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**Growth and division of microalgae
in highly deuterated media**

Ph.D. Thesis

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Annotation

Deuterium-induced changes in morphology and physiology were studied in the model microalgae *Chlamydomonas reinhardtii*, *Desmodesmus quadricauda* and *Parachlorella kessleri*. Optimal growth conditions for deuterated cultures in terms of light intensity and cultivation temperature were determined. Special attention was paid to the changes in cell cycle progression of deuterated microalgae and their ability to divide at different deuterium concentrations. Analysis of the deuterated biomass revealed the presence of highly valuable deuterium-labelled compounds.

Declaration

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

Veronika Kselíková
Třeboň
1. 5. 2023

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List of papers and author's contribution

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

- I. **Kselíková, V.**; Zachleder, V. and Bišová, K. (2021). To divide or not to divide? How deuterium affects growth and division of *Chlamydomonas reinhardtii*. *Biomolecules* 11, 861. (IF = 4.879)
Veronika Kselíková was responsible for planning and conducting of the experiments, analyzing data and manuscript writing. Her contribution was 70 %.
- II. Zachleder, V.; Ivanov, I.N.; **Kselíková, V.**; Bialevich, V.; Vítová, M.; Ota, S.; Takeshita, T.; Kawano, S. and Bišová, K. (2021). Characterization of growth and cell cycle events affected by light intensity in the green alga *Parachlorella kessleri*: a new model for cell cycle research. *Biomolecules* 11, 891. (IF = 4.879)
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Veronika Kselíková participated in planning and conducting of the experiments, analyzing the data and writing the manuscript. Her contribution was 35 %.
- IV. **Kselíková, V.**; Zachleder, V.; Bišová, K. (2022). Analysis of commitment point attainment in algae dividing by multiple fission. In *Plant cell division*, Caillaud, M.-C. (Ed.). Humana, New York, NY, 2022. pp. 89-101.
Veronika Kselíková was responsible for adapting the methodology and manuscript writing. Her contribution was 80 %.
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Co-author agreement

Kateřina Biřov, the supervisor of this Ph.D. thesis and co-author of papers I.-V., fully acknowledges the stated contribution of Veronika Kselkov to these manuscripts.

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RNDr. Kateřina Biřov, Ph.D.

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List of abbreviations

APC	anaphase-promoting complex
ATP	adenosine triphosphate
CDK	cyclin-dependent kinase
CP	commitment point
CYC	cyclin
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
D ₂ O	deuterium oxide
EPA	eicosapentaenoic acid
FC	flow cytometry
FACS	fluorescent activated cell sorting
KIE	kinetic isotopic effect
NMR	nuclear magnetic resonance
PUFA	polyunsaturated fatty acids
RB	retinoblastoma protein
RNA	ribonucleic acid

1. Introduction

1.1. Microalgae as model organisms

Defining a diverse group of algae is not an easy task. Generally, algae are considered aquatic, oxygen-evolving photosynthetic autotrophs that are unicellular, colonial, or composed of filaments or simple tissues (Guiry, 2012). According to AlgaeBase, there are currently more than 165 thousand described algal species, but estimates of the total number of algal species are much higher (Guiry and Guiry, 2022). Algae are found among both the Prokaryota and the Eukaryota. Prokaryotic algae are represented by the phylum Cyanobacteria, with most representatives belonging to the orders Nostocales, Oscillatoriales and Synechococcales. Eukaryotic algae belong largely to the kingdoms Chromista and Plantae. The kingdom Chromista includes several phyla, such as Bacillariophyta, the most numerous phylum representing the biotechnologically important diatoms *Phaeodactylum tricornutum* (*Phaeodactylaceae*) and *Thalassiosira oceanica* (*Thalassiosiraceae*), or the phylum Ochrophyta with *Nannochloropsis* spp. (*Monodopsidaceae*). The kingdom Plantae is represented by several phyla, with Charophyta, Chlorophyta and Rhodophyta comprising the largest number of species. The phylum Charophyta contains both macroscopic and microscopic algae, most of which belong to the classes Charophyceae or Zygnematophyceae. Chlorophyta, or green algae, is a numerous phylum that includes both unicellular and multicellular algae. Several members of Chlorophyta are well-established model organisms such as *Chlamydomonas reinhardtii* (*Chlamydomonadaceae*) or biotechnologically important species such as *Chlorella* spp. (*Chlorellaceae*), *Dunaliella salina* (*Dunaliellaceae*) or *Haematococcus pluvialis* (*Haematococcaceae*). The phylum Rhodophyta or red algae includes both macroscopic and microscopic algae, of which *Porphyridium* spp. (*Porphyridiaceae*) have the greatest biotechnological potential.

The term microalgae encompasses a diverse group of eukaryotic and prokaryotic microscopic algae that are unicellular but may form colonies or chains, in contrast to macroalgae, which are multicellular plant-like organisms. Microalgae thrive in a variety of environments, from marine and freshwater habitats to harsh hot springs, soil and plant surfaces. It is estimated that microalgae are responsible for more than 50 % of global primary productivity (Chen et al., 2021).

The reproductive behavior of microalgae is almost as diverse as the environments they inhabit. Most microalgae are capable of both sexual and asexual reproduction, with the exception of the Cyanophyta, which are characterized by a complete lack of sexual reproduction (Singh et al., 2011). In general, sexual and asexual reproduction alternate during a life cycle. In microalgae, asexual reproduction is usually the predominant form of reproduction, while sexual reproduction is less common and is triggered by environmental stimuli (Muller et al., 2017). Asexual reproduction in unicellular organisms occurs through its simplest form – cell division (mitosis), which results in the formation of two daughter cells. Therefore, it is also called binary fission. Asexual reproduction is evolutionarily older, as the earliest eukaryotic species are thought to have been unicellular haploids that reproduced by mitosis (Wilkins and Holliday, 2009). A key step in the sexual life cycle, meiosis, is thought to have evolved from mitosis (Simchen and Hugerat, 1993). In sexual reproduction, two haploid gametes from opposite mating types fuse to form a diploid zygote. For example, in the model alga *C. reinhardtii*, the gametes have mt^+ and mt^- mating types (Ferris et al., 2002). After the zygote forms and matures, it transforms into a zygospore and germinates. During germination, the zygospore undergoes meiotic division, giving rise to four haploid vegetative cells. For more details on sexual reproduction in microalgae the reader is kindly referred to other works (Nishimura, 2010; Sekimoto, 2017).

1.2. Binary fission

The mitotic cell cycle generally consists of several steps, including growth phase (G1), synthesis phase (S), second growth phase (G2) and mitosis (M), followed by cytokinesis (C) (Howard and Pelc, 1953). The S phase, together with the G2 and M phases, is also referred to as the DNA replication-division sequence (Mitchison, 1971) (Figure 1). Two daughter cells arise from a single mother cell, hence the term binary fission.

The G1 phase is characterized by growth and accumulation of energy reserves that will later support the processes associated with division. Although growth continues to some extent in other phases, it is generally most significant in G1. At the end of the G1 phase, the cell reaches what is called the critical cell size for division, which is about twice the initial size. Control of cell size ensures

maintenance of minimal cell size in daughter cells (Fantès and Nurse, 1977; Jorgensen and Tyers, 2004). At this point, cells acquire the ability to divide without further energy input. This point in time is referred to as the commitment point (CP) for division (John, 1984). In the past, CP has also been called ‘point of no return’ (Moberg et al., 1968), ‘induction of division’ (Šetlík et al., 1972) or ‘transition point’ (Spudich and Sager, 1980), but the meaning remains the same. The analog of CP in mammals is called the restriction point (Zetterberg et al., 1995) or START in yeast (Jorgensen, 2002). The period before the commitment point is sometimes called the pre-commitment period and corresponds to most of the G1 phase. The post-commitment period begins after passing CP and continues with the late G1 phase, sometimes called the pre-S phase to distinguish the parts of G1 before and after CP. The post-commitment period continues with S, G2 and M phases and cytokinesis. The S phase is characterized by DNA synthesis. The amount of DNA in the cell doubles without any change in ploidy level. In G2 phase, the cell is prepared for the upcoming cell division and the proteins required for cell division are synthesized. M phase or mitosis, similar to S phase, is a relatively short period of the cell cycle. Mitosis ensures that the replicated DNA is evenly divided into new nuclei. Mitosis is a multistep process with at least four distinguishable phases: prophase, metaphase, anaphase and telophase. Their detailed role in M phase is beyond the scope of this work and the reader is kindly referred to other sources (Mazia, 1961; Mitchison and Salmon, 2001). M phase is followed by cytokinesis, in which the major cellular components divide into newly forming daughter cells. With the formation of daughter cells, the cell cycle is at its starting point and ready to repeat as long as conditions are favorable (Figure 1). Regulation of cell cycle progression at the molecular level is discussed in detail in chapter 1.5.

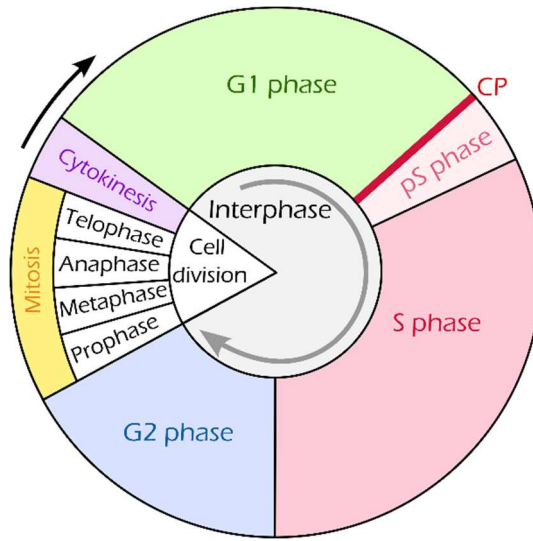


Figure 1: Mitotic cell cycle consisting of growth phase (G1), commitment point (CP), pre-synthesis phase (pS), synthesis phase (S), second growth phase (G2), mitosis (M) and cytokinesis (C). The G1, S and G2 phases are perceived as an interphase to cell division, which consists of mitosis and cytokinesis.

1.3. Multiple fission

Multiple fission (also known as palintomy) is a cell cycle pattern adopted by some microalgae in which a single mother cell divides into more than two daughter cells. The complexity of multiple fission with its overlapping reproductive sequences is difficult to fit into the classical scheme of binary fission, which is perceived as a cycle (Figure 1). Therefore, multiple fission is usually described as a sequence of events that repeat in each new cell, as proposed in the continuum model (Cooper, 1984). Consistent with the continuum model, the cell cycle can be easily represented linearly instead of a traditional circle. The linear representation of binary fission can be seen in Figure 2.

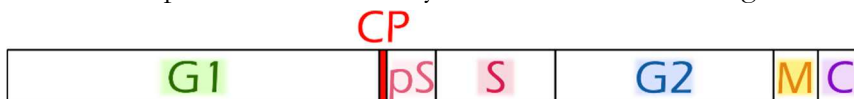


Figure 2: Linear representation of mitotic cell cycle consisting of growth phase (G1), commitment point (CP), pre-synthesis phase (pS), synthesis phase (S), second growth phase (G2), mitosis (M) and cytokinesis (C).

Generally, in the cell cycle of multiple fission, 2^n daughter cells can be formed within a single cell cycle, where n is the number of DNA replication-division sequences. The number of DNA replication-division sequences and thus the number of daughter cells is determined by external conditions and its maximum is species-specific.

Multiple fission is not a uniform mechanism in all algae that have evolved this specific cell cycle pattern. There are at least three described patterns that differ in the coordination of nuclear and cell divisions. Each of them is described below.

1.3.1. *Scenedesmus* cell cycle pattern

The *Scenedesmus* cell cycle pattern, sometimes referred to as the consecutive pattern, is found in some members of the family *Scenedesmaceae*, which are characterized by the formation of coenobia. A coenobium is a colony of daughter cells (autospores) surrounded by a common cell wall and resulting from the division of a single mother cell. Thus, the number of cells in a coenobium corresponds to the number of daughter cells formed within a single cell cycle and reflects external growth conditions. The pattern of the *Scenedesmus* cell cycle has been described in detail in the model organism *Desmodesmus quadricauda*, formerly known as *Scenedesmus quadricauda*. *D. quadricauda* normally divides two or three times in a single cell cycle, forming four- or eight-celled coenobia; however, under certain (stress) conditions, the formation of two-celled coenobia has also been documented (Chia et al., 2016; Zachleder et al., 2002). The *Scenedesmus* cell cycle pattern begins with the growth phase (G1), in which cells grow, accumulate energy-storing molecules and increase in volume. Eventually, cells pass their first commitment point, i. e. they acquire the ability to divide without further growth. After reaching CP, cells are committed to perform the DNA replication-division sequence, consisting of DNA duplication, mitosis and cytokinesis. However, in multiple fission, the DNA replication-division sequence is not performed immediately after reaching CP. Instead, the available energy is used to support another growth phase (G1), which is followed by the attainment of the second CP in a single cell cycle. Such cells would begin the DNA replication-division sequence for production of a four-celled coenobia. However, depending on external conditions, particularly energy availability, they could also begin

a third growth sequence leading to the attainment of the third CP, eventually producing eight-celled coenobia. Thus, general features of the *Scenedesmus* cell cycle pattern include the presence of multiple, partially overlapping growth and DNA replication-division sequences, including overlapping DNA duplications and nuclear divisions, while multiple rounds of cell divisions occur in a short period of time at the very end of the cell cycle for all committed DNA replication-division sequences within the cycle (Bisova and Zachleder, 2014; Zachleder et al., 2016). Therefore, a phase separating nuclear and cell divisions has been proposed for this organism and is referred to as G3 (Zachleder et al., 1997). Thus, the *Scenedesmus* cell cycle pattern is characterized by the consecutive execution of multiple rounds of DNA replication and nuclear division, as evidenced by the presence of multinucleate cells. This situation is schematically illustrated in Figure 3.

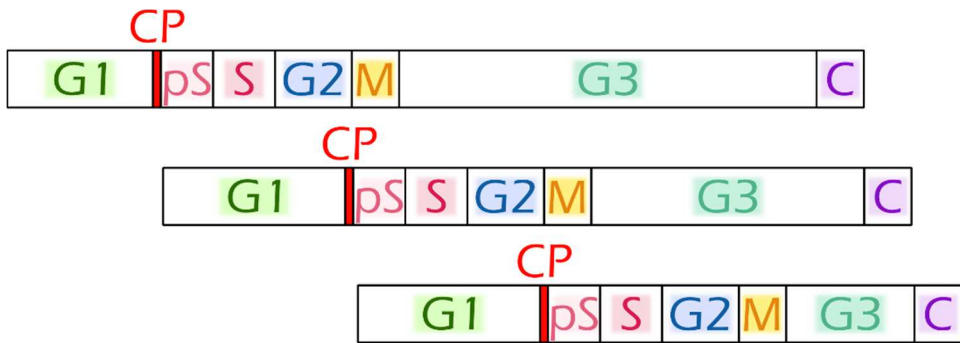


Figure 3: *Scenedesmus* cell cycle pattern of multiple fission. Each bar represents a sequence of partially overlapping growth and reproductive events within a single cell cycle leading to the formation of eight daughter cells (2^n , $n=3$). The individual phases of the cell cycle are as follows: Growth phase (G1), commitment point (CP), pre-synthesis phase (pS), synthesis phase (S), second growth phase (G2), mitosis (M), the phase between nuclear and cell division (G3), and cytokinesis (C). Modified after Zachleder et al. (2016).

1.3.2. *Chlamydomonas* cell cycle pattern

The *Chlamydomonas* cell cycle pattern is found among members of the family *Chlamydomonadaceae*, with *Chlamydomonas reinhardtii* being a well-established model organism for cell cycle studies as well as studies of photosynthesis, ciliogenesis,

photoreceptor functions or pyrenoid structure (Albee et al., 2013; Engel et al., 2015; Foster et al., 1984). The *Chlamydomonas* cell cycle pattern is similar to pattern of multiple fission found in *Scenedesmaceae*, in terms of multiple overlapping growth and DNA replication-division sequences occurring within a cell cycle. However, in the *Chlamydomonas* cell cycle pattern, there is no observable G2 phase and both the S and M phases are realized at the end of the cell cycle and are immediately followed by cytokinesis, which is why the *Chlamydomonas* cell cycle pattern is also referred to as a clustered pattern (Zachleder et al., 2016). Consequently, there is no polynuclear stage. Under optimal growth conditions, *C. reinhardtii* can undergo up to five CPs in a single cell cycle and produce 32 daughter cells (Bisova and Zachleder, 2014; Lien and Knutsen, 1979; Zachleder et al., 2016). Figure 4 shows a schematic illustration of *Chlamydomonas* cell cycle pattern.

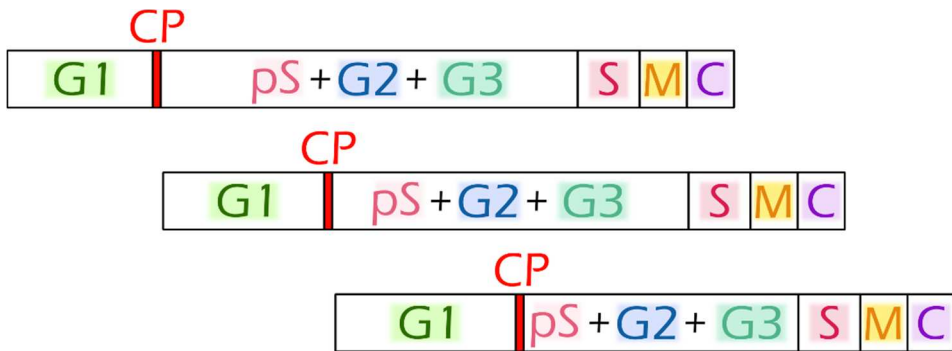


Figure 4: *Chlamydomonas* cell cycle pattern of multiple fission. Each bar represents a sequence of partially overlapping growth and reproductive events within a single cell cycle leading to the formation of eight daughter cells (2^n , $n=3$). The individual phases of the cell cycle are as follows: Growth phase (G1), commitment point (CP), pre-synthesis phase (pS), synthesis phase (S), second growth phase (G2), mitosis (M), the phase between nuclear and cell division (G3), and cytokinesis (C). Obvious G2 and G3 phases are absent in *Chlamydomonas*, but they presumably occur in the extended gap phase between CP and the onset of S phase. Modified after Zachleder et al. (2016).

1.3.3. *Chlorella* cell cycle pattern

The *Chlorella* cell cycle pattern found in *Chlorellaceae* family is similar to the two cell cycle patterns described above. Similar to the *Scenedesmus* cell cycle pattern, DNA replication and nuclear division in *Chlorella* occur shortly after each subsequent commitment point is reached, which is why multinucleate cells can be observed. However, cytokinesis occurs immediately after completion of the last nuclear replication, so eight-nucleated cells would not be observed upon reaching three successive CPs. In this respect, it resembles the *Chlamydomonas* cell cycle pattern, where nuclear division is immediately followed by cytokinesis (Bisova and Zachleder, 2014; Zachleder et al., 2016)(Figure 5).

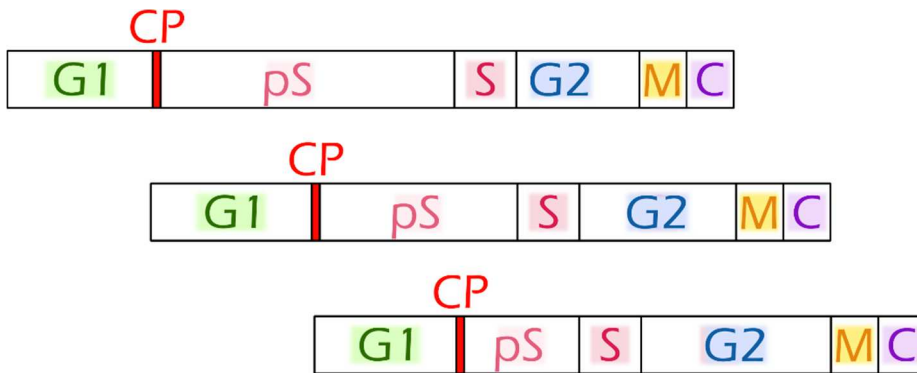


Figure 5: *Chlorella* cell cycle pattern of multiple fission. Each bar represents a sequence of partially overlapping growth and reproductive events within a single cell cycle leading to the formation of eight daughter cells (2^n , $n=3$). The individual phases of the cell cycle are as follows: Growth phase (G1), commitment point (CP), pre-synthesis phase (pS), synthesis phase (S), second growth phase (G2), mitosis (M) and cytokinesis (C). Modified after Zachleder et al. (2021a).

1.3.4. Multiple fission patterns in other microalgae

The multiple fission cell cycle patterns described above are well characterized and widely adopted by model species and biotechnologically important species. However, there are other modifications of multiple fission in microalgae. Recently, the cell cycle pattern of *Haematococcus pluvialis*, a valuable astaxanthin-

producing alga, was described. *H. phuvialis* exhibits a complex life cycle characterized by the presence of five cell types, including gametospores, motile zoospores, immotile aplanospores, daughter cell coenobia and dormant cysts (Kobayashi, 1997; Reinecke et al., 2017). Its cell cycle pattern is described as a hybrid-type cell cycle, with clustered DNA hyperamplification in two S phases, consecutive nuclear division in an M phase and consecutive cell divisions in a cytokinesis followed by a G3 phase in a daughter cell coenobium (Reinecke et al., 2017).

Multiple fission is not only found in green algae but also in red algae such as *Cyanidium caldarium* or *Galdieria sulphuraria* (Jong et al., 2021; Náhlík et al., 2021). The detailed description of the connection between the different phases of their cell cycle remains to be clarified.

1.4. Macromolecular synthesis during the cell cycle

Cell growth, which in algae usually occurs in the G1 phase, is associated with the synthesis of essential macromolecules and cellular structures and can be observed as an increase in cell volume or dry mass of the culture. In synchronous algal cultures (explained in chapter 1.6), such an increase usually has a stepwise character, reflecting a slowdown in macromolecular synthesis at the time of attaining each CP (Bisova and Zachleder, 2014). For all major macromolecules, including DNA as the carrier of genetic information, RNA and proteins necessary for maintaining normal cellular functions and structure, carbohydrates and lipids serving as energy storage molecules, or polyphosphates representing energy and phosphate reserves, a specific course of macromolecule synthesis can be observed during the cell cycle.

The course of DNA synthesis is different in the cell cycle patterns described above. In the cell cycle pattern of *Scenedesmus*, DNA content increases from the early beginnings of the cell cycle with steps corresponding to each successive DNA replication (Zachleder et al., 2016; Zachleder et al., 2002). In the cell cycle pattern of *Chlamydomonas*, DNA content increases rapidly in the final stages of the cell cycle before nuclear and cell divisions, and the final value corresponds to committed duplications (Zachleder et al., 2016; Zachleder et al., 2019a). The cell cycle pattern of *Chlorella* shows a DNA increase with features in common with the two cell cycle patterns mentioned above. DNA replications

are performed in short succession, followed by nuclear and cell divisions once all specified DNA replications are completed. Even when DNA replications are performed in a short time frame, a gradual increase can be observed (Zachleder et al., 2021a).

The increase in both RNA and proteins reflects cellular growth during the cell cycle. The levels of both macromolecules increase continuously during the cell cycle, usually in steps corresponding to a doubling of the initial value for each fixed DNA replication-division sequence. The plateau of RNA and protein content is reached at the time of nuclear and protoplast division, and its value corresponds to the number of fixed duplications (John et al., 1983; Zachleder et al., 2019b; Zachleder and Šetlík, 1982).

In addition to RNA and protein synthesis, the available energy is used to produce starch or lipids, which serve as energy and carbon stores. Their role in the cell cycle is indispensable, as they are used to support division processes. Division processes are driven by energy-storing molecules, especially starch, even when an external energy source is available, as shown by starch degradation in cells dividing in the presence of light (Brányiková et al., 2011; Spudich and Sager, 1980; Torres-Romero et al., 2019). Starch is a biopolymer composed of two main components: amylose and amylopectin, the latter component being predominant in most plant and algal sources. Starch is produced in plastids and stored in the form of starch granules (Pérez and Bertoft, 2010). The course of biosynthesis of starch and lipids in light is similar to that of the above compounds. It begins soon after cell illumination and net quantities increase until reproductive sequences occur. Then, the degradation of energy-storing molecules provides the necessary energy for the completion of the cell cycle (Vítová et al., 2015; Vítová et al., 2011b).

1.5. Cell cycle control

Cell cycle control appears to be highly conserved in eukaryotes. The crucial and challenging task is to coordinate growth and division in such a way that the cell volume is preserved and all essential cell components are passed on to the next generation.

The decision whether to divide or not, and if so, how many daughter cells to form is made upon reaching the commitment point (as defined in chapter 1.2)

close to the end of G1. The perception of commitment in multiple fission is not uniform across the scientific community. There are models that assign individual commitments to each DNA replication-division sequence (Bisova and Zachleder, 2014; John, 1984) as well as models with a single commitment point (Cross and Umen, 2015). Early studies believed in the control and regulation of the first commitment point and its corresponding DNA replication-division sequence, while other DNA replication-division sequences were thought to simply follow at fixed times after the first division (Craigie and Cavalier-Smith, 1982).

While cell size is a single criterion for cell division in yeast, green algae seem to assess their ability to divide in a more complex way. Consistent with the findings from yeast, it was first assumed that cell volume controls the cell cycle in green algae. Early studies considered so-called sizer to control entry into mitosis. Indeed, a size-dependent onset of cell division was observed in *C. reinhardtii*, and consequently the parental cell volume was thought to determine the number of daughter cells, ensuring minimal cell volume after successive rounds of replication and division (Craigie and Cavalier-Smith, 1982). The hypothesis that the sizer controls cell division was later extended to include the timer proposed based on the time interval to the first commitment point in cells incubated at two different temperatures and shifts between them (Donnan and John, 1983). The model proposed by Donnan and John (1983) accounts for the timer to control cell cycle length, while the sizer determines the number of DNA replication-division sequences.

1.5.1. Regulation on molecular level

Our current understanding of cell cycle regulation in microalgae at the molecular level comes mainly from studies with *C. reinhardtii*, as it has a known genome and a well-developed molecular toolbox that makes it an organism of choice. The relationship between cell size and cell division was further elucidated by understanding the role of cyclin-dependent kinases (CDKs) in microalgae. CDKs are highly conserved serine/threonine protein kinases that form functional heterodimers with cyclins serving as regulatory subunits (Joubes et al., 2000). Single CDK controlling cell cycle was identified in *Saccharomyces cerevisiae* (CDC28) and *Schizosaccharomyces pombe* (*cdc2*) (Mendenhall and Hodge, 1998; Moser and Russell, 2000).

Plants, including microalgae, encode multiple CDKs. A-type CDK (CDKA, homologue of yeast single CDK) contains a canonical PSTAIRE motif in the cyclin-binding domain. B-type CDK (CDKB) is a plant-specific CDK and has an altered PPTALRE or PPTTLRE motif corresponding to two subgroups CDKB1 and CDKB2 (Vandepoele, 2002). *C. reinhardtii* encodes homologues of all major plant CDKs (CDKA to CDKE) as well as cyclins. Both CDKA and CDKB found in *C. reinhardtii* are able to complement mutations in yeast single mitotic CDK *cdc2/CDC28* (Čížková et al., 2008). C-type CDKs are thought to be involved in transcription control (Oelgeschlager, 2002). D-type CDKs are CDK-activating kinases (CAKs) (Yamaguchi et al., 2000). E-type CDKs are involved in cell differentiation in multicellular organisms (Wang and Chen, 2004), so their role in unicellular microalgae remains unclear. In addition, four novel CDKs and two cyclins not found in plants were identified in *C. reinhardtii* (Bisova et al., 2005). Potential molecular basis of size hypothesis was found to be CDKG1, a D cyclin-dependent RB kinase, that accumulates in the nucleus of pre-mitotic cells and is progressively diluted with each round of mitotic division in *C. reinhardtii* (Li et al., 2016).

The course of events driving cell division has common features with other eukaryotes and specific features that are only described in *C. reinhardtii*. Common features include activation of mitotic CDK by cyclin binding, which promotes entry into mitosis and consequent cyclin degradation by the anaphase promoting complex (APC), leading to CDK inactivation and termination of division (Atkins and Cross, 2018). The mechanism by which CP is linked to CDK activation at the G2/M transition, often hours away from CP, has yet to be fully understood. Surprisingly, mitotic CDK in plants, including microalgae, is primarily CDKB and not CDKA, although CDKA affects the timing of cell division by inducing the mitotic transcriptome (Atkins and Cross, 2018; Nowack et al., 2012; Tulin and Cross, 2015). CDKA is activated by binding to A-type cyclin and CDKB by binding to B-type cyclin. Only one type of A- and B-type cyclins are present in *C. reinhardtii*, designated CYCA1 and CYCB1, respectively. CDKB protein levels reach a marked peak during the G2/M transition (Bisova et al., 2005; Mironov et al., 1999) as the CDKB-CYCB1 complex is essential for formation of mitotic spindle and nuclear division, but not for DNA replication (Atkins and Cross, 2018; Pecani et al., 2022; Tulin and Cross, 2014). The phase-dependent expression of cyclins is under transcriptional control like

in plants (Mironov et al., 1999). One of the targets for CDK phosphorylation in *C. reinhardtii* is MAT3, a retinoblastoma-related protein, suppressor of the G1 to S passage found in most eukaryotes, where RB binds to the E2F transcription factor, repressing transcription of G1/S-related genes. Phosphorylation of RB by CDKs causes its dissociation from E2F and allows for a G1/S transition. *Chlamydomonas mat3* mutants initiate cell division at abnormally small size and consequently produce small daughter cells (Umen and Goodenough, 2001). However, the effect of MAT3 on transcription levels of E2F-associated genes has not been confirmed, suggesting a possible non-transcriptional role of RB pathway in *C. reinhardtii* (Fang et al., 2006; Olson et al., 2010).

Mitotic exit is enforced by the anaphase-promoting complex (APC), which degrades mitotic B-type cyclins (Glotzer et al., 1991; Pecani et al., 2022). APC defects, in *C. reinhardtii*, lead to metaphase block and nuclear division failure (Cross and Umen, 2015).

1.6. Monitoring cell cycle progression

There are two basic approaches to analyzing cell cycle progression in microalgae: studies on whole populations or observations on single cells. Whole population studies allow the use of powerful biochemical techniques that provide a wide range of information about the cell cycle. However, in a normal population, cells would be at different stages of the cell cycle. To overcome the heterogeneity in the population, the use of synchronous cultures, i. e., cultures in which all cells are at the same stage of their cell cycle, became routine. The first use of synchronous cultures of microalgae dates back to the 1950s, when the first synchronous cultures were prepared by fractional centrifugation (Tamiya et al., 1953). Synchronous cultures can also be obtained by other methods that generally arrest all cells at a particular cell cycle stage. After unblocking, cells continue to grow and divide synchronously, usually for a limited number of cell cycles, unless synchrony is not reinforced. These methods include suboptimal temperature treatment (Zachleder et al., 2019a), nutrient starvation followed by replenishment (Brzezinski et al., 1990) or the use of mitotic inhibitors (Zachleder, 1994). However, all of these agents cause significant stress to cells, so synchronization of microalgae by alternating light and dark periods became the golden standard (Hlavová et al., 2016).

