Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 2285

Single-cell methodologies for ecological and metabolic mapping of mixotrophic microeukaryotes

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ACTA UNIVERSITATIS
UPSALIENSIS
2023

ISSN 1651-6214
ISBN 978-91-513-1847-9
urn:nbn:se:uu:diva-506111

UPPSALA
UNIVERSITET

Dissertation presented at Uppsala University to be publicly examined in Friessalen, Evolutionsbiologiskt centrum, Norbyvägen 14, Uppsala, Friday, 22 September 2023 at 09:15 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Alexandra Z. Worden (GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany).

Abstract
Florenza, J. 2023. Single-cell methodologies for ecological and metabolic mapping of mixotrophic microeukaryotes. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2285. 64 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1847-9.

Mixotrophy in aquatic protists is pivotal for our understanding of aquatic microbial food web dynamics. This thesis is centered around aquatic unicellular mixotrophs, and comprises three methodological approaches aimed to tackle mixotroph ecology at single-cell resolution: the identification of actively feeding mixotrophs in natural samples, the determination of specific interactions among mixotrophs and bacterial prey, and the profiling of two distinct mixotrophic populations based on the gene expression of their constitutive individuals.

First, we investigated the feasibility of cytometrically sorting actively feeding mixotrophs from a natural community. The approach was based on the use of fluorescently labelled feeding tracers (FLTs) in conjunction with chloroplast autofluorescence from the feeding cell to retrieve mixotrophic individuals for subsequent single cell characterization by sequencing of a taxonomic marker gene. The preference for different FLT types showed that for mixotrophs in culture, FLT size was the strongest factor influencing FLT-based capture. This approach was then used to identify actively feeding mixotrophs from a lake water sample. The method proved to be both highly selective and specific and allowed the identification of an active natural mixotrophic community of unexpected diversity.

Secondly, we explored the potential of adapting emulsion, paired-isolation and concatenation PCR (epicPCR) to uncover physical connections between individual unicellular eukaryotes and their associated bacterial cohort. The results from three proof-of-concept experiments, however, did not conform to the expectations and showcased several deficiencies that need to be addressed. Mainly, the frequency of recovered links showed that the protocol, as deployed in our experiments, was prone to yield spurious abundance-driven associations between the eukaryotes and bacteria, since the most abundant bacteria were the ones driving the strongest associations with our test predators. Nevertheless, we identify possible solutions and point to avenues for future development to overcome the current limitations.

Finally, the capability of full-transcript single-cell RNA sequencing was surveyed to provide a reliable transcriptomic landscape of a non-mammalian, non-model eukaryotic organism with no available reference genome. We could show that, while some of the detailed functional information might remain uncharacterized, the workflow provide sufficient raw data to resolve population structure based on expression profiles.

In summary, with varying degrees of success, these attempts to expose and study mixotrophic unicellular eukaryotes demonstrate that the time is ripe to explore the ecology of mixotrophs at single-cell level.

Keywords: mixotrophy, single-cell

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ISSN 1651-6214
ISBN 978-91-513-1847-9
URN urn:nbn:se:uu:diva-506111 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-506111)

The first thing to be undertaken in this weighty work is […] *an inlargement of the dominion of the Senses.*

Robert Hooke, in the preface of his *Micrographia* (1665).

To my family,
those staunch supporters.

Cover image: *Ochromonas triangulata* at 2000× magnification. The image is a composite of four confocal microscopy micrographs featuring the nucleus (red), the chloroplast (green), four food vacuoles (blue) and one bacterium (yellow) which was, presumably, about to be engulfed when the photograph was taken.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Florenza, J.**, Bertilsson, S. (2023) Choice of methodology and surrogate prey are decisive for the quality of protistan bacterivory rate estimates. *Aquatic Microbial Ecology*, 89:43–53.

II. **Florenza, J.**, Divne, A.-M., Bertilsson, S. (2023) Fluorescently labelled prey surrogates in combination with FACS successfully discriminate actively feeding mixotrophs in a lake water sample. *Submitted.*

III. **Florenza, J.**, Tamminen, M., Bertilsson, S. (2019) Uncovering microbial inter-domain interactions in complex communities. *Philosophical Transactions of the Royal Society of London. Biological Sciences*, 374(1786):20190087.

IV. **Florenza, J.**, Pitkänen, O., Bertilsson, S., Tamminen, M. (2023) Testing the epicPCR workflow to reveal eukaryote-prokaryote interactions at single-cell resolution. *Manuscript.*

V. **Florenza, J.***, Jeevannavar, A.*, Divne, A.-M., Tamminen, M., Bertilsson, S. (2023) *De novo* assembled single-cell transcriptomes from aquatic phytoflagellates reveal a metabolically distinct cell population. *Manuscript*.

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Abbreviations

DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FLB	Fluorescently labelled bacteria
FLT	Fluorescently labelled tracer
GFP	Green fluorescent protein
PCR	Polymerase chain reaction
epicPCR	emulsion, paired-isolation and concatenation PCR
OTU	Operational taxonomic unit
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
scRNA-seq	Single-cell RNA sequencing
SDV	Silica deposition vesicle
SSU	Small subunit (of the rRNA operon)

The aquatic microbial food web

The plankton, understood as the living fraction of all suspended matter passively drifting in a water mass (Hutchinson 1967), is a distinctive constituent of aquatic habitats. In the pelagic ecosystem, primary production (i.e. fixation of inorganic carbon into organic matter through photosynthesis) is carried out by planktonic organisms, and the immediate consumers of biomass generated by such primary production are also predominantly planktonic (Lindeman 1942, Sieburth et al. 1978, Azam et al. 1983). In clear analogy to the plant-sustained food webs observed on land, planktonic primary producers are typically referred to as phytoplankton (i.e. plant-like plankton), whereas the term zooplankton (i.e. animal-like) historically referred broadly to biomass consumers. Therefore, autotrophy, or the use of inorganic carbon as the sole carbon source, is a defining attribute of phytoplankton, while heterotrophy, or the

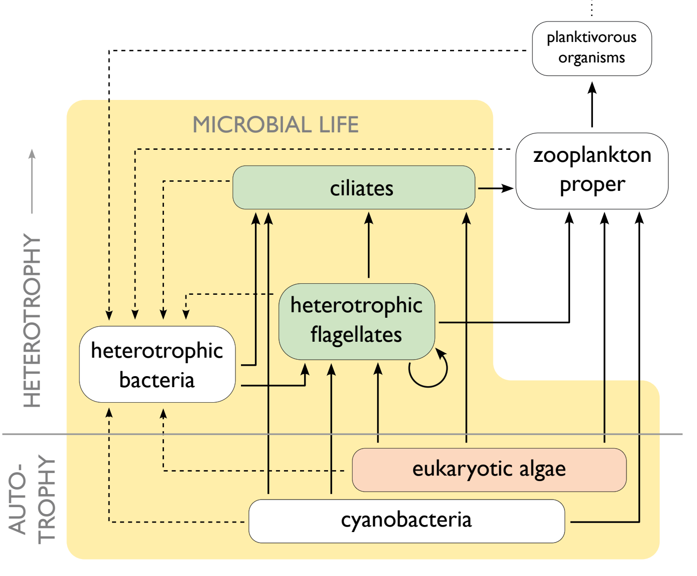

Figure 1. Simplified classical scheme of the pelagic food web in aquatic habitats. Solid arrows depict fluxes of matter mediated by predation, whereas dashed arrows depict fluxes of matter mediated by decomposition. Colored boxes indicate groups where mixotrophy is relevant: green boxes specify the classical heterotrophic components that could behave as autotrophs, whereas red boxes mark classical autotrophic components that could behave as heterotrophs. All the microbial components of the web are collected within a yellow shade. Food web adapted from Azam et al. 1983.

necessary intake of exogenous organic carbon for nutrition, is characteristic of zooplankton.

Phytoplankton, composed of prokaryotic cyanobacteria and eukaryotic algae, both of which are single celled life forms, make up the autotrophic base of the food web in the pelagic zone (Figure 1). With lifestyles ranging from free-living single cells to elaborate colonies of thousands of cells, the size range of phytoplankton is very broad. Following the well-established size-based operational classification of plankton promoted by Sieburth, Smetacek and Lenz (Sieburth et al. 1978, Figure 2), phytoplankton extend over the pico-, nano- and microplankton size fractions (from sub-micron to the millimeter). As a result, a diverse group of consumers, the zooplankton in a broad sense, can benefit directly from grazing upon primary producers. Single-celled heterotrophic flagellates (nano- to microplankton size fractions) and ciliates (microplankton) can all feed directly on phytoplankton, as primary consumers, but also prey on each other as secondary consumers. Actual zooplankton[1] can feed on the larger phytoplankton fraction directly, but also on ciliates and large flagellates, and subsequent predation on zooplankton develops the food web further up the trophic scale. Through the microbial loop, heterotrophic bacteria remineralize suspended decaying organic matter from all trophic levels while a fraction of this pool is recycled back into the system through flagellate and ciliate predation on bacteria (Azam et al. 1983).

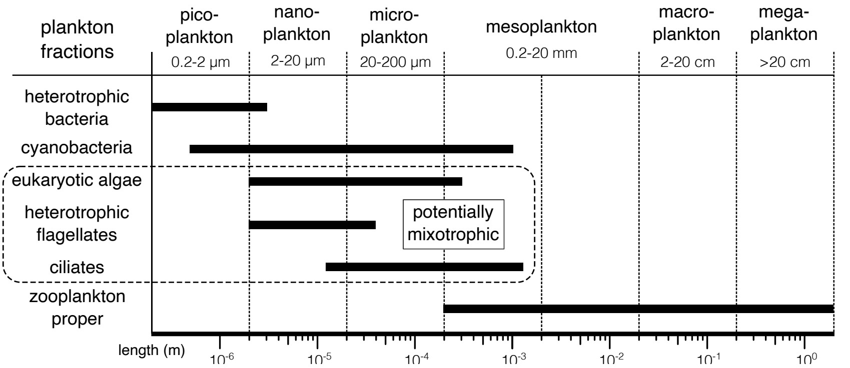

Figure 2. Typical planktonic size fractions, adapted from Sieburth et al. 1978. The length models used as extremes for each fraction are: up to *Flavobacterium* filaments (upper limit of heterotrophic bacteria), from free-living *Prochlorococcus* to *Gloeotrichia* colonies (cyanobacteria), from free-living *Micromonas* to *Volvox* colonies (eukaryotic algae), from *Paraphysomonas* to *Oxyrrhis* (heterotrophic flagellates), and from *Cyclidium* to *Stentor* (ciliates). The length range of multicellular organisms is depicted following Sieburth et al. 1978. All length bars are approximate and should not be taken strictly.

[1] The *actual* zooplankton, or zooplankton proper in Figures 1 and 2, is meant to comprise the planktonic components that are indeed animals, such as cladocerans, rotifers and copepods.

This description of the planktonic food web is indeed greatly simplified, overlooking the allochthonous contribution to the network, the role of dissolved organic carbon and other key nutrients, or the seasonal, spatial and ontogenic factors that can alter the connectivity between food web components. Equally, it is beyond the aim of this thesis to expand quantitatively on the relative importance of the fluxes depicted in Figure 1. However, it summarizes the basic pelagic food web structure that was devised four decades ago and remains relevant for both freshwater and marine ecosystems (e.g. Šimek et al. 2014, Worden et al. 2015). With this summary, it is my intention to highlight that both of the structuring elements of the pelagic food web, which are the classic food chain grounded in microbial primary production and the microbial loop grounded in bacterial heterotrophic nutrition, are in fact mediated by single-celled organisms.

Single-celled organisms have evolved a vast array of nutritional lifestyles. Anoxygenic photosynthesis or chemolithotrophy are two mechanisms of autotrophy that are exclusive to prokaryotes (Madigan et al. 2002). Oxygenic photosynthesis, typical of cyanobacteria, only became a eukaryotic trait after the engulfment, and subsequent endosymbiosis, of a cyanobacterial counterpart by a single-celled eukaryote (De Clerck et al. 2012). This phagocytotic capacity, distinctive of eukaryotic organisms, further enabled secondary and tertiary endosymbiotic events that gave rise to the diversity of autotrophic algae that we see today (Archibald 2012). A mixotrophic lifestyle, combining the phagotrophic uptake of prey with the ability to perform oxygenic photosynthesis within the same cell, is also a key characteristic of many microbial eukaryotes, most of which are planktonic (Raven 1997).

Mixotrophy in aquatic protists

Mixotrophs, in the broadest possible sense, are organisms that are able to combine autotrophy and heterotrophy within the same individual. This is a prevalent nutritional lifestyle among unicellular eukaryotes (Flynn et al. 2013, Li et al. 2022), and can be manifested in multiple ways (Jones 1997, Stoecker 1998, Stoecker et al. 2009, Mitra et al. 2016). Mixotrophic protists, while being able to feed on prey, can benefit from light-driven carbon fixation via their own, innate photosynthetic machinery (constitutive mixotrophy) or by exploiting the machinery of other organisms (acquired mixotrophy) either via specialized symbiosis or by retaining functional chloroplasts from ingested prey (Stoecker et al. 2009, Mitra et al. 2016). In turn, mixotrophic strategies can range from that of a strict phototroph that benefits from prey uptake to obtain essential macronutrients (Tranvik et al. 1989) to that of a strict phagotroph that can supplement growth with energy from light when prey is scarce (Sanders et al. 1990), or needs to ingest photosynthetic prey to take advantage of autotrophic nourishment (Carvalho et al. 2008).

