The role of eukaryotic microbial communities from inland aquatic systems
A metatranscriptomic approach

Inaugural-Dissertation
zur

Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von

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Köln, 2021

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Tag der mündlichen Prüfung: 26 Februar 2021

“A mi norte querido, con sus colores y contrastes, con su mar,
su desierto y montañas; que en la inmensidad parecen vacíos,
en la realidad de detalles y vida inundados,
y en el corazón mil recuerdos atesorados”.

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Contents

List of Figures

List of Tables

Abstract

The role of microbial communities in aquatic ecosystems is essential; they contribute to biogeochemical cycles and the food webs dynamics at various levels. Eukaryotic microbes, found in all major supergroups of the tree of life, are important primary producers, consumers, and decomposers in most aquatic systems. Studying aquatic microbial communities to determine their diversity, functions, and response to adverse environmental conditions is very complex and challenging. Although the increasing high-throughput sequencing efforts in the past years have contributed significantly to diversity estimations in numerous environments, there is still a lack of understanding of these communities' functions and roles in their ecosystems. Small and remote aquatic systems have received less attention.

To understand how microeukaryotes contribute to ecosystem functioning, approaches that combine taxonomic with functional information are needed. In this thesis, I used metatranscriptomics as the main approach to investigate aquatic microeukaryotic communities' functions through gene expression. Two different systems were studied: a small and temperate freshwater pond and a shallow freshwater stream with increasing salinity from a high altitude extreme ecosystem. Specifically, I focused on two main factors affecting these communities: solar radiation and salinity. Light, specifically in the Photosynthetically Active Radiation (PAR) spectrum, is an important factor for the growth of planktonic organisms and modulates key metabolic processes through the diurnal cycles. These diel responses have been studied only on single organisms and in marine or large lake communities. Investigating the microeukaryotic community's activity from a small freshwater system in response to the diurnal cycle revealed that the pond community, mostly dominated by heterotrophs together with Euglenophyta and Chlorophyta, follows similar diurnal dynamics as those described for larger aquatic systems. The gene ontology categories, including photoreception for photosynthesis, defense, and stress mechanisms, dominated during the day. While motility, ribosomal assembly, and other large, energy-consuming processes were restricted to the night.

High solar radiation, broad salinity ranges, high evaporation rates, and temperature variations are characteristics of aquatic systems of the high plateaus in the central

Andes. These ecosystems are subject to extreme seasonal and diurnal fluctuations, to which the inhabiting communities are assumed to be highly adapted. However, the mechanisms evolved by aquatic microorganisms to overcome such conditions are not well understood. Salar de Huasco is a high altitude wetland in the north of Chile, where the diversity and role of the eukaryotic microbial component have not been extensively addressed. I studied how these microeukaryotes are affected by multiple environmental stressors by comparing gene expression patterns along a stream with increasing salinity in the wet (summer) and dry (winter) seasons under different solar irradiance levels. The active communities were dominated by heterotrophic taxa (Ascomycota and Evosea), while phototrophic taxa (mainly Chlorophyta and Bacillariophyta) were less abundant but more variable between sites and seasons. The main environmental factors found to structure the communities were pH, conductivity, and salinity during winter and temperature and PAR during summer. And the most enriched biological processes, as by gene ontology analyses, were related to the mitotic cell cycle, photosynthesis, and stress responses.

To further understand which groups of microeukaryotes affect which types of ecosystem functions in different sites along the salinity gradient, processes were correlated to taxonomic groups. Specifically, I focused on differentially expressed genes belonging to the categories: photosynthesis and carbon fixation, cellular respiration, ion transport, nutrient uptake (including sulfur cycling), and stress response. During summer, Glaucophyta dominated photosynthesis and thus inorganic carbon fixation, while Chlorophyta, Haptophyta, Ochrophyta, and Mucoromycota were the key players for dissolved nitrogen and phosphorus assimilation in the less saline sites. During winter, a higher expression of ion transport and stress response genes in the most saline sites was correlated to heterotrophic groups, which at the same site dominated cellular respiration and sulfur assimilation. The presented data demonstrate that spatial differences (sites along the salinity gradient) were more significant in winter than in summer. And the temporal (wet and dry season) differences by site were most pronounced in the most saline site.

Overall, metatranscriptomics is a promising tool to study eukaryotic microbial communities, particularly in rarely studied and environmentally extremely variable ecosystems. By linking biological processes to biodiversity, more insights into ecosystem functions can be obtained, essential for developing conservation strategies.

Zusammenfassung

Die Rolle der mikrobiellen Gemeinschaften in aquatischen Ökosystemen ist essenziell; sie tragen zu biogeochemischen Kreisläufen und der Dynamik der Nahrungsnetze auf verschiedenen Ebenen bei. Eukaryotische Mikroben, die in allen wichtigen Übergruppen des Lebensbaums zu finden sind, sind wichtige Primärproduzenten, Konsumenten und Zersetzer in den meisten aquatischen Systemen. Das Erforschen aquatischer mikrobieller Gemeinschaften zur Bestimmung ihrer Diversität, Funktionen und Reaktionen auf ungünstige Umweltbedingungen ist sehr komplex und anspruchsvoll. Obwohl die zunehmende Hochdurchsatz-Sequenzierung in den letzten Jahren erheblich zur Abschätzung der Diversität in zahlreichen Umgebungen beigetragen hat, fehlt es immer noch an einem Verständnis der Funktionen und Rollen dieser Gemeinschaften in ihren Ökosystemen. Kleine und abgelegene aquatische Systeme haben weniger Aufmerksamkeit erhalten.

Um den Beitrag der Mikroeukaryoten zur Funktion von Ökosystemen zu verstehen, sind Ansätze erforderlich, die taxonimische mit funktionellen Informationen kombinieren. In dieser Dissertation habe ich Metatranskriptomik als Hauptansatz verwendet, um die Funktionen von aquatischen mikroeukaryotischen Gemeinschaften über die Gen-Expression zu untersuchen. Zwei verschiedene Systeme wurden untersucht: ein kleiner und gemäßigter Süßwasserteich und ein flacher Bach mit ansteigender Salinität aus einem extremen Ökosystem im Hochgebirge. Ich habe mich auf zwei Hauptfaktoren untersucht, die diese Gemeinschaften beeinflussen, konzentriert: Sonnenstrahlung und Salinität.

Licht, insbesondere im Spektrum der photosynthetisch aktiven Strahlung (PAR), ist ein wichtiger Faktor für das Wachstum von planktonischen Organismen und moduliert wichtige Stoffwechselprozesse über die Tageszyklen. Diese tageszeitlichen Reaktionen wurden bisher nur an einzelnen Organismen und in marinen oder großen Seegemeinschaften untersucht. Die Untersuchung der Aktivität der mikroeukaryotischen Gemeinschaft eines kleinen Süßwassersystems als Reaktion auf den Tageszyklus ergab, dass die Teichgemeinschaft, die hauptsächlich von Heterotrophen zusammen mit Euglenophyta und Chlorophyta dominiert wird, einer ähnlichen Tagesdynamik folgt, wie sie für größere aquatische Ökosysteme beschrieben wurde. Die

Gen-Ontologie-Klassen, einschließlich Photorezeption für Photosynthese, Verteidigung und Stressmechanismen, dominierten während des Tages, während Motilität, ribosomaler Aufbau und andere große, energieverbrauchende Prozesse auf die Nacht beschränkt waren.

Hohe Sonneneinstrahlung, große Salinitätsbereiche, hohe Verdunstungsraten und Temperaturschwankungen sind charakteristisch für aquatische Systeme der Hochplateaus in den zentralen Anden. Diese Ökosysteme unterliegen extremen saisonalen und tageszeitlichen Schwankungen, an die die bewohnenden Lebensgemeinschaften vermutlich gut angepasst sind. Tatsächlich sind die evolvierten Mechanismen der aquatischen Mikroorganismen bisher nicht gut verstanden. Der Salar de Huasco ist ein hochgelegenes Feuchtgebiet im Norden Chiles, in dem die Vielfalt und der Beitrag der eukaryotischen mikrobiellen Komponente noch nicht umfassend untersucht wurden. Ich untersuchte, wie diese Mikroeukaryoten durch verschiedene Umweltstressoren beeinflusst werden, indem ich Genexpressionsmuster entlang eines Flusses mit steigendem Salzgehalt in der feuchten (Sommer) und der trockenen (Winter) Jahreszeit unter verschiedenen Sonnenstrahlungswerten verglich. Die aktiven Gemeinschaften wurden von heterotrophen Taxa (Ascomycota und Evosea) dominiert, während phototrophe Taxa (hauptsächlich Chlorophyta und Bacillariophyta) weniger abundant, aber variabler zwischen Standorten und Jahreszeiten waren. Die Faktoren, die hauptsächlich zur Strukturierung der Gemeinschaft beigetragen haben, waren pH-Wert, Leitfähigkeit und Salinität im Winter, und Temperatur und PAR im Sommer. Die biologischen Prozesse, die während der Gen-Ontologie Analyse am häufigsten vertreten waren, gehörten zum mitotischen Zellzyklus, Photosynthese und Stressreaktion.

Um weiter zu verstehen, welche Gruppen von Mikroeukaryoten welche Arten von Ökosystemfunktionen an verschiedenen Probenahmestellen entlang des Salinitätsgradienten beeinflussen, wurden die biologischen Prozesse mit taxonomischen Gruppen korreliert. Insbesondere konzentrierte ich mich auf unterschiedlich exprimierte Gene aus den Kategorien: Photosynthese und Kohlenstofffixierung, Zellatmung, Ionentransport, Nährstoffaufnahme (einschließlich Schwefelkreislauf) und Stressreaktion. Im Sommer dominierten die Glaucophyta die Photosynthese und damit die Kohlenstoff-Fixierung, während die Chlorophyta, Haptophyta, Ochrophyta und Mucoromycota die Hauptakteure für die Assimilation von gelöstem Stickstoff und Phos-

phor in den weniger salzhaltigen Standorten waren. Im Winter korrelierte eine höhere Expression von Ionentransport- und Stressreaktionsgenen an der salzhaltigsten Probenahmestellen mit Heterotrophen Gruppen, die an der gleichen Stelle dominierten die Zellatmung und die Schwefelassimilation. Die präsentierten Daten zeigen, dass die räumlichen Unterschiede (Probenahmestellen entlang des Salinitätsgradienten) im Winter signifikanter waren als im Sommer. Außerdem waren die zeitlichen Unterschiede (Sommer und Winter) an der Probenahmestelle mit der höchsten Salinität am stärksten ausgeprägt.

Insgesamt ist die Metatranskriptomik ein vielversprechendes Werkzeug zur Untersuchung eukaryontischer mikrobieller Gemeinschaften, insbesondere in selten untersuchten und ökologisch extrem variablen Ökosystemen. Durch die Verbindung von biologischen Prozessen und Biodiversität können neue Einblicke in Ökosystem-Funktionen gewonnen werden, die grundlegend sind, um Naturschutz-Strategien zu entwickeln.

Resumen

El papel de las comunidades microbianas en los ecosistemas acuáticos es fundamental; contribuyen a los ciclos biogeoquímicos y a la dinámica de las redes tróficas en varios niveles. Los microbios eucariotas, que se encuentran en todos los principales supergrupos del árbol de la vida eucarionte, son importantes productores primarios, consumidores y descomponedores en la mayoría de los sistemas acuáticos. Estudiar las comunidades microbianas acuáticas para determinar su diversidad, funciones y respuesta a condiciones ambientales adversas es muy complejo y desafiante. Aunque los crecientes esfuerzos de secuenciación masiva en los últimos años han contribuido significativamente a las estimaciones de diversidad en numerosos ambientes, todavía existe una falta de comprensión de las funciones y roles de estas comunidades en sus ecosistemas, siendo los sistemas acuáticos pequeños y remotos los que han recibido menor atención.

Para comprender cómo los microeucariotas contribuyen al funcionamiento del ecosistema, se necesitan enfoques que combinen información taxonómica y funcional. En esta tesis, utilicé la metatranscriptómica como enfoque principal para investigar las funciones de las comunidades microeucariotas acuáticas a través de la expresión génica. Dos sistemas diferentes fueron estudiados: un pequeño estanque de agua dulce templada y un arroyo poco profundo con salinidad creciente en un ecosistema extremo de gran altitud. Específicamente, me enfoqué en dos factores principales que afectan a estas comunidades: radiación solar y salinidad.

La luz, específicamente en el espectro de radiación fotosintéticamente activa (PAR), es un factor importante para el crecimiento de organismos planctónicos y modula los principales procesos metabólicos a través de los ciclos diurnos. Estas respuestas diarias se han estudiado solo en organismos individuales y en comunidades marinas o de grandes lagos. La investigación de la actividad de la comunidad microeucariota en respuesta al ciclo diurno en el pequeño sistema de agua dulce estudiado, reveló que la comunidad del estanque, en su mayoría dominada por heterótrofos junto con Euglenophyta y Chlorophyta, sigue una dinámica diurna similar a la descrita para grandes sistemas acuáticos. Las categorías de ontología de genes, incluida la fotorrecepción para la fotosíntesis, la defensa y los mecanismos de estrés, predominaron

durante el día. Mientras que la motilidad, el ensamblaje ribosomal y otros procesos que consumen gran cantidad de energía se restringieron a la noche.

La alta radiación solar, los amplios rangos de salinidad, de temperatura, y las altas tasas de evaporación son características de los sistemas acuáticos de las altas mesetas en los Andes centrales. Estos ecosistemas están sujetos a extremas fluctuaciones diurnas y estacionales, a las que se estima que las comunidades que lo habitan están adaptadas. Sin embargo, los mecanismos desarrollados por los microorganismos acuáticos para hacer frente a tales condiciones no son bien comprendidos. El Salar de Huasco es un humedal de gran altitud en el norte de Chile, donde la diversidad y la contribución del componente microbiano eucariota al sistema no se han abordado ampliamente. En el presente trabajo estudié cómo estos microeucariotas se ven afectados por múltiples factores de estrés ambiental comparando los patrones de expresión genética a lo largo de un arroyo de agua dulce con un creciente gradiente de salinidad en las estaciones húmeda (verano) y seca (invierno). Las comunidades activas estuvieron dominadas por taxones heterotróficos (Ascomycota y Evosea), mientras que los taxones fototróficos (principalmente Chlorophyta y Bacillariophyta) fueron menos abundantes, pero más variables entre sitios y estaciones. Los principales factores ambientales estructurando las comunidades fueron el pH, la conductividad y la salinidad durante el invierno y la temperatura y la PAR durante el verano. Y los procesos biológicos más enriquecidos, de acuerdo a los análisis de ontología genética, estaban relacionados con el ciclo celular mitótico, la fotosíntesis y las respuestas al estrés.

Para comprender mejor qué grupos de microeucariotas afectan qué tipos de funciones del ecosistema en diferentes sitios a lo largo del gradiente de salinidad, se correlacionaron los procesos con los grupos taxonómicos. Específicamente, me enfoqué en genes expresados diferencialmente que pertenecen a las categorías: fotosíntesis y fijación de carbono, respiración celular, transporte de iones, absorción de nutrientes (incluido el ciclo del azufre) y respuesta al estrés. Durante el verano, Glaucophyta domina la fotosíntesis y, por lo tanto, la fijación de carbono inorgánico, mientras que Chlorophyta, Haptophyta, Ochrophyta y Mucoromycota fueron los principales actores en la asimilación de nitrógeno y fósforo disuelto en los sitios menos salinos. Durante el invierno, una mayor expresión de genes de transporte de iones y respuesta al estrés en los sitios más salinos se correlacionó con grupos heterotróficos, los que en

el mismo sitio dominaron la respiración celular y la asimilación de azufre. Los datos presentados demuestran que las diferencias espaciales (sitios a lo largo del gradiente de salinidad) fueron más significativas en invierno que en verano. Y las diferencias temporales (estación húmeda y seca) por sitio fueron más pronunciadas en el sitio más salino.

En general, la metatranscriptómica es una herramienta prometedora para estudiar las comunidades microbianas eucariotas, particularmente en ecosistemas menos estudiados y con condiciones ambientales extremas. Al vincular los procesos biológicos con la biodiversidad, se puede obtener mayor conocimiento sobre las funciones de los ecosistemas, lo que es esencial para el desarrollo de estrategias de conservación.

General introduction and aims of the study

Aquatic microbial eukaryotes and their role in inland ecosystems.

Eukaryotic microbes or microeukaryotes, referring to those organisms in the microscopic fraction, are mostly unicellular and include protists and fungi (Caron et al. 2009). They are widely distributed in the tree of life, with representatives in all supergroups (Burki 2016). These ubiquitous organisms (Finlay 2002) are found as part of the human or animal microbiota (Parfrey et al. 2011, del Campo et al. 2019), and in diverse terrestrial (Bailly et al. 2007, Hesse et al. 2015, Pulschen et al. 2015) and aquatic systems (Debroas et al. 2017, Machado et al. 2019, Sieracki et al. 2019).

Primary producers, consumers, and decomposers are the three main categories into which microeukaryotic organisms are grouped according to their functions. The microeukaryotic primary producers or phototrophs, commonly called microalgae, can be living in the water column (planktonic), in the soil-water interface, or attached to submerged substrates (benthonic). They are fixing inorganic carbon through photosynthesis; therefore, light is essential as their primary energy source but also to regulate their cellular processes (Falkowski et al. 2004, de los Reyes et al. 2017), releasing organic matter to the systems, which can be used as an autochthonous (Myklestad 1995) source of organic carbon for other microorganisms such as bacteria. Heterotrophic protists, the consumers, prey upon bacteria, primary producers, and other heterotrophic protists to obtain nutrients and energy (Sherr & Sherr 2002, Tarbe et al. 2011). In turn, they are grazed by zooplankton (Gifford 1991, Sautour et al. 2000) and thereby their relevance in the energy transfer in the aquatic food webs (Arndt et al. 2000, Logares et al. 2012, Sommaruga & Kandolf 2014). Fungi are considered the primary decomposers of organic matter in the aquatic systems (Jobard et al. 2012, Wurzbacher et al. 2014, Tant et al. 2015) although generally less addressed.

These functional groups are found in all aquatic ecosystems, including marine and "freshwater" systems, although the correct term to refer to the latter would be inland

waters, as many of them have broad salinities and salt contents of different origins than those from the ocean (Waiser & Robarts 2009). The enormous diversity of inland waters include ponds, streams, rivers, lagoons, and lakes, each of them with several different characteristics (Kruk et al. 2009, Márquez-García et al. 2009, Shurin et al. 2012, Maranger et al. 2018, Fasching et al. 2019, Iniesto et al. 2020), which makes it extremely difficult to find universal rules and patterns that would apply to all inland waters, in terms of communities biodiversity and nutrient cycling, among the most critical factors.

Study approaches and limitations.

The study of aquatic microbes in their environment is very complex and challenging. Determining their diversity and their functions and response to adverse conditions is one of the main aims to address in environmental studies, and it often requires the combination of several targeted or general approaches. The use of culture-dependent technics allows the description and study of microbial isolates, testing several factors influencing their growth and interactions with other species (Hauer & Rogerson 2005, Vaulot et al. 2008, Libkind et al. 2009). Those studies have given us enormous insight into the intriguing microbial world. However, individual microbes' activity under laboratory-controlled conditions cannot reflect the environment's complex interactions and real functions, limiting our microbial ecology knowledge.

According to an old paradigm, only around 1% of microbes in the environment can be cultured under laboratory conditions. This statement has always referred to prokaryotes and has nowadays been debated (Martiny 2019, Steen et al. 2019). Nevertheless, it is important to consider that the culture-independent approaches have increased exponentially over the years based on that limiting premise.

Transmission electron microscopy is among the fundamental approaches that first allowed the identification and role estimation of small eukaryotes in the ocean (Johnson & Sieburth 1982). Furthermore, they continue to be used and give useful inputs in combinations with other techniques. For phototrophic groups, the analysis of pigments via HPLC has been proven to be a fast and convenient method for their abundance estimation based on the different pigment compositions of the different algal groups (Mackey et al. 1996, Schlüter et al. 2016). Also based on their pigments, flow cytometry is a technique that has contributed significantly to study the

abundance and diversity of phototrophic groups of different size fractions (Marie et al. 2010, 2014).

All the mentioned above approaches can be considered suitable for different research areas or questions. Describing morphological aspects of a determined species/group of species and estimating their abundance is the aim (from a simplified perspective). Nevertheless, one cannot determine community structure, metabolisms, species interactions, and environmental response using those approaches. Due to these limitations, is that molecular analysis has become vital with time. Terminal restriction fragment length polymorphism (T-RFLP), denaturing-gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), clone libraries, and quantitative PCR (qPCR) analysis are among the most used molecular methods for years (Zhu et al. 2005, Maturrano et al. 2006, Lima & Sleep 2007), until the next-generation sequencing (NGS) technologies started 15 years ago (Thermes 2014). These advances allowed to start more environmental microbiology studies, increasing the assessment of an unknown diversity in many ecosystems. In the beginning, these studies were much more bacteria-focused. However, in the last ten years, there has been a considerable increase in protist studies, emphasizing that "protists are microbes too" (Caron et al. 2009).

The advances in NGSs also allowed the increase of complete genomes sequencing. Their availability in open databases has been key to identifying closely relative organisms' genes or taxa-related defined functions (McGrath & Katz 2004, Castro-Severyn et al. 2017). The establishment of metabarcoding and metagenomics has allowed high-resolution identification of community structure and the potential of their metabolisms (Cuvelier et al. 2010, Abdelfattah et al. 2018, Bush et al. 2019, Shahraki et al. 2019).

In the era of the "Omic" approaches, vast loads of data have been produced. Even though such big datasets' processing and interpretation remains challenging, we have gained knowledge from diverse environments as we could have never thought. Notwithstanding, the most used approaches aim to identify the community structure, and a community's described diversity does not give insights into their activity and roles in a determined environment. Using metagenomics, the functions can be

predicted by genes' presence but do not imply their expression. And if expressed at what intensity level to consider the real contribution to the environment.

But what are they doing?

While metagenomics mainly focuses on the question "who is there" by describing the gene composition and the diversity of microbial communities, metatranscriptomics has its main focus on the "what are they doing" question, targeting the active fraction of microbial communities that do not necessarily correspond to the most abundant species as determined by metagenomics. Metatranscriptomics is based on the sequencing of RNA (RNAseq), and it can be done with the total RNA molecules of a community (including ribosomal RNA, transfer RNA, interfering RNA, and other non-coding RNAs), or it can target only the messenger RNA (mRNA) from a complex, usually environmental, sample (Poretsky et al. 2009). To study gene expression, i.e., the protein-coding genes, the most suitable approach would be targeting the mRNAs or transcripts, since the amount of ribosomal RNA (rRNA) can be up to 89-90% of the total RNA content, thus leaving mRNA with low sequencing coverage. In the case of eukaryotic organisms, the selection of mRNA, based on the poly(A) sequences present in the 3' extreme of all transcripts, can be done using poly d(T) oligonucleotides.

By targeting only expressed genes, metatranscriptomic has immense potential in comparing spatial and temporal expression profiles of microbial communities under different abiotic factors in a determined time, which is why it is considered a "snapshot" of microbial communities, which has been demonstrated in various studies from terrestrial (Urich et al. 2008, Hesse et al. 2015, Garoutte et al. 2016), to aquatic ecosystems, both marine (Frias-Lopez et al. 2008, Marchetti et al. 2012, Aylward et al. 2015) and freshwater (Vila-Costa et al. 2013, Edgar et al. 2016, Bižic-Ionescu et al. 2018).

Besides the evident difference between detecting the presence of genes and expression of genes, another advantage of metatranscriptomics over metagenomics is that it allows for identifying living organisms only, which is not trivial in environmental microbiology. The persistence of DNA molecules in the environment is much longer than RNA, which is easily degraded; therefore, many species can continue to be detected from extracellular DNA, or recently death cells generating bias in community

composition (Nielsen et al. 2007, Carini et al. 2016). Including the genetic material from dead cells might be relevant for some studies, but when we want to study the functions and processes of a community under determined temporal and spatial conditions, metatranscriptomics would be more appropriate.

Extreme ecosystems and extremophiles.

From an anthropocentric perspective, an extreme ecosystem is defined as one with environmental conditions challenging for life (Dong 2008, Lindgren et al. 2016). Extreme temperatures, pressures, solar radiation, acidity, salinity, to mention some, can indeed be challenging for life as we know it. However, from that point of view, microorganisms are more than often not considered. Moreover, the limits of life for microorganisms are even nowadays not wholly understood. Indeed, we have learned from the past 20-30 years that microorganisms are versatile and that microbial life is possible in so many extreme ecosystems that were previously not imaginable. One explanation for the adapted microbial life in extreme environments is that these resemble the conditions found on early earth and under which microbial life started (Stoeck et al. 2014).

Extremophiles are organisms adapted to extreme ecosystems' environmental conditions and are mainly unicellular organisms (Horikoshi et al. 2011). These can be categorized according to the extreme condition they can tolerate and grow under. Organisms tolerating low or high temperatures are considered psychrophiles or hyperthermophiles, respectively. Barophiles if they can tolerate pressure. Acidophiles and alkaliphiles if they can grow under low or high pH, respectively. Halophiles are organisms growing under high salinity, among some of the most studied categories. Many organisms can also be called poly-extremophiles if they inhabit environments with multiple extreme conditions. Extreme pH, salinity, and exposure to desiccation are conditions with which microbial eukaryotes can cope without a problem. Extreme temperatures, however, are conditions to which prokaryotes are better adapted (Rampelotto 2013, Seckbach et al. 2014). However, studies from inland aquatic extreme ecosystems, considering the microeukaryotic fraction, are scarce.

Remote, extreme ecosystems and factors shaping microeukaryotic communities.

Although essential for primary producers, light (the photosynthetically active radiation, PAR, 400 – 700 nm) can also be considered a stressor when at high intensities (Neidhardt et al. 1998, Dall'Osto et al. 2019, Chiu et al. 2020). Ultraviolet radiation (UVR), the fraction of solar radiation in the spectrum between 100 and 400 nm, is a more substantial stressor for all organisms. From this range, only UV-A (320 – 400) and UV-B (280 – 320) reach the earth's surface, being UV-B the less abundant but most harmful (Reiter & Munzert 1982, Herndl et al. 1997, Allorent et al. 2016). Further, salinity is considered a decisive factor in determining communities' structure and setting limits of life for many organisms, as the costs of osmoregulation are high (Oren 2001).

The Altiplano (high plateau) in the central Andes, South America, at a high altitude (˜ 4000 m a.s.l.), is a region with multiple extreme (poly-extreme) ecosystems (Farias 2020). The high altitude, solar radiation, evaporation, and the cyclic precipitation events, lead to wide salinity ranges (Risacher et al. 2003, Risacher & Fritz 2009), shaping the diversity of the various aquatic systems and salt pans, locally known as "salares" (Aceituno 1997, Squeo et al. 2006). Still, there is scarce information reported from these ecosystems for the eukaryotic microbial component in response to high light, UV radiation, salinity, nor any other biotic/abiotic factor that could help to elucidate the functions of these communities in such extreme environments.

Thesis outline

This thesis contains three chapters. The research focus is the analysis of active eukaryotic microbial communities from inland waters under different environmental conditions. Metatranscriptomics is the main approach used to get insights into the community structure, gene expression, and the predominant processes found as a stress response to environmental factors.

I mainly address the following questions in the following chapters:

• How is the active microeukaryotic community structured?

• What are the primary metabolic processes carried by these communities throughout the daily cycle?

• How do high light and solar radiation affect these main metabolic processes?

• How does an increase in salinity affect the active microeukaryotic communities? And which mechanism are they using to signal stress and adaptation?

• What is the contribution of the eukaryotic microbial communities in Salar de Huasco to this extreme ecosystem?

In **Chapter 1**, I use metatranscriptomics to study a microeukaryotic community from a small freshwater aquatic system. I complement with pigment and carbon analyses to determine the community structure, particularly of the phototrophic fraction, and the functions through the diel cycle. I address the possible day/night rhythmicity in gene expression patterns that has only been explored in large aquatic systems.

In **Chapter 2**, I analyze the metatranscriptomes from samples along a salinity gradient in a high altitude aquatic system under extreme solar irradiance, Salar de Huasco. I evaluated the differential gene expression of the microeukaryotic community to understand the main changes in metabolic processes associated with stress and adaptation to extreme environmental conditions. Further, I investigated if these responses are changing in wet and dry seasons.

In **Chapter 3**, I use gene expression data from Salar de Huasco to evaluate several metabolic processes that were spatially and temporally overexpressed. I specifically

select genes involved in the processes: photosynthesis and carbon fixation, cellular respiration, ion transport, nutrient cycling (including sulfur compounds production), and genes involved in stress response. I correlated the taxonomical groups to these processes to understand the main contributors to the ecosystem functions. An overview of the thesis outline is presented in figure 1.

