Rapport de fin d'étude du projet CYBERI

Biofilms à Cyanobactéries Benthiques toxiques en Rivière : caractérisation, conditions de développement et stratégies d'échantillonnage

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Sommaire

I. Contexte de l'étude

Le projet CYBERI se rapportait aux cyanobactéries benthiques qui prolifèrent au fond de certaines rivières, fixées au substrat, et qui ont causé, depuis le début des années 2000, la mort de chiens ayant fréquenté la Loue située dans le massif du Jura et le Tarn situé au sud du Massif Central (Gugger *et al*., 2005 ; Cadel-Six *et al*., 2007). Il reposait sur le constat que si de nombreux travaux ont été menés au cours des deux dernières décennies sur le déterminisme des proliférations et sur la toxicité des cyanobactéries planctoniques qui se développent dans la colonne d'eau des lacs et des rivières lentes, en revanche très peu d'études ont été réalisées sur les espèces benthiques des rivières (voir en annexe la revue de littérature que nous avons réalisée sur ce sujet ; Quiblier *et al.,* 2013). Ce manque de connaissances explique pourquoi, malgré les risques sanitaires pour l'homme et les animaux associés à la production de neurotoxines par les cyanobactéries benthiques, aucune recommandation concernant leur surveillance et la prise en compte de ces risques toxiques n'a été jusqu'ici proposée par les autorités sanitaires en France.

Trois objectifs avaient été définis dans notre projet :

- Le premier était de tester et de valider différentes stratégies d'échantillonnage pour estimer les biomasses de cyanobactéries benthiques et les risques toxiques associés. Cette partie comprenait notamment l'évaluation d'un nouvel outil (BenthoTorch BBE) permettant une quantification rapide et en temps réel, de la biomasse des microorganismes benthiques photosynthétiques, dont les cyanobactéries.

- Le second était d'identifier, sur la Loue et le Tarn, les biotopes et les conditions environnementales les plus favorables au développement des biofilms à cyanobactéries ainsi que les facteurs qui influencent la production de toxines. Pour ce faire, il était prévu de coupler des études sur le terrain à des approches d'écologie moléculaire et à des dosages de toxines dans le but d'évaluer à la fois la diversité des communautés de cyanobactéries (et des autres bactéries) au sein d'un biofilm et en fonction des sites, et leur potentiel toxique.

- Le dernier objectif était de proposer un guide de bonnes pratiques pour la surveillance des cyanobactéries benthiques et pour l'évaluation des risques toxiques potentiels à l'usage de tous ceux qui seront en charge du suivi de ces microorganismes. Ce guide devait s'appuyer sur les résultats issus des deux premiers objectifs.

Cette étude a reposé sur des travaux menés sur le Tarn et la Loue par deux équipes de recherche parisiennes appartenant à deux instituts : l'*i*EES Paris (ex UMR BIOEMCO) et le MNHN Paris. Une étudiante en thèse, Isidora Echenique, financée sur ce contrat, a travaillé à plein temps sur cette étude dans nos deux laboratoires ; elle soutiendra sa thèse en Avril 2016.

Cette étude s'est également appuyée sur une collaboration avec des collègues néo-zélandais qui travaillent depuis plusieurs années sur ces microorganismes. Les échanges ont été financés dans le cadre d'un programme EGIDE (Programme Dumont d'Urville) qui a permis à nos collègues néo-zélandais de participer à une campagne d'échantillonnage sur la Loue et à une autre sur le Tarn, et qui nous a permis de travailler lors de deux missions sur des rivières néo-zélandaises. Certains des résultats obtenus sur les rivières de Nouvelle-Zélande ont été intégrés dans ce

rapport car ils permettent de compléter nos informations sur ces cyanobactéries et leur toxicité.

II. Calendrier et localisation des échantillonnages réalisés sur la Loue et le Tarn

Les campagnes d'échantillonnage sur la Loue ont été menées en 2012 et celles sur le Tarn en 2013 et 2014. Nous avons choisi de réaliser deux années de suivi sur le Tarn car les biofilms dominés par les cyanobactéries ont été principalement observés dans cette rivière.

Le calendrier des campagnes de terrain a été le suivant :

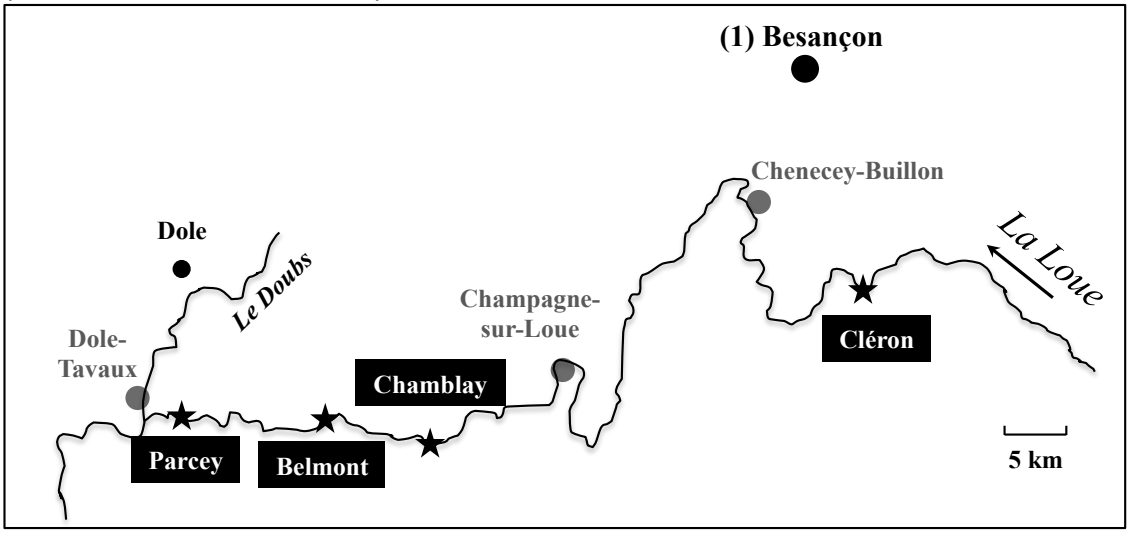
	2012	2013	2014
Loue	5 juin, 10 juillet, 13 août, 10 septembre		
Tarn	Reconnaissance des sites 6-8 août	28 juin, 11 juillet, 5-9* août, 4 septembre	17-18 juin, 16-17 juillet, 18-19 août, 8-9 septembre

* Campagne d'échantillonnage organisée avec nos collègues Néo-Zélandais sur le Tarn et sur la Jonte.

Par ailleurs, une campagne d'observation a été ajoutée sur le Tarn en septembre 2015.

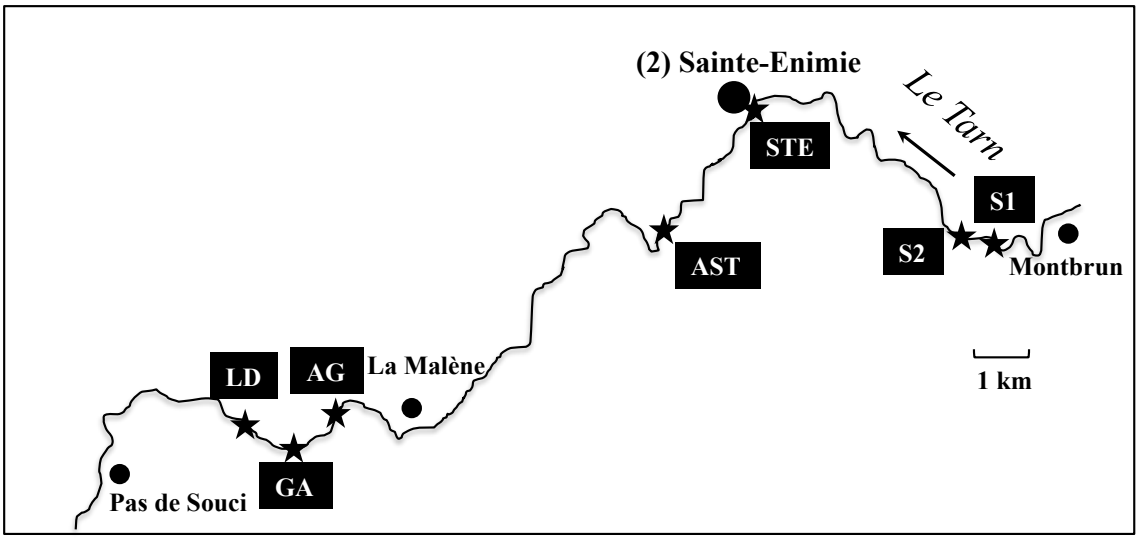
Les stations d'échantillonnages choisies sur les deux sites ont été les suivantes :

- Pour la Loue, quatre points d'échantillonnage ont été retenus, l'un (Cléron) étant situé dans la partie amont de la rivière, juste en aval de Ornans, les trois autres (Chamblay, Belmont et Parcay) étant situés dans la partie aval, en plaine, où des mortalités de chiens avaient été observées en 2003. Par ailleurs, en complément de ces sites qui ont fait l'objet de prélèvements, nous avons également exercé un suivi visuel, accompagné quelques fois de mesures avec la BBE, en trois autres points situés en amont de Cléron (Mouthier, Lods et Ornans).



- Pour le Tarn, un suivi a été réalisé en 2013 et 2014 en canoë avec des prélèvements en plusieurs stations, sur le tronçon Montbrun-St Enimie (voir

descriptif des points dans le tableau suivant). En complément de ce suivi, des points d'échantillonnage supplémentaires ont été ajoutés à l'aval de St Enimie en plusieurs stations, en fonction de la couverture en cyanobactéries. En 2013, une campagne a également été réalisée dans les gorges de la Jonte situées dans la même région ainsi que des observations visuelles dans les gorges de l'Hérault.



Les coordonnées géographiques des sites étudiés ainsi que le détail des campagnes d'échantillonnage à chacun de ces sites est présenté dans le tableau ci-dessous.

Récapitulatif des sites et des dates échantillonnés dans le cadre de ce travail sur la Loue et le Tarn

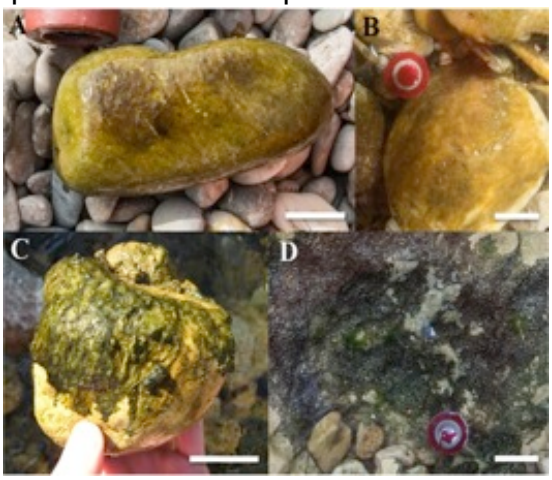
Rivières	Nom des sites	Coordonnées géographiques	Année 2012	Année 2013	Année 2014
Loue	Cléron	47°6'39.99"N; 6°6'32.76"E	05/06;09/07; 14/08;11/09		
	Chamblay	47°0'12.09"N; 5°42'12.38"E	10/07;13/08; 10/09		
	Belmont	47°0'35.39"N; 5°35'42.07"E	10/07;13/08; 10/09		
	Parcey	47°0'52.95"N; 5°29'37.43"E	06/06;10/07; 13/08;10/09		
Tarn	S1	44°20'13.90"N; 3°28'24.35"E		28/06;11/07	
	S2	44°20'23.08"N; 3°28'2.43"E		28/06;11/07; 05/08;04/09	17/06;17/07; 18/08;08/09
	STE	44°21'47.47"N; 3°24'43.58"E		06/08;04/09	18/06;16/07; 18/08;08/09
	AST	44°20'26.39"N; 3°23'12.66"E		06/08	
	AG	44°18'17.60"N; 3°17'53.03"E		07/08;04/09	18/06;16/07; 18/08;08/09
	GA	44°17'57.90"N; 3°17'28.99"E		09/08	
	LD	44°17'53.44"N; 3°16'48.21"E		09/08	

(S1 : Aval Blajoux ; S2 : Castelbouc ; STE : St Enimie (Aval du pont); AST : St Chély du Tarn ; AG : L'Angle (aval de la Malène) ; GA : Gaujac; LD : Les Détroits)

III. Echantillonnage des cyanobactéries benthiques dans les rivières et évaluation de la BenthoTorch BBE

Deux principaux types de biofilms ont été observés dans les rivières étudiées :

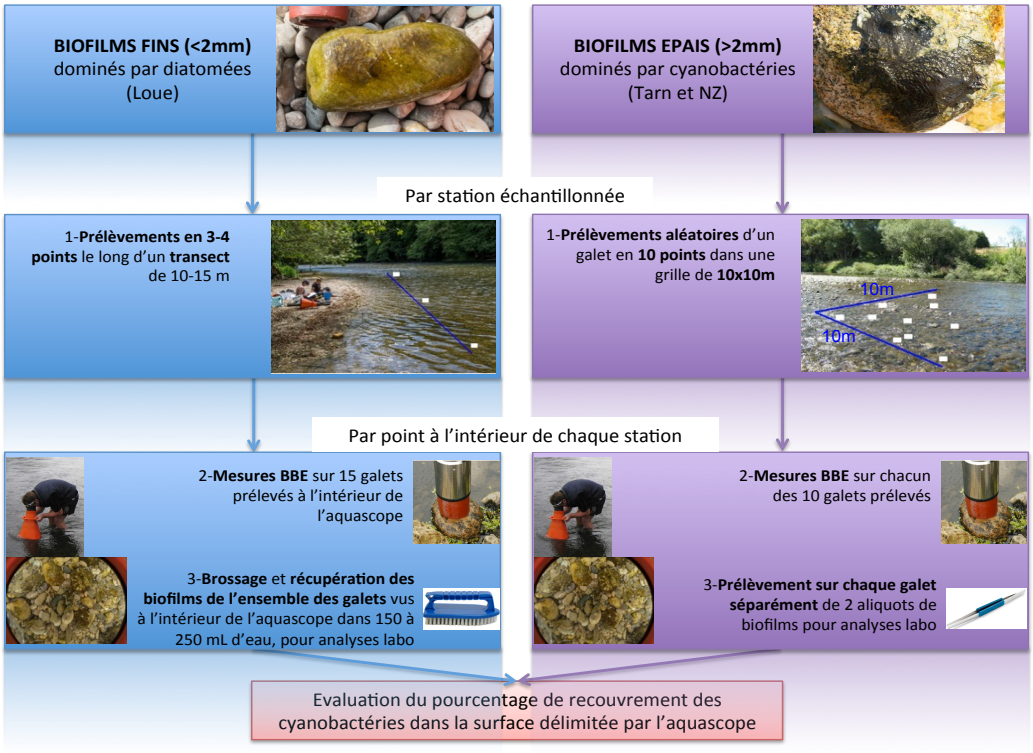
1. Des biofilms fins (moins de deux mm d'épaisseur) qui adhèrent fortement aux substrats auxquels ils sont fixés et qui sont dominés par les diatomées (Photos A & B). Ces biofilms sont ceux qui sont le plus souvent observés dans la Loue et en début de saison dans nos zones d'échantillonnage sur le Tarn.



2. Des biofilms épais (plus de deux mm) de couleur très foncée qui se détachent facilement de leur substrat et qui sont dominés par les cyanobactéries (Photos C & D). Ces biofilms de couleur sombre ont été principalement retrouvés dans le Tarn ; ce sont les mêmes que ceux qui posent problème dans les rivières de Nouvelle-Zélande.

III.1. Stratégies d'échantillonnage

En fonction des caractéristiques de ces biofilms (notamment de leur capacité à adhérer au substrat) et de celles des rivières (profondeur plus importante dans la Loue qui interdisait des prélèvements au milieu de la rivière), nous avons été conduits à définir deux stratégies d'échantillonnage différentes (cf. Figure Synthèse des Protocoles d'Echantillonnage ci-dessous) :



→ **Pour les biofilms fins** (< 2 mm) **de la Loue**, l'échantillonnage était réalisé à chacune des stations en trois ou quatre points (voir exemple ci-dessus à la station de Cléron) alignés le long d'un transect de 10 à 15 m de longueur disposé parallèlement ou perpendiculairement à la rive si la profondeur de la rivière le permettait. A chaque point d'échantillonnage, 15 galets étaient récoltés sur une surface équivalente à celle de la base l'Aquascope (cône en plastique avec une vitre au fond permettant de faire des observations sous l'eau, voir photo ci-dessus). Une mesure était réalisée avec la BenthoTorch sur chacun des 15 galets. Tous les galets visibles dans le cône de l'Aquascope était ensuite récoltés puis brossés pour récupérer les biofilms qui étaient ensuite mis en suspension ensemble dans 150 à 250 ml d'eau de la rivière en vue des analyses en laboratoire.

→ **Pour les biofilms épais** (> 2 mm) **du Tarn dominés par les cyanobactéries**, nous avons adapté le protocole d'échantillonnage mis au point par nos collègues Néo-Zélandais. Cet échantillonnage a reposé, pour chacune des stations étudiées, sur l'utilisation d'une grille de 10m x 10m au sein de laquelle étaient prélevés de façon aléatoire (en générant préalablement des coordonnées aléatoires sur ordinateur), un galet en dix points d'échantillonnage. Sur chacun des galets, une mesure était réalisée avec la BenthoTorch. A l'endroit où la mesure avait été réalisée, un disque de biofilm d'une surface de 4 cm^2 était prélevé pour estimer ultérieurement en laboratoire, la biomasse et pour la caractérisation moléculaire. Un autre disque de biofilm était prélevé à proximité immédiate du premier pour les analyses de toxines.

Lorsque la couverture en cyanobactéries était trop faible, l'échantillonnage aléatoire était abandonné. Dix galets recouverts de cyanobactéries étaient alors recherchés et prélevés sur la station échantillonnée. Le traitement des biofilms des différents galets était alors le même que celui décrit ci-dessus.

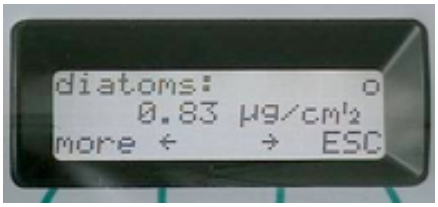
Enfin, en chacun des dix points d'échantillonnage, deux opérateurs réalisaient une estimation visuelle du pourcentage de couverture en cyanobactéries à l'intérieur de la surface définie par l'Aquascope.

Pour conclure, les deux approches que nous avons testées sur les biofilms fins et épais se sont révélées bien adaptées pour réaliser des échantillonnages à visée de recherche mais elles demandent des moyens humains importants ainsi que du temps (une heure par site à trois personnes minimum) qui nous semblent incompatibles avec un suivi opérationnel pour des gestionnaires. C'est la raison pour laquelle nous avons testé un nouvel outil qui devait permettre de déterminer in situ, la biomasse des algues vertes, diatomées et cyanobactéries au sein des biofilms.

III.2. Evaluation de la BenthoTorch BBE

Cet outil est basé sur le même principe que la sonde spectrofluorimétrique Fluoroprobe BBE utilisée depuis une vingtaine d'années pour le suivi du phytoplancton et des cyanobactéries planctoniques dans les plans d'eau et les rivières lentes. A la différence de la Fluoroprobe, le principe de la BenthoTorch repose sur une excitation à trois longueurs d'onde différentes des pigments accessoires des principaux microorganismes

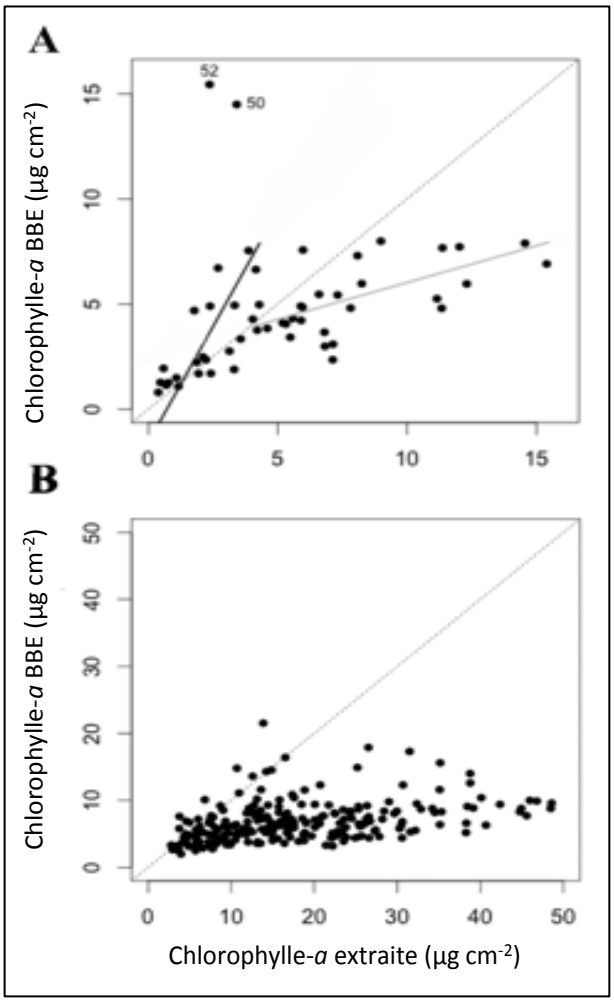
photosynthétiques benthiques (diatomées, algues vertes et cyanobactéries). Sachant que chaque grande classe de ces microorganismes possède une composition pigmentaire qui lui est propre, la sonde détermine les biomasses de chacune d'elles en fonction de la fluorescence mesurée aux différentes longueurs d'onde d'excitation. Les résultats sont exprimés pour chacune des classes en µg de chlorophylle-*a* par unité de surface (cm^2).



L'avantage de cette sonde est de fournir, en moins de 30 secondes, une estimation des biomasses en diatomées, algues vertes et cyanobactéries dans les biofilms. Pour valider ces estimations, nous avons comparé (i) les valeurs de biomasse totale des biofilms (somme des biomasses de chaque classe) données par la sonde à celles obtenues en laboratoire, à partir des échantillons prélevés, après extraction de la chlorophylle-*a* puis dosage en spectrophotométrie et (ii) les estimations données par la sonde de la proportion des algues vertes, diatomées et cyanobactéries au sein des biofilms à des comptages réalisés au microscope et des calculs de biovolumes.

III.2.1. Comparaison des biomasses estimées avec la sonde à celles mesurées après extraction des pigments

Les comparaisons réalisées entre les valeurs de chlorophylle fournies par la BenthoTorch et celles obtenues par extraction des pigments puis dosage spectrophotométrique montrent qu'il existe **une corrélation positive entre les valeurs de biomasse chlorophyllienne totale données par la sonde et celle obtenues par dosage**. Cependant, il apparaît également que :



→ **pour les biofilms fins (A) dominés** par les diatomées, la sonde surestime la biomasse totale des biofilms lorsque cette biomasse est inférieure à 4 µg cm^{-2} alors qu'elle sous-estime la biomasse totale des biofilms lorsque celle-ci est supérieure à 4 µg cm^{-2} (voir les deux droites de régression calculées sur les biomasses inférieures et supérieures à 4 µg cm^{-2}).

→ **pour les biofilms épais dominés par les cyanobactéries (B)**, la sonde sous-estime considérablement les valeurs de la biomasse totale des biofilms en comparaison des valeurs obtenues après extraction de la chlorophylle-*a*. Alors que des valeurs de biomasses atteignant 50 µg cm^{-2} ont été mesurées par spectrophotométrie, ces mêmes valeurs maximales délivrées par la sonde sont le plus souvent inférieures à 10 µg cm^{-2}.

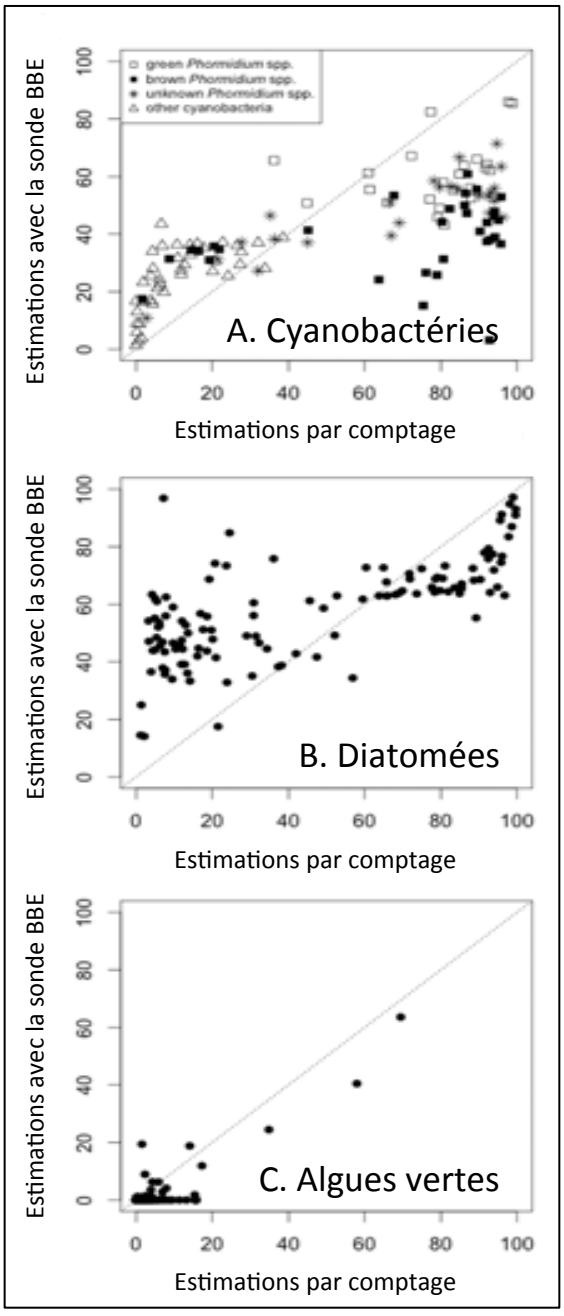
III.2.2. Comparaison des estimations de la composition des biofilms données par la sonde et par comptages au microscope

En ce qui concerne la composition des biofilms, nous avons comparé les estimations des proportions des algues vertes, diatomées et cyanobactéries données par la sonde avec celles résultant d'un comptage des cellules au microscope puis de l'estimation des biovolumes de chaque groupe photosynthétique. La figure ci-dessous montre que :

- **Pour les cyanobactéries (A)**, une surestimation de leurs proportions est observée lorsque ces microorganismes représentent moins de 20% de la biomasse totale. A l'inverse une sous-estimation de la proportion des cyanobactéries est observée lorsque ces microorganismes sont dominants dans les biofilms (>50%). Il faut noter que dans ce dernier cas, les espèces de cyanobactéries présentes au sein des biofilms appartiennent le plus souvent au genre *Phormidium* qui présente en général une pigmentation brune très foncée.

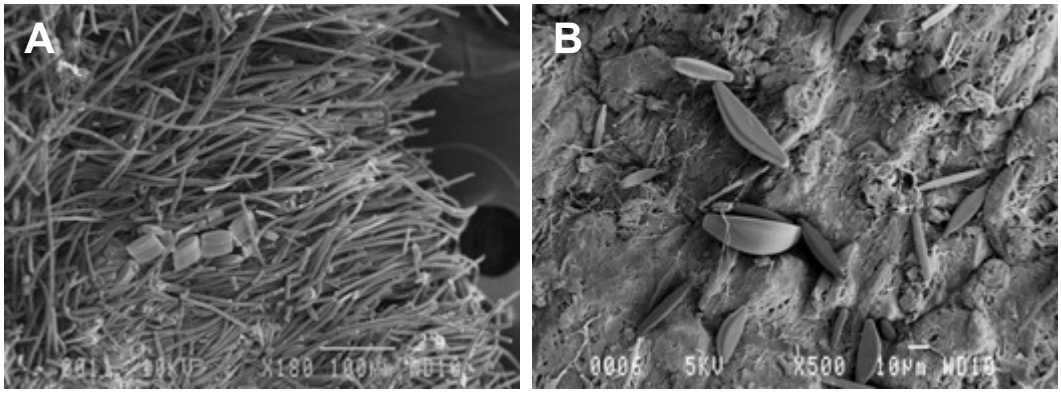
- **Pour les diatomées (B)**, une surestimation de leurs proportions est observée lorsqu'elles représentent moins de 40% de la biomasse totale. En revanche, quand elles sont dominantes dans les biofilms (plus de 60% des cellules comptées), leurs proportions ont tendance à être sous estimées, comme cela avait été également observé pour les cyanobactéries.

- **Pour les algues vertes (C)**, il est plus difficile de donner des conclusions car ces microorganismes représentent le plus souvent une très faible proportion des microorganismes présents au sein des biofilms étudiés. Sur les quelques valeurs disponibles, il semble qu'une sous estimation de leurs proportions est le plus souvent observée avec la sonde.



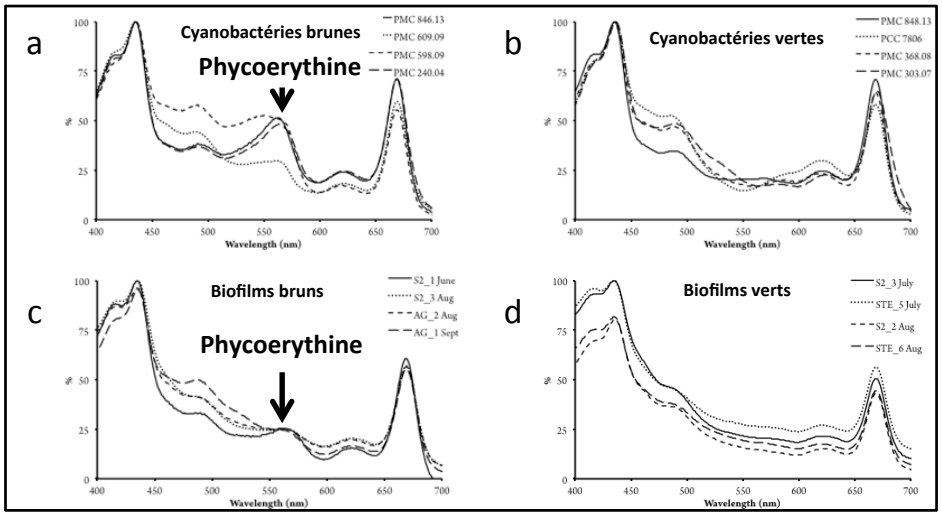
Nous avons ensuite cherché à déterminer pourquoi la sonde détecte mal les cyanobactéries du genre *Phormidium* colorées en brun foncé. Deux hypothèses ont été testées :

- La première était que dans les biofilms épais constitués très majoritairement de filaments de cyanobactéries, les diatomées sont peut être localisées en surface de ces biofilms ce qui conduirait à les surestimer. Les approches réalisées en microscopie à balayage ont montré que ce n'est pas le cas, les diatomées se localisant plutôt dans la partie basale des biofilms (voir photos)



Microscopie à balayage réalisée sur des biofilms à cyanobactéries prélevés dans le Tarn.
A. Structure des biofilms dans leur zone apicale montrant une dominance de filaments de cyanobactéries
B. Structure des biofilms dans la partie basale montrant la présence en nombre de diatomées

- La seconde hypothèse reposait sur la composition pigmentaire des cyanobactéries dominantes dans les biofilms épais. En effet, certaines espèces du genre *Phormidium* sont connues pour contenir de la phycoérythrine (responsable de la couleur brune) qui est un pigment rouge qui pose problème pour les sondes spectrofluorimétriques, comme cela avait déjà été signalé pour les cyanobactéries planctoniques (Leboulanger *et al*., 2002). Sans entrer dans les détails qui ne sont pas nécessaires dans un tel rapport (toutes les informations sont cependant disponibles dans la publication jointe en annexe), il est apparu que les cyanobactéries qui dominent les biofilms épais sont bien des *Phormidium* contenant de la phycoérythrine (voir profils d'absorption ci-dessous) et que les longueurs d'onde des LEDs de la BenthoTorch ne permettent pas d'exciter ces pigments ce qui conduit à sous estimer les biomasses en cyanobactéries et dans le même temps à surestimer celles des diatomées.



Profils d'absorption de cultures de cyanobactéries brunes (avec phycoérythrine) ou vertes (sans phycoérythrine) isolées à partir de biofilms (a et b) et des biofilms complets (c et d)

Pour conclure sur ces tests réalisés avec la BenthoTorch BBE, nous nous attendions à une sous-estimation de la biomasse des biofilms épais car la faible pénétration de la lumière émise par les LEDs de la sonde ne permet qu'une excitation des pigments des microorganismes photosynthétiques occupant la couche supérieure des biofilms. Toutes les cellules localisées plus en profondeur ne sont donc pas prises en compte par la sonde alors qu'elles contribuent aux biomasses estimées après extraction de la chlorophylle puis mesure par spectrophotométrie. Il est en revanche plus surprenant de constater que même pour les biofilms fins, la sonde sous-estime le plus souvent leur biomasse, même s'il existe une corrélation significative entre les valeurs données par la sonde et celles obtenues par mesure de la chlorophylle après extraction.

En ce qui concerne les proportions des deux classes de microorganismes majoritaires (les diatomées et les cyanobactéries) au sein des biofilms du Tarn et de la Loue (mais aussi de ceux de Nouvelle-Zélande), nous avons montré que ces proportions diffèrent significativement selon qu'elles sont estimées par la sonde ou par comptage au microscope puis estimation des biovolumes. D'une façon globale, il apparaît que l'importance relative des diatomées est surestimée par rapport à celle des cyanobactéries, notamment lorsque ces dernières sont dominantes dans les biofilms et qu'elles appartiennent au genre Phormidium.

Ainsi, il apparaît que la BenthoTorch BBE n'est pas, dans sa configuration actuelle, optimisée pour le suivi des biofilms à cyanobactéries dans les rivières. Pour la rendre plus efficace, il serait nécessaire de disposer de LEDs excitant à des longueurs d'onde permettant la détection des Phormidium contenant de la phycoérythrine car ils sont dominants dans les biofilms épais. Des discussions sont en cours avec le fabriquant de la sonde pour réaliser ces adaptations. Par ailleurs, même si ce problème de longueurs d'onde d'excitation était résolu, il demeurerait une sous estimation de la biomasse des biofilms épais pour les raisons exposées ci-dessus. Ce problème ne sous semble cependant pas majeur dans une perspective de suivi opérationnel des biofilms épais dominés par les cyanobactéries par les gestionnaires car dans cette situation, une détection visuelle est très facile à réaliser ce qui rend la quantification de la biomasse moins indispensable. En revanche, l'amélioration de la détection des Phormidium à phycoérythrine permettrait de mieux quantifier leur biomasse au tout début de leur développement, ce qui du point de vue de la surveillance serait très utile. Il serait en effet possible de détecter précocement l'installation des biofilms à cyanobactéries et donc de mieux cibler les sites nécessitant une surveillance renforcée.

IV. Travaux réalisés sur les biofilms de la Loue

IV.1. Conditions de développement des biofilms à cyanobactéries dans la Loue

Les seules données disponibles sur cette question étaient jusqu'ici celles de nos collègues Néo-Zélandais sachant que leurs rivières sont situées dans des contextes très différents que ce soit en terme de géologie, de pressions anthropiques (moindres pollutions par les nutriments par exemple) et dans une moindre mesure de

météorologie. De leurs travaux, il ressortait que les paramètres environnementaux les plus influents sur le développement des biofilms à cyanobactéries étaient la vitesse du courant, la profondeur et la nature du substrat. Nous avons donc considéré prioritairement ces paramètres tout en y ajoutant quelques autres à l'exemple de la température ou des conditions hydrologiques dans les semaines précédents les échantillonnages.

Les quatre sites ayant fait l'objet d'un échantillonnage avaient été choisis car ils présentaient potentiellement, sur la base des connaissances acquises par nos collègues néo-zélandais, des biotopes favorables aux cyanobactéries en terme de profondeur, vitesse du courant, nature du substrat au fond de la rivière et éclairement (voir photos ci-dessous).

Station de Cléron

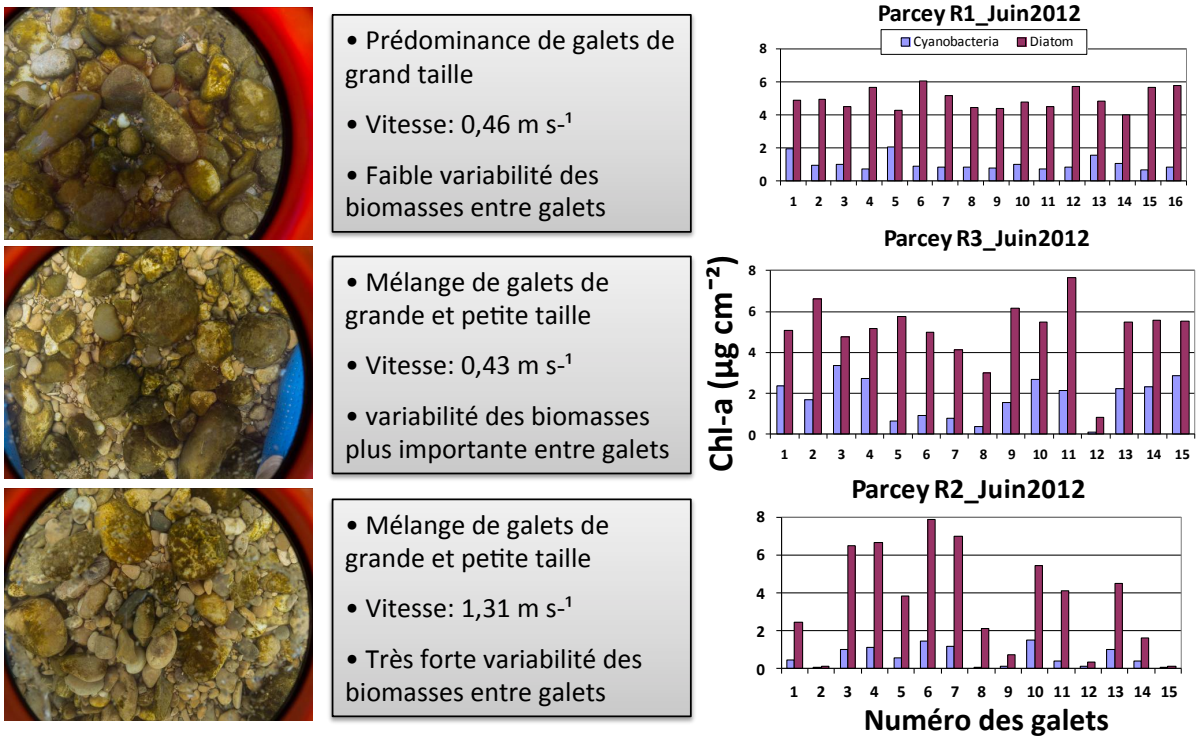
Station de Parcey

Station de Chamblay

Photographies des trois principaux sites échantillonnés dans la Loue. La station de Cléron est localisée dans la partie amont de la rivière, en relief karstique, alors que les deux autres stations sont localisées en aval dans la plaine.

Les valeurs des biomasses des biofilms de la Loue (estimées par dosage de la chlorophylle-*a*) n'ont jamais dépassé 10 µg cm^{-2} de chlorophylle-*a* au cours de nos quatre campagnes d'échantillonnage, quels que soient les sites d'étude. Cette valeur peut être considérée comme faible en comparaison du Tarn où des concentrations en chlorophylle-*a* dépassant 50 µg cm^{-2} ont été observées. Des différences significatives de biomasses ont été enregistrées entre les trois réplicas à l'intérieur d'un même site, mais également entre les sites étudiés.

Lors des échantillonnages réalisés en 2012 sur la Loue, nous avons tout d'abord montré que la taille des galets, combinée à la vitesse du courant, avaient un impact important sur la biomasse des biofilms qui se développent sur ces galets ainsi que l'illustre la figure ci-dessous. Cette figure présente les résultats obtenus sur les trois places d'échantillonnage (ici nommés R1, R2 et R3) d'un transect réalisé à la station de Parcey en juin 2012.



Exemple d'hétérogénéité en fonction de la taille des galets et des vitesses de courant, dans les biomasses chlorophylliennes des diatomées et cyanobactéries estimées sur les 15 galets prélevés sur les trois réplicas d'un transect d'échantillonnage

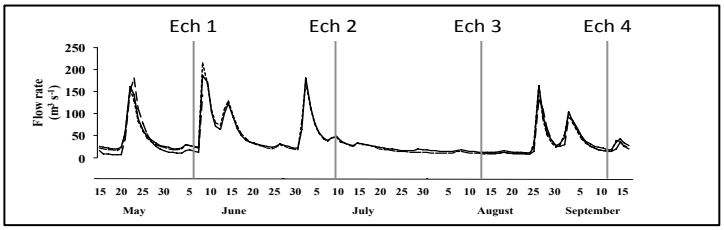
Si les biomasses des biofilms sont équivalentes sur les galets de petite et de grande taille à vitesse de courant modérée (<0,5 m s^{-1}), en revanche seuls les galets de grande taille permettent aux biofilms de se développer à des vitesses supérieures. Ceci s'explique probablement par le fait que, les galets de petites tailles sont entrainés dans des conditions de courant fort, ce qui provoque le détachement des biofilms en formation. Ces observations ont donc confirmé que la vitesse du courant et la nature/taille du substrat sont deux paramètres fondamentaux pour le développement des biofilms en rivières.

IV.2. Etude de l'évolution spatiotemporelle de la composition et de la structure des biofilms de la Loue

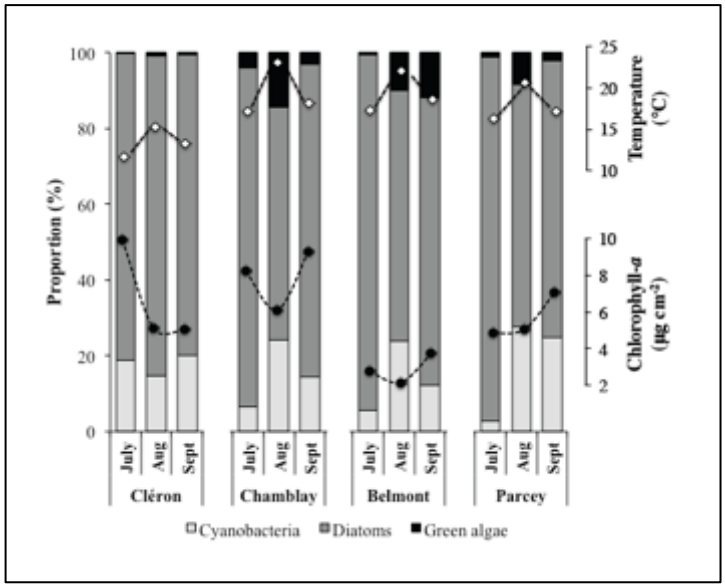
IV.2.1. Approche par microscopie

Dans un premier temps, en nous basant sur des approches reposant sur la microscopie et l'estimation des biomasses chlorophylliennes par spectrophotométrie, il est apparu que tous ces biofilms de la Loue sont largement dominés, en terme de biovolume, par les diatomées. Dans les trois stations situées dans la partie aval de la Loue (Chamblay, Belmont et Parcey), une évolution temporelle est cependant observée dans les proportions de cyanobactéries et d'algues vertes qui montrent

toutes deux une augmentation significative de leurs proportions en août. Cette augmentation peut s'interpréter en regard de l'augmentation de la température de l'eau à cette période mais aussi en liaison avec la longue période d'étiage qui a précédé l'échantillonnage du mois d'août. On remarque cependant que l'été 2012 a connu plusieurs épisodes très pluvieux qui se sont accompagnés de crues peu favorables au développement de biofilms épais. Les différences observées entre la station amont de Cléron et les trois stations situées en aval révèlent l'importance du contexte géologique et hydrologique dans l'évolution saisonnière des biofilms. En effet, la partie amont de la Loue se trouve en zone karstique qui se caractérise notamment par des résurgences d'eau à basse température tout au long de son parcours, expliquant une température d'eau plus froide dans cette partie amont que dans la partie aval. Cette différence dans la température de l'eau entre la station amont et celles situées en aval est très probablement à l'origine d' une part importante des différences observées dans l'évolution saisonnière des biofilms sur ces stations.



Variations du débit dans la Loue en 2012 (Ech 2, Ech 3 et Ech 4 correspondent respectivement à Juillet, Août et Septembre dans la figure du dessous)



Variations de la température de l'eau, de la biomasse totale des biofilms (chlorophylle-*a* par cm^2) et des proportions de diatomées, algues vertes et cyanobactéries aux quatre sites échantillonnés (stations disposées sur la figure d'amont en aval).

En complément de ces résultats obtenus sur les quatre stations ayant fait l'objet de prélèvements, il faut ajouter les observations visuelles réalisées sur les trois sites localisés dans la partie la plus amont de la rivière (Mouthier, Lods et Ornans). Ces observations ont révélé la présence constante de très fortes biomasses de deux algues vertes filamenteuses (*Vaucheria* et *Cladophora*) associées à des taux de couverture des fonds très importants (voir

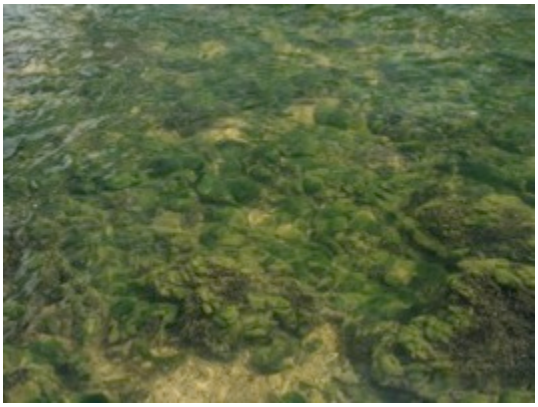
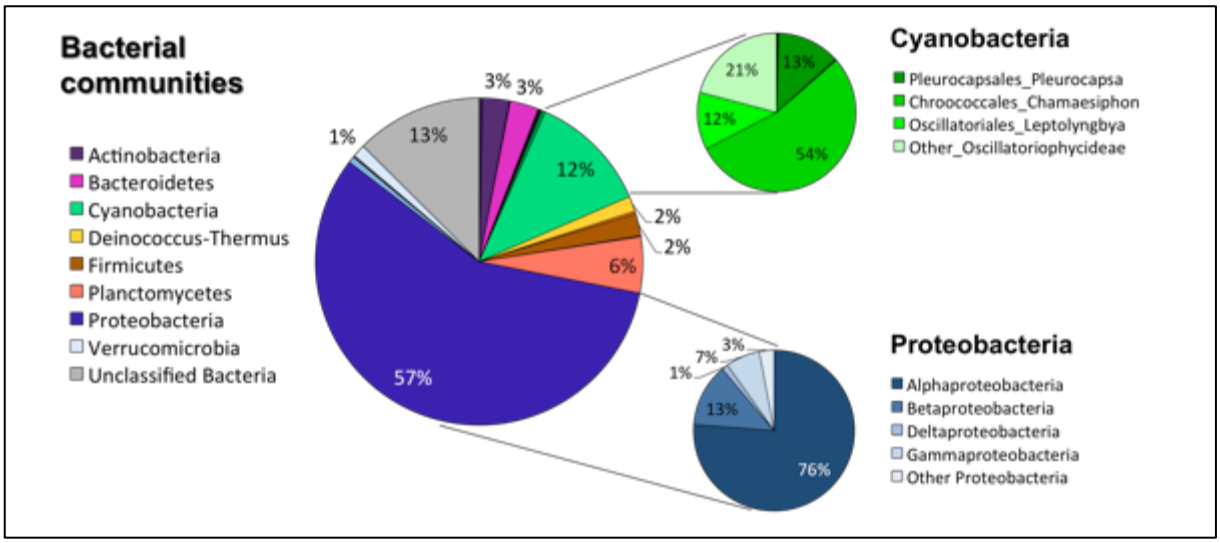


photo ci-contre). Ces biomasses algales étaient cependant décroissantes entre Mouthier et Ornans, ce qui semble traduire un phénomène d'autoépuration dans la rivière.

IV.2.2. Approche par biologie moléculaire

Pour mieux connaître la diversité structurale des communautés microbiennes au sein des biofilms ainsi que les évolutions spatiales et temporelles de leur structure, nous avons réalisé des analyses moléculaires (après séquençage à haut débit) sur les biofilms. Le détail des méthodologies utilisées est fourni dans l'article actuellement soumis à publication, qui figure en annexe.