Direct estimation of cell cycle progression at the population level uses the ability of committed cells (cells that have passed one or more CPs) to divide without further energy input. Samples of the algal culture are taken from the synchronous culture at regular intervals and darkened. After a period of time during which the division processes have been completed, the cells are examined and the percentage of undivided cells and cells that have divided into different numbers of daughter cells is determined. The percentage of cells in each group is plotted against time. The resulting commitment curves or commitment diagrams show the time course of reaching CP(s) in culture (Kselíková et al., 2022c; Zachleder et al., 2016).

Synchronous algal populations that are highly homogeneous can be subjected to a variety of biochemical analyses that provide valuable information about their cell cycle stage. When analyzed repeatedly, they reveal a progression of macromolecular synthesis and cell cycle in culture. For most biochemical methods, there are well-established protocols that can be readily applied to algal biomass. They are usually inexpensive and the results are reliable and reproducible. On the other hand, they are usually time consuming to perform and require a large amount of samples to be analyzed. Modern bulk methods such as mass spectroscopy (MS), gas and high-pressure liquid chromatography, or nuclear magnetic resonance seem to be a good alternative for the identification and quantification of compounds of interest, but they cannot provide information on the spatial localization of different compounds within single cells.

Single cell methods rely on several established and emerging technologies to provide information on the status and/or composition of individual cells. One widely used method that allows high-throughput assessment of single cells is flow cytometry (FC) coupled with fluorescence activated cell sorting (FACS). In general, FACS can be used to assess and sort fractions of cells in the population based on selected parameters. It is advantageously used to determine cell size, the proportion of cells at different cell cycle stages based on their DNA content (Cooper, 2003), or to sort cells based on their lipid content (Terashima et al., 2014). Simple but powerful methods for analyzing cell cycle progression are based on microscopy. Individual cells can be assessed by their size, shape, or specific characteristics such as the presence of a particular substance or the ability to divide. Time-lapse microscopy is particularly useful

in this context because it allows the progression of the cell cycle to be followed. In addition, microscopic methods can be highly automated and coupled with advanced image analysis to increase the throughput of the method (Breker et al., 2016; Jong et al., 2021). Microscopic methods often utilize autofluorescence of the compound of interest or fluorescence from compound-specific labels. However, fluorescent labels can interfere with the native behavior of the labeled molecule and their lifetime is limited. In addition, unlabeled molecules are not visualized (Petibois, 2010). Information on the spatial and temporal relationships between different substances at the single cell level can be obtained using label-free methods, such as Raman microscopy, which combines the advantages of molecular specificity of Raman spectroscopy with the high spatial resolution of optical confocal microscopy (Moudříková et al., 2021; Moudříková et al., 2016). A comprehensive review of single cell imaging techniques has been compiled by Petibois (2010). In addition, the development of sequencing technologies now enables genomic, epigenomic, transcriptomic, or protein profiling of individual cells (Macaulay et al., 2017).

1.7. Biotechnological potential of microalgae

Microalgae are considered one of the most important food and feed producers of the future. Their advantages lie mainly in the ease of cultivation due to their microscopic nature and photoautotrophy, i.e., the ability to use carbon dioxide and light energy to synthesize organic molecules. In addition, the potential of microalgae for biotechnology could be readily enhanced by genetic engineering techniques available for a variety of commercially interesting species, including the green algae *Chlamydomonas reinhardtii* (Kindle et al., 1989), *Chlorella* spp. (Liu et al., 2014; Yan et al., 2019; Yang et al., 2015), *Dunaliella salina* (Simon et al., 2016), heterocont *Nannochloropsis* spp. (Kang et al., 2015; Vieler et al., 2012), red alga *Cyanidioschyzon merolae* (Fujiwara et al., 2015; Sumiya et al., 2014) or the diatoms *Phaeodactylum tricornutum* (Radakovits et al., 2011) and *Thalassiosira pseudonana* (Poulsen et al., 2006).

1.7.1. Protein production

Algal biomass is naturally rich in protein, with protein content averaging between 40% and 60% of dry matter, depending on the species (Becker, 2007;

Li et al., 2021). Algal protein also has a favorable amino acid composition with a high content of essential amino acids suitable for human nutrition (Becker, 2007). In addition to proteins naturally synthesized in microalgae, genetic engineering methods allow the production of recombinant proteins. The most widely used method to introduce heterologous proteins into microalgae is chloroplast transformation, which has several advantages including high plastid DNA copy number, recombination-mediated targeting of foreign DNA sequences and lack of silencing effects (Cutolo et al., 2022). Eukaryotic nature of most microalgal systems offer several advantages over more common bacterial systems; they are able to perform complex post-translational modifications, produce properly folded proteins with multiple domains, or form disulfide bonds within proteins (Yin et al., 2007). In comparison with common eukaryotic production systems, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Pichia pastoris*, microalgae are able to grow photoautotrophically. Recombinant proteins produced in microalgal systems have so far mainly included therapeutic proteins such as antibodies (Gregory et al., 2012; Mayfield et al., 2003) or immunotoxins, i.e. antibodies linked to eukaryotic toxins (Tran et al., 2013a; Tran et al., 2013b).

1.7.2. High-added value bioproducts

In addition to the microalgae-based products already mentioned, microalgae are at the center of the rapidly growing high-added value bioproducts segment. Microalgae are a valuable source of polyunsaturated fatty acids (PUFA) or long-chain fatty acids, which are known for their beneficial effects on human health (Ramesh et al., 2019; Shahidi and Ambigaipalan, 2018). Among omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are particularly valuable for human consumption due to their anti-inflammatory properties, protective role in preventing cardiovascular diseases, involvement in maintaining healthy brain functions and anti-carcinogenic properties (Shahidi and Ambigaipalan, 2018). Microalgal pigments, particularly beta-carotene and astaxanthin, are another valuable commodity. Considerable amounts of beta-carotene are produced by numerous microalgae, with *Dunaliella salina* probably being the most commonly used due to its high yield and culture conditions that minimize the risk of contamination. Beta-carotene is an important component of the human diet as a precursor to vitamin A and retinoids (Harvey and Ben-

Amotz, 2020). Astaxanthin, derived from *Haematococcus pluvialis*, could be used as feed in aquaculture but is mainly marketed as a dietary supplement with antioxidant properties for price reasons (Higuera-Ciapara et al., 2006; Li et al., 2020). Microalgal biomass is also a source of several vitamins important for human nutrition (Brown et al., 1999).

1.7.3. Lipid production

Certain species of microalgae have been shown to be valuable lipid producers, with oil content reaching up to 70% of dry mass in some microalgae (Metting, 1996). Promising microalgae for economically viable lipid production include *Botryococcus braunii* (Li and Qin, 2006), *Nannochloropsis* sp. (Janssen et al., 2019; Ma et al., 2014), *Scenedesmus obliquus* (Sadvakasova et al., 2019) or *Phaeodactylum tricornutum* (Cui et al., 2019) and other emerging species. However, high lipid productivity is often linked to specific, usually stressful, growth conditions that affect biomass productivity (Alishah Aratboni et al., 2019; Li et al., 2011; Mujtaba et al., 2012). Nevertheless, the inverse relationship between biomass and lipid productivity could be reduced by a genetic engineering approach that produces strains with high lipid productivity and little or no growth impairment (Südfeld et al., 2021; Zhang et al., 2014).

1.7.4. Biofuel

Microalgal biomass is considered a promising source for third-generation biofuel production. Mainly because algae have a high potential yield of lipids, rapid growth, low ecological footprint and are able to utilize waste materials as a source of nutrients (Sadatshojaei et al., 2020). Biodiesel derived from microalgae has been reported to be the only renewable biofuel that can completely replace petroleum-derived fuels without reducing the area of agricultural land for food and feed production (Chisti, 2008). In addition, the extraction of lipids from algal biomass during biodiesel production leaves algal biomass that is rich in carbohydrates and can be anaerobically digested to produce biogas. This dual use of algal biomass, along with the high productivity of microalgae in well-established cultivation systems, makes it an energy source of the future (Chisti, 2008). To date, microalgae have not found their way into industrial biofuel production due to various technical and

economic limitations. The current cost of microalgae-based biofuels is higher than that of fossil fuels, and thus biofuels from microalgae are not yet commercially attractive (Alishah Aratboni et al., 2019; Norsker et al., 2011; Venkata Subhash et al., 2022). Significant advances in the cultivation, harvesting, and drying of microalgae, which are highly energy-intensive processes, and increased biomass productivity are essential for improving the feasibility of algae-based biofuels (Venkata Subhash et al., 2022).

1.7.5. Phycoremediation

Another use of microalgae is wastewater treatment, also known as phycoremediation. Wastewater suitable for treatment with microalgae includes wastewater from agricultural, municipal, animal and industrial sources (Lutzu et al., 2021). Microalgae are capable of refining wastewaters from organic pollutants, heavy metals, pesticides or pharmaceuticals (Tolboom et al., 2019). Wastewater is potentially a good medium for microalgae growth, as essential nutrients such as nitrogen and phosphorus are usually present in sufficient quantities. In addition, the resulting biomass can be used, for example, as a feedstock for the production of biopolymers (Lutzu et al., 2021), biodiesel (Rinna et al., 2017), biogas (Ramos-Suárez et al., 2014) or other compounds to create a sustainable circular economy (Lopez-Pacheco et al., 2021).

1.8. Properties of deuterium

Most elements have more than one isotope, that is, a variant of the element with the same number of protons but a different number of neutrons in their nuclei. Isotopes, sometimes called isotopic nuclides, can be divided into two groups: radioactive isotopes, which undergo radioactive decay, and stable isotopes. About two-thirds of the elements exist as multiple stable isotopes, with their number varying from 2 to 10 (Lehmann, 2016). Most biogenic elements also exist as multiple stable isotopes, including hydrogen (^1H , ^2H), carbon (^{12}C , ^{13}C), nitrogen (^{14}N , ^{15}N) and oxygen (^{16}O , ^{17}O , ^{18}O). Lighter isotopes are generally more abundant in nature, while heavier isotopes account for no more than 1 % of the element in question (West et al., 2006).

There is usually little to no difference in the physicochemical properties of isotopes of the same element, with the exception of the stable isotopes

of hydrogen, protium (^1H) with atomic mass one and deuterium (^2H or D) with atomic mass two. This difference is so great that it strongly affects their physicochemical properties. In this work, hydrogen is used as a synonym for protium unless otherwise stated.

The double atomic mass of deuterium compared to protium results in a lower vibrational frequency of the C-D bond compared to the C-H bond, and thus a lower zero-point energy (lowest ground-state vibrational level). Consequently, the activation energy required to reach the transition state for bond cleavage is greater for C-D than for C-H and the reaction rate (represented by the rate constant k) is slower ($k_H > k_D$) (Pirali et al., 2019). The reaction rates are generally lower for bonds with deuterium, C-D has about 7-times lower reaction rates than C-H bond, N-D about 8.5-times lower than N-H bond and O-D bond about 10-times lower than O-H bond (Katz, 1960). As a result, both the thermodynamic and kinetic behavior of the deuterated system differ significantly from that of its protonated counterpart (Price et al., 2013). The difference in reaction rates of isotopically substituted molecules is referred to as the kinetic isotopic effect (KIE). KIE is generally very small for all elements except deuterium, where it is most pronounced due to the largest mass difference (Berg and Strand, 2011). KIE is quantified as the ratio of rate constants for the reaction, k_H/k_D in the case of deuterium. However, to observe KIE of hydrogen substitution in biochemical reactions, the rate-limiting step must involve the formation/breaking of C-H or C-D bonds (Gant, 2014). Such KIE is also referred to as primary KIE, in contrast to the secondary KIE, which is observed when the bond of the isotopically substituted atom is not broken in the reaction, and is generally much smaller (Blake et al., 1975).

Depending on the behavior of the isotopically substituted molecule in the solvent, we can distinguish between the isotopic substitution of hydrogen in non-exchangeable positions, i.e., stable bonds with carbon, or exchangeable positions, i.e., readily available bonds with oxygen, nitrogen, or sulfur (Blake et al., 1975). Therefore, in order to prepare deuterated compounds that retain their deuterated character even in protonated solvents, it is crucial to bond deuterium to carbon atoms.

Deuterium oxide (D_2O) is a water molecule in which both hydrogen atoms have been replaced by deuterium. Such an exchange results in a shift in mass; therefore, deuterium oxide is also known as heavy water. Heavy water also

differs from ordinary water in some physicochemical properties, such as boiling point (100 °C in H₂O versus 101.72 °C in D₂O), freezing point (0 °C in H₂O versus 3.82 °C in D₂O), density at 20 °C (0.9982 in H₂O versus 1.1056 in D₂O) or viscosity (1.005 cP in H₂O versus 1.25 cP in D₂O)(Yang, 2016). D₂O also differs from H₂O in its solvent properties. D₂O exhibits lower solubility of many salts and gasses, i.e., both carbon dioxide and oxygen are about 10% less soluble in D₂O than in H₂O (Katz, 1960). D₂O can be produced by fractional distillation, electrolysis, or the water-hydrogen sulfide process, also called the Girdler sulfide process (Yang, 2016). Nowadays, heavy water is commercially available in high purity and at reasonable cost.

1.9. Biological consequences of deuteration

Deuterium, first discovered in 1932, has a natural abundance of about 0.015 % (Urey et al., 1932; West et al., 2006). Organic compounds are generally depleted in deuterium by 90‰ to 110‰ relative to the surrounding water. The main site of hydrogen fractionation is photosynthesis (Estep and Hoering, 1981; Smith and Epstein, 1970). The extent of hydrogen fractionation varies among organic compounds, reflecting their biosynthetic pathways (Zhang and Sachs, 2007).

Deuteration of living cells elicits a general stress response, as shown by the expression of stress response genes in recent experiments with tumor cell lines (Jandova et al., 2021). Cells exposed to high deuterium concentrations often exhibit common morphological features, including the appearance of abnormally large cells (Chorney et al., 1960; Mosin et al., 2014; Unno et al., 1992). In parallel with this finding, proliferation rates are reduced at high D₂O concentrations (Chorney et al., 1960; Farthing et al., 2017; Opitz et al., 2018; Unno et al., 1992). There are several described mechanisms by which deuterium affects the ability of cells to divide. Deuterium stabilizes tubulin and stimulates microtubule formation from tubulin *in vitro*. However, the products of tubulin assembly were often incompletely folded microtubules or ribbon structures (Chakrabarti et al., 1999). Consequently, deuterium suppresses microtubule dynamics and treadmilling (Panda et al., 2000). *In vivo* experiments confirmed that deuterium negatively affects mitotic spindle assembly and blocks the transition from metaphase to anaphase (Lamprecht et al., 1991).

Based on mathematical modeling, deuterium substitutions in a DNA molecule, as well as deuterium surrounding DNA molecules, increase the probability of the occurrence of open states between DNA bases, indicating a decrease in the stability of the molecular structure of DNA (Dzhimak et al., 2018a; Dzhimak et al., 2018b). Deuterium in the form of D₂O has been shown to affect the structure and possibly the functions of DNA. D₂O inhibits the process of folding ssDNA into specific structures, including G-quadruplexes formed by guanine-rich nucleic acids, which are thought to have important regulatory functions (Jiang et al., 2022; Kouzine et al., 2019).

Other biomolecules are also inevitably affected by deuteration. There are works reporting increased stability of proteins in D₂O (Efimova et al., 2007; Hattori et al., 1965; Verheul et al., 1998). Moreover, D₂O decreases the conformational flexibility of proteins (Cioni and Strambini, 2002) and alters their molecular dynamics (Guzzi, 1999). Compensation of abnormal protein folding in D₂O could be represented by the enhanced expression of heat shock proteins detected in deuterium-resistant mutants of *Chlorella* and yeast (Unno et al., 2005). The solvent isotopic effects of D₂O affect the reaction rates of numerous enzymatic reactions important for homeostasis and survival, including the carboxylation rate of RubisCO, which is 1.7 times slower in pure D₂O (Tcherkez et al., 2013), and proton pumping in cytochrome c oxidase (Salomonsson et al., 2008). Deuteration also affects the stability of phospholipids, the building blocks of cell membranes, resulting in a much less ordered configuration of deuterated lipids (Guard-Friar, 1985).

Substitution of H₂O by D₂O alters both biosignaling and bioenergetic processes in the cell (Hirakura et al., 2011). The mobility of H⁺ in H₂O is 1.41 times that of D⁺ in D₂O (DeCoursey and Cherny, 1997), and proton channels are permeable to D⁺ (deuteron), with a conductance only about 50% that of H⁺ (DeCoursey and Cherny, 1997; Kotyk et al., 1990). H/D exchange slows both ATP synthesis and electron flow in thylakoid membranes, with ATP synthesis more severely impaired, leading to a decrease in the number of ATP molecules formed per electron transferred (de Kouchkovsky et al., 1982). This is consistent with the decreased ATP pools observed in deuterated nerve fibers (Vasilescu and Katona, 1986).

1.10. Deuterated compounds

Microalgae have great potential as a source of deuterium-labelled compounds. They are easy to cultivate and their ability to photosynthesize allows them to incorporate stable isotopes from relatively inexpensive inorganic substrates into higher-value organic compounds (Apt and Behrens, 1999). A variety of microalgal products therefore provide a robust platform for *in vivo* deuterium labelling, including pigments, lipids, carbohydrates or proteins (possibly including heterologous proteins). Several studies have already demonstrated the possibility of producing various deuterated compounds using microalgae (Bhosale et al., 2006; Delente, 1987; Gireesh et al., 2001; Saha et al., 2013). Although cells of unicellular microalgae are equally exposed to deuterium and the extent of labelling is linearly correlated with the amount of deuterium in the growth medium, there is heterogeneity in the amount of incorporated deuterium reflecting the physiological heterogeneity among individual cells (Apt and Behrens, 1999; Berry et al., 2014). This effect is particularly pronounced at higher deuterium concentrations (Berry et al., 2014). Moreover, the extent of deuterium labelling varies among organic compounds, reflecting their biosynthetic pathways and hydrogen isotope fractionation (Zhang and Sachs, 2007).

In recent decades, stable isotope labelled compounds, including deuterated compounds, have found their place in commercial applications and represent a market worth millions of dollars per year (Spolaore et al., 2006). A growing number of companies, such as Cambridge Isotope Laboratories, BOC Sciences, or C/D/N Isotopes, are meeting market demand for stable isotope-labeled reference substances, advanced intermediates, pharmaceutical end products, bioactive compounds and metabolites.

The relative abundance of hydrogen isotopes (delta notation, δ) expresses the variation of an isotopic ratio of an element relative to the isotopic ratio of a standard. Relative abundance of deuterium can be used (usually as part of a multi-element analysis) to track the progress of chemical and enzymatic reactions (Duncan and Millar, 2022; Garg et al., 2017; Yang, 2016), to study current and past hydrologic cycles and climate conditions (Kopeck et al., 2019; Kurita et al., 2004) or to assess the authenticity of food, i.e., to verify its quality and origin (Hermann, 2001; Kelly et al., 2005; Werner and Rossmann, 2015).

The relative abundance of hydrogen isotopes has been shown to be sensitive to the nature of metabolism in bacteria and protists, allowing assessment of the extent of mixotrophy in whole populations (Cormier et al., 2022; Zhang et al., 2009).

Hydrogen-deuterium exchange is a cornerstone of hydrogen/deuterium exchange mass spectrometry (HDX/MS), a key technique for monitoring structural and dynamic aspects of proteins in solution. It relies on the rapid exchange of hydrogen for deuterium in protein regions lacking stable hydrogen bonds, resulting in mass shifts (Konermann et al., 2011; Rand et al., 2014). The use of deuterium in NMR techniques (^2H NMR spectroscopy) is particularly useful for studying the dynamics of lipid membranes and membrane-bound peptides (Strandberg et al., 2009; Yasuda et al., 2015).

Recently, deuteration has also been used in pharmacology, mainly to improve existing drugs. Deuterium substitution in drugs is not expected to change the biochemical potency and specificity of a drug, but it may alter its pharmacokinetic profile, reduce its toxicity, or stabilize chemically unstable stereoisomers (Pirali et al., 2019). Although deuterated drugs are usually prepared by deuterium incorporation into proto-materials, their semisynthesis from perdeuterated compounds produced by microalgae is feasible (Gant, 2014). There is already one deuterated drug (deutetrabenazine/Austedo) approved by the Food and Drug Administration for the treatment of Huntington's disease (Frank et al., 2017). Several deuterated drugs are in various stages of clinical trials, mostly aimed at improving efficacy and reducing adverse effects compared to their non-deuterated counterparts. These include drugs for the treatment of inflammatory and fibrotic diseases (deupirfenidone/LYT-100, Chen et al. (2022)), chronic kidney disease (CTP-499, Braman et al. (2013); Sabounjian et al. (2016)), solid tumors and lymphomas (avadomide/CC-122, Rasco et al. (2019)), Alzheimer-type dementia (AVP-786, Garay and Grossberg (2017)), cystic fibrosis (CTP-656, Harbeson et al. (2017)) and others. The rationale for the use of deuterium in pharmacology, as well as recent developments in the field, have been addressed in other excellent works (Gant, 2014; Pirali et al., 2019).

2. Aims of the thesis

Since the early days of deuterium research, scientists have been interested in how isotopic substitution of hydrogen affects various organisms. Microalgae were among the first organisms tested, probably because they combine both the advantage of ease of cultivation due to their microscopic nature and physiology very similar to that of higher plants. However, the effects of deuteration on the cell cycle have mostly been described as slower growth or cell cycle arrest, depending on the algal species and deuterium concentration. Our goal was to test the cultivation of model microalgae in high deuterium concentration, to describe how deuteration affects growth and/or cell division in model microalgae, and to experimentally test the possibility of producing highly deuterated organic compounds in microalgae.

The main objectives of my work were:

- 1) Test appropriate growth conditions and culture management for cultivation of microalgae in a highly deuterated environment.
- 2) Describe the effects caused by the incorporation of the stable isotope deuterium into algal cells.
- 3) Focus on the alteration/disruption of the cell cycle of green microalgae in highly deuterated medium.

3. Summary

This dissertation consists of five published articles, including four original research papers describing the effects of deuteration on model microalgae and a methodology describing CP assessment in deuterated microalgal cultures.

Article I.

To divide or not to divide? How deuterium affects growth and division of *Chlamydomonas reinhardtii*

We exposed synchronous populations of the model microalga *Chlamydomonas reinhardtii* to high concentrations of D₂O and monitored their cell cycle progression and ability to divide. The results show a concentration-dependent decrease in growth rates. However, deuteration increased the net content of energy storage molecules, such as starch and neutral lipids. We thoroughly analyzed the ability of cells to divide in deuterated media. The proportion of cells capable of dividing decreased to about half in deuterated media, corresponding to the lower kinase activity observed in deuterated cultures. A modified protocol for the commitment point assay, in which deuterated samples were transferred to different concentrations of deuterium oxide after darkening, clearly showed that deuterium affects both growth- and division-related processes. Surprisingly, the ability of cells to divide was reinterpreted after switching to different concentrations of deuterium oxide, further refuting the role of sizer in cell cycle control of *Chlamydomonas reinhardtii*.

Article II.

Characterization of growth and cell cycle events affected by light intensity in the green alga *Parachlorella kessleri*: A new model for cell cycle research

Microalgae grown in deuterated media are under tremendous stress that alters their response to environmental factors such as light. Therefore, we sought to describe the growth and division of *Parachlorella kessleri* in deuterated media under four light intensities ranging from 100 to 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Our results show that both growth and division are affected with increasing deuterium concentration, with division being more affected. While increasing the light intensity in the control culture had a positive effect on growth and division, the opposite was true in the deuterated cultures: increasing the light intensity led to a decrease in growth rates. Such a response is related to the lower

F_v/F_M values observed in deuterated cultures at higher light intensities. Similar to the results of a previous study, deuteration leads to an accumulation of energy storage molecules; in the model microalga *P. kessleri*, these are mainly neutral lipids.

Article III.

Supra-optimal temperature: An efficient approach for overaccumulation of starch in the green alga *Parachlorella kessleri*

Microalgal growth is highly influenced by environmental factors, including temperature, which affects the rate of chemical and enzymatic reactions. At the same time, deuterium elicits kinetic isotope effects, which in most cases reduce the rate of such reactions. Therefore, our goal was to test the optimal temperature for culturing *P. kessleri* in deuterated media and to determine whether increasing the cultivation temperature (within the physiological range) could mitigate the stress induced by deuterium. Interestingly, temperature affected growth and division to varying degrees. Growth of deuterated cultures was faster when the cultivation temperature was increased to 35 °C, but it did not accelerate the onset of division. Increasing the temperature to 39 °C did not improve the performance of the culture, as such a temperature blocked cell division even in the non-deuterated control cultures. Similar to the results described above, neutral lipids were accumulated to a large extent in deuterated cultures and thus represent a potential source of deuterated lipids.

Article IV.

Analysis of commitment point attainment in algae dividing by multiple fission

Our studies with deuterated microalgal cultures showed a clear effect of deuterium on cell cycle progression. Therefore, we needed a method to assess the attainment of the commitment point in deuterated cultures, since the commitment point divides the cell cycle into different phases: the pre-commitment period, characterized by growth, and the post-commitment period, characterized by division-related processes. We regularly sampled cultures grown at different deuterium concentrations and transferred them to different deuterium concentrations after darkening. Thus, the cumulative percentage of cells that passed their commitment point(s) at each transfer suggests

a separate effect of deuterium in the pre-commitment and post-commitment periods.

Article V.

Cultivation of the microalgae Chlamydomonas reinhardtii and Desmodesmus quadricauda in highly deuterated media: Balancing the light intensity

Light is a crucial abiotic factor for the growth of autotrophically cultured microalgae. Therefore, we tested the growth of the model microalgae *Chlamydomonas reinhardtii* and *Desmodesmus quadricauda* in deuterated media at light intensities ranging from 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Interestingly, deuteration reduced saturation light intensity in *D. quadricauda* but not in *C. reinhardtii*. Consequently, an increase in light intensity resulted in an increase in stress in deuterated cultures, as documented by a decrease in the F_v/F_m ratio of the deuterated cultures. Deuteration also increased the relative content of singlet oxygen in *C. reinhardtii* but not in *D. quadricauda*, possibly reflecting their different sensitivity to deuteration. As in previous studies, both microalgae accumulated energy-storing molecules, which is a general trend for deuterium-treated cultures. Using Raman mapping, we demonstrated that detected compounds, such as starch or guanine crystals, were highly deuterated.

4. Results

Article I.

To divide or not to divide? How deuterium affects growth and division of *Chlamydomonas reinhardtii*

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To Divide or Not to Divide? How Deuterium Affects Growth and Division of *Chlamydomonas reinhardtii*

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Abstract: Extensive in vivo replacement of hydrogen by deuterium, a stable isotope of hydrogen, induces a distinct stress response, reduces cell growth and impairs cell division in various organisms. Microalgae, including *Chlamydomonas reinhardtii*, a well-established model organism in cell cycle studies, are no exception. *Chlamydomonas reinhardtii*, a green unicellular alga of the *Chlorophyceae* class, divides by multiple fission, grows autotrophically and can be synchronized by alternating light/dark regimes; this makes it a model of first choice to discriminate the effect of deuterium on growth and/or division. Here, we investigate the effects of high doses of deuterium on cell cycle progression in *C. reinhardtii*. Synchronous cultures of *C. reinhardtii* were cultivated in growth medium containing 70 or 90% D₂O. We characterize specific deuterium-induced shifts in attainment of commitment points during growth and/or division of *C. reinhardtii*, contradicting the role of the “sizer” in regulating the cell cycle. Consequently, impaired cell cycle progression in deuterated cultures causes (over)accumulation of starch and lipids, suggesting a promising potential for microalgae to produce deuterated organic compounds.

Keywords: deuterium; heavy water; cell cycle; multiple fission; commitment point; stress; cell division; *Chlamydomonas reinhardtii*

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1. Introduction

Deuterium (²H or D) is a stable isotope of hydrogen with a natural abundance of 0.015% [1]. The biggest and only difference between the atomic structures of deuterium and protium (¹H) is the extra neutron in the deuterium nucleus. This change in the atomic structure results in unprecedented consequences in its physico-chemical characteristics. Heavier isotopes generally exhibit slower reaction rates, with the phenomenon being described as the kinetic isotope effect. This effect is normally very low [2], but not so with deuterium. As deuterium exhibits the largest relative increase in atomic mass compared to its lighter isotope protium, it also exhibits the strongest kinetic isotope effect observable among stable isotopes of biogenic elements [3]. Exposing living organisms to stable isotopes of other biogenic elements (¹³C, ¹⁵N, ¹⁷O or ¹⁸O) has little or no effect [3]; however, their exposure to deuterium results in various physiological and morphological aberrations. Deuterium disrupts bio-signaling [4] and bio-energetic processes both in mitochondria [5,6] and chloroplasts [7,8]. One of the most pronounced effects of deuterium is the slowing or disruption of growth and cell division in deuterated cells and organisms. This phenomenon has been observed in animals [9,10] and plants [11,12], as well as bacteria [13,14]. Nevertheless, growing microorganisms on deuterated substrates still represents a promising non-synthetic way of obtaining highly deuterated compounds with minimal inputs [15–17].

The ability (or more often the inability) of various organisms to grow in a deuterium-enriched environment has fascinated researchers since the 1930s [18,19]. These early studies were usually unable to distinguish between two separate yet interconnected processes

in the cell—growth and division—which may be affected by deuterium to very different extents. The way to overcome this methodological problem is to choose a model organism that allows a simple separation of growth and cell division. One such class of organism is green alga which, when grown autotrophically, uses light as a source of energy for growth and division. While growth is light-dependent, division itself is light-independent. This feature of green algae is an advantage when used for preparing synchronous populations, i.e., populations in which the majority of cells are in the same stage of their cell cycle. High degrees of synchrony can be attained by simple alternating light and dark regimes of different lengths [20]. This enables effective separation of growth and reproductive events. One well-established model green alga is *Chlamydomonas reinhardtii*, a unicellular alga with two flagella, multiple mitochondria and a single cup-shaped chloroplast. *C. reinhardtii* is of special importance in cell cycle studies because it divides by multiple fission. In the most common type of cell division, binary fission, the cell cycle would follow a sequence of events: growth (G1 phase), synthesis of essential biomolecules including DNA replication and division (S phase), further growth (G2 phase) and nuclear division followed by cytokinesis into 2 daughter cells (M phase) [21]. During multiple fission, a single mother cell divides into 2^n daughter cells, where n is the number of doublings. For *C. reinhardtii*, the most typical number of daughter cells is eight ($n = 3$) at optimal growth conditions [22] and decreases to four ($n = 2$) or two ($n = 1$) in less than optimal conditions [23,24]. This is possible thanks to an overlap in several growth and DNA replication and division sequences within one cycle, which are followed by cytokinesis at the end of the cycle (Figure 1). The G1 phase needs to be long enough to ensure sufficient growth and energy reserves for the upcoming division events. The threshold is governed by the ability of the cell to divide without further energy supply, i.e., in autotrophic organisms if the light supply is stopped. This threshold is reached at the commitment point (CP) in plants and algae. Several CPs are attained within one cell cycle in the multiple fission model. The existence of several CPs allows a maximization of growth as long as the energy source (in autotrophy, light) is available and a postponement of cell division to a time when no external energy source is available. This creates conditions for considerable plasticity that is not only beneficial for the cells but at the same time provides excellent tools for studying the cell cycle.