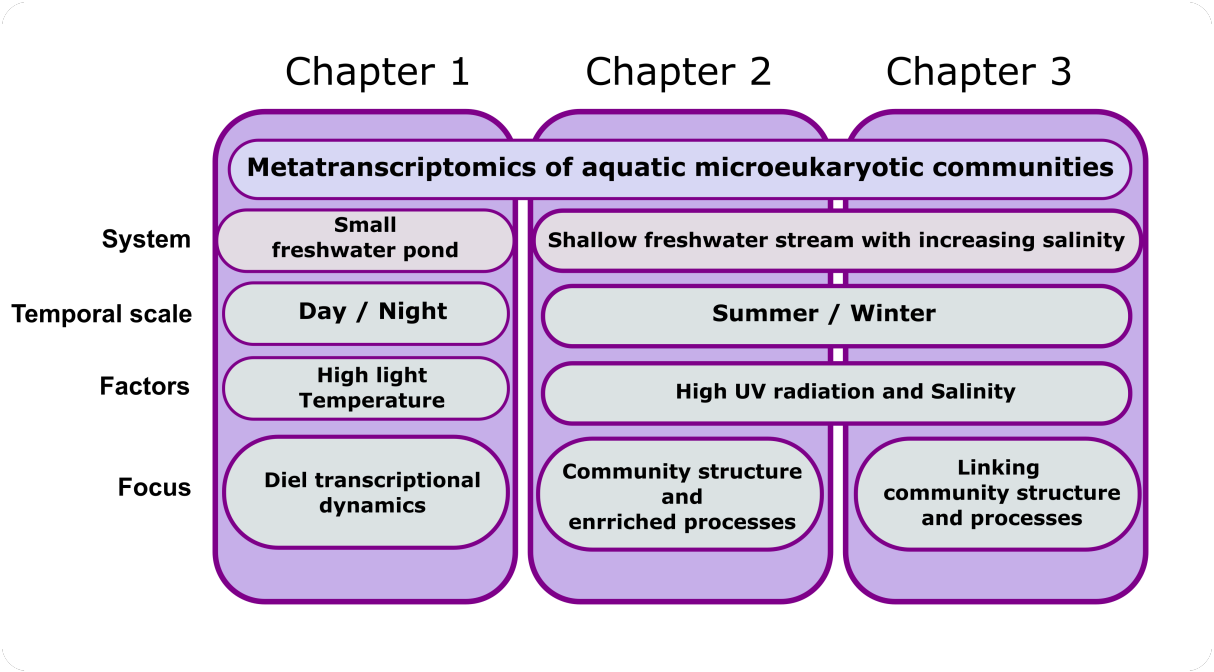

Figure 1: Summary outline of the thesis research -

Chapter 1

Metatranscriptomics From a Small Aquatic System: Microeukaryotic Community Functions Through the Diurnal Cycle

1.1 Abstract

Light is an important factor for the growth of planktonic organisms, and many of them depend on the diurnal light/dark cycle to regulate key metabolic processes. So far, most of the diel responses were only studied in single species or marine and large lake communities. Yet, we lack information on whether these processes are regulated similarly in small aquatic systems such as ponds. Here, we investigated the activity of a microeukaryotic community from a temperate, small freshwater pond in response to the diurnal cycle. For this, we took samples at midday and night during the Central European summer. We extracted pigments and RNA from samples and the sequencing of eukaryotic transcripts allowed us to obtain day and night metatranscriptomes. Differentially expressed transcripts primarily corresponded to photosynthesis-related and translational processes, and were found to be upregulated at midday with high light conditions compared to darkness. Unique gene ontology classes were found at each respective condition. During the day, ontology classes including photoreception for photosynthesis, defense, and stress mechanisms dominated, while motility, ribosomal assembly and other large, energy-consuming processes were restricted to the night. Euglenophyta and Chlorophyta dominated the active phototrophic community, as shown by the pigment composition analysis. Regarding the gene expression patterns, we could confirm that the pond community appears to follow similar diurnal dynamics as those described for larger aquatic ecosystems. Overall, combining pigment analyses, metatranscriptomics, and data on physicochemical factors yielded considerably more insight into the metabolic processes performed by the microeukaryotic community of a small freshwater ecosystem.

1.2 INTRODUCTION

Light plays an important role in structuring microbial communities. For photosynthetic organisms like phytoplankton, light is the source of energy and is therefore crucial for primary production in aquatic systems. Many organisms have the ability to synchronize their metabolism to the light periods and organize cellular processes in response to daily light fluctuations (reviewed in Roenneberg and Merrow, 2005). This rhythmic response of cellular metabolisms to light/dark cycles is known as the circadian clock and in the case of microalgae, the molecular mechanisms have been reported for some model organisms (Mittag et al., 2005; Moulager et al., 2007; Monnier et al., 2010; O-Neill et al., 2011). Gene expression approaches have been key to elucidate the responses of these model organisms to nutrient and light availability (Bailleul et al., 2010; Gifford et al., 2013; Allorent et al., 2016; Jaubert et al., 2017). However, although crucial for the identification of different functions at gene or protein level and the understanding of main biological processes, single organism studies under laboratory conditions hardly picture natural communities that experience unsteady environmental conditions. Thus, metatranscriptomics (transcript sequencing from the whole community) is a more suitable tool to provide a snapshot of the main active organisms and the predominant activities performed by a specific community in response to changing conditions (Vila-Costa et al., 2010; Gifford et al., 2011; Marchetti et al., 2012; Grossmann et al., 2016; Bižic-Ionescu et al., 2018). Nevertheless, metatranscriptomics alone is not sufficient to determine the taxonomic composition of an active community and demands complementary approaches (Shakya et al., 2019). For phytoplankton communities, the analysis of group-specific accessory pigment patterns provides a fast method that is frequently used to identify phytoplankton functional taxonomic composition (Mackey et al., 1996; Sarmento and Descy, 2008; Schlüter et al., 2016).

Previous metatranscriptomic research on the response of microbial communities to the natural day-night cycle has been mainly performed during summer when a diurnal change of temperature, light, and irradiance is pronounced and high biomass of phototrophic microorganism is present. These studies have shown expression patterns that link cellular processes to different times of the day, and that have been related to light availability. Photosynthesis and transporters-related transcripts, as well as transcripts involved in energy production and stress mechanisms, were preferentially expressed during the day. Whereas, carbon fixation, carbohydrates and amino acid synthesis, and cell division are processes that mainly occur during the night (Poretsky et al., 2009a; Vila-Costa et al., 2013; Aylward et al., 2015; Linz et al., 2019).

Yet, most of these studies were done with communities from the open oceans and large lakes, while smaller aquatic ecosystems were so far overlooked.

Such small aquatic systems like ponds are particularly suitable to study the response of natural communities to environmental changes, as isolated systems are more susceptible to small scale environmental variations, these communities are more responsive to dynamic stimuli (Simon et al., 2015; Verbeek et al., 2018). It is of interest, as the global temperatures increase, to study pond systems that are subject to more frequent drought events, and to understand how their planktonic communities will be affected and respond by developing or not an adaptation to drastic environmental changes (Simon et al., 2016).

We hence aimed the present study to investigate the activity of a microeukaryotic community from a small freshwater pond during day and night. We expected the phototrophic fraction of the pond community to be more affected by light changes throughout the day, than the heterotrophic fraction. By measuring community-wide gene expression, we wanted to investigate whether the pond community responds to the diurnal cycle in the same way that has been reported for larger aquatic systems. To do so, we obtained metatranscriptomes together with pigment composition profiles from replicate day and night summer samplings over a month.

1.3 MATERIALS AND METHODS

1.3.1 Sampling

Water samples were taken from a small (2 m$\times$ 2 m, $\sim$1 m depth), artificial pond located in the Botanical Gardens of the University of Cologne, Germany (50°55′31.0″N 6°56′09.4″E) during the summer of 2017 (June–July, with a light-dark ratio of $\sim$16:8 h). Samples were taken on 4 different days within a month. These 4 days were considered as four replicates for each daytime. Daytime samplings were conducted at midday, representing the maximum daily irradiance ($\sim$7 h after sunrise). Nighttime samplings were conducted at 4:30 am with no measurable irradiance ($\sim$ 7 h after sunset). At each sampling point, we determined the following physicochemical parameters in situ: temperature, pH (pH meter Vario. WTW, Germany), photosynthetically active radiation (PAR) in the air (at the pond's surface) and in the water directly beneath the surface (Underwater Spherical Quantum Sensor LI-193. LI-COR Biosciences, United States), dissolved oxygen (DO, HQd portable meter with an optical DO sensor, Hach), and conductivity (Conductivity hand-held meter LF330, WTW, Germany).

1.3.2 Sample Processing

Upon sampling, the collected water was immediately pre-filtered through a 30μm mesh, before 500 mL of pre-filtered water was pumped through $Sterivex^{TM}$cartridges (0.2μm) via a peristaltic pump with sterile Tygon tubing. Up to 2 mL of RNAlater (Qiagen) were added to each cartridge to preserve the samples before storage at $\sim$ 80°C until RNA extraction.
For pigment analyses, replicates of 200 mL of the pre-filtered water were filtered at low light conditions through GF/F filters ∅25 μm with a vacuum pump. Filters were wrapped in aluminum foil and stored at $\sim$ 20°C until acetone extraction and analysis on HPLC according to Ilic (2019). Briefly, filters were placed on vials with 3.5 ml 100% acetone (HPLC grade), sonicated for 2 min and then put on ice for 1 min, these two steps were repeated five times. The vials were then kept at 4°C overnight. On the next day, filters were removed and the extracts were centrifuged at 4500 × g for 15 min and 1 ml of the extracts were used to evaporate to dryness under nitrogen gas. 100 ng of trans-β-apo-8'-carotenal (Sigma Aldrich) was added to the extracts prior to evaporation and used as an internal standard. The evaporated extracts were resuspended in 100 μl of acetone and were transferred to HPLC vials, 25 μl from each sample were injected into the HPLC (Shimadzu Prominence system with a binary pump) onto a Spherisorb ODS2 column (stationary octadecyl-phase C_{18}). The solvent A was methanol: ammonium acetate (1 M): acetonitrile (50:20:30v/v), and solvent B was acetonitrile: ethyl acetate (50:50 v/v). The solvents gradient started at A: 90% and B: 10%, after 2 min A: 90% and B: 10%, after 26 min A: 40% and B: 60%, after 28 min A: 10% and B: 90%, after 30 min A: 10% and B: 90%. Pigments were identified via comparisons of retention times with those of pure standards DHI Water (Høersholm, Denmark). The pigment content from each sample was used to estimate the contribution of different phytoplankton groups to the total chlorophyll a, using the software CHEMTAX (Mackey et al., 1996), and initial matrices with pigment:chl-a ratios previously described for freshwater systems (Sarmento and Descy, 2008; Schlüter et al., 2016). A total of 60 randomized ratio matrices were constructed from the initial ratios for each dataset, and the program was run 60 times. The ratio limits were set to 500. The 10% of matrices with the lowest residual root mean square (RMS) were averaged and used as new input ratio matrices.
For the analysis of the particulate carbon and nitrogen fractions of the pond seston, volumes of 200 mL were filtered through GF/F filters ∅25 mm with a vacuum pump before drying them for 24 h at 60°C. Dried filters were subsequently wrapped into tin capsules and analyzed on a Thermo Flash EA2000 Analyzer (Schwerte, Germany). Soluble reactive phosphorus (SRP)

was determined spectrophotometrically (DR5000 UV-Vis spectrophotometer. Hach, Germany) using the ascorbic acid molybdenum-blue method (Greenberg et al., 1985). For this, 100 mL of each water sample were filtered through GF/F filters and the filtrate collected in an acid-washed flask (5% sulfuric acid), measurements were done in triplicates were used each time. For the dissolved organic carbon (DOC) estimation, 100 mL of water were filtered through a 0.2 μm cellulose membrane filter and was kept in darkness at 4°C until analysis. The measurements were conducted using the Aqualog fluorometer (HORIBA) at excitation wavelengths 250–600 nm and emission wavelengths 250–620 nm, the index calculation was done with the R package staRdom (Pucher et al., 2019). All physicochemical data were tested for normality with the Shapiro-Wilk tests. T-tests or Mann-Whitney rank-sum tests were used to determine significant differences between day and night samples for all parameters. For the relative and absolute pigment composition, two-way ANOVAs (day/night and phyla) were performed, with subsequent pairwise multiple comparisons (Tukey test) for the different sampling dates and phyla. Statistical analyses were performed on R, version 3.6.1 (R Core Team, 2019).

1.3.3 RNA Extraction and Sequencing

Sterivex^{TM} cartridges were thawed on ice, opened and processed as described by Cruaud et al. (2017). Membrane filters were cut into strips to fit on sterile screw-cap 2 mL tubes (CK14 tubes) for the RNA extraction with ReliaPrep kit (Promega), including DNase treatment. The fibrous tissue protocol was applied according to the manufacturer's specification, using the lower amounts of reagents of the protocol, and an initial mechanical disruption of the samples using 0.65 g of 1.4 mm ceramic (zirconium) beads. Total RNA quality (A260/A280 and A260/A230 ratios) were assessed with a Nanodrop photometer, and sample concentrations were measured using the Qubit^{TM} fluorimeter's RNA HS Assay Kit (Invitrogen). RNA integrity (RIN) of the total RNA samples ($\sim$7) was checked with a Bioanalyzer (Agilent Technologies). Samples were poly-A selected for mRNA before cDNA libraries were prepared using the TruSeq^{TM} stranded kit, and 75 bp paired-end sequencing was performed in the Cologne Center for Genomics using the Illumina HiSeq 4000 platform.

1.3.4 Metatranscriptome Analyses

Quality trimming of the sequences (all Phred scores $>$ 30) was done with Trimmomatic 0.36 (Bolger et al., 2014) with parameters LEADING:5 TRAILING:5 MINLEN:70 (Phred 33) and remaining rRNA sequences were removed using Sortmerna 2.1 (Kopylova et al., 2012). Merging

of paired ends sequences done with FLASH (Magoè and Salzberg, 2011) before the assembly gave only ∼30% of overlapping sequences that could be merged from the total raw sequences. Therefore, all sequences were used to perform de novo assemblies with the software Trinity version 2.5.1 (Haas et al., 2013). Raw sequences, samples and replicates individually, were aligned to the reference assemblies using Bowtie2, and then RSEM (Li and Dewey, 2014) was used to estimate transcripts abundances and create gene and isoform count matrices including all replicates and conditions. These matrices were first used to check between replicates and samples correlation, and then to perform differential expression analyses comparing the day and night samples. Differential expression analysis on the transcript level was done with the edgeR package (Robinson et al., 2010) and TMM normalization method (Robinson and Oshlack, 2010), using the default adjusted p-value cutoff of 0.001 for false discovery rate (FDR, Benjamini and Hochberg, 1995). The sequences from the complete day and night assemblies and the differentially expressed transcripts were annotated separately following the Trinotate pipeline (Bryant et al., 2017). This pipeline includes the prediction of coding regions on transcripts (TransDecoder)1, and uses Blastx and Blastp against the Swissprot database to get annotations from both transcripts and predicted proteins. For protein domain prediction, HMMR is used with the PFAM database. Taxonomical assignments, gene ontologies, and KEGG orthology were assigned from these annotations. Gene Ontology enrichment analysis for differentially expressed transcripts was performed using the R package GOseq (Young et al., 2010), the significance for GO categories ($p < 0.05$) was calculated after random resampling to generate a null distribution for each category amongst the over-represented DE transcripts. To find the over enriched GO categories, a 0.05 FDR was used.

1.4 RESULTS

1.4.1 Environmental Parameters, Nutrient Content, and Pigment Composition

The temperatures and PAR (air and water), and the dissolved oxygen measured in situ showed diel rhythmicity, with values that were significantly different for day and night, while this was not the case for pH and conductivity (Table 1.1). From the determined nutrients, only particulate organic carbon (POC) concentrations differed significantly between day and night samples ($p < 0.05$). Particulate organic nitrogen (PON) did not vary greatly between samples, and the same was observed for the C:N ratios. The dissolved organic carbon (DOC) concentration was 11.3 gL^{-1} with an origin mostly from plant degraded material, with low bioavailability (bix = 0.681; fI = 1.38, and hix = 0.887). Soluble reactive phosphorus (SRP) fluctuated between day and night samples, but not significantly throughout the different sampling days (Table 1.1).

Table 1.1: Physicochemical parameters of the pond - (mean ± SD of n = 4 for in situ measured parameters (sampling days), and n = 12 for triplicate nutrient analyses per day); numbers in bold indicate significant differences between day and night determined by t-test.

Parameters		**Day**	**Night**	**Stats**
Temperature [∘C]	Air	**30.70** ±1.75	**18.43** ±1.28	$P < 0.001$
	Water	**24.73** ±0.93	**21.08** ±1.04	$P < 0.05$
PAR [$\mu mol\ s^{-1}m^{-2}$]	Air	**1999.38** ±73.66	**0.12** ±0.08	$P < 0.001$
	Water	**695.25** ±55.67	**0.04**±0.02	$P < 0.001$
pH		6.63 ±0.19	6.39 ±0.06	N.S.
Conductivity [μS cm^{-1}]		50.98 ±1.25	49.50 ±5.78	N.S.
DO [mg L^{-1}]		**3.99** ±1.15	**2.50** ±0.35	$P < 0.05$
Oxygen saturation [%]		**48.09** ±14.28	**28.13** ±4.37	$P < 0.05$
Chl-a [μg L^{-1}]		**3.63** ±1.65	**1.46** ±0.99	$P < 0.001$
POC [mg L^{-1}]		**1.79** ±0.52	**1.25** ±0.56	$P < 0.05$
PON [mg L^{-1}]		0.14 ±0.03	0.11 ±0.05	N.S.
C:N		12.69 ±1.07	11.83 ±1.26	N.S.
SRP [μg L^{-1}]		18.17 ±11.02	14.02 ±6.17	N.S.

N.S. = not significant.

For chlorophyll a content (Chl-a), a significant difference was found between day and night samples ($p < 0.001$). The pigment composition in relation to the Chl a content, analyzed with CHEMTAX, showed that both Euglenophytes and Chlorophytes were the most abundant phototrophic groups in almost all samples(Figura1.1A). The relative abundance of almost all phytoplankton groups and particularly Chrysophytes, Cryptophytes, and Cyanobacteria decreased during the night (Figure1.1A), which also corresponds to a decrease in their absolute abundance in all night samples (data not shown).

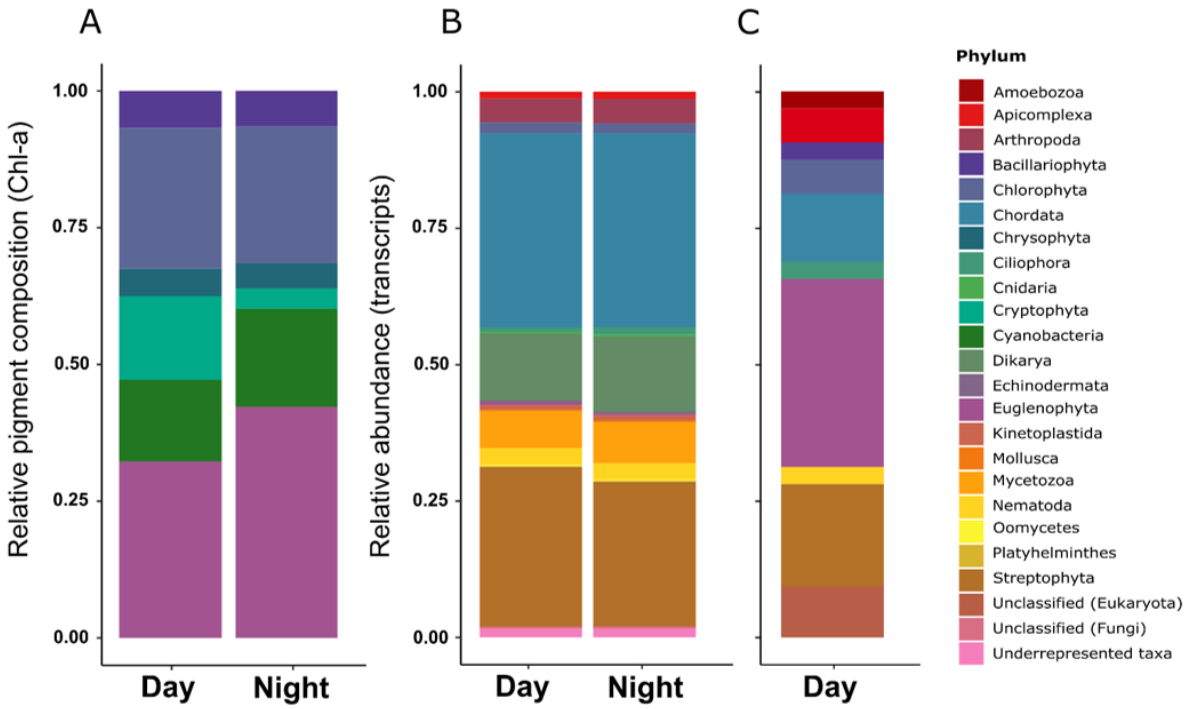

Figure 1.1: Relative taxonomic composition of the pond's microeukaryotic community as derived from three distinct methods/data sets - **(A)** the contribution of phototrophic organisms (microalgae and cyanobacteria) derived from HPLC analyses of accessory photopigments (relative to chlorophyll a) via the matrix-factorization algorithm CHEMTAX (Mackey et al., 1996), **(B)** the general functional annotation of all eukaryotic transcripts obtained by RNA-Seq and determined via Trinotate; and **(C)** the functional annotation of the differentially expressed **(DE)** transcripts found for the RNA-Seq data.

1.4.2 Metatranscriptomes

Each sample yielded 14,792,542 to 16,756,988 reads. Even though the samples were poly-A selected for eukaryotic mRNA, low amounts (5-10%) of rRNA were detected and removed with Sortmerna after quality control (QC). The overall assemblies yielded ∼223,000,000-331,000,000 bases per condition and after the annotation, a total of 763,363 and 509,532 transcripts were identified in the day and night metatranscriptomes, respectively. From the total transcripts assembled, around 40% could be identified as potential proteins once the coding regions were translated, and only ∼15% could be annotated as proteins after performing the protein BLAST against the Swissprot database. From this amount, most of the annotations were assigned to the three ontology categories J (translation, ribosomal structure, and biogenesis), O (post-translational modification, protein turnover, and chaperones), and T (signal transduction mechanisms). A total of 4693 gene ontology (GO) terms were shared between day and night, 5101 were found to be unique during the day and 4388 were unique for night metatranscriptomes. Among the shared GO terms, cellular components like cytoplasm, nucleus, cytosol, membranes, mitochondrion, chloroplast, endoplasmatic reticulum, and Golgi apparatus were found to be highly abundant, as well as molecular functions like ATP-binding, metal ion-binding and RNA-binding (not shown).

The most abundant GO terms found to be unique for day samples were related to cell defense (defense response to bacteria, GO:0042742), stress mechanisms (downregulation of apoptosis, GO:0043066; heat-shock protein binding, GO:0031072), proliferation and growth (mitotic cell cycle, GO:0000278; nuclear membrane, GO:0031965), and photosynthesis-related processes (photosynthesis, GO:0015979; protein-chromophore linkage, GO:0018298). For the night, the most abundant unique terms were related to protein synthesis and cell growth (meiotic cell cycle, GO:0051321; structural constituent of the cytoskeleton, GO:0005200; ribosome biogenesis, GO:0042254; ribosomal small subunit assembly, GO:0000028, and ribosome binding, GO:0043022) and cell movement (dynein light chain binding, GO:0045503, GO:0051959; ATP-dependant microtubule motor activity, GO:0008569; ciliary basal body, GO:0036064; and cilium movement, GO:0003341, Table 1.2).

The general taxonomic assignment from the annotated sequences showed that most of the annotations belonged to the phyla Chordata, Streptophyta, Dikarya, and Mycetozoa, and no great variation was observed for day and night samples, except for a small decrease on the abundances at night compared to day (Figure1.1B). Instead, when observing at the taxonomic assignment

Table 1.2: The most abundant GO terms (>2000 annotations per condition and per million, CPM) found to be unique for day and for night samples are shown. The most common GO terms found for all differentially expressed (DE) transcripts during the day (27) are also shown. Ontology is divided into three main categories, and only counts for unique terms were normalized to the total number of annotations per condition and per million (CPM).

Ontology	**GO terms unique day(CPM)**	**GO terms unique night(CPM)**	**GO terms from DE transcripts**
Cellular component	Kinesin complex (4453)	Ciliary basal body (4841)	Chloroplast thylakoid membrane (5)
	Nuclear membrane (4278)	catalytic step 2 spliceosome (4764)	Cytosol (4)
		lysosomal membrane (4315)	Plasma membrane (4)
		early endosome (4011)	Chloroplast (3)
			Integral component of membrane (3)
			Cellular component Membrane (3)
			Cytoplasm (2)
			Mitochondrial matrix (2)
			Mitochondrion (2)
			Photosystem I (2)
			Plasmodesma (2)
			Proton-transporting ATP
			synthase complex, catalytic core (2)
Biological process	Protein-chromophore linkage (6402)	Ribosomal small subunit assembly (5356)	ATP synthesis
			coupled proton transport (2)
	Defense response to bacteria (4431)	Ribosome biogenesis (4537)	Photosynthesis (2)
	Photosynthesis (4311)	Translational elongation (4325)	Photosynthesis,light-harvesting (2)
	Negative regulation of	Cilium movement (4253)	Response to cytokinin (2)
	apoptotic process (4241)		
	Mitotic cell cycle (4066)	Meiotic cell cycle (4155)	Cell-cell adhesion (2)
	Response to cold (4062)		
	Serine-type	Structural constituent of	Calcium ion binding (3)
	endopeptidase activity (4837)	cytoskeleton (5387)	
Molecular function	Protein serine/threonine	Dynein light intermediate	Electron transfer activity (2)
	phosphatase activity(4475)	chain binding(5367)	
	Calcium-dependent protein	ATP-dependent microtubule motor	GTP binding (2)
	serine/threonine kinase activity (4347)	activity, minus-end-directed (5166)	
	Heat shock protein binding (4311)	Dynein light chain binding (5130)	GTPase activity (2)
	Protein domain specific binding (4183)	Ribosome binding (4516)	Oxidoreductase activity (2)
	Metalloendopeptidase activity (4124)		Proton-transporting ATP synthase activity (2)
			Translation elongation factor activity (2)
			Heparin-binding (2)

from the differentially expressed transcripts during the day (Figure 1C), the phyla Streptophyta and Chordata were still present, but the rest corresponded to less abundant groups from the general taxonomic assignment: Euglenophyta, Chlorophyta, Apicomplexa, Bacillariophyta, Ciliophora, Nematoda, and Amoebozoa, most of which were confirmed by microscopic observation (data not shown).

1.4.3 Expression Patterns

Differential expression (DE) was tested by comparing day and night samples using the initial reference assembly without annotations. We found 45 transcripts to be upregulated during the day. No transcripts were found to be upregulated during the night. From these 45 differentially expressed transcripts, 27 could be functionally annotated. Most of them belonged to the "photosynthesis" category from KEGG Orthology (KO), including light-harvesting complex proteins, photosystem reaction center proteins, oxygen-evolving enhancer proteins, Ferredoxin-NADP reductase, and plastocyanin (Figure 1.2).

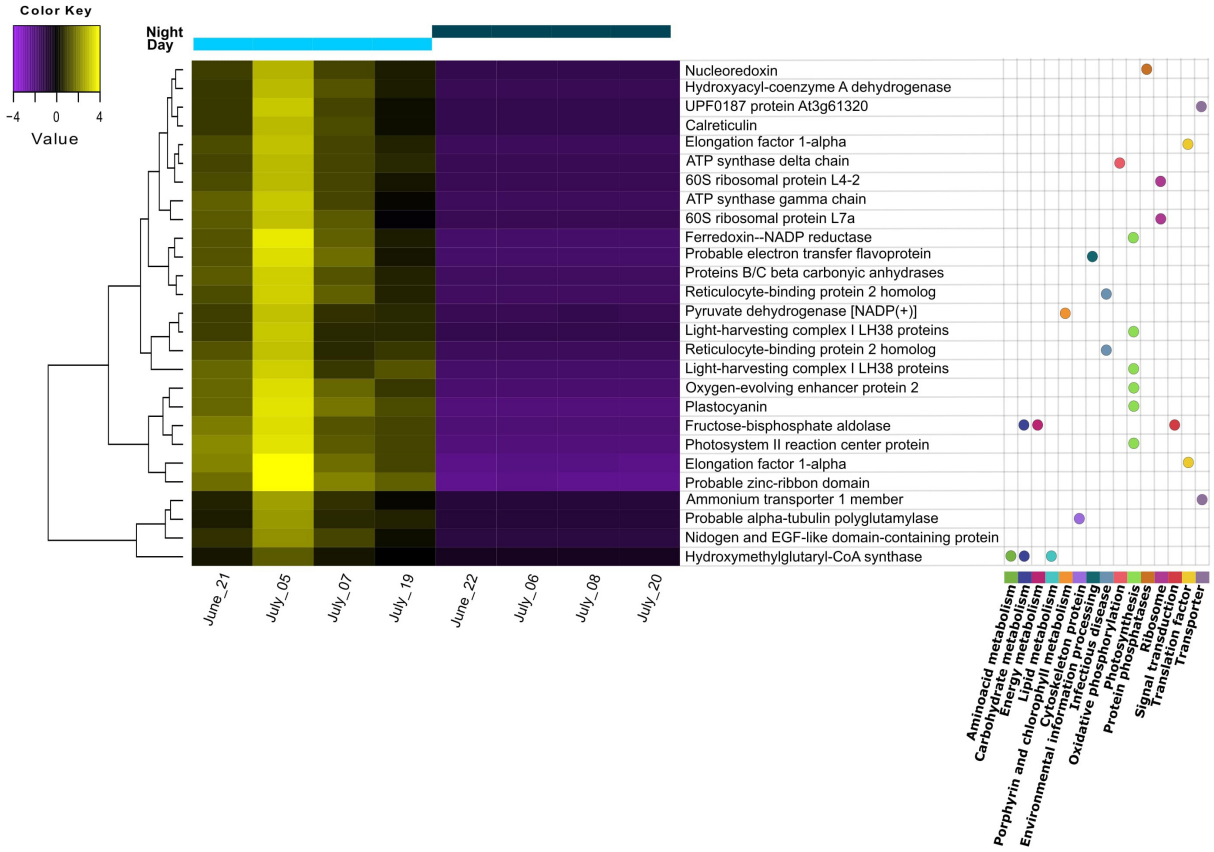

Figure 1.2: Heatmap of differentially expressed transcripts found to be upregulated during the day. - Replicates correspond to different sampling days/nights during summer. Transcripts with annotation are shown with the corresponding pathway from KEGG orthology (KO) assignment. Gene distances were clustered with the Euclidean method. Genes with FDR < 0.01 and at least 2-fold change in expression are shown.

