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Liens entre le parasite d'amphibiens Batrachochytrium dendrobatidis et les biofilms benthiques de lacs de montagne pyrénéens

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# Links between the amphibian parasite *Batrachochytrium dendrobatidis* and benthic biofilms of Pyrenean mountain lakes

## Déclaration (FR)

- Cette thèse est composée de six chapitres, tous écrits en langue anglaise (ortographe britannique). Le résumé, l'introduction, la discussion et la conclusion sont aussi présentées en français. Tous les titres de sections, figures, et tableaux sont indiqués en gras dans le texte et référencées de manière croisée (en maintenant la touche contrôle tout en cliquant sur le mot en gras, le lecteur est renvoyé à la section/figure/tableau correspondant). Tous les travaux présentés dans ce document sont issus de mes recherches, avec les contributions de tierces personnes précisées dans l'avant-propos de chaque chapitre. Le document PDF est archivé et consultable sur these.fr. Les résultats, ou autres composants de ce travail, peuvent être copiés, mentionnés, ou relayés à la condition qu'ils ne soient pas utilisés à des fins commerciales, qu'ils ne soient pas altérés, et qu'ils soient cités de la façon suivante:

Sentenac, Hugo (2023) Liens entre les biofilms benthiques et le parasite amphibian *Batrachochytrium dendrobatidis* dans les lacs de montagne des Pyrénées, *Thèse de doctorat univeristare (Ph.D.)*, Université de Toulouse.

## Declaration (EN)

- This thesis is articulated in six chapters, all written in English (British spelling). The abstract, introduction and conclusion are also reported in French. This document is cross-referenced, with references of sections/figures/tables shown in boldface, meaning that the reader can click (while pressing Ctrl) on the link to be directed to the corresponding illustration. All of the work presented here is my own, with contributions of other people acknowledged in the foreword of each chapter. This document is available on these.fr. Results can be copied, mentioned, distributed, or transmitted on the condition that they are not used for commercial purposes, that they are not altered, and they are properly cited as:

Sentenac, Hugo (2023) Links between benthic biofilms and the amphibian parasite *Batrachochytrium dendrobatidis* in mountain lakes of the Pyrenees, *Doctor of Philosophy (Ph.D.) thesis*, Université de Toulouse.

# Remerciements (FR)

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même bien rigolé. J'exprime ma gratitude à tous ceux qui m'ont aidé à collecter des données là-haut : je pense entre autres à Estelle Ribaud, Pauline Benzi, Nikola Zsolnay. Et aussi un grand merci à toi Solène Caubet pour ton efficacité et ton organisation au labo, tu m'as bien avancé !

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A toute ma famille : mes parents, mes grands-parents, mon frère, ma sœur, ainsi qu'à ma nouvelle belle famille, merci d'avoir été là même dans les bons moments comme dans les mauvais. On va surtout retenir les bons : je pense notamment à ce weekend de mariage n°1, fin octobre 2020, où ce n'était pas gagné d'avance avec ce foutu COVID mais où j'ai passé un week-end inoubliable. Vous voir a toujours été une bouffée d'oxygène et une source de motivation. Sans vous, ça n'aurait pas été la même.

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# Valorisation scientifique / Scientific valorisation

## Publications

*Published*

**Sentenac, H.,** Loyau, A., Leflaive, J., and Schmeller, D.S. (2022) 'The significance of biofilms to human, animal, plant and ecosystem health', *Functional Ecology*, 36(2), 294–313, available: https://doi.org/10.1111/1365-2435.13947.

Valenzuela-Sánchez, A., Azat, C., Cunningham, A.A., Delgado, S., Bacigalupe, L.D., Beltrand, J., Serrano, J.M., **Sentenac, H.,** Haddow, N., Toledo, V., Schmidt, B.R., and Cayuela, H. (2022) 'Interpopulation differences in male reproductive effort drive the population dynamics of a host exposed to an emerging fungal pathogen', *Journal of Animal Ecology*, 91(2), 308–319, available: https://doi.org/10.1111/1365-2656.13603.

Schmeller, D.S., Urbach, D., Bates, K., Catalan, J., Cogălniceanu, D., Fisher, M.C., Friesen, J., Füreder, L., Gaube, V., Haver, M., Jacobsen, D., Le Roux, G., Lin, Y.-P., Loyau, A., Machate, O., Mayer, A., Palomo, I., Plutzar, C., **Sentenac, H.,** Sommaruga, R., Tiberti, R., and Ripple, W.J. (2022) 'Scientists' warning of threats to mountains', *Science of The Total Environment*, 853, 158611, available: https://doi.org/10.1016/j.scitotenv.2022.158611.

*Under review*

**Sentenac, H.,** Valenzuela-Sánchez, A., Haddow, N., Delgado, S., Azat, C., and Cunningham, A.A. (in prep.) 'Accounting for bias in prevalence estimation: the case of a globally-emerging pathogen'; submitted to the *Journal of Applied Ecology*

Vecchiato, M., **Sentenac, H.,** Jaffe, J., and Sainsbury, A.W. (under review) 'Health effects of patagial wing tags in red kites (*Milvus milvus*) in England' submitted to the *Journal of Wildlife Diseases*

**Sentenac, H.,** Loyau, A., Zoccarato, L., Jassey, V.E.J., Grossart, H-P., and Schmeller D.S., (In prep.) 'Biofilm community composition is changing in remote mountain lakes with a relative increase in toxigenic algae' submitted to the journal *Global Change Biology*

## Communications

Oral presentation

**Sentenac, H.,** Valenzuela-Sánchez, A., Haddow, N., Delgado, S., Azat, C., and Cunningham, A.A.'Accounting for bias in prevalence estimation: the case of a globally-emerging pathogen': 1st Global Amphibian & Reptile Disease conference (2022, Knoxville, TN, hybrid)

Poster

**Sentenac, H.,** Valenzuela-Sánchez, A., Haddow, N., Delgado, S., Azat, C., and Cunningham, A.A.'Accounting for bias in prevalence estimation: the case of a globally-emerging pathogen': 69th WDA-EWDA conference (2021, Cuenca, ES, hybrid)

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# Liste des abbreviations/ List of abbreviated terms

AIC: Akaike Information Criterion

AMP: Anti-Microbial Peptides

Ao: *Alytes obstetricans*

ASV: Amplicon Sequence Variant

Bd: *Batrachochytrium dendrobatidis*

BSA: Bovine Serum Albumin

CDD: Consecutive Dry Days (see **Table 10**)

CI: Confidence Interval

Cl: Chloride

CLR: Centered Log-Ratio

CSDI: Cold Spell Duration Index (see **Table 10**)

CWD: Consecutive Wet Days (see **Table 10**)

Df: degrees of freedom

DFTD: Devil Facial Tumour Disease

DNA: Deoxyribo-Nucleic Acid

$dO_2$: dissolved dioxygen

DTR: Daily Temperature Range

EPS: Extra-Polymeric Substances

FD: Frost Days (see **Table 10**)

GDR: Gourg de Rabas (lake)

GDM: Generalised dissimilarity model

GL(M)M: Generalised linear (mixed) model

GSL: Growing Season Length (see **Table 10**)

GPL: Global Panzootic Lineage

HDI: Highest Density Interval

HFA: Home-Field Advantage

K: Potassium

ID: icing days (see **Table 10**)

IUCN: International Union for the Conservation of Nature

LDA: Linear Discriminant Analysis

LEfSe: Linear discriminant analysis Effect Size

LT_PRCP: total precipitations during the winter season (long term) prior to sampling

LT_T: average air temperature during the winter season (long term) prior to sampling

MCMC: Markov Chain Monte Carlo

MHC: Major Histocompatibility Complex

Na: Sodium

N.s.: not significant

OTU: Perational Taxonomic Unit

PCA(PCAMIX): (mixed) Principal Component Analysis

PCoA: Principal Coordinate Analysis

PCR (qPCR): Polymerase Chain Reaction (quantitive, or real-time, PCR)

POP: Persistant Organic Pollutant

PRCP (PRCPTOT): precipitations (total precipitations)

PUFA: Poly-Unsaturated Fatty Acids

recentPRCP: total precipitations over the 3 weeks prior to sampling

recentT: average air temperature over the 3 weeks prior to sampling

rRNA: ribosomal RiboNucleic Acid

SE: Standard Error

$SiO_2$: biogenic silica

SDII: simple precipitation intensity index (see **Table 10**)

SSC: Species Survival Commission

SU: summer days (see **Table 10**)

TC/TOC: Total (Organic) Carbon

TN: Total Nitrogen

TCu: Total Cupper

TDRs: Time decay relationships

Tg/TG: Mean temperature (see **Table 10**) / Tryptone-Glucose

TN: Minimum temperature (see **Table 10**)

TSS: Total Sum Scaling

TX: Maximum temperature (see **Table 10**)

WoS: Web of Science

WSDI: Warm Spell Duration Index (see **Table 10**)

ZE: Zoospore Equivalent

# Glossaire (FR)

Les mots définis ci-dessous apparaissent en **gras** dans le texte principal.

- **Algue :** groupe polyphylétique d'organismes eucaryotiques phototrophiques, uni- ou pluri-cellulaires, n'ayant pas de tige, racine, feuille, ou vascularisaton. Typiquement membres des Chlorophytes (royaume végétal, « algues vertes »), ou de Diatomées (« algues brunes »). Inclut aussi au sens large des organismes procaryotes photosynthétiques tels que les cyanobactéries (« algues bleu-vert » ou cyan).
- **Allélopathie :** phénomène selon lequel un organisme produit et sécrète un ou plusieurs composés capables d'influencer (positivement ou négativement) la survie, la croissance, et/ou la reproduction d'un autre organisme.
- **Amplexus :** type de comportement sexuel propre aux espèces à fécondation externe (par exemple, amphibiens ou limules) selon lequel le mâle saisit la femelle avec ses membres antérieurs, et de façon plus ou moins simultanée, fertilise les œufs au fur et à mesure que la femelle les produit. Chez les amphibiens, en fonction de la région anatomique de la femelle qui est saisie par le mâle, l'amplexus peut-être céphalique, axillaire ou lombaire. Le type d'amplexus est caractéristique pour certains groupes taxonomiques.
- **Assemblage :** ensemble de **taxons** phylogénétiquement liés (par exemple, procaryote) présent au sein d'une communauté mais ne la formant pas exclusivement à eux seuls.
- **Biodiversité :** la diveristé des formes de vies à tous niveaux d'organisation biologiques, allant des gènes, populations, espèces, communautés jusqu'aux écosystèmes. Il y a plusieurs façons de mesurer la biodiversité : la diversité $\alpha$, c'est-à-dire la diversité propre à un site (ou à une **communauté**, un **assemblage** ou une **guilde** ; j'utilise seulement site par simplicité) ; la diversité $\beta$, c'est-à-dire les dissimalarité en termes de composition entre deux sites ; la diversité $\eta$, ou le nombre d'espèces en commun entre un ensemble de sites ; finalement, la diversité $\gamma$, qui est en fait la diversité $\alpha$ de tous les sités combinés.
- **Biofilm :** forme de vie en apparence sessile existant au niveau des interfaces (par exemple, solide-liquide or liquide-gaz) où micro-, meio- et certains macro-organismes cohabitent au sein d'une matrice extracellulaire tri-dimensionnelles composées de susbstance extrapolymériques (EPS). Ces EPS peuvent être produits par les habitants du biofilm (on parle alors de biofilm au sens strict) ou non (biofilm au sens large, par exemple désignant les communautés d'organismes vivant dans le mucus digestif ou cutané de certains animaux).
- **Biologie de la conservation** : l'étude de la conservation des formes vivantes, de leur diversité et des écosystèmes dans leur entièreté.
- **Biorémédiation :** méthode d'assainissement pour traiter n'importe quel milieu contaminé en stimulant la biodégradation effectuée par les microorganismes.
- **Biosphère :** désgine tous les organismes vivants sur Terre.
- **Brumation :** état ralenti, d'inactivité, ou de torpeur propre aux animaux ectothermiques (« à sang froid ») pendant les périodes de faibles températures. Similaire à l'hibernation chez les animaux endothermes.
- **Changement global :** changements environnementaux se produisant à l'échelle planétaire et influençant l'ensemble du système terrestre. Il fait généralement référence aux changements inhérents au développement rapide de la population humaine et de ses activités et englobe le changement climatique, la perte de biodiversité, le changement d'affectation des terres, l'utilisation de l'eau douce, l'acidification des océans, l'appauvrissement de la couche d'ozone et diverses formes de pollution (charge d'aérosols atmosphériques,

pollution chimique, pollution par les nutriments, c'est-à-dire l'altération des flux biogéochimiques de phosphore et d'azote, par exemple).

- **Communauté** : ensemble d'organimes d'espèces différentes (ou n'importe quels autres **taxons**) évoluant au même endroit au même moment. Voir aussi **écologie** pour une définition de l'écologie des communautés. Voir aussi **structure des communautés.**
- **Dysbiose** : terme utilisé pour caractériser un déséquilibre dans un microbiote donné (surtout utilisé dans le contexte des microbiotes des animaux), que ce déséquilibre affecte la composition de la communauté microbienne, ses fonctions ou son homéostasie. L'état de dysbiose est en général associé avec un état malade de l'hôte.
- **Écologie** : étude des relations entre les organismes vivants et leur environnement non vivant. Différent de l'environnementalisme, c'est-à-dire la philosophie, l'idéologie et le mouvement social concernant les préoccupations relatives à la conservation de l'environnement et à l'amélioration de la santé de l'environnement. L'écologie comporte de nombreux sous-domaines. **L'écologie des communautés**, qui étudie la biodiversité et les facteurs qui l'influencent, **l'écologie fonctionnelle**, qui étudie les rôles, ou fonctions, des organismes dans les processus de leurs écosystèmes, et **l'écologie des maladies**, qui étudie les mécanismes, les modèles et les effets des interactions hôte-pathogène, en particulier celles des maladies infectieuses ; et bien d'autres comme l'écologie évolutive etc.
- **Écosystème :** complexe de tous les organismes vivants et de leur environnement non vivant qui interagissent comme une unité fonctionnelle.
- **Effet dilution :** théorie avançant que le risque lié aux maladies infectieuses est d'autant plus réduit que la biodiversité est importante. Son antithèse est l'effet d'amplification, selon lequel le risque lié aux maladies augmente avec la biodiversité.
- **Effet prioritaire :** impact(s) qu'une espèce particulière peut avoir sur le développement de la communauté en raison de son arrivée antérieure sur un site. Peut être inhibiteur ou facilitateur.
- **Endémique :** adjectif qualifiant l'état d'un organisme ne se trouvant que dans une certaine zone géographique. Également utilisé pour une maladie humaine, signifiant dans ce cas la présence constante d'une maladie ou d'un agent infectieux dans une zone géographique ou un groupe de population humaine donné (on parle d'enzootie pour les maladies animales et d'enphytotie pour les maladies végétales).
- **Épidémie :** apparition dans une communauté humaine ou une région de cas d'une maladie infectieuse, d'un comportement spécifique lié à la santé ou d'autres événements liés à la santé dépassant nettement le nombre normal de cas. Le terme épizootie est utilisé pour les maladies animales et le terme épiphytotique pour les maladies végétales.
- **Épidémiologie :** étude de la distribution, de la fréquence et des déterminants des événements liés à la santé. L'éco-épidémiologie est un sous-domaine qui étudie les facteurs environnementaux (biotiques et abiotiques) de ces événements liés à la santé ; elle est très proche de **l'écologie des maladies**, bien que cette dernière tende à se concentrer, en pratique (mais pas en théorie), davantage sur les **maladies infectieuses**.
- **Etats stables alternatifs :** en écologie, cette théorie avance que les écosystèmes peuvent exister sous de multiples états (ensemble de conditions biotiques et abiotiques propres) qui ne sont pas transitionnels, mais stables en termes d'organisation sur des temps écologiques significatifs. Quand ils sont perturbés au delà de leur capacité de résistance et résilience, les écosystèmes peuvent alors transitionner d'un état stable à un autre (changement d'état) dans lequel ils produiront des services écosystémiques potentiellement différents.
- **Guilde :** en écologie, groupe de taxons ayant des rôles ou fonctions similaires dans les écosystèmes.

- **Holobionte :** c'est l'ensemble désignant l'hôte et toutes ses espèces commensales et symbiotiques (vivant dans ou sur l'hôte). Synonyme de superorganisme. Ce terme est de plus en plus utilisé pour indiquer que l'hôte seul n'est pas l'unité soumise à la pression sélective, car les organismes multicellulaires ont probablement toujours évolué avec leur microbiote, ce dernier étant d'une importance capitale pour la **santé** de l'hôte.
- **Homéostasie :** état de stabilité des conditions internes, physiques et chimiques maintenues par les systèmes vivants.
- **Hyperkératose :** désigne l'augmentation de l'épaisseur de la couche cornée, la couche externe de la peau.
- **Hyperplasie :** augmentation du nombre de cellules dans un organe ou un tissu. Ces cellules semblent normales au microscope et ne sont pas nécessairement cancéreuses.
- **Infection :** entrée et développement ou multiplication d'un organisme (appelé agent infectieux) dans un autre organisme, généralement plus grand (appelé hôte). L'infection n'est pas synonyme de maladie infectieuse, car le résultat de l'infection peut ne pas se manifester et être cliniquement inapparent.
- **Intéractions écologiques** : les interactions bidirectionnelles peuvent être classées en six types principaux en écologie (0 dénotant l'absence d'effet, + un effet positif, - un effet négatif) : +/+ mutualisme (ou coopération) ; +/0 commensalisme ; +/- antagonisme (comprend toute forme de consommation : la prédation, l'herbivorie, le parasitisme, le cannibalisme, etc.) ; -/- compétition ; -/0 amensalisme ; 0/0 neutralisme. Notez que la facilitation (une espèce profite d'une autre espèce) comprend le mutualisme, le commensalisme et l'antagonisme.
- **Maladie :** toute anomalie de structure et/ou fonction qui affecte la santé d'un organisme. Les maladies peuvent être infectieuses ou non. Les maladies émergentes (pas nécessairement infectieuses) sont celles dont l'incidence (nombre de cas par unité de temps) et/ou la distribution géographique augmentent.
- **Maladie infectieuse (ou maladie transmissible) : maladie** résultant d'une **infection** ou des effets de produits toxiques résultant de la présence d'un agent infectieux au sein de l'hôte. Les **maladies infectieuses émergentes** sont causées par des agents pathogènes ou des parasites nouvellement évolués (ou l'une de leurs souches nouvellement évoluées), ou des maladies dont la distribution géographique, le spectre d'hôtes et/ou l'incidence (nombre de cas par unité de temps) ont augmenté de manière significative sur une période relativement courte.
- **Métagénomique :** processus à haut débit utilisé pour caractériser le métagénome, c'est-à-dire la collection de génomes et de gènes des membres d'un biote.
- **Métacommunauté :** ensemble de communautés locales qui sont liées par la dispersion de multiples organismes, potentiellement en interaction. La dynamique des patchs (colonisation, compétition), le tri des espèces (dû à l'hétérogénéité environnementale), la dynamique source/puits (effet de masse) et la perspective neutre (processus démographiques stochastiques et capacités de dispersion limitées) sont les cadres centraux de la théorie des métacommunautés.
- **Métataxonomie (ou métabarcoding) :** processus à haut débit utilisé pour caractériser/identifier l'ensemble des membres d'un biote à partir de l'amplification et du séquençage d'un gène marqueur taxonomique.
- **Microbiote** : assemblage de micro-organismes dans un environnement donné.
- **Microbiome :** désigne l'ensemble du microbiote et de son environnement proche. Il peut contenir des organismes autres que microorganismes.
- **Niche :** l'adéquation d'une espèce à une condition environnementale spécifique.

- **Osmorégulation :** régulation active de la pression osmotique des fluides corporels d'un organisme pour maintenir **l'homéostasie** de la teneur en eau de l'organisme ; c'est-à-dire qu'elle maintient l'équilibre des fluides et la concentration des électrolytes (sels en solution qui, dans ce cas, sont représentés par les fluides corporels) pour éviter que les fluides corporels ne deviennent trop dilués ou concentrés.
- **Pandémie/Panzootie :** qualifie une maladie épidémique/épizootique qui a une ampleur mondiale.
- **Parasite :** organisme (historiquement, multicellulaire mais pas nécessairement) qui vit sur ou dans un autre et en tire son énergie. Un parasite n'est pas nécessairement pathogène, c'est-à-dire qu'il ne provoque pas nécessairement de maladie. L'adjectif **parasite** peut qualifier tout organisme (y compris unicellulaire) qui vit aux dépens d'un autre.
- **Partitionnement (ou différenciation ou complémentarité) de niches :** processus par lequel des espèces concurrentes utilisent l'environnement différemment, de manière à pouvoir coexister. Par exemple, différentes espèces de décomposeurs ou détritivores microbiens utiliseront différentes niches, avec différents types de nourriture ou d'autres ressources. Le partage des ressources est l'un des nombreux mécanismes de complémentarité (la facilitation en est un autre ; voir **intéractions écologiques**) entraînant des effets positifs sur la diversité.
- **Pathogène :** organisme (ou adjectif désignant un organisme) capable de provoquer une maladie (littéralement, de provoquer un processus pathologique).
- **Pathogénicité :** le pouvoir d'un organisme de produire une maladie chez un hôte donné. Il existe plusieurs définitions qui se recoupent plus ou moins avec la définition de la virulence. Ici, je désambigüise entre ces deux concepts en considérant la pathogénicité comme la qualité ou l'état d'être pathogène ou non, et la virulence comme le degré de pathogénicité.
- **Périphyton :** mélange complexe et gélatineux d'algues, de cyanobactéries, de micro-organismes hétérotrophes (champignons, micro-animaux et protistes) et de détritus qui sont fixés aux surfaces immergées dans la plupart des écosystèmes aquatiques. Lié au terme allemand *Aufwuchs*. Étymologiquement, ce terme désignait les communautés se développant sur les plantes (épiphyton), mais il a ensuite rallié tous les biofilms se développant sur les plantes, les animaux à carapace et les surfaces non biologiques. De nombreux têtards d'anoures sont des consommateurs (dits « brouteurs ») de périphyton.
- **Polyphylétique :** qualifie un regroupement d'organismes plutôt basé sur leur(s) ressemblance(s) morphologique(s) que sur leur phylogénie. Le groupe n'a pas d'ancêtre commun propre. Opposé à monophylétique, groupe ayant un ancêtre commun qui lui est propre.
- **Propriétés émergentes :** on dit qu'un système a des propriétés émergentes s'il présente des propriétés qui ne peuvent être expliquées par ses composants pris individuellement. À ne pas confondre avec **l'émergence d'une maladie** (augmentation soudaine de l'incidence, de la distribution et/ou de la gamme d'hôtes).
- **Protiste :** terme désignant un groupe **polyphylétique** d'eucaryotes autres que les animaux, les plantes et les champignons, généralement monocellulaires, ou multicellulaires mais alors sans différenciation tissulaire.
- **Protozoaires :** sous-ensemble de protistes hétérotrophes et en général unicellulaire.
- **Quorum-sensing :** régulation de l'expression des gènes en réponse aux fluctuations de la densité de la population cellulaire, principalement par l'intermédiaire de molécules de signalisation chimiques.
- **Redondance fonctionnelle :** synonyme d'équivalence fonctionnelle, reflète le fait que, dans une communauté ou un écosystème, plusieurs groupes taxonomiques ont des fonctions similaires (par exemple, la fixation de l'azote). En pratique, cela signifie que la perte d'un taxon n'aura pas d'impact sur le fonctionnement de l'écosystème tant que d'autres peuvent assumer ses rôles. La redondance sous-tend la résilience

- **Réservoir :** L'habitat naturel d'un agent infectieux ou tout organisme dans lequel un agent infectieux peut théoriquement être maintenu indéfiniment.
- **Résilience :** en écologie, la résilience est la capacité d'un écosystème à répondre à une perturbation ou un dérangement (par exemple, un incendie, une sécheresse) non seulement en résistant aux dommages, mais aussi en étant capable de se rétablir rapidement pour reprendre son état stable initial. Ce n'est pas un synonyme de résistance, mais la résistance est une composante de la résilience.
- **Résistance :** propriété d'un système à rester inchangé. Peut s'appliquer soit à l'hôte dans un contexte épidémiologique, c'est-à-dire la capacité de l'hôte à limiter l'infection ; soit à l'écosystème, c'est-à-dire la capacité à résister aux changements et à rester dans un état stable.
- **Santé :** au sens strict, état d'absence de maladie, de malaise ou de blessure. Dans son sens le plus large, état de bien-être physique, moral et social, et pas seulement synnonyme d'une simple absence de maladie. Le concept de santé s'applique principalement à l'homme et aux animaux vertébrés, mais il a également été utilisé par analogie pour désigner le bon état "normal" d'une entité, par exemple la santé d'un écosystème**.**
- **Service écosystémique :** processus issus de l'écosystème qui profite aux humains, par la fourniture de biens et/ou de bien-être. Opposé de disservice écosystémique.
- **Struture (ou organisation) des communautés :** réfère à la composition taxonomique (par exemple en espèce) et à l'abondance de chaque taxon appartenant à la communauté. La structure, composition ou l'organisation des communautés sont utilisées de manière interchangeable dans ce manuscrit. Cependant, le terme de structure d'une communauté peut aussi comprendre la façon dont les taxons intéragissent entre eux.
- **Succession écologique :** le processus de changement de la structure des taxons d'une communauté écologique au fil du temps. La succession est parfois appelée une sère, elle commence avec une ou quelques espèces pionnières et se termine par un **état stable** ou mature, appelé climax (qui pourrait théoriquement persister indéfiniment s'il n'y avait pas d'événements perturbateurs majeurs).
- **Susceptibilité :** vulnérabilité aux effets de l'infection. Se rapporte à la résistance et à la tolérance.
- **Taxon :** groupe d'une ou plusieurs populations d'organismes considérés par les taxonomistes comme formant une unité, par exemple sous-espèce, espèce, genre, famille, ordre, classe, phylum, royaume, empire.
- **Théorie de l'avantage du terrain (home-field advantage**) : cette théorie postule que les décomposeurs microbiens sont spécialisés dans l'utilisation et la colonisation des substrats qu'ils rencontrent le plus fréquemment, ce qui conduit à prédire que la décomposition de la litière est plus rapide dans le voisinage immédiat de la plante dont elle provient, ou dans une zone dominée par la même espèce végétale.
- **Théorie de la diversité-stabilité :** hypothèse selon laquelle plus une communauté est riche en biodiversité, plus elle est productive et stable dans le temps (résilience), d'après le fait que des communautés stables et productives peuvent utiliser les ressources de manière plus efficace que des communautés moins diverses.
- **Tolérance :** dans un contexte épidémiologique, c'est la capacité à limiter l'effet d'une infection. Ne pas confondre avec résistance, la capacité à limiter l'infection (le nombre d'agent infectieux) en elle-même.
- **Virulence :** le degré de pathogénicité. Elle peut être mesurée par le rapport entre le nombre d'individus développant une maladie clinique et le nombre d'individus exposés à l'infection.
- **Xénobiotique :** substance présente dans un organisme qui n'est pas produite naturellement ou dont on ne s'attend pas à ce qu'elle soit présente dans cet organisme.
- **Zoospore :** spore de certains champignons capable de nager au moyen d'un flagelle.

# Glossary (EN)

Words defined in the glossary appear in **boldface** in the main text.

- **Alga** (*plural* **algae**)**:** term designating a polyphyletic group of phototrophic, eukaryotic, monocellular or multicellular organisms that lack true stems, roots, leaves, and vascular tissue, typically a member of Chlorophyta (plant kingdom, “green algae”) or Diatoms (“brown algae”). In the broadest sense, it also includes prokaryotic organisms able to perform photosynthesis such as cyanobacteria (“blue-green algae”).
- **Allelopathy**: biological phenomenon by which an organism produces one or more chemical substances able to influence (positively or negatively) the survival, growth, and/or reproduction of another organism.
- **Alternative stable states:** in ecology, this theory posits that ecosystems can exist under multiple "states" (sets of unique biotic and abiotic conditions) which are non-transitory, i.e. stable over ecologically-meaningful timescales. When disturbed beyond their capacity of resistance and resilience, ecosystems can transition from one state to another (termed a state shift) in which it will provide more or less, or altogether different ecosystem services.
- **Amplexus:** type of mating behavior exhibited by some externally fertilising species (e.g. amphibians and horseshoe crabs) in which a male grasps a female with his front legs as part of the mating process, and at the same time or with some time delay, he fertilizes the eggs, as they are released from the female's body. In amphibians, depending on the region grasped by the male, the amplexus can be cephalic, axillary or lumbar, and the type of amplexus is characteristic of some taxonomic groups
- **Assemblage:** collection of phylogenetically-related **taxa** present within a community but not forming it exclusively on their own.
- **Biodiversity:** the variety of all life forms, at any level of biological organization, from genes, populations, species, communities to ecosystems. There are several concepts and ways to measure biodiversity: α-diversity, i.e. the diversity in one site (or **community** or **assemblage** or **guild**, I use only site here for clarity); β-diversity, i.e. the differences in diversity between two sites; η-diversity, i.e. the number of species in common between a set of sites, and finally; γ-diversity, which is essentially the α-diversity of all sites in the landscape.
- **Biofilm:** sessile form of life occurring at interfaces (e.g. solid-liquid or liquid-gas) where micro- and other organisms live embedded in a three-dimensional matrix, made out of extrapolymeric substances (EPS). These EPS can be self-producted by biofilm inhabitants (biofilm *sensu stricto*) or not (e.g. animal mucus, biofilm *sensu lato*).
- **Bioremediation:** method of sanitation used to treat any contaminated medium by stimulating microorganism-based biodegradation.
- **Biosphere:** all living organisms developing on Earth.
- **Brumation:** a state or condition of sluggishness, inactivity, or torpor exhibited by ectothermic animals during winter or extended periods of low temperature. The equivalent is hibernation in endothermic animals.
- **Community**: the collection of species (or of any other **taxa**) occurring at same place at a same time. See **ecology** for a definition of community ecology, and **community structure**
- **Community structure (or organisation):** the structure of a community or ecosystem refers to the compositions of taxa belonging to it, and their abundance. It relates to **α-diversity**. Community composition,

community structure or community organisation are used interghangeably here. However, the structure of a community may also encompass the ways its species interact together.

- **Conservation biology:** the study of the conservation of life, its diversity, and its ecosystems, the aim of which is to produce robust evidence and leverage it to better inform conservation practice.
- **Dilution effect theory:** theory predicting that the risks of infectious diseases are reduced with increased biodiversity. The converse theory (disease risks increase with biodiversity) is the amplification effect hypothesis.
- **Disease:** any abnormality in structure and/or function that adversely affects the health of an organism. Can be infectious or not. Emerging diseases are diseases (not necessarily **infectious diseases**) of which the incidence (number of cases per unit of time) or geographical range has increased over a short period of time.
- **Diversity-stability theory**: this theory states that the more diverse a community is, the more stable (resilient) and productive it is, on the basis that more stable and productive communities can use their resources more efficiently than less diverse communities.
- **Dysbiosis**: term used to characterise any imbalance in a given microbiota (especially used for metazoan-associated microbiota), whether it be a change in its composition, in functions, or any disruption of its homeostasis. Dysbiosis is in general associated with a diseased state of the host.
- **Ecological interactions:** two-way interactions can be classified into six main types in ecology (0 denoting no effect, + a positive effect, - a negative effect): +/+ mutualism (or cooperation); +/0 commensalism; +/- antagonism (includes predation, grazing, parasitism, cannibalism and so on); -/- competition; -/0 amensalism; 0/0 neutralism. Note that facilitation (one species benefit from another species) includes mutualism, commensalism, and antagonism.
- **Ecological succession:** the process of change in the species structure of an ecological community over time. The whole series succession is sometimes called a sere, starting with one or few pioneering species and ending with a stable (i.e. in balance with the biotic and abiotic environment) or mature stage, called the climax (which could theoretically persist indefinitely if it was not for major disturbing events).
- **Ecology:** the study of the relationships between living organisms and their non-living environment. Different from environmentalism, i.e. the philosophy, ideology and social movement regarding concerns for environmental conservation and improvement of the health of the environment. Ecology has many subfields. Of interest here are **community ecology**, which investigates biodiversity and the factors influencing it; **functional ecology** which investigates the roles, or functions, that organisms have in the processes of their ecosystems; **disease ecology** which studies the mechanisms, patterns, and effects of host-pathogen interactions, particularly those of infectious diseases; and others such as evolutionary ecology.
- **Ecosystem**: complex of all living organisms and their non-living environment interacting as a functional unit.
- **Ecosystem service:** ecosystem processes that benefit humans, through provision of goods and/or well-being. Opposite of ecosystem disservice.
- **Emergent properties:** a system is said to have emergent properties if it displays properties that cannot be explained by its individual components. Not to be confused with the emergence of disease (see infectious diseases; sudden increase in incidence, distribution, and/or host range).
- **Endemic:** adjective qualifying the state of an organism being found only in a certain geographic location. Also used for a human disease, meaning in that case the constant presence of a disease or infectious agent within a given geographic area or human population group (enzootic is used for animal diseases and enphytotic for plant diseases).

- **Epidemic:** (noun or adjective) the occurrence in a human community or region of cases of an infectious disease, specific health-related behavior, or other health-related events clearly in excess of normal expectancy. Epizootic is used for animal diseases and epihytotic for plant diseases.
- **Epidemiology:** it is the study of the distribution, frequency, and determinants of health-related events. Eco-epidemiology is a subfield investigating the environmental drivers (biotic and abiotic) of these health-related events; it is very close to **disease ecology**, although the latter might focus, in practice (but not in theory), more on **infectious disease**.
- **Functional redundancy**: synonym to functional equivalence, reflects that, in a community or ecosystem, multiple taxonomic groups (e.g. species) have similar functions (e.g. N fixation). Practically, it means that the loss of one taxon will not impact ecosystem functioning as long as other equivalent taxa can take over its roles. Functional redundancy underlies **resilience**.
- **Global change**: environmental changes occurring at a planetary scale, and influencing the whole Earth system. It generally refers to the changes inherent to the rapid development of human population and activities, and encompasses climate change, biodiversity loss, land-use change, freshwater use, ocean acidification, ozone depletion, and various forms of pollution (atmospheric aerosol loading, chemical pollution, nutrient pollution i.e. alteration of biogeochemical flows for instance, of phosphorus and nitrogen).
- **Guild**: group of taxa having similar roles in an ecosystem.
- **Health**: strictly speaking, the state of being free from **disease**, illness or injury. In its broadest sense, state of physical, moral, and social well-being, and not only as the mere absence of disease. The concept of health mainly applies to humans and vertebrate animals, but has also been used as an analogy to refer to the good "normal" state of an entity, e.g. ecosystem health.
- **Holobiont**: the host plus all its commensal and symbiotic species (living in or on the host). Synonym to superorganism. This term is increasingly used to denote that the host alone is not the unit subject to selective pressure, as multicellular organisms have likely always evolved with their microbiota, the latter being of paramount importance to host health.
- **Home-Field Advantage:** this theory posits that microbial decomposers are specialised in using and colonising the substrates that they most frequently encounter, leading to the prediction that litter decomposition is faster in the close vicinity of the plant from which it originates, or in an area dominated by the same plant species.
- **Homeostasis**: state of steady internal, physical, and chemical conditions maintained by living systems.
- **Hyperkeratosis**: refers to the increased thickness of the *stratum corneum*, the outer layer of the skin.
- **Hyperplasia**: increase in the number of cells in an organ or tissue. These cells appear normal under a microscope and are not necessarily cancerous.
- **Infection**: The entry and development or multiplication of an organism (called the infectious agent) in another, usually larger, organism (called the host). Infection is not synonymous of **infectious disease**, as the result of infection may not manifest and may be clinically inapparent
- **Infectious disease** (or communicable disease): **Disease** due to the result of an **infection** or the effects of toxic products that arises from the presence of an infectious agent within the host. **Emerging infectious diseases** are caused by newly evolved pathogens or parasites (or one of their newly evolved strains), or diseases of which the geographical distribution, host range and/or the incidence (number of cases per unit of time) have significantly increased in a relatively short period of time.

- **Metagenomics:** high-throughput process used to characterise the metagenome, i.e. the collection of genomes and gene from members of a biota.
- **Metacommunity**: A metacommunity is a set of local communities that are linked by the dispersal of multiple, potentially interacting, species. Patch dynamics (colonisation, competition), species sorting (due to environmental heterogeneity), source/sink dynamics (mass effect) and neutral perspective (stochastic demographic processes and limited dispersal abilities) are central frameworks of the metacommunity theory.
- **Metataxonomics (or metabarcoding)**: high-throughput process used to characterise/identify all members of a biota based on the amplification and sequencing of a taxonomic marker gene.
- **Microbiota**: assemblage of microorganisms in a given environment.
- **Microbiome**: refers to the microbiota and its their surrounding environment combined. Might contain organisms other than microorganisms.
- **Niche**: the match of a species to a specific environmental condition.
- **Niche partitioning (or differentiation)**: process by which competing species use the environment differently in a way that helps them to coexist. For instance, different microbial decomposer or detritivore species will use different **niches**, with different food types or other resources. Resource partitioning is one of several complementarity mechanisms (facilitation is another one, see **ecological interactions**) leading to positive diversity effects.
- **Osmoregulation**: the active regulation of the osmotic pressure of an organism's body fluids to maintain the **homeostasis** of the organism's water content; that is, it maintains the fluid balance and the concentration of electrolytes (salts in solution which in this case is represented by body fluid) to keep the body fluids from becoming too diluted or concentrated.
- **Pandemic/Panzootic**: qualifies an epidemic/epizootic disease that has a global scale.
- **Parasite**: An organism (historically, multicellular but not necessarily) that lives on or in another and derives its energy therefrom. A parasite is not necessarily pathogenic, i.e. causing disease.
- **Pathogen (pathogenic agent)**: An organism capable of causing disease (literally, causing a pathological process).
- **Pathogenicity**: The power of an organism to produce disease in a given host. Several definitions exist which more or less overlap with the definition of virulence. Here, I disambiguate between these two concepts by considering pathogenicity as the quality or state of being pathogenic or not, and virulence as the degree of pathogenicity.
- **Periphyton**: complex gelatinous mixture of algae, cyanobacteria, heterotrophic microorganisms (fungi, micro-animals and protists), and detritus that is attached to submerged surfaces in most aquatic ecosystems. Related to german term *Aufwuchs*. Etymologically, this denoted communities growing on plants (epiphyton), but then rallied all **biofilms** growing on plants, animals with shells, and non-biological surfaces. Many tadpoles are periphyton grazers.
- **Polyphyletic**: qualifies a grouping of organisms rather based on their morphological resemblance than their phylogeny. The group has no a common ancestor of its own. Opposite of monophyletic, a group having a common ancestor of its own.
- **Priority effect:** impact(s) that a particular species can have on community development due to prior arrival at a site. Can be inhibitive or facilitative.

- **Productivity (or vigor)**: productivity is the rate at which energy is added to the bodies of organisms in the form of biomass. In ecosystems, there are two kinds of productivity: primary productivity, which refers to the energy (mostly solar) fixed by autotrophic communities into organic forms (chemical energy); and secondary productivity, which refers to heterotrophic communities which use the chemical energy produced by autotrophs.
- **Protist**: term designating a polyphyletic group of eukaryotes other than animals, plants and fungi, generally monocellular although sometimes multicellular but with no tissue differenciation.
- **Protozoans**: subgroup of protists, in general single-celled and heterotroph.
- **Quorum-sensing:** Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density, mainly mediated by chemical signal molecules.
- **Reservoir:** The natural habitat of an infectious agent or any organisms in which an infectious agent can theoretically be maintained indefinitely.
- **Resilience**: in ecology, resilience is the capacity of an ecosystem to respond to a perturbation or disturbance (e.g. fire, drought) by not only resisting damage but also being able to recover quickly to resume its original stable state. It is not a synonym of resistance, but resistance is a component of resilience.
- **Resistance**: the property of a system to remain unchanged. Can apply either to host in an epidemiological context, i.e. the ability of the host to limit infection; or to ecosystem, i.e. the ability to resist changes and stay in the stable state.
- **Susceptibility:** vulnerability to the effects of infection. Relates to **resistance** and **tolerance**.
- **Taxon (***plural* **taxa)**: group of one or more populations of an organism or organisms seen by taxonomists to form a unit, e.g. subspecies, species, genus, family, order, class, phylum, kingdom, empire.
- **Tolerance**: in an epidemiologicl context, it is the ability to limit or bear the effect of infection. Not to be confounded with resistance, the ability to limit the infection in itself (i.e. the number of infectious agents).
- **Virulence**: the degree of pathogenicity; the disease-evoking power of an infectious agent in a given host. Can be measured by the ratio of the number of individuals developing clinical illness to the number exposed to infection.
- **Xenobiotic:** a substance found within an organism that is not naturally produced or expected to be present within this organism.
- **Zoospore:** a spore of certain fungi capable of swimming by means of a flagellum.

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# Résumé détaillé (FR)

Les écosystèmes d'eau douce de montagne fournissent des services essentiels à l'humanité, comme la provision d'eau claire, mais sont fortement affectés par les changements globaux anthropogéniques malgré leur apparente isolation. Les biofilms benthiques, communautés d'organismes vivant dans une matrice adhérant aux surfaces immergées, ont des fonctions critiques dans les lacs de montagne : entre autres, ils détoxifient l'eau et forment la base des réseaux trophiques. Toutefois, leur composition et leur biodiversité sont encore mal connues. Aussi, dans les Pyrénées, certaines populations d'amphibiens sont menacées par la chytridiomycose, une maladie infectieuse causée par le champignon zoosporique *Batrachochytrium dendrobatidis* (Bd). Son épidémiologie n'est pas entièrement comprise mais pourrait dépendre des biofilms, abondants dans les lacs d'altitude et constituant la nourriture des têtards.

Ici, j'avais deux objectifs principaux : premièrement, étudier les variations spatio-temporelles de la biodiversité microbienne des biofilms ; deuxièmement, investiguer le potentiel rôle des biofilms dans l'épidémiologie des infections à Bd. Pour ce faire, j'ai réalisé une analyse métataxonomique des assemblages procaryotes et micro-eucaryotes de 230 échantillons de biofilms collectés de 2016 à 2020 dans 26 lacs d'altitude des Pyrénées. En combinant cela avec des données d'infection par Bd de têtards échantillonnés dans les mêmes lacs, j'ai exploré les liens entre la composition des biofilms et la distribution, la fréquence et l'impact populationnel des infections par Bd. En laboratoire, j'ai aussi testé si un biofilm pouvaient affecter le stade libre et infectieux de Bd, la zoospore. Mes hypothèses étaient que la biodiversité des biofilms diminuerait et leur composition changerait au cours de l'étude, que les biofilms des lacs dont les populations d'amphibiens sont moins infectées/impactées contiendraient plus d'antagonistes de Bd que les biofilms d'autres lacs, et que les biofilms produits en laboratoire n'affecteraient pas le nombre de zoospores à moins qu'ils ne contiennent des consommateurs de Bd.

La diversité des assemblages procaryotes et micro-eucaryotes du biofilm a diminué au cours de la période d'étude. Leur composition a aussi changé pendant les cinq années, avec une augmentation des cyanobactéries (organismes possiblement toxinogènes) chez les procaryotes et une diminution des diatomées (organismes indicateurs) chez les micro-eucaryotes. Pris ensemble, ces résultats montrent que les communautés de biofilms benthiques se dégradent avec des implications potentiellement négatives pour tout l'écosystème aquatique et la qualité de l'eau. D'autre part, j'ai constaté que les biofilms des lacs où les amphibiens sont moins infectés et moins impactés présentaient une plus grande abondance d'organismes inhibiteurs ou

consommateurs de Bd. Enfin, j'ai montré que les biofilms, même lorsqu'ils ne contiennent pas de consommateur de Bd mais ne serait-ce qu'une algue phototrophique, peuvent affecter les zoospores de Bd en les inactivant ou en les forçant à s'immobiliser.

Mes recherches transdisciplinaires illustrent les interactions entre la santé environnementale et les santés animale et publique. Les changements environnementaux contemporains détériorent les biofilms, la base même des réseaux alimentaires des lacs de montagne. Ceci pourrait avoir de profonds effets en cascade sur l'ensemble des socio-écosystèmes de montagne, comme l'augmentation potentielle du risque infectieux posé par Bd et la chytridiomycose pour les amphibiens, et de cyanotoxicose pour tous les vertébrés qui fréquentent les lacs de montagne, y compris les humains et le bétail. Si l'on veut que les écosystèmes d'eau douce de montagne continuent à fournir des services plutôt que des disservices, il faudra rapidement identifier et atténuer les facteurs contribuant au changement des biofilms.

# Detailed summary (EN)

Mountain freshwater ecosystems provide essential services to humanity, such as the provision of clean water, but are strongly affected by anthropogenic global change despite their apparent isolation. Benthic biofilms, communities of organisms living in a matrix adhering to submerged surfaces, have critical functions in mountain lakes: among others, they detoxify water and form the basis of food webs. However, their composition and biodiversity are still poorly understood. Also, in the Pyrenees, some amphibian populations are threatened by chytridiomycosis, an infectious disease caused by the zoosporic fungus *Batrachochytrium dendrobatidis* (Bd). Its epidemiology is not fully understood but may depend on biofilms, which are abundant in mountain lakes and form the food of tadpoles.

Here, I had two main objectives: first, to study the spatio-temporal variations of biofilm microbial biodiversity; second, to investigate the potential role(s) of biofilms in the epidemiology of Bd infections. To do this, I performed a metataxonomic analysis of the prokaryotic and micro-eukaryotic assemblages of 230 biofilm samples collected from 2016 to 2020 in 26 Pyrenean mountain lakes. Combining this with Bd infection data from tadpoles sampled in the same lakes, I explored the links between the microbial composition of biofilms and the distribution, frequency and population impacts of Bd infections. In the laboratory, I also tested whether a biofilm could affect the free-living, infectious stage of Bd, the zoospore. My hypotheses were that the biodiversity of biofilms would decrease and their assemblages would change over the course of the study, that biofilms from lakes with less infected/impacted amphibian populations would contain more Bd antagonists than biofilms from other lakes, and that biofilms produced in the laboratory would not affect the number of zoospores unless they contained Bd consumers.

The diversity in both prokaryotic and micro-eukaryotic biofilm assemblages decreased over the study period. Their compositions also changed over the five years, with an increase in cyanobacteria (possibly toxigenic organisms) in prokaryotes and a decrease in diatoms (indicator organisms) in micro-eukaryotes. Taken together, these results show that benthic biofilm communities are degrading with potentially negative implications for the entire aquatic ecosystem and water quality. In addition, I found that biofilms in lakes where amphibians are less infected and less impacted by Bd had a higher abundance of Bd-inhibiting or Bd-consuming organisms. Finally, I showed that biofilms, even when they do not contain Bd consumers but only a phototrophic alga, can affect Bd zoospores by inactivating them or forcing them to immobilise.

My transdisciplinary research illustrates the interactions between environmental health and animal and public health. Contemporary environmental changes are deteriorating biofilms, the very basis of food webs in mountain lakes. This is likely to have profound cascading effects on mountain socio-ecosystems as a whole, such as a potential increase in the risk of Bd infection and chytridiomycosis for amphibians, and cyanotoxicosis for all vertebrates that frequent mountain lakes, including humans and livestock. If mountain freshwater ecosystems are to continue to provide services rather than disservices, the factors contributing to biofilm change will need to be rapidly identified and mitigated.

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*Non french-speaking readers can directly go to page **58** to skip the french introduction.*

# Introduction Générale (FR)

## Concepts de biodiversité, services écosstémiques et liens avec la durabilité

La planète Terre regorge de vie et c'est peut-être sa caractéristique la plus singulière. La vie peut être définie comme "un processus qui se déroule dans des structures organiques hautement organisées et qui se caractérise par le fait d'être préprogrammé, interactif, adaptatif et évolutif", tandis que les êtres vivants sont le système dans lequel ce processus se déroule (Gómez-Márquez 2021). Parce que les êtres vivants s'adaptent et évoluent en réponse à des environnements en constante évolution, une étonnante variété de formes de vie a émergé depuis l'apparition de la vie il y a plus de 3,9 milliards d'années (Betts *et al.* 2018). Cette variété est appelée **biodiversité** et tous les organismes vivants interagissent entre eux et avec leur environnement non-vivant dans une unité fonctionnelle appelée **écosystème** (Mace *et al.* 2005, 2012). Ces interactions entre les organismes d'une part, et entre les organismes et l'atmosphère, la géosphère et l'hydrosphère d'autre part, sous-tendent tous les processus écosystèmiques et constituent l'environnement dont dépendent tous les êtres vivants, y compris nous, les humains (Mace *et al.* 2005). Les processus bénéficiant aux humains sont appelés **services écosystémiques** et comprennent la fourniture de nourriture, d'eau potable, d'air pur, de médicaments, de bois et d'autres processus tels que la pollinisation, la décomposition de la matière organique, la détoxification, la capture du dioxyde de carbone et la régulation des maladies infectieuses, sans oublier les inestimables valeurs spirituelles et culturelles que la nature incarne pour de nombreuses communautés (Mace *et al.* 2012).

Les services écosystémiques sont nécessaires à la survie et à la prospérité de nos sociétés, ainsi qu'à notre **santé** et à notre bien-être (Sentenac *et al.* 2022). En appréciant cela, un problème majeur devient rapidement apparent: la vie et la biodiversité sur Terre disparaissent à un rythme effréné en raison des activités humaines, ce qui menace directement la fourniture de services écosystémiques (Díaz *et al.* 2019). Pour que ces derniers soient durablement fournis, les écosystèmes doivent être en bonne santé, c'est-à-dire qu'ils doivent avoir une **structure de communauté**, une **productivité** et une **résilience** normales (concept de santé des écosystèmes, "Ecosystem Health"; Rapport *et al.* 1998). Ces trois piliers de la santé des écosystèmes impliquent que la plupart des espèces les constituant habituellement doivent être présentes en abondance normale et que la plupart des organismes doivent être en bonne santé.

Les progrès technologiques et en soins de santé ont permis aux humains de prospérer au cours des derniers siècles, mais le développement humain est désormais tel que de nombreux écosystèmes ont été détériorés ou sont en train de l'être (Steffen *et al.* 2015 ; Díaz *et al.* 2019). Cette situation est particulièrement préoccupante si l'on considère que l'homme a évolué et s'est

développé au cours d'une courte période de 11 700 ans (l'Holocène) pendant laquelle la plupart des écosystèmes se trouvaient dans des états relativement stables (voir **États stables alternatifs** dans le glossaire) ; or nous entrons dans une nouvelle époque, l'Anthropocène, caractérisée par des conditions beaucoup plus instables (écosystèmes en mauvaise santé) en raison des **changements globaux** induits par l'homme (Rockström *et al.* 2009 ; Steffen *et al.* 2015). Des écosystèmes instables impliquent une fourniture de services incertaine et/ou éventuellement insuffisante pour que les humains puissent vivre décemment, menaçant ainsi les gains sanitaires des derniers siècles (Queenan *et al.* 2017). Si des changements planétaires se sont également produits et ont provoqué des extinctions massives par le passé, par exemple en raison de l'impact de météorites ou du volcanisme (Bond and Grasby 2017), la sixième extinction de masse en cours est beaucoup plus intense en termes de pertes d'espèces, avec un taux d'extinction environ 1 000 fois supérieur au taux « normal » (Barnosky *et al.* 2011 ; Pimm *et al.* 2014 ; Ceballos *et al.* 2015 ; De Vos *et al.* 2015).

Les facteurs anthropiques ou anthropogéniques actuels du déclin de la biodiversité peuvent être locaux ou globaux, et comprennent le changement climatique, la perte, la fragmentation et la dégradation des habitats, la pollution par les nutriments et les produits chimiques, l'introduction d'espèces exotiques envahissantes, la surexploitation des ressources vivantes et non vivantes, les maladies émergentes et les effets en cascade, entre autres (Diamond 1984; Daszak *et al.* 2000 ; Steffen *et al.* 2015 ; Díaz *et al.* 2019). Tous ces facteurs de déclin sont en fait eux-mêmes, directement ou indirectement, liés à des facteurs interconnectés pouvant être socioculturels, économiques, politiques, institutionnels, technologiques et démographiques, et donc à la racine à la santé et au bien-être de l'homme (Queenan *et al.* 2017 ; Díaz *et al.* 2019). Ainsi, les sociétés humaines doivent changer et trouver des moyens de se développer sans endommager leur environnement immédiat et la biosphère dans sa globalité, pour que les services écosystémiques puissent être durablement fournis et que la santé et le bien-être soient maintenus (Díaz *et al.* 2019). Cela nécessitera des politiques de développement correctement informées par les connaissances écologiques. La vie et les écosystèmes ont évolué pendant des milliards d'années, et comprendre comment des processus écosystémiques, et finalement notre santé, sont affectés par de multiples pressions anthropiques agissant simultanément est extrêmement complexe. Dans ce contexte, des approches holistiques de la santé et de la durabilité sont particulièrement indiquées. L'une de ces approches a déjà été mentionnée, Eco(system) Health (Rapport *et al.* 1998), qui est écocentrique, et une autre, populaire mais plus anthropocentrique, est One Health (Zinsstag *et al.* 2011). Les deux sont convergentes dans une certaine mesure et j'utiliserai une approche mixte tout au long de ce manuscrit (**Figure 1**).

Les domaines scientifiques de **l'écologie des communautés** (Morin 2009), de **l'écologie fonctionnelle** (Calow 1987), de **l'éco-épidémiologie** (Rioux *et al.* 1997) et de la **biologie de la conservation** (Soulé 1985) sont très pertinents ici pour éclairer la prise de décision et les pratiques de conservation efficaces. Parce qu'il est souvent difficile d'observer toutes les espèces d'un site, c'est-à-dire l'ensemble de la **communauté**, les **assemblages** ou **guildes** sont plutôt étudiés avec des concepts tels que **l'α-diversité**, la **β-diversité**, **l'η-diversité**, et enfin, la **γ-diversité** (Whittaker 1960, 1972 ; Fauth *et al.* 1996 ; Hui et McGeoch 2014). De nombreux indices différents existent pour mesurer à la fois l'α-diversité (par exemple, la richesse spécifique, qui ne prend en compte que la présence-absence des taxons alors que l'indice de Shannon ou l'indice Inverse-Simpson prennent également en compte leur abondance) et la β-diversité (par exemple, la dissimilarité de Jaccard qui est basée sur la présence-absence, ou la dissimilarité de Bray-Curtis qui est basée sur l'abondance ; Jaccard 1912 ; Shannon 1948 ; Simpson 1949 ; Bray et Curtis 1957). Le travail présenté ici est transdisciplinaire et fortement ancré dans ces domaines, avec un accent particulier sur les écosystèmes d'eau douce de montagne et plus particulièrement sur l'interaction entre les biofilms benthiques (précisément, les compositions de leurs assemblages procaryotes et micro-eucaryotes), et un parasite fongique aquatique des amphibiens pouvant avoir un impact sur leurs populations.

## Les écosystèmes de montagnes et d'eaux douces

Les montagnes sont des reliefs caractérisés par une topographie accidentée (>200 m de différence d'altitude dans une cellule de 2,5′, résolution de 0,5′) qui occupent environ 12,3 % de la surface terrestre totale hors Antartique (Körner *et al.* 2011). Les montagnes amènent de l'hétérogénéité dans les conditions climatiques, les types de sol et la complexité tridimensionnelle, créant une multitude de **niches** écologiques où les espèces évoluent et se diversifient : par rapport à leur superficie, les montagnes détiennent en effet un niveau élevé de **biodiversité** et d'**endémisme** (Körner 2004; Fjeldså *et al.* 2012 ; Hoorn *et al.* 2013 ; Badgley *et al.* 2017 ; Rahbek *et al.* 2019). Les barrières physiques établies par les montagnes perturbent la circulation des gaz dans l'atmosphère et forcent l'air chargé d'humidité à s'élever et à se refroidir, déclenchant la formation de nuages orographiques et augmentant l'humidité et les précipitations par rapport aux plaines (Roe 2005). L'eau est stockée sous forme de glace et de neige dans les zones sommitales, qui se transforment en eau liquide pendant les saisons chaudes. Les masses d'eau douce sont donc très abondantes en montagne sous forme de cours d'eau mais aussi, en raison de la faible perméabilité du socle cristallin, sous forme de lacs, d'étangs ou de zones marécageuses (Catalan *et al.* 2006).

Alors que les eaux douces (faible salinité) ne représentent que 0,01 % de l'eau sur Terre, elles abritent une biodiversité extraordinaire avec environ 10 % de toutes les espèces connues, dont un tiers des espèces de vertébrés (Dudgeon *et al.* 2006). Malheureusement, la biodiversité des eaux douces est en crise, avec des taux de déclin bien supérieurs à ceux que connaît la biodiversité marine ou terrestre (Dudgeon *et al.* 2006 ; Strayer and Dudgeon 2010 ; Harrison *et al.* 2018 ; Reid *et al.* 2019 ; Tickner *et al.* 2020). Le dernier rapport de l'Indice Planète Vivante a estimé que les populations de vertébrés d'eau douce ont diminué de 83 % [74 % - 89 %] entre 1970 et 2018 (WWF 2022). Les raisons sont multiples et comprennent le changement climatique, la surexploitation (facilitée par le e-commerce), les invasions biologiques, les maladies infectieuses, les efflorescences d'algues nuisibles, l'hydroélectricité, la pollution par les produits chimiques de synthèse, les nanomatériaux et les microplastiques, les perturbations dues à la lumière et au bruit, et les modifications de la chimie de l'eau (par exemple, la salinisation ; Reid *et al.* 2019).

Malgré leur isolation, les écosystèmes d'eau douce de montagne ne font pas exception (Schmeller *et al.* 2018 ; **Figure 2**). En particulier, les impacts du changement climatique sont très forts avec réchauffement plus important qu'en plaine (Rangwala and Miller 2012 ; Pepin *et al.* 2015 ; Schmeller *et al.* 2022). L'augmentation de la température de l'eau et de l'évaporation, le recul des glaciers, la modification des régimes de mélange aquatique, les événements météorologiques extrêmes, ainsi que les modifications de la chimie de l'eau résultant de l'intensification de l'érosion sont des facteurs qui affectent les écosystèmes d'eau douce de montagne (Koinig *et al.* 1998 ; Rangwala and Miller 2012 ; Pepin *et al.* 2015 ; Niedrist *et al.* 2018). Mais d'autres menaces existent (Schmeller *et al.* 2022). La plupart des facteurs de déclin de la biodiversité d'eau douce s'appliquent également au cas des montagnes, à l'exception peut-être du commerce électronique et des perturbations lumineuses et sonores (bien que possibles). Paradoxalement, les eaux douces de montagne ne sont pas à l'abri de la pollution : la topographie des montagnes est telle que des dépôts atmosphériques à longue distance de micropolluants chimiques, y compris des polluants organiques persistants et des oligo-éléments potentiellement dangereux, se produisent (Schmeller *et al.* 2018). La pollution par les pesticides, notamment les herbicides et les médicaments vétérinaires, a également été reportée dans les lacs de montagne (Machate *et al.* 2022, 2023). Les médicaments vétérinaires pénètrent probablement dans ces écosystèmes via le bétail, ce qui est lié à d'autres menaces comme le changement d'affectation des terres. La végétation de montagne a été largement façonnée par l'utilisation des terres par l'homme (Koerner *et al.* 1997), et l'augmentation de la taille des cheptels exerce également une pression sur les lacs et les étangs par la dégradation des rives et l'eutrophisation potentielle (pollution par les nutriments) de masses d'eau naturellement

oligotrophes (Mayer *et al.* 2022 ; Schmeller *et al.* 2022). Les captages d'eau en montagne sont également importants pour la production d'hydroélectricité, l'agriculture, la fabrication de neige, et de nombreux écosystèmes naturels souffrent de ces activités (Schmeller *et al.* 2022). L'introduction d'espèces exotiques envahissantes est également une menace (par exemple, les espèces de plantes aquatiques) qui est très difficile à contrôler dans ces écosystèmes isolés et qui pourrait être exacerbée par le changement climatique (McDougall *et al.* 2011). Les espèces introduites n'ont pas besoin d'être invasives pour causer des dommages étendus dans les lacs de montagne, lorsque leur forte abondance est délibérément maintenue par l'homme grâce à des introductions régulières. C'est le cas des salmonidés et des vairons, non indigènes dans la plupart des lacs d'altitude, qui sont introduits à des fins de pêche (donc à des fins économiques) et affectent considérablement la biodiversité des eaux douces (Miró *et al.* 2018, 2020 ; Miró and Ventura 2020). Les espèces introduites peuvent également être pathogènes pour les organismes indigènes, un phénomène appelé pollution par des agents pathogènes (Cunningham *et al.* 2003). La pollution par les agents pathogènes est un moteur important de l'émergence de nouvelles maladies infectieuses et peut avoir des conséquences considérables (voir la section sur *Batrachochytrium dendrobatidis* pour un exemple d'agent pathogène introduit).

Les lacs de montagne sont donc actuellement très vulnérables. Cela les rend urgents à protéger mais aussi intéressants à étudier, car ils peuvent être considérés comme des sentinelles du changement (Catalan *et al.* 2006 ; Schmeller *et al.* 2018 ; Moser *et al.* 2019 ; Råman Vinnå *et al.* 2021). Ces écosystèmes fournissent un nombre important de services écosystémiques essentiels tant aux personnes vivant à l'intérieur qu'à l'extérieur des montagnes (Grêt-Regamey *et al.* 2012). Il s'agit notamment de la fourniture d'eau potable, avec plus de 50 % des habitants de la Terre dépendant de l'eau provenant de massifs montagneux, mais aussi, entre autres, de bois, de pâturages pour le bétail, de cultures et d'activités récréatives telles que la randonnée, la pêche, la chasse et les sports d'hiver (Viviroli *et al.* 2007, 2020 ; Grêt-Regamey *et al.* 2012 ; Locatelli *et al.* 2017 ; Martín-López *et al.* 2019). Cependant, les impacts du changement global et d'autres activités humaines sur la diversité microbienne des eaux douces de montagne, et les conséquences éventuelles de ces impacts sur la qualité de l'eau, sont encore mal compris car non évalués.

## Micro-organismes et biofilms

Bien qu'invisibles à l'œil nu et, par conséquent, souvent négligés en biologie de la conservation, les micro-organismes forment le socle de la biosphère (Cavicchioli *et al.* 2019 ; Timmis *et al.* 2019). La première forme de vie sur Terre était micro-organismique, et leur

ubiquité des micro-organismes, leur biodiversité et leur abondance sont stupéfiantes (O'Donnell *et al.* 1994 ; Whitman *et al.* 1998 ; Bar-On *et al.* 2018 ; Timmis *et al.* 2019). On estime (avec une incertitude relativement élevée) que les bactéries représentent à elles seules 70 gigatonnes de carbone, soit environ 15 % de la biomasse totale de la Terre ; en comparaison, tous les animaux réunis ne représentent que 2 gigatonnes de carbone et 0,004 % de la biomasse totale (Bar-On *et al.* 2018).

Au cours des deux dernières décennies, l'avènement des technologies de séquençage à haut débit, dits "de nouvelle generation", a révolutionné notre compréhension du **microbiote** et des **microbiomes** (Metzker 2010; Marchesi and Ravel 2015). La métataxonomie (également appelée métabarcodage) a été capitale pour la détermination de la composition du microbiote et s'est fortement appuyée sur l'amplification et le séquençage de gènes marqueurs tels que les gènes de l'ARNr 16S et 18S, suivis d'une attribution taxonomique (Marchesi et Ravel 2015). Les assemblages microbiens contiennent non seulement des représentants du domaine des bactéries, mais aussi d'autres micro-organismes de toutes les branches de l'arbre du vivant, y compris le domaine des archées (les bactéries et les archées forment ensemble les procaryotes) et certains taxons du domaine des eucaryotes, à savoir les micro-eucaryotes (Woese *et al.* 1990). Les micro-eucaryotes comprennent les animaux, les champignons et les plantes microscopiques, ainsi que les eucaryotes unicellulaires appelés protistes. Les protistes constituent un groupe paraphylétique contenant des taxons tels que les Amoebozoa, les Excavata et les Chromoalveolata (Stramenopiles, Alveota, Rhizaria). Il convient toutefois de remarquer que la classification taxonomique du domaine eucaryote, et celle de l'ensemble de l'arbre du vivant par ailleurs, fait encore l'objet de recherches intenses : voir par exemple les travaux de Cavalier-Smith (Roger 2021). La question de savoir si les virus sont des micro-organismes fait également l'objet d'un débat, car il n'y a pas de consensus sur le fait que les virus soient de véritables êtres vivants. Cela dit, étant donné qu'ils sont préprogrammés, interactifs, adaptatifs et évolutifs (Gómez-Márquez 2021), je les considère comme vivants bien qu'ils n'aient pas été abordés dans cette thèse pour des raisons pratiques (aucun gène marqueur facilement disponible).

Les micro-organismes sont essentiels au fonctionnement de tous les écosystèmes sur Terre (Cavicchioli *et al.* 2019 ; Bernardo-Cravo *et al.* 2020), et sont également présents dans des environnements tels que la subsurface profonde où aucun autre organisme n'existe (Phelps *et al.* 1989 ; Lovley and Chapelle 1995). Dans les océans, plus de 90 % de la vie est microbienne et les algues photosynthétiques constituent la base de leurs réseaux alimentaires (Azam and Malfatti 2007; Sunagawa *et al.* 2015 ; 'The Census of Marine Life | Smithsonian Ocean' 2022). Les micro-organismes sont également extrêmement abondants dans les sols, un demi-gramme

pouvant contenir entre 2 000 et 5 000 espèces microbiennes (Schloss and Handelsman 2006). Les micro-organismes y décomposent la matière organique, assurent le cycle des nutriments et rendent les sols fertiles et propices à la croissance des plantes; à cet égard, ils jouent un rôle central dans l'agriculture et la durabilité (Singh *et al.* 2010 ; Bardgett and van der Putten 2014). Une diversité, une abondance et une importance similaires des micro-organismes existent également dans les écosystèmes d'eau marine et d'eau douce (Sigee 2005; Hahn 2006). Même la santé des macro-organismes multicellulaires, y compris les humains, dépend intimement des divers microbiotes qu'ils portent (Clemente *et al.* 2012 ; Bernardo-Cravo *et al.* 2020). Il y a approximativement le même nombre de cellules bactériennes que de cellules humaines dans un corps humain, et on pense qu'un individu de 70 kg porte une biomasse bactérienne de 0,2 kg (Sender *et al.* 2016). Les rôles du microbiote dans le contrôle du développement, de la physiologie et de l'immunologie des humains, des autres animaux et des plantes ont été largement documentés (Lynch 1994; Clemente *et al.* 2012 ; Ezenwa *et al.* 2012 ; Vorholt 2012 ; Fung *et al.* 2017 ; Trevelline *et al.* 2019). Les organismes multicellulaires forment en fait, avec leur microbiote, des superorganismes appelés **holobiontes** (Trevelline *et al.* 2019; Carthey *et al.* 2020). Ainsi, les micro-organismes affectent leur environnement, que ce soit à l'échelle de l'individu multicellulaire, de l'écosystème ou de la planète. Surtout, ils affectent le climat mais sont également affectés par le changement climatique (Cavicchioli *et al.* 2019).

La plupart des micro-organismes ont en fait un mode de vie sessile, intégré dans une matrice de type gélatineuse (**Figure 3**). Ce mode de vie, appelé "**biofilm**", est également l'une des formes de vie les plus anciennes sur Terre (3,3 milliards d'années ; Westall *et al.* 2001). Ce mode de vie a également connu un grand succès car les biofilms sont de manière globale extrêmement abondants (Flemming and Wuertz 2019). Il s'agit de la forme de vie dominante dans de nombreux habitats de la Terre, à l'exception des océans (Flemming et Wuertz 2019). La matrice forme une protection contre les molécules ou les organismes potentiellement dangereux pour les habitants du biofilm ; elle peut être considérée comme une forteresse et le biofilm dans son ensemble, très riche en biodiversité et très organisé, peut être vu comme un mini-écosystème au sein d'un grand écosystème (Watnick and Kolter 2000; Flemming and Wingender 2010; Flemming *et al.* 2016). Les biofilms peuvent être habités par des bactéries, des archées, des virus et une multitude de micro- et méio-eucaryotes tels que des plantes, des diatomées, des animaux et des champignons (Geesey *et al.* 1978 ; Costerton *et al.* 1987 ; Battin *et al.* 2001). La stabilité temporelle et physique offerte par la matrice permet à ces habitants d'interagir de diverses manières, de la compétition à la coopération (Hansen *et al.* 2007 ; Rendueles and Ghigo 2012 ; Flemming *et al.* 2016). Il est intéressant de noter que les biofilms présentent des **propriétés émergentes**, notamment le transfert horizontal de gènes, la sorption

active (capture de matériaux), le transport organisé (à travers les pores, les vides et les canaux au sein du biofilm), la rétention extracellulaire d'eau, de nutriments et d'enzymes, et enfin et surtout, la coopération entre les cellules avec des preuves de division du travail et de comportement coordonné par communication chimique ou électrique (Hansen *et al.* 2007 ; Flemming and Wingender 2010 ; Flemming *et al.* 2016). La matrice et ces propriétés émergentes rendent les biofilms beaucoup plus **productifs** que toute communauté planctonique équivalente (Hansen *et al.* 2007). Par conséquent, les biofilms sont omniprésents et ont été désignés comme la peau microbienne des écosystèmes: une frontière écologique structurante, vivante et dynamique où les matières biotiques et abiotiques sont juxtaposées, transitées, séparées et/ou transformées (ou non ; Battin *et al.* 2016).

Un chapitre entier de cette thèse est consacré aux rôles des biofilms à la fois dans les écosystèmes et dans/sur les organismes multicellulaires (Chapitre 2). Les rôles des biofilms dans les écosystèmes d'eau douce de montagne sont particulièrement intéressants ici. Les biofilms se développent sur pratiquement toutes les interfaces solides-liquides immergées telles que les roches (biofilm épilithique ou épilithon), le bois en décomposition (biofilm épixylique), les grains de sable (épisammon) et les sédiments (épipelon ; Vadeboncoeur and Steinman 2002 ; **Figure 4**). Les biofilms benthiques peuvent dominer, en termes de biomasse, le biote des cours d'eau et autres plans d'eau, car les eaux sont souvent peu profondes et/ou ont une faible turbidité (Vadeboncoeur and Steinman 2002; Besemer 2015). De plus, en raison des conditions extrêmes en montagnes en ce qui concerne la variabilité de la température, le vent, la sédimentation et la disponibilité en nutriments, qui empêchent le développement de la végétation, les biofilms peuvent à eux seuls assurer la production primaire et former la base des réseaux alimentaires (Geesey *et al.* 1978 ; Lock *et al.* 1984 ; Vadeboncoeur and Steinman 2002 ; Rott *et al.* 2006). Ils permettent également la décomposition de la matière organique et le recyclage des nutriments, et peuvent en outre détoxifier l'eau en adsorbant, fixant et métabolisant les polluants (Sabater *et al.* 2002 ; Cardinale 2011). Enfin, leur matrice cohésive permet la rétention d'eau, et assure une bio-stabilisation des sédiments, limitant ainsi l'érosion (Gerbersdorf and Wieprecht 2015).

En revanche, les biofilms d'eau douce peuvent contenir des algues toxigènes, comme les cyanobactéries, qui peuvent sécréter dans l'eau des cyanotoxines nocives pour la plupart des organismes aquatiques sympatriques et autres, y compris les humains (Quiblier *et al.* 2013). Dans les organismes multicellulaires, les agents infectieux peuvent aussi former des biofilms dans les tissus colonisés ou sur les dispositifs médicaux implantés pour mieux résister au système immunitaire ou aux médicaments (section 2.3.1). Ces deux cas illustrent le fait que les

micro-organismes peuvent également affecter négativement la santé d'autres organismes, en leur causant des **maladies**.

## Maladies

Les maladies, ou toutes anomalies de structure et/ou de fonctions affectant la santé d'un organisme, peuvent être infectieuses ou non-infectieuses. Une **maladie infectieuse** est l'effet résultant d'une infection, c'est-à-dire l'entrée et la multiplication d'un agent infectieux exogène dans ou sur un organisme (Porta 2014). Une maladie non infectieuse est le résultat d'autres processus tels que la malnutrition, les processus néosplasiques (tumeur et cancer), les troubles génétiques/physiologiques, l'intoxication par des **xénobiotiques** (par exemple les cyanotoxines, ou toute substance synthétique toxique). Il est également important de distinguer une infection d'une maladie infectieuse, car l'infection peut être non apparente (subclinique) et ne pas provoquer de maladie. Lorsqu'il est infecté mais non malade, un individu hôte peut contribuer grandement à la propagation d'un agent infectieux, ce qui est d'une grande importance lorsqu'on étudie **l'épidémiologie** d'une maladie. Cela renvoie à la différence entre les **agents pathogènes** et les **parasites**. Les agents pathogènes sont des organismes capables de provoquer des maladies, tandis que les parasites sont des organismes qui vivent sur ou dans d'autres organismes et en tirent de l'énergie, sans nécessairement provoquer de maladies. Selon les auteurs, il existe d'autres nuances entre les agents pathogènes et les parasites : certains considèrent que le terme de parasite ne devrait être attribué qu'aux organismes parasites multicellulaires (comme les helminthes ou les tiques), tandis que le terme d'agent pathogène devrait être employé pour les organismes unicellulaires. Comme dans ce travail j'étudie un parasite fongique (multicellulaire) de, et parfois pathogène pour, certaines espèces d'amphibiens, j'ai plutôt utilisé le terme parasite pour indiquer le fait que l'infection n'entraîne pas toujours une maladie. En effet, différents hôtes peuvent avoir des **susceptibilités** différentes à la maladie (Bernardo-Cravo *et al.* 2020). Nous distinguons en outre la **résistance**, c'est-à-dire la capacité de l'hôte à limiter l'infection, et la **tolérance**, c'est-à-dire la capacité à limiter l'effet de l'infection (Råberg *et al.* 2009).

Si j'ai mentionné les maladies émergentes comme un facteur de perte de biodiversité, le parasitisme est un type **d'interactions écologiques** très répandu qui a joué un rôle important dans le façonnement de la dynamique des populations hôtes et dans l'évolution de la biodiversité (Hudson *et al.* 2006 ; Bagchi *et al.* 2014). La grande majorité des maladies ne sont pas à enjeu de conservation. En fait, il a existé pendant longtemps une réticence persistante à reconnaître les maladies infectieuses comme une cause potentielle de déclin des espèces et, plus encore,

d'extinctions. Cela est dû aux modèles théoriques qui prédisent que la pression d'infection diminue nécessairement au fur et à mesure que la population d'hôtes décline, ce qui conduira à l'extinction de l'agent infectieux avant celle de l'hôte (McCallum and Dobson 1995). Cependant, ceci n'est vrai que pour les parasites dont la transmission dépend de la densité d'hôtes, et des modèles plus réalistes ont montré que les extinctions d'hôtes dues aux maladies sont plus probables qu'on ne le pensait, par exemple lorsque les populations d'hôtes sont déjà fragiles et/ou naïves vis-à-vis de l'agent infectieux, lorsqu'il existe des réservoirs environnementaux ou biotiques de cet agent, ou lorsque la transmission ne dépend pas de la densité d'hôtes (par exemple les maladies vectorielles ou sexuellement transmissibles; De Castro et Bolker 2005). Les exemples empiriques ne manquent pas (ou plus), avec entre autres le syndrome du nez blanc pour les chauves-souris d'Amérique du Nord (Hoyt *et al.* 2021), la malaria aviaire pour les oiseaux (Cardualinés) d'Hawaï (van Riper *et al.* 1986 ; Atkinson *et al.* 1995), la Devil Facial Tumour Disease pour les diables de Tasmanie (*Sarcophilus harrisii*; McCallum *et al.* 2007), la maladie virale Ebola pour les grands singes (Leendertz *et al.* 2006), la peste porcine africaine pour les porcs sauvages endémiques d'Asie du Sud-Est (Luskin *et al.* 2021), de multiples maladies pour les coraux (Green and Bruckner 2000; Goldberg and Wilkinson 2004), et les ranaviroses et chytridiomycose(s) pour de nombreux amphibiens (Martel *et al.* 2014 ; Rosa *et al.* 2017 ; Scheele *et al.* 2019), pour ne citer que quelques exemples provenant du règne animal.

Ces maladies sont des **maladies infectieuses émergentes**, c'est-à-dire des maladies causées par des agents pathogènes ou des parasites nouvellement évolués (ou l'une de leurs souches nouvellement évoluées), ou des maladies dont la distribution géographique, le spectre d'hôtes et/ou l'incidence ont augmenté de manière significative sur une période relativement courte (Jones *et al.* 2008). Les maladies infectieuses émergentes représentent une menace sérieuse pour la faune, la flore, les animaux domestiques et la santé humaine (Daszak *et al.* 2000 ; Dobson and Foufopoulos 2001 ; Jones *et al.* 2008 ; Fisher *et al.* 2012). Les facteurs de l'émergence des maladies sont liés aux changements socio-écologiques, et comprennent le changement climatique, les changements technologiques (connectivité et mouvements accrus des humains et des marchandises, liés à la pollution par des agents pathogènes), les changements démographiques, les changements d'utilisation des terres (dégradation et fragmentation des habitats), la pollution par des agents pathogènes (introduction d'espèces parasites invasives), ainsi que les pollutions chimiques et nutritives (Plowright *et al.* 2008 ; Johnson *et al.* 2010b ; McKee *et al.* 2021 ; Baker *et al.* 2022 ; Eby *et al.* 2022). En particulier, les amphibiens ont été et sont toujours plus touchés par les maladies infectieuses émergentes que les autres classes de vertébrés.

## Amphibiens

Les amphibiens constituent une classe de vertébrés ectothermes comprenant 8558 espèces réparties en trois ordres : Anoures (7530 espèces), Urodèles (792 espèces) et Gymnophiones (216 espèces ; Frost 2022). Parmi les vertébrés, les amphibiens sont plus menacés et déclinent plus rapidement que d'autres classes telles que les oiseaux ou les mammifères (Stuart *et al.* 2004 ; Gascon *et al.* 2007), avec pas moins de 41% des amphibiens menacés d'extinction (UICN 2022). Les implications écologiques de ces déclins ne soient pas encore totalement comprises mais les amphibiens requièrent quoi qu'il en soit une attention urgente en matière de conservation, car ils possèdent des traits d'histoire de vie et des processus biologiques uniques (par exemple, un cycle de vie complexe et des stratégies de reproduction très diverses) et sont des espèces clés pour le fonctionnement des écosystèmes (Halliday 2008 ; Hocking and Babbitt 2014). La plupart des espèces d'amphibiens ont deux stades de vie, une larve aquatique (appelée têtard pour les anoures) et un individu terrestre post-métamorphique, qui constituent deux entités écologiques très différentes (Halliday 2008 ; Hocking and Babbitt 2014). Les amphibiens constituent un lien entre les milieux aquatique et terrestre et permettent un flux d'énergie et de matière entre ces deux types d'écosystèmes (Hocking and Babbitt 2014). La biomasse des amphibiens peut atteindre des niveaux très élevés dans leurs habitats aquatiques et terrestres, ce qui en fait des sources de nourriture importantes pour d'autres organismes (Gibbons *et al.* 2006). Cela inclut les serpents, par exemple, et il a été démontré que le déclin des amphibiens peut entraîner le déclin de leurs prédateurs, illustrant ainsi leur importance dans les réseaux alimentaires (Matthews *et al.* 2002 ; Zipkin *et al.* 2020). Cette biomasse très élevée d'amphibiens, plus spécifiquement de têtards d'anoures, est particulièrement pertinente dans les écosystèmes d'eau douce de montagne, qui sont oligotrophes et seraient autrement des écosystèmes relativement peu productifs. Il existe de nombreuses **guildes** de têtards définies en fonction de leur type d'alimentation (et l'anatomie de leurs pièces buccales), mais l'une des plus courantes dans le monde, et également dans les masses d'eau de montagne, est la guilde des brouteurs de biofilms/**périphyton** (McDiarmid and Altig 1999 ; Altig *et al.* 2007). Les têtards broutent les biofilms sur toutes les surfaces immergées, qu'il s'agisse de rochers, de bois, de plantes ou de sédiments. Ce faisant, ils affectent considérablement, du moins dans les cours d'eau tropicaux d'altitude, la structure et la biomasse de la communauté des biofilms, réduisent l'accumulation de sédiments et influencent le cycle des nutriments tels que le phosphore et l'azote (Ranvestel *et al.* 2004 ; Whiles *et al.* 2006 ; Altig *et al.* 2007 ; Hocking and Babbitt 2014). Le déclin des amphibiens et des têtards a considérablement affecté les processus des écosystèmes des cours d'eau dans les régions néotropicales (Ranvestel *et al.* 2004 ; Whiles *et*

*al.* 2006, 2013), et une étude récente dans les cours d'eau de montagne du massif espagnol de Peñalara a montré des conclusions similaires (Alonso *et al.* 2022).

En plus de leur rôle dans le soutien du fonctionnement des écosystèmes, les amphibiens fournissent une variété de services à l'humanité. Ils constituent une source alimentaire importante pour de nombreuses populations humaines, représentant au moins des milliers de tonnes chaque année et une source importante de protéines (Tyler *et al.* 2007 ; Kusrini and Alford 2008 ; Valencia-Aguilar *et al.* 2013 ; Hocking and Babbitt 2014). Les amphibiens ont grandement bénéficié à la médecine humaine et aux connaissances biologiques de diverses manières. Tout d'abord, certaines espèces d'amphibiens comme *Xenopus laevis* sont des espèces modèles importantes pour la recherche sur l'évolution et le développement, et cette espèce a également été utilisée de manière intensive pour produire des tests de grossesse (Tyler *et al.* 2007 ; Hocking and Babbitt 2014). Les amphibiens ont également été largement utilisés pour l'enseignement de l'anatomie et de la physiologie. Ensuite, leurs sécrétions cutanées ont été des sources directes ou des points de départ pour le développement de nombreux produits pharmaceutiques tels que des analgésiques, des médicaments anticancéreux, des antiviraux et des antibiotiques, et les amphibiens sont également utilisés dans la médecine traditionnelle dans diverses parties du monde (Tyler *et al.* 2007 ; Hocking and Babbitt 2014). Il a également été démontré que les larves d'amphibiens carnivores et les individus métamorphosés contrôlent, par la compétition ou la prédation, l'abondance des larves et des adultes d'arthropodes, tels que les moustiques (DuRant and Hopkins 2008; Rubbo *et al.* 2011 ; Valencia-Aguilar *et al.* 2013). Les arthropodes hématophages tels que les moustiques peuvent être des vecteurs d'importantes maladies infectieuses humaines, et on pense que les amphibiens, en régulant les populations d'arthropodes, réduisent les risques inhérents à ces maladies (Valencia-Aguilar *et al.* 2013 ; Hocking and Babbitt 2014). Cela a été récemment démontré par l'augmentation des cas de paludisme en Amérique centrale, suite à de forts déclins des amphibiens (Springborn *et al.* 2022). Enfin, les amphibiens fournissent des services culturels importants qui améliorent le bien-être humain par le biais des loisirs, de la religion, de la spiritualité et de l'esthétique (Tyler *et al.* 2007 ; Hocking and Babbitt 2014).

Les services fournis par les amphibiens sont menacés à l'échelle mondiale par une multitude de facteurs, notamment la perte et la dégradation de l'habitat (déforestation, agriculture, urbanisation et assèchement des zones humides), la pollution par les nutriments et les produits chimiques, la surexploitation (collecte pour les marchés de la nourriture, des animaux de terrarium ou des médicaments), l'introduction d'espèces envahissantes (par exemple, l'introduction de poissons a un impact considérable sur les têtards), le rayonnement ultraviolet B, le changement climatique et les maladies (infectieuses ou non, y compris les

malformations, les syndromes néoplasiques ou les toxicoses ; Halliday 2008). Il convient de noter que les déclins d'amphibiens se sont également produits dans des habitats relativement "vierges" (ou du moins protégés), où il n'y a pas d'empiètement humain ou de pollution, ce qui souligne l'importance de facteurs tels que le changement climatique et les maladies infectieuses (Pounds *et al.* 2006 ; Halliday 2008). Les amphibiens ont été particulièrement touchés par une maladie infectieuse émergente, la chytridiomycose amphibienne.

