are mainly involved in photosynthesis, but they have also shown antioxidant activity in vitro via radical scavenging and, on the contrary, singlet oxygen production under high light conditions, which mitigates their potential use as antioxidant therapeutics (Wada et al. 2013) (Table 10).

Mycosporine-like amino acids (MAAs) are pigments that are widely produced by cyanobacteria (Table 10) and other algae (Jain et al. 2017; Wada et al. 2013). They absorb light in the UV-A and UV-B ranges with a maximum absorbance between 310 and 360 nm (Jain et al. 2017). The primary function of MAAs is to protect cells from damage by absorbing UV and to dissipate energy without generating ROS (Waditee-Sirisattha et al. 2014; Wada et al. 2013). In addition, MAAs show other unique properties. They have been demonstrated to have antioxidant activity through ROS scavenging, are able to protect skin from UV damage, and are involved in osmotic regulation, desiccation, and defense against oxidative and thermal stresses. They are also able to protect fibroblasts against UV-induced cell death (Waditee-Sirisattha et al. 2014; Jain et al. 2017). Jain et al. (2017) stated that two products containing MAAs have been commercialized as sunscreen agents for cosmetics and for use in plastics, paints, and varnishes as a photo stabilizer (Jain et al. 2017).

Lastly, as mentioned above, phycocyanins are antioxidant molecules with the ability to scavenge ROS. In addition to their anti-inflammatory activity, this antioxidant property increases the potential of phycocyanins to be used for pharmaceutical applications (Kuddus et al. 2013).

5.4. Other Metabolites with Potential Beneficial Properties

To close this review on the beneficial activities demonstrated for cyanobacterial metabolites, we highlight a few other compounds that are of potential interest for various fields of application because of their specific features.

For instance, grassystatins-tasiamides constitute a depsipeptide group of related compounds isolated from *Lynghya* and *Symploca* tropical species (Al-Awadhi, Law, et al. 2017; Al-Awadhi et al. 2016; Kwan, Eksioğlu, et al. 2009; Molinski et al. 2012; P. G. Williams et al. 2002; P. G. Williams, Yoshida, Moore, et al. 2003; Mevers, Haeckl, et al. 2014). These metabolites have shown protease inhibitory activity against cathepsin D, cathepsin E, and the β -amyloid precursor protein-cleaving enzyme A (BACE1) for tasiamides B and F (Al-Awadhi et al. 2016; Al-Awadhi, Law, et al. 2017) (Table 11). In addition, these compounds have shown moderate or no cytotoxicity at concentrations higher than required for protease inhibitory activity (Mevers, Haeckl, et al. 2014; Molinski et al. 2012; P. G. Williams et al. 2002). Cathepsin D is an aspartic protease that is localized in the lysosome. This enzyme is considered a biomarker of some forms of metastatic breast cancer because of its related overexpression (Garcia et al. 1996). Cathepsin D has also been shown to promote proliferation and metastasis (Garcia et al. 1996). Cathepsin E, being also an aspartic protease, is mainly localized in immune system cells and, notably, in antigen-presenting cells (Yamamoto et al. 2015). Grassystatin A induces the reduction of antigen presentation in dendritic cells (Kwan, Eksioğlu, et al. 2009), which is correlated with the involvement of cathepsin E in this process and has led to the hypothesis that grassystatin could modulate the immune response. Alzheimer's disease pathogenesis is mediated by the accumulation of amyloid β peptide (A β) in the brain. BACE1 is responsible for A β formation by cleaving the amyloid precursor protein (APP). As a result, BACE1 inhibitors could be promising targets for the development of new therapeutics against Alzheimer's disease (Vassar et al. 1999; Al-Awadhi et al. 2016). Considering

these activities, we assume that members of the grassystatins-tasiamides family constitute promising components for the development of antiproliferative agents, immune response modulatory compounds, and therapeutics for Alzheimer’s disease treatment.

During the process of database construction, we noticed that five metabolite families showed a remarkable ability to bind to cannabinoid receptors (CB1 and CB2). These metabolites were grenadamide (N. Sitachitta and Gerwick 1998), the semiplenamides (B. Han et al. 2003), serinolamide A (Gutiérrez et al. 2011), mooreamide A (Mevers, Matainaho, et al. 2014), and the columbamides (Kleigrew et al. 2015). CB1 and CB2 are cell membrane receptors that belong to the endocannabinoid system (ECS), which is an important part of the human physiological system. It is involved in a wide range of different processes, such as brain plasticity, memory, nociception, appetite regulation, the sleep–wake cycle, the regulation of emotions and stress, and addiction. This ubiquity for the regulation of various vital processes makes exogenous CB1 and CB2 ligands attractive as modulators of this system for the management of pain, diabetes, obesity, cancer, epilepsy, or Alzheimer’s disease, or to develop new anxiolytics (Aizpurua-Olaizola et al. 2017; S. Patel et al. 2017). Columbamides are the most potent CB1/CB2 ligands from cyanobacteria discovered so far (Table 11) (Kleigrew et al. 2015). They are linear acyl amides that have been isolated from *Moorea bouillonii* PNG05-198 using a genome mining approach (Kleigrew et al. 2015). To date, only the CB1-binding and CB2-binding activity of columbamides has been tested, and other investigations are required in order to look deeper into the activity profiles of these molecules, since they still remain promising compounds for therapeutic developments.

Table 11. Other metabolites extracted from our database with promising biomedical potential.

Molecule Family	Chemical Classes	Activity	Producing Organisms	References
Grassystatins-Tasiamides	Depsipeptide	-Protease inhibitory activity	<i>Lyngbya confervoides</i> ,	(Al-Awadhi et al. 2016;
		-Cytotoxic	<i>Symploca</i> sp.,	Al-Awadhi, Law, et al. 2017; Kwan, Eksioglu, et al. 2009;
		-Reduce antigen presentation in dendritic cells	<i>Symploca</i> sp. NHI304, <i>Lyngbya</i> sp. NIH399	Mevers, Haeckl, et al. 2014; Molinski et al. 2012; P. G. Williams et al. 2002; P. G. Williams, Yoshida, Moore, et al. 2003)
Columbamides	Acyl amide	-CB1 and CB2 ligands	<i>Moorea bouillonii</i> PNG05-198	(Kleigrew et al. 2015)

6. Conclusion

In this review, all available information concerning the beneficial activities of natural products of cyanobacteria was gathered. To write this review, a molecular database of the various families of metabolites isolated from cyanobacteria was constructed from the systematic analysis of 670 articles. The derived database represents 260 families of metabolites. It groups various types of information concerning the taxonomy of producing strains, the respective chemical classes, the origin strain habitats,

and the tested/demonstrated activities for each member of the family, together with the related full references.

According to this review, from the above 300 different genera of cyanobacteria (referenced by the taxonomy published by Komarek et al. in 2014) (Komarek et al. 2014), 90 have, so far, been reported to produce bioactive metabolites. Some of them have been shown to produce a high number of compounds, such as those from the genus *Lyngbya-Moorea*, which includes 85 families of metabolites isolated so far. However, the *Lyngbya* genus is a polyphyletic group and its taxonomy position is under revision. This number might be re-evaluated and distributed within distinctive new genera. The genomes of the producing strains are not available in the majority of cases, whereas Shih et al. (2013) demonstrated the large genomic potential of numerous cyanobacteria thanks to the biosynthetic pathways of metabolites highlighted by genome mining analyses (Shih et al. 2013). Therefore, the potential for the discovery of new natural molecules and new biosynthetic pathways from cyanobacteria still remains very important and needs to be systematically explored.

Cyanobacterial metabolites belong to 10 chemical classes (including peptides, alkaloids, terpenes, and lipids), where most of the families of metabolites are peptide derivatives (above 50% of the families). Fourteen different types of activities can be distinguished for cyanobacterial metabolites (e.g., antimicrobial, lethality, cytotoxicity, and antioxidant). The large majority of the components are cytotoxic (110 families), whereas some activities have only been tested rarely, and their occurrence appears to be weakly demonstrated. Globally, no clear correlation has been observed between chemical classes and the specificity of the respective types of bioactivity. Further studies are needed in order to precisely understand the mechanisms of action of cyanobacterial metabolites, which potentially links bioactivity with structural features in order to support the new hypothesis on the biological function of the production of these components for organisms.

Lastly, 47 metabolites isolated from cyanobacteria that present remarkable interest for diverse fields of application were investigated further in the present literature review. For example, hassallidins, which show specific antifungal activity without antibacterial activity, and scytonemin, which has anti-inflammatory properties with no cytotoxicity, were detailed. These metabolites are potentially useful for the development of new concrete applications for cyanobacterial natural products and illustrate the interest in cyanobacteria as a prolific source of bioactive molecules.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Supplementary data S1: Amino acid sequences of cyanopeptolin-like family; S2: Cyanobacterial metabolite database; S3: Detailed of the molecules described. ([en annexe 5 de ce document](#))

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Chapitre II

Description des cyanobactéries issues
des boues thermales de Balaruc-les-
Bains

Chapitre II

DESCRIPTION DES CYANOBACTERIES ISSUES DES BOUES THERMALES DE BALARUC-LES-BAINS

II.1. Contexte de l'étude

Les cures thermales sont utilisées depuis des siècles pour les propriétés curatives qu'elles apportent, notamment pour la guérison de maladies inflammatoires telles que les rhumatismes ou encore des maladies de peau. Bien qu'il soit difficile d'établir précisément l'origine des propriétés thérapeutiques des cures, il est probable qu'il existe un effet « cocktail » apporté par les soins en eux-mêmes, les activités physiques douces pratiquées par le patient et la relaxation induite par la chaleur, l'eau et les massages. Pour les soins par pélothérapie, les propriétés curatives sont dues à la partie non-organique du péloïde (eau thermale et boue argileuse) et à la « bioglée », c'est-à-dire la partie organique de la boue regroupant microalgues, cyanobactéries, bactéries et les molécules qu'elles produisent. Les effets bénéfiques sont en partie dus à la chaleur de l'eau thermale, analgésique, à l'interaction eau/boue qui augmente les échanges d'ions à travers la peau et les propriétés antimicrobienne, anti-inflammatoire, antioxydante ... apportées par les molécules organiques produites par la bioglée (Tateo et al. 2009; Centini et al. 2015; Giorgio et al. 2018).

Plusieurs brevets ont d'ailleurs été publiés au fil des années par les établissements thermaux. En 1971, une société cosmétique italienne publiait un brevet d'invention concernant la préparation de « produits dérivés de flores thermales » pour des usages cosmétiques (Curt Georgi 1971). En France, ce sont les Thermes de Dax qui ont breveté en 2003 un procédé complet de préparation/maturation de péloïdes avec l'ajout de diatomées et/ou de cyanobactéries, ainsi que des bactéries sulforéductrices, mais en évoquant seulement des applications en rhumatologie sans mentionner les principes actifs (Counilh and Gibert 2003). En 2005, les thermes Abano en Italie brevètent, quant à eux, un procédé de maturation des boues favorisant la croissance de la cyanobactérie *Phormidium* sp. ETS05 et sa production d'exopolysaccharides à activité anti-inflammatoire (Lalli et al. 2004; Zampieri et al. 2020).

Les Thermes de Balaruc-les-Bains sont spécialisés dans les soins en rhumatologie et phlébologie. Bien qu'ayant dû abandonner l'utilisation historique du péloïde pour des raisons sanitaires, plusieurs procédés innovants ont été mis en place ces dernières années afin d'apporter des propriétés anti-inflammatoires, antalgiques, drainantes ou décongestionnantes aux curistes souffrant de douleurs articulaires ou de problèmes de circulation sanguine (Thermes de Balaruc-Les-Bains 2019). Néanmoins, nous avons vu que les microorganismes et notamment les cyanobactéries apportent, elles aussi, de nombreux bienfaits grâce à la production de molécules bioactives. Dans cette perspective, il est intéressant pour les Thermes de Balaruc-les-Bains de développer de nouveaux procédés de soins

intégrant des molécules ou des microorganismes isolés des boues thermales de Balaruc-les-Bains. C'est donc en partie pour évaluer cette perspective que ce projet de thèse a vu le jour, avec l'idée d'investiguer le potentiel thérapeutique des neuf souches de cyanobactéries provenant des boues de Balaruc-les-Bains et décrites dans ce chapitre.

Les cyanobactéries sont des productrices prolifiques de métabolites à activités variées. Cependant, il est difficile de corréler la diversité de production de ces métabolites en fonction des souches qui les produisent si celles-ci ne sont pas identifiées ou si elles sont mal assignées taxonomiquement. De plus, à l'heure du Big Data et de la quantité colossale d'informations apportée par les approches omiques, il est particulièrement important d'avoir une affiliation taxonomique solide des souches pour comparer le potentiel métabolomique et le potentiel d'activités des cyanobactéries.

La classification taxonomique des cyanobactéries a fait l'objet de nombreuses révisions et plus de six classifications différentes ont été publiées entre 1892, avec la première classification de Gomont (Gomont 1892), et 1979, avec la séparation des cyanobactéries en cinq sections (I, Chroococcales ; II, Pleurocapsales ; III, Oscillatoriales ; IV, Nostocales ; V, Stigonematales) (Rippka et al. 1979). Ces premières classifications se sont basées essentiellement sur des critères morphologiques tels que l'organisation unicellulaire ou en filament, la forme des cellules et/ou du filament, la présence ou non d'une gaine, la capacité à former ou non des cellules différenciées fixatrices d'azote appelées hétérocytes etc. Néanmoins, ces classifications ont rapidement montré leurs limites avec l'essor des techniques de séquençage moléculaire, de microscopie électronique et avec l'observation que les cyanobactéries sont capables d'adapter rapidement leur morphologie à leurs milieux de vie (Palinska and Surosz 2014). En 1985, Anagnostidis et Komárek débutent une révision profonde de la classification des cyanobactéries basée sur le concept que la classification taxonomique devait refléter l'histoire évolutive des cyanobactéries. Ces travaux, basés sur une approche multiphasique de biologie moléculaire, de phylogénie et de comparaison des ultrastructures, ont abouti à la publication en 2014 d'une classification plus moderne des cyanobactéries (Komarek et al. 2014; Komárek 2016).

Dans le cadre général de la thèse et pour répondre à la seconde question : *Quelle est la taxonomie des souches isolées et le potentiel des souches à produire des métabolites d'intérêt ?*, il est essentiel, de caractériser finement les cyanobactéries étudiées, afin d'apporter des bases solides à l'étude des métabolites et des activités qu'elles produisent. Une approche polyphasique a été utilisée sur les neuf souches de cyanobactéries isolées des boues thermales de Balaruc-les-Bains afin de (i) décrire et assigner taxonomiquement les souches étudiées, (ii) d'analyser la diversité de ces cyanobactéries et de (iii) comparer ces résultats avec les précédentes études menées sur la colonisation du péloïde de Balaruc-les-Bains en 1986 et 1987.

II.2. Résumé

Plusieurs cyanobactéries ont été isolées depuis un bassin de décantation de boues thermales mimant les conditions de maturation du péloïde pendant une période de 7 mois (depuis la colonne d'eau, la surface des boues et le biofilm épilithique recouvrant les parois du bassin) (Hamlaoui 2014). Neuf souches sont maintenues en culture au sein de la collection de cyanobactéries du Muséum National d'Histoire Naturelle (PMC), et ont été décrites en s'appuyant sur des critères morphologiques (par microscopie photonique, microscopie électronique à balayage puis microscopie électronique à transmission) et des analyses moléculaires (séquençage du gène de l'ARNr 16S et de la région ITS entre le 16S et le 23S).

Characterization of cyanobacteria isolated from thermal muds of Balaruc-Les-Bains (France) and description of a new genus and species *Pseudo-chroococcus couteii*

Duval C.¹, Hamlaoui S.¹, Piquet B.², Toutirais G.², Yéprémian C.¹, Reinhardt A.³, Duperron S.¹, Marie B.¹, Demay J.¹, Bernard C.^{1*}

Soumis à *FEMS Microbes*

Cette approche polyphasique a permis la caractérisation des cyanobactéries issues des boues thermales et de mettre en avant leur diversité puisque les souches isolées appartiennent à quatre ordres et neuf genres différents : Synechococcales (*Leptolyngbya boryana*), Oscillatoriales (*Planktothricoides raciborskii*, *Laspinema* sp., *Microcoleus vaginatus*, *Lyngbya martensiana*), Nostocales (*Nostoc* sp., *Aliinostoc* sp., *Calothrix* sp.) et Chroococcales avec la description d'un nouveau genre *Pseudo-chroococcus couteii*. Toutes les affiliations taxonomiques s'appuient sur la phylogénie du gène ARNr 16S et pour certaines souches sur la région ITS (Internal Transcribed Spacer). Elles s'appuient également sur la description morphologique, structurale et ultrastructurale des neuf souches.

Malgré les biais liés à l'isolement, il est intéressant de constater qu'il existe une importante diversité taxonomique dans ce milieu pourtant très spécifique que sont ces boues thermales. En effet, la salinité et une concentration en ions métalliques élevée (Cl⁻ 6,8 g.L⁻¹ ; Na⁺ 3,7 g.L⁻¹ ; SO₄²⁻ 0,9 g.L⁻¹, **Annexe 1**) représentent des conditions potentiellement extrêmes pour certains organismes. Cette richesse, et l'assignation taxonomique des cyanobactéries étudiées, permettent d'appréhender les potentielles propriétés bénéfiques apportées par les métabolites produits par les cyanobactéries de Balaruc-les-Bains.

II.3. Cyanobactéries des boues de Balaruc-les-Bains et potentielles molécules d'intérêt pour les applications des Thermes

Les molécules produites par les cyanobactéries sont très diverses structuralement et ont souvent démontré une ou plusieurs bioactivités différentes (**Chap. I**). Dans le cadre de ces travaux, les familles

de métabolites ont été ciblées pour leurs bioactivités. Notre réflexion s'est basée sur deux points majeurs : (i) les molécules choisies devaient posséder des activités en accord avec les besoins thérapeutiques des Thermes et leurs procédés de soins, et surtout (ii) ne devaient posséder aucune toxicité qui pourrait représenter un risque sanitaire pour les curistes. Ainsi, les molécules présentant des propriétés anti-inflammatoires, antioxydantes, et/ou cicatrisantes et ayant montré une absence de toxicité ont été recherchées.

En effet, les molécules anti-inflammatoires sont particulièrement utilisées pour les applications médicales de nombreuses maladies liées à un dérèglement des processus inflammatoires qui aboutissent à des inflammations chroniques comme le psoriasis, la sclérose en plaque ou la maladie de Crohn. En rhumatologie, l'objectif est de traiter les maladies inflammatoires chroniques telle que la polyarthrite rhumatoïde, ou plus généralement tous les cas d'arthrite, qui sont dus à une atteinte inflammatoire de l'articulation (Hanke et al. 2016). Les molécules antioxydantes, quant à elles, sont utilisées pour combattre la formation de radicaux libres (responsables de l'oxydation des lipides, des protéines et de l'ADN) et plus généralement, le stress oxydatif. Ce dernier est décrit comme étant un déséquilibre des processus naturels de régulation en faveur de la formation de radicaux libres par rapport aux mécanismes de défenses antioxydantes de l'individu. Il serait d'ailleurs impliqué dans de nombreuses maladies telles que l'athérosclérose, certains cancers, les maladies neurodégénératives (Alzheimer, Parkinson, ...) et plusieurs maladies inflammatoires (arthrite, lupus, ...) (Lobo et al. 2010). Enfin, les molécules pro-cicatrisantes sont également particulièrement étudiées grâce à leurs applications cosmétiques et pharmaceutiques. En effet, la cicatrisation est un processus complexe impliquant les mécanismes de l'inflammation, la migration cellulaire des kératinocytes et différents processus de la réponse immunitaire (Lauffenburger and Horwitz 1996; Ridley et al. 2003).

D'après l'analyse bibliographique (**Chap. I**), les capacités de cicatrisation des molécules issues des cyanobactéries ont rarement été étudiées. Néanmoins, récemment, deux molécules, un peptide (NL13) issu d'*Arthrospira platensis* (Sarkar et al. 2020) et un depsipeptide issu de *Moorea bouillonii* (Cai et al. 2018), ainsi que des « vésicules extracellulaires » sécrétées par *Synechococcus elongatus* PCC 7942 (Yin et al. 2019) ont démontré une capacité à augmenter la cicatrisation par stimulation de l'angiogenèse⁴. D'autre part, sept molécules anti-inflammatoires et quatre molécules antioxydantes ont été isolées à ce jour chez les cyanobactéries. Elles peuvent être réparties en deux groupes, les pigments d'une part : chlorophylles, phycobilines, caroténoïdes et scytonémies ; et les métabolites spécialisés d'autre part : aéruginosines, coibacines, honaucines, malyngamides, tolypodiol, ambigol et le groupe des « mycosporine-like amino acids » (MAAs). Parmi toutes ces molécules, seuls les pigments, les aéruginosines et les MAAs, ont montré une activité anti-inflammatoire et/ou antioxydante, sans toxicité associée (**Chap. I, Article 1, partie 5.3**).

⁴ C'est-à-dire la croissance des vaisseaux sanguins

Afin de « prédire » la production potentielle de ces molécules par les cyanobactéries isolées des boues thermales de Balaruc-les-Bains, une analyse de la base de données a été réalisée pour répondre à la question : quel est le potentiel de production de ces molécules ciblées par les cyanobactéries de Balaruc-les-Bains ? Les résultats sont présentés **Tableau 1**.

La plupart des pigments (e.g. chlorophylles, phycocyanines et caroténoïdes) sont retrouvés chez toutes les cyanobactéries puisqu'ils interviennent dans les mécanismes de la photosynthèse. La scytonemine en revanche, un pigment jaune/brun localisé dans la gaine des cyanobactéries, n'a pas de rôle dans la photosynthèse et n'est pas synthétisée par tous les genres (**Figure 3B**). Elle est le plus souvent retrouvée chez des genres soumis à une forte quantité de lumière ou vivant dans des environnements extrêmes (Rajesh Prasad Rastogi et al. 2015). D'après notre analyse bibliographique, la scytonemine a pu être isolée des genres *Nostoc*, *Lyngbya*, *Calothrix* et à partir d'un genre de *Chroococcus* sp.

Les MAAs sont de petits métabolites photoprotecteurs produits par de nombreux organismes (cyanobactéries, microalgues, plantes, bactéries et champignons) et formés par la condensation entre un acide aminé et le 4-hydroxygadusol (**Figure 3C**). Plusieurs MAAs ont été isolés de souches appartenant aux genres très étudiés *Nostoc*, *Lyngbya* et *Calothrix* ; mais également des genres *Leptolyngbya* et *Microcoleus*.

Les honaucines A (**Figure 3E**), B et C ont quant à elles été isolées de *Leptolyngbya crossbyana* HI09-1. Ces molécules ont démontré une baisse de la production de monoxyde d'azote et d'interleukine-1 β (IL-1 β) chez des cellules stimulées par l'ajout de lipopolysaccharides (LPS), et une inhibition de la bioluminescence chez *Vibrio harveyi* signifiant un probable effet sur les mécanismes de quorum-sensing. Enfin, l'aeruginosine-865 (**Figure 3A**) a été isolée chez *Nostoc* sp. Lukešová 30/93, et a montré une capacité à réduire la production des cytokines IL-8 et ICAM-1 médiatrices de la réaction inflammatoire (**Tableau 1**).

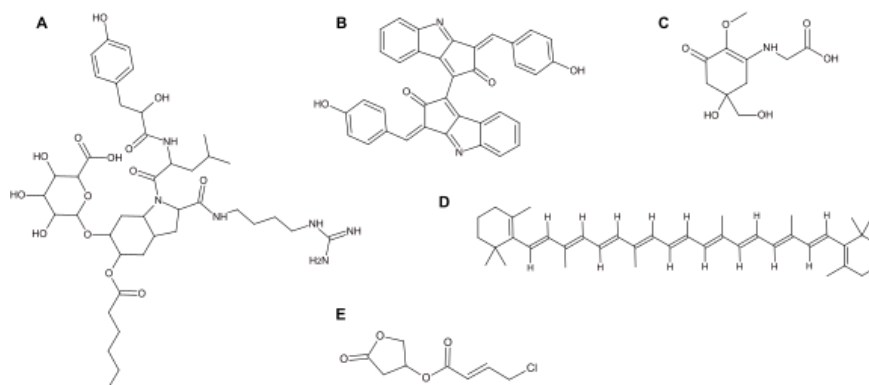


Figure 3. Molécules cibles. (A) aeruginosine-865, (B) scytonemine, (C) mycosporine-glycine, (D) β -carotène, (E) honaucine A

Au-delà de leur production de pigments, les genres *Aliinostoc* et *Laspinema*, nouvellement décrits (Bagchi et al. 2017; Heidari et al. 2018), ainsi que le genre *Planktothricoides* ne sont pas, à ce jour, étudiés pour la production de métabolites bioactifs (**Tableau 1**). D'après les résultats d'analyse de la bibliographie, les cyanobactéries *Lyngbya martensiana* PMC 880.14, *Nostoc* sp. PMC 881.14 et *Calothrix* sp. PMC 884.14, isolées des boues thermales de Balaruc-les-Bains, présenteraient le plus fort potentiel de production de composés aux activités anti-inflammatoires et/ou antioxydantes. Néanmoins, plusieurs travaux ont démontré des différences de production entre des souches appartenant à une même espèce (Kim Tiam et al. 2019). Pour répondre à la question du potentiel thérapeutique des cyanobactéries de Balaruc-les-Bains nous avons choisi de ne pas faire de sélection de souches à cette étape et d'évaluer les propriétés anti-inflammatoires et antioxydantes des neuf cyanobactéries de Balaruc.

Tableau 1. Molécules bioactives extraites de la base de données présentant des propriétés antioxydantes et anti-inflammatoires et produites par des cyanobactéries appartenant aux mêmes genres que les souches étudiées. MAAs: Mycosporine-like amino acids.

Molécules cibles	Caractéristiques	Bioactivités	Genres producteurs	Références
Aeruginosines	Peptide Linéaire NRPS Hydrosoluble	Non cytotoxique Anti-inflammatoire Inhibition protéases (trypsine, thrombine, plasmine)	Nostoc sp. Lukešová 30/93	(Ishida et al. 1999; Matsuda et al. 1996; Fewer et al. 2013; Kapuścik et al. 2013)
Caroténoïdes	Terpénoïde Pigment Liposoluble	Antioxydant Protection vs. UV	Toutes les cyanobactéries	(Karsten and Garcia-Pichel 1996; Stahl and Sies 2003; 2005; Hirschberg and Chamovitz 1994; Joshi et al. 2018; Takaichi et al. 2009)
Chlorophylles	Noyau tétra-pyrrole substitué à la porphyrine Pigment Liposoluble	Antioxydant Antimutagène Chimio préventif Photosensibilisation	Toutes les cyanobactéries	(Lanfer-Marquez et al. 2005; Queiroz Zepka et al. 2019; Ferruzzi and Blakeslee 2007)
Honaucines	Lactone Linéaire Liposoluble	Anti-inflammatoire Non antioxydant Inhibition du quorum-sensing	Leptolyngbya crossbyana HI09-1	(Choi, Mascuch, et al. 2012; Mascuch et al. 2017)
Mycosporine-like amino acids (MAAs)	Cyclohexenone-acide aminé Hydrosoluble	Antioxydant Absorption des UV Protection vs. UV Anti-âge	Calothrix parietina Calothrix sp. Leptolyngbya sp. Lyngbya aestuarii Lyngbya sp. CU2555 Lyngbya sp. Microcoleus chthonoplastes Microcoleus paludosus Microcoleus sp. Nostoc commune Nostoc microscopicum Nostoc punctiforme ATCC 29133 Nostoc sp.	(Rajesh P. Rastogi and Incharoensakdi 2014; F Garcia-Pichel and Castenholz 1993; Shibata 1969; Jain et al. 2017; Kageyama and Waditee-Sirisattha 2018; Ryu et al. 2014; Suh et al. 2003; Matsui et al. 2011; Sakamoto et al. 2019; Karsten and Garcia-Pichel 1996; Sinha et al. 2007)
Phycocyanines	Protéine Pigment Hydrosoluble	Antioxydant Anti-inflammatoire Neuroprotecteur Hepatoprotecteur	Toutes les cyanobactéries	(Ch. Romay et al. 2003; Ramirez et al. 2002; A. Patel et al. 2006; C. Romay et al. 1998)
Phycoérythrines	Protéine Pigment Hydrosoluble	Antioxydant Anti-âge	Nombreuses espèces, mais pas toutes	(Sonani et al. 2014; S. N. Patel et al. 2018; Bryant 1982)
Scytonemines	Alcaloïde Pigment Liposoluble	Anti-inflammatoire Protection vs. UV Inhibition kinases Anti-prolifération Induction de l'autophagie	Calothrix crustacea Calothrix parietina Calothrix sp. Chroococcus sp. Lyngbya aestuarii Lyngbya sp. Lyngbya sp. CU2555 Nostoc commune Vauch Nostoc microscopicum Nostoc parmelioides Nostoc pruniiforme Nostoc punctiforme PCC 73102, ATCC 29133	(C. S. Stevenson et al. 2002; Christopher S Stevenson et al. 2002; Itoh et al. 2013; Pathak et al. 2019; Fleming and Castenholz 2008; Ferran Garcia-Pichel and Castenholz 1991; Rajesh P. Rastogi and Incharoensakdi 2014)

II.4. Article 2

Characterization of cyanobacteria isolated from thermal muds of Balaruc-Les-Bains (France) and description of a new genus and species *Pseudo-chroococcus couteii*

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ABSTRACT

Cyanobacteria are able to synthesize a high diversity of natural compounds that account for their success in the colonization of a variety of ecological niches. Many of them have beneficial properties. The mud from the thermal baths of Balaruc-Les-Bains, one of the oldest thermal baths in France, has long been recognized as a healing treatment for arthro-rheumatic diseases. To characterize the cyanobacteria living in these muds and the metabolites they potentially produce, several strains were isolated from the water column and biofilms of the retention basin and analyzed using a polyphasic approach. Morphological, ultrastructural and molecular (16S rRNA gene and 16S-23S ITS region sequencing) methods were employed to identify nine cyanobacterial strains belonging to the orders Chroococcales, Synechococcales, Oscillatoriales and Nostocales. The combination of morphological and genetic characteristics supported the description of a new genus and species with the type species as *Pseudo-chroococcus couteii*. The high taxonomic diversity in the muds of the thermal baths of Balaruc-Les-Bains along with literature reports of the potential for bioactive metabolite synthesis of these taxa allowed us to hypothesize that some of the metabolites produced by these strains could contribute to the therapeutic properties of the muds from Thermes de Balaruc-Les-Bains.

KEYWORDS

cyanobacteria, thermal mud, taxonomy, polyphasic approach, morphology, ultrastructure.

INTRODUCTION

Cyanobacteria belong to an ancient group of photosynthetic prokaryotes presenting a broad range of cellular strategies, physiological capacities, and adaptations that support their colonization of diverse environments worldwide. Cyanobacteria can even exist in extreme habitats and are able to settle in diverse biotopes such as hot springs. They are also known for their production of natural bioactive compounds (Demay et al. 2019), including some potent toxins (microcystins, anatoxins, saxitoxins) (Bernard et al. 2017). Some of these metabolites have been used for applications in the biotechnology and pharmaceutical fields, which has created an increased interest in the search for new isolates of cyanobacteria. Both the chemical diversity and the related bioactivity must be considered when investigating the application potential of natural products. Demay et al. (2019) concluded that among the 300 different recognized genera of cyanobacteria (referenced by the taxonomy published by Komárek et al. in 2014), 90 have been reported to produce bioactive metabolites. A few taxa are known to be prolific producers of a large set of metabolites. For example the genus *Lyngbya-Moorea*, produces 85 families of the 260 metabolites isolated so far. The majority of species have not been tested, however, and the potential for the discovery of useful natural molecules and new biosynthetic pathways from cyanobacteria remains considerable and needs to be explored.

Thermes de Balaruc-Les-Bains is one of the oldest thermal centers in France and therapeutic application of the mud, whose beneficial effects have been documented since the end of the 19th century, was recognized by the French Health System as a healing treatment for arthro-rheumatic diseases. The beneficial mud is obtained by a process of maturation in which the mud is allowed to settle naturally from a combination of hot spring water and domestic rinse water from mud treatments in a large tank. Two types of biofilms were identified within the maturation basin, one at the surface of the mud and the other on the walls of the basin. Other photosynthetic microorganisms were also described in the water column of the basin. Only two experimental studies have been carried out on mud from Thermes de Balaruc-Les-Bains. The maturation of silt from Thau Pond in Balaruc-Les-Bains was monitored for a few months in 1983, and algal development was observed ten days after formation of a mud mesocosm comprised of *Phormidium* and *Oscillatoria* Cyanobacteria, and some Diatomophyceae (Baudinat 1986). Repeated experiments in 1984 gave similar results except that algal development occurred later, after one month (Baudinat 1986). The second study was carried out in 1987 by Dupuis in experimental mesocosms for thermal mud maturation in the laboratory. In the absence of mud, there was no algal development in water from Balaruc-Les-Bains despite the addition of nutrients. In the mud mesocosms at seven weeks after the experiment started, very thick cyanobacterial mats were observed on walls and bottom, dominated by *Phormidium africanum*, *P. autumnale*, *Lyngbya* sp., *Chroococcus minor* and *Anabaena* sp. These two studies illustrated the importance of cyanobacteria as major actors in the colonization of the mud of Thermes de Balaruc-Les-Bains (Baudinat 1986 & Dupuis 1987).

In the present study, we analyzed the diversity of cyanobacteria isolated from the mud of Thermes de Balaruc-Les-Bains. Nine clonal but non-axenic strains of cyanobacteria were found and their taxonomy was verified before genomic and metabolomic analysis and bioactivities studies on their natural products were conducted. The diversity of the strains was quite high, including members of four orders, Chroococcales, Synechococcales, Oscillatoriales and Nostocales.

MATERIALS AND METHODS

Sampling

Samples for strain isolation were collected twice a month from April 28 to October 13, 2014 (Table 1) from the thermal basins of Balaruc-Les-Bains (43°26'44.0"N 3°40'29.6"E).

The 500 m³ maturation basin (Figure 1a, c and d) received the muddy water from the rinsing of the patients. The slurry was a mixture of water from the thermal springs along with domestic hot water containing the mud particles, which settle to the bottom (Figure 1b and c).

The water in the retention basin was stirred by the continuous influx of rinse water, and the level was kept above the mud by draining excess water to the outside to prevent overflow (Figure 1d). The proper temperature and light levels and nutrient inputs were maintained to ensure that the mud maturation process was optimal. Three types of sampling were carried out from the maturation basin: (i) from the biofilm covering the mud, (ii) from the epilithic biofilm on the walls of the basin where algae development was more extensive and (iii) from the water surface using a 20- μ m membrane filter.



Figure 1. Retention basin of the Thermes de Balaruc-Les-Bains. Views of the mud maturation basin (mb), the water retention basin (rb) and the thermal spring water supply (ts) on February 28th, 2014 (A) and on May 12th, 2014 (B).

Cyanobacterial culture conditions and strain isolation

Samples were inoculated on solid medium (5 or 10 g·L⁻¹ agar) Z8 and Z8-salts (Rippka 1988). Isolations were carried out by repeated transfers (at least three times) of single cells or filaments on solid

or liquid media under an inverted microscope (Nikon ECLIPSE TS100). Viable clones were then cultured in 25 cm² culture flasks (Nunc, Roskilde, Denmark) containing 10 mL of Z8. Cyanobacterial strains were maintained in the Paris Museum Collection (PMC) at 25°C, using daylight fluorescent tubes providing an irradiance of 12 μmol photons·m⁻²·s⁻¹, with a photoperiod of 16h light/8h dark. Isolated strains and cultures were all monoclonal and non-axenic.

Morphological analyses

Morphological analyses of cyanobacterial strains were carried out using an Axio ImagerM2 Zeiss microscope. Photographs of the specimens were taken with an AxioCam MRc digital camera coupled to the microscope and images were processed using the ZEN software (Zeiss). The cell and filament width and length, morphology, color and motility were determined. Strains were identified morphologically using the updated taxonomic literature (Komárek and Anagnostidis 2005; Komárek and Anagnostidis 2007; Komárek and Johansen 2015).

Pigment analysis

Pigment analysis was performed on each strain in triplicate. Ten mL aliquots of culture in exponential growth were filtered through Whatman GF/F filters (Ø 0.7 μm) and freeze-dried. The lipophilic carotenoids and chlorophylls were extracted, analyzed and quantified by HPLC-DAD as described (Ras et al., 2008). The water soluble phycobiliproteins were analyzed following a Standard Operating Procedure (Yéprémian et al. 2017).

Ultrastructural analyses

Cyanobacterial strains were imaged by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described by Parveen et al. (2013), with modifications. Cells or filaments harvested by centrifugation from a growing culture were fixed with 2% (v/v) glutaraldehyde, 2% (v/v) formaldehyde, 0.18 M sucrose, and 0.1% picric acid in 0.1 M Sorensen phosphate buffer (SPB; pH 7.4) for 1 h, at room temperature. The specimens were then washed three times with SPB and post-fixed with 1% osmium tetroxide for 1 h. Afterwards, they were washed with distilled water before being dehydrated in a microscopy-grade ethanol series (30%, 50%, 70%, 90% and 100%), with agitation and centrifugation. For SEM, the samples were pipetted onto glass coverslips on SEM stubs and air dried. They were coated with platinum (Leica EM ACE600 coater) and examined using a scanning electron microscope (Hitachi SU3500, Japan). For TEM, the samples were embedded in epon resin and sectioned at 0.5 μm using an ultra-microtome (Reichert-Jung Ultracut) with a diamond knife and transferred onto 150-mesh copper grids. The prepared sample grids were stained with 2% uranyl acetate in 50% ethanol for 15 min and washed three times in 50% ethanol and twice in distilled water. The copper grids were then dried, examined with a transmission electron microscope (Hitachi HT-7700, Japan), and photographed with a digital camera (Hamamatsu, Japan).

Molecular and phylogenetic analyses

DNA was extracted from cyanobacterial strains using the ZymoBIOMICS DNA mini kit (Zymo Research, CA) following the manufacturer's protocol. Cells were lysed using a Qiagen bead mill for 6 min at maximum speed (30 Hz, 1800 oscillations per minute). The amplification of the 16S rRNA-encoding gene and the 16S-23S internal transcribed spacer (ITS) region was done with the primers and PCR programs described in Cellamare et al. (2018) and Gama et al. (2019), respectively. All PCR reactions were carried out using a GeneTouch thermocycler (Bioer Technology, Hangzhou, China). The extracted and purified PCR products were then sequenced by Genoscreen (Lille, France). Sequences were assembled and corrected using the MEGA version 7.0 software (Kumar et al. 2016) and aligned using MAFFT online service (Kato, et al. 2019). For the 16S rRNA gene, partial sequences with a minimum of 1398 base pairs (bp) from the complete gene sequence (1550 bp) were obtained. For the 16S-23S ITS, partial sequences were obtained with a minimum of 174 bp. Phylogenetic analyses were performed according to three methods: maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML) using the MEGA version 7 software with an equal-to-1000 iterations.

For 16S rRNA gene phylogeny, an overall alignment (n = 129 sequences) was generated, including the newly produced sequences and reference sequences available in GenBank representing Oscillatoriales, Synechococcales, Nostocales and Chroococcales. The selected sequences were all longer than 1200 bp. *Gloeobacter violaceus* PCC 7421 was chosen as outgroup. A cut-off value of 95% of 16S rRNA gene sequence identity was used for genus definition (Komárek 2010).

For the *Chroococcus*-like strain, the 16S-23S ITS phylogeny was performed with representative strains from *Chroococcus*-like, *Inacoccus*, *Cryptococcum*, *Gloeocapsa* (*syn. Limnococcus*) and *Chroococcus*, whose sequences are available in Genbank and (Gama et al. 2019). An overall alignment (n = 21) was generated with the selected sequences, all longer than 1494 bp. *Microcystis aeruginosa* PMC 728.11 was chosen as an outgroup. The generated 16S-23S ITS sequences were used for determination of secondary structure. The conserved regions (D1–D1') were analyzed using the Mfold WebServer (Zuker 2003). Default settings were used, except for the structure drawing mode where the natural angle was selected. Transfer RNA genes were found using tRNAscan-SE 2.0 (Lowe and Chan 2016). The nucleotide sequences reported in this study have been deposited in the National Center for Biotechnology Information database. GenBank accession numbers are listed in the Supplementary table (Table S1).

RESULTS AND DISCUSSION

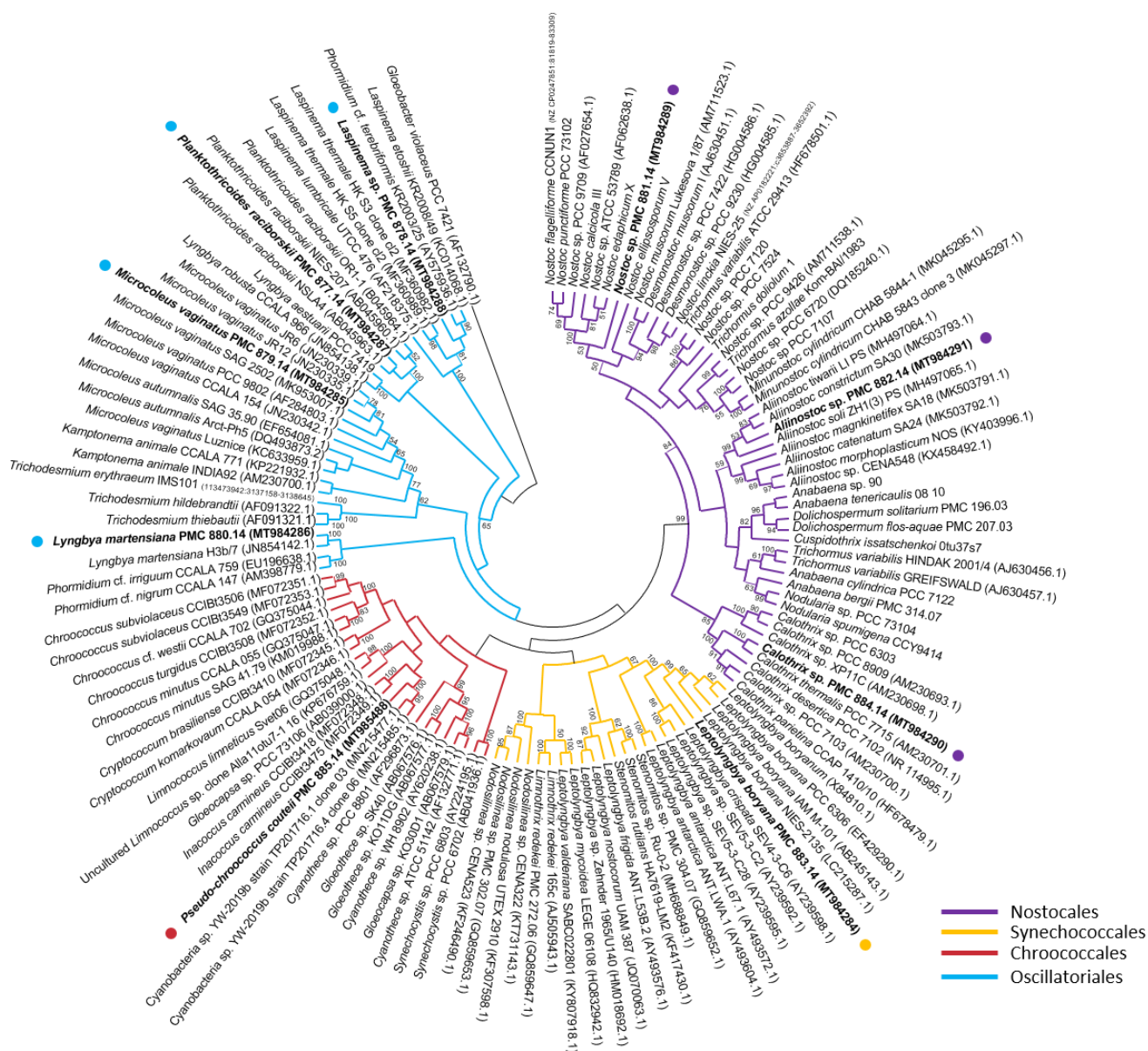
Nine clonal strains of cyanobacteria, living in the mud maturation basin of the Thermes de Balaruc-Les-Bains, were isolated (Table 1). These organisms were representative of the algal communities in (Baudinat 1986; Dupuis 1987). Dupuis (1987) showed that *Phormidium* was the most abundant genus in muds of Balaruc-Les-Bains with the species *Phormidium africanum* as dominant and *P. autumnale*, *Lyngbya sp.* and *Chroococcus* as accompanying species. Baudinat (1986), also showed that the cyanobacteria were dominant, mainly represented by *Phormidium* and *Oscillatoria*. A preliminary study done in 2014 during the mud maturation period showed that the cyanobacterium, *Planktothricoides raciborskii*, was also quite dominant (unpublished data). Other taxa such as *Laspinema sp.*, *Leptolyngbya boryana* and *Nostoc sp.* were observed. The dominance of *Laspinema sp.* was observed on one sampling date, corresponding to a slight increase in salinity of the thermal water. Other cyanobacterial taxa were observed such as *Calothrix sp.* in the mud and *Aliinostoc sp.*, *Chroococcus*-like and *Microcoleus vaginatus* on the walls of the maturation basin. As *Planktothricoides raciborskii* is morphologically very similar to *Phormidium*, we concluded that the dominance of the genus *Phormidium* has been stable since the 1980s.

Table 1. List of cyanobacteria strains isolated from the retention basin of Thermes of Balaruc-Les-Bains and their corresponding strain numbers, habitat (water column or biofilms) and sampling dates. PMC: Paris Museum Collection.

Order	Species	Strain number	Habitat	Sampling date
Chroococcales	<i>Pseudo-chroococcus couteii</i>	PMC 885.14	Epilithic biofilm	23/06/2014
Synechococcales	<i>Leptolyngbya boryana</i>	PMC 883.14	Mud surface	12/05/2014
Oscillatoriales	<i>Planktothricoides raciborskii</i>	PMC 877.14	Mud surface	26/05/2014
	<i>Laspinema sp.</i>	PMC 878.14	Mud surface	23/06/2014
	<i>Microcoleus vaginatus</i>	PMC 879.14	Epilithic biofilm	26/05/2014
	<i>Lyngbya martensiana</i>	PMC 880.14	Epilithic biofilm	23/06/2014
Nostocales	<i>Nostoc sp.</i>	PMC 881.14	Epilithic biofilm	23/06/2014
	<i>Aliinostoc sp.</i>	PMC 882.14	Epilithic biofilm	23/06/2014
	<i>Calothrix sp.</i>	PMC 884.14	Mud surface	12/05/2014

To characterize the nine cyanobacterial isolates, we used morphological, ultrastructural and 16S rRNA and 16S-23S internal transcribed spacer (ITS) gene sequence analyses. Thus, eight cyanobacteria were firmly identified at the genus or species level, while one remained unidentified (PMC 885.14). The phylogenetic trees based on the 16S rRNA gene sequences shared well-supported nodes at the order level regardless of whether the method used was MP, NJ or ML (see the ML tree, Fig. 2, and the three consensus phylogenies, Fig. S2). Some isolates were clearly identified at the species level, while others were only assigned to the genus level because of the large species diversity within the given genus.

The nine new strains belonged to the orders Chroococcales: *Pseudo-chroococcus couteii* (PMC 885.14); Synechococcales: *Leptolyngbya boryana* (PMC 883.14); Oscillatoriales: *Planktothricoides raciborskii* (PMC 877.14), *Laspinema* sp. (PMC 878.14), *Microcoleus vaginatus* (PMC 879.14), *Lyngbya martensiana* (PMC 880.14) and Nostocales: *Nostoc* sp. (PMC 881.14), *Aliinostoc* sp. (PMC 882.14), *Calothrix* sp (PMC 884.14). Identification numbers and habitats of the strains are given in Table 1.



Pseudo-chroococcus couteii (C. Duval, S. Hamlaoui, C. Bernard gen. nov., sp. nov., 2020) (PMC 885.14)

Pseudo-chroococcus couteii (PMC 885.14) was isolated from an epilithic biofilm in the mud maturation basin of Thermes de Balaruc-Les-Bains (Table 1). Examination of samples in the field showed single cells or small colonies of 2-8 cells surrounded by dense, colorless and slightly lamellate mucilaginous envelopes (Fig. 3). Under both natural and laboratory conditions, cells were spherical or hemispherical 14-18 μm in diameter with pale-green, blue-green or grey homogeneous to granulate cell contents (Fig. 3). Cell division occurred by binary fission in three or more planes or irregularly in old colonies. These morphological features are typical of the genus *Chroococcus*. TEM ultrastructure imaging showed a dense mucilaginous layer surrounding four grouped cells (Fig. 3). Thylakoids were fasciculate, as found in *Chroococcus* species.

The 16S rRNA gene sequence analyses showed that the *Pseudo-chroococcus couteii* strain PMC 885.14 had 93% and 92% sequence identity with *Inacoccus* and *Cryptococcum* gen. nov. strains respectively (data not shown). The 16S rRNA sequence data also revealed the polyphyletic nature of the genus *Chroococcus*. The ML phylogenetic tree of the 16S rRNA and the 16S-23S ITS gene sequences (Fig. 4 and Fig. 5) of *Chroococcus* strains produced three main clusters, *Chroococcus*-like, *Inacoccus/Cryptococcum* and *Limnococcus/Gleocapsa*, separated from the *Chroococcus sensu stricto* cluster. The first cluster was mainly composed of three strains of *Inacoccus carmineus* from concrete of Santa Virgínia Park in Brazil and two strains of *Cryptococcum* from thermal springs and soil. The second cluster grouped *Limnococcus limneticus* and *Gleocapsa* sp. 16S rRNA gene sequences. The third *Chroococcus*-like cluster consisted of two sub-clusters: one composed of three marine strains isolated from surface sediments in New Zealand and Hawaii, and one composed of PMC 885.14 (this study) and four freshwater strains from China. The two sub-clusters were well-supported (100% bootstrap support).

Substantial differences were observed among the secondary ITS structures (D1-D1' helices) of the three genera previously mentioned (Fig. 6). The pattern and the number of nucleotides (nt) in the D1-D1' region of *Chroococcus sensu stricto* (58 nt), *Inacoccus carmineus* (63-64 nt), *Cryptococcum* (56-59 nt) and *Chroococcus*-like (48-49 nt) were different. The pattern of the terminal portion of *Pseudo-chroococcus couteii* strain (PMC 885.14) from Thermes de Balaruc-les-Bains was different from that of freshwater strains from China (YW-2019b strain TP20176.5 clone 0.1 and CHA TP20176.4 clone 0.6), with a single loop of 18 nt for *Pseudo-chroococcus couteii* and two loops of 4 nt and 9 nt for the Chinese strains (Fig. 6). A unilateral bulge of 7 nt (CAAUCC) was present in the three strains of the *Chroococcus*-like cluster.

To the best of our knowledge, only four studies to date have examined phylogenetic relationships (16S rRNA) within the genus *Chroococcus* (Komárková et al. 2010; Roldán et al. 2013; Wood et al. 2017; Gamma et al. 2019). The strains studied were all morphologically similar to *Chroococcus* species

in cell shape and organization (Table S2). However, they differed by the color of the sheaths or mucilage (e.g., the mucilage of *Chroococcus* was usually hyaline or yellowish whereas that of *Limnococcus* was described as red or brown) (Gama et al. 2019; Table S2). Wood et al. (2017) stated that within the 51 *Chroococcus*-like 16S rRNA sequences, only five were obtained from isolates, limiting phylogenetic investigations. However, this study included the new 16S rRNA sequences available in GenBank and clearly demonstrated that the *Chroococcus*-like cluster was well-supported and should be described as a new genus.

Pseudo-chroococcus couteii by C. Duval, S. Hamlaoui, C. Bernard gen. nov., sp. nov.

Diagnosis: Solitary cells or small colonies of 2, 4 or 8 cells surrounded by dense, colorless and slightly lamellate mucilaginous envelopes. Cells, spherical or hemispherical 14-18 µm in diameter with pale-green, blue-green or grey homogeneous to granulate cell contents. Cell division occurring by binary fission in three or more planes, or irregularly in old colonies.

Holotype: A formaldehyde-fixed, cryopreserved sample of the strain PMC 885.14 was deposited at the Paris Museum Collection (PMC), Paris, France.

Type strain: A live culture was deposited at the Paris Museum Collection (PMC) as PMC 885.14, with GenBank accession no. MT985488 for the 16S-23S rRNA gene sequence.

Type locality: epilithic biofilm, retention basin of the thermal springs at Balaruc-Les-Bains, France (GPS: 43°26'44.0"N 3°40'29.6"E).

Etymology: the name of the genus, *Pseudo-chroococcus*, refers to the cyanobacterium morphology (colonial) like *Chroococcus sensu stricto*, but, clearly phylogenetically separate in two different clades; *couteii* = named in honor of Prof. Alain Couté, a French phycologist at the National Museum of Natural History in France.

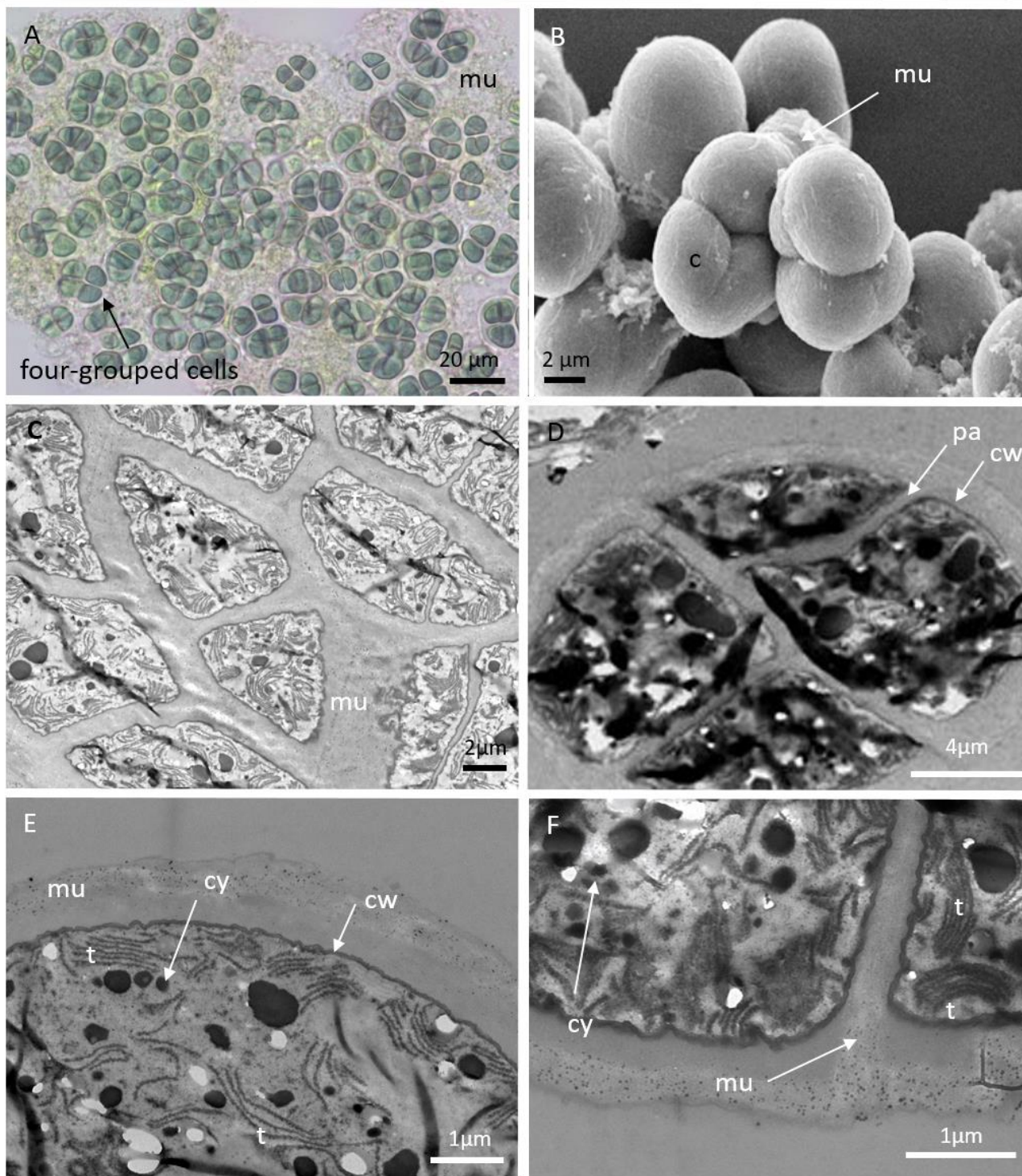


Figure 3. Light microscopy (A), SEM (B) and TEM (C-F) micrographs of *Pseudo-chroococcus couteii* (PMC 885.14) from Thermes de Balaruc-Les-Bains. A: Light microscopy of a packet-form colony, four-celled groups of spherical, very small cells, brownish color with dense mucilaginous sheath around cells. B: external observation of a packet-form colonies C, D, E, F.: the four-grouped cells were surrounded by a dense mucilage. Cells details showing a fasciculate arrangement of thylakoids. Abbreviations: c: colony of four-grouped cells, cy: cyanophycine granules, cw: cell wall, pa: cells partition, mu: mucilage, t: thylakoids.

