Ecology of Aerobic Anoxygenic Phototrophs in Fresh Waters

Cristian Villena Alemany

School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
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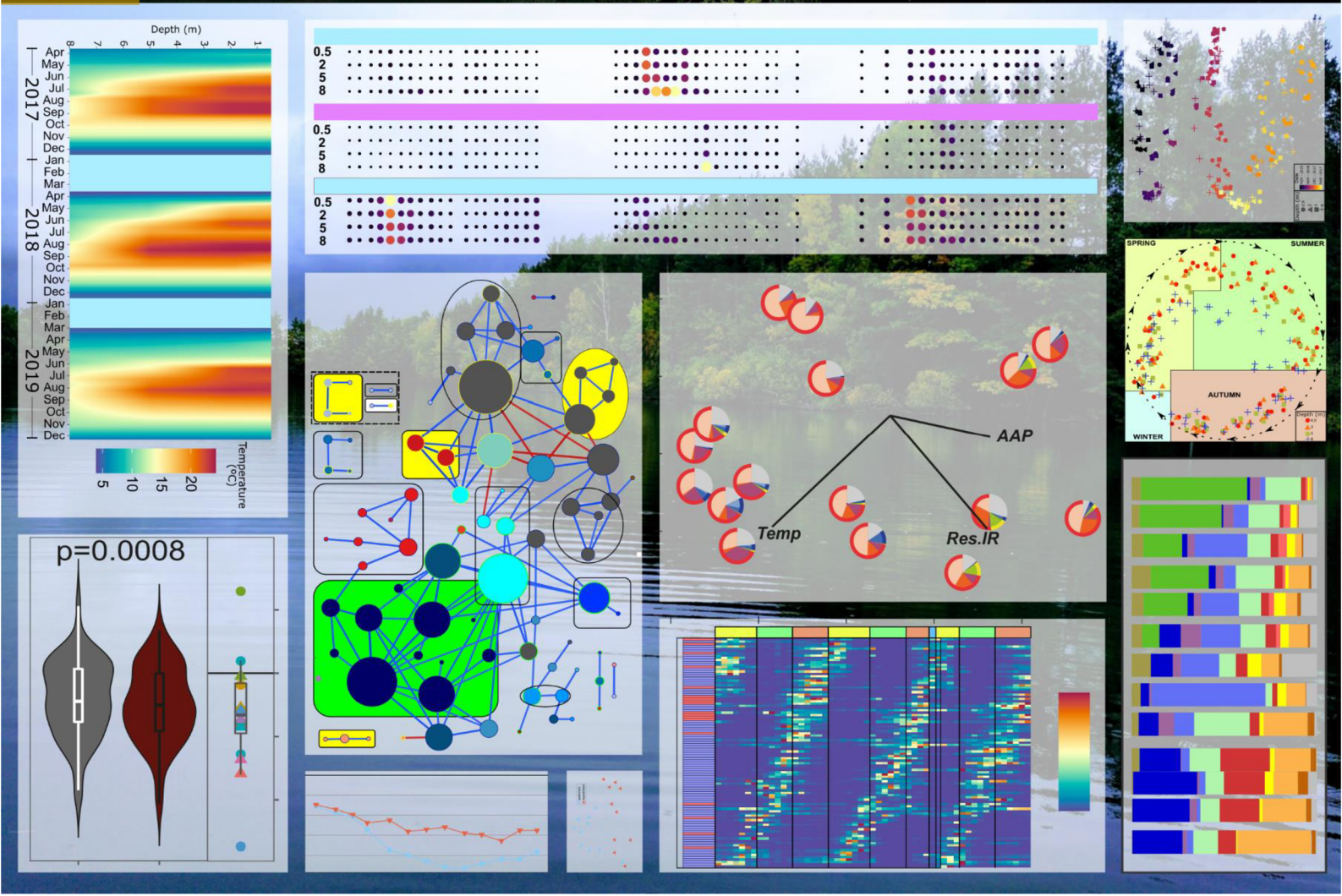

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Ecology of Aerobic Anoxygenic Phototrophs in Fresh Waters

Ph.D. Thesis

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České Budějovice, 2024

This thesis should be cited as:

Villena-Alemany, C., 2024: Ecology of Aerobic Anoxygenic Phototrophs in Fresh Waters, University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 149 pp.

Annotation

Aerobic anoxygenic phototrophic (AAP) bacteria are a functional group that perform anoxygenic photosynthesis in aerobic environments. AAP bacteria are photoheterotrophs, which use organic carbon for their growth but they can additionally supplement their energy requirements by harvesting light. Their discovery challenged the standard paradigm that in aquatic environments there are solely photoautotrophic phytoplankton that use light energy to fix inorganic carbon, and heterotrophic organisms which depend on external organic carbon sources. Since their discovery in marine coastal habitats in Japan in the 1970s, scientists have discussed their distribution, diversity, and environmental role in a wide variety of environments. In this thesis, my research is presented on the ecology of AAP bacteria in a freshwater lake, using molecular, metagenomic and bioinformatic tools for exploring AAP diversity, their role in aquatic ecosystems and the results give insights into the contribution of AAP bacteria to microbial food webs and the carbon cycle in freshwater lakes.

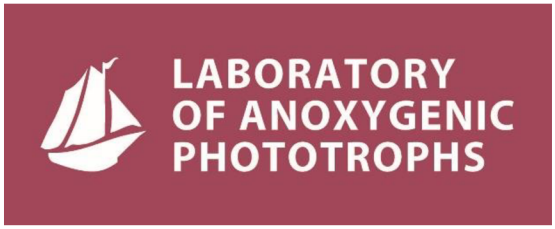
Declaration

I hereby declare that I am the author of this dissertation and that I have used only the sources and literature detailed in the list of references.

Cristian Villena Alemany

České Budějovice, 2024

This thesis was completed as a part of the PhD study program Hydrobiology at the Faculty of Science, University of South Bohemia, in České Budějovice. The experimental part of this work was conducted in the Laboratory of Anoxygenic phototrophs, in Algatech Center, Třeboň, Institute of Microbiology, Academy of Sciences of the Czech Republic.



Financial support

This work was supported by the GAČR (Grant Agency of the Czech Republic) projects *PhotoGemm+* no. 1928778X awarded to Assoc. Prof. Michal Koblížek, Ph.D. and GAČR Junior project *AAPs rule!* no. 18–14095Y awarded to Assoc. Prof. Kasia Piwosz, Ph.D.

Acknowledgements

There are many people to be grateful for all kinds of different support. Firstly, this thesis could not be born without the unconditional support during this whole trip of Michal Koblížek, with corrections, knowledge, discussions, ideas, advices and opportunities. Not just as a Supervisor but also as a good colleague. Sincere Thank You.

As well, I have to thank everybody from my laboratory of Anoxygenic Phototrophs, starting with my buddy Alastair, who helped more than a lot. Of course Jason, Vasil, Jürgen, Mohit, David, Adela, Sahana, Karel and Martina. Thanks for the welcoming, all the help and all the good moments that we have enjoyed. També, Guillem. Que ha sigut com un germà durant tots aquests anys i gràcies al seu incondicional support i a la companyia durant les incomptables anècdotes que em disfrutat. Reviuria cadascuna de elles sense dubtar-ho.

Per descomptat, no podria de cap de les maneres, haver arribat a poder escriure aquesta tesis, si no fós per els impensables sacrificis que han fet, la gegant paciencia que han tingut i el amor i carinyo que la Mare, el Pare, la Uela i Uelo m'han regalat. Gràcies, eternes. Ja puc vendre mantes en Benilloba.

També vuic agraïr la gent de Beniflà i alrededors que m'ha aportat un poc de sí mateixa per fer-me millor persona: al meu germà, Josep, a Jose, Javi, Vicent, Gime i la resta. També a la gent que encara que ja no estan, sempre estaré agraït de haver-me'ls creuat en el camí i d'haver gaudit la vida d'aquella manera plàcida.

Por supuesto, a la gente del laboratorio de Ecología microbiana y molecular de la universidad de Alicante. Pepa, Esther, Borja y Loles, gracias por vuestra ayuda siempre y por todo lo que me habéis enseñado. Cristina, Valentín y Rodrigo, una maravillosa coincidencia haberme encontrado con tan buena gente y haber disfrutado de tan buenos momentos, y los que nos quedan! Y no me olvido de, gracias a quién, no he hecho una tesis de botánica o geología, sino de microbiología. Fer, estaré siempre agradecido por abrir esta puerta, darme la oportunidad y guiarme en el camino de la ciencia.

Furthermore, I would like to be grateful, to the nice people I found during this trip in the different experiments in Croatia, Finland, in Hungary and in Poland, as well as the rest of co-authors that help me with the research. It was a real pleasure to share science and joy with all of you. Mariano, 11 is 10. I també a la meua bessona de tesis, Carlota! Que vas ser com aigua en el desert quan, al principi de la tesis, vas apareixer per traduir-me el que deien els Tesper i per produir un accident naval tan divertit.

Finalmente, a una persona especial, que me ha acompañado durante este camino y ha conseguido que sea mucho más bonito y agradable, convirtiéndose en un pilar indispensable que sustenta mi ciencia i mi vida. Izabela, no existen suficientes palabras para agradecértelo.

Last and one of the most important, I would like to thank another special person that lead all my work, the person that recruited me, that thought me, that gave me uncountable opportunities to grow as a scientist and as a person. She is and will be a great example to follow and I will always be happy for having her as a supervisor. Kasia, thank you very much, from all my hearth.

To all, Gràcies!

List of papers and author's contribution

The thesis is based on the following papers (listed thematically):

I. **Villena-Alemany, C.**, Mujakić, I., Porcal, P., Koblížek, M. & Piwosz, K. (2023) Diversity dynamics of aerobic anoxygenic phototrophic bacteria in a freshwater lake. Environmental Microbiology Reports, 15(1), 60–71. https://doi.org/10.1111/1758-2229.13131 (IF= 4.006).
CVA did the analysis of the sequencing data 90%; prepared figures 70% and wrote the manuscript 60%. Contribution of CVA was 80%.

II. **Villena-Alemany C**., Mujakić I., Fecskeová, L.K., Woodhouse, J., Auladell, A., Dean, J., Hanusová, M., Socha, M., Gazulla, C.R., Ruscheweyh, H.-J., Sunagawa, S., Kavagutti, V., Andrei, A.-S., Grossart, H.-P., Ghai, R.,Koblížek, M., & Piwosz, K. (2023). Phenology and ecological role of Aerobic Anoxygenic Phototrophs in fresh waters. (Manuscript under review in Microbiome, IF= 15.5).
Preprint: https://doi.org/10.1101/2023.11.17.567504
CVA did the conceptualization of the study (20%); prepared the pufM database (50%); performed the formal analysis (80%); participated in the investigation and experimental processes (50%); developed the methodology for the database (50%) and wrote the manuscript (70%). CVA contribution was 75%.

III. Piwosz, K., **Villena-Alemany, C.** & Mujakić, I. Photoheterotrophy by aerobic anoxygenic bacteria modulates carbon fluxes in a freshwater lake. (2022) ISME Journal 16, 1046–1054. https://doi.org/10.1038/s41396-021-01142-2. (IF= 11.217).
CVA participated in the sampling (25%), prepared samples for sequencing (75%), analysed the sequencing data (80%), prepared figures (15%), worked on the writing and commenting on the paper (10%). Contribution of CVA was 35%.

IV. Piwosz, K., **Villena-Alemany, C.**, Całkiewicz J., Mujakić, I., Náhlík V., Dean J. & Koblížek M. (2023). Winners in good times and bad times: Aerobic anoxygenic phototrophic bacteria profit from heterotrophy under carbon-rich and poor conditions. (Manuscript submitted to FEMS Microbial Ecology, IF= 4.519)
CVA participated in formal analysis (20%) and in writing and reviewing (15%). Contribution of CVA was (17.5%).

Co-author agreement

Doc. Kasia Piwosz, Ph.D. supervisor of this Ph.D. thesis, co-author of papers I-II and author of paper III-IV, fully acknowledges the stated contribution of Cristian Villena Alemany to these manuscripts.

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Doc. Kasia Piwosz, Ph.D.

Doc. Michal Koblížek, Ph.D., advisor and guarantor of this Ph.D. thesis, co-author of papers I, II and IV, fully acknowledges the stated contribution of Cristian Villena Alemany to these manuscripts.

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Doc. Michal Koblížek, Ph.D.

Izabela Mujakić, Ph.D., co-author of papers I, II, III and IV, fully acknowledges the stated contribution of Cristian Villena Alemany to these manuscripts.

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Izabela Mujakić, Ph.D.

Table of Contents

1.- Introduction

Aquatic ecosystems belong to the most important environments on Earth. The basis of planktonic freshwater biota is composed of diverse microorganisms: aquatic bacteria as well as unicellular and multicellular eukaryotes. The structure and functioning of these aquatic communities is determined by the interactions between the organisms and their environment, including biotic and abiotic factors. A better understanding of these relationships provides the justification and the proper tools to protect, preserve and, when necessary, recover the invaluable equilibrium that upholds the endurance and sustainability of the biosphere. Furthermore, this understanding is crucial to face the contemporary and future international challenges, such as the global warming, caused by human-induced disturbance in the carbon cycle, environmental pollution, and the loss of diversity. The knowledge for counteracting those environmental impacts is pivotal from an ecological perspective in maintaining the health of the planet.

Prokaryotic microorganisms have a paramount role in the dynamics and functioning of aquatic ecosystems. Bacteria are microscopic organisms that appeared at least 3.5 billion years and have remained as key players in the biosphere continually thereafter (Narbonne 2005). During this time, evolution has been shaping and adapting bacteria to carry out the most versatile metabolic reactions in a plethora of environments. Currently, they comprise approximately 15% of the biomass on Earth (Bar-On et al. 2018) and play fundamental role in the global biogeochemical cycles of elements. Notably, bacteria contribute at least 20-30% to global primary production, and are the only known organisms able to fix nitrogen (N_2), the most abundant yet inaccessible element to the majority of living organisms. Bacteria also play a crucial role in phosphorous cycle (Waterbury et al. 1979; Pisciotta et al. 2010).

Bacteria constitute complex communities that are shaped not only by physicochemical factors, but also by intricate interactions among themselves and with other microorganisms. Community assembly theories seek to elucidate their structures and predict changes in the community composition according to various assembly processes such as drift, dispersal or selection (Rosindell et al. 2011). Bacterial communities in different environments tend to differ in composition and functionality, being adapted to inherent environmental conditions. Each environment hosts multiple roles and numerous biochemical reactions carried out by specific functional groups of bacteria. For instance, photosynthetic

Cyanobacteria are the main prokaryote protagonist for nitrogen and carbon fixation in oxic environments, whereas anoxygenic phototrophs efficiently perform carbon and nitrogen fixation in anoxic environments (Madigan 1995).

1.1.- Phototrophy

Phototrophic organism evolved probably very early during the Earth's history. These organisms harvest light and convert it into chemical energy available for cell metabolism. The captured energy in photoautotrophic species, such as cyanobacteria or purple bacteria, is used for the fixation of inorganic carbon in the process of photosynthesis. Indeed, the majority of organic carbon in the biosphere originates from the reduction of carbon dioxide by photosynthetic organisms. Whether oxygenic or anoxygenic, photosynthesis has significantly shaped the Earth's ecological landscape, leaving distinct marks in the geological record (Rosing et al. 2006).

Oxygenic photosynthesis, present in Cyanobacteria, algae and higher plants, involves both Type-I and Type-II reaction centres (RCs) working in series for building sufficient redox potential to split water, producing oxygen and reducing CO_2. In contrast, anoxygenic photosynthesis is a more ancient process, which operates with a much lower redox potential. Anoxygenic photosynthesis employs either Type-I or Type-II RCs, characterized by differences in structure and electron acceptor. Anoxygenic Type-I RCs are homodimeric (*Psc*A gene) and use ferredoxin, while anoxygenic Type-II RCs are heterodimeric (*puf*L and *puf*M genes) and employ quinones as the electron acceptor (Hohmann-Marriott and Blankenship 2011).

Anoxygenic Type-I reaction centres have been so far identified in anaerobic anoxygenic phototrophs belonging to Bacillota (formerly known as Firmicutes (Oren and Garrity 2021)), Bacteroidota (Chlorobia class, considered before as a Chlorobi phylum) and in the semiaerobic phototrophs belonging to phylum Acidobacteriota (Bryant et al. 2007). Additionally, a Type-I RC has recently been reported from an anaerobic Chloroflexota culture (Tsuji et al. 2020). In spite of that, most of the phototrophic Chloroflexota are semiaerobic or potentially aerobic and contain Type-II RCs (Mehrshad et al. 2018).

Anoxygenic Type-II RCs were initially documented in anaerobic members of the phylum Pseudomonadota (formerly known as Proteobacteria). These bacteria are known by their classic names as purple sulphur (PSB) and purple non-sulphur (PNSB) bacteria. PSB use reduced sulphur compounds as an electron donor to generate reducing power molecules such as NAD(P)H, accumulating sulphur granules in their cytoplasm. In contrast, PNSB utilize reduced organic compounds as an electron donor (Madigan and Jung 2009). Furthermore, numerous species from different phyla demonstrate the capability to perform anoxygenic photosynthesis using Type-II RC in aerobic environments and are acknowledged as the functional group of aerobic anoxygenic photoheterotrophic (AAP) bacteria (Table 1).

Reaction centre	Phylum	Phototrophic Functional groups	Oxygen requirement
Type-I	Bacillota (Firmicutes)	Heliobacteria	Anaerobic
	Bacteroidota (Chlorobia class)	Green sulfur bacteria	Anaerobic
	Acidobacteriota	-	Semiaerobic
		-	Anaerobic**
Type-II	Chloroflexota	GNSB AAPs	Anaerobic Aerobic or semiaerobic
	Pseudomonadota (Proteobacteria)	Purple bacteria	Anaerobic or semiaerobic
		AAPs	Aerobic
	Gemmatimonadetes	AAPs	Aerobic
	Vulcanimicrobiota (Eremiobacteriota)	AAPs	Aerobic
	Myxococcota	AAPs*	Aerobic

Table 1. Distribution of Type-I and Type-II anoxygenic reaction centres across bacterial tree of life. Names in parenthesis indicate former denominations. Abbreviations: GSB (Green sulphur bacteria); GNSB (green non-sulphur bacteria); AAPs (aerobic anoxygenic phototrophs). * indicates group with single cultured representative reported in a preprint (Tsuji et al. 2020); ** indicates potential phototrophic capability predicted from metagenome assembled genomes (MAGs).

1.2.- Discovery and conceptual foundation of AAP bacteria

Despite extensive research, the origin of photosynthesis remains enigmatic. Nonetheless, it is unequivocal that anoxygenic photosynthesis predates oxygenic photosynthesis, as evidenced by stromatolites dating back 3.3 billion years (Des Marais, 2000), a time when Earth was predominantly anoxic with reducing atmosphere and dominated by anaerobic microorganisms. The evolution of oxygenic photosynthesis led to the so called Great oxidation event approximately 2.4-2.1 billion years ago (Bekker et al. 2004). The gradual oxidation of the atmosphere forced the anoxygenic species to

follow 3 possible paths: extinction due to oxygen toxicity; persistence in the anoxic environments (as observed for GSB, PSB and heliobacteria) or the acquirement of oxygen tolerance, gradually adapting to the new oxic environments (Koblížek 2015). The bacteria that adapted to the increasingly oxic conditions, and developed the ability to perform anoxygenic photosynthesis in the presence of oxygen were the beginning of what nowadays are AAP bacteria.

In contrast to the classical purple bacteria, the presence of oxygen for AAP bacteria is neither toxic nor inhibits anoxygenic phototrophy. Indeed, the presence of oxygen is indispensable for growth and the genesis of AAP photosyntetic apparatus. This is due to magnesium-protoporphyrin IX monomethylester cyclase (EC:1.14.13.81), an enzyme that catalyses the conversion of Mg-protoporphyrin IX monomethylester to Mg-divinyl protochlorophyllide, a precursor of bacteriochlorophyll-*a* (BChl*a*) molecule (Figure 1).

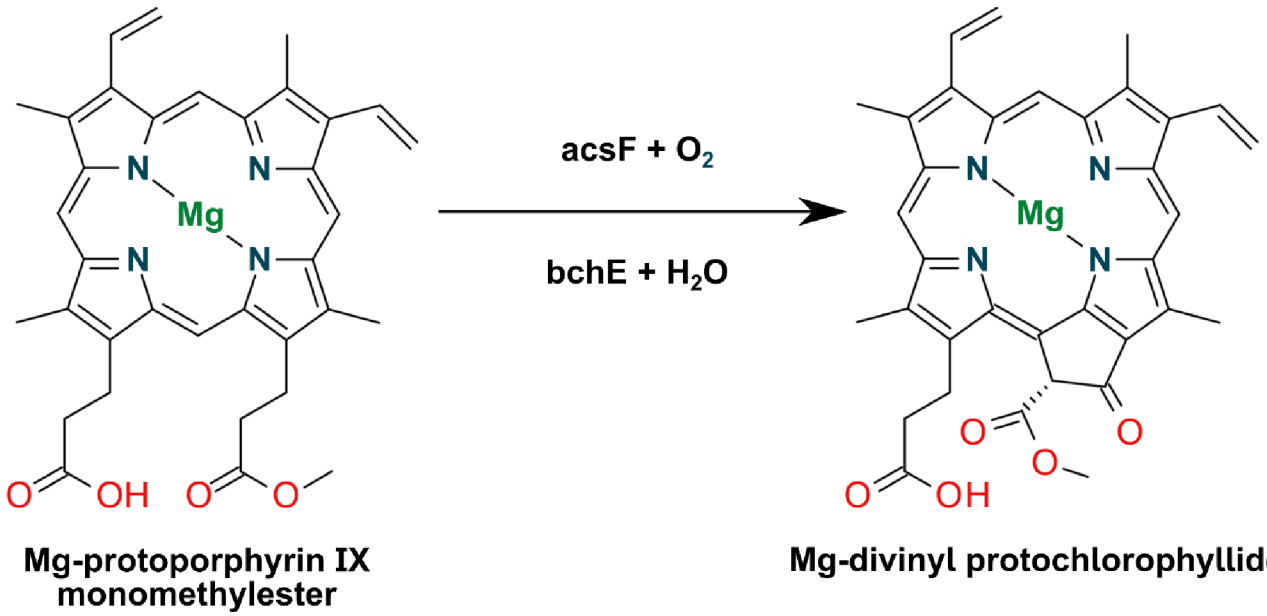

Figure 1. Crucial reaction in the synthesis of the BChl*a* molecule. Conversion of Mg-protoporphyrin IX monomethylester to Mg-divinyl protochlorophyllide using two different forms of the Mg-protoporphyrin IX monomethylester cyclase (EC:1.14.13.81): oxygen dependent *acs*F and anoxygenic *bch*E.

During the BChl*a* synthesis, there are two known forms of this enzyme: an oxygen-dependent form which utilize O_2 to catalyse the reaction, encoded by gene *acs*F, present in AAP bacteria and in some versatile PNSB, and the oxygen-independent form of this enzyme encoded by gene *bch*E that takes an oxygen atom from an H_2O molecule, present in both, purple sulphur and non-sulphur bacteria. Ortholog genes of the *acs*F gene are widely distributed, in oxygenic phototrophs such as cyanobacteria

(Minamizaki et al. 2008), algae (Moseley et al. 2002), and even in higher plants (Tottey et al. 2003). It is hypothesized that the *acs*F gene was transferred from cyanobacteria to PNSB via an horizontal gene transfer event, allowing the latter to proliferate in oxic environments, initiating the evolutionary process leading to the emergence of AAP bacteria (Boldareva-Nuianzina et al. 2013).

Regardless of their taxonomic classification, AAP bacteria share a common metabolic characteristic: they are heterotrophic bacteria that live in oxic environments and obtain energy from light using BChl*a* reaction centres (RC) (Figure 2). The anoxygenic Type-II RC is responsible for converting photons into electrons, channelling them through the quinones pool to the cytochromes for executing cyclic electron transfer. During this process, protons are actively pumped out, contributing to the establishment of electrochemical gradient that, in turn, enables ATP synthase to generate ATP for the metabolism of the cell (Figure 2).

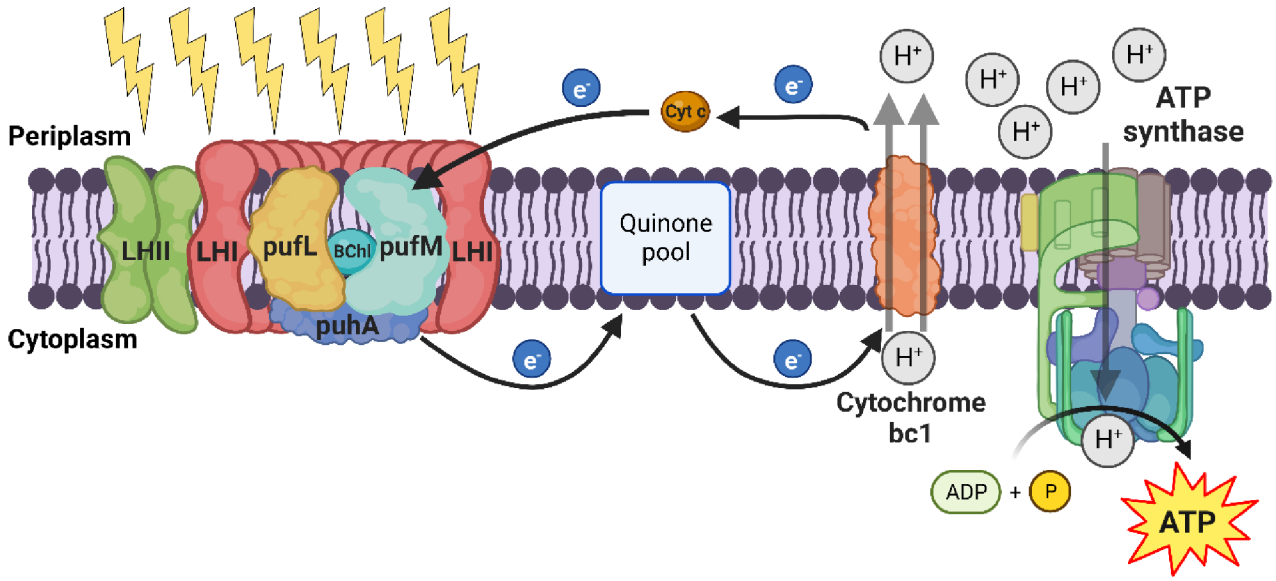

Figure 2. Simplified representation of photosynthetic Type-II RC functioning in AAP bacteria. Antennae complex LHII (not present in all species), LHI containing BChl*a* molecules captures energy from light to transfer it to heterodimeric reaction centre (pufL-pufM held by puhA). Then energy is transmitted to a quinones pool that activates Cytochrome bc1 to pump out 2 protons. Finally, Cytochrome C conducts the electrons back to the reaction centre. This process is also known as cyclic photophosphorylation and creates the electrochemical gradient that provides the required energy for ATP synthase to produce ATP. The Figure was created using BioRender.com.

Despite their phototrophic capability, AAP bacteria are not autotrophic and they predominantly obtain carbon and energy from the oxidation of organic substrates acquired from the environment (Yurkov and Beatty 1998), and the energy derived from the light just complements their metabolic requirements (Hauruseu and Koblížek 2012; Piwosz et al. 2018). However, some unique AAP bacteria exhibit metabolic capabilities that do not fit this definition entirely. For instance, the highly versatile

Roseobacter denitrificans is also able to grow mixotrophically under anoxic conditions and some sporadic MAGs have been found to contain the genes for fixing carbon through Calvin-Benson cycle (Vavourakis et al. 2019; Mujakić et al. 2023). Furthermore, there is one cultured AAP bacteria with the entire gene repertoire for carbon fixation (Yabe et al. 2022). Additionally, the first confirmed case of AAP bacterium combining the anoxygenic Type-II RC with membrane xanthorhodopsin was isolated from an Alpine lake (Kopejtka et al. 2020, 2022). The dual phototrophy synergistically confer photoheterotrophic activity across varying environmental conditions. Nevertheless, these are singular exceptions and the vast majority of AAP bacteria adhere to the acknowledged capabilities aforementioned, with diverse metabolic nuances. AAP bacteria are ubiquitously found in soils and aquatic environments representing a significant fraction of the prokaryotic community.

1.3.- Taxonomic diversity

The first AAP bacterium was isolated by Tsuneo Shiba and Keiji Harashima from the surface of a green macroalgae, *Enteromorpha linza*, in the late 1970s (Harashima et al. 1978). When they analysed the pigments from their aerobic bacterial isolates by a thin-layer chromatography, they noticed a blue spot, which turned out to be BChl*a*. Until then, BChl*a* had only been observed in anaerobic anoxygenic photosynthetic bacteria and exclusively associated with anoxic environments. This first isolated, *OCh 101*, later described as *Erythrobacter longus* (Shiba et al. 1979), established the first genus within the functional group of AAP bacteria (Shiba and Simidu 1982). Subsequently, after the second described genus of AAP species, *R. denitrificans*, many more aerobic strains containing BChl*a* were isolated and affiliated to different genera, families, and orders of, initially, the Pseudomonadota phylum (Shiba and Harashima 1986) and later from diverse other phyla including Chloroflexota, Gemmatimonadota and Vulcanimicrobiota (formerly known as Eremiobacteriota). Recently recovered Myxococcota MAGs have further expanded the list of AAP phyla.

Since the isolation of the first AAP bacterium, the number of described AAP species has been steadily growing. The early discovered AAP bacteria, such as *Sandaracinobacter sibiricus*, *Erythromonas ursincola*, *Erythromicrobium hydroliticum, Erythromicrobium ramosum*, *Erythromicrobium ezovicum* or *Roseococcus thiosulfatophilus* (Shiba and Simidu 1982; Yurkov, Vladimir V. 1992; Yurkov et al. 1993, 1994, 1997), are members of phylum Pseudomonadota. This led to the assumption that AAP

bacteria were essentially members of Pseudomonadota and solely this bacterial phylum harbours AAP bacteria (Yurkov and Hughes 2017). Currently, more than 300 Pseudomonadota AAP species have been cultured and described from Alpha- and Gammaproteobacteria classes (Figure 3), both of which are highly ubiquitous and contribute in variable proportions to marine and freshwater bacterioplankton.

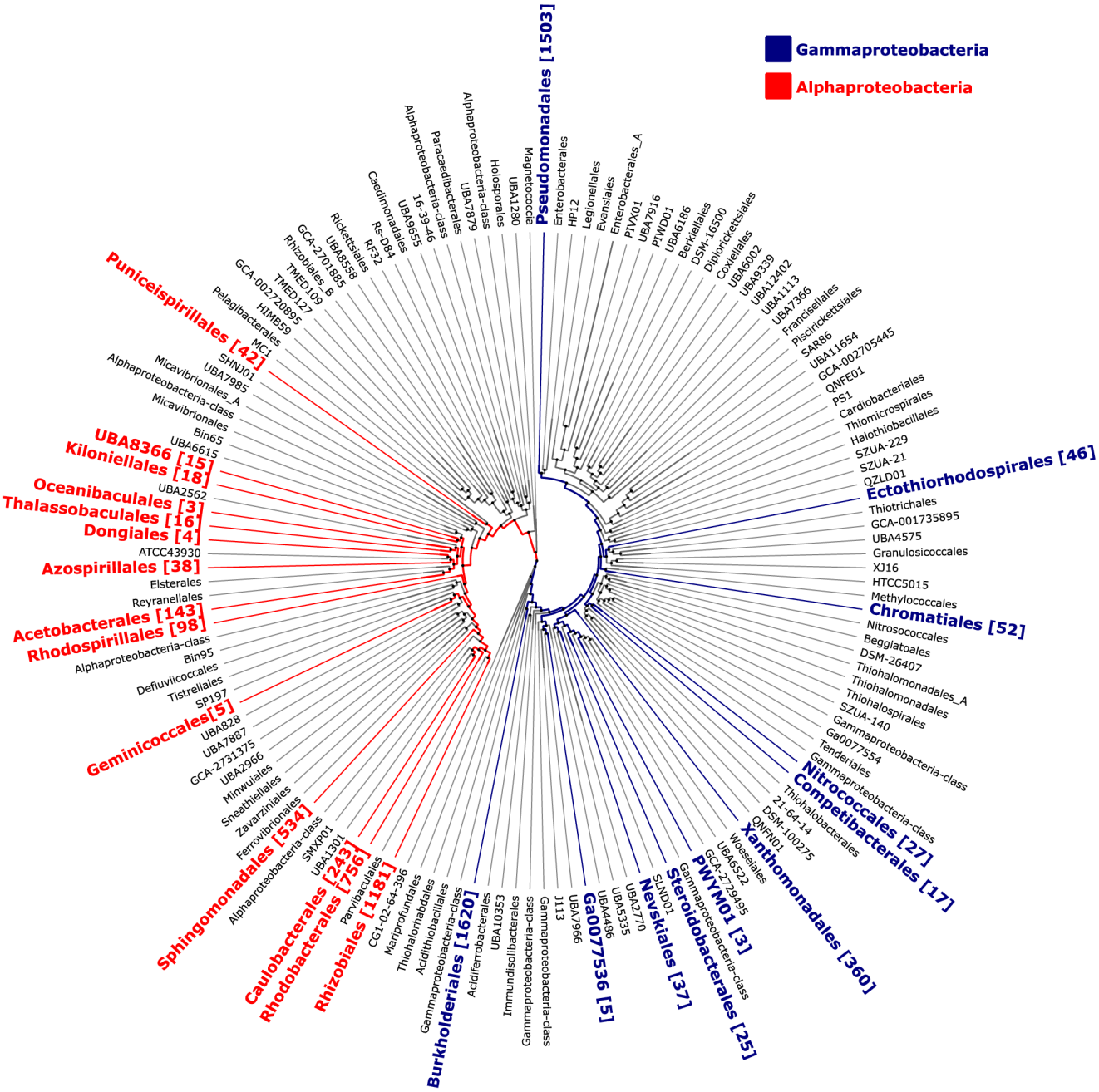

Figure 3. Phylogenomic tree of all Pseudomonadota orders containing genes for anoxygenic photosynthesis modified from Genome Taxonomy Database (GTDB, releaseRS95) and visualized using AnnoTree (version 1.2) (Mendler et al. 2019; Parks et al. 2022). Gammaproteobacteria in blue and Alphaproteobacteria in red. The *puf*M gene has been used as an anoxygenic photosynthesis marker. Numbers between brackets indicate the number of genome representatives within each order.

Within Alphaproteobacteria members, different orders have been described hosting AAP members with Sphingomonadales, Rhodobacterales, Caulobacterales, Rhizobiales and Hyphomicrobiales playing a significant role across a wide variety of environments (Boeuf et al. 2013; Gazulla et al. 2022; Kuzyk et al. 2022). Within the AAP Gammaproteobacteria, the most significant orders are Pseudomonadales, Cellvibrionales and Burkholderiales. The diversity of AAP bacteria in Gammaproteobacteria increased following the reclassification of formerly known Betaproteobacteria class, which might be more abundant in freshwater environments. Their taxonomy, previously based on the 16S rRNA gene sequence, changed after whole genome taxonomic classification, representing a new order of Gammaproteobacteria class called Burkholderiales.

Nevertheless, the prevalence of specific taxonomic groups is different in distinct environments and marine lineages are usually absent from fresh waters. For example, Rhodobacterales, which was the first described order of marine AAP bacteria (Shiba 1991; Wagner-Döbler and Biebl 2006) and other unclassified Alphaproteobacteria are the main representatives of Alphaproteobacteria in marine environments (Gazulla et al. 2023) while the Alphaproteobacteria community composition in freshwaters is more diverse, additionally encompassing Rhizobiales, Sphingomonadales and other orders (Fecskeová et al. 2019). Regarding the Gammaproteobacteria, the difference between marine and fresh waters is more conspicuous. While marine AAP Gammaproteobacteria are mostly Pseudomonadales and unclassified at order level, the Burkholderiales order retains an almost entire hegemony in fresh water lakes (Ferrera et al. 2017b; Gazulla et al. 2022).

However, metabolic capabilities often transcend phylum boundaries, and this is the case for the aerobic anoxygenic phototrophy which is also present in Chloroflexota, Gemmatimonadota, Vulcanimicrobiota and Myxococcota. Phototrophic Chloroflexota, of which the first isolated members came from thermophilic hot springs, was classically assigned as anaerobic green non-sulphur bacteria (Table 1) (Pierson and Castenholz 1974; Hanada et al. 2002; van der Meer et al. 2010). Nonetheless, most of the *puf*M gene sequences assigned to the Chloroflexota phylum originate from uncultured Chloroflexota inhabiting freshwater environments (Mehrshad et al. 2018; Fecskeová et al. 2019), about which the diversity and dynamics remain poorly understood. Furthermore, phototrophic Gemmatimonadota were recently discovered to inhabit fresh waters when the first member was isolated from a freshwater

lake in the Gobi Desert and from a freshwater stream in Greenland (Zeng et al. 2014, 2021). Gemmatimonadota represent the first known event of horizontal gene transfer of entire photosynthetic gene cluster, likely acquired from a Pseudomonadota ancestor (Zeng et al. 2014; Cardona 2016). AAP Gemmatimonadota exhibit great diversity and are found in freshwater lakes and wastewaters (Mujakić et al. 2023). Notably, another lineage of phototrophic Gemmatimonadota was identified in MAGs from soda lake sediments. They contained genes involved in carbon fixation by Calvin cycle (e. g. *rbcL, rbcS,* and phosphoribulokinase) but they have not yet been cultured and are very likely to be anaerobic (Zorz et al. 2019; Vavourakis et al. 2019; Mujakić et al. 2023). Finally, there are 2 more minority phyla in which anoxygenic phototrophy have been discovered only recently. Vulcanimicrobiota, was first isolated from a fumarolic ice cave on a volcano in Antarctica and has emerged as a versatile AAP bacterium remarkably containing a complete gene set involved in the Calvin–Benson–Basham (CBB) cycle, though its carbon fixation capability has not yet been experimentally confirmed (Yabe et al. 2022). Myxococcota, formerly part of Deltaproteobacteria, present potential phototrophic capability, genuinely found in recurrently reconstructed MAGs from a wide variety of environments and sporadically containing ribulose-1,5-bisphosphate carboxylase/oxygenase, key enzyme in carbon fixation (Li et al. 2023).

Expanding the taxonomic spectrum, a member of Acidobacteriota with Type-I RC was isolated from thermophilic biofilms, and described as able to perform photoheterotrophy in aerobic environments (Bryant et al. 2007; Tsukatani et al. 2012; Tsuji et al. 2020). Although it was also cultivated from a eutrophic lake (Kuzyk et al. 2022), it is so far the only isolated Type-I RC performing phototrophy in oxic conditions and its contribution to AAP metabolic group is, according to current studies, negligible. Therefore, AAPs are considered a functional group due to the potential ecological implications arising from their shared metabolic capability conferred by the anoxygenic phototrophy. Thus, study them as a whole provides insights into their dynamics and ecological roles in the environments.

The most common marker used in bacterial community studies, 16S rRNA gene, does not allow for unambiguous identification of AAP bacteria since phototrophic genes have been gained and lost multiple times in closely related species (Kasalický et al. 2018; Kopejtka et al. 2019). Among all the

phototrophic genes, *puf*M (responsible for a subunit of Type-II RC) is one of the most conserved (Nagashima and Nagashima 2013; Imhoff et al. 2018). Consequently, it has been used to study the diversity and composition of AAP communities directly from environments employing amplicon sequencing technique. Notably, combinations of common used primer pairs, puf_UniF – puf_UniR and pufMF – pufM_WAW have been widely used (Béjà et al. 2002; Yutin et al. 2005). In addition to known taxonomic groups, certain phototrophic phylogenetic groups (A-L) were identified according to *puf*M phylogenetic tree (Yutin et al. 2007). Despite the fact that the majority of these phylogroups lack any taxonomically assigned MAGs or cultured representatives, recent phylogenetic analyses have enclosed most of these phylogroups within Rhodobacterales order (phylogroups A-H), Burkholderiales order (phylogroups I) and Halieaceae family (phylogroups K) (Gazulla et al. 2022).

1.4.- Population dynamics

1.4.1.- AAP bacteria abundances

Diverse studies have consistently highlighted the significance of AAP bacteria in different environments and, emphasized their high reported abundances. According to the abundance of marker *puf*M that reached up to 3.5×10^5 *puf*M gene copies per gram of soil (Csotonyi et al. 2010; Tang et al. 2021), AAP bacteria perform a crucial role in biological soil crust development (Tang et al. 2018). Additionally, BChl*a* detection revealed the presence of AAP bacteria in Antarctic soils, permafrost in Tibetan Plateau and from Himalayan soils, where the dissolved organic carbon (DOC) showed a direct correlation with AAP occurrence (Jia et al. 2015; Tahon and Willems 2017; Rehakova et al. 2019). BChl*a* in AAP bacteria can be detected from light absorption and autofluorescence spectra. In this case, two main light absorption peaks are evident: a so called Soret peak in the UV A region and infrared peak(s) (750-900 nm) (Figure 4A). BChl*a* from AAP bacteria is able to emit autofluorescence at 890 nm, which can be registered by epifluorescence microscopy (Figure 4B). Microscopic samples can be excited at 325–370 nm, 450–490 nm and 545–565 nm or using whole light spectrum and the BChl*a* fluorescence emission is detected at wavelengths >850nm. Since chlorophyll-*a* (Chl*a*) autofluorescence spectra emission also encompasses the 890 nm wavelength, cells that show fluorescence emission at 690 and 573 nm, corresponding with Chl*a* and phycoerythrin, respectively, are not counted as AAP bacteria (Cottrell et al. 2006; Mašín et al. 2006; Piwosz et al. 2022) (Figure 4C-D).

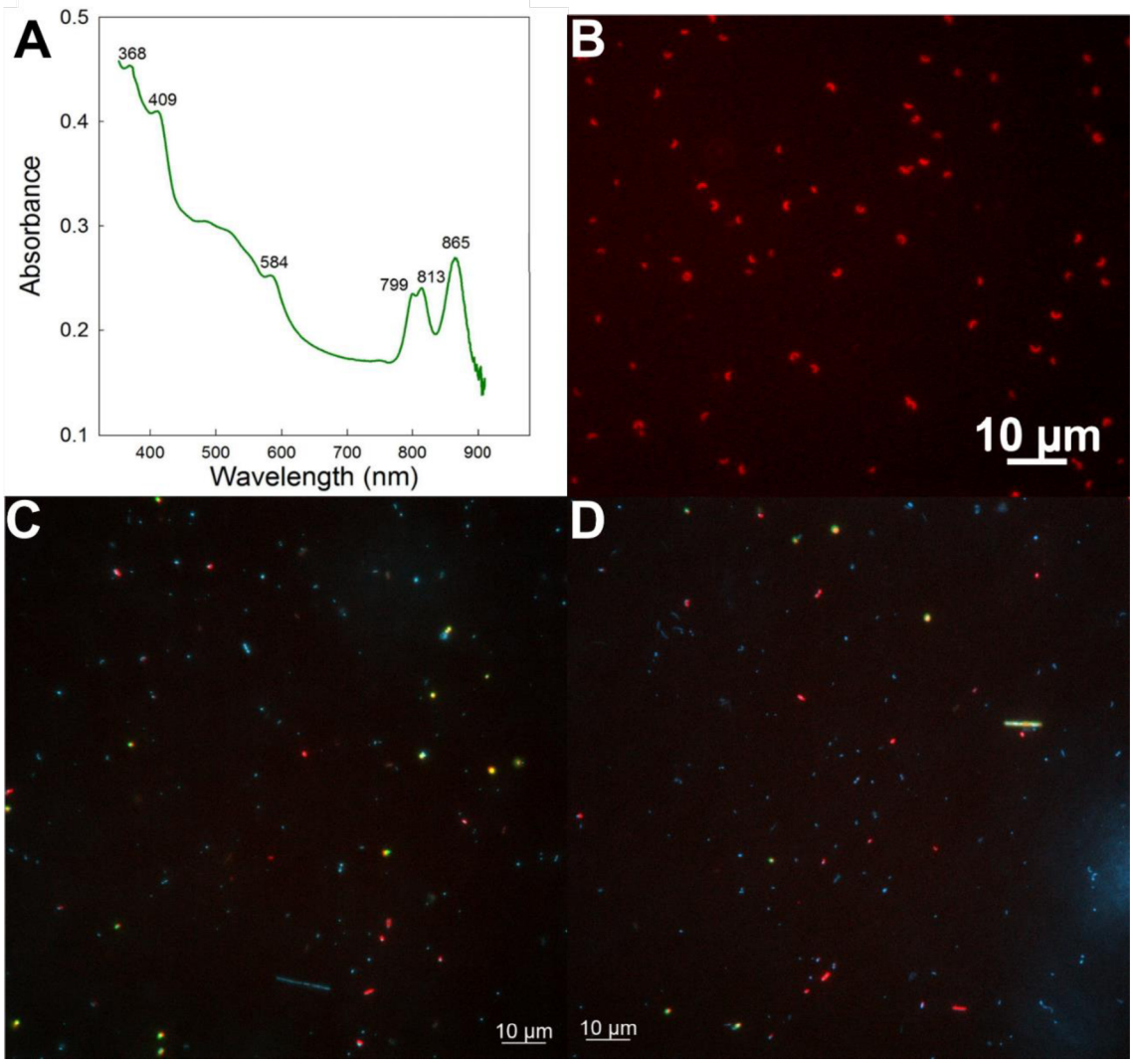

Figure 4. In vivo absorption spectrum of phototrophic *Limnohabitans planktonicus* II-D5T cells (A). Microphotography of infrared emission from BChl*a* autoflorescence in AAP bacterium culture of *Limnohabitans* sp. (B). Overlay microphotographs from environmental fresh water sample combining fluorescence from 4,6-diamidino-2-phenylindole (DAPI) in blue, Chl*a* in green and BChl*a* in red (C-D).

Consequently, the abundance of AAP bacteria has been quantified using epifluorescence microscopy from a plethora of aquatic environments. AAP bacteria represented up to 11% of the total bacteria in the North East Pacific (Kolber et al. 2001) and subsequent investigation revealed AAP bacteria to represent 1-24% of the total prokaryotes in euphotic zones of different seas and oceans (Figure 5). Such discoveries challenged the classical view of marine bacteria as heterotrophic organisms that are solely dependent on organic matter produced by phytoplankton (Kolber et al. 2000; Eiler 2006). Moreover, their relative abundance reached higher values in freshwater environments (up to 29%) and the maximum recorded AAP contribution to bacterial community of 34% in a hypertrophic polyhumic lake (Szabó-Tugyi et al. 2019). Notably, an extraordinary 80 % of microbial biomass assigned to AAP

bacteria was reported from an acidic mountain lake (Mašín et al. 2008). These observed dynamic changes in AAP abundance and contribution to ecosystems are shaped by different environmental and biological variables.