Mixotrophy is an extremely rare feature in multicellular organisms, and is therefore a challenging property to deal with under the classical distinction between phytoplankton and zooplankton presented above (Flynn et al. 2013). Their most obvious effect in the scheme outlined in Figure 1 is the blurring of the divide between auto- and heterotrophy in those mixotrophs that must deploy both strategies to thrive, such as the chrysophyte *Uroglena americana* (Kimura and Ishida 1985) or the ciliate *Laboea strobila* (Stoecker et al. 1988). An additional increase in complexity is generated by the dissociation of those components of the network that harbor mixotrophic members (colored in Figure 1). This is exemplified by eukaryotic algae that can supplement their growth with bacterial ingestion, such as the haptophyte *Chrysochromulina brevifilum* (Jones 1997) or *Cryptomonas* cryptophytes (Tranvik et al. 1989, Marshall and Laybourn-Parry 2002), by plastid-bearing but primarily heterotrophic flagellates, such as the chrysophyte *Poterioochromonas malhamensis* (Sanders et al. 1990), or by plastid retention after prey ingestion (kleptoplastidy) in oligotrich ciliates (Johnson and Beaudoin 2019). Interestingly, however, taxonomy does not predict well mixotrophic behavior, which might be driven rather by biological constraints, illustrated within chrysophytes by the contrasting mixotrophic capabilities in different *Ochromonas* spp. (Lie et al. 2018, Wilken et al. 2020), or by environmental constraints, such as seen in Antarctic cryptophytes that turn only partially to predation of bacterial prey even under severe light limitation conditions (under the ice of perennially ice covered lakes, Marshall and Laybourn-Parry 2002), while *Cryptomonas* sp. was recorded to rely on prey for nutrient acquisition in also light-limited brown humic lakes (Tranvik et al. 1989).

Such levels of intricacy have prompted some authors to advocate for a planktonic food web model in which strict autotrophs or heterotrophs are only special cases of the norm, which is expected to be mixotrophy (Flynn et al. 2013, Mitra et al. 2014). To judge whether this is a better representation of reality than the classical separation will not be addressed further in the current thesis. Nonetheless, this novel proposition on the role of mixotrophy highlights the traditionally overlooked importance of mixotrophs in pelagic habitats and the need to consider mixotrophy as a fundamental strategy to understand the functioning of aquatic food webs.

Mixotrophs, with their bimodal behavior, have the potential to modulate the interplay between the classic food chain and the microbial loop (Mills and Forney 1988, Stockner and Porter 1988, Ptacnik et al. 2016). Accumulating empirical evidence suggests that mixotrophic microeukaryotes can become the dominant fraction of the total eukaryotic phytoplankton community, both in marine (Unrein et al. 2014) and freshwater environments (Bergström and Jansson 2000, Senar et al. 2021), and that they can account for a major proportion of total grazing pressure on bacteria (Bird and Kalff 1986, Zubkov and Tarran 2008, Hartmann et al. 2012). Cases of photoheterotrophy (heterotrophic nutrition coupled to light-driven energy uptake) within mixotrophs can obscure our understanding of carbon fluxes in the pelagic, since photoheterotrophy does not contribute to carbon fixation into the food web (Wilken et al. 2014). Alternatively, mixotrophs can potentially hijack the pool of dissolved nutrients into primary production through bacterial grazing (Mitra et al. 2014), outcompeting both pure auto- and heterotrophs. Finally, mixotrophic nutrition has been revealed as the main strategy supporting coastal blooms of haptophytes, cryptophytes, raphidophytes and dinoflagellates (Legrand et al. 2001, Jeong et al. 2005, Burkholder et al. 2008, Jeong 2011, Yoo et al. 2017, Anschütz et al. 2022). As hinted above, however, the manifestation of mixotrophy is very sensitive to the circumstances of the environment. Thus, lake trophic status (Saad et al. 2016), water temperature (Wilken et al. 2013), concentration of dissolved inorganic carbon (Hansson et al. 2019), light limitation (Wilken et al. 2018) or the requirement of specific nutrients, such as iron (Maranger et al. 1998) or phospholipids (Kimura and Ishida 1985) are all factors that condition the extent of mixotrophy in aquatic habitats. Further fluctuation of these factors over time convey the temporal component of mixotrophy, which has been observed to range from diel (Urabe et al. 2000, Anderson et al. 2017) to seasonal scales (Unrein et al. 2007, Waibel et al. 2019). Combined, all the above demonstrate the relevance of mixotrophy in aquatic ecosystems and illustrate the need to advance our understanding of the ecology of mixotrophic planktonic microorganisms.

Microbes at individual resolution

In a tradition that extends from Ernst Haeckel in the 1870s to Robert Ricklefs in modern times, ecology can be understood as the scientific discipline concerned with the causes and consequences of the interactions between living individuals and between them and the environment they inhabit (Ricklefs and Relyea 2014). Individuals of the same or similar kind constitute natural populations, conform to the notion of species, compose communities organized in trophic guilds and trophic levels across a complex food web, and are further spatially interconnected to shape larger metacommunities. Organisms, species, populations or communities are all levels of biological organization at the focus of ecological research. Nevertheless, at the basis of this hierarchy lies the individual, which can therefore be understood as the fundamental unit of ecology.

Gaining access to information at individual resolution does, however, pose a challenge to the microbial ecologist. Figure 2 highlights that the fundamental components of the pelagic food web are all microscopic, and therefore not readily observable. The illustration on the cover of this thesis is meant to showcase the magnitude of this complication. There, an actual cell of the mixotrophic flagellate *Ochromonas triangulata* is represented. The image is the composite of four micrographs obtained through laser scanning confocal microscopy, each collecting either the nucleus of the cell (in red), its chloroplast (in green), a few food vacuoles (in blue) and a neighboring bacterium (in yellow). As it stands in the cover of the physical book, the illustration is two thousand times larger than the specimen it portrays. In other words, and translating this proportion to the human scale, its dimensions are to reality what Uppsala Cathedral is to a tennis ball. The study of the environment at this scale demands, inevitably, the use of sophisticated techniques to investigate the ecology of such small organisms.

In this thesis, all the methodologies deployed to tackle aquatic microbial mixotrophs rely heavily on two main techniques: flow cytometry and DNA sequencing. Therefore, a brief introduction to each of them is deemed necessary.

Flow Cytometry

Magnifying lenses effectively opened the pathway to microbial research. The mere existence of microbes only became apparent with the development of the microscope during the second half of the 17th century (described in Wollman et al. 2015), and microscopy has since then remained fundamental for most of what we know of the microbial world. However, a microscope is a static system in which the examination of an increasing multitude of individual cells, one by one, quickly becomes a daunting task. In the 1960s, the attempts to couple an optical system to automatically count blood cells suspended in a circulating fluid transcended into the first flow cytometers (Givan 2001). In a sense, then, the flow cytometer can be interpreted as an automated, streamlined refinement of a microscope. In a flow cytometer, a fluidic system carrying the specimens and an optical system designed to detect and probe them are coupled at called a flow cell. The stream, in laminar flow regime, enables the orderly transport of microscopic particles dispersed in an aqueous medium to the interrogation point of the flow cell, where the particles meet a focused light beam, typically from a laser (and therefore of a specific wavelength). Upon encounter, each particle reflects and refracts the light from the laser (light scatter) and, when bearing a fluorescent component sensitive to the frequency of the incident light, reacts to emit fluorescent light. These interactions are precisely recorded individually, and so the particles suspended in an aqueous liquid can be distinguished based on their light scatter properties and fluorescent signatures. Advantageously, a typical flow cytometer enables the reliable scrutiny of samples at the order of thousands of particles per second, one particle at a time (Givan 2001).

Flow cytometry is a well-established tool in microbial ecology, where it was first used four decades ago to profile conveniently autofluorescent[2] phytoplankton from cultures (Trask et al. 1982) and from natural samples (Olson et al. 1985). Its value to the field soon became evident when it enabled the discovery of the picoplanktonic *Prochloroccocus* in the open ocean (Chisholm et al. 1988). The use of fluorescent nucleic acid stains to profile planktonic microorganisms lacking natural fluorescence quickly followed and flow cytometry became a standard tool to enumerate unpigmented planktonic heterotrophic bacteria (Robertson and Button 1989, Del Giorgio et al. 1996, Marie et al. 1997, Gasol and Del Giorgio 2000). Attempts to target the eukaryotic fraction of the nano- and microplankton fraction were also carried out successfully, either via DNA staining in the case of heterotrophic protists in general (Zubkov et al. 2007) and ciliates in particular (Lindström et al. 2002) or via fluorescent dyes targeting food vacuoles in heterotrophic and mixotrophic flagellates (Rose et al. 2004, Carvalho and Granéli 2006). Ingestion rates of

[2] Many photosynthetic algal pigments, such as the ubiquitous chlorophylls but also phycobiliproteins, are naturally fluorescent.

planktonic microeukaryotes have also been monitored using flow cytometry by following the fate of fluorescently labelled prey (Vazquez-Dominguez et al. 1999, Bratvold et al. 2000, Fu et al. 2003).

DNA Sequencing

All living organisms encode their functional potential in their genetic material, which for all prokaryotic and eukaryotic life forms consist of deoxyribonucleic acid (DNA). Living entities reproduce by replicating their DNA and perform their functions by transcribing their DNA into ribonucleic acid (RNA), which can be either functional (non-coding RNAs, such as ribosomal rRNA or transfer tRNA) or is in turn translated to proteins via messenger mRNA. Proteins are, ultimately, the fundamental operative components of living beings (Crick 1970, Keeling 2016). When accessed, DNA and RNA can then be used to infer the identity, evolutionary history, physiology and metabolism of the organisms under investigation. Therefore, the successive developments of DNA sequencing techniques in the 1970s (Sanger et al. 1977) and the polymerase chain reaction (PCR) in the late 1980s (Mullis and Faloona 1987) brought about a revolution to microbial ecology comparable in magnitude to the invention of the microscope.

Currently, DNA sequencing techniques (reviewed in Mardis 2013 and Kraft and Kurth 2020) are so accessible and affordable that most research in environmental microbiology is based upon them (Pedrós-Alió 2006, Xu 2006, López-García and Moreira 2008, Kerkhof and Goodman 2009, Zinger et al. 2011, de Vargas et al. 2015, Moran 2015, Castelle and Banfield 2018). Nowadays it is possible to retrieve genetic information directly from environmental samples in a way that microbial diversity, metabolism and, to some extent, community behaviour can be inferred while circumventing the effort and limitations of microscopic examination and microbial culturing.

Metabarcoding, metagenomics and metatranscriptomics are the most popular approaches due to their relative simplicity. All rely on bulk nucleic acid extraction directly from the environment, either DNA for metabarcoding and metagenomics or RNA for metatranscriptomics[3]. Metabarcoding provides a taxonomic account of the sample. It is based on the selective amplification via PCR of a target DNA sequence which might be present in all organisms of interest but has accumulated enough differences, in evolutionary timescale, to permit the distinction of taxonomic groups. In microbial ecology, the small subunit (SSU) of the rRNA gene is by far the most widely used genetic marker for this purpose, for both prokaryotes (Sinclair et al. 2015) and eukaryotes (Pawlowski et al. 2012), since it is universal, encodes for a universally

[3] RNA-based techniques are also included under this rubric since RNA needs to be retrotrascribed to DNA before it can be sequenced.

conserved function and combines hypervariable with highly conserved regions that reflect both close and distant evolutionary relationships (Olsen et al. 1986, Bhattacharya and Medlin 1995). Publicly accessible SSU rRNA databases that compile the correspondence between sequence and taxonomy, such as the SILVA (Yilmaz et al. 2014) and PR2 databases (Guillou et al. 2013), mediate the taxonomic profiling of the sequencing data. In contrast, in metagenomics the whole nucleic acid extract is directly sequenced (a process known as shotgun sequencing). This way, inferences can be made on the functional potential of the community as a whole (Lepere et al. 2011, Eiler et al. 2014, Hugerth et al. 2015, Peura et al. 2015). For metatranscriptomics, extracted RNA is retrotranscribed to cDNA and sequenced directly (Poretsky et al. 2005) or after target enrichment (He et al. 2010). The subsequent bioinformatic treatment and analysis of the generated sequences is substantially more complex in these cases than for metabarcoding, but ultimately our capacity to infer function from sequence also depends of the existence of good quality databases (Keeling et al. 2014).

However, neither of these approaches can provide information at single cell resolution because they rely on an indiscriminate extraction from the whole sample, and therefore they neglect the boundaries of individual organisms. Although it is in many cases possible to attribute taxonomic identity to many sequences obtained in a meta-omics exploration (Hugerth et al. 2015), it is not possible to link gene identity to cell of origin directly. Therefore, when genotyping natural populations, investigating intraspecific genetic variation, linking metabolic function to taxonomy or targeting species-specific interactions, single cells must be surveyed separately to obtain information at individual level (Stepanauskas and Sieracki 2007, Yoon et al. 2011, Martinez-Garcia et al. 2012, Zaremba-Niedzwiedzka et al. 2013, Annenkova et al. 2015, Spencer et al. 2016, Rengefors et al. 2021).

Single-cell techniques

Both genomic and transcriptomic approaches can be adapted to target the nucleic acid content of a single cell rather than a cell assemblage. Single cell genomics of both prokaryotes (Woyke et al. 2009) and eukaryotes (Yoon et al. 2011) are well established, while single-cell transcriptomics, developed and established on non-microbial eukaryotic cells and heavily dependent on the convenient poly-A tail of eukaryotic mRNA to capture this RNA component specifically, has gained enough momentum to be exported to microbial eukaryotes (Kolisko et al. 2014, Liu et al. 2017).

To achieve single-cell resolution, single cells need to be first physically isolated and their genetic material of interest needs to be subsequently amplified (Woyke et al. 2017). The latter is a necessary condition, since the nucleic acid content in a single cell does not suffice to feed any available sequencing instrument. Regarding the former, strategies to isolate single cells from a sample come in three main families: micromanipulation, fluorescence-activated cell sorting (FACS), and emulsion-based cell encapsulation in microdroplets.

Micromanipulation is based on manually picking single cells while they are being observed under the microscope, either by microcapillary suction (Sassenhagen et al. 2020) or laser microdissection (Mariz et al. 2022). This has the obvious advantage of the visual recognition, but suffers the typical lack of throughput associated with microscope examination. Micromanipulation is not employed in any of the works presented in this thesis and is only mentioned here for the sake of completeness.