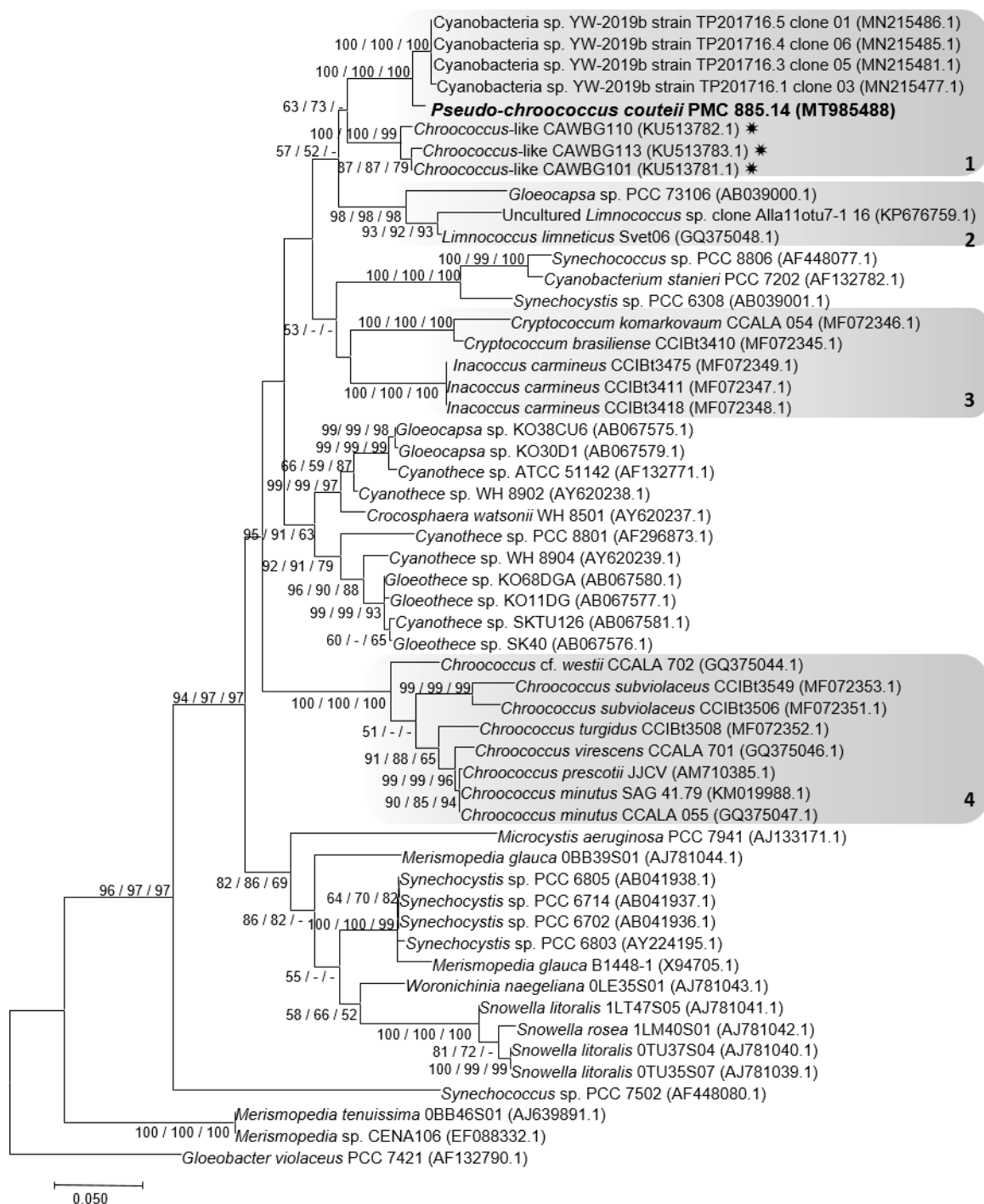


Figure 4. Consensus phylogenetic tree (Maximum likelihood tree presented) based on 16S rRNA-encoding gene sequences (54 sequences, 705 aligned nucleotide positions, GTR+G+I model) of representative cyanobacteria strains belonging to the order Chroococcales. Thermes de Balaruc-Les-Bains's strain is indicated in bold, other species and sequences were obtained from Genbank. *Gloeobacter violaceus* PCC 7421 was used as an out-group. Numbers above branches indicate bootstrap support (>50%) from 1,000 replicates. Bootstrap values are given in the following order: maximum likelihood / neighbor joining / maximum parsimony. (* marine strains; Cluster 1 : *Chroococcus*-like , 2 : *Limnococcus/Gloeocapsa*, 3 : *Inacoccus/Cryptococcus*, 4 : *Chroococcus sensu stricto*)

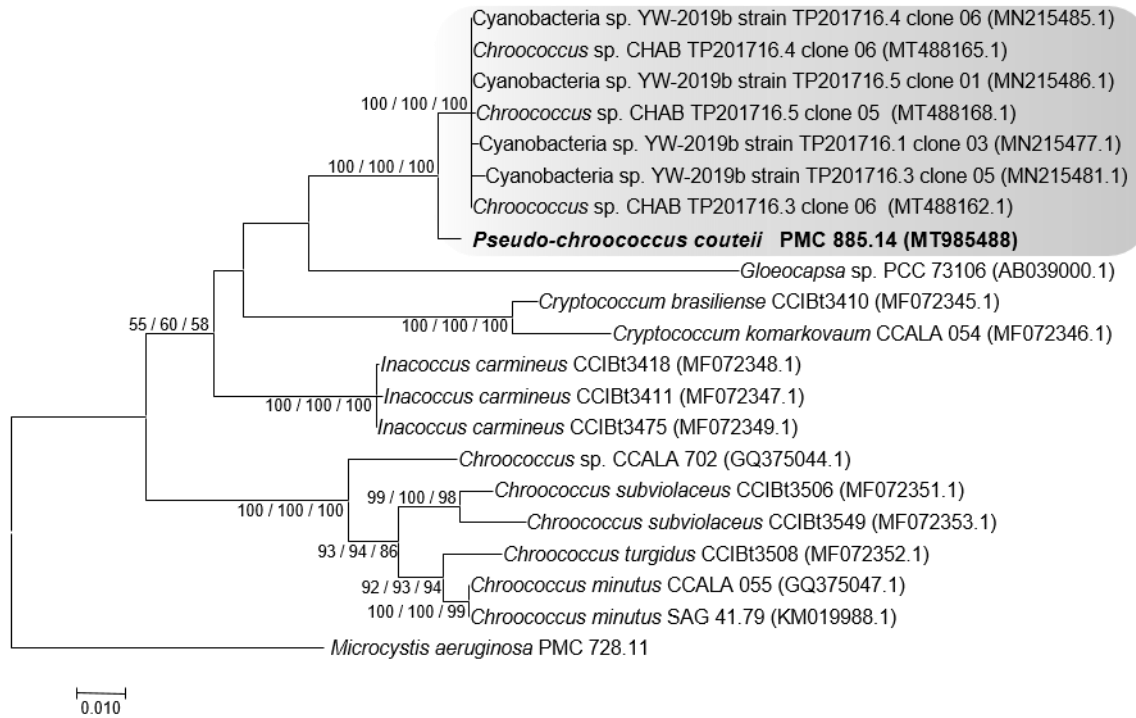


Figure 5. Consensus phylogenetic tree (Maximum likelihood tree presented) based on 16S-23S ITS gene sequences (21 sequences, 1915 aligned nucleotide positions, GTR+G+I model) of representative cyanobacteria strains belonging to the orders Chroococcales, Thermes de Balaruc-Les-Bains's strains (in bold), other species were obtained from Genbank. *Microcystis aeruginosa* PMC 728.11 was used as an outgroup. Numbers above branches indicate bootstrap support (>50%) from 1000 replicates. Bootstrap values are given in the following order: maximum likelihood / neighbor joining / maximum parsimony.

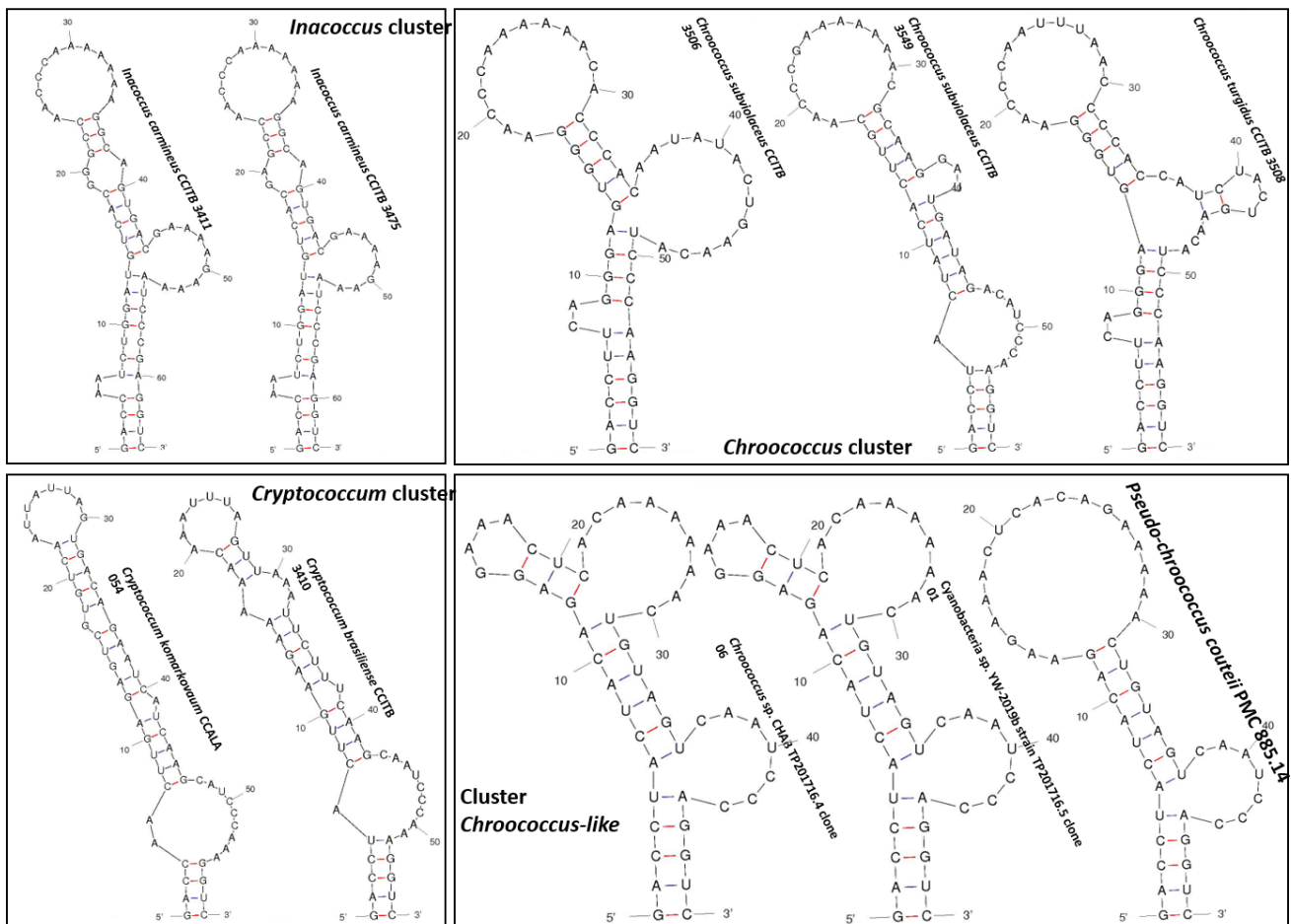


Figure 6. 16S-23S ITS secondary structure of the D1-D1' helices of *Inacoccus*, *Cryptococcus*, *Chroococcus* and *Chroococcus*-like (syn. *Pseudo-chroococcus*) cluster.

Characterization of strains belonging to the Synechococcales

Among the nine studied strains, one strain *Leptolyngbya boryana* (PMC 883.14) belonged to the Synechococcales.

Leptolyngbya boryana (Anagnostidis et Komárek, 1988)

Leptolyngbya boryana (PMC 883.14) was isolated from mats covering the surface of the basin of Thermes de Balaruc-Les-Bains (Fig. 1, Table 1). Examination of samples in the field showed a pale-green, diffluent thallus forming mats (Fig. 7). Under laboratory conditions, the filaments were straight to slightly curved and densely entangled with thin, firm, colorless sheaths. Trichomes were pale to bright blue-green, cylindrical, unbranched, thin, < 3 µm wide and strongly constricted at the ungranulated cross-walls (Fig. 7), motile (hormogonia) or immotile, or with indistinct trembling. Cells were moniliform, shorter than wide, 1.5-2.4 µm wide and 0.9-2.0 µm long with a length:width ratio of 0.76 and well-separated from one another by cross-walls. Cell contents were pale blue-green and homogeneous without gas vacuoles. Apical cells were rounded without calyptra (Fig. 7). A sheath with a thin transparent zone covered the trichome and was clearly visible by TEM. The thylakoids were parietally arranged, concentric and parallel to the cell wall (Fig. 7).

Results based on 16S rRNA gene sequence homology (BLAST), showed that the *Leptolyngbya* strain (PMC 883.14) shared 100% sequence identity with other *Leptolyngbya* strains (data not shown). This result was confirmed by the phylogenetic tree (Fig. 8) where the strain PMC 883.14 was grouped in a well-supported clade including the *Leptolyngbya boryana*, cluster with one *Leptolyngbya foveolarum* sequence (X84808.1) and one *Leptolyngbya boryanum* sequence (X84810.1). The cluster was mainly comprised of strains isolated from benthic freshwater environments.

The morphological features of the *Leptolyngbya boryana* PMC 883.14 strain were very similar to other *Leptolyngbya* species belonging to the same phylogenetic cluster (Table S3) which made it difficult to distinguish. In Komárek and Anagnostidis (2005), *Leptolyngbya foveolarum* was described with the following characteristics: variously curved filaments, sometimes straight and parallel or tangled, rarely pseudo-branched; trichomes pale to bright blue-green 1-2 µm wide; isodiametric cells, rarely longer than wide 0.8-2.2 x 2.5 µm. *Leptolyngbya boryana* was described as follows: curved densely tangled filaments, sometimes pseudo-branched, pale blue-green to colorless, 1.3-2 (3) µm wide, cells more or less isodiametric, somewhat shorter or longer than wide in the main filament 0.6-2 (2.5) x 1.3-3 µm. In other phylogenetic studies, several *Leptolyngbya* species such as *L. foveolarum*, *L. tenerrima*, and *L. angustata* were considered as synonyms of *L. boryana* (Cuzman et al. 2010). As the genus *Leptolyngbya* was defined based on strains with very thin and simple trichomes (0.5-3.5 µm wide), sheaths present or facultative, without gas vesicles and peripherally arranged thylakoids (Komárek and Anagnostidis 2005; Komárek and Johansen 2015), these morphological features were deemed not sufficiently discriminant

to ensure a proper identification; therefore, we consider the strain PMC 883.14 as *Leptolyngbya boryana* based on the former description of this species within the *Leptolyngbya* 16S rRNA gene sequences cluster.

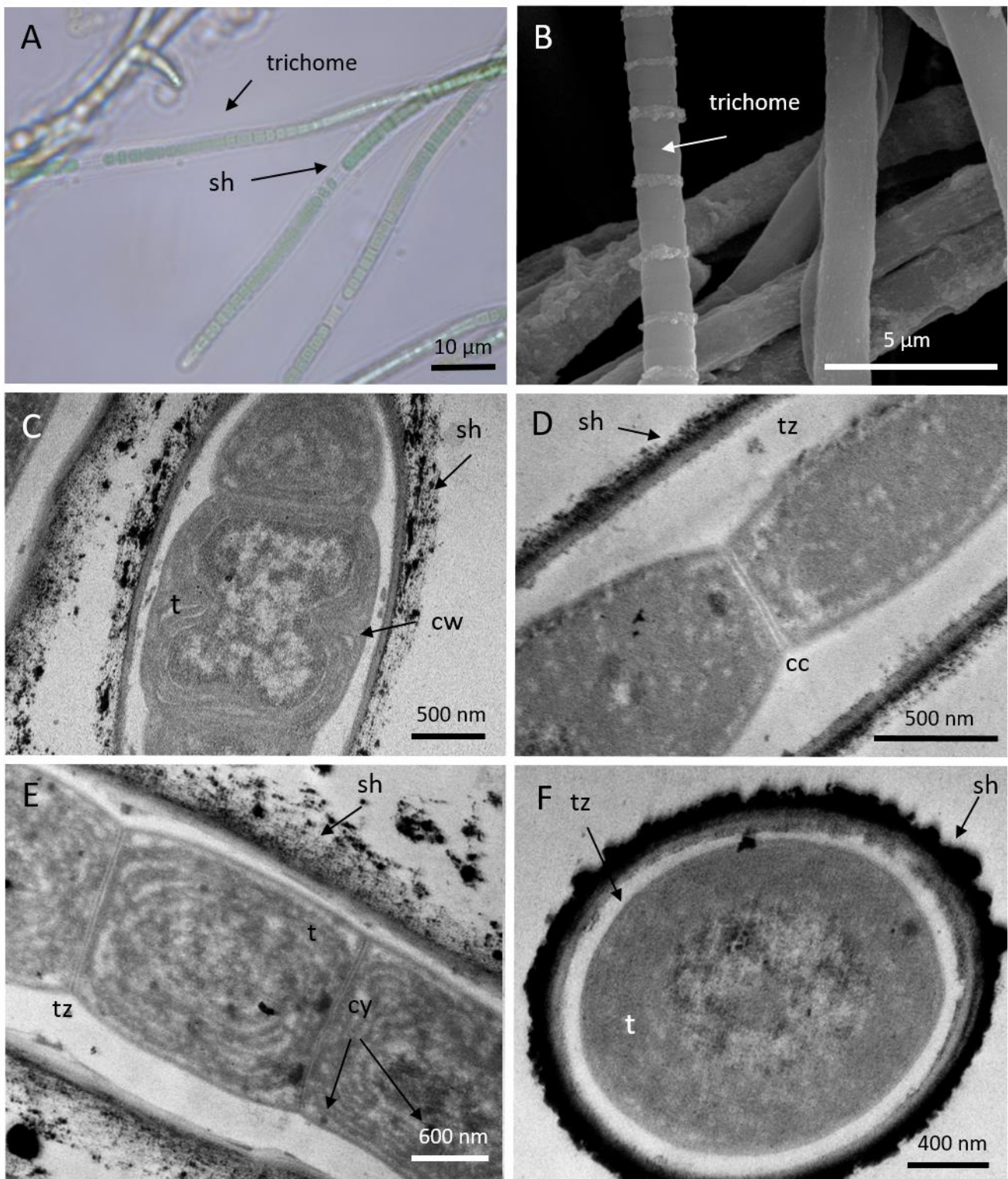


Figure 7. Light microscopy (A, B), MEB (C) and TEM (D-F) micrographs of *Leptolyngbya boryana* (PMC 883.14) from Thermes de Balaruc-Les-Bains. A: entangled filaments and trichomes with sheath. B: observations of trichomes with probable exudates localized on cell junctions. Longitudinal (C-E) and cross sections (F) of trichomes with sheaths showing the ultrastructural details. Abbreviations: cc: cross-wall constriction, cm: cytoplasmic membrane, cy: cyanophycin granules, t: thylakoids, tz: transparent zone.

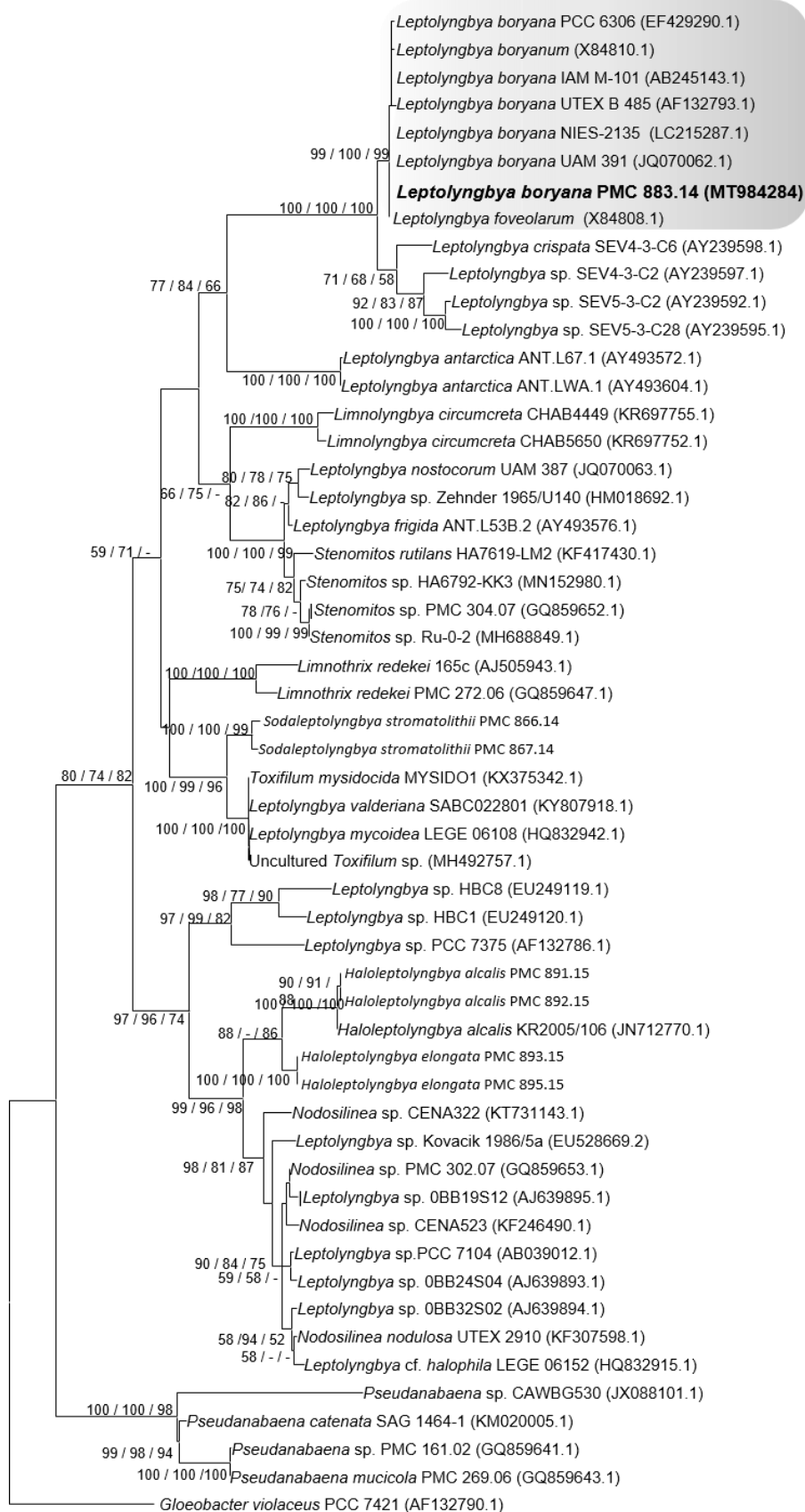


Figure 8. Consensus phylogenetic tree (Maximum likelihood tree presented) based on 16S rRNA gene sequences (54 sequences, 1333 aligned nucleotide position, GTR+G+I model) of representative cyanobacteria strains belonging to the orders Synechococcales, Thermes de Balaruc-Les-Bains's strain is indicated in bold (with brown color), other species were obtained from Genbank. *Gloeobacter violaceus* PCC 7421 was used as an outgroup. Numbers above branches indicate bootstrap support (>50%) from 1000 replicates. Bootstrap values are given in the following order: maximum likelihood / neighbor joining / maximum parsimony.

Characterization of strains belonging to the Oscillatoriales

Among the nine studied strains, four strains belonged to the Oscillatoriales: *Planktothricoides raciborskii* (PMC 877.14), *Laspinema sp.* (PMC 878.14), *Microcoleus vaginatus* (PMC 879.14) and *Lyngbya martensiana* (PMC 880.14).

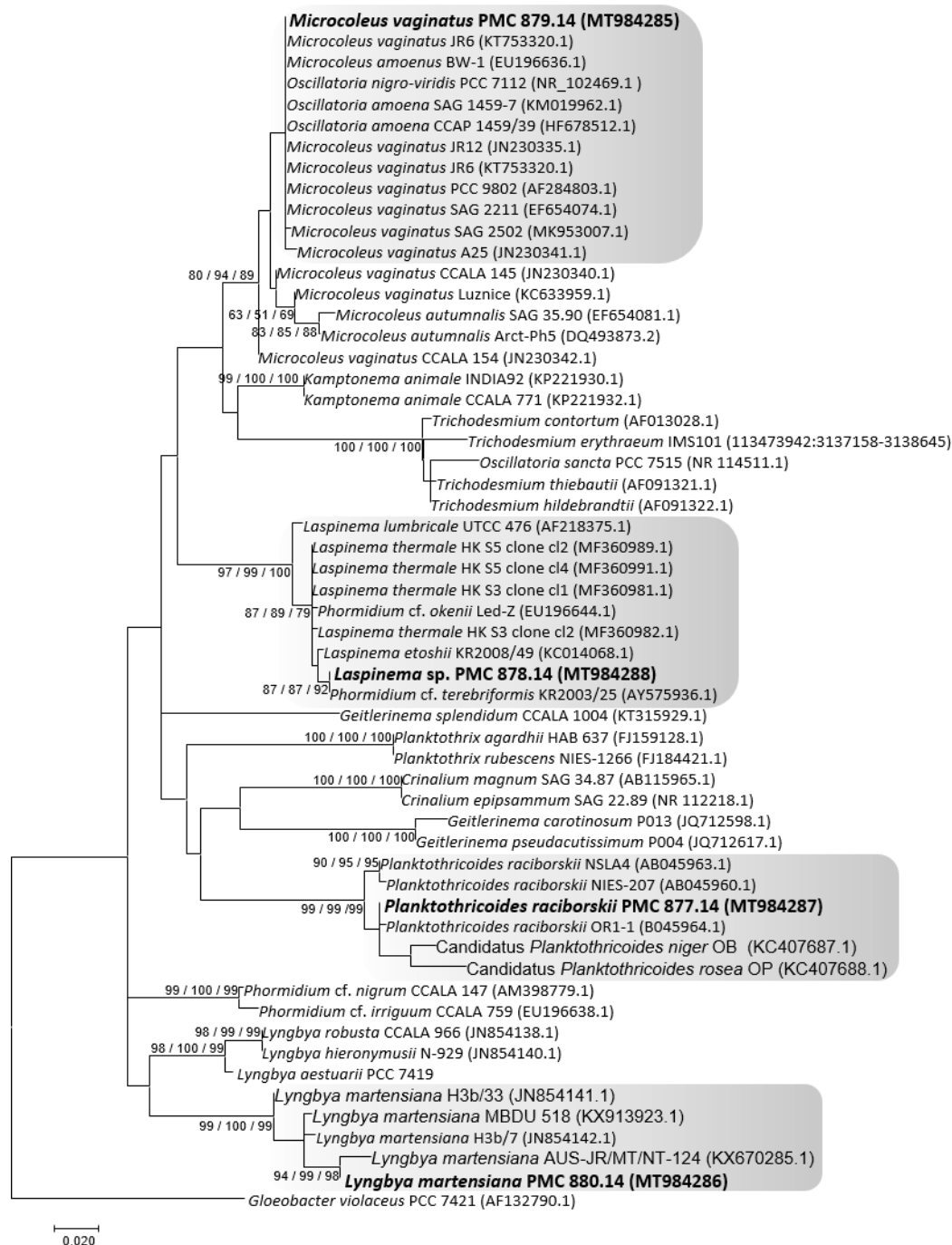


Figure 9. Consensus phylogenetic tree (Maximum likelihood tree presented) based on 16S rRNA gene sequences (57 sequences, 1305 aligned nucleotide positions, GTR+G+I model) of representative cyanobacteria strains belonging to the orders Oscillatoriales, Thermes de Balaruc-Les-Bains's strains are indicated in bold (colored in blue), other species were obtained from Genbank. *Gloeobacter violaceus* PCC 7421 was used as an out-group. Numbers above branches indicate bootstrap support (>50%) from 1000 replicates. Bootstrap values are given in the following order: maximum likelihood / neighbor joining / maximum parsimony.

Planktothricoides raciborskii (Wołosz) (Suda et M. M. Watanabe, 2002)

Planktothricoides raciborskii (PMC 877.14) was isolated from mats covering the mud of the basin (Fig. 1, Table 1). Observations of field samples showed pale-green to brown diffluent thalli (Fig. 10). Trichomes were solitary, pale green to brown, isopolar or heteropolar, cylindrical, variously elongated (100-450 μm). Sheaths were seen occasionally, and were very thin and colorless, with limited motility (gliding). Trichomes were attenuated towards extremities, constricted or not constricted and sometimes slightly bent near the apex (Fig. 10). Cells were cylindrical, shorter than wide, 5-7.3 μm wide and 3.3-5.4 μm long, with a length:width ratio of 0.7. Apical cells were rounded, conical, more or less tapered, sometimes bent but not sharply pointed, without calyptra (Fig. 10; Table S4). Cell content was granular with cyanophycin granules and carboxysomes (polyhedral or dense bodies; Fig. 10). These proteinaceous micro-compartments play a major role in the carbon-fixation process by increasing the CO_2 concentration around RuBisCO, thus promoting carboxylation at the expense of oxygenation. Sheaths were generally thin and some trichomes were associated with bacteria under our laboratory culture conditions. Cross-walls were clearly visible with TEM (Fig. 10) and the thylakoids were arranged radially in cross sections of the trichomes.

The 16S rRNA gene sequence comparisons (BLAST) showed that the PMC 877.14 strain shared $\geq 99\%$ sequence identity with other *Planktothricoides* strains (data not shown). The phylogenetic tree of the sequences of *Planktothricoides* produced two main clusters (Fig. 9). The first cluster was primarily composed of strains isolated from freshwater habitats, the one from Thermes de Balaruc-Les-Bains, one strain from Thailand, one from Australia, one from Japan and one from Singapore. The second cluster grouped the strains *Candidatus* '*Planktothricoides niger*' and *Candidatus* '*Planktothricoides rosea*' from periphyton mats of tropical marine mangroves in Guadeloupe (Guidi-Rontani et al. 2014). The two species, *Candidatus* '*Planktothricoides niger*' and *Candidatus* '*Planktothricoides rosea*', have been distinguished by their phycoerythrin pigment content. The *Planktothricoides* strain from Thermes de Balaruc-Les-Bains (PMC 877.14) contained two dominant phycobilin pigments, phycoerythrin (PE) and phycocyanin (PC), with a PC:PE ratio = 1 (Fig. S1, Table S4). As the color of trichomes differs between field samples and laboratory cultures, we suggest that this strain can either undergo complementary chromatic adaptation (Grossman, 2003) or modify its PC:PE ratio depending on light wavelength or penetration through the biomass. This characteristic is clearly different from the type description of *Planktothricoides raciborskii* (Wołosz.) Suda and M. M. Watanabe (Suda et al., 2002; Komárek et al. 2004), where phycoerythrin was described as absent and complementary chromatic adaptation was not observed. The presence of PE should not be considered as sufficient to assign the strain PMC 877.14 to either *Planktothricoides niger* or *Planktothricoides rosea*, and we considered the phylogenetic data strong enough to label the PMC 877.14 strain as *Planktothricoides raciborskii*.

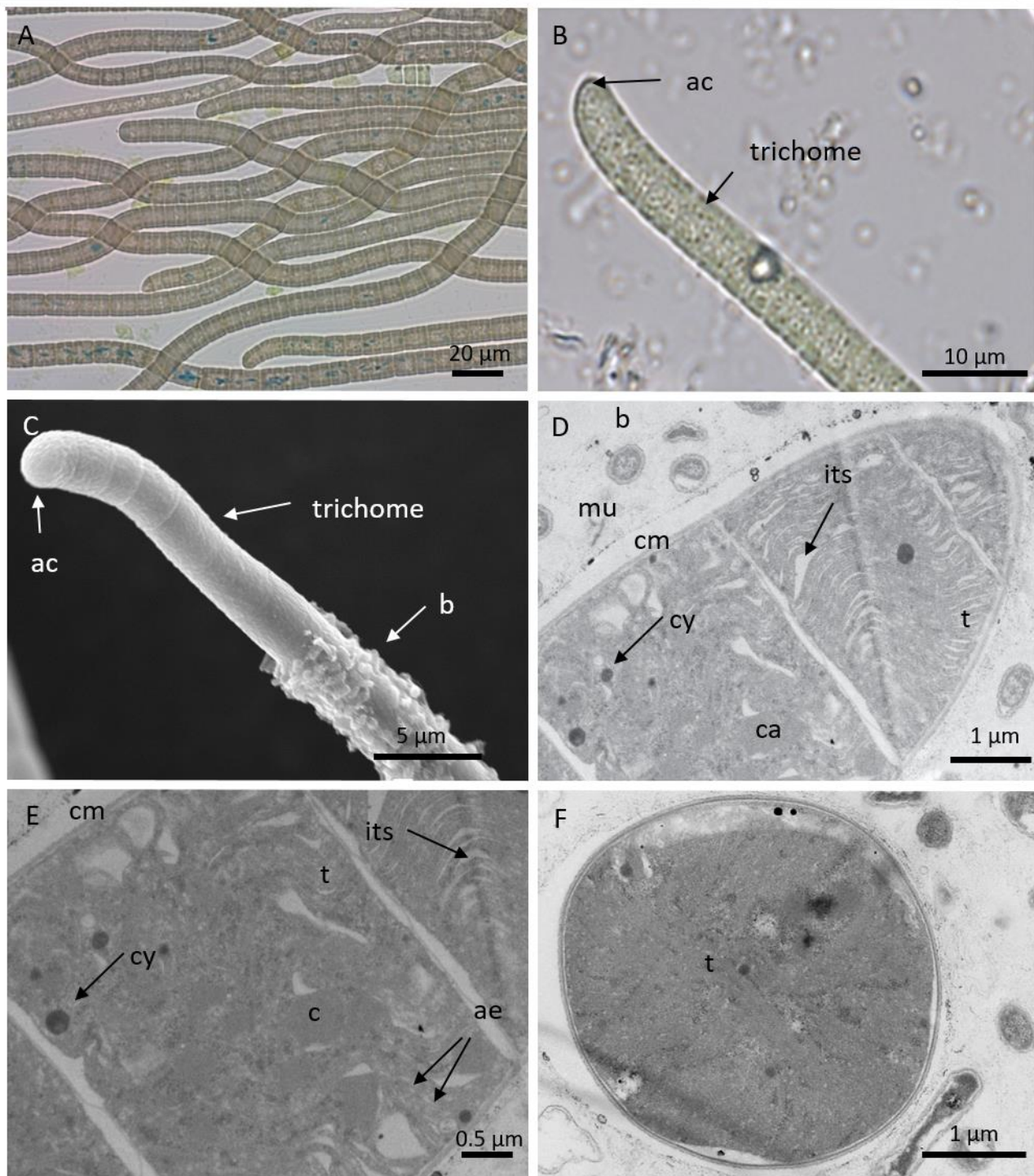


Figure 10. Light microscopy (A, B), SEM (C) and TEM (D-F) micrographs of *Planktothricoides raciborskii* (PMC 877.14) from Thermes de Balaruc-Les-Bains. A: Light microscopy of trichomes from field sample. B: Light microscopy of trichomes in culture. C: trichomes associated or not with bacteria. Longitudinal (C-E) and cross sections (F) of the trichomes showing the ultrastructural details. Abbreviations: ac: apical cell, ae: aerotopes, bact: bacteria, c: carboxysome, cm: cytoplasmic membrane, cy: cyanophycin granules, its: inter thylakoidal space, r: reserves (lipids, polyphosphates, poly- β -hydroxybutyrate), t: thylakoids, vs: clear vesicle.

***Laspinema* sp.** (F. Heidari & T. Hauer, 2018)

The *Laspinema* strain (PMC 878.14) was isolated from the mud covering the maturation basin of Thermes de Balaruc-Les-Bains. Examination of field samples showed a pale green to brown diffluent thallus (Fig. 11). In culture, the strain showed green or blackish green thallus formed mats and its trichomes were bright blue-green often gradually attenuated at the ends, long, straight, not constricted at the finely granulated cross-walls, bent, hooked and intensely motile (Fig. 11). Cells were always shorter than wide, 3.8-4.8 μm wide, 2.3-3.3 μm long with a length:width ratio of 0.7. Sheaths were generally absent (Fig. 11). The apical cells were obtusely-rounded to rounded-conical without calyptra, and often with thickened outer cell walls (Fig. 11).

The thylakoids visible in cross sections of the trichomes were radially arranged with several interthylakoidal spaces (Fig. 11). Other components such as numerous small gas vesicles or aerotopes, which promote flotation of the filaments, carboxysomes, cyanophycin granules (nitrogen reserves), glycogen, lipids, or poly- β -hydroxybutyrate acting as carbon and energy sources, and polyphosphate granules (phosphorus reserves) were observed within the cells

The 16S rRNA gene sequence analysis (BLAST) showed that the PMC 878.14 strain shared $\geq 99\%$ sequence identity with other *Laspinema* sp. strains (data not shown). The phylogenetic tree of *Laspinema* strains had one robust cluster (Fig. 9) composed of several species including *L. lumbricale*, *L. thermale* and *L. etoshii*. Comparison of the morphological characteristics of the different species (Table S5) did not allow us to assign a species name to the studied strain. Thus, strain PMC 878.14 was named *Laspinema* sp. based on morphology (Table S5) and phylogenetics (Fig. 9).

Microcoleus vaginatus (Gomont ex Gomont 1892)

Microcoleus vaginatus (PMC 879.14) was isolated from the wall of the retention basin. The field samples showed a dense green or blackish green thallus (Fig. 12). In culture, the thallus was green or blackish green, forming mats and showing motility (gliding). Trichomes were often gradually attenuated at the ends, with no constrictions at the cross-walls (Fig. 12). The cell wall envelope consisted of a cytoplasmic membrane bounded by relatively thin wall layers. The apical cells were rounded with rounded or truncated calyptra, often with thickened outer cell walls (Fig. 12). Cells were always shorter than wide, 4.7-6.2 μm wide by 2.4-4.8 μm long, with a length:width ratio of 0.7 (Table S6). The thylakoids formed fascicle-like aggregations arranged irregularly in the cells. The cells contained several large cyanophycin granules, some carboxysomes and numerous small gas vesicles or aerotopes (Fig. 12).

The 16S rRNA gene sequence analysis (BLAST) showed that PMC 879.14 shared $\geq 98\%$ sequence identity with other *Microcoleus vaginatus* strains (data not shown). The phylogenetic tree placed the sequence in a well-grouped cluster containing numerous *M. vaginatus* sequences (Fig. 9). Some

sequences belonging to *Oscillatoria amoena* and *O. nigro-viridis* were also grouped in this cluster, probably because they were assigned before the taxonomic revision of this group (Strunecky et al. 2013). According to phylogeny and following the recent taxonomic revision, the strain PMC 879.14 was assigned to *Microcoleus vaginatus*.

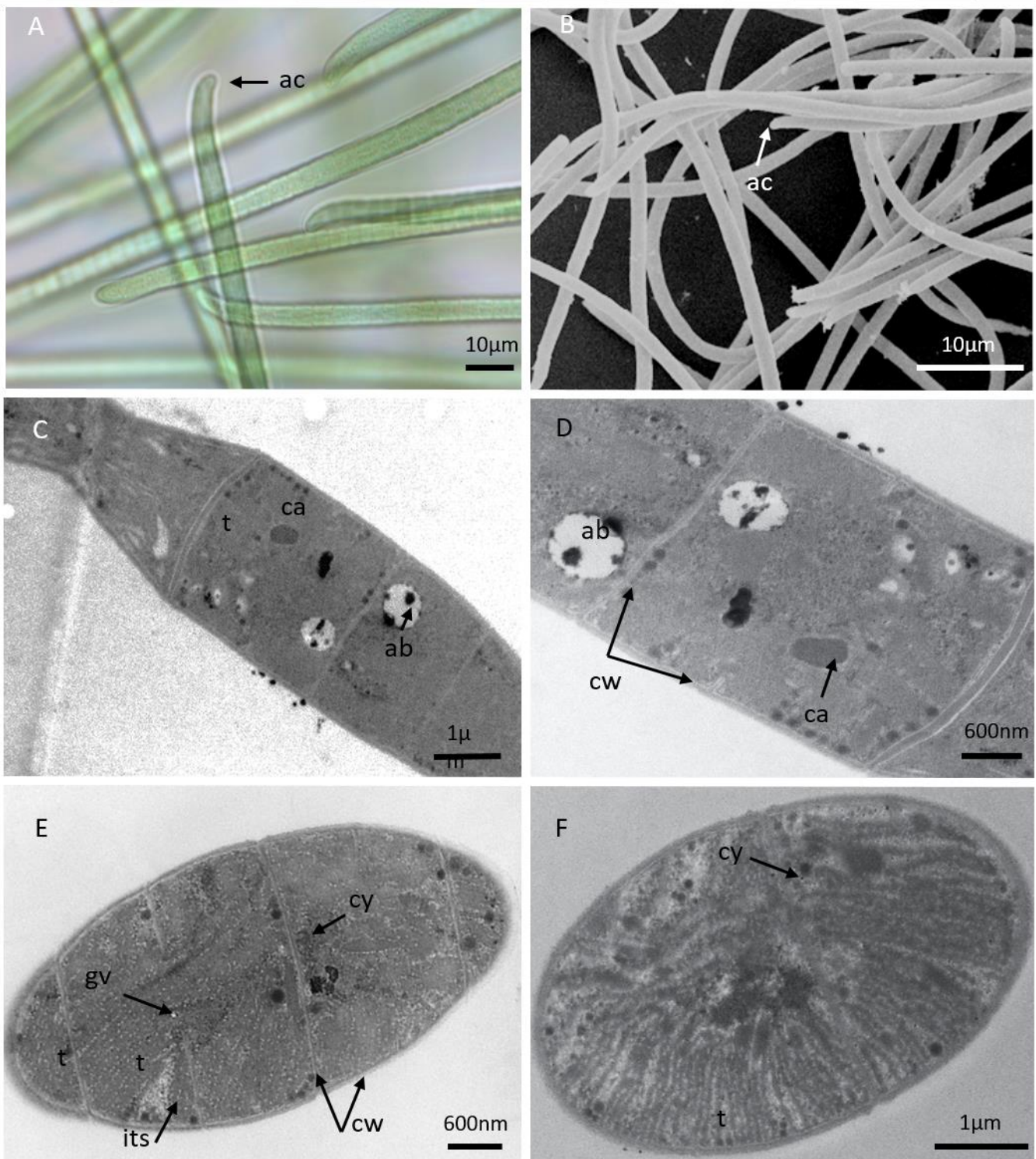


Figure 11. Light microscopy (A), SEM (B) and TEM (C-D-E-F) micrographs of *Laspinema* sp. (PMC 878.14) from Thermes de Balaruc-Les-Bains. A: filaments with typical shape of apical cell. B: observations of filaments. Longitudinal (C-E) and cross sections (F) of trichomes showing the ultrastructural details. Abbreviations: ab: beta-hydroxybutyric acid, ac: apical cell, ca: carboxysome, cy: cyanophycin granule, gv: gas vesicles, its: inter thylakoidal space, t: thylakoids.

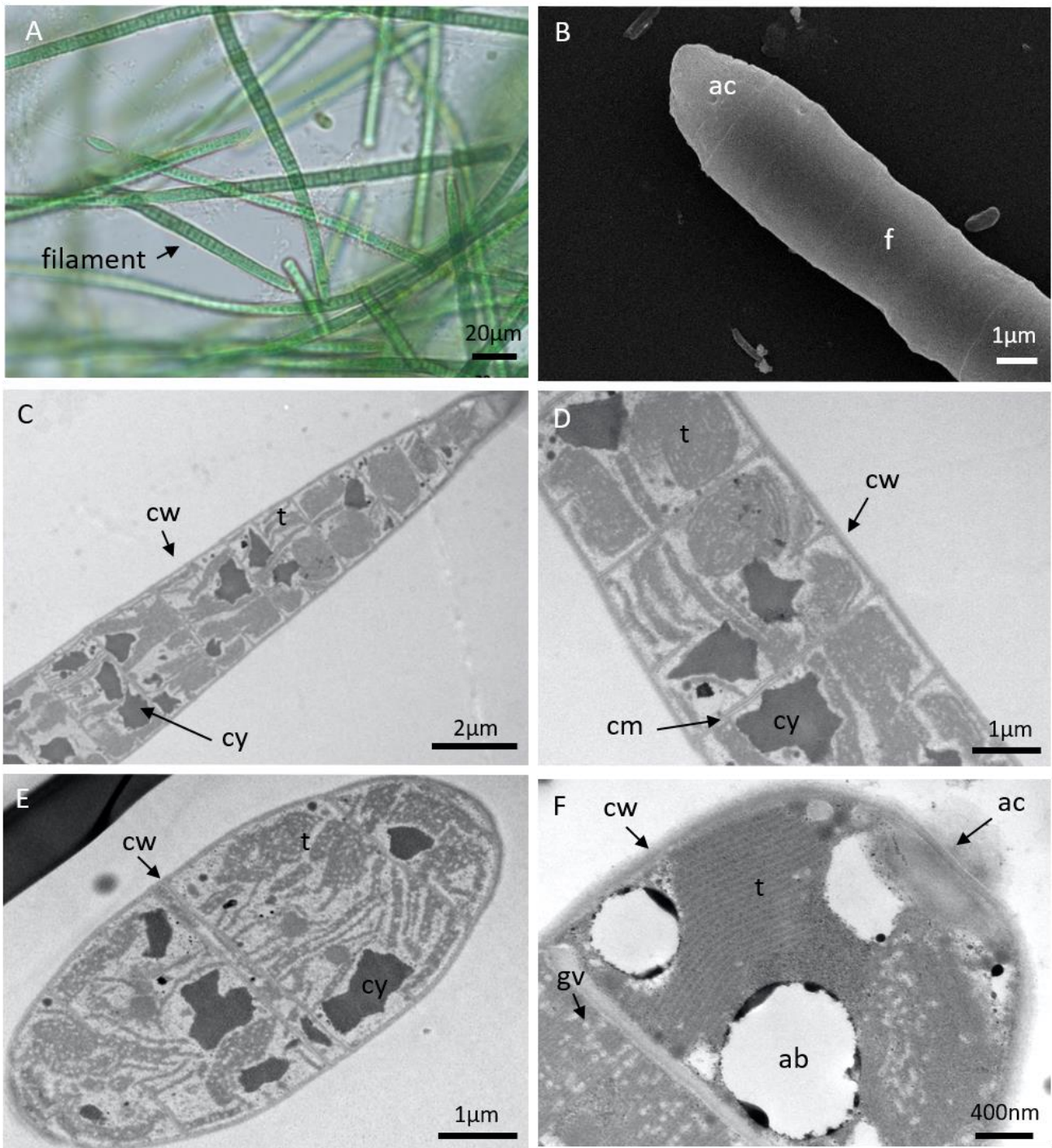


Figure 12. Light microscopy (A), SEM (B) and TEM (C-D-E-F) micrographs of *Microcoleus vaginatus* (PMC 879.14) from Thermes de Balaruc-Les-Bains. A: entangled filaments with sheath. B: filaments with apical cell. Longitudinal (C-D-E-F) of trichomes with sheaths showing the ultrastructural details. Abbreviations: ab: acid beta-hydroxybutyric, ac: apical cell, cy: cyanophycin granule, f: filament, t: thylakoids.

Lyngbya martensiana (Meneghini ex Gomont 1892)

Lyngbya martensiana (PMC 880.14) was isolated from the epilithic biofilm of the retention basin of Thermes de Balaruc-Les-Bains. Field examinations and laboratory cultures showed bright-green thalli composed of tangled filaments, usually arranged in parallel (Fig. 13). Filaments were long, straight or variously flexuous with thick colorless sheaths. Trichomes were cylindrical and not constricted at the

cross-walls. The granular cell contents were pale blue-green. Cells were discoid, shorter than wide, 4.8-6.6 (5.3 ± 0.47) μm wide, 1.2-2.1 (1.6 ± 0.2) μm long with a length:width ratio of 0.3. Apical cells were widely rounded, hemispherical or depressed-hemispherical without calyptra (Fig. 13). The thylakoids formed fascicle-like aggregations arranged irregularly in the cells and situated near walls and cross-walls. Numerous pores penetrating the cross-walls between the cells were clearly visible (Fig. 13). Gas vesicles occurred throughout the cells, which also contained reserve granules and some carboxysomes (Fig. 13).

The 16S rRNA gene sequence analysis showed that PMC 880.14 shared $\geq 96\%$ sequence identity with other *Lyngbya martensiana* strains (data not shown). The phylogenetic tree of the sequences of *Lyngbya martensiana* strains produced one well-grouped cluster (Fig. 9).

The morphological characteristics (Table S7) and the well-grouped *Lyngbya martensiana* cluster allowed us to name the PMC 880.14 strain as *Lyngbya martensiana* (Gomont M. 1892).

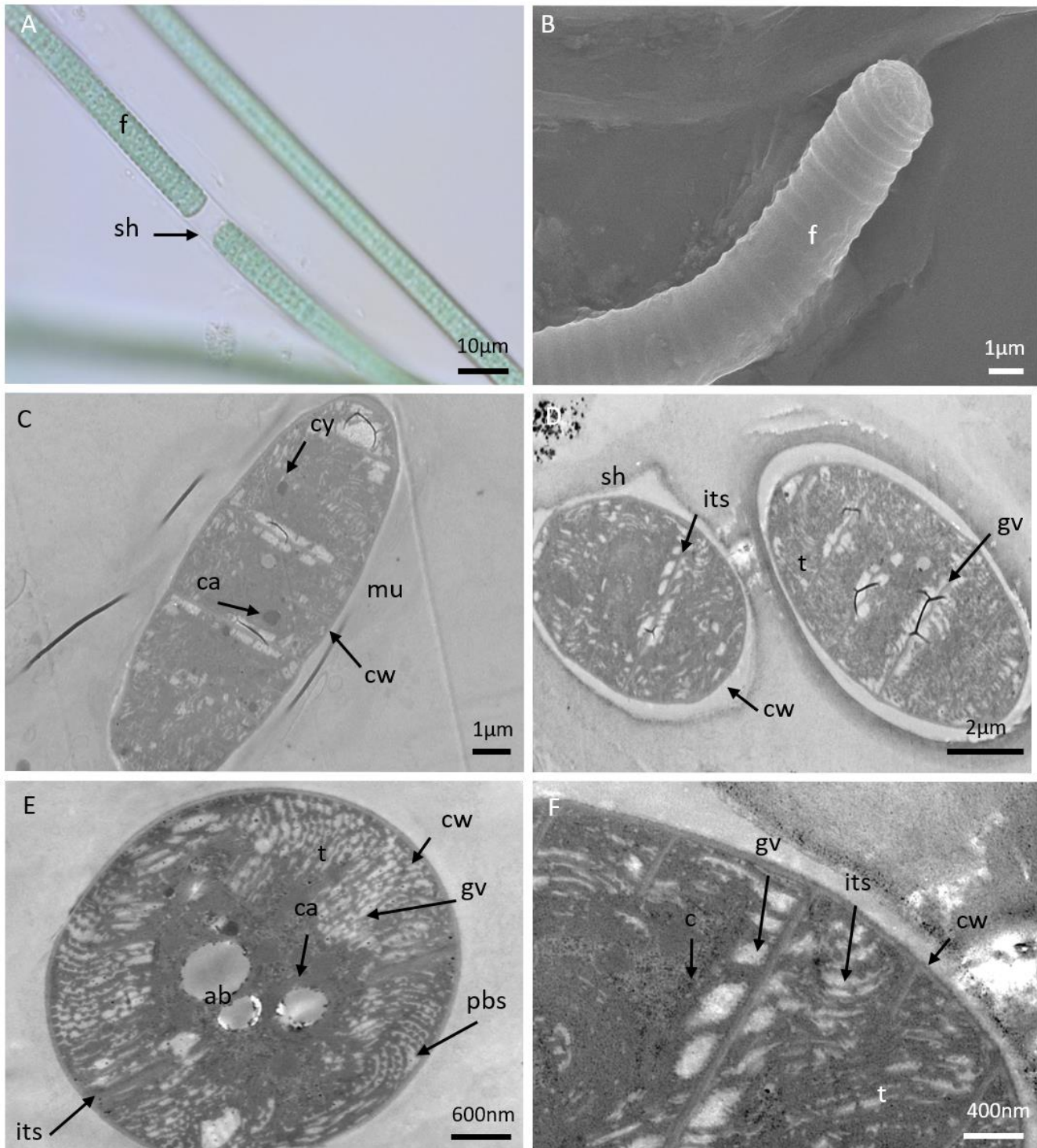


Figure 13. Light microscopy (A), SEM (B) and TEM (C-D-E-F) micrographs of *Lyngbya martensiana* (PMC 880.14) from Thermes de Balaruc-Les-Bains. A: filaments with sheath. B: observation of a filament. Longitudinal section (C-D-F) and cross section showing the ultrastructural details. Abbreviations: ab: beta-hydroxybutyric acid, ca: carboxysome, cy: cyanophycin, cw: cell wall, its: inter thylakoidal space, f: filament, pbs: phycobilisomes (photosynthetic antenna), s: sheath, t: thylakoids.

Characterization of strains belonging to the Order Nostocales

Among the nine studied strains, three belonged to the Nostocales: *Nostoc* sp. (PMC 881.14), *Aliinostoc* sp. (PMC 882.14) and *Calothrix* sp. (PMC 884.14).

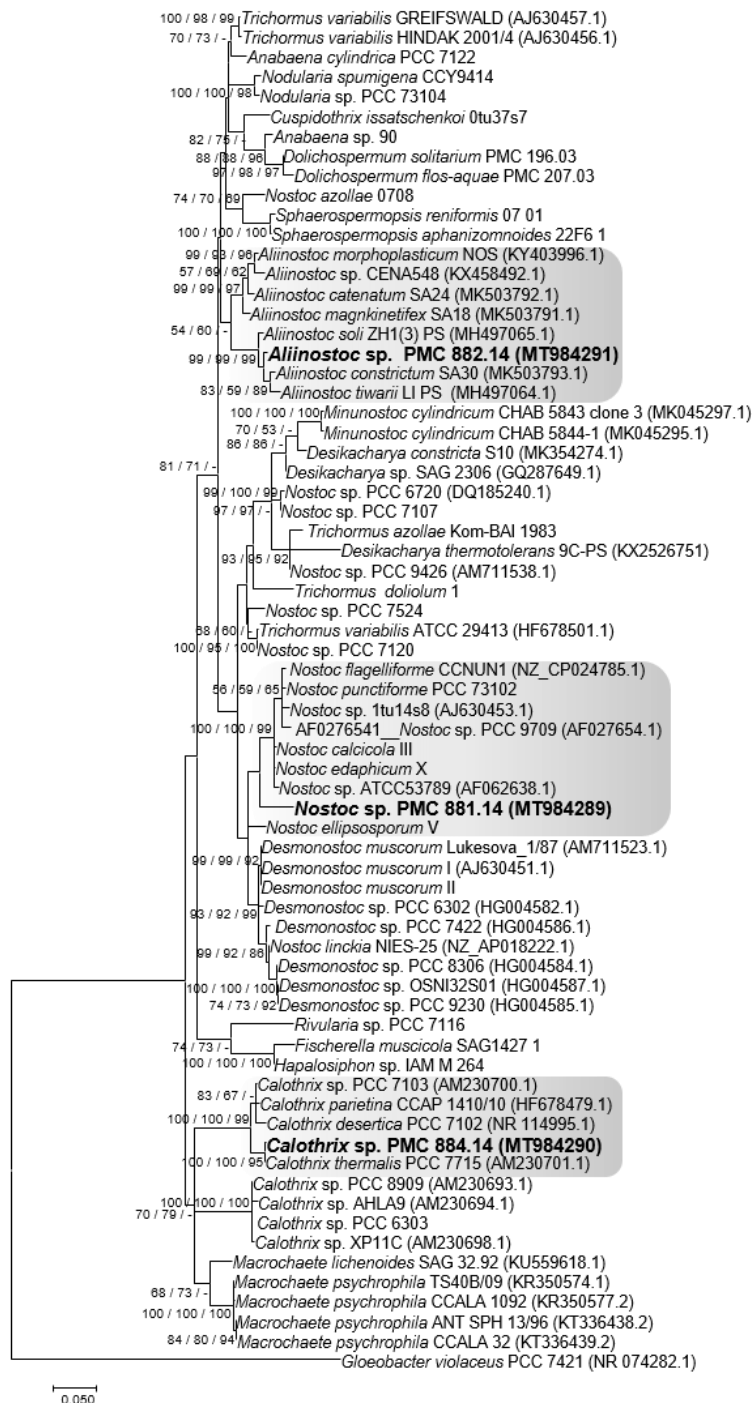


Figure 14. Consensus phylogenetic tree (Maximum likelihood tree presented) based on 16S rRNA gene sequences (69 sequences, 1310 aligned nucleotide position, GTR+G+I model) of representative cyanobacteria sequences (Genbank) belonging to the Nostocales and the studied strains from Thermes de Balaruc-Les-Bains are indicated in bold (with purple color). *Gloeobacter violaceus* PCC 7421 was used as an out-group. Numbers above branches indicate bootstrap support (>50%) from 1000 replicates. Bootstrap values are given in the following order: maximum likelihood / neighbor joining / maximum parsimony.

***Nostoc* sp.** (Vaucher ex Bornet & Flahault, 1886)

Nostoc sp. (PMC 881.14) was isolated from the epilithic biofilm on the wall of the retention basin. Field samples had a dense thallus with blackish green or brownish macroscopic colonies (Fig. 15). These macroscopic colonies resulted from several small, amorphous, fine, thin and mucilaginous colonies. In culture, old colonies (up to 130 μm in diameter) were enveloped by a firm periderm that could break and release young colonies. Within the colonies, the filaments were very densely tangled, flexuous and/or coiled. They were blackish green or brownish, ~ 5 μm wide, highly constricted at cross-walls, uniseriate, unbranched, isopolar, moniliform and/or curled (Fig. 15). Cells were isodiametric, well separated from one another by cross-walls, wider than long, 2.8-5.5 (4.5 ± 0.9) μm wide, 2.6-4.6 (3.4 ± 0.5) μm length with a length:width ratio of 0.7. The apical cells were spherical to conical (data not shown). The heterocytes were 2.6-5.7 (3.4 ± 0.6) μm wide and 2.3-5.1 (3.8 ± 0.7) μm long. Akinetes were never observed during the three years of culture, even when the strain was cultivated in Z8X medium without a nitrogen source to induce stress conditions. An extensive gelatinous matrix was observed resulting in a long internal diffusion path from the surface to the trichomes. A sheath with a periplasmic membrane was clearly visible by TEM (Fig. 15). The thylakoids were fascicular with irregular spherical formations (Fig. 15).

The 16S rRNA gene sequence analysis showed that PMC 881.14 strain shared $\geq 96\%$ sequence identity with other *Nostoc* sp. strains (data not shown). The phylogenetic tree based on 16S rRNA gene sequences showed that the PMC 881.14 strain was grouped into a unique *Nostoc* cluster (Fig. 14) with a high bootstrap value. Within this cluster three sequences were not assigned at the species level, and the five others were assigned to different species: *N. calcicola*, *N. punctiforme*, *N. flagelliforme*, *N. edaphicum*, *N. ellisporum*.

It is now well known that *Nostoc* is a complex genus. The species belonging to *Nostoc* were generally described as microscopic, spherical, oval, ovoid, irregular in their colony form, gelatinous, irregularly clustered, amorphous, dull olive to brownish gelatinous clusters free-living on mud (Table S8). Because of this heterogeneity, it is difficult to clearly differentiate related taxa within the genus based solely on morphology (Singh et al. 2016 and 2020). Previous studies using a polyphasic approach led to the splitting of *Nostoc* into several genera, including *Mojavia*, *Desmonostoc* and *Compactonostoc* (Hrouzek et al. 2003; Řeháková et al. 2007, Cai et al, 2019). It is difficult to separate these groups within *Nostoc sensu lato* based only on morphological characteristics (Table S8). Following the concept that a taxonomic unit (family, genus, species) must be included in only one phylogenetic lineage (Komárek, 2018), the strain PMC 881.14 was considered as a *Nostoc* sp. because our results did not allow us to give any further species assignment to this strain.

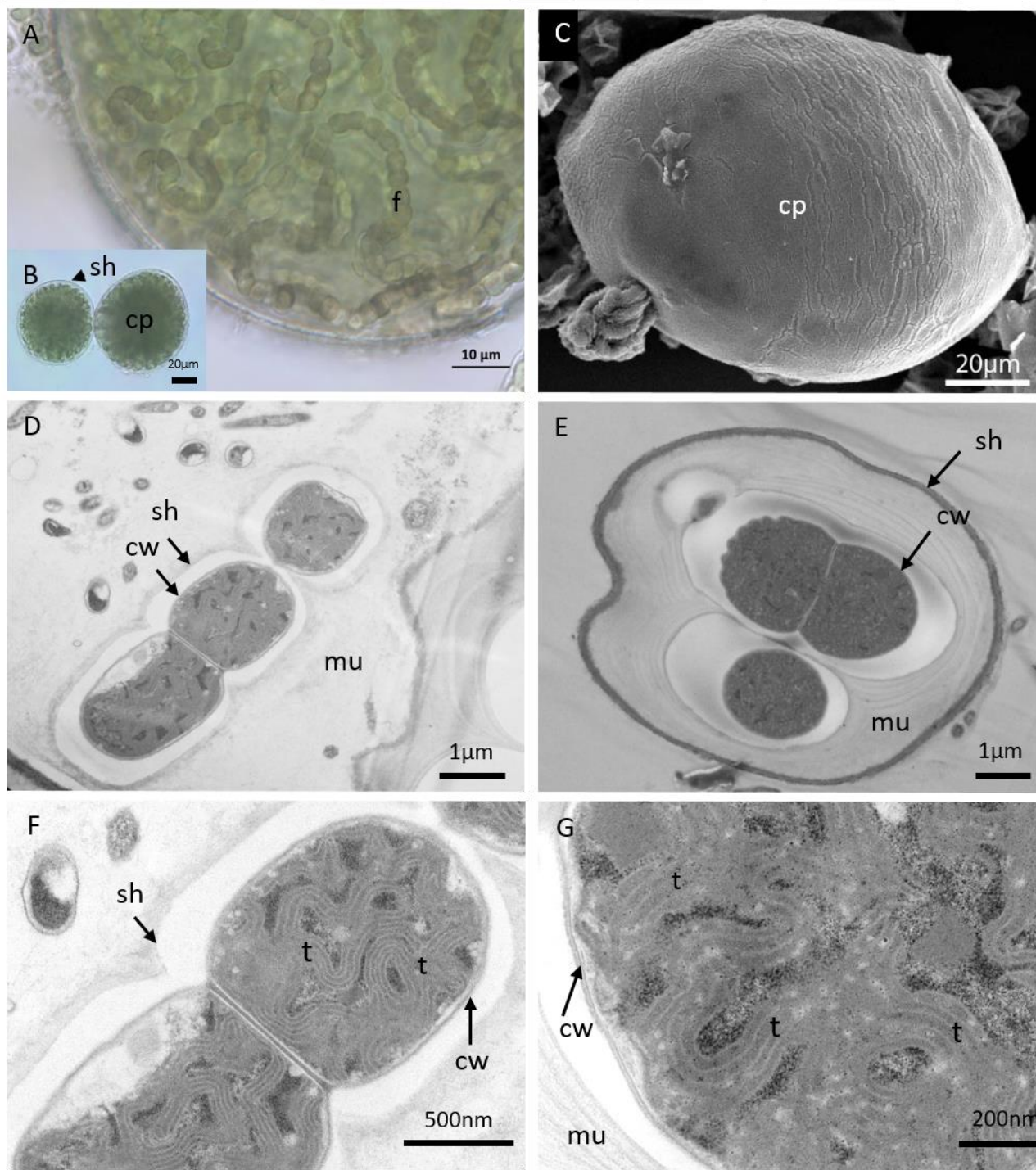


Figure 15. Light microscopy (A-B), SEM (C) and TEM (D-E-F-G) micrographs of *Nostoc* sp. (PMC 881.14) from Thermes de Balaruc-Les-Bains. A: Observation of the filaments located inside the capsule, B: Observation of two colonies, spherical, irregular in their shape, dull olive to brownish-colored and gelatinous. Filaments very densely entangled, flexuous, coiled inside the colonies. C: external observation of a capsule. Longitudinal (D-F-G) and cross sections (E) showing the ultrastructural details. Abbreviations: cp: capsule, cw: cell wall, f: filament, mu: mucilage, sh: sheath, t: thylakoids.

