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Review Article

Recent Advances in Application of Transcriptomics: Research on Heterotrophic and Autotrophic Protists

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Abstract. The application of molecular phylogenetics to research on protists has substantially transformed our understanding of their evolution and systematics. More recently, advances in molecular technology, including high throughput sequencing, has opened new avenues for genomic analyses that elucidate major aspects of protistan biology across all levels of biological organization from cellular to ecosystems. This is a review of recent advances (particularly in the last two decades) of transcriptomic research on heterotrophic and autotrophic protists within three major topics: (i) Physiology and metabolism, (ii) Development and life cycles, and (iii) Environmental and ecological studies. Emphasis is placed on selection of representative research that highlights findings across diverse taxonomic groups within each of the three topics. Examples are drawn from parasitic as well as free-living taxa to provide a broad overview of some of the research strategies, and major findings, that have emerged from application of transcriptomics and related techniques in advancing our understanding of protistan biology.

Keywords: Algae, autotrophy, ecology, ecosystems, functional groups, genomics, heterotrophic protists, metabolism, parasitism, physiology

INTRODUCTION

With the advent of modern molecular genomic research, remarkable advances have been made in almost every domain of biological research across all major taxonomic groups. Most notably, research on protists has been substantially enhanced through high-powered methods of 'Omics' that have contributed to greater depth of understanding of the molecular basis of biological topics from cellular to ecosystems levels. This is a review of representative current research, largely within the last two decades, that specifically addresses transcriptomics research with heterotrophic and autotrophic protists within three broad categories: (i) Physiology and metabolism, (ii) Development and life cycles, and (iii) Environmental and ecological studies. Representative published studies were selected based on online searches using keywords such as 'Transcriptomes and protozoa,' 'Transcriptomes and algae,' 'Transcriptomes and protists.' Additional detailed searches were done adding specific areas of biological inquiry such as 'Transcriptomes and metabolism and protists,' 'Transcriptomes and cell cycle and protists,' or 'Transcriptomes and ecology and protists.' Review of research on heterotrophic protists is presented first followed by

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studies on autotrophic protists. A final section presents conclusions including some suggestions for new approaches to future research.

HETEROTROPHIC PROTISTS

Physiology and metabolism

A relatively small number of citations were found for transcriptomics research on heterotrophic protists within the scope of this review. A summary of findings for free living species is presented first followed by findings for parasitic species.

An interesting study by Gerber *et al.* (2021) examined transcriptome evidence of differentiated encoding of proteins by nuclei at different locations in the amoeboid, plasmodial (syncytial) stage of the slime mold *Physarum polycephalum* (Amoebozoa) in laboratory culture. The plasmodia were grown under different environmental conditions, and single-nucleus RNA-sequencing was used to dissect gene expression heterogeneity among nuclei. Consequently, transcriptome regionality was identified in the plasmodium. These regional differences were associated with proliferation, syncytial substructures, and localized environmental conditions (e.g., recently ingesting oak flakes). Further, the results showed that nuclei are heterogenous in their transcriptional profile, and may process local signals within the plasmodium to coordinate cell growth, metabolism, and reproduction. Morphologically, more nuclei were found in the fan-shaped, expanding front of the plasmodium compared to those in the more posterior network of veins. Overall, the data showed that a single-celled slime mold can control its gene expression in a region-specific manner, while lacking cellular compartmentalization; and suggests that nuclei are mobile processors facilitating local specialized functions. Moreover, nuclei within *Physarum* are mobile and can integrate local signals to coordinate a transcriptional response to dynamic environmental conditions, which enables the syncytium to locally change behavior and morphology.

Hasni *et al.* (2020) examined transcriptomic and proteomic evidence of physiological changes in the amoeboid stage of *Willaertia magna* (a heterolobosean amoeboflagellate) when grown in controlled conditions in a bioreactor. A total of 8,790 transcripts were identified, followed by an analysis of the *Willaertia* proteome, resulting in the identification of 3,561 proteins. Although a substantial number of the proteins (ca. 20%) were not identifiable based on available published data, identifiable proteins included "post-translational modification, protein turnover, chaperones" (14%), "translation, ribosomal structure and biogenesis" (10%), "intracellular tracking, secretion and vesicular transport" (8%) and "signal transduction mechanisms" (8%). However other categories including transcript and proteome data such as "nuclear structure" (0.7% proteins) and "extracellular structure" (0.1% proteins) were weakly represented among *W. magna* transcripts and proteins. More generally, a metabolism study showed that *W. magna* preferentially consumed carbohydrates and fatty acids to grow. Finally, an in-depth analysis showed that *W. magna* produces several enzymes that are probably involved in the metabolism of secondary metabolites.

The physiology and metabolic role of rumen ciliates has been of continuing interest toward better understanding their relationship to the animal host. Wang *et al.* (2019) examined the transcriptome of the rumen ciliate *Entodinium caudatum* to elucidate some of its metabolic features. Among more than 12,000 transcripts, numerous CAZymes (including lysozyme and chitinase) and peptidases were represented in the transcriptome. This study revealed the ability of *E. caudatum* to depolymerize starch, hemicellulose, pectin, and the polysaccharides of bacterial and fungal cell walls, and to degrade proteins. Many signaling pathways, including the ones that have been shown to function in *E. caudatum*, were represented by numerous transcripts. The transcriptome also provided evidence of the expression of genes involved in symbiosis, detoxification of reactive oxygen species, and the electron-transport chain. The presence and expression of the genes involved in the lysis and degradation of microbial cells highlight the dependence of *E. caudatum* on engulfment of other rumen microbes for its survival and growth.

As may be expected, there is increasing interest in studying the transcriptomics of parasitic protists, especially human parasites, and those that form part of the eukaryotic human biome. Among these, some attention has been given to *Entamoeba histolytica*, a parasite of the human intestinal tract with occasional invasion of the liver, forming abscesses. Documenting its sources of nutrition may contribute to a better understanding of its infective and survival strategies.

A general review of current knowledge of transcriptomic analysis of *E. histolytica* and possible explanatory value of these insights for understanding tissue invasion and virulence has been published by Naiyer

et al. (2019). Baumel-Alterzon and Ankri (2014) summarized current evidence of the response of *E. histolytica* to glucose starvation with comparison to other protozoan parasites. They reported that current published evidence indicates that during glucose starvation, *E. histolytica* and also *Entamoeba invadens* downregulate the expression of glycolysis-related genes and mainly use amino acids as their energy source. Among the enzymes which are involved in amino acid catabolism, methionine g-lyase (MGL1), which is involved in the catabolism of sulfur-containing amino acids, is of particular interest. Its expression is up-regulated in *E. histolytica* trophozoites that are glucose-starved, or exposed to oxidative and nitrosative stresses; and in trophozoites, which were isolated from the colon of *E. histolytica*-infected mice.

Moreover, it was discovered that dihydropyrimidine dehydrogenase (DPD), an enzyme that is involved in the degradation of pyrimidines, is essential for the adaptation of *E. histolytica* to a low glucose environment. This observation is supported by the results of previous studies, which found that DPD's expression is upregulated in vitro during long-term and short-term glucose starvation; and in vivo in trophozoites, which were isolated from the colon of *E. histolytica*-infected mice. Presently, there is no other report about DPD induction by glucose starvation in other parasites which suggests that this response is unique to *E. histolytica*.

The transcriptome sequence of *Dientamoeba fragilis*, has been published by Barratt *et al.* (2015). *D. fragilis*, a parasite of the human gastrointestinal tract discovered over a century ago, is an amoeboid stage of a trichomonad flagellate. However, given its morphology and lack of flagella, *D. fragilis* was originally classified as an amoeba. Although it has been generally categorized as a harmless commensal, increasing evidence suggests it can be pathogenic because it is regularly detected in association with gastrointestinal illness. Among the transcript data, there is a large repertoire of protein degrading enzymes. For example, the most abundant transcript was for a cathepsin L-like cysteine peptidase, suggesting that protein degradation is particularly important to cultured *D. fragilis* trophozoites. Peptidases belonging to the MEROPS family 'Mername-AA287 peptidase' were the most highly expressed group of peptidases, represented by 13 cysteine peptidase-like transcripts. There were approximately 300 hits for kinases, suggesting that the kinome of *D. fragilis* is relatively complex. Enzymes detected in high abundance were associated with glycolysis and gluconeogenesis, pyruvate metabolism, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway and starch and sucrose metabolism. Moreover, the carbohydrate metabolic pathways mapped for *D. fragilis* are similar to those of the respective *Trichomonas vaginalis* pathways, further consistent with a taxonomic affinity with trichomonads. In addition to aspects of physiology and metabolism, the authors also discuss their findings in relation to virulence factors and drug implications.

Development and life cycles

Relatively, a substantial number of papers have been published on development and life cycles of heterotrophic protists, particularly parasitic species. A review of free-living protists will be presented first followed by representative research on parasitic species.

The amoebozoan slime mold, *Physarum polycephalum*, has been widely used as a model laboratory organism in cell biological and life cycle studies. Glöckner *et al.* (2008) published a summary of 15,680 sequences, approximately half of the transcribed protein coding genes from the plasmodial stage of the developmental cycle. In addition to the genes for basic metabolism, an unexpected large number of genes involved in sophisticated signaling networks were found; and potential receptors for environmental signals such as light were found.

Overall, among the major categories of transcripts, the following categories were reported: Signal transduction (13.66%), Degradation (4.40%), Transport (4.32%), Cytoskeleton (2.90%), Transcription factor (1.02%), and Cell cycle (0.75%). Abundant domains and all other transcripts accounted for 73% of the transcripts. Comparisons to various other organisms including *Dictyostelium*, the closest relative, revealed that roughly half of the transcribed genes have no detectable counterpart, thus potentially defining species-specific adaptations for *Physarum*.

Further studies on the life cycle of *Physarum polycephalum* examined the transcriptomic changes arising during light-induced sporulation. Barrantes *et al.* (2010) identified the genes associated to the light-induced sporulation in *Physarum*, especially those related to signal transduction. Data was obtained before and after photoinduction using sporulation-competent cells. Within the transcript hit count data, 2,772 displayed significant differential expression (upregulated: 1,623; downregulated: 1,149). Transcripts with valid annotations and significant differential expression were

later integrated into putative networks using interaction information from orthologs.

Gene ontology analysis suggested that most significantly downregulated genes are linked to DNA repair, cell division, inhibition of cell migration, and calcium release, while highly upregulated genes were involved in cell death, cell polarization, maintenance of integrity, and differentiation. In addition, cell death-associated transcripts were overrepresented among the upregulated transcripts. These changes are associated to a network of actin-binding proteins encoded by genes that are differentially regulated before and after light induction.

In research on ciliate life cycles, a comparative transcriptome analyses of the growth (G) and Division (D) stages during the vegetative cell cycle in the monocellular hypotrich ciliate *Pseudokeronopsis erythrina* was published by Xu *et al.* (2020). As is characteristic of ciliates, the cell cycle stages include macronuclear amitosis, micronuclear mitosis, stomatogenesis and somatic cortex morphogenesis, and cytokinesis. The results showed that more than 2,051 significantly differentially expressed genes (DEGs) were detected, among which 1,545 were up-regulated, while 256 were down-regulated at the D stage.

Enriched DEGs during the D stage of the vegetative cell cycle of *P. erythrina* were involved in development, cortex modifications, and several organelle-related biological processes, showing correspondence of molecular evidence to morphogenetic changes for the first time. Several individual components of molecular mechanisms of ciliate vegetative division, the sexual cell cycle and cellular regeneration overlap were reported. The *P. erythrina* cell cycle and division have the same essential components as other eukaryotes, including cyclin-dependent kinases (CDKs), cyclins, and genes closely related to cell proliferation, indicating the conserved nature of this biological process.

Nowak *et al.* (2011) published a functional study of genes essential for autogamy and nuclear reorganization in *Paramecium tetraurelia* during the sexual cycle. Transcriptome analysis revealed four major patterns of gene expression, including two successive waves of gene induction. Functional analysis of 15 upregulated genes revealed four that are essential for vegetative growth, one of which is involved in the maintenance of the somatic macronucleus integrity, and another in cell division or membrane trafficking. Two additional genes, encoding a micronucleus-specific protein and a putative RNA helicase localizing to the old and then to the new macronucleus, are specifically required during sexual processes. In sum, this research provides a proof of principle that genes essential for meiosis and nuclear reorganization can be uncovered following genome-wide transcriptome analysis. In a related paper, Xiong *et al.* (2012) described the first deep sequencing analysis of the transcriptome of *Tetrahymena thermophila* during the three major stages of the life cycle: growth, starvation and conjugation. Using RNA-sequence data, their study significantly improved the genome annotation of this ciliate species and provided a fully comprehensive view of its global transcriptome.