The general gene ontology (GO) assignment for the differentially expressed transcripts (Table 1.2) showed that most of the annotations belonged to parent terms that are expected to be dominant for each of the three GO main categories [cellular component (CC), biological process (BP) and molecular function (MF)], for example, cell and organelle parts including chloroplast, membranes, cytosol, mitochondrion (CC); cellular and metabolic processes like ATP synthesis, photosynthesis, response to cytokinins (BP); catalytic and binding activity like calcium ion-binding, electron transfer activity, GTPase activity (MF), were among the most frequently detected transcripts. From a total of 159 overrepresented ($p < 0.05$) GO terms associated with the DE transcripts, only three GO terms could be identified as enriched (0.05 FDR cutoff). Those were thylakoid (CC), thylakoid membrane (CC), and photosynthetic membrane (CC).

1.5 DISCUSSION

1.5.1 Expression Patterns

We found that most of the differentially expressed transcripts corresponded to photosynthesis-related machinery and processes. Moreover, the only enriched GO terms from the differentially expressed (DE) transcripts were associated with thylakoids and their membranes. Additionally, chloroplasts were among the most abundant GO terms shared between day and night. A high abundance of chloroplast and photosynthesis-related genes has been also reported in previous summer metatranscriptomic studies, with a higher expression around midday (Ji et al., 2018; Linz et al., 2019). Altogether, this shows the daily rhythmicity of photosynthetic organisms and suggests that, according to what we hypothesized, phototrophic microeukaryotes were more responsive to the day/night cycle than the heterotrophic microeukaryotes in the pond. This response was also consistent over the sampling time, as the replicates corresponded to different dates over a month, but would likely be different during winter, with higher expression of adaptation to cold and low-light mechanisms (Edgar et al., 2016). Not surprisingly, no upregulation of photosynthesis-related transcripts was detected under dark conditions. Still, these transcripts were not completely absent during the night (Photosystem I, GO:0009522; photosynthesis light-harvesting, GO:0009765, not shown). This has also been recently reported in other studies, where photosystem I (PSI) gene expression would peak at night, and photosystem II (PSII) would have a peak expression at midday (Davenport et al., 2019; Kolody et al., 2019). These studies indicate that either the expression of the photosynthetic machinery would start at night and peak around midday, or that there would be a constitutive expression for some

genes/isoforms, and others would be only under/overexpressed if stress conditions are present. The latter has been suggested for some antenna proteins of the light-harvesting complex (Stella, 2016).

In the night dataset, we found unique GO terms referring to cytoskeleton structure, ribosome synthesis, and assembly, indicating the importance of these processes for cell growth during nighttime (Raven, 2013). Furthermore, several GO terms involved in the cellular movement were found to be abundant uniquely at night, like dynein, ATP-dependant microtubule motor activity, ciliary basal body, and cilium movement. Dyneins are ATP-driven protein complexes that are responsible for ciliary and flagellar assembly and movement (Pfister et al., 2006), which could indicate either a higher activity of heterotrophic organisms during the night or simply support the occurrence of cell division processes at this time point.

In general, the expression patterns we found are in agreement with previous metatranscriptomic studies showing that during the day, planktonic cells are actively expressing genes used to obtain energy from light and nutrients, for subsequent cellular growth during the night. At night, genes involved in nucleic acid duplication and ribosomes building up will be activated for protein synthesis and cell division processes, to avoid DNA photodamage caused by UV radiation (Poretsky et al., 2009b; Vila-Costa et al., 2013; Broman et al., 2017; Kolody et al., 2019).

1.5.2 Transcripts Taxonomic Assignment

From the taxonomic assignment based on the metatranscriptome, an exclusively marine group (Echinodermata), and also Arthropoda, Chordata, Cnidaria, Mollusca, Mycetozoa, and Platyhelminthes were assigned to our pond community. As we had deliberately excluded most metazoans by pre-filtering our water samples (<30 mm), the occurrence of these taxa was thus unexpected. Nevertheless, it must be taken into account that this taxonomic assignment is based on functional annotations rather than on the commonly used 18S rRNA gene sequences, as the poly-A transcripts selection we used discriminates against ribosomal and prokaryotic RNA. Still, we can infer from these general functional taxonomy data that the annotations belonging to the above-mentioned groups are most probably not exclusive but rather shared functions across taxa, normally biased by the much more comprehensive data available for model organisms (Shakya et al., 2019). The phototrophic fraction was not the most abundant in the pond, as reflected in the metatranscriptomes taxonomical composition. This was also supported by a weakly positive correlation between POC and chlorophyll a ($r2 = 0.5538$), indicating that most of the POC in the pond did not belong to phototrophic organisms. Based on the CHEMTAX

assignment from the pigment analyses, we were able to classify the phototrophic community into the major phytoplankton divisions. It should be noted that this CHEMTAX-based assignment also includes the non-eukaryotic cyanobacteria in the analysis because their marker pigments (echinenone and zeaxanthin) were detected. Excluding this non-eukaryotic contribution to total Chl-a would have been redistributed to the eukaryotic groups, which would have resulted in a biased community composition. Generally, we observed a decrease in most phototrophic groups at night, which could indicate that these groups were grazed by heterotrophs. An alternative explanation is that they were able to perform vertical or horizontal migrations, as it has been shown for several phytoplankton groups that take advantage of surface light and hypolimnetic nutrients through the diurnal cycle (Roenneberg and Mittag, 1996; Shikata et al., 2015). The most abundant phyla in our pond were Chlorophyta and Euglenophyta. The high abundance of Euglenophytes and their differential expression response to the diel cycle suggests that this diverse group of organisms, including mixotrophs, might be the most successful phytoplanktonic group in the pond. This success is perhaps due to their flagella and stigma ("eye spot"), facilitating movement toward best light conditions (phototaxis), but also to their ability to form cysts when environmental conditions deteriorate (Schwartzbach and Shigeoka, 2017).

1.5.3 Environmental Variables

We expected to find diel rhythmicity for the POC and PON content, due to the increasing biomass of primary producers during the day and the nitrogen fixation that typically occurs at night (reviewed in Berman-Frank et al., 2003) but we only found a diel fluctuation on POC. The C:N ratios did not vary significantly between day and night, but the values ($\sim$12) were twice the Redfield ratio (C:N 6.7, Redfield, 1958), which could indicate a moderate nitrogen deficiency in the pond's plankton (Hecky et al., 1993).

Another possible limiting factor for the phototrophic fraction could be the incident light, as direct light was limited by tree shading in the surroundings and floating waterlily (Nymphaea sp.) leaves on part of the pond's surface. Macrophytes such as waterlilies not only limit light for the pond phytoplankton but could additionally be competitors for inorganic nutrients (Bolpagni et al., 2014). The plant material surrounding the pond (allochthonous) explained the high DOC concentrations determined for our pond (11.3 g L$-$1) which are similar to what is found in peatlands (Graeber et al., 2012). Allochthonous DOC is often contributing to the phosphorus input into aquatic systems (Nürnberg and Shaw, 1998). We measured high soluble reactive phosphorus concentrations in the pond ($\sim$15$-$20 mg L$-$1), indicating that phosphorus availability was

not limiting primary production. On the contrary, the high DOC levels from plant origin in the pond could be negatively affecting primary production in the system due to the decreased light incidence, as shown for high DOC-colored lakes (Klug, 2002). Such a simultaneous light and nutrient limitation of the phototrophs would favor the heterotrophic microbial community.

1.5.4 From Functions to the Environment

Our results suggest that the microbial primary producers in the pond were not limited by phosphorus, but rather by nitrogen and/or light availability. The phytoplankton C:N for day and night samples pointed to a deficiency of N in the pond, suggesting that strategies to improve N uptake are needed. One of these strategies is to increase transporter proteins for nutrient uptake, and we can see in our results from DE transcripts and functions that the only identified transporter upregulated during the day is an ammonium transporter. Similarly, in cases were phosphorus is deficient, a general upregulation of different phosphate transporters should be found, as described in previous metatranscriptome studies (Vila-Costa et al., 2013; Zhang et al., 2019), we did not identify this for the pond metatranscriptomes, supporting that P was not limiting in the system.

The phototrophic fraction was the most responsive to high light during midday, which was initially expected as a consequence of a higher phytoplankton abundance during summer with concomitant higher temperature and light availabilities (Schwaderer et al., 2011; Edwards et al., 2016). However, since light availability in the pond was limited not only in time but also in intensity, as a consequence of the high DOC in the water, primary production was reduced. Consequently, the phototrophic fraction was more responsive than the heterotrophic fraction, as they needed acclimation mechanisms in the light-limited system. These mechanisms might involve the enhancement of light-harvesting for energy acquisition and the regulation of stress responses.

Accordingly, we found a high expression of stress response mechanisms like heat-shock proteins (HSP) during the day, indicating cellular stress for the pond's microeukaryotes. HSP are synthesized to protect against protein denaturation when environmental stressors such as high temperature, light, UV, are present (Guo et al., 2015; Sathasivam and Ki, 2019). Apart from light, the temperature was another parameter with pronounced diurnal fluctuations. Considering that light intensity was rather limited, the high expression of HSP might point to temperature stress rather than high light stress. Interestingly, as shown on the heatmap, the highest expression among replicate days for all DE transcripts was observed for July 5th, when the midday

water temperature was the lowest (below 24°C) and PAR values were similar to the other days. We also found a high expression for other stress-related responses: defense response to bacteria and downregulation of apoptosis are GO terms that might indicate defense mechanisms to heterotrophic bacterial infection, although we cannot attribute this stress response to a specific group of organisms in the pond community. An additional indication of stress to temperature and nutrient limitation is the presence of meiosis among the most abundant GO terms during the night, as sexual reproduction in microalgal cells is known to be induced by suboptimal conditions for growth (Borowitzka, 2018, and references therein). The summary of our findings on the pond ecosystem during summer is shown in Figure 1.3.

Overall, for our metatranscriptomics study, we showed that by combining this gene expression approach with other methods such as pigment analyses, and environmental parameters, a better understanding of the system could be obtained. However, the functions identified in our results cannot be attributed to specific organisms, and rather point out to responses from a part of the pond community. To overcome this limitation other approaches such as single-cell transcriptomics should be used, where a higher diversity of the microeukaryotic fraction and specific dynamic interactions can be identified (Ku and Sebé-Pedrós, 2019; Sieracki et al., 2019). We also acknowledge the importance of considering representative time points, as the metabolic processes of a community can change not only at different times of a day but also daily. The results of our study represent the processes carried by a specific fraction of the pond community during the summer, and should therefore not be extrapolated to other seasons where the observed daily expression patterns are likely to differ. To address further questions on the small pond ecosystem in our study, preferably even more parameters and variables should be considered to fully explain the observed expression patterns. This might ultimately help to link the metabolic processes to specific taxonomic groups and give a broader view of the different trophic levels in the pond. We are aware of the importance of, for example, the measurement of particulate phosphorus as it is essential for the calculation of the C:N:P stoichiometry from primary producers and to unveil their specific nutrient requirements and limitations. Also, photosynthesis rates and photoinhibition would have been interesting to determine primary productivity and light/temperature stress responses in the pond, respectively. Likewise, considering the identification of other size fractions would contribute to better understand the trophic interactions in the pond community.

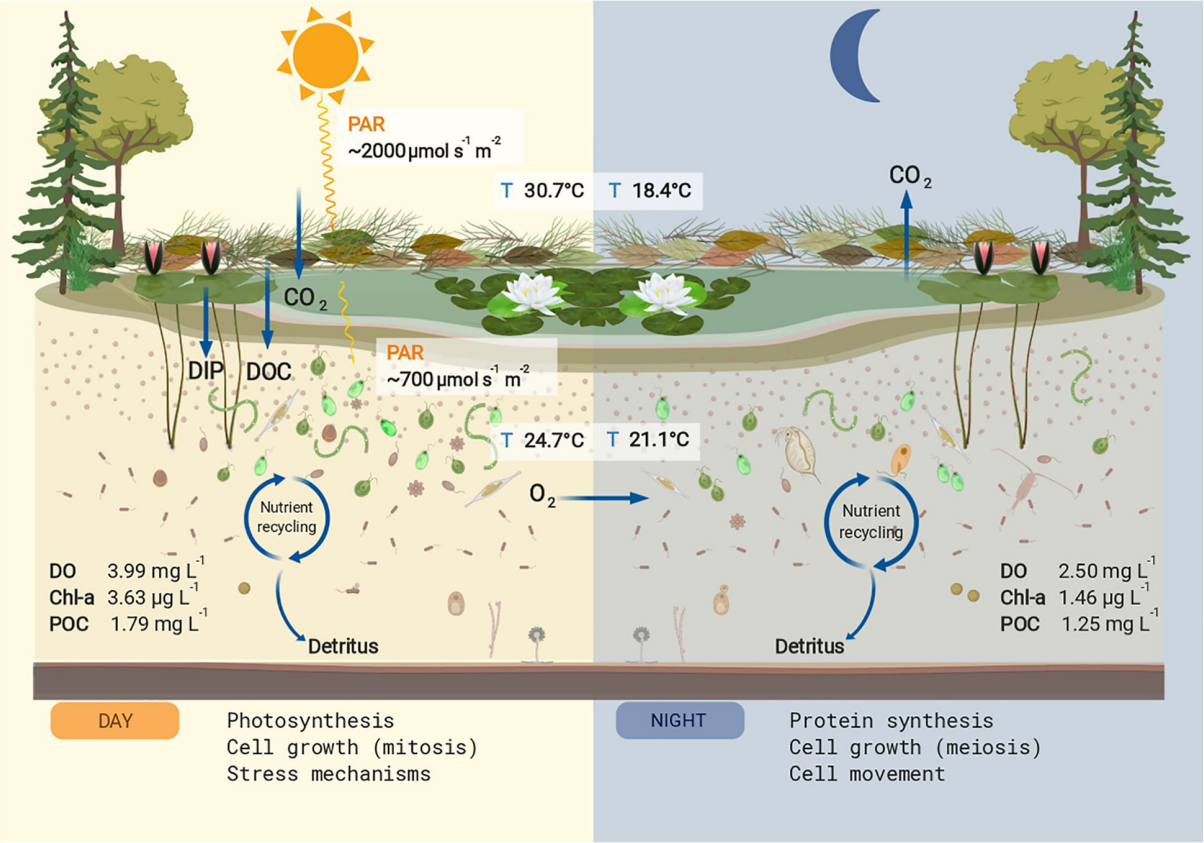

Figure 1.3: Conceptual summary of the studied pond - The mean values for physicochemical parameters that differed significantly between day and night are shown. Nutrient input for dissolved organic carbon (DOC) and dissolved inorganic phosphorus (DIP) are shown in the left panel. During the day the main cellular process upregulated in the microeukaryotic community was photosynthesis, increasing particulate organic carbon (POC), chlorophyll a (Chl a), and dissolved oxygen (DO). The produced and stored energy is used in part for cell growth and stress mechanisms, also upregulated processes during the day. During the night, the oxygen produced via photosynthesis is used for respiration, decreasing the DO concentrations. The main cellular process upregulated at night was protein synthesis, a high energy-consuming task, as well as cell growth and cell movement. The POC and Chl a values decreased at night presumably by grazing effect. This figure was created with BioRender.

1.6 CONCLUSION

Our study revealed that the phototrophic pond microeukaryotes were the less abundant but the most responsive fraction to diurnal fluctuations. This was indicated by the strong phototrophic contribution to the differentially expressed transcripts, the most abundant gene ontology terms shared between day and night samples, and the GO terms found to be unique for the daytime samples. In all cases, photosynthesis-related annotations/processes were prevailing. Furthermore, the expression patterns observed for day and night samples were consistent over the sampling period and were confirmed to be similar to some previous studies in larger aquatic ecosystems, remarking that the diurnal cycles affect the pond community in the same way.
Moreover, combining the metatranscriptomics with pigment analyses allowed us, together with the environmental data, to identify the phototrophic composition on the whole microeukaryotic community and to better understand the main metabolic processes in response to limiting factors for primary production in a small aquatic ecosystem.

Chapter 2

Eukaryotic microbial communities and functions under extreme conditions - a metatranscriptomic approach in a high altitude hypersaline wetland (Salar de Huasco, Chile)

2.1 Abstract

High altitude wetlands are considered extreme ecosystems for life, exposed to high solar radiation, extreme diurnal temperature variations, and high evaporation rates that lead to a broad range of salinities. To understand the mechanisms evolved by aquatic microorganisms to overcome such conditions, functional approaches are necessary. We here used metatranscriptomics to study the aquatic microeukaryotic communities of Salar de Huasco, a high altitude wetland in the north of Chile. We investigated how these communities are affected by multiple environmental stressors by comparing gene expression patterns. Three sampling sites were selected along a freshwater stream with increasing salinity and under different solar irradiance levels during the wet (summer) and dry (winter) season. Heterotrophic taxa (mainly Ascomycota and Evosea) were the most abundant for both sampled seasons, while phototrophic taxa (dominated by Chlorophyta and Bacillariophyta) were less abundant but more variable between subsites. The general expression patterns for samples under lower solar irradiance were similar during the wet and dry seasons. The main environmental factors found to structure the active microeukaryotic communities were pH, conductivity, and salinity in winter, temperature and PAR in summer. The most enriched biological processes from the total gene pool, as by gene ontology analyses, were related to the mitotic cell cycle, photosynthesis, and stress responses.

2.2 INTRODUCTION

Ecosystems with variable low and high environmental conditions, such as temperature, salinity, and solar radiation, among others, are considered extreme habitats (Rothschild & Mancinelli 2001). This definition refers to hostile conditions for life as we know it from an anthropocentric view (Weber et al. 2007, Dong 2008). However, the organisms adapted to extreme environmental conditions (extremophiles) are mainly unicellular and thrive under such conditions (Horikoshi et al. 2011, Seckbach et al., 2014). Thus, studying extreme ecosystems is a valuable tool to establish the limits of life on earth and understand the evolutionary adaptation of many organisms, which could provide insights into future climate scenarios (Rampelotto 2013).

Microbial eukaryotes play essential roles in aquatic systems as primary producers, consumers, and decomposers. They have been found in many extreme ecosystems previously thought to be prokaryote-dominated (Parris et al. 2014, Wang et al. 2014, Kammerlander et al., 2015). Additionally, their diversity estimations have increased significantly in the last years through massive sequencing (Massana et al. 2014, Hu et al. 2015, Debroas et al. 2017, Machado et al., 2019). Although microorganisms' physiological mechanisms to cope with extreme conditions are not yet well understood, and the effect of multiple environmental stressors in a given community is hard to evaluate, previous studies evaluated individual stressors on particular organisms via their metabolic responses. Therefore, we know that solar radiation, and especially UV radiation, cause photoinhibition, production of reactive oxygen species, an increase of pigments production, DNA damage, and alteration of protein synthesis (Häder et al. 2015, Allorent et al. 2016, Puente-Sánchez et al. 2016). An increase in salinity, in turn, may develop tolerance and adaptation mechanisms of cells. These include the upregulation of membrane transporter proteins, accumulation of osmoregulatory solutes, and lipids, amongst some of the described microalgal cell responses (Shetty et al., 2019).

The high altitude wetlands in the Andes region are considered poly-extreme ecosystems (Farias 2020). Not only are they exposed to one of the highest solar irradiance levels in the world ($>$ 1000 W m^{-2}, Cordero et al. 2014, 2016), but they also experience large diurnal changes in temperature and relative humidity. Their deficient yearly precipitation and high evaporation rates, together with the geogenic salts commonly found in the Altiplano (high altitude plateau), lead to wide salinity ranges (Risacher et al. 2003, Risacher & Fritz 2009), which is why these saline basins are locally known as salares. Water availability in these systems is associated with the wet (summer) and dry (winter) seasons, which are regulated by El Niño Southern Oscillation

(ENSO) phenomenon, with more than 50% of annual rainfall during summer (Vuille et al. 2000). The cyclic precipitation events affect the water levels and quality, being crucial for the biodiversity in the Altiplano aquatic systems (Dorador et al. 2003, Márquez-García et al. 2009). One of these systems is Salar de Huasco, a high altitude (3800 m a.s.l.) wetland located in the north of Chile and protected by the international Ramsar convention (Ramsar 2018). It is composed of some freshwater sources, a main shallow saline lagoon, and various surrounding evaporitic ponds, constituting a heterogeneous system with a strong environmental variation in small-scale distances (Risacher & Fritz 2009, Aguilar et al. 2016, Hernández et al. 2016). The extreme daily variation in temperature and solar radiation in Salar de Huasco differ, as precipitations, seasonally (Dorador 2007, Hernández et al. 2016, Molina et al. 2016). During the rainy summer (known as Altiplano winter, Aceituno 1997), the highest solar irradiance and evaporation rates are observed (Risacher et al. 2003, de la Fuente & Meruane 2017), translating into short-term variations in salinity. During winter, i.e., the dry season, the opposite is observed, with lower precipitation rates, solar irradiance, and evaporation. This spatial and seasonal environmental variability shapes the microbial communities in Salar de Huasco and influences their adaptation and resistance to such extreme conditions.

To date, a series of studies in Salar de Huasco have revealed the high diversity of specific bacterial and archaeal groups (Dorador, Busekow, et al. 2008, Dorador, Vila, et al. 2008, Dorador et al. 2010, 2013), and how some of these prokaryotic organisms can overcome the extreme conditions of the system, like UV radiation (Hernández et al. 2016, Molina et al. 2016, Pérez et al. 2017, 2018), and arsenic tolerance (Castro-Severyn et al. 2020). However, although microalgal groups have been reported in different sites of the salar (Hernández et al. 2016), there are no previous studies extensively addressing the diversity and contribution of the complete eukaryotic microbial component in Salar de Huasco.

We here used a metatranscriptomic approach to explore the active microeukaryotic communities of Salar de Huasco. We focused on the structure and functional role of these communities along a freshwater stream with increasing salinity (space) and under different solar irradiance levels during the wet and dry season (time) to understand how these organisms would be affected by the rapid changes in their environment. Despite the limited background knowledge on the microbial eukaryotes of Salar de Huasco, we made several basic assumptions on these communities in the system: (i) The abundance of active organisms should decrease along the salinity gradient. (ii) During the dry season, microeukaryotic communities should be more structured due to fewer short-term environmental disturbances than those found during the wet season. (iii)

Similar expression patterns should be expected for samples taken under similar solar irradiance levels. (iv) The highest photoinhibition is likely to occur under the highest solar irradiance levels and be higher in the freshwater source than the stream's downstream sections.

2.3 MATERIALS AND METHODS

2.3.1 Study site

Salar de Huasco is located at 3800 m a.s.l. from 20° 18'S to 68° 50' W in the Altiplano region in Northern Chile. Several stations in the wetland (H0 – H8) are described by Dorador (2007), of which site H3 was chosen for our study. Site H3, called "El ermitaño" spring, is a groundwater source emerging from an aquifer and running as a shallow stream towards a saline lagoon that starts approximately 200 m downstream with an estimated mean water flow of 23 L/s (DGA, 2010). This spring is the primary source of water for the lagoon. Three subsites were chosen to take water samples: one at the spring where the freshwater reaches the surface (H3-1), one at the beginning of the hypersaline lagoon (H3-3), and one in between (H3-2).

2.3.2 Sampling and processing

The summer sampling was performed on January 22nd, 2017, and the winter sampling was performed on August 9th, 2018. The summer sampling was conducted at 9 am when the solar irradiance was 829 W m^{-2} with a foggy sky and during the solar noon, 12 pm, with a clear sky and an irradiance of 1181 W m^{-2}. Due to weather conditions, winter sampling was only possible at solar noon with a clear sky and solar radiation of 941 W m^{-2}. At each sampling point, the following physicochemical parameters were determined in situ with a multiparameter instrument (3401, WTW): temperature, pH, conductivity, salinity, total dissolved solids (TDS), and dissolved oxygen (DO). Parameters such as solar irradiance, wind velocity and direction, precipitation, atmospheric pressure, and relative humidity were obtained from the monitoring station at Salar de Huasco (http://www.ceazamet.cl/). The summer sampling was done during the rainiest month that year, accumulating 35.9 mm of precipitations. For the winter sampling, the total monthly precipitation was only 2.6 mm. No rainfalls were recorded on sampling days and at least 3-4 days before sampling.

Water samples for RNA sequencing were taken in triplicates at each sampling point. Water samples for nutrient determination were collected in 1 L bottles, with duplicates in each subsite.

Samples were kept cold until spectrophotometrical analysis according to the APHA recommendations (2015).
Upon sampling, the water collected for RNA extraction was pumped through Sterivex^{TM} cartridges (0.2 µm) via a peristaltic pump with a sterile tubing, and RNAlater (Qiagen) was added to the cartridges for the preservation of the samples before storage at -80°C until RNA extraction.

2.3.3 RNA extraction and sequencing

The processing of Sterivex^{TM} cartridges described by Cruaud et al. (2017) was used after thawing them on ice. The membrane filters were cut into strips to fit on sterile screw-cap 2 mL tubes (CK14 tubes). RNA extraction was done with the fibrous tissue protocol from ReliaPrep kit (Promega), including a DNase treatment. The manufacturer's protocol specifications were applied, using the lower amounts of reagents plus an initial mechanical disruption using 1.4 mm ceramic (zirconium) beads. The final elution volume was 20 µl. The total quality of RNA samples (A260/A280 and A260/A230 ratios) were assessed with a Nanodrop photometer, and concentrations were measured using the Qubit^{TM} fluorimeter's RNA HS Assay Kit (Invitrogen). We obtained RNA from each sampling site in triplicates, except for summer morning samples, where some replicates had no measurable RNA: subsite 1 had two replicates, and subsite 3 had only one replicate. RNA integrity (RIN) of all samples was ∼7, as checked with a Bioanalyzer (Agilent Technologies). The mRNA was selected by its poly-A sequences before cDNA libraries were prepared using the TruSeq® stranded kit. Finally, 75 bp paired-end sequencing was performed at the Cologne Centre for Genomics using the Illumina HiSeq 4000 platform.

2.3.4 Metatranscriptome analyses

Quality trimming of the raw sequences (LEADING:5 TRAILING:5 MINLEN:70, Phred >30) was done with Trimmomatic 0.36 (Bolger et al., 2014), and the rRNA sequences were removed with Sortmerna 2.1 (Kopylova et al., 2012). All sequences, per sampling point, were used to perform de novo assemblies with Trinity version 2.5.1 (Haas et al., 2013). Raw sequences from individual replicates were aligned to the reference assemblies using Bowtie2. Subsequently, transcripts abundances were estimated with RSEM (Li & Dewey 2014), and gene and isoform count matrices were created. These matrices were used to check between replicates and sample correlation and to perform differential expression analyses. Differential expression analyses were done on the transcript level with the edgeR package (Robinson et al. 2010) and TMM normalization method (Robinson & Oshlack 2010), using the default adjusted p-value cutoff

of 0.001 for false discovery rate (FDR, Benjamini & Hochberg 1995). The sequences from the complete assemblies and the differentially expressed transcripts were annotated following Trinotate release v3.2.1 (Bryant et al. 2017). This pipeline included predicting coding regions on transcripts (TransDecoder, http://transdecoder.github.io) and used Blastx and Blastp against the Swissprot database to get annotations from both transcripts and predicted proteins. For protein domain prediction, HMMR is used with the PFAM database, and for the clusters of orthologous groups (COG) eggnog is used. Taxonomical assignments and gene ontologies were assigned from these annotations, abundance is referred to transcript abundance. Gene Ontology enrichment analysis for differentially expressed transcripts was performed using the R package GOseq (Young et al. 2010), the significance for GO terms ($p < 0.05$) was calculated after random resampling to generate a null distribution for each category amongst the over-represented DE transcripts, in order to find the over enriched GO category at FDR < 0.01. The top 20 enriched GO terms (with the lowest p-value at FDR < 0.01) from the category' biological processes' were selected and an enrichment factor was calculated as the ratio of the number of enriched DE genes to the total number of annotated genes (per sample) in the category, where a more significant enrichment is observed at a higher value. The generic and uninformative terms were excluded from the top 20 list (i.e., biological process, cellular process, metabolic process, and regulation of cellular metabolism). Also, redundant terms were excluded, keeping a representative term (REVIGO). Canonical correspondence analyses (CCA) were done with the Vegan package (version 2.5-6, Oksanen et al. 2018), using the taxa abundances and the main physicochemical parameters of each subsite for summer and winter sampling, a permutation test was performed to find the significant variables for the model ($p < 0.05$ under 999 permutations). Gene ontology and CCA analyses were done using the R version 3.6.1 (Team 2016).