***Aliinostoc* sp.** (S.N. Bagchi, N. Dubey & P. Singh, 2017)

Aliinostoc sp. (PMC 882.14) was isolated from the epilithic biofilm on the wall of the retention basin. Field samples showed free blue-green filaments (Fig. 16), while

in culture the filaments were flexuous and entangled. The trichomes were pale blue-green, cylindrical, thin ($< 6 \mu\text{m}$ wide), and highly constricted at cross-walls (Fig. 16). Cells were well separated from each other by cross-walls, isodiametric, and longer than wide, $3.4\text{-}5.6$ (4.5 ± 0.6) μm wide by $2.6\text{-}6.4$ (4.6 ± 1.0) μm long with a length:width ratio of 1.0 (Table S9). SEM showed that apical cells were conical (Fig. 16). The heterocytes were $4.1\text{-}6.5$ (5.4 ± 0.5) μm wide by $4.1\text{-}6.8$ (5.4 ± 0.6) μm length and akinetes were $4.5\text{-}7.4$ (5.9 ± 0.8) μm wide by $4.5\text{-}9.0$ (7.2 ± 1.0) μm long (Table S9). No sheath was visible under TEM (Fig. 16). The thylakoids were fascicular with irregular spherical formations (Fig. 16). The cells contained several reserve granules and numerous carboxysomes (Fig. 16). The heterocyst TEM observations showed a few thylakoids and large cyanophycin granules.

The 16S rRNA gene sequence analysis showed that PMC 882.14 shared $\geq 98\%$ sequence identity with other *Aliinostoc* sp. Strains (data not shown). The trees based on 16S rRNA gene sequences placed PMC 882.14 within a well-grouped cluster of *Aliinostoc* sequences (Fig. 14). Within this cluster, several sequences were identified as *Aliinostoc tiwarii*, *A. constrictum*, *A. soli*, *A. magnikinetifex*, *A. catenatum* and *A. morphoplasticum*, mainly based on morphological characteristics (Table S9). *Aliinostoc* sp. Showed close morphological resemblance to the genera *Nostoc* and *Trichormus*. A diacritical feature for *Aliinostoc* is the presence of motile hormogonia with gas vesicles (Bagchi et al. 2017). The authors also stated that filaments must be loosely arranged with variable tendencies for coiling. These organisms were generally isolated from habitats rich in dissolved ions and salts, with high water conductivity. As for the *Nostoc* sp. PMC 881.14, description and morphology alone were of little help in assigning the strain to *Aliinostoc sensu lato* (Bagchi et al. 2017) (Table S9). Based on the rule that a taxonomic unit (i.e. genus, species) must be included in only one phylogenetic lineage (Komárek, 2018), the strain PMC 882.14 was named *Aliinostoc* sp. because our data were not sufficient to give a species assignment.

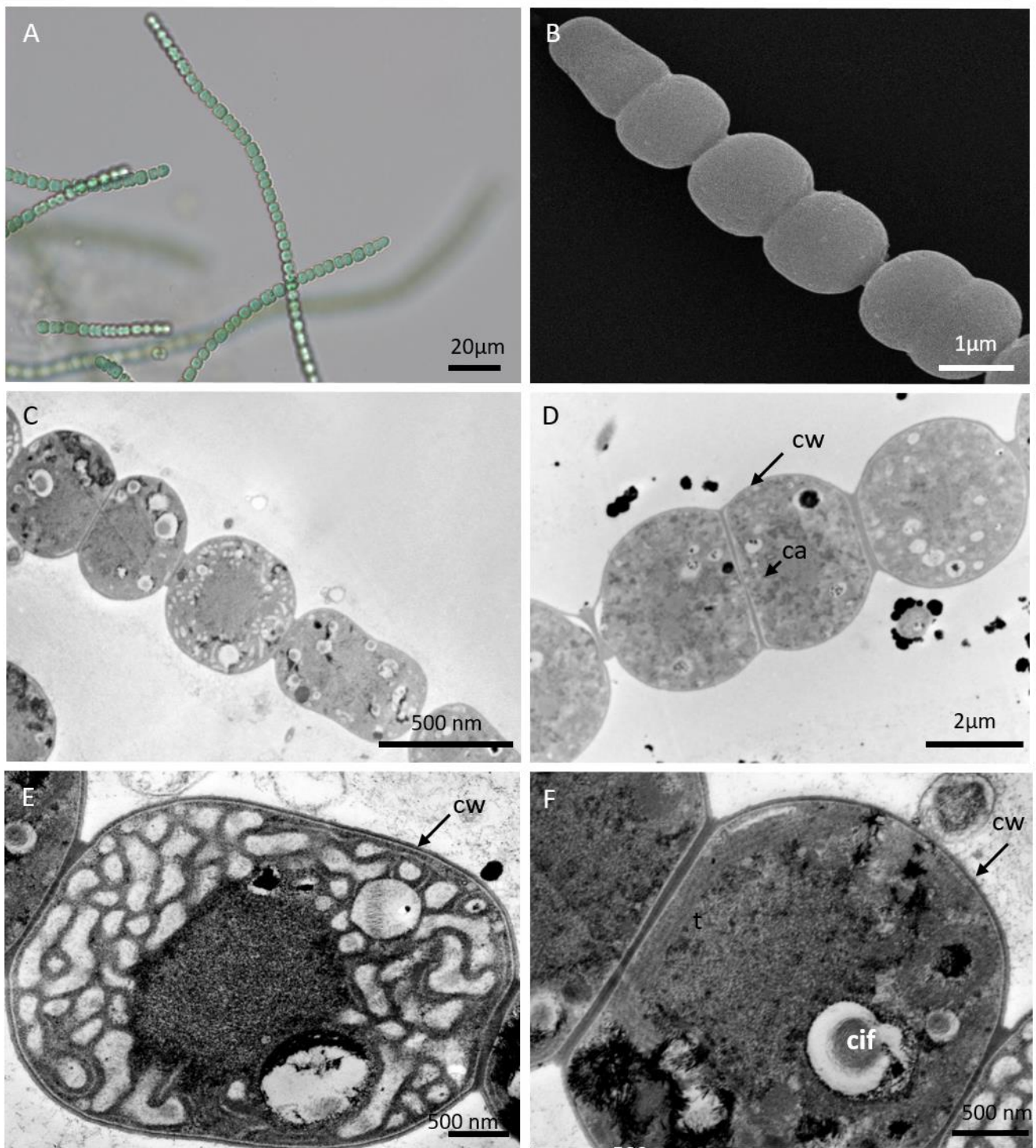


Figure 16. Light microscopy (A), SEM (B) and TEM (C-F) micrographs of *Aliinostoc* sp. (PMC 882.14) from Thermes de Balaruc-Les-Bains. A: Light microscopy of filament from field sample. B: SEM observations of trichome. Longitudinal sections (C-F) of a heterocyte (E) and vegetative cells (C, D, F) showing the ultrastructural details. Abbreviations: ca: carboxysome, cif: carboxysome in formation, cw: cell wall, its: inter thylakoidal space, t: thylakoids.

***Calothrix* sp.** (C. Agardh ex Bornet & Flahaut, 1886)

Calothrix sp. (PMC 884.14) was isolated from the biofilm at the surface of the mud of the retention basin. Observations of field samples showed a brown filamentous thallus basally attached to the substratum and forming thin mats. In culture, the filamentous thallus formed small brownish-green tufts (Fig. 17). Filaments were aggregated, flexuous, heteropolar, widened near the base and narrowed towards the ends, without false branching. The terminal cells were of variable length, conical, bluntly pointed and tapered. The sheath was generally firm and colorless. The trichomes were of different lengths (> 800 μm long), pale blue-green, olive-green to greyish-green, more or less constricted at the cross-walls, and narrowed at the apex: 6.7-10.4 (8.1 ± 0.7) μm wide at the base, 4.7-7.7 (5.8 ± 0.6) μm in the middle and 3.5-5 (4.4 ± 0.4) μm near the ends (Fig. 17, Table S10). Vegetative cells were more or less quadratic, generally shorter than wide in the upper part of the trichome and/or longer than wide in the lower part of the trichome, 5.2-7.9 (6.6 ± 0.7) μm wide by 4.8-6.2 (5.5 ± 0.4) μm long. Heterocytes were basal, single, hemispherical, 4.7-8.2 (6.2 ± 0.9) μm wide by 3.5-7.3 (4.9 ± 0.9) μm long. Akinetes were not observed during the three years the cultures were maintained, even when strain PMC 884.14 was cultivated under stress conditions.

The 16S rRNA gene sequence analysis showed that PMC 884.14 shared $\geq 99\%$ sequence identity with other *Calothrix* sp. strains (data not shown). Phylogenetic trees grouped PMC 884.14 into a coherent *Calothrix* clade (Fig. 14) in which the sequences were mainly assigned to *Calothrix* sp. Recent revision suggested that the genus *Calothrix* should be split into several genera including *Tolypothrix* and *Rivularia* (Berrendero et al. 2016). Based on a polyphasic study of 135 heterocytous tapering cyanobacteria, of which 99 were identified as *Calothrix*, these authors showed that the majority of *Calothrix* sequences were spread among four lineages, two of which were marine, while the other two were freshwater and soil strains (Berrendero et al. 2011, 2016). As the sequences for the *Calothrix* type species (*C. confervicola*) were lacking, and no culture was available in any collection, we could not say which *Calothrix* clade represents the true *Calothrix*. It would be helpful to conduct more phylogenetic analyses to elucidate the phylogeny of the heteropolar, tapering taxa within Nostocales. Based on the rule that a taxonomic unit must be included in only one phylogenetic lineage (Komárek, 2018), PMC 884.14 was assigned to *Calothrix* sp. as this study did not allow us to give a species name to this strain.

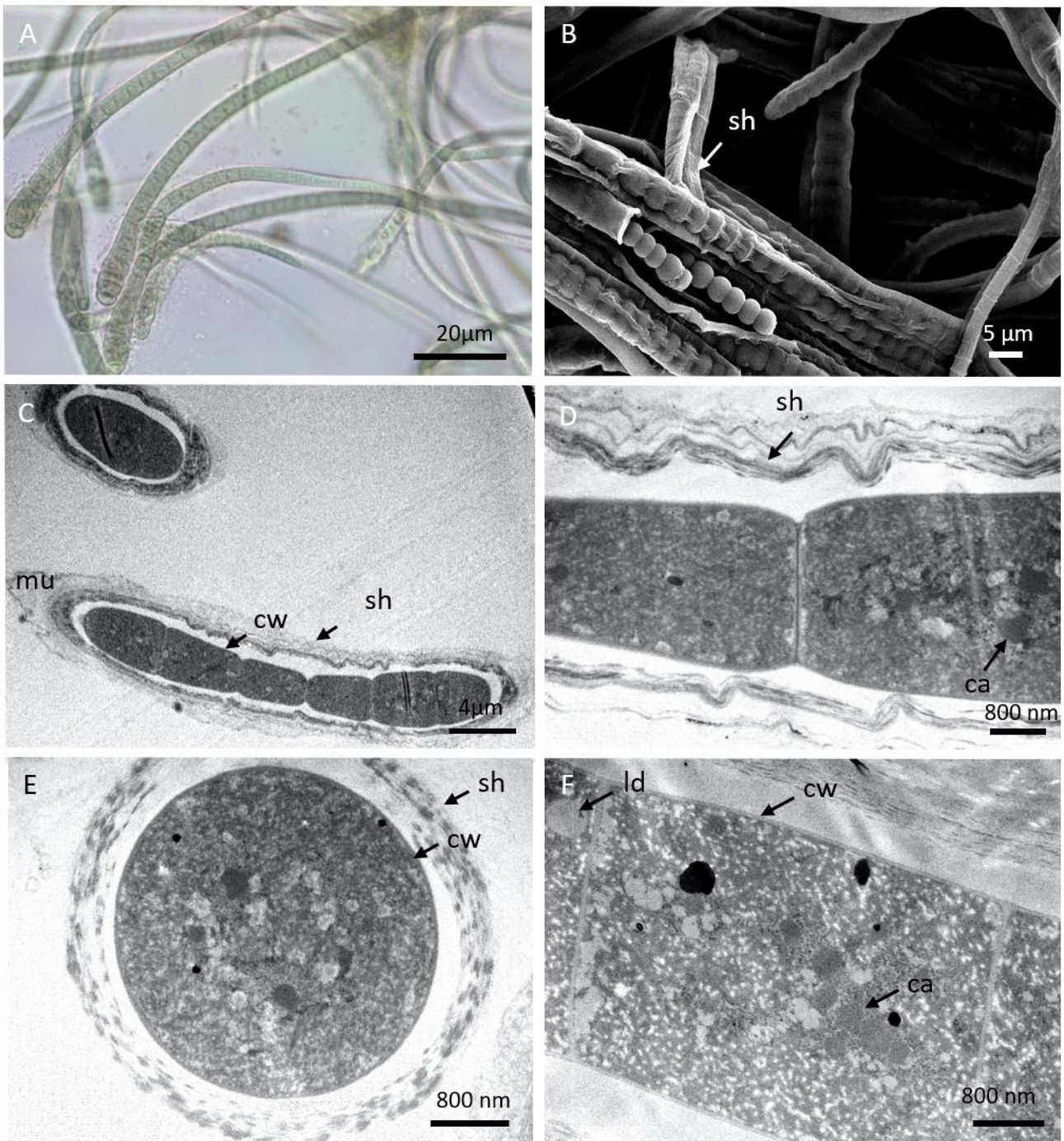


Figure 17. Light microscopy (A), SEM (B) and TEM (C-D-E-F) micrographs of *Calothrix* sp. (PMC 884.14) from Thermes de Balaruc-Les-Bains. A: entangled filaments with sheath. B: observations of filament. Longitudinal (C-D-F) and cross sections (E) of trichomes with sheaths showing the ultrastructural details. Abbreviations: ca: carboxysome, ld: lipid droplet, cw: cell wall, sh: sheath, t: thylakoids, mu: mucilage.

Potential therapeutic properties of cyanobacterial strains from the muds of the thermal springs of Balaruc-les-Bains

A polyphasic approach is still the best way to correctly determine the taxonomy of a new isolate (Komárek 2018, 2020). Using morphological, ultrastructural and molecular methods, the nine cyanobacterial isolates from the muds of Thermes de Balaruc-Les-Bains, were clearly identified as

belonging to the orders Chroococcales: *Pseudo-chroococcus couteii*; Synechococcales: *Leptolyngbya boryana*; Oscillatoriales: *Planktothricoides raciborskii*, *Laspinema* sp., *Microcoleus vaginatus*, *Lyngbya martensiana* and Nostocales: *Nostoc* sp., *Aliinostoc* sp., *Calothrix* sp. This taxonomic diversity along with literature reports of the bioactive metabolite synthesis potential of these taxa allowed us to hypothesize that some of the metabolites produced by these strains may be active in the mud therapy at Thermes de Balaruc-Les-Bains. A recent literature review (Demay et al. 2019) emphasized this great potential (Table S11). All cyanobacteria are known to synthesize pigments such as chlorophylls, phycobiliproteins and carotenoids (Fig. S1), which can have antioxidant or anti-inflammatory properties (Table S11). These pigments were synthesized by the studied isolates in different percentages, depending on the strain and the physiology (Table S11). In addition to pigment production, *Lyngbya*, *Calothrix* and *Chroococcus* strains have attracted further interest because they produce bioactive molecules such as mycosporine-like amino acids (MAAs) and the yellow-brown scytonemin pigments with reported antioxidant, anti-inflammatory and UV-protecting activities. Numerous bioactive compounds from strains belonging to the *Nostoc* genus have also been described, including the anti-inflammatory metabolites aeruginosins and scytonemins. *Planktothricoides*, *Aliinostoc*, *Laspinema* sp., *Microcoleus* and the new genus *Pseudo-chroococcus* were not reported in Demay et al. (2019) to have antioxidant and/or anti-inflammatory activities. Misidentification of Oscillatoriales with their very simple filamentous organization and no diacritical characteristics, occurs frequently and some isolates could have been described as *Phormidium*, *Lyngbya* or other simple organized Oscillatoriales.

CONCLUSIONS

Cyanobacterial taxonomy has changed substantially over the last few years thanks to revisions of the morphological criteria and the use of gene sequencing. To resolve the taxonomic discrepancies between morphological and 16S rRNA data and better assign new isolates of cyanobacteria, the use of whole genome sequencing is warranted. Approximately 1440 cyanobacterial genomes (complete or in progress) are currently available (S. Halary, personal communication) in GenBank (NCBI Datasets “cyanobacteria”, accessed on 9 Sept. 2020). However, this growing number of available genomes may hide the low representativeness of the diversity of cyanobacteria since most sequenced strains belong to the orders Nostocales and Synechococcales with 457 and 600 available assemblies, respectively. For example, very few genomes were available for the genera assigned to Balaruc strains with 2-8 genomes available for *Planktothricoides*, *Lyngbya* and *Microcoleus* and none for *Aliinostoc* and *Pseudo-chroococcus*. Correct assignment of new isolates is essential for understanding the relationships among strains and identifying beneficial therapeutic properties such as those possessed by the mud therapy strains from Thermes de Balaruc-Les-Bains. The isolates maintained in collections will allow in-depth investigation of their potential for production of bioactive metabolites using genomic and biochemical methods.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

SH, CY, CD and CB have worked on the design of the experiments. CD, SH, BP, GT and JD have performed the experiments. CD, SH, BP and CB have performed the data analysis. CD, SH, JD, SD, BM, CY and CB have written and edited the paper.

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DEDICATION

This manuscript is dedicated to the memory of late Professor Alain Couté, who made an outstanding contribution to the taxonomy of cyanobacteria and microalgae in the last decades. His work covers a wide range of studies dealing with photosynthetic microorganisms from marine and freshwater habitats, and also from terrestrial and extreme environments. Alain Couté's contribution will be always relevant and was a driver for shaping this manuscript.

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Chapitre III

Propriétés anti-inflammatoires,
antioxydantes et cicatrisantes des
cyanobactéries de Balaruc-les-Bains

Chapitre III

PROPRIETES ANTI-INFLAMMATOIRES, ANTIOXYDANTES ET CICATRISANTES DES CYANOBACTERIES DE BALARUC-LES-BAINS

III.1. Contexte de l'étude

La recherche de molécules naturelles pour développer des principes actifs a été quelque peu délaissée par les entreprises pharmaceutiques pendant ces vingt dernières années alors que la recherche académique développait de nouvelles techniques permettant de caractériser et de comprendre les mécanismes d'action de ces composés, leurs voies de biosynthèse et leurs rôles écologiques (Beutler 2019; Machado et al. 2017). Historiquement, la découverte de nouvelles molécules bioactives consistait à fractionner un extrait brut présentant une activité recherchée par des méthodes chromatographiques de séparation selon la polarité, la solubilité etc., puis de recommencer ces étapes à partir de la fraction active jusqu'à purifier le composé actif et caractériser sa structure.

Chez les cyanobactéries, cette méthode a été largement utilisée pour la découverte de nouvelles molécules produites par les cyanobactéries avec, par exemple, la cyanobactérine (un antibiotique ciblant spécifiquement les microorganismes photosynthétiques) (Mason et al. 1982) ou encore les scytophycines (cytotoxiques et antifongiques) (M. Ishibashi et al. 1986). Néanmoins, cette méthode bioactivité-centrée s'est avérée désavantageuse puisqu'elle nécessitait une importante quantité de matériel de départ et permettait essentiellement d'identifier les molécules majoritairement produites.

Avec l'essor des techniques « omiques », la recherche concernant les molécules naturelles a pu continuer. La génomique notamment, avec le développement de techniques de séquençage à haut débit et la compréhension des voies de biosynthèse des métabolites spécialisés (NRPS, PKS, RiPPs) (Kehr et al. 2011), a révélé qu'une part importante des gènes de biosynthèse n'était pas affiliée à une molécule décrite (Machado et al. 2017). Cette observation a mené à l'élaboration du concept de « genome mining » qui correspond à une approche « génome centrée » pour la découverte de nouvelles molécules bioactives (Ziemert et al. 2016). Celle-ci consiste en une analyse des génomes à partir d'outils bio-informatiques qui permet la détection et la caractérisation des îlots de gènes de biosynthèse, grâce à leurs séquences conservées. L'outil le plus utilisé par les chercheurs est AntiSMASH, qui permet d'étudier les génomes des bactéries (y compris les cyanobactéries) et des champignons en localisant les îlots de gènes de biosynthèse, en caractérisant l'organisation en domaine pour les synthétases NRPS et PKS, et en réalisant une prédiction du peptide produit via une prédiction des monomères incorporés et ceci par analogie avec les informations contenues dans les bases de données publiques (Blin et al. 2019).

Encart 1 : Les voies de biosynthèse de métabolites chez les cyanobactéries

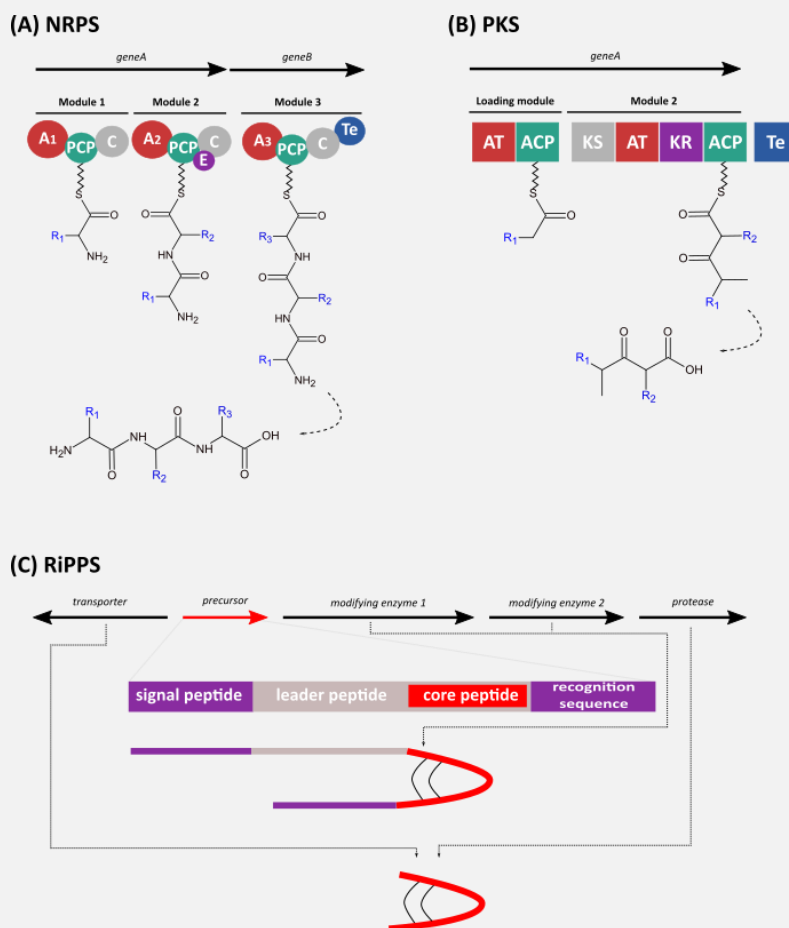


Figure 4. Voies de biosynthèse peptidique chez les cyanobactéries. (A) Synthèse par les synthétases non-ribosomiques (NRPS) ; (B) synthèse par les polycétides synthétases (PKS) ; (C) synthèse ribosomique (RiPPs). Abréviations : A : domaine d'adénylation ; C : domaine de condensation ; PCP : domaine de thiolation (Peptidyl carrier protein) ; Te : domaine de thioestérase ; E : domaine d'épimérisation ; AT : acétyltransférase ; ACP : acyl carrier protein ; KS : domaine de kétoynthase ; KR : domaine de kétoéductase (d'après Kehr et al., 2011).

Il existe trois grandes catégories de voies de biosynthèse chez les cyanobactéries qui leur permettent de synthétiser une grande diversité de métabolites spécialisés (Kehr et al. 2011). La voie NRPS, pour non-ribosomale peptide synthétase, permet notamment de synthétiser des peptides sans utiliser la synthèse ribosomale. Ces synthétases sont des complexes enzymatiques de grande taille organisés en module (Figure 4A), eux même découpés en domaine. Chaque domaine porte une fonction enzymatique qui lui est propre : les domaines d'adénylation (A) sélectionnent et activent un monomère (souvent un acide aminé), les domaines de thiolation (PCP) fixent le peptide en formation et le transfèrent aux domaines de condensation qui vont former les liaisons peptidiques entre chaque monomère. Des domaines accessoires peuvent également être présents dans les modules et servent à modifier le monomère incorporé, on compte les domaines d'épimérisation, de méthylation, de formylation, de cyclisation etc (Marahiel 2009). La synthèse PKS (pour polyketide synthase) quant à elle forme des macrolides, c'est-à-dire différents monomères liés par des liaisons lactones et non peptidiques. A l'instar des NRPS, les synthétases PKS ont une organisation modulaire, chaque module incorporant un monomère distinct. Un module est au minimum composé d'un domaine AT, ACP et KS qui ont des fonctions similaires aux domaines A, PCP et C des NRPS. Des domaines optionnels existent également tels que le domaine kétoéductase (KR), le domaine de déshydratation (DH) (Kehr et al. 2011). Il existe également des voies de biosynthèse hybride qui mêlent domaines NRPS et PKS. La voie RiPPs pour « ribosomally synthesized with post-translational modifications » utilise la synthèse ribosomique classique pour synthétiser un peptide qui est ensuite modifié par une ou plusieurs enzymes (par exemple des hétérocyclases ou des prenyl transférases chez les cyanobactéries) (Martins and Vasconcelos 2015).

Bien que le genome mining permette d'obtenir une vision rapide des métabolites potentiellement produits par une souche, l'utilisation de cette approche n'est que prédictive et nécessite l'utilisation de méthodes supplémentaires pour la découverte de nouvelles molécules. Dans ce cadre, le génome mining est couplé avec

l'analyse chimique du métabolome, c'est-à-dire l'étude de l'ensemble des métabolites présents dans un extrait de la souche étudiée. Deux techniques sont principalement utilisées pour la caractérisation du métabolome : la spectrométrie de masse (MS), qui utilise les propriétés d'ionisation des molécules afin de caractériser leur masse via le rapport masse/charge, et la spectroscopie à résonance magnétique nucléaire (RMN) qui permet l'étude des noyaux atomiques choisis (généralement le proton H⁺) grâce à ses propriétés magnétiques et à son comportement soumis à un champ magnétique. En générale, la RMN est plus utilisée pour caractériser la structure d'un composé et la MS pour obtenir une vision d'ensemble des composés présents dans un échantillon (Machado et al. 2017). Dérivée de la spectrométrie de masse « simple », on retrouve aussi la spectrométrie de masse en tandem (MS/MS) qui permet d'obtenir un spectre de fragmentation pour chacun des ions identifiés au préalable en MS simple, permettant d'obtenir une « signature » pour chacun des composés visant à faciliter son identification.

Ces techniques génèrent une quantité importante de données qui nécessitent l'utilisation d'outils bio-informatiques afin de les décrypter et de les analyser. L'un des principaux outils utilisés pour la caractérisation de nouvelles molécules est la visualisation des données à l'aide de réseaux moléculaires construits par des outils informatiques dédiés : GNPS ou encore MetGem (Nothias et al. 2018; Olivon et al. 2018). La construction du réseau se fait à partir de données MS/MS, en comparant et regroupant ensemble les ions qui partagent des similarités dans leurs spectres de fragmentation. En effet, le profil de fragmentation d'une molécule étant dépendant de sa structure, deux molécules qui partagent des similarités structurales possèdent des spectres de fragmentation similaires.

L'utilisation des différentes méthodes omiques et leur complémentarité ont donc permis à la recherche de nouvelles molécules actives de connaître un regain d'intérêt. En effet, ces techniques permettent l'étude rapide d'échantillons complexes en s'affranchissant des besoins de purification des méthodes plus traditionnelles. Plusieurs approches, couplant les différentes techniques omiques, ont ainsi été développées au fil des années afin d'identifier de nouvelles molécules (Ziemert et al. 2008; Leikoski et al. 2013). Ces méthodes ont d'ailleurs été utilisées avec succès au sein de l'équipe CCE⁵, où l'utilisation d'analyses génomiques des îlots de gènes de biosynthèse et d'analyses LC-MS/MS non ciblées ont permis la caractérisation de la diversité de métabolites produits par plusieurs souches appartenant au genre *Planktothrix* et ont pu mettre en évidence les différences métabolomiques qui peuvent exister entre des cyanobactéries appartenant à un même genre (Kim Tiam et al. 2019).

Un autre challenge de la découverte de molécules bioactives est justement d'identifier leurs activités. En effet, il arrive souvent que l'activité soit perdue ou que l'isolement du composé bioactif soit un échec lors du processus de purification. Cela peut être dû à une dégradation des molécules, une concentration trop faible du composé actif ou encore que l'activité résulte d'une synergie de molécules (Nothias et al. 2018). De plus, il existe une multitude de tests différents pour chaque activité donnée. Par exemple, pour tester une potentielle activité anti-inflammatoire il est possible de mesurer *in vivo* la capacité d'un extrait ou d'une molécule à réduire un œdème induit chez la souris (C. S. Stevenson et al. 2002) ou encore de mesurer *in vitro* le niveau de sécrétion

⁵ « Cyanobactéries, Cyanotoxines et Environnement »

de cytokine pro-inflammatoire (Choi, Mascuch, et al. 2012) jusqu'à identifier le véritable mécanisme d'action de la molécule (Mascuch et al. 2017).

Les chapitres précédents nous ont permis d'estimer le potentiel anti-inflammatoire et antioxydant pour chacune des cyanobactéries isolées des boues thermales de Balaruc-les-Bains. Afin de confirmer ce potentiel, les travaux présentés dans ce chapitre ont pour but de répondre à la question : *quelle est la diversité des molécules produites par les neuf cyanobactéries de Balaruc-les-Bains ?* et quelles sont leurs propriétés anti-inflammatoires, antioxydantes et cicatrisantes ? (**Article 3**).

III.2. Résumé

Pour répondre à ces questions, une approche combinant des analyses de génomique, de métabolomique et de screening de plusieurs bioactivités a été utilisée. Les métagénomomes des neuf cyanobactéries issues des boues thermales de Balaruc-les-Bains ont été séquencés, puis les génomes des cyanobactéries et des bactéries associées ont été séparés et assemblés (en collaboration avec le Dr. S. Halary). Chacun des génomes ainsi obtenus a été étudié dans le but d'identifier les îlots de gènes de biosynthèse de métabolites secondaires et les gènes de biosynthèse des différents pigments anti-inflammatoires et/ou antioxydants à l'aide de la plateforme MicroScope qui intègre différents outils d'analyse des génomes comme antiSMASH et Blast.

Après culture en masse, les cyanobactéries étudiées ont été soumises à deux méthodes d'extraction (eau et méthanol 75%) dans le but d'obtenir un panorama complet des molécules produites par chacune des souches. Ces extraits ont fait l'objet d'une part d'une analyse métabolomique par HPLC-DAD et LC-MS/MS, et d'autre part ont été soumis à plusieurs tests d'activité afin d'identifier leur toxicité, leur potentiel anti-inflammatoire, antioxydant et cicatrisant.

L'activité anti-inflammatoire a été testée en mesurant l'impact des extraits sur la production de quatre cytokines pro-inflammatoires (TNF- α , IL-1 β , IL-6 et IL-8). L'activité antioxydante a été évaluée en mesurant l'impact des extraits sur la sécrétion d'oxyde d'azote, une des espèces de radicaux libres générés en cas de stress oxydatif. Et l'activité cicatrisante a été mesurée sur les kératinocytes humains en suivant la cinétique de cicatrisation d'un tissu lésé en présence des extraits bruts. La cytotoxicité des extraits a également été testée en parallèle sur les trois lignées cellulaires employées pour les tests d'activités afin de valider les tests d'une part et d'évaluer la toxicité potentielle des extraits.

Anti-inflammatory, antioxidant and wound healing properties of cyanobacteria from thermal mud (Balaruc-Les-Bains, France): a multi-approach study

Demay J.^{1,2}, Halary S.¹, Knittel-Obrecht A.³, Villa P.³, Duval C.¹, Hamlaoui S.¹, Roussel T.¹, Yéprémian C.¹, Reinhardt A.², Bernard C.^{1*}, Marie B.^{1*}

Soumis à *Biomolecules*

Les résultats obtenus montrent que les neuf souches sont capables de produire de nombreux métabolites spécialisés avec entre 7 et 30 îlots de gènes de biosynthèse détectés pour chacune des souches. Les gènes de

biosynthèse des différents pigments bioactifs ont également été caractérisés pour chacune des souches ; des différences ont néanmoins été observées pour la phycoérythrine et la scytonémine. Aucune cyanotoxine n'a été détectée chez les neuf cyanobactéries étudiées, que ce soit au niveau des gènes de biosynthèse ou de la production métabolomique. Plusieurs molécules ayant montré des activités anti-inflammatoires et antioxydantes ont pu être détectées au cours de nos travaux comme des caroténoïdes, des phycobilines, ainsi que plusieurs mycosporine-like amino acids (MAAs) et un analogue des aéruginosines. D'autres métabolites ont également été identifiés, notamment des microginines, des microviridines et des anabaenolysines.

Planktothricoides raciborskii PMC 877.14, *Nostoc* sp. PMC 881.14 et *Pseudo-chroococcus couteii* PMC 885.14 ont montré des propriétés anti-inflammatoires de par leur capacité à inhiber la production des cytokines médiatrices de l'inflammation. *Aliinostoc* sp. PMC 882.14, quant à elle, a montré une augmentation de la cicatrisation sur un tissu de kératinocytes. Aucune cytotoxicité n'a été détectée pour les différents extraits, les résultats obtenus sont synthétisés dans le **Tableau 2**. Ces résultats permettent d'obtenir une vision globale des propriétés thérapeutiques des neuf cyanobactéries issues des boues thermales de Balaruc-les-Bains et de supposer un fort potentiel de valorisation pour ces quatre cyanobactéries.

Tableau 2. Synthèse des molécules identifiées et des activités détectées. (MeFA: 7-methoxy-9-methylhexadecadienoic acid. Les couleurs correspondent à l'activité attendue: **antioxydant**; **anti-inflammatoire**; **toxique**; **antimicrobien**; **enzyme inhibition**; **photo-protection**. D'autres activités sont symbolisées par *anti-consommation, ** piégeage du fer, ***stabilisation de membrane)

Cyanobactéries	Ilots de gènes identifiés	Métabolites identifiés	Activités détectées	
			H2O	MeOH
<i>Planktothricoides raciborskii</i> PMC 877.14	Phycocyanobilines Phycoérythrine Chlorophylles Caroténoïdes	Phycocyanobiline Phycoérythrine Chlorophylles Caroténoïdes Sacrolide-like Telbivudine-like Anaephene-like MeFA-like Mueggelone-like * Ypaoamide-like	Anti-inflammatoire Non cytotoxique	Anti-inflammatoire Non cytotoxique
<i>Laspinema</i> sp. PMC 878.14	Phycocyanobilines Chlorophylles Caroténoïdes Heterocyclique cyanobactérie-like	Phycocyanobilines Chlorophylles Caroténoïdes	Pas d'act. détectées Non cytotoxique	Pas d'act. détectées Non cytotoxique
<i>Microcoleus vaginatus</i> PMC 879.14	Phycocyanobilines Chlorophylles Caroténoïdes	Phycocyanobilines Chlorophylles Caroténoïdes Biotérolin- α -glucoside Tanikolide-like	Pro-inflammatoire Non cytotoxique	Pro-inflammatoire Anti-inflammatoire Non cytotoxique
<i>Lyngbya martensiana</i> PMC 880.14	Phycocyanobilines Chlorophylles Caroténoïdes ** Schizokinen-like	Phycocyanobilines Chlorophylles Caroténoïdes	Pro-inflammatoire Non cytotoxique	Anti-inflammatoire Non cytotoxique
<i>Nostoc</i> sp. PMC 881.14	Scytonemine Phycocyanobilines Phycoérythrine Chlorophylles Caroténoïdes MAAs Heterocyst glycolipid	Phycocyanobilines Phycoérythrine Chlorophylles Caroténoïdes MAAs Radiosumine-like MeFA-like	Pro-oxydant Pro-inflammatoire Anti-inflammatoire Non cytotoxique	Anti-inflammatoire Non cytotoxique

<i>Aliinostoc</i> sp. PMC 882.14	Scytonemine				
	Phycocyanobilines		Phycocyanobilines		
	Chlorophylles		Chlorophylles		
	Caroténoïdes		Caroténoïdes		
	MAAs		MAAs		
			Bioplerin- α -glucoside	Cicatrisation	
	Microginine-like		Microginine-like	Pro-inflammatoire	Pas d'act. détectées
	Microviridine-like		Microviridine-like	Anti-inflammatoire	Non cytotoxique
	Amycomycine-like			Non cytotoxique	
	Heterocyte glycolipide				
		***	Hopanoïde-like		
			Gallinamide-like		
<i>Leptolyngbya boryana</i> PMC 883.14	Chlorophylles		Chlorophylles		
	Caroténoïdes		Caroténoïdes	Pas d'act. détectées	Pas d'act. détectées
	Phycocyanobilines		Phycocyanobilines	Non cytotoxique	Non cytotoxique
			Aeruginosines		
<i>Calothrix</i> sp. PMC 884.14	Scytonemine				
	Phycocyanobilines		Phycocyanobilines		
	Phycoérythrine		Phycoérythrine		
	Chlorophylles		Chlorophylles		
	Caroténoïdes		Caroténoïdes	Pro-inflammatoire	Pas d'act. détectées
	MAAs		MAAs	Anti-inflammatoire	Non cytotoxique
	Heterocyte glycolipide			Non cytotoxique	Non cytotoxique
	Cyanobactine-like		Microphycine-like		
	Cylindrocyclophane-like				
<i>Pseudo-chroococcus couteii</i> PMC 885.14	Phycocyanobilines		Phycocyanobilines		
	Phycoérythrine		Phycoérythrine	Anti-inflammatoire	Pas d'act. détectées
	Chlorophylles		Chlorophylles	Non cytotoxique	Non cytotoxique
	Caroténoïdes		Caroténoïdes		

III.3 Article 3



Article

Anti-Inflammatory, Antioxidant, and Wound-Healing Properties of Cyanobacteria from Thermal Mud of Balaruc-Les-Bains, France: A Multi-Approach Study

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Abstract: *Background:* The Balaruc-les-Bains' thermal mud was found to be colonized predominantly by microorganisms, with cyanobacteria constituting the primary organism in the microbial biofilm observed on the mud surface. The success of cyanobacteria in colonizing this specific ecological niche can be explained in part by their taxa-specific adaptation capacities, and also the diversity of bioactive natural products that they synthesize. This array of components has physiological and ecological properties that may be exploited for various applications. *Methods:* Nine cyanobacterial strains were isolated from Balaruc thermal mud and maintained in the Paris Museum Collection (PMC). Full genome sequencing was performed coupled with targeted and untargeted metabolomic analyses (HPLC-DAD and LC-MS/MS). Bioassays were performed to determine antioxidant, anti-inflammatory, and wound-healing properties. *Results:* Biosynthetic pathways for phycobiliproteins, scytonemin, and carotenoid pigments and 124 metabolite biosynthetic gene clusters (BGCs) were characterized. Several compounds with known antioxidant or anti-inflammatory properties, such as carotenoids, phycobilins, mycosporine-like amino acids, and aeruginosins, and other bioactive metabolites like microginins, microviridins, and anabaenolysins were identified. Secretion of the proinflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-8 appeared to be inhibited by crude extracts of *Planktothricoides raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14, and *Pseudo-chroococcus couteii* PMC 885.14. The extract of the *Aliinostoc* sp. PMC 882.14 strain was able to slightly enhance migration of HaCat cells that may be helpful in wound healing. Several antioxidant compounds were detected, but no significant effects on nitric oxide secretion were observed. There was no cytotoxicity on the three cell types tested, indicating that cyanobacterial extracts may have anti-inflammatory therapeutic potential without harming body cells. These data open up promising uses for these extracts and their respective molecules in drugs or thermal therapies.

Keywords: cyanobacteria; thermal mud; natural products; anti-inflammatory; bioactivity

1. Introduction

Cyanobacteria, the so-called blue-green algae, are an ancient lineage of photosynthetic prokaryotes found worldwide. They are notorious for their harmful effects on human health because they produce toxins such as microcystins, anatoxins, and saxitoxins [1–3]. Not all species synthesize cyanotoxins, and cyanobacterial compounds such as pigments and specialized metabolites have been studied for their beneficial actions against cancer and as antioxidants and anti-inflammatories [4,5]. In recent years, the

improvement of genome sequencing techniques and the increasing number of available genomes uncovered the great potential for specialized metabolite production by cyanobacteria [6–9]. Specialized metabolites, also known as secondary metabolites or natural products, are widespread in plants, fungi, and bacteria, but are especially abundant in cyanobacteria [10]. In cyanobacteria, genome exploration has revealed numerous biosynthetic gene clusters belonging to non-ribosomal pathways. These include non-ribosomal peptide synthetase (NRPS), polyketide synthetase (PKS), and members of other ribosomal pathways, such as ribosomally synthesized and post-translationally modified peptide (RiPPs), terpene, or alkaloid pathways [8,11]. Thanks to the diverse metabolic activity of cyanobacteria, their natural products have attracted a great deal of interest in research for new bioactive substances [12–15].

Benthic cyanobacteria especially have shown a wide range of specialized metabolites. This is illustrated by the remarkable number of compounds isolated from the *Lyngbya majuscula-Moorea producens* genus [4,9]. Because they are attached to a substrate, benthic cyanobacteria are subject to intense space competition and to stresses like desiccation, high light intensity, and predation by grazers that forced them to develop a large repertoire of chemical defences [16–18]. Therapeutic thermal mud from the thermal baths of Balaruc-les-Bains (France) has been used to treat various illnesses since ancient Roman times. For joint diseases such as osteoarthritis and rheumatism, the matured thermal mud (peloid) is applied directly to the patient's skin. Two studies were conducted in the 1980s to evaluate the specific algal communities colonizing this peloid, and both showed that diverse genera of microalgae and cyanobacteria have colonized the mud with a predominance of cyanobacteria belonging to the *Phormidium* genus [19,20]. It was hypothesized that compounds produced by this microflora, in association with heat and microelements in the hot spring water, were responsible for the beneficial therapeutic properties of Balaruc-les-Bains mud, as proposed for other thermal baths [21–23]. However, the thermal mud of Balaruc-Les-Bains still remains a relatively unexplored environment, particularly with regard to identifying the therapeutically active compounds produced by the microflora associated with the mud.

In 2014, a new isolation campaign was initiated to ascertain the evolution of the microbial communities in the maturing mud during the five-month summer period. Several species of cyanobacteria and microalgae were isolated and maintained in the Paris' Museum Collection (PMC) [24]. Nine cyanobacteria, belonging to four different orders with different morphotypes (Table 1), were selected for assessment of metabolite production, as they belong to genera considered as prolific producers of potent bioactive compounds. To take advantage of the diverse bioactivities of the compounds produced by cyanobacteria, the anti-inflammatory, antioxidant, and wound-healing potential of the nine strains. (Table 1) were investigated. Numerous compounds produced by these cyanobacteria have been described, including pigments, such as phycobilins, chlorophylls, carotenoids, and scytonemin, and specialized metabolites—mycosporine-like amino acids, aeruginosins, and honaucins [4,25]. The simple screening for activity as a way to identify new therapeutic metabolites has recently been greatly accelerated by improved genome sequencing methods and the development of genome mining to identify and characterize specialized metabolite biosynthetic gene clusters (BGCs) [26]. In consequence, the metagenomes of the present nine Balaruc cyanobacterial strains have been sequenced in-depth and biosynthetic genes for pigments and specialized metabolites have been characterized. Targeted pigment dose studies have been performed and untargeted LC-MS/MS investigations have been developed to determine the diversity of compounds produced by the nine strains. In parallel, crude water and methanol extracts have been tested for anti-inflammatory, antioxidant, wound-healing, and cytotoxic properties to evaluate their potential for further therapeutic development.

Table 1. List of cyanobacterial strains isolated from the Balaruc thermal mud and their corresponding strain reference numbers. PMC: Paris' Museum Collection.

Order	Species	Strain number	BioProject Accession Numbers
Chroococcales	<i>Pseudo-chroococcus couteii</i>	PMC 885.14	PRJNA686263
Synechococcales	<i>Leptolyngbya boryana</i>	PMC 883.14	PRJNA686260
Oscillatoriales	<i>Planktothricoides raciborskii</i>	PMC 877.14	PRJNA686238
	<i>Laspinema</i> sp.	PMC 878.14	PRJNA686242
	<i>Microcoleus vaginatus</i>	PMC 879.14	PRJNA686244
	<i>Lyngbya martensiana</i>	PMC 880.14	PRJNA686257
Nostocales	<i>Nostoc</i> sp.	PMC 881.14	PRJNA686258
	<i>Aliinostoc</i> sp.	PMC 882.14	PRJNA686259
	<i>Calothrix</i> sp.	PMC 884.14	PRJNA686262

2. Materials and Methods

2.1. Biological Material

Nine monoclonal cyanobacteria strains were isolated in 2014 from thermal mud in Balaruc-Les-Bains, France, and have been maintained as non-axenic cultures in the Paris Museum Collection (PMC) (Table 1) [24]. Sampling and taxonomy of the strains were described elsewhere [27]. The isolated cyanobacteria were grown in Z8 medium at 24 °C. During experiments, the cultures were maintained in 1-L Duran bottles with a photon flux density of 12 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR, photosynthetic active radiation) and a 16:8 h light:dark cycle. Cultures were transferred into fresh Z8 medium every six weeks to maintain them in the active growth phase.

2.2. Genome Sequencing and Assembly

After a centrifugation step of 150 mL cultures of each strain, DNA extractions were performed on the pellets using the ZymoBIOMICS DNA mini kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. The Macherey Nagel genomic DNA and total RNA purification kit with NucleoBond AXG20 columns and buffer set III (Macherey- Nagel, PA, USA) was employed for PMC 881.14 and PMC 884.14. Metagenome sequencing was performed by GenoScreen (GenoScreen, Lille, France) using the Nextera XT DNA sample preparation kit (Illumina) for 2 250 bp and using a SMRT2 cell (PacBio) Illumina and Pacbio raw reads were corrected using SPAdes 3.12 and Canu 1.8, respectively, before the assembly performed with Unicycler hybrid-assembler, with default parameters [28–30]. For each new assembly, scaffolds were binned using MyCC (k-mer size = 4, minimal sequence size = 1000) and taxonomically annotated using CAT [31]. Congruent data between both methodologies allowed to characterize the cyanobacteria draft genomes.

2.3. In Silico Analyses

The genome assemblies were integrated on the MicroScope platform v3.14.1 (<https://mage.genoscope.cns.fr/microscope/home/index.php>) [32]. Biosynthetic gene clusters (BGCs) for specific metabolites were identified using the antiSMASH v5.0.0 [33] and MIBiG v1.4 [34] software available through the MicroScope platform. These tools allowed characterization of the gene cluster organization and predicted the molecules potentially produced by comparison of homologies between the query and the BGC sequences annotated in the databases. For each BGC identified, we carefully checked the automatic prediction against the literature on the biosynthetic pathways described to obtain a “curated” annotation of the detected clusters with the highest confidence possible. As the automatic annotation tools did not detect pigment biosynthetic genes, a targeted approach was performed using BLASTp and the search-by-keywords function of the MicroScope platform.

2.4. Pigment Composition Analysis

The pigment composition of the strains was determined in triplicate from 10 mL of culture filtered through Whatman GF/F filters (0.7 μm) and freeze dried. For extracting lipid-soluble pigments like carotenoids and chlorophylls, filtered biomasses were extracted with methanol, pooled, and quantified by HPLC-DAD (as described in Ras et al., 2008) [35]. For determination of the water-soluble phycobiliprotein pigments, filtered biomasses were analyzed following a published procedure [36].

2.5. Metabolite Extraction, Analysis by Mass Spectrometry, and Annotation

For each cyanobacterial strain, biomasses from five successive subcultures (5x500 mL) were collected at the end of the exponential growth and centrifuged at 3220xg for 10 min. The pellets were lyophilized (Freezone 2.5 L, Labconco, Italia) and pooled for metabolite extraction. Two different metabolite extractions were performed with the same pooled samples (200 mg dried weight) to increase the likelihood of identifying all the metabolites: (1) a mild hydrophobic single extraction with MeOH/water, 75%/25%, v/v (solvent A) and (2) a hydrophilic single extraction with ultrapure water acidified with 0.1% formic acid (solvent B). Lyophilized cell pellets were weighed and 1 mg in 100 μL of the extraction solution was sonicated (SONICS Vibra Cell, 130 Watt, 20 Khz) at 80% power three times for two minutes each time (30 s rest between). Prior to analysis, the sonicated extracts were centrifuged at 12,000 g for 10 min at 4 °C. Four microliters aliquots of the supernatants were run on an ultra-high-performance liquid chromatograph (UHPLC Ultimate 3000, Thermo, Waltham, MA, USA) using a Polar Advances II 2.5 pore C18 column (Thermo, Waltham, MA, USA) at a 300 $\mu\text{L min}^{-1}$ flow rate with a linear gradient of acetonitrile in 0.1% formic acid (5–90% for 21 min) coupled with a high-resolution mass spectrometer. Chromatography runs were done in triplicate. The eluted metabolites were analyzed using an electrospray ionization hybrid quadrupole time-of-flight (ESI-QqTOF) high-resolution mass spectrometer (Compact Bruker, Bremen, Germany) in positive simple MS or positive autoMS/MS mode with information-dependent acquisition (IDA), on the 50 to 1500 m/z range at 2 Hz or between 2 and 8 Hz speed, for MS and MS/MS, respectively, according to relative intensity of parent ions, in consecutive cycle times of 2.5 s, with an active exclusion of previously analysed parents. The data were analyzed with the DataAnalysis 4.4 software for internal recalibration (<0.5 ppm) and MGF exports were generated from MS/MS spectra between 1 and 15 min. The raw MS and MS/MS data were analyzed with MetaboScape 4.0 software (Bruker, Bremen, Germany) to automatically search and group together all classical adduct forms ($[\text{M} + \text{H}]^+$, $[\text{M} + 2\text{H}]^+$, $[\text{M} + 3\text{H}]^+$, $[\text{M} + \text{Na}]^+$, $[\text{M} + \text{K}]^+$, and $[\text{M} + \text{NH}_4]^+$) using a threshold value of 0.8 for the co-elution coefficient factor. Metabolite annotation was attempted according to the precise mass of the molecules and their respective MS/MS fragmentation patterns with regards to MS/MS libraries (NIH, GNPS, EMBL, MassBank, ReSpect, etc.) and the CyanoMet database [37] of >2100 cyanobacterial metabolites. Results were confirmed by running 36 commercially available standards of specific cyanobacterial metabolite families: cyanopeptolins, aeruginosins, microginins, anabaenopeptins, aerucyclamides, microcystins, saxitoxins, anatoxins, and cylindrospermopsins.

2.6. Molecular Networking

MetGem v1.2.2 [38] was used to create a molecular network from the MS/MS data (in mgf format) for two out of the nine extracts. The network was created with edges filtered to have a cosine score above 0.65 and more than four matched peaks. Further edges between two nodes were retained in the network only if each of the nodes appeared in each of the other's top ten most similar nodes, respectively. The MetGem database search function was used to screen each spectrum against GNPS and ISDB spectral libraries.

2.7. Bioassays

Activity assays were performed using crude extracts from the same nine cyanobacteria as for metabolomic analysis and the same method with solvents A and B. Methanol extracts were evaporated with a RotaVap R-210 (Büchi, Flawil, Switzerland) and aqueous extracts were lyophilized. Each was then resuspended in DMSO/water (50%/50%) at a concentration of 30 mg mL⁻¹ (dried extract).

The antioxidant potential of the crude extracts was assessed by measuring nitric oxide (NO) production by the macrophage-like cell line, RAW 264.7, stimulated with 5 µg mL⁻¹ lipopolysaccharide (LPS, Sigma L6636). Quercetol was used as the positive antioxidant control and results were expressed as percent inhibition compared to the maximal NO level induced by LPS in 0.1% DMSO [39]. Following 24 h of treatment, NO production was determined indirectly with quantification of nitrite (NO₂⁻) accumulation in culture media using Griess Reaction. Briefly, 100 µL of Griess reagent (Invitrogen G-7921) was added to 100 µL of cellular supernatant samples into 96-well plates (Greiner 655101) and left 30 min at room temperature under shaking. Absorbance at 540 nm was measured using an Envision (Perkin Elmer) microplate reader. The reduction or increase in nitrite levels was calculated by the percentage of absorbance of sample relative to the positive control (5 µg mL⁻¹ LPS with 0.1% DMSO).

Anti-inflammatory activity of the extracts was evaluated by determining their effect on the concentration of the proinflammatory cytokines, TNFα, IL-1β, IL-6, and IL-8, in human peripheral blood mononuclear cells (PBMCs). PBMCs were stimulated with 5 µg mL⁻¹ LPS and the extracts were then added. Cytokine concentration was then measured using HTRF technology with fluorescent antibodies [39]. Dexamethasone was used as positive control and results were expressed as percent inhibition relative to the maximal cytokine secretion induced by LPS.

Cell migration assays were performed using the HaCat cell line of human keratinocytes. Extracts were pipetted onto HaCat monolayers previously wounded and treated to prevent cell proliferation. The plate was then placed into the IncuCyte™ (Essen Bioscience, Ann Arbor, MI) live-cell imaging instrument to measure the cell migration onto the wounded area for 48 h. Fetal bovine serum (FBS) was used as positive control as it enhances cell migration onto the wounded area [40].

Cell viability was also measured during the three previous tests with the WST-1 assay (Ozyme, Saint-Quentin-en-Yvelines, France) following protocol described previously [41] in order to validate the bioassay results and to assess the potential cytotoxicity of the crude extracts. For this test, an extract was considered cytotoxic if the cell viability was less than 70% [39].

3. Results

3.1. Characteristics of the Cyanobacterial Genomes

Results of analysis of the nine cyanobacterial genomes are presented in Table 2. High CheckM completeness scores (>97.1%) indicate that the genomes were mostly complete and well assembled. A low contamination score (<1.4%) was found for all genomes, which means a low risk of potential contamination of assemblies with exogenous sequences. The mean size of the genomes was 7.8 Mb, ranging from 5.8 Mb for the *Pseudo-chroococcus* strain, PMC 885.14, to 13.2 Mb for the *Calothrix* sp., PMC 884.14. An important difference was observed between the numbers of contigs for each strain, which ranged from five contigs for *L. boryana*, PMC 883.14, to 648 for *Nostoc* sp., PMC881.14.

Table 2. Characteristics of assembled genomes of the nine cyanobacteria isolated from thermal mud in Balaruc-Les-Bains obtained from the MicroScope platform. CheckM analysis has been performed for each genome using reference genomes (65-89 for 520-584 lineage-specific markers). CDS : Coding sequences

Genus/Species	Strain	Genome Length (bp)	GC Content	Number of Contigs	Number of CDS	Number of rRNA	Average CDS Length (bp)	CheckM Completeness	CheckM Contamination
<i>P. raciborskii</i>	PMC 877.14	7,392,957	44%	15	7130	12	841.8	99.9 %	0.89 %
<i>Laspinema</i> sp.	PMC 878.14	7,343,542	47%	69	5829	9	1 022.4	99.3 %	1.16 %
<i>M. vaginatus</i>	PMC 879.14	6,904,242	46%	34	6400	6	904.5	99.6 %	0.22 %
<i>L. martensiana</i>	PMC 880.14	6,457,204	40%	132	5904	3	937.8	97.1 %	1.19 %
<i>Nostoc</i> sp.	PMC 881.14	8,003,053	42%	648	7711	3	845.9	99.5 %	1.19 %
<i>Aliinostoc</i> sp.	PMC 882.14	8,136,653	41%	46	7582	12	883.9	98.2 %	0.22 %
<i>L. boryana</i>	PMC 883.14	6,695,177	47%	5	6313	9	929.1	99.4 %	1.02 %
<i>Calothrix</i> sp.	PMC 884.14	13,247,652	39%	46	11,176	15	891.3	99.3 %	1.35 %
<i>P. couteii</i>	PMC 885.14	5,871,606	35%	137	5328	6	946.5	98.5 %	1.31 %

Table 3. The production of pigments with documented antioxidant and/or anti-inflammatory properties were investigated. Targeted annotations of BGC have been performed using Blastp with reference gene cluster and MicroScope research by keyword. “+” and “-“ indicate the detection or not of the genes.

		<i>P. raciborskii</i> PMC 877.14	<i>Laspinema</i> sp. PMC 878.14	<i>M. vaginatus</i> PMC 879.14	<i>L. martensiana</i> PMC 880.14	<i>Nostoc</i> sp. PMC 881.14	<i>Aliinostoc</i> sp. PMC 882.14	<i>L. boryana</i> PMC 883.14	<i>Calothrix</i> sp. PMC 884.14	<i>P. couteii</i> PMC 885.14
Phycobiliproteins	C-Phycocyanin	+	+	+	+	+	+	+	+	+
	Allophycocyanin	+	+	+	+	+	+	+	+	+
	Phycocerythrin	+	-	-	-	+	-	-	+	+
Scytonemin	-	-	-	-	+	+	-	+	-	-
Hydroxylated carotenoids	Cryptoxanthin	+	+	+	+	+	+	+	+	+
	Zeaxanthin	+	+	+	+	+	+	+	+	+
	Caloxanthin	-	+	-	+	-	+	+	+	-
	Nostoxanthin	-	+	-	+	-	+	+	+	-
Myxoxanthophylls	1-hydroxylycopene	+	+	+	+	+	+	+	+	+
	1 ^t -Hydroxy-γ-carotene	+	+	+	+	+	+	+	+	+
	Myxol 2 ^t -pentoside	+	+	+	+	+	+	+	+	+
	2-Hydroxymyxol 2 ^t -methylpentoside	-	+	-	+	-	+	+	+	-
Echinenone	-	+	+	+	+	+	+	+	+	+
Ketocarotenoids	Canthaxanthin	-	+	+	+	+	+	+	+	+
	Adonixanthin	-	+	+	+	+	+	+	+	+

3.2. Pigment Composition

Pigments are some of the primary metabolites that exhibit potent antioxidant and/or anti-inflammatory properties. Thus, we investigated the biosynthetic pathways of the phycobiliproteins, scytonemin, carotenoids, and chlorophylls in the nine strain genomes (Table 3).

Phycocyanin pathways were identified in all nine cyanobacteria, while the phycoerythrin genes were only found in *P. raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14, *Calothrix* sp. PMC 884.14, and *Pseudo-chroococcus couteii* PMC 885.14. Genes for the yellow-brown pigment scytonemin were detected in the genomes of PMC 881.14, *Aliinostoc* sp. PMC 882.14, and PMC 884.14. All the carotenoids biosynthetic genes were identified in *Laspinema* sp. PMC 878.14, *L. martensiana* PMC 880.14, *Aliinostoc* sp. PMC 882.14, *L. boryana* PMC 883.14, and *Calothrix* sp. PMC 884.14. *P. raciborskii* PMC 877.14 lacked the *crtO* gene, suggesting that the strain could not produce echinenone and ketocarotenoid derivatives. The *crtG* gene was not detected in PMC 877.14, PMC 879.14, PMC 881.14, or PMC 885.14, which means that the strains do not have the ability to produce the hydroxylated carotenoids caloxanthin and nostoxanthin, or the myxoxanthophyll 2-hydroxymyxol 2^t-methylpentoside.

Spectrophotometric analysis and HPLC were used to confirm pigment production (Table 4). Phycoerythrin was detected in the four strains in which the biosynthetic genes were identified. The concentration of phycoerythrin in *P. raciborskii* PMC 877.14 was 33.3 $\mu\text{g mg}^{-1}$ (dried weight, DW) compared to 9.9 $\mu\text{g mg}^{-1}$ DW for *Calothrix* sp. PMC 884.14, 3.3 $\mu\text{g mg}^{-1}$ DW for *P. couteii* PMC 885.14 and 2.8 $\mu\text{g mg}^{-1}$ DW for *Nostoc* sp. PMC 881.14. The highest concentration of phycocyanin was extracted from *P. raciborskii* at 32.5 $\mu\text{g mg}^{-1}$ DW followed by *L. boryana* at 24.9 $\mu\text{g mg}^{-1}$ DW and *Aliinostoc* sp. at 21.3 $\mu\text{g mg}^{-1}$ DW). The phycocyanin concentration in extracts of the other strains ranged from 18.4 $\mu\text{g mg}^{-1}$ DW in *Laspinema* sp. to 3.5 $\mu\text{g mg}^{-1}$ DW in *Pseudo-chroococcus couteii*. The largest allophycocyanin producers were *Aliinostoc* sp. and *L. boryana*, with 15.6 and 15.2 $\mu\text{g mg}^{-1}$ DW extracted, respectively. The allophycocyanin concentration in extracts of the other strains was lower, ranging between 7.9 $\mu\text{g mg}^{-1}$ DW for *P. raciborskii* and 2.7 $\mu\text{g mg}^{-1}$ DW for the *P. couteii* strain.

The amount of chlorophylls extracted was also quite variable among the different strains. The largest amount, 35.2 $\mu\text{g mg}^{-1}$ DW, was extracted from *P. raciborskii*, while the lowest was from *Nostoc* sp. PMC 881.14 at 1.5 $\mu\text{g mg}^{-1}$ DW.