## *Batrachochytrium dendrobatidis* et chytridiomycose amphibienne

La chytridiomycose des amphibiens est causée par le champignon *Batrachochytrium dendrobatidis* (Bd ; Berger *et al.* 1998 ; Longcore *et al.* 1999). Elle peut également être causée par *B. salamandrivorans* (Bsal), dont l'expression clinique, le spectre d'hôtes (comprenant principalement des Urodèles) et la distribution géographique connue (hors Asie : Belgique, Pays-Bas et Allemagne, plus récemment Espagne ; il faut toutefois noter que cette distribution géographique réduite est le résultat d'une meilleure mise en application des mesures de biosécurité, de surveillance et de stratégies de mitigation plus efficaces) sont différentes (Martel *et al.* 2013, 2014, 2020). Contrairement à Bsal, Bd a été propagé sur tous les continents où l'on trouve des amphibiens, et est connue pour infecter au moins 1375 espèces (en 2021 ; Olson *et al.* 2021). La chytridiomycose des amphibiens est actuellement une maladie **panzootique**, impliquée dans le déclin d'au moins 501 espèces et l'extinction présumée de 90 espèces (Scheele *et al.* 2019). Elle est, à l'état actuel des connaissances, responsable de la plus grande perte de biodiversité attribuable à une maladie infectieuse, ce qui lui vaut d'être qualifiée à juste titre de pire maladie infectieuse de tous les temps (Gascon *et al.* 2007 ; Scheele *et al.* 2019).

*Batrachochytrium dendrobatidis* est un champignon chytride zoosporique ne produisant pas de mycélium (Phylum Chytridiomycota, Classe Chytridiomycetes, Ordre Rhizophydiales ; Longcore *et al.* 1999). Il a un tropisme pour les cellules kératinisées des amphibiens, c'est-à-dire la peau des adultes et les pièces buccales des têtards (Berger *et al.* 1998, 2005). Son développement nécessite une certaine humidité (la majorité des plus de 700 espèces de chytrides sont aquatiques) et est dépendant de la température : sa croissance est optimale entre 17 et 25°C, nettement plus lente en dessous de 10°C, et la croissance s'arrête complètement au-dessus de 28°C (Piotrowski *et al.* 2004 ; Woodhams *et al.* 2008). Le cycle de vie de Bd dure 4 à 5 jours *in vitro* et on suppose qu'il est similaire *in vivo*, avec deux étapes : le stade infectieux, la zoospore, et l'étape reproductive, la zoosporange (Berger *et al.* 2005 ; **Figure 5**). La zoospore de Bd est uniflagellé, avec un corps de diamètre de 3 à 5 µm et un flagelle mesurant 19-20 µm (Longcore *et al.* 1999). Elle est motile dans l'eau ; elle recherche et envahit le tissu kératinisé

des amphibiens, où elle germe, forme un thalle, colonise la couche profonde de l'épiderme de l'hôte avec des tubes germinatifs et donne naissance à des zoosporanges immatures intracellulaires (Van Rooij *et al.* 2012). Les zoosporanges (40µm) mûrissent au fur et à mesure que les cellules de la peau se différencient vers l'extérieur et chaque zoosporange libère, par un tube de décharge, de nombreuses zoospores (d'une à plusieurs dizaines) dans l'environnement externe (Berger *et al.* 2005). La méthode la plus largement utilisée pour la surveillance de la chytridiomycose des amphibiens repose sur des écouvillonnages buccaux et cutanés non invasifs (respectivement pour les stades larvaire et métamorphosé), suivis de la détection de l'ADN de Bd à l'aide de techniques moléculaires telles que la réaction en chaîne par polymérase quantitative (en temps réel ou qPCR ; Boyle *et al.* 2004 ; Kriger *et al.* 2006 ; Hyatt *et al.* 2007 ; Shin *et al.* 2014).

Bien que Bd n'affecte que la peau des individus métamorphosés, la chytridiomycose est néanmoins létale chez les hôtes sensibles car la peau a des fonctions essentielles chez les amphibiens, comme la respiration et l'osmorégulation (Boutilier *et al.* 1992). En se développant dans les tissus cibles, Bd provoque une **hyperkératose** et une **hyperplasie** (Berger *et al.* 1998). Ce faisant, le champignon perturbe la fonction osmorégulatrice de la peau, entraînant un déséquilibre osmotique (perte d'électrolytes de sodium et de potassium) et finalement un arrêt cardiaque et la mort (Voyles *et al.* 2009 ; Grogan *et al.* 2018). En dehors de la mort subite, les signes cliniques sont très peu spécifiques chez les individus métamorphosés mais comprennent la léthargie, une posture anormale, une perte du réflexe de redressement, une dysecdysie (desquamation anormale de la peau), une décoloration de la peau, un érythème et de l'anorexie (Pessier 2008; Van Rooij *et al.* 2015). Chez les larves d'anoures, cependant, les signes cliniques sont plus spécifiques avec une dépigmentation des disques buccaux (dékératinisation) et même des déformations (œdème des pièces buccales apparaissant gonflées, entièrement rouges ou blanches au lieu d'avoir des rangées de « dents » kératinisées noires ; Fellers *et al.* 2001 ; Smith and Weldon 2007 ; Navarro-Lozano *et al.* 2018)

La transmission de Bd peut se faire directement par contact peau à peau entre amphibiens (par exemple pendant l'**amplexus**) ou indirectement par des zoospores mobiles libérées dans l'eau (Rowley and Alford 2007; Courtois *et al.* 2017 ; Burns *et al.* 2021). La survie des zoospores de Bd dans le milieu extérieur (eau ou substrat humide) n'est pas totalement comprise, mais dans des conditions stériles avec une humidité suffisante, elles peuvent survivre de trois semaines à trois mois en fonction de la quantité de minéraux, et éventuellement de nutriments, disponibles dans le milieu (Johnson and Speare 2003, 2005). Le champignon peut également se fixer et se développer sur des substrats de kératine ou de chitine, comme les plumes d'oiseaux (Johnson et Speare 2005), et même infecter des organismes autres que les

amphibiens, comme les écrevisses (*Procambarus* spp.) ou les nématodes (Shapard *et al.* 2012 ; McMahon *et al.* 2013 ; Brannelly *et al.* 2015).

*Batrachochytrium dendrobatidis* peut infecter les trois ordres d'amphibiens (Olson *et al.* 2021), mais toutes les espèces ni leurs stades de vie ne sont pas sur le même plan en termes de **susceptibilité**. Comme les têtards ne meurent généralement pas de l'infection, qui est limitée aux pièces buccales (Berger *et al.* 1998; Rachowicz and Vredenburg 2004), ils peuvent servir de **réservoirs**, maintenant un pool élevé de zoospores infectieuses dans l'environnement (Briggs *et al.* 2010 ; Walker *et al.* 2010 ; Clare *et al.* 2016b). Les espèces tolérantes, telles que xénope lissse *X. laevis*, qui fait l'objet d'un commerce intense, et l'ouaouaron *Lithobates castesbeianus* (aussi appelé grenouille taureau nord-américaine), agissent également comme des réservoirs et contribuent à la persistance et à la propagation de Bd (Weldon *et al.* 2004 ; Garner *et al.* 2006 ; Van Rooij *et al.* 2015). En fait, l'un des principaux moteurs de l'émergence de la chytridiomycose des amphibiens est l'introduction anthropique d'espèces tolérantes infectées (par exemple par le biais du commerce international d'amphibiens vivants) dans un environnement naïf, ce qui entraîne une pollution par les agents pathogènes (Cunningham *et al.* 2003 ; Daszak *et al.* 2003 ; Garner *et al.* 2006 ; Fisher *et al.* 2009 ; Schloegel *et al.* 2012). Il existe plusieurs lignées de Bd, avec lesquelles certaines espèces semblent avoir co-évolué et sont tolérantes ; cependant, les mouvements anthropiques ont donné lieu à une recombinaison entre souches de différentes lignées et à une **virulence** accrue envers les hôtes amphibiens, notamment avec l'émergence de la lignée panzootique globale (BdGPL ; Rachowicz *et al.* 2005 ; Farrer *et al.* 2011 ; Schloegel *et al.* 2012 ; Rosenblum *et al.* 2013 ; O'Hanlon *et al.* 2018). Le séquençage « génome complet » a montré que Bd, et notamment BdGPL, est originaire de la péninsule sud-coréenne, où les amphibiens sont tolérants ou résistants à l'infection (O'Hanlon *et al.* 2018).

L'épidémiologie des infections à Bd et de la chytridiomycose amphibienne est complexe mais peut être le résultat d'interactions entre quatre catégories de facteurs : ceux liés à l'hôte, au microbiome de l'hôte, à l'agent pathogène et à l'environnement (un cadre théorique connu sous le nom de pyramide des maladies, Bernardo-Cravo *et al.* 2020). La **virulence** de Bd peut varier selon les souches et les lignées (Fisher *et al.* 2009 ; Farrer *et al.* 2011 ; Porta 2014 ; Dang *et al.* 2017 ; Fisher and Garner 2020). Toutes choses égales par ailleurs, les hôtes peuvent être plus ou moins suceptibles à la maladie en fonction de leur état de santé (variation individuelle en raison des co-infections avec d'autres agents pathogènes, et en raison du stade de vie : par exemple, durant la métamorphose le système immunitaire est affaibli), de l'identité de leur population (les populations d'une même espèce peuvent avoir des susceptibilité différentes), ainsi que de l'identité de leur espèce (sensible, tolérante ou résistante ; par exemple le genre

*Speleomantes* est résistant grâce aux sécrétions cutanées de peptides antimicrobiens), ce qui peut être lié à des différences dans l'immunité innée ou adaptative (Rachowicz and Vredenburg 2004; Tobler and Schmidt 2010; Gervasi *et al.* 2013 ; Pasmans *et al.* 2013 ; Baláž *et al.* 2014 ; Bradley *et al.* 2015 ; Savage and Zamudio 2016 ; Voyles *et al.* 2018).

Il a été démontré que le microbiome de l'hôte, en particulier le microbiome de la peau, joue un rôle important dans la détermination de l'occurrence, de l'intensité et de l'issue de l'infection par Bd (voir Bernardo-Cravo *et al.* 2020 pour une revue). D'une part, la présence de certaines bactéries ou micro-eucaryotes, comme les champignons, connus pour inhiber Bd en produisant des peptides antifongiques ou antimicrobiens, rend l'hôte amphibien moins enclin à être infecté et moins susceptible à la maladie (Harris *et al.* 2006 ; Brucker *et al.* 2008 ; Harris *et al.* 2009a ; Kearns *et al.* 2017). L'ajout de probiotiques a même permis de prévenir, ou du moins de réduire, la gravité de la chytridiomycose dans certaines situations (Harris *et al.* 2009b ; Kueneman *et al.* 2016a). Dans certains cas, un microbiote cutané plus riche en diversité a été associé à une résistance ou à des résultats moins sévères de la maladie (Piovia-Scott *et al.* 2017 ; Bates *et al.* 2018), alors qu'on ne sait toujours pas si un microbiote **dysbiotique** (composition anormale, moins riche en diversité) prédispose les hôtes à une infection plus sévère ou est le résultat d'une perturbation par l'infection à Bd (Jani and Briggs 2014 ; Walke *et al.* 2015 ; Jani *et al.* 2017). D'autre part, il a été récemment démontré que non seulement la présence d'inhibiteurs de Bd dans le microbiote cutané, mais aussi l'épaisseur du biofilm cutané qui contient ces micro-organismes était importante pour prévenir l'infection par Bd (Chen *et al.* 2022).

Enfin, les facteurs environnementaux influencent également l'épidémiologie de la chytridiomycose, et tous les autres facteurs. Ils peuvent être divisés en deux sous-catégories, à savoir les facteurs environnementaux biotiques et abiotiques. Les facteurs abiotiques comprennent le climat (température, altitude, précipitations, saisonnalité, humidité, El Niño), les variables liées à l'eau (vitesse du courant, chimie et en particulier salinité, présence de toxiques de synthèse) et l'exposition aux ultraviolets (Pounds *et al.* 2006 ; Kriger *et al.* 2007 ; Kriger and Hero 2007, 2008 ; Rohr and Raffel 2010 ; Walker *et al.* 2010 ; Garner *et al.* 2011 ; Ortiz-Santaliestra *et al.* 2011 ; Rohr *et al.* 2013 ; Raffel *et al.* 2015 ; Clare *et al.* 2016b ; Clulow *et al.* 2018 ; Fisher and Garner 2020). Ces facteurs environnementaux expliquent pourquoi certaines espèces prédites comme étant à faible risque de maladie ont connu des épisodes de mortalité massive lors d'événements saisonniers inhabituels (Baláž *et al.* 2014 ; Clare *et al.* 2016b ; Olson *et al.* 2021). En termes de facteurs environnementaux biotiques, la composition de la communauté influence la probabilité d'infection, avec d'autres espèces d'amphibiens ou d'autres stades de vie qui peuvent servir de réservoirs, ou d'autres espèces ou substrats sur

lesquels Bd peut survivre (Fisher and Garner 2020). Il a été démontré que les zoospores une fois relarguées dans le milieu interagissent avec une variété d'autres organismes dans l'eau. Ces interactions comprennent la consommation par des filtreurs tels que les ciliés, les rotifères, les tardigrades et les crustacés tels que les cladocères, les ostracodes et les copépodes (Buck *et al.* 2011 ; Searle *et al.* 2013 ; Schmeller *et al.* 2014 ; Blooi *et al.* 2017 ; De Troyer *et al.* 2021). La présence de ces espèces dans le zooplancton s'est traduite, tant en laboratoire que sur le terrain, par une réduction de la pression d'infection (Searle *et al.* 2013 ; Schmeller *et al.* 2014).

Dans l'état actuel des connaissances, les déclins graves dus à la chytridiomycose amphibienne se sont principalement produits en Amérique du Sud et centrale, en Océanie et, dans une moindre mesure, en Amérique du Nord, en Afrique et en Europe, mais aucun en Asie (Berger *et al.* 1998 ; Bosch *et al.* 2001 ; Lips *et al.* 2006 ; Schloegel *et al.* 2006 ; Skerratt *et al.* 2007 ; Vredenburg *et al.* 2010 ; Cheng *et al.* 2011 ; Scheele *et al.* 2019 ; Fisher and Garner 2020). En Europe, les espèces d'amphibiens semblent relativement résistantes/tolérantes à la chytridiomycose causée par Bd, et seules quelques épisodes de mortalités massives ont été observées (Garner *et al.* 2005 ; Walker *et al.* 2010). L'infection est répandue, bien qu'elle ne soit pas distribuée de manière aléatoire dans l'espace ou systématiquement synonyme de maladie (Walker *et al.* 2010 ; Miaud 2013 ; Baláž *et al.* 2014). L 'infection par Bd est plus probable dans les familles Alytidae et Bombinatoridae (Baláž *et al.* 2014).

En particulier, l’alyte (ou crapaud) accoucheur, *Alytes obstetricans* (Ao), est très susceptible à la chytridiomycose amphibienne (Bosch *et al.* 2001). C'est l'une des rares espèces à avoir décliné en Europe à cause de cette maladie, mais seulement dans certains endroits, comme dans certains bassins versants des Pyrénées (Bosch *et al.* 2001 ; Tobler and Schmidt 2010 ; Walker *et al.* 2010). Étant très répandus et ayant un développement plastique, les Ao ont été intensivement étudiés pour comprendre l'épidémiologie de la chytridiomycose et les résultats ont montré que les foyers de la maladie sont associés à l'altitude (Walker *et al.* 2010). La température et la saisonnalité sont des facteurs importants, tout comme le fait que les têtards de cette espèce sont des réservoirs très compétents puisqu'ils présentent une métamorphose retardée en altitude, et peuvent rester à l'état larvaire pendant deux, voire trois ans (têtards en **brumation** ; Walker *et al.* 2010 ; Clare *et al.* 2016b). La composition du zooplancton, et plus particulièrement la présence de consommateurs aquatiques de Bd tels que les rotifères et les ciliés, a pu expliqué les variations en termes de prévalence d’infection (Schmeller *et al.* 2014). Cependant, ces éléments ne suffisent pas à expliquer les résultats d'enquêtes de capture-marquage-recapture sur des populations pyrénéennes d'Ao habitant des sites écologiquement et physiquement similaires (proches, et à une altitude semblable; Clare 2014). Ces enquêtes ont montré que, des années après l'émergence (du moins, la première détection) de Bd dans les

Pyrénées en 2002, certaines populations étaient toujours en déclin tandis que d'autres se rétablissaient et devenaient stables bien que toujours infectées. Il a été démontré que les populations d'Ao de ces sites avaient une composition bactérienne cutanée différente, les populations stables ayant des taxons inhibiteurs de Bd plus abondants et généralement un microbiote plus diversifié sur leur peau (Bates *et al.* 2018). Ces différentes populations d'Ao semblent être infectées par la même souche de Bd (Bates *et al.* 2018). Cependant, on ne sait pas si elles présentent des différences dans leur génétique (par exemple, les fréquences des allèles du CMH) qui pourraient potentiellement expliquer ces dynamiques épidémiologiques contrastées, bien que la migration (et donc les échanges de gènes) soient possibles entre ces lacs, en particulier entre Ansabère et Acherito qui sont très proches (environ 1,75 km) mais présentent toujours des dynamiques de maladie différentes.

## Questions à l'origine de ce travail

Alors que les facteurs liés à l'hôte (génétique) doivent encore être étudiés, il est possible que d'autres facteurs environnementaux biotiques puissent expliquer l'épidémiologie particulière de l'infection à Bd et de la chytridiomycose des amphibiens dans les Pyrénées, encore incomplètement comprise. L'hypothèse principale de mon travail est que les biofilms benthiques pourraient en être une autre composante biotique. Les biofilms pourraient avoir un impact sur l'épidémiologie de la chytridiomycose des amphibiens par, au moins, quatre catégories de processus non mutuellement exclusifs. Ces quatre catégories correspondent aux quatre sommets de la pyramide des maladies (Bernardo-Cravo *et al.* 2020 ; **Figure 6**).

- Les biofilms pourraient affecter la santé de l'hôte amphibien. Le biofilm étant une source de nourriture pour les têtards d'alytes, la qualité nutritionnelle des biofilms est de première importance pour la santé des têtards et leur capacité à combattre l'infection avec un système immunitaire efficace. À cet égard, les habitants des biofilms tels que les diatomées sont intéressants car ils sont les principaux producteurs d'acides gras poly-insaturés, tels que les ω-3, qui sont vitaux pour la physiologie et l'immunité d'un large éventail d'organismes, y compris les têtards mais aussi les vertébrés terrestres (Hixson *et al.* 2015). En revanche, d'autres algues comme les algues vertes (Chlorophyta) et les cyanobactéries contiennent moins d'ω-3et ont donc une qualité nutritionnelle moindre (Brett and Müller-Navarra 1997; Guo *et al.* 2015 ; Crenier *et al.* 2019). En fonction de leur composition, les biofilms peuvent donc varier en termes de qualité nutritionnelle, ce qui a un impact sur le système immunitaire de l'hôte. Les biofilms peuvent aussi affecter la santé de l'hôte en raison de leur toxicité, soit en concentrant les polluants (Bonnineau *et al.* 2020 ; Mahler *et al.* 2020), soit par la production de toxines

(Oberemm *et al.* 1999 ; Dao *et al.* 2010). Des cyanobactéries toxigènes vivant dans des biofilms ont en effet été signalées comme étant à l'origine de la mortalité de poissons, de chiens et de bétail en montagne et ailleurs (Mez *et al.* 1997 ; Gugger *et al.* 2005 ; Quiblier *et al.* 2013 ; Wood *et al.* 2020). Les têtards seraient encore plus exposés que les organismes terrestres, puisqu'ils consomment directement les biofilms, et qu'ils peuvent également être impactés par les conséquences de la prolifération des cyanobactéries benthiques, qui désoxygènent l'eau (Wood *et al.* 2020).

- Les biofilms pourraient affecter directement l'agent pathogène (zoospores de Bd). On sait très peu de choses sur les interactions entre les agents pathogènes non humains et les biofilms environnementaux. Pour les agents pathogènes humains, les biofilms peuvent agir soit comme un réservoir, soit comme un puits (Chabaud *et al.* 2006 ; Wingender and Flemming 2011). Les résultats de la recherche sur le microbiote cutané des amphibiens montrent que les zoospores de Bd ont des difficultés à infecter l'hôte lorsque les biofilms cutanés sont épais, ce qui suggère une interférence physique (empêchement d'espace) et/ou chimique (Chen *et al.* 2022). La matrice du biofilm dans l'environnement benthique pourrait être un piège physico-chimique pour les zoospores: physique car son architecture peut être vraiment entrelacée et avoir des streamers, (extensions, **Figure 3**) qui pourraient interférer avec le mouvement des zoospores, et chimique car elle peut contenir des molécules adhésives ou inhibitrices qui, au contact, immobiliseraient ou dommageraient les zoospores de Bd (Rendueles and Ghigo 2012, 2015). Les toxines ou autres molécules sécrétées par les habitants du biofilm dans la colonne d'eau pourraient également affecter les zoospores (**allélopathie** ; Leflaive and Ten-Hage 2007; Wu *et al.* 2011 ; Allen *et al.* 2016).

- Les biofilms pourraient affecter le microbiome de l'hôte. Comme les têtards se nourrissent et sont fréquemment en contact sur des biofilms benthiques, le microbiote du biofilm pourrait affecter les microbiotes cutané, oral et intestinal de l'hôte. Comme mentionné ci-dessus, le microbiote intestinal affecte la santé de l'hôte de diverses manières, tandis que les microbiotes cutané et oral ont une importance dans la prévention de l'infection par le Bd ou la réduction de sa gravité.

- Les biofilms peuvent affecter l'environnement biotique et abiotique. Les biofilms importent des matières organiques et inorganiques par sorption passive ou active pour leurs besoins. En altérant ou même en appauvrissant l'environnement de certains minéraux ou nutriments, les biofilms pourraient indirectement affecter le temps pendant lequel les zoospores sont motiles, c'est-à-dire infectieuses, d'autant plus que leur mouvement est régulé par la chimiotaxie (Johnson and Speare 2003 ; Moss *et al.* 2008 ; Woodhams *et al.* 2008). La composition des biofilms, en tant que base des réseaux alimentaires, pourrait également

influencer celle du zooplancton, qui peut à son tour avoir un impact sur la pression d'infection de Bd pour les amphibiens (Schmeller *et al.* 2014).

Bien que ma thèse explore certains de ces mécanismes, elle vise également à améliorer les connaissances sur la composition des biofilms dans les lacs de montagne en général. Après avoir passé en revue la littérature et illustré l'importance des biofilms pour de nombreux écosystèmes et ceux des lacs et étangs de montagne en particulier, il est clairement apparu que l'acquisition de connaissances sur la composition des communautés de biofilms dans les différents lacs, où les amphibiens ont été suivis dans notre système d'étude, était une condition préalable à toute enquête épidémiologique sur les interactions entre Bd et les biofilms. Par conséquent, j'ai répondu aux questions suivantes, correspondant à mes différents chapitres, en utilisant une combinaison d'études de terrain et d'expériences de laboratoire :

- **Chapitre 2**, intitulé "*L'importance des biofilms pour les santés humaine, animale, végétale et des écosystèmes*" : Que sait-on de l'importance des biofilms pour la santé des organismes multicellulaires et des écosystèmes ?
- **Chapitre 3**, intitulé "*Les communautés microbiennes de biofilms changent dans les lacs de montagne isolés, avec une augmentation de l'abondance relative d'algues toxinogènes*" : Quelle est la composition de la communauté des biofilms benthiques de lacs de montagne ? Varie-t-elle dans l'espace (entre des lacs écologiquement différents) et dans le temps (au sein d'un même lac) ? Existe-t-il des tendances de la biodiversité microbienne cohérentes avec celles observées actuellement pour les macro-organismes ?
- **Chapitre 4**, intitulé "*Premières indications des liens entre biofilms benthiques et épidémiologie des infections à* Batrachochytrium dendrobatidis *dans les Pyrénées"* : Existe-t-il des liens plausibles entre la composition des communautés microbiennes de biofilms et l'épidémiologie des infections à Bd et de la chytridiomycose des amphibiens dans les Pyrénées ?
- **Chapitre 5**, intitulé "*Les biofilms environnementaux peuvent affecter le devenir du stade infectieux d'un agent pathogène emergent*" : Les biofilms ont-ils un impact sur le stade infectieux de Bd en laboratoire ? Si un effet existe, varie-t-il en fonction de la composition du biofilm ? Même un simple biofilm, constitué d'une seule algue autotrophe (sans consommateur), peut-il avoir un effet ?

Comme les lacs de montagne sont actuellement confrontés à de nombreuses pressions anthropiques, j'ai émis l'hypothèse que la biodiversité des procaryotes et celle des micro-

eucaryotes diminueraient au fil du temps, et que la composition de leur assemblage respectif changerait avec une abondance relative accrue d'organismes plus résistants comme les cyanobactéries. En ce qui concerne le chapitre 4, mon hypothèse était que les biofilms dans les lacs avec des populations d'amphibiens moins infectées et/ou moins touchées contiendraient une plus grande diversité, auraient une composition micro-eucaryotique différente avec plus d'espèces de consommateurs potentiels de Bd (ou alors simplement en plus grandes abondances) tels que les rotifères. Enfin, dans le chapitre 5, j'ai émis l'hypothèse que des biofilms très simples (produits par une seule algue phototrophe) cultivés en laboratoire, n'affecteraient pas le nombre de zoospores de Bd au fil du temps, alors que les biofilms cultivés sur le terrain puis ramenés en laboratoire, qui peuvent abriter des consommateurs de Bd, le feraient.

## Système d'étude

Les biofilms ont été échantillonnés dans 26 lacs répartis en six gradients altitudinaux (six vallées), principalement situés dans les Pyrénées françaises de 2016 à 2020 (**Figure 7**). La majorité de ces lacs sont situés au-dessus de 1500 m d'altitude et sont oligotrophes, c'est-à-dire pauvres en nutriments (**Table 1**). Les conditions climatiques sont très différentes entre les gradients, avec à l'Est, Bethmale, Bassies, Arbu-Lers-Estagnon étant les zones les plus chaudes et les plus sèches (influence méditerranéenne), Lescun dans la partie la plus occidentale étant la zone la plus humide (influence océanique), et Fache et Neouvielle, entre les deux, étant les gradients les plus froids et les plus en altitude, avec des précipitations relativement élevées aussi (**Table 1**).

Pour surveiller l'infection par Bd (ainsi que d'autres) chez les amphibiens, des têtards ou des larves de plusieurs espèces ont été échantillonnés, notamment l'alyte accoucheur (Ao), la grenouille commune (*Rana temporaria*), le crapaud épineux (*Bufo spinosus*), le triton palmé (*Lissotriton helveticus*), la salamandre tachetée (*Salamandra salamandra*) et occasionnellement le calotriton des Pyrénées (*Calotriton asper*). Les têtards et les larves sont plus faciles à trouver que les adultes. Étant donné que mon travail se concentre sur la chytridiomycose amphibienne causée par Bd, je n'ai utilisé qu'Ao dans mes données sur les infections. Le crapaud accoucheur est l'espèce sentinelle de cette maladie dans les Pyrénées. *Alytes obstetricans* est un amphibien anoure que l'on trouve communément en France, avec également quelques populations dans le nord de l'Espagne et du Portugal, ainsi qu'en Suisse, en Belgique et en Allemagne, vivant dans les forêts tempérées, les zones semi-arides, les murs, les talus et les pentes avec de petites pierres et une végétation clairsemée (UICN SSC Amphibian

Specialist Group 2022). L'alyte accoucheur se reproduit de préférence dans les étangs et les mares stagnantes permanentes, ou moins fréquemment dans les rivières à faible courant, et présente une forme particulière de soins parentaux : le mâle porte les œufs fécondés sur son dos jusqu'à ce qu'ils soient prêts à éclore, moment auquel le mâle retourne dans l'eau pour libérer les têtards (**Figure 8**). Son statut de conservation sur la liste rouge de l'UICN est "Préoccupation mineure", mais les populations sont généralement en déclin avec des extinctions locales signalées en raison de la perte, la fragmentation et la dégradation de ses habitats, et des maladies infectieuses (chytridiomycose, ranavirose et autres ; IUCN SSC Amphibian Specialist Group 2022).

Les têtards ont été capturés chaque année en été et écouvillonnés pour détecter la présence d'ADN de Bd à partir de 2008. La distribution des têtards d'Ao infectés par Bd est présentée dans la **Figure 9**. Les crapauds accoucheurs ont été trouvés infectés presque chaque année dans les cinq lacs du gradient Lescun : Ansabere, Arlet, Acherito, Lhurs, et Puits d'Arrious. Le lac Acherito est le lieu où la chytridiomycose amphibienne a été détectée pour la première fois dans les Pyrénées en 2002. Depuis lors, dans ces lacs, l'impact de la maladie sur les populations à long terme s'est révélé différent. Les populations d'Ao d'Acherito, de Lhurs et du Puits d'Arrious, après des effondrements suite à l'émergence de la maladie, se sont rétablies et sont maintenant stables, bien que certains individus aient des charges parasitaires de Bd élevées. En revanche, les populations d'Arlet et d'Ansabere n'ont montré aucun signe de rétablissement et il a été difficile d'y trouver le moindre têtard d'Ao ces dernières années.

Dans les autres gradients, des infections à Bd ont été détectées à Fache, Neouvielle et Bethmale mais pas dans les gradients Bassies et Arbu-Lers-Estagnon. Les populations d'Ao de Gourg de Rabas, de Madamète-Haut et de Madamete-Bas (Neouvielle) ont été trouvées infectées en 2009-2010 (et aussi en 2011 pour le premier), puis les populations se sont effondrées. Les têtards et les pontes n'ont été détectés à nouveau, échantillonnés et testés négatifs qu'en 2020 et 2021, ce qui suggère que les populations ont recolonisé les lieux, et ce, sans infection par Bd. Cependant, aucune population d'Ao n'a resurgi à Madamete-Haut, qui est pourtant situé entre Gourg de Rabas et Madamete-Bas. À Fache, Bd a été détecté dans le lac Paradis de 2013 à 2018 et l'étang Vallon en 2015 et 2018, bien que les intensités d'infection aient souvent été faibles, en particulier pour Vallon. A Bethmale, Bd a été détecté à Ayes en 2015 et 2018-19, avec des intensités d'infection et une prévalence élevées. Très peu de têtards d'Ao ont été trouvés en 2020 et 2021, alors que leur nombre paraissait important en 2022. Les analyses n'ont pas encore été effectuées pour ce lac.

Pour toutes les expériences de laboratoire, j'ai utilisé la souche IA043 de Bd isolée à partir d'un individu Ao récemment métamorphosé trouvé mort à Acherito en 2005, et fournie par Matthew Fisher (Imperial College London). Les détails sur la façon dont la souche est maintenue en laboratoire sont donnés au chapitre 5.

# CHAPTER 1

# GENERAL INTRODUCTION

# Chapter 1: General introduction

## 1.1 Biodiversity, ecosystem services and links to sustainability

Planet Earth is teeming with life and this is perhaps its most unique feature. Life can be defined as "a process that takes place in highly organised organic structures and is characterized by being pre-programmed, interactive, adaptative and evolutionary", while living beings are the system in which this process takes place (Gómez-Márquez 2021). Because living beings adapt and evolve in response to ever-changing environments, an astonishing variety of life forms has emerged since life appeared over 3.9 billion years ago (Betts *et al.* 2018). This variety is termed **biodiversity** and all living organisms interact with one another and their non-living environement in a functional unit called **ecosystem** (Mace *et al.* 2005, 2012). These interactions among organisms on the one hand, and between organisms and the atmosphere, geosphere, and hydrosphere on the other hand, underlie all ecosystem processes and constitute the environment on which all living beings, including us, humans, depend (Mace *et al.* 2005). Processes benefiting humans are called **ecosystem services** and include the provision of food, clean water, clean air, medicines, timber, and other processes such as pollination, decomposition of organic matter, detoxification, carbon dioxide capture and the regulation of infectious diseases, not to mention the invaluable spiritual and cultural values for many communities and peoples (Mace *et al.* 2012).

Ecosystem services are necessary for the survival and prosperity of our societies, as well as for our **health** and well-being (Sentenac *et al.* 2022). In appreciating this, a major problem quickly becomes apparent: life and biodiversity on Earth are being lost at a rapid rate due to human activities, which directly threatens the provision of ecosystem services (Díaz *et al.* 2019). For ecosystem services to be continually provided, ecosystems must be healthy, i.e. they must have normal **community structure**, normal **productivity,** and normal **resilience** (concept of Ecosystem Health; Rapport *et al.* 1998). These three pillars of ecosystems health imply that most species usually forming the ecosystem must be present in normal abundance and most organisms must be healthy.

Advances in health care and technology have allowed humans to thrive over the past few centuries, but human development is now such that many ecosystems have been or are being degraded (Steffen *et al.* 2015; Díaz *et al.* 2019). This is of particular concern considering that humans evolved and developed in a short 11,700-year period (the Holocene) during which most ecosystems were in relatively stable states (see **Alternative stable states** in Glossary); but we are entering a new epoch, the Anthropocene, characterized by much more unstable conditions (unhealthy ecosystems) due to human-driven **global change** (Rockström *et al.* 2009;

Steffen *et al.* 2015). Unstable ecosystems mean that the provision of services is uncertain and/or possibly insufficient for humans to live decently, thereby threatening the health gains of the last centuries (Queenan *et al.* 2017). While global changes also occurred and caused mass extinctions in the past, for instance because of meteorite impacts or volcanism (Bond and Grasby 2017), the ongoing sixth mass extinction is estimated to be much more intense in terms of species losses, with an extinction rate about 1,000 times higher than the normal background rate (Barnosky *et al.* 2011; Pimm *et al.* 2014; Ceballos *et al.* 2015; De Vos *et al.* 2015).

Current anthropogenic drivers of declines can be local or global, and include climate change, habitat loss, fragmentation and degradation, nutrient and chemical pollution, the introduction of invasive species, overexploitation of living and non-living resources, emerging diseases, and cascading effects, among others (Diamond 1984; Daszak *et al.* 2000; Steffen *et al.* 2015; Díaz *et al.* 2019). All these drivers of decline are in fact themselves, directly or indirectly, linked to interconnected socio-cultural, economic, political, institutional, technological and demographic factors, and thus to human health and well-being (Queenan *et al.* 2017; Díaz *et al.* 2019). Therefore, human societies must change and find ways to develop without damaging their immediate environment and the **biosphere** as a whole, so that ecosystem services can be sustainably provided and health and well-being maintained (Díaz *et al.* 2019). This will require development policies that are properly informed by ecological knowledge. Life and ecosystems have evolved over billions of years, and understanding how ecosystem processes, and ultimately our health, are affected by multiple anthropogenic pressures acting simultaneously is extremely complex. Holistic approaches to health and sustainability are thus particularly indicated here. One such approach was already mentioned, Ecosystem Health (Rapport *et al.* 1998) which is ecocentric, and another popular one is One Health (Zinsstag *et al.* 2011), which is more anthropocentric. Both are convergent to some extent and I will use a merged approach throughout this manuscript (**Figure 1**).

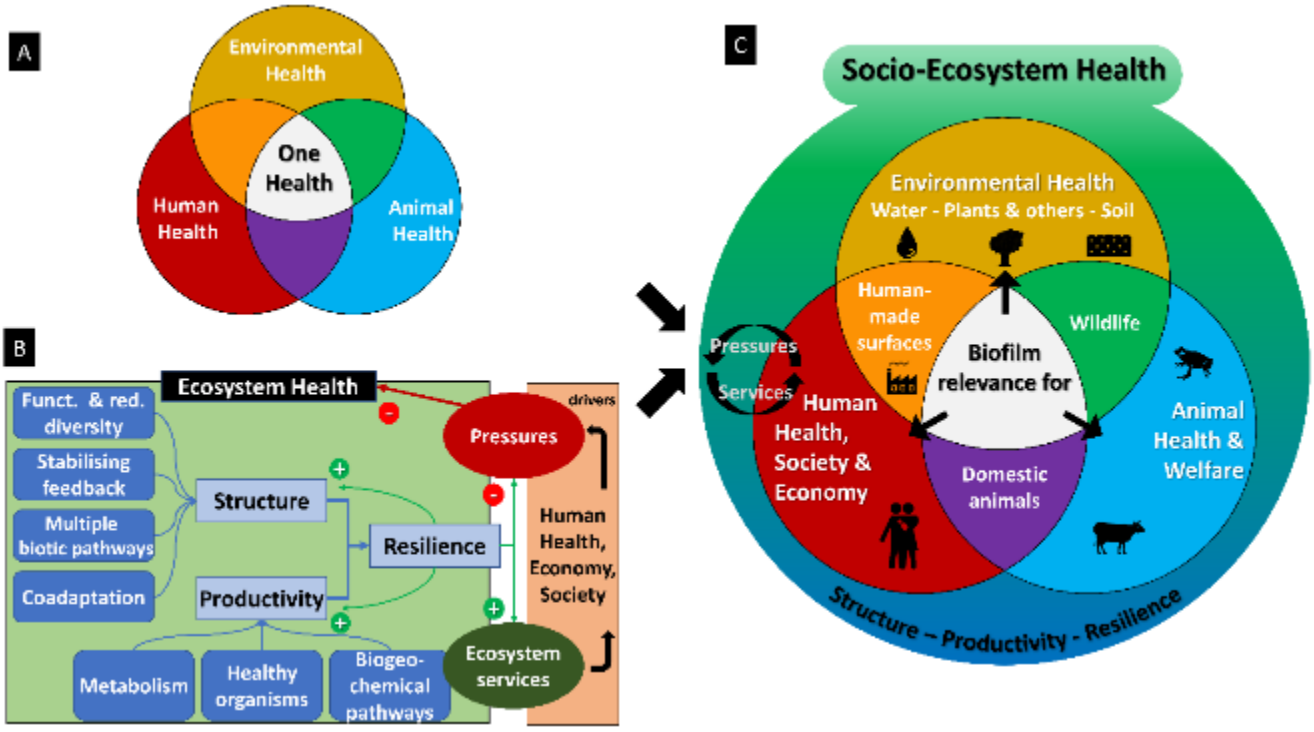


**Figure 1: Holistic approaches to health**. A: diagram of the One Health approach showing that human, animal and environmental health are tightly intertwined. This approach has mainly been used so far to understand the ecological drivers of **emerging infectious diseases** so as to predict and control them. B: conceptualization of ecosystem health, modified from Tett *et al.* (2013), with structure, productivity and resilience being the three pillars conditioning the health of the ecosystem and the delivery of ecosystem services to humanity; abbreviations: 'Funct.': **functional**, 'red.': **redundant diversity**. C: conceptualization of the convergence of the One Health and EcoHealth approaches into our view of a systemic approach to health. We used this latter approach here to review the significance of the biofilm life form to socio-ecosystem health in Sentenac *et al.* (2022).

The scientific fields of **community ecology** (Morin 2009), **functional ecology** (Calow 1987), **eco-epidemiology** (Rioux *et al.* 1997), and **conservation biology** (Soulé 1985) are here highly relevant to informing effective decision making and conservation practices. Because it is often difficult to observe all species in a site, i.e the whole community, **assemblages** or **guild**s are rather studied with concepts such as **α-diversity**, **β-diversity**, **η-diversity**, and finally, **γ-diversity** (Whittaker 1960, 1972; Fauth *et al.* 1996; Hui and McGeoch 2014). Many different indices exist to measure both α-diversity (for example, species richness, which only considers presence-absence of taxa whereas the Shannon index or the Inverse-Simpson index also take the abundance of taxa into account) and β-diversity (e.g. Jaccard dissimilarity which is presence-absence based, or Bray-Curtis dissimilarity which is abundance-based ; Jaccard 1912; Shannon 1948; Simpson 1949; Bray and Curtis 1957). The work presented here is

transdisciplinary and strongly rooted in these fields, with a particular focus on mountain freshwater ecosystems and more particularly on the interplay between the composition of biofilm microbial communities (more precisely, their prokaryotic and micro-eukaryotic assemblages), and an aquatic fungal parasite of amphibians impacting their populations.

## 1.2 Mountains and their freshwater ecosystems

Mountains are landforms characterised by rugged topography (>200 m difference in elevation within a 2.5′ cell, 0.5′ resolution) that occupy about 12.3% of the total terrestrial land area outside Antartica (Körner *et al.* 2011). Mountains provide heterogeneity in climatic conditions, soil types and three-dimensional complexity, creating a multitude of ecological **niches** where species evolve and diversify: relative to their area, mountains indeed hold a high level of biodiversity and **endemism** (Körner 2004; Fjeldså *et al.* 2012; Hoorn *et al.* 2013; Badgley *et al.* 2017; Rahbek *et al.* 2019). The physical barriers established by mountains disrupt the flow of gases in the atmosphere and force moisture-laden air to rise and cool, triggering the formation of orographic clouds and increasing humidity and precipitations relative to lowlands (Roe 2005). Water is stored as ice and snow in the summit areas, which turns into liquid water during the warm seasons. Freshwater bodies are, therefore, very abundant in mountains in the form of streams but also, because of the low permeability of the crystalline bedrock, in the form of lakes, ponds or wetlands (Catalan *et al.* 2006).

While fresh waters (low salinity) only represent 0.01% of the water on Earth, they are home to an extraordinary biodiversity with approximately 10 % of all known species, including one third of vertebrate species (Dudgeon *et al.* 2006). Unfortunately, freshwater biodiversity is in crisis, with rates of decline far greater than those experienced by marine or terrestrial biodiversity (Dudgeon *et al.* 2006; Strayer and Dudgeon 2010; Harrison *et al.* 2018; Reid *et al.* 2019; Tickner *et al.* 2020). The last Living Planet Index report estimated that freshwater populations of vertebrates declined by 83% [74% - 89%] between 1970 and 2018 (WWF 2022). The reasons are multiple and include climate change, overexploitation (facilitated by e-commerce), biological invasions, infectious diseases, harmful algal blooms, hydropower, pollution by synthetic chemicals, nanomaterials and microplastics, light and noise disturbance, and changes in water chemistry (e.g. salinization ; Reid *et al.* 2019).

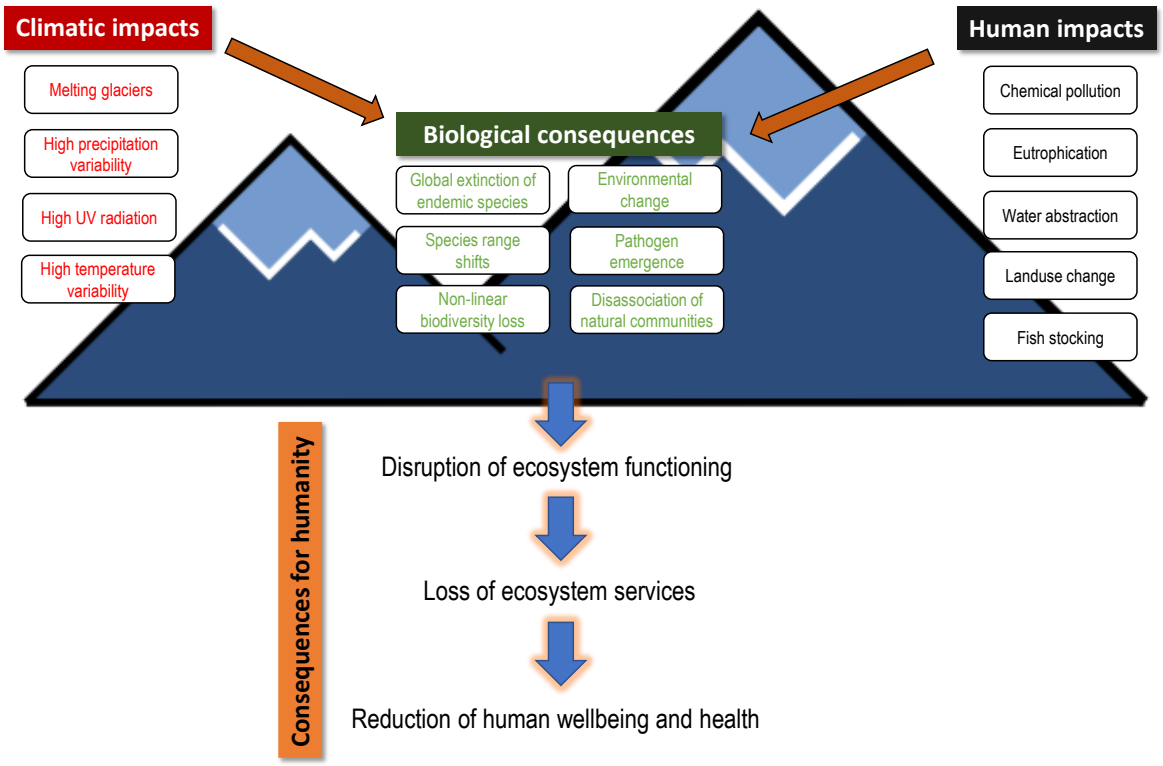


**Figure 2: Threats to mountain ecosystems and their consequences on biodiversity and humanity** (from Schmeller *et al.* 2022).

Despite their remoteness, mountain freshwater ecosystems are no exception (Schmeller *et al.* 2018; **Figure 2**). In particular, the impacts of climate change are very strong and warming is greater in mountains than in lowlands (Rangwala and Miller 2012; Pepin *et al.* 2015; Schmeller *et al.* 2022). Increasing water temperatures and evaporation rates, glacier retreat, alterations of aquatic mixing regimes, extreme weather events, as well as changes in water chemistry resulting from the intensification of erosion, are factors affecting mountain freshwater ecosystems (Koinig *et al.* 1998; Rangwala and Miller 2012; Pepin *et al.* 2015; Niedrist *et al.* 2018). But there are other threats to mountain waters (Schmeller *et al.* 2022). Most of the drivers of freshwater biodiversity declines also apply here, with the possible exceptions of e-commerce and light/ noise disturbance (although possible). Paradoxically, mountain freshwaters are not immune to pollution: the topography of mountains is such that long-range atmospheric deposition of chemical micropollutants, including persistent organic pollutants and potentially harmful trace-elements, occurs (Schmeller *et al.* 2018). Pollution by pesticides, including herbicides and veterinary drugs, has also been reported in mountain lakes (Machate *et al.* 2022, 2023). Veterinary drugs likely enter these ecosystems via livestock, which relates to other threats like land-use change. Mountain vegetation has been largely shaped by

human land-use (Koerner *et al.* 1997), and increasing livestock units also put pressure on lakes and ponds through degradation of shorelines and potential eutrophication (nutrient pollution) of naturally oligotrophic water bodies (Mayer *et al.* 2022; Schmeller *et al.* 2022). Water abstraction in mountains is also important, for hydroelectricity production, agriculture, snowmaking, and many natural mountain freshwater ecosystems are impacted by these activities (Schmeller *et al.* 2022). The introduction of exotic invasive species is also a threat (e.g. aquatic plant species) which is very hard to mitigate in remote freshwater ecosystems and might be exacerbated by climate change (McDougall *et al.* 2011). Introduced species do not need to be invasive to cause widespread damage in mountain lakes, when high abundance is deliberately maintained by humans through regular introductions. This is the case for salmonids and minnows, non-native in most mountain lakes, which are introduced for fishing purposes and substantially affect freshwater biodiversity (Miró *et al.* 2018, 2020; Miró and Ventura 2020). Introduced species can also be pathogenic to native organisms, a phenomenon termed **pathogen** pollution (Cunningham *et al.* 2003). Pathogen pollution is an important driver of emerging **infectious diseases** and can have far-reaching consequences (see section **1.6** for an example of introduced pathogen).

Therefore, mountain lakes are currently very vulnerable. This makes them urgent to protect but also interesting to study, as they can be seen as sentinels of change (Catalan *et al.* 2006; Schmeller *et al.* 2018; Moser *et al.* 2019; Råman Vinnå *et al.* 2021). Mountain ecosystems provide an important number of essential ecosystem services both to people living inside and outside of mountains (Grêt-Regamey *et al.* 2012). These include the provision of clean drinking water, with more than 50% of people on Earth depending on water originating from mountains but also, among others, timber, grazing pastures for livestock, cultivable crops and recreational activities such as hiking, fishing, hunting, winter sports (Viviroli *et al.* 2007, 2020; Grêt-Regamey *et al.* 2012; Locatelli *et al.* 2017; Martín-López *et al.* 2019). However, how global change and other human activities impact mountain freshwater diversity, in particular microbial diversity, and how this affects water quality, is still poorly understood and investigated.

## 1.3 Microorganisms and biofilms

Although unobservable to the naked eyes and, consequently, often overlooked in **conservation biology**, microorganisms support all life in the biosphere (Cavicchioli *et al.* 2019; Timmis *et al.* 2019). Microorganisms were the first life form on Earth, and their ubiquity, biodiversity and abundance are staggering (O'Donnell *et al.* 1994; Whitman *et al.* 1998; Bar-

On *et al.* 2018; Timmis *et al.* 2019). Bacteria alone are estimated (with relatively high uncertainty) to account for 70 gigatons of carbon, that is *c.*15% of the Earth total biomass; in comparison, all animals combined only make up 2 gigatons of carbon and 0.004% of the total biomass (Bar-On *et al.* 2018).

Over the last two decades, the advent of next-generation high-throughput sequencing technologies has revolutionized our understanding of **microbiota** and **microbiomes** (Metzker 2010; Marchesi and Ravel 2015). Of particular interest to the determination of microbiota composition is **metataxonomics** (also called metabarcoding), which has heavily relied on the amplification and sequencing of marker genes such as the 16S and 18S rRNA genes, followed by taxonomic assignment (Marchesi and Ravel 2015). Microbial assemblages contain not only representatives of the domain Bacteria, but also other microorganisms of all branches of the tree of life, comprising the domain Archaea (Bacteria and Archaea form together the Prokaryotes) and some taxa of the domain Eukaryota, namely micro-eukaryotes (Woese *et al.* 1990). Micro-eukaryotes include microscopic animals, fungi and plants, and also unicellular eukaryotes called protists. The protists are a paraphyletic group containing taxa such as Amoebozoa, Excavata and Chromoalveolata (Stramenopiles, Alveota, Rhizaria). However, it should be noted that the taxonomic classification of the eukaryotic domain, and that of the whole tree of life, is still the subject of extensive research: see the work of e.g. Cavalier-Smith (Roger 2021). There is also a debate regarding whether viruses are micro-organisms as there is no consensus on viruses being actual living beings. That said, as they are pre-programmed, interactive, adaptative and evolutionary (Gómez-Márquez 2021), I consider them to be living beings but they were not addressed in this thesis for practical reasons (no marker gene readily available).

Microorganisms are essential to the functioning of all ecosystems on Earth (Cavicchioli *et al.* 2019; Bernardo-Cravo *et al.* 2020), and are also present in environments such as the deep subsurface where no other organisms exist (Phelps *et al.* 1989; Lovley and Chapelle 1995). In oceans, over 90% of life is microbial and photosynthetic algae are the basis of their food webs (Azam and Malfatti 2007; Sunagawa *et al.* 2015; 'The Census of Marine Life | Smithsonian Ocean' 2022). Microorganisms are also extremely abundant in soils, with half a gram able to contain between 2,000 and 5,000 microbial species (Schloss and Handelsman 2006). There, microorganisms decompose organic matter, cycle nutrients and render soils fertile and suitable for plant growth; in this respect, they play a central role in agriculture and sustainability (Singh *et al.* 2010; Bardgett and van der Putten 2014). Similar diversity, abundance and importance of microorganisms also exist in freshwater ecosystems (Sigee 2005; Hahn 2006). Even the health of multicellular macro-organisms, including humans, is intimately dependent on the various

microbiota they carry (Clemente *et al.* 2012; Bernardo-Cravo *et al.* 2020). There are approximately as many bacterial cells as human cells in a human body, and a 70-kg individual is thought to carry a bacterial biomass of 0.2 kg (Sender *et al.* 2016). The roles of microbiota in influencing the development, physiology and immunology of humans, other animals and plants has been widely documented (Lynch 1994; Clemente *et al.* 2012; Ezenwa *et al.* 2012; Vorholt 2012; Fung *et al.* 2017; Trevelline *et al.* 2019). Multicellular organisms actually form, with their microbiota, superorganisms called holobionts (Trevelline *et al.* 2019; Carthey *et al.* 2020). Thus, microorganisms affect their environment, whether it be at a multicellular-individual, ecosystem or global scale. Most importantly, they affect the climate but will also be affected by climate change (Cavicchioli *et al.* 2019).

Most microorganisms actually have a sessile lifestyle, embedded in a gel-like matrix (**Figure 3**). This lifestyle, called '**biofilm'**, is also one of the most ancient life form on Earth (3.3 billion years ;Westall *et al.* 2001). The biofilm way of life has also been very successful as biofilms are globally extremely abundant (Flemming and Wuertz 2019). It is the dominant form of life in many habitats of Earth, except open oceans (Flemming and Wuertz 2019). The matrix acts as a protection against molecules or organisms potentially harmful to biofilm inhabitants; it can be seen as a fortress and the biofilm as a whole, very rich in biodiversity and very organised, can be seen as a mini-ecosystem within the larger ecosystem (Watnick and Kolter 2000; Flemming and Wingender 2010; Flemming *et al.* 2016). Biofilms can be inhabited by bacteria, archaea, viruses and a multitude of micro- and meio-eukaryotes such as plants, diatoms, animals and fungi (Geesey *et al.* 1978; Costerton *et al.* 1987; Battin *et al.* 2001). The temporal and physical stability afforded by the matrix allows these inhabitants to interact in various ways, from competition to cooperation (Hansen *et al.* 2007; Rendueles and Ghigo 2012; Flemming *et al.* 2016). Interestingly, biofilms exhibit **emergent properties**, i.e. properties that would not be observed nor explained by these microorganisms studied alone, including horizontal gene transfer, active sorption (capture of materials), organised transport (through pores, voids and channels within the biofilm), extracellular retention of water, nutrients and enzymes, and last but not least, the cooperation between cells with evidence of division of labour and coordinated behaviour through chemical or electrical communication (Hansen *et al.* 2007; Flemming and Wingender 2010; Flemming *et al.* 2016). The matrix and these emergent properties make biofilms much more productive than any equivalent planktonic communities (Hansen *et al.* 2007). Therefore, biofilms are ubiquitous and were referred to as the microbial skin of ecosystems: a structuring, living and dynamic ecological boundary where both biotic and abiotic matter is juxtaposed, transited, separated and/or transformed (or not; Battin *et al.* 2016).

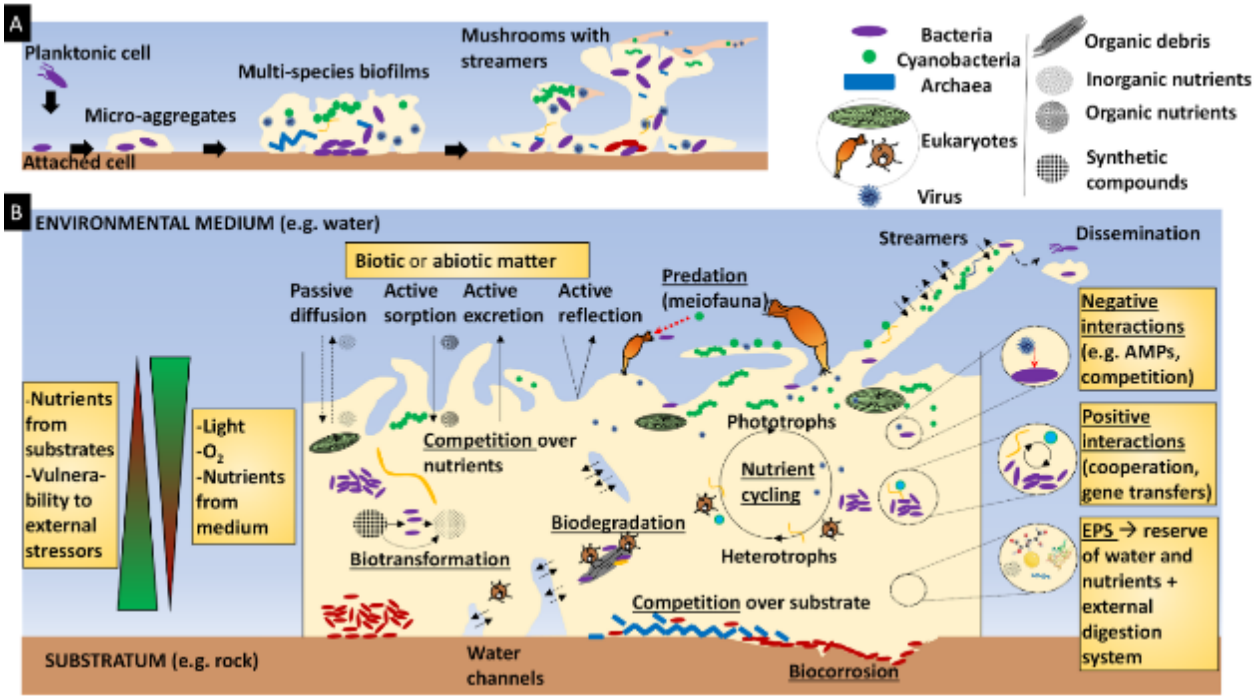


**Figure 3: Structure and properties of biofilms**. A: **ecological successions** (see Glossary) from a single-attached cell to a mature biofilm, with multiple microbial species and streamers to intercept particles. B: Diversity and **emergent properties** conferred by the matrix within a mature biofilm. Abbreviations: AMP antimicrobial peptides, EPS extracellular polymeric substances. From Sentenac *et al.* (2022).

An entire chapter of this thesis is devoted to the roles of biofilm communities both in ecosystems and in/on multicellular organisms (Chapter 2). Of particular interest are the roles of biofilms in mountain freshwater ecosystems. Biofilms grow on virtually all immersed solid–liquid interfaces such as rocks (epilithic biofilm or epilithon), decaying wood (epixylic biofilm), sand grains (episammon) and mud sediments (epipelon ; Vadeboncoeur and Steinman 2002; **Figure 4**). Benthic biofilms can dominate, in terms of biomass, the biota in streams and other water bodies, as waters are often shallow and/or has low turbidity (Vadeboncoeur and Steinman 2002; Besemer 2015). Futhermore, due to the extreme mountain conditions with regard to temperature variability, wind, siltation and nutrient availability, all of which prevent vegetation development, biofilms alone can ensure primary production and form the basis of food webs (Geesey *et al.* 1978; Lock *et al.* 1984; Vadeboncoeur and Steinman 2002; Rott *et al.* 2006). They also allow the decomposition of organic matter and recycling of nutrients, and in addition, can detoxify the water by adsorbing, fixating and metabolising pollutants (Sabater *et al.* 2002; Cardinale 2011). Finally, their cohesive matrix allows water retention, and ensures a bio-stabilisation of sediments, thereby limiting erosion (Gerbersdorf and Wieprecht 2015).

In contrast of these important roles, freshwater biofilms can contain toxigenic algae, such as cyanobacteria, which can secrete in the water cyanotoxins harmful to most sympatric aquatic organisms and others, including humans (Quiblier *et al.* 2013). In multicellular organisms, infectious agents may also form biofilms in colonized tissues or implanted medical devices to better resist the immune system or pharmaceutical drugs (section 2.3.1). Both cases illustrate that microorganisms can also negatively affect the health of other organisms, by causing them disease.

**Figure 4: Presence of a substantial quantity of biofilm on a piece of wood immersed in lake Gourg de Rabas, Pyrenees FR** (© Hugo Sentenac)

## 1.4 Diseases

Diseases, i.e. abnormality of structure and/or functions affecting the health of an organism, can be infectious or non-infectious. An **infectious disease** is the effect resulting from an **infection**, i.e. the entry and multiplication of an exogenous infectious agent in or on an organism (Porta 2014). Non-infectious disease is the result of other processes such as malnutrition, neosplastic processes (tumor and cancer), genetic/physiological disorders,

intoxication by **xenobiotics** (e.g. cyanotoxins, or any toxic synthetic substances). It is also important to distinguish between infection and infectious disease, as infection can be non-apparent (subclinal) and not cause disease. When infected but not diseased, an individual might contribute greatly to the spread of infectious agents, which is of high importance when studying the **epidemiology** of a disease. This relates to the slight difference between pathogens and parasites. **Pathogens** are organisms capable of causing disease, while **parasites** are organisms that live on or in other organisms and derive energy from them, not necessarily causing diseases. Depending on authors, there are further nuances between pathogens and parasites: some consider that the term of parasite should only be assigned to multicellular parasitic organisms (e.g. such as helminths or ticks), while the term pathogen should be employed for unicellular organisms. As in this work I study a fungal parasite of, and only sometimes pathogenic to, some species of amphibians (section 1.6), I rather used the term parasite to denote the fact that infection do not always result in disease. Indeed, different hosts may have different **susceptibility** to disease (Bernardo-Cravo *et al.* 2020). We further distinguish between **resistance**, that is the ability of host to limit infection, and **tolerance**, the ability to limit the effect of infection (Råberg *et al.* 2009).

While I mentioned emerging diseases as a driver of biodiversity loss, parasitism is a widespread type of **ecological interactions** that play an important role in shaping host population dynamics and driving biodiversity and evolution (Hudson *et al.* 2006; Bagchi *et al.* 2014). The vast majority of diseases are not of conservation significance. In fact, there had long been a persistent reluctance to recognise infectious diseases as a potential cause of species declines and, even more so, extinctions. This is due to theoretical models predicting that infection pressure necessarily decreases as the host population declines, leading to the extinction of the parasite before the host (McCallum and Dobson 1995). However, this is only true for parasites of which transmission is density-dependent, and more realistic models have shown that host extinctions due to disease are more likely than previously thought, for instance when the host populations are already fragile and/or naïve to the parasite, when there are environmental or biotic reservoirs of the parasite, or when transmission is not density-dependent but frequency-dependent (e.g. vector-borne or sexually-transmitted diseases; De Castro and Bolker 2005). Empirical examples are not lacking (anymore), with amongst others the White-Nose Syndrome for North American bats (Hoyt *et al.* 2021), avian malaria for Hawaiian Cardualinae birds (van Riper *et al.* 1986; Atkinson *et al.* 1995), the Devil-Facial Tumor Disease (DFTD) for Tasmanian devils (*Sarcophilus harrisii*; McCallum *et al.* 2007), Ebola Virus Disease for great apes (Leendertz *et al.* 2006), African Swine Fever for endemic wild swine of southeast Asia (Luskin *et al.* 2021), multiple diseases for corals (Green and

Bruckner 2000; Goldberg and Wilkinson 2004), and ranaviroses and chytridiomycosis for numerous amphibians (Martel *et al.* 2014; Rosa *et al.* 2017; Scheele *et al.* 2019), to only cite a few examples from the animal kingdom.

These diseases are **emerging infectious diseases**, i.e. diseases caused by newly evolved pathogens or parasites (or one of their newly evolved strains), or diseases of which the geographical distribution, host range and/or the incidence have significantly increased in a relatively short period of time (Jones *et al.* 2008). Emerging infectious diseases represent a serious threat to wildlife, plant, domestic animal and human health (Daszak *et al.* 2000; Dobson and Foufopoulos 2001; Jones *et al.* 2008; Fisher *et al.* 2012). Drivers of disease emergence are linked to socio-ecological changes, and include climate change, technological changes (enhanced connectivity and movements of humans and goods, linked with pathogen pollution), demographic change, land-use change (habitat degradation of fragmentation), pathogen pollution (introduction of invasive parasite species), as well as chemical and nutrient pollutions (Plowright *et al.* 2008; Johnson *et al.* 2010b; McKee *et al.* 2021; Baker *et al.* 2022; Eby *et al.* 2022). In particular, amphibians have been and still are more impacted by emerging infectious diseases than other classes of vertebrates.

## 1.5 Amphibians

Amphibians are a class of ectothermic vertebrates comprising 8,553 species divided into three orders: Anurans (7,530 species), Caudata (792 species) and Gymnophiona (216 species; Frost 2022). Among vertebrates, amphibians are more threatened and are declining faster than other classes such as birds or mammals (Stuart *et al.* 2004; Gascon *et al.* 2007), with as much as 41% of amphibians being threatened with extinction (IUCN 2022). The ecological implications of these declines are not yet fully understood, but amphibians require urgent conservation attention anyway as they possess unique life-history traits and biological processes (e.g. a complex life-cycle, and very diverse reproductive strategies) and are keystone species for ecosystem functioning (Halliday 2008; Hocking and Babbitt 2014). Most amphibian species have two life stages, an aquatic larva (named tadpole for anurans) and a terrestrial post-metamorphic individual, which are two very different ecological entities (Halliday 2008; Hocking and Babbitt 2014). Amphibians provide a link between the aquatic and terrestrial worlds and enable a flow of energy and matter between these two of types of ecosystems (Hocking and Babbitt 2014). Amphibian biomass can reach very high levels in their aquatic and terrestrial habitats, making them important food sources for other organisms (Gibbons *et al.* 2006). This includes snakes, for example, and it was demonstrated that declines in

amphibians can result in the declines of their predators, illustrating their importance in food webs (Matthews *et al.* 2002; Zipkin *et al.* 2020). This very high biomass of amphibian, more specifically of anuran tadpoles, is particularly relevant in mountain freshwater ecosystems, which are oligotrophic and would otherwise be relatively unproductive ecosystems. There are many guilds of tadpoles defined according to their-feeding type (and the anatomy of their mouthparts), but one of the most common worldwide, and also in mountain water bodies, is the biofilm (or **periphyton**) grazer guild (McDiarmid and Altig 1999; Altig *et al.* 2007). Tadpoles graze biofilms on all submerged surfaces, be it rocks, woods, plants, or sediments. In doing so, they considerably affect, at least in upland tropical streams, biofilm community structure and biomass, reduce sediment accumulation and influence the cycling of nutrient such as phosphorus and nitrogen (Ranvestel *et al.* 2004; Whiles *et al.* 2006; Altig *et al.* 2007; Hocking and Babbitt 2014). Declines in amphibian and tadpoles have considerably impacted stream ecosystem processes in the Neotropics (Ranvestel *et al.* 2004; Whiles *et al.* 2006, 2013), and a recent study in mountain streams of the Spanish Peñalara Massif showed similar conclusions (Alonso *et al.* 2022).

In addition to their roles in supporting ecosystem functioning, amphibians provide a variety of ecosystem services to humanity. They are an important food source for many human populations, accounting for thousands of tons each year at least and an important source of proteins (Tyler *et al.* 2007; Kusrini and Alford 2008; Valencia-Aguilar *et al.* 2013; Hocking and Babbitt 2014). Amphibians have greatly benefited human medicine and biological knowledge in various ways. First, some amphibian species like *Xenopus laevis* are important model species for evolutionary and developmental research, and this species was also intensively used to produce pregnancy tests (Tyler *et al.* 2007; Hocking and Babbitt 2014). Amphibians were also widely used for teaching anatomy and physiology. Next, their skin secretions have been direct sources or starting points for the development of many pharmaceuticals such as analgesics, anti-cancer drugs, antivirals and antibiotis, and amphibians are also used in traditional medicine in various parts of the world (Tyler *et al.* 2007; Hocking and Babbitt 2014). Carnivorous amphibian larvae and metamorphosed individuals have also been shown to control, through competition or predation, the abundance of larvae and adults of arthropods, such as mosquitoes (DuRant and Hopkins 2008; Rubbo *et al.* 2011; Valencia-Aguilar *et al.* 2013). Hematophagous arthropods such as mosquitoes can be vectors of important human infectious diseases, and amphibians, by regulating arthropod populations, are thought to reduce risks inherent in these diseases (Valencia-Aguilar *et al.* 2013; Hocking and Babbitt 2014). This has recently been demonstrated by the increase of malaria cases in Central America,

following steep amphibian declines (Springborn *et al.* 2022). Finally, amphibians provide important cultural services that enhance human well-being through recreation, religion, spirituality, and aesthetics (Tyler *et al.* 2007; Hocking and Babbitt 2014).

The services provided by amphibians are under threat globally due to a multitude of factors, including habitat loss and degradation (deforestation, agriculture, urbanization and draining of wetlands), nutrient and chemical pollution, overexploitation (collection for food, pet, or medicinal markets), introduced invasive species (for instance, fish introduction substantially impact tadpoles), ultraviolet-B radiation, climate change, and diseases (infectious or non-infectious, including malformations, neoplastic syndromes or toxicosis; Halliday 2008). It should be noted that amphibian declines have also occurred in "pristine" (or at least, protected) habitats, where there is no human encroachment or pollution, highlighting the importance of drivers such as climate change and infectious diseases (Pounds *et al.* 2006; Halliday 2008). Amphibians have been particularly impacted by one emerging infectious disease, amphibian chytridiomycosis.

## 1.6 *Batrachochytrium dendrobatidis* and the amphibian chytridiomycosis

Amphibian chytridiomycosis is caused by the fungus *Batrachochytrium dendrobatidis* (Bd; Berger *et al.* 1998; Longcore *et al.* 1999). Amphibian chytridiomycosis can also be caused by *B. salamandrivorans* (Bsal) which has a slightly different clinical expression, host range (mostly Caudata), and reduced geographical distribution (outside Asia: Belgium, Netherlands and Germany, more recently Spain; one should note that this reduced geographical distribution is the result of the implementation more effective and stringent biosecurity measures, surveillance, preparedness, and mitigation strategies; Martel *et al.* 2013, 2014, 2020). In contrast to Bsal, Bd has been spread on all continents where amphibians occur, is known to infect at least 1,375 species (as of 2021; Olson *et al.* 2021). Amphibian chytridiomycosis is currently a **panzootic** disease, shown to be involved in the declines of at least 501 species, and the presumed extinctions of 90 species (Scheele *et al.* 2019). It is, to current knowledge, responsible to the greatest loss of biodiversity attributable to an infectious disease, which is why it is rightly called the worst disease of all time (Gascon *et al.* 2007; Scheele *et al.* 2019).

*Batrachochytrium dendrobatidis* is a non-hyphal **zoosporic** chytrid fungus (Phylum Chytridiomycota, Class Chytridiomycetes, Order Rhizophydiales; Longcore *et al.* 1999). It has a tropism for the keratinized cells of amphibians, i.e. the skin of adults and the mouthparts of tadpoles (Berger *et al.* 1998, 2005). Its development requires some moisture (most of the >700 chytrid species are aquatic) and is temperature dependent: it grows best between 17 and 25°C,

significantly slower under 10°C, and growth completely stops over 28°C (Piotrowski *et al.* 2004; Woodhams *et al.* 2008). The life cycle of Bd has a duration of 4-5 days *in vitro* and is assumed to be similar *in vivo*, with two stages: the infective stage, the zoospore, and the reproductive stage, the zoosporangium (Berger *et al.* 2005; **Figure 5**). The zoospore of Bd is uniflagellated (its body measures 3-5 µm diameter and flagellum measures 19-20µm) and motile in the water (Longcore *et al.* 1999); it searches for and invades the keratinized tissue of amphibians, where it germinates, forms a thallus, colonizes the deeper layer of the host epidermis with germ tubes and gives rise to intracellular immature zoosporangia (Van Rooij *et al.* 2012). Zoosporangia (about 40µm) mature as the skin cells achieve their outward differentiation and each zoosporangium releases, through a discharge tube, many (from one to several dozens) zoospores into the external environment (Berger *et al.* 2005). The most widely used method for amphibian chytridiomycosis surveillance relies on non-invasive oral and skin swabs (for larval and metamorphosed stages, respectively), followed by detection of Bd DNA using molecular techniques such as a TaqMan quantitative (real-time) Polymerase Chain Reaction (qPCR) assay (Boyle *et al.* 2004; Kriger *et al.* 2006; Hyatt *et al.* 2007; Shin *et al.* 2014).

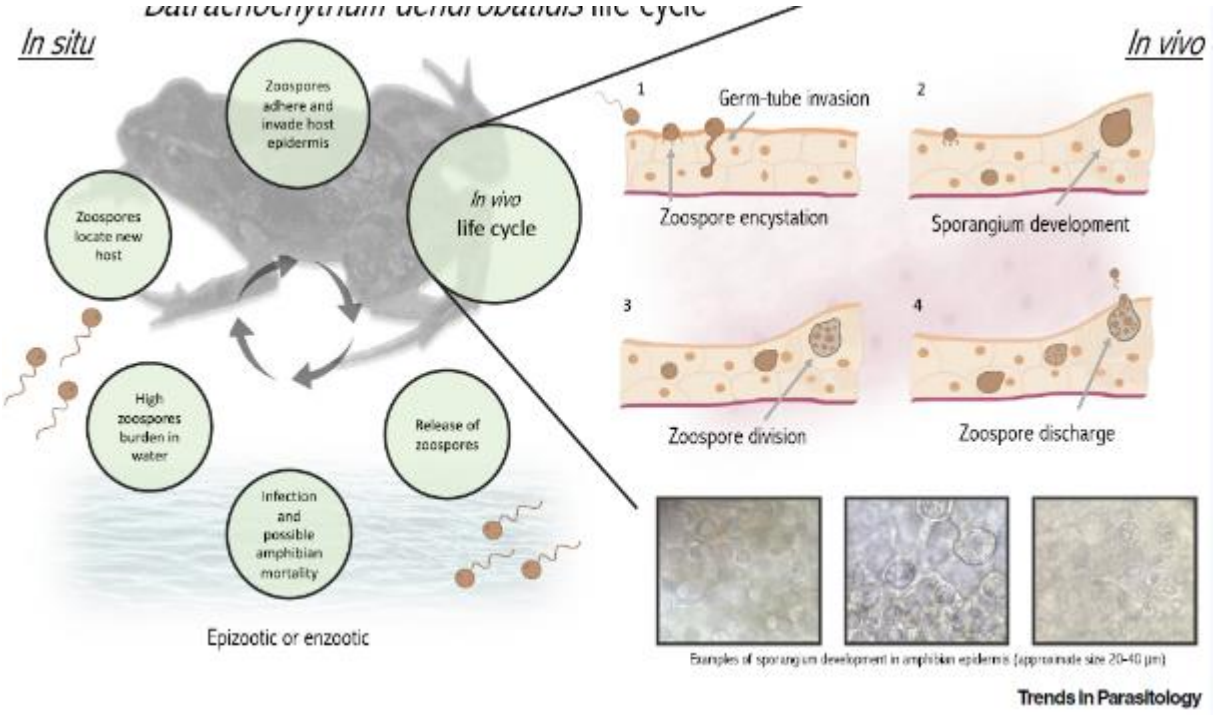


**Figure 5: Batrachochytrium dendrobatidis life cycle** (from Sewell *et al.* 2021).

Although Bd only affects the skin of metamorphosed individuals, chytridiomycosis is nevertheless lethal in susceptible hosts as the skin has essential functions for amphibian

organisms, such as water absorption, respiration, and **osmoregulation** (Boutilier *et al.* 1992). By developing in the target tissues, Bd cause **hyperkeratosis** and **hyperplasia** (Berger *et al.* 1998). In doing so, the fungus disrupts the osmoregulatory function of the skin, leading to osmotic imbalance (loss of sodium and potassium electrolytes) and ultimately cardiac arrest and death (Voyles *et al.* 2009; Grogan *et al.* 2018). Apart from sudden death, clinical signs are very unspecific in metamorphosed individuals but include lethargy, abnormal posture, loss of righting reflex, dysecdysis (abnormal skin shedding), skin discolouration, erythema and anorexia (Pessier 2008; Van Rooij *et al.* 2015). In anuran larvae, however, the clinical signs are more specific with oral disc depigmentation (dekeratinization) and even deformations (oedema of the mouthparts appearing swollen, entirely red or white instead of having black keratinized jaw sheaths and tooth rows; Fellers *et al.* 2001; Smith and Weldon 2007; Navarro-Lozano *et al.* 2018).

Transmission of Bd can occur directly via skin-to-skin contact between amphibians (e.g. during **amplexus**) or indirectly via motile zoospores released in water or moisture (Rowley and Alford 2007; Courtois *et al.* 2017; Burns *et al.* 2021). The survival of Bd zoospores in the external environment (water or moist substrate) is not clearly understood, but under sterile conditions with sufficient moisture, zoopores can survive from three weeks to three months depending on the quantity of minerals, and possibly nutrients, available in the environment (Johnson and Speare 2003, 2005). The fungus can also attach to and grow on keratin or chitin substrates, such as bird feathers (Johnson and Speare 2005), and even infect organisms other than amphibians, such as crayfish (*Procambarus* spp.) or nematodes (Shapard *et al.* 2012; McMahon *et al.* 2013; Brannelly *et al.* 2015).

*Batrachochytrium dendrobatidis* can infect all three orders of amphibians (Olson *et al.* 2021), but not all species or life stages are equally susceptible. As tadpoles do not generally die from infection, which is limited to the mouthparts (Berger *et al.* 1998; Rachowicz and Vredenburg 2004), they can act as **reservoirs**, maintaining a high pool of infective zoospores in the environment (Briggs *et al.* 2010; Walker *et al.* 2010; Clare *et al.* 2016). Tolerant species, such as the heavily traded African-clawed frog *X. laevis* and the North American bullfrog *Lithobates castesbeianus*, also act as reservoirs and contribute to the persistence and propagation of Bd (Weldon *et al.* 2004; Garner *et al.* 2006; Van Rooij *et al.* 2015). In fact, one of the main drivers for the emergence of amphibian chytridiomycosis is the anthropogenic introduction of infected tolerant species (e.g. through the international trade of live amphibians) into naïve environment, leading to pathogen pollution (Cunningham *et al.* 2003; Daszak *et al.* 2003; Garner *et al.* 2006; Fisher *et al.* 2009; Schloegel *et al.* 2012). There are several lineages of Bd, with which some species appear to have co-evolved and are tolerant; however,

anthropogenic movements gave rise to recombination between strains of different lineages and an increased **virulence** towards amphibian hosts, notably with the emergence of the Global Panzootic lineage (BdGPL; Rachowicz *et al.* 2005; Farrer *et al.* 2011; Schloegel *et al.* 2012; Rosenblum *et al.* 2013; O'Hanlon *et al.* 2018). Whole genome sequencing has shown that Bd, and notably BdGPL, originates from the South Korean peninsula, where amphibians are tolerant or resistant to infection (O'Hanlon *et al.* 2018).

The epidemiology of Bd infection and amphibian chytridiomycosis is complex but may be the result of interactions between four categories of factors: those related to the host, the host microbiome, the pathogen and the environment (a theoretical framework known as the disease pyramid, Bernardo-Cravo *et al.* 2020). The **virulence** of Bd can vary between strains and lineages (Fisher *et al.* 2009; Farrer *et al.* 2011; Porta 2014; Dang *et al.* 2017; Fisher and Garner 2020). All else being equal, hosts may be more or less susceptible to disease depending on their current health status (individual variation because of co-infections with other pathogens, and because of life-stage, for example the immune system is weakened during metamorphosis), the identity of their population (populations of the same species have sometimes been shown to be differently susceptible), as well as the identity of their species (susceptible, tolerant, or resistant; for instance the genus *Speleomantes* is resistant thanks to skin secretions of antimicrobial peptides), which may be related to differences in innate or adaptative immunity (Rachowicz and Vredenburg 2004; Tobler and Schmidt 2010; Gervasi *et al.* 2013; Pasmans *et al.* 2013; Baláž *et al.* 2014; Bradley *et al.* 2015; Savage and Zamudio 2016; Voyles *et al.* 2018).

The host microbiome, in particular the skin microbiome, has been shown to play an important role in determining the occurrence, the intensity and the outcome of Bd infection (see Bernardo-Cravo *et al.* 2020 for a review). On the one hand, the presence of certain bacteria or micro-eukaryotes, such as fungi known to inhibit Bd by producing antifungal or antimicrobial peptides, render the amphibian host less prone to infection and less susceptible to disease (Harris *et al.* 2006, 2009a; Brucker *et al.* 2008; Kearns *et al.* 2017). The addition of probiotics has even prevented, or at least, reduced chytridiomycosis severity in some cases (Harris *et al.* 2009b; Kueneman *et al.* 2016a). In some cases, a skin microbiota richer in diversity was associated with resistance or less severe disease outcomes (Piovia-Scott *et al.* 2017; Bates *et al.* 2018), while it remains unclear whether a **dysbiotic** microbiota (abnormal composition, less rich in diversity) predisposes hosts to more severe infection or is the result of a disruption by Bd infection (Jani and Briggs 2014; Walke *et al.* 2015; Jani *et al.* 2017). On the other hand, it was recently shown that not only the presence of Bd inhibitors in the skin microbiota, but also the thickness of the skin biofilm which contains these micro-organisms was important to prevent Bd infection (Chen *et al.* 2022).

Lastly, environmental factors also drive Bd infection and chytridiomycosis epidemiology, and can influence all other factors. They can be further divided into two sub-categories, namely biotic and abiotic environmental factors. Abiotic factors include climate (temperature, altitude, rainfall, seasonality, humidity, El Niño), water-related variables (current velocity, chemistry and in particular, salinity, the presence of synthetic toxicants), and ultraviolet B exposure (Pounds *et al.* 2006; Kriger and Hero 2007, 2008; Kriger *et al.* 2007; Rohr and Raffel 2010; Walker *et al.* 2010; Garner *et al.* 2011; Ortiz-Santaliestra *et al.* 2011; Rohr *et al.* 2013; Raffel *et al.* 2015; Clare *et al.* 2016; Clulow *et al.* 2018; Fisher and Garner 2020). These environmental factors explain why some species predicted to be at low risk of disease experienced mass mortality events during unusual seasonal events (Baláž *et al.* 2014; Clare *et al.* 2016; Olson *et al.* 2021). In terms of biotic environmental factors, community composition, with other amphibian species or life-stages that may act as reservoirs, or other species or substrates on which Bd can survive, influences the probability of infection (Fisher and Garner 2020). Free-living zoospores have been shown to interact with a variety of other organisms in the water. These interactions include consumption by filter-feeders such as ciliates, rotifers, tardigrades and crustaceans such as cladocerans, ostracods, and copepods (Buck *et al.* 2011; Searle *et al.* 2013; Schmeller *et al.* 2014; Blooi *et al.* 2017; De Troyer *et al.* 2021). The presence of such species in the zooplankton translated in reduced infection pressure and prevalence in both laboratory and field settings (Searle *et al.* 2013; Schmeller *et al.* 2014).

Severe declines due to amphibian chytridiomycosis have, based on current knowledge, mainly occurred in South and Central America, Oceania, and to a lesser extent in North America, Africa and Europe, but none in Asia (Berger *et al.* 1998; Bosch *et al.* 2001; Lips *et al.* 2006; Schloegel *et al.* 2006; Skerratt *et al.* 2007; Vredenburg *et al.* 2010; Cheng *et al.* 2011; Scheele *et al.* 2019; Fisher and Garner 2020). In Europe, amphibian species appear to be relatively resilient to chytridiomycosis caused by Bd, and only some mass die-offs have been observed (Garner *et al.* 2005; Walker *et al.* 2010). Infection is widespread, although it not randomly distributed in space or synonymous with disease (Walker *et al.* 2010; Miaud 2013; Baláž *et al.* 2014). Infection by Bd has been shown to be more likely in the families Alytidae and Bombinatoridae (Baláž *et al.* 2014).

