OPTIMIZING RNA EXTRACTION FROM FUNGAL-ALGAL TISSUES FOR TRANSCRIPTOMIC GENE EXPRESSION ANALYSIS

by
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ABSTRACT
MICHEL JOSEPH HOHL: Optimizing RNA Extraction from Fungal-Algal Tissues for Transcriptomic Gene Expression Analysis
(Under the direction of Erik Hom)

Extracting high-quality RNA is critical for downstream applications such a qRT-PCR and RNA-Seq based transcriptomics. Single-stranded RNA is readily susceptible to degradation by environmental RNases, necessitating rapid and sterile homogenization techniques; freezing at -80°C, lysis buffer addition, and short bursts of beadbeating have been shown to increase yields and quality of extracted RNA. Utilizing fungal-algal tissues from the model symbiosis between the filamentous fungi *Aspergillus nidulans* and the green alga *Chlamydomonas reinhardtii*, three different commercial homogenizers and six RNA extractions kits were assessed and an optimized extraction protocol for total RNA from fungal-algal tissues was determined. Using the protocol designed in this study, the relationship between *Chlamydomonas reinhardtii* and the budding yeast *Saccharomyces cerevisiae* when grown under circadian-based light conditions prior to co-culturing will be assessed using RNA-Seq to determine changes gene expression patterns in response to co-culturing under a new light condition to look for synchrony. A phenotypic study of this system was performed and while no significant differences in biomass were observed, it is believed that with the fungus *Neurospora crassa*, a model organism for studying circadian rhythms, a light regimen-dependent phenotypic resonse will be observed.

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LIST OF ABBREVIATIONS

BB	Mini-BeadBeaster-24
CKI/CKII	Casein kinase I and II
CO_2	Carbon dioxide
FP	FastPrep-24 5G
frq	*Frequency* gene
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
$LiCl_2$	Lithium chloride
mL	Milliliter
mm	Millimeter
mg	Milligram
ng	Nanogram
NH_3	Ammonia
NH_4	Ammonium
NO_2	Nitrite
PCR	Polymerase Chain Reaction
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
rpm	Rotations per minute
SB	SoniBeast
TRI	Total RNA Isolation
µE	Microeinsteins
µL	Microliters

INTRODUCTION

Symbiotic relationships are of great relevance in many fields of modern science, including biofuel productions, pharmaceuticals, and ecological engineering. From the mutualistic bacteria in the human intestinal tract to the relationships between corals and marina algae, symbiotic relationships are found everywhere and are crucial to the survival of many different species. Now, researchers are looking into tapping in to these relationships to derive from them natural products for medicinal purposes (Borowitzka, 1995) or to harness the energetic and chemical products of these systems to develop clean energy sources that are self-sustaining and efficient (Patil, et al., 2008). While fuel-cells and pharmaceuticals can be developed and isolated from microalgae and their symbionts, a greater understanding of the molecular genetics of these interactions may allow for even more powerful manipulation of these species to produce carbon products for fuels or for more efficient derivation of natural products for medicine.

RNA-Seq based transcriptomics, a relatively young technology, provides a powerful window into gene expression analysis, which allows researchers to interpret gene function, determine the molecular makeup of cells and tissue, and understand how organisms develop, interact with and respond to the environment and other organisms (Wang, Gerstein, & Snyder, 2009). For many tissues, though, efficient means of extracting RNA from tissues has not been developed to provide efficient results, or

developed at all, like in the case of filamentous fungi (Leite, Magan, & Medina, 2012) and algae. Commercial nucleic acid extraction kits are available for varying tissue types, but most have been optimized for mammalian and bacterial tissues. For RNA, optimization for extraction of total RNA from fungal and algal tissue samples is still necessary and this study defines a protocol to do so. Utilizing commercially available RNA extraction kits and beadbeating homogenizers, an optimal combination of the two was determined to extract high yields of total RNA for analysis using qRT-PCR and RNA-Seq.

The aim of this analysis is to determine the gene expression patterns between the model alga *Chlamydomonas reinhardtii* and the model fungal species *Aspergillus nidulans* and *Neurospora crassa*, as well as the yeast *Saccharomyces cerevisiae*. An synthetic obligate mutualism between these species was previously engineered (Hom & Murray, 2014) which demonstrated that a physical interaction occurs between the alga and the fungus that may facilitate nutrient exchange or circadian rhythm coupling between the organisms (Hom, et al., 2015). To elucidate what is occurring, RNA-Seq is necessary, allowing us to watch gene expression changes over time in reaction to changing circadian rhythms, co-culturing, and nutrient-deficient stress. We plan to investigate the interactions between the algal and the fungal/yeast species above to determine how niche engineering could be applied to biofuel production, and use this system as a model for the creation of other novel synthetic environments such as mass microbial communities in water kefir or the prevention of toxic algal blooms in marine environments.

CHAPTER 1

Fungal-Algal Mutualisms & Circadian Rhythms

A mutualism is defined as a "mutually beneficial association between different kinds of organisms" (Merriam-Webster, 2015). While many species have well defined and understood mutualistic relationships (i.e. Clown Fish & Anemone or Human & Intestinal Microflora), fungal-and algal species exhibit them as well and we are just beginning to fully understand what causes these relationship to develop. Although there are likely many symbioses that exist between different fungal and algal species from many different taxa, this study primarily focuses on the symbiotic relationships that exist between the model green alga *Chlamydomonas reinhardtii* and several model fungal species *Aspergillus nidulans, Neurospora crassa* and *Saccharomyces cerevisiae,* the model yeast species, also known as budding yeast.

When considering circadian rhythms and symbiotic mutualisms, these species are all of particular interest due to their relatively quick growth cycles making cultures easy to grow and the interdependence of mutant fungi/yeast on the alga for nitrogen and the alga on the fungi/yeast for carbon in synthetic systems (Hom & Murray, 2014). In a closed system, *S. cerevisiae* is able to metabolize glucose to carbon dioxide (CO_2) and *C. reinhardtii* is able to reduce nitrite (NO_2) to ammonia (NH_3) (Azuara & Aparicio, 1985), which is the basis for an obligate mutualism between the two organisms (Hom & Murray, 2014). But, the fungi, which have the ability to utilize the NO_2 (Slot & Hibbett, 2007), do

not naturally form a symbiotic mutualism with the alga unless mutant nitrate/nitrite utilizations strains, known as *nit*- strains, are used which then allows for a mutualism to exist (Hom & Murray, 2014). This demonstrates that the loss of gene function in certain species has the ability to be compensated for within a mutualistic relationship (Morris, Lenski, & Zinser, 2012).

Many mutualistic relationships are believed to exist as a whole due to a syntrophic relationship (Hom, Aiyar, Schaeme, Mittag, & Sasso, 2015), and gene loss compensation is also a viable explanation for why these relationships may develop in the first place. While that mechanism on an evolutionary time scale is still unknown, there are some factors we can analyze now that may help explain the interdependence. Within the synthetic fungal-algal mutualism between *A. nidulans* and *C. reinhardtii*, a physical interaction occurs where the algae physically attach to the hyphae of the fungus (Hom & Murray, 2014). This physical interaction is not fully understood but is potentially a mechanism for controlling nutrient exchange between the two organisms, for defense of one or both organisms, or for other unknown reason that will require gene expression experiments to uncover.

However, it has been shown that the cell-wall interactions within the fungal-algal mutualism are a consequence of cell-wall remodeling enzymes that mainly digest the algal wall (Hom & Murray, 2014). Over-degradation of the cell wall may be to the detriment of the alga, but this is believed to be dictated by environmental or genetic factors (Hom, et al., 2015).

1.1 *Chlamydomonas reinhardtii*

The green alga *Chlamydomnas reinhardtii* is a model organism for the study of photosynthesis due to the evolutionary conservation of its photosynthetic mechanism for around 700 million years (Blaby et al., 2014). Its relatively short growth cycle and well-defined genome makes it an excellent candidate for the study of gene function in relation to photosynthesis. The complete genome of *C. reinhardtii* was sequenced in 2007 revealing a 121 megabase (Mb) genome with 17 chromosomes containing around 15,143 protein-coding genes (Merchant et al., 2007). *C. reinhardtii* is often used as a reference system for the study of human ciliary disease since it is possible to isolate the basal bodies and flagella from the alga, which are highly similar to mammalian cilia, for biochemical and proteomic analysis (Blaby et al., 2014). In combination, these two attributes of *C. reinhardtii* allow for the bridging of the plant and animal lineages, which provides a powerful genetic analytical tool in the assessment of undefined genes (Blaby et al., 2014).

While other organisms have also been studied as model organisms for circadian rhythms, which are simply "endogenous biological programs that control metabolic, physiological, and/or behavioral events to occur at optimal phases of the daily cycle" (Mittag, et al., 2005), *C. reinhardtii* is also an ideal candidate for circadian studies due to its multiple circadian driven processes. Circadian rhythms in the green alga have been demonstrated in the processes of phototaxis, chemotaxis, cell division, UV sensitivity, and adherence to glass (Mittag, et al., 2005; Straley & Bruce, 1979). Protein phosphatases 1 and 2A (PP1 & PP2A) have been shown to regulate the circadian rhythms of other model circadian organisms like *Neurospora crassa* and *Drosophila melanogaster,* and

PP1 and PP2A have been localized in the flagellar axoneme of *C. reinhardtii,* where PP1 contributes to control of flagellar motility and PP2A is anchored in the doublet microtubules (Yang, Fox, Colbran, & Sale, 1999). Since various motility functions (i.e. phototaxis & chemotaxis) are linked to circadian rhythms, these two proteins are thought to be regulated by circadian cycles as well.