The use of FACS offers a higher throughput (Thompson et al. 2013, Rinke et al. 2014). A FACS sorter is essentially a flow cytometer in which the sample stream, after flowing through the flow cell, is fragmented into microdrops that encapsulate the particles suspended in the sample. After being generated, the drops are forced to pass between two deflector plates which create an electric field perpendicular to the flow. The key of the system is that those droplets carrying particles of interest, which are in turn cytometrically defined by the user, can be traced very precisely and, when detected, they are electrically charged. Once this occurs, they respond to the electric field between the deflectors and can be physically sorted into a collection vessel. This way, any particle matching any optical properties of interest can be separated from the sample with very high precision (see Figure 3 under Part 1 for a graphical depiction). Since such a system is based on the performance of a flow cytometer, it is capable of sorting hundreds of cells within a few seconds. FACS sorters are commercially available and are the standard tool used for the isolation of microbial single cells (Thompson et al. 2013, Woyke et al. 2017).

Finally, cell encapsulation in emulsion droplets offers the highest possible throughput. In this kind of approaches, an aqueous sample containing a suspension of the cells of interest is emulsified inside an oil phase. This, fractionates the sample into microscopic compartments which, when cell density in the sample is adjusted appropriately, will most likely host a single cell whenever they are occupied (Jönsson and Andersson Svahn 2012). Depending on how the emulsion is generated, two main families of procedures can be identified: methods in which the sample is emulsified using sophisticated microfluidic devices, and microfluidics-free methods which rely on non-channelled mechanical forces. The former allow a high level of control on the emulsion process (permitting, for example, the generation of evenly sized, i.e. monodispersed, droplets), but depend on complex devices that are often not available commercially. The latter typically produce highly polydisperse emulsions (i.e. broad range of droplet sizes) but are readily applicable in any molecular biology laboratory (Hatori et al. 2018). Both FACS and non-microfluidic emulsion-based procedures were employed in the investigations presented here.

Scope of the thesis: identification, interactions, function

In this thesis, the focus has been placed on three methodological approaches that target different aspects of aquatic mixotrophs at the individual level. Broadly, the aims have been the taxonomic identification of actively feeding mixotrophs from a natural sample, the large-scale detection of specific associations between mixotrophs and bacterial prey and the functional characterization of mixotrophic populations based on individual gene expression, respectively. In accordance, the following sections of the thesis are structured in three parts, each part corresponding to one methodology.

In the first part, the results of **Paper I** and **Paper II** are presented. Here I explore the use of fluorescently labelled prey surrogates (hereafter referred to as fluorescently labelled tracers, or FLT) in combination with chlorophyll *a* fluorescence to sort and identify individual mixotrophic cells in a cytometric way. In **Paper I**, the affinity of a mixotrophic flagellate for different types of FLT is examined. In **Paper II**, different types of FLT and one food vacuole tracer are evaluated in their capacity to retrieve actively feeding mixotrophs from a natural freshwater assemblage using FACS.

Paper III and **Paper IV** comprise the second part. **Paper III** describes the potential and applicability of adaptating the emulsion, paired-isolation and concatenation PCR (epicPCR), a non-microfluidic emulsion-based encapsulation procedure, to deliver pairwise linkages between taxonomic markers of unicellular eukaryotes and their physically associated bacterial cohort. In **Paper IV,** preliminary results are presented on the performance of this methodology by using two cultured mixotrophic flagellates as predatory models.

The results of **Paper V** are presented in the third part of the thesis, summarizing the capability of single cell sorting and subsequent single cell mRNA sequencing (scRNA-seq) to recover the transcriptional profile of two distinct populations of a mixotroph in culture.

All parts, being largely independent of each other, are introduced, presented and discussed individually. A brief joint discussion follows, touching upon those aspects common to the three parts. A final conclusions section, integrating the findings of each of them, is included at the end of the thesis.

Part 1. Identification of actively feeding mixotrophs

Mixotrophy is a well-documented trait in many clades across the entire eukaryotic tree of life (Del Campo et al. 2014). As mentioned above, this lifestyle comes in two main modes: on the one hand, acquired phototrophy by predatory phagotrophs is well documented in both freshwater and marine planktonic ciliates (Macek et al. 2006, Schoener and McManus 2012, Johnson and Beaudoin 2019) and in bloom-forming marine dinoflagellates (Stoecker et al. 1997, Carvalho et al. 2008, Nishitani et al. 2012). On the other hand, constitutive mixotrophy has since long also been reported among freshwater chrysophytes (Bird and Kalff 1986, Sanders et al. 1990) and cryptophytes (Tranvik et al. 1989, Marshall and Laybourn-Parry 2002), and also haptophytes (Jones et al. 1994, Granéli et al. 2012, Unrein et al. 2014) and dictyochophytes (Sanders and Porter 1988, Gerea et al. 2016, Li et al. 2021), both freshwater and marine.

Despite the wealth of existing information, novel cases of mixotrophy have recently been documented among marine raphidophytes (Jeong 2011), in an euglenozoan (Yamaguchi et al. 2012), a prasinophyte (Maruyama and Kim 2013), a chlorarachniophyte (Yoo and Palenik 2021) and, only from environmental samples, for several bolidophytes (Frias-Lopez et al. 2009, Li et al. 2022). These findings, combined with the increasing number of reports of unexpected mixotrophic taxa within known mixotrophic groups (Jeong et al. 2005, Yoo et al. 2017, Koppelle et al. 2022) suggest that the taxonomic spread of mixotrophy may be broader than previously assumed, to the point that further exploration almost guarantees novelty (Li et al. 2022).

All these recent, unexpected cases of mixotrophy among taxa previously considered to be non-mixotrophic showcase the weaknesses of evaluating the extent and style of mixotrophy taking place at a given moment in a natural environment when relying on taxonomy. Because of this, it is valuable to include, in the toolkit of the microbial ecologist, a technique that can target mixotrophs naïvely (i.e. without any prior assumption) and that are actively feeding in a given moment.

A FACS-based approach, as described above, could potentially be deployed for such a task. Due to the fluorescent nature of chlorophyll *a*, essential

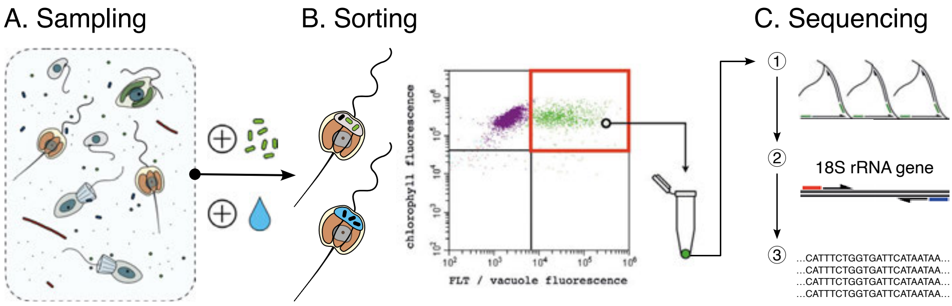

Figure 3. Graphical summary of the procedure presented in Part 1. Initially, a natural sample is incubated with a fluorescent reporter for prey ingestion, either fluorescently labelled tracers (FLTs) or a food vacuole staining agent (A). Based on concomitant fluorescence, mixotrophs can be cytometrically identified and single mixotrophic cells can be sorted (B). After lysis, cell genome is (1) randomly amplified based on multiple displacement amplification (MDA), (2) a portion of the 18S rRNA gene in further amplified by PCR, and (3) the taxonomic marked is sequenced. Sequences can be annotated taxonomically using publicly available databases or phylogenetic trees.

for photosynthesis, and the possibility of tracing prey ingestion using fluorescent cues (Sherr and Sherr 1993, Carvalho and Granéli 2006), the flow cytometry principle of FACS makes it a suitable technique for discriminating mixotrophs based on concomitant signals of chlorophyll and prey ingestion tracers (see Figure 4 below for examples). Using a cell sorter, a large number of individual mixotrophic cells can in this way be identified and captured.

Table 1. The fluorescently labelled tracers (FLTs) used in the studies of Part 1.

Code[a]	**Description**	**Volume (μm^3)**	**Size class**	**Quality class**
BDS-1 / BDS	Polystyrene microspheres, 0.98 µm	0.49 (CV 9%)[b]	large	inert
BDS-.5	Polystyrene microspheres, 0.51 µm	0.070 (CV 9%)[b]	small	inert
FLB-E / FLB	Fluorescently labelled *E. coli* cells	0.76 (CV 41%)	large	dead
FLB-R	Fluorescently labelled *Limnohabitans* sp. cells	0.097 (CV 55%)	small	dead
GFP	*E. coli* TOP10 pQE30-tGFP	0.72 (CV 48%)	large	living

[a] When two labels are provided, these correspond to the encoding used in **Paper I** and **Paper II**, respectively. [b] Based on 3% CV in diameter values declared by the manufacturer.

The idea to be tested in the current thesis was to benchmark and validate this approach by jointly using chlorophyll *a* fluorescence and FLT as bait to capture actively feeding mixotrophs, which in turn could be identified based on downstream sequencing of a taxonomic marker gene (Figure 3). This approach took inspiration from a previous study in which a food vacuole staining agent was used as a fluorescent proxy for prey ingestion (Wilken et al. 2019). To further explore the potential and limitations of this approach, we assessed the impact of the type of FLT on the measurement of feeding rates, therefore assessing to what extent the choice of FLT can influence detection of bacterivore capacity (**Paper I**). Subsequently, we deployed and assessed the feasibility of the method as depicted in Figure 3, while comparing the return of three different FLT types and including food vacuole staining as a tracer for ingestion (**Paper II**). The main characteristics of all FLT types used in both papers are summarized in Table 1.

Results

In **Paper I**, we evaluated the preference for five types of FLT by the cultured mixotroph *Ochromonas triangulata*. We did that by measuring at high precision the rates at which each FLT type was ingested. With the exception of living fluorescent prey (GFP), for which the measurements were not reliable (see **Paper I**, Figure 1B and further discussion in Section 3), ingestion rates associated to large FLT types were significantly higher (Table 2). This phenomenon was apparent regardless of the analytical methodology used for detection and quantification, but was most obvious based on the superior quantitative data from cytometry as compared to microscopy. The differences across FLT quality classes, although significant, were less marked. Based on these observations, we concluded that size, rather than quality, was the main factor responsible for differences in prey preference in *O. triangulata* and, possibly, in other nanoplanktonic flagellates (**Paper I**).

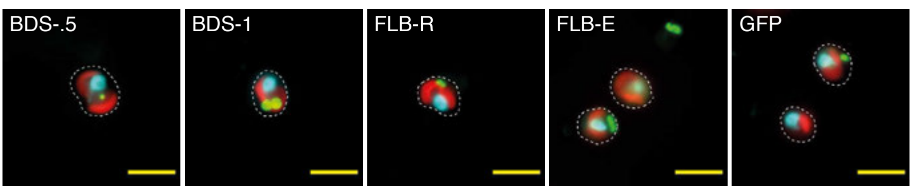

Figure 4. Epifluorescence microscopy images showing examples of ingestion of fluorescently labelled tracers (FLTs) by *Ochromonas triangulata* for all FLTs used in **Paper I**. Each image is a composite depicting the nucleus (cyan), the chloroplast (red) and surrogate prey (green). Cell contours were drawn from the corresponding bright-field images (**Paper I**, Figure 1A). Scale bars: 5 µm.

Table 2. Ingestion rate estimates and their associated standard error for each combination of methodology and fluorescently labelled tracer (FLT). See Table 1 for FLT type descriptions.

FLT type	Ingestion rate (10^{-3} min^{-1})		r^2		P-value	
	Cytometry	Microscopy	Cytometry	Microscopy	Cytometry	Microscopy
BDS-1	7.48 ±0.17	4.68 ±0.54	0.9890	0.8505	<0.001	<0.001
BDS-.5	1.14 ±0.07	1.81 ±0.30	0.9191	0.7372	<0.001	<0.001
FLB-E	9.31 ±0.34	6.34 ±0.55	0.9721	0.9106	<0.001	<0.001
FLB-R	3.26 ±0.10	3.03 ±0.30	0.9779	0.8903	<0.001	<0.001
GFP	0.25 ±0.04	1.91 ±0.61	0.6242	0.4319	<0.001	0.008

In **Paper II**, we compared three different FLT of similar size (those belonging to the *large* size class in Table 1) in their suitability to profile a mixotrophic community from a sample. We included in our comparison a food vacuole staining agent, LysoSensor DND-167, which only fluoresces in acidic conditions and accumulates in acidic organelles, such as food vacuoles. The use of this compound has been reported previously as a good proxy for prey ingestion (Carvalho and Granéli 2006). Most obviously, **Paper II** shows that FACS-mediated isolation was successful in retrieving individual mixotrophs from a natural assemblage.

Dictyochophytes, chrysophytes, dinoflagellates, haptophytes and cryptophytes, all taxa with mixotrophic representatives, accounted for more than 99% of the entire population of FACS-isolated single cells (Figure 5, see **Paper II**, Tables 1 and 2 for a full account of all taxa recovered).

Dictyochophytes dominated the dataset by far, accounting for almost 85% of the total occurrences. Microscopy evidence confirmed two pedinellid morphotypes (one with three chloroplasts and another with six) as highly active bacterivores in the sample (**Paper II**, Figure 2). In contrast, 9 pedinellid OTUs, phylogenetically distinct from any known SSU rRNA pedinellid sequence, were recovered in the single-cell dataset. Since these OTUs represent molecular novelty, we uncovered cryptic molecular diversity within mixotrophic pedinellids, demonstrating the relevance of the approach.

Chrysophytes, the second most abundant taxon (approx. 9% of the total occurrences), illustrate nonetheless one possible weakness of the method.

