Focus on Bdellovibrio and like organisms in lake and marine environments : diversity, abundance and role of the only obligate bacterial predators

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THÈSE

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Zoom sur les *Bdellovibrio* et organismes apparentés en milieux lacustres et marins : diversité, abondance et rôle des seuls prédateurs bactériens obligatoires

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« Sūtor, nē ultrā crepidam»

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Jade A. Ezzedine

Résumé

Les *Bdellovibrio* et organismes apparentés (BALOs) constituent un groupe fonctionnel de bactéries Gram-négatives appartenant aux classes des Oligoflexia et Alpha-proteobactéries. La spécificité principale des BALOs est qu'ils sont des prédateurs bactériens obligatoires. Autrement dit, leur reproduction et croissance dépendent entièrement de la capture d'une proie, ce qui n'est pas le cas des autres bactéries prédatrices pour qui ce mode d'action est facultatif. Caractérisés par deux cycles possibles de reproduction (dit endo- ou épibiotique), les BALOs semblent relativement ubiquistes et leur présence a été rapportée dans des environnements contrastés. A titre d'exemple, ils ont été détectés, parfois avec des abondances pouvant être élevées, dans les sols, les eaux douces et salées ou encore les milieux fortement anthropisés tels que les stations d'épuration des eaux usées. Au vu de leurs attributs, les BALOs pourraient exercer un contrôle biologique significatif sur les populations microbiennes notamment les bactéries pathogènes. A ce jour, la majorité des études portant sur la diversité, la distribution et l'importance quantitative des BALOs a été effectuée dans les sols et les eaux salées. Notre travail a consisté à explorer les eaux douces surtout, représentées ici par les grands lacs naturels profonds péri-alpins (Annecy, Bourget et Léman), mais aussi deux sites marins (SOLA et MOLA dans la baie de Banyuls-sur-mer, Méditerranée nord occidentale) au travers du projet C-BALO. *In fine*, ce dernier a permis d'examiner, à différentes échelles de temps et d'espace, l'ubiquité, la diversité, l'importance quantitative et le rôle fonctionnel potentiel des principales familles de BALOs du groupe des Oligoflexia. Pour ce faire, nous avons employé diverses techniques de biologie moléculaire et de microbiologie. Un travail important a porté sur le dessin et le test de nouveaux couples d'amorces nucléotidiques spécifiques pour les BALOs afin de tenir compte des avancements récents portant sur leur classification et pour une application en séquençage à haut débit et PCR quantitative. Nous avons pu montrer que les *Bdellovibrionaceae, Peredibacteraceae* et *Bacteriovoracaceae* étaient présents dans tous les milieux étudiés et caractérisés par des dynamiques marquées, avec aussi comme constat que les *Peredibacteraceae* constituent la famille la plus abondante, les *Bacteriovoracaceae* étant inversement les moins nombreux. En parallèle, nous avons pu révéler une diversité insoupçonnée chez les BALOs, les *Peredibacteraceae* et les *Bdellovibrionaceae* possédant plus d'une centaine d'OTUs. L'Abondance et diversité des BALOs semblaient toutefois peu dépendantes des variables environnementales (telles que les paramètres physico-chimiques), suggérant l'importance des interactions biotiques à savoir la quantité et qualité des proies, la compétition pour les proies, la prédation par les flagellés et les ciliés, ou encore le parasitisme par les phages, ces derniers s'étant effectivement révélé avoir possiblement une action significative sur la dynamique des prédateurs. Enfin, quelques membres actifs de la famille des *Bdellovibrionaceae* ont pu être isolés du Léman, pointant à nouveau du doigt l'impact potentiel de ce groupe de bactéries dans le contrôle des populations bactériennes, un rôle fonctionnel qu'il reste encore à quantifier.

Abstract

Bdellovibrio and like organisms (BALOs) are a functional group of Gram-negative bacteria belonging to the classes of Oligoflexia and Alpha-proteobacteria. The main characteristic of BALOs is that they are obligate bacterial predators. In other words, their reproduction and growth depend entirely on the capture of prey, which is not the case for other predatory bacteria for which this mode of survival is optional. Defined by two possible cycles of reproduction, referred to as periplasmic or epibiotic growth, BALOs seem relatively ubiquitous and their presence has been reported in contrasting environments. As an example, they have been detected, sometimes with high abundances, in soils, fresh and salt waters, or in highly anthropized environments such as wastewater treatment plants. Given their attributes, BALOs could exert a significant biological control over microbial populations, particularly on pathogenic bacteria. To date, the majority of studies on the diversity, distribution and quantitative importance of BALOs have been carried out in soils and salt water. Our work, through the C-BALO project, has focused on fresh waters, represented here by the natural large and deep peri-alpine lakes (Annecy, Bourget and Geneva), but also two marine sites (SOLA and MOLA in the Bay of Banyuls, NW Med. Sea). C-BALO aimed at examining, on different time and space scales, the ubiquity, diversity, quantitative importance and potential functional role of the main families of BALOs of the Oligoflexia group. To do so, we employed various molecular biology and microbiology techniques. An important work has been performed to design and test a new set of primers specific to BALOs. This work was necesary to take into account recent advances in BALOs classification and for an application in high throughput sequencing and quantitative PCR. Then, we revealed that *Bdellovibrionaceae*, *Peredibacteraceae* and *Bacteriovoracaceae* are present in all the studied habitats. Moreover, they were characterized by different patterns and marked dynamics, with *Peredibacteraceae* being the most abundant family and *Bacteriovoracaceae* the least abundant. In parallel, we were able to reveal an unsuspected diversity within BALOs, especially for *Peredibacteraceae* and *Bdellovibrionaceae*, which encompassed more than a hundred OTUs. Furthermore, abundance and diversity of BALOs seemed to be poorly dependent on environmental variables (*e.g.*, physico-chemical parameters), suggesting the importance of biotic interactions such as quantity and quality of prey, competition for prey, predation by flagellates and ciliates, or parasitism by phages. We showed that the latter possibly have a significant impact on the dynamics of BALOs. Finally, a few active members of the *Bdellovibrionaceae* family were isolated from Lake Geneva, pointing again to the importance of this group of bacteria in the control of bacterial populations, a functional role that has yet to be quantified.

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Liste des abréviations

ADN : Acide désoxyribonucléique
AIC : Akaike information criterion
AICc : Corrected Akaike information criterion
ALBD : Annecy, Léman, Bourget, Dranse
ANOVA : Analysis of variance
AOA : Ammonia-oxidizing archaea
AOB : Ammonia-oxidizing bacteria
AP : Attack phase
ARNr : Acide ribonucléique ribosomal
ARNr 16S : ARN ribosomique de la petite sous unité des ribosomes des procaryotes
BALOs : Bdellovibrio et organismes apparentés
BALO-HI : BALO hôte indépendant
BALO-HD : BALO hôte dépendant
BS: Bootstrap
bp : base pair
BSA : Bovine serum albumin
BV : Bdellovibrio bacteriovorus
$CaCl_2$: Calcium chloride
CARRTEL : Centre alpin de recherche sur les réseaux trophiques des écosystèmes limniques
CCA : Canonical correspondence analysis
$CdCl_2$: Cadmium chloride
Chla : Chlorophyll
CIRM : Centre international de ressources microbiennes
Cl^- : Chloride
Ctot : Total carbon
DCA : Detrended correspondence analysis
DGGE : Denaturing gradient gel electrophoresis
DPANN : Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota and Nanohaloarchaeota
dNTP : Deoxynucleotide triphosphate
DO: Dissolved oxygen
DOC : Dissolved organic carbon
DOM : Dissolved organic matter
EC : Conductivity
EDTA : Acide éthylènediaminetétraacétique
FCM : Flow cytometry
HDNA: High DNA content
HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H-I : Host-independent
hit : Host interaction locus
KCl : Potassium chloride
KO : Kegg orthology

LB : Luria-Bertani broth
LDNA : Low DNA content
LPS : Lipopolysaccharide
$MgCl_2$: Magnesium chloride
MOA : Methane-oxidizing archaea
MOB : Methane-oxidizing bacteria
MPn : Methylphosphonic acid
NaCl : Sodium chloride
NH_4 : Ammonium
NGS : Séquençage nouvelle génération
NMDS : Non-metric multidimensional scaling
NO_3^- : Nitrate
Ntot : Total nitrogen
OD : Optical density
ORF : Cadre de lecture ouvert
OTU : Operational taxonomic unit
PBS : Phosphate-buffered saline
PC : Polycarbonate
PCR : Polymerase chain reaction
P_i : Inorganic phosphate
PLP : Prokaryote-like particles
PO_4-P : Orthophosphates
PP : Posterior probability
Ppart : Particulate phosphorus
Ptot : Total phosphorus
qPCR : Quantitative PCR
RDA : Redundancy analysis
rpm : rounds per minute
rpoB : Sous unité beta de l'ARN polymérase bactérienne
SDS : Sodium dodecyl sulfate
SHL2 : Le point le plus profond du Léman (Lake Geneva). Site de référence pour les suivis.
SiO_2 : Silica
SIP : Stable isotope probing
ssDNA : Single-stranded DNA
SOC : Super optimal broth
SO_4^{2-} : Sulfate
TAC : Water hardness
TACK: Thaumarchaeota, Aigarchaeota, Crenarchaeota, and Korarchaeota
TAE : Tris-acetate-EDTA
TAT : Twin arginine translocation
TCC : Thonon culture collection
TE : Tris-EDTA
Tm : Melting temperature
TN : Total nitrogen
TOC : Total organic carbon

TP : Total phosphorus
Tris : Tris(hydroxyméthyl)aminométhane
VIF : Variance inflation factor
VLP : Virus-like particles

Liste des publications

Revue 1:

Ezzedine JA, Jacquet S. **2019**. Bactéries prédatrices : zoom sur les Bdellovibrio et organismes apparentés (BALOs). *Notes Académiques de l'Académie d'agriculture de France (N3AF).* 2, 1–25 + compléments d'information (7 pages).

Revue 2:

Ezzedine JA, Desdevises Y, Jacquet, S. Bdellovibrio and like organisms: the smallest cellular hunters of the microbial world. Soumis le 15 décembre à *Annual Reviews of Microbiology*

Article 3:

Paix B, **Ezzedine JA**, Jacquet S. **2019**. Diversity, dynamics and distribution of *Bdellovibrio* and like organisms in peri-alpine lakes. *Appl. Environ. Microbiol*.: AEM.02494-18.

Article 4:

Ezzedine JA, Jacas L, Desdevises Y, Jacquet S. **2020**. *Bdellovibrio* and Like Organisms in Lake Geneva: An Unseen Elephant in the Room? *Front. Microbiol*. **11**: 98.

Article 5:

Ezzedine JA, Chardon C, Jacquet S. **2020**. New 16S rRNA primers to uncover *Bdellovibrio* and like organisms diversity and abundance. *J. Microbiol. Methods* **175**: 105996.

Article 6:

Ezzedine JA, Scheifler M, Desdevises Y, Jacquet S. Exploring the diversity and abundance of *Bdellovibrio* and like organisms in four contrasted ecosystems over a year. Soumis le 20 décembre à *The Isme Journal*

Article 7:

Ezzedine JA, Pavard G, Gardillon M, Jacquet S. **2020**. *Bdellovibrio* sp : An Important Bacterial Predator in Lake Geneva ? *J. Microbiol. Biotechnol*. **5**: 1–11.

Article 8:

Janicot A, **Ezzedine JA**, Rasconi S, Domaizon I, Jacquet S. Short-term dynamics of Bdellovibrio and like organisms under stress in Lake Geneva. Soumis le 2 décembre à *Microbial Ecology*

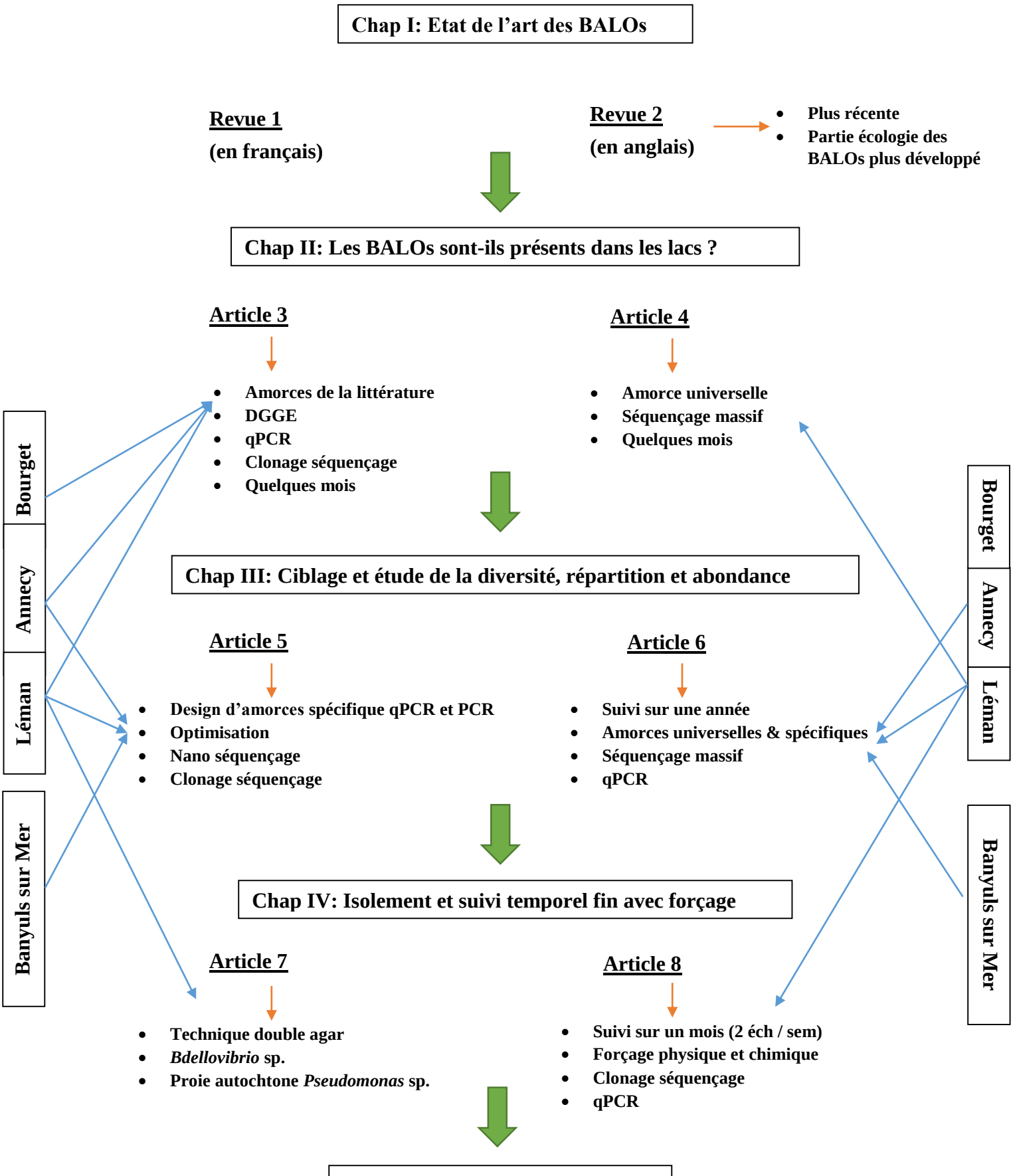
Article 9:

Ezzedine JA, Desdevises Yves, Jacquet S. **2020**. Exploring archaeal and bacterial diversity and co-occurrence in Lake Geneva. *Adv. Oceanogr. Limnol*.

Avant-propos

Le projet C-BALO, financé par INRAE et l'USMB, a consisté à décrypter la diversité, la distribution et l'abondance d'un groupe fonctionnel de bactéries, connu sous le nom de *Bdellovibrio* et organismes apparentés (BALOs) dans divers milieux aquatiques. Tout d'abord, un état de l'art complet des BALOs a été réalisé et est présenté dans les articles de revue 1 et 2 du chapitre I. Ces connaissances nous ont permis d'élaborer notre stratégie d'étude qui est exposée dans les chapitres suivants. Les milieux d'étude du laboratoire d'accueil (UMR CARRTEL) sont les lacs péri-alpins et d'altitude, et les BALOs n'avaient jusqu'alors jamais été recherchés et étudiés dans ces lacs (et plus largement en milieu lacustre). Des lors, des questions basiques telles que savoir s'ils pouvaient être détectés, s'ils étaient présents, s'ils étaient rares ou abondants étaient sans réponse avant ce travail de thèse. Ces questions nous ont poussées à proposer leur étude pour divers environnements et utiliser, dans un premier temps, des outils existants. Ainsi, le chapitre II, à travers l'article 3, révèle notre travail préliminaire de recherche des principales familles de BALOs dans plusieurs lacs au moyen des amorces existantes. Puis dans l'article 4 de ce même chapitre, nous nous sommes servi d'un jeu de données existant (projet TRANSLEM 2014 porté par Stéphan Jacquet) de séquençage pour explorer la présence des BALOs dans le Léman. Ces deux études nous ont permis de faire deux constatations majeures : (i) la nécessité de cibler les BALOs de manière plus fine et plus spécifique, typiquement via le développement de nouvelles amorces respectant la nouvelle classification des BALOs, et (ii) la dynamique des BALOs peut être très marquée d'un mois à un autre justifiant le besoin d'effectuer un suivi temporel plus resserré. Ainsi, nous avons dessiné, testé et utilisé de nouvelles amorces spécifiques des BALOs aussi bien pour la qPCR que le séquençage massif, un travail rapporté dans le chapitre III et l'article 5. Puis ces amorces ont servi à capturer la diversité des BALOs mais aussi de la communauté globale des procaryotes afin de suivre la dynamique temporelle et spatiale des BALOs dans les lacs Léman et d'Annecy ainsi que dans les eaux marines de la baie de Banyuls-sur-Mer (article 6). Pour commencer à approcher véritablement le rôle fonctionnel des BALOs, nous avons finalement tenté d'isoler des BALOs du Léman et effectuer des expériences proie-prédateur (article 7). Pour finir, le déploiement de mésocosmes dans le Léman a permis de travailler à courte échelle de temps (article 8). Au-delà de la production académique rapportée ci-dessus, un effort a aussi été consenti à la vulgarisation scientifique. Cela s'est traduit par l'écriture d'un article pour le magazine *Pour la Science* et de la réalisation de planches de bande dessinée. Ces travaux sont proposés en Annexe

tout comme un autre article, un peu en marge de ce travail, et portant sur les interactions entre bactéries et archées dans le Léman (article 9).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Chap V: Discussion et perspectives

Chapitre I

Etat de l’art sur les BALOs et objectifs de la thèse

Chapitre I : Etat de l'art sur les BALOs et objectifs de la thèse

Les *Bdellovibrio* ou organismes apparentés (BALOs) constituent un groupe particulier de bactéries car elles possèdent la capacité de se nourrir d'autres bactéries. De plus, elles sont qualifiées de prédatrices obligatoires, parce que la condition *sine qua non* de leur survie et de leur croissance repose entièrement sur la capture de proies. Parmi leurs congénères prédatrices, elles sont en fait les seules à avoir cette obligation. Pour mieux les connaitre, la première action effectuée durant cette thèse a consisté à analyser la littérature afin d'obtenir une connaissance globale de ces bactéries prédatrices. Nous avons donc synthétisé les divers aspects caractéristiques des BALOs, à savoir leur diversité (et l'évolution de leur classification), cycle de vie, écologie, prédation et applications. Les résultats de ces recherches bibliographiques se sont traduits par l'écriture de revues scientifiques en français et en anglais. Dans un premier temps, nous avons répondu favorablement à une invitation du journal de « l'Académie d'Agriculture » (N3AF) pour présenter les BALOs. Puis, une revue encore plus étoffée a été écrite et soumise à « *Annual Reviews of Microbiology* ». Ces deux revues constituent logiquement le Chapitre I de cette thèse car ils font un état des lieux des connaissances très complet de tous les aspects connus sur les BALOs mais aussi sur les autres prédateurs bactériens. Faisant directement suite à ces articles, les objectifs de la thèse et les questions auxquelles nous avons tentés de répondre au cours de ce travail sont posés.

Bactéries prédatrices : zoom sur les *Bdellovibrio* et organismes apparentés

Notes Académiques de l'Académie d'agriculture de France
Academic Notes from the French Academy of Agriculture
(N3AF)
Note de synthèse

Bactéries prédatrices : zoom sur les *Bdellovibrio* et organismes apparentés (BALOs)

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1. Introduction

Les microorganismes (virus, bactéries, archées, eucaryotes unicellulaires (« protistes »)…) sont abondants, diversifiés, ubiquistes et ont des rôles très importants dans le fonctionnement des écosystèmes. L'écologie microbienne vise à étudier la diversité et les interactions entre ces microorganismes, mais également entre ces derniers et leur environnement abiotique. Il existe de nombreuses interactions biotiques (qui relèvent de la prédation, du parasitisme, etc.) et parmi celles-ci, certaines ont encore été très peu explorées. C'est le cas pour un groupe remarquable de prédateurs bactériens nommé *Bdellovibrio et organismes apparentés*, connu sous l'acronyme anglo-saxon BALOs pour « *Bdellovibrio And Like Organisms* ». Les BALOs (Figure 1) sont des bactéries Gram négatif, prédateurs obligatoires d'autres bactéries. Dans la majorité des cas, le prédateur se loge dans sa proie (ou hôte) pour former une chambre appelée « bdelloplaste » (Figure 1) qui lui permet de (i) digérer à l'abri le contenu cellulaire de sa proie, (ii) se développer sous la forme d'une cellule filamenteuse, et, *in fine*, (iii) donner naissance à plusieurs « progénitures ». On pourrait parler de parasitoïdes pour ces cellules mais les spécialistes de ce groupe s'accordent plutôt à les définir comme des prédateurs car ces derniers mettent fin à l'activité métabolique de leur hôte en induisant la mort de leur proie dans les 15 minutes suivant l'infection (Chen et Williams, 2012). Et comme tout prédateur dont le besoin est de se nourrir, les BALOs entament à nouveau une recherche dynamique de nouvelles proies, ce qui les différencie des « parasites » *sensu stricto* (voir Encadré 1 dans le complément électronique). Si les BALOs ne sont pas les seuls prédateurs bactériens connus (voir Encadré 2), ils sont les plus étudiés (Harini et al., 2013).

Malgré une distribution qui semble ubiquiste et donc un rôle fonctionnel supposé important ou avéré dans certains cas, très peu d'études ont été consacrées au rôle et à l'impact de ces prédateurs sur la communauté bactérienne des environnements naturels et anthropisés. Les interactions biotiques qui ont été très étudiées pour les bactéries ont surtout été celles impliquant les virus de bactéries (phages ou bactériophages) et les protistes nanoflagellés. En effet, il a souvent été décrit dans la littérature que les bactériophages et les protistes contribuent majoritairement à la mortalité bactérienne et au « turnover » de cette communauté au sein des écosystèmes aquatiques (Miki et Jacquet, 2010). Cependant, certains auteurs avancent aujourd'hui que cette affirmation pourrait être revue légèrement à la baisse. En effet, bien que restant à ce jour l'entité biologique la plus abondante de la biosphère, la concentration des virus a vraisemblablement été surestimée, notamment suite à la découverte des vésicules membranaires extracellulaires qui se confondent avec les virus (Gaudin et al., 2014 ; Soler et al., 2015).

Cette synthèse présente les BALOs et montre leur importance dans les écosystèmes microbiens.

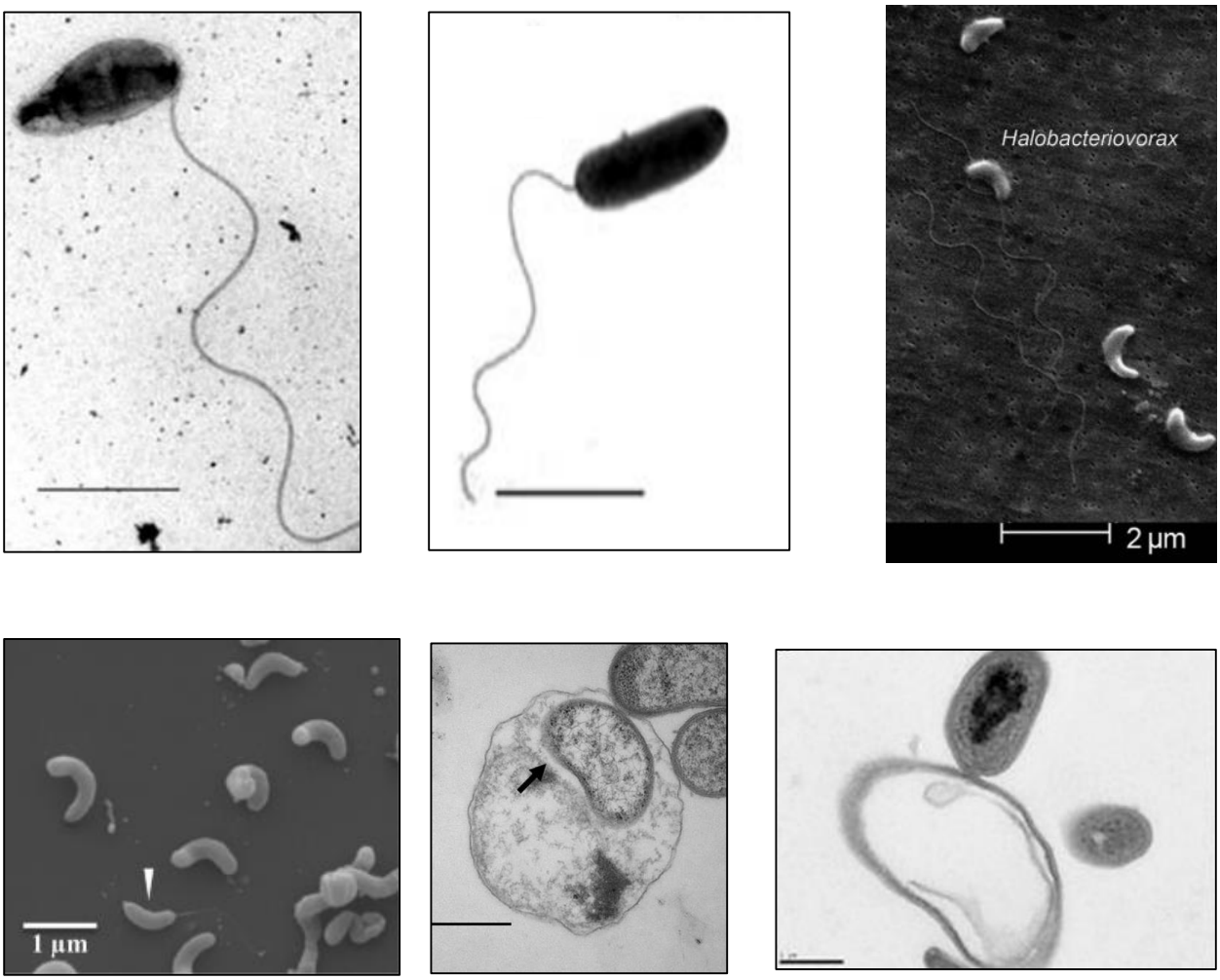


Figure 1 Images de microscopie électronique à transmission et à balayage montrant différents BALOs à l'état planctonique et en phase de prédation. A) *Micavibrio* sp. et B) *Bdellovibrio bacteriovorus* 109J (extrait de Davidov et al., 2006); C) *Halobacteriovorax* sp. (source : Welsh et al., 2016); D) *Bacteriovorax stolpii* (source : Richards et al., 2012); E) Formation d'un bdelloplaste (prédation endobiotique) (source : Chen et al., 2015); F) *B. exovorus* associé à sa proie (prédation épibiotique) (source : Rotem et al., 2014).

2. Caractéristiques générales des BALOs

La découverte des BALOs est le fruit accidentel des recherches menées dans les années 1960 par Stolp dédiées initialement à l'isolement des bactériophages du phytopathogène *Pseudomonas syringae* pv. *phaseolicola*, l'agent responsable de la graisse du haricot. En compagnie de Petzold et Starr, Stolp décrivit et désigna ce groupe de microorganismes formant des plaques autour de bactéries sous le vocable de *Bdellovibirio* (Stolp et Petzold, 1962 ; Stolp et Starr, 1963). Ce mot

dérive de *Bdella*, signifiant sangsue en grec. En effet, le BALO s'attache à sa proie, y pénètre ou non selon l'espèce, et absorbe tel un vampire le contenu cellulaire de sa victime (Harini et al., 2013). Découverts initialement dans le sol, puis dans des milieux différents et variés tels que les environnements lacutres et marins (Schwudke et al., 2001), les BALOs semblent être partout où on les cherche. Les BALOs à l'état planctonique (Figure 1) sont uniflagellés et mobiles, souvent en forme de vibroïde (Davidov et al., 2006; Williams et al., 2015). Leur comportement de prédation est gouverné par le locus *hit* (host interaction). Toutefois, une mutation spontanée de ce locus se traduit par la génération de BALOs hôte-indépendant (BALO-HI) capables de se répliquer par simple fission binaire (Oyedara et al., 2016; Roschanski et al., 2011). Les BALOs ont été détectés et étudiés à l'origine par des méthodes de culture classique de laboratoire, avec leurs avantages mais aussi leurs limites. Cultiver des bactéries a généralement pour résultat de sous-estimer considérablement la diversité réelle. On rapporte d'ailleurs souvent que seulement 1% des microorganismes peut être cultivé et isolé par ces méthodes (Jørgensen et al., 2014). Les BALOs ne font pas exception à cette règle (Van Essche et al., 2011), et il apparait aussi que les BALOs, ne constituent pas un groupe numériquement dominant dans des milieux naturels ouverts (Davidov et al., 2006). De plus, comme la prédation est une nécessité pour la réplication des BALOs, il est aussi primordial d'intégrer dans leur culture des proies qu'ils peuvent infecter. Cette co-culture proie-prédateur a souvent masqué la diversité des BALOs, car certains d'entre eux ne réussissent pas à se nourrir des proies qui leur sont proposées (Chen et al., 2011). Les deux aspects précédemment évoqués ont d'ailleurs pu donner l'illusion d'un rôle mineur des BALOs au sein des communautés bactériennes. L'avènement des méthodes cultures-indépendantes, comme celles utilisant des amorces ADN spécifiques aux BALOs associées par exemple à l'électrophorèse sur gel en gradient dénaturant (DGGE), ont alors révélé l'existence d'une diversité de BALOs plus importante. Mais c'est surtout le clonage suivi du séquençage qui a permis de révéler cette diversité (Davidov et Jurkevitch, 2004). Plus récemment, l'arrivée de techniques encore plus performantes, les techniques de séquençage nouvelles générations (NGS) dites aussi à haut débit, a permis de franchir de nouvelles frontières dans l'analyse des communautés bactériennes en fournissant une grande quantité d'information dans l'identification des phylotypes microbiens (Li et Williams, 2015). Les NGS et les outils bio-informatiques ont relancé ou favorisé les études de la génomique ciblée, autrement dit la métagénétique ou metabarcoding (étude d'un seul marqueur moléculaire), et de la métagénomique (étude de tout le génome). La détection des BALOs peut ainsi se faire en se basant sur le séquençage du « gène » 16S (caractéristique des « procaryotes ») de la petite sous unité ribosomique (ARNr) mais aussi sur le gène de la sous-unité bêta de l'ARN polymérase (*rpoB*) (Pineiro et al., 2004).

Aujourd'hui, et faisant suite aux remaniements réguliers de la taxonomie, les BALOs sont classés en deux groupes polyphylétiques distincts, les Oligoflexia et les α-proteobacteria (pour mieux comprendre la classification des bactéries, voir l'encadré 3). Bien qu'ils soient phylogénétiquement distants, ces groupes partagent des comportements de prédation similaire, à savoir l'obligation de prédation et un cycle de vie composé de deux phases séparées métaboliquement et spatialement (Pasternak et al., 2014), décrites plus loin. Le premier groupe comprend quatre familles appelées *Bdellovibrionaceae, Bacteriovoracaceae, Pseudobacteriovoracaceae, Peredibacteraceae* et *Halobacteriovoraceae,* et le second est constitué d'un genre unique, *Micavibrio* (Pasternak et al., 2014; Koval et al., 2015; McCauley et al., 2015). Parmi les BALOs, l'espèce *Bdellovibrio Bacteriovorus* de la famille des *Bdellovibrionaceae* a été la plus étudiée (Williams et al., 2015). Comparativement à *Escherichia coli*, *B. bacteriovorus* et les BALOs en général sont de plus petite taille. En effet, si *E. coli* mesure 1 x 3 µm (Fenton et al., 2010), *B. bacteriovorus* arbore des dimensions de l'ordre de 0,3 x 1 µm (Baker et al., 2017) et la taille des *Bdellovibrionaceae* varie généralement entre 0,2 à 0,5 µm en largeur, et 0,5 et 2,5 µm en longueur (Crossman et al., 2012). La longueur des *Bacteriovoracaceae* est comprise entre 0,5 et 1,4 µm (Baer et al., 2000) et pour *Halobacteriovoraceae* elle est de 0,6 à 1,0 µm (Crossman et al., 2012). *Micavibrio* possède aussi une petite taille qui est de l'ordre de 0,25-0,4 x 0,5-1 µm (Jurkevitch et Davidov, 2006).

3. Aspects phylogénétiques

Depuis la première caractérisation des BALOs par Stolp et pour les 4 décennies qui ont suivi (voir l'encadré 4), ces prédateurs ont été placés dans un genre unique, *Bdellovibrio* (Davidov et al., 2006). Cette classification est née de deux constats. Le premier était que les bactéries prédatrices présentant le même cycle cellulaire typique étaient incluses automatiquement dans le genre *Bdellovibrio* sans tenir compte de leur relation génétique et malgré de grandes différences dans les taux de GC (Baer et al., 2000). Le second était que la classification taxonomique s'est longtemps appuyée sur le spectre de prédation des BALOs en présence de proies (Sutton et Besant, 1994), quand la co-culture était possible. En raison de la similitude du type de prédation entre *Halobacteriovorax* (BALOs halophile) et *B. bacteriovorus*, les espèces *Halobacteriovorax* ont été classées à l'origine dans le genre *Bdellovibrio* (Enos et al., 2017), et l'appellation *Bdellovibrio* sp. marin a persisté pendant plus d'une décennie (Crossman et al., 2012). On sait aujourd'hui que les BALOs forment en fait des groupes très hétérogènes avec une grande diversité phylogénétique et présentent un spectre de prédation assez large (Davidov et al., 2006). L'analyse des séquences du gène de l'ARNr 16S et l'avènement du séquençage à haut débit ont conduit tout d'abord à une reclassification des *Halobacteriovorax* dans le genre *Bacteriovorax,* avant de finalement les placer

dans leur propre genre (*Halobacteriovorax*) et famille (*Halobacteriovoraceae)* (Enos et al., 2017). La reclassification de la bactérie *Bacteriovorax starri* en *Peredibacter starrii* a été proposée, et la famille des *Peredibacteraceae* est née. Elle comprend des souches d'eau douce et du sol (Pineiro et al., 2004).

Depuis les années 2000, diverses études plus approfondies de la phylogénie basée sur l'analyse des séquences complètes du gène de l'ARNr 16S (dont le nombre de copie varie de 1 à 3 chez les BALOs (Kandel et al., 2014; Pasternak et al., 2014)) et du gène *rpoB* provenant de prédateurs issus d'habitats très variés, ont été menées par plusieurs « bdellovibriologistes ». Ainsi, les BALOs sont aujourd'hui classés en 5 familles, auxquelles il faut rajouter le genre *Micavibrio* (Figure 2). La classification présentée dans cette synthèse (Table 1) est la plus récente et elle est inspirée de Hahn et al. (2017), (Koval et al. (2015) et Rotem et al. (2014). Les 5 familles de BALOs s'insèrent dans la classe des Oligoflexia (à l'origine, membre des delta-proteobacteria). Chaque famille est caractérisée par une espèce ou une souche type. Les deux souches types des *Bdellovibrionaceae* sont *B. bacteriovorus* HD100 et *B. exovorus* JSS. Les *Bacteriovoracaceae* et les *Peredibacteraceae* ne sont représentées que par une seule souche type chacune, respectivement *Bacteriovorax stolpii* UKi2 et *Peredibacter starrii* A3.12. De même, pour les *Pseudobacteriovoracaceae*, avec la souche *Pseudobacteriovorax antillogorgiicola* RKEM611. Enfin, *Halobacteriovorax marinus* SJ et *H. litoralis* JS5 sont les souches types de la famille des *Halobacteriovoraceae*. Le genre *Micavibrio,* quant à lui, s'insère dans la classe des α-proteobacteria et est représenté par deux espèces, *M. admirantus* et *M. aeruginosavorus*. Toutefois, il n'est pas exclu que la classification actuelle des BALOs change encore à l'avenir avec le progrès constant des nouvelles techniques de séquençage et de biologie moléculaire.

La Table 1, présente le nombre de séquences pour chaque groupe de BALO disponible au jour de la rédaction de cet article, dans les trois grandes bases en ligne qui répertorient les séquences bactériennes. La famille des *Bdellovibrionaceae* est la plus fréquente parmi les BALOs dans les environnements terrestres (Oyedara et al., 2016). Bien que les BALOs soient représentés dans la rande majorité des écosystèmes, leurs séquences sont encore faiblement représentées dans les banques de données.

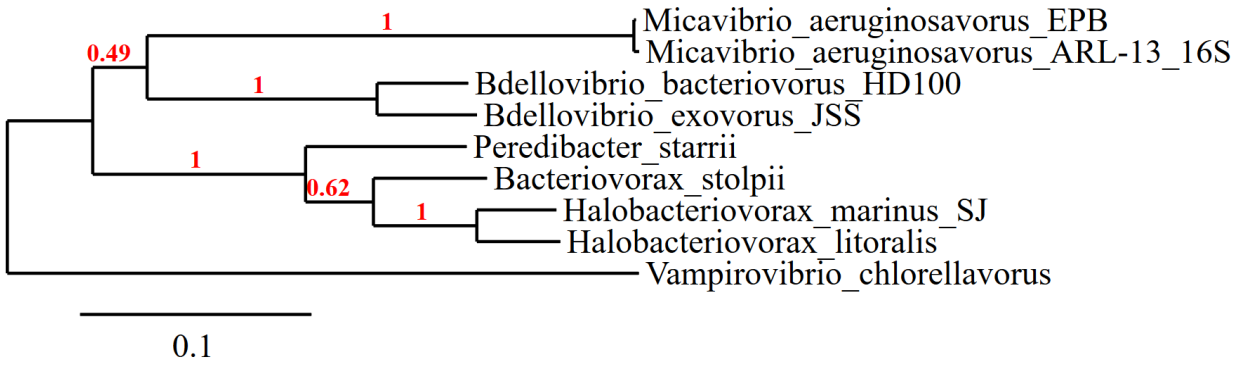


Figure 2 Arbre phylogénétique simplifié des BALOs. Les séquences du gène de la sous unité ribosomique 16S des espèces/souches types de 5 genres de BALOs ont été téléchargées depuis le site www.arb-silva.de (Quast et al., 2013). Le modèle phylogénétique utilisé est GTR+G+I avec 100 bootstraps en maximum de vraisemblance (www.phylogeny.fr) (Dereeper et al., 2008). L'arbre est enraciné avec *Vampirovibrio chlorellavorus*. Concernant l'espèce *Micavibrio admirantus*, aucune séquence n'est répertoriée dans les bases de données classiques tels que NCBI ou arb-silva.

Table 1 Nombre de séquences d'ADNr 16S partielles et complètes pour les 6 genres de BALOs répertoriées dans les principales bases de données : NCBI (Benson et al., 2013), RDP (Cole et al., 2014) et Arb-Silva (Quast et al., 2013).

Genre	NCBI	RDP	Arb-Silva
Bdellovibrio	1865	658	170
Bacteriovorax	904	663	429
Peredibacter	131	91	1
Halobacteriovorax	193	24	7
Micavibrio	818	137	9
Pseudobacteriovorax	7	1	2

4. Cycle de vie

Il existe deux types de cycle de vie, d'une durée de 3,5 à 4 heures (Rogosky et al., 2006) chez les BALOs : le cycle endobiotique ou périplasmique (Figure 3) où le prédateur pénètre et niche à l'intérieur de son hôte, et le cycle épibiotique (Figure 4) où le prédateur s'attache à sa proie mais n'y pénètre pas. Il existe aussi chez certaines formes mutantes de BALOs la possibilité d'un cycle de vie hôte-indépendant (BALO-HI) consistant en une alimentation directe à partir d'un milieu riche sans obligation de prédation. Ce cycle HI a été observé dans la famille des *Bacteriovoracaceae* (Davidov et Jurkevitch, 2004), des *Halobacteriovoraceae* (Crossman et al., 2012) et chez l'espèce *B. bacteriovorus* (BV). Par contre, il semble que ce cycle ne puisse être induit chez d'autres espèces comme *Micavibrio* EPB ou *B. exovorus* JSS (Pasternak et al., 2004). Hormis les BALO-HI, les cycles de vie typiques, endobiotique et épibiotique, se déroulent toujours en deux phases : la phase d'attaque commune aux deux modes, suivie de la phase de croissance et de réplication, différente spatialement et par le nombre de progénitures obtenues.

4.1. Phase d'attaque

La phase d'attaque est conservée parmi toutes les espèces de BALOs, à l'inverse de la phase de croissance. La phase d'attaque est caractérisée par une grande mobilité soutenue par un flagelle polaire, une incapacité à répliquer l'ADN ou à se diviser et une durée de vie courte en absence de proie (Pasternak et al., 2014). Cette phase d'attaque est la mieux étudiée chez la souche *B. bacteriovorus* HD100.

Stage I – *Mécanismes de rencontre du prédateur et de sa proie* : La phase d'attaque, mobile et libre (Chauhan et al., 2009a), est une phase planctonique où la recherche de la proie est aléatoire. Le prédateur, doté d'un unique flagelle polaire (Shatzkes et al., 2016), peut se déplacer rapidement à plus de 50 µm/s (Jashnsaz et al., 2017) pour une vitesse maximale de 160 µm/s (Williams et al., 2015). Chez BV, cette phase mobile fait intervenir différents gènes intervenant dans la motilité, et la synthèse et la structure du flagelle, qui sont localisés sur quatre loci indépendants (Rendulic et al., 2004). Le déplacement du prédateur est aléatoire et la détection de la proie ne dépend pas de molécules de signalisation de type homosérine-lactones (quorum-sensing) pouvant être produites par les bactéries proies (Rendulic et al., 2004). A ce jour, aucune réponse chimiotactique significative n'a été mesurée pour des concentrations de proies inférieures à 10^8 cellules/mL. Il a toutefois été constaté qu'à des concentrations élevées en proies, les prédateurs peuvent s'accumuler chimio-tactiquement autour des proies et de leurs lysats cellulaires (Jashnsaz et al., 2017). La chémotaxie semble donc jouer un rôle modeste dans le ciblage des proies. De plus, l'absence de

récepteur spécifique sur la paroi de la proie corrobore cette hypothèse (Jashnsaz et al., 2017). Que la stratégie de « chasse » bactérienne soit aléatoire est tout à fait concevable car des signaux chémo-attractifs émis par de multiples proies pourraient *in fine* envoyer des messages contradictoires aux prédateurs. Si la rencontre du prédateur avec sa proie est donc fortuite, elle semble être favorisée par l'hydrodynamisme créé par les forces de rotation du flagelle et du corps du prédateur, qui, en plus du déplacement engendré, l'attire et le place (*via* la turbulence) vers des corps inertes dont des proies potentielles gravitant autour de ces corps (Jashnsaz et al., 2017). Bien que le prédateur ne puisse pas se répliquer durant la phase d'attaque, il continue d'absorber les nutriments de l'environnement qu'il utilise pour synthétiser et sécréter une large gamme de protéines et d'enzymes hydrolytiques (Dwidar et Yokobayashi, 2017). Par ailleurs, en l'absence de nutriments, le prédateur peut consommer ses propres composants cellulaires pour se maintenir en vie (Dwidar et al., 2017).

Stage II – *Reconnaissance de la proie et ancrage du prédateur* : Une fois que le prédateur contacte une proie, il s'attache à elle d'abord de façon réversible pour une courte période dite de reconnaissance, puis l'attachement devient irréversible. D'après Rendulic et al. (2004), l'ancrage (ou adhésion) du prédateur sur la proie est gouverné par l'activité de nombreux gènes. Hormis l'existence d'interactions passives entre les membranes extérieures des cellules, du type protéine-protéine et LPS-LPS (lipopolysaccharide, composant essentiel de la face externe de la membrane externe des bactéries), l'adhésion active a lieu *via* l'activité des gènes du pilus. Les pili de type IV ou *fimbriae* réalisent plusieurs fonctions chez les bactéries comme celles de sécréter des protéines d'adhérence ou de permettre au prédateur un type de mobilité particulier, le « twitching ». Le bout du pilus contient une multitude de biopolymères adhésifs qui sont spécifiques de différentes surfaces. Il est supposé que l'ensemble des pili type « twitching » permettent de tirer *B. bacteriovorus* à travers le pore d'entrée généré dans la membrane externe de la proie tout en étant attaché au côté interne de la paroi peptidoglycane.

Stage III – *Pré-invasion et invasion de la proie* : Avant de pénétrer sa cible, le prédateur génère une petite ouverture dans la membrane externe de la couche peptidoglycane de la proie. Cette ouverture se fait grâce à un ensemble d'enzymes hydrolytiques appliquées localement de façon à limiter les dégâts envers la proie. Il est supposé que les gènes responsables de cette fonction codent pour des protéases de type sérine, cystéine, aspartate et métal dépendant (Rendulic et al., 2004).

4.2. Mode endobiontique

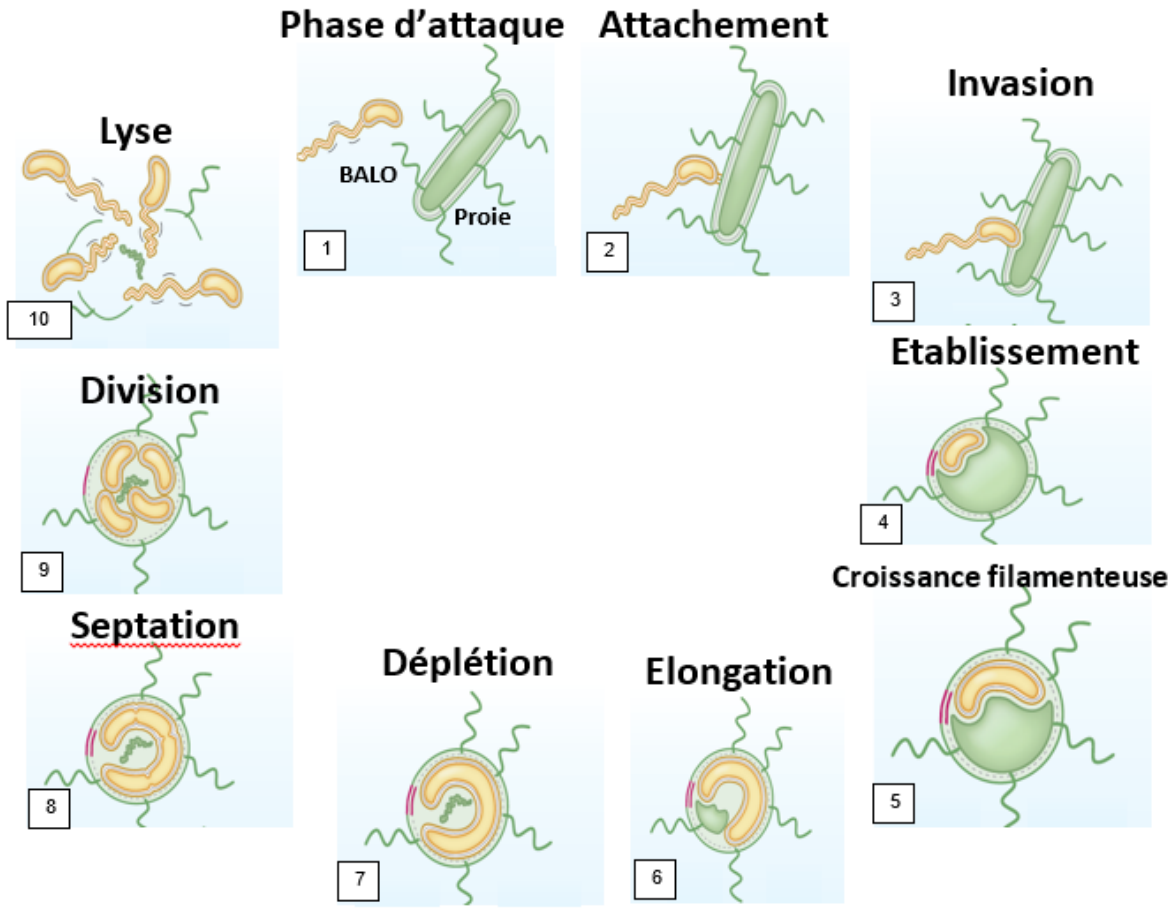
Stage IV – *Formation du bdelloplaste et croissance* : En plus des protéases, Lambert et al. (2015) ont décrit l'activité de la glycanase qui permet de solubiliser les peptidoglycanes de la proie au

début et en cours d'invasion. En effet, deux peptidoglycanes (DD-endopeptidases Bd0816 et Bd3459) rompent les liaisons covalentes entre les chaines de polymère (« decrosslinking ») de la paroi cellulaire en hydrolysant la structure 3-4 peptide crosslinks. Le « crosslinking » est un processus chimique permettant de joindre deux ou plusieurs molécules par liaison covalente, le terme équivalent en français est réticulation. L'action de la DD-endopeptidase est un signal de changement morphologique qui bloque l'entrée de la proie à d'autres prédateurs, éliminant ainsi toute compétition. Une fois entré dans son hôte grâce au système de type pilus IV qui est localisé au pôle non flagellé de la cellule (Fenton et al., 2010), le prédateur se débarrasse de son flagelle. La phase de croissance intrapériplasmique est alors entamée (Chauhan et al., 2009a). Le signal d'occupation généré par la DD-endopeptidase provoque la formation du « bdelloplaste » (Lambert et al., 2015). Précisément, la proie infectée est convertie en une structure hybride proie-prédateur (Van Essche et al., 2011), et la forme de la proie change à cause d'un procédé impliquant une hydrolyse des liaisons peptidiques de la paroi cellulaire et de la dégradation des biopolymères Dans certains cas, la morphologie initiale de la proie peut être maintenue tout au long du processus de prédation (Chen et Williams, 2012). La bactérie proie agit à la fois comme une source de nourriture et comme un habitat (Baker et al., 2017). La forme du bdelloplaste est ronde (Dwidar et Yokobayashi, 2017) ou en croissant de lune (Chen et Williams, 2012). Il constitue aussi une véritable barrière contre les attaques des bactériophages, et protège le couple hybride des conditions physico-chimiques défavorables (Chen et Williams, 2012).

Dans le bdelloplaste, le prédateur secrète un cocktail d'hydrolases, de protéases et de peptidases (Monnappa et al., 2014) pour hydrolyser et consommer les composants cellulaires, protéiques, ARN et ADN de la proie (Oyedara et al., 2016). La modification de la membrane cytoplasmique de la proie par la formation du bdelloplaste augmente la perméabilité pour faciliter l'alimentation du prédateur sur les composés dégradés (Gophna et al., 2006). Ces nutriment sont utilisés pour croître et se répliquer (Dwidar et al., 2017).

Stage V – *Réplication, septation et libération de la progéniture* : Le prédateur se développe sous forme d'un filament au sein de son hôte, puis la longue cellule filamenteuse se divise par segmentation (septation) en 2 à 7 ou en 3 à 6 progénitures de même taille (Dwidar et Yokobayashi, 2017; Fenton et al., 2010). Selon Fenton et al., (2010), la septation et l'élongation filamenteuse pendant la réplication se produisent de manière synchrone pour l'ensemble de la progéniture. Ce synchronisme est même maintenu dans le cas où deux BALOs réussissent à envahir la même proie : les deux prédateurs « s'attendent » pour lyser l'hôte. Ce synchronisme pourrait être expliqué, soit par la diffusion d'un signal entre les prédateurs au sein de la même proie, soit par une réaction simultanée à la déplétion finale du bdelloplaste. Ce cas rare de double ou multi-infection a été

observé uniquement lorsqu'un nombre important de prédateurs est présent pour une quantité limitée de proies. Cependant, les deux BALOs restant en compétition vis-à-vis de la ressource alimentaire, chaque prédateur donnera un nombre de progéniture différent. Lorsque le protoplasme de la proie est consommé entièrement et que la progéniture a atteint la taille maximale, celle-ci



développe un flagelle dans le but de préparer sa sortie. Deux mécanismes de sortie ont été observés, le premier consistant en une rupture enzymatique de la membrane du bdelloplaste (Rendulic et al., 2004) et le second, une libération de la progéniture à travers des pores discrets du bdelloplaste (Fenton et al., 2010).

Figure 3 Schéma illustrant le cycle de prédation et de reproduction endobiotique de *B. bacteriovorus*, souche HD100 (Source : Negus et al., 2017).

4.3. Mode épibiontique

Stage IV bis – *Ancrage extérieur du prédateur à sa proie* : Le genre *Micavibrio*, *B. exovorus* et exceptionnellement *B. bacteriovorus* en présence de bactéries à Gram positif (Iebba et al., 2014 ; Pantanella et al., 2018) sont des prédateurs caractérisés par une phase de croissance épibiotique. Ces prédateurs restent donc attachés à l'extérieur de la proie sans intrusion, tout en consommant les organelles de la proie (Shatzkes et al., 2016). La proie ne s'arrondit pas pour former un

bdelloplaste (Pasternak et al., 2014). *Micavibrio aeruginosavorus* peut s'attacher à sa proie par le côté polaire (pili) non flagellaire mais aussi par le côté non polaire de manière longitudinale. Pour *B. exovorus*, le point d'ancrage ne se fait que du côté polaire. Par ailleurs, plusieurs prédateurs peuvent s'attacher à une même proie (Pasternak et al., 2014).

Stage V bis – *Fission binaire* : A la fin de la consommation du contenu cellulaire, le prédateur épibiotique subit une fission binaire, créant ainsi uniquement deux cellules filles. Cette fission peut se produire de deux façons : soit le BALO reste attaché à sa proie pour rentrer en fission, soit il se détache de sa proie et effectue sa fission indépendamment (Chanyi et al., 2013; Pasternak et al., 2014).

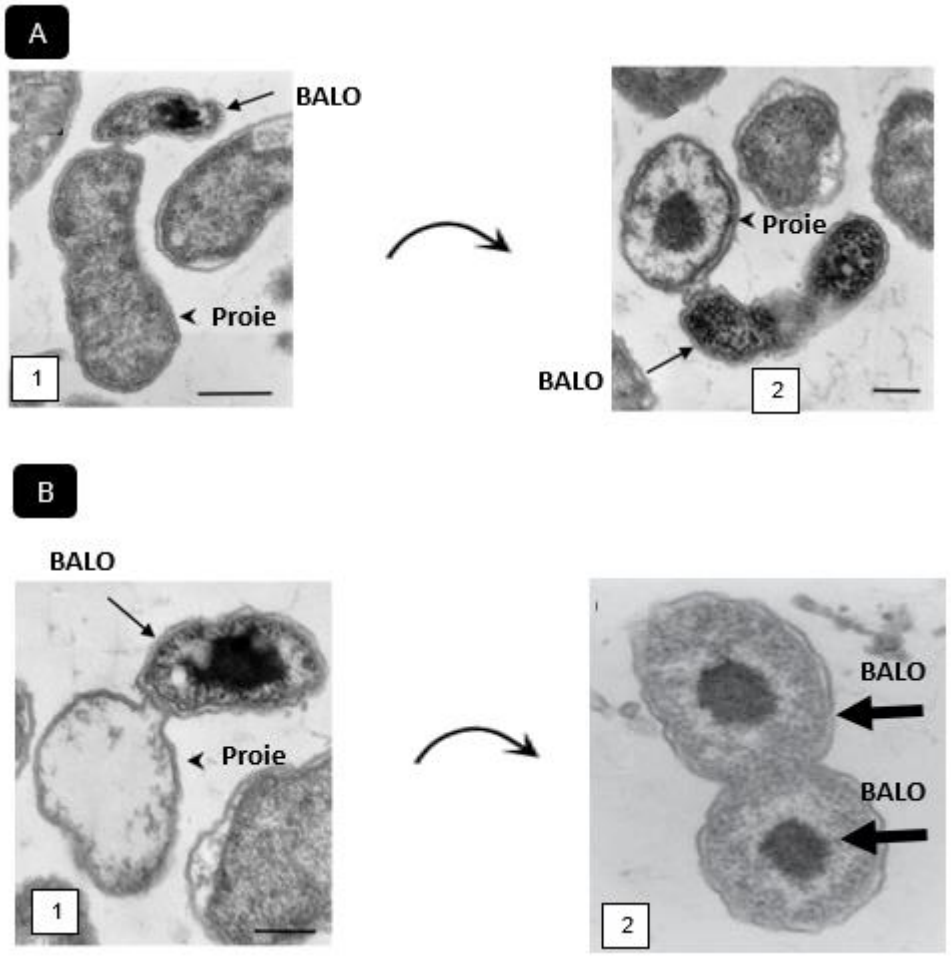


Figure 4 Schéma illustrant les deux cas de figure caractéristiques des BALOs épibiotiques. A) Fission binaire en restant attachée à la proie. B) Fission binaire suivie du détachement de la proie (Source : Chanyi et al., 2013).

5. Prédation

Les BALOs sont des bactéries à Gram négatif, prédatrices d'autres bactéries. Leurs proies de prédilection sont également des bactéries « Gram négatives », certaines étant d'ailleurs des pathogènes pour les plantes, les animaux ou l'Homme (Davidov et al., 2006; Fenton et al., 2010; Rendulic et al., 2004). Par ailleurs, des BALO-HI peuvent émerger spontanément dans certaines cultures de laboratoire. Parmi les BALO-HI certains restent des prédateurs facultatifs alors que d'autres perdent la capacité de prédation (Roschanski et al., 2011). Encore plus surprenant, en présence exclusive de proies Gram positif, *B. bacteriovorus* à l'origine prédateur endobiotique se transforme au bout de quelques heures en prédateur épibiotique (Iebba et al., 2014). Ainsi, chez une même espèce comme *B. bacteriovorus*, trois cycles de vie sont possibles.

5.1. Hôte indépendant, BALOs saprophytique ou axénique

BV peut spontanément manifester un phénotype hôte-indépendant (HI) si deux conditions sont réunies : l'absence totale de proies et, un milieu de culture riche en protéines (milieu complet) (Capeness et al., 2013). On distingue chez les HI deux types de mutants, le type I et le type II. Le type I ou saprophyte est un prédateur facultatif, qui conserve sa capacité d'envahir les bactéries vivantes. Cependant, il a perdu en efficacité de prédation par rapport à la souche sauvage ; les plaques qui se forment autour des proies sont petites et turbides (Roschanski et al., 2011). Les BALOs de ce type peuvent aussi croître en présence d'extraits cellulaires mais en aucun cas dans un milieu axénique (milieu de culture complet dépourvu de proies ou d'extrait cellulaire). Au contraire, les BALOs du type II ou axéniques sont capables de croitre uniquement sur un milieu axénique ou complet. Les types II peuvent être obtenus à partir des types I en mettant ces dernières répétitivement sur des milieux de cultures complets sans ajout d'extrait cellulaire de proies. En général, les expériences au laboratoire ont montré que les BALOs-HI sont composés à 99% du type I et 1% du type II (Roschanski et al., 2011).

Parmi les BALOs hôte dépendant (HD), seule une petite fraction est capable de devenir HI une fois exposée à un milieu de culture riche en protéine (milieu complet) (Capeness et al., 2013). En effet, il faut au moins 10^6 à 10^7 cellules HD pour obtenir quelques cellules HI. L'origine du comportement de prédation de *B. bacteriovorus* dépend de l'intégrité du locus *hit* (« host interaction locus »), qui mesure 959 pb et contient une petite région contenant un seul cadre de lecture ouvert ou ORF dénommé Bd0108 (Cotter et Thomashow, 1992). Le locus *hit* fait partie d'un groupe de gènes responsables de la formation des pili de type IV et de l'adhérence cellulaire. Ces deux éléments sont indispensables pour l'attachement et l'invasion de la proie par le prédateur (Rendulic 2004; Schwudke et al., 2005). Ainsi, chez des mutants HI, la capacité de prédation peut

être restaurée par l'introduction du gène sauvage *hit* (Cotter et Thomashow, 1992). La mutation du locus *hit* est due à une délétion de 42 pb au niveau du gène *bd0108*. Cette mutation impacte le développement des pili Type IVa qui conditionne l'attachement du prédateur à la proie ; ainsi le mauvais développement des pili laisse le phénotype HI prendre le relais (Capeness et al., 2013). Toutefois, certains isolats HI ne possèdent pas de mutation au niveau du locus *hit*. Cotter et Thomashow (1992) ont émis l'hypothèse de l'existence d'une deuxième mutation génératrice de BALOs-HI ailleurs dans le génome. Néanmoins, si on considère la souche *B. bacteriovorus* HD 100 (BV HD100), la majorité des souches HI générées (89%) sont issues de la mutation du gène *bd0108*. En contrepartie, seulement 11% des autres souches BV-HI ont été attribués à un contrôle différentiel de la transcription de l'ADN dans le génome (Wurtzel et al., 2010). Le ou les processus menant à l'apparition du phénotype HI demeurent l'une des questions majeures dans la recherche sur les BALOs (Roschanski et al., 2011).

5.2. Prédateur à Gram positif

Malgré leur préférence pour des proies à Gram négatif, les BALOs sont toutefois susceptibles de cibler, dans certaines conditions, certaines bactéries à Gram positif, comme cela a été montré pour le pathogène *Staphylococcus aureus* impliqué chez des sujets atteints de mucoviscidose (Caballero et al., 2017). En présence de *S. aureus* comme unique proie, *B. bacteriovorus*, prédateur naturellement endobiotique, adopte un mode opératoire différent en cas d'indisponibilité d'autres proies en devenant épibiotique, et ce, après seulement 20 heures (Iebba et al., 2014). Ce temps d'action (comparable à la phase de latence pour la croissance de certaines bactéries, Pantanella et al., 2018) représente le temps nécessaire pour (i) synthétiser des nouvelles enzymes capables d'agir sur la paroi des Gram positives, mais aussi (ii) l'adaptation du prédateur à son nouvel environnement (ici la co-culture avec *S. aureus*). La prédation comme énoncée dans le chapitre « Hôte indépendant, BALOs saprophytique ou axénique », dépend de la formation de pili type IVa et des mécanismes d'adhérences cellulaires. Ces derniers dépendent de l'intégrité du locus *hit*. Pour faire simple, le locus génétique *hit* conditionne la capacité des BALOs, en l'occurrence de BV, à s'attaquer à des proies (Schwudke et al., 2001). Le locus est relié aux gènes *bd0108* et *bd0109* qui par leurs interactions régulent la production de pili chez le prédateur (Capeness et al., 2013). Or l'analyse de la séquence du produit d'amplification spécifique du gène *bd0108* de *B. bacteriovorus* à partir d'échantillons obtenus à différents moments de co-culture de *B. bacteriovorus* et *S. aureus* (Gram +) et de *B. bacteriovorus* et *Pseudomonas aeruginosa* (Gram -) n'a pas révélé de mutations qui peuvent découler du locus *hit* pour expliquer le mode épibiotique de *B. bacteriovorus* en présence de Gram positives (Pantanella et al., 2018).

5.3. Les autres caractéristiques de la prédation des BALOs

Rogosky et al. (2006) ont rapporté que *B. bacteriovorus* peut prédater des cellules mortes dont le contenu cellulaire est intact. Par contre, la présence d'autres bactéries prédatrices, ne servant pas de proies, peut influencer l'efficacité de la prédation (Van Essche et al., 2011). La dynamique entre le prédateur et ses proies est régie par un cycle classique de croissance et de déclin.

Les BALOs ont un spectre de prédation différent d'une espèce à une autre, certaines sont semi-généralistes avec un large spectre de proies, d'autre sont spécialistes, c'est-à-dire restreints à un type de proie bien spécifique, et d'autres sont versatiles ou polyvalents, autrement dit à la fois semi-généralistes et spécialistes. Ce dernier cas constitue un avantage, puisque l'espèce qui l'adopte est moins limitée en termes de choix de proies, et minimise la compétition tout en favorisant sa dominance (Chen et al., 2011). Cependant, être polyvalent ne confère pas forcément au prédateur une prédation plus efficace sur toutes les proies. L'efficacité de prédation change en fonction des proies. Cette tactique constitue un compromis : si le prédateur perd en efficacité, il gagne en nombre de proies potentielles (Chen et al., 2011).
Les bactéries autochtones sont préférées par les prédateurs aux bactéries de culture ou celles venant d'autres habitats (Chauhan et al., 2009a; Pineiro et al., 2004; Wen et al., 2009). En effet, dans un scénario où *B. bacteriovorus* 109J est exposé à plusieurs proies potentielles, en nombre équivalent et de taille comparable, l'infection des proies n'est pas aléatoire et semble dirigée (Rogosky et al., 2006). Ceci peut être expliqué par une plus grande facilité d'attachement qui oriente le prédateur. Ainsi, l'attachement à une proie peut définir le spectre de prédation et attribuer le caractère semi-généraliste ou spécifique au prédateur. Une espèce semi-généraliste peut être considérée comme celle qui possède un « équipement » plus complet pour s'attacher à différents types de proies, alors que les moins équipées ont besoin de conditions particulières et/ou de proies plus spécifiques. L'attachement du prédateur semble donc être affecté par la composition de la paroi de la cellule hôte (Rogosky et al., 2006). La taille de la proie peut aussi être importante dans certaines situations : Chen et al. (2011), en utilisant *Vibrio parahaemolyticus* et *V. vulnificus* (cette dernière étant considérablement plus petite que la première) comme proies pour différentes espèces de *Bacteriovoracaceae*, ont révélé une prédation préférentielle, conditionnée, semble-t-il, par la taille des proies. Malgré l'observation de cette préférence de prédation, aucun mécanisme tel que la présence de sites récepteurs ou de signaux de chimiotaxie n'a été identifiés pour l'expliquer (Rogosky et al., 2006). En général, la chimiotaxie chez les BALOs joue un rôle mineur (Lambert et al., 2015). La question est donc de savoir comment les BALOs arrivent à intercepter leurs proies ? Est-ce que le prédateur et la proie entrent en collision aléatoirement, comme le feraient deux passants distraits dans une rue ? Cette probabilité de rencontre est en réalité extrêmement

faible dans un espace à trois dimensions. Dans un espace à deux dimensions, la probabilité de rencontre devient par contre plus importante. Mais comment passer d'un volume à une surface pour un prédateur aquatique ? Cela semble possible grâce à l'hydrodynamisme. Il est connu que les microorganismes nageurs ayant des faibles nombres de Reynolds sont attirés par les surfaces solides. Le nombre de Reynolds est un nombre sans dimension utilisé en mécanique des fluides. Dans le cas de très faibles nombres de Reynolds, les forces d'inertie liées aux vitesses étant négligeables, les forces visqueuses et les forces de pression s'équilibrent. Cet effet engendre l'accumulation des micro-nageurs et va donc concentrer à la fois proies et prédateurs dans un espace donné. La combinaison des effets hydrodynamiques conduit à un mouvement circulaire à la fois du prédateur et de la proie autour d'un objet, augmentant efficacement la co-localisation des organismes en les confinant à de plus petits volumes ou à des trajectoires dans une dimension. La rencontre aléatoire est rendue probable grâce à l'action de l'hydrodynamisme et son effet réducteur de dimensionalité (Jashnsaz et al., 2017; Prasad, 2017).

La prédation des BALOs peut être altérée voire inhibée par certains produits secondaires d'origine bactérienne. En effet, la prédation de BV HD100 sur *Chromobacterium piscinae* est inhibée. La cause est la production de cyanure par *C. piscinae* lorsque celle-ci est cultivée dans un milieu de culture qui permet de fournir des acides aminés pour la formation du cyanure. A l'inverse dans un milieu caractérisé par une absence d'acides aminés, le relargage de cyanure est presque inexistant. D'une part, le cyanure cause une diminution (jusqu'à la perte) de la motilité du prédateur pendant la phase d'attaque. D'autre part, le cyanure retarde le développement et la lyse du prédateur. De la même manière, l'indole produit par certaines bactéries est toxique pour BV HD100. Par contre, le violacéine qui inhibe la prédation des protistes et nématodes, n'a aucun effet sur BV HD100, et cela même à forte concentration (Mun et al., 2017).

Une résistance phénotypique vis-à-vis des mécanismes de prédation est observée chez certaines proies (Shemesh and Jurkevitch, 2004; Chauhan et al., 2009a). Ce phénomène qui a été largement observé chez les bactéries face à la prédation des protistes (Corno et Jürgens, 2006) existe donc ici avec les BALOs. En effet, dans les expériences de Shemesh et Jurkevitch (2004), l'interaction de *Bdellovibrio* sp. et de *Bacteriovorax* sp. avec leurs proies n'engendre pas l'éradication totale des proies. Ce résultat est expliqué par le développement de formes de résistance par une partie de la population de proies, leur permettant de survivre. Même si le nombre des prédateurs est 3 à 5 fois supérieur au nombre de proies, une sous-population résistante reste toujours en vie. Cette résistance n'est pas génétique mais phénotypique (durcissement de la paroi et/ou élargissement cellulaire), transitoire et réversible. Effectivement, une fois que la pression de prédation s'est estompée, les proies perdent cette capacité de résistance. Comme la résistance à la prédation n'est pas totale, ce mécanisme conduit à la survie à la fois du prédateur et de la proie (Shemesh et Jurkevitch, 2004).

6. Rôle écologique

Dotés d'une grande capacité d'adaptation (Yu et al., 2017), les BALOs, qui peuvent être halophiles ou non halophiles, sont omniprésents dans la nature. On les retrouve dans tous les types d'habitats, naturels et artificiels, sous forme planctonique ou associés à des biofilms (Jurkevitch, 2012a). Les écosystèmes qu'ils occupent sont nombreux et divers : milieux terrestres (sols, plants de rhizosphères), milieux aquatiques (rivières, lacs, mers, océans, estuaires, sédiments, récifs coralliens, étangs, mangroves, etc.), environnements dit extrêmes comme l'Antarctique, les eaux géothermales, le sous-sol océanique, les environnements anoxiques (Davidov et Jurkevitch, 2004; Sutton et Besant, 1994; Williams et al., 2018). On les retrouve encore dans les bassins salés pour la culture de crevettes (Wen et al., 2009), les boues activées et les eaux usées (Aguirre et al., 2017), les intestins d'animaux (humain, esturgeon sibérien, etc.) (Cao et al., 2015; Rendulic et al., 2004), les poumons humains (Iebba et al., 2014), les branchies de crabes bleus (Jurkevitch et Davidov, 2007), les fèces (Schwudke et al., 2001; Van Essche et al., 2011), et même dans les nuages où ils sont d'autant plus abondants que les nuages sont pollués et riches en pathogènes (Amato et al., 2017).

La prédation par les microbes est l'un des moteurs principaux de la mortalité bactérienne dans l'environnement (Johnke et al., 2017). Or, les bactéries sont fondamentales pour l'écologie des environnements en fournissant un support à la production primaire à travers leur rôle dans la chaîne trophique et la minéralisation des nutriments (Azam et al., 1983). Entre autres, les bactéries contrôlent le réservoir de carbone organique dissout, soit par assimilation ou soit par reminéralisation. Ce contrôle varie en fonction de la composition taxonomique et de l'état physiologique des bactéries autochtones, qui dépend de la pression de prédation et des conditions physico-chimiques de l'environnement (Chauhan et al., 2009a). Dans la plupart des écosystèmes, les prédateurs sont constamment en compétition vis-à-vis de la même ressource bactérienne. L'action et l'interaction entre les prédateurs peut conduire à un changement drastique au sein du monde microbien (Johnke et al., 2017) et par extension impacter les services offerts par les bactéries. La grande diversité et le caractère ubiquiste des BALOs impliquent une incidence pouvant être forte sur la structure et la dynamique des communautés microbiennes (Davidov et al. 2004; Williams et al. 2015). Effectivement, il est supposé que les BALOs agissent en tant « qu'équilibreur écologique », dit autrement qu'ils puissent être d'importants régulateurs de la biomasse et de la diversité bactériennes (Iebba et al. 2014; Oyedara et al. 2016; Williams et al. 2015) au même titre que les bactériophages (Jacquet et al., 2010). Malgré cela, on ne sait presque rien sur ce rôle écologique, ce compartiment ayant été très largement ignoré (Williams et al., 2015

; Chauhan et al., 2009a). Bien que les phages et les BALOs participent au recyclage des nutriments *via* la boucle microbienne, les mécanismes de ce recyclage sont très différents. La lyse virale entraine la libération du contenu intracellulaire de la proie dans l'environnement pour servir de nutriments à d'autres bactéries et organismes (Fuhrman et Noble, 1995; Weinbauer et al., 2003). Concernant les BALOs, ils consomment la plupart du contenu cellulaire de leur proie pour leur propre croissance, ne libérant donc que très peu de matière après la lyse de l'hôte. Par contre, ils deviennent riches en nutriments divers. En effet, la cellule proie-hôte (bdelloplaste) peut contenir jusqu'à 7 progénitures, si bien qu'elle devient à son tour une proie potentielle préférentielle pour les phages et les protistes ou métazoaires prédateurs (Williams et al., 2015). Il n'y a que très peu d'études ayant comparé le rôle fonctionnel des BALOs et des phages, et celle de Williams et al. (2015) est riche d'enseignement car ces auteurs ont révélé que les BALOs pouvaient éclipser les bactériophages en nombre de (certaines) bactéries lysées comme *Vibrio parahaemolyticus*. Les auteurs restent toutefois prudents en indiquant que ce résultat pouvait être exceptionnel et donc à relativiser. En effet, il est probable que plusieurs facteurs aient pu interférer dans les résultats de cette étude, comme par exemple le stade cellulaire des proies, sachant que les phages préfèrent les proies en croissance rapide, alors que les BALOs préfèrent les proies en croissance lente ou en phase stationnaire (Chen et Williams, 2012). En outre, le cycle lysogénique des virus peut dissimuler l'efficacité de la lyse virale (Williams et al., 2015).

La pression de prédation peut être différente d'un BALO à un autre, si bien que leurs effets peuvent être très hétérogènes sur la communauté microbienne dans un environnement donné. Ils s'attaquent à une variété de bactéries à l'inverse des bactériophages qui sont hôtes spécifiques (Chen et Williams, 2012). Mais, ils ont tout de même une préférence pour certaines proies selon qu'ils sont semi-généraliste, spécialiste ou versatile. Par ailleurs, il semble qu'il ne soit pas nécessaire que les BALOs soient en forte abondance pour être efficaces, c'est à dire pour observer une baisse significative du nombre de bactéries proies (Williams et al., 2015). Généralement, les abondances de BALOs dans l'environnement sont effectivement relativement faibles, inférieur à 1% (Chauhan et al., 2009a ; Paix et al., 2019). Les BALOs ne dominent pas d'un point de vue numérique, mais peuvent former des populations assez abondantes qui fluctuent en fonction des saisons (Kandel et al., 2014 ; Paix et al., 2019) et dont l'impact peut être significatif dans un espace donné (Kandel et al., 2014). Enfin, les BALOs peuvent être à leur tour consommés et/ou parasités par d'autres organismes tels que les bactériophages (Hashimoto et al., 1970), les protistes flagellés et ciliés (Johnke et al., 2017) et surement le zooplancton métazoaire, en dépit de leur petite taille et leur capacité à se déplacer. Par ailleurs, il ne faut pas oublier qu'il existe aussi d'autres bactéries prédatrices de bactéries susceptibles d'impacter l'ensemble de la communauté microbienne. Les effets de tous les prédateurs et symbiontes parasites formant l'ensemble des interactions biotiques

au sein du monde microbien sont complexes à étudier et restent donc encore largement sous-explorés. La plupart des études ne considèrent que des expériences *in vitro* qui sont souvent très simplifiées et contrôlées. Cette revue est l'occasion de souligner que les études portant sur le rôle fonctionnel des BALOs devraient être encouragées et soutenues.

7. Applications (biotechnologie et médecine)

Au même titre que les virus bactériophages (utilisés dans le cadre de la thérapie phagique, Górski et al., 2018), le comportement de prédation des BALOs fait de ces microorganismes des candidats intéressants pour de nombreuses applications (Figure 5) dans le contrôle biologique de certaines populations bactériennes (Cao et al., 2015). Les bactéries à Gram négatif sont typiquement responsables de plus de 30% des infections acquises à l'hôpital (infections nosocomiales), et elles sont aussi associées à des niveaux de morbidité et de mortalité souvent très élevés dans les unités de soins intensifs (Baker et al., 2017). La raison est que la plupart des patients sont infectés par des bactéries multi-résistantes aux antibiotiques (Monnappa et al., 2014). On comprend ainsi que le recours à des solutions alternatives est aujourd'hui une nécessité, et ce, en dépit du développement et de la mise sur le marché de nouveaux antibiotiques au cours des dernières décennies. Ainsi, la capacité prédatrice des BALOs a été proposée afin de combattre les bactéries à Gram négatif, Gram positif et autres biofilms multi-résistants chez l'Homme, mais aussi chez les animaux et les plantes (Johnke et al. 2017; Sockett et Lambert. 2004). Les BALOs étant présents partout, nous en ingérons sûrement et sans le savoir quotidiennement et de façon inoffensive (Willis et al., 2016). Plusieurs groupes de chercheurs dont Im et al. (2017) ont en effet démontré que ces bactéries prédatrices ne sont pas nocives pour les cultures cellulaires humaines et animales. D'ailleurs, les bactéries prédatrices inoculées dans des modèles animaux tels que les souris, lapins, cochons d'inde, ou encore la poule ne présentent pas de toxicité (Shatzkes et al., 2016). A ce jour, aucune maladie n'a été associée ou attribuée à une infection par des BALOs (Willis et al., 2016). Chez l'Homme ou l'animal, l'absence d'une réponse inflammatoire forte et soutenue en présence de bactéries prédatrices par rapport à d'autres bactéries peut s'expliquer par la composition altérée de la membrane lipopolysaccharidique (LPS) des BALOs. Les LPS chargés négativement, exprimés à la surface des bactéries à Gram négatif induisent des réponses immunitaires innées par l'organisme dans le but de protéger l'hôte contre l'infection. Néanmoins, pour les BALOs, le LPS exprimé est de charge neutre et faiblement immunogène *in vitro* (Willis et al., 2016). En outre, l'activité de prédation des BALOs a été vérifiée *in vitro* sur plus de 100 pathogènes humains (Mun et al., 2017). A titre d'exemple, *B. bacteriovorus* HD100 s'attaque et détruit les pathogènes humains tels que *E. coli, Salmonella* ou encore *Klebsiella pneumoniae* (Im et al., 2017). D'autre

part, l'avantage de l'utilisation des BALOs est l'absence de résistance permanente chez leurs proies vis-à-vis de leurs modes de prédation. En effet, les BALOs envahissent les pathogènes Gram négatifs sans utiliser de système de reconnaissance à base de récepteur, ce qui rend complexe la possibilité pour les proies d'acquérir une quelconque résistance génétique (Willis et al., 2016). Cependant, des résistances liées à de la plasticité phénotypique peuvent apparaître chez les proies, mais sont tout de même réversibles. Clairement, des thérapies employant différentes espèces de BALOs pour diminuer les résistances sont facilement envisageables à la manière des cocktails de phages proposés en thérapie phagique (Chan et al., 2013).

Pour une application réelle dans un hôte humain, animal ou végétal, il faut aller au-delà des systèmes proies-prédateurs étroitement contrôlés dans des solutions tampon de laboratoire classique. Il faut par exemple tenir compte de la présence d'autres espèces bactériennes, faisant office de perturbateur, pour évaluer l'application clinique des BALOs. Dans le corps humain, plusieurs facteurs immunologiques et antimicrobiens tels que les anticorps, les peptides antimicrobiens et les leucocytes peuvent agir sur les bactéries prédatrices. Potentiellement, ces facteurs peuvent perturber ou causer la mort des prédateurs bactériens avant qu'ils n'entament leurs cycles ou comportements de prédation. Pour comprendre ces impacts, deux expériences de prédation de *B. bacteriovorus* sur *K. pneumoniae* (pathogène humain résistant aux carbapénèmes) ont été menées par Baker et al. (2017), l'une dans une solution de tampon classique de laboratoire (*in vitro*) et l'autre dans un sérum humain (*in vitro*). Les résultats ont révélé que *B. bacteriovorus* est capable de réduire la charge de *K. pneumoniae* dans les deux milieux, mais à des échelles temporelles différentes. En effet, dans le sérum humain, le comportement de prédation initial affiche un délai de 19 heures, alors que dans le tampon classique la prédation s'opère de façon rapide et reproductible. Ce délai de prédation est dû à l'incapacité du prédateur à s'attacher à une proie. En effet, en contact avec le sérum humain, *B. bacteriovorus* change de physiologie et passe d'une forme vibroïde à une forme ronde. Cette forme est réversible, mais le prédateur a besoin d'un certain temps pour s'acclimater à son nouveau milieu. En outre, il est possible d'éviter ce délai en pré-exposant au laboratoire le prédateur à du sérum humain avant de l'administrer. Une fois *B. bacteriovorus* adapté à son nouvel environnement, Baker et al. (2017) ont montré que la prédation de ce dernier sur les pathogènes dans le sérum humain est possible. La dynamique de prédation dans le sérum humain est différente de celle dans le tampon de solution de laboratoire classique, mais cette dynamique est variable d'un sérum humain à un autre. Il est nécessaire de mener des expériences à large échelle pour comprendre tous les mécanismes sous-jacents de la prédation dans le sérum humain. Par ailleurs, un autre problème a été constaté : la population de bactéries pathogènes réduite par la prédation de *B. bacteriovorus* réussit à émerger à nouveau au bout de

quelques heures. Cela suggère le développement de résistance par les pathogènes, et cette reprise de croissance peut mettre en péril l'utilisation des BALOs comme bio-agents thérapeutiques (Baker et al., 2017). Cette résistance est la même que celle observée par Shemesh et Jurkevitch (2004) et est liée à la présence de débris suite à l'action de prédation qui « mettent en garde » les proies vivantes.

Im et al. (2017) ont, eux aussi, évalué l'activité bactéricide de *B. bacteriovorus* HD100 dans le sérum sanguin humain contre *K. pneumoniae* et d'autres souches bactériennes associées à des infections, notamment *E. coli* et *Salmonella enterica* sur 24 heures. Leurs tests ont montré que *B. bacteriovorus* HD100 n'est pas sensible au complément sérique (immunité innée) ni à son activité bactéricide. En effet, la viabilité du prédateur est restée stable pendant 24 heures, n'affichant qu'une perte de 33%. Toutefois, la prédation a été inhibée dans le sérum humain à cause de l'osmolalité et de l'albumine. L'activité prédatrice a montré une transition nette entre 200 et 250 mOsm/kg et a été progressivement réduite à mesure que l'osmolalité augmentait. Comme l'osmolalité du sérum sanguin est de 285 à 295 mOsm/kg, les résultats suggèrent ici que la prédation dans les sérums sanguins devrait être sévèrement inhibée en raison de l'osmolalité seule. L'albumine de sérum humain a également agit pour inhiber la prédation en se liant aux cellules prédatrices et en les enrobant complètement, même au niveau du flagelle, empêchant ainsi le prédateur d'attaquer sa proie. Heureusement, les souches de BALOs sont diversifiées et sont présentes dans une multitude d'environnements tels que les eaux salées. Des thérapies utilisant des souches halophiles comme les *Halobacteriovoraceae* plutôt que *B. bacteriovorus* sont à envisager et à étudier à l'avenir. L'encadré 5 du complément électronique propose divers exemples d'applications des BALOs en tant que bio-agents.

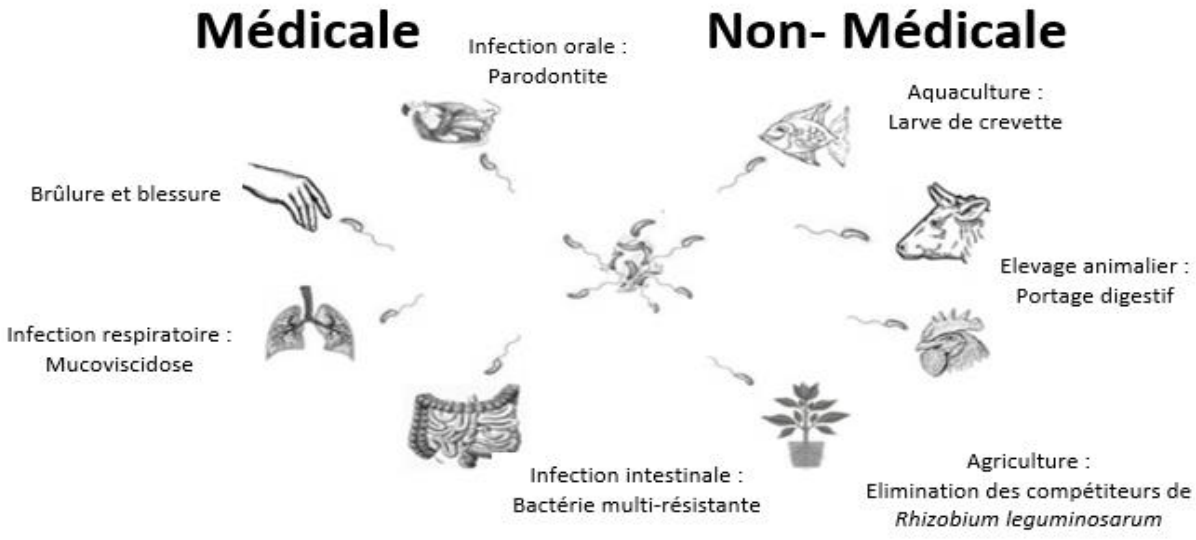


Figure 5 Diverses applications des BALOs comme bioagents (Source : Dwidar et al., 2012)

8. Conclusion

Les *Bdellovibrio* et organismes apparentés (BALOs) représentent un groupe de prédateurs bactériens remarquable, notamment par leur caractère ubiquiste, leur cycle de vie (endo- et/ou épibiotique), leur spectre de prédation (spécialiste ou semi-généraliste), et leur diversité. Ils sont aussi remarquables par leurs capacités d'adaptation aux conditions de leur environnement. Enfin, leur action permet d'imaginer de nombreuses applications dans divers domaines médicaux et biotechnologiques. Toutefois, il reste de nombreux tests à entreprendre pour comprendre le mode d'action des BALOs et les conditions optimales pour la réussite de leurs utilisations notamment thérapeutiques. On comprend mieux dès lors que le nombre de publications et de citations portant sur ce groupe bactérien ait considérablement augmenté au cours des deux dernières décennies (Figure 6), mais force est aussi de constater que la connaissance sur l'écologie de ces microorganismes et de leur rôle fonctionnel dans les environnements naturels reste confidentielle. L'avancée des nouvelles techniques de séquençage à haut débit et de bio-informatique vont permettre de décrypter leur diversité et leurs interactions avec l'environnement biotique et abiotique. C'est tout l'enjeu porté par le projet INRA-USMB C-BALO qui vise à étudier la diversité, la structure et l'abondance des BALOs dans divers environnements aquatiques.

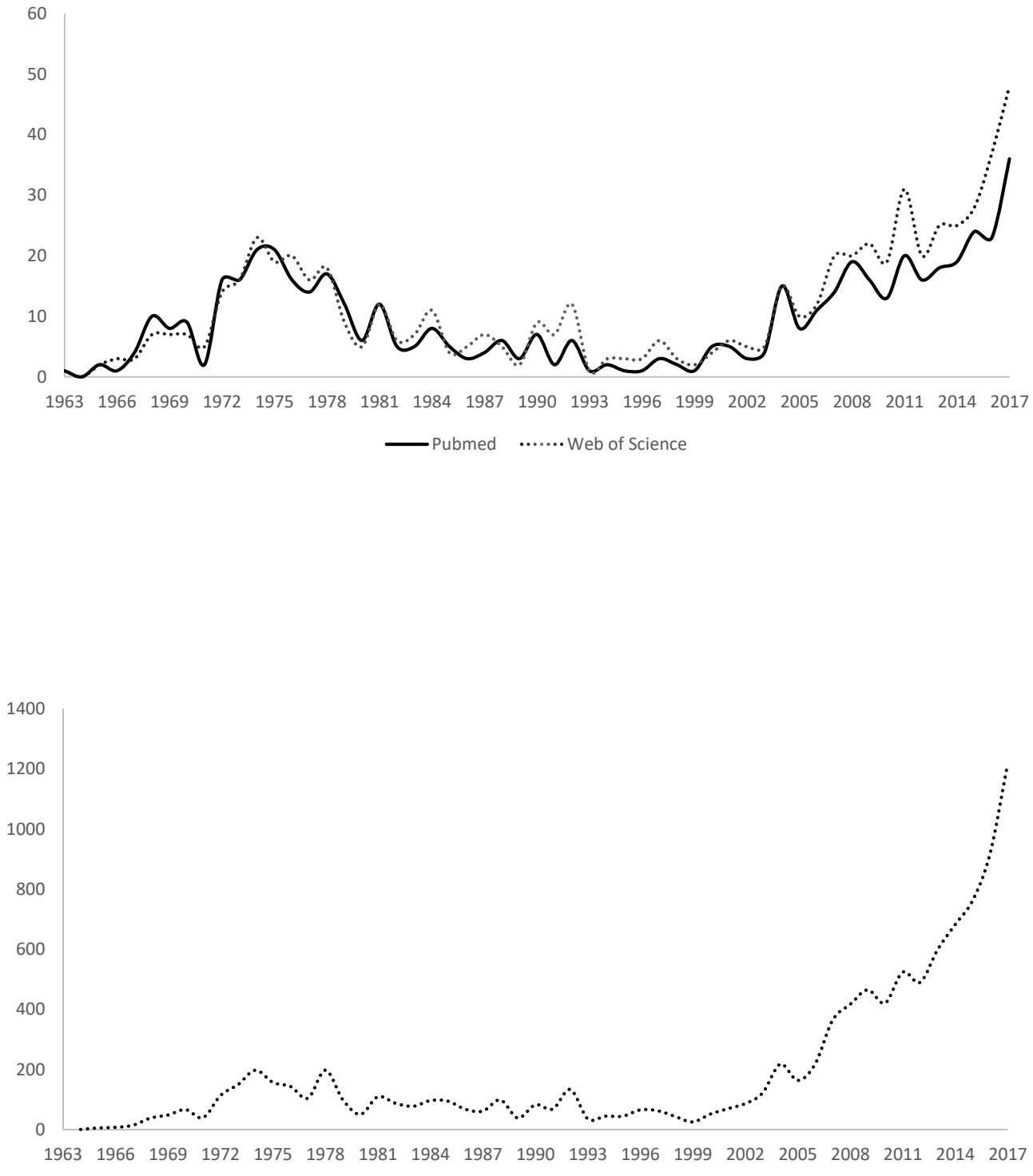


Figure 6 Nombre de publications scientifiques (A) et de citations (B) parues dans *PubMed* et *Web of Science* en relation avec les *Bdellovibrio* et organismes apparentés de 1963 à 2017 inclus. Les critères de recherche ont été le mot-clef : *Bdellovibrio* (ce mot est retrouvé dans l'ensemble des publications traitant des BALOs).

9. Encadrés

Encadré 1 - Prédateur ou parasite ?

Un prédateur est caractérisé par la consommation de plusieurs proies au cours de sa vie. Il est aussi généralement plus grand que sa proie. Le prédateur a également tendance à provoquer la mort de sa proie et, ainsi, évincer sa valeur sélective ou *fitness*. En revanche, pour un microprédateur, les deux dernières règles ne sont pas toujours applicables. Un microprédateur n'est pas forcément plus grand que sa proie et n'induit pas systématiquement la mort de celle-ci, car il peut prélever en petite quantité des repas non létaux.

Un parasite attaque une seule victime par cycle de vie, sans nécessairement éliminer la *fitness* de son hôte. L'hôte est inévitablement plus grand que le parasite. Le parasite au cours d'un stade donné de son cycle de vie ne va normalement pas chercher à sortir de son hôte pour aller en infecter un autre.

A l'instar du parasite, le parasitoïde va infecter un seul hôte par cycle de vie, mais il provoque inévitablement la mort de son hôte. En général, c'est le développement de la progéniture qui met fin à l'hôte.

Concernant les BALOs (*Bdellovibrio* et organismes apparentés), la frontière est mince entre ces différentes catégories. Toutefois les BALOs sont considérés comme des prédateurs, car l'hôte est immédiatement tué, et cela même avant le développement de la progéniture. De plus, le prédateur cherche systématiquement une nouvelle proie à la fin de son cycle. Enfin l'hôte qui a succombé au prédateur ne fait office que d'un réservoir de ressource énergétique, ainsi que d'un environnement inerte, stable osmotiquement et protecteur contre les attaques de prédateurs et les fluctuations des conditions environnementales.

D'après Lafferty K. D., 2002

Encadré 2 - Inventaire des prédateurs bactériens : mode de vie, morphologie et stratégie de prédation

Une bactérie est considérée comme un prédateur dès lors qu'elle tue d'autres microbes et les consomme en tant que ressource nutritionnelle (Velicer *et al.*, 2009). Une dizaine d'espèces bactériennes prédatrices correspondant à divers taxa (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, Chloroflexi, etc.) a été identifiée à ce jour. On les trouve dans les environnements terrestres, aquatiques et extrêmes (sources chaudes, milieux hypersalés, etc.).

Le point commun à tous les prédateurs bactériens est la capacité à dégrader les polymères de leurs proies. Cependant il existe une grande diversité au sein des prédateurs bactériens. Ainsi certaines bactéries sont des prédateurs facultatifs ou obligatoires. De plus, la stratégie de prédation peut se faire individuellement ou en groupe *wolfpacks* (essaims). Enfin certaines cellules ont besoin d'établir un contact cellule-cellule pour initier la prédation. En effet, deux types de contacts peuvent être distingués : le contact endobiotique qui résulte de l'envahissement de la proie par le prédateur (Figure 1, E), et le contact épibiotique (Figure 1, F), qui n'implique pas une intrusion au sein de la proie.

Toutefois d'autres prédateurs ne nécessitent pas de contact et se contentent d'enclencher la prédation à distance par sécrétion d'enzymes lytiques, provoquant ainsi l'éclatement des cellules sensibles à l'action de ces enzymes. Ces différents mécanismes, stratégies et mode de prédation distinguent les BALOs des autres prédateurs bactériens, bien que certaines espèces prédatrices puissent présenter plusieurs stratégies de prédation au cours de leur vie, à savoir le mode épibiotique et le mode endobiotique.

Les prédateurs *Bdellovibrio* spp., *Bacteriovorax* spp. et *Peredibacter* spp. appartenant à la classe des *Oligoflexia* (récemment retirée de la classe des Deltaproteobacteria) (Hahn *et al.*, 2017. *Silvanigrella aquatica* gen. nov., sp. nov., isolated from a freshwater lake, description of *Silvanigrellaceae* fam. nov. and Silvanigrellales ord. nov., reclassification of the order *Bdellovibrionales* in the class *Oligoflexia*, reclassification of the families *Bacteriovoracaceae* and *Halobacteriovoraceae* in the new order *Bacteriovoracales* ord. nov., and reclasssification of the family *Pseudobacteriovoracaceae* in the order *Oligoflexales*. *International Journal of Systematic and Evolutionary Microbiology* **67 (8)**, 2555–2568), ainsi que *Micavibrio* spp. de la classe des *Alphaproteobacteria* ont été réunis au sein du même groupe des *Bdellovibrio and like organisms* (BALOs), puisqu'ils présentent des caractéristiques similaires du point de vue de la morphologie (forme vibroïde) (Figure 1), de la motilité (présence de flagelle), de l'arsenal enzymatique, du mode de prédation individuel (obligatoire pour la nourriture et la multiplication),

et du mécanisme d'attachement à la proie (épibiotique et endobiotique périplasmique ou cytoplasmique).

En plus, les travaux de phylogénie fondés sur la séquence du gène 16S de la petite sous-unité ribosomique renforce la classification des trois espèces modèles de la classe des *Deltaproteobacteria* dans le groupe des BALOs (Figure 2). Par ailleurs, *Micavibrio* ne ressemble à aucun autre prédateur de la classe des *Alphaproteobacteria*. Il s'intègre néanmoins dans le groupe des BALOs par la similitude de son mode d'action, de prédation et sa morphologie.

Ci-dessous sont listés des exemples de prédateurs bactériens n'appartenant pas au groupe des BALOs :

Alphaproteobacteria : *Ensifer adhaerens* : Bactérie du sol, Gram-négatif, en forme de bâtonnet, rencontrée à l'état individuel ou par paire. Elle forme des nodules fixateurs d'azote sur les racines et les tiges de légumineuses. Toutefois elle présente un comportement de prédation lorsque les conditions nutritives deviennent limitantes. *In vitro*, la prédation s'opère par un groupe d'*E. adhaerens* qui s'attache à une proie les uns à côté des autres, à l'image d'une palissade.

Betaproteobacteria : *Cupriavidus necator* : Bactérie du sol en forme de bâtonnet, ayant un comportement de prédation facultative. Sa croissance nécessite la présence de concentrations élevées en cuivre.

Gammaproteobacteria : *Lysobater* : Bactérie à mobilité « glissante » qui prolifère en formant de longues cellules et filaments. La prédation s'effectue en groupe, une stratégie dénommée *wolfpack*. Un contact est établi entre le prédateur et la proie, mais, contrairement aux BALOs, aucune structure d'attachement à la proie n'est observée ou définie.

Deltaproteobacteria : *Myxobacteria* : Bactérie en bâtonnet relativement volumineuse et qui se déplace par glissement dans les sols. La caractéristique la plus marquante de ce groupe est la formation d'essaims (*wolfpack*), ainsi que son mode de vie multicellulaire. La prédation s'opère par un déplacement de l'essaim jusqu'à rencontrer fortuitement une proie. La prédation se déroule en deux temps, d'abord par les cellules mères qui attaquent, piègent et affaiblissent la proie, puis les nouvelles recrues générées principalement par division cellulaire forcent la proie dans un espace contraint afin de la dévorer.

D'après Jurkevitch et Davidov, (2006) et Velicer et Mendes-Soares, (2009)

Encadré 3 - Classification des bactéries

La classification vise à décrire et à regrouper des espèces bactériennes sur la base de caractères similaires. Cette classification apparaît dans deux publications officielles qui sont *The International Journal of Systematic and Evolutionary Microbiology* et le *Bergey's Manual of Systematic Bacteriology*). De plus, *The International Committee on Systematics of Prokaryotes* supervise la nomenclature des procaryotes et détermine les règles de désignation. Historiquement les bactéries ont été classées selon la coloration de Gram (qui permet de mettre en évidence les propriétés de la paroi bactérienne ; la morphologie, la mobilité, la température de croissance, la sporulation, les besoins nutritionnels, etc. De nos jours et depuis la découverte des techniques moléculaires, la taxonomie se fonde sur la constitution chimique (caractéristiques électrophorétiques des protéines) et sur la structure de l'ADN. Cette dernière se fonde sur l'étude du génome, lui-même par l'intermédiaire de techniques telles que le pourcentage en GC (densité et température de dénaturation des bases guanine G et cytosine C), l'électrophorèse de l'ADN en champ pulsé, l'hybridation ADN-ADN, l'hybridation ADN-ARN et le séquençage de l'ARN ribosomique (16S ou/et 23S). Enfin en bactériologie médicale, il existe aussi une autre classification qui se fonde sur les marqueurs épidémiologiques pour caractériser les bactéries à intérêt clinique.

La base de données en ligne *List of Prokaryotic names with Standing in Nomenclature* répertorie et maintien des informations relatives au nom et à la taxonomie des procaryotes. A ce jour, cette base contient 34 phylum bactériens (*Acidobacteria, Actinobacteria, Aquificae, Armatimonadetes, Bacteroidetes, Balneolaeota, Caldiserica, Calditrichaeota, Chlamydiae, Chlorobi, Chloroflexi, Chrysiogenetes, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Dictyoglomi, Elusimicrobia, Fibrobacteres, Firmicutes, Fusobacteria, Gemmatimonadetes, Kiritimatiellaeota, Lentisphaerae, Nitrospira, Planctomycetes, Proteobacteria, Rhodothermaeota, Spirochaetes, Synergistetes, Tenericutes, Thermodesulfobacteria, Thermotogae, Verrucomicrobia*, et une liste de non assignées).

Encadré 4 - Historique et étymologie

En 2000, Baer et al. (2000) ont proposé de transférer les deux espèces *Bdellovibrio stolpii* Uki2 et *Bdellovibrio starrii* A3.12 vers un nouveau genre nommé *Bacteriovorax*. Ce terme est composé de *baktron*, mot grec signifiant petit bâtonnet, et du mot latin *vorax*, exprimant l'action de dévorer. Ainsi *Bacteriovorax stolpii* et *Bacteriovorax starrii* ont vu le jour. Par ailleurs, le terme s*tolpii* provient de Stolp, nom du microbiologiste allemand Heinz Stolp, découvreur du premier BALO.

En 2004, Davidov et Jurkevitch (Davidov Y, Jurkevitch E. 2004. *Diversity and evolution of* Bdellovibrio*-and-like organisms (BALOs), reclassification of* Bacteriovorax starrii *as* Peredibacter starrii *gen. nov., comb. nov., and description of the* Bacteriovorax-Peredibacter *clade as* Bacteriovoracaceae *fam. Nov,* International Journal of Systematic and Evolutionary Microbiology, **54 (5)**, 1439–1452) reclassent l'espèce *B. starrii* dans un nouveau genre *Peredibacter*. Ainsi l'espèce *B. starrii* devient *Peredibacter starrii*. *Peredibacter* dérive du latin *peredere* qui signifie « dévorer » et du néo-latin *bacter* qui dépeint la forme en bâtonnet. Ainsi le terme *Peredibacter* signifie « dévoreur de bactéries ». Le mot *starrii* provient quant à lui de Starr, nom du microbiologiste américain Mortimer P. Starr.

La même année, Baer et al. (2004) proposent à leur tour de reclasser les BALOs marins *Bdellovibrio* sp. en *Bacteriovorax marinus* et *Bacteriovorax litoralis*, *marinus* pour environnement marin et *litoralis* pour la côte. Ensuite ces deux espèces ont été transférées dans un nouveau genre *Halobacteriovorax*. Ce terme est composé du mot grec *hals* ou *haloes,* autrement dit sel, du mot latin *bacterium*, pour la forme en petit bâtonnet, et, enfin, du suffixe latin *vorax*, pour dévoreur. L'ensemble définit les *Halobacteriorax* comme « des dévoreurs de bactéries en milieu halophile ».

Dans un article récent de Hahn *et al.* (2017. *Silvanigrella aquatica gen. nov., sp. nov., isolated from a freshwater lake, description of Silvanigrellaceae fam. nov. and* Silvanigrellales *ord. nov., reclassification of the order Bdellovibrionales in the class Oligoflexia*, reclassification of the families *Bacteriovoracaceae* and *Halobacteriovoraceae* in the new order *Bacteriovoracales* ord. nov., and reclasssification of the family *Pseudobacteriovoracaceae in the order* Oligoflexales, *International Journal of Systematic and Evolutionary Microbiology* **67 (8)**, 2555–2568), la classification des BALOs a encore une fois subi des changements. L'ordre des *Bdellovibrionales* a été transféré de la classe des *Deltaproteobacteria* à la classe des *Oligoflexia*. Cet ordre contenait à l'origine deux familles les *Bdellovibrionaceae* et les *Pseudobacteriovoracaceae*. Aujourd'hui, les *Pseudobacteriovoracaceae* quittent cet ordre pour rejoindre l'ordre des *Oligoflexiales.* De même, l'ordre des *Bacteriovoracales*, contenant les familles *Peredibacteraceae*, *Bacteriovoracaceae* et *Halobacteriovoraceae* a aussi été incorporé à la classe des *Oligoflexia* (Table 2).

Table 2 Classification actuelle des BALOs par Hahn et al. (2017) et Koval et al. (2015).