Three different carotenoids were detected in the nine extracts. β -carotene was identified in all the strains, with concentrations ranging from 3.65 $\mu\text{g mg}^{-1}$ DW for *P. raciborskii* to 0.46 $\mu\text{g mg}^{-1}$ DW for *Calothrix* sp. and *L. martensiana*. Zeaxanthin was detected in extracts of *P. raciborskii*, *Laspinema* sp., *M. vaginatus*, *L. martensiana*, *Aliinostoc* sp., *L. boryana*, and *P. couteii*. Myxoxanthophyll-like pigments were found in six strains, but not in *M. vaginatus*, *L. boryana* and *P. couteii*.

Table 4. Pigment composition and amount ($\mu\text{g}\cdot\text{mg}^{-1}$ DW) of nine cyanobacteria isolated from Balaruc thermal mud.

Pigments	<i>P. raciborskii</i>	<i>Laspinema</i> sp.	<i>M. vaginatus</i>	<i>L. martensiana</i>	<i>Nostoc</i> sp.	<i>Aliinostoc</i> sp.	<i>L. boryana</i>	<i>Calothrix</i> sp.	<i>P. couteii</i>	
	PMC 877.14	PMC 878.14	PMC 879.14	PMC 880.14	PMC 881.14	PMC 882.14	PMC 883.14	PMC 884.14	PMC 885.14	
Carotenoids	Zeaxanthin	1.72	0.38	0.43	0.11	0.00	0.01	0.22	0.00	0.29
	Myxoxanthophyll-like	0.44	0.28	0.00	0.12	0.03	0.32	0.00	0.21	0.00
	β -Carotene	3.65	1.43	1.06	0.46	0.15	1.29	1.31	0.46	0.78
Chlorophylls	Bacteriochlorophyll a	0.54	0.04	0.12	0.04	0.06	0.08	0.07	0.07	0.02
	Chlorophyll a	35.2	9.8	11.2	4.9	1.5	12.3	17.1	9.7	6.7
	Phaeophytin a	0.00	0.09	0.07	0.02	0.01	0.00	0.09	0.04	0.00
Phycobiliproteins	Phycocyanin	32.5 +/- 12.8	18.4 +/- 0.2	12.2 +/- 2.1	6.1 +/- 0.5	2.1 +/- 0.1	21.3 +/- 4.7	24.9 +/- 4.4	9.9 +/- 0.4	3.5 +/- 0.1
	Allophycocyanin	7.9 +/- 3.5	6.6 +/- 0.5	5.8 +/- 1.1	3.7 +/- 0.3	1.5 +/- 0.2	15.6 +/- 3.5	15.2 +/- 3.8	6.7 +/- 0.3	2.7 +/- 0.2
	Phycocerythrin	33.3 +/- 12.1	0.00	0.00	0.00	2.8 +/- 0.2	0.00	0.00	9.9 +/- 0.3	3.3 +/- 0.1

Table 5. Specialized metabolites biosynthetic gene clusters (BGCs) from the nine cyanobacterial strains isolated from Balaruc thermal mud. Automatic annotations have been performed using the MicroScope platform (computing AntiSMASH and MIBiG softwares). *Some clusters have been predicted at the end/start of a contig and are questionable regarding their completeness.

Genus / species	Strain	Nb of clusters predicted	Identified clusters	Reported activity	Unknown/homologous cluster	Unknown/unknown cluster
<i>P. raciborskii</i>	PMC 877.14	7	Phytoene or derivative	Antioxidant	1	5
<i>Laspinema</i> sp.	PMC 878.14	7	Phytoene or derivative	Antioxidant	5	0
			Heterocyclic cyanobactin	Cytotoxic		
<i>M. vaginatus</i>	PMC 879.14	7	Phytoene or derivative	Antioxidant	3	3
<i>L. martensiana</i>	PMC 880.14	9	Phytoene or derivative	Antioxidant	5	1
			Squalene	Antioxidant		
			Schizokinen-like	Iron-chelating activity		
<i>Nostoc</i> sp.	PMC 881.14	20*	Phytoene or derivative (2 x)	Antioxidant	7	10
			Heterocyst glycolipid	n.d.		
<i>Aliinostoc</i> sp.	PMC 882.14	21	Shinorine-like	Antioxidant	6	8
			Microginin-like	ACE inhibition; no cytotoxic		
			Amycomycin-like	Antibiotic		
			Phytoene or derivative (2 x)	Antioxidant		
			Heterocyst glycolipid	n.d.		
			Microviridin-like	Protease inhibitor		
<i>L. boryana</i>	PMC 883.14	16	Phytoene or derivative (5 x)	Antioxidant	8	3
<i>Calothrix</i> sp.	PMC 884.14	27	Cyanobactin	Cytotoxic	14	6
			Mycosporine-glycine-like	Antioxidant		
			Cylindrocyclophane-like	Cytotoxic; antibacterial		
			Phytoene/Lycopene or derivative (3 x)	Antioxidant		
			Heterocyst glycolipid	n.d.		
<i>P. couteii</i>	PMC 885.14	10	Phytoene or derivative (2 x)	Antioxidant	2	6
Total		124	31		51	42

3.3. Specialized Metabolites

Following the analysis of the nine assembled genomes, 124 specialized metabolite biosynthetic gene clusters (BGCs) were retrieved (Table 5 and Tables S1–S9). The numbers of BGCs per strain varied from 7 to 27. Among these 124 BGCs, only 31 were correctly predicted to have the molecule produced by the cluster, representing 24.4% reliable BGC annotation (Table 5, “Identified Clusters” column). The largest number of annotations was obtained for *Aliinostoc* sp. PMC 882.14 and *Calothrix* sp. PMC 884.14, in which the production of six and five metabolites, respectively, was accurately predicted. Among the nine strains, those belonging to the Nostocales order have been shown to present the highest number of BGCs compared to other strains (Table 5). This observation is consistent with the work of Shih and co-workers who showed that heterocytic cyanobacteria tend to exhibit a higher number of BGCs [7]. Automatic annotations accurately predicted the phytoene biosynthetic pathway in all nine cyanobacterial strains, and the pathways of the mycosporine-like amino acids shinorine and mycosporine-glycine for *Aliinostoc* sp. PMC 882.14 and *Calothrix* sp. PMC 884.14. The other BGCs exhibited mostly inaccurate predictions (see Tables S1–S9 for automatic prediction results and detailed organization of the BGCs).

Expert manual annotation also allowed the identification of a schizokinen-like biosynthetic cluster in *L. martensiana* PMC 880.14, from one BGC automatically annotated as siderophore. Heterocyst glycolipid BGCs were identified in the three Nostocales strains (*Nostoc* sp. PMC 881.14, *Aliinostoc* sp. PMC 882.14 and *Calothrix* sp. PMC 884.14). The BGC of ribosomally synthesized peptides with post-translational modifications (RiPPs) of cyanobactins was identified in two strains: *Laspinema* sp. PMC 878.14 and *Calothrix* sp. PMC 884.14. Another RiPP, microviridin, was characterized from *Aliinostoc* sp. PMC 882.14, and shared strong homology with the microviridin K biosynthetic gene cluster. The type I polyketide synthetase (PKS) of amycomycin has been proposed for a PKS gene cluster from *Aliinostoc* sp. PMC 882.14. The cylindrocyclophane-like gene cluster has been identified from an NRPS cluster in *Calothrix* sp. PMC 884.14. Our manual expert annotation also allowed the identification of a microginin BGC from a hybrid NRPS-PKS automatically annotated as the puwainaphycin cluster in *Aliinostoc* sp. PMC 882.14. No cyanotoxin (microcystins, anatoxins, cylindrospermopsins, saxitoxins, etc.) gene clusters were identified in any of the strains. Among the 93 unknown clusters, 51 showed homologous matches with clusters identified in other strains, without characterization of the molecule synthesized, while 42 were fully uncharacterized.

The metabolites putatively identified by the genomic approach were also subjected to LC-MS/MS analysis. A dataset of 3986 analytes was obtained, with their respective intensity in the different extracts (water or methanol) and their respective fragmentation pattern. The molecular network assembled from the analyzed MS/MS is presented in Figure 1 and Figure S2. This type of representation makes it possible to group together analytes in clusters that sharing similarities in their fragmentation spectra and therefore have structural similarities. In these figures, one node represents one analyte with its own mass and fragmentation spectra. The molecular networking performed with the GNPS algorithm allowed us to group 1155 analytes (29%) into 128 clusters containing at least three different analytes. The remaining analytes were classified in pairs or singletons. In Figure 1, nodes have been colored according to the strain from which they were identified. Figure S2 represents the same analysis while nodes have been colored according to their original fraction (water or methanol). Most of the analytes were observed in several strain extracts (Figure 1A) while some were identified exclusively in a single strain (Figure 1B). The same pattern was identified at the cluster level, with several clusters containing multi-species analytes (Figure 1C) and almost 50 strain-specific clusters (Figure 1D). Comparisons with public and in-house spectral MS/MS databases together with the molecular formula database of more than 2100 cyanobacterial metabolites (CyanoMetDB) allowed the

prospective annotation of 193 of those analytes (Figure 1 and Table S10). Primary metabolites have most often been identified, including diverse dipeptides, ribonucleosides, and cofactors (NAD⁺), as well as glycerolipids, polysaccharides, lysophosphatidylcholines and lysophosphoethanolamines. Several mycosporine-like amino acids (MAAs) were also characterized with specific matches to shinorine, mycosporine-glycine and palythine. A few specialized cyanobacterial metabolites were identified, notably aeruginosin-like compounds, anabaenolysins, largamides, microphycins, microviridins, and microginins. According to the propagation principle, one would expect that analytes from the same clusters should belong to the same chemical family.

The annotation details for the targeted list of metabolites of interest from the nine cyanobacterial strains, selected for their potential bioactivities, are given in Table 6. Cyanobacterial pigments are well known for their antioxidant and/or anti-inflammatory properties, and several metabolites with potentially similar bioactivities were identified. Several different MAAs, known to be antioxidant metabolites, were detected in seven strains. Mycosporine-glycine was annotated for PMC 882.14 and PMC 884.14. Shinorine has been detected in *Aliinostoc* sp. PMC 882.14, implying a remarkable antioxidant potential for these two cyanobacteria. One congener of the anti-inflammatory aeruginosin family, aeruginosin TR642, was also annotated in *L. boryana* PMC 883.14, highlighting the anti-inflammatory potential of this strain.

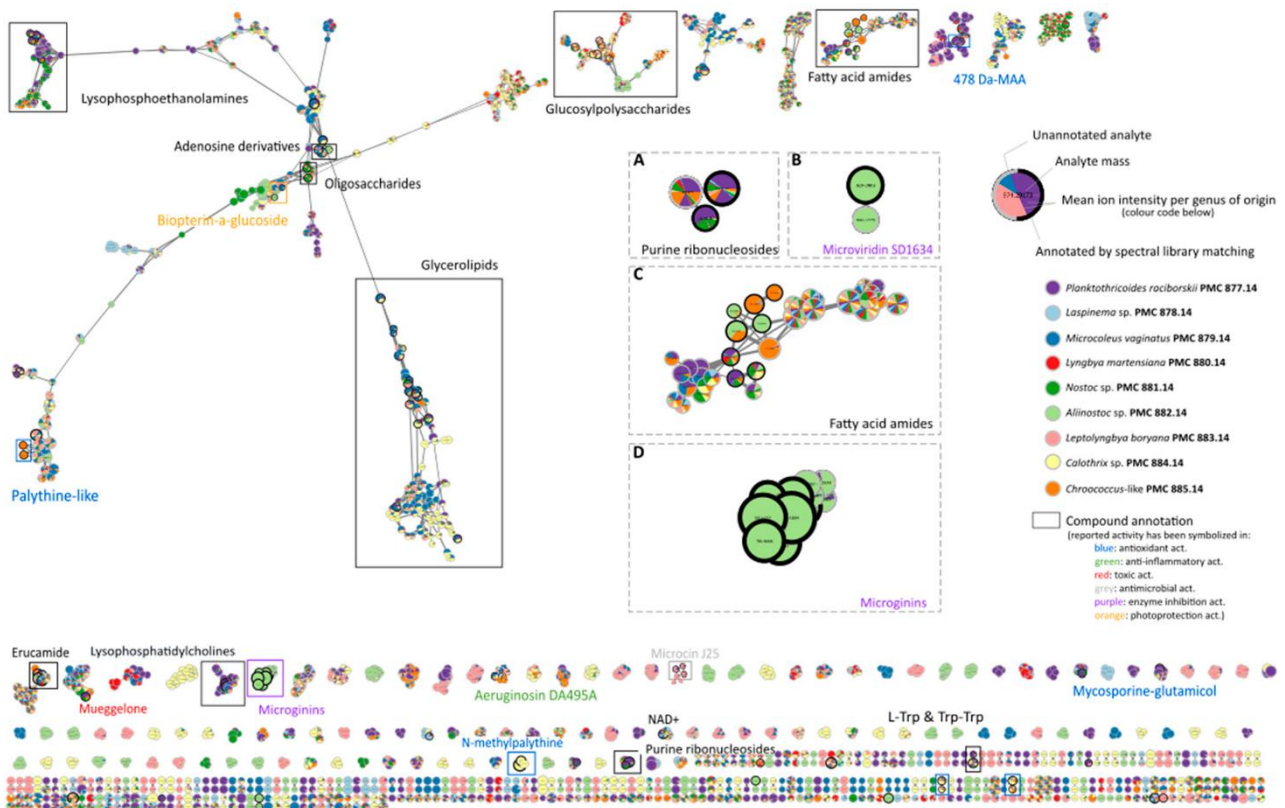


Figure 1. Molecular network of the metabolites produced by nine cyanobacteria isolated from Balaruc's thermal mud. Annotations obtained by untargeted analysis are figured inboxes. Nodes have been colored according to the strain in which they have been identified. (A) Purine ribonucleosides cluster; (B) Microviridins cluster; (C) Fatty acid amides cluster; (D) Microginins cluster.

Table 6. Potential antioxidant and anti-inflammatory compounds identified by HPLC-DAD or LC-MS/MS in the extracts of the nine cyanobacteria strains isolated from Balaruc's thermal mud. MAAs: Mycosporine-like Amino Acids. "+" and "-" indicate the detection or not of the gene clusters.

Class	Compound	<i>P. raciborskii</i> PMC 877.14	<i>Laspinema</i> sp. PMC 878.14	<i>M. vaginatus</i> PMC 879.14	<i>L. martensiana</i> PMC 880.14	<i>Nostoc</i> sp. PMC 881.14	<i>Aliinosoc</i> sp. PMC 882.14	<i>L. boryana</i> PMC 883.14	<i>Calothrix</i> sp. PMC 884.14	<i>P. couteii</i> PMC 885.14
MAAs (antioxidant)	Mycosporine-glycine	-	-	-	-	-	+	-	+	-
	Mycosporine-ornithine	-	-	-	-	+	-	-	-	-
	Nostoc-756	-	-	-	-	+	-	-	-	-
	Shinorine	-	-	-	-	-	+	-	-	-
Carotenoids (antioxidant)	Canthaxanthin	-	-	+	+	-	+	+	+	+
	Myxoxanthophyll-like	+	+	-	+	-	+	-	+	-
	Zeaxanthin	+	+	+	+	-	-	+	-	+
	β -carotene	+	+	+	+	+	+	+	+	+
Chlorophylls (antioxidant)	Chlorophyll a	+	+	+	+	+	+	+	+	+
	Bacteriochlorophyll a	+	-	+	-	-	-	-	-	-
Phycobiliproteins (antioxidant)	Phycocyanin (Anti-inflammatory)	+	+	+	+	+	+	+	+	+
	Allophycocyanin	+	+	+	+	+	+	+	+	+
	Phycoerythrin	+	-	-	-	+	-	-	+	+
	Phycoerythrobilin	-	-	-	-	-	-	-	+	+
Aeruginosins (anti-inflammatory)	Aeruginosin TR642	-	-	-	-	-	-	+	-	-
	Antioxidant	8	6	7	7	7	8	6	9	8
	Anti-inflammatory	1	1	1	1	1	1	2	1	1

3.4. Bioactivity Assessment

Specific bioassays were done to determine the antioxidant, anti-inflammatory and wound healing activities of the cyanobacterial extracts. Both aqueous and methanol extracts were tested at concentrations of 1, 5, 10, and 50 $\mu\text{g mL}^{-1}$ (Table 7).

Antioxidant activity was evaluated by measuring the effect of crude extracts on the amount of the reactive oxygen species, nitric oxide (NO) produced by the murine macrophage cell line, RAW 264.7, subjected to oxidative stress by exposure to lipopolysaccharides (LPS). NO is evolved during normal oxidative metabolism, but an excess of free radicals can cause cell damage and overwhelm protective antioxidant enzymes. A positive antioxidant effect was recorded when an extract reduced the amount of NO produced comparable to the negative control. No significant inhibition of NO formation was seen at the extract concentrations tested. A slight increase in NO production (+20%) was observed for the aqueous extract of *Nostoc* sp. PMC 881.14 at 50 $\mu\text{g mL}^{-1}$ (Table 7), suggesting that this extract might have pro-oxidant capabilities.

Table 7. Antioxidant, anti-inflammatory, and wound-healing activities detected for methanol and aqueous extracts of Balaruc cyanobacteria. - represents no significant effect; - x%, significant inhibiting effect percentage; + x%, significant increasing effect percentage.

		<i>P. raciborskii</i> PMC 877.14	<i>Laspinema</i> sp. PMC 878.14	<i>M. vaginatus</i> PMC 879.14	<i>L. martensiana</i> PMC 880.14	<i>Nostoc</i> sp. PMC 881.14	<i>Aliinostoc</i> sp. PMC 882.14	<i>L. boryana</i> PMC 883.14	<i>Calothrix</i> sp. PMC 884.14	<i>P. couteii</i> PMC 885.14		
Bioassay	µg.mL ⁻¹	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O	MeOH H ₂ O		
NO secretion RAW 264.7 (antioxidant)	1	-	-	-	-	-	-	-	-	-		
	5	-	-	-	-	-	-	-	-	-		
	10	-	-	-	-	-	-	-	-	-		
	50	-	-	-	-	-	+20 +/-2	-	-	-		
Keratinocytes migration HaCaT (wound healing)	1	-	-	-	-	-	-	-	-	-		
	5	-	-	-	-	-	-	-	-	-		
	10	-	-	-	-	-	+22 +/-7	-	-	-		
	50	-	-	-	-	-	-	-	-	-		
Cytokines secretion PBMC (anti-inflammatory)	1	TNF α	-	-	-	-	-	-	-	-	-	
		IL-1β	-	-	-	-	-	-	-	-	-	
		IL-6	-	-	-	-	-	-	-	-	-	
		IL-8	-	-	-	-	-	-	-	-	-	
	5	TNF α	-	-	-	-	-	-	-	-	-	
		IL-1β	-	-20 +/-5	-	-	+34 +/-3	-	+50 +/-8	-	+26 +/-3	
		IL-6	-	-	-	-	-	-	-	-	-27 +/-7	
		IL-8	-	-	-	-	-	-	-	-	-	
	10	TNF α	-	-	-	-	-18 +/-4	-20 +/-4	-	-	-	
		IL-1β	-	-28 +/-2	-	+23 +/-4	+49 +/-6	-	-26 +/-4	+52 +/-7	-	+30 +/-3
		IL-6	-	-	-	-	-	-	-	-	-	-27 +/-4
		IL-8	-	-	-	-	-	-	-	-	-	-
50	TNF α	-19 +/-6	-	-	-	-20 +/-1	-	-	-	-	-	
	IL-1β	-39 +/-9	-57 +/-3	-	+29 +/-3	+61 +/-2	-	+18 +/-3	-45 +/-5	+60 +/-4	-	+26 +/-5
	IL-6	-	-	-	-26 +/-5	-	-	-	-	-23 +/-4	-	-
	IL-8	-	-	-	-	-	-	-	-	-	-	-

The anti-inflammatory properties have been evaluated by measuring specific cytokine secretion by immune cells (PBMCs) exposed to LPS. The secretion of four cytokines, involved in upregulation of the inflammatory response, were assayed: TNF- α , IL-1 β , IL-6, and IL-8 [42,43]. Several aqueous or methanol extracts showed effects on cytokine release, with the majority occurring at the higher concentrations of 10 $\mu\text{g mL}^{-1}$ DW and 50 $\mu\text{g mL}^{-1}$ DW (Table 7). Some of the responses were proinflammatory with an increase in cytokine production, while others showed anti-inflammatory activity and decreased cytokine secretion. Distinct dissimilarities in the cytokine responses were observed in several strains, notably for the aqueous extracts of *Aliinostoc* sp. PMC 882.14 that enhanced IL-1 β secretion and inhibited IL-6 secretion at 50 $\mu\text{g mL}^{-1}$. The methanol extract of *P. raciborskii* PMC

877.14 and the aqueous extract of *P. couteii* PMC 885.14 had the best anti-inflammatory properties as they each simultaneously inhibited the release of two cytokines involved in proinflammatory responses – TNF- α and IL-1 β , and IL-1 β and IL-6 respectively. The greatest inhibition of cytokine secretion was observed with the aqueous extract of PMC 877.14 with a 57% decrease of IL-1 β , compared to the negative control.

The wound-healing activity of the crude cyanobacterial extracts was also evaluated by monitoring the cellular migration of the keratinocyte cell line, HaCaT, for 48 h following injury to the monolayer. The aqueous extract of *Aliinostoc* sp. PMC 882.14 at 10 $\mu\text{g mL}^{-1}$ slightly increased keratinocyte migration by 22% 7 compared to the negative DMSO control, suggesting a beneficial effect of this extract on wound healing (Table 7).

Cell viability was determined on the three cell types used in the bioassays: murine macrophages (RAW 264.7), human keratinocytes (HaCaT), and peripheral blood mononuclear cells (PBMC) (Table S11). This assay has allowed us to assess the viability of the respective cell types used in the bioassays, as well as to evaluate the potential cytotoxicity of the crude extract on these different cell types. No loss of viability of HaCaT human cell line and human PBMC was observed. Murine macrophages RAW 264.7 were apparently more sensitive and showed a slight loss of viability.

4. Discussion

4.1. Genome Quality, Genome Mining, and Molecular Network Analysis

Application of the genome mining approach led to the identification and characterization of biosynthetic gene clusters (BGCs) for natural products. This approach requires high-quality genomic data to confidently annotate the BGCs and excludes doubtful affiliations, especially in the context of genome sequencing and assembly with meta-genomes generated from nonaxenic strains [44]. The median size of the nine cyanobacterial genomes was 7.3 Mb, which is higher than the 5.6 Mb median size of the cyanobacterial genomes referenced in GenBank [45]. However, Alvarenga and co-workers [46] showed that the latter value was biased by the high number of *Prochlorococcus* genomes available wherein the size was < 3 Mb. The GC percentage of our nine genomes (42%) was comparable to reference cyanobacterial genomes, already available on public database such as NCBI's.

The high completeness scores (>97%) obtained for the nine genome assemblies led us to conclude that the draft genomes for all

strains were nearly, if not totally, complete. The low contamination score (<1.4%) indicates that our double binning strategy, which was based on both BLAST and K-mer contig clustering, gives reliable results for obtaining uncontaminated cyanobacterial genomes from nonaxenic monoclonal cultures. According to Parks and co-workers, who developed the CheckM tool, the values of these two scores indicate high quality for the nine cyanobacterial genome assemblies obtained from metagenomic data [47]. Automated tools, such as antiSMASH, together with experts' participation, allowed the annotation of 24% of the 124 identified BGCs. BGCs without annotations were considered orphan gene clusters for which the synthesized metabolite was unknown. However, 52% of these orphan clusters had high homologies with other orphan clusters already observed in other cyanobacteria. This opens up the possibility of global comparative studies of these different strains characterizing their metabolites, as was done for the cyanobactin biosynthesis pathway [48].

4.2. Cytotoxic Properties

We investigated the potential cytotoxicity of the different extracts on the RAW 264.7, HaCat cell lines and on human PBMCs. No significant cytotoxicity was detected on the two last cell types while a slight loss of viability was observed on the more sensitive murine cell line (RAW 264.7) with some extracts. Overall, no cyanotoxins, such as microcystins, anatoxins, saxitoxins, cylindrospermopsins, or nodularins, were identified in the crude extracts and no cyanotoxin BGCs were detected in the nine genome assemblies, suggesting that the nine strains did not present a toxicity risk and are suitable for therapeutic purposes. However, two potential cyanobactin BGCs were identified in *Laspinema* sp. PMC 878.14 and *Calothrix* sp. PMC 884.14. The two clusters represented one gene encoding a heterocyclase enzyme. This may mean that the cyanobactins produced by these strains belong to the group of heterocyclized cyanobactins, which includes patellamides, tenuencyclamides, trichamide, aeruginosamide, and others [11] with potentially cytotoxic activities [49,50]. However, not all cyanobactins exhibit cytotoxic activity and no known cyanobactin compounds were identified in crude extracts of the two strains.

4.3. Antioxidant Properties

Antioxidants have been studied for many years as novel treatments for diseases such as atherosclerosis, osteoarthritis, and neurodegenerative disorders and have been generally recognized as compounds that promote well-being [51]. Pigmented compounds are produced by all cyanobacteria and have shown potentially beneficial properties [52–54]. Biosynthetic genes for phycocyanins and chlorophylls have been detected in the nine cyanobacteria, but their relative amounts varied considerably among the different strains. *Calothrix* sp. PMC 884.14, *L. martensiana* PMC 880.14, *P. couteii* PMC 885.14, and *Nostoc* sp. PMC 881.14 possessed a thick sheath and abundant mucilage that may have affected the pigment extraction efficiency or have biased the measurement of pigment mass by increasing the apparent lyophilisate weight or reducing the number and volume of extracted cells. These observations are consistent with the conclusions of Finkel and co-workers who demonstrated phylogenetic difference in macromolecular composition, notably of chlorophyll *a*

[55]. Phycoerythrin genes have been identified in *P. raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14, *Calothrix* sp. PMC 884.14, and *P. coutteii* PMC 885.14. Phycoerythrin was detected and quantified in these four strains and appeared to be produced constitutively. Carotenoids pathways have also been characterized in the nine assemblies according to the pathways proposed by Kosourov et al. (2016). These authors described sixteen genes coding for enzymes involved in the synthesis of hydroxylated carotenoids, ketocarotenoids, echinenone, and myxoxanthophylls [56]. Of the known carotenoids described in cyanobacteria [57,58], only four have been identified in this study: zeaxanthin, canthaxanthin, myxoxanthophyll-like molecules, and β -carotene (Table 4). Only this last has been detected in all nine strains. Regarding the others, several reasons could explain their non-detection. Some strains did not possess all the genes necessary for the synthesis of certain carotenoids. This is the case for *P. raciborskii* PMC 877.14 which lacks the *crtO* gene encoding a β -carotene ketolase required for canthaxanthin synthesis. This enzyme catalyzes the transformation of β -carotene into echinenone and echinenone into canthaxanthin; thus, this strain is unable to produce canthaxanthin. For the strains possessing all carotenoid biosynthetic genes but appearing to lack carotenoid, it is possible that the amount extracted from the strains is below the detection limit or that some regulatory processes for intermediary products in the linear pathway are inhibiting carotenoid biosynthesis.

Specialized metabolites with potential antioxidant properties have also been detected in the cyanobacterial extracts. Mycosporine-like amino acids (MAAs) are a family of small metabolites identified in fungi, plants, and bacteria, including cyanobacteria, and showing antioxidant and photo-protective activities [59]. BGCs for MAAs have been characterized by automatic annotation tools from *Aliinostoc* sp. PMC 882.14 and *Calothrix* sp. PMC 884.14. Both BGCs contained one demethyl-4-deoxygadusol synthase, one O-methyltransferase, one ATP-grasp domain and one NRPS module described for synthesis of MAAs [60,61]. Both strains produced mycosporine-glycine, the first molecule synthesized from the 4-deoxygadusol precursor. Only PMC 882.14 had the ability to produce shinorine, as this metabolite requires serine incorporation brought about by one adenylation domain. Manual exploration of the *Nostoc* sp. PMC 881.14 genome identified a MAAs gene cluster containing one D-Ala-D-Ala ligase replacing the NRPS gene. This gene cluster organization was similar to that described for *Cylindrospermum stagnale* PCC 7417 [61,62]. While 4-deoxygadusol (4-DG) in PMC 881.14 was synthesized from the shikimate pathway instead of the pentose phosphate pathway, mycosporine-ornithine was detected as in PCC7417.

Unexpectedly, no effects on nitric oxide production were observed for any of the strains despite the antioxidant potential of their pigments and specialized metabolites (Table 6). This absence of effect could be due to low concentration of these pigment molecules in the crude extracts or the potential antagonistic effect of specific inhibitors. Cyanobacteria could also produce oxidative compounds as found for the *Nostoc* sp. PMC 881.14 aqueous extract, in which the cellular oxidative potential increased 20%. This increased NO formation could be caused by the lipopolysaccharide (LPS) content of this strain, even though cyanobacterial LPS is said to be a weak inducer of oxidant activity compared to other Gram-negative bacteria [63,64].

4.4. Wound-Healing Properties

Cell migration is involved in many diseases such as for metastasis and the development of atherosclerosis and arthritis, but is also necessary for normal physiological processes like keratinocyte migration in wound healing or the translocation of leukocytes in the inflammatory response and immune functions [65,66]. The *Aliinostoc* sp. PMC 882.14 extract stimulated slightly keratinocyte migration on the HaCaT cell line. Several cyanobacterial extracts have been studied in recent years for their potential wound-healing effects [67,68], but the mechanism of action of most of these compounds remains unclear. The aqueous extract of *Aliinostoc* sp. PMC 882.14 exhibited a wound healing effect, but did not promote Il-6 secretion. We hypothesized that this mechanism is different from the process observed in *Synechococcus elongatus* PMC 7942 [69], but could be more similar to pathways described for apratyramide, as it induced the secretion of growth factor [70]. Apratyramide, isolated from *Moorea bouillonii*, is a linear depsipeptide of five residues with two terminal N-methylated tyrosines. This terminal organization is quite similar to that of the microginin family [71] and a microginin BGC was identified and several congeners characterized by molecular networking in *Aliinostoc* sp. PMC 882.14 (Figure 1) suggesting the possibility that microginins could be involved in the observed wound healing activity as they share the same tyrosine pattern as apratyramide. Microginins have also been described as protease inhibitors [72–74] and angiotensin converting enzyme inhibitors [75,76]. Phlebitis and other vascular diseases have been cured in the thermal baths of Balaruc-les-Bains since ancient times, and the microginins produced by *Aliinostoc* sp. PMC 882.14 could be involved in these therapeutic properties.

4.5. Anti-Inflammatory Properties

Inflammation is a normal immune process involving a complex network of cells, regulatory pathways and pro- and anti-inflammatory molecular activities to balance the immune response. Defects in immunoregulation can lead to chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, multiple sclerosis, and others [77,78]. Proinflammatory cytokine responses to the crude extracts showed different profiles depending on the strain tested (Table 7). Some extracts showed complex patterns with increasing secretion of one cytokine concomitant with decreased release of another cytokine, making it difficult to draw conclusions about their respective global anti-inflammatory activity. Nevertheless, three strains showed promising anti-inflammatory effects by inhibiting the production of one or more cytokines. Both methanol and water extract of *P. raciborskii* PMC 877.14 showed decreased levels of TNF- α and Il-1 β . As PMC 877.14 is the most important phycocyanin producer of the nine strains from Balaruc-les-Bains, anti-inflammatory properties of the aqueous extract could be attributed to the phycobilin pigment. The aqueous extract of *P. couteii* PMC 885.14 inhibited the secretion of Il-1 β and Il-6. As only low levels of phycocyanin were extracted from this strain, it is likely that another molecule is responsible for this activity. In a recent paper on the therapeutic properties of *Phormidium* sp. ETS05 from thermal mud in Italy, Zampieri et al. reported that hydrosoluble exopolysaccharides demonstrated anti-inflammatory properties without toxicity [23]. The same macromolecules could be responsible for the anti-inflammatory properties of *Pseudo-chroococcus couteii*, which possesses a thick

polysaccharide sheath [27].

Scytonemin BGC has been detected in PMC 881.14. Scytonemin is a lipid-soluble, yellow-brown pigment with anti-inflammatory activity [79]. Unfortunately, we were not able to detect scytonemin or its congeners by our metabolomic analysis and this absence, or non-detection, has been attributed to unfavorable culture conditions for scytonemin production, as scytonemin synthesis has been shown to be upregulated by UV-A exposure [60,79]. As scytonemin could not be responsible for the anti-inflammatory properties of methanol extracts of PMC 881.14 and PMC 877.14, we hypothesized that other molecules must be responsible for the anti-inflammatory properties. Potential candidates could be lipid-derived products as they represent the biggest cluster in our network. This explanation is supported by research from Bruno et al. (2005) who described the anti-inflammatory activities of several galactosyldiacyl-glycerol compounds [80].

4.6. General Comments

The various results obtained in the course of this study open up interesting perspectives in different fields of application and provide insights into the therapeutic mechanisms of thermal mud. In fact, it has been shown that both the “inorganic” and “organic” parts of the peloids confer therapeutic properties to thermal cures [81,82]. To go further in understanding these mechanisms for the thermal mud of Balaruc-les-Bains specifically, it would be interesting to study the metabolome of the strains and their bioactivities under other conditions, including co-culture experiments with the mud/thermal water mixture. This experiment would make it possible to mimic the natural maturation conditions of the peloid and would make it possible to study the whole metabolome and bioactivities of the thermal mud flora as has been done for other thermal baths [83,84]. Moreover, several studies have shown that the metabolome of cyanobacteria varies under co-culture conditions [85,86], allowing the hypothesis that bioactivities (including the antioxidant activity) could be enhanced by synergistic interactions.

While the cyanobacterial extracts did not show cytotoxicity to the cell's types tested, it might be advisable that we tested them against several other cell lines. Because of their transporters, some cell lines could be more sensible to cyanobacterial peptides, which need to be transported into the cell to be toxic. This is the case, for example, of microcystins, which are internalized by liver cells thanks to transporters specific to certain cellular tissues [87]. Beyond the applications sought for balneology, the metabolites identified within the framework of this study could potentially be used in other fields such as cosmetics or pharmaceuticals. It would therefore be interesting to go further in the characterization of the structures and the bioactivities of the whole metabolites. Indeed, cytotoxic compounds, such as cyanobactins or cylindrocyclophanes, have to be investigated in order to develop anticancer drugs when protease-inhibiting molecules (such as microviridins and microginins) can be used in the treatment of vascular diseases, hypertension, or infectious diseases [4].

For future experiments, it could be interesting to purify certain compounds and characterize their structures by HPLC and NMR spectrometry, to characterize their individual activities by other

bioassays (other antioxidant tests, protease inhibition tests) and to follow their production kinetics during the different growth phases of the cyanobacteria strains of interest.

5. Conclusions

Cyanobacteria are prolific producers of natural compounds exhibiting a wide range of chemical structures and activities [4,5]. Although their biological functions remain largely unknown, some compounds have demonstrated their usefulness in pharmacology such as dolastatin 10 for the treatment of lymphoma in Hodgkin's disease [15,88–90]. Here, we investigated the potential beneficial activities of nine cyanobacteria isolated from the thermal mud of Balaruc-les-Bains, France, in terms of the production of antioxidant and/or anti-inflammatory compounds using genomic, metabolomic, and bioactivity analyses. With only 24% of the biosynthetic gene clusters annotated, Balaruc's cyanobacteria constitute a rich source of potentially valuable natural products. The nine strains, which do not express harmful cyanotoxins and did not show cytotoxicity against two human cell types, exhibited beneficial anti-inflammatory and wound-healing properties possibly arising from the identified pigments and specialized metabolites. Although no antioxidant activity was detected, strong anti-inflammatory potential was proven for *Planktothricoides raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14, and *Pseudo-chroococcus couteii* PMC 885.14, and a slight wound-healing function was detected in extracts from *Aliinostoc* sp. PMC 882.14. Antioxidant properties still need to be studied as several strains showed high production of antioxidant pigments, while our assays failed to detect activity. Nevertheless, our study shows a promising potential in further developments for Balaruc's cyanobacteria and opens avenues of future work for applications of the active biomolecules, improvement of their production, and the resolution of their mechanism of action.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2218-273 X/11/1/28/s1>: Figure S1. Consensus phylogenetic tree based on 16S rRNA gene sequences; Figure S2. Molecular network (coloured by extraction methods); Tables S1–S9. BGCs description from the nine strains; Table S10. Pigment biosynthetic genes from the nine strains; Table S11. Results of the cell viability assays; Tables S12–S20. BGCs from associated heterotrophic bacteria.

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Chapitre IV

Régulation de la production de
métabolites en fonction des conditions de
culture

Chapitre IV

REGULATION DE LA PRODUCTION DE METABOLITES EN FONCTION DES CONDITIONS DE CULTURE : *ALIINOSTOC* SP. PMC 882.14

IV.1. Contexte de l'étude

Avant même la production à une échelle industrielle de molécules bioactives à partir de cyanobactéries, il est nécessaire de comprendre la production de la molécule pendant les différentes phases de croissance, en relation avec la physiologie cellulaire. De nombreux facteurs influencent la physiologie cellulaire des cyanobactéries (*e.g.* lumière, température, concentration en nutriments) et influencent, par conséquent, la composition de leurs métabolomes⁶. Par exemple, Puddick et al. 2016 ont démontré qu'une absence d'azote dans le milieu de culture induisait un changement dans la production des variants de microcystines produits par *Microcystis* CAWBG11. En effet, ces conditions de culture favorisent les variants ne contenant pas d'arginine, un acide aminé riche en atome d'azote, ce qui suppose une adaptation dans la sélectivité des synthétases NRPS en fonction des résidus disponibles pour la construction des cyanopeptides (Puddick et al. 2016). D'autres auteurs ont également montré que la production des microcystines- LR, -RR et -YR évoluait en fonction de la croissance des cellules. Les auteurs ont montré que la quantité totale de microcystines était corrélée avec la quantité d'ADN pendant la croissance et que la concentration de microcystines augmentait au moment où la majorité des cellules entraient dans les phases de division cellulaire (Kameyama et al. 2004). A l'échelle du métabolome, Barofsky et al., 2009 ont montré que, chez certaines diatomées, la production de métabolites diffère en fonction des phases de croissance (exponentielle, stationnaire et sénescence). Tous les métabolites n'ont pas le même profil de synthèse, certains étant spécifiques de chacune des phases de croissance et d'autres étant synthétisés constitutivement (Barofsky et al. 2009).

Dans ce contexte, nous nous sommes demandé quel pouvait être l'impact de la phase de croissance et des conditions de culture (quantité lumineuse et température) sur le métabolome des cyanobactéries issues des boues thermales de Balaruc-les-Bains. Afin de répondre à cette question, des travaux préliminaires ont été réalisés par Damien Le Moigne dans le cadre de son stage de master 2 sous mon co-encadrement. Les objectifs étaient de (i) réaliser un suivi cinétique du métabolome de *Aliinostoc* sp. PMC 882.14 issue des boues thermales de Balaruc-les-Bains puis (ii) d'évaluer l'effet des conditions de culture (intensité lumineuse, température) sur le métabolome (Le Moigne 2020). Les résultats obtenus ont permis la rédaction de l'article 4 présenté dans ce chapitre dans le but de répondre à la quatrième question de cette thèse : *quelle est la cinétique de production des métabolites produits par Aliinostoc sp. PMC 882.14 ?*

⁶ L'ensemble des métabolites produits

IV.2. Résumé

Aliinostoc sp. PMC 882.14 est une cyanobactérie issue des boues thermales des Thermes de Balaruc-les-Bains, sélectionnée suite à l'identification sur son génome de 21 îlots de gènes de biosynthèse de métabolites spécialisés (**Chap. 3, Article 3**). Des métabolites tels que la shinorine et la scytonémine ont été identifiés avec des propriétés antioxydantes et anti-inflammatoires. L'évolution globale du métabolome (intracellulaire et extracellulaire) a été suivie au cours d'une cinétique de culture (28 jours) en condition contrôle (milieu Z8, 24°C, 12 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$), une intensité lumineuse (Z8, 24°C, 24 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$), et une température (Z8, 28°C, 12 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) plus élevées.

Conformément aux travaux précédents (**Chap. 3, Article 3**), une extraction aqueuse et une extraction au méthanol ont été utilisées et analysées par LC-MS et LC-MS/MS afin d'analyser le plus grand nombre de métabolites synthétisés. Plusieurs traitements statistiques ont été appliqués afin d'étudier les variations du métabolome (PCA, PLS-DA) et d'identifier les métabolites dont la production variait le plus au cours de la cinétique (MEBA, ANOVA).

The variation of the regulation of cyanobacterial (*Aliinostoc* sp. PMC 882.14) metabolite production is responsive to light intensity and temperature culture conditions

Justine Demay^{1,2*}, Damien Le Moigne^{1*}, Anita Reinhardt², Cécile Bernard¹, Sandra Kim Tiam¹, Benjamin Marie^{1,**}
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Différentes phases de cultures ont été mises en évidence au cours des 28 jours de l'expérience ainsi que la diversité de métabolites produits par *Aliinostoc* sp. PMC 882.14 au cours de ces phases. Parmi cette diversité plusieurs métabolites du métabolisme primaire tel que des dipeptides, des nucléosides et les acides gras ont été identifiés ainsi que des métabolites spécialisés comme les mycosporine-like amino acids (MAAs), des somamides, des microviridines et des microginines.

La forte variation du métabolome mise en évidence est celle en relation avec les différentes phases de croissance de la culture (exponentielle et stationnaire) avec une production majoritaire de la plupart des métabolites dans le compartiment intracellulaire, quelle que soit la phase de croissance. En condition contrôle, plusieurs métabolites appartenant aux mêmes familles montrent des profils d'évolution similaires, laissant supposer des mécanismes de régulation similaires. Des modifications des conditions de lumière ou de température modifient également la production de métabolites (*e.g.* changement dans la cinétique de production par rapport à la condition contrôle). De même, des différences de synthèse ont pu être observées au sein de mêmes familles de molécules supposant des variations de synthèse sous contrôle de la lumière ou de la température. Deux MAAs, la shinorine et la mycosporine-glycine,

produites par *Aliinostoc* sp., voient leur concentration augmenter sous la plus forte intensité lumineuse, en accord avec leur propriété photo-protectrice.

IV.3. Article 4

The variation of the regulation of cyanobacterial (*Aliinostoc* sp. PMC 882.14) metabolite production is responsive to light intensity and temperature culture conditions

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Keywords: cyanobacteria, metabolomics, high-resolution mass spectrometry, secondary metabolite induction, culture conditions

Abstract

Cyanobacteria are microorganisms able to adapt to a wide variety of environmental conditions and abiotic stresses. They produce a very large number of metabolites or natural products that can participate in the adaptation of cyanobacteria to a large range of resources concerning light, temperature, or nutrient availability. The physiological status of cells has also consequences on metabolites or natural product contents. In this study, we aim at characterizing the kinetics of metabolites diversity produced by the cyanobacterium *Aliinostoc* sp. PMC 882.14 during the main growth phases and under light and temperature variations. Metabolomes were analysed by using mass spectrometry (qTOF-MS/MS) combined with untargeted analysis to investigate the significant variations of metabolite production. The growth phase showed significant variations of metabolite production between exponential growth and stationary phases, whatever the tested conditions (control, higher light, or higher temperature). Some of these conditions (*e.g.* higher light or higher temperature) favored certain metabolites synthesis, the different metabolites belonging to the same molecular family presenting similar induction patterns. Among highly regulated metabolites, we observe the presence of mycosporine-like amino acids (MAAs), somamides, microginins, and microviridins analogues. These results reveal interesting regulation properties involved in metabolite production in *Aliinostoc* sp. PMC 882.14 and open up new perspectives in the context of the production of targeted bioactive compounds.

Introduction

Cyanobacteria are gram-negative photosynthetic prokaryotes that can be found in all types of environments including extreme conditions. They play an essential role in the functioning of various ecosystems since they have a key role in primary production by experiencing oxygenic photosynthesis processes, containing both chlorophyll *a* and other more specific pigments such as phycocyanin or phycoerythrin (Whitton and Potts, 2000).

Cyanobacteria produce a wide variety of bioactive metabolites, as more than 1,100 metabolites have been described (Dittmann et al. 2015). This variety of metabolites supports the remarkable ecological capacities of cyanobacteria to colonize or to adapt their metabolism/physiology to such a large variety of environments. Therefore, cyanobacteria are organisms of choice for researching valuable bioactive molecules in various fields, such as pharmacology, food industry or biotechnology (William H Gerwick and Fenner 2013; R. Singh et al. 2017). They also present promising applications in the field of cosmetics thanks to their production of various molecules with photo-protective, antioxidant or anti-inflammatory properties (*e.g.* carotenoids, mycosporine-like amino acids (MAAs), scytonemins, etc.) (R. Singh et al. 2017; Rajesh Prasad Rastogi et al. 2015; Lawrence et al. 2018).

A large portion and diversity of cyanobacterial secondary metabolites are peptides or exhibit peptide structures (Welker and Von Döhren 2006), being synthesized by specific enzymatic biosynthetic machineries (Dittmann et al. 2015), through either ribosomal (ribosomally synthesized and post-translationally modified peptides - RiPPs) or non-ribosomal (non-ribosomal peptide synthase, NRPS, or polyketide synthase, PKS) pathways. However, many other secondary metabolites can present different structure organization and belong to other chemical classes such as alkaloids, terpenes, polysaccharides or even lipids (Demay et al. 2019). Nevertheless, the vast majority of the biological roles of these cyanobacterial secondary metabolites remain unknown (Holland and Kinnear 2013), although certain hypotheses regarding their respective potential biological functions (*e.g.* allelopathy, defense, parasitism/symbiosis, chemical communication, etc.) have been proposed (Leflaive and Ten-Hage 2007).

So far, a large number of studies are available on targeted molecules producing regarding the physiological status and/or growth phases with *in vitro* experimentations approaches (Kameyama et al. 2004). However, only few studies focused on the whole cyanobacterial metabolome and its regulation over time and growing condition parameters (Natumi and Janssen 2020). Interestingly, a pilot study performed on diatoms (Barofsky et al. 2009) demonstrated a clear specificity of the metabolic profiles in regard to the different growth phases (exponential, stationary and senescence phases).

Several factors are supposed to be able to influence the growth of cyanobacteria, including light availability, temperature and nutrient availability. Among them, authors showed that high temperature (around 25°C) improved the growth of the bloom-forming *Microcystis* strains while lack of light or nutrients negatively impacted the growth of cyanobacteria (Jahn et al. 2018; Paerl and Otten 2013; Davis et al. 2009). Beyond its effects on cyanobacterial growth, light is also a key factor driving some metabolites production. It is well known that cyanobacteria are able to set up photo-protection mechanisms such as the production of specific molecules that absorb UV-radiation (e.g. MAAs; Jain et al., 2017) or minimize the production of reactive oxygen species and protect DNA thanks to specific UV-inducible defenses (e.g. scytonemins; Rastogi, et al., 2015).

In the present study, we investigated the whole metabolite production of the cyanobacteria *Aliinostoc* sp. PMC 882.14, isolated from biofilms of thermal mud of Balaruc-Les-Bains. *Aliinostoc* sp. PMC 882.14 has been selected for exhibiting a large number of biosynthetic gene clusters (BGCs) (supplementary table S1) able to produce a wide variety of metabolites valuable in the cosmetic or pharmaceutical sectors, such as MAAs (e.g. antioxidant, anti-UV properties) or microginin, symplostatins and dolastatins (e.g. cytotoxic activities) (supplementary figure S1).

This work aims to describe the diversity of metabolites produced by the strain *Aliinostoc* sp. PMC 882.14 and to evaluate the effect of the *in vitro* culture conditions on the metabolome content using qualitative and quantitative untargeted metabolomic approaches. We focus on the effect of light and temperature variations on the composition of endo- and the exo-metabolites. Regarding the respective influence of the light and temperature increase, we hypothesized that light intensity and temperature modify the production of certain molecules, some of them being especially relevant for cosmetic or pharmaceutical application.

Material and methods

Biological material. *Aliinostoc* sp. PMC 882.14 strain was isolated in 2014 from the thermal mud of Balaruc-les-Bains (France) and kept in collection at the Paris Museum Collection (PMC, Paris). Four large volume (500 mL) of pre-cultures were cultivated in 1-L Durand's bottles up to 46 days in Z8 culture medium, with 12 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ light intensity and 12h:12h light-dark photoperiod. Samples were collected after 33 days for chemical strain characterization. Pre-cultures bottles were thus pooled (on 46th day) in order to obtain a large volume (above 2 L) homogeneous stock solution (D0) that was further used for the experimentation. From this pool, the 1/10 diluted cultures were then inoculated in triplicates in 1-L bottles (initial volume of 500 mL) for experimentations under “control”, “higher light” and “higher temperature” conditions during the 28 following days. The figure 1 represents the global experimental and analytical pipeline developed in this study.

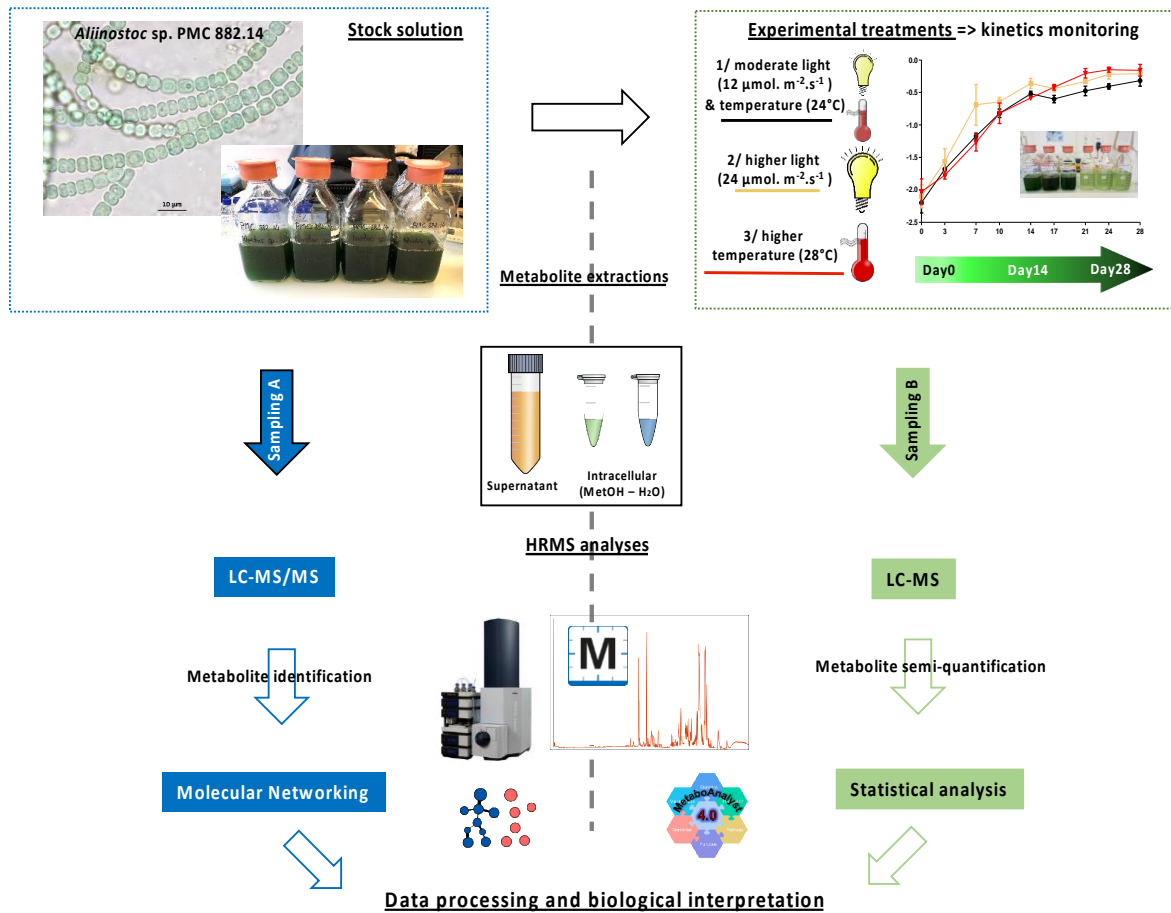


Figure 1. Graphical abstract of the analytical pipeline performed in this study.

Culture conditions and sampling. Experiment was conducted over a 28-days period in “control” (24°C, 12 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$), higher “light” (24°C, 24 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) and higher “temperature” (28°C, 12 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) conditions. Experimental conditions were done in triplicated, under a 12h:12h photoperiod and a constant homogenization maintained by magnetic agitators. Two weekly samples were taken throughout the 28 days (D0, D3, D7, D10, D14, D17, D21, D24, and D28). Each sampling (22 mL) were sub-divided into several parts for *i*) the extraction and the analysis of intra and extra-cellular metabolites (10 mL), *ii*) the biomass monitoring using extraction and quantification of chlorophyll pigments (10 mL), optical density and cell counts (2 mL). Day 0 sampling was performed on the diluted (1/10) stock solution, in triplicate.

Biomass dynamics. Cyanobacterial biomass was followed using: absorbance at 750 nm by *in vivo* spectrophotometric (Cary 500; Varian, U.S.A.), chlorophyll-*a* concentration (Yéprémian et al., 2017) and cell abundance by Malassez’ cell counts (Catherine et al., 2017). Data have been transformed into Neperian logarithm and statistical analysis between treatments and control (ANOVA with Bonferonni’ correction) was conducted with GraphPad Prism 5.01 software.

Metabolite extractions. Samples (10 mL) were centrifuged (10 min, 3,220 g, 4°C). Supernatant was isolated and stored at -20°C in prevision of extracellular metabolites liquid extractions. The pellet was centrifuged and stored at -80°C for at least 24 hours, then the sample lyophilization was performed (-50°C, 0.02 mBar for 16h) (Labconco, Italia). Dry weight was thus determined, and then the two sequential extractions performed (with 100 µL of solvent for 1 mg biomass dry weight). The first extraction of hydrophilic metabolites was carried out with a water/formic acid mixture (0.1%), and a second extraction targeting hydrophobic metabolites was performed with a water/methanol mixture (75%), then the two supernatants were pooled. For both extraction, cell lysis was performed by sonication: 3 cycles of 30 s, 10 s break between each cycle, at 80% of the maximum intensity (SONICS Vibra Cell, 130 Watt, 20 Khz). Samples were then centrifuged (10 min, 13,400 g at 4°C) and the supernatants collected and stored in obscurity at -20°C before mass spectrometry analysis.

Extracellular metabolites present in the supernatant of the culture media (D0, D7, D14, D21, D28 as they corresponding to key moments of the growth phases) were extracted by liquid/liquid extraction with equivalent volume of solvent (100% ethyl acetate) with a separating funnel. Upper organic phase has been collected after homogenization and degassing. Water molecules in the organic phase were removed by the gradual addition of magnesium sulfate powder (MgSO₄). Consecutively to filtration, liquid was dried under air flow, then the dry extracts were re-suspended in a mixture of water/methanol (75%)/formic acid (0.1%) and centrifuged (10 min, 13,400 g, 4°C) before storage at -20°C.

Mass Spectrometry Analysis. Extracts were separated by ultra-high performance liquid chromatography (UHPLC). For each sample, 2 µL was injected and molecules separation was performed by a Polar Advance II 2.5 pore C18 (Thermo) chromatographic column at a flow rate of 300 µL.min⁻¹ under a linear gradient of acetonitrile acidified with 0.1% formic acid (from 5 to 90% in 15 min). Metabolites contents were analyzed using an electrospray ionization hybrid quadrupole time-of-flight (ESI-QqTOF) high resolution mass spectrometer (Compact, Bruker) in the range 50-1500 *m/z*.

Simple MS mode analyses. Compounds were initially analyzed in simple MS positive and negative modes, without quadrupole fragmentation. This mode of analysis notably allows observing a signal intensity of each ion (measured in number of detected counts over the time range necessary for its elution) which is directly proportional to the quantity of ions present in the different samples. This analysis was carried out in order to compare metabolites composition in the three extraction fractions on the pre-culture (representing a total 2,441 intracellular analytes extracted with acidified water or methanol and extracellular analytes, when data were merged), and in the samples collected from the experimentation (for either extra- or pooled intra-cellular fractions, analyzed on positive mode only). MS data were processed using MetaboScape 4.0 software (Bruker, Bremen, Germany) for recalibration of each sample analysis (according to internal standard), peak detection and selection of ions which intensity was greater than 5,000 counts in at least 10% of the set of samples and peak realigned.

Furthermore, different states of charge and adducts were grouped together and the “area-under-the-peak” determined, in order to generate a unique global data matrix containing semi-quantification results for each metabolite, in all analyzed samples peak for each analyte (characterized by the respective mean mass of its neutral form and its corresponding retention time).

MS/MS mode analyses. For qualitative investigation of metabolites, additional ion selection with the quadrupole and fragmentation was carried out in tandem by collision ion dissociation (CID) according to the autoMS/MS analysis, performed in positive mode. The ions with the highest intensities in single MS (MS1) were then selected by the quadrupole and fragmented in a collision cell. Resulting ions of the fragmentation of their respective parent were transferred and detected. This series of MS/MS dataset containing fragment list of all selected ions allow qualitatively the characterization of the diversity of the corresponding metabolites produced by *Aliinostoc* sp. PMC 882.14. through the comparison of the fragmentation profiles. The files containing the fragmentation information for each ion analyzed were exported in mgf format using MetaboScape 4.0 (Bruker) software, before being used for the generation of the molecular network of structural similarity.

Metabolites annotations were made from MS/MS data (mgf file) by generating a molecular network for the comparison of fragmentation profiles using MetGem software (version 1.1.2) and GNPS algorithm (Wang et al., 2016). Public and generalist spectral databases GNPS library, NIH Clinical collections and EMBL metabolomics were used. Annotations were supplemented by a match with CyanoMetDB database, which contains all the raw formulae of the 2,100 cyanobacterial metabolites already described (Jones et al. 2020).

Statistical treatments. MetaboAnalyst 4.0 platform (Chong et al. 2019) was used to perform data matrix normalization (Pareto), Principal Component Analysis (PCA), Partial Least Square Discriminant Analysis (PLS-DA), Multivariate Empirical Bayes Analysis (MEBA) and ANOVA (ANalysis Of VAriance).

Results

Extraction compartment specificity and metabolite diversity of *Aliinostoc* sp. PMC 882.14

The three fractions (intracellular extracted with water (IW) or methanol (IM), and the extracellular supernatant (E)) retrieved from the pre-culture of the strain *Aliinostoc* sp. PMC 882.14 (in control condition) were first investigated in both positive and negative ionization MS mode. Taken all three fractions together (IW, IM and E), 1,971 different ions are observed in positive mode (79% of total ions detected), while 518 ions (20% of the total ions detected) are observed in negative mode, with only 48 ions commonly detected in both positive and negative ionization modes. Accordingly, we assume to focus rationally our investigation on metabolome regulation to the MS analysis performed on the positive mode solely.

Figure 2 exhibits the respective composition (in relative intensity) of the ions analyzed in LC-MS (using merged positive and negative mode dataset) for the three fractions analyzed (IW, IM and E). Figure 2a shows that the three extraction fractions have a very different metabolic composition, especially the extracellular fraction (E) that present a very different metabolic composition from the two intracellular fractions (IW and IM). These observations are also illustrated on the individual plot of the principal component analysis (PCA) (Figure 2b) and the Venn diagram (Figure 2c), indicating that similar numbers of analytes (chemical entities regrouping all observed charge states and adduct forms) are observed in the three fractions (totals of 1596, 1789 and 1693 analytes detected in the E, IM and IW fractions, respectively), 848 analytes (34.7%) being present in the three extracted fractions. On the other hand, these fractions contain 413, 191 and 45 ions that are found exclusively in the E, IW and IM fractions, respectively, representing 16.9%, 7.8% and 1.8% of the total analytes detected. Noticeably, a greater number of common analytes are observed between IW and IM (609 analytes, 24.9%), endorsing the fact that both being obtained from the intracellular compartment, when IM and E fractions share only 194 analytes (7.9%) and the IW and E only 141 ions (5.8%).