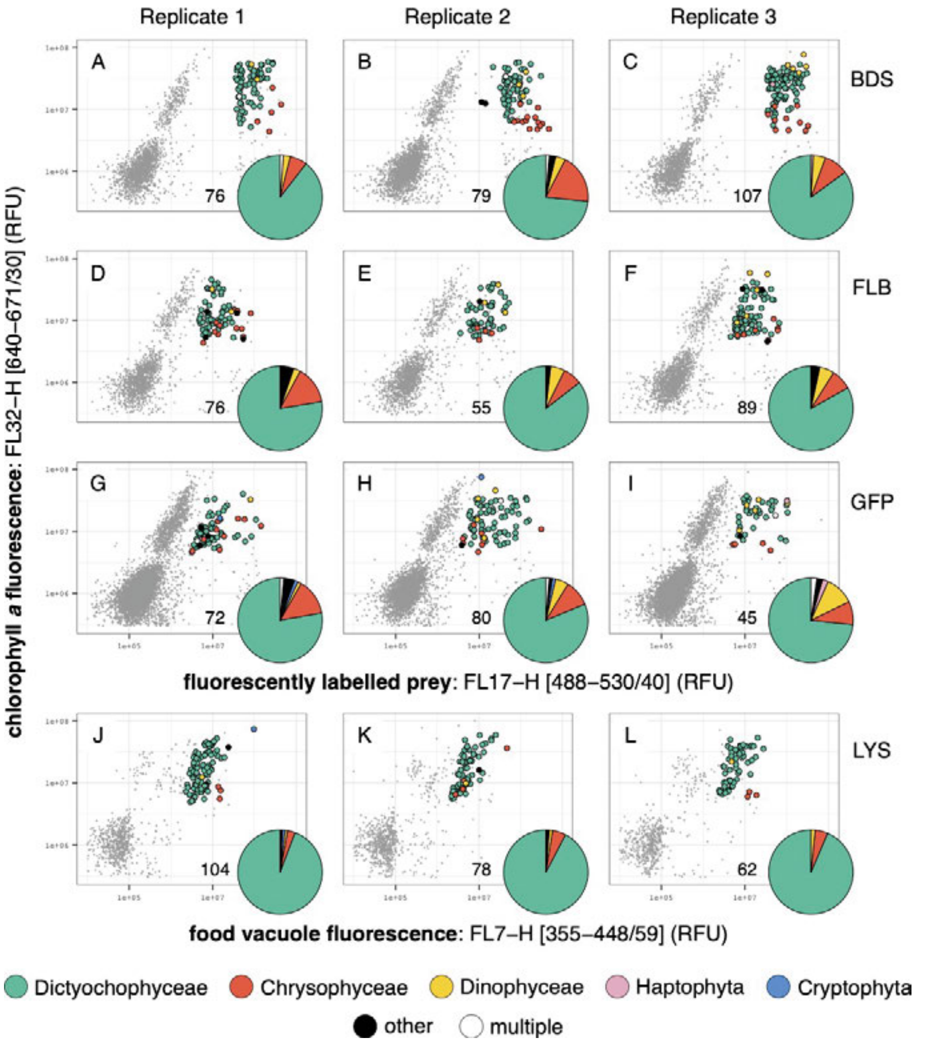

Figure 5. FACS-sorting cytograms showing all the sorted single cells when color-coded based on their taxonomic assignations. Inset pie charts show relative proportion of each taxon, while inset numbers indicate the total number of cells sorted per reporter and replicate. Grey dots represent events of the background population. Gates and gating strategy to discriminate the mixotrophic population are found in **Paper II**. FL7 corresponds to food vacuole fluorescence, FL17 to fluorescently labelled prey fluorescence and FL32 to chlorophyll fluorescence. RFU: relative fluorescence units. Reporters: 1 µm fluorescent microspheres (BDS), fluorescently labelled bacteria from E. coli cells (FLB), GFP-expressing E. coli cells (GFP) and food vacuole staining with LysoSensor Blue DND-167 (LYS).

While all dictyochophyte OTUs represented closely related pedinellids, phylogenetic annotation of the 9 chrysophyte OTUs showed them to be broadly scattered across the phylogeny of this group (**Paper II**, Figure 3). Unlike the dictyochophytes, microscopic evidence of their feeding behavior could not be obtained. In addition, the phylogeny revealed that at least 4 of these OTUs represented unpigmented taxa, and therefore could be false positives. Reasons for why such putative false negatives can be prevalent are discussed below.

Dinoflagellates, the third most abundant taxon (3% of total occurrences) illustrate a case of greater complexity. Represented by a single OTU annotated as *Prorocentrum* sp., this dinoflagellate was almost always recovered (85% of the times) in partnership with a dictyochophyte. This high rate of co-occurrence could be explained by *Prorocentrum* sp. feeding on the abundant pedinellids. If this would have been the case, each doublet would reflect an occasion where either an FLT-fed pedinellid was recently ingested by the dinoflagellate or where the dinoflagellate had ingested both FLT and the pedinellid. We considered these scenarios to be plausible given the reported *Prorocentrum* spp. preference for larger algal prey (Jeong et al. 2005, Johnson 2015).

Regarding differences introduced by prey type, the main differences in output were found to be between the food vacuole reporter (LYS) and all three FLT-based treatments, where the latter yielded similar results (see inset pie charts in Figure 5). The relative frequency of false positives averaged across replicates ($\widehat{fp}$) delivered by the LYS reporter was significantly lower than for that of any other reporters (**Paper II**, Figure 7 and Suppl. Figure 5), while no significant differences were found in $\widehat{fp}$ among FLT-based approaches. The $\widehat{fp}$ for FLTs was found to range, on average, between 14% and 10 % (for the most exclusive and most inclusive scenario, respectively), while LYS $\widehat{fp}$ was between 5% and 3%. Moreover, the larger spread of $\widehat{fp}$ associated to the use of FLTs denoted higher likelihood of generating false positives. LYS, in contrast, delivered consistent results across replicates. We conclude that LysoSensor Blue was more specific, robust and consistent that any of the prey-based reporters.

Discussion

Regarding ingestion proxies, **Papers I and II** combined broadly confirm that prey size, rather than prey quality, might be the major drive of microeukaryote predation over bacterioplankton (Andersson et al. 1986, Chrzanowski and Šimek 1990, Gonzalez et al. 1990, Jürgens and Matz 2002, Schmidtke et al. 2006). The prominent effect of size over quality is more precisely measured and quantified in **Paper I** for a single taxon, and the lack of differences in taxa recovery for similarly sized FLT of differing quality seen in **Paper II** can be interpreted as indirect evidence for predominant size selection.

Contrary to our expectation, food vacuole staining provided the most specific proxy for mixotrophy. Food vacuole stains are ideal tracers because they are comprehensive: while FLT ingestion will only be seen in a subset of the total bacterivorous population (by chance, not all active phagotrophs will feed on an FLT during the experimental incubation), food vacuole tracers will in contrast stain the entire feeding population (and therefore also enable much shorter sorting times). Unfortunately, the use of such physiological stains as proxies for bacterivory can be prone to artifactual signals not related to prey ingestion (Rose et al. 2004, Carvalho and Granéli 2010, Wilken et al. 2019). However, based on our results and on previous observations (Carvalho and Granéli 2006), the use of LysoSensor Blue DND-167 in particular seems to largely be devoid of such artifacts. Specifically, in **Paper II** we avoided the LYS-mediated isolation of diatoms, abundant in the starting community but absent in the single-cell dataset, and known to be susceptible to LysoSensor staining of their silica deposition vesicles (SDVs, Shimizu et al. 2001). This observation could indeed be driven by factors other than the intrinsic performance of LysoSensor Blue, such as low incidence of SDVs at the time of sampling or our failure in lysing diatoms that were FACS-sorted. Nonetheless, the absolute absence of diatoms among sorted cells hints at improved performance of LysoSensor Blue as an optimal food vacuole tracer. In any case, using FLT and LysoSensor jointly, as shown to be practical in **Paper I**, can offer an even more robust alternative despite giving up the increased throughput that LysoSensor alone provides. Both FLTs and LysoSensor are susceptible to artifacts that can be cancelled out when used together to, for example, avoid misclassifications such as those that drove the contended reports of mixotrophic activity in the green algae *Micromonas* spp. (Gonzalez et al. 1993, Sanders and Gast 2012, McKie-Krisberg and Sanders 2014, Wilken et al. 2019, Jimenez et al. 2021), which is most likely not mixotrophic (Jimenez et al. 2021). Beyond bacterivory, the possibility of changing the prey types to fluorescently labelled algae with heat-killed, permanently stained prey (as in Sherr and Sherr 1993), or with living, transformed algae (as in Mix et al. 2018) opens the door to target mixotroph feeding modes beyond bacterivory, such as reported for the *Teleaulax-Mesodinium-Dinophysis* complex (Anschütz et al. 2022).

Paper II shows that the combined signals of a fluorescent proxy for prey ingestion and intrinsic chlorophyll autofluorescence can provide sufficient specificity for successful FACS-mediated recovery of individual actively feeding mixotrophs from aquatic samples. The strategy holds great exploratory potential: since it is taxonomy-agnostic (i.e. its output is not constrained by any taxonomic presumption), it effectively applies a functional filter that couples activity to taxonomy in a way that waives any previous assumption of the feeding behavior of any member of the microbial food web. Following this principle, the approach could provide the means to minimize sampling biases associated with difficulties to recognize diagnostic features of mixotrophy in small cells, which have been at least identified in big-scale marine metabarcoding surveys (Leles et al. 2019). In our experiment, we resolved at least 7 distinct novel mixotrophic phylotypes within pedinellids and revealed the occurrence of a mixotrophic freshwater *Prorocentrum* species. This adds a valuable discovery potential to the method and thus represents an improvement over conventional metabarcoding surveys. Besides, not only does this approach circumvent the need to draw potentially misguided assumptions, but it also highlights taxa that are active feeders at a given moment, offering the chance to uncover feeding patterns over rapidly changing conditions, such as diel feeding cascades or the triggering or halting of ingestion by the same taxa. In addition, the precise single-cell isolation offered by FACS opens the possibility to do single cell inquiry of other traits, at genomic or transcriptomic level, specific for those microeukaryotes that were feeding at the moment of sampling. All of the above demonstrates the relevance of a valuable tool to detect important and unexpected active mixotrophic species in a wider range of aquatic environments, and could easily be coupled to other techniques to describe the finer details of the trophic status of aquatic microbial communities.

Part 2. Microeukaryote interactions with bacterial counterparts

As depicted in Figure 1, interactions between prokaryotes and microbial eukaryotes are fundamental in aquatic environments. Examples of trophic interactions pictured there could include heterotrophic bacteria feeding on carbon compounds in the “phycosphere” surrounding algal cells (Seymour et al. 2017) or bacterivorous protists that recently consumed a prokaryote prey (Weisse et al. 2016). In addition, inter-domain interactions of a nature beyond the trophic arena are also important, such as obligate mutualistic associations between diazotrophs and phytoplankton (Thompson et al. 2012, Gradoville et al. 2021), but also bacterial pathogens such as *Vibrio cholera* infecting and replicating intracellularly inside amoebae (Abd et al. 2005). Large scale statistical analysis of co-occurrence patterns observed in metabarcoding surveys is one possible approach to map interactions between microorganisms. Based on observations in temporal succession, species correlations in space and time can be revealed (either positive or negative) and enable ecological inferences on species associations (Steele et al. 2011, Krabberød et al. 2022). Two obvious limitations of such strategies are that, despite their great discovery potential, they are very sensitive to confounding factors (e.g. nutrient bursts, apparent competition, etc.), and that resolving patterns through correlation cannot reveal causality or the specific interactions at play.

EpicPCR could deliver single cell resolution for such correlative approaches in a high-throughput manner, i.e. retaining the benefits of co-occurrence networks while potentially overcoming some of the limitations. This workflow was originally devised to link function-to-taxonomy in complex bacterial communities (Spencer et al. 2016). In its original version, it successfully provided a taxonomic link based on the 16S rRNA gene to a functional query gene by joining (concatenating) representative fragments of both genomic regions in cells that had been individually encapsulated in polyacrylamide microcompartments (paired-isolation). The central PCR reaction, occurring in emulsion, preserved individuality while enabling high-throughput screening. We tested if these properties could be adopted to identify spatial co-occurrence patterns of different microbial cells, such as the abovementioned physical co-occurrences between bacteria and microscopic eukaryotes. Rather than function-to-taxonomy, interactions would be revealed by linking

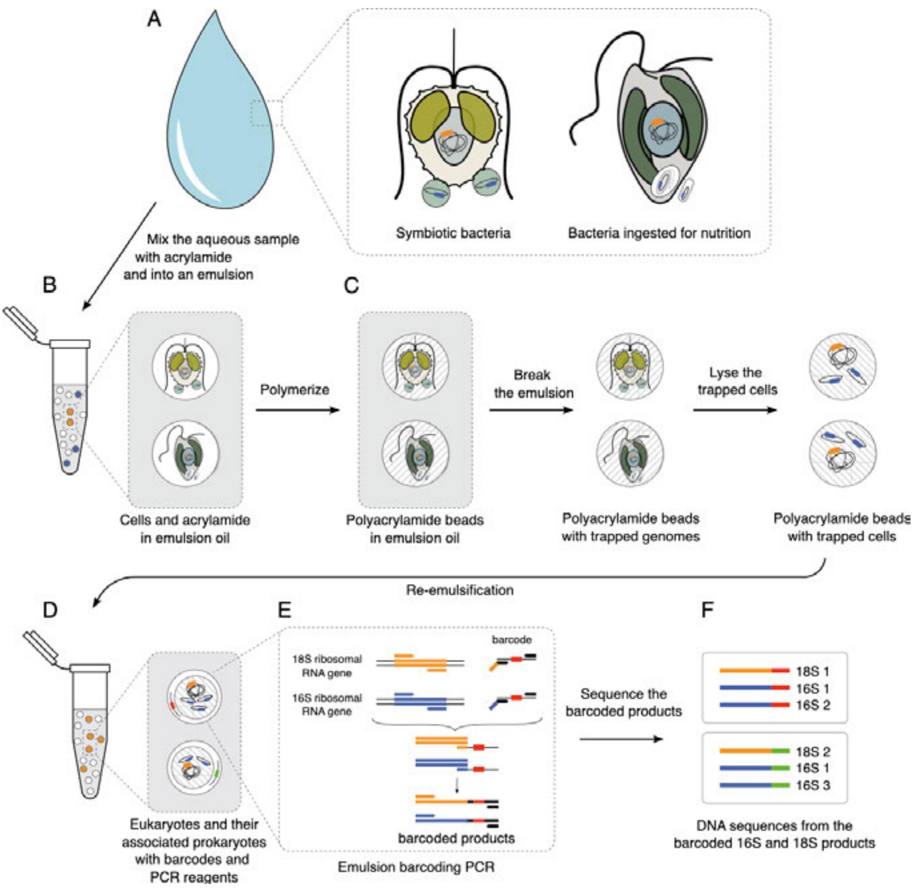

Figure 6. Workflow of epicPCR-based eukaryote-prokaryote taxonomic linkage in its barcoding version. (A) The workflow targets microbial eukaryotes and their physically attached bacteria present in a water sample. (B) Cells in the sample are suspended in an acrylamide solution and emulsified with mineral oil. (C) The emulsion droplets are subsequently polymerized into polyacrylamide beads containing individual cells, which can be exposed to lytic agents without the risk of compartmentalization loss. (D) Polyacrylamide-trapped genomic fragments are re-encapsulated together with barcodes and PCR reagents via a second emulsion. (E) Amplicon barcoding takes place in two steps: first, barcodes and genomic fragments are amplified independently; forward primers for the barcodes bear overhangs complementary to the genomic targets (bridging primers) to promote barcoding. Second, barcoded fragments are further amplified via a standard nested PCR. (F) The barcoded products, encoding 16S-18S associations, are prepared for sequencing.

two taxonomic markers for eukaryotes and prokaryotes, respectively. An extensive discussion on the potential and limitations of this version of epicPCR is laid out in **Paper III**.