Phylum	**Proteobacteria**						
Classe	*Oligoflexia*						
Ordre	*Bdellovibrionales*		*Bacteriovoracales*				*Oligoflexiales*
Famille	*Bdellovibrionaceae*		*Peredibacteraceae*	*Bacteriovoracaceae*	*Halobacteriovoraceae*		*Pseudobacteriovoracaceae*
Genre	*Bdellovibrio*		*Peredibacter*	*Bacteriovorax*	*Halobacteriovorax*		*Pseudobacteriovorax*
Espèce	*bacteriovorus*	*exovorus*	*starrii*	*stolpii*	*marinus*	*litoralis*	*antillogorgiicola*
Souche	HD100	JSS	A3.12	UKi2	SJ	JS5	RKEM611

Phylum	**Proteobacteria**	
Classe	*α-proteobacteria*	
Ordre	*Bdellovibrionales*	
Famille		
Genre	*Micavibrio*	
Espèce	*admirantus*	*aeruginosavorus*
Souche		ARL-13

Encadré 5 - Quelques exemples d'applications des BALOs en tant que bio-agents anti bactériens

Exemple I : La mucoviscidose est une maladie génétique létale (Iebba *et al.*, 2014. *Bdellovibrio* bacteriovorus directly attacks *Pseudomonas aeruginosa* and *Staphylococcus aureus* cystic fibrosis isolates. *Frontiers in Microbiology* **5**, 1–9). L'évolution naturelle de la mucoviscidose est un déclin progressif de la fonction respiratoire des poumons occasionné par un cercle vicieux d'inflammation et de destruction des tissus, enclenché et maintenu par une colonisation bactérienne chronique des voies respiratoires inférieures. *B. bacteriovorus* a été détecté naturellement dans le microbiote des poumons de sujets sains et parvient à survivre dans ces conditions anoxiques. Il a donc été proposé d'inoculer des souches du prédateur au début du stade de colonisation des poumons chez des sujets atteints de mucoviscidose pour contribuer à contrôler la colonisation chronique des deux bactéries dominantes du microbiote, *Pseudomonas aeruginosa* (Gram -) et *Staphylococcus aureus* (Gram +). Effectivement, une fois que les bactéries pathogènes sont installées, rien ne peut les déloger, pas même les antibiotiques. L'action des BALOs semble se traduire par une réduction significative des biofilms de *S. aureus* et de *P. aeruginosa* (Iebba *et al.*, 2014 ; Pantanella *et al.*, 2018. Behaviour of *Bdellovibrio* bacteriovorus in the presence of Gram-positive *Staphylococcus aureus*. *New Microbiologica* **41 (2)**, 145–152).

Exemple II : *B. bacteriovorus* est injecté dans le rhombencéphale (cerveau postérieur) en tant que traitement antibactérien *in vivo* chez des larves de *Danio rerio* (poisson-zèbre) infectées par une souche pathogène humaine résistante à l'antibiotique, *Shigella flexneri*. La survie des animaux infectés est alors significativement améliorée, ainsi que le taux de survie des larves du poisson-zèbre qui augmente de 35%. Le système immunitaire du poisson-zèbre est hautement homologue à celui de l'homme, avec des réponses associées aux neutrophiles et macrophages. Après injection du prédateur dans les larves, le temps de persistance de ce dernier est de 24 heures. En effet, *B. bacteriovorus* est englouti par les neutrophiles et les macrophages du poisson qui se chargent de l'éliminer efficacement après 24 heures, même quand une dose importante du prédateur est apportée. Par contre, malgré ce temps de persistance relativement court, *B. bacteriovorus* est capable de réduire efficacement la charge pathogène *in vivo* avant d'être éliminé par l'action du système immunitaire de l'hôte. De plus, l'action prédatrice ne perturbe pas le système immunitaire, c'est-à-dire que la bactérie ne stimule pas une réponse immunitaire supplémentaire quand une infection est déjà présente ; au contraire on parle d'une réaction synergétique, puisque les leucocytes et *B. bacteriovorus* participent ensemble à l'élimination de *Shigella* (Willis et al., 2016. Injections of predatory bacteria work alongside host immune cells to treat *Shigella* infection in zebrafish larvae. *Current Biology* **26 (24)**, 3343–3351).

Exemple III : La parodontite est une maladie infectieuse polymicrobienne (Van Essche et al., 2011. Killing of anaerobic pathogens by predatory bacteria. *Molecular Oral Microbiology* **26 (1)**, 52–61) qui provoque l'inflammation des tissus de soutien de la dent (parodonte). L'infection est due à de nombreux pathogènes Gram négatifs tels que *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tannerella forsythia, Fusobacterium nucleatum, Capnocytophaga sputigena*, *Eikenella corrodens* et *Actinomyces naeslundii (*Harini et al*., 2013. Bdellovibrio* bacteriovorus: a future antimicrobial *agent? Journal of Indian Society of Periodontology* **17 (6)**, 823.; Van Essche et al., 2011. Killing of anaerobic pathogens by predatory bacteria. *Molecular Oral Microbiology* **26 (1)**, 52–61) qui sont enchâssés dans un biofilm complexe constitutif de la plaque dentaire. Les traitements de la parodontite qui consiste à éradiquer le biofilm par des thérapies conventionnelles comme l'utilisation d'antibiotiques s'avèrent de plus en plus compliqués, et sont souvent inefficaces, voir même déconseillés (Van Essche et al., 2011). En effet, les bactéries développent le plus souvent des résistances aux antibiotiques et les agents antimicrobiens ont du mal à pénétrer le biofilm dentaire. En effet, les bactéries en biofilm sont 1000 fois plus résistantes aux agents antimicrobiens que ceux sous formes libres ou planctoniques (Van Essche et al., 2011). Néanmoins, l'application locale de BALOs permet de réduire spécifiquement le taux de pathogènes à Gram négatif dans la cavité buccale. Les BALOs sont capables de pénétrer profondément dans le biofilm et ainsi de le détruire. De ce fait, il est suggéré de complémenter les bains de bouche classiques par un cocktail de BALOs. Toutefois, il existe quelques inconvénients à leurs utilisations. D'abord, ils n'éliminent pas entièrement les proies même quand la concentration des prédateurs est augmentée, probablement à cause de l'apparition de résistance phénotypique transitoire ou parce que le spectre de prédation de la souche de BALO employée ne permet pas d'éradiquer l'ensemble des pathogènes. De plus, leur activité est affectée par l'état physiologique de leurs proies et par la présence d'autres bactéries qui peuvent contrecarrer leurs activités par encombrement stérique (Van Essche et al., 2011). Enfin, les BALOs sont des bactéries aérobies strictes si bien que leur action est limitée en cas d'absence d'oxygène.

Exemple IV : L'aquaculture en eau douce de crevettes à pattes blanches (*Penaeus vannamei*) en Chine produit annuellement 300 000 tonnes d'animaux à destination alimentaire. Les pathogènes *Vibrio harparahaemolyticus* et *V. cholerae* provoquent des épidémies généralisées et sont responsables d'une perte atteignant les 90%. L'infection de *Vibrio* n'est pas toujours contrôlable par des antibiotiques, et les antibiotiques ont un coût prohibitif et sont mauvais pour l'environnement ainsi que la santé humaine. Or il a été démontré que *B. bacteriovorus* H16 parvient à éradiquer 10 souches différentes de *Vibrio*. En effet, des expériences ont montré une diminution de 99,9% de l'abondance de *V. cholerae* par rapport aux conditions contrôles. Le prédateur a indéniablement un effet protecteur pour la crevette contre les infections à *Vibrio* et le pourcentage

de survie *in vivo* des crevettes augmente de 47,7% à 63,3% (Cao et al., 2015. *Vibrio cholerae* pathogen from the fresh water cultured whiteleg shrimp *Penaeus vannamei* and control with *Bdellovibrio* bacteriovorus. *Journal of Invertebrate Pathology* **130**, 13–20**).** Il a été aussi indéniablement prouvé par Ottaviani et al. (2018) (*Halobacteriovorax* isolated from marine water of the Adriatic sea, Italy, as an effective predator of *Vibrio parahaemolyticus*, non-O1/O139 *V. cholerae, V. vulnificus*. *Journal of Applied Microbiology* **125**, 1199- 1207) et Richards et al., (2012) (Predatory bacteria as natural modulators of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seawater and oysters. *Applied and Environmental Microbiology* **78 (20)**, 7455–7466) que les BALOs marins tel que *Halobacteriovorax* HBXCO1 sont des modulateurs écologiques des populations de *Vibrio* ***sp.*** notamment ***V.*** *parahaemolyticus,* *V. cholerae,* et ***V.*** *vulnificus* chez les bivalves. En effet, certaines souches de ***Vibrio*** s'accumulent naturellement dans les huitres (*Mytilus galloprovincialis* et *Crassostrea virginica*), moules et autres palourdes provoquant des maladies chez l'Homme quand la cuisson des fruits de mer est insuffisante. Les éleveurs n'arrivant pas toujours à décontaminer les bivalves d'une façon efficace, les BALOs ont été considérés comme une bonne solution de contrôle biologique de part une activité de prédation spécifique et rapide (<24 h) même quand une faible concentration de prédateurs est apportée ($<10^8$ CFU). Aujourd'hui, pour que les BALOs marins puissent être utilisés dans ce but, il reste à les isoler et trouver le moyen de les commercialiser.

Exemple V: *B. bacteriovorus* SSB et SKB peuvent être employés comme amplificateurs naturels en agriculture. Les deux souches n'agissent pas sur *Rhizobium leguminosarum* qui vit en symbiose avec les racines des plants de légumineuses et favorise la croissance des plants par fixation d'azote, mais leur action est utile pour éliminer les compétiteurs de *R. leguminosarum* (Oyedara et al., 2016). Isolation of *Bdellovibrio* sp. from soil samples in Mexico and their potential applications in control of pathogens. *Microbiology Open* **5 (6)**, 992–1002).

Exemple VI : Les boues activées biologiques sont principalement constituées de microorganismes et de fibres organiques associées à des particules inorganiques (sel et sable). Le procédé le plus largement utilisé pour le traitement biologique des boues activées génère de grandes quantités de boues résiduelles avec une humidité de plus de 95%. L'incinération, le compostage, l'épandage et l'enfouissement sont les approches les plus couramment utilisées pour la gestion de ces boues. En tenant compte de l'espace de stockage, de l'apport d'énergie, des coûts pour le transport et la manipulation des boues, un processus efficace de déshydratation et de réduction de volume de boues est nécessaire pour les stations d'épuration municipales. Globalement, pour améliorer la déshydratation des boues, les technologies de prétraitement comme l'ultrasonication, l'irradiation aux micro-ondes et l'électrolyse sont généralement appliquées. Des réactifs chimiques de conditionnement sont généralement ajoutés pour perturber la structure des flocs et pour lyser les

cellules afin de libérer l'eau interne. Cependant, ces réactifs diminueraient les performances de décantation des boues et provoqueraient des pollutions secondaires. Une alternative à ces procédés est l'utilisation des BALOs. En effet, en raison de la grande densité de microorganismes dans les boues et de l'abondance des matières organiques, les BALOs y sont naturellement présents. Ainsi, un apport supplémentaire de BALOs permet de perturber la composition des biofilms et par extension la structure des boues, favorisant considérablement leur déshydratation. Le processus de biolyse stimulé par les BALOs présente des avantages opérationnels, économiques, écologiques et hygiéniques (Yu et al. (2017). Isolation and application of predatory *Bdellovibrio*-and like organisms for municipal waste sludge biolysis and dewaterability enhancement. *Frontiers of Environmental Science and Engineering* **11 (1)**, 1–11).

Bdellovibrio and like organisms: The smallest cellular hunters of the microbial world

Soumis à « *Critical Reviews in Microbiology* » le 15 décembre 2020

1. Introduction

Serendipity and vigorous research have been the origin of myriad discoveries and knowledge on microbes since Robert Hooke and Antoni van Leeuwenhoek's description of microscopic organisms from 1665-1683 (Gest, 2004). Decades later, in 1962 in Berlin, Heinz Stolp was conducting experiments on the phytopathogen *Pseudomonas syringae* pv. *phaseolicola*, which is the causative agent of the common bean disease. Initially, Stolp wanted to isolate bacteriophages specific to this species. However, he ran out of 0.2 µm filters and instead used sintered glass, which allowed the passage of bigger microorganisms. Although phages generally form lytic plaques within hours, no plaques were formed within a day. Fortunately, he decided to keep them for two more days and plaques finally occurred. Surprised by this delay, Stolp examined the content of plaques under a microscope and discovered fast-moving cells that tried to attach themselves to other cells (Jurkevitch, 2006a). This was the first observation of a *Bdellovibrio bacteriovorus*, (from the Greek *bdella* or leech (Stolp and Starr, 1963) the first representative of the *Bdellovibrio* and like organism (BALOs), a phylogenetically heterogeneous group of rod or vibrio shaped Gram-negative bacteria that are obligate bacterial predators of other Gram-negative bacteria. Although first isolated from soil, over the years BALOs were detected in almost all aquatic habitats (Fry and Staples, 1976; Williams, 1988; Sutton and Besant, 1994; Jurkevitch and Davidov, 2006; McCauley et al., 2015; Feng et al., 2016).

Since then, researchers from different disciplines have studied on these organisms with a variety of methods, from the simple microscopic description to DNA detection, to reveal their presence and diversity in every possible environment. They rapidly evaluated their ecological impact on other bacterial populations, especially pathogens, in medicine, aquaculture or food processing with the ultimate goal of developing them as effective anti-microbial agent that could complement or replace anti-microbial substances. That being said, despite the effort of many BALOs specialists, we are still missing important information on their life cycle, predation patterns, diversity and ecological role etc. What we uncovered represent surely only the tip of the iceberg. The yearly discovery and growing research projects are proof of the secrets that this group still holds (Fig. 1). As mentioned, specialists working on BALOs are from different scientific fields. Thus, most of this information is not in one place. In addition, many authors chose to focus on one aspect of BALOs. Therefore, the aim of this review is to describe BALOs from various angles while presenting the most important current knowledge and gaps related to these unseen elephants in the room (Ezzedine et al., 2020b).

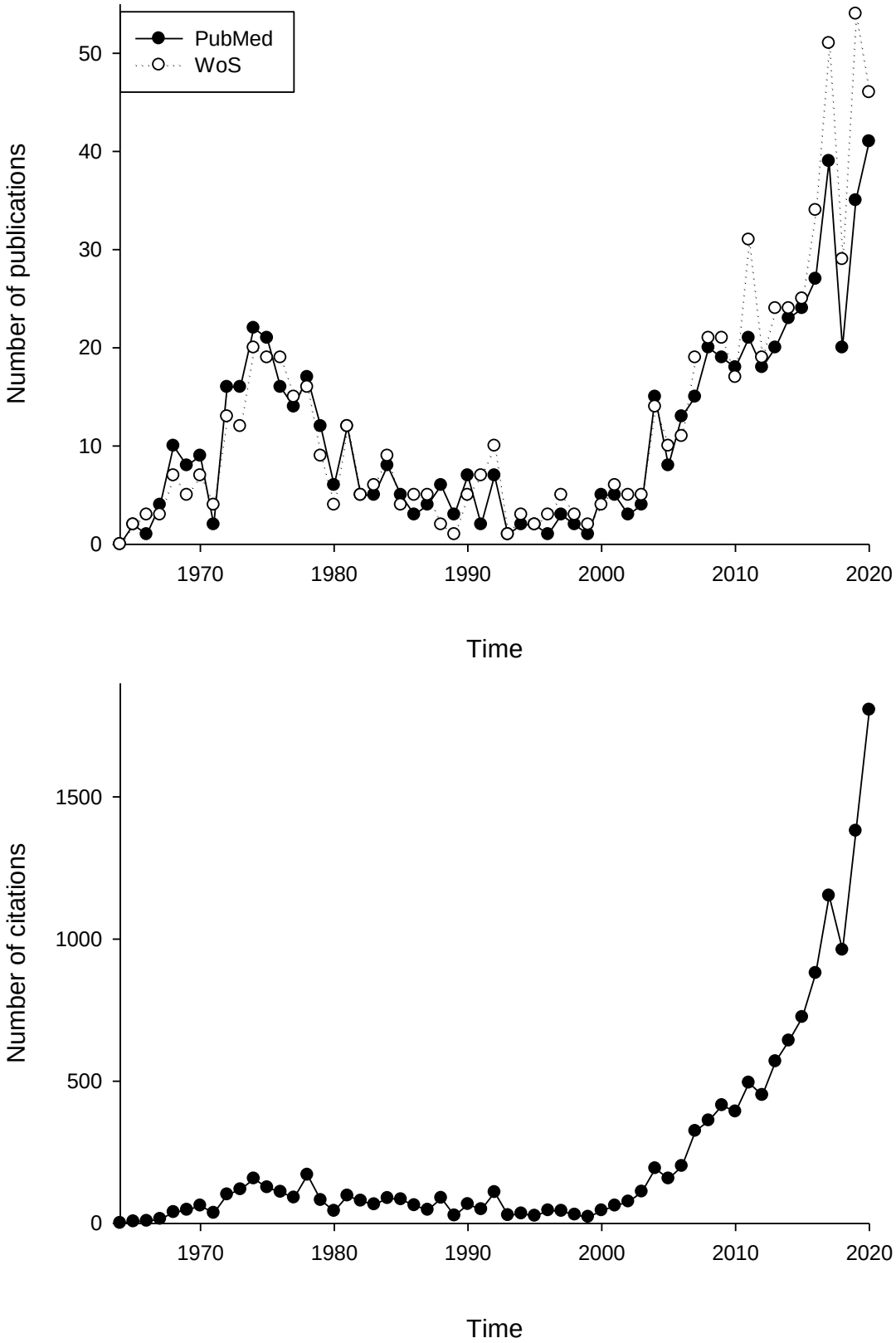


Figure 1 Evolution of the number of publications (up) and citations (down) dealing with BALOs from *Pubmed* and *Web of Science* from 1964 to 2019, using *Bdellovibrio* as a key word.

2. What makes BALOs so special to be in the spotlight?

We usually hear about bacteria causing diseases, helping as probiotics, recycling elements, cleaning up pollution, spoiling foods, or producing antibiotics, but less about bacteria eating other bacteria. BALOs, a group of predatory bacteria, are not the sole predators in the bacterial world. For example, among the Myxobacteria, and when nutrients are scarce, quite a number of species (e.g. *Myxococcus*) are known facultative predators (Jurkevitch and Davidov, 2006). What is particular with BALOs is that they are the only confirmed obligate predators. Indeed, BALOs are designated as predator instead of parasite or parasitoid (Supplementary information 1 for more details). Other predators like *Vampirovibrio chlorellavorus* could also be obligate. But to our knowledge, they are not yet listed in this category. BALOs growth and reproduction rely entirely on consuming other bacteria, paralleling bacteriophages, and acting as a "balancer" or "controller" capable of decreasing the numbers of other bacterial population. Moreover, they are found in many environments, *i.e.,* soil, fresh/saltwater, animal bodies, and polluted water (Markelova, 2002; Shemesh et al., 2003; Davidov and Jurkevitch, 2004; Ezzedine et al., 2020c). However, not all strains can be found at the same time since each has different requirements to thrive (Baer et al., 2004; Pineiro et al., 2013; Kandel et al., 2014; Koval et al., 2015). In addition, while they are not numerically dominant (Davidov et al., 2006a; Kandel et al., 2014; Paix et al., 2019) they are reported to be very effective predators (Williams et al., 2016) with wide or restrained prey range depending on the strain. In the last few years, they have been considered as a serious alternative to antibiotics in light of the rise of resistance developed by pathogenic bacteria (Willis et al., 2016). They are also considered probiotic (Qi et al., 2009; Dwidar et al., 2012; Bratanis et al., 2020). Their predation can occur in two different ways. From the inside through periplasmic (endobiotic) consumption or from the outside through epibiotic consumption. Finally, what makes BALOs special is that they remain difficult to study. Typically, they are more accessible via DNA studies than culture-dependent methods since their replication entirely relies on adequate prey (Davidov et al., 2006a) and this makes their cultivation and isolation complex. In addition, the procedure of isolation and culturing is time consuming, requires multiple steps (Jurkevitch, 2012a) and recovery efficiency greatly varies depending on the BALO's natural abundance. Therefore, the overall volume of research on BALOs is rather small (Shemesh et al., 2003). Altogether, BALOs' molecular operational taxonomic units (MOTUs) are more diverse (Ezzedine et al., 2020b) than what culture-dependent methods can yield. The discovery of new bacteria that can be assigned into BALOs is challenging (Ezzedine et al., 2020c) but not impossible as demonstrated with the recent discovery of three new strains belonging to a new species of *Halobacteriovorax*, namely, *H. vibrionivorans* sp. nov (Ye et al., 2019).

3. BALOs phylogeny

Since the isolation of *Bdellovibrio bacteriovorus* by Stolp and Starr (Stolp and Starr, 1963), and then over the following 4 decades, these predators have been placed in a single genus named *Bdellovibrio* (Davidov et al., 2006a) except for *Micavibrio* spp. which were discovered in 1982 (Lambina et al., 1982). Reclassification started with the use of 16S rRNA gene-based phylogeny led by Henry Williams and his group revealed an extensive diversity (Snyder et al., 2002). They coined the terms *Bdellovibrio*-like organisms and *Bdellovibrio*-and-like organisms (BALOs), to include these phylogenetically diverse but phenotypically and functionally similar bacteria (Snyder et al., 2002; Davidov et al., 2006a). Halotolerant BALOs were removed from the *Bdellovibrio* and placed into another genus, *i.e*., *Bacteriovorax,* and finally classified into their own genus and family, *i.e., Halobacteriovorax* and *Halobacteriovoracaceae* (Enos et al., 2017). Additionally, the reclassification of the bacterium *Bacteriovorax starrii* into *Peredibacter starrii* was proposed, and the family of *Peredibacteraceae* was defined and includes strains issued from freshwater and soil systems (Pineiro et al., 2004). The current classification (Schwudke et al., 2001; Davidov and Jurkevitch, 2004; Koval et al., 2013, 2015a; Hahn et al., 2017) transferred the BALOs from Delta-proteobacteria to the Oligoflexia class of the proteobacteria. BALOs are closely related to Delta-proteobacteria hence their initial classification in this class (Figure 2). The Oligoflexia class contains 3 orders, 5 genera and 5 families (Figure 2). The Bdellovibrionales order encompasses the genus *Bdellovibrio* from the *Bdellovibrionaceae* family with two type species, *B. bacteriovorus* HD100 and *B. exovorus* JSS. The Bacteriovoracales is characterized by 3 families, *Bacteriovoracaceae, Peredibacteraceae* and *Halobacteriovoraceae,* with the respective genera *Bacteriovorax, Peredibacter,* and *Halobacteriovorax* and the respective type species *B. stolpii* Uki2, *P. starrii* A3.12, *H. marinus* SJ and *H. litoralis* JS5. The last order in this class is Oligoflexiales with the family *Pseudobacteriovoracaceae*, the *Pseudobacteriovorax* genus and *P. antillogorgiicola* RKEM611 as the type species. The second class of BALOs is the Alpha-proteobacteria with the type species *Micavibrio aeruginosavorus* ARL-13 and *Micavibrio Admirantus* (Lambina et al., 1982) (Figure 2). Despite *Micavibrio* being in a distant class than the other BALOs, they are still classified as BALOs due to their common characteristics in term of shape and predation. Moreover the *Micavibrio* are still classified as *incertae sedis* Alphaproteobacteria (Davidov et al., 2006b). On the other hand, *Bdellovibrio*, *Peredibacter* and *Micavibrio* found in fresh and salt waters are still not separated into groups in relation to their halotolerance (*i.e.,* halo-*Bdellovibro*, halo-*Peredibacter* or halo-*Micavibrio*) and this issue could be interesting to address. Finally, the current classification heavily relies on phylogenetic trees constructed with the small subunit of the 16S rRNA gene and rpoB RNA polymerase subunit.

Therefore, using whole genome sequencing or at least multilocus analysis could rattle the current phylogeny.

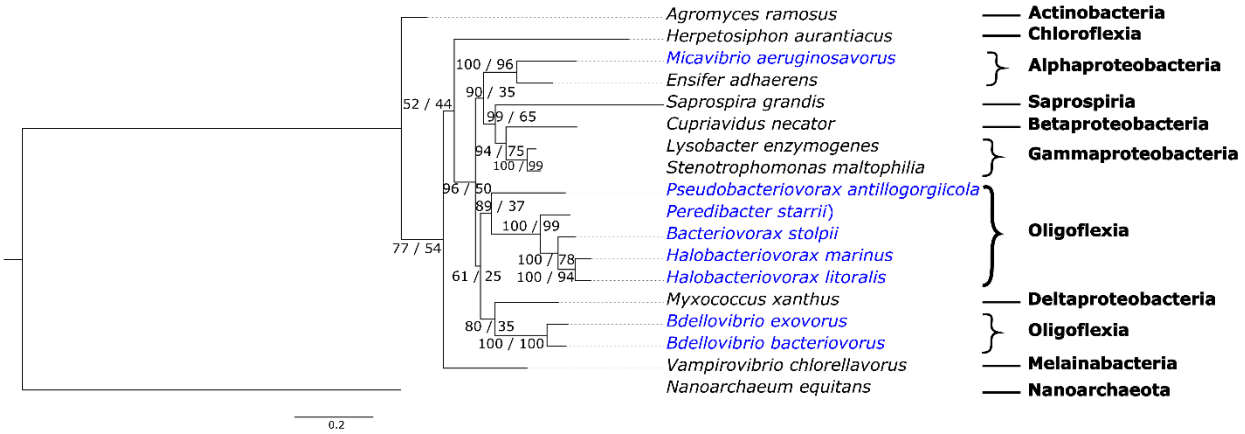


Figure 2 Phylogenetic tree based on sequences of the 16S small ribosomal subunit illustrating the relationship between various obligate or facultative prokaryote predators. The BALO's group (in blue) is composed of both species belonging to the class of Oligoflexia and Alphaproteobacteria. The tree was constructed using the Maximum likelihood (ML) and Bayesian methods. The initial alignment was done with Muscle (1648 positions) and corrected with Globcks v.0.91b (913 positions). The phylogeny model was chosen using the "Akaike Information Criterion 1" (AIC1) and is GTR + I + G with G and I respectively equal to 0.45 and 0.18. The model was obtained with the software "Modelgenerator v.85". The PhyML v.3.1 software was used to generate the ML tree with 100 bootstraps, SPR and BioNJ options. The MrBayes v.3.2.7 software was used to generate the Bayesian tree with 500,000 generation and 25% burn in value options. In order of appearance (top to bottom) the NCBI accession numbers of the sequences are: NR_026165.1; NR_074236.1; DQ186612.1; KY548395.1; AB088636.1; JQ695937.1; NR_036925.1; AB008509.1; KJ685394.1; NR_024943.1; NR_115142.1; NR_102485.1; NR_028724.1; NR_112544.1; EF687743.1; AJ29276.1; NR_104911.1; AJ318041.1.

4. Life cycle of BALOs

BALOs exhibit two types of life cycles (Fig. 3) that last approximately 2 to 3 (Said et al., 2019) or 3.5 to 4 hours (Rogosky et al., 2006), depending on the BALOs strain and the infected prey. One takes place inside the host/prey by forming a bdelloplast (a predator/host structure) and is referred to as the periplasmic (endobiotic) cycle. To our knowledge, BALOs are the only bacterial predators that invade the periplasmic space (Stolp and Starr, 1963; Jurkevitch and Davidov, 2006). The other takes place outside the prey and is called the epibiotic cycle. It was previously thought that epibiotic BALOs have smaller genomes; however, recent findings suggest that epibiotic BALOs are phenotypically diverse and have large genomes as shown with "*Candidatus* Bdellovibrio qaytius*"* (Deeg et al., 2020). Overall, for the periplasmic cycle, one predator cell can infect one prey cell except if predators are abundant and prey are minimal (Fenton et al., 2010; Said et al., 2019). In other words, co-infection is a matter of predator/prey ratio. However, multiple infection of a prey is usually associated to the epibiotic cycle (Pasternak et al., 2014).

Box 1: Predators, micropredators, parasitoids or parasites?

The border is thin between predator and parasitoid for BALOs. According to Poulin (Poulin, 2011), predators, including micropredators, parasitoids and parasites, can be seen as steps on the evolutionary pathway leading to parasitism. This reflects the continuous nature of these concepts and the putative subjectivity in their use that may be context dependent. Parasites and micropredators do not kill their hosts, while predators and parasitoids always do. Parasites and parasitoids use a single host, while predators and micropredators use several. Moreover, parasites, parasitoids and most micropredators are often specific toward their hosts, while many predators use an array of prey. BALOs can be simultaneously described as parasitoids and predators since they exhibit features related to both. For example, they are parasitoids because of the cell/host invasion for replication, and predators since the cell/prey is immediately killed (in contrast to parasitoids or parasites) to serve as a food base without using the cell machinery (Shemesh et al., 2003). The host/prey acts as a reservoir of the energy source and a protective environment against harsh conditions and other predators, and for progeny growth. Progeny can be numerically abundant (depend on prey size) with 2 to 7 or 3 to 6 or more progeny per infected host (Fenton et al., 2010; Dwidar and Yokobayashi, 2017). In the literature, BALOs are referred to as predators and Williams *et al.* (Williams and Piñeiro, 2006) who with tongue-in-cheek called them "the world's smallest hunters.

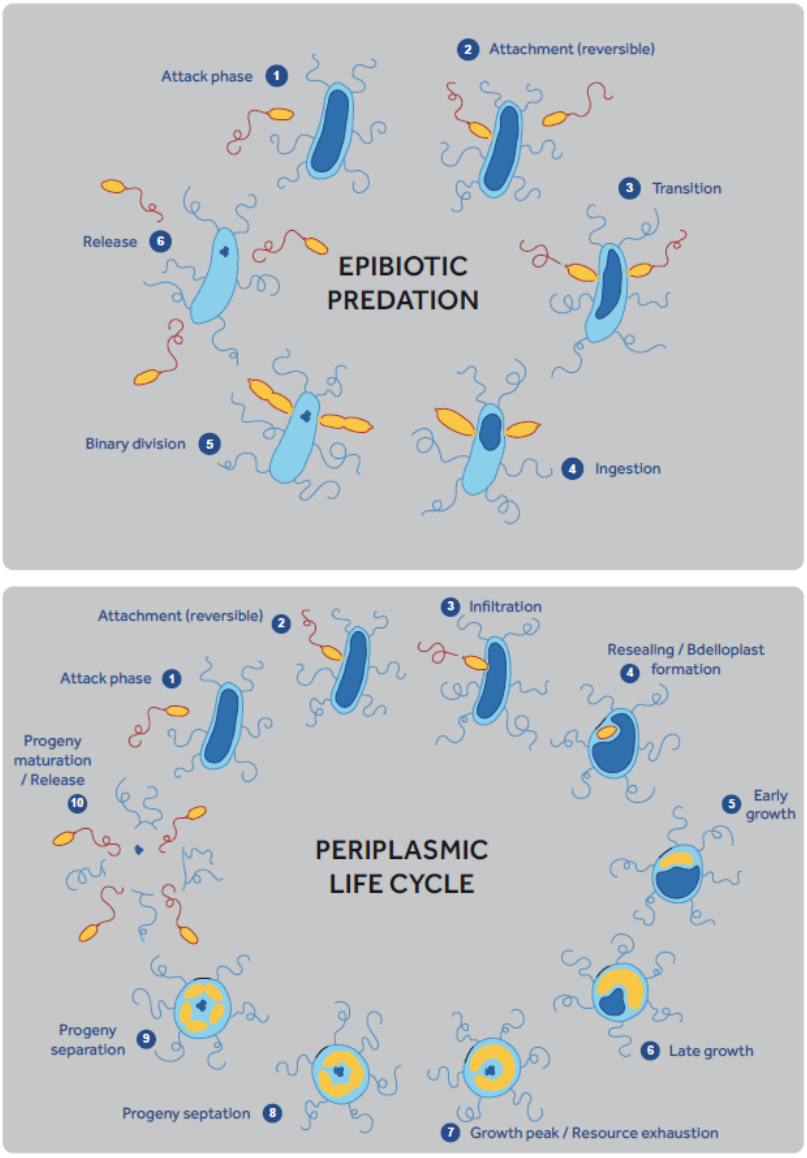


Figure 3 (A) The epibiotic predation mode begins by the attack phase (1) where the BALO meets randomly a prey. Then, a reversible attachment occurs between the two cells (2) during which the BALO checks the prey energetic quality and finally decides to attach irreversibly to it if the predator is satisfied (3). During this transition stage and even before several predators can attach to a same prey. The growth phase of the BALO begins thereafter (4) with the consumption of the prey components. It precedes a few hours later, when the prey cell is depleted, a binary division (5) where the mother BALO gives two daughter cells. At last, each new formed BALO cell acquires a flagellum and releases itself form the dead prey (6), to undergo a new cycle. (B) The periplasmic life cycle is similar to the epibiotic mode for the two first stages (*i.e.* the attack and reversible phases). Then, for the former, an infiltration step occurs (3) where the predator invades the prey by using enzymes to degrade the membrane. Once inside the prey, the BALO loses its flagellum, closes the membrane opening and begins the formation of the bdelloplast (4). An early growth follows (5) where the BALO secretes a cocktail of enzymes enable to degrade the prey components. The predator generates a flagellum and expands in size (6) until resource exhaustion (7). Then, the cell gives birth to several progenies (8) throughout a synchronous septation and these cells divide (9) before the lysis and burst of the prey (10).

Both cycles come in three distinct phases (Rotem et al., 2015); a first phase called “attack phase”, a second phase known as “transition phase” and a third depicted as “growth phase”. The first phase is shared by the two cycles. The second is poorly characterized and was only described in the periplasmic cycle (Rotem et al., 2015). The third (periplasmic *vs* epibiotic) differs in terms of mode, localization, and offspring. The periplasmic life cycle (invasion of the prey and growth as filament) is best studied in *B. bacteriovorus* HD100 and the epibiotic (attached extracellularly and binary replication) is best studied in *B. exovorus* and *Micavibrio* strains. The life cycles of BALOs were only described for a few strains when BALOs were considered a homogenous group. Therefore, the cycle described here cannot be assumed to be identical for all existing BALOs strains. Other strains may exhibit differences with the described cycle in here in term of cycle duration, offspring number and prey invasion. In fact, Williams and Piñeiro (2006) stated that differences in specific stages in the life cycle of BALOs may occur. Furthermore, Said et al. (2019) showed that for the same predator, such as *B. bacteriovorus* HD100, the bdelloplast’s size and shape were different depending on the prey type and size. The same authors also pointed out that the life cycles of BALOs were mainly described by phase contrast microscopy and transmission electron microscopy. However, using these tools, one can neither distinguish ghost cells (lysed preys) from intact bdelloplasts, nor correctly count the number of bdelloplasts and neither count nor differentiate the structure and shape of bdelloplasts. New technologies, such as helium ion microscopy, can offer a more precise description of the life cycle of BALOs (Said et al., 2019) in allowing one to quantify the predator, prey, and bdelloplasts numbers. The cycle described below is mainly inspired from *B. bacteriovorus* life cycle studies.

4.1. Attack phase (AP)

The attack phase during which a motile BALO predator seeks for a prey is phenotypically conserved among all species of BALOs. This phase is characterized by high mobility supported by a polar flagellum and the inability to replicate or divide. The AP cell can absorb nutrients from the environment (*e.g.*, amino acids, glutamates but not sugars), which it used to synthetize and secrete a wide range of proteins and hydrolytic enzymes (Dwidar and Yokobayashi, 2017). In addition, in the absence of nutrients, it is suggested that the predator like other bacteria such as *Aerobacter aerogenes* and *Escherichia coli*, is capable of consuming its own cellular components to keep itself alive (Postgate and Hunter, 1962; Rybkin and Ravin, 1987; Dwidar et al., 2017). In this conformation, the predator has a short lifespan and will eventually starve (Pasternak et al., 2014), unless it can find a suitable prey. To avoid further alterations of its swimming and search patterns,

the predator enter swim-arrest state (Sathyamoorthy et al., 2020). Hence, when a prey is near, the predator can reactivate itself. Three stages can be described in the attack phase.

Stage I – Mechanisms of encounter

The attack, mobile and free phase (Chauhan et al., 2009a) is a planktonic phase where the predator randomly collide with a prey (Jashnsaz et al., 2017). By dint of its single polar flagellum, the predator can move extremely fast and easily reach >50 µm/s (Jashnsaz et al., 2017) and up to 160 µm/s (Williams et al., 2016). This mobile phase in *B. bacteriovorus* involves different motility genes. In fact, four independent loci synthetize and structure the flagellum (Rendulic et al., 2004). BALOs also have gliding motility (A-motility) (Lambert et al., 2011; Milner et al., 2020) coordinated by the ParA homolog protein, which is particularly useful when preying inside a biofilm (Lambert et al., 2011). To date, the predator search has been described as random and not based on the detection of homoserine-lactone (quorum sensing), a signaling molecule that can be produced by prey bacteria (Rendulic et al., 2004) except for a very high concentration of prey (Straley and Conti, 1977; Jashnsaz et al., 2017). However, no significant chemotactic response has been measured for prey concentrated below 10^8 (Jashnsaz et al., 2017) or 10^7 (Lambert et al., 2003) cells mL^{-1}. Therefore, chemotaxis plays a modest role in prey targeting. In addition, no specific receptors have been found on the prey that can serve the predator as an attachment point (Lambert et al., 2006; Jashnsaz et al., 2017). Jashnsaz et al. (2017) mentioned that the random character of BALOs' hunting strategy is entirely conceivable since chemo-attractive signals emitted by multiple preys could ultimately send contradictory messages to predators. Nevertheless, even if the meeting is fortuitous, it seems to be favored by the hydrodynamism created by rotational forces of the predator's flagellum and body, which combine to form the generated moving trajectory that attracts and puts the predator via this turbulence toward inert bodies where potential prey could be orbiting (Jashnsaz et al., 2017). The combination of hydrodynamic effects leads to a circular movement of both the predator and prey around objects and effectively increases the colocation of organisms by confining them to smaller volumes or trajectories in one dimension. A random encounter is thus more probable thanks to the action of hydrodynamics and its dimensionality reducing effect (Jashnsaz et al., 2017; Prasad, 2017).

Stage II – Recognition of the prey and anchoring of the predator

Once the predator intercepts a prey, it attaches to it in a reversible manner over a short period. This attachment is brief and identified as a recognition step, during which the predator may search for binding sites. Thereafter, attachment may become irreversible usually within about 10 minutes

(Said et al., 2019). However, irreversible attachment may only occur in a minority of encounters between a BALO and its prey (Varon and Zeigler, 1978). The anchoring or adhesion of the predator to the prey is governed by the activity of various genes (Rendulic et al., 2004; Duncan et al., 2019). Apart from the existence of passive interactions between the outer membranes of cells, such as protein – protein and LPS – LPS (lipopolysaccharide), active adhesion takes place via the activity of pilus genes. Type IVa and b pili (Evans et al., 2007; Avidan et al., 2017) or fimbriae perform several functions in bacteria such as secreting adhesion proteins or allowing a particular type of mobility in the predator, which is named 'twitching.' The tip of the pilus contains a multitude of adhesive biopolymers, which are specific to different surfaces. It is assumed that all 'twitching' type pili make it possible to draw the predator through the entry pore generated in the external membrane of prey while being attached to the internal side of the peptidoglycan wall. Moreover, once the predator physically interacts with the prey, the latter is almost immediately killed via paralysis of its metabolic machinery (Thomashow and Rittenberg, 1978; Rogosky et al., 2006; Williams and Piñeiro, 2006; Chen and Williams, 2012).

Stage III – Preinvasion and invasion of the prey

The infection phase lasts at least 30 minutes (Said et al., 2019). Before penetrating its target, the predator generates a small opening in the outer membrane of the peptidoglycan layer of the prey. This "door" is created due to a set of hydrolytic enzymes applied locally to limit damages to the prey. Genes responsible for this function code for serine, cysteine, aspartate and metal-dependent proteases (Rendulic et al., 2004).

4.2. Transition phase

Recently, the existence between attack and growth phase of a discrete phase of the periplasmic cycle has been demonstrated (Rotem et al., 2015). It has shown that prey envelope, as well as the cytoplasmic content (soluble fraction) of the cell, are necessary but that each separately is not sufficient to allow the entry of attack cells into growth phase. Each of these two components successively differentially alters gene expression of the predator in attack phase. It is during the transitional phase that the bdelloplast is built. This phase appears as a check point of the cell cycle where the decision to engage in growth is linked to the detection of the cytoplasmic cue. This signal could be used as an indicator of the nutritional value of the prey, to prevent entry into growth phase in the absence of the resources necessary to complete the reproductive cycle.

4.3. Periplasmic life cycle

Stage IV – Formation of the bdelloplast and growth

In addition to proteases, Lambert *et al.* (Lambert et al., 2015) described the activity of glycanase, which permits disruption of the peptidoglycans of the prey at the beginning and during the invasion. Two peptidoglycans (*i.e.*, DD-endopeptidases Bd0816 and Bd3459) break the covalent bonds between the polymer chains ("decrosslinking") of the cell wall by hydrolyzing the 3-4 peptide crosslinks structure. The action of DD-endopeptidase is a signal for morphological change that blocks the prey's entry to other predators and thereby eliminates competition. However, DD-endopeptidases can also attack the predator's peptidoglycans, and the latter has developed protection by using the protein Bd3460 that has an ankyrin repeat domain. This protein will bind with the epitopes of the two endopeptidases to prevent "decrosslinking" of the cell wall of the predator. The bd3460 gene for ankyrin is pre-expressed before the invasion and its expression gradually increases during the invasion. This gene was probably acquired by the predator via horizontal gene transfer from spirochetes (Lambert et al., 2015). Once in its host, the predator can discard its flagellum (Fenton et al., 2010). Then, the periplasmic growth phase can finally begin (Chauhan et al., 2009a). The occupancy signal generated by the DD-endopeptidase prevents entry by successive predators and permits the formation of the bdelloplast (Lambert et al., 2015). Specifically, the infected prey is converted into a hybrid prey-predator structure (Van Essche et al., 2011), and the shape of the prey changes due to hydrolysis of the peptide bonds in the cell wall and degradation of biopolymers. In some cases, the initial morphology of the prey can be maintained throughout the predation process, that is, the size and cell form of the host remain unchanged (Chen and Williams, 2012). Bdelloplast formation is observable after 20 minutes of co-incubation of the predator and prey (Said et al., 2019). The prey bacteria acts both as a food source and as a habitat (Baker et al., 2017). The shape of the bdelloplast, round (Dwidar and Yokobayashi, 2017), crescent moon (Chen and Williams, 2012) or elliptical (Said et al., 2019), can be different depending on the BALOs and the size of the infected prey (Said et al., 2019). In addition, the bdelloplast of *Escherichia coli* has a porous interior (Said et al., 2019). It is osmotically stable (Rendulic et al., 2004), which suggests that the structure of peptidoglycans is partially intact. The peptidoglycans of the prey are modified sequentially to facilitate the formation of the bdelloplast to support the initial invasion of the predator without bursting but also to accommodate the invasion and growth of the predator (Lambert et al., 2015). The bdelloplast also constitutes a real barrier against bacteriophage attack and protects the hybrid couple from unfavorable physico-chemical conditions (Chen and Williams, 2012). In the bdelloplast, the predator secretes a cocktail of hydrolases,

proteases and peptidases (Monnappa et al., 2014) to hydrolyze and consume the cellular, protein, RNA and DNA components of the prey (Oyedara et al., 2016). The modification of the cytoplasmic membrane of the prey by the formation of the bdelloplast increases the permeability to facilitate feeding of the predator on the degraded compounds (Gophna et al., 2006). These nutrients are then used to grow and replicate (Dwidar et al., 2017). All the enzymes involved in the predation and digestion are designated as the predatosome. Among these proteins, *bd0934* and *bd3507* transcripts play a part in the degradation of the host DNA (Bukowska-Faniband et al., 2020).

Stage V – Replication, septation and offspring release

B. bacteriovorus develops in the form of a filament within its host and then the long filamentous cell divides by segmentation (septation) into 2 to 7 or 3 to 6 up to 9 offspring of the same size (Fenton et al., 2010; Dwidar and Yokobayashi, 2017; Said et al., 2019). The number of progeny is correlated with the prey type and size, thus the amount of resources they contain (Said et al., 2019). *Inter alia,* the division is controlled by the ParA homolog (ParAB) and the morphology and proportion of the cell by a DivIVA homolog ($DivIVA_{Bd}$) protein (Milner et al., 2020). According to Fenton et al. (2010), septation and filamentous elongation during replication synchronously occur. The BALOs produce an even or odd number of daughter cells, which indicates control of BALOs in the replication of their DNA (Fenton et al., 2010). The division is synchronous even in the case of multiple offspring (Fenton et al., 2010). This synchrony is even maintained in the case where two predators succeed in invading the same prey: the two wait for each other to lyse the host (Fenton et al., 2010). This synchronism could be explained either by the diffusion of a signal between predators within the same prey or by a simultaneous reaction to the final depletion of the bdelloplast. This rare case of double or multi-infection has been observed only when a large number of predators are present for a limited quantity of prey. However, the two predators remain in competition toward the food resource and each will result in a different number of offspring. When the protoplasm of the prey is completely consumed and the offspring reach their maximum size, they develop a flagellum while producing hydrolytic enzymes. These enzymes dissolve the rest of the peptidoglycan layer in the outer membrane of the wall. Thus, this dissolution of the membrane permits lysis of the bdelloplast (Rendulic et al., 2004), which resembles the lysis of bacteria by bacteriophages. However, another mechanism concerning the release of offspring into the environment has been observed by Fenton *et al.* (Fenton et al., 2010). In this case, there is a release through discrete pores rather than a rupture of the entire outer membrane of the prey or the bdelloplast. Once the daughter cells are outside, they continue to extend in length but not in width, and the cycle will eventually start again (Shatzkes et al., 2016).

4.4. Epibiotic life cycle

Stage IV – External anchoring of the predator to its prey

The genus *Micavibrio* and the species *B. exovorus* are predators characterized by an epibiotic growth phase. Therefore, these predators remain attached to the outside of the prey without intrusion, while consuming the organelles of the prey (Shatzkes et al., 2016). In this scenario, the prey is not transformed into a bdelloplast (Pasternak et al., 2014). *Micavibrio aeruginosavorus* can attach to its prey through the nonflagellar polar side (pili) and longitudinally by the nonpolar side. For *B. exovorus*, the anchor point is only on the polar side. In addition, during the epibiotic cycle, several predators can be seen attached to the same prey (Pasternak et al., 2014).

Stage V – Binary fission

When the prey contents are entirely consumed, the epibiotic predator undergoes binary fission, thus creating only two daughter cells. This fission can occur in two ways: either the predator remains attached to its prey or it detaches itself from the prey and independently performs its fission (Chanyi et al., 2013; Pasternak et al., 2014).

5. BALOs predation strategies

5.1. Predation spectrum and preference

Predation by BALOs has been mainly studied via laboratory isolation and culture using the double layer method (Jurkevitch, 2012a). BALOs are known to mainly prey on Gram-negative bacteria such as *Pseudomonas*, *Vibrio* and *E. coli* (the mechanism behind this preference is not yet elucidated, see supplementary information 2). Prey can be environmental free living bacteria as well as plant, animal or human pathogens (Rendulic et al., 2004; Davidov et al., 2006a; Fenton et al., 2010; Ezzedine et al., 2020c). Not all Gram-negative bacteria are susceptible to predation by a BALO. BALOs can also prey on dead cells with intact cell contents (Rogosky et al., 2006), and more recently, Lee et al. (2018) reported that FD111, a new strain of BALOs, is capable of predating on *Nannochloropsis salina*, which is a single celled eukaryotic alga. Another prey is *Staphylococcus aureus*, which is a Gram-positive bacterium that is reported to serve as a prey for *B. bacteriovorus* when the predator is given enough time to adapt to it (Iebba et al., 2014; Pantanella et al., 2018). However, it is noteworthy that this is still a subject of debate among specialists.

Finally, it has been shown that BALOs favor native bacteria over those of other habitats or cultures (Pineiro et al., 2004; Chauhan et al., 2009b; Wen et al., 2009). Overall, BALO species all display a different predation range; some are generalists with a wide variety of prey and others are specialists, *i.e.*, restricted to a very specific type of prey. Between these two extremes, some BALO species are versatilist hunters, such as *Bacteriovorax* cluster IX (Chen et al., 2011), which can behave as specialist but prey efficiently as generalist. Versatility is an advantage since the predator is less limited in terms of the prey's choice, and also, the competition with other predators is minimized while promoting its dominance (Chen et al., 2011). However, versatility does not necessarily give the predator a more effective predation on all prey. Predation efficiency changes depending on the prey. Thus this tactic is a compromise: if the predator loses efficiency it gains in the number of potential prey (Chen et al., 2011). The predation spectrum is linked to the predator's capabilities of attachment to prey in an irreversible way (because BALOs can attach then detach from nonprey). A generalist predator can be considered as one that has more "equipment" to attach to different types of prey (Rogosky et al., 2006), while the least equipped need special conditions or specific prey. Therefore, predator attachment appears to be affected by the composition of the host cell wall (Rogosky et al., 2006). Moreover, the size of the prey can also influence the predation of BALOs as reported by Chen *et al.* (Chen et al., 2011). In addition to the effect of hydrodynamism described above, Rogosky *et al.* (Rogosky et al., 2006) suggested that *B. bacteriovorus* 109J has a certain level of prey-recognition, which shows that predation efficiency highly depends on the prey type, and a faster attachment by the predator is observed with a preferred prey.

Box 2: A preference for Gram-negative prey?

It is not clear why BALOs preferentially select Gram-negative bacteria. Currently, some hypotheses have been proposed related to the nature of the receptors recognized by BALOs, which appear to be compounds of the outer membrane and possibly also of the periplasm. It has been suggested that when in contact with a potential bacterium prey, biopolymers (pili) of the predator can recognize certain branched sugars (oligosaccharides) on the surface of the bacterial wall, which are only present in Gram-negative bacteria (Dashiff et al., 2011). However, under certain laboratory conditions and in the presence of only Gram-positive bacteria, it has also been reported that some BALOs may prey on these cells. The predator would then change its mode of predation (from periplasmic to epibiotic cycle). Instead of consuming the prey by penetrating it, the predator would consume it from the outside due to the rapid production of new enzymes adapted to the wall of these prey.

5.2. Factors acting on predation

Predation by BALOs can be hampered or inhibited. A steric hindrance can occur from the presence of other predatory bacteria that influence the effectiveness of predation (Van Essche et al., 2011). In addition, the presence of decoy cells or nontarget cells can lead to ineffective predator-prey encounters (Wilkinson, 2001). However, this highly depends on the nature of the decoy species present in the experiment. For example, Gram-positive decoy bacterium, such as *Bacillus subtilis*, can hinder *B. bacteriovorus* predation (Hobley et al., 2006), while *Actinomyces naeslundii* has no effect (Van Essche et al., 2011). Decoy association can decrease or have no effect on the predator predation (Loozen et al., 2015). Overall, Loozen et al. (2015) suggested that BALO's predation efficiency decreases when multiple microorganisms are present. Furthermore, phenotypic resistance to predation mechanisms can arise in multiple prey (Shemesh and Jurkevitch, 2004). In predation experiments lead by Shemesh *et al.* (Shemesh and Jurkevitch, 2004), *Bdellovibrio* spp. and *Bacteriovorax* spp. failed to completely eradicate all prey, even when the predators were 3 to 5 times greater in number than the prey. This is explained by the appearance of a phenotypic, transient and reversible form of resistance (*e.g.*, displaying cell wall hardening or cell enlargement) in a part of the prey population. This resistance can disappear when the predator is removed and these mechanisms allow for the survival of both predators and prey. Moreover, motile or nonmotile prey have different predation outcomes. In fact, motile prey can generate a drag force that slows down the predator entry and killing in comparison to nonmotile prey. Therefore, highly motile prey are more resistant to attacks by BALOs (Duncan et al., 2018). It should be noted that high viscosity does not impact the predation efficiency of BALOs (Sathyamoorthy et al., 2019). Secondary metabolites can also affect the predation efficiency of BALOs, and a number of compounds have been assessed. Cyanide produced by *Chromobacterium piscinae* inhibited *B. bacteriovorus* HD100 predation by decreasing predator motility during the attack phase (Mun et al., 2017). Cyanide and indole (Dwidar et al., 2015) also delayed the development of progeny and prey lysis. However, violacein produced by *C. piscinae* did not inhibit predator activity (Mun et al., 2017). Furthermore, in the presence of environmental pollutants in water, such as cadmium chloride ($CdCl_2$), BALOs can block their growth phase at the terminal stage, which allows them to persist for a long time under adverse environmental conditions. Here, BALOs stay as bdelloplasts rather than adopting the planktonic form until these conditions become more favorable (Markelova and Gariev, 2005). Other pollutants, such as organochlorines (*i.e.*, Benzilan and Thionex), heterocyclic derivatives (*i.e.*, Diazinon), carbamate (*i.e.*, Ravion and Antracol) and organophosphates (*i.e.*, Bromex, Cotnion, and Rogor) were described by Varon and Shilo (Varon and Shilo, 1981) to inhibit the predatory activity of *Bdellovibrio*. Moreover, prey fermentation and catabolism of carbohydrates

leads to a medium level of acidification, which ultimately inhibits and kills the predator (Dashiff et al., 2011). Finally, some prey, such as *Vibrio cholerae*, are reported to display metabolic pathways that influence predation susceptibility (Duncan et al., 2018). For example, *V. cholerae* lacking the O-antigen are easily preyed on by *B. bacteriovorus*. Additionally, the production of toxic substances by the bacterial population are known to shield nontoxic bacterial from predator such as nematode and various protozoa (Jousset, 2012; Johnke et al., 2014). Hypothetically these metabolite can also inhibit BALOs predation. Setting up experimentation using bacteria that produce toxic secondary metabolites in the presence of BALOs would shed more light on BALOs predation and mechanisms.

Predation of BALOs can also be promoted. For example, the growth of *Bdellovibrio* sp. strain BDF-H16 was significantly promoted in the presence of copper sulfate (0.1 and 1.0 $mg.L^{-1}$) in comparison to predators cultivated without copper sulfate. It is likely that copper ions act synergistically with other divalent cations during *Bdellovibrio*-prey interactions (Cao et al., 2018). Moreover, *Bdellovibrio* spp. growth is also promoted with calcium chloride and magnesium sulfate. Ca^{2+} and Mg^{2+} also provide cations for *Bdellovibrio* growth and likely favor the interaction between the predator and prey (Huang and Starr, 1973).

5.3. Obligate predation and host independence

When grown in the absence of prey in complete media, most but not all BALOs are able to form host-independent (H-I) mutants at a frequency of approximately 10^{-6} (Seidler and Starr, 1969). Although they conserve predatory abilities, some BALOs may become nonpredatory in a reversible manner (Varon and Seijffers, 1975). This curious phenotype is a very useful tool for investigating the BALO life cycle as it enables growth of the predators in pure culture (Dori-Bachash et al., 2008) and mutation analyses in genes that are otherwise lethal in wild-type strains. For example, deletions of the structural Type IV pili (a or b) genes cannot be obtained in the obligate predatory wild-type backgrounds but are viable in H-I derivatives (Evans et al., 2007; Avidan et al., 2017). The molecular basis of host-independence is poorly understood. It was first mapped to mutations in a single locus, which is called the "host interaction" or "*hit*" locus (Cotter and Thomashow, 1992). The *hit* locus encodes a rather short protein with an intrinsically disordered protein (IDP) structure (Prehna et al., 2014). It weakly interacts with *Bdellovibrio* Bd0109 and apparently controls Type IV pilus formation and extension (Capeness et al., 2013; Prehna et al., 2014). The *hit* mutants (Type I) make small colonies, require prey extract for growth and often fail during subculture (Cotter and Thomashow, 1992). Secondary spontaneous mutations in *pcnB* or in *rhlB*, which both encode ancillary proteins of the RNA degradosome, enable robust axenic growth and

eliminate the need for prey extract and generate so-called Type II mutants (Roschanski et al., 2011). Early studies have shown that predation relies on two distinct prey-derived cues, the first is involved in promoting prey penetration and establishing the bdelloplast, while the second is needed for cellular growth (Ruby and Rittenberg, 1983; Gray and Ruby, 1990; Thomashow and Cotter, 1992). Thus, Type I and Type II relieve the requirement for the first and second cue, respectively. More recently, Rotem et al. (2015) showed the early cue is located in the prey envelope and a late cue in the prey-soluble fraction, which together delimit a transition between the attack phase to the growth phase and differentially affect gene transcription.

H-I mutants are phenotypically varied and bear various mutations in *hit* (Barel and Jurkevitch, 2001; Capeness et al., 2013). While most H-I strains are mutated in *hit*, approximately 11% are not, which (Wurtzel et al., 2010) suggests additional mutations that also induce host-independence. Furthermore, mutational frequency from Type I to Type II is largely higher than that from wild type to Type I (Cotter and Thomashow, 1992), which suggests that Type II arises from a number of, rather than a unique secondary, mutation (Barel and Jurkevitch, 2001). Therefore, it is notable that mutations in specific diguanylate cyclases (enzymes that synthesize the secondary messenger cyclic-di-GMP) result in an obligate H-I phenotype (*i.e.*, lethal in a wild-type background) or in strains from which H-Is are only extremely and rarely isolated (Hobley et al., 2012a). Currently, no H-I variants have been isolated from epibiotic predators (*B. exovorus* and *Micavibrio* spp.) (Pasternak et al., 2014), and as far as we know, only one was directly isolated from the environment (Hobley et al., 2012a). Collectively, the understanding of the mechanisms leading to and controlling the H-I phenotype may yield important clues on the evolution of predation in BALOs. Another remaining question is to understand if H-I variants are part of all BALO life cycle in nature or are restricted to some genera or strain (Hobley et al., 2012b).

6. Ecological features of BALOs

6.1. Habitats, abundance and distribution of BALOs

In certain occasions, the abundance of BALOs can reach a substantial percentage of the bacterial populations. However, in most cases, BALOs are characterized by low abundances, whether detected by culture-dependent methods or DNA surveys, with the latter is able to recover a larger number of strains. Overall, they do not form dominant populations (Shemesh et al., 2003) unless prey are abundant and are in a closed system (Kandel et al., 2014). For example, the study of marine BALOs is more amenable in estuarine systems rather than in the open ocean (Williams and Piñeiro, 2006). In fact, when using specific primers for the detection of BALOs, BALOs reads and OTUs

(operational taxonomic units) were less frequent in open water, *i.e.,* Banyuls Bay (France) than in peri-alpine lakes (Geneva and Annecy), which constitute smaller and relatively closed systems (Ezzedine et al., 2020a). In addition, the abundance of BALOs which may vary between a few cells to $>10^4$ cells per mL, is affected by the quality of the environment. For instance, polluted clouds (Amato et al., 2017) and polluted river water (Staples and Fry, 1973) are richer in BALOs than their unpolluted counterparts. Other polluted environments, such as sewage, are also rich in BALOs (Staples and Fry, 1973). This is likely due to the presence of a greater number of potential prey. In such environments, BALOs are readily detectable and therefore the probability of isolating them is higher. However, their abundance has not often been quantified. Only Zheng et al. (2008), Kandel et al. (2014), Van Essche et al. (2009) and Iebba et al. (2013) reported abundances using quantitative PCR (qPCR). For example, the count of *Halobacteriovorax* in Baltimore Harbor reached 8,570 copies per mL (Zheng et al., 2008), and for *Bdellovibrio* in the human gut; 2 to 549 copies per mg (Iebba et al., 2013). These reports have been slightly supplemented with data obtained for peri-alpine lakes such as for Lake Geneva where minimum and maximum values were measured to vary between 38 to 1,253 and 5 to 37,270 copies per mL for *Bdellovibrio* and *Peredibacter* respectively (Figure 4) (Ezzedine et al., 2021). In general, the BALO group have great adaptability to different environments (Yu et al., 2017) and they are therefore detected in many places. In natural and anthropic habitats they can be found as free living cells (planktonic), bdelloplast or those associated with biofilm (Markelova and Gariev, 2005; Jurkevitch, 2012a). Furthermore, HI variants, such as *B. bacteriovorus*, are seen in laboratories to form tenacious biofilms on abiotic surfaces (Medina and Kadouri, 2009). To date, BALOs have been reported in soils, rhizosphere plant systems, rivers, lakes, seas, oceans, estuaries, sediments, coral reefs, salty-ponds, mangroves, Antarctica, geothermal waters, oceanic subsoil, anoxic environments, shrimp aquaculture basins, leeches, activated sludge, waste waters, human and animal intestines, human lungs, blue crab gills, feces, and polluted and unpolluted clouds (Sutton and Besant, 1994; Schwudke et al., 2001; Davidov and Jurkevitch, 2004; Rendulic et al., 2004; Williams and Piñeiro, 2006; Davidov et al., 2006a; Wen et al., 2009; Kikuchi et al., 2009; Van Essche et al., 2011; Cao et al., 2015; Williams et al., 2016, 2018; Aguirre et al., 2017; Amato et al., 2017; Paix et al., 2019; Ezzedine et al., 2020b) and more recently in forest leaves (Miura et al., 2019) (Fig. 4). Their presence in so many environments demonstrates the widespread presence of BALOs, but not all strains (as for any bacterium) are found in every type of ecosystem. Many factors influence the distribution of BALOs and their diversity. For example, a salinity above 0.5% is required to detect *Halobacteriovorax* species. Moreover, among the saltwater BALOs, subpopulations are adapted to either low, moderate or extreme salinities (Amat and Torrella, 1989; Williams and Piñeiro, 2006). *Halobacteriovorax* were never reported in freshwater; however, *Bdellovibrio*, *Peredibacter*,

Bacteriovorax and *Micavibrio* DNA are found in fresh and salt waters as shown in our previous studies (Ezzedine et al., 2020a, 2020b). Moreover, qPCR results show that *Peredibacter* is clearly more abundant than *Bdellovibrio* and *Bacteriovorax* and is predominant in the epilimnion of Lake Geneva and the metalimnion of Lake Annecy (Ezzedine et al., 2021). Culture-based approaches or RNA surveys are required to further confirm the presence of active BALOs in these ecosystems. Kandel et al. (2014) also reported the presence of freshwater BALOs (*Bdellovibrio* and *Bacteriovorax*) in a saline mechanical zero discharge system. The presence and abundance of BALOs should only be attributed to active and living individuals. For example, when using a culture-based method, BALOs were reported to be more concentrated in the air-water surface microlayer than below that surface (Williams, 1987). In another study (Ezzedine et al., 2020b) using a DNA survey, the DNA of BALOs were detected at a greater abundance at 200 m than at the surface. These differences are probably due to DNA originating from dead BALOs that may sedimented in the depths, but it cannot be ruled out that active BALOs could be present in these places. Other factors also influence the distribution of BALOs such as oxygen availability and temperature (Schoeffield et al., 1996; Shemesh et al., 2003; Kandel et al., 2014; Williams and Chen, 2020). BALOs are aerobic, but halotolerant strains are reported to tolerate microaerobic conditions and survive anoxic periods as attack phase cells or as bdelloplast (Schoeffield et al., 1996). Moreover, BALOs exhibit seasonal distribution and some BALOs are more abundant or detectable in certain months (Williams et al., 1982; Ezzedine et al., 2020b). In fact, warmer months (late summer and early fall months) were reported to allow a greater recovery of marine BALOs. In winter, marine BALOs were recovered in higher abundance from the sediment (< 5 cm (Fry and Staples, 1976)) than in the water column (Williams et al., 1982). Thus, Williams & Piñeiro (2006) stipulated that BALOs in sediment are similar to "seeds" that allow BALOs to thrive again. Overall, BALOs are reported to be more abundant in sediments and biofilms than in the water column (Williams et al., 1982, 1995). Biofilms contain myriad of bacteria, including prey bacteria, and offer protection from environmental conditions; therefore, BALOs most likely thrive the best in such habitats (Williams et al., 1995; Kelley et al., 1997; Markelova and Gariev, 2005). This is in accordance with Markelova (2002), who suggested that BALOs survive better in harsh environmental conditions (*e.g.*, polluted areas) when they are surface associated compared and more likely as bdelloplast with an arrested stage growth.

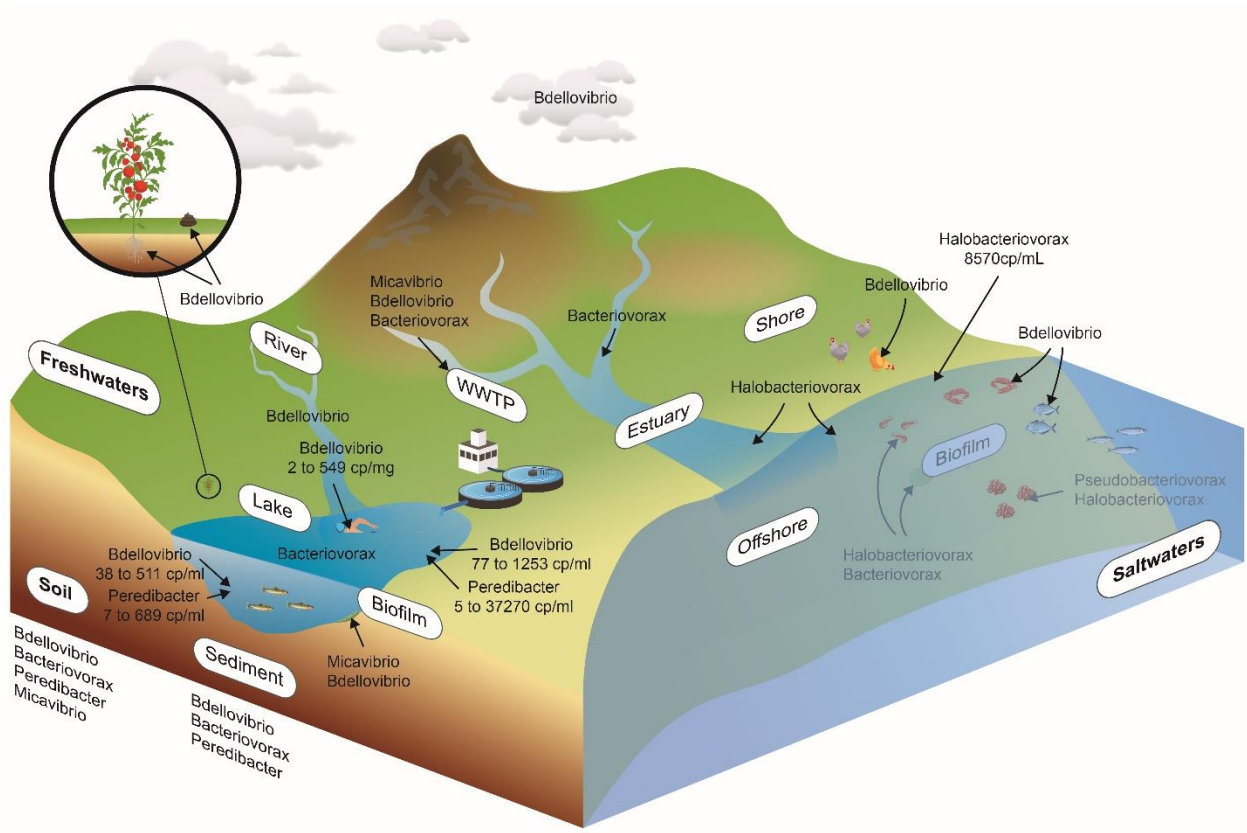


Figure 4 BALOs distribution, presence and abundance (when available) in different types of environments. The list is not exhaustive. Six systems are described: freshwater (with river, sediment and biofilm on rocks), saltwater (with estuary, near and off shore, biofilm on rocks), soil (with ground, rhizosphere and feces), animals (including human, coral, fish, crab and chicken), wastewater treatment plant (WWTP) and clouds. The abundance provided for *Halobacteriovorax* is from samples from Baltimore inner harbor (Zheng et al., 2008). *Bdellovibrio* abundances reported for human are from Iebba *et al.* (Iebba et al., 2013). Finally, the abundances for *Peredibacter* and *Bdellovibrio* are from our recent studies on BALOs in Lake Geneva (Ezzedine et al., 2021). To our knowledge, no other quantitative data is available. However, the presence of BALOs has been detected in the above-mentioned environment by culture or molecular methods. For instance, *Bdellovibrio* and *Bacteriovorax* are readily detected in WWTP and *Halobacteriovorax* in saltwater systems. As shown BALOs are present in multiple environment but not all species can thrive in a same habitat.

Box 3: Statistical methods to identify co-occurring taxa

Traditional culture-methods are not suited for the study of BALOs' ecology (Williams and Piñeiro, 2006). Molecular methods also tend to miss some aspects such as association and functional traits. However, not much has been applied to studies of BALOs, but a number of methods have been developed to connect other interacting taxa such as symbionts and their hosts based on environmental sequencing data. Clasen and Suttle (Clasen and Suttle, 2009) used monotonic multiple-dimensional scaling (MDS) to link viruses from the family *Phycodnaviridae* to their phytoplanktonic hosts from denaturing gradient gel electrophoresis (DGGE) data. While rather crude, this approach allowed them to infer putative new hosts for unknown viruses by analyzing their cooccurrence in the same MDS space. Using sequencing data, network analyses based on correlational approaches have been used on a variety of microbial ecosystems (Vick-Majors et al., 2014). However, they may be biased by the compositional nature of communities and overlook true associations. Machine learning approaches are in development to uncover interaction networks from NGS sequencing data (Vacher et al., 2016). A very recent method has been proposed by Alric *et al.* (Alric et al.) (in press) that infers microbial interactions from environmental NGS sequencing data. This method is a multivariate approach based on predictive cocorrespondence analysis (pCoCA) combined with the computation of distance matrices and randomization tests. It accounts for the variation in community composition among samples while inferring the potential interactions between viruses and microbes and their potential interaction network. These techniques will certainly be useful in shedding light on the ecology of BALOs.

6.2. BALOs as "population balancer" ?

In a system where nutrients (and space) are abundant and unlimited, competition among microorganisms should not exist. However, even in oligotrophic environments, we still find a variety of coexisting microorganisms. It is true that some microorganisms can be favored because of high adaptability. Nonetheless, less adapted organisms can also thrive if they fit well in ecosystems controlled by predation or substance secretion. Predation by microbes is one of the main drivers of bacterial mortality in the environment (Johnke et al., 2017b). In addition, other than being fundamental for ecological balance, predation is important for nutrient acquisition and energy flow since it is present at every trophic level (Shemesh et al., 2003). Key players are protists, bacteriophages, nematodes (mostly in soil) and possibly, predatory bacteria that exert different types of control because of their different hunting strategies, prey size and specificity (Johnke et al., 2014). Thus, regarding their diversity and ubiquity, BALOs participate in their own way in controlling bacterial populations, and by extension impact the reservoir of dissolved organic carbon and the trophic web. Many studies support that BALOs are active and constitute dynamic members of the microbial community (Sutton and Besant, 1994b; Pineiro et al., 2004). In fact, Johnke et al. (2020) suggested that BALOs could act as drivers of microbial alpha-diversity, which indicates that their presence, abundance and richness promote great bacterial diversity by predating highly

abundant species and favoring rare species. Fratamico and Cooke (1996) showed that the genus *Bdellovibrio* can shape the composition of biofilms by reducing the number of cells. Welsh et al. (2016) reported that *Halobacteriovorax* are associated with animal microbiomes and regulate microbiome structure and protect the host from pathogenic bacteria. Feng et al. (2017) stated that *B. bacteriovorus* UP predation reduced >90% of the microbial community abundance and altered their composition in activated sludge. Moreover, *Micavibrio* are thought to influence the abundance and diversity of nitrite-oxidizing bacteria in engineered and natural ecosystems (Dolinšek et al., 2013). Nonetheless, it is still unclear how effective BALOs are as controllers of bacterial populations in comparison to other predators? The answer is still not known since only a limited number of studies have investigated this issue. To the best of our knowledge, only Williams et al. (2016) showed that *Halobacteriovorax* (halophilic BALOs) can eclipse the lytic action of bacteriophages population of the pathogenic bacterium *Vibrio parahaemolyticus*. Johnke et al. (2017a) also showed that predator diversity and spectrum (generalist protist, semispecialist BALOs and specialist bacteriophages) permitted coexistence among all predators. Generalist predators brought balance to the system by predating specialists and avoiding the exhaustion of prey. A comparison between BALO type species (*e.g., Peredibacter vs. Bdellovibrio vs. Bacteriovorax*) and between multiple BALOs, bacteriophages and protists has not been done. Moreover, predators, *i.e.,* BALOs and non-BALOs, are in competition. Hence, the action of and interaction between these predators can lead to a drastic change in microbial diversity (Johnke et al., 2017b). Most likely, predation pressure differs from one BALO to another and for the same BALO toward different prey. For BALOs using similar prey (Shemesh et al., 2003), their effects can be very heterogeneous on the microbial community in a given environment. For example, Said et al. (2019) noticed that the infection rate of *B. bacteriovorus* HD100 is different toward *E. coli* and *Pseudomonas putida.* BALOs tend to be neither true generalists nor strict specialists with a more or less limited prey range (Chen et al., 2011). In addition, they are involved in a cycle where their predation activity is halted for a few hours so they can replicate. On the other hand, protists are generalist grazers of bacteria in marine, aquatic and terrestrial ecosystems with different strategies of predation and a higher capacity of consuming a high number of bacteria over a short period of time (Johnke et al., 2014). Facultative bacterial predators, such as Myxobacteria, are social predators (Morgan et al., 2010) that adopt predation only when resources are scarce. They attack prey from a distance by secreting antibiotics and lytic substances (Sudo and Dworkin, 1972; Morgan et al., 2010). Myxobacteria and BALOs share homologous motility proteins of type IV pili that have evolved and diverged to adapt to each other's strategies (Lowry et al., 2019). Less is known about the relationship between BALOs and Myxobacteria and many questions remain: Do they compete? Do they eat each other? On another note, bacteriophages are highly host-specific

with lytic and lysogenic cycles. Interestingly, bacteriophages can act synergistically with flagellate predation on bacterial mortality (Šimek et al., 2001; Weinbauer et al., 2003; Jacquet et al., 2007) and can also protect bacteria from protists' predation by encoding toxin production in bacteria such as Shiga toxin (Stx), which can harm *Tetrahymena thermophile* (Arnold and Koudelka, 2014). It is not known whether BALOs are affected by bacteriophage's encoding in prey bacteria. Furthermore, not all Gram-negative bacteria are susceptible to predation by a BALO (Williams and Piñeiro, 2006). This may be due to defense mechanisms or simply a prey preference as stated by Rogosky et al. (2006). Additionally, less is known about Gram-positive or non-Gram colored bacteria and Archaea as prey. In fact, predation toward Gram-positive bacteria was reported by (Burger et al., 1968) and more recently by Iebba et al. (2014) and Pantanella et al. (2018), where *B. bacteriovorus* HD100 attached itself to *Staphylococcus aureus* through the epibiotic cycle. Perhaps more convincingly, it has been reported that *B. bacteriovorus* HD100 can degrade Gram-positive *Staphylococcus aureus* biofilms (Im et al., 2018). Waso et al. (2019) also reported that *B. bacteriovorus* PF13 can prey on *S. aureus* and *Enterococcus faecium*. Therefore, predating Gram-positive prey seems possible with the epibiotic cycle and does not generate predation plaques, which implies the use of techniques such as qPCR to monitor the variation of the prey and predator (*e.g.*, the ethidium monoazide quantitative polymerase chain reaction (Waso et al., 2019). It also seems that Gram-positive predation occurs only when specific conditions are met, such as a specific culture broth or buffer, but these results still need to be confirmed and more experiments are needed, especially in natural ecosystems. Even more surprisingly, *Bdellovibrio* strain FD111 has been shown to prey on microorganisms other than prokaryotes, namely, algae such as *Nannochloropsis salina* (Lee et al., 2018). In this case, BALO caused considerable mortality in this alga but it is not known if such a predation exists for other algal species. Ferguson et al. (2014) showed *in vitro* that *B. bacteriovorus* in the presence of *E. coli* and while incubated in a rich nutritive medium, is divided into two subpopulations: the cell population in the inner region of the *E. coli* film shows a hunting phenotype while the cell population in the outer region does not. Overcrowding and high nutrient availability with limited prey appears to favor the existence of two phenotypes of *B. bacteriovorus*. It is not yet known if this can take place in the natural environment, especially in biofilms. More generally, many points require further elucidation concerning the ecology of BALOs in natural systems, such as true predatory capacity and efficiency, the spectrum and effect of predation on the bacterial community, predation strategies in the presence of competitors, and xenobiotics or the nutrient richness of the environment. Therefore, more experiments are needed using a variety of methodological approaches such as microcosms, radioactive marking, culture-based techniques, qPCR, droplet digital PCR (ddPCR) and fluorescent

cytometry to better understand the role of BALOs as “population balancers” (other methods are proposed in supplementary information 3).

6.3. The hunter hunted

Despite their fast movement, small size and predator abilities, BALOs can be consumed and/or parasitized by other organisms. The bdelloplast phase prevents bacteriophage attacks; however, if BALOs are not in this phase it can be infected by bacteriophages as revealed by Hashimoto et al. (1970). Flagellated and ciliated protists (Johnke et al., 2017b) have also been found to prey on BALOs. However, it is reported that BALOs might not constitute the main food source of protists in resource-rich environments (Johnke et al., 2017b). We also hypothesize that metazoan zooplankton can prey on BALOs. Therefore, with such a predation pressure, can BALOs fulfill their role of an “population balancer” in all ecosystems or do only a few ecosystems favor BALOs and therefore are dominated by them?

7. Biotechnological applications of BALOs

7.1. Why use BALOs to fight pathogens?

Like bacteriophages used in phage therapy (Górski et al., 2018), BALOs are found in a variety of environment and are probably ingested safely and unconsciously on a daily basis (Willis et al., 2016). Their predation ability makes them also candidates for many applications dealing with the biological control of some bacterial populations (Sockett and Lambert, 2004; Harini et al., 2013; Cao et al., 2015; Bratanis et al., 2020). In addition, their predation skills have been verified on more than 100 human pathogens (Mun et al., 2017), such as *E. coli, Salmonella* and *Klebsiella pneumonia* (Kadouri et al., 2013; Shatzkes et al., 2016). Gram-negative bacteria are typically responsible for more than 30% of nosocomial infections (Hidron et al., 2008; Baker et al., 2017), and they are often associated with very high levels of morbidity and mortality in intensive care units (Gaynes and Edwards, 2005; Baker et al., 2017) due to bacteria that are multiresistant to antibiotics (Kadouri et al., 2013; Monnappa et al., 2014). Therefore, the use of alternative solutions to control such infections has become a necessity. BALOs have been suggested as a replacement of antibiotics to combat multiresistant pathogens in human and other organisms (Sockett and Lambert, 2004; Johnke et al., 2017b) or as a complement of antibiotic treatments since the genomes of epibiotic and endobiotic BALOs present a large number of antibiotic resistance genes (Pasternak et al., 2014). For example, Marine *et al.* suggested employing *B. bacteriovorus* with antibiotics,

such as trimethoprim, for cotherapy (Marine et al., 2020). Additionally, according to Hobley et al. (2020), *B. bacteriovorus* can be combined with bacteriophages to increase the eradication of *E. coli*. Furthermore, the large number of proteases and other hydrolases in BALOs constitutes a valuable reservoir of enzyme-based antimicrobial substances (Rendulic et al., 2004). Several groups of researchers, including Im et al. (2017), have suggested that these predatory bacteria are not harmful to human and animal cell cultures. In addition, the predatory bacteria inoculated in animal models, such as mice, rats, rabbits, guinea pigs or hens, show no toxicity (Verklova, 1973; Westergaard and Kramer, 1977; Atterbury et al., 2011; Dwidar et al., 2012; Shatzkes et al., 2016). To date, no diseases have been associated or attributed with any BALO infection (Willis et al., 2016). In fact, BALOs such as *B. bacteriovorus* carry out a highly orchestrated and targeted hydrolytic attack on prey bacteria due to type I and II secretion systems and from the protein export system: "Twin arginine translocation" (Tat) (Wang et al., 2011; Rotem et al., 2014). Moreover, the lipopolysaccharide membrane (LPS) of *B. bacteriovorus* is neutral and thus they are weakly immunogenic for humans or animals (Willis et al., 2016). In other words, there is an absence of a strong and sustained inflammatory response in the presence of predatory bacteria, unlike other bacteria, which have negatively charged LPS. Additionally, predators have coevolved alongside prey bacteria and thus encode various predatory enzymes that are difficult to counter with a simple mutation (Negus et al., 2017). Only reversible phenotypic resistance to the predation of BALOs has been previously reported (Shemesh and Jurkevitch, 2004). This resistance probably originates from the release of enzymes from the prey and predator that causes the prey to harden its cell wall or from the accumulation of predation waste that can act as a physical barrier to predator attachment (Shemesh and Jurkevitch, 2004). However, in an *in vivo* circulatory system, an accumulation of waste products that can hinder attachment is not likely to occur (Enos et al., 2017). Furthermore, most BALOs do not replicate outside of the prey and therefore they express few transport proteins and few surface epitopes for recognition by the prey's immune system (Gupta et al., 2016; Monnappa et al., 2016; Negus et al., 2017). Another advantage of their use as antimicrobial agents is that BALOs invade Gram-negative pathogens without using a receptor-based recognition system, which makes it difficult for prey to acquire any genetic resistance (Willis et al., 2016). Finally, multiple strains of BALOs exist and therefore therapies using different species of BALOs to reduce resistance are easily conceivable such as phage cocktails promoted in phage therapy (Chan et al., 2013).

7.2. Understanding pathogen predation of BALOs in vivo

To use BALOs as a biocontrol agent in the near future, on one hand it is essential to characterize if pathogenic prey exhibit predation-resistant phenotypes. On the other hand, it is also necessary to establish a list of prey for each predator since each BALO strain has a different predation spectrum. The evolutionary dynamics of the evolution of the predator genome can be modeled or monitored over the long term to ensure that the BALO strain does not become pathogenic for eukaryotic cells (Enos et al., 2017). However, for an actual application in a human or nonhuman host, it is necessary to go beyond tightly controlled prey-predator systems in conventional laboratory buffer solutions. For example, the presence of other bacterial species, which act as a disruptor, must be considered when evaluating the clinical application of BALOs against simple and mixed infections. Natural environments are very diverse and complex. Moreover, in the human body, several immunological and antimicrobial factors, such as antibodies, antimicrobial peptides and leukocytes, can act on predatory bacteria. These factors can disrupt or cause the death of bacterial predators before they begin their predation cycles. For example, Baker et al. (2017) observed that *B. bacteriovorus* predation on *K. pneumoniae* was delayed since the predators shape changed from vibroid to round when exposed to human serum. However, this form is reversible after acclimation of the predators. In addition, two solutions were offered to avoid such deformity: pre-exposing the predators to human serum or developing resistant strains to human serum by directed evolution over several generations. Additionally, Im et al. (2017) found that the albumin of the human serum inhibits predation by completely coating predatory cells, preventing *in fine* the predator from attacking its prey. These same authors reported that some antibodies in human serum recognize certain BALOs strains or their surface constituents. They also specified that the osmolality of blood serum (285 – 295 mOsm kg^{-1}) severely inhibits nontolerant BALOs strains. Fortunately, there are numerous BALOs strains that are present in a multitude of habitats, including halotolerant BALOs, such as *Halobacteriovorax*. This strain hypothetically could be considered for human serum instead of *B. bacteriovorus* (further experimentation are needed). Another problem was noticed *in vivo* where the population of pathogenic bacteria, which was initially reduced by the predation of *B. bacteriovorus,* succeeded in re-emerging after a few hours. This suggests the development of resistance by pathogens and this resumption of growth can jeopardize the use of BALOs as biotherapeutic agents (Baker et al., 2017). However, the predator-pathogen interaction has been shown to display different outcomes according to ecological conditions (Gallet et al., 2009). To explain the emergence of resistant prey, it is necessary to invoke the hypothesis stating that the presence of cellular debris from predation can disturb the course of predation. Debris can be consumed by prey for its metabolism and therefore promote a resumption of its growth. In addition,

debris that are not used by the prey and therefore remain in the environment can contribute to the emergence of a resistance phenotype (Shemesh and Jurkevitch, 2004; Baker et al., 2017). In addition, the mode of administration of the predator is essential to guarantee the success of eliminating pathogens. Indeed, Shatzkes et al. (2016, 2017) demonstrated that when predators are administered locally to a confined area, such as in rats' lungs rather than in the blood, the predators managed to reduce the pathogen load. The lack of effect in the blood could simply be explained by the inability of predators to locate their prey when directly injected into the vascular system. Alternatively, the authors assumed that the increase in pro-inflammatory cytokines, chemokines, and innate immune cells in the blood due to predatory bacteria and pathogens could eliminate predators before they could act effectively on the pathogen. Thus, therapy using predatory bacteria can be effective for infections occurring in "immune privilege" sites (sites in the body that are able to tolerate the introduction of antigens without provoking an inflammatory immune response), for example, urinary tract infections (Shatzkes et al., 2017). Other examples are listed in the supplementary information 4.

8. Conclusions

(1) Since BALOs were first isolated by Heinz Stolp, many exciting discoveries have been made. However, after 60 years of research and an increasing number of publications and citations on BALOs during the last two decades, a lot still remains to be done on this functional group of bacteria.

(2) Firstly, the few typical strains representing BALOs do not illustrate their actual diversity, which seems much greater as revealed by our recent studies using high throughput sequencing.

(3) Secondly, their role as obligate predators, although this may be of significant importance in many situations and ecosystems, has paradoxically gained low attention. The predation pressure they exert on bacterial communities deserves greater attention to better explain the distribution, dynamics and diversity of bacteria.

(4) In addition, it is possible for BALOs to be used as an ecological indicator of ecosystem status since they become more abundant as other bacteria increase and as shown by the example of wastewater. Their presence in large numbers as bdelloplasts can be related to the presence of polluting substances in the environment.

(5) At last, their capacity to eliminate pathogens and be a serious alternative to antibiotics is still underestimated while they are a potential solution for many biotechnological, veterinary and medical issues. As an example, they can be used as a probiotic or as a way

to eradicate bacteria that grow on food. This should stimulate research on BALOs, especially in more complex systems since simple cultivation or prey-predator experiments alone are no longer sufficient. Research on BALOs needs to explore the entire potential offered by their diversity since most studies have mainly focused on two species, *B. bacteriovorus* and *Halobacteriovorax*. It is necessary to unveil this unseen elephant in the room.

Box 4 Examples of successful applications

Example I

B. bacteriovorus is injected into the rhombencephalon as an *in vivo* antibacterial treatment in larvae of *Danio rerio* (zebrafish) infected with a human pathogen resistant to antibiotics, *Shigella flexneri*. The survival of the infected animals is then significantly improved and the survival rate of the zebrafish larvae increases by 35%. The immune system of zebrafish is highly homologous to humans with responses associated with neutrophils and macrophages. After injecting the predator into the larvae, the persistence time of the latter is 24 h. Indeed, *B. bacteriovorus* is engulfed by the fish's neutrophils and macrophages, which are responsible for effectively eliminating it after 24 hours even when a large dose of the predator is supplied. On the other hand and despite this relatively short persistence time, *B. bacteriovorus* is capable of effectively reducing the pathogenic load *in vivo* before being eliminated by the action of the host's immune system. In this example, a synergistic reaction can be described between leukocytes and *B. bacteriovorus* for the elimination of *Shigella* (Willis et al., 2016).

Example II

Periodontitis is a polymicrobial infectious disease. The infection is caused by many Gram-negative pathogens that are embedded in a complex biofilm referred to as the dental plaque. Periodontitis treatments, which consist of eradicating the biofilm by conventional therapies such as the use of antibiotics, are proving to be increasingly complicated and are often ineffective or not recommended. Bacteria most often develop resistance to antibiotics and antimicrobial agents have important difficulties in penetrating the dental biofilm. Indeed, bacteria embedded in biofilms are 1,000 times more resistant to antimicrobial agents than those found free in the environment. However, the local application of BALOs makes it possible to specifically reduce the rate of Gram-negative pathogens in the oral cavity. BALOs are able to penetrate deeply into the biofilm and destroy it. Therefore, it is suggested to complement classic mouth washing with a cocktail of BALOs. However, more research is required to make this therapy applicable *in vivo* (Van Essche et al., 2011).

Example III

The aquaculture of the freshwater white-legged shrimp (*Penaeus vannamei*) in China produces 300,000 tons of animals annually. The pathogens *Vibrio harparahaemolyticus* and *V. cholerae* cause generalized epidemics and are responsible for a culture loss of up to 90%. *Vibrio* infections are not always controlled by antibiotics. In addition, antibiotics are expensive and deleterious for the environment and human health. Fortunately, it has been demonstrated that *B. bacteriovorus* H16 succeeds in eradicating 10 different strains of *Vibrio*. Experiments conducted with this BALO showed a 99.9% decrease in the abundance of *V. cholerae* compared with control conditions. The

predator undeniably has a protective effect for shrimp against *Vibrio* infections and the percentage of *in vivo* survival of shrimp ranges from 47.7% to 63.3% (Cao et al., 2015).

Example IV

B. bacteriovorus SSB and SKB can be used as natural enhancers in agriculture. The two strains do not act on *Rhizobium leguminosarum*, which lives in symbiosis with the roots of legume plants and promotes their growth by nitrogen fixation. However, *B. bacteriovorus* intervenes in eliminating competitors of *R. leguminosarum* (Oyedara et al., 2016). Another example in agriculture is the use of BALOs to control soft rot diseases in potatoes caused by the bacteria *Pectobacterium* spp. and *Dickeya* spp. Predation by *B. bacteriovorus* was shown to be very effective in controlling the disease (Youdkes et al., 2020).

Example V

Biological activated sludge mainly consists of microorganisms and organic fibers associated with inorganic particles (salt and sand). The most widely used process for the biological treatment of activated sludge generates large quantities of residual sludge with a humidity of more than 95%. Incineration, composting, spreading and landfilling are the most commonly used approaches for the management of this sludge. Considering storage space, energy input, costs for transporting and handling sludge, an efficient process of dewatering and reduction of sludge volume is necessary for municipal wastewater treatment plants. Overall, to improve the dewatering of sludge, pretreatment technologies, such as ultrasonication, microwave irradiation and electrolysis, are generally applied. An alternative to these methods is the use of BALOs. Due to the high density of microorganisms in sludge and the abundance of organic matter, BALOs are naturally present. Thus, an additional supply of BALOs makes it possible to disturb the composition of biofilms and by extension, the structure of the sludge, which considerably promotes their dehydration. The biolysis process stimulated by BALOs has operational, economic, ecological and hygienic advantages (Yu et al., 2017). One last example of the application of BALOs is the use of *B. bacteriovorus* as a pretreatment for rainwater treatment by solar disinfection and solar photocatalysis disinfection (Waso et al., 2020). The combination of both drastically reduced the abundance of *Klebsiella pneumoniae*.

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Objectifs de la thèse

Le projet C-BALO a eu pour objectif de décrypter la diversité des BALOs dans différents environnements aquatiques (lacs vs mer) et de mener une étude comparative de leur structure et composition, abondances et distributions pour ces différents milieux. Un second objectif a été d'isoler et caractériser une ou plusieurs espèces de BALOs afin de tenter d'évaluer leur rôle fonctionnel (interactions biotiques préférentielles). Ce travail a été mené sur deux types de sites principaux d'échantillonnage, deux stations marines de la Baie de Banyuls sur Mer (SOLA et MOLA) et surtout deux grands lacs profonds péri-alpins (les lacs Léman et d'Annecy).

Au cours de cette thèse plusieurs questions ont été posées :

Question 1 : Quelle est la structure et la diversité de la communauté des BALOs au sein des écosystèmes aquatiques ?

Il s'est agi de caractériser les différents groupes de BALOs présents au sein de différents types d'écosystèmes et leur répartition/distribution en fonction des conditions de l'environnement. Des approches de type clonage-séquençage (pour avoir une idée simple de la structuration des communautés) et de séquençage à haut débit (Illumina) ont été utilisées pour répondre à cette première question. Dans ce but, des amorces ciblant l'ADN codant l'ARN 16S des différentes familles de BALOs ont été testées, dessinées et optimisées au sein du plateau de biologie moléculaire du CARRTEL, montrant ainsi pour la première fois l'existence de diverses familles de BALOs en milieu lacustre alpin et en milieu méditerranéen (Baie de Banyuls).

Question 2 : Quelle est l'importance quantitative des BALOs au sein des écosystèmes ?

Il est possible de quantifier l'ensemble des BALOs, comparer leurs abondances et comprendre comment elles se répartissent, au sein de la colonne d'eau, que ce soit en milieu lacustre ou marin. Pour répondre à cette question, l'approche utilisée a été la PCR quantitative. Des amorces spécifiques pour chaque famille ont été testées, dessinées et optimisées.

Question 3 : Peut-on isoler et caractériser des BALOs d'intérêt ?

L'isolement et la caractérisation de BALO à partir de différentes proies permettraient d'obtenir des cultures utiles pour travailler plus finement sur les interactions proie-prédateur et tester par exemple le spectre de prédation de ces BALOs. L'isolement des BALO a été effectué sur boites de Pétri en employant la technique de l'agar double qui permet d'isoler des prédateurs sur des

proies spécifiques. En fonction des échantillons, une étape préliminaire d'enrichissement par diverses proies a été incluse. Le clonage-séquençage du génome des souches isolées a permis de confirmer l'identité de BALOs.

Question 4 : Quelles sont les conditions biotiques et abiotiques, qui peuvent influencer les BALOs ?

En plus des suivis *in situ*, des expériences en mésocosmes ont été réalisées en testant des conditions mimant des changements climatiques (événement extrême avec fort brassage de la colonne d'eau, diminution de l'intensité luminueuse, apports terrigènes par lessivage du bassin versant). Ces expériences ont eu pour objectif de démontrer si les facteurs abiotiques influencent d'avantage les BALOs plutôt que les facteurs biotiques. Les populations de BALOs en fonction des diverses conditions ont été suivies par qPCR. L'énumération et l'analyse de la diversité des différents BALOs en mésocosmes a permis ainsi de mieux comprendre la part des facteurs abiotiques sur la diversité et l'abondance des BALOs.

Chapitre II

Les BALOs sont-ils présents et diversifiés dans les grands lacs péri-alpins ?

Chapitre 2 : Les BALOs sont-ils présents et diversifiés dans les grands lacs péri-alpins ?

Contenu du chapitre

Ce chapitre est composé de deux articles scientifiques qui visent à détecter et explorer la présence, la diversité et l'abondance des BALOs dans les lacs péri-alpins par l'emploi d'amorces nucléotidiques spécifiques et universelles.

Objectifs et résumé de l'article 3

Le premier travail a consisté à vérifier la possibilité de détecter les principales familles de BALOs du groupe des Oligoflexia (*Bdellovibrionaceae*, *Bacteriovoracaceae* et *Peredibacteraceae*) au sein de 3 grands lacs péri-alpins (les lacs Léman, d'Annecy et du Bourget). Avant ce travail, aucune connaissance n'était alors disponible pour les milieux naturels lacustres. Dans ce but, toutes les amorces nucléotidiques disponibles connues pour détecter ces bactéries ont été testées et optimisées dans nos conditions de laboratoire. Puis, le travail de détection et de quantification des BALOs a été effectuée sur une série d'échantillons obtenus à une fréquence mensuelle d'août 2015 à janvier 2016 à différentes profondeurs de la colonne d'eau, entre 2 et 3 m et plus en profondeur, entre 45 et 50 m. Les techniques et outils employés pour cette étude ont été la PCR-DGGE, le clonage séquençage, la PCR quantitative, la phylogénie et des méthodes statistiques. Autre originalité de cette étude, le développement d'une amorce nucléotidique permettant de cibler la famille des *Peredibacteraceae* par PCR quantitative a aussi été effectué. Ainsi en utilisant ce nouveau « primer » et ceux ciblant les autres familles proposées par la bibliographie, nous avons pu montrer que les *Peredibacteraceae* constituent la famille de BALOs la plus abondante, atteignant jusqu'à 7% de l'abondance bactérienne totale en été, et que ces représentants étaient majoritairement distribués en surface. Comparativement, les familles *Bdellovibrionaceae* et *Bacteriovoracaceae* étaient surtout détectées plus en profondeur, et avec des abondances nettement plus faibles. D'autre part, cette étude a révélé que la diversité des BALOs semblait assez limitée avec seulement quelques génotypes détectés, une information somme-toute à considérer avec prudence car obtenue par une méthode d'empreinte moléculaire et donc limitée. Enfin, l'analyse phylogénétique concluait sur le fait que les BALOs des lacs sont phylogénétiquement proches de leurs congénères retrouvés dans les bases de données.

Diversity, dynamics and distribution of *Bdellovibrio* and like organisms in perialpine lakes

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Diversity, Dynamics, and Distribution of *Bdellovibrio* and Like Organisms in Perialpine Lakes

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1. Introduction

Over the last few years, studies on western European large and deep peri-alpines lakes have revealed that these ecosystems harbor a very diverse and dynamic auto- and heterotrophic prokaryotic community (Comte et al., 2006; Debroas et al., 2009; Personnic et al., 2009a; Berdjeb et al., 2011b, 2013; Domaizon et al., 2013; Parvathi et al., 2014). These studies and others have also highlighted that both biotic and abiotic factors are likely to regulate these communities. Among these factors, inorganic nutrients, viruses, nanoflagellates, and other heterotrophic grazers (including ciliates and/or metazooplankton) have been identified as critical players in the dynamics of the abundance, the community composition, or structure patterns (Domaizon et al., 2003; Comte et al., 2006; Personnic et al., 2009b; Berdjeb et al., 2011c, 2013; Thomas et al., 2011; Perga et al., 2013). Clearly, viral lysis and nanoflagellate or ciliate grazing have been observed as important biotic factors involved in bacterial mortality, affecting their abundance with a rate ranging from 10 to 60 % of bacterial loss per day, but also in regulating their (community) structure and/or diversity (Jacquet et al., 2005; Sime-Ngando et al., 2008; Thomas et al., 2011; Meunier and Jacquet, 2015).

Other types of biotic interactions are known to exist but have currently been poorly investigated. They include the interactions between micro- and macroorganisms, the interactions between bacteria and other organisms or the role of eukaryotic pathogens (e.g., fungi) which still remain scarce (Humbert et al., 2009; Mangot et al., 2011; Perga et al., 2013). Another type of biotic interaction that has been largely neglected in aquatic ecosystems is the bacterial predation by other bacteria. To the best of our knowledge, the diversity, abundance, dynamics and functional role of these groups of predators (sometimes also referred to as parasitoids) have never been investigated in alpine lakes so far. Among these predatory bacteria that can belong to several phyla, a "group" is of particular interest towards which this study was directed, i.e. the *Bdellovibrio* and like organisms (BALOs).

BALOs are small bacteria (ranging in size between 0.2- 0.5 µm to 0.5-2.5 µm; (Rendulic et al., 2004)), very motile (moving at up to 160 µm/s; (Chauhan et al., 2009a)) and Gram-negative. To ensure their survival, they hunt for other bacteria, typically Gram-negative cells, making them specific obligate predators. It is noteworthy, however, that recent studies revealed that BALOs can also prey on Gram-positive bacteria (Iebba et al., 2014; Pantanella et al., 2018) when they have enough time to "adapt" to such new types of prey. It seems that the adaptation time is related to the synthesis of necessary enzymes, which grant the predator the capacity to degrade the Gram-positive cell wall. Furthermore, BALOs are ubiquitous and widely distributed in different ecosystems like salt waters, fresh waters, sewage, soils, sediments and they have also been isolated from different animals such as mammals including human guts and feces (Schwudke et al., 2001; Van Essche et

al., 2011; Rotem et al., 2015; Oyedara et al., 2016). So far, their abundance and taxonomic diversity across these various habitats have been unexplored or at least underestimated, largely because of the use of culturing approaches. The use of culture-independent methods, for instance metagenomics, has indeed confirmed that the diversity of cultivated BALOs represents only a small fraction of their diversity (Davidov et al., 2006a).

Bdellovibrio and like organisms are a polyphyletic group and can be found within two different classes: the α-proteobacteria with the genus *Micavibrio* and the Oligoflexia (formerly classified in the δ-proteobacteria) that includes five families: the *Bdellovibrionaceae*, the *Peredibacteraceae*, the *Bacteriovoracaceae*, the *Pseudobacteriovoracaceae* and the *Halobacteriovoraceae* (Koval et al., 2015; McCauley et al., 2015; Rotema et al., 2015; Hahn et al., 2017). The actual BALOs classification is primarily based on four criteria: 1) the16S rRNA gene sequence (Davidov and Jurkevitch, 2004); 2) the sequence of the gene encoding for the β-subunit of bacterial RNA polymerase (*rpoB*) (Pineiro et al., 2004); 3) the percentage of GC and 4) the sodium chloride requirement for growth. Following this, species and/or strain types have been proposed to represent each family. For instance, *Bdellovibrio bacteriovorus* HD100 and *Bdellovibrio exovorus* JSS depict the *Bdellovibrionaceae* family. For the *Peredibacteraceae* it is *Peredibacter starrii* A3.12. For the *Bacteriovoracaceae*, *Bacteriovorax stolpii* Uki2 is the type strain. Finally, *Halobacteriovoraceae* is represented by two type species, exclusively found in salty ecosystems: *Halobacteriovorax marinus* SJ and *Halobacteriovorax litoralis* JS5 (Koval et al., 2015). Comparatively, the genus *Micavibrio* may only represent a minor group within BALOs and is most often represented by *M. admirantus* or *M. aeroginovorus*, which are both epibiotic predators (Rotema et al., 2015).

BALOs have been reported to play an essential role in bacterial ecology by shaping the bacterial community (Shemesh and Jurkevitch, 2004). The general assumption states that BALOs act most likely as an ecological balancer in their environment (Iebba et al., 2014; Oyedara et al., 2016). BALOs are organized in distinct populations under seasonal and spatial segregation; therefore, their action may be continuously modified (Kandel et al., 2014). The understanding of the ecology of this bacterial community remains largely unknown in many aquatic environments, especially natural systems such as large and deep lakes, for which there is almost no data available. Interestingly, a few years ago, the work of Roux et al. (2012) in Lake Bourget, followed by the study of Zhong et al. (2015) for Lakes Annecy and Bourget (France), showed that there is a significant single-stranded DNA virus community in these lakes, the *Microviridae*, which are abundant and diverse. This community displays a boom-bust dynamics, but the correlation between the abundance of these viruses and the abundance of total heterotrophic bacteria remained challenging to establish (Zhong et al., 2015). However, some viruses within the *Microviridae* are

known to infect BALOs such as *B. bacteriovorus* (Brentlinger et al., 2002). Thus, the presence of a relatively abundant and diverse community of ssDNA viruses in peri-alpine lakes could suggest that there is an abundant and diverse community of cellular hosts, including the BALOs. If so, these bacteria, by their potential trophic interactions with other populations of bacteria, could play a significant role in the functioning of the microbial compartment (Pérez et al., 2016; Williams et al., 2016). This is the reason why we decided to examine the existence, throughout the abundance, distribution and diversity, of these bacterial predators in peri-alpine lakes.

Thus, the objective of this pioneering work dealing with freshwater BALOs was to reveal the existence of these bacteria in typical and representative peri-alpine Lakes (Annecy, Bourget, and Geneva) and to address the following questions: (i) Can BALOs be readily detected in these ecosystems? (ii) What are the structure and diversity of the BALOs? (iii) What is the quantitative importance of the leading groups among this community of predatory bacteria? (iv) What are the relationships between the population of the BALOs and heterotrophic bacteria? (v) What environmental factors appear to be important in the regulation of these interactions?

2. Results

2.1.Primer selection

Among the 12 primer sets tested by PCR or qPCR and checked using cloning-sequencing, we chose one primer set for the phylogenetic analysis and another one for qPCR analysis, for each BALO family (Tables 1 and S1). All selected primers were highly specific since all sequences obtained were characterized by more than 96 % identity with different cultured or uncultured bacteria of BALOs family found in databases (not shown). Between the two primers that we designed to quantify 16S rDNA sequence of the *Peredibactereceae* family by qPCR, the couple Per699F (CTGCCTGGACGACTATTGAC) - Per974R (CGGGTTCGTAGGAGTTCAAG) was the best.