1.2 *Neurospora crassa*

The saprotophic, filamentous fungi *Neurospora crassa* has long been studied as a model organism for eukaryotic molecular and biochemical processes. Specifically *N. crassa* has been used to study eukaryotic genetics, metabolism and epigenetic processes (Galagan et al., 2003), mitochondrial function, circadian rhythms, and plant pathology, among others (Davis, 2000). Now, *N. crassa* is one of the most commonly used model organisms mainly due its generalist nature, which has allowed for its application to many different field of biology and chemistry including mycology, medical mycology, pharmacy, and pathology (Davis & Perkins, 2002). Recently, what has allowed for further intensive study of this organism is the sequencing of the complete genome, which is greater than 40 Mb, consisting of 10,082 protein-coding genes with an average length of 1.67 kilobases (kb) covering 44% of the genome (Galagan et al., 2003).

As previously stated, *Neurospora crassa* is a model organism for circadian processes and shares the highly conserved serine/threonine protein phosphatases PP1 and PP2A with *C. reinhardtii* and other model species, which are believed to be involved in circadian regulation. The circadian protein *frequency* (*frq*) in *N. crassa* is regulated by phosphorylation from casein kinase I and II (CKI & CKII), which triggers the

degradation of *frq* (Yang et al., 2004). PP1 influences the stability of *frq* while PP2A opposes the function of PP1, suggesting that these two protein phosphatases act in concert to determine the phosphorylation status of *frq*, which defines the circadian rhythm of *N. crassa* (Yang et al., 2004). There is high possibility that this mechanism is similar to the one that exists in *C. reinhardtii*, which may explain their increased propensity for mutualistic interactions that follow a circadian rhythm as well.

1.3 *Aspergillus nidulans*

The filamentous fungus *Aspergillus nidulans* is also a common model organism other genera of filamentous fungi and has been at the center of cell development and gene regulations research (David, et al., 2008). The complete genome of *A. nidulans* has been sequenced which is one of the main reasons it continues to be a relevant model organism in eukaryotic cell biology studies. The genome size of *A. nidulans* is around 31 Mb organized into 8 chromosomes (David, et al., 2008). While *A. nidulans* is commonly used in concert with *N. crassa* to investigate biomolecular processes, circadian model studies are limited to *N. crassa* due to the lack of *frq* orthologs in *A. nidulans*, which may indicate clocks dictated by other molecular feedback loops (Fuller, Loros, & Dunlap, 2014).

Although in *A. nidulans* a clear circadian clock is not as apparent, it is believed that clock driven cycles may exist (Greene, et al., 2003). One study showed that, although *A. nidulans* conidial development is likely controlled by environmental conditions rather than an endogenous circadian rhythm, unlike other *Aspergillus* species (Greene, et al., 2003), there was some rhythmicity to the expression of the *gdhA* gene in *A. nidulans,*

which encodes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This rhythmic expression of *gdhA* is entrainable and indicates the existence of a potential circadian rhythm in *A. nidulans,* which demonstrates that, like *N. crassa,* its interactions with algal species such as *C. reinhardtii*, may also be related to entrainable circadian rhythms.

1.4 *Saccharomyces cerevisiae*

Also known as the budding yeast, *Saccharomyces cerevisiae* is another classic model organism in eukaryotic cellular and biomolecular studies. The genome of *S. cerevisiae* was the first eukaryotic genome ever completely sequenced and was completed in the early 1990s (Engel et al., 2013). The genome is composed of 16 chromosomes with around 6000 protein-coding genes (Engel et al., 2013) and surprisingly, *S. cerevisiae* shares around 31% of its protein-coding genes with mammals, (Botstein, et al., 1997). This has allowed *S. cerevisiae* to serve as a valuable model for human disease studies as well, including hereditary nonpolyposis colon cancer, neurofibromatosis, ataxia telangiectasia, and Werner's syndrome (Botstein, Chervitz, & Cherry, 1997). Besides modeling human disease, the genome of *S. cerevisiae* has also served as a reference genome, or a scaffold for which other genomes can be modeled and other sequences can be allocated and defined. In fact, the genome is so well understood, current projects are focused on creating the *S. cerevisiae* pan-genome, which would encompass the genetic makeup of all strains and wild isolates (Engel et al., 2013).

Unlike the other fungal species previously discussed, *S. cerevisiae* is not believed to have an innate circadian rhythm and is not a model for circadian-based organisms. But, one study suggest that *S. cerevisiae* actually has entrainable circadian behavior to its

metabolism that is based on light cycle length and light stimulus strength (Eelderink-Chen et al., 2010). This circadian behavior, though, is different in that *S. cerevisiae* has no identified orthologs of the proteins involved with circadian processes like that which is seen in organisms with traditional circadian rhythms like *N. crassa* (Eelderink-Chen et al., 2010). This would imply that there is another mechanism by which *S. cerevisiae* facilitates its circadian behavior and it may be through the release of ammonium (NH_4) or by means of temperature regulation over a 24-hour period. But, much is still unknown about this circadian behavior in budding yeast.

Whether *A. nidulans* and *S. cerevisiae* have an innate circadian rhythm like *C. reinhardtii* and *N. crassa,* or they have an entrainable portion of their biology that can facilitate a circadian pattern, we believe that when combined in co-culture and mutant strains are used where necessary to make the organisms obligate mutualists, a general circadian pattern of interaction will exist and synchrony will be achieved. Other symbionts, such as *Paramecium bursaria* and *Chlorella,* have also demonstrated the interdependence of the two organisms and their circadian clocks on one another and how synchrony is crucial to their relationship (Tanaka & Miwa, 1996). And while the symbiotic relationships between the organisms described above do not occur naturally, by studying the relationships between model organisms and how mutualism can makeup for gene loss, we can extrapolate the information we gather to other natural symbiotic relationships to describe and explain their existence, formation, and persistence.

To fully analyze what is going on in the systems developed, the RNA of the species must be sampled before, periodically throughout, and after experimental parameters are applied to the cultures. These parameters typically include duration of the

experiment, light cycle length and variation, and the magnitude of the light doses. An initial sample is taken to define the system at time zero before samples are taken at equally spaced time intervals for a predetermined amount of time. A final time point is used as an end marker to define total change in gene expression. The RNA is extracted from all of the samples taken to examine gene expression changes in relation to the parameters described above as well as in response to different time points within a circadian pattern. Algal cells attached to fungal hyphae are also distinguished from algal cells that are free-floating in the supernatant; while both are sampled and their RNA is collected simultaneously, differences in gene expression is expected.

For this to be efficient, a succinct protocol is necessary to avoid RNA degradation or contamination and to improve total RNA quality and quantity. Currently, most molecular techniques are optimized for DNA extraction and sequencing, but the field of RNA-Seq based transcriptomics is relatively young and the molecular techniques for isolation and analysis of RNA are still being developed and improved. Utilizing a novel fungal-algal symbiosis (Hom & Murray, 2014), our target study seeks to define an optimal protocol to (i) extract high-quality total RNA from a suite of model fungal-algal systems for RNA-Seq analysis to (ii) define the gene expression patterns of these organisms when grown together in a closed system on a circadian cycle and (iii) to uncover how their physical interactions affect their physiology and gene expression.

CHAPTER 2

RNA Extraction from Fungal-Algal Tissues

Extracting high quality total RNA from a sample is critical for downstream applications in molecular biology such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) or RNA sequencing (RNA-Seq). The single-stranded structure of RNA makes it particularly labile in the environment; it also allows for easy degradation by RNases requiring a necessity for rapid and sterile homogenization techniques. Freezing at -80°C, addition of a lysis buffer to the sample, and short bursts of beadbeating to decrease sample temperature have shown to result in higher yields of total RNA (Leite, et al., 2012). While commercial extraction kits that are currently available have been optimized for mammalian and microbial samples, few have optimized their efficacy on isolating RNA from filamentous fungi (Leite, et al., 2012) or algae.

While some commercial kits claim to be effective on filamentous fungi, the factors that affect the efficacy of these kits as well as downstream applications related to the eluted RNA are poorly characterized. The cell wall of most fungi is composed of β-1,3/1,6-glucans linked to chitin via a β-1,4 glucosidic linkage (Latgé, 2007). Specifically, the cell walls of *S. cerevisiae* and *Aspergillus fumigatus*, a pathogenic cousin of *A. nidulans*, are composed of 3% and 4% β-1,6 glucosidic linkages (Latgé, 2007). With >90% of fungal cell walls composed of these polysaccharides (Latgé, 2007), lysis of

fungal cells to release intracellular contents such as DNA, RNA, and protein requires aggressive mechanical, chemical or enzymatic techniques. The cell wall of *S. cerevisiae* is composed of roughly 1-2% chitin microfibrils while the cell wall of *A. fumigatus* is composed of 10-20% chitin microfibrils (Bowman & Free, 2006), suggesting that different cells may require different homogenization techniques to successfully release intracellular contents. Mechanical homogenization, such as ultrasonication and bead beating, has been found to be the most effective for homogenization of yeast and filamentous fungi (Klimek-Ochab, Brzezińska-Rodak, Żymańczyk-Duda, Lejczak, & Kafarski, 2011). Homogenization with beads, otherwise known as "bead beating," is the high speed shaking of cells in a tube with small beads to break up the cells and release the intercellular contents.

One advantage of bead beating is the significantly reduced possibility of cross-contamination due to machine automation, reducing processing by hand (Leite, et al., 2012). Homogenization via bead beating has shown to be the most effective and consistent at yielding high amounts of nucleic acids from filamentous fungi (Burik, et al., 1999). But, also with filamentous fungi, the quality or quantity of extracted total RNA can be affected by various chemical factors such as secondary metabolite production (Kiefer, Heller, & Ernst, 2000), endogenous RNase release (Sallau et al., 2013; Patyshakuliyeva, Mäkelä, Sietiö, de Vries, & Hildén, 2014), and leftover chemical residue from commercial extraction kits (Fleige & Pfaffl, 2006).