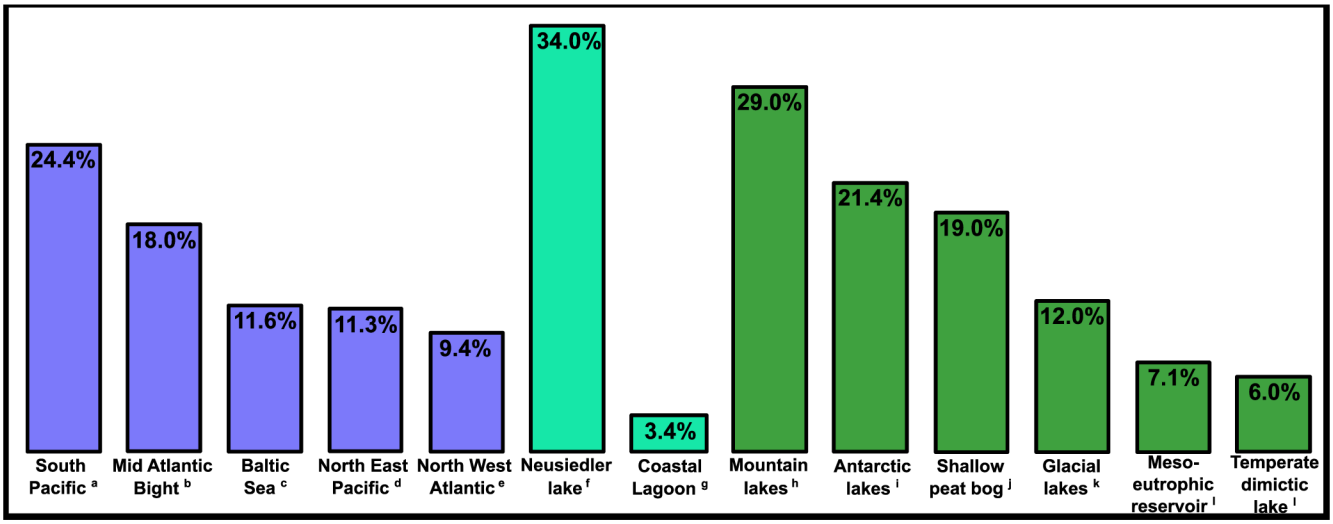

Figure 5. Maximum observed percent contribution of AAP bacteria to total bacterial community according to positive BChl*a* fluorescence cell microscopy counts in marine (blue), polyhumic lake and coastal lagoon (turquoise) and fresh waters (green). References: a (Lami et al. 2007), b (Cottrell et al. 2006), c (Mašín et al. 2006), d (Kolber et al. 2001), e (Sieracki et al. 2006), f (Szabó-Tugyi et al. 2019), g (Lamy et al. 2011), h (Čuperová et al. 2013), i (Medová et al. 2016), j (Lew et al. 2015), k (Mašín et al. 2012) and l (Fecskeová et al. 2019).

1.4.2.- AAP community drivers

Prokaryotic communities are intricately shaped by a myriad of factors including geographic, biological and environmental influences (Hanson et al. 2012). Similarly, AAP populations are moulded by various drivers. For instance, in Antarctic lakes, the low light availability during the winter ice-covered water surface negatively impacted AAP community (Ferrera et al. 2017b). Furthermore, concentration of total phosphorus, Chl*a* and DOC impacted AAP bacteria in freshwater lakes of different trophic statuses (Mašín et al. 2008; Fauteux et al. 2015; Szabó-Tugyi et al. 2019). Moreover, AAP populations undergo substantial grazing pressure, surpassing that of other heterotrophic bacteria due to their larger size (Garcia-Chaves et al. 2016; Szabó-Tugyi et al. 2019).

As a consequence of the aforementioned community drivers, the contribution of AAP bacteria to the ecosystem exhibits strong seasonality, manifesting different community composition dynamics in marine and freshwater environments (Ferrera et al. 2014; Kolářová et al. 2019; Kuzyk et al. 2022). AAP abundance undergo large seasonal oscillations, peaking in spring, summer or autumn and

demonstrating a smaller contribution in winter (Čuperová et al. 2013; Lew et al. 2015; Auladell et al. 2019; Kolářová et al. 2019). Despite the wealth of existing reports, seasonal regulatory mechanisms driving variations in the AAP community composition, and clear drivers of the seasonal changes in AAP abundance in freshwater remain poorly understood.

1.5.- Ecological role of AAP bacteria

Different biotic and abiotic factors have shaped the communities existing in the natural environments during millions of years. Microbial aquatic communities encompass a huge variety of organisms including the most abundant phytoplankton, zooplankton and bacterioplankton that have been living and evolving together, giving rise to yearly repetitive dynamics. These dynamics have been studied and verbally formulated in the Plankton Ecology Group (PEG) model (Figure 6).

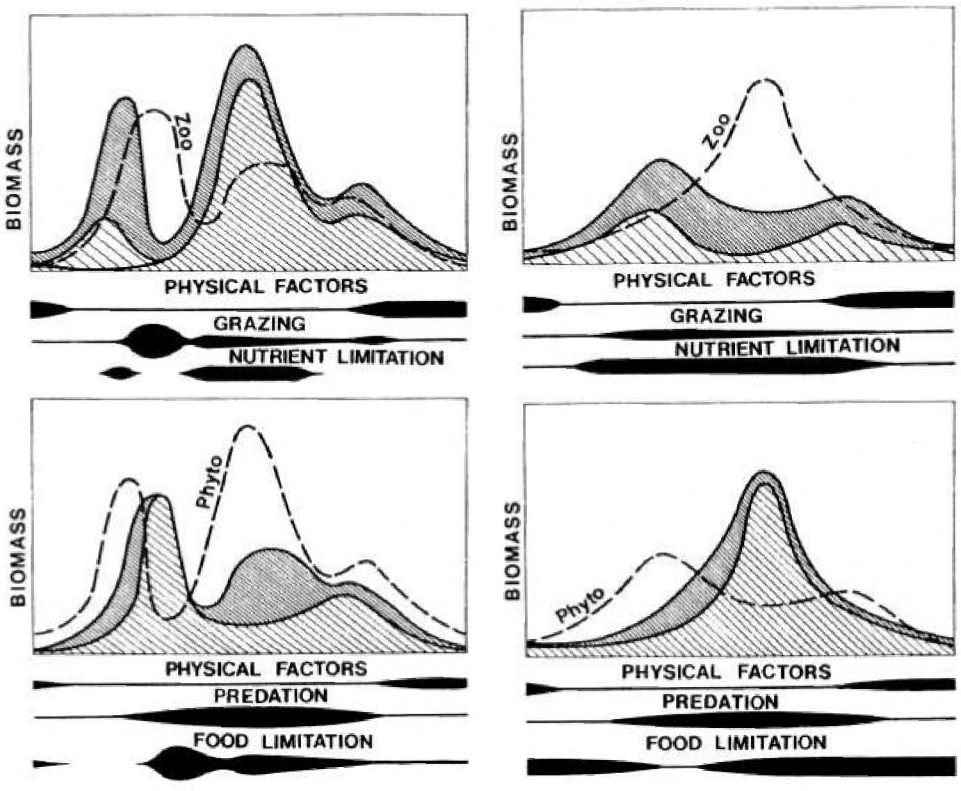

Figure 6. Original graphical representation of the seasonal development of phytoplankton and zooplankton described in the PEG model by Sommer et al., 1986 (Sommer et al. 1986). The seasonal development of the phytoplankton and zooplankton in an idealized eutrophic (left) and oligotrophic (right) scenario. Phytoplankton composition in the upper panels: small algae (dark shading) and large algae (light shade). Zooplankton biomass in the bottom panel: small herbivores (dark shading) and large herbivores (light shading). The horizontal diagrams indicate the periods when major external constraints are important. The Figure was taken from Sommer et al., 1986.

Subsequently, the model has been refined to incorporate more components interacting in the system and additional scenarios (Sommer et al. 2012). In brief, the annual cycle in an ideal temperate freshwater lake initiates with low abundances and activities of the overall microbial community during winter. When temperature rises, the minimal grazing pressure on phytoplankton and rising light availability triggers the spring phytoplankton bloom. Consequently, the rising amount of organic carbon circulating in the ecosystem triggers the population of bacterioplankton, which feeds on DOC (Zeder et al. 2009; Kavagutti et al. 2023), and herbivorous zooplankton that directly feeds on phytoplankton. In synergy with the grazing by zooplankton, a large increase on phytoplankton viruses and cyanophages induce the death of substantial proportion of phytoplankton (Sharoni et al. 2015), leading to the "clear-water" phase. The large zooplankton biomass enhances fish predation, channelling the organic carbon generated by spring phytoplankton bloom to upper trophic levels. Nutrient availability and competition for phosphate becomes a limiting factor by the end of the summer and phosphorus luxury uptake becomes a generous advantage leading to the proliferation of poorly-ingestible and small edible algae that triggers the autumn bloom. With lower temperatures and the reduction of light availability, the community starts decreasing activity and begins overwintering.

Despite ongoing efforts, there still are many functional groups that need to be incorporated into the model, such as viruses or fungi. Indeed, the role of bacterioplankton in this elaborated model has been recently added (Kavagutti et al. 2023; Park et al. 2023). Nevertheless, there is an incommensurable functional diversity within bacteria, with a wide range of metabolisms and different dynamics. For instance, the role of specific functional groups (such as AAP bacteria) and its contribution to the whole microbial community remain excluded in the model.

1.5.1- Impact on microbial communities

Photoheterotrophy confers unique capability to AAP bacteria. By supplying their metabolism with energy from light, AAP bacteria can reduce the amount of carbon that they need to respire for their metabolism thus reducing their carbon requirements (Koblížek et al. 2020) (Figure 7). Additionally, in presence of light, AAP bacteria can increase the carbon assimilation rates, ultimately resulting in higher

growth rates (Piwosz et al. 2018; Koblížek et al. 2020). However, such knowledge derives from cultured experiments and impact of AAP bacteria on microbial respiration and carbon assimilation rates in natural environments remained largely unexplored.

The manner in which the metabolic gain that photoheterotrophy provides to AAP bacteria, reflected in the management of different carbon sources, is also unidentified. There has been hypothesized that AAP bacteria might outperform heterotrophic bacteria by efficiently breaking down high complex carbon sources such as recalcitrant organic matter, potentially allowing them to selectively thrive in oligotrophic conditions (Beatty 2002; Salka et al. 2014; Koblížek 2015).

Finally, taking into consideration the comprehensive presented knowledge: higher assimilation of DOC, greater growth rates leading to substantial biomass, larger cell sizes and high grazing pressure, it is both proposed and imperative to assess AAP bacteria as a key functional group actively involved in the microbial loop and therefore in the constitution of the aquatic microbial communities.

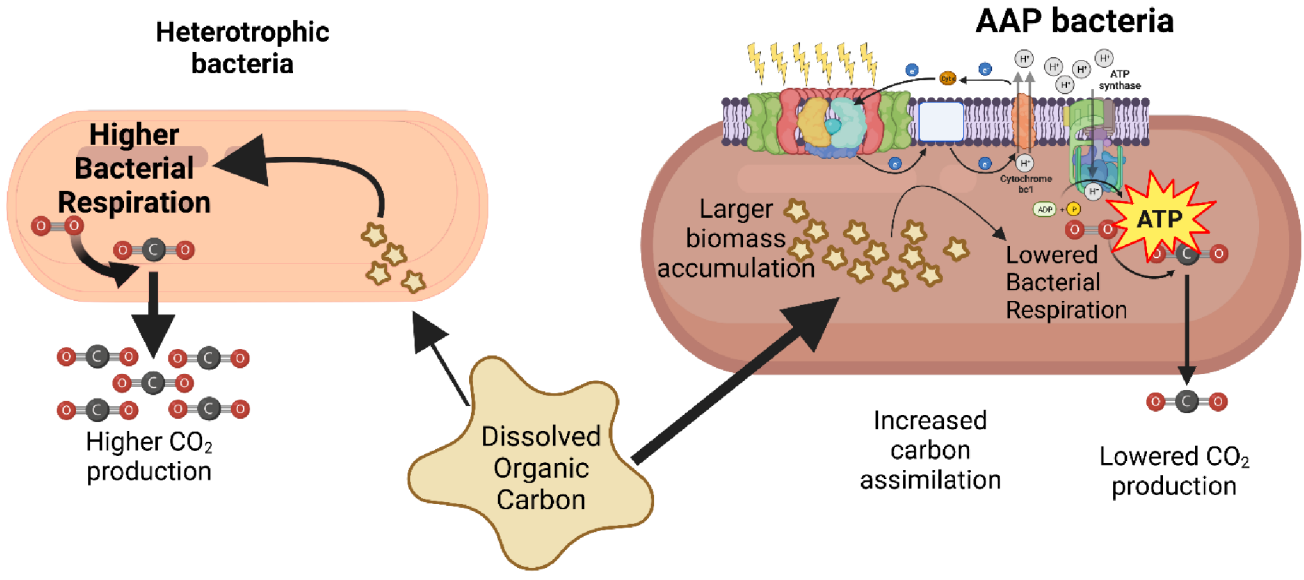

Figure 7. Simplified scheme of the observed attributes of AAP cultures in comparison to heterotrophic bacteria. The Figure was created using BioRender.com.

2.- Research

Chapter I

DOI: 10.1111/1758-2229.13131

BRIEF REPORT

environmental microbiology reports Applied Microbiology International

Diversity dynamics of aerobic anoxygenic phototrophic bacteria in a freshwater lake

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Abstract
Aerobic anoxygenic photoheterotrophic (AAP) bacteria represent a functional group of prokaryotic organisms that harvests light energy using bacteriochlorophyll-containing photosynthetic reaction centers. They represent an active and rapidly growing component of freshwater bacterioplankton, with the highest numbers observed usually in summer. Species diversity of freshwater AAP bacteria has been studied before in lakes, but its seasonal dynamics remain unknown. In this report, we analysed temporal changes in the composition of the phototrophic community in an oligo-mesotrophic freshwater lake using amplicon sequencing of the pufM marker gene. The AAP community was dominated by phototrophic Gammaproteobacteria and Alphaproteobacteria, with smaller contribution of phototrophic Chloroflexota and Gemmatimonadota. Phototrophic Eremiobacteriota or members of Myxococcota were not detected. Interestingly, some AAP taxa, such as Limnohabitans, Rhodoferax, Rhodobacterales or Rhizobiales, were permanently present over the sampling period, while others, such as Sphingomonadales, Rhodospirillales or Caulobacterales appeared only transiently. The environmental factors that best explain the seasonal changes in AAP community were temperature, concentrations of oxygen and dissolved organic matter.

Villena-Alemany C., Mujakić I., Porcal P., Koblížek M. & Piwosz K (2023)

Diversity dynamics of aerobic anoxygenic phototrophic bacteria in a freshwater lake.

Environmental Microbiology Reports 2023; 15: 60–71.

(IF= 4.006)

Chapter I: Context and objectives

The existing characterization of the AAP community composition in freshwater environments relies on snapshot studies, lacking a detailed insight into the dynamic changes that occur throughout the year. Notably, the influence of environmental factors on the seasonal succession of the AAP community, as well as how differently they affect the overall bacterial community is unknown. Subsequently, we tried to seek answers to fill these knowledge gaps by exploring the following questions:

- What are the seasonal dynamics of AAP bacterial community composition in a freshwater lake?
- Which environmental variables drive changes of the AAP bacterial community composition?
- To what extent do these environmental factors affect differently AAP and other heterotrophic bacteria?

Chapter I: Summary

Epilimnion and hypolimnion of meso-oligotrophic freshwater lake Cep were sampled biweekly from April to September to study the dynamics of the AAP community. Amplicon analysis of *puf*M gene provided insights into the composition of the AAP bacterial community, while the total bacterial community was assessed according to 16S rRNA gene amplicon. The relationships between the community composition and environmental variables were assessed with distance-based multidimensional statistical methods.

The study successfully described the diversity dynamics of AAP bacterial community throughout the year revealing the dominance of Gammaproteobacteria and Alphaproteobacteria with a smaller contribution of Chloroflexota and Gemmatimonadota. Phototrophic Vulcanimicrobiota or Myxococcota were not detected. *Limnohabitans*, *Rhodoferax*, Rhodobacterales and Rhizobiales were consistently present through the sampling period, whereas Caulobacterales, Sphingomonadales, Acetobacterales or Rhodospirillales appeared only transiently, peaking at different time of the seasonal succession. Furthermore, the AAP community exhibited distinct temporal patterns in the epilimnion and hypolimnion showing for instance, a higher contribution of Rhodospirillales and Acetobacterales in the epilimnion and Sandarakinorhabdus and Rhodoferax in the hypolimnion.

The same environmental factors explained the variability of community seasonal changes in both, all bacteria and AAP bacteria communities: temperature, concentration of oxygen and dissolved organic

matter. However, the extent of explanation was different with a higher impact of DOC concentration on the AAP community. This indicates a potential differential response of AAP bacteria compared to the overall bacteria concerning DOC concentration.

Additionally, the study uncovered a remarkably large amount of *puf*M gene reads categorized as unclassified, underscoring the still hidden diversity within AAP bacteria. Moreover, time- and depth-related differences in AAP community composition clearly indicated that longer high-frequency sampling studies are necessary to decipher this unexplored diversity and reaffirm the indigenous nature of the observed AAP community in freshwater environments.

Received: 18 March 2022 | Accepted: 19 September 2022

DOI: 10.1111/1758-2229.13131

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Aerobic anoxygenic photoheterotrophic (AAP) bacteria represent a functional group of prokaryotic organisms that harvests light energy using bacteriochlorophyll-containing photosynthetic reaction centers. They represent an active and rapidly growing component of freshwater bacterioplankton, with the highest numbers observed usually in summer. Species diversity of freshwater AAP bacteria has been studied before in lakes, but its seasonal dynamics remain unknown. In this report, we analysed temporal changes in the composition of the phototrophic community in an oligo-mesotrophic freshwater lake using amplicon sequencing of the *puf*M marker gene. The AAP community was dominated by phototrophic Gammaproteobacteria and Alphaproteobacteria, with smaller contribution of phototrophic Chloroflexota and Gemmatimonadota. Phototrophic Eremiobacteriota or members of Myxococcota were not detected. Interestingly, some AAP taxa, such as *Limnohabitans*, *Rhodoferax,* Rhodobacterales or Rhizobiales, were permanently present over the sampling period, while others, such as Sphingomonadales, Rhodospirillales or Caulobacterales appeared only transiently. The environmental factors that best explain the seasonal changes in AAP community were temperature, concentrations of oxygen and dissolved organic matter.

INTRODUCTION

Photoheterotrophic bacteria represent an important component of freshwater bacterioplankton. These organisms harvest light energy but, as they do not fix carbon, require organic carbon to build biomass. Two main groups of aquatic photoheterotrophic bacteria are rhodopsin-containing bacteria and aerobic anoxygenic phototrophic (AAP) bacteria. Rhodopsins are membrane-bound proteins able to use light energy to translocate protons across the membrane. The proton gradient is used for ATP production (Lanyi, 2004; Spudich & Luecke, 2002). In contrast, AAP bacteria harvest light energy using photosynthetic complexes containing bacteriochlorophyll-*a* (BChl-*a*) (Koblížek, 2015; Yurkov & Csotonyi, 2009). Due to the captured energy, AAPs reduce utilization of organic carbon for respiration and increase their biomass yield up to 100% (Hauruseu & Koblížek, 2012; Piwosz et al., 2018). When compared with rhodopsin-containing bacteria, AAP bacteria are more effective in producing energy from light (Kirchman & Hanson, 2013).

AAP bacteria contribute 1–22% of all prokaryote abundance in the euphotic layer of freshwater lakes (Čuperová et al., 2013; Fauteux et al., 2015; Mašín et al., 2008, 2012; Ruiz-González et al., 2013; Ruiz-González et al., 2020). They have, on average, larger cell size and exhibit faster growth and mortality rates than heterotrophic bacteria (Cepáková et al., 2016; Garcia-Chaves et al., 2016). Recently, it has been shown that upon infra-red illumination AAP bacteria reduce total microbial respiration by 15% and increase

microbial production by 6% (Piwosz et al., 2022). All these findings indicate the importance of AAPs in microbial food webs and the freshwater carbon cycle (Fauteux et al., 2015; Koblížek et al., 2005).

The most common representatives of AAP bacteria in freshwaters are members of the order Burkholderiales (Gammaproteobacteria), such as *Limnohabitans*, *Rhodoferax* and *Polynucleobacter* and members of orders Sphingomonadales and Rhodobacterales (Alphaproteobacteria), with a minor contribution of Chloroflexota and Gemmatimonadota (Caliz & Casamayor, 2014; Fecskeová et al., 2019; Kasalický et al., 2018; Martinez-Garcia et al., 2012; Mujakić et al., 2021; Salka et al., 2011). Unfortunately, most of these studies provided only snap-shot information on AAP community composition from a single or infrequent sampling of multiple lakes, even though seasonal time series have shown AAP bacteria undergo large changes in abundance, activity and growth rates (Cepáková et al., 2016; Čuperová et al., 2013; Kolářová et al., 2019). This has likely also a large impact on AAP community composition, which remain unexplored. Therefore, we decided to investigate how the diversity changes during the year.

Here, we applied amplicon sequencing of the 16S rRNA gene and *puf*M marker gene for AAP bacteria to study temporal changes in total bacterial and AAP bacteria community composition in the epi- and the hypolimnion in a shallow, meso-oligotrophic freshwater lake Cep. We hypothesized that the diversity of AAP bacteria would show distinct temporal patterns in the epi- and the hypolimnion, as had been observed for all bacteria (Zemskaya et al., 2020). We also hypothesized that the AAP community would be affected by different environmental factors than the overall bacterial community.

EXPERIMENTAL PROCEDURES

Sampling

Samples were collected from April to September 2016 from oligotrophic Cep lake (48°92′49.24″ N, 14°88′68.11″ E) located in the Třeboň Basin Protected Landscape Area, Czechia. The lake was created in the second half of the 20th century as a result of sand mining and was filled with groundwater seeping from the nearby river Lužnice. Water samples were collected from five depths: 0.5, 2, 5, 7 and 9 m using a Ruttner Water Sampler (model 11.003KC Denmark AS). Water was transported to the laboratory in closed plastic containers, which were pre-rinsed three times with the sampled water and stored in a cooled box. Temperature and oxygen profiles were taken with an EXO1 multi-parameter probe (YSI Inc., Yellow Springs, OH).

Bacteria and AAP bacteria microscopy counts

Samples of 50 ml were fixed with buffered, sterile-filtered paraformaldehyde (Penta, Prague, Czechia) to a final concentration of 1%, and 0.5 ml was filtered onto white polycarbonate filters (pore size 0.2 μm, Nucleopore, Whatman, Maidstone, UK). Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) at concentration of 1 mg L^{-1} (Coleman, 1980). Total and AAP bacterial abundances were determined using an epifluorescence Zeiss Axio Imager.D2 microscope equipped with Collibri LED module illumination system (Carl Zeiss, Jena, Germany). Ten microphotographs were taken for every sample under 325–370 nm excitation and 420–470 nm emission wavelengths for DAPI fluorescence (total bacteria), 450–490 nm excitation and 600–660 nm emission wavelengths for autofluorescence from Chl-*a* (algae and cyanobacteria), and combined 325–370 nm, 450–490 nm, 545–565 nm and 615–635 nm excitation and 645–850 emission wavelengths for autofluorescence from BChl-*a* (AAP bacteria). As some part of Chl-*a* autofluorescence is also visible in the infrared spectrum, only the IR-positive cells that did not show any autofluorescence from Chl-*a* were counted as AAP bacteria (Cottrell et al., 2006).

Nutrients and chlorophyll-*a*

Samples were filtered through glass fibre filters with 0.4 μm nominal porosity (GF-5, Macherey-Nagel, Düren, Germany). Concentrations of soluble reactive phosphorus (SRP) were determined spectrophotometrically (Kopáček & Hejzlar, 1993; Murphy & Riley, 1962). Concentrations of nitrate and ammonium were measured according to Procházková (Procházková, 1959) and Kopáček and Procházková (Kopáček & Procházková, 1993). DOC and dissolved nitrogen (DN) were determined by catalytic thermal combustion at 720°C in combination with chemiluminescence detection by Shimadzu TOC-L equipped with TNM-L Total Nitrogen module (Shimadzu, Kyoto, Japan).

For Chl-*a* measurements, phytoplankton was collected by filtration onto GF-5 glass fibre filters (Macherey-Nagel). The filters were dried of excess water by gently pressing in a paper towel, and flush frozen in liquid nitrogen. Pigments were extracted in acetone-methanol (7:2, v:v) mixture and analysed by HPLC as described in (Piwosz et al., 2020).

DNA isolation

Water (between 250 and 700 ml) was filtered through sterile 0.2 μm Nucleopore Track-Etch Membrane filter units (Whatman, Maidstone, United Kingdom). Filters

were put inside sterile cryogenic vials, flash-frozen in liquid nitrogen and stored at −80°C. DNA extraction was done using PowerWater DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA).

DNA samples were pooled in equimolar concentrations for the subsequent analysis. The epilimnion samples included water collected from 0.5 and 2 m, and the hypolimnion ones from the deeper layers (5–9 m). Such division was based on the temperature profiles.

Bacterial community analysis

Amplicons of 16S rRNA gene were prepared using the primers set 341F-785R (Klindworth et al., 2013). PCR was performed in 20 μl reaction, using Phusion High-Fidelity PCR MasterMix (Thermo Scientific, USA). Reaction conditions were as follow: 98°C for 3 min, 25 cycles at 98°C for 10 s, 60°C for 20 s, 72°C for 20 s and a final extension at 72°C for 3 min. The reactions for each sample were done in triplicates, pooled and purified from the gel using the Wizzard SV Gel and PCR clean system (Promega), and quantified with Qubit dsDNA HS assay. Amplicons were sequenced on Illumina MiSeq (2 × 250 bp) platform at Genomic Service of the Universitat Pompeu Fabra (Barcelona, Spain).

Obtained reads were quality checked using FastQC v0.11.7 (Babraham Bioinformatics, Cambridge, UK). The primer sequences were trimmed using Cutadapt v1.16 (Martin, 2011) and further analysis was done in the R/Bioconductor environment using the DADA2 package (version 1.12.1) (Callahan et al., 2016). Low quality reads were filtered out and cut (dada2::filterAndTrim, truncLen = c(225, 225), maxN = 0, maxEE = c(2,2), truncQ = 2, rm.phix = TRUE, compress = TRUE). Sequences were merged (dada2::mergePairs()), and using removeBimeraDenovo function (method = "pooled"), chimeras were removed and singletons and doubletons were eliminated using Phyloseq (McMurdie & Holmes, 2013) (phyloseq::filter_taxa (ps, function(x) sum(x > 3) > (0.2*length(x)), TRUE)) resulting in 685 amplicon sequence variants (ASV) (Table S1). The final ASV table contained from 18,551 to 71,613 reads per sample (average ± SD: 42,945 ± 14,271). Taxonomic assignment was done in DADA2 (dada2:: assignTaxonomy()) using the SILVA r138.1 database released on 27 August 2020. Graphs were done using phyloseq (McMurdie & Holmes, 2013) and ggplot2 (Wickham, 2009) packages.

AAP bacteria community analysis

The composition of AAP bacteria community was analysed using the *puf*M gene. Amplicons for *puf*M gene were prepared using *puf*M UniF and *puf*M UniR primers (Yutin et al., 2005). The hypolimnion sample from 15/06 could not be amplified and was excluded in further analysis. The PCR was done in a triplicate of 20 μl reaction using Phusion High-Fidelity PCR MasterMix (Thermo Scientific, USA). The conditions were as follows: initial denaturation for 3 min at 98°C, 30 cycles of 98°C for 10 s, 52°C for 30 s, 72°C for 30 s, final elongation at 72°C for 5 min. The obtained triplicate reactions were pooled and amplicons were purified from the gel using the Wizzard SV Gel and PCR clean system (Promega) and quantified with Qubit dsDNA HS assay. Amplicons sequencing was performed on Illumina MiSeq (2 × 250bp) platform by Genomic Service of the Universitat Pompeu Fabra (Barcelona, Spain). Obtained *puf*M gene reads were processed in the same manner as 16S rRNA gene amplicons with different filterAndTrim function values (truncLen = c(130, 130), maxN = 0, maxEE = c(2,2), truncQ = 2, rm.phix = TRUE, compress = TRUE, multithread = FALSE). Final ASV table contained 468 pufM_ASVs and from 15,746 to 84,506 reads per sample (41,524 ± 12,440; Table S2). Taxonomic assignment was done in DADA2 (function: assignTaxonomy[]) using manually curated in-house database. It contained 1475 unique *puf*M sequences, downloaded from the Fungene repository on May 16, 2019 (http://fungene.cme.msu.edu), from metagenomes from the Římov reservoir (Andrei et al., 2019; Mehrshad et al., 2018) and from the Genome Taxonomy database accessed on 16 September 2020 (Parks et al., 2018).

The sequences of 16S amplicons were deposited in the NCBI database under Biosamples SAMN26677261–SAMN26677286 and of *puf*M amplicons under Biosamples SAMN26677246–SAMN26677260 as a part of BioProject PRJNA816466.

Phylogenetic analysis of *puf*M gene

The limitation of the taxonomic assignation of the *pufM* gene amplicons was evident from the initial classification using the default DADA2 algorithm, which failed to assign all Chloroflexota reads and 20% of Proteobacteria reads at class level, and 65% of Alphaproteobacteria reads at order level. Over 50% of reads remain unclassified at the order level after the DADA2 taxonomic assignment. Phylogenetic analysis reduced the contribution of these unclassified Alphaproteobacteria up to 95% in June. Moreover, even though only 5% of Gammaproteobacteria reads remained unclassified at order level, over a quarter of the Burkholderiales reads could not be assigned to the genus level. The *puf*M gene is more variable than 16S rRNA gene and the lack of reference sequences closely related to these unclassified pufM_ASVs in our database hampered a more precise taxonomic assignment.

To partially overcome this limitation and reveal the hidden diversity of freshwater AAP bacteria in the studied lake, we performed a phylogenetic analysis of these unclassified pufM_ASVs.

Amino acid sequences of pufM genes were obtained from the Genome Taxonomy database through AnnoTree tool (Mendler et al., 2019) on 13/09/2021. The sequences with percentage of identity ≤40% to the reference pufM sequence in GTDB were removed as they originated from the *pufL* gene. 14 *puf*M sequences from previous works (Fecskeová et al., 2019; Mujakić et al., 2021; Piwosz et al., 2020; Zeng et al., 2021), which are not shown in GTDB database but they represent a reference point in freshwater environments, were included in the analysis. The unclassified pufM_ASVs were translated to amino acids using the second forward open reading frame. Both the reference *puf*M sequences and the pufM_ASVs were aligned in Geneious v2019.2.3 using ClustalW v2.1 (Larkin et al., 2007). The phylogenetic analysis was done with IQTREE (Trifinopoulos et al., 2016). LG + F + I + G4 was selected as a best-fit nucleotide substitution model by ModelFinder (Kalyaanamoorthy et al., 2017) according to Bayesian information criterion, and the Maximum Likelihood tree was calculated using 1000 ultrafast bootstrap replicates and default settings. The trees were prepared for the publication using iTOL (Letunic & Bork, 2021) and Inkscape v1.01.

Statistical analysis

The statistical relationship between environmental data (Table T3), abundance of all bacteria and abundance of AAP bacteria was analysed by distance based linear models (DistML), distance based Redundancy Analysis (Anderson & Legendre, 1999; Legendre & Andersson, 1999) and non-metric multi-dimensional scaling (nMDS) in the PERMANOVA+ add-on package of the PRIMER7 software (Anderson et al., 2008) (Primer Ltd., Lutton, UK). Environmental variables for each layer were averaged from the corresponding depths (0.5 and 2 m for the epilimnion and 5 to 9 m for the hypolimnion) and normalized. Correlations between different environmental variables were checked using Draftsman plots, and in the case of the strong correlation (absolute value of correlation coefficient >0.7) one of the variables was excluded from the analysis (Table T3). Abundance data for all and AAP bacteria (from microscopic counts), and the amplicon data (after removing singletons and doubletons) were log $(X + 1)$ transformed. The best model was selected using a stepwise selection procedure based on statistical significance (9999 permutations) and the value of the Akaike's Information Criterion (AICc) (Anderson & Legendre, 1999; Legendre & Andersson, 1999).

RESULTS AND DISCUSSION

Environmental variables

Samples were collected from a freshwater lake Cep, from April to September. The environmental conditions were typical for oligo-mesotrophic lakes (Figure S1) indicating that our results are representative for Northern hemisphere temperate lakes (Dodds & Whiles, 2020; Verpoorter et al., 2014). Thermal stratification of the water column was formed in May and present until the end of the sampling. Nevertheless, hypoxic or anoxic conditions were not observed in the hypolimnion (minimal oxygen concentration was 5.69 mg O_2 L^{-1}; Figure S1). Concentrations of nutrients and chlorophyll-*a* (Chl-*a*) were higher in the hypolimnion.

Abundances of total and AAP bacteria

The abundance and dynamics of all bacteria were similar in the epi- and the hypolimnion, with maximum values in July for the epilimnion (4.62×10^6 cells ml^{-1}) and in May for the hypolimnion (5.19×10^6 cells ml^{-1}) (Figure 1A). The abundance of AAP bacteria ranged from 7.11×10^4 to 2.39×10^5 cells ml^{-1} in the epilimnion and from 5.57×10^4 to 2.56×10^5 cells ml^{-1} in

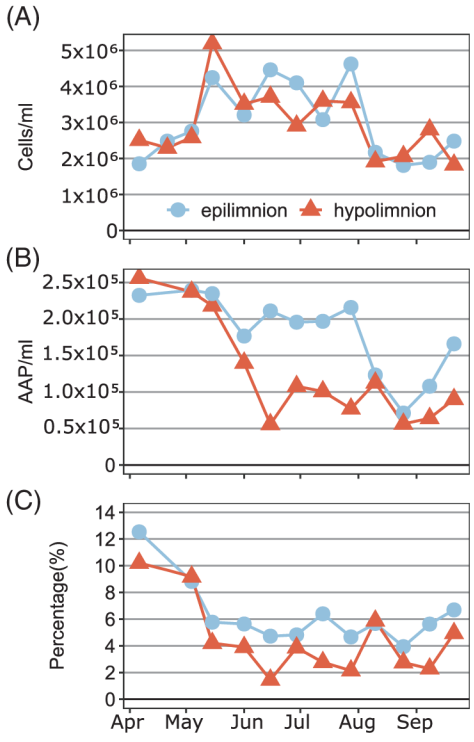

FIGURE 1 Weekly abundances of all prokaryotes (A), AAP bacteria (B) and relative abundance of AAP bacteria in the total bacterial community (C) in the epilimnion (blue-circles and line) and the hypolimnion (red-triangles and line) of Cep lake.

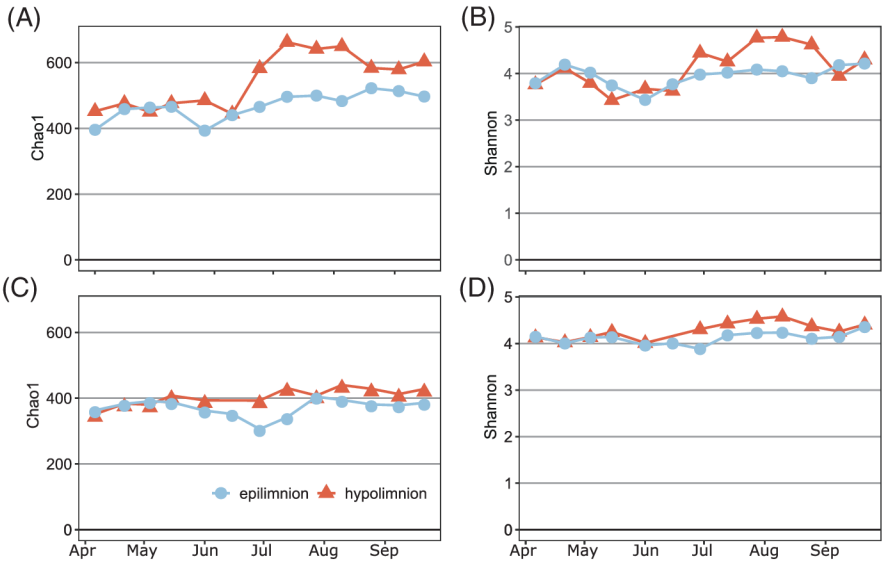

FIGURE 2 Weekly estimates of bacterial diversity in the epilimnion (blue-circles and line) and the hypolimnion (red-triangles and line) of Cep lake. (A) Chao1 index and (B) Shannon index estimated for total bacterial community based on 16S rRNA gene amplicon sequences; (C) Chao1 index and (D) Shannon index estimated for AAP bacterial community based on *puf*M gene amplicon sequences.

the hypolimnion (Figure 1B), with the highest values in April and May in both layers. AAP bacterial abundance decreased in the hypolimnion in June, while in the epilimnion only in August (Figure 1B). The percentage contribution of AAP bacteria to the total bacterial abundance was generally higher in the epilimnion (3.93–12.53%) than in the hypolimnion (1.45–10.20%, Figure 1C). Such temporal patterns of AAP bacteria abundance between in the epi- and the hypolimnion are common in temperate freshwater lakes (Čuperová et al., 2013; Fauteux et al., 2015).

Bacterial community composition

The bacterial alpha diversity was higher in the hypolimnion, especially in summer, based on 16S rRNA gene amplicons (16S_ASVs; Figure 2). The most abundant phyla were, in descending order, Actinobacteriota, Cyanobacteria, Proteobacteria, Bacteroidota, Verrucomicrobiota and Planctomycetota (Figure S2A). Non-metric multidimensional scaling (nMDS) analysis showed that the largest differences in community composition between the two layers were during stratification period from June to September (Figure S2). Since the composition and temporal changes of bacterial community were typical for the oligo-mesotrophic lakes (Cabello-Yeves et al., 2017; Morrison et al., 2017), below we have just focused on phyla known to contain AAP bacteria: Gemmatimonadota, Chloroflexota and Proteobacteria.

The relative abundance of Gemmatimonadota was <1%, which seems to be typical for freshwater lakes (Mujakić et al., 2021). Gemmatimonadota were represented by three 16S_ASVs (Figure 3A), classified as genus *Gemmatimonas* that is common in aquatic habitats (Gołębiewski et al., 2017; Mujakić et al., 2021). Their relative abundance varied substantially in both layers, with the changes in the epilimnion preceding those in the hypolimnion, suggesting a downward propagation of temporal changes (Cram et al., 2015).

Phylum Chloroflexota represented <4% of total 16S rRNA gene sequences (Figure S3A). Genera from class Chloroflexia known to contain *puf*M genes, such as *Roseiflexus*, *Kouleothrix* and UBA965 dominated during spring (Figure 3B). They were followed by the SL56 cluster (Ca. Limnocylindrus, Mehrshad et al., 2018) and they dominated in the epilimnion until the end of the sampling campaign. In the hypolimnion, a noticeable increase of the class Anaerolineae was observed in August and September (Figure 3B), when oxygen concentration was at its lowest. These bacteria are widely spread in different environments (Yamada & Sekiguchi, 2009) and were originally described to be anaerobic (Nakahara et al., 2019; Yamada et al., 2006) but recently an aerobic member containing type-II reaction centre (RC) was assembled from a metagenome (Martinez et al., 2020).

Proteobacteria were the most abundant phylum with known AAP species (Figure S3A). Alphaproteobacteria and Gammaproteobacteria were the dominant classes, with a contribution to the Proteobacteria reads ranging from 23–49% and from 51–77%, respectively.

Temporal patterns of Alphaproteobacteria were quite distinct between the epi- and the hypolimnion (Figure 3C). Caulobacterales, Rhizobiales, Rhodobacterales and Sphingomonadales, orders known to contain AAP species (Imhoff et al., 2019; Kopejtka

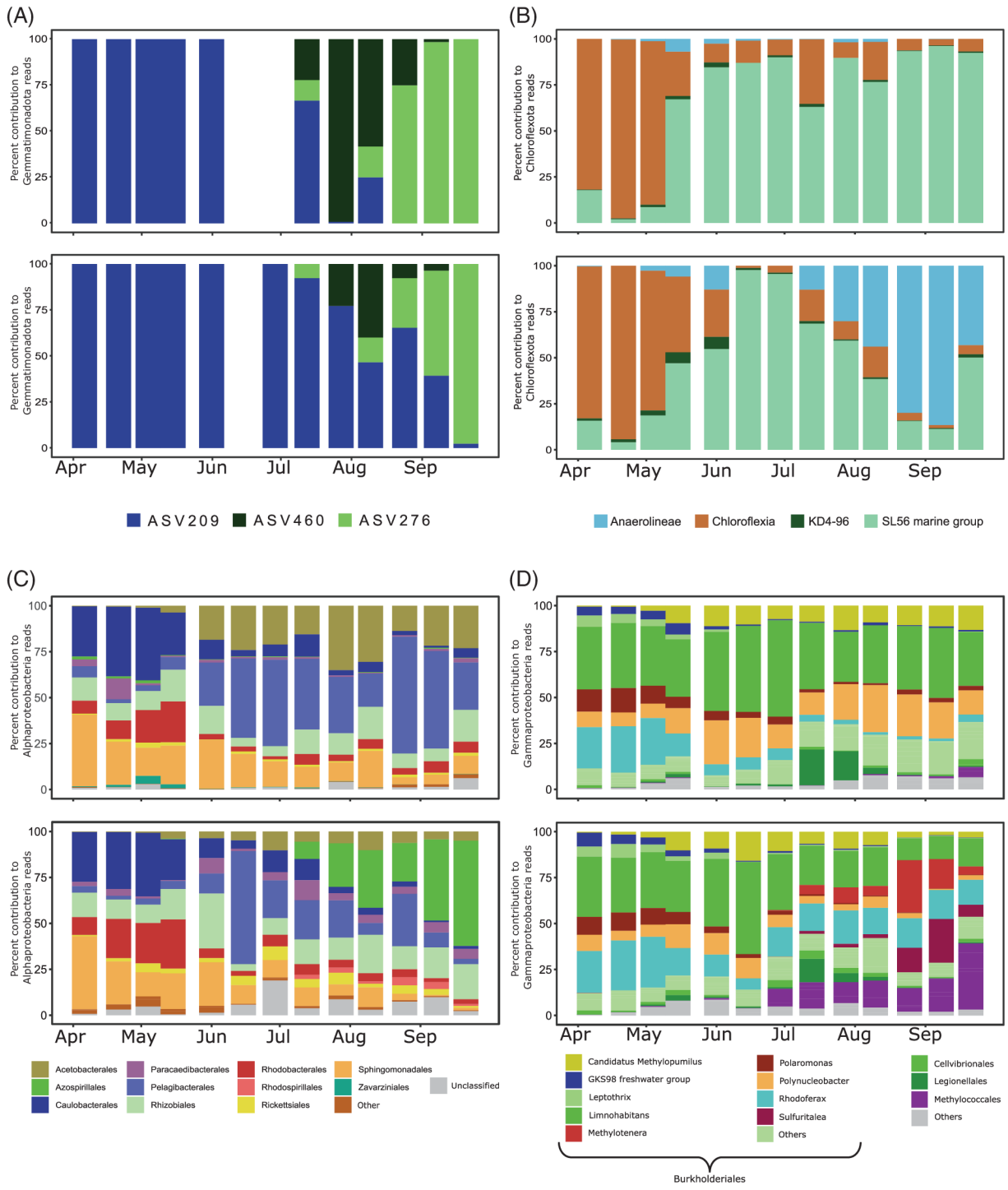

FIGURE 3 Bacterial community composition based on 16S rRNA gene amplicon sequencing in the epilimnion (upper plots in each panel) and the hypolimnion (bottom plots) in Cep lake. Only phyla and classes known to contain AAP species are shown. (A) Percent contribution of ASVs affiliated with Gemmatimonadota to the number of reads coming from Gemmatimonadota; (B) percent contribution of Chloroflexota of classes to the number of reads coming from Chloroflexota, (C) percent contribution of alphaproteobacterial orders to the number of reads coming from Alphaproteobacteria and (D) percent contribution of gammaproteobacterial orders to the number of reads coming from Gammaproteobacteria.

et al., 2017, 2021), dominated in spring, coinciding with the maxim values of AAP bacterial abundance. Pelagibacteriales dominated in the epilimnion and Azospirillales in the hypolimnion from June until the last sampling in September. They have already been reported as a part of microbial communities in a wide variety of freshwater environments (Galachyants et al., 2021; Tsementzi et al., 2019).

Gammaproteobacteria were dominated by the order Burkholderiales (Figure 3D), specifically by genera such as *Limnohabitans*, *Rhodoferax* and *Polynucleobacter*, important members of microbial food webs also known to contain AAP species (Hahn et al., 2012; Jezberová et al., 2017; Kasalický et al., 2018; Salka et al., 2011; Šimek et al., 2005). From the onset of stratification, the Gammaproteobacteria community started to develop differently in the depth layers. The relative abundance of Ca. Methylopumilus and *Polynucleobacter* increased in the epilimnion, while *Methylotenera*, *Sulfuritalea* and order Methylococcales increased in the hypolimnion.

AAP bacteria community composition

The AAP community composition was assessed with amplicon sequencing of the *puf*M gene, which is a routinely used marker for anoxygenic phototrophs containing type-II RC (Koblížek, 2015). The number of *puf*M ASVs (pufM_ASVs) estimated using the Chao1 index and the Shannon diversity index were higher in the hypolimnion than in the epilimnion (Figure 2C, D). Higher bacterial diversity in the hypolimnion seems to be a common feature of freshwater lakes (Shade et al., 2012), and here we demonstrated that this may be also a trend for AAP bacteria, at least in shallow lakes that are oxygenated and illuminated to the bottom. Moreover, AAP community started to diverge between the epi- and the hypolimnion in summer (Figure S2B), as observed also for all bacteria. Nevertheless, the difference in alpha and beta diversity between two layers was less conspicuous for AAP bacteria than for the total bacterial community (Figure 2 and Figure S1).

Gemmatimonadota, Chloroflexota and Proteobacteria were the only phyla detected in our samples, while none of the phototrophic members of Eremiobacteriota and Myxococcota were present in the *puf*M gene libraries (Figure S3B).

The contribution of Gemmatimonadota to the AAP bacteria community was in general below 2%, except in September, when it increased to 9% (Figure S3B). Gemmatimonadota community consisted of three pufM_ASVs (Figure 4A). pufM_ASV69, that clustered in the phylogenetic tree together with the environmental cluster Pg2 (Mujakić et al., 2021) (Figure S4), dominated in spring in the epilimnion and in spring and summer in the hypolimnion (Figure 4A). It was almost replaced by pufM_ASV30 and pufM_ASV209 (Figure S4) that, despite being closely related, showed a distinct seasonal pattern (Figure 4A). Such temporal separation of closely related phylotypes may have resulted from the differences in their physiology, as observed for *G. phototrophica* and *G. groenlandica* (Zeng et al., 2021). Interestingly, the dynamics of Gemmatimonadota by pufM_ASVs resembles that of 16S_ASVs (Figure 3A and Figure 4A). This suggests that pufM_ASV209, pufM_ASV30 and pufM_ASV69 from *puf*M amplicons might correspond to 16S_ASV460, 16S_ASV276 and 16S_ASV209 from 16S rRNA gene amplicons, respectively.

Chloroflexota contributed up to 5% to the *puf*M gene sequences and were more abundant in April and July–August (Figure S3B). They consisted of 5 pufM_ASVs from an uncultured freshwater clade of the Roseiflexaceae family (Mehrshad et al., 2018) that differed only by 1 or 2 amino acids (Figure S5). Despite this high similarity, these Chlorofleoxta ASVs showed distinct dynamics (Figure 4B). Interestingly, the most abundant pufM_ASV18 and pufM_ASV87 were shown to be highly active in August 2016 in the same lake, based on the comparison of DNA and RNA amplicon libraries (Fecskeová et al., 2019). Our results indicate that these Chloroflexota are core members of freshwater AAP bacteria communities over the whole sampling season.

Proteobacteria represented over 90% of all *puf*M reads (Figure S3B). This comprised between 2% and 14% of Alphaproteobacteria, while Gammaproteobacteria were always more than 50%, with a maximum >90% in April (Figure S2B).

Alphaproteobacteria community was similar in both layers in spring, being dominated by Rhodobacterales, Rhizobiales and Caulobacterales (Figure 4C), as observed also for 16S amplicons (Figure 4D). From the onset stratification, Rhodospirillales increased their contribution in the epilimnion, while Sphingomonadales and Rhizobiales showed their maximum contribution in the hypolimnion. Interestingly, the contribution of unclassified reads increased from July, and they reached up to 75% by late September (Figure 4C).

Phototrophic Gammaproteobacteria were more stable compared with other phototrophic phyla, and there was little difference between the epi- and the hypolimnion (Figure 4D). Burkholderiales made up >90% of reads in all samples. *Limnohabitans*, *Polynucleobacter* and *Rhodoferax* were the dominant genera, but 20–25% of the Burkholderiales remained unclassified at genus level. *Limnohabitans* and *Rhodoferax* showed the highest relative abundances in April until June, whereas *Polynucleobacter* showed this in August and September, reaching almost 30% of the Gammaproteobacteria reads in the epilimnion. Phototrophic activity of Burkholderiales may have been relatively low, as they were substantially underrepresented in the active AAP community investigated in August 2016 in the same lake (Fecskeová et al., 2019).