Table 1 Existing and selected primers used in this study[a]

Primer set name	Primer name	Direction	Sequence (5′–3′)	Amplicon size (bp)	Type of analysis	Targeted group	Reference or source
Couple Bde1	BbeF216 BbeR707	Forward Reverse	TTTCGCTCTAAGATGAGTCCGCGT TTCGCCTCCGGTATTCCTGTTGAT	492		*Bdellovibrionaceae*	Van Essche (73)
Couple Bde2	**Bde347F** **Bde549R**	**Forward** **Reverse**	**GGAGGCAGCAGTAGGGAATA** **GCTAGGATCCCTCGTCTTACC**	203	qPCR	*Bdellovibrionaceae*	
Couple Bde3	**Bde529F** **Bde1007R**	**Forward** **Reverse**	**GGTAAGACGAGGGATCCT** **TCTTCCAGTACATGTCAAG**	479	PCR-DGGE & cloning-sequencing	*Bdellovibrionaceae*	Davidov et al. (26)
Couple Bx4	**BxF519** **BxR677**	**Forward** **Reverse**	**CAGCAGCCGCGGTAATAC** **CGGATTTTACCCCTACATGC**	159	qPCR	*Bacteriovoracaceae*	Zheng et al. (74)
Couple Bx5	Bx1442R Bx676F	Reverse Forward	GCCACGGCTTCAGGTAAG ATTTCGCATGTAGGGGTA	767		*Bacteriovoracaceae*	Davidov et al. (26)
Couple Per6	**Per676F** **Per1443R**	**Forward** **Reverse**	**ATTTCACGTGTAGGGGTA** **AGTCACGTCTTAAAATGAAA**	768	PCR-DGGE & cloning-sequencing	*Peredibacteraceae*	
Couple Bct8	**63F** **1378R**	**Forward** **Reverse**	**CAGGCCTAACACATGCAAGTC** **CGGTGTGTACAAGGCCCGGGAACG**	1,316	Nested PCR	*Bacteria* *Bacteria*	Marchesi et al. (75) Heuer et al. (76)
Couple Bct7	**519F** **907R**	**Forward** **Reverse**	**CAGCMGCCGCGGTAATWC** **CCGTCAATTCMTTTRAGTTT**	389	qPCR	*Bacteria* *Bacteria*	Kandel et al. (33)
Couple Bde9	Bd824F Bd1222R	Forward Reverse	ACTTGTTGTTGGAGGTAT TTGTAGCACGTGTGTAG	399		*Bdellovibrionaceae*	Pasternak et al. (77)
Couple Bx10	**Bx341F** **Bx672R**	**Forward** **Reverse**	**CTACGGGAGGCAGCAG** **TACCCCTACATGCGAAATTCC**	332	PCR-DGGE & cloning-sequencing	*Bacteriovoracaceae*	
Couple Per11	**Per699F** **Per974R**	**Forward** **Reverse**	**CTGCCTGGACGACTATTGAC** **CGGGTTCGTAGGAGTTCAAG**	276	qPCR	*Peredibacteraceae*	This study
Couple Per12	Per521F Per754R	Forward Reverse	GAAACTGCGTCTGAAACTGC GCGTCACTGAAGGAGTCAAT	234		*Peredibacteraceae*	

[a]Bold type corresponds to the primers used in this study.

2.2. Abundances and distribution of the BALOs

In the analysis of the absolute abundance of the different BALO families (in copies per mL, measured by qPCR) disregarding the month and the depth, the most represented family of BALOs in the three lakes corresponded to the *Peredibacteraceae*, with an abundance reaching up to 1.62 x 10^5 gene copies per mL. In contrast, *Bdellovibrionaceae* and *Bacteriovoracaceae* were on average 10,000 times lower in abundance than the *Peredibacteraceae*, with maximum concentrations reaching 4 and 1.25 x 10^1 copies per ml, respectively. Compared to total bacteria also quantified using qPCR or FCM, the *Peredibacteraceae* represented up to 7.12 % of total bacteria while *Bacteriovoracaceae* and *Bdellovibrionaceae* accounted for less than 0.05 % of the bacterial community. The highest concentrations were always recorded in the free-living bacterial fraction. No evident seasonal variations were recorded here. When discriminating the three families at the two distinct depths, i.e., the surface (2 m, 2.5 m or 3 m depending on the lake) vs. deeper waters (45 m or 50 m depending on the lake), (i) *Peredibacteraceae* were the most abundant (with 100 to 10000 times more copies per mL than *Bdellovibrionaceae* and *Bacteriovoracaceae*), (ii)

Peredibacteraceae were generally more abundant at the surface compared to depth. On the other hand, we observed an opposite trend for *Bdellovibrionaceae* and *Bacteriovoracaceae*, in particular for Lake Bourget (Fig. 1).

2.3. Relationships between BALOs, total bacteria, and other environmental data

Using the relative abundance of BALOs families and environmental data obtained from the *in situ* surveys of peri-alpine lakes (e.g., http://www6.inra.fr/soere-ola/), a CCA was conducted to assess the relationships between BALOs and their biotic and abiotic environment (Fig. 2). The first two axes of the CCA (CCA1 and CCA2) explained 53.1% of the total variance. *Bdellovibrionaceae* displayed clear links with conductivity ($p<0.05$), and ammonium concentration ($p<0.05$), whereas *Peredibacteraceae* displayed clear links with pH ($p<0.05$), dissolved oxygen ($p<0.05$) and temperature ($p<0.05$). Comparatively to the two other families, no significant relationships were found for the *Bacteriovoracaceae* with any of the environmental factors tested here. Moreover, the two distinct water layers, i.e., the surface (<3 m depending on the lake) vs. deeper waters (>45 m depending on the lake), could be separated (Fig. 2). The analysis suggested that *Bacteriovoracaceae* are more abundant in deep-waters ($p<0.05$) and driven by ecological factors specific to this part of the water column. By contrast, *Peredibacteraceae* were more abundant in near-surface waters ($p<0.05$) and driven by environmental factors more specific to this layer (such as dissolved O_2, chlorophyll *a*, and higher temperature). As for *Bdellovibrionaceae*, the repartition seemed to be less specific to the surface *vs*. the deeper waters ($p > 0.05$).

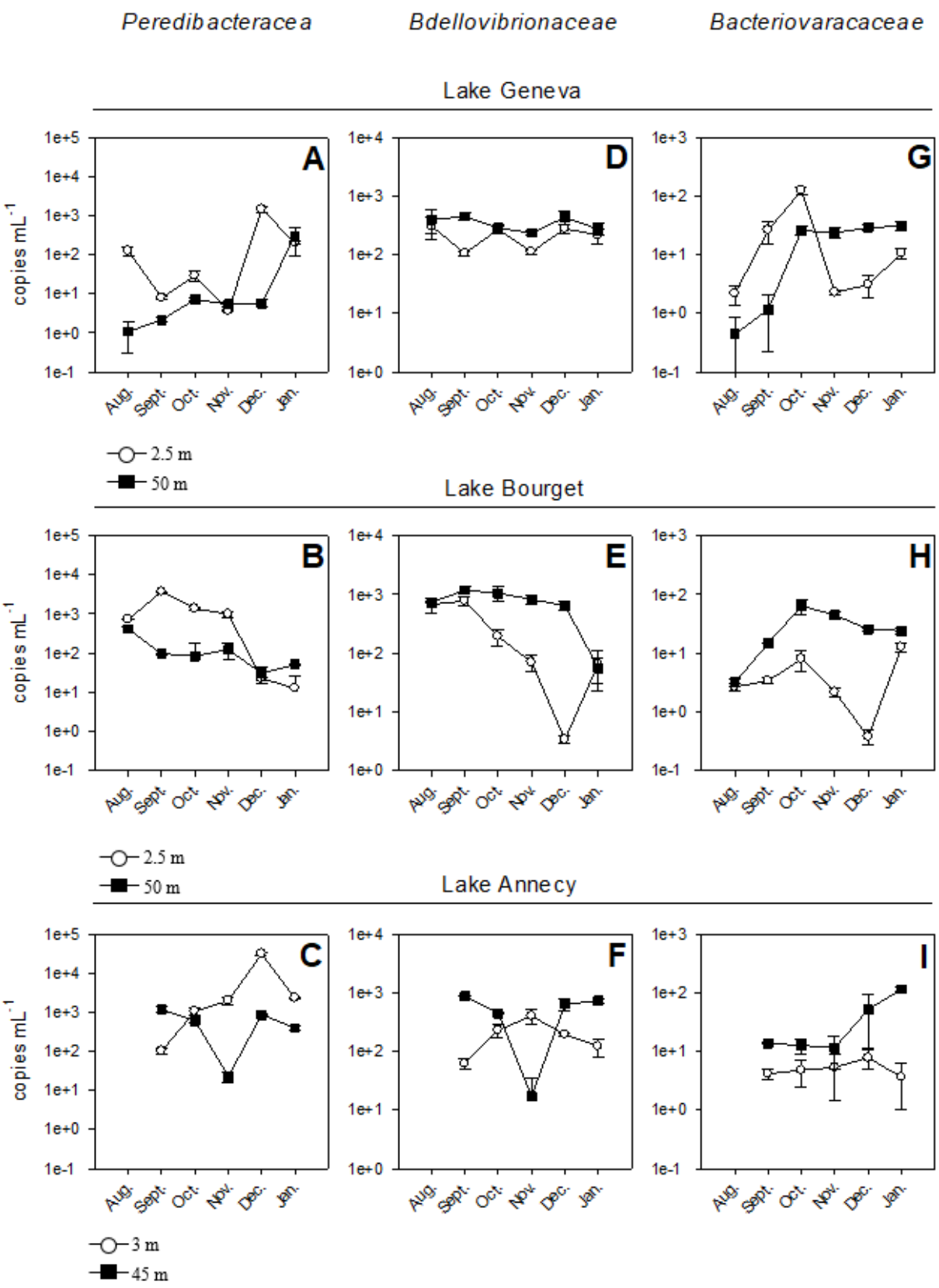


Figure 1 Dynamics of abundance for the different BALOs obtained at two contrasting depths in the three lakes. Each sample was analyzed in duplicates. White circles correspond to surface water (2 m for Lake Bourget, 2.5 m for Lake Geneva, and 3 m for Lake Annecy), whereas dark squares correspond to deep-water (45 m for Lake Annecy and 50 m for Lakes Bourget and Geneva).

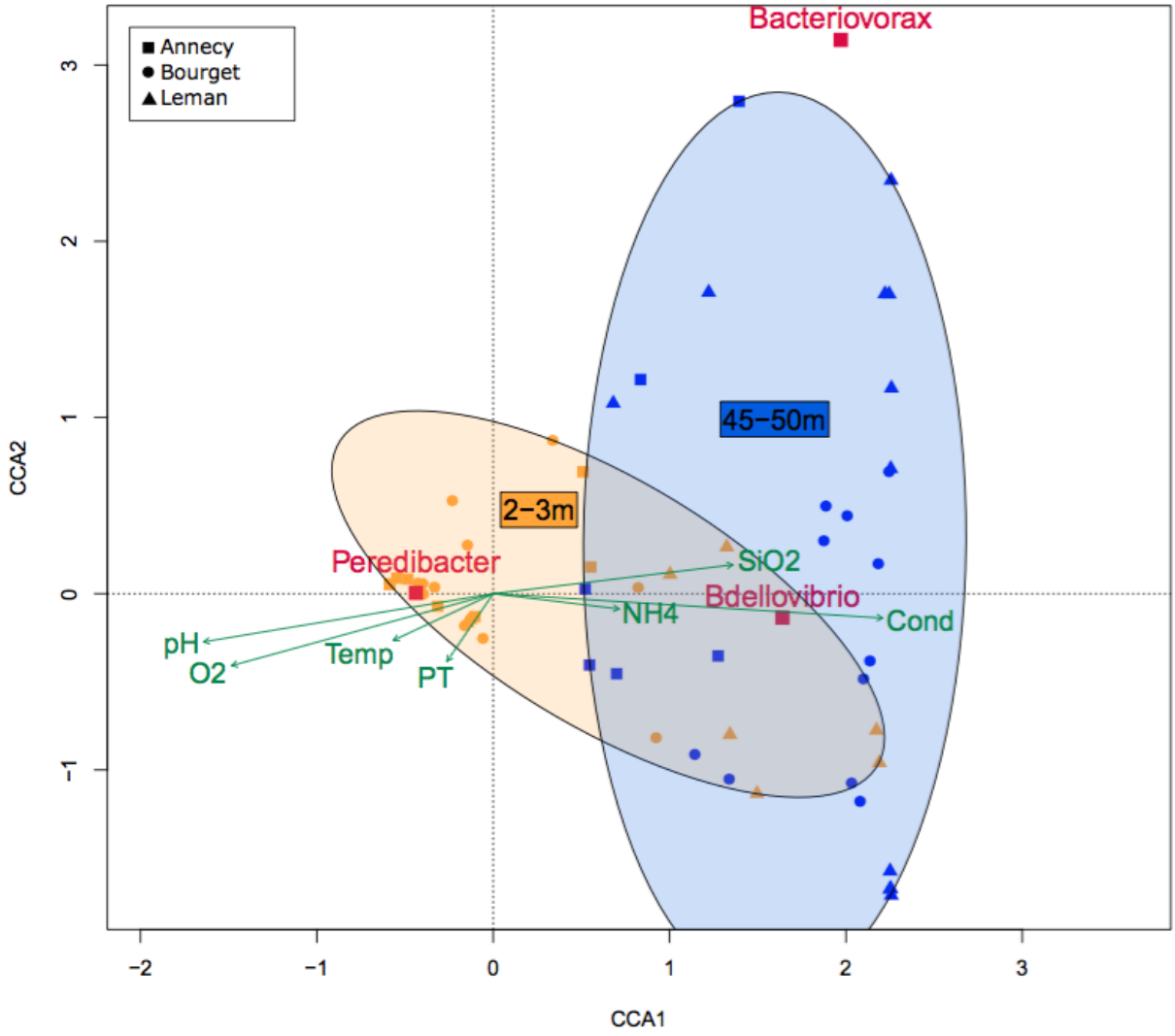


Figure 2 Canonical Correspondence Analysis (CCA) showing the distribution of relative abundance of each group of BALOs (*Bacteriovoracaceae*, *Bdellovibrionaceae*, *Peredibacteaceae*) quantified by qPCR, according to ecological variables measured during samplings. (Temp = Temperature, PT = Total phosphorus NH4 = ammonium, SiO2 = silicate, Cond = Conductivity, O2 = dissolved oxygen)

2.4. Genetic structure

The DGGE analysis revealed only a limited number of bands whatever the BALOs family considered and no seasonal patterns were recorded. Only 1 to 5 major bands could be detected, suggesting a low genotypic diversity. A maximum of three bands was detected for *Bacteriovoracaceae*. One major band with three minor (regarding intensity) bands was observed for *Bdellovibrionaceae*. Two bands were generally observed for *Peredibacteraceae* (see Fig. S1 in the supplemental material).

2.5. Diversity

A cloning-sequencing approach was chosen as a first attempt to assess the genetic diversity of the BALOs. Phylogenetic trees were constructed from 30 sequences based on the 16S rRNA gene of each BALOs family, arising from all the studied lakes. After cleaning, blasting, chimera checking, clustering and aligning, we obtained 16 centroid sequences for the *Bdellovibrionaceae* family, 6 centroid sequences for the *Bacteriovoracaceae* and 8 centroid sequences for the *Peredibacteraceae*. Our results clearly show (in agreement with the DGGE results) that the diversity of each BALO family was relatively low. The phylogenetic tree of the *Bdellovibrionaceae* (Fig. 3) reveals the presence of two distinct clusters. One is related to *Bdellovibrio exovorus* JSS (6 centroid sequences), and the other corresponds to *Bdellovibrio bacteriovorus* and its substrains (10 centroid sequences). For the *Bacteriovoracaceae* also (Fig. 4) two clusters emerged. Our sequences are related to the species *Bacteriovorax stolpii*, but they may constitute two other species. The tree suggests that our sequences fall into two distinct strains with two centroid sequences forming one species and 4 centroid sequences forming the other. For the *Peredibacteraceae* sequences (Fig. 5), one single cluster emerged. All our sequences looked to be closely related to *Peredibacter starrii*. These trees suggest that peri-alpine lakes hold the usual BALOs members found in other ecosystems with maybe some new species when considering *Bacteriovorax*.

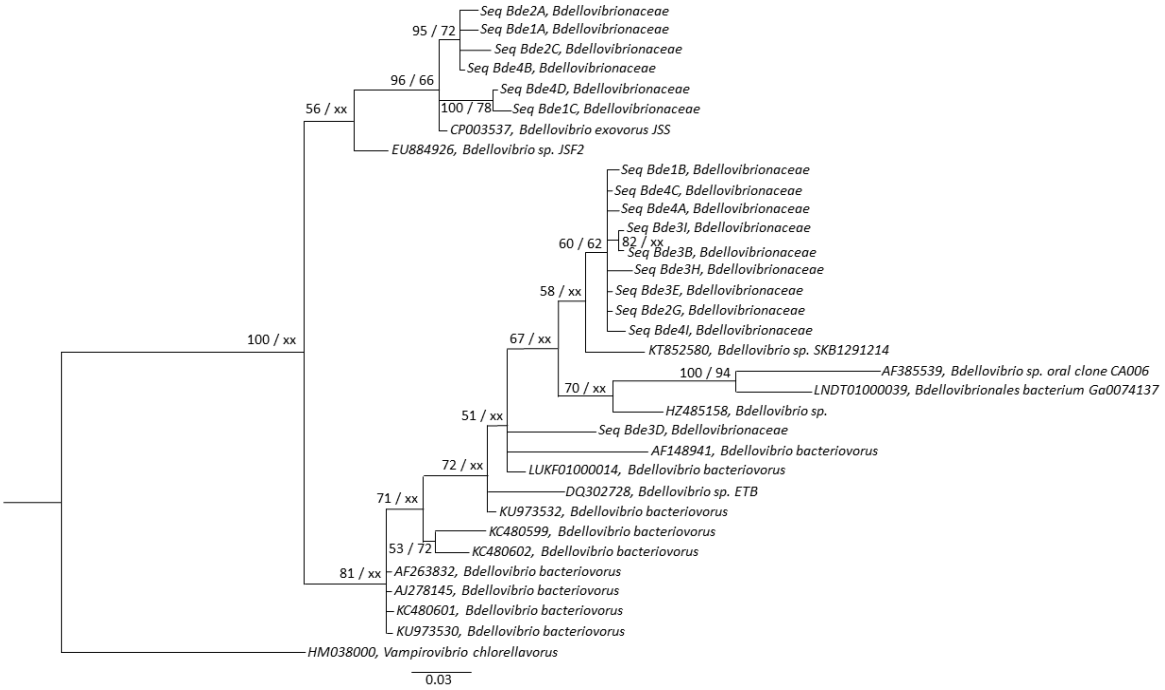


Figure 3 Phylogenetic analysis of 16 centroid sequences of *Bdellovibrionaceae* from Lakes Annecy, Bourget and Geneva based on 16S rDNA Sanger sequencing obtained after curation and clustering, along with 16 other sequences retrieved from arb-SILVA including two type species, *c.a. Bdellovibrio bacteriovorus* and *Bdellovibrio exovorus*. All sequences were aligned using "MUSCLE" via MEGA6. The alignment was trimmed at both ends to eliminate gaps, and then curated with Gblocks resulting in 241 positions from 245 positions. Best-fit model of nucleotide substitution was selected using Jmodeltest-2.1.1. through Akaike model selection strategy, resulting in TIM1 + I + G model. Phylogenetic tree was constructed by the Maximum Likelihood method using PhyML-3.1 and Bayesian inference (GTR + I + G) was conducted using MrBayes 3.2.6 with 5 million generation and a burn-in value of 25%. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). Boostraps below 50 were deleted. *Vampirovibrio chlorellavorus* was used as an outgroup to root the *Bdellovibrionaceae* tree.

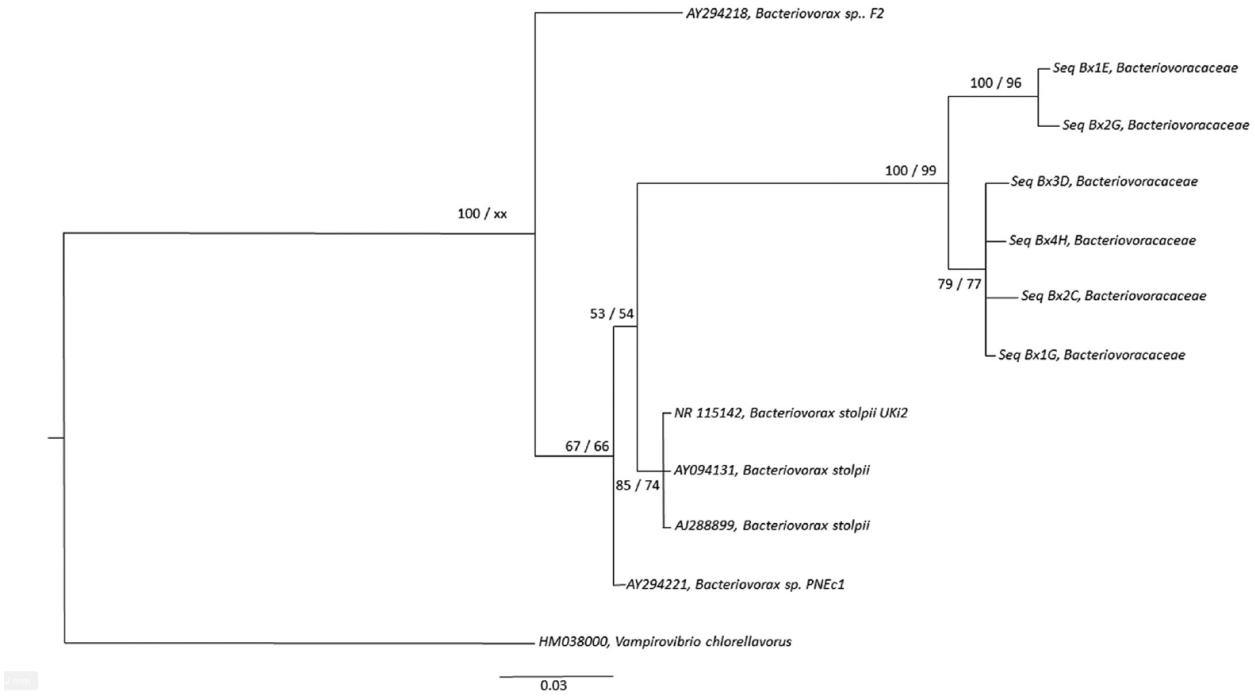


Figure 4 Phylogenetic tree of 16S rRNA *Bacteriovoracaceae* lineage. The tree is based on Maximum Likelihood analysis using PhyML-3.1 and Bayesian inference (GTR + G) analysis using MrBayes 3.2.6 with 2 million generation and a burn-in value of 25%. The tree encompasses 6 centroid sequences of *Bacteriovoracaceae* from Lakes Annecy, Bourget and Geneva based on Sanger sequencing obtained after curation and clustering, along with 5 other sequences retrieved from arb-SILVA including one type species, *c.a.Bacteriovorax stolpii*. All sequences were aligned using "MUSCLE" via MEGA6. The alignment was trimmed at both ends to eliminate gaps, and then curated with Gblocks resulting in 262 positions from 292 positions. Best-fit model of nucleotide substitution was selected using Jmodeltest-2.1.1. through Akaike model selection strategy, resulting in GTR+G model. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). Boostraps below 50 were deleted. *Vampirovibrio chlorellavorus* was used as an outgroup.

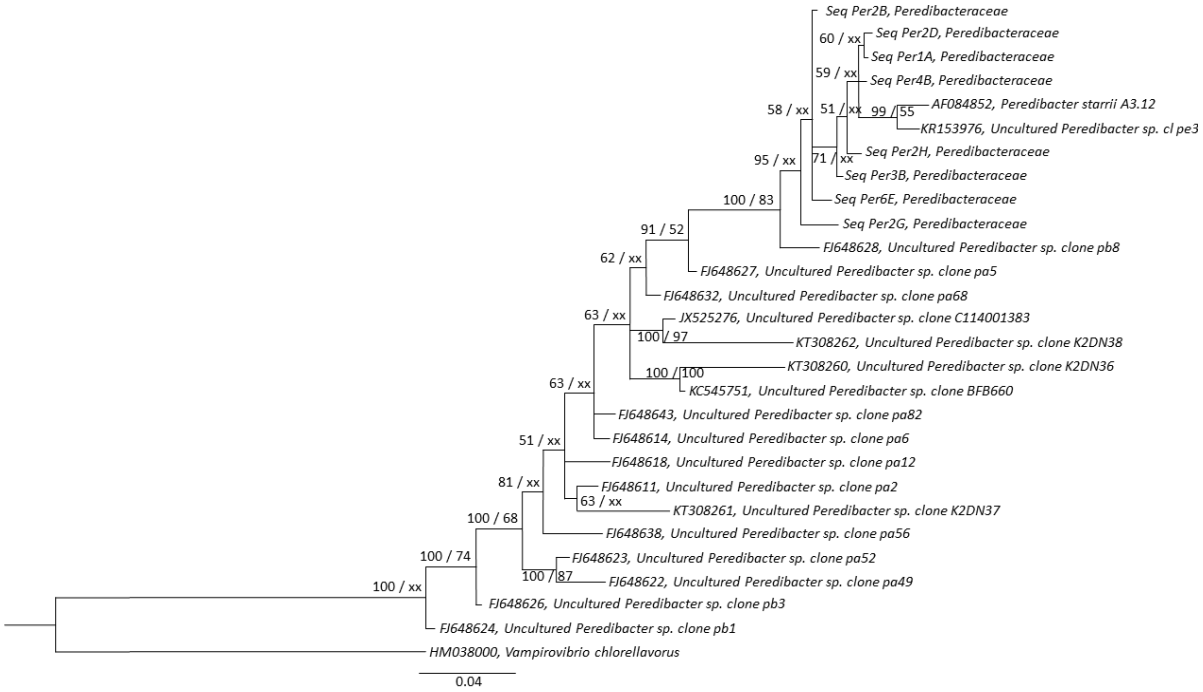


Figure 5 Bayesian tree generated from 16S rDNA dataset of Lake Annecy, Bourget and Geneva with 8 curated and clustered *Peredibacteraceae* centroid sequences originating from Sanger sequencing, along with 19 other sequences retrieved from NCBI (GenBank) including one type species, *c.a. Peredibacter starrii*. These sequences were aligned using "MUSCLE" via MEGA6 software and the alignment was curated using Gblocks, resulting in 544 positions from 570 positions. Bayesian tree was built using MrBayes 3.2.6 with 5 million generation and a burn in value of 25% using GTR + I + G model. As for the ML tree, PhyML-3.1 was used based on Jmodeltest-2.1.1 best substitution model i.e,.: TrN+ I+G. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). Boostraps below 50 were deleted. *Vampirovibrio chlorellavorus* was used as an outgroup to root the *Peredibacteraceae* tree.

3. Discussion

The present study aimed at investigating the existence of some predatory bacteria referred to as BALOs in three large and deep French and Western European alpine lakes. Since this is the first time the existence and the quantitative importance of BALOs were studied in such lake ecosystems, different methods were tested and optimized to assess their diversity and abundance. First of all, we designed a new specific qPCR primer set to target the *Peredibacteraceae*, since no primer existed yet for this recently discovered freshwater BALO family. Then, we quantified and assessed the diversity of three representative groups of BALOs, the *Peredibacteraceae*, the *Bdellovibrionaceae*, and the *Bacteriovoracaceae*, and compared these results to others obtained from a variety of environments. Even if our study revealed unambiguously the presence of BALOs

in large peri-alpine lakes, we are well aware of the limits associated to some of the methodologies chosen and used here (i.e. the DGGE and the cloning sequencing approach), as well as the number of analyzed samples (i.e. only a few depths and a few months). In addition, the primers we used were not degenerate. Therefore, our approach may be too stringent to recover a higher BALOs diversity. *In fine*, this study serves as a pioneering analysis revealing a part of the diversity, distribution, and dynamics of the BALO bacterial community in some freshwater ecosystems.

3.1. BALOs' probable role and diversity in peri-alpine lakes

BALOs were found in each lake and at each depth investigated, whatever the period of the year sampled. While this study did not assess the role of BALOs in the microbial loop and lake functioning, it is already known from other studies that these bacteria are likely to be important bio-agents of mortality (Williams et al., 2016). It is noteworthy however that very few studies have focused on the role and effect of such predatory bacteria on the bacterial community of natural or man-made environments, and, the understanding of bacterial mortality has been mainly and mostly based on the study of viruses and protists so far (Kandel et al., 2014). Unlike viruses and protists, BALOs predation is not dependent on the physiology or the size of the prey (Chauhan et al., 2009a). Additionally, BALOs are ubiquitous in nature (Williams et al., 2016). Thus, predation by BALOs adds a new dimension to the recycling of organic matter through the microbial loop. Both viruses and BALOs recycle nutrients via the microbial loop, however the recycling mechanisms are different. Viral lysis results in the release of the entire intracellular contents of the prey into the environment, while BALOs consume most of the prey content, hence releasing few nutrients in the environment. Saying that, BALOs yield a higher energetic value since they are filled with nutrients, therefore, when other organisms graze on them, the nutrient uptake efficiency is higher (Williams et al., 2016). Regarding their diversity, BALOs form highly heterogeneous groups with a large phylogenetic diversity (Davidov et al., 2006a). We managed to detect in peri-alpine lakes the usual BALOs already found in the current bibliography, although we used a fingerprinting approach for which many biases are associated. We are aware indeed that DGGE bands only reflect the microorganism populations found at relatively high concentration. Additionally, bands can co-migrate in the DGGE gel, thereby the numbers of bands can be over- or underestimated (Berdjeb et al., 2011c). Definitely, a high throughput sequencing approach will reveal better the hidden diversity of these BALOs. Therefore, highly specific primers for each BALOs families should be designed with a fair amount of degeneracy in order to limit non-target region binding but at the same time to maximize taxa detection (Elbrecht et al., 2018). In our study, we used non-degenerate primers for the PCR-DGGE and a cloning sequence approach, therefore we might have missed

some BALOs. Another very important point that we should emphasize about is the taxonomy assignment of BALOs present in 16S databases. Since early 2000, BALOs taxonomy has changed. Baer et al. (2000) reclassified *Bdellovibrio stolpii* and *Bdellovibrio starrii* into a new genus, *Bacteriovorax*. Then Davidov and Jurkevitch (2004), reclassified *Bacteriovorax starrii* as *Peredibacter starrii*, hence creating a new family, the *Peredibacteraceae* family. At the same time Baer et al. (2004) proposed to reclassify salt-water *Bdellovibrio sp.* as *Bacteriovorax marinus* and *Bacteriovorax litoralis*. At last, Koval et al. (2015) redirected salt water BALOs into a new genus *Halobacteriovax*, creating a new family, the *Halobacteriovoraceae*. As a result, these adjustments have caused a few confusions in 16S rRNA databases. Typically, when working with arb-SILVA SSUParc release number 132 (Quast et al., 2013) we encountered *Peredibacter* and *Halobacteriovorax* grouped in the *Bacteriovoracaceae.* Furthermore, some sequences were assigned to *Bdellovibrio sp.* At the beginning of the discovery of BALOs, any found sequence was cataloged under the *Bdellovibrionaceae* family. Lately, some efforts were made to assign correctly these sequences but there is much work to be done. Today again, one cannot determine whether some sequences belong to *Bdellovibrio, Bacteriovorax, Peredibacter,* or *Halobacteriovorax*.

3.2. Peredibacteraceae are the most abundant BALOs family in peri-alpine lakes

The *Bdellovibrionaceae* displayed little diversity in peri-alpine lakes. This result is in agreement with the study of Li and Williams (Li and Williams, 2015) who also reported that the population structure of the *Bdellovibrionaceae* differed from a lake to another. The *Bacteriovoracaceae* seem to be more diverse in saltwater than in freshwater (Davidov and Jurkevitch, 2004)**.** While the *Peredibacteraceae* may be not a much-diversified family according to our study, these bacteria were found in higher concentrations compared to both the *Bdellovibrionaceae* and *Bacteriovoracaceae*. Indeed, the *Peredibacteraceae* isolated from freshwater and soil and described by Pineiro et al. (2004), constituted the most abundant family for all the conditions studied (within the three lakes, depths, different fractions and different sampling periods). This first result suggests that the *Peredibacteraceae* are well adapted to peri-alpine lake ecosystems, either by being a generalist or a versatile hunter regarding the heterotrophic bacteria present or by preying on bigger preys, thus growing faster and making more descendants. The number of preys present in the environment and the differential use of these preys (Kandel et al., 2014) affect the abundance of one population to another. Environmental factors such as temperature and salinity can also affect the distribution and abundance of BALOs families, and here the *Peredibacteraceae* were more correlated to temperature than the *Bdellovibrionaceae* or the *Bacteriovoracaceae*. In addition, it is

known that the presence of a variety of predators such as protists (i.e. the nanoflagellates or the ciliates), metazooplankton and bacteriophages can affect the survival and the growth of bacteria within the ecosystem. In fact, these microorganisms can play a significant role in controlling bacterial populations (Pantanella et al., 2018).

3.3. Low abundance of BALOs may not be indicative of a weak functional role

The study of the abundance of the *Bdellovibrionaceae* and the *Bacteriovoracaceae* in aquaculture systems reported concentrations between 10^3 and 10^6 cells per mL (Kandel et al., 2014). These results combined with our findings could suggest that these two families have a low impact on the community of heterotrophic bacteria in peri-alpine lakes. However, recent studies have also shown that a low abundance of BALOs is not necessarily an evidence towards a lower functional impact on prey dynamics (Richards et al., 2012; Welsh et al., 2016; Williams et al., 2016). Hence, despite the very low abundances of the *Bdellovibrionaceae* and the *Bacteriovoracaceae* we found, their functional role may not be negligible. Moreover, the number of native BALOs in the environment is reported to be low (Chauhan et al., 2009b). In fact, it has been suggested that BALOs rarely dominate continuously from a numerical point of view but form reasonably abundant populations that fluctuate over time (Kandel et al., 2014). For example, a formation of a bacterial hotspot may alter the structure and the abundance of BALOs in an ecosystem at any time. This led Williams et al. (2016) to hypothesize about the "seed bank" theory. The theory implies that when some conditions are met, BALOs could switch from a state of inactive and sparse to a state where they are highly active and abundant, to the point of becoming dominant for a limited period of time. Our previous study about ssDNA viruses and their boom and bust dynamics reinforce this idea (Zhong et al., 2015). Relatively closed ecosystems such as ponds are usually rich in organic matter, resulting in high concentrations of heterotrophic bacteria that can favor the growth of BALOs populations. For example, the number of heterotrophic bacteria in shrimp ponds is 10 to 100 times greater than in natural coastal waters. BALOs react to high prey biomass densities, thus increasing their abundance (Wen et al., 2009), and can become invasive since they have a very high adaptability to different environments (Yu et al., 2017).

3.4. BALOs and environmental factors

Our CCA analysis revealed some significant relationships between the BALOs and some environmental factors, likely to be important to better understand the ecology of the predators. On one hand, we found that the *Peredibacteraceae* displayed clear links with pH, dissolved oxygen

and temperature, and distributed more preferentially in near-surface waters, where waters are warmer, richer in phytoplankton biomass and in bacterial preys (Figure S1). According to Davidov and Jurkevitch (2004), the optimal temperature range of *Peredibacter starrii* is 20-30°C. In summer, the abundance of the *Peredibacteraceae* was clearly higher than in winter or autumn seasons. On another hand, the *Bdellovibrionaceae* seemed to be more sensitive to conductivity and ammonium concentrations. At last, no significant relationships were found for the *Bacteriovoracaceae* with any of the environmental factors tested in our study, while this group was also found to be more abundant in deep water. Unlike *Peredibacter*, *Bacteriovorax stolpii* can handle a wider range of temperature. The optimal temperature range for growth of this species is 15 to 35°C (Baer et al., 2000). In general, environmental factors are undeniably a driving force in bacterial structure, specifically salinity and temperature (Aguirre et al., 2017). However, in the literature, only two factors, i.e., temperature and salinity, have been shown to induce a shift in BALOs structure (Kandel et al., 2014) while other factors seemed to play a minor role in BALOs structure. For instance, (Chen et al., 2018) observed that growth and predation activity of estuarine BALOs were reduced when temperature dropped under 10°C. In parallel, the same trend occurred when salinity reached more than 30 ppt. Excluding temperature and salinity, BALOs may not be directly correlated to conductivity, pH, oxygen, ammonium, chlorophyll or other measurement, but dependent on the presence of prey bacteria. This is what Chauhan et al. (2009b) found with clear positive correlations between BALOs and allochthonous prey bacteria abundances. Van Essche et al. (2011) suggested that BALOs can prey in microaerophilic or anaerobic habitats. They identified a cytochrome oxidase complex (Cytbb3) in *Bdellovibrio bacteriovorus* HD100 strain that eases microaerophilic respiration. Additionally, Sockett and Lambert (2004) indicated that *Bdellovibrio* can utilize other substrates than oxygen such as nitrite or nitric oxide for respiration. Burnham et al. (1976) reported that *Bdellovibrio bacteriovorus* 15143 lyse extracellularly the blue green alga *Phormidium luridum*. The secreted enzymes from the predator inhibited 75% of the algal photosynthesis. Therefore, when chlorophyll *a* measurement are low in the environment, one can expect that among other reasons BALOs enzymes are here and there. To conclude, since the existence and the abundance of preys are more likely to impact BALOs than any other parameters, the next very important step will be to study which heterotrophic bacteria in peri-alpine lakes are associated to the main BALOs species.

3.5. BALOs' provenance and form

While studying an estuarine ecosystem, Williams (1988) observed that BALOs might be of allochthonous origin. BALOs would largely derive from river runoff and wastewater treatment

plant likely to constitute hotspots of concentration. In addition, it was reported that BALOs prefer benthic habitats over the pelagic zone, typically sediments or biofilms formed on small rocks nearshore. These past observations could explain the relatively low abundance of BALOs in peri-alpine lakes sampled in open-water, far from the main tributaries and the littoral zone. It is noteworthy however, that if and when certain conditions are met such as temperature warming and heterotrophic bacteria blossoming, BALOs' abundances will most likely start to increase.

We also want to remind that we used two types of filters to obtain information on both attached (2 µm) and free-living cells (0.2 µm). The attached form implies BALOs undergoing periplasmic (bdelloplast) or epibiotic cycles but also BALOs physically attached to any type of particles. We hypothesized that 2 µm filters would result in more BALOs than the <2 µm fraction, because a bdelloplast can contain at least three progenies and epibiotic BALOs divide into two cells. Nevertheless, the 0.2 µm filter yielded the highest concentrations of BALOs suggesting at first glance that the free-living form is dominant. However, we prefer to point out that this result is to take with caution since we cannot eliminate the possibility that the filtration step as well as the extraction protocol using the GenElute™ Kit may not have mechanically separated aggregates and then lysed the bdelloplasts, yielding *in fine* to false conclusions.

4. Conclusions and perspectives.

Our results have revealed for the first time the presence of bacterial predators belonging to the three main families of BALOs in peri-alpine lakes, with at last for the *Peredibacteraceae*, concentrations reaching relatively high values. These results lead to the conclusion that these bacteria are likely to play a significant role in the functioning of these ecosystems. However, their role remains to be determined. A first perspective of this work is to investigate the interactions between prey and predator, for instance throughout the use of approaches such as next generation sequencing to better capture their diversity and build interaction networks. We assume that using NGS approaches such as 16S metabarcoding combined with high throughput sequencing, will cover more in-depth the BALOs diversity and considerably improve our knowledge regarding these communities. One needs to keep in mind that such methods can also fail to detect taxa at low densities (Caballero et al., 2017). A second perspective is to investigate the action of different environmental factors on prey-predator relationships. To reach this goal, experimental approaches should be carried out with isolates from different strains of BALO families from peri-alpine lake with a spectrum of prey bacteria co-cultured in microcosms. Experiments in micro- or mesocosms could be proposed under different conditions, following the experimental approach proposed by Williams et al. (2016) who used qPCR and SIP after the addition of radiolabelled preys in different conditions. The catalog

and analysis of the diversity of the various BALOs in -cosms would allow to directly correlate the different environmental factors characteristic of the lacustrine environment (such as prey quantity, prey diversity, types of nutrients, etc.) with the distribution of the various BALOs. The study of the abundance, structure, and diversity of BALOs within other matrices, such as biofilms and sediments within the peri-alpine lake environment constitutes another exciting issue, so does the analysis of the possible functional importance of the last group of BALOs not studied in this work, e.g., *Micavibrio*.

5. Materials and Methods

5.1. Study sites and sampling strategy

Sampling was conducted at the reference station of the three largest natural deep lakes in France and Western Europe, i.e., Lakes Annecy, Bourget and Geneva. Different trophic status characterize these ecosystems: mesotrophic for Lake Geneva, oligo-mesotrophic for Lake Bourget, and oligotrophic for Lake Annecy (Jacquet et al., 2012, 2014). The samples were taken at different depths, characteristics of the epi- or the meta-hypolimnion, i.e., 2 or 2.5 m *vs* 50 m for Lakes Bourget and Geneva, and 3 m *vs* 45 m for Lake Annecy. These samples were taken on average once per month for each Lake (except for Lake Annecy) between August 2015 and January 2016. For each depth and sampling site, 1 L of water was filtered successively through two types of 47 mm diameter polycarbonate filters: 2 µm (to obtain the bacterial community attached to particles, epibiotic BALOs attached to prey and periplasmic BALOS within prey), and 0.2 µm (to retrieve only the so-called free-living bacteria, typically BALOs free attack phase). Filters were frozen and kept at -20°C. Both physical and chemical descriptors, as well as total bacterial counts, were obtained as reported in previous studies (Berdjeb et al., 2011b; Zhong et al., 2013; Jacquet et al., 2014; Parvathi et al., 2014). Physical descriptors, nutrients, chlorophyll *a* and other environmental factors including total bacterial abundance using flow cytometry were obtained as previously described (Personnic et al., 2009b; Berdjeb et al., 2011b, 2011c; Thomas et al., 2011).

5.2. DNA extraction and PCR primers

DNA extraction was conducted from filters using the GenElute™ Bacterial Genomic DNA Kit. Different cultures of BALOs, referred to as HD100 and 109J for *Bdellovibrio bacteriovorus* and A3.12 for *Peredibacter starrii* (gently provided by Dr. E. Jurkevitch), used as positive controls for PCR assays (see below) were centrifuged (10 min, 4°C, 13000 g) in order to collect the pellet and extract the DNA. DNA concentrations were quantified and controlled in quality using Nanodrop 1000 Spectrophotometer and QUBIT 3.0 Fluorometer (Thermofischer Scientific) with three replicates for each sample.

Different primers for either PCR or qPCR were selected for their specificity for the 16S rDNA of the three BALOs families, i.e., the *Bdellovibrionaceae*, *Bacteriovoracaceae*, and *Peredibacteraceae*. A total of 12 primers (Table 1) were tested using different PCR and qPCR protocols (TABLE S1). As no qPCR primers for quantifying *Peredibacteraceae* were available in any previous studies, we designed and tested new primers using the NCBI/Primer-BLAST online tool (Ye et al., 2012), the FastPCR software (Kalendar et al., 2017) and the MEGA 6 software (Tamura et al., 2013). 120 existing sequences of 16 rDNA of *Peredibacteraceae* were aligned using clustalW within MEGA 6. A consensus sequence was obtained and used to find specific primers with NCBI/Primer-BLAST, with a high stringency (i.e., primer with at least 3 total mismatches to unintended targets, including at least 2 mismatches within the last 6 bps at the 3' end; targets with 7 or more mismatches to the primer were ignored; the target had a maximum size of 350 bps). Each primer couple designed was then verified by qPCR amplification and cloning-sequencing.

qPCR reactions were performed using the QuantiTect SYBR® Green PCR Kit and with the Rotor-Gene Q thermocycler. Standard curves were established in triplicates using serial dilutions of *E. coli* plasmids containing 16S rDNA sequences of each three families. Linear standards curves were obtained within the range of 10^1 to 10^6 plasmid copies per reaction. The efficacy was 0.99 with an R^2 value of 0.998 and a slope value of -3.32. The specificity of reactions was confirmed by both melting-curve analyses and agarose gel electrophoresis to identify unspecific PCR products. The plasmid copy numbers were calculated using the following formula (Whelan et al., 2003): Copy number = (DNA amount (ng) × 6.022 × 10^{23}) / (length (bp) × 109 × 650).

5.3. FCM

To obtain total bacterial counts, without PCR bias, we used a FACSCalibur flow cytometer as previously described ((Personnic et al., 2009b; Berdjeb et al., 2011b), see supplemental material). Note also that we compared these abundances with qPCR data obtained using the universal primer set for total bacterial counts and obtained a fairly good relationship (r=0.654, not shown).

5.4. DGGE

The BALOs community was analyzed by denaturing gradient gel electrophoresis (DGGE) following the manufacturer's protocol Instruction Manual (C.B.S.-Scientific company.inc, DGGE-2001). One mm thick polyacrylamide gel (6 % [wt./vol] acrylamide in 1 × TAE buffer [40 mM Tris, 20 mM sodium acetate, 1 mM EDTA]; pH adjusted to 7.4) was prepared with a linear formamide/urea gradient ranging from 40 % to 55 % after several tests to find the best gradient. It was overlaid with a non-denaturing stacking gel. Each well was loaded with 15 ng PCR product and 5 µL loading buffer. Electrophoresis was conducted for 16 h at 120 V and 60°C. Subsequently, the gels were stained in darkness for 40 min in 1 × TAE buffer with 2 × SYBR gold solution as specified by the manufacturer. The DGGE profiles were only analyzed visually, because of the low

number of bands obtained and the lack of apparent important diversity (Fig. S1). Each band was then cut under UV with Gel Doc™ XR+ system (Bio-Rad) and conserved in 30 µL of TAE buffer at -20°C. DNA extraction from DGGE band was performed by incubating tubes 20 min at -80°C, 20 min at -4°C and then by centrifugation (10 min, 13000 rpm, 4°C). The supernatant was conserved at 4°C for PCR.

5.5. DNA purification, cloning and sequencing

The DNA of each DGGE band was eluted from the gel slice, after its excision, by adding 100 µl sterile 1 X TAE buffer and heating at 95°C for 15 min. Three microliters of eluted DNA served as template in a 22 µl PCR mixture using the corresponding primer set. The PCRs were performed with the same conditions as the first PCR stage described above. The amplicons were first verified by electrophoresis in a 1.5% agarose gel, then purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare), to finally be cloned into pCR®4-TOPO® vectors using the TOPO TA Cloning® Kit (Invitrogen). Randomly selected clones were sent for sequencing to GATC Biotech (Germany).

5.6. Sequences processing, alignment and phylogenetic analysis

Sequenced DNA from Sanger sequencing required different steps to be cleaned. The same workflow was applied to the sequences of each BALOs family. First, sequences inferior to 100 pb were discarded. Secondly, the remnant of *E. coli* vector at 5' and 3' ends was detected and removed using NCBI BLASTn (Altschup et al., 1990). Then actual BALOs sequences were trimmed at 3' end to remove the poor quality base. Next, sequences that matched other species than BALOs or unknown bacteria, i.e., « uncultured bacterium » were also discarded. Afterward, sequences were dereplicated using Obitools command "OBIUNIQ" (Boyer et al., 2016), checked for chimera sequence using "VSEARCH uchime_denovo" (Rognes et al., 2016) and clustered at 97% identity threshold using the command "CLUSTER FAST" of Usearch (Edgar, 2010). Next, using MEGA 6 software (Ye et al., 2012), centroid sequences were aligned using MUSCLE (Edgar, 2004) and trimmed to equal length. Poorly aligned sequences were discarded. Later, for each family, a reference database was constructed using Arb-SILVA (Quast et al., 2013) and when not enough sequences were found, the NCBI nucleotide database (Benson et al., 2013) was used to complete the database. Each sequence of the database was blasted and confirmed to belong to the chosen BALOs family. Sequences from the reference database and cleaned Sanger sequences were then aligned using MUSCLE and trimmed equally. The alignment was curated using GBLOCKS (Castresana, 2000). The best substitution model was selected using JMODELTEST-2.1.10 (Darriba et al., 2012) with Akaike Information Criterion (AIC) (Akaike H, 1973). Next, Maximum likelihood phylogeny was constructed using PHYML-3.1 (Guindon et al., 2010) with 100 bootstrap replicates, and Bayesian phylogeny inference was made with MRBAYES 3.2.6 (Ronquist et al.,

2012). For each family the same outgroup species was used, i.e., *Vampirovibrio chlorellavorus* (HM038000.1) based on Kandel et al. (2014).

5.7. Accession number(s)

All sequences are found on the GenBank portal with the following identification numbers: MH537943 to MH537970.

5.8. Data analysis of abundance in relation to environmental data

A canonical correspondence analysis (CCA) was performed taking into account only the most significant and non-redundant ecological variables to highlight the relationships between the relative abundances of the three families of BALOs (obtained by qPCR) with environmental factors. The CCA was tested using the Vegan package in R with the following ecological descriptors: temperature, total phosphorus, orthophosphates, nitrates, ammonium, silicon dioxide, dissolved oxygen, chlorophyll *a*, pH, conductivity and only pH, dissolved oxygen, temperature, total phosphorus, conductivity, and ammonium were conserved after forward selection of the variables. A statistical test of the relationship between the abundance of each family with environmental factors was performed with the PERMANOVA test from Vegan.

6. Supplementary data

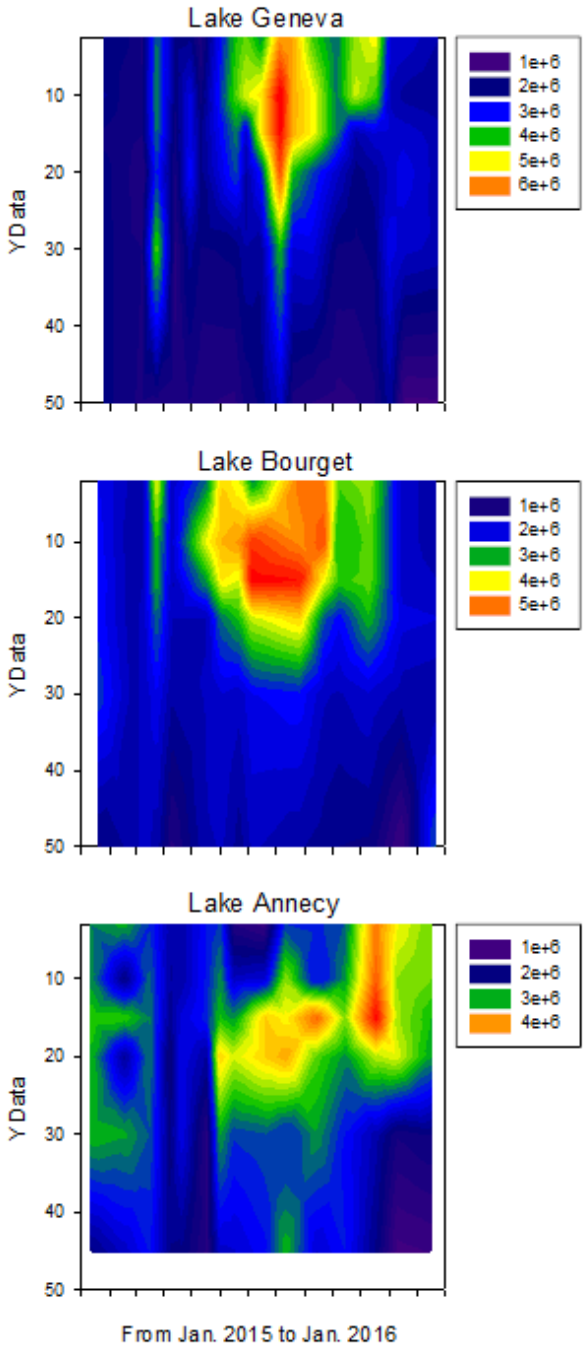


Figure S1 Dynamics and distribution of the heterotrophic bacteria obtained at 6 different depths (i.e., at 2 or 2.5 or 3, 10, 15, 20, 30 and 45 or 50m) for Lakes Geneva, Bourget and Annecy from January 2015 to January 2016. Along the year, cell abundances (cell mL^{-1}) varied for these lakes, between 3.36 x 10^5 and 6.98 x 10^6 cell mL^{-1}, between 3.44 x 10^5 and 5.71 x 10^6 cell mL^{-1} and between 9.62 x 10^5 and 4.02 x 10^6 cell mL^{-1}, respectively.

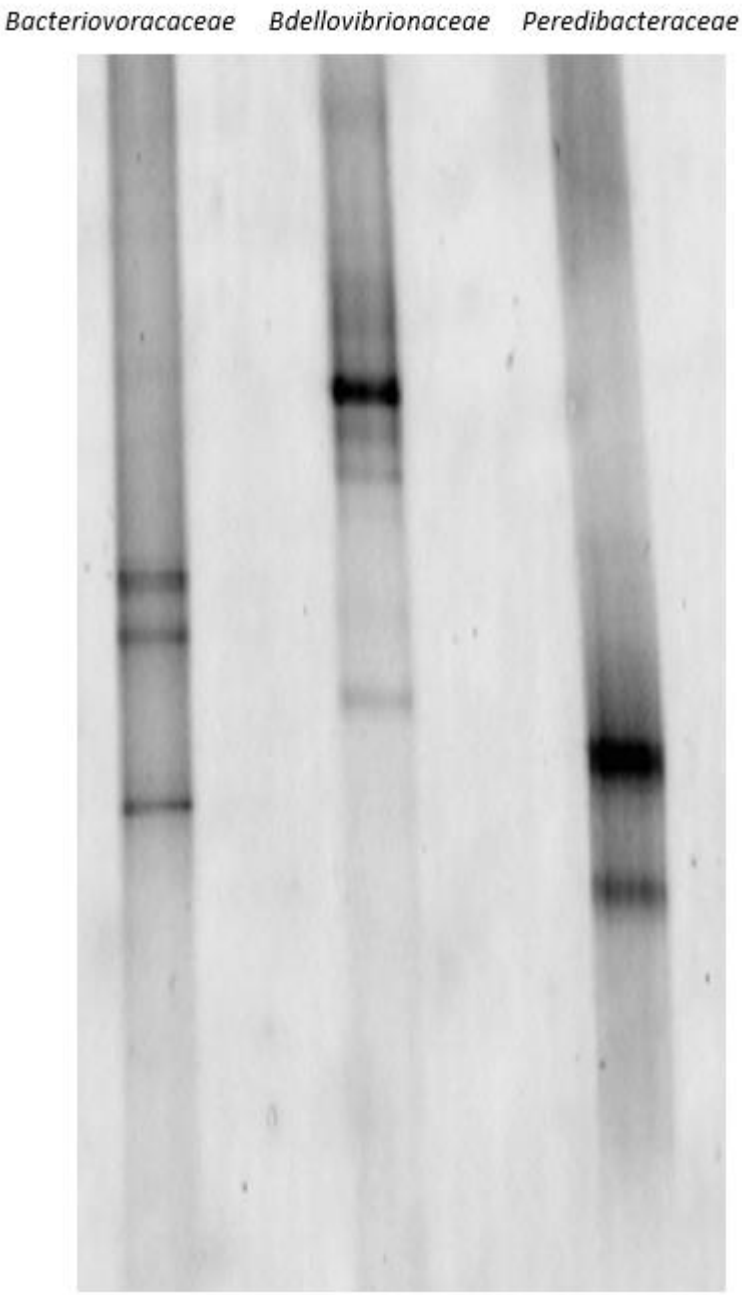


Figure S2 DGGE gel with pooled samples, revealing a limited number of bands for each BALOs family.

Table S1 Tested PCR conditions

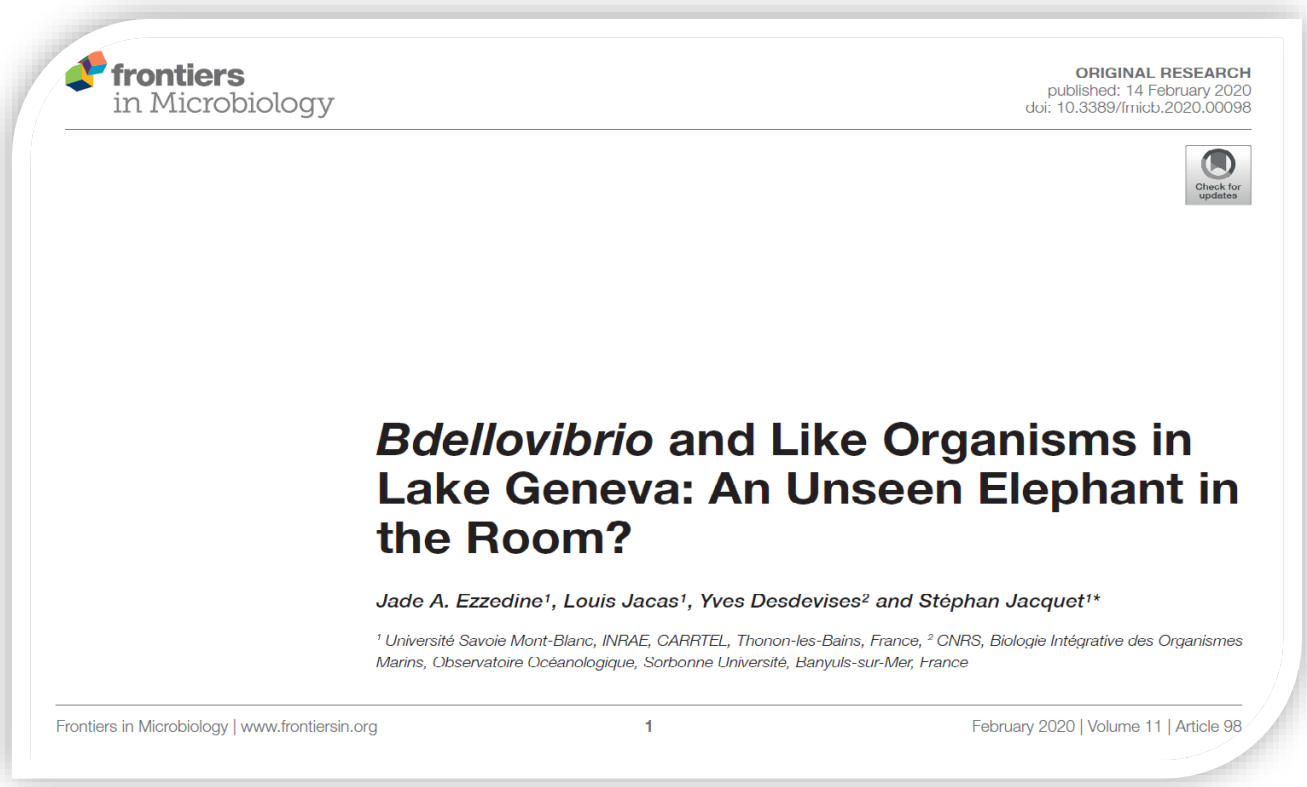
Primer names	Test	Tested PCR conditions		
		Temp of hybridization	DNA volume. *	Number of cycles
Couple Bde1	PCR	49°C, 51°C, 55°C, 56°C, 58°C.	1 and 2 µL	36 cycles
Couple Bde2	PCR	49°C, 51°C, 55°C, 56°C, 58°C.	1 µL	36 cycles
	qPCR	60°C.	1 µL	45 cycles
Couple Bde3	PCR	49°C, 51°C, 55°C, 56°C, 58°C.	1 and 2 µL	36 cycles
Couple Bde9	PCR	48°C, 52°C.	1 µL	36 cycles
Couple Bx4	PCR	49°C, 54°C, 56°C, 58°C.	1 µL	36 cycles
	qPCR	58°C, 60°C.	1 and 2 µL	45 cycles
Couple Bx5	PCR	49°C, 51°C, 55°C, 56°C, 58°C.	1 and 2 µL	36 cycles
Couple Bx10	PCR	48°C, 52°C.	1 µL	36 cycles
Couple Per6	PCR	49°C, 51°C, 55°C, 56°C, 58°C.	1 µL	36 cycles
Couple Bct7	PCR	50°C.	1 µL	36 cycles
	qPCR	52°C.	1 µL	30, 40 & 45 cycles
Couple Bct8	PCR	48°C, 50 and 52°C.	1 µL	36 cycles
Couple Bct8 + Couple Bde3	Nested PCR 1	Couple 8 : 50°C.	1 µL	20 cycles
	Nested PCR 2	Couple 3 : 51°C.	1 µL	20 cycles
Couple Bct8 + Couple Bx10	Nested PCR 1	Couple 8 : 50°C.	1 µL	20 cycles
	Nested PCR 2	Couple 10 : 54°C.	1 µL	20 cycles
Couple Per11	PCR	53°C	1 and 2 µL	36 cycles
	qPCR	53°C	1 and 2 µL	45 cycles
Couple Per12	PCR	53°C	1 and 2 µL	36 cycles

Objectifs et résumé de l'article 4

Suite à l'étude précédente ayant, d'abord, révélé la présence, puis déterminé l'abondance et la distribution des trois familles principales de BALOs du groupe des Oligoflexia dans les lacs périalpins, une deuxième approche a été utilisée pour analyser plus en détail la diversité des BALOs au Léman dans le cadre du projet TRANSLEM. Ce dernier a visé à étudier la diversité des bactéries et des archées aux différentes saisons de l'année (février, juin, août, et novembre), à 3 profondeurs (2, 15, 200 m) et sur 3 sites situés le long d'un transect au milieu du Léman. Le séquençage à haut débit employé a été réalisé *via* un run Illumina Hi-seq employant une amorce 16S ARNr universelle non spécifique. Cela a permis de générer un peu plus de 55 millions de séquences qui ont été traitées par divers outils bio-informatiques. En s'intéressant spécifiquement et en sélectionnant alors les OTUs des bactéries prédatrices, nous avons pu montrer la prédominance des BALOs (0,6% en termes d'abondance relative de reads des bactéries totales), suivies par le groupe des *Saprospiraceae* (0,3%) et les *Myxo*coccales 0,15%). Toutefois, les *Myxococcales* présentaient une plus grande richesse en termes d'OTUs (240 soit 3% des OTUs de bactéries totales) alors que les BALOs étaient représentés par 130 OTUs (1,6%) et les *Saprospiraceae* par 97 OTUs (1,2%). Parmi les BALOs, les *Bdellovibrionaceae* étaient les plus abondants en abondance relative (0,58% des bactéries totales) et en OTUs (70 OTUs soit 0,9%), avec aussi la découverte de ce qui semble être de nouveaux représentants. A noter toutefois que ces chiffres dépendent entièrement du couple d'amorce universelle utilisé qui peut favoriser préférentiellement l'amplification de certains taxons au détriment des autres. La présence des BALOs était relativement homogène sur les trois sites échantillonnés, leur diversité étant toutefois plus élevée en profondeur et marquée par une répartition saisonnière. En effet, les *Peredibacteraceae* étaient relativement abondants en période estivale (un résultat corroborant l'analyse précédemment citée). Enfin, grâce à l'utilisation d'un réseau de co-occurrence, nous avons pu révéler l'existence de liens préférentiels entre les BALOs et 17 autres ordres bactériens, en particulier les Myxococcales (dont beaucoup sont des prédateurs d'autres microbes), pouvant suggérer une large gamme d'interactions biotiques et/ou de proies pour ces prédateurs.

NB : Ce jeu de données TRANSLEM a également permis de regarder de plus près les interactions entre les archées et les bactéries. Cette étude est présentée dans la partie Annexe du manuscrit : « *Exploring archaeal and bacterial diversity and co-occurrence in Lake Geneva* » (article 9).

Bdellovibrio and like organisms in Lake Geneva: An unseen elephant in the room?



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Bdellovibrio and Like Organisms in Lake Geneva: An Unseen Elephant in the Room?

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1. Introduction

In aquatic ecosystems, it is now well known that microbes play many important roles. Viruses, archaea, bacteria, phytoplankton and all other unicellular eukaryotes have been identified as key actors for biomass production, matter and nutrient (re)cycling, biotic interactions (including predation and symbioses such as parasitism) and energy transfer throughout the food webs (Kirchman et al., 1982; Azam et al., 1983; Fuhrman and Noble, 1995; Fenchel, 2008). On one hand, for instance, marine bacteria consume 20 to 60% of the organic carbon from primary production (Azam and Malfatti 2007). Through microbial respiration, the produced dissolved organic matter returns to the atmosphere as CO_2 that will, in turn, serve the photosynthesis process of auto- or mixotrophic organisms (Bolan et al., 2011). On the other hand, dissolved organic matter (DOM) consumed by bacteria is transferred to higher trophic levels, i.e., small flagellates and ciliate protozoans. This microbial loop, to which we can add the lytic action of (bacterio)phages, explains why, in general, the abundance of bacteria remains relatively stable from year to year, whatever the ecosystem studied (Jacquet et al., 2010). If bacteriophage-induced mortality rate and protozoan grazing have been reported as the main biotic pressure on bacterial communities (Fuhrman and Noble, 1995; Pernthaler, 2005), with many consequences on bacterial abundance and composition, transfer of carbon and nutrients to higher trophic levels and, *in fine*, on the whole ecosystem functioning (Motegi et al., 2009; Ram et al., 2014), other biotic interactions such as bacteria predating other bacteria have comparatively received lower attention.

Indeed, among the biological compartments that have been largely neglected, there are the predatory bacteria that depend on other bacteria for their nutrient requirements, growth and survival. Clearly, less is known about these predators present in many bacterial classes (Jurkevitch and Davidov 2006) and which have been proposed as a potential important evolutionary driving force (Erken et al., 2013; Pérez et al., 2016). The bacterial predators can be numerous and diverse in different aspects. Some are facultative hunters, i.e. their hunting behavior is only employed when nutrients become scarce, while others are obligate predators, hunting either in packs or alone. Among these predators, some bacteria are unique obligate predators, the *Bdellovibrio* and like organisms (BALOs).

BALOs have certainly been the most studied group of obligate predatory bacteria (Jurkevitch and Davidov 2006). They are Gram-negative bacteria that have the same hunting mode, characterized by two life cycle strategies: periplasmic life cycle (inside consumption of the prey bacterium) *vs.* epibiotic (external consumption). This group encompass few genera, 6 so far, yet their classification has changed many times over the last two decades. Today, these bacteria are classified in 5 families and 1 order. Originally, the *Bdellovibrionaceae*, *Peredibacteraceae*,

Bacteriovoracaceae, *Halobacteriovoraceae* and *Pseudobacteriovoracaceae*, belonged to the Deltaproteobacteria. Recently, they moved to the Oligoflexia class (Hahn et al., 2017). Only one genus, referred to as *Micavibrio*, is classified differently and belongs to Alphaproteobacteria. Among these obligate predators, *Bdellovibrio bacteriovorus* is by far the most studied BALOs. *B. bacteriovorus* life cycle was described *via* microscopic observation and from a genomic perspective (Rendulic et al., 2004).

BALOs are very similar in shape and size, i.e. a comma shape with a flagellum, measuring between 0.2 to 0.5 µm in width and 0.5 to 2.5 µm in length (Crossman et al., 2013). They grow and reproduce on other Gram-negative bacteria. BALOs are ubiquitous in a multitude of habitats such as soils, salt and fresh waters (Jurkevitch, 2012a). Therefore, they have been reported as possible important bioagents controlling bacterial populations. As a result, BALOs are medically considered as antibiotic replacement giving their hunting behavior towards Gram-negative pathogens (Willis et al., 2016) and indirectly through their reservoir of enzyme-based antimicrobial substances (Rendulic et al., 2004). In addition, BALOs like bacteriophage and protozoans are likely to impact the structure and dynamics of some bacterial populations or communities (Davidov and Jurkevitch 2004; Williams et al. 2016) but studies highlighting this are still missing.

Unlike soil and saltwater, fresh waters especially lakes have been less examined (Paix et al., 2019). It is however hypothesized that these predatory bacteria play, at some extent, important roles in the structure and functioning of lacustrine microbial communities (Chen and Williams 2012). Using data obtained during the TRANSLEM project, a study of the diversity of bacteria and archaea in Lake Geneva from high-throughput sequencing of the 16S rRNA gene, we focused on BALOs in the present work. Our study sheds light on the diversity and distribution of these predators, as well as their relationships with the bacterial community and environmental factors in the largest natural deep lake of Western Europe. We show that these bacteria are phylogenetically diverse in Lake Geneva, suggesting that they can be important players in the (aquatic microbial) game and thus should deserve particular attention.

2. Materials and methods

2.1. Study site

Lake Geneva is a deep and large warm monomictic lake (surface area: 580 km^2; volume: 89 km^3; maximum depth: 309.7 m; mean depth: 152.7 m), located in the western part of the Alps at an altitude of 372 m. Lake Geneva has been monitored since 1974 as a part of a long-term water quality and biological monitoring program. Sampling has been continuously undertaken in the middle of the lake at the deepest point, referred to as SHL2, once or twice a month (Supplementary

Figure 1). This scientific survey revealed the lake has switched from an oligotrophic to eutrophic state with annual phosphorus concentrations reaching 90 µgP L^{-1} in 1979 (Anneville et al., 2002). Thanks to effective management measures, Lake Geneva turned back to a mesotrophic state in the early 2000s with total phosphorus concentrations about 20 µg.L^{-1} in 2010 (Jacquet et al., 2014).

2.2. Sampling strategy

During the TRANSLEM project, we collected water samples at three sites, including the reference station SHL2. These sites referred to as pt2, pt4 (SHL2) and pt6 (Supplementary Figure 1), separated from each other by approximately 14 km, were sampled at four different dates and seasons: February 20 (TL1), June 4 (TL2), August 7 (TL3) and November 20 (TL4) 2014, and at three different depths, i.e. 2, 15 and 200 m. It is noteworthy that pt2 could not be sampled on June 4 due to bad weather conditions. A filtration system was installed on the boat and a volume of 300 mL of each water sample was consecutively filtered through 5, then 2 and finally 0.2 µm polycarbonate filters, 47 mm in diameter (Milipore) to obtain 3 different size fractionations. They were kept at -20°C until DNA extraction.

Descriptors such as temperature, pH, conductivity, chlorophyll *a* and dissolved oxygen concentrations of the water column were measured using a multiparameter probe (Sea & Sun technology GMBH). Transparency was measured using a normalized 25 cm diameter Secchi disk. Nutrients such as total organic carbon (TOC) and nutrient concentrations, i.e., total nitrogen (TN), dissolved ammonium (NH_4-N), dissolved nitrates (NO3-N), total phosphorus (TP), and orthophosphates (PO_4-P) were measured at SHL2 only at the different depths and dates, according to the standard French protocols AFNOR.

2.3. DNA extraction

The filters were subjected to DNA extraction using a homemade protocol with GenEluteTM-LPA (Sigma-Aldrich) solution. The protocol started with a lysis step in Eppendorf tubes by adding 300 µL of TE buffer (TRIS 1M – pH 8, EDTA 0.5M – pH 8) and 200 µL of a lysis solution (TRIS 1M – pH 8, EDTA 0.5M – pH 8 and sucrose 0.7 M). Then, a thermic shock was carried out by placing the tubes at -80°C for 15 min and thawed into a block heater at 55°C for 2 min. Next, 50 µL of a 10% sodium dodecyl sulfate (SDS) and 10 µL of proteinase K (20 mg mL^{-1}) were added to the solution. The solution was incubated at 37°C for 1 h with gentle stirring and placed again in the block heater at 55°C for 20 min. After a quick centrifugation step (13,000 rpm at 4°C for 3 min), the supernatant was collected. Then, 50 µL of sodium acetate (3 M – pH 5.2) and 1 µL of GenEluteTM-LPA (Sigma-Aldrich, 25 µg $µL^{-1}$) were added. One volume of isopropanol was then added and the tubes were centrifuged for 10 min at 12,000 g and 4°C. The supernatant was discarded and 2 washing rounds using ethanol (80%) were carried to purify the DNA. The remaining ethanol was evaporated using a Speed-Vac for 20 min. Finally, 30 µL of TE were added

and tubes were left for 1 h at 37°C. DNA concentration was measured using a NanoDrop 1000 spectrophotometer. Afterwards, all tubes were stored at -20°C until analysis.

2.4. PCR and sequencing

DNA extracts were set at 25 ng μL^{-1}. DNA extracted from the 5 and 2 µm filters were pooled to avoid material loss and used to estimate the attached fraction of the bacteria. Comparatively, the 0.2 < fraction <2 µm corresponded to the free-living bacteria. The PCR amplification of 16S rRNA gene fragments was performed using a tagged forward primer 515F (GTGYCAGCMGCCGCGGTA) (Wang et al. 2009) and a tagged reverse primer 909R (CCCCCGYCAATTCMTTTRAGT) (Wang et al. 2018). The number of samples was 66 and a PCR replicate was made for each sample giving a total of 132 samples. Each sample was identified with a different tag. PCR mixture volume was 30 µL and consisted of (final concentration): 1x buffer, 0.5 mM dNTP, 1.5 mM $MgCl_2$, 0.5 mg mL^{-1} bovine serum albumin (BSA) and 0.75 U Biotaq DNA polymerase (Bioline). In a second step, a unique combination of tagged primers (forward and reverse) was added to each sample. Finally, 1 µL of template DNA (25 ng μL^{-1}) was added. A negative control was included and the PCR program was as follows: 95°C – 2 min, 30 x (94°C – 30 sec, 58°C – 30 sec, 72°C – 30 sec), with a final extension step at 72°C for 5 min. Agarose gel analysis was performed for verification of the PCR products. When samples showed a non-specific band (approximately 550 bp) close to the target band (approximately 450 bp), the target bands were then captured using Pippin prep system (sage science) following the manufacturer's instructions. Then these captured DNA were checked with TapeStation (Agilent 2200) system for size and quality assessment following the manufacturer's instructions. All amplified DNA were quantified using the Quant-iT PicoGreen ds DNA Reagent kit (Invitrogen) and fluorescence was read using the plate reader Fluoroskan Ascent FL. All DNA samples were then pooled as one equimolar tube. DNA were then purified using the Clean PCR kit (CleanNA) according to the manufacturer's instructions to remove dNTP and dimers. Again, the pool was quantified using PicoGreen. Then the pool tube containing the 132 different tagged DNA was sent to the GATC-Eurofins platform for DNA sequencing using Illumina Hiseq 300 paired end technology to get 2 x 150 bp amplicons.

2.5. Bioinformatics pipeline

Two files of raw data (5'-3' and 3'-5') in fastq format were received from the sequencing platform. Files were processed using the pipeline developed by Frederic Mahé known as "Fred's metabarcoding pipeline" found at https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline. It combines programs such as Vsearch (Rognes et al., 2016), Cutadapt (Martin, 2011) and Swarm (Mahé et al., 2015). All default parameters of the pipeline were left unchanged except when mentioned. Briefly, the pipeline starts with merging reads using Vsearch.

Next, Cutadapt is used to demultiplex the sequences. Here, each sequence is assigned to its sample in an individual file by its tag. Primers are also removed and sequence containing ambiguous bases are discarded. In each file, sequences are dereplicated using Vsearch. Then all files are assembled as one file and dereplicated in the process. The Swarm algorithm was then used to cluster the sequences. In this step, the "d" parameter (i.e., the number of different nucleotides between sequences) was changed from 1 to 13 in order to be close to the identity threshold of 97 % which define same species (Schloss and Handelsman, 2005). Next, *de novo* chimera detection was applied to representative sequences of each cluster using Vsearch. The representative sequences were taxonomically assigned to a reference database downloaded from arb-SILVA (release number 132; Quast et al. 2013) and prepared as required. Next, a python script from the pipeline was used to build the final OTU table. The OTU table was then filtered with three different filters. The first one discarded all sequences flagged as chimera. The second filter removed sequences with a quality score lower than 0.0002. The final filter removed OTUs having less than 3 reads in a sample, unless such OTUs were present in 2 or more samples. *In fine*, we considered only OTUs shared among the two PCR replicates.

2.6. Statistical analysis

Statistical analyses and plots were performed using R, version 3.5.0 (R Core Team 2018) and ggplot2 (Wickham et al., 2018). The OTU table was transformed to relative abundance using the "decostand" function from the vegan package (Oksanen et al., 2019). Alpha diversity indices (i.e., Shannon and Pielou) were calculated with the OTUtable package (Linz, 2018). Simpson and inverse Simpson were calculated using the vegan package (Oksanen et al., 2019). The indexes comparison for each condition (Site, Month, Depth and Filter) was performed using the Kruskal-Wallis test. When the p-value of Kruskal-Wallis test was inferior to 0.05 (alpha), a Dunn test was performed with Bonferroni correction (Dinno, 2017). The richness plot found in the supplementary Figure 2 was made using the "rarecurve" function in vegan. An NMDS to illustrate beta diversity was computed using the "metaMDS" function from vegan and also the goeveg package (Goral and Schellenberg, 2017). A Simper test was also performed with vegan to access the contribution of each OTU to the observed dissimilarity between samples. Regarding environmental variables, they were only available for pt4 (SHL2). OTUs found here were extracted and if an OTU had no read in a site, we removed it from the final OTU table. The data was also transformed as log(1 + x) in order to stabilize the OTU dataset. Then, another NMDS with environmental variables and a CCA were performed on that table following the online tutorial of Umer Zeeshan Ijaz (Torondel et al., 2016) found at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html. Co-occurrence network analysis was performed on all sites following Ju Feng's R and python scripts (Ju et al., 2014; Ju and Zhang, 2015; Hu et al., 2017) found at https://github.com/RichieJu520/Co-

occurrence_Network_Analysis. A bacterial co-occurrence network was built with bacterial orders including *Bdellovibrionales* and *Bacteriovoracales*. The network was composed of 92 nodes (bacterial orders) and 461 undirected edges or connections. The bacterial OTU table used for the network was assigned and curated to the order level. Only positive interactions between community members were considered. The Spearman correlation and p-value cutoffs were set to 0.6 and 0.01 in the script. As for the C-score of the network, it was performed with the R package EcoSimR (Gotelli et al., 2015) based on a presence/absence OTU matrix. The visualization and customization of the network was done with Gephi software (Bastian et al., 2009).

2.7. Phylogeny

On one hand, 18 BALOs reference sequences (including type species) of the 16S rRNA (6 for the genus *Bdellovibrio*, 5 for *Peredibacter*, 5 for *Bacteriovorax* and 2 for *Micavibrio*) were downloaded from NCBI (Benson et al. 2013, Supplementary Table 5). On the other hand, the assigned OTU sequences of BALOs we found (31 for the family *Bdellovibrionaceae*, 8 for *Peredibacteraceae*, 6 for *Bacteriovoracaceae* and 9 for the order *Micavibrionales*) were used to construct the phylogenetic tree. All sequences were aligned using the program MUSCLE alignment (Edgar, 2004) in MEGA7 (Kumar et al., 2016). The ends of all sequences were trimmed at 5' and 3', making reference and OTU sequences of equal length. Next, the alignment was improved using Gblocks 0.91b (Castresana, 2000) with "Minimum Length of a block" set to 5. ModelGenerator v.85 (Keane et al., 2006) was used to select the best nucleotide substitution model (GTR + I + G) under corrected Akaike information criterion (AICc) (Akaike 1973) with 4 discrete gamma categories. The tree was built using the Maximum likelihood method with PhyML 3.1 (Guindon et al. 2010) with 100 bootstrap replicates. A Bayesian tree was also reconstructed using MrBayes 3.2.7a (Ronquist et al. 2012) program with 500,000 generations and a burn-in value of 25%.

3. Results

3.1. Diversity

Each raw data file contained 55,679,272 reads with read length equal to 301 bp. The merge paired-reads process yielded 49,545,789 reads. In this step, 11% of reads were lost, and the median read length was 437 bp. After demultiplexing or sorting each read to its sample and after a first dereplication, the number of reads reached 11,079,438, with a median length of 377 bp. The second dereplication of reads after reuniting them in one file gave 6,861,908 reads. The clustering process yielded 127,052 representative sequences, including 107,120 chimeric sequences and 19,391 non-chimeric sequences. The median read length after the clustering was 376 bp. After applying the

aforementioned filter, and after selecting only bacterial taxonomic assignment and curating the ambiguous assignments, the final number of OTUs was 7,987. It is worth mentioning that the median percentage of identity between representative sequences and database (arb-SILVA) sequences for taxonomic assignment was about 97.4%. The lowest and highest identity percentages were 50.3% and 100% respectively, with an average value of 94.8%.

Among (facultative and obligate) predatory bacteria, BALOs constituted the group with the largest number of reads, representing only 0.61% of total bacteria reads. However, the *Myxococcales* order had the largest number of OTUs, i.e., 240 OTUs, representing 3% of total bacterial OTUs. BALOs were in second position with 130 OTUs, representing 1.63% of total bacteria OTUs (Table 1). It is noteworthy here that the detailed analysis of the OTUs revealed the presence of *Halobacteriovorax* (i.e., a marine group) but after a more in-depth inspection of the sequences using NCBI-BLAST, it was clear that these sequences were wrongly assigned. They corresponded to *Bacteriovoracaceae*. *Bdellovibrionaceae* hold the largest number of OTUs, i.e. 70 OTUs. It corresponded also to the higher number of reads (0.585%) among the BALOs (Table 2). At last, when considering the OTUs common to the two replicates, *Bdellovibrionaceae* OTUs remained the most represented (Table 3, supplementary Figure 2).

Table 1 Metabarcoding analysis of 16S rRNA gene sequences revealing all the predatory bacteria found in Lake Geneva through numbers and % of their OTUs and reads, obtained at the three sampled sites and depths all seasons confounded.

Taxa		**OTU count and percentage**	**Reads number and percentage**
***Bdellovibrio* and like organisms**	*Bdellovibrionales*	70 (0.88%)	174712 (0.58538%)
	Bacteriovoracales	33 (0.41%)	5569 (0.01866%)
	Micavibrionales	27 (0.34%)	1998 (0.00669%)
Cellulophaga		1 (0.01%)	70 (0.00023%)
Cytophaga		22 (0.28%)	3323 (0.01113%)
Herpetosiphon		2 (0.03%)	35 (0.00012%)
Lysobacter		1 (0.01%)	58 (0.00019%)
Myxococcales		240 (3.00%)	44017 (0.14748%)
Saprospiraceae		97 (1.21%)	83000 (0.27809%)
Stenotrophomonas		4 (0.05%)	12349 (0.04138%)
Vampirovibrionales		5 (0.06%)	74 (0.00025%)
Total predatory bacteria		502 (6.29%)	325205 (1.08961%)
Total for all bacteria		7987 (100%)	29846037 (100%)

Table 2 Numbers and % of BALOs OTUS and reads obtained at the three sampled sites and depths all seasons confounded.

Taxa of *Bdellovibrio* and like organisms	OTU count and percentage	Reads number and percentage
Bdellovibrionaceae	70 (0.88%)	174712 (0.58538%)
Micavibrionales	27 (0.34%)	1998 (0.00669%)
Bacteriovoracaceae	18 (0.23%)	2089 (0.00700%)
Peredibacteraceae	15 (0.19%)	3480 (0.01166%)
Total *Bdellovibrio* and like organisms	130 (1.63%)	182279 (0.61073%)
Total for all bacteria	7987 (100%)	29846037 (100%)

Table 3 Numbers of OTUs for BALOs only.

Bdellovibrio and like organisms	R1 OTU count	R2 OTU count	R1 read count	R2 read count	Number of shared OTU between R1 and R2	Number of reads for shared OTU between R1 and R2
Bdellovibrionaceae	59	57	87779	86933	31	173393
Micavibrionales	24	24	1044	954	9	1708
Bacteriovoracaceae	16	14	1129	960	6	1860
Peredibacteraceae	13	12	2020	1460	8	3270
Total	112	107	91972	90307	54	180231

3.2. Phylogeny

For the *Bdellovibrionaceae*, OTU 2526 was closely related to *Bdellovibrio exovorus* JSS while OTU 1160 was closer to *B. bacteriovorus* HD100, HD127 and 109J and OTU 3512 to *Bdellovibrio sp* W. OTUs 6069, 2084, 21353, 10400 clustered among these individuals (Figure 1). The other OTUs were apart since they did not cluster with any known *Bdellovibrio* sequences and formed two distinct groups (OTUs 170 and 186 vs OTUs 271, 363, 84, 427, 4206, 16036, 227, 107, 112, 636, 5207, 418, 373, 560, 2506, 9192, 159, 4347, 137, 412, 4535, and 761). For the *Bacteriovoracaceae* two categories were detected. The first group was composed of some *Bacteriovoracaceae* OTUs related to reference sequences of *Bacteriovorax*; it was the case for OTU 600 closely related to *Bacteriovorax stolpii* Uki2 and *Bacteriovorax sp.* F2. The same discrimination was observed for the *Peredibacteraceae:* OTUs 10461 and 1885 were closely related to *Peredibacter starrii* A3.12, OTU 382 to *Peredibacter sp.* BFB6, K2DN38, C114001412 and C114001299. Some OTUs from these families were more difficult to assign: OTU 3405 of *Bacteriovoracaceae* mixed with OTUs 5245 and 5958 of *Peredibacteraceae*, OTU 17966 of *Peredibacteraceae* mixed with OTUs 14998 and 769 of *Bacteriovoracaceae*. At last for the

Micavibrionales OTUs, they were close to the two reference sequences of *Micavibrio sp.* EPB and *M. aeruginosavorus* ARL-13.

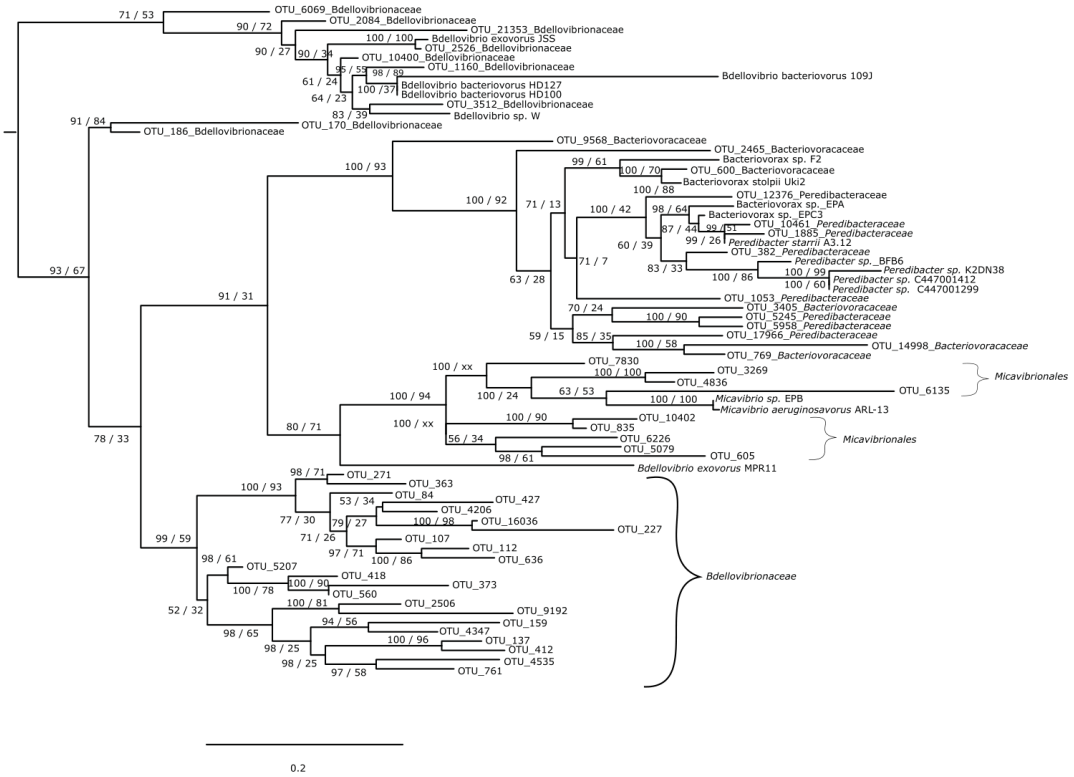


Figure 1 Phylogenetic tree of BALOs environmental sequence with reference database sequence. The tree is based on maximum likelihood analysis and Bayesian inference. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). *Vampirovibrio chlorellavorus* (not shown) was used as an outgroup to root the tree.

3.3. Distribution and dynamics

The relative abundance of BALOs' reads varied with depth and month (Figure 2). At 2 m the *Bdellovibrionaceae* were abundant in November (TL4) in contrast to the other months with 71% of reads, vs. 22% in February (TL1), 1.5% in June (TL2), and 5.5% in August (TL3). This pattern was also observed at 15 and 200 m in November (TL4), with a proportion of 70% and 59.4% of reads respectively. Similarly, *Bacteriovoracaceae* were also abundant at fall, with 97% of reads at 2 m in November. However, June (TL2) revealed a higher abundance of reads at 15 m representing 49.5% and February (TL1) was richer at 200 m with 40.7%. *Peredibacteraceae* were dominant in summer, with 92.9% of reads in August (TL3) at 2 m, 80.5% at 15 m and 42.6% at 200 m. At last,

Micavibrionales reads were merely abundant in June (TL2) at 2 m (93.4%) and 15 m (44.0%). At 200 m, November (TL4) held the highest number of reads, 43.8%.

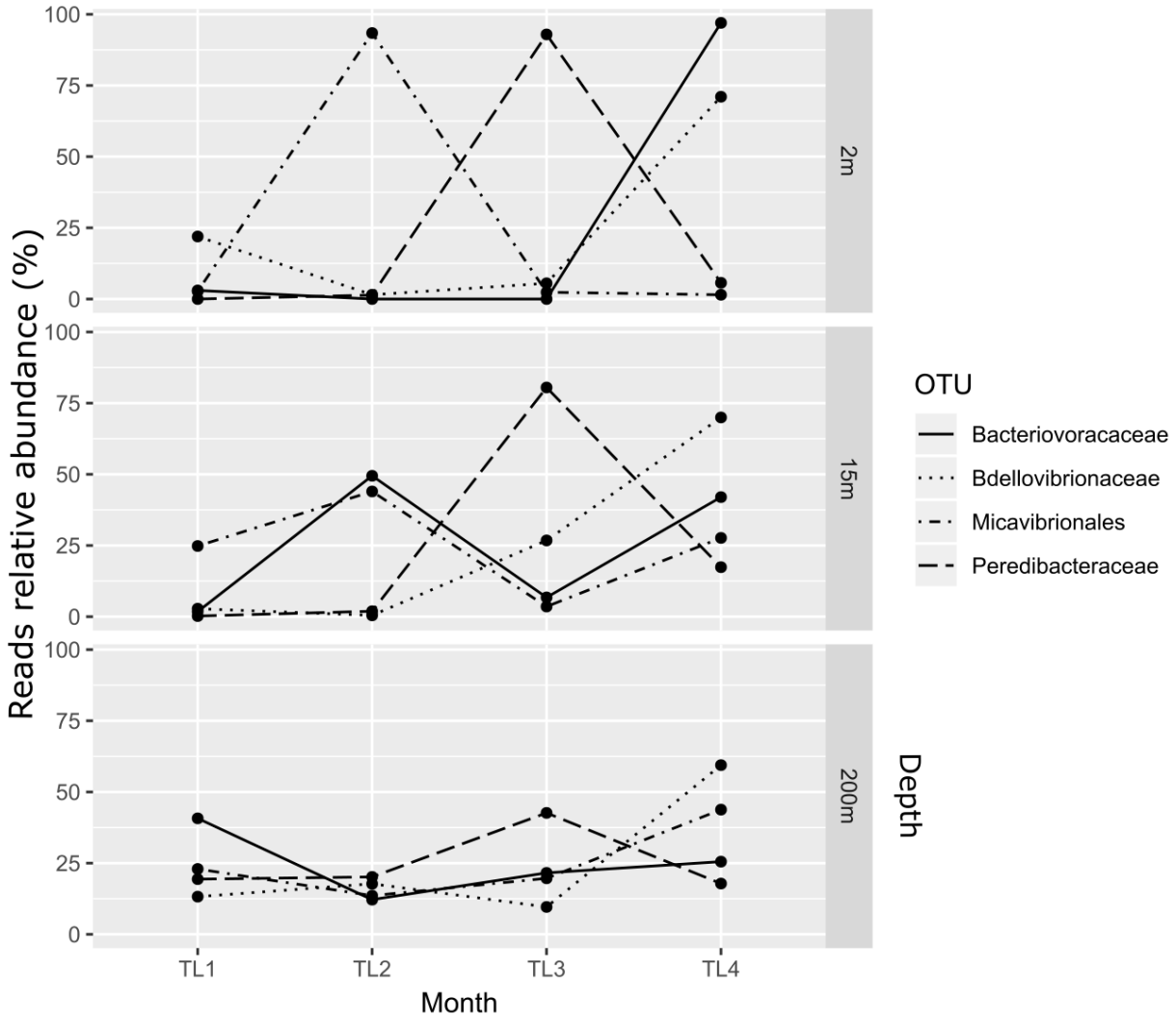


Figure 2 Variation in BALOs relative abundance of reads at the different depths and months.

There was no significant differences for Simpson, Inv-Simpson, Shannon and Pielou indexes between the different sites. A similar result was found when considering the "filter parameter", i.e. when comparing the 0.2 < filter < 2 µm (free-living cells) *vs.* < 2+5 µm (attached and bigger cells) filters. An exception was observed for the Shannon index (p-value = 0.03489), with a higher value for the 0.2 µm filter (Figure 3). Significant differences were observed for Simpson (Inv-Simpson) (p-value = 0.009275) and Shannon (p-value = 0.01315) indexes. The difference was between June (TL2) and November (TL4) with higher values for the November month according to the Dunn test (Simpson p-value = 0.0067 and Shannon p-value = 0.0053). The depth variable also showed differences for Simpson (p-value = 0.005877) and Shannon (p-value = 0.02826) indexes, with 200

m having higher indices values than at 2 m (p-value for Simpson index: 0.0020 and Shannon index: 0.0114) (Supplementary Table 1).

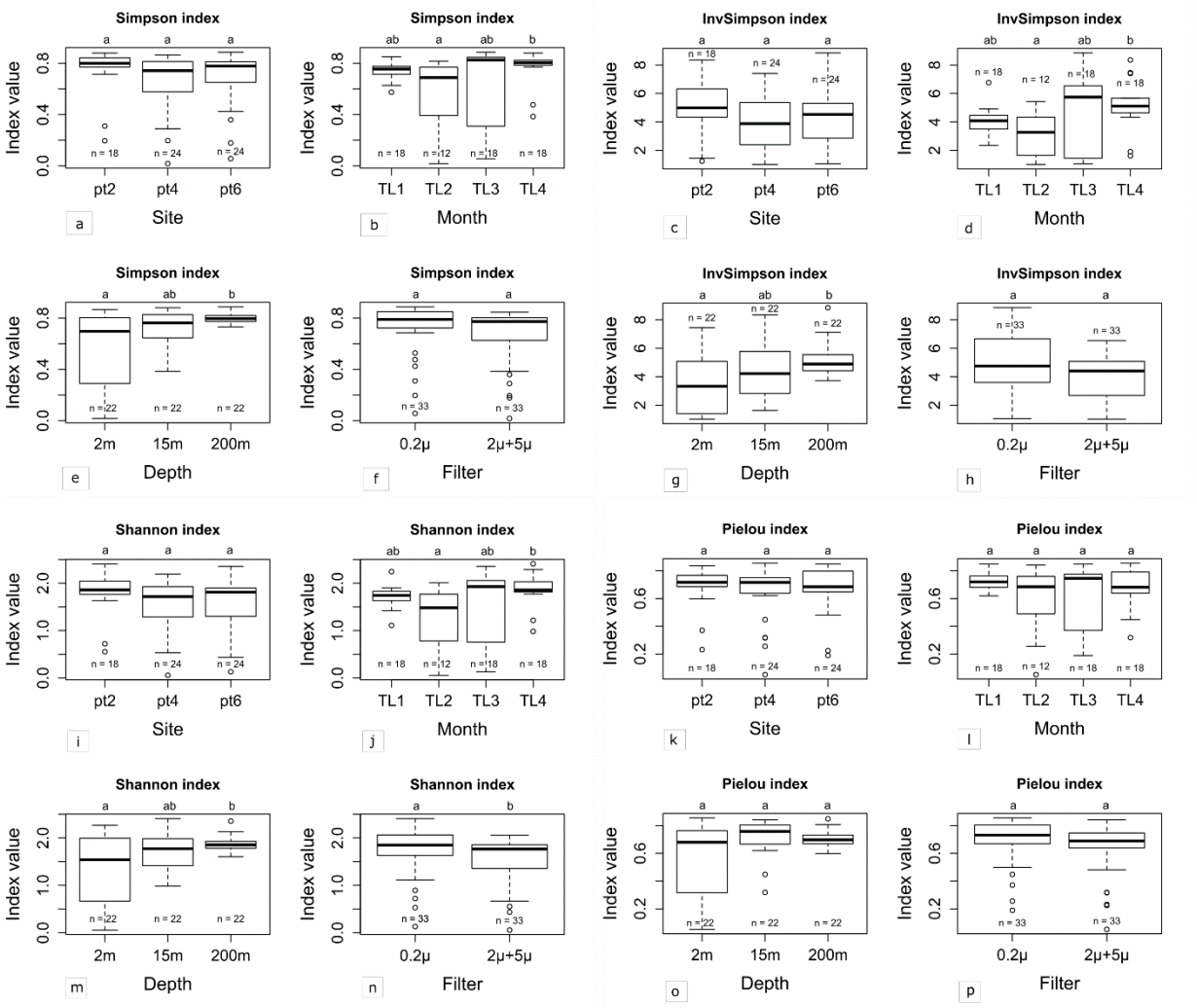


Figure 3 Simpson, InvSimpson, Shannon, and Pielou alpha diversity metrics for site, month, depth and filter variables.

To test the dissimilarities in the BALOs composition among samples, NMDS, ANOSIM and ADONIS were performed (Figures 4-6). Firstly, all sites were investigated, but then, only pt4-SHL2 was specifically examined with a regular NMDS (k= 2) since physicochemical variables were only available for this site which corresponds to the reference station of the lake where the monitoring ecological survey is conducted. A customized NMDS (k= 2) was also performed for pt4-SHL2 (Supplementary Table 2, 3). We observed that depth was a key factor affecting BALOs composition as well as the season. When focusing on pt4-SHL2 where we measured a variety of environmental variables, we highlighted that temperature, turbidity, particulate organic carbon and total phosphorus were important to explain BALOs presence, especially the total phosphorus (for

the OTU 170 at 200 m for the *Bdellovibrionaceae)*. Then, the canonical correspondence analysis (CCA), performed to infer links between BALO's OTUs and environmental variables, extracted a high percentage of variance, 48% for canonical axis 1 and 38% for canonical axis 2 (Figure 7). Correlations between the OTUs and the environmental variables were relatively weak. However, some OTUs found at 200 m (i.e., OTU 769, 2465 of the *Bacteriovoracaceae*, OTUs 271, 16036, 21353 of the *Bdellovibrionaceae*, OTU 5245 of the *Peredibacteraceae* and OTU 4836 of the *Micavibrionales*) were associated with total nitrogen, while others were correlated to a lower extent to conductivity, turbidity and chlorophyll-a concentration.

Weight varied between OTUs to explain their relative importance to discriminate seasons or depths. For season, OTU 605 in *Micavibrionales* was more abundant in February than in June and contributed to 23% of the observed community dissimilarity (Supplementary Table 4). The same finding existed between June and August (22%). OTU 382 in *Peredibacteraceae*, more abundant in August, was the most influential to separate February to August and August to November, with an average dissimilarity of 77% and at 81%, respectively. OTU 227 in *Bdellovibrionaceae* contributed to 19% of the dissimilarity between February (where it was the most abundant) and November community composition and the average dissimilarity between these two months was 79%. Regarding depth, OTU 382 was also the one which contributed the most to the dissimilarity in the community composition between 2 and 15 m, and between 2 and 200 m. However, there was no significant difference ($p=0.8855$, Kruskal-Wallis test). In contrast, the difference was significant when considering OTU 112 of the *Bdellovibrionaceae* responsible for the disparity between 15 and 200 m. Its contribution was about 12%, and it was more abundant at 15 than at 200 m. The average dissimilarity between these depths was 83%.

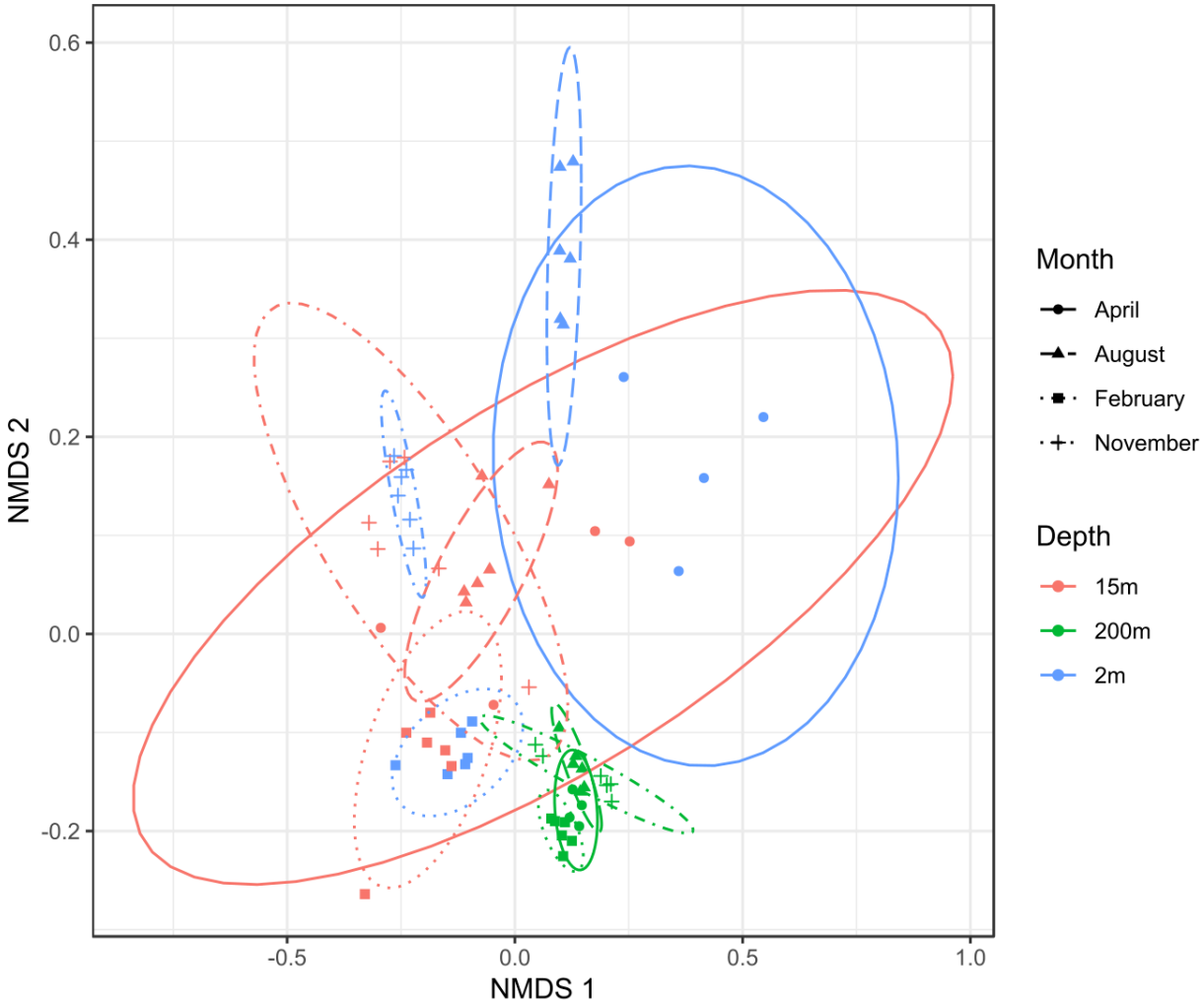


Figure 4 NMDS plot (stress <0.2) for all sites showing that depth and month variables are responsible for the community dissimilarities.