Various steps can be taken to forego these problems. As previously stated, physical factors such as cell wall thickness can be overcome with the use of high-speed bead beating homogenizers, which utilize an automated specific range of motion, speed,

and bead type to lyse difficult samples. Traditional methods of tissue homogenization such as grinding with mortar and pestle under liquid nitrogen or vortex homogenization are rapidly being replaced by new beadbeating technologies as they reduce processing time and opportunity for contamination while increasing tissue disruption and therefore RNA yield. Chemical factors such as release of endogenous RNase and secondary metabolite production can be overcome by the addition of a lysis buffer to the tissue (Sallau et al., 2013), which destabilizes RNases and, if they are tissue specific, will treat the issue of secondary metabolite content in a lysed sample solution.

Compared to the quantity of RNA extraction studies on RNA extraction and analysis from filamentous fungi, green algae, and algae in general for that matter, have even fewer studies (Thanh et al., 2009). As with all tissue homogenization studies, physical and chemical barriers are necessary to address to extract high quality and quantity total RNA. The cell wall of *C. reinhardtii* is rather fragile, composed of 50% sugars (mainly arabinose and galactose), 10% amino acids, and a mixture of other constituents (Miller, et al., 1974). The main complexes that exist in the cell wall of *C. reinhardtii*, and others in the *Chlamydomonas* genus, are hydroxyproline heterooligosaccharides (Miller, et al., 1974). The cell wall of *C. reinhardtii* has also been shown to completely lack cellulose (Roberts, et al., 1972).

The fragility of the cell wall of *C. reinhardtii* dictates that homogenization technique is most likely not an issue that is critical to extracting RNA, as long as contamination is reduced and RNases are inactivated. Beadbeating frozen samples, though, is still the most efficient method at lysing the cell walls of the algae (Kim et al., 2012). But, endogenous chemical release, like in fungi, is an issue that could interfere

with the RNA quality or with the downstream applications and need to be addressed. Green algae, like plants, when lysed can release high contents of polysaccharides, pigments, and other compounds (i.e. polyphenolic compounds, lipids) that cause complications with RNA experiments since these compounds sometimes co-precipitate with RNA (Daohong, et al., 2004; Thanh et al., 2009).

The green alga *Zygnema cruciatum* was found to be resistant to Total RNA Isolation reagent (TRI) based and lithium chloride ($LiCl_2$) RNA extraction and even after modification, pigments released from the algae resulted in inaccurate quantification of RNA on a spectrophotometer, as these pigments are contaminants in RNA measurements (Daohong, et al., 2004). The removal of these pigments may be a crucial step in RNA extraction as they appeared to facilitate RNA degradation, although the pigments were later successfully removed in the aqueous phase when isopropanol, high salt solutions, and $LiCl_2$ were applied (Daohong, Bochu, Biao, Chuanren, & Jin, 2004). So, a protocol, or commercial kit that is designed for algal RNA extraction and can deal with the removal of these pigments may be crucial to extracting high quality RNA.

Another study on the green alga *Ankistrodesmus convolutes* showed that the addition of a buffer to tissue samples helped reduce the issue of polysaccharide and phenol contamination and reduced RNase activity (Thanh et al., 2009). This study also found that RNA extraction is a time-sensitive process that can affect total RNA quality (Thanh et al., 2009). Many of the considerations for algal RNA extraction are extremely similar to that of fungal RNA extraction (i.e. addition of a lysis buffer, minimizing processing time, & bead beating), but they differ in respect to their various chemical complications that may be encountered and may need to be treated individually. While

there are currently a few extraction kits that are specified for filamentous fungi, to our knowledge there are none that are specified for green algae. Plant RNA extraction kits may be viable for algal samples, but as far as we know, they have not been tested on their efficacy on extracting RNA from algal cells.

Considering the aim of this study is to extract total RNA from both fungal and algal tissues simultaneously when they are what is referred to as a "co-tissue," the issue of addressing tissue-specific chemical contaminants may further obscure extraction protocol. Studies that have done extractions on mixed biomasses, which are representatively similar to co-tissues, where one of the components of the biomass is a fungi have shown that organic compounds and humic acids interfere with RNA yield and integrity (Patyshakuliyeva, et al., 2014). Contamination from organic compounds released from *C. reinhardtii* could be a possible complication that could interfere with the integrity of the RNA extracted from *A. nidulans* or the chemistry of a fungal-specific extraction kit, or vice versa as well. Using a model fungal-algal symbiosis defined and engineered by Hom and Murray, this study defines an optimized protocol for extraction of total RNA from a model algal species *Chlamydomonas reinhardtii* (CC1690) and a model fungal species *Aspergillus nidulans* (FGSC A4).

An "optimal" beadbeating-extraction kit pair is defined by analyzing the raw data collected from a matrix of kits and homogenizers (**Appendix A.1**). The homogenizers assessed in this study are the FastPrep-24 5G (MP Biomedicals LLC, Santa Ana, CA), the Mini-BeadBeater-24 (BioSpec Products Inc., Bartlesville, OK) and the SoniBeast (BioSpec Products Inc., Bartlesville, OK). The Fast Prep-24 5G utilizes a vertical tube orientation with a side-to-side bead motion, while the Mini-Beadbeater-24 features a

horizontal tube orientation with a top-to-bottom bead motion. Both of these homogenizers completely homogenize efficiently in around 2 minutes. The third homogenizer, the SoniBeast, utilizes a novel homogenization technique developed in 2015 wherein the samples are vortexed at speeds of 20,000 oscillation/min can be processed in under 30 seconds.

The motion, time, and speed of the homogenizer are just three factors that can affect homogenization efficacy, with other factors being diameter, density, amount of beads, and bead-to-buffer ratio/volume (Hoyt & Doktycz, 2004). For both fungal and algal tissues, the recommended bead type (BioSpec Products, Inc.) is 0.5 millimeter (mm) silica/zirconia beads (Kim et al., 2012). One study that used glass beads to lyse filamentous fungi demonstrated that they were ineffective at lysing fungal tissues (Klimek-Ochab, et al., 2011), so silica/zirconia should be used in lieu of glass beads.

The three homogenizers comprise the vertical vector of the matrix, while the commercial RNA extraction kits comprise the horizontal vector. Traditional methods of chemical RNA extraction utilize phenol-chloroform or TRIzol reagents along with other detergents and buffers to help to lyse cells, wash and isolate RNA, DNA, or proteins, and separate them into a stable solution (Tan & Yiap, 2009). New advances are leading to the use of less harsh chemicals for extraction and elution, shortening the time it takes to extract cellular contents while increasing yield and quality. Newer technologies use solid phase purification via spin columns that are composed of either silica matrices or glass particles, where RNA will adhere to the column after pH and salt concentrations are adjusted (Tan & Yiap, 2009). Extraction kits are also becoming more highly specified for a more diverse array of organisms, species, and cellular substrate.

Presently, there are no specific kits for algal DNA/RNA isolation, and other that include fungal or algal DNA/RNA isolation under plant or microbial (i.e. soil, stool, food, etc.) kits. As more advances in nucleic acid isolation and analysis technology are achieved, it is likely that we will see more highly specified kits for increasingly specific tissue types. After speaking with technical representatives, we utilized the following companies' recommended kits for fungal-algal co-tissues. These kits include the E.Z.N.A. Fungal RNA Mini Kit (Omega Bio-tek Inc., Norcross, Georgia), Plant/Fungal Total RNA Purification Kit (Norgan Biotek Corp., Thorold, Ontario, Canada), RapidPURE RNA Plant Kit (MP Biomedicals), *Quick*-RNA MiniPrep (Zymo Research Corp., Irvine, California), and RNeasy Plant Mini Kit (QIAGEN, Venlo, Limburg, Netherlands). The chemistry and protocol of these kits vary as well as there target tissues, which may affect total RNA yield.

2.1 Materials and Methods

The methods designed for this study define an efficient and effective protocol for RNA extraction across commercial extraction kits and bead beating homogenizers to increase total RNA yield from fungal tissue, algal tissue, and fungal-algal co-tissue. To our knowledge, this is the only study that has assessed both homogenization and purification of RNA from green algae and fungal-algal co-tissues.

2.1.1 *Aspergillus nidulans* Preparation

Spores of the wild type strain of *Aspergillus nidulans* (FGSC A4) were cultured in YM media (**Appendix A.2**) under 90 μE/s of light at 20°C for 4 days on a shaker at 100

rotations per minute (rpm). For the Mini-Beadbeater 24 and the FastPrep-24 5G, hyphae balls were selected for a total biomass of 5mg±1mg and placed in 2.0 mL cryotubes (Neptune Scientific, San Diego, CA). For the SoniBeast, hyphae balls were selected for a total biomass of 2.5mg±0.5mg and placed in 0.6 mL polypropylene graduated tubes with locking lids (Fisher Scientific, Hampton, NH). The hyphae balls were washed twice in CYB basal liquid media (**Appendix A.2**) prior to isolation.

2.1.2 *Chlamydomonas reinhardtii* Preparation

The wild type strain of *Chlamydomonas reinhardtii* (CC1690) was cultured in CYM media (**Appendix A.2**) under 90 μE of light at 20°C on a shaker at 100 rpm. The cells were allowed to grow for 4 days before the culture was spun down at 3500 × g for 2 min in 50 mL polypropylene centrifuge tubes (VWR Intl., Radnor, PA). The supernatant was decanted and the cells were washed twice in CYB basal liquid media (**Appendix A.2**). After resuspension, the cells were spun down once again and a super-concentrated "paste" was made to where 250 microliters (μL) was roughly 5 mg. A wet weight 5mg±1mg of biomass for the Mini-BeadBeater-24 and the FastPrep-24 5G homogenizers and a wet weight of 2.5mg±0.5mg of biomass for the SoniBeast homogenizer were added to the tubes containing the *A. nidulans* tissues.