Comme cela était attendu, une très grande richesse d'espèces a été observée aussi bien dans les communautés bactériennes que dans celles des microalgues ce qui confirme l'extrême complexité des biofilms en rivières. L'analyse des séquences d'ARN 16S obtenues montre que ces biofilms sont composés majoritairement, au niveau de leur fraction procaryote, de bactéries appartenant au phylum des Proteobacteria et notamment d'Alphaproteobacteria (voir figure ci-dessous). Parmi ces dernières, de nombreuses séquences étaient affiliées au groupe des Rhodobactéries qui sont des bactéries potentiellement photosynthétiques qui pourraient donc jouer, avec les cyanobactéries, un rôle important dans la production primaire au sein des biofilms. Il est d'autre part intéressant de constater que de nombreuses séquences correspondent à des espèces capables de fixer l'azote atmosphérique, ce qui pourrait contribuer à soutenir la production primaire et bactérienne dans la Loue. Enfin, chez les cyanobactéries, les séquences les plus abondantes appartiennent au genre *Chamaesiphon* qui est une cyanobactérie épiphyte des plantes et des substrats minéraux. Au contraire du Tarn où les cyanobactéries du genre *Phormidium* (comprises dans « other Oscillatoriophycidea » dans la figure ci-dessous) sont très largement majoritaires dans les biofilms, ce genre est peu présent parmi les séquences des biofilms de la Loue, ce qui confirme les observations visuelles.



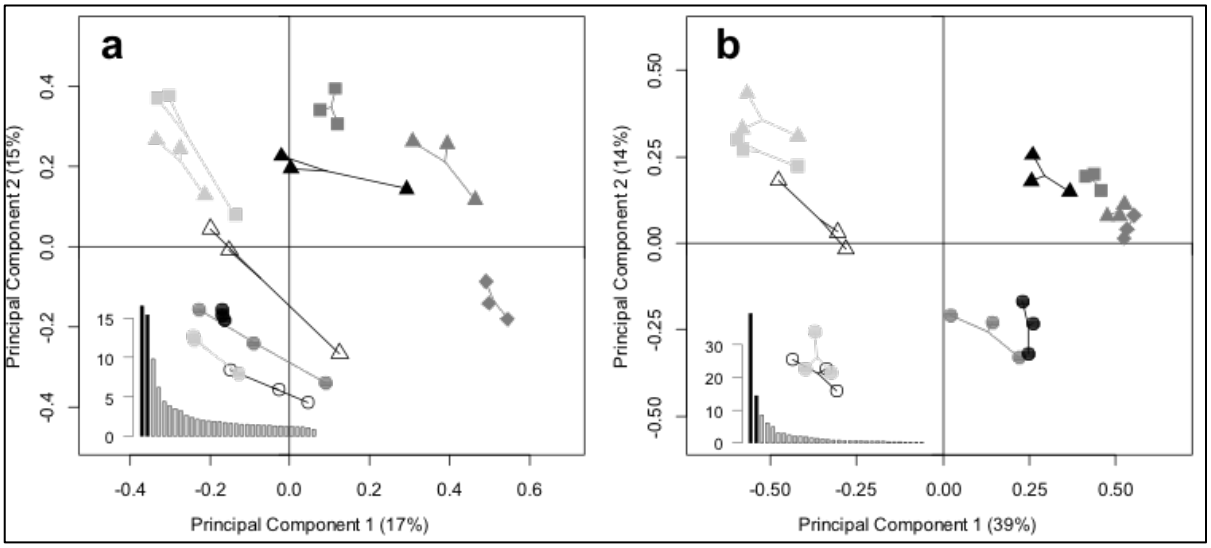
Répartition des différents phyla et classes dans les communautés bactériennes des biofilms de la Loue

Au niveau des communautés eucaryotes, nous avons pu constater les limites de l'approche basée sur l'étude de l'ARN 18S, car les séquences les plus abondantes étaient celles des algues vertes alors que nos comptages au microscope

révèlent très clairement que les diatomées sont les microalgues qui dominent les biofilms de la Loue. En revanche, une analyse réalisée sur les 16S chloroplastiques (les chloroplastes sont les organites qui sont le siège de la photosynthèse chez les eucaryotes photosynthétiques ; ils possèdent un petit génome qui leur est propre et qui ressemble à un génome bactérien car ils sont issus d'une endosymbiose bactérienne) a confirmé les observations microscopiques, à savoir la grande dominance des diatomées dans la communauté eucaryote.

Comme le montre la figure ci-dessous, une analyse comparée de la composition des communautés microbiennes aux quatre stations d'échantillonnage et aux différentes dates d'étude montre très clairement l'existence :

- D'un effet saisonnier qui oppose sur l'axe 1 de l'analyse les communautés procaryotes (a) et eucaryotes (b) de juin et juillet à celles de août et septembre. Cet effet saisonnier est plus marqué pour les communautés eucaryotes que pour celles des procaryotes. Chez ces dernières, les communautés bactériennes de Cléron ne semblent d'ailleurs présenter aucune variation saisonnière (tous les points sont regroupés dans la même zone de la projection), ce qui peut sans doute s'expliquer par les faibles variations observées dans les conditions environnementales à cette station, notamment au niveau de la température de l'eau.
- D'un effet spatial qui sépare la station de Cléron qui est située dans la partie amont de la rivière, des trois stations localisées dans la partie aval (Chamblay, Belmont and Parcey). Cette différence entre la station amont et les trois stations aval qui sont assez proches géographiquement les unes des autres reflète bien les conditions environnementales contrastées existant entre l'amont et l'aval de la Loue. Comme déjà dit précédemment, les contextes géologiques différents de ces stations ont un impact important sur certains paramètres clés ayant un rôle dans l'évolution des biofilms. C'est ainsi qu'au niveau des températures de l'eau par exemple, celles-ci ont varié entre 11 et 15°C à Cléron et entre 15 et 23°C dans la partie aval de la rivière.



Analyses multivariées réalisées sur les données de séquençage des biofilms de la Loue (a) Communautés procaryotes (b) Communautés de microeucaryotes
(cercles = station de Cléron, losanges = Chamblay, carrés = Belmont, triangles = Parcey ; symboles blancs = échantillons prélevés en juin, gris clair = Juillet, gris foncé = août, noir = septembre)

IV.3. Evaluation de la toxicité potentielle des biofilms de la Loue

Nous avons recherché par PCR dans ces mêmes biofilms la présence de gènes impliqués dans la synthèse de deux types de toxines : (i) Les anatoxines qui sont les neurotoxines ayant été impliquées dans les mortalités de chiens observées sur la Loue en 2003 et sur le Tarn depuis le début des années 2000 et (ii) les microcystines qui sont des hépatotoxines produites par de très nombreuses cyanobactéries et qui ont été impliquées dans de nombreux cas d'intoxication dans le monde mais pas sur la Loue. Sur les 39 échantillons ayant fait l'objet d'analyses, aucun échantillon positif à la microcystine n'a été retrouvé sachant que la cible était le gène *mcyA* (Hisbergues *et al.*, 2003). Pour l'anatoxine (dont la cible était le gène *anaOs* décrit par Cadel-Six *et al*., 2009), trois échantillons prélevés à Belmont en juillet et août 2012, et un échantillon prélevé à Chamblay en septembre 2012 se sont révélés positifs par PCR. Ce résultat est en accord avec le fait que les mortalités de chien observées en 2003 étaient survenues au niveau de ces stations aval où les biotopes et les conditions environnementales sont beaucoup plus favorables au développement de cyanobactéries du genre *Phormidium* que celles de la partie amont de la rivière. Ce genre *Phormidium* est en effet connu pour son potentiel à produire les anatoxines. Il faut noter que si la PCR nous donne une indication sur le potentiel des cyanobactéries à synthétiser les toxines recherchées, elle ne nous permet pas cependant d'affirmer avec certitude que ces toxines étaient produites et en quelle quantité était réalisée cette production.

Pour conclure, les résultats obtenus lors de nos campagnes d'échantillonnage réalisées sur la Loue en 2012 montrent que cette rivière présente des biofilms largement dominés par les diatomées comme la plupart des cours d'eau de ce type. Cette dominance des diatomées semble s'expliquer principalement par des températures de l'eau souvent inférieures à 15°C, par des vitesses de courants élevées et par des périodes récurrentes de forts débits dans la rivière qui ont sans doute limité le développement des biofilms lors de notre saison d'échantillonnage ou provoqué leur arrachement. Si des cyanobactéries sont présentes dans les biofilms échantillonnés, elles ne représentent en général que de faibles biomasses, très inférieures à celles que l'on peut trouver dans le Tarn ou dans les rivières Néo-Zélandaises. Toute la partie amont de la rivière localisée dans le massif du Jura semble peu susceptible de connaître des développements massifs de biofilms à cyanobactéries en raison des températures froides de l'eau, de la présence massive d'algues vertes filamenteuses mais aussi de mousses qui sont très compétitives dans ces conditions environnementales (eaux froides et peu éclairées). En revanche, la partie aval de la rivière localisée en plaine (partie qui fait l'objet d'un redressement) semble potentiellement plus favorable au développement de biofilms dominés par les cyanobactéries, notamment d'espèces productrices d'anatoxines, en raison de températures plus élevées de l'eau en été et de vitesses de courant moins élevées qu'en amont qui sont plus favorables au développement de biofilms épais. En particulier, la période de canicule observée en 2003 a pu favoriser de tels développements massifs alors que les épisodes de crues répétés pendant l'été 2012 n'ont pas permis une installation durable de ces biofilms.

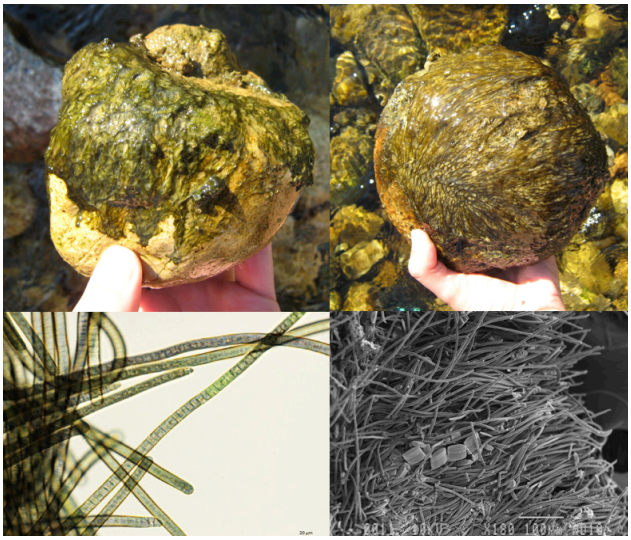
V. Travaux réalisés sur les biofilms du Tarn

V.1. Conditions de développement des biofilms à cyanobactéries dans le Tarn

Pour déterminer quelles étaient les zones de la rivière les plus favorables au développement des biofilms à cyanobactéries, nous avons réalisé plusieurs descentes en canoë de la rivière, ce qui nous donnait accès à l'ensemble de son linéaire.

Dans le Tarn, les biofilms dominés par les cyanobactéries ont presque toujours été observés dans les zones de radier, à l'exemple de celle figurant sur la photo ci-contre. Dans ces radiers qui sont de faibles profondeurs, les cyanobactéries semblent en concurrence avec les diatomées alors que les algues vertes sont surtout présentes sur les bords, là où le courant est quasiment nul.

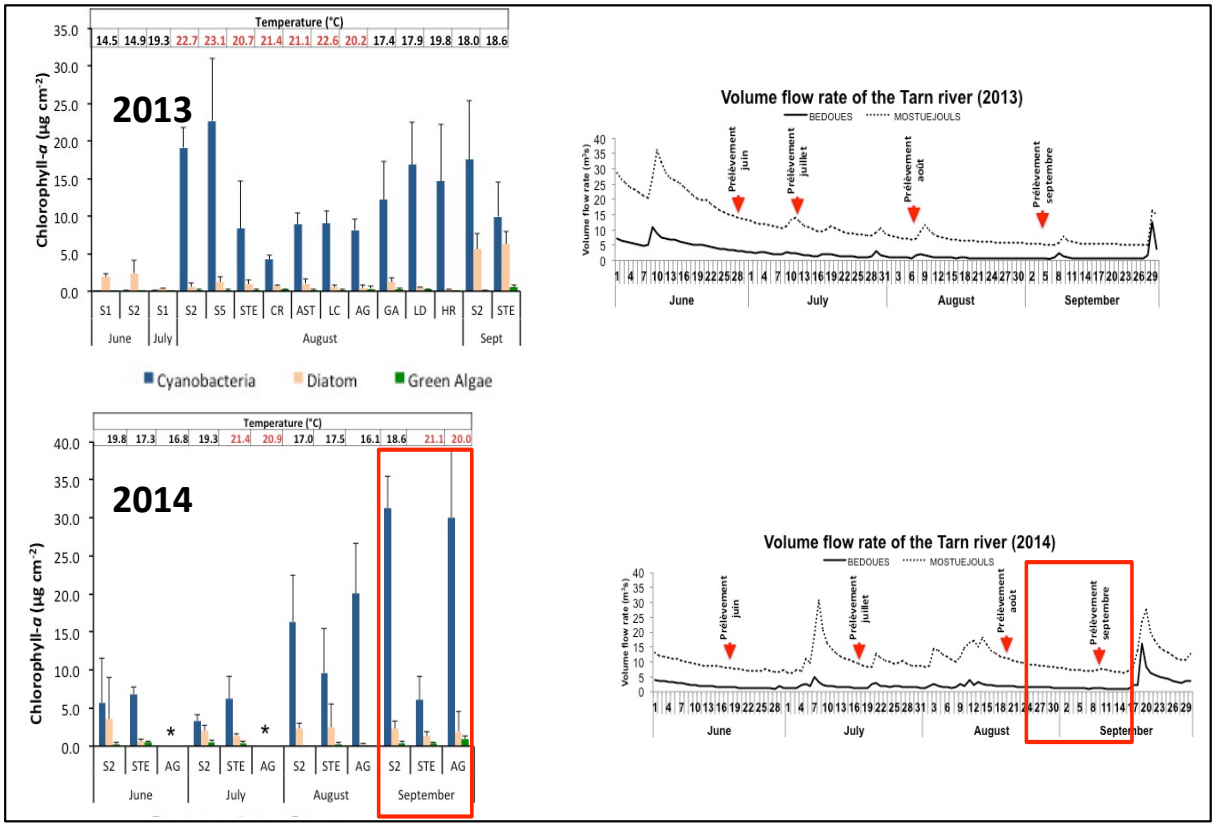
C'est ainsi que, comme l'illustre la photo ci-contre, on peut observer dans certains radiers, de fortes biomasses en algues vertes en bordure (flèche verte), une zone de développement de cyanobactéries plus exposée au courant (flèche noire) et enfin une zone avec des biofilms très fins à diatomées (flèche blanche) dans le chenal principal où le courant est le plus fort.



Les biofilms à cyanobactéries trouvés dans le Tarn peuvent parfois être très épais comme le montre la figure ci-contre. Les cyanobactéries semblent se développer secondairement aux diatomées qu'elles recouvrent progressivement. L'étude de la structure des biofilms en microscopie photonique et électronique confirme qu'ils contiennent une densité de filaments très importante et une présence minoritaire de diatomées lorsque les cyanobactéries se sont installées.

V.1.1. Evolution des biomasses

L'évolution des biomasses de cyanobactéries, diatomées et algues vertes lors des deux années d'échantillonnage ainsi que l'évolution des débits du Tarn sont présentées dans la figure ci-dessous.

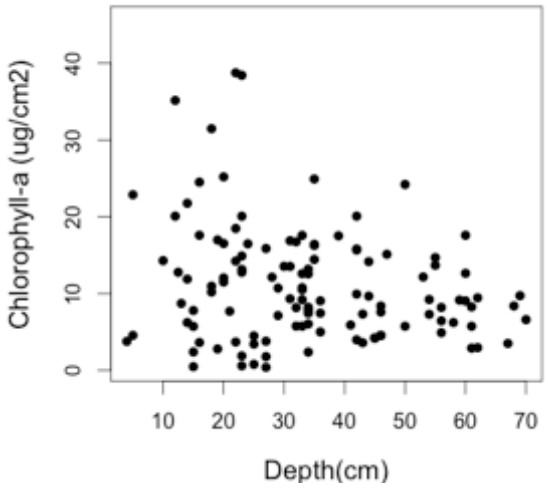


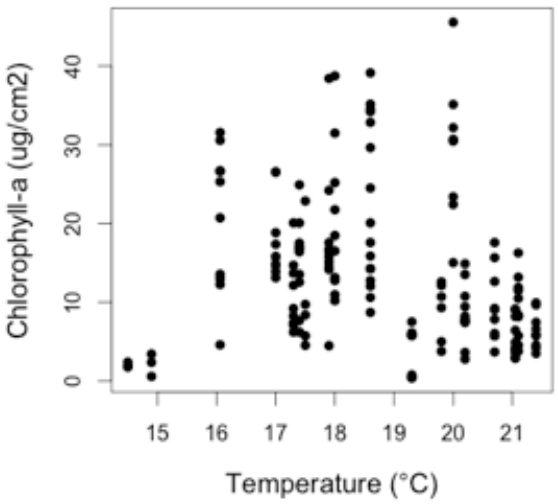
Evolution saisonnière et spatiale des biomasses chlorophylliennes en cyanobactéries, diatomées et algues vertes et variations des débits du Tarn lors des deux années d'échantillonnage (2013 & 2014)

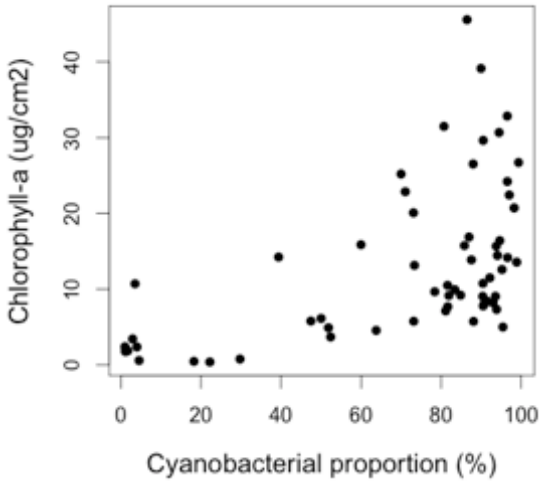
(S2 : Castelbouc ; STE : St Enimie (Aval du pont); AG : L'Angle (aval de la Malène))

Les deux années ont été marquées par des régimes hydrologiques différents, caractérisés par une longue période d'étiage estival en 2013 et par des crues plus ou moins importantes pendant tout l'été 2014. Les cyanobactéries sont largement dominantes en terme de biomasses dans toutes les stations échantillonnées mais il faut se rappeler que ces stations avaient été choisies en raison de leurs caractéristiques favorables au développement de ces microorganismes. Les biomasses peuvent dépasser 50 µg cm^{-2}, ce qui est considérable en comparaison de celles des biofilms de la Loue. Enfin, cette figure montre que les plus fortes biomasses en cyanobactéries sont présentes lors des deux années d'étude, après des périodes d'étiage prolongées et à des températures de l'eau élevées (voir les carrés rouges sur la figure).

A partir de tous nos prélèvements, nous avons ensuite recherché s'il existait des relations significatives entre les biomasses des biofilms, exprimées en unité de chlorophylle-*a* par unité de surface, et différents paramètres telles que la profondeur ou la température de l'eau.







Relations entre la biomasse chlorophyllienne totale par unité de surface et la profondeur à laquelle les biofilms ont été échantillonnés , la température de l'eau et la proportion de cyanobactéries

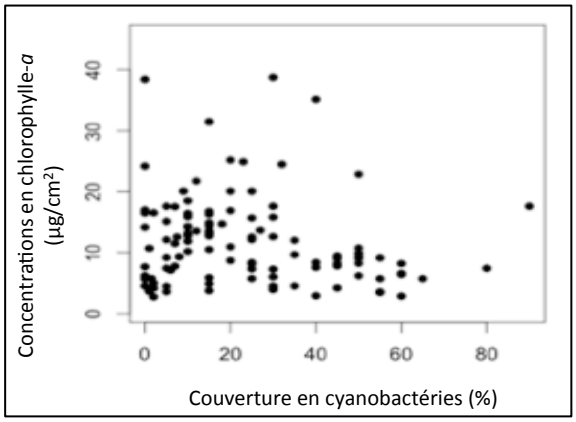
Concernant la profondeur, s'il n'existe pas de relation significative avec la biomasse des biofilms, on peut cependant remarquer que les plus fortes biomasses s'observent à des profondeurs comprises entre 10 et 50 cm. De même, il apparaît que les températures entre 16 et 20°C sont associées aux plus fortes biomasses. Enfin, comme le montre très clairement la dernière figure, les biomasses les plus élevées des biofilms sont observées lorsque les biofilms sont dominés par les cyanobactéries.

Comme l'illustre la photo ci-contre, des zones de développement favorables aux cyanobactéries peuvent devenir favorables aux algues vertes (et inversement) à l'échelle d'une saison estivale et en fonction des variations de la hauteur d'eau et du débit de la rivière. Sur cette photo, on observe que les algues vertes recouvrent les biofilms à cyanobactéries très épais qui s'étaient sans doute développés avant elles sur les galets, lorsque le courant était plus fort dans cette zone.

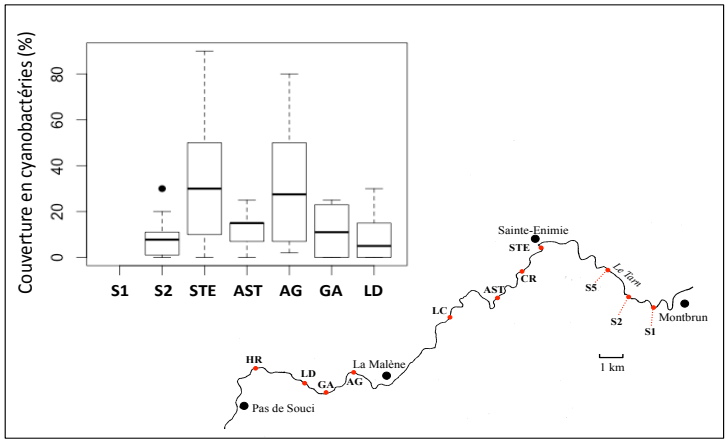
V.1.2. Evolution des couvertures en cyanobactéries

Si les valeurs de biomasses constituent un indicateur d'intérêt, elles ne permettent pas cependant de préjuger de l'étendue de la couverture des biofilms à cyanobactéries au fond de la rivière puisque des biomasses très importantes peuvent par exemple être ponctuellement observées sur un ou quelques galets localisés au milieu d'une zone où tous les autres galets sont dominés par des diatomées ou des algues vertes. La couverture des fonds en cyanobactéries apporte donc une information très complémentaire aux estimations de biomasses en fournissant une estimation de l'étendue de la prolifération dans une zone donnée.

Comme le montre la figure ci-contre, il n'existe pas de corrélation significative entre la biomasse des biofilms (exprimée par unité de surface) et les pourcentages de recouvrement du substrat par les cyanobactéries au fond de la rivière. Ceci traduit le fait que de larges surfaces peuvent être couvertes par des biofilms fins représentant de faibles biomasses par unité de surface mais aussi que de petites surfaces peuvent être couvertes par des biomasses importantes en cyanobactéries.



Les suivis des couvertures en cyanobactéries réalisés sur le Tarn en 2013 et 2014 (voir figure ci-contre) montrent que celles-ci varient entre 0 et 90 % dans les zones d'échantillonnage qui avaient une surface de 100 m^2. Ces couvertures présentent de fortes variations spatiales d'une station à l'autre, ce qui révèle l'importance des facteurs locaux dans le développement des biofilms. Ces résultats montrent également que pour un même site, les couvertures en cyanobactéries peuvent considérablement varier d'un prélèvement à l'autre comme en attestent les écarts types des valeurs obtenues aux stations de St-Enimie (STE, aval du pont) et de L'angle (AG, à l'aval de la Malène), alors que toutes nos observations ont été réalisées en été pendant la période la plus favorable au développement des biofilms à cyanobactéries. Ces variations dans les couvertures en cyanobactéries pour une

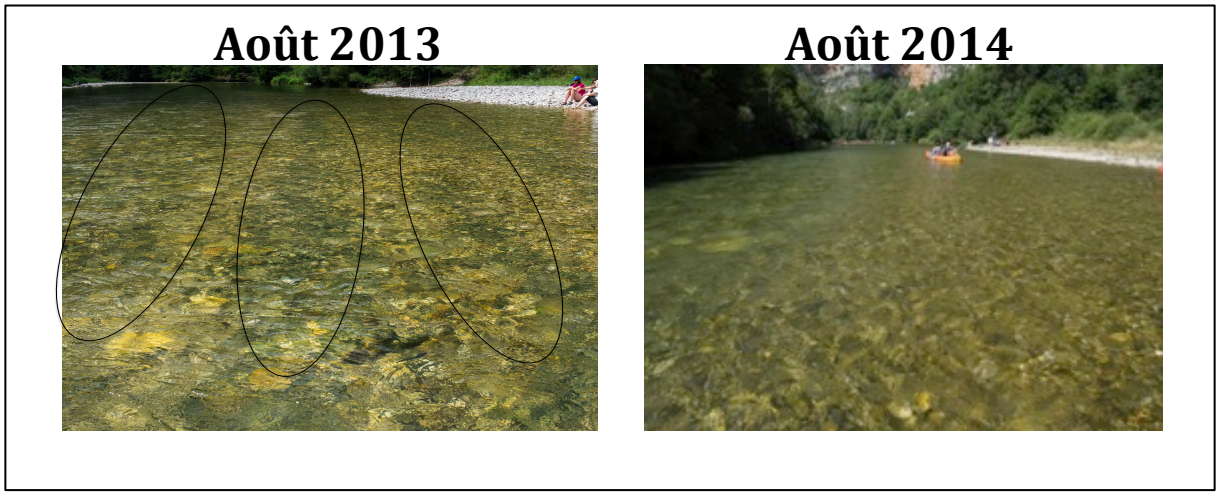


Graphique en box-plot des couvertures en cyanobactéries par site échantillonné

(S1 : Aval Blajoux ; S2 : Castelbouc ; STE : St Enimie (Aval du pont); AST : St Chély du Tarn ; AG : L'Angle (aval de la Malène) ; GA : Gaujac; LD : Les Détroits)

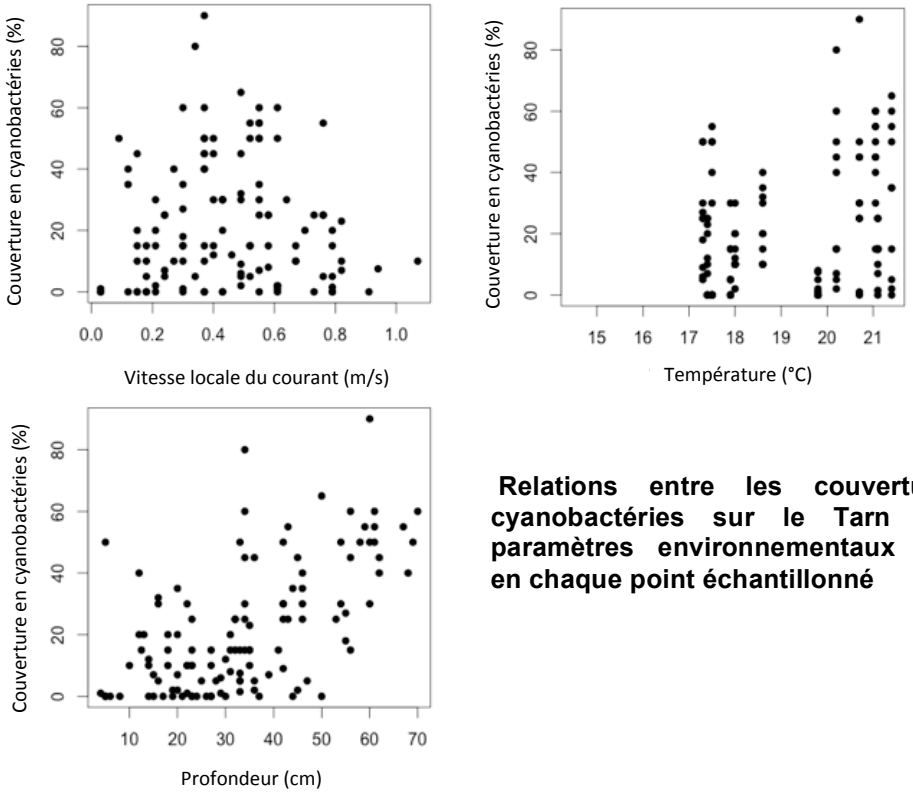
même station traduisent à la fois des variations dans les conditions locales rencontrées au cours d'une même saison et celles survenant d'une année à l'autre.

Pour illustrer ces différences interannuelles qui peuvent survenir sur un même site, les deux photos ci-dessous montrent les couvertures en cyanobactéries observées en août 2013 et 2014 à la station L'Angle à l'aval de la Malène. Il apparaît clairement que les couvertures étaient très importantes en 2013 (en particulier dans les zones entourées par les trois ellipses) alors qu'en 2014 elles étaient quasiment nulles dans ces mêmes zones, sachant que le débit et la hauteur d'eau étaient plus élevés lors de cette seconde année. En revanche, des biomasses importantes étaient observées juste en amont de ce site en 2014.



Développement de biofilms à cyanobactéries au même site d'étude (L'Angle ; à l'aval de la Malène) lors des deux années d'échantillonnage
Les zones de développement des biofilms observées en 2013 sont cerclées en noir sur la photographie

Comme pour la biomasse, nous avons recherché l'existence de corrélations entre les variations des pourcentages de recouvrement en cyanobactéries et celles de différents paramètres environnementaux.



Relations entre les couvertures en cyanobactéries sur le Tarn et trois paramètres environnementaux mesurés en chaque point échantillonné

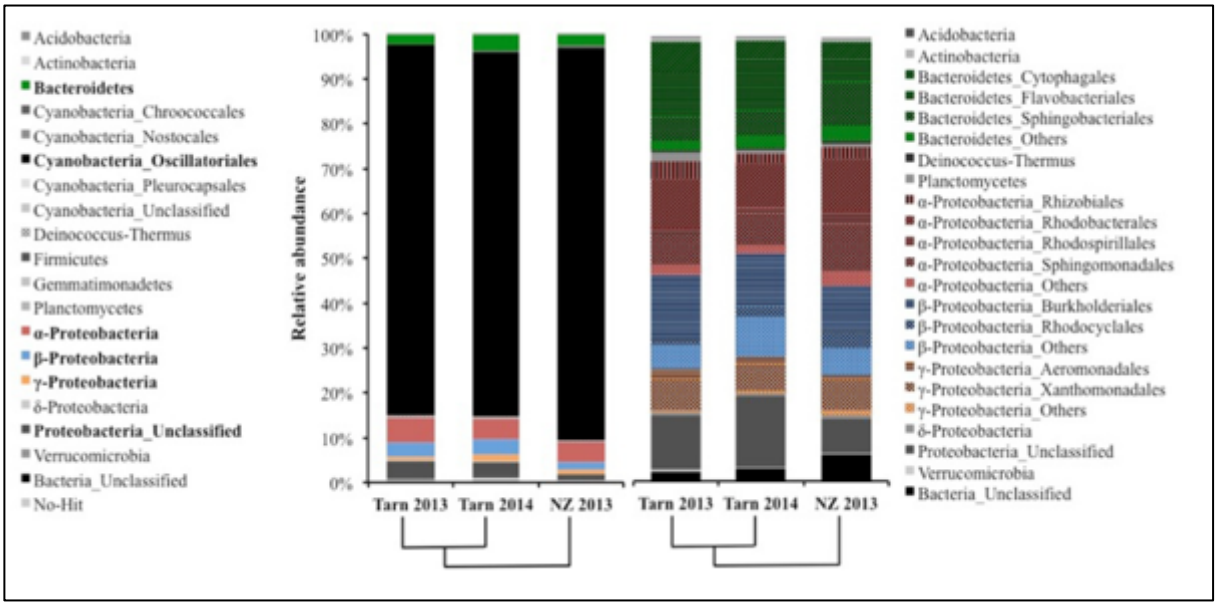
S'il n'existe pas de relation significative entre la couverture en cyanobactéries et la vitesse du courant, on peut quand même remarquer que les couvertures les plus importantes sont observées entre 0,2 et 0,8 m s^{-1} alors que les plus faibles valeurs sont observées de part et d'autre de cet intervalle. Pour les températures, sachant que nous n'avons travaillé qu'en saison estivale, les variations de ce paramètre sont assez limitées. Cependant une tendance à l'augmentation des couvertures en cyanobactéries semble s'observer lorsque la température de l'eau augmente. Enfin, il existe une corrélation significative entre le pourcentage de couverture en cyanobactéries et la profondeur de l'eau, les couvertures en cyanobactéries les plus élevées étant observées aux profondeurs les plus importantes dans la limite de 70-80 cm. Cette observation pourrait s'expliquer par la plus faible luminosité dans les zones plus profondes qui contraindrait les biofilms à se développer sur de faibles épaisseurs (faible biomasse par cm^2 comme vu au paragraphe V.1.1). A contrario, les biofilms plus épais qui se développent dans les zones moins profondes sont plus soumis aux variations du niveau de l'eau et de vitesse du courant ce qui peut limiter leur étalement sur le substrat voire favoriser leur décrochement par patch. Ces hypothèses mériteraient cependant d'être vérifiées. Il faut également nuancer ces observations dans la mesure où, comme cela a été dit plus haut, les biofilms à cyanobactéries ont toujours été observés en zones de radier où les profondeurs maximales restent limitées en comparaison des zones d'eau plus calmes et plus profondes où nous avons souvent pu observer des développements abondants d'algues vertes.

Pour conclure, les résultats obtenus sur les biomasses et les couvertures des biofilms à cyanobactéries ont montré que si les biotopes favorables à leur développement sont désormais connus pour le Tarn (les zones de radiers), il n'en demeure pas moins que les variations dans les conditions environnementales locales vont être déterminantes pour le développement de ces biofilms. En particulier, les variations intra- et interannuelles dans le niveau de l'eau, la vitesse du courant, le débit de la rivière et la température peuvent conduire, pour un même biotope, à des biomasses et/ou à des taux de couverture en cyanobactéries très différents. De même, à une échelle plus globale, si nos données suggèrent que le réchauffement climatique (qui devrait se traduire pour les rivières tel que le Tarn par des températures de l'eau plus élevées et par de longues périodes d'étiages sévères) constitue probablement un processus favorisant les développements de biofilms à cyanobactéries, cet effet sera cependant très variable en fonction des sites et donc des conditions locales. Dans les zones de faibles profondeurs par exemple, une diminution des hauteurs d'eau et des débits ne favorisera pas forcément les cyanobactéries, les algues vertes étant alors très compétitives, alors que dans les zones plus profondes le contraire est attendu.

Nous n'avons pas développé de travaux au cours de cette étude sur le détachement des biofilms car cela aurait nécessité un échantillonnage réalisé à des pas de temps très rapprochés sur des zones bien définies. Ce processus serait cependant important à mieux connaître dans le futur car il est probable que le détachement puis l'échouage et/ou l'accumulation des biofilms dans certaines zones constituent un risque important d'exposition pour les chiens et potentiellement aussi pour de jeunes enfants.

V.2. Etude de l'évolution spatiotemporelle de la composition et de la structure des biofilms du Tarn

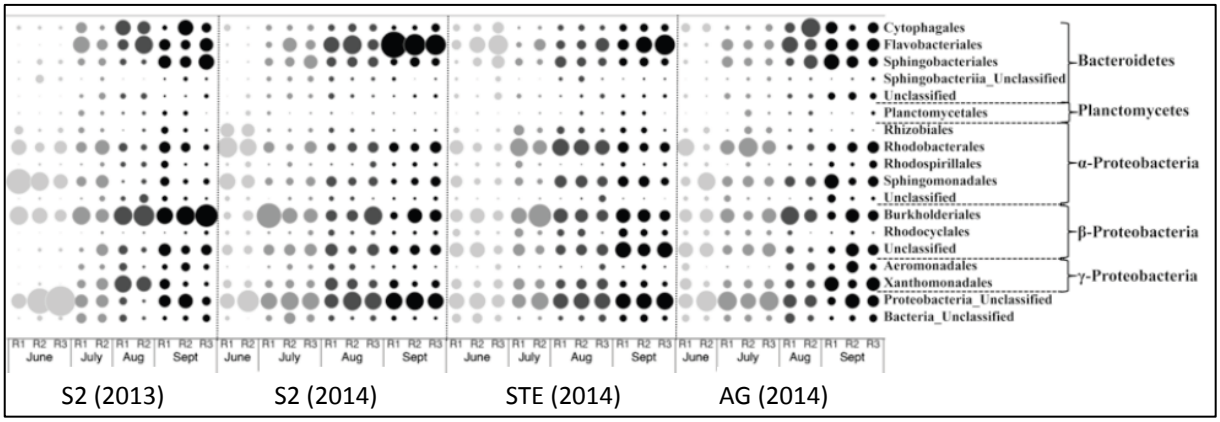
Une approche moléculaire basée sur le séquençage d'un fragment amplifié de l'ARN 16S nous a permis de décrire la composition et la structure des communautés procaryotes (bactéries) des biofilms et de comparer cette composition et structure en fonction des sites et des dates d'échantillonnage. Une comparaison a également été réalisée avec les biofilms prélevés en Nouvelle-Zélande.



Distributions d'abondances relatives des grandes classes de bactéries dans les biofilms du Tarn (campagnes de 2013 et 2014) et dans ceux de Nouvelle Zélande.
Figure de gauche : Toutes bactéries ; Figure de droite : Bactéries sans les cyanobactéries

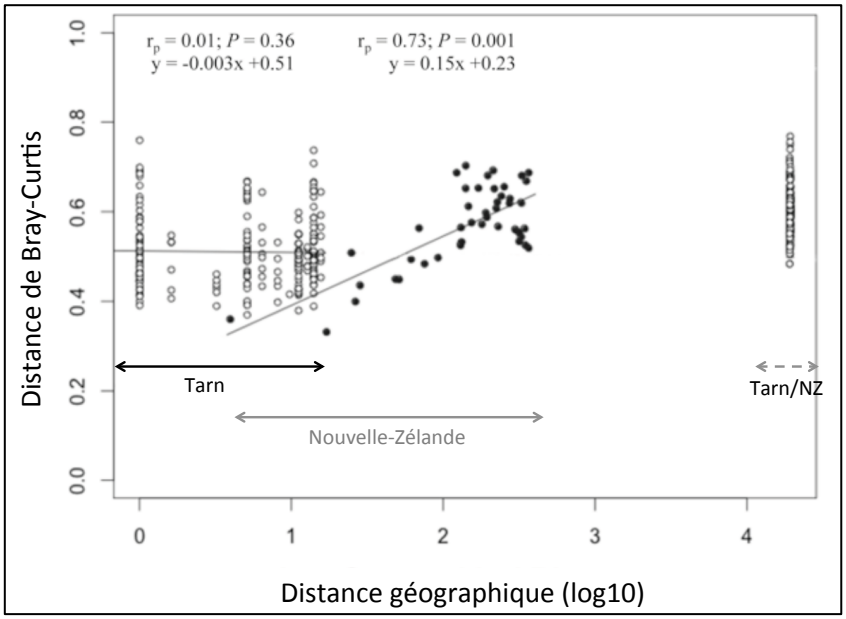
Comme le montre la figure dessus, il apparaît que les communautés bactériennes sont très largement dominées par les cyanobactéries de l'ordre des Oscillatoriales (>80% des séquences ; en noir sur la Figure de gauche) suivies par les Alpha- et Betaproteobacteria. Par ailleurs, la composition globale des biofilms du Tarn est pratiquement identique lors des deux années d'échantillonnage (2013 & 2014) et elle diffère très peu de celle de biofilms récoltés en Nouvelle-Zélande. Lorsqu'un focus particulier est porté sur les séquences autres que celles des cyanobactéries, il apparaît que la composition des communautés bactériennes des biofilms du Tarn présente très peu de variations d'une année à l'autre et qu'elle est quasiment identique à celle des biofilms de Nouvelle-Zélande.

Enfin, les communautés bactériennes associées aux biofilms dominés par les diatomées se distinguent nettement de celles des biofilms à cyanobactéries comme le montre la figure ci-dessous, ce qui suggère une association étroite entre le producteur primaire et les bactéries associées dans le biofilm.



Variations saisonnières dans l'abondance relative des ordres dominants de bactéries à trois stations d'échantillonnage du Tarn lors des deux années d'échantillonnage.
Aux stations S2 (2013 & 2014) et AG (2014), les diatomées étaient dominantes pendant le mois de juin alors qu'à tous les autres points et dates, les cyanobactéries dominaient les biofilms

A un niveau taxonomique plus fin, celui de l'espèce, nous avons constaté que les espèces bactériennes les plus abondantes dans les biofilms sont le plus souvent partagées par les communautés bactériennes du Tarn et de la Nouvelle Zélande. Par ailleurs, comme le montre la figure ci dessous, aucune relation significative n'existe au niveau du Tarn entre la distance geographique existant entre les biofilms et la distance génétique estimée entre leurs communautés bactériennes. En revanche, il existe une relation significative entre ces mêmes distances (géographiques et génétiques) à l'échelle des rivières de la Nouvelle Zélande ce qui signifie que plus les biofilms sont proches géographiquement et plus leurs communautés bactériennes se ressemblent (et inversement). Finalement, il apparaît que les distances génétiques entre les biofilms français et néo-zélandais sont du même ordre de grandeur que celles observées entre les biofilms néo-zélandais éloignés géographiquement, ce qui traduit le fait que ce n'est pas la distance géographique qui explique les différences entre tous ces biofilms mais les conditions environnementales locales (dans le cas contraire, la distance génétique entre les biofilms français et néo-zélandais aurait du être beaucoup plus importante).



Relation entre la distance géographique et la distance génétique des communautés bactériennes des biofilms dominés par les cyanobactéries

Les analyses multivariées non représentées dans ce rapport (mais figurant dans la publication à venir) ont confirmé l'ensemble de ces résultats et notamment (1) les différences observées entre les communautés bactériennes associées aux biofilms dominés par des diatomées et celles des biofilms dominés par les cyanobactéries, et (2) les très fortes ressemblances dans la composition et la structure des communautés bactériennes des biofilms du Tarn et de ceux de la Nouvelle-Zélande, ce qui n'était pas un résultat attendu.

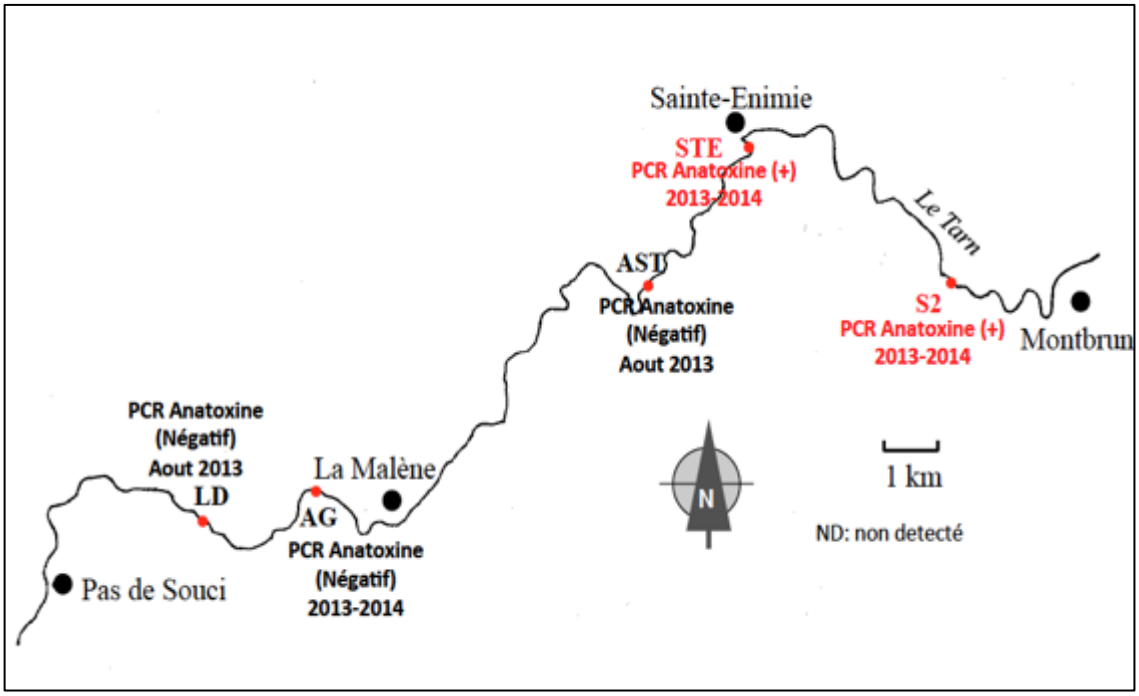
Pour conclure sur cette partie concernant l'étude des communautés bactériennes dans les biofilms du Tarn, nos analyses ont montré que ces communautés dans les biofilms dominés par les cyanobactéries présentent des caractéristiques partagées au niveau de leur structure et de leur composition sur tous les sites d'étude, que ce soit dans le Tarn ou en Nouvelle-Zélande. Ce résultat suggère qu'il existe des associations spécifiques entre les cyanobactéries et les bactéries qui leur sont associées au sein des biofilms avec des conséquences probablement très importantes en terme de fonctionnement des biofilms. Le partage de nombreuses espèces entre les biofilms du Tarn et de la Nouvelle-Zélande et l'absence de structuration biogéographique à large échelle spatiale suggère par ailleurs que des flux de bactéries s'opèrent à ces larges échelles spatiales. Dans le même temps, des variations dans la composition des communautés bactériennes associées aux cyanobactéries ont été observées à l'échelle d'une même station d'échantillonnage sur le Tarn ou en comparant les communautés de plusieurs rivières néo-zélandaises, ce qui traduit très probablement les effets conjugués du stade de maturité du biofilm et des conditions locales sur ces communautés bactériennes.

V.3. Evaluation du potentiel toxique des biofilms à cyanobactéries dans le Tarn

Le potentiel toxique des biofilms à produire des toxines a été évalué par deux approches, la première basée sur la recherche des gènes impliqués dans la synthèse des toxines et la seconde basée sur la recherche et la quantification de ces toxines en spectrométrie de masse.

Concernant l'approche moléculaire, l'utilisation de la PCR a permis de cibler des gènes impliqués dans la synthèse de trois types de toxines, les microcystines, les anatoxines et les saxitoxines. Le choix de cibler ces trois types de molécules a reposé principalement sur l'analyse de la littérature concernant les toxines potentiellement produites par les espèces de cyanobactéries que nous avons mises en évidence dans le Tarn.

Les résultats obtenus pour les anatoxines (neurotoxines identifiées comme responsables des mortalités de chiens observées sur le Tarn et référencées dans la littérature scientifique; Cadel-Six et al., 2007) sont rapportés sur la carte ci-dessous :



Résultats de la recherche par PCR d'un gène impliqué dans la synthèse des anatoxines dans les biofilms du Tarn

(S2 : Castelbouc ; STE : St Enimie (Aval du pont); AST : St Chély du Tarn ; AG : L'Angle (aval de la Malène) ; LD : Les Détroits)

Plus précisément, il apparaît que :

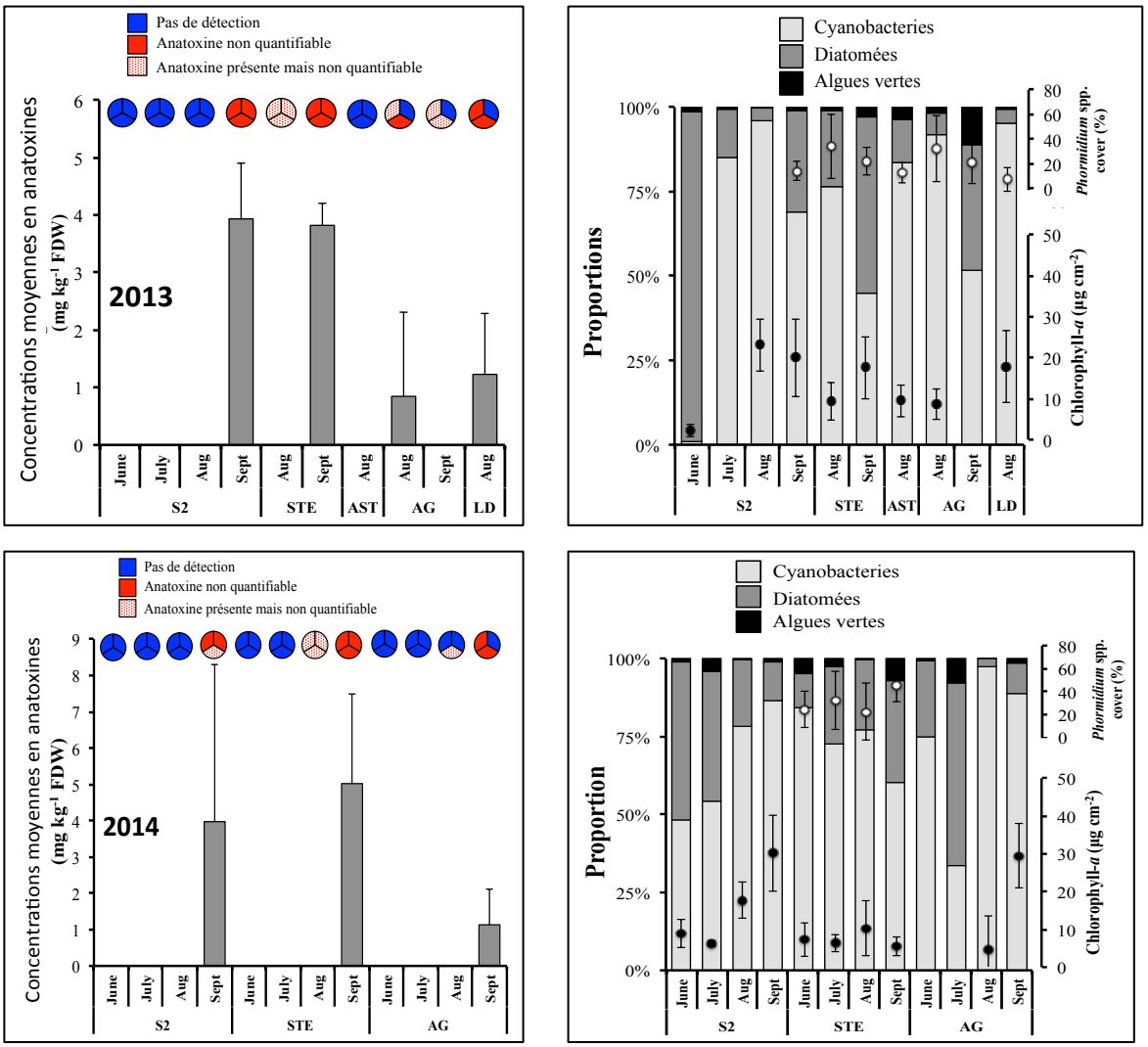
→**Tarn 2013 :** Sur les 30 échantillons prélevés dans cinq sites, huit ont été détectés positifs sur les sites de Castelbouc (S2 ; 2, 4 & 10 septembre) et de St-Enimie (STE ; 2 & 3 août et 4,8 & 10 septembre).