One of the typical features of many free-living heterotrophic protists is an alternation in the life cycle between an active trophont stage and dormant cyst stage. Jiang *et al.* (2019), using transcriptome analysis, examined the molecular mechanism of resting cyst formation in the ciliate *Colpoda aspera* and documented the changes in gene expression between vegetative and encystment stages. Gene annotation and pathway mapping analysis revealed marked changes in biosynthesis, energy metabolism, and autophagy pathways during cyst formation. In addition, some differentially regulated genes were predicted to function in the interconnected cAMP, AMPK, mTOR, and PI3K/AKT signaling pathways, potentially forming a regulatory network for encystment. More generally, this study conducted a large-scale assessment of *Colpoda aspera* genomic resources and provided new insight into the molecular mechanisms underlying cyst formation.

In a general review article, including transcriptomic evidence, Aguilar-Diaz *et al.* (2011) summarized current knowledge of cysts and encystment in protozoan parasites with the goal of identifying optimal targets for new life-cycle interrupting strategies. More particularly, 20 additional studies were identified that used transcriptomics to study parasitic protozoan development or life cycle stages encompassing the following taxa: *Entamoeba* spp. (Gilchrist *et al.* 2008; Husain *et al.* 2011; Jeelani *et al.* 2012; De Cádiz *et al.* 2013; Ehrenkaufer *et al.* 2013; Manna *et al.* 2020); *Giardia* spp. (Faghiri and Widmer 2011; Einarsson *et al.* 2016); *Tritrichomonas foetus* (Huang *et al.* 2013); *Leishmania* spp. (Saxena *et al.* 2007; Inbar *et al.* 2017); *Trypanosoma* spp. (Savage *et al.* 2016; Belew *et al.* 2017; Dos Santos *et al.* 2018); and the malaria parasite, *Plasmodium* spp. (Bozdech *et al.* 2003; Kaiser *et al.* 2004; Howick *et al.* 2019).

Environmental and ecological studies

Caron *et al.* (2017) published a comprehensive review of the merits of using transcriptomics to probe the evolution, ecology, and physiology of marine protists; particularly emphasizing recent developments in our understanding of the ecology, physiology and evolution of protists derived from transcriptomic studies of cultured strains and natural communities. They also addressed how these novel largescale genetic datasets can be used in the future. A general review of the potential for using single-cell transcriptomics in elucidating functional states and interactions in microbial eukaryotes was published by Ku and Sebé-Pedrós (2019), particularly addressing the following: i) different single-cell transcriptomics methodologies with particular focus on microbial eukaryote applications, ii) single-cell gene expression analysis of protists in culture and what can be learned from these approaches, and iii) a perspective on the application of single-cell transcriptomics to protist communities to interrogate not only community components, but also the gene expression signatures of distinct cellular and physiological states, as well as the transcriptional dynamics of interspecific interactions.

Aquatic heterotrophic protists. Krabberød *et al.* (2017) published an informative review on emerging research exploring the oceanic microeukaryotic interactome using genomics and transcriptomics (metaomics). They particularly focused on recent technological developments in metaomics coupled with microfluidics and high-performance computing that are making it increasingly feasible to determine ecological interactions at the microscale. Their review particularly highlighted how the field of marine microeukaryotic ecological interactions can be enhanced using metaomics and related advances in technology.

The potential effects of global warming on the population and molecular responses of an aquatic testate amoeba (*Netzelia tuberspinifera*) was reported by Wang *et al.* (2021). *N. tuberspinifera* is an endemic and sensitive species from East Asia. The data (based on controlled experimental warming in cultures and using transcriptomics) showed that when the temperature was < 25°C, rising temperature triggered the biosynthesis of ribosomes. However, when the temperature was > 25°C, it triggered molecular processes related with cell division, test formation and general biomass increase. Notably, once the temperature exceeded 40°C, *N. tuberspinifera* was unable to survive. Consequently based on this evidence, the authors suggest that the distribution of *N. tuberspinifera* might expand towards higher altitude or latitude regions under global warming.

Benthic foraminifera (Rhizaria), growing in anoxic marine sediments, pose interesting questions how they survive in these oceanic, typically anoxic environments. Two research studies address this issue. Orsi *et al.* (2020) sampled an oxic-anoxic transition zone in marine sediments from the Namibian shelf, where the genera *Bolivina* and *Stainforthia* dominated the foraminifera community. Metatranscriptomics were used to characterize foraminifera metabolism across the different geochemical conditions. Relative foraminifera gene expression in anoxic sediment increased an order of magnitude, which was confirmed in a 10-day incubation experiment simulating the natural anoxic sediments.

The development of anoxia coincided with a 20–40-fold increase in the relative abundance of foraminifera protein encoding transcripts, attributed primarily to those involved in protein synthesis, intracellular protein trafficking, and modification of the cytoskeleton. This indicated that many Foraminifera were not only surviving, but thriving under the anoxic conditions. Their anaerobic energy metabolism was characterized by fermentation of sugars and amino acids, fumarate reduction, and potentially dissimilatory nitrate reduction. Evidence from this study indicated that under anoxia, foraminifera use creatine phosphate (a phosphogen) as an ATP store, allowing reserves of the high-energy phosphate pool to be maintained for sudden demands of increased energy during anaerobic metabolism. This was co-expressed alongside genes involved in phagocytosis and clathrin-mediated endocytosis (CME). Thus, Foraminifera may use CME to engulf and use dissolved organic matter as a carbon and energy source, in addition to ingestion of prey cells via phagocytosis.

In a related study, Gomaa *et al.* (2021) examined gene expression of foraminifera commonly found in severely hypoxic or anoxic sediments and identified metabolic strategies used by this abundant taxon. In field-collected and laboratory-incubated samples, foraminifera expressed denitrification genes regardless of oxygen regime with a putative nitric oxide dismutase, a characteristic enzyme of oxygenic denitrification. A pyruvate : ferredoxin oxidoreductase was highly expressed, indicating the capability for anaerobic energy generation during exposure to hypoxia and anoxia. Near-complete expression of a diatom's plastid genome in one foraminiferal species suggested kleptoplasty, or sequestration of functional plastids, conferring a meta-

bolic advantage despite the host living in darkness, far below the euphotic zone.

Some ciliates are also well adapted to survival in low oxygen environments. Mukhtar *et al.* (2021) reported a transcriptome profile of three *Spirostomum* species in the Class Heterotrichea (*S. ambiguum*, *S. subtilis*, and *S. teres*) and examined response to anaerobic or low oxygen conditions. They particularly probed for the presence of a rhodoquinol-dependent pathway (possibly supporting respiration in low oxygen conditions) as has been reported in species from the class Heterotrichea. Using RNA sequencing, data were obtained by deep molecular investigations on the gene *rquA* for rhodoquinone biosynthesis. Based on transcriptome analysis, two to three RquA proteins were identified in *S. ambiguum*, *S. teres*, and *S. subtilis*, respectively. The presence of a key Motif-I of RquA and mitochondrial targeting signals, also confirmed the identity of these as RquA.

Attention has also been given to salinity adaptations. The osmotic adaptive strategy of two obligate halophilic flagellates (*Halocafeteria seosinensis* and *Pharygomonas kirbyi*) living in hypersaline aquatic environments was studied by Harding *et al.* (2016), who used transcriptomics with the goal of unraveling how the flagellates adapt to the challenges of the hypersaline habitat. The flagellates' cytoplasmic proteomes showed increased hydrophilicity compared with marine protists. These results suggested that these halophilic protists have a higher intracellular salt content than marine protists. However, there was an absence of the acidic signature of "salt-in microbes". Salt-in microbes accumulate intracellular molar concentrations of chloride ions (Cl^-) and potassium ions (K^+) as osmolytes. This suggests that *H. seosinensis* and *P. kirbyi* utilize organic osmolytes to maintain osmotic equilibrium. Additional evidence showed increased expression of enzymes involved in synthesis and transport of organic osmolytes at maximal salt concentration for growth. These included hydroxyectoine and myo-inositol in *H. seosinensis*, suggesting these as possible candidates for the inferred organic osmolytes.

Symbiotic associations of aquatic heterotrophic protists with other microbiota have been investigated among diverse taxa (e.g., testate amoebae, radiolaria, and ciliates). Some representative examples are summarized here. Lhee *et al.* (2020) investigated the endosymbiosis of the testate amoeba *Paulinella micropora* strain KR01containing a plastid acquired by primary endosymbiosis (~124 Ma) resulting in a photosynthetic organelle termed the chromatophore. Analysis of genomic and transcriptomic data resulted in a high-quality draft assembly of size 707 Mb and 32,361 predicted gene models. A total of 291 chromatophore-targeted proteins were predicted in silico, 208 of which comprise the ancestral organelle proteome in photosynthetic *Paulinella* species. These proteins have functions, among others, in nucleotide metabolism and oxidative stress response. Gene coexpression analysis identified networks containing known high light stress response genes as well as a variety of genes of unknown function ("dark" genes). Moreover, the authors characterized diurnally rhythmic genes in this species and found that over 49% are dark, suggesting a need for more intensive analyses of these genes.

Radiolaria, largely found in open ocean locations, are notable for harboring algal symbionts (e.g., Anderson 2014). Liu *et al.* (2019a) analyzed the transcriptome of the large unicellular radiolarian *Thalassicolla nucleata* and elucidated some aspects of the radiolarian symbiotic relationship. They found that *T. nucleata* contained several endobionts including: two dinoflagellate symbionts, one photosymbiont *Brandtodinium* sp., and one putative Peridiniales parasite. Through comparisons of gene expressions of *Brandtodinium* sp. and those of a close relative from a free-living culture, they found that the *Brandtodinium* sp. maintained its photosynthetic activities, but altered its carbon metabolism dramatically as a symbiont *in hospite*. Gene expression data also suggested carbon and nitrogen exchange between the host and photosymbiont and that lectin-glycan interaction might play an important role in host-symbiont recognition.

Among ciliate species containing algal symbionts, Kodama *et al.* (2014) compared gene expression of *Paramecium bursaria*, with and without *Chlorella variabilis* symbionts. *P. bursaria* harbors several hundred green *Chlorella* in its cytoplasm and provides a useful model organism for studying endosymbiotic associations with ciliate hosts. By examining the symbiont-bearing and symbiont-free host transcriptomes, the authors were able to identify differentially expressed genes in the symbiont-bearing *P. bursaria* cells relative to the symbiont-free cells, including heat shock 70 kDa protein and glutathione S-transferase. This research showed that in symbiont-bearing *P. bursaria*, genes for glutathione S-transferase, trans-2-enoyl-CoA, aminotransferases, and ribosomal proteins are down-regulated; and furthermore, genes for Hsp70, transcriptional activator Myb-related proteins, and signal transduction

histidine kinase are up-regulated. More generally, these results contribute to understanding the molecular mechanism for establishment of the secondary symbiosis and for the host evolutionary adaptation to global climate change.

Dohra *et al.* (2015) further explored the amino acid and codon usage of *Chlorella*-bearing *Paramecium bursaria* using transcriptome data. Among other results they found that surface proteins preferentially are composed of smaller amino acid residues like cysteine. Unusual synonymous codon and amino acid usage in highly expressed genes can reflect a balance between translational selection and other factors. A correlation of gene expression level with synonymous codon or amino acid usage was emphasized in genes down-regulated in symbiont-bearing cells compared to symbiont-free cells, suggesting that the strength of translational selection in *P. bursaria* may be related to *P. bursaria–Chlorella* symbiosis. In ciliates such as *P. bursaria*, translational selection on amino acid and codon usage may reflect the presence of large amounts of macronuclear DNAs in the cells.

Endosymbiotic associations in ciliates inhabiting harsh environments such as anoxic sediments may provide adaptive advantages. Hines *et al.* (2018) examined the large, freshwater ciliate *Spirostomum semivirescens*, isolated from the U.K. and Sweden, containing densely packed endosymbiotic algae (closely related to *Chlorella vulgaris*). Analysis of the transcriptome indicated that *S. semivirescens* potentially uses rhodoquinol-dependent fumarate reduction to respire in the oxygen-depleted habitats where it lives, similar to the findings of Mukhtar *et al.* (2021) for deep-dwelling ciliates as reported above. The data also shows that despite large geographical distances (over 1,600 km) between the sampling sites investigated, morphologically-identical species can share an exact molecular signature, suggesting that some ciliate species, even those over 1 mm in size, could have a global biogeographical distribution.