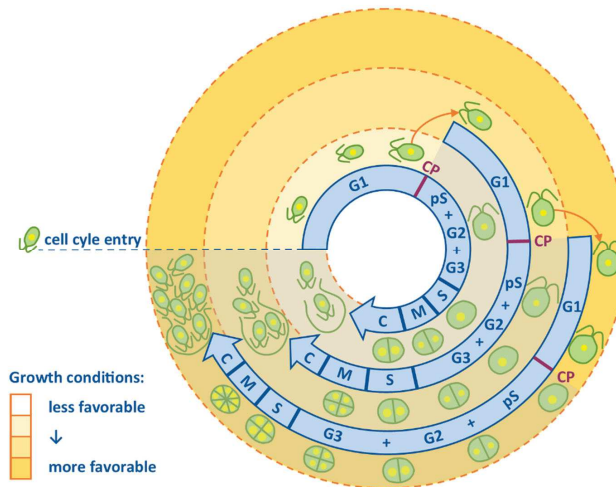


Figure 1. Multiple fission cell cycle in the model alga *Chlamydomonas reinhardtii*. Multiple overlapping growth and DNA replication–division sequences within one cell cycle are demonstrated as individual circle-shaped arrows. The better the growth conditions, the more growth and DNA replication–division sequences will be executed. The innermost ring represents division into two daughter cells. The combination of the innermost and the middle rings will lead to division into four daughter cells. Combination of all the circles will produce eight daughter cells. Each growth and DNA replication–division sequence can be further divided into several phases: G1 characterized by growth of the cell; then cells reach commitment point (CP) characterized by gaining the ability to divide even without further energy supply (shown by the shaded part after CP was reached); pS, the pre-replication phase; S phase during which DNA is replicated; G2 phase spanning from termination of DNA replication to the start of mitosis; M phase during which nucleus is divided; G3 phase between nuclear and cellular division and C phase, when the cell is cleaved. Adapted with permission from Bisova and Zachleder [25]. Copyright 2014 Oxford University Press, on behalf of the Society for Experimental Biology.

2. Materials and Methods

2.1. Organism, Culture Growth Conditions

Chlamydomonas reinhardtii strain 21gr (CC-1690; Chlamydomonas Genetics Center, Duke University, Durham, NC, USA) was cultivated in glass cylinders with an inner diameter of 3 cm and volume of 300 mL. The cylinders were placed in a temperature-controlled water bath and illuminated by incandescent lamps (Dulux L55W/950 daylight, Osram, Munich, Germany). Prior to the experiment, cell cultures were synchronized using alternating light (13 h) and dark (11 h) regimes for at least three cycles. During synchronization, growth conditions were: 30 °C, bubbling with 2% (v/v) CO₂ in air, incident light intensity of 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and cultures were grown in modified HS medium as described by Hlavová, Vítová and Bišová [20]. Synchronized cultures were gently centrifuged at 3000× g for 5 min and re-suspended either in HS medium as described above or in HS medium containing 70% deuterium oxide (D₂O) or 90% D₂O (99.95 atom%, catalog number 300101500, Silantes, Munich, Germany). The growth conditions during experiments were the same as during synchronization except for light intensity, which was reduced to 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Cultures grown in 0% D₂O HS medium, 70% D₂O HS medium or 90% D₂O HS medium were sampled in 2 h, 4 h or 6 h intervals, respectively.

2.2. Assessment of Growth

To determine culture growth, dry mass and optical density were evaluated. For dry mass, 5 mL of the culture were pelleted in pre-weighed tubes at 5000× *g* for 3 min and the supernatant was removed. The pellet was then dried at 100 °C to a constant weight. Optical density was measured at 750 nm. To assess cell counts as well as cell volumes (representing cellular growth), 1 mL of the culture was fixed with 2.5% (*w/v*) glutaraldehyde to a final concentration of 0.25% (*w/v*). These samples were then diluted 20 to 50 times with 0.9% (*w/v*) NaCl to a final volume of 10 mL and measured using a Multisizer 4 (Beckman Coulter, Brea, CA, USA). Cell volume was presented as modal value, i.e., the most common value in the dataset. This representation better reflects the central tendency of the cell size in a population with wide and skewed distribution. Cell division was assessed manually using light microscope.

2.3. Analysis of Commitment Point Attainment

Sampled cultures were diluted 5 times and 10 times using their respective HS media in order to ensure an appropriate cell density. Three microliters of both dilutions were spotted onto plates containing HS medium solidified with 1.5% agar. Every sample was plated on three different plates, containing 0% D₂O, 70% D₂O and 90% D₂O (Table 1). All plates were incubated at 30 °C in the dark for a minimum of 12 h (exact time was determined for every plate by observation of dividing cells). Plates were fixed bottom-up by adding a drop of Lugol's solution (5% KI, 2.5% I) onto the lid of the plate. Fixed plates were stored at 4 °C until analysis. The number of undivided mother cells and mother cells that divided into two, four or eight daughter cells was then counted using a light microscope. Commitment curves represent the cumulative percentage of cells that divided to two, four or eight daughter cells as a function of sampling time. For the detailed protocol, see Kselíková et al. [26].

Table 1. Commitment point (CP) samples as defined by the combination of growth media and media used for dark incubation.

Growth Medium (% D ₂ O)	Medium (% D ₂ O) for Dark Incubation	CP Sample Name
0	0	0–0
	70	0–70
	90	0–90
70	0	70–0
	70	70–70
	90	70–90
90	0	90–0
	70	90–70
	90	90–90

2.4. Kinase Assay

Cultures containing 2×10^7 cells were pelleted, washed with SCE buffer (100 mM sodium citrate, 2.7 mM sodium EDTA, adjusted to pH 7 with citric acid), frozen in liquid nitrogen and stored at −70 °C until analysis. Protein lysates were prepared from pelleted samples as described by Bisova et al. [27], for details see Bišová [28]. The lysates were either used directly for the kinase assay or were purified with CrCKS1 beads [27]. Histone H1 kinase activity was measured in a final volume of 10 µL with either 7 µL of whole cell lysate or the CrCKS1 purified fraction following the protocol of Langan et al. [29]. The master mix consisted of 20 mM HEPES, pH 7.5, 15 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 0.1 mM ATP, 0.2% (*w/v*) histone (cat. number H5505, Sigma-Aldrich, St. Luis, MO, USA) and 0.37 MBq (γ ³²P) ATP. The reactions were carried out at room temperature for 30 min

and then stopped by adding 5 μ L of 5 $\times$ SDS sample buffer (250 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 100 mM DTT, 0.5% (w/v) bromophenol blue) and incubated for 2 min at 98 $^{\circ}$ C. Proteins were separated by SDS-PAGE in 15% gels [30]. Phosphorylated histone bands were visualized by autoradiography and analyzed using a phosphoimager (Storm 860, Molecular Dynamics, Chatsworth, CA, USA). Quantification of phosphorylation was achieved using Image Studio Lite software (ver. 5.2, LI-COR Biosciences, Lincoln, NE, USA). To ensure comparability between samples and experiments, the sums of pixel intensities within the same area were normalized to the background pixel intensity to yield the pixel intensity of the signal. These were further normalized to the pixel intensity of histone bands in the gels stained with Coomassie Brilliant Blue. Resulting values are presented as the sum of pixel intensity [31].

2.5. Quantification of Starch Content

Quantification based on the method of McCready et al. [32] was performed as published previously [33], with modification of the extraction procedure as described below. Two mL of an algal suspension was harvested by centrifugation at 18,000 $\times$ g for 2 min and pellets were frozen at -20 $^{\circ}$ C. Algal cells were disintegrated by vortexing with 300 μ L of glass beads in 500 μ L of distilled water. Pigment extraction was done three times in 1 mL of 80% ethanol for 15 min at 68 $^{\circ}$ C. Each round was followed by centrifugation and removal of the supernatant. For total starch hydrolysis, 1.5 mL of 30% perchloric acid was added to the sediment, the samples were mixed by vortexing and then incubated at room temperature for 15 min. The samples were then centrifuged, and the supernatant was collected. This procedure was repeated three times, yielding 4.5 mL of hydrolyzed starch extract, which was then made up to a total volume of 5 mL. Starch determination with anthrone solution followed the original protocol [33].

2.6. Measurement of Neutral Lipid Content

Neutral lipid content was measured spectrophotometrically in a microplate format following the modified procedure of Takeshita et al. [34]. Aliquots (100 μ L) of the cultures were transferred to a 96-well plate and 5 μ L of freshly prepared Nile Red dye (0.5 mg/mL in DMSO, catalog no. 72485, Sigma-Aldrich, St. Luis, MO, USA) were added to each well. The same amount of Nile Red dye was added to a sample blank consisting of 100 μ L of H₂O. The plate was incubated at room temperature for 15 min. Fluorescence was measured using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) equipped with a 485 nm excitation filter and a 595 nm emission filter. Fluorescence intensity of the samples was normalized using fluorescence intensity of unstained samples and a blank. Standard curves produced from a commercial lipid standard, triolein (catalog no. Y0001113, Sigma-Aldrich, St. Luis, MO, USA) were used to quantify neutral lipids.

2.7. Statistical Analysis

Experiments were performed in three biological replicates. If not stated otherwise, all results are presented as an average and standard deviation ($n = 3$). The modal value was selected to characterize cell volume distribution in the population, as it corresponds to the most common value in the dataset.

3. Results

3.1. Growth of Cultures in Medium Containing D₂O

Cells were synchronized by alternating light/dark regimes prior to the experiment and then grown in continuous light for one cell cycle, i.e., from their birth until completion of cell division. However, some experimental treatments affected the ability of cells to complete their cell division (see below). In such cases, the time point corresponding to cell division maxima was considered to be the end of cell cycle. Cells failing to divide until this point rarely divided later (data not shown). The cell cycle lengths for cultures grown

in 0% D₂O, 70% D₂O and 90% D₂O were established as 16, 36 and 60 h, respectively. The growth of each culture was monitored only during this time span. All the cultures arose from the same initial culture and started from approximately the same cell density. During one cell cycle, the cultures attained a dry mass of 0.85 mg mL⁻¹ in 0% D₂O, 1.05 mg mL⁻¹ in 70% D₂O and 1.08 mg mL⁻¹ in 90% D₂O (Figure 2A). In terms of optical density at 750 nm, the experimental treatments differed in the same manner, reaching maximal values of 1.12 in medium containing 0% D₂O, 1.48 in 70% D₂O and 1.37 in 90% D₂O (Figure 2B). Significant differences in cell counts were also noted, where in 0% D₂O the culture attained 12.3×10^6 cells mL⁻¹ after one cell cycle, i.e., five times the initial cell number, while in 70% D₂O the cell number was less, by approximately one-third, than that in 0% D₂O, reaching 7.9×10^6 cells mL⁻¹, i.e., nearly three times the initial cell number. In 90% D₂O, the cell number after one cell cycle was even less, reaching a maximum of 6.8×10^6 cells mL⁻¹ corresponding to three times the initial cell number. While the cell count decreased with increasing D₂O concentration, the cell volume followed an inverse trend. In 0% D₂O, cells reached a modal cell volume of 330 µm³ at the time of cell division, while in 70% D₂O, the modal cell volume was 430 µm³, and in 90% D₂O, the modal cell volume was 470 µm³ at the time of cell division. The modal cell volume represents a typical cell of the population rather than an average cell. No drop in modal cell volume was observed, because the majority of daughter cells stayed in the division clusters at the end of the experiment and did not immediately release from the mother cell wall. The daughter cells of the control cultures released from the division cluster over several hours in light or in dark. The cells of all of the deuterated cultures stayed in the division clusters for a prolonged period of time (over 10 h). The observed cell numbers and cell volumes pointed to the fact that with increasing D₂O concentrations, the cells grew larger and the cultures attained lower cell numbers.

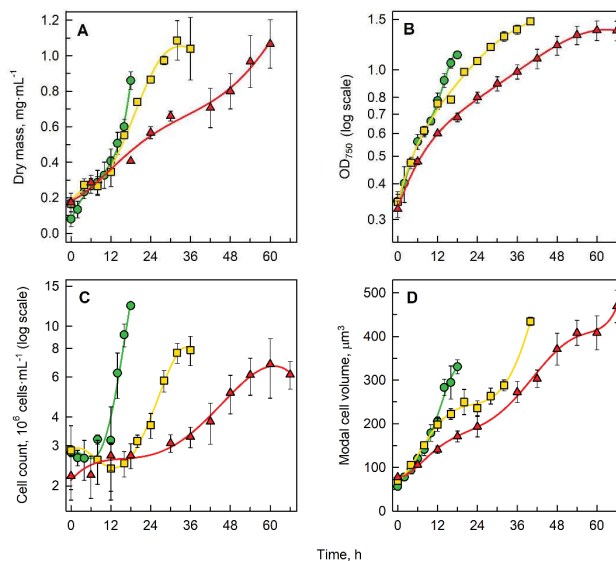


Figure 2. Changes in dry mass (A), optical density at 750 nm (B), cell count (C) and cell volume (D) of *C. reinhardtii* grown in 0% D₂O (green circles), 70% D₂O (yellow squares) and 90% D₂O (red triangles). All cultures were grown and sampled for the duration of one cell cycle. OD: optical density.

3.2. Accumulation of Energy Storing Compounds

The cellular content of energy storing molecules such as starch (Figure 3A) and neutral lipids (Figure 3B) changed with cell cycle progression. The starch content in a synchronous culture of *C. reinhardtii* grown in 0% D₂O reached a maximum of 38 pg·cell⁻¹ in the twelfth hour of the cell cycle (Figure 3A). Then the starch content decreased to 11 pg·cell⁻¹ as the cells in the culture divided. In the culture grown in 70% D₂O, the starch content increased until the sixteenth hour of the cell cycle, when it reached a maximum of 62 pg·cell⁻¹, representing 163% of the value in the control culture, and then decreased to 35 pg·cell⁻¹ with further progression of the cell cycle (Figure 3A). In the culture grown in 90% D₂O, the starch content per cell increased gradually for approximately 24 h, then it reached a plateau at 56 pg·cell⁻¹, representing 147% of the value of the control culture, and did not change any further until the end of the cell cycle (Figure 3A). Different D₂O concentrations in the growth medium also affected the neutral lipid content in the cells (Figure 3B). In 0% D₂O there was no change in neutral lipid content throughout the cell cycle, while both in 70% and 90% D₂O, the lipid content increased considerably with cell cycle progression, reaching a maximum at the end of the cell cycle. In 70% D₂O the maximum neutral lipid content was 5.0 pg·cell⁻¹, in 90% D₂O it was 7.7 pg·cell⁻¹.

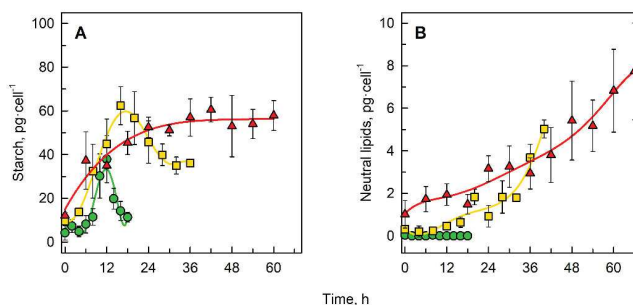


Figure 3. Changes in starch content per cell (A) and neutral lipid content per cell (B) in synchronous cultures of *C. reinhardtii* grown in 0% D₂O (green circles), 70% D₂O (yellow squares) or 90% D₂O (red triangles).

3.3. Kinase Activity and Cell Division

To understand cell division in deuterated cultures, the proportions of cells completing cell divisions were calculated and the kinase activities of the key cell cycle regulator, cyclin-dependent kinase, were established (Figure 4). In medium with 0% D₂O (Figure 4A), 95% of the cells performed cell division, but in 70% D₂O (Figure 4B) and 90% D₂O (Figure 4C) the proportions of cells able to complete cell division were reduced, reaching a maximum of 45% and 60% of the cells, respectively. In terms of histone H1 kinase activity, in the control culture with 0% D₂O, activity increased rapidly prior to cell division, while in both deuterated cultures, the increase in kinase activity was slower and less significant. Nevertheless, the lower kinase activity in deuterated cultures corresponded to the lower proportion of cells finishing their cell division.

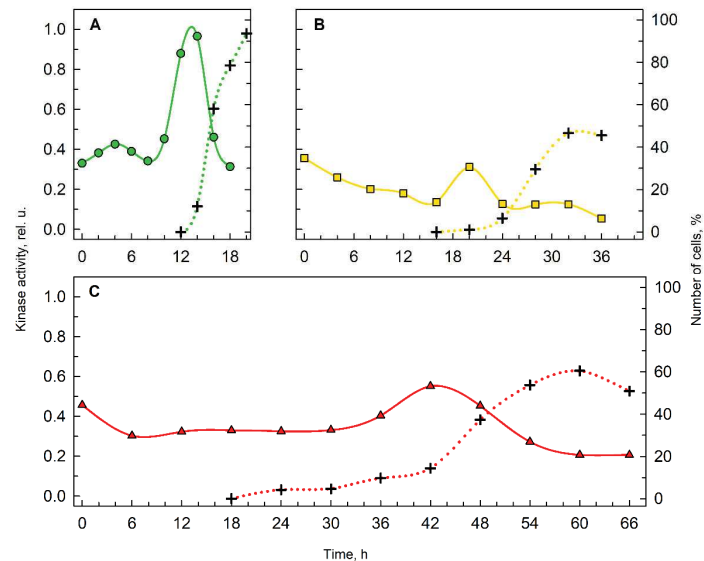


Figure 4. Histone H1 kinase activity in cultures grown in 0% D₂O (A), 70% D₂O (B) and 90% D₂O (C). Kinase activity (solid line, left axis) precedes cell division (dotted line, right axis). One representative replica of the triplicate is shown for each condition.

3.4. The Effect of D₂O on Commitment Point Attainment

To distinguish between the effects of D₂O on growth and/or division, cultures of all experimental treatments were sampled throughout the cell cycle and small aliquots of all concentrations tested were transferred to agar plates and put into the dark until they divided. In this way, all growth processes were stopped, but the processes relating to progression of the cell cycle, DNA replication, nuclear and cell division were not affected. The proportion of cells that were able to divide in the dark, i.e., attain the CP is shown in Figure 5. To simplify the description of CP samples with regard to D₂O concentrations in the growth medium and in the agar plates, their combinations (CP samples) will be designated according to Table 1.

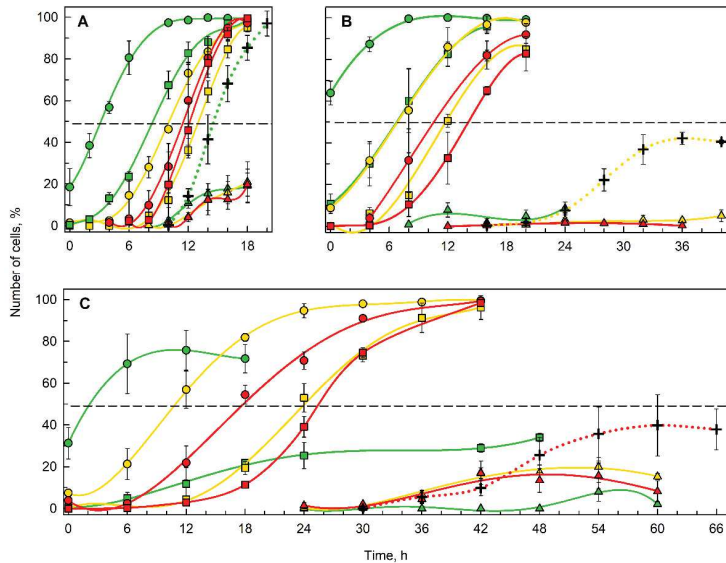


Figure 5. Percentage of cells attaining commitment point (CP) during growth in control medium with 0% D₂O (A), in medium with 70% D₂O (B) and in medium with 90% D₂O (C). Each of the graphs shows CP attainment as the percentage of cells that finished cell division in the dark on plates with 0% D₂O (green lines), 70% D₂O (yellow lines) and 90% D₂O (red lines). Attainment of the first CP (circles), the second CP (squares) and the third CP (triangles) is shown individually as analyzed from plates with 0% D₂O, 70% D₂O or 90% D₂O (green, yellow and red color respectively), e.g., green circles in (A) represent the percentage of cells that attained their first CP after growth in 0% D₂O and division on agar plate with 0% D₂O. The yellow circles in (A) represent the percentage of cells that attained their first CP after growth in 0% D₂O and division on an agar plate with 70% D₂O. The green circles in (B) represent the percentage of cells that attained their first CP after growth in 70% D₂O and division on agar plate with 0% D₂O. Yellow circles in (B) represent the percentage of cells that attained their first CP after growth in 70% D₂O and division on agar plate with 70% D₂O, etc. The percentage of cells that divided during experiments in their respective growth media is represented by a green dotted line for cultures grown in 0% D₂O (A), a yellow dotted line for cultures grown in 70% D₂O (B), and a red dotted line for cultures grown in 90% D₂O (C). The horizontal dashed line represents 50% of cells in the culture and its intersection with individual CP lines gives midpoints of CP attainment.

An increasing D₂O concentration delayed CP attainment for both first and second CPs, ranging from 3.2 h and 8.4 h (first and second CP, respectively) in the 0–0 CP sample to 17.9 h and 25.4 h (first and second CP, respectively) in the 90–90 CP sample (compare Figure 5A,C). Moreover, when the cells were transferred to higher (or lower) concentrations of D₂O upon darkening, the time required for CP attainment became longer (shorter) respectively, for all combinations (compare the positions of the green, yellow and red lines in Figures 5A–C). However, recovery upon transfer to a lower D₂O concentration was limited in the 90–0 CP sample, as the percentage of cells passing both first and second CPs was significantly lower than in the 90–70 and 90–90 CP samples (Figure 5C). Interestingly, only about 40% of the cells were able to divide in both 70% (Figure 5B) and 90% D₂O (Figure 5C), even though they seemed to be committed for division (Figure 5B,C).

While the CP curves in Figure 5 give an overall summary of the D₂O effect on CP attainment, quantification of such effects is summarized in Table 2. Section A (Table 2) quantifies the effect of D₂O on cell cycle progression by comparing CP attainment in the reference 0–0 CP sample and the 70–70 and 90–90 CP samples. Increased D₂O delayed attainment of both CPs in a dose dependent manner, the delay of the first and second CPs

in 70% D₂O represented 115% and 38% increases in time compared with the control, respectively, while in 90% D₂O, the increase was 453% and 196% for first and second CPs, respectively. In Section B (Table 2), the conditions tested differed from their respective reference samples in the D₂O content in the medium used during incubation in the dark, while the D₂O content in each medium used for cultivation in the light was the same. Thus, the specific shift in attainment of the first and second CPs can be attributed mostly to the effect of D₂O on division-related processes. For all combinations tested, the effect of transfer to medium with a different D₂O content was dose dependent, i.e., with transfer to a higher D₂O concentration, the time required to attain both CPs was longer and vice versa. Moreover, the effect of transfer on attainment of both CPs was not the same. In all cases, attainment of the first CP was shifted more (either prolonged or accelerated depending on the nature of the transfer) than the second CP. Interestingly, while the first CP in the 90–0 sample was accelerated by 89% in comparison with the 90–90 sample, the second CP was not reached in the culture (Figure 5C), and therefore the effect of transfer could not be assessed.

Table 2. The effect of D₂O on growth and cell division (section A), or specifically on cell division (section B) in *C. reinhardtii*. The effects are expressed as the proportional (%) change in the time required to attain the first or second commitment points (CP) in comparison with respective reference conditions. Both reference and tested conditions are described as in Table 1, i.e., 0–70 represents sample grown in media with 0% D₂O, which was transferred to the dark to 70% D₂O, etc. NA = effect not analyzed, as the CP was not attained by more than 30% of the cells.

	Reference Conditions	Tested Conditions	Time of 1st CP Midpoint Attainment (% Change)	Time of 2nd CP Midpoint Attainment (% Change)
A	0–0	70–70	+115	+38
		90–90	+453	+196
B	0–0	0–70	+216	+53
		0–90	+259	+45
	70–70	70–0	–120	–41
		70–90	+54	+20
	90–90	90–0	–89	NA
		90–70	–38	–5

3.5. Relative Timeline of Cell Cycle Events

So far, the progression of the cell cycle in deuterated and control cultures has been discussed in terms of individual CP attainments over time. This description is limited by one crucial fact—the cell cycles of the control and deuterated cultures varied significantly in their length. Therefore, information about the relative occurrence of individual cell cycle events within the cell cycle might not be obvious. To overcome this limitation, the timeline showing the occurrence of CP midpoints on the scale of relative cell cycle length was created (Figure 6). This representation allows us to compare the occurrence of such events between individual experimental treatments. For all cultures, both first and second CP attainments were affected by deuterium content during dark incubation in a dose-dependent manner (Figure 6; compare CP attainments within individual growth conditions). The shift in CP midpoint attainment caused by a change in deuterium content during dark incubation was less significant for the second CP than for the first. Furthermore, the timespan between second commitments of cells dividing in 70% D₂O and in 90% D₂O was significantly shorter than the timespan between first commitments of the same cultures.

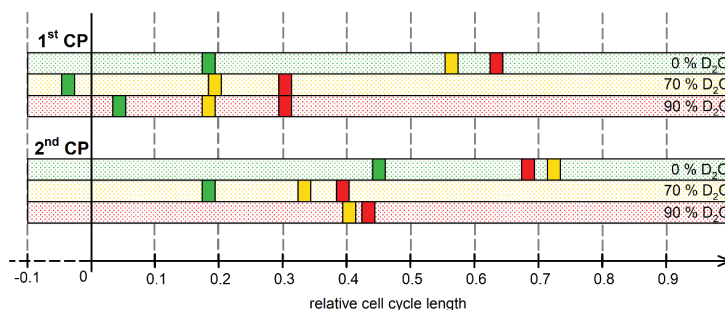


Figure 6. Midpoints of commitment point (CP) attainment in *C. reinhardtii* grown in D₂O represented by relative cell cycle length. The three concentrations of D₂O used for growth in the light are represented by three colored bars for the 1st CP and 2nd CP, respectively. Within each triplet, individual bars represent growth conditions as indicated on the right; green, yellow and red bars are assigned to growth in 0% D₂O, 70% D₂O and 90% D₂O, respectively. Within each of the bars, midpoints of commitment point attainment after transfer to the dark to 0% D₂O (green rectangles), 70% D₂O (yellow rectangles) or 90% D₂O (red rectangles) are displayed, indicating the relative incidence of the event within the cell cycle timeline. The midpoint of the second CP for the culture grown in 90% D₂O and transferred to 0% D₂O was not reached in the culture and therefore it is missing in the diagram (missing green rectangle in lowermost red bar).

4. Discussion

4.1. Growth and Accumulation of Energy-Storing Molecules in Media Containing D₂O

Extensive replacement of protium by deuterium disrupts multiple physiological processes in the cell, including growth and division. The data presented show a decreased growth rate for cultures grown in 70% D₂O or 90% D₂O (Figure 2A–C). Similar findings were reported for *Chlorella* [16,35], *Scenedesmus* [36], *Dunaliella* [37], and *Spirulina* [38], as well as for *Chlamydomonas* itself [39]. Even though most reports show a concentration-dependent relationship between decreased growth rate of the culture and increased deuterium concentration, some studies report a stimulating effect of intermediate D₂O concentrations (usually around 70% D₂O) on observed growth rates [36]. Nevertheless, no such effect was observed in the data presented here. Division in deuterated cultures was accompanied by prolonged time periods (over 10 h) when daughter cells that arose from a single mother cell stayed together in a division cluster. Such structures, palmelloids, i.e., 4 to 16 cells surrounded by a cell wall, are known to originate from cell division without successive degradation of the mother cell wall. Formation of palmelloids is a stress-induced acclimation strategy in *Chlamydomonas* [40]. While growth rate of the cultures generally decreased with increasing D₂O concentration, the opposite was true for cell size (Figure 2D). Enlargement of cells in medium rich in deuterium was previously reported in *Chlorella* [36,41] and *Scenedesmus* [42]. The increased cell size, together with lower cell counts (Figure 2C), suggests a decreased ability of *C. reinhardtii* to perform cell division in media containing 70% D₂O or 90% D₂O (Figures 4 and 5). However, on the basis of these analyses, it is not possible to discriminate between the deuterium effect on cellular growth and cell division (see below).

Apart from an increase in size, growth is characterized by the accumulation of energy-storing molecules. Although *C. reinhardtii* is generally considered a non-oleaginous alga because the carbon flux is primarily directed towards starch [43], lipids can accumulate under specific conditions, e.g., nitrogen or sulfur starvation [44], blockage in starch biosynthesis [45], salinity [46], heat [47] or high light [48] stress. Here, we report increased accumulation of both starch (Figure 3A) and neutral lipids (Figure 3B) in *C. reinhardtii* cultivated in growth medium enriched in deuterium. In a control culture without D₂O,

the starch content per cell reached a maximum at the twelfth hour of the cell cycle, representing 9.5 times the initial value, and then dropped gradually to nearly triple the initial value. The dynamics of cellular starch content reflects cell cycle progression in the control culture. The maximum value at the twelfth hour corresponds to the start of cell division in the culture (Figure 2C). With an increasing fraction of divided cells in the culture (Figure 5A, dotted line), starch was sequentially spent. Since cell division occurred in the light, the starch content did not drop to the initial value. No neutral lipid accumulation was detected in control cultures over the course of the cell cycle (Figure 3B). In both deuterated cultures, the pattern of starch and neutral lipid accumulation was different (Figure 3A,B), even though they arose from the same initial culture as controls and were grown at the same cell density. In 70% D₂O, the cells accumulated about 60% more starch per cell than in the control culture, reaching the peak of starch content about four hours later than in the controls (Figure 3A). This corresponded to the start of cell division in the culture (Figure 5B, dotted line). Nevertheless, the decrease in starch content per cell caused by the proceeding cell division was not as significant as in the control culture, as it was maintained at nearly 60% of the maximal value until the end of the cell cycle (Figure 3A). Similar to the control, this can be partially explained by the fact that division was taking place in the light. Moreover, the proportion of dividing cells in 70% D₂O was only 40% (Figure 5B, dotted line) suggesting that less starch was required for division. In this way, starch was (over)accumulated in such cultures. The neutral lipid content per cell increased, along with starch, in 70% D₂O; however, no decrease that could be attributed to cell cycle progression was apparent (Figure 3B). In 90% D₂O, the starch content per cell reached a plateau at around the 24th hour of the cell cycle (i.e., 12 h later than the control culture) at a value comparable with the starch content in 70% D₂O cultures (Figure 3A). Nevertheless, no decrease in net starch content per cell was observed in this treatment, as cell division in such cultures was spread over a long time, with an average of 40% of cells being able to complete their cell division (Figure 5C, dotted line). Thus, starch production within non-dividing (or already divided) cells could obscure starch consumption in the dividing cells. The (over)accumulation of starch may be a consequence of the greater effect of deuterium on cell division than on cellular growth (for more details see below). The neutral lipid content per cell in 90% D₂O increased gradually to 8 times the initial value (Figure 3B). Notably, the onset of neutral lipid accumulation in both deuterated cultures was detected somewhat later than the onset of starch accumulation (Figure 3A,B). This might be due to the fact that oil synthesis in *Chlamydomonas* occurs only when a carbon supply exceeds the capacity for starch biosynthesis [43]. The same relationship between starch and lipid accumulation was shown for other green algae [49–51]. Deuterated organic compounds such as starch or lipids are highly valued in a wide range of applications [17,52]. They can be produced easily and economically by microalgae using the treatments described here. Understanding the mechanisms governing the (over)accumulation of deuterated starch and lipids can help in optimizing growth conditions for their biotechnological production.