Most of the groups recovered in our time series were also reported in snapshots studies of freshwater lakes (Cepáková et al., 2016; Čuperová et al., 2013; Fecskeová et al., 2019). Here, we show that the composition of AAP community substantially varies in both epi- and hypolimnion, with many groups showing transient occurrence (Figure 4). This clearly indicated that the snapshot studies cannot sufficiently describe their diversity and the season

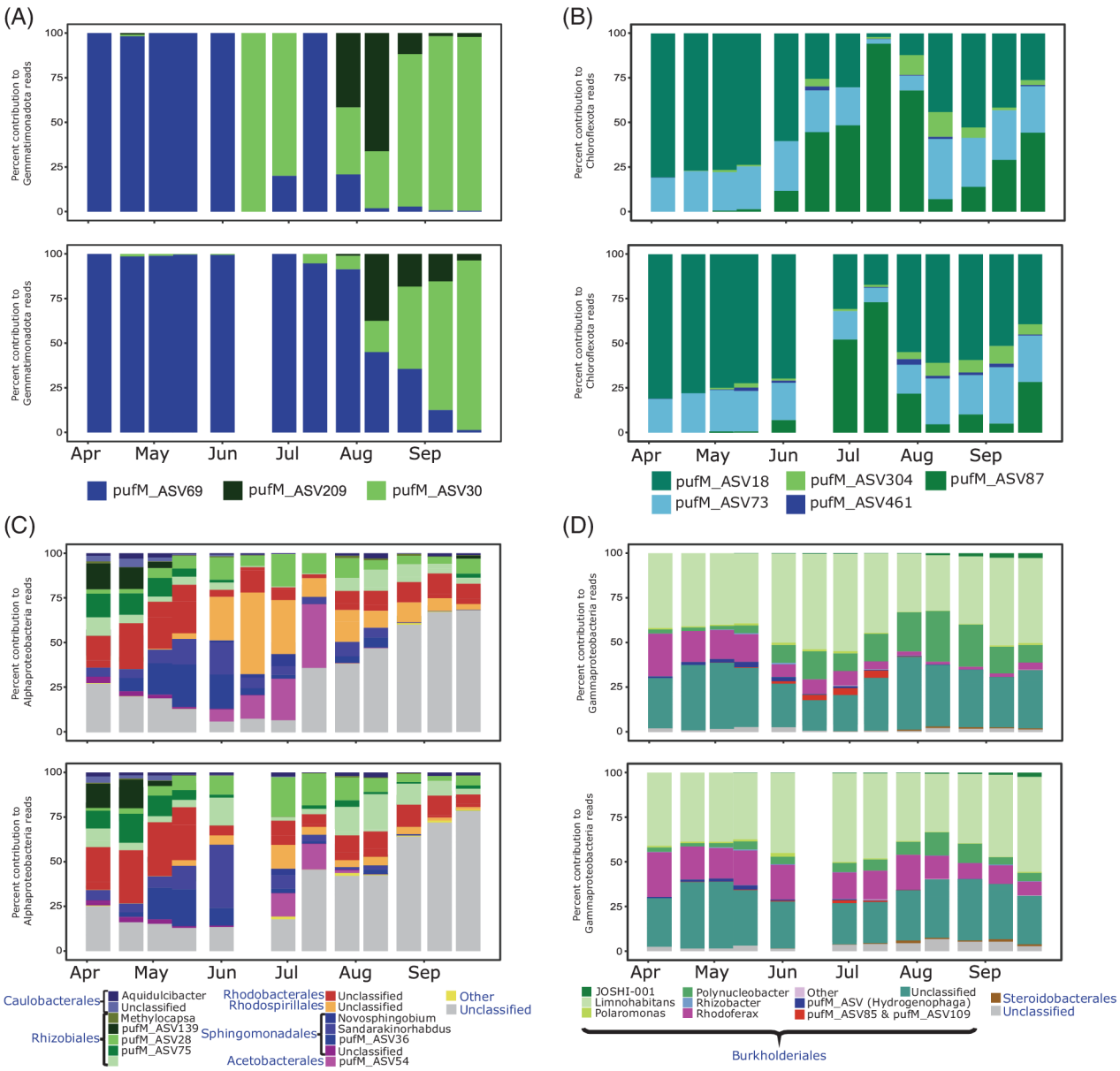

FIGURE 4 AAP bacteria community composition based on *pufM* gene amplicon sequencing in the epilimnion (upper plots in each panel) and the hypolimnion (bottom plots) in Cep lake. (A) Percent contribution of ASVs affiliated with Gemmatimonadota to the number of reads coming from Gemmatimonadota; (B) percent contribution of ASVs affiliated with Chloroflexota to the number of reads coming from Chloroflexota, (C) percent contribution of alphaproteobacterial genera and ASVs to the number of reads coming from Alphaproteobacteria and (D) percent contribution of gammaproteobacterial genera and ASVs to the number of reads coming from Gammaproteobacteria.

of sampling affects the results. As confirmation, a substantial proportion of the reads and pufM_ASVs in our study remained unassigned at genus or order level (Figure 4). This was not just the case for the understudied phyla, such as Chloroflexota and Gemmatimonadota, but also for Proteobacteria (Figure 4C, D), for which diversity in freshwater environments is well described (Ferrera et al., 2017). Furthermore, some pufM_ASVs could not be classified even to the class level. For instance, a group of 20 pufM_ASVs formed a branch between Rhodobacterales (Alphaproteobacteria) and Ectothiorhodospirales (Gammaproteobacteria) (Figure S6). The lack of reference sequences related to the unclassified pufM_ASVs hampered the taxonomic assignation of many of them. These results indicate the high potential of undescribed diversity of AAP bacteria in freshwaters that can be investigated for instance using metagenomics (Mehrshad et al., 2018; Mujakić et al., 2021; Ward et al., 2019).

Relationship with environmental variables

Temporal dynamics of AAP bacteria abundance has been shown to respond to changes in the

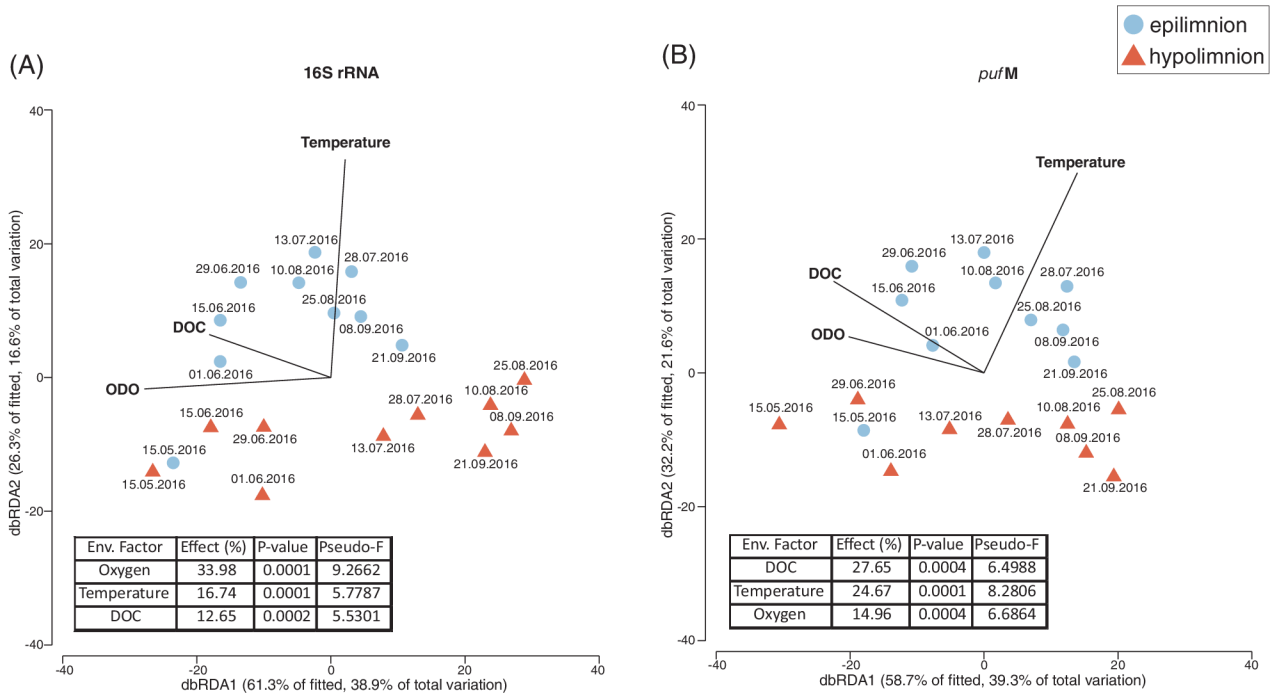

FIGURE 5 Distance-based redundancy analysis biplots relating the observed variability in the composition of communities for (A) all bacteria and for (B) AAP bacteria to the explanatory variables (black lines) in the epilimnion (blue-circles) and hypolimnion (red-triangles). Tables embedded in the graphs show the percentage of variability explained (effect [%]) by individual environmental variables (Env. Factor) and their statistical significance (*p* value and pseudo-F). DOC, dissolved organic carbon; ODO, optical dissolved oxygen.

environmental conditions (Kolářová et al., 2019; Lew et al., 2015; Mašín et al., 2008). However, how they influence the AAP community composition remains mostly unknown. Such information is important as, for example, high photoheterotrophic activity by AAP bacteria was linked to elevated relative abundance of few alphaproteobacterial orders: Caulobacteriales and Sphingomonadales (Piwosz et al., 2022). Thus, we conducted statistical analysis to reveal environmental factors that may have influenced the dynamics of AAP communities.

Distance-based linear models (DistLM) and distance-based redundancy analysis (dbRDA) (Anderson & Legendre, 1999; Legendre & Andersson, 1999) showed that the environmental factors that best explained the variability of the total bacterial community were oxygen, temperature and dissolved organic carbon (DOC) (Figure 5). The variability in the AAP community composition was explained by the same environmental variables but the explanatory power of particular variables differed. Namely, the best explanatory variable was DOC, followed by temperature, whereas oxygen, which explained most of the variability in the total bacterial community was less important for AAP bacteria. These environmental factors have been shown to be determinants in general for shaping bacterial and AAP communities (Ferrera et al., 2017; Mašín et al., 2008; Niño-García et al., 2016). Nevertheless, the observation that this functional group correlates differently with those variables than total bacteria indicates that their response to changing environmental conditions may differ as well.

SUMMARY AND CONCLUSIONS

In this report, we investigated temporal changes in the diversity of bacteria in a freshwater lake, with the focus on AAP bacteria. Our results showed that the AAP community followed different temporal patterns in the epilimnion and the hypolimnion when the lake was stratified. The high number of unclassified reads in the *puf*M libraries indicates that there is still hidden diversity of AAP bacteria within the known bacterial phyla. This was the case not only for less known Gemmatimonadota and Chloroflexota but, surprisingly, also for Alphaproteobacteria, the class to which the first described AAP species belongs (Harashima et al., 1978). Progress in linking this unexpected *puf*M diversity with taxonomic affiliation may be expected with increasing use of metagenomics, targeted single-cell sequencing and culturing of AAP bacteria (Parks et al., 2017; Woyke et al., 2017).

Environmental conditions shape bacterial communities, resulting in temporal changes in their diversity and composition, driven by adaptation that causes physiologic differences between distinct phylotypes. In

contrast to our expectations, the same set of environmental factors explained variability of all and AAP communities but they differed in their importance for total versus functional group. This indicates that AAP bacteria may respond differently to the changing environment, for example to temperature rise and deoxygenation of lakes due to global warming. More information on metabolic capacity of this photoheterotrophic bacteria is needed to fully understand their dynamics.

AUTHOR CONTRIBUTIONS

Cristian Villena-Alemany: analysis of the sequencing data, prepared figures and writing the manuscript; Izabela Mujakić: statistical analysis, prepared the amplicons, prepared a figure and commenting on the manuscript; Petr Porcal: measurement of environmental chemicals and commenting on the manuscript; Michal Koblížek: editing and commenting on the manuscript; Kasia Piwosz: participated in sampling, extracted DNA, supervised analysis of data, editing and commenting on the manuscript.

ACKNOWLEDGEMENTS

Authors thank Jason Dean and Martina Hanusová for help during the sampling campaign, and Alastair T. Gardiner for commenting on the manuscript. This study was supported by project PhotoGemm+ n° 19-28778X from the Czech Science Foundation awarded to Michal Koblížek.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Villena-Alemany, C., Mujakić, I., Porcal, P., Koblížek, M. & Piwosz, K. (2023) Diversity dynamics of aerobic anoxygenic phototrophic bacteria in a freshwater lake. *Environmental Microbiology Reports*, 15(1), 60–71. Available from: https://doi.org/10.1111/1758-2229.13131

Supplementary Figures

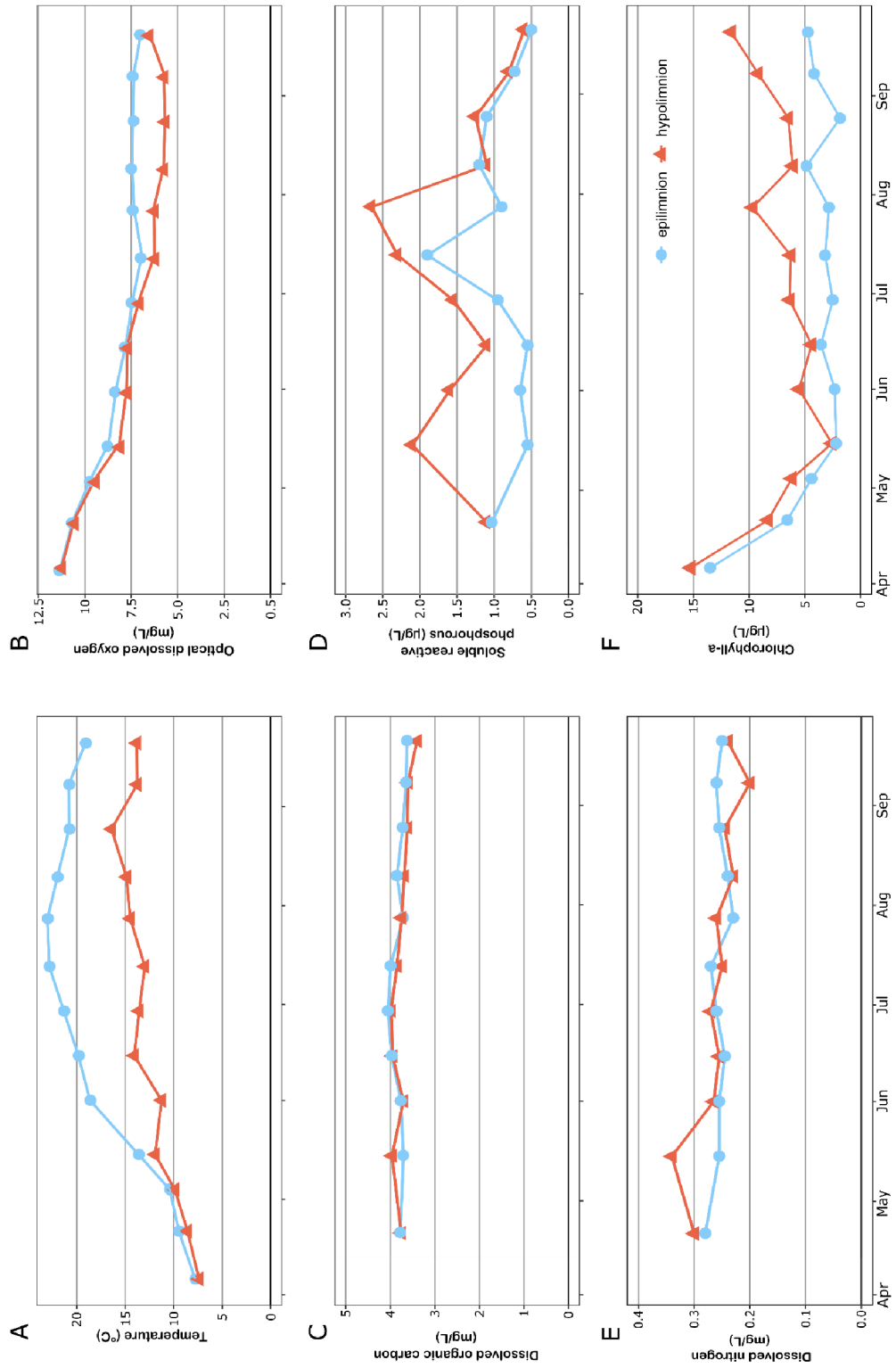

FIGURE S1. Weekly values of environmental variables in epilimnion (blue-circles and line) and hypolimnion (red - triangles and line) of Cep lake. (A) temperature; (B) optical dissolved oxygen; (C) dissolved organic carbon; (D) soluble reactive phosphorus; (E) dissolved nitrogen; and (F) chlorophyll-*a*.

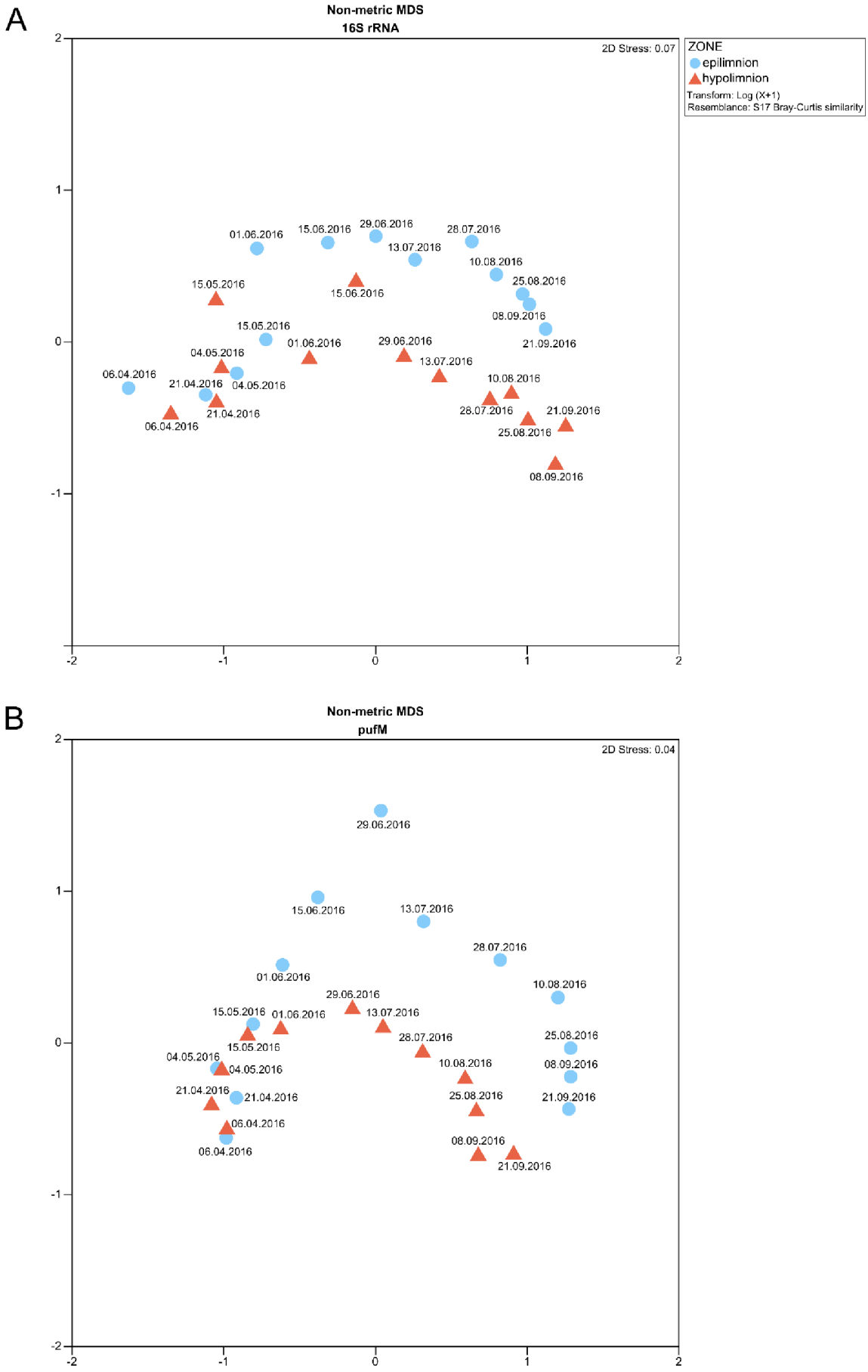

FIGURE S2. Nonmetric multidimensional scaling plots of (A) all bacteria and (B) AAP bacteria based on Bray-Curtis distances.

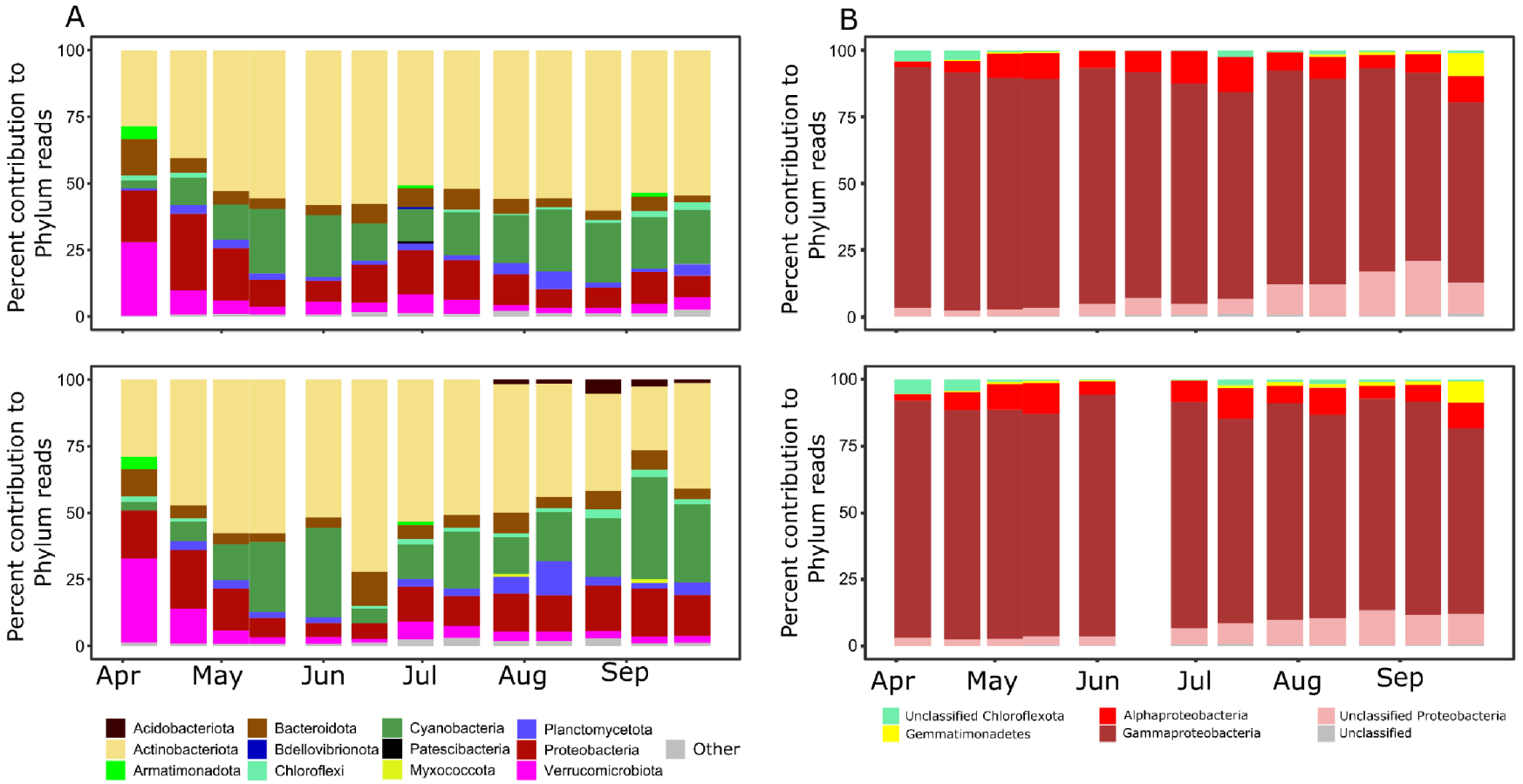

FIGURE S3. Community contribution at phylum level of (A) total bacteria community and (B) AAP bacteria community for epilimnion (top) and hypolimnion (bottom).

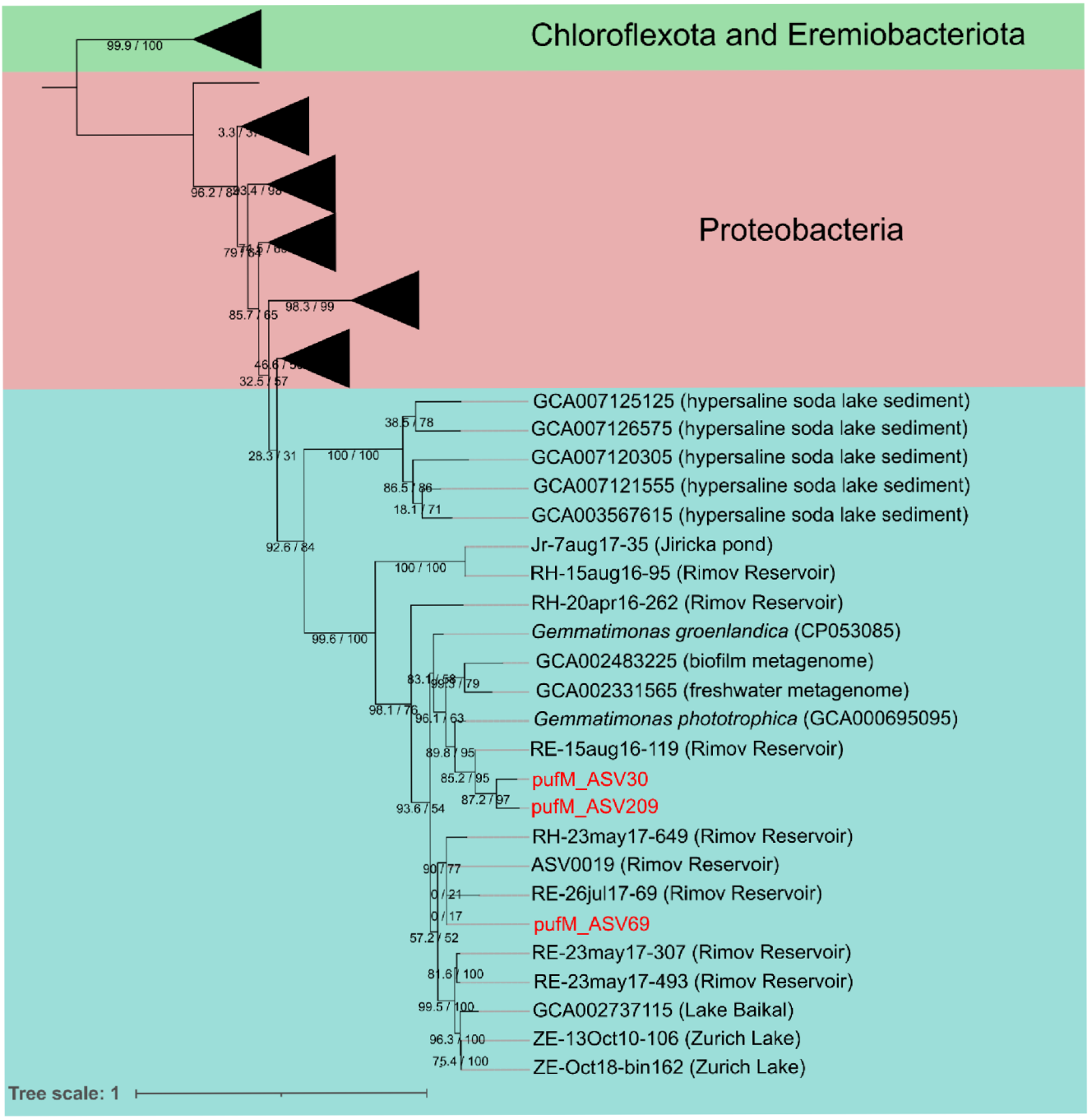

FIGURE S4. Details of Gemmatimonadota sequences distribution in the PufM protein Maximum Likelihood phylogenetic tree computed using LG + F + I + G4 substitution model. The analysis involved pufM amino acid sequences of the reference sequences from Proteobacteria, Chloroflexota, Gemmatimonadota and Eremiobacteriota and the targeted unclassified ASVs.

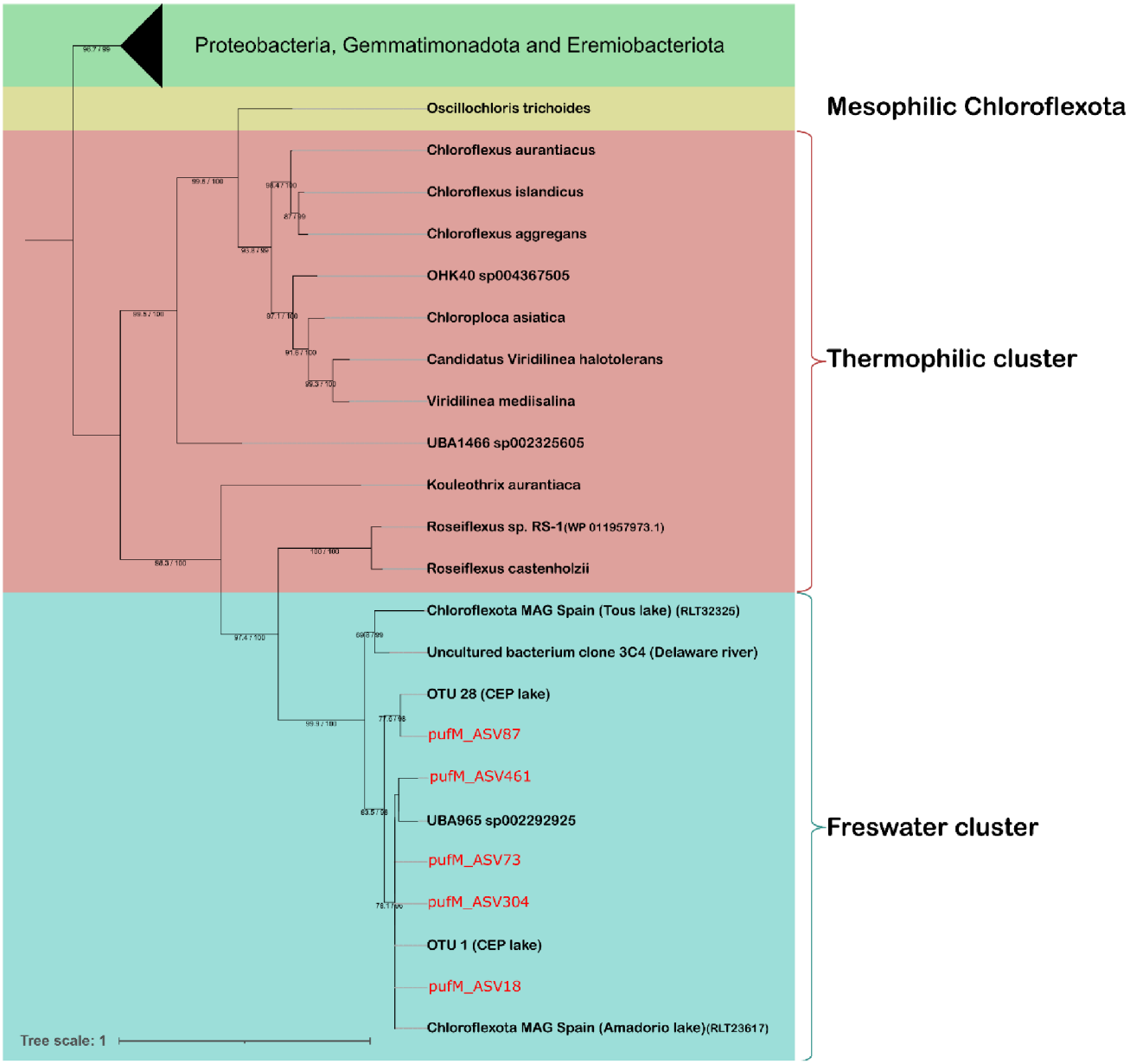

FIGURE S5. Details of Chloroflexota sequences distribution in the PufM protein Maximum Likelihood phylogenetic tree computed using LG + F + I + G4 substitution model. The analysis involved pufM amino acid sequences of the reference sequences from Proteobacteria, Chloroflexota, Gemmatimonadota and Eremiobacteriota and the targeted unclassified.

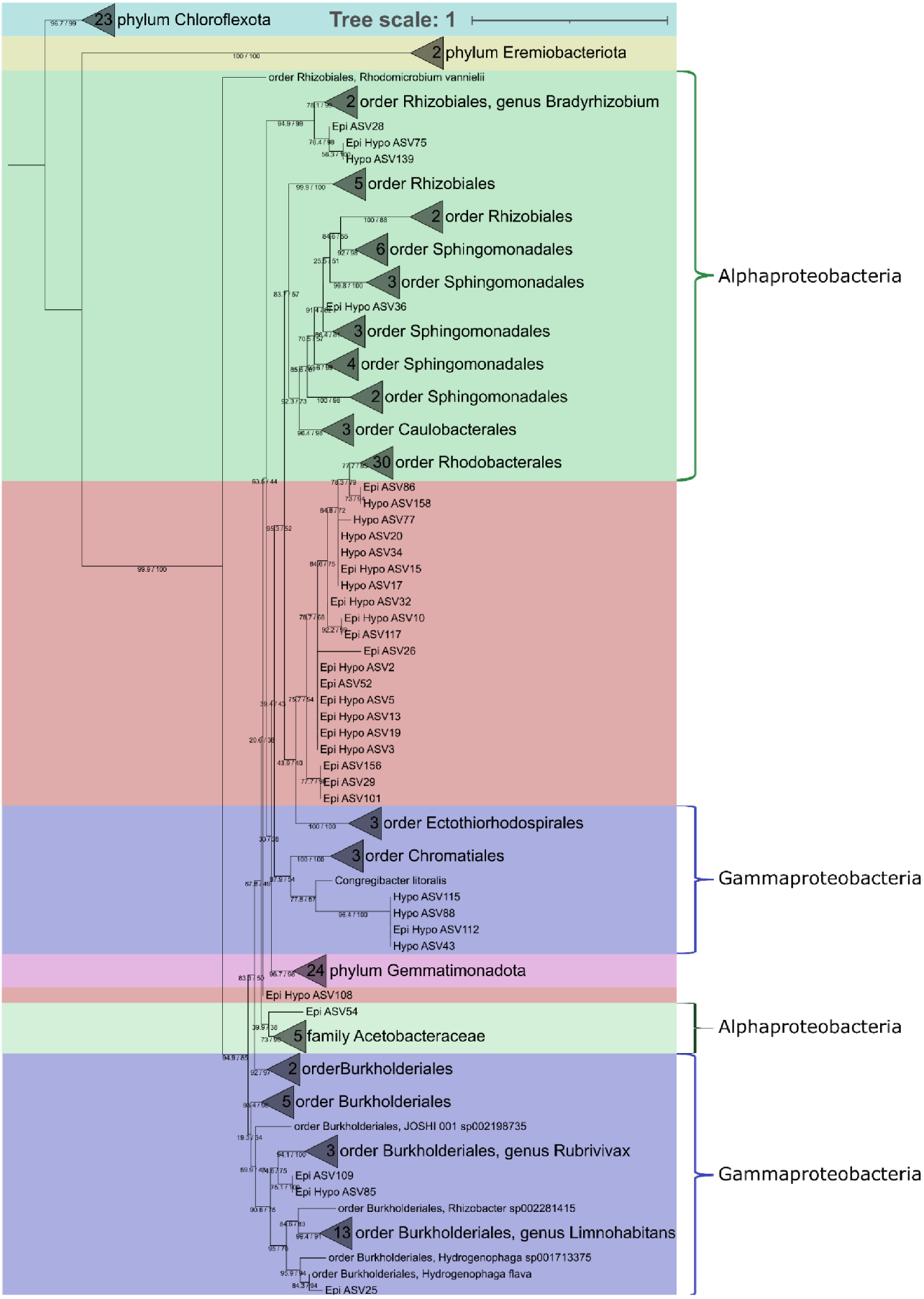

FIGURE S6. Details of Proteobacteria sequences distribution in the PufM protein Maximum Likelihood phylogenetic tree computed using the LG + F + I + G4 substitution model. The analysis involved pufM amino acid sequences of the reference sequences from Proteobacteria, Chloroflexota, Gemmatimonadota and Eremiobacteriota and the targeted unclassified ASVs.

Chapter II

Phenology and ecological role of Aerobic Anoxygenic Phototrophs in fresh waters

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Manuscript under revision in BMC Microbiome Journal
(IF=15.5)

Chapter II: Context and objectives

In the preceding studies, a substantial number of relevant *puf*M gene sequences were identified as taxonomically unclassified, leaving a knowledge gap on the diversity and community composition of AAP bacteria. The low accuracy on the taxonomic classification could be partially attributed to the small amplicon size generated by commonly used primer pairs (UniF-UniR, UniF-pufM_WAW and pufMF-pufM_WAW), producing amplicons shorter than 200 bp. The lower number of variable nucleotide positions in the short amplicon results in fewer amplicon sequence variants (ASVs) artificially reducing diversity and generating ambiguities in the taxonomic assignation that lead to unclassified ASVs. Furthermore, the absence of reference sequences in the database also hampers taxonomic classification of ASVs. For instance, if specific ASV sequence is not present within the reference database, the taxonomic classification will attempt assign it to the most closely related species in the database. In instances where several sequences with different taxonomy in the database closely resemble to the ASV, its taxonomy is resolved as unclassified.

Additionally, it was necessary to understand the seasonal recurrence of the AAP community and its role in aquatic microbial communities. Previous reports indicated that bacterial members within this group present a larger bacterial size and higher activities than the overall bacterial community, peaking in different times of the year and suggesting that AAP bacteria may disproportionately contribute to the upper trophic levels.

The aforementioned known factors and the gaps in our knowledge lead to the formulation of following questions:

- Can we improve the taxonomic classification of the AAP community to reduce unclassified members?

- Does the AAP community display recurrence every year?

- Do AAP abundance patterns differ from those of the overall heterotrophic bacteria?

- What role do AAP bacteria fulfil in aquatic freshwater environments?

Chapter II: Summary

Based on a biweekly 3-year-long sampling of the meso-oligotrophic freshwater Cep lake, the AAP bacterial abundances, their community composition and their responses to the environmental variables were able to be studied. Development of new *puf*M gene primer pair that generate a longer amplicon (450bp) and the construction of the currently largest *puf*M gene database (3363 reference sequences) have substantially improved the taxonomic assignation of *puf*M gene amplicons. Our findings documented the dynamic seasonal changes in AAP community. The annual recurrence of the distinct phylotypes also proves their indigenous freshwater nature. While the majority of AAP bacteria occurred transiently at specific time of the years, a small core of AAP bacteria (22 ASVs, 2% of total ASVs and 4% of average relative abundance) showed a conserved contribution across all depths during whole sampling period. AAP bacteria exhibited a faster response to the spring phytoplankton bloom compared to the overall heterotrophic community, allowing us to contextualize their ecological role in the aquatic PEG model. The high AAP abundance during spring phytoplankton bloom coupled with the significant influence of Chl*a* on AAP community and underscored their crucial role in the recycling of dissolved organic matter released during this bloom. This pointed out their important contribution in the microbial loop, significantly influencing the dynamics of carbon flows in lakes.

Despite of the current progress in disentangling the functional role of AAP bacteria in the microbial functioning of lakes, a direct quantification of the impact of phototrophy in AAP bacteria metabolism was missing. This gap includes understanding how photoheterotrophy alters behaviour of AAP bacteria compared to heterotrophic bacteria.

Phenology and ecological role of Aerobic Anoxygenic Phototrophs in fresh waters

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Short title: Phenology of freshwater AAP bacteria

Key words: fresh waters, aquatic microbial ecology, microbial seasonal succession, long-term sampling, aerobic anoxygenic phototrophs, pufM gene, PEG model, photoheterotrophs.

Abstract

Background

Aerobic anoxygenic phototrophic (AAP) bacteria are heterotrophic bacteria that supply their metabolism with light energy harvested by bacteriochlorophyll-*a*-containing reaction centres. Despite their substantial contribution to bacterial biomass, microbial food webs and carbon cycle, their phenology in freshwater lakes remains unknown. Hence, we investigated seasonal variations of AAP abundance and community composition biweekly across three years in a temperate, meso-oligotrophic freshwater lake.

Results

AAP bacteria displayed a clear seasonal trend with a spring maximum following the bloom of phytoplankton and a secondary maximum in autumn. As the AAP bacteria represent a highly diverse assemblage of species, we followed their seasonal succession using the amplicon sequencing of the *puf*M marker gene. To enhance the accuracy of the taxonomic assignment, we developed new *puf*M primers that generate longer amplicons and compiled the currently largest database of *puf*M gene, comprising 3633 reference sequences spanning all phyla known to contain AAP species. With this novel resource we demonstrated that majority of the species appeared during specific phases of the seasonal cycle, with less than 2% of AAP species detected during the whole year. AAP community presented an indigenous freshwater nature characterized by high resilience and heterogenic adaptations to varying conditions of the freshwater environment.

Conclusions

Our findings highlight the significant contribution of AAP bacteria to the carbon flow and ecological dynamics of lakes and unveil a recurrent and dynamic seasonal succession of the AAP community. By integrating this information with the indicator of primary production (Chlorophyll-*a*) and existing ecological models, we show that AAP bacteria play a pivotal role in the recycling of dissolved organic matter released during spring phytoplankton bloom. We suggest an inclusion of AAP bacterial role into the PEG model and their consideration in further ecological models.

Introduction

Recurrent seasonal changes of aquatic microbial communities are among the best-studied phenomena in freshwater lakes and reservoirs. The Plankton Ecology Group (PEG) model initially described the dynamic interactions between phytoplankton and zooplankton (Sommer et al. 1986) and was later amended with the eutrophic and oligotrophic scenarios and role description of heterotrophic protists (Sommer et al. 2012). Subsequently, the importance of bacterioplankton was revealed, especially during the spring phytoplankton bloom (Zeder et al. 2009; Kavagutti et al. 2023; Park et al. 2023), increasing our understanding of the contribution of microorganisms to the functioning of limnic ecosystems (Reavie et al. 2014). Bacteria represent an important part of aquatic microbial communities. They generate fresh particulate organic matter by utilizing DOC and render it accessible to organisms at upper trophic levels (Pomeroy et al. 2007). However, the role of photoheterotrophic bacteria, which present a significant part of bacterial biomass and activity (Koblížek 2015), remains overlooked.

Aerobic anoxygenic phototrophic (AAP) bacteria are a functional group of photoheterotrophs that rely upon external sources of organic carbon and supplement their metabolism with energy obtained from light through bacteriochlorophyll-*a* (BChl-*a*) type-II reaction centres. This photoheterotrophic life style enables AAP bacteria to reduce their respiration and increase biomass yield in light (Piwosz et al. 2018; Koblížek et al. 2020). Moreover, AAP community show higher growth rates, larger cell sizes and greater activity than heterotrophic bacteria (Mašín et al. 2008; Čuperová et al. 2013; Fauteux et al. 2015; Garcia-Chaves et al. 2016; Ferrera et al. 2017b; Ruiz-González et al. 2020). Photoheterotrophy by AAP bacteria increases carbon transfer efficiency, enlarging the availability of biomass for upper trophic levels and reducing CO_2 emitted to the atmosphere (Piwosz et al. 2022). However, little is known on phenology of AAP community and the absence of exhaustive seasonal sampling hampers the understanding of their role in lakes. AAP bacteria peak during spring in lakes, when they may account for up to 22% of bacteria (Kolářová et al. 2019; Villena-Alemany et al. 2023). Their abundances and diversity dynamics correlate with irradiance, temperature, chlorophyll-*a* (Chl-*a*), oxygen and DOC (Ferrera et al. 2017a; Tomaš et al. 2019; Szabó-Tugyi et al. 2019; Kuzyk et al. 2022; Shi et al. 2022; Villena-Alemany et al. 2023).

One of the obstacles in the study of AAP bacteria is the fact that they do not represent a monophyletic group. On the contrary, phototrophic genes have been gained and lost multiple times in closely related species (Kasalický et al. 2018; Kopejtka et al. 2019). Therefore, AAP species cannot be identified based on the most common marker used in community studies, 16S rRNA gene. Instead, the *puf*M gene, which encodes the subunit M of the anoxygenic type-II reaction centre, has been widely employed to study AAP communities (Béjà et al. 2002; Yutin et al. 2005; Salka et al. 2011; Ferrera et al. 2017b; Tang et al. 2018; Auladell et al. 2019; Fecskeová et al. 2019). However, these studies were unsuccessful in providing a taxonomic assignment for abundant *puf*M phylotypes. This is caused by the low taxonomic resolution of the short amplicon sequences and the lack of a curated reference database. The increased availability of metagenome-assembled and single-cell amplified genomes (MAGs & SAGs) has expanded our knowledge of metabolic potential within multiple bacterial lineages and should allow to establish a comprehensive *puf*M database for amplicon assignment.

To improve the taxonomic assignment, we designed a novel primer set targeting a larger 450 bp region of the *puf*M gene and compiled an extensive database of 3633 non-redundant *puf*M gene sequences from existing genome and metagenome sequence datasets. We applied this novel metabarcoding assay to 215 samples from three years, collected from meso-oligotrophic freshwater Cep lake (Czechia) at biweekly intervals from multiple depths. We hypothesized that the AAP community would show a recurrent seasonal succession, marked by distinct phylotypes reaching abundance peaks under specific environmental conditions. Moreover, we expected that AAP bacteria would exhibit different abundance succession patterns than overall heterotrophic bacteria. Specifically, we surmise that the spring AAP bloom is orchestrated by specific phylotypes, rather than the involvement of the entire AAP community.

Materials and methods

Sampling and measuring environmental variables

Samples were collected biweekly from April 2017 to December 2019 from the freshwater Cep lake (48°92′49.24″N, 14°88′68.11″E). This meso-oligotrophic lake is located in the Třeboň Basin Protected Landscape Area, Czechia, and has an area of 130 ha and a maximum depth of 12 m. Five litres of water were collected from 0.5, 2, 5 and 8 meters using a 3-L Ruttner water sampler (KC Denmark A/S, Denmark) and transported to the laboratory in closed plastic containers in a cooler box, which were pre-rinsed three times with the sampled water. Temperature and oxygen profiles were taken with an EXO1 multi-parameter probe (YSI Inc., Yellow Springs, USA). Total and AAP bacterial abundances were counted using epifluorescence microscopy method as described in Piwosz et al., 2022 (Piwosz et al. 2022). Concentrations of Chl-*a* and BChl-*a* were determined in organic solvent extracts by reversed-phase high-performance liquid chromatography (Fecskeová et al. 2019). The quantification of environmental nutrients was performed as described in Procházková, 1959 (nitrate); Murphy and Riley, 1962 (phosphate); Kopáček and Hejzlar, 1993 (total phosphorous); Kopáček and Procházková, 1993 (ammonia) and Shabarova et al., 2021 (DOC) (Procházková 1959; Murphy and Riley 1962; Kopáček and Hejzlar 1993; Kopáček and Procházková 1993; Shabarova et al. 2021).