Similar ecological relationships of anoxic-dwelling protists with endosymbiotic bacteria have been reported. For example, Beinart *et al.* (2018) used metagenomic and metatranscriptomic sequencing to explore the relationship of a microaerophilic or anaerobic karyorelictid brown ciliate and its prokaryotic symbionts collected from oxygen-depleted sediments in the Santa Barbara Basin (CA, U.S.A.). They particularly assessed the metabolic coupling within this symbiotic consortium. The sequencing confirmed the predominance of deltaproteobacterial symbionts from the Families Desulfobacteraceae and Desulfobulbaceae. The evidence suggested active symbiont reduction of host-provided sulfate, transfer of small organic molecules from host to symbionts and hydrogen cycling among the symbionts. Moreover, patterns of gene expression indicated active cell division by the symbionts, their growth via autotrophic processes and nitrogen exchange with the ciliate host. Overall, this research highlighted the importance of symbiont metabolism to host fermentative metabolism, and thus contributes to the ciliate's success in anoxic and low-oxygen habitats. At a larger scale, it also suggested ciliate-associated prokaryotes play a role in important biogeochemical processes.

Soil protists. Geisen *et al.* (2015) used metatranscriptomics to obtain a census of active protists in 12 mineral and organic soil samples from different vegetation types and climatic zones using small subunit ribosomal RNA transcripts as a marker. They found a broad diversity of soil protists spanning across all known eukaryotic supergroups and discovered a strikingly different community composition than shown previously. Protist communities differed substantially between sites, with Rhizaria and Amoebozoa dominating in forest and grassland soils; while Alveolata were most abundant in peat soils. The Amoebozoa were comprised of Tubulinea, followed with decreasing abundance by Discosea, Variosea and Mycetozoa. Transcripts of Oomycetes, Apicomplexa and Ichthyosporea suggest that soil may be a reservoir of parasitic protist taxa. Further, Foraminifera and Choanoflagellida were ubiquitously detected, indicating that these typically marine and freshwater protists may be autochthonous members of the soil microbiota.

One of the more challenging, yet important, topics of soil biotic interactions encompasses how protist predation especially on bacteria affects soil microbial community composition and ecosystem functioning. Song *et al.* (2015) combined whole genome transcriptome analyses with (live) imaging mass spectrophotometry (IMS) to observe multiple changes in the molecular and chemical dialogues between the bacterium *Pseudomonas fluorescens* and a phagocytic predator, the heterolobosean flagellate *Naegleria americana*. Generally, it is known that elevated densities of microbes in the rhizosphere surrounding plant roots can lead to concomitant increases in the populations and feeding activities of their predators such as heterotrophic protists. Among these, bacterivorous protozoa can have a significant impact on the bacterial community composition in soil and rhizosphere environments.

In this study, Song *et al.* found that lipopeptide (LP) biosynthesis was induced in *Pseudomonas* when grazed by protozoans and LP accumulation transitioned from homogeneous distributions across bacterial colonies to site-specific accumulation at the bacteria-protist interface. Moreover, putrescine biosynthesis was upregulated in *P. fluroescens* when it exhibited predation. When putrescine is present, protozoan trophozoites encyst and it adversely affects cyst viability. Overall, this multifaceted study provided new insights in the common and strain-specific responses among bacteria-protozoa interactions. This includes responses that contribute to bacterial survival in highly competitive soil and rhizosphere environments.

Digestive tract protist symbionts. Digestive tract protist symbionts of terrestrial organisms have received particular attention toward better understanding of their role in host nutrition and environmental interactions. Wang *et al.* (2019) examined the transcriptome of the rumen ciliate *Entodinium caudatum* with the aim of better understanding its metabolic features. Among a large number of transcripts, > 12,000, numerous CAZymes (including lysozyme and chitinase) and peptidases were represented in the transcriptome. This study revealed the ability of *E. caudatum* to depolymerize starch, hemicellulose, pectin, and the polysaccharides of the bacterial and fungal cell wall, and to degrade proteins. Many signaling pathways, including the ones that have been shown to function in *E. caudatum*, were represented by a large number of transcripts. The transcriptome also revealed the expression of the genes involved in symbiosis, detoxification of reactive oxygen species, and the electron-transport chain. The presence and expression of the genes involved in the lysis and degradation of microbial cells highlight the dependence of *E. caudatum* on engulfment of other rumen microbes for its survival and growth.

Additional evidence of the role of rumen ciliates was reported by Feng *et al.* (2020) who examined single-cell transcriptome sequences of rumen ciliates with the goal of better understanding their molecular adaptations to the anaerobic and carbohydrate-rich rumen microenvironment. They studied three rumen ciliates: two entodiniomorphids (*Entodinium furca* and *Diplodinium dentatum*), and one vestibuliferid (*Isotricha intestinalis*). All are monophyletic within the class Litostomatea. As with the prior reviewed study by Wang *et al.* (2019), Feng *et al.* report that carbohydrate-active enzyme (CAZyme) genes were identified in all three species, including cellulases, hemi-cellulases, and pectinases. Overall, the authors suggest that the evidence indicates that all three species have acquired prokaryote-derived genes by horizontal gene transfer (HGT) to digest plant biomass; and includes a significant enrichment of gene ontology categories such as cell wall macromolecule catabolic process and carbohydrate catabolic process, and the identification of genes in common between CAZyme and HGT groups.

Symbiotic protists of insect guts have been of particular interest to protistologists. Nishimura *et al.* (2020) examined the comparative transcriptomics of protists in the hindgut of the wood-feeding termite *Coptotermes formosanus*. Their comparative transcriptomic analysis revealed that the expression patterns of the genes involved in wood digestion were different among species, reinforcing their division of roles in wood degradation. Transcriptomes, together with enzyme assays, also suggested that one of the protists, *Cononympha leidyi*, actively degrades chitin and assimilates it into amino acids. Based on this evidence, the authors suggest that *C. leidyi* contributes to nitrogen recycling. Moreover, this degradation of chitin may inhibit infection from entomopathogenic fungi with chitin-fortified cell walls. Two of the genes for chitin degradation were further shown to be acquired via lateral gene transfer (LGT) implying the importance of LGT in the evolution of symbiosis. Moreover in total, the single cell-based approach successfully characterized the function of each protist in termite hindgut and explained why the gut community includes multiple species.

Aquatic protists: trophic modes and nutrition. Given the importance of productivity and biogeochemical cycles in ecosystems, a considerable amount of attention has been given to elucidating processes of mixotrophy and phagotrophy, particularly in aquatic protists. Liu *et al.* (2016), using RNA-sequencing, examined how gene expression characterizes different nutritional strategies among three mixotrophic protists (*Prymnesium parvum*, *Dinobyron* sp. and *Ochromonas* sp.) under light and dark conditions in the presence of prey.

Gene expression of the obligately phototrophic *P. parvum*, and also *Dinobryon* sp., changed significantly between light and dark treatments; while that of primarily heterotrophic *Ochromonas* sp. was largely unchanged. Gene expression of *P. parvum* and *Dinobryon* sp. shared many similarities, especially in the expression patterns of genes related to reproduction. However, key genes involved in central carbon metabolism and phagotrophy had different expression patterns between these two species, suggesting differ-

ences in prey consumption and heterotrophic nutrition in the dark. Overall, their results provided potential target genes for further exploration of the mechanisms of mixotrophic physiology and demonstrated the potential usefulness of molecular approaches in characterizing the nutritional modes of mixotrophic protists.

McKie-Krisberg *et al.* (2018) performed RNA-Seq transcriptomic analysis for two mixotrophic prasinophytes (*Micromonas polaris* and *Pyramimonas tychotreta*) under dissolved nutrient regimes that altered their ingestion of bacteria prey. The aim was to characterize the transcriptomes of these two non-model phytoflagellates, identify transcripts consistent with phagotrophic activity and assess their differential expression in response to nutrient stress. Though the strains examined were cold-adapted polar isolates, both belong to genera with widespread distribution. Analysis of the transcripts showed many expected expression patterns, including genes involved in photosynthetic pathways and enzymes implicated in nutrient uptake pathways. Moreover, several genes associated with intracellular digestive pathways were actively transcribed in both prasinophytes.

Differential expression analysis showed a larger response in *P. tychotreta*, where 23,373 genes were upregulated and 1,752 were down-regulated in the low nutrient treatment when phagotrophy was enhanced. In contrast, *M. polaris* low nutrient treatments resulted in up-regulation of 314 transcripts, while down-regulating 371. With respect to phagotrophic-related expression, 37 genes were co-expressed in both *P. tychotreta* and *M. Polaris*. Although the response was less pronounced in *M. polaris,* it is consistent with differences in observed ingestion behavior by both species.

Additional studies using transcriptomics examined the effects of nutrient stress (limited N and P) on laboratory cultures of open ocean, bloom forming autotrophic protists (Harke *et al.* 2017) and reported on the differentiated and conserved responses among Bacillariophyta, Dinophyta, and Haptophyta. With increased interest in climate change and increased levels of atmospheric CO_2, Hennon *et al.* (2017) reported on diverse CO_2-induced responses in physiology and gene expression among eukaryotic phytoplankton and found that gene set enrichment analyses revealed shifts in energy, carbon and nitrogen metabolic pathways, though with limited overlap between species in genes and pathways involved when exposed to elevated CO_2.

Phagotrophy in heterotrophic flagellates is of increasing importance in understanding the molecular and physiological processes by which prey organisms are consumed and their biomass incorporated in food webs or remineralized; particularly because flagellates are becoming more widely recognized as major micropredators in some aquatic systems. One of these flagellates is *Cafeteria burkhardae*, widespread in the global oceans. Massana *et al.* (2021) studied this species, grown with the cultured flavobacterium *Dokdonia* sp. as potential prey. The gene expression was compared between exponential and stationary growth phases, which were complemented with three starvation phases induced by dilution, that appeared as intermediate states.

They found distinct expression profiles in each condition and identified 2,056 differentially expressed genes between exponential and stationary samples. Upregulated genes at the exponential phase were related to DNA duplication, transcription and translational machinery, protein remodeling, respiration and phagocytosis; whereas, upregulated genes in the stationary phase were involved in signal transduction, cell adhesion, and lipid metabolism. Furthermore, a few highly expressed phagocytosis genes were identified, like peptidases and proton pumps, which could be used to target this ecologically relevant process in marine ecosystems.

To gain insights into the role of dinoflagellates' plankton population dynamics, community composition, and the flux of carbon through marine planktonic food webs, Rubin *et al.* (2019) using a RNA-Seq approach studied the molecular underpinnings of grazing in dinoflagellates, a group of important heterotrophic protists. The transcriptomic response of *Oxyrrhis marina* was analyzed under fed and starved conditions with three different phytoplankton prey (a diatom, *Isochrysis galbana*, and two strains of the photosynthetic flagellate *Heterosigma akashiwo*). In response to fed and starved conditions, 1,576 transcripts were significantly differentially expressed in *O. marina*. Fed *O. marina* cells upregulated transcripts involved in the synthesis of essential fatty acids and storage carbohydrates, suggesting that the predator was food satiated and excess glucose was being stored as an energy reserve.

Among other responses detected, voltage-gated ion channels were upregulated during grazing, and these ion channels are known to be involved in the detection of mechanical stimuli and the regulation of swimming behavior. Also, kinases were upregulated in *O. marina* that were fed, which can dictate cell shape changes and may be associated with phagocytosis. During starvation, upregulated *O. marina* transcripts included those involved in the degradation of energy-storage molecules like glu-

can 1,4-alpha-glycosidase and those involved in antioxidant activities and autophagy, like acid ceramidase that are associated with the digestion of polar lipids present in cell membranes. Starved *O. marina* also upregulated transcripts with high similarity to proton pumping proteorhodopsins, suggesting that this heterotrophic protist may supplement its energy requirement during starvation with a light harvesting mechanism.

In a broad study of marine heterotrophic stramenopile (MAST) flagellates collected directly from the natural environment and analyzed in laboratory controlled cultures, Labarre *et al.* (2020) investigated the gene expression in a mixed community of the flagellates where bacterivory was promoted. Using fluorescence in situ hybridization and 18S rDNA derived from metatranscriptomics, the taxonomic dynamics were documented during the incubation. The results confirmed an increase in relative abundance of different MAST lineages. Single cell genomes of several MAST species were examined to gain an insight into their most expressed genes, with a particular focus on genes related to phagocytosis. The genomes of MAST-4A and MAST-4B were the most represented in the metatranscriptomes. Moreover, highly expressed genes were identified in these two species involved in motility and cytoskeleton remodeling, as well as many lysosomal enzymes. Particularly relevant were the cathepsins, which are characteristic digestive enzymes of the phagolysosome; and the rhodopsins, perhaps used for vacuole acidification.