4.2. Cell Cycle Progression in Deuterated Cultures

Even though the effect of deuterium on various organisms has been studied since the 1930s [9,19,53], little is known about the extent to which deuterium affects cellular growth and division individually or in combination. Studies focused on microalgal growth in deuterium are often carried out using asynchronous cultures and growth of the culture is assessed by growth parameters such as dry weight, wet biomass, optical density, etc., which do not distinguish between growth in cell volume versus an increase in cell number [16,36]. Therefore, specific procedures that allow separation of the deuterium effect on cellular growth from its effect on cell division, such as CP assay in combination with transfers between D₂O concentrations deployed in this study, were needed to address this question. The results of the CP assay revealed several consequences of deuteration on cell cycle progression of *Chlamydomonas*. These ranged from straightforward and somewhat

expected effects that could be directly observed on the cultures, to more complex effects that could only be discerned by a specific CP assay. The simple effects include prolongation of the cell cycle and effects on kinase activity of key cell cycle regulators, cyclin-dependent kinases (CDKs) (Figure 4). Prolongation of the cell cycle was evident from the course and timing of cell division in cultures with 0% D₂O, 70% D₂O and 90% D₂O (Figure 5; compare the dotted lines). The length of cell cycle in 70% D₂O was doubled, while in 90% D₂O it was 3.3-fold longer than in the control culture. Prolongation of cell cycle is typical for suboptimal growth conditions, i.e., low light intensity [54] or suboptimal temperature [55], as well as application of antibiotics, such as chloramphenicol [56] or cycloheximide [57]. Moreover, the percentage of cells able to finish their cell division is considerably lower in deuterated cultures (Figure 5, dotted lines) and does not increase with prolonged cultivation (data not shown). The activity of CDKs, measured specifically as histone H1 kinase activity, in all experimental treatments, reached the main peak at the onset of cell division in the culture (Figure 4). The decreased kinase activity in deuterated cultures most probably reflects a lower proportion of cells undergoing cell division (Figure 4; compare the dotted lines in A, B, and C). In the control culture, there was an additional peak in histone H1 kinase activity around the fourth hour of the cell cycle (Figure 4A), i.e., at the midpoint of attaining the second commitment point (Figure 5A; green squares). Kinase activity corresponding to the first CP was not observed as the CP was attained very soon after the start of illumination and sampling intervals were too long to observe such a change (Figures 4A and Figure 5A, green circles). No additional peaks of histone H1 kinase activity were apparent in deuterated cultures, probably because of a combination of two factors: (1) the long time it took for the CP to be attained (Figure 5; compare the slope and total length of green commitment curves in A, yellow commitment curves in B and red commitment curves in C), and (2) the longer sampling intervals. Such results are in agreement with the role of cyclin-dependent kinases in the regulation of the cell cycle [27,58,59].

There were at least three phenomena that were observed specifically in the CP assay: (1) temporal shift of CP attainment, (2) difference in time-shifting of the first and second CP, and (3) the recovery from deuterium treatment. The temporal shift of CP attainment was observed in both deuterated cultures (Figures 5 and 6) and comprised two distinct affects, with markedly different biological interpretations. The CPs in cultures grown in D₂O without subsequent shift upon darkening (Figure 5B, yellow lines, and 5C, red lines) were clearly delayed in time compared to controls. However, when plotting midpoints of CP attainment on the timeline of relative cell cycle length (Figure 6), it became clear that the differences between the midpoints of CP attainments were rather minor (Figure 6, rectangles of the same color as the bar they are placed in). In 0%, 70% and 90% D₂O, the first CP was attained at times corresponding to 18, 19 and 30% of the total cell cycle length, respectively, while the second CP was attained after times corresponding to 45, 33 and 43% of the total cell cycle length, respectively (Figure 6). This reflects the effect of D₂O on both growth- and division-related processes, because both pre-commitment and post-commitment periods were prolonged in deuterated cultures. In the 90% D₂O culture, the pre-commitment period was prolonged by up to 30% of the total cell cycle length (compared to 18% and 19% for the control and 70% D₂O cultures, respectively). This suggests that growth-related processes characteristic for pre-commitment periods were more impaired, especially at the higher D₂O concentration. From this perspective, the effect of deuterium resembled the effect of temperature on cell cycle progression, which also affects both pre- and post-commitment periods by similarly changing metabolic rates of both growth- and division-related processes [60]. Furthermore, this suggests that at 90% D₂O, cellular growth is more affected than cell division, and it is thus more sensitive to the presence of deuterium. The second phenomenon observed in the CP assay was a different response of individual CPs to the treatment. Of the two CPs that were attained in the majority of conditions, the first CP was clearly more delayed than the second (Figures 5 and 6). The midpoints of the first CP in 0, 70 and 90% D₂O were attained after 3, 6.7 and

17.7 h, respectively. The midpoints of the second CP were attained after 8.3, 11.9 and 25.3 h, respectively. Thus, the prolongation of time required to attain the first CP in 70% and 90% D₂O represented an approximately 2-fold and nearly 6-fold increase in comparison with the control, respectively. For the second CP, this increase represented an approximately 1.5-fold and 3-fold increase in comparison with the control culture, respectively. Such a difference in the effect of deuterium on individual commitments might suggest their distinctive role and importance in progression of the cell cycle of *Chlamydomonas*. This is in line with the concept of a single decisive CP, the first one, which changes the physiological status of the cell from one unable to divide in the dark to the one able to do so [23,61,62]. In this concept, the additional CPs are considered to be of lesser importance, as supported by the smaller effect of deuterium on the second CP. Apart from the different growth and cell cycle effects there was also an interesting phenomenon of recovery from deuterium treatment. The recovery potential seemed to decrease both with the deuterium concentration and the time spent in deuterium. The effect was particularly clearly seen on the decreased proportion of committed cells after transfer from 90% D₂O to 0% D₂O (Figure 5C, green lines). Even though in the early phases of the cell cycle, transfer to normal conditions seems to alleviate the stress caused by deuterium, prolonged cultivation in D₂O followed by a shift to normal conditions resulted in a worse performance than in a culture deuterated for the whole cell cycle (Figure 5, compare the green and red lines). While a similar phenomenon of failed re-adaptation to normal conditions was also observed in *Chlorella*, it is probably not a general rule, as other microalgae, such as *Scenedesmus*, do not suffer any detrimental effects upon shift from D₂O to H₂O [53]. What needs to be further examined is whether the duration of exposure to D₂O is the only critical factor determining the fate of cells upon transfer from deuterated to normal medium, as it was established that timing of the application matters significantly for other stress-inducing chemical agents [56,63].

4.3. Deuterium Affects Growth- and Division-Related Processes to Different Extents

The results of the modified CP assay, during which CP samples were transferred to different concentrations of D₂O upon darkening, clearly showed that deuterium affects both growth- and division-related processes (Figures 5 and 6, Table 2). The impairment of cellular growth is reflected by the relative prolongation of pre-commitment period in deuterated cultures (Figure 6). It is worth mentioning that while assessing that CP attainment is de facto dependent on cell division, it is unlikely that this would change the interpretation of the results for cellular growth impairment, as this is also supported by a delay in CDK activity in deuterated cultures (Figure 4). Division is affected by deuterium in a concentration-dependent manner (Figures 5 and 6, Table 2, Section B). The effect of D₂O being limited to cell division can be explained by “reinterpretation” of requirements for cell division after transfer, similarly to the temperature shift situation in *Desmodesmus quadricauda* [60], or by deterioration of processes linked specifically to nuclear or cellular division, e.g., dynamic stability of tubulin [64]. Furthermore, our results suggest that transfer of the cells to a lower D₂O concentration upon darkening can accelerate the apparent attainment of CP and vice versa (Figures 5 and 6). This is surprising, as it is widely accepted that the attainment of CP is set by growth via reaching the critical cell size. However, we see significant differences in both the timing and extent of CP attainment in cultures after transfers to lower deuterium concentrations. This contradicts the universal existence of a sizer that interrelates cell size with cell division, and it seems that cellular growth and the cell cycle simply correlate. Similar observations were made by manipulating the cultivation temperature of *Desmodesmus quadricauda*, where requirements for cell cycle progression were “reinterpreted” after a shift to a different temperature, as manifested by changed kinase activity of CDKs [60]. Further studies are needed to prove if such changes in CDK activity occur after a shift between D₂O concentrations.

5. Conclusions

We analyzed the effect of different concentrations of deuterium on growth and cell division in synchronous cultures of *Chlamydomonas reinhardtii*. We observed a concentration-dependent decrease in growth rates of deuterated cultures, which was accompanied by an increase in the net content of energy storage compounds such as starch and neutral lipids. We separated the deuterium effects on cellular growth and cell division by putting the cultures into the dark at different time-points, followed by a CP assay. The CP assay was further modified so that the cultures were transferred to different deuterium concentrations. This novel methodology allowed us to individually assess the effect of deuterium on cell growth and division. Deuteration was found to prolong the duration of the cell cycle in *Chlamydomonas*, change the pattern of kinase activity prior to cell division, and delay CP attainment (especially attainment of the first CP). Both growth- and division-related processes were negatively affected. These findings contribute to our knowledge of cell cycle regulation and coordination of growth and division. Moreover, an understanding of algal growth in a highly deuterated environment, its limitations and patterns of accumulation of energy storage molecules, might be used commercially to cheaply produce highly deuterated organic compounds.

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Article II.

Characterization of growth and cell cycle events affected by light intensity in the green alga *Parachlorella kessleri*: a new model for cell cycle research

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Article

Characterization of Growth and Cell Cycle Events Affected by Light Intensity in the Green Alga *Parachlorella kessleri*: A New Model for Cell Cycle Research

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Abstract: Multiple fission is a cell cycle variation leading to the production of more than two daughter cells. Here, we used synchronized cultures of the chlorococcal green alga *Parachlorella kessleri* to study its growth and pattern of cell division under varying light intensities. The time courses of DNA replication, nuclear and cellular division, cell size, total RNA, protein content, dry matter and accumulation of starch were observed at incident light intensities of 110, 250 and 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. Furthermore, we studied the effect of deuterated water on *Parachlorella kessleri* growth and division, to mimic the effect of stress. We describe a novel multiple fission cell cycle pattern characterized by multiple rounds of DNA replication leading to cell polyploidization. Once completed, multiple nuclear divisions were performed with each of them, immediately followed by protoplast fission, terminated by the formation of daughter cells. The multiple fission cell cycle was represented by several consecutive doublings of growth parameters, each leading to the start of a reproductive sequence. The number of growth doublings increased with increasing light intensity and led to division into more daughter cells. This study establishes the baseline for cell cycle research at the molecular level as well as for potential biotechnological applications, particularly directed synthesis of (deuterated) starch and/or neutral lipids as carbon and energy reserves.

Keywords: cell cycle pattern; energy reserves; growth processes; light intensity; *Parachlorella kessleri*; reproduction events; deuterium; deuterated starch; deuterated lipid

1. Introduction

Growth of algae is affected by a few main factors: (1) temperature, that affects the kinetics of the entire metabolism, (2) the source of energy and (3) the source of carbon. For autotrophically grown algae in nature, the levels of carbon dioxide, their carbon source, are stable. Algae compensate for a lack of sufficient carbon dioxide in the air by exploiting carbon concentration mechanisms [1,2]. Nevertheless, increasing levels of CO_2 will improve growth rates and thus can be used in algal biotechnology. Light and temperature are the two main factors that fluctuate the most and which affect growth rates of light-grown algae. Light serves as the main energy source; with increasing light intensity, growth rates will increase [3,4] until the light reaches inhibitory levels, whereupon growth is negatively affected. In chlorococcal and volvocan algae, including *Parachlorella kessleri*, increasing light intensity will also induce the formation of larger mother cells and lead to the production of more daughter cells [3,4]. The production of more than two daughter

cells is typical for their cell cycle pattern, multiple fission. Such cell cycles consist of several growth steps [5–9] that are separated in time and do not overlap. Their length is affected by growth rate under any given cultivation condition (intensity of light and temperature, no growth occurs in dark). At the end of each growth step, the cells attain a commitment to trigger a sequence of reproductive events, which, in due time, are terminated even in the absence of energy supply, i.e., in the dark [3,4]. The multiple fission cell cycle is characterized by several (n) rounds of reproductive sequence each consisting of DNA replication, nuclear and cellular divisions, which is completed by formation of 2^n daughter cells (for details, see review [3]). The number of daughter cells formed from a single mother cell (division number) depends on light and temperature. It will increase with increase in light intensity as well as with prolongation of light period [3,4], yet it has certain limits. The cell cycle is composed of a growth period of variable length depending on the growth conditions and a reproductive period with a length set by the duration of one or several rounds of reproductive sequences. The two are coordinated and in optimal conditions usually lead to division into 8 or 16 cells. There are at least two distinct types of multiple fission, a consecutive pattern (Scenedesmus—type cell cycle) and a clustered pattern (Chlamydomonas—type cell cycle) [3,6]. These two multiple fission cell cycle patterns share stepwise growth characteristics but differ in timing of individual processes within the reproductive sequences. DNA replication and nuclear divisions for individual reproductive sequences either happen consecutively, one by one (Scenedesmus type) or happen for all the reproductive sequences in a very short time frame at the end of the cell cycle (Chlamydomonas type). Thus, the main morphological difference between the two is the presence (Scenedesmus type) or absence (Chlamydomonas type) of multinuclear stages within the cell cycle.

The chlorococcal alga *Parachlorella kessleri* (formerly *Chlorella kessleri* [10]), has, within a relatively short time period, become frequently used as a model experimental organism for diverse research activities and biotechnological applications [11–23]. It has particularly attracted interest because, depending on growth conditions, it can produce both starch and neutral lipid [16,24]. Both of these energy reserves can be (over)produced, which makes this alga an interesting organism for algal biotechnology. The primary energy store for the alga is starch. It is produced under optimal growth conditions in complete nutrient medium, at physiological values of light intensities and temperatures [16,25]. Moreover, carbon partitioning is affected by stress conditions leading to starch accumulation. Starch can (over)accumulate under nutrient limitation such as nitrogen or sulfur depletion [18,19,26], high light intensity [27] or in the presence of a high concentration of CO₂ [28]. Neutral lipid, the secondary energy reserve, under optimal growth conditions, can be kept relatively low at 1–10% of dry matter (DM) [16,24,25]. It can also (over)accumulate under stress conditions similar to these inducing starch accumulation such as nitrogen, sulfur or phosphorus depletion [18,20,24,26,29], dilution of all the nutrients in medium [19,24] or salt stress [30]. The neutral lipids tend to accumulate later than the peak of starch accumulation [18,24,26]. One of the stress conditions known to induce neutral lipid (over)accumulation is growth in deuterated water [31]. Deuterium, the stable isotope of hydrogen is known to have the highest kinetic effect among the stable isotopes of biogenic elements [31–33]. It affects the entire metabolism and its specific effects range from disrupting signaling [34] and energy producing production in mitochondria and chloroplasts [35,36] to disrupting cell division [37,38]. The treatment with deuterated water is quite expensive and artificial as such high concentrations of deuterated water are never present in nature. Yet, it can be justified for production of fine (bio)chemicals with very high added value. Algae-derived deuterated biomolecules such as carotenoids, lipids, and starch, are of interest both as analytical standards and for metabolic labeling [31,39,40].

Although extensive knowledge has accumulated on starch and neutral lipid production under different conditions, basic information on *P. kessleri* growth under non-limiting growth conditions is missing. It is unclear how growth is affected by external growth conditions such as irradiance and temperature and how its cell cycle is organized. This

obviously hinders further exploitation of this alga for biotechnology. The current work provides baseline information on the effect of different light intensities on growth (RNA, protein, dry matter, cell volume), the reproductive steps of the cell cycle (DNA replication, nuclear division, protoplast fission, formation and liberation of daughter cells), and accumulation of energy and carbon reserves (starch). We aimed to establish the division pattern of *Parachlorella kessleri* as well as the effect of light on progression of the cell cycle. Furthermore, we tested the effect of deuterated water as a specific stressor on both growth and cell cycle progression in *P. kessleri*. Such information can serve as a starting point for further optimization of the alga for biotechnology purposes as well as its exploitation as a novel model in basic research.

2. Materials and Methods

2.1. Organism and Culture

The green unicellular microalga *Parachlorella kessleri* (Trebouxiophyceae, Chlorophyta), (strain CCALA 255), was obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany, Czech Academy of Sciences in Třeboň, Czech Republic (CCALA; <http://www.butbn.cas.cz/ccala/index.php>, accessed on 7 June 2021).

For routine sub-culturing, the cultures were streaked every three weeks onto nutrient medium (see below) solidified by agar (1.5%) and grown on a light shelf at an incident light intensity of $100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ of photosynthetically active radiation.

For experiments, laboratory cultivation units consisted of glass cylinders (inner diameter 36 mm, height 500 mm, volume of suspension 300 mL), or glass vessels, which were flat and rectangular in shape (inner dimensions $400 \times 300 \times 20 \text{ mm}^3$, volume of suspension 2500 mL) (Figure 1). Culture units were placed in a thermostatic water bath ($30 \pm 0.5 \text{ }^\circ\text{C}$) and continuously illuminated by a panel of dimmable fluorescent lamps (DULUX L55W/950 Daylight, OSRAM, Munich, Germany) with maximum incident light intensity of $750 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. They were vigorously mixed with air bubbles containing 2% carbon dioxide (*v/v*) dispersed through a 200 μL micropipette tip fixed to the glass tube placed at the bottom of the cylinder. The aeration in flat vessels was done by bubble stream from a perforated stainless steel tube located at the bottom of the vessel. The flow-rate of the aeration mix was 60 L/h.

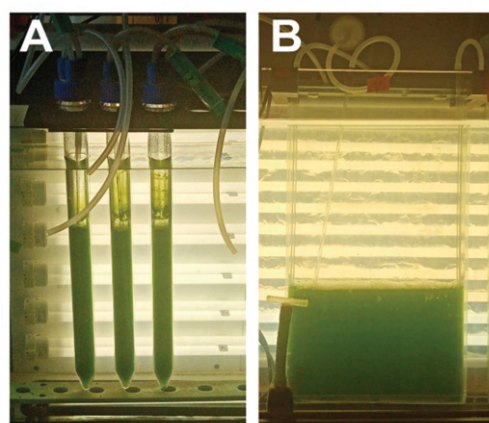


Figure 1. Laboratory photo-bioreactors used for the experiments. (A) glass cylinders (300 mL), (B) plane-parallel cuvettes (2500 mL).

2.2. Mineral Nutrient Medium

The mineral medium was based on the mean content of P, N, K, Mg, and S in algal biomass [41] and had the following initial composition (in mg/L): 2020 KNO₃, 237 KH₂PO₄, 204 MgSO₄·7H₂O, 40 C₁₀H₁₂O₈N₂NaFe, 88 CaCl₂, 0.83 H₃BO₃, 0.95 CuSO₄·5H₂O, 3.3 MnCl₂·4H₂O, 0.17 (NH₄)₆Mo₇O₂₄·4H₂O, 2.7 ZnSO₄·7H₂O, 0.6 CoSO₄·7H₂O, and 0.014 NH₄VO₃ in distilled water [27]. For preparation of medium, 100x concentrated stock solutions of macro-elements and microelements were used. All components were diluted in distilled water and autoclaved for 30 min at 121 °C. The pH was adjusted to 7 with 1 M NaOH.

2.3. Synchronization of Cultures

The cultures were grown under optimal conditions: incident light intensity 500 μmol photons m⁻²s⁻¹, temperature 30 °C, 2% CO₂ in aeration mixture. To set up the synchronization regime, the culture was grown in continuous light until the cell concentration reached approximately 1 × 10⁶ cells mL⁻¹. Next, the culture was put into dark to allow division of all the cells that were capable to do so. Once the cells within the culture were divided, the cultures were put on light and carefully observed by light microscopy for two or three cycles to set the correct length of both the light and dark periods under given conditions. The lengths of light and dark periods were chosen according to the growth parameters of the cells. The time for darkening the cells was when about 10% of cells started their first protoplast fission. The length of the dark period was chosen to allow all cells of the population to release their daughter cells and then the durations of the light and dark periods were kept constant. With the number of light/dark cycles, the synchrony of the cells increased until it was possible to set up the light/dark regime to be used for synchronization. During the synchronization procedure, the cell density was kept below 1 × 10⁶ cells mL⁻¹ by dilution at the end of dark period to prevent cell shading. By this treatment, the synchronization was set to 18 h of light followed by 7 h of dark. This light/dark regime was used throughout the experiments such that the cells were inoculated into the growth media and grown at this light/dark regime until the cell density reached 1 × 10⁶ cells mL⁻¹ whereupon it was kept at this cell density by dilution at the end of dark period until the volume of the culture required for the start of the experiment was reached. The synchronized daughter cells were again diluted to the initial cell density approximately 1 × 10⁶ cells mL⁻¹ and used as inocula for experimental cultures.

2.4. Measurement of Light Intensity

A quantum/radiometer-photometer (LI-COR, Inc., Lincoln, NE, USA) was used. In the culture unit, dimmable fluorescent tubes were used for adjustment of irradiance. To obtain a measure of light energy absorbed by a layer of cell suspension grown at different incident light intensities (I_i) and different optical densities (concentrations of cells), the mean light intensity (I_m) was calculated according to the Lambert-Beer formula: $I_m = (I_i - I_t) / \ln(I_i / I_t)$, where I_i is the incident light intensity measured at the surface of the culture vessel, and I_t is the transmitted light intensity measured at the rear side of the culture vessel.

2.5. Assessment of Nuclear and Cell Division Curves

The proportion of mother cells, and daughter cells was determined by light microscopy in cells fixed in Lugol solution (1 g I, 5 g KI, 100 mL H₂O) at a final concentration 10 μL of Lugol solution per 1 mL of cell suspension.

2.6. Dry Matter Determination

Biomass was separated from the medium by sequential centrifugation of 4 mL of the cell suspension in pre-weighed microtubes at 3000 × g for 5 min; the sediment was dried at 105 °C for 12 h and weighed on an analytical balance (TE214S-OCE, Sartorius, Goettingen, Germany) [27].

2.7. Cell Volume and Number

Cell volume and concentration were measured using a Beckman Coulter Multisizer 4 (Beckman Coulter Life Sciences, Brea, CA, USA) by diluting 50 μ L of fixed (0.2% glutaraldehyde) cell suspension into 10 mL of 0.9% NaCl (*w/v*) electrolyte solution.

2.8. Nuclei and Cell Wall Staining

Nuclei were stained with SYBR Green and observed through a fluorescent microscope using the method described by Vítová, et al. [42], for details see also Hlavová, et al. [43]. Five microliters of freshly defrosted cell pellet were combined with 2.5 μ L of SYBR Green I (cat. no. S7563, ThermoFisher, Waltham, MA, USA) and 2.5 μ L of Fluorescent Brightener 28 (cat. no. 910090, equivalent to Calcofluor, Sigma-Aldrich, St. Luis, MO, USA), vortexed and kept for 5–10 min in the dark at room temperature. Next, the cells were observed using a LSM Zeiss 880 confocal microscope (Carl Zeiss, Jena, Germany) using the following excitation wavelength: chlorophyll—633 nm, Fluorescent Brightener 28—488 nm and SYBR Green I—405 nm.

2.9. Quantum Yield Measurement

Aliquots of 2 mL were withdrawn from the culture and placed into 10 $\times$ 10 mm² plastic cuvettes for 30 min in the dark. Quantum yield was measured using an AquaPen-C AP-C 100 (Photon Systems Instruments, Drasov, Czech Republic).

2.10. Measurement of Neutral Lipid Content

Neutral lipid content was measured spectrophotometrically in a microplate format following the modified procedure of Takeshita, et al. [44]. Aliquots (100 μ L) of the cultures were transferred to a 96-well plate and 5 μ L of freshly prepared Nile Red dye (0.5 mg/mL in DMSO, catalog no. 72485, Sigma-Aldrich, Prague, Czech Republic) were added to each well. The same amount of Nile Red dye was added to a sample blank consisting of 100 μ L of H₂O. The plate was incubated at room temperature for 15 min. Fluorescence was measured using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) equipped with a 485 nm excitation filter and a 595 nm emission filter. Fluorescence intensity of the samples was normalized using fluorescence intensity of unstained samples and a blank. Standard curves produced from a commercial lipid standard, triolein (catalog no. Y0001113, Sigma-Aldrich, St. Luis, MO, USA) were used to quantify neutral lipids.

2.11. Estimation of Bulk RNA, DNA and Protein

2.11.1. Total Nucleic Acids

The procedure of Wanka [45] as modified by Lukavský, et al. [46] was used for the extraction of the total nucleic acids. The samples were centrifuged in 10 mL centrifuge tubes, which also served for storage of the samples. The pellet of algal cells was stored under one mL of ethanol at -20°C . The pellet was extracted 5 times with 0.2 M perchloric acid in 50% ethanol for 50 min at 20°C and 3 times with an ethanol-ether mixture (3:1) at 70°C for 10 min. Such pre-extracted samples can be stored in ethanol at -20°C . Total nucleic acids were extracted and hydrolyzed by 0.5 M perchloric acid at 60°C for 5 h. After hydrolysis, concentrated perchloric acid was added to achieve a final concentration of 1 M perchloric acid in the sample. Absorbance of total nucleic acids in the supernatant was read at 260 nm (A_{260}) and total nucleic acid concentration was calculated based on the calibration with DNA standard of the known concentration treated by the same procedure.

2.11.2. DNA and RNA Determination

The light activated reaction of diphenylamine with hydrolyzed DNA, as described by Decallonne and Weyns [47] was used with the following modification of Zachleder [48]: the diphenylamine reagent (4% diphenylamine in glacial acetic acid, *w/v*) was mixed with the samples of the total nucleic acid extracts in a ratio of 1:1 and the mixture in the test tubes was illuminated from two sides with fluorescent lamps (L 40W F33, Tungsram, Budapest,

Hungary). The incident radiation from each side was $150 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. After 6 h of illumination at 40°C , the difference between the A_{600} and A_{700} nm was estimated. The concentrations of DNA within the samples were set by comparison to the A_{600} and A_{700} nm values of the sample with known DNA concentration treated the same way. The values were normalized to the number of cells extracted. The RNA content was calculated as the difference between the total nucleic acid and DNA content.

2.11.3. Protein Determination

The sediment remaining after nucleic acid extraction was used for protein determination. It was hydrolyzed by 1 M NaOH for 1 h at 70°C . The protein concentration in the supernatant after centrifugation of the hydrolysate (15 min, $5300\times g$, room temperature) was estimated by BCA assay (cat. no. 23225, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's conditions. The same procedure was carried out with a calibration curve set by different concentrations of bovine serum albumin.

2.12. Starch Analyses

Cell pellets containing approximately 2×10^6 cells mL^{-1} were harvested during the cell cycle, washed with SCE buffer (100 mM sodium citrate, 2.7 mM EDTA- Na_2 , pH 7 (citric acid)), snap frozen in liquid nitrogen and stored at -20°C . After thawing the cells, the pellets were disintegrated by adding 300 μL of zirconium beads (0.7 mm in diameter) and vigorously vortexing for 15 min (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA). Depigmentation of the samples was done by adding 1 mL of 80% (v/v) ethanol to the pellet and incubating in a water bath (SW22, Julabo, Seelbach, Germany) for 15 min at 68°C , after which the samples were centrifuged for 2 min at $14,000\times g$ and the supernatant was removed. The depigmentation procedure was repeated 3 to 4 times (or until the pellet was completely discolored).

After that 1 mL of α -amylase from porcine pancreas (Sigma-Aldrich, St. Luis, MO, USA), (0.5 mgL^{-1} (w/v) in 0.1 M sodium phosphate buffer (pH 6.9)) was added to the samples and they were incubated for 1 h at 37°C (FTC 90i, VELP Scientifica, Usmate Velate, MB, Italy). The samples were centrifuged for 2 min at $14,000\times g$, after which the supernatant was used for the quantification of reducing sugars through the DNSA color reaction as described in [49]. In short, 500 μL of supernatant were mixed with 500 μL dinitrosalicylic acid (DNSA) solution (1% (w/v) 3,5- DNSA, 30% (w/v) potassium sodium tartrate tetrahydrate, 20% (v/v) 2M sodium hydroxide) and incubated for 5 min at 105°C on a heat block. Following a cooling period of 10 min at room temperature the mixture was diluted five-fold with distilled water, after which the A_{570} of the samples were measured. The concentration of starch was estimated through a calibration curve of potato starch (Lach-Ner, Neratovice, Czech Republic) digested with α -amylase.

2.13. Statistical Analysis

Experiments were performed in at least three biological replicates. If not stated otherwise, all results are presented as means and standard deviation ($n = 3$).

3. Results

3.1. Light Characteristics

Light intensity and cell growth are interconnected so that increasing cell size and cell numbers will decrease the light availability to the cells and hence affect growth. To establish the relationship between light intensity and dry matter yield, cultures were illuminated at the following incident light intensities: 500, 250, 110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Figure 2). However, the incident light intensity only represents the light at the surface of the bioreactor, which is absorbed by the first layer of cells in the suspension. Instead, mean light intensity provides a biologically meaningful value of light conditions in the cultures. It is calculated from incident and transmitted light intensities (see Methods) and reflects the effect of dry matter density. The dry matter density is a function of cell concentration and

cell size. To avoid the effects of cell density, the same initial biomass density (dry matter, cell size and cell number) was applied at the beginning of the experiments (0 h of the cell cycles) at a given incident light intensity. The initial concentration of dry matter at the beginning of the cell cycle was $100 \mu\text{g mL}^{-1}$ (Figure 2, horizontal axis, vertical dashed line). The mean light intensities, relative to the incident light intensities of 500, 250, 110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, were 360, 180, and 80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ respectively. With ongoing culture growth, dry matter increased until the final values attained at the end of the cell cycles were 1600, 1300 and 400 $\mu\text{g mL}^{-1}$ respectively. This caused an exponential decrease in the mean light intensity so that at the end of cell cycles it had dropped to 160, 85, 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ respectively (Figure 2).

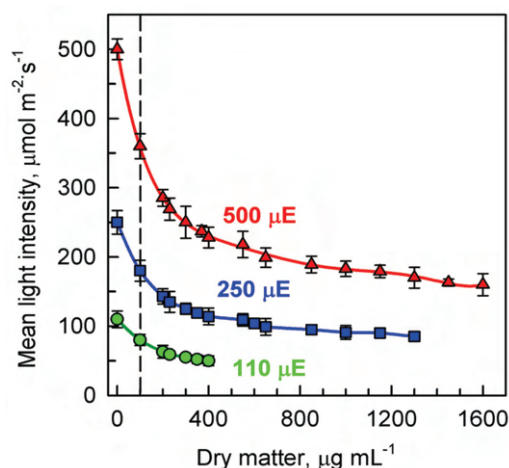


Figure 2. Changes in mean light intensity with increasing dry matter during the cell cycle of synchronous cultures of *Parachlorella kessleri* grown at different incident light intensities of 500, 250, 110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (μE) at a temperature of 30°C . Culture growth was started at the same initial dry matter ($100 \mu\text{g mL}^{-1}$), indicated by the vertical dashed line.

3.2. Time Course of Cell Cycle Characteristics

Daughter cell cultures used as the initial inocula were obtained by synchronization under optimal growth conditions (see Material and Methods). The morphological changes during the cell cycle are shown on the example of synchronized daughter cells grown at the incident light irradiance of $250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Figure 3). Daughter cell cultures consisted of small cells (mean cell volume $25 \mu\text{m}^3$) (Figure 3A, 0 h) with a single tiny nucleus (Figure 4A, 0 h). The cultures at the beginning of the cell cycle sometimes contained both small triangular shaped released daughter cells and divided daughter cells still present in division clusters (Figure 3A). It is common that some of the daughter cells stay connected in the division clusters in dark and immediately after being put to light, but they split into individual daughter cells within 1–2 h of cultivation in light. Other cultures were formed exclusively by individual split cells from the beginning of the experiment (Figure 4A). Neither of these conditions affect the progression through the cell cycle. The cell size increased from the beginning of the cell cycle (Figure 3A, 0 h) to about 14 h when the first protoplast fission into two daughter cells started (Figure 3D, 14 h). Thereafter, within two hours, protoplast fission into four (Figure 3E, 15 h) and then into final eight daughters occurred (Figure 3F, 16 h). The daughter cells formed their own cell walls inside the mother cells (Figure 4D). The mother cell wall was then broken and the daughter

cells were liberated. The cells transferred into dark during protoplast fission formed and liberated the daughter cells as in the light but were not growing any more.