→**Tarn 2014 :** Sur les 35 échantillons prélevés dans trois sites, huit échantillons positifs ont été détectés sur les mêmes sites qu'en 2013, Castelbouc (S2 ; 1 & 2 septembre) et St-Enimie (STE ; 2 juin, 8 & 10 août, 3,4 & 8 septembre).

Aucun signal positif en PCR sur les gènes d'anatoxines n'a été détecté sur les autres sites d'étude.

Pour les microcystines (toxines hépatiques) et les saxitoxines (neurotoxines), aucun signal positif n'a été détecté par PCR sachant qu'il avait été décrit dans la littérature l'existence de souches de *Phormidium* productrices de microcystines (par exemple, Izaguirre et al., 2007) et de saxitoxines (par exemple, Teneva et al., 2005).

Les résultats des dosages d'anatoxines (ATX) dans les biofilms prélevés dans le Tarn sont présentés pour les campagnes d'échantillonnage de 2013 et 2014 dans les figures de gauche ci-dessous :



Résultats des dosages d'anatoxines (valeurs moyennes estimées sur trois échantillons prélevés par site) réalisés sur les biofilms du Tarn récoltés en 2013 et 2014 dans cinq stations d'échantillonnage (Figures de gauche) et rappels des données de biomasses et de composition des biofilms (Figures de droite)

Les camemberts dans la figure de gauche présentent les résultats obtenus sur chacun des trois biofilms analysés par station.

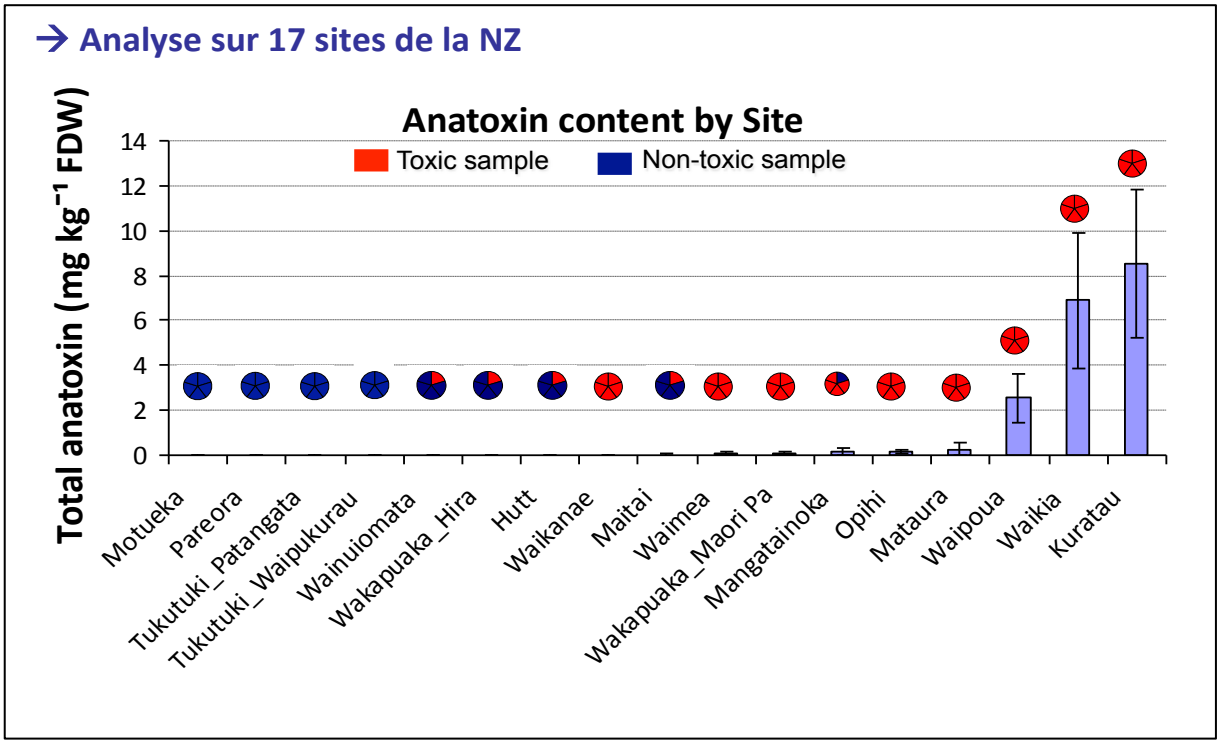
(S2 : Castelbouc ; STE : St Enimie; AST : St Chély du Tarn ; AG : L'Angle; LD : Les Détroits)

De ces quatre figures, il ressort que les concentrations en ATX sont les plus élevées lorsque les biomasses en cyanobactéries sont importantes, en fin de saison estivale (août et septembre 2013 ; septembre 2014). Il faut noter cependant qu'il peut y avoir des exceptions à l'exemple des valeurs élevées de concentrations en ATX observées à la station STE en septembre 2014 alors que les biomasses n'étaient pas particulièrement élevées ou, à l'inverse, des concentrations en ATX faibles mesurées au site AG en septembre 2015 alors que les biomasses étaient très fortes.

Il apparaît également dans ces figures que lorsque ces concentrations en ATX sont élevées, les trois échantillons prélevés sur trois galets différents dans chaque site contiennent en général tous de l'ATX à des concentrations le plus souvent quantifiables. Enfin, il faut noter que les concentrations en ATX sont les plus élevées aux deux sites (S2 et STE) qui avaient été détectés comme étant positifs par PCR

sur un des gènes de synthèse de l'anatoxine. A l'inverse, les analyses PCR étaient restées négatives sur AG et LD alors que de l'ATX a été détectée en faibles concentrations sur ces deux stations.

Il est intéressant de comparer ces résultats obtenus sur le Tarn à ceux obtenus en Nouvelle-Zélande sur ces mêmes anatoxines. Les concentrations en anatoxines (exprimées en mg par kilo de poids sec de biofilm) ont été estimées dans les biofilms collectés sur cinq galets différents par station d'échantillonnage.



Résultats des dosages d'anatoxines réalisés sur les échantillons prélevés dans les rivières de Nouvelle-Zélande
Les camemberts figurant sur chaque site illustrent les résultats obtenus pour chaque galet

Dans cette figure, il faut tout d'abord remarquer l'existence d'une forte hétérogénéité dans les concentrations en ATX des biofilms échantillonnés. Lorsque de l'ATX est détectée, les concentrations mesurées sont du même ordre de grandeur que celles estimées dans les biofilms du Tarn. Ces concentrations peuvent présenter des différences importantes à l'intérieur d'un même site comme en témoignent les intervalles de confiance des valeurs. Si dans les trois sites où les plus fortes concentrations ont été observées (Waipoua, Waikia & Kuratau), les biofilms des cinq galets étudiés étaient positifs, on constate en revanche que pour les sites où les concentrations en anatoxines sont plus faibles, voire très proches de zéro, toutes les situations peuvent s'observer.

Pour conclure sur cette partie concernant la toxicité potentielle des biofilms dominés par une cyanobactérie du genre Phormidum, les résultats obtenus sur le Tarn confirment que ces biofilms possèdent bien un potentiel neurotoxique. Ce potentiel est très variable dans le temps et dans l'espace. Nous avons en effet constaté que les biofilms producteurs de toxines ont été retrouvés dans la seconde partie de l'été (Août et Septembre) et principalement dans deux

stations d'échantillonnage (Castelbouc et St Enimie). En l'état actuel de nos connaissances, il est impossible de déterminer si ces concentrations en ATX élevées mesurées dans les biofilms de ces deux stations en fin d'été s'expliquent simplement par les fortes biomasses en cyanobactéries relevées en ces lieux et à cette période ou s'il existe des facteurs locaux et/ou saisonniers à l'origine de la production de plus fortes quantités d'ATX par les biofilms à Phormidium sur ces stations. Pour rappel, il a par exemple été montré pour des cyanobactéries planctoniques productrices de microcystines, que des successions de génotypes producteurs ou non producteurs de toxines pouvaient survenir dans les populations de Microcystis au sein d'un lac (Briand et al., 2009). Ce type d'information n'est pas disponible pour les cyanobactéries benthiques productrices d'ATX. De nos résultats obtenus dans le Tarn et en Nouvelle Zélande, il apparaît également qu'à l'échelle d'un même site, même si les concentrations « moyennes » en ATX sont très faibles, certains galets peuvent héberger des biofilms contenant des quantités importantes d'ATX.

VI. Recommandations formulées en terme de surveillance

Outre les connaissances que devait apporter ce projet sur les biofilms à cyanobactéries de deux rivières françaises, son deuxième objectif majeur était de formuler des recommandations pour la surveillance des biofilms à cyanobactéries dans les rivières et pour la gestion des situations de crise.

De l'expérience acquise pendant ces trois années d'étude et des nombreux échanges que nous avons eus avec nos collègues Néo-Zélandais, nous voulons dans un premier temps revenir sur quelques résultats qui nous semblent fondamentaux pour nos propositions sur la surveillance :

1. Les rivières favorables au développement de biofilms dominés par les cyanobactéries présentent des caractéristiques communes telles que la présence dominante de faciès d'écoulement peu profonds (< 60 cm), l'alternance de faciès lentiques (vitesse d'écoulement < 30 cm/s) et de faciès lotiques (vitesse d'écoulement > 30 cm/s), un substrat mêlant graviers, galets et blocs (cf Malavoi et Souchon, 2002), et une ouverture de la rivière permettant un bon éclairement.
2. Au sein de ces rivières, les zones de radier dont le substrat est dominé par des galets sont les biotopes les plus favorables au développement des biofilms à cyanobactéries, ce qui permet de cibler ces zones pour la surveillance des cyanobactéries
3. Dans ces biotopes favorables au développement des biofilms à cyanobactéries, les biomasses et les pourcentages de couvertures en biofilms à cyanobactéries peuvent être très variables dans le temps, ce qui impose des contraintes importantes en terme de suivi
4. La BenthoTorch BBE n'est, en l'état actuel de son développement, pas adaptée au suivi des biofilms à cyanobactéries MAIS un apprentissage de la reconnaissance visuelle de ces biofilms est très facile à réaliser
5. Il existe une très grande variabilité spatiale et temporelle dans la toxicité potentielle des biofilms à cyanobactéries qui impose, si l'on veut estimer ce potentiel toxique, de multiplier ces analyses dont le coût est très élevé
6. Enfin, de l'examen de la littérature, il ressort que toutes les mortalités d'animaux, en particulier de chiens, sont survenues après ingestion de biofilms et que les quantités de toxines libres dans l'eau semblent négligeables. Les situations d'exposition aux toxines de cyanobactéries benthiques semblent donc prioritairement concernées :
    - → Les animaux qui vont boire de l'eau ou jouer dans la rivière et qui ingèrent des biofilms dont ils ont provoqué le détachement en les piétinant ou qui se sont détachés tout seul puis accumulés dans la zone de jeu et/ou d'abreuvage.
    - → Les très jeunes enfants qui peuvent être en contact avec les biofilms (i) en portant directement à la bouche des cailloux recouverts de biofilms ou des biofilms détachés qui se sont échoués sur les bords de la rivière et/ou (ii) en portant à la bouche leurs mains souillées par des biofilms.

De l'ensemble de ces connaissances, nous sommes parvenus à la conclusion que le point le plus important dans la gestion des risques liés aux proliférations de cyanobactéries benthiques concerne l'information qui doit

être faite auprès des utilisateurs des rivières. Alors que tout le monde sait que dans beaucoup de forêts françaises poussent des champignons toxiques dont la consommation peut être mortelle, leur présence n'empêche ni les promenades en forêt, ni même le ramassage et la consommation des autres champignons. Le maintien de ces activités reste possible, malgré les risques encourus, grâce aux campagnes d'information et d'éducation réalisées auprès des populations fréquentant ces forêts pour se promener ou pour cueillir des champignons. Pour les mêmes raisons, il est donc raisonnable de penser que même pendant les périodes de développements importants de biofilms à cyanobactéries benthiques, les activités en rivière peuvent être maintenues, à partir du moment où les populations pratiquant ces activités sont informées des risques encourus et des moyens permettant d'éviter de s'exposer à ces risques. C'est aussi ce principe qui a été retenu en Nouvelle-Zélande où les développements de cyanobactéries benthiques toxiques sont beaucoup plus fréquents et beaucoup plus importants qu'en France.

C'est la raison pour laquelle nous recommandons (i) un schéma de surveillance simple basé sur l'estimation de la couverture en cyanobactéries dans les biotopes favorables à leur développement complété par une surveillance visuelle, sur les zones de baignade, de la présence éventuelle de biofilms détachés et (ii) la mise à disposition de flyers dans des structures tels que les campings, les offices de tourisme… et l'installation de panneaux d'information simples et didactiques sur toutes les zones d'accès aux rivières informant les usagers de la présence possible de cyanobactéries benthiques et des risques associés, et donnant les conseils permettant d'éviter une exposition à ces risques. Ces deux recommandations sont développées dans la suite du document.

VI.1. Schéma de surveillance des cyanobactéries benthiques

De tous nos résultats ainsi que de nos échanges avec nos collègues Néo-Zélandais qui ont mis en place une surveillance des cyanobactéries benthiques dans leurs rivières depuis plusieurs années, nous pouvons formuler des premières recommandations sur les outils et méthodologies à utiliser pour le protocole de surveillance.

Sachant que la sonde BBE ne peut être utilisée en l'état pour la surveillance, nous recommandons, après formation des agents à la reconnaissance des biofilms à cyanobactéries, une stratégie de surveillance basée sur l'utilisation de l'Aquascope pour estimer la couverture des fonds en cyanobactéries sur les sites favorables au développement des biofilms.

Cette stratégie est pratiquée en Nouvelle-Zélande sachant qu'en fonction des pourcentages de couvertures, diverses actions sont prises en terme d'analyses (par exemple de recherche de toxines) et d'informations auprès du public.

Le choix de **focaliser la surveillance sur la couverture en cyanobactéries des fonds des rivières** se justifie par le fait qu'il est vraisemblable que plus cette couverture est importante et plus la probabilité de se trouver en contact avec les biofilms augmente. En complément de cette surveillance exercée sur cette couverture des biofilms à cyanobactéries dans leurs zones de développement, nous recommandons **un suivi de la présence de biofilms détachés** (voir photo ci-dessous) **dans les zones de baignade connues.** En effet, même si ces zones de baignades ne constituent généralement pas des zones de développement favorables

au cyanobactéries benthiques (faciès lentiques et plus grandes profondeurs), elles peuvent favoriser l'accumulation de biofilms détachés qui pourraient provenir de zones de développement situées en amont.

Zone d'accumulation de biofilms à cyanobactéries échoués sur les rives du Tarn

La fiche technique détaillant les méthodologies utilisées pour le protocole de surveillance est décrite dans les pages suivantes.

A partir de ce protocole, nous proposons les actions suivantes :

1. **Si des biofilms à cyanobactéries sont détectés dans plus de la moitié des points d'observation (au moins cinq points par site), la fréquence de surveillance de la rivière et des zones de baignade doit devenir bimensuelle.**
2. **Si un grand nombre de biofilms sont découverts échoués sur la berge d'une zone de baignade ou si les couvertures en cyanobactéries sont très importantes (au moins 7 points positifs par site) dans les zones situées juste en amont de ces baignades, en plus de l'information habituelle sur les panneaux, des recommandations devront être communiquées aux parents des très jeunes enfants (moins de 6 ans par exemple) pour les informer des dangers potentiels à les laisser jouer à proximité immédiate de la rivière.**

Ce protocole de surveillance et les décisions qui lui sont associées, devront faire l'objet d'une discussion avec les personnes en charge de la surveillance, l'ARS et les collectivités locales. Aucun dosage de toxines n'est recommandé car nos résultats ainsi que ceux de nos collègues Néo-Zélandais, ont révélé une hétérogénéité spatiale et temporelle très forte dans la toxicité potentielle des biofilms. Dans ces conditions, sauf à procéder à des dizaines d'analyse, il nous parait impossible d'obtenir une estimation vraiment fiable de la toxicité potentielle lors de périodes de forts développements de biofilms à cyanobactéries.

Si nous ne recommandons ni restriction d'usages, ni interdiction d'activités, même en cas de développement très importants de biofilms à cyanobactéries, c'est parce que nous pensons qu'une information bien formulée auprès des usagers de la rivière est suffisante pour prévenir les risques d'exposition humaine et animale. Il est bien évident que si les municipalités ne retenaient pas les propositions d'informations que nous formulons, il serait alors nécessaire de prescrire des restrictions d'usage et même des interdictions d'activités (par exemple la baignade) pour prévenir ces risques d'exposition.

Fiche technique pour l'estimation des couvertures en cyanobactéries benthiques dans leurs zones de développement

Matériel nécessaire

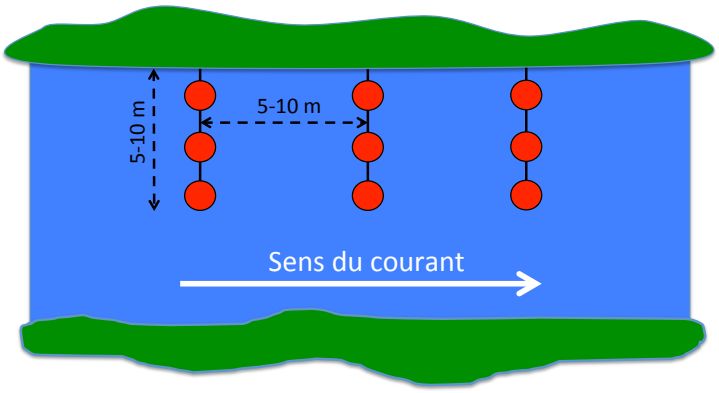
- Aquascope
- Tubes de prélèvements (1,5 ou 2 ml ; 15 ou 50 ml)
- Pinces à bouts plats
- Gants

Zones de surveillance privilégiées

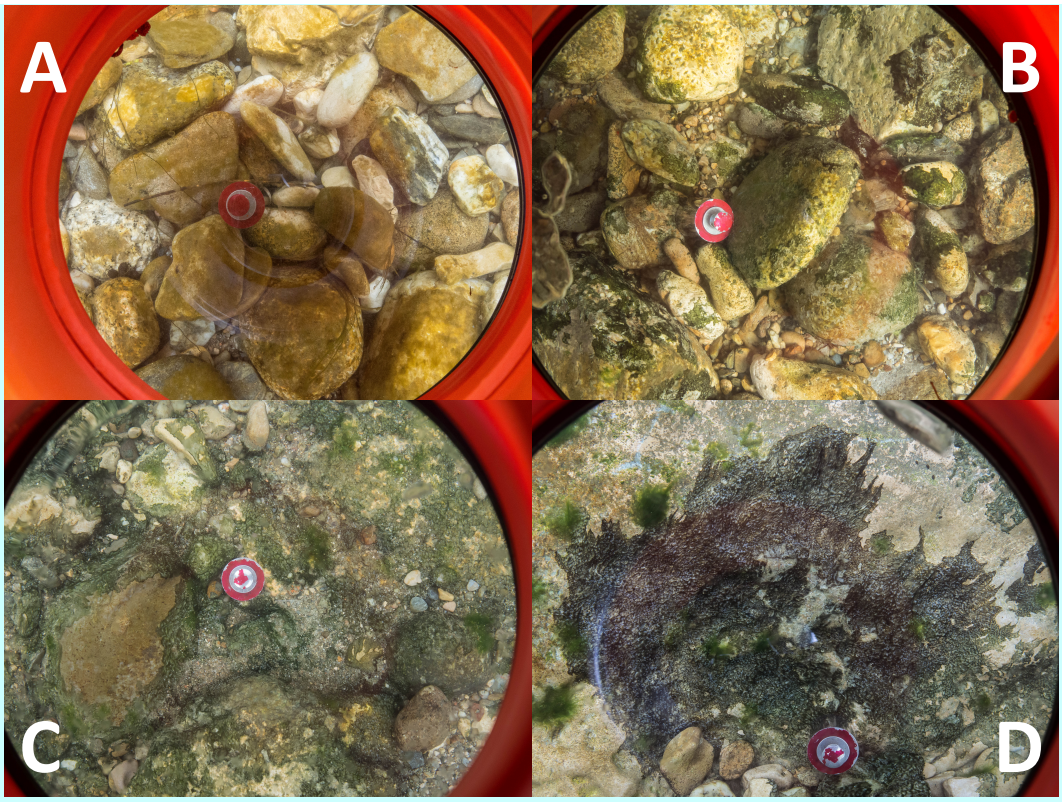
- Radiers

Protocole d'estimation de la couverture en cyanobactéries dans les zones surveillées

Disposition du protocole : L'estimation des couvertures par les biofilms à cyanobactéries est réalisée en trois points le long de trois transects partant d'une berge de la zone surveillée, soit un total de neuf points d'observation. La distance entre les transects dépend de la taille de la zone, sachant qu'ils seront disposés de façon à couvrir au mieux cette zone. De même la longueur des transects et donc l'espacement entre les points d'observation réalisés avec l'aquascope dépend de la largeur de la rivière, sachant qu'il sera fait en sorte que le dernier point du transect soit placé au milieu de la rivière (si la profondeur le permet).



Estimation de la couverture : Pour chaque point de surveillance, il sera déterminé la présence ou non de biofilms à cyanobactéries dans la surface définie par l'Aquascope. Comme le montrent sur les photos ci-après, seront considérés comme positifs les points B, C et D même si leur couverture en cyanobactérie est très différente, et comme négatif le point A.



Protocoles de prélèvements complémentaires

Des prélèvements complémentaires pourront être réalisés dans le but de (i) confirmer par observation au microscope que les biofilms contiennent bien des cyanobactéries s'il existe des doutes et (ii) rechercher la présence d'anatoxines dans des situations où cette information est nécessaire (par exemple d'un point de vue réglementaire si la réglementation évolue).

- Pour les observations au microscope, des échantillons de biofilms seront prélevés avec une pince puis placés dans des tubes Eppendorf de 1,5 ou 2 ml de volume. Un volume d'eau recouvrant les biofilms sera ajouté ainsi que 3 gouttes de Lugol pour fixer le matériel biologique. Les tubes sont ensuite enveloppés dans du papier Aluminium pour les protéger de la lumière et pour le transport (conservation pendant plusieurs semaines).
- Pour le dosage de toxines, les biofilms sont prélevés avec une pince puis placés dans des tubes de 15 ou 50 ml recouverts d'eau, en laissant la moitié du volume vide. Ces tubes sont conservés à 4°C en glacière sur le terrain puis au réfrigérateur au laboratoire avant envoi au froid (conservation pendant 3-4 jours). Ils peuvent être également congelés pour une utilisation ultérieure (conservation pendant plusieurs mois).

Protocole de surveillance des biofilms échoués sur les rives de la rivière

La surveillance des biofilms échoués reposera sur le comptage des biofilms à cyanobactéries sur un linéaire de 10 m en bordure de la zone de baignade.

VI.2. Information auprès des usagers de la rivière

Comme indiqué dans la partie introductive de ce chapitre, nous pensons que l'action la plus efficace pour limiter les risques générés par les proliférations de cyanobactéries benthiques, tout en continuant de maintenir les activités qui s'exercent sur la rivière, est d'informer le plus largement possible les usagers de la rivière.

Cette information doit reposer sur deux actions :

→ La mise à disposition de dépliants dans les syndicats d'initiative, pharmacies, accueil des hôtels et campings, locations de canoës… expliquant la situation et les précautions à prendre pour éviter de s'exposer aux toxines de cyanobactéries benthiques. Il existe déjà un dépliant rédigé par le SAGE et l'Agence de l'Eau Adour-Garonne, une version simplifiée pourrait être également rédigée sous la forme d'un flyer plus facile à lire et à distribuer sur les zones d'activités.

→ La pose de panneaux d'information simples et didactiques dans toutes les zones de baignade et dans celles de départ et d'arrivée des canoës. Un modèle de panneau inspiré de ceux qui sont utilisés pour les rivières de Nouvelle-Zélande, est proposé ci-après.

Si cette information est indispensable chaque année pour le Tarn, elle pourrait être aussi nécessaire dans la partie aval de la Loue lors d'années très sèches comportant des étiages prolongés et probablement dans d'autres rivières présentant des faciès similaires.

ATTENTION-WARNING

Des bactéries toxiques qui peuvent tuer les chiens et rendre malades les humains colonisent parfois le fond de cette rivière en été

Elles forment à la surface des cailloux, des plaques de couleur très foncée qui peuvent se détacher et s'accumuler sur les bords

EVITER DE LES TOUCHER et si cela se produit, rincez-vous les mains dans l'eau de la rivière

Soyez vigilants avec les jeunes enfants qui pourraient les porter à la bouche

TENIR VOS CHIENS EN LAISSE et ne les laissez pas jouer et boire au bord de la rivière.

Si vous constatez que votre chien présente des signes de tremblement et/ ou des difficultés à marcher après avoir joué et/ou bu l'eau de la rivière, emmenez-le rapidement chez un vétérinaire

Logos…

VII. Conclusions générales et perspectives à donner à cette première étude

Les travaux réalisés lors de ce projet CYBERI ont permis de faire progresser nos connaissances sur les conditions de développement et sur la toxicité des biofilms à cyanobactéries dans deux rivières françaises, la Loue et le Tarn. S'il est apparu que la Loue semble être une rivière peu favorable au développement massif de biofilms à cyanobactéries dans des années hydrologiques « normales » pour la région concernée, on ne peut cependant exclure que de tels phénomènes puissent survenir dans la partie aval de la rivière lors d'années très sèches comportant une période d'étiage prolongée. Les mortalités de chiens observées en 2003 dans cette partie aval de la rivière sont d'ailleurs là pour en témoigner (Gugger *et al*., 2005). Pour le Tarn, un risque potentiel associé à ces développements de cyanobactéries benthiques existe chaque année. Les années 2013 et surtout 2014 pendant lesquelles nous avons travaillé, ont été marquées par des étés assez humides, en principe peu favorables au développement de ces biofilms mais malgré cela, des couvertures importantes en cyanobactéries ont été observées sur certains tronçons de la rivière.

Des explorations complémentaires ont été réalisées sur deux autres rivières du Sud du Massif Central, la Jonte et l'Hérault. Elles ont révélé la présence de biofilms dispersés de cyanobactéries benthiques, en quantités plus importantes dans l'Hérault que dans la Jonte qui est une rivière plus fermée (important couvert végétal sur ses bords). Il est donc très probable que d'autres rivières en France, et en particulier dans la partie Sud, puissent connaître des problèmes avec les cyanobactéries benthiques. Cela concerne notamment des rivières ouvertes avec des substrats comportant une granulométrie composée de graviers, galets et blocs et avec une alternance de zones de radiers et de zones de moindre courant.

Parmi les nombreuses questions qui restent ouvertes suite à ces travaux, nous en avons sélectionné trois qui nous semblent prioritaires à étudier.

1. La première de ces questions serait de déterminer, en réalisant deux ou trois campagnes d'échantillonnage estivales dans plusieurs rivières de la moitié Sud de la France, quel est l'étendu de ce problème en France. Ces informations nous permettraient par ailleurs de compléter nos connaissances sur les facteurs favorisant le développement de ces biofilms à cyanobactéries.

2. La seconde question est relative à l'ontogénie des biofilms à cyanobactéries. Il serait en effet particulièrement intéressant de mieux connaître les diverses phases du développement des biofilms à cyanobactéries, de leur initiation jusqu'à leur détachement. Ces connaissances permettraient par exemple de mieux anticiper les périodes de détachement des biofilms et de mieux connaitre les facteurs et processus qui influencent ce détachement.

3. La troisième question s'articule avec la seconde et elle concerne l'étude des liens entre l'hydrodynamique de la rivière, les flux de nutriments et la dynamique de développement des biofilms. Ces connaissances nous permettraient par exemple de comprendre pourquoi certaines zones dans le Tarn sont largement recouvertes de biofilms à cyanobactéries une

année et presque totalement dépourvues de ces biofilms l'année suivante. Par ailleurs, la complexité de ces rivières karstiques qui connaissent des alimentations en eau multiples rend difficile de déterminer si les concentrations en azote et phosphore ont une influence sur le développement des biofilms. Des études basées sur l'utilisation de sondes permettant un enregistrement en continu des concentrations en azote (et peut être bientôt en phosphates) apporteraient probablement des réponses à ces questions.

VIII. Remerciements

Les auteurs de cette étude tiennent à remercier les deux Agences de l'Eau Adour-Garonne (en particulier notre correspondant, J.P. Rebillard) et Rhône-Méditerranée-Corse (en particulier notre correspondant, Lionel Navarro) ainsi que l'ONEMA pour leur soutien financier.

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Enfin, nous n'oublions pas nos collègues Néo-Zélandais (Susie Wood et Mark Heath) dont les connaissances sur ces cyanobactéries nous ont permises de gagner beaucoup de temps dans nos travaux.

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ANNEXES

ANNEXE 1

Synthèse bibliographique réalisée au début de notre étude sur les connaissances disponibles sur les biofilms à cyanobactéries

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Review

A review of current knowledge on toxic benthic freshwater cyanobacteria – Ecology, toxin production and risk management

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ABSTRACT

Benthic cyanobacteria are found globally in plethora of environments. Although they have received less attention than their planktonic freshwater counterparts, it is now well established that they produce toxins and reports of their involvement in animal poisonings have increased markedly during the last decade. Most of the known cyanotoxins have been identified from benthic cyanobacteria including: the hepatotoxic microcystins, nodularins and cylindrospermopsins, the neurotoxic saxitoxins, anatoxin-a and homoanatoxin-a and dermatotoxins, such as lyngbyatoxin. In most countries, observations of toxic benthic cyanobacteria are fragmented, descriptive and in response to animal toxicosis events. Only a limited number of long-term studies have aimed to understand why benthic proliferations occur, and/or how toxin production is regulated. These studies have shown that benthic cyanobacterial blooms are commonly a mixture of toxic and non-toxic genotypes and that toxin concentrations can be highly variable spatially and temporally. Physiochemical parameters responsible for benthic proliferation vary among habitat type with physical disturbance (e.g., flow regimes, wave action) and nutrients commonly identified as important. As climatic conditions change and anthropogenic pressures on waterways increase, it seems likely that the prevalence of blooms of benthic cyanobacteria will increase. In this article we review current knowledge on benthic cyanobacteria: ecology, toxin-producing species, variables that regulate toxin production and bloom formation, their

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impact on aquatic and terrestrial organisms and current monitoring and management strategies. We suggest research needs that will assist in filling knowledge gaps and ultimately allow more robust monitoring and management protocols to be developed.

Contents

1. Introduction

Cyanobacteria are photosynthetic microorganisms found in diverse environments including oceans, freshwater, bare rock and soil (Whitton, 2012). They are believed to have been the first oxygenic photosynthetic organisms on Earth and contributed to the generation of oxygen in the Earth's atmosphere over 3 billion years ago (Rasmussen et al., 2008). They can exist as solitary, free-living cells or as colonies or filaments. These are usually microscopic but populations of them can be visible, for example, as benthic mats, crusts, large gelatinous colonies or blooms.

Cyanobacteria are common in aquatic systems where they grow in the water column (planktonic), aggregated on the water surface (metaphytic), attached to other algae, cyanobacteria or macrophytes (epiphytic), or attached to substrates (benthic). In aquatic environments most cyanobacteria species occur in low concentrations, however, when environmental conditions are favorable, cells can multiply rapidly forming planktonic or benthic blooms. It is well established that eutrophication of water bodies due to agriculture and urbanization, has resulted in the proliferation of some cyanobacteria species (Paerl, 1988). Changes to natural environmental flows in riverine systems (Mitrovic et al., 2011), global warming (Paerl and Huisman, 2009) and water pollution by herbicides (Lürling and Roessink, 2006) could also be responsible for an increase in the frequency and intensity of cyanobacterial blooms.

Cyanobacterial blooms can have detrimental effects on aquatic ecosystems through altering trophic structure and functionality (Havens, 2008), water column deoxygenation leading to fish mortalities, and decreasing water quality (Robarts et al., 2005). An increasing number of cyanobacteria species, including many bloom-forming species, are now known to produce potent toxins that can impact terrestrial and water-based organisms (Codd, 1995; Carmichael, 2001; Wu et al., 2012). The risks posed by cyanotoxins have traditionally been associated with planktonic cyanobacteria (Chorus and Bartram, 1999; Azevedo et al., 2002). As a result, significant scientific attention has been devoted to understanding planktonic cyanobacteria bloom formation (Oliver and Ganf, 2000; Briand et al., 2008, 2009) and to characterizing variables that regulate species diversity and toxin production (e.g., Dittmann et al., 1996, 1997; Sivonen and Jones, 1999; Tillett et al., 2000; Carmichael et al., 2001; Codd et al., 2005; Christiansen et al., 2003). In contrast, there have been few studies on toxic benthic freshwater cyanobacteria despite being linked to numerous animal deaths worldwide (Edwards et al., 1992; Gunn et al., 1992; Mez et al., 1997; Hamill, 2001; Gugger et al., 2005; Wood et al., 2007).

Compared to planktonic species, there are significant knowledge gaps regarding the distribution, toxin production and species composition of toxic benthic cyanobacterial communities (Gugger et al., 2005; Wood et al., 2007). Data on variables that regulate growth and toxin production are also sparse. Consequently information and guidance given on

assessment and management of toxic benthic cyanobacteria is still considered incomplete. This review summarizes the current knowledge on the ecology, toxic production, bloom formation, impact on other organisms and current monitoring and management strategies of freshwater toxic benthic cyanobacteria and identifies areas requiring further investigation. It focuses on benthic mats, which have been associated with poisoning events globally. It proposes future research priorities that will provide valuable information to assist water managers such as local authorities, drinking-water suppliers and government agencies. Although toxic benthic cyanobacteria have been identified in brackish (Herfindal et al., 2005) and marine environments (Paul 2008; Frazão et al., 2010; Méjean et al., 2010; Villeneuve et al., 2012) this review does not cover these organisms or habitats.

2. What cyanobacterial species are abundant in toxic freshwater biofilms dominated by these microorganisms?

Benthic cyanobacterial mats are found in a range of habitats including wetlands, lake littoral zones, wastewater ponds, hypersaline and geothermal ponds, streams and rivers (Congestri et al., 2006; Stal, 2012; Scott and Marcarelli, 2012). The development of cyanobacterial mats in freshwater systems can occur on a variety of substrates, from bedrock to sand, to artificial structures, such as wharf piers and drinking-water intakes, and associated with macrophytes (Mez et al., 1998; Komárek and Anagnostidis, 1999; Smith et al., 2012). Benthic mats usually range from a few mm to several cm thick. When environmental conditions are stable (e.g., constant water flow) this enables continual growth and succession and the mats can become much thicker. In an extreme example Dasey et al. (2005) investigated a toxic cyanobacterial mat that was reported to be over 70 cm in thickness.

Examples of freshwater benthic cyanobacterial mats are shown in Fig. 1. In these mats, the dominant species are usually filamentous Oscillatoriales and among them, the two genera, *Oscillatoria* and *Phormidium*, are often abundant (e.g., Mez et al., 1997, 1998; Gugger et al., 2005; Wood et al., 2007; Heath et al., 2011). In association with these dominant genera, other Oscillatoriales like *Lyngbya*, *Leptolyngbya*, *Microcoleus*, *Tychonema*, *Schizothrix* are commonly observed (Steppe et al., 1996; Mez et al., 1997, 1998; Hitzfeld et al., 2000; Aboal et al., 2005; Gugger et al., 2005). In addition to these typical benthic genera, a toxin-producing *Planktothrix* strain was recently isolated from a biofilm in a New Zealand river (Wood et al., 2010a). Prior to the identification of this benthic strain, *Planktothrix* was considered a planktonic genera. Chroococcales belonging to *Aphanothece* or *Synechococcus* genera have been also found in toxic freshwater benthic biofilms (Dasey et al., 2005; Krienitz et al., 2003; Mohamed, 2008) as well as filamentous nitrogen-fixing cyanobacteria from the group of Nostocales including *Anabaena* and *Scytonema* (Mohamed et al., 2006; Smith et al., 2011).

3. What is known about the ecology of toxic freshwater benthic cyanobacteria and their proliferation?

Benthic cyanobacterial mats are micro-scale ecosystems characterized by fluctuating physico-chemical gradients

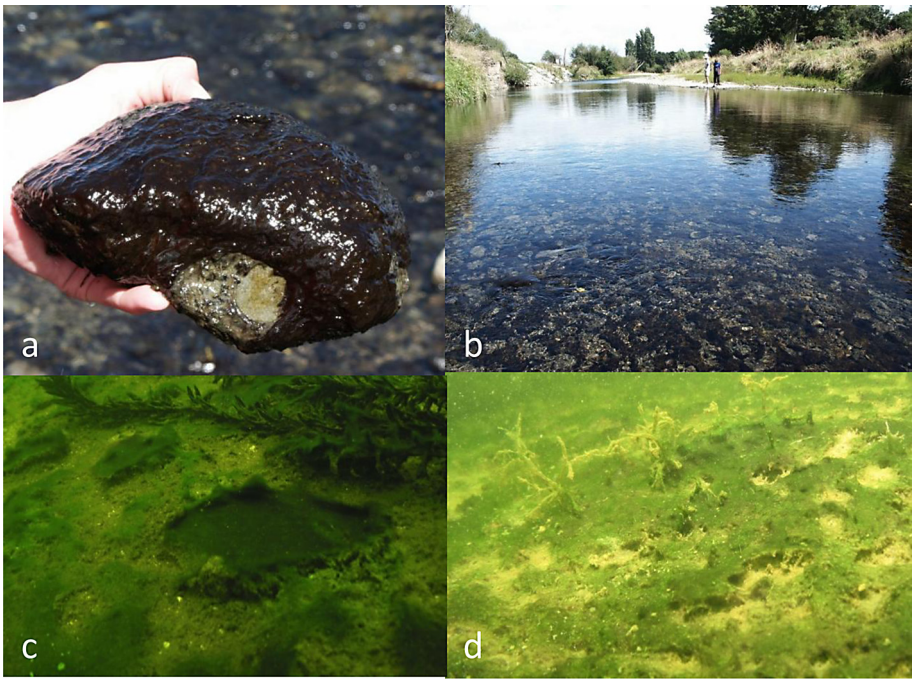

Fig. 1 – (a,b) *Phormidium* mats in the Waipoua River (North Island, New Zealand). Benthic mats in (c) Lake Rotoiti, (d) Lake Okareka (North Island, New Zealand). Photographers: a,b, Susie Wood (Cawthron, New Zealand); c,d, Rohan Wells (National Institute of Water and Atmospheric Research, New Zealand).

among which those of light, oxygen and sulphide are the most conspicuous (Stal, 1995, 2012). Although the mats are dominated by cyanobacteria they contain a diverse assemblage of other organisms including heterotrophic bacteria, photoautotrophic algae and fungi. Ecological interactions occur among all these organisms (Paerl et al., 2000; Scott and Marcarelli, 2012). Many of the filamentous species within cyanobacterial mats are motile, allowing them to move to optimal light and chemical conditions. Additionally, many of the mat-forming species also appear to be adapted to surviving in axenic conditions (see Stal, 2012, for a detailed description of these adaptations).

Benthic cyanobacterial mat formation tends to follow successional stages of development with initial colonization by heterotrophic bacteria and diatoms, followed by cyanobacteria (Stal, 1995, 2012). In some cyanobacterial species when environmental conditions are favorable and as biomass increases, oxygen bubbles, produced through photosynthesis, become trapped among the cyanobacteria and the extracellular polymeric substances they produce, causing the mat to become positively buoyant and eventually detach from the substrate. Cyanobacterial mats are found in a diverse range of habitats and the importance of the plethora of environmental variables that regulate growth varies among these environments and the requirements of the species i.e., some species can fix atmospheric nitrogen or may be more resistant to grazing. A detailed review of the relative importance of environmental controls on cyanobacteria in a variety of benthic habitats is provided in Scott and Marcarelli (2012). They suggest the most important factors controlling growth in benthic environments are physical disturbance (wet/dry cycles, wave action or shear stress), light, temperature, nutrients and grazing. Eutrophication linked to the increase of nitrogen (N) and phosphorus (P) fluxes in water systems is generally considered as one of the major factors that favor the development of planktonic cyanobacterial blooms (reviewed in Chorus and Bartram, 1999; Paerl et al., 2011; Conley et al., 2009; Stal, 2012). In contrast, extensive benthic mats have been found in a range of environments, including oligotrophic environments (e.g., Mez et al., 1998; Wood et al., 2012a; Smith et al., 2012; Scott and Marcarelli, 2012). A likely explanation is that the oligotrophic conditions usually result in high water clarity, allowing sufficient light to reach the benthos and enable cyanobacterial growth (Scott and Marcarelli, 2012). Wood et al. (2012a) studied benthic cyanobacterial communities in three geographically close New Zealand lakes. Although all three lakes had cyanobacterial mats, only mats from one lake (Lake Tikitapu) contained toxins. The species composition of the toxic mats was markedly different from the non-toxic ones, with Nostocales dominating in toxic samples. They suggested that, given the close geographic proximity of the study lakes, abiotic variables such as light attenuation, nutrient supply and water chemistry were most likely to be important in structuring the communities rather than differences in inoculation sources. Mez et al. (1998) investigated environmental parameters that favored the proliferation of toxin-producing benthic cyanobacteria in glacial lakes in Switzerland. They demonstrated that the benthic cyanobacteria were well adapted to the oligotrophic, highly variable environmental conditions, and the short growth period in the lakes.

Few studies have explored variables that regulate toxic benthic cyanobacteria in lentic systems and much of the research in rivers has occurred in New Zealand where toxic *Phormidium* developments are becoming increasingly problematic (Wood and Young, 2012). Wood and Young (2012) demonstrated on rivers that sites with the greatest *Phormidium* coverage had the highest total N:total P ratios (generally greater than 20:1). These sites all had low levels of dissolved reactive P (ca. $<0.01\ mg\ L^{-1}$) indicating that *Phormidium* may have adapted to out-compete other algae/cyanobacteria in these conditions. When New Zealand *Phormidium* cultures were screened for genes involved in nitrogen fixation none were detected (Heath and Wood, unpub. data) and these data correlate with the finding that increased dissolved inorganic N concentrations (ca. $>0.1\ mg\ L^{-1}$) in the water seem to be required before *Phormidium* will bloom. Wood and Young (2012) also suggest, although have yet to prove, that *Phormidium* may be able to obtain P from other sources. For example, a thin layer of fine sediment is commonly observed under well-developed mats and bacterial processes may increase the biological availability of phosphorus in this sediment. Processes such as these may explain how *Phormidium* blooms are able to reach such high biomass in relatively pristine New Zealand rivers. In contrast, benthic cyanobacterial blooms in Spanish rivers have been associated with low total N:total P ratios (Sabater et al., 2003; Vilalta et al., 2003) and oligotrophic to hypertrophic conditions (Loza et al., 2013; Perona and Mateo, 2006). Unfortunately, no toxin analysis was undertaken on these samples.

Heath et al. (2011) demonstrated that water temperature and river flow were the two main factors driving the presence and absence of *Phormidium* mats. They observed a dramatic increase in cyanobacterial development when the river flow decreased below half the yearly average and the temperature was above 14 °C. Subsequently, Heath et al. (unpublished data) showed decreases in summer low flows had only negligible changes to available *Phormidium* habitat using an in-stream habitat assessment and therefore concluded that the frequency of "flushing flows" and not flow that was critical in determining the presence of *Phormidium*. This result is similar to Sabater et al. (2003) who observed a decrease in benthic cyanobacteria in a Spanish river with low flows. This knowledge has led some regional water managers to use changes in river flow (e.g., two weeks without a river flow of three times the long-term median) as an early warning indicator of potential *Phormidium* proliferations (Wood et al., 2009).

An emerging issue that continues to escalate with increasing anthropogenic pressure is the influence of pesticides and herbicides on aquatic benthic microbial communities. The toxic action of these molecules can affect photosynthetic pathways of aquatic organisms, including cyanobacteria. Recent studies suggest that cyanobacteria (planktonic or benthic) could be more resistant to the presence of herbicide than other algae (see review in Villeneuve et al., 2011). Using an experimental approach, Lürling and Roessink (2006) showed that in the presence of the herbicide metribuzin, *Microcystis aeruginosa* out-competed the green algae *Scenedesmus obliquus*. Collectively, these studies demonstrate that the presence of herbicides in aquatic environments could alter benthic communities and promote the proliferation and dominance of potentially toxic cyanobacteria.

Table 1 – Toxic benthic cyanobacteria mats reported in literature and associated toxins. ATX, anatoxin-a; HTX, homoanatoxin-a; Cyn, cylindrospermopsin; Mcy, microcystin; Nod, nodularin; STX, saxitoxin; GTX, gonyotoxin; LTX, lyngbyatoxin; ApTX, aplysiatoxin; ApopTX, apoptogen toxin. nd, non determined; ii, insufficient information.