An interesting comparative study was published by Santoferrara *et al.* (2014) on transcriptomes from marine planktonic mixotrophic and heterotrophic ciliates (i.e., an oligotrich *Strombidium rassoulzadegani* and choreotrich *Strombidinopsis* sp., respectively). Most of the identified genes were related to housekeeping activity and pathways such as the metabolism of carbohydrates, lipids, amino acids, nucleotides, and vitamins. Although *S. rassoulzadegani* uses chloroplasts from its prey (kleptoplasty) to obtain autotrophic nutrition, no genes were found clearly linked to chloroplast maintenance and functioning in the transcriptome of this ciliate. While chloroplasts are known sources of reactive oxygen species (ROS), the authors found the same complement of antioxidant pathways in both ciliates, except for one enzyme possibly linked to ascorbic acid recycling found exclusively in the mixotroph. Contrary to expectations, there were no qualitative differences in genes potentially related to mixotrophy.

Zou *et al.* (2020) published a comparative transcriptomic study examining distinct gene expressions of a model ciliate (*Tetrahymena thermophila*) while feeding on bacteria-free medium (control condition), or when fed a digestion-resistant bacteria or a digestible one (*E. coli*). Comparative analysis of RNA-sequence data showed that, relative to the control, 637 and 511 genes in *T. thermophila* were significantly and differentially expressed in the digestion-resistant bacteria and *E. coli* treatments, respectively. The protistan expression of lysosomal proteases (especially papain-like cysteine proteinases), GH18 chitinases, and an isocitrate lyase were upregulated in both bacterial treatments. The genes encoding protease, glycosidase; and those involving glycolysis, TCA and glyoxylate cycles of carbon metabolic processes were higher expressed when fed digestion-resistant bacteria compared with the *E. coli*. Nevertheless, the genes for glutathione metabolism were more upregulated in the control than those in both bacterial treatments, regardless of the digestibility of the bacteria. The results of this study indicate that not only bacterial food, but also digestibility of bacterial taxa, modulate multiple metabolic processes in heterotrophic protists. Overall, research such as this contributes to a better understanding of protistan bacterivory and bacteria-protists interactions on a molecular basis.

AUTOTROPHIC PROTISTS (ALGAE)

Physiology and metabolism

A substantial amount of research has been devoted to algal physiology and metabolism, particularly the specific topic of algal lipid synthesis. Much of this research appears to be motivated by the possible use of algae in biotechnology to produce environmentally responsible sources of organic compounds to replace the use of fossil sources of petroleum. Less instances of published research were discovered on general topics of physiology and metabolism.

There are two major subtopics within this section on lipid and biomolecular syntheses. The first addresses studies on biosynthesis of macromolecules, particularly lipids and the findings will be organized by taxonomic groups of algae, beginning with “green algae” (Chlorophyceae) where it appears much of the research has been done, followed by studies on diatoms, members of the Bacillariophyceae. The second subtopic addresses transcriptomics research on more general metabolic processes in autotrophic protists.

A general review paper on the use of 'Algal omics' and their potential targets for triacylglycerol (TAG) accumulation was published by Arora *et al.* (2018). Their review specifically was aimed at examining and cataloging systems level data related to stress-induced TAG accumulation in oleaginous microalgae and to inform future metabolic engineering strategies to develop strains with enhanced bioproductivity, which could pave a path for sustainable green energy. A more specific review on use of 'Algal omics' to unlock bioproduct diversity in algal cell biotechnology was published by Guarnieri and Pienkos (2015).

Substantial attention has been given to the role of environmental stressors or inducers on lipogenesis, carbohydrate metabolism and photosynthesis, among other variables – many of the studies apparently motivated by possible biotechnological applications. Although these were laboratory experimental transcriptomic studies, they may have relevance to aquatic ecology because they examined the effects of environmental variables on carbon metabolism and cellular organic storage compounds that may be relevant to ecosystem productivity and food webs.

A list of all algal taxa used in these transcriptomic studies (and author citations) is categorized according to the environmental inducers or stressors that were applied in the experiments:

i) **Nutrient limited (nitrogen**): *Chlorella zofingiensis* (Nordin *et al.* 2020), *Chlorella vulgaris* UPSI-JRM01 (Nordin *et al.* 2020), *Chromochloris zofingiensis* (Liu *et al.* 2019b; Zhang *et al.* 2019), *Dunaliella parva* (Shang *et al.* 2016), *Dunaliella tertiolecta* (Tan *et al.* 2016), *Haematococcus pluvialis* (Huang *et al.* 2019), *Haematococcus pluvialis* (Zhao *et al.* 2020), *Scenedesmus acutus* (Sirikhachornkit *et al.* 2018), *Phaeodactylum tricornutum* (Remmers 2 *et al.* 2018);

ii) **Nutrient limited (phosphorus):** *Scenedesmus* sp. isolated from cyanobacterial-rich culture (Yang *et al.* 2018);

iii) **Nutrient limited (sulfur):** *Chromochloris zofingiensis* (Mao *et al.* 2020b), *Parachlorella Kessleri* **(**Ota *et al.* (2016);

iv) **Glucose supplemented:** *Chlorella zofingiensis* (Huang *et al.* 2016), *Chlorella sorokiniana* (Azaman *et al.*, 2020), *Chromochloris zofingiensis* (Roth *et al.* 2019);

v) **Temperature stress:** *Auxenochlorella protothecoides* UTEX 2341 (Xing *et al.* 2018);

vi) **Light stress:** *Desmodesmus* sp. JSC3 (Yeh *et al.* 2017), *Haematococcus pluvialis* (Hu *et al.* 2020);

vii) **Anaerobic stress:** *Euglena gracilis* (Yoshida *et al.* 2016);

viii) **Salinity stress:** *Chlorella* sp. "SAG-211-18" (Mansfeldt *et al.*, 2016), *Chromochloris zofingiensis* (Mao *et al.* 2020a), *Neodesmus* sp. UTEX 2219-4 (Chang *et al.* 2016), *Nitzschia* sp. (Cheng *et al.* 2014).

Although comparatively fewer studies were found addressing transcriptomics of general metabolism, a broad range of research has been conducted spanning photosynthesis, plant hormonal effects, coral-symbiont interactions, and basic C-metabolic pathways, as summarized below. Ouyang *et al.* (2013) reported transcriptomic analysis of a unique C4-like photosynthesis in the microalga *Myrmecia incisa*. Moreover, in a quest to find additional potential sources of arachidonic acid (ArA), essential for healthy human nervous systems, the authors aimed to gain more molecular information about metabolism pathways, including the biosynthesis of ArA in this non-model microalga. During the course of the study, the C4-like photosynthesis pathway was elucidated, and the biosynthesis pathways of lipid particularly those of ArA and triacylglycerol (TAG) were analyzed in detail. Triacyl glycerides were proposed to be accumulated in oil bodies in the cytosol with the help of caleosin or oil globule-associated proteins. In addition, the carotenoid biosynthesis pathways were discussed.

The role of ethylene (a plant regulatory molecule) in cell wall metabolism, photosynthesis, and abiotic stress responses was studied by Van de Poel *et al.* (2016) in the green alga *Spirogyra pratensis* using mRNA sequencing. They measured changes in gene expression over time in *Spirogyra* filaments in response to an ethylene treatment. At the transcriptional level, ethylene (acting as a plant hormone) predominantly regulated three processes in *Spirogyra*: (1) modification of the cell wall matrix by expansins and xyloglucan endotransglucosylases/hydrolases, (2) down-regulation of chlorophyll biosynthesis and photosynthesis, and (3) activation of abiotic stress responses.

This research confirmed that the photosynthetic capacity and chlorophyll content were reduced by an ethylene treatment and that several abiotic stress conditions could stimulate cell elongation in an ethylene-dependent manner. Moreover, the *Spirogyra* transcriptome had only 10 ethylene-responsive transcription factor (ERF) homologs, several of which are regulated by ethylene.

Mancipe *et al.* (2021) reported a draft genome and transcriptome study of *Scenedesmus glucoliberatum*

PABB004, an unconventional sugar-secreting green alga. *S. glucoliberatum* PABB004 secreted ready-to-use fermentable sugars (glucose and maltose) directly to the extracellular media achieving concentrations greater than 2.7 g L^{-1} of free glucose and 1.2 g L^{-1} of maltose in batch cultures. Significant sugar accumulation occurred over a range from 6.2 to 7.8 pH units. The predicted proteome was compared with other green algae that show different sugar secretion phenotypes aiming to help uncover their common features for simple sugar secretion and those unique to *S. glucoliberatum* PABB004. Comparison of the transcriptome data for *S. glucoliberatum* compared to two species of *Chlamydomonas* (*C. reinhardtii* and *C. variabilis*) showed statistically significant differences; namely, for *S. glucoliberatum* PABB004 vs. *C. reinhardtii* ($p = 2.9\ e^{-5}$) and *S. glucoliberatum* PABB004 vs. *C. variabilis* ($p = 3.8\ e^{-5}$). However, there was no evidence of a difference between *C. reinhardtii* and *C. variabilis* ($p = 0.9$).

Coral-algal relationships are one of the most widely recognized and substantially studied endosymbioses. Mohamed *et al.* (2020) studied the molecular interactions during establishment of this relationship to elucidate processes that have largely been poorly understood. A dual RNA-sequencing approach was used to better understand transcriptional changes in both host and symbiont during the colonization of the coral *Acropora tenuis* by a compatible symbiont strain of *Cladocopium goreaui*; ITS2 type C. A comparison of transcript levels of the in hospite symbiont 3, 12, 48 and 72 hours after exposure to those of the same strain in culture revealed that extensive and generalized down-regulation of symbiont gene expression (including protein synthesis and processing) occurred during the infection process. Included in this transcriptional repression were a range of stress response and immune-related genes. In contrast, a suite of symbiont genes implicated in metabolism was upregulated. Consistent with previous ecological studies, the transcriptomic data suggest that active translocation of metabolites to the host may begin early in the colonization process, and that the mutualistic relationship can be established at the larval stage.

Cells of the green alga *Oophila amblystomatis* form an endosymbiosis with the embryos of the salamander *Ambystoma maculatum*. Burns *et al.* (2017) used de novo dual-RNA sequencing, to compare algae inside the animal cells to those in the egg capsule; and likewise the host salamander cells that harbored intracellular algae to those without algae. This two-by-two-way analysis revealed that intracellular algae exhibit hallmarks of cellular stress and undergo a striking metabolic shift from oxidative metabolism to fermentation. Culturing experiments with the alga showed that host glutamine may be utilized by the algal endosymbiont as a primary nitrogen source. Transcriptional changes in salamander cells suggest an innate immune response to the alga, with potential attenuation of the NF-kB pathway (that links pathogenic signals and cellular danger signals thus organizing cellular resistance to invading pathogens), and metabolic alterations indicative of modulation of insulin sensitivity. In stark contrast to its algal endosymbiont, the salamander cells did not exhibit major stress responses, suggesting that the host cell experience is neutral or beneficial.

Several published studies link metabolic and physiological processes with broader topics such as ecology and related ecosystems variables. Murray *et al.* (2016) published a broad review of current approaches to the functional genetics of dinoflagellates with a particular perspective on implications for ecology and biotechnological applications. More particularly, they emphasize that dinoflagellates occupy an extraordinarily diverse array of ecological niches reflected partially by their success that stems from a suite of functional and ecological strategies, including the production of secondary metabolites with anti-predator or allelopathic impacts, nutritional flexibility, and the ability to form symbiotic relationships. Particularly, current advances in genomics and transcriptomic sequencing approaches have opened new avenues for novel ecological experiments, innovative approaches for monitoring of harmful biotoxins and allowing us to investigate the production of ecologically and economically important compounds.However, Murray *et al.* note that we still generally lack the ability to genetically manipulate species, which would enable the confirmation of biosynthetic pathways and development of novel bioengineering applications.

A de novo transcriptome sequencing and preliminary analysis of the *Euglena gracilis* genome was published by O'Neill *et al.* (2015) that provided a basis for further molecular and functional genomics studies addressing broader basic scientific research and biotechnological applications. The transcriptome contained over 30,000 protein-encoding genes, supporting metabolic pathways for lipids, amino acids, carbohydrates and vitamins, along with capabilities for polyketide and non-ribosomal peptide biosynthesis. The metabolic and environmental robustness of *Euglena* is supported by a substantial capacity for responding to biotic and abi-

otic stress. It has the capacity to deploy three separate pathways for vitamin C (ascorbate) production, as well as producing vitamin E (alpha-tocopherol) and, in addition to glutathione, the redox-active thiols nor-trypanothione and ovothiol.