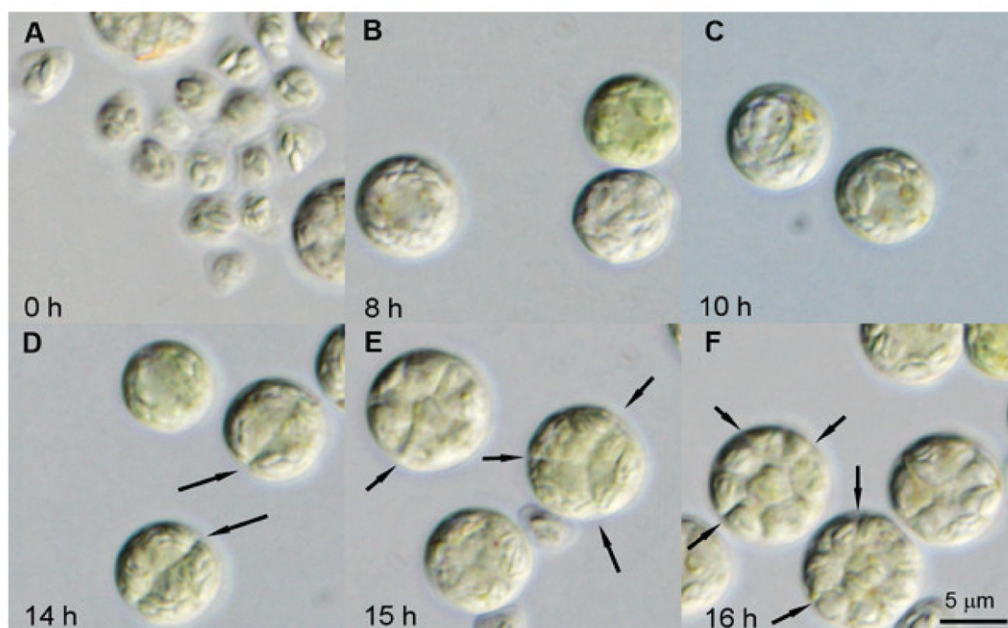


Figure 3. Course of cell cycle progression in synchronized cells of *Parachlorella kessleri* grown at a light intensity of $250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a temperature of 30°C . Light photo-micrographs of daughter cells at 0 (A), 8 h (B) and 10 h (C) and mother cell dividing their protoplast into 2 ((D)—14 h), 4 ((E)—15 h), and into 8 ((F)—16 h). Division furrows are marked by arrows. Scale = $5 \mu\text{m}$.

The nuclear staining showed there was no nuclear division until about 14 h but nuclei increased in size (Figure 4A–C). An increasing size of nuclei indicated an increase in nuclear DNA content (for more details see below). The enlarged, polyploid nuclei underwent multiple consecutive divisions at the very end of the cell cycle. Each of the mitoses was concurrently or immediately followed by chloroplast and protoplast fissions (compare Figure 3D–F and Figure 4C–H).

3.3. Effect of Light Intensities

During cultivation at different light intensities the course of reproductive events was followed, i.e., DNA replication, nuclear division, protoplast fission and daughter cell formation at different light intensities. This was supplemented by assessing different growth parameters such as accumulation of bulk RNA and protein and increased cell size, as well as changes in the level of starch as the primary energy and carbon reserve.

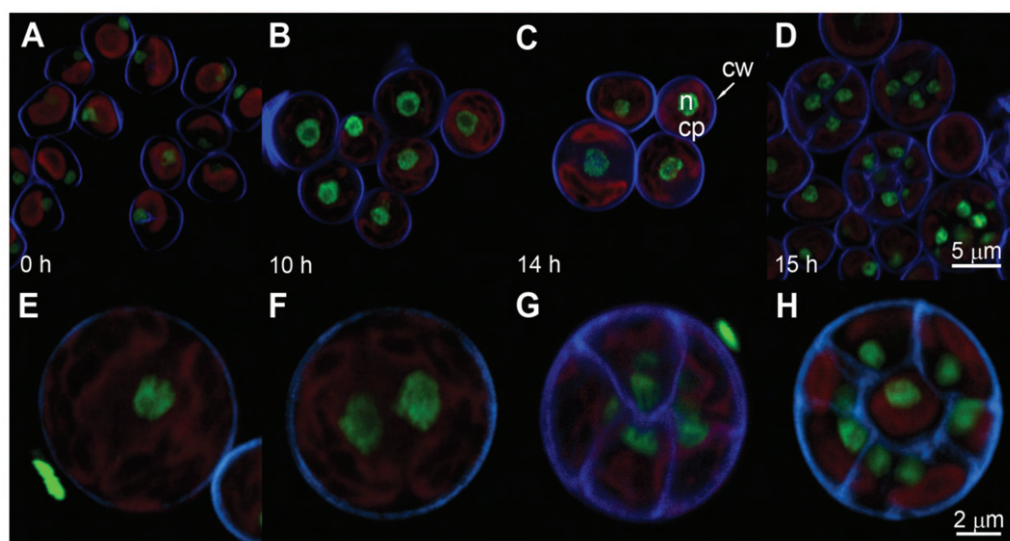


Figure 4. Fluorescent photo-micrographs of synchronized cells of *Parachlorella kessleri* grown in light at $250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a temperature of 30°C at 0 h (A), 10 h (B), 14 h (C) and 15 h (D) of the cell cycle. Magnified cells show dividing nuclei, chloroplasts and protoplasts into 2 (E,F), 8 (G) and 16 (H) daughter cells. To visualize nuclei, the cells were stained by fluorescent dye SYBR Green I dye, the cell walls were stained by calcofluor (Fluorescent Brightener 28) dye. Green stained nucleus, n; red autofluorescence of chloroplast, cp; blue stained cell wall, cw and arrow. Scale is $5 \mu\text{m}$ for (A–D), and $2 \mu\text{m}$ for (E–H).

3.3.1. Sequence of Reproductive Events

The synchronized culture at the beginning of the cell cycle consisted of a single small nucleus (Figure 4A) containing 0.1 pg of DNA (Figure 5A). The sequence of several DNA-duplications started after about 10 h of growth. Individual duplications of DNA (DNA replication rounds) were separated by about a 2-h period without any DNA synthesis and the number of duplications per cell increased with light irradiance applied (Figure 5). At the highest incident light intensity ($500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) four rounds of DNA replication occurred, leading to a 16-fold increase in the DNA content of the daughter cell (from 0.1 pg cell^{-1} to 1.6 pg cell^{-1}) (Figure 5A, triangles). At a medium incident light intensity ($250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$), a 16-fold increase was attained only by about half of the cell population and only three doublings of DNA were performed in the second half of population, as shown by a 12-fold increase in DNA content (Figure 5A, squares). Slow growth at the lowest incident light intensity used ($110 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) enabled only 2 doublings of DNA content and the cells contained only 4-fold higher DNA content compared to their initial values. With decreasing light intensity, the start of consecutive DNA replication rounds was delayed for 2 h at 250 and 4 h at $110 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ when compared to DNA replication in the highest replication light intensity cultures (Figure 5A). Because nuclear division did not follow immediately after DNA, the size of nuclei increased proportionally to the increasing content of DNA, becoming consecutively di-, tetra-, octu- or hexadecaploid but the cells remained uni-nuclear (Figures 4 and 5).

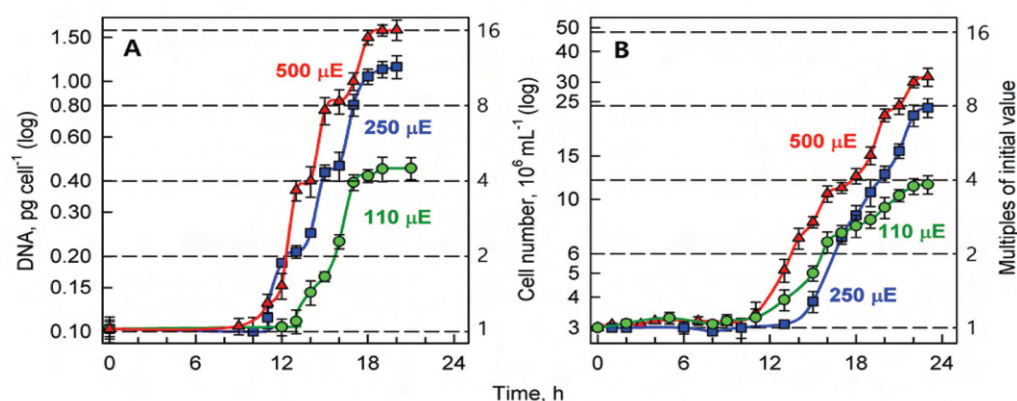


Figure 5. The course of reproductive events in synchronized cultures of *Parachlorella kessleri* at the optimal temperature of 30 °C and different incident light intensities (500, 250, 110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) (red triangles, blue squares, green circles respectively). Panel (A): DNA (pg cell^{-1}), Panel (B): cell number (10^6 mL^{-1}). Horizontal dashed lines indicate doublings of the initial values at the beginning of the cell cycle.

Shortly after all DNA replications were completed, the number of nuclear divisions occurred, corresponding to the preceding number of rounds of DNA replication. This was concomitant with chloroplast division and was immediately followed by protoplast fissions, as only bi-nuclear but no poly-nuclear cells were observed (Figure 4D–H). After completing multiple protoplast fissions, daughter cells were formed and released (Figures 4D and 5B).

In general, light intensity was, at a given temperature, a decisive factor affecting the number of rounds of DNA replication (doublings of DNA content), which were performed during the cell cycle. The number of DNA replication rounds then determined the number of nuclear divisions, the number of protoplast fissions and the number of daughter cells released, corresponding to 2^n , where n is the number of doublings. In *P. kessleri*, at a given temperature, n varies from 1 to 4, and so from 2 to 16 daughter cells could be released at the end of the cell cycle. The number n increased with light intensity. In the experimental conditions tested, the lowest number of daughter cells was 4 but a lower light intensity would be required for the formation of fewer daughter cells.

3.3.2. Sequence of Growth Event

To assess the growth of the cultures, three growth parameters were monitored: bulk RNA content, dry matter and cell volume. All of the growth parameters were the highest at the highest light intensity of 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Figure 6) and decreased both in values and rates with decreasing light intensity, from 250 to 110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Figure 6). The growth events increased in several steps, each of which corresponded to approximately a doubling of the initial/preceding values (indicated by horizontal dashed lines in Figure 6), and led to a 2- to 16-fold increase in the initial values (Figure 6). The growth steps were separated by intervals without growth (4–6 h), as is particularly well documented for an increase in RNA content (Figure 6A). With increasing growth rate, the intervals without growth shortened and, in fast growing cells, sometimes became undistinguishable (Figure 6B, see the first doubling at 500 and 250 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$).

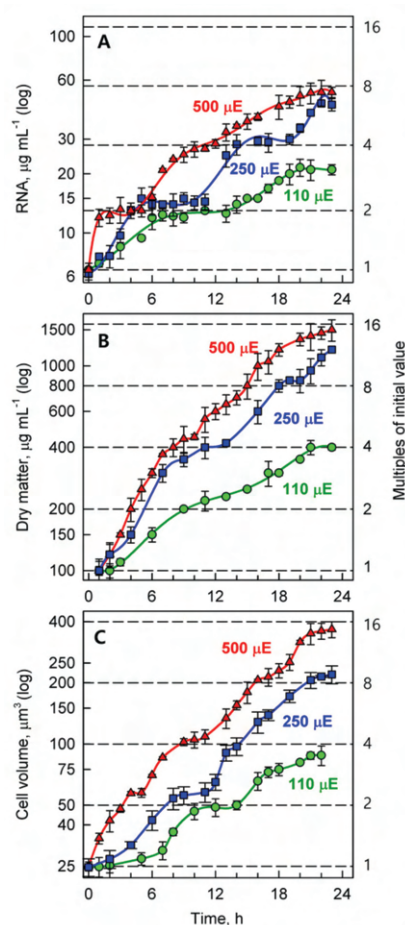


Figure 6. Time course of growth events in the synchronized cultures of *Parachlorella kessleri* grown at the optimal temperature of 30 °C and different incident light intensities (500 (red triangles), 250 (blue squares), 110 (green circles) $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Panel (A): RNA ($\mu\text{g mL}^{-1}$), Panel (B): Dry matter ($\mu\text{g mL}^{-1}$), Panel (C) Cell volume (μm^3). Note the logarithmic scale on the Y axis. Horizontal dashed lines indicate doublings of the initial values.

Only two doublings (4-fold increase) occurred in all growth characteristics at the lowest light intensity (110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) (Figure 6). With increasing light intensity, three doublings of growth parameters were attained at 250 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. At a light intensity of 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, there occurred four doublings in cell volume and dry matter (Figure 6B,C) but only three doublings of RNA content (Figure 6A).

3.3.3. Energy Reserves

The synthesis of starch was affected similarly to cell growth parameters; its content increased with increasing light intensity. At low incident light intensity (110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) the net content of starch only doubled, starting to increase not earlier than in the

second half of the cell cycle (6 h), and after cell division (12 h), its level did not increase further; the starch content remained constant (Figure 7).

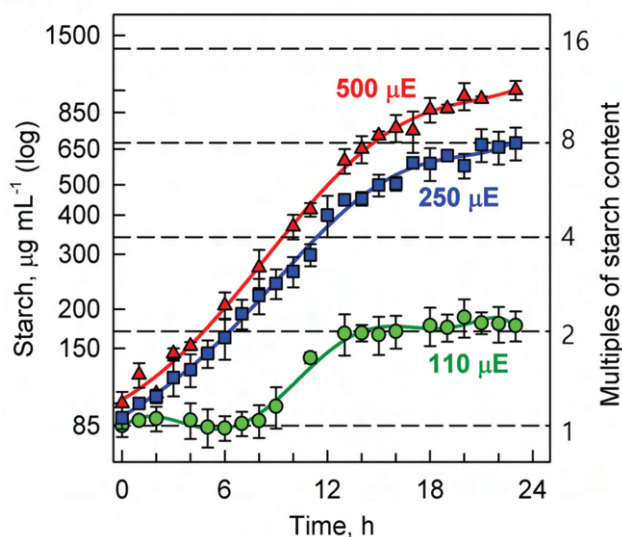


Figure 7. Time course of starch accumulation in synchronized cultures of *Parachlorella kessleri* at the optimal temperature of 30 °C and different incident light intensities (500, 250, 110 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) (red triangles, blue squares, green circles respectively). Horizontal dashed lines indicate doublings of starch content compared with the beginning of the cell cycle.

At higher light intensity, the net starch accumulation started from the very beginning of the cell cycle and continued with increasing rate until cell division (12 h). Then it slowed but did not stop. At the end of the cell cycle, the cells contained 8-fold higher levels of starch at the incident light intensity of 250 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and about 10-fold higher at 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ compared to the initial values (Figure 7).

3.4. Cell Division and Growth at Different Light Intensities in Deuterated Water

As shown in the previous experiments, increasing light intensity caused a rise in both growth rates and the number of daughter cells, suggesting a distinct beneficial effect. Deuterated water has been known to negatively affect growth of all organisms [31,32] by increasing the level of cellular stress. To study its effect on *P. kessleri*, nutrient medium containing increasing concentrations of deuterated water (0, 70, and 99%) was used; this was combined with different incident light intensities (150, 200, 300 and 400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). We analyzed how their combination affected cell division, growth, and the physiological state of the culture (Figure 8).

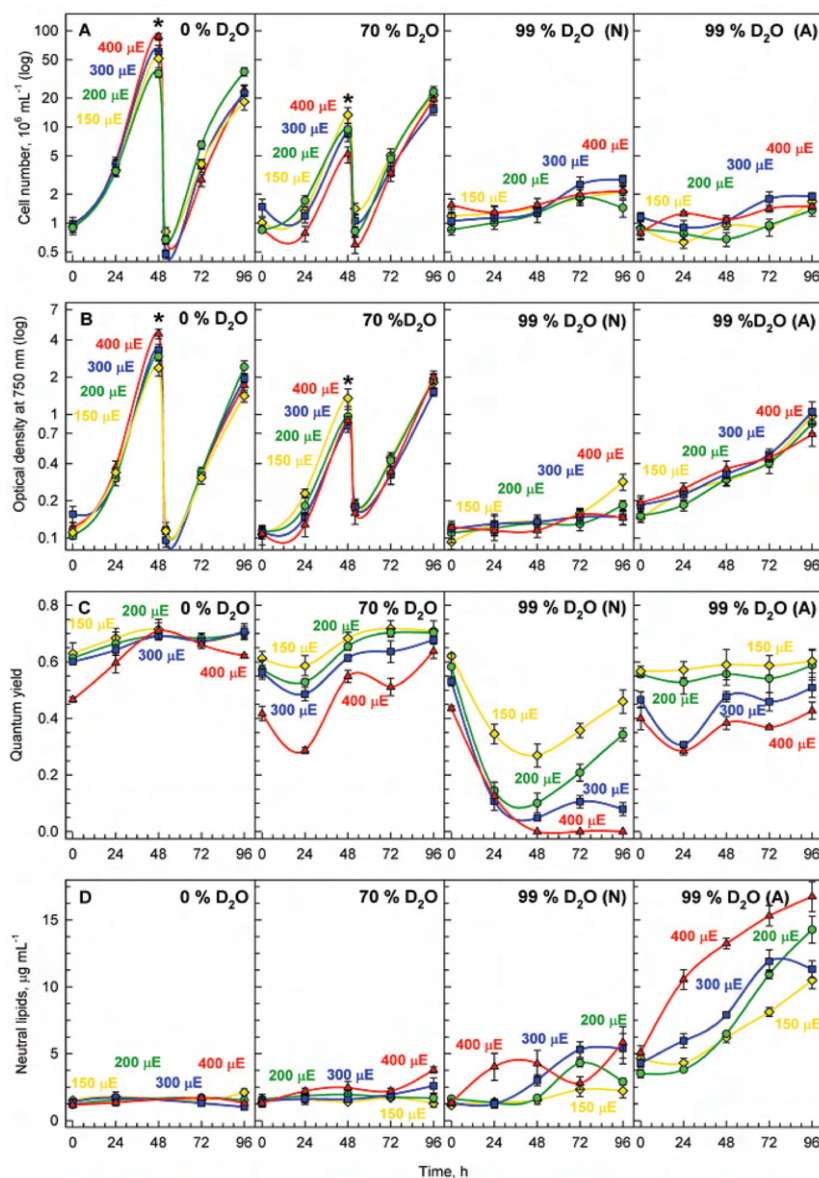


Figure 8. Time course of cell division and growth in synchronized cultures of *Parachlorella kessleri* grown at the optimal temperature of 30 °C, different incident light intensities (400 (red triangles), 300 (blue squares), 200 (green circles), and 150 (yellow diamonds) $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) and different concentrations of deuterated water in the medium (0, 70 and 99%). The cultures transferred directly from control conditions into 99% deuterated water are denoted as “N”, the cultures pre-adapted at the same concentration for one week are denoted as “A”. Panel (A): cell number (10^6 mL^{-1}), Panel (B): Optical density at 750 nm, Panel (C): quantum yield, Panel (D): neutral lipids ($\mu\text{g mL}^{-1}$). Note the logarithmic scale on Y axis for A and B.

3.4.1. Cell Division

In the control culture without any deuterated water in the medium, the cells divided approximately every 24 h and the average number of newly formed daughter cells (division number) increased with increasing incident light intensity from less than 6 to more than 9 daughter cells formed (Figure 8A, Table 1). The cell division number was not affected by initial cell concentration up to approximately 4×10^6 cells mL⁻¹. This corresponds approximately to the cell density of 3×10^6 cells mL⁻¹ used in the experiments above (Figures 2–7). To avoid the decrease in mean light intensity due to cell shading, the cultures were periodically diluted to a concentration of 1×10^6 cells mL⁻¹ once they reached optical density at 750 nm greater than 1. By this sequential dilution approach, we were able to follow four consecutive cell cycles in the control cultures, about three consecutive cell cycles in 70% cultures and about one cell cycle for the 99% cultures (Figure 8A). In the individual cell cycles, there was negligible difference in number of formed daughter cells as is clear from the comparison among the number of daughter cells formed in the first 24 h (and again between 48 and 72 h) and between 24 and 48 h (and again between 72 and 96 h). This also suggested that continuous light did not significantly affect the division patterns. In the presence of 70% *v/v* deuterated water in the medium (70% culture), the division number decreased to approximately 4 under all light intensities (Figure 8A, Table 1). This represented a decrease compared to the control culture in all variants. The decrease was more pronounced with increasing light intensities (Figure 8A, Table 1). Cultivation in 99% *v/v* deuterated water (99% culture) further decreased the division number to below 2 (Figure 8A, Table 1). Similarly to the 70% culture, reduction in the division number was more pronounced with increasing light intensities. To determine whether adaptation of cells to high concentrations of deuterated water could improve the cells reaction, the cultures were pre-adapted to 99% *v/v* deuterated water in the medium prior to the experiment. Such pre-adapted cultures showed similar reductions in division number as the non-adapted culture but the doubling times were shorter compared to the non-adapted culture (Table 1). However, the culture was prone to high death rates at higher light intensities, probably due to extensive oxidative stress.

3.4.2. Cell Growth

Due to the inhibitive high price of deuterated water, cell growth was assessed as changes in optical density at 750 nm, i.e., outside of the main chlorophyll absorbance peaks and corresponding mostly to light scattering (Figure 8B). In the control culture, biomass multiplied more with increasing light intensity and increasing growth rate although it multiplied less than cell number (Figure 8A,B, compare Tables 1 and 2). The mass doubling time shortened with increasing light intensity but the correlation between the two was less pronounced than for cell doubling time. In the 70% culture, mass multiplication was reduced compared to the controls but the reduction was lower than that of the division number (Figure 8A,B, compare Tables 1 and 2). The mass doubling times were comparable for all light intensities and with the exception of the highest light intensity, were higher than the cell number doubling times (Figure 8, compare Tables 1 and 2). In the 99% culture, mass multiplication factor was significantly affected compared to the control cultures and the reduction was clearer with increasing light intensity (Figure 8, Table 2). With the exception of the lowest light intensity, mass multiplication factor was comparable to the corresponding changes in division number (compare Tables 1 and 2). The mass doubling time was reduced to about 10% (light intensity 150 and 200 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) or about 5% (light intensity 300 and 400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) (Table 2). The reduction was thus similar to the reduction in cell division doubling time (compare Tables 1 and 2). In the pre-adapted culture, the reduction in mass multiplication factor followed similar trends as that in the non-adapted culture but the reduction was lower (Figure 8B, Table 2). Furthermore, the reduction was lower than that of division number (compare Tables 1 and 2).

Table 1. Comparison of division number and cell number doubling times for cultures grown in medium containing different concentrations of deuterated water at different light intensities. The average of at least three experiments is shown. Asterisks (*) denote cultures pre-adapted for one week to a given concentration of deuterated water, NA, the calculations are not reliable due to cell death and lysis.

Concentration of Deuterated Water (%)	Light Intensity ($\mu\text{mol Photons m}^{-2}\text{s}^{-1}$)	Division Number	% of Control	Doubling Time	Fold Retardation of Control
0	150	6.96	100	9.98	1
	200	7.44	100	9.05	1
	300	8.27	100	8.79	1
	400	9.35	100	7.98	1
70	150	4.77	68.53	22.80	2.28
	200	4.54	61.00	13.35	1.48
	300	3.92	47.45	11.17	1.27
	400	4.76	50.85	9.28	1.16
99	150	1.33	19.07	117.32	11.75
	200	1.38	18.49	86.27	9.53
	300	1.64	19.88	158.68	18.04
	400	1.14	12.23	145.78	18.28
99 *	150	1.36	19.52	34.32	3.44
	200	1.12	15.05	46.13	5.10
	300	1.20	14.48	NA	NA
	400	1.43	15.31	NA	NA

3.4.3. Stress

From above, it is clear that deuterium causes stress leading to both a decline in cell division and a slowing down of growth. Quantum yield, in dark-adapted samples equivalent to the ratio between maximum fluorescence and variable fluorescence, F_v/F_m , was used to assess the stress levels in individual cultures over time (Figure 8C). In the control culture, the cells experienced stress immediately after being put in light but with time, recovered from it (Figure 8C). In the 70% culture, there was a significant degree of variation in F_v/F_m over time. This was most expressed at the highest light intensity ($400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) (Figure 8C). Firstly, the ratio dropped to very low level as stress was experienced by all cultures. Next, the ratio in all the cultures started to recover suggesting the cells adapted to the stress caused by the treatment. The non-adapted cells grown in the medium containing 99% deuterated water experienced severe stress at all light intensities although it was lower at the lowest light intensity ($150 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) (Figure 8C). Recovery from stress was light intensity dependent and it started only after five days of cultivation (Figure 8). In line with this, the cells pre-adapted to 99% deuterated water showed stress compared to the control culture but for the two lower light intensities (150 and $200 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) it remained similar throughout the experiment (Figure 8). For the two higher light intensities (300 and $400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$), the cultures experienced higher levels of stress but recovered within 48 h (Figure 8C).

Table 2. Comparison of mass multiplication factor and mass doubling times for cultures grown in medium containing different concentrations of deuterated water at different light intensities. Average of at least three experiments is shown. Asterisk (*) denotes cultures pre-adapted for one week to given deuterated water concentration.

Concentration of Deuterated Water (%)	Light Intensity ($\mu\text{mol Photons m}^{-2}\text{s}^{-1}$)	Mass Multiplication Factor	% of Control	Mass Doubling Time (h)	Fold Retardation of Control
0	150	4.33	100	12.79	1
	200	5.65	100	11.65	1
	300	5.54	100	13.65	1
	400	5.74	100	10.60	1
70	150	3.64	84.13	15.29	1.20
	200	3.42	60.52	13.75	1.18
	300	3.32	59.86	16.04	1.18
	400	4.03	70.27	13.24	1.25
99	150	1.83	42.39	118.23	9.24
	200	1.29	22.73	142.92	12.26
	300	1.13	20.43	306.28	22.44
	400	1.05	18.23	309.10	29.17
99 *	150	3.18	73.46	42.53	3.33
	200	2.78	49.20	47.62	4.09
	300	2.78	50.25	49.99	3.66
	400	2.11	36.68	49.09	4.63

3.4.4. Starch and Neutral Lipid Accumulation

Cultures of *P. kessleri* are known to produce varying amounts of starch and neutral lipids in response to stress [16,26,31,50]. We tested whether the combination of increasing light intensity and the presence of increasing concentrations of deuterated water in the medium could induce starch and/or neutral lipid accumulation. The neutral lipid accumulation was negligible in the control cultures grown in 0% D₂O as well as in the 70% cultures (Figure 8D). But it was increased in both the non-adapted and pre-adapted 99% cultures (Figure 8D). In the pre-adapted cultures, there was a higher accumulation of neutral lipids with increasing light intensity (Figure 8D). Due to the inhibitive price of deuterated water, starch accumulation was assessed on cells stained by Lugol solution (Figure 9). In the control cultures, there was more starch produced with increasing light intensities (Figure 9A–D) as is also shown above (Figure 7). In the 70% culture, the accumulation of starch was higher than in the controls and comparable among all the light intensities (Figure 9E–H). In the 99% culture, the highest accumulation of starch was reached at the lowest light intensity (Figure 9I). It was lower at higher light intensities (Figure 9J–L) concomitant with higher neutral lipid accumulation in these cells (Figure 8D). From all the variants, the accumulation of starch was highest in the pre-adapted culture (Figure 9M–P) and it was comparable across the light intensities.

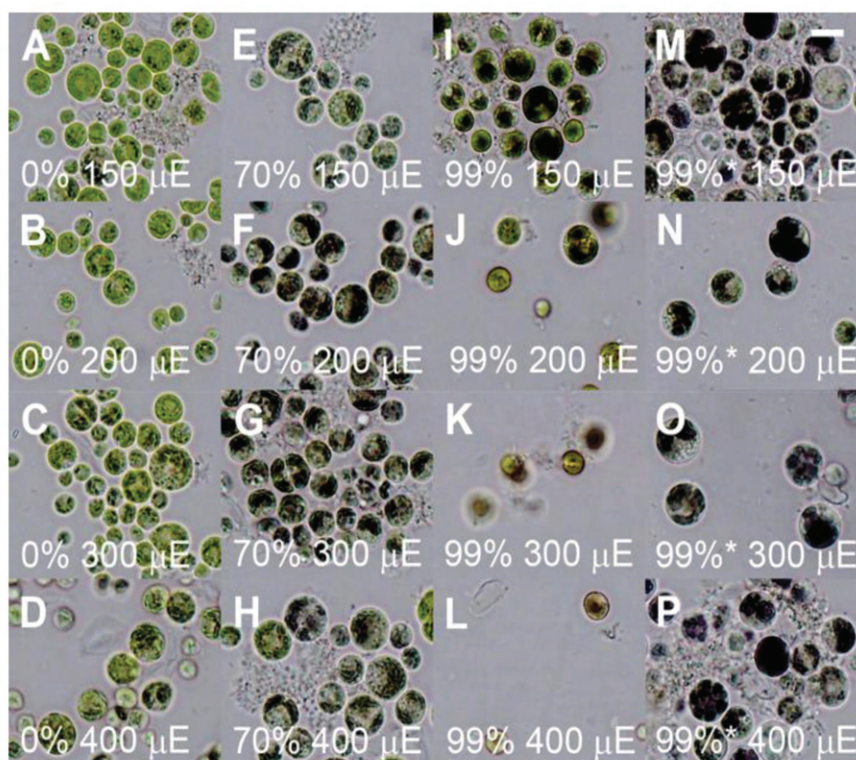


Figure 9. Light photo-micrographs of *Parachlorella kessleri* grown for 3 days at different incident light intensities (400, 300, 200, and 150 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) and different concentrations of deuterated water in the medium (0, 70 and 99%). (A–D): control cultures grown in 0% D_2O , (E–H) cultures grown in 70% D_2O , (I–L) cultures grown in 99% D_2O , (M–P) cultures grown in 99% D_2O and pre-adapted to the same concentration for one week. The cultures pre-adapted to growth at 99% of deuterated water for one week are denoted with asterisk. Scale is 10 μm .