*puf*M gene database

We collected 14,872 *puf*M nucleotide and protein sequences from representative genomes and MAGs available from Genome Taxonomy Database (GTDB) r207 (Parks et al. 2022), Tara Ocean (Delmont et al. 2018), the LIMNOS database compiled from a set of ~1300 freshwater lake metagenomes (PRJEB47226), and from MAG collections publicly available in the NCBI (Kavagutti et al. 2019, 2023; Nayfach et al. 2021; Buck et al. 2021; Chiriac et al. 2022; Serra Moncadas et al. 2023). Bacterial genomes and MAGs taxonomy was determined using GTDB-Tk v2.1.1 (Chaumeil et al. 2022). *puf*M sequences in GTDB r207 were found using hidden Markov model of *puf*M gene (K08929) from the KOFAM database (Aramaki et al. 2020) with a score threshold of 394.57, as described in https://github.com/adriaaula/obtain_gene_GTDB. *puf*M-containing non-redundant MAGs from Tara Oceans (https://doi.org/10.6084/m9.figshare.4902923.v1) were selected using HMMER v3.3.2

(http://hmmer.org/) with a customized *puf*M database from pfam (El-Gebali et al. 2019). The *puf*M sequences were confirmed by Diamond v0.9.24 annotation (Buchfink et al. 2021). Nucleotide *puf*M gene sequences from the LIMNOS database were obtained from open reading frames using Prodigal (Hyatt et al. 2010) and annotated using a custom pipeline incorporating Diamond v0.9.24 (Buchfink et al. 2021) and the KEGG database (Kanehisa 2000). *Puf*M genes were compiled alongside the taxonomy of their associated MAG.

All *puf*M sequences were pooled and duplicated sequences were removed. Protein sequences were aligned with MAFFT v7.453 (--maxiterate 1000 --localpair) (Katoh and Standley 2013) and a maximum likelihood tree was calculated using iqtree2 (Minh et al. 2020) with automatic model selection performed by ModelFinder (Kalyaanamoorthy et al. 2017), and 1000 iterations of ultrafast bootstrapping with 1000 rounds of SH-aLRT testing (-alrt 1000 -B 1000) (Hoang et al. 2018). *puf*L sequences were identified as they formed a long branch. Bona-fide *puf*M sequences were retained (Supplementary File S1), and alignment and phylogenetic trees were redone and visualized using iTOL (Letunic and Bork 2021). The environmental origin of each sequence was obtained manually from source databases (Supplementary File S2).

AAP community analysis by *puf*M gene amplicon sequencing

Between 300 and 1460 ml of water was filtered through sterile 0.2 μm Nucleopore Track-Etch Membrane filters (Whatman®, Maidstone, United Kingdom) that were immediately placed inside sterile cryogenic vials (Biologix Group Limited, Jinan, Shandong China) containing 0.55 g of sterile zirconium beads, flash-frozen in liquid nitrogen and stored at −80 °C until DNA extraction (max. six months). Total nucleic acids were chemically extracted according to Griffiths et al. 2000 (Griffiths et al. 2000) with modifications (Nercessian et al. 2005), re-suspended in 35 μl of DNase and RNase-free water (MP Biomedicals, Solon, OH, USA) and stored at −20 °C. Concentration and quality of the extracts were checked using NanoDrop (Thermo Fisher Scientific).

To improve the accuracy of the taxonomic assignation and reduce the number of unclassified amplicon sequence variants (ASVs), a new primer pair for *puf*M gene was designed. pufM_uniF primer (Yutin et al. 2005) was used as a reverse (pufM_UniFRC in the current study, 5´-RAANGGRTTRTARWANARRTTNCC-3') and pufM_longF was designed ~450 bp upstream (5'-

YGGSCCGWTCTAYSTSGG-3') using a pre-existing database of 1500 sequences (Fecskeová et al. 2019). The specificity and coverage of the new primer pair were tested in comparison to the commonly used *puf*M primers (Béjà et al. 2002; Yutin et al. 2005) against the new *puf*M database. The analysis was done in Geneious (v2023.0.1) with up to three mismatches in the binding region and in both forward and reverse directions. The primers' specificity was also tested separately for Pseudomonadota (formerly known as Proteobacteria), Alphaproteobacteria, Gammaproteobacteria, Gemmatimonadota, Chloroflexota, Myxococcota and Eremiobacterota based on alignments done in Geneious by MUSCLE alignment (v5.1.).

The PCR conditions were optimized using genomic DNA from *Gemmatimonas phototrophica* (Gemmatimonadota), *Sphingomonas glacialis* (Alphaproteobacteria) and *Congregibacter litoralis* (Gammaproteobacteria), and environmental DNA from the current sampling. The final conditions were as follows: initial denaturation for 3 min at 98°C, 35 cycles of 98°C for 15 s, 52°C for 30 s, 72°C for 18 s and final elongation at 72°C for 5 min. Triplicate PCR reactions (20 µL) using Phusion™ High-Fidelity PCR MasterMix (Thermo Fisher Scientific, USA) were pooled and the amplicons of ~450 bp were purified from 1.5% agarose (MP Roche, Germany) gel using the Wizzard SV Gel and PCR clean system (Promega, USA) and quantified with Qubit dsDNA HS assay (Thermo Fisher Scientific, USA). Samples were randomly distributed within two runs to account for the batch effect and sequenced on Illumina Miseq 2 x 300 bp PE (Macrogen, South Korea).

Raw reads were quality-checked using FastQC v0.11.7 (Babraham Bioinformatics, Cambridge, UK). The primer sequences were trimmed and read quality filtered using Cutadapt v1.16 maximum error (-e 0.1), quality cut-off (-q 20) and minimum length (-m 250) (Martin 2011). Reads were truncated using *filterAndTrim* (truncLen = c(220, 220), maxEE=c(2,5), truncQ=2) in the R/Bioconductor environment from DADA2 package v1.12.1 (Callahan et al. 2016). ASVs were constructed and chimeric sequences removed using the method "consensus". ASVs present only in one of the runs were removed from downstream analysis using *intersect* and *subset*. Subsequently, ASVs were aligned in Geneious v2019.2.3 using ClustalW v2.1 (Larkin et al. 2007). Poorly aligned ASVs were confirmed to not be *puf*M with a blast against NCBI non-redundant database (Sayers et al. 2022) and excluded from further analysis. The final dataset consisted of 1588 ASVs (Supplementary File S3, Reference ASV sheet) and 62,729 ± 13,448 reads per sample (mean ± standard deviation, File S3, ASV_table sheet). The

sequences were deposited in the NCBI database under Biosamples SAMN38037304 - SAMN38037518 as a part of BioProject PRJNA970655.

The taxonomic assignment was done through phylogenetic placement using The Evolutionary Placement Algorithm v0.3.5 (Barbera et al. 2019) that placed the ASVs into the phylogenetic tree calculated from the new reference database sequences that were back-translated from protein alignments using trimAl (Capella-Gutiérrez et al. 2009). The taxonomic assignation was handled according to the ASV phylogenetic position using Gappa (Czech et al. 2020) (Supplementary File S3, Taxonomy sheet).

Phytoplankton community analysis based on 16S rRNA gene amplicons

The V3-V4 region of the bacterial 16S rRNA gene was amplified using 341F and 785R primer pair (Klindworth et al. 2013) as described in Piwosz et al., 2022 (Piwosz et al. 2022) The subset of sequences assigned to Chloroplast was extracted and their taxonomy was further affiliated using a curated reference database of the plastidial 16S rRNA gene: PhytoRef (Decelle et al. 2015). Bar plots were visualized using ggplot v3.4.3 (Wickham 2009).

Data and statistical analysis

Unless stated otherwise, all analyses were done in R studio v3.6.1 and were visualized using ggplot2 v3.3.6 (Wickham 2009). Dynamics of environmental and biological variables were interpolated using igraph v1.2.6 and lubridate v1.8.0 (Csardi and Nepusz 2006; Grolemund and Wickham 2011). For addressing the compositional bias of amplicon data (Gloor et al. 2017), principal component analysis was done using centred log ratio (CLR) transformation (Quinn et al. 2019) through *transform* from microbiome package v1.17.42. Community composition bar plots and Alphaproteobacteria, Gammaproteobacteria and Gemmatimonadota bubble plots were done using Phyloseq v1.30.0 (McMurdie and Holmes 2013). The 100 most abundant ASVs were selected and plotted using *plot_heatmap* (McMurdie and Holmes 2013). The occurrence of specific ASVs in spring was tested using analysis of compositions of microbiomes with bias correction in ANCOMBC v2.3.2 (Costea et al. 2014; Lin and Peddada 2020) and plotted using ggplot2 v3.4.3 (Wickham 2009).

Relationships between environmental data and AAP community were analysed using distance-based linear models (DistML) (Legendre and Andersson 1999; Anderson and Legendre 1999) in the PERMANOVA+ add-on package of the PRIMER7 software (Anderson et al. 2008) (Primer Ltd., Lutton, UK). From strongly correlated environmental variables (correlation coefficient > 0.6) only one was selected for further analysis. The model was calculated on the CLR-transformed relative abundance data of AAP bacteria (Quinn et al. 2019), using a stepwise selection procedure. The best model was selected based on statistical significance (9999 permutations) and the value of Akaike's Information Criterion (AICc).

AAP core and network community analyses

ASVs present in more than 80% of the samples from the 3 years and four depths, were considered the AAP lake core microbiome. The percent contribution of each core ASV to their respective maximum percent contribution was calculated and plotted with bubble plots using Phyloseq v1.30.0 (McMurdie and Holmes 2013) and ggplot2 v3.3.6 (Wickham 2009).

SparCC analysis was applied to calculate lake community co-occurrence correlations from the compositional data (Friedman and Alm 2012). Only correlations with pseudo p-value < 0.02 and stronger correlation than ± 0.7 were selected. The network was plotted using Cytoscape v3.9.1 (Shannon et al. 2003).

Time series and trend lines

Interannual trend analysis was done in TTR package v0.24.3 (Ulrich 2022). Raw data on total and AAP bacterial abundances, temperature and Chl-*a* concentrations were averaged for months and depths and transformed into time series assuming annual frequency. They were decomposed into trend, seasonal and random components using *decompose* with default settings. Spearman correlation between interannual trends of AAPs abundance and temperature and Chl-*a* concentrations was done for extracted trend component.

Results

New database and longer amplicons enhance taxonomic assignments of *puf*M ASVs

In order to improve the taxonomic assignment of the *puf*M gene amplicons, we constructed a new reference database containing 3633 *puf*M sequences (> 646 bp) from Pseudomonadota (synonym for Proteobacteria), Gemmatimonadota, Chloroflexota, Eremiobacteriota and Myxococcota (Supplementary File S1). The database includes 529 genera, 114 families, 53 orders, and 9 classes (Supplementary Figure S1) from cultured species and MAGs originating from a wide variety of habitats (Supplementary File S2), mostly from freshwater (2140 sequences) and marine environments (381 sequences).

The newly designed primer set covers 80.5% of the new database at maximum of three mismatches (Supplementary File S4). The older, highly degenerated primer pairs, UniF + UniR, pufMF + pufMR and UniF + pufM_WAW, cover 98.9%, 85% and 96.2%, respectively. However, the amplicon length of the novel primers is about two times longer (~450 bp) allowing for proper taxonomic assignation of more than 95% of the alphaproteobacterial and above 75% of the gammaproteobacterial reads at order level. Additionally, 38 alphaproteobacterial, 36 gammaproteobacterial and 6 Gemmatimonadota genera were detected (Supplementary Figures S2, S3 and S4).

Seasonal changes in Cep lake

Environmental conditions in Cep lake showed seasonal dynamics typical for a temperate freshwater lake (Supplementary File S5). In January and February, the lake was partially frozen and stratified from April/May until September with maximum temperatures >24°C in July and August. The metalimnion was located between 5-8 meter-depth in 2017, and between 2-5-meters in 2018 and 2019. In all three years, the autumnal mixing, characterized by higher values of dissolved oxygen and lower temperatures, was initiated in October (Supplementary Figure S5A-B).

Chlorophyll-*a* measurements varied throughout the year with seasonal maxima representing spring and autumn phytoplankton blooms (Supplementary Figure S5C). The spring phytoplankton bloom

terminated at the onset of stratification, and was composed, according to 16S amplicons affiliated to plastids, mostly by Bacillariophyta and Chrysophyceae (Supplementary Figure S6).

Seasonal dynamics of AAP community composition

The maximal AAP abundances (3.42 - 5.50 × 10^5 cells mL^{-1}) corresponded to 15-20% of the total bacteria (Supplementary Figure S5D-E) and closely proceeded the spring phytoplankton blooms. A second AAP bacterial peak occurred towards the end of summer, before the autumn phytoplankton peak. Alpha diversity of AAP community was lower during the spring abundance peaks and rose during the second part of the year (Supplementary Figure S5F).

The AAP community followed an annual recurrent pattern during the three consecutive years, with a distinction between the epilimnion and hypolimnion communities during the stratified period (Figure 1A). Samples from the same season in different years were more similar to each other than samples from different seasons in the same year, indicating the persistent temporal succession of the community and similar interannual community structure. Distance-based linear models (DistLM) and distance-based redundancy analysis (dbRDA) selected temperature, Chl-*a*, and total, Cyanobacterial, and AAP abundances, to best explain the variability (23.11%) of the AAP community composition (Supplementary File S6). For 2018 and 2019, two years for which nutrient data is available (Supplementary File S5), phosphorous and ammonia increased this explanation by 6.75%, up to 29.86%.

Interestingly, in addition to a seasonal cycle, we observed an interannual variation in AAP community composition (Figure 1B). The decomposition of time series on monthly averaged values for the whole water column showed increasing interannual trends in temperature and AAP abundance during 3 years of sampling, and a decreasing trend in Chl-*a* concentration (Supplementary Figure S7). Trends of temperature and AAPs abundance were significantly correlated (Spearman correlation coefficient rho = 0.8, p-value < 0.0001).

The AAP community was dominated by Gammaproteobacteria over the whole water column, with an average relative contribution exceeding 50% and reaching up to 90% during

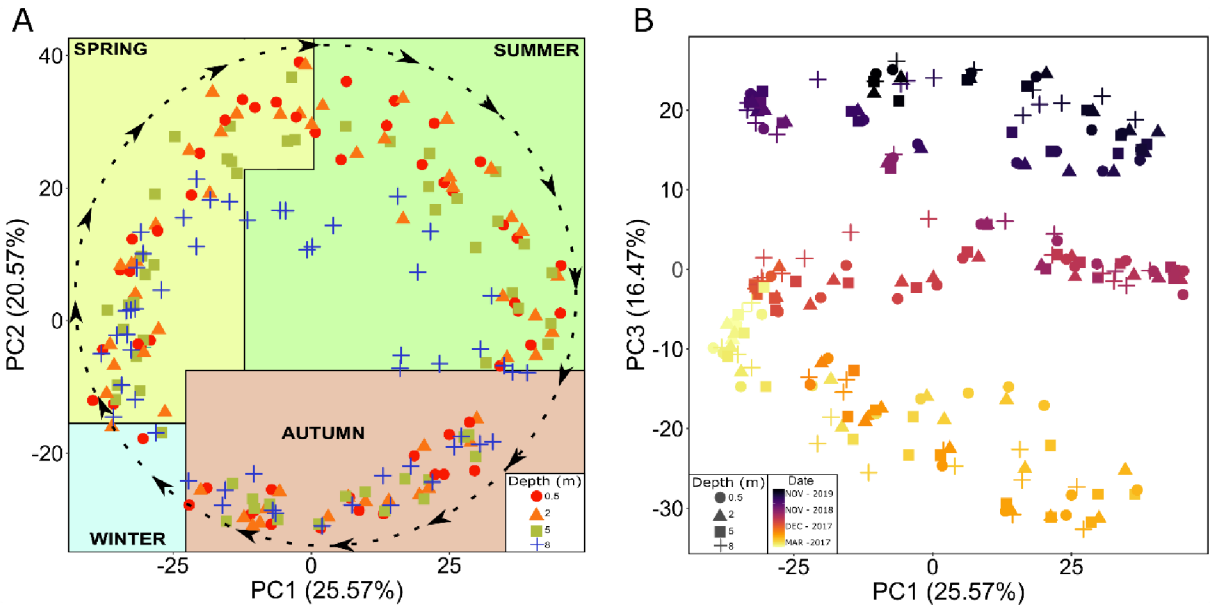


Figure 1: Development of AAP community structure. Principal component analysis of centred log-ratio transformed AAP community composition. Each point represents a sample with 0.5 m (red circle), 2 m (orange triangle), 5 m (green square) and 8 m (blue cross) in PC1 and PC2 axis (A) and 0.5 m (circle), 2 m (triangle), 5 m (square) and 8 m (cross) coloured according to the date of sampling in PC1 and PC3 axis (B). Dashed line and arrows in panel A indicate AAP community succession following an annual chronological direction.

stratification (Supplementary Figure S8). Alphaproteobacteria was the second most abundant class, showing the maxima contributions in spring and autumn, reaching over 50% in the spring of 2018. Classes Gemmatimonadetes (Gemmatimonadota) and Myxococcia (Myxococcota) made up 5% and 2% of the AAP community, respectively. Unclassified Pseudomonadota and unclassified Myxococcota showed transient contributions of <9% and <1% of the AAP community, respectively. Chloroflexota and Eremiobacteriota were not detected.

The 100 most abundant ASVs (based on their average relative abundances) comprised 75 % of the reads and exhibited seasonal recurrence, peaking every year at specific times of the year (Figure 2). The majority of ASVs demonstrated a transient contribution and were generally absent outside their maxima (e. g. ASV28 (Rhizobiales)), while a few showed pronounced relative abundance throughout the year (e. g. ASV4 (*Rhodoferax*)). Interestingly, ASV67 (*Limnohabitans*), whose relative abundance was on average <0.36%, was the sole ASV detected in every sample.

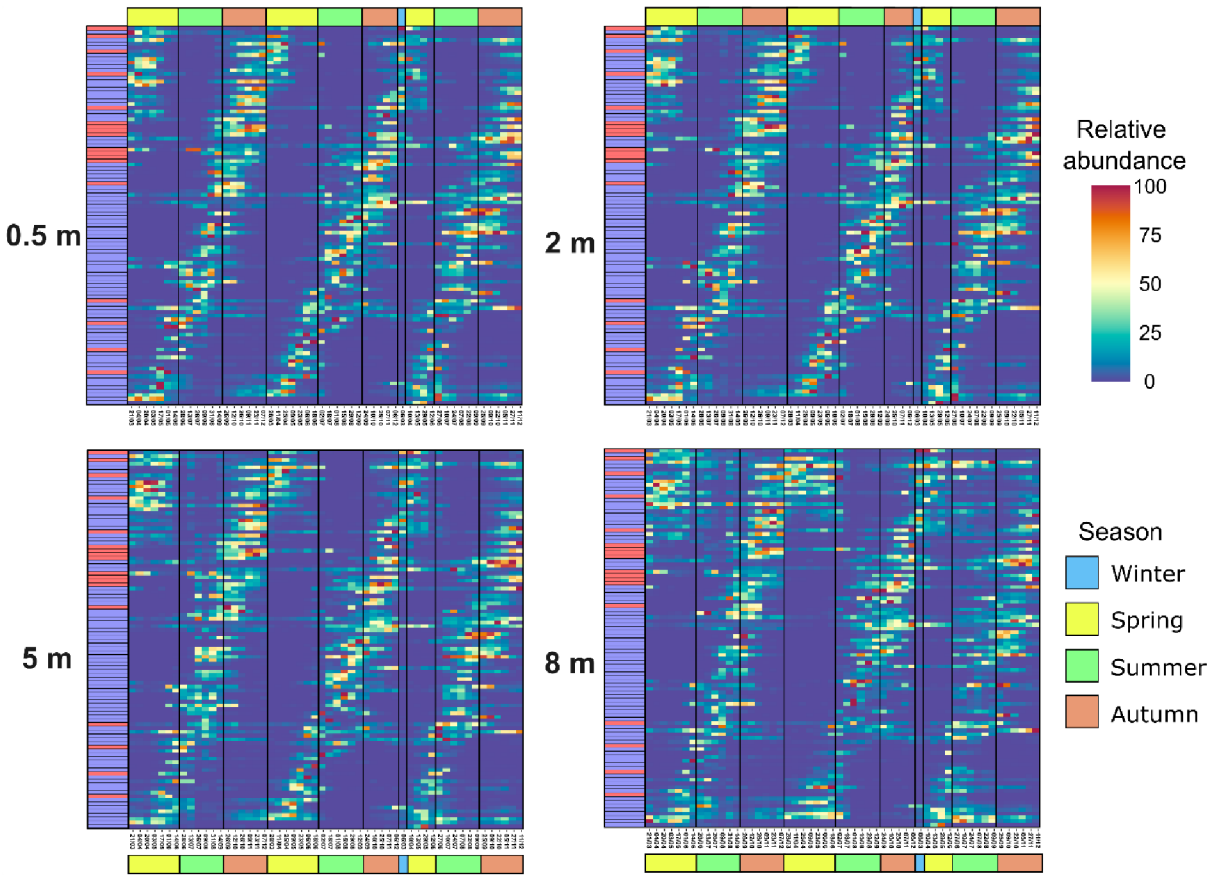


Figure 2. Recurrence of 100 most abundant ASVs. Relative abundance is individually normalised in 0.5 m, 2 m, 5 m and 8 m during the different seasons of the 3-year sampling. Coloured box on the left indicate the taxonomic assignation at class level of each ASV, red for Alphaproteobacteria and blue for Gammaproteobacteria. Colour bar on the top and bottom indicate the seasons of the year.

Out of 1588 *puf*M ASVs (Supplementary File S3, Reference ASV sheet), the stable part of the AAP community (defined here as ASVs present in >80% of the samples) consisted of only 22 ASVs (Supplementary Figure S9): 8 *Rhodoferax*, 4 *Limnohabitans*, 2 *Aestuariivirga*, 1 *Methylobacterium*, 1 *Rubrivivax* and 6 other Burkholderiales. These core ASVs varied largely in their contribution from the most abundant ASV2 (*Aestuariivirga*, with an average relative abundance of 4%) to the least abundant ASV237 (unclassified Burkholderiales, with an average relative abundance of 0.06%). Their seasonal dynamics differed substantially throughout the year and distinct relative abundance patterns were observed even for ASVs from the same genus. For instance, ASV5 and ASV49, both *Rhodoferax*, peaked in autumn and spring, respectively. Similar differences were observed for two *Aestuariivirga*: ASV2 peaked during the spring mixing period (from March to May), while ASV62 showed its highest contribution during the summer stratification. It is noticeable that the core AAP community also included ASVs outside the 100 most abundant, such as ASV115 (*Rhodoferax*) and ASV237 (unclassified

Burkholderiales), which had a low but steady contribution during the whole sampling season. Furthermore, dynamics of some core ASVs, such as ASV31, were different each year.

To identify phylotypes most contributing to the difference in AAP community composition during spring and autumn peaks, we carried out an analysis of compositions of microbiomes with bias correction (Supplementary File S7). Composition of genera and orders contributing to the AAP bacterial peaks was different (Figure 3). Spring peak consisted of a higher

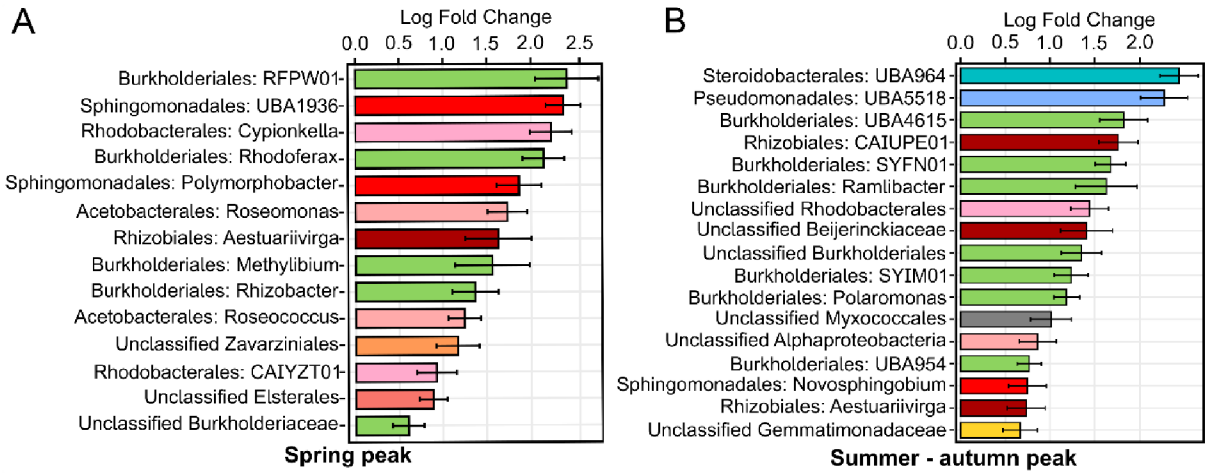


Figure 3. Community composition of AAP abundance peaks. Analysis of compositions of microbiomes showing log fold change values at order and genus level in Spring (A) and in Summer-Autumn peaks (B) with green colour scale for Burkholderiales, blue colour scale for other Gammaproteobacteria, red colour scale for Alphaproteobacteria, yellow for Gemmatimonadota and grey for Myxococcota orders.

prevalence of Alphaproteobacteria versus Gammaproteobacteria genera (9 vs 5), while during autumn, the community was more diverse and included also Gemmatimonadota and Myxococcota. The highest genera contributors to the spring peak (log fold change > 2) were *RFPW01*, *UBA1936*, *Cypionkella* and *Rhodoferax*, while *UBA964* and *UBA5518* (Gammaproteobacteria: Steroidobacterales and Pseudomonadales, respectively) contributed most to the late summer peak. The only genera substantially contributing in both peaks was *Aestuariivirga*.

Network analysis

To study possible interactions between AAP bacteria, we performed a network co-occurrence analysis. The network calculated for the entire lake included 99 ASVs and 139 interactions (92% positive;

Figure 4). Thirteen highly connected nodes with more than six co-occurrence

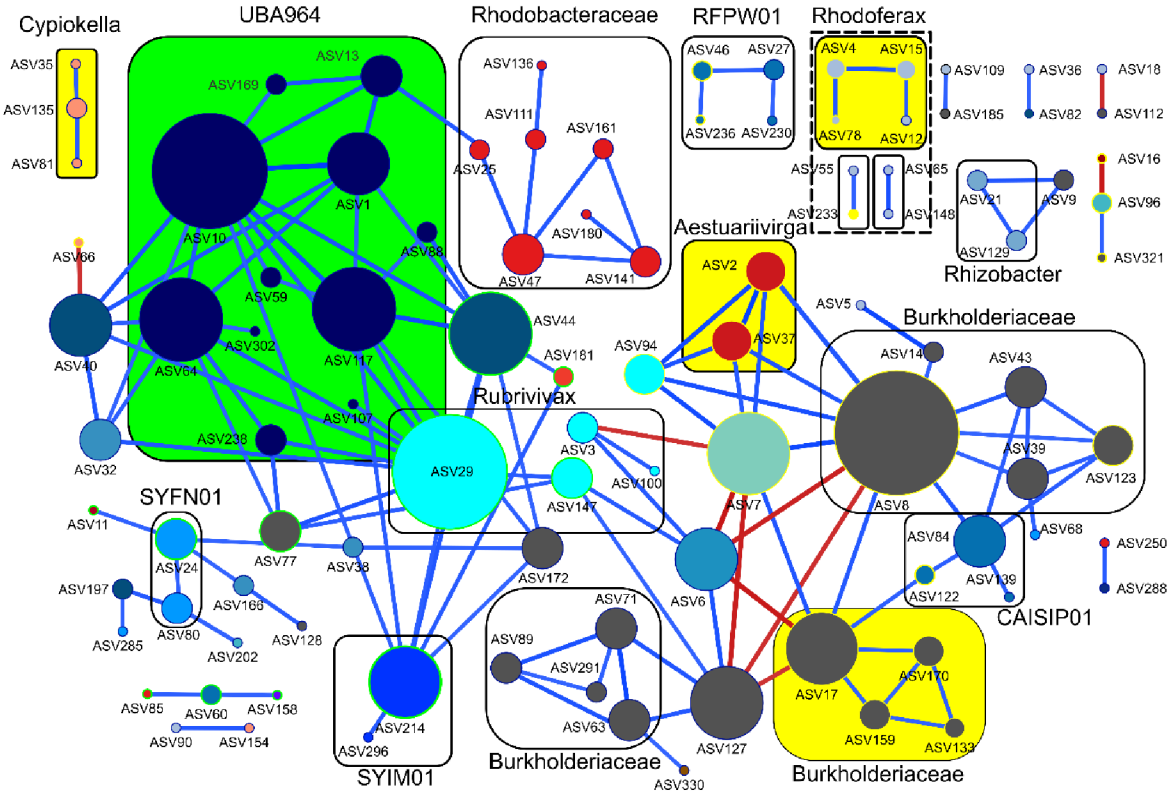


Figure 4. **AAP community co-occurrence correlation network.** The radius of the nodes (ASVs) are directly proportional to the number of significant correlations. Colour of the nodes shows the taxonomy at class level with Alphaproteobacteria in red-orange colour scale, Gammaproteobacteria blue colour scale, unclassified Burkholderiaceae (grey) and Gemmatimonadota (brown). Blue lines indicate positive and red lines negative correlations. Rectangles with solid lines indicate nodes that interact with other nodes from the same taxonomic rank (genus or family) and positive log fold change for Spring AAP peak are highlighted in yellow rectangles fill and yellow node stroke while green rectangles fill and green node stroke for Autumn AAP peak.

correlations were identified as hubs. They belonged to genus UBA964, Steroidobacterales, *Rubrivivax* and unclassified Burkholderiaceae from Gammaproteobacteria. The most connected nodes from Alphaproteobacteria belonged to *Aestuariivirga* and Rhodobacteraceae (4 edges each). The majority of correlations (~60%) occurred between ASVs from the same genus, family or order, creating groups of densely connected nodes (e.g. genus UBA964). Furthermore, some ASVs peaking in spring correlated positively with each other (*Aestuariviirga* and unclassified Burkholderiaceae) in contrast to *Rhodoferax* and *Cypionkella*. A similar pattern was observed for ASVs peaking in summer-autumn.

Discussion

Seasonal succession of planktonic communities in temperate lakes has been intensively studied (Sommer et al. 2012). The PEG model defines the key phases in the annual development of ecological succession as well as the interactions between different organisms in aquatic ecosystems. The annually recurrent phenomena in freshwater lakes include a spring phytoplankton bloom followed by a zooplankton-induced clear-water phase in early summer, a late-summer phytoplankton bloom and a period of low productivity in winter. Recently, seasonal dynamics of heterotrophic bacteria have been incorporated into the model (Park et al. 2023), as they rapidly respond to the transitions in the lakes' pelagic functioning, especially during the phytoplankton bloom (Šimek et al. 2014; Kavagutti et al. 2023; Park et al. 2023). Whilst AAP bacteria have been shown to substantially contribute to the bacterial abundance, biomass and activity in freshwater lakes (Fauteux et al. 2015; Piwosz et al. 2022), they are not considered in the PEG model (Sommer et al. 1986, 2012).

Dynamics of microbial communities are typically investigated by sequencing of the 16S rRNA gene and such analyses do not allow for disentangling the metabolic functionality of bacteria, especially when traits of interest follow a heterogenic pattern of presence within the same taxonomic ranks. This is the case for AAP bacteria, where members from the same genus might or might not contain the ability to carry out anoxygenic photosynthesis (Kasalický et al. 2018). Amplicon analysis of a functional gene may overcome this hindrance, but it requires a comprehensive database with taxonomically assigned reference sequences. Currently, some phylogenetic clades (A-L) cannot be assigned to the genus or even order level (Yutin et al. 2007; Lehours et al. 2018). Moreover, different researchers assemble databases for their environment of interest, with different quality thresholds and criteria (Piwosz et al. 2022; Gazulla et al. 2023), which hampers direct comparison between different studies. Finally, short amplicons obtained with the most commonly used primer combinations, puf_UniF - puf_UniR, puf_UniF – pufM_WAW and pufMF – pufM_WAW (Béjà et al. 2002; Yutin et al. 2005) often do not allow for taxonomic assignment below the class or order level, resulting in a substantial number of unclassified reads (Tang et al. 2018; Galachyants et al. 2021; Villena-Alemany et al. 2023; Gazulla et al. 2023).

Comprehensive database and longer amplicons allow for improved taxonomic assignments of *puf*M ASVs

We constructed the largest curated *puf*M database to date that includes sequences from essentially all environments, with especial high representation of freshwater lakes (Supplementary Figure S1, Supplementary File S1 and S2). The key improvement is that we included only sequences originating from taxonomically assigned genomes and MAGs, excluding all environmental *puf*M sequences classified into phylogroups based on phylogenetic analysis (Yutin et al. 2007). The reason for this exclusion was that phylogenetic trees based on *puf*M gene do not match 16S rRNA phylogeny due to horizontal gene transfer events (Zeng et al. 2014; Kopejtka et al. 2017; Ward et al. 2018). In contrast, taxonomic assignment of genomes and MAGs based on whole genome or 120 selected marker genes is more accurate and consistent (Chaumeil et al. 2019). During our quality control, sequences originating from Bdellovibrionota, Verrucomicrobiota, Omnitrophota, Planctomycetota or Bacteroidota were excluded from the final database. Some members of these phyla were reported to encode *puf*M (Galachyants et al. 2021). However, our manual inspection revealed that their *puf*M gene was present in short contigs, and often found as the only phototrophic gene, which does not warrant phototrophic functionality. Furthermore, *puf*M genes from these phyla did not form a monophyletic clade, suggesting dubious multiple and independent events of horizontal gene transfer. Thus, only members of Pseudomonadota, Chloroflexota, Gemmatimonadota, Eremiobacteriota and Myxococcota were included. Our choice of sequences ensures high quality and allows for future extensions of the database as more metagenomic data is produced and more AAP bacteria are cultured, enhancing its fidelity and functionality. Moreover, as our database contains entire or almost entire *puf*M gene sequences, it can be used for amplicon taxonomy assignment independently of the actual primers used.

The *puf*M gene is one of the most conserved genes from the *puf* operon which codifies the genes for synthesis of the anoxygenic photosynthesis apparatus (Nagashima and Nagashima 2013; Imhoff et al. 2018). Commonly used puf_UniF - puf_UniR, puf_UniF – pufM_WAW and pufMF – pufM_WAW primer pairs (Béjà et al. 2002; Yutin et al. 2005) hybridize on the most conserved regions at the end of the gene, separated by ~110 - 160 bp. They show high coverages (Supplementary File S4) but produce short amplicons that hamper taxonomic assignation below the class or order level resulting in a high

fraction of unclassified reads (Tang et al. 2018; Galachyants et al. 2021; Villena-Alemany et al. 2023; Gazulla et al. 2023). Thus, we designed a new primer set producing longer amplicons to increase the taxonomic resolution, as has been shown for other genes (Jeong et al. 2021). The new primer pair has lower *in silico* coverage against our new database than puf_UniF - puf_UniR (Yutin et al. 2005) (80.5 vs 98.9 %). Some groups, such as Chloroflexota, *Aquidulcibacter* and *Polynucleobacter*, were poorly covered, which may explain their absence in our amplicons. Nevertheless, the number of sequences identified at every taxonomic level increased compared to a previous study in the same lake (Villena-Alemany et al. 2023): 95% of the alphaproteobacterial and above 75% of gammaproteobacterial reads were classified at the order level. Additionally, the number of newly detected genera was substantially higher (80 vs 12) and the Shannon index showed a wider range of diversity (Supplementary Figure S5F), since longer amplicons enable to detect more nucleotide variations, and thus reveal higher diversity, advancing our knowledge on AAP community composition.

Phenology of AAP bacteria and consideration into the PEG model

The seasonal succession of the AAP community revealed differential strategies of adaptation to environmental conditions, unveiling generalist AAP bacteria appearing most of the time, whereas specialists or opportunists showed a transient contribution to the AAP community (Figure 2). Within the generalists, we identified the core AAP community that consistently contributed throughout the seasons for the three consecutive years and across all depths (Supplementary Figure S9). The coexistence of the core AAP community, the dominance of positive correlations in the networks and the numerous correlations between ASVs of similar taxonomic ranks (Figure 4), suggest partial metabolic redundancy within some closely related AAP bacteria that maintain functional kinship. In contrast, the complex seasonal succession pattern indicates that the lake's AAP community is extremely diverse. AAP community represent a large functional repertoire (even within the same genus) allowing for niche speciation *via* temporal succession, facilitating their geographical coexistence. Finally, the 3-year recurrence of the AAP community (Figure 1A) documents its indigenous character in freshwaters and the higher importance of selection over the environmental drift and dispersal processes at short temporal scale (Huber et al. 2020). Changes in the AAP abundance coincided with shifts in their community composition indicating that abundance peaks were caused by specific phylotypes. These

phylotypes differed between both abundance peaks (Figure 3). Generally, AAP community was dominated by Gammaproteobacteria except for spring abundance peaks, when Alphaproteobacteria, that already have shown higher phototrophic activities in spring (Fecskeová et al. 2019), increased their contribution. Additionally, the directional interannual variation of the AAP community (Figure 1B) signifies the evolution of AAPs populations, potentially influenced by changes in environmental and biological variables such as temperature or Chl-*a* (Supplementary Figure S7).

While the PEG model has enhanced our understanding of seasonal patterns, it still does not encompass all aquatic components, such as viruses or specific functional bacterial groups. This includes AAP bacteria, which are characterized by a heterogenic behaviour but still represent an important functional group, fulfilling valuable ecological and biochemical processes in the aquatic environment. For that reason, amending them into the present PEG model will certainly improve our understanding of aquatic community functioning.

Cep lake is representative of a common temperate lake in the northern hemisphere: it is small, shallow and meso-oligotrophic (Verpoorter et al. 2014), thus our conclusions might be applied to other lakes. AAP bacteria play an important role during and shortly after the spring bloom when their abundance and contribution to the total bacterial community were recurrently the highest. This spring AAP abundance peak preceded those from the overall heterotrophic bacteria (Figure 5). The faster response of AAP bacteria highlights that photoheterotrophy confers a distinct metabolic advantage that triggers

their growth in this scenario over other heterotrophic bacteria. Their importance as food for bacterivores in microbial food

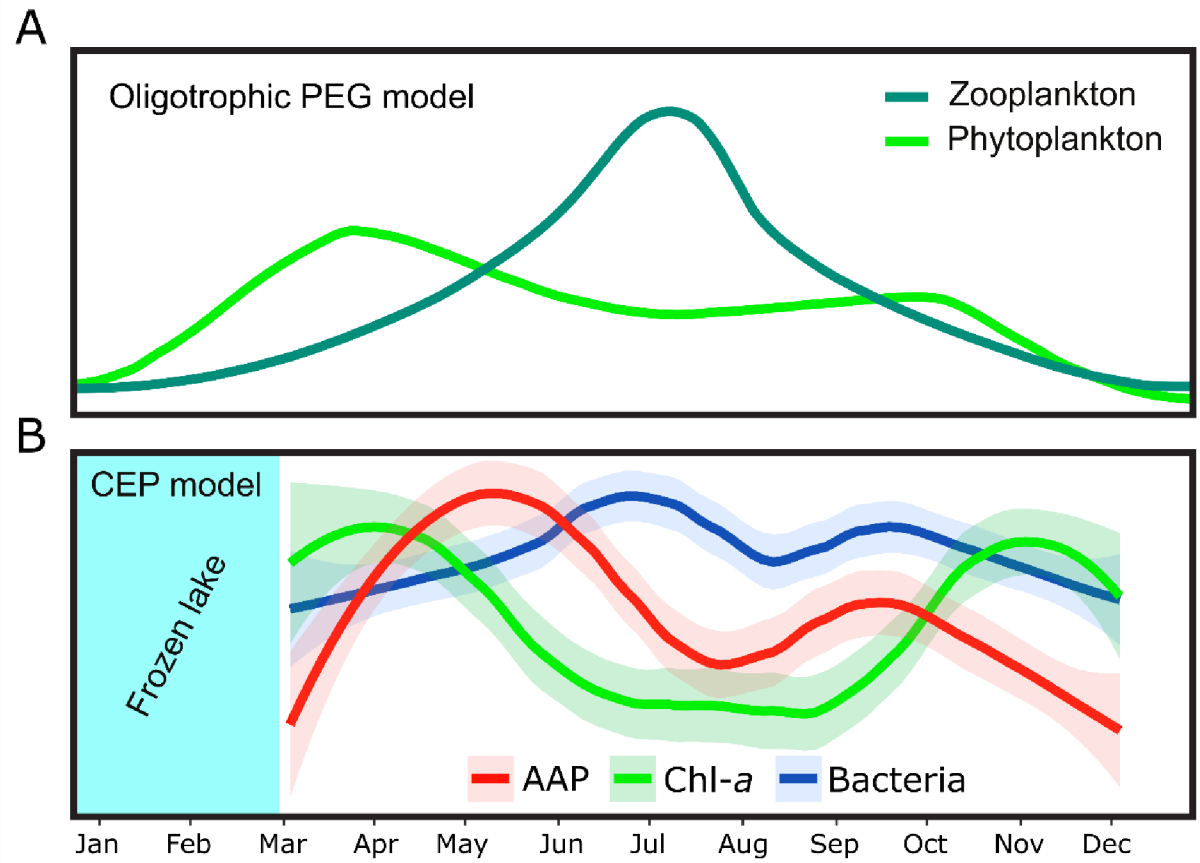


Figure 5. Including AAP bacteria in the PEG model. Annual succession patterns of microbial communities for (A) Phytoplankton and zooplankton according to original PEG model in oligotrophic scenario, and (B) monthly averaged annual succession pattern of AAP abundance, trophic status through Chl-a, and of bacterial abundance. Trend lines are normalized to maxima and minima values for each variable.

web is well documented (Koblížek et al. 2005; Garcia-Chaves et al. 2015; Cepáková et al. 2016) and due to their, on average, larger cell size and higher activity than other heterotrophic bacteria (Fauteux et al. 2015; Garcia-Chaves et al. 2016), they contribute disproportionally to the carbon cycling contrary to their relatively low abundances (Cepáková et al. 2016; Kolářová et al. 2019). Additionally, Chl-*a* concentration has been identified as a variable explaining the dynamics of the AAP bacterial community and it is plausible to assume that the spring abundance peaks of AAP bacteria are triggered by the excess of carbon released by the phytoplankton bloom (mostly diatoms; Supplementary Figure S6) and the lack of grazing pressure after winter. Moreover, AAPs have a highly efficient photoheterotrophic metabolism (Piwosz et al. 2022) increasing secondary bacterial production and disposing more carbon to higher trophic levels *via* the microbial loop. This emphasizes the urgent need for more quantitative studies to further decipher carbon transfers along microbial and classical food webs. The AAP bacterial

peak is terminated by selective and extensive grazing of bacterivorous protists and macrozooplankton that are also present in summer (Šimek et al. 2014; Garcia-Chaves et al. 2015; Fecskeová et al. 2021). The absence of a pronounced AAPs peak following the phytoplankton bloom in autumn (Figure 5) could be attributed to the higher grazing pressure, distinct phytoplankton composition (Supplementary Figure S6), decreasing temperatures (Supplementary File S6), and the decreasing light availability at shorted day length (Sommer et al. 2012; Ferrera et al. 2014; Auladell et al. 2019; Villena-Alemany et al. 2023).

Finally, our study provides novel insight into the ecology of phototrophic Myxococcota. While their average contribution was low, they were recovered during stratification over three consecutive years (Supplementary Figure S8) and constituted a member of the summer-autumn AAPs peak, emphasizing their potential significance in microbial communities during summer as they showed a potentially predatory and photoheterotrophic metabolism(Li et al. 2023).

Conclusions

Our study revealed annual recurrent seasonal patterns of AAP bacteria in a freshwater lake, supporting the conceptual inclusion of this important functional group into the PEG model. The high abundance of AAP bacteria during the spring phytoplankton bloom highlights their crucial role in recycling phytoplankton-derived dissolved organic matter and their role in aquatic food webs, which needs to be further quantified and better understood. Differential contribution patterns of the core community and temporal succession of the AAP community indicate strong competition within AAP bacteria communities, which forces them to conduct temporal niche partitioning in order to geographically coexist. In contrast, positive co-occurrence correlations between closely related AAP bacteria indicated their functional redundancy. Our findings provide unprecedented insights into the phenology of AAP bacteria in temperate freshwater ecosystems.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The dataset supporting the results and conclusions are embedded in the manuscript and available in the Sequence Read Archive (SRA) under the Bioproject accession number PRJNA970655.

Competing interest

All authors declare no conflict of interest.

Funding

The work was funded by Grant Agency of Czech Republic in the project no. 19-28778X awarded to MK, and by the National Science Centre, Poland under the Weave-UNISONO call in the Weave program, project no. 2021/03/Y/NZ8/00076 awarded to KP.

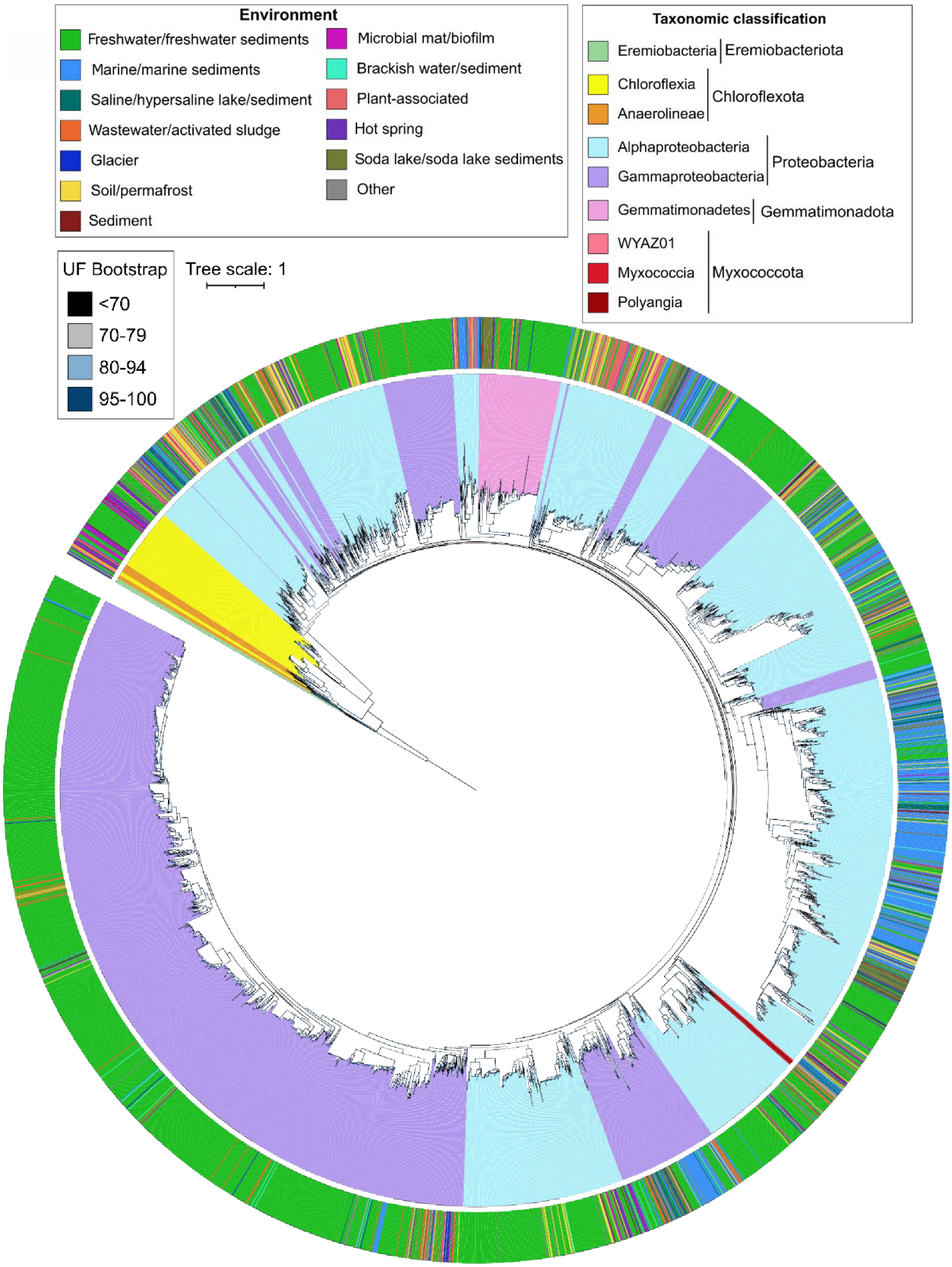
Author's contributions

CVA, KP and MK did the conceptualization; CVA, IM, KP, JW, AA, CRG, MS, HJR, SS, VK, ASA, HPG and RG curated or provided data for pufM database; formal analysis was carried out by CVA, IM, KP, LKF and RG; investigation and experimental processes were done by CVA, IM and KP; methodology of database was developed by RG and CVA; CVA and KP wrote original draft and all the authors helped during the review and editing of the manuscript; JD and MH performed the sampling and measured of environmental variables. All authors have revised and approved the submitted version.