Benthic cyanobacteria observed	Country	Mortality	Screened Toxins	Observed Toxins or Toxicity	Reference
Rivers					
Lyngbya wollei	Australia	ii	Cyn/ATX/STX/ LTX/Mcy/ApTX	Cyn	Seifert et al., 2007
Lyngbya wollei	Canada	ii	Multi-toxin method (ATX/Nod/Mcy/ STX/Cyn)	STX analogs	Lajeunesse et al., 2012
Anabaena subcylindrica, *A. variabilis*, *Calothrix fusca*, *C. parietina*, *Lyngbya epiphytica*, *Nostoc carelum*, *N. muscorum*, *N. spongiforme*, *Oscillatoria angustissima*, *O. formosa*, *O. granulata*, *O. limnetica*, *Pseudanabaena catenata*, *Plectonema buryanum*, *Phormidium corium*, *P. tenue*, *Rivularia bullata*, *Scytonema mirabile*, *S. myochrous*	Egypt	ii	Mcy	Mcy	Mohamed et al., 2006
Phormidium favosum	France	Dogs	Mcy/STX/ATX	ATX	Gugger et al., 2005
Phormidium sp.	France	Dogs	ATX/HTX	ATX/HTX	Cadel-Six et al., 2007
Nostoc muscorum, *Oscillatoria*, *Pseudanabaena*	Morocco	ii	Mcy	Mcy	Oudra et al., 2009
Planktothrix	New Zealand	Dog	ATX/HTX/Mcy/ Cyn/Nod/STX	Mcy	Wood et al., 2010a
Phormidium or Oscillatoria sp.	New Zealand	ii	ATX/Mcy/STX	Mcy	Wood et al., 2006
Phormidium autumnale	New Zealand	Dogs	ATX/HTX/Cyn/ Mcy/STX	ATX/HTX	Wood et al., 2007; Heath et al., 2010, 2011
Oscillatoria like genus	New Zealand	Dogs	ATX/Mcy/PSPs	ATX	Hamill, 2001
Schizothrix lateritia, *Phormidium fragile*, *P. foveolarum*, *Pleurocapsa minor*, *Calothrix parietina*, *Tolypothrix distorta*	Spain	ii	Microtox method	Yes (nd)	Aboal et al., 2002
Rivularia psp., *Schizothrix fasciculata*, *Tolypothrix distorta*, *Phormidium splendidum*	Spain	ii	Mcy	Mcy	Aboal et al., 2005
Oscillatoriaceae?	USA, Ontario	Dogs	ATX	ATX	Puschner et al., 2008
Lakes, Reservoirs, Ponds					
Phormidium sp., *Leptolyngbya frigida*, *Nostoc* sp., *Nodularia* sp.	Antarctica	ii	Mcy/Nod	Mcy/Nod	Hitzfeld et al., 2000
Phormidium, *Oscillatoria*, *Lyngbya*, *Nostoc*, *Nodularia*, *Anabaena*	Antarctica	ii	Mcy	Mcy	Jungblut et al., 2006
Nostoc sp.	Antarctica	ii	Mcy	Mcy	Wood et al., 2008
Mixed species mats	Antarctica/ Arctic	ii	Mcy	Mcy	Kleinteich et al., 2012
Phormidium formosum, *P. amoenum*	Australia	Mice	Mouse bioassay	nd	Baker et al., 2001
Aphanothece, *Chroococcales*	Australia	ii	Mcy	Mcy	Dasey et al., 2005
Anabaena sp. (+*Nostoc* sp., *Calothrix* sp.)	Baltic Sea	ii	Mcy/Nod/ ApopTX	ApopTX	Herfindal et al., 2005
Fischerella	Brazil	ii	Mcy	Mcy	Fiore et al., 2009
Oscillatoria	Ireland	Dogs	ATX	ATX	James et al., 1997
Phormidium terebriformis, *Oscillatoria willei*, *Spirulina subsalsa*, *Synechococcus bigranulatus*	Kenya	Lesser flamingos	Mcy/ATX	Mcy/ATX	Krienitz et al., 2003
Phormidium	Netherlands	Dogs	ATX/HTX/Cyn/ STX/GTX	ATX/HTX	Faassen et al., 2012
Nostoc	New Zealand	ii	ATX/Mcy/STX	Mcy	Wood et al., 2006
Scytonema sp.	New Zealand	ii	STX/GTX	STX	Smith et al., 2011
Scytonema sp.	New Zealand	ii	STX/GTX	STX/GTX	Smith et al., 2012

Table 1 – (*continued*)

Benthic cyanobacteria observed	Country	Mortality	Screened Toxins	Observed Toxins or Toxicity	Reference
Leptolyngbya sp., *Microcoleus paludosus*, *Cyanothece* sp., *Phormidium murrayi*, *Nostoc* sp., *Calothrix* sp.	New Zealand	ii	ATX/HTX/ Mcy/Nod	Nod	Wood et al., 2012b
Microcoleus vaginatus, *Synechococcus lividus*, (+*Chroococcus minutus*, *Fischerella thermalis*, *Oscillatoria limosa*, *Phormidium tenue*, *Plectonema boryanum*, *P. mucicola*, *Schizothrix calcicola*, *Calothrix thermalis*)	Saudi Arabia	ii	Mcy	Mcy	Mohamed, 2008
Oscillatoria	Scotland	Dogs	ATX	ATX	Edwards et al., 1992
Phormidium sp., *Oscillatoria* sp., *Lyngbya* sp., *Tolypothrix distorta*, *Calothrix parietina*, *Rivularia biasolettiana*	Spain	ii	Mcy	Mcy	Aboal and Puig, 2005
Phormidium sp., *P. tenue*, *Geitlerinema* sp., *Leptolyngbya fragilis*, *Oscillatoria* sp., *O. limosa*, *Pseudanabaena sp.*, *P. catenata*	Spain	ii	Mcy	Mcy	Hurtado et al., 2008
Oscillatoria limosa, *Phormidium konstantinosum*	Switzerland	Cattle	Mcy	Mcy	Mez et al., 1997,1998
Lyngbya wollei	USA, Tennessee	ii	STX/GTX	STX	Carmichael et al., 1997
Oscillatoriales	USA, South California	ii	Mcy	Mcy	Izaguirre et al., 2007

Table 2 – Benthic cyanobacterial species in which toxin production has been confirmed through uni-cyanobacterial strain isolation, culturing and toxin testing. NA, not available.

Toxin	Species (culture code)	Reference
Microcystin	*Planktothrix* sp. (CYN60,61)	Wood et al., 2010a
	Anabaena subcylindrica (NA)	Mohamed et al., 2006
	Anabaena variables (NA)	Mohamed et al., 2006
	Nostoc spongiforme (NA)	Mohamed et al., 2006
	Plectonema boryanum (NA)	Mohamed et al., 2006
	Phormidium corium (NA)	Mohamed et al., 2006
	Rivularia biasolettiana (NA)	Aboal et al., 2005
	Rivularia haematites (NA)	Aboal et al., 2005
	Phormidium splendidum (NA)	Aboal et al., 2005
	Tolypothrix distorta (NA)	Aboal et al., 2005
	Phormidium sp. (11 strains)	Izaguirre et al., 2007
Cylindrospermopsin	*Lyngbya wollei* (NA)	Seifert et al., 2007
	Oscillatoria sp. (PCC 6506)[a]	Mazmouz et al., 2010
Saxitoxins	*Lyngbya wollei* (NA)	Yin et al., 1997
	Scytonema cf. *crispum* (UNFS10)	Smith et al., 2011, 2012
Homo/anatoxin-a	*Oscillatoria* sp. (PCC6506,6407, 6412, 9029, 9240)[a]	Aráoz et al., 2005
	O. formosa (PCC10111)[a]	
	Oscillatoria sp. (PCC 10601,10702,10608)	Cadel-Six et al., 2007
	Oscillatoria sp. (NA)	Edwards et al., 1992
	Phormidium autumnale (many strains)	Wood et al., 2012b
		Heath et al., 2010
	Phormidium favosum (PMC240.04)	Gugger et al., 2005
Unknown	*Limnothrix* (AC0243)	Humpage et al., 2012a

a The habitat (i.e., benthic or planktonic) from which these strains were isolated is unknown, but based on their growth characteristics they are likely to be benthic species.

4. Which toxins are produced by freshwater benthic cyanobacteria and which species produce these toxins?

Most of the known cyanotoxins have been identified from benthic cyanobacteria including the hepatotoxic microcystins, nodularins and cylindrospermopsins, and the neurotoxic saxitoxins, anatoxin-a and homoanatoxin-a (e.g., Gugger et al., 2005; Izaguirre et al., 2007; Wood et al., 2007, 2010a,b; Seifert et al., 2007; Smith et al., 2011; Table 1 and 2). Toxicity that could not be attributed to any of the known cyanotoxins has also been identified. For example, extracts from *Phormidium* aff. *formosum* in Upper Paskeville Reservoir (Australia) were lethal by intraperitoneal injection into mice (400 mg kg^{-1}; Baker et al., 2001) and authors observed that clinical symptoms of toxicity on mice and chemical characteristics were different from those of the known major cyanotoxins. Likewise, Humpage et al. (2012a) results suggested that *Limnothrix* AC0243 (benthic for at least part of its life cycle), which is acutely toxic to mice, is able to produce a novel toxin named "Limnothrixin". Based on published research it appears that microcystins and homo/anatoxin-a are the most commonly detected cyanotoxins among benthic cyanobacteria (Tables 1 and 2). This may be an artifact of sampling biases in toxin analysis. Among the 37 studies listed in Table 1, samples were screened 26 and 16 times for microcystins and homo/anatoxin-a, respectively. Screening for saxitoxins was performed 13 times and only 7 and 5 times for cylindrospermopsin and nodularin respectively. These differences can partly be attributed to the fact that routine tests for microcystins are more readily available. Additionally, many studies on benthic cyanobacteria have been reactive (i.e., undertaken in response to animal deaths) as opposed to systematic surveys, and therefore the testing undertaken is based on the symptoms of poisoning or results of autopsies and samples are not all tested for all toxins. Most benthic samples/mats generally consist of multiple species, therefore isolation of single filaments, culturing and toxin testing of pure uni-cyanobacterial strains is required to identify with certainty the toxin-producing species. Only a limited number of studies have done this (Table 2). Collectively these studies confirm the production of toxins by species from the orders Oscillatoriales and Nostocales. To our knowledge, uni-cyanobacterial cultures of freshwater benthic toxin-producing Chroococcales and Stigonematales have not been established.

Multiple benthic species have been cultured and found to produce microcystins (Table 2). Mohamed et al. (2006) and Aboal et al. (2005) identified a range of microcystin-producing benthic species, however, these were relatively minor parts of the benthos. Wood et al. (2010a) investigated a dog death linked to ingestion of a benthic "algal" mat at the Waitaki River (New Zealand) and using molecular techniques identified it as *Planktothrix*. Toxin testing identified the presence of a range of microcystins variants many of which were similar to those produced by planktonic *Planktothrix* species (Wood et al., 2010a). Izaguirre et al. (2007) isolated 17 benthic Oscillatoriales strains from drinking-water reservoirs in southern California (USA) and 11 of these strains were found to contain high concentrations of microcystins. Sequencing of the partial 16S rRNA gene showed the producers had the highest similarity to an unidentified Oscillatoriales and *Phormidium*.

One of the earliest toxic benthic cyanobacteria identified was the saxitoxin-producing *Lyngbya wollei* (Carmichael et al., 1997; Yin et al., 1997). This species has been shown to produce various saxitoxins, including decarbamoyl saxitoxins (dcSTX) and decarbamoyl gonyautoxins (dcGTX2, dcGTX3), as well as six novel analogs (Onodera et al., 1997). Blooms of saxitoxin-producing *L. wollei* have now been reported from other locations including Florida springs (Foss et al., 2012) and the St. Lawrence River (Canada; Lajeunesse et al., 2012). Additionally, this species also produces cylindrospermopsin and its analog, deoxy-cylindrospermopsin (Seifert et al., 2007) and the dermatoxin lyngbyatoxin which can cause skin irritation, rashes and gastrointestinal problems (Paerl and Otten, 2013). Recently, samples collected from the metaphyton of a drinking-water reservoir and a small eutrophic lake in New Zealand returned positive results for saxitoxin when screened using a Jellett PSP Rapid Test Kit. This prompted the isolation of a culture of *Scytonema* cf. *crispum* and saxitoxin production was confirmed (Smith et al., 2011). A survey of other lakes and further cultures demonstrated this species also produced the variants GTX 1–5, neosaxitoxin, dcSTX and dcGTX2/3 (Smith et al., 2012).

Anatoxin-a and/or homoanatoxin-a production has been associated with benthic cyanobacteria since the first reported dog deaths from consumption of benthic mats in the early 1990s (Edwards et al., 1992). A culture of *Oscillatoria* sp. from this early study tested positive for anatoxin-a, although no rigorous morphological or molecular analyses were undertaken. Using morphological and molecular characterization of cultures subsequent studies have identified new benthic homo/anatoxin-a producers from the Oscillatoriales including *Phormidium favosum* (Gugger et al., 2005), *Phormidium autumnale* (Heath et al., 2010) and a variety of *Oscillatoria* sp. including *Oscillatoria formosa* (Aráoz et al., 2005; Cadel-Six et al., 2007). The original source locations (albeit they were all freshwater) and habitat of many of the strains given in Aráoz et al. (2005) is unclear, however, based on their growth characteristics, they are presumed to be benthic. Mazmouz et al. (2010) recently demonstrated that in addition to anatoxin-a and homoanatoxin-a, *Oscillatoria* sp. (PCC6506) also produces cylindrospermopsin.

In addition to culture-based studies, research on environmental benthic samples indicates the presence of many additional toxic benthic cyanobacteria species. Following the death of 100 cattle in alpine sites in south-eastern Switzerland, Mez et al. (1997) discovered that cyanobacteria dominated mats on the surface of sediments and on submerged rocks contained high concentrations of microcystins. The mat samples yielding the highest microcystin contents consisted mainly of *Oscillatoria limosa* and *Phormidium konstantinosum*, however, it was not possible to identify the microcystin-producing species among strains isolated from these mats. Additionally, the symptoms suggested neurotoxins were also present, however, no tests for these compounds were undertaken. In New South Wales (Australia), large benthic aggregations (referred to as cyanobacterial ooze) were observed in sheltered embayment in Myall Lake (Dasey et al., 2005). The cyanobacterial ooze comprised a mixture of species from the order Chroococcales and was dominated by two *Aphanothece* morphotypes. Molecular, immunological and

chemical analysis of cyanobacterial ooze suggested that cyanobacteria within it had the potential to produce microcystin or closely related products but attempts to definitively identify the producer(s) were not undertaken. Benthic *Nostoc* spp. have also been demonstrated to be likely microcystin producers in samples from a New Zealand lake (Wood et al., 2006) and in a massive growth of the benthic mat-forming *Nostoc muscorum* in Oukaïmeden River located at 2600 m altitude in the High-Atlas mountains of Marrakech (Oudra et al., 2009). Following the mysterious deaths of the Lesser Flamingos in the hot springs at the shore of Lake Bogoria (Kenya) cyanobacterial mats were investigated and microcystins and anatoxin-a were detected (Krienitz et al., 2003). These mats were dominated by *Phormidium terebriformis*, *Oscillatoria willei*, *Spirulina subsalsa* and *Synechococcus bigranulatus*, although the species responsible for toxin production was not definitively confirmed. Hurtado et al. (2008) found a diverse assemblage of cyanobacteria in their investigation of sediments from the La Contraparada water treatment plant in the city of Murcia (Spain) where microcystins were discovered. The samples were dominated by Oscillatoriales, particularly *Phormidium tenue*, *Pseudanabaena catenata* and *Geitlerinema* sp. Microcystins and nodularins have also been identified from the often expansive benthic mats that dominate in Polar freshwater ecosystems (Hitzfeld et al., 2000; Jungblut et al., 2006; Wood et al., 2008; Kleinteich et al., 2012). Hitzfeld et al. (2000), Jungblut et al. (2006) and Kleinteich et al. (2012) did not identify the species responsible for microcystin production, whereas Wood et al. (2006, 2008) demonstrated a *Nostoc* species was the microcystin producer in some of their samples. Hitzfeld et al. (2000) also suggested that the presence of nodularins was likely to be due to *Nodularia* sp., which were abundant in the nodularin positive samples. Recently, nodularins were detected by Wood et al. (2012a) in benthic mats collected from a small New Zealand freshwater lake.

Multiple studies have identified homo/anatoxin-a in benthic mats dominated by *Oscillatoria* sp. or *Phormidium* (Table 2) and these have commonly been associated with dog deaths. In some cases the toxin-producing species have been cultured and identified (Table 2). However, culturing and species level identification is not always undertaken (e.g., Puschner et al., 2008; Faassen et al., 2012) and it is likely that more homo/anatoxin-a producing *Oscillatoria* or *Phormidium* will be identified as research continues.

5. What is known about spatial and temporal variability and regulation of toxin production by benthic cyanobacteria?

Currently, the underlying reasons for toxin production by benthic, as well as planktonic, cyanobacteria remain unclear. There have been a number of studies of planktonic cyanobacteria, which have explored; (i) the regulation of toxin production (reviewed in Chorus and Bartram, 1999; Neilan et al., 2013); (ii) variables that regulate the proportions of microcystin and non-microcystin-producing cells in planktonic blooms (Vézie et al., 1998; Vaitomaa et al., 2003; Kurmayer et al., 2004; Briand et al., 2008, 2009; Sabart et al., 2009, 2010); and (iii) the ecological function of the toxins (e.g., Schatz et al., 2007; Gan et al., 2011; Zilliges et al., 2011). In contrast only a few studies have investigated variability and factors that may regulate toxin production by benthic species either in nature or in culture.

Analysis of *Phormidium* dominated mats in New Zealand rivers have revealed a wide variability in homo/anatoxin-a concentrations (Wood et al., 2007; Heath et al., 2010). Wood et al. (2010b) sampled seven rivers and showed spatial variability in homo/anatoxin-a concentration within 10×10 m grids. Of the seven sites sampled, there was only one site where all 15 samples collected contained detectable levels of homo/anatoxin-a. At three sites, both toxic and non-toxic samples co-occurred and in some instance these mats were less than 1 m apart. They speculated that the most likely reason for this variability was that toxic and non-toxic genotypes co-occurred. Using culture-based studies Wood et al. (2012c) conclusively proved this and also found that homo/anatoxin-a concentration varied 100 fold among strains isolated from the same mat. This demonstrates that in addition to the relative abundance of toxic genotypes, variations in toxin-producing capability can influence toxin concentrations in *Phormidium* mats.

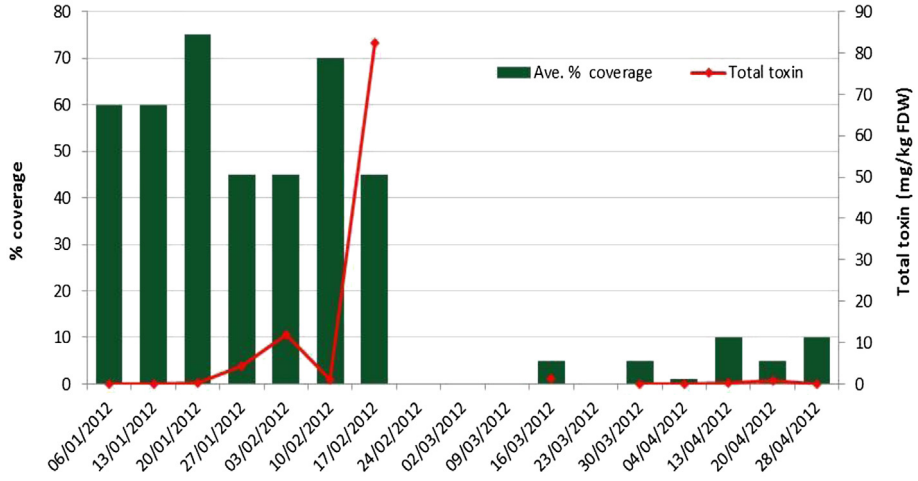

Fig. 2 – Weekly total anatoxin concentrations (presented as mg/kg of freeze dried material) and *Phormidium* mat cover in the Makakahi River (North Island, New Zealand). Figure modified from Wood and Young (2012).

Heath et al. (2011) undertook a 12 months study at eight sites on two rivers and observed that homo/anatoxin-a occurrence was restricted to water temperatures above 13.4 °C and suggested that the toxin-producing strains may have "outcompeted" non-toxic *Phormidium* strains under these conditions. Multiple studies in New Zealand rivers (Heath and Wood, 2010; Heath et al., 2011) showed there is no correlation between toxin concentrations and percentage *Phormidium* coverage (Fig. 2). In all of these studies marked changes in toxin concentrations were observed within short periods (i.e., one week; Fig. 2). The most likely reason, is that toxic and non-toxic genotypes co-occur in the *Phormidium* mats and that the relative amount of each genotype can vary rapidly and this affects the toxin concentration in each sample.

Laboratory studies on cyanobacteria have shown that environmental factors and growth phase can induce changes toxin concentration, but usually by a factor of no more than three or four (Sivonen and Jones, 1999). The large majority of these studies have focused on planktonic species. Tracking growth of benthic mat-forming cyanobacteria to generate standard growth curves and determine toxin production is challenging. The methods used for monitoring planktonic cyanobacterial growth i.e., mixing the culture and sampling a known volume of media, cannot be undertaken on cultures of mat-forming benthic species without disturbing growth patterns. Additionally, cell counts of sub-samples are meaningless for estimating the entire biomass as growth is not homogenous within culture vessels.

Smith (2012) developed a method to investigate cyanotoxin quota in liquid cultures of benthic mat-forming cyanobacteria through their growth cycle. The method involves setting up multiple culture vessels, inoculating each with a known mass and harvesting triplicate vessels at each time point. Smith (2012) used this method to study the effect of iron (40–4000 $\mu g\,L^{-1}$) and copper (2.5–250 $\mu g\,L^{-1}$) on growth and anatoxin – a quota in *P. autumnale* (CYN50). Anatoxin-a concentrations varied throughout the growth curve with maximum quota correlated with *P. autumnale* spreading completely across the substrate. Growth rates were significantly affected by copper and iron concentrations, however, no statistically significant difference in anatoxin-a quota was observed.

Yin et al. (1997) investigated the influence of light, temperature and nutrient concentrations on the growth and saxitoxin production in *L. wollei*. Optimum production of both biomass and toxins occurred at 26 °C and a light intensity of 11 or 22 $\mu mol\,m^{-2}\,s^{-1}$. Compared to control levels, lower PO_4-P and NO_3-N and increased calcium produced the greatest biomass and saxitoxin concentrations.

6. What are the negative impacts of the developments of benthic cyanobacteria biofilms?

6.1. Impacts in aquatic ecosystems and organisms

The negative impacts cyanobacterial blooms can have on ecosystem functioning are well known (e.g., Havens, 2008). For example, it has been shown that dense planktonic cyanobacterial blooms can result in deoxygenation of the bottom waters underneath blooms (Robarts et al., 2005), however, little data are available for benthic cyanobacteria.

There have been few studies that have investigated how toxic benthic cyanobacterial mats impact aquatic ecosystem health or biodiversity. Studies on other mat-forming periphyton have shown significant effects on abundances and composition of aquatic species, for example, when thin diatom mats are present caddisflies and mayflies commonly dominate the benthos, but as filamentous green take over elmid beetles, snails, midges, and ostracods dominated. (e.g., Larned et al., 2007; Suren et al., 2003). A recent study in New Zealand (Wood and Heath, unpub. data) within four rivers found a higher density of macroinvertebrates in samples collected from toxic benthic mats compared to those collected from areas without mats. Diptera were particularly prominent.

Aboal et al. (2000, 2002) found that, cyanobacterial mats were usually associated with macroinvertebrate communities with low diversity in Spanish streams. They suggested that the heavily encrusted algal community were not palatable or could not be processed by herbivores. Their data also indicated that cyanobacterial toxicity contributed to the differences in diversity and density, especially when the water was cold.

Cyanotoxins produced by freshwater planktonic species have been shown to accumulate in many aquatic organisms (Ibelings and Havens, 2008). The majority of these studies have focused on microcystins. Because of the high instability of anatoxin-a (Stevens and Krieger, 1991) and the lack of human casualties, it is considered of less concern than other cyanotoxins. However, given the prevalence of homo/anatoxin-a in benthic cyanobacteria, further studies on this toxin are required. In addition to bioaccumulating in organisms, cyanotoxins can have negative effects on aquatic organisms ranging from severe liver damage, to oxidative stress, reduced growth and reproductive success (Malbrouck and Kestemont, 2006; Ibelings and Havens, 2008). Aboal et al. (2002) undertook palatability/toxicity tests on a range of macroinvertebrates and demonstrated evidence for hepatotoxic and neurotoxic effects on diverse taxonomic groups including Ephemeroptera, Plecoptera and Trichoptera. Little data is provided on how these experiments were undertaken or the types of responses observed. No testing was undertaken to determine if the toxin bio-accumulated within the organisms.

Benthic cyanobacteria that dominated mats in a small New Zealand lake were found to contain nodularin (Wood et al., 2012b). Using an in-lake feeding experiment with isotopically labeled ^{13}C benthic mats Wood et al. (2012b) confirmed the mats were consumed by freshwater crayfish. The hepatopancreas of all crayfish tested positive for nodularin and low concentrations were detected in the tail tissue of two individuals. Wood et al. (2012b) suggest that benthic mats may need to be considered as a potential source of cyanotoxins in future freshwater food-web studies.

6.2. Impacts to terrestrial organisms

Terrestrial mammals seem less resistant to cyanotoxins than aquatic organisms. For example, the intraperitoneal injection LD_{50} for microcystin range from 50 to 300 μg microcystin-

LR kg^{-1} for mammals (depending on the species tested; Sivonen and Jones, 1999) whereas, the LD_{50} for microcystin measured on carp and perch are 500 and 1500 μg microcystin-LR kg^{-1}, respectively (Rabergh et al., 1991; Ibelings et al., 2005). Thus, it is not surprising that the impacts on terrestrial organisms are more widely reported than for aquatic species. Cyanotoxins produced by toxic benthic cyanobacteria growing in ponds, lakes and rivers have been responsible for the death of dogs, flamingos and cattle (Table 1). These animal toxicosis episodes have been caused by homo/anatoxin-a and microcystins. To date, there are no documented cases of deaths or poisonings related to saxitoxin, nodularin or cylindrospermopsin-producing benthic cyanobacteria. Curiously, dogs appeared to be the animal most affected by the toxins produced by benthic cyanobacteria (Table 1). It is not clear if dogs are more sensitive to these compounds compared to other animals, or if they are attracted to the mats by odor and taste compounds such as geosmin or 2-methylisoborneol (Izaguirre and Taylor, 2007; Jähnichen et al., 2011). Alternatively, they may be exposed to more benthic mat material as they tend to drink from the shallow littoral areas of rivers and lakes where detached mats can accumulate. Following the death of two dogs on the Loue River (France), Gugger et al. (2005) estimated that consumption of 6 and 60 g of toxic anatoxin-a-containing mats would have been sufficient to kill the 2.5 and 25 kg dogs, investigated in their study.

There have been no reported human fatalities from toxins produced by benthic mats. In New Zealand, anecdotal reports of human illnesses associated with recreational activities in rivers containing *Phormidium* mats have been observed. In one instance, a young child was taken to hospital with severe stomach pains after swimming in a river and *Phormidium* mats collected from the site were later tested and found to contain high levels of homo/anatoxin-a (Wood and Young, 2012). However, there was no conclusive evidence to prove that the *Phormidium* had caused the observed symptoms.

6.3. *Impacts in drinking-water supplies and risks associated*

Until recently, little was known about the release of cyanotoxins from benthic cyanobacteria into the water column (i.e., the extracellular component). This information is vital to the management of toxic benthic cyanobacteria particularly for drinking-water supplies. Toxic benthic cyanobacteria growing on the bottom substrate of a freshwater body, while healthy, are a limited health risk as the majority of cyanotoxins are likely to be intracellular and thus not being released into the water. Under certain environmental conditions these mats may detach from the substrate, potentially releasing massive pulses of cyanotoxins into the water as the mats degrade. Currently sampling techniques e.g., taking grab samples of water near the intake, would miss these pulses and thus significantly underestimate the risk posed by benthic cyanobacteria.

Wood et al. (2011) recently used an *in situ* technique known as solid phase adsorption toxin tracking technology (SPATT) and developed a method specifically for detecting homo/anatoxin-a in rivers over an extended period. They undertook a field trial in a river experiencing a *Phormidium* bloom and detected homo/anatoxin-a in all SPATT bags demonstrating that cyanotoxins are released into the water from benthic cyanobacterial mats. During the experiment total homo/anatoxin-a concentrations of 21.7 μg L^{-1} were also detected in one 'grab' sample taken directly from the river. The toxin concentrations in this samples would have exceeded the provisional maximum acceptable values in drinking water set by the New Zealand Ministry of Health (6 μg L^{-1} for anatoxin-a and 2 μg L^{-1} for homoanatoxin-a; Ministry of Health of New Zealand, 2005).

During a year-long study of benthic cyanobacteria in Spanish streams, Aboal et al. (2002) detected extracellular microcystins in all samples. The maximal values were relatively low (0.7 μg L^{-1}). They occurred at the end of summer and were correlated with the highest intracellular microcystin concentrations. Mez et al. (1998) tracked extracellular microcystins in two lakes with benthic cyanobacterial mats and detected concentrations up to 2 μg L^{-1} (total microcystin). By using SPATT, Wood et al. (2012b) demonstrated that extracellular nodularins sourced from benthic cyanobacterial mats were present in a New Zealand lake. These studies demonstrates that release of toxins from benthic cyanobacteria needs to be considered and that concentrations that may be hazardous to humans and animals especially if the water is used for drinking. These data in concert with the identification of toxic benthic cyanobacteria in a drinking-water sources in other studies (Izaguirre et al., 2007; Mohamed et al., 2006; Smith et al., 2012) highlight the largely unconsidered risk they may pose to human health.

In addition to the potential problems posed by toxins synthesized by benthic cyanobacteria in drinking-water supplies, taste and odor compounds can also be problematic (Izaguirre and Taylor, 2007; Jähnichen et al., 2011). The most commonly reported compounds are geosmin and 2-methylisoborneol (Watson, 2003; Smith et al., 2008; Jähnichen et al., 2011). Not all cyanobacteria produce these compounds and it is not correlated with toxin production. In Llobregat River (Spain), the production of geosmin was maximal in free-floating mats (Sabater et al., 2003). Sabater et al. (2003) suggested that the meiofauna, particularly well developed in free-floating mats, could enhance their degradation and thus the release and diffusion of these compounds into the water.

7. Guidelines for monitoring, managing and determining the health risks of benthic cyanobacteria

Guidelines for monitoring and management of planktonic cyanobacteria in recreational use and drinking-water supplies are well developed (e.g., Australian National Health and Medical Research Council, 2008; Chorus, 2012), however, there is currently little information available on how to sample, monitor and manage benthic cyanobacteria. Most of the sampling (e.g., 'grab' or 'depth integrated' samples) and analysis methods (e.g., cell counts) do not provide meaningful data when trying to determine the extent and risks associated with benthic cyanobacteria.

The health risks posed by benthic cyanobacteria are becoming more apparent and several countries recognize this

Table 3 – Alert-level framework for benthic cyanobacteria as given in the New Zealand guidelines for managing cyanobacteria in recreational fresh waters (Wood et al., 2009).

Alert level	Actions
Surveillance (green mode) Up to 20% coverage of potentially toxigenic cyanobacteria attached to substrate.	• Undertake fortnightly surveys between spring and autumn at representative locations in the water body where known mat proliferations occur and where there is recreational use.
Alert (amber mode) 20–50% coverage of potentially toxigenic cyanobacteria attached to substrate.	• Notify the public health unit. • Increase sampling to weekly. • Recommend erecting an information sign that provides the public with information on the appearance of mats and the potential risks. • Consider increasing the number of survey sites to enable risks to recreational users to be more accurately assessed. • If toxigenic cyanobacteria dominate the samples, testing for cyanotoxins is advised. If cyanotoxins are detected in mats or water samples, consult the testing laboratory to determine if levels are hazardous.
Action (red mode) *Situation 1:* Greater than 50% coverage of potentially toxigenic cyanobacteria (see Table 1) attached to substrate; or *Situation 2:* up to 50% where potentially toxigenic cyanobacteria are visibly detaching from the substrate, accumulating as scums along the river's edge or becoming exposed on the river's edge as the river level drops.	• Immediately notify the public health unit. • If potentially toxic taxa are present then consider testing samples for cyanotoxins. • Notify the public of the potential risk to health.

in national documents. A Scottish Executive Health Department (2002) document on cyanobacteria acknowledges the significance of benthic cyanobacteria and states that "*swimmers may be in contact with benthic cyanobacteria after a storm breaks off clumps of filaments, or cyanobacterial mats naturally detach from the sediment and are accumulated on shore lines*", however, no guidance on how to assess or manage benthic cyanobacteria is provided. Recent dog deaths in The Netherlands caused by toxic benthic *Phormidium* (Faassen et al., 2012) have raised awareness of benthic cyanobacteria in this country. They now acknowledge that the "*assumption that blooms, scums and benthic mats of cyanobacteria other than the listed five genera are harmless is unjustified and seems in conflict with the basic principles of the Bathing Water Directive*" (Chorus, 2012).

To our knowledge, only two countries have developed guidelines for monitoring and managing the risks associated with benthic cyanobacteria. New Zealand has introduced national guidelines for the management of risks linked to cyanobacteria in recreational waters with a section devoted to benthic cyanobacteria in rivers (Wood et al., 2009). They acknowledged that the threshold values given in the guidelines are based on preliminary research, and it is anticipated that these will require further refining as knowledge and monitoring tools improve. The benthic specific section provides guidance on identifying high-risk water bodies/sites, how to collect representative samples and undertake site surveys. The alert-level framework for benthic cyanobacteria from this document is given in Table 3. The values are based on the percentage coverage determined after undertaking a site survey. This involves assessing cyanobacterial coverage at five points on four transects which are perpendicular to the riverside. Under certain environmental conditions, or as benthic mats become thicker (and bubbles of oxygen gas become entrapped within them), mats will detach from the substrate and may accumulate along river edges. During these events the risk to human and animal health is increased due to the accessibility of the cyanobacterial mats to river users. The highest risks to water users are likely to be via ingestion of water containing detached mats and/or direct contact with these cyanobacterial mats. The risk associated with both types of contact is likely to rise as the abundance and/or number of detachment events increases. Thus the guidelines use cyanobacterial abundance and the occurrence of mats visibly detaching from the substrate to determine the alert-level status. No toxin concentrations are given in the guidelines as these can be very variable both spatially and temporally (Wood et al., 2010b; Heath et al., 2011; Wood and Young, 2012). However, toxin testing is encouraged to help make informed risk assessments.

Recent research in the San Juan River in Cuba has identified mat-forming benthic *Phormidium* and *Oscillatoria* (Chorus, 2012). This has prompted Cuba to develop benthic cyanobacterial guidelines initially for water bodies used for recreational purposes. To date they have only been applied at local scale and have not been adopted nationally. The decision chart for benthic cyanobacteria is very similar to New Zealand, with the key difference being that the "Surveillance – Alert Level" is "*Up to 40% coverage of cyanobacteria attached to substrate*" and "Alert – Alert Level" is "*20% coverage of potentially toxigenic cyanobacteria attached to substrate*" (Chorus, 2012).

8. Conclusion: what are the knowledge gaps and research needs?

During the last decade numerous animal poisonings related to toxic benthic cyanobacteria have been reported. There is now

increasing concern regarding the health risk benthic cyanobacteria pose to human health particularly when affected water bodies are used as a drinking-water source. In most countries observations of toxic benthic cyanobacteria are fragmented, and have generally been descriptive in response to an animal toxicosis event. As climatic conditions change and anthropogenic pressures on waterways intensify, it seems likely that the prevalence of toxic benthic cyanobacteria will increase. Current knowledge gaps limit the ability to provide comprehensive advice to water managers, particularly information on how to sample, monitor and manage these events. We suggest that further studies are needed and that these should include:

8.1. *New tools for monitoring of benthic cyanobacteria and their toxins*

Monitoring of benthic cyanobacteria needs to be improved to enable faster and more efficient sampling and analysis. The use of fluorometric probes for studying planktonic cyanobacteria has enabled rapid and cost-effective acquisition of data on cyanobacterial biomass (Leboulanger et al., 2002). Similar tools have recently been adapted for benthic environments, however, further evaluation is required before they can be incorporated into monitoring programs. Using this type of probe, initial data generated in our laboratories (Echenique-Subiabre et al., unpublished data) suggests the technique is promising, at least for studying the initial stages of biofilm development.

Tools based on the use of aerial sensors could be useful for obtaining a rapid assessment of benthic cyanobacteria coverage in rivers or lakes. Remote sensing has enabled high spatial resolution of the distribution of planktonic cyanobacteria in lakes (e.g., Hunter et al., 2009; Odermatt et al., 2012). To the best of our knowledge, this technology has never been tested to study benthic cyanobacteria distribution. Benthic cyanobacteria often have distinctive colorations and it might be possible to estimate the coverage of biofilms by using airborne systems fitted with sensors or cameras.

Rapid, semi-quantitative tests are available for some cyanotoxins (e.g., microcystins) and these have proved valuable for identification of these compounds immediately after sample collection (Humpage et al., 2012b). Given the high prevalence of homo/anatoxin-a in benthic mats, a similar test for these toxins may enable more rapid risk assessment of benthic blooms.

8.2. *Research that explores environmental variables impacting the development of benthic cyanobacteria*

Further studies identifying environmental variables that regulate the development of toxic benthic cyanobacteria are required. Long-term data sets are needed as well as *in situ* experimental approaches, such as the use of nutrient substrate plates. It is essential to acquire a greater understanding of the processes that occur between the mats and; (i) the substrate they attach to (i.e., their ability to source nutrients from sediments) and, (ii) the water column interface (i.e., nutrient uptake rates). Further knowledge on interactions between all the species composing benthic biofilms are required. Recent advances in next-generation sequencing (NGS) enable now entire microbial communities to be characterized (Huber et al., 2007; Huse et al., 2008; Parfenova et al., 2013). Pyrosequencing approaches targeting the 16S rRNA and the 18S rRNA gene sequences, will allow the diversity of benthic biofilms to be characterize and enable interactions among these species to be explored. Sequencing approaches targeting highly variable molecular markers (e.g., intergenic spacer region), will provide new data on the population genetics of benthic cyanobacteria and assist in understanding the development of these populations at different temporal and spatial scales (for planktonic cyanobacterial examples see Briand et al., 2009; Sabart et al., 2009).

8.3. *Research that explores variables that regulate toxin production*

To understand parameters that regulate toxin production careful field studies and more laboratory experiments are necessary. Quantitative molecular techniques, such as quantitative PCR, should be used to determine the genotype composition (i.e., the amount of toxin and non-toxic cells within a sample; Neilan et al., 2013). Collecting these data from environmental sample sets would provide an opportunity to explore whether there are specific variables (e.g., temperature, oxygen availability) that trigger toxic/non-toxic strains to out-compete each other or cause up-regulation in toxin production. This information could ultimately help in predicting periods of highest animal and human health risk. Field observation should be followed up with laboratory studies where individual parameters can be manipulated and the response in growth and toxin production monitored.

8.4. *Understand the impacts of toxic benthic cyanobacteria and cyanotoxins produced on other aquatic communities*

Further research is required to determine what metabolic and/or functional links exist within mats between cyanobacteria and other microorganisms (e.g., bacteria, and algae) and what variables control these interactions. The impacts of toxic benthic cyanobacteria on aquatic animals (for example macroinvertebrates), need further investigating. The toxicity of the mats may have direct impacts on these organisms (i.e., reduced growth rates, reproductive success) or alternatively they may have indirect impacts through changing trophic links, habitat and food sources. These concerns are equally applicable to larger organisms such as fish and freshwater crayfish where toxins may bioaccumulate and effect physiological processes, or large expanses of benthic mats may limit the access to spawning zones or reduce feeding habitat.

8.5. *Development of mathematical models for improving the management of risks*

Increasing research on benthic cyanobacteria will identify more toxin-producing species and these data will assist water managers in identifying risk. Continued studies investigating drivers of benthic bloom formation and toxin production may ultimately lead to the development of models that can be used

to predict times of highest risk and assist in streamlining monitoring regimes. Given the great diversity of habitats that benthic cyanobacteria inhabit and the range of species, these models may need to be specific for each environment.

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ANNEXE 2

Manuscrit en cours de révision pour Water Research sur l'utilisation de la sonde BBE pour surveiller les cyanobactéries

Application of a Spectrofluorimetric Tool (bbe BenthoTorch) for Monitoring Potentially Toxic Benthic Cyanobacteria in Rivers

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Highlights

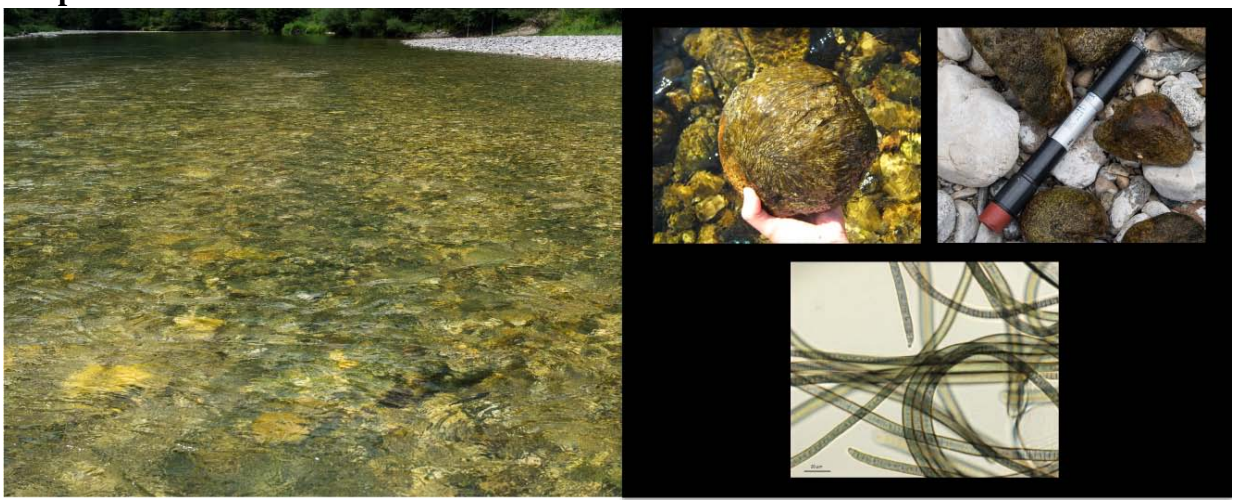
- This is the first time bbe BenthoTorch is tested on cyanobacterial dominated biofilms
- We performed an analysis of the reliability of the bbe BenthoTorch in natural conditions from a range of biomasses and also we include laboratory cultures.
- We identify the major limitation of the BenthoTorch and we propose optimization of the tool according to the results obtained

Abstract

Over the last decade reports of animal poisoning following accidental consumption of neurotoxin-producing benthic cyanobacteria (mainly *Phormidium* spp.) have increased. There is a need for rapid and cost-effective tools to survey benthic cyanobacteria. In this study we assessed the performance of the BenthoTorch, a fluorometric probe that provides *in situ* estimations of cyanobacteria, diatoms and green algae biomass in biofilms. Biofilms (n = 288) were analysed from two rivers in France and eight in New Zealand. Pearson correlations between chlorophyll-*a* measured using the BenthoTorch and spectrophotometry were stronger for thin (<2 mm) compared to thick (>2 mm) biofilms (r_p = 0.76 and 0.52 respectively; p < 0.001). When cyanobacteria represented less than 50% of the total biomass (based on biovolumes), microscopic and BenthoTorch compositional estimations were strongly correlated (r_p = 0.73, p < 0.001). Conversely, there was no correlation when cyanobacteria exceeded 50% of the total biomass. Under this scenario diatoms were overestimated. Our results suggest that the observed biases occur because the BenthoTorch only measures the upper biofilm layer and it underestimates the biomass of phycoerythrin-containing cyanobacteria. To improve the performance of this sensor and render it a useful tool for a rapid evaluation of benthic cyanobacterial biomass in rivers, we propose that: (i) the algorithms based on the LEDs responses currently available on this tool need revision, (ii) new excitation wavelengths should be included that allow the fingerprints of phycoerythrin-containing cyanobacteria to be discriminated, and (iii) a sensor that penetrates the biofilms is needed to obtain more accurate estimates of cyanobacterial biomass.

Keywords: Benthic cyanobacteria monitoring, BenthoTorch, chlorophyll-*a*, *Phormidium* spp., Phycoerythrin

Graphical Abstract



Introduction

Monitoring freshwater microalgae to improve basic knowledge on their ecology, or for more applied programs, such as surveying potentially toxic cyanobacteria is challenging. Microalgae often exhibit complex spatial and temporal variability and extensive sampling is required. Even when such sampling can be undertaken, the cost and time associated with analysis can be prohibitive. Traditionally, extraction and laboratory-based assessment of chlorophyll-*a* (Chl-*a*) using spectrometry has been used as a proxy for total microalgal biomass but this approach provides no information on community composition. Light microscopy is commonly used to identify and enumerate microalgae. Distinguishing genera or species using morphological characteristics is difficult, laborious, and requires a great deal of expertise (Vuorio et al., 2007).

Over the last two decades, new "real-time" tools have been developed to monitor microalgae communities using *in situ* fluorometry (Beutler et al., 2002; Parésys et al., 2005; Pinto et al., 2001). These tools offer a cost-effective alternative to traditional methods, returning a benefit that justifies the initial investment. For example, the FluoroProbe (bbe Moldaenke, Germany) is able to discriminate the major phytoplanktonic groups (Cryptophytes, cyanobacteria, diatoms, dinoflagellates and green algae) in mixed environmental populations based on fluorescence intensity of Chl-*a* at 680 nm, following sequential light excitation by five light-emitting diodes (Beutler et al., 2002). The benefits and limitations of fluorometric tools for phytoplankton species have been well tested and documented for phytoplankton species, in particular studies have focused on bloom-forming cyanobacteria (Catherine et al., 2012; Escoffier et al., 2014; Kring et al., 2014; Leboulanger et al., 2002; Zamyadi et al., 2012).

In contrast to phytoplankton communities, the development of rapid assessment tools

for monitoring benthic algae has received limited attention. Traditionally benthic communities have not been associated with adverse effects on aquatic environments or human and animal health. However, in the last decade toxin producing benthic cyanobacteria have been responsible for numerous animal mortalities worldwide and there is a pressing need for new methods to assess their abundance and biomass (Quiblier et al., 2013). Recently, a new fluorometric tool, the BenthoTorch (bbe Moldaenke, Germany) was developed for monitoring and characterizing phytobenthic communities. An initial evaluation of the BenthoTorch was performed by Kahlert and McKie (2014), however, this previous study mainly consider thin biofilms often dominated by diatoms and did not evaluate the potential of the BenthoTorch for monitoring biofilms of varying thickness dominated by potentially toxic cyanobacteria.

In this study, we assessed the performance of the BenthoTorch on a range of cyanobacteria-dominated biofilms in two countries, France and New Zealand. The BenthoTorch results were compared to those obtained by microscopic identification and enumeration, and by Chl-*a* extraction and subsequent spectrophotometry measurements. To evaluate the influence of the secondary pigment composition on the biomass estimations we investigated the performance of the BenthoTorch using monoclonal cultures of cyanobacteria displaying various pigmentations.

Materials and Methods:

Sampling strategy

Cyanobacterial biofilms were collected from ten shallow rivers in France and New Zealand. Previous studies had identified the presence of potentially toxic benthic cyanobacteria in these rivers (Cadel-Six et al., 2007; Gugger et al., 2005; Wood et al., 2007). In France, samples were collected monthly at four sites in the Loue River between June and September 2012, and seven sites in the Tarn River between June and September 2013 and 2014. In New Zealand, nine sites located in eight rivers were sampled in February 2013 (Fig. 1).

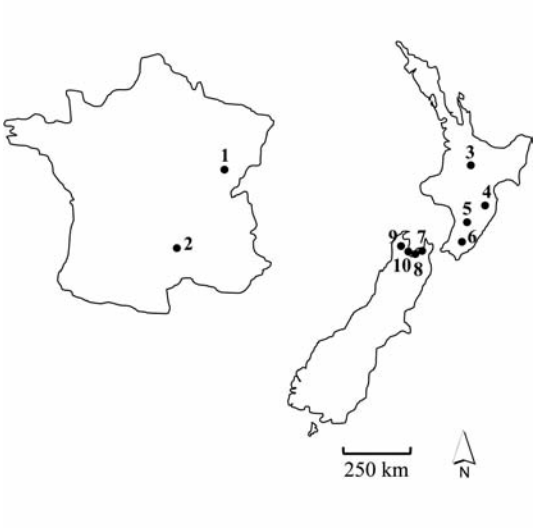


Figure 1. Geographic location of sampling sites. France: (1) Loue, and (2) Tarn rivers. New Zealand: (3) Kuratau, (4) Tukituki, (5) Mangatainoka, (6) Waipoua, (7) Wakapuaka, (8) Maitai, (9) Motueka, and (10) Waimea rivers.

Two different sampling approaches were implemented depending on the accrued biomass, visual estimation of biofilm thickness, and how strongly they were adhered to the substrate. Method 1 was used at sites where biofilms were thin (<2 mm) and their removal was difficult or impossible using tweezers. These biofilms were generally light brown or occasionally dark brown or green. These are hereafter referred to as "thin biofilms" (Fig. 2 a,b). At each site, three or four points on a transect parallel to the water's edge and positioned one to two meters from the shoreline were sampled. At each point, all cobbles (4 to 15 cm length) visible when the riverbed was observed with an underwater viewer (707 cm^2) were collected. A measurement of photosynthetic microorganism biomass was performed on 10 to 15 of the cobbles using the BenthoTorch. The cobbles were then scrubbed and biomass collected in 150 to 250 mL of river water. Aliquots (5 mL) were filtered (GF/C Whatman) and the filter stored chilled in the dark, and subsequently frozen (-20 °C) for later Chl-*a* analysis. Subsamples (1 mL) were fixed with Lugol's iodine solution and stored at 4 °C for later microscopic identification and enumeration.

Method 2 was used at sites where biofilms were thick (>2 mm) and they were easy to remove from the substrate with tweezers. They were predominantly dark green or brown. These biofilms are hereafter referred to as "thick biofilms" (Fig. 2 c,d). A 10 m × 10 m grid was set up in riffle habitat (shallow regions of the river with fast flows) from which 10 cobbles were sampled randomly (Wood et al., 2010). Photosynthetic microorganism biomass was measured on each cobble using the BenthoTorch. The biofilm in the area (7.07 cm^2) where the BenthoTorch measurement was made was removed with tweezers, transferred to a Falcon tube (15 mL), stored chilled in the dark, and subsequently frozen (-20 °C) for later Chl-*a* analysis. A

small (ca. 1 cm^2) section of the adjacent biofilm was fixed with Lugol's iodine solution and stored at 4 °C for later microscopic identification and enumeration.

Method 1 was used for all biofilms from the Loue River and those from June and July 2013 in the Tarn River. The remaining biofilms from the Tarn River and all New Zealand rivers were sampled using Method 2.

Figure 2. Examples of biofilms in French rivers: (a,b) thin diatom dominated-biofilms; (c,d) thick cyanobacterial-dominated biofilms. Scale bar = 4 cm.