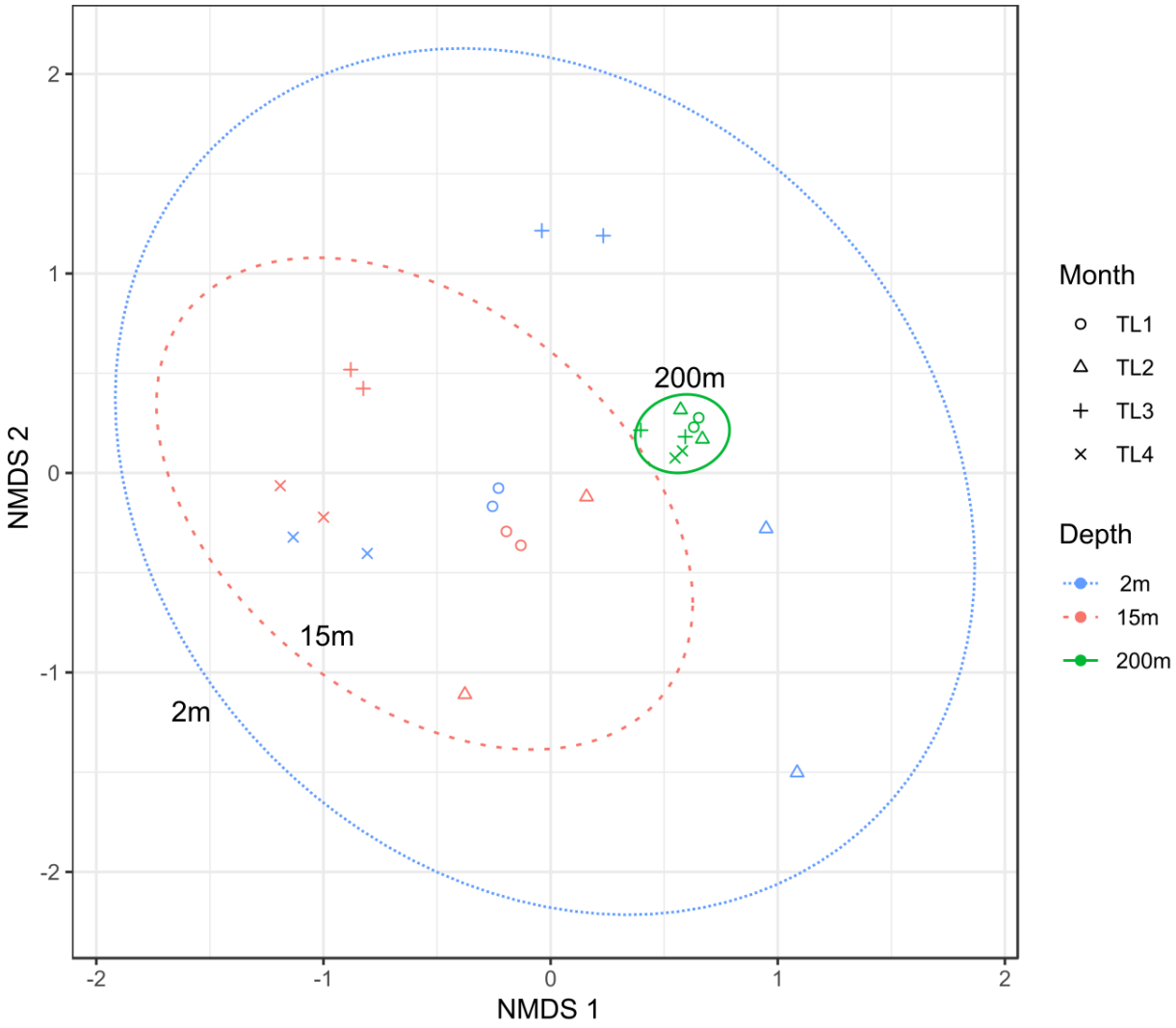


Figure 5 NMDS plot (stress <0.2) for pt4-SHL2 site showing that depth and month variables are responsible for the community dissimilarities.

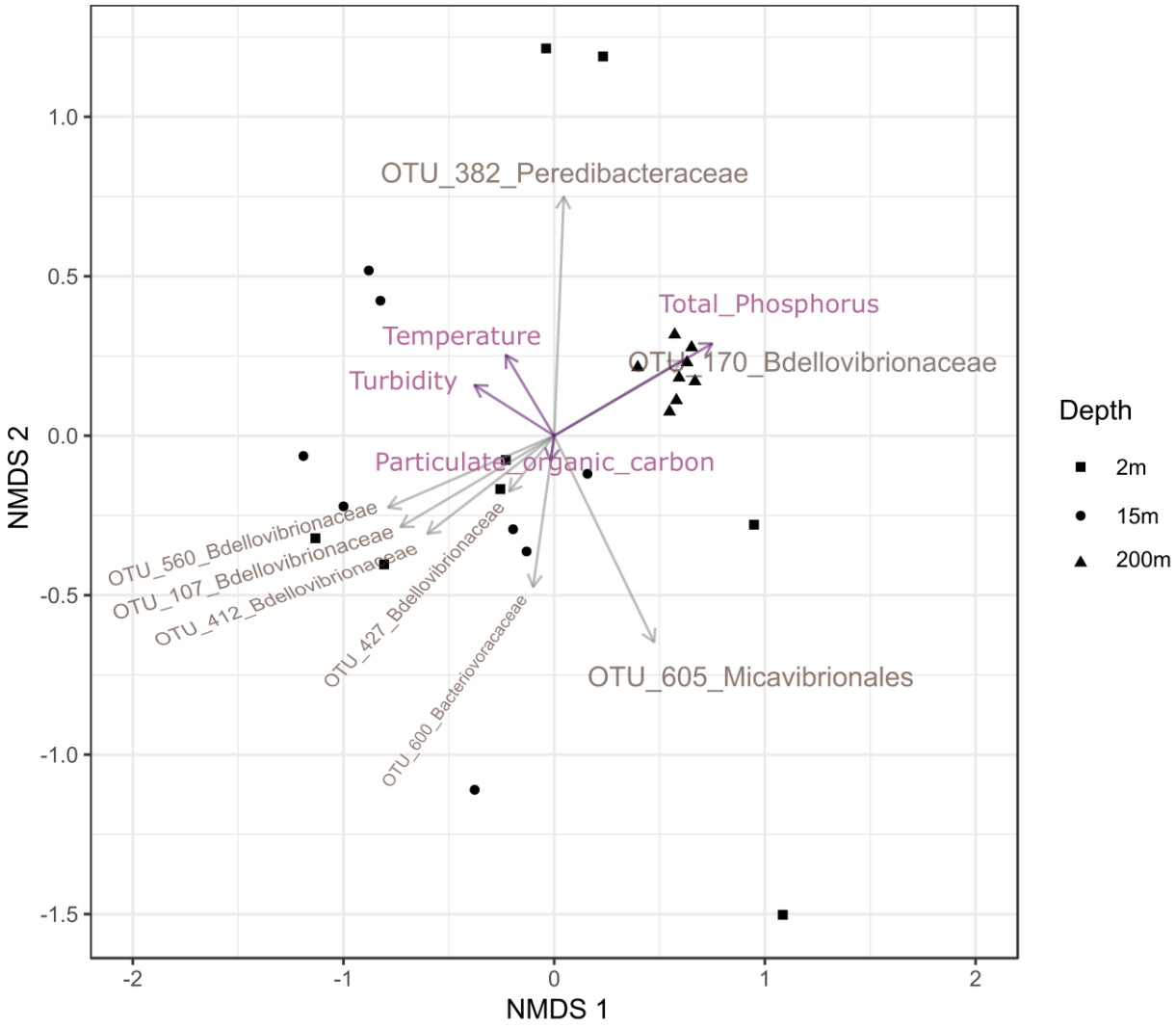


Figure 6 Custom NMDS (stress <0.2) for pt4-SHL2 site with the abiotic descriptors.

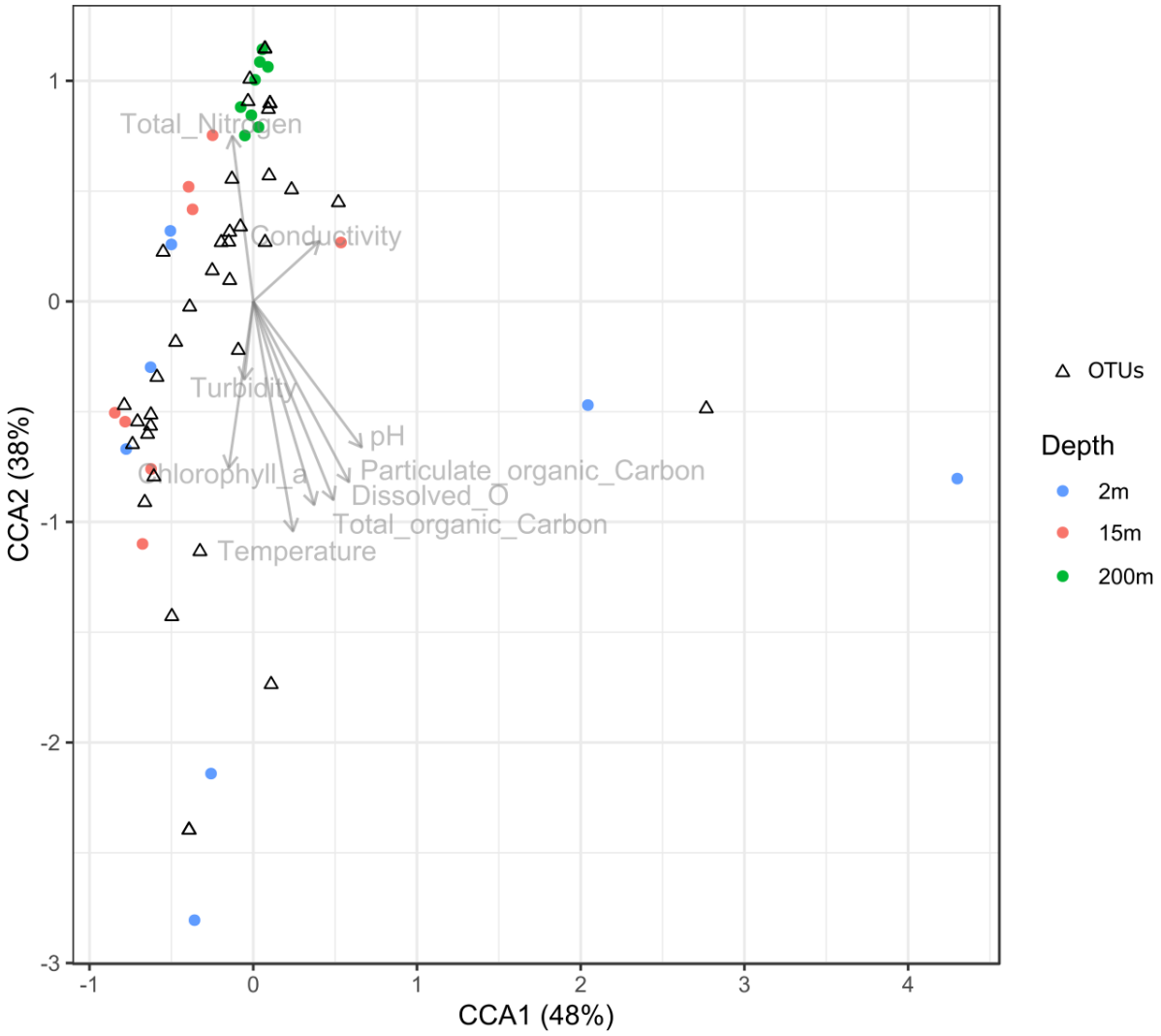


Figure 7 Canonical Correspondence Analysis (CCA) diagram showing the ordination of OTUs along the first two axes and their correlation with environmental variables.

3.4. Relationships between BALOs and other bacteria

We focused only on strong (Spearman's $\rho > 0.6$) and significant (p-value <0.01) positive correlations between the different bacterial groups (Figure 8). It is noteworthy that *Micavibrionales* did not pass this cutoff. The checkerboard score (C-score) test that was performed to measure the overall co-occurrence in the bacterial OTU table confirmed that the co-occurrence network was nonrandom. Indeed, the observed C-score (21.103) was higher than the mean value ($C\text{-score}_{mean}$ =20.538, p-value < 0.001) expected under the null model. There were 17 positive undirected connections between BALOs and other bacteria, the size of each node being proportional to the number of links (Figure 8). These bacteria were from a variety of orders: *Oligoflexales, Chthonomonadales, Campylobacterales, Phycisphaerales, Solibacterales, Bradymonadales, Methylococcales, Chlamydiales, Bacteroidales, Hydrogenedentiales, Myxococcales, Desulfarculales, Saccharimonadales, Omnitrophales, Planctomycetales, Solirubrobacterales,* and *Legionellales*. The majority of these bacteria are Gram-negative except the *Solirubrobacterales,* which is a Gram-positive. On the other hand, no pieces of information dealing with Gram aspects were found for *Hydrogenedentiales, Saccharimonadales* and *Omnitrophales*. It is worth mentioning that BALOs were positively correlated with the *Myxococcales,* which are known to include facultative predators.

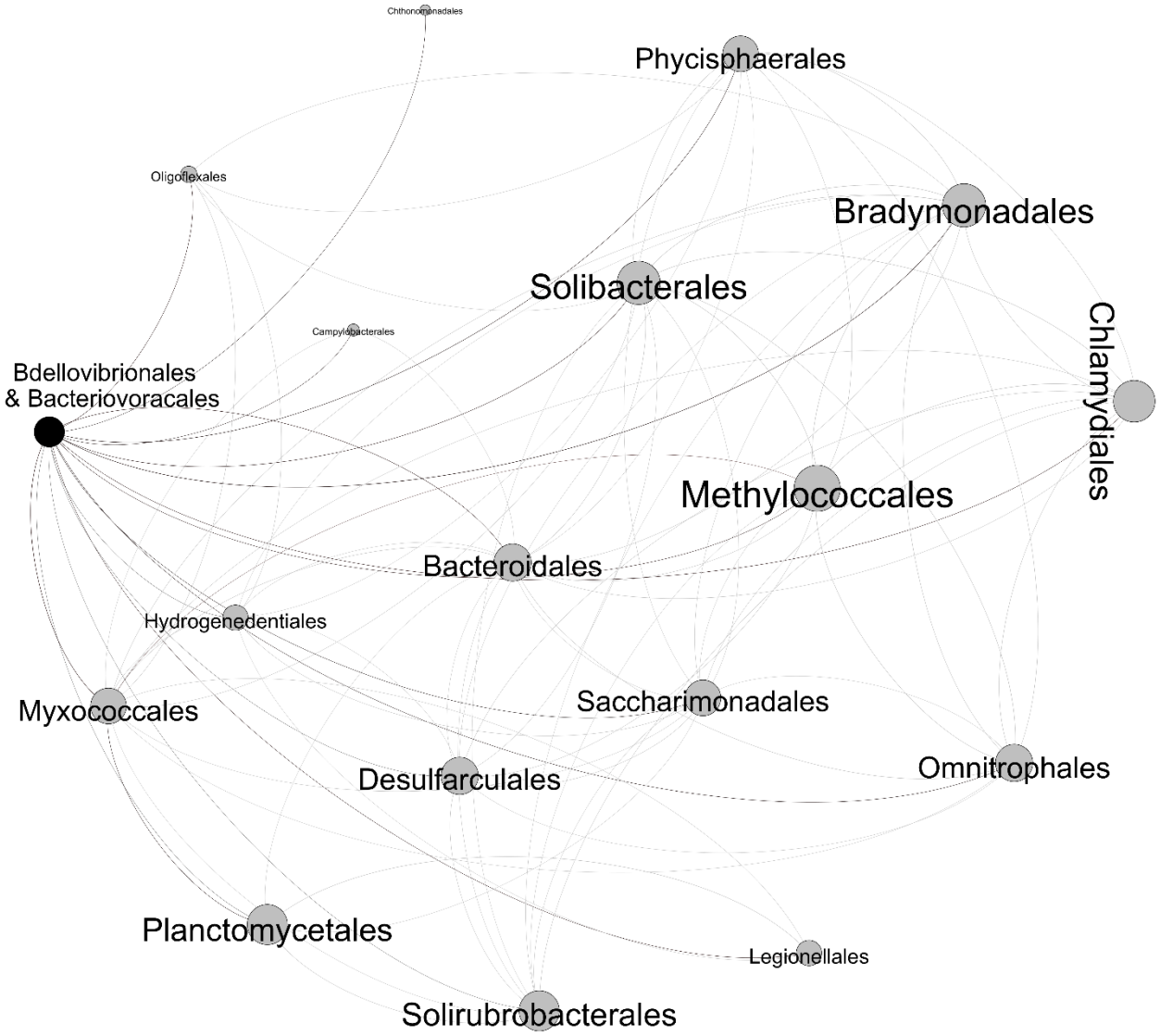


Figure 8 Co-occurrence network revealing the positive relationships between the BALOs and the other bacterial orders.

4. Discussion

Our study focused on a specific bacterivorous bacterial group while we are aware that a large variety of predatory bacteria exists and are all likely to play different but significant roles in aquatic ecosystems. The advantage of studying BALOs is that they are represented by a relatively few "species", are the only group of obligate predators, and they have been well defined since the early 2000's (Baer et al. 2000, 2004; Davidov and Jurkevitch 2004; Koval et al. 2015; Hahn et al. 2017). It remains however that, although BALOs are clearly more described and studied (with >500 publications according to PubMed, October 2019), the importance of their role in natural ecosystems is still unclear.

Using 16S rRNA universal primers targeting bacteria, we focused on the different bacterial predators of bacteria in Lake Geneva. We found that the highest number of OTUs was assigned to *Myxococcales*, followed by BALOs and *Saprospiraceae*. BALOs also included the largest number of reads among all predatory bacteria and seemed to be a relatively homogenous group since the alpha diversity was not significantly different from one site to another. BALOs' diversity did not change dramatically over-time, while their diversity (Shannon and Simpson indexes) were higher in August than in the other months. Temperature could be a key factor explaining such difference since it is known that BALOs have a limited growth and predation activity at low temperature (<10°C) in the water column and sediment (Williams 1988; Sutton et al. 1994; Kandel et al. 2014; Williams et al. 2016). However, temperature was unlikely responsible for the difference in BALOs' diversity between summer and fall since it was relatively constant across the two periods. Rather, the difference could be explained by phytoplankton structure and concentrations, since they provide various nutritive substrates for heterotrophic bacteria, some of them being possible prey for BALOs. A higher phytoplankton biomass recorded in November was indeed associated to higher bacterial concentrations at that time (Rimet, 2015; Jacquet unpublished), and likely to changes in the bacterial community composition (Berdjeb et al., 2011a), then explaining the increase in BALOs diversity.

While more than half of the sequences obtained for Lake Geneva could be associated to known clusters (Benson et al., 2013) some BALOs were new and constituted new independent clusters. It is noteworthy however that these last OTUs were "out" of the clustering algorithm of Swarm used with a clustering threshold (d) of 13, which is the maximum value of differences allowed between two sequences (Mahé et al., 2015). The median length of BALOs sequence was 376 bp, corresponding to 96.6% of similarity. We are aware that the definition of a "species" is subject to many discussions, and recently, Edgar (2018) suggested to update the identity threshold from 97% (Schloss and Handelsman, 2005) to 99% for full length sequences and to 100% for the V4

hypervariable region of the 16S ribosomal RNA. Therefore, we cannot exclude that our threshold is the best to define *sensu stricto* our BALOs sequences as species. By the way, we noticed that *Bacteriovoracaceae* and *Peredibacteraceae* OTUs clustered together, which might be due to short sequence length and to the identity threshold used.

BALOs diversity was higher at depth than surface suggesting these predators occupy a variety of ecological niches (Paix et al. 2019) including cold and dark waters, as phages, maybe because they correspond to sedimenting particles or because they can live on sedimenting material or preys present there. Environmental factors such as temperature, turbidity, particulate organic carbon and merely total phosphorus explained the variation of certain BALOs OTU at 200 m while for others, it was total nitrogen, conductivity, turbidity and Chlorophyll *a*. Such a disparity among BALOs was also recorded within the seasons. That being said, it is unclear whether environmental variables or prey may better explain BALOs diversity. Since BALOs are obligate predators, we assume that BALOs are more limited by preys than by environmental variables. Indeed, BALOs have been discovered everywhere even in extreme environment (Jurkevitch and Davidov, 2006) such as deep-sea sediment (Williams et al., 2018) and according to Kandel et al., (2014), apart from temperature and salinity, environmental parameters do not offer more insight in explaining BALOs structure. It is also noteworthy to mention here that bacteria diversity was higher at 200 m than at 2 and 50 m (data not shown), likely to offer a higher panoply of potential preys.

As BALOs have different predatory spectrum, some cells being generalists, others specialists, and again others versatilist (Chen et al., 2011), this could explain why we observed the dominance of *Bdellovibrionaceae* in winter in surface, *Micavibrionales* at 15 m and *Bacteriovoracaceae* at 200 m. Comparatively *Micavibrionales* were dominant in summer at 2 and at 15 m along with the *Bacteriovoracaceae* whereas it was *Bdellovibrionaceae* and *Peredibacteraceae* at 200 m. *Peredibacteraceae* were dominant in August whatever the depth and finally November was associated to *Bdellovibrionaceae* at 2 m along with *Bacteriovoracaceae* at 15 and 200 m with *Micavibrionales*. Such dynamics were reported elsewhere (Kandel et al. 2014) and the dominance in summer of the *Peredibacteraceae* was also consistent with our previous study revealing the dominance and high abundance of this group in summer (Paix et al. 2019).

Aside from being the most abundant in terms of reads and OTUs among other predators, BALOs (except for *Micavibrionales*) and *Myxococcales* co-occurred and were positively correlated. No other predators showed similar correlations with BALOs. The rest of the network was mainly composed by Gram-negative bacteria that all may be potential prey for BALOs and *Myxococcales* or simply groups co-varying in response to abiotic factors. This last hypothesis is supported by a recent work where we have isolated new *Bdellovibrio* strains from Lake Geneva that were only able to grow on *Pseudomonas*-like preys (Ezzedine et al., 2020c). It is clear however that further

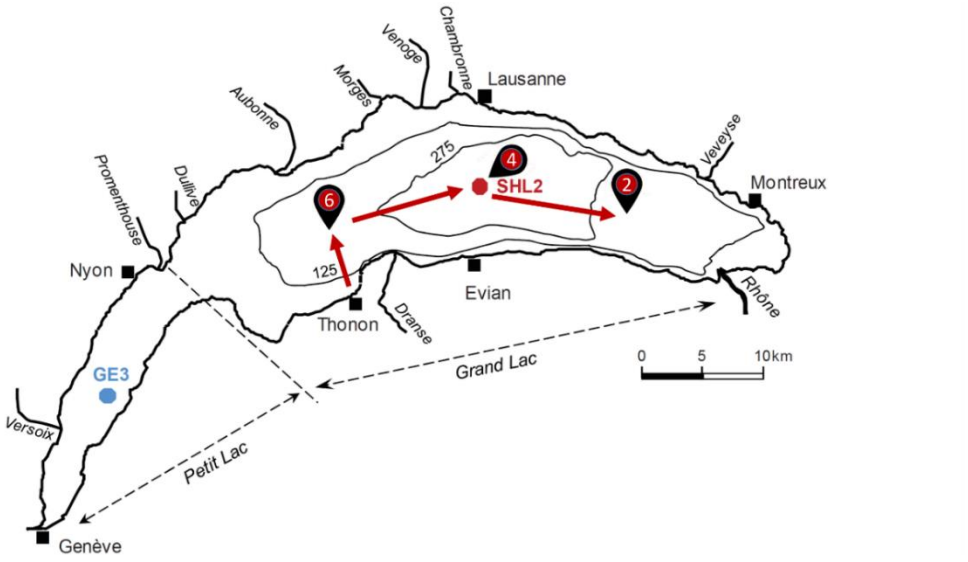
work is now required to investigate predator-prey relationships. Kandel et al (2014) reported that BALOs along with *Myxococcales* were highly abundant in aquaculture zero-discharge systems among other predators. In the same kind of ideas, BALOs were again classified into the Deltaproteobacterial families with the *Myxococcales* (myxobacteria) order a few years ago. In addition, Lowry et al. (2019) reported that *Bdellovibrio bacteriovorus* and *Myxococcus xanthus* shared homologous motility proteins of type IV pili that evolved and diverged to make BALOs a lone predator and *Myxococcus* a social predator. Morgan et al. (2010) described BALOs and myxobacteria as Gram-negative hunter specialists. We then wonder if BALOs and *Myxococcales* are somehow connected to each other or if competition for food has forced them to change their predatory strategy. Myxobacteria can be abundant (Curtis et al., 2007), have a social hunting strategy (wolfpack) *via* swarming motility (Spormann et al., 1999) and employ chemotaxis-like pathways (Berleman et al., 2008). They attack their prey from distance by producing antibiotics and lytic compounds that kill and decompose it (Sudo and Dworkin, 1972; Morgan et al., 2010). In contrast, BALOs are single, obligate and fast swimming predators (Jurkevitch and Davidov 2006) with a majority being periplasmic (endobiotic) predators. Moreover, chemotaxis was not demonstrated to be used by BALOs, rather, they randomly collide with their prey (Jashnsaz et al., 2017). BALOs's lytic enzymes are only produced when a prey is invaded (endobiotic) or anchored (epibiotic). Therefore, the impact of BALOs and Myxobacteria in Lake Geneva is likely to be different.

As a conclusion, we believe that despite their low abundance, BALOs can be highly efficient predators (Williams et al. 2016) and could play, at some time and depth, a significant role on structuring the prokaryotic community composition in Lake Geneva. Further studies will have to support this hypothesis.

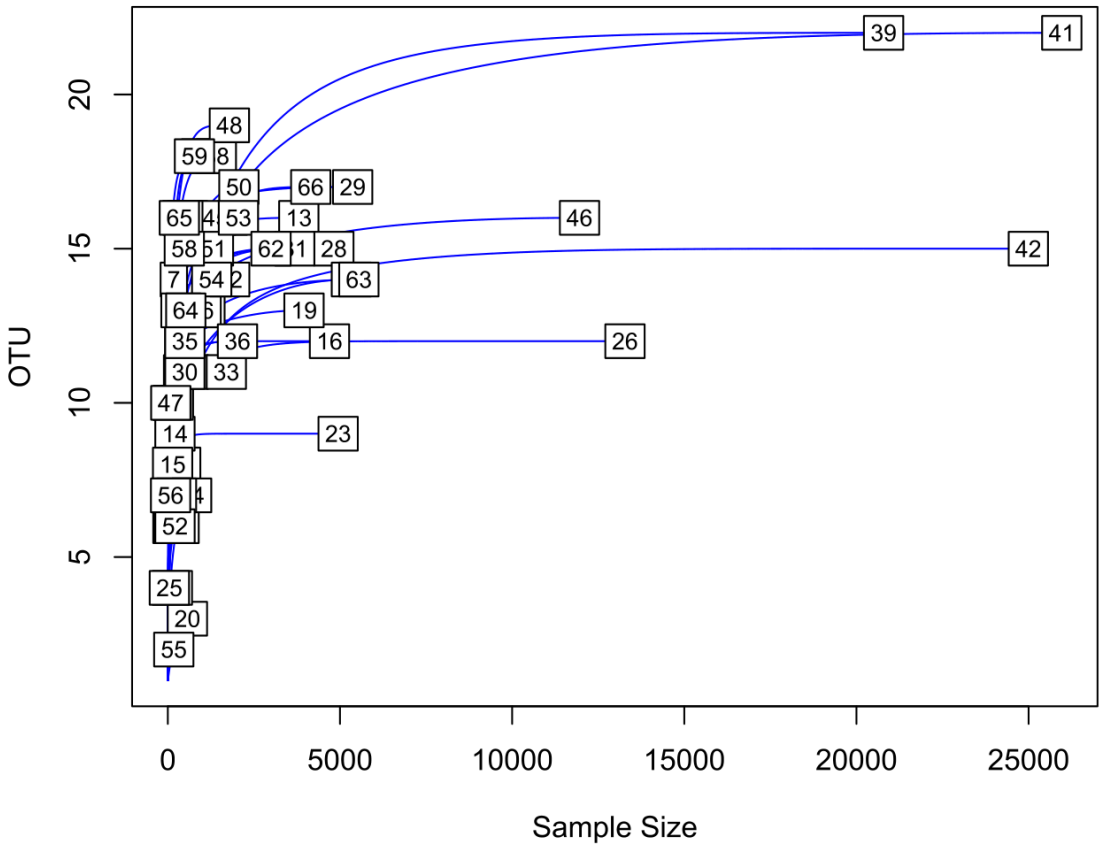
5. Data Availability Statement

BALOs generated sequences used to construct the phylogenetic tree can be found under NCBI accession numbers: MN617094 to MN617147.

6. Supplementary data



Supplementary Figure 1 Map of Lake Geneva and coordinates of the different sampling points selected during TRANSLEM: Site 2 (N 46° 26.206 / E 006° 46.848), Site 4-SHL2 (N 46° 27.207 / E 006° 35.654) and Site 6 (N 46° 25.061 / E 006° 24.957).



Supplementary Figure 2 Number of BALO reads and OTUs per sample. Sample 41 (November - pt4 - 15m - 5µm+2µm) owns the highest number of reads (25,964) and OTUs (22). Comparatively, Sample 55 (August - pt6 - 2m - 0.2µm) has the lowest number of OTUs, *i.e.*, 2. Sample 25 (June - pt6 - 15m - 5µm+2µm) has the lowest count of reads, *i.e.*, 44. At last, OTU_84_Bdellovibrionaceae was almost everywhere, in 62 out of the 66 samples.

Supplementary Table 1 Results of the Kruskal-Wallis and Dunn tests performed on alpha diversity indexes in order to detect significant differences between the tested variables (Site, Month, Depth, and Filter). The results show a significant difference for the Month (α= 5%; p-value= 0.009275) and Depth (α= 5%; p-value= 0.0020) variable for Simpson and InvSimpson index. Dunn test indicates that the differences lie within the June and November (α= 5%; p-value= 0.0067) communities for the Month variable, and within 2m and 200m (α= 5%; p-value= 0.002) for the Depth variable. Similar results were obtained for Shannon index. In addition, a significant difference was detected for the filter variable (α= 5%; p-value= 0.03489).

α diversity index	Condition tested	Kruskal-Wallis test (p-value)	Dunn test (p-value)	
Simpson and InvSimpson	Site	0.1768		
	Month	0.009275 *	0.0067 *	June ≠ November
	Depth	0.005877 *	0.0020 *	2 m ≠ 200 m
	Filter	0.1192		
Shannon	Site	0.1346		
	Month	0.01315	0.0053 *	June ≠ November
	Depth	0.02826	0.0114 *	2 m ≠ 200 m
	Filter	0.03489	0.0174 *	0.2µm ≠ 2µ+5µm
Pielou	Site	0.6982		
	Month	0.7756		
	Depth	0.1037		
	Filter	0.08455		

Supplementary Table 2 Results of ANOSIM and ADONIS tests. The tests were performed for the three sampled sites (pt2, pt4-SHL2 and pt6) along with the Month, Depth and Filter variable in order to validate the visual interpretation of the NDMS. The results show a significant difference within the Month (α= 5%; p-value= 0.001) and Depth (α= 5%; p-value= 0.001) for all samples sites.

	Site	Month	Depth	Filter
Anosim (p-value)	0.947	0.001 **	0.001 **	0.423
Adonis (p-value)	0.891	0.001 **	0.001 **	0.543

Supplementary Table 3 ANOSIM and ADONIS tests performed for the pt4-SHL2 site only, along with the Month, Depth and Filter variable in order to validate the visual interpretation of the NDMS. The results show a significant difference within the Month (α= 5%; p-value= 0.001) and Depth (α= 5%; p-value= 0.001) variables.

	Month	**Depth**
Anosim (p-value)	0.001 **	0.001 **
Adonis (p-value)	0.001 **	0.001 **

Supplementary Table 4 Results of the Simper test

Group		Most influential OTU	Kruskal-Wallis (p-value)	Simper cumulative contributions	Average abundances in each compared treatment	Average dissimilarity between the two treatment
Month	February – June	605_Micavibrionales	1.508e-07 ***	23%	February < June	80%
	February – August	382_Peredibacteraceae	6.661e-06 ***	21%	February < August	77%
	February - November	227_Bdellovibrionaceae	7.568e-06 ***	19%	February > November	79%
	June - August	605_Micavibrionales	1.508e-07 ***	22%	June > August	80%
	June - November	605_Micavibrionales	1.508e-07 ***	22%	June > November	83%
	August - November	382_Peredibacteraceae	6.661e-06 ***	20%	August > November	81%
Site	pt2 - pt4	227_Bdellovibrionaceae	0.9722	11%	pt2 < pt4	73%
	pt2 – pt6	382_Peredibacteraceae	0.3048	13%	pt2 < pt6	74%
	pt4 – pt6	382_Peredibacteraceae	0.3048	11%	pt4 < pt6	77%
Depth	2m – 15m	382_Peredibacteraceae	0.08855	17%	2m > 15m	77%
	2m – 200m	382_Peredibacteraceae	0.08855	14%	2m > 200m	88%
	15m – 200m	112_Bdellovibrionaceae	3.68e-09	12%	15m > 200m	83%
Filter	0.2µm - 2µ+5µm	382_Peredibacteraceae	0.8934	11%	0.2µm > 2µ+5µm	75%

Supplementary Table 5 Accession numbers of the sequence downloaded from NCBI to construct the phylogenetic tree

Sequence name	Accession number
Bdellovibrio bacteriovorus strain HD 127	AJ29276.1
Bdellovibrio sp. W strain ATCC 27047	AJ292518.1
Bdellovibrio bacteriovorus strain 109J	M61234.1
Bdellovibrio bacteriovorus strain HD100	NR_027553.1
Bdellovibrio exovorus strain JSS	EF687743.1
Bdellovibrio exovorus strain MPR11	MH230062.1
Peredibacter sp. Uncultured clone K2DN38	KT308262.1
Peredibacter sp. Uncultured clone C114001412	JX525305.1
Peredibacter sp. Uncultured clone BFB660	KC545751.1
Peredibacter sp. Uncultured clone C114001299	JX525192.1
Peredibacter starrii strain A3.12	NR_024943.1
Bacteriovorax stolpii strain Uki2	NR_115142.1
Bacteriovorax sp. EPC3	AY294222.1
Bacteriovorax sp. EPA	AY294220.1
Bacteriovorax sp. F2	AY294218.1
Bacteriovorax sp. strain DSM 12778	NR_042023.1
Micavibrio aeruginosavorus strain ARL-13	DQ186612.1
Micavibrio sp. EPB	DQ186613.1
Vampirovibrio chlorellavorus strain ICPB 3707	NR_104911.1

Conclusion du chapitre II

Au travers de ce chapitre, nous avons pu révéler que les BALOs sont bien présents dans les grands lacs péri-alpins, confirmant, semble-t-il, le caractère ubiquiste de ce type de bactéries. Grace à l'utilisation d'un couple d'amorces bactériennes universel, tous les genres de BALOs (mais aussi d'autres bactéries prédatrices) ont pu être été détectés dans le Léman. Grace aux couples d'amorces dédiés à la qPCR nous avons pu mettre en évidence que le genre *Peredibacter* est le plus abondant dans les lacs péri-alpins, suivi de loin par les *Bdellovibrio* et *Bacteriovorax*. Ces résultats étaient très prometteurs mais il manquait à ces travaux une anlayse plus fine et détaillée de la diversité génotypique des familles de BALOs, ainsi que de leurs abondnaces et distributions sur une période de temps plus consèqente. L'étape qui a suivi consista à créer et optimiser des amorces nucléotidiques spécifiques au BALOs et de les étudier sur une échelle de temps plus longue (une année complète).

Chapitre III

Quelle est la diversité réelle des BALOs et leurs abondances dans divers environnements ?

Chapitre III : Quelle est la diversité réelle des BALOs et leurs abondances dans divers environnements ?

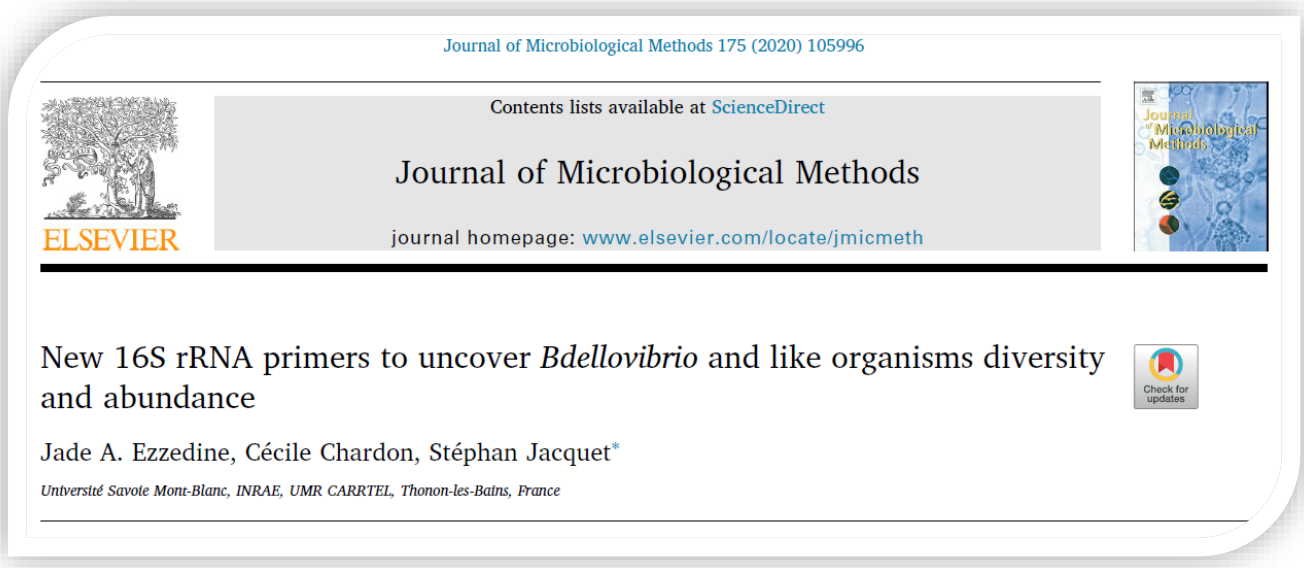
Contenu du chapitre

Ce chapitre est composé de deux articles scientifiques révélant (i) le travail réalisé pour dessiner, tester et utiliser de nouvelles amorces nucléotidiques spécifiques pour les différents genres de BALOs en accord avec la classification et les techniques de biologie moléculaire actuelles, puis (ii) l'étude de la diversité, distribution et abondance des BALOs et de la communauté procaryotique globale sur une année complète pour les lacs Léman et d'Annecy, et les sites MOLA et SOLA de la Baie de Banyuls sur Mer.

Objectifs et résumé de l'article 5

Une utilisation appropriée et spécifique des amorces nucléotidiques est importante pour évaluer la diversité et l'abondance des BALOs. Ainsi, afin d'être en accord avec la classification changeante des BALOs et des progrès techniques de biologie moléculaire, un travail important de développement, test et validation de nouveaux primers destinés au séquençage à haut débit et pour la PCR quantitative a été réalisé, avec pour objectif de décrypter en profondeur la diversité et les abondances de certains BALOs pour une variété d'écosystèmes. Ainsi, des couples de primers ont été conçus et testés avec succès pour le séquençage à haut débit des genres *Bdellovibrio* et *Peredibacter*, et pour la PCR quantitative pour le genre *Bacteriovorax*. D'autres couples de primers ciblant les abondances des genres *Peredibacter* et *Halobacteriovorax* ont également été dessinés et testés, révélant qu'ils sont fonctionnels. Cependant, ces primers nécessitent encore des optimisations car il existe peu de représentants de *Peredibacter* et *Halobacteriovarax* dans les bases de données pour dessiner des amorces hautement fiable. Ce travail n'a pas permis d'obtenir des couples d'amorces hautement spécifiques pour les autres genres de BALOs même si elles restent plus fiables pour la détection de tous les genres de BALOs comparativement aux amorces universelles. Suite à la création et validation de ces amorces, un premier résultat très original a été obtenu avec la détection du groupe des *Peredibacter* en milieu marin, ce dernier n'ayant à ce jour été décrit que dans les eaux douces. Si ce résultat est avéré et que la présence d'individus actifs de ce groupe est réelle dans les eaux côtières marines, une nouvelle remise à jour de la classification des BALOs pourrait encore être proposée. D'autres résultats portant sur les abondances des différentes familles de BALOs, pour une variété d'écosystèmes, ont confirmé que ce groupe des *Peredibacter* semble être le plus abondant dans les lacs péri-alpins. Prédominant en surface (2-3 m) dans le Léman, ce groupe s'est avéré plus abondant en profondeur à Annecy (45 m). Comparativement, les *Bdellovibrio* présentent une abondance globale inférieure à celle des *Peredibacter* mais leurs dynamiques sont assez similaires (sauf à 200 m). Le groupe des *Bacteriovorax,* quant à lui, est globalement moins important, détecté à Annecy, beaucoup moins au Léman et encore moins dans les eaux côtières marines testées. Chaque groupe a révélé des dynamiques très différentes suivant les écosystèmes, en lien, semble-t-il, avec la qualité et l'abondance des proies plutôt qu'avec des facteurs physico-chimiques.

New 16S rRNA primers to uncover *Bdellovibrio* and like organisms diversity and abundance



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New 16S rRNA primers to uncover *Bdellovibrio* and like organisms diversity and abundance

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1. Introduction

Among predatory bacteria, some are facultative and others are obligate predators (Jurkevitch and Davidov, 2006). The only known obligate predators belong to a group referred to as the *Bdellovibrio* and like organisms (BALOs). These Gram-negative bacterial cells are relatively small, rod-shaped and motile, and solely survive in natural ecosystems by predating other bacteria, here again Gram-negative bacteria (Davidov et al., 2006a; Jurkevitch, 2006b). Owing to their way of life and their ubiquitous distribution (Williams and Piñeiro, 2006), BALOs are suggested to act as an important "ecological balancer" on microbial communities (Iebba et al., 2013), sometimes comparable to the action of bacteriophages and/or protozoan grazers. It is noteworthy, however, that not all BALOs strains thrive in the same habitat; some may be excluded from some ecosystems (Williams and Piñeiro, 2006) such as the halo-tolerant family of *Halobacteriovoraceae* that have never been reported in freshwaters.

To study BALOs' diversity and abundance, molecular biology tools are required such as PCR-sequencing and quantitative PCR (qPCR). The application of these technics required the use of primers that can target specifically the different representatives of the BALOs. Indeed, the use of universal primers is not adapted since they cannot detect all prokaryotes (Baker et al., 2003; Ben-Dov et al., 2006; Klindworth et al., 2013). Besides, most BALOs are known to be cryptic rather than numerous in natural ecosystems (Kandel et al., 2014; Williams et al., 2016). Therefore, the use of universal primers is likely to overlook the diversity and abundance of less abundant bacteria such as the BALOs group (Ezzedine et al., 2020b).

Previous studies from eminent colleagues reported the design and use of primers targeting some BALOs (Jurkevitch and Ramati, 2000; Davidov et al., 2006a; Van Essche et al., 2009). During the recent years, however, the reclassification of BALOs and the development of novel sequencing technologies have rendered difficult the use of "old" primers and we attempted the challenge of designing a new generation of primers. Briefly, since 2000's the classification of BALOs evolved to encompass today two classes (Baer et al., 2000, 2004; Davidov and Jurkevitch, 2004; Koval et al., 2015; Hahn et al., 2017). The first class corresponds to the Oligoflexia (formerly δ-proteobacteria) and includes 5 genera: *Bdellovibrio, Bacteriovorax, Halobacteriovorax, Pseudobacteriovorax* and *Peredibacter*. The second class, the α-proteobacteria, holds a single genus, *i.e.*, *Micavibrio*. Regarding the sequencing technologies, we moved from the Sanger method to the era of high-throughput methods such as the Illumina sequencing approaches, capable of generating a huge amount of sequence data (Bleidorn 2017).

When looking into the bibliography to search for an adequate set of primers for BALOs, more specifically those targeting the 16S rRNA gene, three findings can be reported. Firstly, there are

no qPCR primers for each BALO genus. Secondly, one BALO primer can amplify multiple other BALOs. This is probably due to the old classification where multiple BALO species have been encompassed in the same genus. For example, *Bacteriovorax* primers 519F and 677R (Zheng et al., 2008) amplify also *Halobacteriovorax in silico*. Thirdly, the amplicon obtained from available PCR primers exceeds 400 bp (supplementary Table 1). For instance, the pair of primers Per 676F and Per 1443R targeting *Peredibacter* yield amplicons of 770 bp (Davidov et al., 2006a). Hence, these primers cannot be used for Illumina MiSeq 2 x 250 bp sequencing.

Here, we report the design of new primers compatible with qPCR and Illumina MiSeq sequencing approaches for a more in-depth analysis of a functional bacterial group still largely unknown in a variety of ecosystems.

2. Materials and Methods

2.1. BALOs primer design workflow for Illumina sequencing technology

The workflow explained below was applied to all primers designed for each BALO genus and subsequent PCR/MiSeq sequencing. However, for the sake of clarity, primers designed for *Halobacteriovorax* are given as an example thereafter. Firstly, the software Primer-Blast (Ye et al., 2012) was used to design primer pairs. *Halobacteriovorax* type species sequence of the 16S rRNA gene, *i.e., H. marinus* strain SJ (supplementary Table 2), was used as template to generate primers. The parameters set for the design were as follows: PCR product size between 350 and 400 bp to be compatible with a Miseq 2 x 250 bp run; primer melting temperature (Tm) with a minimum of 59°C, an optimal of 62°C and a maximum of 65°C; primer specificity stringency with at least 2 total mismatches to unintended targets, including at least 2 mismatches within the 5 base pairs at 3' ends; primer size between 18 and 24 bp with an optimal of 20 bp; primer GC content ranging from 40 to 60%; max poly-x set to 4 and the software asked to output 20 pairs of primers. The other parameters were left unchanged. In the next step, primers were placed in 3 sets of alignment made with Geneious 11.1.5 (https://www.geneious.com) to verify their specificity to the *Halobacteriovorax* genus. To get the first set of aligned sequences, the R Primer-Miner package (Elbrecht and Leese, 2017) was used to download all sequences of *Halobacteriovorax* from NCBI (Benson et al., 2013). For instance, *Halobacteriovorax* were downloaded using keywords such as "16s", "16S", "*Halobacteriovorax*", "*Halobacteriovoraceae"*. The script downloaded the sequences that met the requirements and then the sequences were dereplicated and clustered at a 97% identity threshold using Vsearch (Rognes et al., 2016). Clustered sequences were then verified by reassigning them with Mothur (Schloss et al., 2009) to arb-SILVA (Quast et al., 2013) database release r132. Unassigned or miss-assigned sequences were removed. The second set of alignment

was composed of 30 bacteria containing BALO type species and non-BALO sequences (supplementary Table 3), also downloaded from NCBI (Benson et al., 2013). These sequences were used to check if the designed primers amplify other bacteria than the targeted BALO genus. The third and final set of alignment was composed of BALO type species only. For example, *Halobacteriovorax* type species *H. marinus* SJ and *H. litoralis* JS5 were aligned together. A consensus sequence with 25% variability was created with these type species. Then the consensus sequence was mapped to the verified sequence of *Halobacteriovorax* and the bacterial alignment to see better where nucleotides were different. The 20 primers generated by Primer-Blast (Ye et al., 2012) were mapped to the three alignments as follows. Firstly, the primers were mapped to the sequences of the 30 bacteria found in the supplementary table 3. If the primer pairs matched regions of other bacteria than the targeted BALO, these primers were removed from the candidates. If the primer was specific enough (*i.e.*, both the forward and reverse sequences were very specific or, at minima, the forward or the reverse was highly specific), they were mapped to the type species alignment to see whether the primer could be degenerated to target all type species. For instance, one primer matched completely a region of *H. marinus* but needed to degenerate in the 6th position (M for A or C) to target also *H. litoralis*. Then, the primer was mapped to the *Halobacteriovorax* clustered and verified sequences to see how many other sequences the primer could target and if the primer needed further degeneration but without altering its specificity. The primer that seemed to be specific was tested to a bigger database containing bacterial sequences *via* the online tool TestPrime (Klindworth et al., 2013) of arb-SILVA (Quast et al., 2013). The "maximum number of mismatches" was set to 0. Furthermore, these primers were tested for their secondary structure. When possible the following rules were respected for hairpins, self-dimer and cross dimer: Hairpins, 3' end with a ΔG of -2 kcal/mol and an internal value with a ΔG of -3 kcal/mol; Self Dimer, 3' end with a ΔG of -5 kcal/mol and an internal with a ΔG of -6 kcal/mol; Cross Dimer, 3' end with a ΔG of -5 kcal/mol and an internal with a ΔG of -6 kcal/mol. Hairpin and self-dimer were checked using the online tool OligoAnalyzer (https://www.idtdna.com/OligoAnalyzer/). As for cross dimer, they were checked using NetPrimer from Biosoft (http://www.premierbiosoft.com/netprimer/). As a final step, an *in silico* PCR was performed with the primers on the type species sequence using the program SerialCloner 2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html). Among the 20 primers selected for each BALO genus, we selected the best 5 primer pairs for each BALO and ordered 3 of them from GATC/Eurofins. The list of the selected primers can be found in Table 2 and supplementary Table 1.

2.2. BALOs primer design workflow for quantitative PCR

The design of qPCR (SYBR Green) primers for *Bacteriovorax*, *Halobacteriovorax*, *Peredibacter* and *Micavibrio* was inspired by Thornton and Basu (2011). Primer3 web 4.1.0 (Untergasser et al., 2012) was used to design the primers. A type sequence of a BALO genus (supplementary Table 2) was used as template with the following parameters: product size range from 80 to 150 bp (shorter amplicons lengths gives higher PCR efficiencies (Thornton and Basu, 2011)); number of primers to return set to 20; primer size from 18 to 24 bp with an optimal size of 20 bp; primer Tm with a minimum of 60°C, a maximum of 65°C and an optimal of 62°C; maximum Tm difference set to 2°C; SantaLucia 1998 for table of thermodynamic; product Tm with an optimal of 50°C; primer GC% from 40 to 65% with an optimal of 60%; max self-complementarity set to 4; 3 for max 3'self-complementarity; 4 for max pair complementarity; 3 for max 3'pair complementarity; 3 for max poly-X; concentration of divalent cations set to 3.5; 0.2 for dNTPs concentration; objective function penalty weights for primers with Tm Lt = 1, GT = 1; Size Lt = 1, Gt = 1, self-complementary = 3, 3' self-complementary = 3, #N's = 2; and finally objective function penalty weights for primer pairs are product Tm Lt = 1, Gt = 1, Tm difference = 2, any complementary = 3 and 3' complementary = 3. Then, the generated 20 primers were checked for similarity and mapped on an alignment of 30 non-BALO bacteria and BALOs (supplementary Table 3) to verify their specificity (as detailed in the section above). Next, the suitable primers were mapped on the sequences of the targeted BALOs as detailed above. *A contrario* to the PCR primers, the qPCR primers were not degenerated to keep their specificity as much as possible. Primers were also checked with TestPrime (Klindworth et al., 2013) from arb-SILVA for specificity. Appropriate primers had their secondary structure verified with the online tool "Beacon Designer Free Edition" (http://www.premierbiosoft.com/qpcr/). When possible, primers with hairpin, cross dimer and self-dimer were discarded if their ΔGs were < -3.5 kcal/mol and/or if they tended to have 3 bp matched at the 3' end. At last, selected primers were verified *in silico* with Serial Cloner 2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html) and the generated amplicon was copied/pasted to UNAFold for secondary structure check (https://eu.idtdna.com/UNAFold). The temperature was set to 60°C and Mg concentration to 3 mM. Once again, when possible, amplicons with Tm superior to 60°C (temperature of hybridization) were discarded. Same as before, we ordered 3 primers out of 5 to test them in the laboratory. The final list of the designed primers can be found in Table 1 and supplementary Table 1.

2.3. Bdellovibrio and like organisms (positive control) strains and culture

To test the primers *in vitro* we tried to acquire some BALOs strains to serve as positive control. *Bdellovibrio bacteriovorus* HD100 and 109J as well as *B. exovorus*, *Micavibrio aeruginosavorus* ARL-13 and *Peredibacter* sp. were courtesy obtained from Prof. Jurkevitch Edouard laboratory. *Halobacteriovorax* sp. was kindly sent by Prof. Williams Henry N. laboratory. Except for *Halobacteriovorax* sp. all strains were cultured and multiplied using the double-layer agar method with suitable prey as recommended by Jurkevitch (2012). We were not able to acquire *Bacteriovorax* strains. We also used a *Bdellovibrio* sp. previously obtained from Lake Geneva (Ezzedine et al., 2020a). At last, a mock community sample was also constituted by pooling all available BALOs DNA, latter referred to as BALOs mix (supplementary Table 4).

2.4. Negative control of bacteria strain and culture

In order to test primers' specificity, negative controls were made with some bacterial strains (*i.e.*, *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 10536, *Hafnia alvei* ATCC 13, *Pseudomonas fluorescens* ATCC 13525 and *P. putida* ATCC 12633) purchased from the "Centre International de Ressources Microbiennes" (CIRM) (https://www6.inra.fr/cirm_eng/). *P. aeruginosa* was kindly sent by Prof. Jurkevitch. These bacteria were cultured on liquid LB medium (Trypton 10 g, Yeast extract 5 g, NaCl 10 g) and incubated at 25°C under low shaking conditions (200 rpm). *Vibrio parahaemolyticus* was kindly sent by Prof. Williams but not cultured. We also used *Pseudomonas* sp. previously isolated from Lake Geneva (Ezzedine et al., 2020a). At last, a mix of all the (negative control) bacterial strain DNA was also prepared (supplementary Table 4).

2.5. Environmental samples for PCR and qPCR tests

Four types of samples were used in amplifying the designed primers for PCR. A first sample corresponded to a pool of filtered water (*i.e.*, water filtered on 0.2 µm PC filter) from Lake Geneva taken at 2.5, 50, 200 m in February, May, July and November. A second mixed sample of water originated from Lake Annecy, also filtered on 0.2 µm PC filter, was taken at 3 and 45 m, in February, May, July and November. The third sample was a mixture of filtered water samples from the MOLA station in NW coastal Mediterranean Sea sampled offshore Banyuls-sur-mer (France) at 2, 80 and 200 m in March, April, July and November. The last sample was taken at another reference station in Banyuls bay, *i.e.*, SOLA, and was a mix of filtered waters taken at 2 and 24 m, in February, May, July and November. Samples used for qPCR tests were also from different locations covering a range of salinities (<1, ~15 and >35 g/L): Lake Geneva (taken at 2.5, 50 and 200 m on June 30th and July 30th 2019 and mixed to obtain a unique pool), the estuary of Arcachon bay (France) near Audenge (taken in April as a single sample), and in the English Channel close to the marine biological station of Roscoff.

2.6. DNA extraction

Before DNA extraction, BALOs pure cultures were filtered through 0.45 µm pore filter to remove other cells *i.e.,* prey. The other cultures (negative bacteria control) were not filtered, since they do not require a co-culture with other microorganisms for growth. Environmental samples were all filtered at 0.2 µm as mentioned before. Then <0.45 µm BALOs filtrates, culture of "negative control bacteria" and environmental samples filtrates were subjected to DNA extraction using a homemade protocol with GenEluteTM-LPA (Sigma-Aldrich) solution. Firstly, all samples were centrifuged for 3 min at 6,000g and 4°C and the supernatant was discarded. Then, 300 µL of TE buffer (TRIS: 1M – pH8, EDTA: 0.5M – pH8) were added to the pellet. Next, a lysis step was performed by adding 200 µL of lysis solution (TRIS: 1M – pH8, EDTA: 0.5M – pH8 and sucrose: 0.7M). After a thermic shock at -80°C for 15 min and at 55°C for 2 min, 50 µL of 10% sodium dodecyl sulfate (SDS) as well as 10 µL of proteinase K (20 mg/mL) were added. Samples were then incubated at 37°C for 1 h with gentle stirring, and placed in a heating block at 55°C for 20 min. After a quick centrifugation step (13,000 rpm at 4°C for 3 min), the supernatant was collected. Then, 50 µL of sodium acetate (3M – pH 5.2) and 1.5 µL of GenEluteTM-LPA (Sigma-Aldrich, 25µg/µL) was added. Next, one volume of isopropanol was added and the tubes were centrifuged for 10 min at 12,000 g and 4°C. Following this step, two rounds of ethanol (80%) washing was carried out to clean the DNA pellet. The remaining ethanol was evaporated using a SpeedVac for 20 min. Finally, 30 µl of TE was added and samples were incubated at 37°C for 1 h to let the pellet gently dissolve into the TE buffer. DNA concentration was measured using NanoDrop 1000 spectrophotometer. For DNA concentration superior to 25 ng/µL, a dilution was performed. All DNA preparations were stored at -20°C until analysis.

2.7. PCR amplification (primers optimization and Nano MiSeq run preparation)

We used a gradient of temperature for PCR conditions and determined that the optimum annealing temperature for BALO DNA amplification was around 58-60°C. The chosen protocol for BALOs amplification consisted of a PCR mixture volume set at 25 µL with reagent final concentration as follows: 1x buffer, 0.2 mM dNTP, 3mM $MgCl_2$, 0.3 mg/mL bovine serum albumin (BSA), 0.2 mM of Forward and Reverse primer, 0.625 U Biotaq DNA polymerase (Bioline) and 1 µL of DNA template concentrated at 25 ng/µL. Negative control and when possible a positive control (BALO isolates) were included in the protocol. The PCR program adapted from Davidov et al. (2006) was as follows: 94°C – 5 min, 35 x (94°C – 1 min, 58°C – 1 min, 72°C – 3 min) and with a final extension step at 72°C for 5 min. Primers Bd pP2, Mica pP5, Bx pP3, Per pP1 and Hbx pP2 (Table 2) were thought to be the most promising primers for BALOs specific amplification. Since these primers are meant for sequencing *i.e.*, Miseq, we chose to test them directly *in situ via* a Nano Miseq run. Thus, following the instructions of the sequencing platform, the samples were prepared

and sent to GenoToul (GeT-PlaGe, INRAE, Toulouse, France). In brief, 25 samples were sent to GenoToul but only 18 were successfully sequenced. The successful samples were: Bd pP2 amplification of Lake Geneva, Annecy, SOLA and mock community for *Bdellovibrio*; Per pP1 amplification of Lake Geneva, Annecy, MOLA and SOLA for *Peredibacter*; Bx pP3 amplification of Lake Geneva and Annecy for *Bacteriovorax*; Hbx pP2 amplification of Lake Geneva, MOLA and SOLA for *Halobacteriovorax* and finally Mica pP5 amplification of Lake Geneva, Annecy, MOLA, SOLA and mock community for *Micavibrio* (see Results).

2.8. qPCR amplification, cloning-sequencing and taxonomic assignation

The Rotor-Gene Q machine (Qiagen) and a SYBR Green PCR kit (Qiagen) were used to test BALO qPCR primers. After optimization, the volume of the reaction was set to 25 µL and the reagent final concentration was 1x for SYBR Green master mix, 0.2 µM for forward and reverse primers, 0.3 mg/mL for BSA and 1 µL of template DNA. The qPCR program was 95°C – 15 min, 40 x (95°C – 45 sec, 62°C – 45 sec, 72°C – 45 sec) and 60 to 95°C with +1°C every 5 sec. After many tests, qPCR primers Bx qP5, Hbx qP4, Per qP5, Mica qP1 and Mica qP4 (Table 1) were selected as the best candidates to get the specific amplification of dedicated BALOs. The qPCR products generated using these primers were purified with GE healthcare illustra GFX according to the manufacturer's instructions and then cloned using TOPO TA Cloning kit (Thermo Fisher Scientific) following the manufacturer's recommendation. The cloning here is meant to reveal the specificity of each set of primers. For primer Per qP5 targeting *Peredibacter*, 20 clones were selected. As for primers Bx qP5, Hbx qP4, Mica qP1 and qP4 targeting *Bacteriovorax*, *Halobacteriovorax* and *Micavibrio* respectively, 19 clones were chosen. The inserts were sequenced by Sanger technology at GATC/Eurofins. The obtained sequences were dereplicated using Vsearch (Rognes et al., 2016) and then the taxonomic assignment was carried using NCBI BLASTn (Altschul et al., 1990).

2.9. Bioinformatics pipeline

The Nano-Miseq paired-end sequencing of the 18 samples from section 2.7 resulted in two files R1 and R2, each contained 400,519 reads in fastq format. The files were processed using the Frederic Mahé pipeline found at https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline. The Text box number 1 in the supplementary data describe briefly the workflow of the pipeline. The OTU tables (available on the Zenodo repository) were filtered by removing chimera sequences, singletons, sequences with less than 90% identity to the database and sequences with a < 0.0002 quality score. The figures used for the analysis of the reads were drawn on R version 3.5.0 (R Core Team, 2018) with ggplot2 (Wickham et al., 2018).

2.10. Phylogeny

The assigned qPCR clones sequences for each set of primer were phylogenetically related to BALO reference sequences. Supplementary Table 5 shows the reference BALOs sequences used in building these trees. All the alignment files are available in the Zenodo repository cited in the "Data accessibility" statement. Assigned qPCR clones and reference sequences were first aligned together using MUSCLE algorithms *via* MEGA7 (Kumar et al., 2016). The ends of all sequences were trimmed at 5' and 3' to make the aligned sequences of equal length. The alignment was only improved for *Bacteriovorax* qPCR amplicons using Gblocks 0.91b program (Castresana, 2000), where it kept 43 positions from 87, with "Minimum Length of a block" set to 5. Gblocks were not used on *Peredibacter* (1297 positions) and *Halobacteriovorax* (1103 positions) alignment, since alignment contained too many gaps, which made Gblocks discard many positions. Next, ModelGenerator v.85 (Keane et al., 2006) was used to select the best nucleotide substitution model with discrete gamma categories set to 4. Corrected Akaike information criterion (AICc) (Akaike, 1973) defined our model selection. The models used for *Bacteriovorax*, *Halobacteriovorax* and *Peredibacter* trees were K80 + G (0.46), GTR + G (0.29) and TrN + G (0.44), respectively. The constructed trees were built using the Maximum likelihood method (100 bootstrap replicates) with PhyML 3.1 (Guindon et al., 2010) and Bayesian method (500,000 generations and 25 % burn-in value) using MrBayes 3.2.7a (Ronquist et al., 2012). The same workflow was applied to the assigned OTUs of the Nano MiSeq sequencing run. For the OTUs assigned to *Bdellovibrio*, *Bacteriovorax*, *Halobacteriovorax* and *Micavibrionales,* Gblocks kept respectively 192 out of 342, 251 out of 277, 197 out of 343 and 224 out of 376 positions. Gblocks was not used on *Peredibacter* alignment because it removed too many positions. The best nucleotide substitution model for *Bdellovibrio*, *Bacteriovorax*, *Halobacteriovorax, Micavibrionales* and *Peredibacter* selected under AICc were GTR + G (0.67), GTR + G (0.41), TrN + I (0.36) + G (0.54), TrN + I (0.33) + G (0.39) and GTR + G (0.40), respectively. The ML phylogeny was constructed with 100 bootstraps and the Bayesian phylogeny was run with 2 million generations and a 25% burn-in value.

3. Results

3.1 qPCR primers specificity check and Sanger sequencing results

Primers for qPCR were designed to grasp the abundances of BALOs in environmental ecosystems. The amplicons were chosen to be short in order to get high PCR efficiencies. All the selected primers were first checked for specificity *in vitro* on targeted BALOs, not-targeted BALOs and other bacterial strains using qPCR amplification *i.e.*, melting-curves. Then, the most specific

among them were chosen to have their amplicons sequenced using the Sanger method (Table 1). The assignment of the sequences of the clones confirmed whether these primers are perfectly specific or not to the targeted BALOs as first observed with qPCR amplification.

For *Bacteriovorax*, *in vitro* amplification of the primer Bx qP5 showed no amplification of bacterial strains (negative control) and other BALOs that were not *Bacteriovorax* (not shown). In addition, Lake Geneva sample was amplified by Bx qP5. Despite not having a positive control, the *in vitro* result seemed encouraging. Indeed, after sequencing the Lake Geneva sample and assigning the clones, the amplification proved to be very specific to *Bacteriovorax*. *In fine*, all clones were assigned to *Bacteriovorax* sp. according to NCBI BLASTn (not shown). Therefore, the specificity of Bx qP5 primers toward *Bacteriovorax* is 100% (Table 1). Moreover, the phylogenetic tree (supplementary Figure 1) confirmed that the assigned clones were phylogenetically close to *Bacteriovorax stolpii* Uki2 and *Bacteriovorax.* sp. F2.

For *Peredibacter*, although Per qP5 primer did not amplify the positive control, a good specificity was also observed (supplementary Figure 2). Typically, there was no non-specific amplification and all clones were assigned to *Peredibacter* sp (not shown). Therefore, the specificity value of this set of primer is 100% (Table 1). The phylogenetic tree (supplementary Figure 3) shows that clone 9 is phylogenetically related to *Peredibacter* species. However, the other clones were not resolved on the tree. In this case, the phylogenetic tree did not confirm the result as seen previously with primer Bx qP5.

The exact *in vitro* results can be reported for *Halobacteriovorax* when using the primer Hbx qP4 (not shown). Amplicons from the Bay of Arcachon were sequenced and resulted in 83% of specificity toward *Halobacteriovorax* (Table 1). Briefly, 1 clone was empty, 15, 2 and 1 clone was assigned to *Halobacteriovorax* sp., Alteromonadales and *Bacteriovorax* sp. respectively. Here, the assigned clones of *Halobacteriovorax* were phylogenetically closer to *Peredibacter* species rather than *Halobacteriovorax* (supplementary Figure 4).

Finally, the Mica qP1 primer for *Micavibrio* showed high specificity for the positive control and Lake Geneva water sample after 12-17 cycles, but non-specific amplification appeared after approximately 30 cycles (not shown). The non-specific amplification was at the same temperature when observing the melt curve (not shown). The assignation result of the 19 clones was as follows: 3, 2 and 14 clones matching with *Inquilinus*, *Micavibrio* and "Uncultured bacterium", respectively. Said differently, only 10.5% of the clones were identified as *Micavibrio* (Table 1). The second tested primer, Mica qP4 also amplified *Micavibrio* positive control after 12 cycles but, here again, non-specific amplification occurred with a larger number of cycles. The results of the assignment of the generated amplicons were all identified as *Brevundimonas sp* and none as *Micavibrio.* Hence, Mica qP4 had 0% specificity toward *Micavibrio* (Table 1).

Table 1 BALOs potential primer sets designed for quantitative PCR (qPCR)

Primer qPCR	*E. coli* location (bp)	Sequence (5'-3')	Target BALOs	Product length (bp)	No. of target BALOs detected	Specificity (%)[a]
Bx qP5 F Bx qP5 R	421 482	Fw CGGTCTGTAAAGCTCTGTTAATGT Rv GGTGCTTCCTCTATGTGTACCA	*Bacteriovorax*	~ 84	19 (from 19 clones)	100
Hbx qP4 F Hbx qP4 R	220 279	Fw CCAAATGATGAGCCTGCGTAG Rv TCTCAGACCAGCTAAGCATCG	*Halobacteriovorax*	~ 80	15 (from 18 clones)	83.3
Per qP5 F Per qP5 R	627 696	Fw AAACTGCGTCTGAAACTGCT Rv TGTTCCTTCACATCTCTACGGA	*Peredibacter*	~ 91	20 (from 20 clones)	100 *
Mica qP1 F Mica qP1 R	737 808	Fw ACTGGACTGGTATTGACGCT Rv TAGCACACATCGTTTACGGC	*Micavibrio*	~ 91	2 (from 19 clones)	10.5
Mica qP4 F Mica qP4 R	1301 1413	Fw TCAGATTGTCCTCTGCAACTC Rv TCAGGTAGAACCAACTCCCA	*Micavibrio*	~ 132	0 (from 19 clones)	0

a. Specificity = (Number of non-target strains undetected / Total number of clones) x 100

*. The BLASTn assigned all the clones as *Peredibacter*, however when constructing phylogenetic tree only one clone seems to be closely related to *Peredibacter* species. The other clones could not be resolved correctly on the tree

3.2 PCR primers specificity check and 2x250 NanoV2 MiSeq results

To study BALOs diversity, we designed primer sets compatible with PCR/Miseq 2 x 250 bp. The selected primers (Table 2 and supplementary Table 1) were first tested in PCR conditions with targeted BALOs (positive control, when available), other BALOs and non-BALOs bacteria to select the most specific and promising primer pairs (Table 2). Then, since these promising primers are intended for sequencing, the amplified amplicons were sequenced with Nano-Miseq to check how they perform and to validate or not their specificity toward the targeted BALOs. The sequencing of the 18 samples generated 400,519 reads. The reads were analyzed per primer set and OTUs tables were generated.

Table 2 BALOs potential primer sets designed for Illumina sequencing MiSeq (or PCR)

Primer PCR	*E. coli* location (bp)	Sequence (5'-3')	Target BALOs	Product length (bp)	No. of target BALOs detected	Specificity (%)[a]
Bd pP2 F Bd pP2 R	186 481	Fw TGCGGMTCTAGGGGTYAAAG Rv CGATCCTTTCTTRCAKGGTACMTT	*Bdellovibrio*	~ 291	121 (from 121 OTUs)	100
Per pP1 F Per pP1 R	1024 1349	Fw TGCCCGCAAGGGAATGTAGT Rv GGAGCGTGCTGATCTCCGAT	*Peredibacter*	~ 346	91 (from 91 OTUs)	100
Bx pP3 F Bx pP3 R	584 874	Fw GCGGACCTGCAAGTCAGATG Rv CGTACTTCCCAGGCGGAACA	*Bacteriovorax*	~ 311	69 (from 148 OTUs)	46.6
Hbx pP2 F Hbx pP2 R	253 607	Fw GGTGGGGTAAYGGCCTACCA Rv CGRGGTTGAGCCCCGAGATT	*Halobacteriovorax*	~ 375	7 (from 157 OTUs)	4.5
Mica pP5 F Mica pP5 R	132 500	Fw TGCCCTTAGGTGCGGAACAA Rv GGCACGAAGTTAGCCGGAG	*Micavibrio*	~ 349	27 (from 224 OTUs)	12.1

a. Specificity = (Number of non-target strains undetected / Total number of OTUs) x 100

Among the selected primers for *Bdellovibrio*, primer set Bd pP5 amplified not only *in vitro* some BALOs but also other organisms such as *Pseudomonas* sp. (supplementary Figure 5). *A contrario,* primer set Bd pP2 amplified only BALOs especially *Bdellovibrio* spp. Disregarding of amplifying the *Micavibrio* DNA and not seeing any band appearing when environmental DNA was tested, Bd pP2 was retained for Nano MiSeq 2 x 250 sequencing. It is noteworthy that this primer did not amplify DNA from the MOLA sample so that no sequences were obtained for this site. However, it successfully amplified DNA from Lakes Geneva and Annecy, the Mock sample (mix of BALO DNA) and SOLA. The bioinformatics analysis resulted in 121 OTUs, all assigned without exception to the *Bdellovibrio* genus. Hence, Bd pP2 specificity value is 100% toward *Bdellovibrio* (Table 2). Also, the phylogenetic tree (supplementary Figure 6) confirmed the assignment results with a majority of OTUs closely related to *Bdellovibrio*. Furthermore, the bar plot in Figure 1 (left) shows that the *Bdellovibrio* individuals in the mock community were well detected. In addition, Lake Geneva holds the highest number of raw reads for the *Bdellovibrio.* Lake Annecy classified as second and the SOLA station displayed fewer reads.

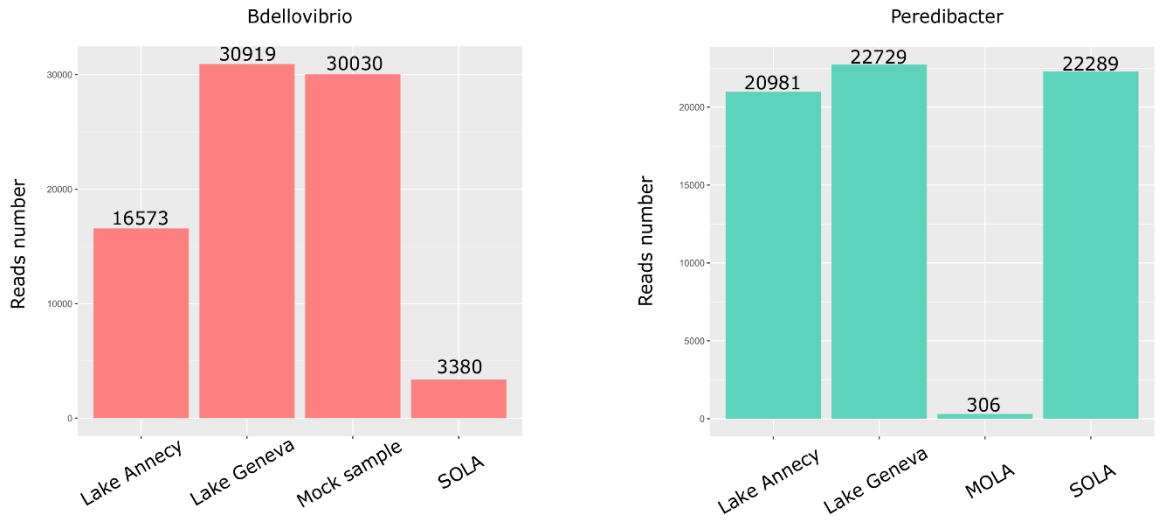


Figure 1 Number of *Bdellovibrio* (left) and *Peredibacter* (right) raw reads in different environments amplified respectively by the designed primer Bd pP2 (Bd p186F – p481R) and Per pP1 (Per p1024F – p1349R). *Bdellovibrio* is highly detected in Lake Geneva as in the mock sample (pool of BALOs DNA). However, SOLA is characterized by a lower number of *Bdellovibrio* raw reads compared to other ecosystems. Since no other species were detected the *Bdellovibrio* primer set is specific. As for *Peredibacter*, the number of raw reads is almost equivalent in the 3 ecosystems *i.e.* Lake Annecy, Lake Geneva and SOLA, while MOLA yielded fewer reads. The disparity in the number of reads could be from three origins: sequencing depth, primers behavior and rarity of the targeted DNA. Here again, the primer set used in detecting *Peredibacter* is specific.

Despite not amplifying the *in vitro* positive control (*Peredibacter* sp. DNA) and showing amplification to the isolated *Bdellovibrio* sp. from Lake Geneva (Ezzedine et al., 2020a), the *Peredibacter* primer Per pP1 was the most specific to BALOs DNA in comparison to the two other ordered primers (supplementary Figure 1). *In silico*, the amplicon size obtained was around 346 bp. In some samples, non-specific bands on the agarose gel were observed (not shown) above or below the targeted size. However, the environmental samples from Lake Geneva and the Bay of Arcachon were amplified around 350 bp. Therefore, the primer set Per pP1 was tested by sequencing. The mock sample was not sequenced since no amplification was visible when constructing the libraries. The results of the sequencing and the bioinformatics analysis gave 91 OTUs which were all assigned to the *Peredibacter* genus. Thus, the specificity of Per pP1 toward *Peredibacter* is 100% (Table 2). Once more, the phylogenetic tree (supplementary Figure 7) agreed with the assignment results. However, some OTUs revealed to be phylogenetically related to *Halobacteriovorax* and *Bacteriovorax* sp. EPA and EPC3. Furthermore, when analyzing the number of reads generated upon the detection of *Peredibacter* DNA (Figure 1 right), the results showed that Lake Geneva, Lake Annecy and the marine SOLA station had high and approximately equal number of raw reads. On the contrary, only a small amount of reads were amplified for MOLA sample.

For the *Batceriovorax* primers set we did not test them on a positive control since we could not obtain one. However, after analyzing the agarose gel of each primer set (supplementary Figure 1), primer Bx pP3 revealed itself as the most specific primer set toward the tested BALOs strains. Despite amplifying BALOs DNA other than *Bacteriovorax* the primer Bx pP3 was considered as promising. Also we noted, that some non-specific bands were visible but as previously explained for *Peredibacter*, they were above or below the targeted size (not shown). The sequencing was carried and the results showed that the primer amplified not only *Bacteriovorax* but also *Peredibacter* at high quantity, especially for the sample from Lake Annecy (Figure 2 left). This result was surprising since no band was visible on the electrophoresis gel when *Peredibacter* DNA was tested. On the other hand, for Lake Geneva, *Bacteriovorax* was amplified almost equally as *Peredibacter* in terms of raw reads (Figure 2 right) and OTUs. Aside from amplifying *Bacteriovorax* and *Peredibacter*, we noted that two sequences were assigned to *Bdellovibrio* and 6 to other bacteria. In summary, the specificity of Bx pP3 toward *Bacteriovorax* is at 46.6% (Table 2). The phylogenetic tree (supplementary Figure 8) was in accordance with these results. The assigned OTUs clustered either with *Peredibacter* or *Bacteriovorax*. However, some OTUs seemed to be related more to *Halobacteriovorax* than to *Bacteriovorax* or *Peredibacter*.

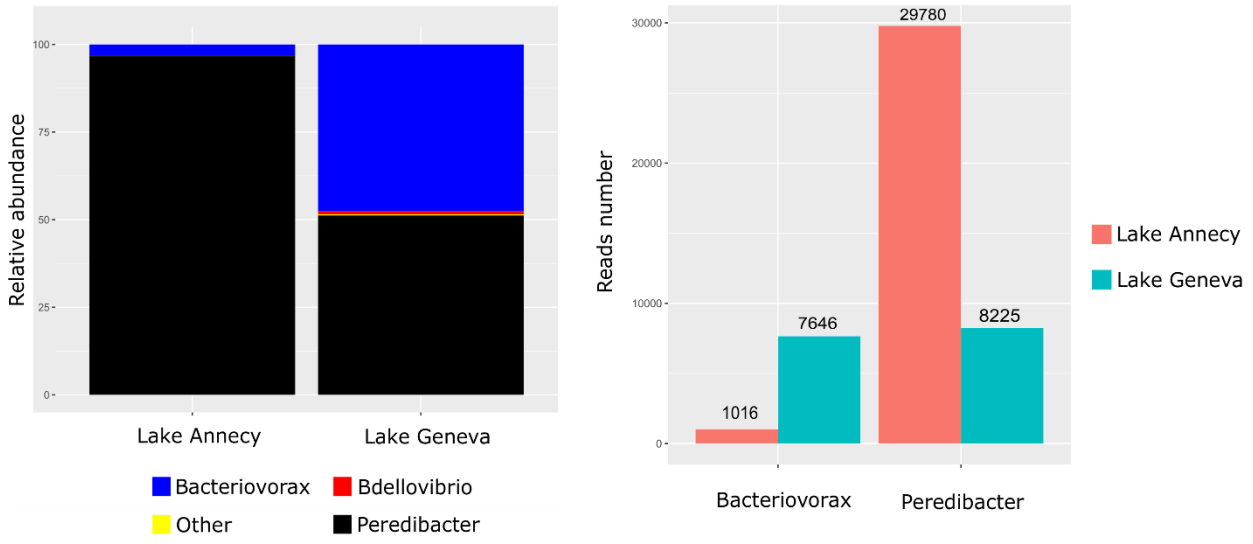


Figure 2 Specificity of the designed primer Bx pP3 (Bx p584F – p874R) toward *Bacteriovorax* in different environments (left) illustrated with relative abundance. We can note that Bx pP3 amplified not only *Bacteriovorax* but also *Peredibacter* especially in Lake Annecy. Other bacteria and other BALOs are not significantly amplified. The bar plot on the right shows the distribution of raw reads using Bx pP3. *Bacteriovorax* is more amplified in Lake Geneva than in Lake Annecy. However, *Peredibacter* reads are overall more abundant and especially in Lake Annecy. In conclusion, the primer set Bx pP3 is only half specific toward *Bacteriovorax*.

On one hand, the *Halobacteriovorax* primer set Hbx pP2 did not amplify non-BALO DNA (not shown). In another hand, Hbx pP2 did not amplify the positive control sample containing *Halobacteriovorax* sp. DNA. Also, it amplified were it should not *i.e.*, Lake Geneva. Results from sequencing revealed that it mainly amplified other bacteria and BALOs than *Halobacteriovorax* (Figure 3 left). Among the few detected BALOs, Figure 3 (right) revealed that *Bacteriovoracaceae*, *Peredibacter* and *Bacteriovorax* were overall more detected than the *Halobacteriovorax* itself. Again, few reads and OTUs (7 out of 157) were assigned to *Halobacteriovorax*. Besides, some OTUs were not assigned to *Halobacteriovorax* but to "Marine BALOs" (6 OTUs). In short, the specificity of Hbx pP2 is only at 4.5% toward *Halobacteriovorax* (Table 2). A phylogenetic tree (supplementary Figure 9) was constructed with both *Halobacteriovorax* and "Marine BALOs" reads. In majority, *Halobacteriovorax* reads clustered with *Halobacteriovorax marinus* SJ but two were placed next to *Micavibrio*. Furthermore, the "Marine BALOs" were not phylogenetically related to *Halobacteriovorax* but *Bacteriovorax* and *Peredibacter*.

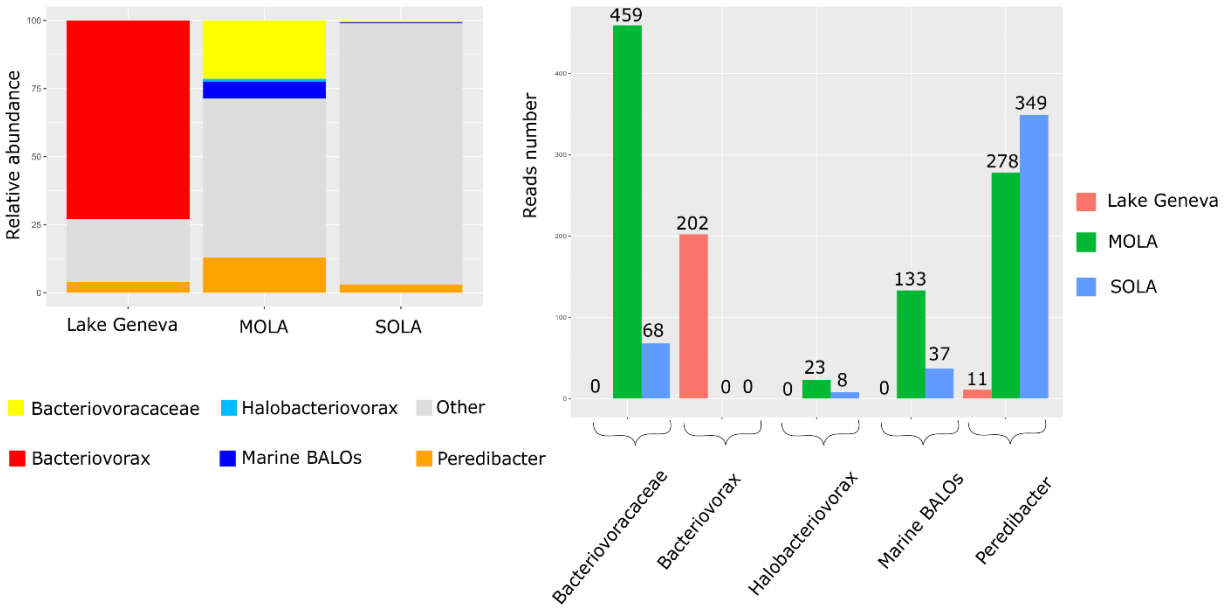


Figure 3 Specificity of the designed primer Hbx pP2 (Hbx p253F – p607R) toward *Halobacteriovorax* or/and marine BALOs in different environments (left) with relative abundance. *Halobacteriovorax* reads are majority detected at MOLA. Few sequences are detected at SOLA and none in Lake Geneva. Unassigned marine BALOs were also detected in MOLA and SOLA. Overall, the primer amplifies more other bacteria than *Halobacteriovorax*. The bar plot on the right shows the distribution of raw reads number of detected BALOs in the three selected ecosystems. *Halobacteriovorax* and marine BALOs DNA are poorly detected. *Bacteriovoracaceae* and *Peredibacter* are more detected in the three different environments than *Halobacteriovorax*. In conclusion, this primer set is not specific to *Halobacteriovorax*.

At last, the *Micavibrio* primer Mica pP5 revealed *in vitro* a specific amplification for the *Micavibrio* DNA (*i.e.*, the positive control). However, results from the sequencing revealed that other bacteria than *Micavivibrio* were also amplified. The *Micavibrio* DNA was exclusively amplified in the mock sample as shown in Figure 4 (left). However, only a few *Micavibrio* were detected in the 4 natural environmental samples. Among the 224 obtained OTUs, only 27 were assigned to *Micavibrionales*. Besides, unlike previous assignments, *Micavibrio* OTUs were only assigned to the order level *i.e. Micavibrionales* except of the first OTU which represented the *Micavibrio* used in the mock sample. Also, this OTU was poorly detected in natural samples. However, other *Micavibrionales* seemed to be more detected. According to Figure 4 (right), the SOLA station revealed a higher number of reads for *Micavibrionales* than the other ecosystems. Overall, the specificity of Mica pP5 toward *Micavibrio* in environmental samples is 12.1%. The phylogenetic tree (supplementary Figure 10) showed that the majority of *Micavibrionales* detected OTUs were closely related to *M. aeruginosavorus* ARL-13 and *Micavibrio* sp EPB, especially OTUs 372, 340, 440, 400, 145 and 200. However, some OTUs were shown to be phylogenetically closer to *Bdellovibrio exovorus* MPR11 than *Micavibrio*.

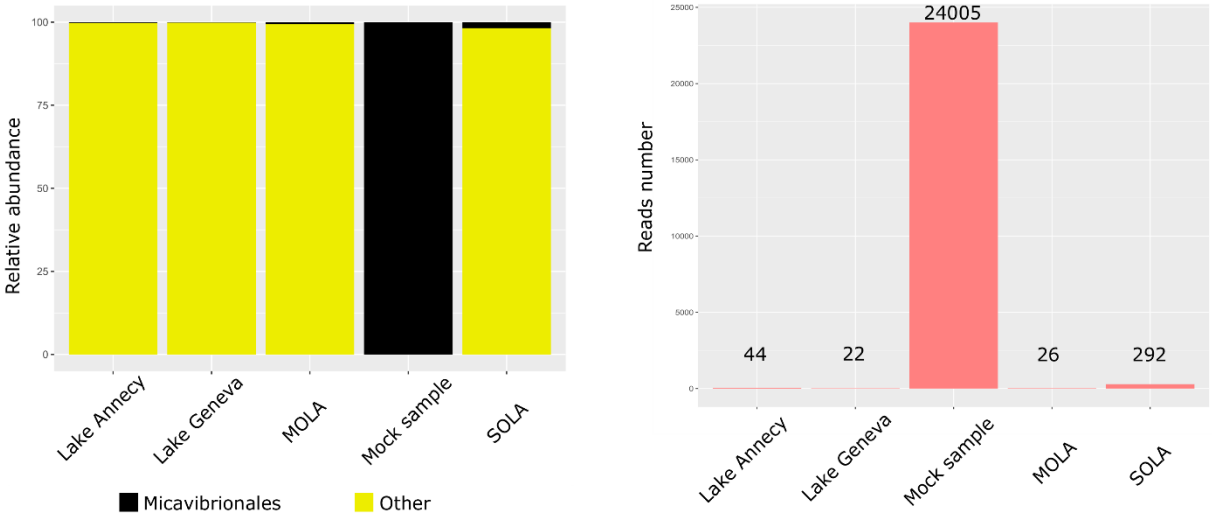


Figure 4 Primer Mica pP5 (Mica p132F – p500R) significantly amplify *Micavibrionales* when they are abundant in the mock sample. The primer set is very specific towards the *Micavibrionales* despite the presence of other BALOs. *A contrario*, we can see that in the environmental samples *Micavibrionales* are not abundantly amplified. The bar plot on the right shows that *Micavibrionales* raw reads numbers are more numerous at SOLA than in other natural ecosystems. The detection of *Micavibrio* is poorly undertaken by this set of primers.

4. Discussion

To study BALOs diversity and abundance with the current classification of the *Bdellovibrio* and like organisms group and the new technological advances, we designed and tested *in silico* and *in vitro* new specific primers for both qPCR and PCR/MiSeq sequencing. Most importantly, we used Sanger method (qPCR) and Nano Miseq (PCR) sequencing to reveal what exact taxa hid behind the obtained amplicons. Hence, attributing a specificity value for each promising set of primers. By designing specific primers, amplification biases were minimized and the recovery of BALOs taxa maximized (Elbrecht et al., 2019). Globally, some of our primers worked properly for the different tested ecosystems and we are confident they could be applied successfully to other systems, even if further testing and optimization may be required.

We successfully designed qPCR primers for *Bacteriovorax* as the taxonomic assignment and phylogenetic results showed. For *Halobacteriovorax* and *Peredibacter* their specificity is as good as the NCBI taxonomic assignment indicated since a large number of the sequences of the clones could not be resolved phylogenetically. Therefore, the use of *Halobacteriovorax* and *Peredibacter* set of primers should be handled with utmost caution. On the other hand, *Micavibrio* qPCR set of primer Mica qP1 and Mica qP4 showed very poor and no specificity at all toward *Micavibrio*. Thus, these primers should not be used to study *Micavibrio* abundance. Finally, we did not design qPCR primers for *Bdellovibrio* because of the primer pair Bd347–Bd549, made by Van Essche et al., 2009 (supplementary Table 1), is already specific for *Bdellovibrio*. Also, the group of *Pseudobacteriovorax* was not considered here since, to the best of our knowledge, this group was only reported to be associated with the octocoral *Antillogorgia elisabethae* that inhabit tropical waters (McCauley et al., 2015). It is noteworthy that no standard curves were used or optimized since the main aim of the study is to design primers and not yet reveal the abundances of BALOs in the considered ecosystems. On the other hand, as an explanation as to why some clones sequences for *Peredibacter* and *Halobacteriovorax* could not be resolved on the phylogenetic trees is that the length of the sequences obtained from Sanger sequencing is short, typically around 80 bp. The sequences could be also of poor quality. Most importantly, the alignment of the sequenced clones, with the reference sequences generated too many gaps. The alignment files were deposited in the Zenodo repository so that the reader can independently judge the quality of the alignment. Lastly, the location of these primers according to TestPrimer from arb-SILVA on the 16S sRNA gene of *E. coli* is represented in Figure 5. *In fine*, Bx qP5 primers are named Bx q421F and Bx q482R, Hbx qP4 primers are named Hbx q220F and Hbx q279R, and Per qP5 primers are named Per q627F and Per q696R.

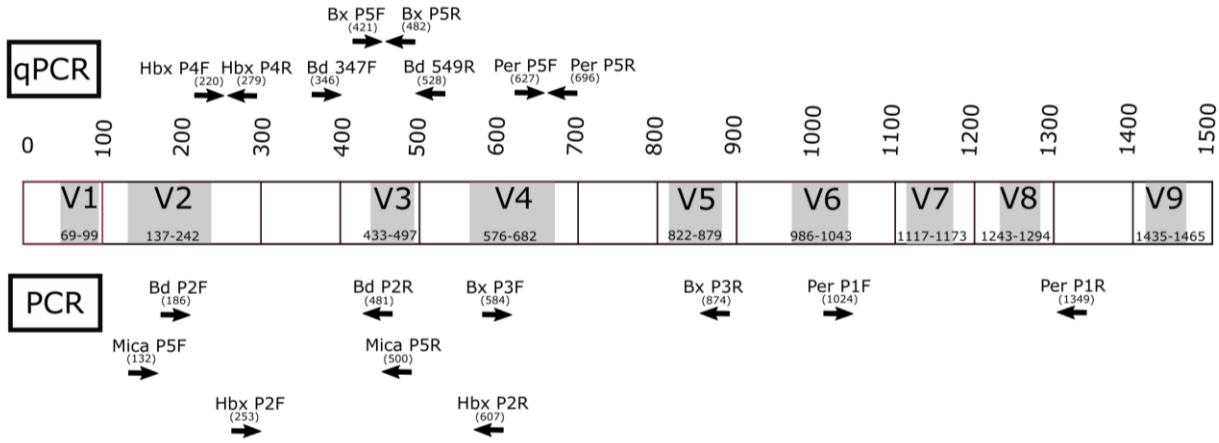


Figure 5 Primer positions on the 16S rRNA gene of *E. coli*. F and R symbols represent forward and reverse primer respectively. Under each primer name, the position based on the 16S of *E. coli* is given according to the TestPrime tool from arb-SILVA (Quast et al., 2013). For example, the forward qPCR primer of *Bacteriovorax* named Bx qP5F is positioned at 421 bp of *E. coli* 16S rRNA gene. The hypervariable regions V1 to V9 (gray rectangle) are positioned according to Chakravorty et al. (2007).

Some primers were also successfully designed for Illumina Miseq. Here again we did not consider *Pseudobacteriovorax*. For OTUs clustering we used the program Swarm with "d = 1" in order to get a better taxonomic resolution to detect larger genetic diversity among BALOs. *Bdellovibrio* (Bd pP2) and *Peredibacter* (Per pP1) primers were very successful according to the OTU assignments and the phylogenetic trees. Indeed, 100% of OTUs amplified by Bd pP2 and Per pP1 were assigned to *Bdellovibrio* and *Peredibacter*, respectively. *Bacteriovorax* primer (Bx pP3) was only half-good since it could also detect *Peredibacter*. That being said, *Bacteriovorax* DNA is well present and detected in the environmental samples. On the other hand, the *Halobacteriovorax* primer (Hbx pP2) was not solely specific to *Halobacteriovorax* but also to other BALOs and bacteria. Although this set of primers should not be used to grasp the diversity of *Halobacteriovorax*, these primers most likely can reveal more *Halobacteriovorax* related sequences than using a universal set of primer. The *Micavibrio* primer (Mica pP5) amplified 100% *Micavibrio* in the mock sample containing all available BALOs strains in our laboratory. This suggests that the primer specifically amplified *Micavibrio* and not other BALO strains. However, in a natural ecosystem *Micavibrio* was less amplified compared to the tremendous amplification of other bacteria. This either can suggest that this primer is not that specific and that the bacterial strains that we used as negative controls were not enough to demonstrate that the primer is not specific as we thought. It can also suggest that *Micavibrio* is weakly present in the natural environment so that the primer may amplify another target. In the light of these elements, the behavior of this primer set is instable, thus the repeatability of the results from an environment to

another can be compromised. Again, how that set of primer can perform in comparison to a universal primer set toward the detection of *Micavibrio* need an answer. Figure 5 represents the position of the primers on *E. coli* 16S rRNA gene. *In fine*, we renamed Bd pP2, Bx pP3, Per pP1, Hbx pP2, Mica pP5 as Bd p186F and Bd p481R, Bx p584F and Bx p874R, Per p1024F and Per p1349R, Hbx p253F and Hbx p607R, Mica p132F and Mica p500R, respectively.

Our work was challenging for several aspects and we faced a variety of issues. First, we could not get all BALOs strains (positive control) for *in vitro* tests *e.g.*, *Bacteriovorax stolpii*. We also acquired only one positive strain as a positive control, thus not very representative of the diversity of a BALO genus. As we are aware that this is not sufficient to predict *in vitro* specificity, we then selected the best set of primers and sequenced their amplification. Therefore, not having a positive control or even failing to amplify it does not discredit or invalid the experiment since the analysis of primers specificity was carried on environmental samples *via* sequencing. To prove even more, that positive control was only used to get a general idea about *in vitro* specificity, two examples can be given. The first is that we managed to design a very specific qPCR set of primers for *Bacteriovorax* without a positive control. The second is that despite showing a very promising specificity toward *Micavibrio*'s positive control *in vitro*, the designed primers set for qPCR and PCR failed as demonstrated by the sequencing results to specifically amplify *Micavibrio*. On the other hand, difficulties lied in using uncurated databases (uncertain or wrong taxonomic assignments) with sequences originating from various environments and from different sequencing technologies. This last main problem was recently highlighted by Lydon and Lipp (2018) who reported that sequences of *Pseudoalteromonadaceae* were wrongly placed in the order of the *Vibrionales* and *vice versa* in Greengenes (v13_5 and 13_8; DeSantis et al., 2006) database. 68 published articles have however based their results on these erroneously assigned sequences. The same issue can be reported here since many BALO sequences found at NCBI were/are still under the *Bdellovibrio* name. Also, a confusion between *Bacteriovorax*, *Peredibacter* and *Halobacteriovorax* is still visible, and some sequences are assigned to the wrong species. For example, *B. exovorus* MPR11 does not phylogenetically relate to *Bdellovibrio* but to *Micavibrio* as also observed in all our phylogenetic trees. The same problem was also observed for sequences assigned to *Bacteriovorax* sp. EPC3 and EPA. These sequences clustered better with *Peredibacter* than *Bacteriovorax*. Furthermore, OTUs assignment *via* our pipeline was also challenging since we observed a different taxonomic assignment in the arb-SILVA new release version 138 from the agreed BALOs classification reported by Baer et al. (2000, 2004), Koval et al. (2015), and Hahn et al. (2017). Currently, Arb-SILVA follows this taxonomic assignment where BALOs class is Bdellovibrionia and not Oligoflexia (*e.g.*, Bacteria |Bdellovibrionota | Bdellovibrionia | Bacteriovoracales). Additionally, the last level in the taxonomic assignment does not always belong

to the correct genus of BALOs (*e.g.* |Bacteriovorax|Bdellovibrio_sp._SD1 and Peredibacter|Bacteriovorax_sp._EPC3).

Our results highlighted other important issues. Firstly, samples issued from the marine SOLA and MOLA sites were difficult to amplify with the different primers. For instance, we had to use a sample from the Bay of Arcachon for *Halobacteriovorax* qPCR primers tests since no or low amplification was obtained at MOLA and SOLA. In addition, the Nano MiSeq sequencing showed that fewer reads were detected for these sites, especially for MOLA. We first believed that the problem was associated with PCR inhibitors present in the sample. However, after making a dilution test with qPCR (not shown) we observed that the diluted samples (1/2, 1/4, 1/10, 1/20, 1/40) appeared after the undiluted sample, suggest that the problem was elsewhere. Most likely BALOs are less abundant in the Bay of Banyuls than in Lakes Geneva and Annecy, and more globally in marine than in freshwaters. If so, the relatively low abundance of BALOs and their DNA may have biased the amplification. This could explain the result observed for Mica p132F – p500R that amplified the *Micavibrio* DNA in the mock sample but not in the environmental samples. Furthermore, *Bacteriovorax* primer p584F - p874R might also be concerned with most likely *Peredibacter* being more present than *Bacteriovorax* as reported by Paix et al. (2019). Finally, primers' performance could be related to other reasons that we did not explore here such as DNA polymerase (Śpibida et al., 2017), the number of PCR cycles or the cell GC content (Elbrecht et al., 2019).

The Nano Miseq revealed interesting results regarding the presence of some BALOs in certain ecosystems. To begin, as expected *Halobacteriovorax* DNA was not detected in the freshwater system *i.e.*, Lake Geneva (Figure 3). However, *Peredibacter* DNA was detected in the marine ecosystem. Indeed, in SOLA with the primer set Per pP1 (Figure 1 right), 22289 reads assigned to *Peredibacter* were detected. This result is surprising, according to Piñeiro et al. (2008) the only recognized species of *Peredibacteraceae* are found in freshwater and soil environments. The SOLA site located in Banyuls bay is influenced by fluvial contributions from the Rhone, coastal rivers such as the Tech or Têt and locally episodic floods from the Baillaury in Banyuls Bay. Therefore, *Peredibacter* detected DNA could be from allochthonous origin. Alternatively, this could be some freshwater resistant representative that managed to adapt to salinity. The same logic could be applied to *Bdellovibrio* since we detected 3380 reads of the latter at the SOLA site (Figure 1 left). Furthermore, with Hbx pP2 we found for some sequences the assignment "uncultured marine bacterium" at the last level of the taxonomic assignment. Historically, marine BALOs are initially referred to as "marine *Bdellovbirio*" before being named *Halobacteriovorax*. However, the levels before that assignment indicated that these OTUs belong most probably to *Bacteriovoracaceae* or *Peredibacter*. We also tested these sequences *via* NCBI BLASTn (Altschup et al., 1990) and

approximately found the same assignment. These results of taxonomic assignment might be due to erroneous affiliation of some sequences in the database. Alternatively, it can also bring other elements to the existence of probable other halo-tolerant BALOs, which could be proposed as new members of a halo-*Peredibacter*, halo-*Bdellovibrio* group. In fact, in the classification of BALOs, there is no distinction between fresh and saltwater *Bdellovibrio*, *Peredibacter* and *Micavibrio*. Finally, these results are very interesting and open doors for further research for determining whether other halo-tolerant BALOs can thrive in saltwater.

A final word is that we purposely listed all designed primers set in the supplementary Table 1 so that other researchers could avoid losing time in testing such sequences or could eventually improve them.

5. Conclusion

Our study aims to participate in the study of the diversity and abundance of BALOs genera except *Pseudobacteriovorax* in natural ecosystems. Therefore, we designed primers for qPCR and PCR/Miseq. We propose new validated primers to detect specifically *Bacteriovorax* abundance and *Bdellovibio* and *Peredibacter* diversity. As discussed, the qPCR primer set for *Peredibacter* and *Halobacteriovorax* is to be used with caution since they are as good as the taxonomic assignment could tell. Also, a word of caution is appropriate to the use of primer set Bx pP3 to unravel *Bacteriovorax* diversity since it can also detect *Peredibacter*. Finally, primer pairs for *Halobacteriovorax* and *Micavibrio* failed to be specific. *In fine*, these validated tools will be very useful to better assess the distribution, dynamics and diversity of this functional bacterial group and highlight the ecology of the BALOs in a variety of ecosystems.

6. Data accessibility

The majority of the OTUs sequences are available at NCBI under the accession numbers: MT177341 to MT177658. Clones sequence from qPCR are shorter than 150 bp and were not admitted on NCBI. Regardless, these sequences along with all alignment files, OTU tables obtained from the different tested primers are available on the Zenodo repository website (https://doi.org/10.5281/zenodo.3706495).

7. Supplementary data

Supplementary Table 1 Designed and tested primers for Illumina sequencing MiSeq (or PCR) and quantitative PCR (qPCR).

Primer	Usage	Sequence 5'-3'	Target BALOs	Product length (bp)
Bd pP3 F Bd pP3 R	PCR	Fw TGAGTACTAGTGGCGCACGG Rv GCGTCGCTGCATCAGRGTTT	*Bdellovibrio*	~ 311
Bd pP5 F Bd pP5 R	PCR	Fw YCCGAAAGCGTGGGGATCAA Rv AGCCATGCAGCACCTGTGWA	*Bdellovibrio*	~ 300
Bd529 F * Bd1007 R *	PCR	Fw GGTAAGACGAGGGATCCT Rv TCTTCCAGTACATGTCAAG	*Bdellovibrio*	~ 481
Bd347 F ** Bd549 R **	qPCR	Fw GGAGGCAGCAGTAGGGAATA Rv GCTAGGATCCCTCGTCTTACC	*Bdellovibrio*	~ 190
Per pP3 F Per pP3 R	PCR	Fw CGCAAGGGCTCATGCTGAAA Rv AACAACGCTTGCACCCTTCG	*Peredibacter*	~ 349
Per pP4 F Per pP4 R	PCR	Fw GGAAACCCTGACGCAGCAAC Rv GCCTCCGGTGTTCCTTCACA	*Peredibacter*	~ 348
Per qP3 F Per qP3 R	qPCR	Fw CGGATCAGCAAGTCAGATGTG Rv CCTCCGACATTCTAGACTAGCA	*Peredibacter*	~ 80
Per qP4 F Per qP4 R	qPCR	Fw GACGAGACTGCCTGGGTTAA Rv CGCGCCATTGTATTACGTGT	*Peredibacter*	~ 96
Per 699 F *** Per 974 R ***	qPCR	Fw CTGCCTGGACGACTATTGAC Rv CGGGTTCGTAGGAGTTCAAG	*Peredibacter*	~ 276
Bx pP4 F Bx pP4 R	PCR	Fw GATGGGCCTGCGGGACATTA Rv AACAACGCTTGCACCCTTCG	*Bacteriovorax*	~ 330
Bx pP5 F Bx pP5 R	PCR	Fw GAAACCCTGACGCAGCAACG Rv TCGCCTCTGGTGTTCCATCG	*Bacteriovorax*	~ 350
Bx qP1 F Bx qP1 R	qPCR	Fw ACTTCGGTCTGTAAAGCTCTGT Rv CCGGTGCTTCCTCTATGTGT	*Bacteriovorax*	~ 90
Bx qP2 F Bx qP2 R	qPCR	Fw GCGTGAGTGAGGAAGGACTT Rv TGTGTACCATCAAACAATCGGC	*Bacteriovorax*	~ 91
Hbx pP1 F Hbx pP1 R	PCR	Fw TGCCGMGTGAGTGAGGAAGG Rv TCCTGTTTGCTACCCACGCT	*Halobacteriovorax*	~ 391
Hbx pP5 F Hbx pP5 R	PCR	Fw TGGCTCAGAACGAACGCTGK Rv GCATTGCTGCGTCAGGSTTT	*Halobacteriovorax*	~ 392
Hbx qP3 F Hbx qP3 R	qPCR	Fw GGAGAAATTCTGGCTAATACCGC Rv CGCAGGCTCATCATTTGGTAAA	*Halobacteriovorax*	~ 100
Hbx qP5 F Hbx qP5 R	qPCR	Fw GAATGCCTCTCCTTGGAAGC Rv GCATCGTAGTCATGGTAGGC	*Halobacteriovorax*	~ 90
Mica pP3 F Mica pP3 R	PCR	Fw RGGAAACYCTGATCCAGCCA Rv CCTACGCCACTGGTGTTCCT	*Micavibrio*	~ 329
Mica pP4 F Mica pP4 R	PCR	Fw CAACGAGCGCAACCCTCATC Rv AGCGTACCGCCTTCAGGTAG	*Micavibrio*	~ 346
Mic431 F * Mic996 R *	PCR	Fw AAGCTCTTTTAGGTGTGAAA Rv TGAAAGTCAAAAGGAGGAT	*Micavibrio*	~ 558
Mica qP5 F Mica qP5 R	qPCR	Fw ACCTTACCTACTCTTGTATCCTCC Rv GGGACTTAACCCAACATCTCAC	*Micavibrio*	~ 124

Fw : forward ; Rv : reverse ; * : Davidov et al., 2006; ** : Van Essche et al., 2009; *** : Paix et al., 2019

Supplementary Table 2 Type species sequences of BALOs used to generate primers with Primer-Blast and Primer3

Genus	**Type species**	**NCBI accession number**
Halobacteriovorax	*H. marinus* strain SJ	NR_102485.1
Bacteriovorax	*B. stolpii* strain Uki2	NR_115142.1
Peredibacter	*P starrii* strain A3.12	NR_024943.1
Micavibrio	*M. aeruginosavorus* strain ARL-13	DQ186612.1
Bdellovibrio	*B. bacteriovorus* strain HD100	NR_027553.1

Supplementary Table 3 Bacteria genera used to verify BALOs primers specificity *in silico*

Non-BALOs	**BALOs**
Aeromonas aquatic WCHAH045096	*Bacteriovorax stolpii* Uki2
Aggregatibacter actinomycetemcomitans ANH9381	*Bdellovibrio bacteriovorus* HD100
Bacillus cereus FRA000041	*Bdellovibrio exovorus* JSS
Campylobacter rectus RM3267	*Halobacteriovorax litoralis* JS5
Capnocytophaga sputigena ATCC 33612	*Halobacteriovorax marinus* SJ
Eikenella corrodens ATCC 23834	*Micavibrio aeruginosavorus* EPB
Escherichia coli MPEC EX-33	*Micavibrio aeruginosavorus* ARL-13
Escherichia coli MPEC Y-69	*Peredibacter starrii* A3.12
Escherichia coli MN900682.1	
Fusobacterium nucleatum CTI-2	
Klebsiella aerogenes JCM 1235	
Klebsiella aerogenes A20-1	
Myxococcus xanthus DK 1622	
Porphyromonas gingivalis ATCC 33277	
Prevotella intermedia JCM 12248	
Pseudomonas aeruginosa ATCC 27853	
Proteus mirabilis NCTC 11938	
Serratia liquefaciens ATCC 27592	
Staphylococcus aureus RA20	
Streptococcus mutans AB294730.1	
Streptococcus sanguinis ATCC 29667	
Vampirovibrio chlorellavorus ICPB 3707	

Supplementary Table 4 Cultured bacteria (BALOs and non-BALOs) in the laboratory to extract their DNA for primers specificity tests.