2.4 RESULTS

2.4.1 Environmental parameters

The water temperature differed significantly ($p < 0.05$) between summer morning (13.7 ± 0.44) and summer noon (19.3 ± 1.93) sampling, and between summer morning and winter noon (16.2 ± 0.81, $p < 0.01$) sampling. The same was observed for the pH values, where summer morning (6.55 ± 0.39) differed significantly from summer noon (8.04 ± 0.34, $p < 0.05$) and winter noon (8.14 ± 0.51, $p < 0.05$) samplings. However, the values between summer noon and winter noon sampling did not differ significantly. Nonetheless, an increase in pH, conductivity, and salinity was observed along the stream for all sampling times, and an increase in temperature was observed along the stream during summer sampling at noon (Table 2.1). In winter sampling, an increase of dissolved oxygen (DO) was observed along the salinity gradient, and an increasing concentration of ammonium, phosphate, sulfate, and chloride was also found towards the most saline subsite. For sodium, potassium, calcium, silica, and nitrite, a decline was found in the concentrations in subsite H3-2 with respect to both subsite H3-1 and H3-3.

Table 2.1: Environmental parameters measured in site H3 from Salar de Huasco.

† Data obtained from CEAZAmet monitoring station, ∗ Data from summer season published by Molina et al, 2018 and Eissler et al, 2019.

Sampling time/season	Site	Air temperature [°C]	Water temperature [°C]	pH	Conductivity [µS cm^{-1}]	Salinity (PSU)	DO [mg L^{-1}]	Solar irradiance [W m^{-2}] †	Wind velocity and direction [m s^{-1}]†	Atmospheric pressure [hPa]†	Relative humidity [%]†	Monthly Precipitation [mm] †
Summer morning		7.9							1.0 – 3.2, SE	649.7	81.2	35.9
	H3-1		14.2	6.3	765	0.4	4.62*	829				
	H3-2		13.4	6.4	613	0.3	-					
	H3-3		13.5	7.0	26800*	23.7*	4.7*					
Summer noon		16.3							6.8 – 8, SW	648.4	40.9	35.9
	H3-1		17.9	7.9	565	0.3	4.62*	1181				
	H3-2		18.5	7.8	587	0.3	-					
	H3-3		21.5	8.4	26800*	23.7*	4.7*					
Winter noon		8.2							1.2 – 2.4, S – SW	651.2	27.4	2.6
	H3-1		15.3	7.6	580	0.2	5	941				
	H3-2		16.8	8.5	12600	7.2	6.9					
	H3-3		16.6	8.4	38600	24.3	7.1					

2.4.2 Taxonomic assignment from functional annotation

The relative abundance of functionally active taxa present at each sampling site in both sampling seasons showed that the entire microeukaryotic community was dominated by heterotrophs (Figure 2.1). The most abundant groups were Ascomycota and Evosea (mainly "slime molds"). All heterotrophic groups had similar abundances for summer and winter samples, except for Choanozoa, which was more abundant during winter than in summer at subsites H3-2 and H3-3. Among autotrophs (including mixotrophs), the most abundant groups were Chlorophyta and Bacillariophyta. During summer, Bacillariophyta increased along the stream, while Chlorophyta decreased. In winter, the opposite pattern was observed. Euglenophyta was detected in all summer samples, but not in winter samples. On the contrary, Dinophyta were mostly present at subsites H3-2 and H3-3 in winter samples and almost no detectable in summer samples, as also seen on the canonical correspondence analysis (Figure 2.2). Haptophyta were present in all samples with increasing abundances along the stream, and Ochrophyta (Raphidophyceae, Xanthophyceae) decreased in abundance along the stream's course both in summer and winter. The groups with the lowest abundances (less than 0.05% relative abundance in all subsites) were Bigyra, Foraminifera, Tubulinea, and Zoopagomycota, among the heterotrophic groups, and Rhodophyta among the autotrophic groups. The Cercozoa phylum was composed of both autotrophic (Chlorarachniophyceae) and heterotrophic (Imbricatea) taxa at low abundances.

The taxonomic assignment of the differentially expressed genes (Figure 2.5) was also dominated by Ascomycota and Evosea in all subsites and seasons, except in site H3-1 during summer, where Chlorophyta were the most abundant. The relative abundance of the taxa overexpressing genes in response to the salinity gradient (Figure 2.5 A) was similar to the general taxonomic assignment (Figure 2.1). However, the differentially expressed genes in the same site (different sampling times) showed a different taxonomic assignment pattern (Figure 2.5 B). For site H3-1, summer samples were dominated by Chlorophyta and winter samples by Ascomycota. In site H3-3, there was a higher relative abundance of Bacillariophyta, Ochrophyta, and Haptophyta during winter sampling than summer. Furthermore, summer samples (morning and noon) had a higher relative abundance of Euglenophyta, Dinophyta, and Choanozoa overexpressing genes with respect to winter.

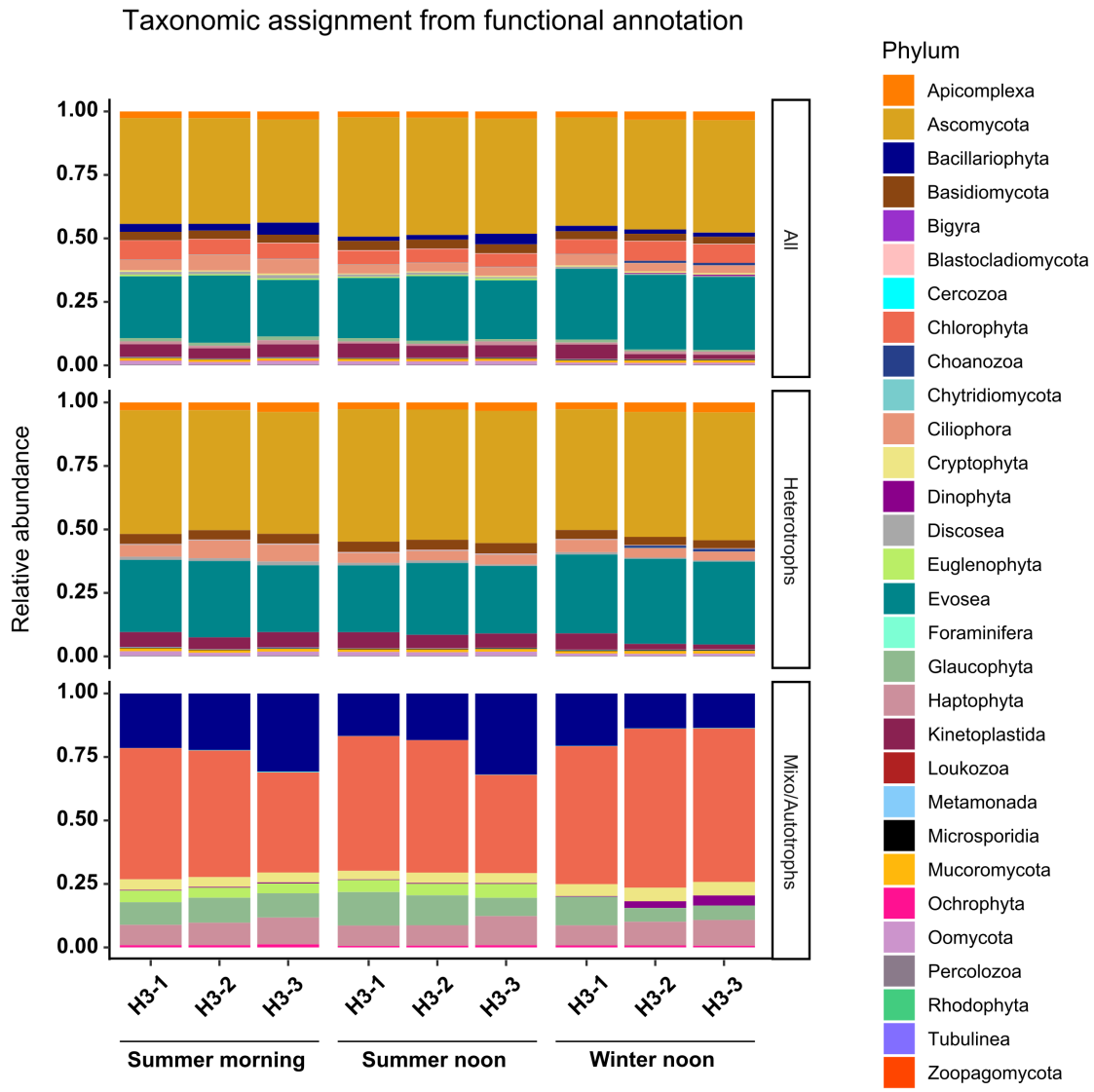

Figure 2.1: Relative taxonomic composition from RNA-Seq.
Functional annotation of the complete set of microeukaryotic transcripts in all sampling sites for summer and winter samples (upper panel), heterotrophic groups only, and mixo/autotrophic groups.

2.4.3 Correlation analysis of environmental variables and taxa

When comparing summer and winter samples using the taxa abundances and the basic physicochemical parameters with a canonical correspondence analysis (CCA, Figure 2.2), we observed how summer and winter samples clustered separately. All environmental variables displayed in the triplot were significant (pH, temperature, salinity, and conductivity, $p < 0.01$; PAR, $p < 0.05$). These constrained variables explained 91% of the dataset's total variance, with CCA1 explaining 60% and CCA2 explaining 18% of the variance. The first axis separated along with pH, conductivity, and salinity, which appeared as the main drivers for the winter microeukaryotic community at subsites H3-2 and H3-3. In these subsites, we observed the low-abundance phyla Tubulinea, Rhodophyta, and Cercozoa, and is where the Dinophyta and Choanozoa groups had their highest abundances when compared to the other sites. The second axis separated along with temperature and PAR, primary drivers of the summer community, grouping in the bottom panel all morning samples (the lowest temperature and PAR values of all samples) and also the most saline noon summer site (H3-3), all having the highest abundances of the phyla Haptophyta, Bacillariophyta, Ochrophyta, and Euglenophyta. The noon subsite H3-1 (summer and winter) and noon subsite H3-2 (summer) are grouped with the lowest conductivity and salinity values and high temperature and PAR in the upper panel. The low-abundance phyla Foraminifera was only found in subsite H3-1 during winter noon, and Bigyra had their highest abundances at subsites H3-1 and H3-2 during summer. In summer, subsites H3-1 and H3-2 appeared more close to each other than to subsite H3-3. In winter, subsite H3-1 clustered apart from subsites H3-2 and H3-3, mostly due to their conductivity and salinity.

2.4.4 Metatranscriptomes summary

The RNA sequencing yielded $\sim$ 20 M reads per sample, the replicates were assembled, and between 100,000 and 600,000 transcripts were obtained from each assembly. A small percentage of bacterial sequences were found ($\sim$10%), and a high percentage of metazoans ($\sim$30%) were discarded for the present analyses. Between 260 and 20,416 transcripts were found as differentially expressed and classified as microeukaryotic sequences. The classification of the annotated microeukaryotic transcripts into the COG categories showed that its abundance decreased along the stream, having the least abundant annotations in site H3-3, regardless of the season (Figure 2.3). The most abundant category in all samples was "Translation, ribosomal structure, and biogenesis", followed by "Posttranslational modification, protein turnover, chaperones", and

"Signal transduction mechanisms". The rest of the categories varied in abundance for the different sampling times or seasons.

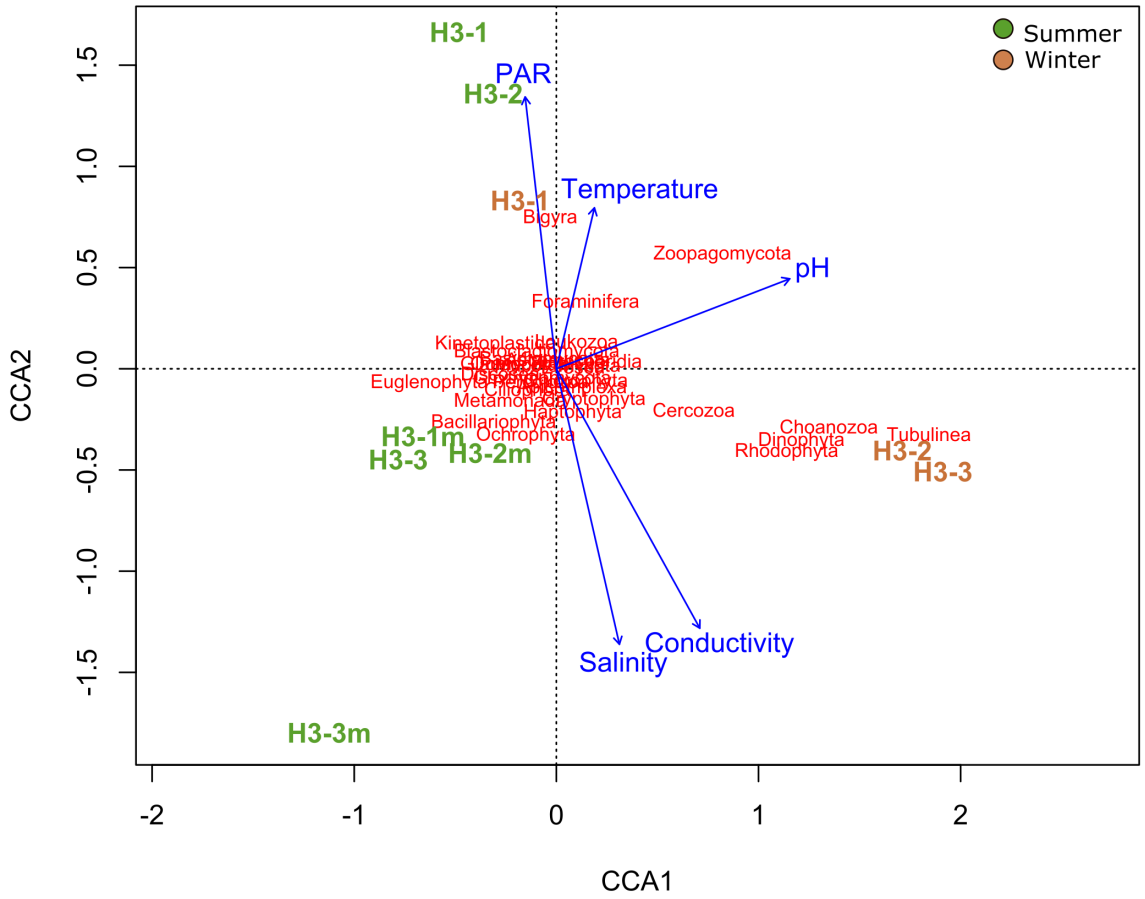

Figure 2.2: Canonical correspondence analysis (CCA) - triplot using environmental parameters and taxonomic assignment from annotations at the phylum level for summer and winter samples taken at morning (m) and noon. The arrows correspond to significant environmental variables that explain the variance of the microeukaryotic community for both seasons. The first two axes accounted for 60% ($p < 0.01$) and 18% ($p < 0.05$) of the explained variance, respectively.

2.4.5 Gene expression analyses

Differential expression analyses were performed in two ways: grouping by sampling time and grouping by site. In the first approach, all summer morning samples (low solar irradiance) were compared with one another, and the same was done with all summer noon samples (high solar irradiance) and all winter noon samples (low solar irradiance) to see changes along the salinity gradient. In the second approach, all samples from the same site were compared with one another, having three different sampling times (summer morning, summer noon, and winter noon) in each site, to see the changes in expression under different solar irradiance.

With the first approach, we found 418 transcripts with annotations to be differentially expressed for summer morning samples, with the highest amount found in the source water H3-1 in contrast to subsite H3-2. In the case of summer samples taken at noon, 260 transcripts with annotations were differentially expressed, with most of them present at subsite H3-3 in contrast to subsite H3-2. For winter (only sampled at noon), we found 20416 transcripts with annotations to be differentially expressed and distributed in all three subsites. When looking at the gene expression's overall clustering, we found subsites H3-2 and H3-3 clustered together for summer morning and winter noon samples. For summer noon samples, subsites H3-1 and H3-2 clustered together, leaving H3-3 as the most dissimilar (Figure 2.4). With the second approach, we found 491 differentially expressed transcripts with annotations in subsite H3-1, with the highest amount found in winter samples than summer morning samples. For H3-2, we found 1997 transcripts to be differentially expressed, with most of them in the summer morning and summer noon samples compared to winter noon samples. For the site H3-3, 14954 transcripts were differentially expressed, with most of them found in summer morning and summer noon compared to winter noon samples. The overall gene expression clustering showed that all summer samples clustered together and apart from winter samples.

When we performed gene ontology enrichment analyses on the sets of differentially expressed genes and their annotations, we only found enriched GO terms in DEGs from a few site/time/season combinations for the first approach (by time/condition). For the second approach (by site), the gene ontology enrichment analyses on the sets of differentially expressed genes could not be completed. Only site H3-1 during summer morning and winter noon, and site H3-3 during summer noon and winter noon presented enriched GO terms. Since we were

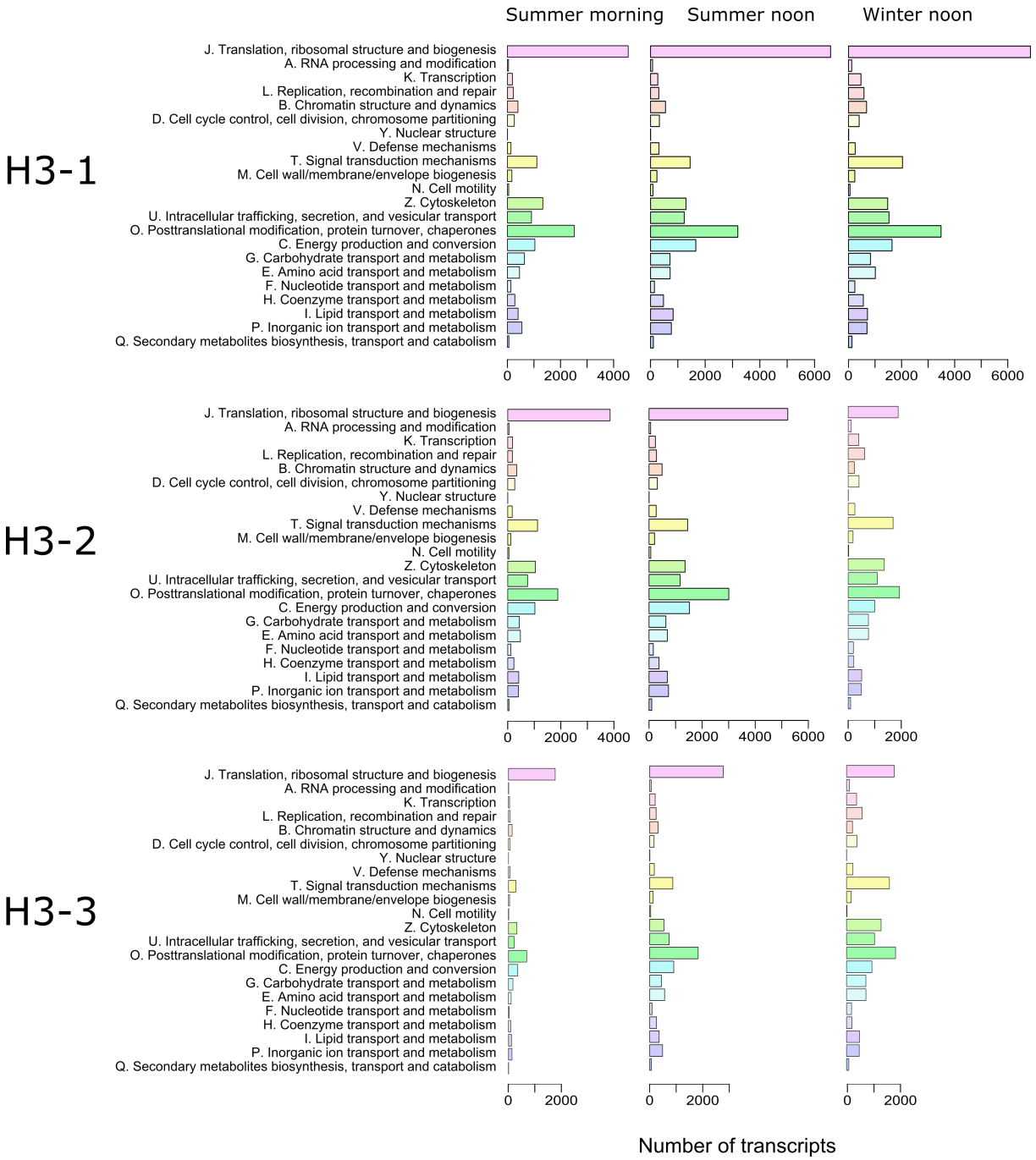

Figure 2.3: Categories of orthologous groups from the eggNOG database - The total amount of microeukaryotic transcripts with annotations are shown.

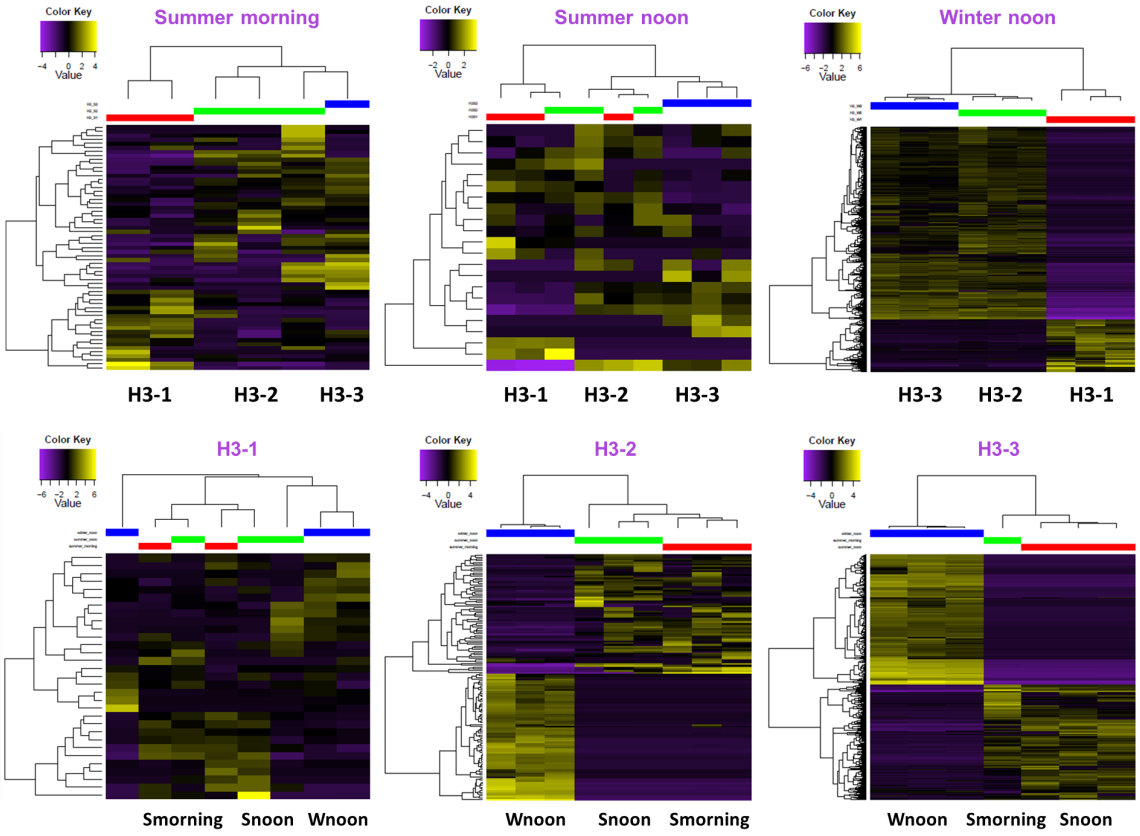

Figure 2.4: Heatmaps of differentially expressed transcripts - Along the salinity gradient (up) and at different sampling times (down) for all summer and winter samples. Only genes with microeukaryotic annotation and at least a 2-fold change in expression are shown (false discovery rate (FDR) < 0.01).

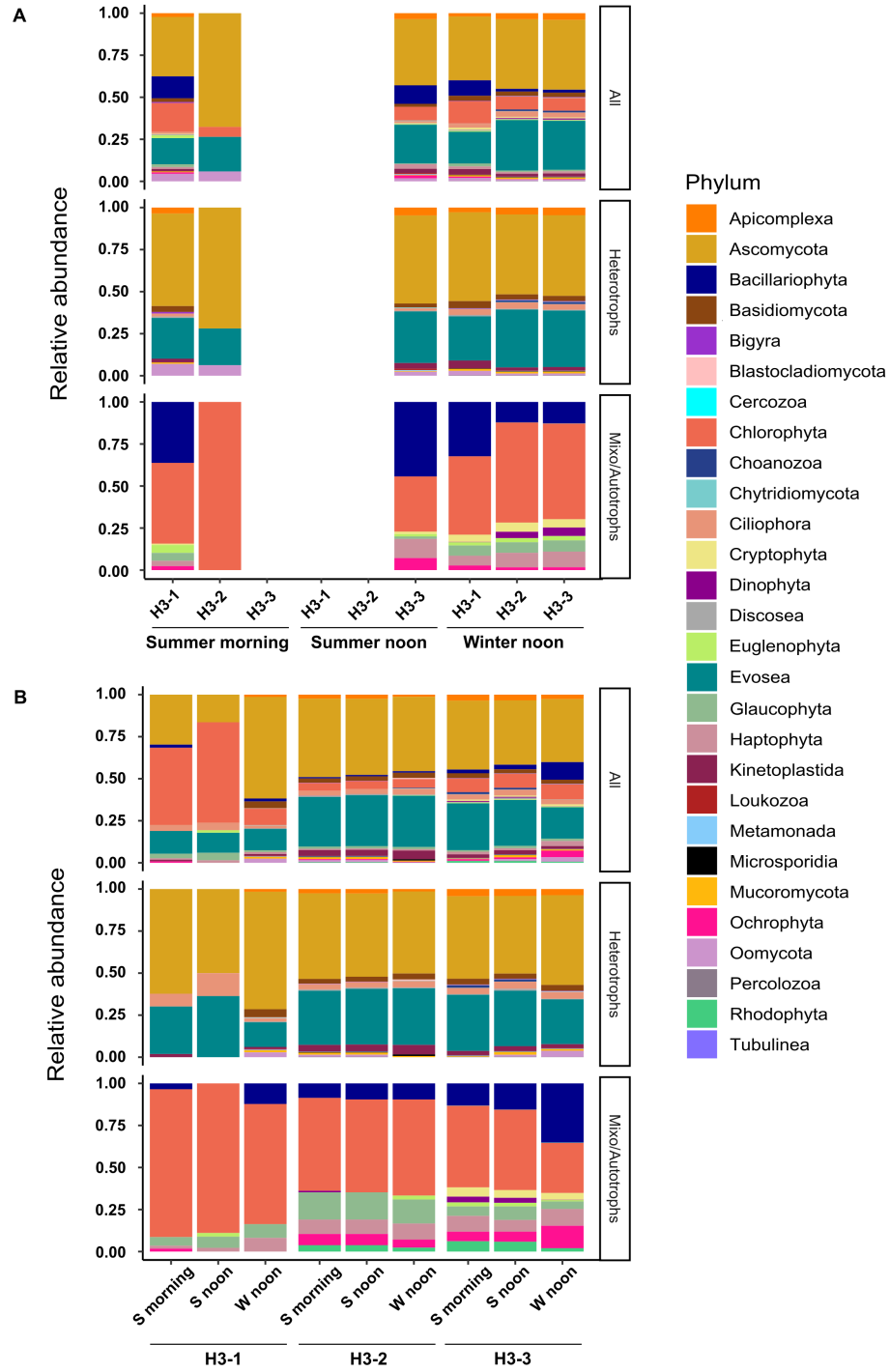

Figure 2.5: Relative taxonomic composition from differentially expressed transcripts Along the salinity gradient (**A**), comparing the different sites sampled simultaneously, and (**B**) under different solar irradiance, comparing the same sites at a different sampling time. No differentially expressed transcripts were found for the sites where bars are missing.

interested in the overall metabolic functions performed by the microeukaryotic communities and their differences across sampling time and season, we here report the enriched GO terms from one of the three main categories, "Biological Process". We subsequently created two lists with the most significant and most enriched results (Figure 2.6).