Culture conditions

Three benthic and three planktonic non-axenic freshwater cyanobacterial strains from the Paris Museum Collection (PMC) and from the Pasteur Culture Collection (PCC), known to contain phycocyanin and/or phycoerythrin, were selected for pigment extraction (Table 1). Two additional *Phormidium* strains (PMC 846.13 and PMC 848.13), isolated from the Tarn River from samples collected in 2013, were also utilized for pigment extraction. Isolation of these two strains was carried out as described previously (Yéprémian et al., 2007), with some modifications. Biofilm samples were inoculated on semi-solid Z8 medium (Kotai, 1972) (5 g L^{-1}), after migrating by phototactism towards a single light source for 24 h. At least 10 individuals per sample were isolated under an inverted microscope. These trichomes were then transferred sequentially into Z8 liquid medium in 96-well or 48-well plates. Pure isolates were maintained in Erlenmeyer flasks.

Table 1. Cyanobacterial strains used in this study

Genera/species	Strain number	Origin	Habitat type	Color
Phormidium favosum	PMC 240.04	Loue River, France	benthic	brown
Phormidium sp.	PMC 846.13	Tarn River, France	benthic	brown
Phormidium sp.	PMC 848.13	Tarn River, France	benthic	green
Planktothrix agardhii	PMC 368.08	Lake Enghien, France	planktonic	green
Planktothrix rubescens	PMC 598.09	Lake Bourget, France	planktonic	brown-red
Microcystis aeruginosa	PCC 7806	Lake Braakman, Netherlands	planktonic	green
Leptolyngbya foveolarum	PMC 303.07	Stairs border, Limoges, France	benthic	green
L. foveolarum	PMC 609.09	Guadeloupe Island, Caribbean, France	benthic	brown

Triplicates of each culture were maintained in Z8 media at 25 °C and under 17 μmol m^{-2} s^{-1} (16:8 h light-dark photoperiod). To establish pigment extraction, cultures (50 mL) were sampled weekly for 4 weeks. In parallel, all cultures were also grown on semi-solid agar (5 g L^{-1}; 6 cm diameter) in triplicate for 2 weeks to facilitate the development of growth across a solid surface and thus enable direct BenthoTorch measurements.

Chlorophyll-a extraction and analyses of environmental biofilm samples

Glass fiber filters (from biofilms collected using Method 1) or direct biomass samples (Method 2) were extracted with 90% or absolute methanol respectively (10 mL) in Falcon tubes (15 mL) covered with aluminium foil. The tubes were shaken manually, vortexed (30 s), incubated (24 h, 4 °C) and centrifuged (10 min, 3220 × *g*). The absorbance of the supernatant was measured with a spectrophotometer (Agilent Technologies Cary 60 UV-vis, USA). The absorbance of samples collected using Method 1 was measured at 665 nm, and for Method 2 an additional absorbance was measured at 652 nm. A correction for turbidity at 750 nm was applied and the equations of Talling and Driver (1963) and Porra et al. (1989) were adapted to calculate Chl-*a* concentrations (μg cm^{-2}) for each method respectively.

BenthoTorch measurements

The BenthoTorch was applied directly to the surface of submerged biofilms, in the rivers, ensuring that the foam pad around the diodes shadowed the biofilms from extern light. The fluorescence excitation was undertaken by seven diodes (LEDs) that emitted light at three wavelengths (470, 525, and 610 nm). An additional LED of 700 nm is used to compensate the

effects of background reflection. The BenthoTorch measures the resulting fluorescence of Chl-*a* emitted at 680 nm. Results of measurements are displayed in a lateral screen after 10 s. Data produced by the BenthoTorch are given for three photosynthetic groups: cyanobacteria, diatoms, and green algae. The calculation of the respective biomasses of the photosynthetic groups is via an algorithm based on the fluorescence responses to all different excitation wavelengths. The calculation algorithms are based on characteristic spectral fingerprints (fluorescence spectral signature) for each photosynthetic group. Biomasses are expressed as a Chl-*a* equivalent per unit of surface (μg cm^{-2}). The pre-programmed factory settings of the BenthoTorch were used for all measurements and total Chl-*a* concentrations were deduced by summing biomasses values for the three photosynthetic groups. For Method 1, average Chl-*a* values were calculated for each sampled point.

Pigment extraction for absorption and in vivo fluorescence measurements

Pigment extraction and corresponding absorption spectra were undertaken on samples from eight monoclonal cyanobacteria cultures belonging to four genera (Table 1) and on 29 samples from environmental biofilms, to assess the possible influence of secondary pigments, such as cyanobacterial phycobiliproteins, on the performance of the BenthoTorch. It is well known that different cyanobacterial species can contain different proportions of phycocyanin (PC) and phycoerythrin (PE) (Grossman et al., 1993; Lohscheider et al., 2011; Rippka et al., 1979; Tandeau de Marsac, 1977). Therefore, for this part of the study we compared "green" (associated with potentially PC dominant) and "brown" (associated with potentially PE dominant) cultures or biofilms samples. Biofilm samples were collected in the Tarn River using Method 2, during the summer of 2014, and stored chilled prior to analysis. A total of 20 green biofilms and nine brown biofilms were collected. The cyanobacterial cultures and biofilms were also grouped according to their color. Green cultures included: *Leptolyngbya foveolarum* PMC 303.07, *Microcystis aeruginosa* PCC 7806, *Phormidium* sp. PMC 848.13, and *Planktothrix agardhii* PMC 368.08. Brown cultures included: *L. foveolarum* PMC 609.09, *Phormidium* sp. PMC 846.13, *Phormidium favosum* PMC 240.04 and *Planktothrix rubescens* PMC 598.09.

Pigments (carotenoids, Chl-*a*, PC and PE) were extracted together according to Palinska et al. (2011) and an analysis of the absorption spectra of monoclonal cultures and natural biofilm samples was performed. Weekly subsamples (2 to 6 mL of planktonic cyanobacteria) were centrifuged (10 min, 3220 × *g*). For natural biofilms, a subsample (50 mg) of fresh

biomass was used. Samples were extracted in 2 to 5 mL of buffer containing 50 mM Tris/hydrochloric acid (pH 8.0), 250 mM sodium chloride, and 10 mM EDTA. Cells were lysed using ultrasonication. Samples were incubated in 5% Triton (30 min, 28 °C) to allow extraction of hydrophobic pigments including chlorophylls and carotenoids. Samples were then centrifuged (10 min, 3220 × *g)* and absorbance of the supernatant measured from 350 to 750 nm in a spectrophotometer (Agilent Technologies Cary 60 UV-vis, USA).

In addition, fluorescence measurements were performed *in vivo* with a Fluoroprobe (bbe Moldaenke, Germany) on two green (*L. foveolarum* PMC 303.07 and *M. aeruginosa* PCC 7806), and two brown cyanobacterial cultures (*P. favosum* PMC 240.04 and *P. rubescens* PMC 598.09). These enable us to compare the fluorescence emission patterns of Chl-*a* at 680 nm when excited at the five different wavelengths available on the Fluoroprobe (470, 525, 570, 590 and 610 nm).

Microscopic analysis

Lugol's iodine preserved samples were homogenized briefly (Ultra-Turrax T25 IKA, Germany, 3 × 2 s at 9.5 min^{-1}) to break up filaments, but avoid cell damage. The samples were diluted in Milli-Q water and identification and enumeration performed using a photonic microscope (200× magnification, Nikon Optiphot-2, Japan), and a Malassez chamber (Marienfeld, Germany). All cells contained in 25 squares of the Malassez chamber were counted and analysis of triplicate aliquots undertaken. Species identification was performed only for cyanobacteria and followed Komarek and Anagnostidis (2005). The other cells were only identified as belonging to diatom or green algae. The length and the width (or diameter for filaments) of the cells from the three photosynthetic groups were estimated during the counting. For diatoms and green algae, the height was estimated according to literature (Olenina et al., 2006). For all species, biovolumes were then calculated according to Olenina et al. (2006) and Hillebrand et al. (1999).

Data analysis

Statistical analyses were performed in R software (version 3.1.0 R Core Team 2014, Vienna, Austria). Normality was checked using the Shapiro-Wilk test. Skewness and kurtosis were estimated by D'Agostino and Anscombe-Glynn tests respectively (package "moments"; Komsta and Novomestky, 2012). When significant departures from normality were identified, data were normalized using Box-Cox transformation (package "car"; Fox et al., 2014; see

Supporting Information Table S1). Outliers where identified with the Bonferroni outlier test (package "car") and homoscedasticity checked with Breusch-Pagan test (package "lmtest"; Hothorn et al., 2014).

Differences between Chl-*a* values obtained with the spectrophotometer and BenthoTorch measurements were identified using Paired *t* tests (package "stats" of base R) and the relationship analyzed by type II linear regression (package "lmodel2"; Legendre, 2013) using the major axis method (see Supporting Information Table S2). In order to identify the change point of the relationship between Chl-*a* values obtained after extraction and from BenthoTorch measurements, the non-parametric Pettitt test was applied using XL STAT version 2011 (Addinsoft, France). As no normalization was possible with the proportion data estimated from microscopy observations and from BenthoTorch measurements, relationships for each photosynthetic group were analysed by type II linear regression using ordinary least squares (see Supporting Information Table S3). Additional analyses of the residuals were performed with Wilcoxon test to compare deviations from the 1:1 theoretical relationship. Cyanobacterial proportion differences between those calculated from microscopy observations and those from BenthoTorch measurements were determined using the non-parametric Wilcoxon test (package "stats" of base R). Correlation coefficients presented correspond to Pearson correlation (r_p).

Results

Comparison of chlorophyll-a concentrations measured using spectrophotometry and BenthoTorch

A total of 50 thin- and 238 thick-biofilms were analyzed separately for Chl-*a* using spectrophotometry and the BenthoTorch. Total Chl-*a* concentrations measured by spectrophotometry ranged between 0.4 and 48.6 μg cm^{-2} compared to 0.8 and 21.5 μg cm^{-2} using the BenthoTorch (Fig. 3).

For the thin biofilms (Fig. 3a), there was no significant difference between Chl-*a* values measured using spectrophotometry and the BenthoTorch (Paired *t* test, $p = 0.81$), and a strong correlation ($r_p = 0.76$, $p < 0.001$) was observed between total Chl-*a* concentrations estimated by the two approaches (Fig. 3a). Most of the Chl-*a* concentrations values estimated were below 10 μg cm^{-2} (86.5% spectrophotometry, and 96.2% BenthoTorch) and they never exceeded 15.4 μg cm^{-2}. BenthoTorch Chl-*a* values never exceeded 8 μg cm^{-2} (except two outliers), and a knee

point was detected in the Chl-*a* relationship values below and above 4 μg cm^{-2} as measured by spectrophotometry (Fig. 3a; Pettitt test, $p < 0.01$). For Chl-*a* values below the change point a strong correlation was obtained ($r_p = 0.77$, $p < 0.001$). The correlation was weak when Chl-*a* values exceeded 4 μg cm^{-2} ($r_p = 0.48$, $p = 0.008$). Equations used are provided in the supporting information (Table S2).

The Chl-*a* concentrations for thick biofilms measured using spectrophotometry were significantly different from those obtained from the BenthoTorch (Fig. 3b; Paired *t* test, $p < 0.001$). However, a significant correlation was found between concentrations obtained by the two approaches (Fig. 3b; $r_p = 0.52$, $p < 0.001$), even when there was a clear underestimation of the Chl-*a* concentrations using the BenthoTorch. The Chl-*a* concentrations measured using spectrophotometry were up to 48.6 μg cm^{-2}, while almost all values measured using the BenthoTorch (89.9%) were below 10 μg cm^{-2} and never exceeded 21.5 μg cm^{-2} (Fig. 3b).

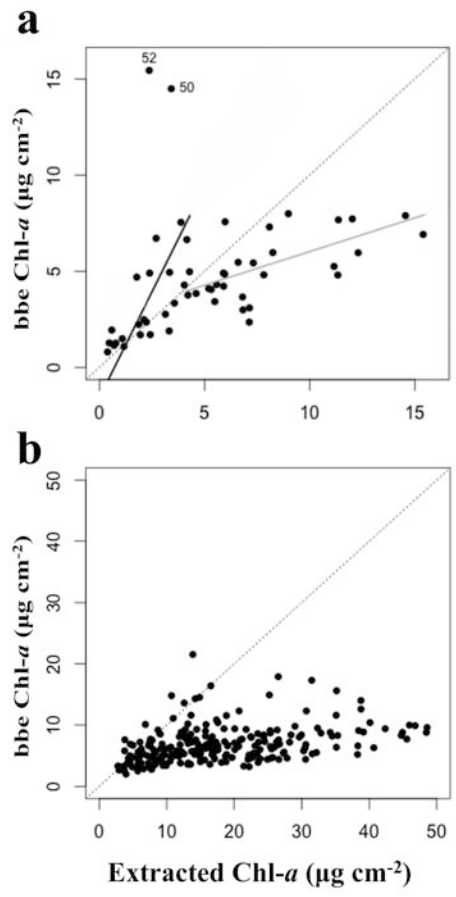


Figure 3. Relationship between chlorophyll-*a* (Chl-*a*) values obtained using the BenthoTorch (bbe) and spectrophotometry after methanol extraction: (a) thin biofilms sampled using Method 1; Samples marked as 50 and 52 were excluded from statistical analysis; Black and grey regression lines correspond to values below and above 4 μg cm^{-2} of extracted Chl-*a* respectively; (b) thick biofilms sampled using Method 2. Dashed lines correspond to the 1:1 relationship.

Efficiency of BenthoTorch for assessing taxonomic composition

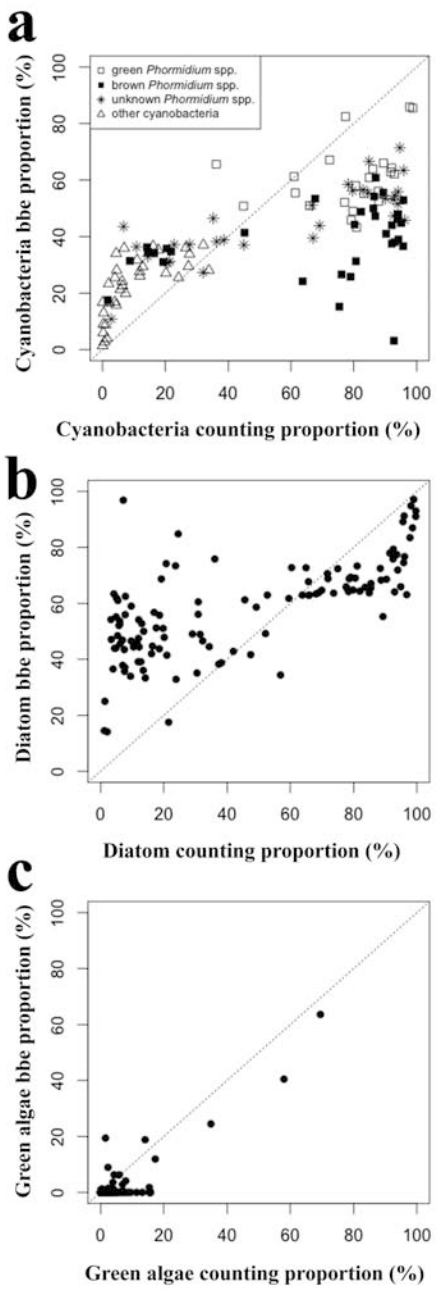
A total of 120 biofilm samples were analysed microscopically for their taxonomic composition and the proportions of the three main photosynthetic groups (cyanobacteria, diatoms and green algae) were calculated based on their respective total biovolume. Almost all the thin biofilms were dominated by diatoms (Table 2, mean 82.9%, max. 99.7%), while thick biofilms were mainly dominated by cyanobacteria (mean 74.2%, max. 98.8%), followed by diatoms (mean 21.7%, max. 96.8%) and green algae (mean 4.1%, max. 69.5%).

Table 2. Proportion (%) of each photosynthetic group calculated from chlorophyll-*a* concentrations measured by the BenthoTorch and biovolumes estimated by the microscopic analysis. Values for Method 1 and 2 are presented separately (CV% = Coefficient of variation in percentage).

		Min.	Max.	Mean	Median	CV (%)
Method 1						
Cyanobacteria	**BenthoTorch**	2.7	43.6	27.6	30.8	35.2
	Microscope	0.2	38.6	13.2	11.9	75.9
Diatoms	**BenthoTorch**	55.3	97.2	71.6	68.7	13.5
	Microscope	45.6	99.7	82.9	84.9	15.4
Green algae	**BenthoTorch**	0.0	8.9	0.8	0.0	237.2
	Microscope	0.0	15.9	3.9	1.6	131.5
Method 2						
Cyanobacteria	**BenthoTorch**	1.3	95.3	50.5	51.5	28.7
	Microscope	0.0	98.9	74.2	83.2	35.1
Diatoms	**BenthoTorch**	4.7	96.9	48.0	47.9	28.1
	Microscope	1.2	96.8	21.7	13.6	96.8
Green algae	**BenthoTorch**	0.0	63.6	1.5	0.0	504.9
	Microscope	0.0	69.5	4.1	0.8	274.5

Further in-depth analysis of the cyanobacterial component of the thin biofilm samples identified that 69% were dominated by *L. foveolarum* (Rabenhorst ex Gomont) Anagnostidis et Komárek 1988, and 31% by *Phormidium* cf. *amoenum* Kützing 1843 ex Anagnostidis et

Komárek 1988. Among the 75 thick biofilms from the Tarn and New Zealand rivers, two species (*P.* cf. *amoenum* and *Phormidium autumnale* [Agardh] Trevisan ex Gomont 1892 sensu stricto pro typo) were dominant in 96% of the samples.



When cyanobacteria represented less than 50% of the total biovolume, their proportions were strongly correlated with those estimated from BenthoTorch measurements (Fig. 4a and see Supporting Information Table S3; $r_p = 0.73$, $n = 58$, $p < 0.001$). When cyanobacteria exceeded 50%, the proportions estimated by the two methods were non-correlated (Fig. 4a; $r_p = 0.16$, $n = 62$, $p = 0.21$). There was also a highly significant deviation from the 1:1 theoretical relationship (Wilcoxon test of residuals, $p < 0.001$) compared to the values below 50% indicating the BenthoTorch was underestimating cyanobacteria.

Conversely, for diatoms, a strong relationship (Fig. 4b; $r_p = 0.73$, $n = 56$, $p < 0.001$) was found between BenthoTorch and microscopy estimations when their proportions were greater than 40%. When diatoms represented less than 40% of the total biovolume, there was no correlation ($r_p = 0.14$, $n = 64$, $p = 0.27$). Diatom proportion appeared to be overestimated by the BenthoTorch. The deviation of the data was higher when diatom biovolumes represented less than 40% (Wilcoxon test of residuals, $p < 0.001$).

The proportions of green algae were generally very low (<20% of the total biovolume) in the biofilms (Fig. 4c). When green algae represented more than 10% of the total biovolume as estimated from microscopy

Figure 4. Relationship between the proportions of: (a) cyanobacteria, (b) diatoms and (c) green algae (n=120) estimated using the BenthoTorch (bbe) and based on biovolume proportions determined microscopically. Dashed lines correspond to the 1:1 relationship. In (a) cyanobacterial community dominated by green *Phormidium* spp. (open squares), brown *Phormidium* spp. (solid squares), unknown *Phormidium* spp. (asterisk) and other cyanobacteria (open triangles) are identified.

observations, correlation with the BenthoTorch estimations were strong and significant (r_p = 0.95, n = 12, p < 0.001). When proportions were less than 10%, no correlation was observed and data suggested an underestimation by the BenthoTorch (r_p = 0.17, n = 108, p = 0.06).

Influence of cyanobacteria phycoerythrin content on BenthoTorch performance

Analysis of the pigment absorption spectra revealed the presence of a PE peak (~560 nm) in all brown cultures and biofilm samples (Fig. 5a,b). No PE was detected in the green cultures or biofilms samples (Fig. 5d,e). Green and brown cultures and biofilm samples shared the same peaks at ~435 nm and 665 nm (Chl-*a*), ~487 nm (carotenoids) and ~620 nm (PC). In addition, *in vivo* fluorescence emission recorded at 680 nm with the bbe Fluoroprobe also showed two different response patterns (Fig. 5c,f). A peak of fluorescence emission was observed for brown cyanobacteria when exciting at 525 nm while the fluorescence emission was low for green ones. The responses with the other excitation wavelength offered by the Fluoroprobe (470, 570, 590 and 610) were similar between brown and green cyanobacteria.

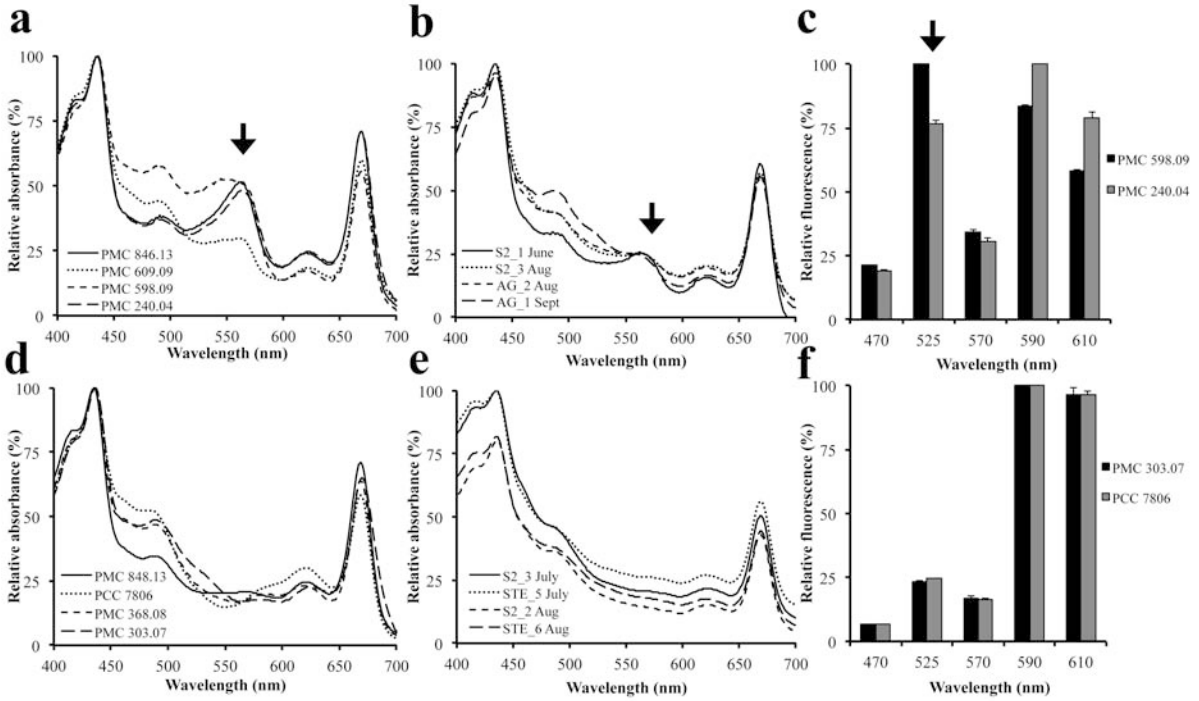


Figure 5. Absorption spectra of (a) brown and (d) green cyanobacterial cultures; (b) brown and (e) green cyanobacterial biofilms and *in vivo* fluorescence emission of chlorophyll-*a* measured with the bbe Fluoroprobe of (c) brown and (f) green cyanobacterial cultures. Arrows show phycoerythrin peak. Error bars represent the standard deviation.

A comparison between the efficiency of the BenthoTorch and data from microscopy observations for detecting cyanobacteria in cultures and biofilms samples containing or lacking

PE (Fig. 6) showed a large difference between brown PE-containing (71% ± 6.4 SD for cultures; 47% ± 17.2 SD for biofilms) and green PE-lacking (16% ± 21.5 SD for cultures; 17% ± 17.3 SD for biofilms) cyanobacteria.

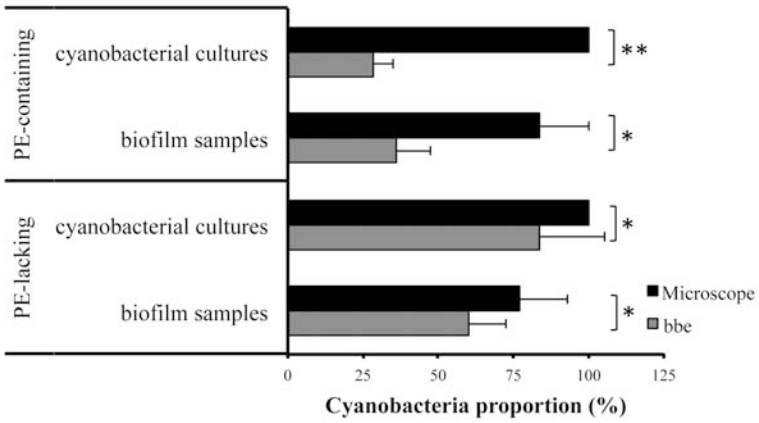


Figure 6. Comparison of cyanobacteria proportions obtained using the BenthoTorch (bbe) and assessed using microscopy on phycoerythrin (PE)-containing (n = 12) and PE-lacking (n = 11) cyanobacterial cultures, and PE-containing (n = 9) and PE-lacking (n = 20) biofilm samples. Wilcoxon test was performed to compare methods (*p < 0.01; **p < 0.001). Error bars represent the standard deviation.

Discussion

The main objective of this study was to assess the BenthoTorch performance's as a tool for rapid field evaluations of benthic cyanobacterial proliferations in rivers. Measurements performed on biofilms from French and New Zealand rivers demonstrated that the BenthoTorch efficiency is strongly influenced by the thickness of biofilms and the composition of algal communities. When analyzing thin biofilms (<2 mm), the results from the BenthoTorch and methanol extraction/spectrophotometry analysis of Chl-*a* were very similar. Conversely, for thick biofilms (>2 mm), the BenthoTorch significantly underestimated biomass. Our findings on thin biofilms support the recent study by Kahlert and McKie (2014) who tested the BenthoTorch on phytobenthic communities from 24 oligotrophic streams in Sweden. These authors also found a strong correlation between biomass data obtained by the BenthoTorch with those of conventional methods. However, in all instances the biofilms they assessed had very low biomasses (ca. 0.5 µg Chl-*a* cm^{-2}) compared to the range of biomass that was studied here (0.4 and 48.6 µg Chl-*a* cm^{-2}).

To our knowledge, no other studies have assessed the performance of the BenthoTorch on thick biofilms, but previous studies on phytoplankton communities have reported an underestimation of biomasses when using the bbe Fluoroprobe in eutrophic ecosystems experiencing dense cyanobacterial blooms (Catherine et al., 2012; Gregor and Marsálek, 2004;

Rolland et al., 2010). Authors reported that restricted light penetration, in particular when working on colonial planktonic cyanobacteria, was thought to be one of the main reasons for the underestimation of biomass in these systems (Gregor and Marsálek, 2004). It is likely that the underestimation of the biomass of benthic communities by the BenthoTorch, observed here for thick biofilms, resulted from: (i) the difficulty of the light emitted by the sensor to penetrate the full depth of the biofilm, and (ii) possible self-shading (of the fluorescence emitted) linked to the cells density into the biofilm that could partially limit the detection by the BenthoTorch sensor. The manufacturer of the BenthoTorch specifies that the penetration of light is restricted to the upper layer and the fluorescence response reflects signals from a 0.1 mm thick layer at the surface of a fixed carrier (bbe moldaenke Home Page, 2014). The reality is probably more complex as the surface and the general structure of biofilms is often very heterogeneous, particularly when they become thicker, as observed in our study. *Phormidium* filaments were commonly density packed and entangled in the thick biofilms observed in the rivers studied (see supporting Information Fig. S1), suggesting that only a fraction of the biofilm biomass is reached by the light emitted from the BenthoTorch.

The data produced by the BenthoTorch for the evaluation of photosynthetic groups composition showed a clear underestimation of cyanobacteria, particularly for thick biofilms dominated by *Phormidium*. It is well known that some *Phormidium* species contain phycoerythrin (Marquardt and Palinska, 2007; Palinska and Marquardt, 2008; Palinska et al., 2011; Soni et al., 2010). The patterns of absorbance and fluorescence of natural biofilms dominated by cyanobacteria and cultures observed in this study demonstrate that green and brown samples can be distinguished by the presence/absence of PE. Comparisons between BenthoTorch measurements and microscopic observations clearly show that the probe underestimates the biomasses of PE-containing cyanobacteria and overestimates diatoms. This under/overestimation of cyanobacteria and diatoms in biofilms dominated by *Phormidium* was not due to an accumulation of diatoms at the biofilms surface (see SEM analyses, Supporting Information Fig. S1). Inconsistencies in the data provided by the probe could arise from a miss-interpretation of the fluorescence signal. For example, our fluorescence analyses highlighted a fluorescence peak at 525 nm in PE-containing cyanobacteria, in agreement with a previous study (Leboulanger et al., 2002) and not in PE-lacking cyanobacteria. The fluorescence of diatoms (at 525 nm) is greater than that of green cyanobacteria (Carpentier et al., 2013), and we suggest that the underestimation of cyanobacteria in thick biofilms dominated by PE-containing cyanobacteria and the overestimation of diatoms could be linked to the fluorescence response at

525 nm. This bias could probably be corrected by: (i) revising the algorithms used by the BenthoTorch to estimate biomasses of the different photosynthetic groups from the fluorescence response at different wavelengths; or (ii) by including a further diode corresponding to a wavelength which would improve the ability to discriminate brown cyanobacteria from diatoms. This modification has already been incorporated on the Fluoroprobe used for monitoring planktonic algal population. In this sensor five diodes emit light at 490, 525, and 610 nm, the same wavelengths utilized in the BenthoTorch, but also at 570 and 590 nm. Despite these two additional diodes, an underestimation of PE-containing *Planktothrix rubescens* by the Fluoroprobe was observed by Leboulanger et al. (2002) leading them to define a specific fingerprint in the probe software. This approach cannot be undertaken in the BenthoTorch as the software is not directly accessible by the end-user, and therefore new fingerprints and revision of algorithms can only be made by the manufacturer.

Other factors that may affect *in situ* measurements undertaken with the BenthoTorch could include the environmental conditions within the biofilms and the resulting variations of the physiological state of the photosynthetic community. It is well known that environmental conditions such as light and nutrient availability can affect the physiological state of the photosynthetic community and thus, pigment content and fluorescence response can change (Escoffier et al., 2014; Grossman et al., 1993; Rolland et al., 2010). No evaluations of the physiological state of the biofilms were performed during our study, however, we suggest that these variations would have only had a minor effects on the BenthoTorch measurements compared to the bias described above for thick biofilms.

Our study has led to contrasting conclusions on the validity of using the BenthoTorch for monitoring potentially toxic benthic cyanobacteria in rivers. The usability of the data depends on: (i) the relative proportion of diatoms and cyanobacteria; (ii) the thickness and the phase of development; and (iii) the nature of the cyanobacteria (PE-containing or lacking) that dominates the biofilms. Three main scenarios were distinguished: (i) when biofilm are thin and dominated by diatoms, the BenthoTorch can efficiently measure their biomass; (ii) if the cyanobacteria proliferation is mainly due to a PE-lacking species, the BenthoTorch can at least initially, correctly estimate their biomass; and (iii) if a cyanobacteria proliferation is dominated by PE-containing species, the BenthoTorch will greatly underestimated their biomass, and thus the degree of risk posed to river users is underrated. It is noteworthy that when mature and thick cyanobacterial-dominated biofilms are present, these are usually easily recognizable

macroscopically and qualified river managers may be able to make accurate assessments of risk without a tool such as the BenthoTorch.

Conclusion

This study demonstrates that the BenthoTorch can be used for the quantification of the biofilm biomass, but only at the early stages of their development. In contrast, this tool is not well suited for monitoring mature and thick biofilms with high biomass or for biofilms dominated by PE-containing cyanobacteria, such as *Phormidium* spp. To improve the performance of this tool and render it useful for rapid evaluation of benthic cyanobacterial biomass in rivers, we suggest that; (i) algorithms based on the responses of the different LEDs currently available in this tool are revised, (ii) an excitation wavelength that allow fingerprints of phycoerythrin-containing cyanobacteria to be distinguished is included, and (iii) an adjustable sensor capable of penetrating and performing measurements through the entire depth of thick biofilms is developed.

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SUPPLEMENTAL INFORMATION

Supplemental Table 1. Summary statistics of extracted (ext_Chl-*a*) and BenthoTorch measured (bbe_Chl-*a*) of chlorophyll-*a* in µg cm^{-2} before and after transformation. Values for Method 1 and 2 are represented separately (*p*-values of each statistical test are presented in bracket).

	ext_Chl-*a*1	**ext_Chl-*a*2**	**bbe_Chl-*a*1**	**bbe_Chl-*a*2**
Min.	0.39	2.78	0.80	2.00
Max.	15.4	48.57	15.44	21.52
Mean	5.30	17.56	4.66	6.71
Median	4.45	16.93	4.30	6.20
Skewness	0.88 (0.09)	0.83 (0.001)	1.58 (0.01)	1.52 (<0.001)
Kurtosis	3.20 (0.47)	3.17 (0.45)	6.73 (0.001)	6.52 (<0.001)
Shapiro test	0.92 (0.003)	0.93 (<0.001)	0.87 (<0.001)	0.89 (<0.001)
Transformed data (Box-Cox transformation)				
Transformation	$(x^{0.39}-1)/0.39$	$(x^{0.27}-1)/0.27$	$(x^{0.23}-1)/0.23$	$(x^{-0.097}-1)/-0.097$
Skewness	-0.08 (0.87)	-0.04 (0.85)	-0.01 (0.98)	0.001 (0.996)
Kurtosis	2.42 (0.41)	2.28 (<0.001)	2.9 (0.81)	2.90 (0.91)
Shapiro test	0.98 (0.67)	0.99 (0.02)	0.97 (0.34)	0.996 (0.83)

Supplemental Table 2. Type II linear regression model applied to the relationship between extracted and BenthoTorch measured chlorophyll-*a* (bbe/ext Chl-*a*). Values for Method 1 and 2 are represented separately (*p*-values of each statistical test are presented in bracket; MA = Major Axis regression; BP = Breusch-Pagan).

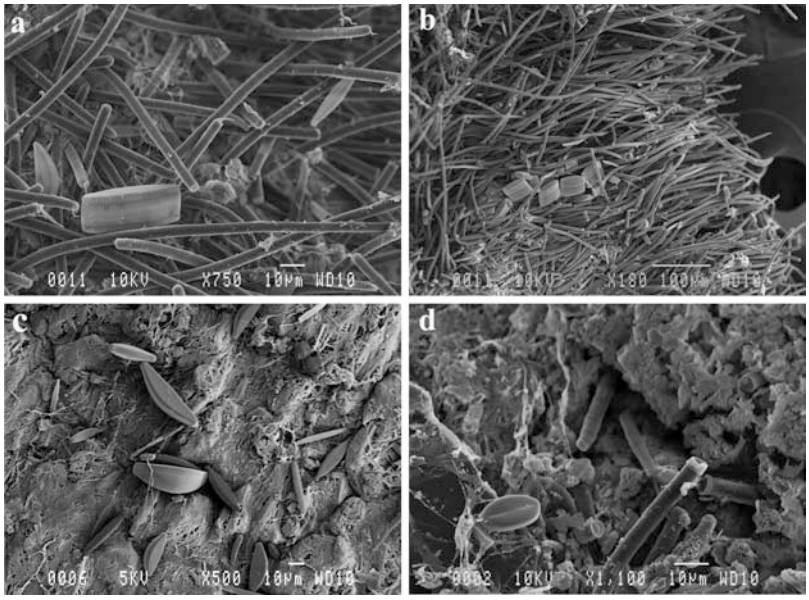
Relationship	Data	Method	Equation	R^2	BP test
bbe/ext Chl-*a* 1	Raw	MA	y = 0.46x + 1.79	0.50 (<0.001)	0.17 (0.68)
	Normalized	MA	y = 0.83x + 0.49	0.58 (<0.001)	0.03 (0.85)
bbe/ext Chl-*a* 2	Raw	MA	y = 0.12x + 4.52	0.17 (<0.001)	0.31 (0.58)
	Normalized	MA	y = 0.14x + 1.07	0.27 (<0.001)	1.32 (0.25)

Supplemental Table 3. Type II linear regression model applied to the relationship between biovolume and BenthoTorch proportion raw data (bbe/microscope) for each photosynthetic group (*p*-values of each statistical test are presented in bracket; OLS = Ordinary Least Squares regression; BP = Breusch-Pagan).

Relationship	Values	Method	Equation	R^2	BP test
Cyanobacteria bbe/microscope	less 50% (*n* = 58)	OLS	y = 0.68x + 17.74	0.53 (<0.001)	0.09 (0.77)
Cyanobacteria bbe/microscope	more 50% (*n* = 62)	OLS	y = 0.24x + 30.86	0.03 (0.21)	0.16 (0.69)
Diatom bbe/microscope	less 40% (*n* = 64)	OLS	y = 0.21x + 45.39	0.02 (0.27)	0.02 (0.90)
Diatom bbe/microscope	more 40% (*n* = 56)	OLS	y = 0.56x + 24.68	0.54 (<0.001)	0.40 (0.53)
Green algae bbe/microscope	less 10% (*n* = 108)	OLS	y = 0.19x + 0.22	0.03 (0.058)	0.19 (0.66)
Green algae bbe/microscope	more 10% (*n* = 12)	OLS	y = x – 11.49	0.90 (<0.001)	0.07 (0.78)

Materials and Methods: Scanning electron microscopy

Selected biofilm samples (Tarn River, August 2013) were fixed in the field with Sorensen's phosphate buffer containing 2.5% Glutaraldehyde. Biofilms were dehydrated in a series of aqueous ethanol solutions (30-100%) and then critical point dried (Emitech K850, UK) using liquid carbon dioxide as the substitution medium. Samples were mounted on metal stubs, sputtered with gold and observed under scanning electron microscope (SEM; Jeol 840A, Japan; Technique platform of electron microscopy and microanalyses from the Paris Natural History Museum).



Supplemental Figure 1. Scanning electron microscopic images of biofilms dominated by *Phormidium* spp. from the Tarn River samples: (a,b) top; (c,d) underside of biofilm.

ANNEXE 3

Manuscrit en cours de préparation sur les données obtenues sur la Loue

Deciphering biodiversity and interactions between bacteria and micro-eukaryotes inside epilithic biofilms of a French river

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Manuscript in preparation

Abstract

Epilithic river biofilms are complex matrix-enclosed communities harbouring a great diversity of prokaryotic and eukaryotic microorganisms. They are subjected to dynamic environmental conditions that change over time and space, that shape the structure and composition of these benthic communities. In order to better understand composition and functioning of epilithic river biofilms and the relative impact of environmental factors, we described the spatio-temporal variations and interactions in the structure and composition of bacterial and micro-eukaryotic communities of stream biofilms. In this goal, we performed a study on natural samples collected in at least four sampling sites during a summer season in a French river. In addition to the characterization of prokaryote and micro-eukaryote communities by using a 454-pyrosequencing approach on 16S and 18S rRNA gene fragments, biofilm biomasses were assessed by spectrophotometric measurement of the chlorophyll-*a* and the phytobenthic community charaterized by microscopic examination. First, we have shown that the assessment of micro-algal communities composition by 18S rRNA sequencing leads to a strong overestimation of the relative abundance of green algae when comparing with microscopic counting, while the assessment provided by the chloroplastic 16S rRNA gene was in agreement with microscope examination. Second, we found the same patterns of spatio-temporal changes in bacterial and micro-eukaryotic communities. Interstingly, significant differences were found in the temporal evolution of microbial communities from the most upstream sampling station compared to downstream stations. These differences can be explained by the geological and hydrological river contexts that have a strong impact on the water temperature. Finally, this work has also provided interesting findings on the interactions occurring between phototrophic and heterotrophic microorganisms and a putative competition between *Rhodobacter* and the dominant photosynthetic microorganisms (cyanobacteria and diatoms).

KEYWORDS: River biofilms, benthic communities, coocurrence network

Introduction

Epilithic river biofilms are complex matrix-enclosed communities living attached to various substrates, including for example rocks or plants. Inside these biofilms, which can be described as real landscapes (Battin et al., 2007), prokaryotic and eukaryotic microorganisms are closely associated. Numerous works have already been published on the diversity of these communities and on the ontogenesis of biofilms. For example, it is now well established that bacteria mainly belonging to Betaproteobacteria (Araya et al., 2003; Manz et al., 1999) but also diatoms (Battin et al., 2003; Besemer et al., 2007), have a fundamental role in the colonisation of the substrates by producing Extracellular Polymeric Substance (EPS) (Stolz, 2000). These pionners microorganisms allow to prepare the establishment of the next arrivals, including heterotrophic and phototrophic microorganisms (Roeselers et al., 2007) such as bacteria belonging to Alphaproteobacteria and Bacteroidetes (Manz et al., 1999), cyanobacteria, microalgae (Barranguet et al., 2005; Brasell et al., 2015; Roeselers et al., 2007), and other components (*i.e.* archea, fungi, protozoa, little metazoan and viruses; Battin et al., 2007; Beraldi-Campesi et al., 2012; Besemer, 2015).

From a functional point of view, cyanobacteria, diatoms, and green algae are recognized as principal primary producers in periphyton (Lamberti, 1996; Roberts et al., 2004) but other potential photosynthetic bacterial taxa are also frequently detected in stream biofilms such as purple bacteria (*e.g.* Anderson-Glenna et al., 2008; Beraldi-Campesi et al., 2012; Bricheux et al., 2013; Drury et al., 2013). Among them, *Rhodobacter* genus is able to growth in anaerobic (phototrophy) and aerobic (chemoheterotrophy) conditions (Blankenship, 2014). More globally, all the autotrophic microorganisms have been described as the principal producers of organic matter that is used by heterotrophic or mixotrophic microorganism in biofilms (Romaní and Sabater, 1999; Romani et al., 2004), while allochtonous organic matter seems to contribute for a minor part as carbon sources for them (Kamjunke et al., 2015). Finally, predators as protozoa exploiting biofilms as food source are the drivers of the carbon transferring to higher trophic levels (Risse-Buhl et al., 2012). Thus, multiple interactions occur into microbial biofilms knowing that the very short cellular distances occurring between species facilitate direct interactions between microorganisms (Leflaive et Ten-Hage, 2007).

In most of the papers dealing with the microbial communities from stream biofilms, algal and bacterial components have been mainly described separately. In one hand, the algal component has attracted the attention of numerous studies dealing with the use of these microorganisms as bioindicators for the assessment of the water quality (Fetscher et al., 2014;

Kelly and Whitton, 1998; Stevenson and Smol, 2003; Visco et al., 2015) and for the identification of the environmental factors and processes impacting biofilm development in lotic environments (Biggs, 1996). On the other hand, numerous works have been performed on the composition of bacterial communities in periphytic biofilms by using 16S rRNA fingerprint methods and more recently next generation sequencing approaches (Besemer et al., 2012), with the goal to better understand the spatial and temporal variations occurring in these communities (Jackson et al., 2001; Lyautey et al., 2005; Margulies et al., 2005).

To our knowledge, the only paper dealing simultaneously with both components (prokaryote and eukaryote) has been published by Bricheux et al. (2013). This study based on a 454-pyrosequencing approach, aimed to characterize the microbial diversity by testing different sets of primers on biofilms growing on glass substrates in one station of a French river. Consequently, there is no data based on the use of high-throughput sequencing describing simultaneously the spatiotemporal variations in the structure and composition of bacterial and micro-eukaryotic communities of stream biofilms despite the interest of such an approach in order to identify the relative impact of environmental factors on these two communities and the putative interactions occurring between them. In this goal, we have performed a study on natural samples collected in at least four sampling sites during a summer season in a French river located in the eastern part of France, the Loue River. In addition to the characterization of eukaryote and prokaryote communities by using a 454-pyrosequencing approach on 16S and 18S rRNA gene fragments, biofilm biomasses and microscopic examination of principal photosynthetic microorganisms were also assessed in order to compare molecular and microscope identification. Finally, several physicochemical parameters were also recorded with the aim to better understand the variations occurring in microbial communities.

Material and Methods

Sites and samples collection

Samples were collected during summer 2012 (5^{th}-6^{th} June, 9^{th}-10^{th} July, 13^{th}-14^{th} August and 10^{th}-11^{th} September) in four sites located along the Loue River in France (Figure 1; *i.e.* from June to September for Cléron and Parcey and in July and August for Belmont and Chamblay). At each site, three points on a transect parallel to the water's edge and positioned one to two meters from the shoreline were sampled. At each point, all cobbles (4 to 15 cm length) contained in a known area defined by the surface of an underwater viewer (707 cm^2) were

collected. The cobbles were then scrubbed and the biomass collected in 250 ml of the river water. Aliquots (5 ml) were filtered for chlorophyll-*a* (GF/C Whatman; n = 36, no samples in June) and DNA extraction (Polycarbonate 0.2 μm GTTP Millipore; n = 39, no sample from Chamblay in July due to bad climate conditions), then stored chilled in the dark, and subsequently frozen (-20°C) for later analysis in the laboratory. Subsamples (1 ml; n = 36, no samples in June) were fixed with lugol solution and stored at 4°C for later microscopic identification and enumeration.

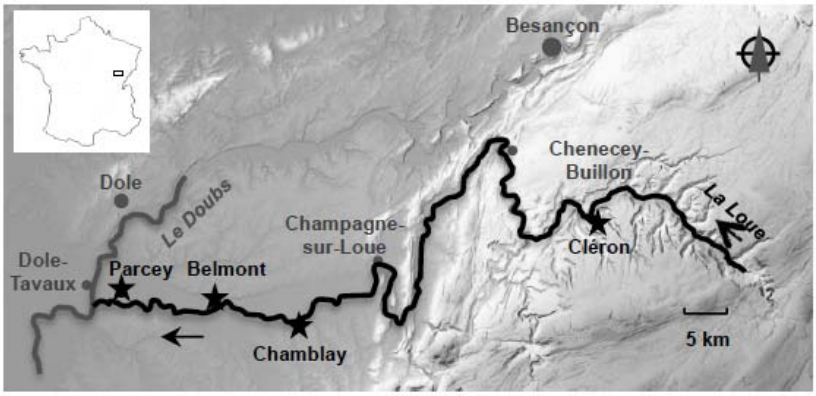

Figure 1. Geographic locations of the sampling sites (represented by stars) from the Loue River. Arrows represent the flow direction. Lighter zones represent altitudes higher than 300 meter above sea level.

Environmental and physico-chemical parameters

Local water temperature, pH, current velocity and depth were measured at each sampling point for each sampling date. In addition, flow rate was obtained from three survey stations (Chenecey-Buillon, Champagne-sur-Loue and Parcey; Figure 1) from Hydro France (http://hydro.eaufrance.fr). Precipitations were acquired for Chenecey-Buillon and Dole-Tavaux stations (Figure 1) from Info climat (http://infoclimat.fr). Moreover, phosphates (PO_4), dissolved oxygen (DO) and suspended matter concentrations as well as, conductivity, pH, and turbidity were obtained for station Chenecey-Buillon from the Port Douvot depuration station.

Chlorophyll-a extraction and quantification

Glass fiber filters were extracted in darkness with 90% methanol (10 ml) in falcon tubes (15 ml). The tubes were shaken manually, vortexed (30 sec), incubated (24h, 4°C) and centrifuged (10 min, 3220 ×*g*). Absorbance of the supernatant was measured at 665 nm with a correction for turbidity at 750 nm, using a spectrophotometer (Agilent Technologies Cary 60 UV-vis, USA). Talling and Driver (1963) equation was adapted to calculate chlorophyll-*a* (Chl-*a*)

concentrations ($\mu g.cm^{-2}$) from the known sampling area defined by the surface of an underwater viewer (707 cm^2).

Counting procedure and identification of photosynthetic microorganisms

Lugol's iodine preserved samples were homogenized and were diluted in Milli-Q water. Identification and enumeration of the samples were performed using a photonic microscope (200× magnification, Nikon Optiphot-2, Japan), and a Malassez chamber (Marienfeld, Germany). Cells were only identified as belonging to cyanobacteria, diatoms or green algae. Cells from other taxonomic groups were not considered as they were always in ultra-minority. All cells contained in 25 squares of the Malassez chamber were counted and analysis of triplicate aliquots undertaken. The length and the width (or diameter for filaments) of the cells from the three photosynthetic groups were estimated during the counting. For diatoms and green algae, the height was estimated according to literature (Olenina et al., 2006). For all species, biovolumes were then calculated according to Olenina et al. (2006) and Hillebrand et al. (1999).