In particular, the common-midwife toad, *Alytes obstetricans* (Ao), is very susceptible to amphibian chytridiomycosis (Bosch *et al.* 2001). It is one of the few species to have declined in Europe due to this disease, but only in some locations, like in some catchments of the Pyrenees (Bosch *et al.* 2001; Tobler and Schmidt 2010; Walker *et al.* 2010). Being widespread and developmentally plastic, Ao have been intensively studied to understand chytridiomycosis epidemiology and results showed that disease outbreaks are associated with altitude (Walker *et*

*al.* 2010). Temperature and seasonality are important factors, as is the fact that tadpoles of this species are very competent reservoirs since they exhibit delayed metamorphosis in altitude, and can remain as larvae for two or even three years (overwintering tadpoles able to enter and survive a **brumation**; Walker *et al.* 2010; Clare *et al.* 2016b). The composition of the zooplankton, and more particularly, the presence of aquatic consumers of Bd such as rotifers and ciliates explained some of the variation in infection prevalence between sites (Schmeller *et al.* 2014). However, these elements alone fail to explain the results of capture-mark-recapture surveys on Pyrenean Ao populations inhabiting distinct sites that are similar in terms of altitude and close to each other geographically (Clare 2014). These surveys showed that, years after Bd emergence (at least, after the first detection) in the Pyrenees in 2002, some populations were still declining while others recovered and got stable. The Ao populations of these sites were also shown to have different skin bacterial composition, with stable populations having more abundant Bd inhibitory taxa and generally a more diverse microbiota on their skin (Bates *et al.* 2018). These different Ao populations appear to be infected by the same strain of Bd (Bates *et al.* 2018). However, it is not known if they exhibit differences in their genetics (e.g. MHC allele frequencies), or in their demographic traits (Valenzuela-Sánchez *et al.* 2022) which could potentially explain these contrasting disease dynamics, although migration (and thus gene exchanges) are possible between these lakes, particulary between Ansabere and Acherito which are very close (roughly 1.75 km) but still exhibit different disease dynamics.

## 1.7 Questions driving this work

While further host factors (genetics) remain to be investigated, it is possible that other biotic environmental factors might explain the peculiar epidemiology of Bd infection and amphibian chytridiomycosis in the Pyrenees, still incompletely understood. The overarching hypothesis of my work is that benthic biofilms could be one further biotic component of Bd infection dynamics. Biofilms could impact the epidemiology of amphibian chytridiomycosis by, at least, four non-mutually exclusive categories of processes. This four categories correspond to the four vertices of the disease pyramid (Bernardo-Cravo *et al.* 2020; **Figure 6**).

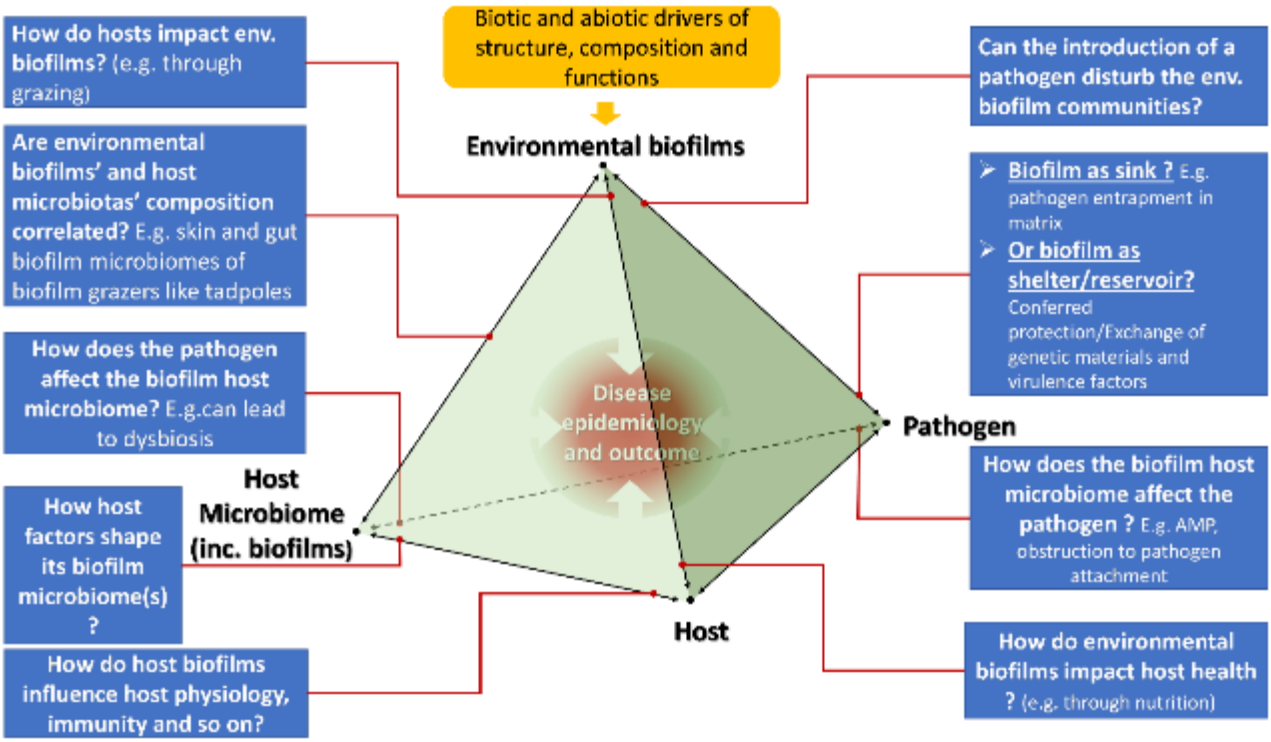


**Figure 6: Implications of biofilms growing on non-biological surfaces in infectious disease epidemiology**. The disease pyramid concept (Bernardo-Cravo *et al.*, 2020) was used to schematised these implications. Disease expression, outcome, and epidemiology are the results of dynamic processes under controls from the host (e.g. innate and acquired immune system), the **pathogen** (e.g. **virulence**), the host microbiomes (e.g. pathogen interference, or full permeability), and the environment (e.g. temperature). Environmental biofilms are part of the latter factors, and we argue that they can influence disease expression, epidemiology and outcome through diverse mechanisms (blue boxes). Abbreviations: AMP antimicrobial peptides. From Sentenac *et al.* (2022).

- Biofilms could affect the health of the amphibian host. As biofilm is a food source for *Alytes* (and many other) tadpoles, the nutritional quality of biofilms is of primary importance for their health and their ability to combat infection with an effective immune system. In this respect, biofilm dwellers such as diatoms are of interest as they are the primary producers of poly-unsaturated fatty acids (PUFA), such a ω-3, which are vital for the physiology and immunity of a wide range of organisms, including tadpoles but also terrestrial vertebrates (Hixson *et al.* 2015). In contrast, other algae such as green algae (Chlorophyta) and cyanobacteria contain fewer PUFA and therefore have a lesser nutritional quality (Brett and Müller-Navarra 1997; Guo *et al.* 2015; Crenier *et al.* 2019). Depending on their composition, biofilms can vary in terms of nutritional quality, which in turn impacts the host immune system. Biofilms can also affect host health through toxicity, either by concentrating pollutants (Bonnineau *et al.* 2020; Mahler *et al.* 2020) or also by the production of toxins (Oberemm *et*

*al.* 1999; Dao *et al.* 2010). Biofilm-dwelling toxigenic cyanobacteria have been indeed reported to cause mortalities of fish, dogs and cattle in mountains (Mez *et al.* 1997; Gugger *et al.* 2005; Quiblier *et al.* 2013; Wood *et al.* 2020). Tadpoles would be even further exposed than terrestrial organisms, as they directly consume the biofilms, and also they may be impacted by the consequences of biofilm cyanobacteria blooms, which deoxygenate the water (Wood *et al.* 2020).

- Biofilms could directly affect the pathogen (Bd zoospores). Very little is known on the interactions between non-human pathogens and environmental biofilms. For human pathogens, biofilms can either act as a reservoir or a sink (Chabaud *et al.* 2006; Wingender and Flemming 2011). Results from research on amphibian skin microbiota show that Bd zoospores have difficulty infecting the host when skin biofilms are thick, suggesting physical (space premption) or chemical interference (Chen *et al.* 2022). The biofilm matrix in benthic environment may also be a physico-chemical traps for zoospores: physical because its architecture can be really intertwined and have streamers (extensions, **Figure 3**) that may interfere with the movement of zoospores, and chemical because it may contain either adhesive or inhibitory molecules that, upon contact, would immobilise or damage Bd zoospores (Rendueles and Ghigo 2012, 2015). The toxins or other molecules secreted by biofilm inhabitants in the water column could also affect zoospores (**allelopathy**; Leflaive and Ten-Hage 2007; Wu *et al.* 2011; Allen *et al.* 2016).
- Biofilms could affect the host microbiome. As tadpoles feed and are in contact with benthic biofilms almost permanently, the microbiota of the biofilm could affect the skin, oral, and gut microbiota of the host. As mentioned above, the gut microbiota affects host health in a variety of ways, while skin and oral microbiota have importance in preventing Bd infection or reducing its severity.
- Biofilms could affect the biotic and abiotic environment. Biofilms import organic and inorganic matter by passive or active sorption for their needs. By altering or even depleting the environment of certain minerals or nutrients, biofilms could indirectly affect the time during which zoospores are motile, i.e. infective, especially as their movement is regulated by chemotaxis (Johnson and Speare 2003; Moss *et al.* 2008; Woodhams *et al.* 2008). The composition of biofilms, as the basis of food webs, could also influence that of the zooplankton, which can in turn impact Bd infection pressure for amphibians (Schmeller *et al.* 2014).

Although my thesis aims to explore some of these mechanisms, it also has the purpose to improve the knowledge on biofilm community composition in mountain lakes in general. After reviewing the literature and illustrating the importance of biofilms for many ecosystems and those of mountain lakes and ponds in particular, it became clear that acquiring knowledge

about biofilm community composition in the different lakes, where amphibians were monitored in our study system, was a prerequisite for any epidemiological investigations of the interactions between Bd and biofilms. Therefore, I answered the following questions, corresponding to my different chapters, using a combination of field studies and laboratory experiments:

- **Chapter 2**, entitled "*The significance of biofilms to human, animal, plant and ecosystem health*": What is known (and not known) about the importance of biofilms for the health of multicellular organisms and ecosystems?
- **Chapter 3**, entitled "*Biofilm community composition is changing in remote mountain lakes with a relative increase in toxigenic algae":* What is the community composition of benthic biofilms? Does it vary in space (between ecologically-different lakes) and in time (within a lake)? Are there trends in microbial biodiversity consistent with those currently observed for macro-organisms?
- **Chapter 4**, entitled "*First insights into the links between benthic biofilms and the epidemiology of* Batrachochytrium dendrobatidis *infection in the Pyrenees*": Are there any plausible links between biofilm community composition and the epidemiology of Bd infections and amphibian chytridiomycosis in the Pyrenees?
- **Chapter 5**, entitled "*Environmental biofilms can affect the fate of the free-living stage of a globally-emerged pathogen":* Do biofilms have an impact on the infective stage of Bd in laboratory settings? If an effect exists, does it vary with the composition of the biofilm? Can even simple biofilm, made out of one autotrophic alga (no consumer), have an effect?

Because mountain lakes are currently facing many anthropogenic pressures, I hypothesized that prokaryote and micro-eukaryote biodiversity would decline over time, and that the composition of their respective assemblage would change with an increased relative abundance of more resistant organisms such as cyanobacteria. With regard to Chapter 4, my hypothesis was that biofilms in lakes with less infected and/less impacted amphibian populations would contain greater diversity, have different micro-eukaryotic composition with more species of, or more abundant, potential Bd consumers such as rotifers. Finally, in Chapter 5, I hypothesized that very simple biofilms (produced by only one phototrophic alga) grown in the laboratory, would not affect the number of Bd zoospores over time, whereas biofilms grown on the field, which may harbour Bd consumer, would.

## 1.8 Introduction to the study system

Biofilms were sampled in 26 lakes spread into six altitudinal gradients, mainly located in the French Pyrenees from 2016 to 2020 (**Figure 7**). The majority of these lakes are located above 1,500m asl, and are oligotrophic, i.e. poor in nutrients (**Table 1**). Climatic conditions are quite different between the gradients, with in the East, Bethmale, Bassies, Arbu-Lers-Estagnon being the hottest and driest areas (Mediterranean influence), Lescun in the westernmost part being the wettest (oceanic influence), and Fache and Neouvielle, in between, being the coldest and highest areas, with relatively high precipitations too (**Table 1**).

To monitor Bd (and other) infection in amphibians, tadpoles or larvae of several species were sampled, including the common-midwife toad (Ao), the common frog (*Rana temporaria*), the spiny toad (*Bufo spinosus*), the palmate newt (*Lissotriton helveticus*), the fire salamander (*Salamandra salamandra*) and occasionally the Pyrenean brook newt (*Calotriton asper*). Tadpoles and larvae are easier to find that adults. Given that my work focuses on amphibian chytridiomycosis caused by Bd, I only used Ao in my infection data. The common-midwife toad is the sentinel species for this disease in the Pyrenees. *Alytes obstetricans* is an amphibian commonly found across France, with also some population in northern Spain and Portugal, and in Switzerland, Belgium and Germany, living in temperate forests, semi-arid areas, walls, embankments, and slopes with small stones and sparse vegetation (IUCN SSC Amphibian Specialist Group 2022). Common-midwife toads preferentially breed in permanent stagnant ponds and pools, or less frequently in slow moving rivers, and exhibits a peculiar form of parental care: the male carries the fertilized eggs on its back until they are ready to hatch, at which point the male returns to the water to release the tadpoles (**Figure 8**). Its IUCN Red list conservation status is “Least Concern”, but populations are generally in decline with local extinction reported due to habitat loss, fragmentation and degradation, and infectious diseases (chytridiomycosis, ranavirosis and others; IUCN SSC Amphibian Specialist Group 2022).

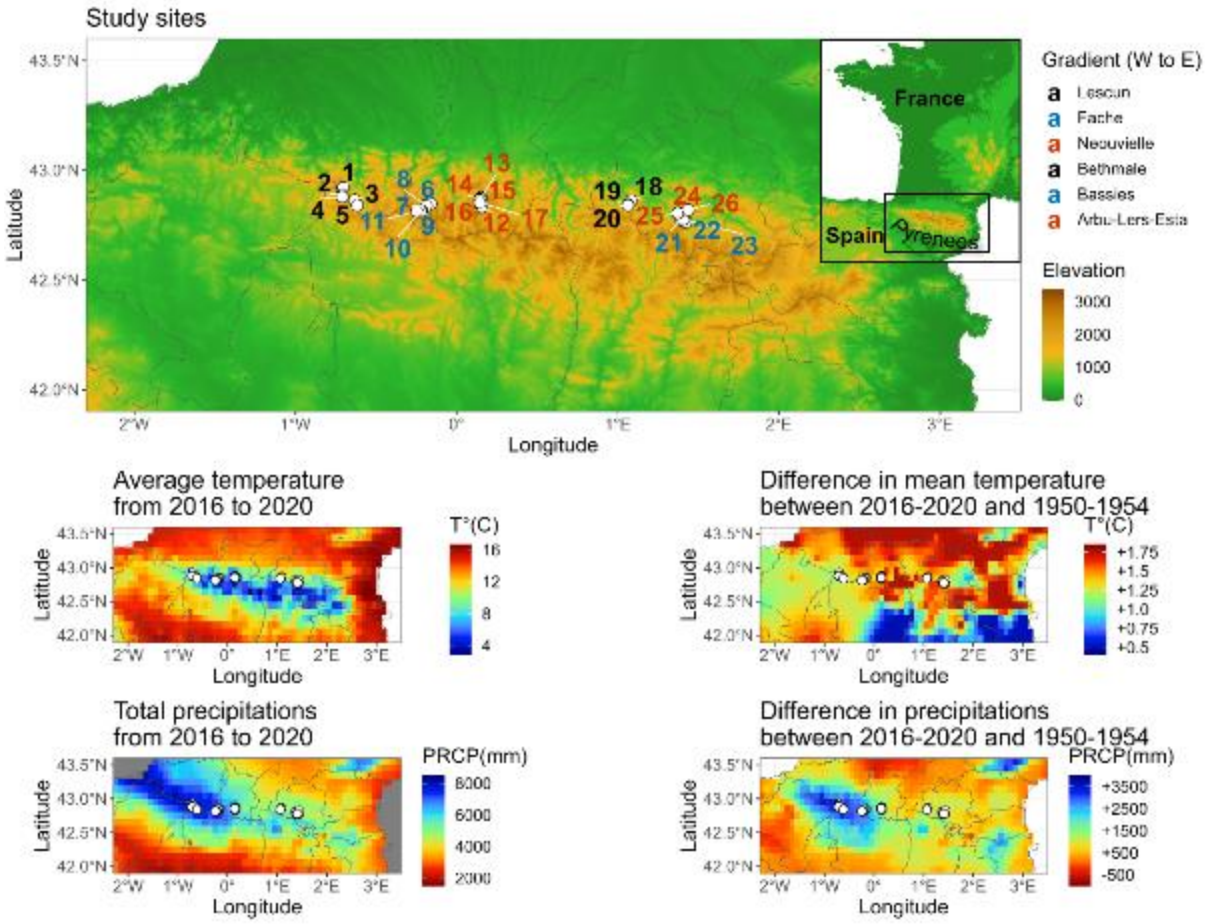


**Figure 7: Location and climatic conditions of the lakes in which biofilms were sampled. A:** Lakes are located in six altitudinal gradients, from West to East, their legend keys can be found in **Table 1 B:** Average annual temperature during the study period from 2016 to 2020. **C:** Difference between the mean annual temperature of the periods 2016-2020 vs. 1950-1954. **D:** Total sum of precipitations from 2016 to 2020. **E:** Difference between the total sum of precipitations of the periods 2016-2020 vs. 1950-1954.

**Table 1**: **Description of the study sites.** Abbreviations: area: lake size in hectares; Hardness is defined the sum of electrolytes Calcium and Magnesium in mg/L; TC: total organic carbon in mg/L. TN: total nitrogen in mg/L. Ntot: number of amphibians captured and swabbed since 2008. Nb pos is the number of amphibians for which Bd DNA has been detected. Prev: prevalence (number of positive individuals / numbers of captured individuals). Mean is the mean infection intensity (Bd parasite load, in ZE), respectively.

| # | Lake | gradient | altitude | area | pH | Hardness | TC | TN | Ntot | Nb_pos | Prev | mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Lhurs | Lescun | 1697 | 3.59 | 8.72 | 25.48 | 4.79 | 0.29 | 1441 | 631 | 0.44 | 46.56 |
| 2 | Ansabere | Lescun | 1850 | 0.21 | 8.38 | 14.87 | 3.2 | 0.3 | 1605 | 657 | 0.41 | 63.53 |
| 3 | Puits d'Arrious | Lescun | 1880 | 0.26 | 7.71 | 2.88 | 3.96 | 0.82 | 1675 | 800 | 0.48 | 193.45 |
| 4 | Acherito | Lescun | 1880 | 7.46 | 8.67 | 21.04 | 4.61 | 0.83 | 1166 | 676 | 0.58 | 175.16 |
| 5 | Arlet | Lescun | 1974 | 3.46 | 9.23 | 10.1 | 3.95 | 0.7 | 2175 | 1408 | 0.65 | 675.51 |
| 6 | Paradis | Fache | 1609 | 0.42 | 8.94 | 12.93 | 2.87 | 1.13 | 227 | 37 | 0.16 | 0.34 |
| 7 | Embarrat | Fache | 2180 | 0.01 | 7.76 | 5.86 | 4.52 | 0.26 | 203 | 0 | 0 | 0 |
| 8 | Vallon | Fache | 2215 | 0.05 | 7.93 | 7.51 | 2.52 | 0.4 | 210 | 19 | 0.09 | 0.01 |
| 9 | Petite-Fache | Fache | 2301 | 0.38 | 7.99 | 8.86 | 2.72 | 1.04 | NA | NA | NA | NA |
| 10 | Grande-Fache | Fache | 2422 | 0.87 | 7.35 | 4.46 | 3.5 | 0.61 | 201 | 0 | 0 | 0 |
| 11 | Fache-Espagne | Fache | 2522 | 0.64 | 7.15 | 3.12 | 6.55 | 0.42 | 140 | 0 | 0 | 0 |
| 12 | Laquettes | Neouvielle | 2087 | 3.06 | 7.6 | 6.74 | 2.69 | 0.32 | NA | NA | NA | NA |
| 13 | Coueyla-Gran | Neouvielle | 2159 | 0.59 | 8.09 | 7.84 | 2.71 | 0.2 | NA | NA | NA | NA |
| 14 | Madamete-Bas | Neouvielle | 2307 | 0.17 | 7.91 | 5.89 | 4.67 | 1.01 | 176 | 48 | 0.27 | 9.02 |
| 15 | Pecheur | Neouvielle | 2310 | 0.59 | 8.29 | 7 | 2.96 | 0.53 | NA | NA | NA | NA |
| 16 | Madamete-Haut | Neouvielle | 2374 | 0.31 | 8.22 | 8.86 | 2.96 | 0.5 | 47 | 0 | 0 | 0 |
| 17 | Gourg de Rabas | Neouvielle | 2400 | 1.326 | 7.62 | 2.64 | 3.12 | 0.19 | 224 | 65 | 0.29 | 19.96 |
| 18 | Bethmale | Bethmale | 1063 | 2.91 | 8.96 | 41.66 | 1.36 | 0.41 | NA | NA | NA | NA |
| 19 | Ayes | Bethmale | 1714 | 1.87 | 8.71 | 26.69 | 6.55 | 0.19 | 302 | 26 | 0.09 | 2.52 |
| 20 | Bellonguere | Bethmale | 1907 | 0.17 | 8.25 | 33.1 | 3.75 | 0.95 | 158 | 0 | 0 | 0 |
| 21 | Labant | Bassies1 | 1600 | 0.46 | 7.41 | 3.1 | 3.64 | 0.75 | 45 | 0 | 0 | 0 |
| 22 | Mort | Bassies1 | 1651 | 0.86 | 7.46 | 4.56 | 6.29 | 0.59 | 31 | 0 | 0 | 0 |
| 23 | Alate | Bassies1 | 1865 | 2.13 | 6.95 | 0.85 | 3.24 | 0.79 | 86 | 0 | 0 | 0 |
| 24 | Lers | Bassies2 | 1272 | 8.21 | 8.73 | 14.21 | 4.09 | 0.29 | 181 | 0 | 0 | 0 |
| 25 | Estagnon | Bassies2 | 1314 | 0.25 | 7.61 | 14.93 | 5.31 | 0.64 | 41 | 0 | 0 | 0 |
| 26 | Arbu | Bassies2 | 1737 | 5.01 | 7.23 | 2.27 | 3.21 | 0.69 | 214 | 0 | 0 | 0 |

**Figure 8:** ***Alytes obstetricans*** : overwintered tadpoles (left, ©Franck Gilbert), metamorphosing individual (middle, ©Dirk Schmeller) both captured during fieldwork, and adult male carrying eggs (right, ©Christian Fisher).

Tadpoles were captured every year in summer and swabbed for the presence of Bd DNA from 2008 onwards. The distribution of Bd infected Ao tadpoles can be found in **Figure 9**. Common-midwife toads have been found infected almost every year in the five lakes of gradient Lescun: Ansabere, Arlet, Acherito, Lhurs, and Puits d'Arrious. Acherito is where amphibian chytridiomycosis was detected for the first time in the Pyrenees in 2002. Since then, in these lakes, the long-term population impact of the disease has been different. Populations of Ao in Acherito, Lhurs, and Puits d'Arrious, after collapses following emergence, recovered and are now stable, despite some individuals having high Bd loads. In contrast, populations from Arlet and Ansabere have shown no signs of recovery and it has been arduous to find any Ao tadpoles there in recent years.

In other gradients, Bd infections have been detected in Fache, Neouvielle, and Bethmale but not in the Bassies and Arbu-Lers-Estagnon gradients. The Ao populations of Gourg de Rabas, Madamete-Haut and Madamete-Bas (Neouvielle) were found infected in 2009-2010 (and also in 2011 for the first one), and then populations collapsed. Tadpoles and clutches were only detected again, sampled and tested negative, in 2020 and 2021, suggesting that populations resurged there without Bd infection. However, no Ao population resurged in Madamete-Haut, while this lake is located in between Gourg de Rabas and Madamete-Bas. In Fache, Bd was detected in lake Paradis in 2013-2018 and Vallon in 2015 and 2018, although infection intensities were often low, especially for Vallon. In Bethmale, Bd was detected in Ayes in 2015 and 2018-19, with high infection intensities and prevalence. Very few Ao tadpoles were found in 2020 and 2021, while Ao tadpoles were abundant in 2022. Diagnostic testing by qPCR is pending for these years.

For all laboratory experiments, I used the Bd strain IA043 isolated from a recently metamorphosed Ao individual found dead in Acherito in 2005, and kindly provided by Matthew Fisher (Imperial College London). Details on how the strain is maintained are given in Chapter 5.

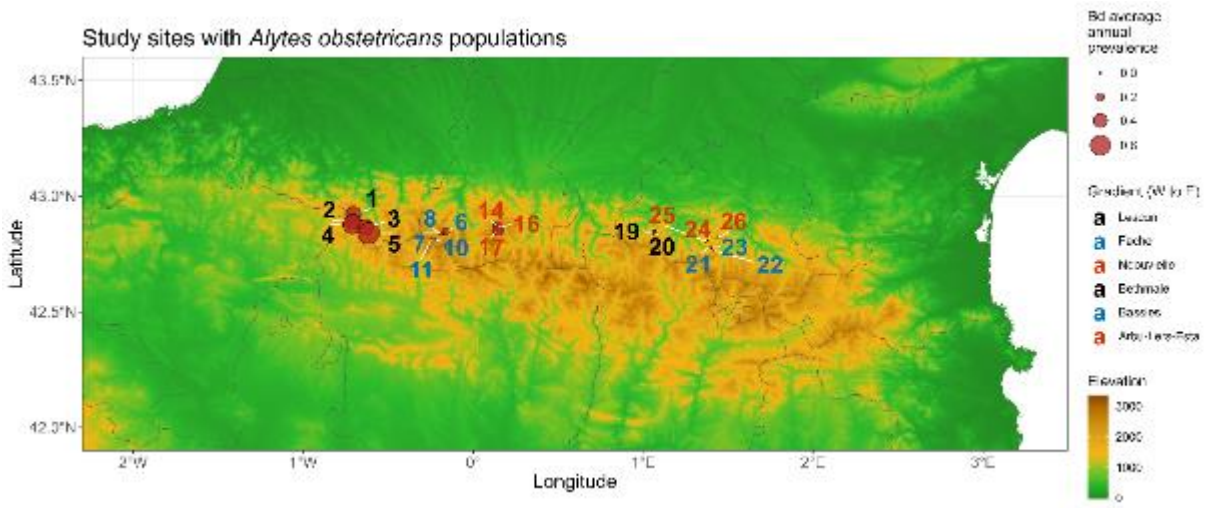


**Figure 9**: **Location of the study sites with *Ao* populations, and mean annual prevalence of Bd infection.** Infections by Bd are widespread in lakes from gradient Lescun, where Bd was first detected in Acherito (4) in 2002. Lake keys can be found in the following tables.

# CHAPTER 2

# THE SIGNIFICANCE OF BIOFILMS TO HUMAN, ANIMAL, PLANT AND ECOSYSTEM HEALTH

## Foreword of Chapter 2

This chapter was published in the journal *Functional Ecology* as

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Biofilms are ubiquitous on Earth but a consequence of this is that biofilm research is very disparate and often carried out in disciplinary silos. Furthermore, biofilms are poorly known from the general public. My goal with this review was to shed light on the main functions of microbial communities living in biofilms (whether the matrix is self-produced by microorganisms or not) in multicellular organisms and ecosystems, and on important knowledge gaps.

I was particularly interested in reviewing what was known i) benthic biofilms in mountain lakes and ii) on the interactions between pathogens (or any microorganisms not being a biofilm dweller) and biofilms growing on non-biological surfaces.

# Chapter 2: The significance of biofilms to human, animal, plant and ecosystem health


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I formulated ideas, with help of D.S.S. and A.L., and wrote the first draft of this manuscript. I drew the figures. All authors critically contributed to revisions. I corresponded with the editors.

## Abstract


Biofilms are matrix-enclosed communities that represent the most dominant and active mode of microbial life on Earth. Because biofilms are inherently more productive than any equivalent planktonic community, they are of great relevance to all environments they inhabit. However, their existence and importance are still poorly known by the general public, conservation practitioners, and environmental policymakers. Most microorganisms of multicellular organisms (including humans, animals and plants) occur in the form of true biofilms or biofilm-like structures that play vital roles in their development, physiology and immunity. Conversely, some biofilms can have a negative effect on host health. Biofilms growing on non-biological surfaces are essential components of many terrestrial and marine ecosystems: they form the basis of food webs and ensure nutrient cycling and bioremediation in natural systems. However, environmental biofilms can promote the persistence of human pathogens, produce harmful toxins, foul and corrode surfaces in natural and man-made settings; all of which can have significant health and economic implications. There is a knowledge gap about the roles of biofilms in the epidemiology of wildlife emerging infectious diseases, yet these pose a major threat to public health, biodiversity and

sustainability. The drivers of global environmental change all affect biofilm structure and functions. The consequences for host and ecosystem health are, however, poorly understood. While the concept of a healthy microbiome (as opposed to dysbiosis) is emerging in medicine and conservation biology, the concept of a healthy biofilm remains to be defined in environmental sciences. Here, we use an integrative approach to (i) review current knowledge on the roles of biofilms growing on biological and non-biological interfaces for the health of multicellular organisms and ecosystems, and (ii) provide future research directions to address identified knowledge gaps. Giving the biofilm life form its full importance will help understand the effects of global environmental change on these communities and, in turn, on human, animal, plant and ecosystem health.

## 2.1 Introduction

The overall importance of microorganisms for the health of multicellular hosts and ecosystems is becoming increasingly evident (Clemente *et al.* 2012; Cavicchioli *et al.* 2019; Bernardo-Cravo *et al.* 2020). The advent of new technologies such as high-throughput sequencing made the study of the specific diversity of microorganisms (the "**microbiota**"), their genomes and their surrounding environment, which together form the **microbiome**, widely available (Marchesi and Ravel 2015). Microorganisms are now considered the bedrock of biodiversity, playing a pivotal role in global change biology (Cavicchioli *et al.* 2019; Zhu and Penuelas 2020). Yet, the fact that most of them live in complex communities attached to an interface in a three-dimensional gel-like matrix, known as **biofilms** (Flemming and Wuertz 2019), is still poorly recognised in research, conservation practices, and environmental policies. We believe this oversight hinders a good grasp of the relationships between the structures and functions of microbial communities in various environments, thus limiting a thorough understanding of the determinants of host and ecosystem health.

Compared to planktonic microorganisms, the biofilm is a very different life form: the gel-like matrix allows for the retention of water and nutrients, and also provides its inhabitants with protection against physical, chemical or biological aggressions and changing environmental conditions (Flemming and Wingender 2010; Flemming *et al.* 2016). This temporal and physical stability likely explains why biofilms were found to be one of the oldest, dating back 3.3 billion years ago (Westall *et al.* 2001), and most successful life forms, prevailing in almost all habitats on Earth (Battin *et al.* 2016; Flemming and Wuertz 2019). Biofilm research is located at the nexus of many fields of life sciences, including microbiology and functional ecology (Battin *et al.* 2003), biodiversity and evolution (Hansen *et al.* 2007;

Besemer 2015), biogeochemistry (Battin *et al.* 2016), biotechnology (Edwards and Kjellerup 2013), human and veterinary medicines, and public health (Costerton *et al.* 1999; Mah and O'Toole 2001; Clutterbuck *et al.* 2007). Biofilms provide, therefore, an ideal opportunity for transdisciplinary and integrative research, for instance in health-related sectors (Queenan *et al.* 2017). In the last decades, several fully-integrated health approaches have emerged (**Figure 1**), including the convergent One Health and the Ecosystem Health (EcoHealth) initiatives (Zinsstag 2012), within which **health** is understood in its broadest sense, including physical, moral, and social well-being, and not only as the mere absence of disease. Health has strong socio-ecological determinants and is therefore intimately linked to ecosystem health, sustainability and global change (**Figure 1**). A healthy ecosystem is defined here as a **productive** system, **resilient** against external pressures, and capable of maintaining its **structure** and services to humans (Rapport *et al.* 1998; Tett *et al.* 2013).

However, to date, there is no comprehensive review on the numerous health implications of the biofilm life form for humans, animals, plants and ecosystems. Because biofilm research is very disparate (**Figure 10**), we deem here that using a holistic approach to health is appropriate for analysing the all-encompassing relevance of biofilms. In doing so, we demonstrate their global significance, unite the highly contrasting fields of biofilm research, and identify knowledge gaps and areas for further biofilm investigations that will contribute to improving our understanding of host and ecosystem health. We also provide a definition of a healthy biofilm, which we believe is essential to properly detect, monitor and mitigate the effects of global change on these communities.

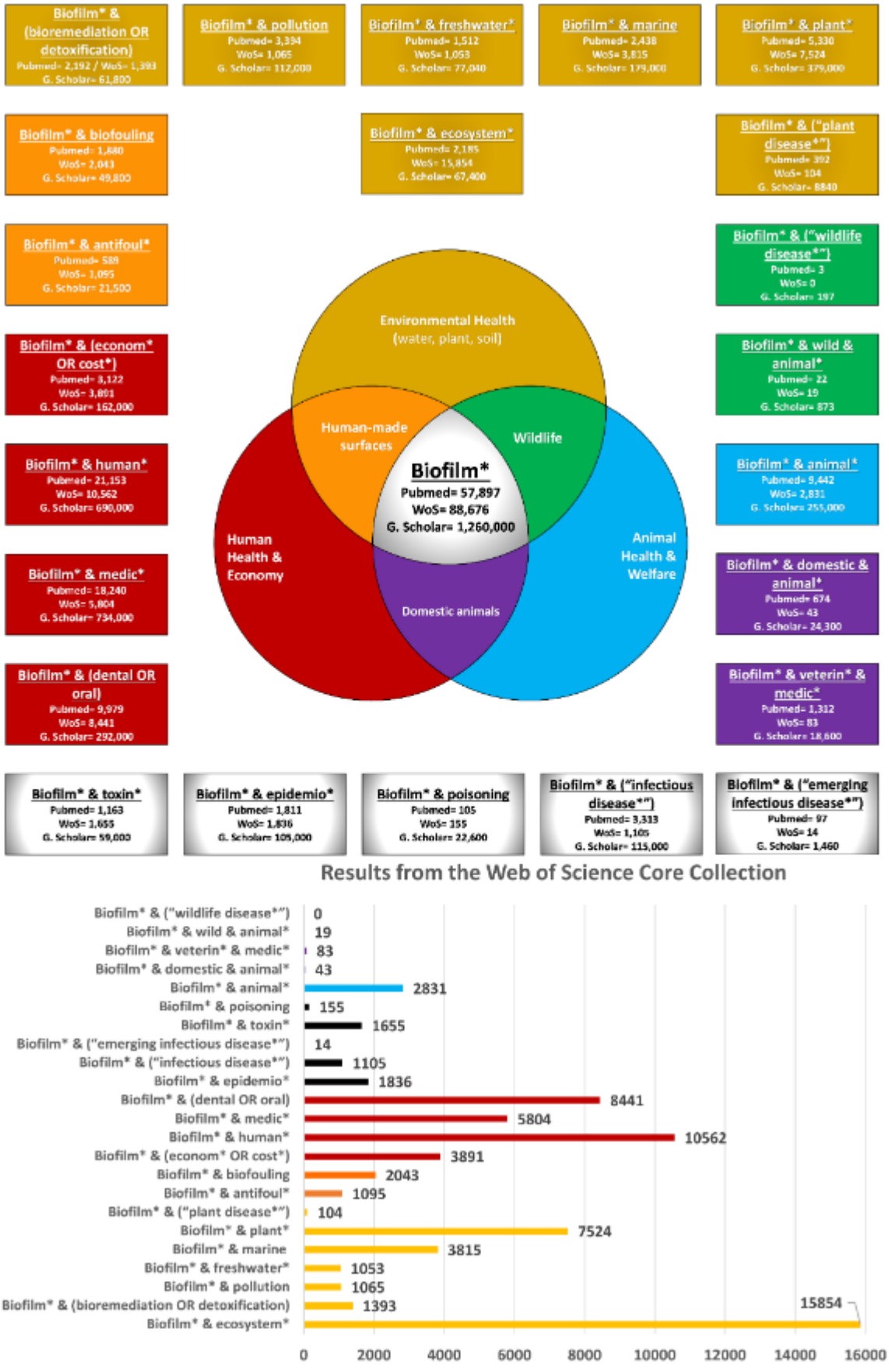


**Figure 10: Current biofilm research efforts in the scientific literature.** Research efforts were measured by results in different search engines, namely Google Scholar (G. Scholar), Pubmed and the Web of Science (WoS) Core Collection. Results from the latter are represented in a bar plot for better

visualization. Biofilm research is unevenly distributed with most research directed to human medicine, biofilm and plants, and the economic costs of biofilms to human societies. Regarding infectious diseases, most studies focused on the biofilms of human and zoonotic diseases. Very little is known of the roles of environmental biofilms in the epidemiology of wildlife infectious diseases (whether biofilms can act as a sink or a source of pathogens).

## 2.2 Definition and properties of biofilms

Biofilms are generally defined as matrix-enclosed populations or communities of organisms adherent to an interface (usually solid–liquid, but also liquid–liquid, liquid–gas and solid–gas) and/or to each other (i.e. non-attached aggregates of cells, often termed flocs; Costerton *et al.* 1987; Hall-Stoodley *et al.* 2004). In the strictest sense (hereafter biofilm *sensu stricto*), the matrix in which biofilm microorganisms live is self-produced and made out of extracellular polymeric substances (EPS, Vert *et al.* 2012). These EPS contain polysaccharides, nucleic acids, proteins, lipids, other carbohydrates, and any element pertaining to the medium in which biofilms occur (Flemming and Wingender 2010). Flemming and Wuertz (2019) expanded the definition of biofilms (hereafter biofilm *sensu lato*) to include the mucosa-associated microbiota of animals, whose epithelia may produce mucus (e.g. on the lining of the digestive tract or on the skin). Contrary to biofilms *sensu stricto*, polysaccharides in biofilms *sensu lato* are secreted by the host glands and not by microorganisms. However, this broad definition of biofilms has the advantage of drawing a clear line between, on the one hand, microorganisms living aggregated in a matrix, and on the other hand, planktonic ('non-sessile') or attached individual microorganisms (Flemming and Wuertz 2019). The continuum between a single attached cell and the different types of microbial aggregates forms an **ecological succession** (**Figure 3**A).

Biofilm research has long lacked a theoretical framework, but now is solidly rooted in ecological theory built upon work from Battin *et al.* (2007). Landscape and **metacommunity** ecology are now central tenets of biofilm research, which help understand community assembly and the processes generating biodiversity within biofilm and how this relates to biofilm functioning, productivity and resilience (Battin *et al.* 2007; Besemer *et al.* 2012; Nemergut *et al.* 2013; Besemer 2015). Not only does ecological theory help understand biofilm biology, but biofilm research in turn can also help inform ecological theory (Prosser *et al.* 2007; Flemming *et al.* 2016; Feng *et al.* 2017). There are, as a matter of fact, many similarities between biofilms, forests or corals: all form biogenic habitat, providing a 3-D structure and a somewhat enclosed environment within which processes and internal interactions are enhanced, and through which

external interactions are filtered (e.g. resources or invasive species; Flemming *et al.* 2016). Therefore, insights from macroecology has considerably improved the way biofilms are understood, but in turn, biofilms provide a model to understand **emergent properties** of biological systems and also the opportunity to test hypotheses (which can sometimes be inaccessible from the study of other organisms; e.g. the **diversity-stability hypothesis**), and the roles of different **ecological interactions** in the structuring of communities (Ponge 2005; Battin *et al.* 2007; Flemming *et al.* 2016).

**Box 1: Estimation of biofilm biomass and productivity**

We estimated biofilm biomass on Earth as a proxy of their productivity. We used estimates from Bar-On *et al.* (2018) and Flemming and Wuertz (2019) to do so. The latter only estimated the total number of Bacteria and Archaea on Earth ($1.2x10^{30}$), and that in biofilm (ranging from $3.5x10^{29}$ to $9.1x10^{29}$, depending on whether the deep Earth subsurface is included or not). Biofilm Archaea and Bacteria thus represent from 29% to 76% of all bacteria and archaea cells on Earth. Now, Bar-on *et al.* (2018) estimated that bacteria biomass was 70 gigatons of carbon (Gt C) and that of Archaea 7 Gt C, making 77Gt C combined (note that there is a more than 10-fold uncertainty in the archaea biomass estimate). Therefore, we could draw an estimate of the biomass of Archaea and Bacteria in biofilm on Earth: 22-59 Gt C. They also estimated that the total Earth biomass was 550Gt C, so Archaea and Bacteria in biofilms would represent from 4 to 11% of all biomass on Earth. This estimate of biofilm biomass is conservative as other biofilm inhabitants, and above all, EPS, are not considered here. In comparison, all animals represent 0.4% (2/550 Gt C).

Doubtless, the most distinctive feature of a biofilm is the presence of a 3-D matrix that allows a steady juxtaposition of microorganisms next to one another for prolonged periods of time (Flemming *et al.* 2016). As a result, biofilms are more organised and productive than any equivalent planktonic community (Hansen *et al.* 2007): the biomass (productivity) of the Archaea and Bacteria cells in biofilms was conservatively estimated at 4 to 11% of the total biomass on Earth (**Box 1**; Lock *et al.* 1984; Flemming and Wingender 2010). Biofilms exhibit emergent properties such as enhanced horizontal gene transfer, including that of antimicrobial resistant genes, but also active sorption (capture, facilitated by streamers), transport (through pores, voids, and channels) and extracellular retention of water, nutrients, and enzymes (**Figure 3**B). Above all, the cooperation between cells (social interactions), through chemical and electrical communication, may be the most surprising property, with signs of division of labour

and coordinated behaviour, thought to be typical of multicellular organisms (**Figure 3**B; Hansen *et al.* 2007; Flemming and Wingender 2010; Flemming *et al.* 2016). However, while the biofilm matrix grants increased survival, it also enhances competition between inhabitants (Rendueles and Ghigo 2015; Flemming *et al.* 2016). The growth and maturation of biofilms over time both result in the appearance of environmental micro-niches and gradients within the matrix, with a centre generally containing less dioxygen ($O_2$), fewer nutrients and more waste products (that is, more stressful conditions; Rendueles and Ghigo 2015). This environmental heterogeneity selects for diversity within and among species through **niche partitioning** (Boles *et al.* 2004; Singer *et al.* 2010; Cardinale 2011; Rendueles and Ghigo 2015; Flemming *et al.* 2016). Biofilms can form complex communities including prokaryotes such as bacteria (e.g. cyanobacteria) and archaea; as well as eukaryotes like plants, diatoms, fungi, other unicellular eukaryotes, small metazoans (i.e. animals) and viruses (**Figure 3**; Geesey *et al.* 1978; Costerton *et al.* 1987; Battin *et al.* 2001). Mechanisms of competition within biofilms are numerous but can be divided in two groups: exploitative vs. interference competitions (Rendueles and Ghigo 2015). Exploitative competition refers to indirect interactions between organisms, whereby one microorganism prevents access and/or limits the use of resources (e.g. $O_2$, iron) by another microorganism. Interference competition corresponds to more direct mechanisms such as predation; inhibition of growth, communication or biofilm colonisation; or induction of biofilm dispersal (Rendueles and Ghigo 2015). Colonisation and dispersal are important biofilm processes (Battin *et al.* 2007; McDougald *et al.* 2012; Flemming *et al.* 2016). Many microorganisms can live in both biofilm and planktonic lifestyles and can switch from one to the other, the planktonic form being used to disseminate and colonise new environments, while the biofilm form allows persistence. The processes of biofilm formation and, to a lesser extent, detachment involve close communication between cells through density-dependent signalling and gene-expression synchronisation, a phenomenon known as **quorum-sensing** (Solano *et al.* 2014). Biofilm detachment can also be caused by external forces (allogenic processes, e.g. shear stress) or by the death of deep layer cells (autogenic processes, **Figure 3**B). Such cooperation/competition and colonisation/dispersal mechanisms are essential to understand processes of biofilm assembly. They will also determine whether biofilms might act as a source or a sink for pathogens, a key question from a one health perspective (Wingender and Flemming 2011; Rendueles and Ghigo 2015).

All in all, biofilms are very complex and heterogenous entities in space (gradients creating microenvironments), time (maturation and succession of stages), composition (both in species and EPS) and functions (Flemming and Wingender 2010; Battin *et al.* 2016; Flemming *et al.* 2016; Flemming and Wuertz 2019). These properties are controlled by a broad range of

physical, biogeochemical and ecological factors, all of which are driven by environmental processes (Battin *et al.* 2016; **Figure 11**). Biofilms can be seen as mini-ecosystems *per se* with their own community structure, productivity and resilience to external pressures, which can reach **alternative stable states** (Flemming *et al.* 2016). For instance, one study revealed that biofilm communities were able to recover faster to stable performance (i.e. better resilience) after pH shock when diversity was initially high (indicating **functional redundancy**) and competition between the two dominant genera (*Geobacter* and *Methanobrevibacter*) was low (Feng *et al.* 2017). Therefore, concepts from ecological theories (such as the metacommunity, ecological interactions and successions, niche partitioning, **priority effects**), are essential to comprehend the biology and ecology of biofilms (Battin *et al.* 2007, 2016; Besemer *et al.* 2012; Besemer 2015). Only then will we be able to fully understand the key roles that biofilms play in their environment, whether it be a multicellular host or an ecosystem (Battin *et al.* 2003, 2016; Peter *et al.* 2011; Besemer *et al.* 2012; Besemer 2015). Here, we review the roles of biofilms communities and the ways these might be impacted by global change. We distinguish between biofilms growing on non-biological surfaces ('environmental biofilms') and biofilms growing on biological surfaces (more particularly multicellular organisms, i.e. 'host-associated biofilms'). Making these distinctions and using this terminology might reflect the biases from different research perspectives, but these distinctions are helpful to demonstrate the different lenses that can be used when considering the functional importance of biofilms.

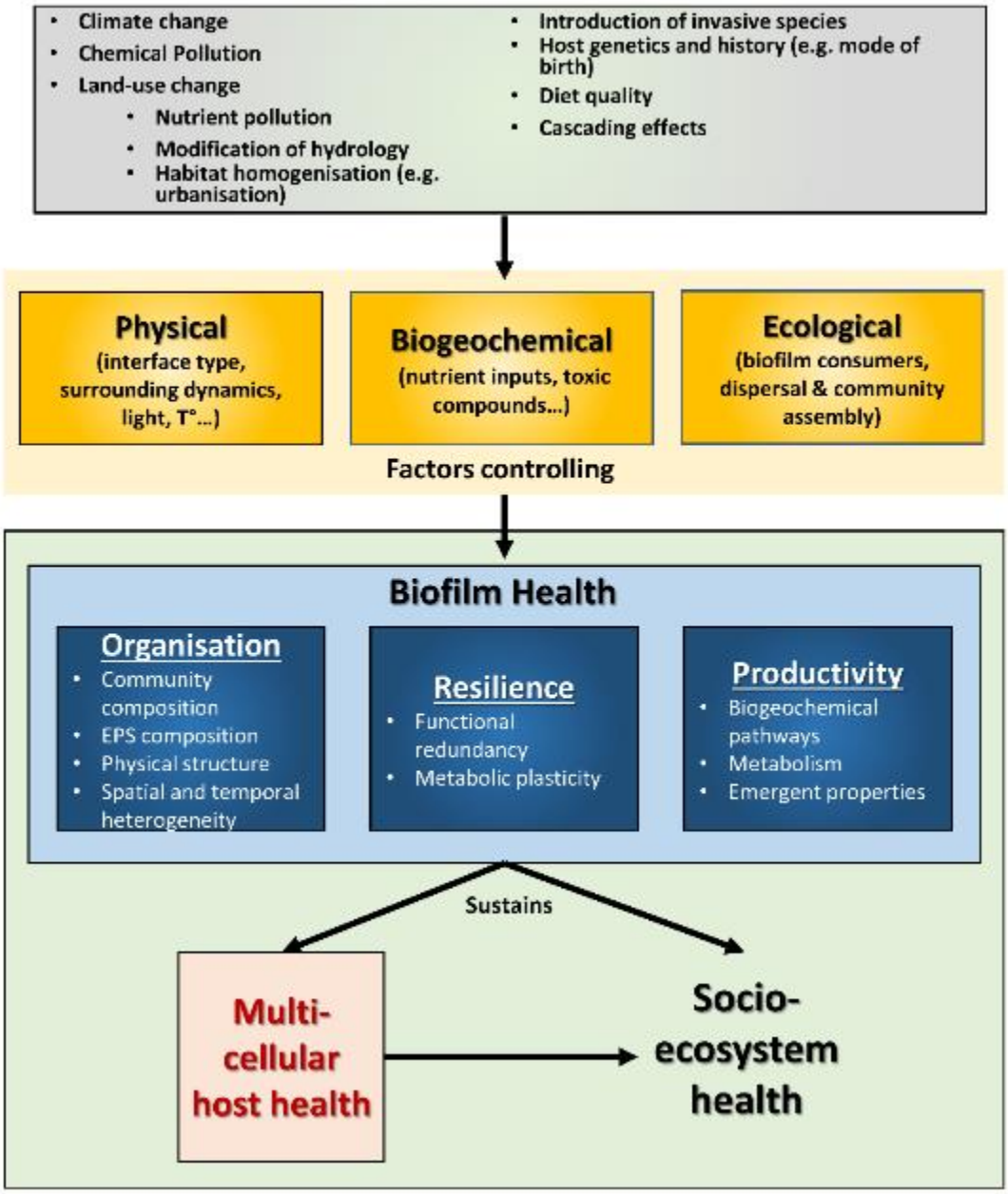


**Figure 11: Factors controlling biofilm health.** A: broad range of physical, biogeochemical and ecological factors (yellow box) can drive biofilm health and have further implications on the system where biofilms occur (green box; see also **Figure 12** and **Figure 13**). These factors are themselves impacted by drivers of global environmental change and/or host-related factors (grey box).

## 2.3 Importance of biofilms growing on biological interfaces

### 2.3.1 For Human and Animal Health

Biofilms are problematic in human and veterinary medicine because bacteria or other parasites can form biofilm on host tissues or implanted medical biomaterials, and cause chronic infections (Costerton *et al.* 1999; Hall-Stoodley *et al.* 2004; Clutterbuck *et al.* 2007). Pathogenic biofilms inherently allow their microorganisms to evade host immune defences and resist

antimicrobial agents (Mah & O'Toole, 2001). They can also act as a source of pathogenic bacteria that can disperse and colonise new tissues (**Figure 12**; Fleming and Rumbaugh 2018). Notable diseases associated with biofilms include periodontitis, osteomyelitis, and cystic fibrosis pneumonia in humans (Costerton *et al.* 1999), chronic ear infections in dogs, and mastitis in cattle (Olson *et al.* 2002; Melchior *et al.* 2006; Moreira *et al.* 2012). Human and other animal patients are particularly exposed to biofilm nosocomial infections when they are in an immunodeficient state or in intensive care, and require the use of indwelling medical devices (Lynch and Robertson 2008). Biofilm-associated infections on animals can seriously threaten human livelihoods and economy, for example by disrupting milk production (**Figure 12**; Melchior *et al.* 2006), and can pose a direct threat to human health since the involved microorganisms are often zoonotic (e.g. *Staphylococcus aureus*; Clutterbuck *et al.* 2007; Zambori *et al.* 2013). Since most antibiotics used in veterinary medicine are or were also used in human medicine, animal biofilms largely contribute to the emergence of antimicrobial-resistant pathogens, a major threat to public health (Roca *et al.* 2015).

However, there is increasing evidence that biofilms are also essential to human and animal health, even though the literature is sometimes unclear as to whether biofilms are considered *sensu stricto* or *sensu lato*. For example, dental biofilms are subject to much research, both on oral health and disease (Marsh 2006). True biofilms in healthy women have been shown to exist in the urogenital tract and to act as an ecological barrier against pathogens by preventing their adherence to cells (Chan *et al.* 1985; Domingue *et al.* 1991), while more recent studies suggest that commensal (see **ecological interactions**) vaginal lactobacilli prevent the growth of uropathogens like *Neisseria gonorrhoeae* by acidifying the environment (Graver and Wade 2011). The existence of biofilms *sensu stricto* on the walls of the vertebrate digestive tract is more controversial. Their presence is confirmed in animal species but consensus for humans is missing (Palestrant *et al.* 2004; Swidsinski *et al.* 2005; Lebeer *et al.* 2011; von Rosenvinge *et al.* 2013; de Vos 2015). Some medical researchers detected true biofilms in the healthy human colon, arguing that direct observations were previously precluded due to the difficulty of preserving the matrix after sampling (Palestrant *et al.* 2004; Sonnenburg *et al.* 2004; Bollinger *et al.* 2007). However, the presence of biofilms on food particles inside the digestive tracts was demonstrated multiples times, both in humans and animals, suggesting that biofilms play significant digestive roles (Costerton *et al.* 1987; Macfarlane and Macfarlane 2006; Macfarlane and Dillon 2007; Walker *et al.* 2008).

If one considers biofilms in their broadest sense, their importance for human and animal health becomes even more obvious. A substantial part of their microbiota lives attached and/or incorporated within the mucus of the digestive tract, explaining why they persist in such an

ever-changing environment (**Figure 12**; Sonnenburg *et al.* 2004; Martens *et al.* 2018). There is increasing evidence of the roles of the gut microbiota in shaping the behaviour, development, physiology and immunity of the host; conversely, **dysbiosis** is implicated in the pathogenesis of multiple infectious and non-infectious diseases such as obesity, irritable bowel disease, type-2 diabetes, certain cancers of the digestive tract, cardiovascular diseases, and autoimmune syndromes and allergies (Round and Mazmanian 2009; Clemente *et al.* 2012; Ezenwa *et al.* 2012; Sampson and Mazmanian 2015; Fung *et al.* 2017; Levy *et al.* 2017). For instance, a study showed that early disruption by antibiotics of the gut microbiome of Cuban tree frog tadpoles (*Osteopilus septentrionalis*) had subsequent consequences for the resistance of the adult host to intestinal worm infection, possibly due to an impairment of the immune system development in early life stages (Knutie *et al.* 2017). The normal gut microbiome is also thought to act as a barrier against pathogens; however, the specific mechanisms underlying this barrier effect remain in general little understood (Reid *et al.* 2001; Bass *et al.* 2019). As an example, the role of two specific genes (yiaF and bssS) in limiting the colonisation of a mouse commensal-*Escherichia coli* gut biofilm by the two pathogens *Klebsiella pneumoniae* and diarrheagenic enteroaggregative *E. coli* was demonstrated, but precise mechanisms are to be elucidated (Da Re *et al.* 2013). Skin microbiomes (mucosomes) have also been shown to be relevant for host health (**Figure 12**; Ross *et al.* 2019), but have rarely been considered as biofilms while they are contained in skin mucus in many vertebrates taxa such as fish and amphibians (Shephard 1993; Toledo and Jared 1993; Flemming and Wuertz 2019). Exceptions include the integument of marine animals, such as corals and crustaceans, often colonised by biofilms *sensu stricto* which may be detrimental by limiting light, gas, and nutrient availability and/or beneficial by interfering with other predators and parasites (Qian *et al.* 2007; Wahl *et al.* 2012).

Biofilms roles in protecting against pathogens are of paramount importance since emerging diseases pose a threat to humanity, biodiversity, and sustainability (Daszak *et al.* 2000; Fisher *et al.* 2012). Most of these diseases originate in wildlife (Jones *et al.* 2008). As an illustration, hard corals are keystone species for marine ecosystem health, on which a substantial part of humanity relies for food (Moberg and Folke 1999). Yet corals are threatened by a range of factors, including climate change and emerging infectious diseases, the former having a substantial impact on the latter (Harvell *et al.* 2002). Ritchie (2006) found that the mucus from healthy Elkhorn coral (*Acropora palmata*) had a significantly higher protective effect against certain pathogens than mucus sampled from heat-stressed corals. There is also growing evidence that the occurrence, severity, and outcome of the panzootic amphibian chytridiomycosis in susceptible amphibians are correlated with the composition of skin

microbial communities: a diverse skin microbiome being associated with enhanced survival and dysbiosis with the disease status (Bernardo-Cravo *et al.* 2020).

Much remains unclear about the determinants (host genome, diet and lifestyle) and functions of metazoan-associated biofilms (Huttenhower *et al.* 2012; Heintz-Buschart and Wilmes 2018). However, ancestral and modern human gut microbiomes were shown to differ substantially (Blaser and Falkow 2009; David *et al.* 2014; Sonnenburg and Sonnenburg 2019). Homogenisation of habitat, sedentariness, industrialisation of food production, increased use of antibiotics caused, amongst others, losses of commensal microbes and have led to dysbiosis and a rise of non-infectious chronic diseases in human populations, such as obesity. However, it remains difficult to disentangle the relative contributions of each factor as all occurred more or less simultaneously with industrialisation (Blaser and Falkow 2009; David *et al.* 2014; Flandroy *et al.* 2018; Sonnenburg and Sonnenburg 2019). Insights from animal research confirmed this trend: captivity, pollution, land-use change, and climate change, among other factors, generate dysbiosis and have negative effects on animal health, with substantial implications for conservation (Bass *et al.* 2019; Trevelline *et al.* 2019; West *et al.* 2019). For instance, warming of the environment led to gut dysbiosis and reduced growth in *Ololygon perpusilla* tadpoles (Greenspan *et al.* 2020), while **xenobiotics** (e.g. lead) were also shown to alter the structure and functions of the human and animal gut microbiome (Maurice *et al.* 2013; Gao *et al.* 2017).

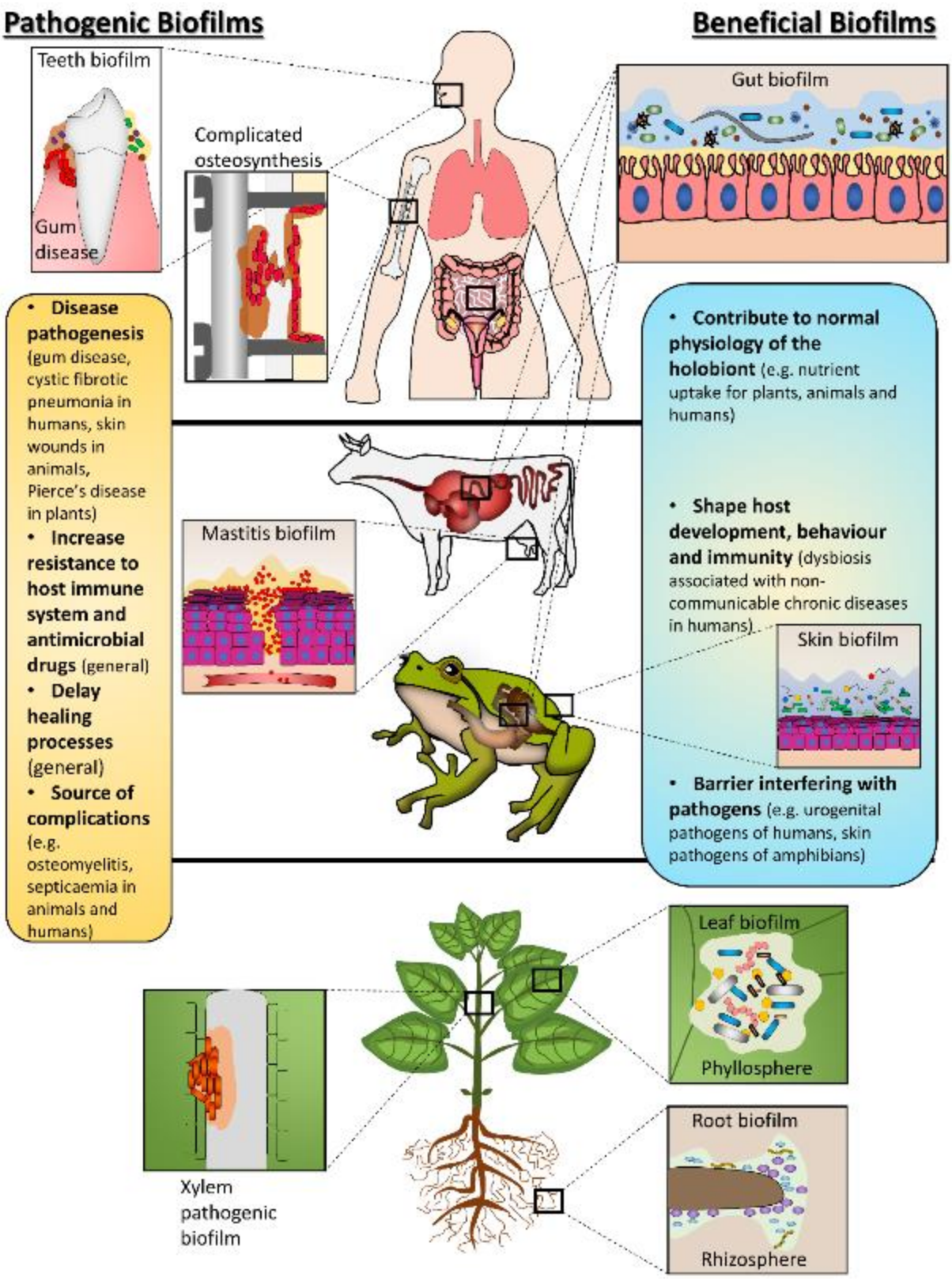


**Figure 12: Occurrence and roles of biofilms in disease (left) and health (right) for a human host, other animal hosts and a plant host.** Biofilms are involved in the pathogenesis of some diseases including gum disease and secondary osteomyelitis (here a complication of a humeral fracture osteosynthesis is shown) in humans, mastitis in cattle, and Pierce's disease in plants. True beneficial biofilms occur in plants (rhizosphere and phyllosphere), around food particles ingested by humans and

animals (e.g. ruminants), and on the lining of the urogenital tract of humans. Biofilms *sensu lato* occur on the gut of animals and humans and on the skin of animals with mucus (e.g. amphibians, fish).

### 2.3.2 For the Health of Plants and Other Macro-organisms

Terrestrial plants constitute the highest biomass on Earth (Bar-On *et al.* 2018), and their physiology and health fundamentally depend on the existence of symbiotic microbial communities which are mainly in the form of biofilms *sensu stricto* (Morris and Monier 2003; Flemming and Wuertz 2019; Rodriguez *et al.* 2019). Pathogenic biofilms exist in or on terrestrial plants (Morris and Monier 2003; Rudrappa *et al.* 2008), but mostly biofilms are considered beneficial to the plant host (Vandenkoornhuyse *et al.* 2015; Compant *et al.* 2019; Rodriguez *et al.* 2019). Two biofilm communities are important for terrestrial plants: the leaf microbiome or phyllosphere (Vorholt 2012), and the root microbiome or rhizosphere (**Figure 12**; Lynch 1994). There is strong evidence that the phyllosphere plays vital roles for terrestrial plants in terms of growth, functioning and fitness. These include resistance to biotic and abiotic stressors, as well as nutrient acquisition and cycling (Delmotte *et al.* 2009; Kembel *et al.* 2014). Although the concept of **Home-field advantage** (HFA) has long been considered to deal with soil microbial communities only, recent evidence shows that the phyllosphere plays significant roles in driving HFA effects through priority effects (Fanin *et al.* 2021). The aboveground phyllosphere would therefore impact ecosystem functioning by influencing nutrient dynamics and carbon cycling (Fanin *et al.* 2021). The rhizosphere is equally important for plant diversity, stability and interactions with other plants and organisms (e.g. fungi), thereby mediating biomass productivity. It is also important for enhancing tolerance and resilience to stress factors and affects root decomposition (Lynch 1994; Knief *et al.* 2012; Zhou *et al.* 2020a).

Plant biofilm communities offer the opportunity, through inoculation and enrichment with key microorganisms, to sustainably stimulate production and **bioremediation** (Compant *et al.* 2019). Emerging diseases of plants are also a serious threat to biodiversity, food security and the related economy (Anderson *et al.* 2004; Fisher *et al.* 2012) and both the healthy phyllosphere and rhizosphere have been shown to protect the host from pathogens, either through interference or by driving immunity (Rudrappa *et al.* 2008; Castrillo *et al.* 2017; Pérez-de-Luque *et al.* 2017). This provides opportunities for plant disease management (Poudel *et al.* 2016). As an example, *Bacillus subtilis* is widely used as a biocontrol agent and one study showed that it protected *Arabidopsis* sp. roots from the pathogen *Pseudomonas syringae* by forming biofilms and producing surfacin, an antimicrobial agent (Pal Bais *et al.* 2004). There has been growing awareness of the potentially adverse cascading effects that global change

could have on ecosystems through changes in plant-associated biofilms (Vacher *et al.* 2016; Delavaux *et al.* 2019). A meta-analysis of 135 studies concluded that elevated $CO_2$ had overall a positive influence on root-associated bacteria and fungi (Compant *et al.* 2010). Regarding pollution, however, Jumpponen and Jones (2010) showed that phyllosphere fungal communities of *Quercus macrocarpa* significantly differed between urban and non-urban environments due to air pollution. The functional implications of such shifts are still poorly understood, yet, the suppression of the phyllosphere microbiota by antibiotic applications led to major shifts in the plant host metabolome (Gargallo-Garriga *et al.* 2016). As to aquatic plants, they are also almost systematically covered by biofilms, with similar beneficial and detrimental roles than for aquatic animals (Schiel and Foster 2006; Wahl *et al.* 2012; Egan *et al.* 2013; Dang and Lovell 2016).

The ability of biofilms to protect plant or metazoan hosts against pathogens confirms that the traditional disease triangle, often mentioned in disease ecology, should be extended to a disease pyramid with the host-associated microbiome as a fourth vertex (Bernardo-Cravo *et al.* 2020). The biofilm microbiomes form, with their host, a superorganism termed **holobiont** (Egan *et al.* 2013; Vandenkoornhuyse *et al.* 2015; Carthey *et al.* 2020). Increasingly, experts argue that conservation medicine and biology would benefit from a paradigm shift to view not the host but the holobiont as a unit subject to selective pressures, like those from global change (Trevelline *et al.* 2019; Carthey *et al.* 2020). However, many knowledge gaps subsist (**Table 2**). For instance, it has yet to be determined whether biofilms grow on and play significant roles for other multicellular organisms, such as macroscopic fungi. It also remains difficult to predict how the interactive effects of e.g. climate change, pollution, and land-use change will affect host health through their impacts on biofilms. Consideration of host-associated biofilms in future research, but also in conservation practices and developmental policies, will be essential to preserve host and ecosystem health in the context of global change.

**Table 2: Identified areas for further research on the structure and functions of biofilms growing on biological** (host-associated biofilms) **and on non-biological surfaces** (environmental biofilms, see also **Figure 6**)

| **Host-associated biofilms (growing on biological surfaces)** |
|---|
| • Determine whether biofilms *sensu stricto* on the lining of the human gut exist<br>• Determine whether biofilms exist on multicellular fungi<br>• Study the specific mechanisms underlying biofilm barrier effect against pathogens *in situ*<br>• Further study the determinants of host-associated biofilm community structure and functions<br>• Further study the mechanisms of resilience of host-associated biofilms that prevent dysbiosis<br>• Grasp the functional implications (using OMICS approaches) of the changes induced by climate change, habitat loss and pollution (taken separately and together) on animal and plant health through their effects on the associated biofilms<br>• Explore the virome of host-associated biofilms (taxonomy, abundance, functions and importance for host health) |
| **Environmental biofilms (growing on non-biological surfaces)** |
| • Study the roles of environmental biofilms in infectious disease epidemiology, especially with regards to the fate of pathogens of global importance such as SARS-CoV-2 and *Batrachochytrium dendrobatidis*<br>• Study the roles of environmental biofilms in non-infectious disease epidemiology, e.g. in toxin production (including at low level), and explore the nutritional importance of biofilms for consumer health (in particular when biofilm transition from one state to another following a disturbance, e.g. diatom-dominated towards cyanobacteria-dominated)<br>• Impacts of cocktail of toxicants or nanoparticules at ecologically-relevant concentrations on *in situ* biofilm structure and functions<br>• Study the specific effects of stressors other than physical and chemical (biological stressors such as invasive species) on biofilms<br>• Pursue multiple stressor research (interactive effects of warming, change in hydrology, habitat loss and degradation, nutrient and chemical pollution, and other stressors) especially in regards with impact on functions and consequences for ecosystem health<br>• Explore the virome of environmental biofilms (taxonomy, abundance, biogeography, roles and importance of virus for ecosystem health), especially in freshwater ecosystems<br>• Further test the hypothesis of diversity-stability in various environments, exploring the mechanisms of resilience (existence of functional redundancy, niche partitioning, cooperation, competition) that prevent state shift (“dysbiosis”)<br>• Further study biofilms (structure and functions) other than the periphyton, such as the neuston, the plastisphere, marine snow and so on |

## 2.4 Importance of biofilms growing on non-biological interfaces

### 2.4.1 For water and soil health

Biofilms are the dominant mode of life in all habitats on Earth except open oceans (**Figure 13**; Battin *et al.* 2016; Flemming and Wuertz 2019). Battin *et al.* (2016) qualified biofilms as 'the microbial skin' of landscapes: a structuring, living and dynamic ecological boundary where transition, contact, exchange but also separation of biotic or abiotic matter occur. The soil biomass is dominated by bacteria and fungi mostly in the form of biofilms (Cai *et al.* 2019; Flemming and Wuertz 2019) and this soil microbiome is critical for ecosystem functioning: it provides structure and stabilisation to friable soils and maintains soil fertility by rendering key nutrients available to plants and other soil organisms (Ahmad *et al.* 2017; Fierer 2017). Freshwater microbiomes are constantly inoculated by soil microorganisms via surface runoffs or subsurface flows (**Figure 13**; Battin *et al.* 2016). Biofilms develop on virtually all immersed solid–liquid interfaces such as rocks (epilithic biofilm or epilithon), decaying wood (epixylic biofilm), sand grains (episammon), and mud sediments (epipelon; **Figure 13**; Vadeboncoeur and Steinman 2002). The terms **periphyton** and periphytic biofilms cover all these meanings, including epiphyton (biofilms on plant organisms). Benthic biofilms can be, in terms of biomass, the dominant component in many freshwater ecosystems, especially when the water is shallow and/or has low turbidity (Vadeboncoeur and Steinman 2002; Besemer 2015). Biofilms also develop on artificial substrates, such as plastics and on other interfaces than solid–liquid like the neuston layer at aquatic-air interface (i.e. pellicle) of marine and freshwater bodies (Flemming and Wuertz 2019).

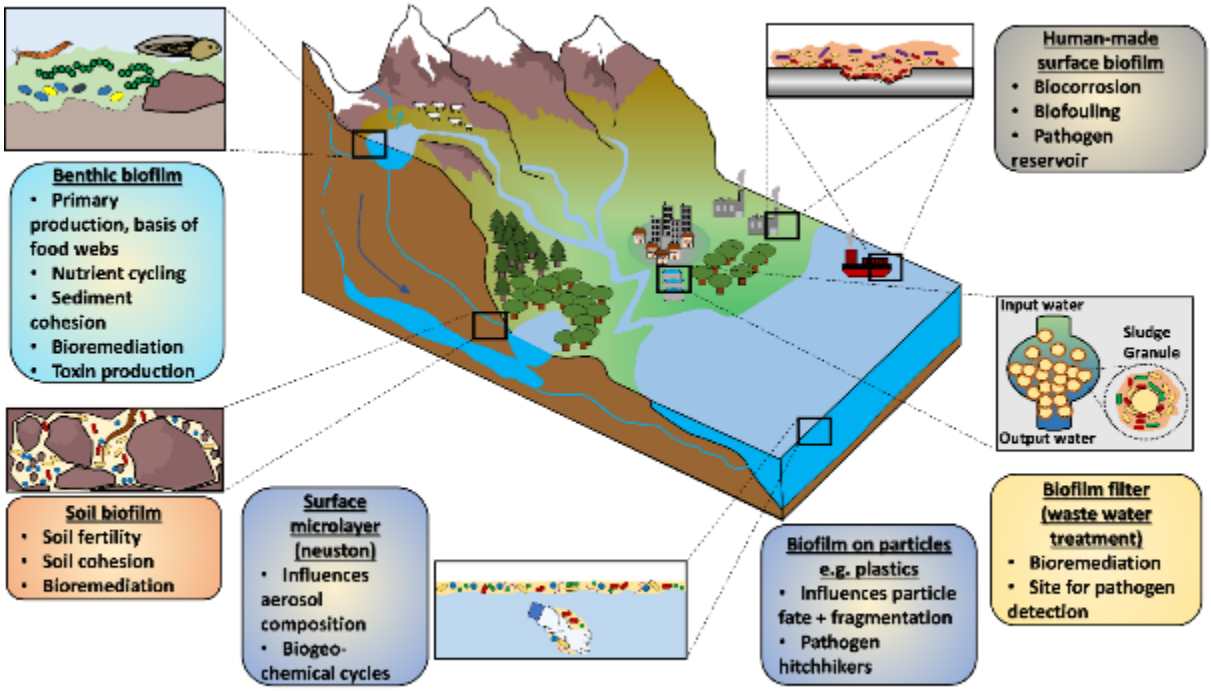


**Figure 13: Occurrence and importance of biofilms at the scale of a landscape.** Environmental biofilms can be seen as the microbial skin of landscapes as they are ubiquitous in many ecosystems including (i) terrestrial ecosystems: in freshwaters with benthic and hyporheic biofilms, the neuston of lakes, in the soil and the deep continental subsurface (not illustrated); (ii) marine ecosystems, with the ocean surface microlayer, on particles such as plastics and other debris, the upper sediment layer (not illustrated) and the deep oceanic subsurface (not illustrated); (iii) human-made surfaces like pipelines, boats or biofilm filters in waste water treatment.

It is increasingly evident that biofilms regulate carbon fluxes in ecosystems, such as boreal peatlands and streams (Battin *et al.* 2016; Wyatt *et al.* 2021). In these ecosystems, the phototrophic community of biofilms ensures primary production, and their heterotrophic community ensures the decomposition and cycling of carbon and other nutrients (Battin *et al.* 2003; Bartrons *et al.* 2012; Besemer 2015; Wu 2017a). Biofilms also form a very important trophic level, being the sometimes unique basis of the food webs (Hecky and Hesslein 1995; Vadeboncoeur and Steinman 2002; Rott *et al.* 2006; Weitere *et al.* 2018). This is particularly true for oligotrophic (e.g. alpine or boreal) ecosystems where conditions in regard to temperature variability, wind, siltation and nutrient availability are extreme and prevent vegetation development (Geesey *et al.* 1978; Lock *et al.* 1984; Vadeboncoeur and Steinman 2002; Rott *et al.* 2006). Biofilms provide the living environment for many primary consumers

and are grazed by both invertebrates and vertebrates such as tadpoles (Hecky and Hesslein 1995; Füreder *et al.* 2003; Rott *et al.* 2006). The trophic ecology of biofilms and their consumers is of particular interest given their relevance in carbon cycling: the absence of top-down controls by animals against biofilm herbivory was shown to increase carbon dioxide emissions in boreal peatlands, highlighting the cascading effects of the loss of biodiversity (Wyatt *et al.* 2021). Particularly, boreal ecosystems are expected to experience changes in resource availability through climate change, shifting biofilm communities towards heterotrophy under certain conditions (Myers *et al.* 2021).

Another important function of periphytic biofilms in fast-flowing waters is to substantially increase water retention in quiescent areas (Battin *et al.* 2003). Increasing water retention allows longer exchange of organic particles and enhances the ability of a system to catch and retain usually-limiting nutrients such as phosphorus and nitrogen. In both lotic and lentic freshwater ecosystems, biofilms act as a buffer for these nutrients and contribute therefore to biogeochemical and ecological processes (Battin *et al.* 2003; Lu *et al.* 2016; Wu 2017b). Biofilms also ensure the biostabilisation of sediments, thus limiting erosion (Gerbersdorf and Wieprecht 2015). Active denitrifying genes were also found in biofilms, suggesting they also have an important role in regulating the global nitrogen cycle, which has been substantially disrupted by the massive use of nitrogen-based fertilizers for food production, emissions from fossil fuel combustion, and the increasing number of humans and livestock (Gruber and Galloway 2008; Vila-Costa *et al.* 2014). Such activities led to eutrophication and acidification of aquatic ecosystems on a global scale; this is why nutrient enrichment is considered a type of pollution which affect biofilms, but that biofilms can also help mitigate. For example, periphyton biofilms were shown to decrease the levels of total nitrogen and phosphorus in polluted rivers (Liu *et al.* 2016; Wu 2017c). Nutrient enrichment otherwise leads to algal blooms which can have devastating impacts on aquatic ecosystems by producing harmful toxins, like microcystins, and depleting the water from resources such as nutrients, dioxygen and light (Huisman *et al.* 2018). It was shown both *in-* and *ex-situ* that periphyton biofilms made of diatoms and bacteria could control cyanobacterial blooms by i) producing water-soluble allelochemicals (see **allelopathy**) that inhibit photosynthesis of planktonic cyanobacteria; and ii) to a lesser extent, competing for key nutrients (Wu *et al.* 2011). Periphyton biofilms could be used as a safe and practical bio-measure to limit algal blooms and restore the health of hyper-eutrophic aquatic ecosystems, following provision of substrates such as macrophytes (Wu *et al.* 2011; Liu *et al.* 2016). Periphyton biofilms were also shown to remove the most common microcystin through adsorption and biodegradation, with important implications for water quality, animal and human health (Wu *et al.* 2010).

Chemical pollution is also a major threat to global health since an ever-increasing number of synthetic compounds are entering soils and water bodies, thereby decreasing water quality even in remote mountain areas (Schwarzenbach *et al.* 2006; Schmeller *et al.* 2018). Pollution can have an impact on biofilm growth and biomass, species composition, and on biofilm functions (Sabater *et al.* 2007; Proia *et al.* 2013a; Boyero *et al.* 2019). However, to date most studies have focused on one type of biofilm (on one single substrate) and one type of contaminant exposed at one (often high) concentration in experimental settings (Bonnineau *et al.* 2020). Little is known about the impacts that cocktails of compounds present at low concentrations (i.e. natural conditions), have on the structure and functions of biofilms. Proia *et al.* (2013a) showed that multi-toxin pollution led to significant change in biofilm structure and functions *in situ*, with an increase in autotrophic biomass but a decrease in photosynthetic efficiency. Analgesics and anti-inflammatories had major effects on biofilm responses, notably by decreasing the ratio green algae/cyanobacteria (Proia *et al.* 2013a). Biofilms can reduce freshwater and soil pollution by absorbing nutrients or synthetic pollutants into their matrix, thus detoxifying the surrounding medium (Sabater *et al.* 2002; Cardinale 2011). Many toxicants can be captured and sometimes metabolised in environmental biofilms; these include Persistent Organic Pollutants (POP), but also other pesticides, heavy metals, human and veterinary drugs, industrial effluents, nanomaterials, and plastics (Kohušová *et al.* 2011; Sánchez-Pérez *et al.* 2013; Wu 2017d, 2017e, 2017f; Tang *et al.* 2018; Shabbir *et al.* 2020). Multispecies biofilms may generally be more efficient in remediation than single-species biofilms due to synergistic interactions (Breugelmans *et al.* 2008).

Our review shows that biofilms buffer natural ecosystems against impacts of synthetic molecules, nutrient pollution and cyanobacterial blooms (Edwards and Kjellerup 2013; Wu 2017g, 2017c). However, drivers of global change impact these important ecosystem services. For instance, Cardinale (2011) showed that reduction of flow heterogeneity in stream mesocosms and, subsequent absence of diverse niches, led to loss of biodiversity within biofilms and lower removal of polluting nitrate. Similar results were found with glucose (Singer *et al.* 2010). The diversity within biofilms reflects the diversity of their habitats, therefore habitat loss and degradation might have great consequences on biofilms functions within their ecosystems. In mesocosms experiments associated with long-term field studies, Baulch *et al.* (2005) demonstrated that warming alone consistently led to increased bacterial densities and metabolic rates. However, the extent of these changes differed depending on the initial communities, with effects of substrate types (natural rocks vs. tiles) as well as biofilm age (Baulch *et al.* 2005). Generally, *in situ* effects of global warming and other stressors are difficult to predict because effects are not always additive, but rather synergistic or antagonistic (Baulch

*et al.* 2005; Romero *et al.* 2018). On river benthic biofilms, for example, the effects of the herbicide triclosan were far worse when biofilms were also subject to drought episodes (Proia *et al.* 2013b). More multiple-stressor research is needed to understand how biofilm and the aquatic biota will respond to global change conditions.

Biofilms have short generation times, are ubiquitous and sensitive to toxicants and other stressors (pH, salinity, nutrient pollution), meaning that their initial micro-ecosystem (in terms of community structure, productivity and resilience) can definitely shift towards another stable system (see theory of **Alternative stable systems**) more adapted to the new local conditions (e.g. resistance to a pollutant; Burns and Ryder 2001; Sabater *et al.* 2007; Wu 2017h). This "ecological memory" made biofilms reliable indicators to assess and monitor the health of aquatic ecosystems, with the development of many structurally-based or functionally-based methods (Burns and Ryder 2001; Sabater *et al.* 2007; Wu 2017h). In a recent survey monitoring pesticide occurrence in 54 Californian small streams, biofilms were shown to contain, in average, four times as many current-use pesticides like pyrethroids as in streambed sediments (Mahler *et al.* 2020). As biofilms adsorb toxicants and form the basis of aquatic food webs, they make toxicants bioavailable to consumers, thus posing a risk to their health (see part **2.4.3**). In conclusion, not only are biofilms the sentinels of aquatic ecosystems, they are also a critical control point to monitor and understand the fate of toxicants in food webs (Bonnineau *et al.* 2020; Mahler *et al.* 2020).

### 2.4.2 For the anthroposphere

Biofilms also develop on man-made surfaces and pose difficulties in industrial settings because they produce deleterious metabolites, clog filters and pipes, foul surfaces, and corrode metals. Biofilms have wrought havoc in oil, gas and nuclear industries by degrading pipelines and heat exchangers (**Figure 13**; Costerton *et al.* 1987; Beech and Gaylarde 1999). This poses a direct threat to human health and economy, since biofouling and biocorrosion increase the likelihood of industrial accidents and cost human societies trillions of US dollars in repair or prevention (Beech and Gaylarde 1999; Procópio 2019). Environmental biofilms are also major sources of concern and expense for maintaining good hygiene standards in clinical settings, due to their inherent resistances to biocides and antimicrobial agents (Costerton *et al.* 1999; Hall-Stoodley *et al.* 2004).

However, the ability of biofilms to adsorb, transform or degrade synthetic compounds is also useful *ex situ*. Biofilms have been extensively employed as biotechnology to achieve or

increase bioremediation in a cost-effective manner, especially in the treatment of wastewater (**Figure 13**; Singh *et al.* 2006; Edwards and Kjellerup 2013; Wu 2017i). As an example, periphyton biofilms were shown to effectively remove the azo-dye methyl orange from textile industrial wastewater (Shabbir *et al.* 2017). Given the growing number of humans and cities on Earth, these biofilm-based approaches may help mitigate pollution at a global scale.

### 2.4.3 For epidemiology

Environmental biofilms can also affect the incidence, distribution, and determinants of any health-related events, be it infectious or not. Toxin production has also been acknowledged for biofilm cyanobacteria and can pose a threat to human and animal health (Mez *et al.* 1998; Wood *et al.* 2012; Quiblier *et al.* 2013). The environmental conditions leading to toxin production are not yet fully understood. However, eutrophication, global warming, water pollution, and human alteration of water flow were reported to increase the frequency and intensity of these events (Quiblier *et al.* 2013). Toxic blooms are often only detected after mass mortalities occur, that is when toxin production is very high. The effects of low exposure are unknown in natural conditions, although experimental findings have shown effects of low concentrations of toxins on growth and reproduction on some crustaceans, fish and amphibians (Oberemm *et al.* 1999; Dao *et al.* 2010).