Acknowledgements

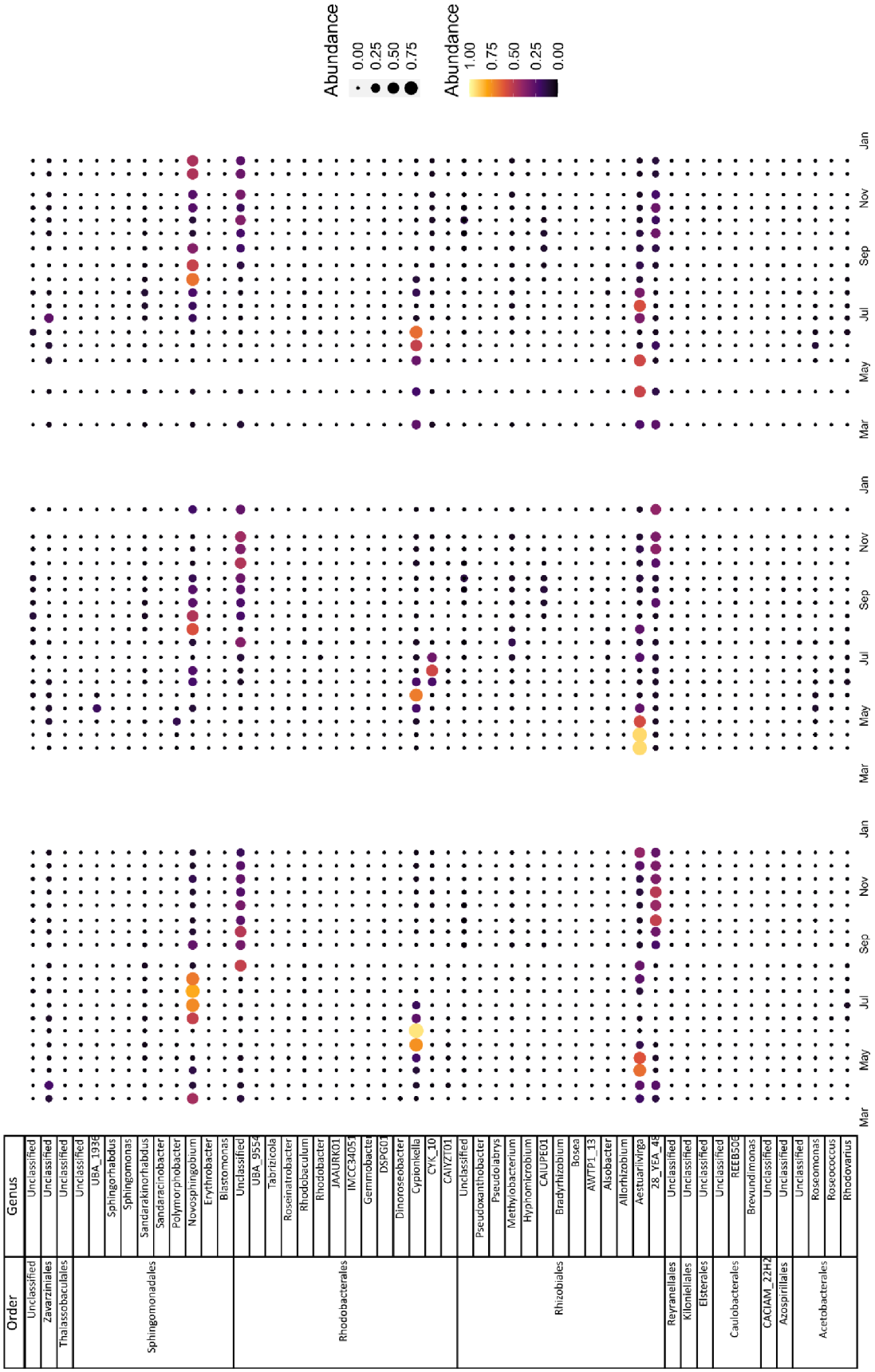
The authors want to thank Jürgen Tomasch, Esther Rubio Portillo and Iva Stojan for the insights into the code for data analysis. The Limnos metagenomic dataset was enabled by technical and financial support of the technician team at IGB Stechlin and the Leibniz foundation, respectively.

Supplementary Figures



Supplementary Figure S1: Maximum likelihood phylogenetic tree of *puf*M gene sequences of the constructed database. Outer ring represents the environment of origin and the colour of the clades between branches and the outer ring shows the taxonomic classification of the sequences at class level. Colour of the branches refers to the ultrafast bootstrap values.

A


Alphaproteobacteria community 0.5 m
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Thalassobaculales
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Sphingorhabdus
Sphingomonas
Sandarakinorhabdus
Sandaracinobacter
Polymorphobacter
Novosphingobium
Erythrobacter
Blastomonas
Rhodobacterales
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Roseinatrobacter
Rhodobaculum
Rhodobacter
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IMCC34053
Gemmobacter
DSPG01
Dinoroseobacter
Cypionkella
CYK_10
CAIYZT01
Rhizobiales
Unclassified
Pseudoxanthobacter
Pseudolabrys
Methyloblacterium
Hyphomicrobium
CAIUPE01
Bradyrhizobium
Bosea
AWTP1_13
Afipia
Allorhizobium
Aestuariivirga
28_YEA_48
Reyranellales
Unclassified
Kiloniellales
Unclassified
Elsterales
Unclassified
Caulobacterales
Unclassified
REEB506
Brevundimonas
CACIAM_22H2
Unclassified
Azospirillales
Unclassified
Unclassified
Acetobacterales
Roseomonas
Roseococcus
Rhodovarius
Mar
May
Jul
Sep
Nov
Jan
Mar
May
Jul
Sep
Nov
Jan
Mar
May
Jul
Sep
Nov
Jan
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0.75
Abundance
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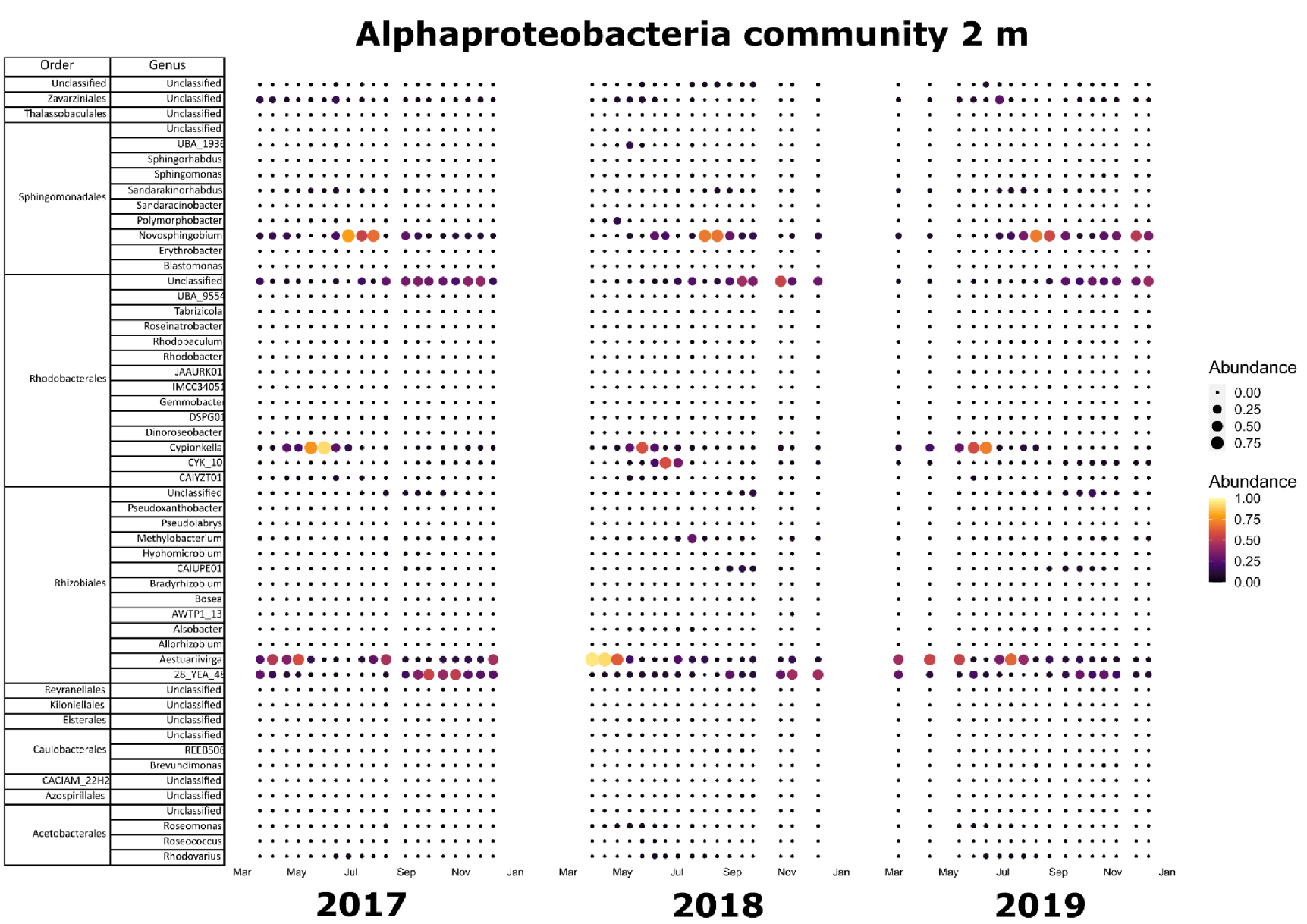
0.25
0.00

B


Alphaproteobacteria community 2 m
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Genus
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Thalassobaculales
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Sphingomonas
Sandarakinorhabdus
Sandaracinobacter
Polymorphobacter
Novosphingobium
Erythrobacter
Blastomonas
Sphingomonadales
Unclassified
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Tabrizicola
Roseinatrobacter
Rhodobaculum
Rhodobacter
JAAURK01
IMCC34051
Gemmobacter
DSPG01
Dinoroseobacter
Cypionkella
CYK_10
CAIYZT01
Rhodobacterales
Unclassified
Pseudoxanthobacter
Pseudolabrys
Methylobacterium
Hyphomicrobium
CAIUPE01
Bradyrhizobium
Bosea
AWTP1_13
Alsobacter
Allorhizobium
Aestuariivirga
28_YEA_48
Rhizobiales
Reyranellales
Unclassified
Kiloniellales
Unclassified
Elsterales
Unclassified
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REEB506
Brevundimonas
Caulobacterales
CACIAM_22H2
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Azospirillales
Unclassified
Unclassified
Roseomonas
Roseococcus
Rhodovarius
Acetobacterales
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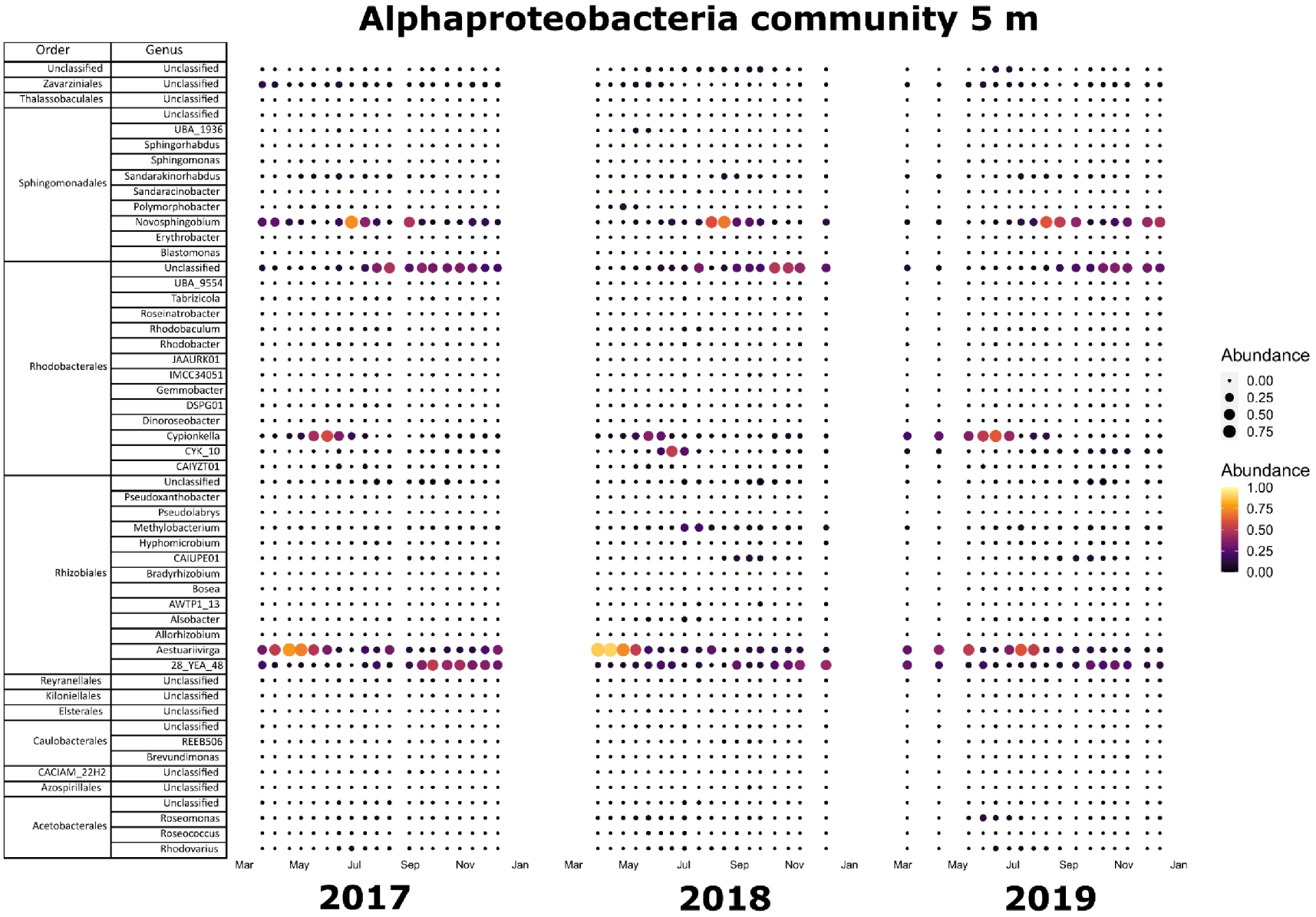
0.25
0.00

C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Genus
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Sphingorhabdus
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Sandarakinorhabdus
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Tabrizicola
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Gemmobacter
DSPG01
Dinoroseobacter
Cypionkella
CYK_10
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Rhizobiales
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Pseudolabrys
Methylobacterium
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Bradyrhizobium
Bosea
AWTP1_13
Alsobacter
Allorhizobium
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28_YEA_48
Reyranellales
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Caulobacterales
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REEB506
Brevundimonas
CACIAM_22H2
Unclassified
Azospirillales
Unclassified
Acetobacterales
Unclassified
Roseomonas
Roseococcus
Rhodovarius
Mar
May
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Nov
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2017
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Abundance
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Abundance
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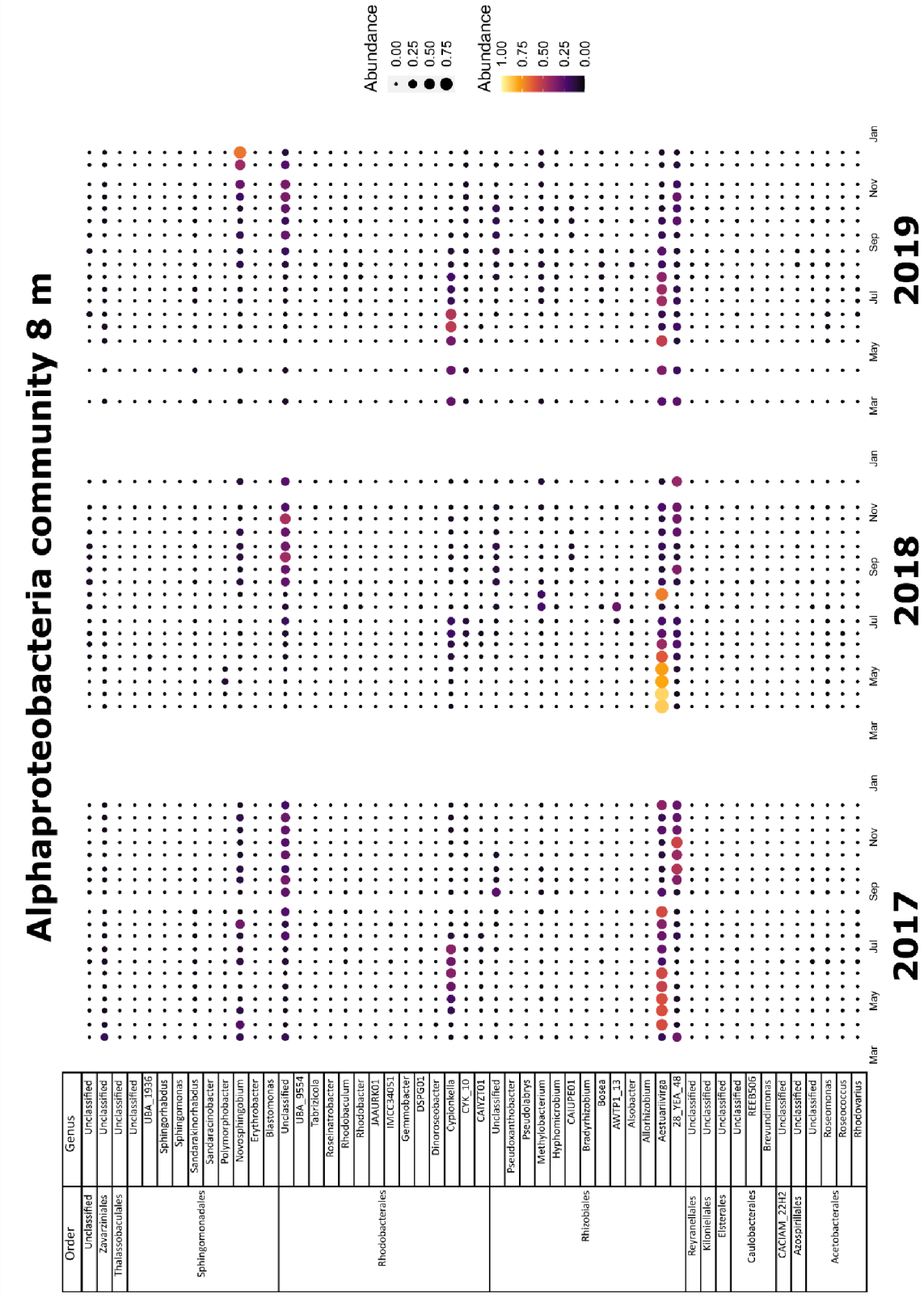
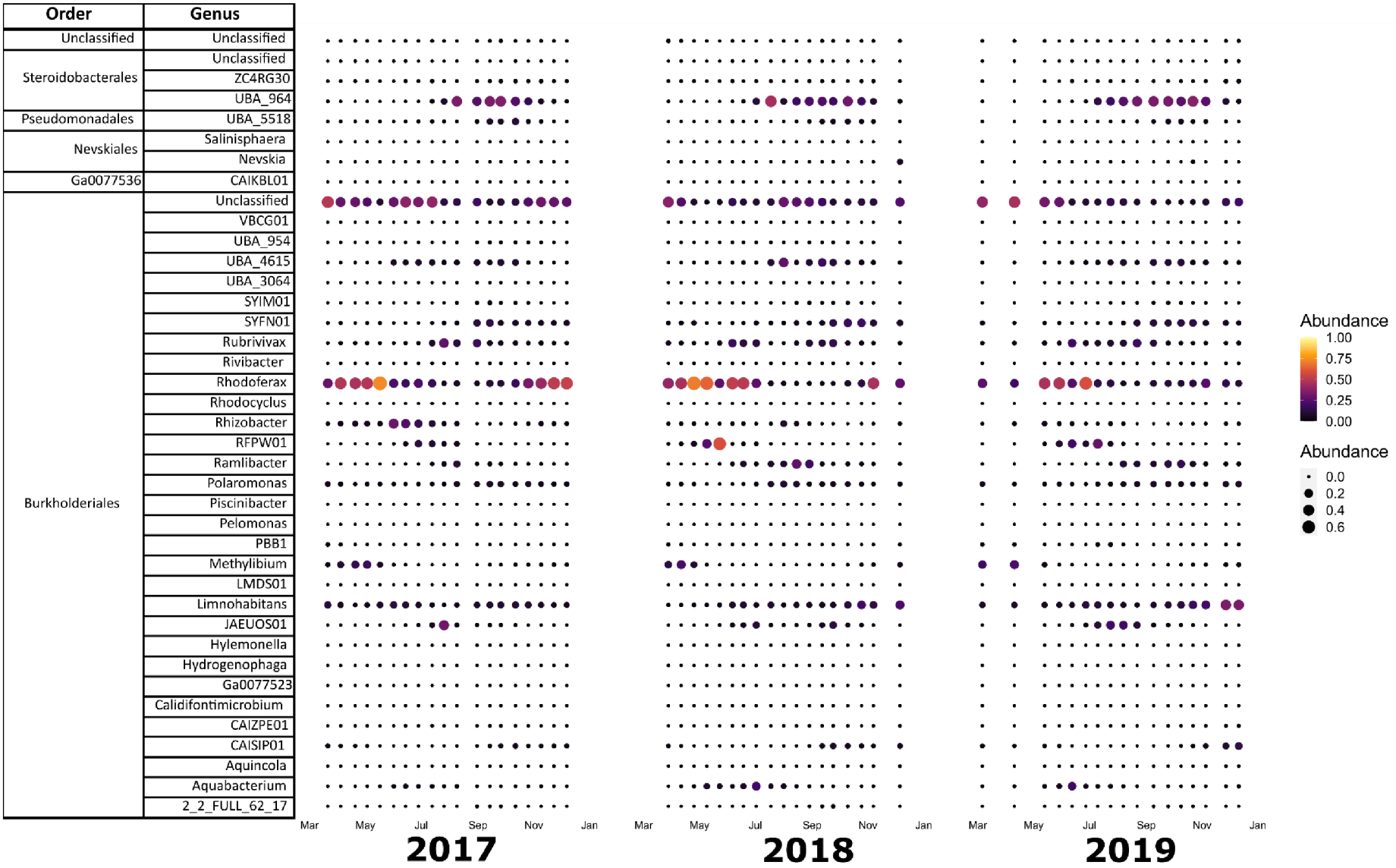


Supplementary Figure S2: Alphaproteobacteria community composition at order and genus level for 3-year sampling at 0.5 (A), 2 (B), 5 (C) and 8 m depth (D). Larger size and brighter colours are directly proportional to the relative contribution of each genus to the total Alphaproteobacteria community.

A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Unclassified
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UBA_5518
Nevskiales
Salinisphaera
Nevskia
Ga0077536
CAIKBL01
Burkholderiales
Unclassified
VBCG01
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UBA_3064
SYIM01
SYFN01
Rubrivivax
Rivibacter
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Rhizobacter
RFPW01
Ramlibacter
Polaromonas
Piscinibacter
Pelomonas
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Methylibium
LMDS01
Limnohabitans
JAEUOS01
Hylemonella
Hydrogenophaga
Ga0077523
Calidifontimicrobium
CAIZPE01
CAISIP01
Aquincola
Aquabacterium
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Mar
May
Jul
Sep
Nov
Jan
2017
2018
2019
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Abundance
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0.2
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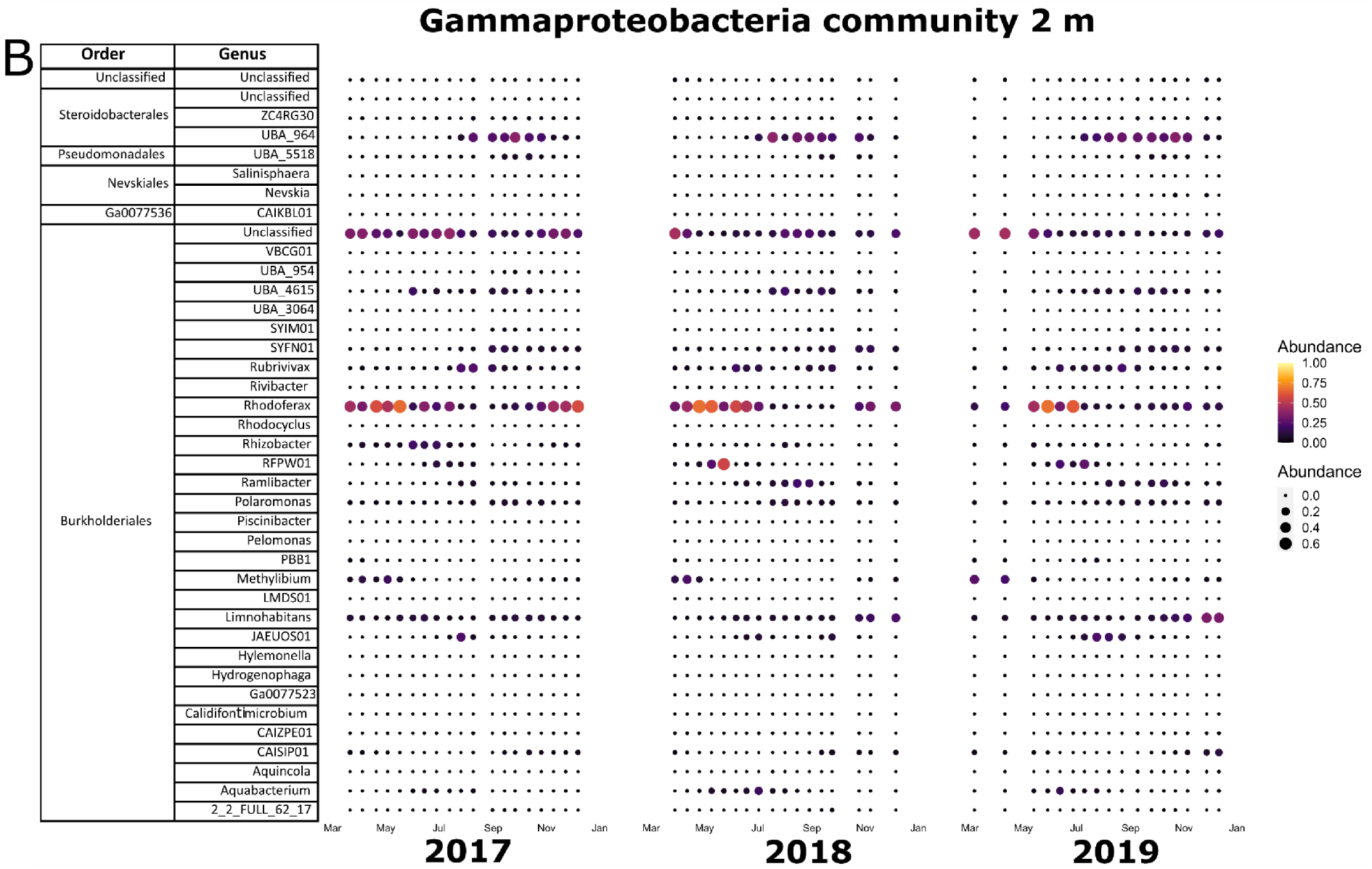
Gammaproteobacteria community 2 m
B
Order
Genus
Unclassified
Unclassified
Steroidobacterales
Unclassified
ZC4RG30
UBA_964
Pseudomonadales
UBA_5518
Nevskiales
Salinisphaera
Nevskia
Ga0077536
CAIKBL01
Burkholderiales
Unclassified
VBCG01
UBA_954
UBA_4615
UBA_3064
SYIM01
SYFN01
Rubrivivax
Rivibacter
Rhodoferax
Rhodocyclus
Rhizobacter
RFPW01
Ramlibacter
Polaromonas
Piscinibacter
Pelomonas
PBB1
Methylibium
LMDS01
Limnohabitans
JAEUOS01
Hylemonella
Hydrogenophaga
Ga0077523
Calidifontimicrobium
CAIZPE01
CAISIP01
Aquincola
Aquabacterium
2_2_FULL_62_17
Mar
May
Jul
Sep
Nov
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2017
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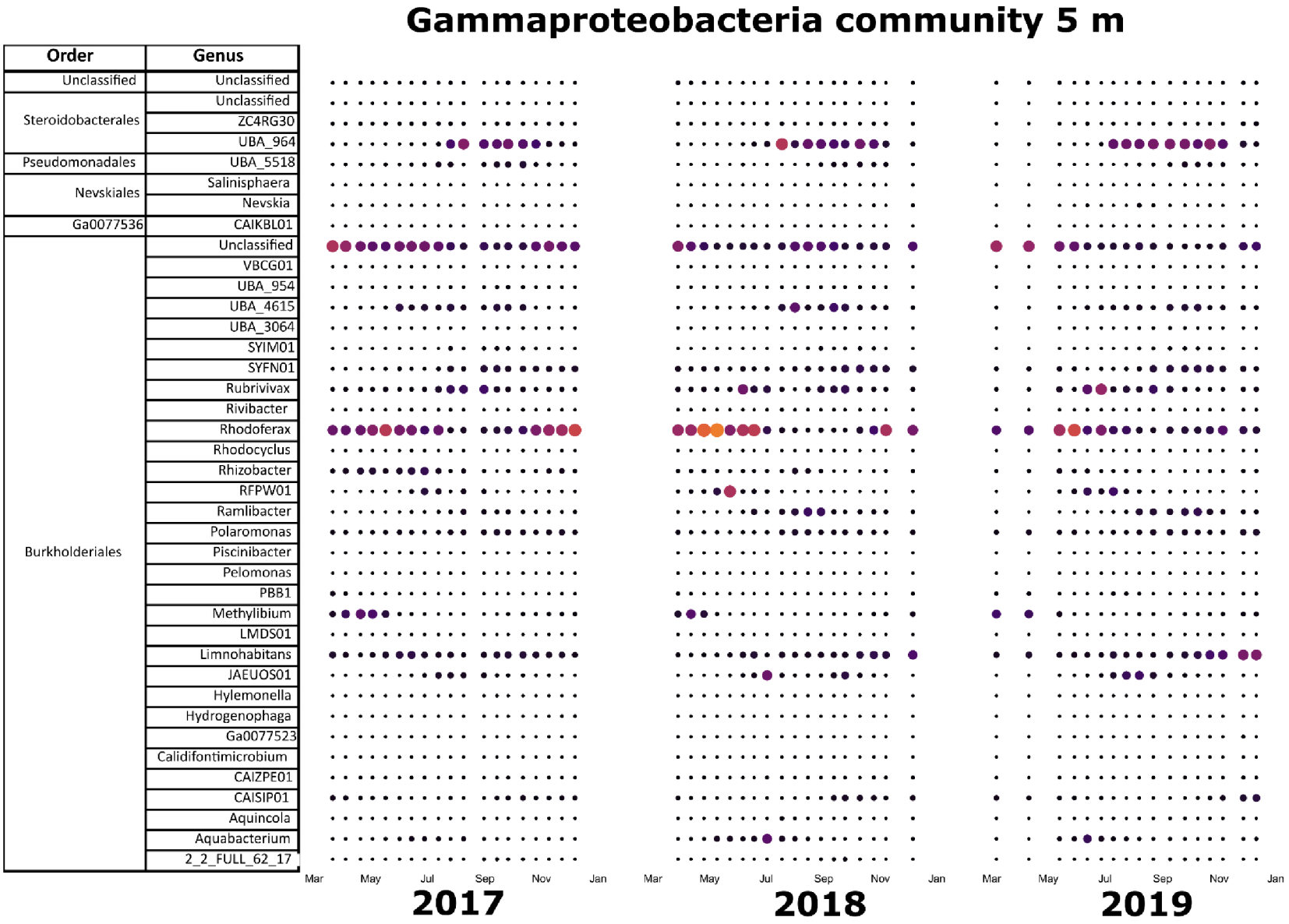
C


Gammaproteobacteria community 5 m
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Genus
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Steroidobacterales
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UBA_5518
Nevskiales
Salinisphaera
Nevskia
Ga0077536
CAIKBL01
Burkholderiales
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SYIM01
SYFN01
Rubrivivax
Rivibacter
Rhodoferax
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Rhizobacter
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LMDS01
Limnohabitans
JAEUOS01
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CAIZPE01
CAISIP01
Aquincola
Aquabacterium
2_2_FULL_62_17
Mar
May
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2017
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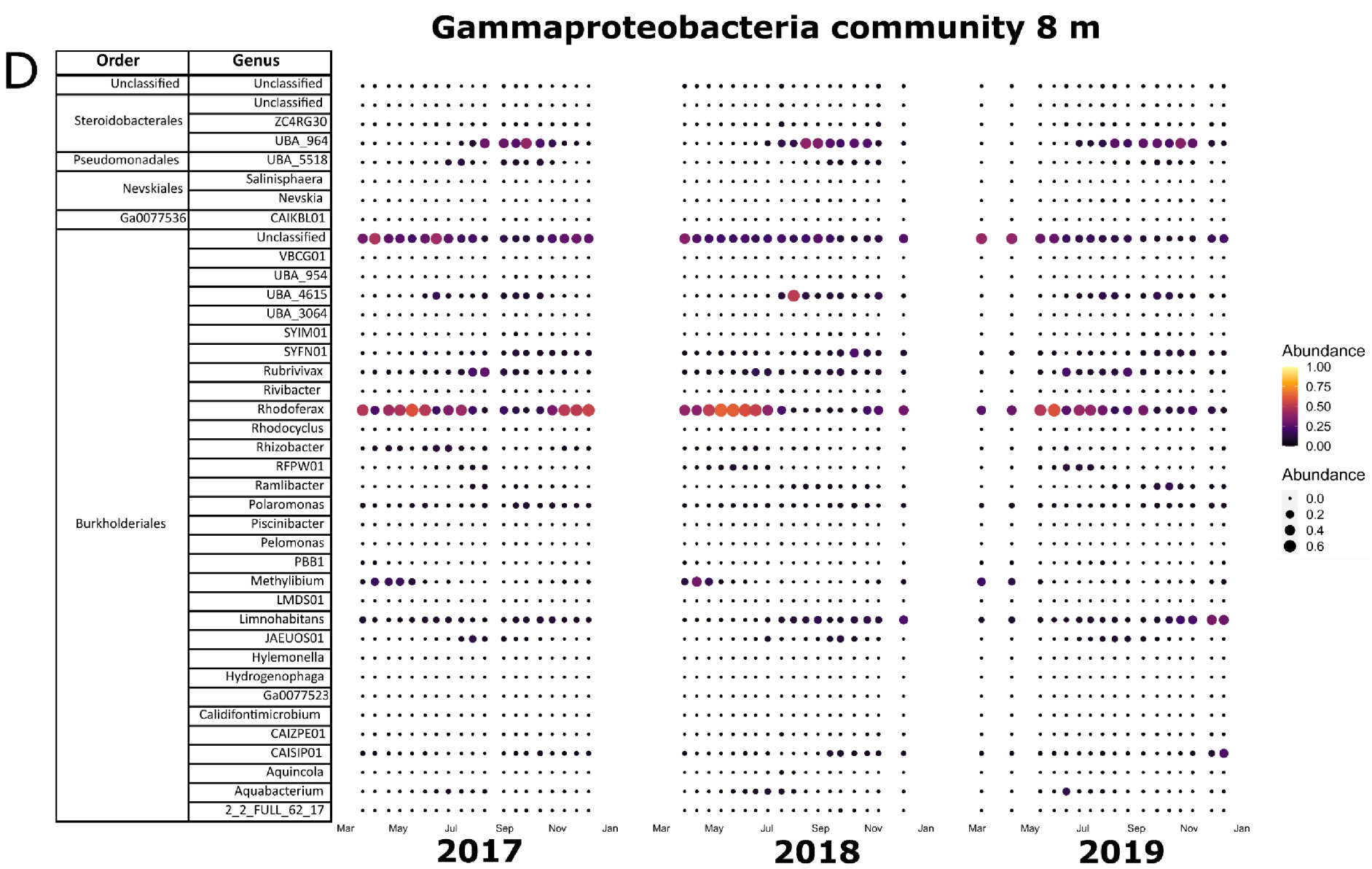
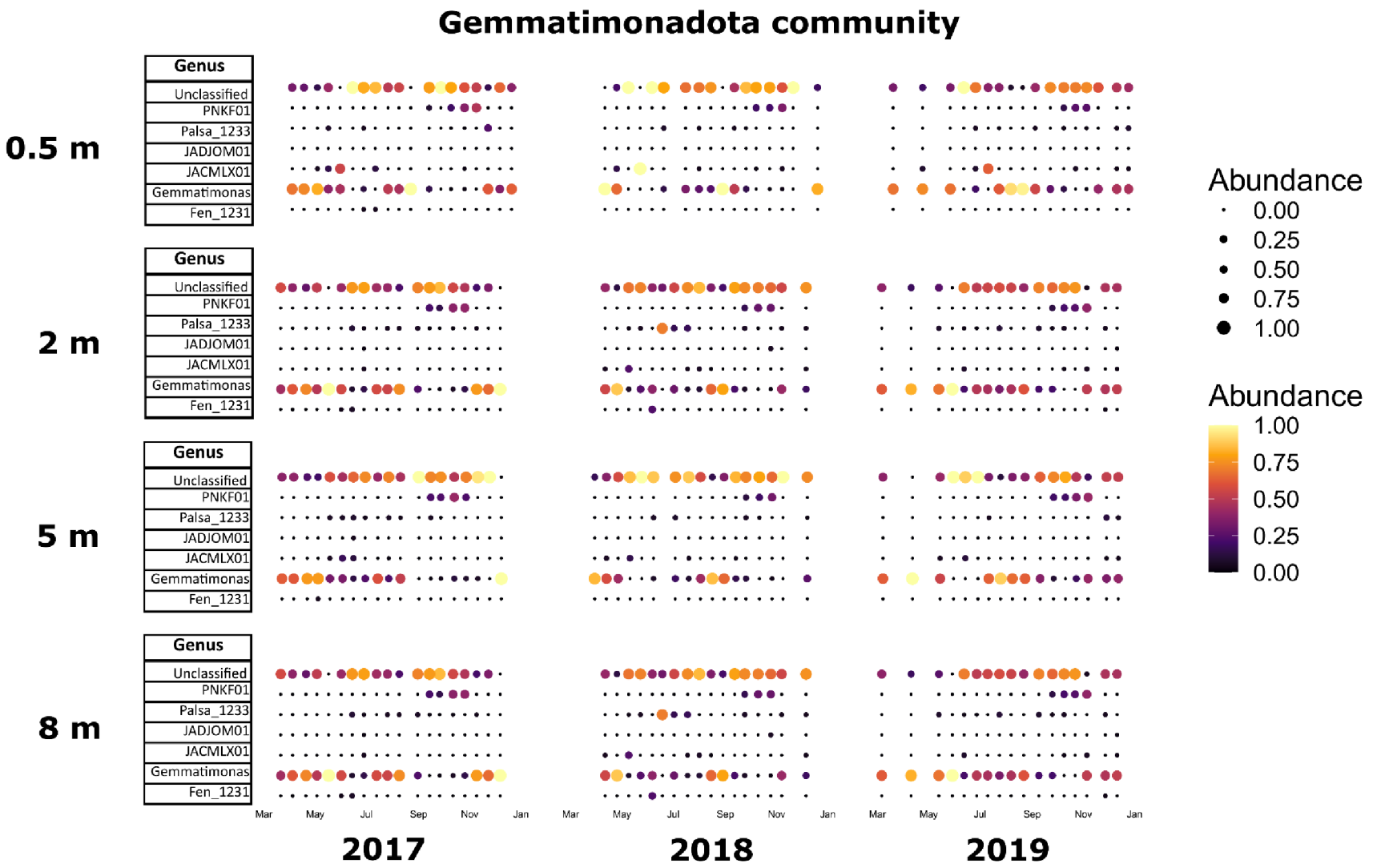
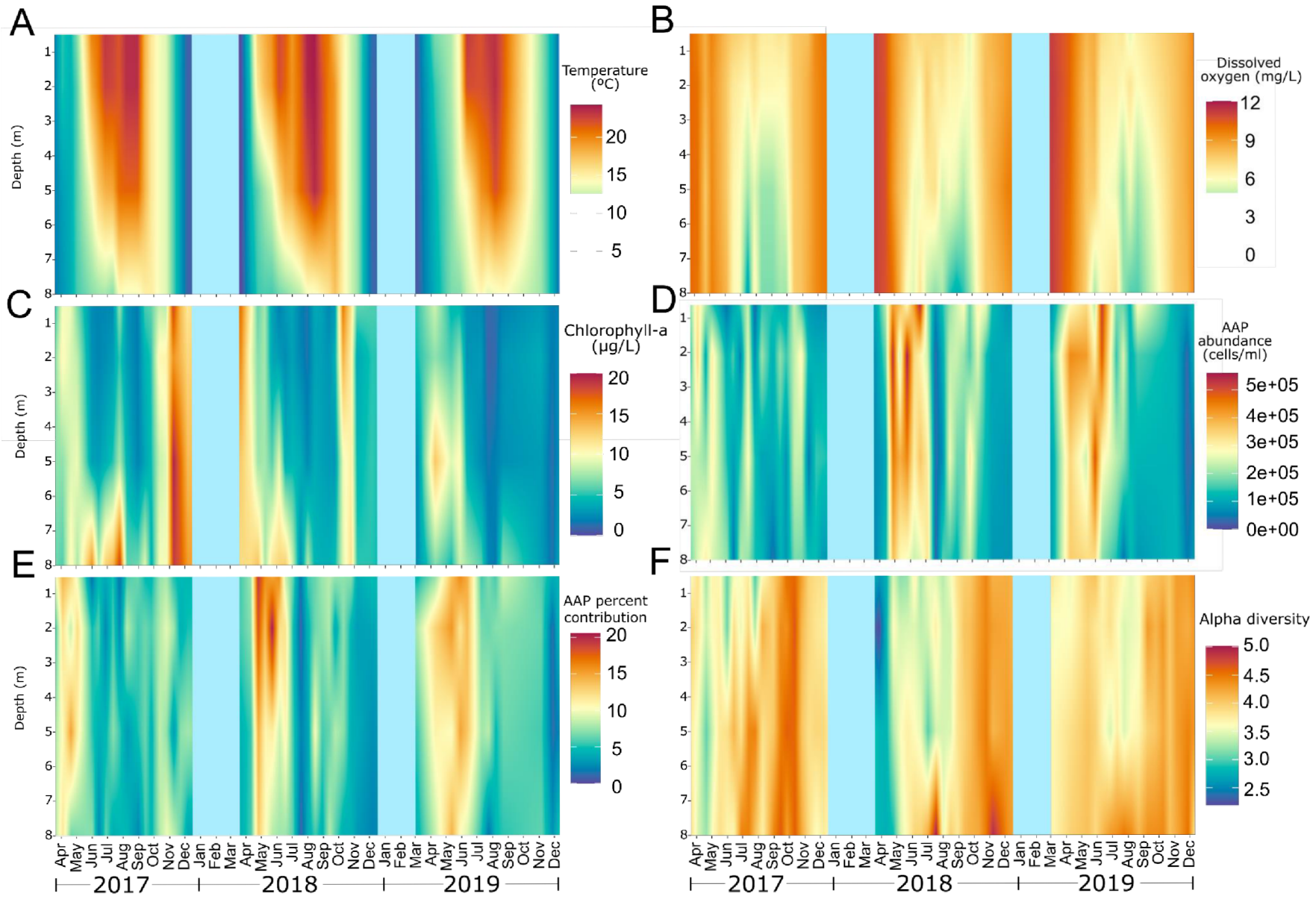


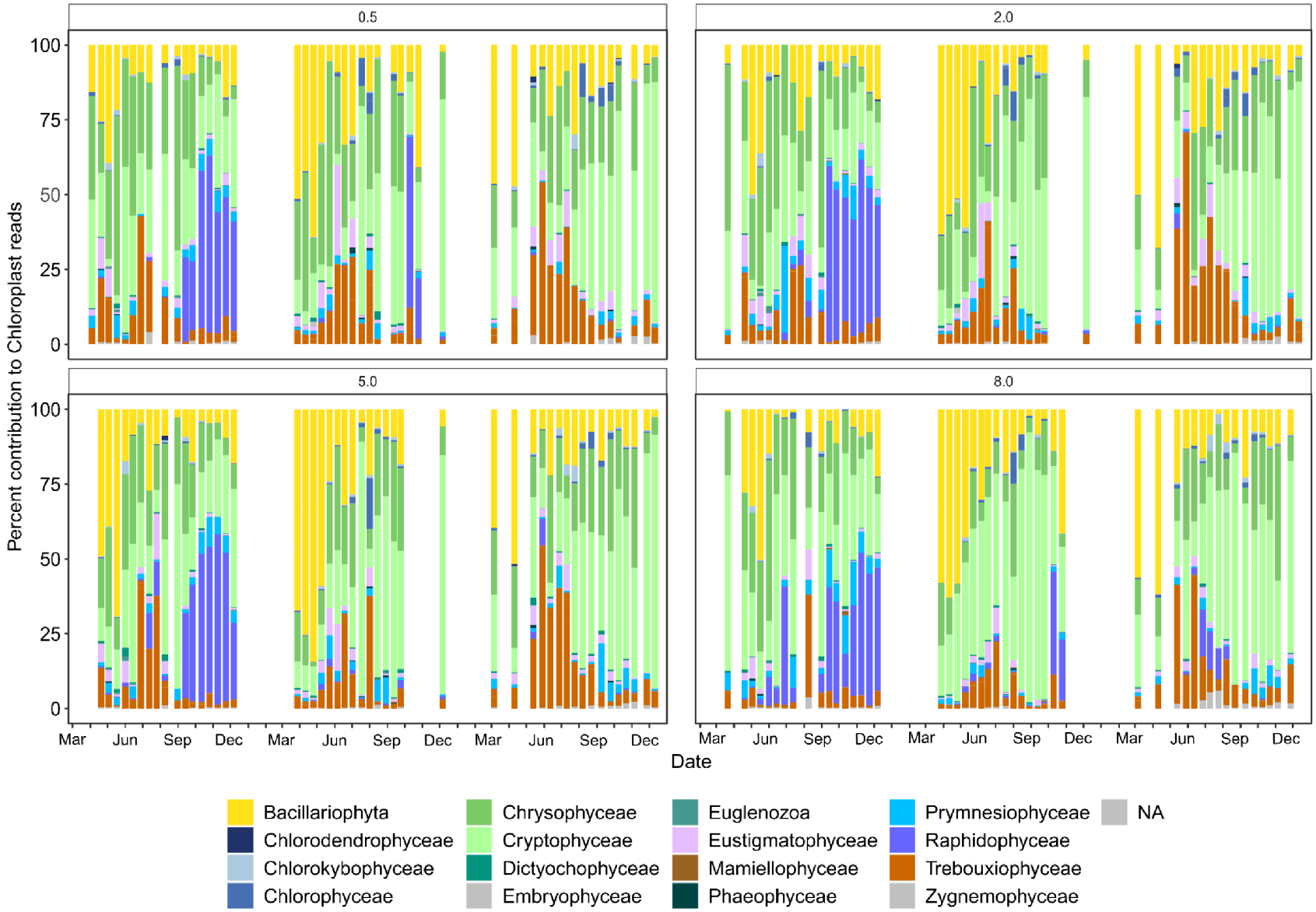
Supplementary Figure S3: Gammaproteobacteria community composition at order and genus level for 3-year sampling at 0.5 (A), 2 (B), 5 (C) and 8 m depth (D). Larger size and brighter colours are directly proportional to the relative contribution of each genus to the total Gammaproteobacteria community.