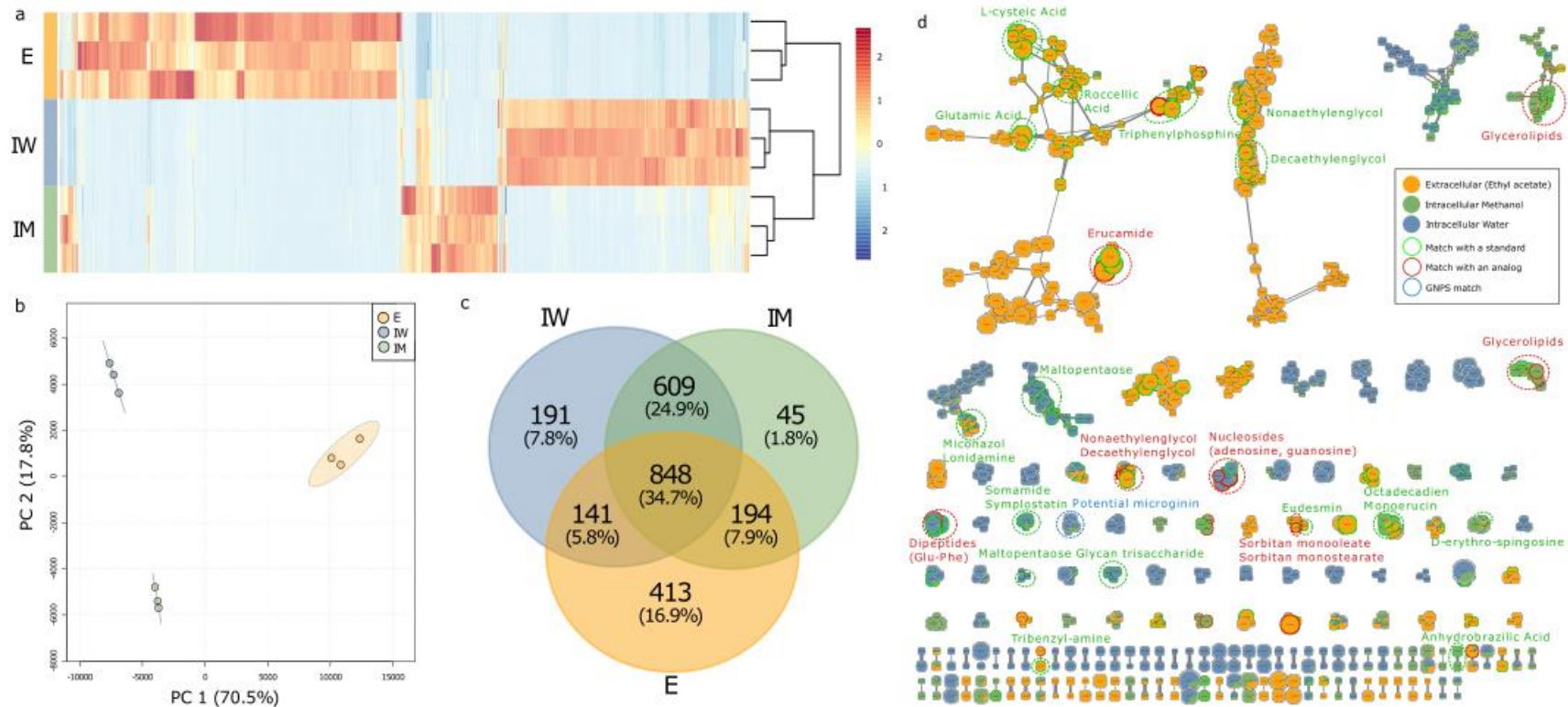


Figure 2. Metabolic composition of the three extraction fractions (triplicates) illustrated a) by a heatmap with hierarchical classification, b) by a Principal Component Analysis, c) by a Venn diagram and by d) molecular network representation of the molecules detected in each one of the three extraction fractions. E, extracellular; IW, intracellular-water; IM, intracellular-methanol. On molecular network, the dots represent the different ions analyzed in autoMS/MS (merged positive and negative mode dataset). The colors of the dots correspond to the fraction in which the analytes have been detected. The annotations were made with the MetGem 1.2.1 software (red and green dotted circles) or with the GNPS web tool (blue dotted circle) using public spectral databases. Only clusters of at least two nodes are represented. here

Further fragmentation experiments were performed on these three cellular fraction using LC-MS/MS positive and negative ionization modes. Out of the total 2,441 analytes detected with simple LC-MS experiments, 1,530 analytes could have been analyzed by LC-MS/MS. These MS/MS fragmentation data was used for metabolite annotation (supplementary table S2) and molecular network construction (Figure 2d). In this network, each analytes is represented by a node which diameter depends on its respective total ion intensity, the color area indicating the corresponding portion of the cellular fractions in which it has been detected. Clouds group together ions that present similar MS/MS fragmentation profiles according to MetGem algorithm. The most important clusters are constituted by molecules isolated in the supernatant and comprise molecules possessing COOH acid groups (cepteic, roccellic, glutamic acids). Remarkably, this E fraction also contains various fatty acids (roccellic acid) and amino derivatives of fatty acids (erucamide). On the other side, the intracellular metabolites that were isolated with water (polar, in blue) and methanol (non-polar, in green), comprise, respectively, hydrophilic compounds, presenting noticeable osidic residues such as nucleosides, malto-pentose and glycan tri-saccharides, or more hydrophobic molecules (mainly lipids), such as glycerolipids or erythro sphingosines. Other components presenting more intermediary hydrophobicity were retrieved in both IM and IW fractions, and remarkably not in the supernatant, comprising, among others, dolastatins, microginins and various amino acids or peptides.

Dynamics of metabolite production under control, higher “light” and higher “temperature” conditions

Influence of growth on metabolite production

During the 28 days of PMC 882.14 *Aliinostoc* sp. culture, the 750 nm absorbance (Figure 3), the chlorophyll *a* concentration and the cell abundance (supplementary figure S2) showed a characteristic growing pattern with an exponential phase (from D0 to D14), then a stationary phase (after D14). Although, these two phases were being observed whatever the three culture conditions. The comparison of the different growth curves with those obtained under control conditions (24°C, 12 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) shows few significant variations (ANOVA, $p < 0.001$) with a slightly higher growth of *Aliinostoc* exposed to “light” (24 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at D7 or D14 (exponential phase) or to “temperature” (28°C) from D7 to D28 (stationary phase).

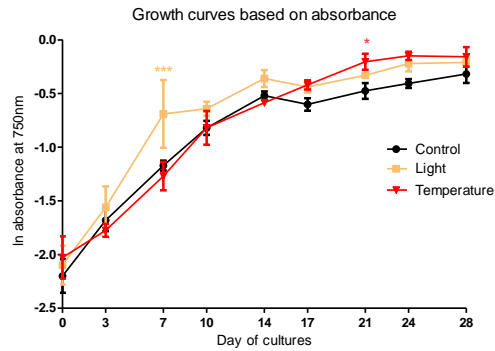


Figure 3. Cell growth curves of *Aliinostoc* sp. PMC 882.14 according to different culture conditions (“control” 24°C/12 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, higher “light” 24 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, higher “temperature” 28°C) according to the number of culture days considering the *in vivo* absorbance at 750 nm (mean \pm SD). Significant differences between control and treatments (high light, high temperature) were assessed by the ANOVA ($p < 0.05$) corrected with Bonferroni’s test.

Regarding the intracellular metabolome (pooled IM and IW fractions), the variations of the global metabolome composition during the 28 days of culture have first been explored with un-supervised multivariate analysis (PCA, principal component analysis) as illustrated in figure 4, for control condition only (Fig. 4a) or for all three experimental conditions (Fig. 4b). A progressive shift of the global metabolome among the duration of the culture was observed. Although the replicated measurements illustrate a reliable replication of the experiments, it also shows a progressive disparity increase between triplicates through the growing kinetic and/or under the different experimental conditions, in a lesser extent. On the other hand, the points corresponding to the first days of sampling (D0, D3 and D7) are very close to each other, suggesting that only little metabolic composition changes occur during the first seven days of the culture. Interestingly, the PCA analysis mostly discriminated the samples from day 0 to day 14 according to the component 1 (representing 58 and 45% of the total variance), when the component 2 (10 and 11% of the total variance) clearly discriminates samples from day 14 to day 28, suggesting that 2 distinguishable metabolome regulation events (metabolic phase 1 = 0-14 days and metabolic phase 2 = 14-28 days) could be distinguished along this 28-day kinetics.

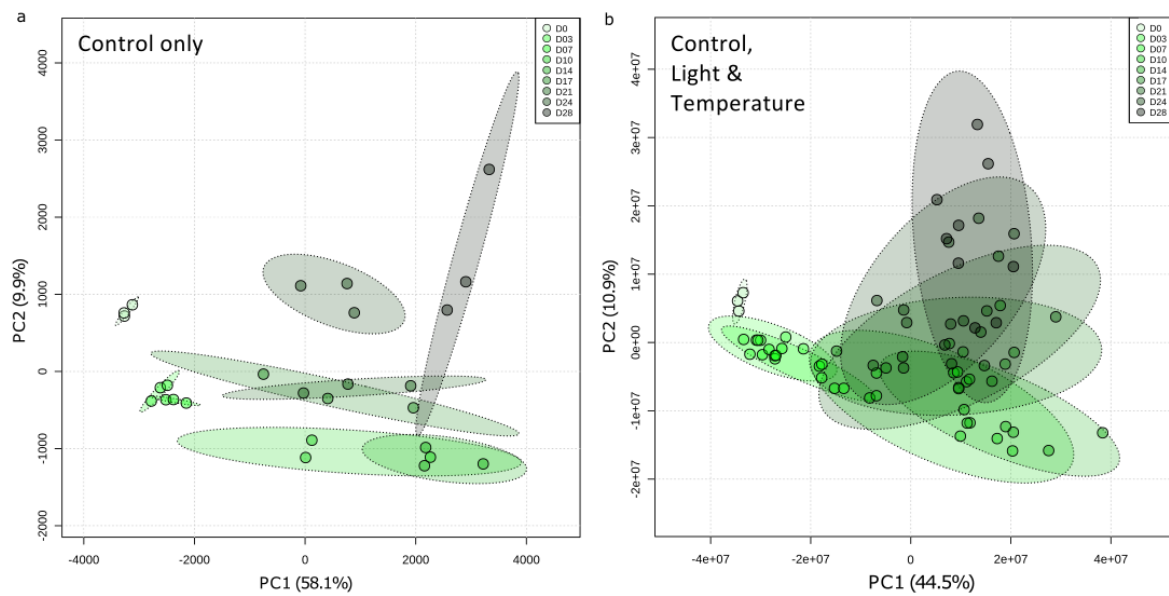


Figure 4. Principal Component Analysis (PCA) representing the evolution of the intracellular metabolic composition of *Aliinostoc* sp. PMC 882.14 as a function of number of culture days a) under the control condition, b) under control, higher light and higher temperature conditions. Each point represents a culture replicate. The green color gradient reflects the temporal scale of the cultures.

Supervised multivariate analysis (*i.e.* partial least square discriminate analysis, PLS-DA) was then similarly conducted considering the days of sampling and represented on supplementary figure S3, for control samples only (Fig. S3a) and for the samples from the three experimental conditions (Fig. S3b). These individual plot PLS-DA representations exhibit very similar patterns than those observed previously for PCA (with relatively similar %age of component 1-2 contributions too), and illustrate the good performance ($R^2_{\text{cumulative}}$) and predictability ($Q^2_{\text{cumulative}}$) scores. The list of the analytes that are contributing the most to the sample discrimination through the culture kinetics (variable of importance in the projection, VIP score >2) are represented on supplementary figures S3c and S3d. Interestingly, the list of variables that are the most discriminate samples during the kinetics are globally the same when considering the samples from the control condition only, or those from the three experimental conditions (illustrated by red squares). Overall, and except for some rare examples, these intracellular metabolites globally present a net increase of their relative intracellular concentrations, suggesting that these compounds are accumulated within the cells during the culture kinetics (Figure 5, supplementary figure S4 and supplementary figure S5). The observation of the individual analyte variations illustrates this global increase tendency over the time of culture, and also allows to distinguish three principal patterns comprising: *a*) one-step progressive increase at day 10, as observed for some microginins, some dolastatins or the adenosine (Fig. 5a), *b*) two-step progressive increase at days 3 and 17, as observed for microviridin 1724, for example (Fig. 5b), or *c*) a late, but important, increase occurring at day 24, such as for the monoacylglycerol 16:1 (Fig. 5c). These observations suggest that various regulation processes controlling the intracellular content of those components may occur during the culture kinetics,

depending to the molecule family and depending on their respective biosynthesis/consumption balance within the cyanobacteria.

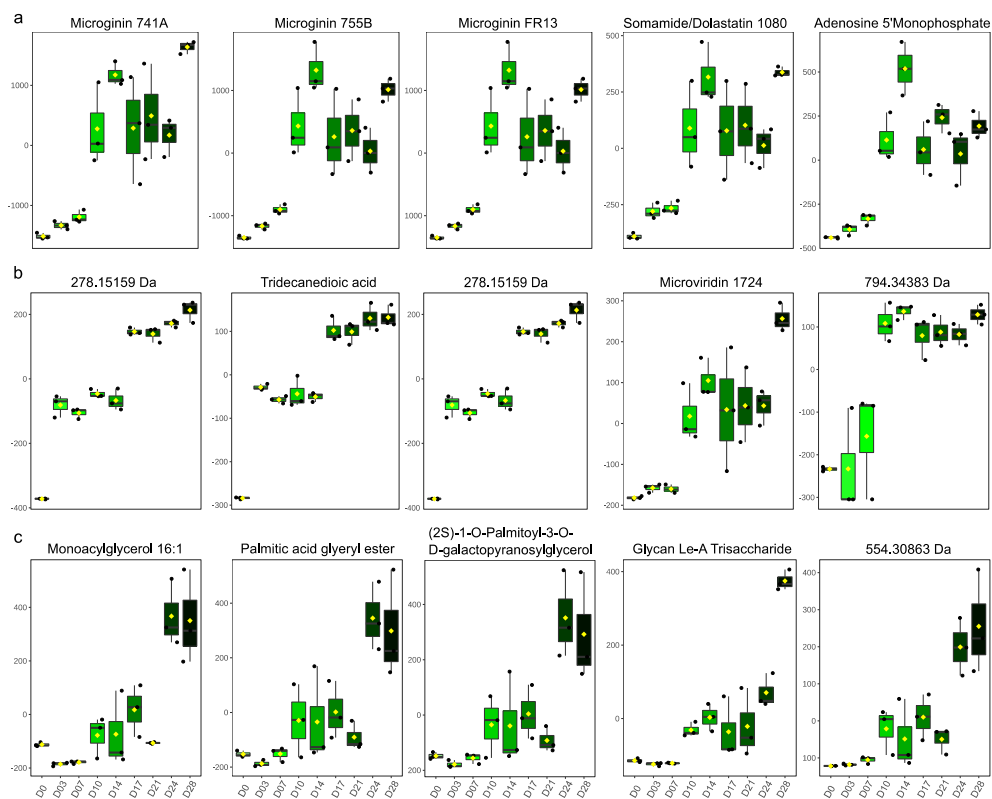


Figure 5. Box-plots representing a selection of 15 among the 38 intracellular molecules (exhibiting VIP scores > 2), which explain the most of the differences between the different days of culture. The three main patterns are illustrated here with molecules whose relative intensity a) increases promptly at day 14, b) increases by forming a transitional step between days 10 and 21, c) increases mostly at the end of kinetics (days 24 and 28).

Variation in metabolite production under higher “light” and higher “temperature”

In order to identify the metabolites which relative quantity are the most affected by the different culture conditions, data were analyzed by a multivariate approach specifically dedicated to process time series investigation (MEBA, multivariate empirical bayes analysis (Tai and Speed, 2006)). This approach highlights variables for which quantity variations over the time diverge the most between the different experimental conditions and was supported by two ways ANOVA investigations ($P < 0.001$), revealing a cross-effect of the experimental conditions and the culture duration. The variation profiles of the metabolites presenting the best MEBA scores are illustrated in figure 6 (supplementary figure S7).

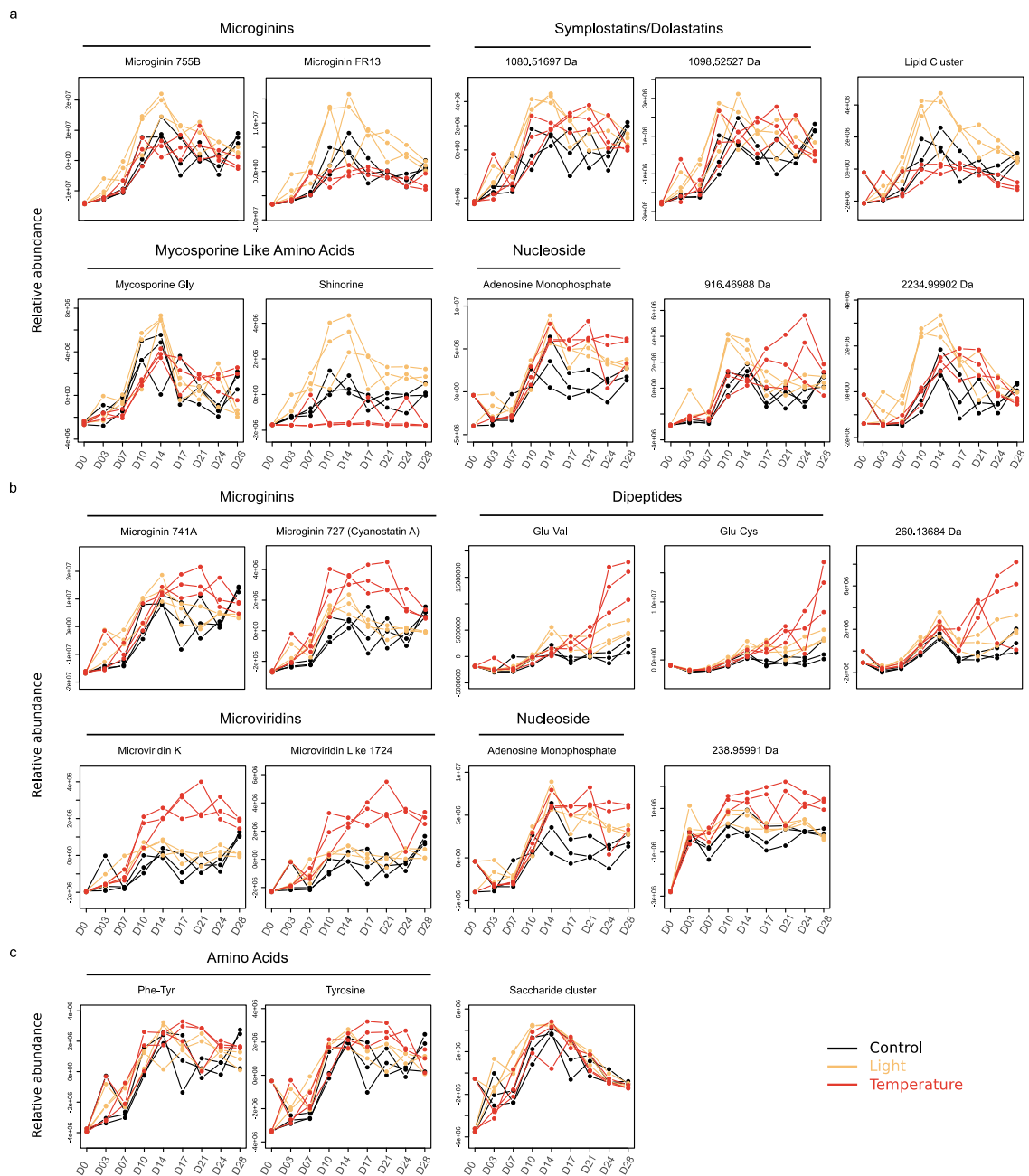


Figure 6. Relative abundance profiles of a selection of 22 analytes among the 48 presenting the best MEBA (Multivariate Empirical Bayes Analysis) classification scores, further confirmed by two ways ANOVA ($p < 0.001$). a) Metabolites with a higher intracellular concentration in the higher “light” condition than in the control. b) Metabolites with a higher intracellular concentration in the higher “temperature” condition than in the control. c) Metabolites presenting more complex pattern of concentration when compared to the control. Each line representing a different replicated culture.

Figure 6a shows metabolites whose intracellular relative concentrations is greater under higher “light” conditions when compared to the control. Overall, these metabolites follow a quite similar trend, characterized by an increase until day 14, then a slight decrease. This comprises two microginins and

two dolastatins, both presenting very similar patterns. For these components, this peak of maximal concentrations observed at day 14 was also observed under control condition, but appears to be less marked. Interestingly, one of the metabolites belonging to the MAAs family, the shinorine (a potential photo-protector), exhibited a larger intracellular accumulation under (higher) light condition than in control, when almost no signal was detectable under the higher temperature. Figure 6b shows the metabolites whose intracellular content is greater under the (higher) temperature condition compared to the control. As exemplified for 2 microginins, 2 microviridins, as well as for adenosine monophosphate and an unknown metabolite of 238.95991 Da (neutral mass), we observed an initial relative concentration increase (up to day 10) then a stabilization until day 28. Quite different profiles were observed for the dipeptides Glu-Val, Glu-Cys as well as for the unknown 260.13684-Da metabolite that present important concentration increase after day 21. It appears interesting to notice that, the maximum of the increase of the intracellular metabolite concentration seemed to be induced by higher light condition after 10-14 days of culture, then it decreased. Differently, the temperature-induced increases occurred latter during the kinetics (from day 14), but appeared to be much more stable until day 28. This observation may traduce that for these components quite different mechanisms of cellular concentration regulation may occur between the light and temperature concentration-increase inductions.

On the other side, similar analyses were attempted for the extracellular analytes (Supplementary figure S8-S11) and lead to the observation that: *i*) the extracellular metabolome presented a net temporal variation along the time series, with important heterogeneity between replicates that appears through latter culture days (supplementary figure S8); *ii*) the experimental factors (higher “light” and higher “temperature”) seemed to have a limited effect on the extracellular metabolome variation (supplementary figure S9); *iii*) the analytes that were the most discriminating the most the extracellular metabolome of the time series were the same when considering only the control conditions or all conditions together (supplementary figure S10); *iv*) these extracellular components corresponded mostly to unknown molecules, that failed to be annotated, and that presented rather global increases or decreases during the time series (supplementary figure S11); *v*) no perceptible metabolite transfer from the intracellular compartment to the extracellular one could had been observed through the investigated time series covering both exponential and stationary growing phases.

Discussion

Analysis of the molecular network of metabolites of the *Aliinostoc* sp. PMC 882.14 indicates the presence of numerous generalist cellular metabolites such as dipeptides, nucleosides, or fatty acids, as well as, more specific molecules of cyanobacteria such as analogs of MAAs, somamides, microviridins and microginins. Somamides are part of the class of cyclo-depsipeptides and have been isolated in particular from cyanobacteria of the genus *Schizotrix* and *Lyngbya* (Atta-ur-Rahman, 2015), but this family of molecules has been poorly described so far. The *Aliinostoc* sp. PMC 882.14 also produces

different variants of microginins (supplementary figure S1). These molecules are also secondary metabolites of linear peptide structure produced by the NRPS/PKS hybrid biosynthetic pathway (Zervou et al., 2020), that can lead to the formation of a large diversity of structural variants by a single strain (Süssmuth and Mainz, 2017). To date, more than 90 variants of microginins have been already referenced in databases (Jones et al., 2020), isolated mainly from cyanobacteria of the genus *Microcystis* and *Planktothrix* but also from some cyanobacteria belonging to the genus *Nostoc* (Tikhonova et al., 2017).

The monitoring of the metabolome variation through 28 days of culture under different experimental conditions indicates that the time of culture is basically the first factor affecting the relative composition of both intra- and extra-cellular contents of *Aliinostoc* sp. PMC 882.14. This result is illustrated in figure 4 since the samples corresponding to the different points of the time series are discriminated along component 1 for samples ranging from day 0 to day 14, then along component 2 for latter samples. This progressive temporal change of the intracellular metabolite contents across 2 distinguishable phases (day 0-14 and day 14-28) are in perfect correspondence with growth phases (exponential phase from day 0 to day 14, then stationary phase) observed from the monitoring of the cell proliferation within the cultures (figure 3 and supplementary figure S2).

A closer look at the variables (the analyte semi-quantification) mostly responsible of these global metabolome variations (supplementary figure S3) and at their respective relative concentration variations through the time series (figure 5; supplementary figure S4-S5) reveals a global increase in the relative intracellular contents for these components. It also highlights and even more subtle regulation events, implicating different biosynthesis, accumulation and consumption modalities according to the different components (figure 5a-c). Interestingly, metabolites that annotations correspond to molecules belonging to the same molecule families present very similar and reliable variation patterns, claiming for the occurrence of homogeneous regulation processes through the different variants of these molecule families. While certain analytes, such as microginins exhibit a prompt and important relative-concentration increase occurring at the end of the exponential phase then a stabilization, other molecules, such as microviridins, rapidly increase their relative concentrations in two steps occurring at the beginning of the exponential phase (from day 0 to day 3), then during the late stationary phase (Days 21-28). Differently, other metabolites present mainly a net increase during the late stationary phase (days 24-28).

The physical *stimuli* induced by higher “light” ($24 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) or higher “temperature” (28°C) conditions seems to have a different cellular impacts, as our results indicate, as a biological consequence, that the accumulation of intracellular metabolites differ in term of quantity and kinetics, as illustrated in figure 6. In the present case, metabolites that are induced by light appear then induced earlier and faster than those induced by temperature through the culture time series presently explored.

Indeed, the intracellular concentration of microginin 755B and microginin FR13, symplostatins/dolastatins, MAAs (mycosporine-glycine and shinorine) as well as certain un-annotated metabolites appear to be increased under higher “light” conditions ($24 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) compared to the “control” ($12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), especially during the maximum of induction occurring at days 10-14, corresponding to the end of the exponential phase (Figure 6a). One can here hypothesize that these metabolites could play a role in reducing the oxidative stress effects potentially induced by higher light conditions (Tonk et al., 2005).

On the other hand, other compounds present net intracellular concentration increases under higher “temperature” (28°C), with comparison to control (24°C) condition, occurring during the early (from day 14) or the late (from day 24) parts of the stationary phase, depending on the molecule family, as exemplified with microginin 741A, microginin/cyanostatin A, microviridin K and microviridin 1724, 2 dipeptides, Glu-Val and Glu-Cys (Figure 6b). In contrast to light-induced metabolites, the concentrations of the temperature-induced metabolites appear to be less numerous and to increase only at the end of the exponential phase, or even later at the end of the experiment. The difference of only 4°C that discriminates the control (24°C) and higher “temperature” condition (28°C) therefore appears to have less general impact on the metabolite production and the cellular growing of *Aliinostoc* sp. PMC 882.14, than a light increase from 12 to $24 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

The strong increase in the concentrations of two MAAs (shinorine and mycosporine-glycine) in under high-light condition is consistent with the putative photo-protective role of MAAs against UV-B radiations (Řezanka et al. 2004). However, so far, the physiological function of MAA production in cyanobacteria MAAs may be multiple or more generalist, as they may also act as anti-oxidants or osmo-protectors (Kageyama and Waditee-Sirisattha 2018).

These variations in the intracellular metabolite relative contents can result from the summation of different additive or antagonist phenomena. Thus, the variation in observed intracellular contents for a given metabolite can be either the consequence of *i*) a modulation of its biosynthesis rate depending of the physiological status of the cells, *ii*) a variation in its degradation rate, or *iii*) a change of its excretion rate by the cells through the extracellular compartment; this latter supposition seems not to occur in the present case as none apparent metabolite transfer from the intra- to the extra-cellular compartment could have been revealed by the analyses.

Adenosine monophosphate is a primary metabolite which exhibits in the present experiment higher relative concentrations during the stationary phase for both higher light and temperature conditions compared to the control (Figure 6). This apparent accumulation could be the result of the degradation of ADP (adenosine diphosphate) and ATP (adenosine triphosphate), which may indicate an increase of energy consumption occurring under stress. However, to confirm this hypothesis, it would be necessary to specifically quantify the ratios between the amounts of AMP (adenosine monophosphate), ADP and

ATP, as these latter components were not retrieved by the present un-targeted metabolomics pipeline based on variable extraction using differential analysis.

Furthermore, all the metabolites belonging to the same chemical family do not necessarily exhibit identical variation profiles according on the different culture conditions. This is remarkably the case for some microginin variants. Differences in the ratios of variants of secondary metabolites in other cyanobacteria have already been described, especially in the case of response to light stress (Tonk et al. 2005). In their study, Tonk and co-workers (2005) observed in *Planktothrix agardhii* a light-stress induced change in the ratio of the different variants of microcystins, cyanobacterial-specific secondary metabolites produced by NRPS/PKS complexes, similar to those responsible of microginin biosynthesis. The variations of the intracellular availability of the substrates necessary for the biosynthesis of these compounds could be an explanation for such differences. Indeed, microginins 755B, 741 and microginin/cyanostatin A structures differ in the composition of central amino acid (alanine, serine or valine, respectively), and the production of one variant or another one can vary depending on the affinity of elongation enzymes to the substrates and of their respective availability.

Conclusion

This study provides an initial picture of chemical diversity of metabolites produced by the cyanobacterium *Aliinostoc* sp. PMC 882.14, and its production regulation under different culture conditions. This strain is characterized by the presence of numerous BGCs in its genome, and the consecutive production of numerous and specific metabolites such as various analogues of somamides/dolastatins, microginins, microviridins and MAAs.

The phase of culture appears to play a key role in the metabolic composition, with remarkable variations occurring between the different growth phases (*i.e.* exponential *vs* stationary). The culture conditions also appear to have significant effects on the dynamic of the metabolic composition, and should be further considered, especially in the context of bioactive compound production for valorisation purposes. Indeed, the production of such potentially bioactive components, such as shinorine, somamides/dalostatins and microginins, by *Aliinostoc* sp. PMC 882.14, represent great potential for further bioactivity screening.

Acknowledgements

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Chapitre V

Discussion générale et Perspectives

Chapitre V

DISCUSSION GENERALE ET PERSPECTIVES

V.1. Synthèse des résultats, discussion et perspectives

Les cyanobactéries sont capables de vivre dans des milieux variés (*e.g.* riches ou pauvres en nutriments, forte salinité, forte intensité lumineuse, ...). Ces capacités d'adaptation se traduisent notamment par des caractéristiques cellulaires (*e.g.* possibilité de fixer l'azote à l'aide des hétérocytes) et par des caractéristiques chimiques (*e.g.* production de molécules photo-protectrices, de sidérophores ou de métabolites antimicrobiens qui leur sont propres (Whitton and Potts 2012; Kurmayer et al. 2016)).

C'est la recherche de nouvelles molécules naturelles pour des applications pharmaceutiques qui a permis d'étudier l'incroyable diversité de métabolites produits par les cyanobactéries, notamment grâce au développement de différentes techniques à haut débit de type « omiques ». Ces techniques ont permis l'étude à grande échelle des génomes et des molécules produites par les cyanobactéries, ce qui a conduit à une compréhension accrue de la diversité structurale, des voies de biosynthèse et des modes d'actions de ces composés (Tidgewell et al. 2010; Dittmann et al. 2015; Salvador-Reyes and Luesch 2015).

Dans ce contexte, il semble intéressant d'étudier spécifiquement la composition métabolomique des cyanobactéries provenant d'environnements particuliers tels que les boues thermales. En effet, ces boues (ou péloïdes) abritent, en plus de l'argile et de l'eau thermale, de nombreux microorganismes potentiellement responsables de bienfaits thérapeutiques. C'est pourquoi, dans le cadre de la collaboration de recherche entre les Thermes de Balaruc-les-Bains et le Muséum National d'Histoire Naturelle, les travaux menés au cours de cette thèse ont eu pour but la caractérisation de la diversité des molécules et de la bioactivité associée chez neuf cyanobactéries isolées des boues thermales de Balaruc-les-Bains.

Ces travaux ont été organisés en plusieurs volets complémentaires répondant aux différentes problématiques liées à la caractérisation du potentiel thérapeutique de ces souches de cyanobactéries. L'ensemble des questions et résultats majeurs obtenus lors de cette thèse sont représentés **Figure 5**. Dans ce chapitre, la discussion est organisée en trois parties autour des principaux résultats portant sur : 1/ la diversité générale des métabolites produits par les cyanobactéries et leurs activités, 2/ la caractérisation (taxonomique, métabolomique et du potentiel bioactif) des cyanobactéries issues des boues thermales de Balaruc-les-Bains, et 3/ les perspectives de valorisation pour la bio-production des molécules bioactives.

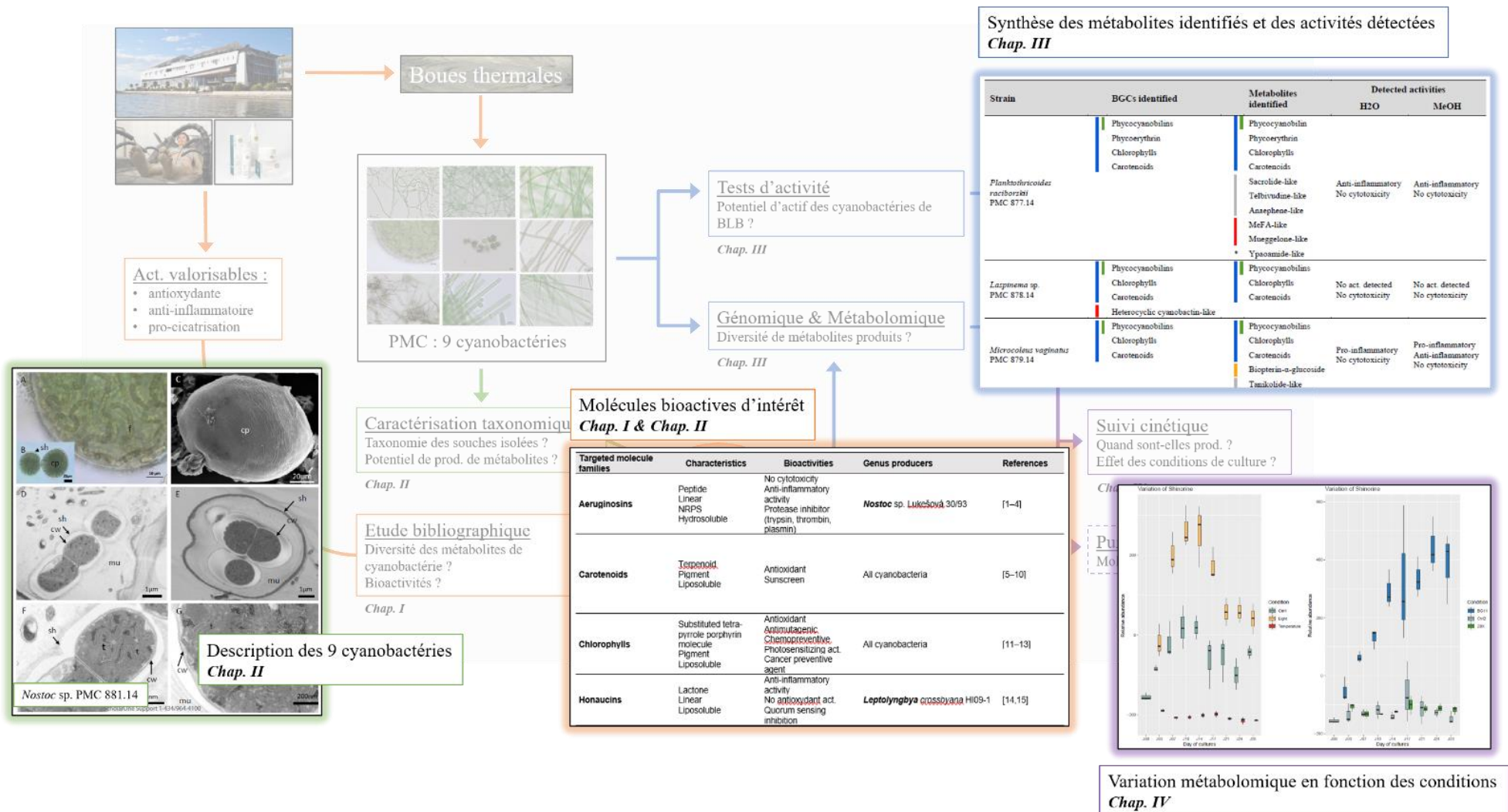


Figure 5. Schéma conceptuel des objectifs et résultats obtenus

V.1.1. Diversité des métabolites produits par les cyanobactéries et leurs activités

Ces dernières années, de nombreux articles de revue ont compilé les informations concernant les métabolites isolés de cyanobactéries. La plupart se sont concentrés sur la description des structures chimiques (Tidgewell et al. 2010; Mi et al. 2017; Bertocchi et al. 1990) ou des activités observées (Zahra et al. 2020; Vijayakumar and Menakha 2015; Tânia K Shishido et al. 2015; R. Singh et al. 2017; Ali Shah et al. 2017). Des bases de données de molécules, telles que MarinLit⁷, existent et regroupent différents *corpus* de métabolites décrits dans la littérature. Néanmoins, ces bases de données restent le plus souvent incomplètes (ou parfois spécifiques à un milieu ou à un organisme/taxa) et ne font que rarement mention des résultats des tests d'activités effectués par ailleurs.

Il nous est donc apparu nécessaire, d'obtenir une vision globale des connaissances déjà disponibles sur les métabolites produits par les cyanobactéries et les bioactivités associées en construisant une base de données regroupant les différentes informations disponibles à propos de ces métabolites de cyanobactéries marines, terrestres ou d'eau douce. Ainsi, les données de près de 670 publications publiées depuis les années 70s jusqu'à fin 2018 ont été analysées, 1630 métabolites ont été décrits et regroupés en 250 familles de molécules apparentées entre elles soit *via* leur voie respective de biosynthèse, lorsque cette dernière est connue, ou soit *via* leur homologie/analogie structurale (étant supposé que des structures similaires partageraient la même origine biosynthétique). Cette synthèse (**Chap. 1**) a permis de confirmer l'importante diversité structurale illustrée précédemment (Le Manach et al. 2019; Dittmann et al. 2015) avec plus d'une dizaine de classes chimiques différentes répertoriées (peptide, lipide, polysaccharide, alcaloïde, terpène, macrolide, ...). Malgré ces structures très différentes, la majorité des métabolites décrits appartiennent à la classe des peptides ou de leurs dérivés (lipopeptide, depsipeptide, ...). Cette observation est probablement reliée aux nombreux complexes de biosynthèse peptidiques utilisant des voies non ribosomales (NRPS, PKS) ou ribosomales (RiPPs) qui ont été identifiées chez les cyanobactéries (Dittmann et al. 2015; Kehr et al. 2011).

Peu de revues mentionnent la diversité des espèces ou souches de cyanobactéries productrices de métabolites spécialisés, les dernières recherches datant des travaux de Tidgewell et al. 2010. Comme ces auteurs, nous avons montré qu'une très grande majorité des métabolites a été isolée à partir de quelques souches appartenant à l'ordre des Oscillatoriales, même si, depuis 2010, la proportion des souches appartenant aux Synechococcales a augmenté. Au niveau du genre, la diversité relative est faible puisque seulement 90 genres sur les 300 de cyanobactéries décrits par Komárek et al. 2014 ont montré la production d'un ou plusieurs métabolites. Ces chiffres ouvrent le champ des possibles quant à la découverte de nouvelles molécules à partir de genres ou d'espèces peu ou sous-étudiés. Il est à noter également que certaines initiatives de recherche, comme celle portée par l'International Cooperative

⁷ <http://pubs.rsc.org/marinlit/>

Biodiversity Group (ICBG) et centrée sur l'étude des cyanobactéries marines, ont permis la caractérisation de nombreux composés bioactifs (environ la moitié des métabolites référencés) alors que les cyanobactéries terrestres ou d'eau douce ne dépassent pas 30% des molécules (**Chap. I, Article 1**).

Au niveau des bioactivités observées, 14 grandes catégories d'activités (*e.g.* activités antimicrobiennes, activités toxiques, capacité à inhiber diverses enzymes) sont distinguées pour les métabolites de cyanobactéries. La plupart des activités recherchées le sont dans le but de développer de nouvelles molécules pharmaceutiques, et ont été mises en évidence à travers des tests de cytotoxicité (potentiel anticancéreux) et/ou de tests antibactériens (développement d'antibiotiques). La plupart des molécules décrites ont pu montrer plusieurs activités différentes, pouvant ainsi compliquer le développement d'applications thérapeutiques directes et nécessitant des manipulations pour favoriser l'une ou l'autre de ces bioactivités et éviter d'éventuels effets secondaires non ou peu souhaitables (LaMonte et al. 2017).

L'analyse bibliographique portant sur les métabolites isolés à partir des cyanobactéries et leurs bioactivités a permis de choisir des métabolites à activités bénéfiques, au regard des soins exercés par les Thermes de Balaruc-les-Bains. Cet état de l'art actualise les connaissances sur les métabolites de cyanobactéries et permet de décrire le panorama des activités « bénéfiques » des molécules potentiellement exploitables dans des perspectives de valorisation et de production.

Néanmoins, ce travail représente un « instantané » de la bibliographie auquel manquent les informations des articles publiés depuis fin 2018. Il est donc nécessaire de réaliser une veille bibliographique sur ce sujet afin de continuer d'incrémenter la base de données avec les nouvelles molécules décrites et de mettre à jour les diverses informations recueillies (révision taxonomique des souches, tests d'activités ...). Il conviendrait également de rendre accessible cette base de données *via* une interface web publique qui pourrait être directement incrémentée par les utilisateurs travaillant sur les métabolites de cyanobactéries et leurs activités. Une autre option consisterait à ce que les données collectées puissent être intégrées dans une base de données généraliste comme ChEMBL⁸ ce qui permettrait de bénéficier de la structure de gestion de cette base de données et de leurs serveurs, mais nécessiterait de se plier aux exigences de format et de contenu, ce qui pourrait requérir un effort spécifique de mise à jour des données collectées.

V.1.2. Diversité des cyanobactéries de Balaruc-les-Bains

V.1.2.a. Diversité taxonomique des cyanobactéries isolées des boues thermales

Les travaux menés au cours de cette thèse font suite à une campagne d'isolement des microorganismes photosynthétiques colonisant les boues thermales des Thermes de Balaruc-les-Bains dans des conditions mimant la maturation du péloïde historiquement utilisé pour ses propriétés

⁸ <https://www.ebi.ac.uk/chembl/>

thérapeutiques (Hamlaoui 2014). En effet, les boues thermales de Balaruc-les-Bains représentent un environnement particulier dont la diversité des microorganismes restait à décrire. Cette étude, menée en 2014, a permis de montrer une dominance des cyanobactéries par rapport aux autres microalgues au sein de ces boues, laissant supposer que les cyanobactéries et les molécules qu'elles produisent seraient en partie responsables des bienfaits thérapeutiques apportés par les cures ; le lien entre les cyanobactéries et les effets bénéfiques ayant par ailleurs été proposé pour d'autres établissements thermaux utilisant la pélothérapie (*e.g.* les Thermes de Dax, en France, et les Thermes d'Abano, en Italie) ((Counilh and Gibert 2003; Curt Georgi 1971; Lalli et al. 2004).

Neuf cyanobactéries ont donc été isolées avec succès puis maintenues en culture au sein de la collection vivante de cyanobactérie du Muséum National d'Histoire Naturelle⁹. En 2014, l'affiliation taxonomique initiale de ces cyanobactéries a été réalisée sur la base de critères uniquement morphologiques. Néanmoins, comme nous avons pu le remarquer lors de notre analyse bibliographique, il est important d'affilier de façon cohérente les cyanobactéries étudiées avec le reste de la communauté scientifique (Komárek 2016). De plus, à l'heure du développement des techniques omiques et du « big data », il est apparu essentiel d'affilier finement les souches au regard de la phylogénie afin de générer des données correctement affiliées qui puissent être intégrées aux bases de données publiques.

Enfin, les neuf souches issues de Balaruc-les-Bains appartenant à la collection vivante de cyanobactéries du Muséum National d'Histoire Naturelle, ces travaux permettent de compléter les informations au sujet des souches référencées. Ils ont été réalisés en collaboration avec les différents membres de l'équipe CCE en utilisant plusieurs approches (*e.g.* biologie moléculaire, description morphologique et ultrastructurale) (**Chap. II**).

Il est intéressant de constater que les communautés de microalgues des boues thermales de Balaruc-les-Bains sont stables depuis plusieurs décennies, si l'on se base sur les observations morphologiques. En effet, Baudinat (1986) et Dupuis (1987) ont identifié dans un premier temps une dominance de cyanobactéries appartenant aux genres *Phormidium* et *Oscillatoria*, puis dans un second temps une dominance des espèces *Phormidium africanum*, *Phormidium autumnale*, *Lyngbya* sp., *Chroococcus minor* et *Anabaena* sp. (Baudinat 1986; Dupuis 1987). Morphologiquement, ces genres et/ou espèces présentent des caractéristiques morphologiques identiques aux cyanobactéries étudiées au cours de la thèse avec, par exemple, l'espèce *Pseudo-chroococcus couteii* PMC 885.14 qui présente les mêmes critères morphologiques que le genre *Chroococcus*. Toutefois, la séquence de l'ARNr 16S montre une affiliation clairement différente des cyanobactéries *Chroococcus* qui nous a conduit à la description d'un nouveau genre et espèce. De même, *Aliinostoc* sp. PMC 882.14 a des critères morphologiques identiques à ceux de l'espèce *Anabaena* sp. observée par Dupuis en 1987; et les Oscillatoriales *Planktothricoides raciborskii* PMC 877.14, *Microcoleus vaginatus* PMC 879.14 et *Lyngbya martensiana* PMC 880.14

⁹ <https://mcam.mnhn.fr/fr/collection-de-cyanobacteries-et-microalgues-vivantes-pmc-alcp>

sont identiques morphologiquement à *Phormidium africanum*, *Phormidium autumnale* et *Lyngbya* sp., respectivement.

Ces résultats montrent l'importance de l'utilisation d'une approche polyphasique pour une affiliation taxonomique précise des cyanobactéries. Si l'on compare ces trois études sur plusieurs décennies, les boues thermales de Balaruc-les-Bains semblent colonisées par les mêmes morphotypes, voire les mêmes espèces de cyanobactéries. Cette hypothèse apparaît difficile à confirmer car les cyanobactéries observées en 1986 et 1987 n'ont pas été isolées et maintenues en culture. Mais ces observations ouvrent la réflexion sur la pérennité des communautés de cyanobactéries capables de se développer dans ce milieu particulier et sur la spécificité de l'écosystème microbien constitué par les boues thermales qui pourraient induire une sélection de ces cyanobactéries, en relation avec des conditions physico-chimiques de cet environnement : la salinité de l'eau thermale ? La composition de l'argile ? Les conditions lumineuses ? La température ? Ou bien l'historique de colonisation et de sa stabilité ? Ces paramètres sont en effet constants à l'échelle d'une année si l'on considère que les conditions climatiques sont comparables d'une année sur l'autre en raison de l'origine des eaux thermales et représentent les conditions qui rendent spécifiques l'écosystème microbien des boues thermales de Balaruc-les-Bains.

Pour terminer, quant à l'affiliation des souches de cyanobactéries, la caractérisation taxonomique approfondie des cyanobactéries doit systématiquement être entreprise, quel que soit le programme de recherche ou de valorisation, afin d'incrémenter de façon précise les bases de données publiques permettant par la suite des comparaisons fiables des résultats entre les différentes cyanobactéries et d'entreprendre à plus grande échelle la phylogénie ou de la comparaison des génomes de ces organismes.

V.1.2.b. *Cyanosphère associée aux cyanobactéries en culture*

Les cyanobactéries isolées des boues de Balaruc-les-Bains sont maintenues en cultures monoclonales et non-axéniques dans la collection de cyanobactéries du Muséum (PMC). Les cultures sont donc issues d'un seul individu (cellule ou filament) qui s'est ensuite divisé et multiplié tout en maintenant la flore bactérienne associée. En effet, il a été démontré que la diversité bactérienne était spécifique à chaque genre de cyanobactérie de la collection PMC (Zhu et al. 2016). Ainsi, chaque souche de cyanobactérie, et plus particulièrement celles qui produisent une quantité importante de mucilage, possède sa propre « cyanosphère » ou « phycosphère » composée de divers microorganismes associés.

Dans le cadre de cette thèse, la cyanosphère a été mise en avant lors du séquençage des métagénomes avec l'identification de séquences appartenant à des bactéries hétérotrophes associées à chaque souche de cyanobactérie en culture (e.g. jusqu'à six génomes différents de bactéries hétérotrophes pour un seul génome de cyanobactérie (Halary et al., *in prep.*). La visualisation en microscopie électronique à balayage et à transmission a mis en évidence ces bactéries hétérotrophes de petites tailles et de formes

diverses, localisées à la surface des filaments ou au sein du mucilage (Arthur Marme, Master 1). A titre d'illustration, une planche des observations réalisées avec *Planktothricoides raciborskii* PMC 877.14 est présentée en **Annexe 2**.

Suite à ces observations, des travaux de métabarcoding à partir du séquençage de fragment du gène codant pour l'ARNr 16S ont été menés afin d'identifier les communautés bactériennes associées à chacune des cyanobactéries de Balaruc-les-Bains en culture. Entre 3 et 8 phyla différents ont pu être identifiés pour chacune des souches de cyanobactéries de Balaruc (pour *P. raciborskii* PMC 877.14 et *L. martensiana* PMC 880.14, respectivement), avec une majorité de bactéries hétérotrophes appartenant aux Bacteroidetes et aux Proteobacteria. Ces résultats concordent avec ceux obtenus en 2009 par des chercheurs finlandais montrant que la plupart des bactéries hétérotrophes associées aux cyanobactéries appartiennent aux phyla des Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes et Deinococcus-Thermus (Berg et al. 2009). Avec une telle diversité de bactéries associées aux cyanobactéries en culture, il est apparu nécessaire de vérifier le potentiel de production de métabolites de ces communautés associées.

Les différentes séquences obtenues grâce aux données de métagénomique ont donc été assemblées pour chaque phylum puis les assemblages ainsi obtenus ont été traités par différents outils bio-informatiques d'annotation automatique afin de détecter les éventuels îlots de gènes de biosynthèse (**Annexe 7, article 3, Table S12-20**). Il existe une grande disparité en terme de nombre d'îlots de gènes de biosynthèse en fonction des phyla avec plus d'une dizaine d'îlots détectés pour les assemblages de certaines Proteobacteria. Néanmoins, les îlots annotés codent la plupart pour des protéines impliquées dans la synthèse terpénique et notamment un grand nombre de gènes intervenant dans la synthèse de différents caroténoïdes. Ces travaux propres à la caractérisation des bactéries hétérotrophes associées aux neuf souches de cyanobactéries et à leur contenu génique notamment font actuellement l'objet de la rédaction d'un article (Halary et al., *in prep.*).

Ces travaux exploratoires abordent de nouvelles questions quant aux rôles respectifs joués par la cyanobactérie et les bactéries hétérotrophes associées. Ils questionnent également sur les interactions entre ces différents phyla. L'association profite probablement aux deux protagonistes, comme dans les symbioses, notamment *via* différents échanges de « biens communs ». Cette hypothèse est supportée par l'étude de Louati et al., en 2015, montrant que certaines communautés bactériennes sont plus ou moins favorisées en fonction de la cyanobactérie dominant le consortium (Louati et al. 2015). D'autres études ont également montré que certaines bactéries hétérotrophes sont capables d'augmenter la croissance des cyanobactéries quand d'autres, à l'inverse, sont capables d'inhiber leur croissance (Berg et al. 2009).

Du point de vue de la bio-production de molécules d'intérêt, il est important d'évaluer le rôle de ces communautés associées sur la physiologie générale de la culture mais également sur la production

métabolique des cyanobactéries. En effet, il a été montré que certaines bactéries isolées de lacs fréquemment colonisés par les cyanobactéries étaient capables de dégrader des métabolites de cyanobactéries (*e.g.* microcystines et nodularines) (Rapala et al. 2005; Berg et al. 2009). D'autre part, il a été démontré chez des microalgues que l'utilisation de bactéries associées pendant les processus de production de biocarburants pouvait permettre une meilleure efficacité du processus, *via* des échanges de nutriments ou éléments minéraux entre les deux protagonistes (vitamine, matière organique, fer, ...) ou permettre la dégradation de la biomasse résiduelle pour produire de l'éthanol (Yao et al. 2019).

Comme perspective, il serait intéressant de poursuivre la caractérisation des communautés associées aux cyanobactéries de Balaruc-les-Bains, en culture. En effet, à l'instar des bactéries sulfo-réductrices utilisées par les Thermes de Dax (Counilh and Gibert 2003), ces bactéries pourraient également apporter des bienfaits ou être utile à la croissance des cyanobactéries dans le cadre de la production de molécules bioactives. Des analyses des métagénomes sont en cours afin de caractériser les fonctions portées par chacun des membres du consortium. A ces analyses génétiques, l'isolement des différentes bactéries associées pourrait permettre de cultiver et d'affilier taxonomiquement ces espèces et de pouvoir mieux les caractériser (au niveau des génomes, métabolomes, ...).

V.1.2.c. Diversité de métabolites produits par les cyanobactéries de Balaruc-les-Bains

Les différents volets de cette étude ont permis de mettre en évidence la diversité des molécules aux propriétés anti-inflammatoires, antioxydantes et cicatrisantes potentiellement produites par les cyanobactéries de Balaruc-les-Bains. Ces résultats ont pu être obtenus à travers deux niveaux d'analyse, génomique et métabolomique, accompagnés de la synthèse des connaissances acquises lors de la construction de la base de données de métabolites bioactifs.

Deux « catégories » de molécules candidates ont été décrites pour leur potentiel antioxydant et anti-inflammatoire : d'une part les pigments (*e.g.* phycobilines, chlorophylles, caroténoïdes, scytonémine) et d'autre part certains métabolites spécialisés (*e.g.* aéruginosines, honaucines) (**Tableau 1**). Peu de travaux, en revanche, ont étudié les capacités cicatrisantes des composés produits par les cyanobactéries. Une étude évoque cette activité pour la phycocyanine, en association avec une hausse de la viabilité cellulaire (Sevimli Gur et al. 2013) et deux autres décrivent des molécules aux propriétés cicatrisantes isolées à partir de *Moorea bouillonii* et *Arthrospira platensis* (l'apratyramide et l'adenosyl homocysteinase, respectivement) (Cai et al. 2018; Sarkar et al. 2020).

Les deux approches complémentaires d'analyse des génomes et du métabolome ont permis d'évaluer le potentiel de production pigmentaire des cyanobactéries de Balaruc-les-Bains. Ces analyses nous ont permis de mettre en évidence la capacité de quatre souches à produire de la phycoérythrine avec ces propriétés antioxydantes (*P. raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14, *Calothrix* sp. PMC 884.14 et *P. couteii* PMC 885.14), capacité confirmée ensuite par extraction et quantification des

différents pigments. Trois cyanobactéries (*Nostoc* sp. PMC 881.14, *Aliinostoc* sp. PMC 882.14 et *Calothrix* sp. PMC 884.14) possèdent également le matériel génétique pour produire la scytonémine, un pigment anti-inflammatoire, même si malheureusement nos analyses LC-MS/MS n'ont pas permis de la quantifier. La synthèse de ce pigment est inductible par les rayons ultraviolets (UV) (Rajesh Prasad Rastogi et al. 2015). Il est possible que l'absence ou la faible proportion de rayons UV des lumières LED utilisées pour la culture en masse dans les conditions du laboratoire, n'ait pas induit la synthèse de ce pigment.

Par ailleurs, bien que toutes les souches possèdent le matériel génétique nécessaire à la synthèse des pigments photosynthétiques et des caroténoïdes, de fortes différences ont été observées en terme de quantités de pigment extraites à unité de biomasse égale, exprimée en poids de matière lyophilisée (e.g. 2,1 $\mu\text{g}\cdot\text{mg}^{-1}$ DW de phycocyanine extraite chez *Nostoc* sp. PMC 881.14 et 32,5 $\mu\text{g}\cdot\text{mg}^{-1}$ DW pour *P. raciborskii* PMC 877.14). Celles-ci peuvent provenir de différences dans l'efficacité de l'extraction en elle-même pour les différentes souches. En effet, à plusieurs reprises au cours de ces travaux de thèse, des difficultés à broyer les biofilms formés par certaines souches ont été rencontrées et tout particulièrement pour *Nostoc* sp., *Calothrix* sp., et *L. martensiana*. Les difficultés d'extraction expliqueraient ce relatif manque d'efficacité.

Les neuf cyanobactéries de Balaruc-les-Bains présentent néanmoins un potentiel bioactif anti-inflammatoire et antioxydant. *P. raciborskii* PMC 877.14 représente le meilleur candidat pour la bio-production de pigment de par la quantité importante de pigments extraite (e.g. $>30 \mu\text{g}\cdot\text{mg}^{-1}$ DW de phycocyanine extraite). Cette quantité est néanmoins plus faible que la quantité extraite de « Spiruline » (*Arthrospira* spp.), la cyanobactérie utilisée à ce jour dans l'industrie agroalimentaire, avec près de 80 $\mu\text{g}\cdot\text{mg}^{-1}$ DW de phycocyanine extraite (Doke 2005). Toutefois, les conditions de culture au laboratoire n'ont pas été optimisées pour la production de cette molécule et les procédés d'extraction utilisés sont différents entre ces deux études.

L'étude des îlots de gènes de biosynthèse a montré un fort potentiel de production de métabolites spécialisés avec une dizaine d'îlots en moyenne pour chacune des neuf cyanobactéries. Les trois Nostocales (*Nostoc* sp. PMC 881.14, *Aliinostoc* sp. PMC 882.14 et *Calothrix* sp. PMC 884.14) sont celles qui possèdent le plus grand nombre avec plus de 20 îlots identifiés dans leur génome, supposant une diversité de métabolites produits plus importante. En terme de biosynthèse, la majorité des îlots codent pour des synthétases NRPS, PKS, ou des synthétases hybrides NRPS-PKS. La diversité de molécules synthétisées par ces îlots n'est d'ailleurs qu'en partie élucidée puisque la plupart restent orphelins de produits de biosynthèse et n'ont pas pu être reliés à la production d'une molécule en particulier. Malgré ces inconnues, de nombreux îlots de gènes de biosynthèse ont montré de fortes homologues avec des îlots identifiés chez d'autres cyanobactéries (e.g. chez *L. boryana* PMC 883.14, 8 îlots sur 16 sont homologues à ceux identifiés chez *L. boryana* NIES-2135, **article 3, Table S7**) ouvrant

la possibilité de réaliser des études comparatives entre ces souches, ce qui permettrait de pouvoir ainsi relier les îlots de gènes de biosynthèse avec les métabolites produits.

Comme montré dans le chapitre 3, plusieurs métabolites spécialisés potentiellement bioactifs ont été identifiés dans les extraits des neuf cyanobactéries (**Tableau 2**). Nous avons pu noter la présence de plusieurs mycosporine-like amino acids (MAAs), déjà connues pour leur propriétés photo-protectrices et antioxydantes, qui viennent s'ajouter au potentiel anti-inflammatoire, antioxydant et cicatrisant apporté par les pigments. Néanmoins, d'autres métabolites spécialisés ont pu être identifiés, tel que des cyanobactines, des microginines ou des microviridines qui pourraient apporter des activités non recherchées ou difficilement valorisables (*e.g.* cytotoxicité, inhibition de différentes enzymes, activités antimicrobiennes, ...).

En perspective, il reste à décrire structurellement les différents variants des molécules identifiées. Par exemple pour la famille des microginines, produites par *Aliinostoc* sp. PMC 882.14, qui représentent un groupe de 14 analytes des réseaux moléculaires, il serait intéressant de chercher à purifier et à élucider la structure chimique du variant majoritaire notamment par des approches de RMN (Kenneth E. Milligan et al. 2000). D'un point de vu génomique, il serait intéressant de pouvoir être capable d'inactiver certains gènes de biosynthèse puis d'analyser leur métabolome par comparaison avec la souche « sauvage » *via* des approches de réseaux moléculaires, ce qui devrait permettre l'identification des métabolites synthétisés par ces nouveaux gènes de biosynthèse encore inconnus.

V.1.2.d. *Potentiel des activités observées des cyanobactéries de Balaruc-les-Bains*

Le quatrième volet de ces travaux de thèse a consisté en l'évaluation des activités mesurées pour les extraits bruts des neuf cyanobactéries. Les différents tests de bioactivités ont été réalisés en collaboration avec la Plateforme de chimie biologique intégrative de l'Université de Strasbourg (PCBiS). L'utilisation des approches non ciblées portant sur les extraits bruts, qui représentent un cocktail de molécules non purifiées, a permis d'obtenir un panorama des métabolites produits par les cyanobactéries. En effet, avant même d'envisager toutes perspectives de valorisation, il est nécessaire d'identifier les souches pouvant présenter un intérêt, tout en cherchant à minimiser le risque de présenter des effets néfastes (exploré ici par les activités de cytotoxicité). Néanmoins, il est apparu difficile en fonction des ressources et du temps disponible pour mener à bien ces travaux de multiplier les tests de bioactivité, d'une part parce que ces tests restent coûteux et d'autre part car le test de fractions purifiées nécessite à la fois un grand nombre d'essais ainsi qu'une grande quantité de matériel purifié à tester. Dans le contexte de cette thèse, les extraits bruts ont été testés pour leurs propriétés anti-inflammatoires, antioxydantes et cicatrisantes avec, en parallèle, l'effet de ces extraits sur la viabilité cellulaire des lignées utilisées.

Le seuil de toxicité significatif n'a pas été atteint sur les trois lignées cellulaires testées (RAW 264.7, HaCaT, PMBC). Ces observations s'accordent avec le fait qu'aucune cyanotoxine n'ait été détectée (i.e. MCs, STXs, ANTXs, CYN) aussi bien par les approches génomique que métabolomique. En revanche, des questions se posent sur les activités et/ou la fonctionnalité des variants produits par ces souches pour certaines familles de molécules connues pour leur cytotoxicité, comme c'est le cas par exemple de certains variants de cyanobactines produits par *Laspinema* sp. et *Calothrix* sp. L'hypothèse est que soit les variants sont produits en trop faible quantité pour être toxiques, soit que les lignées sélectionnées ne sont pas sensibles à ces composés.