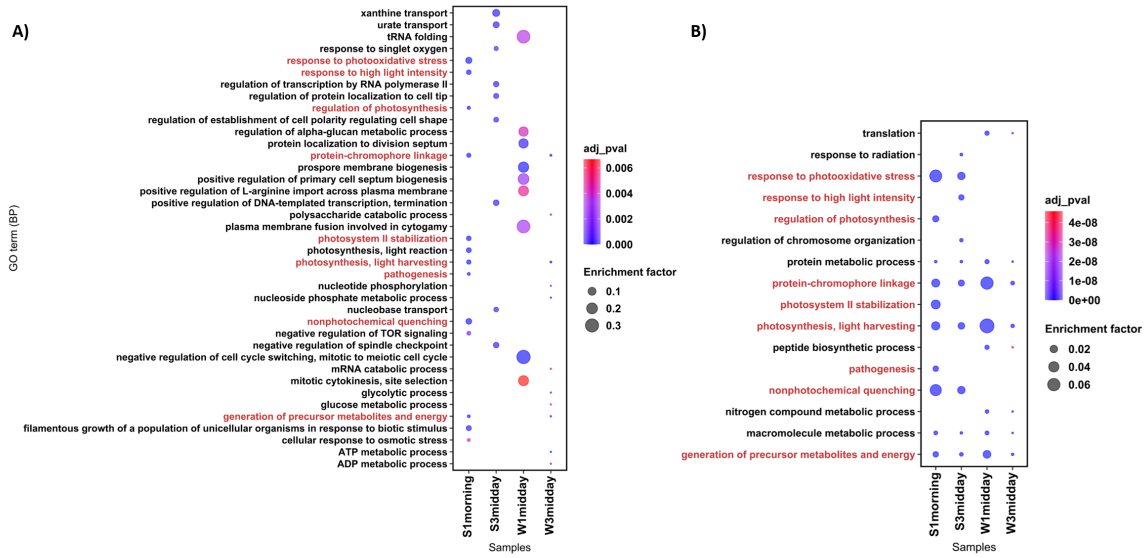

Figure 2.6: Dot plot of enriched gene ontology (GO) terms - (with p-value and adjusted p-value (FDR) < 0.01) from the differentially expressed genes (DEG) of summer (S) and winter (W) samples. Showing a reduced list of the top highest enriched GO terms (**A**) and top significant enriched GO terms (**B**). In both cases, only the category Biological Process (BP) was considered. The terms present in both, most enriched and most significant, are highlighted in red.

Amongst the top significant enriched GO terms (p-value and FDR < 0.001, Figure 2.6b), we found for source water during summer morning (S1 morning) terms mainly related to response to photooxidative stress, regulation of photosynthesis, protein-chromophore linkage, photosystem II stabilization, light-harvesting, non-photochemical quenching, and pathogenesis. For site H3-3 during summer noon (S3 noon), the top significant terms were related to response to high light intensity, response to oxidative stress, protein-chromophore linkage, light-harvesting, non-photochemical quenching, and regulation of chromosome organization. For winter noon site H3-1 (W1 noon), protein-chromophore linkage, light-harvesting, generation of precursor metabolites and energy, nitrogen compound metabolic process, and translation were among the represented

c)

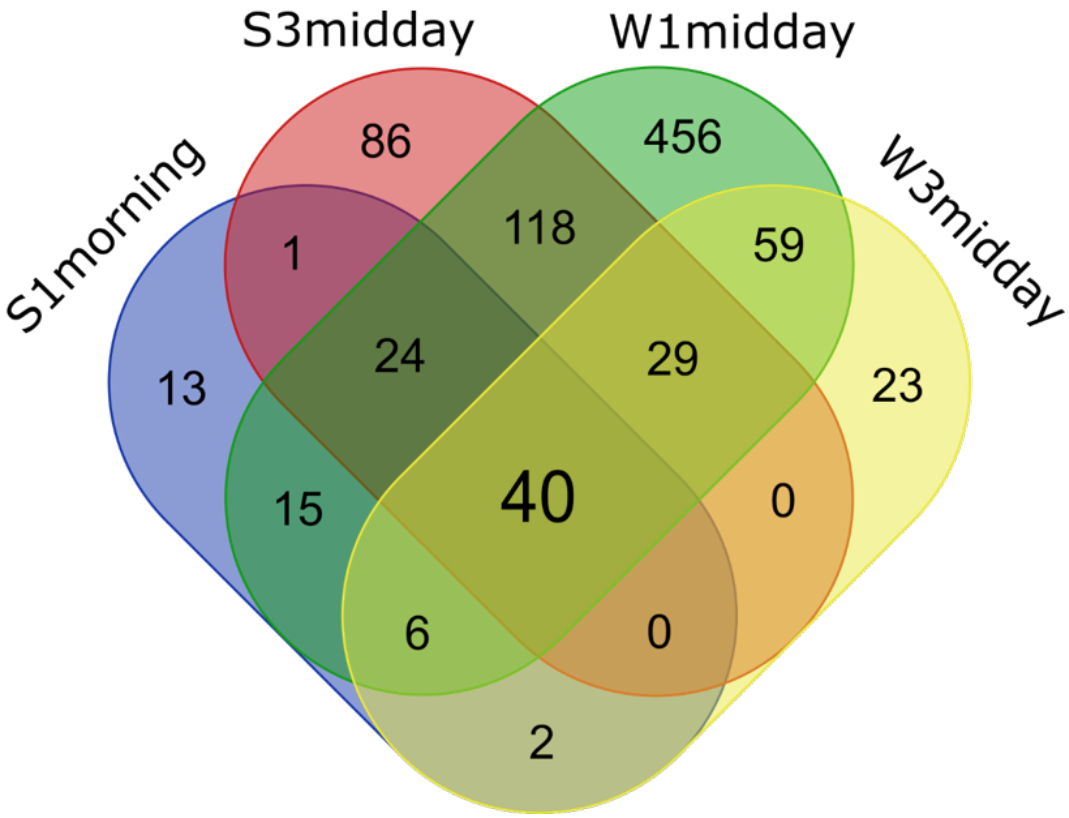

Figure 2.7: Venn diagram showing the shared GO terms - resulting from the enrichment analysis comparing samples along the salinity gradient. The total numbers of GO terms were 102, 298, 747, and 159 for summer subsite H3-1 (S1 morning), summer subsite H3-3 (S3 noon), winter subsite H3-1 (W1 noon), and winter subsite H3-3 (W3 noon), respectively. Only 40 terms were shared among all samples.

biological processes. For site H3-3 during winter noon (W3 noon), we found the same terms as in site H3-1, but less enriched (Figure 2.6b). The list of the highest enriched terms (Figure 2.6a) contains less generic terms from the Biological Process category than the observed in the list in Figure 2.6b. Some shared terms were found among both lists, being the most enriched and most significant terms (highlighted in red).

Only 40 enriched GO terms were shared among all samples (Figure 2.7C, Table S1). Most of the shared terms were related to photosynthesis (photosystem I, photosystem II, plastid thylakoid membrane, chloroplast thylakoid membrane, light-harvesting complex, chlorophyll-binding), and protein metabolism (protein-containing complex, membrane protein complex, protein modification, protein-chromophore linkage). The only shared enriched term between site H3-1 and site H3-3 during summer sampling was the "downregulation of TOR signaling," which was not found in the enriched terms from winter samples. In winter, however, we found 59 unique terms not detected as enriched during summer. Most of these terms were related to nucleobase and phosphate-containing compounds (ribonucleotide metabolism, ribonucleoside binding, purine nucleotide metabolism, ribose phosphate metabolism, nucleoside phosphate metabolism, nucleoside-triphosphatase activity, among others). Some enriched terms found only in one sample, with no similar terms in the rest of the samples, are highlighted in Figure 2.8. Cellular response to osmotic stress and response to toxic substances were terms only found to be enriched at subsite H3-1 in summer; xanthine transport, nucleobase transport, vacuolar transport, and salt transmembrane transporter activity were enriched terms only found in summer at subsite H3-3; tRNA folding, prospore membrane biogenesis, plasma membrane involved in cytogamy, L-arginine import, regulation of alpha-glucan metabolism, cellular response to anoxia, chitin metabolism, sulfate assimilation, and proton transmembrane transport, among others, were terms found only in winter at subsite H3-1; mRNA catabolic process, glucose metabolism, organophosphate metabolism, trichocyst, and peroxisome were only found in winter at subsite H3-3.

2.5 DISCUSSION

2.5.1 Community composition along the salinity gradient during the wet and dry season

Salinity is a critical barrier considered to shape microbial communities. A decrease in diversity has been described with increasing salinity, as the osmoregulatory adaptation is energetically

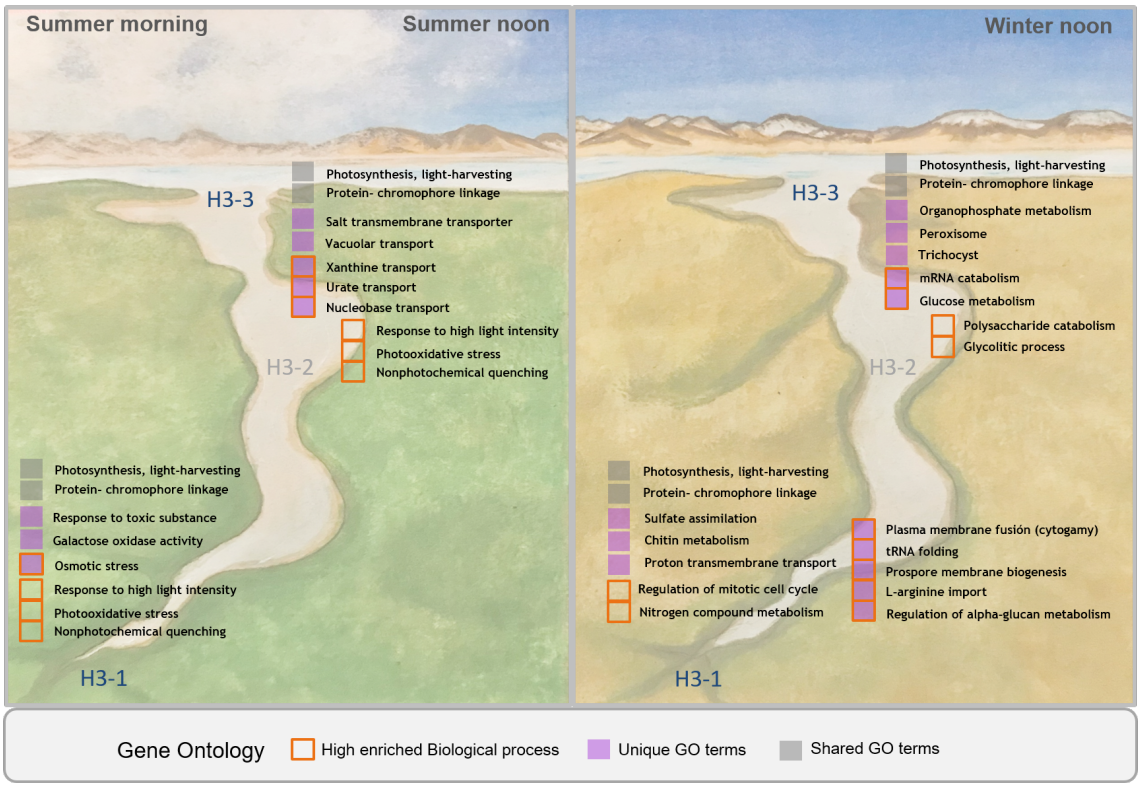

Figure 2.8: Summary scheme - showing the results of gene ontology enrichment analyses for summer and winter samplings at Salar de Huasco. No enriched GO terms were found for site H3-2.

costly (Oren 2001, Ji et al. 2019). Therefore, we expected to find a decrease in the active microeukaryotic groups along the salinity gradient, which was confirmed by the assemblies' general taxonomical assignment. Both the relative (and absolute, not shown) abundance of most active groups decreased along the salinity gradient. Bacillariophyta were the only exception during summer, and Choanozoa, along with Dinophyta during winter. Those groups had a higher abundance at the most saline site. Additionally, we expected to find a more structured community during winter (dry season) than during the rainy summer, where water levels increase and short-termed salinity changes are observed. The general taxonomic composition exhibited only low variance along the stream for both seasons; therefore, the microeukaryotic community did not appear to be more structured in winter than in summer. However, the main differences among seasons were observed on some lesser abundant taxa, such as choanozoans and dinophytes, which were more abundant in winter and appeared to be more influenced by environmental parameters (in this case, pH and salinity, as shown in the CCA). Dinophytes and choanozoans can tolerate even higher salinities than the reported ($\sim$ 24 PSU) for site H3-3 (Filker et al. 2017, Annenkova et al. 2020). Indeed, a new choanoflagellate species ("Salpingoeca" huasca sp. Nov.) described from the hypersaline lagoon ($\sim$150 PSU) in Salar de Huasco presented variable salinity tolerance (Schiwitza et al. 2018). For other groups such as euglenophytes and kinetoplastids, temperature appeared to be the main factor influencing their distribution, with higher abundances during summer, as shown in the CCA. This pattern has also previously been reported in other systems (Poniewozik & Juráň 2018). Chlorophytes and diatoms were among the most abundant phototrophic groups in all samples. Diatoms are an essential food source for two species of flamingoes inhabiting Salar de Huasco and other wetlands in the Altiplano region (Siefeld et al. 1998, Mascitti & Kravetz 2002). They are one of the most abundant microalgal groups in these systems, suggesting high adaptability to hypersaline waters and high UV radiation (Márquez-García et al. 2009, Rivera & Cruces 2009, Adiconis et al. 2013, Ramirez et al. 2015, Hernández et al. 2016). Hence, it is not surprising to find the highest abundance during summer towards the lagoon.

Within the heterotrophic microeukaryotes, Ascomycota made up almost 50% of the taxonomic assignments in all samples. Ascomycota are the most abundant fungal group and highly ubiquitous, with a crucial saprotrophic role in wetlands (Stephenson et al. 2013). In Salar de Huasco, their abundance might relate to a large number of macrophytes, dominated by Oxychloe andina, that form the peatlands surrounding the spring and mid-saline waters and constitute a major source of organic matter (Squeo et al. 2006). For Evosea, which accounted for up to 28% of the

assignments, there is no substantial information from aquatic ecosystems in the Andes region. Slime molds are mostly present in plants and soil and have been identified from several localities in northern Chile (Lado et al. 2007) and at high altitudes in the Tibetan Plateau (Liu et al. 2019). However, little has been reported on aquatic slime molds (Lindley et al. 2007, Tamayama & Keller 2013), suggesting that only the microscopic stages were found in the sampled sites in Salar de Huasco, or they were possibly soil or benthic contribution. The latter could also be the case for many of the identified taxa in our study, owing to the shallowness of the system, the stream direction, varying wind velocity, and presence of birds, all of which can be factors contributing to the dispersal of microorganism (Kristiansen 1996, Finlay 2002, Tesson et al. 2018). Diatoms, for example, identified as abundant in several high altitude aquatic ecosystems were part of the periphyton community that could be found on the sediment float as a result of strong winds and shallowness (Sylvestre et al. 2001, Cabrol et al. 2007)

A high phylogenetic novelty of microeukaryotes is expected from inland saline waters (Triadó-Margarit & Casamayor 2013). A higher resolution method in the taxonomic assignment (i.e., 18S rRNA) would be needed to evaluate this postulate. With our functional taxonomic classification in Salar de Huasco, we show only the phyla, and most of them have also been described in other ecosystems with varying salinity ranges (Triadó-Margarit & Casamayor 2013, Wang et al. 2014, Filker et al. 2015).

2.5.2 Differential expression in response to salinity

The enriched GO terms found at the saline site H3-3 during summer noon sampling ("Salt transmembrane transporter activity" and "Vacuolar transport") suggest osmotic stress, as the accumulation of osmolytes, maintaining the ion balance through membrane transporters and vacuoles, indicates a fast response to salinity stress in microalgae (Weiss et al. 1991, Raven & Doblin 2014). During summer, the frequent rain events cause short-term salinity changes, which might be the observed effect.

Conversely, during winter, the microeukaryotic communities at site H3-3 might be more adapted to increased salinity, as shown by the enriched GO terms ("Polysaccharide catabolism", "Glycolytic process", "Glucose metabolism", and "Peroxisome"). Strategies including lipid production and accumulation from different pathways (i.e., starch degradation and polar lipids degradation, reviewed by Shetty et al. 2019) indicate a long-term adaptation response to increased salinity.

Furthermore, at the freshwater site H3-1, we found the biological process "Cellular response

to osmotic stress" to be enriched only during the morning in summer and "Proton transmembrane transport" during winter, which may reflect osmolarity differences between the aquifer and the spring water in site H3-1. Further, the upregulation of cell division processes ("negative regulation of cell cycle switching, mitotic to meiotic cell cycle" and "plasma membrane fusion involved in cytogamy", among the most enriched GO terms) indicates the switching to an active metabolism after emergence from the aquifer.
However, at the same site, we found an upregulation of spore formation processes. Many microorganisms can form spores or cysts to ensure their spread and survival in the system under harsh conditions (Hellebust 1985, Urushihara 2009, Filippidou et al. 2019, Velez 2019). Thus, as some microeukaryotes were activating, others were forming spores.

2.5.3 Differential expression under different light and solar radiation levels

We expected high photoinhibition of the phototrophic groups from samples collected at noon (i.e., maximum solar irradiance) compared to the samples collected in the morning. In Salar de Huasco, this had previously been shown for cyanobacteria (Dorador, Vila, et al. 2008), with lower abundances at noon. However, they were found to recover quickly after solar irradiance decreased in the afternoon (Molina et al. 2016). In this study, we found photosynthesis among the most enriched processes in samples from both seasons; still, "response to radiation" and "response to high light intensity" were among the most significantly enriched processes found only at noon during summer. Additionally, according to the light history hypothesis of Hernández et al. 2016, we expected higher photoinhibition in the source waters at site H3-1 compared to the downstream sites. Organisms with prolonged exposures to solar radiation (i.e., in ponds, Trench-fiol & Fink 2020) might be better adapted to this condition than organisms that recently emerged from the aquifer with no previous solar exposition (Hernández et al. 2016). Some of the enriched processes found at the freshwater site H3-1 indicated photoinhibition in the morning sampling from summer (response to photooxidative stress, response to high light intensity, regulation of photosynthesis, photosystem II stabilization, and non-photochemical quenching), but not in winter. We expected a similar general expression in response to comparable solar irradiance levels during summer morning and winter noon samples. Indeed, we observed from DE analyses that in both seasons, sites H3-2 and H3-3 clustered separately from the freshwater site H3-1, which is in contrast to the samples collected in summer at noon. However, as shown in the CCA, the microeukaryotic communities from summer are more influenced by PAR than the winter communities, where pH is a more critical environmental factor.

2.6 CONCLUSION

We here provide the first eukaryotic metatranscriptomes from a high altitude Andean system and show how they respond to the steep environmental gradients that characterize Salar de Huasco. The heterotrophic taxa dominated over mixo- and phototrophic taxa in the active communities from this extreme ecosystem. However, primary production was the most enriched function, as supported by gene ontologies. We also revealed different responses to increased salinity during the wet and dry seasons, suggesting that winter communities are better adapted to higher salinities. Overall, our study contributes essential information on the microeukaryotic communities of Salar de Huasco. As such, this study forms a basis for future research on specific taxonomic groups, their responses to multiple environmental stressors, and their contributions to ecosystem functioning.

Chapter 3

The role of microeukaryotic groups for functional processes in a polyextreme high altitude aquatic system

3.1 ABSTRACT

Eukaryotic microorganisms are key players for almost all types of ecosystem functions in aquatic systems, and are therefore extensively studied in marine and fresh waters. However, extreme and remote environments have been less explored. High altitude aquatic systems experience extreme seasonal and even diurnal fluctuations in environmental conditions, such as temperature, salinity, and solar irradiance. To understand how microeukaryotes contribute to ecosystem functioning in such extreme environments, approaches that combine taxonomic with functional information are needed. We here present a metatranscriptome study from Salar de Huasco, a high altitude wetland in the North of Chile, with a marked salinity gradient. From eukaryotic mRNA sequencing data, we specifically investigated which groups of microeukaryotes affect which types of ecosystem functions in different sites along the salinity gradient in the wet and dry seasons. We found that, for example, during summer, Glaucophyta dominate photosynthesis and thus inorganic carbon fixation. Chlorophyta, Haptophyta, Ochrophyta, and Mucoromycota were the key players for dissolved nitrogen and phosphorus assimilation at the less saline sites. During winter, a higher expression of ion transport and stress response genes at the more saline sites was correlated to heterotrophic groups, which at the same sites dominated cellular respiration and sulfur assimilation. Our data demonstrate that spatial differences were greater in winter than in summer, and temporal differences by site were most pronounced at the most saline site. Overall, metatranscriptomics is a novel and promising tool to link biodiversity and ecosystem functions for microeukaryotic communities, particularly in less studied and environmentally extremely variable ecosystems.

3.2 INTRODUCTION

A big challenge in microbial ecology is to properly understand the taxonomic and functional complexity of microbial communities, with respect to all the factors and interactions that simultaneously affect those communities. Including microbial community structure and functional gene data into ecosystem processes models has improved these models (Graham et al. 2016). Therefore, by studying the functions and processes carried by a group of organisms, we can get better insights into their roles and contribution to the whole ecosystem.

To evaluate functional groups' activity in the environment, a widely used approach has been studying functional genes encoding for crucial enzymes through DNA microarrays and qPCR, such as ribulose bisphosphate carboxylase (RuBisCO) in carbon fixation, nitrate, and ammonium transporters (Ward 2008). However, these targeted methods do not evaluate gene composition and expression from complex samples in diverse ecosystems. Further, the use of degenerate primers to account for the nucleotide sequence variance causes amplification bias (Gaby & Buckley 2017). High throughput next-generation sequencing (NGS) methods can tackle this issue to some extent (Stewart 2013, Li et al. 2017), with metatranscriptomics being the most powerful tool to link microbial processes to their respective environments.

Among the most valuable ecosystems, wetlands are globally significant carbon sinks, and they provide various ecosystem services (Mitsch et al. 2015). Supporting primary productivity, nutrient cycling, and habitat provision to aquatic and terrestrial species are among the most evident, though essential services. Many of the ecosystem processes behind these services are driven by microorganisms, thus giving them a critical role in aquatic systems (Durance et al. 2016).

The high altitude wetlands in the South American Andes region provide habitat for auquenid herds (llama, vicugna) and water birds like flamingos (Squeo et al. 2006, Caziani et al. 2007) despite their extreme environmental conditions. The extreme temperatures, salinities, and high solar radiation are among the most prominent factors shaping these remote ecosystems' high microbial diversity (Maturrano et al. 2006, Dorador et al. 2013, Albarracín et al. 2015, Scott et al. 2015).

One of these Andean wetlands is Salar de Huasco, located in the north of Chile. It comprises various ponds, groundwater sources, and very shallow streams and lagoons (Dorador et al. 2008). The system has naturally high sulfate concentrations and broad salinity ranges (Risacher et al. 2003) and undergoes strong seasonal (wet and dry season) and diurnal (temperature, solar irradiance) changes in environmental conditions (De La Fuente & Niño 2010, Hernández et

al. 2016). Bacterial and archaeal groups are the main players in nitrogen cycling, being more abundant in the less saline sites of Salar de Huasco (Dorador, Busekow, et al. 2008, Dorador et al. 2010, Molina et al. 2018). However, the complete eukaryotic microbial component and their specific roles in nutrient cycling and other ecosystem functions have so far not been evaluated in this system, nor any other high altitude Andean wetland.

Besides their role as primary producers, eukaryotic phytoplankton are the primary biogenic source of sulfur, producing dimethylsulfoniopropionate (DMSP). This compound serves as a sulfur and carbon source to marine plankton (Yoch et al. 2001) and, when broken down, produces the gas dimethyl sulfide (DMS). This volatile compound is released into the atmosphere, forming part of cloud condensation nuclei and potentially affecting climate by increasing the albedo effect (Charlson et al. 1987, Vogt & Liss 2009). The most studied genera in the ocean DMSP breakdown and the liberation of DMS is Roseobacter (Wagner-Döbler & Biebl 2006), although some fungal species have also been reported to degrade DMSP to DMS (Todd et al. 2009, Curson et al. 2011). DMSP is considered an osmolyte molecule; although its role in microalgal cells is not yet fully understood, it is presumed to help keep the cell protected at high salinities (Kiene et al. 2000, Lyon et al. 2016, Bullock et al. 2017). Interestingly, the production and uptake of DMS are also affected by UV radiation (Slezak et al. 2007, Vallina & Simó 2007, Harada et al. 2009, Galí et al., 2013).

The broad salinity gradients, the high solar radiation levels, and high sulfate concentrations in Salar de Huasco, together with the detection of Roseobacter species among the most abundant in the salar (Dorador 2007, Dorador et al. 2013), led us to hypothesize that eukaryotic phytoplanktonic groups in Salar de Huasco could be producing DMSP as an osmolyte in saline waters. Therefore, DMS and its oxidized form dimethyl sulfoxide (DMSO) could be found from DMSP microbial degradation. These compounds have been detected in non-marine environments, although their origin is not entirely clear (Hu et al. 2007, Sela-Adler et al. 2016, Du et al. 2017, Steinke et al. 2018). Therefore, the analysis of these sulfur compounds combined with metatranscriptomics would support our hypothesis.

Using metatranscriptomics, we aimed to provide a snapshot of the relevant processes driven by aquatic microeukaryotes in Salar de Huasco during the wet and dry seasons. By selecting groups of differentially expressed genes involved in representative processes, we ultimately aimed to link them to the taxonomic groups driving these functions in the salar, and therefore, provide a better picture of the microeukaryotes' contribution to the ecosystem processes in Salar de Huasco in particular, and high altitude wetlands in general.

3.3 MATERIALS AND METHODS

3.3.1 Sample collection and processing

Sampling was performed during the wet season (Summer, January 2017), at 9 am (shortly after dawn) and around noon (maximum irradiance), and dry season (Winter, August 2018), when only sampling around noon was possible. Three sites: H3-1, H3-2, and H3-3 (described in the previous chapter), were sampled each time along a salinity gradient in Salar de Huasco. Physicochemical parameters were determined in situ at each sampling point (temperature, pH, conductivity, salinity, total dissolved solids (TDS), and dissolved oxygen (DO)). Water samples for RNA sequencing were taken in triplicates at each sampling point. Upon sampling, the water collected for RNA extraction was pumped through SterivexTM cartridges (0.2 µm) via a peristaltic pump with a sterile tubing, and to preserve the samples, RNAlater (Qiagen) was added before storage at -80°C until RNA extraction. The RNA extraction and sequencing were done as described in the previous chapter.
Water samples for the analysis of dimethylsulfoniopropionate (DMSP) and dimethyl sulfoxide (DMSO) were taken only on the most saline site H3-3 during summer sampling (morning and noon) with six replicates each. During winter sampling, samples (6 replicates each) were collected from each site (3) at noon for DMSP/DMSO measurements.

3.3.2 DMSP and DMSO analysis

The water samples were processed in situ; half of the replicates (3) were filtered through 0.45 µm syringe filters to remove particulate DMSP/DMSO. The second half of the samples remained unfiltered to measure total DMSP/DMSO (dissolved + particulate). All samples were sparged for 15 min with Nitrogen gas to remove DMS and other volatiles. After this, samples were transferred to 50 ml Falcon tubes, where sodium hydroxide (NaOH) pellets were added to each sample to convert DMSP into DMS. The tubes were sealed gas-tight with minimal headspace and stored at 4°C and dark conditions until measurement. Samples were measured at the Helmholz Centre for Ocean Research (GEOMAR), Kiel (Germany) with a gas chromatograph coupled to a sulfur-selective flame photometric detector (GC 6000 vega series 2; capillary column CP-SIL 5CB for sulfur, 30 m×0.32 mm ID, FPD 800, CE instruments), as described in Zindler et al. (2012, 2013). Calibrations were made with DMS standards (liquid DMS diluted in ethylene glycol), and each sample was measured in triplicates. First, samples were purged in-vial for 10 minutes in a custom-made purge and trap system with helium as the carrier gas. The DMS

was preconcentrated and then desorbed by heating the trap, injecting the sample into the gas chromatograph. After measuring the DMS from DMSP, the DMSO present in the samples was reduced to DMS by using sodium borohydride pellets (NaBH4), and it was measured using the same method as for DMS from DMSP. The peak areas were transformed into concentration values using the corresponding calibration functions; the DMS values were subtracted from DMSPd to obtain the DMSPp concentrations. By subtracting the dissolved DMSP (DMSPd, filtered samples) from the total concentrations measured (DMSPt, unfiltered samples), the particulate or microbial-produced DMSP (DMSPp) concentrations were obtained.