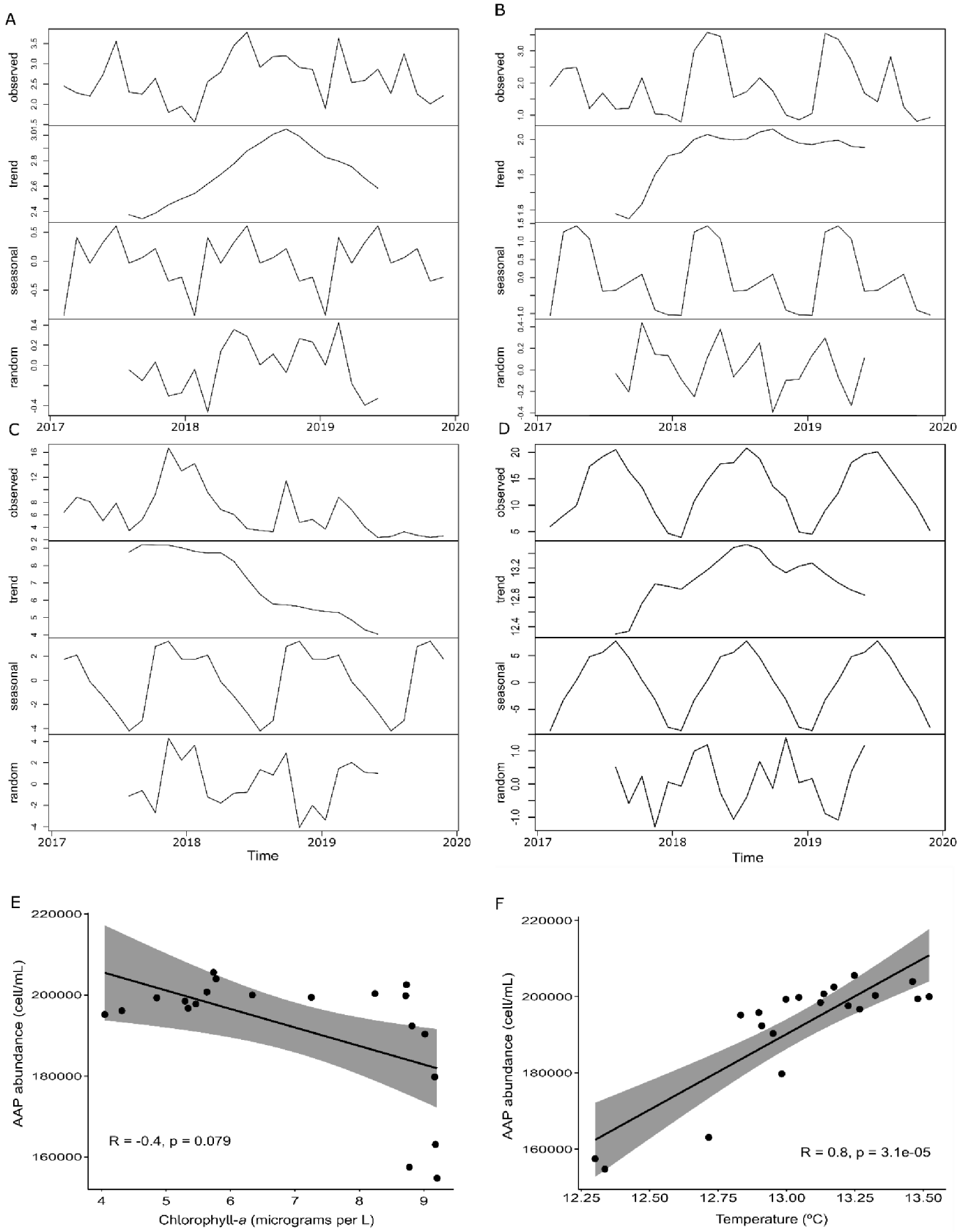
Supplementary Figure S4: Gemmatimonadota community composition at order and genus level for 3-year sampling at 0.5 (A), 2 (B), 5 (C) and 8 m depth (D). Larger size and brighter colours are directly proportional to the relative contribution of each genus to the total Gemmatimonadota community.



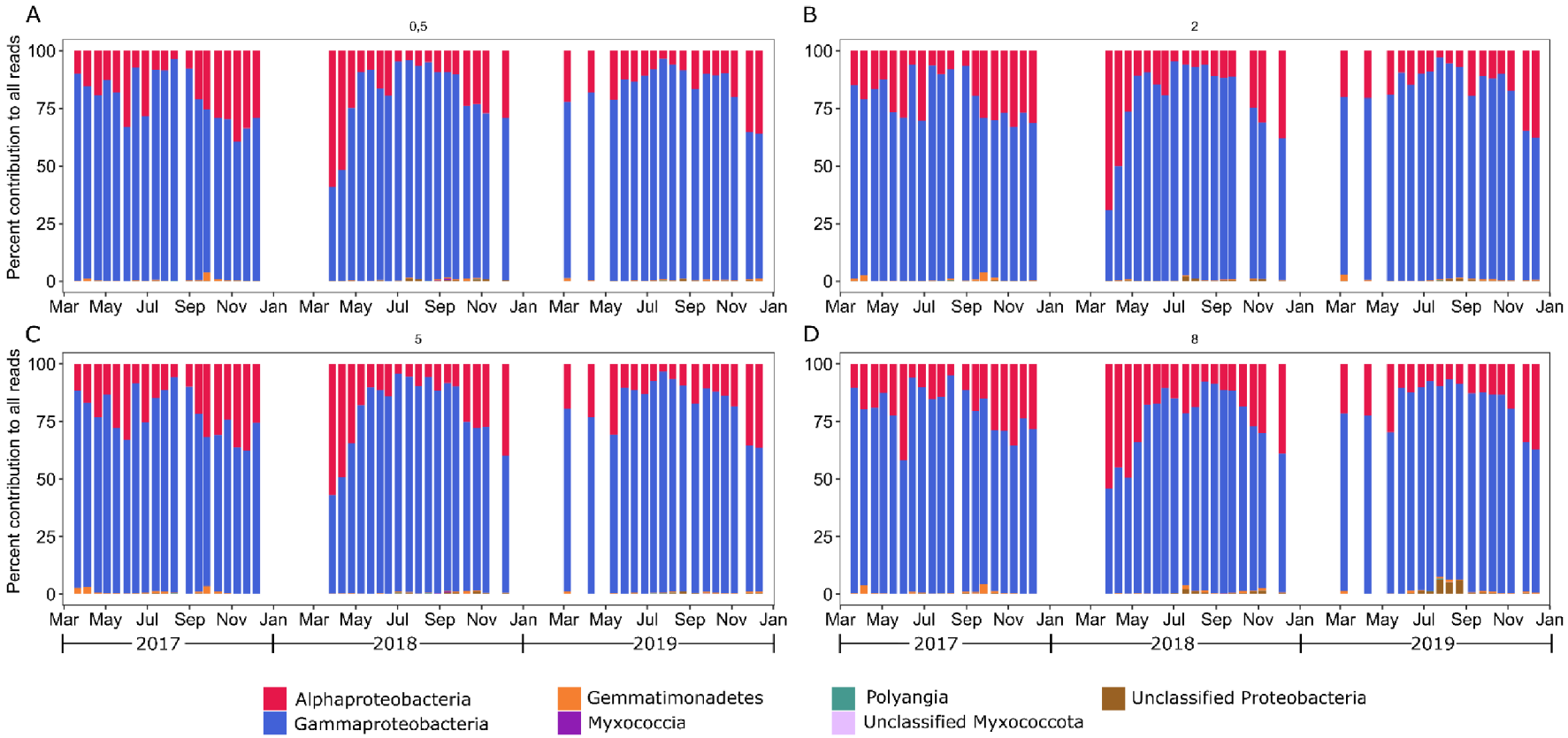
Supplementary Figure S5: Environmental and biological variables for 8 meters' depth profile during 3-year sampling in CEP lake. Temperature (A), AAP abundance (B), dissolved oxygen (C), percentage contribution to total bacterial community (D), Shannon alpha diversity values (E), and Chlorophyll-*a* (F). Light-blue vertical bands represent lack of sampling due to frozen lake surface.



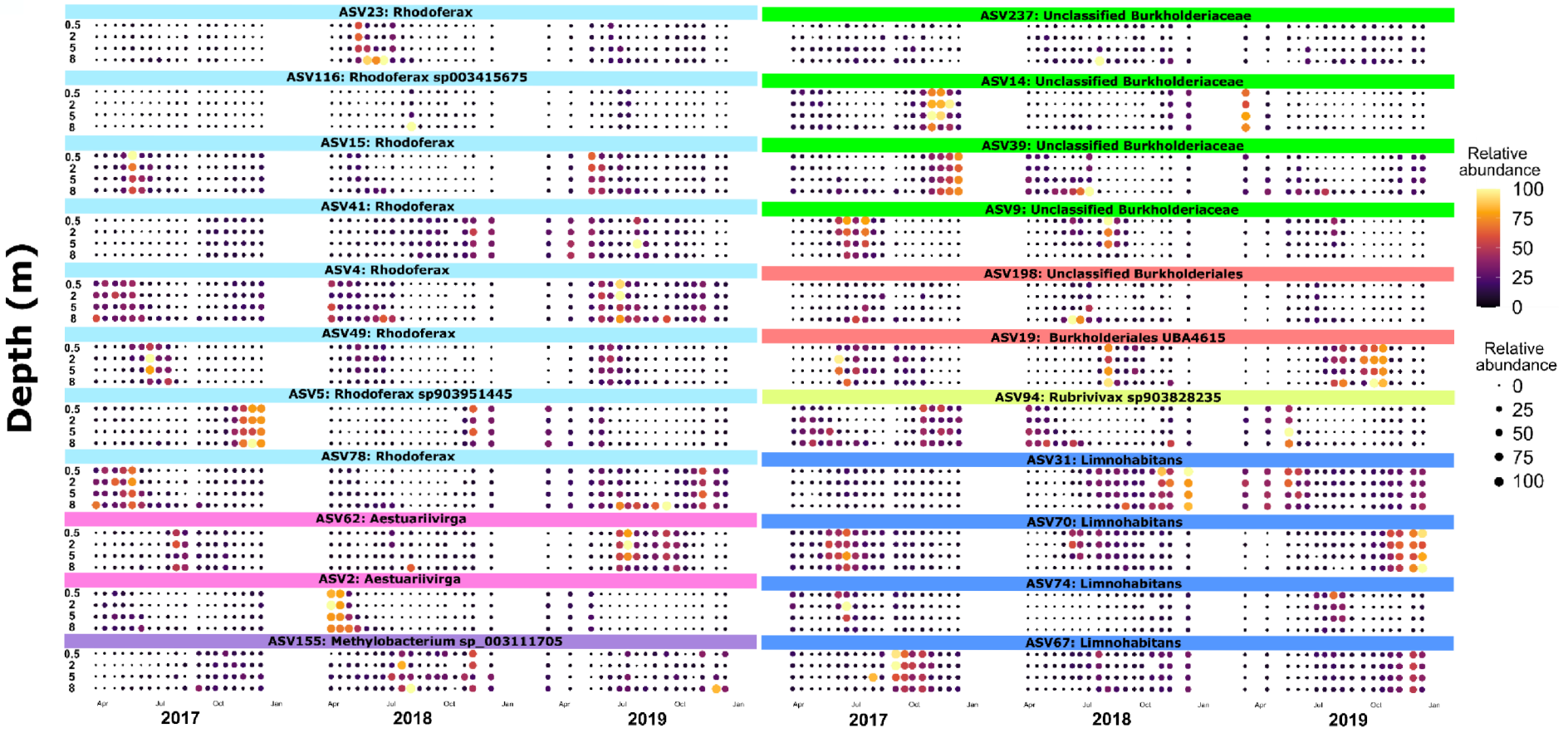
Supplementary Figure S6: Phytoplankton chloroplast-based community composition at class level for 0,5, 2, 5 and 8 meters' depth during 3-years temporal series.



Supplementary Figure S7: Decomposition of additive time series for bacterial abundance (A), AAP abundance (B), chlorophyll-*a* concentration (C) and temperature (D). Analysis was done in the TTR package version 0.24.3 (R version 4.2.0). Spearman correlation of the decomposed trends between AAP abundance and chlorophyll-*a* (E) and AAP abundance and temperature (F). R: spearman's rho value, p: p-value.



Supplementary Figure S8: AAP bacteria community composition according to *puf*M gene taxonomic assignment at class level for 0,5 (A), 2 (B), 5 (C) and 8 meters' depth (D) during 3-year sampling campaign.



Supplementary Figure S9: Individually normalized relative abundance of the 22 core AAP ASVs during 3 years in 4 depths. Brighter colors and bigger dots indicate larger contribution to the AAP bacterial community. ASVs are clustered according to taxonomic classification at the maximum possible level (genus, family or order).

Supplementary Files (online)

Supplementary File S1: Nucleotide *puf*M gene sequence of the database in fasta format.

Supplementary File S2: ID of the *puf*M gene sequences from the database and their environment of origin.

Supplementary File S3: File containing all the information from the amplicon sequence analysis. Reference ASVs (IDs and sequences), ASV table (ID and abundance on each sample) and Taxa (ID and taxonomic assignation of each ASV).

Supplementary File S4: Primer coverage comparison of the most commonly used *puf*M gene primer pairs and the newly designed one with 0, 1, 2 and 3 mismatches (MM). Numbers represent the percentage of sequences from the *puf*M gene database covered for different phyla and classes.

Supplementary File S5: Sample identification number (Sample name) and all the environmental and biological variables measured.

Supplementary File S6: Draftsman plot correlation of the environmental and biological variables, samples removed due to lack of environmental variables, marginal and sequential test for DistLM for 3 years and 2 years (includes also nutrients).

Supplementary File S7: Positive log fold change (lfc) values, standard error (SE) at genus and ASV levels for the spring and autumn AAP abundance peaks with the p- and q-values (p, q).

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Chapter III

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Photoheterotrophy by aerobic anoxygenic bacteria modulates carbon fluxes in a freshwater lake

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Lakes are a significant component of the global carbon cycle. Respiration exceeds net primary production in most freshwater lakes, making them a source of CO_2 to the atmosphere. Driven by heterotrophic microorganisms, respiration is assumed to be unaffected by light, thus it is measured in the dark. However, photoheterotrophs, such as aerobic anoxygenic photoheterotrophic (AAP) bacteria that produce ATP via photochemical reactions, substantially reduce respiration in the light. They are an abundant and active component of bacterioplankton, but their photoheterotrophic contribution to microbial community metabolism remains unquantified. We showed that the community respiration rate in a freshwater lake was reduced by 15.2% (95% confidence interval (CI): 6.6–23.8%) in infrared light that is usable by AAP bacteria but not by primary producers. Moreover, significantly higher assimilation rates of glucose (18.1%; 7.8–28.4%), pyruvate (9.5%; 4.2–14.8%), and leucine (5.9%; 0.1–11.6%) were measured in infrared light. At the ecosystem scale, the amount of CO_2 from respiration unbalanced by net primary production was by 3.69×10^9 g CO_2 lower over these two sampling seasons when measured in the infrared light. Our results demonstrate that dark measurements of microbial activity significantly bias the carbon fluxes, providing a new paradigm for their quantification in aquatic environments.

The ISME Journal; https://doi.org/10.1038/s41396-021-01142-2

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Piwosz K, Villena-Alemany C. and Mujakić I. (2022)

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The ISME Journal 2022; 16: 1046-1054.

(IF= 11.0)

Chapter III: Context and objectives

Lakes systematically impact global carbon cycle, acting as a source of carbon dioxide to the atmosphere because respiration exceeds net primary production. However, the conventional approach for calculating carbon fluxes relies on microbial respiration measurements, conducted in the dark in order to mitigate the influence of photosynthesis-generated oxygen. Notably, diverse AAP cultures have shown to reduce their respiration and increase carbon assimilation in the light. In this context, the effect of photoheterotrophic organisms that exhibit differential behaviour in dark and light conditions is overlooked. Despite these findings, there is not direct measurement of the impact of photoheterotrophic metabolism in environments. Furthermore, it remains unexplored whether AAP bacteria reduce overall microbial respiration.

Consequently, we wanted to answer the following questions:

- Does phototrophy by AAP bacteria induce a reduction in microbial respiration?
- Does phototrophy by AAP bacteria enhance organic compounds assimilation rates?
- Does the impact of phototrophy by AAP bacteria vary throughout the year?

Chapter III: Summary

Water samples from the surface of freshwater oligotrophic Cep lake were taken monthly over a span of two years. Microbial respiration and organic matter assimilation rates were measured in dark and in infrared light. Infrared light can be specifically harvested by bacteriochlorophyll-containing RC and it does not impact oxygenic photosynthesis. Indeed, carbon fixation exhibited no increase in the infrared treatment compared to the dark. In contrast, AAP bacteria strongly responded by using the energy harvested from infrared light to supply their metabolisms.

Despite constituting less than 20% of the bacterial community, AAP bacteria reduced the entire bacterial community respiration by 15.2%. Additionally, they exhibited significantly higher rates of glucose, pyruvate and leucine assimilation. Altogether, our findings underscore that generalized microbial respiration measurements in the dark overestimate microbial respiration by undervaluing

AAP bacteria metabolisms. Furthermore, our study reveals that the phototrophy of AAP bacteria enhances dissolved organic matter uptake rates, contributing to increased carbon efficiency transference to upper trophic levels.

While our results shed light on the impact of phototrophy on the carbon respiration and assimilation by AAP bacteria to the lake community and the potential influence on the carbon cycle, there are still many uncertainties about how different sources of organic carbon directly impact the metabolism of AAP bacteria.

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The ISME Journal; https://doi.org/10.1038/s41396-021-01142-2

INTRODUCTION

Alleviating consequences of the climate change requires a profound understanding of the global carbon cycle. Lakes are an important component of the carbon cycle: their annual burial of organic carbon in sediments exceeds that of oceans [1], but they are still a net source of CO_2 to the atmosphere at a rate of about 0.9 Pg C y^{-1} (range 0.7–1.3 Pg C y^{-1}) [2]. Almost 70% of this surplus CO_2 production is driven by microbial heterotrophic respiration [3]. Microbial respiration in lakes is predicted to increase as climate change progresses, which may slow down the rate of carbon sequestration [1]. Therefore, an accurate estimate of microbial respiration is crucial for precise global carbon fluxes assessments.

Microbial respiration is typically considered to be independent of light [4]. This assumption has been challenged by the discovery that photoheterotrophic bacteria, such as rhodopsin-containing bacteria and aerobic anoxygenic phototrophic (AAP) bacteria, are abundant in aquatic environments [5, 6]. AAP bacteria use bacteriochlorophyll (BChl)-containing reaction centers to harvest energy from light but cover most of their energy requirements through respiration. Both processes compete for quinone pool on the same membrane. In consequence, respiration gets reduced with the increasing light intensity [7]. Moreover, photochemical ATP production enhances the anabolism, resulting in more efficient incorporation of organic matter and increased bacterial growth efficiency [8, 9].

AAP bacteria comprise up to 30% of total bacteria in the epilimnion during spring and summer [10, 11]. They are on average larger and more active than heterotrophic bacteria [12], exhibit fast growth rates and high susceptibility to grazing, substantially contributing to aquatic food webs and bacterial production [10]. Cultured AAP species exposed to light decrease their respiration by 75% and increase biomass yield by 50% [7, 8]. Light exposure also raises the proportion of active AAP bacteria in freshwater lakes [12]. However, the benefits of light-harvesting by photoheterotrophic bacteria in situ, especially of highly active AAP bacteria, remain unquantified. Measurements of microbial respiration in light are hampered by the oxygen production by photoautotrophic phytoplankton, the inhibitory effect of UV radiation, and the photooxidation of organic matter [13, 14]. Consequently, the rates are typically measured in the dark [15], which biases such estimates in times of an elevated abundance of photoheterotrophs.

This study aimed to quantify the contribution of photoheterotrophic metabolism by AAP bacteria to the carbon fluxes in a freshwater lake. Their BChl-containing reaction centers have an absorption maximum at ~870 nm, which allowed us to use infrared light in the experimental incubations. Phytoplankton and all other microorganisms perceived such conditions as lightless, allowing direct comparison of rates from the IR and conventional dark measurements. We hypothesized that the respiration rate would be lower, while assimilation rates of organic compounds

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Received: 29 April 2021 Revised: 11 October 2021 Accepted: 12 October 2021
Published online: 20 November 2021

would be higher in the IR light compared to the dark. Moreover, we expected that the effect would vary seasonally with changing environmental conditions and the community composition of all bacteria and of AAP bacteria. We demonstrated that dark measurements of microbial activity significantly bias the estimates of the carbon fluxes in the studied lake, providing a new paradigm for its quantification in aquatic environments.

MATERIALS AND METHOD

Sampling

Water was sampled from Cep lake in Czechia, at a regular sampling site of 10 m depth (48.944 °N, 14.877 °E). The lake originates from sand mining in the 1970–80s. It is a permanent meso-oligotrophic (chlorophyll-a concentrations ranged from 1.4–16.4 μg L^{-1}) seepage reservoir filled with groundwater penetrating from the nearby river Lužnice. The lake area is about 1.16 km^2, with the maximum depth about 11–12 m. These characteristics are representative for most of temperate and boreal lakes [16].

Samples were collected every four weeks from April till October in 2018, and from April till November in 2019. Ten liters of water were collected from 0.5 m depth using a Ruttner Water Sampler (model 11.003KC Denmark AS). Temperature and oxygen profiles were taken with an EXO1 multi-parameter probe (YSI Inc., Yellow Springs, USA). Water was transported to the laboratory within 2 h from the collection in a closed container made from high-density polyethylene, rinsed three times with the sampled water and stored in a cooled box.

Nutrients

Samples were filtered through glass fiber filters with 0.4 μm nominal porosity (GF-5, Macherey-Nagel, Düren, Germany). Concentrations of soluble reactive phosphorus (SRP) were determined spectrophotometrically [17, 18]. Concentrations of nitrate and ammonium were measured according to Procházková [19] and Kopáčkek and Procházková [20]. Dissolved organic carbon (DOC) and dissolved nitrogen (DN) were measured with a TOC 5000 A analyzer (Shimadzu, Kyoto, Japan).

Pigments

Seston from 1.43 to 3.65 L of water was collected onto GF-5 glass fiber filters (diameter 47 mm, Macherey-Nagel). The filters were dried of excess water by gently pressing in a paper towel, and flush frozen in liquid nitrogen. Pigments were extracted and analyzed by HPLC as described in Piwosz et al. [21].

Net primary production (NPP) and community respiration (oxygen measurements)

Oxygen concentration was measured with the Winkler method [22]. It was chosen because it allows O_2 concentration to be measured directly in the water without the need to consider carbonic equilibrium, which is the case when changes in CO_2 concentration is measured [23]. Samples were unfiltered to avoid the removal of particle-associated bacteria and also of free-living AAP bacteria, which tend to be larger than average freshwater bacteria [12]. Glass stoppered Winkler type oxygen bottles (115 mL nominal capacity, VTR glass, Prague, Czechia) were filled with the sampled water directly from the sampler via a rubber tube. Each bottle was first rinsed three times and then filled without the formation of air bubbles. Water was allowed to overflow the neck of the bottle for about 1 min, and the bottle was closed with a glass stopper to avoid air bubbles. The closed bottles were kept in the dark in a cooled box. On the shore, three bottles were selected as T0, and 1.2 mL of manganese (II) chloride solution (concentration 3 mol L^{-1}) was addded, followed by the addition of 1.2 mL of a mixture containing 4 mol L^{-1} of sodium iodide solution and 8 mol L^{-1} of sodium hydroxide solution. These samples were processed in the laboratory within 3 h. The remaining bottles were incubated for 24 h at in situ temperature in the IR-box prepared from the MAKROLON IR polycarbonate sheet (4 mm thickness, Professional Plastics, Inc. Fullerton, CA, USA). These panels have a maximum transmittance of 90% in the infrared region 850–2000 nm, 50% at 780 nm, and 0% <740 nm. Dark bottles were wrapped in tinfoil to cut off all irradiance. Illumination was provided with 40 W incandescent (tungsten filament) lightbulbs delivering approx. 50 Wm^{-2} of IR irradiance, which according to our measurements corresponds to IR irradiation on a sunny day. Bottles for NPP were incubated in white light next to the IR-box. To ensure stable temperature and avoid overheating, the incubations were done in a 60 L water bath and the temperature was controlled with a CTB 06 C cryostat (LABIO a.s., Prague, Czechia).

The incubations were terminated by adding manganese (II) chloride and sodium iodide – sodium hydroxide solutions, as described above for the T0 samples. The bottles were incubated for 1 h in a fridge (4 °C). Subsequently, 2.4 ml of 50% H_2SO_4 was added, and samples were immediately titrated with 0.01 mol L^{-1} $Na_2S_2O_3$ solution. The precise concentration of the $Na_2S_2O_3$ solution was determined each time by titrating the KIO_3 standard. For the samples collected in 2018, titrations were done manually: the samples were titrated until the solution turned pale yellow. Then, 1 mL of 0.1% starch solution was added, and the titration continued until full decolorization was achieved. In 2019, samples were titrated to a monotonic equivalence point using Metrohm 877 Titrino plus equipped with a double Pt-wire coulometric electrode (Herisau, Switzerland). Respiration rates were calculated as a difference between oxygen concentrations at the end of the incubation and T0 samples.

To calculate the balance between the NPP and respiration measured in the dark and in the IR light, we assumed that one mole of O_2 produced or consumed was equivalent to one mole of CO_2. Such assumption may not be always accurate for respiration measurements, but considering the high variability of a respiratory quotient in freshwaters, we decided to adopt the most frequent choice [3]. Values of daily NPP were calculated based on the length of light time from sunrise to sunset on the sampling day (Equation 1 in Supplementary File S1). Values of daily dark respiration were taken directly from the measurements, while values of the daily IR-respiration were calculated as an average weighted for the length of light time to take into account that the effect of light was only during the light time (Equation 2 in Supplementary File S1). Rates were integrated over the duration of the season with water temperatures >10 °C according to our measurements (180 days, Equation 3 in Supplementary File S1). Subsequently, the differences in the Cep Lake's carbon budget for the surface layer (down to 0.5 m depth) were calculated by multiplying the integrated values by the volume of this layer (Equation 4 and 5 in Supplementary File S1).

HCO_3^- incorporation

Triplicated water samples (32 mL) were incubated for 3.2–5.2 h in the IR light and the dark at in situ temperature, as described for respiration. Total activity added to each bottle was measured from 1 mL aliquot of the incubated sample that was transferred to a scintillation vial containing 20 μl of 5 mol L^{-1} NaOH (to prevent a loss of ^{14}C-bicarbonate). Thirty mL of sample was filtered through 2.5 μm nitrate cellulose filters (Pragopor, Prague, Czechia, diameter 25 mm). Five mL of the filtered water was collected and subsequently filtered through a 0.17 μm nitrate cellulose filter. The resulting cell-free filtrate, which contained ^{14}C-DOC was collected. The filtration was done at a low vacuum (0.02 MPa) to avoid cell breakage. The total CO_2 assimilation rate was calculated as the sum of all these fractions.

The filters were kept in an HCl-saturated atmosphere for 24 h at room temperature in a fume hood. They were placed in

scintillation vials and dissolved in 1 mL of ethyl acetate (Penta, Prague, Czechia). Then, 5 mL of Ultima Golt LLT scintillation cocktail (PerkinElmer, Waltham, MA, USA) was added. Five mL of cell-free filtrates were acidified by adding 100 µL 5 mol L^{-1} HCl to volatilize non-incorporated $H^{14}CO_3$ and incubated 24 h at room temperature in a fume hood. Then, 10 mL of the scintillation cocktail was added. Finally, 5 mL of the scintillation cocktail was added to the total activity samples. Subsequently, the samples were gently mixed and left in the dark for 48 h. The radioactivity in the samples was measured using a Tri-Carb 2810 TR scintillation counter (PerkinElmer).

To estimate carbon fluxes (µmol C L^{-1} h^{-1}), a fraction of the added $H^{14}CO_3$ incorporated or released was corrected for the incubation time and multiplied by the concentration of total dissolved inorganic carbon (DIC). The DIC concentration was calculated based on temperature, pH, and alkalinity measurements (Inolab pH 720, WTW Xylem Inc. Rye Brook, NY, US) determined by Gran titration.

Assimilation of organic monomers

The difference between microbial activity in the IR light and dark was also estimated based on assimilation rates of radiolabeled glucose, pyruvate, leucine and thymidine (American Radiolabeled Chemicals, St. Louis, MO, USA). Tritiated glucose (specific activity (SA): 2220 GBq $mmol^{-1}$), leucine (SA: 4440 GBq $mmol^{-1}$) and thymidine (SA: 2275.5 GBq $mmol^{-1}$) were added to 5 mL samples to a final concentration of 5 nmol L^{-1}. ^{14}C-pyruvate (SA: 2.035 GBq $mmol^{-1}$) was added to a final concentration of 10 nmol L^{-1}. Trichloroacetic acid (TCA) was added to the killed controls to a final concentration of 1%. Samples were incubated for 1 h in the dark and IR light as described for respiration. The incubations were terminated as the killed controls and kept at 4 °C in the dark until processed within <4 h. Biomass was collected onto 0.17 µm nitrate cellulose filters as described for HCO_3^- incorporation. The filters were washed twice with 2.5 mL of ice-cold 5% TCA, and then twice with 2.5 mL of ice-cold 80% ethanol [24]. They were placed in the scintillation vials and air-dried overnight. Dried filters were dissolved in 1 mL of ethyl acetate, and 5 mL of Ultima Golt LLT scintillation cocktail (PerkinElmer) was added. Samples were gently mixed and left in the dark for 48 h. The radioactivity in the samples was measured using a Tri-Carb 2810 TR scintillation counter (PerkinElmer).

Bacterial and AAP abundance

Samples of 50 mL were fixed with buffered, sterile-filtered paraformaldehyde (Penta, Prague, Czechia) to a final concentration of 1%, and 0.5 mL was filtered onto white polycarbonate filters (pore size 0.2 µm, Nucleopore, Whatman, Maidstone, UK). Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) at concentration of 1 mg L^{-1} [25]. Total and AAP bacterial abundances were determined using an epifluorescence Zeiss Axio Imager.D2 microscope equipped with Collibri LED module illumination system (Carl Zeiss, Jena, Germany). Ten microphotographs were taken for every sample under 325–370 nm excitation and 420–470 nm emission wavelengths for DAPI fluorescence (total bacteria), 450–490 nm excitation and 600–660 nm emission wavelengths for autofluorescence from Chl-*a* (algae and cyanobacteria), and combined 325–370 nm, 450–490 nm, 545–565 nm and 615–635 nm excitation and 645–850 emission wavelengths for autofluorescence from BChl-*a* (AAP bacteria). As some part of Chl-*a* autofluorescence is also visible in the infrared spectrum, only the IR-positive cells that did not show any autofluorescence from Chl-*a* were counted as AAP bacteria [26].

DNA extraction

Between 300 and 1460 mL of water were filtered through sterile 2 µm and 0.2 µm Nucleopore Track-Etch Membrane filter units (Whatman). The filters were put inside sterile cryogenic vials (Biologix Group Limited, Jinan, Shandong China) containing 0.55 g of sterile zirconium beads, flash-frozen in liquid nitrogen and stored at −80 °C. Total nucleic acids were extracted within a month following the protocol by Nercessian et al. [27]. Lysis buffer (75 µl of 10% sodium dodecyl sulfate (Tokyo Chemical Industry CO, LTD., Tokyo, Japan), 75 µl of 10% N-Lauroylsarcosine (Sigma–Aldrich, St. Louis, USA), 750 µl of phenol-chloroform-isoamyl alcohol (25:24:1; AppliChem GmbH, Darmstadt, Germany) and 750 µl of 10% hexadecyltrimethylammonium bromide (CTAB; Sigma-Aldrich) in 1.6 M NaCl and 240 mM potassium-phosphate-buffer, pH = 8) was added to the vials and they were vortexed for 10 min. After centrifugation for 10 min at 4 °C and 16,000 x g, supernatant was mixed carefully with the equal volume of chloroform (PENTA s.r.o., Prague, Czechia). After the second centrifugation, the supernatant was mixed with two volumes of 30% Poly(ethylene glycol) (PEG; Sigma–Aldrich) in 1.6 M of NaCl, and incubated for 2 h in the dark at 4 °C, followed by centrifugation for 90 min at 4 °C and 17,000 x g. The pellet was washed with 70% ethanol (VWR International S.A.S., Fontenay-sous-Bois, France) and centrifuged again for 1 min. Extracted DNA was re-suspended in 35 µl of DNase and RNase-free water (MP Biomedicals, Solon, OH, USA) and stored at −20 °C. Concentration and quality of the extracts were checked using NanoDrop (Thermo Fisher Scientific).

Bacterial community composition

The V3-V4 region of bacterial 16S rRNA gene was amplified using 341F and 785R primers [28]. PCR was performed in triplicate 20 µL reactions using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, USA) with the following reaction conditions: 98 °C for 3 min, 25 cycles at 98 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s, and a final extension at 72 °C for 3 min. The triplicate product reactions for each sample were pooled andpurified from the gel using the kit Wizard SV Gel and PCR Clean-Up System (Promega, USA), and sequenced on Illumina MiSeq (2 × 250 bp) platform of the Genomic Service of the Universitat Pompeu Fabra (Barcelona, Spain).

Initial analysis, performed as described below, indicated that the communities in both fractions were similar for each sampling day (Bray-Curits similarity >65% except for the samples from 9th May and 29th Aug 2018 (10% each), 1st Aug 2018 (21%), 25th Nov 2018 (1%), and 14th Aug 2019 (22%), Supplementary Fig. S1A). Thus, we decided to concatenate the fastaq files and analyze both fractions together as the total community. This also facilitated statistical analysis, as the activity rates were measured for the whole community without fractionations.

Reads quality was evaluated using FastQC v0.11.7 (Babraham Bioinformatics, Cambridge, UK). After primer sequences trimming using Cutadapt [29] (v1.16), the number of reads per sample ranged from 49,354 to 188,942. Subsequent analyses were done in the R/Bioconductor environment using the dada2 package (version 1.14.1) [30]. Forward and reverse reads were truncated to 225 bp and low quality sequences were filtered out with the filterAndTrim function (truncLen = c(225, 225), maxN = 0, maxEE = c(2, 2), truncQ = 2), which reduced the number of reads per sample to range from 30,190 to 143,552. After merging and chimera removal using the removeBimeraDenovo function, 4,893 amplicon sequence variants (ASV) were obtained. Rare ASVs (not seen >3 times in at least 20% of the samples) were removed, which reduced the number of ASVs to 658, and the number of reads to 14,613–69,046 per sample. Taxonomic assignment was done using SILVA 138.1 database [31, 32] released on August 27, 2020. ASVs identified as Chloroplast or Cyanobacteria were excluded from the analyses, giving the final number of 546 ASVs and from 10,819 to 54,799 reads per sample. The bacterial community composition graphs were done using phyloseq [33] and ggplot2 [34] packages.

AAP community composition

The composition of AAP community was analyzed by amplicon sequencing of *pufM* gene encoding the M subunit of bacterial type-2 reaction centers. This gene is routinely used for diversity studies of AAP bacteria [35].

*Puf*M gene amplicons (approx. 245 bp) were prepared using pufM_UniF (5′-GGN AAY YTN TWY TAY AAY CCN TTY CA-3′) and pufM_WAW (5′-AYN GCR AAC CAC CAN GCC CA-3′) primers [36]. PCR was performed in triplicate 20 µL reactions using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, USA) with the following reaction conditions: 98 °C for 3 min, 27 cycles at 98 °C for 10 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The triplicate product reactions for each sample were pooled and gel purified using the kit Wizard SV Gel and PCR Clean-Up System (Promega, USA). The sequencing was performed on the Illumina MiSeq platform (2 × 150 bp) at Macrogen, South Korea.

The fastq files were concatenated as described for bacteria communities. The Bray-Curtis similarity between two fractions for each sampling day was >70%, except for the samples from 1st Aug 2018 (47%) and 14th Aug 2019 (18%, Supplementary Fig. S1B).

The samples were analyzed as described for bacterial communities. The number of reads per sample ranged from 192,360 to 239,418 after the cutadapt trimming. Forward and reverse reads were truncated to 130 bp, and the number of reads per sample after the quality filtering and denoising ranged from 189,432 to 235,311. Merging the forward and reverse reads with mergePairs function created 12,692 ASVs and reduced the number of reads to 183,136–221,281 per sample. The chimera removal lowered the number of ASVs to 1816, and the number of reads to 159,451–208,679. Rare ASVs (not seen >3 times in at least 20% of the samples) were removed, which resulted in the final 566 ASVs, and a number of reads ranging from 155,915 to 203,021 per sample. A manually curated taxonomic database was used for taxonomic assignment following the naïve Bayesian classifier method [37]. It contained 1580 unique *puf*M sequences, downloaded from the Fungene repository on May 16, 2019 (http://fungene.cme.msu.edu [38]), from metagenomes from the Římov Reservoir [39, 40] and from the Genome Taxonomy database accessed on September 16, 2020 [41].

Statistical analysis

Linear mixed-effects models were calculated in R (version 3.6.2) using lme function from the nlme package (version 3.1.143) on untransformed activity data and $\log_{10}$ transformed environmental variables [42]. Models' parameters were estimated using maximum likelihood method and their significance was tested with ANOVA. Relationships between the activity measures, the environmental variables and the composition of AAP communities were investigated with distance-based linear models (DistLM) [43, 44] in Primer (version 7.0.13) with PERMANOVA + 1 add on (e-Primer, Plymouth, UK) [45]. The sequence reads were transformed with the varianceStabilizingTransformation function of the DESeq2 package [46] (version 1.14.1, blind = FALSE, fitType = "mean").

Data accessibility

The sequences of 16S and pufM amplicons that support the findings of this study have been deposited in the EMBL database as the BioProject with the accession number PRJEB41596, together with most of the environmental metadata. The scripts and the remaining data supporting the results are included in the Supplementary Material.

RESULTS AND DISCUSSION

Microbial activity was the highest during spring phytoplankton maxima, and in summer at temperatures >20 °C (Fig. 1 and Supplementary Fig. S2). The difference between the activity rates measured in the dark and IR light varied over the sampling period and were the highest in April and May in both years (Fig. 1).

The average respiration rate was 15.2% (95% CI: 6.6–23.8%, p value = 0.0008) higher in the dark (average (95% CI): 0.30 (0.25–0.35) µmol O_2 L^{-1} h^{-1}) than in the IR light (0.27 (0.22–3.20) µmol O_2 L^{-1} h^{-1}; Fig. 2A). To exclude the possibility that the observed difference was due to oxygen production by phytoplankton, we also measured incorporation of $H^{14}CO_3^-$ in the dark and the IR light. The bicarbonate incorporation rates were about 5-fold lower than respiration rates (Fig. 1B) and did not differ significantly between the dark and IR light (p value = 0.5646, Fig. 2B). Thus, the lower respiration rates measured in the IR light can be attributed to the photoheterotrophic activity of AAP bacteria and not due to oxygen production by phytoplankton.

The decrease in the respiration rate in the IR light compared to dark was driven by production of ATP from the light energy harvested by the reaction centers, which results in a more efficient metabolism as the organic carbon can be redirected from catabolism to anabolism [7]. Consequently, increased assimilation rates of sources of organic carbon should be observed in the IR light. Such differences ought to be especially conspicuous for molecules that can be directly channeled either to anabolic or catabolic reactions depending on the cells' requirements for ATP production, e.g., glucose and pyruvate [8]. In agreement with this prediction, the average assimilation rate of glucose was 18.1% (7.8–28.4%, p value = 0.0009) higher in the IR light (0.67 (0.51–0.82) nmol C L^{-1} h^{-1}) than in the dark (0.55 (0.42–0.68) nmol C L^{-1} h^{-1}; Fig. 2C). The most noticeable difference was measured in April 2018, when the assimilation rate in the IR light was 9-fold higher than in the dark (Fig. 1C). Likewise, the assimilation rate of pyruvate was enhanced by 9.5% (4.2–14.8%; p value = 0.0006) in the IR light, and averaged at 13.94 (11.10–16.78) nmol C L^{-1} h^{-1} for the IR light, and at 12.67 (10.40–14.95) nmol C L^{-1} h^{-1} for the dark measurements (Fig. 2D).

We also compared assimilation rates of leucine (Fig. 1E). This molecule is used by bacteria for protein synthesis and is routinely applied to measure bacterial secondary production in aquatic environments [24]. The average assimilation rate of leucine in the IR light (0.39 (0.23–0.55) nmol C L^{-1} h^{-1}) was 5.9% (0.1–11.6%; p value = 0.0461) higher than in the dark (0.36 (0.20–0.51) nmol C L^{-1} h^{-1}, Fig. 2E). This further supports the notion that the lower respiration rates in the IR light allowed for more efficient metabolism and increased biomass production by the microbial community. Finally, we also used thymidine (Fig. 1F), which is routinely applied to measure rates of DNA synthesis and to determine growth rates of aquatic bacteria [47]. The exposure to the IR light did not significantly affect thymidine assimilation rates (p value = 0.5601), which were even slightly lower in the IR (0.11 (0.08–0.14) nmol C L^{-1} h^{-1}) than in the dark conditions (0.12 (0.09–0.15) nmol C L^{-1} h^{-1}, Fig. 2F). This agrees with the previous observations that the thymidine assimilation rate is independent of irradiance in a cultured AAP species [8], and might result from the fact that DNA synthesis occurs in evening hours [48].

The effect of the IR light on microbial activity, as compared to dark measurements, varied seasonally (Fig. 1). Such dynamics in aquatic environments often correlate with environmental variables, such as temperature [11]. Thus, we augmented the linear mixed-effects models with measured physico-chemical (temperature, pH, inorganic N and P, dissolved organic carbon), and biological (bacterial and AAP numbers, photosynthetic pigments) variables (Supplementary Fig. S2) to test their correlations with the activity rates, and also to test whether such relationship had been affected by light. Respiration rates, both those measured in the dark and in the IR light, correlated positively only with BChl-*a* concentrations (Fig. 3A), indicating overall high contribution of BChl-*a* containing AAP bacteria to the total community respiration, a phenomenon observed also in other lakes [11, 12]. The slope of this relationships was similar for the dark and the IR rates,

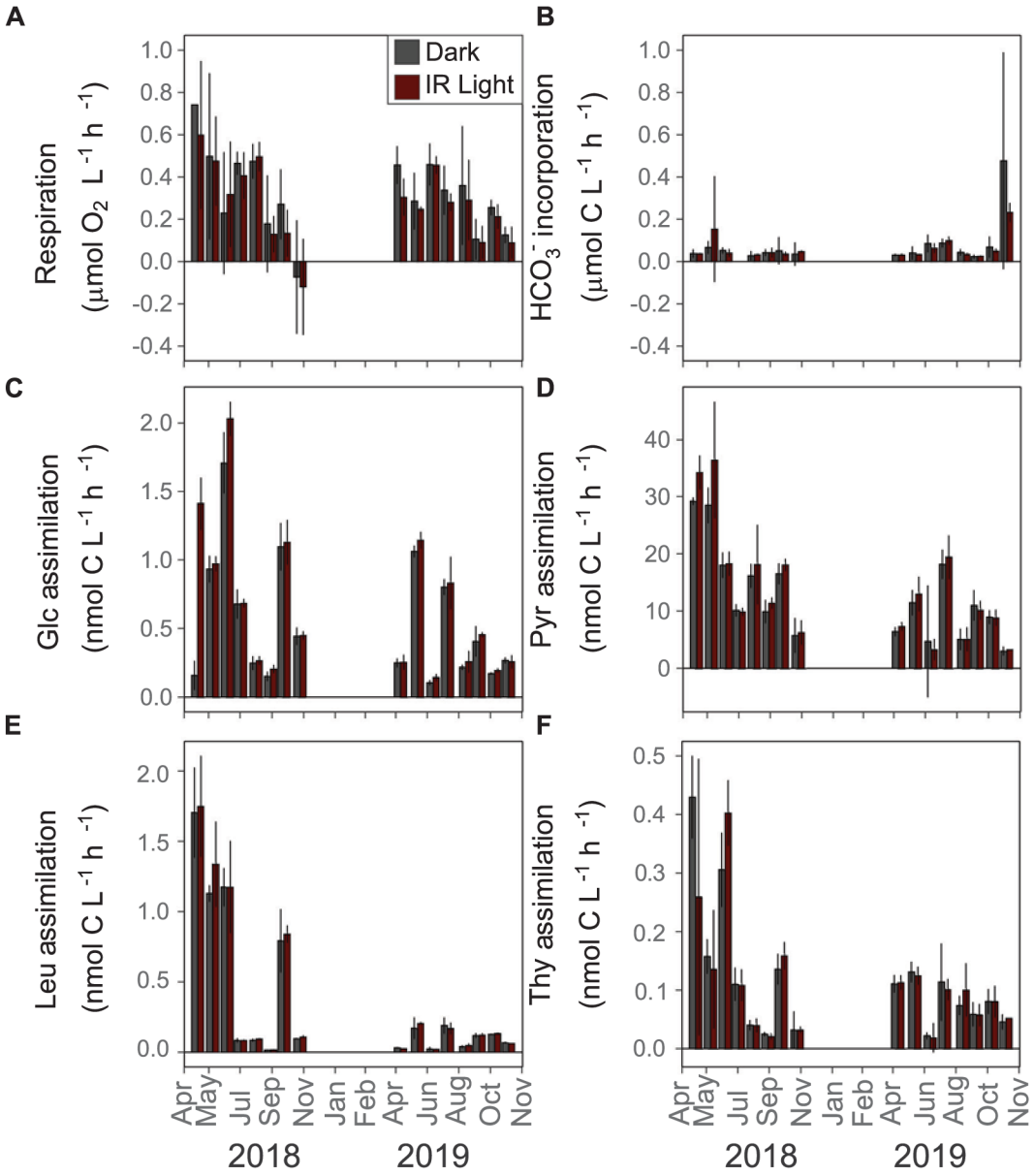

Fig. 1 Microbial activity in the surface layer of Lake Cep measured in the dark (dark gray) and the IR light (dark red). A respiration rate; (**B**) incorporation rate of $H^{14}CO_3^-$; (**C**) assimilation rate of glucose; (**D**) assimilation rate of pyruvate; (**E**) assimilation rate of leucine; (**F**) assimilation rate of thymidine. Bar plots show mean values of triplicate measurements; error bars indicate 95% confidence intervals. Scales on *Y* axes differ between the panels except for panels (**A**) and (**B**).

likely reflecting the fact that the rate of ATP production by photophosphorylation depends, among others, on the number of BChl-*a* containing reaction centers that generate electrons flow [8]. AAP species differ with regard to the number of BChl-*a* molecules in reaction centers [49], which may explain the lack of significant correlation between the concentrations of BChl-*a* and the absolute abundances of AAP bacteria (Pearson correlation test, p value = 0.05902, $R^2 = 0.4665878$, $t = 2.0431$), and the concentrations of BChl-*a* and the relative abundances of AAP bacteria (p value = 0.07377, $R^2 = 0.4445804$, $t = 1.9223$). Therefore, the lack of direct relationship between the respiration rates and AAP bacteria abundance does not contradict our conclusion on their importance in total microbial respiration.

Assimilation rate of glucose negatively correlated with concentrations of ammonia, however, the correlation was stronger for the rates measured in IR light than those measured in the dark (Fig. 3B). This indicates that although such relationship was important for both heterotrophic and photoheterotrophic bacteria assimilating glucose, it affected AAP bacteria more strongly. In agreement with this interpretation, glucose assimilation rates measured in the IR light (but not in the dark) correlated positively with the relative abundance of AAP bacteria (Fig. 3C). Similarly, the correlations between the relative AAP abundance and assimilation rates of pyruvate (Fig. 3D) differed for dark and IR light measurements. The significantly steeper slopes of these correlations for the rates measured in IR light indicate not only the overall high importance of AAP bacteria for assimilation of glucose and pyruvate, but also that their contribution was additionally enhanced in the light. This supports attributing the observed differences between the dark and the IR rates of microbial activities to photoheterotrophic metabolism by AAP bacteria.