4. Discussion

Parachlorella kessleri is a biotechnologically relevant algal species that, depending on the growth conditions, is able to (over)produce both starch and neutral lipids [16,26]. A cell cycle division block has been shown to be one of the mechanisms leading to both starch and neutral lipid production [16,26,50]. However, the pattern of cell division under optimal non-stressed growth conditions remained unknown. Here, we characterized the cell cycle pattern and studied the effect of light intensity on cell cycle progression. To do so, we used synchronized cultures where all the cells within the population were in approximately the same phase of the cell cycle. This is evidenced by stepped increases in DNA content in the entire population and the sharp increases in cell number (Figure 5A,B). Furthermore, the cell volume is giving an idea how the cell size increased within the population with time (Figure 6C). For non-synchronous cultures, it would be expected instead that the lines describing the changes in cell numbers and DNA content will be parallel with abscissa as both processes would be running continuously in the cell population. Moreover, we characterized the effect of different light light intensities on growth, including the levels of

energy reserves. Finally, we tested how cell division and growth were affected by the presence of deuterium. The synchronization by light/dark periods used here is a natural process. The algae dividing by multiple fission evolved so that they exploit the alternating light/dark periods of solar irradiation to grow during the light phase and divide during the dark phase [3,4]. Still, once all the reproductive processes were completed, the cells would divide irrespective if they were in dark or on light (Figures 5B and 8A) [51,52]. Once the culture is synchronized it will stay this way for some time depending on the conditions. In alternating light/dark regimes respecting the cell natural division patterns, the cultures will stay synchronized almost indefinitely. In continuous light, the synchrony will be lost over time, yet, for some time, the divisions will happen at the same time as long as the light conditions are maintained (Figure 8A). Interestingly, although the algae evolved to adapt to the alternating light/dark cycles their cell cycles are not necessarily controlled by circadian rhythms. The link between circadian clock and cell cycle has been established in cyanobacteria [1] and mammalian cells [2–4], including cancer cells [4,5]. However, its existence has been a controversial topic in the field of algae dividing by multiple fission. Early reports studying multiple fission cell cycle in *Chlorella* sp. routinely present and use synchronizing experiments with non-circadian duration [6,7]. It has been established that multiple fission cell cycle is regulated by a combination of size and timer in *Chlamydomonas reinhardtii* [4,8–11], but the timer is not circadian. Similar results were also established for another green alga *Desmodesmus quadricauda* [12]. There were reports suggesting involvement of circadian gating of cell division in *Chlamydomonas reinhardtii* [13]. Yet, a comprehensive testing of multiple fission cell cycles at wide range of light intensities and temperatures disregarded the existence of circadian gating in this organism [14,15]. In establishing the synchronization regime, we respected the cell and culture behavior at given growth conditions. The cells were darkened when they started to divide and were re-illuminated when they completed cell division. The synchronization regime we established was in agreement with synchronization regimes used for a relative alga *Chlorella* sp. [6,7].

Division into more than two daughter cells is common among green algae. Such a multiple fission cell cycle is performed in different species according to different patterns. All patterns share several common features: (1) they are composed of several growth steps, (2) the growth steps run consecutively, (3) completion of each growth step (n , n ranges from 1 to 10) by doubling the initial biomass will allow for a start of a single reproductive step, (4) each reproductive step or sequence starts with commitment point (CP) and consists of processes of the standard cell cycle; DNA replication, nuclear division, cell division including the gap phases (G1, G2), (5) several (n) reproductive steps are running concurrently, (6) cell division is postponed until completion of the final reproductive step and runs simultaneously for all of the reproductive steps leading to a release of 2^n daughter cells. Two main distinct types of multiple fission cell cycle, consecutive pattern (*Scenedesmus*—type) and clustered pattern (*Chlamydomonas*—type) [3,4,6,53] differ in time succession of the reproductive sequences (Figure 10). In each consecutive cell cycle, the attainment of CP is followed shortly by the phase for preparation of DNA replication (pre-S), DNA replication (S), phase of preparation of nuclear division (G2), nuclear division (M), and finally, preparation of protoplast fission (G3) and protoplast fission (C) (Figure 10). This is terminated by formation and release of daughter cells. Such a sequence of events, and the reproductive sequence, repeats after each doubling of cell biomass, i.e., completion of the growth step. Within one multiple fission cell cycle there will be several consecutive doublings of DNA, each followed by a G₂ phase and then by nuclear division (Figure 10). The overlap among individual reproductive sequences will result in bi-, tetra- and then octa-nuclear cells. Each nuclear division is followed by a G₃ phase of different duration and thereafter by a corresponding number of protoplast fissions and release of daughter cells [3,4,6,53] (Figure 10A). In the clustered cell cycle pattern, no rounds of DNA replication nor nuclear divisions occur during the majority of the cell cycle. The cells sequentially undergo several growth steps and increase their size correspondingly to the number of growth doublings steps, but they remain haploid and uni-nuclear until

the very end of the cell cycle. Then occurs several rounds of DNA replications, each of them immediately followed by corresponding nuclear division. These are followed again nearly immediately by protoplast fission and terminated by the formation of daughter cells. During the entire cell cycle, no polyploid or multi-nuclear stages were present [3,4] (Figure 10B). Besides the two main cell cycle patterns, there could be a combination of these such as the cell cycle pattern of the alga *Haematococcus pluvialis* (class Chlorophyceae, order Volvocales) designated “Haematococcus—type cell cycle” [54]. *H. pluvialis* firstly undergoes DNA replication for all initiated reproductive sequences, which corresponds to the number of daughter cells formed. This way it becomes polyploid. Thereafter it repeatedly divides nuclei becoming poly-nuclear. The poly-nuclear cells divide their protoplasts and form non-flagellated daughter cells inside the mother cell wall. Finally, flagellated daughter cells are released [54] (Figure 10C). The cell cycle of *P. kessleri*, as described in this manuscript, is characterized by multiple rounds of DNA replication (Figure 5). In contrast to the *Haematococcus* or *Scenedesmus* pattern, none of the early DNA replications were followed by nuclear division; this only occurred once all replication rounds were terminated. Consequently, the cell became successively polyploid with a single large nucleus (Figure 4).

From the experiments presented here, the status of the polyploid DNA is not clear. In principle, the replicated copies could stay together in a single chromosome as they do in the polytene chromosome in *Drosophila melanogaster*, or in plants [55,56], or they could undergo endomitosis so that the nuclei contain multiple sets of chromosomes [57]. At the end of the cell cycle, the number of nuclear divisions corresponding to the number of preceding DNA replications are triggered (Figures 3 and 4).

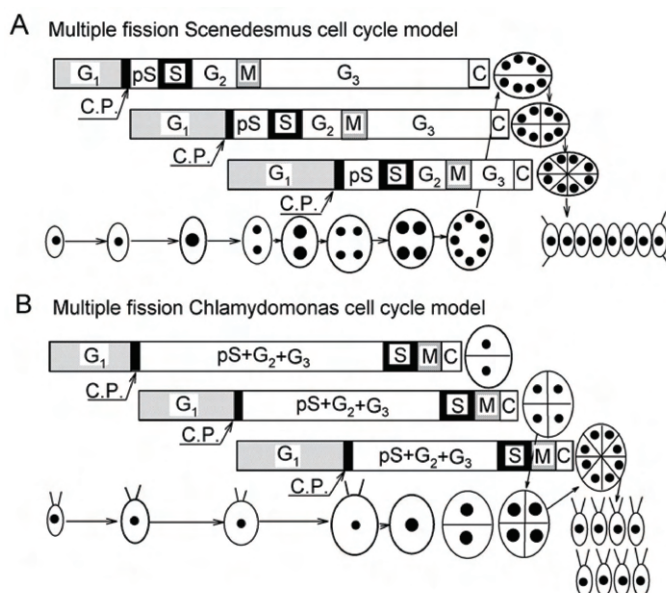


Figure 10. Cont.

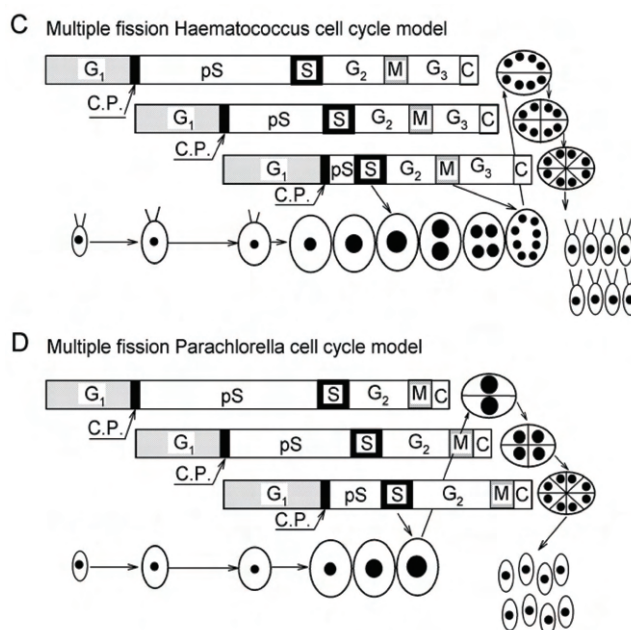


Figure 10. Schematic diagram of growth and the cell cycle in a single cell of different algae dividing into 8 daughter cells. (A): *Desmodesmus* (*Scenedesmus*) *quadricauda*, (B): *Chlamydomonas reinhardtii*, (C): *Haematococcus pluvialis*, (D): *Parachlorella kessleri*. The schematic pictures reflect increasing cell size. The full circles inside the cells illustrate the size and number of nuclei. Larger circles indicate a doubling of DNA. Ellipsoids and lines represent protoplast division. Three bars indicate three overlapping growth and reproductive sequences terminated by division into 2, 4, and 8 daughter cells, respectively. Pre-commitment period (G1): the period until threshold critical cell size for commitment to divide (CP) is reached and CP is attained. Post-commitment period consists of pS—the pre-replication phase between CP attainment and the beginning of DNA replication. The processes required for initiation of DNA replication are assumed to happen during this phase. S: DNA replication takes place. G2: the phase between the termination of DNA replication and the start of mitosis (M). Processes leading to the initiation of mitosis are assumed to take place during this phase. G3: the phase separating mitosis from cellular division, which is clearly visible in some algae dividing by multiple fission. The processes leading to cellular division are assumed to take place during this phase. C: the phase during which cell cleavage (protoplast fission) and daughter cell formation occurs. Modified after Zachleder, Schläfli and Boschetti [6], Zachleder and van den Ende [8], Reinecke, Castillo-Flores, Boussiba and Zarka [54] and Zachleder, Bišová and Vitová [3].

Each nuclear division is immediately followed by protoplast fission without any intervening G3 phase. In this way no poly-nuclear cells could be observed during the *Parachlorella* cell cycle, similar to the pattern established in *Chlamydomonas* [3,4]. Thus, cells of *P. kessleri* divide by another unique hybrid cell cycle pattern, which is a combination of features of all described patterns of cell cycles in species dividing by multiple fission [3,54] (Figure 10D).

The final biomass content of *P. kessleri* at the end of the cell cycle, measured as the time course of RNA, dry matter and cell volume (Figure 6), increased with light intensity. It multiplied 4-fold within one cell cycle at the lowest irradiance ($110 \mu\text{mol photons m}^{-2}\text{s}^{-1}$), 12-fold at $250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and 16-fold at the highest irradiance ($500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$).

$\text{m}^{-2}\text{s}^{-1}$). Cell growth at any given light intensity did not occur as a continuous increase in the given growth parameter. Rather, it occurred in steps, each of which corresponded to a doubling of the value of the preceding one. The steps were separated by a short interval without (or with only a slight) increase in growth characteristics. With increasing light intensity, growth rate within individual steps increased, the intervening interval without growth shortened and the number of steps increased (Figure 6). These rules were particularly well expressed in the time courses of RNA accumulation (Figure 6A), but they can also be followed for cell volume (Figure 6C) and less so for dry mass (Figure 6B). Such growth with non-overlapping and time-separated steps is typical for multiple fission cell cycle patterns in the majority of chlorococcal and volvocaeal algae [3,4,6,8].

Starch accumulates in light via photosynthesis. Under unstressed growth conditions, it serves as a main source of energy and carbon for *P. kessleri* cells kept in the dark or under insufficient light conditions [16,26]. The starch content measured in our experiments represents the net starch content, which is a result of starch synthesis and consumption. Thus clearly, it is an underestimate of the real level of starch synthesis and newly made starch content. The net starch synthesis was negligible in the early part of the cell cycle for the cultures grown at the lowest light intensity ($110 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) (Figure 7), probably due to the balance between material and energy supply from photosynthesis and their use in synthetic processes [3]. The net starch content as well as its rate of synthesis grew proportionally to the increasing light intensity (Figure 7). The final net content of starch, a 2-fold increase at the lowest light intensity ($110 \mu\text{mol photons m}^{-2}\text{s}^{-1}$), an 8-fold increase at $250 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a 10-fold increase at the highest irradiance ($500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$), were lower than those of RNA (Figure 7, see above). Since nuclear and cellular divisions are the key consumers of starch, even under light conditions [3,27,53,58,59], we assume that part of the newly made starch is immediately spent for reproductive processes. This would also explain why there seems to be significantly less net starch content (only a 10-fold increase) compared to a 16-fold increase in dry mass and cell volume at the highest light intensities. The lower starch levels might be caused by the higher number of reproductive sequences and higher number of daughter cells formed (Figure 5), which naturally requires more energy. The increase in consumption processes also explains the plateau of net starch at the end of the cell cycle in all experimental variants (Figure 7). Moreover, it is known that rates of photosynthesis (and consequently rates of starch synthesis) decrease or are stopped during cell division [60,61]. Neutral lipids represent the other energy reserve of *P. kessleri* [16,26]. In the majority of our experiments, the levels of neutral lipids were negligible (not shown) as they are expected to be synthesized in response to stress conditions [16,26]. This is particularly true for the later phases of stress after starch was (over)produced and is being replaced by neutral lipids as the secondary energy reserve [16,26]. In line with this, we detected more starch in the 70% deuterated culture than in the control but the accumulation of neutral lipid was, for the majority of the experiment, negligible. In contrast, higher levels of neutral lipids were found in the cultures grown in 99% D_2O (Figures 8D and 9). Moreover, the increase in the amount of neutral lipids increased with light intensity and was most strongly manifested in the cultures pre-adapted to 99% D_2O (Figure 8D). The same cultures also accumulated the most starch (Figure 9), suggesting they are stressed but still able to cope with the stress levels [16,26].

Cultivation in increasing concentrations of deuterated water showed a distinct effect on cell division and cell growth, with growth being less affected than cell division (Figure 8, compare A and B, compare Tables 1 and 2). In the presence of deuterated water, the response to different light intensities was opposite to its effect in normal water, i.e., the increasing light intensity decreased growth rates (Figure 8). This was probably due to the combined effect of light and deuterium stress on the cells and is particularly clearly seen for the non-adapted 99% culture (Figure 8C). There was a significant degree of variability of Fv/Fm ratio, which suggested some of the cultures acclimated to the presence of deuterium. The adaptation process was faster in 70% cultures where it took approximately

48 h to recover the Fv/Fm ratios in the lower light intensities but 96 h at the highest light intensity. In 99% cultures, the cultures at the two lower light intensities adapted by 96 h of the treatment, whilst the ones at the higher light intensities (300 and 400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) were unable to recover and eventually died (Figure 8C). Clearly, the cells were able to adapt even to the highest concentrations of deuterium as it is evident from the cultures adapted at 99%. Such cultures did not show a decline in Fv/Fm ratios at lower light intensities. At higher light intensities (300 and 400 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$), there was a drop in Fv/Fm ratios comparable to that in 70% cultures at the highest light intensities (Figure 8C). Yet, such cultures were able to recover the initial Fv/Fm values within two days (Figure 8C). Interestingly, there was only a small effect of light intensity on the number of daughter cells in different concentrations of deuterated water, further supporting the smaller effect of deuterium on growth. Although mass multiplication was less affected than division number in all variants, the mass doubling time was similar for all light intensities (Table 2) and it approximately correlated with cell number doubling time of the same culture (compare Tables 1 and 2). The cells show striking resilience to the presence of deuterated water. In the presence of 70% of deuterated water they recovered from the stress within 48 h (Figure 8C). They were even able to cope quite well with 99% deuterated water provided they are pre-adapted to this concentration (Figure 8C). How growth in 99% deuterated water for a prolonged period of time affects cell physiology is a matter for future research. Particularly interesting will be the mechanisms allowing cells to cope with the majority of their hydrogen being replaced by deuterium as is the case of pre-adapted 99% cultures. Due to the inhibitive high price of deuterated water, the treatment with deuterated water is quite expensive. It can be justified in the field of basic research to understand the effects of deuterium on the cell metabolism. Understanding the stress reaction is important even though high concentrations of deuterated water are never present in nature. As stable isotopic labeling is extensively used to analyze cell metabolism [31,32,62] it is crucial to disentangle the effects of the deuterium tracer in the cells to discriminate its effect from the effect of the treatment. Furthermore, although treatment with deuterated water would be too expensive to be used in large scale algal biomass production it is fully justified for production of very high added value products such as deuterated compounds [31,39,40]. The individual isolated biomolecules can be used as analytical standards or as a well-defined source of deuterium for stable isotopic labeling. Indeed, algae were shown to serve as a commercial source of fully deuterated sugars and amino acids [63], proteins [64], chlorophylls [39,65], and carotenoids [39]. The fully deuterated algal biomass arising from the experiments presented here can be used for production of deuterated starch and, to a certain extent, neutral lipids but also other cellular components.

5. Conclusions

We describe a novel pattern of multiple fission cell cycle. The pattern utilized by cells of *P. kessleri* consists of multiple rounds of DNA replication, which, after completion of the last one, are followed by successive nuclear divisions. Each of the nuclear divisions was immediately followed by cell division. Thus, morphologically, the cells became sequentially polyploid but not polynuclear. In this way, the cell cycle pattern is yet another combination of established patterns of multiple fission cell cycles. Cultivation under increasing light intensities led to an increase both in growth rate and the number of growth steps completed by the cells, leading to a higher number of daughter cells being formed. Growth of *P. kessleri* in different concentrations of heavy water slowed growth and progression of the cell cycle, with cell division being more affected than cell growth. The inhibitory effect of heavy water was more pronounced with increasing light intensity. The highest concentration of heavy water (99%) caused a prolonged stress that led to the accumulation of neutral lipids. However, the stress levels can be decreased by pre-adaptation of the cells to high concentrations of heavy water. Our results establish *P. kessleri* as a novel model of the cell

cycle for basic research as well as to set a baseline for extension of its biotechnological potential for the production of deuterated compounds.

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Article III.

Supra-optimal temperature: an efficient approach for overaccumulation of starch in the green alga

Parachlorella kessleri

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Article

Supra-Optimal Temperature: An Efficient Approach for Overaccumulation of Starch in the Green Alga *Parachlorella kessleri*

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Abstract: Green algae are fast-growing microorganisms that are considered promising for the production of starch and neutral lipids, and the chlorococcal green alga *Parachlorella kessleri* is a favorable model, as it can produce both starch and neutral lipids. *P. kessleri* commonly divides into more than two daughter cells by a specific mechanism—multiple fission. Here, we used synchronized cultures of the alga to study the effects of supra-optimal temperature. Synchronized cultures were grown at optimal (30 °C) and supra-optimal (40 °C) temperatures and incident light intensities of 110 and 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The time course of cell reproduction (DNA replication, cellular division), growth (total RNA, protein, cell dry matter, cell size), and synthesis of energy reserves (net starch, neutral lipid) was studied. At 40 °C, cell reproduction was arrested, but growth and accumulation of energy reserves continued; this led to the production of giant cells enriched in protein, starch, and neutral lipids. Furthermore, we examined whether the increased temperature could alleviate the effects of deuterated water on *Parachlorella kessleri* growth and division; results show that supra-optimal temperature can be used in algal biotechnology for the production of protein, (deuterated) starch, and neutral lipids.

Keywords: microalgae; *Parachlorella kessleri*; starch; supra-optimal temperature; cell cycle; energy reserves; growth processes; reproduction events; deuterium; deuterated starch; deuterated lipid

1. Introduction

Light and temperature are two crucial factors affecting algal growth and division, both in natural habitats and in biotechnological applications. For autotrophically grown organisms, there is a physiological range of light intensities and temperatures that support growth and division, and light intensities and temperatures below the threshold are not sufficient. On the other hand, light intensities and temperatures above this range are increasingly stressful, and will inhibit cell division and/or growth. The duration of light, along with its intensity and spectral composition will, in autotrophically grown algae, affect growth rates [1–11], but will not affect light-independent processes such as cell division, which uses internal energy stores for its support [1,5–9,11]. In contrast, temperature is much less specific, as it will affect all metabolic processes, including “dark” ones such as cell division; thus, its effects reach beyond setting growth rates. In principle, within the physiological range, a 10 °C increase in temperature will increase metabolic

rate twofold [12]. Increasing temperature will thus speed up growth, as well as individual processes leading to cell division, and consequently shorten the duration of the cell cycle, as shown in different algae such as *Chlorella ellipsoidea* [11], *Chlamydomonas reinhardtii* [13], *Chlamydomonas eugametos* [14], and *Desmodesmus quadricauda* [15]. However, individual metabolic processes are differentially sensitive to various stresses [16], including temperature [17], which significantly affects how cells react to changes in temperature. In particular, cell division seems to be more sensitive to temperature increases than growth does [17,18]; this has peculiar consequences; there are threshold temperatures that will only slightly or not at all affect cell growth, but will completely block cell division. The effects of such supra-optimal temperatures were described in the green algae *Chlamydomonas reinhardtii* [17] and *Chlorella vulgaris* [18].

The chlorococcal alga *Parachlorella kessleri* (formerly *Chlorella kessleri* [19]) is a biotechnologically promising algal species [20–29] that divides by multiple fission. Its cell cycle consists of multiple rounds of DNA replication that, after completion of the last one, are followed by successive nuclear divisions. Each of the nuclear divisions is immediately followed by cell division. Morphologically, the cells become sequentially polyploid but not polynuclear [30]. Within a single cell cycle, the mother cell can give rise to 2, 4, 8, or 16 cells; this is consistent with the established scheme of multiple fission, where a single mother cell can divide into 2^n daughter cells—where n is the number of rounds of started reproductive sequences (DNA replication, nuclear division, cell division) [31]. Increasing light intensity will increase both growth rate and the number of reproductive sequences started, and thus, the number of daughter cells being formed from a single cell [30].

The primary energy and carbon store of *P. kessleri* is starch, which is produced under optimal growth conditions in complete nutrient medium and at physiological values of light intensity and temperature [23,32]. Starch is exclusively located in the chloroplasts in the form of starch grains of different numbers and sizes. In the autotrophically grown algal cell, starch reserves serve mostly as a buffer to supply a stable carbon and energy source. This is indispensable, as in nature the energy supply from photosynthesis varies with sunlight intensity during the day, and is absent during nights. The buffering role of starch is crucial for cell reproduction. The processes of DNA replication, along with nuclear and cellular divisions, are of importance in the life of a cell and so they depend on a constant and reliable supply of energy and carbon. Indeed, the majority of starch produced by cells is degraded exclusively for cell reproduction [33], and this is true even for cells growing in continuous light [34]. This was evidenced in synchronized cultures of different algal species, where net starch increased to some maximal value during the cell cycle, and subsequently was nearly completely degraded during nuclear and cellular division at the end of the cell cycle [33,35,36]. The cellular starch content at any given time is the net result of starch synthesis and utilization; thus, decreasing starch content can be caused by lower starch synthesis, by increased consumption, or by a combination of both factors. It seems that a decrease in starch content at the time of cell division is caused by a combination of increased starch spending for cell reproduction and a minimum photosynthetic rate specific for this period [35]. Furthermore, a blockage of cell division processes as the primary starch consumers will lead to starch (over)accumulation. This has been established for different stress conditions, such as the application of inhibitors [36], nutrient starvation [24,25,32,36], high light intensity [32], the presence of a high concentration of CO_2 [37], or supra-optimal temperatures [18], and it should be explored biotechnologically [36,38]. If stress conditions are prolonged, starch reserves will gradually start to be replaced by neutral lipids—the secondary energy store of *P. kessleri* [23,24,32,39]. Although neutral lipids under optimal growth conditions are maintained at a relatively low level, at 1–10% of dry matter (DM) [23,39,40], they can also (over)accumulate under stress conditions similar to those inducing starch accumulation, such as nitrogen, sulfur, or phosphorus depletion [24,26,32,39,41], dilution of all the nutrients in the medium [25,39] or salt stress [42]. The ability to (over)produce both starch and neutral lipids makes *P. kessleri* a useful model for applications in biotechnology. A peculiar source of stress for *P. kessleri* is cultivation

in deuterium, which leads predominantly to the accumulation of starch [30], but also that of neutral lipids [43]. Deuterium—the stable isotope of hydrogen—is known to have the highest kinetic effect among the stable isotopes of biogenic elements [43–45]. Deuterium in deuterated water is known to increase cell stress via several mechanisms [43,44], including greater bond dissociation and activation energies [46]. Its presence affects the entire metabolism, and its specific effects range from disrupting signaling [47] and energy production in mitochondria and chloroplasts [48,49] to disrupting cell division [50,51]. Given the very low concentrations of deuterium (or deuterated water) in nature, its application in high concentrations is artificial, and is also quite expensive. However, deuterated molecules are used extensively as analytical standards and for metabolic labeling [43,52,53], and deuterated compounds are exploited in pharmacology [44,54–57]. Therefore, production of algal-derived deuterated compounds is justified for the production of fine (bio)chemicals with very high added value. Indeed, algal-derived deuterated biomolecules such as sugars, proteins, carotenoids, lipids, and starch can be used commercially [43,52,53,58–60].

Here, we supplement the extensive knowledge that has accumulated on starch and neutral lipid production under different conditions in *P. kessleri* by providing baseline information on the effects of supra-optimal temperature on cell reproduction (DNA replication, cell division) and growth (RNA, protein, dry matter, cell volume). Particular focus has been placed on the production of starch and lipids as the primary and secondary energy stores. Furthermore, we analyze the effects of a high concentration of deuterium on cell growth and reproduction, as well as the production of deuterated starch and lipids. The results of this study can be exploited as a starting point for further optimization in larger scale biotechnological production, but will also widen the *P. kessleri* portfolio as a model for basic research.

2. Materials and Methods

2.1. Organism and Culture

The green unicellular microalga *Parachlorella kessleri* (Trebouxiophyceae, Chlorophyta) (strain CCALE 255) was obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany, Czech Academy of Sciences in Třeboň, Czech Republic (CCALA; <https://ccala.butbn.cas.cz/>, accessed on 7 June 2021).

For routine sub-culturing, the cultures were streaked every three weeks onto nutrient medium (see below) solidified by agar (1.5%), and grown on a light shelf at an incident light intensity of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation.

2.2. Mineral Nutrient Medium

The mineral medium was based on the mean content of P, N, K, Mg, and S in algal biomass [61], and had the following initial composition (in mg/L): 1100 KNO_3 , 237 KH_2PO_4 , 204 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 40 $\text{C}_{10}\text{H}_{12}\text{O}_6\text{N}_2\text{NaFe}$, 88 CaCl_2 , 0.83 H_3BO_3 , 0.95 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3.3 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.17 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 2.7 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.014 NH_4VO_3 in distilled water [36]. For the preparation of the medium, 100x concentrated stock solutions of macroelements and microelements were used. All components were diluted in distilled water and autoclaved for 30 min at 121 °C. The pH was adjusted to 7 with 1 M NaOH.

2.3. Culture Conditions

Two types of cultivation units were used for experiments: (1) glass cylinders (inner diameter 36 mm, height 500 mm, volume of suspension 300 mL), and (2) flat and rectangular glass vessels (inner dimensions 400 × 300 × 20 mm, volume of suspension 2500 mL). The first type of the units was used for routine growth, synchronization, and small-scale experiments with deuterated water; the second type of the units was used for large-scale experiments. Culture units were placed in a thermostatic water bath, where the temperature was set and automatically maintained at the values stated in the Results section for each experiment. The vessels were illuminated from one side with a panel of dimmable fluorescent lamps (DULUX L55W/950 Daylight, OSRAM, Munich, Germany).

For the experiments, the incident light intensity was set either to 110 or 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of photosynthetically active radiation. The cultures were vigorously mixed with air bubbles containing 2% carbon dioxide (*v/v*) dispersed through a 200- μL micropipette tip fixed in a glass tube placed at the bottom of the cylinder. The aeration in the flat vessels was performed via bubble stream from a perforated stainless steel tube located at the bottom of the vessel. The flow rate of the aeration mix was 60 L/h.

2.4. Synchronization of Cultures

Cultures were synchronized under optimal conditions: incident light intensity 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, temperature 30 °C, 2% CO_2 (*v/v*) in aeration mixture. The cultures were initiated by inoculating algal cells directly from the culture plates into the liquid medium. Such cultures were synchronized by alternation of 18 h light and 7 h of dark, as established earlier [30]. During the synchronization procedure, the cell density was kept below 1×10^6 cells mL^{-1} by dilution at the end of the dark period, in order to prevent cell shading. This regime was maintained for several days (about 3 cycles) until the required culture synchrony was reached. Once the cultures were synchronized, they were grown for approximately one more cycle at the same cell density until the volume of the culture required for the start of the experiment was reached. The synchronized daughter cells were again diluted to the initial cell density of approximately 1×10^6 cells mL^{-1} , and used as inocula for experimental cultures.

2.5. Management of Deuterated Cultures

The synchronized inocula for the deuterated water experiments were centrifuged at $3000 \times g$ for 5 min and resuspended in a medium containing the desired concentration of deuterated water. The cultures were cultivated in a semi-batch mode, controlled by the optical density at 750 nm (OD_{750}). Upon reaching an OD_{750} of 0.4, the cultures were diluted with fresh medium with corresponding D_2O content to the initial value of OD_{750} . This way, growth unlimited by nutrients and light was achieved.

2.6. Measurement of Light Intensity

The dimmable fluorescent tubes were used for the adjustment of the incident irradiance. The light intensity was measured using a quantum/radiometer/photometer (LI-COR, Inc., Lincoln, NE, USA). Incident light intensity (I_i) was measured at the surface of the culture vessel, while the transmitted light intensity was measured at the rear side of the culture vessel (I_t). The different values of incident (I_i) and transmitted (I_t) light intensities at different optical densities given by cell size and concentrations were used to calculate the mean light intensity (I_m)—i.e., light energy absorbed by a layer of cell suspension—according to the Lambert–Beer formula: $I_m = (I_i - I_t)/\ln(I_i/I_t)$.

2.7. Assessment of Cell Division Curves

The proportion of mother cells and daughter cells was determined by light microscopy in cells fixed in Lugol's solution (1 g I, 5 g KI, 100 mL H_2O) at a final concentration of 10 μL of Lugol's solution per 1 mL of cell suspension.

2.8. Dry Matter Determination

Biomass was separated from the medium by centrifugation of 4 mL of the cell suspension in pre-weighed microtubes at $3000 \times g$ for 5 min; the sediment was dried at 105 °C for 12 h and weighed on an analytical balance (TE214S-OCE, Sartorius, Goettingen, Germany) [36].

2.9. Cell Volume and Number

Cell volume and number were measured using a Beckman Coulter Multisizer 4 (Beckman Coulter Life Sciences, Indianapolis, IN, USA) by diluting 50 μL of fixed (0.2% glutaraldehyde) cell suspension into 10 mL of 0.9% NaCl (*w/v*) electrolyte solution.

2.10. Quantum Yield Measurement

Aliquots of 2 mL were withdrawn from the culture and placed into 10 × 10-mm plastic cuvettes for 30 min in the dark. Quantum yield was measured using an AquaPen-C 100 (Photon Systems Instruments, Drasov, Czech Republic).

2.11. Neutral Lipids and Starch Staining

For neutral lipids and starch staining, 1 mL of culture was sampled and stored for up to several hours at 4 °C until analysis. Staining was conducted on microscopy slides prior to observation. For starch staining, 20 µL of culture was combined with 0.5 µL of Lugol's solution (1 g I, 5 g KI, 100 mL H₂O) and observed immediately via light microscope (model BX51, Olympus, Tokyo, Japan). Neutral lipids were stained with the addition of 0.5 µL of freshly prepared Nile red dye (0.5 mg/mL in DMSO, catalog no. 72485, Sigma-Aldrich, Prague, Czech Republic) to 20 µL of culture, directly on the microscopic slide. Such slides were incubated at room temperature for 20 min in the dark. Stained cells were observed using a microscope (model BX51, Olympus, Tokyo, Japan) equipped with a mercury lamp and filter combination U-MNU2 (360–370 nm excitation and >515 nm barrier filter). Microphotographs were taken with a DP72 camera (Olympus, Tokyo, Japan).