2.1.3 Homogenization

The tubes containing the tissues were immediately placed in the -80°C freezer after isolation inside of a metal freezing block for at least 15 min. After removal from the freezer, a 1:2 ratio of 0.5 mm Zirconia/Silica beads to lysis buffer were added to each

tube. For the 24-well homogenizers 0.1 mL of beads were added along with 200µL of the provided lysis buffer. For the SoniBeast, 30µL of beads were added along with 60µL of lysis buffer. Three tubes for each homogenizer-kit combination were removed from the freezing block and placed in the homogenizers. Optimization speeds and times for each of the machines is as follows:

- **MP Biomedicals' FastPrep-24 5G (FP):** 6.0 m/sec. for 2 x 45 seconds with 30 seconds between cycles
- **BioSpec Products' Mini-Beadbeater-24 (BB):** 3450 strokes/min. 2 x 45 seconds with 30 seconds between cycles
- **BioSpec Products' SoniBeast (SB):** 20,000 oscillations/min. for 30 seconds

After the homogenization cycle was complete, the tubes were removed from the homogenizers and placed immediately back into a metal freezing block in the 4°C refrigerator to cool the samples. The remaining volume of lysis buffer was added to the tubes and the provided commercial extraction protocol was followed through to elution of the RNA in 50µL elution buffer. Each homogenizer-kit combination was assessed in triplicate.

2.1.4 Fluorometric Analysis

Total RNA eluted in 50µL elution buffer was quantified using a Quantus Fluorometer (Promega Corp., Madison, WI) and the Qubit RNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA). Assay samples were prepared in 0.5mL PCR tubes (Axygen

Inc., Corning, NY) with 1μL from each eluted sample of RNA. Total concentration of RNA was calculated in ng/μL according to the manufacturer's protocol and total RNA per tissue (ng/mg) was then determined.

2.2 Results and Discussion

Tissue samples were analyzed using the Qubit HS RNA Assay to determine the amount of total RNA (ng RNA/mg tissue) extracted from the fungal-algal co-tissue by homogenizer-extraction kit combinations to determine which combination is optimal for extraction (**Figure 1; Appendix A.1**). Our results showed that the homogenizer that yielded the highest average total RNA across all of the extraction kits was BioSpec's SoniBeast with 11,867±241 ng/mg total RNA, and the extraction kit that yielded the highest average total RNA was Zymo Research's *Quick*-RNA MiniPrep with 11,963±326 ng/mg total RNA (**Figure 2**).

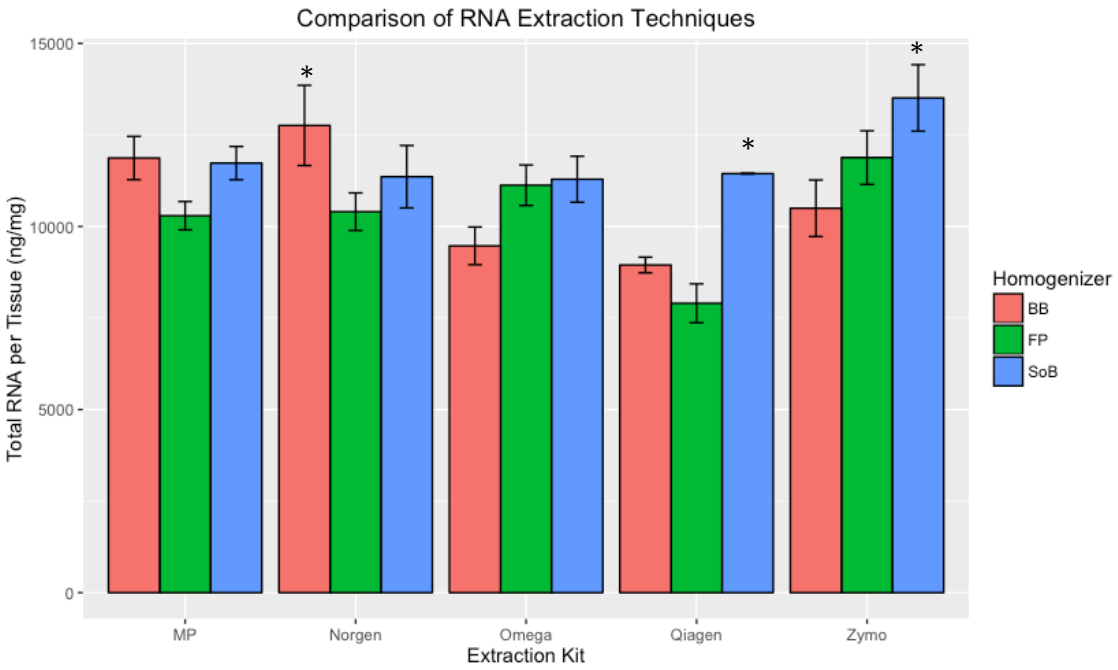

Figure 1: Average total RNA extracted from commercial extraction kits and homogenizer combinations. Three replicates of fungal-algal co-tissue for each homogenizer-kit combination were isolated and total RNA was extracted. The averages of each combination are shown above measured in nanograms (ng) of total RNA per milligram (mg) tissue. Each sample for the FastPrep-24 5G and the Mini-BeadBeater-24 contained 10mg±2mg tissue and each sample for the SoniBeast contained 5mg±1mg tissue. Standard error bars indicate the deviation from the mean of each group of averages and an asterisk indicates statistical significance determined from a two-way ANOVA and the Scheffe test. Statistical significant was only reported in the combination of kit and homogenizer significantly increased total RNA yield.

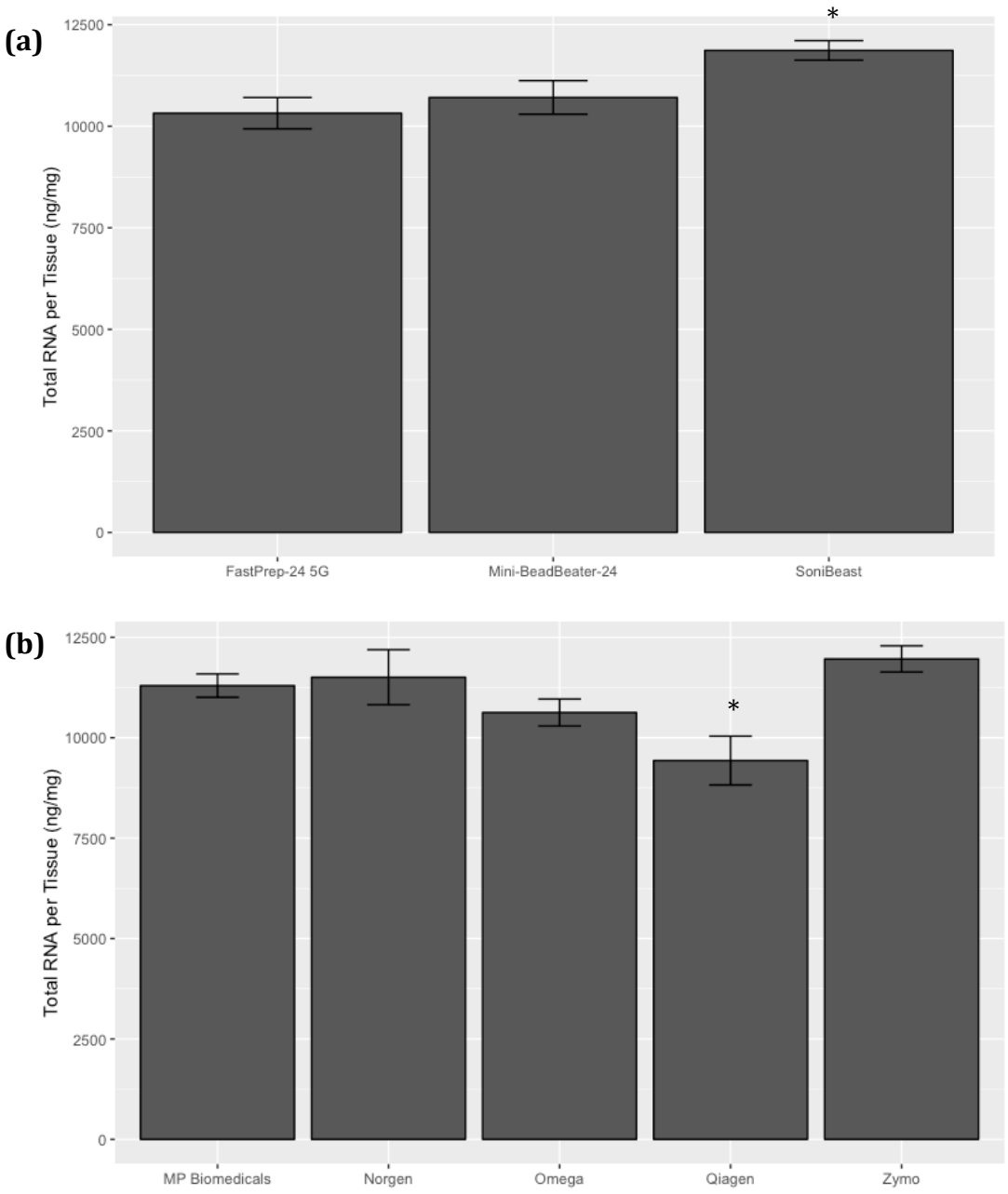

Figure 2: Average total RNA extracted from commercial extraction kits & homogenizers. (a) Total RNA in ng/mg tissue extracted from the homogenizers across all extraction kits. Each average is comprised of three replicates from each of the extraction kits for a total of 15 samples per homogenizer. **(b)** Total RNA in ng/mg tissue extracted from the commercial extraction kits across all homogenizers. Each average is comprised of three replicates from each of the homogenizers for a total of 9 samples per extraction kit. Standard error bars indicate the deviation from the mean of each group of averages and an asterisk indicates statistical significance determined from a two-way ANOVA and the Scheffe test.

A two way ANOVA was performed to determine if there was a significant interaction between kits and homogenizers exists. The means and standard deviations are presented in (**Appendix A.1**). The two-way ANOVA showed the existence of a highly significant main effect for the extraction kits ($F(4,30)= 7.08$, $p<0.05$; $\omega^2=0.24$) and for the homogenizers ($F(2,30)= 7.89$, $p<0.05$; $\omega^2=0.14$). The interaction between kit and homogenizer also produced a significant result ($F(8,30)= 3.37$, $p<0.05$; $\omega^2=0.19$) with an omega squared value for the combined effect ($\omega^2=0.559$) that suggests 55.9% of the variability is related to the combined effect of the homogenizers with the kits.