Interestingly, while catabolic processes correlated with BChl-*a* concentration, anabolic were related with relative abundance of AAP bacteria. The former relationship can result from direct

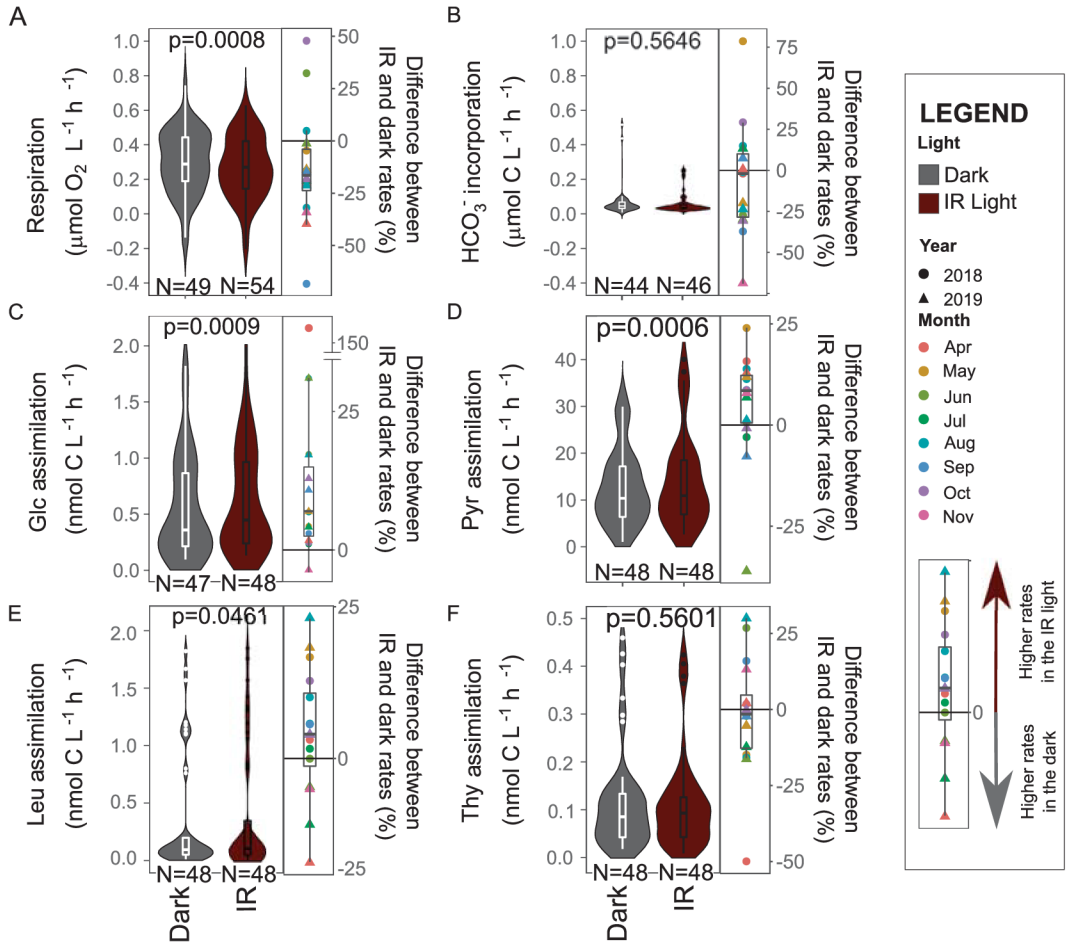

Fig. 2 Difference between microbial activity measured in the dark and IR light. Violin plots with a box-plot inside compare the distribution of the data for the dark and IR measurements (p: *p* value from the ANOVA test of significance of the linear mixed-effects model with the date as the random variable. N: number of independent measurements). Box-plots show percent difference between mean rates measured in the dark and in the light ($N = 16$) for each sampling day. **A** respiration rate; (**B**) incorporation rate of $H^{14}CO_3^-$; (**C**) assimilation rate of glucose; (**D**) assimilation rate of pyruvate; (**E**) assimilation rate of leucine; (**F**) assimilation rate of thymidine.

dependence of photophosphorylation rate on electrons production on reaction centers that supplements the catabolic ATP production via respiration [7, 8], as explained above. On the other hand, the positive relationship between the anabolic metabolism and the relative abundance of AAP bacteria might indicate their generally high ability to assimilate organic compounds. The use of organic molecules substantially varies between different bacterial lineages [50]. Even closely related strains of an important freshwater AAP species *Limnohabitans planktonicus*, II-D5 and 2KL-16 [51], show different growth response to the same organic substrates [52]. This indicates that in situ microbial photoheterotrophic activity may also depend on AAP community composition [21, 53], which was tested statistically. The distance based linear model (DistLM) that best explained variability in the AAP community composition included AAP abundance ($p = 0.0025$, Pseudo-F = 3.9203, 21.9% of the explained variability), water temperature ($p = 0.0001$, Pseudo-F = 4.8571, 21.2% of the explained variability), and respiration rate measured in the IR light ($p = 0.0005$, Pseudo-F = 3.3375, 12.4% of the explained variability). The respiration rate measured in the dark was not statistically significant factor in any of the calculated models, even when the rates in the IR light were intentionally excluded from the analysis. Higher AAP abundance and respiration rate in the IR light were associated with an increased contribution of alphaproteobacterial orders Caulobacterales (genus *Aquidulcibacter*) and Sphingomonadales (genus *Sandarakinorhabdus*) and genus *Rhodoferax* (Burkholderiales, Gammaproteobacteria) to AAP communities (Fig. 4A). In contrast, genus *Limnohabitans* and other unclassified Burkholderiales [54] showed higher relative abundance in summer or in autumn, when the differences between the microbial activity measured in the dark and in the IR were the lowest (Fig. 1, Fig. 4A, Supplementary Fig. S3). The observed variability in the bacterial community was explained in 23.1% by the abundance of AAP bacteria ($p = 0.0006$, Pseudo-F = 4.2097) and in 17.8% by temperature ($p = 0.0001$, Pseudo-F = 3.9078; Fig. 4B). This confirms the overall importance of photoheterotrophy in the community metabolism, extend of which depends not only on the abundance of AAP bacteria, but also on the taxonomic composition of their communities.

The key result of our study is that the dark incubations substantially overestimate microbial respiration and underestimate microbial production rates in lake surface waters, challenging a current paradigm for measuring microbial activity in aquatic environments. Moreover, we demonstrated high importance of photoheterotrophy by AAP bacteria in freshwater pelagic environments, confirmed over two years using different, independent measures of microbial in situ activity, and pointed to the factors that may drive it. However, AAP bacteria are not the only photoheterotrophic microorganisms in freshwater lakes. Many bacteria produce ATP in light using rhodopsin. Actinobacteriota from acI clade, a group that often dominates bacterial communities in freshwater lakes [55], contains actinorhodopsin [56, 57]

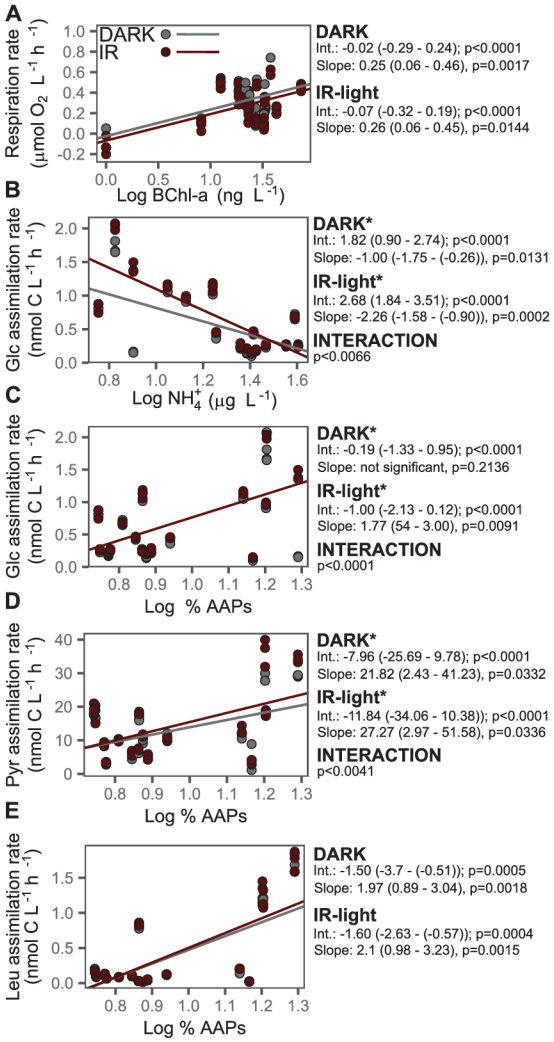

Fig. 3 Correlations between microbial activity rates and environmental variables. Average and 95% confidence intervals are given for intercept (Int.) and slope values. Star indicates an interaction with the light (i.e. significantly different correlation coefficients for the rates measured in the dark and the IR light). Correlations between (**A**) concentrations of bacteriochlorophyll-a and respiration rate; (**B**) concentrations of ammonia and assimilation rates of glucose; and between relative abundance of AAP bacteria and assimilation rate of glucose (**C**), pyruvate (**D**) and leucine (**E**).

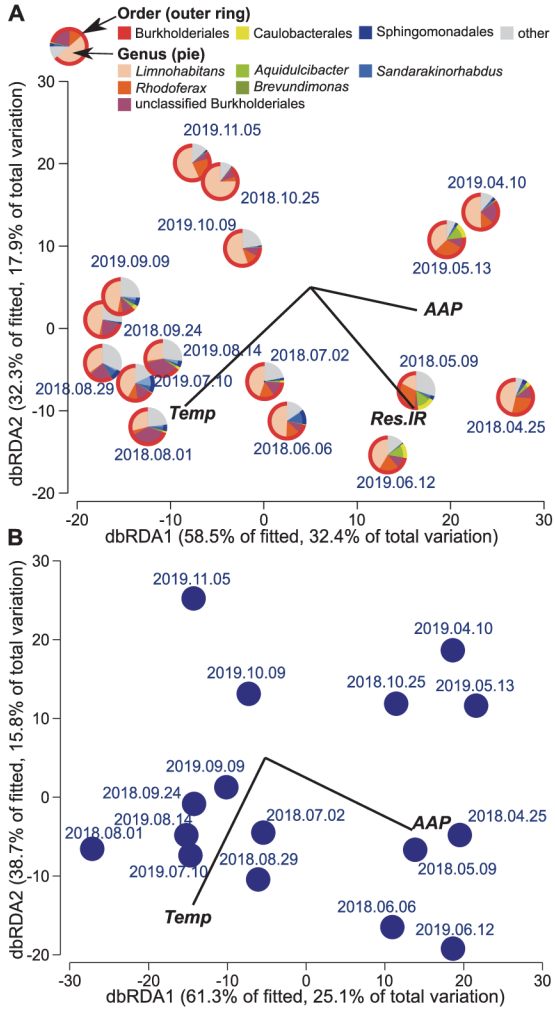

Fig. 4 Relationships between bacterial and AAP community composition and environmental variables. Distance-based redundancy analysis biplots relating the observed variability in the composition of communities of AAP (**A**) and all bacteria (**B**) to the explanatory variables (black lines). The pie charts in the panel (**A**) show relative abundance of the top three AAP orders (bar plots showing composition of AAP and bacterial communities can be found in Supplementary Figs. S3 and S4). AAP: abundance of AAP bacteria, Res.IR: respiration rate in the IR light, Temp: water temperature.

that have ion-pumping activity in green light and are highly expressed in situ [58]. The net energy gain from rhodopsin-based phototrophy is lower than from bacteriochlorophyll-based photosystems of the AAP bacteria [59]. Nevertheless, their contribution to the lake photoheterotrophy might have been substantial, as Actinobacteriota were a dominant group of bacteria in both sampling season (Supplementary Fig. S4A). Unfortunately, actinorhodopsins absorb green light that is also used by oxygenic photoautotrophs. Thus, their photoheterotrophic activity cannot be as easily measured by applying a specific light wavelength as in the case of AAP bacteria. In our previous experiment, we used (3-(3,4-dichlorophenyl)−1,1-dimethylurea) to block photosystem II in oxygenic phototrophs without affecting bacteria [21]. However, a strong inhibition of the dark bacterial respiration was observed in this treatment, indicating some detrimental effect on bacterial activity and hampering the use of such approach to environmental studies. Picocyanobacteria are another group of microorganisms that assimilate amino acids in the dark, as observed for marine *Prochlorococcus* [60]. This does not seem to be the case for picocyanobacteria in lakes [50], whose communities in our study were dominated by an obligate photoautotroph *Cyanobium* [61]. Finally, the fact that we had to use IR light to avoid confounding effect from oxygenic photosynthesis might have lowered photoheterotrophy even by AAP bacteria, as they can also use light in the visible spectrum (400–600 nm). Taken together, our results are the lower estimates for the importance of the photoheterotrophy in freshwater lakes.

Nevertheless, they alter the current understanding of carbon fluxes at the ecosystem level. For instance, the balance between the net primary production (NPP) and bacterial respiration (BR) determines whether a system is heterotrophic, i.e., releases CO_2 to the atmosphere (NPP < BR), or autotrophic, i.e., absorbs CO_2 from the atmosphere (NPP > BR). This balance is negative for most freshwater lakes, making them an important source of CO_2 to the atmosphere at the global scale [1]. However, as we have shown here, dark measurements overestimate microbial respiration, resulting in biased carbon budget for lakes. Calculations of the carbon fluxes for the surface layer of the Lake Cep indicated that it is heterotrophic both in

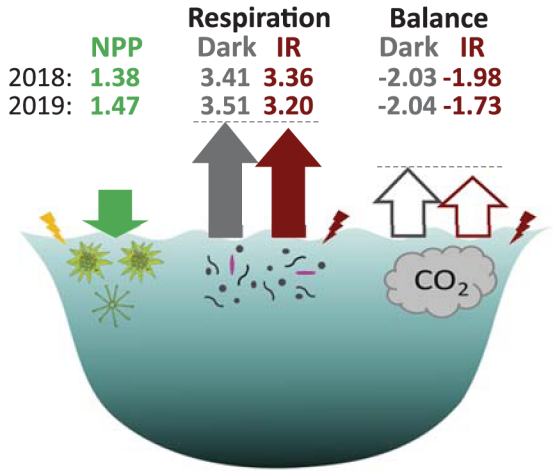

Fig. 5 Comparison of carbon fluxes for the surface layer of the Lake Cep measured in the dark and the infrared light. CO_2 fluxes (10^{10} g CO_2 over the season with water temperature >10 °C) from net primary production (NPP, green color) and respiration (Res) for the surface layer (0.5 m) of the whole area of the lake Cep based on dark (gray color) and infrared (red color) measurements. Lengths of the arrows are scaled to reflects the differences, and the dash lines are meant to aid direct comparison between the arrows. The difference between the excessive CO_2 from the respiration based on dark vs infrared (IR) measurements was 0.54×10^9 g CO_2 in 2018 and 3.15×10^9 g CO_2 in 2019, summing up to 3.69×10^9 g CO_2 over these two years. Lightning indicate energy from light, pink microbe indicates AAP bacteria.

case of dark and IR measurements (Fig. 5). However, the amount of excessive CO_2 during months with water temperature >10 °C (April-October) was significantly lower for the respiration measurements in the IR light compared to dark (p value = 0.0255). The estimates of CO_2 released during these months based on the IR measurements were lower by 0.54×10^9 g CO_2 in 2018 and by 3.15×10^9 g CO_2 in 2019 (Fig. 5). These numbers illustrate the potential impact of photoheterotrophic activity by AAP bacteria in freshwater lakes. More measurements of microbial activity in the IR light instead of in the dark, and especially in habitats where AAP bacteria are abundant, such as the coastal areas of the ocean or mountain lakes [35], will improve estimates of microbial respiration and production, providing a comprehensive understanding of the role of photoheterotrophy in the global carbon cycle.

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ACKNOWLEDGEMENTS

Authors thank Michal Koblížek for providing the idea of discriminating between the AAP and bulk bacterial activity using IR light, Petr Šmilauer for assistance with statistical analysis, Vít Náhlík and Jason Dean for help during the sampling campaign, Petr Porcal for measuring nutrients concentrations, Kasia Huzarska from malaidea.pl for drawing of phytoplankton (Fig. 5), Alastair Gardiner, Michaela M. Salcher, and Josefa Antón for commenting on the paper. This study was supported by a junior project no. 18–14095Y from the Czech Science Foundation awarded to KP.

AUTHOR CONTRIBUTIONS

KP conceived the project, performed measurements of respiration and assimilation rates of radioactive compounds, microscopic analysis, analyzed the data, prepared the figures, and wrote the paper. CVA helped with sample collections and writing and commenting on the paper, prepared the samples for sequencing, analyzed the sequencing data and prepared figures. IM helped with sample collections, did the DNA extractions, and commented on the paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41396-021-01142-2.

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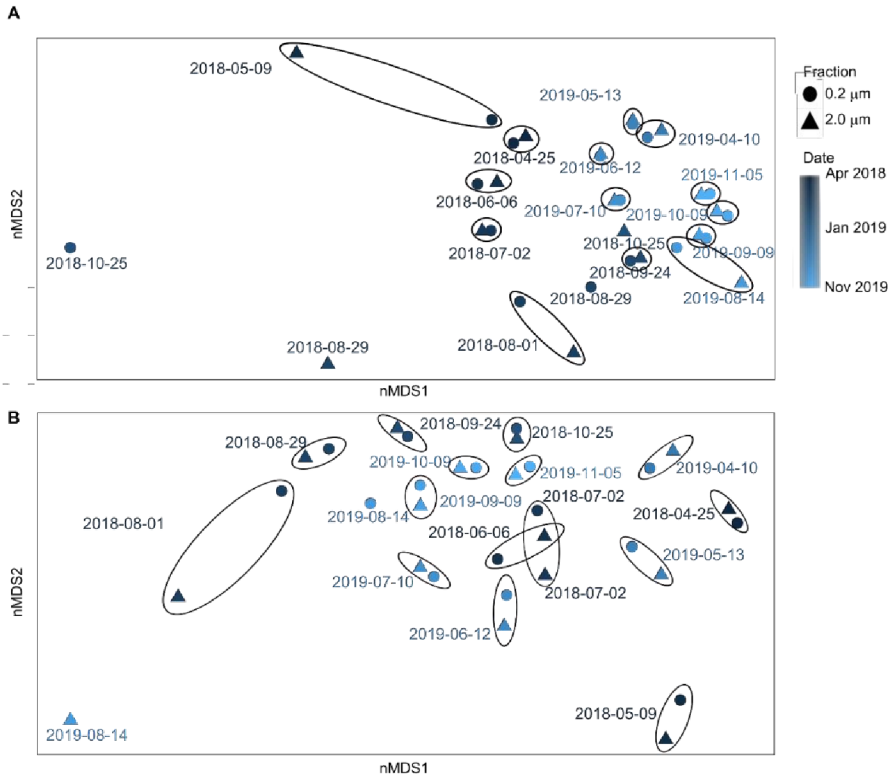
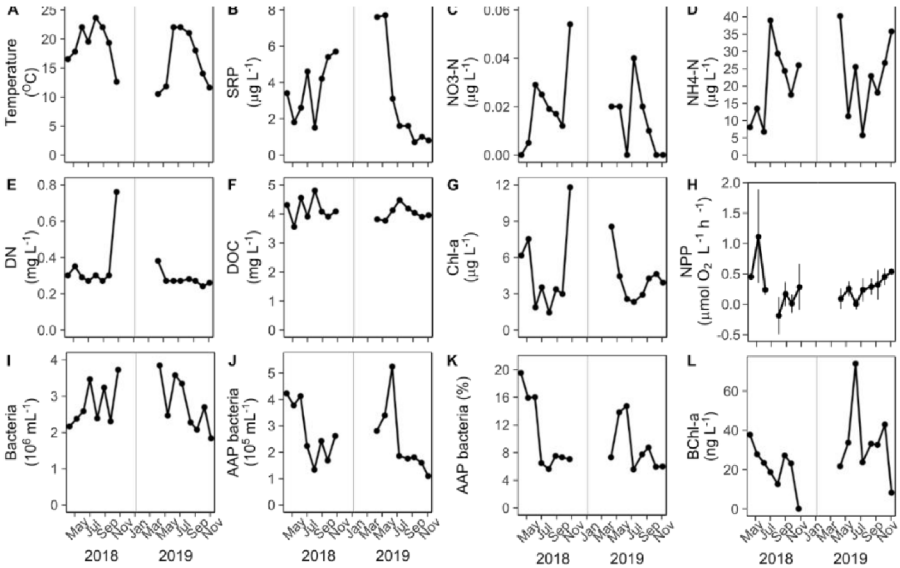
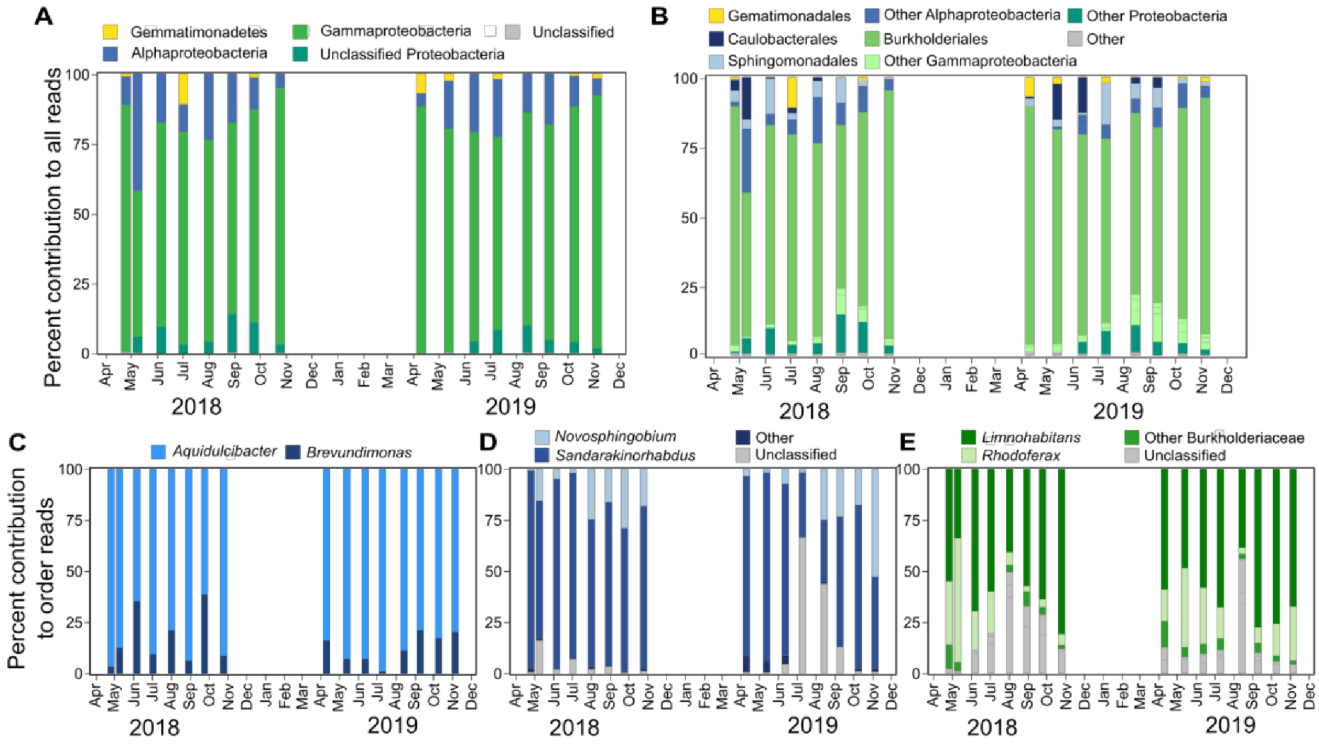
Supplementary Figures



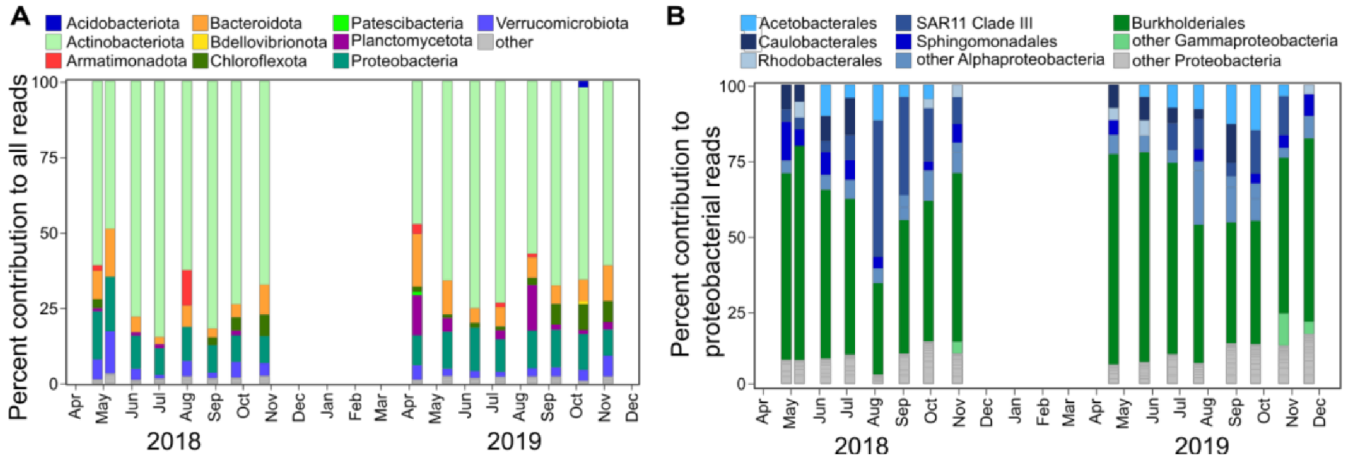
Supplementary Figure S1. Nonmetric multidimensional scaling plots of total (A) and AAP (B) bacterial communities in different size fractions. Plots were calculated using Bray-Curtis distances.



Supplementary Figure S2. Environmental variables. A: Temperature; B: concentration of soluble reactive phosphorous (SRP); C: concentration of nitrate (NO3-N); D: concentration of ammonia (NH4-N); E: concentration of disolved nitrogen (DN); F: concentration of disolved oranic carbon (DOC); G: concentration of chlorophyll-*a* (Chl-a); H: Net primary production (NPP). Dots show mean values of triplicate measurements, error bars indicate 95% confidence intervals; I: abundance of all bacteria; J: abundance of AAP bacteria; K: relative abundance of AAP bacteria; L: concentration of bacteriochlorophyll-*a* (Bchl-*a*).



Supplementary Figure S3. Composition of AAP bacteria communities (based on the *puf*M gene amplicons). A: percent contribution of clases to the total number of reads in the sequencing libraries; B: percent contribution of orders to the total number of reads in the sequencing libraries; C: percent contribution of genera to the number of reads coming from order Caulobacterales (Alphaproteobacteria); D: percent contribution of genera to the number of reads coming from order Sphingomonadales (Alphaproteobacteria); E: percent contribution to the number of reads coming from order Burkholderiales (Gammaproteobacteria).



Supplementary Figure S4. Composition of bacterial communities (based on 16S rRNA gene amplicons). A: percent contribution of the top 10 most abundant phyla to the total number of reads in the sequencing libraries; B: percent contribution of proteobacterial orders to the number of reads coming from Proteobacteria.

Chapter IV

Winners in good times and bad times: Aerobic Anoxygenic Phototrophic bacteria profit from heterotrophy under carbon-poor and rich conditions

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Manuscript under revision in FEMS Microbial Ecology
(IF=4.519)

Chapter IV: Context and objectives

Understanding how the nature of carbon compounds triggers the phototrophic capability in AAP bacteria is a persistent topic of discussion and research. AAP bacteria may have an advantage over other heterotrophic bacteria in oligotrophic environments, requiring less carbon for their metabolism due to the additional energy gained from light. Conversely, phototrophy confers an advantage to AAP bacteria over other heterotrophic bacteria in carbon-rich conditions, facilitating better assimilation and faster growth rates. Additionally, the extra energy obtained from light may allow AAP bacteria to more efficiently use low energy carbon compounds and complex carbon molecules such as recalcitrant carbon (rDOC). To address this recurrent discussion, we aimed to investigate the following questions:

- Do AAP bacteria thrive in carbon-limiting conditions?
- Does energy produced from light empower AAP bacteria to access low-energy or recalcitrant carbon sources more efficiently?

Chapter IV: Summary

Bacterial communities from oligotrophic freshwater Cep lake were sampled in summer and autumn to experimentally test the aforementioned questions. These bacterial communities underwent three different carbon regimes: control, carbon limitation and recalcitrant carbon (rDOC - lignin) in summer or low energy carbon (acetate) in autumn. All three conditions were replicated in dark and in infrared light to trigger AAP bacteria phototrophy.

Our results, consistent with previous studies, showed that AAP bacteria is a highly heterogenetic group. Since their community composition is continually changing so do their responses with regard to the carbon sources. In Spring, the AAP bacterial community showed a positive response to the carbon-limited condition indicating that AAP bacteria efficiently thrives in environments with lower carbon concentrations, typical of oligotrophic environments but they outcompete heterotrophic bacteria when carbon is available. Conversely, the AAP community did not profit from phototrophy in October when their growth was not affected by infrared light. Finally, AAP bacteria suffered negative impact from low-energy carbon and rDOC sources, suggesting potential ecosystem-level implications as human-

induced lake-browning continues to rise. This last observation was unexpected and the potential mechanisms remain a mystery at the moment.

Winners in good times and bad times: Aerobic anoxygenic phototrophic bacteria profit from photoheterotrophy under carbon-rich and poor conditions

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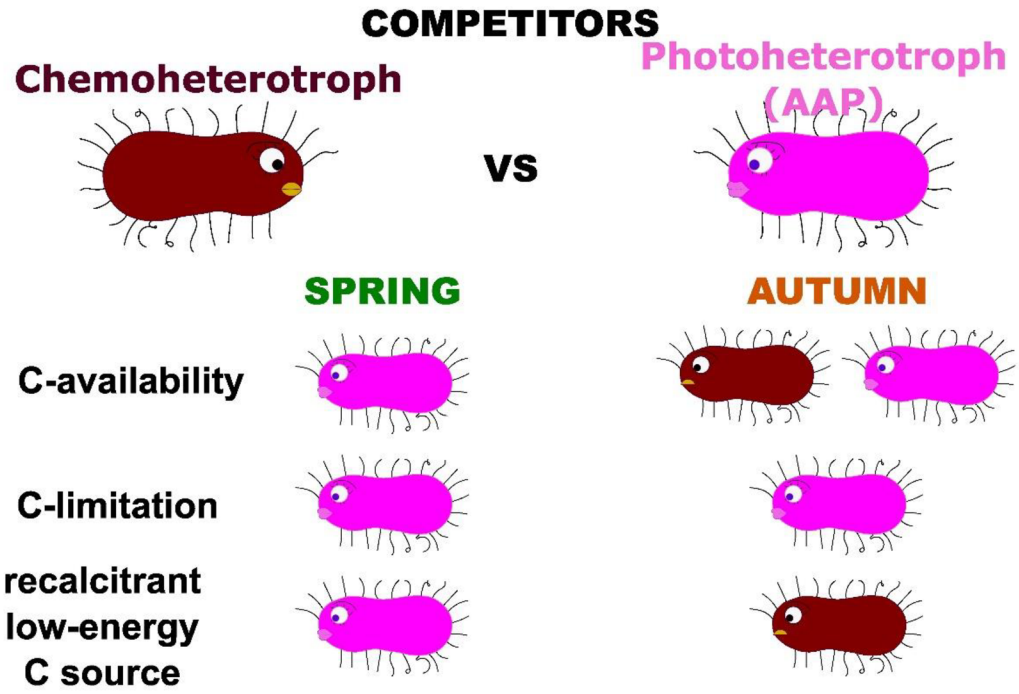
Keywords: Aerobic anoxygenic phototrophic bacteria, microbial ecology, freshwater lakes, carbon limitation, lignin, acetate



Abstract

Aerobic Anoxygenic Phototrophic (AAP) bacteria are an important component of freshwater bacterioplankton. They can support their heterotrophic metabolism with energy from light, and by that enhance their growth efficiency. Based on results from cultures, it was hypothesized that photoheterotrophy provides an advantage under carbon limitation and facilitates access to recalcitrant or low-energy carbon sources. However, verification of these hypotheses for natural AAP communities has been lacking. Here, we conducted whole community manipulation experiments and compared the growth of AAP bacteria under carbon limited and with recalcitrant or low-energy carbon sources under dark and light conditions to elucidate how they profit from photoheterotrophy. We found that it depends on the season. In spring, AAP bacteria induce photoheterotrophic metabolism under carbon limitation but they outperform heterotrophic bacteria when carbon is available. This effect seems to be driven by physiological responses rather than changes at the community level. In autumn photoheterotrophy is less beneficial. In both seasons, AAP bacteria responded negatively to recalcitrant or low-energy carbon sources in light. This unexpected observation may have ecosystem-level consequences as lake browning continues. In general, our findings contribute to the understanding of the dynamics of AAP bacteria observed in pelagic environments.

Introduction

Photoheterotrophic bacteria are an abundant part of bacterioplankton. These organisms depend on organic matter for their growth but they can supplement their energy requirements by light. One of the main photoheterotrophic groups is Aerobic Anoxygenic Phototrophic (AAP) bacteria, which harvest light by bacteriochlorophyll and carotenoid molecules bound to photosynthetic complexes to produce ATP via cyclic photophosphorylation (Okamura *et al.*, 1986, Yurkov & Beatty, 1998). Therefore, when illuminated, AAP bacteria can minimize the use of organic substrates for respiration and utilize them instead for building biomass (Hauruseu & Koblížek, 2012, Piwosz *et al.*, 2018b, Koblížek *et al.*, 2020).

AAP bacteria were discovered in coastal marine waters (Shiba *et al.*, 1979, Shiba *et al.*, 1991). Later, they were also found to be common in the open ocean (Kolber *et al.*, 2001), where they typically represent 1-10% of total bacteria (reviewed in Koblížek 2015). Initially, it was hypothesized that the photoheterotrophy represents an advantage in nutrient-poor oceans, which seems to be correct for rhodopsin-containing photoheterotrophs, but AAP bacteria prefer more productive coastal areas (Gómez-Consarnau *et al.*, 2019, Vrdoljak Tomaš *et al.*, 2019).

AAP bacteria are also commonly found in freshwater lakes, representing from < 1 to > 30% of total bacteria, with strong seasonality (Yurkov & Gorlenko, 1990, Masin *et al.*, 2008, Čuperová *et al.*, 2013, Lew *et al.*, 2015, Kolářová *et al.*, 2019). AAP cells are on average larger than heterotrophic bacteria, thus they contribute more to the total bacterial biomass than their abundance alone would indicate (Fauteux *et al.*, 2015). They are also more active and have higher growth rates (Cepáková *et al.*, 2016; Garcia-Chaves *et al.*, 2016) thus they play an important role in the microbial food webs both as consumers of phytoplankton-derived dissolved organic matter (Piwosz *et al.*, 2020) and as a food source for bacterivores (Ruiz-González *et al.*, 2020). Moreover, infra-red (IR) light exposure (selectively absorbed by AAP bacteria) reduced the total microbial respiration and enhanced total microbial production (Piwosz *et al.*, 2022). The extent of the effect varied over the season. The driving factors likely included seasonal succession of phytoplankton, which may significantly affected the spectrum and availability of organic substrates in the lake, and large seasonal changes in AAP community composition, which may have affected its functioning (Villena-Alemany *et al.*, 2023a). However, how the additional energy from light is utilized to provide an advantage for AAP bacteria in

the environment remains unknown. In addition to the aforementioned hypothesis on survival in the oligotrophic environment, it was also suggested that photoheterotrophy may help AAP bacteria to access low energetic and recalcitrant carbon sources (Salka *et al.*, 2014, Koblížek, 2015).

Here, we conducted the whole microbial community manipulation experiment from a freshwater lake to test hypotheses that (i) the additional energy from light provides AAP bacteria additional energy under carbon limiting conditions, and (ii) the additional energy from light allows AAP bacteria to access low energetic or recalcitrant carbon sources. The incubations were done in the dark and in the near-infrared (IR) light (λ>800 nm), which selectively excited the infra-red *in vivo* absorption band of bacteriochlorophyll (Kasalický *et al.*, 2018, Kopejtka *et al.*, 2020). To all other microorganisms, both conditions were perceived as dark. We followed the bulk growth rates of heterotrophic and AAP bacteria in dark vs IR-light in the conditions of (i) organic carbon limitation, (ii) low energetic or recalcitrant organic carbon source, and (iii) natural organic carbon availability (control). We expected that in the conditions that favour photoheterotrophic metabolism, AAP bacteria would grow faster in the IR light and as a result they would increase their contribution to the total bacterial abundance Moreover, to account for the metabolic differences between different AAP phylotypes, we also followed the changes in their community.

Material and methods

Setting up the experiment

We conducted two experiments: in June and October 2018, to account for seasonal differences in bacterial and AAP community composition (Villena-Alemany *et al.*, 2023a). Water was collected from 0.5 m depth of the meso-oligotrophic freshwater lake Cep (48°55'29.7"N, 14°53'12.5"E) using a Ruttner Water Sampler (model 11.003KC Denmark AS) on the 20th of June 2018 and the 1st of October 2018. It was transported to the laboratory within half an hour in a closed plastic container, which was pre-rinsed three times with the sampled water and stored in a cool box.

Two different treatments were prepared: carbon limitation (C-limited) and recalcitrant organic carbon source (lignin) in June and low-energy organic carbon source (acetate) in October (Fig. 1). For the C-limited treatment, the untreated water from the lake was diluted at a 1:4 ratio with unamended sterile inorganic basal (IBM) medium (Hahn *et al.*, 2003). For the lignin/acetate treatments, the untreated water from the lake was diluted 1:4 with a sterile IBM medium containing 2.5 mg L^{-1} of dissolved lignin (in the June experiment) or 3.0 mg L^{-1} of acetate (in October). The media were prepared during the week before the experiment. They were filtered through a 0.2 μm filter and autoclaved. As a control, we used the untreated water from the lake diluted 1:4 with sterile filtered lake water that was collected three days before the experiment. It was sequentially prefiltered through a 20 μm plankton net, 0.2 μm filter, and 1-litre Stericup® Filter Units with a membrane pore size of 0.1 μm (Millipore, Merck) and kept in the dark at 4°C until the experiment. The dilution allowed for an increase in the carbon availability for bacteria and to reduce the grazing pressure from protistan grazers.Each treatment was divided into six 2-litre portions that were incubated in the dark or IR light at *in situ* temperature (21°C in June and 17°C in October) in triplicates. Samples were taken every 12 hours in June and every 24 hours in October.

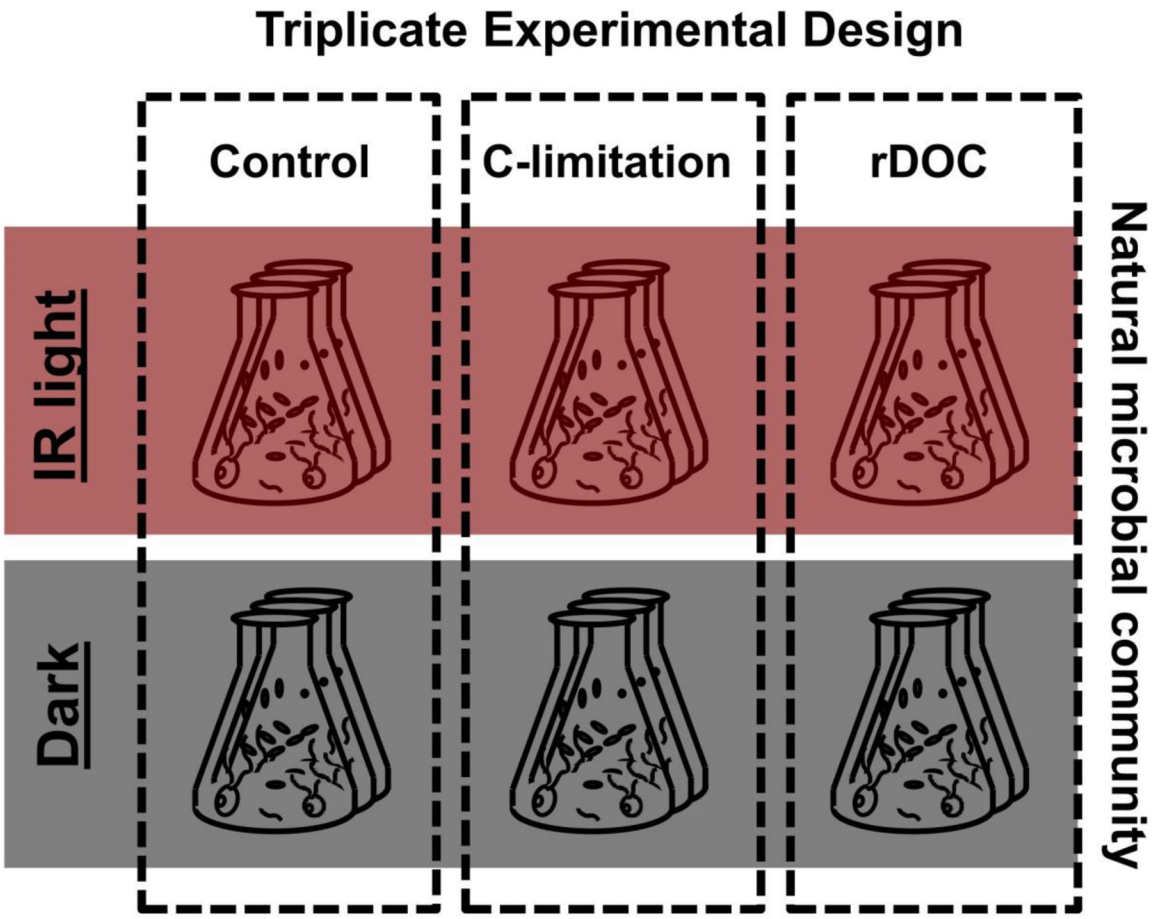


Figure 1. Experimental design. Natural microbial communities were diluted 1:4 with sterile filtered lake water (Control), sterile inorganic medium (C-limited) or sterile inorganic medium containing lignin (in June) or acetate (in October) as carbon source, and incubated in the dark or under infrared illumination (IR light). All treatments were performed in triplicate.

Bacterial and AAP abundance

Samples of 50 mL were fixed with buffered, sterile-filtered paraformaldehyde (Penta, Prague, Czechia) to a final concentration of 1%, and 0.5 mL was filtered onto white polycarbonate filters (pore size 0.2 µm, Nucleopore, Whatman, UK). Cells were stained with 4',6-diamidino-2-phenylindole at a concentration of 1 mg L^{-1} (Coleman, 1980). Total and AAP bacterial abundances were determined using an epifluorescence Zeiss Axio Imager.D2 microscope (Cottrell *et al.*, 2006, Villena-Alemany *et al.*, 2023a). The abundance of heterotrophic bacteria was calculated as the difference between the total bacteria and AAP bacteria.

DNA extraction, amplicon preparation and sequencing

About 350 mL of water was filtered onto sterile 0.2 μm Nucleopore Track-Etch Membrane filters (Whatman, Maidstone, United Kingdom). Filters were placed inside sterile cryogenic vials containing 0.55 g of sterile zirconium beads, flash-frozen in liquid nitrogen and stored at −80 °C until DNA extraction (< 6 months). Total nucleic acids were chemically extracted according to Griffiths et al. (2000) with modifications (Nercessian et al. 2005). Extracted DNA was re-suspended in 35 μl of DNase and RNase-free water (MP Biomedicals, Solon, OH, USA) and stored at −20 °C. The concentration and quality of the extracts were checked using a NanoDrop (Thermo Fisher Scientific). A pure culture of *Dinoroseobacter shibae* was used as a control for cross-contamination between the samples.

Amplicons for the *puf*M gene (a marker gene for AAP bacteria) were prepared using *puf*M UniF and *puf*M UniR primers (Yutin *et al.*, 2005). The PCR conditions were as follows: initial denaturation for 3 min at 98°C, 30 cycles of 98°C for 10 s, 52°C for 30 s, 72°C for 30 s, and final elongation at 72°C for 5 min. PCR was performed in 20 μL triplicate reactions using Phusion™ High-Fidelity PCR MasterMix (ThermoFisher Scientific, USA).

The triplicate reactions for each sample were pooled and purified from the gel using the Wizzard SV Gel and PCR clean system (Promega) and quantified using the Qubit dsDNA HS assay (ThermoFisher Scientific). Amplicons were pooled in equimolar amounts and sequenced on the Illumina MiSeq (2x250 bp) platform at the Genomic Service of the Universitat Pompeu Fabra (Barcelona, Spain).

Analysis of amplicon data

Reads were quality-checked using FastQC v0.11.7 (Babraham Bioinformatics, Cambridge, UK). Primer sequences were trimmed in Cutadapt v1.16 (Martin, 2011). Subsequent analyses were done in the R/Bioconductor environment using the DADA2 package (version 1.12.1) (Callahan *et al.*, 2016). *puf*M sequences were processed and assigned using reference database and methods described in Villena-Alemany *et al.* (2023b). The contamination in the *D. shibae* culture was about 1%. To remove this contamination, the final ASVs tables consisted of ASVs with the sum of reads in all samples > 10 and present in at least two replicates in a treatment at a given time point, or with the sum of reads in all samples > 10 and present in at least three time points in a given replicate in a treatment (Piwosz *et al.*, 2018a).

Statistical analysis

Growth rates were calculated as linear fit coefficients on abundance data transformed with natural logarithm. Differences between incubation in the dark and IR treatment at the end of the experiment were tested with the Welsh t-test. The p-value was adjusted for multiple tests using Bonferroni correction, and the significance of the results was assumed for $p<0.01$. The distribution of the data was tested with the Shapiro-Wilk test. The changes in ASVs' reads abundance between control and C-limited, and control and Lignin/Acetate treatments in the IR light at the end of the experiments were tested using the DESeq function (test="Wald", fitType="parametric") from DESeq2 package (version 1.36.0). All analyses were done in Rstudio for Windows (version 2023.03.1+446; R version 4.2.0 (The R Core Team, 2015).

Data availability

The sequences were deposited in the EMBL database under Biosamples ERS17465032-ERS17465210 and ERS17468627 in the BioProject PRJEB71033.Count data are available as Supplementary Table S1.

Results

June experiment

Heterotrophic bacteria grew the fastest in the control treatment (IR light: 0.31±0.03 d^{-1}, dark: 0.25±0.05 d^{-1}, $p=0.57$), where they almost doubled within 56 h (Fig. 2a). The growth rate was

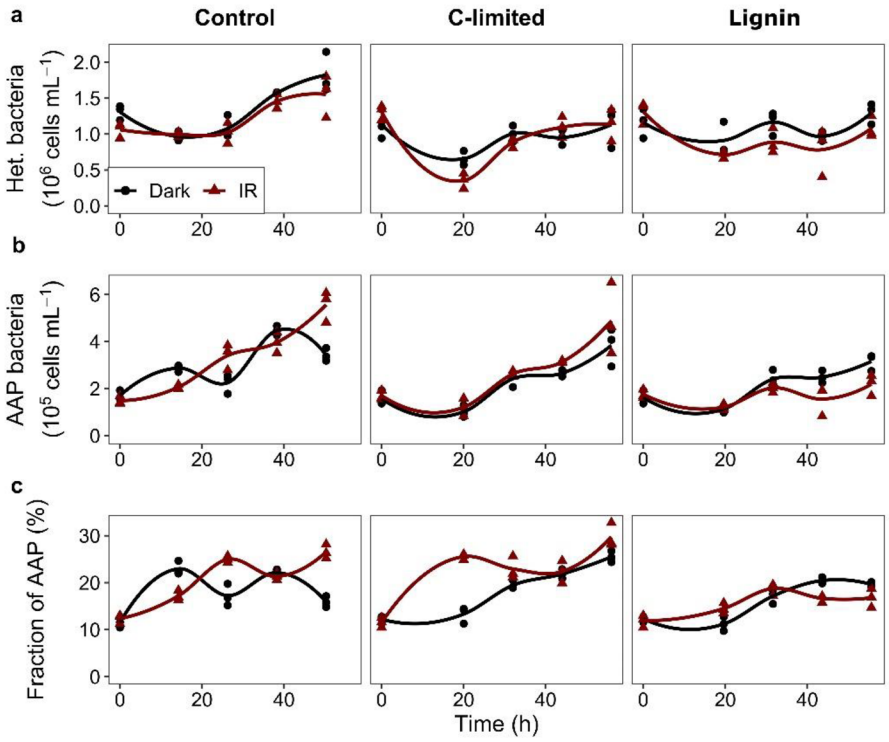


Figure 2. Abundance of heterotrophic bacteria (a); AAP bacteria (b) and contribution of AAP bacteria to total bacterial numbers (c) in the June experiment. Values for each triplicate are shown as points, and the line was fitted locally using the loess function from the ggplot2 package in R.

slower in the C-limited (IR light: 0.26±0.13 d^{-1}, dark: 0.16±0.03 d^{-1}, p=0.37), while in the Lignin treatment they almost did not grow (IR light: -0.05±0.06 d^{-1}, dark: 0.12±0.02 d^{-1}, p=0.016).