The epicPCR workflow adapted to target eukaryote-prokaryote interactions is depicted in Figure 6. This is a modified version of the workflow featured in

Paper III, in which 18S-16S rRNA gene linkage is described via the fusion of the two amplicons into a single concatenate. In Figure 6, the barcoding version of the workflow is pictured. In this version, beads carrying the genetic material of eukaryotic single cells and their associated cohorts are emulsified together with a unique barcode. During emulsion PCR, the barcodes are amplified using primers with overhangs matching both 16S and 18S rRNA gene priming regions, while the taxonomic markers are amplified independently (Figure 6D).

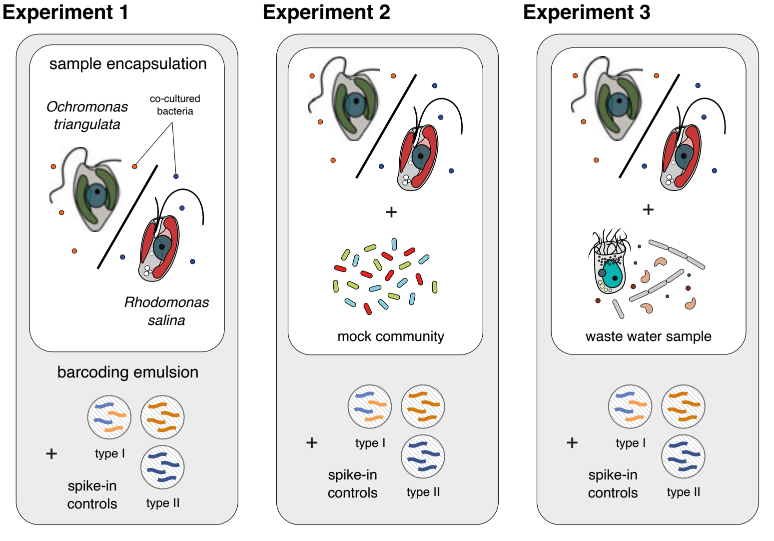

Figure 7. Diagrams showing the components of each of the three experiments. The white area comprises the elements that were encapsulated together during the first emulsion step in each experiment. The grey area comprises the elements that were combined during the second emulsion prior to the barcoding PCRs. Fragments in either blue or yellow hues represent artificial DNA molecules bearing 16S or 18S primer binding sites, respectively, in the synthetic controls.

To test the feasibility of the epicPCR approach to connect eukaryotes to their bacterial associates, we used two mixotrophic flagellates in non-axenic, unialgal culture (the chrysophyte *O. triangulata* and the cryptophyte *Rhodomonas salina*) as model phagotrophic eukaryotes (**Paper IV**). Predatory associations between the eukaryote and their co-cultured bacterial communities were our target. Mixotrophy was key for our proof of concept because both flagellates, being constitutive mixotrophs, were expected to produce the association between the nuclear 18S rRNA gene and the plastid 16S rRNA gene in addition to bacterial 16S rRNA genes that would reveal their feeding preferences.

In addition, synthetic internal controls were included in our experiments. These were polyacrylamide beads carrying artificial DNA sequences with primer-binding sites complementary to the primers used during the emulsion PCR step. Two types of controls were used: type I controls carried either 16S or 18S rRNA primer-binding sites in the same bead. Type II control beads, in contrast, combined two different molecules, each presenting binding sites for either 16S or 18S rRNA gene primers, into the same bead.

Three exploratory experiments were performed (Figure 7). In the first set, either of the two mixotrophs were encapsulated on their own (each with their co-cultured bacterial community) and the workflow was carried out adding the synthetic controls to the second emulsion mixture. In the second set, the mixotrophs were encapsulated jointly with an independently grown bacterial community in which all members were known (bacterial mock, see **Paper IV**, Table 1). In the third set, a wastewater sample was encapsulated together with the mixotrophs.

Results

In most cases, the most frequent associations between mixotroph and bacterial sequences were those that involved the most abundant bacterial taxa, as revealed by the abundance of 16S rRNA gene sequences in the combined experimental incubations. (**Paper IV**, Table 4 and Figures 3 and 4, not included here due to their large size). This is most evident in experiment 2, where the strongest associations correspond to those seen between eukaryotes and the

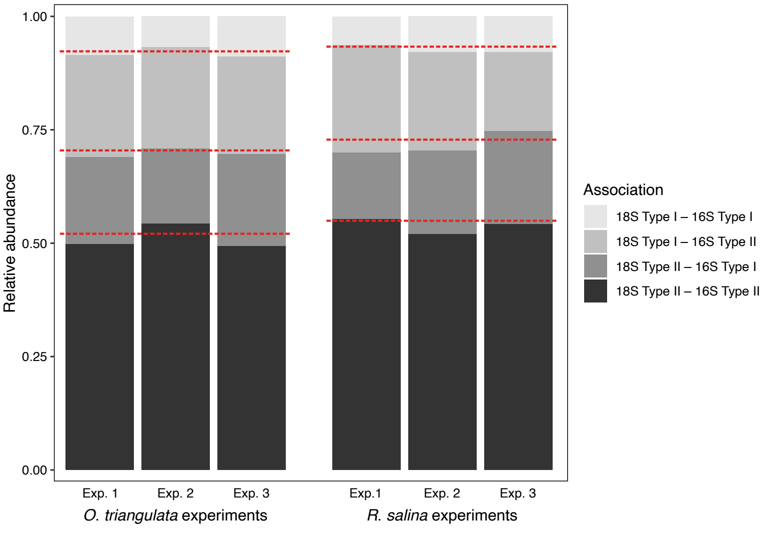

Figure 8. Relative abundances of type I and type II synthetic control associations for each experiment. Dashed red lines indicate the expected proportions of each association, averaged for each experiment set, if synthetic control associations would respond exclusively to their amplicon abundance.

most abundant members of the mock community. This suggests loss of individuality at some point during the experimental protocol.

This pattern was even stronger when analyzing the recovered associations between type I and type II controls. Among these, 18S rRNA Type II – 16S rRNA Type II was the most frequent pairing, followed by both 18S Type I – 16S Type II and 18S Type II – 16S Type I at approximately one third of the frequency of the first, and 18S Type I – 16S Type I at one seventh (Figure 8). This is in stark contrast with the experimental expectation, in which the 18S Type I – 16S Type I association should be the only recovered interaction among controls since these are the only controls that should co-occur in encapsulation droplets (**Paper IV**, Figure 1). Instead, more than 90% of the recovered association among controls corresponded to those that should have been avoided. Remarkably, the association between synthetic controls reflected very closely the expected proportions if they would be exclusively driven by molecular abundance (red lines in Figure 8), signifying that they followed perfectly an abundance-driven scenario. The patterns in Figure 8 are decisive to confirm loss of individuality during our attempt and, hence, we could not obtain any evidence for single-cell specificity in our experiments.

Discussion

Although our experiment appeared to have lost the single-cell resolution, the barcoding strategy deployed in **Paper IV** was successfully implemented. This strategy represents an improvement to the original workflow grounded in amplicon concatenation, as described in **Paper III**. Barcoded amplicons instead of concatenates allow not only 16S-18S rRNA linkage, but also 18S-18S, expanding the interaction landscape that can be uncovered with the technique. For example, it could contribute to the exploration of potential for non-constitutive mixotrophy, since it recovers the association between the nuclear 18S rRNA and the plastid 16S rRNA gene in a plastid bearing eukaryote. Thus, kleptoplastidic eukaryotes could potentially be revealed by occasional association with the plastid 16S gene of another species, and in turn known mixotrophs could be revealed as actively feeding when their 18S rRNA gene comes associated with specific bacterial taxa (Anschütz et al. 2022).

However, the workflow as presented here is far from mature. Inter-domain associations in our experiment were not seen to harbor specific linkages, but rather seemed to be driven by the abundances of the bacterial counterparts. The recovery patterns of the associations of synthetic controls, matching perfectly an abundance driven scenario, confirmed this apparent loss of individuality during the experimental protocol. Two different phenomena might explain the patterns observed, one related to sample encapsulation (Figure 6B) and the other related to barcoding and subsequent PCR reactions (Figure 6D and onwards).

Eukaryotes, no matter how small, will be on average one order of magnitude larger than bacteria. At the same time, bacteria are one or two orders of magnitude more abundant than the eukaryotes, both in non-axenic cultures (as those used in **Paper IV**, see **Paper I** or **Paper V,** where *O. triangulata* was also used, for further details) and in environmental samples. The epicPCR workflow, as deployed in **Paper IV**, relies on sample encapsulation after emulsifying the sample via vortexing. This generates a highly polydisperse emulsion, and therefore the whole range from sub-micron to >100 μm in diameter water-in-oil polyacrylamide containers will be formed. The smallest droplets are of minor concern, since they can only encapsulate free-living bacteria and thus not generate spurious associations. Larger eukaryote cells, on the other hand, can only be encapsulated in correspondingly large droplets. Given that bacterial abundances are way higher than that of eukaryotes, and that bacterial cells are much smaller, the chances of paired encapsulation of a eukaryote with random bacteria that are not directly physically associated is high. However, since frequency distributions of droplet sizes cannot be predicted due to the polydisperse nature of the encapsulation stage, the probability of random occupancy by free-living bacteria as a function of droplet size cannot be estimated. To fix this issue, monodisperse (i.e. all droplets of the same size) emulsions prior to polymerization (depicted in Figure 6B and 6C) would be a pre-requisite. This would imply adopting microfluidic-mediated emulsion strategies at the expense of ease of implementation, although easily operated microfluidic alternatives exist (Kim et al. 2017).

The second phenomenon behind the apparent loss of compartmentalization can be crosstalk caused by non-barcoded amplicons generated during the second emulsion (as partially amplified products that have not completed the second PCR step during the second emulsion, Figure 6E) that pass to the nested PCR stage. A nested step is needed because the barcoding PCR is an inefficient reaction. This reaction requires a complex PCR setup, since 7 primers in total are needed for an optimal yield in the reaction. However, the reverse primers amplifying the genomic fragments are technically not needed and could thus be omitted. In this scenario, with only the forward primer annealing to the target, linear amplification of these fragment would ensue. This would result in lower yields that would constrain the output of the barcoding step, but at the same time would prevent the translation of a vast array of potentially non-barcoded fragments that become spuriously barcoded during the nested PCR, thus ruining sample compartmentalization. Related to the loss of compartmentalization, inefficient or insufficient blocking strength during blocking PCR might also leave behind a considerable amount of partial, non-barcoded products from the barcoding PCR that would misguide the nested amplification.

Although technical limitations exist, avenues for further development, such as using monodisperse emulsions, barcoded gel beads (Macosko et al. 2015) and/or split-pool barcoding (Rosenberg et al. 2018), remain open. The vast exploratory potential of the technique warrants further research to overcome technical constraints.

Part 3. Physiological state and functional potential

Studies targeting gene expression in microeukaryotes at single cell levels are scarce, and mostly devoted to either yeast model organisms (Nadal-Ribelles et al. 2019, Saint et al. 2019) or pathogenic apicomplexans, such as *Plasmodium* (Poran et al. 2017, Reid et al. 2018, Howick et al. 2019) and *Toxoplasma* (Xue et al. 2020). To my knowledge, only two publications exist that offer insight into microeukaryote gene expression beyond either of these lineages: one exploring the feasibility of expression-targeted scRNA-seq in two small flagellates, the haptophyte *Prymnesium parvum* and the dinoflagellate *Karlodinium veneficum*, both of which are mixotrophic (Liu et al. 2017), and another one uncovering successive stages of infection in the autotrophic haptophyte *Emiliania huxleyi* by its specific giant virus (Ku et al. 2020). Both of these examples rely on the availability of either good reference genomes or an integrated reference transcriptome to which scRNA-seq reads can be mapped to build single cell expression profiles. Such resources, however, might not be available (as would for example be the case for most of the organisms captured by the methodology presented in **Paper II**). In such scenarios, an *ad hoc* transcriptome reference can still be built by assembling *de novo* the combined set of mRNA reads from the total pool of cells in the single cell dataset under scrutiny.