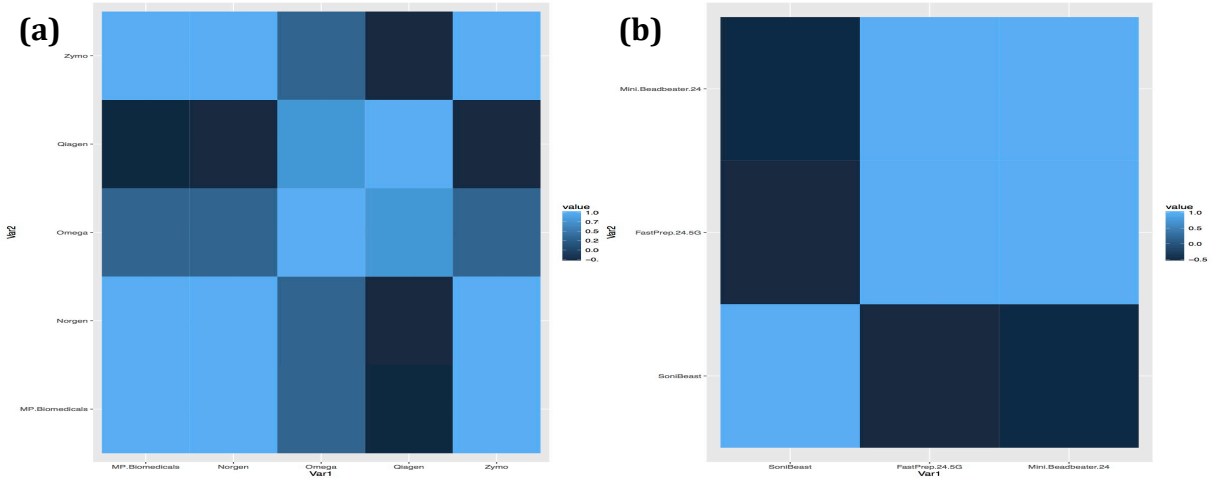

Figure 3: Correlation heat maps of inverse p-values for extraction kits and homogenizers. The inverse p-values (1/p) obtained from a two-way ANOVA were plotted using a correlation heat map in R. **(a)** The inverse p-values obtained from the interaction of the extraction kits to determine statistical significance. The main effect between MP Biomedicals & Qiagen, Norgen & Qiagen, and Zymo & Qiagen are all statistically significant. **(b)** The inverse p-values obtained from the interaction of the commercial homogenizers to determine statistical significance. The main effect between the Mini-BeadBeater-24 & SoniBeast and the FastPrep-24 5G & SoniBeast are statistically significant.

For the extraction kits, there were significant main effects between MP Biomedicals & Qiagen, Norgen & Qiagen, and Zymo & Qiagen (**Figure 3a**). From this

data generated by the two way ANOVA and the data presented in **Figure 2a**, we conclude that these three kits are significantly more effective at extracting total RNA from fungal-algal tissue than was Qiagen's extraction kit. Although there was not an extraction kit that was consistently more efficient regardless of homogenizer, some significant interactions were observed when analyzed using the Scheffe procedure: Norgen's kit was more effective on the Mini-BeadBeater-24 when compared to the FastPrep-24 5G ($p=0.043$), Qiagen's kit was more effective on the SoniBeast when compared to both the Mini-BeadBeater-24 ($p=0.030$) and the FastPrep-24 5G ($p=0.002$), and Zymo's kit was more effective on the SoniBeast than on the Mini-BeadBeater-24 ($p=0.007$).

For the commercial homogenizers, there were significant main effects between the Mini-BeadBeater24 & SoniBeast and the FastPrep-24 5G & SoniBeast (**Figure 3b**) determined by the two way ANOVA, which when combined with the data presented in **Figure 2b**, shows that, by using the Scheffe procedure, the SoniBeast is significantly more efficient at extracting RNA from fungal-algal tissues than these two homogenizers. While yielding a significant main effect when compared to the other homogenizers, the SoniBeast is also significantly faster at sample processing than the other two homogenizers, processing fungal-algal samples in 30 seconds while the other homogenizers require 2 minutes to process samples. Another important factor is cost, and the SoniBeast, priced at $1800, is significantly cheaper than other high-speed commercial homogenizers which can cost anywhere from $2500-$10,000. While there is a trade-off between speed and number of samples (the SoniBeast only allows for 12 samples to be processed at a time), the SoniBeast is the optimal homogenizer as defined by this study;

increased sample RNA yield, reduction of cost, and reduction of processing time all indicate that the novel homogenizer, the SoniBeast, is optimized for RNA extraction procedures.

The results of this study show that while no individual extraction kit is significantly more efficient than another, there exists significant interactions between kits and homogenizers, which we propose derive from the orientation of the homogenization plate which affects bead motion inside the tubes. This effect may be due to viscosity differences in detergents used in lysis buffers, some of which tend to foam during homogenization, potentially interfering with bead-to-tissue contact either by slowing down bead movement or providing a barrier for the tissue. The FastPrep-24 5G and the SoniBeast both feature vertically facing homogenization plates while the Mini-Beadbeater-24 features a horizontally facing homogenization plate (**Figure 4**) Further studies into efficient RNA extraction methods should take this interaction between homogenization plate orientation and lysis buffer chemistry into account as it may improve total yields for labs that already have a purchased homogenizer.

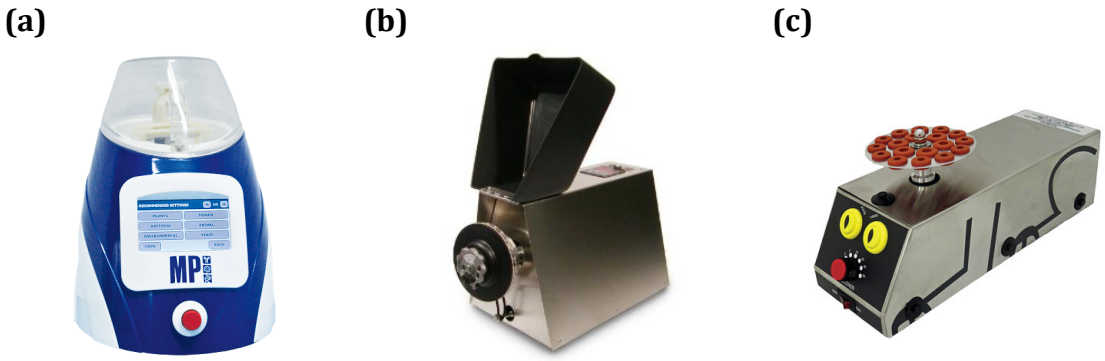

Figure 4: Commercial homogenizers. (a) MP Biomedical's FastPrep-24 5G, which features a vertically facing homogenization plate and can hold up to 24 samples. **(b)** BioSpec Products' Mini-Beadbeater-24, which features a horizontally facing homogenization plate and can hold up to 24 sampless. **(c)** BioSpec Products' SoniBeast, which features a vertically facing homogenization plate and can hold up to 12 samples.

The main finding from this study is that the newest homogenizer, the SoniBeast, which utilizes a vortexing motion at 20,000 oscillations per minute, significantly increases the amount of RNA extracted when compared to the other homogenizers regardless of extraction kit. The SoniBeast is also the most consistent homogenizer (**Table A-1)** with a standard deviation of 924 ng/mg, which is slightly than half that of the other two homogenizers. The SoniBeast allows for reduction in cost, reduction in processing time, increase in RNA yield, and consistency, with the only trade-off being the low number of samples that can be processed at a time. While we tested for fungal-algal tissues, other tissues may require more studies on this relatively new homogenizer to optimize a protocol for RNA extraction.

Future studies stemming from this initial methods study include determining the quality of the RNA extracted from these fungal-algal tissues and determining the ratio of fungal:algal tissue. This will allow us to determine how efficient the protocol is at removing inhibitory substrates that may interfere with downstream applications, extracting even stoichiometric ratios of RNA from different cell types in the samples, and preserving the integrity of the RNA from homogenization to elution. Now that an RNA extraction protocol has been optimized for this system, the next step toward defining the gene expression patterns of the model fungal-algal mutualism is to perform phenotypic assessment studies to determine the effects, if any at all, of circadian rhythms on co-cultures of *C. reinhardtii* and eith *A. nidulans, S. cerevisiae,* or *N. crassa.*

CHAPTER 3

Effects of Circadian Light Cycles on *S. cerevisiae* & *C. reinhardtii*

A capacity for obligate mutualism has been demonstrated to exist between the budding yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii* (Hom & Murray, 2014), and is based on a nutrient cross-exchange. The yeast, *S. cerevisiae*, metabolizes glucose ($C_6H_{12}O_6$) to carbon dioxide (CO_2), which is then photosynthetically used by *C. reinhardtii*. The alga, *C. reinhardtii*, metabolizes nitrite (NO_2^-), releasing ammonia (NH_3), which can be utilized by *S. cerevisiae*. An obligate mutualism between the two species will form in a closed system.

Entrainable circadian rhythms are suggested to exist in *C. reinhardtii* and *S. cerevisiae* demonstrates circadian patterned metabolic processes. So, we hypothesize that: (i) when obligately mutualistic cultures grown under different light cycle regimens are combined, the need for mutual nutrient release may require adaptive synchronization that may result in a lag in mutualistic co-culture growth, that (ii) when cultures from the same light are combined they will have a have increased growth from the lack of necessity to synchronize their cycles, and that (iii) these differences will be detectable from phenotypic, empirical observations of total co-culture biomass.

3.1 Materials and Methods

The methods designed for this study have allowed for the phenotypic assessment of the effects of differentially entrained circadian rhythms on the budding yeast *S. cerevisiae* and the green alga *C. reinhardtii* on their mutualistic interaction when combined in co-culture to determine if circadian synchrony affects co-culturing or if asynchrony can be corrected by co-culturing.