The effect of IR light on AAP bacteria was evident in the control treatment: their growth rate was almost twice as fast in the IR light than in the dark (0.66±0.02 d^{-1} vs 0.37±0.07 d^{-1}, respectively, $p < 0.006$). This resulted in a higher abundance (IR light: 5.57±0.66×10^5 cells ml^{-1}, dark: 3.43±0.27×10^5 cells ml^{-1}, $p<0.009$) and contribution of AAP bacteria (IR light: 26.6±1.5%, dark: 15.9±1.2%, $p<0.0005$) in the IR light at the end of the experiment (Fig. 2b, c). A steady growth of AAP bacteria was also observed in the C-limited treatment in both dark and IR light conditions, but the difference in growth rate (0.55±0.06 d^{-1} in IR light and 0.55±0.04 d^{-1} in dark, p=0.56), abundance (IR light: 4.07±0.55 d^{-1} ×10^5 cells ml^{-1}, dark: 3.84±0.81×10^5 cells ml^{-1}, $p>0.35$) and contribution (IR light: 29.8±2.6%, dark: 25.5±1.2%, $p<0.043$) was insignificant. Furthermore, the differences in growth rate, abundance and contribution of AAP bacteria between the control and C-limited treatments in the IR light were insignificant. Interestingly, the growth of AAP bacteria was slower in IR light in the Lignin treatment (IR light: 0.12±0.09 d^{-1}, dark: 0.43±0.02 d^{-1}, p=0.01), which resulted in their slightly higher abundances (IR

light: 2.19±0.45×10^5 cells ml^{-1}, dark: 3.16±0.36×10^5 cells ml^{-1}, p>0.02) and contributions (IR light: 16.8±2.0%, dark: 19.6±0.5%, p=0.13) in the dark. The growth of AAP bacteria in the IR light was significantly slower in the Lignin treatment compared to the control (p=0.003), resulting in their lower abundances (p=0.0015) and contribution (p=0.0017) at the end of the experiment. AAP bacteria grew more than twice as fast as heterotrophic bacteria in the IR light in all treatments, indicating that they profited from photoheterotrophy under all of these conditions.

The changes in AAP community composition were minor and only several ASVs significantly altered their relative abundance in C-limited and Lignin treatments compared to the control at the end of the experiment in the dark but not in the IR light (Fig 3, Supplementary Table S2). C-limitation induced a relative increase of *Novosphingobium* and *Methylobacterium* (Alphaproteobacteria), and *Limnohabitans* (Gammaproteobacteria) compared to the control treatment. Members of the genus *Limnohabitans* were also affected by Lignin treatment, with different ASVs either profiting or losing in these conditions (Fig. 3c).

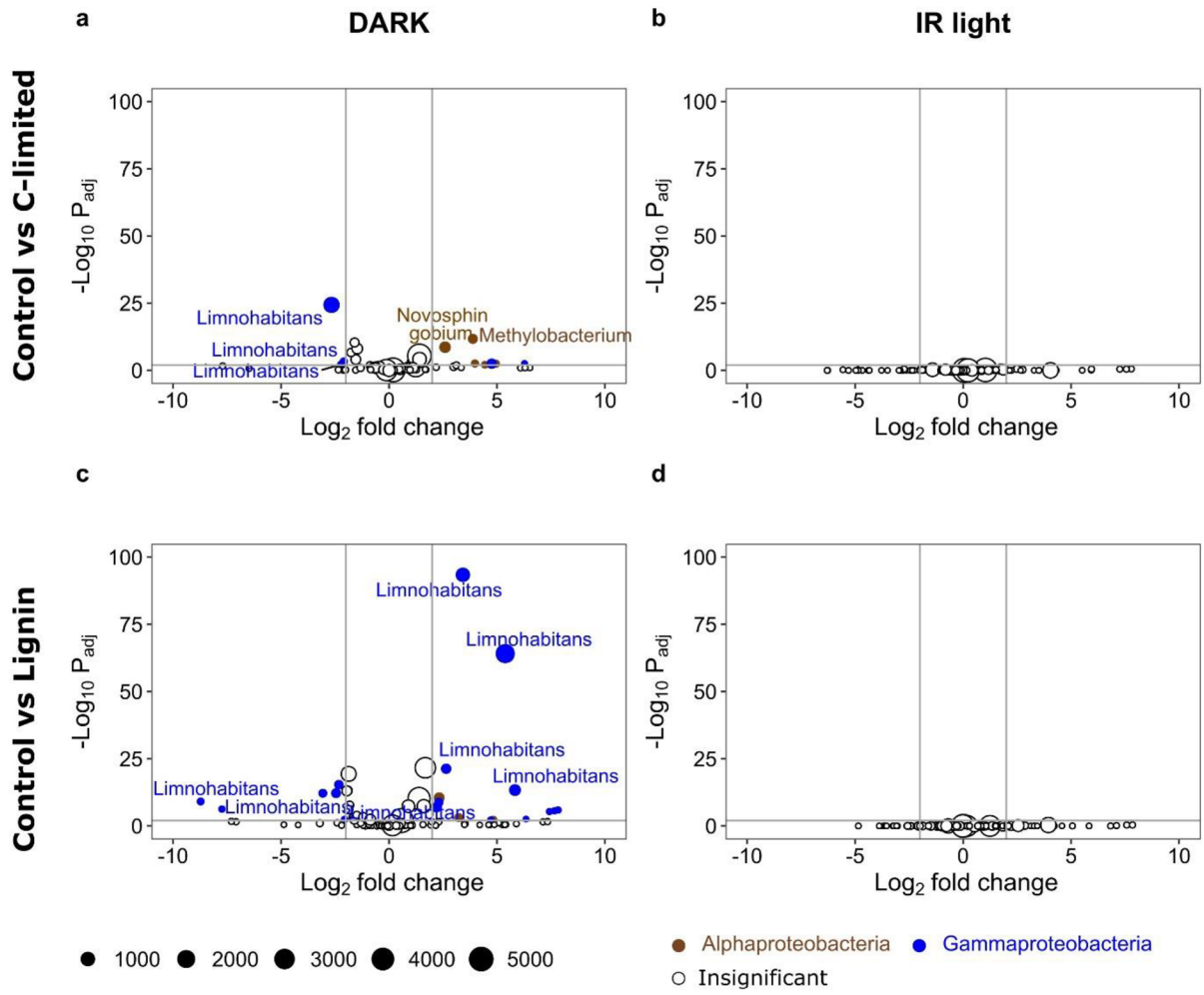


Figure 3. Vulcan plots showing the ASVs with significantly different (adjusted p-value < 0.01, Log2 fold change > |2|) relative abundance at the end of the experiment between (a-b) control and C-limited treatments, and (c-d) control and Lignin treatments in dark (a, c) and IR light (b, d) in June experiment. Negative Log2 fold change value (x axes) indicates that the read count of an ASV was lower in the experimental treatment than in the control, and the positive that it was higher. Vertical grey lines show Log2 fold change values of -2 and 2, horizontal grey lines show significance level (adjusted p-value < 0.01). Bubble size corresponds to the mean number of reads in both compared treatments, colours show the Class affiliation for significant ASVs (brown – Alphaproteobacteria, blue – Gammaproteobacteria, white – insignificant).

October experiment

The growth patterns in the October experiment were different than in June. Heterotrophic bacteria grew fastest in the C-limited treatment regardless of the light conditions (IR light: 0.16±0.02 d^{-1}, dark: 0.17±0.01 d^{-1}, p=0.71), reaching similar abundance at the end of the

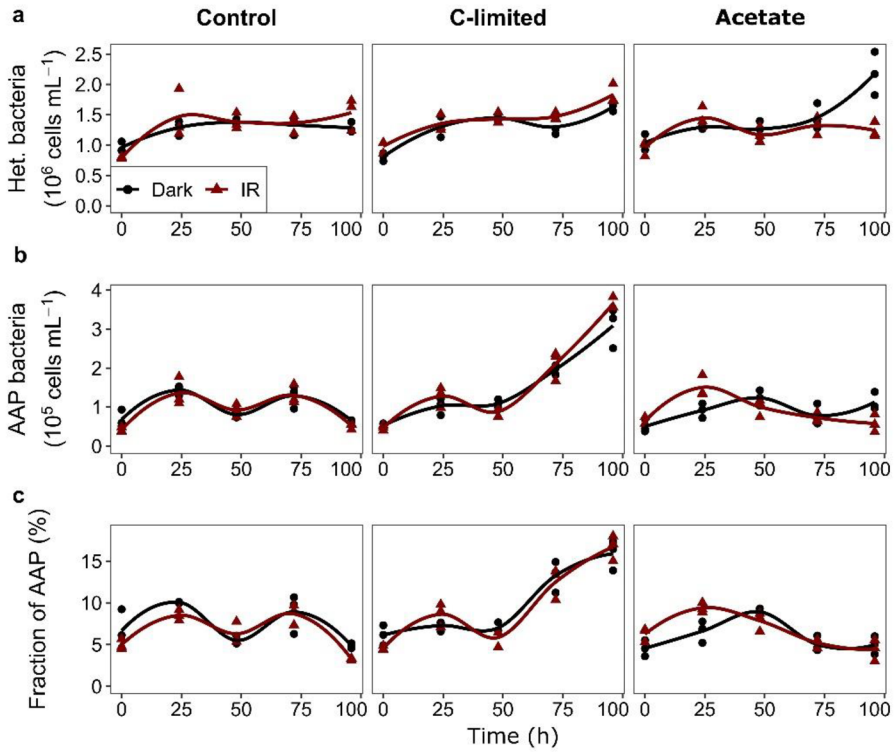


Figure 4. Abundance of heterotrophic bacteria (a); AAP bacteria (b) and contribution of AAP bacteria to total bacterial numbers (c) in the October experiment. Values for each triplicate are shown as points, and the line was fitted locally using loess from the ggplot2 package in R.

experiment (Fig. 4a). Their growth rate was twice as fast in the IR light as in the dark in the control treatment (IR light: 0.12±0.04 d^{-1}, dark: 0.06±0.03 d^{-1}, p=0.06), but the abundance at the end of the experiment was similar (IR light: 1.59±0.27×10^6 cells ml^{-1}, dark: 1.35±0.09×10^6 cells ml^{-1}, p=0.13). In contrast, in the Acetate treatment, heterotrophic bacteria grew only in the dark (growth rate IR light: -0.04±0.05 d^{-1}, dark: 0.16±0.01 d^{-1}, p=0.01), reaching much higher abundances at the end of the experiment (IR light: 1.30±0.15×10^6 cells ml^{-1}, dark: 2.29±0.36×10^6 cells ml^{-1}, p=0.01).

The growth rate of AAP bacteria was close to 0 in the control treatment, both in the IR light (0.04±0.05 d^{-1}) and dark (-0.01±0.03 d^{-1}, p=0.11), and their abundance and contribution to total bacterial abundance did not change (Fig. 4b, c). In contrast, they grew rapidly in the C-limited treatment in both dark and light conditions (growth rate IR light: 0.46±0.05 d^{-1}, dark: 0.42±0.07 d^{-1}, p=0.02, Fig. 4b) and their contribution to the total bacterial abundance tripled (IR light: 16.7±1.5%, dark: 15.9±1.8%, p<0.02, Fig. 4c). Their growth rate, final abundance and contribution were significantly higher in the C-limited treatment than in the control (p<0.003). Finally, in the Acetate treatment, AAP bacteria grew in the dark (0.15±0.10 d^{-1}) but decreased in the IR light (-0.37±0.02 d^{-1}, p=0.006), resulting in twice

lower abundance in the IR light at the end of the experiment (IR light: 0.58±0.22×10^5 cells ml^{-1}, dark: 1.13±0.22×10^5 cells ml^{-1}, p=0.02). However, even though the growth rate was significantly lower in the Acetate than in the control (p=0.002), their abundance and contribution at the end of the experiment did not differ between these treatments (p>0.6).

The growth rate of heterotrophic bacteria compared to AAP bacteria in the IR light did not differ in the control treatment (p=0.94), was significantly lower in the C-limited treatment in IR light (p= 0.0023), and was significantly higher in the IR light in the Acetate treatment (p=0.003).

As observed in June, only several ASVs significantly changed their relative abundance during the experiment both in the dark and IR light (Supplementary Table S3). In the control treatment, *Limnohabitans* and *Hydrogenophaga* increased, while Methylobacteriaceae and Gemmatimonadaceae decreased, especially in the IR treatment. In the C-limited and Acetate treatments, the ASVs that increased were affiliated mainly with *Hydrogenophaga*, while those that decreased included Methylobacteriaceae, Gemmatimonadaceae, Pseudomonadales UBA5518 and other Burkholderiaceae (Supplementary Table S3). The number of ASVs that showed significantly different relative abundance at the end of the experiment between the control and C-limited or Acetate treatments was lower than that observed within the treatments between T0 and T end (Supplementary Table S3). *Hydrogenophaga* increased in the C-limited and Acetate treatments compared to the control both in the dark and IR light, while *Limnohabitans* and *Sandarakinorhabdus* decreased but only in the dark (Fig. 5).

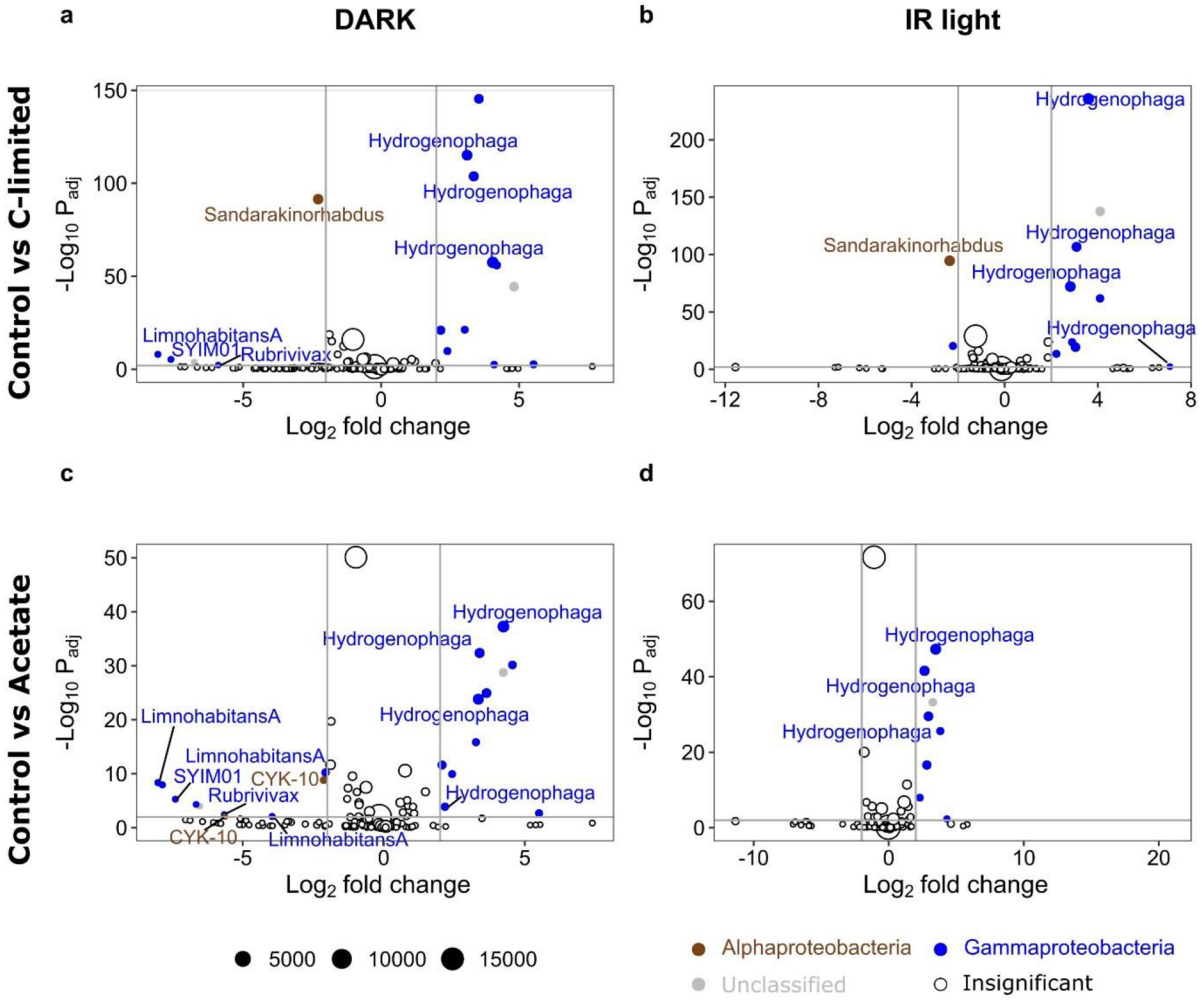


Figure 5. Vulcan plots showing the ASV with significantly different (adjusted p-value < 0.01, Log2 fold change > |2|) relative abundance at the end of the experiments between (a-b) control and C-limited treatments, and (c-d) control and Acetate treatments in dark (a,c) and IR light (b, d) in the October experiment. Negative Log2 fold change value (x axes) indicates that the read count of an ASV was lower in the experimental treatment than in the control, and the positive that it was higher. Vertical grey lines show Log2 fold change values of -2 and 2, horizontal grey lines show significance level (adjusted p-value < 0.01). Bubble size corresponds to the mean number of reads for both compared treatments, colours show the Class affiliation for significant ASVs (brown – Alphaproteobacteria, blue – Gammaproteobacteria, grey – unclassified, white – insignificant).

Discussion

When AAP bacteria were discovered to be abundant in the Northeast Pacific, it was assumed that their ability to use light to produce ATP to support their heterotrophic metabolism helps them to survive in the oligotrophic environment of the ocean (Kolber *et al.*, 2001). However, later observations of their phenology and distribution indicated otherwise: higher concentrations were observed in more eutrophic coastal waters during or shortly after the phytoplankton bloom (Auladell *et al.*, 2019, Vrdoljak Tomaš *et al.*, 2019). A similar pattern emerged from freshwater studies (Kolářová *et al.*, 2019,

Villena-Alemany *et al.*, 2023b), questioning the initial assumption. It was also suggested that additional energy from light can facilitate access to recalcitrant complex organic polymers or low-energy carbon sources, such as lignin or acetate (Koblížek, 2015). However, experimental support for these statements comes mostly from experiments with cultured species (Kuzyk *et al.*, 2023), and the question of how AAPs utilize their photoheterotrophy in natural environments remains open.

The key finding of this study is that the profit from photoheterotrophic nutrition differed between the seasons. The positive response of AAP bacteria to IR light only in the control in the June experiment (Fig. 2b, c) suggests that additional energy from light allows them to successfully compete with heterotrophic bacteria at the time of surplus DOC availability in spring. This result contradicts the initial hypotheses (Kolber *et al.*, 2001), but agrees with the fact that many freshwater AAP genera (*e.g.* *Limnohabitans*) are considered copiotrophs (Chiriac *et al.*, 2023, Park *et al.*, 2023). Furthermore, in June AAP bacteria grew faster than the heterotrophic bacteria in all treatments, providing experimental support for the recently suggested inclusion of AAP bacteria in the Phytoplankton Ecology Group (PEG) model as super competitors profiting from spring phytoplankton bloom (Villena-Alemany *et al.*, 2023b). However, the negligible growth of AAP bacteria in the control treatment in the October experiment indicates that this advantage is irrelevant in autumn (Fig. 4b, c) when the AAP community may represent different gene repertoires and thus distinct functionalities(Villena-Alemany *et al.*, 2023b). In general, AAP bacteria seem to be less active and abundant in autumn than in spring (Kolářová *et al.*, 2019, Piwosz *et al.*, 2022, Villena-Alemany *et al.*, 2023a).

The growth of AAP bacteria in the C-limited treatment was similar in the IR light and the dark in both seasons (Fig. 2 and 4), indicating that they did not profit from photoheterotrophy in such conditions. However, this was contradicted by the observation that they grew much faster than heterotrophic bacteria, increasing their contribution to the total bacterial community up to threefold. This incongruence suggests a dual role of photoheterotrophic metabolism for AAP bacteria: On one hand, carbon limitation triggers the production of BChl-*a* to aid their survival (Kolber *et al.*, 2001, Koblížek, 2015, Kopejtka *et al.*, 2020, Kuzyk *et al.*, 2023). On the other, photoheterotrophy seems to be the most advantageous under carbon availability, when a more efficient metabolism allows them to grow faster than heterotrophic bacteria, as discussed above.

An unexpected result was the negative response of the AAP bacteria to the Lignin treatment in June and the Acetate treatment in October (Fig. 2, 3). This indicates that both complex polymers (lignin) and low-energy monomers (acetate) are disadvantageous for freshwater AAP bacteria. This negative relationship, for which the mechanism remains yet unknown, may have serious consequences for lake functioning. Currently, many temperate lakes in the Northern Hemisphere are affected by browning, resulting from the increase in terrestrial dissolved organic matter (DOM) (Williamson *et al.*, 2015) which is often recalcitrant for bacteria (Kritzberg *et al.*, 2004). Browning is predicted to continue as the atmospheric acid deposition decreases and due to climate change (Meyer-Jacob *et al.*, 2019), affecting pelagic food webs (Williamson *et al.*, 2015) and increasing CO_2 flux to the atmosphere (Kritzberg *et al.*, 2020). While some AAP bacteria, such as *Sphingomonas* sp. strain FukuSWIS1 from the acidic lake Grosse Fuchskuhle (Salka *et al.*, 2014), seem to be adapted to conditions prevailing in humic and brown lakes, our results indicate that overall AAP bacteria may be negatively impacted by recalcitrant or low energy carbon sources. This effect may be more pronounced in spring when terrestrial DOM inputs, potentially higher due to river runoff, could hamper their photoheterotrophy, thus decreasing the efficiency of carbon assimilation and lowering its availability for higher trophic levels (Piwosz *et al.*, 2022, Villena-Alemany *et al.*, 2023b). Further experiments employing a wider variety of recalcitrant and low-energy compounds are needed to confirm and understand this effect.

AAP community composition shows recurrent seasonal patterns in freshwater lakes (Villena-Alemany *et al.*, 2023b), which are driven by changes in environmental conditions, such as temperature and DOC concentration (Villena-Alemany *et al.*, 2023a). The minor changes in the AAP community composition under IR light observed here indicate that the response to the treatments could have been driven by a physiological change in specific AAP phylotypes switching to phototrophic metabolisms rather than a community-level response (Fecskeová *et al.*, 2019, Piwosz *et al.*, 2020). For instance, the only genus that had significantly increased its relative abundance in IR light was *Hydrogenophaga* in October (Fig. 5 b, d). Members of this genus were reported to oxidize hydrogen as an energy source (Willems *et al.*, 1989), which may have interesting implications for the functional role of AAP bacteria in freshwaters. In addition, numerous ASVs affiliated with *Limnohabitans* either increased or decreased

in Lignin treatment in June (Fig. 3c), which may indicate niche separation between closely related AAP species (Villena-Alemany *et al.*, 2023b).

Interestingly, more ASVs changed their relative abundance under dark conditions throughout the experiment, especially in June (Figs. 3, 5). This suggests that dark incubations which are commonly used in experimental design to minimise the effect of primary producers (Šimek *et al.*, 2020, Fecskeová *et al.*, 2021), may exaggerate the community-level responses compared to light treatments (Piwosz *et al.*, 2020). This observation aids in arguments that dark incubations provide biased insights into the activity of freshwater bacterioplankton (Piwosz *et al.*, 2022).

Conclusions

Our experimental evidence indicates that although AAP bacteria's ability to use light as a supplementary energy source is induced under carbon limitation, they profit from photoheterotrophy when carbon is available. However, this advantage over heterotrophic bacteria depends on the season and may be more pronounced in springtime. This effect also seems to be driven by physiological responses rather than changes at the community level. These findings contribute to our understanding of the dynamics of AAP bacteria observed in pelagic environments. Finally, the observation of the negative effect of lignin and acetate on AAP bacteria opens a new research topic in their ecology, as it may have ecosystem-level consequences as lake browning continues.

Funding

This research was in part funded by the National Science Centre, Poland project no. 2021/42/E/NZ8/00163 and project no. 2021/03/Y/NZ8/00076 under the Weave-UNISONO call in the Weave programme, both awarded to KP.

Acknowledgements

Authors Contribution (CRediT): KP: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft; CVA: Formal Analysis, Writing – review & editing; JC: Data curation, Formal Analysis, Writing – review & editing; IM: Investigation, Writing – review & editing, VN: Investigation, Writing – review & editing; JD: Investigation, Writing – review & editing; MK: Conceptualization, Funding acquisition, Writing – review & editing.

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3.- Discussion and conclusions

In the current thesis, four primary aspects of AAP bacteria have been comprehensively studied and discussed. Firstly, the diversity and abundance dynamics of the AAP community. Subsequently, the impact of these dynamics on the lake microbial communities' development. Additionally, we studied how the metabolic capability of phototrophy in AAP bacteria impacts carbon cycle. Our findings demonstrate that they reduce respiration, thereby decreasing carbon dioxide release into atmosphere, while simultaneously increasing carbon assimilation and enhancing its availability for upper trophic levels. Finally, we studied the relationship between carbon quality, quantity and the growth of the AAP community. This revealed that the composition of AAP community, determined by seasonal succession, constrains their response to various carbon sources: carbon limitation, low energy carbon and recalcitrant carbon.

For first time, we were able to show the recurrence of AAP bacteria community composition in freshwaters, constituted of a huge amalgam of genera from mainly Gamma- and Alphaproteobacteria, and with a minor contribution from Gemmatimonadota, Chloroflexota and Myxococcota. This achievement is partially due to the enlarged database hereby presented that contains the largest published collection of *puf*M genes and will help researchers to achieve a consensus on the taxonomic classification of AAP bacteria according to *puf*M gene sequences. Nonetheless, there are still many gaps in our knowledge regarding these topics that represent an exciting puzzle to solve. For instance, with regard to the AAP diversity, in spite of the obtained improvement in the taxonomic classification by the longer primers and the new *puf*M gene database, there are several environmental sequences that could not be taxonomically classified due to lack of representative genomes and taxonomic consensus on the reference sequences of the *puf*M gene database. It is, therefore, important to continue enlarging the database with more reference sequences from different environments, which will unambiguously lead to a reduction in the number of still unclassified sequences in further studies. Here, I feel it is necessary to highlight that the newly designed long amplicon primers have substantially optimized the study of the AAP diversity. One thing notable from the design of the pufM_longF primer (Figure 8) is that it points out a conserved region of the *puf*M gene that enables the production of longer amplicons and so

improved taxonomic assignment. After the *puf*M database construction, we can say with confidence that the coverage of pufM_longF primers still has further room for improvement by including more degenerated nucleotides and so increasing the amount of *puf*M sequences that can be retrieved. Currently, the pufM_longF primer contains 6 degenerated nucleotides whereas the most used *puf*M primer with the highest coverage, UniF, encompasses 10 degenerated nucleotides.

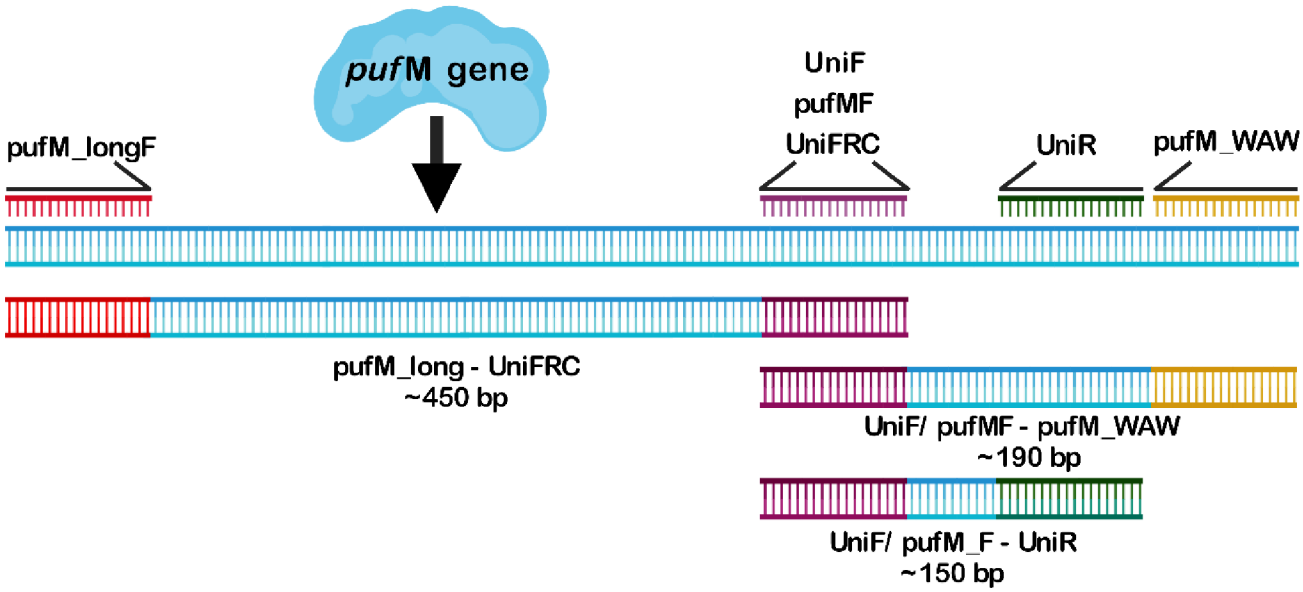

Figure 8. Representation of the amplicon generated from pufM gene by the most commonly used primers. pufMF from Beja et al., 2002; UniF, UniR and pufM_WAW from Yutin et al., 2005 and pufM_longF and UniFRC from Villena et al., submitted. The Figure was created using BioRender.com.

Furthermore, the new *puf*M gene database presented here contains information about the environment of origin of each sequence, allowing for the adaptation of primers to achieve the highest coverage for a targeted environment of interest.

With regard to thoroughly describing the effect that phototrophic metabolism in AAP bacteria has on the natural environment, great advances were achieved in my thesis. For instance, it should prove extremely useful for future ecological and carbon cycle related studies that the overall microbial respiration reduction in infrared light performed by photoheterotrophic metabolism of AAP bacteria has been properly quantified. Also, as a result of the energy harvested from light, AAP bacteria can capture more carbon than with purely heterotrophic metabolism (dark conditions), suggesting an important role in rendering carbon to the upper trophic levels and so boosting the microbial loop. We

have shown that this is well exemplified during the spring AAP peak, triggered by the release of DOC from the phytoplankton bloom and including the annual succession of AAP bacteria into the microbial ecological PEG model (Figure 9).

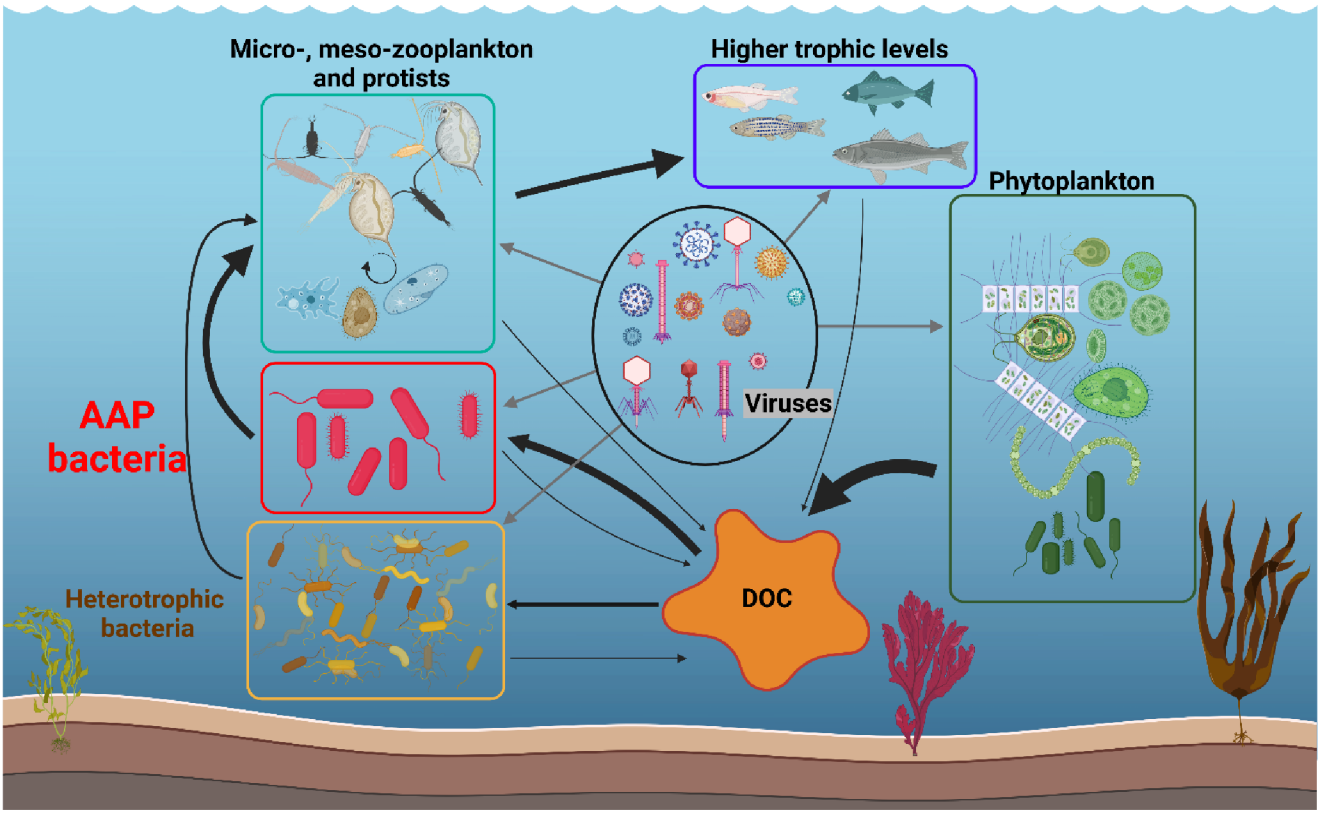

Figure 9. Highly simplified model of interactions between organisms of the aquatic communities. Due to the simplification, several components, such as fungi or parasites, have been excluded and the intricate community structure and interactions occurring within protists and zooplankton has been reduced to a single level. Black arrows indicate carbon flow and grey the viral interactions. DOC: dissolved organic carbon. The Figure was created using BioRender.com.

However, precise and accurate quantifications of the carbon flow circulating through these process needs to be assessed. For instance, the photoheterotrophic activity was uneven during the sampling season, likely being impacted by the highly dynamic AAP community composition, thus, indicating a differential contribution of specific phylotypes to the photoheterotrophic activity. A similar phenomenon was observed regarding the quality of organic carbon consumed by AAP bacteria and the impact that it has on their growth. Samples from different seasons responded differently to same condition highlighting the discrepancy on AAP community performance and their role in the environment. This variance in the AAP community behaviour provides a potential explanation of the contradictory roles traditionally assigned to AAP bacteria regarding the usage of low energy compounds, recalcitrant carbon or opportunistic peaking on phytoplankton bloom exudates.

All the above mentioned statements lead to one of the main conclusions of the current thesis. AAP bacteria are extremely diverse, showing a wide range of metabolic functionalities fulfilling distinct roles in the environment and the best commonality among them is their phototrophic capability. This includes differential phototrophic activity and distinct response to the existing carbon sources. Metabolic single cell studies will shed light on the specific phylotypes responsible of the higher phototrophic activities and will allow the description of specific carbon metabolism in AAP bacteria.

From the observed resilient recurrence of AAP bacteria, we can conclude that the freshwater AAP community is highly adapted to the seasonal succession and that they potentially play an important role by enhancing microbial loop during spring phytoplankton bloom. Nevertheless, the trigger phenomena enhancing the second peak of AAP bacteria in autumn still need to be studied.

Finally, emphasizing the future implications of global carbon cycle with regard to AAP bacteria is crucial. On the one hand they reduce their respiration and subsequently the release of carbon dioxide to the atmosphere. However, this phenomenon still needs to be studied in other lakes and rivers and the largest aquatic environments on Earth, the seas and the oceans. On the other hand, AAP bacteria have demonstrated limited utilization of recalcitrant carbon but the largest abundances and biomass of AAP bacteria have been recorded in polyhumic and acidic lakes. Compounding this, the human-induced destruction of habitats has led to lake brownification, through the introduction of humic substances, altering the primary production and ultimately impacting AAP bacteria and their substantially unveiled contribution to the global carbon cycle.

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5.- ***Curriculum Vitae***

Cristian Villena Alemany

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Date of Birth: 07 of September, 1993.

Place of Birth: Gandia, València, Spain.

ORCID: https://orcid.org/0000-0002-6158-1879

Research Gate: https://www.researchgate.net/profile/Cristian-Villena-Alemany-2

Areas of compentence: aquatic microbial ecology, molecular biology, microbial diversity, metagenomics, environmental microbiology, genomics, anoxygenic photosynthesis and limnology among others.

Education

September 2019 – Current

Ph.D. study in Laboratory of Anoxygenic Phototrophs, Algatech Center, Institute of Microbiology, Czech Academy of Sciences, Czechia.
Ph.D. study in Hydrobiology, Department of Ecosystem Biology, University of South Bohemia, Czechia.
Thesis: *Ecology of Aerobic Anoxygenic Phototrophic Bacteria in Fresh Waters*, supervised by Doc. Kasia Piwosz, Ph.D. and Doc. Michal Koblížek, Ph.D.

September 2018 – June 2019

Master in Biotechnology for health and sustainability, University of Alicante, Sant Vicent del Raspeig, País Valencià, Spain. Thesis: Virus-host interactions in *Haloquadratum walsbyi*, under supervision of: Prof. Fernando Santos, Ph.D. in laboratory of Microbial ecology, from Physiology, Genetic and Microbiology department of University of Alicante, País Valencià, Spain.

September 2014 – July 2018

Bachelor in Biology, University of Alicante, Sant Vicent del Raspeig, País Valencià, Spain. Thesis: Isolation and characterization of halophile Bacteroidetes viruses, under supervision of: Prof. Fernando Santos, Ph.D. in laboratory of Microbial ecology, from Physiology, Genetic and Microbiology department of University of Alicante, País Valencià, Spain.

Professional experience

July 2019 – Current

Laboratory of Anoxygenic Phototrophs, Algatech Center, Institute of Microbiology, Czech Academy of Sciences, Czechia.

March 2020 – May 2020

RNA SARS-CoV-2 detection from Jindřichův Hradec and České Budějovice hospitals during 1st wave of global pandemic using RT-qPCR.

January 2019 – June -2019

Laboratory of Microbial ecology, from Physiology, Genetic and Microbiology department of University of Alicante, País Valencià, Spain. (Master thesis)

January 2018 – May 2018

Laboratory of Microbial ecology, from Physiology, Genetic and Microbiology department of University of Alicante, País Valencià, Spain (Bachelor thesis)

May 2018 – June 2018

Practicum in University of Alicante, Sant Vicent del Raspeig, País Valencià, Spain. Report: Detection of new halophile Bacteroidetes using targeted PCR, under Supervision of: Prof. Fernando Santos, Ph.D. in laboratory of Microbial ecology, from Physiology, Genetic and Microbiology department of University of Alicante, País Valencià, Spain.

Presentation at conferences

12th – 15th September 2023, Budapest, Hungary. Central and East European Symposium of microbial ecology (CEESME).

Oral presentation: **Villena-Alemany C**, Mujakić I, Feckesova L. K., Woodhouse J, Auladell A, Socha M., Gazulla C.R., Ruschewehy H.J., Sunagawa S., Kavagutti V., Andrei S, Grossart H.P., Ghai R., Koblížek M. and Piwosz K. "Exploring the ecological significance of aerobic anoxygenic photoheterotrophic bacteria in freshwater ecosystems."

21st – 25th August 2023, Helsinki, Finland. Baltic Sea Science congress 2023.

Poster: Vanharanta M., Piiparinen J., Santoro M., **Villena-Alemany C.**, Grossart H.-P., Labrenz M., Piwosz K., Spilling K. "Microbial remineralization processes during the post-spring bloom excess phosphate conditions in the northern Baltic Sea"

20th – 25th August 2023, Tartu, Estonia. Symposium of aquatic microbial ecology (SAME17).

Oral presentation: **Villena-Alemany C**, Mujakić I, Feckesova L. K., Woodhouse J, Auladell A, Socha M., Gazulla C.R., Ruschewehy H.J., Sunagawa S., Kavagutti V., Andrei S, Grossart H.P., Ghai R., Koblížek M. and Piwosz K. "Exploring the ecological significance of aerobic anoxygenic photoheterotrophic bacteria in freshwater ecosystems."

7th – 8th December 2022, České Budějovice, Czechia. 2nd USB Conference of Doctoral Students.

Oral presentation: **Villena- Alemany C**. "Have you ever heard about anoxygenic photosynthesis? An overview of its important role in freshwaters."

14th – 19th August 2022, Lausanne, Switzerland. International Symposium of Microbial Ecology (ISME18).

Poster: Mujakić I., Cabello-Yeves P.J., **Villena-Alemany C.**, Piwosz K., Picazo A., Camacho A., Koblížek M. "Multi-environment ecogenomics analysis of cosmopolitan phylum Gemmatimonadota."

14th – 19th August 2022, Lausanne, Switzerland. International Symposium of Microbial Ecology (ISME18).

Poster: **Villena-Alemany C.**, Mujakić I., Koblížek M. and Piwosz. "Diversity dynamics of Aerobic Anoxygenic Phototrophic bacteria in a freshwater lake."

26th May 2022, Třeboň, Czechia. Algatech Poster Session.
Poster: **Villena-Alemany C.**, Mujakić I., Koblížek M. and Piwosz K. "Seasonal changes of freshwater Aerobic Anoxygenic Phototrophs community composition."

21st – 22nd October 2021, Warsaw, Poland. EMBO Young Scientist's Forum.
Poster: **Villena-Alemany C.**, Mujakić I., Koblížek M. and Piwosz K. "Aerobic anoxygenic photoheterotrophic bacteria composition community in oligotrophic freshwater lake."

11th – 12th November 2020, ISME Virtual Microbial Ecology Summit.
Poster: Piwosz K., **Villena-Alemany C**. "Contribution of photoheterotrophy by aerobic anoxygenic phototrophic bacteria to freshwater community metabolisms."

23rd – 25th May 2018, Matalascañas, Huelva, Spain. National network of extremophile microorganisms (RedEx).
Oral presentation: Sánchez R., Aldeguer B., **Villena-Alemany C**., Gangloff V., Santos F. and Antón J. "Isolation of new halophile Bacteroidetes using alternative culture methods."

International Stays and Experiments

10th – 14th July 2023, 1-week experiment in Laboratory of Microbiology, Institute of Oceanography and Fisheries, Split, Croatia with Prof. Danijela Šantić.

13th – 18th May 2023, 1-week experiment in Laboratory of Microbiology, Institute of Oceanography and Fisheries, Split, Croatia with Prof. Danijela Šantić.

February – April 2023, 2.5-month internship in Department of Fisheries Oceanography and Marine Ecology, National marine research institut, Kołłątaja 1, 81-332 Gdynia, Poland.

7th – 15th May 2022, 1 week PCR screening experiment in ELTE department of microbiology, in Prof. Tamás Felföldi, Ph.D. laboratory.

June 2021, 1-month experiment in Tvarminne Zoological Station, University of Helsinki, Tvarminne, Hanko. Mesocosm experiment in baltic sea: Effect of decreasing inorganic N:P ratio on the plankton community - INN:PP.

January – May 2021, 5-month internship in Molecular and microbial ecology laboratory, University of Alicante, Valencian Country, Spain.

4th – 8th November 2019, Barcelona, Catalonia, Spain. "Data manipulation, analysis, and visualization in R using Tidyverse", organized by Transmitting Science: https://www.transmittingscience.com

Scientific Project participation involved

PhotoGemm+ no. 1928778X from the Grant Agency of the Czech Republic (GAČR) awarded to Assoc. Prof. Michal Koblížek, Ph.D.

AAP rule! Junior project no. 18–14095Y from the Czech Science Foundation awarded to Assoc. Prof. Kasia Piwosz, Ph.D.

European's union Horizon 2020 project. AQUACOSM – H2020-INFRIA-Project N° 731065 - Transnational Access Report awarded to Kasia Piwosz, Ph.D.

Fellowships and awards

September 2019 – February 2024, Ph.D. study scholarship from University of South Bohemia.

September 2023, Fellowship from CEESME - ISME committee to attend CEESME in Budapest, Hungary.

August 2023, Fellowship from SAME17 organizing committee to attend Symposium of aquatic microbial ecology (SAME17).

February 2023, Best presentation award in Ph.D. conference of Hydrobiology and Ecology, in Vodnany.

December 2022, Best Presentation award in 2nd USB Conference of Doctoral Students

January – June 2021 and February – April 2023, Erasmus + Traineeship from Ministry of Education in Czechia.

February 2021, 3rd Best presentation award in Ph.D. conference of Hydrobiology and Ecology, in Vodnany.

October 2019, Transmitting Science fellowship for the course "Data manipulation, analysis, and visualization in R using Tidyverse": https://www.transmittingscience.com

Teaching experience

January 2023, 1 week lectures on KBE 760 Biology of Microorganisms in laboratory practices for 15 hours.

January 2022, 1-day lecture on KBE 760 Biology of Microorganisms in laboratory practices for 3 hours.

Language skills

Catalan (mother tongue)
Spanish (mother tongue)
English (C1)

Academic Publications

Stojan I., Šantić D., **Villena-Alemany C.**, Trumbić Ž., Matić F., Vrdoljak Tomaš A., Lepen Pleić I., Piwosz K., Kušpilić G., Ninčević Gladan Ž., Šestanović, S., Šolić M. (Manuscript under review) Distribution, diversity, and community composition of Aerobic Anoxygenic Phototrophs on a spatiotemporal scale in the central Adriatic Sea.

Vanharanta M., Santoro M., **Villena-Alemany C.**, Piiparinen J., Piwosz K., Grossat H.-P., Labrenz M., Spilling K. (Manuscript in prep.) Microbial remineralization processes during post-spring-bloom excess phosphate in the northern Baltic Sea.

Villena-Alemany C., Mujakić, I., Fecskeova, L.K., Woodhouse, J., Auladell, A., Dean, J., Hanusova, M., Socha, M., Gazulla, C.R., Ruscheweyh, H.-J., Sunagawa, S.Andrei, A.-S., Grossart, H.-P., Ghai, R., Koblizek, M., & Piwosz, K. (Manuscript under review) Phenology and ecological role of Aerobic Anoxygenic Phototrophs in fresh waters. Preprint doi: https://doi.org/10.1101/2023.11.17.567504

Piwosz, K., **Villena-Alemany, C.**, Całkiewicz J., Mujakić, I., Náhlík V., Dean J. & Koblížek M. (2023) (Manuscript under review) Winners in good times and bad times: Aerobic anoxygenic phototrophic bacteria profit from heterotrophy under carbon-rich and poor conditions.

Mujakić I., Cabello-Yeves PJ., **Villena-Alemany C.**, Piwosz K., Rodriguez-Valera F., Picazo A, Camacho A., Koblížek M. Multi-environment ecogenomics analysis of the cosmopolitan phylum Gemmatimonadota. Microbiol Spectr. 2023 Sep 21; 11(5):e0111223.doi: 10.1128/spectrum.01112-23

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