In addition to basic metabolic research studies using transcriptomics, some significant attention has been given to algal nutrition (particularly mixotrophy and phagotrophy) and their potential roles in food webs and carbon pathways in ecosystems. A survey of some of the diverse topics published is included here. A comprehensive transcriptome analysis of the nutritional strategies and phylogenetic relationships among chrysophytes was published by Beisser *et al.* (2017). Among the taxa analyzed were *Poteriospumella lacustris*, *Poterioochromonas malhamensis*, *Dinobryon* sp., *Synura* sp. *Ochromonas/Spumella* strains, *Dinobryon* sp., *Epipyxis* sp. and *Uroglena* sp. Chrysophytes are important model species and are important grazers of bacteria-sized microorganisms. They are also primary producers. Consequently, a more thorough documentation of their genomics was undertaken to provide more basic information for ecological and ecophysiological studies of ecosystem dynamics.

Beisser *et al.* used transcriptomes to determine relations between the trophic mode (mixotrophic vs. heterotrophic) of the protists and gene expression. They reported an enrichment of genes for photosynthesis, porphyrin and chlorophyll metabolism for both phototrophic and mixotrophic strains that can perform photosynthesis. Genes involved in nutrient absorption, environmental information processing and various transporters (e.g., monosaccharide, peptide, lipid transporters) were present or highly expressed only in heterotrophic strains that have to sense, digest and absorb bacterial food. Additional evidence was presented on the phylogenetic implications based on the transcriptomic evidence.

Wilken *et al.* (2020) developed an experimental system to study responses of mixotrophic protists to availability of living prey and light, and used it to characterize contrasting physiological strategies in two stramenopiles in the genus *Ochromonas* (Isolate CCMP1393 and CCMP2951). They found that the oceanic isolate CCMP1393 is an obligate mixotroph, requiring both light and prey as complementary resources. Interdependence of photosynthesis and heterotrophy in CCMP1393 comprises a significant role of mitochondrial respiration in photosynthetic electron transport. In contrast, coastal isolate CCMP2951 is a facultative mixotroph that can substitute photosynthesis by phagotrophy, and hence grow purely heterotrophically in darkness. In contrast to CCMP1393, CCMP2951 also exhibited a marked photoprotection response that integrates non-photochemical quenching and mitochondrial respiration as an electron sink for photosynthetically produced reducing equivalents. Facultative mixotrophs similar to CCMP2951 might be well adapted to variable environments, while obligate mixotrophs similar to CCMP1393 appear capable of resource efficient growth in oligotrophic ocean environments; overall this provides finer details on niche differentiation that can impact food webs and lead to opposing carbon cycle roles.

The prymnesiophyte *Prymnesium parvum* is a globally distributed phototrophic protist of considerable ecological importance. *P. parvum* occurs in ecosystems across a wide range of salinities and environmental conditions, possesses a complex life history including haploid and diploid stages, and exhibits mixotrophic capacities. Moreover, the combined photosynthetic and heterotrophic capabilities of *P. parvum* enable the alga to form ecosystem disruptive algal blooms. Hence a more detailed understanding of the molecular basis for its remarkable adaptive properties is warranted. Thus, Liu *et al.* (2015) examined the gene expression of *P. parvum* in response to prey availability. They used RNA-Sequencing technology to study changes in gene transcription of *P. parvum* in three treatments with different microbial populations available as potential prey: axenic *P. parvum* (no prey), bacterized *P. paruvm*, and axenic *P. parvum* with ciliates added as prey. Thousands of genes were differentially expressed among the three treatments. Most notably, transcriptome data indicated that *P. parvum* obtained organic carbon, including fatty acids, from both bacteria and ciliate prey for energy and cellular building blocks. The data also suggested that different prey provided *P. parvum* with macro-and micro-nutrients; namely, organic nitrogen in the form of amino acids from ciliates, and iron from bacteria. However, both transcriptomic data and growth experiments indicated that *P. parvum* did not grow faster in the presence of prey despite the gains in nutrients; although, algal abundances of this algal flagellate attained in culture were slightly greater in the presence of prey.

Li *et al.* (2020a) used transcriptomic analysis combined with physiological assays to determine the effects of mixotrophic nutrition compared to autotrophic nutrition in cultures of *Asterarcys* sp. SCS-1881 (Scenedesmaceae Family). The expression level of three rate-limiting enzymes for glycolysis (hexoki-

nase, 6-phosphofructokinase and pyruvate kinase) were up-regulated in the microalga under mixotrophic conditions when compared to autotrophic conditions, indicating that the mixotrophy conditions enhanced glycolysis to provide the cells with an advanced carbon skeleton, as well as more ATP and NADPH. The expression levels of 6-phosphoglucono-lactonase, which is the rate-limiting enzyme for the pentose phosphate pathway, were all down-regulated under mixotrophic conditions when compared to autotrophic conditions. Triacylglycerol synthesis pathway was attenuated under mixotrophic conditions. The action of two key enzymes mediating nitrate assimilation, including nitrate reductase (which converts nitrate to nitrite) and nitrite reductase (which converts nitrite to ammonium), were up-regulated under mixotrophic conditions in comparison to autotrophic conditions.

In summary, Mixotrophic cultures of *Asterarcys* sp. SCS-1881 had a significantly higher biomass concentration and cell count than those under autotrophic conditions. Mixotrophy tended to increase protein content, which might play an important role in the consumption of excess energy. Under mixotrophic conditions, the cultures had less of a need for light energy, and also showed enhanced glycolysis and photosynthetic carbon fixation, which ensured an efficient supply of energy and carbon skeleton for rapid growth of the microalga under mixotrophic conditions.

Burns *et al.* (2015) studied the phago-mixotrophic mode of nutrition in the bacterivorous green alga *Cymbomonas tetramitiformis*, a marine prasinophyte, that is one of only a few green algae that still retain an ancestral particulate-feeding mechanism, while harvesting energy through photosynthesis. Using genomic and transcriptomic methods they found that a number of unusual metabolic pathways (for the Chloroplastida) are predicted for *C. tetramitiformis*, including pathways for Lipid-A and peptidoglycan metabolism. Comparative analyses of the predicted peptides of *C. tetramitiformis* to sets of other eukaryotes revealed that nonphagocytes are depleted in a number of genes, a proportion of which have known function in feeding.

In addition, their analysis suggested that obligatory phagotrophy is associated with the loss of genes that function in biosynthesis of small molecules (e.g., amino acids). Furthermore, *C. tetramitiformis*, and at least one other phago-mixotrophic alga, therefore, are unique compared with obligatory heterotrophs and nonphagocytes in that both feeding and small molecule synthesis-related genes are retained in their genomes. These results suggest that early, ancestral host eukaryotes that gave rise to phototrophs had the capacity to assimilate building block molecules from inorganic substances (i.e., prototrophy).

Life Cycle and Development

Approximately 20 published research studies were identified that dealt with life cycle and development of autotrophic protists. To sharpen the focus, studies reviewed here will include those dealing with major phases of development during vegetative and reproductive cell cycles.

Vegetative cell cycles and development. Studies involving stages of development during growth or physiological changes associated with metabolic and maturational stages are particularly addressed in this section. Ashworth *et al.* (2013) published a detailed physiological and transcriptomic survey to measure the recurrent transcriptional changes that characterize typical diatom growth in batch cultures of the planktonic diatom *Thalassiosira pseudonana*. Roughly 40% of the transcriptome varied significantly and recurrently, reflecting large, reproducible cell-state transitions between four principal states: (i) "dawn," following 12 h of darkness; (ii) "dusk," following 12 h of light; (iii) exponential growth and nutrient repletion; and (iv) stationary phase and nutrient depletion.

The authors noted that increases in expression of thousands of genes at the end of the reoccurring dark periods (dawn), including those involved in photosynthesis (e.g., ribulose-1,5-bisphosphate carboxylase oxygenase genes rbcS and rbcL). The authors concluded that this implied large-scale anticipatory circadian mechanisms at the level of gene regulation. There were repeated shifts in the transcript levels of hundreds of genes encoding sensory, signaling, and regulatory functions that accompanied the four cell-state transitions. This provided a preliminary map of the highly coordinated gene regulatory program under varying conditions. Several putative light sensing and signaling proteins were associated with recurrent diel transitions, suggesting that these genes may be involved in light-sensitive and circadian regulation of cell state. These results begin to explain, in comprehensive detail, how the diatom gene regulatory program operates under varying environmental conditions.

Transcriptome analyses of the benthic diatom *Seminavis robusta* by Blicke *et al.* (2021) highlighted adaptations supporting a benthic lifestyle. Nearly 90% of expressed protein-coding genes and 66.9% of expressed

long intergenic non-coding RNAs showed significant expression oscillations and were predominantly phasing at night with a periodicity of 24 h. Phylostratigraphic analysis found that rhythmic genes are enriched in highly conserved genes, while diatom-specific genes are predominantly associated with midnight expression. Integration of genetic and physiological cell cycle markers with silica depletion data revealed potential new silica cell wall-associated gene families specific to diatoms.

Additionally, they observed genes with a remarkable semidiurnal (12-h) periodicity. Moreover, the expansion of putative circadian transcription factors may reflect adaptations to cope with highly unpredictable external conditions. Altogether, these results provided new insights into the adaptations of diatoms to the benthic environment and should serve as a valuable resource for the study of diurnal regulation in photosynthetic eukaryotes.

Li *et al.* (2019a) published a laboratory culture study of *Haematococcus pluvialis* with the aim of improving our understanding of the complex metabolic changes, especially astaxanthin biosynthesis, occurring during different growth phases: (i) fast-growing vegetative cell proliferation, (ii) transformation of intermediate cells and (iii) formation of resting non-motile cells. Seven specified developmental points with perceptible color difference were sampled and sequenced using high throughput sequencing. Comparative transcriptome profiling was employed to identify the differentially expressed genes. After pairwise comparisons, 2,674 DEGs were identified. Seventeen unigenes related to carotenoid biosynthesis were actively transcribed according to KEGG pathway analysis, as well as more detailed changes in lipid metabolism across the varied life stages.

Hovde *et al.* (2015) reported the draft genome sequence of *Chrysochromulina tobin* (Prymnesiales), and analyzed transcriptome data collected at seven time points over a 24-hour light/dark cycle. The nuclear genome of *C. tobin* is small (59 Mb), compact (~40% of the genome is protein coding) and encodes approximately 16,777 genes. Genes important to fatty acid synthesis, modification, and catabolism showed distinct patterns of expression when monitored over the circadian photoperiod; and they detected major groups of genes with the largest expression changes, including: i) genes whose expression peaked midway through the dark period, ii) those expressed at the end of the dark cycle, iii) genes expressed at the beginning of the light period, and iv) over representation of ribosomal subunit gene expression that occurred from the middle to the end of the day.

Diel gene transcription in *Chlamydomonas reinhardtii* was studied by Panchy *et al.* (2014) who reported that ~50% of the annotated genes exhibited cyclic expression. These cyclic expression patterns indicate a clear succession of biological processes during the course of a day. Among 237 functional categories enriched in cyclically expressed genes, 90% were phase-specific, including photosynthesis, cell division, and motility-related processes. To better understand the cis regulatory basis of diel expression, putative cis-regulatory elements were identified that could predict the expression phase of a subset of the cyclic transcriptome.

In a further study of *Chlamydomonas reinhardtii*, Zones *et al.* (2015) devised a highly synchronous photobioreactor culture system with frequent temporal sampling to characterize genome-wide diurnal gene expression in *Chlamydomonas*. Over 80% of the measured transcriptome was expressed with strong periodicity, forming 18 major clusters. Genes associated with complex structures and processes, including cell cycle control, flagella and basal bodies, ribosome biogenesis, and energy metabolism, all had distinct signatures of coexpression with strong predictive value for assigning and temporally ordering function. Significantly, the frequent sampling regime revealed meaningful fine-scale phase differences between and within subgroups of genes and enabled the identification of a transiently expressed cluster of light stress genes. Coexpression was further used both as a data-mining tool to classify and/or validate genes from other data sets related to the cell cycle and to flagella and basal bodies, and to assign isoforms of duplicated enzymes to their cognate pathways of central carbon metabolism.