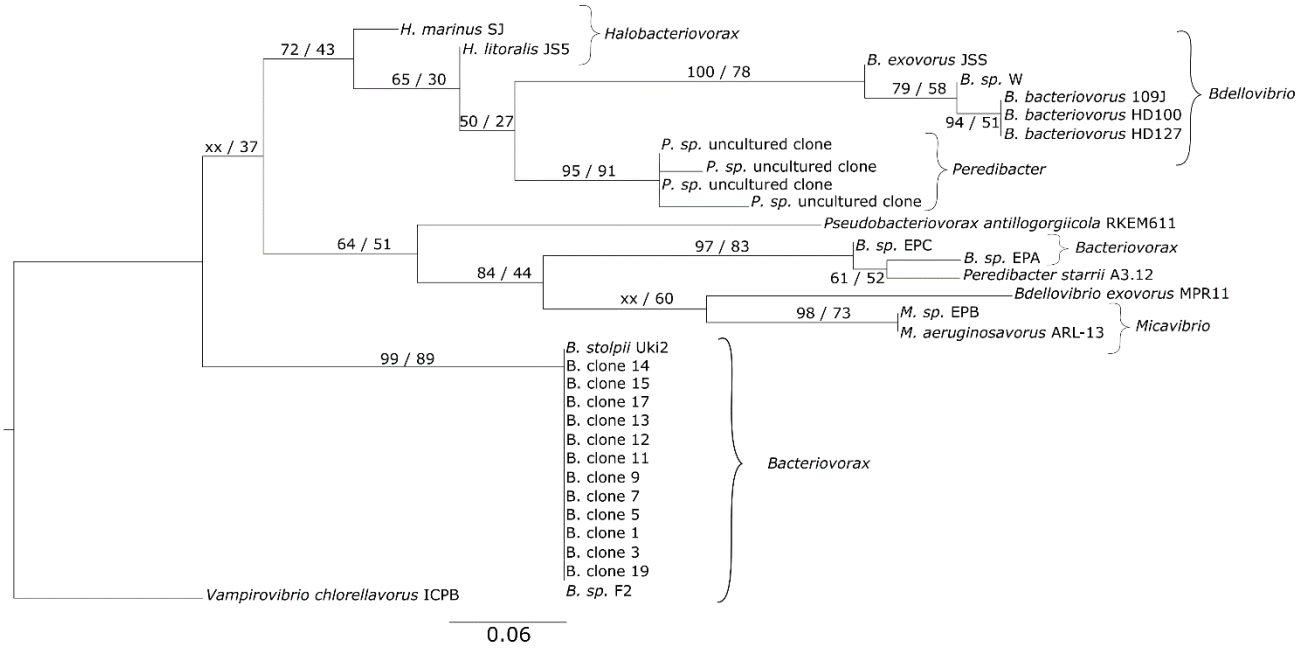
	Bacteria name
BALOs (positive control and Mock community sample)	*Bdellovibrio bacteriovorus* HD100
	Bdellovibrio bacteriovorus 109J
	Bdellovibrio exovorus (unknown strain)
	Micavibrio aeruginosavorus ARL-13
	Peredibacter sp. (unknown strain)
	Halobacteriovorax sp. (unknown strain)
	Bdellovibrio sp. (isolated species from Lake Geneva)
Non-BALOs (negative control and bacterial mix)	*Citrobacter freundii* ATCC 8090
	Escherichia coli ATCC 10536
	Hafnia alvei ATCC 13
	Pseudomonas fluorescens ATCC 13525
	Pseudomonas putida ATCC 12633
	Vibrio parahaemolyticus (unknown strain)
	Pseudomonas sp. (isolated from Lake Geneva)

Supplementary Text Box 1 Workflow of the bioinformatics pipeline

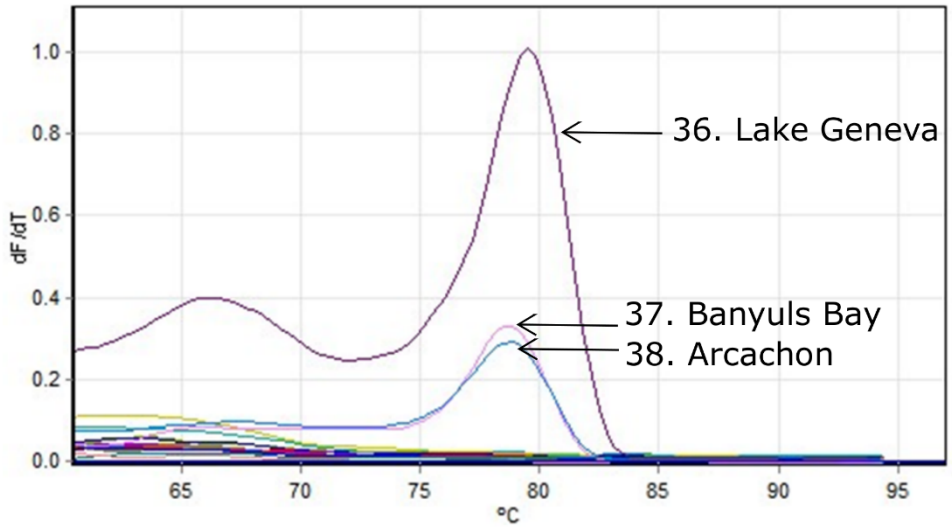
The pipeline chains up different programs *i.e.* Vsearch (Rognes et al., 2016), Cutadapt (Martin, 2011), Stampa and Swarm (Mahé et al., 2015). All default parameters of the pipeline were left unchanged including the "d = 1" for the OTUs clustering. The pipeline was run independently 5 times to analyze the files (R1 and R2) with each designed primers. Briefly, the pipeline starts with merging reads with Vsearch. The merged reads were then demultiplexed using a tag list with cutadapt. In this step, the primer sequences were also trimmed and reads containing ambiguous bases were discarded. Next, each demultiplexed file were dereplicated independently using Vsearch. Then, all files were concatenated and dereplicated using again Vsearch. The Swarm algorithms with "d = 1" was then used on the dereplicated sequences. The obtained representative sequences were checked *de novo* for chimera detection using also Vsearch. Disregarding the nature of the sequence, chimera or not chimera, all the sequences were taxonomically assigned using arb-SILVA (release number 138; Quast et al., 2013) *via* the Stampa program. Finally, a Python script from the pipeline combined all the obtained files to build an OTU table (OTU table per primer).

Supplementary Table 5 Accession numbers of BALOs sequences downloaded from NCBI to construct the different phylogenetic trees for qPCR and PCR, clones and OTUs.

Sequence name	Accession number
Bdellovibrio bacteriovorus strain HD127	AJ29276.1
Bdellovibrio sp. W strain ATCC 27047	AJ292518.1
Bdellovibrio bacteriovorus strain 109J	M61234.1
Bdellovibrio bacteriovorus strain HD100	NR027553.1
Bdellovibrio exovorus strain JSS	EF687743.1
Bdellovibrio exovorus strain MPR11	MH230062.1
Peredibacter sp. Uncultured clone K2DN38	KT308262.1
Peredibacter sp. Uncultured clone C114001412	JX525305.1
Peredibacter sp. Uncultured clone BFB660	KC545751.1
Peredibacter sp. Uncultured clone C114001299	JX525192.1
Peredibacter starrii strain A3.12	NR024943.1
Bacteriovorax stolpii strain Uki2	NR115142.1
Bacteriovorax sp. strain EPC3	AY294222.1
Bacteriovorax sp. strain EPA	AY294220.1
Bacteriovorax sp. strain F2	AY294218.1
Micavibrio aeruginosavorus strain ARL-13	DQ186612.1
Micavibrio sp. strain EPB	DQ186613.1
Pseudobacteriovorax antillogorgiicola strain RKEM611	KJ685394.1
Halobacteriovorax marinus strain SJ	NR102485.1
Halobacteriovorax litoralis strain JS5	NR028724.1
Vampirovibrio chlorellavorus strain ICPB 3707	NR104911.1



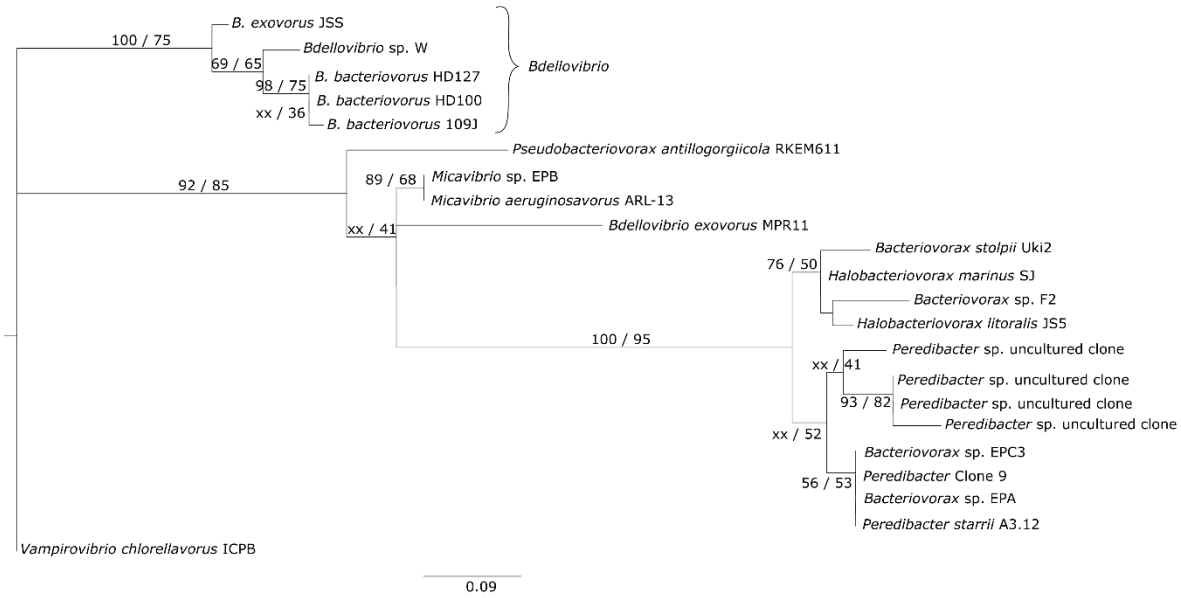
Supplementary Figure 1 Phylogenetic tree of qPCR amplicons amplified with the Bx qP5 primer (Bx q421F - q482R). The 12 dereplicated amplicons sequences assigned as *Bacteriovorax* according to NCBI are phylogenetically very close to *Bacteriovorax stolpii* Uki2 and *Bacteriovorax* sp. F2. Prior to the tree construction, the alignment of the sequences was corrected using Gblocks; 43 positions were kept from 87. The tree is based on maximum likelihood and Bayesian methods. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). The tree is rooted using the outgroup *Vampirovibrio chlorellavorus*.



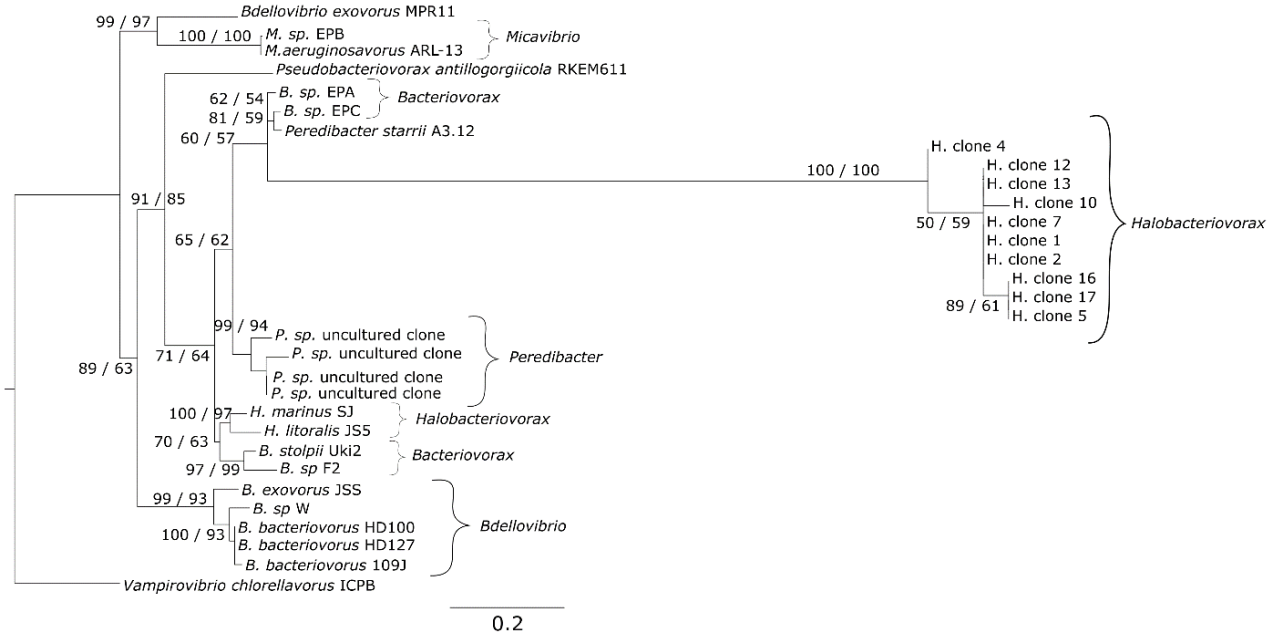
No.	Color	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4
21		21_P5_Per_Halobacteriovorax_pos_control		62.3	80.3	85.3	90.0
22		22_P5_Per_Micavibrio_neg_control		63.5	72.3	79.5	84.2
23		23_ P5_Per_Bdellovibrio_HD100_neg_control		73.2	77.5	83.8	89.3
24		24_P5_ Per_Peredibacter_pos_control		63.5	77.5	82.0	87.3
25		25_P5_Per_Exovorus_neg_control		63.0	74.7	78.2	83.2
26		26_ P5_Per_Isolated-Bdellovibrio-Geneva_neg_control		76.0	83.8	90.8	
27		27_P5_Per_Ecoli		63.3	72.7	78.5	84.0
28		28_P5_Per_Pa		65.5	75.2	79.0	83.0
29		29_P5_Per_Pf_neg_control		64.8	72.7	77.5	85.7
30		30_P5_Per_Pp_neg_control		65.5	73.7	81.2	88.2
31		31_P5_Per_Citrobacter_neg_control		63.5	74.7	79.5	84.8
32		32_P5_Per_Hafnia_neg_control		64.5	74.3	78.5	88.0
33		33_P5_Per_Bacterial_strains_MIX_neg_control		63.0	76.7	81.5	90.0
34		34_P5_PER_Isolate_bacteria_Geneva_neg_control		62.5	80.0	86.8	
35		35_P5_Per_Vibrio_parahaemolyticus_neg_control		61.8	73.0	78.7	83.5
36		36_P5_Per_Lake_Geneva_sample		66.0	79.5	84.7	87.0
37		37_P5_Per_Banyuls_bay_sample		65.8	71.5	78.7	86.0
38		38_P5_Per_Arcachon_sample		67.3	78.7	87.0	91.7
39		39_Water_NTC_1		63.5	67.0	73.2	77.5
40		40_ Water_NTC_2		65.5	75.7	85.5	

Supplementary Figure 2 Melt curve for qPCR primer Per qP5 showing no amplification for positive control *Peredibacter* sp. and other BALOs or bacterial strains that are *Peredibacter*. Lake Geneva, Banyuls and Arcachon samples are amplified. Amplicons of Lake Geneva are cloned and sequenced using the Sanger method. 21: *Halobacteriovorax* sp.; 22: *Micavibrio aeruginosavorus*; 23: *Bdellovibrio bacteriovorus* HD100; 24: *Peredibacter* sp.; 25: *Bdellovibrio exovorus*; 26: *Bdellovibrio* sp. isolated from Lake Geneva; 27: E. coli; 28: *P. aeruginosa*; 29: *P. fluorescens*; 30: *P. putida*; 31: *Citrobacter freundii*; 32: *Hafnia alvei*; 33: A mix of bacterial strain containing *Citrobacter freundii*, *Escherichia coli*, *Hafnia alvei*, *P. fluorescens*, *P. putida* and *P. aeruginosa*;

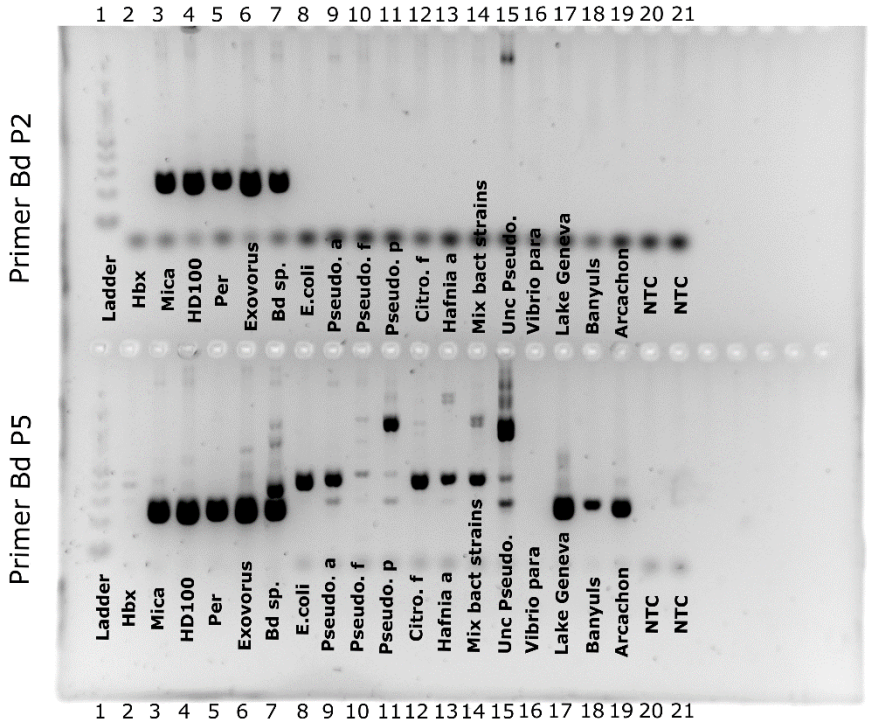
34: Isolated prey bacteria from Lake Geneva; 35: *Vibrio parahaemolyticus*; 36: Total DNA from Lake Geneva sample; 37: Total DNA from Banyuls sample; 38: Total DNA from Arcachon sample; 39: First NTC qPCR mix without DNA; 40: Second NTC qPCR mix without DNA.



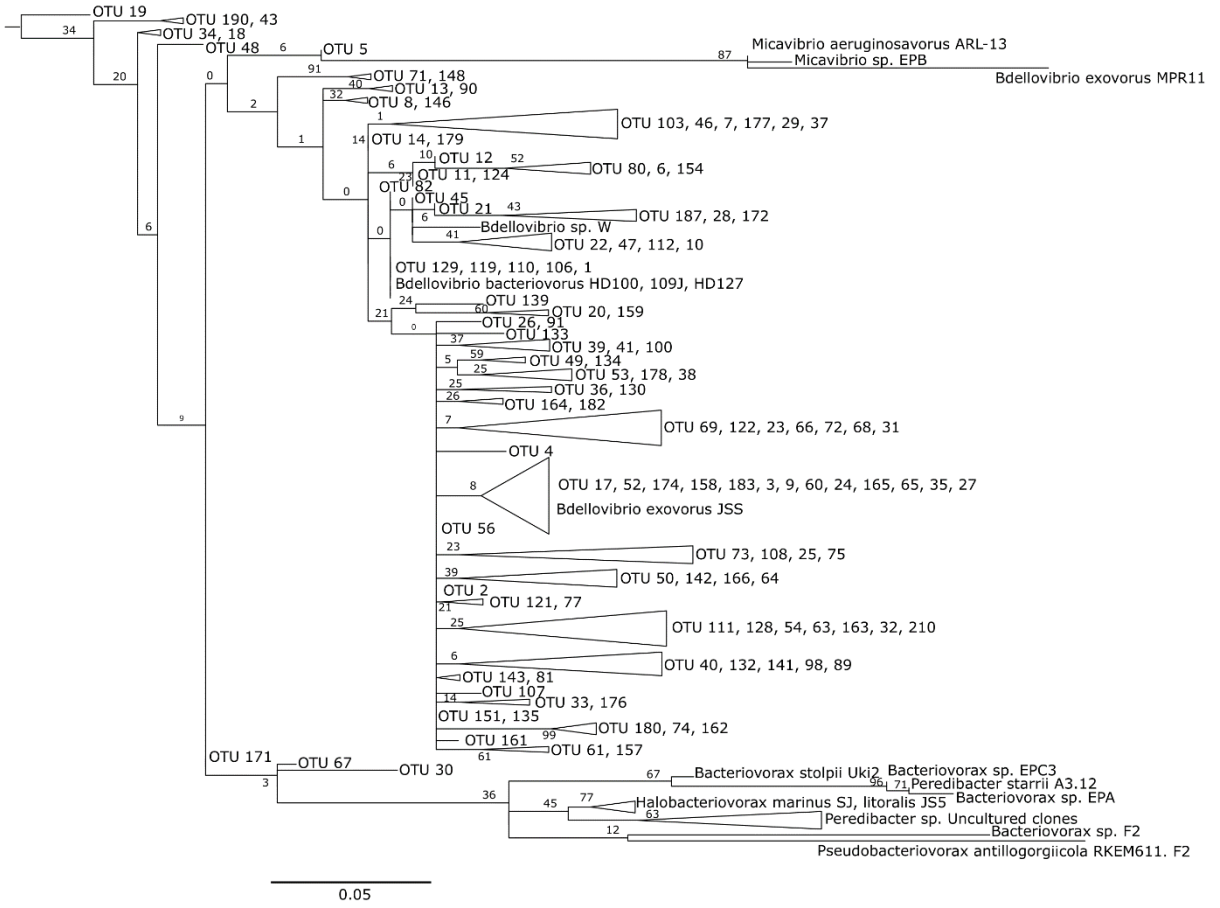
Supplementary Figure 3 Phylogenetic tree of qPCR amplicons amplified with the Per qP5 primers. Clones 1, 2, 3, 4, 5, 6, 7, 18 and 19 (not shown) assigned to *Peredibacter* according to NCBI could not be resolved on the tree. They are phylogenetically distant from *Peredibacter*. Only clone number 9 could be resolved and clustered with *Peredibacter starrii* A3.12. The short length of the sequences and the numerous gap in the alignment rendered difficult the construction of a solid phylogeny. 1297 positions with gaps were counted. Also, the high number of gaps made the use of Gblocks problematic since the program removed too many positions. The tree is based on maximum likelihood and Bayesian methods. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). The tree is rooted using the outgroup *Vampirovibrio chlorellavorus*.



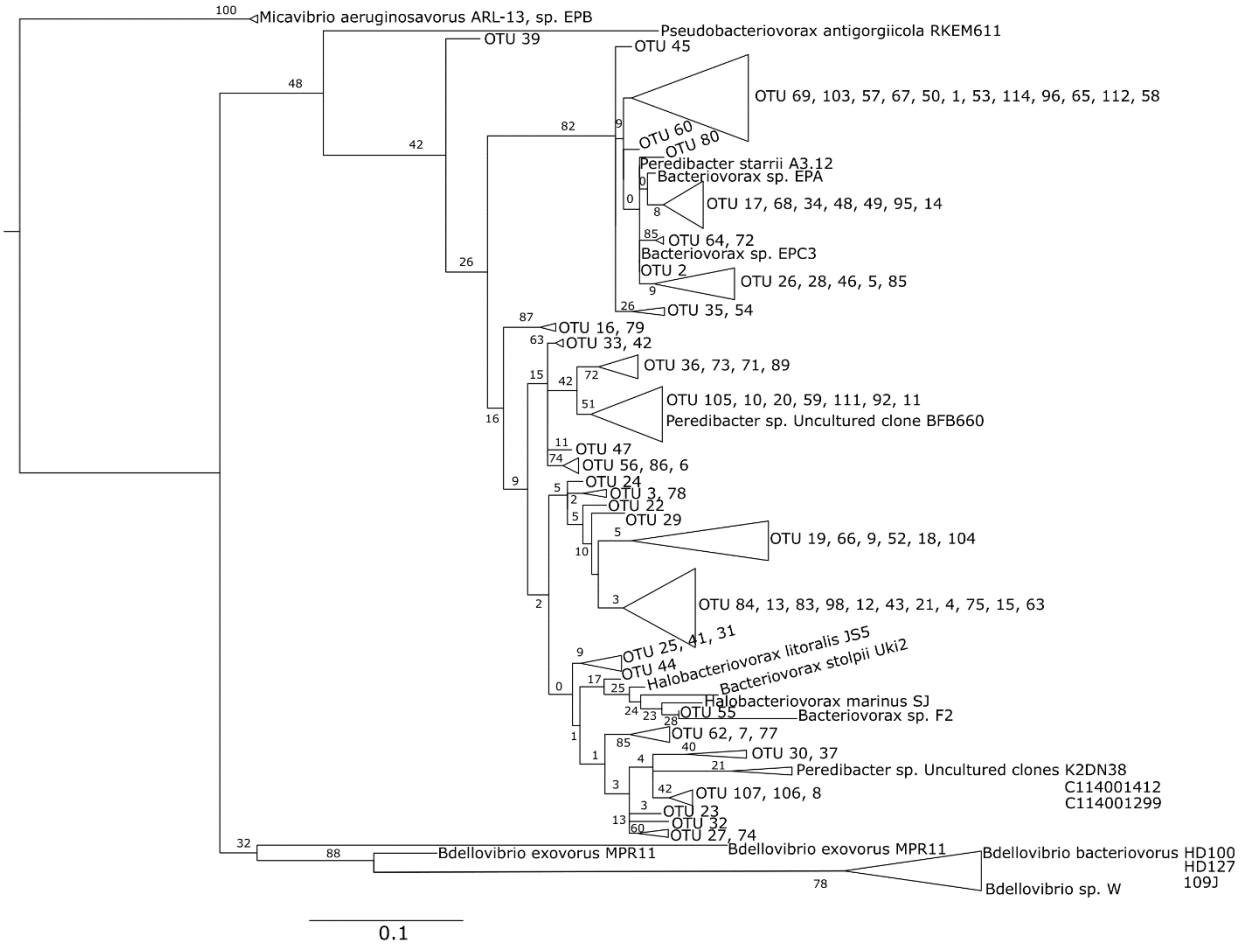
Supplementary Figure 4 Phylogentic tree of qPCR amplicons amplified with the Hbx qP4 primer. The 10 dereplicated sequences assigned to *Halobacteriovorax* according to NCBI are phylogenetically closer to *Peredibacter* species. The number of gaps is high in the alignment. The number of positions in the sequence alignments is 1103. The tree is based on maximum likelihood and Bayesian methods. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). The tree is rooted using the outgroup *Vampirovibrio chlorellavorus*.



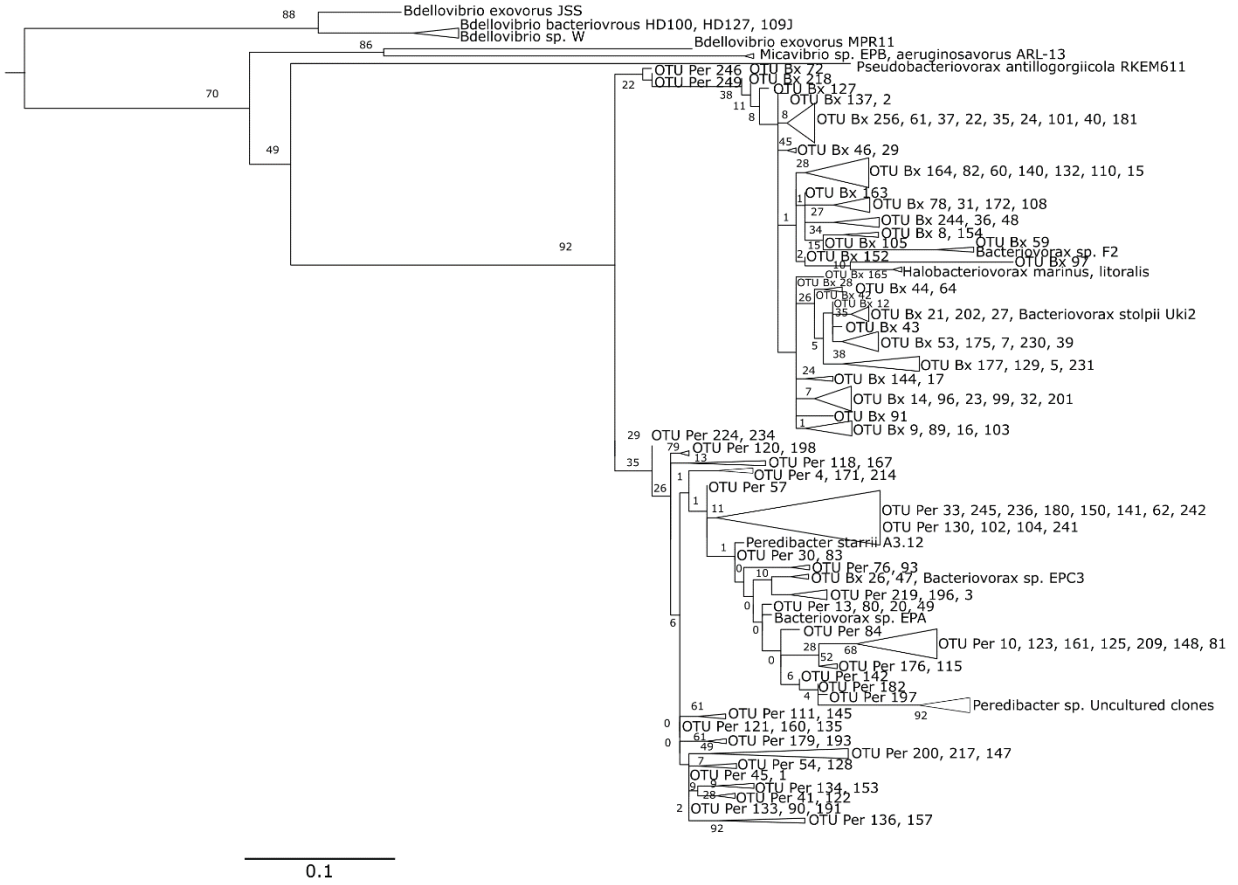
Supplementary Figure 5 Gel electrophoresis of DNA amplified by the designed primers for *Bdellovibrio* genus. The Bd pP5 amplifies not only BALOs but also other organisms such as *Pseudomonas* sp. However, primer Bd pP2 amplifies only BALOs organisms especially the *Bdellovibrio*. DNA in environmental samples was not amplified by Bd pP2 in this test.1. Ladder; 2. *Halobacteriovorax* sp.; 3. *Micavibrio aeruginosavorus* ARL-13; 4. *Bdellovibrio bacteriovorus* HD100; 5. *Peredibacter* sp.; 6. *Bdellovibrio exovorus*; 7. Lake Geneva isolated *Bdellovibrio* sp.; 7. *Escherichia coli*; 8. *Pseudomonas aeruginosa*; 10. *P. fluorescens*; 11. *P. putida*; 12. *Citrobacter freundii*; 13. *Hafnia alvei*; 14. Mix of bacterial organisms non-BALOs; 15. Lake Geneva isolated *Pseudomonas* sp.; 16. *Vibrio parahaemolyticus*; 17. extracted DNA from Lake Geneva; 18. extracted DNA from Banyuls bay; 19. extracted DNA from the Bay of Arcachon; 20. NTC (no template control); 21. NTC.



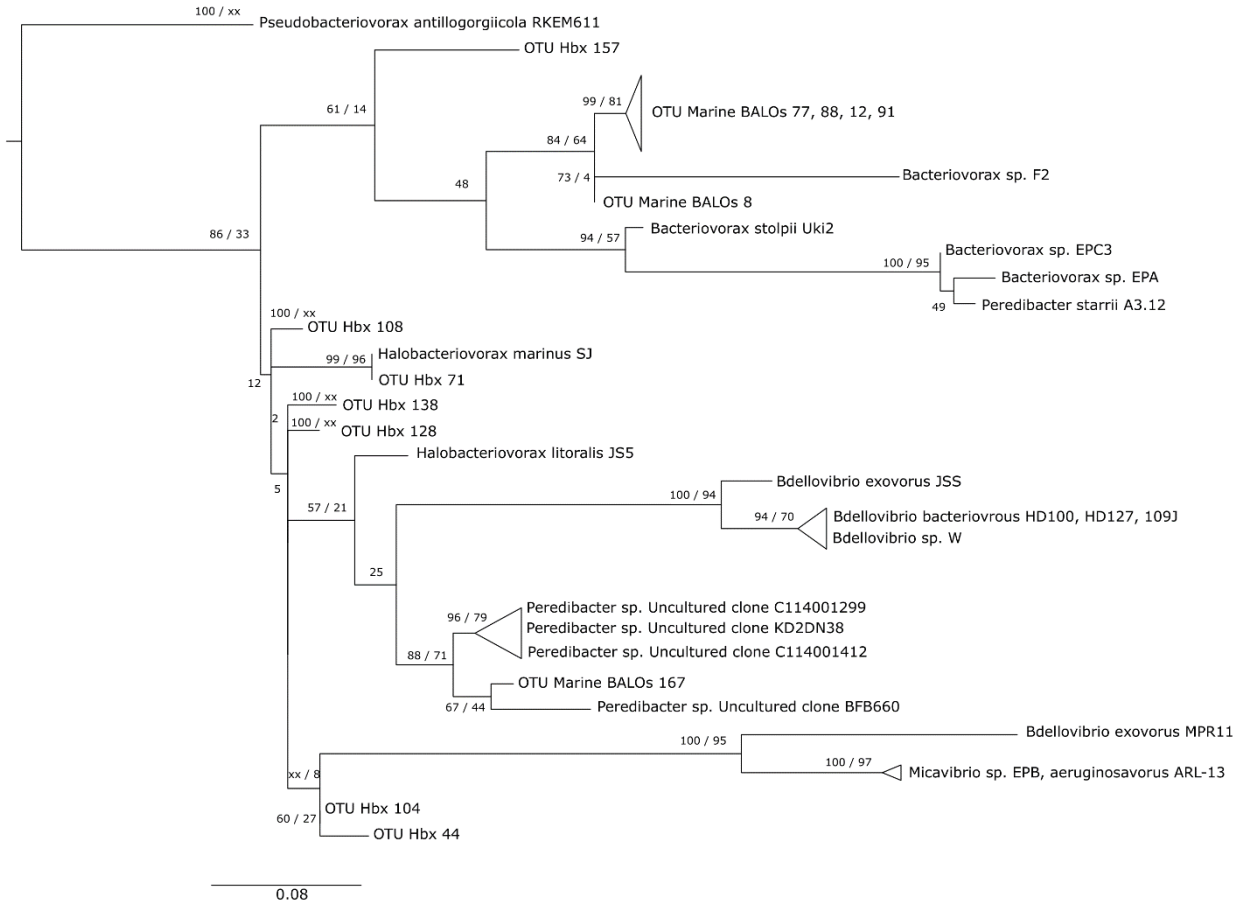
Supplementary Figure 6 Phylogenetic tree of sequences obtained with the Bd pP2 PCR primers and bioinformatics pipeline. The OTUs are bioinformatically assigned to the genus *Bdellovibrio* without exception. The alignment was curated with Gblock, where 192 positions were kept from 342. The tree is based on maximum likelihood analysis and Bayesian inference. Bootstrap values are shown but not the Posterior probability (PP) which is above 50%. *Vampirovibrio chlorellavorus* (not shown) was used as an outgroup to root the tree. The majority of the OTUs are phylogenetically more related to *Bdellovibrio* than other BALOs genera. This result is in accordance with the assignment results. According to the tree, *Bdellovibrio* OTUs are very diverse.



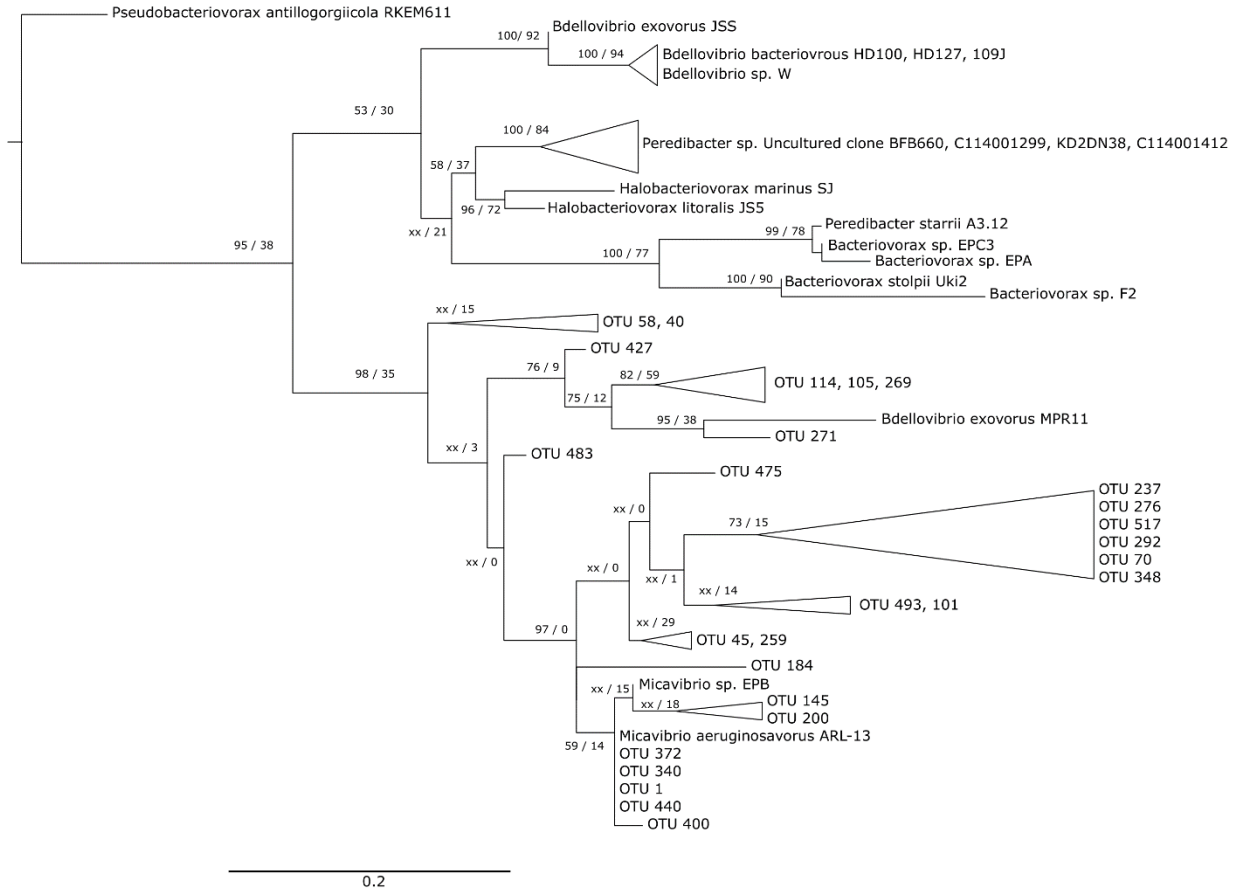
Supplementary Figure 7 Phylogenetic tree of amplicons amplified by the Per pP1 PCR primers and assigned according to the bioinformatics pipeline to the genus *Peredibacter* without exception. The alignment was not corrected with Gblock, and the position number is 326. The tree is based on maximum likelihood analysis and Bayesian inference. Bootstrap values are shown but not the Posterior probability (PP) which is above 50%. *Vampirovibrio chlorellavorus* (not shown) was used as an outgroup to root the tree. The majority of the OTUs are phylogenetically more related to *Peredibacter* except for OTU 44 and 55 which clustered with *Halobacteriovorax* genus. *Bacteriovorax* sp. EPA and EPC3 are most likely not correctly identified in the database as *Bacteriovorax*. They seemed to be more related to *Peredibacter* genus than *Bacteriovorax.* Therefore, OTUs near these two individuals belong most likely to the *Peredibacter* genus and not to *Bacteriovorax.* The phylogenetic results are in majority in accordance with the bioinformatics assignment. *Peredibacter* OTUs are very diverse according to the tree.



Supplementary Figure 8 Phylogenetic tree of amplicons amplified with Bx pP3 PCR primers and assigned in majority according to the bioinformatics pipeline to the genus *Bacteriovorax* and *Peredibacter*. The alignment was curated with Gblock, where 251 positions were kept from 277.The tree is based on maximum likelihood analysis and Bayesian inference. Bootstrap values are shown but not the Posterior probability (PP) which is above 50%. *Vampirovibrio chlorellavorus* (not shown) was used as an outgroup to root the tree. As the assignment results showed that, Bx P3 PCR primers are not completely specific to the *Bacteriovorax* genus. The primers are capable of amplifying also the *Peredibacter* genus. OTUs seemed to cluster in three groups, one related to *Bacteriovorax*, the other to *Halobacteriovorax* and the last one to *Peredibacter*. The presence of three clusters may be related to phylogenetic difficulties to construct a solid tree with short (251 bp) sequences. However, one can also hypothesize that the assignment of the OTUs sequence may have also encountered some difficulties owing to the arb-SILVA incomplete taxonomy for the *Bdellovibrio* and like organisms.



Supplementary Figure 9 Phylogenetic tree of amplicons amplified with Hbx pP2 PCR primers and assigned according to the bioinformatics pipeline to different organisms. Here, the tree shows only individuals assigned to the *Halobacteriovorax* genus and to "Marine BALOs", other assigned bacteria are not represented. The alignment was curated with Gblock, where 197 positions were kept from 343.The tree is based on maximum likelihood analysis and Bayesian inference. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). *Vampirovibrio chlorellavorus* (not shown) was used as an outgroup to root the tree. "Marine BALOs" OTUs such as 77, 88, 12, 91 and 8 clusters more closely to the *Bacteriovorax* genus. However, others like OTU 167 cluster with the *Peredibacter* genus. Also, OTUs like 104 and 44 are more related to the *Micavibrio* genus than any other BALOs genus. OTUs assigned to *Halobacteriovorax* with the exception of OTU 157 are related to *Halobacteriovorax marinus* and *litoralis*. The assignment of OTUs to "Marine BALOs" and their presence far from the halophilic BALOs raise the question of the probability of the presence of other marine BALOs that are not part of the *Halobacteriovorax* genus and which are not yet identified as halotolerant.



Supplementary Figure 10 Phylogenetic tree of amplicons amplified with Mica pP5 PCR primers and assigned according to the bioinformatics pipeline to Micavibrionales. The alignment was curated with Gblock, where 224 positions were kept from 376. The tree is based on maximum likelihood analysis and Bayesian inference. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). *Vampirovibrio chlorellavorus* (not shown) was used as an outgroup to root the tree. All OTUs are closely related to *Micavibrio* sequence found in the database. The *Bdellovibrio exovorus* MPR11 is more closely related to *Micavibrio* than *Bdellovibrio*. This organism might have been wrongly assigned to *Bdellovibrio exovorus* species.

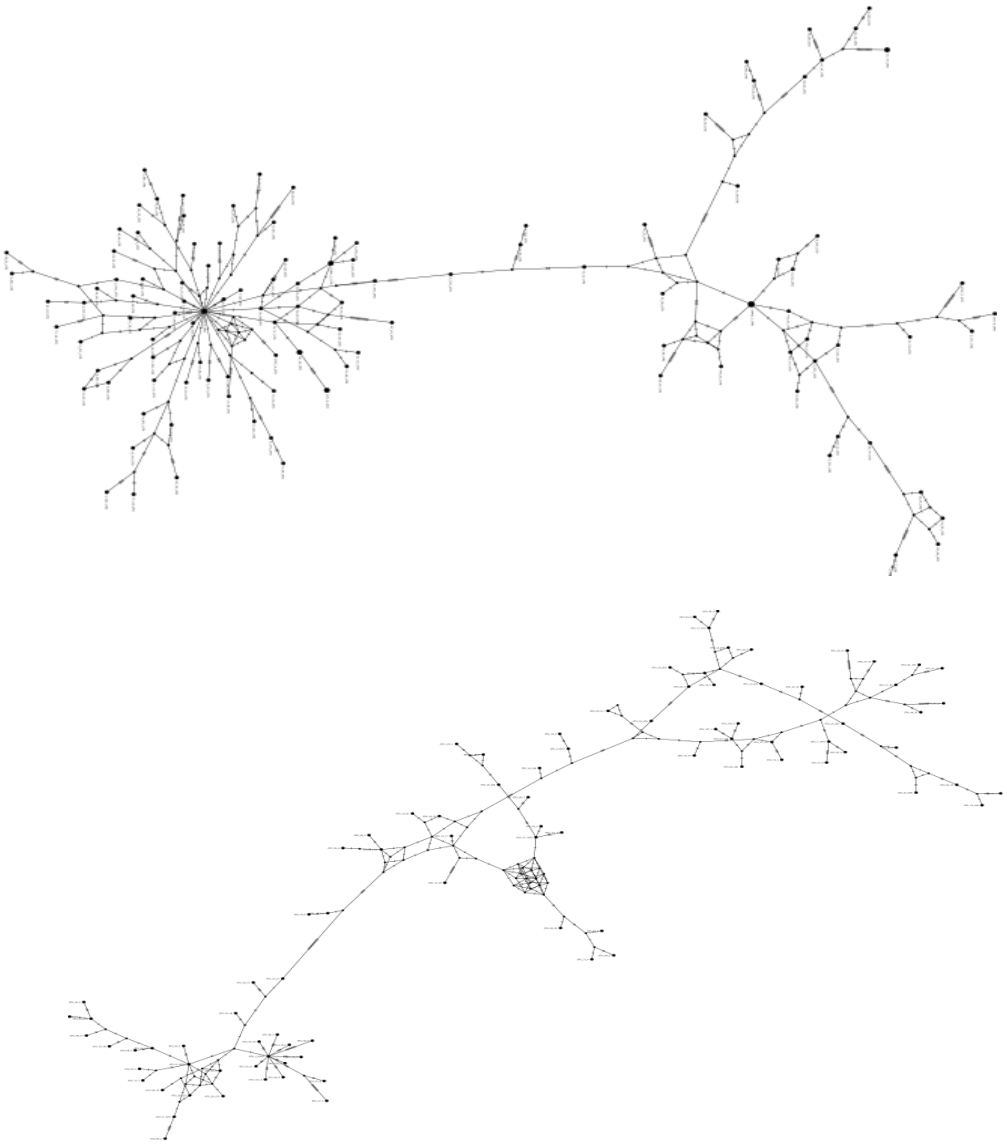
Constats

Pour capturer uniquement la diversité des BALOs, autrement dit l'éventail des genres et espèces qui caractérisent ce groupe fonctionnel de bactéries, il est nécessaire d'utiliser des couples d'amorces spécifiques et des nouvelles techniques de séquençage à haut débit. Dans le cadre de cette thèse, un effort important a donc été entrepris pour concevoir des couples d'amorces spécifiques aux différents genres ou familles de BALOs, pouvant de plus être utilisées avec la technologie Illumina Miseq 250 pb x 2 qui offre une profondeur de séquençage de 12 millions de reads chez Eurofins/GATC. Sur 5 couples d'amorces ciblant les 5 genres ou familles de BALOs différents, nous avons réussi à valider deux couples qui amplifient spécifiquement le genre *Bdellovibrio* et *Peredibacter*. Toutefois, l'amplification de nos cibles dans les sites marins, SOLA et MOLA, était plus difficile. Ainsi, pour le couple d'amorce ciblant les *Bdellovibrio*, les échantillons du site MOLA n'ont pas pu être séquencés, alors que quelques reads (3380) ont été obtenus pour le site SOLA. Comparativement, les échantillons des lacs Léman et d'Annecy ont « produit » respectivement 16573 et 30919 reads. Concernant, le couple d'amorce *Peredibacter*, la tendance était très similaire pour MOLA avec l'obtention de 306 reads. Par contre, et à l'inverse de la situation précédente, le groupe des *Peredibacter* était bien détecté au site SOLA avec 22289 reads. Pour les lacs Léman et d'Annecy, 20981 et 22729 reads ont été obtenus. Concernant les autres couples d'amorces, la spécificité n'était pas assez bonne. Ainsi, le couple d'amorces ciblant *Bacteriovorax* a amplifié la même quantité d'ADN de *Bacteriovorax* et *Peredibacter*. De plus, aucune amplification n'a pu être obtenue pour les sites SOLA et MOLA. Pour les deux derniers couples d'amorces ciblant les *Halobacteriovorax* et *Micavibrio*, la spécificité était orientée vers des bactéries autres que les BALOs. Ces amorces n'étaient donc pas bonnes. Sur la base de ces résultats, plusieurs constatations peuvent être faites.

- Nous n'avons pas réussi à étudier la diversité de tous les BALOs avec nos amorces, obligeant de recourir à l'emploi d'une paire d'amorce universelle, peut être différente de celle proposé dans l'article (515F-909R), permettant aussi d'identifier les autres porcaryotes partageant les mêmes niches écologiques que les BALOs.
- Il semble qu'allouer 12M de reads lors du séquençage pour un ou deux couples d'amorces spécifiques est excessif. En effet, le séquençage Nano-miseq à 1M de reads a montré une profondeur satisfaisante pour chaque couple d'amorce, ce que l'analyse des séquences par l'intermédiaire du logiciel POPART a confirmé. Les figures ci-après révèlent en effet l'existence de seulement quelques séquences distinctes ou dominantes *vs* un nuage d'autres séquences avec quelques nucléotides de différences qui gravitent autour. La diversité génétique semble donc modeste chez les *Bdellovibrio* et *Peredibacter*. A noter toutefois

que ces observations sont peut-être dépendantes du couple d'amorce utilisé et de sa portée de détection, sans oublier les erreurs d'amplification issues des PCR et séquençage.

- Amplifier les BALOs dans les sites SOLA et MOLA semble représenter un vrai défi, l'assemblage de plusieurs échantillons n'ayant pas permis d'amplification. Comme les tests d'inhibitions en qPCR effectués ont révélé un profil d'amplification normal, l'hypothèse la plus simple serait d'avancer que les BALOs sont simplement faiblement présents dans la Baie de Banyuls, à la différence de la Baie d'Arcachon. Comme décrit dans le chapitre I, les BALOs sont plutôt bien présents dans les systèmes fermés, les sédiments et biofilms, surement là où les proies sont très abondantes.

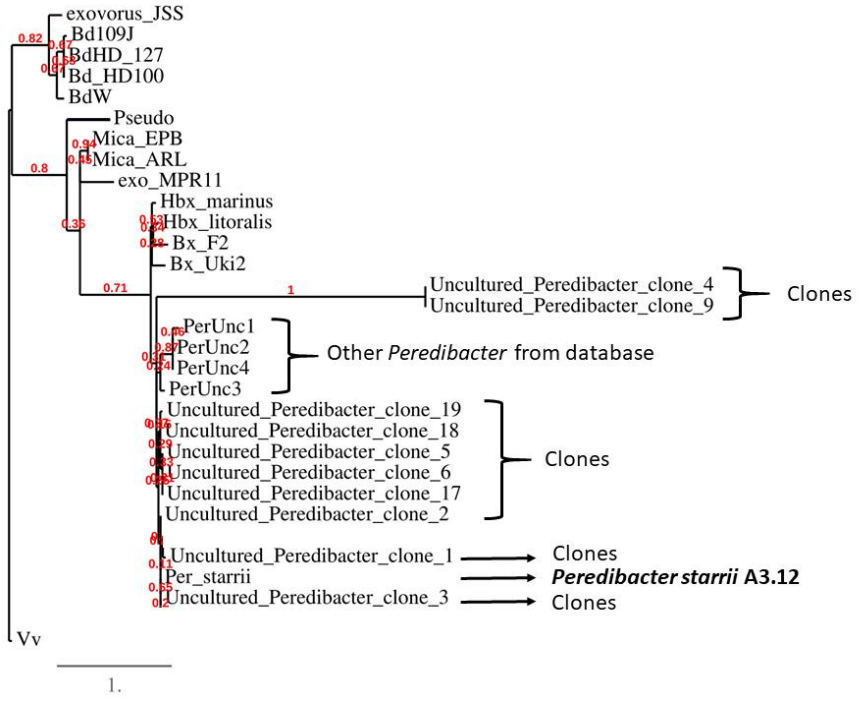


Figures 1 & 2: Diversité des séquences de *Bdellovibrio et* de *Peredibacter* illustrée par le logiciel POPART

Avant le confinement imposé au cours de l'année 2020 dans la lutte contre la pandémie au COVID-19, deux séries de séquençage étaient prévus pouvant contenir 192 échantillons (4 écosystèmes x 1 réplicat x 1 filtre 0,2µm x 2 profondeurs x 2 couples d'amorces x 12 mois). La première série devait être composée des échantillons amplifiés avec les couples d'amorces spécifiques *Bdellovibrio* et *Peredibacter*, la seconde avec le couple d'amorce universel 16S rRNA 515F – 806R ciblant les bactéries totales et l'amorce semi-spécifique *Bacteriovorax*. Cela permettait en théorie d'obtenir une profondeur de séquençage de 62500 reads. Cependant, la compagnie Eurofins/GATC a changé son type de prestation après le confinement, avec des runs à 96 échantillons (125000 reads par échantillon) pour le même prix ! *In fine*, nous avons opté pour deux run, l'un ciblant les *Bdellovibrio* et *Peredibacter* pour les lacs Léman et d'Annecy, et l'autre pour le couple d'amorce universel 515F – 909R (car les expérimentations en laboratoire avec le couple 515F – 806R n'ont pas donné des résultats fiables) avec les 4 écosystèmes.

Erratum :

Les séquences issues de la qPCR (clones) de *Peredibacter* et *Halobacteriovorax* sont dans le mauvais sens c'est-à-dire en 3'-5' au lieu de 5'-3'. Cette erreur a été corrigé en remettant les séquences dans le bon sens 5'-3'. De ce fait l'alignement des séquences clones qPCR *Peredibacter* avec les séquences de références est meilleur. Pour les séquences qPCR *Halobacteriovorax* l'alignement reste médiocre. Les arbres phylogénétiques pour les séquences qPCR (clones) des deux genres avec les séquences de références ont été refait en prenant en compte le nouvel alignement. On observe que les clones issus de l'amplification qPCR de *Peredibacter* se regroupent mieux avec les séquences *Peredibacter* de référence (figure ci-dessous). Ce résultat confirme la solidité des primers conçu pour mesurer l'abondance de *Peredibacter* en qPCR. A l'inverse, l'arbre phylogénétique avec les clones *Halobacteriovorax* ne semble pas s'améliorer (non montré). De ce fait, il est nécessaire d'optimiser encore le couple de primer pour la mesure de *Halobacteriovorax* en qPCR.



Arbre phyogénétique des clones de *Peredibacter* obtenus par amplifiaction en qPCR avec les séquences de références BALOs

Objectifs et résumé de l'article 6

Le *Bdellovibrio* et organismes apparentés (BALOs) sont des prédateurs bactériens obligatoires d'autres bactéries Gram négatives, connues pour occuper diverses niches écologiques. Cependant, une grande variété d'écosystèmes n'a été que très peu explorée jusqu'à présent. Dans la présente étude, nous avons tout d'abord exploré l'abondance de trois familles de BALOs appartenant à la classe des Oligoflexia (c'est-à-dire les *Bdellovibrionaceae*, *Peredibacteraceae* et *Bacteriovoracaceae*) sur une année et à différentes profondeurs de la colonne d'eau de deux lacs (Léman et et lac d'Annecy) et de deux sites marins (SOLA et MOLA, Baie de Banyuls-sur-mer, mer Méditerranée nord occidentale). Les *Peredibacteraceae* étaient une nouvel fois le groupe dominant presque partout, sauf en profondeur dans le lac Léman où les *Bdellovibrionaceae* étaient plus nombreuses. La diversité des *Bdellovibrionaceae* et des *Peredibacteraceae*, évaluée à l'aide d'une nouvelle génération d'amorces spécifiques, révélait que ces familles sont caractérisées par une centaine d'OTUs. D'une part, il était constaté que les OTUs dominants étaient présents simultanément dans les deux lacs et, d'autre part, que d'autres OTUs appartenaient spécifiquement à chaque environnement. Enfin, abondance et diversité des BALO étaient faiblement corrélées aux variables physico-chimiques, suggèrant de manière indirecte l'importance d'autres facteurs ou processus, comme les relations biotiques supposées de jouer un plus grand rôle dans la dynamique des BALOs.

Exploring the diversity, abundance and structure of *Bdellovibrio* and like organisms in four contrasted ecosystems over a year

Soumis à «Microorganisms».

1. Introduction

Among microorganisms inhabiting aquatic systems, a large variety of micropredators remains poorly known. These micropredators belong to various groups (i.e. metazooplankton, ciliates, flagellates, fungi, bacteria) and are facultative and/or obligatory hunters of other bacteria (Jurkevitch and Davidov, 2006). For example amongst heterotrophic bacteria, Myxobacteria are facultative predators when food resources become scarce (Thiery and Kaimer, 2020). By contrast, there is one group that consists of obligate predators of Gram-negative bacteria: there are themselves small Gram-negative bacteria from the Oligoflexia and Alpha-proteobacteria, and are referred to as "*Bdellovibrio* and Like Organisms" (named BALOs thereafter).

BALOs are reported to be widely distributed in nature and man-made habitats (Rotem et al., 2014). Since these small bacterial hunters can also be abundant in some favorable situations (typically where preys are abundant), they have been proposed as an important potential "population balancer" (Shemesh et al., 2003; Iebba et al., 2014) or a "driver of bacterial alpha diversity" (Johnke et al., 2020). Indeed, BALOs could limit competition and favor rare taxa by eliminating dominant species. Additionally, this functional group of bacteria has been reported to exert strong potential effects on bacterial pathogens (Mun et al., 2017). Thus, numerous applications using BALOs are being developed (Bratanis et al., 2020), in particular in medicine (Iebba et al., 2014; Willis et al., 2016), aquaculture (Li et al., 2014; Cao et al., 2015) and in the food industry (Fratamico and Whiting, 1995; Fratamico and Cooke, 1996) with the goal to reduce or eliminate pathogens. However, these bacteria remain largely underrated and their ecology (particularly in natural aquatic ecosystems) is still poorly documented.

The goal of the present study is to shed light on the dynamics of BALOs in natural aquatic environments, based on a full year of sampling in four contrasted ecosystems: two freshwater sites (in alpine lakes) and two marine sites from the Bay of Banyuls (NW Med. Sea). Based on the recent BALOs classification (Koval et al., 2015; Hahn et al., 2017), we explored BALOs' diversity using two runs of high throughput sequencing (HTS): one targeting all prokaryotes including all BALOs, the second focusing on *Bdellovibrionaceae* and *Peredibacteracaceae*. In parallel, using qPCR, we determined the abundance and distribution of the three main families of BALOs within Oligoflexia. To the best of our knowledge, this is the first comparative study using both HTS and qPCR in natural environments characterizing BALOs diversity, abundance and structure. We aimed at answering the following questions: Are BALOs globally abundant in freshwater and saltwater ecosystems? Which BALOs dominate these ecosystems? Does high throughput sequencing (HTS) fathom the diversity of each BALOs genera?

2. Materials and Methods

2.1. Study sites, sampling and environmental descriptors

An annual water sampling, once a month, was carried out in four different environments for which long-term ecological surveys exist. The locations investigated were Lakes Geneva (SHL2; 46°27'9.72 N - 6°35'19.4 E) and Annecy (GL; 45°52’23.42 N) and two marine sites of the Bay of Banyuls-sur-Mer (North Western Mediterranean Sea), referred to as SOLA (42°29'300 N - 03°08'700 E) and MOLA (42°27'205 N - 03°32'565 E). Sampling of Lakes Geneva and Annecy took place from February 2018 to January 2019 and from March 2018 to February 2019 for SOLA and MOLA. The sampled depths were 2.5, 50 and 200 m for Lake Geneva, 3 and 45 m for Lake Annecy, 3 and 24 m for SOLA and 2, 80 and 200 m for MOLA. Because of bad weather conditions, one sample could not be obtained (200 m in January for MOLA). Two replicates of a 2-liter volume of water from each sampled depth were serially filtered over 5 and 2 µm polycarbonate (PC) filters. Then, one liter of the < 2 µm filtered water was filtered on a 0.2 µm PC filter. All filters were kept and stored at -80°C until DNA extraction. In parallel, physico-chemical descriptors were measured at each site from the information system OLA (Rimet et al., 2020) for lakes (https://si-ola.inra.fr/si_lacs/) and the national observation service SOMLIT for MOLA and SOLA (https://www.somlit.fr/).

2.2. Molecular analyses

DNA extraction was performed from the 0.2 µm filters and these extracts were firstly used for quantitative PCR (qPCR) with specific sets of primers for *Bdellovibrionaceae*, *Peredibacteraceae* and *Bacteriovoracaceae* for all sites. *Halobacteriovoracaceae* abundances were also measured, but only for the marine sites. Secondly, the extracts were used to assess BALOs’ diversity using HTS. One run used two specific sets of primers for *Bdellovibrionaceae* and *Peredibacteraceae* for which we analyzed the samples corresponding to lake surface and bottom taken each month. The second run used a universal (unspecific) pair of primers. The sequence reads were analyzed using the Frederic Mahé pipeline found at https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline. Statistical analyses were performed using R (R Core Team, 2018) and the phylogenetic analysis for BALOs and other bacteria with PhyML (Guindon et al., 2010) and MrBayes (Ronquist et al., 2012). A more complete description can be found in the supplementary data section (Supplementary Text 1).

3. Results

3.1. BALOs abundance, distribution and dynamics

Both abundances and distribution of the BALOs were variable across the ecosystems examined (Figure 1). Because some BALOs were not detected or well amplified, particularly at SOLA and MOLA sites, we focused on samples in which enough measurements were obtained. Overall, when considering all depths and months combined, *Peredibacteraceae* dominated the lakes in terms of abundance (with concentrations up to 71,700 and 31,137 copy.mL^{-1} for Lakes Geneva and Annecy, respectively), followed by the *Bdellovibrionaceae* (reaching up to 20,944 and 3,856 copy.mL^{-1} for Lakes Geneva and Annecy, respectively) (Supplementary Figure 1). By contrast, *Bacteriovoracaceae* were in general in low abundance or undetected; in Lake Annecy for example, maximal concentrations reached 1,250 copy.mL^{-1}. BALOs were more abundant in surface waters (reaching up to 80,550 copy.mL^{-1}) for Lake Geneva, followed by the 50 m depth (with 10,003 copy.mL^{-1}) compared to deeper waters (3,582 copy.mL^{-1}). However, it was the reverse for Lake Annecy, for which the abundance at 45 m could reach 26,153 copy.mL^{-1} vs 10,089 copy.mL^{-1} at the surface. BALOs abundances (Copy mL^{-1}) in the four studied ecosystems at all depths and throughout the year can be found in the Supplementary Table 2.

The abundance of BALOs varied over the year and across ecosystems, showing various dynamics (Supplementary Figure 2). In Lake Geneva, the seasonal patterns observed for *Bdellovibrionaceae* were rather similar at 2.5, 50 and 200 m except in July and October where high values were reached at 2.5 m. The same type of patterns was observed for the *Peredibacteraceae* but at different months, *i.e*. April and October. The *Bacteriovoracaceae* dynamics were more difficult to interpret, with abundances that remain relatively constant along the year. For Lake Annecy, the different bacterial groups exhibited quite different dynamics. A first difference was that the abundance was higher at 45 m than at 3 m. Secondly, BALOs abundance declined significantly in surface during spring and early summer to increase again starting August. The opposite trend was observed at 45 m, where the abundance of BALOs increased from winter until summer and then began to decrease in fall. For MOLA and SOLA sites, the evolution of *Bdellovibrionaceae* was difficult to describe due to missing points. The *Peredibacteraceae* seemed to increase in June and July and decreased from January. A similar trend exists for this last group at SOLA, but the decrease started a little bit earlier, in December (Supplementary Figure 2).

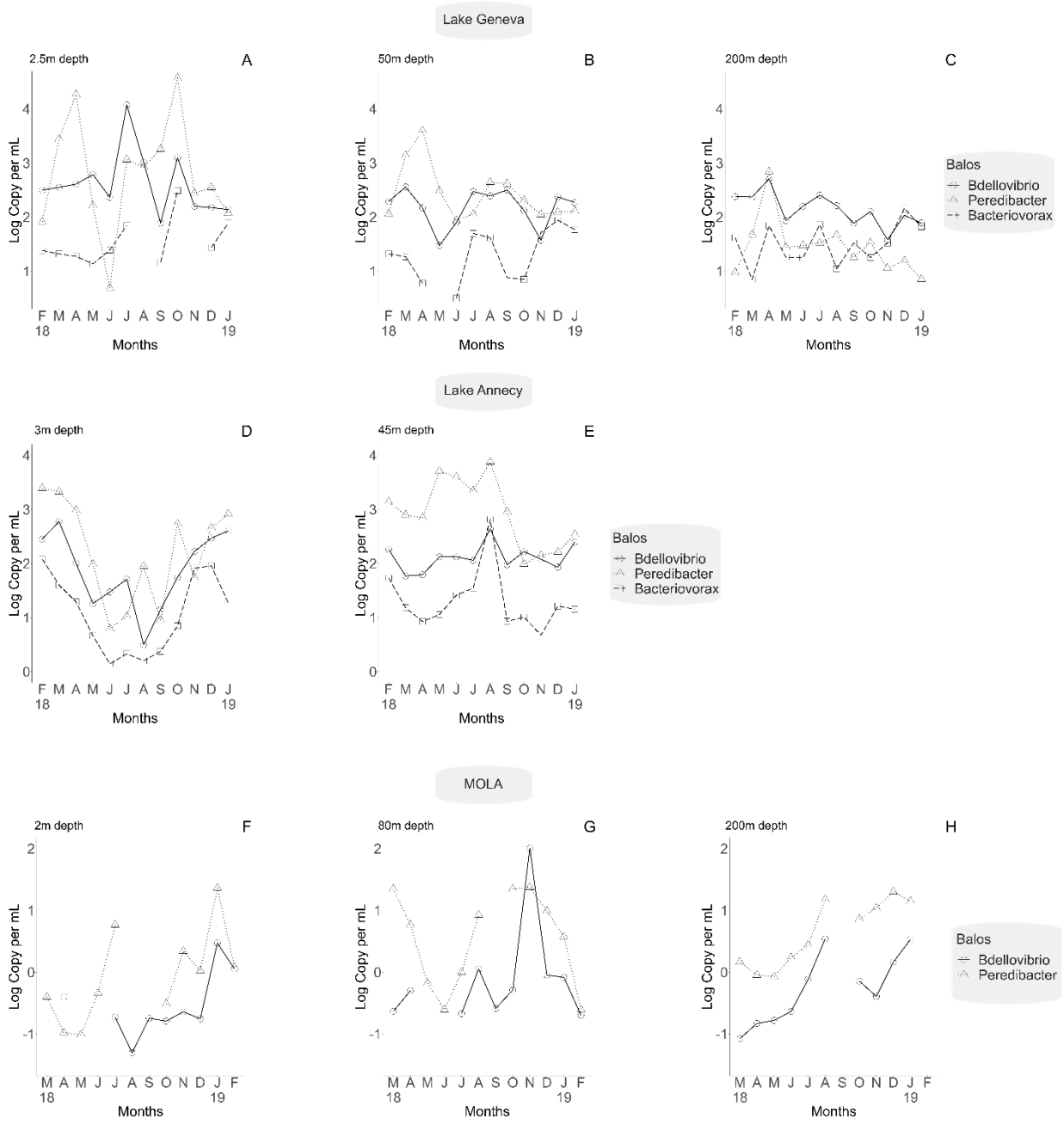


Figure 1: Distribution and dynamics of *Bdellovibrio*, *Peredibacter* and *Bacteriovorax* abundances (copy per mL) over one year in Lakes Geneva and Annecy and at the offshore marine station MOLA. The gaps reflect a lack of amplification of the target BALOs or values that were outside the standard curves.

When looking at the mean abundance of BALOs for each depth during the 12 months of sampling (Figure 2), both *Bdellovibrionaceae* and *Bacteriovoracaceae* were not significantly different between depths in Lake Geneva (*p*-value = 0.06 and 0.95). An opposite trend was observed for the *Peredibacteraceae* (*p*-value = 0.0004) with a significant difference between surface or intermediate waters and 200 m depth (2.5-200 m *p*-value = 0.0003; 50-200 m *p*-value = 0.003) and significantly higher abundances in the upper layer. When comparing *Bdellovibrionaceae, Peredibacteraceae* and *Bacteriovoracaceae,* the mean abundances at 2.5 m of the latter was significantly lower from the mean abundance of the other genera *(p*-value = 0.0017 and 0.0008). By contrast, there was no difference between the mean abundances of *Bdellovibrionaceae* and *Peredibacteraceae* at 2.5 m (*p*-value = 1). At 50 m, similar results than above were observed (*p*-value = 0.001 between *Bacteriovoracaceae* and *Bdellovibrionaceae*; *p*-value = 0.0001 between *Bacteriovoracaceae* and *Peredibacteraceae*). At 200 m the mean abundances between *Bdellovibrionaceae* and the two other families were also significantly different (*p*-value = 0.0004 between *Bdellovibrionaceae* and *Peredibacteraceae; p*-value = 0014 between *Bdellovibrionaceae* and *Bacteriovoracaceae*). However, the mean abundance was not different between *Peredibacteraceae* and *Bacteriovoracaceae* at 200 m (*p*-value = 1). In Lake Annecy, mean abundances at 3 and 45m were not significantly different for *Bdellovibrionaceae* and *Bacteriovoracaceae* (*p*-value = 0.4357 and 0.3572) but they were for *Peredibacteraceae* (*p*-value = 0.03407). The mean abundance at 3 m was significantly different between the different BALOs (*p*-value = 0.00357), in particular between *Bacteriovoracaceae* and *Peredibacteraceae* (*p*-value = 0.00314) and between *Bacteriovoracaceae* and *Bdellovibrionaceae* (*p*-value = 0.04410). The mean abundances at 45 m were all significantly different between the 3 families (*p*-value = 0.000007), i.e. between *Bdellovibrionaceae* and *Bacteriovoracaceae* (*p*-value = 0.0150), *Bdellovibrionaceae* and *Peredibacteraceae* (*p*-value = 0.0334), and between *Bacteriovoracaceae* and *Peredibacteraceae* (*p*-value = 0.00002).

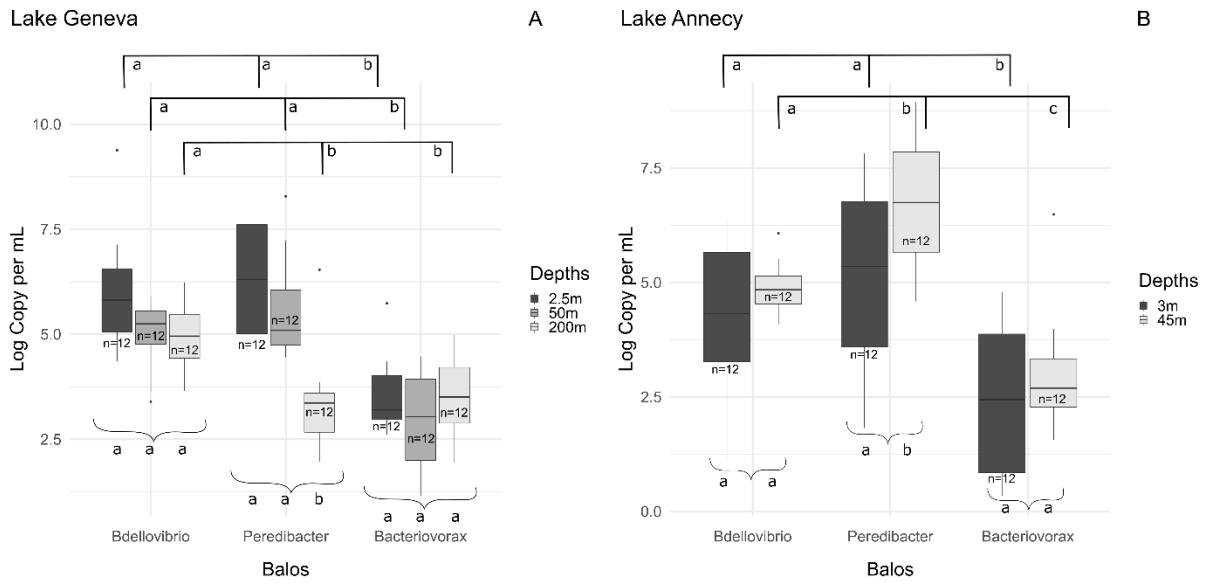


Figure 2: Mean values of the different BALOs at the different depths sampled in Lakes Geneva and Annecy. Brackets are used to show comparison for a BALO between the sampled depth and square-brackets for comparison between BALOs for a same depth. Letters represent the statistical significance ($p < 0.05$) of differences between the studied groups.

3.2. Relationships between BALOs and environmental variables

The first and second axis of the RDA explained for Lakes Geneva and Annecy 45% and 14%, and 61% and 13% of variability, respectively (Figure 3). In Lake Geneva, *Bdellovibrionaceae* were positively related to high values of temperature, pH, total organic carbon and low concentration of nitrate and sulfate. *Peredibacteraceae* variability was associated to high concentrations of chlorophyll and dissolved oxygen, and to low total phosphorus concentration. In contrast, *Bdellovibrionaceae* were more related to high concentrations of sulfate in Lake Annecy while for *Peredibacteraceae* it was high concentrations of nitrate and reactive silica. *Bacteriovoracaceae* was positively related to high concentrations of chlorine and total nitrogen, and to low values of temperature, total and dissolved organic carbon.

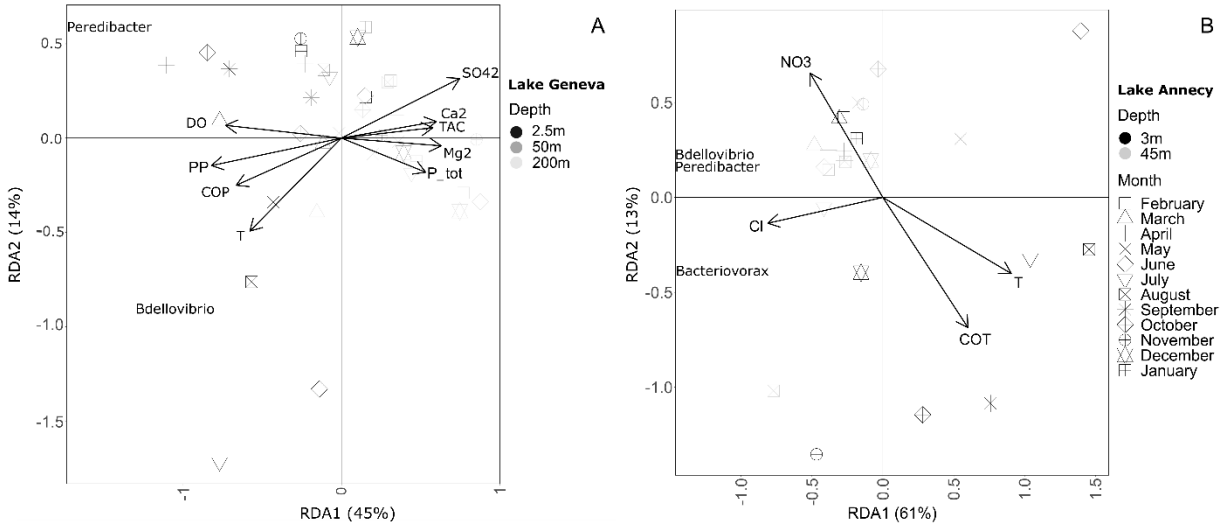


Figure 3: RDA triplots showing the relationship between BALOs and environmental variables in Lake Geneva and Annecy. In Lake Geneva, the first and second axis explained 45 and 14% of the variability, respectively. In Lake Annecy, the first and second axis explained 61 and 13% of the variability, respectively. Arrows indicate the direction and magnitude of variables. Ca2: Calcium ion ; Mg2: Magnesium ion ; Cl: chloride ; PP: Particulate Phosphorus ; P_tot: Total Phosphorus ; SO42: Sulfate ion ; TAC: Water Hardness ; DO: Dissolved Oxygen ; COP: Particulate Organic Carbon ; T: Temperature ; NO3: Nitrate ; COT: Total Organic Carbon.

3.3. OTUs diversity and structure

We used two strategies to capture BALOs' diversity: one with specific pairs of primers targeting *Bdellovibrionaceae* (186F-481R) and *Peredibacteraceae* (1024F-1349R) and the other with a universal set of primer (515F-909R) to encompass all bacteria (Supplementary Text 2). We obtained 110 OTUs for *Bdellovibrionaceae* and 109 OTUs for *Peredibacteraceae* with the specific primers while the "universal" run yielded 34, 5, 13 and 3 OTUs for *Bdellovibrionaceae, Peredibacteraceae, Bacteriovoracaceae* and Micavibrionales. Rarefaction curves revealed that the number of OTUs obtained whatever the method did not reach a plateau (Supplementary Figure 3). The OTUs in *Bdellovibrionaceae* and *Peredibacteraceae* with the higher number of reads (dominant OTUs) were both found in the two lakes. However, lesser abundant OTUs were more often found only in lakes. Overall, Lakes Geneva and Annecy shared 58 OTUs for *Bdellovibrionaceae* and 32 OTUs for *Peredibacteraceae* (Supplementary Figure 4). *Bdellovibrionaceae* OTUs clustered phylogenetically closer to *Bdellovibrio* reference sequences namely *B. bacteriovorus* (*e.g.*, OTUs 12, 21, 42, 47 and 194) and *B. exovorus* (*e.g.*, 4, 8, 24, 46, and 83) (Supplementary Figure 5). The other *Bdellovibrio* OTUs clustered far from the latter but not with other BALOs reference sequences. *Peredibacteraceae* OTUs were closely related to *Peredibacter* namely *P. starrii* (Figure 4). OTUs composition in *Bdellovibrionaceae* and *Peredibacteraceae* were different in the lakes (NMDS: K = 2, stress = 0.13; Supplementary Figure 6. Adonis: $p = 0.003$), but not according to depth and month in each lake, except for Lake Annecy with depth (p-value = 0.30, 0.20, 0.04 and 0.78). *Bdellovibrionaceae* and *Peredibacteraceae* OTUs relative abundance and environmental variables in Lake Geneva correlated positively (*e.g.*, temperature (T) and ammonium (NH_4^+)) and negatively (*e.g.*, chloride (Cl^-) and chlorophyll (chla)) to environmental factors but again these relations were not significant. The same pattern was found for Lake Annecy OTUs (Supplementary Figure 7).

Using the universal primer set, OTUs assigned to Oligoflexia (*e.g.* OTUs 490 and 920) were not always placed next to BALOs type species and other OTUs (*e.g.* 11439) were placed next to *Silvanigrellaceae*. Also, some *Bdellovibrionaceae* OTUs (*e.g.* 676 and 1044) were distant from *Bdellovibrio* type species. By contrast, OTUs in *Peredibacteraceae*, *Bacteriovoracaceae* and Micavibrionales were placed next to their type species. Regarding the quality of the run, only SOLA (and not MOLA) data could be analyzed. Oxygen (O; p-value = 0.005), nitrite (NO_2; p-value = 0.05) and chlorophyll (chla; p-value = 0.017) were identified as influential environmental factors (Supplementary Figure 7).

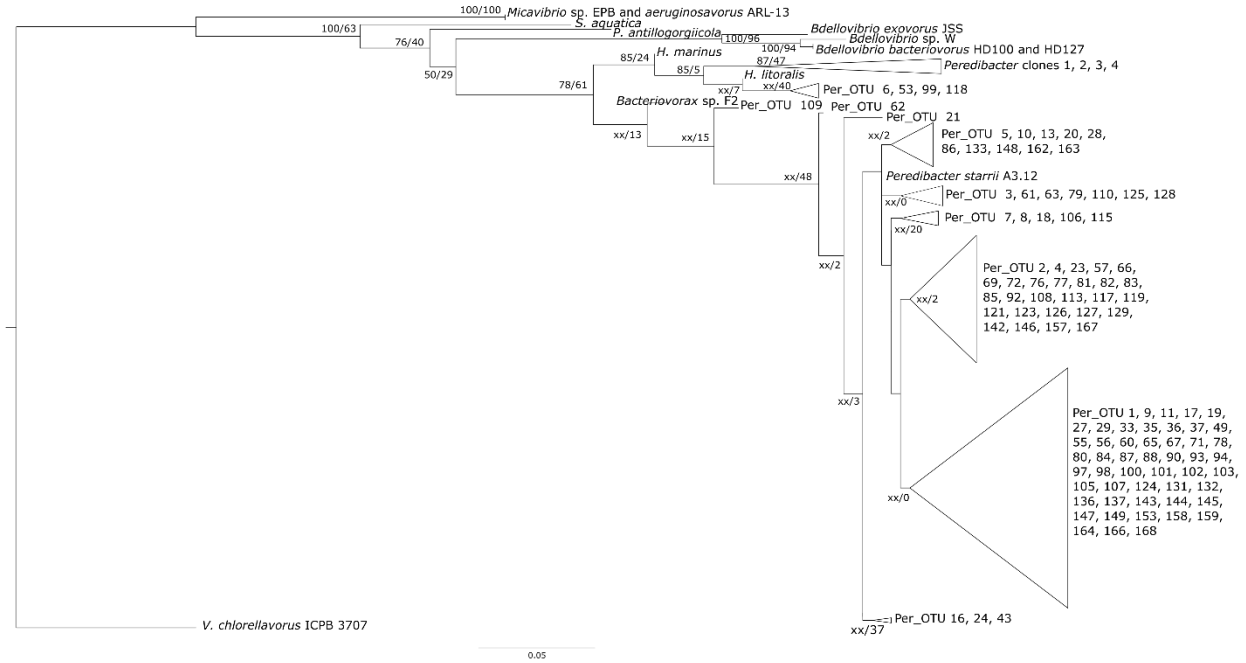


Figure 4: Phylogenetic tree of *Peredibacter* OTUs amplified with Per F1024 – R1349 in Lakes Geneva and Annecy. The tree is based on maximum likelihood and Bayesian methods with the evolutionary model K80 + G (1.11). The alignment curated with Gblocks yielded 268 positions. Posterior probability / bootstrap values are shown at each node when possible. The tree is rooted using the outgroup *Vampirovibrio chlorellavorus*. Most *Peredibacter* OTUs are phylogenetically related to the type species *P. starrii* A3.12.

4. Discussion

BALOs are fascinating bacteria because they are the only known prokaryotic hunters with the requirement to find bacterial preys to grow and reproduce [5]. While their use in a variety of fields has been proposed, as potential or efficient biological-based therapeutic agents against bacterial pathogens [10], their ecology remains largely underexplored, especially in natural aquatic systems [22]. However, such microbial predation might be an essential biotic interaction, as many others, in the maintenance of ecological balance [7, 8]. This study is thus original because our results shed light on both abundance, distribution and diversity on the main BALOs' families belonging to the Oligoflexia class in contrasted ecosystems, i.e. fresh vs marine, lakes vs coastal waters.

The first important result is that we could find all BALO types, whatever the environment studied. Amongst them, some families could reach relatively high concentrations and various dynamics were also recorded. It seems that this functional group of bacteria can be found in a wide variety of natural or manmade environments and we confirm here that it is likely ubiquitous. When comparing the two lakes, we observed that BALOs were concentrated in surface waters of Lake Geneva whereas higher abundances were measured deeper in Lake Annecy. Overall, the genus *Peredibacter* was the most abundant BALO representative, with a preference for the upper layers in both lakes. Comparatively, *Bdellovibrio* were globally more abundant at depth, especially in Lake Geneva. At last, whatever the system or depth examined, *Bacteriovorax* was much less abundant than *Peredibacter* and *Bdellovibrio*. Such a distribution was already observed in a past study [22] and may suggest that each BALO type could have niche preference. Three main peaks of BALOs' abundance were recorded in lakes, between March and April, between July and August and between October and November. These observations were consistent with previous studies (Sutton and Besant, 1994a; Williams and Piñeiro, 2006) and coincided with the peaks of the total bacteria measured by flow cytometry (Supplementary Figure 9). It is assumed here that this relationship could be attributed to favorable conditions, likely to allow efficient growth and development of the bacteria, both prey and predator, the latter depending on the former. It is noteworthy that these peaks of abundances were also observed at 45 m in Lake Annecy, but the dynamics was different than that in surface, perhaps because *Peredibacter* were more abundant at depth in this lake. Moreover, the first peak observed at 3 m in Lake Annecy was observed in winter, highlighting again the capacity of these bacteria to develop and occupy very different niches and periods, as observed elsewhere, for instance in arctic marine sediments (Davidov and Jurkevitch, 2004). It is possible also that BALOs, being active swimmers (Jashnsaz et al., 2017), can migrate from one layer to another where competition is lower and/or prey more available. More generally, despite of preying on a wide range of bacteria, it is likely that each BALO may have different

effects on various hosts in such mixed microbial assemblages, which are characteristics of natural ecosystems. For the marine site MOLA, *Peredibacter* was also the dominant BALO whatever the depth sampled while *Halobacteriovorax* was difficult to detect, either because this group was poorly represented or because of methodological bias. We believe the first hypothesis was the good one since we could obtain good amplification for this group in other marine or estuarine waters (not shown).

The multivariate analyses did not reveal strong relationships between environmental factors and the distribution of BALOs. However, at SOLA, dissolved oxygen, chlorophyll and nitrite concentrations had significant relationships with BALOs. Also, temperature was likely important to explain *Bdellovibrio* abundances in Lake Geneva, and those of *Bdellovibrio* and *Peredibacter* in Lake Annecy. Most surprisingly, the potential relationship of chloride (known to be a biocide at high levels) with *Bacteriovorax* in Lake Annecy would deserve confirmation using laboratory experiments. At that time, we can only hypothesize that BALOs might have developed a certain resistance to chloride while other competitors, other bacterial predators, did not (Khan et al., 2019). It is noteworthy here that Huang and Starr (Huang and Starr, 1973) reported that some cations, magnesium and calcium, could play a role in predator-prey interaction. Globally, both the variability of BALOs' abundance and diversity was poorly explained by environmental variables, as already mentioned before (Varon and Shilo, 1981). The exception comes with salinity that may explain the exclusive presence of the halophilic BALO *Halobacteriovorax* in salt waters and some pollutants that can halt and favor BALOs to adhere to surfaces [33, 34].

It was globally hard to detect BALOs in the Bay of Banyuls, using either the primers dedicated to qPCR or the ones for PCR and HTS. We believe that BALOs abundance was in fact relatively low or diluted in the vast space of the Bay, since the role of inhibitors was unlikely (according to specific conducted tests), and because BALOs are generally more dominant in closed systems, sediments or biofilms compared to the open water column (Williams and Piñeiro, 2006; Kandel et al., 2014). A way to work around this could be the use of Droplet Digital PCR (ddPCR), which allows a better detection of rare targets.

Based on obtained OTUs, *Peredibacter* and *Bdellovibrio* could be numerous, suggesting these groups are relatively diverse, a feature already shown in a previous study (Ezzedine et al., 2020a). However, one must keep in mind that the number of generated OTUs can vary greatly with the used algorithms. Here, we used swarm algorithm (Mahé et al., 2015) that produce fine-scale molecular OTUs. When looking at the shared and unshared OTUs in the two lakes, dominant OTUs with a high number of reads of *Bdellovibrio* and *Peredibacter* were shared, while OTUs with a low number of reads were not shared and most likely inferred to each environment. We therefore hypothesize that some BALOs could adapt to different environments, while others are only adapted

to the environment in which they are located. Finally, the presence of *Peredibacter* and *Bdellovibrio* in the lacustrine and marine environments arose our interest in studying their genetic structure in order to find the adaptations processes that allow them to exist in both environments. Detecting and isolating active individuals of these groups could be the premise of separating *Peredibacter* and *Bdellovibrio* into fresh and salt-water clades. For that, it would be necessary to isolate active individuals, as the detection of DNA alone is not enough and could be issued from elsewhere (Ezzedine et al., 2020a). A shotgun sequencing and genetic annotation would help to characterize the adaptation of each one. In the laboratory, manipulating a variety of prey from fresh or marine waters could also be interesting to analyses whether some differences exist in terms of efficiency and predation spectrum. Clearly, what is the predatory behavior of BALOs in complex natural habitats is still to be discovered.

5. Conclusion

The present study showed that BALOs are present and sometimes relatively abundant and probably very diverse in lakes and marine ecosystems. Clearly, our results extend our knowledge on BALOs and confirm they can occupy diverse ecological niches. Among them, *Peredibacter* was the dominant group and likely to be driven by biotic variables and interactions, not investigated here. The most important questions are still pending: what is the role of these bacteria and to what extent do they influence composition and dynamics of the bacterial community? The next step is to perform dedicated experiments to quantify BALOs' predation, to assess how it is comparable to other biotic pressure (e.g. viral lysis, flagellate or ciliate grazing) and how it can influence the carbon cycle in aquatic ecosystems.

6. Data availability

The raw files (R1 and R2), tags list, unfiltered and filtered OTUs table of the specific and universal run datasets obtained in this study have been deposited at Zenodo's depository under DOI number: 10.5281/zenodo.4293824. Also, most of the OTU sequences used to construct phylogenetic tree can be found at NCBI GenBank under accession numbers: MW299511:MW299708 and MW302902:MW302988.

7. Supplementary data

7.1. DNA extraction

The two replicates of the 0.2 µm filters were subjected to DNA extraction using a homemade protocol. First, all samples were centrifuged for 3 min at 6,000 g and 4°C. The supernatant was discarded. Second, 300 µL of TE buffer (TRIS: 1 M – pH 8, EDTA: 0.5 M – pH 8) were added to the pellet. Next, a lysis step was performed by adding 200 µL of lysis solution (TRIS: 1 M – pH 8, EDTA: 0.5 M – pH 8 and sucrose: 0.7 M). After, a thermic shock was performed at -80°C for 15 min and samples were immediately thawed into a block heater at 55°C for 2 min. 50 µL of 10% sodium dodecyl sulfate (SDS) as well as 10 µL of proteinase K (20 mg/mL) were added. Samples were then incubated at 37°C for 1 h with gentle stirring, and placed in a heating block at 55°C for 20 min. After a quick centrifugation step (13,000 rpm at 4°C for 3 min), the supernatant was collected. Then, 50 µL of sodium acetate (3M – pH 5.2) and 1.5 µL of GenEluteTM-LPA (Sigma-Aldrich, 25µg/µL) was added. Next, one volume of isopropanol was added and the tubes were centrifuged for 10 min at 12,000 g and 4°C. Following this step, two rounds of ethanol (80%) washing were carried out to clean the DNA pellet. The remaining ethanol was evaporated using a SpeedVac for 20 min. Finally, 30 µl of TE was added and samples were incubated at 37°C for 1 h to let the pellet gently dissolve into the TE buffer. DNA concentration was measured using NanoDrop 1000 spectrophotometer. For DNA concentration superior to 25 ng/µL, a dilution was performed. All DNA preparations were stored at -20°C until analysis.

7.2. qPCR standard curves

One replicate of the 0.2 µm PC filters were used to measure the abundances of *Bdellovibrio*, *Peredibacter*, *Bacteriovorax* in Lake Geneva, Lake Annecy, SOLA and MOLA at all sampled depth with qPCR machine Rotor-Gene Q (Qiagen). *Halobacteriovorax* abundance were also measured in SOLA and MOLA. The set of primer used to measure BALOs abundance are Bd 347F – 549R (Van Essche et al., 2009) (*Bdellovibrio*), Per 627F – 696R (Ezzedine et al., 2020a) (*Peredibacter*), Bx 421F – 482R (Ezzedine et al., 2020a) (*Bacteriovorax*) and Hbx 220F – 279R (Ezzedine et al., 2020a) (*Halobacteriovorax*). Standards were prepared using identified clones as stated in our previous study (Ezzedine et al., 2020a). In brief, plasmid were extracted and purified using NucleoSpin Plasmid kit (Macherey-Nagel) according to the manufacturer's instructions. Plasmids were then digest with BamH I restriction enzyme following manufacturer's instructions. Then digested plasmid concentration were measured using Quant-iT PicoGreen ds DNA Reagent kit (Invitrogen) and fluorescence was read using the plate reader Fluoroskan Ascent FL. Number of copies for each BALOs clone were calculated using the following formula :

$$Number\ of\ copies = \frac{DNA\ concentration}{(Insert\ size + Plasmid\ size) \times 660} \times 6.02 \times 10^{23}$$

Then serial dilution was conducted from 10^9 to 10^0 copies. Diluted DNA from 10^7 to 10^0 were used in duplicate and amplified by BALOs qPCR set of primers to constitute the standard curve. Two control were added each time. Lake samples *i.e.,* Geneva and Annecy were integrated in the same run. Likewise for marine samples *i.e.,* SOLA and MOLA. The qPCR mixture volume was 25 µL and consisted of (final concentration): 1 X Master Mix (QuantiTect SYBR Green PCR kit, Qiagen), 0.3 mg mL^{-1} of BSA, 0.2 µM of forward and reverse primers and 1 µL of template DNA (25 ng μL^{-1}). The program used was as follow: 95°C – 15 min, 40 × (95°C – 45 s, 60°C – 45 s, 72°C – 45 s), + 1°C every 5 s from 60 to 95°C. The standard curve parameters for *Bdellovibrio* for Lakes were R^2 = 0.99916 and efficiency = 0.95. As for marine samples, R^2= 0.99752 and efficiency = 0.91. The threshold was set to 0.02. In the same logic and order for Lakes and marine samples, *Peredibacter* standard curve parameters were R^2 = 0.999762, efficiency = 0.91, R^2 = 0.99895, efficiency 0.92 and threshold set to 0.015. For *Bacteriovorax* R^2 = 0.99784, efficiency = 0.93, R^2 = 0.99968, efficiency = 0.95 and threshold set to 0.02. Finally, for *Halobacteriovorax* (SOLA and MOLA samples only), R^2 = 0.99714, efficiency = 0.99 and threshold set to 0.02. For all environmental samples amplified with BALOs primers, those that failed to amplify or were outside (lower) the standard curve were not considered in the analysis. BALOs abundances were obtained in copy per reaction and were transformed to copy per milliliter using the fowling formula:

$$Copy\ per\ milliliter = \frac{\frac{Copy\ per\ reaction}{Dilution\ factor\ for\ 25\ ng\ \mu L^{-1}} \times DNA\ elution\ volume}{Filtered\ volume\ on\ 0.2\ \mu m}$$

7.3. PCR and Next-generation sequencing

Two sequencing run were prepared and only one of the two replicates were sequenced. One utilized universal prokaryote primers and the second employed BALOs specific primers. The first, totalizing 95 samples, involved extracted DNA of Lake Geneva, Annecy, SOLA and MOLA from surface (2, 2.5, 3 m) and bottom (24, 45 and 200 m). All samples were amplified by polymerase chain reaction (PCR) using a combination of tags attached to universal primers 515F (Wang and Qian, 2009) (GTGYCAGCMGCCGCGGTA) and 909R (Wang et al., 2018) (CCCCCGYCAATTCMTTTRAGT). Hence, each sample could be discriminated by the tagging of forward and reverse primers. PCR mixture volume was 50 µL and consisted of (final

concentration): 1 U buffer, 0.4 mM dNTP, 2 mM $MgCl_2$, 0.4 mg mL^{-1} bovine serum albumin (BSA) and 1 U Biotaq DNA polymerase (Bioline). In a second step, a unique combination of tagged primers (0.2 µM) was added to each sample. Finally, 1 µL of template DNA (25 ng $µL^{-1}$) was added. Negative controls were included and the PCR program was as follows: 95°C – 2 min, 30 × (94°C – 30 s, 58°C – 30 s, 72°C – 30 s), with a final extension step at 72°C for 5 min. Agarose gel analysis was performed for verification of the PCR products. This revealed that non-specific bands were close to the expected band (~ 400 bp). The 95 samples were quantified using the Quant-iT PicoGreen ds DNA Reagent kit (Invitrogen) and fluorescence was read using the plate reader Fluoroskan Ascent FL. Samples belonging to each environment were pooled in an equimolar way. Each pool was checked on agarose gel. Then, under UV light, expected band was cut using a sterile scalpel. The excised band was purified using "Illustra GFX Gel Band Purification Kit" following the manufacturer's instructions. Then each pool was measured using PicoGreen to make a single equimolar pool containing 1000 ng DNA.

The second run consisted of 96 samples from the amplification of Lakes Geneva and Annecy DNA from surface and bottom by specific primers for *Bdellovibrio* (Bd F186 - R481) and *Peredibacter* (Per F1024 – R1349) (Ezzedine et al., 2020a). The same workflow was applied to the resulting amplicons, however primer dimers were highly present so we did not measure each sample with Picogreen. Therefore, no equimolar pools were made for Bd Geneva, Bd Annecy, Per Geneva and Per Annecy. The band of interest was excised as described before from these pool and purified. Then each purified sample was measured using Picogreen to constitute a final equimolar pool.

Pool 1 (universal Geneva, Annecy, SOLA and MOLA) and Pool 2 (Bd Geneva, Bd Annecy, Per Geneva and Per Annecy) were sent to the GATC-Eurofins platform for DNA sequencing using Illumina Miseq 250 bp paired end technology (12M reads package).

7.4. Bioinformatic pipeline

R1 and R2 fastq files of each pool were processed using Frederic Mahé pipeline found at https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline. Briefly, the pipeline uses multiple program such as Vsearch (Rognes et al., 2016), Cutadapt (Martin, 2011), Swarm and Stampa (Mahé et al., 2015). All default parameters of the pipeline were left unchanged unless stated otherwise. OTUs were created using Swarm with "d=1" for the first pool and second pool. All OTUs were taxonomically assigned with arb-SILVA database release number 138 (Quast et al., 2013). OTUs tables were filtered by removing chimera sequences, singletons, sequences with less than 90% identity to the database and sequences with a quality score inferior to 0.0002.

7.5. Statistics

For the abundance data (copy.mL^{-1}), the values were transformed using *log(x)*. All graphs were built *via* ggplot2 (Wickham et al., 2018) using the transformed values. The "vegan" package

(Oksanen et al., 2019) was used to analyze BALOs abundance with environmental variables. First, a DCA (detrended correspondence analysis) was performed on Lake Geneva and Annecy data (DCA axis lengths < 3). The test determined that RDA (redundancy analysis) was the adequate choice for the analysis (Powell, 2018). In addition, "Envfit" function and "corrplot" package (Wei et al., 2017) were used to select significant environmental variables. The RDA significance was tested using ANOVA (analysis of variance).

For OTUs, Non-metric multidimensional scaling (NMDS), Permutational multivariate analysis of variance using distance matrices (Adonis), Canonical correspondence analysis (CCA) and rarefaction curves were performed and draw using the R vegan package (Oksanen et al., 2019). Here, CCA were performed since the DCA > 3. Also, the significance of the CCA was tested using ANOVA and Variance inflation factors (VIF) were calculated for environmental variable where only VIF <10 were selected. Venn diagram were drawn using the online tool found at http://bioinformatics.psb.ugent.be/webtools/Venn/.

7.6. Phylogeny

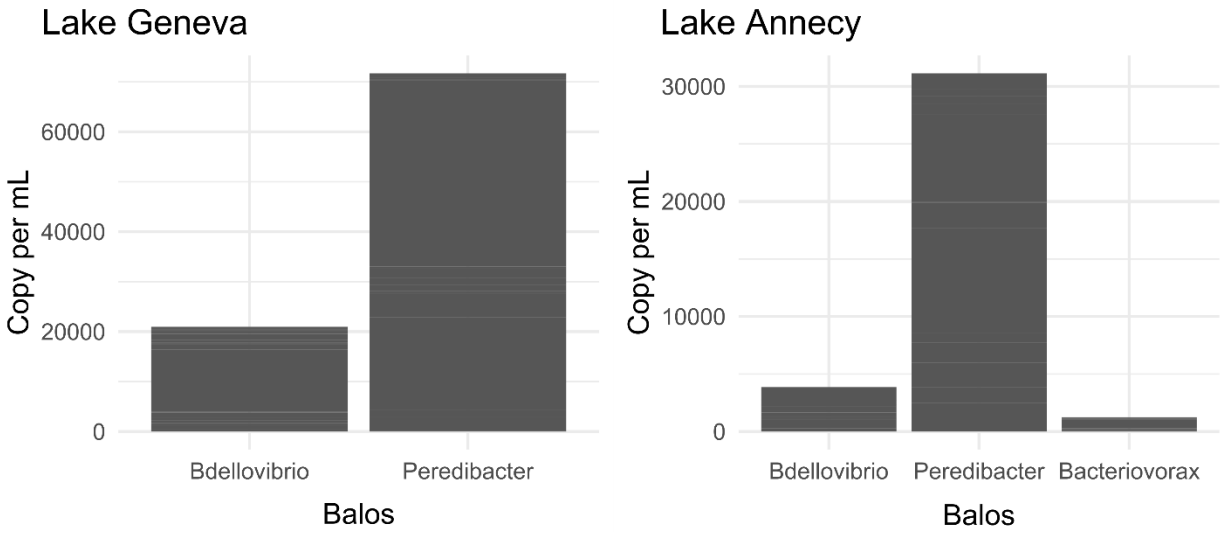
Phylogenetic trees were constructed using BALOs OTUs obtained by specific and universal set of primers and BALOs references sequences (Supplementary Table 1). OTUs obtained with the universal set of primers assigned to Oligoflexia and *Silvanigrellaceae* were also included in the phylogeny. Briefly, OTUs and reference sequences were first aligned and visualized together using MUSCLE (Edgar, 2004) and MEGA X (Kumar et al., 2018). Then, both ends of the sequences were trimmed, making them of equal length. For specific and universal primers, the alignment generated respectively 229, 235, and 389 positions for *Bdellovibrio, Peredibacter* and BALOs (OTUs from universal amplification). The alignments were next curated with Gblocks 0.91b (Castresana, 2000), yielding respectively 221, 268 and 353 positions. ModelGenerator v.85 (Keane et al., 2006) was used to select the best nucleotide substitution model with discrete gamma categories set to 4. Akaike information criterion (AIC) (Akaike H, 1973) was used for model selection. The models used to compute *Bdellovibrio, Peredibacter* and BALOs trees were respectively GTR + G (0.29), K80 + G (1.11), GTR + G (0.52) + I (0.30). The trees were then built using Maximum likelihood (100 bootstrap replicates) with PhyML 3.1 (Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, 2010) and Bayesian method (1 to 1.5 millions generations and 25 % burn-in value) using MrBayes 3.2.7a (Ronquist F, Teslenko M, Mark PVD, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, 2012).