Periphyton biofilms are an important food resource for many species, but biofilm nutritional quality can vary depending on their composition, for example with regard to the content of PUFA (ω-3). The latter are essential for many species, including humans, to ensure a correct metabolism and a competent immune system (Boëchat *et al.* 2011; Guo *et al.* 2015; Hixson *et al.* 2015). Due to stressors, biofilm composition often shifts from diatom-dominated towards cyanobacteria-dominated, with a decrease in ω-3 content (Hixson *et al.* 2015; Leflaive *et al.* 2015; Crenier *et al.* 2019). The implications of such a reduced nutritional value have rarely been explored in practice for consumer health and infectious disease susceptibility. However, Crenier *et al.* (2019) showed that a composition shift could impact the growth and survival of a crustacean species. Similarly, the fact that biofilms can adsorb pollutants can be deleterious for biofilm consumers, depending on the fate of pollutants therein (Bonnineau *et al.* 2020). Microplastics are one example, being able to impair periphyton growth and cause adverse health effects to tadpoles consuming these contaminated biofilms (Boyero *et al.* 2019).

It is possible that these biofilm-related diseases (deficiencies in key nutrients, toxin or chemical poisoning) influence the dynamics of wildlife and plant infectious diseases (**Figure 6**). Rarely were microorganisms sympatric with pathogens considered as biotic environmental

drivers of infection and disease (but see Johnson *et al.* 2010a; Schmeller *et al.* 2014; Bernardo-Cravo *et al.* 2020). Yet, elimination of human-pathogens from biofilms was demonstrated, following predation by biofilm-associated protozoans or biofilm grazers (**Figure 3**); inactivation by bacteriophage, or by antimicrobial substances secreted by other microorganisms; or competition with other bacterial inhabitants for nutrients and substrates (Stevik *et al.* 2004; Langmark *et al.* 2005; Chabaud *et al.* 2006; Skraber *et al.* 2007). In contrast, some pathogens are known to survive, aggregate and even replicate in or on biofilms (Langmark *et al.* 2005; Searcy *et al.* 2006; Skraber *et al.* 2007). This is the case for numerous human water-borne pathogens, including viruses, bacteria and protozoans (Hall-Stoodley and Stoodley 2005; reviewed in Wingender and Flemming 2011). The behaviour and fate of pathogenic microorganisms within biofilms appear to depend on the properties of the pathogen, those of the biofilm, the nature of the substrate, and environmental parameters including those influencing desorption (Langmark *et al.* 2005; Stott and Tanner 2005; Skraber *et al.* 2007). For instance, oocysts of *Cryptosporidium parvum* appear to remain only at the surface of the biofilm without being incorporated and are released back in the aquatic medium up to 40 days after adhesion (Searcy *et al.* 2006). Other studies have shown that oocyst attachment could reach a saturated level in biofilms, varying with biofilm roughness, which itself reflected environmentally-driven changes in the biofilm community (Wolyniak *et al.* 2009; Wolyniak-DiCesare *et al.* 2012). How global change impacts these processes is still not yet clarified. The role of environmental biofilms as pathogen reservoirs has also been mostly studied for human and zoonotic pathogens, but very rarely for those specific to animals and plants. Only two studies were found for non-human pathogens: the first revealed that epilithic biofilms, including in high-elevation streams, harboured the plant pathogen *P. syringae* (Morris *et al.* 2007); the other showed that tank biofilms could serve as a reservoir of infection for *Mycobacterium chelonae*, a pathogen of zebrafish (*Danio rerio* ; Chang *et al.* 2019).

Other biofilms may show the same or similar processes. The ocean surface microlayer (**Figure 13**), containing the neuston, covers up to 70% of the Earth's surface (Flemming and Wuertz 2019). It is three to five times richer in microorganisms than the rest of the water column and likely drives important biogeochemical processes (Engel *et al.* 2017). As every rising (aerosol) or incoming particle must pass through the surface microlayer, the neuston participates in aerosol enrichment in microorganisms and plays a role in the diffusion of pathogens (Aller *et al.* 2005). There is also a neuston community in freshwater bodies which seems to play similar roles (Hervas and Casamayor 2009). Other types of biofilms exist, notably in the marine environment, such as marine snow (Simon *et al.* 2002) and biofilms forming on microplastics (plastisphere) or other debris (Harrison *et al.* 2014; Kooi *et al.* 2017). These have

been shown to be relevant in epidemiology as they are involved in the spread of pathogens (e.g. *Vibrio parahaemolyticus*; Kirstein *et al.* 2016) and harmful toxin-producing species (e.g. dinoflagellates; Masó *et al.* 2003).

There are many knowledge gaps regarding environmental biofilms (**Table 2**). While it is known that viruses (like other microorganisms) exist in biofilms and play huge roles in aquatic ecosystems (Sutherland *et al.* 2004; Jacquet *et al.* 2010), there is still no metabarcoding nor metabolomic methods for viruses that would allow a better understanding of the virome within biofilms, especially in freshwater ecosystems (Marchesi and Ravel 2015). Given the social and economic importance of the COVID-19 pandemic and other viral epidemics, the study of the biofilm virome represents a promising avenue for research, as biofilms can form reservoirs for enteric human-pathogenic viruses (Wingender and Flemming 2011; Von Borowski and Trentin 2021). Current knowledge gaps on the interaction between biofilms and pathogens stand in stark contrast to the major impediment to sustainable development posed by increasingly numerous emerging infectious diseases. For instance, *Batrachochytrium dendrobatidis*, causative agent of the panzootic amphibian chytridiomycosis, is an aquatic chytrid fungus but its interactions with biofilms are unknown. Doubtless, environmental biofilms are relevant in the ecology of some pathogens and the disease pyramid concept provides an interesting framework to find and test hypotheses (**Figure 6**). Besides, the biofilm life form promotes the exchange of antibiotic resistance genes, but most research on antibiotic resistance focuses on planktonic bacteria in man-made ecosystems (Hausner and Wuertz 1999; Balcázar *et al.* 2015). Antimicrobial resistance occurrence has increased in environmental microbiomes following inputs of human and animal resistant microbes, and exposure to synthetic chemicals (Zhu and Penuelas 2020). Biofilms could be used as a preferred detection site of antibiotic resistant genes to assess the risk of this threat to public health, especially in mountain ecosystems which provide water to billions of human beings (Balcázar *et al.* 2015).

We note a sizeable knowledge gap regarding effects of biological stressors on biofilms, such as human-mediated introduction of exotic species like fish which are often introduced in usually fishless high-elevation lakes, with already known impacts on some taxa (Miró *et al.* 2018). However, the effects of fish introduction on biofilms remain unknown. Besides, pathogenic (potentially antimicrobial-resistant) or toxin-producing microorganisms may be introduced with fish or livestock (Hunter and Thompson 2005; Espunyes *et al.* 2021). Here, biofilms could serve as detection site for pathogen pollution as done in human disease epidemiology to monitor environmental circulation of bacteria, protists and viruses (Bauman *et*

*al.* 2009; Wingender and Flemming 2011). Finally, understanding the interactions of all combined stressors on biofilms and the cascading effects on ecosystem will arguably be the central challenge for future biofilm research (Pesce *et al.* 2018; Romero *et al.* 2018; Chaumet *et al.* 2019a, 2019b).

## 2.5 Conclusions

It is essential that biofilm research is not neglected to identify, understand and mitigate the interactive impacts of global environmental change on host and ecosystem health through its effects on biofilms. To do so, we first need to capture the inherent natural variability –relative to community structure, physical structure, and functions– of biofilm communities to define what is a 'normal' biofilm in living organisms and ecosystems (**Figure 11**). This will require advances in both technological and theoretical frameworks. Advances in sequencing technology and functional metagenomics will shed further light on biofilm composition and functions. From a theoretical standpoint, we deem it useful to apply the analogy of health to biofilms, as done for ecosystems (Rapport *et al.* 1998; Tett *et al.* 2013). The concept of a 'healthy' biofilm, i.e. the state of a system that, given its location, its substrate, its age (stage of succession) and the surrounding environmental conditions, contains an appropriate functional diversity with all the expected organisms and their associated EPS. Such a biofilm would be productive, resilient against external pressures, and capable of maintaining its organisation and functions. One could argue that host-pathogenic biofilms or toxin-producing periphytic biofilms could then be considered healthy because there are very productive. However, these biofilms should be considered unhealthy because they usually appear as a result of disturbances such as anthropogenic excessive loading (Wu 2017j), over a relatively short period of time, and are often dominated by a single species.

The theory of alternative stable states is central in the concept of biofilm health, as when challenged beyond their resilience, biofilm transition to other stable state with different community structure and functions. A healthy biofilm would reflect the stable state that is optimal for, and indicative of, the good health of the larger system it inhabits, be it a multicellular host or an entire ecosystem. For instance, unhealthy biofilms with less biodiversity, such as host microbiome in **dysbiosis**, are less resilient and might be more permeable to pathogen invasion (Feng *et al.* 2017; Keesing and Ostfeld 2021). The concept of healthy microbiomes has already been used in a medical context to designate a functional microbiota associated with a healthy host (Clemente *et al.* 2012; Huttenhower *et al.* 2012). We argue that the same should be applied to environmental biofilms and ecosystems, respectively.

Future research should produce more evidence supporting the diversity-stability hypothesis in the diverse environments that biofilms inhabit. The concept of biofilm health would help raise awareness about biofilm importance, promote scientific outreach, unify the disciplinary silos of biofilm research in medical and environmental sciences and thus facilitate transdisciplinary studies.

# CHAPTER 3

# BIOFILM COMMUNITY COMPOSITION IS CHANGING IN REMOTE MOUNTAIN LAKES WITH A RELATIVE INCREASE IN TOXIGENIC ALGAE

## Foreword of Chapter 3

Little is known on the composition of prokaryotic and micro-eukaryotic assemblages within benthic biofilm communities of mountain lakes, although biofilms are essential in their functioning. They form the basis of food webs, but can also have, under certain circumstances leading to toxin production, a negative impact on other organisms including humans and animals. Mountain lakes are also strongly impacted by global change and human activities. Any change in the composition of biofilm communities might have considerable consequences on their functions. For all these reasons, biofilms require urgent monitoring. The aims of this study were i) to shed light on the composition of benthic microbial biofilms in different mountain lakes and its environmental drivers ii) to monitor the temporal trends in biofilm biodiversity; iii) to investigate the trends in keystone taxa such as diatoms or potentially harmful ones such as cyanobacteria. Such knowledge was needed before investigations on the epidemiological importance of biofilms in the context of amphibian chytridiomycosis could be undertaken. Furthermore, our findings have broad implications with likely negative and profound consequences for the health of mountain lakes, the supply of clean drinking water, or the use of these lakes for recreational activities. This warrants further biofilm monitoring studies but also more function-oriented ones.

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# Chapter 3: Biofilm community composition is changing in remote mountain lakes with a relative increase in toxigenic algae


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- Contribution

I performed fieldword in 2020, all the laboratory work, analysed the data, with help of A.L., D.S.S. and V.E.J.J., and wrote the first draft of the manuscript, with contributions from A.L., D.S.S., L.Z. and V.E.J.J. A.L., D.S.S. and H-P.G. conceived the study. D.S.S. and A.L. supervised and performed the fieldwork (biofilm sampling) from 2016 to 2020. L.Z performed the bioinformatics processing. All authors contributed to the final version of the manuscript.

## Abstract


It is widely recognised that mountain lakes are strongly impacted by global change. Productive benthic biofilms composed of bacteria, archaea, protists and other micro-eukaryotes are crucial for the functioning of mountain lakes. Yet little is known about the effects of global change on mountain lake biofilm communities. Combining the analyses of metataxonomic data on 16S and 18S rRNA genes with climatic and environmental data enabled us to investigate global change effects on the composition of biofilm prokaryotic and micro-eukaryotic assemblages in a 5-years monitoring program of 26 Pyrenean lakes (2016-2020). Since 1950, the climate has drastically changed at our study sites with an average increase of 1.75°C. Our results show that successions of both prokaryotic and micro-eukaryotic assemblages of biofilm communities

significantly diverged between 2016 to 2020. In particular, we observed an increase in richness and relative abundance of cyanobacteria, including toxigenic cyanobacteria, and a decrease in diatom richness and relative abundance. Temporal changes in microbial assemblages of benthic biofilms were driven by climatic conditions and related water chemistry. The variability in biofilm composition between lakes was mostly explained by water pH and hardness, both of which increase in Pyrenean lakes due to intensified rock weathering as a result of climate change. Given the predicted climate trends, the composition of benthic biofilm communities is likely to further change in the future with difficult-to-predict consequences for mountain lake ecosystems and an alarming increase in health risks both for humans and animals.

## 3.1 Introduction

Biodiversity is dramatically declining due to human activities (Butchart *et al.* 2010; Johnson *et al.* 2017; Díaz *et al.* 2019). Anthropogenic impacts have mainly been analysed for animals and plants, but much less attention has been paid to microbial communities (Cavicchioli *et al.* 2019; Wu *et al.* 2022). This is in stark contrast to their quantitative and qualitative importance for ecosystem functioning, as microbes are very abundant and diverse, play essential roles in many biogeochemical processes, and support the health of multicellular organisms and ecosystems (Bar-On *et al.* 2018; Cavicchioli *et al.* 2019; Timmis *et al.* 2019; Zhu and Penuelas 2020). Therefore, microbes play a crucial role in the response of ecosystems to global environmental changes and, consequently, in their ability to keep providing goods and services to human populations (Cavicchioli *et al.* 2019; Bernardo-Cravo *et al.* 2020; Sentenac *et al.* 2022).

One of the most important goods provided through microbial processes is clean drinking water. Mountains have been named ‘the water towers of the world’, as more than half of the human population currently depends on water from mountains with an increasing tendency (Grêt-Regamey *et al.* 2012; Viviroli *et al.* 2020). Mountains and their freshwater ecosystems are highly vulnerable to global change, including climate change, nutrient and synthetic chemical pollution, land-use change, and the introduction of invasive species, among others (Schmeller *et al.* 2022). This vulnerability directly threatens the quantity of water that mountains can deliver (Thompson *et al.* 2005; Immerzeel *et al.* 2020; Schmeller *et al.* 2022). However, we still know little about how global change affects the water quality of mountain lakes through its impacts on aquatic microbiomes, in particular benthic biofilm microbial communities.

Benthic biofilms are very diverse and highly organised matrix-enclosed communities which form the basis of food-webs and one of the most dominant microbial life form in mountain freshwater ecosystems (Vadeboncoeur and Steinman 2002; Füreder *et al.* 2003; Rott *et al.* 2006; Battin *et al.* 2016; Sentenac *et al.* 2022). As such, biofilms exert important controls on water quality by ensuring organic matter decomposition, primary production, nutrient cycling and by acting as a buffer against both synthetic and nutrient pollutants (Rott *et al.* 2006; Vadeboncoeur *et al.* 2008; Vila-Costa *et al.* 2014; Wu 2017; Sentenac *et al.* 2022). Diatoms are normally abundant and important biofilm autotrophs because they produce nutrients such as polyunsaturated fatty acids (PUFA), which are vital for a wide range of organisms to meet physiological requirements (Brett and Müller-Navarra 1997; Torres-Ruiz *et al.* 2007; Guo *et al.* 2015). In oligotrophic ecosystems such as mountain lakes, PUFA are scarce and diatoms even more essential. However, they are very sensitive to environmental changes and easily replaced by other more resistant photoautotrophs such as green algae and cyanobacteria, which are of lower dietary quality (Müller-Navarra *et al.* 2004; Guo *et al.* 2015; Leflaive *et al.* 2015; Crenier *et al.* 2019).

In addition, cyanobacteria can, under certain conditions, significantly deteriorate water quality and ecosystem health, either by proliferating and outcompeting other lifeforms in regard to oxygen, light or nutrients, or by producing cyanotoxins that can have acute or chronic toxic effects for many organisms living in or dependent on aquatic ecosystems (Oberemm *et al.* 1999; Dao *et al.* 2010; Wood *et al.* 2012, 2020; Quiblier *et al.* 2013; Chorus and Welker 2021; Fastner *et al.* 2023). The impacts of cyanobacteria on the health of domestic and wild animals is considerable, with mass mortalities often reported (Mez *et al.* 1997; Gugger *et al.* 2005; Roegner *et al.* 2014; Huisman *et al.* 2018; Fastner *et al.* 2023). In contrast to planktonic cyanobacteria, little is known on the ecology of toxigenic (i.e. toxin-producing) benthic cyanobacteria (Anses 2020; Wood *et al.* 2020; Pokrzywinski *et al.* 2021; Fastner *et al.* 2023). However, deaths of cattle and dogs due to ingestion of water containing cyanotoxins produced by biofilm cyanobacteria, or of cyanobacteria-laden biofilms flocs (i.e. loose pieces of floating biofilms) have already been observed, including in mountain areas, where swimming is often banned for this reason (Mez *et al.* 1997, 1998; Gugger *et al.* 2005; 'Cyanobactéries Alerte' 2022). Therefore, toxigenic benthic cyanobacteria represent a threat to human, animal and aquatic ecosystem health, as well as to local economies, and there is an urgent need to monitor benthic biofilm communities in mountain lakes to assess and prevent any potential health risks.

Yet, the structure of benthic biofilm communities in mountain ecosystems, and their trends in the context of global change, are relatively little studied compared to other microbiomes (Wang *et al.* 2022). To date, the main focus has been on prokaryotic assemblages,

sometimes complemented by particular taxa of micro-eukaryotes (fungi or diatoms). Most studies have remained purely descriptive or highly restricted in their temporal and spatial scales, attempting to predict climate change effects through space-for-time substitution (Bartrons *et al.* 2012; Teittinen *et al.* 2017; Yeh *et al.* 2019). These shortcomings may restrict our understanding on how microbial communities and ecosystems respond to global environmental change. In particular, benthic biofilms of mountain lakes offer an excellent opportunity to fill this knowledge gap (Catalan *et al.* 2006; Schmeller *et al.* 2018; Moser *et al.* 2019), as climate change impacts are particularly pronounced in mountains and represent one of the most important threats to mountain lake biodiversity (Pepin *et al.* 2015; Schmeller *et al.* 2022). Climate change leads to increased water temperatures and evaporation, glacier retreat, alterations of aquatic mixing regimes, as well as changes in water chemistry mainly related to increased rock weathering and glacier runoff (Koinig *et al.* 1998; Rangwala and Miller 2012; Marcé *et al.* 2015; O'Reilly *et al.* 2015; Pepin *et al.* 2015; Niedrist *et al.* 2018; Woolway *et al.* 2020). All of these environmental changes are likely to have considerable consequences for biodiversity and structure of benthic biofilm microbial assemblages.

Here, we used metataxonomic data on the 16S and 18S rRNA genes to study the successions of prokaryotic and micro-eukaryotic assemblages in biofilms over a five-year period in 26 mountain lakes spread across six different elevational gradients of the French Pyrenees. We analysed the climatic and water chemistry variables driving the composition of microbial assemblages of benthic biofilms in mountain lakes and examined whether they varied temporally. We hypothesized that, due to environmental modifications, biofilm assemblages would diverge over time with a potential loss of biodiversity. Our study is the first to simultaneously assess the temporal trends and environmental drivers of prokaryotic and micro-eukaryotic assemblages of benthic biofilm communities in mountain lakes, with the aim to provide deeper insights on biodiversity and water quality of these sensitive ecosystems under ongoing global change.

## 3.2 Materials and Methods

### 3.2.1 Biofilm taxonomic and community data

We sampled biofilms of 26 lakes from six altitudinal gradients located from West to East on the French side of the Pyrenees (**Figure 7**, **Table 1**). Lakes were visited in summer: twice in 2016; thrice in 2017 and 2018, and once both in 2019 and 2020 (for only a subset of the lakes in 2020; **Table 5**). At each visit, epilithic (i.e. growing-on-rock) biofilms were sampled (n=235) at the same location in each lake, with a metal spatula (disinfected with

chlorhexidine and rinsed with sterile water) by scraping four or five rocks located at a depth of 15-30 cm. Samples were directly frozen on dry ice. Biofilm DNA was extracted and purified from 400 mg of thawed sample using the NucleoSpin Soil kit™ (Macherey-Nagel™, Düren, Germany) according to the manufacturer's protocol. DNA quality was checked with a Nanodrop ND-1000 spectrophotometer (230/260nm and 260nm/280nm absorbance ratios, Nanodrop Technologies LLC™). We amplified the V3-V4 region of the 16S rRNA gene (F: 5′-CCTACGGGNGGCWGCAG and R: 5′-GACTACHVGGGTATCTAATCC; Klindworth *et al.* (2013)) and the V8-V9 region of the 18S rRNA gene (F:5'-ATAACAGGTCTGTGATGCCCT and R: 5'-CCTTCYGCAGGTTCACCTAC; Bradley *et al.* (2016)), respectively, by Polymerase Chain Reaction (PCR). Our PCR mix contained 12.5µL MyTaq™, 1µL of 1µM solution of both forward and reverse primer, 0.5µL of Bovine serum albumin, and 10µL of diluted template to obtain exactly 20ng of DNA in each well. The PCR conditions were as follows: 95°C for 3 minutes, 30 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s, with a final extension step at 72°C for 5 minutes. The PCR products were then sent to an independent research platform, GeT Biopuces (https://get-biopuces.insa-toulouse.fr/), which performed the indexing PCR and amplicon sequencing. Briefly, PCR products were cleaned using Agencourt AMPure XP™ and submitted to a 10-cycle PCR according to the standard Illumina protocol (95°C for 180s, 10 cycles x [95°C for 30s, 55°C for 30s, 72°C for 30s] and 72°C for 300s) with sample-specific Illumina-Nextera Index primers. Products were cleaned, quantified and diluted to include 140ng of DNA in the pool. Finally, the library was sequenced on a MiSeq Illumina platform (2x250bp V3).

Demultiplexing and the removal of primer and adapter sequences was performed using Cutadapt v3.4 (Martin 2011). Additional trimming, formation of contiguous sequences, identification of unique amplicon sequence variants (ASVs), and chimera removal were performed in R v4.2.0 (R Core Team 2022) using the DADA2 v1.20.0 pipeline (Callahan *et al.* 2016). Taxonomy of ASVs of both 16S and 18S rRNA genes was assigned using SINA v1.7.2 and the SILVA 138.1 reference database (Pruesse *et al.* 2012; Quast *et al.* 2013). For the 16S rRNA library, ASVs unclassified at the class level, or (mis)classified as eukaryotes, chloroplasts, or mitochondria were removed using the *phyloseq* package (McMurdie and Holmes 2013). For the 18S rRNA library, unclassified ASVs at the third taxonomic rank and Metazoan taxa belonging to Vertebrata, Arthropoda, Platyhelminthes, Annelida, and Mollusca were removed. We used rarefaction to 3897 and 1856 reads for the 16S and 18S rRNA datasets, respectively, resulting in four 16S and six discarded 18S rRNA gene sequence samples (**Figure 18**). We obtained 231 prokaryotic and 229 micro-eukaryotic sample libraries. We used the function avgdist() of package *vegan* (Oksanen *et al.* 2022) with 100 iterations to compute an

average Bray-Curtis dissimilarity based on 100 rarefied datasets, and used this as an index for pairwise β-diversity (Cameron *et al.* 2021). We computed the Inverse-Simpson index to represent α-diversity with the package *microbiome* (Lahti and Shetty 2012), and the richness and relative abundance of indicator taxa such as all cyanobacteria, potentially toxigenic cyanobacteria, and diatoms with package *phyloseq*. Potentially toxigenic cyanobacteria were identified as such when we detected cyanobacterial genera (the SILVA database rarely allows identification down to species level) known to produce cyanotoxins while living in biofilms (Quiblier *et al.* 2013; Wood *et al.* 2020). Note that all analyses presented below were also run without rarefaction but normalised with total sum scaling (TSS; McKnight *et al.* 2019; Lin and Peddada 2020). Significant results were similar and are not presented here.

### 3.2.2 Environmental metadata

Conductivity, pH and dissolved oxygen were measured with a multi-parameter probe (WTW™ Multi 3420 SET G). Dataloggers (Hobo pendant 64K®) monitored temperature on an hourly basis in each lake. Total organic carbon and total nitrogen concentrations were determined in the laboratory from unfiltered water with a Shimadzu™-TC-L analyser, and total phosphorus concentrations with a SECOMAM™-Uvi-Light-XT. Concentrations of major anions and cations were assessed with filtered water using ThermoScientific ion-chromatographs Dionex™ ICS-5000+ and Dionex™ DX 120, respectively. Biogenic silica was determined with an ALPKEM™ IV flow analyser. Finally, we also determined concentrations of fourteen trace elements using ICP-MS (Agilent Technologies™ 7500ce and Element XR™; **Figure 19**). We excluded many variables that contained too many zeros and/or missing values (see **Figure 19** for the number of measurements per variable). We retained hardness, pH, chloride (hereafter denoted Cl), sodium (Na), potassium (K), total copper (TCu), total organic carbon (TC), total nitrogen (TN), and biogenic silica ($SiO_2$). Conductivity was discarded as it was highly correlated with hardness ($r_{19} = 0.97$, $p < 0.001$). In our selected variables, missing values were filled with Multivariate Imputation by Chained Equation (R package *MICE*): i) using the method *2L.lmer* for hardness, chloride and potassium, with time as fixed effects and lake as random effects, because a temporal trend in each lake was detected for these variables (**Appendix**: Environmental changes in the studied Pyrenean lakes); ii) using again the same method for pH, but with hardness and time in the fixed effects, as hardness and pH were initially correlated ($r_{48} = 0.62$, $p < 0.001$); iii) using the default method *predictive mean matching* for other variables for which no trend was initially detected (Buuren and Groothuis-Oudshoorn 2011). Because pH and hardness were not independent, we clustered them together with the R package *ClustOfVar* (Chavent *et al.* 2011). The latter is a method for clustering correlated

variables in the best way possible, by creating one synthetic variable per cluster. The synthetic variable is the first principal component obtained with the mixed Principal Component Analysis (PCAMIX) method performed on a set of variables in a same cluster. Here, we obtain a synthetic variable, termed pH-hardness (r = -0.96 with pH and hardness).

Using public climate data on daily precipitation and air temperature (E-OBS dataset from the EU-FP6 project UERRA; https://www.uerra.eu) as well as our water temperature data, we described climate changes in the Pyrenees and more specifically in each altitudinal gradient under study (see **Appendix:** Environmental changes in the studied Pyrenean lakes for details). For other analyses, we also computed with these data several climatic variables for each sample: i) the mean air temperature from the 15$^{th}$ of November to the 1$^{st}$ of June to capture long-term temperatures (winter and spring) prior to sampling (denoted LT_T), ii) the sum of precipitations over this period (denoted LT_PRCP), and iii) the mean air and water temperatures over three weeks prior to sampling. As both were highly correlated (Pearson's r =0.73, $p < 0.001$), they were clustered using *ClustOfVar* in a synthetic variable denoted recentT (r=0.93 with mean air and water temperatures). We also computed iv) the sum of precipitations over three weeks prior to sampling (denoted recentPRCP), and v) the average daily temperature range over three weeks prior to sampling (being right-skewed, it was log-transformed and denoted as ln.wDTR).

In total, we retained 14 environmental predictors (that were z-transformed): recentPRCP, recentT, LT_T, LT_PRCP, ln.wDTR, pH-hardness, $SiO_2$, TC, TN, Cl, Na, K, TCu, and lake area.

### 3.2.3 Statistical analyses

#### 3.2.3.1 Temporal trends in biofilm biodiversity.

We used eight (generalised) linear mixed models ((G)LMMs) with the following eight response variables: i) the Inverse-Simpson index of the prokaryotic and ii) micro-eukaryotic assemblages (α-diversity; gamma distribution and a log link function) and iii) the richness of cyanobacteria, iv) potentially toxigenic cyanobacteria and v) diatom (normal distribution, identity link function), vi) the relative abundances of cyanobacteria and vii) potentially toxigenic cyanobacteria (beta distribution, logit link function), and viii) the relative abundance of diatoms (logit-transformed data, normal distribution, identity link function). For each model, we fitted random slope and intercept models with time since beginning of the study as sole fixed effect and and lake as random slope and intercept (except for cyanobacteria richness: only a random intercept model was fitted due to singular convergence with the random slope model). We used the R packages *glmmTMB* (Brooks *et al.* 2017) and *lmerTest* to fit GLMM and LMM,

respectively, both estimated using restricted maximum likelihood. For all model fits, residuals diagnostics were assessed with the *Performance* (Lüdecke *et al.* 2021) and *DHARMa* packages (Hartig 2022). Since climate change was not homogenous between western (lakes of Lescun, Fache, Neouvielle) and eastern gradients (Bethmale, Bassies, Arbu-Lers-Estagnon), we tested whether the temporal trends for the eight above-mentioned response variables differed between these two groups, by fitting similar models with the interaction between time and location (West or East, same specification otherwise).

To explore within-lake temporal trends in β-diversity (i.e. biofilm community succession divergence over time), we used two approaches. First, we determined time-decay relationships (TDRs) according to Guo *et al.* (2018). We partitioned our time series data into subset windows of one year (four windows: 2016-17, 2017-18, 2018-19, 2019-20), two years (2016-18, etc.), three years, and four years and restricted pairwise comparisons to samples taken in a same lake at the same period of the year. The rationale for this was to get rid of sub-seasonal variation as climatic conditions vary quickly in mountains between late June and early September. We defined three periods within a year, which basically correspond to our three sampling campaigns: early summer (before mid-July), mid-summer (mid-July to end August), and late summer (September onward). Basically, TDRs consist of fitting a random-slope and intercept LMM with logarithmic β-similarities (S or 1-Bray-Curtis dissimilarity) as the response variable and logarithmic temporal distance (dT) as the explanatory variable, with one slope and one intercept estimated for each lake. The TDR model estimates a general slope *v* (the TDR value), which is commonly reported to assess the temporal turnover rate of community successions (Guo *et al.* 2018). We then also looked whether these temporal turnover rates differed between the Western and Eastern groups, by adding the interaction between the logarithmic temporal distance and location.

Second, we used a Global Dissimilarity Model (GDM, Ferrier *et al.* 2007) with the temporal approach of Blois *et al.* (2013) and comparisons restricted to samples belonging to the same lake and period (as above). Further details on GDM fundamentals are given in the next section. To determine the environmental drivers associated with the temporal dissimilarity, we included in the model our 14 retained environmental predictors. We used the gdm.varImp() function of package *gdm* to assess the respective importance and significance of each predictor, as well as the significance explained by the full model (see next section, Fitzpatrick *et al.* 2022).

#### 3.2.3.2 Environmental drivers of biofilm biodiversity

We used the 14 retained environmental variables as fixed effects in eight (G)LMMs with the same eight response variables and specification detailed in the previous section, but

only with lake random intercept models (no random slopes, as it was then not possible to fit the models). After fitting, collinearity was assessed with the package *performance* to ensure that the Variance Inflation Factor was low (VIF <5) for each predictor, which led us to exclude elevation from the pool of variables due to systematically great VIF when fitted with LT_T and LT_PRCP. We then fitted eight other models with only location (East or West) as categorical predictor and lake as random effect.

We used GDMs to determine the environmental factors explaining between-lake variations in the prokaryotic and micro-eukaryotic assemblages. GDMs relate biological dissimilarity $d_{ij}$ (Bray-Curtis dissimilarity) to environmental and spatial ('geographical') distances $\boldsymbol{\eta}$ between samples i and j using a GLM link function (equation 1).

$$d_{ij} = 1 - e^{-\eta} \quad (1)$$

In this equation, $\eta$ is the sum across all predictor variables (the 14 environmental plus spatial distance) of the absolute differences in the model transformed predictor values (by default, I-spline basis functions fit using non-negative least squares regression) between samples *i* and *j* (Mokany *et al.* 2022). The significance of the model and the predictors, as well as predictor importance, were determined using the gdm.varImp() function with n=1,000 permutations. Model (or predictor) significance is determined using bootstrapped p-values when the table is permuted (only that predictor permuted). Predictor importance is measured as the percent decrease in deviance explained by the GDM fit to the unpermuted table and the deviance explained by a model fit with only that predictor permuted. We fitted a GDM by restricting the pairwise dissimilarity matrix to comparisons across samples taken at the same period (as defined above) of the same year, to obtain the drivers of between-lake β-diversity and eliminate the influence of season and time (results were similar with the full pairwise dissimilarity matrix). Because spatial distance as well as precipitations and temperature variables were included in GDMs, we could see whether differences in assemblage composition existed or not between western and eastern gradients. We also draw Principal Coordinate Analysis (PCoA) ordination plots to visualise potential compositional differences between these two groups with packages *vegan* and *ggordiplot* (Quensen 2021).

## 3.3 Results

### 3.3.1 Climatic changes

We used climdex indices to monitor climatic changes from 1950 to 2021 (see **Table 10** for more precise definition of Climdex variables). The annual mean temperature has increased

by 1.75°C in all our gradients since 1950 (**Figure 40** and **Table 11**). However, other aspects of climate change differ markedly between eastern (gradients Arbu-Lers-Estagnon, Bassies, Bethmale) and western sites (Neouvielle, Fache, Lescun). For the eastern sites, the annual maximum temperature has significantly increased (between 1.75 and 2.45°C, depending on the spatial gradients) as well as the annual number of extremely hot days (between 9 and 13 days), and the duration of warm spells (between 8 and 9 days) from 1950 to 2021. The average annual daily temperature range has either been stable (Bethmale) or has increased (between 1.26 to 1.75°C in Arbu-Lers-Estagnon and Bassies, respectively, **Figure 40**). The pattern is the opposite for our western sites, with stagnation of the annual maximum temperature, the number of extremely hot days, and duration of warm spells per year over the last 70 years. In contrast, the annual minimal temperatures (TNn) have increased between 3.85 and 4.76°C. As a consequence, the annual daily temperature range have considerably decreased (between 2.73 and 4°C, **Figure 40**).

Concerning precipitations, there has been an increasing trend in the sum of annual precipitations, but it was only significant at our westernmost sites (from 1950 to 2021, increase of 220 mm in Neouvielle, 410 mm in Fache and 595 mm in Lescun), both in terms of quantity and intensity (reflected by climdex indices SDII, R10mm, R20mm, R95ptot, R99ptot). At our eastern sites, the sums of annual precipitations have been stable, but the precipitation intensities have significantly decreased over time (between 1.12 and 1.33 mm per rainy day), meaning that it has rained more often than in the past, but less heavily (**Figure 41)**. The annual maximum number of consecutive dry days has significantly decreased (between 4.7 and 7.8 days) and that of consecutive wet days has increased at all sites (between 2.7 and 4.9 days).

Throughout our study period (2016-2020), climdex indices have followed generally similar trends than for the period 1950-2021. The mean annual temperature and total precipitations have increased over the years (**Figure 42** and **Figure 43, Table 12**). Based on air temperature and in-situ logged water temperature data, our model shows that average annual water temperatures in the ice-free period significantly increased in almost all our lakes from 2007 to 2021 (**Figure 44**). This increase was significantly greater for eastern sites than for western sites (**Table 13**). Yet, average annual water temperatures remained generally stable during the study period (2016-2020; **Figure 45**).

While no trend was detectable in any of the lakes when taken alone, some water chemical variables showed significant trends when the whole dataset was analysed, or when data from western vs. eastern lakes were compared, with lake as random effect (**Figure 46**, **Figure 47** and **Figure 48**, **Table 13**). Hardness and potassium had a significant overall positive

trend, whereas chloride had a significantly negative temporal trend (**Table 13)**. These trends did not significantly differ between western and eastern sites (**Table 13**). There were no significant overall temporal trends for sodium. Trends in other chemical variables could not be assessed due to too many missing data points. Because hardness has increased over the study period and pH is correlated to hardness, the synthetic variable pH-hardness, negatively correlated to both of these variables, has decreased from 2016 to 2020.

### 3.3.2 Taxonomic overview

We obtained 231 prokaryotic and 229 micro-eukaryotic libraries from 26 Pyrenean lakes during the sampling period 2016-2020, for which we identified a total of 35,118 and 13,410 ASVs after rarefaction, respectively. Cyanobacteria and Proteobacteria (now called Pseudomonadota) were the two main phyla in the biofilm prokaryotic assemblages, followed by Bacteroidota, Actinobacteriota, Planctomycetota, and Verrucomicrobiota (**Figure 20**). The corresponding dominant classes were Cyanobacteriia, Alphaproteobacteria, Gammaproteobacteria, Bacteroidia and Actinobacteria (**Figure 21**). The micro-eukaryotic communities were dominated by three supergroups: the Archaeplastida (i.e. plants, in particular the green algae belonging to the class of Chlorophyceae), the Stramenopiles-Alveolata-Rhizaria (SAR, with notably the Diatomea class), and the Amorphea, a vast supergroup gathering the animal and fungal kingdoms (**Figure 23** and **Figure 24**).

### 3.3.3 Temporal trends in biofilm diversity and indicator taxa

The Inverse-Simpson index (α-diversity) of the prokaryote assemblage decreased over time ($p = 0.008$), but not that of micro-eukaryotes ($p = 0.554$, **Table 3, Figure 14**). The richness and relative abundance of cyanobacteria increased significantly over time (respectively, $p < 0.001$ and $p = 0.001$). Similarly, the richness ($p = 0.003$) and relative abundance ($p < 0.001$) of toxigenic cyanobacteria increased over time (**Figure 24**). In contrast, diatom richness and relative abundance significantly decreased over time ($p = 0.025$, $p = 0.032$, **Table 3, Figure 14**).

Time-decay analyses revealed that similarities between biofilm compositions (same lake, same period over different years) significantly decreased with increasing temporal distance, both for prokaryotic and micro-eukaryotic assemblages ($p = 0.004$, $p = 0.037$; **Table 3, Figure 14**). The temporal GDM supported these results: temporal differences were a significant and important predictor of prokaryotic β-diversity (importance = 29.31, $p = 0.024$: deviance explained by the full model = 13.03%, model $p = 0.001$), and marginally significant,

with relatively high importance, for micro-eukaryotic β-diversity (importance = 18.31, p = 0.098, 13.76%, model p < 0.001, **Figure 15**). The temporal dissimilarity of prokaryotic assemblages was also driven by long-term precipitations (importance = 31.14, p = 0.025) and that of micro-eukaryote assemblages by pH-hardness (importance = 29, p = 0.042, **Figure 15**). For none of the response variables studied are there significant differences in the temporal trends of the western and eastern sites.

**Table 3: Summary statistics of the temporal trends in biofilm diversity.** Temporal trend estimates (β or ν) are not back-transformed, and time was z-transformed prior to fitting. GLMM fitted with *glmmTMB* uses infinite degrees of freedom to compute the z statistics and p-values.

| Response variable | Overall temporal trend (β or ν)<br>East vs. West | 95% CI | Conditional $R^2$ | Marginal $R^2$ | statistics | p |
|---|---|---|---|---|---|---|
| Prokaryotic Inverse-Simpson index | -0.16 | [-0.27, -0.04] | 0.30 | 0.04 | z = -2.64 | **0.008** |
| | 0.16 | [-0.07, 0.38] | 0.31 | 0.04 | $t_{227}$ = 1.4 | 0.163 |
| Micro-eukaryotic Inverse-Simpson index | -0.03 | [-0.12, 0.06] | 0.17 | 1.40e-3 | z = -0.59 | 0.554 |
| | 0.02 | [-0.16, 0.21] | 0.18 | 1.59e-3 | $t_{225}$ = 0.22 | 0.827 |
| Cyanobacteria richness | 7.52 | [3.83, 11.20] | 0.51 | 0.03 | $t_{190}$ = 4.02 | **<0.001** |
| | 0.10 | [-7.93, 8.13] | 0.52 | 0.09 | $t_{18.9}$ = 0.03 | 0.979 |
| Cyanobacteria relative abundance | 0.21 | [0.08, 0.33] | 0.49 | 0.12 | z = 3.28 | **0.001** |
| | -0.08 | [-0.33, 0.18] | 0.51 | 0.12 | $t_{227}$ = 0.58 | 0.561 |
| Toxigenic cyanobacteria richness | 3.40 | [1.62, 5.18] | 0.44 | 0.04 | $t_{10.9}$ = 3.77 | **0.003** |
| | 1.03 | [-2.95, 5.01] | 0.46 | 0.08 | $t_{19.5}$ = 0.54 | 0.593 |
| Toxigenic cyanobacteria relative abundance | 0.23 | [0.10, 0.36] | 0.29 | 0.07 | z = 3.39 | **<0.001** |
| | -0.01 | [-0.28; 0.27] | 0.31 | 0.07 | $t_{226}$ = -0.06 | 0.955 |
| Diatom richness | -2.45 | [-4.44, -0.45] | 0.61 | 0.02 | $t_{19.6}$ =-2.42 | **0.026** |
| | 1.59 | [-2.87, 6.05] | 0.62 | 0.06 | $t_{21.8}$ = 0.74 | 0.467 |
| Diatom relative abundance | -0.17 | [-0.32, -0.02] | 0.72 | 0.02 | z = -2.30 | **0.032** |
| | 0.19 | [-0.13, 0.51] | 0.73 | 0.03 | $t_{22}$ = 1.22 | 0.236 |
| Prokaryotic similarity (TDR) | -0.42 | [-0.69, -0.16] | 0.41 | 0.05 | $t_{23}$ = -3.18 | **0.004** |
| | -0.01 | [-0.59, 0.58] | 0.41 | 0.06 | $t_{20.3}$ = 0.02 | 0.983 |
| Micro-eukaryotic similarity (TDR) | -0.29 | [-0.55, -0.03] | 0.53 | 0.04 | $t_{24.5}$ =-2.20 | **0.037** |
| | -0.27 | [-0.83, 0.30] | 0.54 | 0.05 | $t_{21.9}$ = 0.98 | 0.337 |

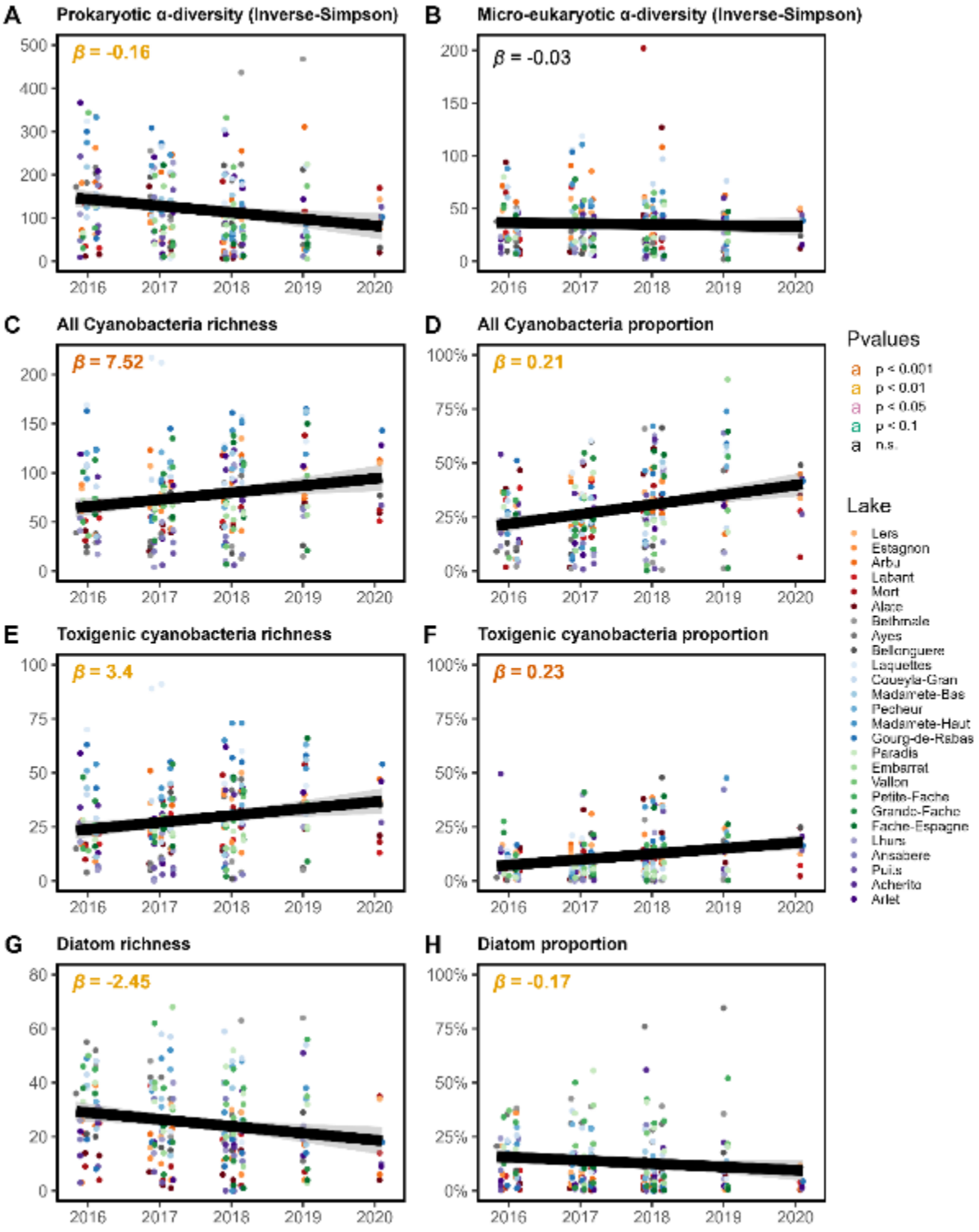
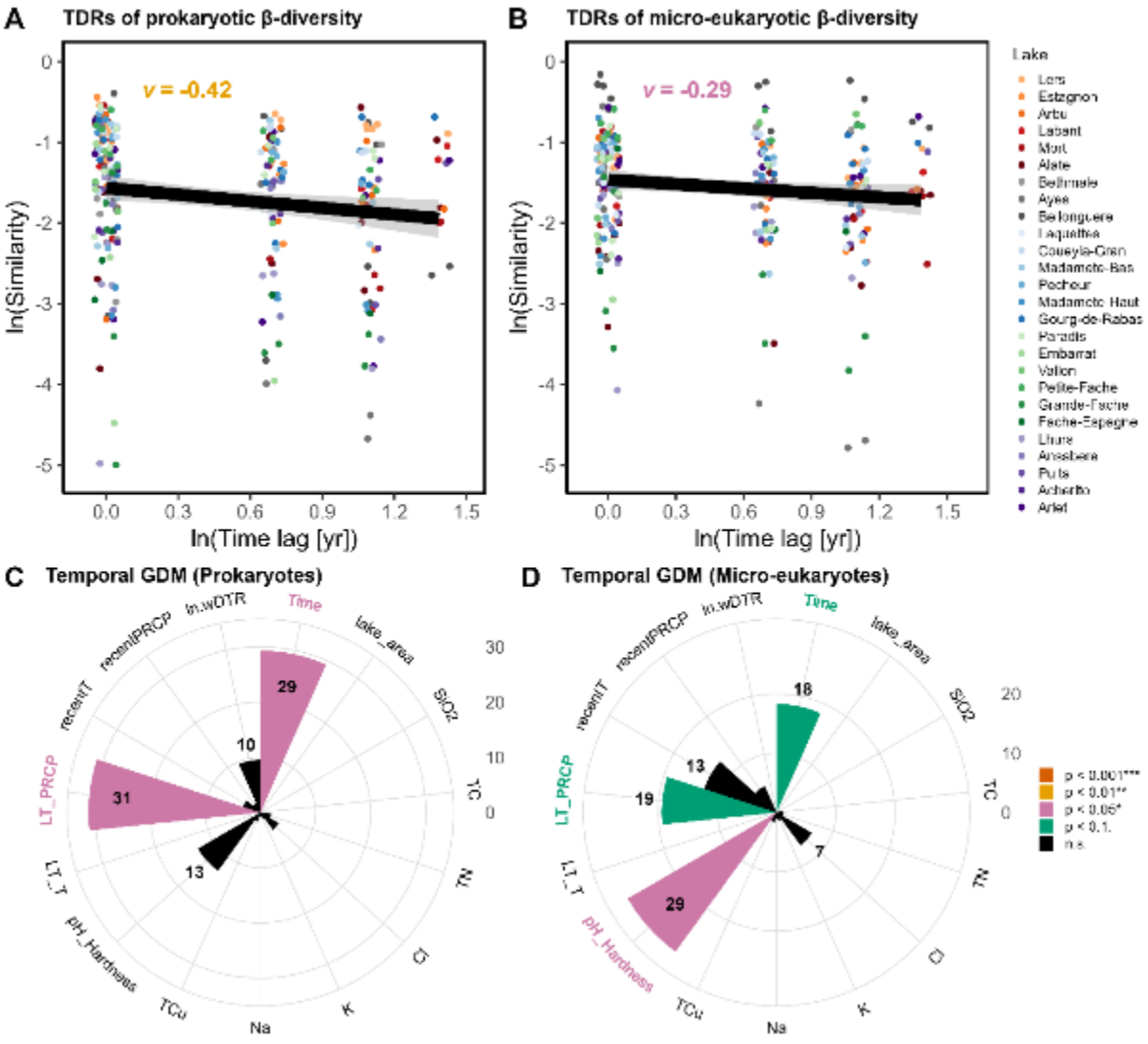



**Figure 14: Temporal trends in biofilm α-diversity (A-B) and in indicator taxa richness and relative abundance (C-H) in the period from 2016-2020.** The regression line is shown in black, with 95% confidence intervals in grey shade and its estimated slope indicated by β, colored in function of its p-value. Coefficients β are shown untransformed (i.e. not back transformed, see methods for respective models and their link functions). Lakes are sorted by gradient, by increasing altitude within a gradient: Arbu-Lers-Estagnon: orange shades; Bassies: red shades; Bethmale: grey shades; Neouvielle: blue shades; Fache: green shades; Lescun: purple shades.




**<u>Figure 15:</u> Temporal trends in biofilm β-diversity determined by Time-Decay Relationships (TDRs, A, B), and importance of time and other environmental predictors in driving intra-lake variation in biofilm microbial composition, determined by Generalised Dissimilarity Models (GDM, C and D).** The absolute value of coefficient ν (TDR) represents the microbial turnover of community succession (see methods; it is basically the slope of the regression line): ν is significant for both prokaryotes and micro-eukaryotes. Significance levels are shown in colours. Dissimilarity was measured with the Bray-Curtis index (Similarity = 1 - dissimilarity). Temporal GDMs control for the effects of season and lake. Only importance score superior to 5% are displayed. <u>Abbreviations</u>: lake_area: Lake size, $SiO_2$: Biogenic silica, TC: Total Organic Carbon, TN: Total Nitrogen, Cl: Chloride, K: Potassium, Na: Sodium, Cu: Copper; pH-hardness: synthetic variable (r = -0.96 with pH and hardness, LT_T: temperatures during winter prior to sampling, LT_PRCP: precipitations during winter prior to sampling, recentT: synthetic variable grouping mean air and water temperatures over 3

weeks prior to sampling (r=0.73), recentPRCP: same with precipitation variables, ln.wDTR: average water daily temperature range over 3 weeks prior to sampling (log-transformed).

### 3.3.4 Environmental drivers of biofilm diversity and indicator taxa

There was no significant impact of the 14 tested environmental predictors on the prokaryotic Inverse-Simpson index (**Figure 16**). Micro-eukaryotic α-diversity was positively related to pH-hardness ($p = 0.006$) and lake area ($p = 0.010$, **Table 4**, **Figure 16**). Cyanobacteria richness was positively affected by lake area ($p = 0.001$) and negatively by long-term temperature ($p < 0.001$), chloride ($p = 0.027$), copper ($p = 0.013$) and long-term precipitations ($p = 0.033$). Cyanobacterial relative abundance was associated positively with lake area ($p = 0.035$), and negatively with copper ($p = 0.037$), and long-term temperatures ($p = 0.044$). The richness of potentially toxigenic cyanobacteria was negatively affected by long-term temperatures ($p < 0.001$), chloride ($p = 0.041$), and copper ($p = 0.042$), and positively affected by lake area ($p = 0.009$). Toxigenic cyanobacteria proportion was negatively influenced by chloride ($p = 0.033$). Diatom richness was positively affected by biogenic silica ($p < 0.001$), and negatively by pH-hardness ($p = 0.001$), long-term precipitations ($p = 0.004$), and recent temperatures ($p = 0.037$). Diatom proportion was also negatively affected by pH-hardness ($p = 0.002$), recent temperatures ($p = 0.016$) and potassium ($p = 0.033$). The availability of biogenic silica was linked to an increase in diatom relative abundance ($p = 0.013$, **Table 4**, **Figure 16**). There were no significant differences between the western and eastern sites for any of response variables.

Environmental GDM analyses of between-lake prokaryotic β-diversity (deviance explained = 27.41%, model $p < 0.001$) showed that Bray-Curtis dissimilarity was greater with increasing differences in pH-hardness (importance = 55.89, $p < 0.001$) and, to a lesser extent, with geographic distance (4.34, $p < 0.001$) and recent temperatures beteen samples (5.2, $p = 0.08$). Similar results were found for the micro-eukaryotic assemblage (deviance explained = 25.16%, model $p < 0.001$), with pH-hardness (importance = 50.65, $p < 0.001$) and geographic distance (importance = 2.61, $p = 0.004$) being the predictors explaining between-lake dissimilarity (**Figure 17**). For both prokaryotic and micro-eukaryotic assemblages, climatic variables and distance accounted for little variation in composition, showing that biofilm microbial compositions from Western and Eastern sites are somewhat similar, which was supported by PCoA plots where western and eastern sites widely overlap (**Figure 25**).

**Table 4: Summary of all significant environmental drivers of biofilm diversity.** Estimates β are not back-transformed (see methods for link functions), and variables were z-transformed prior to fitting. CI: confidence intervals; C.$R^2$: conditional $R^2$; M.$R^2$: marginal $R^2$.

| Environ-mental drivers | Response variable | β | 95% CI | C. $R^2$ | M. $R^2$ | statistics | p |
|---|---|---|---|---|---|---|---|
| pH-Hardness | Micro-eukaryotic Inverse-Simpson index | 0.18 | [0.06, 0.31] | 0.2 | 0.15 | z = 2.86 | 0.004 |
| | Diatom richness | -5.08 | [-7.84, -2.31] | 0.51 | 0.35 | $t_{27.5}$ = -3.61 | 0.001 |
| | Diatom proportion | -0.43 | [-0.68, -0.18] | 0.61 | 0.28 | $t_{45.5}$ = -3.37 | 0.002 |
| | Micro-eukaryotic within-lake dissimilarity (GDM) | / | / | 0.14, model p < 0.001 | | Importance = 29 | 0.042 |
| | Prokaryotic between-lake dissimilarity (GDM) | / | / | 0.27, model p < 0.001 | | Importance = 55.89 | < 0.001 |
| | Prokaryotic between-lake dissimilarity (GDM) | / | / | 0.25, model p < 0.001 | | Importance = 50.65 | < 0.001 |
| Lake area | Micro-eukaryotic Inverse-Simpson index | 0.18 | [0.05, 0.32] | 0.2 | 0.15 | z = 2.68 | 0.007 |
| | Cyanobacteria richness | 16.54 | [7.77, 25.32] | 0.50 | 0.33 | $t_{20.5}$ = -3.71 | 0.001 |
| | Cyanobacteria relative abundance | 0.20 | [0.01, 0.39] | 0.53 | 0.29 | z = 2.11 | 0.035 |
| | Toxigenic cyanobacteria richness | 6.08 | [1.93, 10.22] | 0.45 | 0.25 | $t_{20.8}$ = -2.89 | 0.009 |
| Biogenic Silica ($SiO_2$) | Diatom richness | 5.80 | [2.91, 8.68] | 0.51 | 0.35 | $t_{18.1}$ = 3.96 | < 0.001 |
| | Diatom proportion | 0.39 | [0.11, 0.68] | 0.61 | 0.28 | $t_{21.8}$ = 2.72 | 0.013 |
| Chloride (Cl) | Cyanobacteria richness | -7.10 | [-13.37, -0.82] | 0.50 | 0.33 | $t_{215.4}$ = -2.23 | 0.027 |
| | Toxigenic cyanobacteria richness | -3.07 | [-6.01, -0.12] | 0.45 | 0.25 | $t_{215}$ = -2.05 | 0.041 |
| | Toxigenic cyanobacteria relative abundance | -0.23 | [-0.43, -0.02] | 0.22 | 0.14 | $t_{189.6}$ = -2.15 | 0.033 |
| Cupper (TCu) | Cyanobacteria richness | -5.60 | [-9.98, -1.21] | 0.50 | 0.33 | $t_{215.4}$ = -- 2.51 | 0.013 |
| | Cyanobacteria relative abundance | -0.13 | [-0.25, -7.90e-03] | 0.53 | 0.29 | z = -2.09 | 0.037 |
| | Toxigenic cyanobacteria richness | -2.14 | [-4.20, -0.08] | 0.45 | 0.25 | $t_{215}$ = -2.05 | 0.042 |
| Potassium (K) | Diatom proportion | -0.18 | [-0.35, -0.01] | 0.61 | 0.28 | $t_{213.8}$ = -2.14 | 0.033 |
| Winter temperatures (LT_T) | Cyanobacteria richness | -22.82 | [-33.54, -12.09] | 0.50 | 0.33 | $t_{56.9}$ = -4.19 | < 0.001 |
| | Toxigenic cyanobacteria richness | -9.33 | [-14.38, -4.28] | 0.45 | 0.25 | $t_{57.9}$ = -3.64 | < 0.001 |
| | Cyanobacteria relative abundance | -0.26 | [-0.52, -6.19e-03] | 0.53 | 0.29 | z = -2.01 | 0.044 |
| Winter precipitations (LT_PRCP) | Cyanobacteria richness | -8.21 | [-15.73, -0.69] | 0.50 | 0.33 | $t_{146.3}$ = -- 2.15 | 0.033 |
| | Diatom richness | -4.16 | [-6.97, -1.35] | 0.51 | 0.35 | $t_{132.3}$ = -2.92 | 0.004 |
| | Prokaryotic within-lake dissimilarity (GDM) | / | / | 0.13, model p = 0.001 | | Importance = 31.1 | 0.025 |
| Recent temperatures (recentT) | Diatom richness | -2.12 | [-4.12, -0.13] | 0.51 | 0.35 | $t_{213.6}$ = -2.10 | 0.037 |
| | Diatom proportion | -0.18 | [-0.32, -0.03] | 0.61 | 0.28 | $t_{212.5}$ = -2.43 | 0.016 |

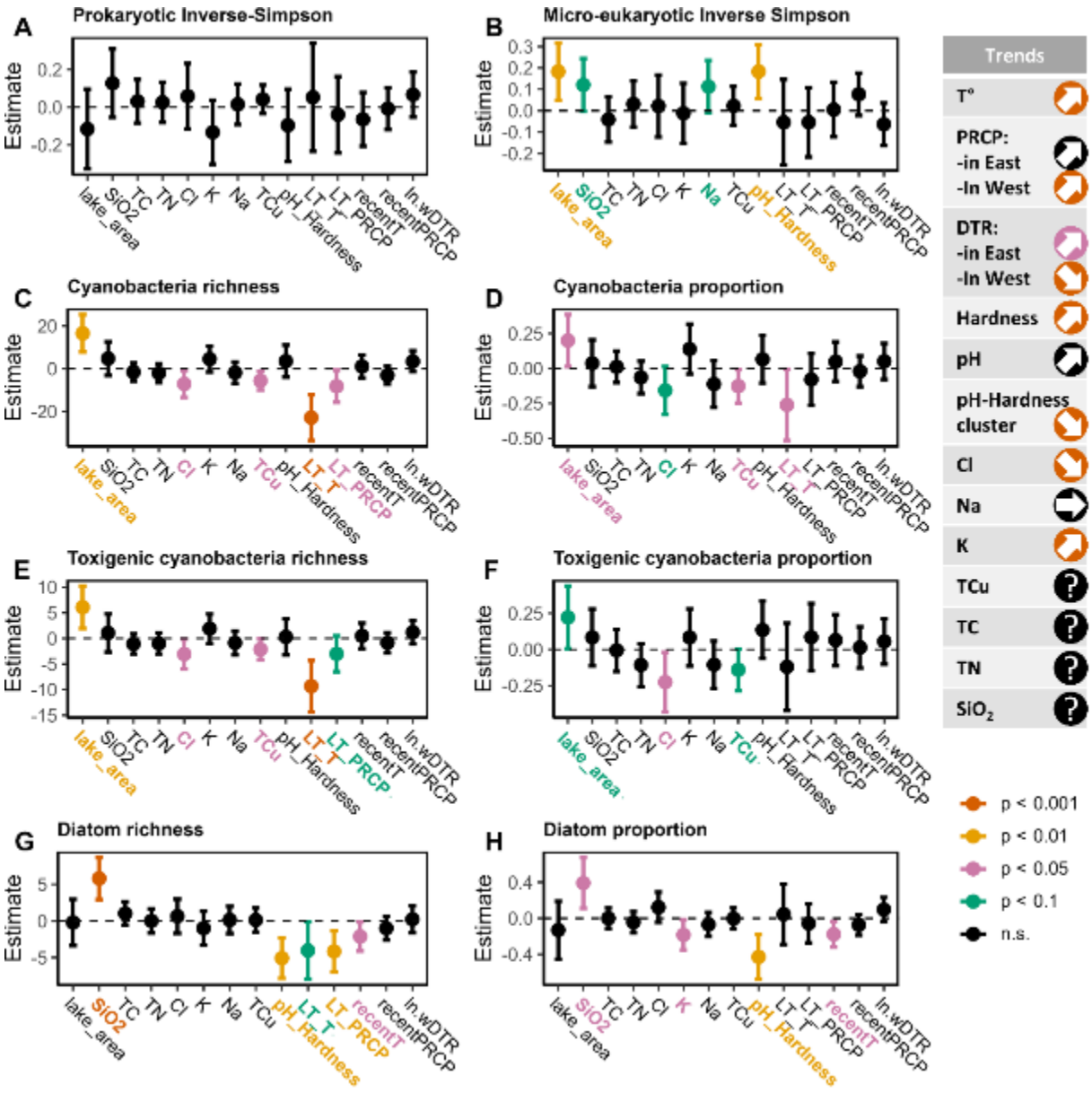



**Figure 16: Environmental drivers of the Inverse Simpson indices of prokaryotic (A) and micro-eukaryotic biofilm (B) assemblages, and of the richness and relative abundance of indicator taxa (C-H).** Random (Lake)-intercept model outputs are displayed, with points and segments representing the mean and 95% confidence intervals, respectively. Estimates are displayed untransformed, i.e. on the log scale for A and B, and on the logit scale for D, F, H. Temporal trends over the last decades or years (depending on the data available) for each of the 14 environmental predictors are displayed on the right-hand side. While trends in pH could not be assessed in our study due to lack of data, we draw an increasing trend as i) pH is positively correlated to hardness, which is significantly increasing in our lakes, ii) other studies show that pH is increasing in the Pyrenees (Curtis *et al.* 2009).

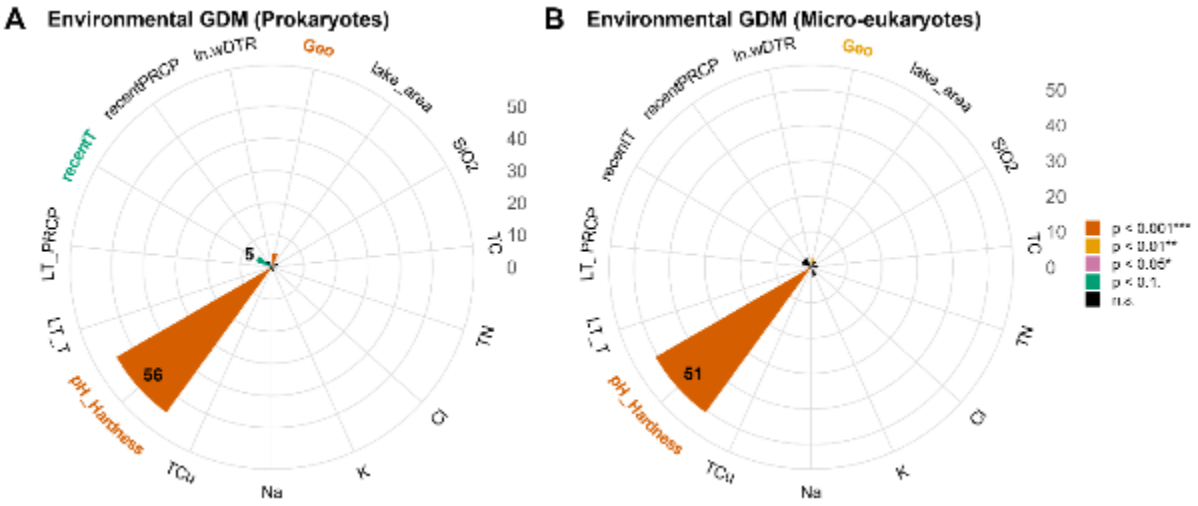



**Figure 17: Importance and significance of environmental predictors of between-lake variations in prokaryotic (A) and micro-eukaryotic (B) biofilm composition, explained by GDM.** Comparisons were restricted to samples taken at the same time in our study (same year, same season) to control for temporal effects. Dissimilarity was measured with the Bray-Curtis index. Same abbreviations as in Figure 2, with "Geo" (geographical distance between samples) instead of time.

## 3.4 Discussion

We studied the composition of prokaryotic and micro-eukaryotic assemblages of benthic biofilm communities in 26 mountain lakes located in the French Pyrenees from 2016 to 2020 and linked it to climate and environmental factors. First, we showed that, since 1950, climate change has changed drastically in the Pyrenean mountain range, but not homogenously between eastern and western lakes, while important water chemistry variables such as hardness have generally increased in most lakes, regardless of these spatial changes in climatic variables. Our study revealed that successions of prokaryotic and micro-eukaryotic assemblages in benthic biofilms have diverged significantly in most sites over the course of our five-year study, with a significant increase in the richness and proportion of toxigenic cyanobacteria relative to other prokaryotes and a decrease in the richness and proportion of diatoms relative to other micro-eukaryotes. Community divergence and loss of biodiversity over time were generally more pronounced for prokaryotes than for micro-eukaryotes, consistent with previous studies showing that global change factors do not systematically lead to a decline in microbial diversity but almost ineluctably to pronounced shifts in community composition and structure (Guo *et al.* 2018; Zhou *et al.* 2020b). Because we also found that water acidity and hardness were the main determinants of benthic biofilm community composition, our results suggest that the ongoing modifications in lake water chemistry in the context of global change have caused

these structural shifts in biofilm communities of Pyrenean mountain lakes. While the presence and rise in richness and relative abundance of potentially toxigenic cyanobacteria does not necessarily mean an increase in toxin production, it does indicate profound disturbances at the ecosystem level with potentially increased health risks. Combined with a decline of sensitive taxa such as diatoms, indicators of high-water quality, this supports the idea that ongoing global change in the Pyrenees is leading to important alterations in microbial communities of benthic biofilms in mountain lakes, with indication of structural shifts toward new ecological states (Oleksy *et al.* 2020a, 2020b). We see here strong evidence of declining ecosystem health of mountain lakes in the Pyrenees. Such changes in biofilms may result in a reduction in water quality with profound negative consequences for aquatic food webs, animal and human health, as well as local economies, and shed light on the possible impacts of ongoing global change in lakes of other mountain ranges around the world.

The relative increase in potentially toxigenic benthic cyanobacteria observed in our study is of particular concern for water quality as cyanotoxin poisoning presents a non-negligible risk for humans and animals (Roegner *et al.* 2014; Huisman *et al.* 2018). Most studies and monitoring to date have focused on toxins from planktonic cyanobacteria (Huisman *et al.* 2018), and our results are consistent with global trends in lake phytoplankton: cyanobacteria blooms and toxin production build up as climate and anthropogenic pressures on water bodies increase (Huisman *et al.* 2018; Ho *et al.* 2019; Hayes *et al.* 2020). In our benthic biofilms, we observed a high proportion of *Phormidium* and, to a lesser extent *Oscillatoria*, two cyanobacteria genera responsible for numerous animal deaths following biofilm and/or cyanotoxin ingestion in French mountain areas (Mez *et al.* 1997; Gugger *et al.* 2005; **Figure 24**). *Phormidium* and *Oscillatoria* species are able to produce microcystins, anatoxin-a and cylindrospermopsin (ANSES, 2020). These cyanotoxins are known to have adverse health effects that depend on the toxin and target species (Dodds and Whiles 2020). However, in humans, acute exposure induces various digestive, respiratory and/or neurological disorders that can lead to death, while chronic exposure is linked with an increased incidence of liver cancer (Roegner *et al.* 2014; Colas *et al.* 2021). Symptoms are similar in other vertebrates, and mass mortalities due to cyanotoxin poisoning have been reported in multiple occasions for wild animals (Roegner *et al.* 2014). Mountain lakes are popular recreational areas and often the sole source of water for livestock in mountain pastures (Mayer *et al.* 2022; Schmeller *et al.* 2022). Therefore, our findings have considerable implications for public health as well as local economies, including tourism and farming, in the Pyrenees. Locals and tourists (e.g. anglers and hikers) may be orally exposed to cyanotoxin poisoning by drinking contaminated water, eating fish, or unintentionally ingesting water during recreational activities (Buratti *et al.* 2017).

The potential presence of cyanotoxins would also decrease the water quality of mountain lake water and would ultimately raise the costs inherent in drinking water production. Temporal and spatial patterns of cyanotoxin concentrations, as well as of both planktonic and biofilm cyanobacteria abundance, are therefore urgently needed to better predict the risks cyanobacteria may pose to the health of humans, animals and mountain ecosystems as a whole.

In addition to the potential toxic risks associated with cyanobacteria, the observed changes in mountain lake biofilms may also have nutritional consequences due to the relative decline in diatoms. Diatoms are a high-quality food resource in biofilms (Brett and Müller-Navarra 1997; Torres-Ruiz *et al.* 2007; Guo *et al.* 2015). Few organisms can synthetise these nutrients and most terrestrial vertebrates, for example, are consequently directly or indirectly dependent on aquatic ecosystems to obtain PUFA (Hixson *et al.* 2015). Previous work has shown that a decline in diatoms combined with an increase in cyanobacteria, following exposure to stressors (phosphorus and silver), led to biofilms with different PUFA profiles which, in turn, negatively affected the growth and survival of *Gammarus fossarum*, a crustacean biofilm grazer (Crenier *et al.* 2019). In our study system, the observed decline in diatoms, combined with the increase in cyanobacteria, could therefore reduce the availability of these fatty acids with difficult to predict, adverse cascading effects on all organisms in aquatic food webs and beyond.

Our study indicates that climate change has been pronounced in the Pyrenees over the last decades, with various direct and indirect impacts on mountain lake biofilms. High recent temperatures prior to sampling negatively affected diatom richness and relative abundance and spatial (between-lake) variation in prokaryotic assemblages. Warm, dry winters were also associated with lower richness of cyanobacteria, toxigenic cyanobacteria and diatoms. The different climatic conditions between western and eastern gradients, although exacerbated by climate change, do not appear to drive the spatial dissimilarity in biofilm composition, unlike pH and hardness. Climate change could also indirectly impact microbial biofilms through modifications of lake water chemistry due to altered erosion rates, cryosphere melt and consequent water mixing regimes (Koinig *et al.* 1998; Catalan *et al.* 2013; Woolway *et al.* 2020). Depending on the lithology of the catchment and the initial levels of alkalinity and base cations, water pH can decrease (acidification; Marchetto *et al.* 1995; Skjelkvåle and Wright 1998; Camarero *et al.* 2009) or increase (basification; Curtis *et al.* 2009; Jeannin *et al.* 2016). In the Pyrenees, sediment analysis showed that diatom-inferred pH has increased in the last century, probably because of intensified climate-induced rock weathering (Curtis *et al.* 2009). Rock weathering has also been associated with a significant increase in water hardness and conductivity in other alpine lakes in the last three decades (Rogora *et al.* 2020). Our study

confirms these relationships and identifies pH and water hardness as the main determinants of biofilm microbial composition. Hardness and pH had significant effects on micro-eukaryotic α-diversity, notably on diatom richness and relative abundance, and were the main drivers of spatial dissimilarity and temporal dissimilarity in biofilm compositions.

Our results suggest that, if climate-change induced basification and changes in water hardness continue to occur in Pyrenean lakes, the prokaryotic and micro-eukaryotic assemblages of microbial biofilm communities will further change. Together with other factors, increasing temperatures, chemical pollution (Machate *et al.* 2022), eutrophication through livestock and atmospheric deposition (Camarero *et al.* 2009; Oleksy *et al.* 2020a; Mayer *et al.* 2022), invasive species and fish introductions (Gacia *et al.* 2018; Moser *et al.* 2019), we expect to see a further rise in benthic cyanobacteria, including toxigenic ones. Future studies need to assess other aspects of biofilms, e.g. changes in their biomass or in EPS, to determine when blooms of toxigenic benthic cyanobacteria may occur. Furthermore, when mountain lakes experience substantial benthic cyanobacteria blooms (Kravtsova *et al.* 2014; Vadeboncoeur *et al.* 2020), regardless of their toxicity, oxygen levels at the sediment-water interface and even in the water column can drop dramatically, leading to hypoxia or even anoxia. Oxygen deprivation has profound consequences for aquatic food webs, as all higher trophic levels are affected and even shifts in prokaryotic communities and their functions can be expected (Brune *et al.* 2000; Schuster *et al.* 2021). Additionally, dense phytoplankton or benthic filamentous blooms may decrease light penetration to the bottom and further impact benthic flora and fauna with cascading effects to the entire lake ecosystem (Brothers *et al.* 2013).

In conclusion, our study indicates that the quality of biofilm and water in French Pyrenean mountain lakes has rapidly decreased in the recent past, increasing health risks for livestock, humans and wild animals. Therefore, mitigating the effects of global change on mountain aquatic ecosystems, in particular on their benthic microbial communities, must be considered a top priority in order to avoid serious health risks for billions of people worldwide and to protect mountain environments as an important component of our global life support system.

## Supplementary materials for Chapter 3

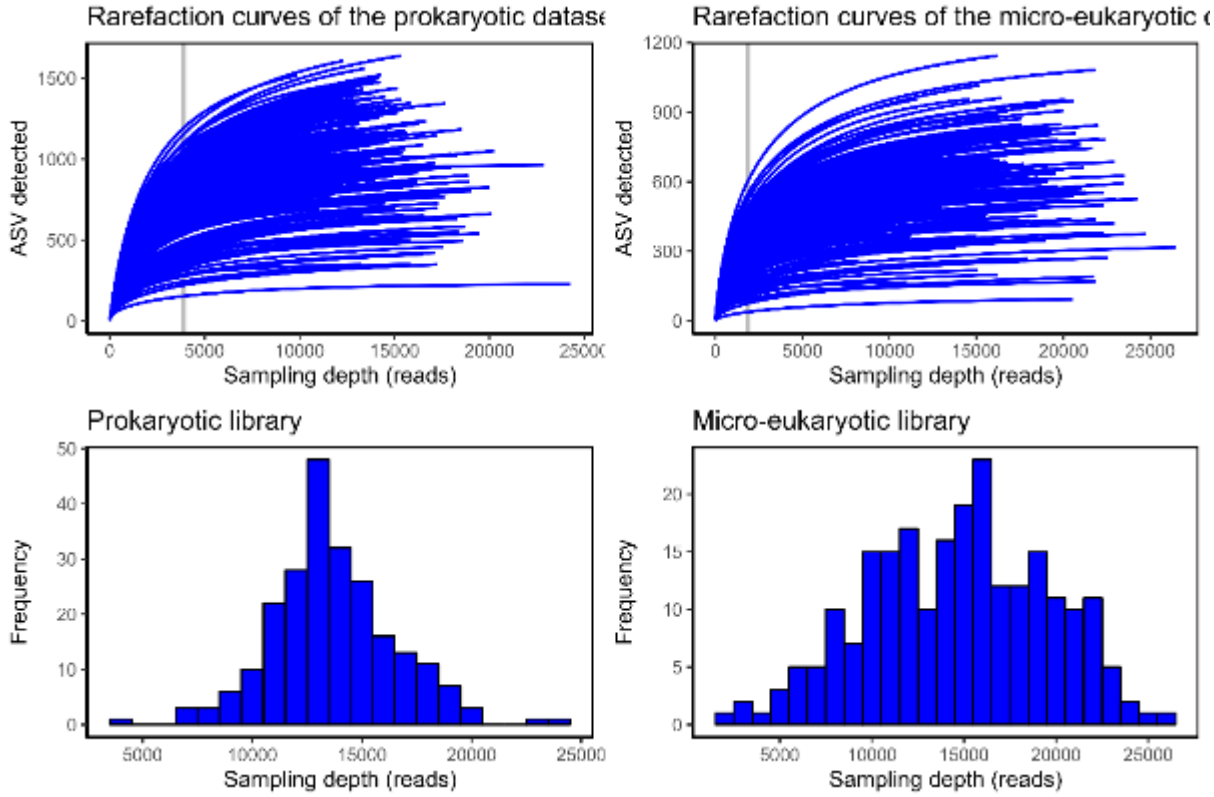



**Figure 18: Rarefaction curves (upper) and histograms (lower) of the prokaryotic and micro-eukaryotic datasets (left and right respectively).** Grey vertical lines indicate the depth used for rarefaction (3897 and 1856 reads, respectively). Four and six samples, inferior to the thresholds, were discarded.

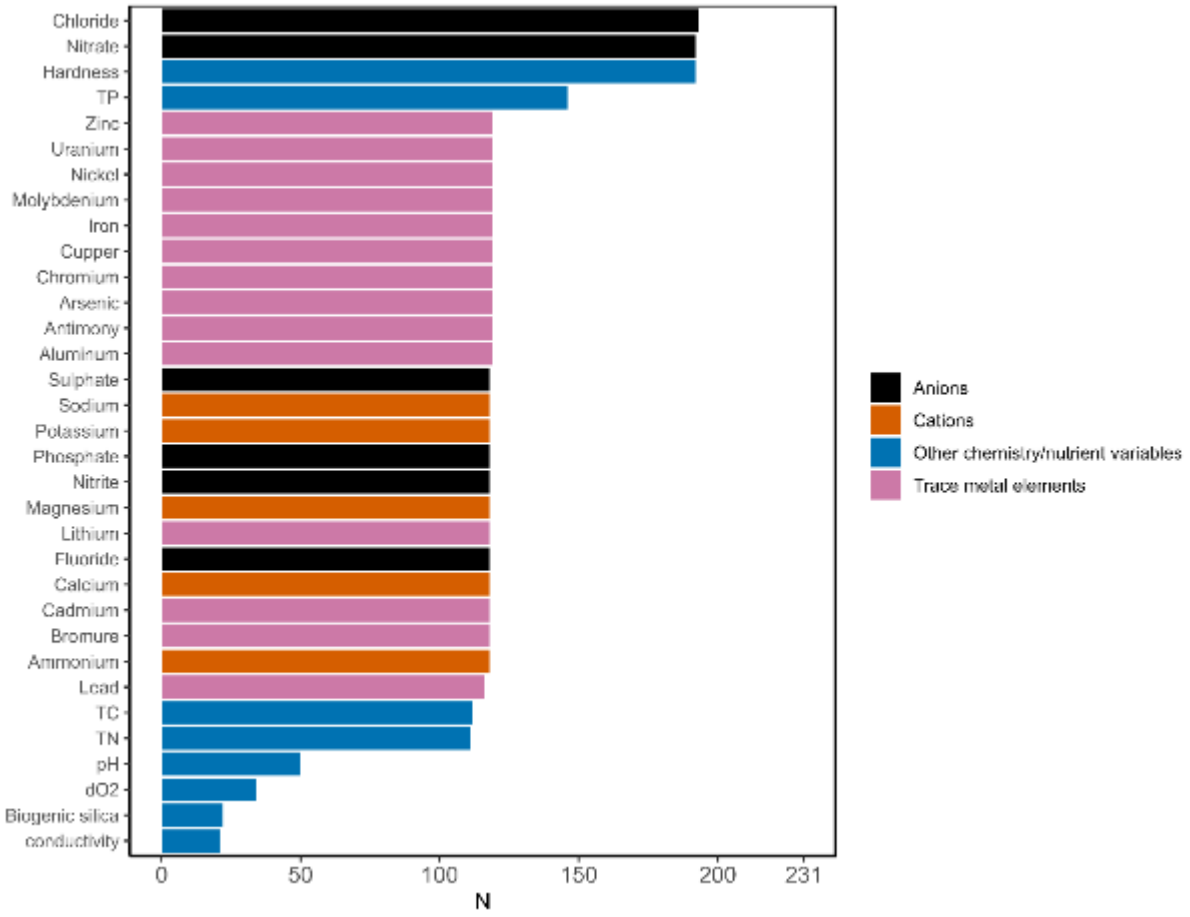



**Figure 19: Number of measurements of water chemistry variables in this study.** Abbreviations: TP: total phosphorus, TC: Total Organic Carbon, TN: Total Nitrogen, dO2: dissolved oxygen

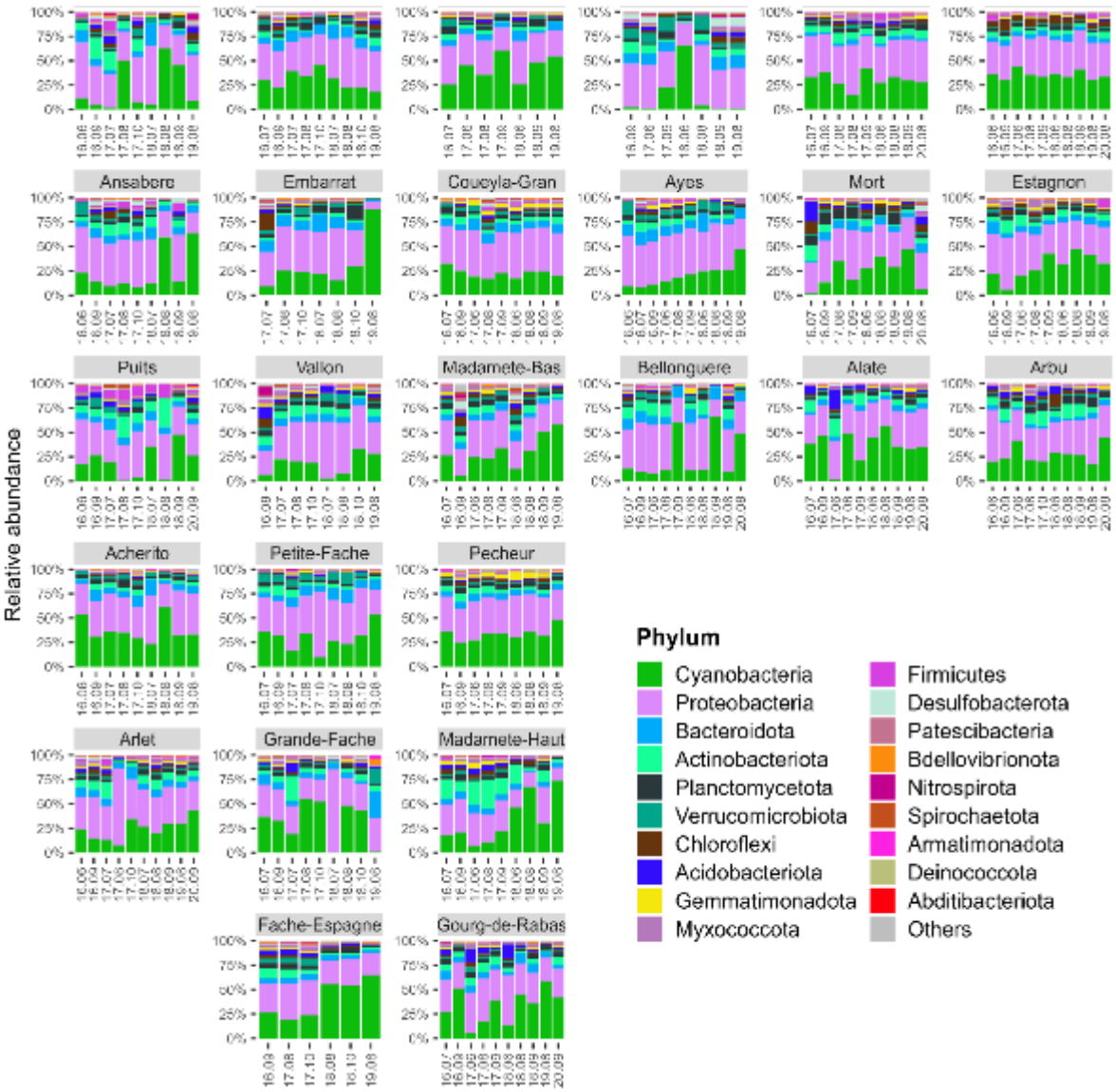



**Figure 20: Composition bar plot (relative abundance) of the prokaryotic biofilm assemblages (determined by metabarcoding of the 16S rRNA gene) of the 231 biofilm samples sequenced in this study, represented at the Phylum level.** Each facet is a lake with all its sample (sorted in chronological order: dates on the x-axis, 16.06 means June 2016), and each column contains all the lakes of a same gradient, sorted by increasing elevation (top = lowest). The 19 most abundant phyla were selected with this plot (the 25 others are grouped in the category "Others"). Phyla are sorted by total abundance from bottom to top of each barplot, with legends arranged accordingly.