A transcriptome study of cell cycle regulation in two dinoflagellates (*Lingulodinium polyedrum* and *Symbiodinium* sp.) that possess 'budding yeast cell cycle pathway components' was published by Morse *et al.* (2016) who found that most yeast cell cycle regulators have homologs in these dinoflagellates. This suggested that the yeast model is appropriate for understanding regulation of the dinoflagellate cell cycle. The dinoflagellates, however, lacked several components essential in yeast, but a comparison with a broader phylogenetic range of protists revealed these components are usually also missing in other organisms. Lastly, phylogenetic analyses show that the dinoflagellates contain at least three cyclin-dependent kinase (CDK) homologs (be-

longing to the CDK1, CDK5 and CDK8 families), and that the dinoflagellate cyclins belong exclusively to the A/B type.

In the unicellular red alga *Cyanidioschyzon merolae*, the S and M phases of the cell mitotic cycle occur at night. Fujiwara *et al.* (2020) examined how diel transcriptomic changes in metabolic pathways are related to the cell cycle and identified all genes for which mRNA levels change depending on the stage of the cell cycle. In addition, they compared transcriptomic changes between the wild type with normal diel cycle and transgenic lines, in which the cell cycle was uncoupled from the diel cycle by the depletion of either cyclin-dependent kinase A or retinoblastoma-related protein.

Among the nucleus-encoded genes, the mRNA levels of 1,979 genes exhibited diel transcriptomic changes in the wild type. Of these, the periodic expression patterns of 454 genes were abolished in the transgenic lines, suggesting that the expression of these genes is dependent on cell cycle progression. The periodic expression patterns of most metabolic genes, except those involved in starch degradation and de novo deoxyribonucleotide triphosphate synthesis, were not affected in the transgenic lines, indicating that the cell cycle and transcriptomic changes in most metabolic pathways are independent of the diel cycle.

The haptophyte *Emiliania huxleyi* is one of the most abundant calcifying phytoplankton species in the ocean, playing an important role in global carbon fluxes, with an interesting life cycle alternating between haploid and diploid phases (haplo-diplontic). Von Dassow *et al.* (2009) published a transcriptome analysis of the functional differentiation between haploid and diploid cells. Relatively more is known about the diploid calcifying cells, but less is known about the haploid cells. However, they have been hypothesized to allow persistence of the species between the yearly blooms of diploid cells.

The haploid and diploid transcriptomes showed a dramatic differentiation, with approximately 20% greater transcriptome richness in diploid cells than in haploid cells; and only ca. 50% of transcripts were estimated to be common between the two phases. The major functional category of transcripts differentiating haploids included signal transduction and motility genes. Diploid-specific transcripts included Ca^{2+}, H^{+}, and HCO_3^{-} pumps. Potential factors differentiating the transcriptomes included haploid-specific Myb transcription factor homologs and an unusual diploid-specific histone H4 homolog. Greater transcriptome richness in diploid cells suggests they may be more versatile for exploiting a diversity of rich environments; whereas, haploid cells are intrinsically more streamlined.

Sexual reproduction and cell cycles. The complex relationship between cell cycle and reproductive stages in algae has received increasing attention using genomic transcriptomic analyses. Klein *et al.* (2017) examined transcriptomic evidence of differentiation in different cell types in the large, obligately autotrophic, colonial green alga *Volvox carteri* that exhibits sexual dimorphism. In the asexual mode of reproduction, both male and female algae contain approximately 2,000 small, terminally differentiated, bi-flagellate somatic cells embedded in the surface of a transparent sphere of glycoprotein-rich, extracellular matrix (ECM). In addition, approximately 16 large reproductive germ cells (called gonidia) are positioned slightly below the surface of the spheroidal colony. *V. carteri* shows a complete division of labor between the two cell types – small, flagellated somatic cells and large, immotile reproductive cells.

Thus, *V. carteri* provided a unique opportunity to study this example of multicellularity and cellular differentiation at the molecular level. Overall, the results demonstrate an extensive compartmentalization of the transcriptome between the two cell types. More than half of all genes show a clear difference in expression between somatic and reproductive cells. This study constitutes the first transcriptome-wide RNA-Sequence analysis of separated cell types of *V. carteri* focusing on gene expression. The high degree of differential expression indicates a strong differentiation of cell types despite the fact that *V. carteri* diverged relatively recently from its unicellular relatives.

Further examination of differences in gonadial and somatic cells of *V. carteri* using transcriptomics was reported by Matt and Umen (2018) who made a comprehensive characterization of the gonidial and somatic transcriptomes *V. carteri* of to uncover fundamental differences between the molecular and metabolic programming of these cell-types. They found extensive transcriptome differentiation between the two cell-types, with somatic cells expressing a more specialized program overrepresented in younger, lineage-specific genes; and gonidial cells expressing a more generalist program overrepresented in more ancient genes that shared striking overlap with stem cell-specific genes from animals and land plants.

Additionally, directed analyses of different pathways revealed a strong dichotomy between cell-types with gonidial cells expressing growth-related genes and somatic cells expressing an altruistic metabolic

program geared toward the assembly of flagella, which support organismal motility. Moreover the somatic cells expressed conversion of storage carbon to sugars, which act as donors for production of extracellular matrix (ECM) glycoproteins whose secretion enables massive organismal expansion. *V. carteri* orthologs of diurnally controlled genes from *C. reinhardtii*, a single-celled relative, were analyzed for cell-type distribution and found to be strongly partitioned, with expression of dark-phase genes overrepresented in somatic cells and light-phase genes overrepresented in gonidial cells. This result is consistent with cell-type programs in *V. carteri* arising by cooption of temporal regulons from a unicellular ancestor.

Species of brown algae also have alternation of generations cycling between haploid and diploid phases, in some cases exhibiting dimorphism with different morphological features between the two phases. Lipinska *et al.* (2019) analyzed the nature and extent of genome-wide, generation-biased gene expression in four species of brown algae with contrasting levels of dimorphism between life cycle generations. The proportion of the transcriptome that is generation-specific is broadly associated with the level of phenotypic dimorphism between the life cycle stages. Importantly, the results revealed a remarkably high turnover rate for life-cycle-related gene sets across the brown algae, and highlights the importance not only of co-option of regulatory programs from one generation to the other; but also of a role for newly emerged, lineage-specific gene expression patterns in the evolution of the gametophyte and sporophyte developmental programs in this major eukaryotic group. Furthermore, this study showed that generation-biased genes display distinct evolutionary modes, with gametophyte-biased genes evolving rapidly at the coding sequence level; whereas, sporophyte-biased genes tend to exhibit changes in their patterns of expression.

Martins *et al.* (2013) provided additional evidence, using transcriptomics, of sex-biased gene expression in the brown alga *Fucus vesiculosus* that exhibits alternation of generations. Overall, the results suggest constraint on female-biased genes (possible pleiotropy), and less constrained male-biased genes, mostly associated with sperm-specific functions. More generally, the results supported the growing contention that males possess a large array of genes regulating male fitness, broadly supporting findings in evolutionarily distant heterogametic animal models.

In a more specific and detailed analysis of alginate polymerization and secretion in brown algae, Shao *et al.* (2019) studied kelp (*Saccharina japonica*), and used transcriptomic data mining, via profile analysis and weighted gene co-expression network analysis (WGCNA), to identify gene families correlated with specific stages of the alginate secretion. This included genes related to development, modules correlated with traits, and potential hub genes responsible for alginate and mannitol bio-synthesis. Based on sequencing of genes in 30 kelp samples from different stages and tissues, the authors deduced that ribosomal proteins, light harvesting complex proteins and "imm upregulated 3" gene family are closely associated with the meristematic growth and kelp maturity.

Moreover, 134 and 6 genes directly involved in alginate and mannitol metabolism were identified, respectively. Overall, the authors concluded that the network of co-responsive DNA synthesis, repair and proteolysis are presumed to be involved in alginate polymerization and secretion; while upstream, light-responsive reactions are important for mannitol accumulation in meristem of kelp. The transcriptome analysis provided new insights into the transcriptional regulatory networks underlying the biosynthesis of alginate and mannitol during *S. japonica* development.

Garcia-Jiménez and Robaina (2015) published a perspective article highlighting the need for further research on reproduction in red algae at the molecular level, especially in recognition that these organisms have some of the most complex life cycles known in living organisms. Subsequently, Garcia-Jiménez *et al.* (2018) published an analysis of the transcriptome of the red seaweed *Grateloupia imbricata* with emphasis on reproductive potential. The results showed the presence of transcripts required for the uptake of glycerol, which is a specific carbon source for in vitro culture of *G. imbricata*. Further analyses uncovered nucleotide sequences that are involved in polyamine-based biosynthesis, polyamine degradation, and metabolism of regulatory compounds such as jasmonates and ethylene. Polyamines, ethylene and methyl jasmonate are plant growth regulators that elicit the development and maturation of cystocarps and the release of spores from seaweeds.

Environmental and ecological studies

A substantial number of studies were discovered online that used transcriptomics involved in environmental and ecological topics for autotrophic protists. Some representative examples will be reviewed in five subsections: i.) Light effects, ii.) Temperature stress,

iii.) Salinity stress, iv.) Nutrient effects, and v.) Desiccation adaptations.

Light effects. The effect of light intensity and spectral quality has been investigated using transcriptomics. Li *et al.* (2019b) analyzed regulation of gene expression during photoacclimation to high irradiance levels in *Dunaliella salina* a member of the Chlorophyceae. This is an attractive model autotrophic, flagellated protist for laboratory studies because changes in irradiance level during cell growth affect the organization and structure of the photosystem and the composition of pigments. The RNA of *D. salina* was sequenced to investigate the transcriptomic response of the organism after transitioning from normal light conditions to higher light intensity.

Genes encoding for enzymes involved in photosynthesis were down-regulated; whereas, genes involved in the metabolism of carotenoid and triacylglycerol were upregulated. Genes encoding for photoprotective enzymes related to reactive oxygen species scavenging and to the xanthophyll cycle were also upregulated at higher irradiance levels. Concurrently, Photosystem II activity and chlorophyll content were reduced, carotenoids content and neutral lipids increased as light intensity increased, suggesting that the photosynthetic apparatus was reduced and photoprotection mechanisms were activated.

Furthermore in a follow-up study, Li *et al.* (2019c) examined carotenoid synthesis in *D. salina* under red and blue light. Cultures of *D. salina* were exposed to red (660 nm) and blue (450 nm) light. The cell growth, total carotenoid content, and transcriptomes were analyzed. Growth was enhanced by illumination with red light, but blue light did not promote algal growth. In contrast, the total carotenoid content increased under both red and blue light. Six transcripts encoding for blue light receptor cryptochrome were identified, and transcripts involved in the carotenoid metabolism were up-regulated under both red and blue light. Transcripts encoding for photoprotective enzymes related to the scavenging of reactive oxygen species were up-regulated under blue light.

Nan *et al.* (2018) reported a transcriptomic analysis of the effects of different light intensities on the growth and transcriptomics of a typical freshwater rhodophyte (*Sheathia arcuata*) that is an important component of freshwater microflora. Different gene expression patterns were caused principally by different irradiances, considering the similarity in water conditions of the sampling site where the specimens were collected. Comparison results of gene expression levels under different irradiances revealed that photosynthesis-related pathways were significantly up-regulated under the weak light. Molecular responses leading to improved photosynthetic activity include the transcripts corresponding to antenna proteins (LHCA1 and LHCA4), photosynthetic apparatus proteins (PSBU, PETB, PETC, PETH and beta and gamma subunits of ATPase) and metabolic enzymes in the carbon fixation pathway. Along with photosynthesis, other metabolic activities were also regulated to optimize the growth and development of *S. arcuata* under appropriate sunlight. Protein-protein interactive networks revealed that the most responsive up-expressed transcripts were ribosomal proteins.

The response of three Southern Ocean phytoplankton species to ocean acidification and light availability was studied using transcriptomics by Beszteri *et al.* (2018). They report that ocean acidification (OA) and high light was found to negatively affect the three Antarctic key species *Phaeocystis antarctica* (haptophyte), and *Fragilariopsis kerguelensis* and *Chaetoceros debilis* (diatoms). To examine the underlying physiological response at the transcriptomic level, the three species were grown under ambient and elevated pCO_2 combined with low or high light. RNA sequencing revealed that the haptophyte (*P. antarctica*) was much more tolerant towards OA than the two diatoms, because only these showed distinct OA-dependent gene regulation patterns.

Under ambient pCO_2, high light resulted in decreased glycolysis in *P. antarctica*. Contrastingly, upregulation of genes related to cell division and transcription as well as reduced expression of both cata-and anabolic carbon related pathways were seen in *C. debilis*. OA in combination with low light led to reduced respiration, but also surprisingly to higher expression of genes involved in light protection, transcription and translation in *C. debilis*. Though not affecting *P. antarctica*, OA combined with high light also caused photosensitivity in both diatoms.