Supplementary Table 1: Accession numbers of BALOs and other bacteria sequences downloaded from NCBI used to build the phylogenetic trees.

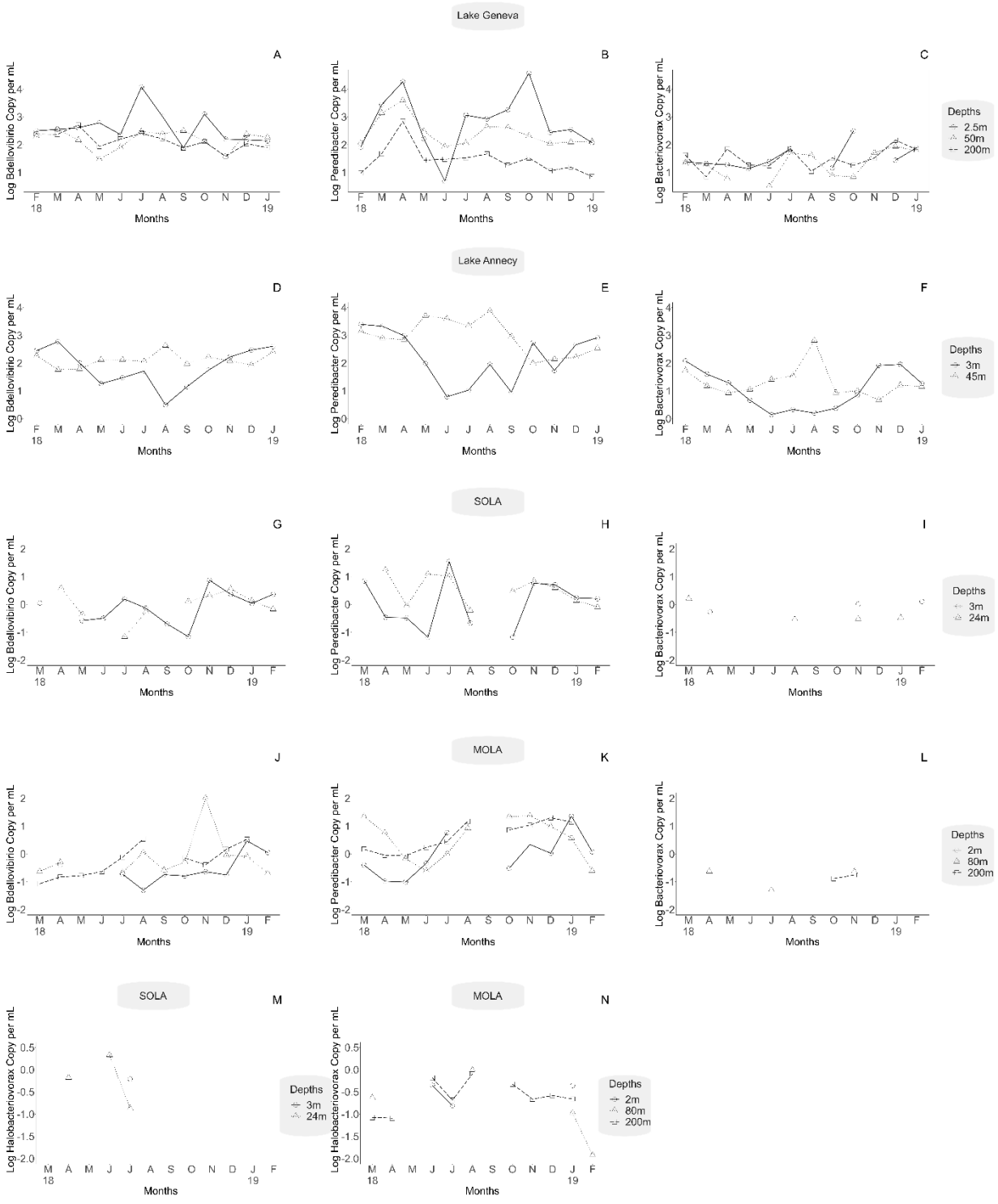
Sequence name	Accession number
Bdellovibrio bacteriovorus strain HD127	AJ29276.1
Bdellovibrio sp. W strain ATCC 27047	AJ292518.1
Bdellovibrio bacteriovorus strain 109J	M61234.1
Bdellovibrio bacteriovorus strain HD100	NR027553.1
Bdellovibrio exovorus strain JSS	EF687743.1
Peredibacter sp. Uncultured clone 1	KT308262.1
Peredibacter sp. Uncultured clone 2	JX525305.1
Peredibacter sp. Uncultured clone 3	KC545751.1
Peredibacter sp. Uncultured clone 4	JX525192.1
Peredibacter starrii strain A3.12	NR024943.1
Bacteriovorax stolpii strain Uki2	NR115142.1
Bacteriovorax sp. strain F2	AY294218.1
Micavibrio aeruginosavorus strain ARL-13	DQ186612.1
Micavibrio sp. strain EPB	DQ186613.1
Pseudobacteriovorax antillogorgiicola strain RKEM611	KJ685394.1
Halobacteriovorax marinus strain SJ	NR102485.1
Halobacteriovorax litoralis strain JS5	NR028724.1
Silvanigrella aquatica strain MWH-Nonnen-W8red	NR157754.1
Vampirovibrio chlorellavorus strain ICPB 3707	NR104911.1

Supplementary Table 2: BALOs abundances in copy per mL in the four studied ecosystems for all the sampled depth throughout the year. Minimal, maximal and mean values that are highlighted in gray were calculated with missing values.

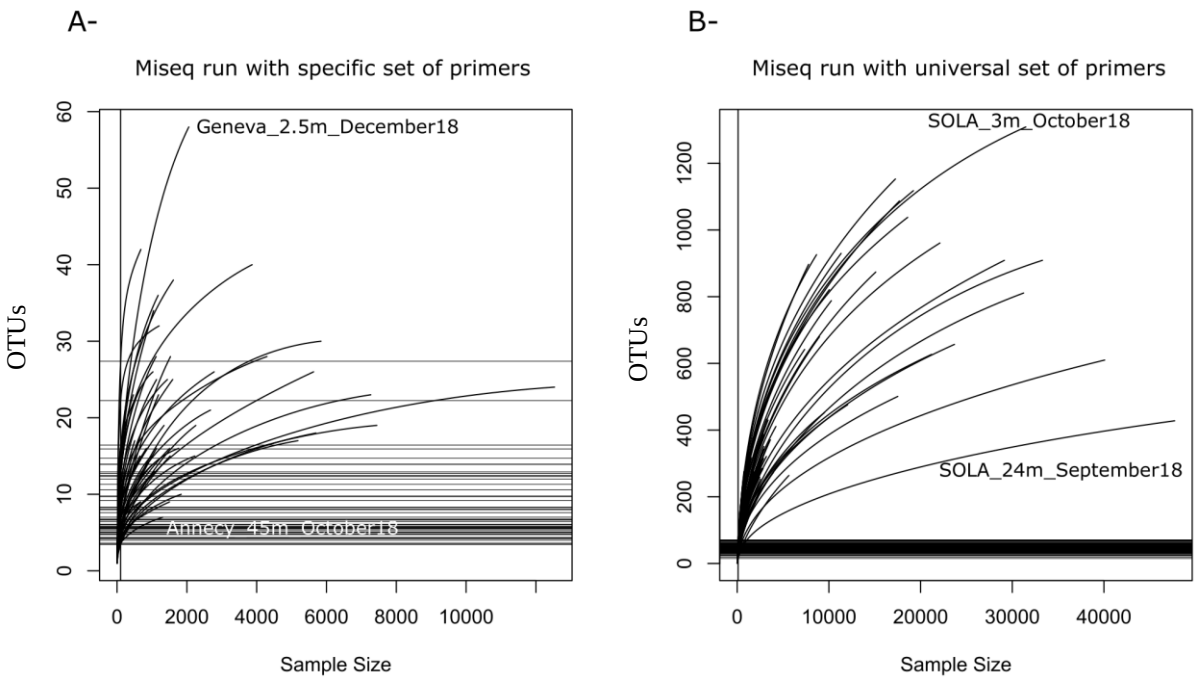
	Lake Geneva (Copy mL^{-1})			**Lake Annecy (Copy mL^{-1})**			**SOLA (Copy mL^{-1})**			**MOLA (Copy mL^{-1})**		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Bdellovibrio	30	11,858	582	3	598	161	0.07	7	2	0.05	102	4
Peredibacter	5	37,270	1,992	6	7,604	1,297	0.07	35	6	0.1	24	7
Bacteriovorax	3	310	45	1	655	52	0.3	2	0.8	0.05	0.2	0.1
Halobacteriovorax							0.1	2	0.9	0.01	1	0.3



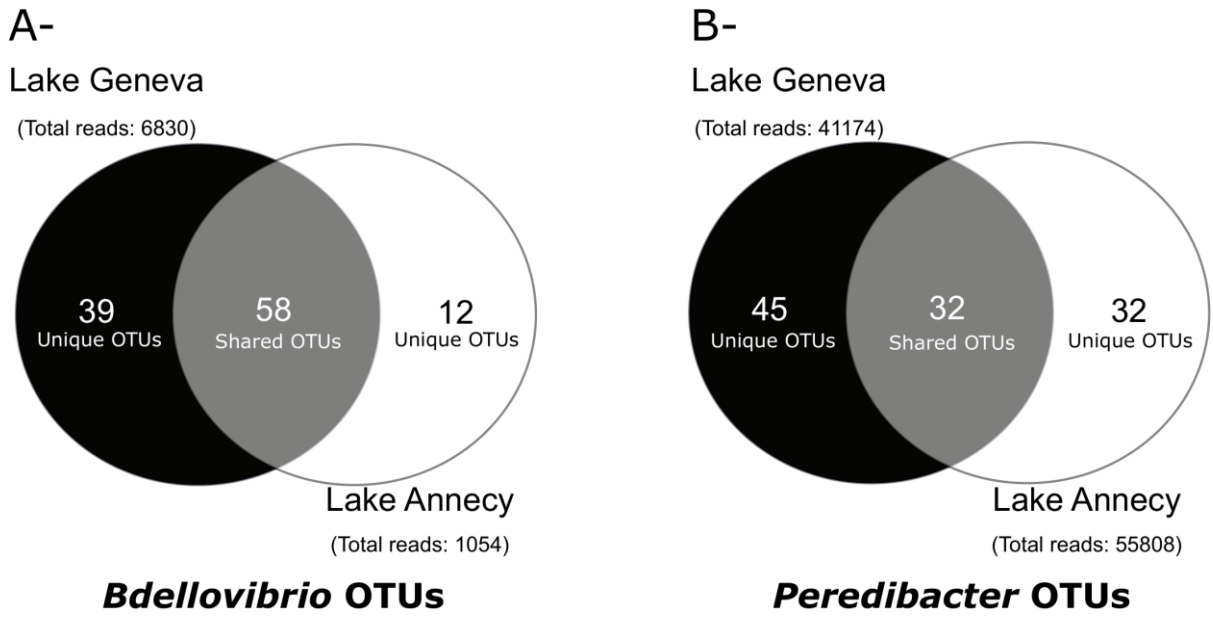
Supplementary Figure 1: Sum of BALOs abundance in copy per mL in Lake Geneva and Annecy for all sampled depth and months. The barplots show that *Peredibacter* dominated in number in each ecosystem.



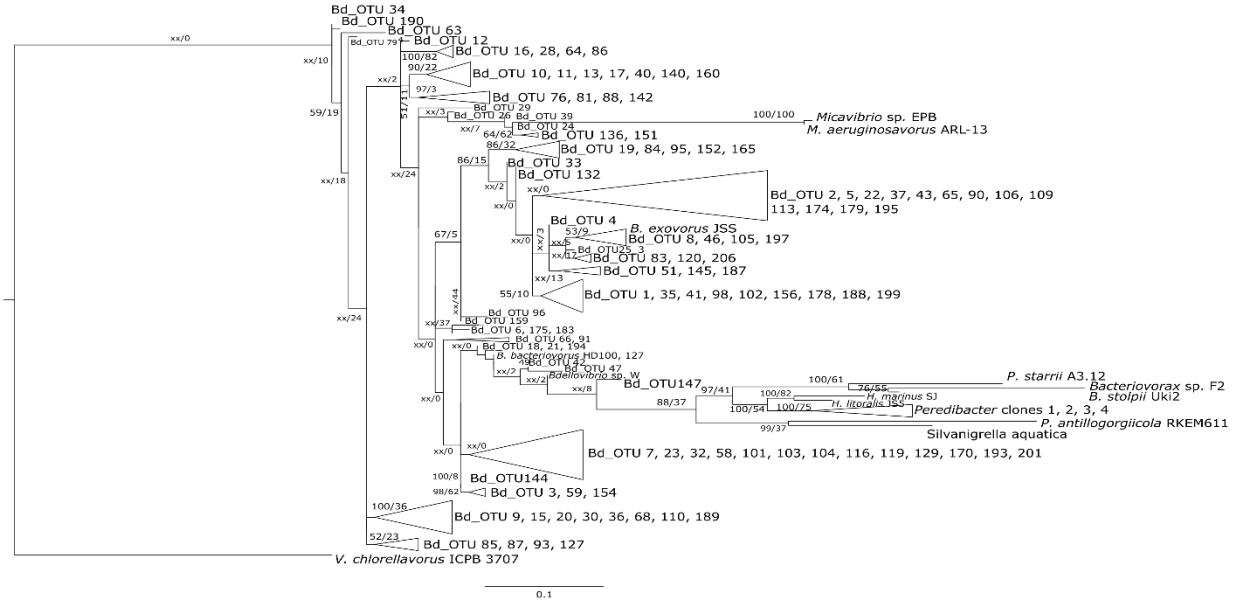
Supplementary Figure 2: Distribution and dynamics of *Bdellovibrio*, *Peredibacter*, and *Bacteriovorax* in the water column sampled in the four studied ecosystems.



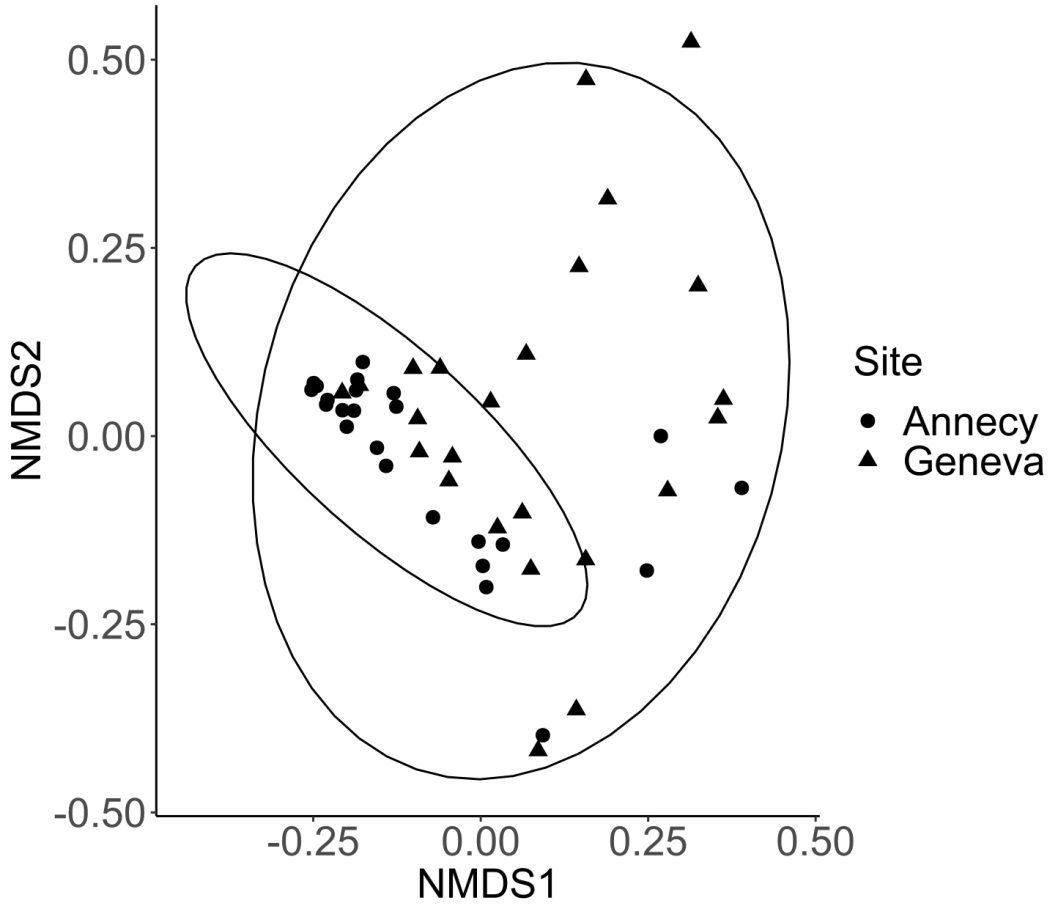
Supplementary Figure 3: Rarefaction curves for the samples of the "specific" and "universal" run computed from BALOs OTUs (A) and bacteria OTUs (B).



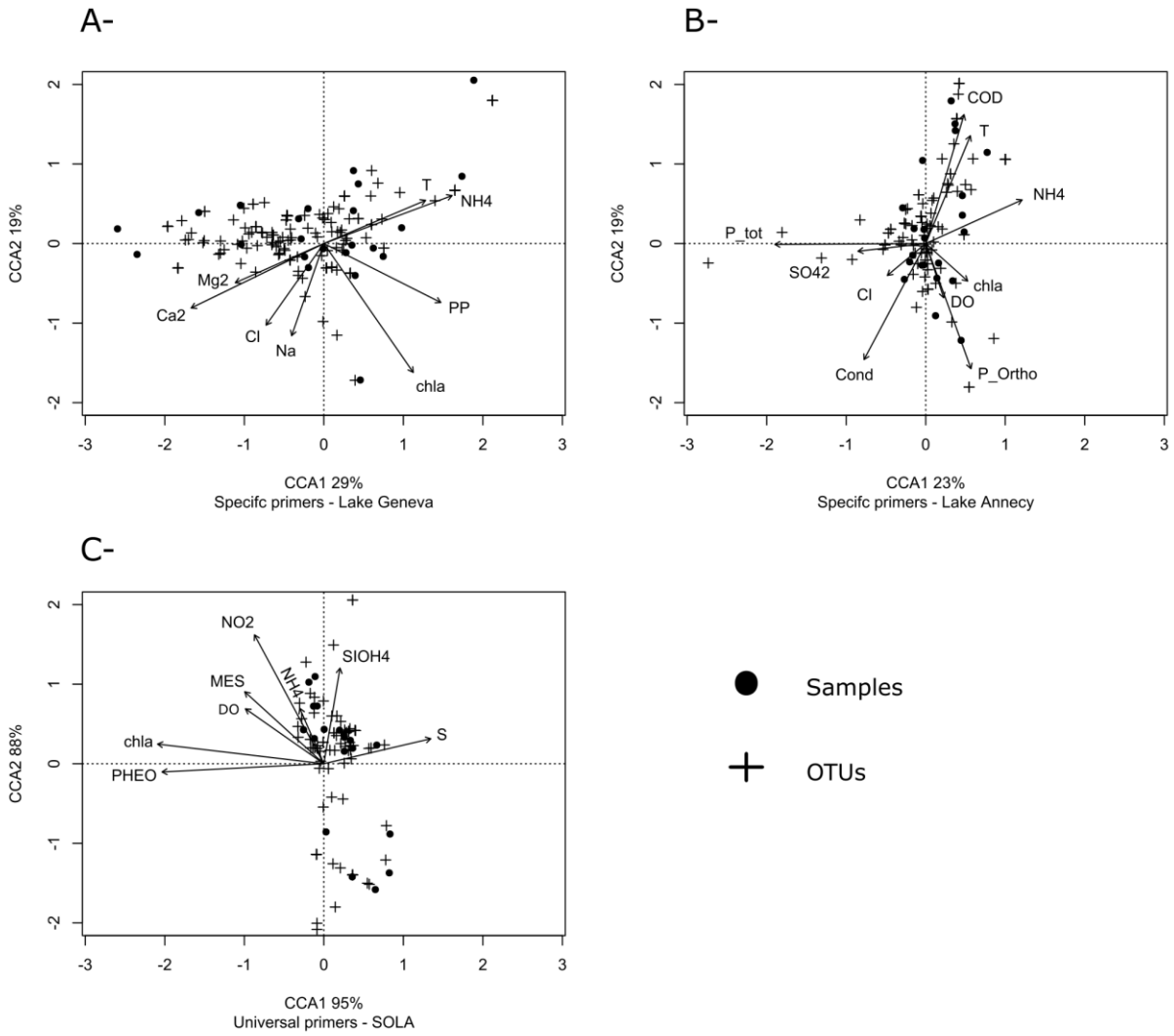
Supplementary Figure 4: Venn diagram showing shared and unshared *Bdellovibrio* (A) and *Peredibacter* (B) OTUs in Lakes Geneva and Annecy.



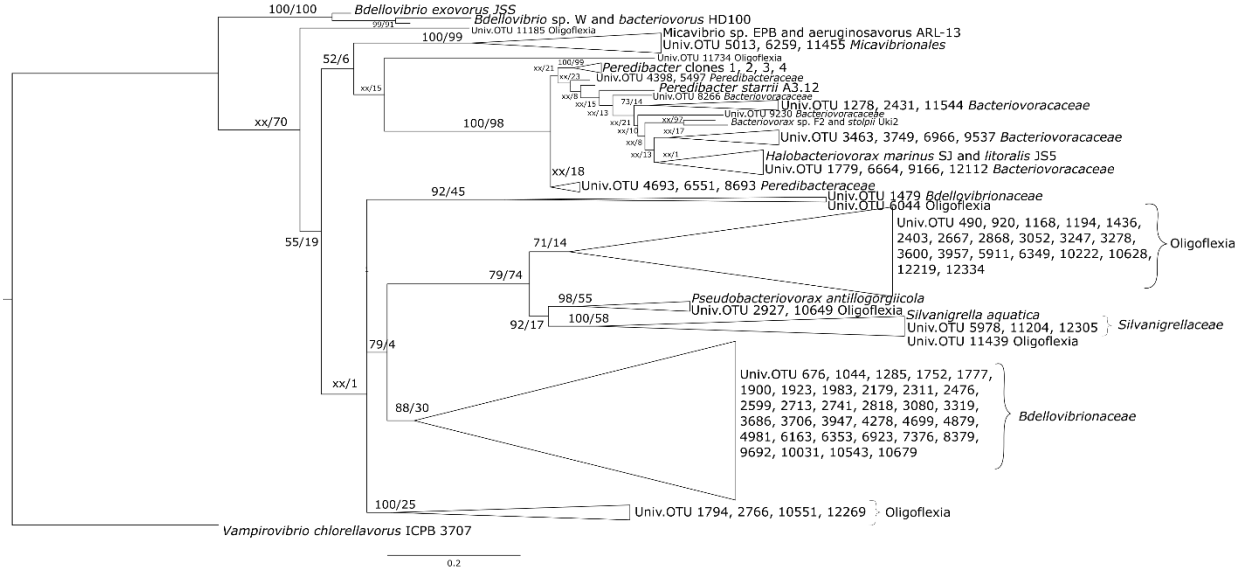
Supplementary Figure 5: Phylogenetic tree of *Bdellovibrio* OTUs amplified with Bd F186 - R481 in Lakes Geneva and Annecy. The tree is based on maximum likelihood and Bayesian methods. The alignment curated with Gblocks yielded 221 positions. The substitution model used is GTR + G (0.29). Posterior probability values / bootstrap values are shown at each node when possible. The tree is rooted using *Vampirovibrio chlorellavorus*.



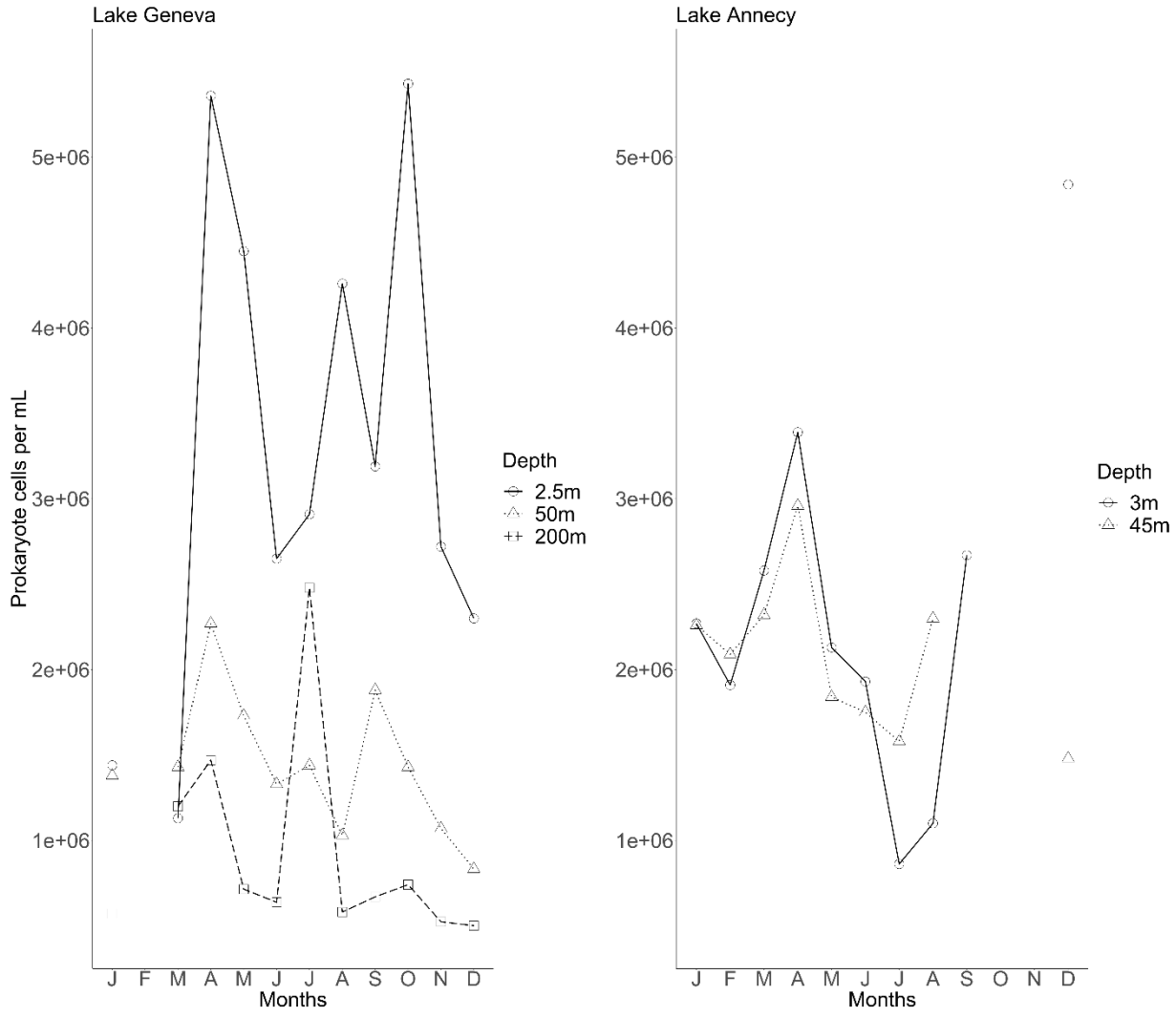
Supplementary Figure 6: Nonmetric Multidimensional Scaling (NMDS) showing the placement of the samples from the specific Miseq run (*Bdellovibrio* and *Peredibacter*) in the ordination space (stress = 0.13) with 95%ellipses. Permutational Multivariate Analysis of Variance (Adonis) showed that OTUs are statistically different from each other between the two lakes (*p*-value = 0.003) and between the two sampled depths of Lake Annecy (*p*-value = 0.04).



Supplementary Figure 7: Canonical Correspondence Analysis (CCA). Ordination diagram of the BALOs community data with environmental variables. The CCA model of Lakes Geneva and Annecy are not significant according to ANOVA. However, SOLA's CCA model is significant (*p*-value = 0.05). Ca2: Calcium ion ; Mg2: Magnesium ion ; Cl: chloride ; Na: Sodium ; chla: Chlorophyll ; PP: Particulate Phosphorus ; NH4: Ammonium ; P_tot: Total Phosphorus ; SO42: Sulfate ion ; Cond: Conducitivity ; P_Ortho: Orthophosphate ion ; DO: Dissolved Oxygen ; COD: Dissolved Organic Carbon ; T: Temperature ; NO2: Nitrite ; SIOH4: Silicate ; S: Salinity ; PHEO: Phaeopigments ; MES: Suspended particles.



Supplementary Figure 8: Phylogenetic tree of BALOs, Oligoflexia and *Silvanigrellaceae* OTUs amplified with the universal set of primer F515 – R909 in Lakes Geneva and Annecy and the marine sites SOLA and MOLA. The tree is based on maximum likelihood and Bayesian methods. The alignment curated with Gblocks yielded 389 positions. The substitution model used is GTR + G (0.52) + I (0.30). Posterior probability values / bootstrap values are shown at each node. The tree is rooted using *Vampirovibrio chlorellavorus*.



Supplementary Figure 9: Evolution of the prokaryote-like particle abundances in Lakes Geneva and Annecy in the water column from January to December 2018 as measured by flow cytometry. Gaps represent missing measurements.

Conclusion du chapitre III

Après avoir développé et optimisé les outils moléculaires nécessaires pour capturer une partie de la diversité et de l'abondance des BALOs, nous avons pu suivre la dynamique de quelques familles dans des systèmes aquatiques diversifiés. Il semble confirmé que le genre *Peredibacter* est le plus abondant, plus particulièrement en milieux lacustres, un résulat pouvant suggérer l'importance fonctionnel de ce groupe auquel il faudrait s'intérsser plus finement à l'avenir *via* l'isolement et la mise en culture d'individus actifs et de leurs proies. Ce chapitre a également révélé que les BALOs étudiés semblent dépendre assez peu des variables environnementales physico-chimiques mesurées, la température pouvant toutefois avoir un effet indirect assez marqué, sur les proies qui profitent alors aux prédateurs. Il apparait très clairement qu'il faudrait s'intéresser aux proies et à d'autres compartiments microbiens (et viraux), les interactions biotiques semblant à l'évidence très importantes pour expliquer la diversité, l'abondance et la distribution des BALOs. Des efforts d'isolement et des analyses à court terme sont donc importants et nous avons tenté de les réaliser en partie.

Chapitre IV

Un écosystème modèle, le Léman ?

Chapitre IV : Un écosystème modèle, le Léman ?

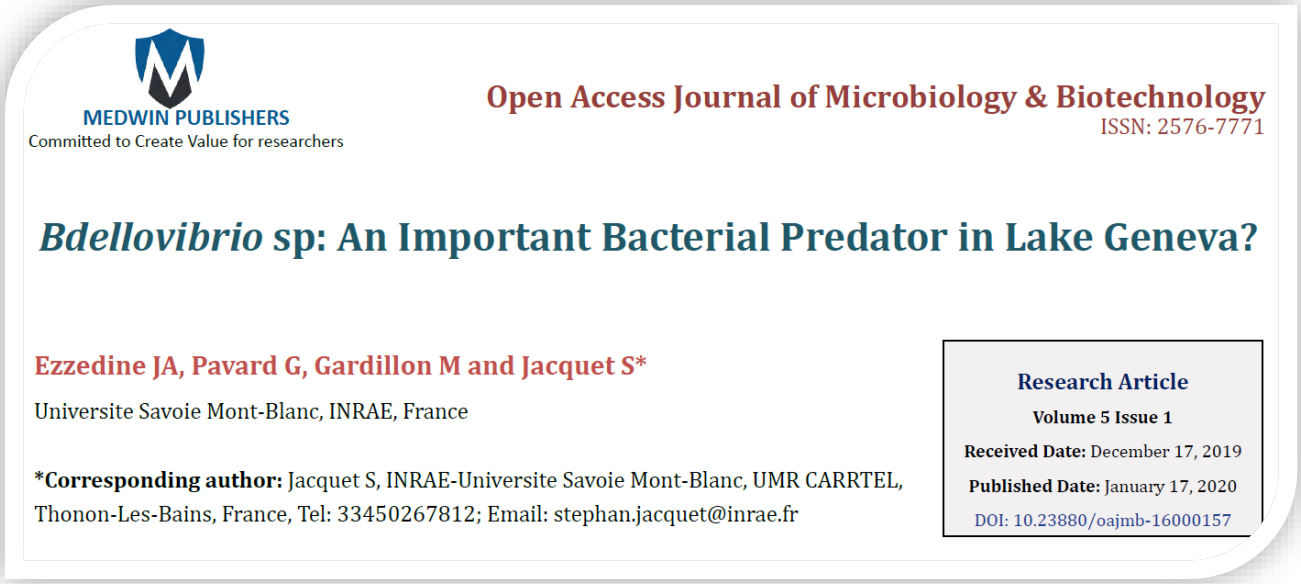
Contenu du chapitre

Ce chapitre est composé de deux articles scientifiques. Le premier s'intéresse à la dynamique à court terme des BALOs lors d'une expérience menée pendant 4 semaines au moyen de mésocosmes. Le second article porte sur l'isolement et la carcatérisation d'un BALO et de ses proies dans le Léman.

Objectifs et résumé de l'article 7

Parce que les différentes interactions entre les microorganismes et leur environnement, ainsi que leurs rôles fonctionnels, constituent un enjeu crucial, tenter d'isoler et caractériser certains microorganismes peut présenter un intérêt scientifique fort. Ainsi avons-nous fait un effort pour tenter d'isoler un ou plusieurs BALOs de nos lacs. Dans ce but, nous avons tout d'abord sélectionné trois « types » de bactéries hôtes possibles: des bactéries isolées des lacs périalpins à partir d'un mélange d'eaux provenant des lacs Léman, d'Annecy et du Bourget et de la Dranse (ALBD), des bactéries issues d'une sélection de cultures de microalgues lémaniques non axéniques de la Thonon Culture Collection (TCC) et, enfin, des bactéries bancarisées commandées comme *Escherichia coli*, *Pseudomonas fluorescens*, *Hafnia alvei*. Après avoir cultivé l'ensemble de ces bactéries proies potentielles, nous en avons sélectionné et caractérisé une dizaine (9 de type Gram- et 1 Gram+). Ces proies ont été utilisées pour enrichir et isoler un ou plusieurs BALOs à partir d'eau prélevée dans le Léman à différents moments de l'hiver et du printemps. Un prédateur bactérien a été isolé, du genre *Bdellovibrio*, associé aux proies T1 (TCC) et A4 (ALBD) identifiées comme étant des *Pseudomonas* sp. suite à leur caractérisation par clonage séquençage. Des expériences de spectre de prédation sur le BALO isolé et d'autres en culture dans notre laboratoire ont également révélé que les *Bdellovibrio exovorus* semblent croitre sur d'autres pathogènes bactériens comme *Aeromonas salmonicida salmonicida*, une bactérie responsable de la furonculose chez les salmonidés notamment l'omble chevalier retrouvé dans les lacs péri-alpins.

Bdellovibrio sp: An important bacterial predator in Lake Geneva?



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Bdellovibrio sp: An Important Bacterial Predator in Lake Geneva?

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Research Article

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1. Introduction

Aquatic microorganisms, *i.e.*, viruses, bacteria, archaea and unicellular eukaryotes, are abundant, diversified, and play major roles in the functioning of ecosystems (Azam et al. 1983, Fuhrman 1999, Pernthaler 2005, Cavicchioli et al. 2019). Microbiologists have long relied on the principle that microorganisms existed only if they were cultivable (Handelsman, 2004). However, it is well known now that only a very small fraction (<1%) of these microorganisms are likely to grow on laboratory conditions (Jørgensen et al., 2014). This is not really surprising when one knows that unicellular organisms may live, grow and reproduce in a variety of environments where a multitude of parameters may intervene both in terms of quantity and quality (*e.g.,* light, temperature, nutrients, pH, etc…) not to mention the biotic interactions (predation, parasitism, etc…). In fact, the huge diversity of microorganisms has been highlighted mainly by the advent and use of tools derived from molecular biology: PCR, cloning-sequencing, metagenomics, high-throughput sequencing. It remains, however, that the different interactions between these microorganisms and their environment, as well as their functional roles, often have to be explored, and, for this purpose, isolation based methods still represent a scientific interest. This is typically the case for many bacteria (comprising a large microbial community, consisting of species considered pathogenic to humans, animals and plants, as well as beneficial species that interact with other organisms, not to mention their various ecological roles), for instance the *Bdellovibrio* and like organisms (BALOs).

BALOs are composed of Gram-negative bacteria, and they are obligate predatory bacteria of other Gram-negative bacteria. Two life cycles exist among BALOs, the endobiotic cycle where the prey is shaped as a bdelloplast and consumed from the inside, *vs.* a periplasmic cycle with an external consumption of the prey (Jurkevitch and Davidov, 2006). BALOs are composed of 5 families: the *Bdellovibrionaceae,* the *Peredibacteraceae,* the *Bacteriovoracaceae,* the *Halobacteriovoraceae* and the *Pseudobacteriovoracaceae,* all belonging to the Oligoflexia class (Hahn et al., 2017). A little bit apart, one genus, the *Micavibrio*, belongs to the Alpha-proteobacteria class. In general, BALOs are morphologically similar and look like a comma shape form with a flagellum, and measure between 0.2 to 0.5 µm in width and 0.5 to 2.5 µm in length (Crossman et al., 2013). BALOs have different predation strategies, some are generalists with a broad spectrum of prey, while others are specialized on a few prey (Chen et al., 2011). The high diversity and ubiquitous nature of BALOs has implications on the structure and dynamics of microbial communities (Davidov and Jurkevitch 2004; Williams et al. 2016). Indeed, it is assumed that BALOs may act as an "ecological balancer". In other words, they could regulate bacterial biomass and diversity like bacteriophages and small protists do (Iebba et al., 2014; Williams et al., 2016). BALOs in freshwater ecosystems have been poorly studied (Cao et al. 2015; Chen et al.

2012; Kandel et al. 2014; Li and Williams 2015; Sar et al. 2015; Van Essche et al. 2009), and this is particularly true for lakes.

Recently, we proved the existence of BALOs in large and deep peri-alpine lakes (Paix et al. 2019). Using a cloning-sequencing (Sanger) approach and quantitative PCR, we found that, while the *Peredibacteraceae* family was represented mainly by a single species (*Peredibacter starrii*), it could represent up to 7% of the total bacterial cell abundances. Comparatively, the abundances of two other families (*i.e.*, the *Bdellovibrionaceae* and *Bacteriovaracaceae*) were significantly lower. In addition, the distribution in the water column was very different between the three groups suggesting various life strategies/niches: *Peredibactereacea* dominated near the surface while *Bdellovibrionaceae* and *Bacteriovaracaceae* were more abundant at greater depth.

On the basis of these results and to go deeper in the knowledge about the possible importance of BALOs in the microbial functioning of lakes, we tried to isolate and characterize new BALOs as well as their potential prey from Lake Geneva. Our aims were thus multiple and consisted in: (I) isolating, growing and identifying potential BALOs and prey from different origins, and (ii) defining host-range for the predators.

2. Material and Method

2.1. Prey isolation, selection, and culturing from different origins

Prey used in this study were from four different origins. Firstly, five standard reference (Gram-) bacteria were ordered from the "Centre International de Ressources Microbiennes (https://www6.inra.fr/cirm_eng/)" (CIRM): *Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 10536, *Hafnia alvei* ATCC 13337, *Pseudomonas fluorescens* ATCC 13525 and *P. putida* ATCC 12633. These bacteria were cultured on liquid LB medium (Trypton 10 g, Yeast extract 5 g, NaCl 10 g) and incubated at 25°C under low shaking at 200 rpm. The second source of potential prey was obtained from a mixed sample of water issued from Lakes Geneva, Bourget and Annecy and of the Dranse River (ALBD). From each source, 100 ml of water was collected and mixed. The third source of bacteria was obtained from 25 non-axenic cultures of freshwater microalgae, all isolated from Lake Geneva and stored in the "Thonon Culture Collection" (https://www6.inra.fr/carrtel-collection_eng/) hold in our laboratory (Suppl. Table 1). Briefly, 3 ml of culture were collected and mixed together. For the two latter sources, samples were filtered through microfiber filters (Whatman, GF/F, 47 mm), and polycarbonate filters of 5 and 2 µm (ipPoRE). Then filtrates were distributed in Falcon tube of 50 ml and centrifuged at 12,000 g for 20 min. The pellet was dissolved with 3 ml of PBS 1X (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g). Finally, 1 ml were distributed into 3 flasks containing 30 ml of LB medium and

incubated for 48 h at 25°C under low shaking at 200 rpm. After incubation, the cell concentration was estimated by flow cytometry (not shown). The cultures were then diluted 10 fold up to 10^{-7} in PBS 1X, and 100 µl of each dilution was spread on LB agar plate (15 g/l) and incubated for 24 h at 25°C. Isolated colonies so obtained of different shapes and colors were selected and cultured in liquid LB medium for 24 h at 25°C under low shaking at 200 rpm. Then, the culture was centrifuged at 5,000 g at 4°C for 10 min and the supernatant was discarded. The pellet was dissolved in a few milliliter of HM buffer (HEPES 6 g, 15g agar, 6 ml $CaCl_2$ (0.5 M), 3.33 ml $MgCl_2$ (0.6 M)) to obtain a concentration of about 10^{10} cells per ml in order to be used for BALOs enrichment. The cell concentration was obtained by reading optical density (OD) at 600 nm. Each culture was colored using a Gram stain kit (Sigma-Aldrich) according to the manufacturer's instructions. Finally, the species *Aeromonas salmonicida salmonicida* SA28 recently characterized and isolated from farmed Arctic charrs (*Salvelinus alpinus*) infected with furonculosis (Jacquet unpublished) was also tested as a potential prey. Specific Fur medium (Tryptone 10 g, yeast extract 5 g, NaCl 2.5 g, Tyrosine 1.0 g) was used to grow the species.

2.2. Sampling and enrichment of BALOs

A few litters of water mixed with biofilms on rocks and sediment were sampled on April 2, 2019 in a coastal area of Lake Geneva next to our research institute. Immediately, the sample was filtered as above and 100 ml was used for the enrichment step (see below), with 5 ml of each concentrated prey sources (CIRM, ALBD, TTC). A negative control was prepared with filtered sterile lake water to which prey were added. All flasks were placed in a hot chamber at 25°C and gently shacken at 200 rpm. Each flask was examined daily for a decrease in turbidity, a marker of bacterial prey concentration decrease. Also, a drop of each sample was observed under phase-contrast microscopy for fast moving cells, a marker of BALO presence.

2.3. Predator isolation and growth on double-layered agar plates

We applied the protocol proposed by Jurkevitch (2012) to isolate BALOs. 50 ml of each enriched culture were centrifuged at 500 g for 5 minutes at 4 °C. The supernatant was then centrifuged at 27,000 g for 20 minutes at 4 °C. The pellet was taken up with 3 mL of HM buffer and filtered through 1.2 µm. This filtrate containing the possible predators was used to achieve a dilution range of 10 to 10^{-4} in a final volume of 5 mL of HM buffer. 100 µL of each dilution were added to 4 mL of molten HM top agar (0.5%), supplemented with 300 µL of prey at 10^{10} cells/ml. For each dilution of predator, one type of prey was added. After mixing it rapidly, the HM top was poured on a Petri dish containing 20 mL of solid HM (15%). Once the top HM was solidified, the dishes were sealed with Parafilm and incubated at 25°C. After 5 to 8 days of incubation, the presence of transparent halos of a few millimeters (resembling lysis plagues) testified of the consumption of the tested prey. These predation plates were then removed by means of a 1 ml pipettor tips having been

previously cut by a sterile scalpel. The agar piece was then resuspended in 500 µL of HM buffer. The presence of BALOs in the suspension was confirmed by microscopy observation. When fast moving cells were detected, the suspension was filtered through 0.45 µm filter. This isolation step was repeated three times to obtain a "purified" strain of BALO. Finally, half of the suspension was stored in glycerol (20%) at -80°C and half was processed for DNA extraction.

2.4. DNA extraction

DNA extraction was performed on both prey and predators. For BALOs, liquid medium were filtered with 0.45 µm pore size filters in order to remove larger microorganisms such as prey cells. All samples were then centrifuged for 3 min at 6,000 g and at 4°C. The supernatant was discarded and the pellet was used for DNA extraction. We used a homemade protocol combined with GenEluteTM-LPA (Sigma-Aldrich) solution. Briefly, the protocol began with a lysis step by adding 300 µL of TE buffer (TRIS: 1M - pH8, EDTA: 0.5M - pH8) and 200 µL of lysis solution (TRIS: 1M - pH8, EDTA: 0.5M - pH8 and sucrose: 0.7 M). After a thermic shock at -80°C for 15 min and at 55°C for 2 min, 50 µL 10% sodium dodecyl sulfate (SDS) and 10 µL of proteinase K (20 mg/mL) were added. The sample was incubated at 37°C for 1 h with gentle stirring and placed again in the block heater at 55°C for 20 min. After a quick centrifugation step (13,000 rpm at 4°C for 3 min), the supernatant was collected, and 50 µL of sodium acetate (3M – pH 5.2) plus 1 µL of GenEluteTM-LPA (Sigma-Aldrich, 25µg/µL) were added. One volume of isopropanol was then added and the tubes were centrifuged for 10 min at 12,000 g and 4°C. Two rounds of ethanol (80%) washing were carried to purify the pellet of DNA. The remaining ethanol was evaporated using the SpeedVac for 20 min. Finally, 30 µL of TE were added and tubes were left for 1 h at 37°C. DNA concentration was measured using NanoDrop 1000 spectrophotometer. When DNA concentration was superior to 25 ng/µL, a dilution was carried. All tubes were stored at -20°C until analysis.

2.5. PCR amplification

PCR amplification of the 16S rRNA gene were performed using specific primers (Bd529F and Bd1007R for *Bdellovibrio*, Bac676F and Bac1442R for *Bacteriovorax* and Per676F and Per1443R for *Peredibacter*, Davidov et al. 2006), as well as universal primers targeting bacteria (515F and 909R, Wang et al. 2009, 2018). Adapted from Davidov et al. (2006), the specific amplification PCR mixture volume was at 25 µL and consisted of (final concentration): 1x buffer, 0.2 mM dNTP, 3 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin (BSA) and 0.625 U Biotaq DNA polymerase (Bioline). In a second step, primers (Forward and Reverse) for each BALO genus were added to the different mixtures. Finally, 1 µL of template DNA (BALOs extracted DNA) concentrated at 25 ng/µL was added. A negative control was included and the PCR program was as follows: 94°C – 5 min, 30 x (94°C – 1 min, 58°C – 1 min, 72°C – 3 min), and with a final extension step at 72°C for 5 min. For the universal amplification, the mixture volume was at 25 µL and consisted of (final

concentration): 1x buffer, 0.4 mM dNTP, 2 mM $MgCl_2$, 0.4 mg/mL bovine serum albumin (BSA), 0.2 µM of forward primer 515F, 0.2 µM of reverse primer 909R and 0.5 U Biotaq DNA polymerase (Bioline). Finally, 1 µL of template DNA concentrated at 25 ng/µL were added. A negative control was included and the PCR program was as follows: 95°C – 2 min, 30 x (94°C – 30 sec, 58°C – 30 sec, 72°C – 30 sec), and with a final extension step at 72°C for 5 min. Agarose gel analysis was performed for verification of PCR products.

2.6. Cloning, Sanger sequencing and taxonomic assignment

When plaques were detected, both the predator and the prey were extracted, amplified and cloned for sequencing. Prior to cloning, PCR products were purified using GE healthcare illustra GFX kit according to the manufacturer's instructions. The purified products were then measured using a Nanodrop 1000 spectrophotometer. Cloning was conducted using TOPO TA Cloning kit (Thermo Fisher Scientific) following manufacturer's recommendation. Briefly, ligation step was carried in 6 µL volume consisting of 1 µL of salt solution, 1 µL of topo vector and 4 µL of purified PCR product. The mix was incubated for 20 min at ambient temperature. After the incubation time, the mix was put in icy water to stop the ligation process. Next, the transformation process was performed by adding 2 µL of the ligation product in 50 µL of competent *E. coli*. A thermic shock was executed in ice water for 10 min, immediately thawed into a block heater at 42°C for 40 sec, and then again in ice for 3 min. After, 250 µL of SOC medium were added. The sample was incubated at 37°C for 1h30 with gentle stirring. Then, 50, 80 and 100 µL of *E. coli* were streaked on agar plate. The next day, white clones were selected for DNA amplification. White clones were heated at 95°C for 10 min in order to burst the DNA from *E. coli* cell. 1 µL of DNA was pipetted and added to a PCR mix of 29 µL composed of: 1x buffer, 2 mM dNTP, 2 mM $MgCl_2$, 0.1 µM of forward primer M13F (-20) (5'-GTAAAACGACGGCCAG-3'), 0.1 µM of reverse primer M13R (5'-CAGGAAACAGCTATGAC-3') and 0.6 U Biotaq DNA polymerase (Bioline). A negative control was included and the PCR program was as follows: 94°C – 10 min, 30 x (94°C – 60 sec, 55°C – 60 sec, 72°C – 60 sec), and with a final extension step at 72°C for 10 min. Agarose gel analysis was performed for PCR products verification. With these PCR products, 2 plates of 96 wells were prepared and sent to GATC / Eurofins Genomics for single end Sanger sequencing. 10 clones for each prey and 16 clones for each predator with universal and specific primer were sent. Taxonomic assignment of sequences obtained from Sanger sequencing was performed online with NCBI BLASTn (Altschup et al., 1990).

2.7. Phylogeny

On one hand, 8 *Bdellovibrio* reference sequences (including type species) of the 16S rRNA gene were downloaded from NCBI (Benson et al. 2013, supp. Table 2) and *Vampirovibrio chlorellavorus* was used here as an outgroup. On the other hand, 30 assigned sequences of

Bdellovibrio (Cloning-sequencing) were used to construct the phylogenetic tree. To begin, all sequences were aligned visually using the program MEGA7 (Kumar et al., 2016) via MUSCLE alignment (Edgar, 2004). The alignment was trimmed at both ends in order for all the sequences to fully overlap with each other (255 positions with gaps). The generated file was then formatted to Phylip and Nexus to be respectively used in PhyML 3.1 (Guindon et al. 2010) and MrBayes 3.2.7a (Ronquist et al. 2012). ModelGenerator v.85 (Keane et al., 2006) was used to select the best nucleotide substitution model under corrected Akaike information criterion (AICc) (Akaike 1973) with "Number of discrete gamma categories" set to 4. The program returned TIM + G as the best substitution model. The tree was built under Maximum likelihood (ML) method using the PhyML 3.1 (Guindon et al. 2010) program with "Tree topology search operation" set to SPR and with 100 bootstraps. Then the Bayesian tree was constructed using MrBayes 3.2.7a (Ronquist et al. 2012) program with 500,000 generations and a burn-in value of 25%. In the final ML tree, "Posterior" probability (PP) and "Boostraps" (BS) values (PP/BS) were added respectively at each node when possible.

2.8. Data accession numbers

The *Bdellovibrio* sequences are available in the GenBank database with the following accession numbers: MN556920 to MN556941.

2.9. Predatory spectrum experiment

For each possible predator, a predatory spectrum (host-range) experiment was carried by presenting one prey at a time for the predator in the double-layered agar plate. Two replicates were set for each experiment. The *Bdellovibrio exovorus* JSS strain was also tested with a variety of prey.

3. Results

3.1. Characterization of the prey and predators

All colonies isolated from the ALBD sample and selected for the isolation were Gram-negative bacteria. For the TCC samples, 4 colonies were identified as Gram-negative and one colony was Gram-positive (Table 1). Following the isolation procedure to obtain one or several predators, samples for which turbidity was reduced were then sub-sampled and observed using microscopy. For some of them, we could detect fast moving cells, likely to be BALOs, so that a more in-depth analysis was performed. *In fine,* predators referred to as T1 and A4 displayed distinct plaques. After PCR amplification, cloning and sequencing the taxonomic assignation confirmed that they were BALOs, more particularly *Bdellovibrio* sp. Indeed, following the molecular approach using the specific primers, 13 out of 16 sequences for both A4 and T1 could be associated to *Bdellovibrio*

sp. while only 4 for T1 and 0 for A4 when using the bacterial universal primer. The phylogenetic analysis revealed that A4 and T1 were in fact the same microorganism (Fig. 1). On the other hand, all clones associated to the prey and also referred to as A4 and T1 were *Pseudomonas* sp. Prey referred to as A5 and A3 were assigned to *Acinetobacter* sp. and *Pseudomonas* sp, respectively, while A5 and A3 predators could not be isolated and identified despite the observation of discrete plaques.

Table 1 Gram coloration of isolated prey colonies from ALBD and TCC samples.

ALBD colonies		TCC colonies	
A1	*Gram-negative*	T1	*Gram-negative*
A2	*Gram-negative*	T2	*Gram-negative*
A3	*Gram-negative*	T3	*Gram-positive*
A4	*Gram-negative*	T4	*Gram-negative*
A5	*Gram-negative*	T5	*Gram-negative*

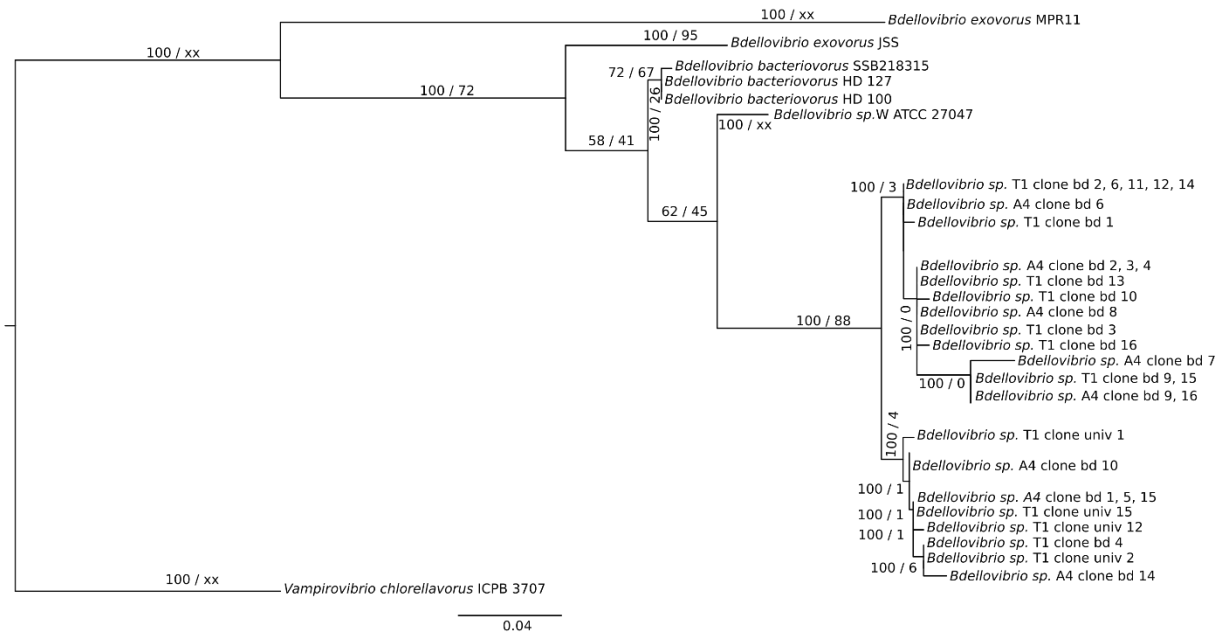


Figure 1 Phylogenetic analysis of 16S rRNA of *Bdellovibrio* clones obtained after Sanger sequencing. Phylogenetic tree was constructed by the maximum likelihood method using PhyML-3.1 and Bayesian inference was conducted with MrBayes 3.2.7.a. Posterior probability (PP) values followed by bootstrap values are added to the left of a node when possible (PP/BS). *Vampirovibrio chlorellavorus* was used as an outgroup to root the tree.

3.2. Predation spectrum (host-range experiment)

The experiment was carried with *Bdellovibrio* sp. T1, A4 and *B. exovorus*. Typical plaques were observed with T1 (*Pseudomonas* sp.), A4 (*Pseudomonas* sp.*;* Fig. 2), *P. fluorescens* and A3 (*Pseudomonas* sp.). However, no plaques were detected when using the T4, *C. freundii*, *E. coli*, *H. alvei*, T3 and A5 (Table 2) and other prey. In addition, plaques were observed for all the *Bdellovibrio* tested with *A. salmonicida salmonicida.*

Table 2 Predation experiment with *Bdellovibrio* sp. T1 and *B. exovorus.*

Predator	Prey	Predation
Bdellovibrio sp. T1	T1 (*Pseudomonas* sp.)	+++
	A4 (*Pseudomonas* sp.)	+++
	Pseudomonas fluorescens	++
	A3 (*Pseudomonas* sp.)	+
	T4 (Gram-negative)	-
	Aeromonas salmonicida salmonicida SA28	+
	Citrobacter freundii	-
	Escherichia coli	-
	Hafnia alvei	-
	T3 (Gram-positive)	-
	A5 (*Acinetobacter* sp.)	-
Bdellovibrio exovorus	*Aeromonas salmonicida salmonicida* SA28	+

(-) No predation. (+++) multiple and distinct predation plaques. (++) moderate and distinct predation plaques. (+) few predation plaques

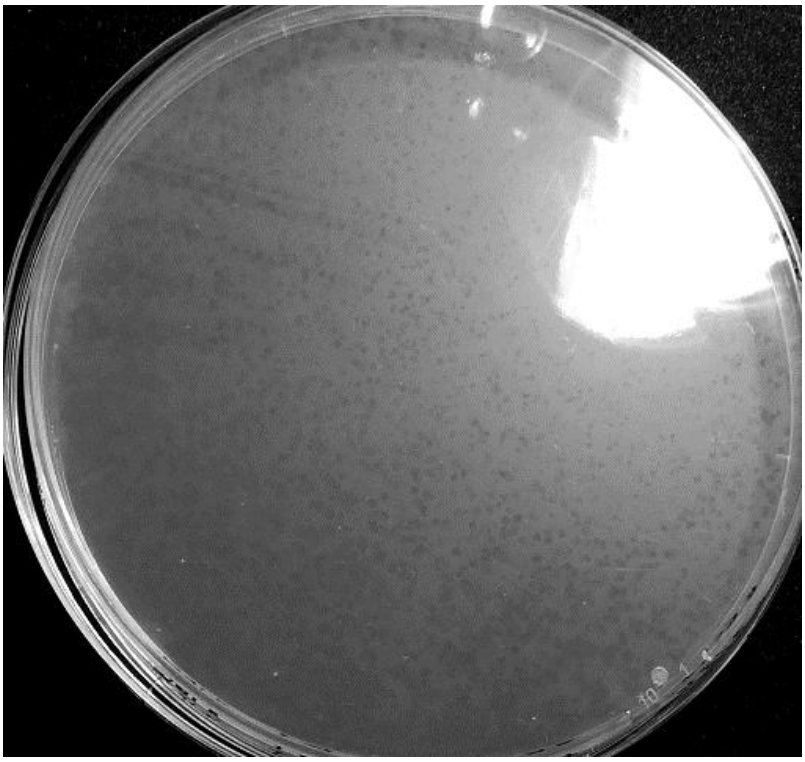


Figure 2 Predation experiment between *Bdellovibrio* sp. T1 and A4 prey (*Pseudomonas* sp.) showing distinct small plaque typical of a predation process.

4. Discussion

BALOs have been mainly studied and isolated in marine and terrestrial environments (Sutton and Besant, 1994; Jurkevitch, 2006; Williams et al., 2018). Hence, knowledge gaps for fresh waters are important, especially for (peri-alpine) lakes. In a previous work, we found that *Peredibacteracaceae* seem to be the most abundant BALO family in Lakes Geneva, Annecy and Bourget and these bacteria were mostly localized in surface (Paix et al., 2019). In another study, we revealed that *Bdellovibrionaceae* reads were the highest in Lake Geneva (Ezzedine et al. submitted). The different results clearly suggested that BALOs can be numerically and functionally important in Lake Geneva. Thus, isolating, characterizing and maintaining one or several BALOs in culture may help to determine their importance in this lake and beyond.

We were moderately successful since we only obtained one *Bdellovibrio* sp. representative despite an important effort to try to obtain several bacterial predators. We obtained it from a mixture of water sediment and biofilms from the shore. Thus, we do not know if we selected a benthic or a pelagic species, a free-living population or an attached group. It is noteworthy, however, that it has been reported that BALOs can be more present in surface biofilms and sediments than in the water column (Williams et al. 1995; Williams and Pineiro. 2006). We also chose a location with natural

clean water and far from any wastewater treatment pipes, and this may have reduced our success to isolate different populations. Indeed, some authors reported that BALOs are more concentrated in the waters at the outlet of wastewater treatment plants (Aguirre et al., 2017). However, polluted waters have also been reported by Markelova et al. (2002, 2005) to drive BALOs into entering a bdelloplast state in order to survive. At last, while we tried to be not too limiting in terms of prey range, our selection remained relatively low (with only 10 different species or strains) and this may also have contributed to a low isolation success.

We obtained however very interesting results. Firstly, the only predators we could isolate were *Bdellovibrio*. The reason behind this result, specifically why this genus and not another BALO is difficult to explain since we used only one sample, from one location, and one date. On one hand, maybe the coastal environment of Lake Geneva at this period of time is richer with *Bdellovibrio* than in other BALOs. In another hand, *Bdellovibrio* spp could be the easiest BALOs to isolate since they dominate existing isolates. As optimal temperature for growing *Peredibacter* is between 20 and 30°C (Davidov and Jurkevitch, 2004), *Bacteriovorax* between 15 and 35°C (Baer et al., 2000), and *Bdellovibrio* between 28 and 30°C (Fratamico and Whiting, 1995; Jurkevitch, 2006b; Sar et al., 2015), our temperature incubation about 25°C was unlikely to explain our result. The isolated *Bdellovibrio* sp. was characterized by small, numerous and distinct predation plaques, smaller but globally similar to those of *Bdellovibrio* cells growing on *Pseudomonas corrugata* (Jurkevitch, 2006b). In another hand, plaques were different in size and shape from *Bdellovibrio* growing on *E. coli*. isolated by Sar et al. (2015) from Nigerian freshwater bodies. Such results are not surprising since Jurkevitch (2006) stated that both the shape and size of predation plaques depend on the nature of the predator, which produces them. Since these predators belong to different environments, each one may have a different predation strategy or simply behave differently in different laboratory conditions.

Secondly, we found that the predator had a narrow host range with a clear preference for *Pseudomonas* spp. Indeed, the isolated *Bdellovibrio* seemed to be a specialist since predation plaques were mainly observed with *Pseudomonas*, *i.e.*, *P. fluorescens* ATCC 13525, T1, A4 and A3 prey that were also *Pseudomonas* sp. species. This predation activity against *Pseudomonas* sp is interesting when one knows that different species or strains of this genus can carry enteric diseases for humans. Unlike Iebba et al. (2014) and Pantanella et al. (2018) who reported that *B. bacteriovorus* may predate on Gram-positive bacteria (in case of nutrient deficiency), such a situation was not observed here. In any case, further experiment should be carried to evaluate the impact of BALOs predation on a largest variety of prey.

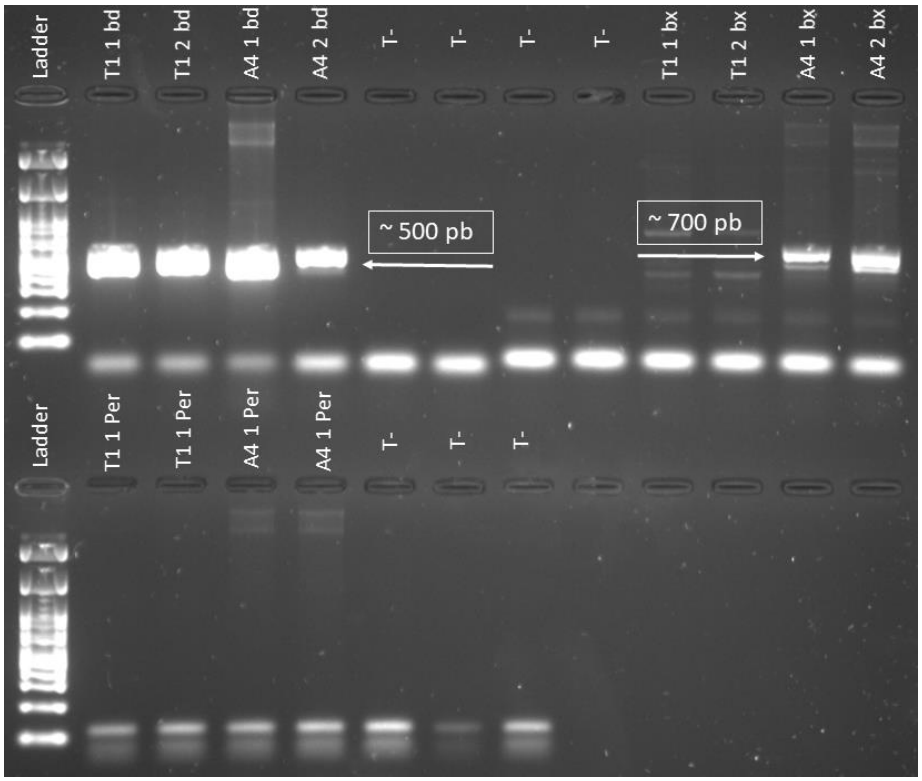
Bdellovibrio could also predate on *A. salmonicida salmonicida* (here a strain recently isolated in our lab from Arctic charr). *Bdellovibrio exovorus*, which is an epibiotic predator with

no bdelloplast formation, was shown to grow on a few variety of prey including *Caulobacter crescentus, Acinetobacter johnsonii* RS3-17, *A. junii* RS3-41, *Delftia acidovorans* RS3-16, and *Aeromonas hydrophila* AS12a (Koval et al. 2013 ; Chanyi et al. 2013). The appearance of predation plaques on *A. salmonicida salmonicida* SA28 reminds thus the result obtained with *A. hydrophila.* Such a result is promising since bacterial predators of bacteria could be used as bioagents controlling specifically some toxigenic bacterial strains, among which the ones that are multi-resistant to antibiotics, for instance in fish farming. Richards et al. (2012), Cao et al. (2015) and Ottaviani et al. (2018) reported that *B. bacteriovorus* H16 and *Halobacteriovorax* HBXCO1 predated *Vibrio* species that infect and kill shrimps (*Penaeus vannamei*) and bivalves. In addition, Schwudke et al. (2001) showed a correlation between the health status of domestic animals and the abundance of BALOs. The more BALOs are present, the less these animals were exposed to pathogens. Here, a new potential application is highlighted here for salmonids infected by Furonculosis that can cause a significant mortality in a variety of species, including salmons, and in the peri-alpine lakes the Arctic charr (Braden et al., 2019).

5. Conclusion

The isolation and characterization of a new obligate bacterial predator of bacteria highlights the necessity to interest to this functional group of bacteria and probably to other (non-obligate) predators to better understand the functioning of microbial ecosystems. As also proposed in a parallel study for Lake Geneva, we believe that BALOs constitute an important group of bacteria likely to play key roles in the structure, dynamics and diversity of the heterotrophic bacterial community, constituting thus an unseen elephant in the room (Ezzedine et al. 2020a). In the future, new attempts of isolation will be required as well as the conduction of experiments to precisely define mortality rates induced by such predators.

6. Supplementary data



Supplementary Figure 1 Gel photo showing amplification at ~500 pb when using the specific primer of *Bdellovibrionaceae* on T1 and A4 predators DNA extract. In addition another amplification was observed at ~700 pb when using *Bacteriovoracaceae* specific primer on A4 predator DNA extract. However, no amplification is detected when using *Peredibacter* specific primer on T1 and A4 predators DNA extract.

Supplementary Table 1 List of cultured phytoplankton from which prey bacteria were isolated.

Class	**Family**	**Genus Species**	**Source**	**ID in TCC**
Chlorophyceae	Ulotrichaceae	*Ulothrix sp.*	Lake Geneva	TCC1a
Cyanophyceae	Phormidiaceae	*Planktothrix rubescens*	Lake Geneva	TCC13
Cyanophyceae	Synechococcaceae	*Synechococcus rhodobaktron*	Lake Geneva	TCC33
Zygophyceae	Desmidiaceae	*Staurastrum sebaldii*	Lake Geneva	TCC106
Chlorophyceae	Oocystaceae	*Mychonastes homosphaera*	Lake Geneva	TCC108b
Chlorophyceae	Scenedesmaceae	*Scenedesmus serratus*	Lake Geneva	TCC110
Chlorophyceae	Chlamydomonadaceae	*Chlamydomonas intermedia*	Lake Geneva	TCC113
Chlorophyceae	Scenedesmaceae	*Scenedesmus acutus*	Lake Geneva	TCC116
Chlorophyceae	Hydrodictyaceae	*Pediastrum duplex*	Lake Geneva	TCC120
Chlorophyceae	Dictyosphaerioideae	*Botryococcus protuberans*	Lake Geneva	TCC123
Chlorophyceae	Volvocaceae	*Eudorina elegans*	Lake Geneva	TCC125
Chlorophyceae	Coelastraceae	*Coelastrum reticulatum*	Lake Geneva	TCC129
Chlorophyceae	Oocystaceae	*Mychonastes sp.*	Lake Geneva	TCC136-1
Diatomophyceae	Bacillariaceae	*Nitzschia palea*	Lake Geneva	TCC139-3
Chlorophyceae	Prasiolaceae	*Stichococcus bacillaris*	Lake Geneva	TCC145-7
Chlorophyceae	Chlorellaceae	*Chlorella vulgaris*	Lake Geneva	TCC213
Chlorophyceae	Chlorellaceae	*Monoraphidium contortum*	Lake Geneva	TCC223
Diatomophyceae	Fragilariaceae	*Fragilaria crotonensis*	Lake Geneva	TCC365
Diatomophyceae	Fragilariaceae	*Fragilaria perminuta*	Lake Geneva	TCC743
Chlorophyceae	Scenedesmaceae	*Scenedesmus costatus*	Lake Geneva	TCC744
Diatomophyceae	Achnanthidiaceae	*Achnanthidium minutissimum*	Lake Geneva	TCC746
Diatomophyceae	Fragilariaceae	*Fragilaria perminuta*	Lake Geneva	TCC747
Diatomophyceae	Achnanthidiaceae	*Achnanthidium minutissimum*	Lake Geneva	TCC748
Diatomophyceae	Fragilariaceae	*Fragilaria perminuta*	Lake Geneva	TCC749
Diatomophyceae	Achnanthidiaceae	*Achnanthidium straubianum*	Lake Geneva	TCC833

Supplementary Table 2 Accession numbers of the sequence downloaded from NCBI to construct the phylogenetic tree.

Sequence name	Accession number
Bdellovibrio bacteriovorus strain HD 127	AJ292760.1
Bdellovibrio sp. W strain ATCC 27047	AJ292518.1
Bdellovibrio bacteriovorus strain 109J	M61234.1
Bdellovibrio bacteriovorus strain HD100	NR_027553.1
Bdellovibrio bacteriovorus strain SSB218315	KT807464.1
Bdellovibrio exovorus strain JSS	EF687743.1
Bdellovibrio exovorus strain MPR11	MH230062.1
Bdellovibrio exovorus strain KL8	NR_115142.1
Vampirovibrio chlorellavorus strain ICPB 3707	NR_104911.1

Objectifs et résumé de l'article 8

Les microorganismes étant caractérisés par des dynamiques rapides, il est critique de pouvoir les étudier à des échelles de temps courtes pour mieux appréhender leur rôle au sein des écosystèmes. Cet enjeu a été relevé au travers de la réponse des BALOs à un stress environnemental simulant un ou plusieurs événements extrêmes au sein de mésocosmes au Léman. Le projet MESOLAC, dans lequel s'est inscrit ce travail, a eu pour but d'étudier l'impact d'un événement extrême estival sur les communautés planctoniques du Léman. L'expérience, rendue possible par le déploiement de 9 mésocosmes dans le lac, a consisté à simuler un épisode de fortes précipitations engendrant un apport significatif de matière organique dissoute terrigène, la brunification des eaux (abattement de la lumière) et un brassage important des eaux de surface. Pendant 3 semaines au mois de juillet 2019, deux types de traitements simulant des impacts d'intensité différente ont été appliqués et la dynamique des bactéries planctoniques, en particulier celles des BALOs, a été suivie. Echantillonnées tous les 4 jours en moyenne, les dynamiques d'abondances des trois familles de BALOs étudiées (*Bacteriovoracaceae, Peredibacteraceae* et *Bdellovibrionacaceae*) ont été globalement faiblement impactées par les différents traitements. Ceci dit, l'abondance des BALOs a été légèrement favorisée par le traitement le moins extrême et faiblement réduite par le traitement le plus extrême par rapport au contrôle. Les mesures des abondances de BALO ont révélé tout au long de l'étude une plus forte abondance des *Peredibacteraceae (*pouvant atteindre dans certaines conditions près de 10^4 cellule/mL), par rapport aux *Bdellovibrionacaceae* et aux *Bacteriovoracaceae.* Si les différentes familles de BALOs ont présenté des réponses légèrement différentes aux traitements appliqués, aucune modification importante sauf pour *Bacteriovoracaceae* n'a toutefois été enregistrée dans les dynamiques observées, les tendances générales étant plutôt similaires et les paramètres mesurés expliquant peu ces dynamiques. Cette étude a surtout mis en lumière que se sont les variations au niveau de la pression de prédation et des proies qui constituent le plus surement les raisons expliquant ces dynamiques, peut être aussi celui de la lyse virale. L'avenir est clairement à l'étude spécifique et orienté de ces interactions biotiques.

Short-term dynamics of Bdellovibrio and like organisms in Lake Geneva in response to a simulated climatic extreme event

Soumis à « *Microbial Ecology* » le 2 décembre 2020.

1. Introduction

Among microbial predators, *Bdellovibrio* and like organisms (BALOs) are obligate predatory bacteria with a broad spectrum of prey that are mainly gram-negative bacteria. BALOs have been reported to act as "population-balancers" (Iebba et al., 2013) and "microbial alpha diversity drivers" (Johnke et al., 2020), highlighting their potential important roles in microbial ecosystems. They are classified into two distinct polyphyletic clades, Oligoflexia and Alphaproteobacteria, and are characterised by two well-described modes of reproduction (Jurkevitch and Davidov, 2006; Koval et al., 2015; Hahn et al., 2017). BALOs are found in many habitats such as soil and aquatic environments (Sutton and Besant, 1994; Jurkevitch and Davidov, 2006; Williams et al., 2018), and they have recently been discovered and studied in peri-alpine lakes (Paix et al., 2019; Ezzedine et al., 2020a, 2020b, 2020c). In general, the water column is not a favourable environment for the multiplication of BALOs, which thrive better in biofilms, sediments, or closed environments such as in aquaculture (Williams and Piñeiro, 2006; Kandel et al., 2014; Li et al., 2014). Given their predation obligation and distribution in nature and diversity, BALOs may have a strong impact on the dynamics and structure of bacterial communities. However, studies related to the ecological impact of BALOs on microbial communities are scarce.

BALOs have been seldom studied in freshwater lakes. Recently, these bacteria have been shown to favour eutrophic conditions, both in terms of abundance and diversity, due to a higher number and diversity of potential prey (Chauhan et al., 2009b). It is noteworthy that BALOs have also been detected in less nutrient-replete lakes, such as Lake Geneva, Bourget, and Annecy, characterised by meso- to oligotrophic status (Paix et al., 2019; Ezzedine et al., 2020a, 2020b). However, in these lakes, BALOs have been studied away from shore, at reference stations of the lakes, on a monthly or seasonal time scale, i.e., at a temporal scale unsuited to studying microbial predator dynamics. In Lake Geneva, probably one of the most studied lakes regarding BALOs, *Bdellovibrionaceae* seems to be the most diverse family in terms of OTUs, while *Peredibacteraceae* are the most abundant, as measured by quantitative PCR (qPCR). It was also found that potential prey mainly belonged to the genus *Pseudomonas* (Chauhan et al., 2009b; Ezzedine et al., 2020c) are predated by *Bdellovibrionaceae*.

Assessing the dynamics of BALOs and the impact of environmental forcing on this functional group can be significantly facilitated by analysing them on short time scales and under relatively controlled experimental conditions. This is what *in situ* deployed mesocosms can typically provide, as they constitute an ideal experimental tool, intermediate between microcosms commonly used in laboratories and *in situ* ecological surveys (Mcallister et al., 1961; Antia et al., 1963; Egge and Heimdal, 1994). In the past, *in situ* deployed mesocosms have proved to be a relevant type of

instrumentation for observing changes in complex planktonic communities while controlling environmental conditions such as brightness, concentrations of nutrients or dissolved gases, water mixing, and predatory effects, among others. (Mostajir et al., 1999; Thingstad and Rassoulzadegan, 1999; Jacquet et al., 2002b; Havskum et al., 2004). We conducted a mesocosm experiment in Lake Geneva, designated project "MESOLAC", which aimed at analysing the response of surface planktonic populations to extreme events caused by global climatic change. The latter has already resulted in an increase in the number and strength of the so-called extreme events, including storms, heavy rainfall, floods, or drought (Lavell et al., 2012). Ecosystems are likely to be massively impacted by such events, and lakes are no exception (Jenny et al., 2020). For example, Woolway and Merchant (Woolway and Merchant, 2019) reported alterations in the thermal regime of lakes, which could result in planktonic diversity reduction and the inability of lake ecosystems to return to their original state due to the loss of their functional redundancy. Therefore, addressing issues regarding lacustrine functions and their resilience is of major importance to better understand the impact of climate change, including global warming, on their state and evolution in the near future. The focus of this work was to study i) the dynamics and diversity of three main BALO families belonging to the Oligoflexia group (e.g*., Bdellovibrionaceae*, *Bacteriovoracaceae*, and *Peredibacteraceae)* on a short time scale and ii) their response to simulated extreme events and abiotic and biotic factors. We hypothesised that the addition of dissolved organic carbon (simulating an input of carbon from the watershed in response to runoff from the catchment), light reduction, and mixing (simulating stormy conditions) would stimulate bacterial growth and thus the predation by bacterial predators such as BALOs with specificities according to the different families constituting this functional group.

2. Materials and Methods

2.1 Experimental set up

The MESOLAC experiment was conducted on Lake Geneva over 4 weeks in July 2019. Nine experimental mesocosms (**Supplementary Figure 1)** were placed approximately 100 m away from the coast and at a depth of 7 m near the Alpine Centre for Research on Lake Ecosystems and Food Webs (CARRTEL, Thonon les Bains; 46°22′09.1″ N, 6°27′15.3″ E). An ecological anchoring system, set up by professional scuba divers, including the corresponding author, ensured the fixation of each mesocosm and good preservation of the bottom (i.e., sediments and plants) of the lake. Mesocosms consisted of reinforced polyethene bags of 4.5 m height and 1.4 m diameter at their upper level with a finishing conical part towards the bottom (Insinööritoimisto Haikonen Oy,

Norra Paippis, Finland). The mesocosms were supported at every meter by a plastic frame and a double system of buoys on top. All bags were filled with water simultaneously on the same day (1 July) within a few hours and were untouched for 3 d to acclimate. The total volume of water in each mesocosm was approximately 4 m^3. The experiment started on 4 July, designated T0. The experimental design included three treatments, each replicated thrice. Each bag was covered by filters (Lee filters) applied on the surface of the mesocosm so that they were all treated similarly and to protect embedded water from bird droppings and other external elements. The control treatment (C) consisted of minimal light reduction (i.e., 5%). Treatment M consisted of a medium stress situation, i.e., 30% light reduction, dissolved organic carbon (DOC; using autoclaved peat soil extract) at a concentration ×1.5 times that of the control and regular manual mixing for 5 min daily for 2 weeks. Treatment H consisted of a shorter but stronger intensity stress simulation. For 5 d, light was reduced by ~85%, DOC concentration was increased 5-fold (i.e., ~6 mg L^{-1}), and daily mixing was performed for 15 min. After 5 d, treatment H was exposed to the control conditions. More details can be found in (Tran-Khac et al., 2019).

2.2. Sampling strategy and analyzed parameters

Water samples were collected at a depth of 2 m in each bag, with a 2- to 4-day interval between 4 and 22 July 2019 (i.e., on 4, 8, 11, 16, 18, and 22 July). Physicochemical parameters such as pH, dissolved oxygen concentration, turbidity, phosphorus, and nitrogen concentrations were measured following standard protocols (Rimet et al., 2020). Meteorological data were collected daily via the CLIMATIK platform, which is reserved for INRAE research. For DNA analysis, 200–350 mL of water was filtered through 0.2-µm PC filters. All filters were stored at -20°C until DNA extraction. Water samples were also taken for delayed flow cytometry analyses and consisted of 2 mL of water fixed with glutaraldehyde (15 min, 0.5%), flash-frozen in liquid nitrogen, and stored at -80°C.

2.3. DNA extraction

The 0.2-µm filters were subjected to DNA extraction using a inhouse protocol. In a 2-mL Eppendorf tube, 300 µL of TE buffer (TRIS, 1 M [pH 8] and EDTA, 0.5 M [pH 8]) was added to each filter. Next, a lysis step was performed by adding 200 µL of lysis solution (TRIS, 1M [pH 8]; EDTA, 0.5 M [pH 8], and sucrose: 0.7 M). After a thermal shock at -80°C for 15 min and at 55°C for 2 min, 50 µL of 10% sodium dodecyl sulfate (SDS), and 10 µL of proteinase K (20 mg/mL) were added. The samples were then incubated at 37°C for 1 h with gentle stirring and placed on a heating block at 55°C for 20 min. After a quick centrifugation step (13,000 rpm at 4°C for 3 min), the supernatant was collected. Afterwards, 50 µL of sodium acetate (3 M [pH 5.2]) and 1.5 µL of 25 µg/µL GenEluteTM-LPA (Sigma-Aldrich, St Louis, MO, USA) was added. Subsequently, one volume of isopropanol was added, and the tubes were centrifuged for 10 min at 12,000 × *g* at 4°C. Following this step, two rounds of ethanol (80%) washing were carried out to clean the DNA pellet.

The remaining ethanol was evaporated using a SpeedVac for 20 min. Finally, 30 µL of TE was added, and samples were incubated at 37°C for 1 h to allow the pellet to gently dissolve into the TE buffer. The DNA concentration was measured using a NanoDrop 1000 spectrophotometer. For DNA concentrations greater than 25 ng/µL, a dilution was performed. All DNA preparations were stored at -20°C until further analysis.

2.4. Quantification of BALOs by qPCR

Qiagen's Rotor-Gene Q machine and QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) were used to amplify the BALOs. Standard curves were prepared using identified clones of *Bdellovibrionaceae, Peredibacteraceae,* and *Bacteriovoracacea* from our previous study (Ezzedine et al., 2020a). Briefly, plasmids were extracted and purified using a NucleoSpin Plasmid kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Plasmids were then digested using a BamHI restriction enzyme (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions. The digested plasmid concentrations were measured using a Quant-iT PicoGreen ds DNA Reagent kit (Invitrogen, Carlsbad, CA, USA), and fluorescence was read using a Fluoroskan Ascent FL plate reader. The number of copies for each BALO clone was calculated using the following formula:

$$Number\ of\ copies = \frac{DNA\ concentration}{(Insert\ size + Plasmid\ size) \times 660} \times 6.02 \times 10^{23}$$

Serial dilutions were then conducted from 10^9 to 10^0 copies. Diluted DNA from 10^7 to 10^0 was duplicated and amplified by the BALOs qPCR set of primers to construct the standard curve. The list of primers used to amplify the 16S rDNA gene is shown in **Supplementary Table 1** (Van Essche et al., 2009; Ezzedine et al., 2020a). All primer sets for BALO amplification were optimised in our previous study (Ezzedine et al., 2020a). Two controls were added at each time point. The *Bdellovibrionaceae* standard curve had an efficiency of 0.92, R^2 of 0.996, and the threshold was set to 0.02. For *Peredibacteraceae,* the efficiency was 0.87, R^2 was 0.87, and the threshold was fixed at 0.015. For *Bacteriovoracaceae,* the efficiency was 0.96, R^2 was 0.993, and the threshold was set to 0.02. The qPCR mixture volume was 25 µL and consisted of (final concentration): 1 X Master Mix (QuantiTect SYBR Green PCR kit, Qiagen), 0.3 mg mL^{-1} of BSA, 0.2 µM of forward and reverse primers, and 1 µL of template DNA (25 ng μL^{-1}). The cycling parameters were as follows: 95°C for15 min followed by 40 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s, with + 1°C every 5 s from 60 to 95°C. For all environmental samples, those that failed to amplify or were outside the standard curve limits were not considered in the analysis. BALO abundances

were obtained in copy per reaction and were transformed to copy per millilitre of filtered water using the following formula:

$$Copy\ per\ milliliter = \frac{\frac{Copy\ per\ reaction}{Dilution\ factor\ for\ 25\ ng\ \mu L^{-1}} \times DNA\ elution\ volume}{Filtered\ volume\ on\ 0.2\ \mu m}$$

2.5. Flow cytometry analysis

We used a FACSCalibur flow cytometer (BD BioSciences, Franklin Lakes, NJ, USA) to determine the total prokaryote abundance. After each water sample was thawed at ambient temperature, 2.5 µL was added to 245 µL filtered (<0.02 µm) TE buffer and 2.5 µL of SYBR Green I (diluted 10,000 times). The sample was then heated for 10 min at 75°C before the FCM analysis (Jacquet et al., 2013b). "Listmode" files were exported and analysed using CYTOWIN (Vaulot et al., 1989). The analysis provided information on prokaryote-like particles (PLPs), which encompassed two subgroups designated "high" (HDNA) and "low" DNA content (LDNA), indicating possibly active and less productive/active bacterial populations, respectively (Lebaron et al., 2001). Virus-like particles (VLPs) and the virus-to-prokaryote ratio (VPR) were also analysed (Marie et al., 1999).

2.6. Statistics

Statistical analyses were performed using R software version 3.6.3 (R Core Team, 2018), and figures were produced using the ggplot2 package (Wickham et al., 2018) or ggpubr (Kassambara, 2020). One-way ANOVA ($p < 0.05$) or Kruskal-Wallis test followed by Dunn's test (Alexis Dinno, 2017) or Tukey's HSD post hoc tests were performed to compare the mean values of each treatment throughout the experiment. NMDS and Adonis tests were also performed to test the differences between groups, i.e., replicates, treatments, and dates. Spearman's rank correlation test was conducted to examine the correlation between variables, and a correlogram was drawn using the corrplot package (Wei et al., 2017) following the Antoine Soetewey code (https://statsandr.com/blog/correlation-coefficient-and-correlation-test-in-r/). The correlogram shows the correlation coefficient for all pairs of variables (with more intense colours for more correlations), and correlations not statistically significant are represented by a white box. Moreover, to visualise the variation in microbial community structure and the relationship between environmental variables and sample clusters, we performed a redundancy analysis (RDA). All RDAs performed were significant according to the ANOVA test and chosen according to the detrended correspondence analysis (DCA) (Ramette, 2007). RDA was performed using the Vegan package (Oksanen et al., 2019). Environmental parameters were selected using the envfit function

($p < 0.05$). Multicollinear variables were removed using variance inflation factors (VIF) (Oksanen et al., 2019).

3. Results

3.1. BALOs abundance and dynamics

qPCR measurements indicated that *Peredibacteraceae* abundance was the highest, followed by *Bdellovibrionaceae* and *Bacteriovoracaceae*. The abundance of *Bdellovibrionaceae* (**Figure 1A**) in the H and C treatments seemed to follow the same decreasing pattern until day 14, with *Bdellovibrionaceae* being less abundant in treatment H. The abundance of *Bdellovibrionaceae* slightly increased in treatment M until day 4 before decreasing as the others. *Bdellovibrionaceae* abundance varied from 2.14×10^2 to 2.13×10^2 copies mL^{-1} in C, from 0.40×10^2 to 0.95×10^2 copies mL^{-1} in H, and from 0.83×10^2 to 1.03×10^2 copies mL^{-1} in M. While statistical analyses revealed that treatments H and M were different (Dunn's test p-value = 0.02), no significant difference was observed between treatments C and H, or between C and M (**Figure 2A**). *Peredibacteraceae* (**Figure 1B**) and *Bacteriovoracaceae* (**Figure 1C**) dynamics showed a similar pattern to those of *Bdellovibrionaceae*, with a decrease in abundance that was more marked in *Bacteriovoracaceae. Peredibacteraceae* varied from 4.73×10^3 to 2.59×10^2 copies mL^{-1} in C, from 9.61×10^2 to 0.82×10^2 copies mL^{-1} in H, and from 2.46×10^3 to 1.88×10^2 copies mL^{-1} in M. For *Bacteriovoracaceae,* abundance varied from 1.44×10^3 to 5.12 copies mL^{-1} in C, from 4.19 $\times 10^2$ to 5.11 copies mL^{-1} in H, and from 10.59×10^2 to 9.12 copies mL^{-1} in M. Kruskal-Wallis test showed no significant difference between treatments for both *Peredibacteraceae* (p-value = 0.19) and *Bacteriovoracaceae* (p-value = 0.34). The Adonis test confirmed that the treatments were not responsible for the disparity (p-value = 0.196). Moreover, the same test confirmed that the replicate mesocosms did not differ (p-value = 0.588). However, differences existed between dates (p-value < 0.01), and three different clusters could be defined (**Figure 3**): days 0, 4, 7, and 12–18.

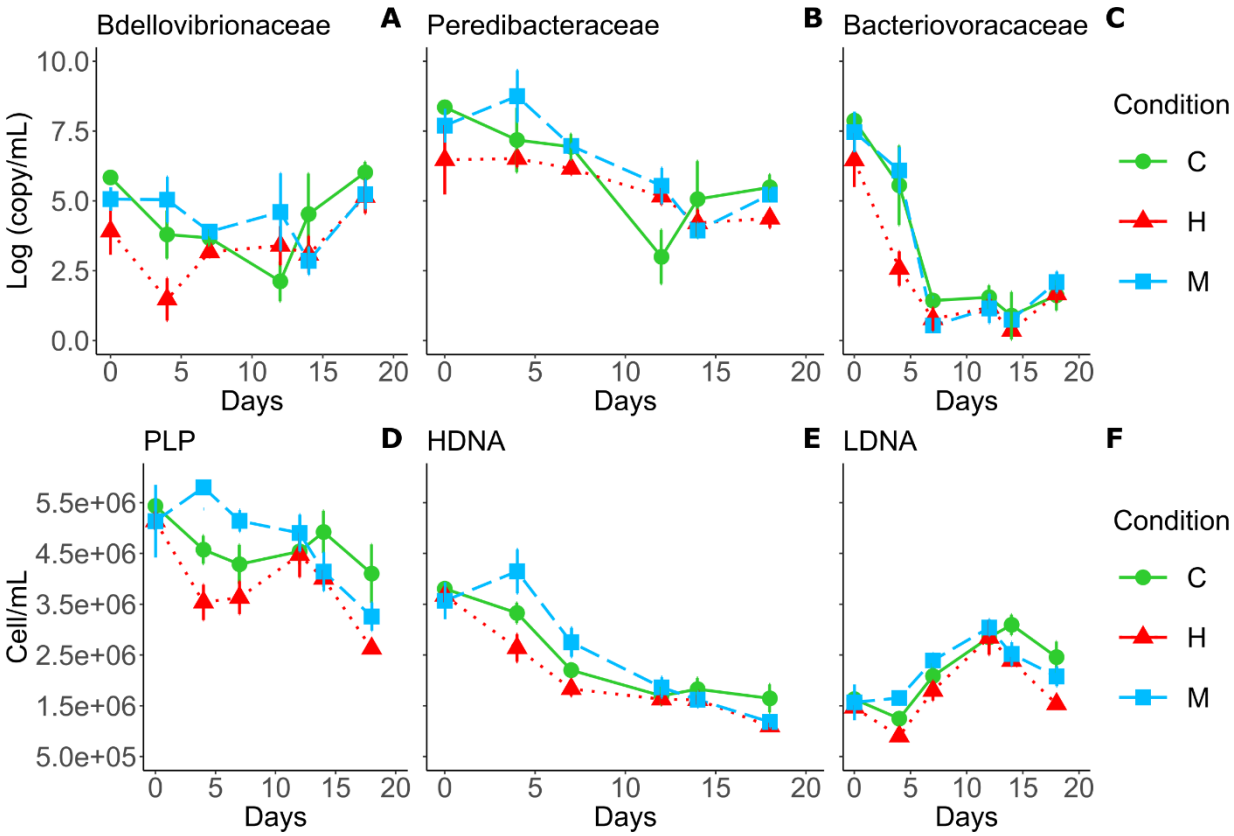


Figure 1 : Dynamics of BALOs as measured by qPCR using adequate set of primers that amplify the 16S rRNA gene of each family (A-B-C) and heterotrophic prokaryotes as measured using Flow cytometry (D-E-F) for the 3 treatments (Control (C)- green circle, Moderate (M)- red triangle, and High (H)- blue square). PLP: Prokaryote-like particles; HDNA: High DNA content; LDNA: Low DNA content.

3.2. Relationships with abiotic factors

The dynamics of the main environmental parameters during the experiment are described in **Supplementary Text Box 1** and presented in **Supplementary Figure 2**. On the one hand, the correlogram (white boxes for non-significant correlation; **Figure 4**) shows that *Bdellovibrionaceae,* unlike the other two BALOs, displayed very few relationships with environmental variables. Correlations were observed between pH (ρ = 0.30) and water hardness (ρ = 0.27). On the other hand, *Peredibacteraceae* and *Bacteriovoracaceae* were positively and significantly correlated with water hardness, nitrite (NO_2^-), total nitrogen (N_{tot}), phosphate (PO_4^{3-}), total phosphorus (P_{tot}), chloride (Cl^-), temperature, and particulate phosphorus (P_{part}), and negatively correlated with ammonium (NH_4^+), silica (SiO_2), and chlorophyll *a* (Chl *a*). The RDA, which explained 65% of the variance (**Figure 5A**), showed that *Bdellovibrionaceae* correlated positively with water hardness, pH, and day 18; *Peredibacteraceae* with nitrite (NO_2^-) and chloride (Cl^-), and *Bacteriovoracaceae* with temperature and day 0. The correlogram confirmed these observations. Overall, environmental parameters explained, to some extent, the variation in abundance observed for *Peredibacteraceae* and *Bacteriovoracaceae* but not for *Bdellovibrionaceae*.

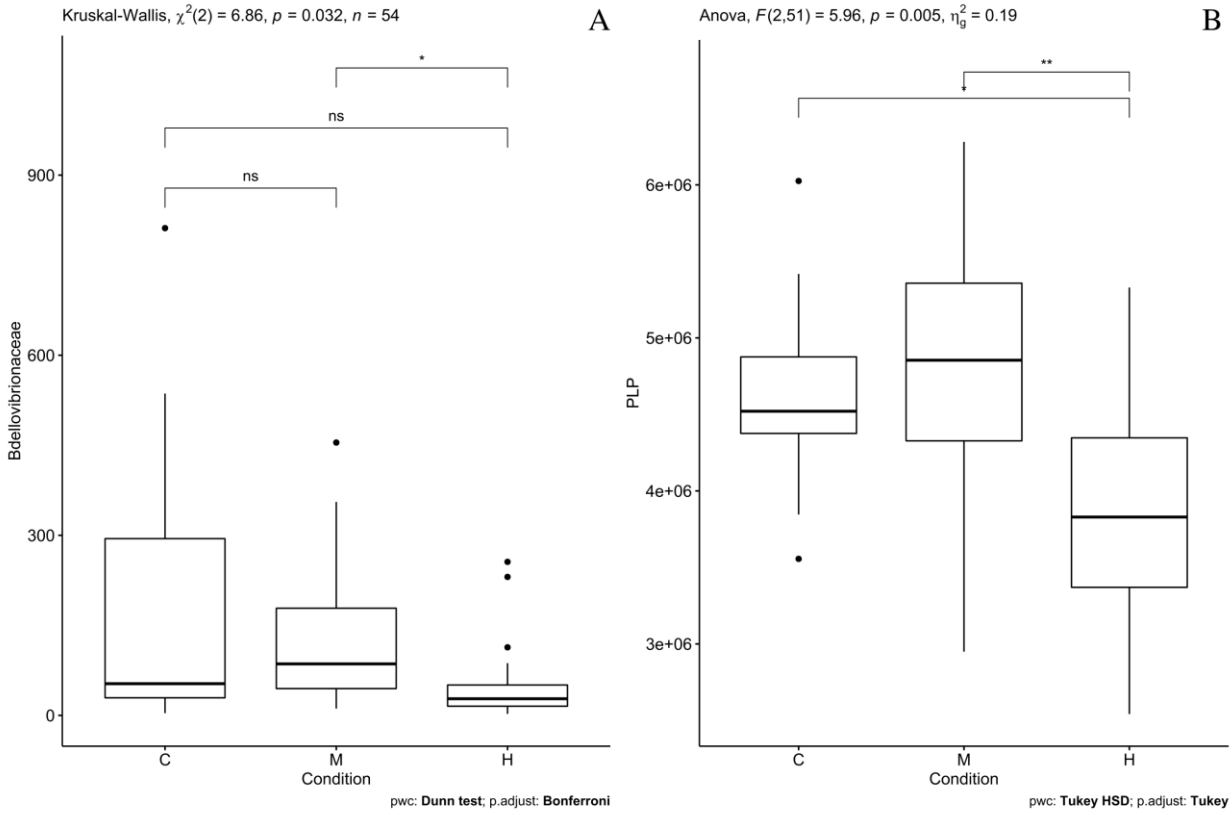


Figure 2 : Box plot of *Bdellovibrionaceae* abundances measured by qPCR (A) and prokaryote-like particles (PLP) measured by Flow cytometry (B) for each treatment over time. The solid line in each box plot corresponds to the median value. The letters C, M and H denote the treatment types, i.e. control, moderate and high, respectively. The statistical tests used are displayed at the top of the figures. The significance of the tests is represented by stars and the non-significance by the letters “ns”.

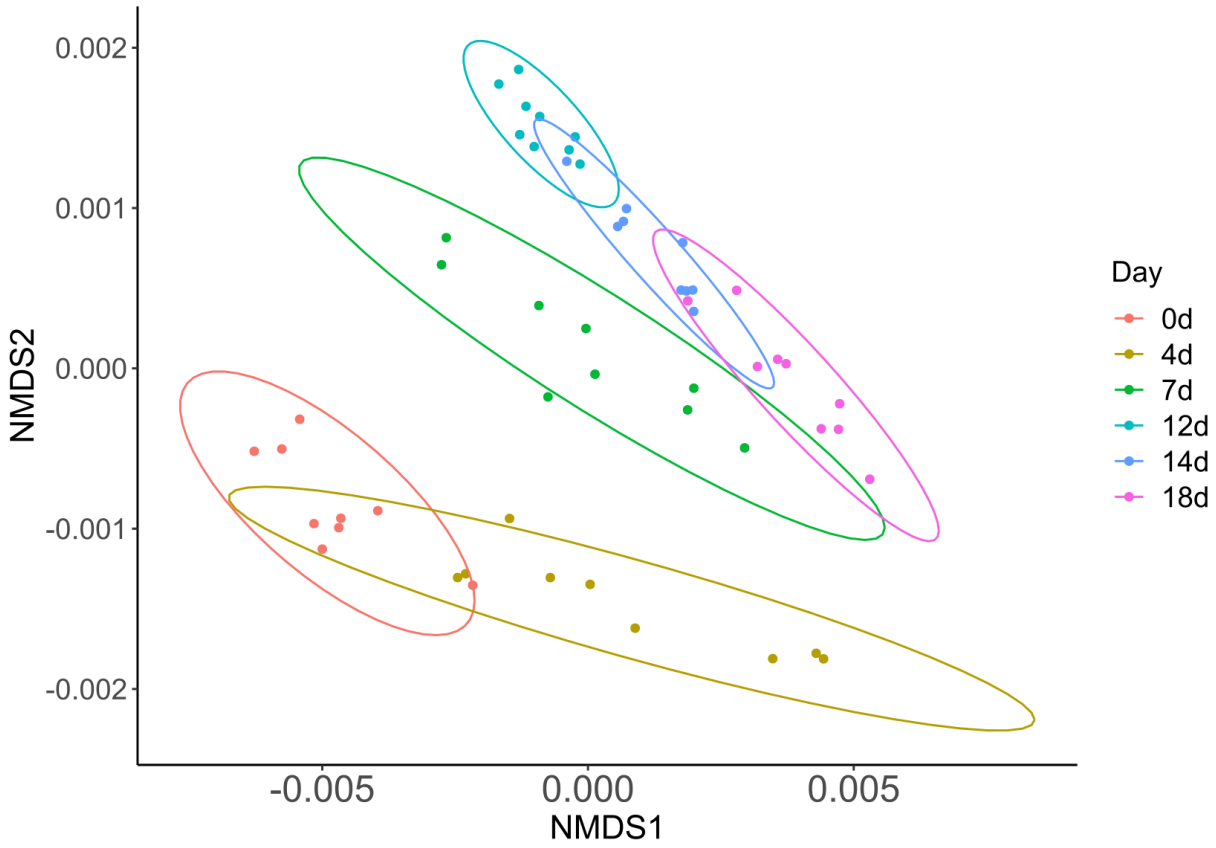


Figure 3 : Non metric multidimensional scaling (NMDS) showing the place of the samples according to dates in the ordination space with 95% ellipses (k = 2; stress = 0.00009). Permutational multivariate analysis of variance (Adonis) showed that the dates are statistically different.

3.3. Relationships between biotic variables

The correlogram (**Figure 4**) suggested that *Bdellovibrionaceae* was not significantly correlated with PLP, HDNA, or LDNA. Contrastingly, both *Peredibacteraceae* and *Bacteriovoracaceae* had a significant positive relationship with PLP and HDNA and a significant negative relationship with LDNA. More specifically, *Peredibacteraceae* was more correlated with HDNA (ρ = 0.81) than *Bacteriovoracaceae* (ρ = 0.60), whereas the opposite was true for LDNA (ρ = -0.51 vs -0.66). Furthermore, *Peredibacteraceae* had a superior relationship with PLP (ρ = 0.53) than *Bacteriovoracaceae* (ρ = 0.30). Considering the evolution of PLP, HDNA, and LDNA during the experiment (**Figure 1D, 1E, 1F**), we observed that the trend was generally similar to that observed for BALOs. The curve of the M treatment was slightly higher than that of the C and H treatments. Moreover, except for LDNA, abundance tended to decrease during the experiment—LDNA abundance increased during the experiment until days 13–14. PLPs (**Figure 1-D**) decreased during the experiment in all mesocosms, from 5.44×10^6 to 4.10×10^6 cells mL^{-1} for C, from 5.14×10^6 to 2.63×10^6 cells mL^{-1} for H, and from 5.13×10^6 to 4.26×10^6 cells mL^{-1} for M. The same pattern was also observed for the HDNAs (**Figure 1-E**) with a decrease in abundance from 3.80×10^6 to 1.65×10^6 cells mL^{-1} for C, from 3.67×10^6 to 1.10×10^6 cells mL^{-1} for H, and from 3.65×10^6 to 1.18×10^6 cells mL^{-1} for M. The LDNA dynamics were completely different (**Figure 1-F**) since the proportion of this group increased significantly during the experiment (from 1.63×10^6 to 2.5×10^6 cells mL^{-1} for C, from 1.47×10^6 to 1.58×10^6 cells mL^{-1} for H, and from 1.59×10^6 to 2.00×10^6 cells mL^{-1} for M). It is also noteworthy that the HDNAs were more abundant than the LDNAs at the beginning of the experiment for all treatments, which was reversed at the end. The ANOVA test showed that HDNA and LDNA mean abundances were not significantly different between the treatments (p-value = 0.37 and 0.11, respectively). However, treatments impacted PLP, and differences were significant between C and H (p-value = 0.01) and between M and H (p-value = 0.007). As shown in **Figures 1D** and **2B**, the H treatment had less PLP than C and M. Moreover, the correlogram **in Figure 4** shows that HDNA and PLP were positively and significantly correlated (ρ = 0.77). In contrast, LDNA was not significantly correlated with PLP and was significantly and negatively correlated with HDNA. PLP, LDNA, and HDNA had 8, 12, and 14 positive or negative significant relationships with environmental variables, respectively. LDNA had mostly negative relationships with environmental parameters, whereas HDNA had mostly positive relations. The RDA (**Figure 5B**), which explained 70% of the variance in PLP, HDNA, and LDNA, revealed that PLP correlated positively with water hardness and day, HDNA with nitrite and chloride, and LDNA with chlorophyll *a* and day 12.