DNA extraction

The DNA extraction procedure was based on mechanical and chemical extraction and was adapted from the procedure described in Massana et al. (1997). A quarter of each polycarbonate filters were resuspended in 1.1 ml of lysis buffer (50 mM Tris-HCl, 40 mM EDTA, 0.75 M sucrose) and were crushed using Lysing matrix E tubes in a FastPrep homogenizer (MP Biomedicals). A lysozyme digestion (0.5 mg/ml) was performed for 45 min at 37°C. This was followed by a second lysis with sodium dodecyl sulphate (SDS, 1%) and proteinase K (0.2 mg/ml, Sigma-Aldrich) for 1h30 at 55°C. Then, the samples were centrifuged at 13000 ×g for 5 min at 4°C. The supernatant was collected and an equal volume of phenol-chloroform-isoamyl alcohol was added. After centrifugation for 10 min, the supernatant was recovered and an equal volume of chloroform was added. Following another centrifugation, sodium acetate (10% of the recovered volume) and isopropanol (60% of the recovered volume) were added to the supernatant to precipitate the nucleic acids. The samples were stored at -20°C overnight. The DNA was then pelleted by centrifugation at 13000 ×g for 30 min at 4°C. The pellet was washed with cold 80% ethanol, dried and resuspended in ultrapure water. The nucleic acid

concentration was then determined by spectrophotometry (Nanodrop 1000, Thermo Fischer Inc, USA) at a wavelength λ = 260 nm. Samples were then stored at -20°C.

PCR and pyrosequencing

A region of the 16S rRNA gene including the V3-V4 region was selected for tag pyrosequencing. This region was amplified using the bacterial forward primer 563F (Claesson et al., 2010), which also included the Roche 454 pyrosequencing adapter FLX A and a unique 10 bp barcode. The bacterial reverse primer 907rM (Schauer et al., 2003) was also used and included the Roche 454 pyrosequencing adapter FLX B adaptor. The PCR reaction volume of 50 µl contained 1X Phusion HF Buffer, 0.6 mM of BSA, 1 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide, 0.4 µM of each primer, 1U of Phusion HF Polymerase (Thermo scientific, EU Lithuania), 10 ng of DNA template, and complete up to 50 µl with nuclease-free water. Polymerase chain reactions (PCR) were performed under the following conditions: 98°C for 2 min; 30 cycles of 98°C for 10 sec, 52°C for 30 sec and 72°C for 1 min; followed by 72°C for 10 min.

For 18S rRNA gene sequencing, primers including the V4 region were used, P45F and P47R (Dorigo et al., 2002) following the pyrosequencing procedure described above. The PCR reaction volume of 50 µl contained 1X Phusion HF Buffer, 1 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide, 0.4 µM of each primer, 1U of Phusion HF Polymerase (Thermo scientific, EU Lithuania), 10 ng of DNA template, and complete up to 50 µl with nuclease-free water. PCR were performed under the following conditions: 98°C for 1 min; 30 cycles of 98°C for 10 sec, 57°C for 30 sec and 72°C for 50 sec; followed by 72°C for 10 min.

Each DNA extract was amplified by three replicate PCR reactions, which were then pooled. These PCR products were then purified using MinElute PCR purification kit (Qiagen, Venlo, The Netherlands). The amount of DNA in each sample was quantified using the Qubit dsDNA HS assay (Invitrogen, Carlsbad, CA, USA). Finally, the PCR products were combined together in equimolar amounts and sequenced using the Genome Sequencer GS FLX Titanium 454 of GATC Biotech (Roche Company Branford, CT, USA).

Bioinformatics analysis

The 454 pyrosequencing of the 16S and 18S rRNA gene produced 644,701 and 392,109 raw sequences respectively. All sequences were cleaned by applying PANGEA trimming (Giongo et al., 2010) with a quality threshold (>23), a minimum sequence length of 270 bp for 16S rRNA and 200 bp for 18S rRNA genes and removal of sequences with error in the forward primer. The remaining sequences were clustered using USEARCH (Edgar, 2010) at 97% and 95% similarity threshold for bacterial and eukaryotic sequences respectively (Caron et al., 2009) and were then grouped according to different taxonomic levels using the SILVA base (Pruesse et al., 2007). The process was automated by PANAM (http://code.google.com/p/panamphylogeneticannotation) that constructs phylogenetic trees for taxonomic annotation (Taib et al., 2013), the assignment method used was LCA (Lowest Common Ancestor). After the removal of low-quality sequences and singleton, a range of 10,117 to 28,112 sequences were obtained for bacterial communities and a range of 885 to 16,261 sequences were obtained for eukaryotic communities. For further analyses, from a total of 33 successfully analysed biofilm samples, only 10,116 and 884 sequences per sample were used respectively for 16S and 18S rRNA gene analyses. For the bacterial communities, within the 333,828 sequences analysed (10,116 sequences per sample), 13,799 OTU (Operational Taxonomic Unit) were obtained. For the eukaryotic communities, within the 29,172 sequences analysed (884 sequences per sample), 519 OTU were obtained. The eukaryotic community was also characterized by the analysis of the 15,764 16S rRNA gene sequences affiliated to chloroplast (SILVA base), which were then classified according to the phylogenetic assigned by BLAST on NCBI. OTUs were classified as abundant (≥1%; Pedrós-Alió, 2006), intermediate abundant (<1% and >0.01%; Mangot et al., 2012) and rare (≤0.01%; Galand et al., 2009). Eukaryotic and cyanobacterial classifications were updated respectively according to the latest revised classification from Adl et al. (2012) and Castenholz (2001).

Statistical analysis

All statistical analyses and figures were performed using the statistical software package R 2.15.1 (R Development Core Team, Vienna, Austria). Only significant differences at $P < 0.05$ were considered.

Sampling site and date effects on biomasses (Chl-*a*, cyanobacteria, diatoms and green algae), diversity indices and abundance of microbial groups were analysed using 2-factor ANOVA tests, followed by Tuckey tests.

Diversity indices (richness, Chao1, Shannon and Evenness) and rarefaction curves were calculated using the package vegan (Dixon, 2003). Correlation coefficients between bacterial and micro-eukaryotic diversity indices were calculated with "cor" function using spearman method. The beta diversity was also estimated; conceptually Beta diversity is the extent of change of community composition (Whittaker, 1960) among sites within a geographical area of interest. In this study we apply the beta diversity to the OTU level in order to measure the variation between sites and dates of the bacterial and eukaryotic communities. Following the procedure described by Baselga (2010), beta diversity was estimated on presence-absence data (normalized) with the pairwise Sorensen dissimilarities to compare the spatial and temporal variation of the beta diversity. In addition beta diversity was partitioned in turnover (OTU replacement; β_{sim}) and nestedness (OTU loss; β_{sne}) using betapart package (Baselga et al., 2015).

Three hellinger transformations were first done on 454-pyrosequencing normalized data (bacterial sequences data set without OTU identified as chloroplasts, bacterial sequences data set identified as chloroplasts and micro-eukaryotic sequences data set) and then, principal component analyses (PCA), and co-inertia analyses were performed using the Ade4TkGUI software (Thioulouse and Dray, 2007). Permutational Multivariate ANOVA (PERMANOVA), and Mantel tests were performed to test for differences in bacterial and micro-eukaryotic (16S rRNA chloroplasts and 18S rRNA) communities among the different sampling sites and dates using vegan package (Oksanen et al., 2016).

Network analysis

To determine associations among the dominant bacterial and micro-eukaryotic OTU (>1% of the total sequences for at least one of the 33 samples) and environmental data, we calculated Spearman correlations using the SparCC method (Sparse Correlations for Compositional data; available at https://bitbucket.org/yonatanf/sparcc; Friedman and Alm, 2012) among relative abundances of dominant bacterial and eukaryotic OTU (based on 454 pyrosequencing data set of the 16S bacterial and chloroplastic rRNA gene sequences) and environmental data (local water temperature, current velocity, pH and global water flow rate) for all the samples (n=33).

The resulting matrix of correlation coefficients was transformed in R using the function "exportNetworkToCytoscape" of the WGCNA library. Only correlations < -0.5 and > 0.5 were considered as statistically significant. Co-occurrence networks were then visualized using Cytoscape software (Shannon, 2003) that depicts data sets as nodes (dominant bacterial and eukaryotic OTU) connected by edges (significant positive or negative correlations).

Results

Environmental conditions

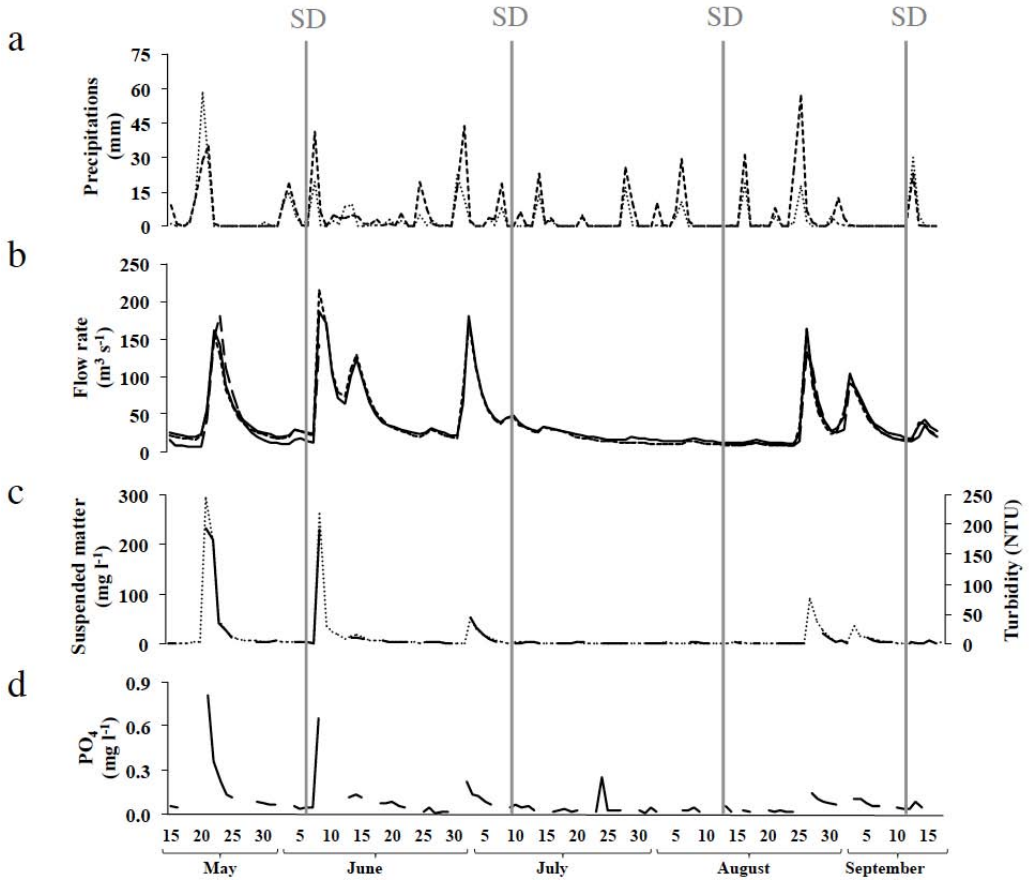

Figure 2. (a-b) Environmental parameters measured during the sampling period at different stations from the Loue River (Chenecey-Buillon station = small dash, Champagne-sur-Loue station = solid line, Parcey station = long dash and Dole-Tavaux station = dots); (c-d) Physico-chemical parameters at Chenecey station (suspended matter and phosphates = solid lines; turbidity = dots). SD = Sampling Date on the four sampling sites.

Precipitations and flow rates showed the same patterns at different stations along the Loue River (Figure 2a-b). Four major events of high precipitations (20/05, 07/06, 01/07 and 24/08; see Figure 2a) were recorded and followed by episodes of high flow rates in the river. At the same time, an increase of suspended matters and turbidity, as well as an increase of phosphates

concentrations were also detected in the Chenecey-Buillon station (Figure 2c-d). Only one period of low and stable water flow was observed, between mid-July and the end-August. Conductivity (464 µS cm^{-1} ± 21) and pH (8.0 ± 0.1) remained stable over the study. Dissolved oxygen concentrations were also stable (9.8 mg l^{-1} ± 0.9) except a decrease observed between the 20 to 25th of August (7.3 mg l^{-1} ± 0.9), at the end of the low-flow period (data not shown). Data resulting from the measurements performed on the different sampling sites are summarized in Table 1. While pH was relatively stable among the different sampling sites and dates, we observed a spatial and temporal variability in water temperature. Indeed, water temperature was lower in Cléron than in the other sites and regarding the variations among dates, as expected under this latitude, the higher temperatures were registered in August.

Table 1. Environmental and physico-chemical data measured at each sampling site and date

Sampling site	Sampling date	Water depth (cm)	Water pH	Water temperature (°C)	Current velocity ($m.s^{-1}$)
Cléron	June	NA	8.7	11.7	0.1 ± 0.2
	July	NA	8.6	11.6	0.7 ± 0.1
	August	29 ± 6	8.6	15.3	0.3 ± 0.2
	September	32 ± 3	8.7	13.3	0.5 ± 0.3
Chamblay	August	46 ± 5	8.4	23.0	0.3 ± 0.0
Belmont	July	43 ± 3	8.5	17.3	0.6 ± 0.2
	August	46 ± 12	8.3	22.1	0.3 ± 0.1
Parcey	June	NA	8.8	15.5	0.7 ± 0.5
	July	22 ± 4	8.5	16.3	0.3 ± 0.1
	August	25 ± 1	8.3	20.6	0.3 ± 0.1
	September	34 ± 15	8.5	17.2	0.6 ± 0.2

Values (± s.d.) corresponded to means of three replicates environmental measurements

Variations in the biomasses and structure of the photosynthetic microbial communities in the biofilms

Total Chl-*a* values ranged between 2 and 10 µg.cm^{-2} (Figure 3). The higher values were found at the end of the sampling period (September), except for Cléron where the Chl-*a* concentrations were higher in July. In addition, Belmont showed significant lower biomasses compared to Cléron and Chamblay (2-factor ANOVA tests; $P < 0.01$; Supplemental Table 1).

When looking at the spatial and temporal evolution of the structure of biofilm photosynthetic communities (assessed from the estimation of biovolumes of cyanobacteria, diatoms and green algae under microscope), it appears that these communities were always dominated by diatoms, followed by cyanobacteria and then by green algae (Figure 3). Conversely to diatoms, cyanobacterial proportions significantly increased in August/September in three of the four sampling sites (Chamblay, Belmont and Parcey) (2-factor ANOVA tests; *P*

< 0.001). In the same way, there was also an increase in green algae proportions in August (2-factor ANOVA tests; $P < 0.05$).

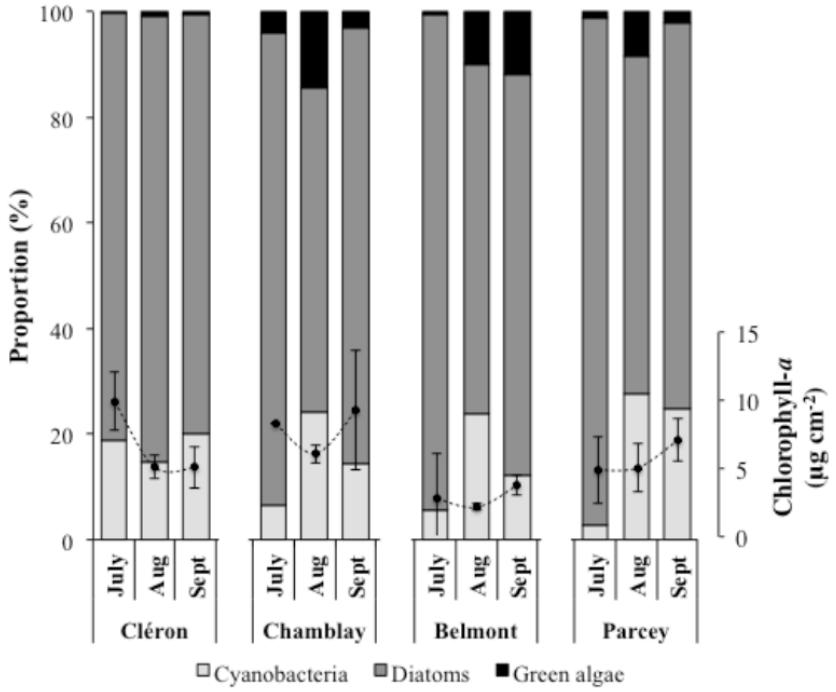

Figure 3. Spatiotemporal variations in the main photosynthetic taxonomic groups from the Loue River biofilms estimated by microscopic counting (stacked histogram) compared to total Chlorophyll-*a* (black circles) estimated by spectrophotometric measurment. Abbreviations: Aug, August; Sept, September. Error bars represent standard deviation.

Molecular study on the diversity of bacterial and micro-eukaryotic communities within biofilms

After normalisation, 10,116 and 884 sequences per sample were used respectively for 16S and 18S rRNA gene analyses. The rarefaction curves and Chao1 have shown that less sequencing depth was required to capture micro-eukaryotic diversity than to capture bacterial diversity (Supplemental Figure 1, Supplemental Table 2), nevertheless our data let us to describe an important fraction of the bacterial community. According to classic used diversity indices (*i.e.* richness, Shannon and Evenness), the bacterial community within the Loue River biofilms displayed a larger richness and diversity than the micro-eukaryotic community among the different sampling sites and dates (Supplemental Table 2). When comparing all the sampling sites, no significant correlation was found in the variations of the richness and diversity in bacterial and microeukaryote communities (data not shown).

When looking at the temporal evolution of the beta diversity in bacterial and micro-eukaryotic communities, our analysis revealed that total beta diversity values were always high (>0.6) and displayed not significant difference in regard to the sampling dates (Figure 4a,b). In the same way, only small changes were found in the relative contribution of turnover and

nestedness, meaning that changes in microbial communities from one sampling date to another were mainly due to OTU replacements inside these communities.

Regarding to the sampling sites (Figure 4c,d), the beta diversity displayed a decreasing trend from upstream to downstream for both communities but this decrease was only statistically significant for the eukaryotic community ($P < 0.05$, ANOVA). Interestingly, the relative contribution of turnover and nestedness in the values of the total beta diversity was different in bacterial and eukaryotic communities, meaning that OTU loss has a greater contribution in beta diversity of these latter than in bacterial communities.

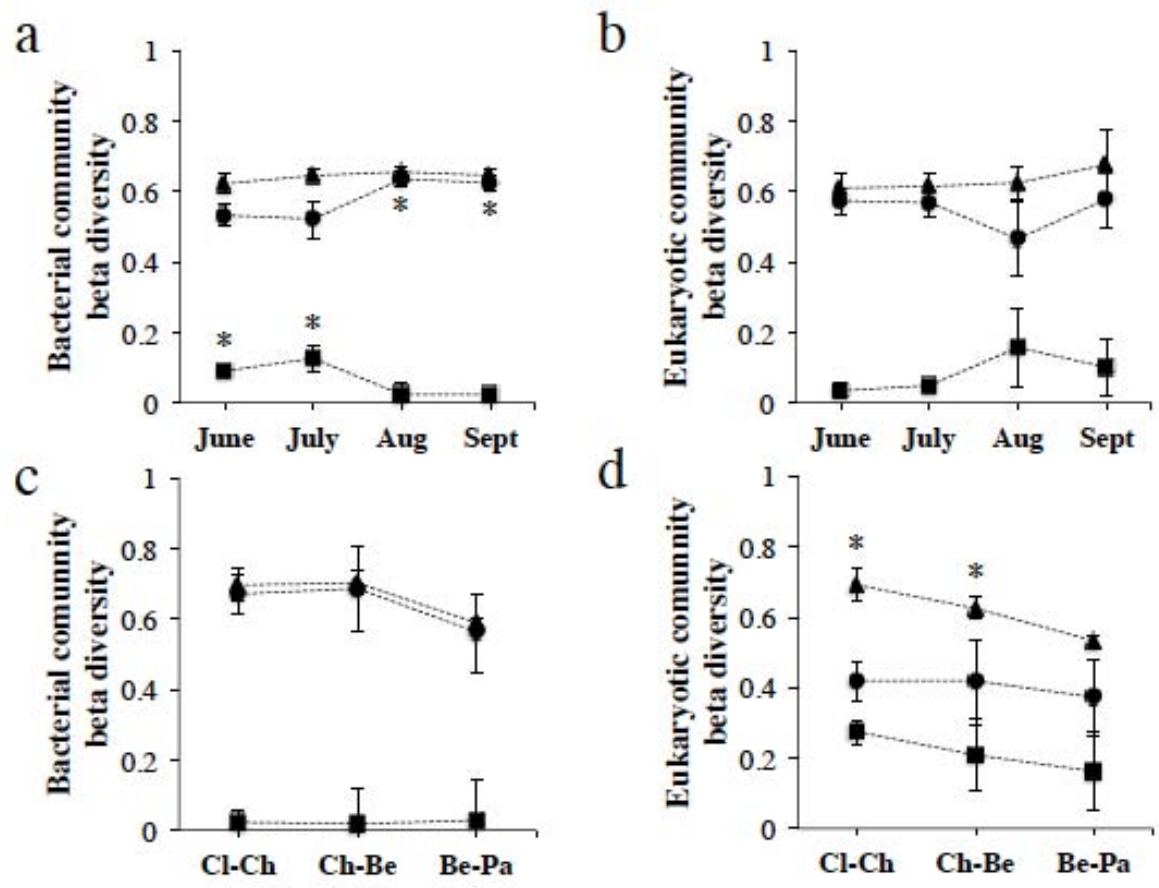

Figure 4: Temporal variation of the beta-diversity based on OTU comparison between Cléron and Parcey for bacterial (a) and eukaryotic (b) communities; Spatial variations of the beta-diversity in August based on OTU comparison in bacterial (c) and micro-eukaryotic (d) communities. Total beta-diversity = triangles, turnover = circles and nestedness = squares. Abbreviations: Aug, August; Sept, September; Cl, Cléron; Ch, Chamblay; Be, Belmont; Pa, Parcey.

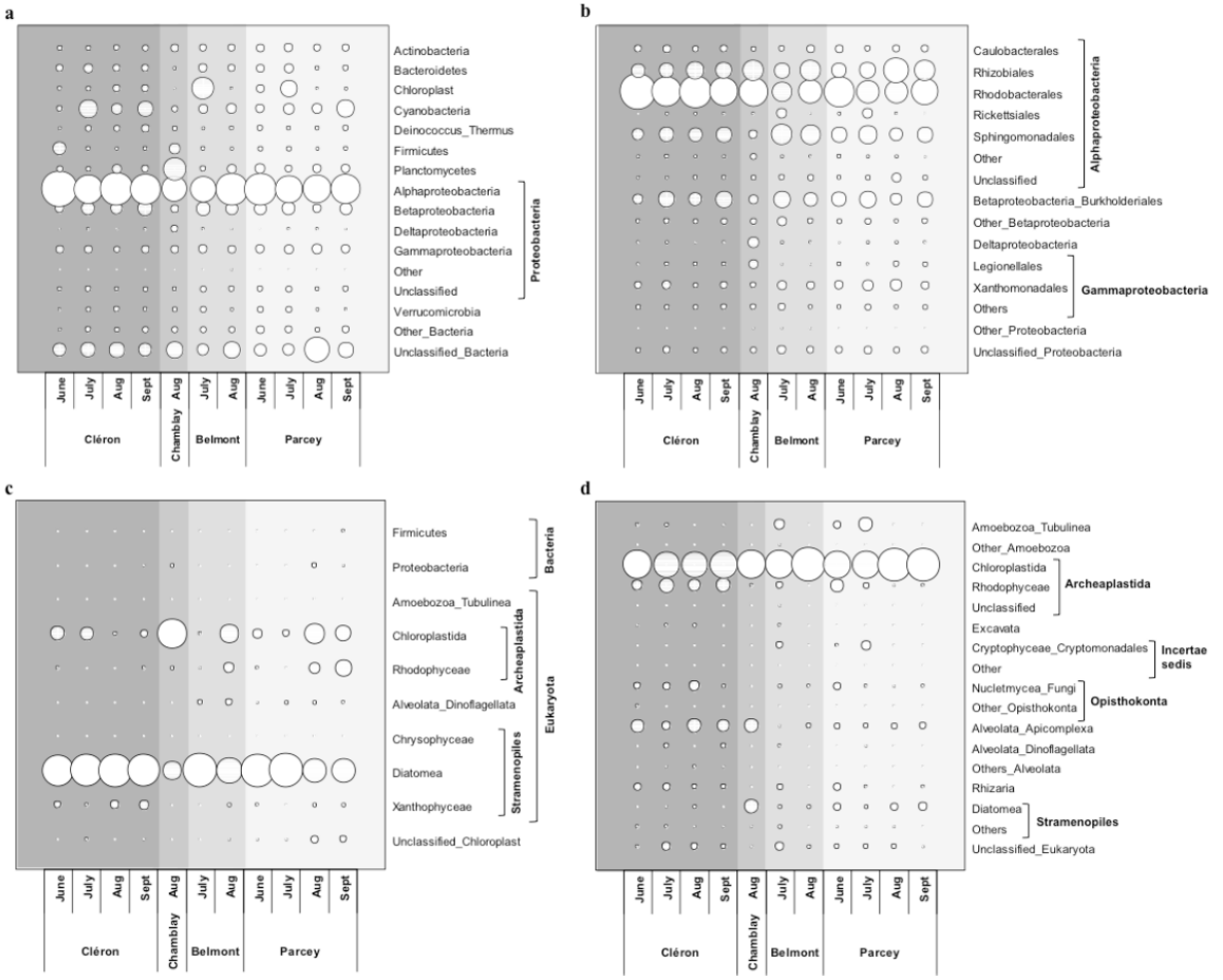

Figure 5. Spatio-temporal variations in the composition of the bacterial (a), Proteobacterial (b), micro-eukaryotic (c) and chloroplastic (d) communities based on 16S (a,b,d) and 18S (b) rRNA gene sequences. Circles represent the mean proportions of microbial sequences in the three replicates for each sampling site and date. Chloroplastic 16S rRNA gene sequences (SILVA base) were classified according to the phylogenetic assignment by BLAST on NCBI. Abbreviations: Aug, August; Sept, September.

Molecular study on the composition and structure of bacterial and micro-eukaryotic communities within biofilms

Pyrosequencing data revealed that the bacterial communities were dominated by Proteobacteria (58%) followed by cyanobacteria (7%) and Planctomycetes (5%) (Figure 5a, Supplemental Table 3). Among Proteobacteria, the Rhodobacterales order (Alphaproteobacteria) contained more than 40% of the Proteobacteria sequences (Figure 5b, Supplemental Table 3). When looking at the relative abundance of the bacterial OTU, 43% of all the sequences belonged to the nine most abundant OTU (>1% of all sequences for all the samples), and six of these nine OTU belonged to the Rhodobacterales order (Supplemental Table 3). The rest of the abundant OTU were represented by cyanobacteria (*Chamaesiphon* sp.) and two other were unclassified. According to the phylogenetic assignment by BLAST on NCBI, these two unclassified

bacterial OTU displayed 98% and 93% sequence similarity with cyanobacteria (*Chamaesiphon* sp.) and Rhizobiales order respectively.

From the sequencing of the 18S rRNA, it appeared that Chloroplastida (68%), totally represented by Chlorophyta, were dominant in the micro-eukaryotic community from the Loue River biofilms, followed by Alveolata (8%, principally represented by Apicomplexa), Rhodophyceae (7%), Stramenopiles (4%), Tubulinea (3%), Fungi (3%), Rhizaria (2%) and Chryptophyceae (1%) (Figure 5d, Supplemental Table 4). Moreover the 15 abundant OTU contained 90% of all the sequences, seven of them belonging to Chlorophyta (Supplemental Table 4).

The composition of the micro-eukaryotic community was also characterized by a BLAST analysis, which was performed on the 15,764 16S rRNA gene sequences affiliated to chloroplast. In contrast with results from 18S rRNA sequencing, 91% of the chloroplastic sequences were affiliated to Diatomea while only 4% were affiliated to Chloroplastida (Figure 5c, Supplemental Table 5). Diatomea represented 17 of the 19 abundant OTU, while the rest the abundant OTU were affiliated to Chlorophyta and Dinoflagellata (Supplemental Table 5).

Consequently, these two analyses performed on the eukaryotic communities from the Loue River provide very contrasting findings on the relative proportions of diatoms and green algae in our samples, knowing that microscopic enumerations had highlighted a large dominance of diatoms as assessed by chloroplastic sequences.

Comparison of the bacterial and micro-eukaryotic biofilm communities among sampling sites and dates

Principal component analyses (PCA) were performed on the distribution of all OTU for both bacterial and micro-eukaryotic communities (based on 16S rRNA normalized data set without chloroplastic sequences, 18S rRNA and 16S rRNA chloroplastic normalized data sets respectively) (Figure 6). In the three PCA, significant effects of sampling site, sampling date and interaction between these two factors ($P < 0.001$, PERMANOVA) were detected.

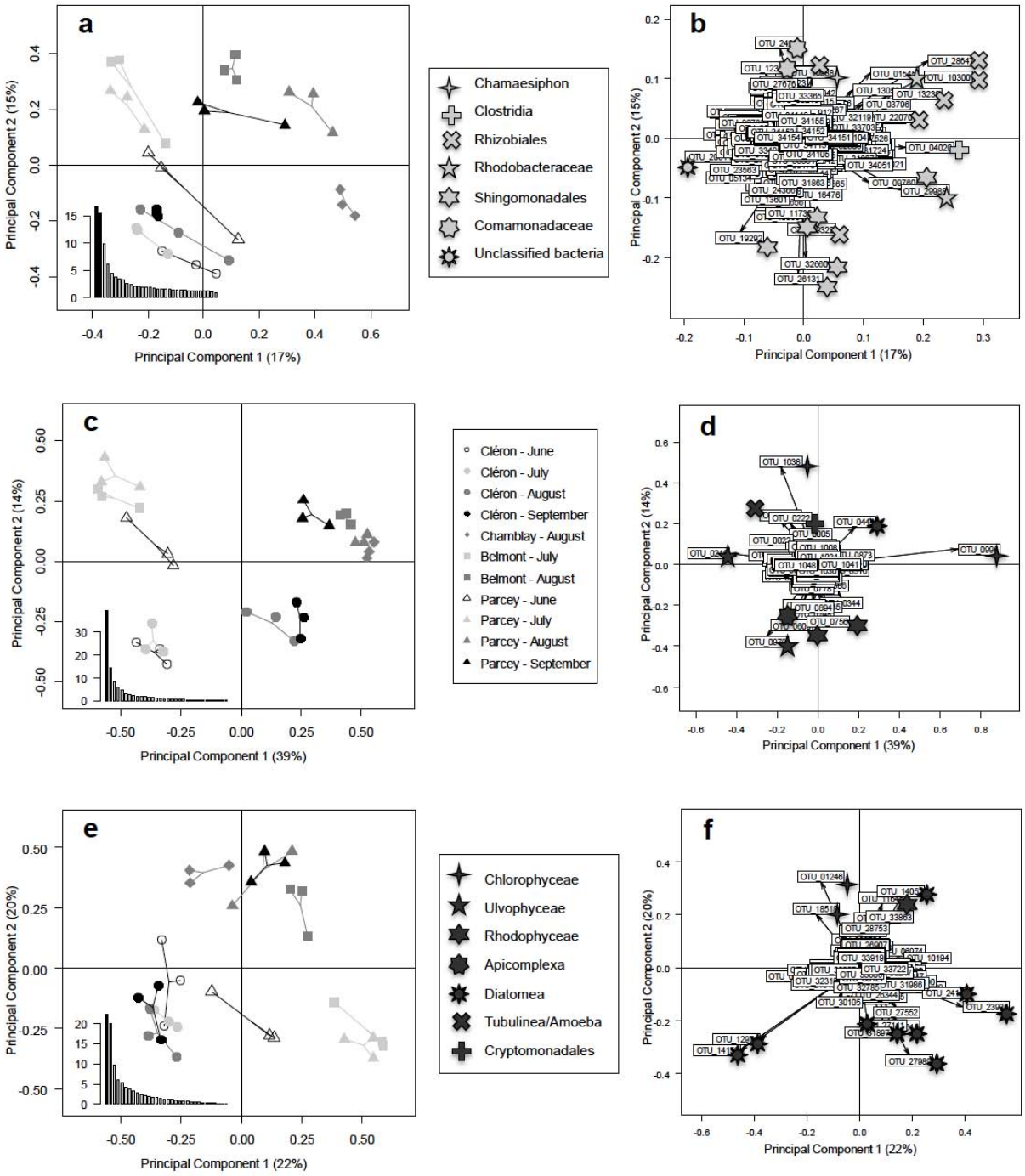

Figure 6: Principal component analyses (PCA) performed on 454-pyrosequencing data of the 16S (a,b,e,f) and 18S rRNA gene sequences (c,d), representing respectively the diversity of the bacterial communities (a,b), eukaryotic (c,d) and chloroplastic (e,f) communities among the different sampling sites and dates. a,c,e represent samples distribution in the two first principal components and b,d,f represent the OTU contribution in PCA.

First, a clear distinction was found between microbial communities sampled in June/July and those sampled in August/September on the first axes of 16S rRNA bacterial PCA (Figure 6a) and 18S rRNA PCA (Figure 6c) and on the second axis of 16S rRNA chloroplastic PCA

(Figure 6e), excepted for the bacterial and chloroplastic sequences of the upstream sampling station of Cléron, which were grouped together. More in details, a significant increase of Rhizobiales and Chloroplastida (particularly members of Chlorophyceae) was found in August/September compared to July while numerous bacterial and micro-eukaryotic groups such as for example Acidobacteria, Betaproteobacteria, Ulvophyceae and Amoeba displayed a significant decrease in August/September (ANOVA and Tuckey tests, Supplemental Table 6).

Second, it appeared on the first axis of 16S rRNA chloroplastic PCA and on the second axes of 16S rRNA bacterial and 18S rRNA PCA that the bacterial and micro-eukaryotic communities displayed spatial differentiation, in particular between Cléron and the others sampling sites (Figure 6). More in details, significant differences were found between Cléron and the other sampling sites in the relative abundance of some microbial groups such as Rhodobacteraceae and Rhodophyceae, which were overrepresented in Cléron and conversely Acidobacteria, Chlorophyceae and Stramenopiles which were significantly underrepresented in in this station (ANOVA and Tuckey tests, Supplemental Table 6). While PCA analysis based on 16S rRNA chloroplastic sequences was more representative of microscopic observations that discriminated mostly the abundant Diatomea OTU, PCA analysis based on 18S rRNA sequences was also important to show spatiotemporal differences in microbial communities for Archeaplastida OTU (Chlorophyceae, Ulvophyceae and Rhodophyceae) and non photosynthetic micro-eukaryotics OTU (Apicomplexa, Amoeba and Cryptomonadales). These three complementary analyses have shown thus similar spatiotemporal differences within samples. Indeed, co-inertia analyses performed among 16S rRNA bacterial data, 16S rRNA chloroplastic data and 18S rRNA data, revealed significant correlations between all these data ($P < 0.001$, Mantel tests).

Cooccurences occurring among the dominant bacterial and eukaryotic OTU within biofilms and environmental data

To assess putative microbial interactions within biofilms sampled in the Loue River among the different sites and dates and also with environmental conditions, a cooccurence analysis was performed. Among the 103 dominant bacterial and micro-eukaryotic OTU analyzed (based on 16S rRNA bacterial and chloroplastic sequences), 97 of them displayed many positive and negative correlations drawing in this way a complex cooccurences network (Figure 7).

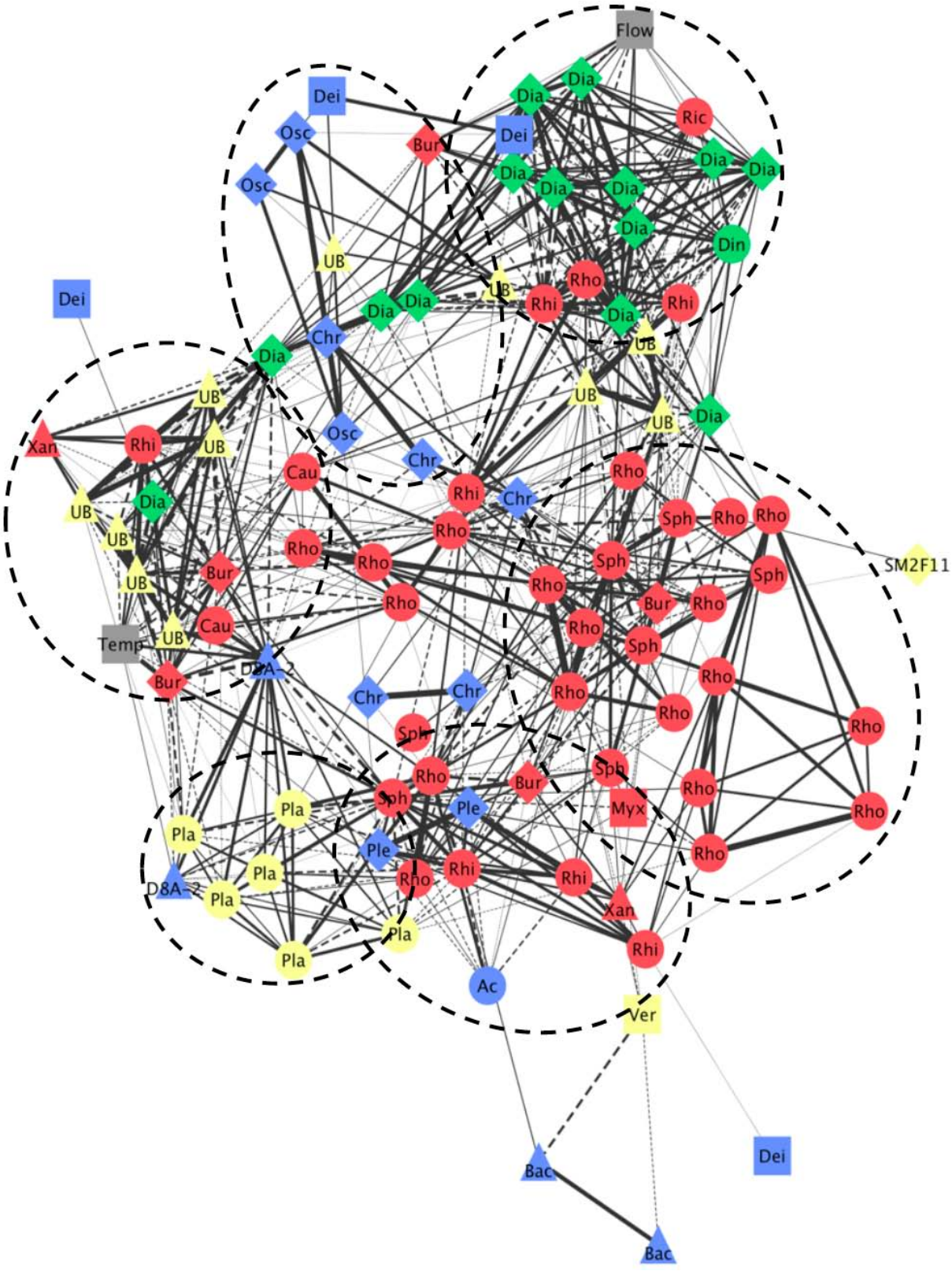

Figure 7. Cooccurences networks based on Spearman correlations among the relative abundances of the dominant bacterial and micro-eukaryotic OTU (based on bacterial and chloroplastic 16S rRNA) and environmental data (local water temperature, current velocity, pH and global water flow rate) for all the sampled biofilms. Only significant correlations were represented (< -0.5 or > 0.5). Circles highlight six main OTU groups. Symbols represents the different microbial OTU identified as follow: blue circle for Acidobacteria, blue diamonds for Cyanobacteria, blue squares for Deinococcus-Thermus, blue triangles for Firmicutes, yellow circles for Planctomycetes, red circles for Alphaproteobacteria, red diamonds for Betaproteobacteria, red squares for

Deltaproteobacteria, red triangles for Gammaproteobacteria, yellow diamond for SM2F11, yellow squares for Verrucomicrobia, yellow triangles for unclassified Bacteria, green circle for Alveolata and green diamonds for Stramenopiles; dash and solid lines represent negative and positive correlations respectively; line thickness is proportional to the value of correlations between two nodes, thick and thin lines correspond respectively to high (near |1|) and low (near |0.5|) correlations.

Abbreviations: Ac, Acidimicrobiales; Chr, Chroococcales; Osc, Oscillatoriales; Ple, Pleurocapsales; Dei, Deinococcales; Bac, Bacilliales; Pla, Planctomycetales; Cau, Caulobacterales; Rhi, Rhizobiales; Rho, Rhodobacterales; Ric, Rickettsiales; Sph, Sphingomonadales; Bur, Burkholderiales; Myx, Myxococcales; Xan, Xanthomonadales; Ver, Verrucomicrobiales; UB, unclassified bacteria; Dino, Dinoflagellata; Dia, Diatomea; Flow, global water flow rate; Temp, local water temperature.

Among these 103 dominant OTU, more than 60% of them are facultative or obligatory phototrophic microorganisms (Rhodobacterales, Rhizobiales, Rickettsiales, Sphingomonadales, Cyanobacteria, Dinoflagellata and Diatomea). When looking at all the significant interactions detected between OTU into the network, it appeared that more positive correlations (61%) were observed between OTU than negative ones. When looking at the positive interactions, six main OTU groups were identified into the network and were largely dominated by one taxonomic group of microorganisms (for example the group I of diatoms). Concerning the negative correlations, most of them occurred between OTU belonged to phylogenetically not related classes as for example those found between Diatomea and some Alphaproteobacteria OTU (Rhodobacterales and Rhizobiales). Interestingly, when considering cyanobacteria, these microorganisms displayed strong positive interactions between them and also with OTU belonging to Burkholderiales, Shingomonadales and Rhizobiales orders and negative interactions with OTU belonging to Rhodobacterales order (Supplemental Table 7).

Finally, when considering environmental variables, only global flow rate and local water temperature displayed significant correlations with some microbial OTU (Figure 7). In particular, a positive correlation was found between global flow rate and the presence of Diatoms.

Discussion

Deciphering the structure and composition of microbial communities inside river biofilms and the interactions occurring among them, and identifying the impact of the main environmental factors and processes on these communities are of particular importance for a better understanding of the functioning of riverine biofilms. In this way, it seems essential to consider together bacterial and micro-eukaryotic communities because both are functionally closely associated in the organic matter producing-recycling processes. In this global framework, our

study on the Loue River biofilms has provided new insights on these relationships occurring between prokaryote and micro-eukaryote communities.

The first interesting finding provided by our study was to show that if as expected, the richness and diversity of bacterial communities was always higher than those of micro-eukaryotes communities, the spatiotemporal variations of these two indices in the bacterial communities were not correlated to those occurring in the microeukaryote communities. On the other hand, a decrease in the beta-diversity of these two communities was found from the upstream part to the downstream part of the river, in agreement with previous findings of Besemer et al. (2013). Moreover, the same global patterns of spatiotemporal variations were detected in the structure of bacterial and micro-eukaryotic communities. All together, these findings suggest that changes occurring in the environmental conditions have an impact on both bacterial and micro-eukaryote communities but that the variations in the richness and diversity of these communities cannot be considered as a good indicator of this impact. This can be probably explained by the fact that even if high-throughput sequencing approaches allow us to better describe the rare biosphere, the estimation of richness and diversity in microbial communities remains problematic (Haegeman et al., 2013).

Among the changes occurring in both communities (bacteria and micro-eukaryotes), it is interesting to consider the evolution in the proportions of cyanobacteria, diatoms, and green algae. Except for the sampling station of Cléron, the increase of water temperature associated with a decrease of water velocity in August and September leads to a concomitant increase of cyanobacterial and green algae and a decrease of diatoms proportions in the downstream stations. These findings are in agreement with other studies describing diatoms as « cool season species » and cyanobacteria as « warm/hot season species » (Coles and Jones, 2000; Sabater et al., 2003; van der Grinten et al., 2005). Interestingly, we found that microbial communities from Cléron sampling site displayed less variations in the seasonal evolution of their composition compared to those sampled in the downstream stations, knowing that in the Loue river, the upstream part is located in a karst landscape while the downstream part is located in a river plain (Calinon, 1955; Chapuis et al., 2006). Moreover, in the upstream part of the river, the water temperature was at least 4-5°C lower than in the downstream part and water temperature in Cleron station displayed less temporal variations due to multiple cold water resurgences occurring along this part of the river. All together, these findings support the primordial role of water temperature in the evolution of the composition of all biofilm

microbial communities, and not only on diatoms and cyanobacteria. These findings showed also that it is necessary to take into account the geological and hydrological contexts of the sampling stations when working along such a river.

One of other striking point of this study concern the high number of reads assigned to bacteria that are potentially able, in addition to cyanobacteria, to contribute to primary production inside these river biofilms. Indeed, 45% of the bacterial reads correspond to potential photosynthetic microorganisms belonging to Rhodobacteraceae, Rhizobiales, Sphingomonadales and Comamonadaceae groups, confirming previous studies highlighting, for example, the large abundance of purple non-sulfur bacteria (e.g. Anderson-Glenna et al., 2008; Beraldi-Campesi et al., 2012; Bricheux et al., 2013; Drury et al., 2013). Even if most of these bacteria can also be considered as heterotrophic (Blankenship, 2014), their photoautotrophic metabolism depends on the environmental conditions (anaerobic conditions for example), this finding suggest that it might be interesting to estimate their real contribution to primary production.

Concerning the multiple interactions occurring between microorganisms inside river biofilms, the fact that the strongest positive interactions occurred between OTU belonging to the same taxonomic group (for example between diatoms) suggests that environmental conditions leading to the high abundance of a given group allow also to promote and maintain a high richness inside the group. This finding is interesting to consider knowing that it has been shown that species that are phylogenetically closely related, generally display a high functional redundancy (*e.g.* Martiny et al., 2013) and this functional redundancy supports biodiversity and ecosystem function (*e.g.* Wohl et al. 2004). When looking more carefully at interactions occurring between photosynthetic microorganisms (cyanobacteria and microalgae) and bacteria, the two most impressive results concern the fact that cyanobacterial and diatoms OTU displayed negative correlations with Rhodobacterales OTU but positive correlations with OTU belonging to Burkholderiales and Sphingomonadales orders. Knowing that bacteria belonging to these two orders are known for their high ability to degrade complex organic matter (*e.g.* Cerniglia, 2003; Pérez-Pantoja et al., 2012), their high abundance in biofilms dominated by diatoms or cyanobacteria suggest that they might play a major role in the degradation of organic matter produced by these benthic photosynthetic microorganisms. On the other hand, the negative correlations registered with *Rhodobacter* OTU might reflect a competition with cyanobacteria and diatoms for acess to light.

From a more technical point of view, we identified two main issues concerning our molecular approach. The first one concerns the contrasting results obtained on the structure and composition of micro-eukaryotic communities when using 454-pyrosequencing of the 18S rRNA gene and microscope counting. The over-representation of Chlorophyta in the 18S rRNA sequences might be potentially explained by the number of copies of the rRNA operon into the cells. Indeed, while the number of 16S rRNA gene copies per cell varies from one to fifteen among the different bacteria (Klappenbach et al., 2001; Větrovský and Baldrian, 2013), the number of 18S rRNA gene copies per cell varies from one to hundreds of thousands among the different eukaryotes (Gong et al., 2013; Prokopowich et al., 2003; Zhu et al., 2005). Furthermore, Zhu et al. (2005) and Godhe et al. (2008) highlighted a significant relationship between cell biovolume and the number of rRNA gene copies per cell in marine phytoplanktonic communities. In addition to this problem concerning the number of rRNA copies, it cannot be excluded that the underrepresentation of diatoms in molecular approach can be partly explained by difficulties of breaking the shells during DNA extraction as suggested also by Medinger et al. (2010). On the other hand, the assessment of the structural diversity of the micro-eukaryotic communities using chloroplast 16S rRNA gene was much more congruent with our microscopic observations. Shi et al. (2011) have summarized that when studying the diversity of picoeukaryote communities in marine environments, studies using the 18S rRNA gene were heavily biased towards heterotrophic cells, while targeting plastid genes such 16S rRNA, *psbA* or specific 18S rRNA gene primers could be more effective strategies. The second technical issue concerns the high number of "unclassified bacteria" (2238 OTU, representing from 5 to 33% of the total bacterial communities) detected among the different samples knowing that the taxonomic assignation of all OTU was performed by LCA (Lowest Common Ancestor) method on SILVA database (http://www.arb-silva.de/aligner). It has been shown by Taib et al. (2013) that LCA provides more accurate assignation than nearest neighbor (NN) method, whatever the taxonomic level considered (from kingdom to genus). However, this method will assign as "unclassified" any OTU sequence surrounded by two contrasting phylogenetic contexts (e.g. two differing phyla, class, order or genus). When looking at these unclassified OTU using SeqMatch (RDP base, http://rdp.cme.msu.edu/seqmatch), most of them (64%) were identified as Proteobacteria and only 7% have any assignation with RDP.