Pour les différentes bioactivités ici étudiées, on observe que l'activité potentielle prédite par nos analyses et nos connaissances quant à l'activité des différentes familles de composés, n'est pas systématiquement confirmée par les résultats des tests d'activités. En effet, chacune des souches est capable de produire des molécules antioxydantes et anti-inflammatoires, il aurait donc été logique de détecter ces activités pour les extraits. Des facteurs de concentration ou sensibilité des tests pourraient expliquer de telles incongruences, mais d'éventuels effets antagonistes des différents composés présents au sein des extraits le pourraient également.

Concernant les activités anti-inflammatoires, les résultats restent difficiles à interpréter. L'action des effets sur les cytokines pro-inflammatoires varient d'une cytokine à l'autre (**Article 3, Table 7**), par exemple l'extrait méthanol de *M. vaginatus* PMC 879.14 inhibe la sécrétion d'IL-1 β et active celle d'IL-6, questionnant les mécanismes moléculaires et cibles d'action des molécules actives de ces extraits. Certaines cyanobactéries peuvent toutefois présenter une réponse plus claire de par l'inhibition de la sécrétion de plusieurs cytokines sans en activer d'autres. Ainsi, *Planktothricoides raciborskii* PMC 877.14 paraît confirmer son potentiel anti-inflammatoire pour les deux types d'extraits (aqueux et méthanol) qui semblent clairement bioactif, ou encore l'extrait méthanol de *Nostoc* sp. PMC 881.14 et l'extrait aqueux de *Pseudo-chroococcus couteii* PMC 885.14. Il est d'ailleurs intéressant de constater que les deux types d'extraits sont susceptibles de montrer des activités anti-inflammatoires, suggérant une diversité des composés impliqués. Si l'activité de l'extrait aqueux de *P. raciborskii* est potentiellement dû à la quantité plus importante de phycocyanine extraite, cette explication ne convient pas pour *P. couteii* pour qui 10 fois moins de phycocyanine a été extraite (**Article 3, Table 4**). Il est donc intéressant de se demander quelles molécules pourraient être responsables de ces activités. Comme évoqué dans la partie « discussion » de l'article 3, certains dérivés de lipides ou encore des exopolysaccharides (EPS) produits par *Phormidium* sp. ETS05, isolée de boues thermales en Italie ont montré des activités anti-inflammatoires (Zampieri et al. 2020; Bruno et al. 2005). Il serait alors judicieux, dans le but d'identifier les molécules responsables de ces activités anti-inflammatoires, de fractionner les extraits des souches de Balaruc de manière à isoler les fractions contenant les EPS ou les lipides et de réaliser à nouveau les tests d'activités en parallèle des réseaux moléculaires selon la méthode développée par l'équipe de W. Gerwick (Naman et al. 2017).

Concernant l'activité cicatrisante, seul l'extrait aqueux d'*Aliinostoc* sp. PMC 882.14 a montré un résultat positif avec une augmentation de 22% de la cicatrisation pendant la période du test. Comme l'activité d'une molécule dépend de sa capacité à se lier à son récepteur cible et dépend donc de sa structure, l'hypothèse proposée (**Article 3**) est que ces propriétés pourraient être portées par certaines microginines produites par *Aliinostoc* sp., présentant des similitudes structurales avec l'apratyramide isolée de *Moorea bouillonii* (**Figure 6**) (Cai et al. 2018).

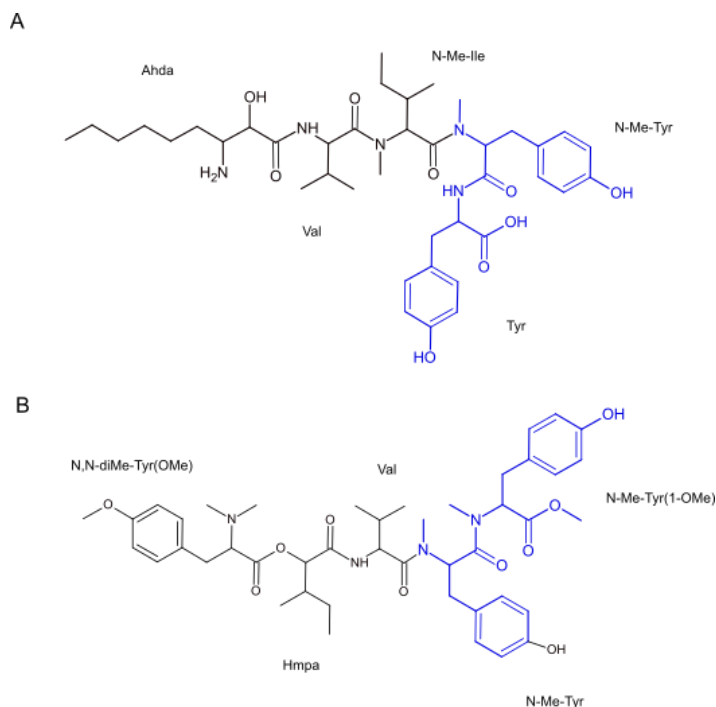


Figure 6. (A) Structure prédite de la microgine produite par *Aliinostoc* sp. PMC 882.14. (B) Structure de l'apratyramide isolée de *Moorea bouillonii*

Cette hypothèse pourrait se vérifier en isolant ces microginines et en testant l'activité de cicatrisation de la fraction purifiée ou encore en comparant la structure prédite de différents variants de microginines d'*Aliinostoc* sp. à l'aide des nouveaux outils bio-informatiques de prédiction de l'activité comme l'outil MuSSeL (Alberga et al. 2019).

La troisième série de tests visait à déterminer les propriétés antioxydantes des extraits des cyanobactéries de Balaruc. Aucun effet antioxydant n'a pu être mis en évidence, et ce malgré le nombre important de molécules détectées et référencées pour cette activité. Pour expliquer ce résultat, l'hypothèse proposée est que la concentration de ces molécules dans les différents extraits ait été trop faible et/ou que des effets antagonistes pourraient intervenir entre plusieurs molécules comme par exemple avec les lipopolysaccharides (LPS) connus pour augmenter le stress oxydatif (I. Stewart et al. 2006). Afin de confirmer ou d'infirmer cette absence d'activité antioxydante, il serait intéressant de réaliser d'autres tests d'activité ciblant d'autres molécules candidates impliquées dans les mécanismes du stress oxydatif. Il existe plusieurs tests *in vitro* comme le test DPPH (2,2-diphényl-1-picrylhydrazyl), qui fait partie des radicaux libres qui sont « piégés » par les molécules antioxydantes pour lutter contre

le stress oxydatif, ou encore d'autres tests *in vivo*, comme notamment l'observation de l'inhibition de la production d'espèce réactive de l'oxygène (ROS) chez le ver *Caenorhabditis elegans* (S. N. Patel et al. 2018).

Pour finir, il serait intéressant d'envisager d'autres tests afin d'évaluer l'action des cyanobactéries sur d'autres cibles intéressantes pour la valorisation. En effet, les soins pratiqués aux Thermes sont orientés sur les problèmes articulaires, pour lesquels des molécules anti-inflammatoires sont utiles, mais également contre les maladies du système circulatoire telles que les phlébites. Dans ce contexte, il serait intéressant de tester des activités comme l'inhibition de la thrombine et de l'enzyme de conversion de l'angiotensine (ACE), deux molécules impliquées dans les mécanismes de la coagulation et pouvant être mises en œuvre dans les thérapies dirigées contre les maladies vasculaires. Plusieurs métabolites produits par les cyanobactéries ont d'ailleurs déjà montré ce type d'activité inhibitrice, notamment certaines cyanopeptolines (inhibition de la thrombine) et certaines microginines (inhibition de l'ACE) (Zervou et al. 2020; Bonjouklian et al. 1996).

V.1.2.e. Variation de la production des métabolites en fonction des conditions : *Aliinostoc* sp. PMC 882.14

Considérant que chez de nombreux microorganismes la production de métabolites varie en fonction des phases de croissance et des conditions de culture (Barofsky et al. 2009; Natumi and Janssen 2020), il est essentiel d'étudier l'importance de ces paramètres sur le contrôle de production de métabolites d'intérêt par les cyanobactéries de Balaruc-les-Bains. En effet, lorsqu'il est question de bio-production d'une molécule, il est nécessaire de connaître les conditions de culture optimales pour sa production notamment : quand est-elle produite en quantité la plus importante ? Et quelles sont les conditions favorables pour en obtenir le maximum ? La souche *Aliinostoc* sp. PMC 882.14 a été sélectionnée pour le grand nombre d'îlots de gènes de biosynthèse de métabolite susceptibles de varier (21 îlots) et pour l'identification de plusieurs métabolites photo-protecteurs et donc probablement photo-induits (*e.g.* shinorine, scytonémine) (**Chap. IV**). Ces travaux (D. Le Moigne, Master 2) nous ont permis de mettre en évidence qu'au-delà de la variation métabolomique induite par les conditions de culture testées (lumière, température), c'est la phase de croissance et donc l'état physiologique qui impacte le plus le métabolome d'*Aliinostoc* sp. PMC 882.14.

À l'échelle des familles de métabolites, les différents variants n'ont pas forcément tous le même profil de production (**Annexe 3, Annexe 4**) Certains variants sont produits en quantité supérieure sous des conditions de température plus élevée (*e.g.* palythine) alors que d'autres composés apparaissent produits en plus grande quantité en condition de lumière plus élevée (*e.g.* shinorine). Ces résultats ouvrent des questions concernant les modes de régulation de la biosynthèse de ces composés. Par exemple, une seule voie de biosynthèse a été décrite à ce jour pour la synthèse des MAAs (Pathak et al. 2019; Balskus and Walsh 2010) (la famille de métabolite à laquelle appartiennent la shinorine et la

palythine), il est intéressant de se demander quels sont les procédés qui, en réponse à des conditions particulières, conduisent à la synthèse préférentielle de l'une ou l'autre de ces molécules. Certains gènes sont-ils surexprimés ? Peut-on reconnaître des séquences régulatrices au voisinage des îlots de gènes de biosynthèse concernés ? D'autres gènes que ceux identifiés interviennent-ils ? Ou est-ce une question de disponibilité de substrat ? En effet, lorsque l'on compare les différences structurales des MAAs produites par *Aliinostoc* sp. (**Figure 7**), on peut imaginer une voie de biosynthèse unique capable de se moduler en fonction des besoins de la cellule et qu'il pourrait exister des « priorités » de synthèse en fonction : i/ des substrats disponibles ou, ii/ des conditions « stressantes » nécessitant la synthèse rapide de molécules « protectrices ».

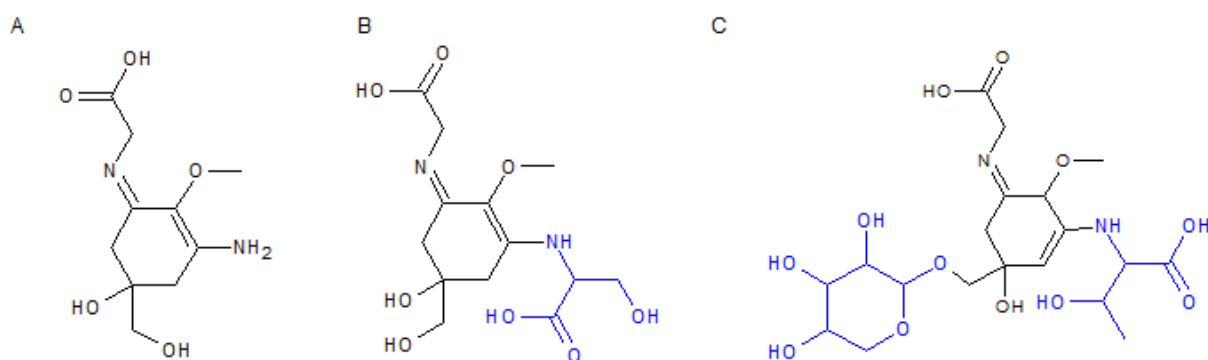


Figure 7. MAAs identifiées chez *Aliinostoc* sp. PMC 882.14. (A) Palythine; (B) Shinorine; (C) 478-MAA

Ces considérations sont également de première importance pour des perspectives de valorisation. En effet, la palythine ou la shinorine sont des molécules antioxydantes et photo-protectrices qui ont prouvé leurs bienfaits (Lawrence et al. 2018) et font désormais l'objet de travaux de recherche utilisant des cyanobactéries modifiées génétiquement pour augmenter la production de ces molécules d'intérêt (G. Yang et al. 2018). Des différences de production de métabolites en fonction du type de milieu de culture utilisé ont été observées (**Annexe 3, Annexe 4**), au cours d'une expérimentation pilote menée sur un cycle de repiquage. Par exemple, la shinorine serait produite en quantité plus importante lorsque la souche est cultivée dans le milieu BG11 par comparaison au milieu Z8 dans lequel elle est cultivée précédemment. Mais ces travaux mériteraient de pouvoir être maintenant répétés sur plusieurs cycles.

Les résultats obtenus lors de ces travaux de thèse ouvrent d'autres perspectives, notamment concernant la production pigmentaire. Il serait intéressant d'étudier l'évolution de la production de certains pigments selon les phases de croissance et les conditions de culture (qualité de la lumière qui peut jouer sur la production de phycoérythrine *via* l'adaptation chromatique). Comme discuté dans le paragraphe précédent, il serait intéressant de coupler les analyses LC-MS/MS à des tests d'activité sur différents points de la cinétique de culture des souches afin d'évaluer si l'activité évolue en parallèle de la production de certains composés en particulier.

Les conditions (quantité lumineuse et température) ayant été volontairement choisies pour ne pas provoquer de « stress » pour *Aliinostoc* sp. PMC 882.14, il serait intéressant d'observer le comportement des cyanobactéries de Balaruc et de leurs métabolomes sous des pressions environnementales plus drastiques : absence d'alternance de phase jour/nuit (lumière continue), forte quantité de lumière, température élevée ... D'autres paramètres, comme la salinité du milieu de culture, pourraient également être testés étant donné que l'eau thermale de Balaruc-les-Bains contient des quantités spécifiques de sels (**Annexe 1**) bien supérieures au milieu de culture actuel des souches.

Enfin, une autre perspective serait d'observer « l'acclimatation » des souches à de nouvelles conditions en incluant une série de repiquages successifs afin d'étudier les processus d'adaptation lors des étapes de réensemencement avant transfert vers une échelle industrielle de bio-production.

V.1.3. Perspective de valorisation

Étant donné la nature de cette thèse CIFRE et la collaboration de recherche entre les Thermes de Balaruc-les-Bains et le Muséum National d'Histoire Naturelle, les travaux menés au cours de cette thèse ont été conçus pour ouvrir, en plus des questions abordées, des perspectives en terme de valorisation. En effet, cette thèse a permis d'évaluer au sens large le potentiel des différentes cyanobactéries issues des boues thermales pour des applications thermales thérapeutiques. Parmi ces applications, deux principaux modes d'utilisation semblent envisageables : l'utilisation de la biomasse « entière » d'une part, et l'utilisation de molécules bioactives extraites des cyanobactéries d'autre part. Les différents challenges et pistes de réflexion sont détaillés afin de favoriser l'un ou l'autre de ces procédés.

V.1.3.a. Valorisation de la biomasse

La pélothérapie¹⁰ a été utilisée depuis plusieurs siècles à Balaruc-les-Bains. Si aujourd'hui les normes sanitaires obligent les établissements accueillant du public à contrôler parfaitement les composants des boues, il serait intéressant de mettre au point un procédé reproduisant les bienfaits apportés par la maturation du péloïde en ajoutant au mélange boue/eau thermale de la biomasse de cyanobactéries, originaires des boues de Balaruc-les-Bains, et cultivées dans des conditions contrôlées. Il suffirait de développer un procédé de culture optimale pour la croissance des cyanobactéries (et des métabolites bioactifs) puis de conditionner cette biomasse (filtrage, pressage, congélation ou lyophilisation) avant de l'intégrer en quantité contrôlée (broyée ou non) dans le mélange boue/eau thermale (Mourelle et al. 2017).

La problématique de cette approche est de développer ce procédé de culture pour la ou les cyanobactérie(s) que l'on souhaite ajouter aux boues. Ce procédé doit être réfléchi pour produire la

¹⁰ Processus utilisant des boues mûries contenant différents microorganismes dans les soins thermaux

quantité de biomasse nécessaire pour respecter la concentration en cyanobactéries à incorporer au procédé, et les travaux à envisager dépendant du type de culture à mettre en place.

Si c'est une culture en milieu solide, il faut évaluer les capacités de croissance des cyanobactéries sélectionnées pour ce mode de culture et optimiser l'accès au nutriment. Cette technique favorise la croissance des cyanobactéries en biofilm et permet de récolter facilement la biomasse par un « raclage manuel » du support.

Si à l'inverse, la culture liquide des cyanobactéries de Balaruc est conservée, il faudrait envisager une étude d'augmentation des volumes de culture afin de disposer de plus grandes biomasses. Cette méthode nécessite d'évaluer la capacité des cyanobactéries à se multiplier, de mettre en place un procédé de récolte efficace (par filtration, pressage ou centrifugation), d'optimiser l'accès aux ressources (lumière plus ou moins accessible dans l'ensemble du bioréacteur ; utilisation de lame UV ; supplémentation ou non des minéraux nécessaires à la croissance) et éventuellement de maintenir la culture à son optimal de croissance en contrôlant les paramètres physico-chimiques (e.g. lumière, température).

Pour les deux méthodes, la meilleure façon d'ensemencer les cultures est à définir ainsi que la meilleure façon de s'assurer de la qualité et de la pérennité de l'inoculum, notamment en terme de proportion de bactéries hétérotrophes associées. D'un point de vue contrôle sanitaire et qualité, différents points de contrôle seront à mettre en place afin de s'assurer de la non-contamination des cultures (hors cyanosphère) et éventuellement de la teneur en métabolites d'intérêt.

Dans ce cadre, il serait intéressant d'utiliser un mélange de plusieurs cyanobactéries isolées des boues thermales (par exemple *P. raciborskii*, *Nostoc* sp., *Aliinostoc* sp. et *P. couteii*) dans l'optique de s'approcher au plus près des propriétés historiques du péloïde des Thermes de Balaruc-les-Bains et chercher à potentialiser les différentes bioactivités de ces souches.

V.1.3.b. *Production et purification de molécules bioactives*

L'utilisation de molécules bioactives pures peut paraître plus adaptée à des procédés pharmacologiques ou cosmétiques. En effet, une fois bien caractérisés, l'activité et le mode d'action d'une molécule n'évoluent pas, au contraire de la biomasse composée de cellules de cyanobactéries vivantes qui peuvent évoluer lors des différentes cultures. Néanmoins, l'utilisation de molécules pures, notamment en pharmacologie, est particulièrement difficile du fait de l'obtention des agréments nécessaires à l'utilisation de nouvelles molécules. Si le processus de validation semble plus rapide dans les industries cosmétiques puisque leur commercialisation ne nécessite pas d'autorisation préalable de mise sur le marché, il est nécessaire de réaliser plusieurs études pour respecter la réglementation et construire le dossier d'information du produit qui doit attester de sa sécurité pour le consommateur (ANSM 2014).

Au-delà de ces travaux qui consistent principalement en des tests d'activités visant à connaître les effets de la molécule, il faut dans un premier temps optimiser le procédé de production de la ou des molécule(s) cible(s). Cela peut nécessiter, à l'instar des travaux relatés au chapitre IV, de savoir à quel moment de la cinétique de culture le composé est produit et quelles sont les conditions optimales pour sa production. Cela peut comprendre également la mise au point d'un procédé d'extraction et de purification efficace du composé bioactif.

Une fois ces points fixés, il faut développer un procédé de production à grande échelle étant donné que la proportion de molécule purifiée est souvent faible par rapport à la biomasse (par exemple la phycocyanine extraite à partir de *P. raciborskii* de $33\mu\text{g}\cdot\text{mg}^{-1}$ de poids sec). Plusieurs procédés existent pour produire de la biomasse, le choix du procédé se fait donc en fonction de la valeur marchande de la molécule à produire. Un avantage des cyanobactéries repose sur le fait qu'elles sont autotrophes, c'est-à-dire qu'elles sont capables de produire leur propre matière organique grâce à la photosynthèse et aux nutriments minéraux. Les cyanobactéries ont donc essentiellement besoin de lumière et de relativement peu de nutriments par rapport aux autres bactéries (Apel and Weuster-Botz 2015).

Pour la production de molécules à faible valeur ajoutée comme les biocarburants, le procédé de production doit être le moins coûteux possible afin d'être rentable. Ainsi, le procédé de production le moins coûteux est probablement celui utilisé un peu partout dans le monde pour la culture de la Spiruline (*Arthrospira platensis*). Ce procédé consiste à faire croître la cyanobactérie à l'air libre dans de grandes structures de culture. Les limites de ce système sont qu'il nécessite des conditions favorables constantes (suffisamment d'ensoleillement, température variant peu et non extrême) et qu'il n'est pas transposable à toutes les espèces de cyanobactéries. De plus, ce mode de culture peut être sujet à la contamination par d'autres espèces de cyanobactéries ou de microalgues (certaines potentiellement toxiques) qui viendrait altérer le rendement de production de la molécule ciblée ou l'innocuité du matériel produit (Zahra et al. 2020; Furmaniak et al. 2017).

La seconde alternative est la culture en bioréacteurs fermés selon le même principe que la production de biomasse évoquée précédemment. Ce procédé est très pratique puisqu'il permet un contrôle quasi total sur la production car les différents paramètres peuvent être suivis en temps réel. Néanmoins il peut s'avérer coûteux en énergie puisqu'il faut produire de la lumière, chauffer à la température optimale et éventuellement agiter la culture de façon mécanique ou par bullage, et ne convient donc qu'à la production de molécules à haute valeur ajoutée (Zahra et al. 2020).

V.2. Conclusion générale

Les travaux menés au cours de cette thèse CIFRE en collaboration entre les Thermes de Balaruc-les-Bains et le Muséum National d'Histoire Naturelle ont permis l'évaluation des propriétés anti-

inflammatoires, antioxydantes et pro-cicatrisantes potentielles de neuf cyanobactéries isolées des boues thermales de Balaruc-les-Bains.

Par une approche multiphasique regroupant génomique, métabolomique et tests de bioactivité, et également au regard de la littérature, nous avons pu mettre en évidence le haut potentiel de production de molécules bioactives de quatre cyanobactéries en particulier : *Planktothricoides raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14, *Pseudo-chroococcus couteii* PMC 885.14 (activités anti-inflammatoires) et de *Aliinostoc* sp. PMC 882.14.

Ces résultats ouvrent de nombreuses perspectives tant dans le domaine de la valorisation industrielle avec le développement d'applications thérapeutiques, que dans le domaine de la recherche avec l'apport de nouvelles questions sur le rôle des métabolites de cyanobactéries, leur régulation, ou encore le rôle des bactéries associées au sein de la cyanosphère. Ces travaux ont également permis de montrer l'importance de la validité et la mise à jour des connaissances, notamment taxonomiques, référencées dans les bases de données à l'heure du séquençage haut-débit et de l'essor des approches omiques.

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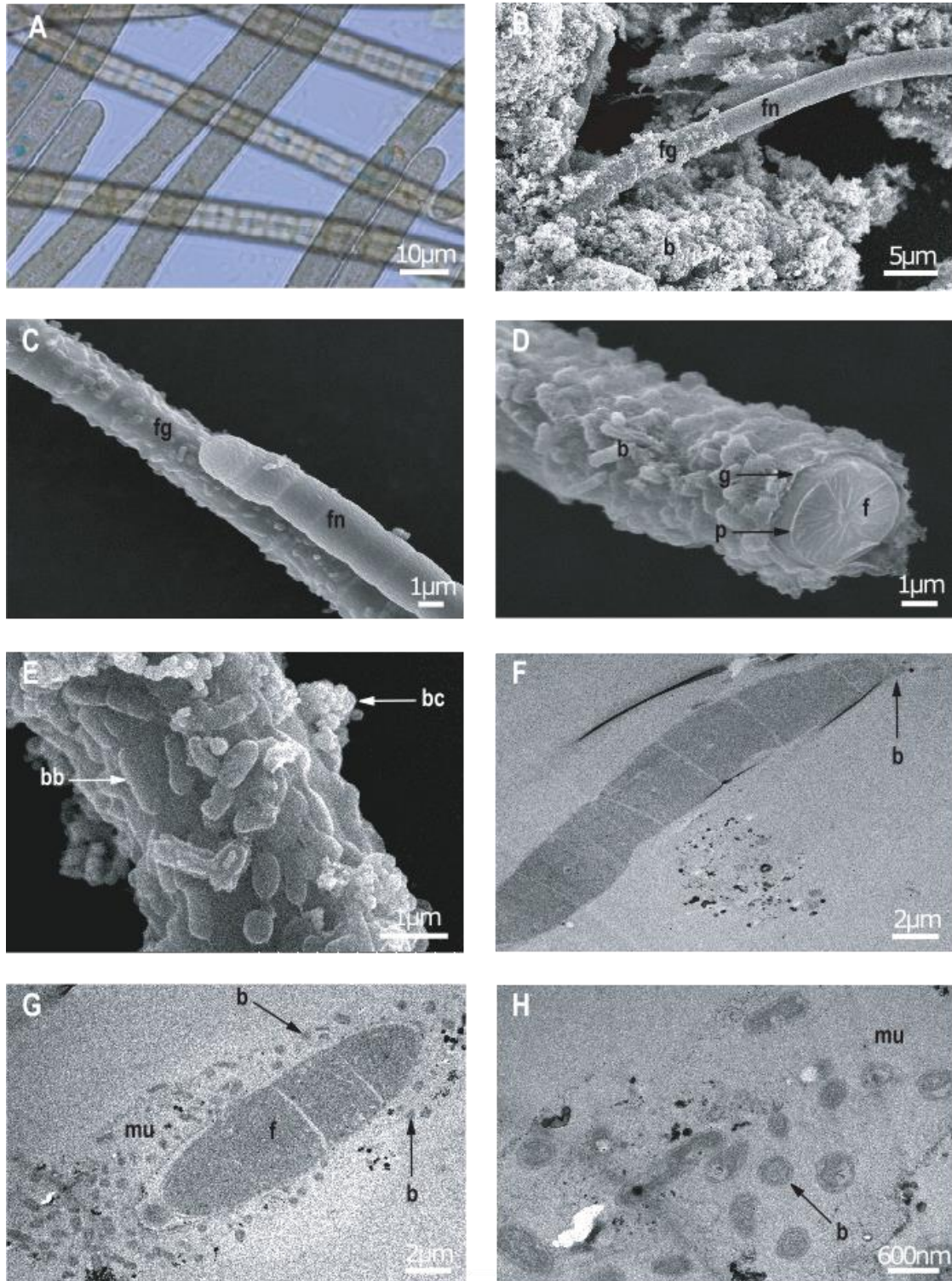
Structure to Genes and Vice Versa.” *Applied and Environmental Microbiology* 74 (6): 1791–97.
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Annexes

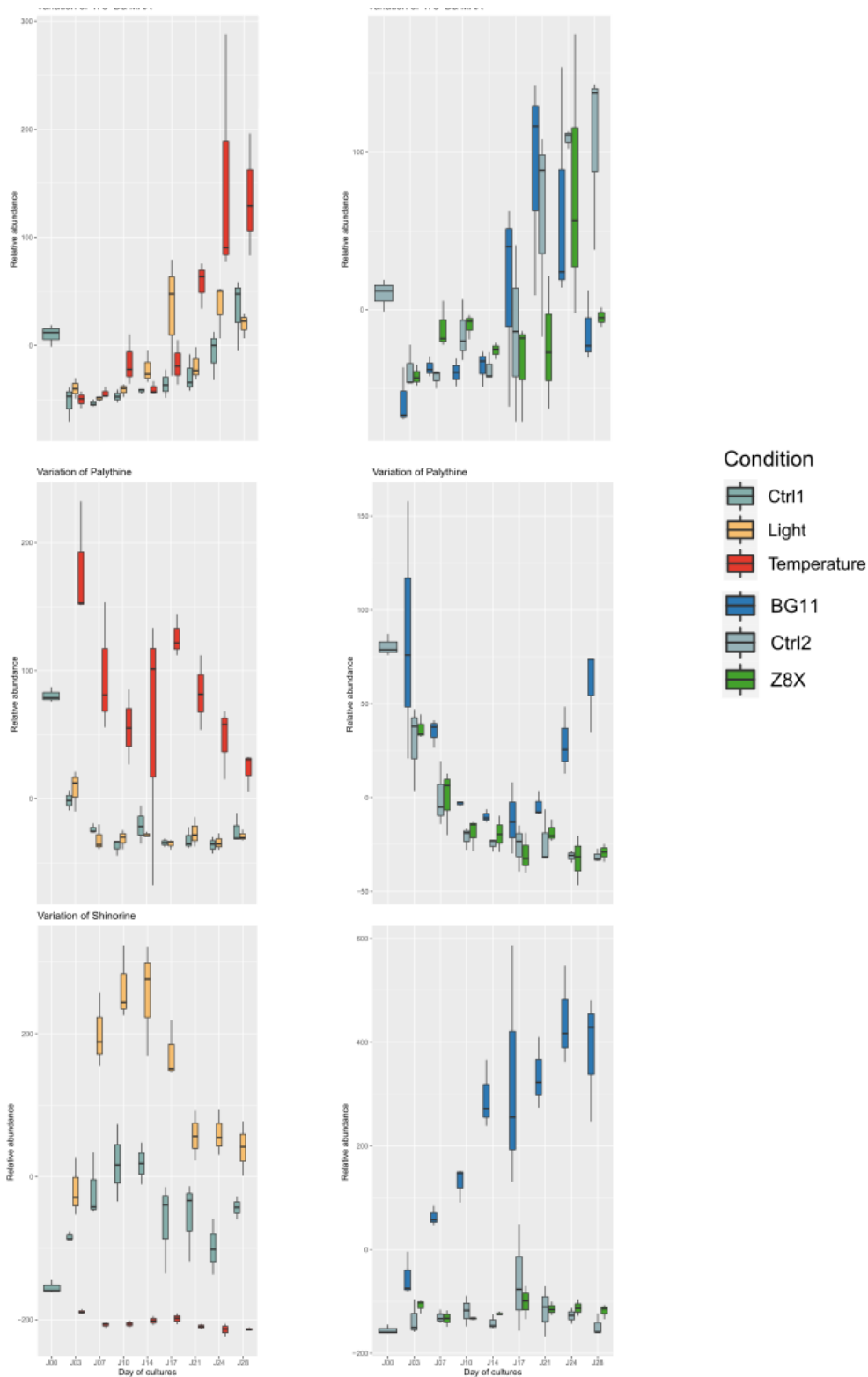
Annexe 1. Composition ionique des eaux thermales de Balaruc-les-Bains

Cations mg.L ⁻¹		Anions mg.L ⁻¹	
Calcium, Ca ²⁺	560	Bicarbonates, HCO ₃ ⁻	550
Magnésium, Mg ²⁺	380	Chlorure, Cl ⁻	6 800
Sodium, Na ⁺	3 700	Sulfates, SO ₄ ²⁻	900
Potassium, K ⁺	120	Fluor, F ⁻	0,35
Fer, Fe ²⁺	0,10	Brome, Br ⁻	0,7
Manganèse, Mn ²⁺	0,042		
Aluminium, Al ³⁺	0,02		

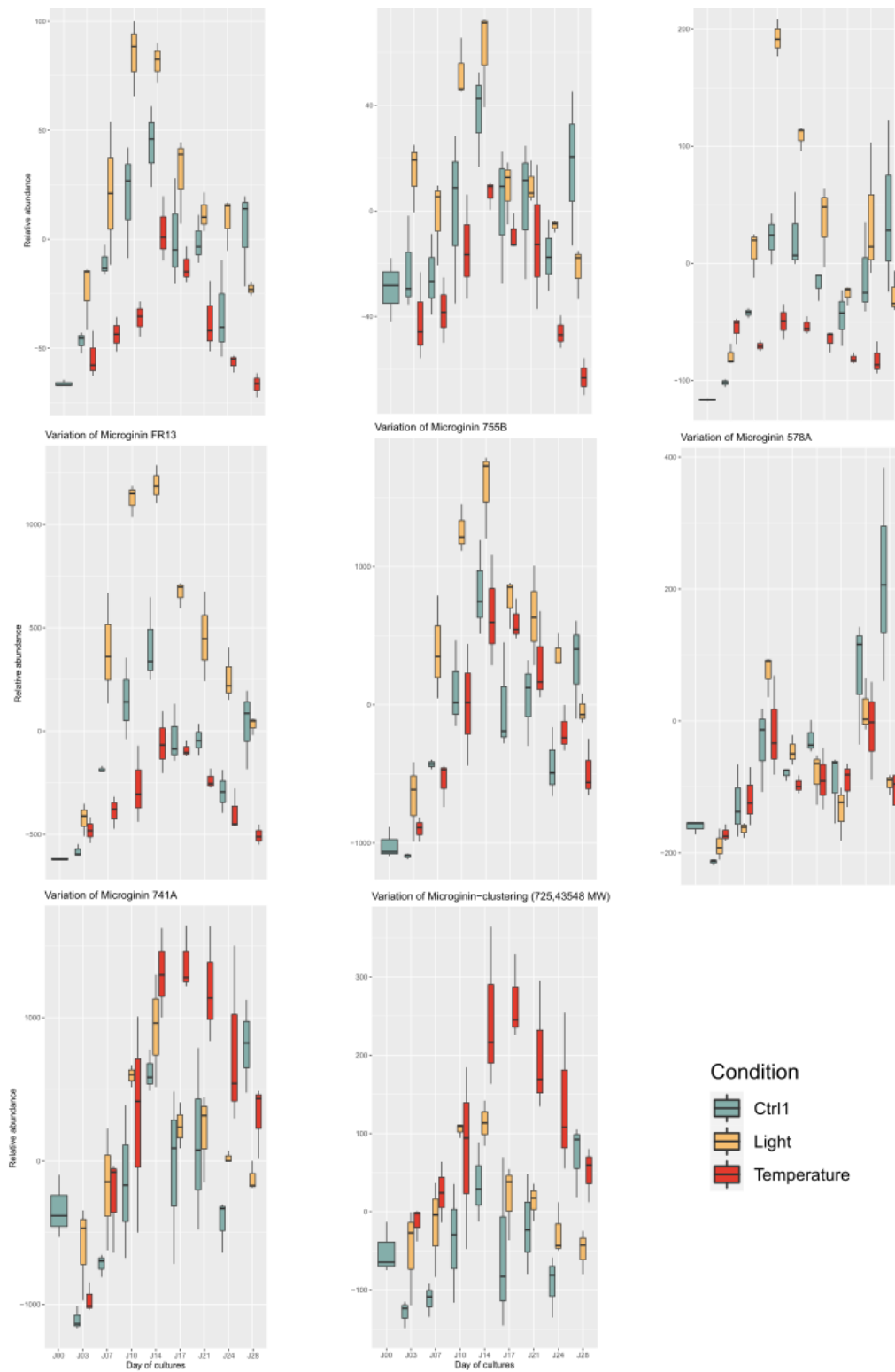
Annexe 2. Observation de la cyanobactérie *Planktothricoides raciborskii* PMC 877.14 et des communautés bactériennes associées. A: observation au microscope photonique de filaments de *P. raciborskii*; B-C-D-E: observation au MEB de filaments de *Phormidium nigrum* sans gaine (fn), avec gaine (fg) et de bactéries (b), associées aux filaments, en forme de coques (bc) ou de bacilles (bb); F-G: observation au MET d'une coupe longitudinale de filaments de *P. raciborskii* avec ou sans mucilage et des bactéries associées. g: gaine, p: paroi, mu: mucilage, f: filament.



Annexe 3. Évolution de l'abondance relative des métabolites de la famille des MAAs chez *Aliinostoc* sp. PMC 882.14.



Annexe 4. Évolution de l'abondance relative des métabolites de la famille des microginines chez *Aliinostoc* sp. PMC 882.14



Annexe 5 : Supplementary materials – Article 1

3 supplementary tables

Table S1. Amino acid sequences of the cyanopeptolin-like family (capture d'écran, table complète disponible <https://www.mdpi.com/1660-3397/17/6/320#supplementary>)

Ref	Strain	Variant name	Core aa sequence							Side chain sequence			
			1st residue	2nd	3rd	4th	5th	6th	7th	8th	9th		
Bonjoukian_19f	<i>Microchaete</i> A90720A		L-Thr	L-Arg	L-Ahp	L-Leu	L-Thr	L-N-MeTyr	L-Val	D-Leu	3-O-sulfated		Ahp: 3-amino-6-hydroxy-2-piperidone
Harada_1993	<i>Microcystis</i> Aeruginopeptin 228-A		Thr	Tyr	Ahp	Thr	Thr	N-MePhe	Ile	Gln	Hpla		
Harada_1993	<i>Microcystis</i> Aeruginopeptin 228-B		Thr	ThTyr	Ahp	Thr	Thr	N-MePhe	Ile	Gln	Hpla		Hpla : 4-hydroxyphenyl-lactic acid
Harada_2001	<i>Microcystis</i> Aeruginopeptin 917S-A		Thr	Tyr	Ahp	Leu	Leu	N-MeTyr	Ile	Gln	Hpla		ThTyr : Tetrahydroxytyrosine
Harada_2001	<i>Microcystis</i> Aeruginopeptin 917S-B		Thr	ThTyr	Ahp	Leu	Leu	N-MeTyr	Ile	Gln	Hpla		
Harada_2001	<i>Microcystis</i> Aeruginopeptin 917S-C		Thr	Leu	Ahp	Leu	Leu	N-MeTyr	Ile	Gln	Hpla		
Harada_1993	<i>Microcystis</i> Aeruginopeptin 95-A		Thr	Tyr	Ahp	Thr	Thr	N-MePhe	Ile	Gln	Thr		
Harada_1993	<i>Microcystis</i> Aeruginopeptin 95-B		Thr	ThTyr	Ahp	Thr	Thr	N-MePhe	Ile	Gln	Thr		
Fujii_2002	<i>Anabaena</i> s1 Anabaenopeptilide 202A		L-Thr	L-Hty	L-Ahp	L-Thr	L-Thr	L-N-dlMeTyr	L-Ile	L-Glu	N-formyl L-Pro		
Fujii_2002	<i>Anabaena</i> s1 Anabaenopeptilide 202B		L-Thr	L-Hty	L-Ahp	L-Thr	L-Thr	L-N-Chloro-MeTyr	L-Ile	L-Glu	N-formyl L-Pro		
Fujii_2002	<i>Anabaena</i> s1 Anabaenopeptilide 90A		L-Thr	L-Hty	L-Ahp	L-Thr	L-Thr	L-N-dlMeTyr	L-Ile	L-Gln	N-formyl		
Fujii_2002	<i>Anabaena</i> s1 Anabaenopeptilide 90B		L-Thr	L-Hty	L-Ahp	L-Thr	L-Thr	L-N-Chloro-M-Ile	L-Ile	L-Gln	N-formyl		
Okumura_2009	<i>Planktothrix</i> Cyanopeptolin 880		Thr	Hty	Ahp	Ile	Ile	N-Phe	Ile	L-Gln	2-Ome-GA		2-Ome-GA : E-O-methyl-D-glyceric acid res
Von Eiert_2015	<i>Microcystis</i> Cyanopeptolin 954		L-Thr	L-Leu	L-Ahp	L-Phe	L-Phe	L-N-chloro-M	L-Val	Gln	sulfated 2-Ome-GA		
Okumura_2009	<i>Planktothrix</i> Cyanopeptolin 960		Thr	Hty	Ahp	Ile	Ile	N-Phe	Ile	L-Glu	2-Ome-GA		
Martin_1993	<i>Microcystis</i> Cyanopeptolin A		L-Thr	L-Arg	Ahp	L-Leu	L-Leu	N-MePhe	L-Val	L-Asp	HA		HA : Hexanoic acid
Martin_1993	<i>Microcystis</i> Cyanopeptolin B		L-Thr	L-Lys	Ahp	L-Leu	L-Leu	N-MePhe	L-Val	L-Asp	HA		
Martin_1993	<i>Microcystis</i> Cyanopeptolin C		L-Thr	L-N-MeLys	Ahp	L-Leu	L-Leu	N-MePhe	L-Val	L-Asp	HA		
Martin_1993	<i>Microcystis</i> Cyanopeptolin D		L-Thr	L-N-dlMeLys	Ahp	L-Leu	L-Leu	N-MePhe	L-Val	L-Asp	HA		
Jakobi_1995	<i>Microcystis</i> Cyanopeptolin S		L-Thr	L-Arg	Ahp	L-Ile	L-Ile	L-N-MePhe	L-Ile	SulfGlc			SulfGlc : Sulphated glyceric acid
Jakobi_1996	<i>Microcystis</i> Cyanopeptolin SS		L-Thr	L-Arg	Ahp	L-Ile	L-Ile	L-N-MePhe	L-Ile	diSulfGlc			
Choi_2008	<i>Aphanocaps</i> Cyanopeptoline CB071		L-Thr	L-Arg	L-Ahp	L-Ile	L-Ile	L-N-O-dlMeTyr	L-Val	Glu	HA		
Matern_2003	<i>Scytonema</i> / Hofmannolin		Thr	Tyr	Ahp	O-MeTyr	O-MeTyr	N-MeTyr	Val	Glu	Hmv		Hmv : 2-hydroxy-3-methylvaleric acid
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide A		Hmp	L-Leu	Ahp	L-Ile	L-Ile	N-MeTyr	L-Val	Acetic acid	L-Citrulline		Hmp : 3-hydroxy-4-methyl-proline
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide B		Hmp	L-Leu	Ahp	L-Ile	L-Ile	N-MeTyr	L-Val	Acetic acid	L-Citrulline		
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide C		Hmp	L-Leu	Ahp	L-Ile	L-Ile	L-N-O-dlMeTyr	L-Val	Acetic acid	L-Citrulline		
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide D		Hmp	L-Leu	Ahp	L-Ile	L-Ile	L-N-O-dlMeTyr	L-Val	Acetic acid	L-Citrulline		
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide E		L-Thr	L-Hphe	Ahp	L-Thr	L-Thr	L-N-MeTyr	L-allo-Ile	L-Ser	L-Pro	BA	
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide F		L-Thr	L-Hphe	Ahp	L-Thr	L-Thr	L-N-MeTyr	L-Val	L-Ser	L-Pro	BA	
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide G		L-Thr	L-Hphe	Ahp	L-Thr	L-Thr	L-N-MePhe	L-Val	L-Ser	L-Pro	BA	
Mehner_2008	<i>Nostoc insul</i> Insulaeptolide H		L-Thr	L-Hphe	Ahp	L-Thr	L-Thr	L-N-MePhe	L-allo-Ile	L-Ser	L-Pro	BA	
Gallegos_2018	<i>Symploca</i> s1 Jizanpeptin A		L-Thr	L-Lys	L-Ahp	L-Ile	L-Ile	L-N-O-dlMeBr	L-Ile	D-Val	Gac-sulfate		Gac-sulfate : 3-O-sulfated glyceric acid
Gallegos_2018	<i>Symploca</i> s1 Jizanpeptin B		L-Thr	L-Lys	L-Ahp	L-Ile	L-Ile	L-N-O-dlMeBr	L-Ile	D-Val	Mgs		Mgs : 2-O-methylated glyceric acid sulfate
Gallegos_2018	<i>Symploca</i> s1 Jizanpeptin C		L-Thr	L-Lys	L-Ahp	L-Ile	L-Ile	L-N-O-dlMeBr	L-Ile	L-Val	Mgs		
Gallegos_2018	<i>Symploca</i> s1 Jizanpeptin D		L-Thr	L-Lys	L-Ahp	L-Ile	L-Ile	L-N-O-dlMeBr	L-Ile	L-Ile	Mgs		
Gallegos_2018	<i>Symploca</i> s1 Jizanpeptin E		L-Thr	L-Arg	L-Ahp	L-Ile	L-Ile	L-N-O-dlMeBr	L-Ile	D-Val	Mgs		

Table S2. Cyanobacterial metabolite database (capture d'écran des 15 premières entrées ; table complète disponible <https://www.mdpi.com/1660-3397/17/6/320#supplementary>)

Metabolite family	Abbreviated Synonyms	Variants	Chemical class: Structure	Metabolic pathway	Order	Family	Genus	Species	Strain	Note	Ecosystem	Site
1,8-dihydroxy-4-methylanthraquinone		1	Aromatic corr Cyclic	NA	Nostocales	Nostocaceae	<i>Nostoc</i>	<i>Nostoc comi</i>	EAWAG 122b	Environmenta	Terrestrial	Switzerland
2,5-dimethylododecanoic acid		2	Fatty acid	NA	Oscillatoriales	Oscillatoriaceae	<i>Lyngbya</i>	<i>Lyngbya sp.</i>	<i>Lyngbya</i> a6	Environmenta	Marine	Australia
2-methylisoborneol	MIB	1	Terpene Cyclic	Terpene synthase	Synechococci	Pseudanabaenaceae	<i>Pseudanaba</i>	<i>Pseudanaba dqt15</i>		Freshwater		California
4-hydroxy-7-methylindan-1-one		1	Aromatic corr Cyclic	NA	Nostocales	Nostocaceae	<i>Nostoc</i>	<i>Nostoc comi</i>	EAWAG 122b	Environmenta	Terrestrial	Switzerland
6,11-epoxyisodaucane		1	Terpene Cyclic	NA	Nostocales	Rivulariaceae	<i>Calothrix</i>	<i>Calothrix sp.</i>	PCC 7508	Axenic	Sphagnum bo	Switzerland
6-cyano-indolocarbazoles		2	Alkaloid Cyclic	NA	Nostocales	Nostocaceae	<i>Nostoc</i>	<i>Nostoc spha</i>	EX-5-1	Environmenta	Soil	Hawaii
9-Ethyliminomethyl-12-(morpholin-4-yl)-EMTAHDACA		1	Carboxylic aci Cyclic	NA	Nostocales	Nostocaceae	<i>Nostoc</i>	<i>Nostoc sp.</i>	MGL001	Unialgal cultu	Freshwater	India
Abietic acids		2	Terpene Cyclic	NA	Oscillatoriales	Oscillatoriaceae	<i>Microcoleus</i>	<i>Microcoleus lacustris</i>	LEGE : 10388	NA	Freshwater ; f	Japan
Acetylphycins		2	Macrolide Cyclic	NA	Oscillatoriales	Oscillatoriaceae	<i>Oscillatoria</i>	<i>Oscillatoria acutissima</i>		Environmenta	Freshwater	Hawaii
Aerucydamides	Aerucyclamid	4	Peptide Cyclic	NA	Chroococcales	Microcystaceae	<i>Microcystis</i>	<i>Microcystis</i>	PCC 7806	Environmenta	Freshwater	The Netherlar
Aeruginazoles	Aeruginazole	3	Peptide Cyclic	NA	Chroococcales	Microcystaceae	<i>Microcystis</i>	<i>Microcystis aeruginosa</i>		Environmenta	Freshwater	Israel
Aeruginoguanidines	Aeruginogua	3	Peptide Mixed	NA	Chroococcales	Microcystaceae	<i>Microcystis</i>	<i>Microcystis</i>	NIES-98, 91, 91	Axenic	Freshwater	Japan
Aeruginosamide	Aeruginosarr	3	Peptide Linear	Cyano bactin (RIPPs)	Chroococcales	Microcystaceae	<i>Microcystis</i>	<i>Microcystis</i>	PCC 9432	Freshwater	Freshwater	Canada ; Engl
Aeruginosins	AER	55	Peptide Linear	NRPS	Chroococcales	Microcystaceae	<i>Microcystis</i>	<i>Microcystis</i>	a PCC 7806 ; CV	Axenic ; asser	Freshwater;M;	Czech Repu

Lethality-toxic Test	Neurotoxicity Test	Hepatotoxicity Test	Dermal toxicity Test	Cytotoxicity Test	Anti-inflamma Test	Antioxidant Test	Antiviral Test	Antialgal Test
ND/NT	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
1 VS <i>Lemma m</i>	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
0 VS <i>Chironom</i>	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	1 VS KB, LoVo c	0	ND/NT	0	1 VS HSV-II
ND/NT	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
0 VS <i>Artemia s</i>	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	ND/NT	0	ND/NT	0	ND/NT
1 VS <i>Thamno c</i>	0	ND/NT	0	1 VS murine Lev	0	ND/NT	0	0 VS <i>Chlorella</i>
ND/NT	0	ND/NT	0	0 VS L6 rat myo	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	0 Not mentione	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	1 VS P388 murir	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	1 VS A2780 hurr	0	ND/NT	0	ND/NT
ND/NT	0	ND/NT	0	0 Impedance m	1 AlphaLISA ass	ND/NT	0	ND/NT

Antibacterial Test	Antifungal Test	Antitumor Test	Antiprotozoal Test	Other enzyme Test	Protease inhib Test	Others proper Mode of actio
1 VS <i>Bacillus c</i>	ND/NT	0	ND/NT	0	ND/NT	0
ND/NT	0	ND/NT	0	ND/NT	0	0
1 VS <i>Microcyst</i>	ND/NT	0	ND/NT	0	1 VS TRPA1 cha	0
1 VS <i>Bacillus c</i>	ND/NT	0	ND/NT	0	ND/NT	0
ND/NT	0	ND/NT	0	ND/NT	0	0
ND/NT	0	ND/NT	0	ND/NT	0	0
1 VS <i>Escherich</i>	ND/NT	0	ND/NT	0	ND/NT	0
1 VS <i>Staphyloc</i>	ND/NT	0	ND/NT	0	ND/NT	0
1 VS <i>Synechroc</i>	ND/NT	0	ND/NT	0	ND/NT	0
ND/NT	0	ND/NT	0	1 Anti-prolifera	0	0
ND/NT	0	ND/NT	0	ND/NT	0	0
1 VS <i>Staphyloc</i>	ND/NT	0	ND/NT	0	ND/NT	0
ND/NT	0	ND/NT	0	ND/NT	0	0 Not mentione
ND/NT	0	ND/NT	0	ND/NT	0	0
ND/NT	0	ND/NT	0	ND/NT	0	0
ND/NT	0	ND/NT	0	ND/NT	0	1 VS Trypsin, Thrombin, Plasmin

Table S3. Detailed informations about the activities of metabolites described (capture d'écran des 7 premières entrées ; table complète disponible <https://www.mdpi.com/1660-3397/17/6/320#supplementary>)

Positive point	Molecule families	Variants	Chemical classes	Activity	Test targets	Producing organisms
Specific antibacterial (cyanobacteria) activity	Abietic acids	Dehydroabietic acid	Terpene	Antibacterial No lethality No anti-fungal	<i>Synechococcus nidulans</i> <i>Artemia salina</i> <i>Chlorella vulgaris</i>	<i>Plectonema radiatum</i> LEGE 06105 <i>Nostoc</i> sp. LEGE 06077 and LEGE 07365 <i>Chroococcidiopsis</i> sp. LEGE 06174 <i>Synechocystis</i> sp. LEGE 06079 <i>Synechocystis salina</i> LEGE 06099 <i>Lepolyngbya ectocarpi</i> LEGE 11425 <i>Nodosilinea</i> sp. LEGE 13457 <i>Nodosilinea nodulosa</i> LEGE 07084
Anti-inflammatory activity with no associated cytotoxicity	Aeruginosins	Aeruginosin 102A-B, 298-A, -B, 98-A, -B, -C, 101, 89-A, -B, 865 Aeruginoside 126A-B	Peptide	Anti-inflammatory activity Protease inhibitor No cytotoxicity	Alpha-LISA assay Trypsin Thrombin Plasmin HeLa HepG2	<i>Microcystis aeruginosa</i> NIES-98, NIES-298, NIES-101, NIES-89; <i>Microcystis viridis</i> NIES-102 <i>Planktothrix agardhii</i> CYA 126/8; <i>Nodularia spumigena</i> CCY9414; <i>Nostoc</i> sp. <i>Labeovia</i> 3093
1. Broad-spectrum antimicrobial 2. COX inhibitor (potential anti-inflammatory development)	Ambigols	Ambigol A-C	Alkaloid	Anti-fungal Antiprotozoal Lethal activity Cytotoxicity Enzyme inhibitor	<i>Chlorella fusca</i> <i>Bacillus subtilis</i> <i>Bacillus megaterium</i> <i>Mycobacterium tuberculosis</i> <i>Penicillium oxalicum</i> <i>Ustilago violacea</i> <i>Microbotryum violaceum</i> <i>Trypanosoma cruzi</i> <i>Trypanosoma rhodesiense</i> <i>Plasmodium falciparum</i> <i>Biomphalaria glabrata</i> L6 rat myoblasts Cyclooxygenase HIV-1 reverse transcriptase	<i>Fischerella ambigua</i> 108b
Cytotoxicity associated with cell membrane permeabilization	Anabaenolysins	Anabaenolysin A, B	Lipopeptide	Cytotoxic Antifungal Haemolytic activity Ability to permeabilize cell membranes	Jurkat cells HL-60 NB4 IPC-81 Ewing SH5Y-5Y HeLa U87 NRK cells and Primary hepatocytes <i>Candida albicans</i>	<i>Anabaena</i> sp. XPORK 15F; <i>Anabaena</i> sp. XSPORK 27C
1. Antiviral against Chikungunya's virus 2. Inhibition of the potassium channels	Aplysatoxins	Aplysatoxin Oscillatoxin A Nhantrangan A-B Debromoplysatoxin Anhydroaplysatoxin 3-methoxyaplysatoxin Neo-debromoplysatoxin A-B	Alkaloid	Antiviral Dermatitis and swimmer itch agents Cytotoxic Potent tumour-promoting activity	CHIKV (in infected SJCRH30 cells)	<i>Lyngbya majuscula</i> ; <i>Schizothrix calcicola</i> ; <i>Oscillatoria nigro-viridis</i> ; <i>Trichodesmium erythraeum</i>
Actin microfilament disrupting activity	Aurilides	Aurilide B, C L-arginamide A-C Oubamide Patau amide	Depsideptide	Cytotoxicity Lethal activity Anti-swarming Antiprotozoal Induce loss of microfilament network	NCI-H460 neuro-2a KB cells HeLa S3 P-388 <i>Artemia salina</i> <i>Pseudomonas aeruginosa</i> PA01 <i>Plasmodium falciparum</i> NF54	<i>Lyngbya majuscula</i> ; <i>Okeania</i> sp. ; <i>Lyngbya</i> sp.
1. Antiviral with potent selective activity for enveloped viruses 2. Low anticoagulant activity	Codium spirulinum	None	Polysaccharide	Antiviral Low anticoagulant activity No cytotoxicity	HIV-1 HSV-1 human cytomegalovirus (HCMV) measles virus mumps virus influenza A virus HeLa HEL Vero cells MDCK MT-4	<i>Arthrospira platensis</i>

Annexe 6 : Supplementary materials – Article 2

2 supplementary figures

11 supplementary tables

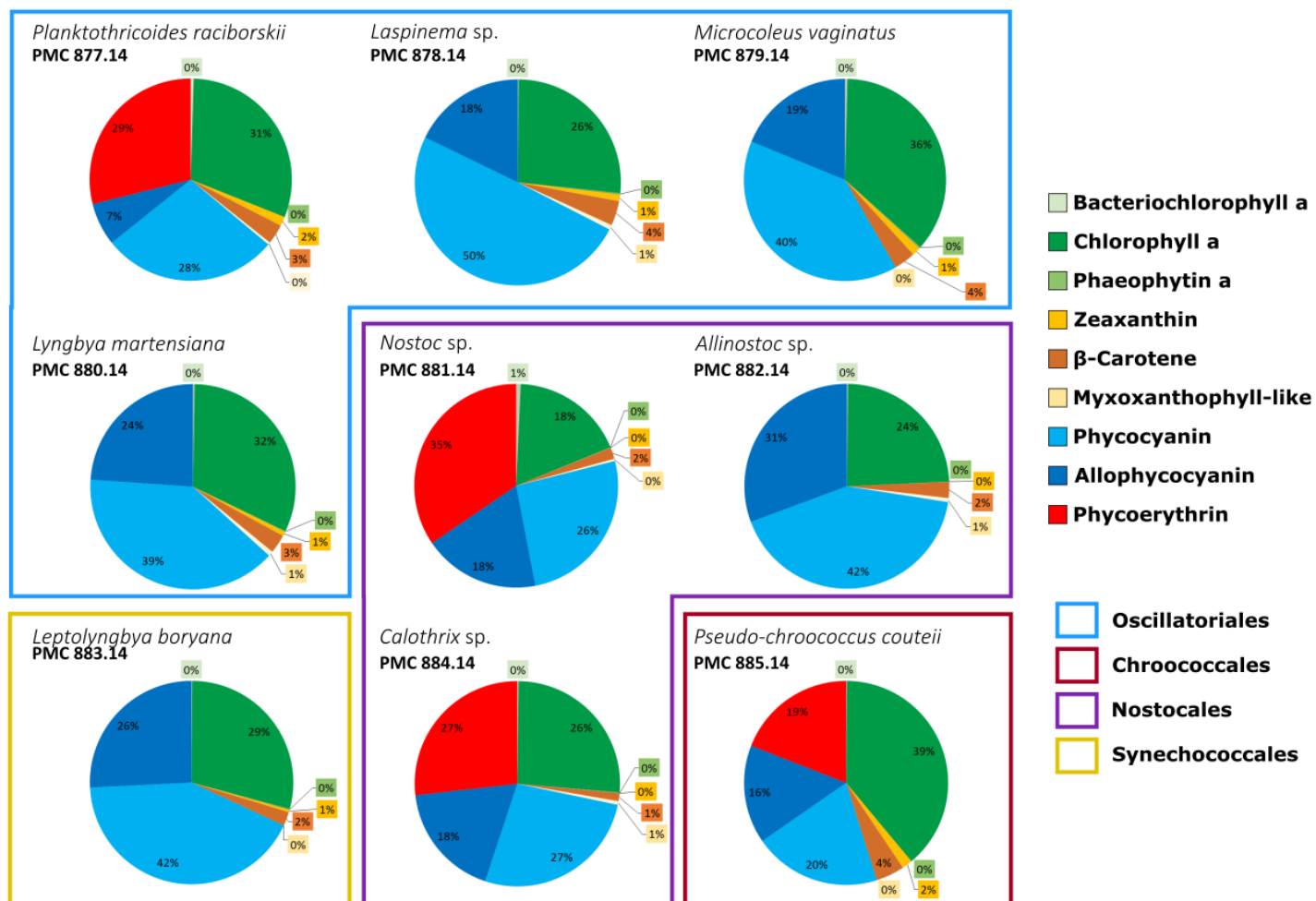


Figure S1. Pigments composition (%) of the nine studied strains, isolated from Thermes de Balaruc-Les-Bains. PMC: Paris Museum Collection, Museum National of Natural History.

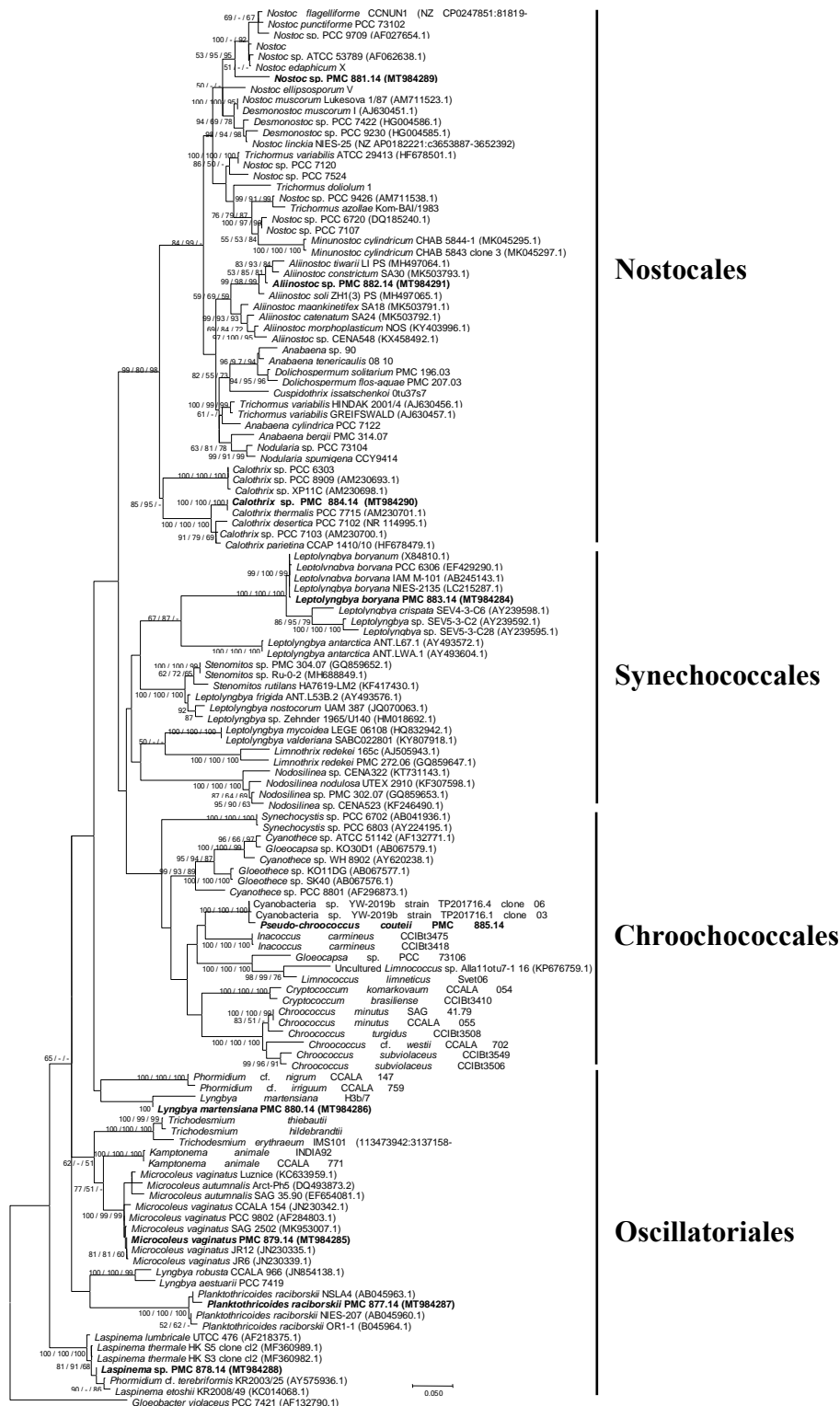


Figure S2. Consensus phylogenetic tree based on 16S rRNA gene sequences of representative cyanobacteria strains (129 sequences, 1446 aligned nucleotide positions, GTR+G+I model) belonging to the orders Oscillatoriales, Nostocales, Chroococcales, Synechococcales and the studied strains from Thermes de Balaruc-Les-Bains (in bold). *Gloeobacter violaceus* was used as outgroup. Numbers above branches indicate bootstrap support (>50%) from 1000 replicates. Bootstrap values are given in the following order: maximum likelihood / neighbor joining / maximum parsimony.