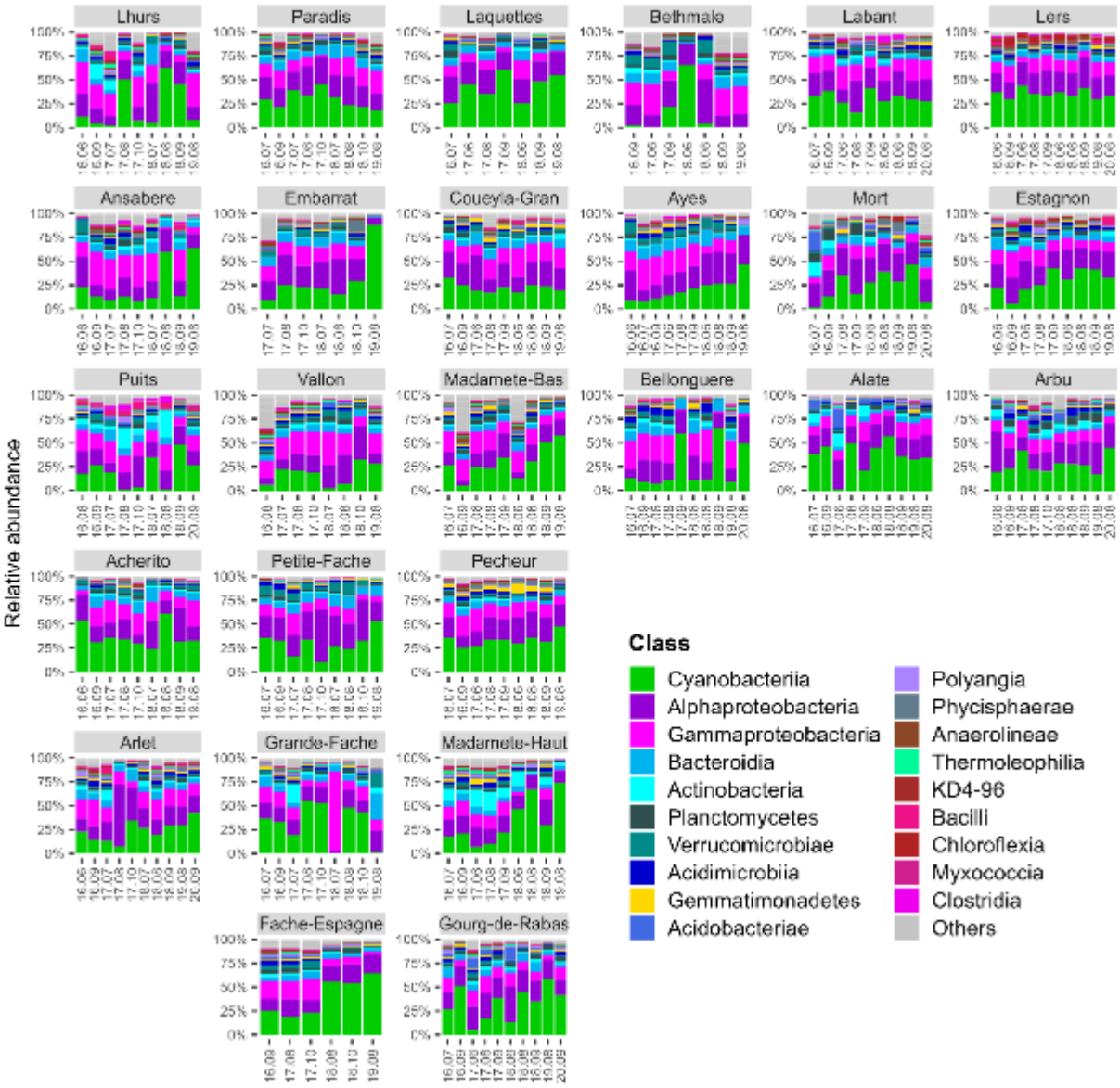



**Figure 21: Same plot as Figure 20 but represented at the Class level. The 19 most abundant classes were selected in this plot (the 117 other classes were grouped in "Others" and coloured in grey).** Classes are sorted by total abundance from bottom to top of the bar plot, with legends arranged accordingly. As much as possible, classes belonging to a certain Phylum tend to have similar colours that this Phylum has in **Figure 20**.

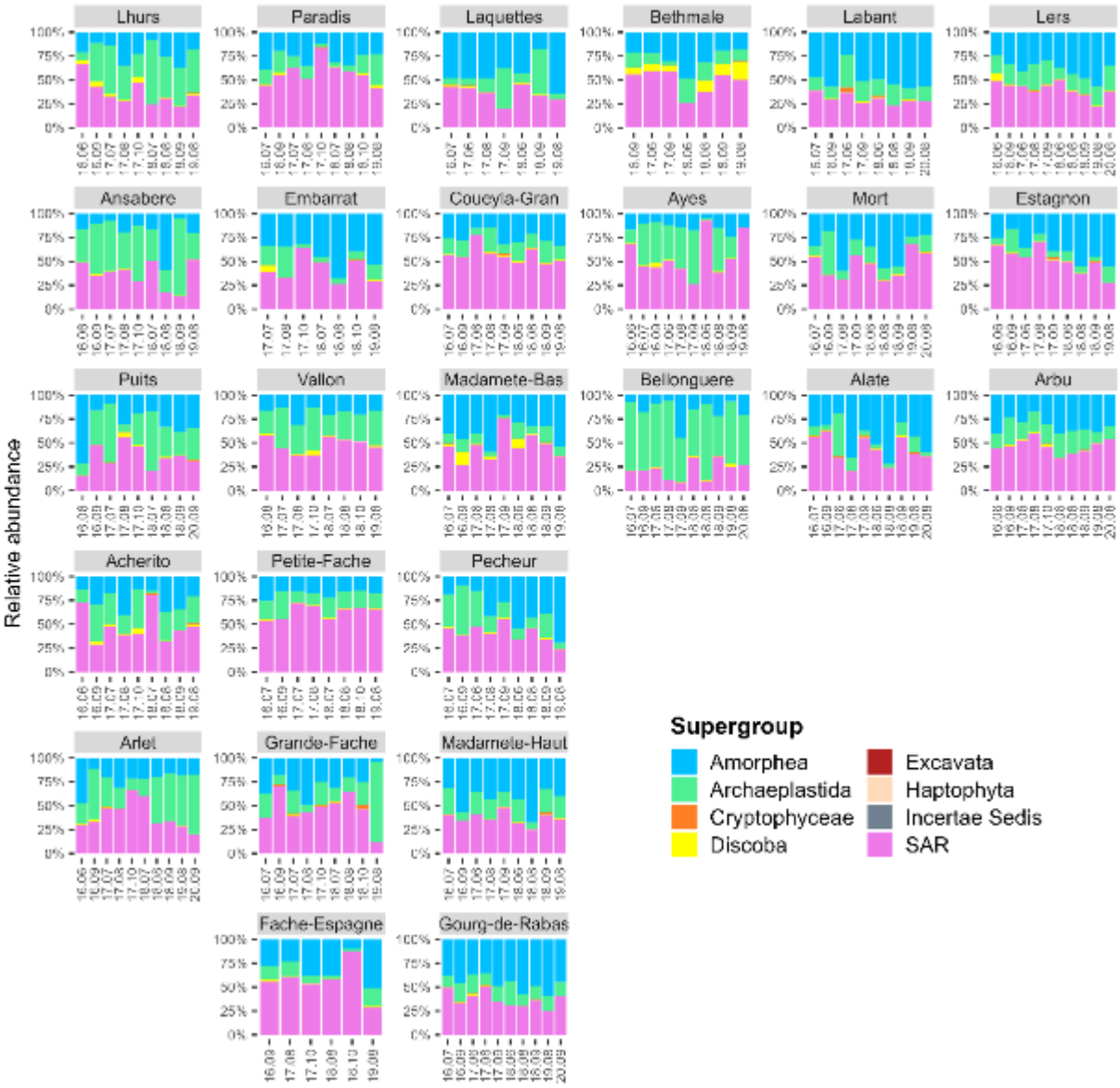



**Figure 22: Composition bar plot (relative abundance) of the micro-eukaryotic biofilm assemblages (determined by metabarcoding the 18S rRNA gene) of the 229 biofilm samples sequenced in this study (two-bad-quality samples were removed compared to the previous plots), represented at the Supergroup level.** Each facet is a lake with all its sample (sorted in chronological order), and each column contains all the lakes of a same gradient)

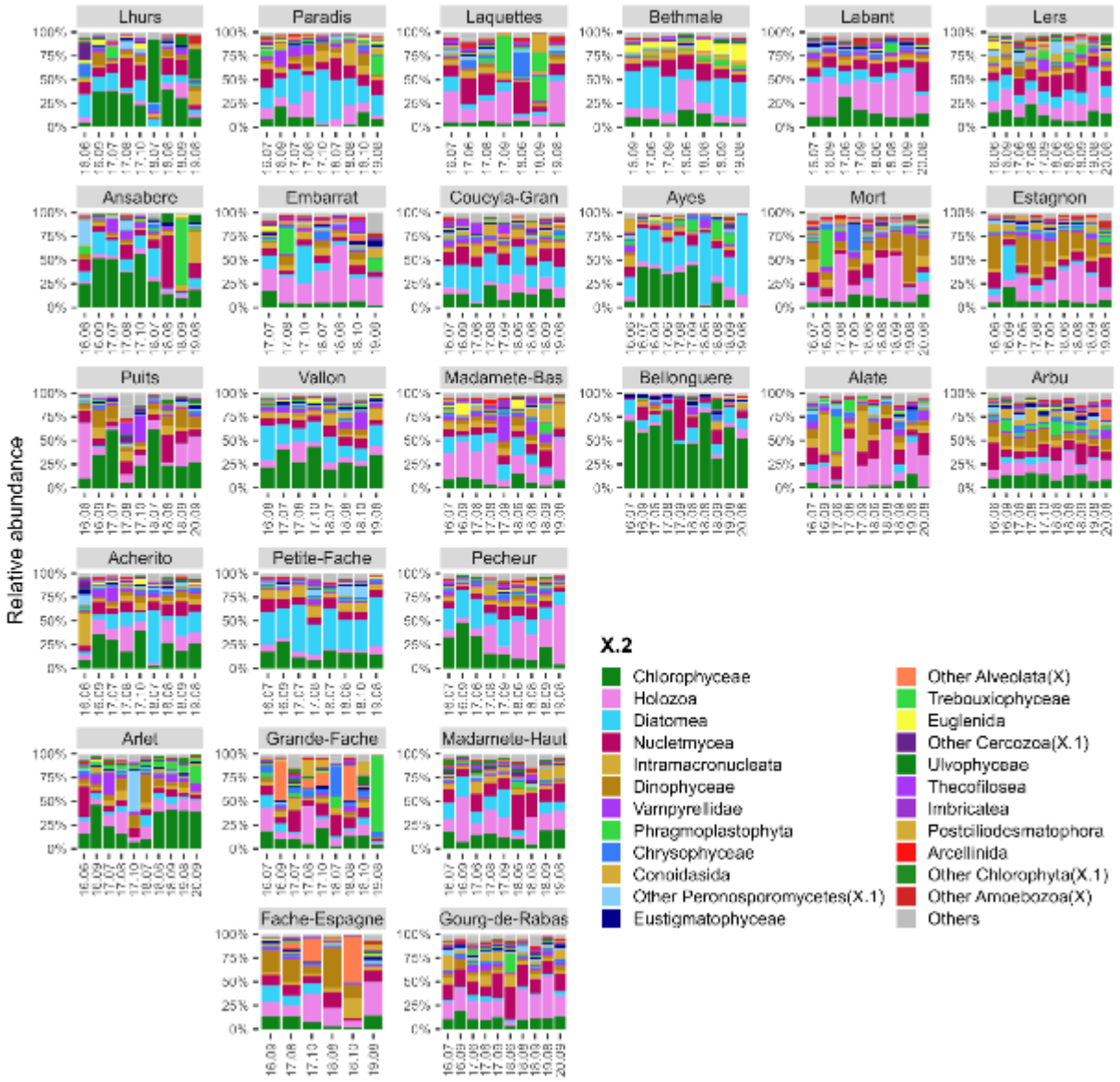
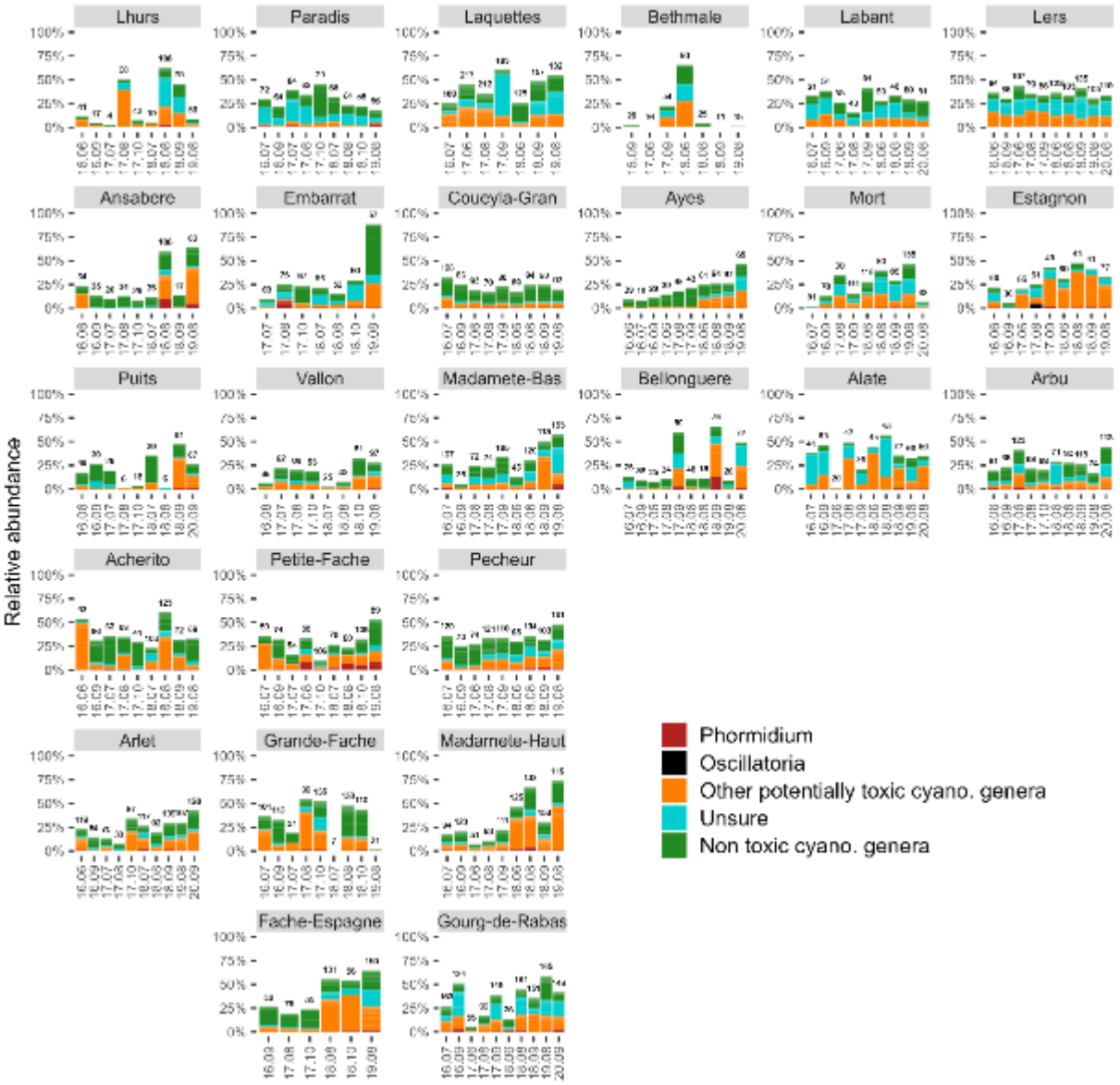



**Figure 23: Same bar plot as in Figure 22 but coloured at a deeper taxonomic resolution (X.2 level).** Taxa are sorted by total abundance from bottom to top of the barplot, with legends arranged accordingly. All plant organisms (supergroup Archaeplastida) are in shades of green (Chlorophyceae being the most abundant); Amorphea are in shades of pink/red with Holozoa (containing animals), Nucletmycea (Fungi) and Amoebozoa; all Alveolates are in shades of brown/coral, all Stramenopiles in shades of blue (including Diatoms in cyan), and Rhizaria in shades of purple; finally, the only member of Discobas (Euglenida) is in yellow. Only the top 25 taxa are displayed, the 79 others are grouped in category "Others". Some ASVs were not defined up to the X.2 level, therefore, we classified them as 'Other- last defined level' (shown in brackets in the legends).




**<u>Figure 24:</u> Bar plot showing the relative abundance and richness of all cyanobacteria in prokaryotic assemblages, coloured in function to the known ability of their genera to produce cyanotoxins. Genera *Phormidium* and *Oscillatoria* were also specifically coloured as they were identified, while living in biofilms, to be the cause of animal deaths in French mountain areas.** The category "Unsure" denotes cyanobacteria ASVs undefined at the genus level, but the family of which may produce cyanotoxins. The number above each bar represents the number of cyanobacteria ASVs detected in each sample. Facets are arranged in the same way as in previous figures.

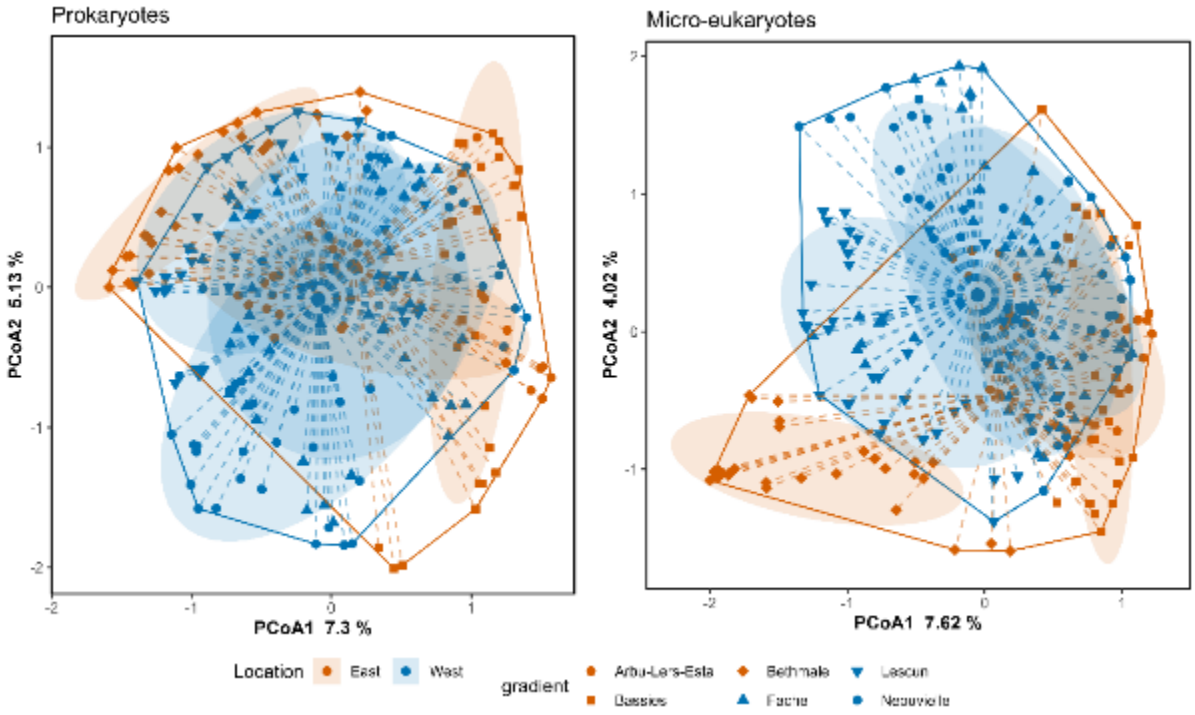



**Figure 25: Two-dimensional ordination of Principal Coordinate Analysis (PCoA) of prokaryotic and micro-eukaryotic assemblages of biofilm microbial communities from mountain lakes.**

**Table 5: Dates of sample collection for each lake**. All samples were taken in summer (early = campaign 1, i.e before mid-July: mid = campaign 2, i.e. between mid-July and end of August; late = campaign, i.e. September onwards). Samples in red were excluded from both prokaryotic and micro-eukaryotic libraries; in brown, samples excluded from the micro-eukaryotic library (unsufficient sampling depth).

| Lake | season | 2016 | 2017 | 2018 | 2019 | 2020 |
|---|---|---|---|---|---|---|
| Arbu | Early | 30/06/2016 | 14/06/2017 | 24/06/2018 | | |
| | Mid | | 08/08/2017 | 03/08/2018 | 08/08/2019 | 25/08/2020 |
| | Late | 08/09/2016 | 03/10/2017 | 22/09/2018 | | |
| Lers | Early | 30/06/2016 | 14/06/2017 | 24/06/2018 | | |
| | Mid | | 08/08/2017 | 03/08/2018 | 08/08/2019 | 25/08/2020 |
| | Late | 08/09/2016 | 27/09/2017 | 22/09/2018 | | |
| Estagnon | Early | 30/06/2016 | 14/06/2017 | 24/06/2018 | | |
| | Mid | | 08/08/2017 | 03/08/2018 | 08/08/2019 | |
| | Late | 08/09/2016 | 27/09/2017 | 22/09/2018 | | |
| Labant | Early | 18/07/2016 | 17/06/2017 | 18/06/2018 | | |
| | Mid | | 17/08/2017 | 10/08/2018 | | 25/08/2020 |
| | Late | 27/09/2016 | 27/09/2017 | 20/09/2018 | | |
| Alate | Early | 18/07/2016 | 09/06/2017 | 18/06/2018 | | |
| | Mid | | 17/08/2017 | 10/08/2018 | 10/08/2019 | 25/08/2020 |
| | Late | 27/09/2016 | 27/09/2017 | 20/09/2018 | | |
| Mort | Early | 18/07/2016 | | 18/06/2018 | | |
| | Mid | | 17/08/2017 | 10/08/2018 | 10/08/2019 | 25/08/2020 |
| | Late | 27/09/2016 | 27/09/2017 | 20/09/2018 | | |
| Bellonguere | Early | | 15/06/2017 | 25/06/2018 | | |
| | Mid | 25/07/2016 | 09/08/2017 | 04/08/2018 | 01/08/2019 | 27/08/2020 |
| | Late | 09/09/2016 | 26/09/2017 | 21/09/2018 | | |
| Ayes | Early | 01/06/2016 | 15/06/2017 | 25/06/2018 | | |
| | Mid | 25/07/2016 | 09/08/2017 | 04/08/2018 | 01/08/2019 | |
| | Late | 09/09/2016 | 26/09/2017 | 21/09/2018 | | |
| Bethmale | Early | | 15/06/2017 | 25/06/2018 | | |
| | Mid | | 09/08/2017 | 04/08/2018 | 01/08/2019 | |
| | Late | 10/09/2016 | 26/09/2017 | 21/09/2018 | | |
| Laquettes | Early | | 21/06/2017 | 27/06/2018 | | |
| | Mid | 26/07/2016 | 10/08/2017 | | 16/08/2019 | |
| | Late | | 25/09/2017 | 24/09/2018 | | |
| Gourg de Rabas | Early | | 21/06/2017 | 27/06/2018 | | |
| | Mid | 26/07/2016 | 10/08/2017 | 06/08/2018 | 16/08/2019 | |
| | Late | 12/09/2016 | 25/09/2017 | 24/09/2018 | | 08/09/2020 |
| Madamete Haut | Early | | 21/06/2017 | 27/06/2018 | | |
| | Mid | 26/07/2016 | 10/08/2017 | 06/08/2018 | 16/08/2019 | |
| | Late | 12/09/2016 | 25/09/2017 | 24/09/2018 | | |
| Madamete Bas | Early | | 21/06/2017 | 27/06/2018 | | |
| | Mid | 26/07/2016 | 10/08/2017 | 06/08/2018 | 16/08/2019 | |
| | Late | 12/09/2016 | 25/09/2017 | 24/09/2018 | | |
| Pecheur | Early | | 21/06/2017 | 27/06/2018 | | |
| | Mid | 26/07/2016 | 10/08/2017 | 06/08/2018 | 16/08/2019 | |
| | Late | 12/09/2016 | 25/09/2017 | 24/09/2018 | | |
| Coueyla Gran | Early | | 21/06/2017 | 27/06/2018 | | |
| | Mid | 26/07/2016 | 10/08/2017 | 06/08/2018 | 16/08/2019 | |
| | Late | 12/09/2016 | 25/09/2017 | 24/09/2018 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Paradis | Early | 07/07/2016 | 05/07/2017 | 05/07/2018 | | |
| | Mid | 06/09/2016 | 20/08/2017 | 11/08/2018 | 23/08/2019 | |
| | Late | | 06/10/2017 | 01/10/2018 | | |
| Embarrat | Early | | 07/07/2017 | 06/07/2018 | | |
| | Mid | | 21/08/2017 | 12/08/2018 | 22/08/2019 | |
| | Late | | 06/10/2017 | 01/10/2018 | | |
| Vallon | Early | | 07/07/2017 | 06/07/2018 | | |
| | Mid | 03/08/2016 | 21/08/2017 | 12/08/2018 | 22/08/2019 | |
| | Late | | 06/10/2017 | 01/10/2018 | | |
| Petite Fache | Early | 07/07/2016 | 06/07/2017 | 06/07/2018 | | |
| | Mid | 06/09/2016 | 21/08/2017 | 12/08/2018 | 23/08/2019 | |
| | Late | | 06/10/2017 | 02/10/2018 | | |
| Grande Fache | Early | 07/07/2016 | 06/07/2017 | 06/07/2018 | | |
| | Mid | 06/09/2016 | 21/08/2017 | 12/08/2018 | 23/08/2019 | |
| | Late | | 06/10/2017 | 02/10/2018 | | |
| Fache Espagne | Mid | 06/09/2016 | 21/08/2017 | 12/08/2018 | 23/08/2019 | |
| | Late | | 06/10/2017 | 02/10/2018 | | |
| Lhurs | Early | 21/06/2016 | 03/07/2017 | 02/07/2018 | | |
| | Mid | | 13/08/2017 | 14/08/2018 | 04/08/2019 | |
| | Late | 19/09/2016 | 08/10/2017 | 26/09/2018 | | |
| Ansabere | Early | 22/06/2016 | 04/07/2017 | 03/07/2018 | | |
| | Mid | | 14/08/2017 | 15/08/2018 | 02/08/2019 | |
| | Late | 20/09/2016 | 09/10/2017 | 27/09/2018 | | |
| Acherito | Early | 22/06/2016 | 04/07/2017 | 03/07/2018 | | |
| | Mid | | 14/08/2017 | 15/08/2018 | 02/08/2019 | |
| | Late | 20/09/2016 | 09/10/2017 | 27/09/2018 | | |
| Puits d'Arrious | Early | 23/06/2016 | 04/07/2017 | 03/07/2018 | | |
| | Mid | | 15/08/2017 | 15/08/2018 | | 03/09/2020 |
| | Late | 21/09/2016 | 09/10/2017 | 27/09/2018 | | |
| Arlet | Early | 23/06/2016 | 04/07/2017 | 03/07/2018 | | |
| | Mid | | 15/08/2017 | 15/08/2018 | 03/08/2019 | 03/09/2020 |
| | Late | 21/09/2016 | 09/10/2017 | 27/09/2018 | | |

# CHAPTER 4

# FIRST INSIGHTS INTO THE LINKS BETWEEN BENTHIC BIOFILMS AND THE EPIDEMIOLOGY OF *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTIONS IN THE PYRENEES

## Foreword of chapter 4

Here, I used data from the previous chapter to explore whether the composition of prokaryotic and micro-eukaryotic assemblages of benthic biofilms could play a role in the epidemiology of Bd infections or the impact of amphibian chytridiomycosis on Ao populations. I defined epidemiologically different categories of Ao populations (lakes) and compared them in pairs, using a systematic approach. For each pairwise comparison, I compared the α-diversity, β-diversity, and differentially-abundant taxa, as well as the abundance of functionally important taxa (e.g. toxigenic cyanobacteria or potential predators). Biofilms from lakes with less infected or impacted populations tended to harbour more potential consumers of Bd and/or Bd-inhibitory bacteria. This means that biofilms have the potential to be a relevant epidemiology factor influencing Bd infection dynamics. However, further analyses of this dataset, as well as other approaches, are needed to definitely answer the question. I wish to point out that this is chapter for which I had the least time. As a result, it is less accomplished than others.

# Chapter 4: First insights into the links between benthic biofilms and the epidemiology of *Batrachochytrium dendrobatidis* infections in the Pyrenees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- Contribution:

I formulated ideas, performed laboratory work (biofilm DNA extraction and Bd infection data of 2019 and 2020), data analysis and wrote the first draft of this manuscript. A.L. and D.S.S. collected data and performed the rest of the laboratory work. All authors contributed to this version.

## Abstract


*Batrachochytrium dendrobatidis* (Bd), causative agent of the panzootic amphibian chytridiomycosis, is widespread in France, but disease-driven declines have been described in populations of the common-midwife toad *Alytes obstetricans* (Ao) only in some montane lakes of the Pyrenees. The reasons why Bd infections and disease impacts are site-specific are not yet fully understood. Here, we investigate whether benthic biofilms, essential components of these lakes, are an important factor in epidemiological processes. To this end, we used small subunit (SSU) rRNA metabarcoding and Bd infection data from 2008 to 2020 to compare the composition of prokaryotic and micro-eukaryotic assemblages between biofilms sampled in lakes with Ao populations exhibiting different disease dynamics. We performed four sets of comparisons of biofilms from lakes with Ao populations: i) systematically infected but stable vs. declining; (ii) systematically infected vs. sporadically infected; (iii) systematically infected vs. uninfected; (iv) sporadically infected vs. uninfected. For each pairwise comparisons, we compared the α-diversity, β-diversity, the relative abundance of functionally important taxa (toxigenic cyanobacteria, nutritious diatoms, and potential Bd consumers), and differentially abundant taxa identified by linear discriminant analysis effect size (LEfSe) between groups.

Neither the α-diversity of prokaryotic and micro-eukaryotic biofilm assemblages, nor the relative abundance of diatoms, toxigenic cyanobacteria or potential Bd consumers, significantly differed between the two groups of each pairwise comparisons. However, we found that biofilm assemblages differed in terms of β-diversity in cases (i) and (ii), with multiple differentially abundant taxa. Some of these, more abundant in less infected/impacted lakes, are known to be either Bd-inhibitory on the amphibian skin or *in vitro*, or to be consumer of Bd. Of particular interest are sessile rotifers, previously identified as effective Bd consumer in the plankton of the study lakes, which were here more abundant in less impacted lakes. Therefore, our results suggest that benthic biofilms could contribute to the frequency and impact of Bd infections, and call for further studies to confirm and better explain the mechanisms involved.

## 4.1 Introduction

Widlife **emerging infectious diseases** are a threat to biodiversity and in particular to amphibians (Daszak *et al.* 2000, 2003; Fisher *et al.* 2012). This is mainly due to the **panzootic** amphibian chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd, Berger *et al.* 1998; Longcore *et al.* 1999; Scheele *et al.* 2019; Fisher and Garner 2020). Amphibian chytridiomycosis was shown to be implicated in the declines of more than 500 species and 90 species extinctions, making it the worst infectious disease in terms of biodiversity attrition (Scheele *et al.* 2019). It is generally recognised that Bd infection and chytridiomycosis dynamics are regulated by four interconnected categories of factors: host-related (species susceptibility, individual immunity etc), host-microbiome-related (composition, diversity), pathogen-related (e.g. strain virulence), and environment-related factors (Bernardo-Cravo *et al.* 2020). For instance, **susceptibility** depends on host species identity and life stage (Tobler and Schmidt 2010; Baláž *et al.* 2014). Different Bd lineages and strains have also different **virulence** (Farrer *et al.* 2011; Dang *et al.* 2017). Abiotic environmental conditions like temperature have a huge influence on Bd and the hosts (Piotrowski *et al.* 2004; Ribas *et al.* 2009). Biotic factors have been shown to explain the epidemiology of Bd infections in some systems, as the infective stage of Bd, the zoospore,[2] is free-living and can be consumed in the aquatic medium, thus reducing infection pressure (Buck *et al.* 2011; Searle *et al.* 2013; Schmeller *et al.* 2014; De Troyer *et al.* 2021). Finally, host microbiomes, in particular the skin microbiome, have been demonstrated to play an important role in infection prevention and disease severity (Bernardo-Cravo *et al.* 2020; Fisher and Garner 2020).

In Europe, the impacts of amphibian chytridiomycosis caused by Bd have not been as dramatic as in the Americas and Australia (Scheele *et al.* 2019). Infections by Bd are widespread but disease-related mass mortalities appear to be limited to mountain populations of susceptible host species (Walker *et al.* 2010). In particular, the common-midwife toad, *Alytes obstetricans* (Ao), has proved highly susceptible to amphibian chytridiomycosis and some of its population have declined in the French Pyrenees (Clare 2014; Clare *et al.* 2016; Bates *et al.* 2018). Individuals of this species, especially tadpoles, serve as sentinels for monitoring Bd infection: tadpoles are very efficient reservoirs, rarely die from infection unlike metamorphosing and metamorphosed individuals, and their tendency to delay metamorphosis for up to three years at alttitude allows Bd to be maintained over time (Walker *et al.* 2010; Clare *et al.* 2016). Second, disease outbreaks have been shown to be associated with altitude, showing the importance of temperature (Walker *et al.* 2010).

Infections are geographically clustered in the Pyrenees. Infections mainly cluster in lakes around Lescun in Vallée d'Aspe (western Pyrenees), where chytridiomycosis was detected for the first time on Ao in 2002 at lake Acherito. Later, Bd infections were detected in some lakes of both the Neouvielle and Cauterets massifs in the central Pyrenees, but only sporadically. More recently, Ao have been found infected in a lake near Bethmale in the eastern Pyrenees. Zooplankton composition, and more specifically, the presence of Bd consumers such as rotifers and ciliates could explain variation in prevalence (Schmeller *et al.* 2014). *Alytes obstetricans* have been found infected almost each year near Lescun since the monitoring began in 2008. This explains why most of what is known of Bd infection epidemiology comes from this place. Even there, where lakes are located approximately at a same altitude and have a similar climate, different disease impacts on populations are observed with some still declining (in lakes Arlet and Ansabere) while other populations have resurged and are now stable (lakes Acherito, Lhurs, Puits d'Arrious; Bates *et al.* 2018). Populations of these sites were also shown to have different skin bacterial composition, with stable Ao populations having Bd inhibitory taxa and generally a more diverse microbiota on their skin (Bates *et al.* 2018). A same strain of Bd, with supposedly the same virulence, appear to infect all populations (Bd GPL IA043, Bates *et al.* 2018). It is not yet known if Ao populations of these lakes are differentially susceptible, e.g. based on their genetics. However, that populations of Acherito and Ansabere are differentially impacted, while likely being able to exchange genes through migration (the two lakes are very close), does not support this hypothesis. Therefore, it is worth investigating the importance of other biotic environmental factors such as benthic biofilms.

In shallow mountain lakes, most microorganisms live in benthic matrix-enclosed communities called biofilms. This diverse communities can dominate lake ecosystems in terms

of biomass, and form the basis of their food-webs (Sentenac *et al.* 2022). Tadpoles of Ao extensively feed on benthic biofilms (Altig and Johnston 1989; Altig *et al.* 2007). Biofilms are also known to interact with plant and human pathogens (Wingender and Flemming 2011; Sentenac *et al.* 2022). Thus, benthic biofilms may impact *Bd* infection dynamics through the following mechanisms, among others. First, biofilms could impact the health and immune system of the hosts (amphibians) via trophism (mechanism 1), depending on their nutritional quality or toxicants they contain. Diatoms are considered a very high-quality food resource for biofilm grazer, whereas cyanobacteria are considered a medium-to-low food resource, as they contain fewer polyunsaturated fatty acids (PUFA) essential for a competent immune system (Brett and Müller-Navarra 1997; Guo *et al.* 2015; Hixson *et al.* 2015; Crenier *et al.* 2019). Biofilms can concentrate pollutants and cyanobacteria in the biofilm can contain or secrete cyanotoxins within the matrix, which may also negatively impact consumer health (Quiblier *et al.* 2013; Bonnineau *et al.* 2020; Mahler *et al.* 2020; Wood *et al.* 2020). Second, biofilm cyanobacteria could directly impact host health without the host consuming the biofilm, as cyanotoxins can be secreted in the water column and have toxic effects via contact only (mechanism 2; Oberemm *et al.* 1999; Dao *et al.* 2010; Quiblier *et al.* 2013; Wood *et al.* 2020). While other biofilm inhabitants also produce molecules e.g. for communication or allelopathy (Wu *et al.* 2011; Allen *et al.* 2016), to our knowledge, only cyanotoxins can adversely affect vertebrate health. Third, biofilms could harbour Bd inhibitors, predators or consumers, such as sessile filter-feeders like ciliates or rotifers (mechanism 3; Eisenmann *et al.* 2001; Mialet *et al.* 2013). Known microbial Bd consumers include some ciliates, rotifers, and tardigrades (and also larger invertebrates) and the presence of such sessile consumers in benthic biofilms may eventually reduce infection pressure to amphibian as observed in the zooplankton (Schmeller *et al.* 2014).

Here, using taxonomic data on benthic biofilm communities and Bd infection data of Ao populations in Pyrenean lakes, we aimed to test whether the composition and diversity of prokaryotic and micro-eukaryotic assemblages differ between epidemiologically contrasting sites and to find evidence for the existence of the above-mentioned mechanisms by which biofilms could explain the epidemiology of Bd infection dynamics. We compared biofilms (i) between lakes with stable Ao populations and lakes with declining ones in Lescun; (ii) between lakes with systematically-infected (in Lescun) and lake with sporadically infected Ao populations (Neouvielle and Cauterets); (iii) between lakes with systematically infected and lakes with uninfected Ao populations; (iv) between lakes with sporadically infected and lakes with uninfected Ao populations. For each pairwise comparison, we first compared the α-diversity and β-diversity, then assessed whether the differentially abundant taxa were potential

Bd inhibitors, predators or consumers (evidence for mechanism 3) or potentially nutritiously important taxa (diatoms or cyanobacteria; mechanism 1), and finally compared the relative abundance of all potential Bd microbial consumers combined (ciliates, rotifers, and tardigrades; mechanism 3) and the relative abundance of known toxigenic cyanobacteria between the two groups (mechanisms 1 and 2).

## 4.2 Materials and Methods

### 4.2.1 Biofilm community data

We used a subset of the biofilm metataxonomic dataset described in Chapter 3, restricted to the 21 lakes where *Alytes obstetricans* (Ao) individuals were sampled for detection of Bd (see next section, **Figure 9**). Briefly, benthic biofilms were sampled on rock at 20-30cm depth from 2016 to 2020 in the French Pyrenees. Taxonomic data were obtained by metabarcoding of the 16S and 18S rRNA genes, which are markers used to determine the composition of prokaryotic and micro-eukaryotic assemblages of microbial communities, respectively. Lakes were visited once, twice or thrice per year: in general, two samples were collected per lake in 2016 (early and late summer), three in 2017 and 2018, and one in 2019 and 2020 (**Table 5**). Taxonomy of Amplicon Sequence Variants (ASVs) of both 16S and 18S rRNA genes was assigned using SINA v1.7.2 and the SILVA 138.1 reference database (Pruesse *et al.* 2012; Quast *et al.* 2013). Note that certain taxa were recently renamed, like Proteobacteria by Pseudomonadota; here, we kept the SILVA nomenclature for consistency. For the prokaryotic library, ASVs unclassified at the class level, or classified as eukaryotes, chloroplasts, or mitochondria were removed using the *phyloseq* package (McMurdie and Holmes 2013) in the R environment (R Core Team 2022). For the micro-eukaryotic library, unclassified ASVs at the superkingdom or superphylum (third taxonomic rank) and Metazoan taxa belonging to Vertebrata, Arthropoda, Platyhelminthes, Annelida, and Mollusca were removed. To normalise sample libraries to the same number of reads, repeated rarefaction to 3897 and 1856 reads was used for the prokaryotic and micro-eukaryotic datasets, respectively.

We used the function avgdist() of package *vegan* (Oksanen *et al.* 2022) with 100 iterations to compute an average Bray-Curtis dissimilarity based on 100 rarefied datasets, and used this as an index for pairwise β-diversity. The Inverse-Simpson index was used to represent α-diversity and calculated with package *microbiome* (Lahti and Shetty 2012). The relative abundance of potential microbial Bd consumers, i.e. ciliates, tardigrades and rotifers, and that of potentially toxigenic cyanobacteria, were computed in R with packages *phyloseq* and *tidyverse-dplyr* (Wickham *et al.* 2022). Toxigenic cyanobacteria genera were identified with

data from Quiblier *et al.* (2013). Visualisation of community data was performed with Principal Coordinate Analysis (PCoA) ordination with packages *vegan*, *ggplot2* (Wickham 2016), *ggvegan* (Simpson 2019) and *ggordiplot* (Quensen 2021). Heatmaps were drawn with *ggplot2* and *tidytext* (Silge and Robinson 2016).

### 4.2.2 *Batrachochytrium dendrobatidis* infection data

We used data from a Pyrenean-wide monitoring survey of Bd infection conducted since 2008 and still ongoing (data from 2021 and 2022 pending). This dataset covers many Pyrenean waterbodies, including the lakes where biofilms were sampled and in which several amphibian species are known to occur. We only used infection data from Ao tadpoles, for their role of sentinels. Each year, at each lake visit, the presence of Ao tadpole was visually assessed, tadpoles gently captured either by hands or nets, and a maximum of 30 tadpoles were sampled for Bd using a dry, sterile rayon-tipped swab (MW100, Medical & Wire Equipment Co$^{TM}$). The swab was rubbed against the keratinized jaw sheaths approximately 10 times. Swabs were immediately frozen on sites on dry ice and stayed so (-25°C freezer) until DNA extraction. Basic rules of biosecurity were applied on all circumstances: a unique pair of gloves was used for each set of tadpoles (from a same lake), and footwear as well as all equipment in contact with water or amphibians were disinfected by spraying Virkon®, away from water bodies, between each lake. Permits for capture were granted by the Direction régionale de l'Environnement, de l'Aménagement et du Logement (DREAL) of regions Occitanie and Nouvelle-Aquitaine, by the Parc National des Pyrénées, and the Instituto Aragonés de Gestión Ambiental.

Deoxyribonucleic Acid (DNA) was extracted from skin swabs following the Prepman Ultra$^{TM}$ protocol and the presence of Bd DNA was detected using the validated TaqMan qPCR assay targeting the ITS1/5.8S region of the Bd genome, with extractions diluted 1:10 and including bovine serum albumin (BSA) to decrease PCR inhibition (Boyle *et al.* 2004; Hyatt *et al.* 2007; Garland *et al.* 2010; O.I.E. 2019). Negative controls and quantitation standards at 0.1, 1, 10 and 100 zoospore genomic equivalents (ZE) were run in duplicates on each qPCR plate. The standards were made with a Bd strain belonging to the Global Panzootic Lineage (GPL, ref. IA043) cultured from a dead *Alytes obstetricans* metamorph collected from Ibon Acherito, Spain. Two qPCR assays were run for each sample, and a sample was considered positive if both assays amplified (i.e. showed a clear sigmoid curve) and yielded a value superior to 0 ZE. When qPCR duplicates were conflicting, two new assays were rerun from the same diluted extraction. A sample was considered positive if it showed again at least one clear positive qPCR

result. The infection intensity (quantification of amplified DNA by the qPCR), a proxy for Bd load (burden), was used untransformed (not corrected for dilution) and are presented in ZE.

### 4.2.3 Categorisation of lake infection status

Depending on their infection history and the trends of their Ao populations, lakes were assigned to different categories. First, the five lakes from Lescun were classified as "systematically infected". We defined an "uninfected" category, for the six lakes in which Bd was never detected (Arbu, Lers, Embarrat, Fache-Espagne, Grande-Fache, Bellonguère), and a "sporadically infected" category for three lakes where Bd was detected on Ao for a limited period of time (Gourg de Rabas, Madamète-Bas, Vallon). Of these, Gourg de Rabas and Madamete-Bas experienced a pronounced amphibian chytridiomycosis epizootic from 2009-2011, after which no Ao were found during a few years. Healthy individuals and clutches were found again in recent years, with no detection of Bd infection in 2020 (data for 2021 and 2022 pending). Regarding Vallon, in 2015 most captured tadpoles were found infected but at very low level of infection. One individual out of 30 was again found very weakly infected in 2018. Since then, we always found 30 uninfected individuals every year.

Even if Bd infection is systematic in the five Lescun lakes, there are differences in terms of infection dynamics and disease long-term impacts on their Ao populations. Bates *et al.* (2018), based on the capture-mark-recapture data of Clare (2014), categorized Lhurs, Acherito and Puits d'Arrious as "enzootic" because their populations, after severe declines coinciding with the emergence of Bd, have now recovered to stable abundance levels in spite of infection. On the other hand, Ao populations of Arlet and Ansabère do not show any sign of recovery, are continually declining and even potentially extinct (no Ao tadpoles have been seen in the last two years in these two lakes), which Bates *et al.* (2018) qualified as "epizootic". As all sites can be considered **enzootic** since Bd is constantly detected, we employ a different terminology, namely "Ao-declining" vs. "Ao-stable" sites.

There is uncertainty about the "uninfected" lakes. It is difficult to know whether Ao are uninfected because Bd never reached the populations of these lakes or, in the case it has, because environmental conditions are not favourable to the free-living infective stage of Bd and we did not detect it during the short period(s) when it was present. There is now extensive evidence that Bd is an emerging pathogen introduced from Asia (Walker *et al.* 2010; O'Hanlon *et al.* 2018). That said, it is often considered that Bd is now, years or even decades after emergence, ubiquitous in France since infectious have been detected throughout the country (Miaud 2013). In the Pyrenees, there is a relatively high likelihood that hikers or anglers might

unknowingly carry Bd from one lake to another. This is why we included uninfected lakes in our study.

### 4.2.4 Analytical and statistical workflow

To determine whether benthic biofilm composition is linked to Bd epidemiology, i.e. to Bd infection occurrence and/or frequency, and/or impact on Ao populations, we compared biofilm communities between different groups in pairs. We first compared biofilms from Ao-declining sites of Lescun (Arlet, Ansabère) with those of Ao-stable sites (Lhurs, Acherito, Puits), for which we have the most data (comparison 1). Then, we compared biofilms from Lescun sites, where infection was systematic, with biofilms from sites where Bd infection was sporadic (even potentially cleared) and where Ao populations have recovered (Vallon, Gourg de Rabas and Madamete-Bas; comparison 2). Next, we compared biofilms from systematically infected sites with those of uninfected sites (Arbu, Lers, Embarrat, Fache-Espagne, Grande-Fache, Bellonguère; comparison 3), and finally, biofilms from sporadically infected sites with those of uninfected sites (comparison 4).

In all cases, we examined whether α- and β-diversity of both prokaryotic and micro-eukaryotic biofilm microbial assemblages were different between groups at the ASV level. First we looked at α-diversity, fitting linear mixed models (LMM) with package *lmerTest* (Kuznetsova *et al.* 2017)), with the Inverse-Simpson index as response variable, the grouping variable (e.g. Ao-declining vs Ao-stable) as fixed effect and lake as random effect. Second, to investigate compositional differences between groups, we calculated their respective multivariate dispersion, i.e. the mean distance of each sample to the group centroid, with function betadisper (Anderson 2006). We then tested for significantly different multivariate dispersion with a permutation test (function permutest). We implemented permutational multivariate analysis of variance, i.e. PERMANOVA or non-parametric ANOVA (distance-based), with function adonis2 to test if community composition significantly differed among groups (i.e. difference in location between groups; Anderson 2001). It is acknowledged that PERMANOVA may result in misleadingly low p-values and high false positive rates when multivariate dispersion is significantly heterogenous between groups (Anderson 2001, 2006). However, PERMANOVA is the method least sensitive to dispersion effect compared to other methods like ANOSIM or MRPP (Anderson 2001). Further, it is not because multivariate dispersion is significantly different that PERMANOVA results are necessarily inconclusive, as true differences in location between differently-dispersed groups might still exist (Schloss 2008). Therefore, we complemented the PERMANOVA results with a two-dimensional

ordination (PCoA) to assess whether communities were different. For all permutation tests, pairwise comparisons were restricted to biofilm samples taken at the same sampling campaign, i.e. at roughly the same period to exclude the effect of time (argument strata). We also tested whether the average intra-lake biofilm stability (i.e. intra-lake biofilm's multivariate dispersion) was different between the two groups, that is whether biofilms from lakes belonging to one group were more unstable (more dispersed) on average than biofilms from lakes of the other group. We used LMM with the distance of a sample to its lake centroid (calculated with function betadisper) as response variable, the grouping variable as fixed effect and lake as random effect.

We further analysed whether groups were characterized by differentially-abundant taxa using linear discriminant analysis (LDA) effect size (LEfSe; Segata *et al.* 2011). We used for this the Galaxy web application of the Huttenhower's laboratory (http://huttenhower.sph.harvard.edu/galaxy/) and imported the results into R. LEfSe determines whether the relative abundance of a taxon is significantly different between groups. It first applies a Kruskal-Wallis test to each taxon in the dataset, to know whether it is discriminating between groups; then it performs a Wilcoxon Rank Sum test only to these discriminating taxa to know if they are differentially abundant between its own subclass (here, the lake where it has been found) and all subclasses (lakes) of the other group. Finally, LDA is performed on the retained taxa (if any) to provide their effect size, i.e. how characteristic they are of the group. We only retained taxa with LDA score >2 and $p < 0.05$ for both Kruskal-Wallis and Wilcoxon-tests (these are the default settings). We applied LEfSe at each taxonomic resolution, that is from the ASV level to the genus, family, order, class and phylum levels to characterise differences in the composition of prokaryotic assemblages between biofilm communities of the two groups, and that, for each of the four pairwise comparisons. We did the same for micro-eukaryotic assemblages, with the difference that the taxonomy is not homogenised compared to that of prokaryotes. This means that the micro-eukaryotic ASV table provided by SILVA is not organised in a fixed number of columns such as Phylum, Class, Order, Family, Genus, ASV. Thus, a same column can contain information from, for instance, the phylum for ASV1, the genus for ASV2, the superorder for ASV3, the subfamily of ASV4 etc. To circumvent this, we applied LEfSE on each column and retrieved information on the identified taxa *a posteriori* using the NCBI taxonomy browser (to know whether the identified taxon is a genus, or a family, or an order etc). We then drew bar plots for each comparison at each taxonomic resolution for each of the prokaryotic and micro-eukaryotic assemblages.

To test whether biofilm communities between groups are functionally different in their ability to consume Bd zoospores, we computed the relative abundance of potential micro-eukaryotic Bd consumers, in which we include all ciliates, tardigrades and rotifers, and

compared it between groups. We used LMM with the same parametrization as above (grouping variable as fixed effect and lake as random effect). We did the same with the relative abundance of toxigenic cyanobacteria to find evidence of potential toxicity towards amphibians, using GLMM with Beta distribution of error and logit link fitted with package *glmmTMB* (Brooks *et al.* 2017). Note that we did not perform a similar analysis to find evidence for nutritional quality (comparing the relative abundance of all cyanobacteria and diatoms), because it would be redundant: LEfSe would already have identified these taxa if they were differentially abundant between groups.

## 4.3 Results

### 4.3.1 Comparison 1: biofilms from Ao-declining vs. biofilms from Ao-stable lakes

We found no significant difference in α-diversity between biofilms with declining Ao populations and biofilms with stable Ao populations (for prokaryotes, $p = 0.141$, for micro-eukaryotes $p = 0.313$). With respect to β-diversity, we observed significant heterogeneity in multivariate dispersions for both prokaryotic ($p = 0.013$) and micro-eukaryotic assemblages ($p = 0.002$), with biofilms from Ao-stable lakes being on average more dispersed than biofilms from Ao-declining lakes. However, intra-lake biofilm stability was not significantly different between groups, for both prokaryotes ($p = 0.530$) and micro-eukaryotes ($p = 0.092$). The PERMANOVA indicated significant compositional differences in both prokaryotic and micro-eukaryotic assemblages between groups ($p < 0.001$ for both). Visualisation on the two first axes of PCoA ordinations showed a consequent non-overlapping area, especially for micro-eukaryotes, supporting the PERMANOVA result (**Figure 26**).

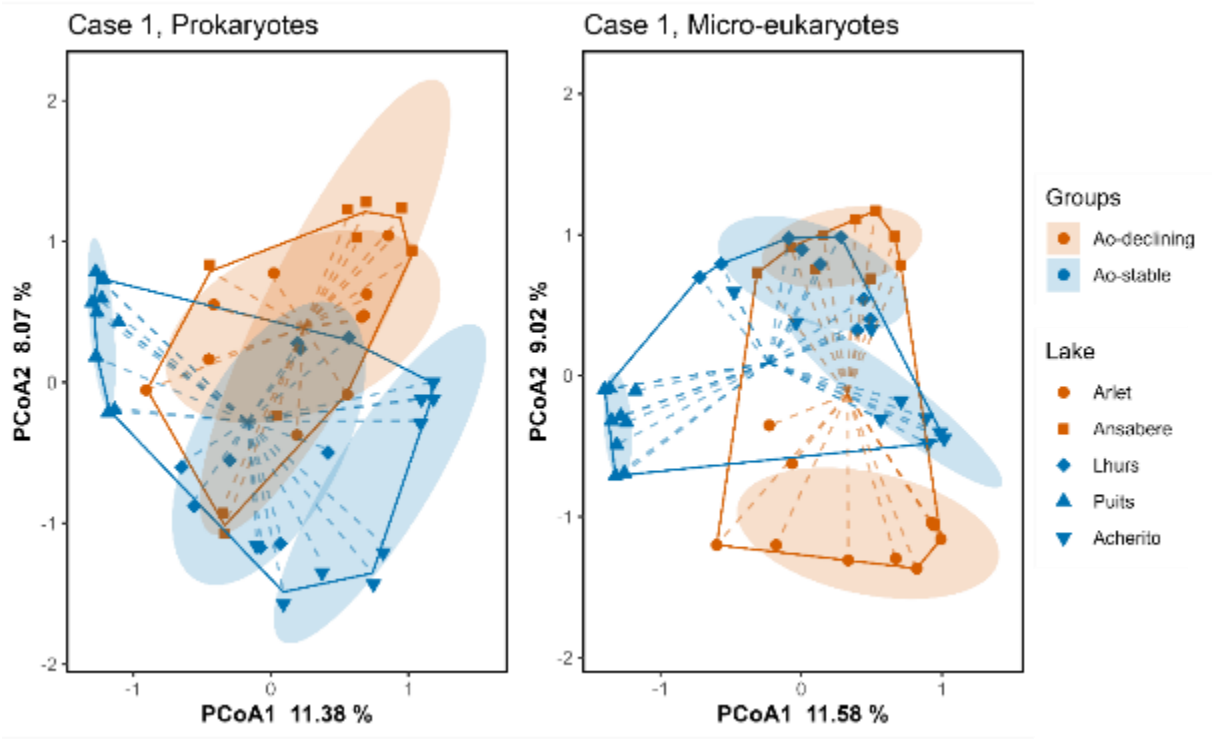



**Figure 26: Ordinations of benthic biofilms prokaryotic (left) and micro-eukaryotic (right) assemblages in comparison 1 (biofilms from sites with Ao-stable vs. Ao-declining populations).** The first two axes of Principal Coordonate Analysis are displayed, with their respective eigenvalues. Points represent samples. Bold lines represent the hull of each group: the less the hulls overlap, the more dissimilar the groups. Dotted lines represent the distance to the group centroid, indicating group β-dispersion. Filled ellipses contain 75% of the data for each lake, and show intra-lake β-dispersion (the smaller, the more stable).

The results from differential abundance analysis (LEfSe) showed that biofilm communities from Ao-declining sites were characterised by a high number of taxa. Fourty-five discriminating ASVs for this group were found, of which 37 were prokaryotic and 8 were micro-eukaryotic ASVs (**Figure 27**, **Figure 28**). We also found 11 prokaryotic genera, the most discriminating being *966-1*, *Methylibium*, *Roseomonas* and *Ahniella* (all Proteobacteria), and *Geminocystis PCC-6308*, a cyanobacterium). At the family level, LEfSe detected 8 prokaryotic families, including Nitrosomonadaceae and Cyanobacteriaceae, and one micro-eukaryotic family, the Sellaphoraceae (diatom). It also detected four prokaryotic orders, namely OPB41, PeM15, Acetobacterales and an unidentified Thermodesulfovibrionia, and one micro-eukaryotic order of Ciliates, Peniculia; two prokaryotic classes, Thermodesulfovibrionia, and KD4_96, and another micro-eukaryotic class, Phytomyxea. Finally, one fungal phylum Aphelidea, was also discriminating for biofilm from lakes with declining Ao populations (**Figure 27**, **Figure 28**).


**<u>Figure 27:</u> Relative abundance of differentially abundant prokaryotic taxa in comparison 1 (biofilms from sites with Ao-stable vs. Ao-declining populations).** Points indicate the median for

each lake, and the bars indicate the interquartile range (the 75th minus the 25th percentiles, containing 50% of the data). The grey vertical line represents the limit of detection, corresponding to the relative abundance of one read (i.e. $1/3{,}897 = 2.57*10^{-4}$, since all library sizes equal 3,897 reads after rarefaction: relative abundances under this limit of detection are meaningless as they would correspond to value between 0 and 1 read, which is not possible). Taxa are sorted by LDA scores as in LEfSe plots, with most discriminating taxa for Ao-declining group at the top, and most discriminating taxa for the Ao-stable group at the bottom.

The biofilm communities of Ao-stable lakes were characterised only by two micro-eukaryotic ASVs, both belonging to class Dinophyceae (Dinoflagellates). However, there were three discriminating prokaryotic genera including the cyanobacterial genus *Tychonema* CCAP-1459-11B, the proteobacterial genus *Brevundimonas*, and the planctomycete genus *Zavarzinella*; and one micro-eukaryotic genus, *Rhogostoma* (because the latter is the only representant of family Rhizaspididae and order Cryomonadida, this family and this order were also found differentially abundant). We then found one discriminating family (Rhizaspididae), two prokaryotic orders, Propionibacteriales and Pseudomonadales; and one micro-eukaryotic order, Cryomonadida. At higher taxonomic levels, the LEfSe analysis identified one subclass of rotifers, Bdelloidea, two classes of fungi named Leotiomycetes and LKM 15, and, finally, two micro-eukaryotic phyla, Chaetophorales (Chlorophyta) and Rotifera (Animals; **Figure 27**, **Figure 28**).

Neither the relative abundance of all potential consumers of *Bd* zoospore (Ciliates, Rotifers and Tardigrades combined), nor that of toxigenic cyanobacteria, were significantly different between the two groups ($p = 0.198$, $p = 0.92$, respectively).

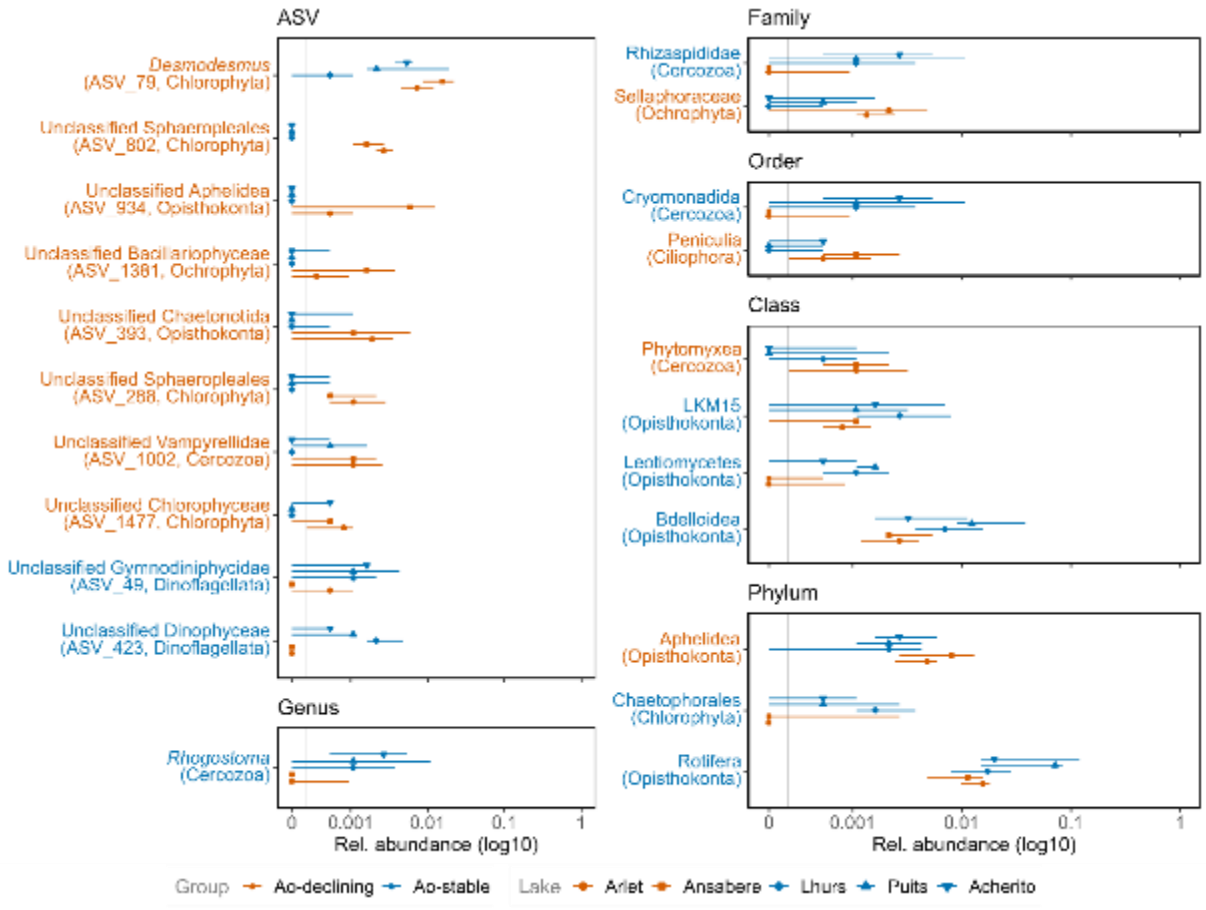



**Figure 28: Relative abundance of differentially abundant micro-eukaryotic taxa in comparison 1 (biofilms from sites with Ao-stable vs. Ao-declining populations).** Same legends as **Figure 27** except that the limit of detection is 1/1,856 (all micro-eukaryotic libraries were rarefied to 1,856, so relative abundances under 1/1,856 are meaningless).

### 4.3.2 Comparison 2: biofilms from systematically infected lakes vs biofilms from sporadically infected sites

The prokaryotic α-diversity was not significantly different between groups ($p = 0.142$) but the micro-eukaryotic one was significantly higher in biofilms from sporadically infected lakes ($p = 0.029$). There was significant heterogeneity in multivariate dispersions between the two groups for the prokaryotic ($p = 0.020$) but not the micro-eukaryotic assemblages ($p = 0.853$). The PERMANOVA showed that biofilm assemblages were significantly different between the two groups for both prokaryotes and micro-eukaryotes ($p < 0.001$ in both cases). From the PCoA, these results seem to be valid as there is little overlap between groups (**Figure 29**). Intra-lake biofilm stability was not significantly different between the two groups for both prokaryotes ($p = 0.194$) and micro-eukaryotes ($p = 0.813$).

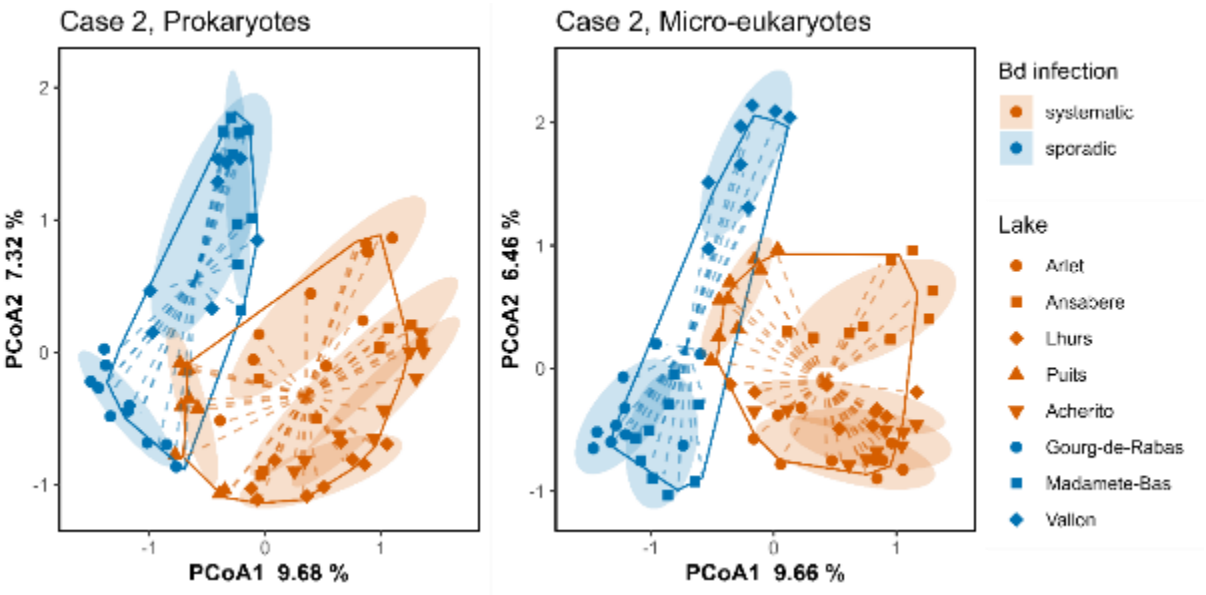


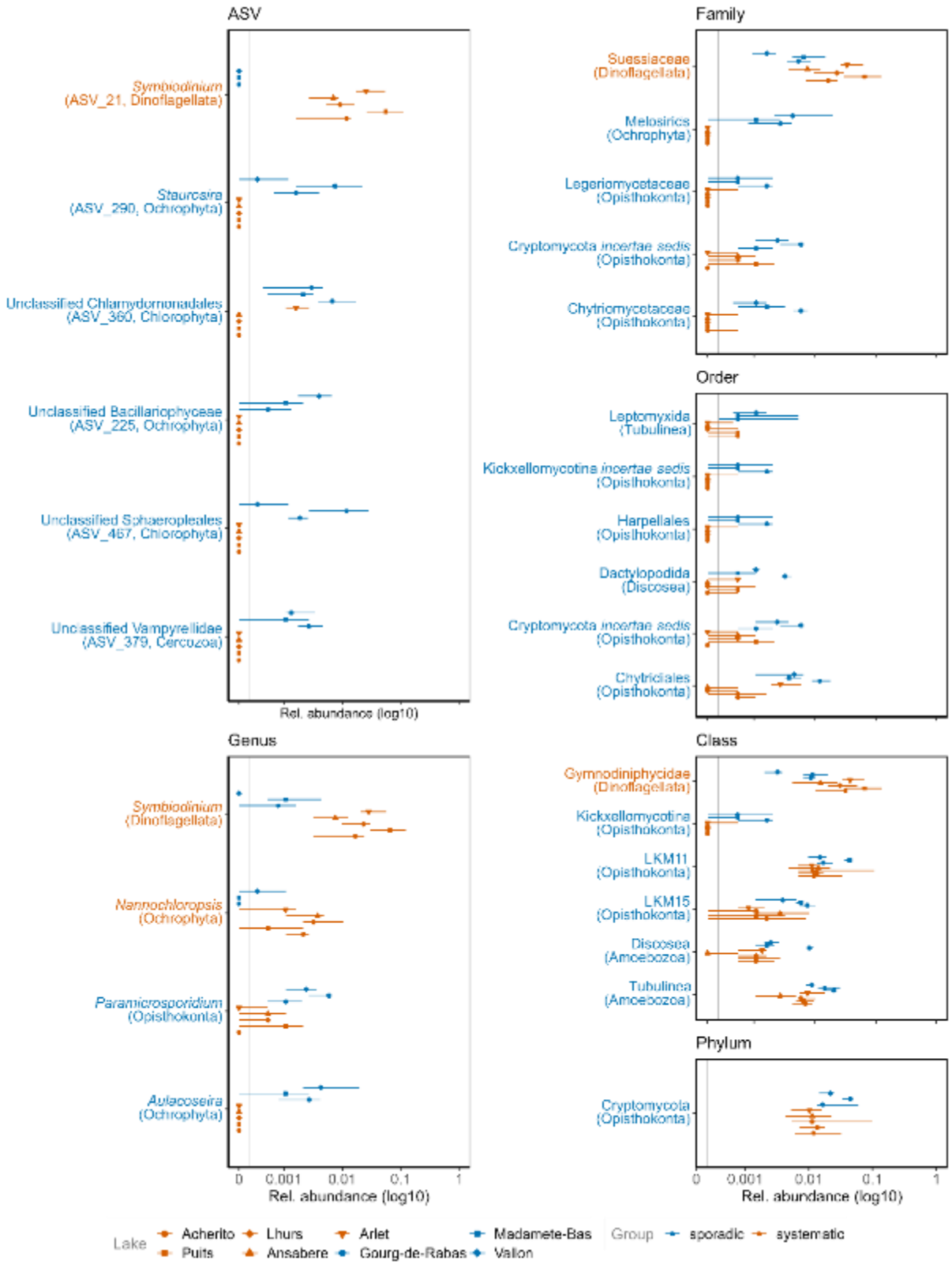

**Figure 29: Ordinations of benthic biofilms prokaryotic (left) and micro-eukaryoic assemblages in comparison 2 (biofilms from sites with systematically vs. sporadically Bd infected populations).** Same legends as **Figure 26**.

Biofilms from lakes with systematically infected Ao (Lescun) were characterized by one prokaryotic ASV belonging to genus *Bacillus* and one micro-eukaryotic ASV from genus *Symbiodinium*. At the genus level, five prokaryotic and two micro-eukaryotic genera were identified. The LEfSe analysis further identified six prokaryotic families and one micro-eukaryotic (Suessiaceae) family, three prokaryotic orders (Xanthomonadales, Bacillales, Steroidobacteriales), the micro-eukaryotics subclass Gymnodiniphycidae and the prokaryotic class Bacilli (**Figure 30**, **Figure 31**).


**Figure 30: Relative abundance of differentially abundant prokaryotic taxa in comparison 2 (biofilms from sites with systematically vs. sporadically Bd infected populations).** Same legends as **Figure 27**.

Biofilms from sporadically infected lakes (Gourg de Rabas, Madamete-Bas, and Vallon) were characterised by 13 prokaryotic and 5 eukaryotic ASVs. We found eight prokaryotic and two eukaryotic genera (*Paramicrosporidium*, and the diatom *Aulacoseira*), as well as six prokaryotic and three micro-eukaryotic families (Chytriomycetaceae, Legeriomycetaceae, Melosirids). At higher taxonomic levels, LEfSe identified six prokaryotic and four micro-eukaryotic orders (Chytridiales, Harpellales, Dactylopodida, Leptomyxida), four micro-eukaryotic classes (the amebozoans Tublinea and Discosea, and the fungi LKM11 and LKM15), the fungal subphylum Kickxellomycotina and phylum Cryptomycota (**Figure 30**, **Figure 31**). Interestingly, biofilms from Madamete-Bas and Gourg de Rabas, but not Vallon, had significantly higher abundance of the subclass Bdelloidea and phylum Rotifera than the biofilms from the five lakes of Lescun ($p = 0.007$).

Neither he relative abundance of all potential *Bd* zoospore consumers (Ciliates, Rotifers and Tardigrades combined) nor that of toxigenic cyanobacteria were significantly different between the two groups ($p = 0.342$ and $p = 0.676$, respectively).




**<u>Figure 31:</u> Relative abundance of differentially abundant micro-eukaryotic taxa in comparison 2 (biofilms from sites with systematically vs. sporadically Bd infected populations).** Same legends as **<u>Figure 27</u>** except that the limit of detection is 1/1,856.

### 4.3.3 Comparison 3: biofilms from systematically infected lakes vs. biofilms from uninfected lakes

There was no significant difference in α-diversity between groups (for prokaryotes, p = 0.141, for micro-eukaryotes p = 0.313). The multivariate dispersions of prokaryotic biofilm communities were not significantly different (p = 0.076), but those of micro-eukaryotic biofilm communities were significantly different (p = 0.002). The PERMANOVA showed that prokaryotic and micro-eukaryotic biofilm assemblages were different between the two groups (p = 0.001), but the PCoA ordination indicated that the two groups largely overlapped, suggesting that they may not be different (**Figure 32**). Intra-lake biofilm stability was not significantly different between the two groups for both prokaryotes (p = 0.619) and micro-eukaryotes (p = 0.657).

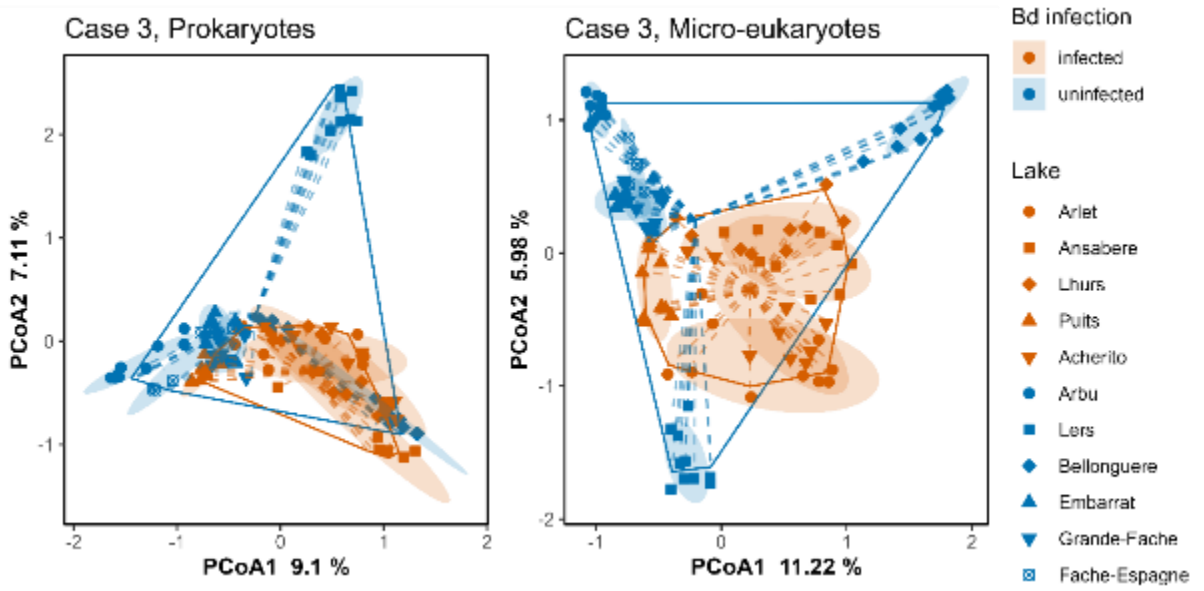



**Figure 32: Ordinations of benthic biofilms prokaryotic (left) and micro-eukaryoic assemblages in comparison 3 (biofilms from sites with Bd infected vs. uninfected populations).** Same legends as **Figure 26**.

No prokaryotic taxon was differentially abundant between biofilms of the two groups. Only one eukaryotic ASV, belonging to genus *Symbiodinium* (the entire genus was also discriminating), was found to be characteristic of biofilms systematically infected lakes (**Figure 33**).

Neither the relative abundance of all potential *Bd* zoospore consumers (Ciliates, Rotifers and Tardigrades combined) not that of toxigenic cyanobacteria were not significantly different between the two groups (p = 0.770 and p = 0.36, respectively).

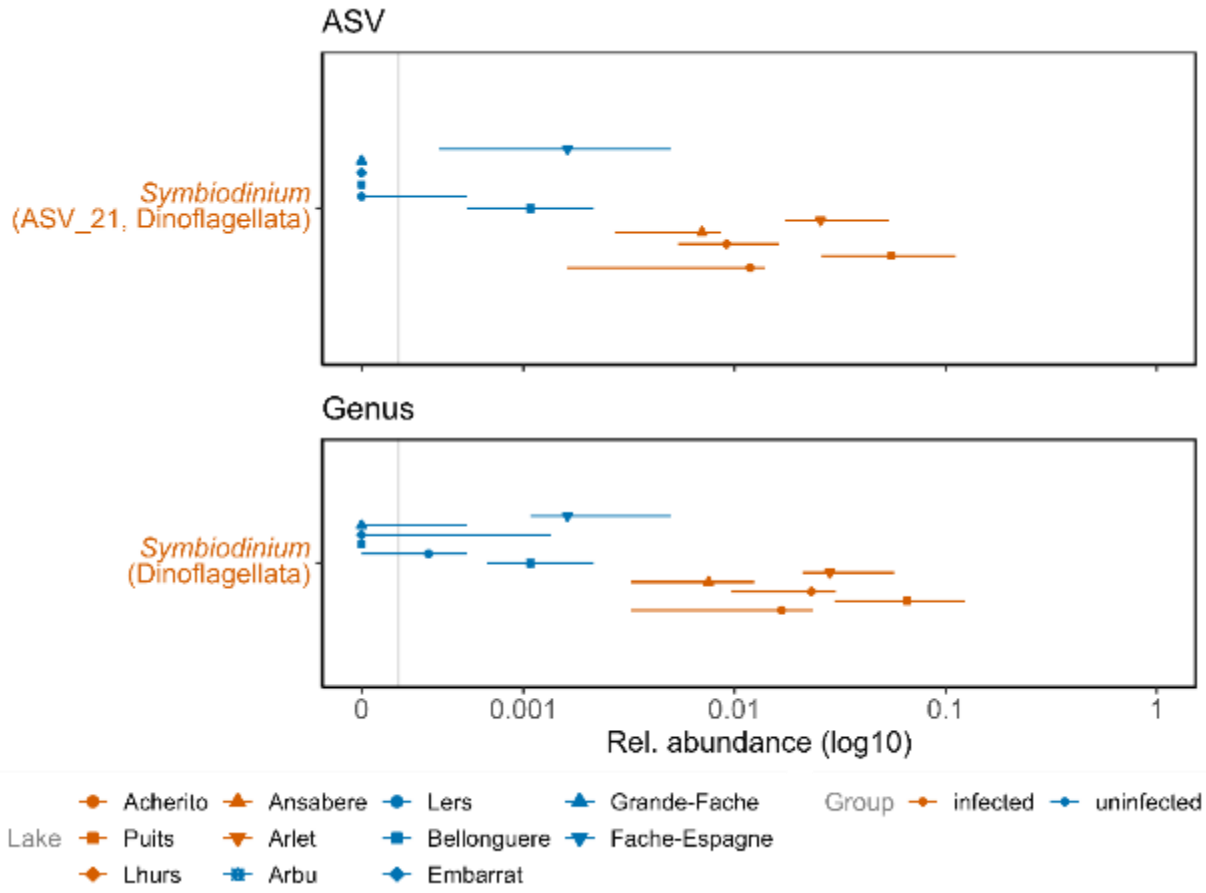



**Figure 33: Relative abundance of differentially abundant taxa in comparison 3 (biofilms from sites with Bd systematically infected vs. uninfected populations).** Same legends as **Figure 27** except that the limit of detection is 1/1,856.

### 4.3.4 Comparison 4: biofilms from sporadically infected lakes vs. never-infected lakes

The prokaryotic α-diversity was significantly greater in biofilms of the sporadically infected group than biofilm of the infected group ($p = 0.050$), but not for micro-eukaryotic α-diversity ($p = 0.481$). For both prokaryotic and micro-eukaryotic assemblages, the overall dispersion of biofilms from the uninfected sites was greater than that from biofilms of the sporadically infected group ($p = 0.001$ in both cases). The PERMANOVA showed that both prokaryotic and micro-eukaryotic assemblages were different between the two groups ($p = 0.001$). The PCoA ordination indicated that biofilms from sporadically infected lakes were not dissimilar to biofilms from uninfected lakes (**Figure 34**). Biofilm within-lake stability was not significantly different between groups (prokaryotes: $p = 0.731$, micro-eukaryotes: 0.873).

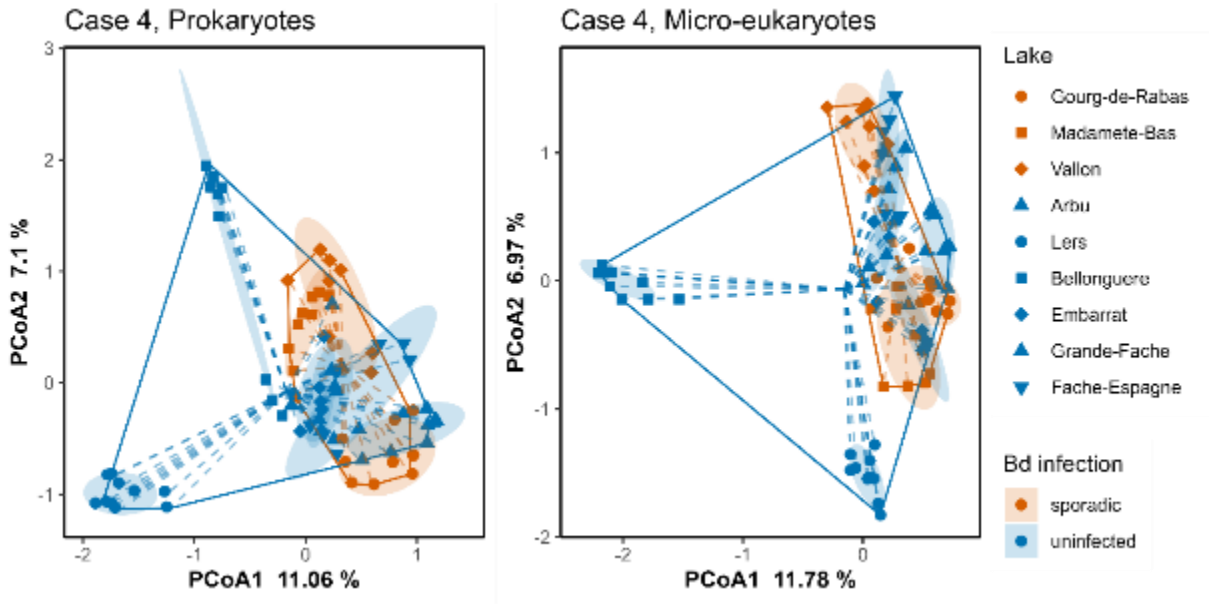



**Figure 34: Ordinations of benthic biofilms prokaryotic (left) and micro-eukaryoic assemblages in comparison 4 (biofilms from sites with Bd sporadically infected vs. uninfected populations).** Same legends as **Figure 26**.

Biofilm communities from uninfected lakes had no discriminating taxa. Biofilms from sporadically infected lakes were characterised by two ASVs (one uncultured Gemmatimonadaceae, and one chlorophyta from order Sphaeropleales), one family of diatoms (Melosirids, only represented by genus *Aucoseira*, also significant), and one family of Proteobacteria named Paracaedibacteraceae (**Figure 35**).