In conclusion, this study has revealed several important findings that concern the structural and functional diversity of biofilms in rivers and the putative interactions occurring between the numerous OTU living together inside biofilms. Among the main questions raised

by this study, it would be very interesting to better known the relative contribution of all photosynthetic microorganisms in the primary production of these river biofilms knowing the high abundance of some groups like *Rhodobacter* that have not been considered until yet as major contributor to primary production in these ecosystems. One very important finding of this work was also to reveal that the different hydrological and geological contexts of the Loue River have an indirect impact on the seasonal variations occurring in the structure and composition of microbial communities by the way of their direct impact on the water temperature. Finally, this work also allowed us to remind that data provided by molecular approaches must be considered with caution and validated, when possible, by other approaches such as microscopic examination.

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SUPPLEMENTAL INFORMATION

Supplemental tables

Supplemental Table 1. Statistical analysis results of the effect of both sampling site and date on biofilm composition and diversity indices

	Site	Date	Site:Date
Chl-*a*	0.0005 ***	0.07 ^{ns}	0.04 *
Cyanobacterial biomass	0.03 *	0.22 ^{ns}	0.004 **
Diatom biomass	0.00004 ***	0.0005 ***	0.12 ^{ns}
Green algae biomass	0.0000001 ***	0.005 **	0.007 **
Cyanobacterial proportion	0.0009 ***	0.00001 ***	0.0007 ***
Diatom proportion	0.0003 ***	0.0000002 ***	0.0002 ***
Green algae proportion	0.0000003 ***	0.000008 ***	0.0001 ***

Significance of 2-factor ANOVA tests: ns, *, ** and *** indicate not significant and significant at levels of 0.05, 0.01 and 0.001 respectively.

Supplemental Table 2. Diversity indices in the bacterial (16S rRNA gene sequences) and micro-eukaryotic (18S rRNA gene sequences) communities

		16S diversity indices				18S diversity indices			
Site	Sampling date	Richness	Chao1	Shannon	Evenness	Richness	Chao1	Shannon	Evenness
Cléron	June	1218 ± 106	2381 ± 41	5.0 ± 0.4	0.70 ± 0.06	84 ± 14	138 ± 3	2.9 ± 0.3	0.65 ± 0.05
	July	1307 ± 290	2550 ± 840	5.5 ± 0.2	0.77 ± 0.01	99 ± 20	144 ± 16	3.3 ± 0.4	0.71 ± 0.05
	August	1658 ± 145	3342 ± 316	5.8 ± 0.0	0.78 ± 0.00	75 ± 14	133 ± 33	2.6 ± 0.3	0.60 ± 0.05
	September	1911 ± 227	3897 ± 253	6.0 ± 0.2	0.80 ± 0.02	81 ± 7	154 ± 17	2.4 ± 0.0	0.55 ± 0.01
Chamblay	August	1912 ± 348	3436 ± 842	6.0 ± 0.3	0.79 ± 0.03	27 ± 3	40 ± 12	1.3 ± 0.2	0.41 ± 0.03
Belmont	July	1984 ± 251	3856 ± 557	6.2 ± 0.4	0.81 ± 0.03	102 ± 13	161 ± 40	3.0 ± 0.2	0.66 ± 0.03
	August	2142 ± 202	4265 ± 439	6.4 ± 0.2	0.83 ± 0.01	57 ± 16	101 ± 39	1.6 ± 0.3	0.40 ± 0.06
Parcey	June	1797 ± 172	3554 ± 557	6.0 ± 0.4	0.80 ± 0.05	78 ± 27	140 ± 10	2.6 ± 0.8	0.60 ± 0.13
	July	2196 ± 221	4376 ± 542	6.5 ± 0.2	0.84 ± 0.01	91 ± 10	147 ± 4	2.8 ± 0.3	0.62 ± 0.04
	August	1885 ± 239	3841 ± 489	5.9 ± 0.2	0.78 ± 0.02	44 ± 18	84 ± 36	1.4 ± 0.3	0.38 ± 0.04
	September	2165 ± 275	4716 ± 1410	6.1 ± 0.0	0.81 ± 0.00	51 ± 14	93 ± 39	2.0 ± 0.1	0.50 ± 0.03

Supplemental Table 3. Taxonomic composition of the bacterial community in the Loue River biofilms based on the normalized 16S rRNA gene sequences characterized by pyrosequencing

			Reads	Total	Number of OTU at the 97% cut-off			
Phylum	Class	Order	(%)	seq nb	Total	Abundant	Interm.	Rare
Acidobacteria	Acidobacteria		0.3	932	161		6	155
Actinobacteria	Acidimicrobiia		1.6	5302	287		28	259
	Actinobacteria		0.6	2033	242		13	229
	Thermoleophilia		0.3	974	121		4	117
	Others		0.1	220	20		2	18
Armatimonadetes	Armatimonadia		0.1	254	23		2	21
Bacteroidetes	Cytophagia	Cytophagales	0.5	1559	125		9	116
	Flavobacteria	Flavobacteriales	1.4	4668	137		33	104
	Sphingobacteria	Sphingobacteriales	0.9	3094	290		24	266
	Others		0.0	28	11			11
BD1-5			0.0	97	21			21
Caldiserica			0.0	1	1			1
Candidate division OD1			0.1	231	49			49
Candidate division OP11			0.0	99	16		1	15
Candidate division SR1			0.0	35	8			8
Candidate division TM7			0.0	60	25			25
Candidate division WS3			0.0	2	1			1
Candidate division WS6			0.0	65	9			9
Chlamydiae			0.1	437	93		2	91
Chlorobi			0.0	48	11			11
Chloroflexi			0.3	1017	188		4	184
CK-1C4-19			0.0	7	1			1
Chloroplast			4.7	15764	190		41	149
Cyanobacteria	Chroococcales		4.0	13300	111	1	16	94
	Pleurocapsales		1.0	3219	28		5	23

	Oscillatoriales		2.4	7878	169		22	147
	Nostocales		0.0	8	2			2
	Others		0.0	107	16			16
Deinococcus-Thermus	Deinococci		1.5	5117	129		10	119
Fibrobacteres	Fibrobacteria		0.3	835	6		2	4
Firmicutes	Bacilli		1.1	3621	187		12	175
	Clostridia		1.0	3213	244		12	232
	Others		0.1	292	37		1	36
Fusobacteria			0.0	77	11		1	10
Gemmatimonadetes			0.0	100	25			25
JL-ETNP-Z39			0.0	3	1			1
Lentisphaerae	Lentisphaeria		0.0	20	7			7
Nitrospirae	Nitrospira		0.0	27	11			11
Planctomycetes	Planctomycetacia		5.2	17278	1309		102	1207
	Others		0.1	402	91		1	90
Proteobacteria	Alphaproteobacteria	Caulobacterales	1.6	5208	263		30	233
		DB1-14	0.0	142	33			33
		Rhizobiales	9.7	32296	1735		112	1623
		Rhodobacterales	24.0	80152	526	6	97	423
		Rhodospirillales	0.5	1535	108		10	98
		Rickettsiales	0.7	2405	220		15	205
		Sphingomonadales	7.4	24825	685		78	607
		Unclassified	0.9	2965	352		16	336
	Betaproteobacteria	Burkholderiales	6.1	20427	773		72	701
		Methylophilales	0.1	466	16		2	14
		Neisseriales	0.1	221	25		2	23
		Nitrosomonadales	0.1	289	49		3	46
		oca12	0.0	139	49			49
		Rhodocyclales	0.2	655	95		5	90
		SC-I-84	0.2	531	30		4	26

		Others	0.4	1416	129		7	122
	Deltaproteobacteria		0.6	1842	270		3	267
	Gammaproteobacteria	Chromatiales	0.2	532	17		4	13
		Legionellales	0.6	2103	315		9	306
		NKB5	0.2	774	143		1	142
		Pseudomonadales	0.3	969	71		6	65
		Xanthomonadales	2.2	7395	214		35	179
		Others	0.2	774	129		5	124
	Others		0.1	268	62		1	61
	Unclassified		1.5	4980	450		24	426
SM2F11			0.7	2172	58		13	45
Spirochaetes			0.0	25	3			3
Tenericutes	Mollicutes		0.1	318	30		3	27
TM6			0.0	135	45			45
Verrucomicrobia	Verrucomicrobiae		1.1	3737	145		17	128
	Others		0.1	455	102		2	100
WCHB1-60			0.0	18	5			5
Unclassified Bacteria			12.4	41235	2238	2	88	2148
Total			100	333828	13799	9	1017	12773

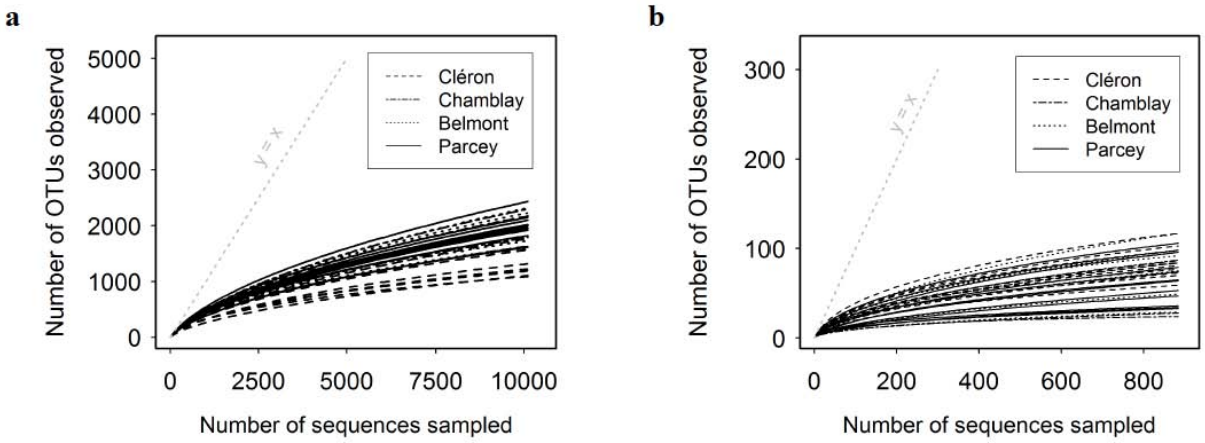
Supplemental Table 4. Taxonomic composition of the micro-eukaryotic community in the Loue River biofilms based on the normalized 18S rRNA gene sequences characterized by pyrosequencing

Taxonomic Affiliation			Reads (%)	Total seq nb	Number of OTUs at the 95% cut-off			
					Total	Abundant	Interm.	Rare
Amoebozoa	Discosea	Longamoebida	0.0	1	1			1
	Tubulinea	Euamoebida	3.1	904	23	1	11	11
	Unclassified		0.1	32	2		2	
Archeaplastida	Chloroplastida	Chlorophyta	67.7	19735	99	7	51	41
	Rhodophyceae	Bangiales	0.1	26	4		2	2
		Florideophycidae	0.0	2	2			2
		Unclassified	7.1	2069	11	3	4	4
	Unclassified		0.2	52	10		4	6
Excavata	Discoba	Discicristata	0.1	43	9		1	8
	Metamonada	Fornicata	0.3	98	3		2	1
		Parabasalia	0.0	3	2		1	1
Incertae sedis	Cryptophyceae	Cryptomonadales	1.1	333	6	1	1	4
		Unclassified	0.0	4	2			2
	Unclassified		0.0	4	1			1
Opisthokonta	Holozoa	Choanomonada	0.1	18	4		2	2
		Ichthyosphorea	0.1	23	3		2	1
	Nucletmycea	Fungi	2.7	785	90		19	71
		Rozella	0.1	21	9		1	8
SAR	Alveolata	Apicomplexa	7.3	2121	52	2	19	31
		Ciliophora	0.1	36	10		3	7
		Dinoflagellata	0.6	167	11		3	8
		Protalveolata	0.1	30	5		2	3
		Unclassified	0.0	10	5			5
	Stramenopiles	Bicosoecida	0.0	14	4		2	2
		Chrysophyceae	0.1	30	3		2	1
		Diatomea	3.6	1048	16	1	7	8
		Dictyochophyceae	0.1	25	1		1	
		Eustigmatales	0.0	1	1			1
		Hyphochytriales	0.1	30	2		1	1
		Labyrinthulomycetes	0.0	1	1			1
		Peronosporomycetes	0.2	61	6		1	5
		Unclassified	0.1	32	8		3	5
	Rhizaria	Cercozoa	2.3	660	30		14	16
		Unclassified	0.0	5	1		1	
Unclassified			2.6	748	82		25	57
Total				29172	519	15	187	317

Supplemental Table 5. Taxonomic composition of the microbial community in the Loue River biofilms based on the chloroplast 16S rRNA gene sequences characterized by pyrosequencing

Taxonomic Affiliation				Reads (%)	Total seq nb	Number of OTUs at the 97% cut-off			
						Total	Abundant	Interm.	Rare
Bacteria	Firmicutes	Clostridia		0.0	2	1			1
	Proteobacteria	Betaproteobacteria	Burkholderiales	0.0	1	1			1
		Gammaproteobacteria	Xanthomonadales	0.1	15	8		1	7
Eukaryota	Amoebozoa	Tubulinea	Euamoebida	0.0	2	1			1
	Archeaplastida	Chloroplastida	Charophyta	1.3	203	9		6	3
			Chlorophyta	2.9	455	44	1	24	19
		Rhodophyceae	Composogonales	0.0	1	1			1
			Florideophycidae	0.8	120	6		4	2
			Porphyridiophyceae	0.0	4	1		1	
			Unclassified	0.2	34	1		1	
	SAR	Alveolata	Dinoflagellata	1.6	256	1	1		
		Stramenopiles	Chrysophyceae	0.0	2	2			2
			Diatomea	91.4	14406	82	17	56	9
			Xanthophyceae	1.1	177	10		7	3
Unclassified				0.5	86	22		6	16
Total					15764	190	19	106	65

Supplemental figures



Supplemental Figure 1: Rarefaction curves for bacterial (a) and micro-eukaryotic (b) communities. Rarefaction curves were calculated using the package vegan based on normalized data (10116 and 884 reads per sample respectively for bacterial and micro-eukaryotic communities). The three replicates of each sites and dates were represented. Samples from: Cléron = dash, Chamblay = dash/dot, Belmont = dot and Parcey = continuous line.

ANNEXE 4

Manuscrit soumis très prochainement sur les données obtenues sur le Tarn

Variability in bacterial communities associated with *Phormidium*–dominated biofilms in rivers: A comparison across geographical and temporal scales

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Manuscript in preparation

Abstract

Phormidium-dominated biofilms have increased in prevalence and severity in numerous rivers worldwide. They are receiving growing scientific and public attention as *Phormidium* can produce potent toxins and accidental ingestion of biofilms or contaminated water has been linked with multiple animal mortalities. Physicochemical parameters been identified as important in causing proliferations, however little is known about the structure and role of the associated microbial communities. We hypothesised that a core set of bacteria would be identified in *Phormidium*-dominated biofilms regardless of their origin, and that local conditions would led to geographical segregation of bacterial communities (BCs) at OTU level. *Phormidium*-dominated biofilms were sampled in shallow rivers in France (n = 1, over a period of 4 months) and New Zealand (n = 8, one point in time), and 16S rRNA meta-barcoding using the Illumina MiSeq platform used to characterise BC. Bacterial communities were dominated by Alphaproteobacteria (27%), Betaproteobacteria (21%), and Bacteroidetes (24%). At the OTU level, we observed a seasonal pattern structuring BC composition in relation to the increase of cyanobacterial biomasses. At the intra-river scale (Tarn River in France), no significant relationship was observed between Bray-Curtis dissimilarity and geographic distance. This is probably explained because of a constant bacterial dispersion by flow from upstream and downstream sites. Conversely, at the inter-river scale (New Zealand rivers), a geographic structuration of bacterial communities according to distance was revealed by a strong and significant distance-decay pattern ($r_p = 0.73$; $P < 0.001$). When comparing both bacterial communities from Tarn and New Zealand rivers (inter-country scale) more than 25% of the OTU were shared and these OTU resulted to be the most abundant and widely distributed. In addition, Bray-Curtis dissimilarities were of the same range of variation. All these results suggest that spatial structuration of New Zealand bacterial communities associated to *Phormidium*-dominated biofilms are mostly a result from local environmental conditions than from dispersion limitation.

Keywords: bacterial benthic communities, Cyanobacteria, spatial and temporal analysis

Introduction

Microbial biofilms in shallow rivers perform critical ecological functions and processes such as nutrient recycling, nitrogen fixation, biodegradation of xenobiotics, and they are important primary producers (Battin et al., 2008, 2003; Romani et al., 2004; Vercraene-Eairmal et al., 2010). The biofilms are usually comprised of a large range of species, but are often dominated by diatoms, green algae or cyanobacteria (Allan and Castillo, 2007; Lamberti, 1996; Roberts et al., 2004). Cyanobacteria dominated biofilms can be problematic as many species are known to produce natural toxins (cyanotoxins) that pose a risk to human and animal health (Quiblier et al., 2013). In particular, species within the genera *Phormidium* have been documented to produce anatoxins and ingestion of these has led to numerous canine poisonings worldwide (Cadel-Six et al., 2007; Gugger et al., 2005; Wood et al., 2007).

Variables leading to *Phormidium* dominance in microbial biofilms are not well understood despite the increasing scientific attention on the environmental factors and processes that determine biofilm biodiversity in stream ecosystems (Besemer, 2015). Many studies describe shifts in benthic algal dominance associated with changing environmental conditions, such as nutrient, light, temperature and flow regime (Allan and Castillo 2007; DeNicola, 1996). Similarly, physicochemical parameters such as hydrodynamics, water temperatures and nutrient availability have been linked to *Phormidium* proliferations (Heath et al., 2011, 2015; Loza et al., 2014; Wood et al., 2015). These previous studies have tended to focus on physicochemical drivers and have not characterised the bacterial communities (BC) associated with *Phormidium* proliferations. To date, only one study has examined and characterised the BC associated with *Phormidium*-dominated biofilms (Brasell et al., 2015). This study showed the BC associated with *Phormidium*-dominated biofilms generally consisted of Alphaproteobacteria, Betaproteobacteria and Sphingobacteria and that the BC displayed variations in their composition depending on their phase of development. However, due to the lack of data collected on other rivers, it is not possible to determine if BC associated with *Phormidium*-dominated biofilms share a common composition and structure. Such a relationship could indicate the existence of specific links between bacteria and *Phormidium* within biofilms.

The links between microbial primary producers and heterotrophic bacteria in aquatic biofilms have received considerable attention, leading to an increased understanding of biofilm ontogeny (*e.g.* Barranguet et al., 2005; Bott, 1996; Fischer, 2003; Geesey et al., 1978; Haack and McFeters, 1982; Pohlon et al., 2010; Roeselers et al., 2007). Moreover, in the global

framework of the phycosphere (*e.g.* Bell and Mitchell, 1972; Cai et al., 2014; Cole, 1982; Louati et al., 2015), it has become apparent that an increased knowledge of interactions occurring between cyanobacteria and bacteria will lead to a better understanding of the dynamics of these communities. It will also provide new insights in to the spatio-temporal changes occurring in their structural and functional diversity.

The overarching aim of this study was to gain a greater understanding in the composition and processes structuring BC within *Phormidium*-dominated biofilms. We hypothesized that; (i) if close interactions exist between heterotrophic bacteria and *Phormidium* in these biofilms, there would be strong similarities between the dominant orders in BC within *Phormidium*-dominated biofilms regardless of their origin (inter-country comparison), (ii) that there will be a significant relationship between BC dissimilarity and geographic distance (inter-river comparison), and (iii) BC composition at a high taxonomic level (phylum/order) would remain similar over a two years period, although some shifts would be observed as biofilms transition through different successional stages (intra-river comparison).

To address these hypotheses *Phormidium*-dominated biofilms were sampled in shallow rivers in France (n = 1, over a period of 4 months) and New Zealand (n = 8, one point in time), and 16S rRNA meta-barcoding using the Illumina MiSeq platform used to characterise BC.

Material and Methods

Sites and sample collection

Samples were collected from nine shallow rivers in France and New Zealand. Previous studies had identified the presence of potentially toxic benthic cyanobacteria in these rivers (Cadel-Six et al., 2007; Gugger et al., 2005; Wood et al., 2007). In France, samples were collected at five sites (T1 to T5; Figure 1) in the Tarn River between June and September 2013 and 2014. Flow rate was obtained from two survey stations: Bedoues located 28 km upstream, and Mostuejouls located 38 km downstream from Sainte-Enimie, respectively from hydro France (http://hydro.eaufrance.fr).

In New Zealand, ten sites located in eight rivers were sampled in February 2013 (Figure 1). Sites TP and TW were both located in the Tukituki River (North Island) and sites WN and WMP in the Wakapuaka River (South Island; Figure 1). The substrates at all sites were

dominated with cobble and boulder (6 to 26 cm length), except at the Kuratau River (site K ; Figure 1), where sand was the most abundant substrate.

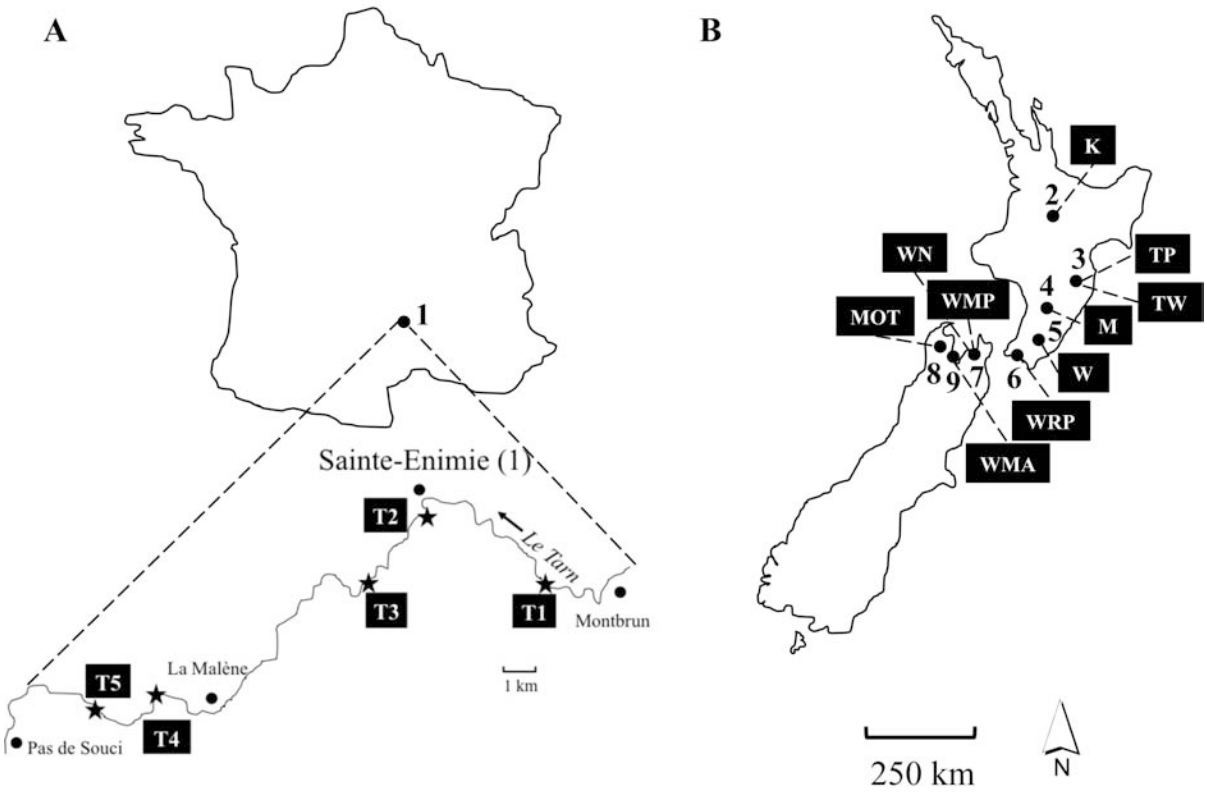

Figure 1. Sample sites (T1-T5) located in France at Tarn River (A) and New Zealand (B) Kuratau River (K; 2), Tukituki River (TP and TW; 3), Mangatainoka River (M; 4), Waipoua River (W; 5), Wainuiomata River (WRP; 6), and in the South Island at Wakapuaka River (WN and WMP; 7), Motueka River (MOT; 8), and Waimea River (WMA; 9). Countries are to scale.

A grid (10 m × 10 m) was set up in riffle habitat (shallow region of river with fast flow). *Phormidium*-dominated biofilm coverage was estimated at 10 randomly selected points using an underwater viewer. At each sampling point a single cobble with a *Phormidium*-dominated biofilm was sampled (Wood et al., 2010). Biofilms were removed using sterile tweezers. Subsamples were taken for; DNA extraction, a known area (7.07 cm^2) for chlorophyll-*a* (Chl-*a*) extraction, and a known area (1 cm^2) for later microscopic identification and enumeration (preserved immediately with Lugol's iodine solution). Samples were stored chilled in the dark, and subsequently frozen (-20°C), except for the samples for microscope analysis which were stored at 4°C.

For site T1 in June 2013, where low *Phormidium*-dominated biofilm coverage was observed (<5% of the river substrate), three points on a transect parallel to the water's edge and positioned one to two meters from the shoreline were sampled. At each point, all cobbles (5 to

20 cm length) visible in a single field of view of an underwater viewer (707 cm^2) were collected. The cobbles were scrubbed and the biomass collected in 150 mL of river water. Aliquots (5 mL) were filtered for Chl-*a* (GF/C Whatman) and DNA extraction (Polycarbonate 0.2 μm GTTP Millipore). Samples were stored chilled in the dark, and subsequently frozen (-20°C) for later analysis in the laboratory. Subsamples (1 mL) were fixed with Lugol's iodine solution and stored at 4°C for later microscopic identification and enumeration.

Chlorophyll-a extraction

Direct biomass samples or glass fiber filters (from biofilms collected at site T1 in June 2013) were extracted with absolute methanol or 90% methanol respectively (10 mL) in Falcon tubes (15 mL) covered with aluminum foil. The tubes were shaken manually, vortexed (30 s), incubated (24 h, 4°C) and centrifuged (10 min, 3220 × *g*). The absorbance of the supernatant was measured with a spectrophotometer (Agilent Technologies Cary 60 UV-vis, USA). The absorbance of samples was measured at 652 and 665 nm. A correction for turbidity at 750 nm was applied and the equation of Porra et al., 1989 was adapted to calculate Chl-*a* concentrations (μg cm^{-2}) for all the samples except for site T1 where the equation of Talling and Driver (1963) was used.

Enumeration of the photosynthetic community

Lugol's iodine preserved samples were homogenized briefly (Ultra-Turrax T25 IKA, Germany, 3 × 2 s, 9.5 min^{-1}) to break up filaments, but avoid cell damage. The samples were diluted in Milli-Q water and identification and enumeration performed using a photonic microscope (200× magnification, Nikon Optiphot-2, Japan), and a Malassez chamber (Marienfeld, Germany). All cells contained in 25 squares of the Malassez chamber were counted and analysis of triplicate aliquots undertaken. Species identification was performed only for cyanobacteria (Komarek and Anagnostidis, 2005). The other cells were only identified as belonging to diatom or green algae. The length and the width (or diameter for filaments) of the cells were measured. For diatoms and green algae, the height was estimated according to literature (Olenina et al., 2006). For all species, biovolumes were then calculated following published formula (Hillebrand et al., 1999; Olenina et al., 2006).

Molecular analysis of biofilm communities

Triplicates subsamples from each site were lyophilized (FreeZone2.5, Labconco, USA) and DNA extracted from ca. 50 mg using a Power Biofilm® DNA Isolation Kit (MOBIO, USA) following the manufacturer's instructions. Nucleic acid concentration was determined by spectrophotometry (Nanodrop 1000, Thermo Fischer Inc, USA) and samples stored at -20°C. A region of the 16S rRNA gene (~394 bp) including the variable region 3-4 was amplified using the bacterial-specific primers 515F (Caporaso et al., 2011) and 909R (Wang and Qian, 2009) in a commercial facility (Research and Testing Laboratories, Lubbock, TX, USA) using Illumina MiSeq sequencing technology.

Bioinformatics analysis

The Illumina MiSeq sequencing of the 16S rRNA gene produced 2,085,469 raw sequences. All sequences were processed by the Research and Testing Laboratories, according to their pipeline (http://www.researchandtesting.com/docs/Data_Analysis_Methodology.pdf, last updated 11/07/2014). Briefly, after denoising (*i.e.* removing of short sequences and singletons) and chimera checking, the remaining sequences were clustered using UPARSE (Edgar, 2013) at 97% similarity threshold. The centroid sequences of each cluster were run against the RDP classifier database (Wang et al., 2007). The number of read per sample ranged from 604 to 57,391. Of the 95 biofilm samples sequenced, 92 were selected for further analysis, rarefying (randomly down-sampling) resulted in a total of 820,456 16S rRNA sequences (8,918 sequences per sample), which clustered in 2,198 Operational Taxonomic Units (OTU). A second analysis of sequences was performed after removing cyanobacterial and No-Hit (CNH) sequences. The number of sequences ranges between 32 to 30,560 per sample and a total of 82 samples were retained (at least two replicates per sampling site). Rarefying resulted in 32,964 16S rRNA sequences (402 sequences per sample), which clustered in 1004 OTU.

OTU were classified as abundant when they comprised ≥1% (Pedrós-Alió, 2006) of the total sequences, intermediate when between <1% and >0.01% (Mangot et al., 2012), and rare when ≤0.01% (Galand et al., 2009).

Statistical analysis

Statistical analyses were performed using the statistical software package R 2.15.1 (R Development Core Team, Vienna, Austria). Differences were considered significant when $P < 0.05$. The effect of sampling site and date on biomasses (Chl-*a*, cyanobacteria biovolume proportions and percentage *Phormidium* spp. coverage) and abundance of microbial taxa were analysed using Kruskal-Wallis non parametric test, followed by a Dunn post-hoc test (package "dunn.test") using a Bonferroni adjusted *P*-value for multiple comparison using rank sums.

Diversity indices (richness, Chao1, Shannon and Evenness) and rarefaction curves were calculated using "vegan" package (Dixon, 2003). Hellinger transformations were undertaken on Illumina sequencing data. Beta-diversity was calculated to measure the variation in BC among sites and dates using Bray-Curtis dissimilarities performed in abundance transformed (normalized) data using "vegan" package (Dixon, 2003) at order and OTU level. Analysis was also undertaken on presence-absence data at the OTU level using Sorensen dissimilarity index in the "betapart" package (Baselga et al., 2015). Principal component analyses (PCA) were performed using the Ade4TkGUI software (Thioulouse and Dray, 2007) in order to visualise samples distribution based on their OTU composition. Permutational Multivariate ANOVA (PERMANOVA; Anderson, 2005) using PERMANOVA+ (Anderson et al., 2008) and PRIMER v7 (Clarke and Gorley, 2015) was performed on Bray-Curtis dissimilarities to test for differences in BC among sites and between sampling times (countries, sites and dates) including factorial and hierarchical (nested) designs and tests of interactions (Anderson, 2001; McArdle and Anderson, 2001). Because of the un-balanced design (unequal number of sites or replicates), a type III sum of squares (partial) was performed. Permutation (999) of residuals under a reduced model was used (Anderson, 2005). A PERMANOVA analysis was used to quantify the magnitude of the variation at each spatial/temporal scale using the estimated components of variation (ECV) as a percentage of the total variation. Finally, the relationship between geographical distance and Bray-Curtis dissimilarity was performed using the "fossil" package (Vavrek, 2012), longitude and latitude coordinates were transformed to kilometres distances in straight line. Mantel statistic based on Pearson's product-moment correlation ("vegan" package) was performed between dissimilarity matrix and permutation (999) used to evaluate statistical significance.

Results

Spatiotemporal variations in the composition, biomasses and coverage of biofilm photosynthetic communities

Microscopic analysis demonstrated the biofilms were dominated by cyanobacteria (principally *Phormidium* sp.), followed by diatoms and green algae (Figure 2). This pattern was observed in all samples except; (i) June site T1 (2013-2014) and July site T4 (2014) in the Tarn River, France, and (ii) site WMA in the Waimea River, New Zealand. At these sites diatoms were dominant and *Phormidium* spp. coverage was always below 5%.

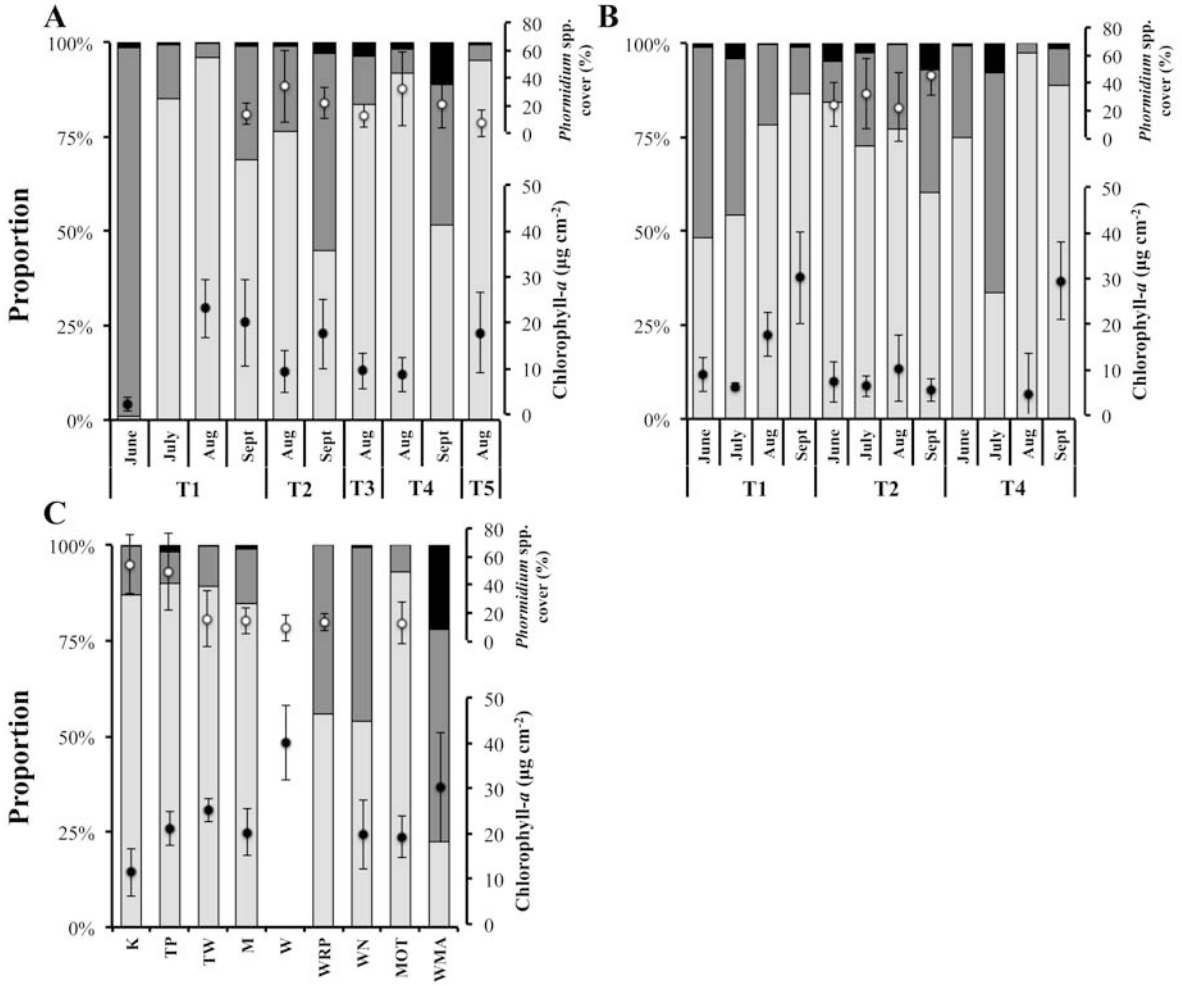

Figure 2. Average composition of dominant photosynthetic microorganisms of biofilm samples. Cyanobacteria = light grey, diatoms = grey and green algae = black. Mean of the total Chlorophyll-*a* content and percentage *Phormidium* spp. cover from Tarn River in 2013 (A), 2014 (B) and New Zealand rivers in 2013 (C). Percentage *Phormidium* spp. cover < 5% are not presented. Error bars are standard deviations.

The proportions of cyanobacteria did not vary markedly among the five sampling sites in the Tarn River in 2013 and 2014 (Figure 2A & B and Table S1), however, the biomasses (expressed in µg of Chl-*a* per cm^2) and the percentage of *Phormidium* cover varied

significantly among sites during the two sampling years (Kruskal-Wallis test, all $P < 0.007$; Figure 2A & B and Table S1). Temporal variations were also observed for these parameters from June to September, for example Chl-*a* concentrations increased over the summer period (Figure 2A & B and Table S1). The lowest cyanobacterial proportions were observed in June 2013 and July 2014 (Figure 2A-B), which were taken shortly after two large flow events (Figure S1). In New Zealand rivers, significant differences were observed in cyanobacterial proportions, total biomasses and percentage *Phormidium* cover among sampling sites (Kruskal-Wallis test, all $P < 0.04$; Figure 2C and Table S1).

Spatio-temporal comparison of the composition and structure of bacterial communities estimated at high taxonomic ranks

Analysis of the 16S rRNA gene sequences, showed the biofilms were dominated by cyanobacteria (>85% of reads), mainly belonging to Oscillatoriales in particularly *Phormidium* (Figure 3). Excluding CNH sequences, the most abundant bacterial phyla were Proteobacteria (ave. 70%) and Bacteroidetes (ave. 24%) (Figure 3). Within Proteobacteria Alphaproteobacteria (ave. 27%) and Betaproteobacteria (ave. 21%) and to a lesser extent Gammaproteobacteria (ave. 9%) were most prominent. Unclassified Proteobacteria also accounted for a high proportion (ave. 12%) of the reads in this phylum. Within these three classes, most of the sequences were classified in the orders of Rhodobacterales/Sphingomonadales, Burkholderiales and Xanthomonadales, respectively. Bacteroidetes were mainly represented by the orders Flavobacteriales (Tarn River) and Sphingobacteriales (New Zealand rivers; Figure 3 and Table S2). At these high taxonomic ranks, the bacterial structure and composition in biofilms were similar during the two sampling years in Tarn River (Bray Curtis dissimilarity = 0.32) and when comparing Tarn River and New Zealand rivers BC (Bray Curtis dissimilarity = 0.36; Figure 3, Table S4).

Analysis of the spatio-temporal shifts in the relative abundance of dominant bacterial orders (≥1%; excluding CNH) of the Tarn River (Figure 4A), showed that the communities collected from T1 station in June 2013 and 2014 and in T4 station in June 2014 were different from all the others samples, in particular among orders belonging to Bacteroidetes. As previously mentioned, these biofilms were dominated by diatoms not cyanobacteria. Temporal variations were observed at order level. Rhodobacterales showed the highest relative abundances between June-July (Figure 4A) and mainly diminished between August-September.

In contrast, Flavobacteriales showed the highest relative abundances at the end of the sampling period (Figure 4A).

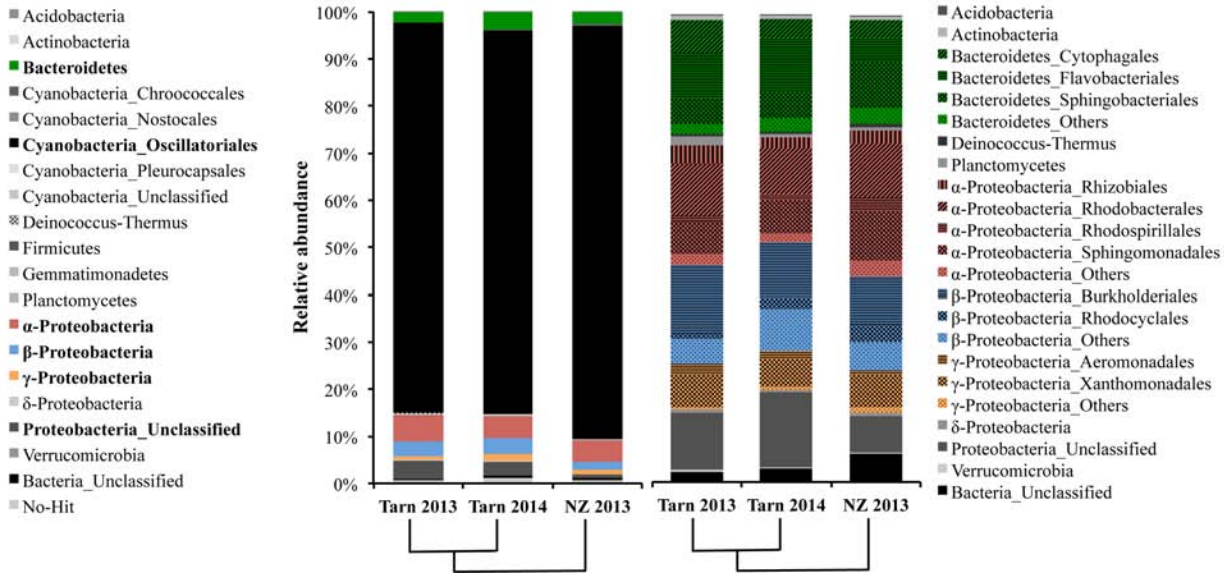

Figure 3. Relative sequence abundance of the dominant bacterial phyla/order composing cyanobacterial biofilms from each sampling campaign. On the left all phyla and on the right excluding cyanobacteria phyla and No-Hit. At the bottom cluster analysis of the microbial diversity at the order level.

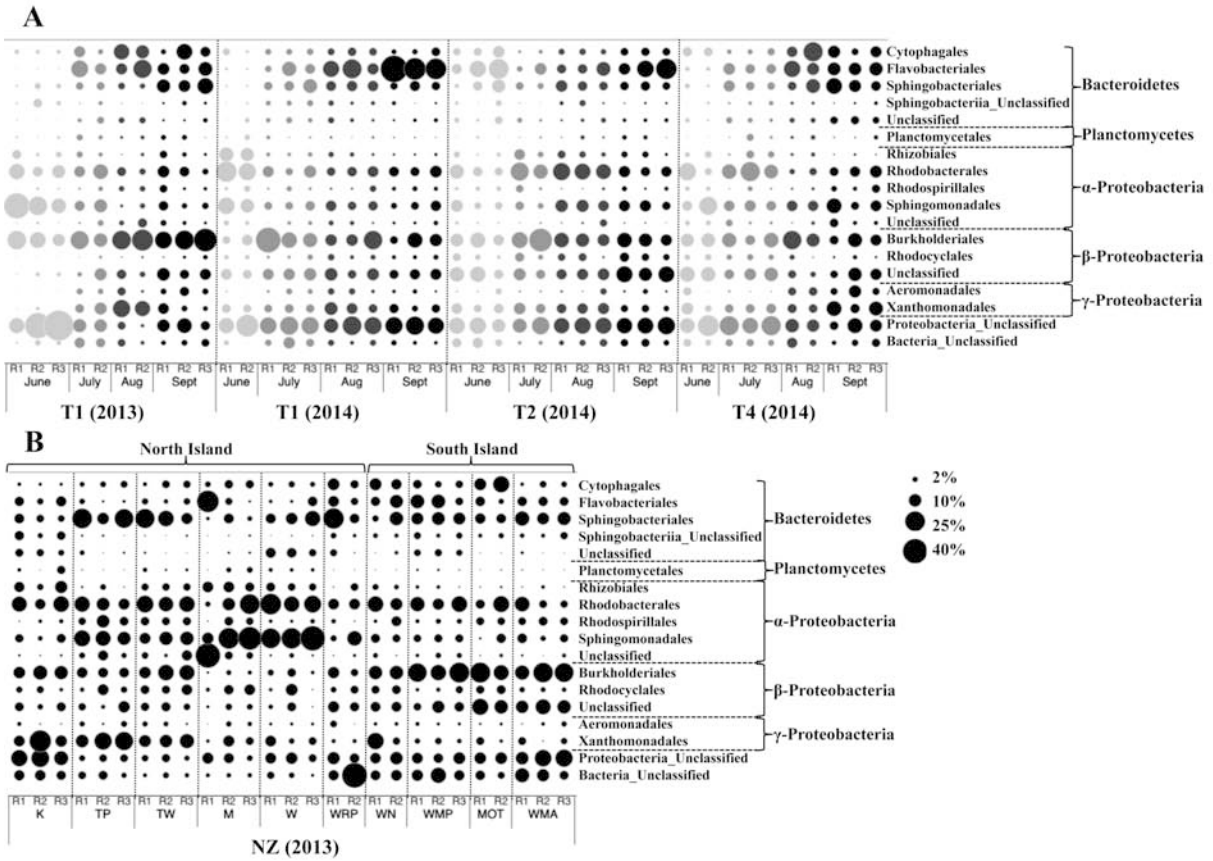

Figure 4. Spatiotemporal variation of the most abundant (≥1%) bacterial orders associated with *Phormidium*-dominated biofilms from the Tarn River at site T1 in 2013 and T1, T2 and T4 in 2014 (A) from June (light grey) to September (black) and from New Zealand Rivers (B). R1, R2 and R3 correspond to replicates.

In New Zealand rivers, the BC sampled at the same river (sites TP and TW at Tukituki River; and sites WN and WMP at Wakapuaka River) were similar at the order level (Bray-Curtis dissimilarity = 0.15 and 0.19 respectively; Table S4). The Bray Curtis dissimilarity values were higher when comparing the sites located at different rivers (Figure 4B). In particular, BC from South Island river sites were more homogeneous (Bray-Curtis dissimilarity = 0.22, Table S4) than those from North Island river (Bray-Curtis dissimilarity = 0.36). The overall BC dissimilarity between the North and South Islands was 0.38.

Spatio-temporal comparison of the composition and structure of bacterial communities at the OTU level

Two analyses were performed on the 16S rRNA gene sequences dataset, one including all sequences (8918 sequences per sample) and a second excluding CNH sequences (402 sequences per sample). As expected, the rarefaction curves were more asymptotic when considering all the sequences but the use of 402 sequences per sample accounted for the majority of the BC richness (Figure S2). The rarefaction curves suggest a higher richness in BC from the Tarn River, compared to those from New Zealand rivers (Figure S2; Table S3).

Of the 2198 OTU identified when all sequences were included, 534 OTU (24.3%) were shared by all samples (Figure 5A). These OTU contained a very high number of reads compared to those when Tarn or New Zealand rivers were analysed separately and similar results were obtained when excluding CNH sequences (Figure 5B).

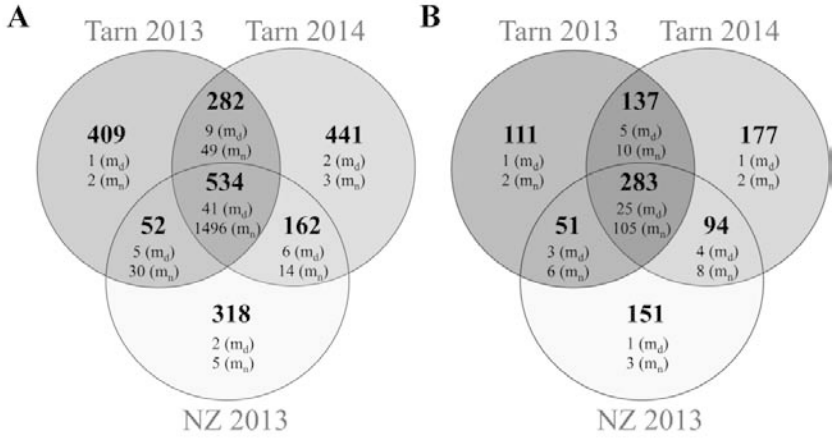

Figure 5. Venn diagram of 16S rRNA OTU general composition of biofilm samples from the Tarn (2013-2014) and New Zealand rivers. (A) All OTU, (B) excluding cyanobacteria and No-Hit OTU. Abbreviations refer to the median (m_d) and mean (m_n) of the number of sequences per OTU.

There was a clear relationship between the mean number of reads per OTU and the number of samples in which each OTU was found (Figure S3). At the genus level, the most abundant bacterial OTU (≥ 1% of total reads) were represented by *Rhodobacter*, *Pedobacter*,

Silanimonas, *Flavobacterium*, *Hydrogenophaga*, *Runella*, *Tolumonas* and *Sphingobacterium* (Table S5).

Principal Component Analysis (PCA) on the OTU sequences dataset when excluding CNH revealed a weak separation between BC associated with biofilms from New Zealand and the Tarn River (Figure 6A). The inertia values of the two first axes of the analysis are very low (<19%), supporting this suggestion. Analysis of the different components of the variance (Table 1), indicated that most of the variance was due to differences occurring at a short geographic scale (variations between sites or variations between replicates, which are include in residual variances). Less than 12% of the variations were due to the country origin. When all OTUs were considered (including CNH), a clear distinction was found between BC found in biofilms dominated by diatoms or by cyanobacteria (data not shown).