3.1.1 Culture Preparation

Cultures of *S. cerevisiae* (Y) and *C. reinhardtii* (C) were grown in YM and CYM media respectively (**Appendix A.2**) under 90 μE/s of light on a shaker at 100 rpm for 48 hours and were at least of the target density of 2×10^5 cells/mL. Three different light treatments were applied to the cultures of these organisms during growth and this period is referred to as the light training period. One light treatment (A) exposed one set cultures to 12 hours of continuous light from 8 a.m. to 8 p.m., another (B) exposed a different set cultures to 12 hours of continuous light from 8 p.m. to 8 a.m., and the final treatment (24) exposed yet a third set of cultures to 12 hours of light given in random doses varying in duration (1-7min) over a 24 hour period (**Figure 5**). For the random light treatment, when a dose of light was applied, an equivalent period of darkness followed before another light dose was applied.

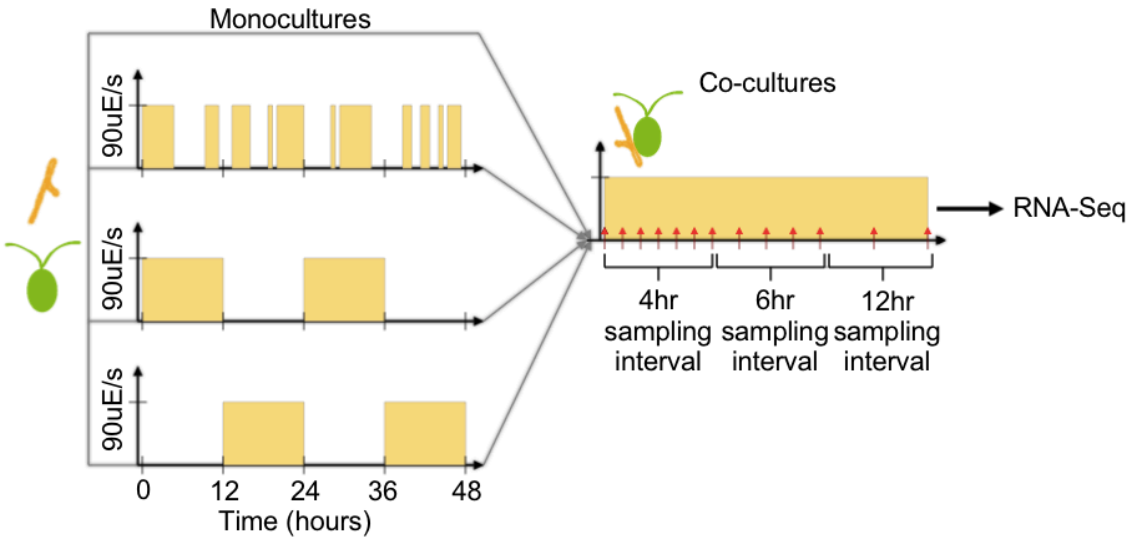

Figure 5: Experimental scheme for light entrainment of monocultures of and sampling of co-cultures. Cultures of *S. cerevisiae* and *C. reinhardtii* were grown as monocultures according to the three light conditions show for 48 hours. Cultures were then combined across all three treatments and allowed to grow in co-culture under 24 hours of light for 72 hours with sampling intervals for the first day at every 4 hours, the second day at every 6 hours, and the third day every 24 hours. If phenotypic differences were observed, RNA will be extracted to be sequenced via RNA-Seq.

3.1.2 Co-culture Preparation

All cultures were removed from the shakers and pelleted via centrifugation at 3500 × g for 2 min in 50 mL centrifuge tubes (VWR Intl.) before the supernatant was poured off. The pellet was resuspended in 1X CYB salts (**Appendix A.2**) and pelleted once more. The pellet was then resuspended in 40 mL of CYM media (**Appendix A.2**) and a cell count was obtained using the Luna Dual Fluorescence Cell Counter (Logos Biosystems, Inc., Annandale, VA). All cultures were diluted to 2×10^5 cells/mL with CYM media. From there, 500µL of algae and 500µL of yeast were added to 2mL screw cap microcentrifuge tubes (Neptune Scientific, San Diego, CA). Upon combination of algal and yeast cultures, nine different treatment groups were formed (**Table 1**). All tubes were placed under constant 24-hour light after monoculture combination and were

allowed to grow for 72 hours before the experiment was terminated. Three replicates were assessed for each treatment group.

Table 1: Differential light treatment group combinations for phenotypic assessment under constant light conditions. Nine treatment groups resulted from the cross of each of the light treatment groups for both the *C. reinhardtii* and *S. cerevisiae*. These co-cultures were allowed to sit under 24-hour light for 3 days after combination according to this table before their phenotypic differences were assessed.

Key: A = a.m. light B = p.m. light 24 = random light		***Saccharomyces cerevisiae* (Y)**		
		YA	**YB**	**Y24**
***Chlamydomonas reinhardtii* (C)**	**CA**	[1] CA × YA	[2] CA × YB	[3] CA × Y24
	CB	[4] CB × YA	[5] CB × YB	[6] CB × Y24
	C24	[7] C24 × YA	[8] C24 × YB	[9] C24 × Y24

3.1.3 Sampling

For the first 24 hours, samples were taken at 4-hour intervals from all treatment groups. When sampled, the tubes were inverted to distinguish between the algae physically attached to the yeast and those in the supernatant. For the second 24 hours, samples were every 6 hours and for the final 24 hours, samples were taken every 12 hours. After 72 hours, 300 μL of sample from each of the nine treatment groups was placed into a 96-well culture plate (VWR Intl.) and the samples were centrifuged at 2000 × g for 2 min. After centrifugation, an image of the 96-well plate was obtained from an Epson Perfection V850 Pro scanner (Epson America, Inc., Long Beach, CA). These images were used to determine if there were phenotypic differences exhibited by the

different treatment groups, whether as a result of light entrainment, co-culturing, or length of time under constant light.

3.2 Results and Discussion

After the tubes were removed, an image was taken of the relative biomass that was generated from all light condition combinations (**Table 1**). For the experiment involving the co-culturing of *S. cerevisiae* and *C. reinhardtii*, no significant phenotypic difference was observed after 72 hours of combined growth after removal from their respective light conditions. A side study was performed where the algae and the yeast were allowed to grow following the same experimental procedure as before. After co-culturing, the tubes containing the organisms were allowed to grow for 72 hours before their relative biomass was observed and again no significant differences existed (**Figure 6**). This is most likely explained by the fact that *S. cerevisiae* does not have an innate circadian clock, rather it has an entrainable metabolic circadian pattern; while this entrainable pattern was thought to be enough to cause differences in relative biomass in response to changing circadian light treatments, further studies into the gene-level expression changes may be necessary to extract this response.

This experiment was also performed with *A. nidulans* and *C. reinhardtii* following the same protocol as described previously for the study with *S. cerevisiae.* Initially, no significant differences in relative biomass were observed, so the side study was performed also, where relative biomass was observed after 72 hours. Again, no significant differences were observed between treatment groups and we believe this to be

due to the weak evidence that *A. nidulans* has may have a circadian rhythm, but it also may just feature circadian patterns like that in *S. cerevisiae*.

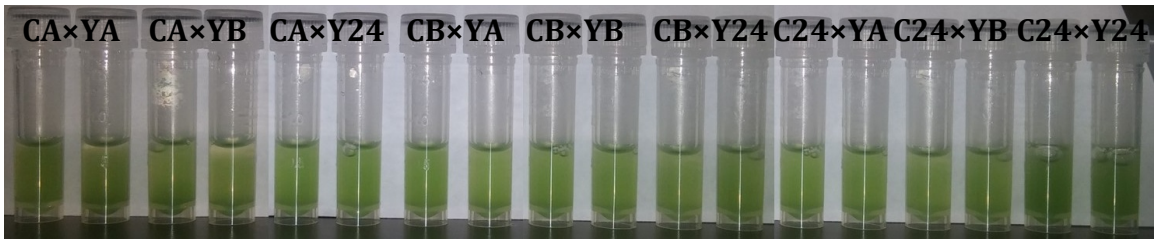

Figure 6: Total biomass generated from *S. cerevisiae* and *C. reinhardtii* phenotypic study. Before combination, cultures of algae (C) and yeast (Y) were grown under varying light cycles: A = a.m. light, B = p.m. light, 24 = random light intervals. After combination, nine different treatment groups resulted as shown above and were grown under constant light (for all treatments, hυ=90μE). No relative difference in total biomass was observed between any of the nine treatment groups, leading us to reject out hypothesis, from a phenotypic standpoint, but molecular analysis from RNA-Seq may uncover a gene expression reaction.

We expect that if this study is performed with *N. crassa* that a response, assessed by relative biomass, will be observed since both *C. reinhardtii* and *N. crassa* have well documented, innate circadian rhythms. If a response is observed, it will allow for the continuation of this study into its next steps, which includes qPCR analysis and eventually RNA-Seq based analysis of gene expression. Based on the data we obtained, it may require RNA-Seq analysis to uncover a reaction to the treatments applied on the molecular level in this experiment. The next steps will be to assess the affects of *N. crassa* in this experiment, determine whether there are other factors that must be modified to observe a reaction in the system, and determine whether molecular analysis is the correct pipeline for determining how these organisms affect each other and are effected by circadian growth conditions.

CONCLUSION

Defining the gene expression patterns of the synthetic model fungal-algal system (Hom and Murray, 2004) is a key component to the foundation for future studies into the engineering of symbiotic communities or mutualisms and the understanding of the mechanisms, both physical and molecular, that cause them to function. This specific understanding of a model system created from two well-studied model organisms allows for applications to many extant symbioses that may have conserved evolutionary traits that are related to these organisms. And, a basic understanding of how niche engineering can be accomplished through molecular techniques allows for the manipulation of species to produce potential products such as probiotics, biofuels, and pharmaceuticals.