The present study also allowed us to examine VLP dynamics that were found to be opposite to those of BALOs and prokaryotes (**Supplementary Figure 3**). VLP abundance increased more in H than in the C and M treatments; however, the ANOVA test revealed that the differences were not significant (p-value = 0.5). Moreover, the correlogram (**Figure 4**) suggested the absence of a significant correlation between VLP and BALOs. No significant correlations were observed between VLP and PLP, HDNA, or LDNA. When considering the virus-to-prokaryote ratio (VPR), H favoured an increase in VPR at the beginning of the experiment (until day 12; **Supplementary Figure 3B**), and statistical analyses indicated a significant difference between H and M, but not between C and H or between C and M. The correlogram (**Figure 4**) indicated significant and negative correlations between VPR and *Peredibacteraceae* (ρ = -0.49) and *Bacteriovoracaceae* (ρ = -0.32), but not with *Bdellovibrionaceae*. VPR also displayed a strong negative relationship with PLP (ρ = -0.86) and HDNA (ρ = -0.67), but not with LDNA.

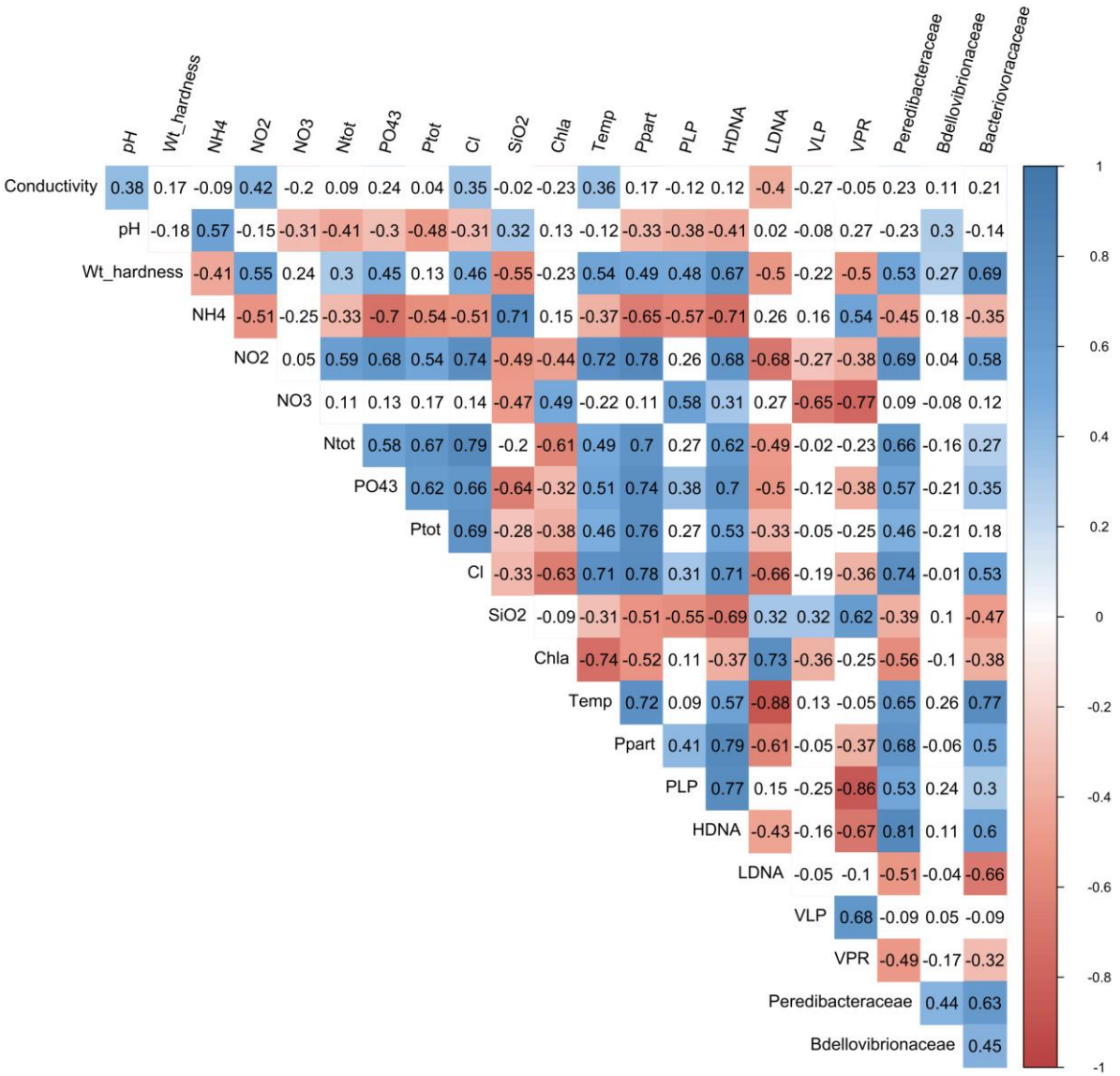


Figure 4 : This correlogram is based on 21 observations that represent environmental variables, PLPs, VLPs and BALOs. Positive and negative correlation are displayed in blue and red, respectively. The intensity of the color is proportional to the correlation coefficient. The white boxes indicate that the correlation is not significantly different from 0 at the 5% level tested with a correlation test. Abbreviation are as follows: ammonium (NH_4^+), chloride (Cl^-), chlorophyll *a* (Chla), nitrate (NO^3-), nitrite (NO_2^-), particulate phosphorus (Ppart), silica (SiO_2), sulfate (SO_4^{2-}), phosphate (PO_4^{3-}), total carbon (Ctot), total phosphorus (Ptot), total nitrogen (Ntot), temperature (temp), water hardness (Wt hardness), prokaryote-like particles (PLP), high DNA content (HDNA), low DNA content (LDNA), virus-like particles (VLP) and virus-to-prokaryote ratio (VPR).

4. Discussion

With global climate change, episodic extreme events (such as floods and violent storms) are expected to become more frequent, causing changes in lake subsidies and exposing them to nutrient pulses and transfer imbalances. The effects of allochthonous dissolved organic matter inputs on aquatic organisms, biotic interactions and metabolic processes feedback, and the impact on the littoral versus pelagic communities remain largely underexplored. Our experimental approach made it possible to simulate such a scenario with the main aim of investigating the response of a specific functional group of bacteria.

To study microbial interactions, especially predator-prey relationships, the time scale is important. For BALOs, predation is known to be rapid under controlled and favourable conditions, but the impacts of bacterial predators on bacterial prey dynamics, distribution, and composition under natural conditions have been poorly investigated until now. Using a 2- to 4-day sampling strategy and an outdoor mesocosm approach, we aimed to assess, for the first time, the dynamics of the main BALO families belonging to Oligoflexia, highlighting their response to abiotic and biotic factors.

After observing the BALO dynamics and performing statistical analyses, it appears that the simulated effects did not have a strong impact on BALO abundance. Although the M treatment slightly increased BALO abundance and the H treatment reduced it, the mean abundances of each treatment were not significantly different from the control. The only significant difference was observed for *Bdellovibrionaceae* between the H and M treatments, whose dynamics changed over time. In other words, BALOs were not affected by the stresses or modifications caused by extreme events. When the treatment effects are not considered, it is clear that each family of BALOs displayed a very different dynamic, suggesting a variety of interactions and responses to forcing. Many factors and/or processes could be responsible for these differences (e.g., the predation spectrum, prey presence/absence, resistance genes or other attributes, etc.). The *Peredibacteraceae* family was the most abundant among the studied BALOs. Despite their abundance decreasing slightly during the experiment, this was consistent with our previous studies on BALOs in Lake Geneva (Paix et al., 2019; Ezzedine et al., 2020c, 2020b, 2020a) suggesting that this group is the dominant BALO, especially in lake surface waters. In contrast, *Bacteriovoracaceae* were less abundant and decreased strongly during the experiment, while *Bdellovibrionaceae* abundance remained relatively stable.

Bdellovibrionaceae displayed only relationships with pH and water hardness. It has been established in literature that some bacterial species are vastly dependent on pH (Stockner and Shortreed, 1991; Ruber et al., 2018), but to our knowledge, no study has shown the direct effect of

pH on BALO dynamics. Moreover, pH varied only slightly during the experiment. Laboratory experiments should be conducted to test whether pH could influence the abundance and dynamics of *Bdellovibrionaceae*. *Peredibacteraceae* had marked relationships with nitrite and chloride ions, but here again, there are no studies that show a positive or negative relationship between these two variables, which also varied very little during the experiment. For the chloride ion, Roeßler et al. (2003) (Roeßler et al., 2003) demonstrated that chloride ions are not necessary for growth in 44 different bacterial strains, except in very salty environments. Naturally, this is not the case for Lake Geneva. The only ions reported to be beneficial to the growth of BALOs are calcium and magnesium ions because they are important for BALO penetration into prey (Huang and Starr, 1973). For nitrite, *Peredibacteraceae* may be associated with this variable because they can consume nitrite-oxidising bacteria. For *Bacteriovoracaceae,* temperature seemed to be important, and literature has indicated temperature's impact on the abundance of BALOs (Schoeffield et al., 1996; Kelley et al., 1997; Williams and Piñeiro, 2006; Kandel et al., 2014). *Bacteriovoracaceae* abundance decreased with increasing temperature; however, the temperature difference in the present study was 3°C, which is small considering that BALOs generally tolerate a wide temperature range (Davidov and Jurkevitch, 2004; Jurkevitch and Davidov, 2006). A study by Kandel et al. (2014) (Kandel et al., 2014) showed that a variation of 4°C in a closed system (aquaculture) does not affect BALO abundance. In general, it takes large temperature fluctuations, as in natural systems, to observe effects on the BALO population.

Amongst the prokaryotes (PLP) are bacteria with high (HDNA) and low (LDNA) DNA content, some of which might be considered potential BALO prey. The first question we sought to address was whether there was enough prey for BALOs in the mesocosms, and if so, who consumes what? First, to answer these questions, we eliminated the treatment effect. The statistical tests' results did not show significant differences in the abundances of HDNA and LDNA. On the other hand, there was a significant difference in PLP abundances between C and H and M and H. The events that the H mesocosms were exposed to induced a significant decrease in PLPs compared with the M treatment and the control. This result was the opposite of what we expected. Our initial hypothesis was that our experimental conditions, especially the increase in dissolved organic carbon, would enhance bacterial growth and reproduction if temperature and inorganic nutrients were not limiting at that time, and by extension, promote the growth of BALOs and other bacterial predators. In fact, BALOs have been reported to be more abundant and diverse in eutrophic lakes owing to the abundance and diversity of the population of prey bacteria (Chauhan et al., 2009b). To explain the overall absence of significant differences between treatments, we first surmised that the commercial peat-based solution used as the organic carbon source was inadequate. Heterotrophic bacteria might not have efficiently assimilated the organic carbon in the prepared solution. Second,

one cannot rule out the possibility that a weak increase in organic matter input will have little or no effect on prokaryotic communities, as already shown elsewhere (Kankaala et al., 2010). Third, it is possible that other elements were limiting for prokaryotic growth (Jacquet et al., 2002a). However, it was not the case for inorganic nutrients because ammonium, nitrite, nitrate, nitrogen, and phosphorus concentrations were at sufficient concentrations to sustain microorganism growth (Tran-Khac et al., 2019). Regardless of the small decrease in PLPs, their abundance remained relatively high during the experiment. In general, the PLPs and HDNAs decreased slightly over time. Conversely, LDNA slightly increased. Regarding the decrease in PLPs and HDNAs, we surmised that the disruption in the number of prokaryotes, and by extension of potential prey, especially the most active one, HDNA, caused a decrease in BALO abundance. BALOs also obviously did not benefit from the increase in LDNAs, which contain less nucleic acid. Indeed, BALOs consume the entire cell content of their prey. BALOs generally seek out prey that can support their growth and reproduction. BALOs are known to carry out a control step of the host cell resource via reversible attachment to the wall (Rendulic et al., 2004). Therefore, we hypothesised that LDNAs do not represent suitable prey for BALO development. This hypothesis was supported by the correlogram results (**Figure 4**). *Bacteriovoracacacae,* and *Peredibacteraceae,* more for the latter, showed a strong positive correlation with PLPs, especially HDNAs, and conversely, they both had a negative relationship with LDNAs. Thus, a decrease in PLPs, particularly HDNAs, could have disturbed BALO dynamics in the mesocosms. *Bdellovibrionaceae* was not significantly correlated with PLP, HDNA, or LDNA. Other factors seem to explain the dynamics over time. Furthermore, BALOs are known to mainly hunt for gram-negative bacteria; however, not all gram-negative bacteria are susceptible to BALO predation. Moreover, BALOs can be generalist, specialist, or versatilist hunters. While generalists can feed on multiple prey types, specialists are only capable of feeding on one type or a limited number of strains (Shemesh et al., 2003), while versatilists behaviour as specialists but prey as generalists (Chen et al., 2011). Such a variety of strategies could also explain the important decline of *Bacteroidaceae,* the relative stability of *Bdellovibrionaceae,* and the entre-deux for *Peredibacteraceae*. On the other hand, the decrease in HDNA and especially PLP was partly explained by the treatments, but it seemed that other factors were also at play, particularly for the increase in LDNA. The increase in LDNA was strongly and positively correlated with chlorophyll *a* and negatively correlated with temperature. These two factors seemed to play a role in LDNA dynamics, despite their variations being minimal. Furthermore, LDNA was negatively correlated with HDNA, implying that the dynamics of one probably influenced that of the other. For the decrease of PLP, especially HDNA, the environmental variables of the correlogram and RDA did not appear to show a clear pattern. Both were positively correlated and showed several other

correlations with other factors. In contrast, LDNA had more negative relationships with the environmental variables.

Other interactions may explain the dynamics of *Bdellovibrionaceae*, PLP, and HDNA. Among the other parameters, we were able to study the dynamics of viruses (PLP). According to literature, some bacteriophages are capable of infecting BALOs (Hashimoto et al., 1970), and in Lake Geneva PLPs, and HDNAs (Personnic et al., 2009b; Jacquet et al., 2013a). PLP dynamics were observed to increase over time. Statistical tests did not show any treatment effect on PLP abundance. Despite the relative increase in VLPs, there was no correlation with BALOs or PLPs, HDNA, and LDNA. The presence and abundance of VLPs had no visible impact on the prokaryotic community and BALOs. In contrast, the virus-to-prokaryote ratio (VPR), which can be used as a proxy for host-parasite interactions (Parikka et al., 2017), showed the opposite trend. This ratio increased strongly in the H treatment, with statistical analysis showing a significant difference between the H and M treatments. This suggests that phages affected prokaryotic growth and dynamics in the H treatment through cell lysis. In contrast, the M and C treatments did not seem to favour a "boom" of phages. We also noted a negative correlation between VPRs and *Peredibacteraceae* and *Bacteriovoracaeceae*, but no significant correlation with *Bdellovibrionaceae*. Finally, we observed that *Peredibacteraceae* and *Bacteriovoracaceae* appeared to share common trends, and these two families were positively correlated with each other, and *Bdellovibrionaceae* had a weaker positive correlation. The current study could not explain *Bdellovibrionaceae* dynamics.

We strongly believe that factors other than those measured here, such as the importance and role of other predators, e.g., heterotrophic nanoflagellates, ciliates, and metazooplankton, could be determinants of the observed effect. These groups could likely exert considerable control over bacteria, explaining the observed dynamics of both PLPs and BALOs. It would be interesting to specifically target BALO prey using an adequate set of qPCR primers instead of looking at the general trend of prokaryotes. However, we only identified one BALO prey species in Lake Geneva, i.e., *Pseudomonas* spp. (Ezzedine et al., 2020c). Further efforts to pinpoint prey strain and type are needed to fully understand BALO dynamics in Lake Geneva and peri-alpine lakes.

5. Conclusions

Bacteria play key roles in ecosystem functioning, as they are involved in nutrient cycling and are prey for a variety of predators, among other functions. Therefore, it is essential to accurately predict their relationships with other species and general patterns governing their adaptation to the environment. Here, we shed some light on a group of bacteria that are still poorly understood in terms of their ecology, particularly in natural freshwater systems such as lakes. Although BALOs

were not significantly affected by our treatment conditions, which were supposed to mirror the advent of extreme events on the surface waters of Lake Geneva, they were shown to be a dynamic community, and that constitutive families of this functional group exhibited different patterns. As the main environmental physicochemical parameters measured in the present study did not seem responsible for the observed dynamics, the importance of biotic interactions is proposed. These interactions were likely predation and parasitism through the action of phages and protozoans, but also possibly the scarcity of specific prey for BALOs. Next steps should include more (biotic) factors, focus on the quantity and quality of prey, and examine different types of predation on both total and specific groups of bacteria.

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7. Supplementary data

Supplementary Figure 1 : Design of MESOLAC. The mesocosms were anchored in front of the hydrobiological station CARRTEL of INRAE at Thonon-les-Bains. Each tested condition was reproduced 3 times: M for moderate and H for High. A control (C) set up was also included in the design

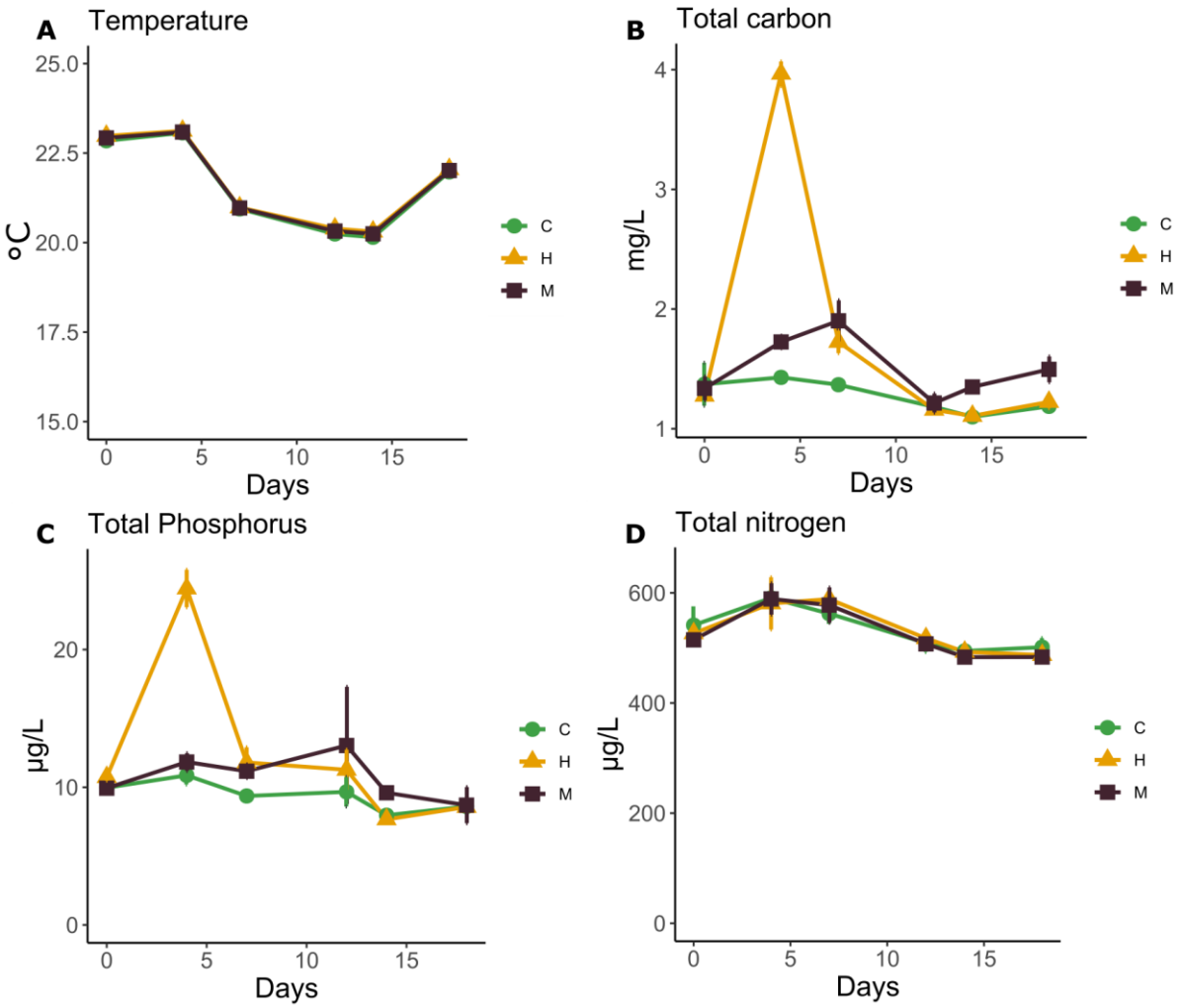
Supplementary Table 1 : List of primer pairs used to amplify the 16S rRNA gene of BALOs by qPCR.

qPCR set of primers	Sequence (5'-3')	Target BALOs	Amplicon size (bp)	Reference
Bd347 F	GGAGGCAGCAGTAGGGAATA	*Bdellovibrionaceae*	190	Van Essche *et al.*, (2009)
Bd549 R	GCTAGGATCCCTCGTCTTACC			
Bx421 F	CGGTCTGTAAAGCTCTGTTAATGT	*Bacteriovoracaceae*	84	Ezzedine *et al.*, (2020)
Bx482 F	TGCCGMGTGAGTGAGGAAGG			
Per627 F	AAACTGCGTCTGAAACTGCT	*Peredibacteraceae*	91	
Per696 R	TGTTCCTTCACATCTCTACGGA			

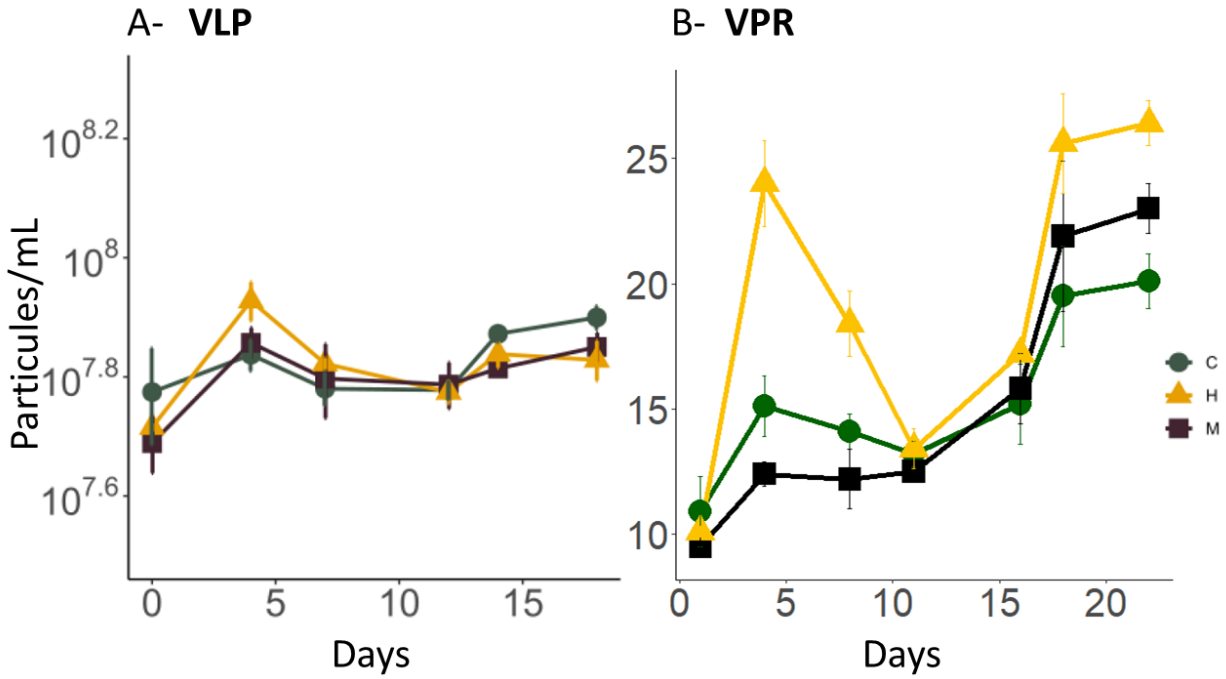
Text Box 1 : Evolution of the main environmental parameters

The local weather varied significantly during the MESOLAC experiment. Air temperatures fluctuated between 14.5 and 33.3°C (hourly averages). Rainfalls were recorded on the 2nd, 3rd, 11th and 17th days of the experiment and ranged from 0.5 to 8.5 mm. Wind gusts measured on the 7th, 8th, 14th, 24th, 25th days reached up to 23 m s^{-1}.

Inside the mesocosms and for all treatments, the water temperature varied in a lower range than air temperature. On the first day of the experiment, the temperature was ~22.9°C, then it decreased between day 4 and 14 from ~23°C to ~21.2°C, and reached ~22°C on the 18th day (**Supplementary Fig. 2-A**). Peat inputs led to an increase of the total organic carbon (TOC) concentration from ~1.3 to ~4 mg L^{-1} in the 4th day of the experiment for treatment H. As for treatment M, it increased to a maximum of 1.9 mg L^{-1} on the 7th day of the experiment. Then, TOC was reduced to reach approximately the values of the C treatment at the end of the experiment (**Supplementary Fig. 2-B**). Total phosphorus concentration for treatment H also showed a similar profile, with a strong increase until day 4 (from about 10 to 25 µg L^{-1}). M treatment seemed rather constant, apart from an increase on day 12 with ~12 µg L^{-1}) (**Supplementary Fig. 2-C**). The total nitrogen concentration kept a similar profile in all treatments with an increase on the 4th day from ~500 to 600 µg L^{-1} (**Supplementary Fig. 2-D**). Finally, pH ranged from 7.9 to 8.6.



Supplementary Figure 2 : Evolution of the main environmental parameters in regards to the different treatments tested in the mesocosms. A) Water temperature (°C); B) Total organic carbon (mg L^{-1}); C) Total Phosphorus (µg L^{-1}); D) Total Nitrogen (µg L^{-1}).



Supplementary Figure 3 : A. In contrast to PLPs, VLPs increased along the experiment. In treatment C, VLPs went from 5.95×10^{7} to 7.94×10^{7} Particles mL^{-1}. In treatment H, VLPs peaked on July 8th to 8.47×10^{7} Particles mL^{-1} but decreased slightly thereafter. In treatment M, VLPs also increased until day 4, from 4.88×10^{7} to 7.20×10^{7} Particles mL^{-1}. On day 18, the abundance was 7.08×10^{7} Particles mL^{-1}. At the end, VLP abundance was higher in C than in the two other treatments. B. The virus-to-prokaryote ratio (considered as a proxy of the interactions between bacteria and phages) also increased along the experiment from ~10 to >25 depending on the treatment considered. VPR was, most of the time, higher in the treatment H.

Conclusion du chapitre IV

L'isolement des BALOs s'est avéré ardu. Il serait nécessaire de consacrer plus de temps et de matériel à cela. Travailler sur des sites plus riches en BALOs telles que les sorties d'effluents dans le lac ou encore à partir de biofilms pourrait faciliter cette tache. De plus, il semble que certains genres de BALOs, notamment les *Bdellovibrio*, soient plus faciles à isoler et cibler ce groupe pourrait donc être une première solution pour aller plus loin. Enfin, les interactions biotiques expliquant à l'évidence mieux la dynamique des BALOs (comparativement aux variables environnementales), des expériences ciblées devraient être mises en place pour quantifier la prédation.

Chapitre V

Discussion et perspectives

Chapitre V : Discussion et perspectives

La présence des BALOs

La diversité des BALOs tout comme leur distribution et dynamique spatio-temporelle, notamment au travers des abondances cellulaires, restent très peu connues pour les environnements naturels, en particulier les milieux dulçaquicoles. La majorité des connaissances émane de l'étude des sols, des eaux salées ou encore des stations d'épuration. Notre projet a donc comblé un vide important et permis d'alimenter les connaissances sur les BALOs pour des écosystèmes encore peu explorés. Cela a été rendu possible via la technique de séquençage à haut débit et la quantification de copies de gènes par l'outil moléculaire qPCR. Cette stratégie d'étude a aussi été relativement originale car les BALOs ont été très largement étudiés jusqu'à maintenant par des techniques d'isolement et de comptage de plaque de lyse, ne révélant que très partiellement leur dynamique et leur diversité. Ainsi, ce travail de thèse aura révélé que les BALOs sont présents et relativement abondants et/ou diversifiés dans les grands lacs péri-alpins (Léman, Annecy et Bourget), dans la baie de Banyuls, près de la côte ou plus au large, ou encore dans d'autres environnements qui nous ont servi pour effectuer des tests, à savoir la baie d'Arcachon et la Manche près de Roscoff.

Nos travaux ont montré que les abondances des BALOs peuvent être relativement importantes à certaines périodes de l'année. En effet, ces abondances sont susceptibles d'atteindre des pics durant les mois ou saisons les plus chaudes, la température semblant être un paramètre clef surtout pour la croissance et disponibilité des proies. Les BALOs semblent assez présents mais certains d'entre eux, typiquement les *Peredibacter,* semblent être les plus abondants, en particulier dans les lacs. Ce résultat est très intéressant car on s'est surtout focalisé par le passé, et encore aujourd'hui, sur les *Bdellovibrio*. A l'inverse, les *Bacteriovorax* semblent être les moins abondants. A ce stade nous ne pouvons pas expliquer pourquoi les *Peredibacter* sont les plus abondants mais nous pouvons formuler plusieurs hypothèses en lien avec les facteurs biotiques car nos résultats ont montré que les facteurs abiotiques n'expliquent pas ou peu la dynamique des BALOs. Il pourrait donc s'agir d'une efficacité de prédation plus performante que celle des autres familles grâce à un spectre de prédation large ou spécifique à une proie très abondante dans les milieux étudiés. Il pourrait s'agir d'une capacité de compétition meilleure chez *Peredibacter* vis-à-vis des autres prédateurs pour une ou plusieurs proies. Il n'est pas impossible également que *Peredibactcer* soit moins ciblé par les attaques des protistes ou des bactériophages. Enfin, une aptitude génétique ou environnementale

favorisant le développement de *Peredibacter* n'est pas à exclure. On comprend bien ici que le champ des possibles est important et les perspectives d'étude passionnantes et très nombreuses. Les travaux menés au cours de cette thèse ont également permis de révéler que les BALOs sont assez diversifiés en termes d' « espèce » comme le souligne la centaine d'OTUs obtenue pour *Bdellovibrio* et *Peredibacter*. Pour la génération de ces OTUs, nous avons utilisé le programme Swarm avec comme paramètre « d=1 » (Mahé et al., 2015). Ce programme permet d'obtenir une meilleure résolution taxonomique pour détecter une plus grande diversité génétique parmi les BALOs. D'autres programmes ou algorithmes existent et auraient pu être testés comme UCLUST (Edgar, 2010) ou celui utilisant les variants de séquence d'amplicon (ASV ou ESV) (Callahan et al., 2017) qui se popularise dans le milieu scientifique, sans forcément faire l'unanimité.

Notre étude reste exploratoire et il serait intéressant de poursuivre cette analyse sur plusieurs années, dans différentes conditions environnementales, divers habitats, etc…. Même si les BALOs semblent être « ubiquistes », cela reste à démontrer. A titre d'exemple, nos résultats ont révélé que *Halobacteriovorax*, le genre adapté et retrouvé uniquement dans les eaux salées, n'était pas abondant dans la baie de Banyuls alors qu'il était détecté de manière plus importante dans la baie d'Arcachon. La colonne d'eau n'est aussi peut-être pas le meilleur habitat pour étudier les BALOs, les isoler, les caractériser et déterminer l'importance de leur rôle fonctionnel. Ainsi, les biofilms, les sédiments, les sites côtiers proches des embouchures de rivière ou des stations d'épuration pourraient abriter une plus grande diversité et/ou abondance de BALOs. Un projet est en cours de construction (ANR internationale) pour aller dans ce sens et explorer en profondeur le Léman, un site que l'on peut désormais définir comme un des lacs les plus étudiées en ce qui concerne ces bactéries (voir plus loin).

Notre projet ne s'est intéressé qu'aux Oligoflexia et des différentes familles que l'on trouve dans ce groupe. Il existe toutefois un autre genre de BALO, *Micavibrio*, auquel il faudrait s'intéresser à l'avenir. C'était dans notre projet initial mais faute d'avoir réussi à dessiner une paire d'amorces suffisamment spécifique, nous avons dû abandonner ce projet. Leur ADN a toutefois été détecté dans les lacs Léman et d'Annecy ainsi que dans les sites marins MOLA et SOLA, ce dernier milieu était d'ailleurs le plus riche en *Micavibrio*. Les échantillons traités dans le cadre du projet TRANSLEM ont également révélé que les OTUs et nombre de reads de *Micavibrio* pouvaient égaler ceux de *Bacteriovoracaceae* dans le Léman.

Enfin, un point qui semble très intéressant est que la présence de certains BALOs, à la fois dans les milieux d'eaux salées et douces, typiquement *Peredibacter*, suggère une grande adaptation de

certains BALOs pour divers milieux ou une diversité encore mal appréciée. Il serait intéressant de pouvoir isoler des individus actifs dans les différents milieux étudiés, puis les caractériser pour éventuellement proposer une nouvelle phylogénie séparant des *Peredibacter* halophiles vs. dulçaquicoles.

Le développement nécessaire de nouvelles amorces nucléotidiques

Ce travail de thèse a été très riche et devrait permettre à d'autres scientifiques d'avancer sur l'étude de ce groupe fonctionnel de bactéries, notamment grâce aux nouvelles amorces ADN que nous avons dessinées, testées et validées. Plusieurs raisons nous ont poussées à développer de nouvelles amorces nucléotidiques. La première est que certaines techniques de biologie moléculaire, comme la DGGE et le clonage-séquençage et qui ont été employées dans deux de nos études, ont souligné leur limite pour révéler toute la diversité des BALOs. La seconde raison a été le constat que les paires de primers existantes et publiées dans la littérature ne tenaient pas forcement compte de la classification la plus récente des BALOs et encore moins des nouvelles techniques de séquençage. Une partie importante de ce projet a donc consisté à développer des paires d'amorces, compatibles avec la technique Illumina Miseq, un travail initialement prévu pour l'ensemble des familles ou genre du groupe des BALOs. Les amorces qui ont servi pour le séquençage ont été dégénérées afin de maximiser la capture des taxons sans pour autant diminuer leur spécificité. Les amorces dédiées à la qPCR ont, quant à elles, été construites pour répondre au « cahier des charges » de cette technique, à savoir des séquences courtes et non-dégénérées pour une meilleure fiabilité de quantification. *In fine*, nous avons eu du succès pour certaines familles de BALOs mais aussi des échecs pour d'autres, qui ont été soit aspécifiques soit dans l'incapacité de détecter la cible recherchée. Cette détection a d'ailleurs été un problème majeur dans la conception de ces amorces. En effet, nous avons utilisé les échantillons des eaux des milieux ciblés dans ce projet et il s'est avéré que certains d'entre eux contenaient très peu de matériel malgré le regroupement de plusieurs dates et profondeurs pour constituer un pool « concentré », et ce comparativement à un pool d'ADN bactérien de laboratoire (« mock community »). L'échec a été le plus marqué pour les amorces ciblant spécifiquement *Micavibrio* ou *Halobacteriovorax.* Il est possible que nos amorces aient pu être sensibles au milieu étudié ou alors véritablement à la très faible abondance de la cible. Clairement, nous sommes bien conscients que pour optimiser ces amorces, il faudrait travailler sur plusieurs systèmes et allouer un effort important pour isoler et caractériser de nouveaux BALOs, tant il est vrai que les bases de données ne contiennent que très peu de représentants des *Peredibacter*, *Micavibrio* et *Bacteriovorax.* A noter que dans ces bases de données, même celles « curated », le taux d'erreur d'assignation et de classification reste important.

Le rôle des BALOs

Ces bactéries prédatrices obligatoires, qui attaquent et digèrent d'autres bactéries, jouent surement un rôle important dans la structuration des populations microbiennes. Ce rôle n'a pour autant pas été beaucoup étudié jusqu'alors. Les BALOs sont connus pour consommer des pathogènes bactériens mais aussi, peut être, et de manière plus surprenante, des algues (Lee et al. 2018), les enzymes de *B. bacteriovorus* ayant en effet été montrées par le passé capables d'inhiber 75% de la photosynthèse chez *Phormidium luridum* (Burnham et Stelak, 1976). Les BALOs ne semblent pas constituer une biosphère rare, même si leur abondance est généralement faible. Cette faible abondance n'est de toute façon pas la preuve d'un impact fonctionnel qui serait moindre et certaines études ont en effet rapporté que les BALOs sont des prédateurs efficaces, capables de réduire une population de proie en quelques heures, au même titre que l'action de certains bactériophages (Williams et al. 2016). Toutefois, il est clair qu'aujourd'hui, la question de savoir si, quand et comment les BALOs sont aussi ou plus efficaces que d'autres prédateurs bactériens (flagellés, ciliés, virus) reste à déterminer.

Les BALOs sont connus pour avoir différentes stratégies de prédation, certains étant généralistes ou spécialistes alors que d'autres sont dits versatiles. Par comparaison, les protistes sont plutôt des prédateurs généralistes alors que les bactériophages sont clairement des spécialistes. La prédation des BALOs est donc multiple et donne une dimension accrue aux contrôles des populations mais aussi aux flux de matière. Si les bactériophages, via la lyse virale, entrainent la libération d'une grande partie du contenu intracellulaire dans l'environnement, les BALOs consomment la majeure partie du contenu des proies, libérant au final peu de nutriments. Par contre, la proie contenant la progéniture du prédateur (le bdelloplaste) a une valeur énergétique plus élevée, cette progéniture étant caractérisée par plusieurs individus, chacun regorgeant de nutriments. Parallèlement, les interactions biotiques impliquant les BALOs sont susceptibles d'être multiples mais les prédateurs des BALOs sont en fait peu connus. On sait que certains phages peuvent les parasiter et que des protistes peuvent les consommer. Mais, à quelle vitesse les BALOs sont-ils consommés ? Constituent-ils eux même une proie de choix ? etc….

Dans le cadre de cette étude, au travers de l'isolement, l'analyse de l'abondance et de la distribution des BALOs, ou encore de l'analyse de leur diversité et de réseaux de cooccurrence, le Léman a été l'écosystème le plus étudié, permettant de commencer à apprécier le rôle possible de certaines de ces bactéries. Parmi les BALOs, *Peredibacter* s'est révélé être le plus abondant, laissant à penser que ce genre puisse jouer un rôle écologique plus important que les *Bdellovibrio*. Chaque prédateur

ayant potentiellement un spectre et une efficacité de prédation différente, nous ne pouvons que proposer cette hypothèse, qui ne pourra être testée qu'expérimentalement. Plus d'informations ont été obtenues pour les *Bdellovibrio.* Des *Bdellovibrio* périplasmiques pouvaient consommer *Pseudomonas* spp. alors que les épibiotiques avaient une préférence pour *Aeromonas salmonicida salmonicida*. Les deux bactéries proies précitées sont connues pour provoquer des maladies chez l'Homme et les salmonidés. Il serait donc possible que certains *Bdellovibrio* puissent rendre un service à l'Homme en participant à la réduction de certains pathogènes potentiels ou avérés. Ce résultat mériterait d'être creusé car proposer une solution alternative à l'utilisation des antibiotiques, une thérapie bactérienne, dans les pisciculture de salmonidés, pourrait être très porteuse. Les liens marqués qui ont été trouvés entre les BALOs et certaines bactéries Gram-négatives laissent à penser qu'il existe des interactions privilégiées au premier rang duquel le fait que certaines bactéries puissent effectivement être des proies pour les BALOs et soient donc, en partie, contrôlées par ces derniers. Par exemple, si certains BALOs consomment certains membres des Methylococcales, il est possible d'imaginer un impact sur le cycle du méthane. Cette hypothèse reste à démontrer, mais rappelons que *Micavibrio,* par exemple, est connu pour prédater *Nitrospira* spp. et potentiellement perturber le processus de nitrification (Dolinšek et al., 2013). Les Myxobacteria s'étant aussi révélées être très présentes au Léman et dans le réseau de co-occurrence, il est possible qu'une importante compétition existe entre ces prédateurs bactériens ou au contraire qu'un effet synergique induise un impact fort sur les communautés bactériennes servant de proie.

A l'origine du projet, nous avions bon espoir de quantifier une partie de ce rôle fonctionnel. Isoler des BALOs a été plus compliqué que prévu et nous n'avons pas encore eu le temps pour utiliser d'autres méthodes pour mieux apprécier la mortalité induite par les BALOs.

Perspectives

C-BALO a été un projet novateur et ambitieux, fournissant *in fine* de nombreux résultats originaux (10 publications ou synthèses en 4 ans) et nous positionnant parmi les leaders de l'étude de ces bactéries si singulières. De nombreux points d'ombre demeurent et il est donc critique de poursuivre l'étude de ce groupe fonctionnel. Les perspectives sont nombreuses, en particulier celles de comprendre la place des BALOs au sein des réseaux trophiques, leurs rôles et effets sur les dynamiques et stabilité des écosystèmes microbiens. Les questions auxquelles il faudrait s'atteler à l'avenir pourraient être les suivantes :

1) Quel est l'impact de la prédation des BALOs sur la structure et la composition des communautés bactériennes ?
2) Comment, quantitativement et qualitativement, les BALOs contribuent-ils directement (par prédation) et indirectement (par exemple en décomposant les flocs/biofilms) à la mortalité bactérienne et à la libération de nutriments, c'est-à-dire au turnover bactérien ?
3) Quelles relations entretiennent-ils avec les autres micro-prédateurs (comme d'autres bactéries prédatrices comme les Myxobacteria), les phages et les protistes ?

La réponse à ces questions ne pourra être obtenue que par l'utilisation d'approches complémentaires, expérimentales et systémiques, dans des conditions contrôlées de laboratoire et *in situ* (le Léman étant privilégié car constituant aujourd'hui le lac le plus riche en informations suite à C-BALO). Pour déterminer le rôle et l'impact des BALOs comme agents de mortalité cellulaire, leur importance sur les flux de nutriments, un effort devra être continué puis mené sur la quantification et l'identification précise des prédateurs ainsi que sur les dynamiques d'interactions proie-prédateur. Cela sera effectué en continuant de combiner le séquençage à haut débit et la qPCR (ddPCR pour *Bacteriovorax*), mais sur des échelles de temps courtes, afin de suivre et d'identifier les prédateurs et leur dynamique de manière précise. L'approche computationnelle en corrélant statistiquement les fluctuations/co-occurrences en termes d'abondance des populations Gram- à celles des populations de BALOs sera aussi poursuivie.

D'autres méthodologies pourraient être mises en œuvre. La technique FISH a déjà été appliquée au genre *Bdellovibrio*, via le développement d'une sonde oligonucléotidique. Seulement deux études ont été publiées avec cette approche (Mahmoud et al. 2007 & Szabo et al. 2017) ; elle pourrait être complétée et étendue à la cytométrie en flux et tri cellulaire actif. Les populations ainsi triées (single cell approach) seraient séquencées pour révéler la composition des populations inter réagissant, et donc des relations proie-prédateur. Une autre approche appelée EPIC-PCR (Emulsion, Paired Isolation and Concatenation – PCR), permettant d'isoler des cellules pour une caractérisation génomique facilitée (Spencer et al. 2016), serait également intéressante à tester, car permettant de couvrir des interactions directes entre cellules. Enfin, le flux de nutriments entre des proies marquées et les prédateurs pour identifier les prédateurs actifs pourrait être révélé par la technique SIP (stable isotope probing) déjà utilisée par Chauhan et al. (2009a) et Dolinšek et al. (2013) pour les BALOs.

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Annexes

Annexe 1 : Article de vulgarisation autour des *Bdellovibrio* et organismes apparentés

Annexe 2 : Bande déssiné autour des *Bdellovibrio* et organismes apparentés

Annexe 3 : Un article scientifique qui étudie la diversité et l'intéraction des bactéries et archées du Léman (projet TRANSLEM)

Annexe 1

Objectif et résumé

Un travail de vulgarisation a été conduit via l'écriture d'un article de synthèse en collaboration avec la revue scientifique française *Pour la Science*. Notre objectif a été de faire connaitre les BALOs à un large public, scientifique et personnes non spécialistes.

Ces bactéries qui en dévorent d'autres

L'ESSENTIEL

> Les Balo sont de petites bactéries qui se nourrissent exclusivement d'autres bactéries.

> Contrairement à d'autres bactéries prédatrices, elles chassent en solitaire.

> Depuis quelques années, on s'aperçoit que les Balo sont présents partout où on les cherche et que ce sont sûrement de puissants régulateurs écologiques.

> Leurs applications potentielles sont multiples, notamment dans la lutte contre l'antibiorésistance.

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Ces bactéries qui en dévorent d'autres

1. Introduction

Au beau milieu du Léman, le plus grand lac naturel profond d'Europe occidentale, l'attaque a été foudroyante. Peu de chance d'en réchapper ! La victime est une bactérie aquatique, *Limnohabitans planktonicus*, dont la taille ne dépasse pas deux micromètres. Sa prédatrice… est aussi une bactérie, plus petite. Du genre *Peredibacter*, elle se délecte de ses semblables. La proie, qui se nourrissait de matière organique dissoute libérée par le phytoplancton alentour, n'a rien senti venir. Force est de constater que la loi de la jungle s'applique aussi au monde microbien.

Les bactéries aquatiques jouent un rôle fonctionnel clé au sein des écosystèmes, ce qui n'empêche pas qu'elles soient les proies de prédateurs ou de parasites très efficaces. Outre les bactériophages ou phages – des virus mangeurs de bactéries –, qui se sont révélés, au cours des trois dernières décennies, un facteur majeur de régulation de l'abondance des bactéries dans les milieux aquatiques et de leur diversité, d'autres prédateurs, comme les organismes unicellulaires flagellés, les ciliés ou certains métazoaires appartenant au zooplancton (ou plancton animal), sont bien connus des biologistes. Les bactéries qui se nourrissent de leurs consoeurs le sont moins, mais depuis peu, on s'accorde à y voir un groupe qui compte aussi dans le contrôle des populations bactériennes. Certaines sont regroupées dans un groupe fonctionnel, les *Bdellovibrio* et organismes apparentés ou Balo (pour *Bdellovibrio and like organisms*).

Depuis quelques années, grâce aux nouvelles techniques de séquençage et de manipulation génétique, de bio-informatique et de microscopie, on commence à mieux connaître ces bactéries et leur impact écologique. Au point d'en faire de sérieux candidats dans la lutte antibactérienne en aquaculture, mais aussi en agriculture et en médecine.

2. Une découverte fortuite

En sciences, le hasard est parfois un facteur important d'innovation. La poussée d'Archimède, la radioactivité, la pénicilline, l'ADN et nombre d'autres avancées sont ainsi les fruits de découvertes fortuites. C'est aussi le cas des Balo. Un jour de 1962, l'année de la mort de Marilyn Monroe et de l'arrestation de Nelson Mandela, le microbiologiste allemand Heinz Stolp menait une expérience à l'Institut de bactériologie de Berlin pour isoler des bactériophages spécifiques des bactéries *Pseudomonas syringae* pv. *phaseolicola*, responsables de la graisse du haricot. Durant ce travail, Stolp, à court des filtres qu'il utilisait pour obtenir des échantillons non contaminés par des bactéries, décida de les remplacer par des filtres en verre fritté, bien qu'ils soient moins sélectifs et susceptibles de laisser passer des cellules de taille supérieure à celle de la maille de ses filtres habituels (0,2 micromètre).

Pour détecter des bactériophages, une méthode consiste à observer sur boîte de culture ce que l'on appelle des « plages de lyse », c'est-à-dire des trous qui se forment rapidement au sein des colonies bactériennes en réponse au succès de l'infection virale des bactéries. Stolp a donc laissé incuber ses boîtes de bactéries avec ses échantillons en espérant y observer le lendemain des plages de lyse. Cependant, ce ne fut pas le cas, aucun trou n'était visible. Naturellement, Stolp aurait dû se débarrasser de ces boîtes, mais il les conserva deux jours supplémentaires. Or, contre toute attente, des plages de lyse tardives apparurent ! Intrigué, Stolp examina ces trous au microscope. Et ce qu'il vit fut une incroyable surprise : des bactéries se déplaçant à vive allure, s'attachant parfois à certaines cellules… jusqu'à les détruire. Des bactéries prédatrices de bactéries !

En 1963, après avoir isolé et étudié ces microorganismes, Stolp et son collaborateur Mortimer Starr, de l'université de Californie à Davis, les ont nommées *Bdellovibrio*, du grec *Bdella* signifiant « sangsue » (l'attachement du Balo à sa proie et la succion du contenu cellulaire rappelant le mode d'action des sangsues) et *vibrio*, qui renvoie à la forme en virgule de ces microorganismes. Depuis le premier *Bdellovibrio* de Stolp, plusieurs équipes ont découvert divers autres Balo, les dotant en général d'un nom synonyme de « dévoreur ». Jusque dans les années 2000, cependant, ce domaine de recherche est resté assez confidentiel : entre 1972 et 1980, seules une vingtaine de publications sur les Balo ont vu le jour, puis le sujet a été délaissé. Mais à partir des années 2000, dix à vingt articles scientifiques sur les Balo ont paru chaque année, surtout sur *Bdellovibrio bacteriovorus*. L'année 2017 a comptabilisé à elle seule plus d'une trentaine de publications liées à leur étude. De fait, alors que l'on recherchait des alternatives à l'utilisation des antibiotiques, devenus inopérants face aux bactéries multirésistantes, on s'est aperçu que les Balo, comme les phages, avaient un grand potentiel antimicrobien.

3. Petites mais redoutables

Les Balo sont des bactéries dites « à Gram négatif », c'est-à-dire caractérisées par une enveloppe particulière à double membrane *(voir l'encadré page ci-contre)*. Équipés d'un flagelle lorsqu'ils ne sont pas attachés à leur proie, ils sont par ailleurs très mobiles. En effet, un tel prédateur est capable de se déplacer à 160 micromètres par seconde, ce qui n'est pas négligeable pour des organismes de 0,5 à 2,5 micromètres de longueur et de 0,2 à 1 micromètre de largeur, soit sensiblement plus petits que les bactéries *Escherichia coli*, longues d'environ 3 micromètres. Mais petit ne signifie pas inoffensif ! Bien au contraire, ces prédateurs redoutables s'attaquent à de nombreux hôtes quels que soient leur physiologie, leur taille ou leur pedigree, contrairement aux bactériophages, qui ont souvent un hôte bien spécifique. Ainsi, une grande proie ne les impressionne point et, d'ailleurs, certains Balo infectent plus rapidement les grandes cellules que les petites.

Découverts initialement dans le sol, puis dans divers milieux aquatiques, les Balo semblent être partout où on les cherche : océans, mers, eaux côtières, estuaires, rivières, lacs, bassins d'aquaculture, stations d'épuration, eau d'irrigation, sols variés, rizières, rhizosphère et même fèces animales. De fait, si, en nombre, ils sont loin de dominer leur milieu de vie, ils n'en restent pas moins suffisamment nombreux pour être aisément détectables par les techniques routinières de biologie moléculaire.

Au premier abord, on pourrait voir dans les Balo des parasitoïdes – des organismes qui se développent dans ou sur un autre organisme et finissent par le tuer. Mais les spécialistes de ce groupe les définissent plutôt comme des prédateurs, car comme ces derniers, un Balo induit rapidement la mort de sa proie (généralement dans les quinze minutes suivant l'attaque), bien avant le développement de sa progéniture. Mais, à l'inverse des grands prédateurs, la majorité des Balo ne consomment pas leur victime à la vue de tous. Si certains restent à l'extérieur de leur proie (prédation épibiotique), la plupart l'utilisent comme un refuge temporaire, un habitat osmotiquement stable et regorgeant de nutriments (prédation endobiotique). Ce faisant, les Balo se protègent des variations des conditions environnementales et des attaques des bactériophages. Une fois qu'ils se sont multipliés dans l'hôte, leur progéniture détruit sa paroi et se retrouve dans le milieu environnant, prête à entamer son cycle *via* la prédation d'un nouvel hôte *(voir l'encadré pages 66 et 67).*

4. Des bactéries à Gram-négatif

Les Balo sont des bactéries à Gram négatif et se nourrissent… de bactéries à Gram négatif. Pourquoi ? La réponse n'est pas claire. La distinction entre bactéries à Gram positif et négatif ne constitue ni un critère fonctionnel ni un critère de classification, mais provient de la coloration dite de Gram. Du nom de son inventeur, le bactériologiste danois Hans Christian Gram (1853-1938), ce protocole de coloration des membranes bactériennes fait apparaître les bactéries à Gram négatif en rose et celles à Gram positif en violet. Aujourd'hui, on sait que ces couleurs correspondent à deux structures distinctes de l'enveloppe bactérienne et que certains antibiotiques, par exemple, fonctionnent mieux sur telle ou telle structure. Celle des bactéries à Gram négatif *(ci-contre en haut)* està double membrane organisée en trois parties. En périphérie, la membrane externe est composée de phospholipides et de protéines intrinsèques, notamment de transport. Les bactériesà Gram positif *(ci-contre en bas)* en sont dépourvues. Puis, un espace dit périplasmique comportant la paroi – une couche de peptidoglycanes relativement mince, contrairement à celle des bactéries à Gram positif – sert notamment de stockage d'enzymes et de nutriments. Enfin, la membrane

plasmique, commune aux deux types de bactéries, est très semblable à la membrane externe, mais contient de nombreux autres complexes protéiques d'une importance vitale pourle métabolisme.

Pour expliquer pourquoi les Balo se nourrissent préférentiellement de bactéries à Gram négatif, une hypothèse est que les Balo reconnaissent des composants de la membrane externe, voire du périplasme. Il a en effet été suggéré qu'au contact d'une proie potentielle, des biopolymères du prédateur reconnaîtraient certains sucres ramifiés (des oligosaccharides) à la surface de la paroi bactérienne, présents uniquement chez les bactéries à Gram négatif. Toutefois, on a aussi proposé que, dans certaines conditions de laboratoire, en présence uniquement de bactéries à Gram positif, certains Balo se nourrissent de ces dernières. Le prédateur changerait alors de mode de prédation. Au lieu de consommer la proie enla pénétrant, il la consommerait de l'extérieur, grâce à la production rapide de nouvelles enzymes adaptées à la paroi de ces proies. Néanmoins, ce scénario n'a pas encore été observé.

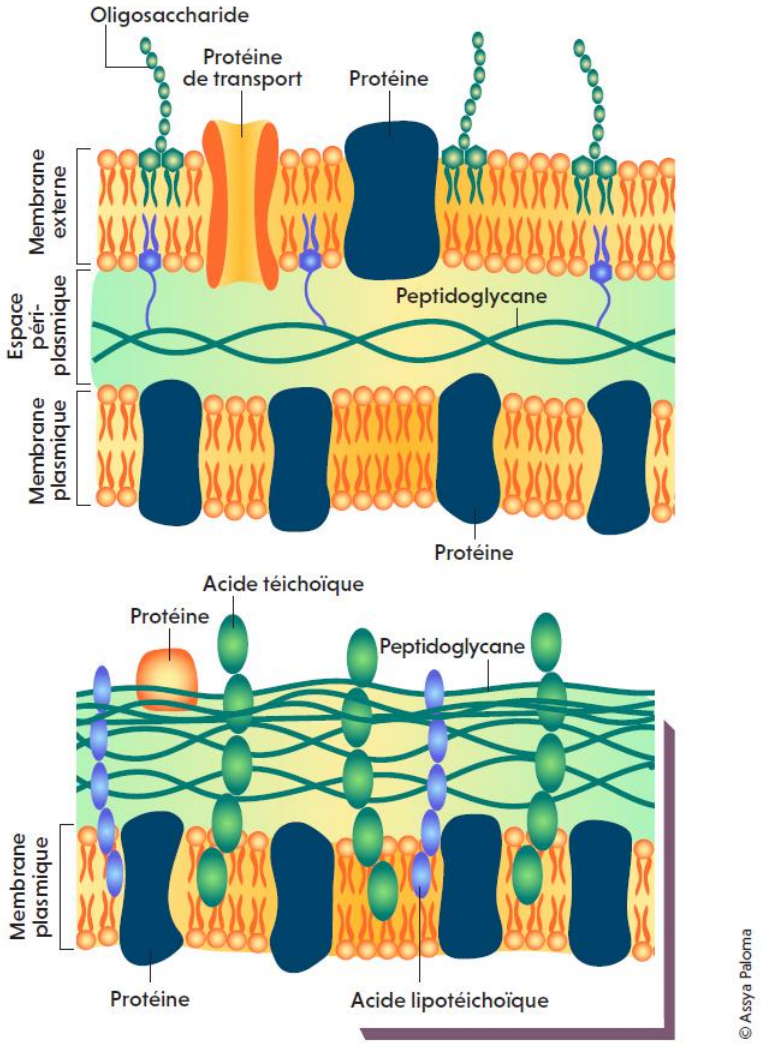

Figure 1 : Les membranes des bactéries Gram-négative et positive

5. Une chasse en solitaire

Dans l'arbre du vivant, les Balo ne constituent pas un groupe monophylétique : ils n'ont pas d'ancêtre commun à eux seuls *(voir l'encadré page 68)*. Ce qui fait qu'un Balo est un Balo n'est donc pas tant lié à sa signature génétique qu'à son mode de fonctionnement. De fait, la morphologie et les stratégies de prédation uniques des Balo en font un groupe à part des autres bactéries prédatrices. D'abord, ce sont des prédateurs « obligatoires » : ils sont incapables de vivre dans des milieux dépourvus de proie. Cela dit, en laboratoire, diverses équipes ont observé qu'en l'absence de proie, mais placés dans un milieu très riche en nutriments, certains types de Balo étaient capables de se nourrir du milieu nutritif.

Ensuite, la stratégie de prédation et de croissance des Balo – individuelle et en mode endobiotique ou épibiotique – est une marque bien caractéristique. Le cycle endobiotique consiste en une appropriation complète de la proie. En effet, le Balo infecte son hôte et, dans son élan, empêche toute autre infection de celui-ci par d'autres Balo *via* l'émission d'un signal d'occupation (une molécule dédiée). En laboratoire, un cas de multi-infection a quand même été observé avec un nombre de Balo largement supérieur à celui des proies, mais cela reste anecdotique. Le cycle épibiotique, de son côté, est plus « partageur » : plusieurs Balo peuvent s'attacher à la paroi d'une proie et consommer simultanément son contenu. Les autres prédateurs, comme *Myxobacteria* ou *Lysobacter*, se différencient par une prédation facultative – ils ne s'attaquent aux autres bactéries que lorsque les ressources en nutriments sont au plus bas – et par une stratégie de chasse en meute *(voir l'encadré page 69)*. Par ailleurs, les études de l'ADN des Balo les séparent clairement des autres prédateurs bactériens.

6. Un large choix de proies

En matière de proie, la majorité des études ont confirmé que les Balo préfèrent se nourrir de bactéries à Gram négatif. Et si on leur donne le choix, ils consomment d'abord celles qu'ils rencontrent habituellement dans leur milieu de vie. Leurs proies sont de toutes sortes, pathogènes ou commensales des plantes, des animaux ou des humains. Toutefois, il y a quelques années, une équipe de l'université Sapienza, à Rome, sous la direction de Serena Schippa, a observé un nouveau phénomène : le Balo *Bdellovibrio bacteriovorus* arrive à cibler et à consommer des bactéries à Gram positif. En effet, en présence de proies à Gram positif comme *Staphylococcus aureus*, *Bdellovibrio bacteriovorus*, prédateur naturellement endobiotique, finit par s'adapter à son nouveau repas. En une vingtaine d'heures, il change de stratégie et opte pour une prédation

épibiotique. Le temps nécessaire pour consommer *S. aureus* est comparable à une phase de latence durant laquelle le prédateur synthétise un nouvel arsenal d'enzymes hydrolytiques capables d'agir sur la paroi des bactéries à Gram positif. Plus surprenant encore, *Bdellovibrio bacteriovorus* est aussi capable de s'en prendre à des cellules mortes dont le contenu cellulaire est intact.

Le spectre de prédation des Balo varie selon les représentants. Certains sont semi-généralistes, avec un large choix de proies consommables, tandis que d'autres sont plutôt spécialistes d'un type de proie. Il arrive aussi que des Balo soient versatiles, c'est-à-dire à la fois semi-généralistes et spécialistes, optant pour un comportement ou l'autre selon l'environnement. Une stratégie gagnante ? Pas sûr. Certes, elle offre un avantage indéniable : la bactérie qui la pratique est moins limitée par la compétition et est donc apte à dominer la population de Balo. Cependant, l'efficacité de prédation est plus faible et un Balo spécialiste d'une proie la consomme naturellement plus vite qu'un versatile… Les populations de Balo varient donc en fonction des proies présentes dans le milieu.

Toutes ces caractéristiques confèrent aux Balo une grande facilité d'adaptation aux milieux qu'ils colonisent et expliquent sans doute pourquoi on en trouve dans tous les milieux naturels et anthropisés. Cette ubiquité et le contrôle qu'ils opèrent sur des populations bactériennes entières en font de puissants régulateurs écologiques. En milieu aquatique, notamment, leur impact sur les bactéries hétérotrophes (non photosynthétiques) ne doit plus être négligé.

7. Le cycle de vie des BALO

Les Balo ont développé deux cycles de vie différents. Certaines bactéries, comme *Peredibacter starrii* et *Bdellovibrio bacteriovorus*, ont un cycle de reproduction endobiotique, c'est-à-dire qui se déroule à l'intérieur de l'hôte *(voir page ci-contre le cycle de* Bdellovibrio bacteriovorus*)*, tandis que d'autres, comme *Bdellovibrio exovorus*, ont un cycle de reproduction épibiotique : le prédateur reste en dehors de sa proie *(ci-dessous le cyclede* B. exovorus*).* Les deux cycles durent entre trois heures et demie et quatre heures.

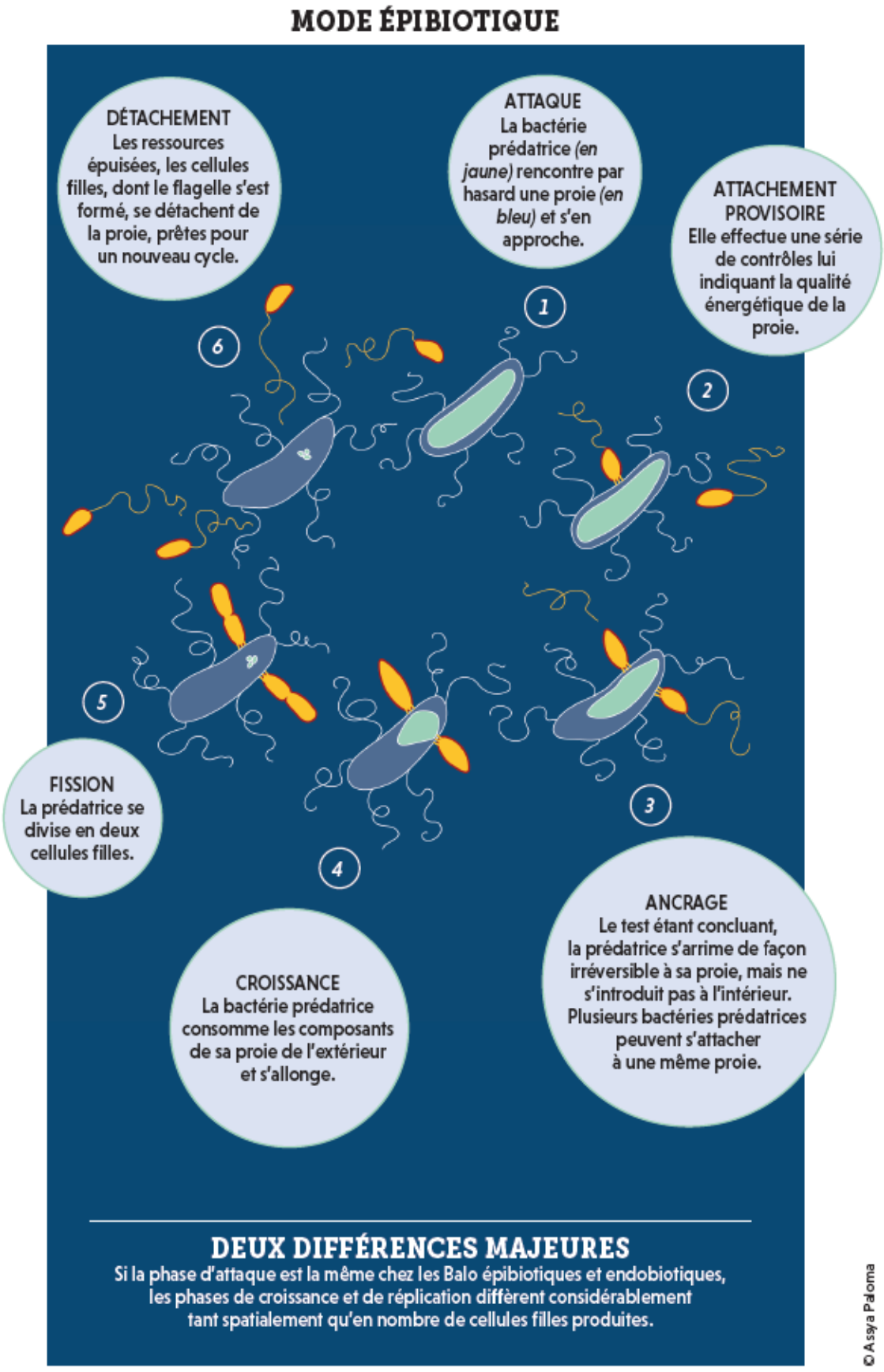

Figure 2 : Cycle épibiotique de certains BALO

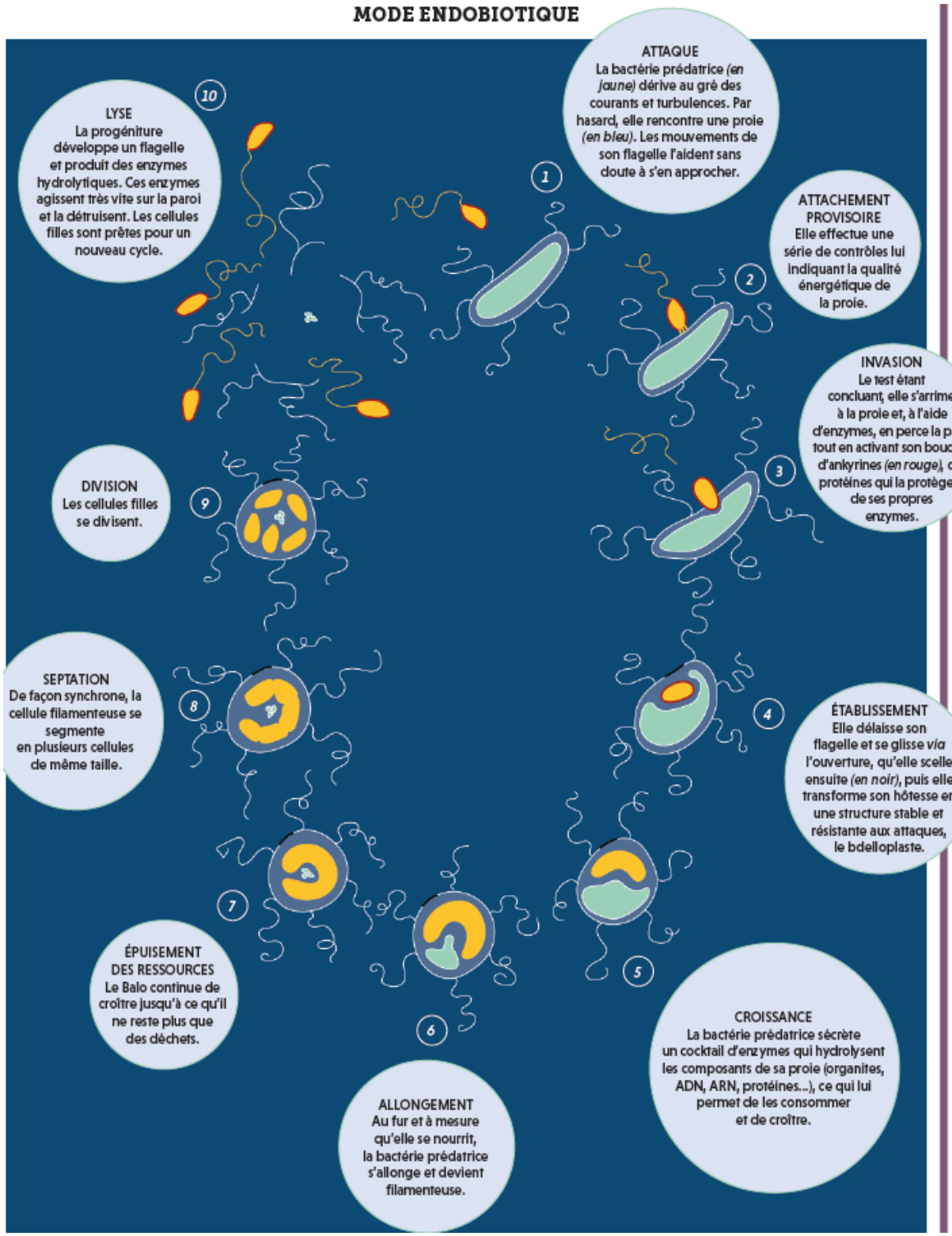

Figure 3 : Cycle endobiotique ou périplsmique des BALO

8. Une classification mouvante

La classification des bactéries évoluant sans cesse, celle des Balo a subi divers remaniements au cours de la dernière décennie. De nos jours, l'identification des bactéries et de leurs liens de parenté se fonde principalement sur l'étude du gène de l'ARNr 16S, une sous-unité du ribosome, le complexe qui synthétise les protéines dans les cellules. Cette sous-unité a en effet l'avantage d'être présente chez toutes les bactéries et de présenter une structure moléculaire très bien conservée dont l'évolution est très lente. On commence par extraire l'ADN des bactéries. Puis, des séquences d'ADN nommées amorces, reconnaissant des zones très conservées du gène, permettent d'amplifier par PCR (réaction de polymérisation en chaîne) une grande partie du gène, qui peut alors être séquencé. Les données sur sa séquence sont alors comparées avec des bases de données. Selon les auteurs, pour que deux bactéries appartiennent à la même « espèce », leur degré d'homologie doit être supérieur à 97 %, voire à 99 %.

Sur la base de telles analyses, on s'est aperçu que les Balo constituent un groupe paraphylétique : dans l'arbre du vivant, ils ne sont pas seuls sur leur branche. Leur dernier ancêtre commun est aussi celui d'autres bactéries. En effet, ils appartiennent à deux classes distinctes. La première, nommée Oligoflexia, qui comporte cinq familles de Balo composées d'une ou plusieurs « espèces » répertoriées *(voir ci-dessus)*, regroupe aussi de nombreuses autres bactéries *(non représentées)*. Par exemple, la famille des *Halobacteriovoraceae*, typique des environnements halophiles (salés), est actuellement constituée de deux représentants, *Halobacteriovorax marinus* et *H. litoralis*. La seconde classe, « sans famille », contient un genre unique du nom de *Micavibrio*, représenté par deux « espèces », *M. aeruginosavorus* et *M. admirantus (non montré)*.

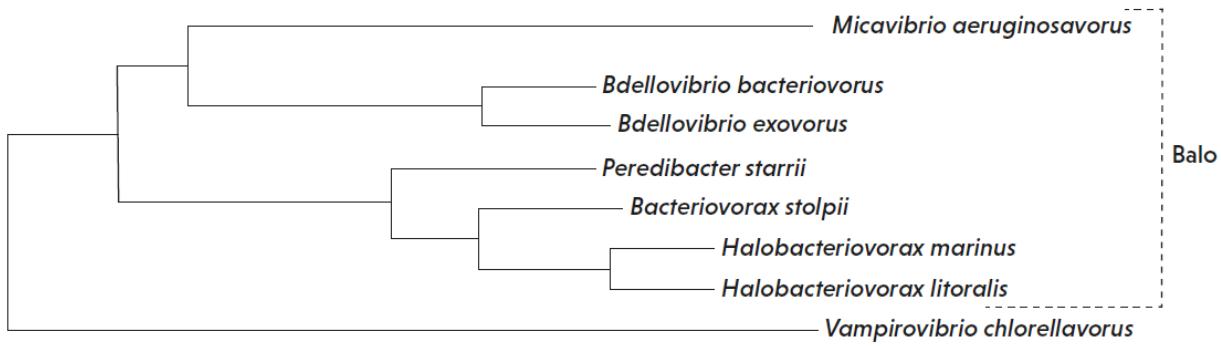

Figure 4 : Phylogénie des BALO

9. Des régulateurs non négligeables des écosystèmes

Les bactéries hétérotrophes jouent en effet un rôle central dans le réseau trophique de tout écosystème, c'est-à-dire dans le réseau des chaînes alimentaires qui y sont en jeu. Elles recyclent la matière organique dissoute dans le milieu, issue du phytoplancton, en la consommant. Puis elles deviennent le repas de protozoaires (animaux unicellulaires) – des flagellés et des ciliés –, qui transfèrent ainsi cette biomasse aux niveaux trophiques supérieurs.

Découverte dans les années 1970-1980 par Farooq Azam, de l'université de Californie de San Diego, et Tom Fenchel, de l'université de Copenhague, qui cherchaient alors à décrire le rôle des bactéries dans les cycles du carbone et des éléments nutritifs du milieu, cette « boucle microbienne » a une conséquence importante : toute altération qu'elle subit à des répercussions sur les niveaux trophiques supérieurs. Longtemps, on a pensé que les bactériophages et les protozoaires étaient les principaux acteurs de la mortalité bactérienne et du renouvellement de cette communauté au sein des écosystèmes aquatiques. De fait, des études antérieures suggéraient que les virus étaient l'entité la plus abondante sur Terre, certaines avançant qu'un millilitre d'eau océanique contenait de 10^5 à 10^9 particules virales. Cependant, en 2014, une découverte a changé la donne : Steven Biller, de l'institut de technologie du Massachusetts, aux États-Unis, et ses collègues ont montré que les vésicules membranaires extracellulaires – des vésicules de 50 à 200 nanomètres de diamètre que sécrètent les cellules des trois domaines du vivant (archées, bactéries et eucaryotes) – sont bien plus abondantes dans les écosystèmes marins qu'on ne le pensait *(voir l'encadré page 71)*. Or ces vésicules se confondent facilement au microscope avec les virus sans queue.

Depuis, le nombre et l'impact des virus sur les populations bactériennes sont donc revus un peu à la baisse. Et outre les bactériophages et les protistes, un nouvel acteur commence à prendre de l'ampleur : les bactéries prédatrices d'autres bactéries et, en particulier, les Balo. Certains chercheurs avancent l'hypothèse que, malgré leur infériorité en nombre par rapport aux bactériophages, ils seraient tout de même efficaces pour contrôler les populations bactériennes, voire plus efficaces encore dans certaines situations, les bactériophages ne pratiquant qu'une prédation spécifique.

Ces dernières années, avec la montée de la résistance des bactéries aux antibiotiques, les Balo, comme les bactériophages, sont ainsi devenus des candidats intéressants pour de nombreuses applications dans le contrôle de populations bactériennes, qu'il s'agisse de combattre des bactéries pathogènes des humains, des animaux ou des cultures.

10. Des vésicules sous-estimées

Dès les années 1960, on s'est aperçu que les bactéries et les cellules eucaryotes (c'est-à-dire comportant un noyau) sécrétaient des vésicules membranaires. Depuis, on sait que c'est aussi le cas des archées, le troisième domaine du vivant. Il s'agit donc d'un mécanisme très conservé au fil de l'évolution. Et beaucoup plus répandu qu'on ne le pensait, comme l'ont observé Steven Biller, de l'institut de technologie du Massachusetts, et ses collègues en 2014 en montrant que les cyanobactéries *Prochlorococcus* produisent d'étonnantes quantités de ces vésicules dans leur milieu naturel.

Il s'agit de petits compartiments clos formés à partir de membranes cellulaires, sécrétés dans le milieu extérieur ou intervenant dans le trafic intracellulaire. De formes, tailles, compositions et fonctions variées (défense contre certaines infections, protection antivirale, libération de facteurs de virulence, matériel de transport génétique, moyen de communication entre organismes, leurre, nourriture pour organismes unicellulaires, inhibiteur de croissance…), ces vésicules sont produites par une diversité de mécanismes. Dans leur environnement, elles sont capables d'interagir avec des cellules et même des virus aquatiques, avec lesquels il arrive qu'elles soient confondues. En effet, de taille comparable (entre 40 et 230 nanomètres), elles sont parfois présentes en nombre très élevé (supérieur à 10^6 vésicules par millilitre). Les virus aquatiques sont donc probablement un peu moins nombreux qu'on ne le pensait, et leur impact sur l'écosystème plus faible. Les bactéries prédatrices de bactéries y ont dès lors toute leur place.

11. Les autres bactéries prédatrices

Outre les Balo, on connaît une dizaine de bactéries prédatrices, appartenant à diverses branches de l'arbre phylogénétique des bactéries (Proteobacteria, Chloroflexi…). Le point commun à tous ces prédateurs bactériens est la capacité à dégrader les polymères de leurs proies. *Ensifer adhaerens*, par exemple, est une bactérie du sol qui, en temps normal, forme des nodules fixateurs d'azote sur les racines et les tiges de légumineuses. Mais lorsque les conditions nutritives deviennent limitantes, il lui arrive de présenter un comportement de prédation. C'est aussi le cas de *Cupriavidus necator*, une autre bactérie du sol qui devient prédatrice quand sa croissance est limitée faute de concentrations élevées en cuivre. Contrairement à ces dernières, *Lysobater* est un genre bactérien dont la prédation s'effectue en groupe, une stratégie dénommée « meute de loups ». Mais contrairement aux Balo, quand le contact est établi entre le prédateur et la proie, aucune structure d'attachement à la proie n'est observée ou définie. Chez *Myxobacteria*, la prédation s'opère aussi de manière groupée, par un déplacement de l'essaim jusqu'à la rencontre fortuite d'une proie. La

prédation se déroule en deux temps : d'abord, les cellules mères attaquent, piègent et affaiblissent la proie, puis les nouvelles recrues, produites principalement par division cellulaire, la contraignent dans un espace réduit et la dévorent. Quand les nutriments sont rares, cependant, les bactéries *Myxobacteria* s'agrègent en une structure latente de plusieurs dizaines de micromètres qui favorise leur survie *(ci-contre)*.

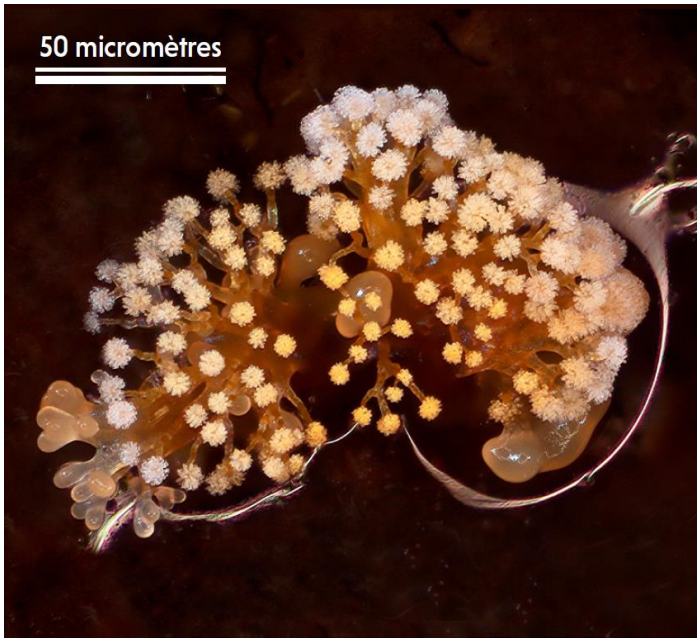

Figure 5 : Agrégation de myxobacteria

12. Une piste contre les bactéries résistantes

De fait, chez l'humain, les bactéries à Gram négatif sont responsables de plus de 30 % des infections contractées à l'hôpital (infections nosocomiales). Elles sont aussi associées à une morbidité et une mortalité souvent très élevées dans les unités de soins intensifs, car la plupart des patients concernés sont atteints de souches résistantes aux antibiotiques. En 2015, en Europe, le Centre européen de prévention et de contrôle des maladies a encore recensé près de 700 000 infections dues à des bactéries résistantes aux antibiotiques, lesquelles ont entraîné 33 000 décès. Et chez l'animal, si l'utilisation des antibiotiques a diminué dans certains pays européens depuis 2005 (dont la France), de grandes disparités subsistent entre les pays, d'après l'étude européenne JIACRA II. La situation est donc tout aussi préoccupante, d'autant que les résistances à certains antibiotiques comme les quinolones – traitements contre la salmonellose et la campylobactériose chez l'humain – sont associées à leur utilisation chez l'animal. Le recours à des solutions alternatives est donc une nécessité.

À ce jour, plusieurs expériences avec les Balo se sont déjà révélées prometteuses contre des maladies humaines et animales. La mucoviscidose, notamment, est une maladie génétique létale au cours de laquelle une colonisation bactérienne chronique des voies respiratoires inférieures déclenche et maintient un cercle vicieux d'inflammation et de destruction des tissus qui entraîne le déclin progressif de la fonction respiratoire. Or *Bdellovibrio bacteriovorus* a été détecté dans le microbiote des poumons de sujets sains, signe que ce Balo y survit et n'y est *a priori* pas toxique. En 2014, l'équipe de Serena Schippa a donc examiné si ce prédateur était capable de se nourrir des deux principales bactéries responsables de la colonisation des voies respiratoires dans la mucoviscidose, *Pseudomonas aeruginosa* (à Gram négatif) et *Staphylococcus aureus* (à Gram positif). Après avoir prélevé ces bactéries chez des patients atteints de la maladie, l'équipe les a mises en culture, puis a testé le Balo sur chacune. Après 24 heures, même si *B. bacteriovorus* avait marqué une légère préférence pour la bactérie à Gram négatif, elle avait réduit les biofilms de chaque bactérie de plus de 70 %. L'inoculation de *Bdellovibrio bacteriovorus* au début du stade de colonisation des poumons pourrait donc contribuer à contrôler cette colonisation chronique.

13. Des BALO contre la parodontite

Les Balo constituent aussi une piste pour lutter contre la parodontite, une maladie infectieuse polymicrobienne qui provoque l'inflammation des tissus de soutien de la dent (le parodonte). L'infection est due à de nombreuses bactéries pathogènes à Gram négatif, comme *Porphyromonas gingivalis* et *Aggregatibacter actinomycetemcomitans*, qui sont enchâssées dans un biofilm complexe, la plaque dentaire. Les traitements de la parodontite consistant à éradiquer le biofilm par des thérapies classiques comme l'utilisation d'antibiotiques se révèlent de plus en plus compliqués et souvent inefficaces, voire déconseillés. Mais en 2011, l'équipe de Wim Teughels, à l'université catholique de Louvain, en Belgique, a montré que diverses souches de Balo étaient capables d'attaquer, en culture, six sortes de bactéries pathogènes de la plaque dentaire et de réduire ainsi considérablement leur population, qu'elles soient cultivées seules ou ensemble, mélangées à des bactéries à Gram positif de la plaque ne faisant pas partie du menu des Balo. Toutefois, des études complémentaires sont nécessaires dans des conditions se rapprochant plus de l'environnement buccal, notamment parce que les bactéries pathogènes de la plaque dentaire sont anaérobies, tandis que les Balo ont besoin d'oxygène pour vivre. En 2019, Romeo Patini, de l'université catholique du Sacré-Coeur, à Rome, et ses collègues ont en effet montré qu'en conditions anaérobies, les Balo ne se développent pas suffisamment pour attaquer efficacement certaines de ces bactéries pathogènes.

Des expériences menées *in vivo* chez la larve du poisson-zèbre suggèrent par ailleurs que le Balo *Bdellovibrio bacteriovorus* pourrait aider à combattre une souche de la bactérie *Shigella flexneri* résistante à deux antibiotiques, la streptomycine et la carbénicilline. Les bactéries *Shigella,* à Gram négatif, font partie des agents pathogènes causant la dysenterie. Selon l'Organisation mondiale de la santé, elles sont responsables de plus d'un million de décès chaque année dans le monde, principalement dans les pays en développement. En 2016, Alexandra Willis, de l'Imperial College London, et ses collègues ont montré qu'en injectant *B. bacteriovorus* dans le rhombencéphale (la partie postérieure du cerveau) de larves de poissons-zèbres infectées par *Shigella flexneri*, les Balo augmentaient de 35 % leur taux de survie, agissant conjointement avec le système immunitaire des larves avant que celui-ci n'élimine *B. bacteriovorus* à son tour. Le système immunitaire du poisson-zèbre présentant de grandes similarités avec celui de l'humain, cette stratégie fonctionnerait-elle aussi chez ce dernier ?

14. Protéger les élevages de crevettes

Outre les humains, l'aquaculture et l'élevage sont tout aussi concernés par l'essor des bactéries antibiorésistantes. Chez les crevettes à pattes blanches *(Penaeus vannamei)* notamment, élevées en eau douce dans divers pays d'Amérique et d'Asie, plusieurs bactéries du genre *Vibrio,* comme *V. parahaemolyticus* et *V. cholerae* (le bacille du choléra), provoquent des épidémies généralisées et sont responsables d'une perte atteignant parfois 90 % de l'élevage, l'infection n'étant pas toujours contrôlable par des antibiotiques. Or en Chine, notamment, l'aquaculture produit annuellement 300 000 tonnes de ces crevettes à destination alimentaire.

Dans ce cas encore, les Balo offrent une piste intéressante. En 2015, Haipeng Cao, de la Shanghai Ocean University, et ses collègues, sont parvenus à éradiquer dix souches pathogènes différentes de *Vibrio* en culture en les incubant avec le Balo *Bdellovibrio bacteriovorus*. De plus, le prédateur a indéniablement pour les crevettes un effet protecteur contre les infections à *Vibrio* : en reproduisant l'expérience *in vivo,* les chercheurs ont observé que le taux de survie des crevettes augmentait de plus de 60 %.

Il semble par ailleurs que les bactéries prédatrices ne soient pas nocives pour les cultures cellulaires humaines ou animales, aucune maladie n'ayant été associée ou attribuée à une infection par des Balo. Contrairement à ce que l'on observe avec d'autres bactéries ou des bactériophages employés en thérapie phagique, ils ne déclenchent qu'une faible réaction inflammatoire et, à ce jour, aucune résistance permanente n'a été décelée chez leurs proies.

Les Balo sont encore peu étudiés malgré le grand potentiel qu'ils recèlent et le nouvel engouement qu'ils suscitent. On est encore loin des 6 990 articles que le portail Pubmed a référencés sur les bactéries *Staphylococcus* pour la seule année 2018 ! Néanmoins, il ne fait plus aucun doute que ces bactéries prédatrices gagneront en importance dans un futur proche, tant leurs applications potentielles sont nombreuses *(voir la figure page ci-contre)*. Il reste cependant encore beaucoup à comprendre le rôle des Balo dans les milieux naturels, qui n'ont encore jamais été étudiés de façon globale.

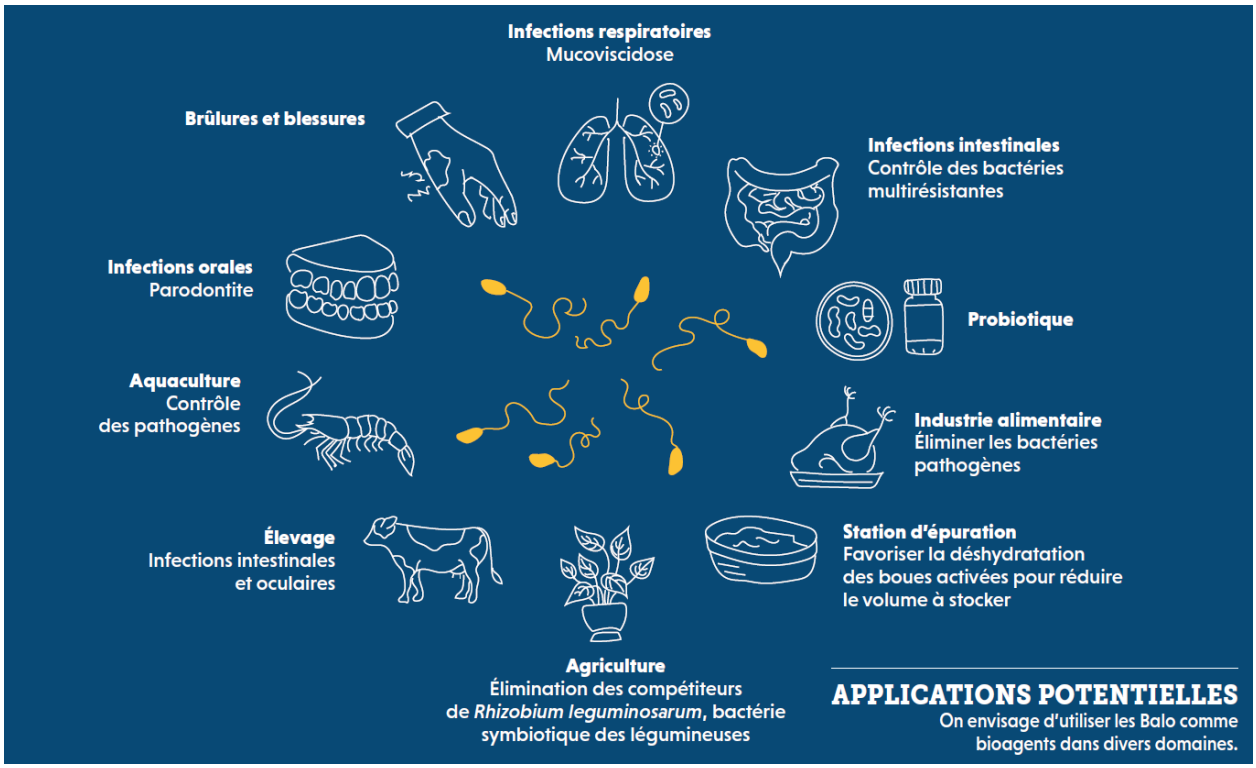

Figure 6 : Applications diverses des BALO en médecine, vétérinaire, aquaculture etc.

15. Plein de BALO dans les lacs périalpins

Aujourd'hui, les nouvelles techniques de séquençage à haut débit et de la bio-informatique offrent la possibilité de décrypter la diversité de ces bactéries et leurs interactions avec l'environnement. Fin 2017, nous avons ainsi lancé un projet dans ce sens, visant à étudier la diversité, la structure, l'abondance et les rôles des Balo dans divers écosystèmes aquatiques. Nos premiers résultats sont déjà très prometteurs. Nous avons découvert que certains prédateurs bactériens étaient parfois très abondants dans les grands lacs périalpins. La famille des Peredibacteraceae – en particulier la bactérie *Peredibacter starrii*, majoritaire –représentait parfois jusqu'à 7 % de l'abondance totale

des bactéries, tandis que les deux autres familles analysées, les Bdellovibrionaceae et les Bacteriovoracaceae, restaient très peu représentées. Ces trois familles occupaient d'ailleurs des zones différentes de la colonne d'eau, les Peredibacteraceae dominant près de la surface, tandis que les Bdellovibrionaceae et les Bacteriovoracaceae étaient plus abondantes en profondeur. Stratégies de vie différentes ? Niches écologiques distinctes ? L'avenir nous le dira. Une chose est sûre, la prochaine fois que vous vous baignerez dans le lac Léman, vous ne le regarderez pas de la même façon.

16. Bibliographie

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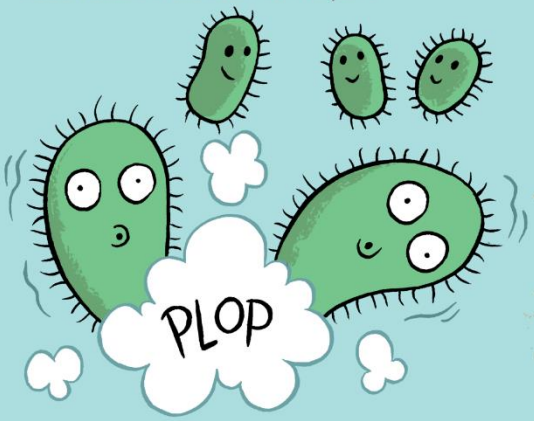
Annexe 2

Objectif

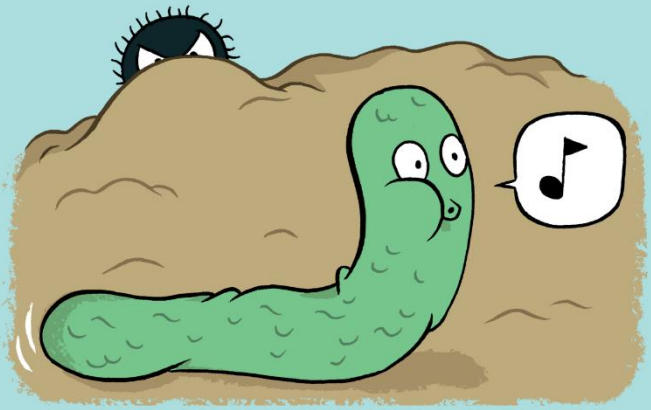
Quoi de mieux qu'une petite bande dessinée humoristique pour faire connaitre le monde microbien et ici des bactéries largement méconnues ? Nous avons demandé à deux artistes, Peb et Fox, aguéris à la vulgarisation des sciences, de nous aider à réaliser ce projet. Nous avons proposé le scenario et avons par la suite travaillé à la mise en images. Ci-dessous, le projet dans sa dernière version.

Bande dessinée

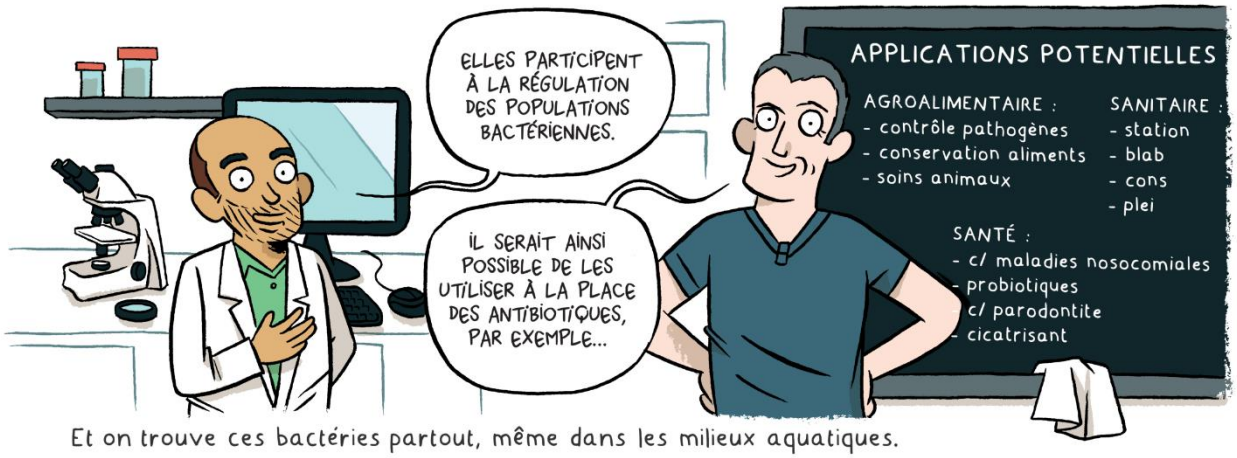
Pour se reproduire, les bactéries ont pour habitude de se diviser en deux nouveaux individus identiques.



Chez certaines bactéries, cette reproduction ne peut se faire qu'en se nourrissant de la force vitale d'une autre bactérie.

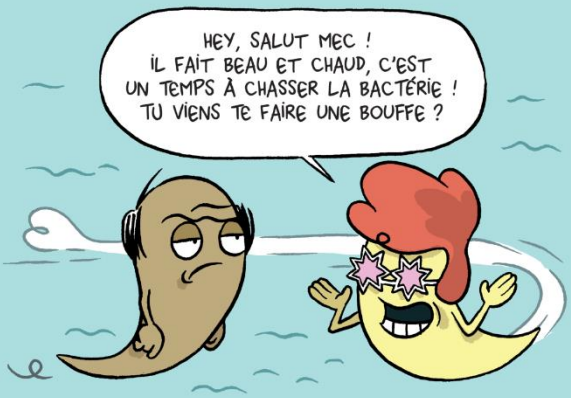


Ces bactéries, qui en mangent donc d'autres, sont dites prédatrices. Certaines sont même des prédateurs obligatoires, car, sans proies, elles ne peuvent survivre. Cette particularité fait qu'il pourrait être intéressant de les utiliser comme agents antimicrobiens dans divers domaines.



Et on trouve ces bactéries partout, même dans les milieux aquatiques.

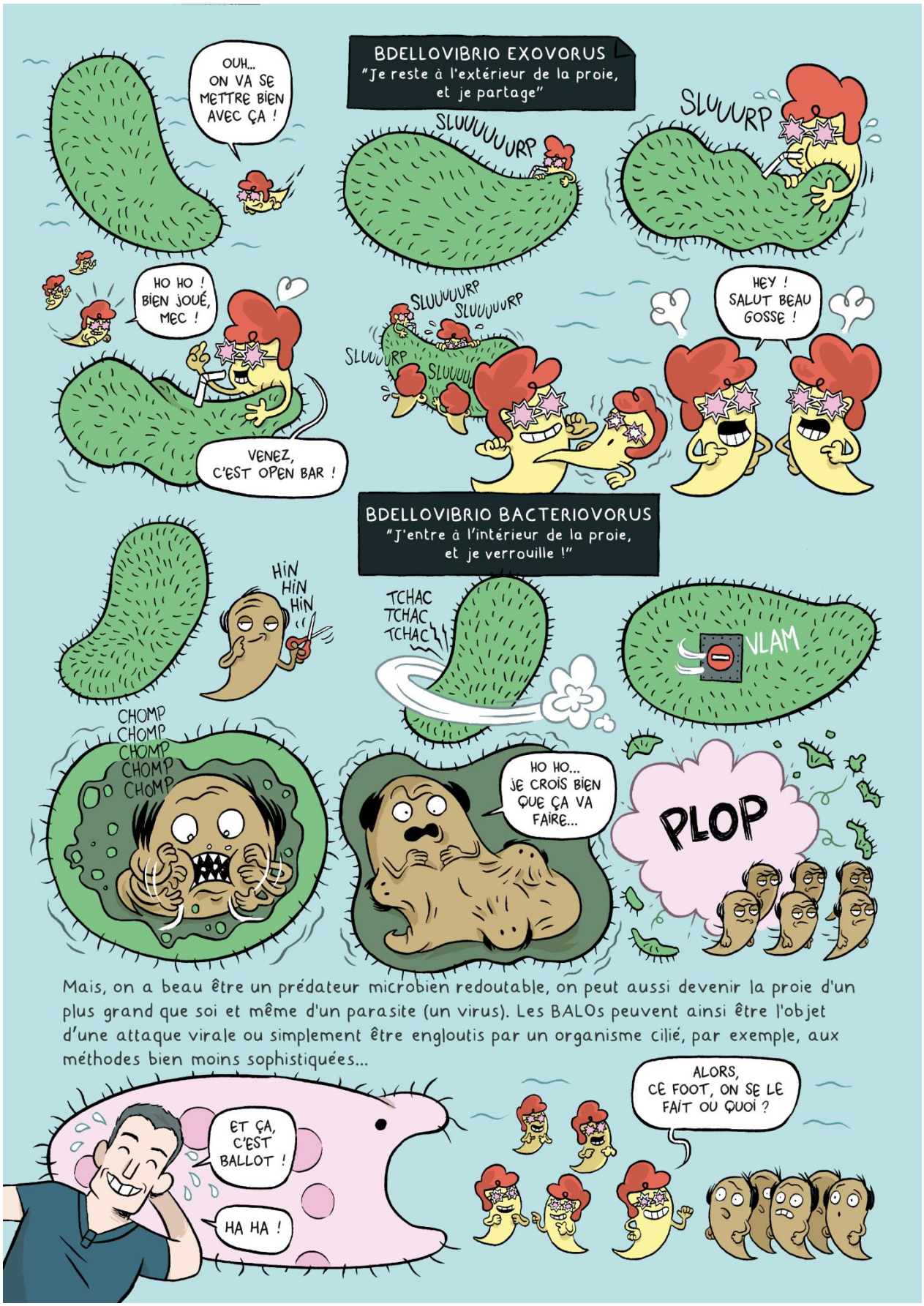
Les bactéries prédatrices obligatoires, bien que plus petites que leurs proies, sont très rapides, ce qui fait d'elles des chasseurs microbiens redoutables.



On les appelle BALOs (Bdellovibrio et organismes apparentés).

Cependant, elles n'ont pas toutes le même art de vivre !

Ainsi, chacune a son propre mode de reproduction, se nourrit et se reproduit d'une manière qui lui est propre...

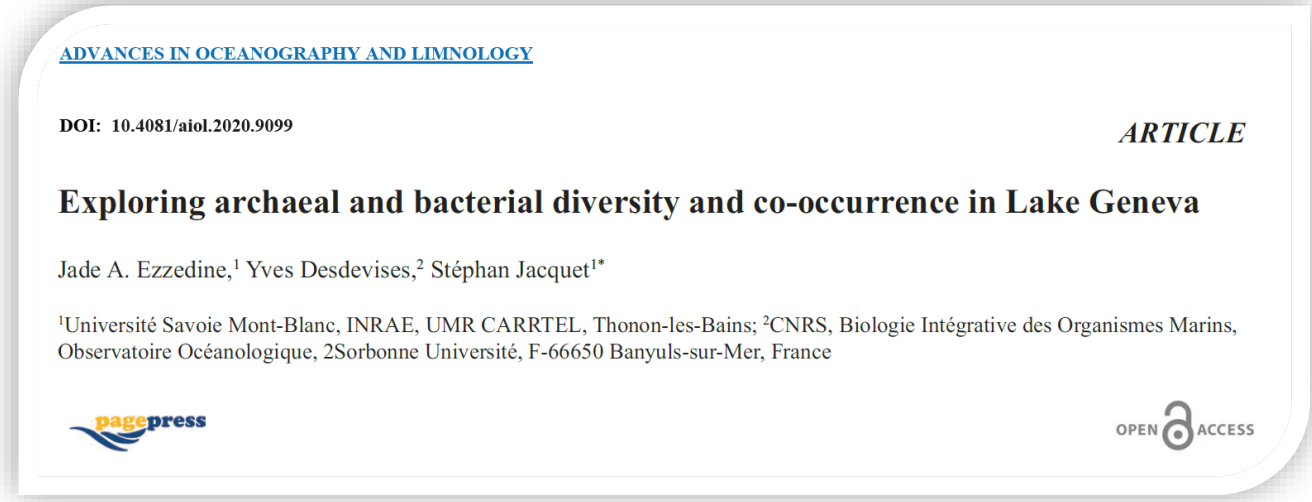

OUH... ON VA SE METTRE BIEN AVEC ÇA !
BDELLOVIBRIO EXOVORUS
"Je reste à l'extérieur de la proie, et je partage"
SLUUUUURP
SLUUURP
HO HO ! BIEN JOUÉ, MEC !
VENEZ, C'EST OPEN BAR !
SLUUUURP
SLUUUURP
SLUUURP
SLUUUU
HEY ! SALUT BEAU GOSSE !
BDELLOVIBRIO BACTERIOVORUS
"J'entre à l'intérieur de la proie, et je verrouille !"
HIN HIN HIN
TCHAC TCHAC TCHAC
VLAM
CHOMP CHOMP CHOMP CHOMP CHOMP
HO HO... JE CROIS BIEN QUE ÇA VA FAIRE...
PLOP
Mais, on a beau être un prédateur microbien redoutable, on peut aussi devenir la proie d'un plus grand que soi et même d'un parasite (un virus). Les BALOs peuvent ainsi être l'objet d'une attaque virale ou simplement être engloutis par un organisme cilié, par exemple, aux méthodes bien moins sophistiquées...
ET ÇA, C'EST BALLOT !
HA HA !
ALORS, CE FOOT, ON SE LE FAIT OU QUOI ?