3.3.3 Functional analysis

The analyses presented here are based on annotated transcripts that were differentially expressed, as spatially (different sites, same time) and temporally (same site, different times) evaluated. These differentially expressed transcripts were obtained from the metatranscriptome analyses done in the previous chapter. Briefly, the eukaryotic mRNA sequences were quality trimmed, remaining rRNA sequences removed, and sequences from biological replicates were used to perform de novo assemblies. The raw sequences were mapped back to the assemblies, and count matrices were obtained to perform differential expression analyses at the transcript level. The resulting differentially expressed transcripts were used for annotation via the Trinotate pipeline (release v3.2.1, Bryant et al. 2017). Finally, the transcript annotations (Blastx against the Swissprot database) and the PFAM annotations were used to estimate the abundance of genes involved in basal metabolisms like photosynthesis and carbon fixation, cellular respiration, ion transport, and nutrient uptake, including sulfur cycling. We also included a category for stress response to evaluate differences between wet and dry seasons. Selecting the differentially expressed genes belonging to the above-mentioned categories, allowed us to narrow down the number of genes from the total pool dominated by transcription-related annotations (ribosomal proteins, polymerases, transcription factors).
Analyses were performed with R version 3.6.1 (RStudio Team 2016). Pairwise correlation (Spearman) were calculated with the package corrplot (Taiyun & Simko 2017) between the selected processes and the phyla assigned to genes in the processes categories. A non-metric multidimensional scaling (NMDS) ordination analysis based on Bray-Curtis distances was performed with the Vegan package (Oksanen et al. 2018) to compare similarities among the Pfam annotations and environmental variables in different sites and seasons.

3.4 RESULTS

3.4.1 Sulfur compounds

The presence of both DMSP and DMSO could be confirmed for all subsites: total DMSP (DMSPt) concentrations in winter samples increased with salinity, as did sulfate concentrations (0.67 ± 0.14, 1.64 ± 0.14, 3.04 ± 0.67 nmol $L^{-}1$, Figure 3.1 A). In the summer samples, DMSPt was higher at noon than at dawn. However, summer measurements showed considerable variance among replicates, possibly due to degradation of the samples during the extended storage between sampling and analysis. Total DMSO concentrations were significantly higher than DMSPt in all samples (Figure 3.1 C).Only particulate DMSP (p) could be determined from winter samples in all three sites (Figure 3.1 B) No genes related to DMSP production or breakdown were found within our eukaryotic metatranscriptomes. Only one bacterial gene (dmdC) involved in the demethylation of DMSP to produce methylmercaptopropionate (MMPA) was found.

3.4.2 Genes and processes

By season

In winter, more changes in gene expression were found along the salinity gradient (spatial) than in summer, with the highest upregulated genes found at the more saline sites H3-2 and H3-3 (Figure 3.2 A) They corresponded to ion transport genes (calcium-permeable stress-gated cation channel, high-affinity nickel transport, high- and low-affinity potassium transport, manganese transporter), and nutrient uptake (ammonium transporter and nitrogen regulatory protein). At site H3-1, Calvin cycle protein and high osmolarity signaling protein were expressed most strongly. During summer, phosphate permease and light-harvesting complex stressed-related proteins were the highest expressed genes at site H3-1. Calvin cycle protein was the most up-regulated at site H3-2 and a boron transporter gene at site H3-3.

By site

When looking at the temporal changes in gene expression, comparing one site in the different seasons, the highest upregulation of the selected processes is found at site H3-3 (Figure 3.2 B) At this site during summer, the most expressed genes were involved in ion transport and nutrient uptake (boron transporter, MFS transporter, vacuolar cation-chloride transporter, sodium-sulfate cotransporter, nitrite reductase), stress response (high osmolarity signaling protein), and sulfur

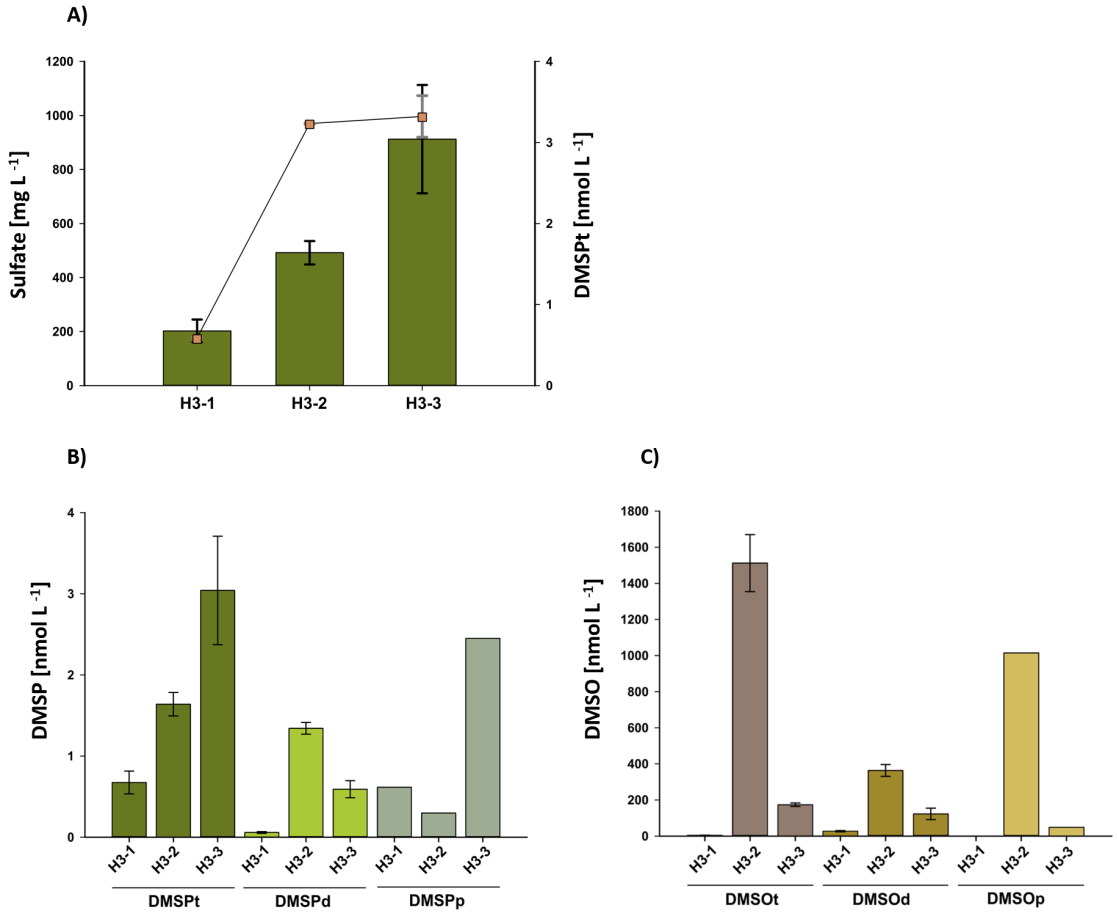

Figure 3.1: Concentration of DMSP,DMSPt and DMS - (A) Concentrations (mean + SE, n = 2 – 3) of total DMSP (t) together with sulfate concentrations (mean + SE, n = 2) measured from the same samples. (B) DMSPt, compared to dissolved DMSP (d), and particulate DMSP (p) measured from winter samples. (C) concentrations (mean + SE, n = 2 – 3) of total DMSO (t), dissolved DMSO (d), and particulate DMSO (p) from winter samples.

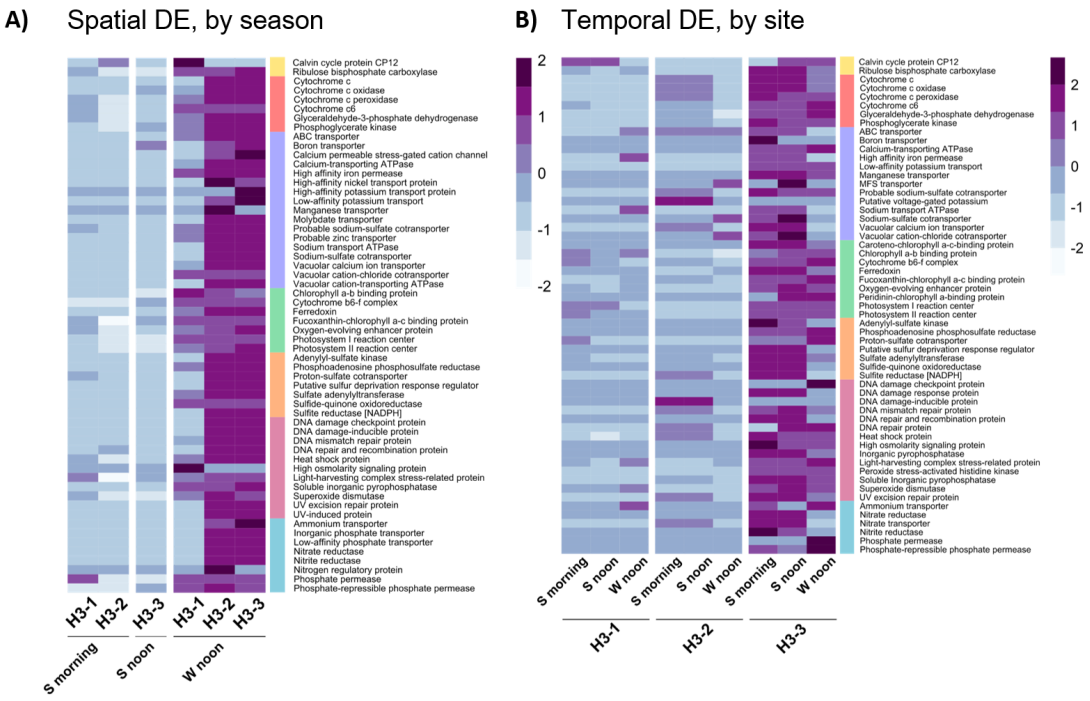

Figure 3.2: Functional heatmaps - Heatmaps display groups of genes involved in the following processes: carbon fixation, cellular respiration, ion transport, nutrient uptake, photosynthesis, sulfur cycling, and stress response. A) Gene annotations from spatial differentially expressed transcripts; different sites were compared simultaneously (summer morning, summer noon, or winter noon). B) Gene annotations from temporal differentially expressed transcripts; same site (H3-1, H3-2, or H3-3) compared at different sampling times. Intensity corresponds to log2 normalized counts.

cycling (adenylyl-sulfate kinase). At this site during winter, the higher expression corresponded to DNA damage checkpoint protein and two phosphate transporters (phosphate permease, and phosphate-repressible phosphate permease). At site H3-1, Calvin cycle protein was highly expressed during summer, and iron, sodium, and ammonium transporters during winter. For site H3-2, potassium transporter and DNA damage-inducible protein were the most upregulated during summer. And during winter, ion transport genes were the most expressed (MFS transporter, vacuolar cation-chloride transporter, sodium-sulfate cotransporter, ABC transporter).

3.4.3 Processes and phyla

By season

When correlating the taxonomic groups from the functional annotation with the categories of processes, we found a higher number of correlations during winter (Figure 3.3, upper panel)and at site H3-3 (Figure 3.3, lower panel). During winter, stronger positive correlations than during summer were found between the phyla and processes. All correlations refer to positive correlations unless stated otherwise. The phyla Charophyta, Chlorophyta, Dinophyta, Euglenophyta, Basidiomycota, Blastocladiomycota, and Kinetoplastida, had significant positive correlations to all processes, while Bacilliariophyta, Ciliophora, and Oomycota had weaker correlations to all processes (Figure 3.3, upper panel). Negative correlations were found for Glaucophyta and Rhodophyta to all processes, which was not the case in summer. During summer, Chlorophyta, Haptophyta, Ochrophyta, and Mucorophyta were the groups with a stronger correlation to nutrient uptake processes. Cellular respiration, ion transport, and stress response were processes with the strongest correlations to Mucoromycota and Blastocladiomycota. In contrast, Glaucophyta were the only phototrophic phylum with a strong correlation to photosynthesis and carbon fixation, together with the heterotrophic phylum Kinetoplastida. The latter had a significant positive correlation to Glaucophyta (Figure 3.4).

By site

Most of the phyla were positively correlated to stress response, ion transport, and cellular respiration at site H3-1, except for Euglenophyta and Dinophyta, both negatively correlated with those factors. Bacillariophyta were strongly correlated to sulfur cycling at site H3-1 and H3-2 but weakly correlated at H3-3. Ascomycota and Basidiomycota were also strongly correlated to sulfur cycling at both H3-1 and H3-3. Ciliophora were strongly correlated to sulfur cycle only at site H3-3. Dinophyta appear strongly correlated to photosynthesis and carbon fixation at

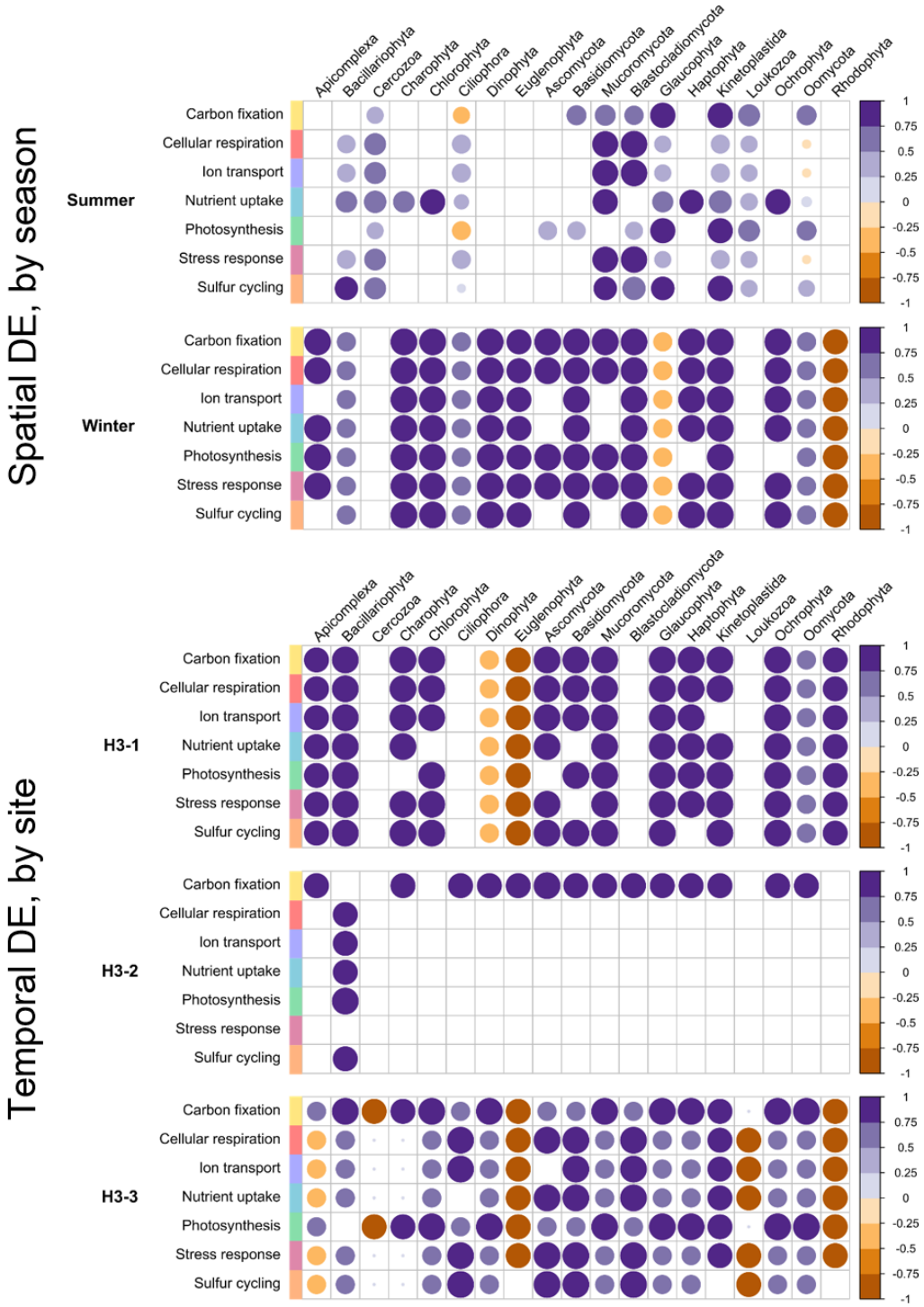

Figure 3.3: Correlogram of processes and phyla from the functional assignment - as by season (upper panel) or by sampling site (lower panel). Only significant correlations are displayed, positive correlations in purple and negative correlations in orange. The color intensity and circle size are proportional to the correlation coefficients (Spearman correlation).

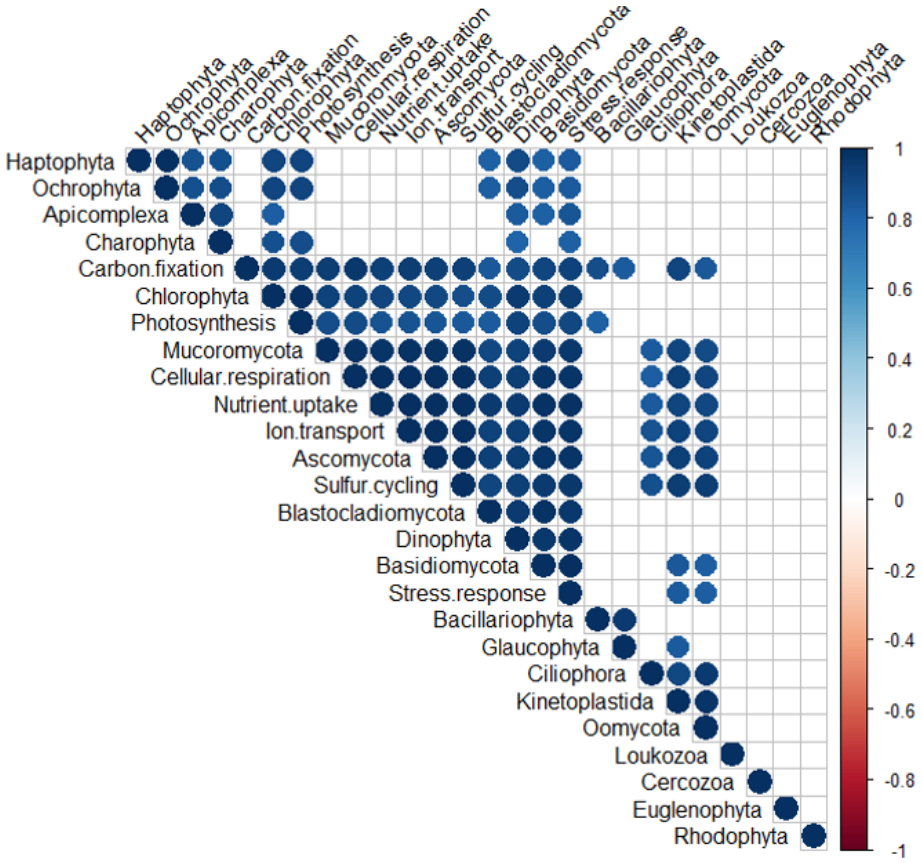

Figure 3.4: Complete correlogram - of pairwise comparison among phyla and processes. Only significant correlations ($p < 0.01$) are displayed. A positive correlation (Spearman) is shown in Blue.

site H3-3 and negatively correlated to all processes at site H3-1. At site H3-3, the phototrophic groups Chlorophyta, Dinophyta, Glaucophyta, Haptophyta, and Ochrophyta had the same correlation patterns, with the strongest correlations to photosynthesis and carbon fixation. The heterotrophic groups Oomycota and Mucuromycota also had a strong correlation to photosynthesis and carbon fixation, due to their correlation to phototrophic groups and not directly to the processes. For example, Ascomycota and Mucoromycota had a significant ($p < 0.01$) strong positive correlation with Dinophyta. And Oomycota had a significant positive correlation with Glaucophyta (Figure 3.4).
At the intermediate site H3-2, we could identify significant positive correlations of most phyla to carbon fixation. Only Bacilliarophyta had strong correlations with the rest of the processes, except for stress response.

3.4.4 Gene functions to the environment

The clustering of the protein domain annotations (Pfam) associated with the differentially expressed group of genes and the environmental parameters showed no significant correlation to the environmental variables in a non-metric multidimensional scaling (NMDS). The plot separated the summer and winter samples, although with considerable overlap. A higher overlap was observed for the spatial with respect to the temporal comparisons at the respective sites (Figure 3.5).

3.5 DISCUSSION

As shown in the previous chapter, diatoms were among the most abundant phototrophic groups in Salar de Huasco. Additionally, Fungi and Amoeba dominated the active microeukaryotic community at all sampled sites in both wet and dry seasons. Diatoms are found in many aquatic systems with varying salinity in the Altiplano region (Márquez-García et al. 2009, Rivera & Cruces 2009, 2018, Ramirez et al. 2015). Fungal groups reported in high altitude ecosystems present high resistance to UV radiation (Libkind et al. 2009, Pulschen et al. 2015). Both groups are expected to be highly adapted to the extreme conditions in the salar and thus be the main contributors to the system's functions. Diatoms, as important primary producers, were expected to dominate photosynthesis, carbon fixation, and sulfur cycling (Norici et al. 2005), and diverse fungal groups, essential for nutrient cycling through organic matter degradation (Gadd & Raven 2010), would be relevant in nitrogen assimilation. Further, by linking the stress

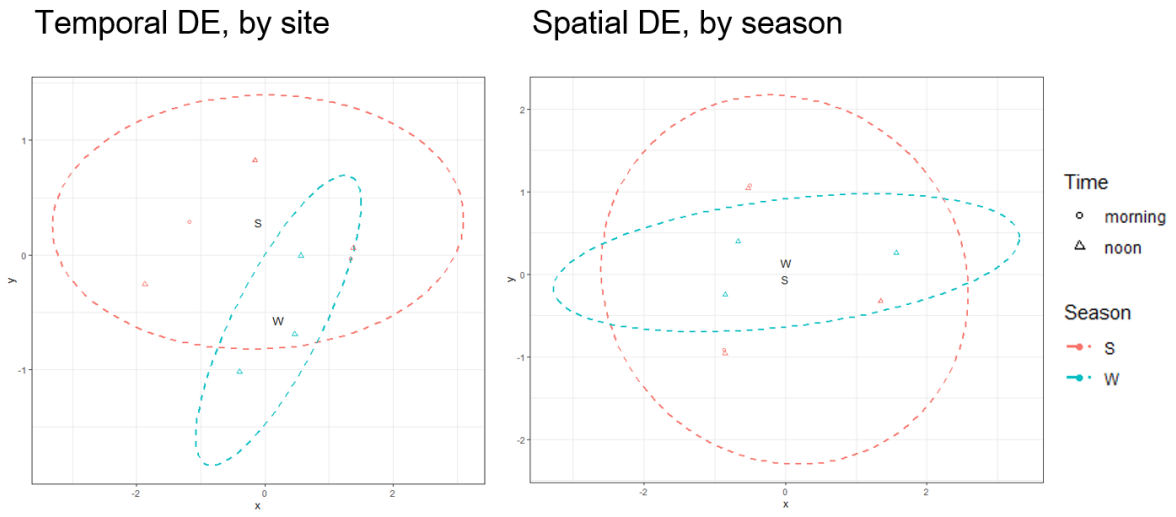

Figure 3.5: Non-metric multidimensional scaling (NMDS) - ordination of Pfam annotations from differentially expressed transcripts and environmental parameters in 3 different sampling sites during summer (S) and winter (W). Ellipses were built with 95% confidence.

response processes to specific taxonomical groups, the seasonal differences could be evaluated and confirm that the winter community is better adapted to increased salinity, as suggested in the previous chapter.

3.5.1 Responses to salinity and solar radiation stress

We found that stress response and carbon fixation were the most upregulated processes and strongly correlated to most taxa. Moreover, these processes were strongly correlated with each other.

Stress response genes were more strongly expressed at the more saline sites during summer (H3-3) and winter (H3-2 and H3-3) sampling. However, Bacilliariophyta and most phototrophic phyla only correlated weakly to stress response at site H3-3, suggesting adaptation to increased salinities. In diatoms, as for other microalgal groups, various responses may account for these adaptation strategies, such as glycerol and lipid accumulation, as well as DMSP production in marine species (Lyon et al. 2011, Cheng et al. 2014, Kettles et al. 2014a). We found a membrane protein involved in response to high osmolarity (Reiser et al. 2000) and several ion transporters overexpressed in the most saline sites (sodium-sulfate, potassium, chloride, calcium,

and ABC transporters). These are all known to contribute to the regulation of cellular homeostasis (Borowitzka 2018). An increase in cytosolic calcium levels, for example, has been recently found to alleviate the oxidative stress induced by salinity stress on microalgal species and further result in lipid accumulation (Qiao et al. 2020). Calcium-transporting ATPase and calcium permeable stress-gated cation channel were genes highly expressed at site H3-3 during winter. Thus, a decrease in oxidative stress and increased lipid accumulation, a long-term adaptation response to increased salinity (Shetty et al. 2019), would support that the phototrophic groups are more adapted to increased salinities during winter, as proposed in the previous chapter.
Diatoms, highly abundant and among the adapted groups to increased salinities in Salar de Huasco, were surprisingly not the main contributors to photosynthesis and carbon fixation as expected. Instead, less abundant groups from the active microeukaryotic communities had a stronger correlation to primary production; Glaucophyta during summer, and Charophyta, Dinophyta, Euglenophyta, Haptophyta, and Ochrophyta during winter. Even some of the abundant heterotrophic groups (Ascomycota, Kinetoplastida, Apicomplexa) were significantly correlated with photosynthesis and carbon fixation, which is likely influenced by their strong association with phototrophic taxa (Banos et al. 2020, Kilias et al. 2020). This, in turn, might be explained by their use of organic compounds excreted by phototrophs into the water column (Myklestad 1995, Jobard et al. 2012). The fact that more groups were associated with primary production during winter correlates with higher photoinhibition during summer. Indeed, it was shown in the previous chapter that photooxidative stress was a predominant process in summer samples. Stress through UV radiation in microalgae can lead to impairment of photosynthesis, lipid peroxidation, and protein and DNA damage. Photosystem stabilization, nonphotochemical quenching, protein folding, DNA repair, and response to oxidative stress are among the most described compensatory mechanisms (Puente-Sánchez et al. 2016, Liu et al. 2019). Further, pigments and mycosporine-like amino acids (MAA) are important photoprotective compounds, the latter widespread in microalgae, yeast, copepods, and bacteria (Tartarotti & Sommaruga 2006, Sonntag et al. 2007, Libkind et al. 2009). In the Altiplano, a bacterial UV resistome was described (Kurth et al. 2015), along with some defense mechanisms against the high UV radiation, such us photolyases and cryptochromes, antioxidative enzymes, and pigment production (Albarracín et al. 2013, 2015, Pérez et al. 2017, Rasuk et al. 2017, Portero et al. 2019). However, there is no information reported from these systems for the eukaryotic microbial response to high light and UV radiation. We here reported DNA repair mechanisms, superoxide dismutase enzymes,

light-harvesting complex stress-related proteins, heat shock proteins among the higher expressed genes at the most saline sites, and predominantly during noon summer sampling.

3.5.2 Sulfur cycling

Sulfur is essential for the growth of all living organisms (reviewed by Beinert 2000). For primary producers, it is an integral part of the photosystem, required for enzymes of the Calvin cycle, and an important sink of energy obtained through photosynthesis, as sulfate must be activated for its assimilation (reviewed by Norici et al. 2005). We thus expected primary producers to dominate the sulfur cycling processes in Salar de Huasco. However, this was only found at site H3-2 with a strong correlation of sulfur cycle genes to Bacilliariophyta. At the most saline site, Fungi were responsible for most of the sulfate assimilation.

Another relevant role of sulfur in photosynthetic organisms is the production of DMSP as an osmoregulatory compound under changing salinities, as mentioned above (Kettles et al. 2014b, Lyon et al. 2016). This function has been shown for diverse species in the marine phytoplankton and had not been reported for inland aquatic systems. The few available genes (Todd et al. 2009, 2016, Alcolombri et al. 2015, 2017, Curson et al. 2017) of marine species involved in the production and breakdown of DMSP were not found in our metatranscriptomes, except for one bacterial gene involved in the DMSP demethylation pathway (Reisch et al. 2011). Nevertheless, DMSP and DMSO were detected in all samples from both seasons, a novel and relevant result for high altitude aquatic systems where the production and metabolizing pathways for DMSP and DMSO are yet unknown.

The origin of DMS in non-marine systems is not yet precisely elucidated. It has been detected in salt marshes, where bacteria oxidize it to DMSO (Zeyer et al. 1987), and in hypersaline mats, where its production was attributed to hydrogen sulfide and organic carbon from photosynthesis (with no presence of DMSP), further indicating the photochemical oxidation to DMSO as the major DMS sink in the water column (Visscher et al. 2003). We thus suggest that the high concentrations of DMSO found in the salar might result from DMS photodegradation due to the extremely high solar irradiance. Further, similarity analyses of protein sequences to the known eukaryotic enzymes involved in DMSP production together with DMSP measurements would be advisable to clarify the origin of DMSP in Salar de Huasco.