2.12. Neutral Lipids Quantification

Neutral lipids content was measured spectrophotometrically in a microplate format following the modified procedure of Takeshita, et al. [62]. Aliquots (100 µL) of the cultures were combined with 5 µL of freshly prepared Nile red dye (0.5 mg/mL in DMSO, catalog no. 72485, Sigma-Aldrich, Prague, Czech Republic) in a 96-well plate. The same amount of Nile red dye was added to a sample blank consisting of 100 µL of H₂O. The plate was incubated at room temperature in the dark for 15 min. Fluorescence was measured using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) equipped with a 485-nm excitation filter and a 595-nm emission filter. The fluorescence intensity of the samples was normalized using the fluorescence intensity of unstained samples and a blank. Standard curves produced from a commercial lipid standard—triolein (catalog no. Y0001113, Sigma-Aldrich, St. Luis, MO, USA)—were used to quantify neutral lipids.

2.13. Estimation of Bulk RNA, DNA, and Proteins

2.13.1. Total Nucleic Acids Extraction

The procedure of Wanka [63], as modified by Lukavský, et al. [64], was used for the acidic extraction of total nucleic acids. After the removal of small-molecular-weight contaminants, the total nucleic acids were hydrolyzed with 0.5 M perchloric acid, and the absorbance of total nucleic acids in the supernatant was read at 260 nm (A_{260}). Total nucleic acid concentration was calculated based on calibration with a DNA standard of known concentration treated using the same procedure, and normalized to the number of cells in the sample.

2.13.2. DNA and RNA Determination

The light-activated reaction of diphenylamine with hydrolyzed DNA, as described by Decallonne and Weyns [65], was used with the modifications of Zachleder [66]. The concentrations of DNA within the samples were set by comparison to the A_{600} and A_{700} nm values of the sample with known DNA concentration treated using the same procedure. The values were normalized to the number of cells extracted; the RNA content was calculated as the difference between the total nucleic acid and DNA contents.

2.13.3. Protein Determination

The sediment remaining after nucleic acid extraction was used for protein determination; it was hydrolyzed with 1M NaOH for 1 h at 70 °C. The protein concentration in the supernatant after centrifugation of the hydrolysate (15 min, 5300 × g, room temperature) was estimated by BCA assay (cat. no. 23225, Thermo Fisher Scientific, Waltham, MA, USA)

according to the manufacturer's specifications. The same procedure was carried out with a calibration curve set by different concentrations of bovine serum albumin.

2.14. Starch Analyses

Net starch content was determined as described by Zachleder, et al. [30]. In short, the cells were disintegrated by vortexing with zirconium beads, and the pigments were removed via the addition of 80% (v/v) ethanol to the pellet, followed by incubation for 15 min at 68 °C. The procedure was repeated until the pellets were completely discolored. After that, 1 mL of α -amylase from porcine pancreas (Sigma-Aldrich, St. Luis, MO, USA), (0.5 mgL⁻¹ (w/v) in 0.1 M sodium phosphate buffer (pH 6.9)) was added to the samples, and they were incubated for 1 h at 37 °C (FTC 90i, VELP Scientifica, Usmate Velate MB, Italy). Starch content was determined in the supernatant using DNSA color reaction for the quantification of reducing sugars [67]. The concentration of starch was estimated through a calibration curve of potato starch (Lach-Ner, Neratovice, Czech Republic) digested with α -amylase.

2.15. Statistical Analysis

Experiments were performed in at least three biological replicates, and the mean values were used to construct the graphs in SigmaPlot version 11 (Jandel Scientific Software, Erkrath, Germany). Regression curves of various orders were applied to fit the data. If not stated otherwise, all results are presented as means and standard deviation ($n = 3$). MS Excel 2016 was used to compute one- and two-way ANOVA. A p -value < 0.05 was considered to be significant.

3. Results

3.1. Growth in Normal Water

Synchronized cultures of *Parachlorella kessleri* were grown at optimal (30 °C) and supra-optimal (40 °C) temperatures, at low (110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and high (500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) incident light intensities. The reproductive events (DNA replication, cellular division) (Figure 1), growth processes (RNA and protein synthesis, dry matter, and cell volume) (Figure 2), and accumulation of energy reserves (starch and lipids) (Figure 3) were followed within the time interval corresponding to two consequent cell cycles (48 h) in the control culture.

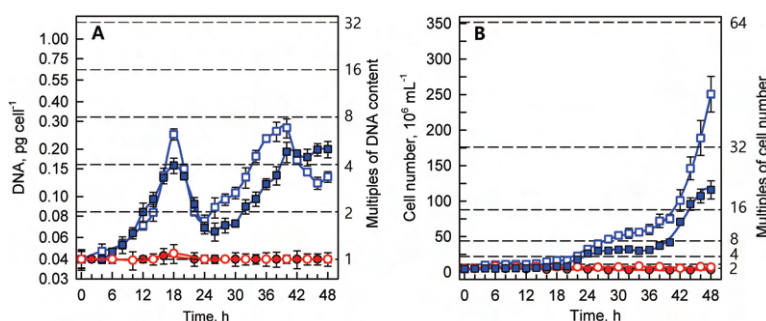


Figure 1. The course of reproductive events in the synchronized cultures of *Parachlorella kessleri* grown at temperatures of 30 °C and 40 °C (blue and red symbols and lines, respectively), and at incident light intensities of 500 and 110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (empty and full symbols, respectively). (A) DNA (pg cell⁻¹), and (B) cell number (10⁶ mL⁻¹). Horizontal dashed lines indicate the number of doublings of the initial values at the beginning of the cell cycle (0 h). Multiples of these values are given on the right ordinate. Data are presented as means $\pm$ SE. The differences between 30 °C and 40 °C are statistically significant at $p < 0.001$ (two-way ANOVA) in both A and B.

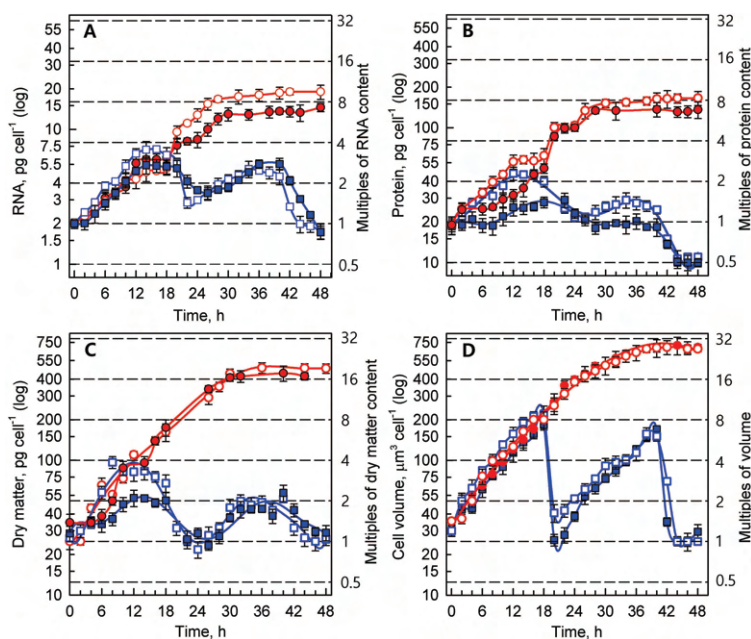


Figure 2. Time course of growth events in synchronized cultures of *Parachlorella kessleri* grown at 30 °C and 40 °C (blue and red symbols and lines, respectively), and at incident light intensities of 500 and 110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (empty and full symbols, respectively). (A) RNA (pg cell^{-1}), (B) protein (pg cell^{-1}), (C) dry matter (pg cell^{-1}), and (D) cell volume ($\mu\text{m}^3 \text{cell}^{-1}$). Horizontal dashed lines indicate the number of doublings of the initial values at the beginning of the cell cycle (0 h). Multiples of these values are given at right ordinate. Data are presented as means $\pm$ SE. The differences between 30 °C and 40 °C are statistically significant at $p < 0.001$ (two-way ANOVA) in both A and B.

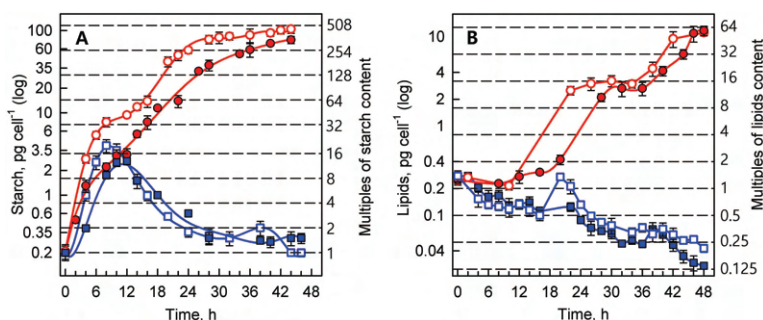


Figure 3. The course of changes in energy reserves in synchronized cultures of *Parachlorella kessleri* at temperatures of 30 °C and 40 °C (blue and red symbols and lines, respectively), and at incident light intensities of 500 and 110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (empty and full symbols, respectively). (A) Starch (pg cell^{-1}), and (B) lipids (pg cell^{-1}). Horizontal dashed lines indicate the number of doublings of the initial values at the beginning of the cell cycle (0 h). Multiples of these values are given on the right ordinate. Data are presented as means $\pm$ SE. The differences between 30 °C and 40 °C are statistically significant at $p < 0.001$ (two-way ANOVA) in both A and B. The differences between different light intensities in A are statistically significant at $p < 0.05$ (two-way ANOVA).

3.1.1. Reproductive Events

Daughter cells that were released during the dark period were uninucleate, containing about 0.04 pg of DNA per nucleus. At the optimal temperature, the first replication round started after about 8 h and was completed at the 12th h of growth in light at both light intensities. The second replication round was terminated by the 18th h, when cell division started. The extent of multiplication differed between the two light intensities, as the DNA content increased about fourfold at low light intensity, and almost eightfold at high light intensity. The content of DNA per cell became correspondingly reduced during cell division between the 20th and 24th hours, when the cell numbers increased about eightfold and fourfold at high and low light intensities, respectively (Figure 1A). In the second cell cycle, the completion of DNA replication again shortly preceded the division of cells, attaining a maximum DNA content per cell just before the release of the daughter cells (Figure 1A,B). At high light intensity, DNA replication occurred earlier and to a greater extent than at the low light intensity. Similarly to the first cell cycle, nearly three replication rounds of DNA occurred at high light intensity, followed by division into mostly eight daughters, while at lower light intensity only two replication rounds and division into four daughter cells occurred during the cell cycles (compare Figure 1A,B).

The reproductive events were completely inhibited in the cultures grown at the supra-optimal temperature (40 °C), which was demonstrated by constant values of DNA content (Figure 1A), and number of cells (Figure 1B) corresponding to the initial values at the beginning of the first cell cycle.

3.1.2. Growth Processes

Growth was characterized as changes in the cellular content (pg cell⁻¹) of total RNA (Figure 2A), proteins (Figure 2B), dry matter (Figure 2C), and cell volume (μm³ cell⁻¹) (Figure 2D). Total RNA content (Figure 2A) in the first cell cycle increased approximately threefold at the low light intensity and almost fourfold at high light intensity within 18 h of growth in cells grown at 30 °C, reaching a maximum just before cellular division, and then decreasing with time as the cells divided to a value corresponding to the RNA content of the daughter cells (Figure 2A). A similar behavior was also noted in the second cell cycle, with the maximum being reached after 36 h, i.e., 18 h after cell division started. However, the rate of RNA content increase was slower in the second cell cycle than in the first one, probably due to a decrease in the mean light intensity (Table 1) due to an increase in cell concentration by the end of the first cell cycle (Figure 2A).

Table 1. Changes in mean light intensities experienced by the cultures during the experiment at different incident light intensities and temperatures.

Incident Light Intensity	Mean Light Intensity (μmol photons m ⁻² s ⁻¹)			
	110 μmol photons m ⁻² s ⁻¹		500 μmol photons m ⁻² s ⁻¹	
Temperature	30 °C	40 °C	30 °C	40 °C
Time (h)	0	70 ± 5	75 ± 5	332 ± 15
	24	56 ± 5	54 ± 7	274 ± 10
	42	50 ± 5	50 ± 5	186 ± 10
	48	50 ± 5	50 ± 5	177 ± 10

Similar kinetics were also observed in the time courses of protein content (Figure 2B), dry matter (Figure 2C), and cell volume (Figure 2D) at 30 °C. The growth characteristics (RNA, protein, and dry matter) were slowed, and attained a lower maximum content at the lower light intensity of 110 μmol photons m⁻² s⁻¹ than at the higher intensity of 500 μmol photons m⁻² s⁻¹. Within the second cell cycle, the differences between these variants were less apparent, and growth rate was suppressed by an increase in cell concentration, and a consequent decrease in mean light intensity (Table 1). Cell volume multiplied about

eightfold (Figure 2D) in proportion to the number of daughter cells liberated at the end of both cell cycles at both light intensities (Figure 1B).

Completely different kinetics of growth processes occurred in cultures grown at 40 °C. As described above, the reproductive processes (events) at this temperature were inhibited (Figure 1), but the growth processes continued undisturbed for the duration of two cell cycles of the control cultures grown at 30 °C (Figure 2). The cells reached a giant size corresponding to about five doublings (32-fold increase) in cell volume within 36 h (Figure 2D). Within the same time period, protein content attained three doublings (8-fold increase; Figure 2B), dry matter attained four doublings (16-fold increase, Figure 2C), and RNA content increased 8-fold (Figure 2A). The proportions of different components of the cells differed compared to control cells.

3.1.3. Energy Reserves

The biochemical analyses of starch content solely detect net starch, i.e., the total amount of starch produced in light minus starch consumed in light. Cells of the control culture grown at high light intensity increased their starch content to about 16 times the daughter cells' content within 12 h of growth in the first cell cycle (Figure 3A). At the end of the cell cycle, the starch was extensively degraded, until it reached the values present in the daughter cells. At lower light intensity ($110 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), accumulation of starch to its maximal value during the growth phase was slower, and it attained a lower maximum than in the culture grown at high light intensity ($500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Figure 3A). In the second cell cycle, the synthesis of starch decreased due to an increase in cell concentration caused by mother cell divisions (Figure 1B). This slowed the accumulation of starch so that it only covered the requirements of metabolism, but did not accumulate (Figure 3A).

Due to inhibited reproductive processes at 40 °C, the net starch content did not decrease at the time of cell division in controls. Consequently, starch overaccumulated to between 508- and 254-fold compared to the initial content in the daughter cells, and to about 32-fold the maximum values found in the control cells (Figure 3A). The rate of accumulation at the lower light intensity was slightly slower, and the final content of overaccumulated starch was lower ($77 \text{ vs. } 104 \text{ pg cell}^{-1}$) (Figure 3A).

The secondary energy reserve, neutral lipids, did not accumulate at the optimal temperature of 30 °C. The lipid content remained fairly constant, and decreased during the second cell cycle (Figure 3B). Contrary to growth at 30 °C, lipid reserves at 40 °C increased from the middle of the first cell cycle until the end of the second cell cycle (Figure 3B). Lipid accumulation was delayed at low light intensity, but the final values—about a 64-fold increase compared to the initial values—were comparable between both light intensities (Figure 3B).

3.2. Growth in Deuterated Water

Stress caused by growth at the supra-optimal temperature affected the cell composition, and significantly increased the content of both starch and lipids. Since increased temperature increases kinetic energy—leading to a seeming decrease in both dissociation and activation energies, which are both affected by the presence of deuterium—we tested whether the combination of increased temperature and the presence of deuterium would affect cell growth and accumulation of energy reserves. Synchronized cultures of *P. kessleri* were grown at the optimal temperature of 30 °C and two higher temperatures: 35 °C, and 39 °C. In contrast to the experiments described above, for the experiments in deuterated water, the temperature of 39 °C was used as the supra-optimal one; this was due to the fact that combination of the higher temperature (40 °C) and deuterated water strongly affected cell vitality and viability, but the effect of 39 °C on deuterated cultures (and control cultures) was similar to the effect of 40 °C. As high light intensity, in combination with the presence of deuterium, increases cellular stress [30], only a low light intensity of $110 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was used for these experiments. Due to the inhibitory high price of

deuterated water, only cellular division was followed, and growth was assessed by changes in optical density at 750 nm (OD_{750}) and cell volumes (Figure 4). The stress caused to the photosynthetic apparatus was estimated as quantum yield.

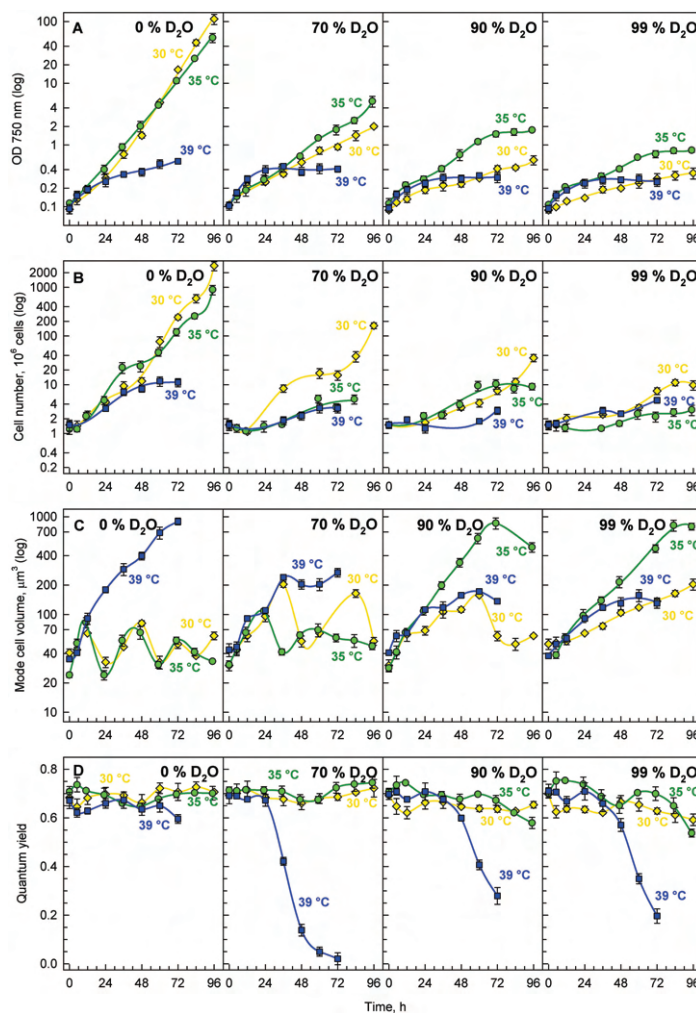


Figure 4. Time course of cell division and growth in synchronized cultures of *Parachlorella kessleri* grown at an incident light intensity of $110 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and temperatures of 30 °C (yellow diamonds), 35 °C (green circles), and 39 °C (blue squares), with different concentrations of deuterated water in the medium (0, 70, 90, and 99%). (A) Optical density at 750 nm, (B) cell number (10^6 mL^{-1}), (C) modal cell volume, and (D) quantum yield. Note the logarithmic scale on the Y-axis for (A–C). Note that the cultures were maintained in semi-batch mode. However, both panels (A) and (B) show recalculated values multiplied by individual dilution factors. Thus, they represent a projection of the growth in semi-batch cultivation mode to the continuous growth. This way, the plots remain easily readable.

3.2.1. Growth and Division

Synchronous cultures of *P. kessleri* grown in deuterated water showed a concentration-dependent decrease in their growth rate (Figure 4A). Apart from decreased growth rates, changes in cell numbers (Figure 4B) indicated decreased division rates in deuterated cultures. This was further supported by observed changes in mode cell volume (Figure 4C), which reflected cell cycle progression in synchronous cultures. At 30 °C, the culture in 0% D₂O (Figure 4C, first panel, yellow line) completed three full cell cycles, as illustrated by three sequences of cell volume increase and decrease, while there were only two cell cycles completed in 70% D₂O (Figure 4C, second panel, yellow line), one full cell cycle in 90% D₂O (Figure 4C, third panel, yellow line), and only growth—not followed by cell division—which was characteristic for the majority of cells in 99% D₂O (Figure 4C, fourth panel, yellow line).

Interestingly, the effects of temperature varied between different deuterated cultures. In the control culture, temperatures of 30 and 35 °C showed similar performance, both in terms of growth (Figure 4A, first panel) and cell cycle progression (Figure 4B,C, first panels), while at a temperature of 39 °C cell division was clearly inhibited, as documented by the occurrence of abnormally enlarged cells at 39 °C (Figure 4C, first panel, blue line), accompanied by significantly lower cell numbers (Figure 4B, first panel, blue line). This phenotype recapitulates what is described in more detail above. In deuterated cultures, the best performance, in terms of growth measured as OD₇₅₀, was obtained at 35 °C (Figure 4A, green lines). However, such a performance was mainly caused by an increase in cell volume under such conditions—especially in 90% and 99% D₂O (Figure 4C, green lines). At the same time, division was more adversely affected by cultivation at 35 °C (Figure 4B, green lines), as fewer cells were produced during cultivation. The adverse effect of cultivation at 39 °C was especially pronounced in quantum yields (Figure 4D, blue lines), which points to severe impairment of photosynthetic efficiency in such cultures.

In order to quantify and compare the effects of different deuterium concentrations (and their combination with tested temperatures), mass doubling time and cell number doubling time were calculated (Table 2). Mass doubling time increased with increasing D₂O content in the growth medium, thus proving the adverse effect of D₂O on growth. At the same time, cell number doubling time also increased with increasing D₂O content in the medium, thus proving the adverse effect of deuterium on division. This trend applied to all temperatures tested; however, at 39 °C, it was the weakest, as such a temperature already poses severe stress—even for the control, undeuterated culture. Moreover, the effect of increasing D₂O concentration on growth seemed to be more pronounced than the effect on cell division at 30 °C, but not at 35 and 39 °C (Table 2).

3.2.2. Energy Reserves

The combined effects of different deuterium concentrations with optimal and supra-optimal temperatures affected both starch and neutral lipid accumulation. After 24 h of cultivation, the starch content was increased in all variants except for the control culture grown at 30 °C (Figure 5, compare A with B–D and I–L). The degree of starch accumulation at 30 °C generally increased with increasing D₂O concentration—except for 99% D₂O, which contained the lowest amount of starch among cultures grown at 30 °C (Figure 5, compare panels A–D); at 39 °C, the net starch content was comparable in all variants (Figure 5I–L). The neutral lipids content was negligible in the control cultures grown in 0% D₂O (Figure 5E), but it started to accumulate in some of the cells grown at 39 °C (Figure 5M), thus repeating the behavior described above (Figure 3). The deuterium-treated cultures had a higher content of neutral lipids, and their content increased with increases in both temperature and deuterium concentration from 70% D₂O to 90% D₂O (Figure 5). The cells grown at the highest deuterium concentration (99% D₂O) contained slightly less neutral lipids than those grown in 90% D₂O, but both of the cultures contained neutral lipids in almost all cells at 39 °C, which was in contrast to 70% D₂O, where only about half of the cells contained detectable neutral lipids (Figure 5, compare O and P with N).

Table 2. Comparison of mass and cell number doubling times for cultures grown in media containing different concentrations of deuterated water at different temperatures. The average of at least three experiments is shown.

Concentration of Deuterated Water (%)	Temperature (°C)	Mass Doubling Time (h)	% of Control	Cell Number Doubling Time (h)	% of Control
0	30	9.58	100.00	8.85	100.00
	35	10.66	100.00	9.99	100.00
	39	28.18	100.00	24.84	100.00
70	30	22.97	239.67	14.18	160.25
	35	17.01	159.54	28.90	289.35
	39	36.48	129.46	63.35	255.00
90	30	32.23	336.30	21.00	237.37
	35	24.01	225.27	36.35	363.91
	39	43.92	155.86	73.63	296.38
99	30	42.34	441.87	35.35	399.54
	35	35.32	331.37	92.33	924.32
	39	50.61	179.58	63.51	255.66

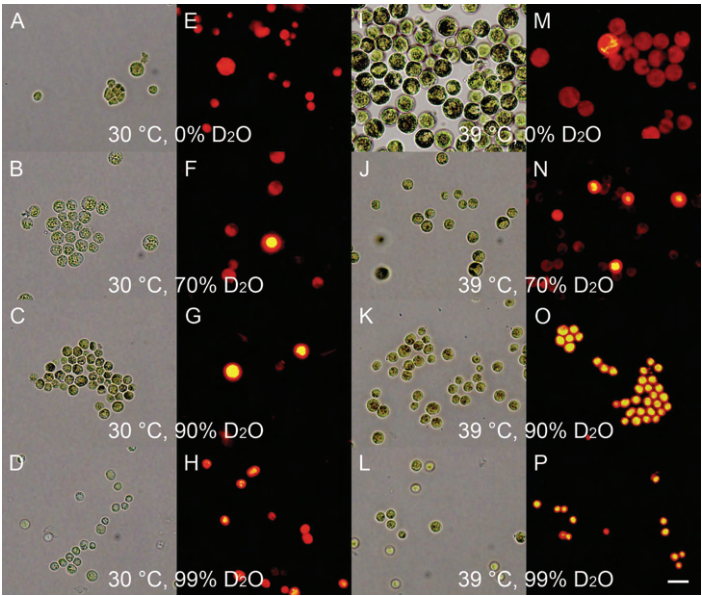


Figure 5. Light and fluorescence photomicrographs of synchronized cultures of *Parachlorella kessleri* grown for 24 h at an incident light intensity of 110 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, temperatures of 30 °C and 39 °C, and different concentrations of deuterated water in the media (0, 70, 90, and 99%). (A,E,I,M) control cultures grown in 0% D₂O, (B,F,J,N) cultures grown in 70% D₂O, (C,G,K,O) cultures grown in 90% D₂O, and (D,H,L,P) cultures grown in 99% D₂O. In (A–D) and (I–L) the cells were stained with Lugol’s solution to visualize starch; for (E–H) and (M–P) the cells were stained with Nile red to visualize neutral lipids. Scale is 10 μm .

4. Discussion

Parachlorella kessleri is an alga with emerging biotechnological potential, particularly because it can produce both starch as a primary energy store and neutral lipids as a secondary one [23,32]. Here, we used synchronized cultures of *P. kessleri* to analyze the effects of supra-optimal temperature on the production of starch and neutral lipids, both in the presence of normal (hydrogenated) water and of deuterated (heavy) water. We chose to use synchronized cultures where all of the cells within the cultures were in the same phase of the cell cycle. This allowed us to identify any subtle differences in the behavior of the supra-optimal-temperature-treated cultures and, thus, better characterize the effects.

The application of different light intensities to the control culture at 30 °C led to different degrees of multiplication of DNA, and to the production of different numbers of daughter cells: four daughter cells at low light intensity, and eight daughter cells at high light intensity (Figure 1). Interestingly, the number of daughter cells produced was not significantly affected by the decreasing light intensities in the second cycle. At the low light intensity, about 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the beginning of the first and 56 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the beginning of the second cell cycle were both sufficient for division into four daughter cells (Table 1). Similarly, for division into eight cells, a sufficient mean light intensity was 332 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the beginning of the first cell cycle and 274 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the beginning of the second. This ability to divide into multiple daughter cells, even at low light intensities, allows the alga to grow and divide at high cell densities when the majority of incoming light is shaded [68,69], making it a highly efficient species for algal biotechnology [70,71]. Although the number of daughter cells produced was not affected, the different incident light intensities affected the timing of the reproduction processes, so that DNA replication and cell divisions in the second cell cycle were delayed by 4 and 2 h, respectively (Figure 1). Moreover, the daughter cells produced at the end of the second cell cycle were smaller than those at the end of the first cycle, and contained fewer proteins than the cells at the beginning of the experiment (Figure 2). As the RNA and starch contents per cell stayed approximately the same at 30 °C (Figures 2 and 3), the cell composition was clearly altered by light intensity. Furthermore, cells at high light intensity were, in both cell cycles, richer in protein compared to the low-light-grown cells (Figure 2B). This phenomenon might be exploited when a protein-rich algal biomass is required [68,72]. Altogether, this documents the plasticity of the multiple fission lifestyle, which allows the cells to slightly prolong the growth phase in order to be able to reach a critical cell size for starting another reproductive sequence, and to maintain the reproduction potential at the expense of producing slightly smaller daughter cells with altered cell compositions. Such growth and metabolic plasticity of algae allows them to thrive both at low light intensities [68,69], and in the harsh conditions of a desert, with very high light intensity and extreme fluctuating temperatures [73–76].

Growth at the supra-optimal temperature led to a blockage of DNA replication and cell division (Figure 1), an approximately 30-fold increase in cell size (Figure 2D), and overaccumulation of both starch and lipids (Figure 3). The cell cycle arrest at supra-optimal temperatures has been established both in the related alga *Chlorella vulgaris* [18] and in the distantly related *Chlamydomonas reinhardtii* [17,77]. However, the nature of the blockage seems to be different in *P. kessleri*, since no DNA replication was detected (Figure 1), which is in contrast to both *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, where (at least some) DNA replication occurred and the cell cycle blockage took place at the level of nuclear (and cellular) division(s) [17,18,77]. Indeed, it has been established that individual cell reproduction processes are sensitive to stress in an order opposite to their occurrence in the cell cycle, with cell division being the most sensitive process [16]. Thus, the observed behavior may also be caused by the supra-optimal temperature used, and if a lower temperature is used, DNA replication might not be affected. In the experiments presented, both supra-optimal temperatures of 39 °C and 40 °C caused the same blockage of cell division (Figures 1 and 4), suggesting that the temperature of 40 °C is well above the threshold required to cause the cell cycle blockage. However, the effects of temperature

are very precise and subtle, so a 1 °C variation in temperature can have the quite distinct effect of only a partial blockage [17,77]. Of the three effects of supra-optimal temperature treatment, the most striking was the approximately 32-fold overaccumulation of starch compared to the highest content reached in the control cells, and the approximately 508-fold increase compared to the initial cell content. The accumulation was very fast, as close to maximal values were reached within about 24 h at the high light intensity, and within about 36 h at the low light intensity (Figure 3A). At the optimal temperature, the net accumulation of starch was slower, and reached lower levels at the low light intensity; this phenomenon was even more pronounced at the supra-optimal temperature (Figure 3). This stresses the importance of light intensity for starch (over)production, and is consistent with the established knowledge in the field [38]. The starch overaccumulation at supra-optimal temperatures was consistent with the previous experiments on the related *Chlorella* sp. K [78], *Chlorella vulgaris* [18] and distantly related *Chlamydomonas reinhardtii* [17,77]. The overaccumulation of neutral lipids was delayed by about 16 hours compared with that of starch (Figure 3). This fits with the two-step production of starch, followed by neutral lipids, as stress conditions are prolonged [23,24,32,39]. Lipid production also proved to be light-dependent, as it was about six hours delayed at the low light intensity (Figure 3B). Although the lipids overaccumulated to about 64 times the initial values, their final cell content was much lower than that of starch (Figure 3), making starch the predominant component of the cell. Indeed, cell composition was strikingly affected by the supra-optimal temperatures. At the high light intensity, the cell volume (Figure 2D) increased about 30-fold, dry matter (Figure 2C) increased about 19-fold, RNA content more than 8-fold (Figure 2A), and protein content by about 32-fold (Figure 2B). Together with a massive 508-fold increase in starch, this made the biomass significantly enriched in starch and proteins. In general, starch (over)production can be attained either through increasing starch production by improving growth conditions (high light, high CO₂ concentration) [36,37] or by decreasing starch consumption—mainly by blocking cell reproduction as the biggest starch consumer under nutrient-limiting conditions [24–26,32,39,41]. Indeed, a blockage of cell division has emerged as an effective tool to (over)produce starch [79]. Improved starch production by optimizing growth conditions, combined with decreasing its consumption due to inhibition of the major consumers, should lead to superior starch (over)accumulation. Rapid and extensive starch (over)production by supra-optimal temperature treatment was shown in the green alga *Chlamydomonas reinhardtii* in both laboratory-scale [17] and pilot-scale photobioreactors [38]. Compared to *Chlamydomonas reinhardtii*, *P. kessleri* grows at lower light intensities, and is able to reach very high cell densities [70,71]. Together with the ability to produce significant amounts of neutral lipids via a simple alteration of conditions, this makes it an excellent organism for algal biotechnology. The experiments presented here provide a means for the fast and reliable overproduction of starch by increasing the temperature to a supra-optimal one. Such a treatment is very simple, but a rapid and controllable increase in temperature, and maintenance of that temperature, might be complicated at a very large scale. Alternatively, the treatment can be used in closed pilot-scale reactors [38], where temperature control is more feasible. Such production would only be justifiable for high-added-value compounds, such as stable isotopically labeled ones.