In **Paper V**, we aimed to test such an approach. For this purpose, we used full-length mRNA sequencing, based on poly-A capture of eukaryotic mRNA, to explore and analyze expression profiles of *O. triangulata*, for which there is no reference genome currently available.

Single cells were FACS-sorted from two distinct growth stages during its life cycle in batch culture, either at the early exponential growth phase or at the onset of the stationary phase. Cells from the former group were steadily growing at a rate of 0.6 doublings per day at the time of sampling, and are hereon referred to as fast-growing cells. In contrast, cells from the latter group were growing at a rate linearly declining by a factor of 0.08 (0.2 doublings per day at the time of sampling), and are referred to as slow-growing cells. The transition from fast to slow growth occurred around day 5-6 after inoculation and was associated to a depletion of the fast-growing bacteria in co-culture with *O. triangulata* (**Paper V**, Supplementary Figure 1), which transitioned into a persistent community concurrently with *O. triangulata* transition into linear growth.

Results

De novo assembly of filtered and quality trimmed reads pooled across all cells produced a total transcriptome of ~166 000 transcripts. Such a size was deemed unrealistic for a small sized unicellular phytoflagellate (Liu et al. 2017). Accordingly, a requirement of presence in at least 2 cells was set as a threshold to accept a transcript in the assembly. This requirement reduced the transcriptome size to 60 307 transcripts, which might still have represented an inflated number compared to the ~20 000 transcripts reported for other *Ochromonas* strains (Lie et al. 2018) but accounted for the best non-informed arbitrary threshold that we could accept. The resulting transcriptome was therefore chosen as the basis for read mapping, generating the transcript expression table supporting all subsequent analysis.

Clustering of individual gene expression profiles reproduced the two expected populations but also revealed the existence of an unexpected third group present at both sampling occasions (Figure 9). This population, given the lack of a priori information, is referred to as cryptic.

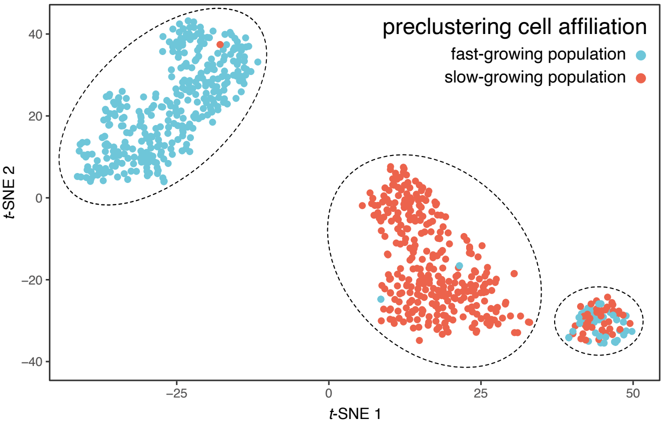

Figure 9. t-SNE visualization of Ochromonas triangulata populations. Each dot represents one cell color-coded based on its sampling origin –either the fast-growing (cyan) or slow-growing (red) populations. Clusters following expression profiles and identified by DBSCAN are enclosed by dotted lines.

Read support per cell (i.e. the number of reads supporting gene expression per cell) was high in all three cases, with median values above one million reads per cell and minimum values above 100 000 reads per cell. At the same time, transcriptome coverage was above 75% for all populations. These observations suggested that the cryptic population was not artifactual but rather a consequence of a biological phenomenon. The cryptic population was associated to low transcriptome richness (as compared to the other two populations), and less than 1% of the total transcripts were unique to this population.

When attempting to functionally annotate the transcripts against the UniProt database, only 30% of all transcripts could be assigned biological meaning. In the case of the cryptic population, only 42 of 183 genes uniquely expressed in this group could be annotated. Of these, 22 carried prokaryotic annotations (discussed below). Nonetheless, many of the genes that could be annotated encoded enzymes that take part in widespread and fundamental metabolic pathways across multiple taxa (such ubiquitous pathways are well represented in the databases). Differential expression analysis of this group, although limited to a small subset of transcripts, revealed key metabolic differences between the fast and slow growing stages (Figure 10). A total of 5

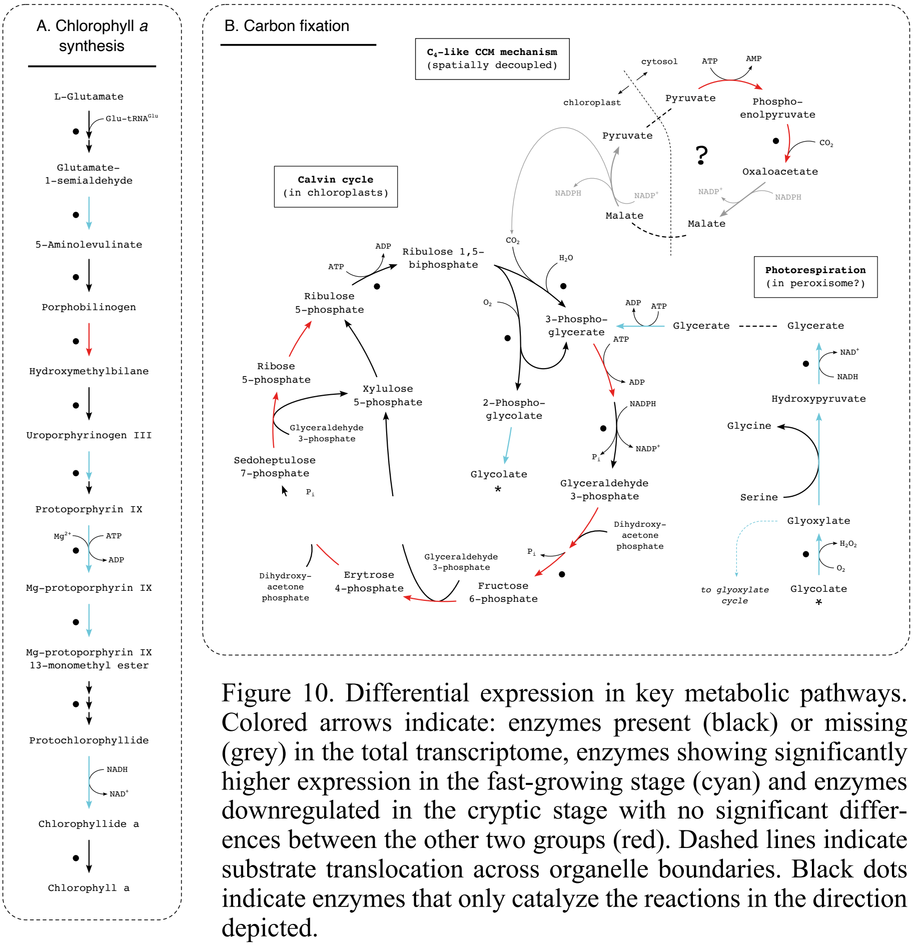

Figure 10. Differential expression in key metabolic pathways. Colored arrows indicate: enzymes present (black) or missing (grey) in the total transcriptome, enzymes showing significantly higher expression in the fast-growing stage (cyan) and enzymes downregulated in the cryptic stage with no significant differences between the other two groups (red). Dashed lines indicate substrate translocation across organelle boundaries. Black dots indicate enzymes that only catalyze the reactions in the direction depicted.

enzymes involved in the synthesis of chlorophyll *a* were upregulated in the fast-growing stage, including magnesium chelatase, which catalyzes the first committed step of chlorophyll *a* production, and protochlorophyllide reductase, which generates chlorophyllide *a*, the immediate precursor of chlorophyll *a*.

In addition, upregulation of photorespiration enzymes in the fast-growing stage (Figure 10B) suggested high CO_2 fixation activity associated to these cells, since this it is a compensatory mechanism to counteract the efficiency loss in carbon fixation due to the oxidase activity (i.e. the oxidation of ribulose 1-5-biphsphate into 2-phosphoglycolate and 3-phosphoglycerate, in competition with CO_2 addition to ribulose 1,5-biphosphate to generate 2 molecules od 3-phosphoglycerate) of rubisco.

Complementary to the functional analysis of the data, an ancillary component of the workflow that we explored was the possibility to taxonomically annotate each of the sorted cells taking advantage of rRNA leakage into the dataset. By doing so, we were able to correctly identify 88% of the cells in the dataset as *O. triangulata*, with the exception for those belonging to the cryptic stage. This could either be a technical consequence of limited rRNA recovery for this group (4% of its total read pool, whereas for the other two populations the rRNA fraction represented more than 20% of the total) or a consequence of the biological character of this population.

Discussion

The question of what the cryptic population might represent remains open. Based on the identity of recovered transcripts, it was impossible to ascertain which biological mode or state this population could represent. This highlighted the problem of underrepresentation of protist sequences in publicly available annotation databases, which has already been diagnosed by the microbial ecology community (Keeling et al. 2014). General downregulation of transcriptional activity (**Paper V**, Figure 5) and carbohydrate catabolism pathways (**Paper V**, Figure 6C) may suggest that these are cells in their way to encystment. This would be compatible with LysoSensor Blue positive signal (used to cytometrically sort single cells prior to library preparation and sequencing) since *Ochromonas* spp. and other chrysophytes make use of acidic SDVs to build up the silica walls prior to encystment (Hibberd 1977, Andersen 1982).

Alternatively, this cryptic population could possibly represent infected or senescent cells that have been colonized by an invading bacterial community. In this case, eukaryotic mRNA levels would be very low and much of the RNA recovered would correspond to non-polyadenylated prokaryotic mRNA that leaked into the dataset in a way similar to rRNA leakage. This scenario, however, is difficult to reconcile with LysoSensor fluorescence, which in **Paper**

V was precisely used as a means of discriminating living *O. triangulata* cells (see **Paper V**, Supplementary Figure 2). A combination of both scenarios is also a plausible explanation.

In our workflow, prokaryotic transcripts are typically considered as a nuisance because the transcriptome was assembled from the total read pool, without having the precaution of identifying and eliminating reads of probable prokaryotic origin. This could however be done to circumvent this potential confounder, for example, through the implementation of *k*-mer analysis algorithm prior to assembly (Wood and Salzberg 2014). Discriminating prokaryotic from eukaryotic reads would, in turn, bear the collateral advantage of exposing inter-domain interactions at both taxonomic and functional level, essentially representing an alternative to the method presented in **Paper IV**. Despite the risk of erratic prokaryotic functional information in the dataset, the possibilities of exploring prokaryote-eukaryote interactions from this experiment deserve further scrutiny in the future.

The feasibility of the single cell transcriptome analysis for mixotrophic eukaryotes, and microeukaryotes in general, is nevertheless made obvious, and so is its capability to uncover hidden populations (i.e. cell populations that would we averaged out in an integrated approach such as metatranscriptomics) based on the expression levels of individual cells. The capacity of this strategy to uncover population structure within a species or within a functionally defined assemblage based on transcript recovery, dissociated of the biological significance of each transcript (as shown in Figure 9), is independent of a genomic reference and is therefore its main asset. The availability of a reference will of course only be advantageous, but population structure can be resolved independent of it.

While its capacity for detailed mechanistic characterization of functional states is limited because of deficiencies in public functional annotation databases, the potential for discovery of unexpected populations remains a key asset of scRNA-seq. The combination of 18S rRNA annotation with expression-driven population clustering can be without doubt a powerful tool to characterize community structure from natural samples beyond metabarcoding approaches.

Overall discussion

Overall, these studies show, with diverse levels of success, that DNA-based single cell resolution is an achievable reality to infer the ecology of aquatic microeukaryotes in their natural context. However, there are certain limitations, common to all methodologies presented here, that are worth mentioning.

A most obvious constraint to the applicability of single-cell techniques as presented in this thesis is the wide array of morphologies that microbial eukaryotes can display. Both size, as illustrated in Figure 2, but also shape contribute to this. Size is problematic because FACS cannot deal with organisms that are too big. The upper size limit eventually depends on the specific instrument used, but the boundary is typically located between 40 and 60 µm in length (the rule of thumb being one third of the diameter of the nozzle, Thompson et al. 2013). This includes most pelagic microeukaryotes, but excludes some of potential importance (e.g. mid-sized ciliates, Lischke et al. 2015). Shape can pose a challenge because anything that departs in excess from a spherical shape will cause problems when encapsulated via emulsion, which generates spherical droplets. Pennate diatoms or *Ceratium*-like dinoflagellates, important in both marine and freshwater environments, exemplify this issue. At the expense of throughput, micromanipulation remains as a flexible enough technique to target a broader catalogue of sizes and shapes that either FACS or emulsion-based approaches cannot accommodate.

Cell density can also be limiting. Whether cytometrically or emulsion-based, organisms that occur in low densities will be hard to retrieve because sample volumes that can be handled by these techniques are necessarily low (see **Paper I**, Section 4 for a deeper discussion on this issue). Sample concentration strategies (such as presented in Hinga et al. 1979), at the expense of the representability of the sample, might be unavoidable when scarce organisms are targeted. Microscopy, which allows the screening of larger sample volumes deposited onto membrane filters, still offers indispensable contributions in this regard.

Finally, all the methodologies presented are relatively sophisticated observational techniques, and therefore their capability to target mechanistic explorations is limited (even scRNA-seq, alhough based on gene expression, cannot really be used for mechanistic inference in the absence of a well curated reference genome). Nonetheless, all of them hold great exploratory potential at the scale relevant for microorganisms.

Concluding remarks

The studies composing this thesis introduce, using mixotrophy as the golden thread, three methodologies that deal with multiple facets of microeukaryote ecology at single-cell resolution. A summary of the main contributions of each of them now follows.

In Part 1, FACS-based sorting of actively feeding mixotrophs, based on tracing ingestion with fluorescent reporters in addition to flagellate autofluorescence, is shown to be selective and highly specific for mixotrophy. We successfully deploy fluorescently labelled prey surrogates for this purpose, providing an extra level of precision to support the use of fluorescent food vacuole tracers (Wilken et al. 2019). The technique offered a hint of its potential by resolving unexpected cryptic diversity within mixotrophic algae.