Temperature stress. Considerable attention has been given to transcriptomic studies of temperature effects on algae, both at relatively low temperatures or at elevated temperatures related to the normal temperature range in the habitat of the species. Hwang *et al.* (2008) examined the differentially expressed genes using cDNA chip analysis and transcriptomic evidence for the Antarctic alga *Chaetoceros neogracile* maintained in laboratory cultures between 4 and 10°C. Among

the 1,439 probes, 21.5% were differentially regulated (≥ two-fold) by the temperature upshift within three days. Up-regulation was more prominent among cytoprotective genes; whereas, down-regulation was featured in photosynthetic genes. A third of the differentially expressed genes had an unknown function or no similarity to known genes, highlighting their potential importance as a resource to identify key players in the acclimation response of polar algae under thermal stress.

Transcriptome analysis also revealed novel aspects of temperature-responsive, coordinated changes in the abundance of specific mRNAs, along with the rapid establishment of molecular homeostasis in polar algae. Although a large number of *C. neogracile* genes responded rapidly to heat-imposed stress, acclimatory homeostasis was quickly established in the transcriptome within three days of thermal stress. Even within 15 min. after transferring the cells to 10°C conditions, eighty-three percent (n = 113) of the differentially transcribed genes were up-regulated; while 17% (n = 24) were down-regulated at 10°C relative to 4°C after 15 min. With prolonged culturing at 10°C for up to one hour, the number of differentially expressed genes increased to 239. Unexpectedly, a small set of genes encoding fucoxanthin chlorophyll a/c-binding proteins were rapidly up-regulated by thermal stress, implying that they have different roles other than light harvesting.

Ice-binding proteins (IBPs) have been identified in ice-associated algae indicating that these proteins may be essential for survival in icy habitats. To explore the generality of these findings, and probe for possible origins of IBPs, Raymond and Remias (2019) studied *Kremastochrysopsis austriaca* (Chrysophyceae), an Austrian snow alga that is not closely related to any of the ice-associated algae examined so far. They reported that *K. austriaca* also produced IBPs, although their activity was weak. Sequencing the algal genome and the transcriptomes of cells grown at 1 and 15°C revealed three isoforms of a type 1 IBP. In agreement with their putative function, the three isoforms were strongly up-regulated by one to two orders of magnitude at 1°C compared to 15°C. In a phylogenetic tree, the *K. austriaca* IBPs were distant from other algal IBPs, with the closest matches being bacterial proteins. These results suggest that the *K. austriaca* IBPs were derived from a gene that was acquired from a bacterium unrelated to other IBP donor bacteria.

Liu *et al.* (2016) examined the acclimation of Antarctic *Chlamydomonas* sp. ICE-L to the sea-ice environment using transcriptomic analysis, and also reported evidence that many genes in the ICE-L transcriptome have high similarity to the genes from Antarctic bacteria. These include genes that encode polyunsaturated fatty acid synthesis enzymes (promoting fluidity of the cell membranes under freezing temperatures), molecular chaperon proteins, and cell membrane transport proteins. These ICE-L genes are apparently acquired through horizontal gene transfer from its symbiotic microbes in the sea-ice brine. The presence of these genes in both sea-ice microalgae and bacteria indicated the biological processes they involved in are possibly contributing to ICE-L success in sea ice. Moreover, differential gene expression analysis at the transcriptome level of ICE-L indicated that genes that are associated with post-translational modification, lipid metabolism, and nitrogen metabolism are responding to the freezing treatment.

Zhang *et al.* (2019) conducted transcriptome sequencing of two Antarctic psychrophilic green algae (*Chlamydomonas* sp. ICE-L and *Tetrabaena socialis*) and performed positive selection and convergent substitution analyses to investigate the two species molecular convergence and adaptive strategies against extreme cold conditions. The results revealed considerable shared positively selected genes and significant evidence of molecular convergence in the two Antarctic psychrophilic algae. Significant evidence of positive selection and convergent substitution were detected in genes associated with photosynthetic machinery, multiple antioxidant systems, and several crucial translation elements in Antarctic psychrophilic algae.

An arctic *Chlorella*-Arc sp., exhibiting eurythermal adaptive mechanisms, was studied in laboratory conditions by Song *et al.*, (2020) using transcriptome analysis of differentially expressed gene profiles under high temperature, low temperature, and control temperature treatments at 24°C, 3°C and 15°C. Transcripts for photosynthesis, carotenoid biosynthesis, carbon fixation in photosynthetic organisms, pentose phosphate pathway, polysaccharide biosynthesis and fatty acid metabolism were found to be highly represented in response to thermal/cold stress. Low temperature induced up-regulation of genes encoding photosystem components, including PsbB (CP47), PsbC (CP43), PsbA (D1), PsaA and PsaB to make up for the loss of light harvesting antenna pigments to maintain normal photochemical activity under low temperature. The coordinated up-regulation of key enzymes in gluconeogenesis and polysaccharide synthesis, and down-regulation of lipid biosynthesis, sup-

port the hypothesis that *Chlorella*-Arc employs a flexible allocation of carbon between sugars and lipids to acclimate to the thermal/cold stress.

Unusually harsh geographic locales beyond polar regions offer unique opportunities to study algal adaptations to extreme cold. Zhang *et al.* (2020) published a comprehensive transcriptome comparative analyses of two *Oocystis* algae (*Oocystis marina* and *Oocystis* sp. LXD-20) growing on the Qinghai-Tibet Plateau (QTP). Despite the ecological importance of *Oocystis* algae on the QTP, the genetic mechanisms of the adaptations of these algae to this high-altitude environment are poorly understood. The authors' comparative transcriptomic and evolutionary analyses were undertaken to reveal the adaptive strategies of the algae.

The results identified 348 positively selected genes in *Oocystis* algae, and functional analyses indicated that many of these positively selected genes were associated with adaptation to abiotic stresses, such as antioxidative response, DNA repair mechanisms, translation, and post-translational modifications. Also the authors identified the cold-responsive and UV-B-responsive genes in *O. marina* and *Oocystis* sp. LXD-20, and revealed the transcriptional regulation strategies under stress conditions. Among the specific findings, functional analyses showed that upregulated unigenes were significantly enriched in pathways associated with genetic information processing, such as "proteasome," "ubiquitin-mediated proteolysis," "spliceosome," "mRNA surveillance pathway," "RNA transport," and "protein export". The proteasome and ubiquitin-mediated proteolysis have a vital role in plant development and adaptation to environmental stresses, including cold, drought, salinity, and nutrient deprivation.

The effects of elevated temperatures above the normal for the scleractinian coral dinoflagellate symbiont (*Symbodinium* sp.), inhabiting coral reefs, was examined using transcriptomics to determine possible mechanisms contributing to coral bleaching with increasing temperatures of oceanic water (Gierz *et al.*, 2017). Cultures were exposed to elevated temperatures (average 31°C) or control conditions (24.5°C) for a period of 28 days. Whole transcriptome sequencing of *Symbiodinium* cells on days 4, 19, and 28 were used to identify differentially expressed genes under thermal stress. A large number of genes representing 37.01% of the transcriptome (~23,654 unique genes) with differential expression were detected at no less than one of the time points. Consistent with previous studies of *Symbiodinium* gene expression, changes across the transcriptome were low, with 92.49% differentially expressed genes at ≤ 2-fold change. The transcriptional response included differential expression of genes encoding stress response components such as the antioxidant network and molecular chaperones, cellular components such as core photosynthesis machinery, integral light-harvesting protein complexes and enzymes such as fatty acid desaturases. Differential expression of genes encoding glyoxylate cycle enzymes were also found, representing the first report of this in *Symbiodinium*.

The effects of elevated temperatures on thermotolerant *Ulva prolifera* and the role of salicylic acid (SA) in thermotolerance regulation was studied by Fan *et al.* (2017). Complementary transcriptome and proteome analyses were performed with *U. prolifera* grown at 35°C and with the addition of SA at high temperature. The results indicated that SA alleviated the high-temperature stimulus partially through antioxidant related proteins that were up-regulated, jasmonic acid signal pathways enhancement, increased Ca^{2++}-binding proteins and photosynthesis-related proteins, elevated antioxidant enzyme activities and changed photosynthesis index.

With the aim of better understanding the possible evolutionary origin of land plants from filamentous algae, de Vries *et al.* (2020) studied heat stress response in the closest algal relatives of land plants, and found conserved stress signaling circuits. They assumed that transition from aquatic to land existence included likely increases in thermal stress. The effect of heat stress was studied in *Mougeotia* and *Spirogyra*, two representatives of Zygnematophyceae – the closest known algal sister lineage to land plants. Heat stress induced pronounced phenotypic alterations in their plastids. The global differential gene expression responses triggered by heat were examined. Each organism had its own distinct gene expression profile; less than one-half of their shared genes showed concordant gene expression trends. Nevertheless, they detected common signature responses to heat such as elevated transcript levels for molecular chaperones, thylakoid components, and (corroborating their metabolomic data) amino acid metabolism. They also uncovered the heat-stress responsiveness of genes for phosphorelay-based signal transduction that links environmental cues, calcium signatures and plastid biology.

Salinity stress. Given the globally significant differences in salinity among widely different aquatic environments and the adaptive challenges for organisms living at the boundaries between freshwater and marine

environments a relatively large number of studies addressed salinity tolerance and stress responses among algae. Illustrative examples for a variety of taxonomic groups are reviewed here.

Abdellaoui *et al.* (2019) examined the effects of salt stress on gene expression in *Chlorella vulgaris*. Transcriptome analysis was performed for the *C. vulgaris* response to salt stress (1% and 3% NaCl) applied for different times (2 hours and 4 hours). The number of upregulated genes after four hours of salinity stress was greater than the number of downregulated genes, suggesting that the alteration of gene expression may be related to a mechanism of adaptation to a high-salinity environment. Further analyses revealed that numerous biological pathways were affected by salt stress. Among the upregulated pathways, the cytoplasmic calcium signaling pathway, which is involved in the regulation of homeostasis, was highly upregulated. Genes involved in the photosystem I light-harvesting pathway were downregulated under salt stress.

In a further study of green alga responses to salinity stress, Wang *et al.* (2018) documented salt stress responding genes in *Chlamydomonas reinhardtii* using transcriptomics. Physiological evidence indicates that saline stress increases intracellular peroxide levels and inhibits photosynthetic-electron flow in *C. reinhardtii*. In this study, the transcriptome analysis of short-term acclimation to salt stress (200 mM NaCl for 24 hours) was performed in *C. reinhardtii*. A total of 10,635 unigenes were identified as being differently expressed by RNA-sequencing, including 5,920 up-and 4,715 downregulated unigenes. A series of molecular cues were screened for salt stress response, including maintaining the lipid homeostasis by regulating phosphatidic acid, acetate being used as an alternative source of energy for solving impairment of photosynthesis; and enhancement of glycolysis metabolism to decrease the carbohydrate accumulation in cells.

The salinity tolerant green alga (*Dunaliella salina*) is widely studied as a model laboratory protist. As a follow-up to research on transcriptomics of salt stress response by Li *et al.* (2019c) who showed that salinity stress upregulated key genes in photosynthesis and glycerol metabolism, consistent with results by Fang *et al.* (2017), Gao *et al.* (2019) analyzed salinity stress responsive miRNAs and target genes in this species. Their goal was to better characterize the molecular basis for its euryhaline responses. Analysis of miRNA expression in *D. salina* under salinity stress found that 49 miRNAs showed significant differences in expression. For the first time in *D. salina*, 745 target genes, regulated by 194 miRNAs, were validated by degradome sequencing. Further analyses showed that these miRNA target genes are involved in a variety of molecular biological regulation processes, such as signal transduction, material transport, transcriptional regulation and protein processing. In combination with transcriptome sequencing results, 14 differentially expressed miRNAs and 87 differentially expressed target genes were found to be negatively correlated in expression. Further analysis showed that mmu-miR-466, dme-miR-2493, mmu-mir-669h, dre-mir-29a and dme-mir-9388 play an important role in osmoregulation in response to high salinity stress in *D. salina*.

Two species of *Chara*, a typical freshwater Streptophyte (green alga), were studied by Phipps *et al.* (2021) for transcriptome response to salt stress; particularly in relation to transport mechanisms in *Chara longifolia* (salt-tolerant) and *Chara australis* (salt-sensitive). During a time-course study after transfer from freshwater to salt water, transcriptomes verified that a cation transporter (HKT), a Na^+ /H^+ antiport (NHX), H^+-ATPase (AHA), and a Na^+-ATPase (ENA) were involved in the response to changes in salinity.