3.5.3 Spatial and temporal variation in nutrient cycling

We found the major spatial and temporal differences in nutrient uptake processes. Heterotrophic taxa highly correlated to nutrient uptake processes at the most saline site. There, phosphate and ammonium transporters were predominantly upregulated during winter, which correlated with the highest ammonium and phosphate concentrations found at this site (Table 4.1). High ammonium uptake by ammonia-oxidizing bacteria (AOB) was previously reported for the saline lagoon (Molina et al., 2018). Further, the highest concentrations of phosphate previously recorded at the same site (Dorador et al. 2010, Hernández et al. 2016, Molina et al. 2018, Eissler et al. 2019) were positively correlated to conductivity and nitrate (Eissler et al. 2019) and to microbial activity (Hernández et al. 2016). Therefore, the lagoon's primary producers would benefit from the nitrate resulting from the AOB nitrification process, explaining the low nitrate concentrations at site H3-3 (Table 4.1) and the high expression of nitrate reductase enzymes. Moreover, the high phosphate concentrations correlate to the higher primary production at the lagoon (de la Fuente 2014) and the consequent organic matter recycling performed by bacterial and fungal groups. In particular, Ascomycota, Basidiomycota, and Blastocladiomycota were strongly correlated to nutrient uptake.

So far, we reported the main processes upregulated in the microeukaryotic communities from Salar de Huasco and considered salinity and solar radiation as important structuring factors of these communities (together with pH, conductivity, and temperature as found in the previous chapter). However, the combination of the environmental factors with the availability of nutrients in Salar de Huasco might better explain the community structure and the ecosystem processes.

3.6 CONCLUSION

Studies of eukaryotic microorganisms in aquatic systems of the Altiplano are scarce and do not provide information on this group's contribution to functional processes in these ecosystems. We here identified the main processes driven by microeukaryotes along a stream in Salar de Huasco and could attribute them to specific functional groups adapted to the extreme conditions. Further, our results show that spatial dissimilarities in the microeukaryotes' central metabolic processes along the salinity gradient were higher during winter, and temporal dissimilarities in the same sites were most pronounced at the most saline site. Overall, this approach gives insights into stress and resistance mechanisms of the microeukaryotic community in response to

the extreme environmental conditions of the salar and how they could affect the main biological processes, which could be used for further estimations of ecosystem processes.

General Discussion and conclusions

The three chapters presented in this thesis explored aquatic microeukaryotic communities from inland systems, focusing on their interaction with environmental parameters and their functional role in their ecosystems. The results shed light on how metabolic processes in small aquatic systems are regulated throughout the diel cycle, how communities differ spatially and temporally in composition and function, and how these two aspects can be linked to understanding the ecosystem functioning better.

The specific results have already been discussed in the corresponding chapters. Here, I would like to briefly discuss these results and gain some insight into the ecosystem functions.

Both studied systems were shallow but distinctive in water composition (from fresh to hypersaline), although they presented some similarities at the very general level. The pond and the stream in Salar de Huasco were sampled during summer, with the year's highest solar radiation levels. Although much higher in Salar de Huasco, the PAR measured at the pond during noon was equivalent to that of morning samplings in the salar. Both ecosystems receive nutrient inputs from the land; through rain events and the surrounding vegetation; however, shallow and small systems like the pond are more susceptible to fluctuations (Simon et al. 2015). Further, a dominance of heterotrophs over phototrophs was found in both systems. The most abundant taxa represented in the active community correspond to Fungi. Additionally, the less abundant taxa showed to be essential for the main ecosystem's functions. These characteristics are further discussed down.

In shallow aquatic systems, the sediment-water interface is important for the activity of benthonic organisms. Microphytobentos, including microeukaryotic algae and cyanobacteria, are important primary producers in these systems (Macintyre et al. 1996). However, if inorganic nutrients are limiting, they compete with heterotrophic bacteria for these resources (Grover 2000). On the other hand, if carbon is limiting the growth of these benthic bacteria, they might benefit from the microphytobenthos exudates (Coveney & Wetzel 1989), which can enhance the bacterial production if light conditions are optimal for the primary producers (Rier & Stevenson 2002). In Salar de Huasco, only one study has reported bacterial secondary production (Hernández et al. 2016). The study showed that in isolated ponds, with more prolonged exposure to UV radiation and thus higher adaptation from microorganisms, the bacterial production was higher than in the streams-source waters. Further, this productivity also correlated to higher microalgal biomass.

When there is a higher nutrient input to the water, then phytoplanktonic primary producers would benefit from microphytobenthos. In streams like in Salar de Huasco, there is a continuous input of nutrient from diverse sources; organic matter from water birds and auquenids might contribute to the ammonia and phosphate input, while the rain events (although scarce most of the year) together with the upwelling water from the aquifer, contribute to the input of inorganic nutrient and salts (carbonates, sulfates) of volcanic origin and characteristic of the Altiplano region (Risacher et al. 2003). Therefore, the phytoplanktonic community might not be nutrient-limited and be favored over the benthonic community. However, the extreme solar radiation and the increased salinity might be an essential limiting factor in the system. Still, even if the phytoplanktonic primary producers would thrive under extreme conditions, the high abundance of heterotrophic groups such as fungi, amoeba, ciliates, kinetoplastids would regulate their growth and thereby contribute to the nutrient cycling in the system. Indeed, I found that heterotrophic taxa groups correlated strongly to the nutrient uptake process, specifically to nitrogen and sulfur assimilation. At the same time, the high abundance of heterotrophic groups, mostly fungi, amoeba, and ciliates, might be due to several reasons. In the case of fungal groups, they are highly adapted to extreme UV radiation (Libkind et al. 2009, Pulschen et al. 2015), and salinity (Velez 2019). For ciliates, an increased abundance might be not only because of their resistance to extreme conditions, but most likely because of the lack of abundant microinvertebrates in the saline waters.

Furthermore, an important factor supporting heterotrophy dominance could be a higher input of allochthonous over autochthonous DOM to the system. It has been shown that the organic material exudated by primary producers cannot always fulfill the complete demands of heterotrophs in aquatic systems, and if the systems are also receiving inputs of allochthonous DOM, the growth of the heterotrophic groups will be enhanced and, thus, the system dominated by heterotrophy. This might be the case for both studied systems; in the pond, high DOC concentrations were determined as from plant origin, and in Salar de Huasco, most of the DOM might be the contribution of the peatlands surrounding the stream. In the pond, the high DOC concentrations coloring the water also caused light limitation for the phototrophic community.

How far we have come and future challenges

Forty years ago, the concept of the microbial loop was introduced (Azam et al. 1983). Before that, the role of microorganisms in diverse ecosystems, through energy transfer in the food webs, had been completely overlooked. Back then, it was unimaginable to think about how tiny organisms invisible to the naked eye could contribute enormously to so many processes on the

planet. It is even less feasible to think about how we can investigate the organisms-ecosystems relations through sequences of their genetic material!

It was pointed out previously how challenging it can be studying natural microbial communities to understand their taxonomic and functional complexity concerning all the factors and interactions that simultaneously affect them. Thus, this continues to be one of the significant challenges in microbial ecology.

Notwithstanding, there have been great advances in describing microbial (both prokaryotic and eukaryotic) communities of diverse ecosystems (Fonseca & Bicudo 2008, Vaulot et al. 2008, de Vargas et al. 2015, Aguilar et al. 2016), some of them previously considered uninhabitable (Stoeck et al. 2014, Rasuk et al. 2016, Brown & Fritz 2019, Pérez et al. 2020). Thus, the question "who is there?" has been largely addressed for most types of ecosystems. The question "what are they doing?" has been less addressed from natural communities. Moreover, it has only been possible through the NGS advances, first with the use of functional genes to target specific functions in a community (Ward 2008, Reisch et al. 2011), and more recently through the use of metatranscriptomics, metaproteomics, and metabolomics to a less extent (Alexander et al. 2015, Hamilton et al. 2017, Krohn-Molt et al. 2017, Lau et al. 2018). The third question, "who is doing what?" remains the less addressed. Despite having new tools to investigate this question, it is difficult to get an accurate interpretation with a lack of previous studies to compare to.

In this thesis, specifically for the extreme ecosystem studied, I addressed all three questions regarding the complete microeukaryotic community: "who is there?", "what are they doing?", and most importantly, "who is doing what?" This last question is the one that would take us to a broader level of understanding of the ecosystem's functions.

Although this study could not sufficiently answer this last question, it certainly provided a bunch of (until now lacking) information on the system's microeukaryotic component, and it will continue to open new and more specific questions.

The focus of the thesis was on the interaction of microeukaryotes with the environment. However, a relevant factor to consider for further studies would be the interaction between these communities' species.

Conclusions

The studied aquatic systems, although very different, presented similarities: heterotrophs dominated both, and the less abundant phototrophic fraction contributed significantly to the systems processes. Further, the communities from Salar de Huasco were better adapted to the increased salinity during winter than the summer communities, as indicated by the stress responses to salinity and solar radiation. Moreover, most of the phototrophic groups exhibited fewer stress responses than the heterotrophic groups. The results demonstrate that the spatial differences in Salar de Huasco (sites along the salinity gradient) were more significant in winter, and the temporal (wet and dry season) differences by site were most pronounced in the most saline site.

Overall, metatranscriptomics is a promising tool to study eukaryotic microbial communities, particularly in rarely studied and environmentally extremely variable ecosystems. By linking biological processes to biodiversity, more insights into ecosystem functions can be obtained, essential for developing conservation strategies.

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Supplementary Information

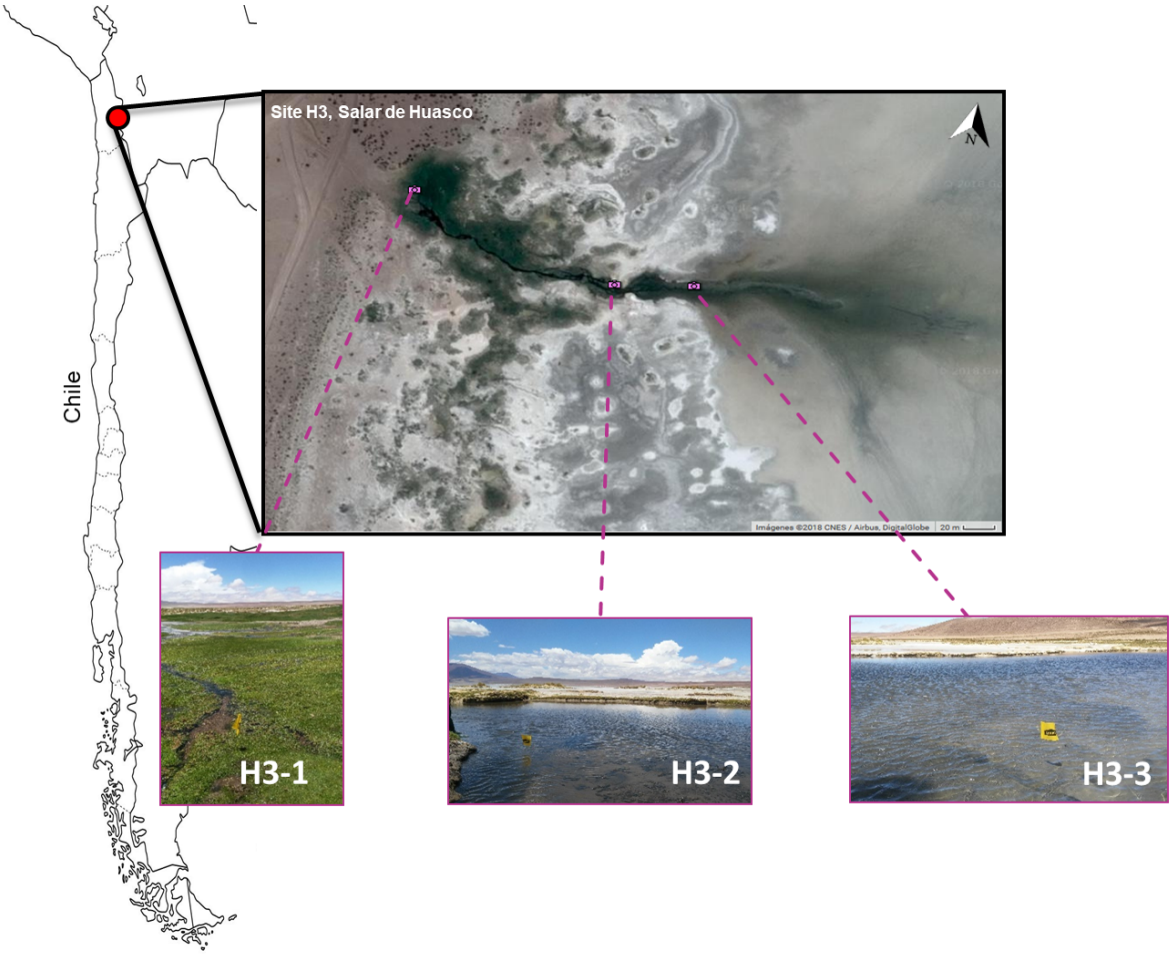

Figure 3.6: Location of the sampling sites at Salar de Huasco, in the north of Chile. H3-1 corresponds to the freshwater source, H3-2 is part of a shallow stream and has intermediate salinity, and H3-3 is at the beginning of a hypersaline lagoon. (Pictures by Patrick Fink)

Table 3.1: Nutrient content of the sampled sites at Salar de Huasco.

	H3-1		H3-2		H3-3	
Nitrite [µg L^{-1}]	1.19	-	0.95	-	2.12	-
Nitrate [µg L^{-1}]	0.05	-	0.14	± 0.01	0.19	-
Ammonium [µg L^{-1}]	1.25	± 0.88	51.88	± 3.54	93.13	± 3.54
Phosphate [µg L^{-1}]	36.57	± 1.01	132.57	± 8.08	155.43	± 8.08
Sulfate [mg L^{-1}]	176.68	± 1.49	970.24	± 2.24	996.62	± 76.87
Silica [mg L^{-1}]	91.60	-	33.42	± 0.06	129.77	-
Sodium [mg L^{-1}]	648.47	± 1.96	291.03	± 4.57	918.67	± 2.77
Potassium [mg L^{-1}]	42.28	± 0.07	11.22	± 0.29	59.89	± 0.10
Magnesium [mg L^{-1}]	37.49	± 0.77	50.15	± 0.37	53.11	± 1.09
Calcium [mg L^{-1}]	58.56	± 0.21	21.10	± 0.56	82.95	± 0.29
Chloride [mg L^{-1}]	63.81	-	4,176.01	-	7,224.71	-

Figure 3.7: Pictures of Salar de Huasco during the summer and winter sampling. (Summer picture by Patrick Fink)

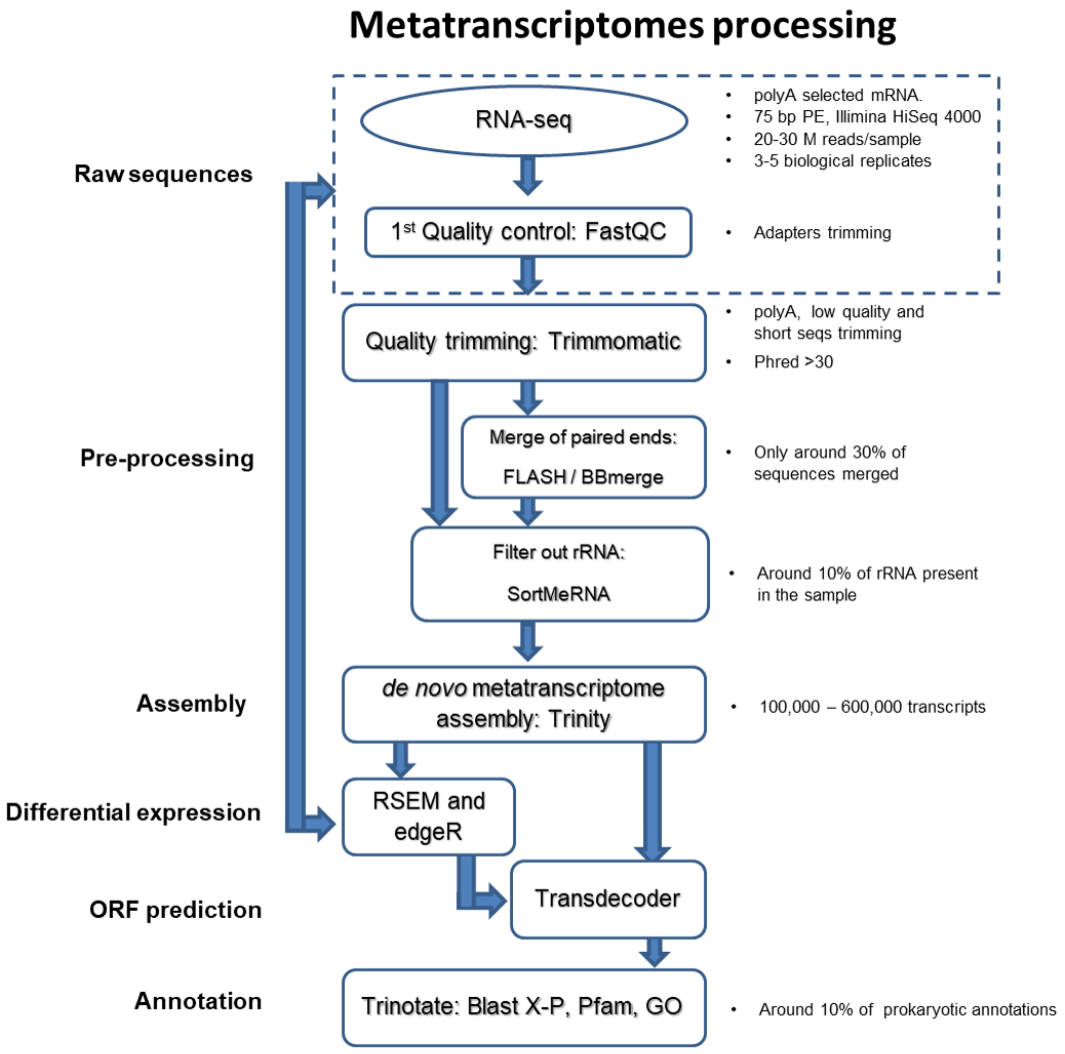

Figure 3.8: Methodology flow chart of the metatranscriptomes analyses.

Table 3.2: Enriched GO terms, found shared or unique between the sampling sites. Annotations correspond to differentially expressed genes along the salinity gradient.

Samples	Shared	GO terms	
S1morning	1	negative regulation of TOR signaling	
S3noon			
S1morning	2	molecular function	
W3noon		plastid	
S1morning	6	chloroplast	
W1noon		organic substance biosynthetic process	
W3noon		oxidation-reduction process	
		oxidoreductase activity	
		polysaccharide catabolic process	
		ribonucleoprotein complex	
S1morning	15	carbohydrate metabolic process	
W1noon		cell wall organization	
		cell wall organization or biogenesis	
		cytoplasmic translation	
		external encapsulating structure organization	
		extracellular region	
		extrinsic component of membrane	
		filamentous growth	
		heme binding	
		interspecies interaction between organisms	
		multi-organism process	
		other organism part	
		oxygen evolving activity	
		pathogenesis	
		polysaccharide metabolic process	
S1morning	24	cation binding	regulation of generation of precursor metabolites and energy
S3noon		energy quenching	regulation of photosynthesis
W1noon		integral component of membrane	regulation of photosynthesis, light reaction
		intrinsic component of membrane	response to abiotic stimulus
		membrane	response to high light intensity
		metal ion binding	response to light intensity
		nonphotochemical quenching	response to light stimulus
		oxidoreductase complex	response to oxidative stress
		photosynthesis, light harvesting in photosystem I	response to photooxidative stress
		photosystem II oxygen evolving complex	response to radiation
		photosystem II stabilization	response to stimulus
		protein-DNA complex	response to stress
S3noon	29	ADP metabolic process	nucleoside diphosphate phosphorylation
W1noon		anion binding	nucleoside phosphate binding
W3noon		ATP generation from ADP	nucleotide binding
		catabolic process	nucleotide phosphorylation
		cell part	nucleus
		cellular component	organelle part
		chloroplast part	plastid part
		cytoplasmic part	protein disulfide isomerase activity
		glycolytic process	purine nucleoside diphosphate metabolic process
		hydrolase activity	purine ribonucleoside diphosphate metabolic process
		intracellular membrane-bounded organelle	ribonucleoside diphosphate metabolic process
		intracellular organelle part	small molecule binding
		intracellular part	thylakoid part
		membrane-bounded organelle	intramolecular oxidoreductase activity, transposing S-S bonds
		nucleoside diphosphate metabolic process	
S1morning	40	binding	metabolic process
S3noon		biological process	nitrogen compound metabolic process
W1noon		carbohydrate catabolic process	organelle
W3noon		catalytic activity	organic cyclic compound binding
		cellular macromolecule metabolic process	organic substance metabolic process
		cellular metabolic process	organonitrogen compound metabolic process
		cellular process	photosynthesis
		cellular protein metabolic process	photosynthesis, light harvesting
		cellular protein modification process	photosynthetic membrane
		chlorophyll binding	photosystem
		chloroplast thylakoid membrane	photosystem I
		cytoplasm	photosystem II
		generation of precursor metabolites and energy	plastid thylakoid membrane
		heterocyclic compound binding	primary metabolic process
		intracellular organelle	protein metabolic process
		ion binding	protein modification process
		light-harvesting complex	protein-chromophore linkage
		macromolecule metabolic process	protein-containing complex
		macromolecule modification	tetrapyrrole binding
		membrane protein complex	thylakoid membrane

Samples	Shared	GO terms	
W1noon	59	2 iron, 2 sulfur cluster binding	glyceraldehyde-3-phosphate dehydrogenase (NAD(P)+) phosphorylating activity
W3noon		amide biosynthetic process	glyceraldehyde-3-phosphate dehydrogenase (NAD+)phosphorylating activity
		ATP metabolic process	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
		biosynthetic process	peptide biosynthetic process
		carbohydrate derivative metabolic process	peptide metabolic process
		cellular amide metabolic process	photosystem I reaction center
		cellular aromatic compound metabolic process	purine nucleoside monophosphate metabolic process
		cellular biosynthetic process	purine nucleoside triphosphate metabolic process
		cellular macromolecule biosynthetic process	purine nucleotide metabolic process
		cellular nitrogen compound biosynthetic process	purine ribonucleoside monophosphate metabolic process
		cellular nitrogen compound metabolic process	purine ribonucleoside triphosphate metabolic process
		electron transfer activity	purine ribonucleotide metabolic process
		GTPase activity	purine-containing compound metabolic process
		heterocycle metabolic process	pyrophosphatase activity
		hydrolase activity, acting on acid anhydrides	pyruvate metabolic process
		intracellular non-membrane-bounded organelle	ribonucleoside binding
		intramolecular oxidoreductase activity	ribonucleoside monophosphate metabolic process
		large ribosomal subunit	ribonucleoside triphosphate metabolic process
		macromolecule biosynthetic process	ribonucleotide metabolic process
		non-membrane-bounded organelle	ribose phosphate metabolic process
		nucleic acid binding	ribosome
		nucleobase-containing compound metabolic process	RNA binding
		nucleoside binding	RNA metabolic process
		nucleoside monophosphate metabolic process	rRNA metabolic process
		nucleoside phosphate metabolic process	rRNA processing
		nucleoside triphosphate metabolic process	structural constituent of ribosome
		nucleoside-triphosphatase activity	structural molecule activity
		nucleotide metabolic process	translation
		organic cyclic compound metabolic process	translation factor activity, RNA binding
		organonitrogen compound biosynthetic process	
S3noon	118	ascospore formation	regulation of cellular biosynthetic process
W1noon		biological regulation	regulation of cellular macromolecule biosynthetic process
		carbohydrate derivative binding	regulation of cellular metabolic process
		cell cortex of cell tip	regulation of cellular process
		cell cortex of growing cell tip	regulation of chromosome organization
		cell cycle process	regulation of chromosome segregation
		cell division site	regulation of chromosome separation
		cell redox homeostasis	regulation of DNA-templated transcription in response to stress
		cellular component organization	regulation of endoplasmic reticulum unfolded protein response
		cellular response to heat	regulation of establishment of cell polarity regulating cell shape
		CENP-A containing chromatin	regulation of establishment or maintenance of cell polarity
		chromatin	regulation of establishment or maintenance of cell polarity regulating cell shape
		chromosomal part	regulation of gene expression
		cytosol	regulation of macromolecule biosynthetic process
		deactivation of mitotic spindle assembly checkpoint	regulation of macromolecule metabolic process
		DNA binding	regulation of metabolic process
		DNA-binding transcription factor activity	regulation of metaphase/anaphase transition of cell cycle
		DPS complex	regulation of mitotic cell cycle phase transition
		fungal-type vacuole	regulation of mitotic metaphase/anaphase transition
		fungal-type vacuole membrane	regulation of mitotic nuclear division
		intracellular signal transduction	regulation of mitotic sister chromatid segregation
		meiotic cell cycle process	regulation of mitotic sister chromatid separation
		membrane organization	regulation of nitrogen compound metabolic process
		membrane part	regulation of nuclear division
		molecular function	regulation of nucleic acid-templated transcription
		negative regulation of cell cycle phase transition	regulation of nucleobase-containing compound metabolic process
		negative regulation of cellular component organization	regulation of organelle organization
		negative regulation of chromosome organization	regulation of primary metabolic process
		negative regulation of chromosome segregation	negative regulation of metaphase/anaphase transition of cell cycle
		negative regulation of chromosome separation	negative regulation of mitotic metaphase/anaphase transition
		negative regulation of mitotic cell cycle phase transition	positive regulation of DNA-templated transcription, termination
		negative regulation of mitotic nuclear division	positive regulation of establishment of cell polarity regulating cell shape
		negative regulation of mitotic sister chromatid segregation	positive regulation of establishment or maintenance of cell polarity regulating cell shape
		negative regulation of mitotic sister chromatid separation	positive regulation of metaphase/anaphase transition of cell cycle
		negative regulation of nuclear division	positive regulation of mitotic metaphase/anaphase transition
		negative regulation of organelle organization	positive regulation of termination of RNA polymerase II transcription
		negative regulation of sister chromatid segregation	positive regulation of transcription from RNA polymerase II promoter in response to heat stres
		negative regulation of spindle checkpoint	positive regulation of transcription from RNA polymerase II promoter in response to stress
		non-growing cell tip	regulation of protein localization to cell tip
		nuclear chromatin	regulation of RNA biosynthetic process
		nuclear chromosome part	regulation of RNA metabolic process
		phosphate-containing compound metabolic process	regulation of sister chromatid segregation
		phosphorelay response regulator activity	regulation of termination of RNA polymerase II transcription
		phosphorylation	regulation of transcription by RNA polymerase II
		positive regulation of biosynthetic process	regulation of transcription from RNA polymerase II promoter in response to oxidative stress
		positive regulation of cellular biosynthetic process	regulation of transcription from RNA polymerase II promoter in response to stress
		positive regulation of chromosome segregation	regulation of transcription, DNA-templated
		positive regulation of chromosome separation	reproductive process in single-celled organism
		positive regulation of macromolecule biosynthetic process	response to singlet oxygen
		positive regulation of mitotic sister chromatid separation	ribonucleotide binding
		positive regulation of protein localization to cell tip	RNA polymerase II proximal promoter sequence-specific DNA binding
		positive regulation of transcription by RNA polymerase II	RNA polymerase II regulatory region DNA binding
		protein phosphatase type 1 complex	RNA polymerase II regulatory region sequence-specific DNA binding
		proximal promoter sequence-specific DNA binding	sexual sporulation
		purine nucleotide binding	sexual sporulation resulting in formation of a cellular spore
		purine ribonucleoside triphosphate binding	sporulation
		purine ribonucleotide binding	sporulation resulting in formation of a cellular spore
		regulation of biological process	storage vacuole
		regulation of biosynthetic process	transcription regulatory region sequence-specific DNA binding