In addition, prey size was observed to be the main driver for prey selection among constitutive mixotrophs. This was made apparent through predator preference for the different prey surrogates, measured quantitatively for a marine mixotrophic nanoflagellate in culture, and seen qualitatively for a number of mixotrophic flagellates from a freshwater sample.

In Part 2, the results from the first attempt to massively tackle eukaryote-prokaryote interactions did not conform to the expectations, since single-cell resolution was lost during the procedure. However, it is worth stressing the pioneering nature of this attempt. Improvements in cell encapsulation and PCR in emulsion, both plausible solutions, are identified for future development.

In Part 3, we show that single-cell transcriptomics can be used successfully in organisms lacking a reference genome by successfully recovering the population structure of a feeding mixotroph. Although, without a reference, the capacity of the method to provide a mechanistic snapshot is proven limited, its suitability to reveal population structure is demonstrated as its major asset. To this end, taking advantage of rRNA leakage, typically disregarded as a nuisance, is key.

Future perspectives

Specific to the methodologies described here, an obvious extension of this thesis is to combine the strategies presented in Parts 1 and 3. Following single-cell sorting of living mixotrophic protists, as shown in **Paper II**, a full-transcript scRNA-seq workflow could be deployed as demonstrated in **Paper V**. The combination of taxonomic annotation based rRNA leakage plus metabolic profiling based on mRNA capture would prove to be a powerful tool to map the functional structure of the actively feeding mixotroph community in the sample to an unprecedented level of detail.

The extension of the FACS-mediated mixotroph isolation to not only reveal taxonomic identity, but also reconstruct full single-cell genomes is of course possible (Yoon et al. 2011, Rinke et al. 2014, Troell et al. 2016). A genomic exploration of the cryptic diversity uncovered in **Paper II** is underway and will be studied in the future.

Further exploration of prokaryote-eukaryote interactions from the dataset in **Paper V**, or from a similar dataset, offers also great potential. Such an exploration, based on RNA rather than DNA, has the potential to support in a single experiment the exploratory power of **Papers II, IV and V** combined.

The aquatic environment is by default well suited to all these techniques, which require the sample to be in particulate suspension in an aqueous medium. The adaptation, whenever possible, of these methodologies to lesser studied habitats (such as wetlands or extreme environments) could pave the path to discovery. Furthermore, beyond genetic surveying, the isolation strategies presented here can be adapted to cell cultivation (Pereira et al. 2011) or measurement of individual physiological parameters (Behrendt et al. 2020).

In general, this thesis highlights, through mixotrophy, that the integration of single cell techniques into the overall toolkit of the microbial ecologist is a necessary step towards a deeper understanding of microbial life. This thesis shows that the time is ripe and the future fertile to tackle the ecology of mixotrophs (and any other feature that can be fluorescently tagged!) at the scale that matters most: organismal resolution.

Summaries

Summary in English

The aquatic habitats, such as lakes or the ocean, are teeming with microorganisms. In fact, in the open waters of the ocean or in the central part of a lake, microorganisms compose the base of the food web either by generating biomass through photosynthesis or by recycling the debris from decaying organic matter back into the system. Among all these microbes naturally occurring in those habitats, mixotrophs are an integral part.

Mixotrophs, defined broadly, are eukaryotic organisms that combine the capacity to ingest prey (i.e. feed like an animal) with the capacity to perform photosynthesis (i.e. feed like a plant). They come in multiple forms: some mixotrophs carry their own innate photosynthetic machinery, while others exploit the machinery of other organisms either via specialized symbiosis or by retaining functional chloroplasts from the prey they ingest. Besides, mixotrophic strategies can range from that of a strict phototroph that benefits occasionally from prey uptake to just obtain essential nutrients to that of a strict phagotroph that can supplement growth with energy from light when prey is scarce. Whether any of these strategies can flourish will, in turn, depend on the temperature of the water, the amount of CO_2 or specific nutrients dissolved in it, the penetration depth of light or the abundance of prey, among other factors.

These organisms are not novelty, since they are known since long, but their ecological relevance in aquatic habitats has been traditionally considered anecdotic. However, starting in the late 80s examples of mixotrophy had been steadily accumulating, both among families of microorganisms in which mixotrophy was already documented, but also in groups were it was unknown. All these observations have led to an increasing recognition, especially during the last decade, that mixotrophs are fundamental components of the aquatic environment and therefore they must be considered as such if we are to fully understand the functioning of aquatic ecosystems.

However, mixotrophs are single-cell organisms and, hence, they are microscopic. As a consequence, we need to rely on sophisticated techniques to observe mixotrophs at the level of the individual. Unavoidably, single-cell techniques have to be employed to study their identity, behaviour or interactions at their own scale. In this thesis, I have explored three different methodologies that could contribute to the study of different aspects of mixotrophy at such scale.

In the first methodology, the aim was to improve our capacity to reveal which mixotrophs are actively feeding in a given moment in an aquatic sample. To this purpose, I made use of artificial fluorescent tracers for prey ingestion that, together with the fluorescent signal of chlorophyll naturally present in these organisms, could be used to specifically recognize feeding mixotrophic individuals. We could show that this strategy was very precise in fulfilling its purpose, and with it we were able to uncover an unexpected level of diversity within active mixotrophs in a lake water sample.

For the second methodology, the aim was to adapt an existing procedure to massively identify individual interactions between eukaryotes and their bacterial associates. This procedure, based on the emulsion of the sample in an oily phase, attains individuality by trapping each eukaryotic cell inside a single emulsion droplet. This way, each eukaryote and their accompanying bacteria can be revealed by looking at the DNA content, coming from each organism in the association, of each droplet. To tests the feasibility of this approach, we used two bacterivorous mixotrophic organisms as a testbed, since these would exhibit both the interaction with their prey and the signal from their chloroplasts, which, like any other chloroplast, has evolutionary origins in bacteria. Our attempt, the first in trying to adapt this technique to this purpose, was unfortunately not successful, since we lost the capacity to trace the specific content of each droplet in the emulsion. Nonetheless, we could identify several weak spots of the process susceptible to be improved, and we hope it will just be the initial step in an eventual successful adaptation of the technique.

Finally, the third methodology was based on the individual extraction of the mRNA of single mixotrophic organisms. Since the mRNA composition can be used as a proxy to the physiological state of a given cell, our aim was to uncover functional variation within a population of a non-model mixotroph kept in culture. We could only partially achieve this, since we barely obtained anecdotal evidence of the mixotrophic behaviour of this species, but most importantly, thanks to the single-cell resolution, we provided a way to expose the structure of the mixotrophic population (i.e. which cells resemble or differ from each other) that could be readily applied to resolve population structure in natural samples.

Overall, in this thesis we see that it is possible, with varying degrees of success, to tackle the ecology of mixotrophs at individual resolution. Single cell techniques are a mature reality that can be included in the toolkit of the microbial ecologist, holding great discovery potential because they offer a view of the microbial world that was only glimpsed before.

Sammanfatning på svenska

Akvatiska livsmiljöer såsom sjöar och hav, kryllar av mikroorganismer. I öppna vattenmassor utgör dessa mikroorganismer basen för näringsväven, antingen genom att producera ny biomassa genom fotosyntes eller alternativt genom att frisätta och återvinna näring från dött organiskt material (detritus) och återbörda denna resurs till näringsväven. Det är väl känt att akvatiska mikroorganismer uppvisar en stor funktionell mångfald, och en särskilt svårstuderad grupp med synbart stor betydelse i dessa näringsvävar är de så kallade mixotroferna. I generella termer är mixotrofer eukaryota organismer som kombinerar förmågan att få i sig bytesdjur (dvs livnära sig genom predation) med förmågan att utföra fotosyntes (dvs livnära sig på solljus som en växt). De finns i flera olika former: vissa mixotrofer bär med sig sitt eget fotosyntetiska maskineri medan andra mixotrofer kan dra nytta av andra organismers fotosyntetiska organeller via specialiserad symbios eller genom att stjäla och internalisera kloroplaster från byten som de får i sig. Dessutom kan mixotrofa strategier variera från strikt fototrofi som enbart använder predation för att få i sig nödvändiga näringsämnen, till strikta fagotrofer som endast under speciella omständigheter, exempelvis när bytes-tillgången är begränsad, övergår till att tillgodose en del av sitt energibehov genom att dra nytta av solljuset. Vilken av dessa processer som dominerar kan även bero på miljöfaktorer såsom vattnets temperatur, tillgång till CO2 eller andra specifika näringsämnen, ljusförhållanden samt tillgång på bytesdjur.

Det är ingen nyhet att det finns organismer som har förmågan att kombinera dessa båda olika livsstilar. Förekomst av mixotrofer har varit känt sedan länge, men vilken ekologisk betydelse de har i akvatiska livsmiljöer är ändå ganska sparsamt och anekdotiskt utforskat. Alltsedan slutet av 80-talet har man dock kunnat se en stadigt ökande mängd exempel på mixotrofi, både bland grupper av mikroorganismer där mixotrofi redan är välkänd och väl dokumenterad, men även i grupper av mikroorganismer där man inte hade belägg för denna typ av flexibel ekologisk funktion. Denna ökande kunskap och talrika exempel har format nuvarande paradigm där mixotrofer är centrala och betydelsefulla komponenter i akvatiska näringsvävar och att vi måste ha en god förståelse av deras ekologi för att förstå akvatiska ekosystem.

Ett betydande hinder för att studera mixotrofer är att de är encelliga organismer och därför mikroskopiska. Som en konsekvens av detta behöver vi använda oss av sofistikerade tekniker och verktyg för att kunna observera mixotrofers mångfald, funktion och interaktioner på individnivå och koppla detta till den specifika mikroskopiska livsmiljö som de utsätts för

I denna avhandling har jag utforskat tre olika metoder som skulle kunna användas för att studera mixotrofer och mixotrofi på mikroskopisk skala ner till enstaka celler. Syftet med den första metoden var att kunna identifiera vilka specifika mixotrofer som aktivt livnär sig genom predation i ett givet ögonblick, och göra detta med precision och utan att förlita sig på renodling.

För att möjliggöra detta studerade jag hur olika fluorescerande partiklar (plastkulor och fluorescensinmärkta bakterier) kan användas för att påvisa predation och även hur en sådan signal och i samma organism, kan kopplas till fluorescens från klorofyll som finns naturligt i fotosyntetiserande organismer, vilket då skulle kunna möjliggöra identifiering av aktivt mixotrofa individer. Mina studier visar att denna metod kan fungera mycket bra för detta syfte och genom att analysera ett naturligt sjövatten med metoden kunde vi identifiera oväntat stor mångfald av aktiva mixotrofer.

Syftet med den andra metod-ansatsen var att utan odling beskriva interaktioner mellan enstaka eukaryoter och deras fysiskt associerade bakterier, och att göra detta i stor skala för att få en övergripande bild av näringsvävens sammansättning och interaktioner såsom predation. Metoden utgick från en redan etablerad teknik som baseras på emulsion av ett vattenprov i en olja för att på detta sätt skapa individualitet genom att fånga varje enskild eukaryot cell i en separat emulsionsdroppe. Till skillnad från tidigare studier använde vi sedan dessa emulsioner för att med DNA-analys beskriva inte bara den in fångade eukaryota cellens identitet, utan även dess medföljande bakterier. För att utveckla metoden och studera genomförbarheten användes två mixotrofa eukaryota mikroorganismer som modellorganismer. Dessa mixotrofer livnär sig på bakterier och förväntas därför vara fysiskt associerade till såväl de bakterier som äts som den egna signalen från kloroplaster, som likt andra kloroplaster har sitt evolutionära ursprung från bakterier. Mina studier var ett första försök att anpassa denna teknik för detta specifika ändamål, men framgången var tyvärr begränsad då efterföljande analys visade att vi synbarligen inte kunde behålla mixotrofernas individualitet i emulsionen och de efterföljande analysstegen. Icke desto mindre identifierades flera svaga punkter i metoden som med enkla medel kan förbättras. Förhoppningsvis kan vidare utvecklingsarbete leda till att denna metod kan bli ett värdefullt verktyg för att studera fysiska associationer på mikroskopisk skala.

Avhandlingens tredje metodansats utgår från individuell extraktion av mRNA från enstaka mixotrofa mikroorganismer. Eftersom sammansättningen på cellens mRNA kan användas för att beskriva dess fysiologiska tillstånd, var målet att avslöja funktionell variation inom en genetiskt likvärdig population av mixotrofer som odlades i renkultur. Detta mål kunde endast delvis uppnås, då den sekvensbaserade information som erhölls bara gav begränsad information om artens mixotrofa beteende, men viktigare var att vi tack vare encellsupplösningen, kunde blottlägga populations-strukturen (dvs vilka celler som liknar eller skiljer sig från varandra med avseende på vilka gener som uttrycks) och att denna metodansats äv en kan användas för att studera funktionell populationsstruktur i naturliga prover.

Sammanfattningsvis visar jag i denna avhandling att det är möjligt att studera mixotrofers ekologi med individuell upplösning. Det finns tveklöst vidare utmaningar och begränsningar, men denna typ av utforskande enkelcellsmetoder kan nu inkluderas i den mikrobiella ekologens verktygslåda och har potential att ge oss en ny bild av mikrobiell mångfald som tidigare bara kunde skymtas.

Resum en català

Els hàbitats aquàtics, com llacs o mars, estan plens de microorganismes. De fet, a les aigües obertes de l'oceà o a la part central d'un llac, els microorganismes composen la base de la xarxa tròfica, ja sigui generant biomassa a través de la fotosíntesi o bé reciclant la matèria orgànica en descomposició retornant-la al sistema. De tots aquests microbis presents de manera natural en aquests hàbitats, els mixòtrofs en són una part integral.