Annexe 3

Objectifs et résumé

Cet article a été fait dans la continuité de l'analyse du projet TRANSLEM décrit dans l'article 4, mais en s'intéressant plus spécifiquement aux interactions possibles entre bactéries et archées. Le jeu de données généré par séquençage massif (Illumina Hiseq) a permis de décrire pour la première fois la diversité des bactéries et archées dans le Léman, ainsi que leurs interactions possibles, un aspect jusqu'alors jamais examiné. Nos résultats ont révélé que les bactéries sont diversifiées et que les phylums des Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Plantomycetes et Proteobacteria dominent en termes de reads et OTUs. A l'inverse, les archées étaient faiblement représentées dans le jeu de donnée (9% des reads) et beaucoup moins diversifiées. Ainsi, les Thaumarchaeota représentaient 97,4% du total des reads des archées. Bien qu'elles soient moins diversifiées que les bactéries, les archées dominantes attribuées aux phylums des Thaumarchaeota et Nanoarchaeota ont montré des liens forts avec les bactéries nitrifiantes et d'autres bactéries (comme suggérés par les réseaux de co-occurrence et les prévisions de profils de fonction). *In fine*, nous avons proposé que les OTU d'archées soient très probablement impliquées dans les cycles de l'azote et du méthane, formant un consortium avec d'autres bactéries qui exploitent également ces élements et les transforment dans les couches profondes du lac. Ces associations syntrophiques ou mutualistes probables suggéraient que les archées dominantes partagent avec certaines bactéries une niche commune pour des bénéfices mutuels.

Exploring archaeal and bacterial diversity and co-occurrence in Lake Geneva



ADVANCES IN OCEANOGRAPHY AND LIMNOLOGY

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ARTICLE

Exploring archaeal and bacterial diversity and co-occurrence in Lake Geneva

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1. Introduction

In aquatic ecosystems, microorganisms play a variety of important roles at the foundation of the microbial food webs as prey, predators or parasites, but also as actors in decomposing organic matter and (re)cycling nutrients. Among such microorganisms, archaeal contribution to the microbial loop and the interactions involved between archaea and other prokaryotes have been partially examined so that many pieces of information still remain to be explored and discovered (Braga *et al.*, 2016; Parada and Fuhrman, 2017; Seyler *et al.*, 2019). For a long time, archaea were believed to only inhabit extreme environments. High temperature, salinity and extreme anaerobiosis conditions were thought to be obligate prerequisite for archaeal growth. Therefore, possible links between archaea and other prokaryotes, typically bacteria, were not examined because not expected in such habitats where bacterial growth is improbable or even not possible (Seyler *et al.*, 2019). For the last two decades, however, it has been shown that archaea can be found everywhere (Fuhrman and Davis, 1997; Robertson *et al.*, 2005) including pelagic and surface freshwater ecosystems (Hugoni *et al.*, 2013; Casamayor, 2017). Along the last two decades, archaea were even reported to be as abundant as bacteria in marine sediments (Wurzbacher *et al.*, 2017) or dominant in deep ocean prokaryotic communities (Varela *et al.*, 2008). Archaea, like bacteria, are important players in biogeochemical cycles, *i.e.* the carbon and nitrogen cycles. Moreover, they are the only life forms to operate some fundamental processes such as methanogenesis (Cavicchioli, 2011) which is a very important function of anaerobic lake waters and sediments (Koizumi *et al.*, 2004; Bomberg *et al.*, 2008). Archaea are also known to oxidize ammonia (Könneke *et al.*, 2005), degrade ammonium urea and, like bacteria, to operate the nitrification process (Alonso-Sáez *et al.*, 2012).

Operating comparable or same functions may logically generate a competition between archaea and bacteria. Therefore, a variety of factors including nutrient availability is likely to structure differently these communities (Berdjeb *et al.*, 2011, 2013). This does not exclude that they can coexist in a non-limiting environment for growth with different responses to resource availability and/or predation pressure by viruses or planktonic grazers (Seyler *et al.*, 2019). It is noteworthy, however, that archaeal growth seems to be favored over bacteria in oligotrophic environments (Vuillemin *et al.*, 2019). Still today, the relationships between archaea and bacteria remain largely unknown. In the microbial realm, we can find relationships such as mutualism, amensalism, competition or elimination for example through antibiotics secretion. Therefore, the outcomes of such relations can be described as positive, neutral or negative (Pacheco and Segrè, 2019). For instance, we can cite the parasitic life style for Pacearchaeota and Woesearchaeota with bacteria (Ortiz-Alvarez and Casamayor, 2016). Another contact dependence has also been reported for two

archaea, where *Nanoarchaeum equitans* directly depends on *Igniococcus hospitalis* for its survival (Hu *et al.*, 2018). This could be explained by the fact that some archaea have a compact genome (Koonin and Wolf, 2008) and limited metabolic capabilities (Ortiz-Alvarez and Casamayor, 2016) so that they could need a partnership to compensate. Another example is provided by some nitrogen-fixing archaea, designated as a single entity which is known to engage an interaction with the bacterial genus *Desulfosarcina* (Dekas *et al.*, 2009).

The aim of this study was to assess, for the first time using high throughput sequencing, the diversity of both bacterial and archaeal assemblages in Lake Geneva and to investigate the potential relationships between these two communities using co-occurrence network analysis and functional profile prediction. We tested the hypothesis that archaea and bacteria may share similar niches and display significant links for a variety of ecological or biogeochemical functions.

2. Methods

2.1. Study site

Lake Geneva is a deep and large warm monomictic lake (surface area: 580 km^2; volume: 89 km^3; maximum depth: 309.7 m; mean depth: 152.7 m), located in the western part of the Alps at an altitude of 372 m. Lake Geneva has been monitored since 1974 as a part of a long-term water quality and biological monitoring program. Sampling has been continuously undertaken in the middle of the lake at the deepest point, referred to as SHL2, once or twice a month. This scientific survey revealed that the lake has switched from an oligotrophic to eutrophic state with annual phosphorus concentrations reaching 90 µg L^{-1} in 1979 (Anneville *et al.*, 2002). Thanks to effective management measures, Lake Geneva turned back to a mesotrophic state in the early 2000s with total phosphorus concentrations about 20 µg L^{-1} in 2010 (Jacquet *et al.*, 2014).

2.2. Sampling strategy

During the TRANSLEM project, we collected water samples at three sites, including the reference station SHL2. These sites referred to as pt2, pt4 (SHL2) and pt6 (Supplementary Fig. S1), separated from each other by approximately 14 km, were sampled at four different dates and seasons: February 20 (TL1), June 4 (TL2), August 7 (TL3) and November 20 (TL4) 2014, and at three different depths, *i.e.* 2, 15 and 200 m. It is noteworthy that pt2 could not be sampled on June 4 due to bad weather conditions. A filtration system was set on the boat and a volume of 300 mL of each water sample was filtered through 5 (to select all prokaryotes and avoid clogging of the 2-µm filter), 2 and 0.2 µm polycarbonate 47 mm filters (Millipore). Samples were then kept at -20°C until DNA extraction.

Descriptors such as temperature, pH, conductivity, chlorophyll *a* and dissolved oxygen concentrations of the water column were measured using a multiparametric probe (Sea & Sun technology GMBH). Transparency was measured using a normalized 25 cm diameter Secchi disk. Total organic carbon (TOC), total nitrogen (TN), dissolved ammonium (NH_4-N), dissolved nitrates (NO3-N), total phosphorus (TP) and orthophosphates (PO_4-P) were measured only at pt4, at the different depths and dates, according to the standard French protocols AFNOR.

2.3. DNA extraction

The filters were subjected to DNA extraction using a homemade protocol with GenEluteTM-LPA (Sigma-Aldrich) solution. The protocol started with a lysis step in Eppendorf tubes by adding 300 µL of TE buffer (TRIS 1M – pH 8, EDTA 0.5M – pH 8) and 200 µL of a lysis solution (TRIS 1M – pH 8, EDTA 0.5 M – pH 8 and sucrose 0.7 M). Next, a thermic shock was carried out by first placing the tubes at -80°C for 15 min and then thawed into a block heater at 55°C for 2 min. After, 50 µL of a 10% sodium dodecyl sulfate (SDS) and 10 µL of proteinase K (20 mg mL^{-1}) were added to the solution. The solution was incubated at 37°C for 1 h with gentle stirring and placed again in the block heater at 55°C for 20 min. After a quick centrifugation step (13,000 rpm at 4°C for 3 min), the supernatant was collected. Then, 50 µL of sodium acetate (3 M – pH 5.2) and 1 µL of GenEluteTM-LPA (Sigma-Aldrich, 25 µg $µL^{-1}$) were added. One volume of isopropanol was then added and the tubes were centrifuged for 10 min at 12,000 g and 4°C. The supernatant was discarded and 2 washing rounds using ethanol (80%) were carried to purify the DNA. The remaining ethanol was evaporated using a Speed-Vac for 20 min. Finally, 30 µL of TE were added and tubes were left for 1 h at 37°C. DNA concentration was measured using a NanoDrop 1000 spectrophotometer. The amount of DNA extracted from 5 µm size filters ranged between 4.25 and 138.09 ng $µL^{-1}$ with an average of 45.37 ng $µL^{-1}$. For the 2 µm filters, the minimum, maximum and mean concentrations were 6.71, 166.77, and 59.75 ng $µL^{-1}$, respectively. For the 0.2 µm filters such minimal, maximal and mean concentrations were 2.93, 215.18 and 66.89 ng $µL^{-1}$ respectively. Afterwards, all tubes were stored at -20°C until analysis.

2.4. PCR and sequencing

Total DNA extracts were set at 25 ng $µL^{-1}$. Total DNA extracted from the 5 and 2 µm filters were pooled since we had low amplification when considering only 2 µm filters. 0.2 µm filter was considered as it is. The PCR amplification of 16S rRNA gene fragments was performed using universal set of primers. The primers, namely forward primer 515F (GTGYCAGCMGCCGCGGTA) (Wang and Qian, 2009) and reverse primer 909R (CCCCGYCAATTCMTTTRAGT) (Wang *et al.*, 2018) had tags attached to them. It is noteworthy here that we checked, using TesPrime (not shown), that this set of primers had a good coverage

toward prokaryotes. The final number of samples was 66 and a PCR replicate was made for each sample giving a total of 132 samples. Each sample was identified with a different tag. PCR mixture volume was 30 µL and consisted of (final concentration): 1x buffer, 0.5 mM dNTP, 1.5 mM $MgCl_2$, 0.5 mg mL^{-1} bovine serum albumin (BSA) and 0.75 U Biotaq DNA polymerase (Bioline). In a second step, a unique combination of tagged primers (forward and reverse) was added to each sample. Finally, 1 µL of template DNA (25 ng μL^{-1}) was added. A negative control was included and the PCR program was as follows: 95°C – 2 min, 30 x (94°C – 30 sec, 58°C – 30 sec, 72°C – 30 sec), with a final extension step at 72°C for 5 min. Agarose gel analysis was performed to check the PCR products. When samples showed a non-specific band (approximately 550 bp) close to the target band (approximately 450 bp), the target bands were then captured using Pippin prep system (sage science) following the manufacturer's instructions. These captured DNA were checked with TapeStation (Agilent 2200) system for size and quality assessment following the manufacturer's instructions. All amplified DNA were quantified using the Quant-iT PicoGreen ds DNA Reagent kit (Invitrogen) and fluorescence was read using the plate reader Fluoroskan Ascent FL. All DNA samples were then pooled as one equimolar tube. DNA was then purified using the Clean PCR kit (CleanNA) according to the manufacturer's instructions to remove dNTP and dimers. Again, the pool was quantified using PicoGreen. Then, the pool tube containing the 132 different tagged DNA was sent to the GATC-Eurofins platform for DNA sequencing using Illumina HiSeq paired end technology to get 2 x 250 bp amplicons.

2.5. Bioinformatics pipeline

Two paired fastq files, referred to as R1 (forward sequences) and R2 (reverse sequences), were received from the sequencing platform. Files were processed using the pipeline developed by Frederic Mahé which combines Vsearch (Rognes *et al.*, 2016), Cutadapt (Martin, 2011) and Swarm (Mahé *et al.*, 2015) and reported at https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline. All default parameters of the pipeline were left unchanged except when mentioned. Briefly, the pipeline started with merging reads of R1 and R2 files using Vsearch. Next, Cutadapt was used to demultiplex the sequences according to a list of tags. Here, each sequence was assigned to its sample in an individual file by its tag. Primers were also removed and sequences containing ambiguous bases were discarded. In each file, sequences were dereplicated using Vsearch. All files were then assembled as one file and dereplicated in the process. The Swarm algorithm was then used to cluster the sequences. The "d" parameter (*i.e.* the number of different nucleotides between sequences) was changed from 1 to 13 in order to be close to the identity threshold of 97% which describes same species. For the calculation, we took into account the median length of sequences, which is 377 bp. Next, *de novo* chimera detection was applied to representative sequences of each cluster using Vsearch. Using Stampa (Sequence Taxonomic

Assignment by Massive Pairwise Alignments), the representative sequences were taxonomically assigned to a reference database downloaded from arb-SILVA (release number 132; (Quast *et al.*, 2013)) and prepared as required. The final OTU table was built using the python script provided in the pipeline. Then, multiple filtering was carried out on the OTUs table and these steps were named "default", "stringent", and "shared". In the "default" step, the OTU table was filtered by removing chimera, low quality sequences (< 0.0002) and OTUs with less than 3 reads in a sample, unless they were present in 2 or more samples. After examining the data, we applied the "stringent" filter. It consisted of keeping OTUs with 10 or more reads present in 2 or more samples, removing OTUs with an identity score lower than 90% to arb-SILVA and discarding unassigned OTUs. Finally, we applied the last filter, "shared", where we kept OTUs that are common between the PCR replicates. On the other hand, we intended to study microorganisms attached to particles, *i.e*. those captured on the 2 µm filter *vs* the free living part collected between 2 and 0.2 µm. However, due to technical issues, we chose to associate reads of all filters (5+2 µm and 0.2 µm), providing prokaryotes of all size. At the end, after applying all the aforementioned filters and association, only 33 out of 132 samples were analyzed.

2.6. Statistical analysis

Statistical analyses and plots were performed using R, version 3.5.0 (R Core Team, 2019) with the ggplot2 package (Wickham *et al.*, 2018). The OTU table was transformed to relative abundance using the "decostand" function from the vegan package (Oksanen *et al.*, 2019). Thus, all statistical analysis was performed using relative abundance. Alpha diversity indices (*i.e*. Shannon, Pielou, Chao1) were calculated with the OTUtable package (Linz, 2018). Simpson and inverse Simpson were computed using the vegan package (Oksanen *et al.*, 2019). The indexes comparison for each condition (Site, Month, and Depth) was performed using ANOVA or Kruskal-Wallis test according to the data distribution after initial testing with Shapiro and Bartlett test. When the p-value of ANOVA or Kruskal-Wallis test was inferior to 0.05 (alpha), a Tukey HSD or a Dunn test with Bonferroni correction (Alexis Dinno, 2017) were performed. An NMDS to illustrate beta diversity was computed using the "metaMDS" function from vegan and also the goeveg package (Goral and Schellenberg, 2017). Also, Adonis and Anosim (Oksanen *et al.*, 2019) tests were performed to analyse whether the groups observed were significantly different. Moreover, a Simper test (Oksanen *et al.*, 2019) was performed to assess the contribution of each OTU to the observed dissimilarity between samples. Regarding environmental variables, they were only available for pt4. OTUs of pt4 were extracted and if an OTU had no read in that site, the OTU was removed from the final OTUs table. The raw data was transformed as log(1 + x) in order to stabilize variances, and converted to relative abundance in order to perform a CCA following the online tutorial of Umer Zeeshan Ijaz (Torondel *et al.*, 2016) found at

http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html. Redundant environmental variables were removed after calculating a variance inflation factor (VIF). Any environmental variable with VIF superior to 10 were deleted. Co-occurrence network analysis was performed on all sites for OTUs and phylum-class clusters following Ju Feng's R and python scripts (Ju *et al.*, 2014; Ju and Zhang, 2015; Hu *et al.*, 2017) found at https://github.com/RichieJu520/Co-occurrence_Network_Analysis. Only positive interactions between community members were considered. The Spearman correlation and p-value cutoffs were set to 0.9 and 0.01 in the script. As for the C-score of the network, it was performed with the R package EcoSimR (Gotelli *et al.*, 2015) based on a presence/absence OTU matrix. The visualization and customization of the network was done with Gephi software (Bastian *et al.*, 2009). Finally, we used the computational method called pangenome-based functional profiles (PanFP) (Jun *et al.*, 2015) to infer functional profiles for bacterial and archaeal classes. The software can be found at (https://github.com/srjun/PanFP). Generated KOs where then analyzed using KEGG website (https://www.genome.jp/kegg/ko.html) (Kanehisa *et al.*, 2016). Pairwise intersection of KOs between archaea and bacteria were obtained from the website Molbiotools (http://www.molbiotools.com/listcompare.html). At last, the total shared KOs were calculated using the dedicated Bioinformatics and Evolutionary Genomics website tool, available at http://bioinformatics.psb.ugent.be/webtools/Venn/.

2.7. Sequence data

Paired end fastq files, tags list, unfiltered bacterial and archaeal OTU tables can be found at Zenodo repository website following this link: http://doi.org/10.5281/zenodo.3678385

3. Results

The sequencing results along with the details regarding archaeal and bacterial reads and OTUs can be found in the Supplementary text 1 and Supplementary Figures S2-S8.

3.1. Archaeal diversity and distribution

The archaeal reads and OTUs represented 9% and 4% of the dataset, respectively. OTU 2, assigned to Nitrososphaeria (Thaumarchaeota), constituted 97.4% of archaeal reads and was the second OTU to hold the highest number of reads among bacterial and archaeal OTUs. Overall, 5 phyla and 7 classes were detected after taxonomic assignment. Furthermore, Thaumarchaeota was dominant in all samples (Supplementary Fig. S4) followed by Nanoarchaeaeota, mostly present in all samples and characterized by a lower number of reads. Archaeal richness illustrated with the Chao1 index (Supplementary Fig. S5) was similar to bacteria, and the highest values for this index were obtained

at 200 m depth (p-value = 1.859^{-05}). Evenness (Pielou index) was significantly different between seasons (p-value = 0.01758), especially between winter (February) and summer (August), but not between depths (Supplementary Table S1). Archaeal diversity calculated with Shannon, Simpson and InvSimpson indices (Fig. 1-A and Supplementary Table S1) also revealed significant differences between depths (p-value = 0.006761), especially between near surface and deep waters (p-value = 0.0028). Similarly, depth was a factor explaining the difference in OTUs composition among samples as showed by the NMDS (Fig. 2-A), Anosim (p-value = 0.001) and Adonis (p-value = 0.003) (Supplementary Table S2). Undoubtedly, one OTU contributed significantly to the beta diversity between treatments, *i.e.* OTU 2 (Thaumarchaeota, Nitrososphaeria), in particular regarding depth (p-value = 0.006761) since it drove respectively more than 45%, 47% and 40% of the difference between 2 and 15 m, 2 and 200 m and 15 and 200 m for the entire community (Supplementary Table S3). Finally, the CCA (Fig. 3) performed on SHL2 OTUs revealed that these 77 OTUs were positively correlated to total phosphorus, especially at 200 m, and also to conductivity, total nitrogen and ammoniacal nitrogen. In contrast, archaeal OTUs were negatively correlated to chlorophyll *a*, dissolved oxygen and total organic carbon.

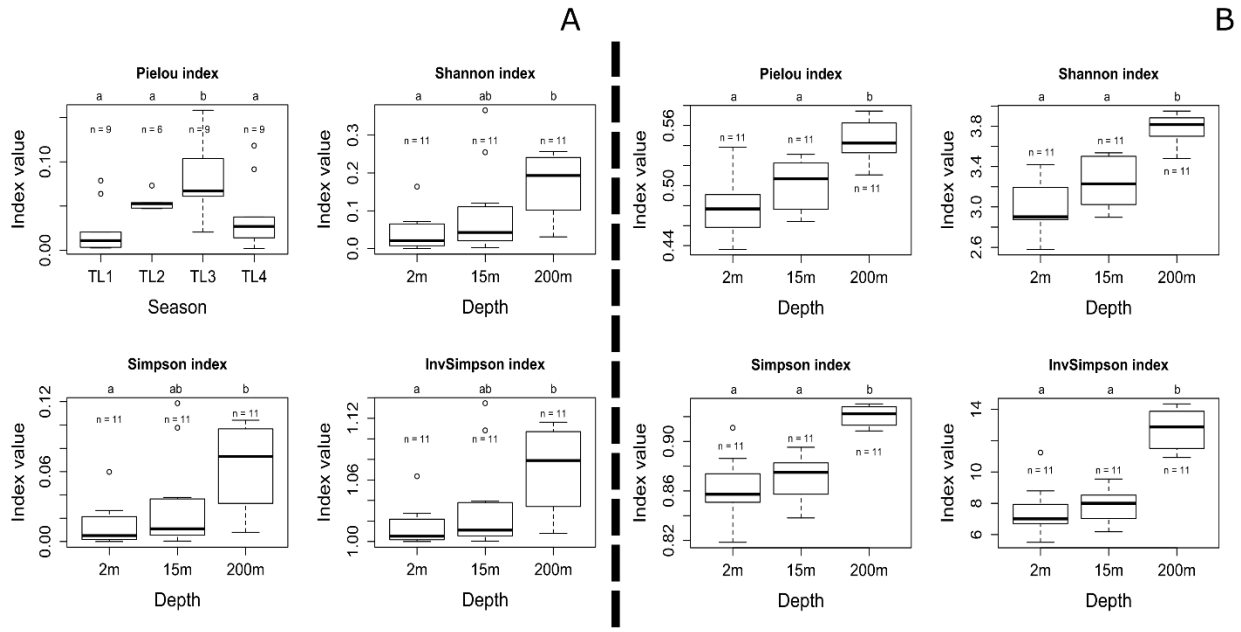

Fig. 1 Boxplot diagram comparing alpha diversity indices for archaea (panel A) and bacteria (panel B) (only significant results for indices are shown). For archaea, significant differences (p-value < 0.01) are detected among the depth and season (TL1: February; TL2: June; TL3: August; TL4: November) variables. For bacteria, significant differences (p-value < 0.01) are detected among depths. N is the number of samples; letters a and b correspond to significant differences.

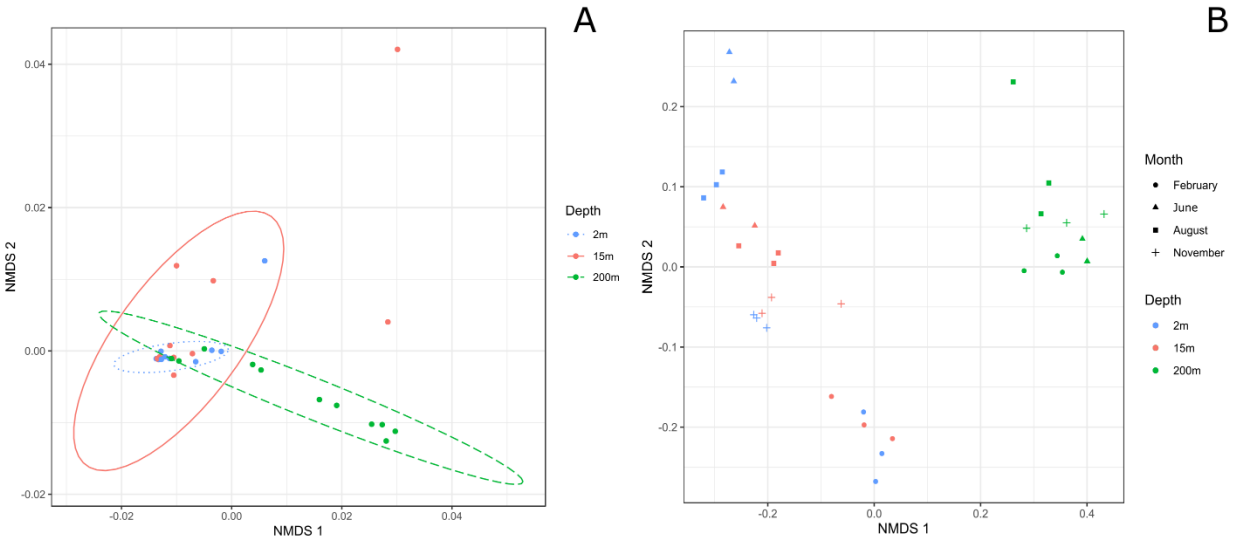

Fig. 2 NMDS plot illustrating separation of samples based upon differences in archaeal (panel A; stress value 0.03) and bacterial (panel B; stress value 0.05) OTUs structure. Shape and color correspond respectively to different seasons February (TL1), June (TL2), August (TL3) and November (TL4), and depths (2, 15 and 200 m). For archaea, ellipses indicate 95% confidence intervals of OTUs grouped by different depths. For bacteria, the ellipses could not be generated.

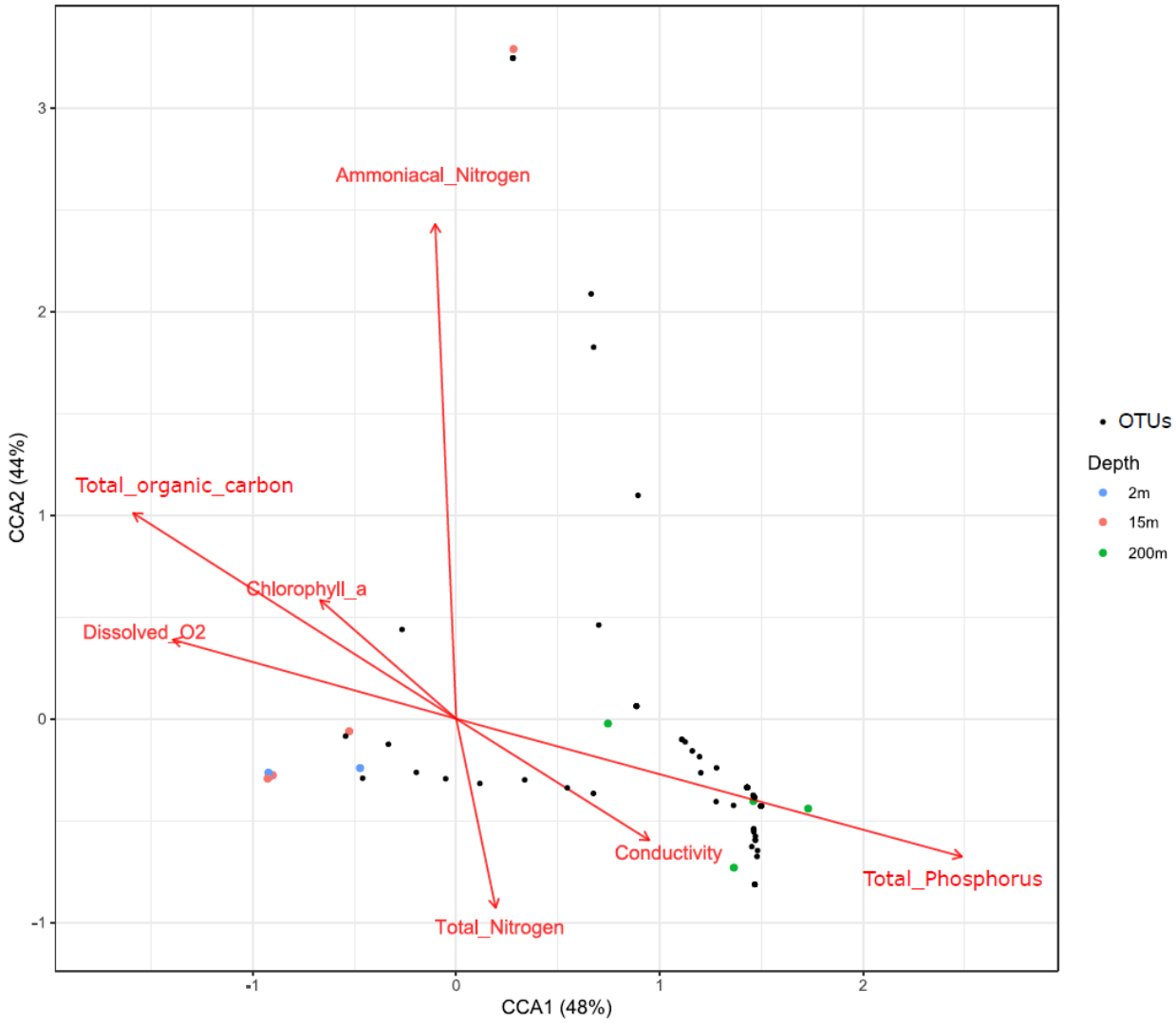

Fig. 3 CCA illustrating the separation of archaeal OTUs and samples based upon physicochemical descriptors. The CCA was only performed on pt4 site were physicochemical variables were obtained. 12 samples and 77 archaeal OTUs were used in this analysis.

3.2. Bacterial diversity and distribution

41 bacterial phyla and 110 classes were assigned according to the pipeline. Alphaproteobacteria (Proteobacteria) yielded the highest number of OTUs (*i.e*. 411) (Supplementary Fig. S6) and Actinobacteria the highest number of reads, *i.e*. 8,948,606 (Supplementary Fig. S7). Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Planctomycetes and Proteobacteria were highly present in all samples (Fig. 4). Samples at 200 m contained a higher phylum diversity than any other sample. Indeed, when looking at the richness and diversity indices performed on the OTUs, the results showed a significant difference according to depth, while no significant diversity shifts were observed for sites or seasons (Supplementary Table S4). Samples at 200 m displayed higher and significant values for all indices (Chao1 p-value= 7.710^{-10}, Pielou p-value= 4.706^{-6}, Shannon p-value= 4.322^{-08}, Simpson p-value= 4.757^{-08} and InvSimpson p-value= 2.335^{-05}) compared to the two other depths (2 and 15 m) (Fig. 1-B and Supplementary Fig. S8). All investigated sites were not significantly different in OTUs composition (Fig. 2-B) which was affected mainly by depth (Anosim and Adonis p-value were respectively 0.001 and 0.001) and by season (Anosim and Adonis p-value were respectively 0.006 and 0.011) (Supplementary Table S5). The Simper test (Supplementary Table S6) indicated that OTUs 4 and 5, assigned to the class Anaerolineae (Chloroflexi) and Oxyphotobacteria (Cyanobacteria) respectively, drove significantly the beta diversity. Indeed, OTU 4 contributed (p-value = 1.714^{-05}) to the disparity between surface and depth (up to 11%), as well as between 15 and 200 m depth (up to 12%). In addition, the average dissimilarity between 2 and 200 m was 65% and it was about 62% between 15 m and 200 m. OTU 5 contributed significantly (p-value = 0.0003058) to the disparity between February and the 3 other months (June 14%, August 14% and November 15%). The CCA (Fig. 5) that was performed on 1964 OTUs found at pt2, revealed that the variance is drawn in all directions. On one hand, total phosphorus revealed to be well linked to the OTUs present at 200 m. On the other hand, total nitrogen, chlorophyll *a*, conductivity and dissolved oxygen were linked to bacterial OTUs present at 2 and 15 m.

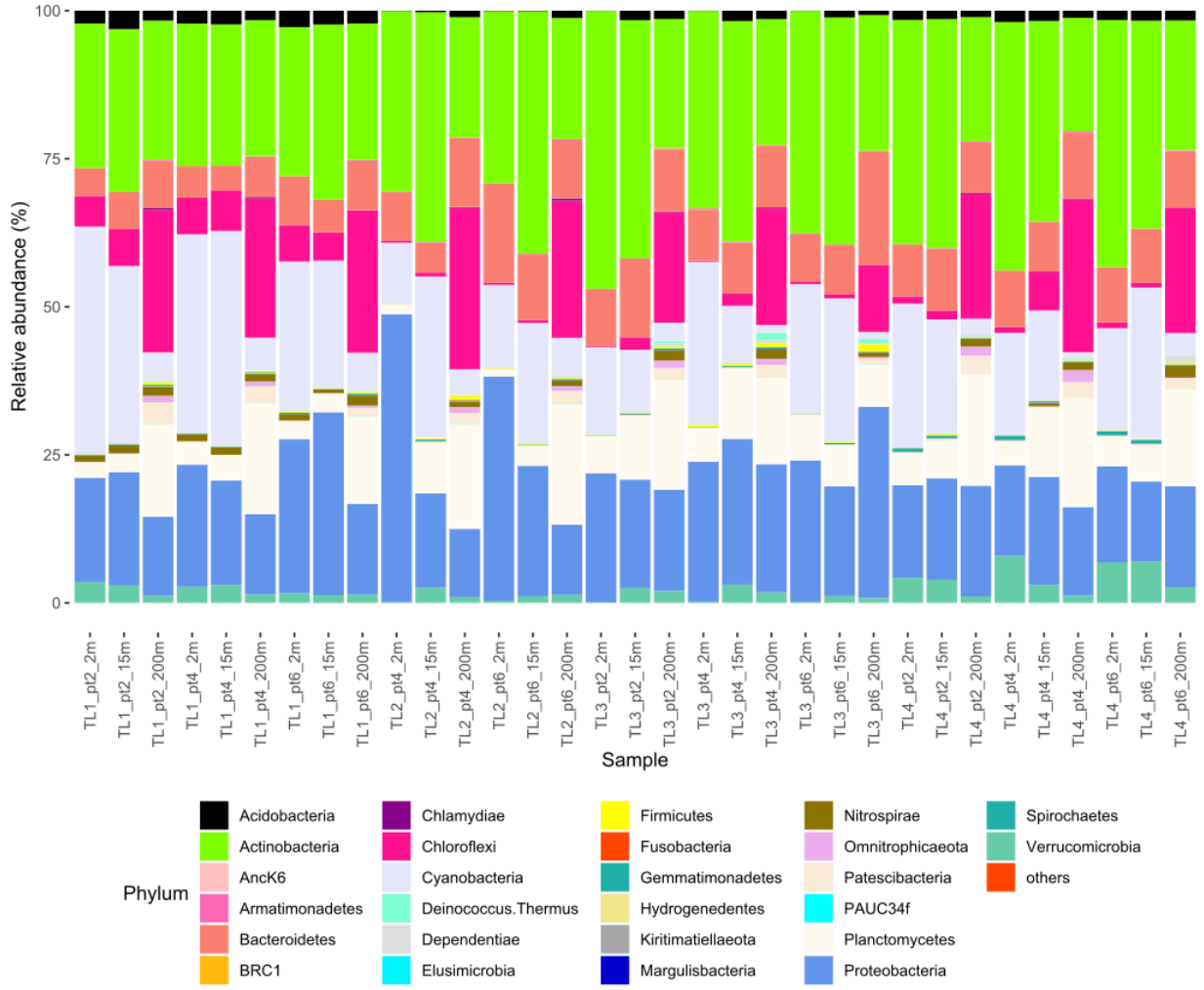

Fig. 4 Stacked histogram of bacterial phylum. Phylum abundances are represented by read relative abundance per sample. Phyla with less than 5% of read relative abundance in a sample are merged and identified as “others”.

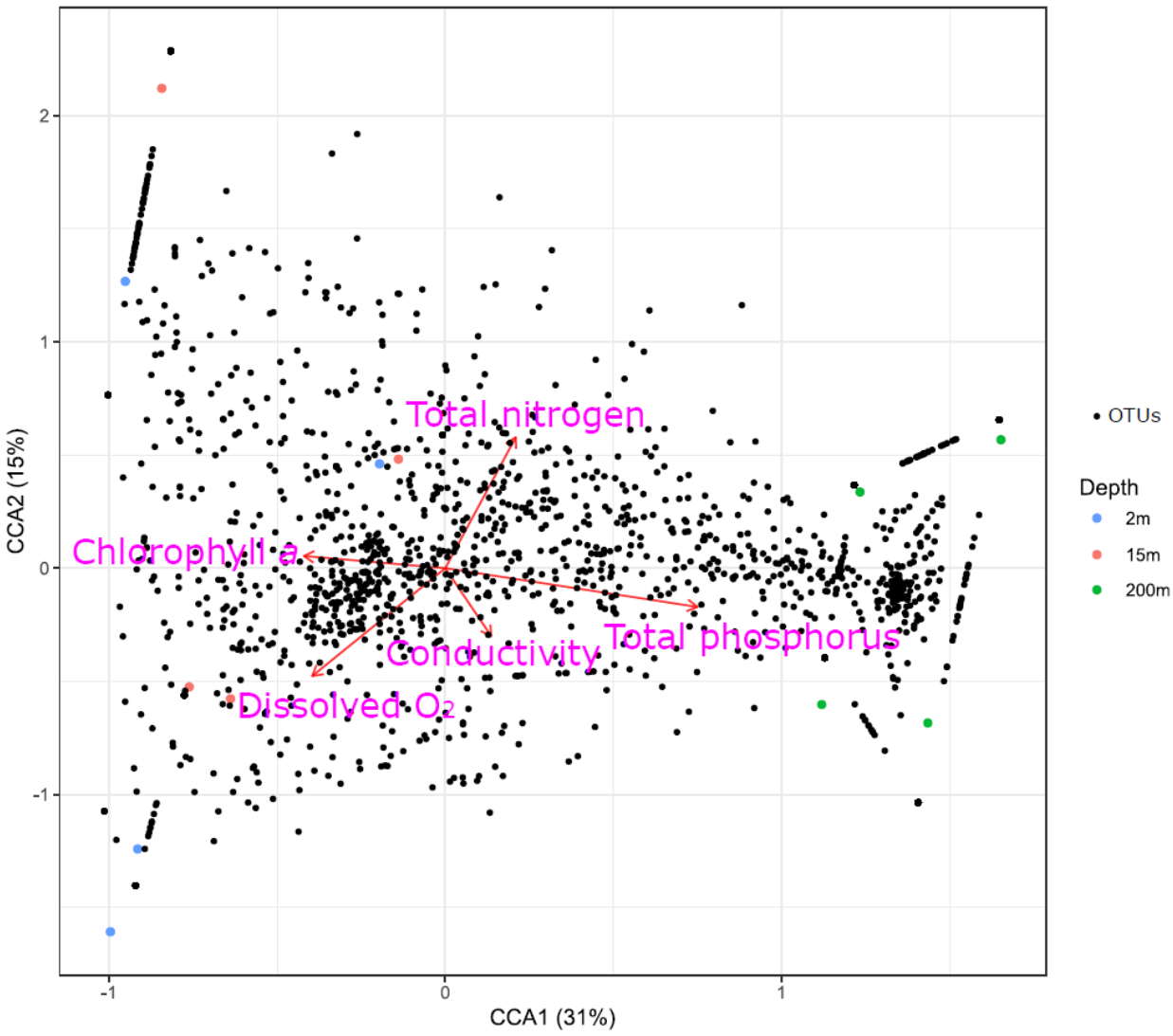

Fig. 5 CCA illustrating the separation of bacterial OTUs and samples based upon physicochemical variables. The CCA was only performed on pt4 site were physicochemical variables were obtained. 12 samples and 1,964 bacterial OTUs were used in this analysis.

3.3. Relations between archaea and bacteria

To assess possible interactions between bacteria and archaea we used two different and complementary approaches performed on the OTUs tables: a co-occurrence network analysis to determine significant connections between microbes and a functional profile prediction analysis to go further.

The co-occurrence network (made of 260 and 24 nodes and 1230 and 33 undirected edges, for bacteria and archaea respectively) revealed connections among and between bacterial and archaeal OTUs. We only reported here strong (correlations ≥ 0.9) and significant (p-value < 0.01) links. The C-score test with sim9 algorithm confirmed that the network was non-random. Indeed, the observed C-score (10.523) was higher than the mean value (c-score mean = 10.321, p-value < 0.001) expected under the null model. On one hand, the archaeal OTU 2 assigned to the genus *Nitrosopumilus* (Thaumarchaeota, Nitrososphaeria, Nitrosopumilales, *Nitrosopumilaceae*) was linked to 8 bacterial OTUs. These OTUs were identified as *Anaerolineaceae* (Chloroflexi, Anaerolineae, Anaerolineales, OTU 4), BSV26 (Bacteroidetes, Ignavibacteria, Kryptoniales, OTU 10), CL500-3 (Planctomycetes, Phycisphaerae, Phycisphaerales, *Phycisphaeraceae*, OTU 19), *Nitrospira* (Nitrospirae, Nitrospira, Nitrospirales, *Nitrospiraceae*, OTU 25), IMCC26256 (Actinobacteria, Acidimicrobiia, OTU 46), SAR11 clade (Proteobacteria, Alphaproteobacteria, OTU 124), Elsterales (Proteobacteria, Alphaproteobacteria, OTU 135), and *Methylomonaceae* (Proteobacteria, Gammaproteobacteria, Methylococcales, OTU 149). On the other hand, OTU 67 identified as the archaeal class Woesearchaeia (Nanoarchaeaeota) was linked to 4 bacterial OTUs (Fig. 6). These OTUs are identified as BSV26 (Bacteroidetes, Ignavibacteria, Kryptoniales, OTU 10), Peribacteria (Patescibacteria, Gracilibacteria, OTU 188), P2-11E (Chloroflexi, OTU 417) and KD4-96 (Chloroflexi, OTU 587). At the class level (Supplementary Fig. S9), Nitrososphaeria (Thaumarchaeota) was only linked to Anaerolinae (Chloroflexi). The Woesearchaeia (Nanoarchaeaeota) class maintained 3 links which were P2-11E (Chloroflexi), KD4-96 (Chloroflexi) and Gracilibacteria (Patescibacteria) and a new link with JG30.KF.CM66 (Chloroflexi) appeared. The C-score test also confirmed that this network is non-random with observed C-score (5.1653) being higher than the mean value (c-score mean = 4.4757, p-value < 0.001) expected under the null model.

The prediction of functional profiles from lineages (phylum – class) that are involved in the co-occurrence network were only possible for Thaumarchaeota - Nitrososphaeria and its associated bacteria *i.e.* Chloroflexi – Anaerolineae; Bacteroidetes – Ignavibacteria; Planctomycetes – Phycisphaerae; Nitrospirae – Nitrospira; Actinobacteria – Acidimicrobiia; Proteobacteria – Alphaproteobacteria; Proteobacteria – Gammaproteobacteria (Supplementary Fig. S10). No

functional profiles were found by the software for Nanoarchaeaeota – Woesearchaeia. Nitrososphaeria had the minimum number of functional orthologs (KO=736), and Gammaproteobacteria the highest (*i.e.* 5217). Overall, the archaeal class Nitrososphaeria had less KOs involved in metabolic pathways, enzymes, carbon, sulfur and pyruvate metabolism, but methane and nitrogen metabolism. Moreover, we calculated the shared number of KOs among all these class and found a value of 186. When looking at the pairwise intersections (Supplementary Fig. S11) Nitrososphaeria had the less shared KOs with Alpha and Gammaproteobacteria (Jaccard index = 0.1231 and 0.0989, respectively), while most KOs were shared among Alpha and Gammaproteobacteria (Jaccard index = 0.6833). This result showed that some pathways may be lacking in Nitrososphaeria.

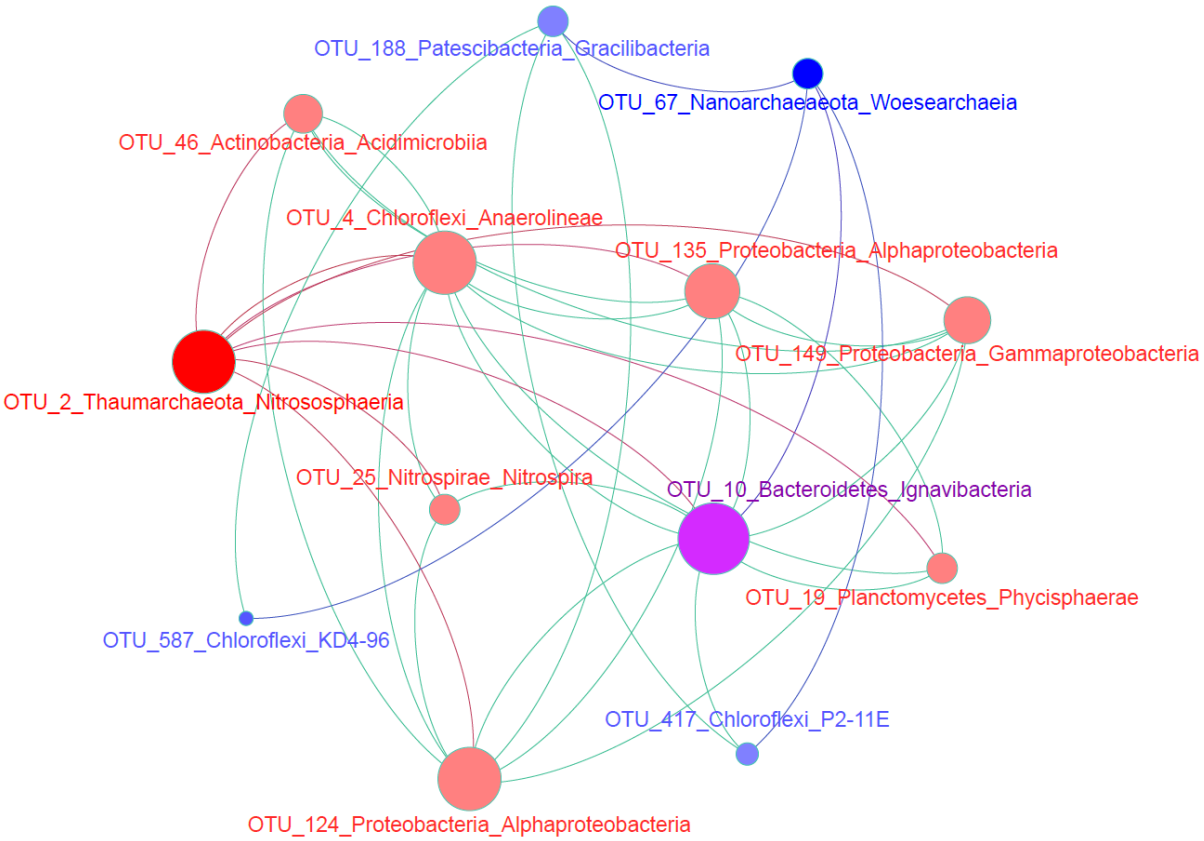

Fig. 6 Co-occurrence network between bacteria and archaea OTUs. Only OTUs that passed the cutoff (correlation coefficient ≥ 0.9 and p-value < 0.01) are illustrated. Only positive relationship are shown. Red and salmon color are the connections from the archaeal OTU 2 to its associate bacteria. While blue colors represents the connections between archaeal OTU 67 and its associate bacteria. The purple color indicates that both archaeal OTU shared a connection with a bacterial OTU.

4. Discussion

4.1. The choices we made shape our observations

We investigated the genetic diversity of bacteria and archaea at four months representing the different seasons of the year 2014. We are aware that the time scale of this study was relatively short (*i.e*. four dates) compared to other studies (Bomberg *et al.*, 2008; Hugoni *et al.*, 2013; Parada and Fuhrman, 2017) and the relatively low number of sampled sites, depths and months might not be representative to illustrate all the complex diversity of both archaea and bacteria in Lake Geneva. Using Illumina HiSeq, although being very useful to assess deeply the 16S rRNA-based genetic diversity of these prokaryotes, an important issue could be the primer specificity since we noted that the pair of primers targeting the V4-V5 region used in our study preferably amplified Actinobacteria and Thaumarchaeota. The *in silico* results showed that these primers had a good taxonomic resolution, covering 80.2% and 98.8% of the taxa at 0 and 3 mismatches respectively. It is noteworthy, however, that the behavior of primer pairs *in vitro vs in silico* may greatly differ as demonstrated in a previous study (Ezzedine *et al.*, 2020). Ideally, different universal primers should be tested with the same sequencing technology to compare the results.

It is also noteworthy that the taxonomic assignment is the result of bioinformatics algorithms, a procedure not perfect that can result in a variety of bias. Also, the sequence identity threshold for 16S rRNA OTUs can induce misidentification. As stated in the literature, sequences with 95% identity are likely to represent a same genus, whereas sequences with 97% identity may represent a same species (Schloss and Handelsman, 2005; Schloss *et al.*, 2009). In this study, we used OTUs, set the threshold at 96.6% and chose to analyze the taxonomic assignment to the genus level, whenever possible. Indeed, the Swarm algorithm was then used to cluster the sequences. The "d" parameter (*i.e.* the number of different nucleotides between sequences) was changed from 1 to 13 in order to be close to the identity threshold of 97% which describes same species. For the calculation, we took into account the median length of the sequences that was 377 bp. In addition, we chose stringent cutoff to filter the OTUs tables and considered only shared OTUs among PCR replicates.

As we explored relationships between dominant archaea OTUs and other bacterial OTUs *via* a co-occurrence network analysis, we have to remind here again that it was constructed using a stringent cutoff (> 0.9) in order to only reveal significant and strong correlations. In addition, we made the choice by focusing on positive correlation. The co-occurrence network along with the detected functional profiles from 16S rRNA (and not from metagenome) are presented as a suggestion based on strong statistical analysis.

Regardless, this study sheds light on both bacterial and archaeal diversity in Lake Geneva using a high throughput sequencing approach and highlights, for this large and deep lake, possible relationships between prokaryotic communities, known to play a variety of key roles in aquatic ecosystems.

4.2. Archaeal and bacterial diversity

Our data set was mainly represented by two archaeal phyla, Thaumarchaeota and Nanoarchaeaeota, while archaea are generally more diversified in aquatic ecosystems (Kozubal *et al.*, 2013; Evans *et al.*, 2015; Vanwonterghem *et al.*, 2016; Eme *et al.*, 2017). Other phyla were in fact detected, such as Euryarchaeota, Diapherotrites and Crenarchaeota but they were clearly not dominant and only found with a low number of reads and OTUs. Also, the diversity was found to be higher in deep-water (*i.e.* 200 m). Globally, it has been reported that archaea may represent less than 10% of the microbial population in freshwater habitats (Bomberg *et al.*, 2008; Bahram *et al.*, 2019). Our results are in accordance with these previous studies since archaea reads represented only 9% of the whole sequencing data set. This result could be related to the archaeal genome which is compact with a low number of ribosomal copies (Koonin and Wolf, 2008; Angly *et al.*, 2014), that may lead to underestimation of archaeal abundance (Wurzbacher *et al.*, 2017). In addition, the quite generic primers we used could have played a part in overlooking the archaeal diversity. As a matter of fact, (Bahram *et al.*, 2019) proved that employing an adequate pair of specific primers enabled to get accurate estimate of archaeal diversity by detecting readily, the Asgard, the TACK and the DPANN superphyla in a variety of ecosystems. That being said, (Haller *et al.*, 2011) in their study of bacterial and archaeal communities at different contamination levels, using a cloning-sequencing approach also in Lake Geneva, reported that Euryarchaeota were mainly found in contaminated sediments, rich in organic matter. (Eme *et al.*, 2017) found that ammonia oxidizing archaea (AOA) Thaumarchaea living in the neighboring Lake Bourget, another (French) peri-alpine lake, were favored when ammonia concentrations were the lowest and during winter. For the same lake, other studies reported that archaea can occupy different ecological niches due to the influence of different abiotic and biotic factors. Indeed, archaeal OTUs were shown to be structured by factors such as ammoniacal nitrogen, total nitrogen and total phosphorus (Berdjeb *et al.*, 2013; Pollet *et al.*, 2018). At last, (Parada and Fuhrman, 2017) reported that depth and seasonality influenced archaeal community composition, and that archaeal group selected different ecotypes depending on how they fit to environmental conditions at each depth. Overall, this suggests that archaea have preferential niches and that Thaumarchaea of Lake Geneva are most probably adapted to thrive at the three sampled depths and the studied months, compared to other groups. For instance, OTU 2

that contributed to sample dissimilarity also held the highest number of reads for archaea and the second in the data set. This OTU was assigned to Candidatus *Nitrosopumilus* from the family of the *Nitrosopumilaceae* (Thaumarchaeota), a genus reported to be ubiquitous in marine environments and an important ammonia oxidizing archaea.

Bacteria were much more diverse than archaea. Actinobacteria, Cyanobacteria, Proteobacteria, Chloroflexi and Bacteroidetes dominated this community. This result agrees with previous studies which identified most of these groups as predominant phyla of freshwater systems (Glöckner *et al.*, 1999; Debroas *et al.*, 2009; Newton *et al.*, 2011; Zwirglmaier *et al.*, 2015). As for the archaea, depth mainly explained the differences found in the bacterial diversity and the highest values in bacteria richness and diversity were also found at 200 m depth (with a dominance of Actinobacteria). No difference in richness, diversity and evenness was found among the different sampling sites. However, the season also played a role since bacterial OTUs found at 2 and 15 m (but not those at 200 m) were segregated by months. OTU 5 (Oxyphotobacteria) was the most influential OTU to drive the beta diversity within seasons. OTU 4 (Anaerolineae) was more present at 200 m and it drove the beta diversity between 2 and 200 m, and 15 and 200 m. Physicochemical descriptors explained 41% of the bacterial OTUs distribution present at pt4. Total phosphorus explained well the presence of some OTUs at 200 m, a result reminding the study of (Berdjeb *et al.*, 2011) who reported that the bacterial community structure present in the hypo- and epilimion in two other peri-alpine lakes, Bourget and Annecy, was affected mainly by bottom-up factors regardless of the local environmental conditions of these lakes.

4.3. Potential relationships between prokaryotes

A variety of relationships was found between the prokaryotes, suggesting potential ecological and/or biogeochemical interactions.

The archaeal OTU 2 assigned to the phylum – class; Thaumarchaeota – Nitrososphaeria co-occurred with 8 bacterial OTUs. Recently, in the release of SILVA SSU database 138 (December 16, 2019), Nitrososphaeria was transferred to the Crenarchaeota phylum. However, the analysis was performed with database 132 and prior to the release of the new database version, explaining why we maintained the former classification. The detection of functional profiles revealed that Nitrososphaeria is less equipped in term of pathway in comparison to associate bacteria. This is expected since archaea possesses a compact genome. In addition, only few KOs were common between Nitrososphaeria and the other bacteria, suggesting that some functions are missing in the archaeal genome. These results explained probably why Nitrososphaeria co-occurred with other bacteria. The archaeal OTU 2 (*i.e. Nitrosopumilus*), mentioned above, co-occurred with the

bacterial OTU 25 assigned to *Nitrospira* (Nitrospirae). This relationship is coherent with the finding of (Parada and Fuhrman, 2017) who also reported a correlation between marine Thaumarchaea and ammonia oxidizing bacteria (AOB) such as *Nitrospina*, both responsible for nitrification. Archaeal OTU 2 displayed also a link with the bacterial OTU 19, identified as CL500-3 (*i.e*. a Planctomycetes). These bacteria were already reported to be associated to AOA such as Thaumarchaeota in oxygenated hypolimnion of deep freshwater lakes (Okazaki *et al.*, 2017). *Nitrosopumilus* is known to operate the methane cycle and produce methylphosphonic acid (Carini *et al.*, 2014). Methane-oxidizing archaea (MOA) and bacteria (MOB) such as α- and γ-proteobacteria (Hanson and Hanson, 1996; Lüke *et al.*, 2010) can either produce or consume methane. These microorganisms are essential for the methane release and rate in the atmosphere. Therefore, the occurrence between the archaeal OTU 2 and the bacterial OTU 4 assigned to *Anaerolineaceae* (Chloroflexi) makes sense, with the possibility that *Anaerolineaceae* provide organic acid such as acetate to other microorganisms like acetoclastic methanogens. Moreover, *Anaerolineaceae* could form a syntrophic cooperation with some archaea involved in methanogenic degradation of alkanes (Lüke *et al.*, 2010; Liang *et al.*, 2015). Similarly, we observed MOB co-occurrence through OTU 149 assigned to the *Methylomonaceae* (Gammaproteobacteria). Furthermore, a mutualistic interaction might be occurring between archaea OTU 2 and bacteria OTU 124 assigned to SAR11 clade (Alphaproteobacteria). (Parada and Fuhrman, 2017) A link between some Thaumarchaea and SAR groups such as SAR406 and SAR86 was also found in the ocean. In fact, *Nitrosopumilus* produce methylphosphonic acid (MPn), which is decomposed by phosphate-starved microorganisms such as SAR11 clade (chemoheterotrophic bacteria) that produce CH_4 from MPn when P_i (inorganic phosphate) starved (Carini *et al.*, 2014). This was also suggested by the functional profile where Nitrososphaeria, Alpha and Gammaproteobacteria shared 526 to 536 from 736 KOs pointing out that the bacterial class lack some pathways that can be compensated with an interaction with Nitrososphaeria. Finally, the co-occurrence with OTU 10 assigned to BSV26 (Kryptoniales) and OTU 46 identified as IMCC26256 (Acidimicrobiia) could be ecologically relevant since these two OTUs were originally isolated together from extremely acidic environments or geothermal sites were archaea blossom (Ludwig *et al.*, 2012; Hu *et al.*, 2017).

The second most important archaeal OTU (*i.e.* OTU 67) showed important relationships with bacterial OTUs 10, 188, 417 and 587. OTU 67 was assigned to the class Woesearchaeia of the phylum Nanoarchaeaeota. Woesearchaeota are widely spread in diverse environments and (Liu *et al.*, 2018)reported a syntrophic relationship between Woesearchaeota and other methanogenic archaea. This syntrophic and/or mutualistic partnership emerged from deficiencies in metabolic pathways within the Woesearchaeota class. (Liu *et al.*, 2018) also suspected that Woesearchaeota

develop a partnership with some bacterial methanogens, proposing a Woesearchaeota-methanogens consortium, likely to influence methane formation. However, these authors lacked reliable bacterial data to analyze collectively these microorganisms and prove this assumption. Our results support their finding. A relationship also existed with Chloroflexi OTU 417 (P2-11E), 587 (KD4-96) and to JG30.KF.CM66 (Supplementary Fig. S9). In fact, Chloroflexi possess a wide diversity of metabolisms (e.g. aerobic respiration, denitrification and phototrophy), and ecological roles, but are best known as photoheterotrophs (Ward *et al.*, 2018). They are reported to be dominant and abundant in methanogenic reactors (Bovio *et al.*, 2019). Recently, (Ward *et al.*, 2019) reported that within the Chloroflexi phylum some of them such as OHK40 are capable of methane oxidation (photomethanotrophy). We cannot pinpoint the exact need or relationship between OTU 67 and the other bacterial OTUs but a syntrophic or/and a mutualistic link is very probable between them. This could explain the abundance of Chloroflexi observed at 200 m (Fig. 1) where oxygen concertation was found to be lower than at the surface (*i.e*. 7 vs 9-10 mg L^{-1}). Indeed, if some Chloroflexi OTUs can produce methane they are likely to occur in less oxic layers since methanogenesis is not favored where oxygen is abundant (Carini *et al.*, 2014), and, as pointed out by (Mayr *et al.*, 2019), MOB can occupy different niches according to oxygen and methane concentration. At last, the co-occurrence observed with OTU 188 assigned to *Peribacteria* (Gracilibacteria) also make sense since the latter lack some pathways and is predicted to be either a symbiont or closely dependent on other community members for key building blocks (Sieber *et al.*, 2019).

5. Conclusion

In parallel to studies dealing with physiology, biodiversity, biological indication and assessment, assessing interactions between microorganisms in pelagic communities are of great ecological interest but still remain poorly documented in lakes. Using Illumina HiSeq combined with 16S metabarcoding *via* universal primer, co-occurrence networks and functional profile predictions, we showed that Lake Geneva is booming with connections, and our results suggest that some OTUs co-occur for scavenging or sharing similar ecological niches for mutual benefits. Sequencing different functional genes and conducting experiments with isolated organisms could help to better understand the relationship between key “species” and biotic interactions sustaining the microbial ecosystem functioning.

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Author Contributions

SJ designed the study and collected the samples. JE performed bioinformatics and statistical analysis. JE and SJ analyzed and interpreted the results. JE and SJ wrote the manuscript with revisions by YD.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Supplementary data

Supplementary Text 1: Sequencing results

Following sequencing, each raw data file (R1 forward and R2 reverse sequence) contained 55,679,272 reads. After merging, we obtained 49,545,789 paired end reads. At this step, 11% of reads were lost, and the median read length was 437 bp. After demultiplexing, and after a first dereplication, the number of reads reached 11,079,438, with a median length of 377 pb. The second dereplication of reads after reuniting them in one file gave 6,861,908 reads. The clustering process yielded 127,052 representative sequences, including 84.3% chimeric sequences and 15.3% non-chimeric sequences. However, when considering abundance into account, this corresponded to 1.3% chimeras and 98.7% non-chimeras. The median read length after the clustering was 376 pb.

For the archaeal OTU table, we obtained with the default filter, 631 OTUs and 2,868,729 reads. After applying the stringent filter, this number fell down to 194 OTUs and 2,862,662 reads. In addition, only 112 OTUs (2,858,335 reads) were common to both replicates. After applying all these filters, we only lost 0.4% of archaeal reads. Overall, 5 phyla and 7 classes were detected after taxonomic assignment. Woesearchaeia (Nanoarchaeaeota) had the highest number of OTUs (*i.e.* 91), but was classed second regarding the number of reads that was 67,646. Nitrososphaeria (Thaumarchaeota) followed with 11 OTUs, corresponding however to 2,790,583 reads. For Methanomicrobia (Euryarchaeota), Iainarchaeia (Diapherotrites), Bathyarchaeia (Crenarchaeota), Methanobacteria (Euryarchaeota), and Micrarchaeia (Diapherotrites) the number of OTUs varied between 1 and 3, with 3 to 43 reads (Supplementary Fig. S2 and S3).

For the bacterial OTU table, the "default" step gave 7,987 bacterial OTUs and 29,846,037 reads. After applying the "stringent" step we obtained 3,712 OTUs and 29,502,677 reads. This step resulted in a loss of only 1.15% in read number. The "shared" filter yielded 2,674 OTUs and 29,383,517 reads. Only 0.4% of reads were lost between these two steps, for a total loss of 1.55%. In general, 41 bacterial phyla and 110 classes were assigned according to the pipeline. Alphaproteobacteria (Proteobacteria) yield the highest number of OTUs (i.e. 411) (Supplementary Fig. S6), followed by Bacteroidetes (Bacteroidia) (*i.e.* 400). The Gammaproteobacteria (Proteobacteria) class followed with 381 OTUs, the Deltaproteobacteria (Proteobacteria) with 223 OTUs, the Planctomycetacia (Planctomycetes) with 122 OTUs, and the Oxyphotobacteria (Cyanobacteria) with 110 OTUs. Actinobacteria was characterized by the highest number of reads, *i.e.* 8,948,606. It was followed by the Oxyphotobacteria (Cyanobacteria) with 4,699,878 reads, the Gammaproteobacteria (Proteobacteria) with 2,220,792 reads, the Anaerolineae (Chloroflexi) with 2,209,038 reads, the Bacteroidetes (Bacteroidia) with 2,076,034 reads and the Alphaproteobacteria

(Proteobacteria) with 1,955,836 reads. 48 bacterial classes had less than 1,000 reads (Supplementary Fig. S7).

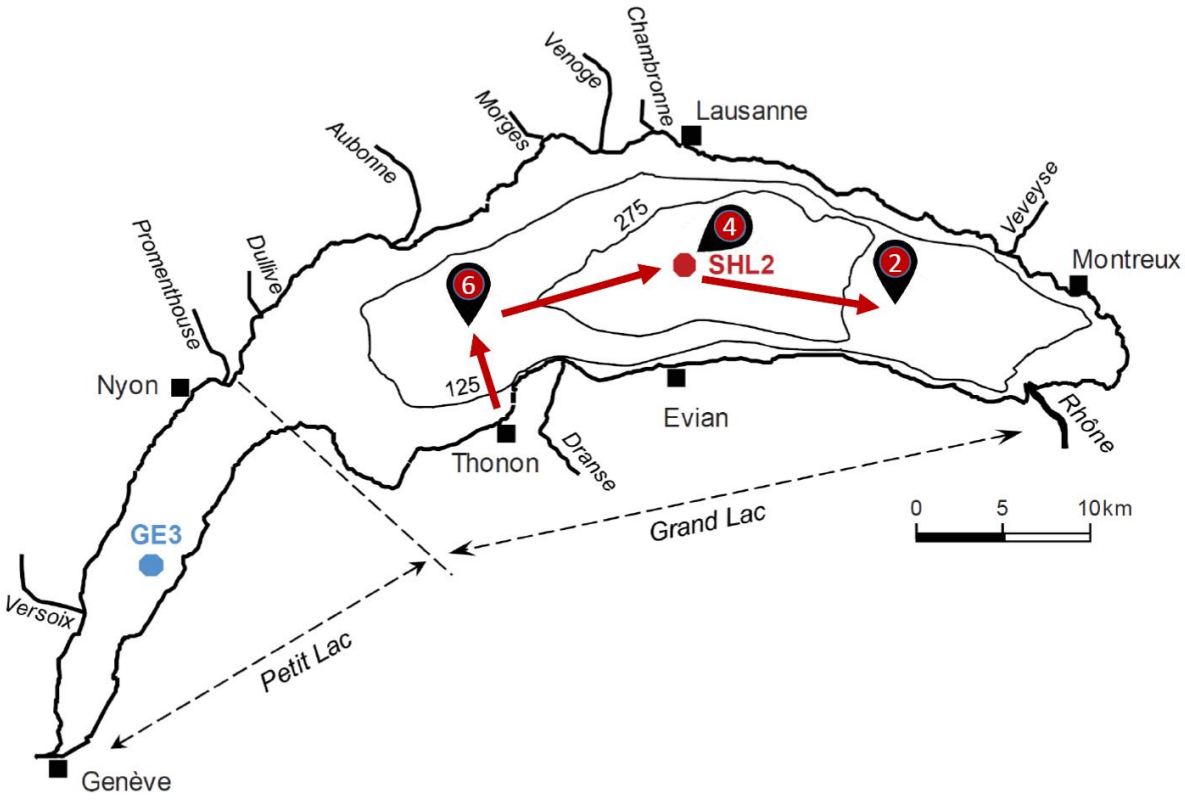

Fig. S1 Map of Lake Geneva and coordinates of the different sampling points selected during TRANSLEM: Site 2 (N 46° 26.206 / E 006° 46.848), Site 4-SHL2 (N 46° 27.207 / E 006° 35.654) and Site 6 (N 46° 25.061 / E 006° 24.957).

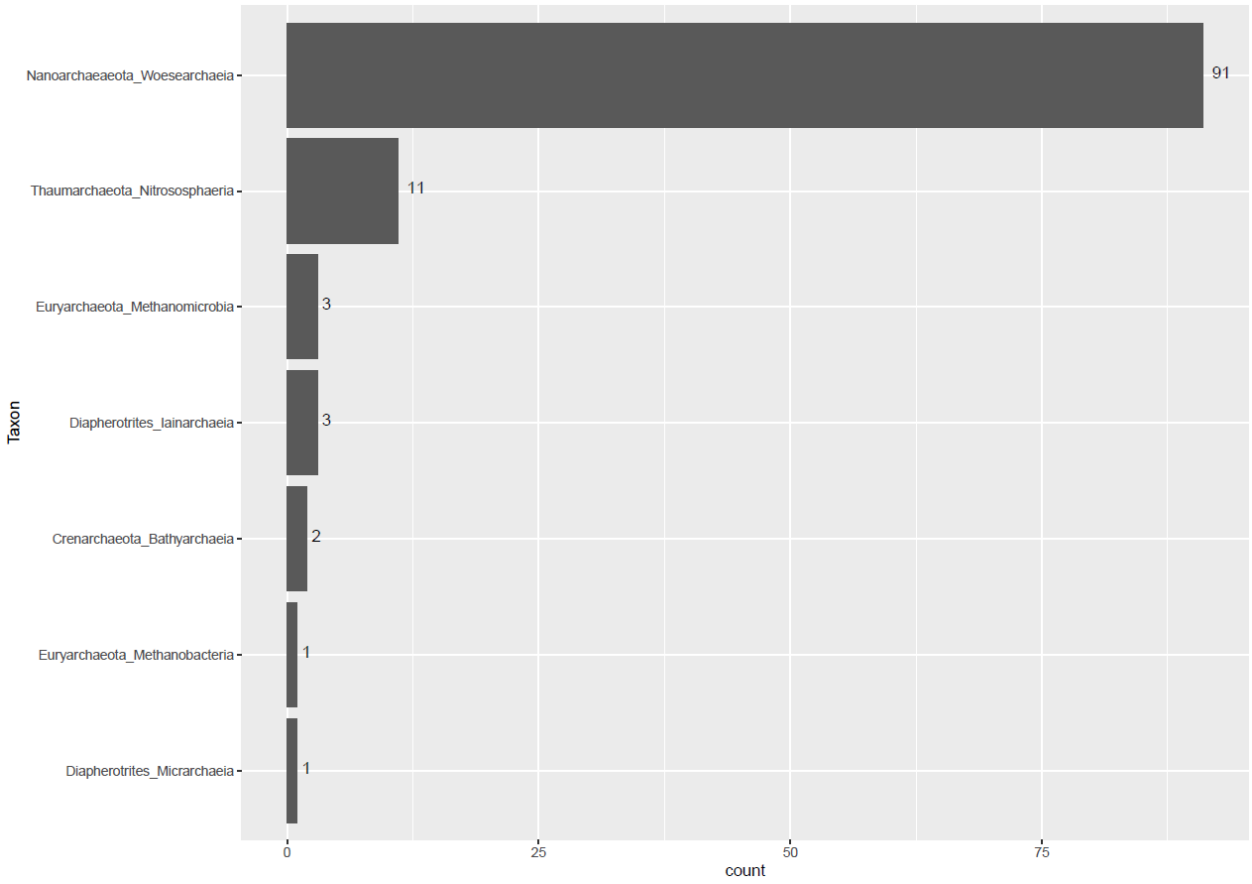

Fig. S2 Number of OTUs per archaeal class.

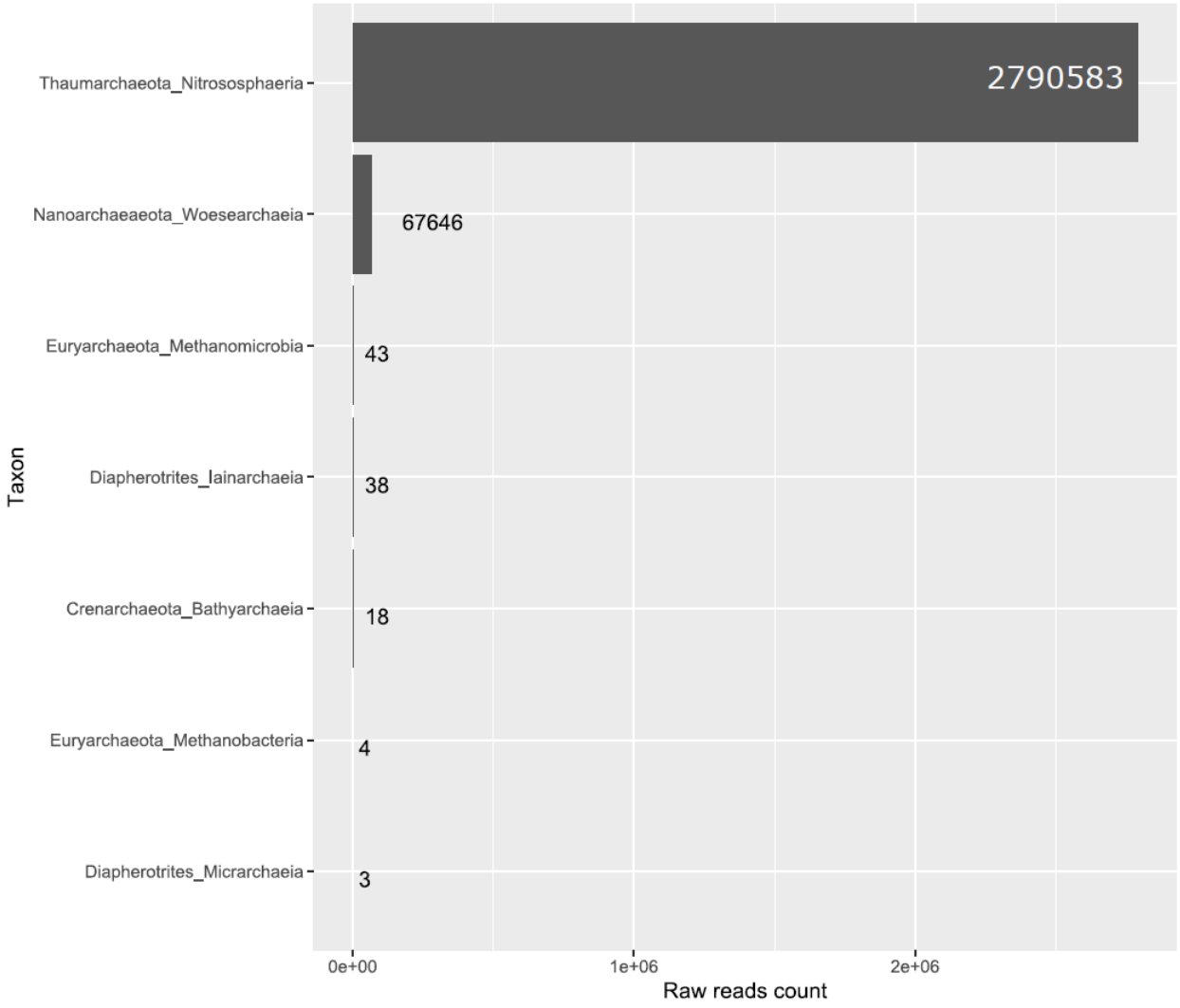

Fig. S3 Number of raw reads per archaeal class.

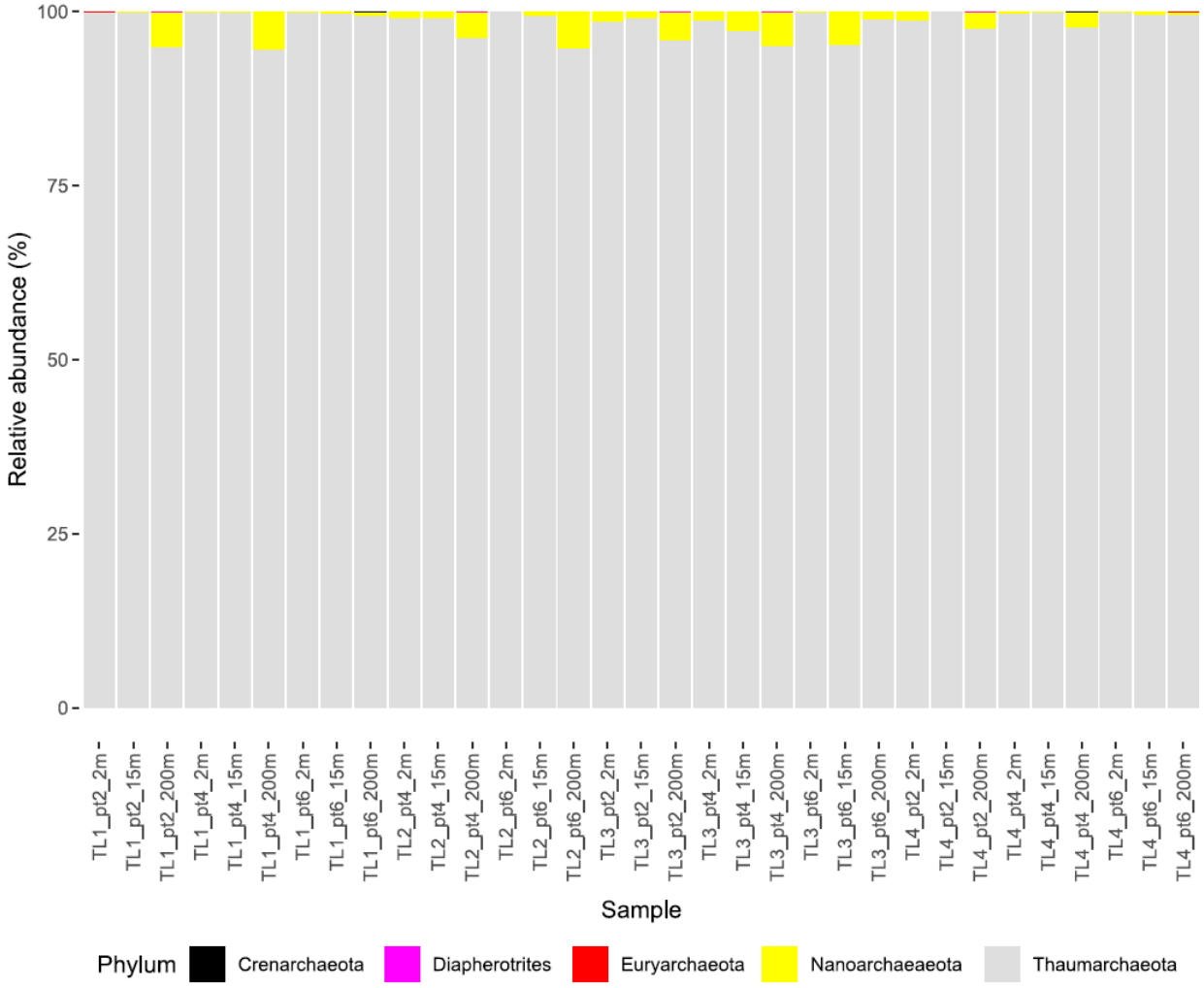

Fig. S4 Stacked histogram of archaeal phylum. Phylum abundances are represented by reads relative abundance per sample. Thaumarchaeota is dominant in all samples.

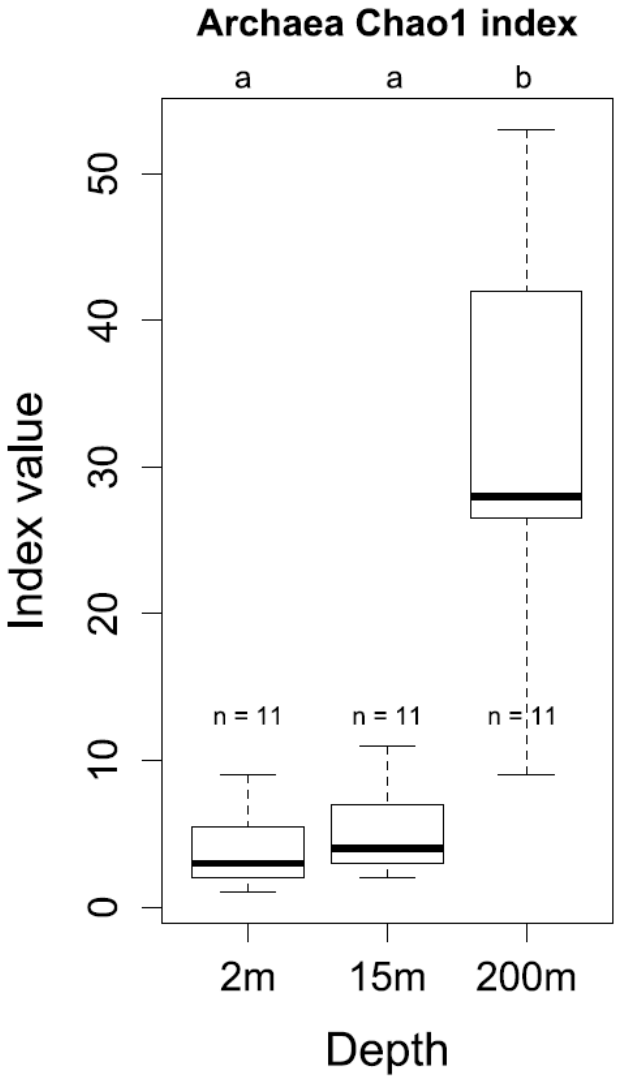

Fig. S5 Chao1 richness index for archaea showing significant differences between depths. N indicate the number of samples ; letters a and b correspond to significant differences.

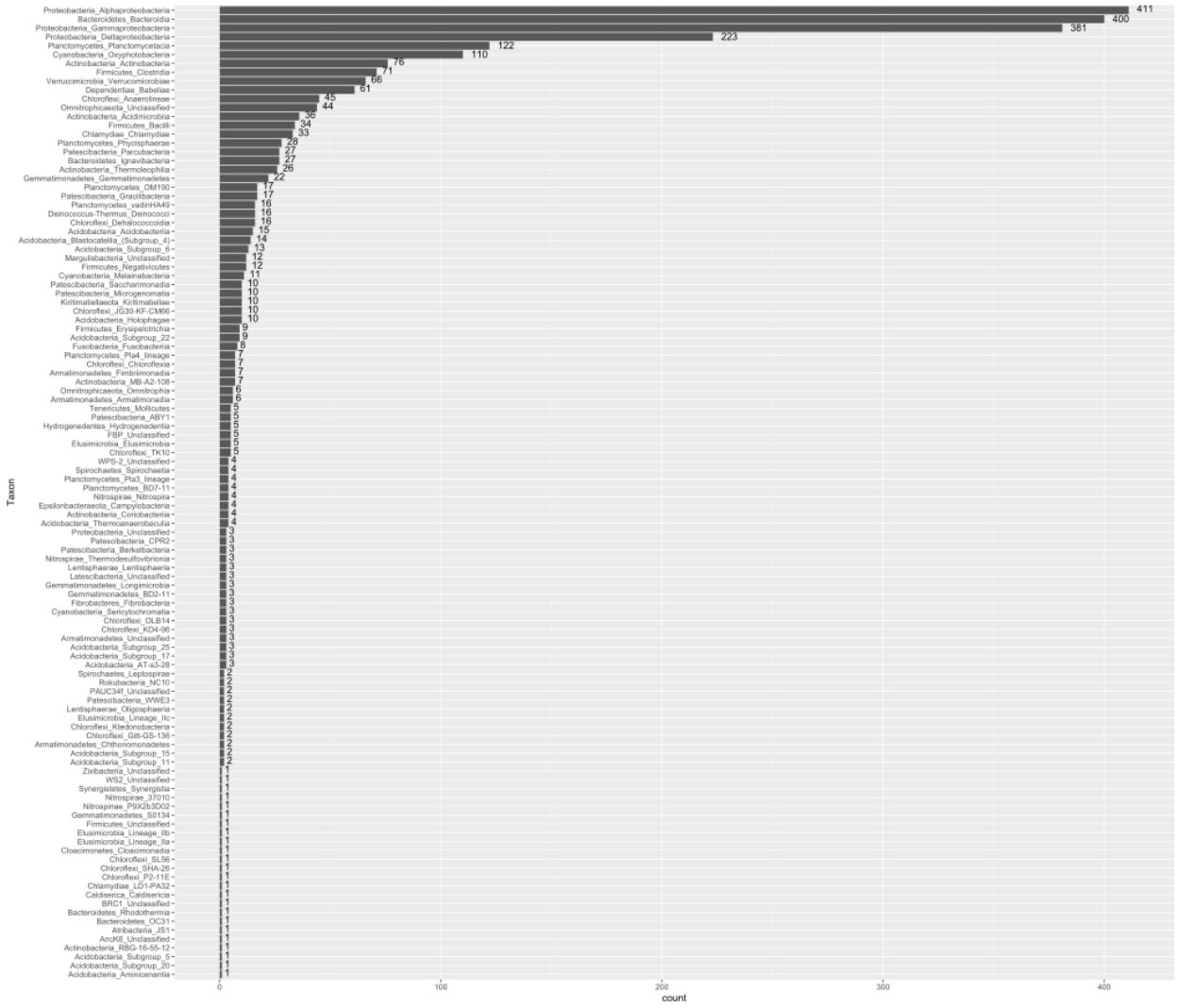

Fig. S6 Number of OTUs per bacterial class.

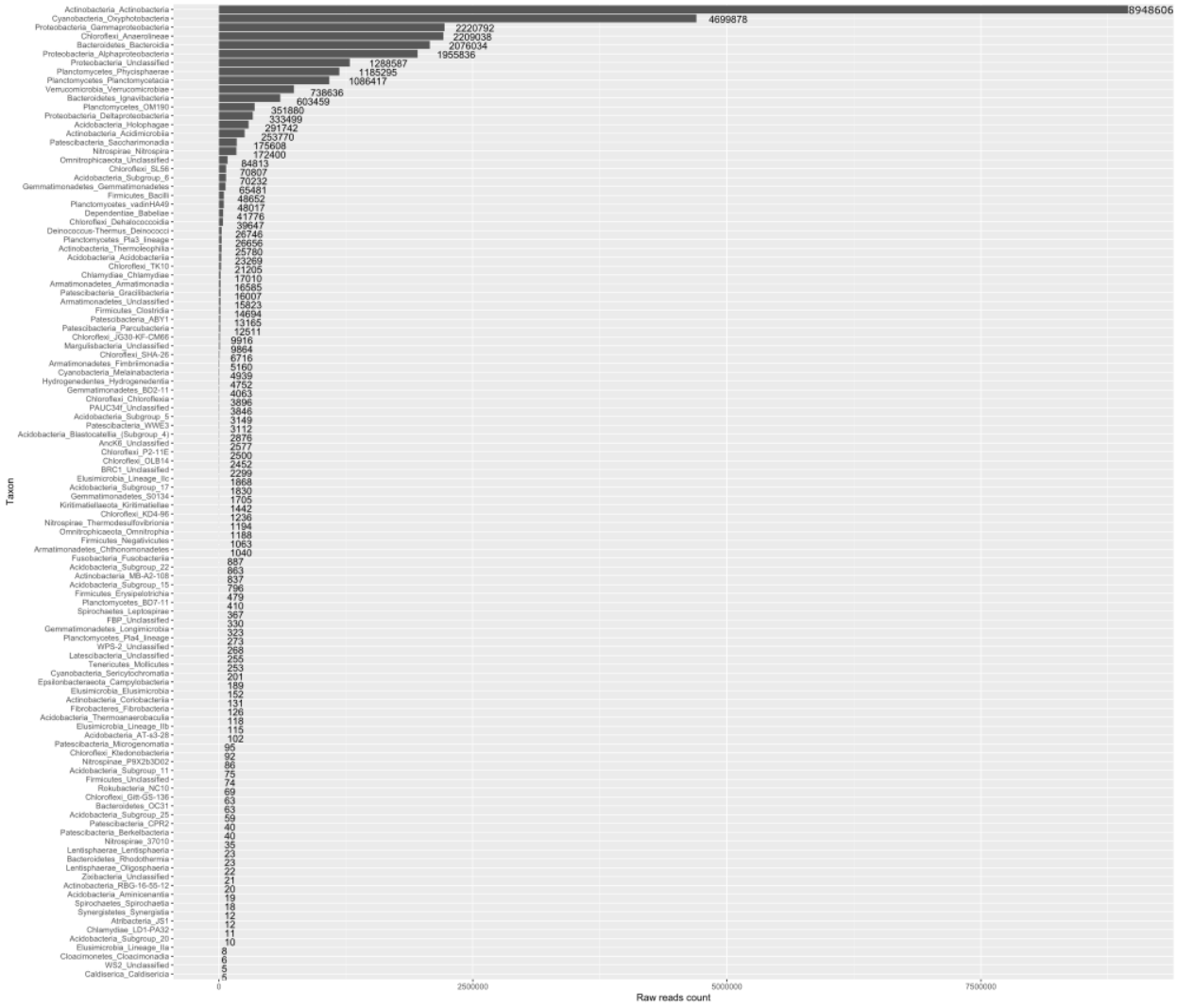

Fig. S7 Number of raw reads per bacterial class.

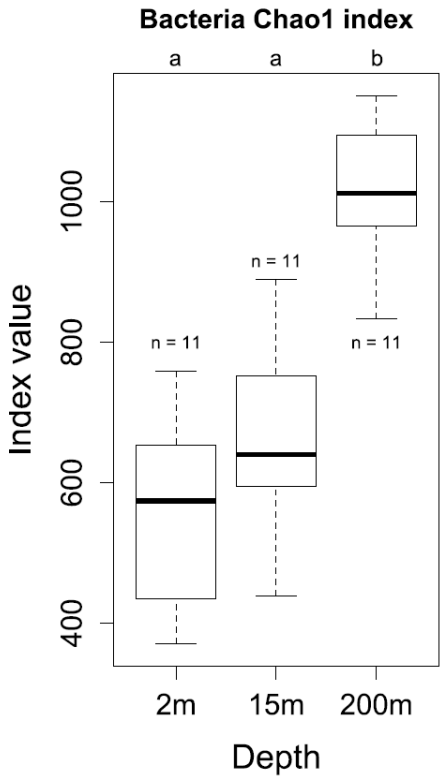

Fig. S8 Chao1 richness index for bacteria showing significant differences between depths. N indicate the number of samples and the letters a and b show where the differences are.

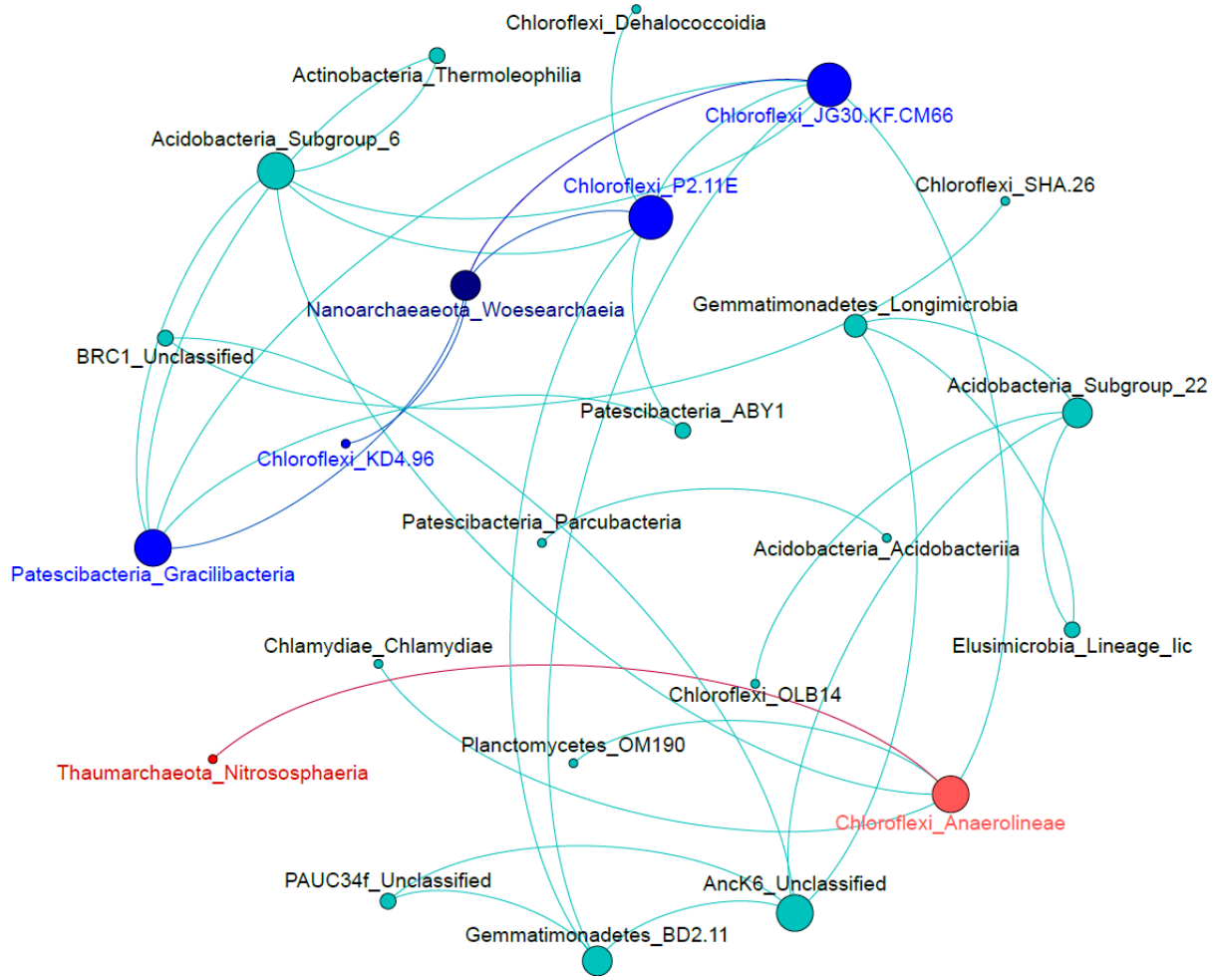

Fig. S9 Co-occurrence network between archaeal and bacterial phyla. Only phylum that passed the cutoff (correlation coefficient ≥ 0.9 and p-value < 0.01) are shown.

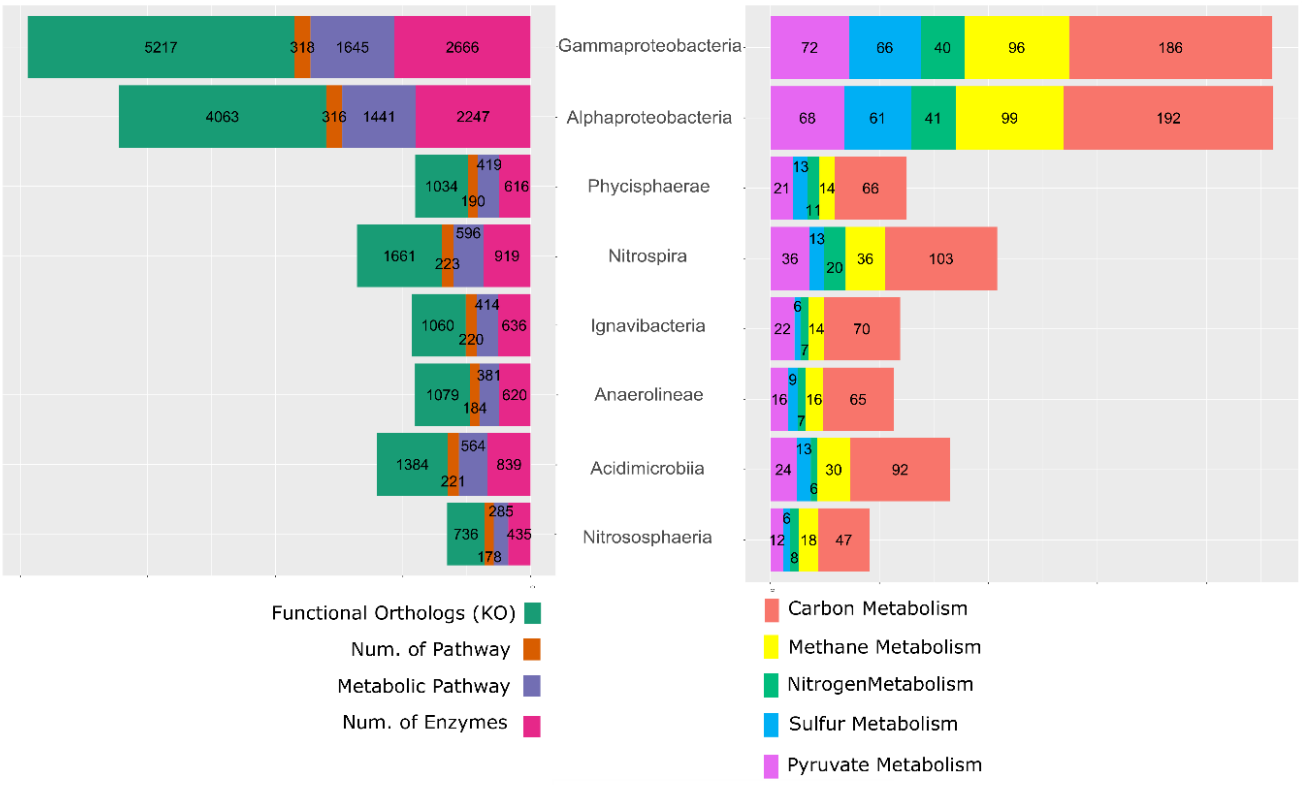

Fig. S10 Functional prediction based on PanFP software of the archaeal OTU 2 Nitrososphaeria and its bacteria associates showed in the co-occurrence network (Fig. 6). The archaea Nitrososphaeria is shown to possess less functional orthologs and pathway than its bacterial associates except for the nitrogen and methane metabolism.

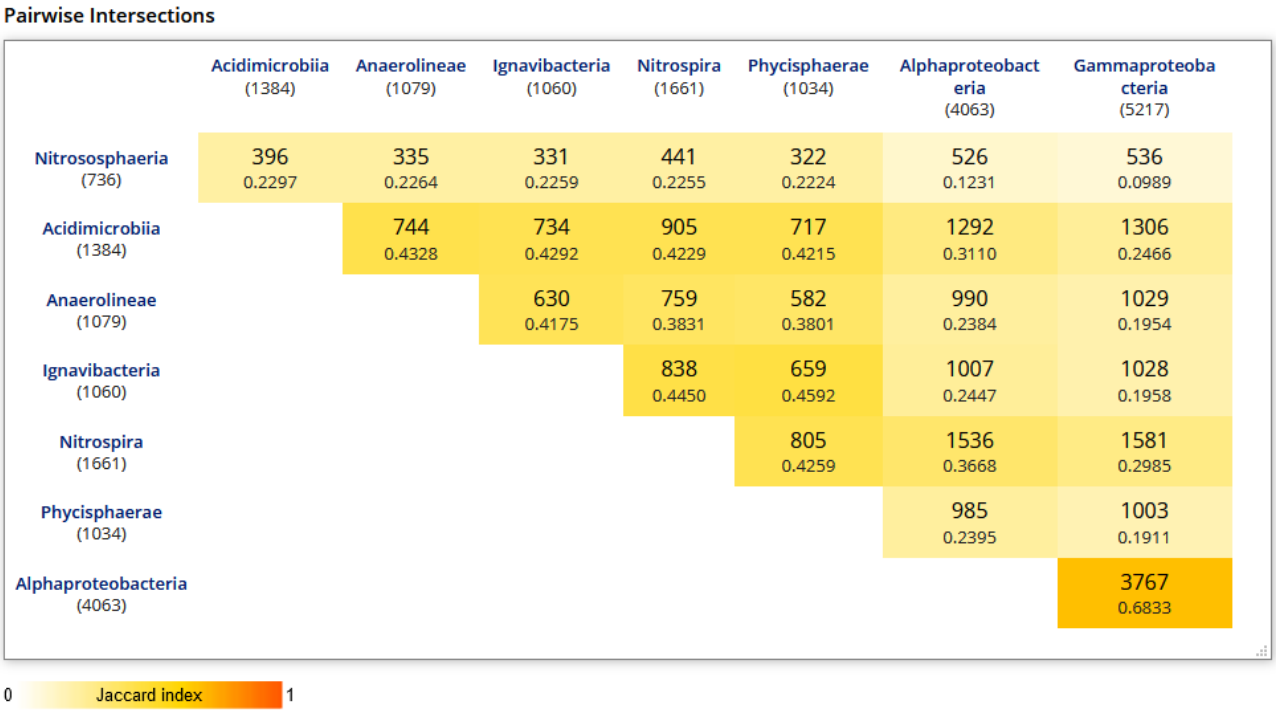

Fig. S11 Pairwise intersection comparison between the numbers of KOs generated *via* PanFP software. The presented class are from the co-occurrence network of Figure 6.

Table S1 Results of the statistical analyses on richness and diversity indices for archaeal OTUs table.

Chao1	Site	Month	Depth
Kruskal p-value	0.5268	0.3121	1.859^{-05} ***
Dunn test			2 m ≠ 200 m (0.0000) 15 m ≠ 200 m (0.0006) 2 m = 15 m (0.5)
Pielou	Site	Month	Depth
Kruskal p-value	0.4466	0.01758*	0.6221
Dunn test		TL1 ≠ TL3 (0.0084*)	
Shannon	Site	Month	Depth
Kruskal p-value	0.3802	0.1672	0.006761
Dunn test			2 m ≠ 200 m (0.0028) 15 m = 200 m (0.0605) 2 m = 15 m (0.4)
Simpson/ InvSimpson	Site	Month	Depth
Kruskal p-value	0.3579	0.1792	0.006761***
Dunn			2 m ≠ 200 m (0.0028) 15 m = 200 m (0.0605) 2 m = 15 m (0.4)

Table S2 Anosim and Adonis results performed on the Bray-Curtis dissimilarity matrix generated from the archaeal OTUs table in order to validate the NMDS graph.

	Site	Month	Depth
Anosim p-value	0.227	0.137	0.001***
Adonis p-value	0.592	0.26	0.003***

Table S3 Simper test results and significance test analysis for archaeal OTUs table.

	Group	Most influential OTU	Kruskal-Wallis (p-value)	Simper cumulative contributions	Average abundances in each compared treatment	Average dissimilarity between the two treatment
Month	February – June	2_Thaumarchaeota_Nitrososphaeria	0.1792	48%	February > June	2%
	February – August	2_Thaumarchaeota_Nitrososphaeria	0.1792	45%	February > August	3%
	February - November	2_Thaumarchaeota_Nitrososphaeria	0.1792	46%	February < November	2%
	June - August	2_Thaumarchaeota_Nitrososphaeria	0.1792	43%	June > August	3%
	June - November	2_Thaumarchaeota_Nitrososphaeria	0.1792	45%	June < November	2%
	August - November	2_Thaumarchaeota_Nitrososphaeria	0.1792	43%	August < November	3%
Site	pt2 - pt4	2_Thaumarchaeota_Nitrososphaeria	0.3579	43%	pt2 = pt4	3%
	pt2 – pt6	2_Thaumarchaeota_Nitrososphaeria	0.3579	45%	pt2 > pt6	2%
	pt4 – pt6	2_Thaumarchaeota_Nitrososphaeria	0.3579	46%	pt4 > pt6	2%
Depth	2m – 15m	2_Thaumarchaeota_Nitrososphaeria	0.006761***	45%	2m > 15m	2%
	2m – 200m	2_Thaumarchaeota_Nitrososphaeria	0.006761***	47%	2m > 200m	3%
	15m – 200m	2_Thaumarchaeota_Nitrososphaeria	0.006761***	40%	15m > 200m	3%

Table S4 Results of the statistical analyses on richness and diversity indices for bacterial OTUs table.

Chao1	Site	Month	Depth
Anova p-value	0.619	0.5945	7.71^{-10}***
Tukey multiple comparisons test			2 m ≠ 200 m (0.000000) 15 m ≠ 200 m (0.0000003) 2 m = 15 m (0.07)
Pielou	Site	Month	Depth
Anova p-value	0.8347	0.4889	4.706^{-06} ***
Tukey multiple comparisons test			2 m ≠ 200 m (0.000004) 15 m ≠ 200 m (0.0006579) 2 m = 15 m
Shannon	Site	Month	Depth
Anova p-value	0.8597	0.4149	4.322^{-08} ***
Tukey multiple comparisons test			2 m ≠ 200 m (0.000000) 15 m ≠ 200 m (0.0000210) 2 m = 15 m (0.07)
Simpson	Site	Month	Depth
Anova p-value	0.9225	0.6342	4.757^{-08} ***
Tukey multiple comparisons test			2 m ≠ 200 m (0.000016) 15 m ≠ 200 m (0.000001) 2 m = 15 m (0.6)
InvSimpson	Site	Month	Depth
Kruskal p-value	0.916	0.6285	2.335^{-05} ***
Dunn test			2 m ≠ 200 m (0.0000) 15 m ≠ 200 m (0.0003) 2 m = 15 m (0.8)

Table S5 Anosim and Adonis results performed on the Bray-Curtis dissimilarity matrix generated from the bacterial OTUs table.

	Site	Month	Depth
Anosim p-value	0.982	0.006 ***	0.001 ***
Adonis p-value	0.975	0.011 **	0.001 ***

Table S6 Simper test results with differences tested by statistical analysis for bacterial OTUs table.

Group		Most influential OTUs	Kruskal-Wallis (p-value)	Simper cumulative contributions	Average abundances in each compared treatment	Average dissimilarity between the two treatment
Month	February – June	5_Cyanobacteria_Oxyphotobacteria	0.0003058 ***	14%	February > June	55%
	February – August	5_Cyanobacteria_Oxyphotobacteria	0.0003058 ***	14%	February < August	54%
	February - November	5_Cyanobacteria_Oxyphotobacteria	0.0003058 ***	15%	February < November	49%
	June - August	3_Cyanobacteria_Oxyphotobacteria	0.3754	9%	June < August	50%
	June - November	4_Chloroflexi_Anaerolineae	0.1434	8%	June < November	51%
	August - November	4_Chloroflexi_Anaerolineae	0.1434	8%	August < November	48%
Site	pt2 - pt4	5_Cyanobacteria_Oxyphotobacteria	0.4996	9%	pt2 > pt4	47%
	pt2 – pt6	5_Cyanobacteria_Oxyphotobacteria	0.4996	8%	pt2 > pt6	48%
	pt4 – pt6	4_Chloroflexi_Anaerolineae	0.6618	8%	pt4 > pt6	49%
Depth	2m – 15m	5_Cyanobacteria_Oxyphotobacteria	0.8098	13%	2m > 15m	39%
	2m – 200m	4_Chloroflexi_Anaerolineae	1.714^{-05} ***	11%	2m < 200m	65%
	15m – 200m	4_Chloroflexi_Anaerolineae	1.714^{-05} ***	12%	15m > 200m	62%

7. References

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