Samples	Unique	GO terms	
S1morning	13	pectate lyase activity	
		iron ion binding	
		oxidoreductase activity, acting on the CH-OH group of donors, oxygen as acceptor	
		filamentous growth of a population of unicellular organisms in response to biotic stimulus	
		carbon-oxygen lyase activity, acting on polysaccharides	
		galactose oxidase activity	
		cellular component	
		filamentous growth of a population of unicellular organisms	
		cellular response to osmotic stress	
		intracellular	
		thylakoid	
		photosynthesis, light reaction	
		response to toxic substance	
S3noon	86	adenyl nucleotide binding	mRNA cleavage and polyadenylation specificity factor complex
		adenyl ribonucleotide binding	positive regulation of nitrogen compound metabolic process
		anatomical structure formation involved in morphogenesis	positive regulation of nucleobase-containing compound metabolic process
		ATP binding	positive regulation of RNA biosynthetic process
		attachment of mitotic spindle microtubules to kinetochore	positive regulation of RNA metabolic process
		attachment of spindle microtubules to kinetochore	positive regulation of transcription, DNA-templated
		autophagosome lumen	protein binding
		bounding membrane of organelle	protein serine/threonine phosphatase activity
		catalytic complex	PTW/PP1 phosphatase complex
		cell	purine nucleobase binding
		cellular response to stimulus	purine nucleobase transmembrane transporter activity
		cellular response to stress	purine nucleobase transport
		double-stranded DNA binding	pyridine nucleotide metabolic process
		glutathione hydrolase complex	pyridine-containing compound metabolic process
		lysosomal microautophagy	regulation of cell cycle checkpoint
		mitochondrion	regulation of cellular component organization
		mRNA cleavage factor complex	regulation of chromatin organization
		negative regulation of binding	regulation of chromatin silencing
		negative regulation of cell cycle checkpoint	regulation of DNA recombination
		negative regulation of cellular process	regulation of DNA-templated transcription, termination
		negative regulation of chromatin organization	regulation of establishment of protein localization to chromosome
		negative regulation of chromatin silencing	regulation of gene silencing
		negative regulation of gene silencing	regulation of mitotic cell cycle spindle assembly checkpoint
		negative regulation of phospholipid biosynthetic process	regulation of mitotic spindle checkpoint
		negative regulation of phospholipid metabolic process	regulation of phospholipid biosynthetic process
		negative regulation of TORC1 signaling	regulation of spindle checkpoint
		nicotinamide nucleotide metabolic process	regulation of translation in response to stress
		nuclear envelope organization	regulatory region nucleic acid binding
		nucleobase binding	response to heat
		nucleobase transmembrane transporter activity	RNA polymerase II CTD heptapeptide repeat phosphatase activity
		nucleobase transport	salt transmembrane transporter activity
		nucleoside-triphosphatase regulator activity	sequence-specific double-stranded DNA binding
		organelle membrane	transcription regulatory region DNA binding
		oxidoreduction coenzyme metabolic process	transferase activity
		phosphorus metabolic process	ubiquitin-like protein transferase activity
		polyphosphate metabolic process	urate transmembrane transporter activity
		positive regulation of cellular metabolic process	urate transport
		positive regulation of chromosome organization	vacuolar lumen
		positive regulation of gene expression	vacuolar transport
		positive regulation of gene expression, epigenetic	vacuolar transporter chaperone complex
		positive regulation of macromolecule metabolic process	vacuole fusion, non-autophagic
		positive regulation of metabolic process	xanthine transmembrane transporter activity
		positive regulation of nucleic acid-templated transcription	xanthine transport
W3noon	23	autophagosome membrane	
		cellular catabolic process	
		coenzyme binding	
		cofactor binding	
		extracellular non-membrane-bounded organelle	
		glucose metabolic process	
		glycosome	
		macromolecule catabolic process	
		microbody	
		mRNA cap binding	
		mRNA catabolic process	
		NAD binding	
		NADP binding	
		organic acid metabolic process	
		organic substance catabolic process	
		organophosphate metabolic process	
		oxidoreductase activity, acting on the aldehyde or oxo group of donors	
		oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	
		oxidoreductase activity, acting on the CH-CH group of donors	
		oxoacid metabolic process	
		peroxisome	
		RNA 7-methylguanosine cap binding	
		trichocyst	

Samples	Unique	GO terms	
W1noon	456	active ion transmembrane transporter activity	ATPase activity, coupled to transmembrane movement of ions, rotational mechanism
		actomyosin contractile ring, proximal layer	cell morphogenesis involved in conjugation with cellular fusion
		amino sugar biosynthetic process	energy coupled proton transport, down electrochemical gradient
		amino sugar catabolic process	establishment or maintenance of bipolar cell polarity regulating cell shape
		amino sugar metabolic process	establishment or maintenance of cell polarity regulating cell shape
		aminoglycan catabolic process	maintenance of transcriptional fidelity during DNA-templated transcription elongation
		aminoglycan metabolic process	maintenance of transcriptional fidelity during DNA-templated transcription elongation from RNA polymerase III promoter
		anchored component of membrane	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, LSU-rRNA,5S)
		anchored component of plasma membrane	monovalent inorganic cation transmembrane transporter activity
		antioxidant activity	negative regulation of cell cycle switching, mitotic to meiotic cell cycle
		arginine catabolic process to glutamate	negative regulation of cytoplasmic mRNA processing body assembly
		arginine catabolic process to proline	negative regulation of induction of conjugation with cellular fusion
		arginine catabolic process to proline via ornithine	negative regulation of microtubule polymerization or depolymerization
		ascospore-type prospore membrane assembly	negative regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay
		asexual sporulation	negative regulation of nuclear-transcribed mRNA poly(A) tail shortening
		ATP biosynthetic process	nuclear membrane biogenesis involved in mitotic nuclear division
		ATP synthesis coupled proton transport	oxidoreductase activity, acting on diphenols and related substances as donors
		ATPase coupled ion transmembrane transporter activity	oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor
		blue light photoreceptor activity	oxidoreductase activity, acting on diphenols and related substances as donors, with copper protein as acceptor
		blue light signaling pathway	oxidoreductase activity, acting on iron-sulfur proteins as donors, NAD or NADP as acceptor
		box H/ACA snoRNA 3'-end processing	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, another compound as one donor, and incorporation of one atom of oxygen
		box H/ACA snoRNA processing	oxidoreductase activity, acting on superoxide radicals as acceptor
		budding cell apical bud growth	photosystem II oxygen evolving complex assembly
		budding cell bud growth	plasma membrane fusion involved in cytogamy
		C4-dicarboxylate transport	plasma membrane of cell tip
		calcium-mediated signaling	plastid envelope
		carbohydrate biosynthetic process	plastid thylakoid lumen
		carbohydrate import across plasma membrane	plastoglobule
		carbon fixation	plastoquinol--plastocyanin reductase activity
		carbon-carbon lyase activity	poly(A) binding
		carboxy-lyase activity	poly(U) RNA binding
		cation transmembrane transport	poly-purine tract binding
		cation transmembrane transporter activity	poly-pyrimidine tract binding
		cation transport	positive regulation of (1->3)-beta-D-glucan biosynthetic process
		cation-transporting ATPase activity	positive regulation of cell cycle
		Cdc42 GTPase complex	positive regulation of cell cycle phase transition
		Cdc42 protein signal transduction	positive regulation of cell cycle process
		cell cortex part	positive regulation of cellular amide metabolic process
		cell cortex region	positive regulation of cytoplasmic translation
		cell division site part	positive regulation of exonuclease activity
		cell morphogenesis involved in conjugation	positive regulation of exoribonuclease activity
		cell septum	positive regulation of L-arginine import across plasma membrane
		cell tip	positive regulation of mitotic cell cycle
		cell wall	positive regulation of mitotic cell cycle phase transition
		cellular bud	positive regulation of mitotic nuclear division
		cellular bud neck	positive regulation of nuclear division
		cellular bud neck septin collar	positive regulation of nuclease activity
		cellular component assembly	positive regulation of penicillin biosynthetic process
		cellular component biogenesis	positive regulation of penicillin metabolic process
		cellular component organization or biogenesis	positive regulation of primary cell septum biogenesis
		cellular detoxification	positive regulation of protein complex disassembly
		cellular oxidant detoxification	positive regulation of translational elongation
		cellular protein-containing complex assembly	positive regulation of translational initiation
		cellular response to anoxia	positive regulation of translational termination
		cellular response to blue light	post-mRNA release spliceosomal complex
		chitin binding	posttranscriptional regulation of gene expression
		chitin biosynthetic process	prospore membrane
		chitin catabolic process	prospore membrane biogenesis
		chitin deacetylase activity	prospore membrane leading edge
		chitin metabolic process	prospore membrane spindle pole body attachment site
		chitin synthase activity	protein heterodimerization activity
		chloroplast envelope	protein lipoylation
		chloroplast thylakoid lumen	protein localization to cell cortex
		chromatin assembly or disassembly	protein localization to cell cortex of cell tip
		cleavage apparatus septin structure	protein localization to cell tip
		conidium formation	protein localization to division septum
		conjugation	protein localization to growing cell tip
		copper ion binding	protein localization to new growing cell tip
		cortical dynamic polarity patch	protein tag
		cytochrome b6f complex	protein-containing complex assembly
		cytochrome-c peroxidase activity	protein-containing complex binding
		cytokinesis, site selection	protein-containing complex subunit organization
		cytokinetic process	protein-DNA complex assembly
		cytoplasmic microtubule plus-end	protein-DNA complex subunit organization
		cytoplasmic ribonucleoprotein granule	proton export across plasma membrane
		cytoplasmic stress granule	proton transmembrane transport
		cytoplasmic translational elongation	proton transmembrane transporter activity
		cytoskeleton	proton-transporting ATP synthase activity, rotational mechanism
		cytosolic large ribosomal subunit	proton-transporting ATP synthase complex, catalytic core F(1)
		cytosolic part	proton-transporting two-sector ATPase complex, catalytic domain
		cytosolic small ribosomal subunit	proton-transporting two-sector ATPase complex, proton-transporting domain
		deacetylase activity	proton-transporting V-type ATPase, V0 domain
		defense response to bacterium	purine nucleoside binding
		defense response to other organism	purine nucleoside monophosphate biosynthetic process
		dense fibrillar component	purine nucleoside triphosphate biosynthetic process
		detoxification	purine nucleotide biosynthetic process
		division septum	purine ribonucleoside binding

Samples	Unique	GO terms	
W1noon		DNA binding, bending	purine ribonucleoside monophosphate biosynthetic process
		DNA packaging complex	purine ribonucleoside triphosphate biosynthetic process
		DNA replication-independent nucleosome organization	purine ribonucleotide biosynthetic process
		DNA-templated transcription, elongation	purine-containing compound biosynthetic process
		endopeptidase activity	RAVE complex
		envelope	reactive oxygen species metabolic process
		establishment of localization	reductive pentose-phosphate cycle
		establishment of RNA localization	regulation of (1->3)-alpha-glucan biosynthetic process
		establishment or maintenance of actin cytoskeleton polarity	regulation of (1->3)-alpha-glucan metabolic process
		establishment or maintenance of cell polarity	regulation of actin cortical patch localization
		establishment or maintenance of cytoskeleton polarity	regulation of alpha-glucan biosynthetic process
		external encapsulating structure	regulation of alpha-glucan metabolic process
		ferredoxin-NADP+ reductase activity	regulation of amino acid import across plasma membrane
		fumarate transmembrane transporter activity	regulation of amino acid transmembrane transport
		fungal biofilm matrix	regulation of asexual sporulation
		fungal-type cell wall	regulation of cell cycle
		fungal-type cell wall biogenesis	regulation of cell cycle phase transition
		fungal-type cell wall organization	regulation of cell cycle process
		fungal-type cell wall organization or biogenesis	regulation of cell morphogenesis
		fungal-type cell wall polysaccharide biosynthetic process	regulation of cell septum assembly
		fungal-type cell wall polysaccharide metabolic process	regulation of cell shape
		glucosamine-containing compound biosynthetic process	regulation of cell wall (1->3)-alpha-glucan biosynthetic process
		glucosamine-containing compound catabolic process	regulation of cell wall (1->3)-alpha-glucan metabolic process
		glucosamine-containing compound metabolic process	regulation of cell wall (1->3)-beta-D-glucan biosynthetic process
		glutathione-disulfide reductase activity	regulation of cellular amide metabolic process
		glyoxysome	regulation of conidium formation
		G-protein beta/gamma-subunit complex binding	regulation of conjugation
		growing cell tip	regulation of conjugation with cellular fusion
		GTP binding	regulation of cytokinesis
		GTPase complex	regulation of cytokinetic process
		guanyl nucleotide binding	regulation of cytoplasmic translation
		guanyl ribonucleotide binding	regulation of cytoplasmic translational elongation
		heterocycle biosynthetic process	regulation of cytoplasmic translational initiation
		heterotrimeric G-protein complex	regulation of division septum assembly
		host cell nucleus	regulation of exonuclease activity
		host cell part	regulation of exoribonuclease activity
		host intracellular membrane-bounded organelle	regulation of fungal-type cell wall (1->3)-alpha-glucan biosynthetic process
		host intracellular organelle	regulation of fungal-type cell wall (1->3)-alpha-glucan metabolic process
		host intracellular part	regulation of L-arginine import
		hydrogen sulfide biosynthetic process	regulation of L-arginine import across plasma membrane
		hydrogen sulfide metabolic process	regulation of leucine import
		hydrolase activity, hydrolyzing O-glycosyl compounds	regulation of leucine import across plasma membrane
		hyphal cell wall	regulation of mitotic cell cycle
		hyphal growth	regulation of mitotic cytokinesis
		incipient cellular bud site	regulation of mitotic cytokinetic process
		inorganic cation transmembrane transport	regulation of mRNA stability involved in response to oxidative stress
		inorganic cation transmembrane transporter activity	regulation of mRNA stability involved in response to stress
		inorganic diphosphatase activity	regulation of nuclease activity
		inorganic ion transmembrane transport	regulation of penicillin biosynthetic process
		intracellular organelle lumen	regulation of penicillin metabolic process
		intrinsic component of plasma membrane of cell tip	regulation of photosynthesis, dark reaction
		ion transmembrane transport	regulation of primary cell septum biogenesis
		ion transmembrane transporter activity	regulation of reductive pentose-phosphate cycle
		ion transport	regulation of response to endoplasmic reticulum stress
		isomerase activity	regulation of ribonuclease activity
		large ribosomal subunit rRNA binding	regulation of strand invasion
		lipoate synthase activity	regulation of translation
		lipoate-protein ligase activity	removal of superoxide radicals
		localization	reproductive fruiting body development
		malate synthase activity	respiratory chain
		mannan endo-1,6-alpha-mannosidase activity	response to bacterium
		mannose transmembrane transporter activity	response to cycloheximide
		mating projection tip	response to temperature stimulus
		maturation of LSU-rRNA	ribonucleoprotein complex assembly
		medial cortex septin ring	ribonucleoprotein complex binding
		medial membrane band	ribonucleoprotein complex biogenesis
		membrane biogenesis	ribonucleoprotein complex subunit organization
		membrane fusion	ribonucleoprotein granule
		membrane-enclosed lumen	ribonucleoside monophosphate biosynthetic process
		microtubule bundle	ribonucleoside triphosphate biosynthetic process
		microtubule cytoskeleton	ribonucleotide biosynthetic process
		microtubule plus-end	ribose phosphate biosynthetic process
		mismatch repair complex binding	ribosomal large subunit assembly
		mitochondrial inner membrane	ribosomal small subunit assembly
		mitochondrial membrane	ribosomal subunit
		mitochondrial part	ribosome binding
		mitotic cytokinesis, site selection	ribosome biogenesis
		mitotic cytokinetic process	ribulose-1,5-bisphosphate carboxylase/oxygenase activator activity
		mitotic septin complex	ribulose-bisphosphate carboxylase activity
		mitotic spindle pole body	RNA 3'-end processing
		monooxygenase activity	RNA biosynthetic process
		monophenol monooxygenase activity	RNA polymerase II preinitiation complex assembly
		monovalent inorganic cation transport	RNA polymerase III preinitiation complex assembly
		mRNA 3'-UTR binding	RNA processing
		mRNA binding	RNA transport
		mRNA metabolic process	rRNA binding
		mRNA pseudouridine synthesis	SCF ubiquitin ligase complex
		mRNA transport	SCF-dependent proteasomal ubiquitin-dependent protein catabolic process

Samples	Unique	GO terms	
W1noon		MutSalpha complex binding	second-messenger-mediated signaling
		ncRNA metabolic process	septin collar
		ncRNA processing	septin complex
		negative regulation of cell cycle	septin cytoskeleton
		negative regulation of cell cycle process	septin cytoskeleton organization
		negative regulation of conjugation	septin ring
		negative regulation of conjugation with cellular fusion	septin ring assembly
		negative regulation of exonuclease activity	septin ring organization
		negative regulation of exoribonuclease activity	sequence-specific DNA binding
		negative regulation of gene expression	serine hydrolase activity
		negative regulation of microtubule polymerization	serine-type endopeptidase activity
		negative regulation of mRNA catabolic process	serine-type peptidase activity
		negative regulation of nuclease activity	single-stranded DNA-dependent ATP-dependent DNA helicase complex
		negative regulation of reductive pentose-phosphate cycle	single-stranded RNA binding
		negative regulation of ribonuclease activity	site of polarized growth
		negative regulation of RNA catabolic process	small GTPase mediated signal transduction
		negative regulation of RNA metabolic process	small nucleolar ribonucleoprotein complex
		negative regulation of transcription by RNA polymerase II	small ribosomal subunit
		nickel cation binding	SNAP receptor activity
		nuclear membrane biogenesis	SNARE binding
		nuclear SCF ubiquitin ligase complex	SNARE complex
		nuclear ubiquitin ligase complex	snRNA modification
		nucleic acid metabolic process	snRNA pseudouridine synthase activity
		nucleic acid transport	snRNA pseudouridine synthesis
		nucleobase-containing compound biosynthetic process	spindle pole body
		nucleobase-containing compound transport	spore-bearing structure development
		nucleobase-containing small molecule metabolic process	sporocarp development
		nucleoside monophosphate biosynthetic process	sporocarp development involved in sexual reproduction
		nucleoside triphosphate biosynthetic process	static microtubule bundle
		nucleosome	sterol regulatory element binding protein cleavage
		nucleosome assembly	stromule
		nucleosome binding	succinate:fumarate antiporter activity
		nucleosome organization	sulfate assimilation
		old cell tip after activation of bipolar cell growth	sulfate assimilation via adenylyl sulfate reduction
		old growing cell tip	sulfate assimilation, phosphoadenylyl sulfate reduction by phosphoadenylyl-sulfate reductase (thioredoxin)
		organelle envelope	sulfate reduction
		organelle fusion	superoxide dismutase activity
		organelle inner membrane	superoxide metabolic process
		organelle lumen	Tea1 cell-end complex
		organelle membrane fusion	thylakoid lumen
		ornithine-oxo-acid transaminase activity	transcription elongation from RNA polymerase II promoter
		peptidase activity	transcription preinitiation complex assembly
		peptidase activity, acting on L-amino acid peptides	translation elongation factor activity
		pexophagy	translational elongation
		phosphoadenylyl-sulfate reductase (thioredoxin) activity	translational frameshifting
		phosphoglycerate kinase activity	transmembrane transport
		phosphotransferase activity, carboxyl group as acceptor	transmembrane transporter activity
		photorespiration	transport
		photosynthesis, dark reaction	transporter activity
		photosynthetic electron transport chain	tRNA folding
		photosynthetic electron transport in photosystem I	vacuole inheritance
		photosynthetic electron transport in photosystem II	vesicle fusion
		photosystem II assembly	yeast-form cell wall

Record of achievement and publications

Chapter 1:

Metatranscriptomics from a small aquatic system: microeukaryotic community functions through the diurnal cycle. Trench-Fiol S and Fink P (2020) Front. Microbiol. 11:1006. doi: 10.3389/fmicb.2020.01006

Samplings and processing, as the complete metatranscriptome analyses for the results described in this chapter were exclusively performed by me or under my direct supervision. Patrick Fink was involved in conceiving the study, discussing the design, and has critically read and contributed to the published manuscript.

Chapter 2:

Eukaryotic microbial communities and functions under extreme conditions – A metatranscriptomic approach in a high altitude hypersaline wetland (Salar de Huasco, Chile). Trench-Fiol S, Dorador C, and Fink P.

In preparation

Two sampling campaigns were performed in Chile to obtain the dataset described in this chapter, one was carried out by Patrick Fink (summer) and one by me (winter). The processing of the samples and the complete metatranscriptome analyses were performed exclusively by me or under my direct supervision. Patrick Fink was involved in technical discussions for the sampling campaigns and has critically read and contributed to the manuscript in preparation. Cristina Dorador contributed to the summer sampling and has critically read the manuscript in preparation.

Chapter 3:

The role of microeukaryotic groups for functional processes in a polyextreme high altitude aquatic system. Trench-Fiol S, Hengst M, Dorador C, Marandino C, and Fink P.

In preparation

The dataset described in this chapter, corresponds to the same samples used for chapter II. The analyses of the metatranscriptomic data, and the sulfurous compounds measurement were performed exclusively by me or under my direct supervision. Patrick Fink was involved in technical discussions and has critically read and contributed to the manuscript in preparation. Christa Marandino provided the analytical facilities and guidance for the analyses of sulfurous compounds. Martha Hengst and Cristina Dorador contributed to the summer sampling and have critically read the manuscript in preparation.

Erklärung zur Dissertation

gemäß der Promotionsordnung vom 12. März 2020

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

[1]Trench-Fiol S and Fink P (2020) Metatranscriptomics from a small aquatic system: microeukaryotic community functions through the diurnal cycle. Front. Microbiol. 11:1006.

[1] entspricht Chapter I

Köln, den 26. Februar 2022

Stephanie Trench Fiol

Declaration for the doctoral thesis

According to the doctoral regulations published on 12th March 2020

According to the doctoral regulations published on 12th March 2020

"I hereby declare that I have completed the present dissertation independently and without the use of any aids or literature other than those referred to. All passages that have been taken, either literally or in a sense, from published and unpublished works, are marked as such. I declare that this dissertation has not been submitted to any other faculty or university; that - apart from the partial publications and included articles and manuscripts listed below - it has not yet been published, and that I will not publish the dissertation before completing my doctorate without the permission of the Ph.D. Committee. I am aware of the terms of the doctoral regulations. In addition, I hereby declare that I am aware of the "Regulations for Safeguarding Good Scientific Practice and Dealing with Scientific Misconduct" of the University of Cologne, and that I have observed them during the work on the thesis project and the written doctoral thesis. I hereby commit myself to observe and implement the guidelines mentioned there in all scientific activities. I assure that the submitted electronic version is identical to the submitted printed version".

Partial publications of the thesis:

[1]Trench-Fiol S and Fink P (2020) Metatranscriptomics from a small aquatic system: microeukaryotic community functions through the diurnal cycle. Front. Microbiol. 11:1006.

[1] corresponds to Chapter I

Cologne, the 26th of February 2022

Stephanie Trench Fiol

Acknowledgements

Firstly, I want to acknowledge my supervisor PD Dr. Patrick Fink. Thank you for accepting me as your student even when you didn't know me and I still didn't have a clear structure of the project but the idea and the desire to apply for the scholarship and come abroad. I think this process has been an adventure, with lows and highs, not only for me. Thanks for understanding and supporting; for always trying to guide me in the right direction, even if it was not an easy task considering my different background and expertise. Through conversations and discussions, I have learned so much all these years. And I admire your enthusiasm and passion for science, which motivated me many times.

I also want to thank Prof. Dr. Hartmut Arndt, for accepting to be the second reviewer of my thesis in such a short-term notice. I hope we can have an interesting discussion about the salares.

I am very grateful to Dr. Martha Hengst and Dr. Cristina Dorador, who taught me so much and guided me during my studies in Antofagasta, both helped me to create this project and supported my scholarship application.

Sampling campaigns in extreme ecosystems would not be possible without the help of a great team. Muchas gracias to everyone who contributed to the hard work in Salar de Huasco, especially: Johanna Cortés, Pablo Arán, Eduardo Acosta, Nicolas Miranda, Jonathan Garcia, Alejandro Angel, Chris Harrod.

To Prof. Dr. Eric von Elert, thank you for allowing me to be part of your workgroup and providing me with what I needed for work. Also, for your kindness to me and the delicious Christmas dinners prepared with your lovely wife.

Thomas von Einem and Katja Preuss, who were always there for any question or request I had, thank you. I enjoyed the mensa and coffee time, and I am grateful for always learning new german words with you. To Sascha Dopf, who came later but still was so helpful and always having a good disposition, thank you.

Also thanks to Carlos Sanchez, although we didn't share so much, I always appreciated la buena onda de otro latino en el grupo. Thank you for accepting being part of my thesis committee.

The most important part of the workgroup: all the Ph.D. collegues (half of you already made it through!). Thank you Dr. Christian Bubu Burberg, Jacqueline Lange, Dr. Sandra Klinthworth, Meike Hahn, Christian the first Werner. Ihr seid alle echt Toll! I appreciated the

great atmosphere, the Karneval celebrations, the Christmas wreath crafting and the Glühwein, and the moments and laughs for stupid and nerdy things. I learned a lot of aquatic ecology thanks to you!

To Dr. Maja Illic, Dr. Alessandra Iannino, and Jessica Titocci, my Ph.D. sisters, I am so glad I met you! It was definitely not only being "ausländer" what brought us closer but something else. Gracias Alex, my desk neighbor, for making me feel welcomed at the beginning by talking to me in Spanish and also explaining everything I asked. Thank you Maja bonita for many talks (really your thing!), for making me practice mein Deutsch mit dir, and for your hugs and delicious sweets. Thank you bella Jess, for your joy and kindness and so many details you had with me. I also enjoyed dancing with you and learning about the delicious Italian food. I treasure so many great moments (the not so great as well, is part of our story!) shared with the three of you.

This project and thesis would not have been possible without the awarded doctoral fellowship "DAAD/BECAS Chile" by the Deutscher Akademischer Austausch Dienst and the Chilean National Research and Development Agency (ANID).

To my beloved family:

Mamá, I am the resourceful person I am thanks to you. Siempre eres la primera en mi lista de agradecimientos, porque aún no alcanzan las palabras para agradecerte por tanto en mi vida: me inspiraste a ser una persona respetuosa con las personas y la naturaleza, responsable, motivaste desde pequeña mi curiosidad y mi creatividad, entre tantas cosas. Todas me han servido no solo como científica, sino en todos los aspectos de mi vida, y se han transformado en fortalezas.

A mi amada abuela, que aunque me hagas mucha falta y no compartas este logro conmigo en la tierra, sé que estarías muy orgullosa de mi, como siempre, y dándome aliento para llegar a esta meta. Siempre sentí tu apoyo en cada decisión que tomara.

A mi padre le agradezco por mi gran fortaleza, por su apoyo en los estudios y en la búsqueda de este sueño de venir a estudiar al extranjero.

Hermanitos queridos: Caroline, Benji y Nico. Los adoro y los tengo siempre presente en mi vida, gracias por su apoyo y preocupación y por mis adorados sobrinitos.

Last but not at all least:

to my partner Alfonso and my engine of life, Arantxita. Ambos me ayudaron a mantener la motivación al final de esta aventura. Continuaron dándome empujoncitos y recordándome que era capaz de lograr lo que me propusiera. Aguantaron y experimentaron conmigo mis altos y bajos (y muchos bajos últimamente), y me ayudaron a sobrellevarlo en tantas formas. No habría logrado entregar esta tesis en tiempo record sin los cafecitos, las frazaditas, los chocolatitos, los abracitos y todo lo necesario para mantenerme en el camino tras mi objetivo, gracias.

Arantxita, mi amorcita, eres mi compañerita de vida y aventuras. Y realmente mi mayor motor de vida: el que me impulsa siempre a ir por más, a buscar lo mejor, y nunca rendirme. Soy la mujer que soy gracias a tí en muchos sentidos.

Este logro también es suyo.

Stephanie Trench Fiol

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2017 - 2021 **Promotion in Biologie Fach**, Universität zu Köln, Deutschland
Titel: "*The role of microbial eukaryotic communities from inland aquatic systems, a metatranscriptomic approach focussed on solar radiation and salinity.*" (In English)

2009 - 2014 **B.Sc. in Biotechnologie**, Universidad de Antofagasta, Chile. (Fünfjahresabschluss)
Titel: "*Epibiont bacterial microbiome associated to Octopus mimus (Gould, 1852) mucus: Caracterization of the first inmunity bareer for the aquaculture sustainability of the north octopus*" (In Spanish)

2004 - 2007 **High school.** Colegio Parroquial Padre Negro, Caldera, Chile. Und Colegio Cervantino, Copiapó, Chile.
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Trench-Fiol S. und Fink, P (2019) "*Extreme irradiance levels in a high-altitude ecosystem affect functional gene expression profiles in aquatic microeukaryotes"* (Vortrag, in English); ASLO Aquatic Sciences Meeting. San Juan, Puerto Rico.

Trench-Fiol S., Fink P., und Dorador C. (2018) "*Metatranscriptomics from a high-UV aquatic system reveals the contribution of microeukaryotes to the sulfur cycle"* (Poster, in English); ISMSM5 International Symposium on Microbial Sulfur Metabolism. Wien, Österreich.

Auszeichnungen

2018 **Tagung Beihilfe** von „Federation of European Microbiological Societies (FEMS)" für das International symposium of sulfur microbes (ISMSM).

2016 - 2021 **Promotionsstipendium** „DAAD/BECAS Chile" von der Deutscher Akademischer Austauschdienst, und die Chilenische Nationale Agentur für Forschung und Entwicklung (ANID), mit einem 6-monatigen deutschkurs.

2014 - 2015 **Forschungsförderung** für „Diplomarbeit oder Titulationstätigkeit" der Universidad de Antofagasta.

2014 **English Sprachkurs** „CORFO English Course" der chilenischen Regierung. 200-Stunden-Kurs plus TOEIC-Test.