Els mixòtrofs, definits àmpliament, són organismes eucariotes que combinen la capacitat d'ingerir preses (és a dir, alimentar-se com un animal) amb la capacitat de fer la fotosíntesi (és a dir, alimentar-se com una planta). Se'ls pot trobar de múltiples formes: alguns posseeixen la seva pròpia maquinària fotosintètica, mentre que d'altres s'aprofiten de la maquinària d'altres organismes que o bé acullen en simbiosi o bé dels quals retenen els cloroplasts després d'haver-los ingerit. A més, les estratègies de mixotrofia poden anar des de la d'un fotòtrof estricte que es beneficia ocasionalment de la ingesta de preses per a obtenir nutrients específics, fins la d'un fagòtrof estricte que suplementa el seu creixement amb energia solar quan la presa escasseja. Que qualsevol d'aquestes estratègies floreixin dependrà, al seu torn, de la temperatura de l'aigua, de la quantitat de diòxid de carboni o de nutrients específics dissolts en ella, de la profunditat de penetració de la llum o de l'abundància de preses, entre altres factors.

Aquests organismes no suposen una novetat, ja que són d'antuvi coneguts, però la seva rellevància ecològica a hàbitats aquàtics ha estat tradicionalment considerada anecdòtica. Tot i això, molts exemples de mixotrofia s'han anat acumulant des de finals dels anys 80, tant dins de famílies de microorganismes en les que ja s'havia documentat abans com en grups en què era desconeguda. Totes aquestes observacions han dut a reconèixer, especialment durant la darrera dècada, que els mixòtrofs són components fonamentals de l'ambient aquàtic y, per tant, han de ser considerats com a tal si volem entendre plenament el funcionament dels ecosistemes aquàtics.

Tanmateix, els mixòtrofs son organismes unicel·lulars i, per tant, són microscòpics. Com a conseqüència d'això, depenem de sofisticades tècniques d'observació per a poder estudiar la seva identitat, comportament o interaccions a la seva pròpia escala. En aquest tesi, he explorat tres metodologies diferents que podrien contribuir a l'estudi de diversos aspectes de la mixotrofia a tal escala.

A la primera metodologia, l'objectiu va ser millorar la nostra capacitat per a revelar quins mixòtrofs depreden activament en un moment donat en una mostra d'aigua. Per aquest propòsit, vaig utilitzar indicadors fluorescents d'ingestió que, juntament amb el senyal fluorescent de la clorofil·la que existeix de forma natural en aquests organismes, podrien ser utilitzats per a reconèixer, específicament, mixòtrofs que estan depredant. Fent servir aquesta estratègia vam poder complir l'objectiu de forma precisa, y a més vam ser capaços de revelar uns nivells de diversitat inesperats entre mixòtrofs actius en una mostra d'aigua lacustre.

Quant la segona metodologia, l'objectiu va ser adaptar un procediment ja existent per a descobrir de forma massiva interaccions individuals entre organismes eucariotes i els seus bacteris associats. Aquest procediment, basat en l'emulsió de la mostra en una fase oliosa, aconsegueix la individualitat atrapant cada cèl·lula eucariota dins una gota de l'emulsió. Així, cada eucariota y els seus bacteris acompanyants poden ser associats llegint el contingut d'ADN de cada gota. Per a avaluar l'aplicabilitat d'aquest plantejament, es van utilitzar dos mixòtrofs bacterívors com a model, donat que aquests organismes exhibirien tant la interacció amb la seva presa com la senyal amb el seu cloroplast (que, com qualsevol altre cloroplast, té el seu origen evolutiu en els bacteris). Aquest intent, el primer en mirar d'adaptar la tècnica per a aquest propòsit, va ser desafortunadament fallit perquè es va perdre la capacitat de rastrejar el contingut específic de cada gota de l'emulsió. No obstant això, vam poder identificar diversos punts dèbil al llarg del procés que són susceptibles de millora, i esperem que aquest sia només el primer pas en la, en última instància, exitosa adaptació de la tècnica.

Finalment, la tercera metodologia es va basar en l'extracció individual d'ARN missatger de cèl·lules individualment aïllades. Donat que la composició d'aquesta fracció de l'ARN es pot utilitzar com a representativa de l'estat fisiològic d'una cèl·lula, el nostre objectiu va ser descobrir els mecanismes de variació funcional dins d'una població d'un mixòtrof en cultiu. Aquest objectiu es va poder complir de manera parcial, ja que només vam obtenir pistes superficials sobre el comportament mixotròfic d'aquest espècie. De manera més rellevant, gràcies a la resolució de l'experiment a nivell cel·lular, vam facilitar una manera d'exposar l'estructura d'una població cel·lular mixotrófica (és a dir, quines cèl·lules s'assemblen més o menys entre elles) que podria aplicar-se fàcilment per a resoldre l'estructura de poblacions naturals.

En general, aquesta tesi mostra que és possible, si bé amb diversos nivells d'èxit, escometre l'ecologia dels mixòtrofs a escala individual. Tècniques a una sola cèl·lula són una realitat madura que pot ser inclosa en el conjunt d'eines de la ecologia microbiana, proveint un gran potencial de descobriment perquè ofereixen una visió del món microbià que abans només s'havia albirat.

Resumen en español

Los hábitats acuáticos, como lagos o mares, están llenos de microorganismos. De hecho, en las aguas abiertas del océano o en la parte central de un lago, son los microorganismos los que componen la base de la red trófica, ya sea generando biomasa a través de la fotosíntesis o bien reciclando la materia orgánica en descomposición de vuelta al sistema. De todos estos microorganismos presentes de manera natural en estos hábitats, los mixótrofos son una parte integral.

Los mixótrofos, definidos ampliamente, son organismos eucariotas que combinan la capacidad de ingerir presas (es decir, alimentarse como un animal) con la capacidad de hacer la fotosíntesis (es decir, alimentarse como una planta). Se los puede encontrar en múltiples formas: algunos poseen su propia maquinaria fotosintética, mientras que otros se aprovechan de la maquinaria de otros organismos que o bien albergan en simbiosis o bien cuyos cloroplastos retienen después de haberlos ingerido. Además, las estrategias de mixotrofía pueden ir desde la de un fotótrofo estricto que se beneficia ocasionalmente de la ingesta de presas para obtener nutrientes específicos, hasta la de un fagótrofo estricto que suplementa su crecimiento con energía solar cuando la presa escasea. Que cualquiera de estas estrategias florezca dependerá, a su vez, de la temperatura del agua, de la cantidad de dióxido de carbono o nutrientes específicos disueltos en ella, de la profundidad de penetración de la luz o de la abundancia de presas, entre otros factores.

Estos organismos no suponen ninguna novedad, ya que se conocen desde antiguo, pero su relevancia ecológica en hábitats acuáticos ha sido tradicionalmente considerada anecdótica. Sin embargo, muchos ejemplos de mixotrofía se han ido acumulando desde finales de los años 80, tanto en familias de microorganismos en las que ya se había documentado antes como en grupos en los que era desconocida. Todas estas observaciones han llevado a reconocer, especialmente durante la última década, que los mixótrofos son componentes fundamentales del ambiente acuático y, por tanto, deben ser considerados como tales si queremos entender plenamente el funcionamiento de los ecosistemas acuáticos.

No obstante, los mixótrofos son organismos unicelulares y, por consiguiente, son microscópicos. Como consecuencia de esto, dependemos de sofisticadas técnicas de observación para poder estudiar su identidad, comportamiento o interacciones a su propia escala. En esta tesis, he explorado tres metodologías diferentes que podrían contribuir al estudio de distintos aspectos de la mixotrofía a tal escala.

En la primera metodología, el objetivo fue mejorar nuestra capacidad para identificar qué mixótrofos depredan activamente en un momento dado en una muestra de agua. Para ello, utilicé indicadores fluorescentes de ingestión que, junto con la señal fluorescente de la clorofila que existe de forma natural en estos organismos, podrían ser usados para reconocer, específicamente, mixótrofos que están fagocitando. Empleando esta estrategia pudimos cumplir el objetivo de forma precisa, y además fuimos capaces de revelar unos niveles de diversidad inesperados entre mixótrofos activos en una muestra de agua lacustre.

En la segunda metodología, el objetivo fue adaptar un procedimiento ya existente para descubrir de forma masiva interacciones individuales entre organismos eucariotas y sus bacterias asociadas. Este procedimiento, basado en la emulsión de la muestra en una fase oleosa, consigue la individualidad atrapando cada célula eucariota dentro de una gota de la emulsión. De esta forma, cada eucariota y sus bacterias acompañantes pueden ser asociadas leyendo el contenido de ADN de cada gota. Para evaluar la aplicabilidad de este planteamiento, se usaron dos mixótrofos bacterívoros como modelo, ya que estos organismos exhibirían tanto la interacción con su presa como la señal con su cloroplasto (que, como cualquier otro cloroplasto, tiene su origen evolutivo en bacterias). Este intento, el primero en tratar de adaptar la técnica a este propósito, fue desafortunadamente fallido ya que se perdió la capacidad de rastrear el contenido específico de cada gota de la emulsión. No obstante, pudimos identificar varios puntos débiles en el proceso que son susceptibles de mejora, y esperamos que este sea sólo el primer paso en la, en última instancia, exitosa adaptación de la técnica.

Finalmente, la tercera metodología se basó en la extracción individual de ARN mensajero de células individualmente aisladas. Dado que la composición de este ARN se puede usar como representativa del estado fisiológico de una célula, nuestro objetivo fue descubrir los mecanismos de variación funcional dentro de una población de un mixótrofo en cultivo. Este objetivo se pudo cumplir de forma parcial, ya que solamente obtuvimos pistas marginales sobre el comportamiento mixotrófico de esta especie. De manera más relevante, gracias a la resolución del experimento a nivel celular, facilitamos una manera de exponer la estructura de una población celular mixotrófica (es decir, qué células se parecen más o menos entre ellas) que podría aplicarse fácilmente para resolver la estructura de poblaciones naturales.

En general, esta tesis muestra que es posible, si bien con variados grados de éxito, abordar la ecología de los mixótrofos a escala individual. Técnicas a una sola célula son una realidad madura que puede ser incluida en el conjunto de herramientas de la ecología microbiana, proveyendo de un gran potencial de descubrimiento porque ofrecen una visión del mundo microbiano que antes solo se había vislumbrado.

Acknowledgements

I would like to start acknowledging **Stefan Bertilsson**, my supervisor. If one is to sort all the people one interacts with during a thesis between those who make it easier and those who make it harder, Stefan clearly falls within the latter. Without discussion. And yet, he deserves my most genuine appreciation. He has given me almost absolute freedom, offered guidance at key moments, and been supportive throughout. The timeline, and arguably the content, of this thesis have perhaps suffered by such freedom, but this feels significantly counterbalanced by the immense amount of knowledge that I mined during this time and, likely, would have otherwise remained hidden. Now, in the end, I appreciate it more than ever. For that, and for his endless patience, I am very grateful.

To **Manu Tamminen** and **Fabien Burki**, co-supervisors, I am also much indebted. Especially to Manu, who offered me a grip when I was somewhat lost, welcomed me in his lab and into his research, and got his hands dirty to teach me make emulsions in eppendorf tubes. His enthusiasm, breadth of knowledge and view of the trade are an inspiration. Fabien, in the background, has always been constructive whenever I knocked on his door. I appreciate that.

From Manu's group, I thank **Olli Pitkänen** for his solid support in the lab and **Aditya Jeevannavar**, very much indeed, with a knack for bioinformatics and essential contributor in Part 3.

At the Microbial Single Cell Genomics facility, **Anna-Maria Divne** and **Claudia Bergin** belong definitely to those people who make it easier. I thank both of them for their guidance, patience and open attitude to figure out new things, and I very much thank Anna-Maria, whom I ended up working more closely, for her commitment to my projects. She did all the sorting, sometimes in very demanding circumstances.

This thesis was a part of the EU-funded SINGEK project. I would like to sincerely thank **Elena Torrecilla** and **Ramon Massana**, the steering forces behind SINGEK, for making it a reality. I am also grateful to **Colomban de Vargas** and **Sarah Romac** for hosting and guiding me in Roscoff, in a SINGEK-enabled collaboration. What I did there never crystalized into a chapter of the thesis, but it is actually echoed in every of its papers. And to **Gavin Lucas** and **Tobias Maier**, it didn't seem like it at the time, but much of what I learned from them helped me write this whole piece. To all the fellow

SINGEK PhDs, I'm really grateful for all the fun. Whenever we met, it felt like a big family reuniting.

Throughout all this time, I have been a member of the Limnology division. I won't list everyone here, I'd simply say that I can't think of anyone I shared my time with in this department that, in one way or another, has not influenced this thesis. However, most remarkably and because of Limno, I am now familiar with vertical migration, lake browning, methane ebullition and legacy effects; I've become acquainted with the Secchi disc, the Schmidt stability, the Redfield ratio and the Van Krevelen diagram; I have sampled from thousand liter mesocosms to seventy milliliter culture flasks; and I have learned about perch, *Daphnia*, *gubbslem* and actinobacteria. Such an extensive research landscape is hosted in Limno. I am immensely grateful for the opportunity to have seen all this.

In a way, this thesis is the culmination of an academic pathway that started in high school, when I became convinced that I would devote my coming years (decades) to study some of the natural aspects of reality. Therefore, it seems appropriate to remember now two people that were instrumental to me during those beginnings: **Robert Casals**, my history teacher, and **Luis Pueyo**, my physics teacher. Their influence is still fresh to this very day. Also, **Elvira Gómez**, my tutor during my chemistry stint, gave me the cue that thankfully clicked me forward.

Beyond the academic, there are two people, **Helena Parra** and **Fernando Chaguaceda**, who have been intrinsic parts of my journey. They have been invaluable supports in virtually everything that mattered during all this years. My debt to them is enormous and multifaceted. They have my deepest, everlasting gratitude.

Finally, I dedicate this thesis to my closest family. In strict order of appearance, these are my grandfather **José**, my grandmother **Flor**, my father **Josep**, my mother **Nuria**, my uncle **José Javier** and my cousin **Míriam**. They are just there, at the base, like they've always been. I love them so much.

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Editor: The Dean of the Faculty of Science and Technology

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