In this study, we have defined a protocol, which optimizes RNA extraction from fungal and algal tissue, which allows for further research into the molecular mechanisms of these species as well as transcriptomic analysis of the interaction between them as symbionts. With the studies that are slated to follow this, we plan to determine the molecular effects of circadian growth conditions on these model organisms and how that may cause them to synchronize with a symbiont, growing, then, synergistically, or how mutualistic dependence affects how they interact physically and biochemically. For this, RNA-Seq analysis will be the method of choice for analysis once we have determined phenotypically that a response is occurring in these species to changing conditions when cultured in the lab.

APPENDICES

Appendix A.1 Total RNA Extracted Matrix

Table A-1: Total RNA extracted from each of the three samples for homogenizer-kit combinations. Total RNA was measured in ng RNA/mg fungal-algal tissue from each of the three replicates for each combination. Total biomass was around 10mg±1mg for each sample. Standard deviations for each average of the combinations are shown as well as the standard error for the average of each homogenizer and extraction kit.

	Zymo Research	Qiagen	Omega Bio-tek	MP Biomedicals	Norgen Biotek	Grand Average
FastPrep-24 5G	12172	8927	11679	11036	10930	
	12979	7627	10018	10113	9378	
	10495	7153	11683	9734	10904	
Average	11882	7902	11127	10295	10404	10322
Std. Deviation	1267	919	960	670	889	1495
Mini-BeadBeater 24	9564	8648	10454	12944	12139	
	12030	8832	9247	10900	11250	
	9899	9365	8711	11765	14888	
Average	10497	8948	9471	11870	12759	10709
Std. Deviation	1338	372	893	1026	1897	1599
SoniBeast	11745	11424	12478	12637	13022	
	14033	11464	11045	11240	10202	
	14755	11445	10347	11313	10852	
Average	13511	11444	11290	11730	11358	11867
Std. Deviation	1572	20	1086	786	1477	934
Grand Average	11963	9432	10629	11298	11507	10966
Std. Error	979	1820	1007	872	1185	804

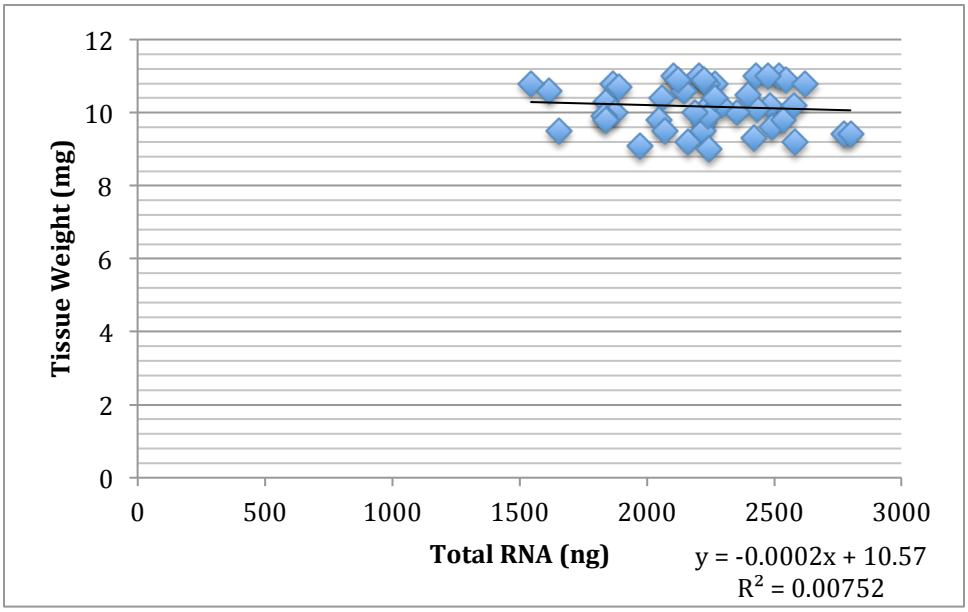

Figure A-1: Total RNA extracted per milligram of tissue. Extracted RNA was graphed against tissue weight to demonstrate that there was no bias towards larger tissues having greater contents of RNA. The slope of the line (m=-0.0002) indicates an insignificant correlation.

Appendix A.2 Media Ingredients

Table A-2: Fungal, algal, and yeast growth media recipes. Recipes for media made in 1L quantities for fungal (YM), algal (CYM), and yeast (YM) liquid cultures. Basal media is used as for washing of tissues to remove excess NO_2 or NH_4 before co-culturing to ensure that there is not bias due to chemotaxis from the organisms after they are combined.

Media	Volume (For 1L of medium)	Ingredient
CYB (Basal Liquid Medium) [1X]	958 mL	d_2H_2O
	40 mL	CYB Salts [25X]
	1 mL	P Stock [1000X]
	1 mL	Vitamin Stock [1000X]
CYB Salts [25X]	925 mL	d_2H_2O
	25 mL	Ca^{2+} Stock [1000X]
	25 mL	Mg^{2+} Stock [1000X]
	25 mL	Trace Elements Stock [1000X]
CYM + 100 mM glucose + 10mM NO_2 [1X]	908 mL	d_2H_2O
	40 mL	CYB Salts [25X]
	45 mL	Glucose Stock [25X]
	10 mL	NO_2 Stock [100X]
	1 mL	P Stock [1000X]
	1 mL	Vitamin Stock [1000X]
YM + 100mM glucose + 10mM NH_4 [1X]	908 mL	d_2H_2O
	40 mL	CYB Salts [25X]
	45 mL	Glucose Stock [25X]
	10 mL	NH_4 Stock [500X]
	1 mL	P Stock [1000X]
	1 mL	Vitamin Stock [1000X]

Appendix A.3 Two-Way ANOVA Results

Table A-3: Descriptive statistics and results from a two way ANOVA test. (a) Means, standard deviations, sample sizes, and groups assessed in two way ANOVA. **(b)** Results obtained from two way ANOVA showing the main effects of Factor 1 (Kit), Factor 2 (Machine), and the interaction between F1 and F2 (Kit × Machine). The p-values were used to determine statistical significance of the main effect and the ω^2 values were used to determine the amount of variation that was due to each of the three factors assessed by the two way ANOVA test. F values that were higher than F_{crit} also indicated statistical significance.

(a)

Two-way ANOVA					
Summary					
Response	*RNA*				
Factor #1	*Kit*	*Fixed*			
Factor #2	*Machine*	*Fixed*			
Descriptive Statistics					
Factor	*Group*	*Sample size*	*Mean*	*Variance*	*Standard Deviation*
Kit	*MP*	9	11,298.10844	1,100,129.33489	1,048.8705
Kit	*Norgen*	9	11,507.06361	2,694,556.58828	1,641.51046
Kit	*Omega*	9	10,629.13918	1,484,655.32617	1,218.46433
Kit	*Qiagen*	9	9,431.71402	2,729,449.75691	1,652.10464
Kit	*Zymo*	9	11,963.44184	3,172,425.38181	1,781.13037
Machine	*BB*	15	10,709.02991	3,245,328.17232	1,801.47944
Machine	*FP*	15	10,321.93034	2,574,945.45891	1,604.66366
Machine	*SoB*	15	11,866.72	1,669,468.92867	1,292.0793
Kit x Machine	*MP x BB*	3	11,869.62585	1,052,568.78731	1,025.94775
Kit x Machine	*MP x FP*	3	10,294.50538	448,725.28389	669.8696
Kit x Machine	*MP x SoB*	3	11,730.1941	618,399.36389	786.38373
Kit x Machine	*Norgen x BB*	3	12,759.06225	3,597,764.10383	1,896.77729
Kit x Machine	*Norgen x FP*	3	10,403.79906	790,059.78204	888.85307
Kit x Machine	*Norgen x SoB*	3	11,358.32953	2,180,179.81429	1,476.5432
Kit x Machine	*Omega x BB*	3	9,470.69423	797,366.48944	892.9538
Kit x Machine	*Omega x FP*	3	11,126.87181	921,900.27111	960.15638
Kit x Machine	*Omega x SoB*	3	11,289.85149	1,179,944.71841	1,086.2526
Kit x Machine	*Qiagen x BB*	3	8,948.30795	138,653.96813	372.36268
Kit x Machine	*Qiagen x FP*	3	7,902.44024	843,882.25245	918.63064
Kit x Machine	*Qiagen x SoB*	3	11,444.39387	403.00674	20.07503
Kit x Machine	*Zymo x BB*	3	10,497.45927	1,788,951.72077	1,337.517
Kit x Machine	*Zymo x FP*	3	11,882.03522	1,605,183.20525	1,266.95825
Kit x Machine	*Zymo x SoB*	3	13,510.83102	2,470,348.76567	1,571.73432

(b)

ANOVA							
Source of Variation	*SS*	*d.f.*	*MS*	*F*	*p-level*	*F crit*	*Omega Sqr.*
Factor #1 (Kit)	34,789,002.25619	4	8,697,250.56405	7.07695	0.00039	2.68963	0.23809
Factor #2 (Machine)	19,382,337.52192	2	9,691,168.76096	7.8857	0.00177	3.31583	0.13489
Factor #1 + #2 (Kit x Machine)	33,198,730.51605	8	4,149,841.31451	3.37672	0.00705	2.26616	0.18624
Within Groups	36,868,663.06642	30	1,228,955.43555				
Total	124,238,733.36057	44	2,823,607.57638				
Omega squared for combined effect	0.55923						

BIBLIOGRAPHY

[1] Azuara, M. P., & Aparicio, P. J. (1985). Spectral dependence of Photoregulation of inorganic nitrogen metabolism in Chlamydomonas reinhardii1. , *77*(1), . Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1064463/

[2] Blaby, I. K., Blaby-Haas, C. E., Tourasse, N., Hom, E. F. Y., Lopez, D., Aksoy, M., … Prochnik, S. (2014). The Chlamydomonas genome project: A decade on. *Trends in Plant Science*, *19*(10), 672–680. doi:10.1016/j.tplants.2014.05.008