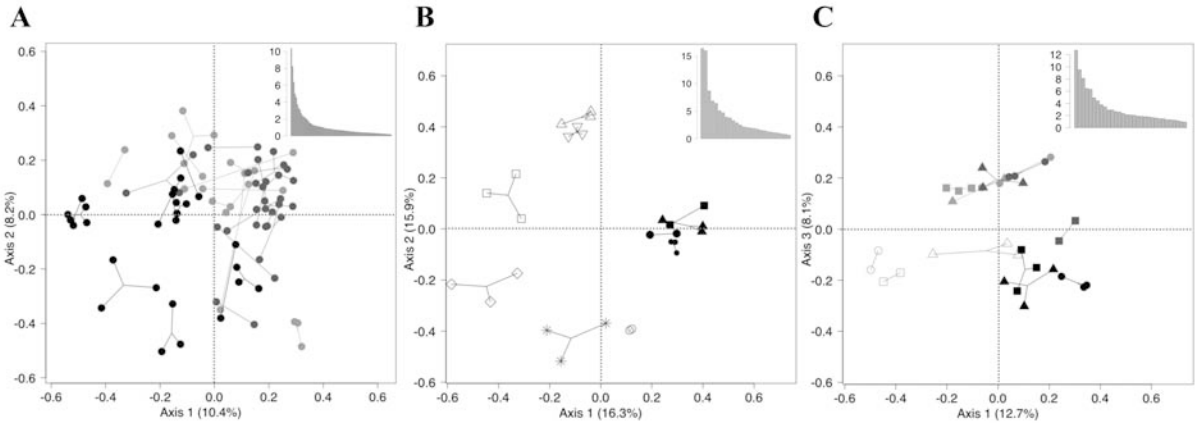

Figure 6. Principal Component Analysis (PCA) of bacterial 16S rRNA OTU abundance excluding cyanobacteria and No-Hit OTU sequences of biofilm samples from: all sampling campaigns (A), New Zealand rivers (B) and the Tarn river in 2014 (C). In (A) Sampling campaigns are represented in light grey, dark grey and black for Tarn 2013, 2014 and New Zealand rivers respectively. In (B) asterisks, open: triangles, inverted triangles, squares, diamonds and circles represents north sites K, TP, TW, M, W and WRP respectively; while black circles, small circles, squares and triangles represents south sites WN, WMP, MOT and WMA respectively. In (C) circles, triangles and squares represents site S2, STE and AG respectively, while the colours gradient from white to black follow the months June to September.

A PCA analysis of the BC of New Zealand biofilms, revealed a clear distinction on the first axis between biofilms from the South Island, which were tightly grouped (Sorensen dissimilarity = 0.56) and those from the North Island, which were much more dispersed (Sorensen dissimilarity = 0.74; Figure 6B; Table S4). Interestingly, biofilms from the North Island followed a gradient of ordination from north to south on the second axis of the analysis, excepted for site K.

No significant difference was found between the two sampling years for the Tarn biofilms (Nested PERMANOVA, $P = 0.105$; Table 1). However significant differences were detected among months ($P < 0.005$; Table 1), contributing to 20.1% of the total variance. The ACP performed on the BC sampled in 2014 in the Tarn River (Figure 6C) confirmed the existence of seasonal variations with a distinction among samples from June, July-August and September.

Table 1: PERMANOVA results for the different comparison within and between sampling campaigns excluding cyanobacterial and No-Hit (CNH) OTU. A total of 999 permutations were performed. Site(Country), Month(Year) or Site(Island) means that the term on the left is nested inside the term in brackets. Asterisk denotes interaction between factors. df: degrees of freedom, MS: means of squares, ECV %: percentage of estimated components of variance. Bold values: $P < 0.05$

Sampling Campaigns	Source	Excluding CNH				
		df	MS	Pseudo-F	*P*	ECV %
Tarn August 2013 & New Zealand 2013	Country	1	7426.8	2.30	**0.007**	11.6
	Site(Country)	13	3460.2	2.86	**0.001**	37.4
	Residual	23	1211.3			51.0
	Total	37				100
New Zealand 2013	Island	1	6520.2	1.89	**0.036**	11.0
	Site(Island)	8	3594.9	3.17	**0.001**	39.7
	Residual	17	1134.7			49.3
	Total	26				100
Tarn 2013 & 2014	Year	1	5458.9	1.57	0.105	5.0
	Month(Year)	6	4023.3	2.75	**0.001**	20.1
	Residual	47	1465.3			74.9
	Total	54				100
Tarn 2014	Site	2	2543.4	1.24	0.215	2.7
	Month	3	3541.7	1.72	**0.021**	11.0
	Site*Month	6	2068.2	1.80	**0.001**	20.3
	Residual	20	1147.3			66.0
	Total	31				100

No significant relationship between BC dissimilarities and geographical distances were found among Tarn River sites when all sampling sites/dates were considered (Figure 7). A weak relationship (Mantel test, $r_p = 0.32$; $P = 0.04$) was observed when only one sampling date (August 2013) was considered (data not shown). There was a highly significant relationship among BC dissimilarities and geographical distances for New Zealand rivers (Mantel test, $r_p = 0.73$; $P = 0.001$; Figure 7). The Bray-Curtis dissimilarity values found among geographically close samples from the Tarn River were in the same order as those from the most geographically distant samples from New Zealand rivers (Figure 7), as well as the Bray-Curtis

dissimilarities between New Zealand and France (Figure 7; Average Bray-Curtis dissimilarity values presented in Table S4).

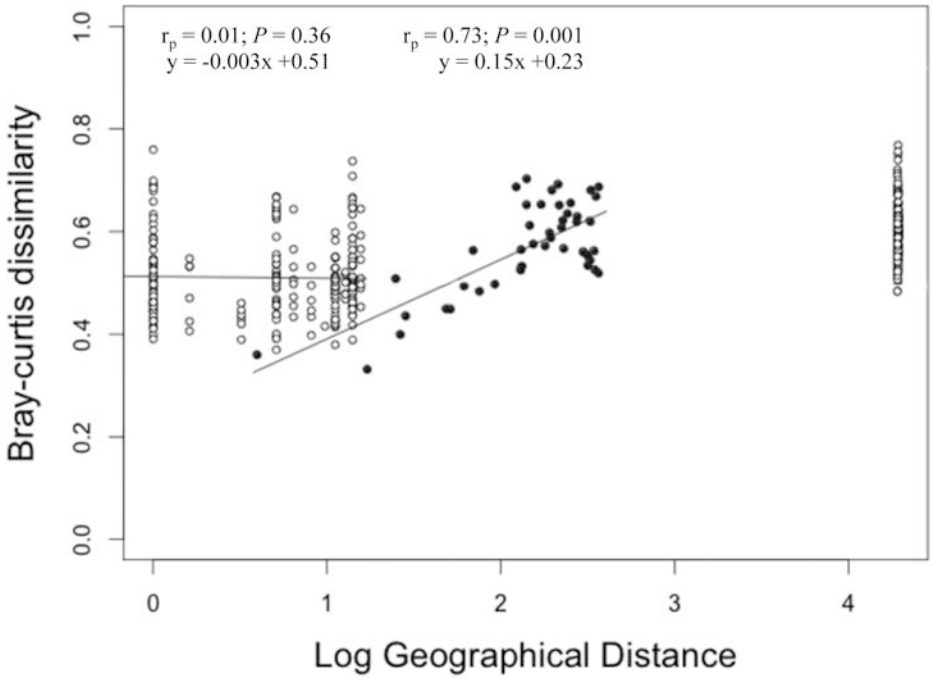

Figure 7: Geographic distance and Bray-Curtis dissimilarity relationship in Tarn River (open circles on the left), New Zealand rivers (black circles in the middle) and between Tarn and New Zealand rivers (open circles at the right) of bacterial 16S rRNA OTU abundance of pooled replicates excluding cyanobacterial and No-Hit sequences. Mantel statistics are based on Pearson's product-moment correlation.

Discussion

Deciphering the interactions between heterotrophic and autotrophic bacteria in freshwater ecosystems is essential for improving knowledge on the diversity and functioning of these complex communities. Proliferations of potentially toxic planktonic and benthic cyanobacteria have increased in their prevalence globally (Quiblier et al., 2013; Harke et al., 2016). There is growing evidence for complex exchanges among BC and planktonic cyanobacteria (Bertilsson and Jones, 2003; Cole, 1982; Paerl and Pinckney, 1996). However, to date only one study (Brasell et al. 2015) has investigated BC associated with *Phormidium*-dominated biofilms, despite increased reports of extensive proliferations and their involvement in numerous animals mortalities (Quiblier et al., 2013). The aim of this study was to characterise and compare BC in *Phormidium*-dominated biofilms collected at a range of spatial and temporal scales to improve

understanding of their structural diversity and the factors and processes shaping their composition.

The dominant taxonomic groups associated with *Phormidium*-dominated biofilms were very similar when examined at the phylum, class and order levels. All BCs were dominated by species belonging to Proteobacteria (Alpha- and Betaproteobacteria) and Bacteroidetes. Previous research has highlighted that the dominance of Proteobacteria and Bacteroidetes is also a common feature of bacterial biofilms in streams (Anderson-Glenna et al., 2008; Besemer, 2015; Besemer et al., 2012), lakes (Parfenova et al., 2013) and marine intertidal cyanobacterial mats (Severin et al., 2010). In the present study, we observed a co-dominance of Alpha-, Betaproteobacteria and Bacteroidetes, suggesting that these taxonomic groups play an important role in structuring *Phormidium*-dominated biofilm communities. A comparisons of the BC associated with *Phormidium*-dominated biofilms in this study with those associated with bloom-forming cyanobacteria in lakes (e.g. Cai et al., 2014; Eiler and Bertilsson, 2004; Louati et al., 2015; Parveen et al., 2013) shows a marked difference in the relative abundance of Alpha-, Betaproteobacteria and Bacteroidetes. This indicates that the habitat (benthic *versus* pelagic) is a greater driving force in structuring BC than the presence of cyanobacteria.

When the data was evaluated at a finer taxonomic scale (genus and OTU), the BC associated with biofilms dominated by diatoms in Tarn River were clearly differentiated from those in *Phormidium*-dominated biofilms. Previous research has shown that diatom associated BC are largely dominated by Alphaproteobacteria in marine environments (Amin et al., 2012), in epilithic lake biofilms (Bruckner et al., 2008), and in river biofilms (Zancarini et al., unpublished data – Article 1). This findings collaborates our findings from *Phormidium*-dominated biofilms (see previous paragraph), and suggests that BC are to at least some extend dependant on the key phototroph in the biofilms, and the resulting micro-environmental conditions. Becker et al. (2014) showed that the Dissolved Organic Matter (DOM) produced by diatoms and cyanobacteria is very different, which potentially has a substantial influence on heterotrophic communities utilising this for growth. Wagner et al. (2014) also found that the quality of the DOM impacted the functional and structural diversity of BC in hyporheic biofilms. *Phormidium*-dominated biofilms can be several millimetres thick and form very cohesive biofilms (Wood et al., 2015). Recent research has shown that this creates conditions within the biofilm (e.g., pH, dissolve oxygen, nutrient and metal concentrations) that are very different from those of the overlying water (Wood et al., 2015). It is likely that this has a

substantial impact on the BC composition. As *Phormidium*-dominated biofilms develop these within-biofilm conditions probably become more intense and this may explain why marked shifts in BC have been observed during different successional phases (Brasell et al., 2015).

We demonstrated a positive relationship between the abundance of reads per OTU and the number of samples in which they were retrieved. This indicates that the most abundant OTU are widely distributed and that they constitute the core species of the BC associated with *Phormidium*-dominated biofilms in rivers. A similar positive relationship has been described by Humbert et al. (2009) for pelagic BC from lakes and Dougal et al. (2013) for BC in the large intestine of horses. As members of the core species, it is probable that these OTU play a major role in the basic functioning of BC of biofilms while satellite species distributed in a restrictive number of samples, are probably involved in the adaptation to local environmental conditions. Many of the dominant OTU in the biofilms examined in this study are well known for their ability to degrade complex compounds such as recalcitrant humic substances or organic contaminants (*Sphingomonas* and *Hydrogenophaga*; Glaeser and Kämpfer, 2014; Willems, 2014) or large organic polymeric proteins and polysaccharides (*Flavobacterium* and *Runella*; McBride, 2014; McBride et al., 2014). Others have been shown to be involved in the nitrogen cycle, including nitrogen fixation and the denitrification (for example OTU from *Rhodobacter*, *Sphingomonas*, *Azonexus* and *Hydrogenophaga*; Glaeser et Kämpfer, 2014; Oren, 2014; Pujalte et al., 2014; Willems, 2014). During the early stages of development of the *Phormidium*-dominated biofilms (in June and July in Tarn River), numerous reads belonging to *Rhodobacter* genus were identified. This genus is metabolically diverse (from anaerobic phototrophy and photoautotrophy to aerobic chemoheterotrophy) (Pujalte et al., 2014). The high abundance of these purple bacteria (including also OTUs belonging to Burkholderiales, Rhizobiales, Rhodocyclales, Rhodospirellales, Rhodobacterales and Sphingomonadales) raises questions regarding their potential contribution to primary production in river biofilms. Although extreme fluctuations in dissolved oxygen have been shown to occur in *Phormidium*-dominated biofilms (Wood et al., 2015), it is not possible to determine to the extent to which *Phormidium* causes these shifts or associated BC such as *Rhodobacter*. Consequently we are not able to determine which kind of metabolism these OTUs display in biofilms.

There was a strong relationship between the Bray-Curtis dissimilarities and geographical distances between BC among the New Zealand biofilms. This association has been reported previously in BC from stream biofilms (Fierer et al., 2007; Lear et al., 2013) and

in other environments such as lake sediments (Xiong et al., 2012) and soil (Ge et al., 2008). Soininen et al. (2007) performed a meta-analysis on more than 400 dissimilarity/similarity-distance relationships, and concluded that this relationship unites several ecological processes such as dispersal propensity and environmental structuring. Our results, which showed that the dissimilarities between French and New Zealand BC were in the same order as those found between the most distant rivers in New Zealand, suggests that local environmental conditions are probably the major factor in driving the dissimilarities-distances relationship found in New Zealand BC. Isolation by distance, due to limited ability to disperse, would have led to very high dissimilarities values when comparing France and New-Zealand BC. This hypothesis that environmental conditions are critical is supported by the partitioning analysis of the variance showing that variations in the BC composition were mainly explained by variations at short geographical scales and by seasonal variations. For the Tarn River, temporal variations were observed in BC structure at order and OTU level, revealing a possible effect of successional changes in biofilm BC, as observed by Brasell et al. (2015)

The results from the present study suggest that most of the variation observed was due to differences between replicates within a site. The reviewed by Ramette and Tiedje (2007), corroborates this findings through demonstrating that within many prokaryotic communities, micro-scale variations generally exceed large-scale variations. Besemer et al. (2009) showed that for river biofilms heterogeneous flow and resources can generate various microhabitats with complex architecture and differentiated BC and and Lear et al. (2008) demonstrated greater variations in BC on different faces of individual stream rocks compare to whole rocks sampled within a stream. This variability has prompted several new microbial concepts including the suggestion that "Patch dynamics" a concept usually used to defined variability at within a stream, occurs in microbial communities (Pringle et al., 1988), and the notion that biofilms need to be considered as landscapes (Battin et al., 2007). Further fine-scale with *Phormidium*-dominant biofilm studies are recommended to complement to the large-scale comparison performed in this study.

In conclusion, this study has demonstrated that; (i) the structure and composition of BC associated with biofilms display significant differences dependant on the dominant phototroph (*Phormidium* or diatom), (ii) the structure and composition of BC associated with *Phormidium*-dominated biofilms in France and New Zealand is similar. This finding supports the hypothesis for the existence of close interactions between heterotrophic bacteria and *Phormidium* in these

biofilms, (iii) BC from New Zealand rivers display a distance-decay pattern, and (iv) if similar BC were observed between a period of two years sampling, seasonal variations structured BC at the Tarn River. A significant challenge for the future is to establish to what extent the recent increase in benthic cyanobacteria proliferations in river worldwide is due to perturbations of BC in biofilms, or whether it is a result of changes to environmental factors and processes that promoting the development of cyanobacteria, with subsequent consequences on the BC.

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SUPPLEMENTAL INFORMATION

Tables

Supplemental Table 1. Statistical analyses of cyanobacterial proportion, chlorophyll-a and percentage *Phormidium* cover of the Tarn (2013 and 2014) and New Zealand River sites and months. Only significant differences (P < 0.05) are presented.

	Tarn 2013		Tarn 2014		Tarn 2013-2014	New Zealand 2013	
Variable	Kruskal-Wallis test (*P*)	Dunn test (*P*)	Kruskal-Wallis test (*P*)	Dunn test (*P*)	Kruskal-Wallis test (P)	Kruskal-Wallis test (*P*)	Dunn test (*P*)
Cyanobacterial proportion (%)	Month (<0.001)	June-July (0.042) June-Aug (0.002) Aug-Sept (0.002)	Month (0.021)	June-July (0.034) July-Aug (0.012)	June (0.014) Sept (0.012)	Site (0.035)	MOT-WMA (0.026)
Chlorophyll-*a* (µg cm^{-2})	Site (<0.001)	T1-T3 (0.009) T1-T4 (0.002) T4-T5 (0.032)	Site (<0.001)	T1-T2 (<0.001) T1-T4 (0.042) T2-T4 (<0.001)	T2 (0.002) T4 (<0.001)	Site (<0.001)	K-TW (0.003) K-W (<0.001) K-WMA (<0.001)
	Month (0.001)	June-Aug (0.015) June-July (0.001) Aug-Sept (0.032)	Month (<0.001)	July-Aug (<0.001) July-Sept (0.001)	June (0.007)		TP-W (0.013) M-W (0.007) W-WN (<0.001) W-MOT (0.001)
Phormidium cover (%)	Site (0.006)	T2-T5 (0.003) T4-T5 (0.022)	Site (<0.001)	T1-T2 (<0.001)	T1 (<0.001)	Site (0.001)	K-WMP (0.006) K-WMA (0.004)
			Month (0.004)	June-Sept (0.002) Aug-Sept (0.030)	Sept (<0.001)		TP-WMP (0.021) TP-WMA (0.015)

Supplemental Table 2. Taxonomic composition of the bacterial community of the biofilms (excluding CNH) from this study based on the normalized 16S rRNA gene sequences characterized by Illumina sequencing approach.

Taxonomic Affiliation			Reads	Total	Number of OTUs at the 97% cut-off			
Phylum	**Class**	**Order**	**(%)**	**seq nb**	**Total**	**Abundant**	**Interm.**	**Rare**
Acidobacteria	Acidobacteriia	Acidobacteriales	0.1	37	11		2	9
		Unclassified	0.0	9	3		1	2
	Holophagae	Holophagales	0.1	23	3		1	2
Actinobacteria	Actinobacteria	Acidomicrobiales	0.0	4	3			3
		Actinomycetales	0.5	167	15		8	7
		Solirubrobacterales	0.0	8	2		1	1
		Unclassified	0.1	49	6		2	4
	Unclassified		0.0	9	4			4
Aquificae	Unclassified		0.0	1	1			1
Armatimonadetes	Armatimonadia	Armatimonadales	0.0	13	1		1	
Bacteroidetes	Bacteroidia	Bacteroidales	0.3	85	4		2	2
	Cytophagia	Cytophagales	4.9	1621	75	1	29	45
	Flavobacteriia	Flavobacteriales	8.9	2920	45	2	29	14
		Unclassified	0.3	115	4		1	3
	Sphingobacteriia	Sphingobacteriales	6.8	2246	67	2	30	35
		Unclassified	1.0	343	17		12	5
	Unclassified		1.4	460	36		19	17
Chloroflexi	Anaerolineae	Anaerolineales	0.0	9	1		1	
	Caldilineae	Caldilineales	0.0	2	2			2
	Chloroflexia	Herpetosiphonales	0.0	1	1			1
	Unclassified		0.0	8	4			4
Deferribacteres	Deferribacteres	Unclassified	0.0	11	1		1	
Deinococcus-Thermus	Deinococci	Deinococcales	0.4	124	3		3	
Fibrobacteres	Fibrobacteria	Fibrobacterales	0.0	3	1			1
Firmicutes	Bacilli	Bacillales	0.1	23	2		1	1
		Lactobacillales	0.0	4	2			2

	Clostridia	Clostridiales	0.1	22	7		2	5
		Unclassified	0.0	5	3			3
Fusobacteria	Fusobacteriia	Fusobacteriales	0.0	10	1		1	
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	0.1	40	2		1	1
Planctomycetes	Phycisphaerae	Phycisphaerales	0.0	5	1		1	
	Planctomycetia	Planctomycetales	1.0	328	60		17	43
		Unclassified	0.0	13	7			7
	Unclassified		0.1	28	11		2	9
Proteobacteria	Alphaproteobacteria	Caulobacterales	0.7	228	10		8	2
		Rhizobiales	2.9	963	54		31	23
		Rhodobacterales	10.8	3551	30	2	12	16
		Rhodospirillales	1.9	616	23		7	16
		Rickettsiales	0.1	31	13		2	11
		Sphingomonadales	8.0	2630	65	1	33	31
		Unclassified	1.9	619	10	1	1	8
	Betaproteobacteria	Burkholderiales	12.2	4011	43	5	22	16
		Methylophilales	0.4	140	4		1	3
		Neisseriales	0.2	55	6		3	3
		Nitrosomonadales	0.0	1	1			1
		Procabacteriales	0.0	2	1			1
		Rhodocyclales	2.2	718	15		8	7
		Unclassified	6.5	2144	31	2	15	14
	Deltaproteobacteria	Bdellovibrionales	0.0	10	5			5
		Desulfobacterales	0.0	7	1			1
		Desulfovibrionales	0.0	1	1			1
		Desulfuromonadales	0.0	11	5			5
		Myxococcales	0.2	73	12		6	6
		Syntrophobacterales	0.0	1	1			1
		Unclassified	0.3	111	15		7	8
	Gammaproteobacteria	Acidithiobacillales	0.0	8	1		1	

		Aeromonadales	1.6	541	3	1	1	1
		Chromatiales	0.1	22	3		2	1
		Enterobacteriales	0.1	24	1		1	
		Legionalles	0.1	18	9		1	8
		Methylococcales	0.0	12	4		1	3
		Oceanospirillales	0.0	1	1			1
		Pseudomonadales	0.4	133	10		6	4
		Thiotrichales	0.0	7	2			2
		Xanthomonadales	6.4	2118	35	2	18	15
		Unclassified	0.1	39	3		1	2
	Oligoflexia	Oligoflexales	0.0	2	1			1
	Unclassified		12.2	4034	73	3	30	40
Spirochaetes	Spirochaetia	Spirochaetales	0.0	5	2			2
Verrucomicrobia	Opitutae	Opitutales	0.1	39	8		1	7
	Verrucomicrobiae	Verrucomicrobiales	0.1	29	6		1	5
	Unclassified		0.1	24	3		1	2
Unclassified			3.8	1239	87		32	55
Total				32964	1004	22	421	561

Supplemental Table 3. Diversity indexes of the bacterial communities based on 16S rRNA gene sequences (excluding CNH).

Sampling Campaign	Site	Month	Richness	Chao1	Shannon	Evenness
Tarn 2013	T1	June	72 ± 6	138 ± 15	3 ± 0.5	0.7 ± 0.1
		July	122 ± 6	193 ± 17	4.1 ± 0.02	0.9 ± 0.004
		August	74 ± 21	126 ± 48	3.2 ± 0.4	0.8 ± 0.04
		September	102 ± 8	182 ± 32	3.9 ± 0.2	0.8 ± 0.03
	T2	August	125 ± 27	208 ± 71	4 ± 0.3	0.8 ± 0.04
		September	124 ± 33	222 ± 119	4.2 ± 0.3	0.9 ± 0.02
	T3	August	119 ± 5	183 ± 16	4.1 ± 0.07	0.9 ± 0.01
	T4	August	98 ± 11	178 ± 30	3.7 ± 0.2	0.8 ± 0.03
		September	92 ± 18	181 ± 27	3.7 ± 0.3	0.8 ± 0.03
	T5	August	100 ± 8	182 ± 41	3.7 ± 0.2	0.8 ± 0.03
Tarn 2014	T1	June	92 ± 11	233 ± 146	3.7 ± 0.07	0.8 ± 0.01
		July	138 ± 22	332 ± 146	4.1 ± 0.5	0.8 ± 0.08
		August	129 ± 22	269 ± 61	4.2 ± 0.2	0.9 ± 0.02
		September	103 ± 6	194 ± 16	3.6 ± 0.2	0.8 ± 0.05
	T2	June	109 ± 3	205 ± 27	4 ± 0.07	0.9 ± 0.01
		July	103 ± 4	160 ± 9	3.8 ± 0.2	0.8 ± 0.05
		August	138 ± 16	278 ± 89	4.3 ± 0.2	0.9 ± 0.02
		September	110 ± 29	195 ± 80	3.9 ± 0.4	0.8 ± 0.05
	T4	June	83 ± 2	147 ± 18	3.6 ± 0.04	0.8 ± 0.01
		July	129 ± 14	275 ± 61	4.1 ± 0.1	0.8 ± 0.01
		August	137 ± 2	293 ± 74	4.3 ± 0.09	0.9 ± 0.02
		September	105 ± 26	208 ± 27	3.9 ± 0.4	0.8 ± 0.04
NZ 2013	K	February	118 ± 8	217 ± 28	4.1 ± 0.1	0.9 ± 0.01
	TP	February	64 ± 4	103 ± 15	3.4 ± 0.2	0.8 ± 0.05
	TW	February	71 ± 11	119 ± 41	3.4 ± 0.003	0.8 ± 0.03
	M	February	78 ± 29	138 ± 62	3.2 ± 0.7	0.7 ± 0.1
	W	February	90 ± 7	173 ± 24	3.4 ± 0.3	0.8 ± 0.06
	WRP	February	91 ± 5	141 ± 11	3.7 ± 0.3	0.8 ± 0.05
	WN	February	102 ± 16	164 ± 49	3.9 ± 0.3	0.9 ± 0.03
	WMP	February	119 ± 5	244 ± 16	4.1 ± 0.09	0.9 ± 0.02
	MOT	February	69 ± 7	92 ± 16	3.5 ± 0.05	0.8 ± 0.01
	WMA	February	87 ± 4	137 ± 14	3.8 ± 0.07	0.9 ± 0.01

Supplemental Table 4. Beta-diversity at different sampling scales (excluding CNH). Bray-Curtis dissimilarity was calculated as average from abundance data at order (≥1% most abundant) and OTU level, Hellinger transformations were performed on abundance at OTU level. Sorensen dissimilarity was calculated from presence-absence data at OTU level.

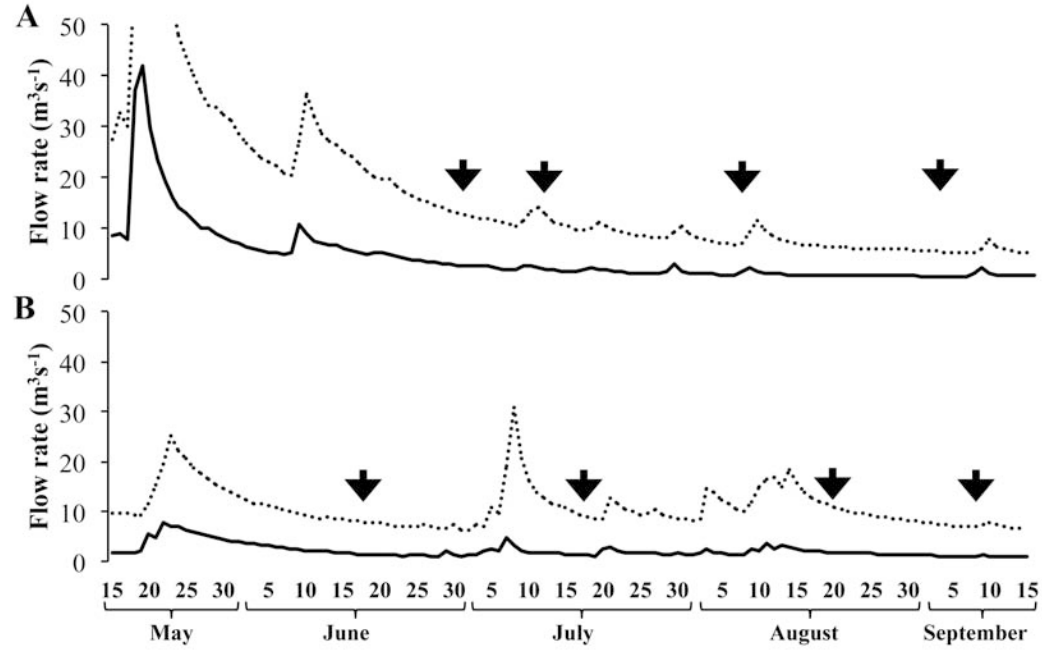
Sampling Campaigns	Comparison	Beta-diversity		
		Order level	OTU level	
		Bray-Curtis (non transformed)	Bray-Curtis (transformed)	Sorensen
Tarn 2013	All	0.34	0.51	0.77
	Intra-river (Tarn August 2013)	0.32	0.50	0.64
	Intra-site (T1)	0.38	0.60	0.68
Tarn 2014	All	0.30	0.48	0.78
	Intra-site (T1)	0.35	0.49	0.61
	Intra-site (T2)	0.25	0.44	0.58
	Intra-site (T4)	0.31	0.51	0.63
Tarn 2013 & 2014	All	0.32	0.51	0.87
New Zealand 2013	All	0.38	0.57	0.80
	Nord Island	0.36	0.61	0.74
	South Island	0.22	0.45	0.56
	Intra-river (Tukituki)	0.15	0.33	0.43
	Intra-river (Wakapuaka)	0.19	0.36	0.37
Tarn & New Zealand	All	0.36	0.55	0.91
	Tarn 2013 & New Zealand 2013	0.37	0.60	0.88
	Tarn 2014 & New Zealand 2013	0.36	0.60	0.88
	Tarn Aug 2013 & New Zealand	0.39	0.60	0.85

Supplemental Table 5. First 50 most abundant bacterial OTU from this study (excluding CNH).

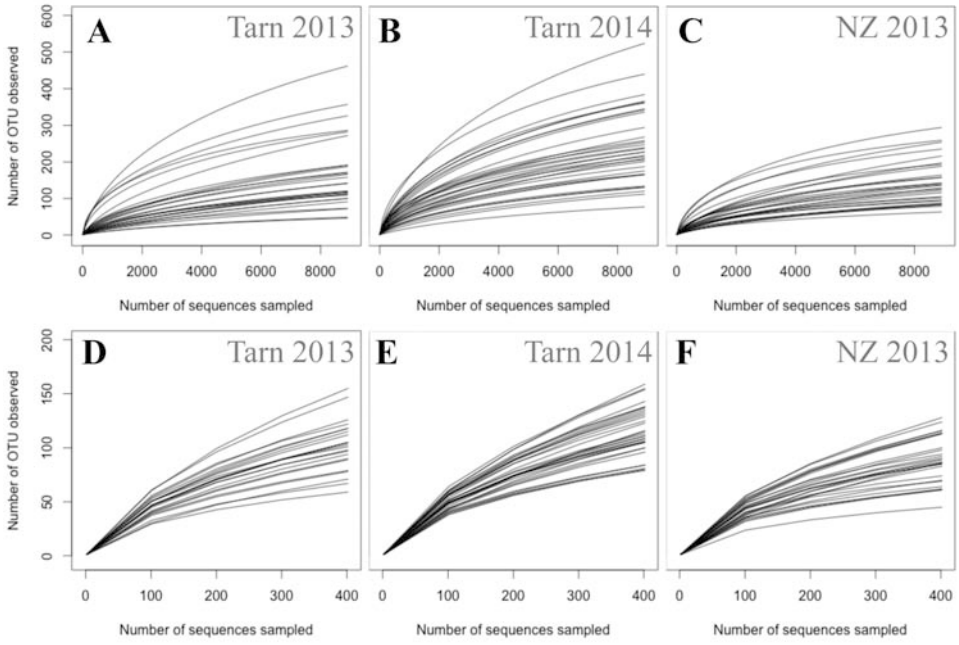
Taxonomic Affiliation					Reads	Total
Phylum	**Class**	**Order**	**Family**	**Genus**	**(%)**	**seq nb**
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobacter*	6.3	2093
Proteobacteria	Unclassified				4.6	1517
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Unclassified		3.3	1075
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	3.2	1046
Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified		3.2	1039
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Silanimonas*	2.9	955
Proteobacteria	Betaproteobacteria	Unclassified			2.9	952
Bacteroidetes	Flavobacteriia	Flavobacteriales	Unclassified		2.7	893
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	2.3	745
Proteobacteria	Unclassified	Unclassified			2.2	728
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobacter*	2.2	726
Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified		2.2	712
Proteobacteria	Alphaproteobacteria	Unclassified			1.8	599
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Hydrogenophaga*	1.8	599
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Runella*	1.8	578
Proteobacteria	Betaproteobacteria	Unclassified			1.7	575
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Hydrogenophaga*	1.5	493
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Tolumonas*	1.4	467
Proteobacteria	Unclassified				1.4	463
Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified		1.3	426
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	1.1	352
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Unclassified		1.0	344
Unclassified Bacteria					0.9	283
Bacteroidetes	Cytophagia	Cytophagales	Unclassified		0.8	259
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	*Azonexus*	0.8	253
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	*Zoogloea*	0.8	253
Proteobacteria	Unclassified				0.7	232

Proteobacteria	Betaproteobacteria	Unclassified			0.7	230
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Lysobacter*	0.6	212
Proteobacteria	Unclassified				0.6	210
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.6	199
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	0.6	198
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Roseococcus*	0.6	195
Unclassified Bacteria					0.6	188
Proteobacteria	Unclassified				0.6	187
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	0.5	173
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Unclassified		0.5	169
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	0.5	157
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Arcicella*	0.5	155
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Lysobacter*	0.5	151
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Variovorax*	0.4	145
Bacteroidetes	Flavobacteriia	Flavobacteriales	Unclassified		0.4	144
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobacter*	0.4	143
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Unclassified		0.4	136
Proteobacteria	Betaproteobacteria	Methylophilales	Unclassified		0.4	135
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.4	125
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium*	0.4	125
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*	0.4	124
Proteobacteria	Betaproteobacteria	Unclassified			0.4	122
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Spirosoma*	0.4	120
Total					68	22400

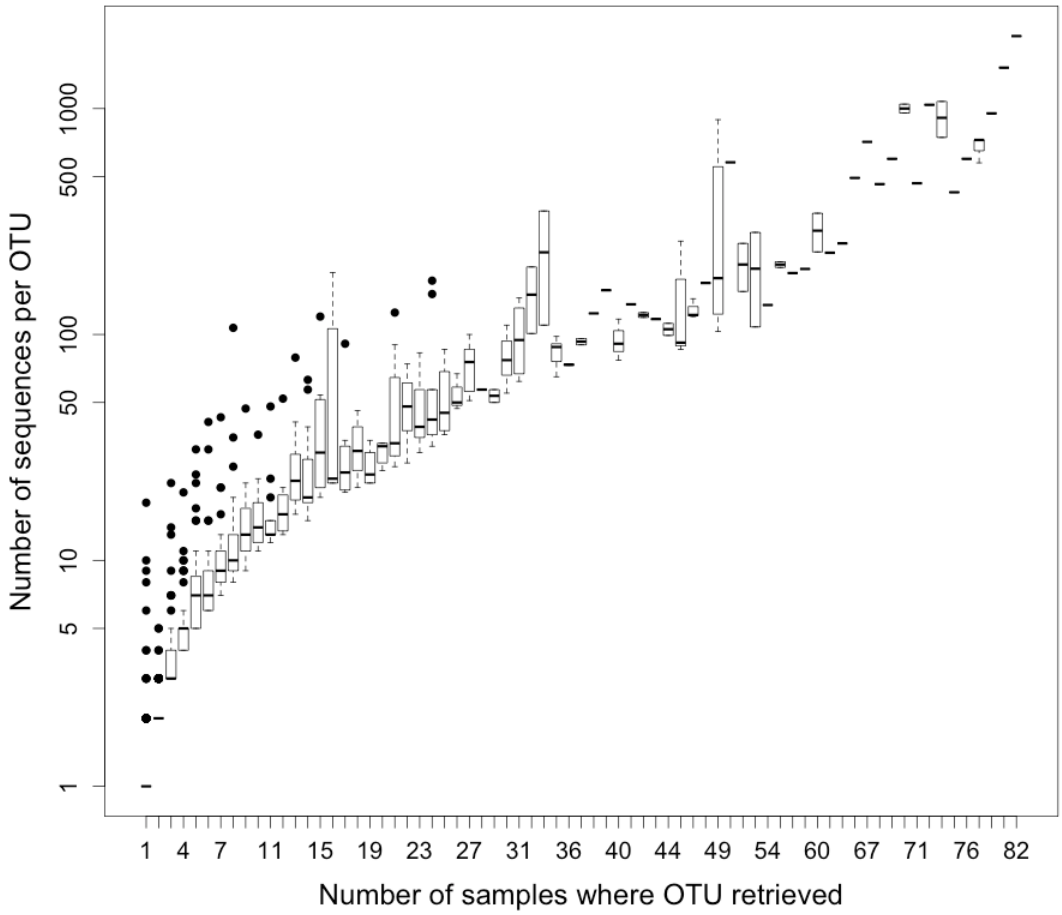
Supplemental Figures



Supplemental Figure 1. Flow rates of Tarn River in 2013 (A) and 2014 (B) measured at Bedoues (doted line) located 28 km upstream and Mostuejouls (solid line) located 38 km downstream from Sainte-Enimie respectively. Arrows show sampling days.



Supplemental Figure 2. Rarefaction curves of Tarn and New Zealand rivers of all 16S rRNA gene sequences (A-C) and excluding cyanobacteria and No-Hit OTU sequences (D-F).



Supplemental Figure 3. Bacterial 16S rRNA OTU occurrence and sequence number per biofilm sample excluding cyanobacteria and No-Hit OTU sequences.

ADDITIONAL RESULTS

Environmental parameters

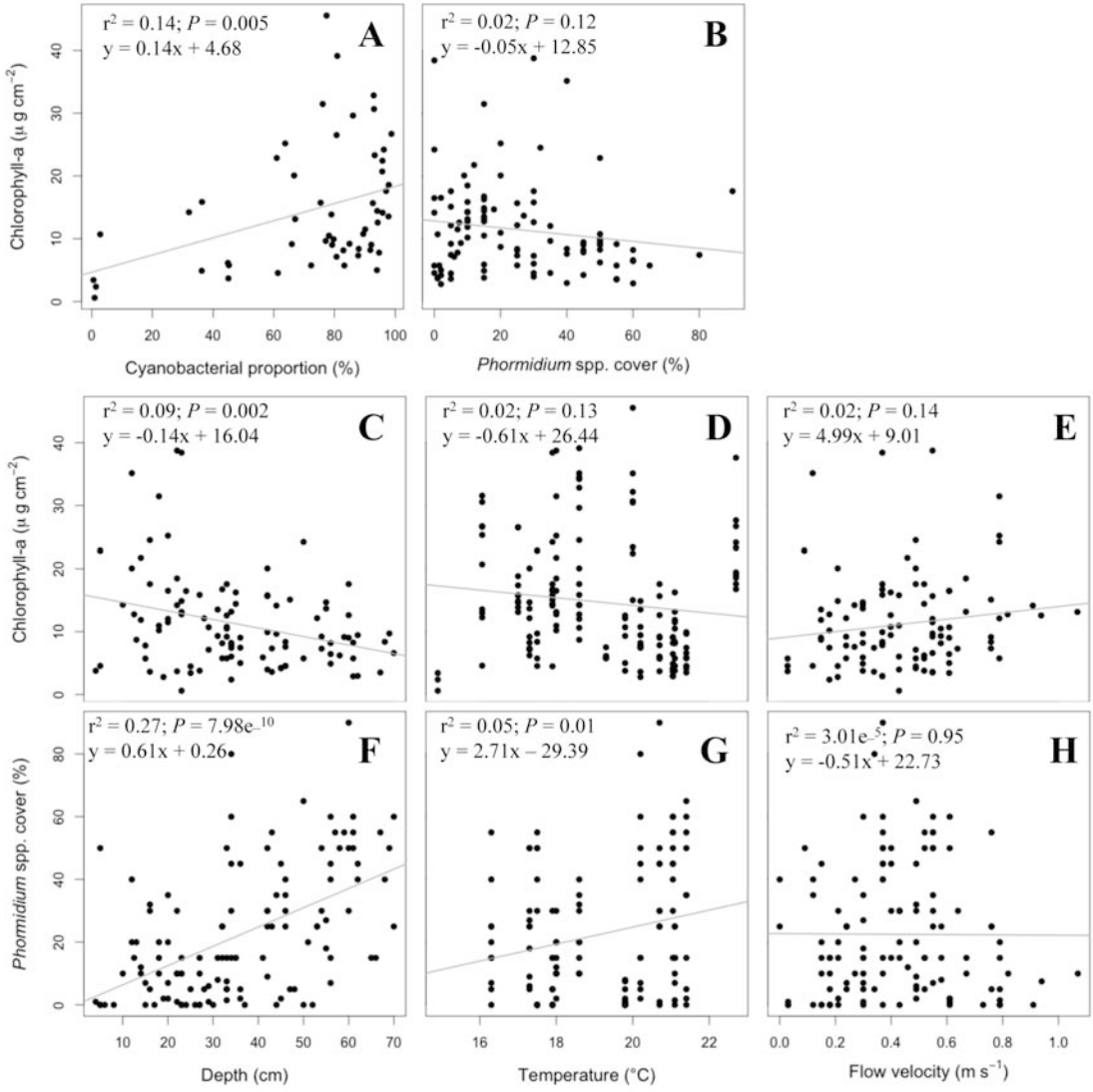
Water temperature, pH, flow velocity and depth were measured at each sampling in the Tarn River (France).

Additional Table 1. Physico-chemical and biological parameters of sites at the Tarn River.

Site	Month/Year	Area (cm^2)	Current velocity ($m\ s^{-1}$)	Depth (cm)	pH	T (°C)	Chl-a ($\mu g\ cm^{-2}$)	Cyanobacteria proportion (%)	Diatom proportion (%)	Green algae proportion (%)	Phormidium cover (%)
T1	June 2013	ND	0.4 ± 0.2	27.3 ± 5.9	8.1	14.9	2.1 ± 1.4	1 ± 0.4	97.7 ± 1.6	1.4 ± 1.5	less 5
	July 2013	ND	ND	ND	ND	ND	ND	84.9 ± 15.3	14.5 ± 15	0.6 ± 0.4	less 5
	August 2013	221.8 ± 42.5	ND	ND	7.8	22.7	23.1 ± 6.4	96 ± 2.5	3.6 ± 2.7	0.3 ± 0.2	less 5
	September 2013	147.7 ± 72.5	0.6 ± 0.2	19.8 ± 2.9	7.9	18.0	19.9 ± 9.5	69 ± 6.4	30.2 ± 6.2	0.8 ± 1.1	13.9 ± 7.8
	June 2014	145.8 ± 53.9	0.6 ± 0.2	20.3 ± 13	8.8	19.8	8.9 ± 3.7	48 ± 64	50.8 ± 63	0.8 ± 1.1	less 5
	July 2014	187.6 ± 34.5	ND	ND	ND	19.3	6.2 ± 0.7	54.1 ± 15.7	41.8 ± 15.6	4.1 ± 1.3	less 5
	August 2014	160.5 ± 122.1	ND	ND	8.1	17.0	17.7 ± 4.9	78.4 ± 2.7	21.5 ± 2.7	0.1 ± 0.1	less 5
	September 2014	146 ± 63.1	ND	ND	ND	18.6	30.2 ± 10.1	86.6 ± 6	12.5 ± 5	0.9 ± 1.1	less 5
T2	August 2013	132.6 ± 54.7	0.3 ± 0.2	38.9 ± 15.2	7.9	20.7	9.3 ± 4.6	76.6 ± 27.3	22.4 ± 26.3	1 ± 1.1	34.1 ± 26
	September 2013	177.1 ± 86.1	0.3 ± 0.2	16.1 ± 5.4	7.8	18.6	17.5 ± 7.6	45 ± 18.9	52 ± 18	3 ± 1.2	22.2 ± 11.3
	June 2014	256.2 ± 137.3	0.4 ± 0.2	48.3 ± 10.9	8.6	17.3	10.6 ± 4.5	84.5 ± 3.6	10.8 ± 5.9	4.7 ± 2.6	24.5 ± 16.1
	July 2014	191.4 ± 58.7	0.3 ± 0.2	47.1 ± 12.6	8.6	21.4	6.4 ± 2.3	72.7 ± 9.9	24.8 ± 8.3	2.5 ± 1.9	32.2 ± 25.1
	August 2014	185.9 ± 44.2	0.3 ± 0.2	38.3 ± 22	8.1	17.5	10.3 ± 7.3	77.4 ± 14.4	22.2 ± 14	0.4 ± 0.4	22.5 ± 24.6
	September 2014	138.5 ± 65.6	0.5 ± 0.1	54 ± 7.7	ND	21.1	5.5 ± 2.5	60.5 ± 22	32.7 ± 22.6	6.9 ± 1.9	45.5 ± 14.2
T3	August 2013	190.3 ± 79.3	0.5 ± 0.2	27.9 ± 11.9	8.1	21.1	9.4 ± 4	83.7 ± 6	12.7 ± 7.2	3.5 ± 5.2	12.9 ± 8.5
T4	August 2013	105.4 ± 57.3	0.3 ± 0.1	31.3 ± 14.5	8.0	20.2	8.6 ± 3.8	92 ± 2.6	6.2 ± 4.3	1.8 ± 1.8	31.9 ± 26.8
	September 2013	98 ± 61.3	0.2 ± 0.2	57.4 ± 7.2	7.9	16.3	ND	51.9 ± 23.7	36.9 ± 18.8	11.3 ± 13.3	20.7 ± 16.7
	June 2014	ND	ND	ND	8.6	16.8	ND	75.1 ± 25.9	24.2 ± 25.3	0.7 ± 0.6	less 5
	July 2014	ND	ND	ND	ND	20.9	ND	33.8 ± 37.1	58.3 ± 36	7.9 ± 4.2	less 5
	August 2014	128 ± 65.5	ND	ND	8.0	16.1	20.5 ± 9.1	97.4 ± 1.6	2.5 ± 1.5	0 ± 0	less 5
	September 2014	163.5 ± 124.3	ND	ND	ND	20.0	29.5 ± 8.6	88.7 ± 9.9	10 ± 10	1.3 ± 0.6	less 5
T5	August 2013	150.1 ± 49.9	0.6 ± 0.2	35.5 ± 9.9	8.1	17.9	17.7 ± 8.7	95.4 ± 1.2	4.1 ± 0.8	0.5 ± 0.4	7.5 ± 9.8

Additional Table 2. Physico-chemical and biological parameters of New Zealand river.

Site	Current velocity (m s^{-1})	Depth (cm)	Chl-a (µg cm^{-2})	Cyanobacteria proportion (%)	Diatom proportion (%)	Green algae proportion (%)	Phormidium cover (%)
K	ND	ND	11.9 ± 5.2	87.1 ± 4.9	12.8 ± 4.9	0.1 ± 0.1	56 ± 20.7
TP	ND	ND	21.6 ± 3.8	90.1 ± 4.8	8.2 ± 3.5	1.7 ± 1.3	51 ± 27.5
TW	ND	ND	25.6 ± 2.5	89.3 ± 3	10.6 ± 2.9	0.1 ± 0.1	17.2 ± 19.6
M	ND	ND	20.8 ± 5.1	84.6 ± 3.8	14.3 ± 3.8	1.1 ± 0.9	16.2 ± 9.2
W	ND	ND	40.7 ± 8.3	ND	ND	ND	11 ± 13.4
WRP	0.5 ± 0.1	15.6 ± 0	ND	55.7 ± 21.8	44.3 ± 21.9	0 ± 0	15 ± 6.1
WN	0.5 ± 0.1	16 ± 0	20.2 ± 7.6	54.1 ± 47.1	45.4 ± 46.2	0.5 ± 0.9	less 5
WMP	0.4 ± 0.3	18.8 ± 0.1	ND	ND	ND	ND	less 5
MOT	1.1 ± 0.1	28 ± 0	19.8 ± 4.6	93.2 ± 2.5	6.8 ± 2.5	0 ± 0	14.2 ± 14.5
WMA	0.5 ± 0.3	13.8 ± 0	30.7 ± 12.1	22.3 ± 16.3	55.6 ± 23.4	22.1 ± 11.2	less 5



Additional Figure 1. Relationship of Chlorophyll-*a* and *Phormidium* spp. cover with environmental parameters of the Tarn River (2013 and 2014).

When looking at the relationship between environmental parameters (depth, flow velocity, temperature, flow velocity) and biotic parameters (total biomass, proportion of cyanobacteria

and *Phormidium* cover), there was a positive correlation between total biomass and the proportions of cyanobacteria (Figure 1 A), between *Phormidium* cover and temperature (Figure 1 G). Interestingly, if there was a negative correlation between depth and total biomass (Figure 1 C), we found at the same time, a positive correlation between depth and *Phormidium* cover (Figure 1 F), meaning that this cyanobacterium was able to develop thin biofilms in deep areas of the river.