Neither the relative abundance of all potential predators (Ciliates, Rotifers and Tardigrades combined) not that of toxigenic cyanobacteria were not significantly different between the two groups ($p = 0.559$).

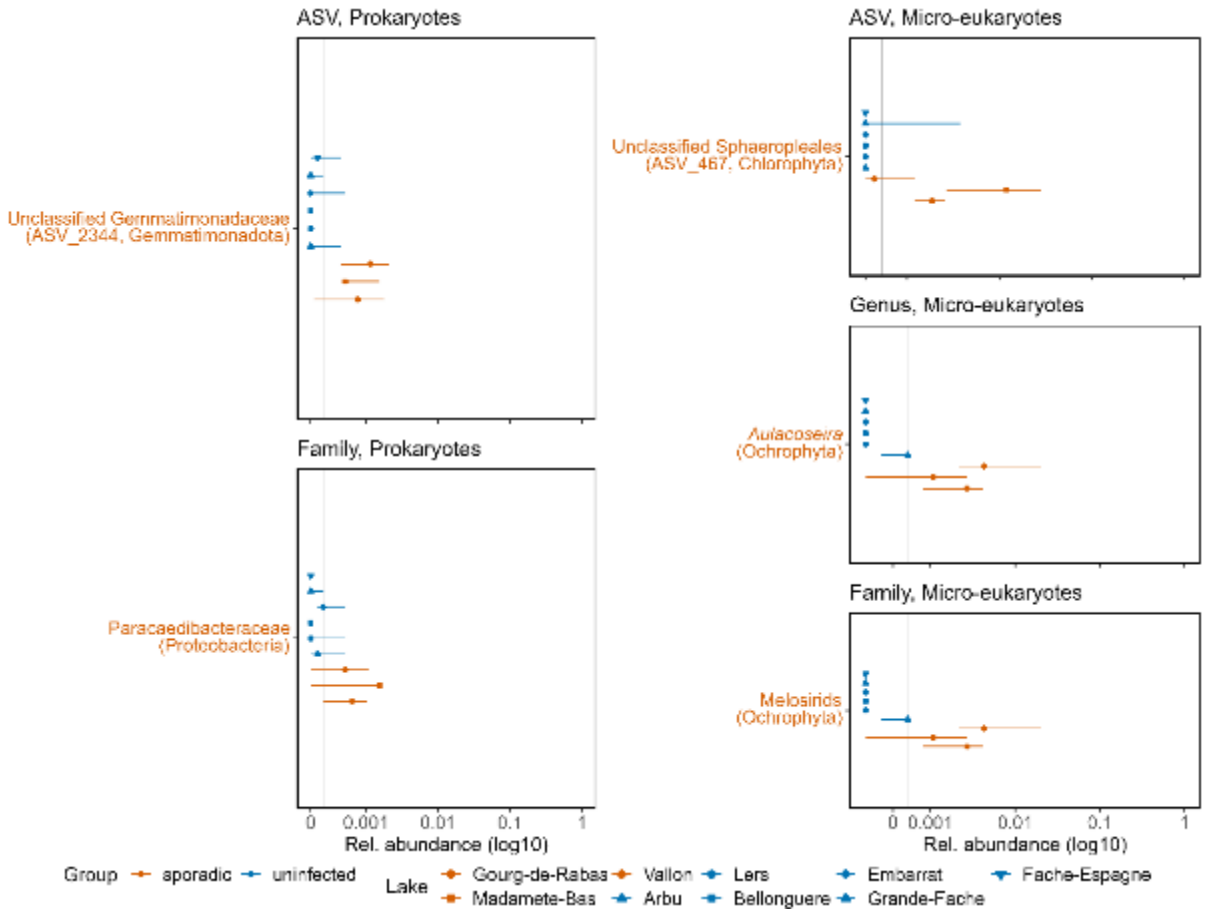



**Figure 35: Relative abundance of differentially abundant taxa in comparison 4 (Sporadic vs. uninfected).** Same legends as **Figure 27** (the limit of detection is 1/1,856 for micro-eukaryotes).

## 4.4 Discussion

Our study examined the potential links between the prokaryotic and micro-eukaryotic assemblages of benthic biofilm microbial communities and some epidemiological aspects of *Batrachochytrium dendrobatidis* infections in lakes of the French Pyrenees. Our results indicated that differences in biofilm composition correlate to some extent with differences in amphibian chytridiomycosis impact on Ao populations (comparison 1) and with the ability of Bd to infect Ao persistently on a year-to-year basis or only sporadically (comparison 2). These compositional differences were partly explained by more potential predators, inhibitors or consumers of Bd in lakes with less infected or impacted Ao populations. However, we found little evidence that differences in biofilm composition explain whether Bd has or has not infected Ao in a lake (comparisons 3 and 4).

We found that biofilms from systematically infected lakes and biofilms from sporadically infected lakes on the one hand, and biofilms from Ao-declining and Ao-stable lakes on the other hand, differed in terms of community composition (β-diversity). These

differences in β-diversity were driven by numerous prokaryotic and micro-eukaryotic taxa that were differentially abundant between these two pairs of groups. Of particular interest are the discriminating features of Ao-stable lake biofilms which contained a higher abundance of ASVs belonging to the phylum Rotifera and, more precisely, the class Bdelloidea. Rotifers are known consumers of *Batrachochytrium* fungi, including Bd (Schmeller *et al.* 2014; Stegen *et al.* 2017). Monogont rotifers of the genus *Lecane* have been shown to occur in the zooplankton of some of the lakes studied here, and to effectively reduce the pool of infective zoospores in experimental settings (Schmeller *et al.* 2014). Bdelloid rotifers are important biofilm-dwellers (Majdi *et al.* 2012; Mialet *et al.* 2013) and we show here that they can have high relative abundance in some mountain lake biofilms. Bdelloids are also very efficient at consuming Bd zoospores (Loyau, unpublished data) and could partly explain the different disease dynamics in Lescun lakes. They were also in high relative abundance in Madamete-Bas and Gourg de Rabas, but not in Vallon, explaining why Bdelloid rotifers were not identified by our differential abundance analysis (LEfSe) in the sporadically infected group.

Biofilms from Ao-stable lakes revealed additional discriminating taxa, such as the proteobacterial genus *Brevundimonas*. *Brevundimonas* has been isolated from the skin of multiple amphibian species from different continents, and repeatedly shown to inhibit Bd in culture conditions (Woodhams *et al.* 2015). Similarly, these biofilms were characterized by high abundance of Pseudomonadales, an order of Proteobacteria of which several genera are inhibitory to Bd in culture, and were also found to be negatively associated with Bd infection on *Rana cascadae* skin sampled in the Trinity Alps (USA, California; Woodhams *et al.* 2015; Kueneman *et al.* 2017). Another order, Propionibacteriales, was found in biofilms of Ao-stable lakes. Propionibacteriales belong to Actinobacteria (also known as Actinomycetes), a class known to i) have a substantial potential in producing antimicrobial compounds (Bérdy 2005), ii) be associated with Bd resistance in the case of *R. sierrae* in Sierra Nevada (Jani and Briggs 2014) and multiple species in Panama (Rebollar *et al.* 2016b) and iii) be Bd inhibitory *in vitro* (Woodhams *et al.* 2015). *Rhogostoma*, the unique representative of family Rhizaspididae and order Cryomonadida in our dataset (all found discriminating), is a cercozoan genus with some species known to feed on unicellular fungi (Dumack *et al.* 2017). The two characteristic micro-eukaryotic ASVs of Ao-stable lakes are unidentified dinoflagellates from class Dinophyceae, which are mixotroph and can feed with phagocytosis (Stoecker 1999). Finally, we also found two discriminating fungal classes, LKM15 (Cryptomycota or Rozellomycota) and Leotiomycetes (Ascomycota, Pezizomycotina). Many fungi inhabiting the amphibian skin microbiome have been shown to be protective against Bd infection (Kearns *et al.* 2017). Pezizomycotina, including Leotiomycetes, were negatively correlated with Bd infection in wild

*R. cascadae* (Kueneman *et al.* 2017). All abovementioned taxa could inhibit, predate or consume Bd zoospores and thus have the potential to reduce the impacts of Bd infections on Ao populations in systematically-infected lakes, although controlled experiments are needed to confirm this.

We also found a variety of taxa of interest that discriminate sporadically-infected lake biofilms and systematically infected lakes with, for instance, genus *Rhizobacter* (family ) and an ASV belonging to family SC-I-84. Both belongs to Burkholderiales, an order found to be highly represented in the skin microbiome of Bd-resistant individuals of wild *Anaxyrus boreas* found in high-elevation wetlands of Colorado (Kueneman *et al.* 2016b). Comamonadaceae, in particular, have been shown to inhibit Bd *in vitro* (Woodhams *et al.* 2015). Biofilms from sporadically infected lake were characterized by many fungal taxa, namely the entire phylum Cryptomycota (represented by classes LKM11 and LKM15 and also the genus *Paramicrosporidium, incertae sedis* class), the classes Chytridiales (represented by family Chytriomycetaceae; Chytridiales is the sister class of Rhizophydiales, to which Bd belongs) and Harpellales (represented by family Legeriomycetaceae), and the subphylum Kickxellomycotina. Little is known about these fungi and the antimicrobial metabolites they may produce but it is not impossible they can affect Bd (Kearns *et al.* 2017). Many Planctomycetota taxa, including orders Isoshpaerales and Gemmatales, were shown to produce antimicrobial compounds with high antifungal activity (Graça *et al.* 2016). The *Aetherobacter* genus is also famously known to produce aetheramides, which are effective antiviral (anti-HIV) and antifungal molecules (Gerstmann and Kalesse 2016). Vampyrellids (Rhizaria Cercozoa) can also feed on eukaryotes (Hess and Suthaus 2022). There were also two Amoebozoa classes, Tubulinea (represented by order Leptomyxida) and Discosea (represented by order Dactylopodida) which were more abundant in biofilms of sporadically infected sites, with at least Leptomyxida being able to prey on unicellular fungi (Chakraborty *et al.* 1983). All these taxa could explain why Bd infections are less frequent in lakes Gourg de Rabas, Madamete-Bas, and Vallon compared to lakes around Lescun.

From our taxonomic data; there is little evidence that biofilms influence frequency or impact of Bd infections based on spatial difference in nutritional quality (mechanism 1). Indeed, we did not find that diatoms as a whole were more abundant in less infected or impacted group or that cyanobacteria as a whole were more abundant in more infected or impacted groups. We did find that a family of diatoms, Melosirids, and the genus *Aucaloseira* in particular, were significantly more abundant in sporadically-infected lakes compared to systematically-infected lakes (comparison 1). It is unknown if these taxa are peculiar in their ability to synthetise highly nutritional PUFA. Regarding the potential toxicity of biofilms with regards to the amphibian

host (mechanisms 1 and 2), we again did not find much evidence because toxigenic cyanobacteria were not differentially abundant between groups in any comparison. Some cyanobacterial taxa were differentially abundant, but more abundant in the less infected or impacted groups, which does not support our hypotheses. Other approaches than metabarcoding are needed to really test the existence of mechanisms 1 and 2 (nutritional quality and cyanobacteria-related toxicity).

Few differentially abundant taxa were identified in comparisons 3 and 4, which might be because the uninfected group includes many lakes from ecologically and geographically different areas of the Pyrenees, in contrast to the systematically-infected group which contains geographically clustered lakes of which the biofilms are more likely to harbour organisms in common. This indicates that differential abundance analysis should also be performed lake by lake to identify the biomarkers of biofilms from each lake, and not only those common to a group of lakes, as lakes could have different characteristic taxa that are similar in functions (here, consuming Bd zoospores), which could explain why they have the same infection status. Furthermore, comparisons with the uninfected group may be limited in their interpretation, as it is not certain that Bd has actually reached the Ao populations in these lakes. In other words, we should not take these lakes as negative controls if we are not sure that they are truly "repelling" Bd. From here, there are two ways to interpret our results. On the one hand, if we assume that Bd has reached amphibian populations of the uninfected lakes but cannot infect them because of environmental conditions, then our results indicate that this is not related to biofilm composition. Indeed, we found few discriminating biomarkers and communities between groups were not very dissimilar in comparisons 3 and 4. On the other hand, if we assume that Bd has never reached these lakes, then it is not surprising that we have found no discriminating taxon in comparisons 3 and 4, because the biofilms of these lakes have, so to say, never been "tested" against Bd. Because we have numerous discriminating features between biofilms from lakes with different Bd infection dynamics (of which we are certain, comparisons 1 and 2), our results are rather in favour of the second interpretation. Care should be taken, however, as many other factors come into play in determining the occurrence, frequency and impact of Bd infection (Bernardo-Cravo *et al.* 2020; Fisher and Garner 2020).

Only negative correlations between biofilm taxa and Bd have been discussed here (i.e. biofilm taxa from lakes with less frequent infections or infections with less severe impact), but positive correlations with Bd should be investigated too as it is possible that some taxa might negatively affect Bd consumers which would allow the zoospores to be more abundant and infect more amphibians. Network analysis could be performed on these datasets to explore both positive and negative correlation patterns between Bd and other microbes (Barberán *et al.* 2014;

Rebollar *et al.* 2016a). Unfortunately, Bd is not referenced in the SILVA database, and we have detected 245 unidentified or uncultured Rhizophydiales ASVs in our micro-eukaryotic dataset, among which the 18S rRNA gene sequence of Bd was not found. In the absence of metabolomics or metatranscriptomics approaches, the functions of biofilms communities could be predicted with inference tools such as Piphyllin and KEGG, and functional profiles could then be compared between groups (Kanehisa *et al.* 2008; Iwai *et al.* 2016). Even so, our study would still remain correlational by nature, and laboratory experiments are necessary to further determine if and how biofilms can impact Bd itself, and/or amphibian tadpoles, and, in turn, contribute to the dynamics of amphibian infections. Such experiments would also shed light on aspects of biofilms that we did not investigate here, such as the effects of biofilm biomass on Bd, which relates to both the abundance of microorganisms (we only can compare relative abundance of microbes in our study) and that of the gel-like matrix they produce. The three-dimensional architecture of the matrix, and its chemical composition (EPS), could also affect Bd zoospores (Flemming and Wingender 2010; Sentenac *et al.* 2022). From our personal field observations, we could see that the matrix quantity is highly variable between lakes but not necessarily correlated to the richness of micro-organisms found within this matrix. For instance, Arlet biofilms have in average one of the highest species richness of all biofilms both in prokaryotic and micro-eukaryotic assemblage but it is arduous to gather a decent amount of biofilm compared to other lakes.

In conclusion, we found some evidence that biofilms could play a role in Bd infection epidemiology in the French Pyrenees. Biofilms from lakes in which Ao populations are less frequently infected by Bd, or less impacted by amphibian chytridiomycosis, contain micro-organisms that are known to consume, predate or inhibit Bd or other fungi. Further experimental studies should be conducted to demonstrate direct causality and elucidate the mechanisms involved. Amphibians are keystone species, and there is increasing evidence that their disease-driven declines have far-reaching consequences on the structure and functioning of their ecosystems (Whiles *et al.* 2006, 2013; Zipkin *et al.* 2020; Alonso *et al.* 2022). This in turn can jeopardise the delivery of ecosystem services, such as the provision of clean water and the regulation of vector-borne diseases, and affect human health and wellbeing (Springborn *et al.* 2022). Therefore, elucidating Bd infection epidemiology is important to prevent further losses and subsequent implications to socio-ecosystems.

# CHAPTER 5

# ENVIRONMENTAL BIOFILMS CAN AFFECT THE FATE OF THE FREE-LIVING STAGE OF A GLOBALLY-EMERGED PATHOGEN

## Foreword of Chapter 5

This chapter presents my experimental work on the interactions between Bd zoospores and various benthic biofilms. Most of the laboratory work has been performed by Master students under my supervision. I personally wish to thank them for their work, as well as my laboratory and the Fondation pour la Recherche sur la Biodversité (FRB) for having selected my grant proposals, which allowed to complement my field-based research with laboratory experiments. We tested the effects of simple monospecific biofilms (three different phototrophic algae), a simple multispecific biofilm (these three algae combined), and more complex (natural) biofilms on the number of zoospores over time. We have encountered some difficulties in finding appropriate protocols in each case, and the experiments regarding the field-grown biofilms need to be redone. However, we have shown that even monospecific biofilms can affect Bd zoospores. Again, our results warrant further studies to find out whether biofilms actually reduce the infection pressure for amphibians by inactivating zoospores, or whether biofilms only make them encyst on surfaces, waiting for conditions to improve, which would imply that the infection pressure is not reduced for tadpoles grazing on surfaces.

# Chapter 5: Environmental biofilms can affect the fate of the free-living stage of a globally-emerged pathogen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I formulated ideas, with help of J.L., D.S.S., and A.L. R.G. performed preliminary experiments, with my help. A.C. performed experiments of setting 1, and some experiments of setting 2, with help of S.C and me. S.C also performed experiments of setting 3. I performed fieldwork with A.C., R.G., D.S.S and A.L. I performed the statistical analyses and wrote the first draft of this manuscript. H.S., A.L. and D.S.S. contributed to the final version.

## Abstract


Microbial biofilms are ubiquitous and very productive in freshwater ecosystems, yet their interactions with aquatic pathogens remain little studied. Here, we investigated the fate of the free-living infective stage of *Batrachochytrium dendrobatidis* (Bd), a fungal parasite responsible for global amphibian declines, when brought into contact with benthic biofilms. To test this, we used laboratory-grown biofilms composed of either one or three phototrophic algae, or biofilms grown in the wild with a complex organisation. Our results clearly demonstrate that, compared to controls without biofilm, the presence of biofilms increases the mortality rate and/or encystment rate of Bd zoospores in the aquatic environment. The laboratory-grown multispecies biofilm had the highest effect compared to other monospecific and field biofilms. Even in the absence of biofilm-dwelling predators of Bd, biofilms can impact zoospores. Further studies are needed to identify the mechanisms by which biofilms affect Bd zoospores (e.g., physico-chemical interference of the polymeric matrix, allelopathic effects, or nutrient depletion), and to determine whether this results into a reduced infection pressure for amphibians.

## 5.1 Introduction

Emerging infectious diseases of humans, animals, and plants can substantially impact economy, biodiversity and ecosystem functioning (Daszak *et al.* 2000; Jones *et al.* 2008; Fisher *et al.* 2012). A good understanding of host and pathogen (or parasite) ecology is thus essential to elucidate the determinants of infection and disease and provide effective mitigation strategies (Plowright *et al.* 2008; Langwig *et al.* 2015; Parratt and Laine 2016; Rothenburger *et al.* 2017). However, compared to the impacts of abiotic environmental factors on pathogens, the impacts of biotic environmental factors have rarely been studied, while pathogens that have a free-living stage must cope in the environment with many sympatric organisms and the metabolites they produce, and survive these interactions, to potentially infect a new host (Thieltges *et al.* 2008; Johnson *et al.* 2010a; Leisner and Haaber 2012; Schmeller *et al.* 2014; Vaerewijck *et al.* 2014).

These sympatric organisms include a plethora of microbes, the majority of which live in matrix-enclosed communities, named biofilms; biofilms are extremely diverse, abundant, and essential to the functioning of many ecosystems on Earth (Battin *et al.* 2016; Cavicchioli *et al.* 2019; Flemming and Wuertz 2019; Bernardo-Cravo *et al.* 2020; Sentenac *et al.* 2022). Despite this, our knowledge of how pathogens interact with biofilms remains very limited (Sentenac *et al.* 2022). Most of what is known relates to pathogens causing human-water borne diseases in artificial aquatic environments. On the one hand, some biofilms are known to harbour pathogens from predators and biocides, and serve as a reservoir for parasitic protozoans, pathogenic bacteria, or viruses (Hall-Stoodley and Stoodley 2005; Searcy *et al.* 2006; Morris *et al.* 2007; Skraber *et al.* 2007; Wingender and Flemming 2011; Chang *et al.* 2019). On the other hand, biofilms can constitute a sink for pathogens, where the latter can be i) consumed by organisms living in or on the biofilms, e.g. filter-feeders, biofilm grazers, ii) inactivated by hyperparasites, bacteriophages, or allelopathy, i.e. by antimicrobial substances secreted by other microorganisms, iii) entrapped due to the physical and chemical interference of the matrix, composed of a multitude of adhesive extracellular polymeric substances (EPS), and finally, iv) outcompeted with regards to nutrient, light, gas or substrate availability, by other biofilm inhabitants (Stevik *et al.* 2004; Chabaud *et al.* 2006; Skraber *et al.* 2007; Rendueles and Ghigo 2012, 2015). This competition extends even outside the matrix, as biofilms capture nutrients in the water column by active or passive sorption and can produce antimicrobial compounds therein (Sentenac *et al.* 2022). Although some knowledge has been gained from the study of human-water borne diseases, there remains a wide knowledge gap about the roles of environmental biofilms in the ecology of emerging infectious diseases of wildlife and plants (Sentenac *et al.* 2022).

One of the most relevant examples of emerging infectious diseases impacting biodiversity and entire ecosystems on a large scale is that of the amphibian chytridiomycosis caused by *Batrachochytrium dendrobatidis* (Berger *et al.* 1998; Longcore *et al.* 1999; Whiles *et al.* 2013; Scheele *et al.* 2019; Zipkin *et al.* 2020). This disease is the most destructive ever recorded, with at least 500 amphibian species suffering severe declines or extinctions due to its global spread (Scheele *et al.* 2019). *Batrachochytrium dendrobatidis* is an aquatic fungus that parasitizes the keratinized cells of amphibians, namely the mouthparts of larval stages and the skin of metamorphosed individuals (Berger *et al.* 1998). Infection causes a lethal disease in metamorphosed individuals of susceptible species, but larval stages rarely die from infection (Garner *et al.* 2009).

The infective stage of Bd is a free-living uniflagellated zoospore (body: 3-5 µm, flagellum 19-20µm; (Longcore *et al.* 1999)) that is motile and able to swim in aquatic environments to find a new host via chemotaxis (Moss *et al.* 2008; Garmyn *et al.* 2012), allowing transmission of the pathogen even without physical contact with an infected host (Courtois *et al.* 2017; Burns *et al.* 2021). But the ability of Bd zoospores to infect a new host is constrained by the distance they can swim and the time before they die or encyst, which is ultimately dependent on biotic and abiotic environmental conditions. In the laboratory, zoospores are known to generally swim less than two centimeters and encyst before 24 hours in sterilised water at 23°C (Piotrowski *et al.* 2004), although this might be influenced by the molecules present in the water (chemotaxis), or the current, in natural conditions (Moss *et al.* 2008; Lam *et al.* 2011; Courtois *et al.* 2017). The length of the Bd life cycle is much dependent on temperatures, and zoospores are more active and encyst later at low temperatures (10°C) than they do in the optimal temperature range between 17 and 25°C, while at warmer temperatures they die and tend to encyst more rapidly (Woodhams *et al.* 2008). Another study revealed that the length of the Bd life cycle also depends on nutrient availabilty: when availability is low, zoospores encyst sooner and do not necessarily develop into zoosporangia to complete the cycle, in contrast to when nutrients are more abundant (Johnson and Speare 2003). In this state of arrested or slowed development (no more swimming zoospores were visible), encysted zoospores were shown to still be viable (i.e. infective); however, the more nutrients at the beginning, the longer they remained viable, for up to seven weeks (Johnson and Speare 2003)

Regarding biotic environmental factors, we know from the well-studied amphibian skin microbiome that both the microorganisms living in host-associated biofilms and the thickness of these biofilms can negatively impact Bd infectivity, through production of antifungal compounds (allelopathy) and physical preemption, respectively (Harris *et al.* 2006, 2009a, 2009b; Brucker *et al.* 2008; Kearns *et al.* 2017; Piovia-Scott *et al.* 2017; Chen *et al.* 2022).

However, little is known about the impacts of sympatric planktonic microorganisms and sessile biofilms when zoospores live freely outside the host. Searle *et al.* (2013) found fewer Bd zoospores in their experiments when the density of green algae was high in the water column, suggesting physical interference or allelopathy. Schmeller *et al.* (2014) also showed that differences in aquatic zooplankton diversity and abundance, with the presence of micro-predators consuming Bd zoospores, could explain the disparities in Bd infection prevalence in different lakes of a same mountain range. In the water column, a number of sympatric zooplankton organisms including ciliates, rotifers, tardigrades and crustaceans such as cladocerans, ostracods, and copepods, can predate Bd zoospores and reduce infection pressure on amphibians (Buck *et al.* 2011; Searle *et al.* 2013; Schmeller *et al.* 2014; Blooi *et al.* 2017; De Troyer *et al.* 2021).

To the best of our knowledge, there have been no studies on the interactions between Bd zoospores and environmental biofilms. Yet, those two elements are bound to interact with each other, especially in freshwater ecosystems where both biofilms and amphibians are important and abundant (Schmeller *et al.* 2018; Sentenac *et al.* 2022). Biofilms can be the most productive compartment of the biota in shallow and clear freshwater bodies, and tadpoles of many amphibian species extensively feed on them (Altig and Johnston 1989; Altig *et al.* 2007). This is particularly relevant in mountain freshwater ecosystems, where Bd and chytridiomycosis have had dramatic impacts on numerous amphibian species, with tadpoles and other amphibian larvae often playing a central epidemiological role of reservoir as they do not die from infection and can overwinter for several years (Woodhams *et al.* 2008; Briggs *et al.* 2010; Walker *et al.* 2010; Wells 2010; Medina *et al.* 2015; Scheele *et al.* 2019).

Here, we aimed to test two hypotheses: i) that biofilms increase the rate at which Bd zoospores disappear from the water column following encystment or death, and ii) that different biofilms cause zoospores to disappear from the water column at different rates. We expected that biofilms could induce encystment of Bd zoospores by allelopathy or nutrient depletion, and/or inactivate them by several mechanisms, including physico-chemical interference (binding or entrapment) of the biofilm matrix which would immobilise zoospores, by allelopathic effects, or by consumption by biofilm dwellers, such as filter-feeders. We grew several biofilms in laboratory settings, introduced a known number of zoospores in the water column, and monitored their concentration over a one-day period. We used four different biofilms produced by phototrophic algae known to occur in natural biofilms, namely one cyanobacterium, two different diatoms, and a mix of these three species to test the effects of simple biofilms (without predators) on zoospores. We then tested, using a similar protocol, the effects of more complex biofilms, potentially containing predators.

## 5.2 Methods

### 5.2.1 Zoospore culture and overall design

For all experiments, we used the isolate IA043 of BdGPL, obtained from a recently metamorphosed individual of *Alytes obstetricans*, found dead in Ibon Acherito, Pyrenees, in 2005, and kindly provided by M. Fisher (Imperial College London). All manipulations were performed under a laminar flow hood. This isolate was maintained in a TG liquid medium (1% Tryptone and 0.32% Glucose) by serial passage approximately every week (roughly 50 passages at the time of the experiments), at a temperature of 19°C. After one week of development, approximately three mL of mature liquid cultures were deposited on agar gels (1% Tryptone, 0.32% Glucose, 1% Agar) which were then sealed with parafilm® to maintain humidity. After five to six days at 19°C, we used one to three mL of sterile water to rinse the surface of the agar gels, waited 30 s for zoosporangia to release zoospores, and filtered the supernatant with a 10-µm mesh to only collect zoospores. The zoospore concentration of the resulting solution was assessed by averaging four counts on a Thoma haemocytometer under light microscopy (x100 magnification). For each count, a volume of 13µL was introduced in the chamber, and all motile zoospores were counted in all squares of the haemocytometer. Only motile zoospores were counted, as non-attached zoospores do not appear to develop into thalli in solution (i.e. unattached, in which case they are not viable; Woodhams *et al.* 2008). This solution was then used to introduce a known number of Bd zoospores in each container of our experimental settings (**Figure 36**). We measured zoospore concentrations at t+0h (time of zoospore introduction), and then regularly, in general at t+1h, t+3h, t+6h and t+23h, by sampling the water column four times at random places above the biofilm. All manipulations were carried out at room temperature.

### 5.2.2 Main experimental designs

#### 5.2.2.1 Setting 1: Laboratory-grown biofilms with fixed composition

We exposed Bd zoospores to simple biofilms grown in six-well sterile cell culture plates (Corning® Costar® 3506, made of non-pyrogenic polystyrene previously treated to increase cell attachment). By simple, we mean that we controlled the identity of the species producing the biofilm. We selected three autotrophic algae: *Nitzschia palea*, *Mayamea permitis* (both diatoms), and *Leptolyngbya sp*. (a cyanobacterium) for their ability to quickly grow a biofilm in laboratory conditions, and because cyanobacteria and diatoms are building blocks of mountain benthic biofilms (Chapter 3). Each species was initially kept and grown separately in an algal bank at 19°C and a photoperiod of 16 h light/8h dark (light intensity: 30-40 µmol.s$^{-}$

$^{1}$.m$^{-2}$), in the nutritive medium COMBO for diatoms and BG11 for the cyanobacterium (Stanier *et al.* 1971; Kilham *et al.* 1998). We harvested each alga separately in a 50 mL sterile Falcon® tube. For diatoms, Falcon® tubes were first vortexed for five minutes to break cell aggregates and cell concentration was then estimated with a Malassez counting chamber under the microscope (magnification x100). In each well, we introduced six mL of *M. permitis* (5 x $10^6$ cells/mL), or six mL of *N. palea* (2.5 x $10^6$ cells/mL, as *N. palea* is roughly twice as large as *M. permitis*). For *Leptolyngbya* sp., we used a highly-efficient disperser (Ultra-Turax T25, ®Janke & Kunhel, Ika Labortechnik) during one minute. Recalcitrant aggregates were manually removed and cell concentration was then estimated with a spectrophotometer (wavelength of 663 nm) to obtain six mL of a solution with an absorbance between 0.290 and 0.295. We also produced a biofilm containing a mix of the three algae (hereafter called "Mix"), by introducing into each biofilm well six mL of a mix solution (*M. permitis* of 5x$10^6$ cells/mL, *N. palea* of 2.5 x $10^6$ cells/mL, and *Leptolyngbya* sp. an absorbance of 290-295 at 663nm).

We used two six-well plates with, for each, three biofilm wells and three control wells (**Figure 36**). In the control wells was placed a volume of six mL of the corresponding medium used to grow the algae. The medium of the Mix controls consisted of two mL of BG11 plus four mL of COMBO. Biofilms were left to grow on the bottom of the wells at 19°C, under light intensity of 80 µmol.s$^{-1}$.m$^{-2}$ (photoperiod: 14h light/10h dark), and parafilm sealed cover, during seven days, with the medium renewed every two days during week days (every three days over the week end), by meticulously collecting three mL of the water column and reintroducing three mL of the appropriate medium (one mL of BG11 and two mL of COMBO for wells of treatment "Mix"). The experiment started when three mL of zoospore solution were introduced into each well (biofilms and controls). To ensure that the total volume remains six mL, the corresponding volume was previously removed from the well water column.

#### 5.2.2.2 Setting 2: Field-grown biofilms with complex composition

In these experiments, we tested the effects of natural, supposedly more complex biofilms. To do so, we used two by two cm ceramic tiles which we immersed in multiple outside ponds for at least six months. Tiles were placed in summer 2021 in mountain lakes Arlet (42° 50' 25.368" N, 0° 36' 54" W), Puits d'Arrious (42° 51' 50.508" N; 0° 38' 0.348" W), Paradis (42° 50' 56.1"N 0° 09' 37.3"W), Gourg de Rabas (42° 51' 8.964" N, 0° 8' 42.2124" E), Arbu (42° 49' 13.632" N, 1° 26' 16.303" E), and in an artificial pond in Cazavet (42° 59' 49.245" N, 1° 2' 9.326" E). Except from Cazavet, information about biofilm composition can be found in **Figure 20-23**. The tiles were recovered one day before the beginning of the experiment, and were placed in tight boxes on humidified paper towels, carefully transported to the laboratory and

stored at 4-6°C until the experiment began. Each tile was placed in a small Petri dish (BD Falcon® 351008, 35mm diameter, 10mm height, made of untreated non-pyrogenic polystyrene). At the beginning of the experiment, biofilms were covered with three mL of zoospore solution in the dish. Zoospores were then counted as in the previous experiment. For each treatment, we used three tiles to have as many biofilm replicates and three controls. The controls consisted of Petri dish filled with three mL of sterile ultra-pure water (**Figure 36**).

#### 5.2.2.3 Setting 3: Laboratory-grown biofilms, with complex composition

Because zoospores disappeared much faster in experiments of setting 2 compared to setting 1, including in the controls, we slightly changed the protocol of setting 2. Instead of using ultrapure water to rinse zoospores from agar gels, we used mineral water (Volvic®). Due to logistic constraints, we used a complex biofilm grown in the laboratory to have more replicates available and more biomass compared to field-grown biofilms. We set similar ceramic tiles in 2L tanks filled with previously dechlorinated tap water. We placed in the water column a net containing 10 g of shredded dead oak leaves, for at least three weeks under the same lighting conditions as in setting 1. Shredded leaves inoculated organic nutrients and a variety of microorganisms in the water column. These dead leaves were sampled in July 2022 outside the laboratory (43° 33' 27.705" N, 1° 34' 12.324" E). After three weeks, a considerable biofilm grew and four tiles were individually placed in a Petri dish with three mL of BG11. We waited three days before starting the experiments, in order to let the biofilms recover from the transfer from the tank to the dish. Then, dishes were emptied and 3 mL of zoospore solution was poured, marking the beginning of the experiment. We replicated this a second time, making a total of eight biofilm replicates and eight controls (**Figure 36**).

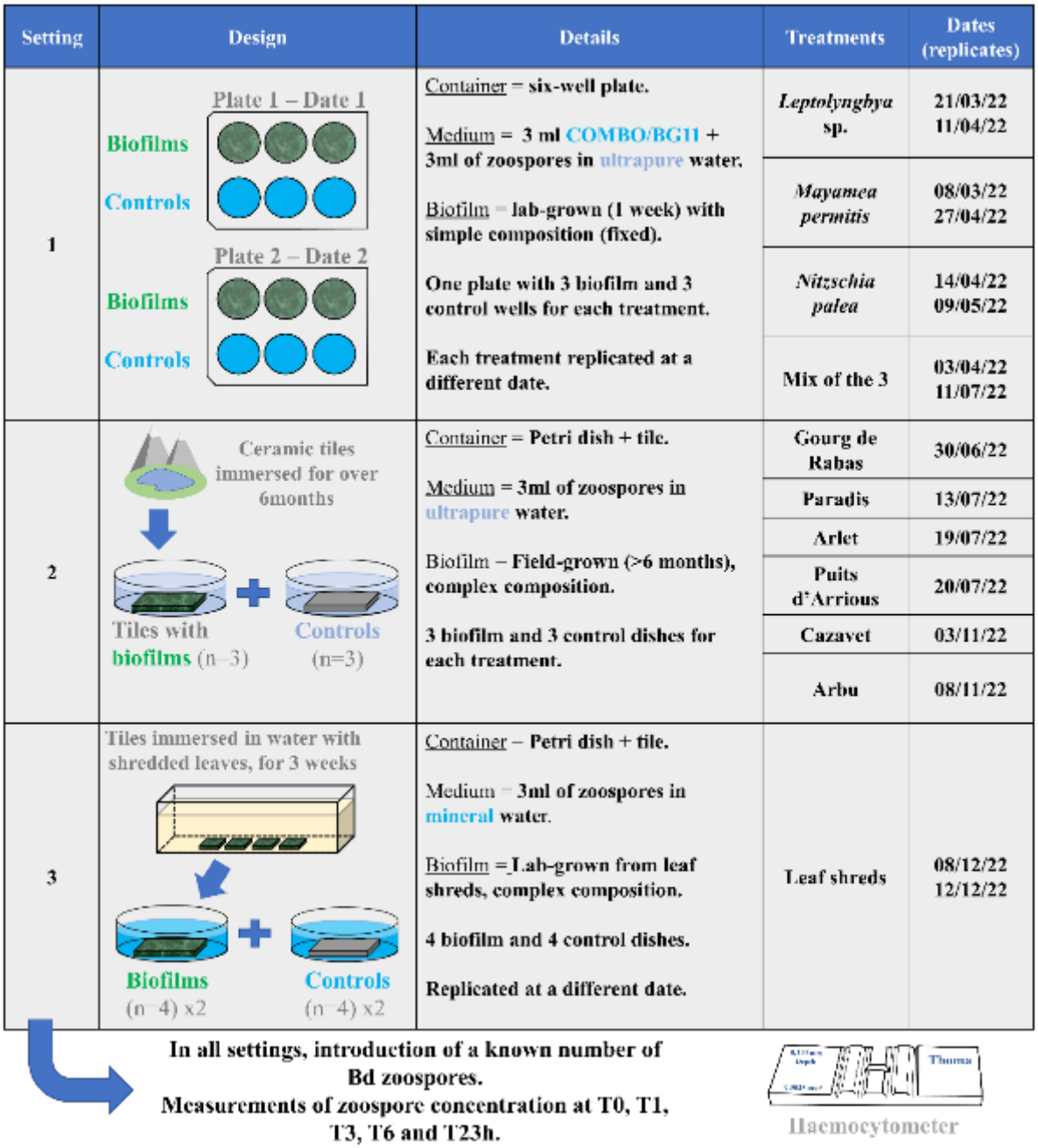


**Figure 36: Summary of the three experimental designs (settings) employed in this study.** In setting 1, either COMBO and/or BG11 has been used depending on the algae being grown (see Methods). Abbreviations: 6m: 6 months.

### 5.2.3 Statistical analysis

All numerical analyses were performed with the software R v4.2.0 (R Core Team 2022). The number/concentration of Bd zoospores over time followed an exponential decay law, meaning that it decreases at a rate proportional to its current value (Woodhams *et al.* 2008). Over this short period of time, only mortality and encystment (on the sides of the wells or maybe

in the biofilms) can affect zoospore concentration (Woodhams *et al.* 2008). Therefore, the rate of change of the concentration of zoospore can be described by:

$$\frac{dZ(t)}{dt} = -(m_z + f_z).Z(t) \qquad (1a)$$

Where Z(t) is the concentration in motile zoospores at time t, $m_z$ is the mortality rate constant of zoospore and $f_z$ their encystment rate constant. The zoospore disappearance constant λ can be defined as

$$\lambda = m_z + f_z \qquad (2)$$

which means that equation (1) can be written:

$$\frac{dZ(t)}{dt} = -\lambda.Z(t) \qquad (1b)$$

One primitive function of equation 1b is

$$Z(t) = Z(0).\mathrm{e}^{-\lambda.t} \qquad (3)$$

Equation (3) is the classic formula descrbing an exponential decay law and, using this equation, we fitted a non-linear model to all our data. We used the function glns() of the R package *nlme* (Pinheiro and Bates 2000). We initially aimed to fit a mixed model with date as random effects to account for the fact that replicates were not made simultaneously, and "well_ID" (or Petri dish_ID for complex biofilms) to account for the fact that observations from the same well/dish are not independent. However, the random effect specification (function nlme(), with one or the two abovementioned random effects) led to convergence errors; therefore, we fitted model with fixed effects only. We also fitted three models to the datasets corresponding to the three different settings, and there, it was possible to include random effects (except for setting 2), and results (significance of pairwise comparisons between treatments) were nearly identical. For clarity, we only present results of the global model.

The model estimates two parameters for each treatment (i.e. each kind of biofilm and its respective control): the intercept Z(0), i.e. the initial zoospore concentration, and the zoospore disappearance constant λ. We tried different variance structure and selected the best model using the corrected Akaike Information Criterion (AICc, Sakamoto *et al.* 1986). We found that allowing the variance to vary exponentially with time and treatment (specification of the parameter *weights = varExp(form = ~ Time|treatment*) greatly improved model fits.

We compared the model estimates of the zoospore disappearance constants λ from each treatment two by two with the function emmeans of the eponym package (Lenth 2022). First, we compared the estimated zoospore disappearance constant of each treatment with that of its control. Then, we compared the zoospore disappearance constant of the different controls with

each other. They were often significantly different. Therefore, to compare zoospore disappearance between biofilm treatments, we corrected each λ by its control (i.e. $\lambda_{weighed} = \lambda_{biofilm} - \lambda_{control}$). The parameter $\lambda_{weighed}$ is automatically calculated by *emmeans* when comparing biofilm and control disappearance rates. We extracted the weighed disappearance constants $\lambda_{weighed}$ and conducted pairwise comparisons on them, again with *emmeans*. In all cases, we used the Šidák correction to adjust p-values for multiple comparisons (Šidák 1967).

### 5.2.4 Additional experiment: comparisons of different media

To test whether the media used in the different settings influence zoospore concentrations and our ability to detect biofilm effects, if any, we conducted a small experiment where zoospores were introduced into three different media and their concentration monitored over time. We used ultra-pure water, COMBO, and mineral water (Volvic®). Agar gels with Bd were rinsed with these media, and the resulting solutions were randomly placed in wells of two six-well plates, with a total of three wells per medium. We repeated this a second time, at another date, using Petri dishes instead of the six-well plates, with three dishes per medium. A mixed linear model was fitted to these data using function nlme. The same specification as above was used (including the variance structure), except that a variable "medium-experiment ID" (e.g. COMBO -1$^{st}$ experiment, COMBO - 2$^{nd}$ experiment, ultra-pure -1$^{st}$ experiment, etc) was added as random effect. *Emmeans* was again used to provide statistical significance on pairwise comparisons.

## 5.3 Results

### 5.3.1 Main experiments

We fitted an exponential decay model to our data (**Figure 37**) estimating the zoospore disappearance constant λ for each treatment and its controls (**Figure 38**, **Table 6**). The zoospore disappearance constant was systematically greater in the presence of biofilms than in controls in settings 1 and 3 ($p < 0.0001$ in all cases except for *M. permitis*, $p = 0.005$). In setting 2, only with biofilms from Gourg de Rabas ($p < 0.0001$) and Paradis ($p = 0.021$) did zoospores disappear significantly faster than with the controls (**Figure 38**, **Table 6**). Even within a same setting, in which controls were realised in a similar way, the disappearance constants of controls were often significantly different (**Figure 38**, **Table 7**). This was especially true within setting 2, where controls had very high disappearance constants (from 0.082 to 0.723 $h^{-1}$), much greater than those observed in other settings (from 0.035 to 0.116 $h^{-1}$, **Table 7**).

After weighing, the biofilm consisting of a mix of the three algal species (Mix) in setting 1 had the greatest effect on zoospore disappearance ($\lambda_{Mix\_weighed}$ = 0.736 $h^{-1}$), followed by that of *Leptolyngbya* sp. (0.393 $h^{-1}$), the biofilm produced from shredded leaves (0.286, setting 3), *M. permitis* (0.209 $h^{-1}$, setting 1), Paradis (0.194 $h^{-1}$, setting 2) and, lastly, *N. palea* (setting 1) and Gourg de Rabas (setting 2; both 0.186 $h^{-1}$; **Figure 38**, **Table 8**). The disappearance constant of the "Mix" was significantly higher than that of all other biofilms (all p-values < 0.0001), and there were many other significant differences between the different abilities of biofilms to cause zoospores to disappear from the water column (**Figure 38**, **Table 8**).

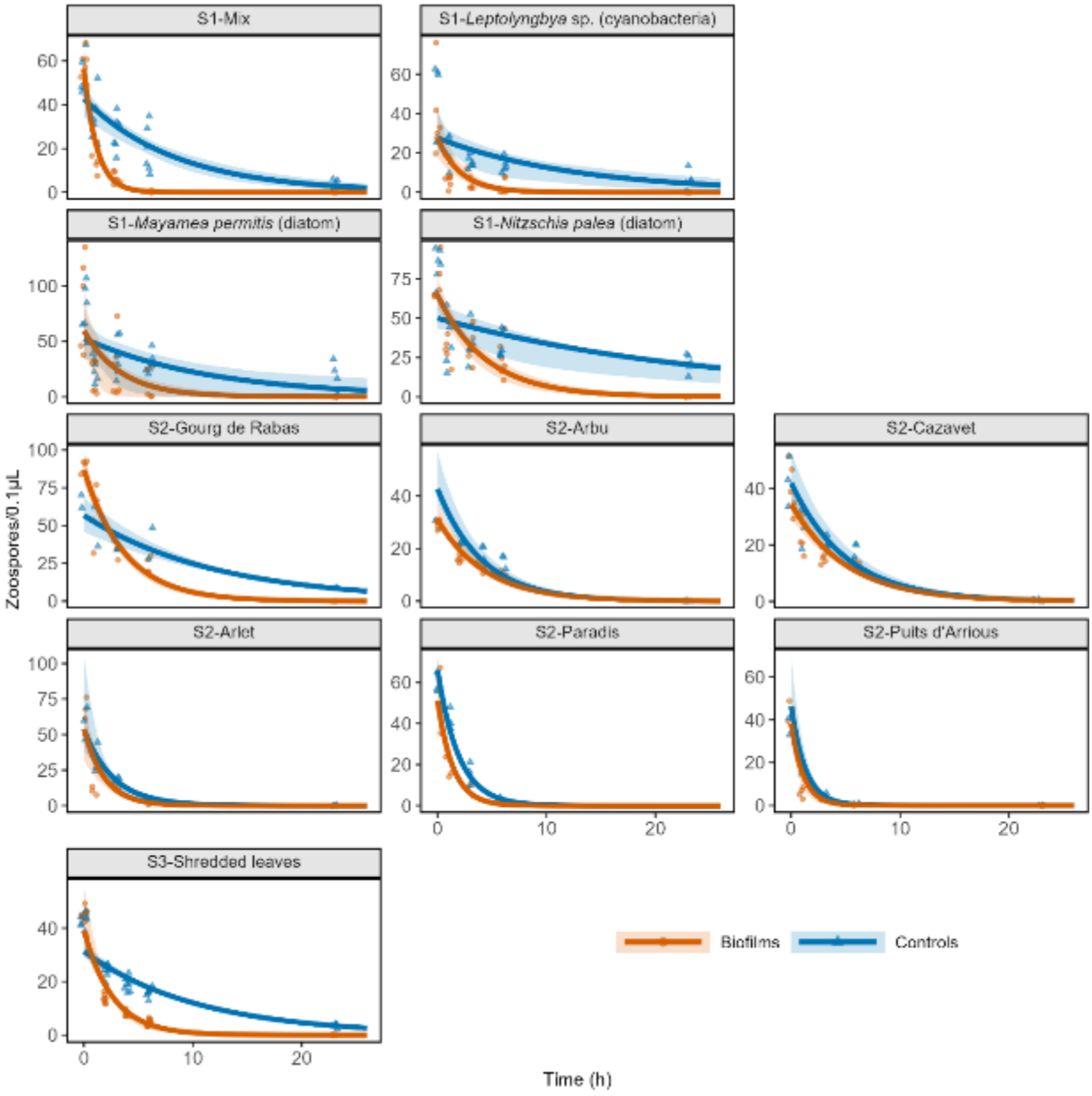


**Figure 37: Evolution of zoospore concentration with time when exposed to different biofilms.** Dots represent the data points (disks are for biofilms, triangles for controls), the solid lines are the fitted curves (exponential decay law, $Z = Z_0.e^{-\lambda t}$), and shaded areas are the 95% confidence intervals around the fitted values. Abbreviations S1, S2, S3 correspond to the settings 1, 2, 3 respectively.

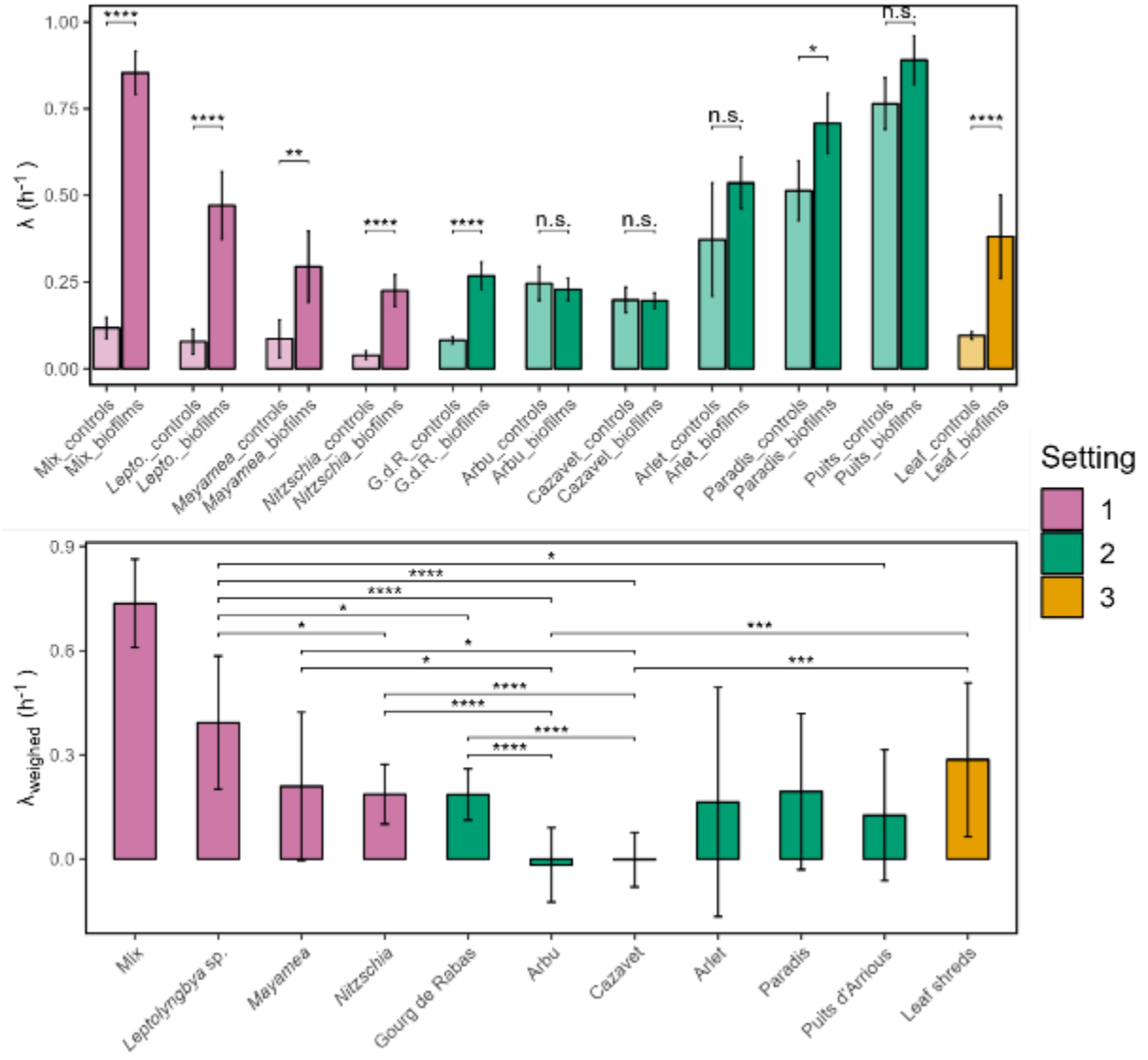


**Figure 38: Uncorrected (top) and corrected (bottom; $\lambda_{weighed} = \lambda_{biofilm} - \lambda_{control}$) zoospore disappearance constants for each treatment.** Significance levels are displayed: $p < 0.0001$ "****", $p < 0.001$ "***", $p < 0.01$ "**", $p < 0.05$ "*", $p < 0.1$ ".", $p > 0.1$ "n.s.". P-values were adjusted for multiple comparisons with the Šidák correction. For the bottom plot, comparisons between Mix and other treatments are not shown, but always extremely significant ($p < 0.0001$). Abbreviation: G.d.R. = Gourg de Rabas; Puits = Puits d'Arrious.

### 5.3.2 Additional experiment

Zoospores disappeared significantly faster in ultra-pure water than in mineral water ($p = 0.006$) and COMBO ($p = 0.029$). There was no significant difference in disappearance constant between COMBO and mineral water ($p = 0.835$) although zoospores declined slower in the latter (**Figure 39**, **Table 9**).

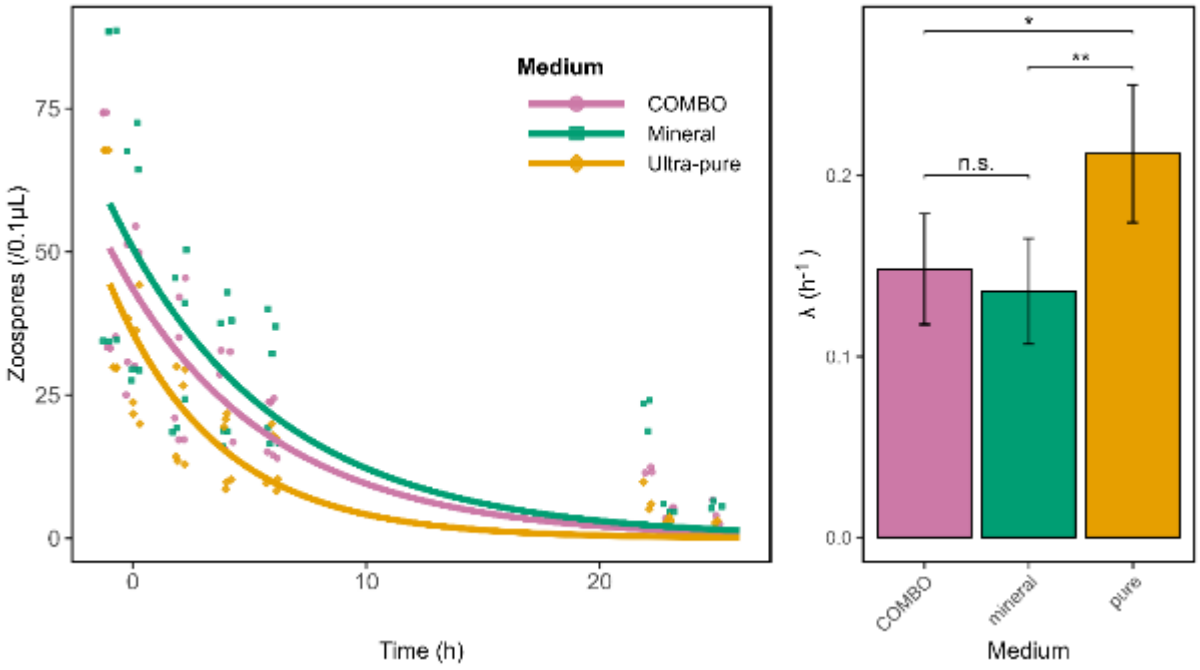


**Figure 39: Zoospore disappearance in different media containing different quantities of electrolytes.**

## 5.4 Discussion

Our results support both our hypotheses: that benthic biofilm can affect the fate of Bd zoospores in the water column by increasing the rate at which they disappear, and that distinct biofilms, in terms of the identity and number of species producing the matrix, have different ability to do so. The disappearance constants of biofilms were significantly greater than those of the controls in many of the tested biofilms. This demonstrates that environmental biofilms, even when they do not contain Bd consumers (biofilms from setting 1, all significantly effective), can influence zoospores by mechanisms that still remain to be determined. Although further experiments are needed to determine whether our results translate into an effective reduction in infection pressure for amphibians, our study is the first to demonstrate the existence of interactions between environmental biofilms and *Batrachochytrium dendrobatidis*.

The effects on zoospore concentration of biofilms from settings 1 and 3 were very clear. However, this was not the case for the majority of field-grown biofilms (setting 2), except Gourg de Rabas. In this setting, the uncorrected disappearance constants were high, but so were those of the controls, with disappearance constants about three to 20 times as great as the controls in the laboratory biofilm experiments. Essentially, the controls indicate the basal rate at which zoospore disappear in their environment, and these high disappearance constants suggest that something negatively affected them during most of the experiments in setting 2 but

not in settings 1 and 3. We suspected that this was related to temperature variations when the extremely high temperatures of July 2022 (when some the experiments in setting 2 were carried out) caused the cooling system of the laboratory to fail on at least two occasions. High temperatures are known to increase zoospore mortality rates, thereby inflating the disappearance constants (Woodhams *et al.* 2008). However, while temperature may well explain some variation in disappearance constants, it fails to explain why these high rates of disappearance persisted in fall 2022 (Arbu and Cazavet) when temperatures were cooler. Part of the explanation might lie in the chemical composition of the water column. In settings 1 and 3, the medium was made of a mix of ultra-pure water and algal medium (COMBO, BG11 or both), and mineral water respectively. In contrast, the medium in setting 2 consisted in ultra-pure water only. We showed in our additional experiment that ultra-pure water alone as a medium was somewhat unfavourable to zoospores. Both mineral water and algal media contain many more electrolytes than ultra-pure water (Stanier *et al.* 1971; Kilham *et al.* 1998). Our results support findings of Johnson and Speare (2003) which showed that Bd zoospores remained active longer in autoclaved lake water than in tap or ultra-pure water, suggesting that electrolytes and nutrients are important for zoospores to remain motile. Faced with more adverse conditions in ultra-pure water, it is possible that zoospores encysted more rapidly, and thus their disappearance rate increased. However, our additional experiments did not reproduce the high disappearance rates observed during setting 2 experiments. The type of container (surface-treatment to increase cell attachment for six-well plates but not Petri dishes) could play a role, but results from setting 3 experiments did not support this hypothesis, as there were no differences between replicates (two replicates have been done at different times, one with six-well plate, one with Petri dish). We think that results from setting 2 experiments should be taken with caution, as the high basal zoospore disappearance precluded the observation of the biofilm effect, if any. These experiments should be repeated with mineral water as medium.

Our experiments with simple laboratory-grown biofilms clearly show that the presence of a biofilm that contains no Bd consumer, but only one phototrophic alga, affects the fate of sympatric zoospores. This means that either physical and/or chemical interference of the matrix, allelopathy and/or nutrient depletion increase the mortality and/or encystment rates of zoospores. Our design does not allow to distinguish between the mechanisms at play here, nor does it allow us to know whether zoospores are actually inactivated, or attached to the sides of the wells or on the biofilm in a state of slowed/arrested development until conditions improve (Johnson and Speare 2003). Further study should elucidate this point, for instance by sampling at variable times after the experiment the lateral walls of the wells and a chunk of a biofilm which was in contact with Bd, and reintroduce that in the TG medium to examine whether a

new population of Bd develops, in which case Bd would still be viable (Johnson and Speare 2003). This is crucial to determine whether biofilms do contribute in reducing infection pressure for amphibians. Indeed, our results would not necessarily translate into a decreased infection risk if zoospores have encysted to persist in a state of slower development until conditions improve in the biofilm since tadpoles feed on them for most of their lives (Altig *et al.* 2007; Wells 2010). In this case, tadpoles would still be largely exposed to Bd when feeding on biofilms, and the latter would be a source and not a sink of infection.

The different composition of biofilms tested here were not equivalent in their ability to cause zoospore to disappear. Interestingly, the disappearance constant of the biofilm formed by the three algal species cultivated together was very significantly greater than that of all other biofilms, including those of its species taken alone. It is well known that multispecies biofilms can exhibit emergent properties, that is properties that cannot be explained by its single components, such as the increased sorption and the transport of nutrients (Flemming *et al.* 2016). Increased diversity in biofilms has been repeatedly shown to increase biofilm efficiency in removing and degrading organic and inorganic nutrients and chemicals from the water column (Sabater *et al.* 2002; Singer *et al.* 2010; Cardinale 2011; Peter *et al.* 2011; Burmølle *et al.* 2014). It is possible that the multispecies biofilm here was more efficient at catching and using nutrients, which would deplete the water column and force zoospores to encyst. Multispecies biofilms were reported to attain a higher biomass than monospecies biofilm in similar conditions (Murga *et al.* 1995; Burmølle *et al.* 2006; Chen *et al.* 2022), which may mean that such biofilms adsorb and use more nutrients from the water colum. The matrix of the multispecies biofilm can also have different properties than monospecies biofilms, in terms of architecture (roughness), of EPS composition, or in quantity produced (Murga *et al.* 1995; Elias and Banin 2012). While the significantly greater disappearance constant of the multispecies biofilm clearly suggests this biofilm is different in some aspects, we did not examine the composition of the multispecies biofilms at the end of the experiments nor compare their architecture and biomass.

Regarding monospecific biofilms only, the cyanobacterium *Leptolyngbya* sp. had more effects on zoospores than the two diatoms. Different monospecific biofilms, in terms of composition, were also shown to differentially retain *Cryptosporidium parvum* oocysts on their surface, but biofilm roughness (under environmental forcing) was shown to be the determinant factor explaining these differences (Searcy *et al.* 2006; Wolyniak-DiCesare *et al.* 2012). *Leptolyngbya* is known to produce filaments, and these filaments could physically interfere with the movement of zoospores. Alternatively, it can also produce toxic metabolites with

allelopathic effects that could induce either death or encystment (Leflaive and Ten-Hage 2007; Leão *et al.* 2009; Allen *et al.* 2016).

The multispecies biofilm from Gourg de Rabas and that produced from shredded leaves also made zoospores disappear significantly quicker than their controls. However, we expected an even higher disappearance constant for a biofilm that contains dozens if not hundreds of microbial species (at least in the case of Gourg de Rabas, Chapter 3). The disappearance constant of the biofilm from Gourg de Rabas was equivalent to that of the *N. palea* monospecific biofilm, the least effective biofilm tested in the laboratory. One explanation to the low disappearance constant of the natural biofilm may be that very little biomass, i.e. matrix, was visible on the ceramic tile compared laboratory experiments. Gourg de Rabas is a high-elevation oligotrophic lake (2400m, total organic carbon < 3mg/L, total nitrogen <0.5 mg/L and total phosphorus often undetectable) allowing very low productivity. Despite that, the biofilm from Gourg de Rabas still had an effect on zoospores, and predation by organisms attached to the biofilm may be one likely process at play here, given the very low biomass of the biofilm. Indeed, many organisms known to predate Bd zoospores in the plankton, such as rotifers and ciliates, frequently live in a sessile way in the biofilms, where they filter-feed on planktonic organisms (Weitere *et al.* 2003, 2018; Kathol *et al.* 2009; Mialet *et al.* 2013). This is the case of bdelloid rotifers, the DNA of which was shown to occur in Gourg de Rabas biofilms (Chapter 4).

We demonstrated that environmental benthic biofilms can affect the fate of Bd zoospores in the water column, and that their composition is an important factor in the strength of these effects. Further studies should determine whether zoospores only encyst more rapidly or are killed when in presence of biofilms, and if so, by what mechanisms, to know if biofilms can reduce infective pressure for amphibians. Benthic biofilms are currently undergoing significant compositional changes in natural settings (Chapter 3), and such experiments are needed to know if these changes will alter the epidemiology of amphibian chytridiomycosis and cause further damage to biodiversity and ecosystems.

# Supplementary Materials for chapter 5

**Table 6: Estimates of the zoospore disappearance constant λ of each treatment and its control, and of their respective difference ($\lambda_{weighed} = \lambda_{biofilm} - \lambda_{control}$).** SE = Standard error, df = degrees of freedom, CI = 95% confidence intervals.

| setting | Treatment | λ | SE | df | Lower CI | Upper CI | t ratio | p-value |
|---|---|---|---|---|---|---|---|---|
| | **λ** | | | | | | | |
| S1 | Mix controls | 0.117 | 0.016 | 447 | 0.087 | 0.148 | 7.479 | < 0.0001 |
| S1 | Mix biofilms | 0.853 | 0.032 | 447 | 0.791 | 0.915 | 27.055 | < 0.0001 |
| S1 | *Leptolyngbya* sp. controls | 0.078 | 0.018 | 447 | 0.042 | 0.114 | 4.274 | < 0.0001 |
| S1 | *Leptolyngbya* sp. biofilms | 0.471 | 0.050 | 447 | 0.373 | 0.569 | 9.470 | < 0.0001 |
| S1 | *Mayamea permitis* controls | 0.086 | 0.028 | 447 | 0.031 | 0.140 | 3.075 | 0.002 |
| S1 | *Mayamea permitis* biofilms | 0.295 | 0.052 | 447 | 0.192 | 0.397 | 5.666 | < 0.0001 |
| S1 | *Nitzschia palea* controls | 0.039 | 0.006 | 447 | 0.027 | 0.051 | 6.531 | < 0.0001 |
| S1 | *Nitzschia palea* biofilms | 0.225 | 0.023 | 447 | 0.180 | 0.270 | 9.769 | < 0.0001 |
| S2 | Arbu controls | 0.246 | 0.025 | 447 | 0.198 | 0.295 | 9.937 | < 0.0001 |
| S2 | Arbu biofilms | 0.229 | 0.016 | 447 | 0.197 | 0.261 | 14.167 | < 0.0001 |
| S2 | Arlet controls | 0.372 | 0.083 | 447 | 0.209 | 0.536 | 4.488 | < 0.0001 |
| S2 | Arlet biofilms | 0.536 | 0.038 | 447 | 0.461 | 0.611 | 14.098 | < 0.0001 |
| S2 | Cazavet controls | 0.198 | 0.019 | 447 | 0.162 | 0.235 | 10.597 | < 0.0001 |
| S2 | Cazavet biofilms | 0.197 | 0.011 | 447 | 0.175 | 0.218 | 17.814 | < 0.0001 |
| S2 | Gourg de Rabas controls | 0.082 | 0.005 | 447 | 0.073 | 0.092 | 16.261 | < 0.0001 |
| S2 | Gourg de Rabas biofilms | 0.268 | 0.020 | 447 | 0.229 | 0.307 | 13.577 | < 0.0001 |
| S2 | Paradis controls | 0.514 | 0.044 | 447 | 0.427 | 0.600 | 11.697 | < 0.0001 |
| S2 | Paradis biofilms | 0.707 | 0.044 | 447 | 0.621 | 0.794 | 16.120 | < 0.0001 |
| S2 | Puits d'Arrious controls | 0.765 | 0.038 | 447 | 0.690 | 0.839 | 20.160 | < 0.0001 |
| S2 | Puits d'Arrious biofilms | 0.890 | 0.036 | 447 | 0.820 | 0.961 | 24.838 | < 0.0001 |
| S3 | Leaf shreds controls | 0.095 | 0.005 | 447 | 0.085 | 0.106 | 17.793 | < 0.0001 |
| S3 | Leaf shreds biofilms | 0.381 | 0.061 | 447 | 0.261 | 0.501 | 6.225 | < 0.0001 |
| | $\lambda_{weighed} = \lambda_{biofilm} - \lambda_{control}$ | | | | | | | |
| S1 | Mix | 0.736 | 0.035 | 447 | 0.636 | 0.836 | 20.884 | < 0.0001 |
| S1 | *Leptolyngbya* sp. | 0.393 | 0.053 | 447 | 0.242 | 0.543 | 7.412 | < 0.0001 |
| S1 | *Mayamea* | 0.209 | 0.059 | 447 | 0.041 | 0.377 | 3.541 | 0.005 |
| S1 | *Nitzschia* | 0.186 | 0.024 | 447 | 0.119 | 0.254 | 7.824 | < 0.0001 |
| S2 | Gourg de Rabas | 0.186 | 0.020 | 447 | 0.128 | 0.244 | 9.105 | < 0.0001 |
| S2 | Arbu | -0.017 | 0.030 | 447 | -0.101 | 0.067 | -0.581 | 1 |
| S2 | Cazavet | -0.002 | 0.022 | 447 | -0.064 | 0.060 | -0.085 | 1 |
| S2 | Arlet | 0.164 | 0.091 | 447 | -0.096 | 0.423 | 1.793 | 0.569 |
| S2 | Paradis | 0.194 | 0.062 | 447 | 0.017 | 0.370 | 3.121 | 0.021 |
| S2 | Puits d'Arrious | 0.126 | 0.052 | 447 | -0.023 | 0.274 | 2.413 | 0.165 |
| S3 | Leaf shreds | 0.286 | 0.061 | 447 | 0.111 | 0.460 | 4.648 | < 0.0001 |

**Table 7: Pairwise comparisons of the zoospore disappearance constant λ between controls.** P-values were adjusted for multiple comparisons using Šidák correction. SE = Standard error, df= degrees of freedom, CI = 95% confidence intervals, GdR = Gourg de Rabas, Puits = Puits d'Arrious.

| $\lambda_{Control\ 1}$ | $\lambda_{Control\ 2}$ | estimate | SE | df | Lower CI | Upper CI | t ratio | p-value adj. |
|---|---|---|---|---|---|---|---|---|
| Arbu | Arlet | -0.126 | 0.087 | 447 | -0.415 | 0.162 | -1.457 | 1 |
| Arbu | Cazavet | 0.048 | 0.031 | 447 | -0.056 | 0.151 | 1.543 | 0.999 |
| Arbu | GdR | 0.164 | 0.025 | 447 | 0.080 | 0.248 | 6.475 | < 0.0001 |
| Arbu | Leaf shreds | 0.151 | 0.025 | 447 | 0.066 | 0.235 | 5.950 | < 0.0001 |
| Arbu | Leptolyngbya | 0.168 | 0.031 | 447 | 0.065 | 0.271 | 5.458 | < 0.0001 |
| Arbu | Mayamea | 0.161 | 0.037 | 447 | 0.036 | 0.285 | 4.305 | 0.001 |
| Arbu | Mix | 0.129 | 0.029 | 447 | 0.031 | 0.227 | 4.390 | < 0.001 |
| Arbu | Nitzschia | 0.207 | 0.025 | 447 | 0.122 | 0.292 | 8.136 | < 0.0001 |
| Arbu | Paradis | -0.267 | 0.050 | 447 | -0.435 | -0.099 | -5.303 | < 0.0001 |
| Arbu | Puits | -0.518 | 0.045 | 447 | -0.669 | -0.367 | -11.440 | < 0.0001 |
| Arlet | Cazavet | 0.174 | 0.085 | 447 | -0.109 | 0.458 | 2.047 | 0.902 |
| Arlet | GdR | 0.290 | 0.083 | 447 | 0.013 | 0.567 | 3.488 | 0.029 |
| Arlet | Leaf shreds | 0.277 | 0.083 | 447 | 0.000 | 0.554 | 3.332 | 0.05 |
| Arlet | Leptolyngbya | 0.294 | 0.085 | 447 | 0.011 | 0.577 | 3.463 | 0.032 |
| Arlet | Mayamea | 0.287 | 0.088 | 447 | -0.005 | 0.578 | 3.276 | 0.061 |
| Arlet | Mix | 0.255 | 0.084 | 447 | -0.026 | 0.536 | 3.019 | 0.137 |
| Arlet | Nitzschia | 0.334 | 0.083 | 447 | 0.056 | 0.611 | 4.009 | 0.004 |
| Arlet | Paradis | -0.141 | 0.094 | 447 | -0.454 | 0.172 | -1.504 | 1 |
| Arlet | Puits | -0.392 | 0.091 | 447 | -0.696 | -0.088 | -4.298 | 0.001 |
| Cazavet | GdR | 0.116 | 0.019 | 447 | 0.051 | 0.181 | 5.975 | < 0.0001 |
| Cazavet | Leaf shreds | 0.103 | 0.019 | 447 | 0.038 | 0.168 | 5.288 | < 0.0001 |
| Cazavet | Leptolyngbya | 0.120 | 0.026 | 447 | 0.033 | 0.207 | 4.592 | < 0.001 |
| Cazavet | Mayamea | 0.113 | 0.034 | 447 | 0.001 | 0.225 | 3.355 | 0.046 |
| Cazavet | Mix | 0.081 | 0.024 | 447 | -0.001 | 0.162 | 3.311 | 0.054 |
| Cazavet | Nitzschia | 0.159 | 0.020 | 447 | 0.094 | 0.225 | 8.117 | < 0.0001 |
| Cazavet | Paradis | -0.315 | 0.048 | 447 | -0.474 | -0.156 | -6.605 | < 0.0001 |
| Cazavet | Puits | -0.566 | 0.042 | 447 | -0.707 | -0.425 | -13.388 | < 0.0001 |
| GdR | Leaf shreds | -0.013 | 0.007 | 447 | -0.038 | 0.012 | -1.751 | 0.99 |
| GdR | Leptolyngbya | 0.004 | 0.019 | 447 | -0.059 | 0.068 | 0.227 | 1 |
| GdR | Mayamea | -0.003 | 0.028 | 447 | -0.098 | 0.091 | -0.114 | 1 |
| GdR | Mix | -0.035 | 0.017 | 447 | -0.090 | 0.020 | -2.120 | 0.856 |
| GdR | Nitzschia | 0.044 | 0.008 | 447 | 0.017 | 0.070 | 5.569 | < 0.0001 |
| GdR | Paradis | -0.431 | 0.044 | 447 | -0.579 | -0.284 | -9.754 | < 0.0001 |
| GdR | Puits | -0.682 | 0.038 | 447 | -0.810 | -0.555 | -17.827 | < 0.0001 |
| Leaf shreds | Leptolyngbya | 0.017 | 0.019 | 447 | -0.046 | 0.081 | 0.904 | 1 |
| Leaf shreds | Mayamea | 0.010 | 0.028 | 447 | -0.085 | 0.104 | 0.342 | 1 |
| Leaf shreds | Mix | -0.022 | 0.017 | 447 | -0.077 | 0.033 | -1.329 | 1 |
| Leaf shreds | Nitzschia | 0.056 | 0.008 | 447 | 0.030 | 0.083 | 7.049 | < 0.0001 |
| Leaf shreds | Paradis | -0.418 | 0.044 | 447 | -0.566 | -0.271 | -9.455 | < 0.0001 |
| Leaf shreds | Puits | -0.669 | 0.038 | 447 | -0.797 | -0.542 | -17.471 | < 0.0001 |
| Leptolyngbya | Mayamea | -0.008 | 0.033 | 447 | -0.119 | 0.104 | -0.226 | 1 |
| Leptolyngbya | Mix | -0.039 | 0.024 | 447 | -0.120 | 0.041 | -1.630 | 0.998 |
| Leptolyngbya | Nitzschia | 0.039 | 0.019 | 447 | -0.025 | 0.103 | 2.041 | 0.905 |
| Leptolyngbya | Paradis | -0.436 | 0.048 | 447 | -0.594 | -0.277 | -9.155 | < 0.0001 |
| Leptolyngbya | Puits | -0.686 | 0.042 | 447 | -0.827 | -0.546 | -16.302 | < 0.0001 |
| Mayamea | Mix | -0.032 | 0.032 | 447 | -0.138 | 0.075 | -0.993 | 1 |
| Mayamea | Nitzschia | 0.047 | 0.029 | 447 | -0.048 | 0.142 | 1.642 | 0.997 |
| Mayamea | Paradis | -0.428 | 0.052 | 447 | -0.601 | -0.255 | -8.228 | < 0.0001 |
| Mayamea | Puits | -0.679 | 0.047 | 447 | -0.836 | -0.522 | -14.424 | < 0.0001 |
| Mix | Nitzschia | 0.079 | 0.017 | 447 | 0.023 | 0.135 | 4.677 | < 0.001 |
| Mix | Paradis | -0.396 | 0.047 | 447 | -0.552 | -0.241 | -8.495 | < 0.0001 |
| Mix | Puits | -0.647 | 0.041 | 447 | -0.784 | -0.510 | -15.764 | < 0.0001 |
| Nitzschia | Paradis | -0.475 | 0.044 | 447 | -0.622 | -0.327 | -10.713 | < 0.0001 |
| Nitzschia | Puits | -0.726 | 0.038 | 447 | -0.854 | -0.598 | -18.903 | < 0.0001 |
| Paradis | Puits | -0.251 | 0.058 | 447 | -0.444 | -0.058 | -4.324 | 0.001 |

**Table 8: Pairwise comparisons of the zoospore disappearance constant $\lambda_{weighed}$ between treatments.** P-values were adjusted for multiple comparisons using Šidák correction. SE = Standard error, df= degrees of freedom, CI = 95% confidence intervals, GdR = Gourg de Rabas, Puits = Puits d'Arrious.

| λweighed $_1$ | λweighed $_2$ | estimate | SE | df | Lower CI | Upper CI | t ratio | p-value adj. |
|---|---|---|---|---|---|---|---|---|
| Arbu | Arlet | 0.181 | 0.096 | 447 | -0.139 | 0.501 | 1.885 | 0.967 |
| Arbu | Cazavet | 0.015 | 0.037 | 447 | -0.107 | 0.138 | 0.418 | 1 |
| Arbu | GdR | 0.203 | 0.036 | 447 | 0.083 | 0.323 | 5.644 | < 0.0001 |
| Arbu | Leaf_shreds | 0.303 | 0.068 | 447 | 0.075 | 0.530 | 4.439 | < 0.001 |
| Arbu | Leptolyngbya | 0.410 | 0.061 | 447 | 0.208 | 0.612 | 6.754 | < 0.0001 |
| Arbu | Mayamea | 0.226 | 0.066 | 447 | 0.006 | 0.446 | 3.426 | 0.036 |
| Arbu | Mix | 0.753 | 0.046 | 447 | 0.600 | 0.906 | 16.365 | < 0.0001 |
| Arbu | Nitzschia | 0.203 | 0.038 | 447 | 0.077 | 0.330 | 5.356 | < 0.0001 |
| Arbu | Paradis | 0.211 | 0.069 | 447 | -0.018 | 0.440 | 3.067 | 0.119 |
| Arbu | Puits | 0.143 | 0.060 | 447 | -0.057 | 0.343 | 2.385 | 0.621 |
| Arlet | Cazavet | -0.166 | 0.094 | 447 | -0.478 | 0.147 | -1.764 | 0.989 |
| Arlet | GdR | 0.022 | 0.094 | 447 | -0.290 | 0.334 | 0.235 | 1 |
| Arlet | Leaf shreds | 0.122 | 0.110 | 447 | -0.245 | 0.489 | 1.108 | 1 |
| Arlet | Leptolyngbya | 0.229 | 0.106 | 447 | -0.123 | 0.581 | 2.170 | 0.818 |
| Arlet | Mayamea | 0.045 | 0.109 | 447 | -0.317 | 0.408 | 0.417 | 1 |
| Arlet | Mix | 0.572 | 0.098 | 447 | 0.246 | 0.898 | 5.848 | < 0.0001 |
| Arlet | Nitzschia | 0.023 | 0.094 | 447 | -0.292 | 0.337 | 0.239 | 1 |
| Arlet | Paradis | 0.030 | 0.110 | 447 | -0.338 | 0.398 | 0.273 | 1 |
| Arlet | Puits | -0.038 | 0.105 | 447 | -0.388 | 0.313 | -0.359 | 1 |
| Cazavet | GdR | 0.187 | 0.030 | 447 | 0.088 | 0.287 | 6.293 | < 0.0001 |
| Cazavet | Leaf shreds | 0.287 | 0.065 | 447 | 0.070 | 0.505 | 4.410 | < 0.001 |
| Cazavet | Leptolyngbya | 0.395 | 0.057 | 447 | 0.204 | 0.585 | 6.890 | < 0.0001 |
| Cazavet | Mayamea | 0.211 | 0.063 | 447 | 0.001 | 0.420 | 3.352 | 0.047 |
| Cazavet | Mix | 0.738 | 0.041 | 447 | 0.600 | 0.876 | 17.821 | < 0.0001 |
| Cazavet | Nitzschia | 0.188 | 0.032 | 447 | 0.081 | 0.296 | 5.836 | < 0.0001 |
| Cazavet | Paradis | 0.196 | 0.066 | 447 | -0.024 | 0.415 | 2.974 | 0.157 |
| Cazavet | Puits | 0.128 | 0.057 | 447 | -0.061 | 0.316 | 2.260 | 0.742 |
| GdR | Leaf shreds | 0.100 | 0.065 | 447 | -0.116 | 0.316 | 1.543 | 0.999 |
| GdR | Leptolyngbya | 0.207 | 0.057 | 447 | 0.018 | 0.396 | 3.647 | 0.016 |
| GdR | Mayamea | 0.023 | 0.062 | 447 | -0.185 | 0.231 | 0.373 | 1 |
| GdR | Mix | 0.550 | 0.041 | 447 | 0.415 | 0.686 | 13.515 | < 0.0001 |
| GdR | Nitzschia | 0.001 | 0.031 | 447 | -0.104 | 0.105 | 0.019 | 1 |
| GdR | Paradis | 0.008 | 0.065 | 447 | -0.210 | 0.226 | 0.124 | 1 |
| GdR | Puits | -0.060 | 0.056 | 447 | -0.246 | 0.127 | -1.066 | 1 |
| Leaf shreds | Leptolyngbya | 0.107 | 0.081 | 447 | -0.163 | 0.378 | 1.321 | 1 |
| Leaf shreds | Mayamea | -0.077 | 0.085 | 447 | -0.360 | 0.207 | -0.899 | 1 |
| Leaf shreds | Mix | 0.450 | 0.071 | 447 | 0.214 | 0.686 | 6.359 | < 0.0001 |
| Leaf shreds | Nitzschia | -0.099 | 0.066 | 447 | -0.319 | 0.120 | -1.507 | 1 |
| Leaf shreds | Paradis | -0.092 | 0.087 | 447 | -0.383 | 0.199 | -1.051 | 1 |
| Leaf shreds | Puits | -0.160 | 0.081 | 447 | -0.428 | 0.109 | -1.980 | 0.934 |
| Leptolyngbya | Mayamea | -0.184 | 0.079 | 447 | -0.448 | 0.081 | -2.317 | 0.688 |
| Leptolyngbya | Mix | 0.343 | 0.064 | 447 | 0.131 | 0.555 | 5.393 | < 0.0001 |
| Leptolyngbya | Nitzschia | -0.206 | 0.058 | 447 | -0.400 | -0.013 | -3.554 | 0.023 |
| Leptolyngbya | Paradis | -0.199 | 0.082 | 447 | -0.471 | 0.073 | -2.437 | 0.569 |
| Leptolyngbya | Puits | -0.267 | 0.074 | 447 | -0.515 | -0.019 | -3.587 | 0.02 |
| Mayamea | Mix | 0.527 | 0.069 | 447 | 0.298 | 0.756 | 7.667 | < 0.0001 |
| Mayamea | Nitzschia | -0.023 | 0.064 | 447 | -0.235 | 0.189 | -0.357 | 1 |
| Mayamea | Paradis | -0.015 | 0.086 | 447 | -0.301 | 0.270 | -0.177 | 1 |
| Mayamea | Puits | -0.083 | 0.079 | 447 | -0.346 | 0.179 | -1.054 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mix | Nitzschia | -0.550 | 0.043 | 447 | -0.691 | -0.408 | -12.924 | < 0.0001 |
| Mix | Paradis | -0.542 | 0.071 | 447 | -0.780 | -0.304 | -7.594 | < 0.0001 |
| Mix | Puits | -0.610 | 0.063 | 447 | -0.820 | -0.400 | -9.686 | < 0.0001 |
| Nitzschia | Paradis | 0.008 | 0.066 | 447 | -0.214 | 0.229 | 0.113 | 1 |
| Nitzschia | Puits | -0.060 | 0.057 | 447 | -0.252 | 0.131 | -1.052 | 1 |
| Paradis | Puits | -0.068 | 0.081 | 447 | -0.338 | 0.202 | -0.837 | 1 |

**Table 9: Results of the additional experiment**. For the contrasts, p-values were adjusted with Tukey's post-hoc test.

| | Estimate | SE | df | Lower CI | Upper CI | t ratio | p-value adj. |
|---|---|---|---|---|---|---|---|
| $\lambda_{Medium}$ | | | | | | | |
| COMBO | 0.148 | 0.015 | 106 | 0.118 | 0.179 | 9.616 | < 0.0001 |
| mineral | 0.136 | 0.015 | 106 | 0.107 | 0.165 | 9.236 | < 0.0001 |
| Ultra-pure | 0.212 | 0.019 | 106 | 0.174 | 0.250 | 11.052 | < 0.0001 |
| Contrasts ($\lambda_1 - \lambda_2$) | | | | | | | |
| COMBO - mineral | 0.012 | 0.021 | 106 | -0.038 | 0.063 | 0.573 | 0.835 |
| COMBO – ultra-pure | -0.064 | 0.025 | 106 | -0.122 | -0.005 | -2.594 | 0.029 |
| mineral – ultra-pure | -0.076 | 0.024 | 106 | -0.134 | -0.019 | -3.144 | 0.006 |

# CHAPTER 6
# DISCUSSION AND PERSPECTIVES

# Chapter 6: Discussion and perspectives

My thesis addresses aspects of microbial community ecology and disease epidemiology, exploring some links between these two fields in the context of mountain lake ecosystems. While the importance of host-assiociated microbial communities to host health is becoming increasingly apparent and acknowledged (Clemente *et al.* 2012; Ezenwa *et al.* 2012; Sampson and Mazmanian 2015; Fung *et al.* 2017), that of environmental microbial communities (i.e. outside the host) have rarely been considered, let alone evaluated. Yet, micro-organisms surround hosts and fuel a multitude of ecosystem processes that, in turn, can benefit or harm hosts, or benefit or harm their pathogens or parasites. Thus, environmental microbial communities are bound to affect host health and may influence the epidemiology of their infectious diseases in some ways. Here, using a combination of field studies and laboratory experiments, I provided the first answers to the question of whether benthic biofilm communities play a role (or some roles) in Bd infection dynamics in amphibian populations. I also generated new knowledge on the determinants and status of biofilm biodiversity in mountain freshwater ecosystems. Although some work remains to be done to elucidate the epidemiological roles of biofilms with regards to amphibian chytridiomycosis, I give here some perspectives for further research.