Table S1. List of GenBank accession numbers. PMC: Paris Museum Collection.

Order	Species	Strain number	16S	16S -23S
Chroococcales	<i>Pseudo-chroococcus couteii</i>	PMC 885.14	-	MT985488
Synechococcales	<i>Leptolyngbya boryana</i>	PMC 883.14	MT984284	-
Oscillatoriales	<i>Planktothricoides raciborskii</i>	PMC 877.14	MT984287	-
	<i>Laspinema</i> sp.	PMC 878.14	MT984288	-
	<i>Microcoleus vaginatus</i>	PMC 879.14	MT984285	-
	<i>Lyngbya martensiana</i>	PMC 880.14	MT984286	-
Nostocales	<i>Nostoc</i> sp.	PMC 881.14	MT984289	-
	<i>Aliinostoc</i> sp.	PMC 882.14	MT984291	-
	<i>Calothrix</i> sp.	PMC 884.14	MT984290	-

Table S2. Comparison of the morphological features of *Pseudo-chroococcus couteii* PMC 885.14 strain with other Chroococcales (*Gleocapsa*, *Limnococcus*, *Inacoccus*, *Cryptococcum*) genera (Fig. 4). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Vegetative cell				Reference
				Shape	Sheath	Color	Size min.-max. (mean), μm	
<i>Pseudo-chroococcus couteii</i>	PMC 885.14	France	Epilithic biofilm	More or less spherical, oval, hemispherical after division	Colorless, slightly lamellate	Pale green, blue-green or grey	14-17.8 (15.6)	This study
Cyanobacteria sp. YW-2019b	TP201716.1 clone 03 (MN215477.1)	China	nd	nd	nd	nd	nd	Institute of Hydrobiology site web
<i>Gleocapsa</i> sp.	PCC 73106 (AB039000.1)	Switzerland	Sphagnum bog	nd	nd	nd	nd	PCC site web collection
<i>Limnococcus limneticus</i>	Svet06 (GQ375048.1)	Czech Rep	Fishpond Svet plankton	nd	nd	nd	7-11	Komárková et al. 2010
<i>Inacoccus carmineus</i> sp. nov.	CCIBt3411 (MF072347.1)	Brazil (Santa Virginia Park)	Terrestrial, on concrete.	Hemispherical to rounded	Hyaline to intense red-colored smooth or rarely lamellate	Purple brownish to green-brownish	4.7–11.9 (6.7)	Gama et al. 2019
<i>Inacoccus carmineus</i> sp. nov.	CCIBt3475 (MF072349.1)	Brazil (Ilha do Cardoso State Park)	Wet Rock	Hemispherical to rounded	Hyaline to intense red-colored smooth or rarely lamellate	Purple brownish to green-brownish	4.7–11.9 (6.7)	Gama et al. 2019
<i>Cryptococcum komarkovaum</i>	CCALA 054 (MF072346.1)	Unknown	Aquatic, thermal spring	Hemispherical to rounded solitary or rarely grouped in few-celled colonies	Hyaline, smooth or rarely lamellate	Blue-green to yellowish-green	8.7–13.9 (10.8)	Gama et al. 2019
<i>Cryptococcum brasiliense</i>	CCIBt3410 (MF072345.1)	Brazil (Ilha do Cardoso State Park)	Terrestrial, on dry soil among pebbles	Hemispherical to rounded solitary or rarely grouped in few-celled colonies	Hyaline, smooth or rarely lamellate	Green to blue-green	8.3–15.5	Gama et al. 2019

Table S3. Comparison of the morphological features of the *Leptolyngbya boryana* sp. PMC 883.14 strain with other *Leptolyngbya* species belonging to the same phylogenetical cluster (Fig. 8). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Trichome					Vegetative cell			Reference
				Color	Sheath	Shape	Shape of terminal cell	Width min.-max. (mean) μm	Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm	
<i>Leptolyngbya boryana</i>	PMC 883.14	France	Freshwater, benthic, biofilms	Pale to bright blue-green	Thin, colourless, attached to the trichome	Straight, curved, distinctly constricted,	Rounded hemispherical	1.5-2.4	Moniliform, shorter than wide	1.5-2.4 (2.1 \pm 0.24) length:width ratio = 0.75	0.9 - 2 (1.52 \pm 0.27)	This study
<i>Leptolyngbya boryana</i>	PCC 6306 (EF429290.1)	nd	Freshwater, metaphytic, lentic	Blue-green	Absent	Long, straight or wavy, distinctly constricted, false branching	Rounded	nd	More or less isodiametric	length:width ratio = 0.78	length:width ratio = 0.78	Chakraborty et al. 2019
<i>Leptolyngbya boryana</i>	UAM 391 (JQ070062.1)	Spain Guadarrama river	Freshwater, benthic, biofilms	Intense blue-green or pale color	Hyaline, homogeneous sometimes thick, separated from the filament	Strongly constricted, false branching	Rounded	nd	nd	2.1–3.2	1.2–3.6	Loza et al. 2013
<i>Leptolyngbya boryana</i>	IAM M-101 (AB245143.1)	Japan	Freshwater	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>Leptolyngbya boryana</i>	NIES-2135 (LC215287.1)	Japan	Freshwater	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>Leptolyngbya foveolarum</i>	1964/112 (X84808.1)	nd	nd	nd	Presence of a sheath	Constrictions at the cross-walls	nd	nd	nd	3.8	3.8	Nelissen et al. 1996
<i>Leptolyngbya boryanum</i>	PCC 73110 (X84810.1)	nd	Freshwater	nd	Variable	Constrictions at the cross-walls	nd	nd	nd	1.9 – 2.6	1.9 – 2.9	Nelissen et al. 1996

Table S4. Comparison of the morphological features of the *Planktothricoides raciborskii* PMC 877.14 strain with other *Planktothricoides* species belonging to the same phylogenetical cluster (Fig. 9). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Trichome			Vegetative cell		Reference			
				Color	Sheat	Shape	Shape of terminal cell	Width min.-max. (mean) μm		Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm
<i>Planktothricoides raciborskii</i>	PMC 877.14	France	Freshwater, mud	Pale-green, brown	Thin, colourless	Straight +/- curved	Rounded conical	5-7.3 (2020)	Cylindrical, shorter than wide	5-7.3 (6 \pm 0.56)	3.3-5.4 (4.48 \pm 0.46)	This study
Candidatus <i>Planktothricoides rosea</i>	OP (KC407688.1)	Guadeloupe	Marine mangrove Mat on thesediment from the periphyton	Pink	Thin, clear	Straight slightly waved	Slightly rounded without calyptra	5–10	Disk-shaped greater in width than in length	5–10	1.25-2.5	Guidi-Rontani et al. 2014
Candidatus <i>Planktothricoides niger</i>	OB (KC407687.1)	Guadeloupe	Marine mangrove Mat on the sediment from the periphyton	Black	Thin, clear	Straight slightly waved	Slightly rounded without calyptra	5–10	Disk-shaped greater in width than in length	5–10	1.25-2.5	Guidi-Rontani et al. 2014
<i>Planktothricoides raciborskii</i>	OR1-1 (B045964.1)	Thailand Bangkok	Freshwater planktic in lakes	Pale blue-green yellow-green	nd	Straight attenuated at the ends	Rounded without calyptra	nd	nd	5.4–2.2 (Group IV) : 5.4–12.2	(Group IV) : 1.7–6.7 (3.3 \pm 0.6)	Suda et al. 2002
<i>Planktothricoides raciborskii</i>	NSLA4 (AB045963.1)	Australia New South Wales Lake Alexandrina	Freshwater planktic in lakes	nd	nd	Straight attenuated at the ends	nd	nd	nd	(Group IV) 5.4–12.2	(Group IV) : 1.7–6.7	Suda et al. 2002
<i>Planktothricoides raciborskii</i>	NIES-207 (AB045960.1)	Japan Ibaraki Lake Kasumigaura	Freshwater planktic in lakes	Pale blue-green yellow-green	nd	Straight attenuated at the ends	Tapered, bent	nd	nd	5.4–9.8 (Group IV) 5.4–12.2	(Group IV) : 1.7–6.7	Suda et al. 2002
<i>Planktothricoides</i> sp.	SR001 (NZ_LIUQ01000113.1)	Singapore	Freshwater, planktic reservoir	nd	nd	Straight attenuated at the ends	Attenuated without calyptra	nd	Cylindrical, shorter than wide	(8.13 \pm 0.92 width: length ratio 1.68 (\pm 0.65)	5.48 (\pm 2.01)	Te et al. 2017

Table S5. Comparison of the morphological features of the *Laspinema* sp. PMC 878.14 strain with other *Laspinema* and *Phormidium* species belonging to the same phylogenetical cluster (Fig. 9). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Trichome			Vegetative cell			Reference		
				Color	Sheath	Shape	Shape of terminal cell	Width min.-max. (mean) μm	Shape		Width min.-max. (mean), μm	Length min.-max. (mean), μm
<i>Laspinema</i> sp.	PMC 878.14	France	Benthic on mud freshwater, brackish waters	Bright blue-green	Lacking sheath	Straight, nutated at the end	Obtuse-conical, rounded-conical, without calyptra	3.8 - 4.8	Shorter than wide	3.8 - 4.8 (4.26 \pm 0.27)	2.3 - 3.3 (2.87 \pm 0.28)	This study
<i>Laspinema thermale</i>	HK S5 clone 2 (MF360989.1)	Iran, Mahallat	Thermal spring	Blue-green or olive green	Thin, inconspicuous	Straight in natural environment forming benthic mats, slightly constricted on cross walls	Rounded, conical, shortly bent	3 – 4 (5)	Shorter than wide	3 – 4 (5)	1 – 2,4	Heidari et al. 2018
<i>Laspinema lumbricale</i>	UTCC 476 (AF218375.1)	Antarctica	Saline pond on McMurdo Ice Shelf	nd	nd	nd	Rounded, straight	nd	nd	5 – 6 (7)	2 - 4	Casamatta et al. 2005
<i>Laspinema etoshii</i>	KR2008/49 (KC014068.1)	Namibia Etosha National Park, Okandeka Spring	Hyposaline water puddles springs and wet soil	Pale bright blue-green	Hyaline, colorless	Solitary, straight or curvy, not or slightly constricted	Round, conical; straight or bent without calyptra	nd	Mainly wider than long	5.5 \pm 1.5	3.0 \pm 1.0	Dadheech et al. 2013
<i>Phormidium cf. okenii</i>	Led-Z (EU196644.1)	Czech Republ Lednice na Moravě park	a pond, benthos	Dark blue-green	Delicate colourless	Gradually narrowed towards end, distinctly constricted at cross-walls	Bent, elongated usually rounded	nd	Slightly shorter than wide or isodiametrical, sometimes longer than wide	5.6-6.5 (3.7-7.5 ; 6.1) cell length/width ratio 0.5-0.8	nd	Lokmer 2007
<i>Phormidium cf. terebiformis</i>	KR2003/25 (AY575936.1)	Kenya, Hot spring, Lake Bogoria,	Hyposaline hot springs	nd	nd	Solitary, straight to undulating Constricted	Rounded without calyptra	nd	Mainly isodiametric	5.5 \pm 0.5	3.0 \pm 1.0	Dadheech et al. 2013

Table S6. Comparison of the morphological features of the *Microcoleus vaginatus* PMC 879.14 strain with other *Microcoleus* species or strains belonging to the same phylogenetical cluster (Fig. 9). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Trichome			Vegetative cell			Reference		
				Color	Sheath	Shape	Shape of terminal cell	Width min.-max. (mean) μm	Shape		Width min.-max. (mean), μm	Length min.-max. (mean), μm
<i>Microcoleus vaginatus</i>	PMC 879.14	France	Epilithic biofilm	Bright blue green	Thin, firm	Straight, cylindrical	Elongated, capitate, with rounded or truncated calyptra	4.7 - 6.2	Shorter than wide	4.7 - 6.2 (5.63 \pm 0,36) 5 (2014)	2.4 - 4.8 (3.68 \pm 0.52) 2 (2014)	this study
<i>Microcoleus autumnalis</i>	SAG 35.90 (EF654081.1)	Switzerland, brook in Verzascatal	Freshwater	nd	nd	nd	nd	nd	nd	nd	nd	SAG site web collection
<i>Microcoleus autumnalis</i>	Arct-Ph5 (DQ493873.2)	Canada, Ellesmere Island	Mineral soil	nd	Few filaments possessed	Single filaments or mucilaginous colonies	Rounded calyptra	8 – 9	nd	3 – 4	2 – 4	Elster et al. 1997
<i>Microcoleus vaginatus</i>	CCALA 154 (JN230342.1)	Japan, East Asia	Cooling tower	nd	nd	nd	nd	nd	nd	nd	nd	Strunecky et al. 2013
<i>Microcoleus vaginatus</i>	JR12 (JN230335.1)	Antarctica James Ross Island	Gray-black mats biofilm on rocks	nd	nd	nd	nd	nd	nd	nd	nd	Strunecky et al. 2013
<i>Microcoleus vaginatus</i>	JR6 (JN230335.1)	Antarctica James Ross Island	Periphyton in wetlands close Lachman Lake	nd	nd	nd	nd	nd	nd	nd	nd	Strunecky et al. 2013
<i>Microcoleus vaginatus</i>	A25 (JN230341.1)	Tennessee, North America	Soil	nd	nd	nd	nd	nd	nd	nd	nd	Strunecky et al. 2013
<i>Oscillatoria amoena</i>	SAG 1459-7 (KM0119962.1)	Germany, Bot. Gard. Univ. Bonn	Freshwater	nd	nd	nd	nd	nd	nd	nd	nd	SAG site web collection
<i>Microcoleus vaginatus</i>		Cosmopolitan	Subaerophytic, on soils, stones, dried mud, waterfalls, littoral of lakes, rarely submersed in stagnant and flowing waters	Bright blue green or olive green to dirty green	Colourless,	Trichome long not constricted	Capitate, bluntly rounded, with conical, obtuse-conical or hemispherical calyptra	(2,5) 3 - 7 (9?)	Shorter than wide	2 - 5 (6,7)	nd	Komárek & Anagnostidis 2005

Table S7. Comparison of the morphological features of the *Lyngbya martensiana* PMC 880.14 strain with other *Lyngbya martensiana* belonging to the same phylogenetical cluster (Fig. 9). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Trichome			Vegetative cell			Reference		
				Color	Sheath	Shape	Shape of terminal cell	Width min.-max. (mean) μm	Shape		Width min.-max. (mean), μm	Length min.-max. (mean), μm
<i>Lyngbya martensiana</i>	PMC 880.14	France	Epilithic biofilm	Pale blue-green, olive green	Thick, colourless	Cylindrical straight, flexuous	Widely rounded, hemispherical without calyptra	4.8 - 6.6	Shorter than wide	4.8 - 6.6 (5.3 \pm 0.47)	1.2 - 2.1 (1.6 \pm 0.2)	This study
<i>Lyngbya martensiana</i>	AUS-JR/MT/NT-124 (KX670285.1)	India	Rice Field soil	Blue green	Colourless, firm, thick	Entangled and interwoven, long flexible, straight	Rounded without calyptra, not capitate	6-10	Cells $\frac{1}{2}$ -1/4 times long as broad		1.5 – 2.5	Thajamambi et al. 2016
<i>Lyngbya martensiana</i>	MBDU 518 (KX913923.1)	India	Marine	nd	nd	nd	nd	nd	nd	nd	nd	Genbank site web
<i>Lyngbya martensiana</i>	H3b/7 (JN854142.1)	Guatemala, Lake Atitlan	Periphytic freshwater	Bright blue green	Firm, smooth from outside	Cylindrical, straight or slightly bent, coiled, or screw-like coiled	Not attenuated	9	nd	(6.2) 7 – 7.5 (8.7)	1.4	Komárek et al. 2013 Turicchia et al. 2009

Table S8. Comparison of the morphological features of the *Nostoc* sp. PMC 881.14 strain with other *Nostoc* species belonging to the same phylogenetical cluster (Fig. 14). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Colony morphology		Vegetative cell		Akinete			Heterocyte		Reference	
				Sheath and shape	Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm	Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm	Shape	Width min.-max. (mean), μm		Length min.-max. (mean), μm
<i>Nostoc</i> sp.	PMC 881.14	France	Epilithic biofilm	nd	nd	2.8 – 5.5 (4.5)	2.6 – 4.6 (3.4)	not observed	nd	nd	+/- sub spherical	2.6 – 5,7 (3.4)	2.3 – 5.1 (3.8)	This study
<i>Nostoc linckia</i>	IAM M-251	Japan	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Genbank site web
<i>Nostoc muscorum</i>	II	Czech Republic	Field	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Rajaniemi et al 2005
<i>Nostoc ellipsosporum</i>	V	Czech Republic	Field	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Rajaniemi et al 2005
<i>Nostoc edaphicum</i>	X	Czech Republic	Field (salty soils)	Spherical or oval sometimes irregular colourless or yellowish brown	Barrel shaped	3 - 4.2	nd	not observed			Mainly terminal, rarely intercalary	nd	nd	Singh et al 2020
<i>Nostoc calcicola</i>	III	Czech Republic	Field	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Rajaniemi et al 2005
<i>Nostoc</i> sp.	1tu14s8	Finland	Lake	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Rajaniemi et al 2005
<i>Nostoc</i> sp.	ATCC 53789	Scotland	lichen thallus	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Genbank site web
<i>Nostoc punctiforme</i>	PCC 73102	Australia	Root section, Marozamia sp. (Humid soil or cycad tubercles)	Dark blue green or blackish spherical sub aerophytic	Barrel shaped to spherical to ellipsoidal	2.6 – 5.5 (3 - 5)	nd	Present	nd	nd	Barrel shaped to +/- sub spherical	4 -6.5 (diameter)		Singh et al 2020

Table S9. Comparison of the morphological features of the *Aliinostoc* sp. PMC 882.14 strain with other *Aliinostoc* and *Trichormus* species belonging to the same phylogenetical cluster (Fig. 14). no: not observed. nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Sheath	Filament		Vegetative cell		Akinete		Heterocyte		Reference		
					Shape of terminal cell	Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm	Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm	Shape		Width min.-max. (mean), μm	Length min.-max. (mean), μm
<i>Aliinostoc</i> sp.	PMC 882.14	France	Epilithic biofilm	nd	Conical	Spherical /cylindrical	3.4 - 5.7 (4.5)	2.7 - 6.5 (4.6)	oval	4.6 - 7.4 (5.9)	4.5 - 9.1 (7.2)	Spherical, 4.1 - 6.5 (5.4) intercalary	1.2 - 6.9 (5.4)	This study	
<i>Aliinostoc constrictum</i>	SA3O	Iran	Paddy field	thin hyaline	nd	spherical / cylindrical	3.1 - 5.6	2.5 - 6.5	no	nd	nd	spherical to 3.0 - 6.8 oblong	6.5 - 8.0	Kabirnatay et al. 2020	
<i>Aliinostoc soli</i>	ZH1(3)	India, Pachmarhi	Soil dwelling	thin hyaline	Bluish green mats with leathery texture	Barrel shaped	3.2 - 3.7	3.6 - 4.0	Present	nd	nd	spherical	3.7 - 4.9	3.8 - 4.3	Saraf et al. 2018 Kabirnatay et al. 2020
<i>Aliinostoc tiwari</i>	LI PS	India, Mumbra	Freshwater	thin hyaline	Greenish blue mats with soft texture	Barrel shaped	5.2 - 5.6	5.3 - 5.6	Present	nd	nd	spherical	3.7 - 4.9	3.8 - 4.3	Saraf et al. 2018 Kabirnatay et al. 2020
<i>Trichormus dolium</i>	1	nd	nd	nd	Conical	nd	1.6 - 3.0 (2.3)		lenticular	1.9 - 3.7 (2.7)	3.5 - 6.5 (4.6)				Rajaniemi et al 2005
<i>Trichormus variabilis</i>	KINDAK 2001/4	Russia	Soil	nd	Conical	nd	2.1 - 9.6 (6.7)	nd	Oval, slightly compressed in the middle	5 - 7.4 (6.9)	8 - 14.8 (11.3)	nd	nd	nd	Rajaniemi et al 2005
<i>Trichormus azollae</i>	Kom-BAI	nd	nd	nd	Rounded	nd	1.4 - 5.3 (4)	nd	oval	7.5 - 7.5 (7.5)	12.5 - 15 (14.5)	nd	nd	nd	Rajaniemi et al 2005
<i>Nostoc</i> sp.	PCC 7120	nd	nd	nd	nd	nd	3 - 3.5		no	nd	nd	nd	nd	nd	
<i>Anabaena oscillarioides</i>	BO HINDAK 1984/43	Canada	nd	nd	Conical	nd	2.3 - 5.2 (3.8)	nd	no	nd	nd	nd	nd	nd	Rajaniemi et al 2005
<i>Minunostoc cylindricum</i>	CHAB 5843	China	Small seven-hole Scenic area	blue green gelatinous	Cylindrical	nd	2.5 - 3.8 (3.2)	nd	no	nd	nd	no	nd	nd	Cai et al. 2019

Table S10. Comparison of the morphological features of the *Calothrix* sp. PMC 884.14 strain with other *Calothrix* species belonging to the same phylogenetical cluster (Fig. 14). nd: not determined.

Taxonomic assignment	Strain	Origin	Habitat	Vegetative cell		Akinete		Heterocyte		Reference	
				Shape	Width min.-max. (mean), μm	Length min.-max. (mean), μm	Shape	Shape	Width min.-max. (mean), μm		Length min.-max. (mean), μm
<i>Calothrix</i> sp.	PMC 884.14	France	Epilithic biofilm	nd	5.2 – 7.9 (6.6 \pm 0.7)	4.8 – 6.2 (5.5 \pm 0.4)	not observed	hemispherical	4.7-8.19 (6.2 \pm 0.9)	3.48-7.33 (4.9 \pm 0.9)	This study
<i>Calothrix thermalis</i>	PCC 7715	France	Thermal spring	Isodiametric to longer than wide cells	nd	nd	Present	nd	nd	nd	PCC site web collection
<i>Calothrix desertica</i>	PCC 7102	Chile	Soil, fine desert sand	nd	nd	nd	nd	nd	nd	nd	PCC site web collection
<i>Calothrix</i> sp.	PCC 7103	USA	Herbarium material of <i>Anacystis montana</i>	nd	nd	nd	nd	nd	nd	nd	PCC site web collection
<i>Calothrix parietina</i>	CCAP 1410/10	England	Freshwater	nd	nd	nd	nd	nd	nd	nd	Genbank site web

Annexe 7 : Supplementary materials – Article 3

2 supplementary figures

11 supplementary tables

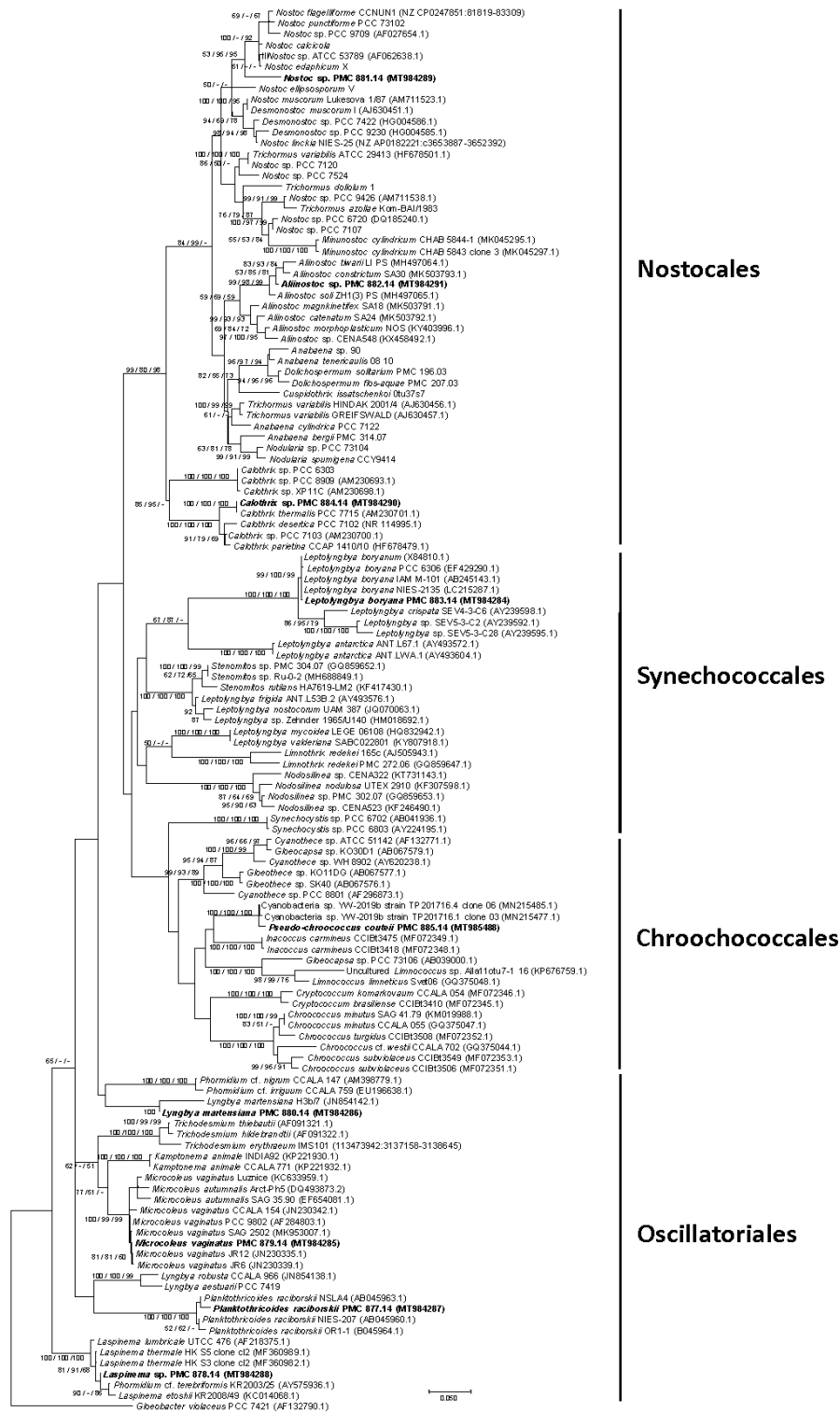


Figure S1. Consensus phylogenetic tree based on 16S rRNA gene sequences of representative cyanobacteria strains belonging to the orders Oscillatoriales, Nostocales, Chroococcales and Synechococcales, Balaruc-les-Bains strains (in bold) and one outgroup (*Gloeobacter violaceus*). Numbers above branches indicate bootstrap support (>50%) from 1000 replicates. Bootstrap values are given in the following order: neighbor joining/maximum likelihood/maximum parsimony.

Table S1. Biosynthetic gene clusters from *Planktothricoides raciborskii* PMC 877.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C877_NODE_2 - Region 1	2 058 429	2 068 638	10 209	Bacteriocin	-	-	Unknown product but homolog BGC	-
2	C877_NODE_2 - Region 2	2 476 893	2 523 309	46 416	NRPS	-	-	Unknown (no homolog)	-
3	C877_NODE_2 - Region 3	2 668 068	2 709 384	41 316	NRPS-like	-	-	Unknown (no homolog)	-
4	C877_NODE_2 - Region 4	2 782 211	2 824 994	42 783	NRPS-like	-	-	Unknown (no homolog)	-
5	C877_NODE_4 - Region 1	103 736	126 882	23 146	Lanthipeptide	-	-	Unknown (no homolog)	-
6	C877_NODE_10 - Region 1	48 223	58 600	10 377	Ectoine	-	-	Unknown (no homolog)	-
7	C877_NODE_15 - Region 1	37 588	58 517	20 929	Terpene	S-layer glycan saccharide	13.00%	Validated (manually curated)	Phytoene (carotenoids)

Confidence level

Low

Medium

High

Homologous with uncharacterized cluster

Table S2. Biosynthetic gene clusters from *Laspinema* sp. PMC 878.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C878_NODE_8 - Region 1	33 145	85 231	52 086	NRPS	Cyanopeptin (cyanopeptolin-)	75%	Unknown product but homolog BGC	-
2	C878_NODE_8 - Region 2	127 431	135 191	7 760	Bacteriocin	-	-	Unknown product but homolog BGC	-
3	C878_NODE_8 - Region 3	203 805	214 038	10 233	Bacteriocin	-	-	Unknown product but homolog BGC	-
4	C878_NODE_8 - Region 4	560 936	581 862	20 926	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids precursors)
5	C878_NODE_13 - Region 1	166 470	209 289	42 819	NRPS-like	Anacyclamide	21%	Unknown product but homolog BGC	-
6	C878_NODE_14 - Region 1	368 689	401 669	32 980	RIPPS/Cyanobactin / b	Tenucyclamide A/Tenucyclin	85%	Validated (manually curated)	Heterocyclin cyanobactin-like
7	C878_NODE_46 - Region 1	1	57 418	57 417	NRPS-T-IPKS	Nostopeptolide	50%	Unknown product but homolog BGC	-

Table S3. Biosynthetic gene clusters from *Microcoleus vaginatus* PMC 879.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C879_NODE_4 - Region 1	1 061 051	1 104 041	42 990	NRPS-like	Anabaenopeptin NZ857/nostoc	100%	Unknown (no homolog)	-
2	C879_NODE_6 - Region 1	253 113	274 042	20 929	Terpene	-	-	Validated (manually cur)	Phytoene (carotenoids)
3	C879_NODE_6 - Region 2	855 090	896 958	41 868	Resorcino	Desotamide	9%	Unknown product but h	-
4	C879_NODE_6 - Region 3	961 866	1 040 080	78 214	NRPS	Puwainaphycins	40%	Unknown product but h	-
5	C879_NODE_6 - Region 4	1 095 604	1 111 897	16 293	RIPPS	-	-	Unknown product but h	-
6	C879_NODE_11 - Region 1	520 507	577 761	57 254	NRPS	Micropeptin	25%	Unknown (no homolog)	-
7	C879_NODE_23 - Region 1	1	78 685	78 684	transAT-PKS	Luminaolide	0	Unknown (no homolog)	-

Table S4. Biosynthetic gene clusters from *Lyngbya martensiana* PMC 880.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C880_NODE_44 - Region 1	1	34 099	34 098	Terpene	Malleobactin (NRPS)	7%	Validated (manually cur)	Phytoene (carotenoids)
2	C880_NODE_80 - Region 1	89 303	112 713	23 410	Bacteriocin/Lanthipep	-	-	Unknown product but h	-
3	C880_NODE_106 - Region 1	45 183	67 488	22 305	Lassopeptide	-	-	Unknown product but h	-
4	C880_NODE_125 - Region 1	18 697	28 930	10 233	Bacteriocin	-	-	Unknown product but h	-
5	C880_NODE_127 - Region 1	32 392	49 251	16 859	Siderophore	-	-	Validated (manually cur)	Schizokinen
6	C880_NODE_128 - Region 1	28 176	77 399	49 223	NRPS	Cyanopeptin	75%	Unknown product but h	-
7	C880_NODE_142 - Region 1	52 908	73 837	20 929	Terpene	Trichamide (cyanobactin)	18%	Validated (manually cur)	Phytoene (carotenoids)
8	C880_NODE_231 - Region 1	37 446	47 679	10 233	Bacteriocin	-	-	Unknown product but h	-
9	C880_NODE_1578	1	8 029	8 028	Bacteriocin	-	-	Unknown (no homolog)	-

Table S5. Biosynthetic gene clusters from *Nostoc* sp. PMC 881.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C881_NODE_77 - Region 1	1	88 771	88 770	NRPS/T1PKS/Bacteriocin	Nostophycin	27%	Unknown product but h	-
2	C881_NODE_132 - Region 1	24 582	45 514	20 932	Terpene	-	-	Validated (manually cur	Phytoene (carotenoids)
3	C881_NODE_151 - Region 1	3 676	14 392	10 716	Bacteriocin	-	-	Unknown (no homolog)	-
4	C881_NODE_268 - Region 1	1	29 757	29 756	T1PKS	-	-	Unknown (no homolog)	-
5	C881_NODE_275 - Region 1	8 666	18 956	10 290	Bacteriocin	-	-	Unknown (no homolog)	-
6	C881_NODE_281 - Region 1	1	37 735	37 734	NRPS/T1PKS	Chondrochloren	33%	Unknown product but h	-
7	C881_NODE_300 - Region 1	7 522	18 376	10 854	Bacteriocin	-	-	Unknown product but h	-
8	C881_NODE_328 - Region 1	1	33 447	33 446	T1PKS/hgIE-KS	Heterocyst glycolipids	100%	Validated	Heterocyst glycolipids
9	C881_NODE_379 - Region 1	1	28 956	28 955	NRPS	Anabaenopeptin	43%	Unknown (no homolog)	-
10	C881_NODE_394 - Region 1	12 176	27 485	15 309	Cyanobactin	Piricyclamide	25%	Uncertained	Cyanobactin-like
11	C881_NODE_396 - Region 1	1	27 204	27 203	NRPS	Nostophycin	18%	Unknown (no homolog)	-
12	C881_NODE_427 - Region 1	1	25 425	25 424	NRPS	-	-	Unknown (no homolog)	-
13	C881_NODE_522 - Region 1	3 473	21 216	17 743	Terpene	-	-	Validated (manually cur	Phytoene (carotenoids)
14	C881_NODE_537 - Region 1	1	17 625	17 624	Terpene	-	-	Unknown product but h	-
15	C881_NODE_574 - Region 1	10 827	19 081	8 254	Bacteriocin	-	-	Unknown (partial)	-
16	C881_NODE_651 - Region 1	1	16 581	16 580	Phosphonate	-	-	Unknown (partial)	-
17	C881_NODE_793 - Region 1	1	12 472	12 471	Bacteriocin (microvirid	6,6'-oxybis(2,4-dibromophenc	21%	Unknown (no homolog)	-
18	C881_NODE_970 - Region 1	1	8 621	8 620	Lanthipeptide	-	-	Unknown (partial)	-
19	C881_NODE_1168 - Region 1	1	5 446	5 445	Terpene	-	-	Unknown (partial)	-
20	C881_NODE_1419 - Region 1	1	3 049	3 048	Indole	-	-	Unknown (partial)	-

Table S6. Biosynthetic gene clusters from *Alinostoc* sp. PMC 882.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C882_NODE_3 - Region 1	15 640	37 498	21 858	NRPS-like	Shinorine	100%	Validated	Shinorine
2	C882_NODE_3 - Region 2	245 505	310 129	64 624	NRPS/T1PKS/microviridin	Puwainaphycins	60%	Validated (manually curated)	Microginin
3	C882_NODE_3 - Region 3	640 730	684 201	43 471	NRPS-like	-	-	Unknown (no homolog)	-
4	C882_NODE_3 - Region 4	1 403 211	1 493 957	90 746	NRPS/T1PKS	Hapalosin	40%	Unknown (no homolog)	-
5	C882_NODE_3 - Region 5	1 568 034	1 637 956	69 922	NRPS	Cyanoheptin	75%	Unknown (no homolog)	-
6	C882_NODE_7 - Region 1	82 074	126 088	44 014	T1PKS	Amycomicin	62%	Uncertain	Amycomycin-like
7	C882_NODE_7 - Region 2	166 759	210 645	43 886	NRPS	Micropeptin	62%	Unknown (no homolog)	-
8	C882_NODE_7 - Region 3	264 995	311 442	46 447	T1PKS/bacteriocin	Jamaicamide	15%	Unknown (no homolog)	-
9	C882_NODE_7 - Region 4	404 145	473 658	69 513	NRPS	Nostopeptolide	37%	Unknown (no homolog)	-
10	C882_NODE_7 - Region 5	560 196	581 026	20 830	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
11	C882_NODE_9 - Region 1	145 215	197 610	52 395	T1PKS/hgIE-KS	Heterocyst glycolipids	85%	Validated	Heterocyst glycolipids
12	C882_NODE_9 - Region 2	335 457	345 690	10 233	Bacteriocin	-	-	Unknown product but homolog BGC	-
13	C882_NODE_9 - Region 3	364 924	406 075	41 151	Ladderane	-	-	Unknown product but homolog BGC	-
14	C882_NODE_12 - Region 1	482 095	502 947	20 852	Microviridin (bacteriocin)	Microviridin K	62%	Validated	Microviridin
15	C882_NODE_13 - Region 1	45 863	138 173	92 310	NRPS/T1PKS	Nostophycin	27%	Unknown product but homolog BGC	-
16	C882_NODE_14 - Region 1	225 206	244 913	19 707	Terpene	6,6'-oxybis(2,4-dibromophend)	21%	Unknown product but homolog BGC	-
17	C882_NODE_14 - Region 2	305 160	326 092	20 932	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
18	C882_NODE_15 - Region 1	193 638	269 628	75 990	NRPS	Puwainaphycins	40%	Unknown product but homolog BGC	-
19	C882_NODE_15 - Region 2	270 364	315 220	44 856	T1PKS	Carbamidocyclophane	30%	Uncertain	Cylindrocyclophane-like
20	C882_NODE_16 - Region 1	202 309	253 885	51 576	NRPS/betalactone	-	-	Unknown (no homolog)	-
21	C882_NODE_18 - Region 1	65 575	76 344	10 769	Bacteriocin	Welwitindolinone	6%	Unknown product but homolog BGC	-

Table S7. Biosynthetic gene clusters from *Leptolyngbya boryana* PMC 883.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C883_NODE_1 - Region 1	83 669	104 948	21 279	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
2	C883_NODE_1 - Region 2	112 313	156 329	44 016	NRPS	-	-	Unknown product but homolog BGC	-
3	C883_NODE_1 - Region 3	248 779	253 596	4 817	Bacteriocin	-	-	Unknown product but homolog BGC	-
4	C883_NODE_1 - Region 4	1 408 656	1 418 889	10 233	Bacteriocin	-	-	Unknown product but homolog BGC	-
5	C883_NODE_1 - Region 5	1 619 886	1 638 156	18 270	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
6	C883_NODE_1 - Region 6	1 905 423	1 929 318	23 895	Lasso peptide	-	-	Unknown product but homolog BGC	-
7	C883_NODE_1 - Region 7	3 038 383	3 059 201	20 818	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
8	C883_NODE_2 - Region 1	66 511	86 277	19 766	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
9	C883_NODE_2 - Region 2	253 355	292 807	39 452	Bacteriocin/lanthipept	-	-	Unknown (no homolog)	-
10	C883_NODE_2 - Region 3	790 251	830 822	40 571	NRPS-like	Heme D1	11%	Unknown product but homolog BGC	-
11	C883_NODE_2 - Region 4	969 702	1 011 851	42 149	NRPS	-	-	Unknown product but homolog BGC	-
12	C883_NODE_2 - Region 5	1 341 612	1 362 619	21 007	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
13	C883_NODE_2 - Region 6	1 988 527	2 031 662	43 135	NRPS-like	-	-	Unknown product but homolog BGC	-
14	C883_NODE_2 - Region 7	2 588 904	2 642 774	53 870	Bacteriocin/NRPS	Cyanopeptin (NRPS)	75%	Unknown product but homolog BGC	-
15	C883_NODE_2 - Region 8	2 950 929	2 995 203	44 274	NRPS	-	-	Unknown (no homolog)	-
16	C883_NODE_16 - Region 1	128 363	151 761	23 398	Lanthipeptide/bacteriocin	-	-	Unknown (no homolog)	-

Table S8. Biosynthetic gene clusters from *Calothrix* sp. PMC 884.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C884_NODE_1 - Region 1	81 420	110 742	29 322	Lasso peptide	-	-	Unknown product but homolog BGC	-
2	C884_NODE_1 - Region 2	373 531	383 734	10 203	Bacteriocin	-	-	Unknown product but homolog BGC	-
3	C884_NODE_1 - Region 3	947 694	970 253	22 559	Terpene	Malleobactin	7%	Validated (manually curated)	Phytoene (carotenoids)
4	C884_NODE_1 - Region 4	1 058 565	1 087 796	29 231	LAP/cyanobactin	Trichamide	45%	Validated (manually curated)	Cyanobactin-like
5	C884_NODE_1 - Region 5	1 430 708	1 486 356	55 648	oligosaccharide/terpe	-	-	Unknown product but homolog BGC	-
6	C884_NODE_1 - Region 6	1 658 997	1 701 884	42 887	NRPS	Hexone-palmythine-serine/hex	57%	Validated	MAAs
7	C884_NODE_1 - Region 7	2 383 265	2 404 230	20 965	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
8	C884_NODE_1 - Region 8	2 469 690	2 489 983	20 293	Microviridin	-	-	Unknown (no homolog)	-
9	C884_NODE_1 - Region 9	3 026 023	3 070 112	44 089	TIPKS	Carbamidocyclophane	38%	Validated	Cylindrocyclophane
10	C884_NODE_1 - Region 10	3 127 148	3 262 787	135 639	NRPS/TIPKS	Nostopeptolide	50%	Unknown (no homolog)	-
11	C884_NODE_2 - Region 4	2 133 166	2 176 396	43 230	NRPS-like	Cylindrocyclophane	12%	Unknown product but homolog BGC	-
12	C884_NODE_2 - Region 5	2 618 846	2 642 049	23 203	Lanthipeptide/bacteri	-	-	Unknown product but homolog BGC	-
13	C884_NODE_3 - Region 1	340 357	360 905	20 548	Phenazine	-	-	Unknown product but homolog BGC	-
14	C884_NODE_3 - Region 2	911 043	931 963	20 920	Microviridin	Microviridin K	25%	Unknown product but homolog BGC	-
15	C884_NODE_3 - Region 3	1 317 677	1 340 549	22 872	Lasso peptide	Nostophycin	36%	Unknown product but homolog BGC	-
16	C884_NODE_3 - Region 4	1 513 993	1 524 304	10 311	Bacteriocin	-	-	Unknown product but homolog BGC	-
17	C884_NODE_3 - Region 5	1 537 496	1 579 958	42 462	NRPS-like	-	-	Unknown (no homolog)	-
18	C884_NODE_5 - Region 1	285 211	326 362	41 151	Ladderane	-	-	Unknown product but homolog BGC	-
19	C884_NODE_5 - Region 2	813 707	837 030	23 323	Indole	Staurosporine	13%	Unknown product but homolog BGC	-
20	C884_NODE_5 - Region 3	863 596	885 716	22 120	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids)
21	C884_NODE_7 - Region 1	181 332	261 872	80 540	NRPS-like/hgIE-KS/TIP	Heterocyst glycolipids	85%	Validated	Heterocyst glycolipids
22	C884_NODE_7 - Region 2	290 786	333 878	43 092	NRPS-like	Nocuolin A	19%	Unknown product but homolog BGC	-
23	C884_NODE_11 - Region 1	2 126	12 251	10 125	Bacteriocin	-	-	Unknown (no homolog)	-
24	C884_NODE_18 - Region 1	259 581	299 134	39 553	NRPS-like	Anabaenopeptin NZ857/nost	100%	Unknown product but homolog BGC	-
25	C884_NODE_22 - Region 1	175 498	195 959	20 461	Phenazine	X	X	Unknown product but homolog BGC	-
26	C884_NODE_22 - Region 2	242 813	275 695	32 882	NRPS	X	X	Unknown (no homolog)	-
27	C884_NODE_28 - Region 1	89 411	153 168	63 757	TIPKS/NRPS	Jamaicamide	23%	Unknown (no homolog)	-

Table S9. Biosynthetic gene clusters from *Pseudo-chroococcus couteii* PMC 885.14

Cluster	Contig	From	To	Size (bp)	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C885_NODE_15 - Region 1	262 433	369 084	106 651	NRPS/T1PKS	Nostophycin	27%	Unknown product but homolog BGC	-
2	C885_NODE_26 - Region 1	108 837	171 757	62 920	NRPS-like/T1PKS	Puwainaphycins	30%	Unknown (no homolog)	-
3	C885_NODE_32 - Region 1	93 483	143 228	49 745	NRPS	-	-	Unknown (no homolog)	-
4	C885_NODE_43 - Region 1	115 568	136 380	20 812	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids precursor)
5	C885_NODE_44 - Region 1	114 952	136 889	21 937	Terpene	-	-	Uncertained	Terpene derivative
6	C885_NODE_60 - Region 1	1	81 263	81 262	NRPS/LAP/thiopeptide	Cyanopeptolin (NRPS)	57%	Unknown (no homolog)	-
7	C885_NODE_62 - Region 1	76 055	90 934	14 879	Terpene	-	-	Validated (manually curated)	Phytoene (carotenoids precursor)
8	C885_NODE_73 - Region 1	1	13 578	13 577	Lanthipeptide	-	-	Unknown (no homolog)	-
9	C885_NODE_102 - Region 1	10 331	41 567	31 236	Ladderane	-	-	Unknown (no homolog)	-
10	C885_NODE_213 - Region 1	1	6 434	6 433	NRPS	-	-	Unknown (no homolog)	-

Table S10. Biosynthetic genes for pigment production

Species	Strain number	C-phycoerythrin						allo-phycoerythrin						phycoerythrin					
		alpha chain <i>cpcA</i>	beta chain <i>cpcB</i>	linker polypep <i>cpcC</i>	linker polypep <i>cpcD</i>	linker polypep <i>cpcE</i>	linker polypep <i>cpcF</i>	Phycobilisome <i>cpcG</i>	Phycobilisome <i>cpcH</i>	Phycobilisome <i>cpcI</i>	alpha chain <i>cpcA</i>	beta chain <i>cpcB</i>	Phycobilisome <i>cpcC</i>	Phycobilisome <i>cpcD</i>	Allophycococcal subunit <i>cpcE</i>	beta chain <i>cpcF</i>	beta chain <i>cpcG</i>	Phycobilisome <i>cpcH</i>	Phycobilisome <i>cpcI</i>
<i>Planktothricoides racibolii</i>	PMC 877.14	++	++	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+
<i>Laspinema</i> sp.	PMC 878.14	+	+	+	-	+	-	-	+	+	+	-	-	+	+	-	-	-	-
<i>Microcoleus vaginatus</i>	PMC 879.14	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-
<i>Lyngbya martsensiana</i>	PMC 880.14	+	+	+	+	+	-	-	+	+	+	-	-	+	+	-	-	-	-
<i>Nostoc</i> sp.	PMC 881.14	+	+	+	-	+	+	-	+	+	+	-	-	+	+	+	+	+	++
<i>Alinostoc</i> sp.	PMC 882.14	+	+	+	+	+	-	-	+	+	+	-	-	+	+	-	-	-	-
<i>Leptolyngbya boryana</i>	PMC 883.14	++++	++++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Calothrix</i> sp.	PMC 884.14	++	++	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
<i>Pseudo-chroococcus coulteri</i>	PMC 885.14	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+

Species	Strain number	Scytonemin															
		<i>scyA</i>	<i>scyB</i>	<i>scyC</i>	<i>scyD</i>	<i>scyE</i>	<i>scyF</i>	<i>tyrA</i>	<i>frnE</i>	<i>aroB</i>	<i>trpE</i>	<i>trpC</i>	<i>trpA</i>	<i>tyrP1</i>	<i>trpB</i>	<i>trpD</i>	<i>aroG</i>
<i>Planktothricoides racibolii</i>	PMC 877.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Laspinema</i> sp.	PMC 878.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Microcoleus vaginatus</i>	PMC 879.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Lyngbya martsensiana</i>	PMC 880.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Nostoc</i> sp.	PMC 881.14	++	+	+	+++	+++	+	+	+	+	+	+	+	+	+	+	+
<i>Alinostoc</i> sp.	PMC 882.14	+	+	+	++	++	+	+	+	++	++	+	+	+	+	+	+
<i>Leptolyngbya boryana</i>	PMC 883.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Calothrix</i> sp.	PMC 884.14	+	+	+	+++	+++	+	+	+	+	++	++	+	++	++	+	++
<i>Pseudo-chroococcus coulteri</i>	PMC 885.14	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-

Species	Strain number	Carotenoids																
		Geranylgeranyl <i>crtE</i>	Phytoene synthase <i>crtB</i>	15-cis-phytyl <i>crtP</i>	Beta-carotene <i>crtQ</i>	Beta-carotene <i>crtH</i>	cis-carotene <i>crtI</i>	Lycopene cyclase <i>cruA</i>	Lycopene cyclase <i>cruP</i>	1,2'-hydratase <i>cruF</i>	2'-O-glycosyltransferase <i>cruG</i>	3,4'-desaturase <i>crtD</i>	GDP-fucose synthase <i>wcaG</i>	Beta-carotene <i>crtR</i>	Beta-carotene <i>crtO</i>	2,2'-beta-hydroxylase <i>crtG</i>	Lycopene cyclase <i>crtL</i>	Beta-carotene <i>crtW</i>
<i>Planktothricoides racibolii</i>	PMC 877.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
<i>Laspinema</i> sp.	PMC 878.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Microcoleus vaginatus</i>	PMC 879.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Lyngbya martsensiana</i>	PMC 880.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Nostoc</i> sp.	PMC 881.14	+	+	+	+	+	+	+	+	+++	+	+	+	+	+	+	+	+
<i>Alinostoc</i> sp.	PMC 882.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Leptolyngbya boryana</i>	PMC 883.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Calothrix</i> sp.	PMC 884.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+++
<i>Pseudo-chroococcus coulteri</i>	PMC 885.14	+	+	+	+	+	+	+	+	+	+	+	+++	+	+	+	+	+/+

+	presence of one gene copy
++	presence of two gene copies
+++	presence of three gene copies
++++	presence of four gene copies
-	absence of gene copy

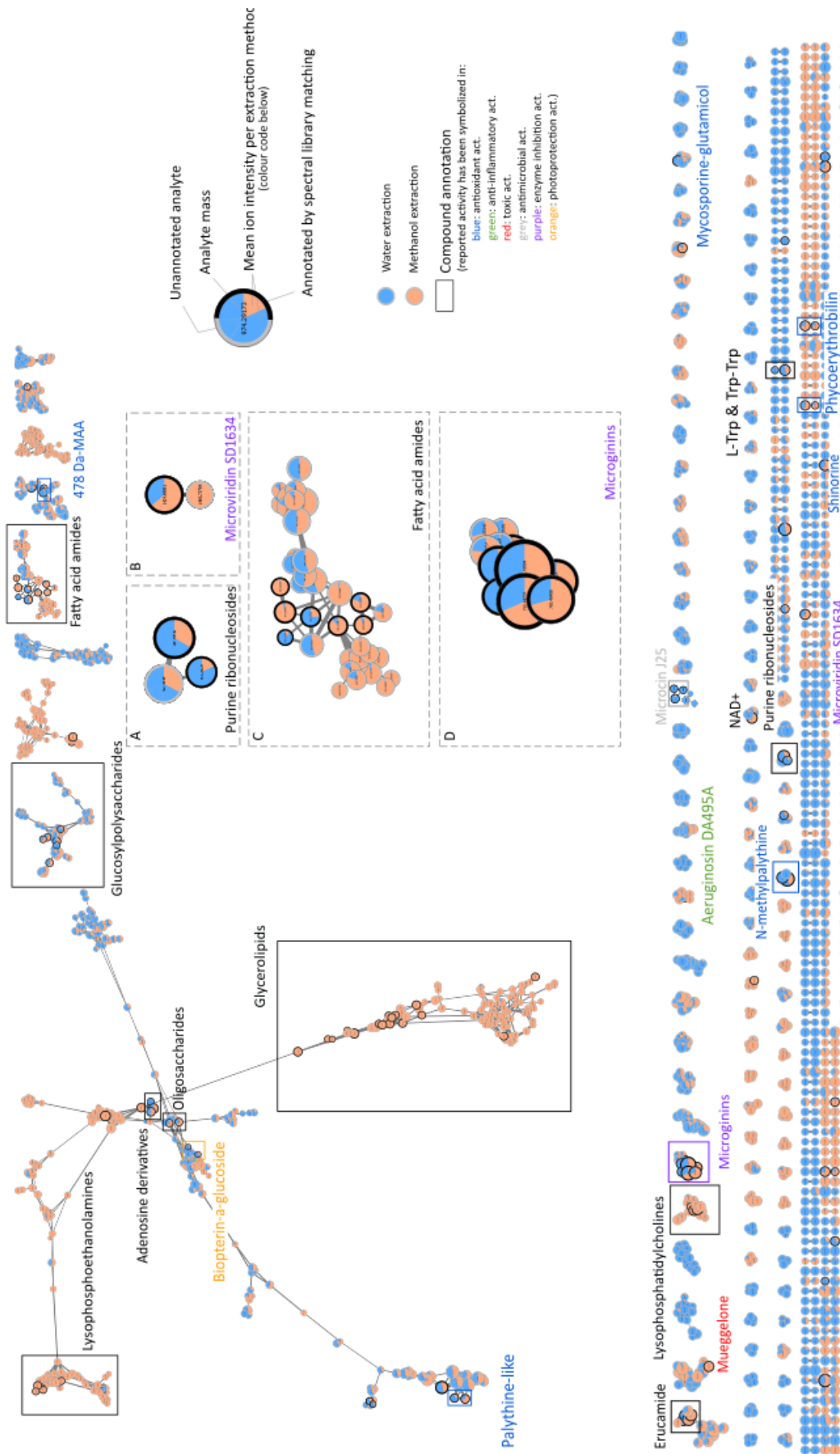


Figure S2. Molecular network of the metabolites produced by the nine cyanobacteria isolated from Balaruc thermal mud. Annotations obtained by untargeted analysis are figured inboxes. Nodes have been coloured according to the fraction of the extraction in which they were detected.

Table S11. Cell viability bioassays were assessed on the three cell lines used for other activity tests (RAW 264.7; HaCaT; PMBC cell lines). The reliable toxicity threshold is set at a 40% decrease in cell viability.