The oceanic microalga (*Nannochloropsis oceanica*) member of the Eustigmatophyceae was studied by Guo and Yang (2015) to identify changes in gene activity using transcriptomics when cultured cells were transferred from seawater to freshwater. Differentially expressed genes were mainly assigned to the degradation of cell components, ion transportation, and ribosomal biogenesis. These findings indicated that the algal cells degrade their components (mainly amino acids and fatty acids) to yield excessive energy (ATP). This energy source is used to maintain cellular ion (mainly K^+ and Ca^{2+}) homeostasis; while the depletion of amino acids and ATP, and the reduction of ribosomes, attenuate the protein translation and finally slow down the cell growth.

To more fully understand how diatom species respond to salinity variations, Bussard *et al.* (2017) used integrated physiological assays and measurements of morphological plasticity with a functional genomics approach to examine the regulatory changes that occur during the acclimation to salinity in the estuarine diatom *Thalassiosira weissflogii*. Cells exposed to different salinity regimes for a short-term (ST) or long-term (LT) treatment presented adjustments in their carbon fractions, silicon pools, pigment concentrations and/or photosynthetic parameters. Salinity-induced altera-

tions in frustule symmetry were observed only in the LT cultures. Whole transcriptome analyses revealed a down-regulation of nuclear and plastid encoded genes during the LT response and identified only a few regulated genes that were in common between the ST and LT responses. Consequently, the authors proposed that in diatoms, one strategy for acclimating to salinity gradients and maintaining optimal cellular fitness could be a reduction in the cost of transcription.

Desiccation stress. Some attention has been given to desiccation stress among algae. Peredo and Cardon (2020), following up on prior evidence that desiccation tolerance (DT) genes are upregulated in some tissues of desiccation tolerant plants, examined comparative transcriptomics of desiccation-tolerant and intolerant green algae to determine if the same suite of DT genes is upregulated in the desiccation-tolerant algae. They used three closely related aquatic and desert-derived green microalgae in the family Scenedesmaceae and capitalized on extraordinary desiccation tolerance in two of the species, contrasting with desiccation intolerance in the third.

Results showed that during desiccation, all three species increased expression of common protective genes. However, the feature distinguishing gene expression in DT algae was extensive down-regulation of gene expression associated with diverse metabolic processes during the desiccation time course, suggesting a switch from active growth to energy-saving metabolism. This widespread downshift did not occur in the desiccation-intolerant taxon. These results showed that desiccation-induced up-regulation of expression of protective genes may be necessary, but is not sufficient, to confer desiccation tolerance. The data also suggested that desiccation tolerance may require induced protective mechanisms operating in concert with massive down-regulation of gene expression controlling numerous other aspects of metabolism.

Development of desiccation resistance in *Zygnema circumcarinatum* in experimental laboratory cultures was analyzed using transcriptomics by Rippin *et al.* (2017). Cultures of *Z. circumcarinatum* grown in liquid medium or on agar plates were desiccated at ~86% relative air humidity until the effective quantum yield of PSII [Y(II)] ceased. In general, the response to dehydration was much more pronounced in *Z. circumcarinatum* cultured in liquid medium for 1 month compared with filaments grown on agar plates for 7 and 12 months. When cultured on solid medium, the alga became acclimated to dehydration much better, and an increase in desiccation tolerance was clearly correlated to increased culture age. Moreover, gene expression analysis revealed that photosynthesis was strongly repressed upon desiccation treatment in the liquid culture; while only minor effects were detected in filaments cultured on agar plates for 7 months. Otherwise, both samples showed induction of stress protection mechanisms such as reactive oxygen species scavenging (early light-induced proteins, glutathione metabolism) and DNA repair as well as the expression of chaperones and aquaporins. Additionally, *Z. circumcarinatum* cultured in liquid medium upregulated sucrose-synthesizing enzymes and strongly induced membrane modifications in response to desiccation stress.

Huang *et al.* (2021), using transcriptome, proteome, and metabolome analyses, examined the response of a red alga (*Pyropia haitanensis*) to intertidal desiccation during low tides when blades of the alga were exposed to desiccation stresses. Experimental treatments included single stress (SS) of desiccation, and triple stresses (TS) of desiccation, high-temperature, and high-light. Differentially expressed genes (DEGs), differentially expressed proteins (DEPs), and differentially accumulated metabolites (DAMs) were identified and further analyzed in pairs. The results showed that several pairs of DEGs/DEPs, DEGs/DAMs, and DEPs/DAMs participated in glyoxylate and dicarboxylate metabolism, and carbon fixation in photosynthetic organisms. Moreover, several pairs of DEGs/DAMs were significantly enriched in ether lipid metabolism. Correlated DEGs/DAMs of glyoxylate and dicarboxylate metabolism were significantly enriched under both stressful conditions, and correlated DEGs/DAMs and DEPs/DAMs of carbon fixation in photosynthetic organisms were significantly enriched under SS and TS conditions, respectively.

The authors speculated that the plasma membrane responded first to intertidal desiccation and activated stress signal transduction by degradation of phospholipid. Organic acids (malate and succinate) and amino acids (glutamate, aspartate, and alanine), involved in osmoregulation, were then synthesized and accumulated to scavenge reactive oxygen species (ROS). Further evidence was presented on the changes in energy metabolism and photosynthesis that accompanied adaptive mechanisms to avoid desiccation stress, especially with higher illumination and possible heat stress encountered during intertidal exposure of the alga.

Additional evidence of the effects of intertidal stress on algae was investigated by Salavarría *et al.* (2018) who reported a transcriptome analysis of the brown alga

Macrosystis integrifolia (Phaeophyceae) using samples collected from the intertidal zone during low tide (considered as abiotic stressed) and samples collected from the subtidal zone (considered as control). A total of 535 unigenes (271 upregulated and 264 downregulated) showed significantly altered expression between the stressed and control samples. In the abiotic-stressed condition, three categories of genes were upregulated: i) relative expression levels of genes associated with antioxidant defenses (vanadium-dependent bromoperoxidase, glutathione S-transferase, lipoxygenase, serine/threonine-protein kinase, aspartate Aminotransferase, HSPs), ii) water transport (aquaporin), and iii) photosynthesis (light-harvesting complex) protein. Whereas, in the non-stressed control condition, most of the genes predominantly involved in energy metabolism were overexpressed: NADH-ubiquinone oxidoreductase/NADH dehydrogenase, NAD(P)H-Nitrate reductase, long-chain acyl-CoA synthetase, and UDP-n-acetylglucosamine pyrophosphorylase.

Conclusions and Future Prospects for Research

The conceptual framework for this review of the current status of transcriptomic research with heterotrophic and autotrophic protists contained three main themes: i) *Physiology and metabolism*, ii) *Development and life cycles*, and iii) *Environmental and ecological studies*. The aim was to characterize the current status of the field encompassing research across cell biological to environmental and ecological levels of biological organization. The search of the literature particularly focused on publications in the last two decades to largely address the most recent advances. As a result, some of the earlier pioneering work was not included in this review. However, much of the research cited in this review contains literature citations that link back into earlier historical contributions to the field.

The objective of this review of the literature was to be representative, as much as possible, of research published within the above three themes. This review does not provide a quantitative representation of the amount of published research within the scope of the three themes. Nonetheless, it became increasingly clear that a relatively larger amount of research has been done with autotrophic protists compared to heterotrophic protists. Some of the emphasis on autotrophic protists can be traced to increasing interest in biotechnology of algae, particularly using algal cultures as a way to improve lipid production for possible commercial alternatives to production of fossil petroleum fuels. Moreover, some autotrophic protists may be more easily isolated and brought into laboratory culture, and thus there could be more available for transcriptomic research.

This emphasis on autotrophic protists is especially apparent in the number of published studies in the section on Physiology and metabolism of autotrophic protists, where numerous papers using a wide range of algal species examined algal metabolism under a variety of environmental variables, with the goal of accounting for the amount of organic compounds (particularly lipids) that were produced. Some of this research also has significance for ecology and environmental studies, because studies of biomass production (although largely focused on lipids) also included other related organic compounds as well as metabolic correlates of the environmental variables that were used in the culturing studies.

Other areas of strong research development also emerged while reviewing aquatic and biological oceanic research with algae, where transcriptomics and related molecular genetic studies provided important evidence of how variations in geographic location, water mass properties and other environmental variables influenced the physiology and ecological status of important primary producers in these locales. With increasing interest in the role of viruses, especially in aquatic environments, particularly giant viruses, additional transcriptomic research on protist-virus interactions is warranted; and there is increasing evidence of the significance and productive potential in this line of research (e.g., Ku *et al.* 2020).

Relatively fewer studies on physiology and metabolism of heterotrophic protists were discovered; and as may be expected a good number of these were related to biomedically important taxa such as pathogenic amoebae, ciliates and flagellates. Of greater concern, is the relatively less published transcriptomic research on environmental and ecological studies of heterotrophic protists. Currently, the significant role of heterotrophic protists in terrestrial and aquatic habitats is well established both from a purely scientific perspective as well as for applications in agriculture, commercial aquatic endeavors including fisheries, and preservation of natural environmental quality. Several leading examples of application to soil protist ecology were cited above (e.g., Geisen *et al.* 2015; Song *et al.* 2015), as well as prior published recommendations for broader applications to soil microbiological research (Prosser 2015).

Heterotrophic protist predators transfer nutrients to higher trophic levels in aquatic and soil food webs

(de Ruiter *et al.* 1995; Crotty *et al.* 2012). Predation by heterotrophic protists also stimulates microbial activity and nutrient cycling via the microbial loop, thus enhancing growth and activity of autotrophic protists in aquatic and soil ecosystems (Bonkowski and Clarholm 2012; Caron 2005).

Application of modern transcriptomics in laboratory microcosm and model ecosystem studies can contribute substantially to our understanding of the complex interactions among heterotrophic and autotrophic protists in aquatic and in terrestrial ecosystems; for example, by examining how differentially expressed genes (DEGs) change when the model ecosystems vary in terms of the number, taxonomic composition, and functional roles of protists that are included in the experimental set up. Moreover, varying abiotic factors in conjunction with the foregoing biotic variables can add another dimension increasing the relevance of these laboratory studies to understanding the natural environment. Further research in the natural environment to explore these relationships with appropriate emphasis on heterotrophic and autotrophic protists can be highly productive in correlating laboratory and field-based evidence using transcriptomics.

The marked efficiency and substantial volume of transcripts produced by high throughput sequencing can yield massive amounts of gene transcript data, often presented as heat maps or other data-rich representations. While these are concise ways of presenting high density data, it can be a challenge to interpret the results more broadly beyond cellular biology to population, community and ecosystem levels. Increasing use of network analyses (Proulx *et al.* 2005), particularly based on rich data sets generated by molecular genetic evidence (e.g., transcriptomics and metabolomics), promises to provide deeper insights into the complexities of the roles of heterotrophic and autotrophic protists in natural and laboratory studies of microbial physiological ecology, including the contributions by prokaryotes (Ren *et al.* 2017; Mo *et al.* 2021).

With an increasing volume of published transcriptomic data on autotrophic and heterotrophic protists, there is an opportunity for additional metatranscriptomic research studies; particularly if there is improved access to digital libraries and compendiums such as A *Global Ocean Atlas of Eukaryotic Genes* (Carradec *et al.*, 2018) and *The Marine Microbial Eukaryotic Transcriptome Sequencing Project* (Keeling *et al.*, 2014) that aims to assemble, functionally annotate, and make publicly available eukaryotic microbial transcriptomes, especially for expanding their use in marine ecology.

One of the major challenges for research, particularly in terrestrial ecosystems, is to more fully account for encysted versus trophic stages when doing transcriptomic research studies. Considerable interest has been given to analyzing molecular genetics of the encystment process (e.g., Schapp and Schilde 2018), but there is less evidence of attention to the molecular genetics of different fractions of protists that are in varying stages of activation (trophic vs. encysted) within ecosystems, particularly in relation to major abiotic and biotic variables.

Many freshwater and estuarine heterotrophic protists, in contrast to open ocean species, encyst when conditions become less favorable for growth; either due to drying of temporary aquatic habitats, or when prey are less available. For example, the latter is particularly true of amoeboid protists that are abundant and active in aquatic environments when there is sufficient floc or large particulate suspended matter where they can attach, locomote, and feed on available surface-associated prey, but become encysted in the absence of floc-related prey (e.g., Anderson 2011). Although this topic has been researched rather substantially using conventional techniques such as microscopic analyses, there is a substantial advantage to applying modern molecular genetic techniques to more fully document the metabolic state of protists in varying aquatic environmental conditions.

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