[3] Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology*, *7*(1), 3–15. doi:10.1007/bf00003544

[4] Botstein, D., Chervitz, S. A., & Cherry, M. J. (1997). Yeast as a model organism. , *277*(5330), . Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3039837/ Bowman, S., & Free, S. (2006). The structure and synthesis of the fungal cell wall. *BioEssays : news and reviews in molecular, cellular and developmental biology*., *28*(8), 799–808. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16927300

[5] Bowman, S., & Free, S. (2006). The structure and synthesis of the fungal cell wall. *BioEssays : news and reviews in molecular, cellular and developmental biology*., *28*(8), 799–808. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16927300

[6] Burik, van, Schreckhise, R., White, T., Bowden, R., & Myerson, D. (1999). Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Medical mycology*., *36*(5), 299–303. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10075499

[7] Daohong, W., Bochu, W., Biao, L., Chuanren, D., & Jin, Z. (2004). Extraction of total RNA from Chrysanthemum containing high levels of phenolic and carbohydrates. *Colloids and Surfaces B: Biointerfaces*, *36*(2), 111–114. doi:10.1016/j.colsurfb.2004.05.014

[8] David, H., Özçelik, İ. Ş., Hofmann, G., & Nielsen, J. (2008). Analysis of Aspergillus nidulans metabolism at the genome-scale. *BMC Genomics*, *9*(1), 163. doi:10.1186/1471-2164-9-163

[9] Davis, R. H. (2000). *Neurospora: Contributions of a model organism*. United Kingdom: Oxford University Press.

[10] Davis, R. H., & Perkins, D. D. (2002). Neurospora: A model of model microbes. *NATURE REVIEWS | GENETICS*, *3*, . Retrieved from https://web.stanford.edu/group/neurospora/pdfs/nrgDavis%26Perkins.pdf

[11] Eelderink-Chen, Z., Mazzotta, G., Sturre, M., Bosman, J., Roenneberg, T., & Merrow, M. (2010). A circadian clock in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences*, *107*(5), 2043–2047. doi:10.1073/pnas.0907902107

[12] Engel, S. R., Dietrich, F. S., Fisk, D. G., Binkley, G., Balakrishnan, R., Costanzo, M. C., … Cherry, J. M. (2013). The reference genome sequence of Saccharomyces cerevisiae: Then and now. *G3: Genes|Genomes|Genetics*, *4*(3), 389–398. doi:10.1534/g3.113.008995

[13] Fleige, S., & Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-pCR performance. *Molecular Aspects of Medicine*, *27*(2-3), 126–139. doi:10.1016/j.mam.2005.12.003

[14] Fuller, K. K., Loros, J. J., & Dunlap, J. C. (2014). Fungal photobiology: Visible light as a signal for stress, space and time. *Current Genetics*, *61*(3), 275–288. doi:10.1007/s00294-014-0451-0

[15] Galagan, J., Calvo, S., Borkovich, K., Selker, E., Read, N., Jaffe, D., … Birren, B. (2003). The genome sequence of the filamentous fungus Neurospora crassa. *Nature.*, *422*(6934), 859–68. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12712197

[16] Greene, A. V., Keller, N., Haas, H., & Bell-Pedersen, D. (2003). A Circadian oscillator in Aspergillus spp. Regulates daily development and gene expression. *Eukaryotic Cell*, *2*(2), 231–237. doi:10.1128/EC.2.2.231-237.2003

[17] Han, J. W., Yoon, M., Lee, K. P., & Hoon Kim, G. (2007). Isolation of Total RNA from a Freshwater Green Alga, Zygnema cruciatum, Containing High Levels of Pigments. *ALGAE*, *22*(2), 125–129. doi:10.4490/algae.2007.22.2.125

[18] Hom, E. F. Y., & Murray, A. W. (2014). Niche engineering demonstrates a latent capacity for fungal-algal mutualism. *Science*, *345*(6192), 94–98. doi:10.1126/science.1253320

[19] Hom, E. F. Y., Aiyar, P., Schaeme, D., Mittag, M., & Sasso, S. (2015). A chemical perspective on Microalgal–Microbial interactions. *Trends in Plant Science*, *20*(11), 689–693. doi:10.1016/j.tplants.2015.09.004

[20] Hoyt, P. R., & Doktycz, M. J. (2004). Optimized beadmilling of tissues for high-throughput RNA production and microarray-based analyses. *Analytical Biochemistry*, *332*(1), 100–108. doi:10.1016/j.ab.2004.04.025

[21] Kiefer, E., Heller, W., & Ernst, D. (2000). A simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. *Plant Molecular Biology Reporter*, *18*(1), 33–39. doi:10.1007/bf02825291

[22] Kim, B.-H., Ramanan, R., Cho, D.-H., Choi, G.-G., La, H.-J., Ahn, C.-Y., … Kim, H.-S. (2012). Simple, rapid and cost-effective method for high quality nucleic acids extraction from different strains of Botryococcus braunii. *PLoS ONE*, *7*(5), e37770. doi:10.1371/journal.pone.0037770

[23] Klimek-Ochab, M., Brzezińska-Rodak, M., Żymańczyk-Duda, E., Lejczak, B., & Kafarski, P. (2011). Comparative study of fungal cell disruption—scope and limitations of the methods. , *56*(5), . Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3189342/

[24] Latgé, J.-P. (2007). The cell wall: A carbohydrate armour for the fungal cell. *Molecular Microbiology*, *66*(2), 279–290. doi:10.1111/j.1365-2958.2007.05872.x

[25] Leite, G. M., Magan, N., & Medina, Á. (2012). Comparison of different bead-beating RNA extraction strategies: An optimized method for filamentous fungi. *Journal of Microbiological Methods*, *88*(3), 413–418. doi:10.1016/j.mimet.2012.01.011

[26] Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., … Grossman, A. R. (2007). The Chlamydomonas genome reveals the evolution of key animal and plant functions. , *318*(5848), . Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2875087/

[27] Merriam-Webster (2015). In *Merriam-Webster*. Retrieved from http://www.merriam-webster.com/dictionary/mutualism

[28] Miller, D. H., Mellman, I. S., Lamport, D. T. A., & Miller, M. (1974). THE CHEMICAL COMPOSITION OF THE CELL WALL OF CHLAMYDOMONAS GYMNOGAMA AND THE CONCEPT OF A PLANT CELL WALL PROTEIN. *The Journal of Cell Biology*, *63*(2), 420–429. doi:10.1083/jcb.63.2.420

[29] Mittag, M., Kiaulehn, S., & Johnson, C. H. (2005). The Circadian clock in Chlamydomonas reinhardtii. What is it for? What is it similar to?. *PLANT PHYSIOLOGY*, *137*(2), 399–409. doi:10.1104/pp.104.052415

[30] Morris, J. J., Lenski, R. E., & Zinser, E. R. (2012). The black queen hypothesis: Evolution of Dependencies through Adaptive gene loss. , *3*(2), . Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3315703/

[31] Patil, V., Tran, K.-Q., & Giselrød, H. R. (2008). Towards sustainable production of Biofuels from Microalgae. *International Journal of Molecular Sciences*, *9*(7), 1188–1195. doi:10.3390/ijms9071188

[32] Patyshakuliyeva, A., Mäkelä, M. R., Sietiö, O.-M., de Vries, R. P., & Hildén, K. S. (2014). An improved and reproducible protocol for the extraction of high quality fungal RNA from plant biomass substrates. *Fungal Genetics and Biology*, *72*, 201–206. doi:10.1016/j.fgb.2014.06.001

[33] Roberts, K., Gurney-Smith, M., & Hills, G. J. (1972). Structure, composition and morphogenesis of the cell wall of Chlamydomonas reinhardi. *Journal of Ultrastructure Research*, *40*(5-6), 599–613. doi:10.1016/s0022-5320(72)80046-7

[34] Sallau, A. B., Henriquez, F., Nok, A. J., Ibrahim, S., Sommerville, C., & Roberts, C. (2013). Aspergillus niger-specific ribonucleic acid extraction method. *Journal of Yeast and Fungal Research*, *4*(5), 58–62. doi:10.5897/JYFR2013.0107

[35] Slot, J. C., & Hibbett, D. S. (2007). Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: A Phylogenetic study. *PLoS ONE*, *2*(10), e1097. doi:10.1371/journal.pone.0001097

[36] Straley, S. C., & Bruce, V. G. (1979). Stickiness to glass: Circadian changes in the cell surface of Chlamydomonas reinhardi. *PLANT PHYSIOLOGY*, *63*(6), 1175–1181. doi:10.1104/pp.63.6.1175

[37] Tan, S. C., & Yiap, B. C. (2009). DNA, RNA, and protein extraction: The past and the present. *Journal of Biomedicine and Biotechnology*, *2009*, 1–10. doi:10.1155/2009/574398

[38] Tanaka, M., & Miwa, I. (1996). Significance of Photosynthetic products of symbiotic Chlorella to establish the Endosymbiosis and to express the mating reactivity rhythm in Paramecium bursaria. *Zoological Science*, *13*(5), 685–692. doi:10.2108/zsj.13.685

[39] Thanh, T., Omar, H., Abdullah, M. P., Chi, V. T. Q., Noroozi, M., Ky, H., & Napis, S. (2009). Rapid and effective method of RNA isolation from green Microalga Ankistrodesmus convolutus. *Molecular Biotechnology*, *43*(2), 148–153. doi:10.1007/s12033-009-9182-8

[40] Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: A revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57–63. doi:10.1038/nrg2484

[41] Yang, P., Fox, L., Colbran, R., & Sale, W. (1999). Protein phosphatases PP1 and PP2A are located in distinct positions in the Chlamydomonas flagellar axoneme. *Journal of cell science.*, *113*, 91–102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10591628

[42] Yang, Y., He, Q., Cheng, P., Wrage, P., Yarden, O., & Liu, Y. (2004). Distinct roles for PP1 and PP2A in the Neurospora circadian clock. *Genes & development.*, *18*(3), 255–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14871927