## 6.1 On biofilms in general

In Chapter 2, using a holistic approach to health and sustainability, I illustrated how biofilm microbial communities can impact on the health of various biological levels of organisation, from multicellular individuals to entire ecosystems (Sentenac *et al.* 2022). I have endeavoured to break down disciplinary silos to provide translational knowledge (Ciesielski *et al.* 2017). In doing so, I highlighted many knowledge gaps in biofilm research, in particular that little was known on the roles of biofilms in the epidemiology of infectious diseases, apart from human waterborne diseases in the context of drinking water distribution systems (Wingender and Flemming 2011). This is the case for wildlife and plant emerging diseases, including the amphibian chytridiomycosis. Using the disease pyramid framework, I argued, however, that biofilms could influence disease dynamics in a variety of ways, impacting the host, the pathogen, and/or their environment (Bernardo-Cravo *et al.* 2020). Given the ubiquity and abundance of biofilms (Battin *et al.* 2016; Flemming and Wuertz 2019; Sentenac *et al.* 2022), this work can apply to many host-parasite systems, not only to Bd-amphibians, or not only to those parasites that have a free-living stage. As a matter of fact, most of what is known about

the interactions between biofilms and pathogens/parasites relates to viruses or bacteria (Hall-Stoodley and Stoodley 2005; Wingender and Flemming 2011), and not to pathogenic fungi, while the latter are of substantial concern for sustainability (Fisher *et al.* 2012). More than just reviewing the existing literature, I leveraged existing ecological theories such **as the theory of alternative stable states** to coin the concept of biofilm health, characterized by their structure, productivity, and resilience, to help guiding future research and scientific outreach with a theoretical, easily comprehensible framework (which I implicitly used later on).

## 6.2 On biofilms in Pyrenean mountain lakes

Most of my work in this thesis has focused on analysing the composition of prokaryotic and micro-eukaryotic assemblages of benthic biofilm communities of Pyrenean mountain lakes (Chapter 3). I produced many results, and only reported the most interesting and robust ones. For instance, I analysed the characteristics of lakes that are most unstable (β-dispersion) or most prone to decline in biofilm prokaryotic α-diversity. However, because I only have a "population" of 26 lakes, these results lacked robustness and were not included. In Chapter 3, I first characterized the ways climate change manifested itself in our study sites, with more precipitations and smaller daily temperature range (because temperatures do not drop as low as they did in the past) in western sites, while it is getting considerably hotter and the daily temperature range increased in eastern sites (greater maximal temperatures). These differences in climate change do not seem to drive the spatial dissimilarities in biofilm compositions nor the rate at which these compositions change over time. Indeed, I found that, within a same lake, the compositions of the prokaryotic and micro-eukaryotic assemblages in the biofilms became increasingly dissimilar as the time between samples increased (more pronounced for prokaryotes), indicating a shift in biofilm community structure. I also demonstrated that, in general, biofilms lost prokaryotic, but not micro-eukaryotic, biodiversity over time. To understand more precisely how these changes are reflected in taxonomic composition, I studied the temporal trends in richness and relative abundance of indicator taxa such as cyanobacteria and diatoms. I found that the richness and relative abundance of cyanobacteria generally increased over time in prokaryotic assemblages, while the opposite occurred for diatoms in micro-eukaryotic assemblages.

High-throughput sequencing data are compositional: the total number of reads detected for each sequence is limited by the capacity of the sequencing instrument and data are converted to proportions (relative abundance; Gloor *et al.* 2017). This means that, taking our results and the example of cyanobacteria, we do not know whether they have actually increased over time

in absolute abundance, or whether they have remained stable while other prokaryotic organisms have decreased, or whether they decreased over time but at a lower rate than other prokaryotic organisms. All these scenarios would lead to the same observations. In this respect, other methods that allow absolute quantification of microoganisms, e.g. with flow cytometry, are needed to confirm the observed trends (Props *et al.* 2017; Hamard *et al.* 2021). Furthermore, our methological approach does not allow for a direct analysis of prokaryotic and micro-eukaryotic assemblages *in tandem*, whereas it would have been useful here to know whether cyanobacteria increased at the expense of diatoms (since it actually is the same biofilm community). This is due to the fact that the marker genes used for the metabarcoding of prokaryotes and micro-eukaryotes are different (16S vs. 18S rRNA genes, respectively). That said, it is acknowledged that diatoms are sensitive organisms, while cyanobacteria are tolerant to many stressors and have a tendency to proliferate under some conditions adverse to other micro-organisms (Morin *et al.* 2016; Huisman *et al.* 2018; Wood *et al.* 2020; Ossyssek *et al.* 2022). Following stress, cyanobacteria tend to replace diatoms in freshwater biofilms (Leflaive *et al.* 2015; Crenier *et al.* 2019). In other threatened ecosystems, such as coral reefs, cyanobacterial biofilms are also known to replace less tolerant organisms (de Bakker *et al.* 2017; Ford *et al.* 2018). Taken together, our results suggest that biofilm health in mountain lakes is deteriorating as benthic biofilm compositions are changing towards more cyanobacteria-dominated communities. I found no evidence of a complete compositional shift towards a new stable state in any of the lakes (Abreu *et al.* 2020; Wang *et al.* 2021), which might be due to the relatively short duration of the study (5 years). I have discussed at length, in Chapter 3, the implications of such compositional changes for the local economies (impact on recreational activities and farming) and the health of human, animal, and the entire mountain freshwater ecosystem.

As to why these biofilms have changed, our study is field-based and observational, thus correlational in essence. We are consequently unable to disentangle the respective impacts of each anthropogenic or natural pressure on mountain lakes. However, the threats of mountain freshwater ecosystems are relatively well known (Schmeller *et al.* 2018, 2022), with climate change considered the number one threat as its impacts are stronger in elevation than in lowlands (Rangwala and Miller 2012; Pepin *et al.* 2015; Schmeller *et al.* 2022). We imputed these compositional shifts (at least some of them) to climate change effects because, based on the data and other results available, this seemed the most parcimonious explanation. Indeed, other studies showed that some mountain lakes, specifically those of the Pyrenees, were prone to basification due to the increasing melting ice, the alteration of mixing regime, and the intensification of erosion as a result of climate change (Curtis *et al.* 2009; Rogora *et al.* 2020).

Yet, we found that water pH and hardness, more than geographic location, were the most important factors explaining between-lake differences in the composition of both prokaryotic and micro-eukaryotic assemblages. This led us to argue that the climate change effects on water chemistry (increasing pH and hardness) were driving change in biofilm microbial composition.

However, the observed compositional changes might be the result of a combination of stressors acting simultaneously as, unfortunately, there are many other pressures than climate change in mountains ecosystems. Chemical pollution is one of them (Schwarzenbach *et al.* 2006; Schmeller *et al.* 2018; Machate *et al.* 2023). A recent study in some of the lakes studied here found evidence of contamination by a wide range of synthetic compounds (more than 150 different ones), including insecticides diazinon and permethrine, in concentrations so high that it could drive acute toxic risks for crustaceans but not algae (Machate *et al.* 2022). Pollution effects on biofilm composition in our study system should be assessed (Sabater *et al.* 2007; Proia *et al.* 2013a). Except from the negative effects of copper effects on the richness and relative abundance of cyanobacteria, I did not find evidence in my analyses of a trace element impacting the α-diversity or β-diversity of prokaryotic or micro-eukaryotic assemblages, but more data should be collected. Similarly, I investigated whether the presence of fish, livestock and tourism could have any effects on biofilm biodiversity, but found no association whatsoever. I used indices created in the past by other members of my research team, based on on-site observations and data from the Federation de Peche (fishing federation). All these variables are qualitative, so rather coarse, and more data is needed to test the effects of these factors. I also looked at whether livestock presence and abundance are associated with the concentrations of nitrite, nitrate, or total organic nitrogen detected in lake water, but again there was no associations. I think that interventional, rather than observational, studies are warranted here to assess the existence and strenght of these anthropogenic pressures, for instance by comparing the composition of biofilm communities before and after fish removal (although this is still not yet on the agenda in the French Pyrenees because of economico-political reasons, it will be implemented in some lakes for research purposes; https://www.biodiversa.eu/2022/10/25/fishme/) and/or partial exclusion of livestock/humans. The Limno Pirineus Life project reported promising results in this respect (http://www.lifelimnopirineus.eu/sites/default/files/laymanen_0.pdf). First, the elimination of minnows led to marked increase in the abundance of crustaceans, a decrease in phytoplankton biomass, and a decrease in water turbidity. Second, complete exclusion of livestock from threatened habitats like tufa-forming spring, or partial exclusion by the construction of watering troughs to reduce livestock attendance in wetlands or lake shorelines, greatly limited habitat

degradation. Similarly, to reduce the impact of tourism, elevated wooden platforms have been built to limit the impact of trampling on the wetlands.

There are weaknesses in the study of Chapter 3 of which I am fully aware. Many environmental variables had missing data points, and although I found ways to deal with that, this greatly complicated variable selection, susbsequent analyses and the robustness of our results, as least with regards to the analyses of environmental drivers (but not temporal trends; Altman and Bland 2007; Buuren and Groothuis-Oudshoorn 2011). Similarly, in terms of design flaws, biofilms were very intensively sampled in the first three years (2016, 2017, 2018) compared to the last two years (2019 and 2020), and we have data only for a subset of the lakes in 2020. I do have biofilm data from all lakes in 2020, but not from natural substrates. This is because I had also placed artificial substrates for other purposes on which I was focusing at that time. I did not systematically sample biofilms from natural substrates, which I regret. For the sake of consistency, I only analysed biofilms from natural substrates, but if the unbalanced design proves to be a major object of criticism and if the substrates are not an important determinant of biofilm composition, I could use those samples to render the design more balanced. In 2021 and 2022, we also collected many biofilm samples both on natural and artificial substrates. These samples are not yet sequenced, but will be soon and if needs be, can strengthen our time-series data. For these sequencing runs, we should include positive controls for better reproducibility, as the lack of controls in the present study could also be an area for criticism (Ravel and Wommack 2014; Schloss 2018a; Hornung *et al.* 2019).

Reproducibility in microbiome research has been questioned due to the existence of plethora of methods, and the lack of consensus on them, used to preprocess and analyse this type the data (McMurdie and Holmes 2014; Gloor *et al.* 2017; Weiss *et al.* 2017; Lin and Peddada 2020). I have ensured that my results are reproducible by adopting good coding and data management pratices (Schloss 2018b). However, there are many preprocessing/normalisation methods and many indices both for α- and β-diversity. I used rarefaction as a normalisation technique but some say this is not an acceptable one (McMurdie and Holmes 2014; Weiss *et al.* 2017). However, other researchers disagree (Schloss 2018b; Cameron *et al.* 2021). I repeated all analyses without rarefaction, but by normalising with total sum-scaling (TSS; results not shown), i.e. dividing each ASV abundance in a sample by the total library size of the sample (McKnight *et al.* 2019; Lin and Peddada 2020), or by applying the centered-log transformation (CLR; Gloor *et al.* 2017). Results and conclusions were the same for all analyses (TDR, GDM, temporal trends in α-diversity and the richness and relative abundance of indicator taxa), and so were they when I used other α-diversity indices such as the Shannon index or species richness or Chao 1. I wanted to use the weighted Unifrac as

alternative to Bray-Curtis dissimilarity for β-diversity measure, because the former better accounts for phylogenetic differences (Lozupone and Knight 2005; Lozupone *et al.* 2007). Yet, I was hampered by a lack of computational power and time to produce a phylogenetic tree from my taxonomic datasets.

## 6.3 On Bd and biofilm *in situ*

In Chapter 4:, I found some evidence that, in natural conditions, biofilms could potentially reduce the frequency and impacts of Bd infections on *Alytes obstetricans*, notably by harbouring zoospore consumers, predators or inhibitors. However, the results are highly descriptive and correlational. Further work is required to strengthen them. Given the time constraints to perform the analyses of this chapter, my goal was first to adopt a broad systematic approach with which I could detect the possible existence of major differences in community composition between sites with different epidemiology. This is why I first worked on a community level, by studying the α- and β-diversity, used differential abundance analysis to identify which taxa was differentially represented between the epidemiologically-different sites, and looked whether the identified taxa could be consistent with the epidemiology.

A difficulty in this study was to define pertinent epidemiolical categories and to correctly assign each lake. Lake assignment was based on the Bd infection data from the Pyrenean monitoring conducted since 2008. As detailed in the discussion of Chapter 4, the definition and inclusion of an "uninfected" category in the comparisons posed some problems in that we do not know (i) if these lakes are truly uninfected, as we may have missed infection since observational uncertainty in disease ecology is generally high (Lachish and Murray 2018); (ii) if they are uninfected because Bd was never introduced in their amphibian populations, or (iii) uninfected because environmental conditions preclude Bd infections to be sustained over time. In this study, we assumed the latter hypothesis, but if this hypothesis is wrong, then using uninfected lakes will lead to incorrect results.

On the other hand, me might have the converse problem, namely classifying a lake as (sporadically) infected while it is not infected at all. Vallon is one such example, where Ao have been found infected at very low levels. At such levels, the possibility of false-positives is relatively high, and this is why some research teams have adopted stringent criteria such as considering a qPCR assay positive only if it yields a result over 0.1 ZE and shows a clearly-sigmoid amplification curve, and a sample is considered positive if and only if there are consensus between qPCR duplicate assays, i.e. both should be either positive negative, and if not, the duplicates are rerun. If there is no consensus after two reruns, then the sample is

considered negative (Soto-Azat *et al.* 2013a; Hudson *et al.* 2016; Valenzuela-Sánchez *et al.* 2017). Here, we do not apply such criteria: a qPCR assay is considered positive when the result is over 0 ZE and show a clear sigmoid amplification curve. Also, a sample is considered positive even if rerun qPCR duplicates show only one positive assay. Such practices may increase the likelihood of having false positives bu this might not have any influence on the results because I used a categorical variable (a lake is infected or not). It migh become a problem if we use quantitative variable such as prevalence for later analysis.

I ended up exluding from my analyses some lakes of which the infection status was or is unsure. First, there is Ayes, near Bethmale, where Ao populations suffered an epizootic in 2018-19, after which we found only 3 tadpoles in 2020 (uninfected, but because of high temperatures, very few Bd infection were found that year, including in Lescun), none in 2021. Plenty were found in 2022 but I was not aware of that when I did the analyses: More time is needed to we know whether its populations are now systematically infected or not. Second, at Paradis, Ao have been found infected in 2013, 2015, 2017, and 2018, and while tadpoles were found uninfected in 2019 and 2020, we found no tadpole thereafter. Finally, there is the case of Madamete-Haut, where Ao were sampled in 2010, tested uninfected, and never found again since then, while major epizootics hit two nearby lakes, Gourg de Rabas and Madamete-Bas at that time. I think some data are missing in the dataset because Courtois *et al.* (2017) reported many infections in Madamete-Haut in 2010. In any case, as no Ao were detected since then in this lake contrary to Gourg de Rabas and Madamete-Bas, I was reluctant to classify it into the same category. Likely, more capture-mark-recapture studies in combination with disease monitoring are needed to know if these populations (and others) are actually declining because of amphibian chytridiomycosis or other pressures. In the meantime, biofilms from these lakes could be used for finer-scale analyses (see below).

Another problem with this Bd infection dataset is that it is very unbalanced: most data points are in the five lakes around Lescun where Bd infections occur almost every year. In all other lakes, there are much fewer data points, which can be due to a lower sampling effort or to the fact that Ao tadpoles were not found at the moment of fieldwork, either because they were not detected (bad meteorological conditions, or they tend to hide under rocks in the presence of fish) or because there were truly none as a result of extirpation or migration (once metamorphosed, individuals are fully terrestrial and cryptic; thus, the timing of the fieldwork is important). Variation in host detectability can be a substantial problem in disease ecology (Faustino *et al.* 2004; McClintock *et al.* 2010; Lachish and Murray 2018; Valenzuela-Sánchez *et al.* 2019). That this dataset is imbalanced means that I have very few infection data points matching with my taxonomic data points on biofilm compositions (2016 to 2020) for lakes

other than those around Lescun. This prevents me from doing finer-scale analyses, such as studying the relation between biofilm composition and prevalence or the average infection intensity of the population. I will be able to do that only for Lescun lakes or others like Ayes where the population exhibited infections during the period 2016-2020.

## 6.4 On Bd and biofilm *ex situ*

The aim of my experimental work (Chapter 5) was to determine whether benthic biofilms could affect Bd zoospores in controlled settings, and provide a proof of concept warranting further investigations. Although seemingly straightforward, these experiments represent a substantial amount of work, and have not been without obstacles. They notably require synchronisation between the production of zoospores and that of biofilms. However, there always is a random component regarding zoospore production, as it is very sensitive to bacterial and fungal contamination and the available Bd strain do not produce consistently the same numbers of zoospores despite being maintained under the same conditions. Without enough zoospores to begin with, the experiments are pointless because there is then little chance that we can observe a difference between the temporal trends in zoospore concentration in wells with and without biofilms. A better understanding of the cycle of this strain would make these experiments much easier to plan and conduct. For example, it would be good to know exactly when to harvest zoospores to get maximum yields, so that we can homogenise the initial concentrations of zoospores in all treatments and all replicates (Woodhams *et al.* 2008). The production of field-grown biofilms has also proved difficult, with little biomass occurring on most of tiles, even when left up to a year in the lakes. We will need more biomass to have a decent chance to show if biofilms from mountain lakes have an effect on Bd zoospores. The substrate itself is not a problem as in the laboratory, biofilms grow very well on them. In order to increase the chances of having a high biomass of biofilm in the field, one could put low-mesh nets around the tiles to prevent grazing by large invertebrates and even amphibian larvae or small fish (Füreder *et al.* 2003; Alonso *et al.* 2022).

The analysis of the data was another encountered problem. The modelling framework used here (exponential decay law) had already been used in a previous study, but after a logarithmic transformation of the data in order to work with linear models (Woodhams *et al.* 2008). I found that directly fitting non-linear models was preferable because it allows a more straightforward and transparent analysis. Logarithmic transformation of zeros is mathematically impossible, yet zeros were frequent in this dataset (at t+23h) contrary to that of Woodhams *et al.* (2008). Adding a constant was thus necessary if we wanted to operate in a

linear framework but I found that the choice of this constant greatly influenced the estimaton of the zoospore disappearance rate. I also noticed that an exponential decay law did fit the data quite well when zoopores were exposed to biofilms, but less well in the controls where there was more variability. This is quite evident for instance in **Figure 37**. I do not think this is a major problem as far as conclusions are concerned, as "all models are wrong" to some extent (Box 1976), and in my opinion these ones are still a good way of interpreting the data as we are only interested in the rate of declines relative to other treatments, not in the exact prediction of the data. However, we could not replicate, for instance in experiments of setting 3, the high disappearance constant often observed in experiments of setting 2. We also found quite a lot of variation between replicates of a same treatment, especially for controls (for instance between controls of date 1 and date 2 of treatement *Mayamea permitis*). This indicates that i) our environmental conditions are not perfectly controlled and we should pay more attention to them, for instance by monitoring room temperatures more closely; ii) more replicates of these experiments (we repeated the experiments only once per treatement and only for settings 1 and 3) are needed to capture the variation caused by these environmental conditions. The last area of improvement regarding the design would be to perform a measurement in between t+6h and t+23h: it would be good to have data around t+12h too but this need some logistic adaptations.

We did not expect to observe any effect of very simple (monospecific) biofilms on Bd zoospores. This demonstrates that mechanisms other than consumption of Bd zoospores by biofilm inhabitants (which was our main hypothesis) are at play. I have several ideas to investigate this further and disentangle which potential mechanisms really inactivate zoospores or force them to encyst. Nutrient or electrolyte depletion might well be possible. Actually, we provide further evidence that a medium deprived from electrolytes is detrimental to zoospores, or at least, to zoospore activity (Johnson and Speare 2003). Biofilm could deplete the water column of electrolytes and nutrients, explaining why zoospores disappeared faster when exposed to biofilms than in controls. When no electrolytes were in the medium (setting 2 with ultrapure water), there was in general little difference between biofilms and controls. Measuring the concentrations of the electrolytes in the water at regular time during the course of the experiment, in control and in biofilm wells (after filtrating zoospores out), would be a good way to test this hypothesis. To test the existence of allelopathy, we could take a solution of algae (e.g. *Leptolyngbya* sp.), remove the algae by filtration to keep only the secreted molecules, and expose the zoospores to that solution.

It would then be essential to determine whether zoospores are truly inactivated or only encysted and waiting in a state of arrested development for conditions to improve (Johnson and Speare 2003). Woodhams *et al.* (2008) estimated the disappearance constant exactly like us,

but by counting also the dead (immotile) zoospores in the haemocytometer, could determine the mortality rate and, with a simple deduction, the encystment rate too. While we could have imitated this, the presence of biofilms in our experiments made this inappropriate, as zoospores could be trapped in the matrix, or lysed because of antimicrobial substances. Consequently, these dead zoospores would not have been counted in the haemocytometer, and we would have largely overestimated the encystment rate and underestimate the mortality one. I think it is better to directly test whether zoospores are still viable by reintroducing them in the tryptone-glucose medium as described in the chapter. The major obstacle here would be to introduce bacteria with the biofilm chunk (even our monospecific algal cultures were not axenic) which would rapidly colonise the entire culture medium and would overcome Bd. To circumvent this, I suggest introducing broad-spectrum antibiotics such as penicillin associated with streptomycin as done by previous studies (Piotrowski *et al.* 2004; Woodhams *et al.* 2008).

## 6.5 Synthesis and concluding remarks

With this doctoral work, I have improved the knowledge on benthic biofilms of mountain lakes and their potential roles in the infections by an amphibian parasite of conservation significance. It is essential that biofilms, and more generally, microbial communities, are not neglected by researchers and conservation practitioners if we are to best preserve the outstanding biodiversity of mountain freshwater ecosystems and the invaluable services they provide to humanity. My results clearly show that benthic biofilm communities are currently changing in mountain lakes with the increasing dominance of cyanobacteria which are potentially harmful to other sympatric organisms. The respective importance of global change drivers for these compositional shifts should be assessed in more details. It would now also be appropriate to know whether the functions of biofilms change with these compositional shifts. To do so, we first need to know what their functions are. Yet, as shown in chapter 2, there are still grey areas for example in our knowledge of how biofilms function with regard to the health of sympatric aquatic macroorganisms. I tried to shed light on this by focusing on the interactions between benthic biofilms and Bd in the rest of my thesis.

My findings are consistent with the hypothesis that biofilms play a role in the epidemiology of Bd infections and amphibian chytridiomycosis, although further studies are necessary to confirm this and elucidate the mechanisms at play. While the zooplankton helps explain if and to what extent (prevalence) a population gets infected (Schmeller *et al.* 2014), benthic biofilms, from my results, may help explain the long-term impact of the disease on Ao population (stable vs. declining). However, caution must be taken as my results provide

circumstancial evidence at best. While we focus on environmental epidemiological factors, other ones could explain the peculiar Pyrenean epidemiology of amphibian chytridiomycosis too. The importance of some of these factors have not yet been assessed here: I have already mentioned the genetics (CMH genes) of the populations, but interpopulation differences in demographic life-history traits could also explain the variation in impacts on amphibian populations, as shown in other systems including Bd-amphibian ones (Valenzuela-Sánchez *et al.* 2021, 2022).

In my opinion, an appropriate way to move forward would be first to continue the work presented in chapter 5, by repeating the experiments to make the results more robust, and by determining whether or not zoospores are inactivated or still infective. To complement this, I think it is essential at some point to carry out mescosm experiments, where tadpoles are reared with or without biofilms (different biofilm could be tested) and infected with a same number of Bd zoospores. In fact, I have already obtained ethical approval for such experiments, and done all the required trainings. I hope to be able to do these experiments in the future. The major obstacle would be to get a sufficient number of tadpoles that are relatively similar in age and health status to draw robust conclusions from experiences.

If, with all of this combined, we do not find evidence that biofilms negatively affect Bd zoospores, e.g. if zoospores are not inactivated but just in an immotile state of arrested development and still infective, then biofilms should perhaps be considered as a source of, rather than a protection from, infections for tadpoles. In contrast, if we have evidence that biofilms negatively affect Bd zoospores, thereby reducing infection pressure and positively impacting the health of tadpoles, then we should determine whether different biofilms have the same effects, and whether the changes in biofilm structure observed *in situ* would impair this role of protection. From my results, there were some indications that, all else being equal (i.e. in a similar setting, see Chapter 5 setting 1), a biofilm with more biodiversity would have more impact on the zoospores with potentially a greater protective effect for the tadpoles. Again, the last assertion remains to be demonstrated, but if true, this would support the **dilution effect theory** (Keesing *et al.* 2006, 2010) and also, because amphibian chytridiomycosis greatly disrupts ecosystems, the **diversity-stability theory** (Goodman 1975; McCann 2000). In that regard, the consequences of the loss of prokaryotic biodiversity shown in chapter 3 should be assessed.

That being said, the generality of the dilution effect theory has been the subject of intense debate among disease ecologists (Randolph and Dobson 2012; Lafferty and Wood 2013). The validity of this theory seems to depend on the scale, the context and the system considered as

many host-parasite systems are not affected by biodiversity; e.g. those where parasiste (*sensu lato*, also including viruses) are directly transmitted, specialist, and without free-living stages, intermediate hosts or vectors (Rohr *et al.* 2019). In many (but not all) Bd-amphibian systems, there is often support for the dilution hypothesis with a reduced infection risk when biodiversity is high at the host level (Searle *et al.* 2011; Becker *et al.* 2014; Venesky *et al.* 2014; James *et al.* 2015), the zooplankton level (Schmeller *et al.* 2014), and the skin microbiota level (Piovia-Scott *et al.* 2017; Bates *et al.* 2018). But actually, most of these studies show that, rather than species diversity, it is the presence of one or some particular species in the community that drives the reduction (or amplification) of disease risks: for example, the presence of a filter-feeding amphibian tadpole (a different guild of tadpoles than periphyton grazing; Venesky *et al.* 2014) or ciliates or rotifers in the community (all rotifers and ciliates are not equally competent at consuming Bd; Schmeller *et al.* 2014). Here, I found that the presence and relative abundance in biofilms of rotifers and, most particularly, bdelloids matched to some extent the patterns of chytridiomycosis impacts observed in some Pyrenean Ao populations. These bdelloid rotifers are filter-feeding biofilm dwellers (Majdi *et al.* 2012; Mialet *et al.* 2013) and very competent at consuming Bd (Loyau, unpublished data). Knowing this, we should test in the abovementioned experiments if biofilms with bdelloid rotifers reduce the infection pressure more than biofilms without these rotifers. Then, we should also examine which kind of biofilm community, or other environmental conditions, bdelloid rotifers tend to use the most, and whether the observed changes in mountain lake biofilms lead to a decline in bdelloid and other rotifers. If such potential Bd consumers decline or are extirpated, the risks of infections by Bd and disease would increase for amphibians.

However, rotifers are not microorganisms *sensu stricto*, but rather benthic meio-organisms, i.e. organisms between 42 and 500 µm (Mialet *et al.* 2013). One object of criticism here is that our sampling of biofilms might not be representative enough of organisms larger than microorganisms that occur in the lake benthos. For this reason, microbial ecologists often remove DNA sequences belonging to metazoans from their micro-eukaryotic library. In chapters 3 and 4, I removed large metazoans such as arthropods, vertebrates etc, but kept those small metazoans that are part of the meiobenthos such as rotifera and tardigrada. This comes with the assumption that our sampling was as representative as possible of the true diversity of the meiobenthos of each lake. While we scraped four or five rocks to make a biofilm sample, these rocks were often found close to each other at the scale of a lake; I suggest that several rocks from disctinct areas of a lake should be sampled and then pooled to constitute a more representative biofilm sample (this is also the goal of placing the other artifical substrates: I

systematically placed three replicates per lake, in order to test whether the location within a lake influences the composition of biofilm microbial assemblages).

Whatever the roles of biofilms with regards to Bd zoospores, biofilms still could negatively impact amphibian chytridiomycosis epidemiology, that of other infectious diseases, and the health of tadpoles in general through other mechanisms such as toxicity or nutrition. Exploring these mechanisms would further increase knowledge in mountain freshwater ecology and disease epidemiology. First, the risks of toxin production by biofilms should be assessed in more details in mountain lakes by screening both biofilms and the water column for cyanotoxins. The determinants of toxin production should also be investigated to examine links, for instance, with climate change, fish stocking or livestock effluents. Toxin production poses a substantial health risk to tourists, livestock and other animals depending on mountain waters, and would also increase the costs of producing clean drinking water. Second, it would be interesting to have more information on the production, distribution and abundance of ω-3 PUFA in freshwater ecosystems in the Pyrenees, and to relate this to the health of amphibans (in particular, their Bd infection status) and ecosystems as a whole. I would then recommend to assess the temporal trends in both toxin production and PUFA in the current context where biofilm microbial compositions are changing.

Finally, on top of investigating at the effects of biofilms on amphibian health as discussed above, one could investigate the effects of amphibian larvae on biofilm communities. That is, how grazing affects the structure and functions of biofilm communities. Importantly, to investigate whether amphibian loss alters lake benthic biofilm biomass, structure and functions, as witnessed in mountain streams (Alonso *et al.* 2022). If my hypotheses are validated, one could expect a potential vicious circle taking place in mountain lakes. Indeed, with increasingly stronger anthropogenic pressures, biofilms are changing towards potentially less biodiverse communities, devoid of functionally important organisms (e.g. Bd consumers or nutritious algae) and only containing suboptimal ones for amphibians (less nutritious, or even toxigenic algae). These changes in biofilm might translate into an increased risk of Bd infection and chytridiomycosis (other pathogens and diseases should be investigated as well), as well as risks of cyanotoxicosis, for amphibians which could cause their populations to decline further and further with eventually possible extirpations. The resulting decline or complete absence of amphibian larvae, i.e. biofilm grazers, in mountain lakes would then mean that a top-down control on biofilm accrual is lost, with the possibility that the most tolerant algae would further

proliferate at the expense of other more sensitive biofilm inhabitants. Of course, many links in these chains of events remains to be demonstrated.

My work can be used to inform conservation practices, and the management of mountain water resources, not only in the Pyrenees but also in other mountain ranges. To stop biofilms from changing and for many other reasons, mitigating climate change is an utmost priority. While this is a societal and political choice, it is also important to act locally on other anthropogenic pressures on mountain lakes, as I believe they also impact biofilm communities. This includes, for instance, controlling tourism, eradicating non-native fish or at least stopping their systematic introduction for fishing purposes, and limiting the impact of livestock. All of this must be done through regulation but also education and scientific outreach. This would reduce the pressures on mountain lake ecosystems and give their organisms more time to adapt to other pervasive pressures such as warming. Implementing these actions seems at first sight to be socially difficult, but has been possible elsewhere (e.g. in the Spanish Pyrenees; http://www.lifelimnopirineus.eu/sites/default/files/laymanen_0.pdf) and is actually straightforward (at least, more achievable) compared to the global efforts required to mitigate climate change. This underscores the need for transdisciplinary approaches to development with contributions from, among others, the stakeholders such as farmers; representatives of the community and politicians; environmentalists; social scientists; human, plant, and veterinary epidemiologists; and ecologists.

# Discussion et perspectives (FR)

Ma thèse aborde des aspects de l'écologie des communautés microbiennes et de l'épidémiologie des maladies, en explorant certains liens entre ces deux domaines dans le contexte des écosystèmes des lacs de montagne. Alors que l'importance des communautés microbiennes de l'hôte pour sa santé est de plus en plus évidente et reconnue (Clemente *et al.* 2012 ; Ezenwa *et al.* 2012 ; Sampson and Mazmanian 2015 ; Fung *et al.* 2017), celle des communautés microbiennes environnementales (c'est-à-dire en dehors de l'hôte) a rarement été considérée, et encore moins évaluée. Pourtant, les micro-organismes entourent les hôtes et forment leur environnement ; ils alimentent également une multitude de processus écosystémiques qui, à leur tour, peuvent bénéficier ou nuire aux hôtes, ou bénéficier ou nuire à leurs pathogènes ou parasites. Ainsi, les communautés microbiennes environnementales sont vouées à affecter la santé des hôtes et pourraient influencer d'une manière ou d'une autre l'épidémiologie de leurs maladies infectieuses. Ici, en utilisant une combinaison d'études de terrain et d'expériences en laboratoire, j'ai fourni les premières réponses à la question de savoir si les communautés de biofilms benthiques jouent un rôle (ou certains rôles) dans la dynamique de l'infection par Bd dans les populations d'amphibiens. J'ai aussi généré de nouvelles connaissances sur les déterminants et le statut de la biodiversité des biofilms dans les écosystèmes d'eau douce de montagne. Bien qu'il reste encore du travail à effectuer pour élucider les rôles épidémiologiques des biofilms en ce qui concerne la chytridiomycose des amphibiens, je donne ici quelques perspectives pour les recherches futures.

## Sur les biofilms en général

Dans le chapitre 2, en utilisant une approche holistique de la santé et de la durabilité, j'ai illustré comment les communautés microbiennes de biofilms peuvent avoir un impact sur la santé de divers niveaux d'organisation biologique, des individus multicellulaires aux écosystèmes entiers (Sentenac *et al.* 2022). Je me suis efforcé de briser les silos disciplinaires pour fournir des connaissances translationnelles (Ciesielski *et al.* 2017). Ce faisant, j'ai mis en évidence de nombreuses lacunes de connaissances dans la recherche sur les biofilms, en particulier le fait que l'on savait peu de choses sur les rôles des biofilms dans l'épidémiologie des maladies infectieuses autre qu'humaines dans le contexte des systèmes de distribution d'eau (Wingender and Flemming 2011). C'est le cas des maladies émergentes de la faune et de la flore, dont la chytridiomycose des amphibiens. En utilisant le cadre de la pyramide des maladies, j'ai cependant soutenu que les biofilms pouvaient influencer la dynamique des

maladies de diverses manières, en ayant un impact sur l'hôte, l'agent pathogène et/ou leur environnement (Bernardo-Cravo *et al.* 2020). Étant donné l'ubiquité et l'abondance des biofilms (Battin *et al.* 2016 ; Flemming and Wuertz 2019 ; Sentenac *et al.* 2022), ce travail peut s'appliquer à de nombreux systèmes hôte-parasite, pas seulement à Bd et aux amphibiens, ou pas seulement aux pathogènes qui ont un stade de vie libre. En fait, la plupart des connaissances sur les interactions entre les biofilms et les agents pathogènes concernent des virus ou des bactéries (Hall-Stoodley and Stoodley 2005; Wingender and Flemming 2011), et non les champignons parasites pathogènes, alors que ces derniers constituent une préoccupation importante pour la durabilité (Fisher *et al.* 2012). Plus qu'un simple examen de la littérature existante, j'ai exploité les théories écologiques actuelles telles que celle des **états stables alternatifs**, pour caractériser le concept de santé des biofilms défini avec une structure, une productivité et une résilience qui leur sont propres. Ceci aidera à orienter les recherches futures et la vulgarisation scientifique avec un cadre théorique facilement compréhensible (que j'ai implicitement utilisé par la suite).

## Sur les biofilms des lacs pyrénéens

La plupart de mes travaux dans cette thèse ont porté sur l'analyse de la composition des assemblages procaryotiques et micro-eucaryotiques des communautés de biofilms benthiques des lacs de montagne pyrénéens (chapitre 3). J'ai produit de nombreux résultats, et n'ai rapporté que les plus solides. Par exemple, j'ai analysé les caractérisitques des lacs les plus instables (β-dispersion) ou les plus enclins au déclin de l'α-diversité procaryote du biofilm. Cependant, comme je ne dispose que d'une population de 26 lacs, ces résultats manquaient de robustesse et n'ont pas été inclus. Dans le chapitre 3, j'ai d'abord caractérisé les façons dont le changement climatique s'est manifesté dans nos sites d'étude, avec plus de précipitations et une amplitude de température quotidienne plus faible (parce que les températures ne descendent pas aussi bas que par le passé) dans les sites occidentaux, alors qu'il fait nettement plus chaud et que l'amplitude de température quotidienne a augmenté dans les sites orientaux (températures maximales plus importantes). Ces différences dans le changement climatique ne semblent pas être ni à l'origine des dissemblances spatiales entre les biofilms, ni à celle de la vitesse à laquelle ces compositions changent. En effet, j'ai constaté qu'au sein d'un même lac, les compositions des assemblages de procaryotes et de micro-eucaryotes dans les biofilms devenaient de plus en plus dissemblables à mesure que le temps entre les échantillons augmentait (de manière plus prononcée pour les procaryotes). J'ai également démontré qu'en général, les biofilms perdaient avec le temps de la biodiversité procaryote, mais pas micro-eucaryote. Pour comprendre plus

précisément comment ces changements se reflètent dans la composition des assemblages, j'ai étudié les tendances temporelles de la richesse et de l'abondance relative de taxons indicateurs tels que les cyanobactéries et les diatomées. J'ai constaté que la richesse et l'abondance relative des cyanobactéries augmentaient généralement au fil du temps dans les assemblages procaryotes, tandis que le contraire se produisait pour les diatomées dans les assemblages micro-eucaryotes.

Les données de séquençage à haut débit sont compositionnelles : le nombre total de détection dans chaque échantillon est limité par la capacité de l'instrument de séquençage : s'il y en a plus dans l'un, il y en a moins dans l'autre ; les données sont donc exprimées en proportion (ou abondance relative; Gloor *et al.* 2017). Pour nos résultats, cela signifie que, en prenant l'exemple des cyanobactéries, nous ne savons pas si elles ont réellement augmenté au fil du temps en termes d'abondance absolue, ou si elles sont restées stables alors que d'autres organismes procaryotes ont diminué, ou encore si elles ont diminué au fil du temps mais à un rythme plus faible que les autres organismes procaryotes. Tous ces scénarios aboutissent à la même observation. À cet égard, d'autres méthodes permettant une quantification absolue des microoganismes, par exemple avec la cytométrie de flux, sont nécessaires pour confirmer les tendances observées (Props *et al.* 2017 ; Hamard *et al.* 2021). Par ailleurs, notre approche méthodologique ne permet pas une analyse directe des assemblages procaryotes et micro-eucaryotes *en tandem*, alors qu'il aurait été utile ici de savoir si les cyanobactéries ont augmenté au détriment des diatomées (puisqu'il s'agit en fait de la même communauté de biofilms). Ceci est dû au fait que les gènes marqueurs utilisés pour le métabarcoding des procaryotes et des micro-eucaryotes sont différents (gènes 16S vs 18S rRNA, respectivement). Cela dit, il est reconnu que les diatomées sont des organismes sensibles, alors que les cyanobactéries sont tolérantes à de nombreux facteurs de stress et ont tendance à proliférer dans certaines conditions défavorables aux autres micro-organismes (Morin *et al.* 2016 ; Huisman *et al.* 2018 ; Wood *et al.* 2020 ; Ossyssek *et al.* 2022). Suite à un stress, les cyanobactéries ont tendance à remplacer les diatomées dans les biofilms d'eau douce (Leflaive *et al.* 2015 ; Crenier *et al.* 2019). Dans d'autres écosystèmes menacés, comme les récifs coralliens, les biofilms cyanobactériens sont également connus pour remplacer des biofilms constitués d'organismes moins tolérants (de Bakker *et al.* 2017 ; Ford *et al.* 2018). Pris ensemble, nos résultats suggèrent que la santé des biofilms dans les lacs de montagne diminue alors que les compositions des biofilms benthiques divergent vers des communautés de plus en plus dominées par les cyanobactéries. Je n'ai trouvé de preuve d'un changement complet de composition vers un nouvel état stable dans aucun des lacs (Abreu *et al.* 2020 ; Wang *et al.* 2021). Cela pourrait être dû à la durée relativement courte de l'étude (5 ans). J'ai longuement discuté, au chapitre 3, des implications de tels changements

de composition pour les économies locales (impact sur les activités récréatives et l'agriculture) et la santé des humains, des animaux et de l'ensemble de l'écosystème d'eau douce de montagne.

Quant à savoir pourquoi ces biofilms ont changé, notre étude est observationnelle, donc corrélationnelle par essence. Elle n'est donc pas en mesure de démêler les impacts respectifs de chaque pression anthropique ou naturelle sur les lacs de montagne. Cependant, les menaces qui pèsent sur les écosystèmes d'eau douce de montagne sont relativement bien connues (Schmeller *et al.* 2018, 2022). Le changement climatique est considéré comme la menace numéro une car ses impacts sont considérés comme plus forts en altitude qu'en plaine (Rangwala and Miller 2012; Pepin *et al.* 2015 ; Schmeller *et al.* 2022). Nous avons imputé ces changements de composition (du moins certains d'entre eux) aux effets du changement climatique car, sur la base des données et des autres résultats disponibles, cela semblait l'explication la plus parcimonieuse. En effet, d'autres études ont montré que certains lacs de montagne, notamment ceux des Pyrénées, étaient susceptibles de se basifier en raison de la fonte croissante des glaces, de la modification des régimes de mélange et de l'intensification de l'érosion dans le contexte du changement climatique (Curtis *et al.* 2009 ; Rogora *et al.* 2020). Or, nous avons constaté que le pH et la dureté de l'eau, plus que l'emplacement géographique, étaient les facteurs les plus importants expliquant les différences entre les lacs dans la composition des assemblages de procaryotes et de micro-eucaryotes. Cela nous a conduit à affirmer que les effets du changement climatique sur la chimie de l'eau (augmentation du pH et de la dureté) provoquaient des changements dans la composition microbienne du biofilm.

Cependant, les changements de composition observés pourraient être le résultat d'une combinaison de facteurs de stress agissant simultanément. En effet, il existe de nombreuses autres pressions que le changement climatique dans les écosystèmes de montagne. La pollution chimique est l'une d'entre elles (Schwarzenbach *et al.* 2006 ; Schmeller *et al.* 2018 ; Machate *et al.* 2023). Une étude récente a trouvé, dans certains des lacs étudiés ici, des preuves de contamination par un large éventail de composés synthétiques (plus de 150 molécules différentes), notamment les insecticides diazinon et perméthrine, à des concentrations si élevées qu'elles pourraient entraîner des risques toxiques aigus pour les crustacés mais pas pour les algues (Machate *et al.* 2022). Les effets de la pollution sur la composition des biofilms dans notre système d'étude doivent être évalués (Sabater *et al.* 2007 ; Proia *et al.* 2013a). À l'exception des effets négatifs du cuivre sur la richesse et l'abondance relative des cyanobactéries, je n'ai pas trouvé de preuve d'impact d'un élément trace métallique sur l'α-diversité ou la β-diversité des assemblages procaryotes ou micro-eucaryotes dans mes analyses, mais davantage de données devraient être collectées. De même, j'ai cherché à savoir si la présence de poissons, de bétail et de tourisme pouvait avoir des effets sur la biodiversité

microbienne des biofilms, mais je n'ai trouvé aucune association. J'ai utilisé des indices créés dans le passé par d'autres membres de mon équipe de recherche, basés sur des observations de terrain et les données des Fédérations de Pêche. Toutes ces variables sont qualitatives, donc plutôt « grossières », et davantage de données sont nécessaires pour tester les effets de ces facteurs. J'ai également cherché à savoir si la présence et l'abondance du bétail sont associées aux concentrations de nitrites, aux nitrates ou à l'azote organique total détectés dans l'eau du lac, mais là encore, aucune association n'a été constatée. Je pense que des études interventionnelles plutôt qu'observationnelles sont justifiées ici pour évaluer l'existence et la force de ces pressions anthropiques ou anthropogéniques, par exemple en comparant la composition des biofilms avant et après le retrait des poissons (bien que cela ne soit pas encore à l'ordre du jour dans les Pyrénées françaises pour des raisons économico-politiques, cela sera mis en œuvre dans certains lacs à des fins de recherche ; https://www.biodiversa.eu/2022/10/25/fishme/) et/ou l'exclusion partielle du bétail et/ou des humains. Le projet Limno Pirineus Life a rapporté des résultats prometteurs à cet égard (http://www.lifelimnopirineus.eu/sites/default/files/laymanen_0.pdf). Premièrement, l'élimination des vairons auparavant introduits a entraîné une augmentation marquée de l'abondance des crustacés, une diminution de la biomasse du phytoplancton et une baisse de la turbidité de l'eau. Ensuite, l'exclusion complète du bétail des habitats menacés comme les sources pétrifiantes avec formation de tuf, ou l'exclusion partielle du bétail grâce à la construction d'abreuvoirs pour réduire la fréquentation dans les zones humides ou sur les rives des lacs, a permis de limiter considérablement la dégradation des habitats. De même, pour réduire les impacts du tourisme, des plateformes surélevées en bois ont été construites pour limiter l'impact du piétinement sur les zones humides.

L'étude présentée dans le chapitre 3 présente des faiblesses dont je suis pleinement conscient. De nombreuses variables environnementales présentaient des données manquantes et, bien que j'aie trouvé des moyens d'y remédier, cela a grandement compliqué la sélection des variables, les analyses ultérieures ainsi que la robustesse de nos résultats (Altman and Bland 2007; Buuren and Groothuis-Oudshoorn 2011). De même, en termes de défauts de désign, les biofilms ont été échantillonnés de manière très intensive au cours des trois premières années (2016, 2017, 2018) par rapport aux deux dernières années (2019 et 2020), et nous ne disposons de données que pour un sous-ensemble de lacs en 2020. J'ai bien des données sur les biofilms de tous les lacs en 2020, mais pas provenant des substrats naturels. En effet, j'avais également placé des substrats artificiels pour d'autres fins sur lesquelles je me concentrais à ce moment-là. Je n'ai donc pas systématiquement échantillonné les biofilms des substrats naturels, ce que je regrette. Par souci de cohérence, je n'ai analysé que les biofilms provenant de substrats

naturels, mais si le déséquilibre au niveau du design s'avère être une critique majeure et si les substrats ne sont pas un déterminant important de la composition des biofilms, je pourrais utiliser ces échantillons pour rendre le désign plus équilibré. En 2021 et 2022, nous avons également collecté de nombreux échantillons de biofilms à la fois sur des substrats naturels et artificiels. Ces échantillons ne sont pas encore séquencés, mais le seront bientôt et pourront, le cas échéant, renforcer nos données de séries temporelles. Pour ces séquençages, nous devrions inclure des contrôles positifs pour une meilleure reproductibilité, car l'absence de contrôles dans la présente étude pourrait aussi être critiquée (Ravel and Wommack 2014; Schloss 2018a; Hornung *et al.* 2019).

La reproductibilité dans la recherche sur les microbiomes a été remise en question en raison de l'existence d'une pléthore de méthodes, et de l'absence de consensus à leur sujet, utilisées pour prétraiter et analyser ce type de données (McMurdie and Holmes 2014; Gloor *et al.* 2017 ; Weiss *et al.* 2017 ; Lin and Peddada 2020). J'ai veillé à ce que mes résultats soient reproductibles en adoptant de bonnes pratiques de codage et de gestion des données (Schloss 2018b). Cependant, il existe de nombreuses méthodes de prétraitement/normalisation et de nombreux indices tant pour la α- que pour la β-diversité. J'ai utilisé la raréfaction comme technique de normalisation : certains disent que ce n'est pas une technique acceptable (McMurdie and Holmes 2014 ; Weiss *et al.* 2017), tandis que d'autres maintiennent l'intérêt de son utlisation (Schloss 2018b; Cameron *et al.* 2021). J'ai répété toutes les analyses sans raréfaction, mais en normalisant en divisant chaque abondance d'ASV dans un échantillon par la taille totale de la bibliothèque de l'échantillon (TSS ; McKnight *et al.* 2019 ; Lin and Peddada 2020). Les résultats et les conclusions étaient les mêmes pour toutes les analyses (TDR, GDM, tendances temporelles de la α-diversité et de la richesse et de l'abondance relative des taxons indicateurs), et il en était de même lorsque j'utilisais d'autres indices d'α-diversité tels que l'indice de Shannon ou la richesse des espèces. Je voulais utiliser l'Unifrac pondéré comme alternative à la dissimilarité de Bray-Curtis pour la mesure de la β-diversité, car le premier rend mieux compte des différences phylogénétiques (Lozupone and Knight 2005; Lozupone *et al.* 2007). Mais, j'ai été gêné par un manque de puissance de calcul et de temps pour produire un arbre phylogénétique à partir de mes données taxonomiques. La transformation centrée-log ratio (CLR) pourrait également être utilisée comme technique de normalisation (Gloor *et al.* 2017).

## Sur les biofilms et Bd *in situ*

Dans le chapitre 4, j'ai trouvé quelques preuves en faveur du fait que les biofilms peuvent potentiellement réduire la fréquence et les impacts des infections à Bd sur *Alytes obstetricans*, notamment en hébergeant des consommateurs, des prédateurs ou des inhibiteurs de zoospores. Cependant, les résultats sont hautement descriptifs et corrélationnels et un travail supplémentaire conséquent est nécessaire pour les renforcer. Étant donné les contraintes de temps pour effectuer les analyses de ce chapitre, mon objectif était d'abord d'adopter une approche systématique large avec laquelle je pourrais détecter l'existence éventuelle de différences majeures dans la composition des communautés entre des sites ayant une épidémiologie différente, au lieu de tester des hypothèses spécifiques. C'est pourquoi j'ai d'abord travaillé à l'échelle de la communauté, en étudiant l'α- et la β-diversité. J'ai utilisé des techniques d'analyse d'abondance différentielle pour identifier quels taxons étaient différentiellement représentés entre les sites épidémiologiquement différents, et ai ensuite regardé si ces taxons pouvaient potentiellement expliquer l'épidémiologie observée

Une difficulté dans cette étude a été de définir des catégories épidémiologiques pertinentes et d'assigner correctement chaque lac. L'affectation des lacs a été basée sur les données d'infection par Bd issues du suivi pyrénéen réalisé depuis 2008. Comme détaillé dans la discussion du chapitre 4, la définition et l'inclusion d'une catégorie de lacs " non infectés " dans les comparaisons posent problèmes dans la mesure où nous ne savons pas (i) si ces lacs sont réellement non infectés, car nous pouvons avoir manqué une infection étant donné que l'incertitude observationnelle en écologie des maladies est généralement élevée (Lachish et Murray 2018) ; (ii) s'ils ne sont pas infectés parce que Bd n'a jamais été introduit dans leurs populations d'amphibiens ; ou (iii) s'ils ne sont pas infectés parce que les conditions environnementales empêchent les infections par Bd de se maintenir dans le temps. Dans cette étude, nous avons supposé la dernière hypothèse, mais si cette hypothèse est fausse, alors l'utilisation de lacs non infectés conduira à des résultats incorrects.

D'autre part, je pourrais avoir le problème inverse, à savoir classer un lac comme étant (sporadiquement) infecté alors qu'il ne l'est pas du tout. Vallon est un exemple, où les Ao ont été trouvés infectés à des charges parasitaires très basses. À de tels niveaux, la possibilité de faux positifs est relativement élevée, et c'est pourquoi certaines équipes de recherche ont adopté des critères stricts tels que considérer un test qPCR comme positif uniquement s'il donne un résultat supérieur à 0,1 ZE et présente une amplification clairement sigmoïde. De plus, un échantillon est considéré comme positif si et seulement s'il y a consensus entre les duplicats qPCR : les deux doivent positifs ou négatifs. Sinon les duplicats sont réanalysés, et s'il n'y a pas

de consensus après deux réanalyses, alors l'échantillon est considéré comme négatif (Soto-Azat *et al.* 2013a ; Hudson *et al.* 2016 ; Valenzuela-Sánchez *et al.* 2017). Ici, nous n'appliquons pas de tels critères : un test qPCR est considéré comme positif lorsqu'il est supérieur à 0 ZE et qu'il présente une amplification clairement sigmoïde, et un échantillon est considéré comme positif même si les qPCR initialement non-consensuelles en doublon ne montrent qu'un seul résultat positif une fois réanalysés. Bien que ces pratiques augmentent la probabilité d'avoir des faux-positifis, ce n'est peut-être pas un problème ici parce que j'ai utilisé une variable catégorielle (un lac est infecté ou non), mais cela en pourrait devenir un si nous utilisons des variables quantitatives comme la prévalence pour une analyse ultérieure. Je montre dans l'annexe 2 (Supplementary materials) que de telles différences dans les critères de positivité peuvent conduire à des estimations différentes de la prévalence d'infection à partir d'un même jeu de données.

J'ai fini par exclure de mes analyses certains lacs dont le statut est incertain. Tout d'abord, il y a Ayes, près de Bethmale, où les populations d'Ao ont subi une épizootie en 2018-19, après laquelle nous n'avons trouvé que 3 têtards en 2020 (non infectés, mais en raison des températures élevées, très peu d'infections par Bd ont été trouvées cette année-là, y compris à Lescun), et aucun en 2021. Beaucoup ont été trouvés en 2022 mais je n'étais pas au courant de cela quand j'ai fait ces analyses. Il faut encore du temps pour savoir si ses populations sont maintenant systématiquement infectées ou non. Ensuite, au lac Paradis, des Ao ont été trouvés infectés en 2013, 2015, 2017 et 2018, et si des têtards ont été trouvés non infectés en 2019 et 2020, nous n'avons trouvé aucun têtard par la suite. Enfin, il y a le cas de Madamete-Haut, où des Ao ont été échantillonnés en 2010, testés non infectés, et jamais retrouvés depuis, alors que des épizooties majeures ont touché deux lacs voisins, Gourg de Rabas et Madamete-Bas à cette époque. Je pense que certaines données sont manquantes dans le jeu de données car Courtois *et al.* (2017) ont rapporté de nombreuses infections à Madamete-Haut en 2010. En tout cas, comme aucun Ao n'a été détecté depuis lors dans ce lac, contrairement à Gourg de Rabas et Madamete-Bas, j'ai hésité à le classer dans la même catégorie. Il est probable que d'autres études de capture-marquage-recapture combinées à la surveillance des maladies soient nécessaires pour savoir si ces populations (et d'autres) sont réellement en déclin à cause de la chytridiomycose des amphibiens ou d'autres pressions. En attendant, les biofilms de ces lacs pourraient être utilisés pour des analyses à plus petite échelle (voir ci-dessous).

Un problème majeur de ce jeu de données sur les infections par Bd est qu'il est très déséquilibré : la plupart des données se trouvent dans les cinq lacs autour de Lescun où les infections par Bd se produisent presque chaque année. Dans tous les autres lacs, il y a beaucoup moins de données, ce qui peut être dû à un effort d'échantillonnage plus faible ou au fait que les

têtards d'Ao n'ont pas été trouvés au moment des campagnes de terrain, soit parce qu'ils n'ont pas été détectés (inexpérience de l'observateur, mauvaises conditions météorologiques, ou à cause de leur tendance à se cacher sous les rochers en présence de poissons), soit parce qu'il n'y en a vraiment pas eu à la suite d'une disparition ou d'une migration (une fois métamorphosés, les individus sont entièrement terrestres et cryptiques ; le moment où sont effectuées les sorties terrain est donc important). La variation de la détectabilité des hôtes peut constituer un problème important en écologie des maladies (Faustino *et al.* 2004 ; McClintock *et al.* 2010 ; Lachish and Murray 2018 ; Valenzuela-Sánchez *et al.* 2019b). Le fait que ce jeu de données soit déséquilibré signifie que je dispose de très peu de données d'infection correspondant exactement à mes données taxonomiques sur les biofilms (2016 à 2020) pour les lacs autres que ceux autour de Lescun. Cela m'empêche de faire des analyses à plus fine échelle, comme étudier la relation entre la composition du biofilm et la prévalence ou l'intensité d'infection moyenne de la population. Je ne pourrai le faire que pour les lacs de Lescun ou d'autres comme Ayes où la population a présenté des infections au cours de la période 2016-2020.

## Sur les biofilms et Bd *ex situ*

L'objectif de mon travail expérimental (chapitre 5) était de déterminer si les biofilms benthiques pouvaient affecter les zoospores de Bd dans des environnements contrôlés, et de fournir une preuve de concept justifiant des investigations supplémentaires. Bien qu'apparemment simples, ces expériences représentent une quantité non négligeable de travail, et n'ont pas été sans obstacles. Elles nécessitent notamment une synchronisation entre la production de zoospores et celle de biofilms. Cependant, la production de zoospores comporte toujours une part d'aléatoire, car elle est très sensible aux contaminations (bactériennes ou fongiques) et la souche de Bd à disposition ne produit pas toujours le même nombre de zoospores, même si maintenue dans les mêmes conditions. Sans suffisamment de zoospores au départ, les expériences sont inutiles car il y a alors peu de chance que nous puissions observer une différence entre les tendances temporelles de la concentration de zoospores dans les puits avec et sans biofilm. Une meilleure compréhension du cycle de cette souche rendrait ces expériences beaucoup plus faciles à planifier et à réaliser. Par exemple, savoir exactement quand récolter les zoospores pour en obtenir le maximum permettrait de standardiser le nombre de zoospores introduits à t0 entre chaque réplicat (Woodhams *et al.* 2008). La production de biofilms cultivés sur le terrain s'est également avérée difficile, avec peu de biomasse sur la plupart des tuiles, même lorsqu'elles ont été laissées jusqu'à un an dans les lacs. Nous aurons besoin de plus de biomasse pour avoir une chance décente de montrer si les biofilms des lacs

de montagne ont un effet sur les zoospores de Bd. Le substrat en lui-même n'est pas un problème car, en laboratoire, les biofilms s'y développent très bien. Afin d'augmenter les chances d'avoir une biomasse élevée de biofilm sur le terrain, on pourrait mettre des filets à mailles basses autour des tuiles pour empêcher la consummation par les grands invertébrés et même les larves d'amphibiens ou les petits poissons (Füreder *et al.* 2003 ; Alonso *et al.* 2022).

L'analyse des données a été un autre problème rencontré. Le cadre de modélisation utilisé ici (loi de décroissance exponentielle) avait déjà été utilisé dans une étude précédente, mais après une transformation logarithmique des données afin de travailler avec des modèles linéaires (Woodhams *et al.* 2008). J'ai trouvé que directement ajuster des modèles non linéaires était préférable car il permet une analyse simple et transparente. La transformation logarithmique des zéros est mathématiquement impossible, mais les zéros étaient fréquents dans ce jeu de données (à t+23h) contrairement à celui de Woodhams *et al.* (2008). Par conséquent, l'ajout d'une constante était nécessaire si l'on voulait opérer dans un cadre linéaire, mais j'ai constaté que le choix de cette constante influençait grandement l'estimation du taux de disparition des zoospores. J'ai remarqué qu'une loi de décroissance exponentielle correspondait assez bien aux données lorsque les zoospores étaient exposées aux biofilms, mais moins bien dans les contrôles. Ceci est tout à fait évident par exemple dans la **Figure 37**. Je ne pense pas qu'il s'agisse d'un problème majeur en ce qui concerne les conclusions, car "tous les modèles sont faux" dans une certaine mesure (Box 1976), et à mon avis, ceux-ci restent une bonne façon d'interpréter les données, car nous sommes seulement intéressés par le taux de disparition par rapport aux autres traitements, et non par la prédiction exacte des données. Cependant, nous n'avons pas pu reproduire, par exemple dans les expériences de la configuration 3, la constante de disparition élevée souvent observée dans les expériences de la configuration 2. Nous avons aussi trouvé beaucoup de variations entre les réplicats d'un même traitement, surtout pour les contrôles (par exemple entre les contrôles de la date 1 et de la date 2 du traitement *Mayamea permitis*). Cela indique que i) nos conditions environnementales ne sont pas parfaitement contrôlées et que nous devrions y prêter plus d'attention, par exemple en surveillant de plus près la température des pièces ; ii) un plus grand nombre de répétitions de ces expériences (nous n'avons répété les expériences qu'une seule fois par traitement et seulement pour les configurations 1 et 3) est nécessaire pour saisir la variation causée par ces conditions environnementales.

Nous ne nous attendions pas à observer un quelconque effet des biofilms très simples (monospécifiques) sur les zoospores de Bd. Cela démontre que des mécanismes autres que la consommation des zoospores par les habitants du biofilm (ce qui était notre principale hypothèse) sont en jeu. J'ai plusieurs idées pour approfondir cette étude et démêler les

mécanismes potentiels qui inactivent réellement les zoospores ou les forcent à s'enkyster. L'épuisement des nutriments ou des électrolytes pourrait bien être possible. En fait, nous fournissons des preuves supplémentaires qu'un milieu privé d'électrolytes est préjudiciable aux zoospores, ou du moins, à l'activité des zoospores (Johnson et Speare 2003). Le biofilm pourrait appauvrir la colonne d'eau en électrolytes et en nutriments, ce qui explique pourquoi les zoospores ont disparu plus rapidement lorsqu'elles étaient exposées aux biofilms que dans les témoins. Lorsque peu d'électrolytes étaient présents dans le milieu (configuration 2 avec de l'eau ultrapure), il y avait en général peu de différence entre les biofilms et les témoins. Mesurer les concentrations d'électrolytes dans l'eau à des moments réguliers au cours de l'expérience, dans les puits de contrôle et dans les puits de biofilms (après avoir filtré les zoospores), serait un bon moyen de tester cette hypothèse. Pour tester celle de l'allélopathie, on pourrait prendre une solution d'algues (par exemple *Leptolyngbya* sp.), retirer les algues par filtration pour ne garder que les molécules sécrétées, et exposer les zoospores à cette solution.

Il serait alors essentiel de déterminer si les zoospores sont réellement inactivées ou seulement attachées en attendant, dans un état de développement lent ou arrêté, que les conditions s'améliorent (Johnson et Speare 2003). Woodhams *et al.* (2008) ont estimé la constante de disparition exactement comme nous, mais en comptant également les zoospores mortes (immobiles) dans l'hémocytomètre, ils ont pu déterminer le taux de mortalité et, par simple déduction, le taux d'attachement également. Nous aurions pu imiter cette méthode, mais la présence de biofilms dans nos expériences la rendait inappropriée, car les zoospores pouvaient être piégées dans la matrice, ou lysées à cause de substances antimicrobiennes. Par conséquent, ces zoospores mortes n'auraient pas été comptées dans l'hémocytomètre, et nous aurions largement surestimé le taux d'attachement et sous-estimé celui de mortalité. Je pense qu'il est préférable de tester directement si les zoospores sont encore viables en les réintroduisant dans le milieu tryptone-glucose comme décrit dans le chapitre. L'obstacle majeur ici serait d'introduire des bactéries avec le morceau de biofilm (même nos cultures d'algues monospécifiques n'étaient pas axéniques) qui coloniseraient rapidement tout le milieu de culture, et domineraient Bd. Pour pallier à cela, on pourrait envisager d'introduire des antibiotiques à large spectre comme la pénicilline associée à la streptomycine (Piotrowski *et al.* 2004 ; Woodhams *et al.* 2008).

## Synthèse et conclusions

Grâce à ce travail de doctorat, j'ai amélioré les connaissances sur les biofilms benthiques des lacs de montagne et leurs rôles potentiels dans les infections par un parasite amphibien à

enjeu de conservation. Il est essentiel que les biofilms, et plus généralement les communautés microbiennes, ne soient pas négligés par les chercheurs et les praticiens de la conservation si nous voulons préserver au mieux la biodiversité exceptionnelle des écosystèmes d'eau douce de montagne et les services inestimables qu'ils rendent à l'humanité. Mes résultats montrent clairement que les communautés de biofilms benthiques sont en train de changer dans les lacs de montagne avec une dominance accrue de microorganismes plus tolérants mais potentiellement néfastes pour les organisms sympatriques. L'importance respective des facteurs du changement global dans ces modifications compositionnelles devrait être évaluée plus en détail. Il serait maintenant aussi opportun de savoir si les fonctions des biofilms changent avec ces modifications de structure. Pour ce faire, nous devons d'abord savoir quelles sont, ou étaient, leurs fonctions. Or, comme le montre le chapitre 2, il existe encore des zones d'ombre, par exemple dans notre connaissance du fonctionnement des biofilms par rapport à la santé des macro-organismes aquatiques sympatriques. J'ai essayé de faire la lumière sur ce point en me concentrant sur les interactions entre les biofilms benthiques et Bd dans la suite de ma thèse.

Mes résultats sont cohérents avec l'hypothèse selon laquelle les biofilms jouent un rôle dans l'épidémiologie des infections à Bd et de la chytridiomycose des amphibiens, bien que d'autres études soient nécessaires pour la confirmer et élucider les mécanismes en jeu. Alors que le zooplancton aide à expliquer si et dans quelle mesure (prévalence) une population est infectée (Schmeller *et al.* 2014), les biofilms benthiques, d'après mes résultats, peuvent eux aider à expliquer l'impact à long terme de la maladie sur la population d'Ao (stable ou en déclin). Cependant, il faut être prudent car mes résultats fournissent au mieux des preuves circonstancielles. Bien que nous nous concentrions sur les facteurs épidémiologiques environnementaux, d'autres facteurs pourraient également expliquer l'épidémiologie particulière observée dans les Pyrénées pour la chytridiomycose amphibienne. L'importance de certains de ces facteurs n'a pas encore été évaluée ici : j'ai déjà mentionné la génétique des populations, mais les différences interpopulationnelles dans les traits démographiques d'histoire de vie (nombre d'œufs par femelle, recrutement) pourraient également expliquer la variation des impacts sur les populations d'amphibiens, comme cela a été montré dans d'autres systèmes hôte-parasite (Valenzuela-Sánchez *et al.* 2021, 2022).

A mon avis, une façon appropriée d'avancer serait d'abord de continuer le travail présenté dans le chapitre 5, en répétant les expériences pour rendre les résultats plus robustes, et en déterminant si les zoospores sont inactivées ou encore infectieuses. En complément, je pense qu'il est indispensable à un moment donné de réaliser des expériences de méscosme, où les têtards sont élevés avec ou sans biofilms (différents biofilms pourraient être testés) et infectés par un même nombre de zoospores de Bd. En fait, j'ai déjà obtenu l'approbation éthique pour

de telles expériences, et fait toutes les formations requises. J'espère pouvoir réaliser ces expériences à l'avenir. L'obstacle majeur ici est d'obtenir un nombre suffisant de têtards relativement similaires en âge et en état de santé pour tirer des conclusions robustes des expériences.

Si, avec tout cela combiné, nous ne trouvons pas de preuve que les biofilms affectent négativement les zoospores de Bd, par exemple si les zoospores ne sont pas inactivées mais juste dans un état ralenti de développement et toujours infectieuses, alors les biofilms devraient être considérés comme une source d'infections, ou un élément neutre, plutôt qu'un élément protecteur pour les têtards. En revanche, si nous avons la preuve que les biofilms affectent négativement les zoospores de Bd, en réduisant la pression infectieuse et donc en ayant un impact positif sur la santé des têtards, alors nous devrions déterminer si différents biofilms ont les mêmes effets, et si les changements dans la structure des biofilms observés i*n situ* altèrent leur rôle dans la réduction de la pression infectieuse. D'après mes résultats, il y a quelques indications que, toutes choses étant égales par ailleurs (c'est-à-dire dans un contexte similaire, voir chapitre 5, cadre 1), un biofilm avec plus de biodiversité aurait plus d'impact sur les zoospores avec potentiellement un plus grand effet protecteur pour les têtards. Encore une fois, cette dernière affirmation reste à démontrer, mais si elle est vraie, cela soutiendrait la théorie de **l'effet de dilution** (Keesing *et al.* 2006, 2010) et aussi, parce que la chytridiomycose des amphibiens perturbe fortement les écosystèmes, **la théorie de la diversité-stabilité** (Goodman 1975; McCann 2000).. À cet égard, il convient d'évaluer les conséquences de la perte de la biodiversité procaryote observées au chapitre 3.

Cela dit, la généralité de la théorie de l'effet de dilution a fait l'objet d'un débat intense parmi les écologues de la santé (Randolph and Dobson 2012; Lafferty and Wood 2013). La validité de cette théorie semble dépendre de l'échelle, du contexte et du système considéré car de nombreux systèmes hôte-parasite ne sont pas affectés par la biodiversité; par exemple ceux où le parasite (*sensu lato*, incluant également les virus) est directement transmis, spécialisé, et sans stade de vie libre, hôte intermédiaire ou vecteur (Rohr *et al.* 2019). Dans de nombreux systèmes Bd-amphibiens (mais pas tous), cette hypothèse est souvent soutenue avec un risque sanitaire moindre lorque la diversité au niveau des hôtes (Searle *et al.* 2011 ; Becker *et al.* 2014 ; Venesky *et al.* 2014 ; James *et al.* 2015), du zooplancton (Schmeller *et al.* 2014) et du microbiote cutané (Piovia-Scott *et al.* 2017 ; Bates *et al.* 2018) est plus importante. Mais en fait, la plupart de ces études montrent que, plutôt que la diversité des espèces, c'est la présence d'une ou de quelques espèces particulières dans une communauté qui conduisent à la réduction (ou à l'amplification) des risques de maladie : par exemple, la présence d'un têtard amphibien de type filtreur (une autre guilde de têtards que les consommateurs de biofilms ; Venesky *et al.*

2014) ou de ciliés ou de rotifères se nourrisant aussi par filtration, dans la communauté (tous les rotifères et ciliés n'ont pas la même compétence pour consommer Bd ; Schmeller *et al.* 2014). Ici, j'ai constaté que la présence et l'abondance relative dans les biofilms de rotifères et, plus particulièrement, de bdelloïdes correspondaient dans une certaine mesure aux schémas d'impacts de la chytridiomycose observés dans certaines populations pyrénéennes d'Ao. Ces rotifères bdelloïdes sont des habitants de biofilms, filtrant l'eau pour se nourrir (Majdi *et al.* 2012 ; Mialet *et al.* 2013) et sont très compétents pour consommer les zoospores de Bd (Loyau, données non publiées). Sachant cela, nous devrions tester dans les expériences mentionnées ci-dessus si les biofilms avec des rotifères bdelloïdes réduisent la pression d'infection plus que d'autres biofilms dépourvus de ces rotifères. Ensuite, nous devrions également examiner quel type de communauté de biofilms, ou d'autres conditions environnementales, les rotifères bdelloïdes ont tendance à affectionner le plus, et si les changements observés dans les biofilms des lacs de montagne conduisent à un déclin des bdelloïdes et d'autres rotifères. Si ces consommateurs potentiels de Bd diminuent ou disparaissent, les risques d'infections par Bd et de maladies augmenteraient pour les amphibiens.

Cependant, les rotifères ne sont pas des micro-organismes *sensu stricto*, mais plutôt des méio-organismes benthiques, c'est-à-dire des organismes entre 42 et 500 µm (Mialet *et al.* 2013). Un objet de critique ici est que notre échantillonnage de biofilms pourrait ne pas être assez représentatif pour les organismes plus grands que les microorganismes. Pour cette raison, les écologistes microbiens retirent souvent les séquences d'ADN appartenant aux métazoaires de leur bibliothèque micro-eucaryote. Dans les chapitres 3 et 4, j'ai supprimé les métazoaires de grande taille comme les arthropodes, les vertébrés, etc., mais j'ai conservé les « petits » métazoaires qui font partie du meiobenthos comme les rotifères et les tardigrades. Ceci en partant du principe que notre échantillonnage était aussi représentatif que possible de la véritable diversité du meiobenthos de chaque lac. Bien que nous ayons gratté quatre ou cinq roches pour constituer un échantillon de biofilm, ces roches ont souvent été trouvées proches les unes des autres à l'échelle d'un lac ; je suggère que plusieurs roches provenant de zones distinctes d'un lac soient échantillonnées à l'avenir puis regroupées pour constituer un échantillon de biofilm plus représentatif (c'est également le but de la pose des autres substrats artificiels : j'ai systématiquement placé trois réplicats par lac, afin de tester si l'emplacement dans un lac influence la communauté microbienne du biofilm).

Quel que soit le rôle des biofilms par rapport aux zoospores de Bd, les biofilms pourraient toujours avoir un impact sur l'épidémiologie de la chytridiomycose des amphibiens et celle d'autres maladies infectieuses en impactant la santé des têtards en général. Tout d'abord, les risques de production de toxines par les biofilms devraient être évalués plus en détail dans les

lacs de montagne en recherchant les cyanotoxines à la fois dans les biofilms et dans la colonne d'eau. Les déterminants de la production de toxines devraient également être étudiés afin d'examiner les liens, par exemple, avec le changement climatique, l'introduction volontaire de poissons ou les effluents du cheptel. La production de toxines présente un risque sanitaire important pour les touristes, le bétail et les autres animaux dépendant des eaux de montagne, et si elle est avérée, augmenterait également les coûts de production d'eau potable. Deuxièmement, il serait intéressant d'avoir plus d'informations sur la production, la distribution et l'abondance des acides gras polyinsaturés ω-3 dans les écosystèmes d'eau douce des Pyrénées, et de les mettre en relation avec la santé des amphibiens (en particulier, leur statut d'infection par Bd) et des écosystèmes dans leur ensemble. Il serait ensuite opportun d'évaluer les tendances temporelles à la fois de la production de toxines et des ω-3 dans le contexte des compositions microbiennes des biofilms divergeant avec le temps.

Enfin, une dernière piste de recherche serait, en plus de l'étude des effets des biofilms sur la santé des amphibiens comme indiqué ci-dessus, d'étudier les effets des larves d'amphibiens sur les communautés de biofilms. Par exemple, comment le broutage affecte la structure des communautés et les fonctions des biofilms. Il est important d'étudier si la disparition des amphibiens modifie la biomasse, la structure et les fonctions des biofilms de lacs d'altitude, comme cela a été observé dans certains torrents de montagne (Alonso *et al.* 2022). Si mes hypothèses sont validées, on pourrait voir potentiellement un cercle vicieux s'installer dans les lacs de montagne : avec des pressions anthropiques de plus en plus fortes, les biofilms évoluent vers des communautés potentiellement moins diversifiées, dépourvues d'organismes fonctionnellement importants (par exemple les consommateurs de Bd ou les algues nutritives) et ne contenant que des organismes sous-optimaux pour les consommateurs (algues moins nutritives, voire toxigènes). Ces changements dans le biofilm pourraient se traduire par un risque accru de cyanotoxicose et/ou d'infection par Bd et de chytridiomycose pour les amphibiens (d'autres agents pathogènes devraient également être étudiés), ce qui pourrait entraîner un déclin de plus en plus important des populations d'amphibiens, voire leur disparition. Le déclin ou l'absence totale de larves d'amphibiens, c'est-à-dire de brouteurs de biofilms, dans les lacs de montagne signifierait alors qu'un contrôle descendant sur l'accumulation de biofilms est perdu, avec la possibilité que les algues les plus tolérantes continuent à proliférer aux dépens d'autres habitants du biofilm plus sensibles. Bien entendu, de nombreux maillons de ces chaînes d'événements restent à démontrer.

Mon travail peut être utilisé pour informer les pratiques de conservation et la gestion des ressources en eau de montagne, non seulement dans les Pyrénées mais aussi dans d'autres chaînes de montagnes. Pour empêcher ou au moins ralentir le changement des communautés de biofilms et, bien sûr, pour de nombreuses autres raisons, l'atténuation du changement climatique est une priorité absolue. Bien qu'il s'agisse d'un choix politique et de société, il est également important d'agir localement sur d'autres pressions anthropiques des lacs de montagne, car je pense qu'elles ont également un impact sur les communautés de biofilms. Cela inclut, par exemple, le contrôle du tourisme, l'éradication des poissons non indigènes ou du moins l'arrêt de leur introduction systématique à des fins de pêche, et la limitation de l'impact du bétail, par la réglementation mais aussi par l'éducation et la sensibilisation scientifique. Cela permettrait de réduire les pressions sur les écosystèmes des lacs de montagne et donnerait à leurs organismes plus de temps pour s'adapter à d'autres pressions plus pernicieuses comme le réchauffement des températures. La mise en œuvre de ces actions semble à première vue difficile sur le plan social, mais elle a été possible ailleurs (par exemple dans les Pyrénées espagnoles ; http://www.lifelimnopirineus.eu/sites/default/files/laymanen_0.pdf) et est en fait simple (en tout cas plus atteignable) comparée aux efforts requis pour limiter le changement climatique. Cela souligne le besoin d'approches transdisciplinaires du développement avec des contributions, entre autres, des parties prenantes telles que les agriculteurs, les représentants de la communauté, les politiciens, les environnementalistes, les spécialistes des sciences sociales, les épidémiologistes humains et vétérinaires, et les écologues.

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# Appendix: Environmental changes in the studied Pyrenean lakes

## Introduction

Mountain freshwater ecosystems are strongly impacted by global change factors, including pollution (Vilanova *et al.* 2001; Machate *et al.* 2022) and climate change (Rangwala and Miller 2012; Mountain Research Initiative EDW Working Group 2015; Schmeller *et al.* 2022). The latter is currently considered the greatest threat to mountain ecosystems (Schmeller *et al.* 2022). However, climate change can take many forms and result in, e.g. warmer temperatures, modified precipitation regimes, an increase of extreme weather events, or any combination of these. As a result, it can affect different mountain ranges in various ways, and even distinct localities within the same mountain range, such as mountain lakes, could be differently impacted. Furthermore, climate change, through impacts on the cryosphere, on rock weathering rates, on hydrology and notably on mixing regimes, affects lake water chemistry (Koinig *et al.* 1998; O'Reilly *et al.* 2015; Woolway *et al.* 2020). Here, using publicly available climate datasets, we characterise climate change on 26 lakes spread across the French Pyrenees from 1950 to 2021. We complemented these data with locally recorded water temperature data and water chemistry data by examining the trends in water temperature and chemistry. Describing climate change is essential to better understand its impacts on mountain biodiversity and in particular on biofilm communities.

## Materials and Methods

We retrieved daily minimum, mean, and maximum air temperatures and precipitations from 1950 to 2021 using the Europe-wide E-OBS gridded (0.1 deg) datasets (Version 25.0e, Cornes *et al.* (2018)). We then computed for all 26 lakes (spread into six gradients) the mean annual temperature and several annual climdex indices with the R-package *climdex.pcic* (David Bronaugh for the Pacific Climate Impacts Consortium 2020; **Table 10**). By sampling water on sites, we also retrieved information on water chemistry: pH, hardness, major ions and explored their temporal trends (see Chapter 3). Dataloggers (Hobo pendant 64K®) were placed from 2007 to 2021 in the 26 lakes in which biofilms were sampled for the main study. Temperature was recorded on an hourly-basis. However, some dataloggers were lost from one visit to another (stolen or destroyed), and others malfunctioned due to battery loss. As a result, our dataset contained missing values for some sites and/or periods of time. We used Multivariate Imputation by Chained Equation (R package *MICE*) with a random forest model, calibrated with the air temperature dataset and the available temperature data, to impute the missing values

of the water temperature dataset (Buuren and Groothuis-Oudshoorn 2011). Dataloggers showed a temperature of 0°C when water froze, although air temperatures could go well below 0. As a result, the imputations of water temperatures during winter were not reliable, and we used the mean temperature during the ice-free period (June-October, when all our samples were taken) as variable to determine trends in water temperatures.

To describe the effects of climate change in our study sites, we determined the temporal trends of each climatic variables (mean annual temperature and other annual climdex indices, **Table 10**) during the period 1950-2021 and during our study period (2016-2020). On the one hand, we determined an overall trend, pooling all sites together; on the other hand, we determined site-specific trends. Because all lakes from a same gradient are located in a same grid, we randomly selected one lake per gradient and used the interaction between time and gradient (as a proxy for site) in the model, to avoid pseudoreplication. To analyse the overall trend, we used random-intercept linear mixed models in R (R Core Team 2022), package *lmerTest* (Kuznetsova *et al.* 2017), with the above-mentioned variables as response, and time (in years) as explanatory variables with site as a random factor. To analyse gradient-specific trends, we used linear models with the same response variables but with the interaction between time and gradient as explanatory variables. Function *emtrends* of package emmeans was then use to extract the temporal coefficients for each gradient, and compute their p-values which were adjusted by Tukey's Honest Significant Difference posthoc test (Lenth 2022). Regarding water temperature, we used the same kind of model but only during the period 2007-2021, and included each lake as they have their own temperature regime; that is, we used the interaction time*lake instead of time*gradient to determine local trends.

We also explored the temporal trends (global and local) of water chemistry variables (the most complete ones in our dataset being hardness, chloride, and potassium) from 2016 to 2021. We included data from 2021 values to better assess trends, although outside of our study period. We applied the same statistical approach, with generalized linear mixed models (gamma distribution and a log link function) instead of linear mixed models, with package *glmmTMB*, when assessing the global trends (Brooks *et al.* 2017).

## Results

Results are presented in the main text.

## Conclusion

Since 1950, the climate has changed markedly in our study sites, but while temperatures have on average warmed everywhere, there are sizeable differences depending on the localisation of the lakes. Furthermore, although more data are needed to confirm these trends, climate change appears to be associated with modifications in the chemistry of water chemistry in our sites. These observations are consistent with previous studies investigating the impact of climate change on mountain lake water chemistry (Curtis *et al.* 2009; Rogora *et al.* 2020).