Bioassay	$\mu\text{g.mL}^{-1}$	<i>P. ractiborskii</i> PMC 877.14		<i>Laspinema</i> sp. PMC 878.14		<i>M. vaginatus</i> PMC 879.14		<i>L. martensiana</i> PMC 880.14		<i>Nostoc</i> sp. PMC 881.14		<i>Altitostoc</i> sp. PMC 882.14		<i>L. boryana</i> PMC 883.14		<i>Calothrix</i> sp. PMC 884.14		<i>P. couteii</i> PMC 885.14	
		MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O	MeOH	H ₂ O
RAW 264.7	1	-	-	-31 +/- 2%	-	-	-33 +/- 1%	-	-38 +/- 1%	-	-32 +/- 2%	-	-	-	-	-	-	-	-
	5	-	-	-32 +/- 2%	-	-	-34 +/- 5%	-32 +/- 1%	-41 +/- 0%	-	-36 +/- 3%	-	-	-	-	-	-	-	-
	10	-	-	-30 +/- 2%	-	-	-28 +/- 6%	-35 +/- 1%	-37- /2%	-	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-28 +/- 2%	-	-	-28 +/- 3%	-	-	-32 +/- 1%	-	-	-	-	-	-
Cell Viability HaCaT	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PBMC	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table S12-S14. BGCs from heterotrophic bacteria associated to Balaruc cyanobacteria in culture

Strain_Phylum	Nb_seq	Total_size (bp) > 2Mb			
C877_Bacteroidetes	8	2940501	3 Mb		
<i>P. raciborskii</i> PMC 877.14	15	7391557	7 Mb		
C877_Proteobacteria	7	3625685	4 Mb		
Region	Cluster type	From	To	Automatic annotation	Similarity
C877_Bacteroidetes					
Region 1.1	terpene	375,588	393,393	carotenoid	28%
Region 1.2	terpene	1,478,242	1,499,378		
C877_Proteobacteria					
Region 1.1	T3PKS	642,994	684,064	Stilbene synthase	
Region 1.2	betalactone	1,038,946	1,061,957		
Region 1.3	terpene	1,328,349	1,352,625	zeaxanthin	66%
Region 1.4	bacteriocin	3,397,096	3,407,962		

Strain_Phylum	Nb_seq	Total_size (bp) > 2Mb			
<i>Laspinema</i> sp. PMC 878.14	69	7336742	7 Mb		
C878_Proteobacteria	45	12013276	12 Mb		
Region	Cluster type	From	To	Automatic annotation	Similarity
C878_Proteobacteria					
Region 2.1	bacteriocin	172,817	182,664	N-tetradecanoyl tyrosi	6%
Region 2.2	bacteriocin	889,564	900,409		
Region 3.1	terpene	1,491,126	1,515,402	zeaxanthin	66%
Region 4.1	T3PKS	827,476	868,507		
Region 5.1	bacteriocin	493,475	504,341		
Region 7.1	T3PKS	344,592	385,671		
Region 8.1	terpene	324,954	345,886	zeaxanthin	66%
Region 9.1	terpene	168,596	189,771		
Region 11.1	betalactone	94,369	117,380		
Region 17.1	terpene	40,031	61,775		
Region 17.2	NAGGN	117,470	132,105		
Region 20.1	terpene	31,184	52,260	carotenoid	100%

Strain_Phylum	Nb_seq	Total_size (bp) > 2Mb			
C879_Bacteroidetes	15	1758668	2 Mb		
<i>M. vaginatus</i> PMC 879.14	34	6900942	8 Mb		
C879_Proteobacteria	11	10840780	11 Mb		
Region	Cluster type	From	To	Automatic annotation	Similarity
C879_Bacteroidetes					
Region 1.1	terpene	362,114	383,250	bacillomycin D	20%
C879_Proteobacteria					
Region 1.1	lassopeptide	59,674	82,047		
Region 1.2	resorcinol	877,084	918,226		
Region 1.3	terpene	2,262,269	2,285,875	carotenoid	50%
Region 2.1	NRPS	605,873	649,721	quinolobactin	13%
Region 2.2	acyl_amino_a	737,440	803,530		
Region 2.3	bacteriocin	1,021,910	1,033,529		
Region 2.4	hserlactone	1,127,881	1,148,627		
Region 2.5	terpene	1,515,604	1,536,849		
Region 2.6	terpene	1,616,641	1,637,678	carotenoid	100%
Region 3.1	acyl_amino_a	132,511	193,269		
Region 4.1	terpene	960,247	984,488	carotenoid	25%
Region 7.1	bacteriocin	30,208	40,167		
Region 7.2	bacteriocin	407,492	418,376		

Table S15. BGCs from heterotrophic bacteria associated to Balaruc cyanobacteria in culture (suite)

Strain_Phylum	Nb_seq	Total_size (bp)	> 2Mb		
C880_Bacteroidetes	1803	14271057	14 Mb		
<i>L.martensiana</i> PMC 880.14	132	6444104	6 Mb		
C880_Gemmatimonadetes	25	4542236	5 Mb		
C880_not classified	1059	7386760	8 Mb		
C880_Planctomycetes	216	6159044	6 Mb		
C880_Proteobacteria	2151	21578483	21 Mb		
C880_Verrucomicrobia	80	12198660	12 Mb		
Region	Cluster type	From	To	Automatic annotation	Similarity
C880_Bacteroidetes					
Region 1.1	T3PKS	293,918	334,997		
Region 1.2	terpene	339,573	360,415	carotenoid	100%
Region 6.1	terpene	2,827	24,395		
Region 175.1	T3PKS	1	13,353		
Region 230.1	terpene	1	10,860	carotenoid	42%
Region 355.1	terpene	1	7,739	carotenoid	42%
Region 861.1	T3PKS	1	3,334		
C880_Gemmatimonadetes					
Region 5.1	NRPS-like	187,071	230,421		
Region 7.1	terpene	193,343	214,425		
Region 15.1	terpene	1	20,921		
C880_not classified					
Region 29.1	terpene	1	13,674		
Region 35.1	terpene	24,420	35,162		
Region 169.1	terpene	1	12,890		
Region 257.1	bacteriocin	1	7,548		
Region 353.1	terpene	1	4,883		
Region 483.1	bacteriocin	1	3,127		
Region 687.1	terpene	1	1,905	carotenoid	100%
Region 729.1	bacteriocin	1	1,754		
C880_Planctomycetes					
Region 2.1	T1PKS	31,216	77,608		
Region 20.1	T3PKS	1	28,411		
Region 39.1	NRPS-like	6,091	45,069		
Region 41.1	terpene	7,010	28,959		
Region 73.1	NRPS-like	1	25,289		
Region 119.1	terpene	1	12,278		
C880_Proteobacteria					
Region 1.1	NAGGN	713,809	728,444		
Region 1.2	terpene	763,228	784,972		
Region 2.1	arylpolyene	394,378	435,577	APE Vf	10%
Region 12.1	bacteriocin	1	8,037		
Region 15.1	bacteriocin	85,427	96,302	N-tetradecanoyl tyros	6%
Region 17.1	terpene	66,118	87,110	(2R,3S,3'S)-2-hydrox	33%
Region 55.1	terpene	32,030	49,799		
Region 70.1	terpene	7,126	30,699	carotenoid	41%
Region 71.1	bacteriocin	36,514	42,106		
Region 76.1	terpene	29,535	40,909		
Region 105.1	terpene	5,249	28,846	carotenoid	83%
Region 128.1	NRPS	1	29,985		
Region 216.1	arylpolyene	1	22,177		
Region 218.1	TfuA-related	3,735	22,140		
Region 241.1	hserlactone	1	11,963		
Region 437.1	hserlactone	1	12,908		
Region 440.1	hserlactone	1	10,780		
Region 525.1	NRPS-like	1	10,675		
Region 542.1	betalactone	1	10,217		
Region 548.1	hserlactone	1	10,071	gentamicin	5%
Region 592.1	bacteriocin	1	6,019		
Region 706.1	T1PKS	1	7,029		
Region 779.1	NRPS-like	1	6,096		
Region 870.1	lassopeptide	1	5,070		
Region 926.1	terpene	1	4,511		
Region 952.1	lassopeptide	1	4,300		
Region 1137.1	terpene	1	3,149		
Region 1442.1	bacteriocin	1	2,095		
Region 1578.1	hserlactone	1	1,788		
Region 1612.1	T3PKS	1	1,730		
C880_Verrucomicrobia					
Region 2.1	NRPS-like	116,797	158,402		
Region 2.2	NRPS	224,523	269,115		
Region 4.1	terpene	553,632	574,498		
Region 5.1	terpene	227,102	248,130		
Region 7.1	ladderane	110,407	151,651		
Region 7.2	NRPS, T1PKS	259,992	337,273		
Region 13.1	NRPS, T1PKS	265,464	318,599	hapalosin	40%
Region 16.1	terpene	201,844	226,655		
Region 18.1	T3PKS	214,687	255,856		
Region 20.1	terpene	149,329	170,411		
Region 26.1	NRPS	86,648	130,541	alkylpyrone-407 / alk	4%
Region 27.1	NRPS	32,628	76,076		
Region 31.1	T1PKS	21,768	69,462		
Region 37.1	terpene	74,137	95,882		
Region 47.1	T3PKS	12,087	53,277		

Table S16, S17. BGCs from heterotrophic bacteria associated to Balaruc cyanobacteria in culture (suite)

Strain_Phylum	Nb_seq	Total_size (bp) > 2Mb			
<i>Nostoc</i> sp. PMC 881.14	648	7938353	8 Mb		
C881_not_classified	401	5727335	6 Mb		
C881_Proteobacteria	1146	27227936	27 Mb		
Region	Cluster type	From	To	Automatic annotation	Similarity
C881_not_classified					
Region 1.1	terpene	3,440	24,639	carotenoid	100%
Region 3.1	hserlactone	25,338	46,168		
Region 6.1	terpene	53,307	75,001		
Region 13.1	terpene	12,844	31,992	carotenoid	50%
Region 13.2	terpene	82,338	96,558		
Region 35.1	T3PKS	15,650	43,374	alkylresorcinol	100%
Region 48.1	terpene	1	18,017	carotenoid	100%
Region 53.1	acyl_amino_acids,hserlactone	1	24,545	N-acyl alanine	100%
C881_Proteobacteria					
Region 1.1	indole	493,985	515,277		
Region 3.1	bacteriocin	379,145	389,993		
Region 4.1	bacteriocin	319,562	330,437	N-tetradecanoyl tyrosine	6%
Region 8.1	T3PKS	123,988	165,082		
Region 8.2	lassopeptide,hserlactone	237,236	268,428	gellan polysaccharide	7%
Region 9.1	NRPS-like,T1PKS,NRPS	138,763	190,304		
Region 11.1	bacteriocin	98,030	108,233		
Region 14.1	betalactone	48,038	78,075		
Region 15.1	betalactone	89,411	117,657		
Region 21.1	terpene	80,393	101,568		
Region 24.1	hserlactone	99,764	120,381		
Region 31.1	terpene	1	17,854		
Region 48.1	arylpolyene	86,390	107,690	APE Vf	15%
Region 63.1	bacteriocin	60,916	71,743		
Region 71.1	hserlactone	48,161	68,766		
Region 72.1	terpene	70,521	81,478		
Region 74.1	arylpolyene	56,279	80,794		
Region 86.1	T3PKS	38,925	75,251		
Region 102.1	T3PKS	23,940	64,183		
Region 126.1	hserlactone	4,857	25,534		
Region 127.1	NRPS-like,T1PKS	11,928	53,614		
Region 153.1	terpene	1	15,121		
Region 159.1	NRPS,T1PKS	1	34,712	capsular polysaccharide	3%
Region 172.1	betalactone	4,591	36,403		
Region 177.1	indole	7,504	29,399		
Region 206.1	betalactone	3,708	30,194		
Region 265.1	bacteriocin	16,007	26,596		
Region 303.1	NAGGN	4,891	19,669		
Region 315.1	terpene	2,215	21,746		
Region 418.1	TfuA-related	1	15,131	kanamycin	7%
Region 428.1	terpene	1	14,403		
Region 460.1	NRPS-like	1	12,926		
Region 574.1	terpene	1	8,260		
Region 595.1	terpene	1	7,749		
Region 756.1	terpene	1	3,799		
Region 781.1	NRPS-like	1	3,473		

Strain_Phylum	Nb_seq	Total_size (bp) > 2Mb			
C882_Armatimonadetes	5	7360037	7 Mb		
<i>Aliinostoc</i> sp. PMC 882.14	46	8132153	8 Mb		
C882_Proteobacteria	41	9857661	10 Mb		
C882_Verrucomicrobia	2	8907			
Region	Cluster type	From	To	Automatic annt	Similarity
C882_Armatimonadetes					
Region 1.1	terpene	3,473,218	3,494,117		
Region 2.1	terpene	520,350	541,288		
C882_Proteobacteria					
Region 1.1	T3PKS	1,084,056	1,125,144		
Region 1.2	terpene	2,132,398	2,156,643	carotenoid	40%
Region 2.1	hserlactone,la	748,957	779,728		
Region 3.1	bacteriocin	83,075	93,911		
Region 4.1	terpene	853,021	877,388	carotenoid	33%
Region 5.1	terpene	21,401	46,225	carotenoid	33%
Region 5.2	lassopeptide	161,384	184,705	lipopolysacch	8

Table S18-S20. BGCs from heterotrophic bacteria associated to Balaruc cyanobacteria in culture (suite)

Strain_Phylum	Nb_seq	Total_size (bp)	> 2Mb		
C883 Armatimonadetes	4	3575512	4 Mb		
C883 Chloroflexi	6	5641484	6 Mb		
<i>L. boryana</i> PMC 883.14	5	6694777	7 Mb		
C883 Proteobacteria	72	6645423	7 Mb		
Region	Cluster type	From	To	Automatic an	Similarity
C883 Armatimonadetes					
Region 3.1	terpene	86,458	107,399		
C883 Chloroflexi					
Region 1.1	terpene	901,874	921,928		
Region 1.2	other	1,107,827	1,148,516		
Region 1.3	hglE-KS, T1PK	1,303,737	1,356,127	heterocyst gly	57%
Region 1.4	terpene	1,544,356	1,565,345		
Region 2.1	indole	305,205	326,479		
Region 3.1	other	473,594	514,370		
C883 Proteobacteria					
Region 1.1	lassopeptide	295,974	318,417		

Strain_Phylum	Nb_seq	Total_size (bp)	> 2Mb		
C884 Bacteroidetes	4	1942276	2 Mb		
<i>Calothrix</i> sp. PMC 884.14	46	13243152	12 Mb		
C884 Proteobacteria	132	11961935	13 Mb		
Region	Cluster type	From	To	Automatic an	Similarity
C884 Bacteroidetes					
Region 1.1	terpene	426,153	446,203		
Region 1.2	betalactone, T	615,389	677,072		
Region 1.3	terpene	805,203	830,425	carotenoid	42%
C884 Proteobacteria					
Region 1.1	terpene	389,387	410,448		
Region 2.1	betalactone	202,681	225,687		
Region 2.2	hserlactone, la	299,115	329,821	gellan polysac	7%
Region 9.1	betalactone	224,667	253,201	platensimycin	5%
Region 15.1	terpene	65,369	86,283	(2R,3S,3'S)-2	33%
Region 20.1	NRPS-like	57,913	100,525		
Region 24.1	bacteriocin	89,655	99,743		
Region 24.2	bacteriocin	128,860	139,735	N-tetradecano	6%
Region 34.1	T3PKS	69,104	110,198		
Region 47.1	terpene	11,922	32,728		
Region 51.1	phosphonate	7,632	48,498		
Region 54.1	T1PKS	42,795	79,016		
Region 77.1	terpene	1	11,589		
Region 96.1	arylpolyyene	3,425	26,566	xanthomonadi	14%
Region 104.1	terpene	1	16,316		

Strain_Phylum	Nb_seq	Total_size (bp)	> 2Mb		
C885 Bacteroidetes	30	7094005	7 Mb		
<i>Pseudo-chroococcus couteii</i> PMC 885.1	137	5858006	5 Mb		
C885 Proteobacteria	52	18208582	18 Mb		
C885 Verrucomicrobia	76	4044644	4 Mb		
Region	Cluster type	From	To	Automatic an	Similarity
C885 Bacteroidetes					
Region 1.1	terpene	939,489	964,724	carotenoid	42%
Region 1.2	betalactone	1,873,792	1,904,496	belactosin A /	8%
Region 1.3	bacteriocin	2,923,911	2,936,076		
Region 2.1	terpene	607,832	628,671	carotenoid	28%
Region 3.1	terpene	82,152	103,288	bacillomycin I	20%
C885 Proteobacteria					
Region 1.1	betalactone	2	25,094	fengycin	13%
Region 1.2	betalactone	2,480,073	2,506,146	lipopolysacch	5%
Region 1.3	terpene	3,203,551	3,223,720	carotenoid	100%
Region 1.4	terpene	3,760,742	3,782,246		
Region 2.1	terpene	593,773	613,219		
Region 2.2	terpene	937,884	958,891	carotenoid	100%
Region 2.3	NRPS-like, T1	1,177,234	1,228,154		
Region 3.1	bacteriocin	135,147	145,992		
Region 3.2	hserlactone	568,025	589,985		
Region 4.1	NAGGN	1,560,492	1,575,214		
Region 5.1	terpene	52,141	73,166		
Region 5.2	bacteriocin	1,389,174	1,400,076		
Region 8.1	terpene, T1PK	92,078	216,175	BE-43547A1 /	20%
Region 8.2	arylpolyyene	309,552	350,742	xanthomonadi	14%
Region 9.1	T3PKS	96,418	137,455		
Region 13.1	terpene	18,159	38,890	conglobatin	10%
Region 14.1	bacteriocin	112,837	123,682		
Region 17.1	bacteriocin	64,156	75,010		
Region 22.1	T1PKS	7,683	56,706		
Region 23.1	lassopeptide, t	16,414	61,965		
C885 Verrucomicrobia					
Region 10.1	bacteriocin	80,997	91,884		
Region 13.1	NRPS, T1PKS	39,524	73,831		

Annexe 8 : Supplementary materials – Article 4

2 supplementary tables 11 supplementary figures

Table S1. Biosynthetic gene clusters of *Aliinostoc* sp. PMC 882.14

Cluster	Contig	From	To	Cluster type	Automatic annotation	Similarity	Annotation status	Expert annotation
1	C882_NODE_3_1	15 640	37 498	NRPS-like	Shinorine	100%	Validated	Shinorine
2	C882_NODE_3_2	245 505	310 129	NRPS/T1PKS	Puwainaphycins	60%	Validated (manually curated)	Microginin
3	C882_NODE_3_3	640 730	684 201	NRPS-like	X	X	Unknown (no homolog)	X
4	C882_NODE_3_4	1 403 211	1 493 957	NRPS/T1PKS	Hapalosin	40%	Unknown (no homolog)	X
5	C882_NODE_3_5	1 568 034	1 637 956	NRPS	Cyanopeptin	75%	Unknown (no homolog)	X
6	C882_NODE_7_1	82 074	126 088	T1PKS	Amycomycin	62%	Uncertained	Amycomycin-like
7	C882_NODE_7_2	166 759	210 645	NRPS	Micropeptin	62%	Unknown (no homolog)	X
8	C882_NODE_7_3	264 995	311 442	T1PKS/bacteriocin	Jamaicamide	15%	Unknown (no homolog)	X
9	C882_NODE_7_4	404 145	473 658	NRPS	Nostopeptolide	37%	Unknown (no homolog)	X
10	C882_NODE_7_5	560 196	581 026	Terpene	X	X	Validated (manually curated)	Phytoene (cartenoid precursor)
11	C882_NODE_9_1	145 215	197 610	T1PKS/hglE-KS	Heterocyst glycolipids	85%	Validated	Heterocyst glycolipids
12	C882_NODE_9_2	335 457	345 690	Bacteriocin	X	X	Unknown product, but homolog BGC	X
13	C882_NODE_9_3	364 924	406 075	Ladderane	X	X	Unknown product, but homolog BGC	X

14	1	C882_NODE_12_	482 095	502 947	Microviridin	Microviridin K	62%	Validated	Microviridin
15	1	C882_NODE_13_	45 863	138 173	NRPS/TIPKS	Nostophycin	27%	Unknown product, but homolog BGC	X
16	1	C882_NODE_14_	225 206	244 913	Terpene	6,6'-oxybis(2,4-dibromophenol)	21%	Unknown product, but homolog BGC	X
17	2	C882_NODE_14_	305 160	326 092	Terpene	X	X	Validated (manually curated)	Phytoene (cartenoid precursor)
18	1	C882_NODE_15_	193 638	269 628	NRPS	Puwainaphycins	40%	Unknown product, but homolog BGC	X
19	2	C882_NODE_15_	270 364	315 220	TIPKS	Carbamidocyclophane	30%	Uncertained	Cylindrocyclophane-like
20	1	C882_NODE_16_	202 309	253 885	NRPS/betalactone	X	X	Unknown (no homolog)	X
21	1	C882_NODE_18_	65 575	76 344	Bacteriocin	Welwitindolinone	6%	Unknown product, but homolog BGC	X

antiSMASH betalactone = betalactone containing protease inhibitor

Confidence level

Low
Medium
High
Homologous with uncharacterized BGC

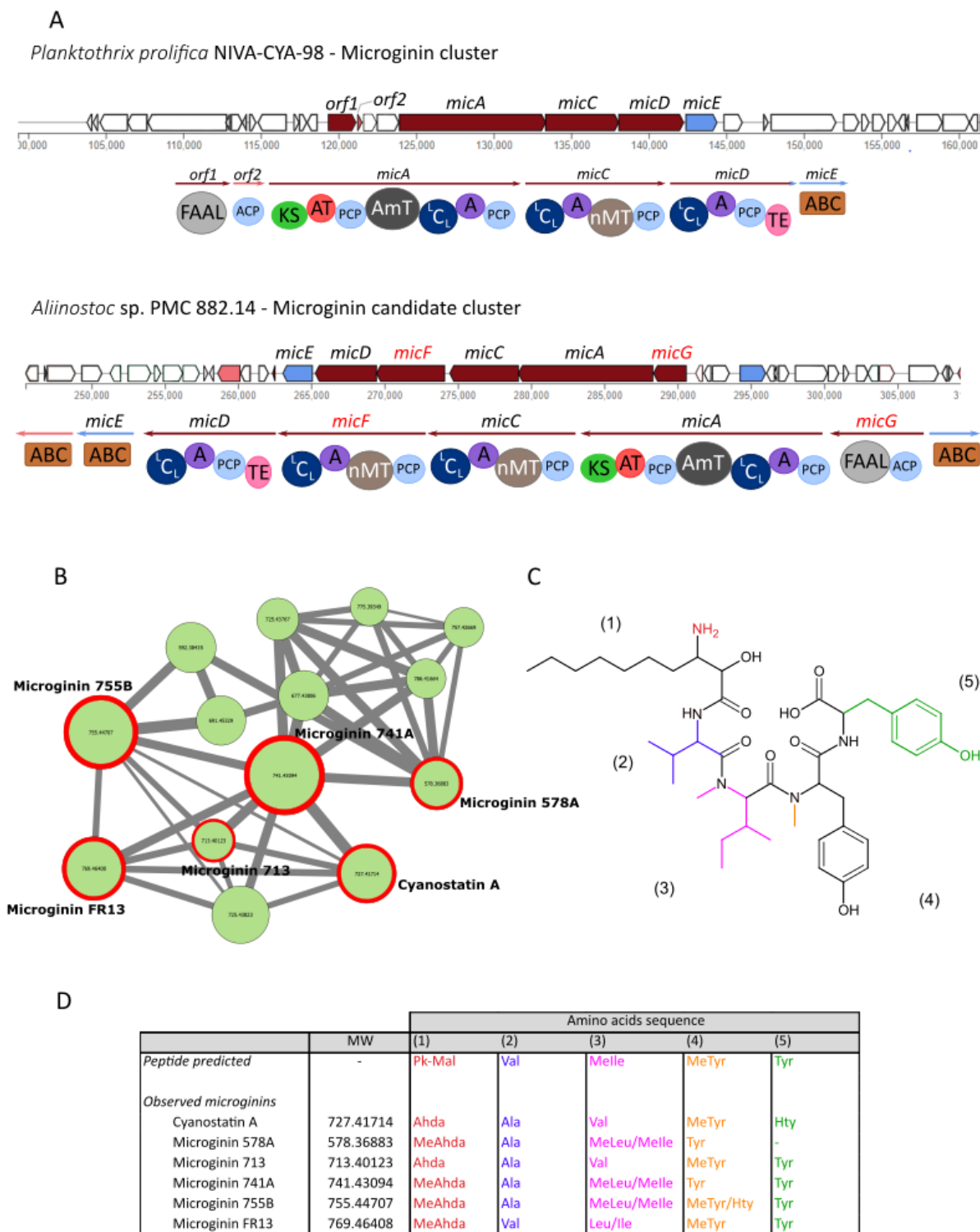


Figure S1. Production of microginins shown for *Aliinostoc* sp. PMC 882.14. A). Comparison of the microginin reference cluster of *Planktothrix prolifica* NIVA-CYA 98 and the microginin-candidate gene cluster from PMC 882.14. Both syntheses are shown under the BGCs representation. Core biosynthetic genes are represent in dark red, additional biosynthetic genes in pink and transport-related genes in blue. (Abr. FAAL: fatty acyl-AMP ligase domain; ACP: acyl-carrier protein; KS: ketosynthase domain; AT: acyltransferase domain; PCP: peptidyl-carrier protein domain; LCL: condensation domain linking a L-amino acid to a peptide ending with a L-amino acid; A: adenylation domain; nMT: nitrogen methyltransferase; TE: thioesterase domain; ABC: ATP-binding cassette transporter; AmT: aminotransferase domain); B) Microginin cluster obtained by molecular network analysis. Six variants were identified (circled in red); C) Predicted structure of the microginin produced by the hybrid NRPS/PKS synthase identified in PMC 882.14; D) Amino acid sequences of the predicted peptide and the six identified variants.

Table S2. *Aliinostoc* sp. PMC 882.14 list of metabolite annotation.

PEPMASS (Da)	RT (min)	METABOLITE NAME	MOLECULAR FORMULA
128.04865	45.1 3	(1E)-1-hydroxy-4-methoxy-penta-1,4-dien-3-one	C ₆ H ₈ O ₃
147.0535	63.0 5	(1-formyl-2-methoxy-ethyl)carbamic acid	C ₅ H ₉ NO ₄
154.03995	774. 99	(2-keto-1-pyridyl)carbamic acid	C ₆ H ₆ N ₂ O ₃
181.07489	478. 48	Tyrosine	C ₉ H ₁₁ NO ₃
202.11176	496. 17	N-[2-(1H-Indol-3-yl)ethyl]acetamide	C ₁₂ H ₁₄ N ₂ O
203.12735	68.0 4	Aphanorphine	C ₁₃ H ₁₇ NO
204.07962	834. 87	7-formyl-3-methoxy-5-methylindanone	C ₁₂ H ₁₂ O ₃
204.13724	565. 78	(3R)-3,10-dihydroxydecanoic acid	C ₁₀ H ₂₀ O ₄
218.16788	855. 93	Anaephene A	C ₁₅ H ₂₂ O
219.10549	840. 22	N-Phenyl-1-naphthylamine	C ₁₆ H ₁₃ N
224.18924	669. 6	(-)-anaferine	C ₁₃ H ₂₄ N ₂ O
228.17219	773. 96	(-)-7-methoxydodec-4(E)-enoic acid	C ₁₃ H ₂₄ O ₃
229.08956	69.3 8	caerulomycin A	C ₁₂ H ₁₁ N ₃ O ₂
244.10588	116. 39	Palythine	C ₁₀ H ₁₆ N ₂ O ₅

244.16856	792. 88	Tridecanedioic acid	$C_{13}H_{24}O_4$
245.08669	110. 12	Mycosporine glycine	$C_{10}H_{15}NO_6$
246.12134	129. 55	Glu-Val	$C_{10}H_{18}N_2O_5$
260.13684	257. 54	Glu-Cys	$C_8H_{14}N_2O_5S$
267.09664	108. 67	Adenosine	$C_{10}H_{13}N_5O_4$
270.08422	107. 28	3-acetyl-2'-deoxyuridine	$C_{11}H_{14}N_2O_6$
278.0931	142. 77	Pukeleimide A	$C_{13}H_{14}N_2O_5$
292.20365	751. 33	Mueggelone	$C_{18}H_{28}O_3$
292.20392	657. 91	Mueggelone	$C_{18}H_{28}O_3$
296.23457	884. 96	7-methoxy-9-methylhexadecadienoic acid	$C_{18}H_{32}O_3$
308.19869	676. 33	Sacrolide A	$C_{18}H_{28}O_4$
328.26104	781. 76	Monoacylglycerol 16:1	$C_{19}H_{36}O_4$
328.26106	792. 94	Undecanedioic acid disec-butyl ester	$C_{19}H_{36}O_4$
328.26115	713. 84	Monoacylglycerol 16:1	$C_{19}H_{36}O_4$
330.27651	844. 59	Palmitic acid glyceryl ester	$C_{19}H_{38}O_4$
332.12179	78.9 9	Shinorine	$C_{13}H_{20}N_2O_8$

347.06289	108. 5	Adenosine 5'-monophosphate	C ₁₀ H ₁₄ N ₅ O ₇ P
352.26111	714. 01	Monolinolenin	C ₂₁ H ₃₆ O ₄
358.15268	478. 47	Phe-Tyr	C ₁₈ H ₂₀ N ₂ O ₄
383.27814	478. 45	acetylleucyl-leucyl-norleucinal (tripeptide)	C ₂₀ H ₃₇ N ₃ O ₄
389.12706	55.9 8	S-(2-methyl-1-oxobut-3-en-2-yl)glutathione	C ₁₅ H ₂₃ N ₃ O ₇ S
399.1391	136. 82	Biopterin-a-glucoside	C ₁₅ H ₂₁ N ₅ O ₈
399.13919	110. 45	Biopterin-a-glucoside	C ₁₅ H ₂₁ N ₅ O ₈
461.2138	57.8	L-Ala-gamma-D-Glu-meso-Dap-D-Ala	C ₁₈ H ₃₁ N ₅ O ₉
478.1799	162. 6	478-Da MAA	C ₁₉ H ₃₀ N ₂ O ₁₂
492.32944	844. 59	(2S)-1-O-Palmitoyl-3-O-?-D-galactopyranosylglycerol	C ₂₅ H ₄₈ O ₉
492.32944	766. 61	(2S)-1-O-Palmitoyl-3-O-?-D-galactopyranosylglycerol	C ₂₅ H ₄₈ O ₉
496.19052	121. 41	Glycan Le-A Trisaccharide	C ₂₀ H ₃₅ NO ₁₅
552.26118	774. 92	H-DL-Tyr-DL-Ser-DL-Arg-DL-Gln-OH	C ₂₃ H ₃₆ N ₈ O ₈
578.36814	454. 92	Microginin 578A	C ₃₀ H ₅₀ N ₄ O ₇
586.27888	606. 86	Phycoerythrobilin	C ₃₃ H ₃₈ N ₄ O ₆
592.3837	459. 91	Gallinamide A	C ₃₁ H ₅₂ N ₄ O ₇

606.39927	470. 79	Microginin 606A	$C_{32}H_{54}N_4O_7$
612.15235	108. 18	glutathione disulfide	$C_{20}H_{32}N_6O_{12}S_2$
691.45206	488. 5	Microginin 591	$C_{36}H_{61}N_5O_8$
707.53322	769. 92	Triterpene	$C_{41}H_{73}NO_8$
713.39995	438. 61	Microginin 713	$C_{37}H_{55}N_5O_9$
721.54883	778. 66	Bacteriohopanetetrol	$C_{42}H_{75}NO_8$
725.43589	481. 46	Microginin 725	$C_{39}H_{59}N_5O_8$
727.41564	443. 12	Microginin 727 (Cyanostatin A)	$C_{38}H_{57}N_5O_9$
727.41576	469. 36	Microginin 727 (Cyanostatin A)	$C_{38}H_{57}N_5O_9$
741.43029	478. 45	Microginin 741A	$C_{39}H_{59}N_5O_9$
755.44607	483. 11	Microginin 755B	$C_{40}H_{61}N_5O_9$
755.44679	499. 67	Microginin 755B	$C_{40}H_{61}N_5O_9$
769.46212	492. 57	Microginin FR13	$C_{41}H_{63}N_5O_9$
771.4414	467. 09	Microginin 771	$C_{40}H_{61}N_5O_{10}$
916.46988	430	Unknown 916	$C_{47}H_{64}N_8O_{11}$
934.47916	429. 94	Somamide/Dolastatin 934	$C_{47}H_{66}N_8O_{12}$

1080.5169 7	617. 89	Somamide/Dolastatin 1080	$C_{56}H_{72}N_8O_{14}$
1098.5252 7	617. 9	Somamide/Dolastatin 1098	$C_{57}H_{70}N_{12}O_{11}$
1122.4599 7	599. 27	Aminoglycodise analog	$C_{54}H_{70}N_6O_{20}$
1152.4436 7	617. 89	Somamide/Dolastatin 1152	$C_{73}H_{56}N_{10}O_5$
1634.7017 6	417. 91	Microviridin SD1634	$C_{75}H_{98}N_{18}O_{24}$
1724.7374 4	447	Microviridin 1724	$C_{84}H_{100}N_{20}O_{21}$

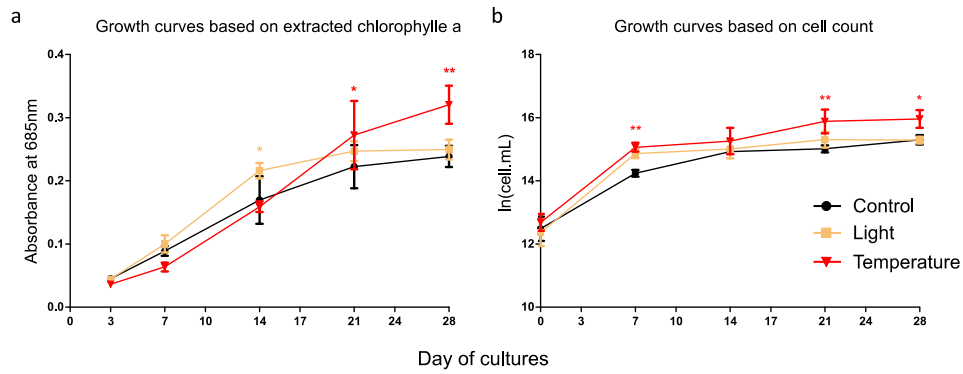


Figure S2. Cell growth curves of *Aliinostoc* sp. PMC 882.14 according to different culture conditions (control, high light, high temperature) considering the a) chlorophyll a concentration, and the b) cell abundance (mean \pm SD). Significant differences between control and treatments (high light, high temperature) were assessed by the ANOVA ($p < 0.05$) corrected with Bonferroni's test.

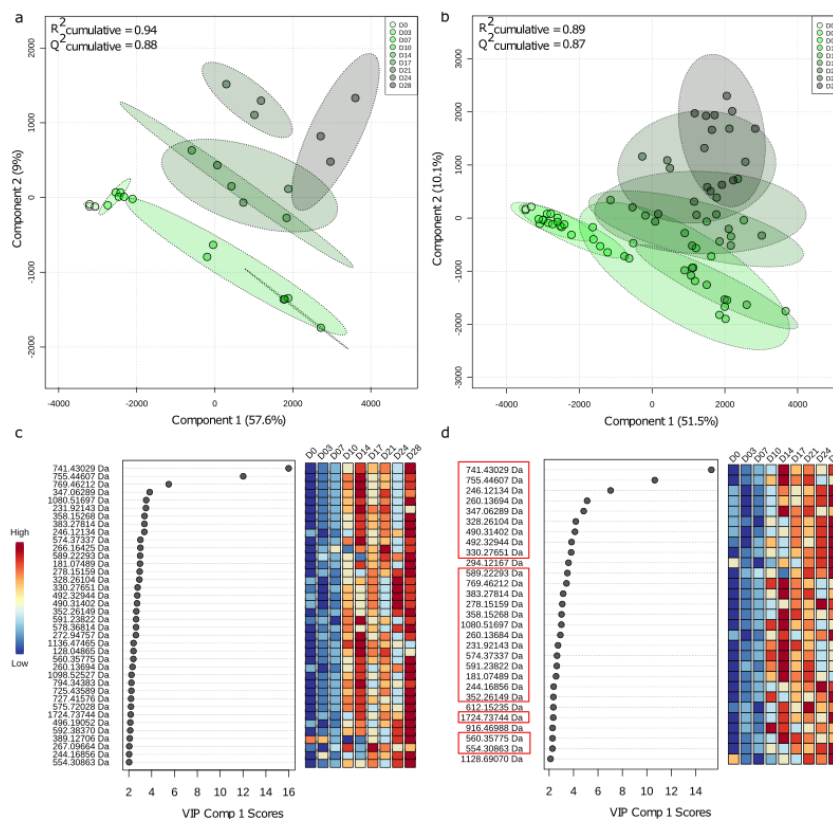


Figure S3. Partial Least Square Discriminant Analysis (PLS-DA) and corresponding VIP list describing the variation of the intracellular metabolic composition through the different time of culture sampled for controls (a-c) and all conditions (b-d). Individual score plots (a and b) and the variables (c and d) best explaining the differences in metabolic composition between the different culturing days (comprising 38 and 29 metabolites exhibiting VIP scores > 2 , respectively; Supplementary figure S3 and S4). Red rectangles (d) highlight 25 common metabolites also observed in PLS-DA performed only with the sample control dataset.

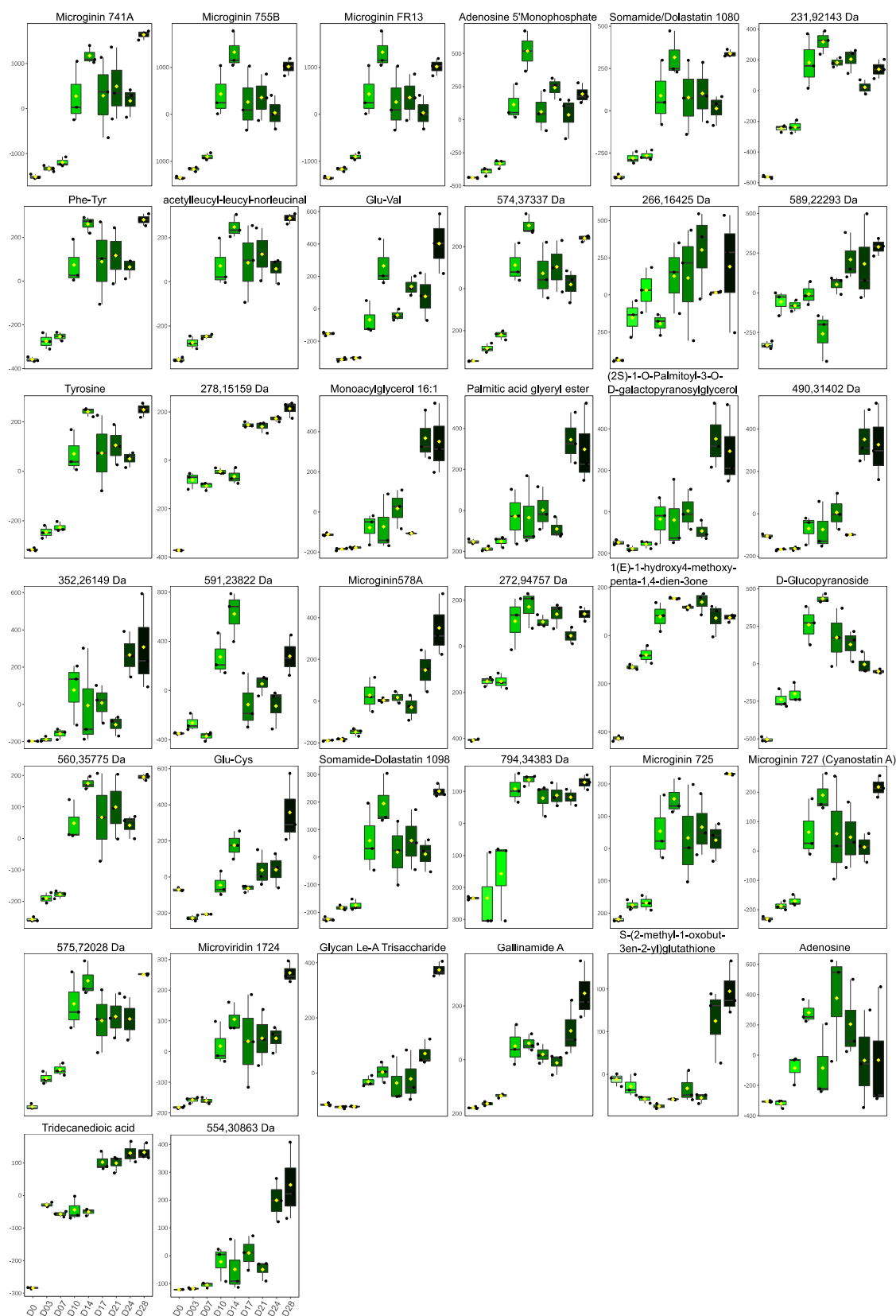


Figure S4. Box-plots of all the 38 intracellular metabolites presenting VIP scores >2 , and that best explain the intracellular metabolite variation during the time series under control conditions only, according to PLS-DA analysis.

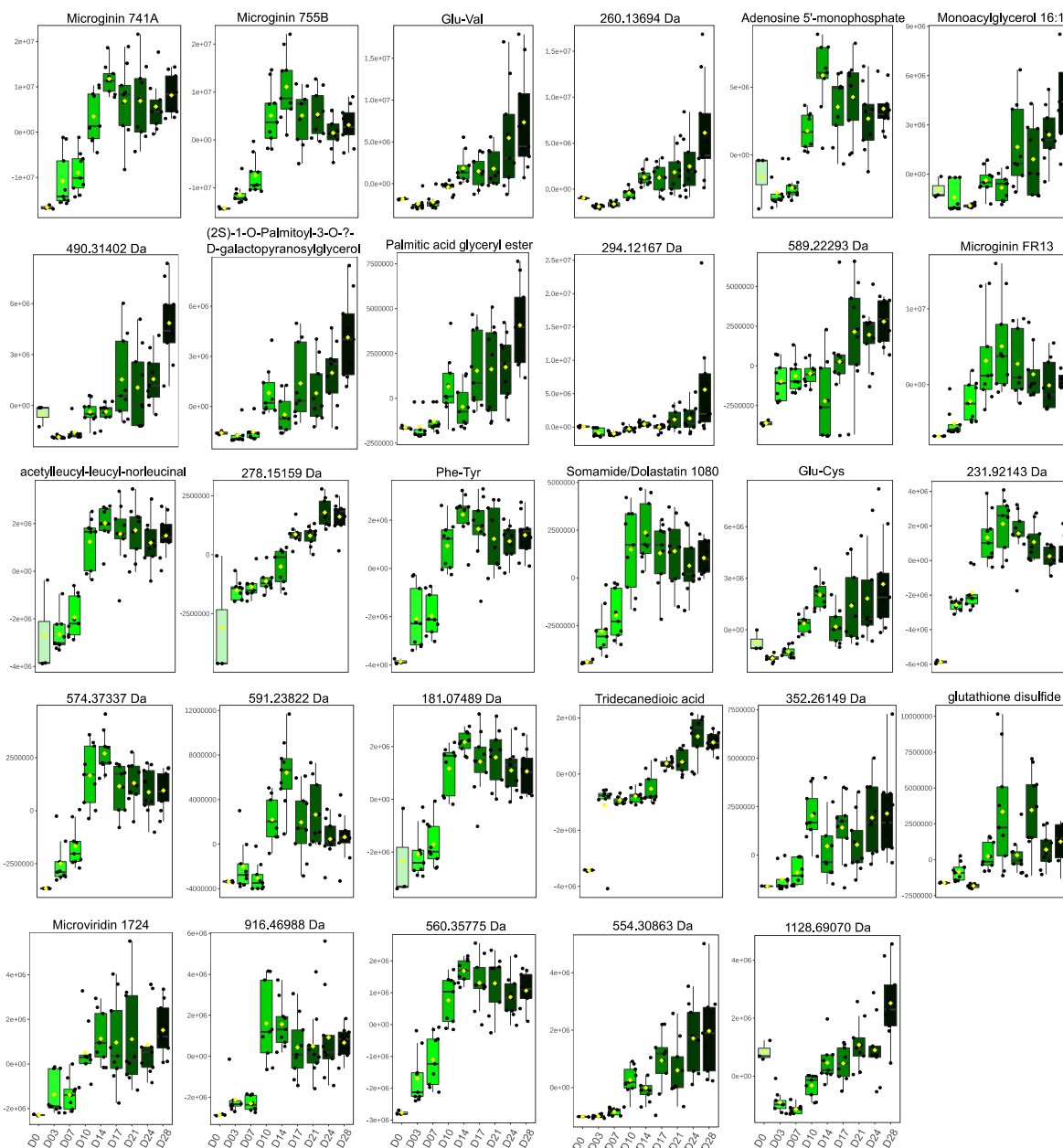


Figure S5. Box-plots of all the 29 intracellular metabolites presenting VIP scores >2 , and that best explain the intracellular metabolite variation during the time series under control, high light and high temperature conditions, according to PLS-DA analysis

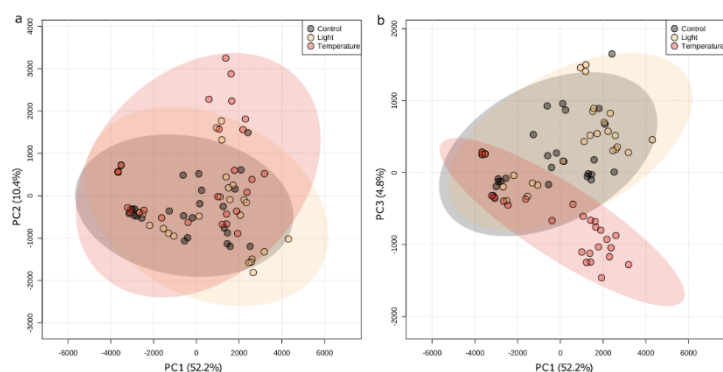


Figure S6. Principal Component Analysis (PCA) representing the intracellular metabolic composition of *Aliinostoc* sp. PMC 82.14 as a function of the culture conditions (control, light, temperature) according to components 1-2 (a) and components 1-3 (b). Each point represents a culture replicate.

Annotation	MW (Da)	RT (s)	Condition.adj. p.	Significativité Anova condition	MB.statistics	Meba Rank
Symplostatin/Dolastatin 1080	1080,51697	617,89	9,75E-17	*	10,755	1
	574,37337	483,15	1,41E-23	*	8,767	2
	591,23822	126,39	2,34E-14	*	8,4507	3
	383,27814	478,45	2,06E-16	*	8,3909	4
Phe-Tyr	358,15268	478,47	6,12E-18	*	4,2673	5
Symplostatin/Dolastatin 1098	1098,52527	617,9	1,55E-14	*	4,1965	6
Adenosine monophosphate	347,06289	108,5	3,25E-17	*	2,974	7
Tyrosine	181,07489	478,48	6,93E-16	*	2,9718	8
	916,46988	430	3,29E-17	*	2,7954	9
Microginin 755B	755,44607	483,11	1,22E-20	*	2,7903	10
Microviridin Like 1724	1724,73744	447	7,14E-15	*	2,569	11
Mycosporine gly	245,08273	67,05	3,71E-15	*	2,1385	12
Microginin 741A	741,43029	478,45	2,85E-19	*	1,9134	13
Microginin Cyanostatin A	727,41576	469,36	1,85E-18	*	1,0855	14
Microviridin K	1634,70176	417,91	5,55E-19	*	0,54826	15
	560,35775	478,46	5,13E-24	*	0,23169	16
Glu-Cys	260,13694	233,62	7,80E-16	*	-0,02492	17
	231,92143	45,66	1,32E-26	*	-0,19771	18
	589,22293	136,33	3,18E-08	*	-0,34041	19
	397,29385	483,13	7,06E-19	*	-0,47849	20
Glu-Val	246,12134	129,55	1,40E-18	*	-0,8154	21
Microginin FR13	769,46212	492,57	9,84E-12	*	-1,1977	22
Lipid Cluster	588,38921	492,62	1,03E-11	*	-1,7025	23
	492,32944	844,59	2,50E-12	*	-1,7978	24
	238,95991	45,36	1,72E-22	*	-1,9585	25
	496,19052	121,41	1,19E-07	*	-2,2128	26
	808,35856	483,18	1,41E-23	*	-2,6115	27
	210,10298	775,05	4,67E-04	*	-2,7509	28
	725,43589	481,46	5,59E-13	*	-3,5675	29
	555,13068	108,69	3,41E-17	*	-3,6947	30
	2234,99902	617,93	7,69E-16	*	-3,8586	31
	575,72028	617,9	4,40E-13	*	-3,9654	32
	794,34383	478,53	4,21E-16	*	-4,2866	33
	272,94757	45,78	6,16E-23	*	-4,317	34
	260,13684	257,54	6,38E-11	*	-4,3709	35
	328,26104	781,76	3,30E-15	*	-4,5952	36
	779,37852	478,48	4,06E-23	*	-4,8149	37
	267,09664	108,67	1,84E-05	*	-5,1143	38
	330,27651	844,59	3,32E-12	*	-5,1681	39
	461,2138	57,8	1,18E-05	*	-5,5018	40
	352,26149	775,35	3,25E-09	*	-6,301	41
	1643,30459	454,44	1,98E-09	*	-6,4183	42
	793,39387	483,24	1,84E-13	*	-7,2629	43
	1128,81511	377,47	3,50E-12	*	-7,4437	44
Saccharide cluster	1136,47465	617,94	2,15E-15	*	-7,4493	45
	249,93189	45,75	4,38E-27	*	-7,4845	46
	592,3837	459,91	9,71E-16	*	-7,839	47
Shinorine	332,12179	78,99	3,65E-10	*	-7,9429	48

Figure S7. List of metabolites presenting top-48 MEBA scores and respective annotations. The variation profiles of a selection of 22 of these compounds (underlined in yellow) are shown on figure 6.

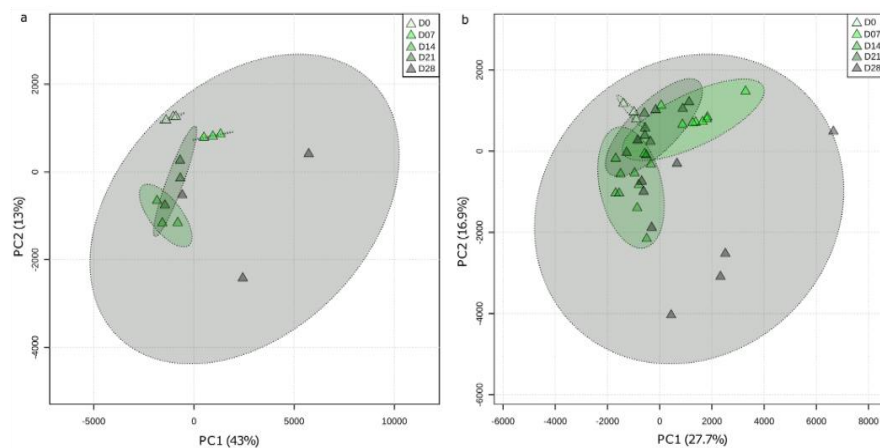


Figure S8. Principal Component Analysis (PCA) representing the evolution of the extracellular metabolic composition of *Alinostoc* sp. PMC 882.14 according to the time of culture a) under the control condition, and b) under all conditions. Each point represents a culture replicate.

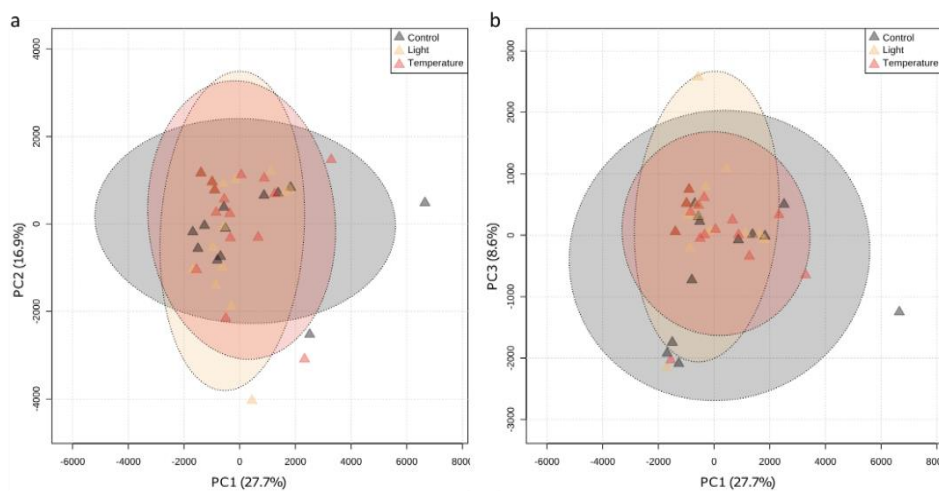


Figure S9. Principal Component Analysis (PCA) representing the evolution of the extracellular metabolic composition of *Alinostoc* sp. PMC 882.14 according to experimental factors a) for components 1-2, and b) components 1-3. Each point represents a culture replicate.

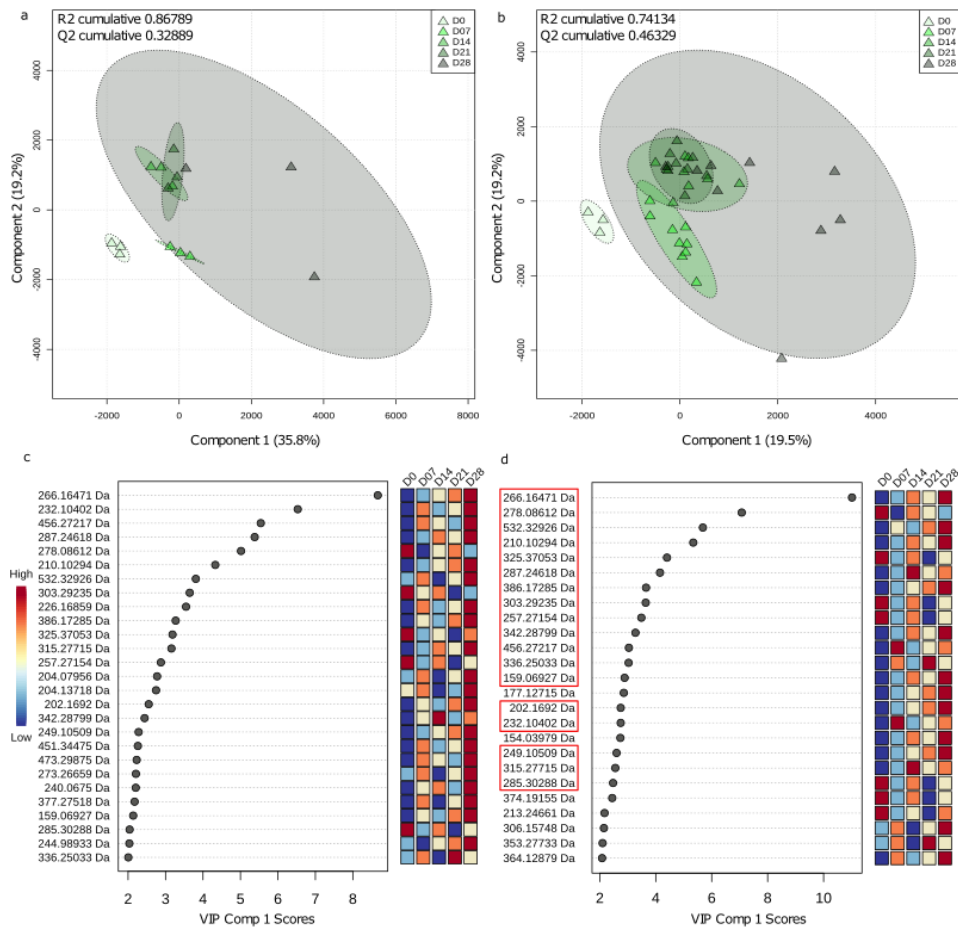


Figure S10. Partial Least Square Discriminant Analysis (PLS-DA) and corresponding VIP list describing the variation of the extracellular metabolic composition through the different culture time sampled for controls (a-c) and all conditions (b-d). Individual score plots (a and b) and the variables (c and d) best explaining the differences in metabolic composition between the different culturing days (27 and 25 metabolites with VIP scores > 2, respectively). Red rectangles (d) highlight common metabolites also observed in PLS-DA performed only with the sample control dataset.

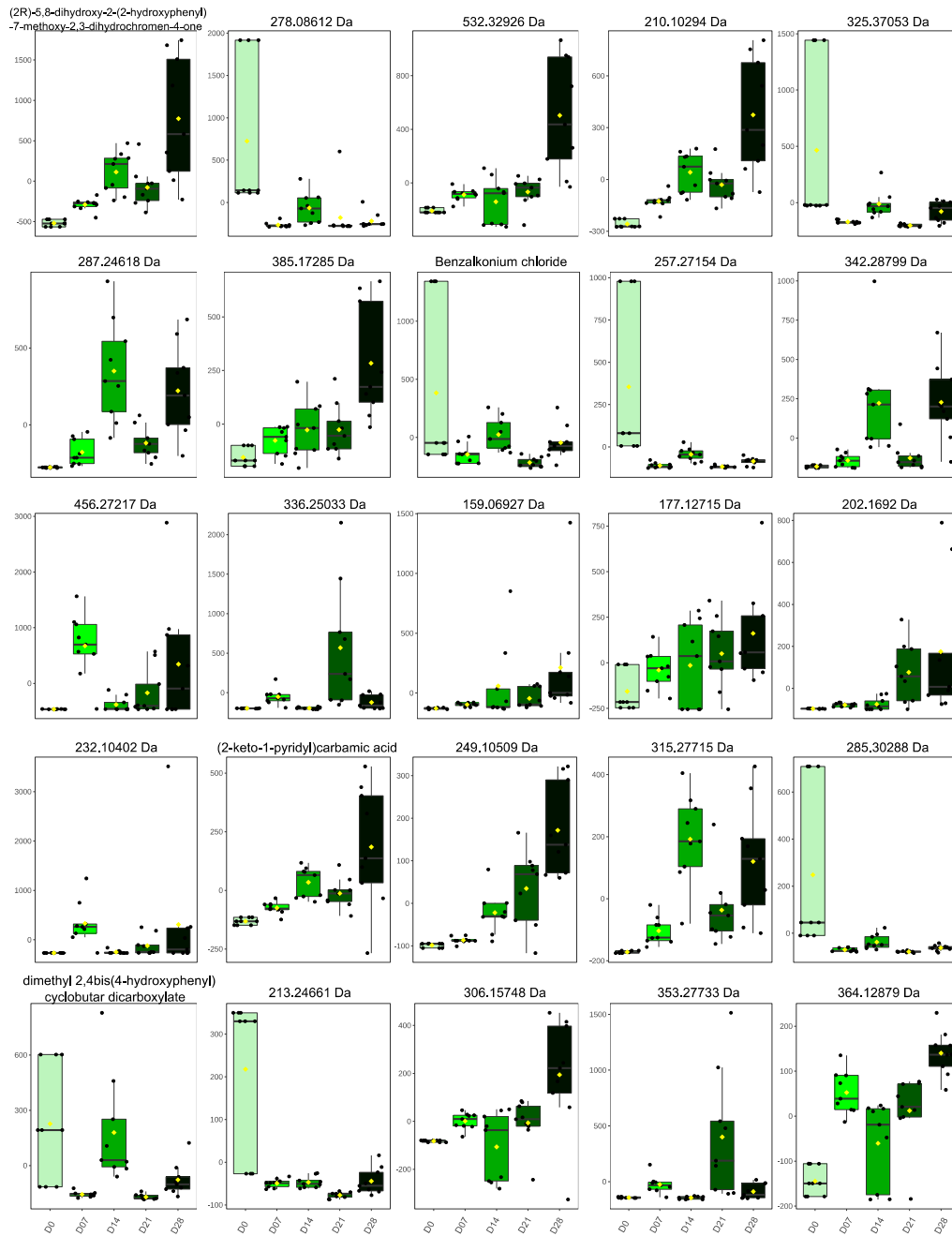


Figure S11. Box-plots of all the 25 extracellular metabolites presenting VIP scores >2 , and that best explain the intracellular metabolite variation during the time series under control, high-light and high-temperature conditions, according to PLS-DA analysis. Notice that for some analytes (*e.g.* 278.08612 Da, 325.37053 Da, benzalkonium chloride, 342.28799 Da, ...) the triplicates of D0 samples correspond to triplicates of 1/10-diluted 46-days old pre-cultures, that may explain the such large variability observed between those replicates for these components.

Résumé :

Les cyanobactéries sont des microorganismes procaryotes capables de réaliser la photosynthèse oxygénique comme source d'énergie pour leur métabolisme et capable de coloniser divers écosystèmes (e.g. mers, rivières, lacs, sol, milieux extrêmes ...). Elles sont également connues pour produire de nombreuses molécules bioactives (toxines, antimicrobiens, anti-inflammatoires, ...) aux structures variées. Ainsi, ces dernières années, les cyanobactéries ont fait l'objet de nombreux projets de recherche visant à découvrir et caractériser de nouveaux produits naturels exploitables dans les domaines pharmaceutiques, cosmétiques, biotechnologiques et agroalimentaires. Dans ce contexte, les travaux menés au cours de cette thèse se sont intéressés à la diversité des molécules bioactives produites par les cyanobactéries isolées des boues thermales de Balaruc-les-Bains (France). En effet, ces travaux s'intègrent dans le cadre d'une Convention Industrielle de Formation par la Recherche (CIFRE) avec les Thermes de Balaruc-les-Bains, un établissement thermal historique spécialisé dans les soins de rhumatologie et de phlébologie.

Les objectifs principaux de ces travaux ont donc été de caractériser la diversité de molécules produites par les neuf cyanobactéries isolées des boues thermales puis d'estimer les propriétés anti-inflammatoires, antioxydantes et cicatrisantes des extraits bruts de ces cyanobactéries. Afin de répondre à cette problématique, une revue bibliographique des connaissances sur les métabolites produits par les cyanobactéries et leurs activités référencées a été réalisée. Son analyse a permis de questionner l'origine taxonomique des cyanobactéries productrices de métabolites ainsi que la diversité chimique et de bioactivité démontrée. Dans un second temps, la caractérisation globale des neuf cyanobactéries issues des boues thermales a été réalisée à travers l'affiliation taxonomique des souches étudiées et l'analyse globale du génome et du métabolome de chacune à l'aide de plusieurs méthodes haut-débit de type « omique ».

Ces travaux ont permis de se figurer la forte diversité de métabolites produits par les cyanobactéries avec plus de 1400 métabolites décrits à ce jour dans la littérature. Ils ont également permis de caractériser l'incroyable richesse de microorganismes capables de se développer au sein d'un environnement spécifique tel que les boues thermales. Enfin, un haut potentiel de production de molécules bioactives a pu être identifié pour les neuf cyanobactéries de Balaruc-les-Bains. Quatre souches ont d'ailleurs montré des résultats prometteurs au regard des activités détectées : *Planktothricoides raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14 et *Pseudo-chroococcus couteii* PMC 885.14 pour leur potentiel anti-inflammatoire et *Aliinostoc* sp. PMC 882.14 pour son potentiel cicatrisant.

Par ces différentes approches, ces travaux de thèse ont permis de mettre en avant l'important potentiel thérapeutique des cyanobactéries dans le cadre du développement d'applications pour les cures thermales et ouvrent à de futurs projets d'optimisation de la production de ces molécules bioactives. Outre les perspectives de valorisation industrielle, ces travaux ouvrent également sur des questions autour des phénomènes de régulation de la synthèse de ces métabolites et sur le rôle des bactéries hétérotrophes associées en culture aux cyanobactéries.

Mots clés : cyanobactéries, métabolites, molécules, génomique, métabolomique, bioactivité, anti-inflammatoire, antioxydant, cicatrisant, boues thermales

Abstract :

Cyanobacteria are prokaryotic microorganisms capable of carrying out oxygen photosynthesis as a source of energy for their metabolism and capable of colonizing various ecosystems (e.g. seas, rivers, lakes, soil, extreme environments ...). They are also known to produce numerous bioactive molecules (toxins, antimicrobials, anti-inflammatory ...) with various structures. Thus, in recent years, cyanobacteria have been the subject of numerous research projects aimed at discovering and characterizing new natural products that can be used in the pharmaceutical, cosmetic, biotechnological and agri-food industries. In this context, the work carried out during this thesis focused on the diversity of bioactive molecules produced by cyanobacteria isolated from the thermal mud of Balaruc-les-Bains (France). Indeed, this work is part of an Industrial Convention for Training through Research (CIFRE) with the Balaruc-les-Bains Thermes, a historical thermal establishment specialized in rheumatology and phlebology.

The main objectives of this work were to characterize the diversity of molecules produced by the nine cyanobacteria isolated from the thermal mud and to estimate the anti-inflammatory, antioxidant and healing properties of the raw extracts of these cyanobacteria. In order to answer this problem, a bibliographical review of the knowledge on the metabolites produced by the cyanobacteria and their referenced activities was carried out. Its analysis allowed to question the taxonomic origin of cyanobacteria producing metabolites as well as the chemical diversity and demonstrated bioactivity. In a second step, the global characterization of the nine cyanobacteria from thermal mud was carried out through the taxonomic affiliation of the strains studied and the global analysis of the genome and metabolome of each one using several high throughput methods of "omic" type.

This work revealed the high diversity of metabolites produced by cyanobacteria, with more than 1400 metabolites described in the literature to date. They have also made it possible to characterize the incredible richness of microorganisms capable of developing within a specific environment such as thermal mud. Finally, a high potential for the production of bioactive molecules has been identified for the nine cyanobacteria of Balaruc-les-Bains. Four strains have shown promising results with regard to the activities detected: *Planktothricoides raciborskii* PMC 877.14, *Nostoc* sp. PMC 881.14 and *Pseudo-chroococcus couteii* PMC 885.14 for their anti-inflammatory potential and *Aliinostoc* sp. PMC 882.14 for its healing potential.

Through these different approaches, this thesis work has highlighted the important therapeutic potential of cyanobacteria in the development of applications for thermal cures and opens the way to future projects to optimize the production of these bioactive molecules. In addition to the prospects for industrial applications, this work also opens up questions concerning the phenomena of regulation of the synthesis of these metabolites and the role of heterotrophic bacteria associated in culture with cyanobacteria.

Key words: cyanobacteria, metabolites, molecules, genomics, metabolomics, bioactivity, anti-inflammatory, antioxidant, wound healing, thermal mud