## Figures

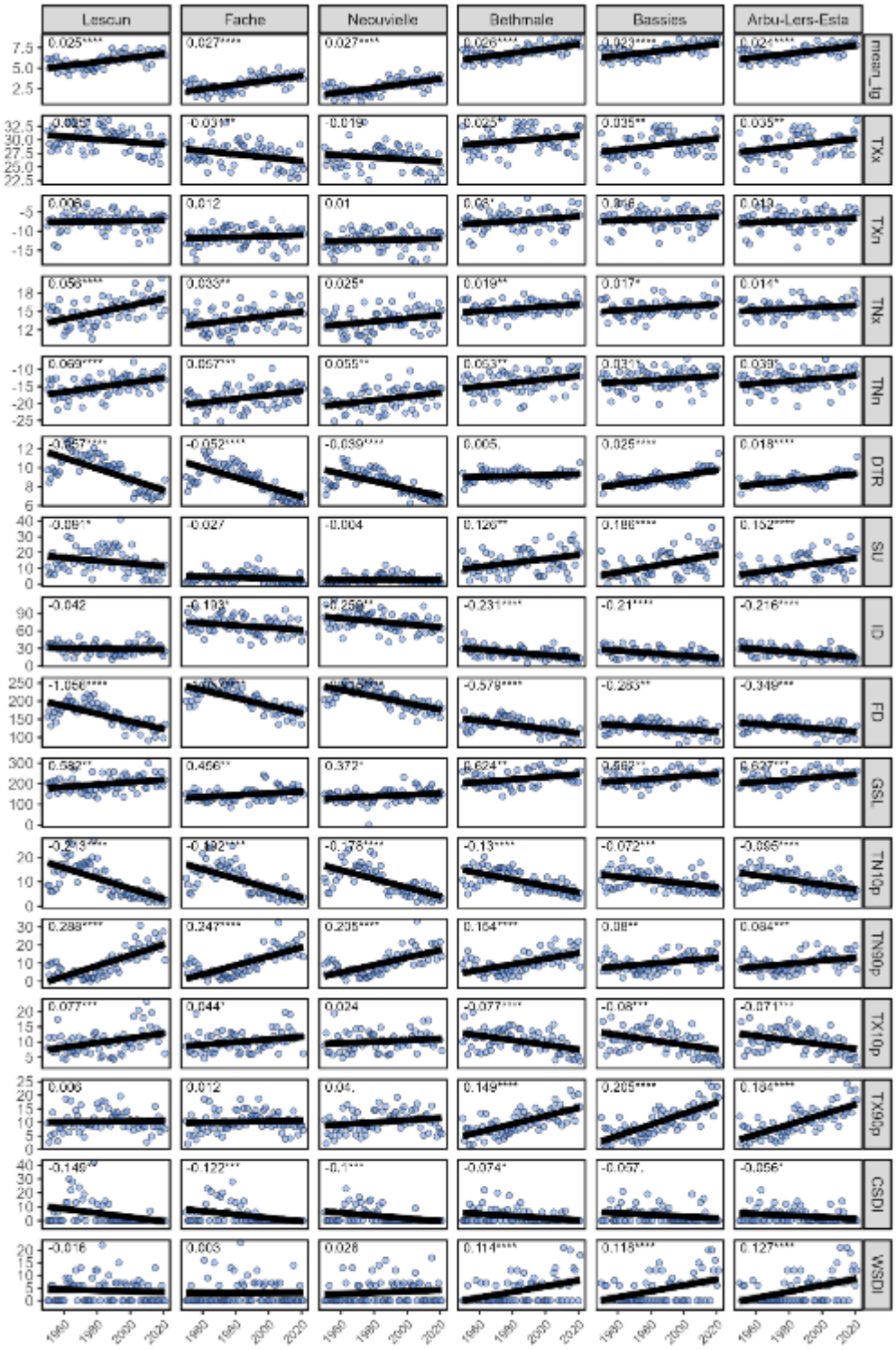


**Figure 40: Local temporal trends of the temperature-related Climdex indices from 1950 to 2021 in the six studied altitudinal gradients.** Gradients are sorted from West to East. The slope coefficient of the linear regression line is displayed in each facet, with its significance ($p < 0.0001$: "****", $p < 0.001$: "***", $p < 0.01$: "**", $p < 0.05$: "*", $p < 0.1$: ".", $p > 0.1$: "ns"). P-values of each gradient temporal trend were determined with the function stat_poly_eq of package ggpmisc (Aphalo 2022) following the linear model: Variable ~ year*gradient, but they are not adjusted for multiple comparisons. See **Table 11** for adjusted p-values

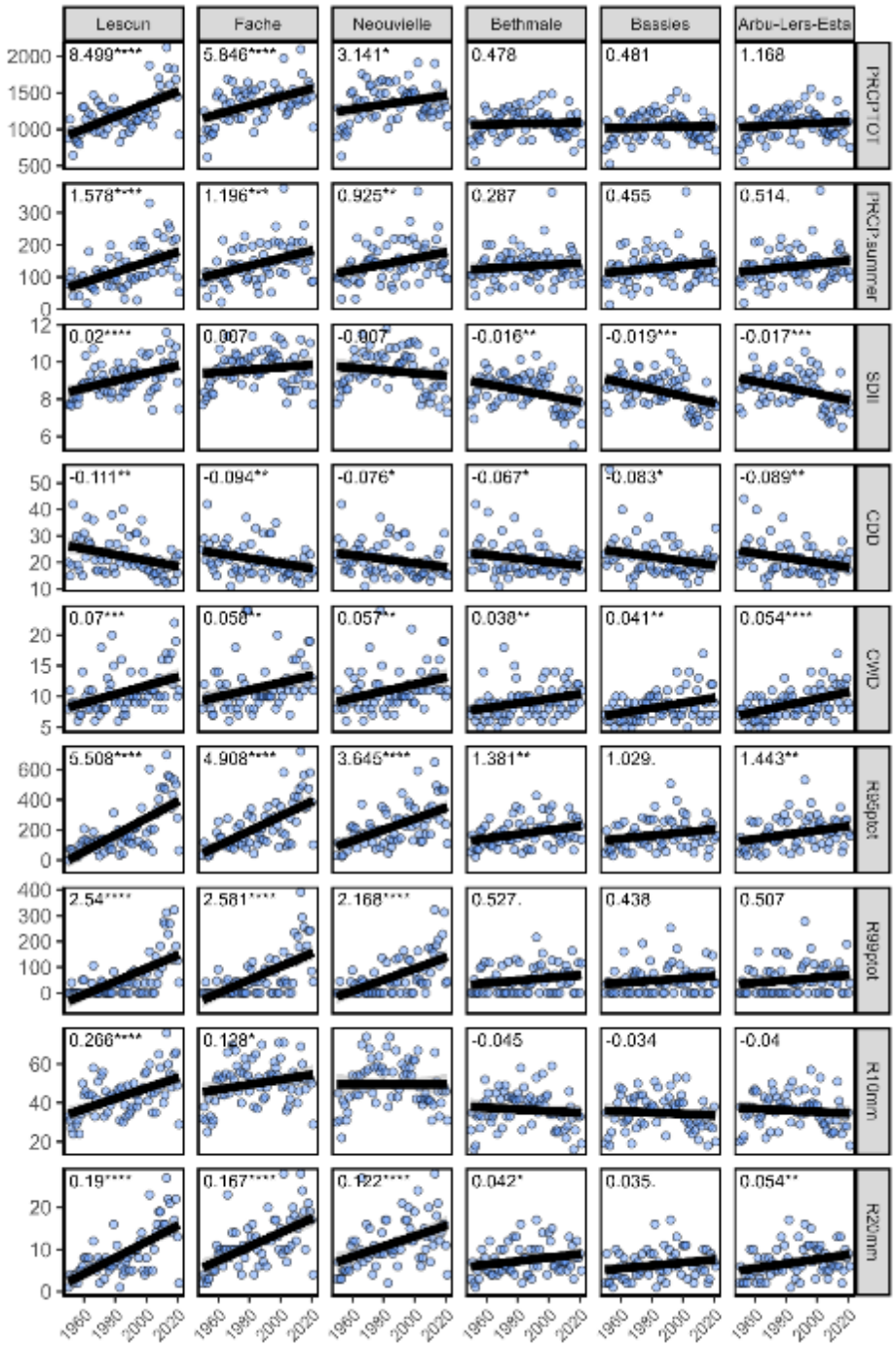


**Figure 41: Local temporal trends of the precipitation-related Climdex indices from 1950 to 2021 in the six studied altitudinal gradients.** Gradients are sorted from West to East. The slope coefficient of the linear regression line is displayed in each facet, with its significance ($p < 0.0001$: "****", $p < 0.001$: "***", $p < 0.01$: "**", $p < 0.05$: "*", $p < 0.1$: ".", $p > 0.1$: "ns"). P-values of each gradient temporal trend were determined with the function stat_poly_eq of package ggpmisc (Aphalo 2022) following the linear model: Variable ~ year*gradient, but they are not adjusted for multiple comparisons. See **Table 11** for adjusted p-values


**Figure 42: Local temporal trends of the temperature-related Climdex indices from 2016 to 2020 in the six studied altitudinal gradients.** Gradients are sorted from West to East. The slope coefficient of the linear regression line is displayed in each facet, with its significance ($p < 0.0001$: "****", $p < 0.001$: "***", $p < 0.01$: "**", $p < 0.05$: "*", $p < 0.1$: ".", $p > 0.1$: "ns"). P-values of each gradient temporal trend were determined with the function stat_poly_eq of package ggpmisc (Aphalo 2022) following the linear model: Variable ~ year*gradient, but they are not adjusted for multiple comparisons. See **Table 12** for adjusted p-values.

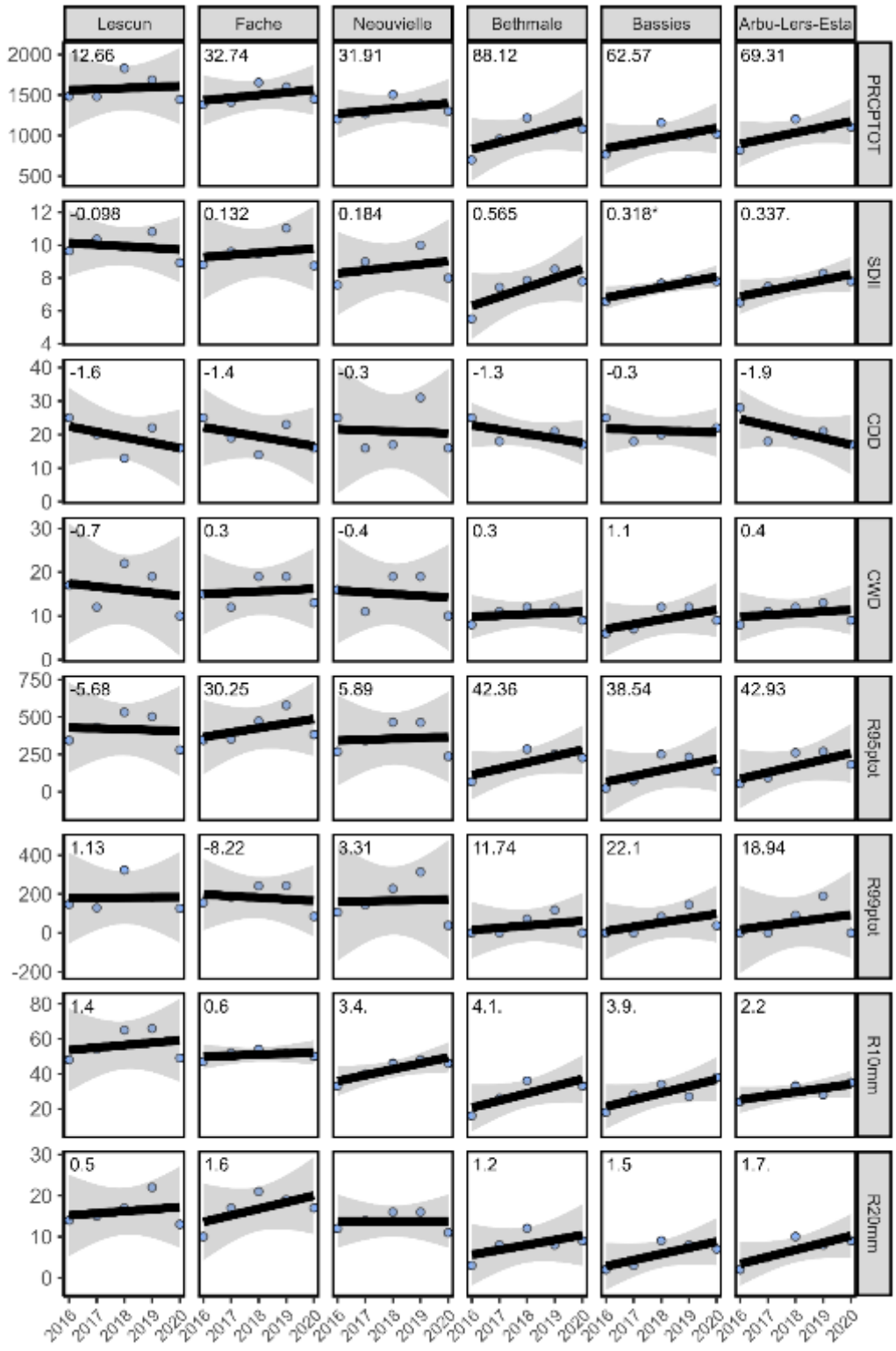


**Figure 43: Local temporal trends of the precipitation-related Climdex indices from 2016 to 2020 in the six studied altitudinal gradients.** Gradients are sorted from West to East. The slope coefficient of the linear regression line is displayed in each facet, with its significance ($p < 0.0001$: "****", $p < 0.001$: "***", $p < 0.01$: "**", $p < 0.05$: "*", $p < 0.1$: ".", $p > 0.1$: "ns"). P-values of each gradient temporal trend were determined with the function stat_poly_eq of package ggpmisc (Aphalo 2022) following the linear model: Variable ~ year*gradient, but they are not adjusted for multiple comparisons. See **Table 12** for adjusted p-values

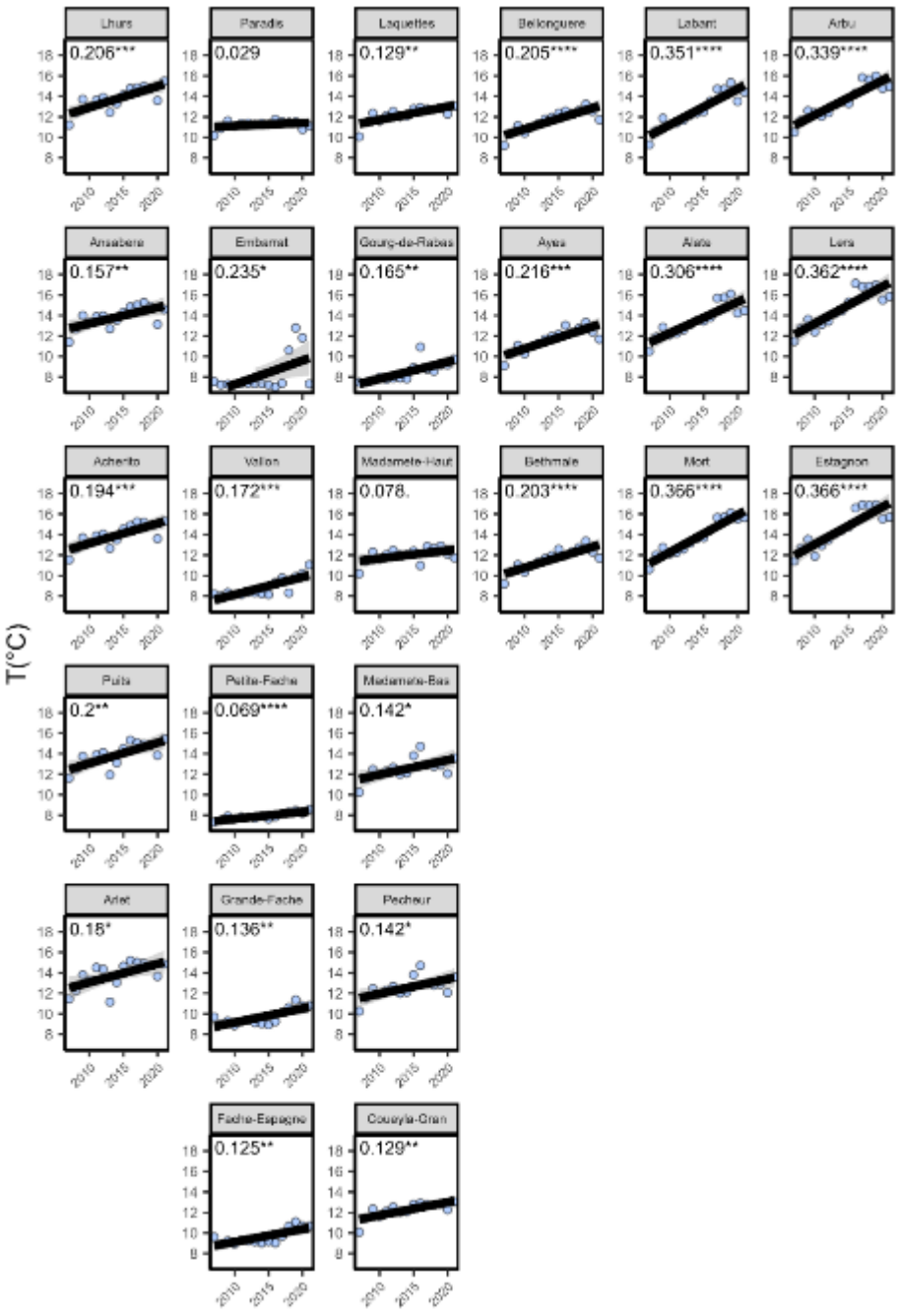


**Figure 44: Local temporal trends of annual average water temperature during the ice-free period (June-October) from 2007 to 2021.** These data are inferred from in-site water temperature datalogger and air temperature data. We only present temperatures in the ice-free period as dataloggers do not register temperatures correctly when water is frozen. Therefore, winter water temperatures of dataloggers are not well correlated to air temperatures, resulting in spurious inferences for missing winter water temperatures. Gradients are represented in columns from West to East, within which lakes are placed by increasing elevation. The slope coefficient of the linear regression line is displayed in each facet, with its significance ($p < 0.0001$: "****", $p < 0.001$: "***", $p < 0.01$: "**", $p < 0.05$: "*", $p < 0.1$: ".", $p > 0.1$: "ns"). P-values of each gradient temporal trend were determined with the function stat_poly_eq of package ggpmisc (Aphalo 2022) following the linear model: Variable ~ year*gradient, but they are not adjusted for multiple comparisons. See **Table 13** for adjusted p-values

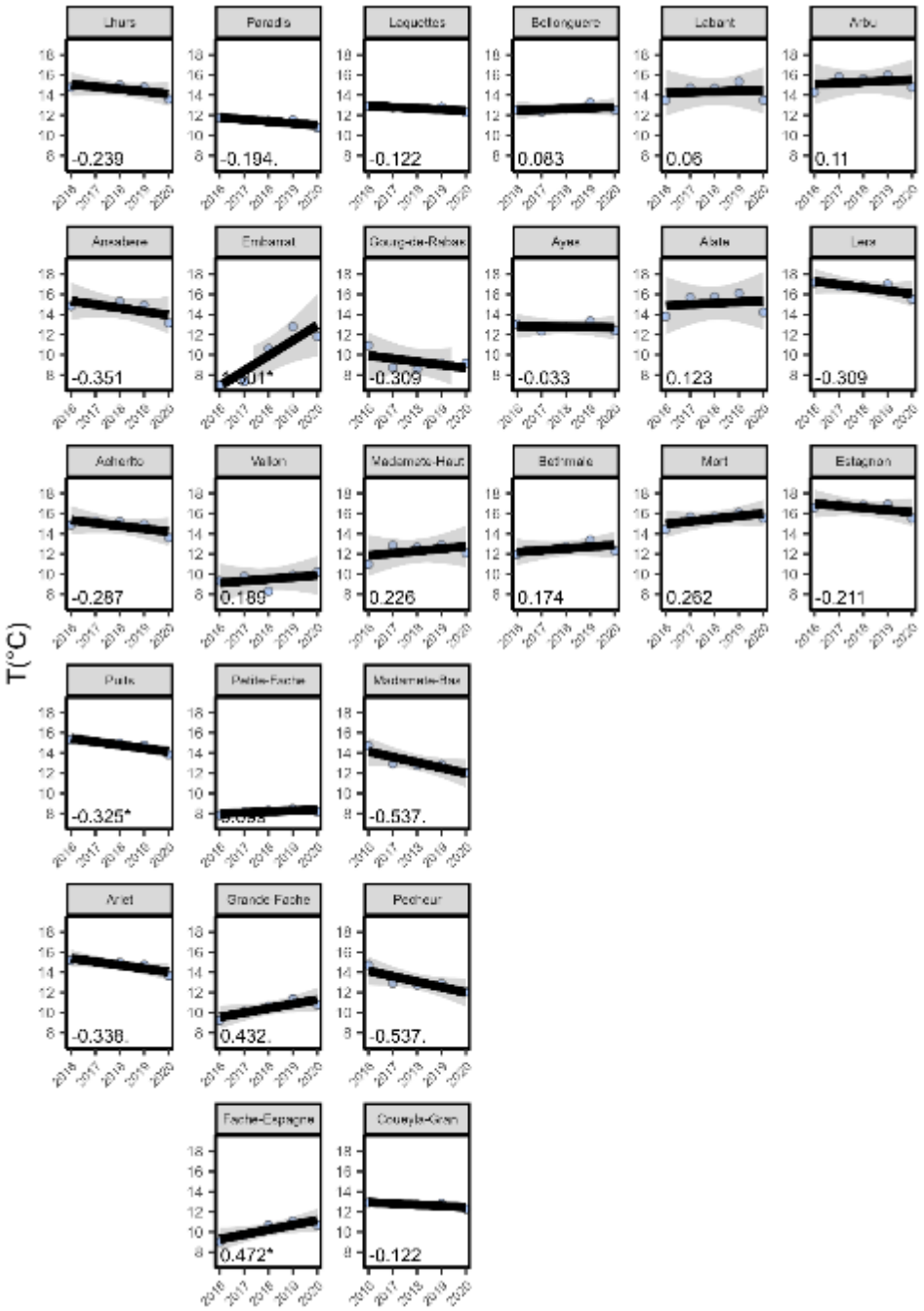


**Figure 45: Local temporal trends of annual average water temperature during the ice-free period (June-October) from 2016 to 2021.** Same legends as previous figure.

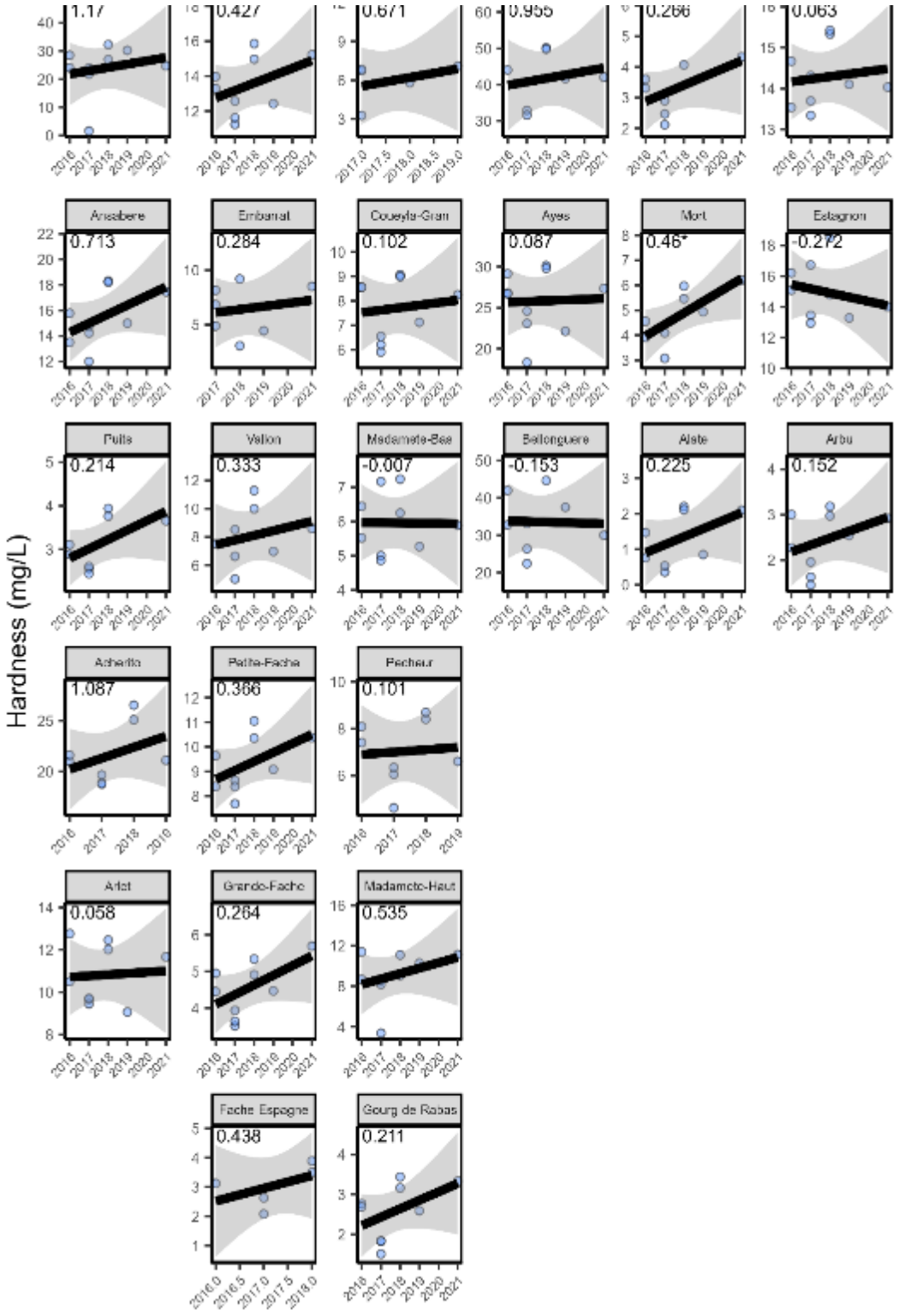


**Figure 46: Local temporal trends in water hardness ($Ca^{2+}$ + $Mg^{2+}$) over the course of the study period.** Gradients are represented in columns from West to East, within which lakes are placed by increasing elevation. The slope coefficient of the linear regression line is displayed in each facet, with its significance (p < 0.0001: "****", p < 0.001: "***", p < 0.01: "**", p < 0.05: "*", p < 0.1: ".", p > 0.1: "ns"). P-values of each gradient temporal trend were determined with the function stat_poly_eq of package ggpmisc (Aphalo 2022) following the linear model: Variable ~ year*gradient, but they are not adjusted for multiple comparisons. See **Table 13** for adjusted p-values.

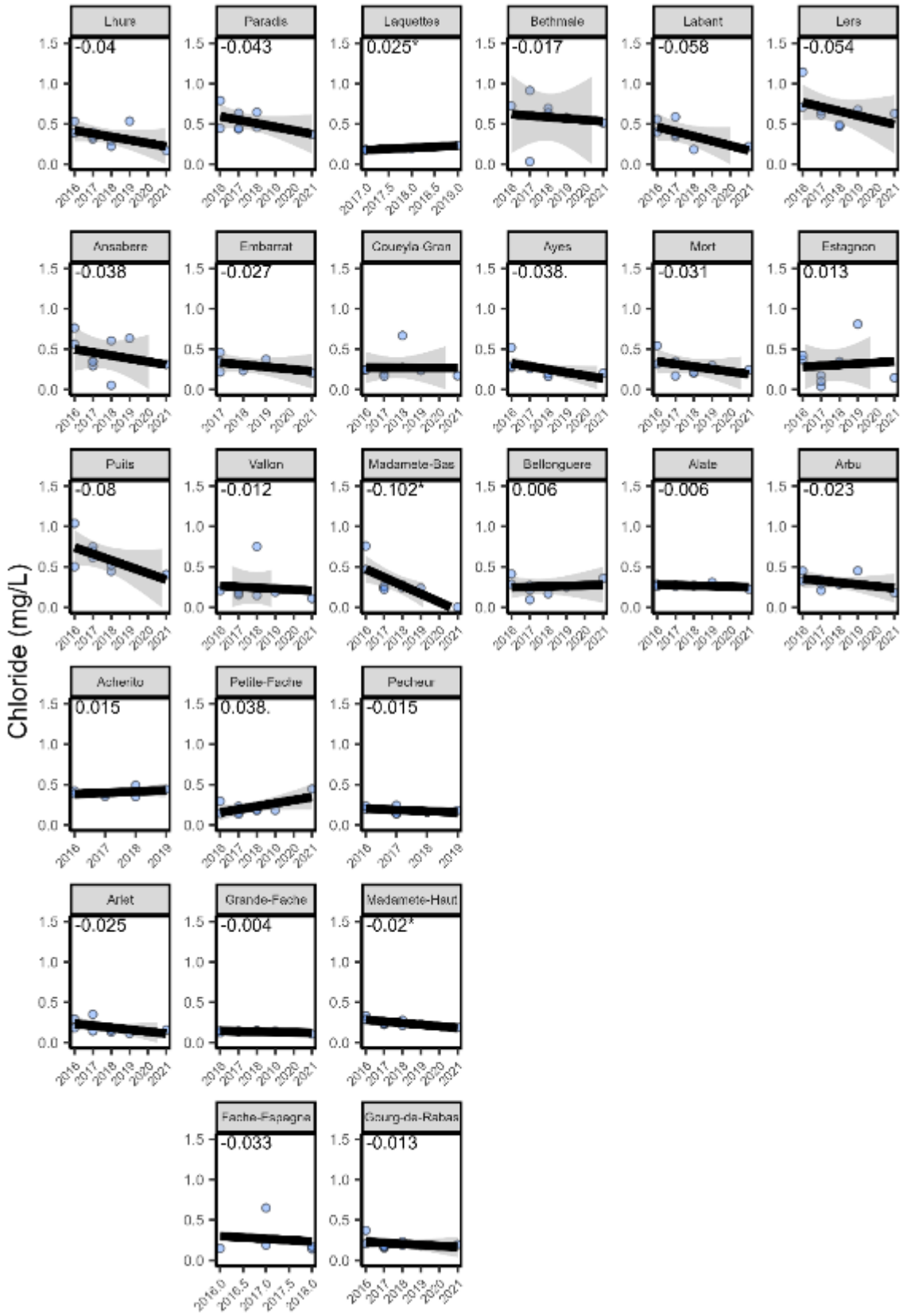


**Figure 47: Local temporal trends in Chloride (mg/L) over the course of the study period.** Same legends as in **Figure 46**.

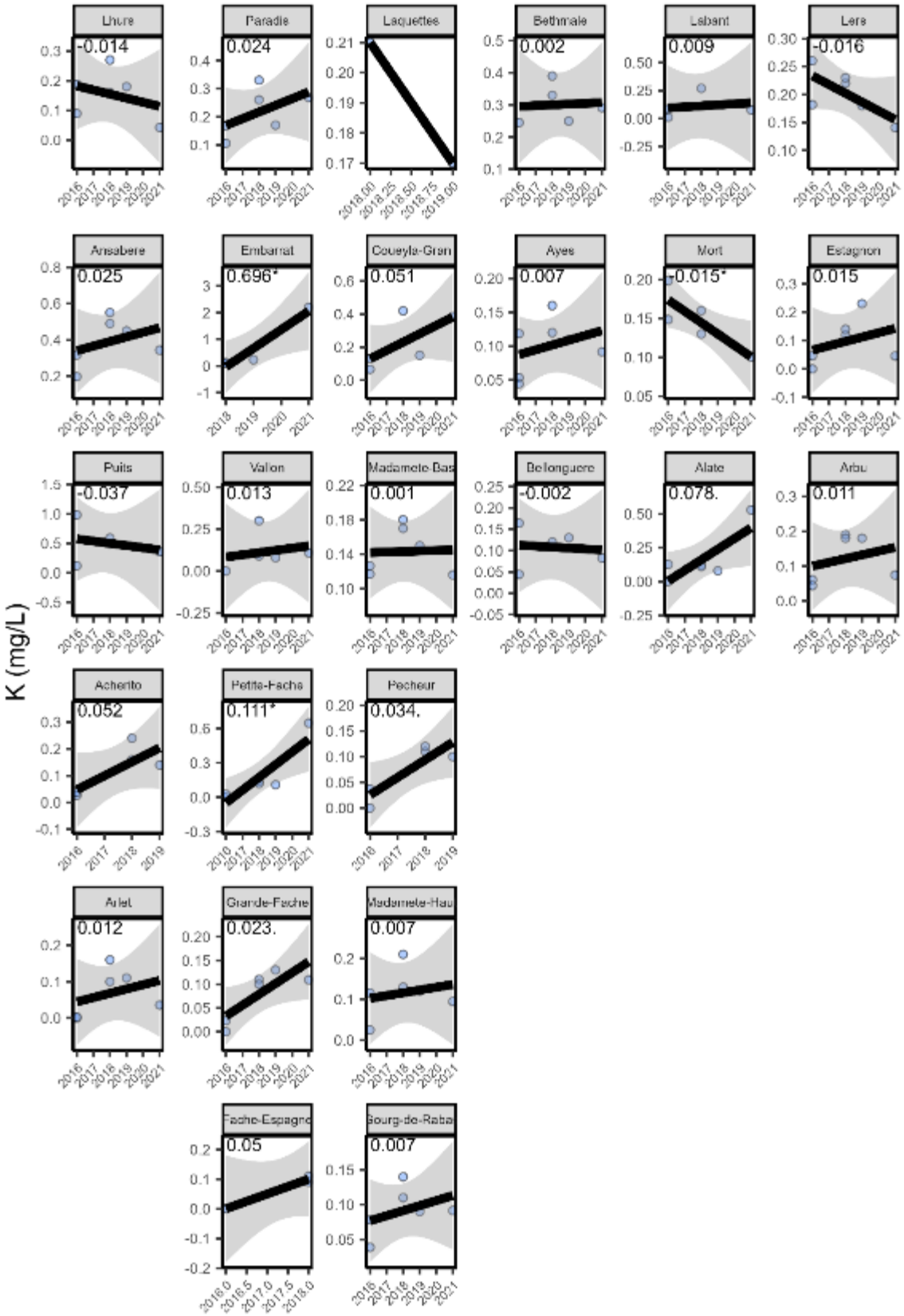


**Figure 48: Local temporal trends in Potassium (mg/L) over the course of the study period.** Same legends as in **Figure 46**.

# Tables

## Table 10: Meaning of the Climdex indices

| Climdex index | Abbreviation | Definition |
|---|---|---|
| Average annual temperature | Mean_tg | |
| Number of frost days | FD | Annual count of days when TN (daily minimum temperature) < 0°C |
| Number of icing days | ID | Annual count of days when TX (daily maximum temperature) < 0 °C |
| Number of summer days | SU | Annual count of days when TX (daily maximum temperature) > 25°C |
| Growing season length | GSL | Annual* count between the first span of at least 6 days with daily mean temperature TG >5 °C and the first span after July 1st (Jan 1st in SH) of 6 days with TG <5 °C. |
| Maximum value of daily maximum temperature | TXx | The maximum of all TX within a year, i.e. the warmest temperature of that year |
| Minimum value of daily minimum temperature | TNn | The minimum of all TN within a year, i.e. the coldest temperature of that year |
| Maximum value of daily minimum temperature | TNx | The maximum of all TN within a year, i.e. the minimum temperature during the warmest day of the year |
| Minimum value of daily maximum temperature | TXn | The minimum of all TX within a year, i.e. the maximum temperature of the coldest day of the year |
| Average Daily Temperature Range | DTR | Mean of the subtraction of all TX-TN in a year |
| Percentage of days when TN < 10th percentile | TN10p | Percentage of days in a year when TN is < to the 10th percentile of all TN from the base period 1961-1990 |
| Percentage of days when TX < 10th percentile | TX10p | Percentage of days in a year when TX is < to the 10th percentile of all TX from the base period 1961-1990 |
| Percentage of days when TN > 90th percentile | TN90p | Percentage of days in a year when TN is > to the 90th percentile of all TN from the base period 1961-1990 |
| Percentage of days when TX > 90th percentile | TX90p | Percentage of days in a year when TX is > to the 90th percentile of all TX from the base period 1961-1990 |
| Warm spell duration index | WSDI | Annual count of days with at least 6 consecutive days when TX > 90th percentile of all TX from the base period1961-1990 |
| Cold spell duration index | CSDI | Annual count of days with at least 6 consecutive days when TN < 10th percentile of all TN from the base period1961-1990 |
| Annual total precipitation | PRCPTOT | The sum of all RR (daily precipitation) in a year |
| Simple precipitation intensity index | SDII | The annual total precipitation in mm divided by the number of wet days (i.e. days when RR>1mm) |
| Annual count of days when PRCP ≥ 10mm | R10mm | |
| Annual count of days when PRCP ≥ 10mm | R20mm | |
| Annual total PRCP when RR > 95th percentile | R95p | The sum of all RR > 95$^{th}$ percentile of RR from the base period 1961-90 |
| Annual total PRCP when RR > 99th percentile | R99p | The sum of all RR > 99$^{th}$ percentile of RR from the base period 1961-90 |
| Consecutive dry days | CDD | Maximum number of consecutive days with RR < 1mm |
| Consecutive wet days | CWD | Maximum number of consecutive days with RR ≥ 1mm |

**Table 11: Summary statistics for each model: Climatic variables ~ time * gradient (local trends) or Climatic variables ~ time only (overall trend), from 1950 to 2021.** P-values for each gradient (local trend) were adjusted for multiple comparisons with Tukey's honest significant difference posthoc test with package *emmeans* (Lenth 2022). Significance: **** p < 0.0001, *** p < 0.001; ** p < 0.01, * p< 0.05, n.s. p > 0.05

| variable | gradient | Year trend | SE | df | t.ratio | p.value |
|---|---|---|---|---|---|---|
| mean_tg | Overall | 0.025 | 0.001 | 425 | 17.184 | < 0.001 |
| | Lescun | 0.025 | 0.004 | 420 | 6.85 | < 0.001 |
| | Fache | 0.027 | 0.004 | 420 | 7.477 | < 0.001 |
| | Neouvielle | 0.027 | 0.004 | 420 | 7.539 | < 0.001 |
| | Bethmale | 0.026 | 0.004 | 420 | 7.207 | < 0.001 |
| | Bassies | 0.023 | 0.004 | 420 | 6.354 | < 0.001 |
| | Arbu-Lers-Estagnon | 0.024 | 0.004 | 420 | 6.479 | < 0.001 |
| FD | Overall | -0.706 | 0.046 | 425 | -15.241 | < 0.001 |
| | Lescun | -1.056 | 0.108 | 420 | -9.808 | < 0.001 |
| | Fache | -1.057 | 0.108 | 420 | -9.822 | < 0.001 |
| | Neouvielle | -0.915 | 0.108 | 420 | -8.502 | < 0.001 |
| | Bethmale | -0.579 | 0.108 | 420 | -5.379 | < 0.001 |
| | Bassies | -0.283 | 0.108 | 420 | -2.626 | 0.009 |
| | Arbu-Lers-Estagnon | -0.349 | 0.108 | 420 | -3.241 | 0.001 |
| SU | Overall | 0.057 | 0.014 | 425 | 3.975 | < 0.001 |
| | Lescun | -0.091 | 0.033 | 420 | -2.738 | 0.006 |
| | Fache | -0.027 | 0.033 | 420 | -0.825 | 0.41 |
| | Neouvielle | -0.004 | 0.033 | 420 | -0.108 | 0.914 |
| | Bethmale | 0.126 | 0.033 | 420 | 3.809 | < 0.001 |
| | Bassies | 0.186 | 0.033 | 420 | 5.612 | < 0.001 |
| | Arbu-Lers-Estagnon | 0.152 | 0.033 | 420 | 4.568 | < 0.001 |
| ID | Overall | -0.192 | 0.025 | 425 | -7.68 | < 0.001 |
| | Lescun | -0.042 | 0.061 | 420 | -0.684 | 0.495 |
| | Fache | -0.193 | 0.061 | 420 | -3.173 | 0.002 |
| | Neouvielle | -0.259 | 0.061 | 420 | -4.243 | < 0.001 |
| | Bethmale | -0.231 | 0.061 | 420 | -3.797 | < 0.001 |
| | Bassies | -0.21 | 0.061 | 420 | -3.438 | < 0.001 |
| | Arbu-Lers-Estagnon | -0.216 | 0.061 | 420 | -3.543 | < 0.001 |
| GSL | Overall | 0.537 | 0.072 | 425 | 7.493 | < 0.001 |
| | Lescun | 0.582 | 0.176 | 420 | 3.3 | 0.001 |
| | Fache | 0.456 | 0.176 | 420 | 2.588 | 0.01 |
| | Neouvielle | 0.372 | 0.176 | 420 | 2.112 | 0.035 |
| | Bethmale | 0.624 | 0.176 | 420 | 3.54 | < 0.001 |
| | Bassies | 0.562 | 0.176 | 420 | 3.188 | 0.002 |
| | Arbu-Lers-Estagnon | 0.627 | 0.176 | 420 | 3.555 | < 0.001 |
| TXx | Overall | 0.003 | 0.005 | 425 | 0.693 | 0.489 |
| | Lescun | -0.025 | 0.011 | 420 | -2.281 | 0.023 |
| | Fache | -0.031 | 0.011 | 420 | -2.873 | 0.004 |
| | Neouvielle | -0.019 | 0.011 | 420 | -1.75 | 0.081 |
| | Bethmale | 0.025 | 0.011 | 420 | 2.29 | 0.023 |
| | Bassies | 0.035 | 0.011 | 420 | 3.209 | 0.001 |
| | Arbu-Lers-Estagnon | 0.035 | 0.011 | 420 | 3.173 | 0.002 |
| TNx | Overall | 0.027 | 0.003 | 425 | 7.861 | < 0.001 |
| | Lescun | 0.056 | 0.008 | 420 | 6.641 | < 0.001 |
| | Fache | 0.033 | 0.008 | 420 | 3.935 | < 0.001 |
| | Neouvielle | 0.025 | 0.008 | 420 | 3.027 | 0.003 |
| | Bethmale | 0.019 | 0.008 | 420 | 2.25 | 0.025 |
| | Bassies | 0.017 | 0.008 | 420 | 2.052 | 0.041 |
| | Arbu-Lers-Estagnon | 0.014 | 0.008 | 420 | 1.624 | 0.105 |
| TXn | Overall | 0.015 | 0.005 | 425 | 2.808 | 0.005 |
| | Lescun | 0.006 | 0.013 | 420 | 0.467 | 0.641 |
| | Fache | 0.012 | 0.013 | 420 | 0.903 | 0.367 |
| | Neouvielle | 0.01 | 0.013 | 420 | 0.727 | 0.468 |
| | Bethmale | 0.03 | 0.013 | 420 | 2.195 | 0.029 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Bassies | 0.016 | 0.013 | 420 | 1.185 | 0.237 |
| | Arbu-Lers-Estagnon | 0.019 | 0.013 | 420 | 1.376 | 0.17 |
| TNn | Overall | 0.051 | 0.006 | 425 | 7.915 | < 0.001 |
| | Lescun | 0.069 | 0.016 | 420 | 4.4 | < 0.001 |
| | Fache | 0.057 | 0.016 | 420 | 3.625 | < 0.001 |
| | Neouvielle | 0.055 | 0.016 | 420 | 3.488 | < 0.001 |
| | Bethmale | 0.053 | 0.016 | 420 | 3.368 | < 0.001 |
| | Bassies | 0.031 | 0.016 | 420 | 2.003 | 0.046 |
| | Arbu-Lers-Estagnon | 0.039 | 0.016 | 420 | 2.474 | 0.014 |
| TN10p | Overall | -0.147 | 0.01 | 430 | -15.256 | < 0.001 |
| | Lescun | -0.213 | 0.023 | 420 | -9.248 | < 0.001 |
| | Fache | -0.192 | 0.023 | 420 | -8.345 | < 0.001 |
| | Neouvielle | -0.178 | 0.023 | 420 | -7.749 | < 0.001 |
| | Bethmale | -0.13 | 0.023 | 420 | -5.649 | < 0.001 |
| | Bassies | -0.072 | 0.023 | 420 | -3.115 | 0.002 |
| | Arbu-Lers-Estagnon | -0.095 | 0.023 | 420 | -4.131 | < 0.001 |
| TX10p | Overall | -0.014 | 0.008 | 430 | -1.704 | 0.089 |
| | Lescun | 0.077 | 0.019 | 420 | 4.136 | < 0.001 |
| | Fache | 0.044 | 0.019 | 420 | 2.354 | 0.019 |
| | Neouvielle | 0.024 | 0.019 | 420 | 1.306 | 0.192 |
| | Bethmale | -0.077 | 0.019 | 420 | -4.124 | < 0.001 |
| | Bassies | -0.08 | 0.019 | 420 | -4.31 | < 0.001 |
| | Arbu-Lers-Estagnon | -0.071 | 0.019 | 420 | -3.824 | < 0.001 |
| TN90p | Overall | 0.176 | 0.011 | 430 | 16.146 | < 0.001 |
| | Lescun | 0.288 | 0.025 | 420 | 11.324 | < 0.001 |
| | Fache | 0.247 | 0.025 | 420 | 9.702 | < 0.001 |
| | Neouvielle | 0.205 | 0.025 | 420 | 8.074 | < 0.001 |
| | Bethmale | 0.154 | 0.025 | 420 | 6.074 | < 0.001 |
| | Bassies | 0.08 | 0.025 | 420 | 3.158 | 0.002 |
| | Arbu-Lers-Estagnon | 0.084 | 0.025 | 420 | 3.299 | 0.001 |
| TX90p | Overall | 0.099 | 0.009 | 430 | 10.882 | < 0.001 |
| | Lescun | 0.006 | 0.02 | 420 | 0.295 | 0.768 |
| | Fache | 0.012 | 0.02 | 420 | 0.579 | 0.563 |
| | Neouvielle | 0.04 | 0.02 | 420 | 1.951 | 0.052 |
| | Bethmale | 0.149 | 0.02 | 420 | 7.327 | < 0.001 |
| | Bassies | 0.205 | 0.02 | 420 | 10.068 | < 0.001 |
| | Arbu-Lers-Estagnon | 0.184 | 0.02 | 420 | 9.047 | < 0.001 |
| WSDI | Overall | 0.062 | 0.012 | 430 | 5.338 | < 0.001 |
| | Lescun | -0.016 | 0.028 | 420 | -0.577 | 0.564 |
| | Fache | 0.003 | 0.028 | 420 | 0.1 | 0.921 |
| | Neouvielle | 0.026 | 0.028 | 420 | 0.946 | 0.345 |
| | Bethmale | 0.114 | 0.028 | 420 | 4.094 | < 0.001 |
| | Bassies | 0.118 | 0.028 | 420 | 4.268 | < 0.001 |
| | Arbu-Lers-Estagnon | 0.127 | 0.028 | 420 | 4.562 | < 0.001 |
| CSDI | Overall | -0.093 | 0.014 | 430 | -6.866 | < 0.001 |
| | Lescun | -0.149 | 0.033 | 420 | -4.476 | < 0.001 |
| | Fache | -0.122 | 0.033 | 420 | -3.669 | < 0.001 |
| | Neouvielle | -0.1 | 0.033 | 420 | -3.016 | 0.003 |
| | Bethmale | -0.074 | 0.033 | 420 | -2.239 | 0.026 |
| | Bassies | -0.057 | 0.033 | 420 | -1.724 | 0.086 |
| | Arbu-Lers-Estagnon | -0.056 | 0.033 | 420 | -1.696 | 0.091 |
| DTR | Overall | -0.017 | 0.003 | 425 | -6.603 | < 0.001 |
| | Lescun | -0.057 | 0.005 | 420 | -12.045 | < 0.001 |
| | Fache | -0.052 | 0.005 | 420 | -11.003 | < 0.001 |
| | Neouvielle | -0.039 | 0.005 | 420 | -8.391 | < 0.001 |
| | Bethmale | 0.005 | 0.005 | 420 | 1.052 | 0.293 |
| | Bassies | 0.025 | 0.005 | 420 | 5.304 | < 0.001 |
| | Arbu-Lers-Estagnon | 0.018 | 0.005 | 420 | 3.932 | < 0.001 |
| RX1day | Overall | 0.257 | 0.029 | 425 | 8.954 | < 0.001 |
| | Lescun | 0.479 | 0.068 | 420 | 7.061 | < 0.001 |
| | Fache | 0.441 | 0.068 | 420 | 6.49 | < 0.001 |
| | Neouvielle | 0.336 | 0.068 | 420 | 4.947 | < 0.001 |
| | Bethmale | 0.1 | 0.068 | 420 | 1.477 | 0.141 |
| | Bassies | 0.078 | 0.068 | 420 | 1.154 | 0.249 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Arbu-Lers-Estagnon | 0.109 | 0.068 | 420 | 1.6 | 0.11 |
| RX5day | Overall | 0.395 | 0.056 | 425 | 7.098 | < 0.001 |
| | Lescun | 0.898 | 0.131 | 420 | 6.852 | < 0.001 |
| | Fache | 0.721 | 0.131 | 420 | 5.496 | < 0.001 |
| | Neouvielle | 0.526 | 0.131 | 420 | 4.015 | < 0.001 |
| | Bethmale | 0.11 | 0.131 | 420 | 0.839 | 0.402 |
| | Bassies | 0.007 | 0.131 | 420 | 0.057 | 0.955 |
| | Arbu-Lers-Estagnon | 0.109 | 0.131 | 420 | 0.831 | 0.406 |
| SDII | Overall | -0.005 | 0.002 | 425 | -2.45 | 0.015 |
| | Lescun | 0.02 | 0.005 | 420 | 3.966 | < 0.001 |
| | Fache | 0.007 | 0.005 | 420 | 1.363 | 0.174 |
| | Neouvielle | -0.007 | 0.005 | 420 | -1.425 | 0.155 |
| | Bethmale | -0.016 | 0.005 | 420 | -3.152 | 0.002 |
| | Bassies | -0.019 | 0.005 | 420 | -3.709 | < 0.001 |
| | Arbu-Lers-Estagnon | -0.017 | 0.005 | 420 | -3.338 | < 0.001 |
| R10mm | Overall | 0.046 | 0.023 | 425 | 1.983 | 0.048 |
| | Lescun | 0.266 | 0.055 | 420 | 4.829 | < 0.001 |
| | Fache | 0.128 | 0.055 | 420 | 2.312 | 0.021 |
| | Neouvielle | 0 | 0.055 | 420 | -0.009 | 0.993 |
| | Bethmale | -0.045 | 0.055 | 420 | -0.811 | 0.418 |
| | Bassies | -0.034 | 0.055 | 420 | -0.616 | 0.538 |
| | Arbu-Lers-Estagnon | -0.04 | 0.055 | 420 | -0.727 | 0.467 |
| R20mm | Overall | 0.102 | 0.01 | 425 | 10.23 | < 0.001 |
| | Lescun | 0.19 | 0.023 | 420 | 8.148 | < 0.001 |
| | Fache | 0.167 | 0.023 | 420 | 7.144 | < 0.001 |
| | Neouvielle | 0.122 | 0.023 | 420 | 5.242 | < 0.001 |
| | Bethmale | 0.042 | 0.023 | 420 | 1.784 | 0.075 |
| | Bassies | 0.035 | 0.023 | 420 | 1.49 | 0.137 |
| | Arbu-Lers-Estagnon | 0.054 | 0.023 | 420 | 2.315 | 0.021 |
| CDD | Overall | -0.087 | 0.014 | 430 | -6.272 | < 0.001 |
| | Lescun | -0.111 | 0.034 | 420 | -3.249 | 0.001 |
| | Fache | -0.094 | 0.034 | 420 | -2.768 | 0.006 |
| | Neouvielle | -0.076 | 0.034 | 420 | -2.233 | 0.026 |
| | Bethmale | -0.067 | 0.034 | 420 | -1.963 | 0.05 |
| | Bassies | -0.083 | 0.034 | 420 | -2.436 | 0.015 |
| | Arbu-Lers-Estagnon | -0.089 | 0.034 | 420 | -2.609 | 0.009 |
| CWD | Overall | 0.053 | 0.007 | 425 | 8.148 | < 0.001 |
| | Lescun | 0.07 | 0.016 | 420 | 4.38 | < 0.001 |
| | Fache | 0.058 | 0.016 | 420 | 3.621 | < 0.001 |
| | Neouvielle | 0.057 | 0.016 | 420 | 3.546 | < 0.001 |
| | Bethmale | 0.038 | 0.016 | 420 | 2.372 | 0.018 |
| | Bassies | 0.041 | 0.016 | 420 | 2.594 | 0.01 |
| | Arbu-Lers-Estagnon | 0.054 | 0.016 | 420 | 3.392 | < 0.001 |
| R95ptot | Overall | 2.985 | 0.251 | 425 | 11.907 | < 0.001 |
| | Lescun | 5.508 | 0.58 | 420 | 9.504 | < 0.001 |
| | Fache | 4.908 | 0.58 | 420 | 8.469 | < 0.001 |
| | Neouvielle | 3.645 | 0.58 | 420 | 6.289 | < 0.001 |
| | Bethmale | 1.381 | 0.58 | 420 | 2.382 | 0.018 |
| | Bassies | 1.029 | 0.58 | 420 | 1.775 | 0.077 |
| | Arbu-Lers-Estagnon | 1.443 | 0.58 | 420 | 2.49 | 0.013 |
| R99ptot | Overall | 1.46 | 0.144 | 430 | 10.146 | < 0.001 |
| | Lescun | 2.54 | 0.335 | 420 | 7.576 | < 0.001 |
| | Fache | 2.581 | 0.335 | 420 | 7.699 | < 0.001 |
| | Neouvielle | 2.168 | 0.335 | 420 | 6.467 | < 0.001 |
| | Bethmale | 0.527 | 0.335 | 420 | 1.571 | 0.117 |
| | Bassies | 0.438 | 0.335 | 420 | 1.306 | 0.192 |
| | Arbu-Lers-Estagnon | 0.507 | 0.335 | 420 | 1.512 | 0.131 |
| PRCPTOT | Overall | 3.269 | 0.499 | 425 | 6.557 | < 0.001 |
| | Lescun | 8.499 | 1.175 | 420 | 7.234 | < 0.001 |
| | Fache | 5.846 | 1.175 | 420 | 4.976 | < 0.001 |
| | Neouvielle | 3.141 | 1.175 | 420 | 2.674 | 0.008 |
| | Bethmale | 0.478 | 1.175 | 420 | 0.407 | 0.684 |
| | Bassies | 0.481 | 1.175 | 420 | 0.409 | 0.683 |
| | Arbu-Lers-Estagnon | 1.168 | 1.175 | 420 | 0.994 | 0.321 |

## Table 12: Same as Table 11 but restricted to the study period (2016-2020)

| variable | gradient | Year trend | SE | df | t.ratio | p- |
|---|---|---|---|---|---|---|
| mean_tg | Overall | 0.138 | 0.034 | 23 | 4.042 | < 0.001 |
| | Lescun | 0.171 | 0.093 | 18 | 1.846 | 0.081 |
| | Fache | 0.132 | 0.093 | 18 | 1.427 | 0.171 |
| | Neouvielle | 0.070 | 0.093 | 18 | 0.757 | 0.459 |
| | Bethmale | 0.137 | 0.093 | 18 | 1.477 | 0.157 |
| | Bassies | 0.154 | 0.093 | 18 | 1.663 | 0.114 |
| | Arbu-Lers-Estagnon | 0.165 | 0.093 | 18 | 1.775 | 0.093 |
| FD | Overall | -4.500 | 1.464 | 23 | -3.073 | 0.005 |
| | Lescun | -4.100 | 3.965 | 18 | -1.034 | 0.315 |
| | Fache | -7.000 | 3.965 | 18 | -1.765 | 0.094 |
| | Neouvielle | -4.900 | 3.965 | 18 | -1.236 | 0.232 |
| | Bethmale | -2.500 | 3.965 | 18 | -0.630 | 0.536 |
| | Bassies | -5.300 | 3.965 | 18 | -1.337 | 0.198 |
| | Arbu-Lers-Estagnon | -3.200 | 3.965 | 18 | -0.807 | 0.43 |
| SU | Overall | -0.367 | 0.769 | 23 | -0.477 | 0.638 |
| | Lescun | 0.000 | 2.045 | 18 | 0.000 | 1 |
| | Fache | 0.900 | 2.045 | 18 | 0.440 | 0.665 |
| | Neouvielle | 0.500 | 2.045 | 18 | 0.244 | 0.81 |
| | Bethmale | -2.000 | 2.045 | 18 | -0.978 | 0.341 |
| | Bassies | -0.200 | 2.045 | 18 | -0.098 | 0.923 |
| | Arbu-Lers-Estagnon | -1.400 | 2.045 | 18 | -0.684 | 0.502 |
| ID | Overall | -2.267 | 1.160 | 23 | -1.954 | 0.063 |
| | Lescun | -3.400 | 3.152 | 18 | -1.079 | 0.295 |
| | Fache | -3.500 | 3.152 | 18 | -1.111 | 0.281 |
| | Neouvielle | -3.000 | 3.152 | 18 | -0.952 | 0.354 |
| | Bethmale | -0.700 | 3.152 | 18 | -0.222 | 0.827 |
| | Bassies | -1.500 | 3.152 | 18 | -0.476 | 0.64 |
| | Arbu-Lers-Estagnon | -1.500 | 3.152 | 18 | -0.476 | 0.64 |
| GSL | Overall | 4.217 | 2.959 | 23 | 1.425 | 0.168 |
| | Lescun | 3.100 | 8.070 | 18 | 0.384 | 0.705 |
| | Fache | 5.700 | 8.070 | 18 | 0.706 | 0.489 |
| | Neouvielle | 9.000 | 8.070 | 18 | 1.115 | 0.279 |
| | Bethmale | 2.000 | 8.070 | 18 | 0.248 | 0.807 |
| | Bassies | 3.400 | 8.070 | 18 | 0.421 | 0.679 |
| | Arbu-Lers-Estagnon | 2.100 | 8.070 | 18 | 0.260 | 0.798 |
| TXx | Overall | 0.585 | 0.249 | 23 | 2.344 | 0.028 |
| | Lescun | 0.661 | 0.686 | 18 | 0.964 | 0.348 |
| | Fache | 0.614 | 0.686 | 18 | 0.895 | 0.382 |
| | Neouvielle | 0.277 | 0.686 | 18 | 0.404 | 0.691 |
| | Bethmale | 0.702 | 0.686 | 18 | 1.024 | 0.32 |
| | Bassies | 0.601 | 0.686 | 18 | 0.876 | 0.392 |
| | Arbu-Lers-Estagnon | 0.653 | 0.686 | 18 | 0.952 | 0.354 |
| TNx | Overall | 0.572 | 0.206 | 23 | 2.782 | 0.011 |
| | Lescun | 0.529 | 0.566 | 18 | 0.935 | 0.362 |
| | Fache | 0.657 | 0.566 | 18 | 1.162 | 0.261 |
| | Neouvielle | 0.601 | 0.566 | 18 | 1.063 | 0.302 |
| | Bethmale | 0.352 | 0.566 | 18 | 0.622 | 0.542 |
| | Bassies | 0.624 | 0.566 | 18 | 1.103 | 0.284 |
| | Arbu-Lers-Estagnon | 0.667 | 0.566 | 18 | 1.179 | 0.254 |
| TXn | Overall | 0.246 | 0.227 | 23 | 1.084 | 0.289 |
| | Lescun | 0.324 | 0.616 | 18 | 0.526 | 0.606 |
| | Fache | 0.577 | 0.616 | 18 | 0.936 | 0.362 |
| | Neouvielle | 0.361 | 0.616 | 18 | 0.586 | 0.565 |
| | Bethmale | -0.037 | 0.616 | 18 | -0.060 | 0.953 |
| | Bassies | 0.176 | 0.616 | 18 | 0.286 | 0.778 |
| | Arbu-Lers-Estagnon | 0.073 | 0.616 | 18 | 0.118 | 0.907 |
| TNn | Overall | 0.254 | 0.310 | 23 | 0.820 | 0.421 |
| | Lescun | 0.579 | 0.850 | 18 | 0.681 | 0.505 |
| | Fache | 0.263 | 0.850 | 18 | 0.309 | 0.761 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Neouvielle | -0.143 | 0.850 | 18 | -0.168 | 0.868 |
| | Bethmale | 0.286 | 0.850 | 18 | 0.336 | 0.741 |
| | Bassies | 0.219 | 0.850 | 18 | 0.258 | 0.8 |
| | Arbu-Lers-Estagnon | 0.322 | 0.850 | 18 | 0.379 | 0.709 |
| TN10p | Overall | -0.391 | 0.222 | 23 | -1.764 | 0.091 |
| | Lescun | -0.266 | 0.611 | 18 | -0.436 | 0.668 |
| | Fache | -0.451 | 0.611 | 18 | -0.738 | 0.47 |
| | Neouvielle | -0.508 | 0.611 | 18 | -0.832 | 0.416 |
| | Bethmale | -0.477 | 0.611 | 18 | -0.781 | 0.445 |
| | Bassies | -0.444 | 0.611 | 18 | -0.727 | 0.477 |
| | Arbu-Lers-Estagnon | -0.202 | 0.611 | 18 | -0.330 | 0.745 |
| TX10p | Overall | 0.025 | 0.174 | 23 | 0.143 | 0.887 |
| | Lescun | -0.065 | 0.465 | 18 | -0.139 | 0.891 |
| | Fache | 0.091 | 0.465 | 18 | 0.197 | 0.846 |
| | Neouvielle | 0.406 | 0.465 | 18 | 0.872 | 0.394 |
| | Bethmale | 0.107 | 0.465 | 18 | 0.229 | 0.822 |
| | Bassies | -0.277 | 0.465 | 18 | -0.595 | 0.559 |
| | Arbu-Lers-Estagnon | -0.113 | 0.465 | 18 | -0.242 | 0.811 |
| TN90p | Overall | 1.328 | 0.452 | 23 | 2.937 | 0.007 |
| | Lescun | 1.290 | 1.236 | 18 | 1.043 | 0.311 |
| | Fache | 1.093 | 1.236 | 18 | 0.884 | 0.388 |
| | Neouvielle | 0.850 | 1.236 | 18 | 0.687 | 0.501 |
| | Bethmale | 1.318 | 1.236 | 18 | 1.066 | 0.301 |
| | Bassies | 1.962 | 1.236 | 18 | 1.587 | 0.13 |
| | Arbu-Lers-Estagnon | 1.454 | 1.236 | 18 | 1.176 | 0.255 |
| TX90p | Overall | 0.374 | 0.448 | 23 | 0.836 | 0.412 |
| | Lescun | 0.082 | 1.150 | 18 | 0.071 | 0.944 |
| | Fache | -0.157 | 1.150 | 18 | -0.137 | 0.893 |
| | Neouvielle | -1.000 | 1.150 | 18 | -0.870 | 0.396 |
| | Bethmale | 1.135 | 1.150 | 18 | 0.987 | 0.337 |
| | Bassies | 1.162 | 1.150 | 18 | 1.011 | 0.325 |
| | Arbu-Lers-Estagnon | 1.025 | 1.150 | 18 | 0.891 | 0.384 |
| WSDI | Overall | 0.483 | 0.718 | 23 | 0.673 | 0.508 |
| | Lescun | 0.600 | 1.972 | 18 | 0.304 | 0.764 |
| | Fache | 0.600 | 1.972 | 18 | 0.304 | 0.764 |
| | Neouvielle | 0.700 | 1.972 | 18 | 0.355 | 0.727 |
| | Bethmale | -0.500 | 1.972 | 18 | -0.254 | 0.803 |
| | Bassies | 0.800 | 1.972 | 18 | 0.406 | 0.69 |
| | Arbu-Lers-Estagnon | 0.700 | 1.972 | 18 | 0.355 | 0.727 |
| DTR | Overall | -0.054 | 0.037 | 23 | -1.464 | 0.157 |
| | Lescun | 0.031 | 0.096 | 18 | 0.329 | 0.746 |
| | Fache | -0.091 | 0.096 | 18 | -0.948 | 0.356 |
| | Neouvielle | -0.156 | 0.096 | 18 | -1.634 | 0.12 |
| | Bethmale | -0.033 | 0.096 | 18 | -0.342 | 0.736 |
| | Bassies | -0.038 | 0.096 | 18 | -0.396 | 0.697 |
| | Arbu-Lers-Estagnon | -0.035 | 0.096 | 18 | -0.371 | 0.715 |
| RX1day | Overall | 0.423 | 1.534 | 23 | 0.276 | 0.785 |
| | Lescun | -2.200 | 3.926 | 18 | -0.560 | 0.582 |
| | Fache | -3.210 | 3.926 | 18 | -0.818 | 0.424 |
| | Neouvielle | -1.520 | 3.926 | 18 | -0.387 | 0.703 |
| | Bethmale | 3.200 | 3.926 | 18 | 0.815 | 0.426 |
| | Bassies | 3.860 | 3.926 | 18 | 0.983 | 0.339 |
| | Arbu-Lers-Estagnon | 2.410 | 3.926 | 18 | 0.614 | 0.547 |
| RX5day | Overall | 1.720 | 2.056 | 23 | 0.836 | 0.412 |
| | Lescun | -1.590 | 4.961 | 18 | -0.320 | 0.752 |
| | Fache | -4.310 | 4.961 | 18 | -0.869 | 0.396 |
| | Neouvielle | -3.100 | 4.961 | 18 | -0.625 | 0.54 |
| | Bethmale | 6.740 | 4.961 | 18 | 1.359 | 0.191 |
| | Bassies | 5.060 | 4.961 | 18 | 1.020 | 0.321 |
| | Arbu-Lers-Estagnon | 7.520 | 4.961 | 18 | 1.516 | 0.147 |
| SDII | Overall | 0.240 | 0.100 | 23 | 2.405 | 0.025 |
| | Lescun | -0.098 | 0.249 | 18 | -0.395 | 0.698 |
| | Fache | 0.132 | 0.249 | 18 | 0.527 | 0.604 |
| | Neouvielle | 0.184 | 0.249 | 18 | 0.740 | 0.469 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Bethmale | 0.565 | 0.249 | 18 | 2.266 | 0.036 |
| | Bassies | 0.318 | 0.249 | 18 | 1.274 | 0.219 |
| | Arbu-Lers-Estagnon | 0.337 | 0.249 | 18 | 1.352 | 0.193 |
| R10mm | Overall | 2.600 | 0.680 | 23 | 3.821 | < 0.001 |
| | Lescun | 1.400 | 1.727 | 18 | 0.811 | 0.428 |
| | Fache | 0.600 | 1.727 | 18 | 0.347 | 0.732 |
| | Neouvielle | 3.400 | 1.727 | 18 | 1.968 | 0.065 |
| | Bethmale | 4.100 | 1.727 | 18 | 2.374 | 0.029 |
| | Bassies | 3.900 | 1.727 | 18 | 2.258 | 0.037 |
| | Arbu-Lers-Estagnon | 2.200 | 1.727 | 18 | 1.274 | 0.219 |
| R20mm | Overall | 1.083 | 0.375 | 23 | 2.892 | 0.008 |
| | Lescun | 0.500 | 0.972 | 18 | 0.514 | 0.613 |
| | Fache | 1.600 | 0.972 | 18 | 1.646 | 0.117 |
| | Neouvielle | 0.000 | 0.972 | 18 | 0.000 | 1 |
| | Bethmale | 1.200 | 0.972 | 18 | 1.234 | 0.233 |
| | Bassies | 1.500 | 0.972 | 18 | 1.543 | 0.14 |
| | Arbu-Lers-Estagnon | 1.700 | 0.972 | 18 | 1.749 | 0.097 |
| CDD | Overall | -1.133 | 0.511 | 28 | -2.216 | 0.035 |
| | Lescun | -1.600 | 1.488 | 18 | -1.075 | 0.296 |
| | Fache | -1.400 | 1.488 | 18 | -0.941 | 0.359 |
| | Neouvielle | -0.300 | 1.488 | 18 | -0.202 | 0.842 |
| | Bethmale | -1.300 | 1.488 | 18 | -0.874 | 0.394 |
| | Bassies | -0.300 | 1.488 | 18 | -0.202 | 0.842 |
| | Arbu-Lers-Estagnon | -1.900 | 1.488 | 18 | -1.277 | 0.218 |
| CWD | Overall | 0.167 | 0.446 | 23 | 0.374 | 0.712 |
| | Lescun | -0.700 | 1.189 | 18 | -0.589 | 0.563 |
| | Fache | 0.300 | 1.189 | 18 | 0.252 | 0.804 |
| | Neouvielle | -0.400 | 1.189 | 18 | -0.336 | 0.74 |
| | Bethmale | 0.300 | 1.189 | 18 | 0.252 | 0.804 |
| | Bassies | 1.100 | 1.189 | 18 | 0.925 | 0.367 |
| | Arbu-Lers-Estagnon | 0.400 | 1.189 | 18 | 0.336 | 0.74 |
| R95ptot | Overall | 25.715 | 11.946 | 23 | 2.153 | 0.042 |
| | Lescun | -5.680 | 31.231 | 18 | -0.182 | 0.858 |
| | Fache | 30.250 | 31.231 | 18 | 0.969 | 0.346 |
| | Neouvielle | 5.890 | 31.231 | 18 | 0.189 | 0.853 |
| | Bethmale | 42.360 | 31.231 | 18 | 1.356 | 0.192 |
| | Bassies | 38.540 | 31.231 | 18 | 1.234 | 0.233 |
| | Arbu-Lers-Estagnon | 42.930 | 31.231 | 18 | 1.375 | 0.186 |
| R99ptot | Overall | 8.167 | 10.130 | 23 | 0.806 | 0.428 |
| | Lescun | 1.130 | 27.382 | 18 | 0.041 | 0.968 |
| | Fache | -8.220 | 27.382 | 18 | -0.300 | 0.767 |
| | Neouvielle | 3.310 | 27.382 | 18 | 0.121 | 0.905 |
| | Bethmale | 11.740 | 27.382 | 18 | 0.429 | 0.673 |
| | Bassies | 22.100 | 27.382 | 18 | 0.807 | 0.43 |
| | Arbu-Lers-Estagnon | 18.940 | 27.382 | 18 | 0.692 | 0.498 |
| PRCPTOT | Overall | 49.552 | 17.020 | 23 | 2.911 | 0.008 |
| | Lescun | 12.660 | 44.706 | 18 | 0.283 | 0.78 |
| | Fache | 32.740 | 44.706 | 18 | 0.732 | 0.473 |
| | Neouvielle | 31.910 | 44.706 | 18 | 0.714 | 0.485 |
| | Bethmale | 88.120 | 44.706 | 18 | 1.971 | 0.064 |
| | Bassies | 62.570 | 44.706 | 18 | 1.400 | 0.179 |
| | Arbu-Lers-Estagnon | 69.310 | 44.706 | 18 | 1.550 | 0.138 |

**Table 13: Summary of the models for the mean annual water temperature in the ice-free period (water_tg), hardness, chloride, potassium and sodium.** Significance:**** p < 0.0001, *** p < 0.001; ** p < 0.01, * p< 0.05, ns p > 0.05

| variables | Lake | year.trend | SE | df | t.ratio | p.value |
|---|---|---|---|---|---|---|
| Water_tg | Overall | 0.2 | 0.01 | 362.99 | 20.222 | < 0.001 |
| | Arbu | 0.339 | 0.045 | 338 | 7.478 | < 0.001 |
| | Lers | 0.362 | 0.045 | 338 | 7.983 | < 0.001 |
| | Estagnon | 0.366 | 0.045 | 338 | 8.074 | < 0.001 |
| | Labant | 0.351 | 0.045 | 338 | 7.743 | < 0.001 |
| | Alate | 0.306 | 0.045 | 338 | 6.749 | < 0.001 |
| | Mort | 0.366 | 0.045 | 338 | 8.058 | < 0.001 |
| | Bellonguere | 0.205 | 0.045 | 338 | 4.52 | < 0.001 |
| | Ayes | 0.216 | 0.045 | 338 | 4.759 | < 0.001 |
| | Bethmale | 0.203 | 0.045 | 338 | 4.472 | < 0.001 |
| | Laquettes | 0.129 | 0.045 | 338 | 2.837 | 0.005 |
| | Gourg de Rabas | 0.165 | 0.045 | 338 | 3.634 | < 0.001 |
| | Madamete-Haut | 0.078 | 0.045 | 338 | 1.728 | 0.085 |
| | Madamete-Bas | 0.142 | 0.045 | 338 | 3.131 | 0.002 |
| | Pecheur | 0.142 | 0.045 | 338 | 3.131 | 0.002 |
| | Coueyla-Gran | 0.129 | 0.045 | 338 | 2.837 | 0.005 |
| | Paradis | 0.029 | 0.045 | 338 | 0.642 | 0.521 |
| | Embarrat | 0.235 | 0.045 | 338 | 5.184 | < 0.001 |
| | Vallon | 0.172 | 0.045 | 338 | 3.796 | < 0.001 |
| | Petite-Fache | 0.069 | 0.045 | 338 | 1.52 | 0.129 |
| | Grande-Fache | 0.136 | 0.045 | 338 | 3 | 0.003 |
| | Fache-Espagne | 0.125 | 0.045 | 338 | 2.755 | 0.006 |
| | Lhurs | 0.206 | 0.045 | 338 | 4.551 | < 0.001 |
| | Ansabere | 0.157 | 0.045 | 338 | 3.456 | < 0.001 |
| | Acherito | 0.194 | 0.045 | 338 | 4.275 | < 0.001 |
| | Puits | 0.2 | 0.045 | 338 | 4.402 | < 0.001 |
| | Arlet | 0.18 | 0.045 | 338 | 3.966 | < 0.001 |
| hardness | Overall | 0.042 | 0.013 | Inf | 3.31 | < 0.001 |
| | Lers | 0.063 | 0.737 | 162 | 0.085 | 0.932 |
| | Estagnon | -0.272 | 0.737 | 162 | -0.369 | 0.713 |
| | Arbu | 0.152 | 0.737 | 162 | 0.206 | 0.837 |
| | Labant | 0.266 | 0.783 | 162 | 0.34 | 0.735 |
| | Mort | 0.46 | 0.746 | 162 | 0.617 | 0.538 |
| | Alate | 0.225 | 0.746 | 162 | 0.302 | 0.763 |
| | Bethmale | 0.955 | 0.824 | 162 | 1.159 | 0.248 |
| | Ayes | 0.087 | 0.695 | 162 | 0.126 | 0.9 |
| | Bellonguere | -0.153 | 0.739 | 162 | -0.207 | 0.836 |
| | Laquettes | 0.671 | 1.843 | 162 | 0.364 | 0.716 |
| | Coueyla-Gran | 0.102 | 0.737 | 162 | 0.138 | 0.89 |
| | Madamete-Bas | -0.007 | 0.737 | 162 | -0.01 | 0.992 |
| | Pecheur | 0.101 | 1.204 | 162 | 0.084 | 0.933 |
| | Madamete-Haut | 0.535 | 0.737 | 162 | 0.725 | 0.469 |
| | Gourg de Rabas | 0.211 | 0.737 | 162 | 0.287 | 0.775 |
| | Paradis | 0.427 | 0.737 | 162 | 0.579 | 0.563 |
| | Embarrat | 0.284 | 0.919 | 162 | 0.309 | 0.758 |
| | Vallon | 0.333 | 0.802 | 162 | 0.415 | 0.678 |
| | Petite-Fache | 0.366 | 0.737 | 162 | 0.497 | 0.62 |
| | Grande-Fache | 0.264 | 0.737 | 162 | 0.358 | 0.72 |
| | Fache-Espagne | 0.438 | 1.97 | 162 | 0.222 | 0.824 |
| | Lhurs | 1.17 | 0.737 | 162 | 1.587 | 0.114 |
| | Ansabere | 0.713 | 0.737 | 162 | 0.967 | 0.335 |
| | Puits | 0.214 | 0.777 | 162 | 0.276 | 0.783 |
| | Acherito | 1.087 | 1.204 | 162 | 0.904 | 0.368 |
| | Arlet | 0.058 | 0.737 | 162 | 0.079 | 0.937 |
| Cl | Overall | -0.071 | 0.022 | Inf | -3.293 | < 0.001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Lers | -0.054 | 0.031 | 163 | -1.726 | 0.086 |
| | Estagnon | 0.013 | 0.031 | 163 | 0.419 | 0.676 |
| | Arbu | -0.023 | 0.031 | 163 | -0.736 | 0.463 |
| | Labant | -0.058 | 0.033 | 163 | -1.737 | 0.084 |
| | Mort | -0.031 | 0.032 | 163 | -0.989 | 0.324 |
| | Alate | -0.006 | 0.031 | 163 | -0.182 | 0.856 |
| | Bethmale | -0.017 | 0.035 | 163 | -0.496 | 0.621 |
| | Ayes | -0.038 | 0.03 | 163 | -1.267 | 0.207 |
| | Bellonguere | 0.006 | 0.032 | 163 | 0.196 | 0.845 |
| | Laquettes | 0.025 | 0.079 | 163 | 0.323 | 0.747 |
| | Coueyla-Gran | -0.001 | 0.031 | 163 | -0.032 | 0.975 |
| | Madamete-Bas | -0.102 | 0.031 | 163 | -3.239 | 0.001 |
| | Pecheur | -0.015 | 0.051 | 163 | -0.292 | 0.771 |
| | Madamete-Haut | -0.02 | 0.031 | 163 | -0.642 | 0.522 |
| | Gourg de Rabas | -0.013 | 0.031 | 163 | -0.417 | 0.677 |
| | Paradis | -0.043 | 0.031 | 163 | -1.378 | 0.17 |
| | Embarrat | -0.027 | 0.039 | 163 | -0.686 | 0.494 |
| | Vallon | -0.012 | 0.034 | 163 | -0.339 | 0.735 |
| | Petite-Fache | 0.038 | 0.031 | 163 | 1.217 | 0.225 |
| | Grande-Fache | -0.004 | 0.031 | 163 | -0.137 | 0.892 |
| | Fache-Espagne | -0.033 | 0.084 | 163 | -0.393 | 0.695 |
| | Lhurs | -0.04 | 0.031 | 163 | -1.26 | 0.21 |
| | Ansabere | -0.038 | 0.031 | 163 | -1.215 | 0.226 |
| | Puits | -0.08 | 0.033 | 163 | -2.413 | 0.017 |
| | Acherito | 0.015 | 0.051 | 163 | 0.301 | 0.764 |
| | Arlet | -0.025 | 0.031 | 163 | -0.783 | 0.435 |
| | Overall | 0.181 | 0.039 | Inf | 4.649 | < 0.001 |
| | Lers | -0.016 | 0.027 | 88 | -0.575 | 0.567 |
| | Estagnon | 0.015 | 0.027 | 88 | 0.561 | 0.576 |
| | Arbu | 0.011 | 0.027 | 88 | 0.396 | 0.693 |
| | Labant | 0.009 | 0.028 | 88 | 0.324 | 0.747 |
| | Mort | -0.015 | 0.027 | 88 | -0.535 | 0.594 |
| | Alate | 0.078 | 0.027 | 88 | 2.88 | 0.005 |
| | Bethmale | 0.002 | 0.032 | 88 | 0.076 | 0.94 |
| | Ayes | 0.007 | 0.025 | 88 | 0.284 | 0.777 |
| | Bellonguere | -0.002 | 0.027 | 88 | -0.081 | 0.935 |
| | Laquettes | -0.04 | 0.163 | 88 | -0.245 | 0.807 |
| | Coueyla-Gran | 0.051 | 0.027 | 88 | 1.856 | 0.067 |
| | Madamete-Bas | 0.001 | 0.027 | 88 | 0.022 | 0.982 |
| K | Pecheur | 0.034 | 0.043 | 88 | 0.793 | 0.43 |
| | Madamete-Haut | 0.007 | 0.027 | 88 | 0.249 | 0.804 |
| | Gourg de Rabas | 0.007 | 0.027 | 88 | 0.266 | 0.791 |
| | Paradis | 0.024 | 0.027 | 88 | 0.872 | 0.386 |
| | Embarrat | 0.696 | 0.047 | 88 | 14.763 | < 0.001 |
| | Vallon | 0.013 | 0.032 | 88 | 0.417 | 0.678 |
| | Petite-Fache | 0.111 | 0.027 | 88 | 4.069 | < 0.001 |
| | Grande-Fache | 0.023 | 0.027 | 88 | 0.839 | 0.404 |
| | Fache-Espagne | 0.05 | 0.071 | 88 | 0.707 | 0.482 |
| | Lhurs | -0.014 | 0.027 | 88 | -0.505 | 0.615 |
| | Ansabere | 0.025 | 0.027 | 88 | 0.925 | 0.357 |
| | Puits | -0.037 | 0.028 | 88 | -1.318 | 0.191 |
| | Acherito | 0.052 | 0.043 | 88 | 1.209 | 0.23 |
| | Arlet | 0.012 | 0.027 | 88 | 0.432 | 0.667 |
| | Overall | 0.011 | 0.023 | Inf | 0.48 | 0.631 |
| | Lers | -0.015 | 0.134 | 88 | -0.115 | 0.909 |
| | Estagnon | 0.004 | 0.134 | 88 | 0.033 | 0.974 |
| | Arbu | 0 | 0.134 | 88 | 0.003 | 0.997 |
| Na | Labant | 0.006 | 0.139 | 88 | 0.043 | 0.966 |
| | Mort | -0.095 | 0.134 | 88 | -0.707 | 0.481 |
| | Alate | 0.027 | 0.134 | 88 | 0.199 | 0.843 |
| | Bethmale | -0.027 | 0.157 | 88 | -0.17 | 0.865 |
| | Ayes | 0.005 | 0.123 | 88 | 0.041 | 0.967 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Bellonguere | 0.014 | 0.134 | 88 | 0.102 | 0.919 |
| | Laquettes | -0.37 | 0.806 | 88 | -0.459 | 0.647 |
| | Coueyla-Gran | 0.024 | 0.134 | 88 | 0.176 | 0.861 |
| | Madamete-Bas | -0.02 | 0.134 | 88 | -0.146 | 0.884 |
| | Pecheur | 0.056 | 0.212 | 88 | 0.262 | 0.794 |
| | Madamete-Haut | -0.016 | 0.134 | 88 | -0.115 | 0.908 |
| | Gourg de Rabas | -0.002 | 0.134 | 88 | -0.012 | 0.991 |
| | Paradis | -0.007 | 0.134 | 88 | -0.053 | 0.958 |
| | Embarrat | 0.152 | 0.233 | 88 | 0.654 | 0.515 |
| | Vallon | -0.169 | 0.157 | 88 | -1.078 | 0.284 |
| | Petite-Fache | 0.08 | 0.134 | 88 | 0.594 | 0.554 |
| | Grande-Fache | 0.009 | 0.134 | 88 | 0.065 | 0.949 |
| | Fache-Espagne | 0.184 | 0.349 | 88 | 0.529 | 0.598 |
| | Lhurs | -0.017 | 0.134 | 88 | -0.127 | 0.899 |
| | Ansabere | -0.015 | 0.134 | 88 | -0.112 | 0.911 |
| | Puits | 0 | 0.139 | 88 | 0.003 | 0.997 |
| | Acherito | 0.083 | 0.212 | 88 | 0.392 | 0.696 |
| | Arlet | 0.004 | 0.134 | 88 | 0.028 | 0.977 |

**TITLE :** Links between the amphibian parasite *Batrachochytrium dendrobatidis* and benthic biofilms of Pyrenean mountain lakes

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**SUMMARY :**

Here I studied the biodiversity of prokaryotic and micro-eukaryotic assemblages in benthic biofilms of 26 Pyrenean lakes from 2016 to 2020, to explore links with amphibian chytridiomycosis, a disease of conservation significance caused by *Batrachochytrium dendrobatidis* (Bd). My results show that biofilms have changed rapidly with a decline in biodiversity and an increase in algae potentially detrimental to water quality and public health. I found that biofilms in lakes where amphibians are less impacted by the disease contained more Bd antagonistic organisms, and demonstrated experimentally that a biofilm can affect the fate of Bd, suggesting a potential role for biofilms in the epidemiology of chytridiomycosis. My research illustrates the interdependence between environmental health and animal and human health, all of which are threatened by global changes in mountain socio-ecosystems.

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**KEYWORDS:** pathogens, cyanobacteria, diatoms, microbial ecology, metabarcoding, zoospores.

**AUTEUR :** Hugo SENTENAC

**TITRE :** Liens entre le parasite d'amphibiens *Batrachochytrium dendrobatidis* et les biofilms benthiques de lacs de montagne pyrénéens

**DIRECTEUR DE THESE :** Dirk SCHMELLER & Adeline LOYAU

**LIEU ET DATE DE SOUTENANCE :** Toulouse, le 28/04/2023

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**RESUME :**

J'ai étudié ici la biodiversité des assemblages procaryotes et micro-eucaryotes des biofilms benthiques de 26 lacs pyrénéens de 2016 à 2020, pour en explorer les liens avec la chytridiomycose amphibienne, maladie à enjeu de conservation causée par *Batrachochytrium dendrobatidis* (Bd). Mes résultats montrent que les biofilms ont changé rapidement avec un déclin de la biodiversité et une augmentation des algues potentiellement délétères pour la qualité de l'eau et la santé publique. J'ai constaté que les biofilms des lacs où les amphibiens sont moins impactés par la maladie contenaient plus d'organismes antagonistes de Bd, et ai démontré expérimentalement qu'un biofilm peut affecter le devenir de Bd, suggérant un potentiel rôle des biofilms dans l'épidémiologie de la chytridiomycose. Mes recherches illustrent l'interdépendance entre la santé environnementale et les santés animale et humaine, toutes menacées par les changements globaux dans les socio-écosystèmes de montagne.

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**MOTS-CLES:** agent pathogène, cyanobactéries, diatomées, écologie microbienne, metabarcoding, zoospores.

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**DISCIPLINE ADMINISTRATIVE :** Ecologie fonctionnelle

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