The experiments on the combined effects of deuterium and temperature had a double rationale: Firstly, from a basic research point of view, it is intriguing to analyze the combination of deuterium, which increases bond dissociation and activation energies [46], with increased temperature, which is known to increase kinetic energy and, thus, speed up general metabolism. Secondly, (over)production of deuterated compounds such as starch, proteins, and/or neutral lipids is biotechnologically relevant, as these are very high-added-value products that can be exploited both as biochemical standards and in stable isotope-labeling experiments [43,52,53]. When starting the experiments, it was not clear which of the two phenomena would prevail. We assumed that the temperature increase might decrease the effect of the presence of deuterium, and at the same time that

the deuterium might prevent the blockage of cell division observed in hydrogenated water at supra-optimal temperatures. As it turned out, some of our assumptions were confirmed, while others were not. Cultivation in increasing concentrations of deuterated water showed a concentration-dependent decrease in growth rates and cell division (Figure 4A,B). Interestingly, although the difference in growth rates between 30 °C and 35 °C in the control was negligible (Figure 4A, first panel), the cultivation at 35 °C promoted growth in all deuterated cultures (Figure 4A, second to fourth panels, green circles). This partially confirmed the prediction that increased temperature might alleviate the effects of deuterium on the cells. However, the effect was specific only to growth, and was not reflected in cell division (Figure 4B, green circles), as the cells remained large (Figure 4C, green circles). This implies that although the effects of deuterium on metabolism in general can be partially compensated for by temperature, the deuterium-induced problems with cell division are either more sensitive or more specific. A further increase in temperature to 39 °C inhibited both cell growth and division (Figure 4A,B, blue squares). The effect of supra-optimal temperatures was more detrimental to the deuterated cultures than to the controls, suggesting that the alleviating effect of 35 °C was replaced with a strictly stress effect. Due to the combination of the two stresses, the same temperature considered to be supra-optimal in control cultures might be above this threshold for highly deuterated cultures. Thus, temperature in deuterated cultures showed aspects of hormesis—a phenomenon where low concentrations of a compound are beneficial but higher levels cause stress [80,81]. The significant stress caused to the deuterated cells by the supra-optimal temperatures was further supported by a rapid decrease in the Fv:Fm ratio in all deuterated cultures after about 36 h of the experiment (Figure 4D, second to fourth panels, blue squares). This was in contrast to the control cultures at the same temperatures (Figure 4D, blue squares), which were not significantly affected. The cultures of *P. kessleri* showed striking resilience to the presence of deuterium, as they were not significantly stressed by growth in very high deuterated water concentrations for more than three days (Figure 4D, yellow and green lines). Only on the fourth day of cultivation did the cultures at the two highest deuterated water concentrations (90% and 99%) start to be stressed (Figure 4D, third and fourth panels). However, the absence of cell division at the higher deuterated water concentrations allowed (over)accumulation of starch, although the temperature did not seem to have a promoting or inhibiting effect in such cultures (Figure 5, compare B–D with J–L). The combination of supra-optimal temperatures and growth in deuterated water promoted the accumulation of neutral lipids (Figure 5, panels N–P). These experiments confirmed that the effect of supra-optimal temperatures is a general phenomenon not only in different organisms [17,18,77], but also in different growth conditions. If desired, such treatment can be used to (over)produce deuterated starch and, interestingly, also deuterated neutral lipids. Due to limitations in maintaining supra-optimal temperatures and the inhibitive high prices of deuterated water, such treatment is conceivable only at small scales for the production of stable isotopically labeled compounds [43,52,53]. This is consistent with the proposed algal production of deuterated sugars and amino acids [58], proteins [59], chlorophylls [52,60], and carotenoids [52]. Supra-optimal temperature seems to be a promising tool for a rapid increase in starch (and neutral lipid) content, compared to the slower effect of nutrient starvation [43]. Furthermore, it offers recovery of other deuterated cell components.

5. Conclusions

We describe the effects of supra-optimal temperatures on synchronized cultures of the green alga *P. kessleri*. Supra-optimal temperatures block DNA replication and cell division, but do not affect cell growth. Moreover, due to the absence of cell division, starch and neutral lipids overaccumulate to levels 508-fold and 64-fold those of the initial values in the daughter cells. This leads to the production of large cells with altered cell compositions, consisting mostly of proteins and starch. We tested whether increased cultivation temperatures could alleviate the effects of deuterium in heavy water. Temperature showed

a hormetic effect, as a temperature increase over optimum improved cell growth, but the supra-optimal temperatures caused extensive stress. The cultures were strikingly resilient to the stress caused by heavy water, and were able to grow and divide even at 90% D₂O, although they were only able to grow but not divide at 99% D₂O. The supra-optimal temperature treatment, when applied to cultures grown in heavy water, had similar effects as in normal water, and led to the production of cells with increased starch and neutral lipid contents. Our results establish new treatments for the overaccumulation of starch (and proteins) in *P. kessleri*, and they also prove that such treatment can be used to produce deuterated starch, proteins, and neutral lipids for biotechnological purposes.

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Article IV.

Analysis of commitment point attainment in algae dividing by multiple fission

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Chapter 5

Analysis of Commitment Point Attainment in Algae Dividing by Multiple Fission

Veronika Kselíková, Vilém Zachleder, and Kateřina Bišová

Abstract

This work represents a detailed guide for commitment point analysis in microalgae dividing by multiple fission. The method is based on allowing the committed cells to divide in favorable conditions in the dark. This protocol offers a strategy to monitor cell cycle progression, both in control cultures and cultures treated with compounds affecting cell cycle length and/or progression. As the variety of such compounds is wide, our aim was to make the protocol easily modifiable to various research aims. The technique is easy to follow, low-cost, does not require any special equipment and offers reliable results in a reasonable time. The protocol offers step-by-step instructions, explains the theory behind these steps and offers solutions to some of the problems that may arise during the procedure.

Key words Cell cycle, Cell division, *Chlamydomonas*, Commitment point, Green algae, Multiple fission

1 Introduction

Cell division is one of the key processes in cell biology. The most common division model is binary fission, where one mother cell gives rise to 2 daughter cells. The cell cycle of the binary fission model follows G1, S, G2, and M phases, as described by Howard and Pelc [1]. From the very beginning of cell cycle research, green microalgae were recognized as valuable model organisms [2] because they can be easily synchronized using alternating light and dark periods to very high levels of synchrony. In green algae, cell division may adopt two patterns: binary fission as described above or multiple fission, in which 2^n daughter cells arise from a single mother cell, where n is the number of divisions within one cell cycle. In the case of the multiple fission cell cycle model, the phases of the cell cycle can be described as a growth sequence (G1) and DNA replication-division sequences (including S, G2, and M phases) [3]. During the growth phase, cells absorb light

energy, form energy reserves and increase their size until achieving a distinct time period denoted as commitment point (CP). The CP marks the moment, from which the cell is “committed” to divide; in other words, the cell is able to divide without further energy supply and growth [4]. Autotrophically grown cells in their pre-CP and post-CP periods can therefore be distinguished by their ability to perform cell division in the dark [5]. In the cell cycle of algae dividing by multiple fission, multiple growth and DNA replication-division sequences overlap. When light is available, growth continues, multiple CPs are attained, and multiple DNA-replication-division sequences occur. The cell divisions of the early DNA-replication-division sequences are postponed until the end of the cell cycle. Moreover, there are at least two types of multiple fission cell cycle among green microalgae: Scenedesmus type and Chlamydomonas type (Fig. 1). In the Scenedesmus type, the cells replicate DNA and divide their nuclei shortly after attaining CP (s) and multinuclear cells are formed. All cell divisions that were committed during the cell cycle are then performed almost simultaneously at the end of the cell cycle [6]. The Chlamydomonas cell cycle type is also characterized by attaining multiple CPs in one cell cycle but in contrast to Scenedesmus type, no multinuclear cells are formed. In this case, DNA replications and nuclear divisions are postponed to the end of the cell cycle and each round of DNA replication and nuclear division is immediately followed by cell division. The intriguing nature of the cell cycle and multiple fission was thoroughly reviewed by Bišová and Zachleder [7] and Zachleder et al. [6].

There are many procedures or agents that affect cell cycle length and/or progression in green microalgae. Altering the light intensity used to illuminate the culture is the easiest way to do so. With decreasing light intensity, the energy supplied to individual cells in the culture decreases, hence lowering the growth rate of the autotrophically grown culture. Temperature alterations work in a similar manner; generally a lower temperature will slow growth [8] and decrease the number of DNA replication-division sequences in the cell [9]. The tolerated temperature range is species specific. An increase in temperature to sub-lethal levels might stop the cell cycle altogether. Nevertheless, this arrest is reversible upon a shift to physiological conditions [10]. Apart from physical methods of altering the cell cycle length/progression, there are numerous chemical substances that have an effect on the cell cycle. These may be represented by specific inhibitors [11, 12] or by compounds with a more diverse effect such as metal ions [13] or heavy water (D_2O), i.e., water containing a heavier isotope of hydrogen, deuterium. In the case of D_2O , the growth rates are lower and division is (partially) blocked in a concentration-dependent manner [14]. This protocol was originally developed to monitor the effects of D_2O on the growth and division of green microalgae.

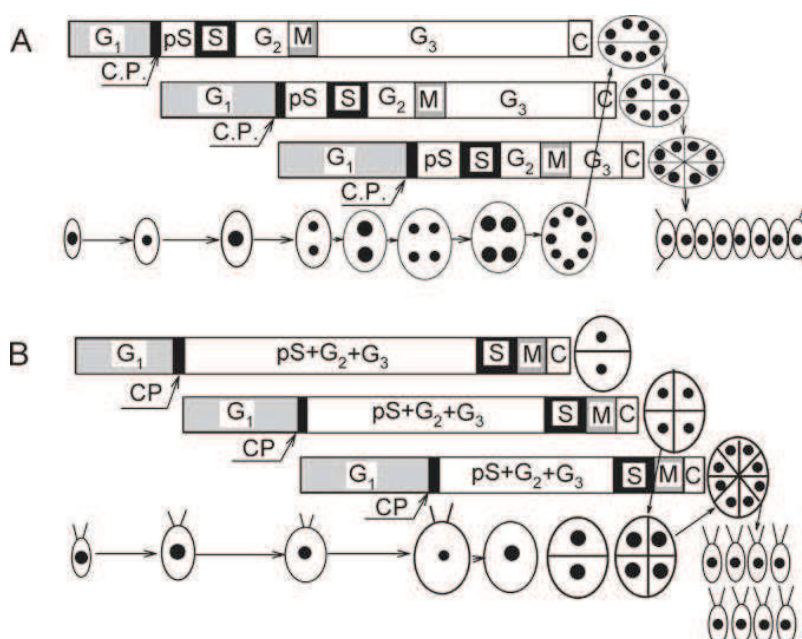


Fig. 1 Schematic diagram of multiple fission to 8 daughter cells in *Scenedesmus* type (a) and *Chlamydomonas* type (b) cells. Individual growth and DNA replication-division sequences are schematically represented by three partially overlapping bars describing individual cell cycle phases. Below, increase in cell size is indicated as well as the number of nuclei within the cell (number of black dots within cells) and DNA replication (size of black dots within cells). The difference between *Scenedesmus* and *Chlamydomonas* type in respect to timing of nuclei and cell divisions is apparent. Modified after Zachleder et al. [6]

The aim of this work is to enable the wider scientific community to analyze commitment point attainment in green microalgae by providing step-by-step instructions. The method described in this protocol is based on allowing the committed cells to divide under favorable conditions in the dark. To distinguish which cells are committed at a specific time point, samples from the synchronous, autotrophically grown culture are transferred to an agar plate in the dark. By putting the samples in the dark, the energy supply to the cells is stopped and as a result no more growth is possible. Therefore, only cells that have reached their CP(s) while in the light, are able to undergo cell division(s). Moreover, the cells are immobilized on agar, so that daughter cells rising from a single mother cell remain close together. The proportions of the cells that passed their first, second, third, or every other CPs can be then easily recognized. The protocol is designed to follow CP(s) attainment in control and two treated cultures (with treatment affecting cell cycle length and/or progression). In addition, the effects of treatments are analyzed individually in the pre-CP and post-CP periods. The protocol is then supplemented with additional notes that offer further explanations of instructions as well as useful tips for optimizing the procedure to individual needs.

2 Materials

2.1 Organisms

A wide variety of algal species dividing by multiple fission can be used to study the cell cycle. In our laboratory, we have been routinely using wild-type strains listed below. Nevertheless, a similar protocol might be used for other organisms and/or strains; however, it must be kept in mind that the use of different strains and different growth conditions might result in changes in the cell cycle length and progression. Therefore, the protocol and growth conditions need to be optimized for every organism individually.

2.1.1 *Chlamydomonas reinhardtii* strain 21gr

C. reinhardtii strain 21gr (listed as CC-1690) was obtained from Chlamydomonas Resource Center, University of Minnesota, St. Paul, MN.

2.1.2 *Desmodesmus quadricauda*, syn. *D. communis*, strain Greifswald/15

D. quadricauda strain Greifswald/15 was obtained from Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany of the AS CR, Třeboň.

2.1.3 *Parachlorella kessleri* strain NIES-2152

P. kessleri strain NIES-2152 was obtained from Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan.

2.2 Medium

2.2.1 *Chlamydomonas reinhardtii* strain 21gr

C. reinhardtii was cultivated in HS medium [15] with modifications described by Hlavová et al. [16]. For plate preparation, 15 g/l agar was used to solidify the medium.

2.2.2 *Desmodesmus quadricauda*, syn. *D. communis*, strain Greifswald/15

D. quadricauda was cultivated in half concentration ŠS medium [17]. This modification and detailed instructions on its preparation are described by Hlavová et al. [16]. Solid media were prepared using 15 g/l agar.

2.2.3 *Parachlorella kessleri* strain NIES-2152

P. kessleri was cultivated using half concentration of ŠS medium as described in Subheading 2.2.2.

2.3 Growth Conditions

The cells of all strains used in this study were grown in a cylindrical glass vessel with an inner diameter of 2.5 cm and a volume of 300 ml. The vessels were submerged in a tempered aquarium and illuminated from one side by fluorescent tubes with adjustable light intensity. For synchronization, the suggested light intensity is $500 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ to prevent shading of the cells and temperature of 30 °C. The culture was aerated and mixed by bubbling with 2% (v/v) CO_2 in air, with a flowrate of approximately 1.3 l h^{-1} .

3 Methods

3.1 Preparing Synchronous Cultures

1. Inoculate 300 ml of the fresh medium (*see Note 1*) suitable for a chosen organism with about a full-loop of the culture from a plate.
2. Place the inoculated culture in a cylindrical glass vessel in a tempered aquarium and let it grow in light for about 24 h. For suggested light intensity and temperature, go to Subheading 2.3.
3. Darken the culture for about 10–12 h, enabling all the cells that reached their commitment point(s) while in the light, to divide and release their daughter cells (*see Note 2*).
4. Place the culture in light again and check the cells under a microscope at regular intervals.
5. When approximately 10% of the cells undergo their first protoplast division, place the culture into the dark.
6. Repeat **steps 3** and **4** for at least three consecutive cycles. The level of synchrony in the population should increase with every cycle.
7. Continue repeating **steps 3** and **4** until the synchrony reaches about 95% of the whole population. For further information on prolonged synchronization, *see Note 3*. The light/dark intervals established during this process can be then used with advantage in further experiments as they are consistent for a specific organism under given growth conditions.

3.2 Affecting Cell Cycle Length/Progression

In this protocol, the scheme for a control (C) and two experimental lines (T1, T2) treated with the cell cycle-affecting agent will be presented. As this protocol was developed to monitor the effect of D₂O on growth and division of microalgae, we assume that a cell cycle-affecting agent is a substance present in the growth medium. The protocol presumes following each experimental treatment in biological triplicate within a single experiment. However, such a setup is optional and alternatively only a single biological replicate per each treatment can be analyzed.

1. Prepare a synchronous culture following the instructions in Subheading 3.1.
2. Count the number of cells in 1 ml of the culture.
3. Establish the volume of synchronous culture needed to start each of the experimental lines. The final cell density should be between 1×10^6 and 1.5×10^6 cells·ml⁻¹ for each experimental line (*see Note 4*).

4. Transfer the volume of the synchronous culture established in **step 3** to Falcon tubes and centrifuge gently at $1700 \times g$ for 5 min.
5. Remove the supernatant and resuspend each tube in the desired volume of the cultivation media—control and both treatments (*see Note 5*).
6. Place the cultures prepared in **step 5** in the growth vessels and let them grow in the light under selected growth conditions.
7. Sample 1 ml of the culture into 1.5 ml tubes at regular intervals (*see Note 6*) both from control and treated cultures to monitor progression of their cell cycle.
8. After each sampling, progress immediately to assessing CP attainment.

3.3 Assessing Commitment Point (CP) Attainment

1. Immediately after sampling, aliquot each of the samples into a 96-well plate to dilute them (*see Note 7*). If working in biological triplicates, we suggest to work on C, T1 and T2 triplicates individually, as it reduces the risk of error. Dilute all samples both 5 and 10 times to a final volume of at least 100 μ l. It is important to dilute the samples with their respective medium (C, T1 or T2). The dilution ensures that there will be an optimal number of cells to be analyzed.
2. With a multichannel pipette, transfer 3 μ l of the diluted sample from each well onto plates (*see Note 8* for further information on plate preparation) creating spots. A sample from each well is transferred onto three plates—with control medium and both treated media, creating a set of three plates (Fig. 2).
3. Place all three plates in the dark at 30 °C (*see Note 9*).
4. Repeat **steps 1–3** for each triplicate of all experimental treatments. The combinations shown in Fig. 3 will be created.
5. Repeat **steps 2–4** at every sampling time. At each time point, transfer diluted samples to the set of all three plates (C, T1, T2). Using a set of fresh plates for every sampling time is recommended (for exceptions, *see Note 10*).
6. When all the committed cells have divided (*see Note 11* and Subheading 3.4), turn the plate(s) bottom-up and fix the plate(s) by putting a drop of Lugol's solution (5% elemental iodine and 10% of potassium iodide in water) on the lid of the plate. The iodine vapors effectively fix the cells on the plate in several minutes.
7. Score the spots on all plates using a light microscope (*see Note 12*). For tips on storing the plates until analysis, *see Note 13*. Depending on cell density, choose which spots are better to be evaluated (either 5- or 10-times diluted culture). Generally, several groups of cells will be distinguished: (a) the cells that

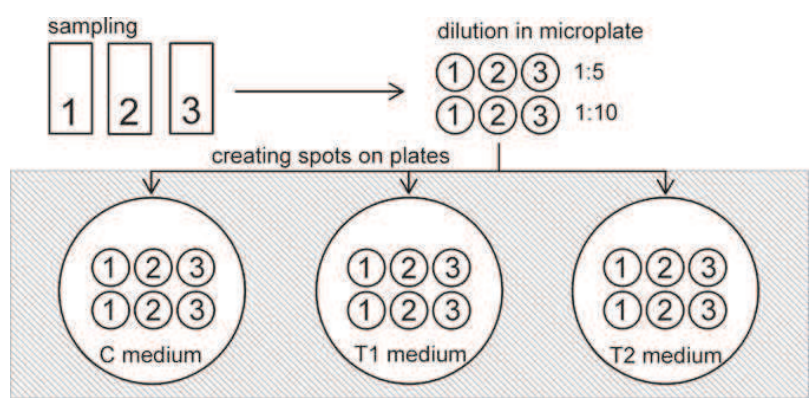


Fig. 2 A schematic diagram of a workflow during sampling for attainment of CP. Here, sampling three biological replicates (1–3) is shown, followed by their dilution with the corresponding medium in the microplate. The diluted samples are then transferred on agar plates with control (C) medium, and media corresponding to experimental treatments (T1, T2). The plates are then incubated in the dark as represented by the gray background

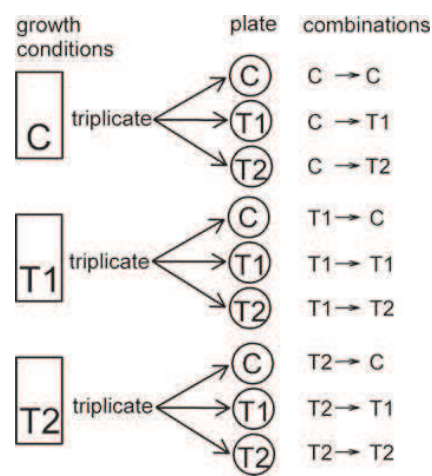


Fig. 3 An overview of the combinations of experimental line-plate created when sampling for attainment of CP. C represents the control untreated culture, T1 and T2 represent individual experimental treatments. The cross-comparison of the treatments during growth and division allows one to differentiate between processes affected by the treatment

did not divide, (b) the cells that divided once, forming 2 daughters, (c) the cells that divided twice, forming 4 daughters, (d) the cells that divided three times, forming 8 daughters, etc. The percentage of cells in each group out of the total number of scored cells has to be established for every combination of experimental line and plate and at each sampling time

(for more thorough explanations, *see* **Note 14**). The cumulative percentage of cells that attained their first, second, and every other consecutive CP can be then plotted as a function of sampling time.

3.4 Establishing Fixation Times for Plates

(in preliminary experiment, for explanation *see* **Note 15**)

1. Start the experimental cultures (C, T1, T2) as described in Subheading 3.2.
2. Sample at least 4 times during the cell cycle as described in **step 7**, Subheading 3.2.
3. At each sampling time, transfer the diluted samples onto plates as described in **steps 1–5** of Subheading 3.3. The only modification to this process is that at every sampling time, the cells should be transferred to multiple (n) plate sets (one set being C, T1, and T2 plates). Therefore, n sets of identical plates will be created at every sampling time (Fig. 4).
4. Each set (C, T1, and T2 plate) of identical plates created in the previous step will be fixed at n different time points of plate incubation (*see* **Note 15**).
5. The proportion of cells that divided into daughter cells is then counted across n fixation times for each combination of experimental line and plate. The best fixation time is then selected as the time when the proportion of divided cells stops to increase with further incubation of the plate.

4 Notes

1. In principle, synchronization of algal cells requires a medium enabling only autotrophic growth, thus preventing the cells from growing in the dark. For this reason, the TAP medium routinely used for cultivation of a wide range of algal species is not suitable because it contains acetate, thus serving as an external carbon source in the dark. On the other hand, both HS and ŠS media, as suggested in this protocol, comply with this requirement.
2. The cells divide in the dark and form daughter cells, which are then released from the mother cell. Sometimes, the release of daughter cells might not proceed in the dark. Such a culture can be put into light without any consequences and the daughters will be released shortly upon illuminating the culture.
3. Sometimes synchronization might take more cycles or the cycles might be longer on their own. In such cases, we recommend to regularly check culture density. In dense cultures, the light availability to individual cells decreases, thus the light/

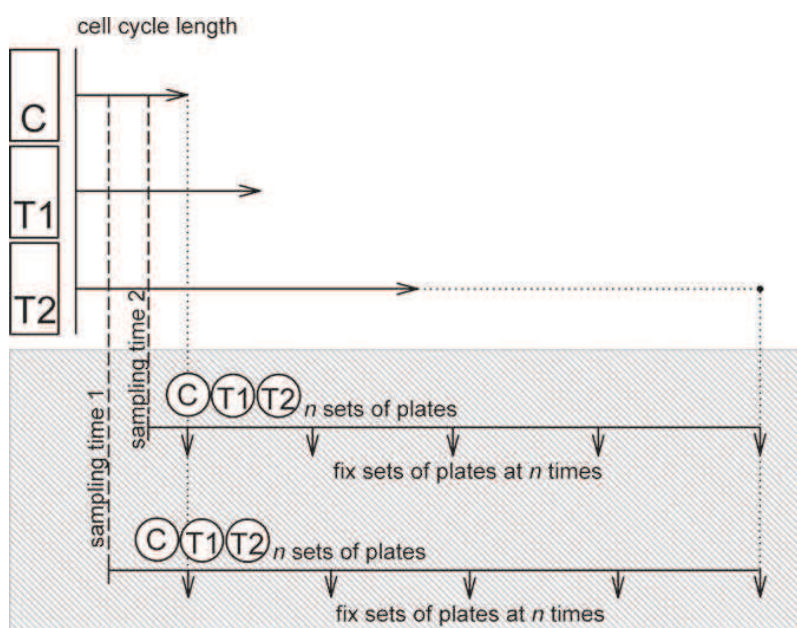


Fig. 4 A schematic overview of establishing fixation times in plates for each experimental line-plate combination. C represents the control untreated culture, T1 and T2 represent individual experimental treatments. Rectangles C, T1, and T2 represent individual cultures, and their cell cycle length is indicated by the length of the adjacent horizontal arrow. Two sampling times are shown in the diagram, represented by dashed vertical lines at two different time points during the cell cycle. In principle, n sets of plates (one set being plates with C, T1, and T2 medium), where n corresponds to the number of fixation times, will be created at every sampling time. One of these sets will then be fixed at each of the n tested fixation times individually, e.g., for testing five fixation times, five sets of plates (with C, T1, and T2 medium) will be created. We suggest testing at least 5 fixation times, ranging from the shortest cell cycle among experimental lines, to double the length of the longest cell cycle within experimental cultures (interval between vertical dotted lines). Other fixation times are to be distributed evenly over the given time interval. The same flow of action will be repeated at the second and every other sampling time tested

dark intervals might be changing over time and the synchrony will be gradually decreasing. It is therefore advised to dilute the cultures regularly. The maximum cell concentration for dilution depends on the growth conditions. Under growth conditions that we routinely use in the laboratory ($30\text{ }^{\circ}\text{C}$, $500\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 2% (v/v) CO_2), we maintain the cells at a maximum cell density of $1 \times 10^6\text{ cells}\cdot\text{ml}^{-1}$ by dilution at the beginning of each cell cycle. Generally, the best time to dilute the culture is just before it is put in the light, when the daughters are already formed.

4. It is strongly advised to increase the estimated cell concentration by about 10% because during centrifugation some cells will be lost. The gentle centrifugation used in this step needs to be a

compromise between losing as few cells as possible but not damaging the remaining cells in any way.

5. The **steps 4** and **5** of Subheading **3.2** are only applicable if all the medium needs to be replaced to induce the effect of the cell cycle-affecting agent, e.g., the water in the medium needs to be replaced with heavy water. If your treatment does not require this, just resuspend the established volume of the synchronized culture in the normal growth medium and add the cell cycle-affecting agent afterwards.
6. The appropriate sampling intervals may vary greatly, even between different treatments of one organism. The main point is to have at least 8–10 time points spread evenly throughout the cell cycle of individual treatments. Hence, we suggest to establish the time required until the completion of cell division in each of the experimental treatments and then to set respective sampling intervals for each of them prior to the experiment.
7. It is convenient to dilute the samples in a 96-well plate as it allows processing more samples at once, keeps the samples organized and makes it easy to transfer the samples onto plates using a multichannel pipette in the following step.
8. Three sets of plates should be prepared—one for each experimental treatment. We highly recommend to use 50 mm Petri dishes, as their size is adequate for placing 6–12 spots. One plate can therefore be used at up to 2 consecutive sampling times. The layer of the medium on the plate should be thin in order to make further evaluation easier. Filling the plates with medium up to 1/4 of their depth (approximately 7 ml for 50 mm plates) is sufficient and should not prevent evaluation. To keep a track of all the plates and spots, paper stencils in the size of the plate with 6 or 12 spots and all the necessary information can be attached to the bottom of each plate by double-side tape.
9. In experiments following long cell cycles, there is a risk of excess drying of plates during incubation. Placing a container with water in the incubator can increase humidity and help to prevent the plates from drying out.
10. If it is desired to decrease the number of plates used (some component of the medium is expensive, not enough space for incubating plates, etc.), one plate can be used for two consecutive sampling times under certain restrictions: firstly, it is crucial to minimize illumination of the plate during the second sampling and secondly the sampling times are not far from each other as this would result in different times of fixation for each of them.

11. There are two contradictory requirements for plate incubation. First, the plates need to be incubated long enough for the committed cells to divide, and second, the longer the plates are incubated at 30 °C, the higher the risk of the plates drying out. Therefore, the most suitable time to fix the plates should be established. There are two ways to do this. First, the cells on the plates can be checked at regular intervals and the proportion of divided cells can be established over time. When the proportion of divided cells does not increase any more, it is time to fix the plates. The drawbacks of this approach are excess manipulation of the plates during incubation, which inevitably leads to illumination of the plates during the dark, and the amount of time needed to check the plates regularly. We suggest to take the second approach, in which the fixation times are established in a preliminary experiment (described in Sub-heading 3.4).
12. The plates can be inserted under the microscope as they are, or the agar layer with the spots can be cut out and transferred onto the slide. By cutting the spots out, the observer has a better access to the spots without a rim of the plate getting in the way of the objective and better illumination of the sample. On the other hand, cutting the agar layer requires extra time and skills, as the thin layer of the agar is prone to tear.
13. The fixed plates can be stored at 4 °C for up to 2 weeks. Nevertheless, it is advised to score the spots on plates as soon as possible. With prolonged storing, the plates dry out and scoring becomes more difficult.
14. The cumulative percentage of cells attaining a specific number of CP(s) is based on counting the percentage of cells dividing to a corresponding number of daughters. At least a hundred cells per sample should be scored. Nevertheless, one has to bear in mind that the CP(s) are attained in a consecutive manner; in order to attain the third CP, the cell needs to go through first and second CPs first. For example, every cell giving rise to 4 daughter cells attained 2 consecutive CPs. Therefore, the cumulative percentage of the cells attaining second CP will be a sum of the percentage of cells dividing to 4 daughters (those that attained 2 CPs) and the cells that divided to 8 daughters (because those also went through second CP) and every cell that divided to more than 8 daughters.
15. The fixation time (= the duration of plate incubation in dark) represents the minimal time needed to finish the cell cycle (= divide and create daughter cells). It must be kept in mind that the fixation times may differ greatly for each combination of experimental line and plate shown in Fig. 3. Therefore, the best times to fix the plates must be selected for each of the

combinations. We suggest testing the fixation times ranging from those that would be sufficient to complete the shortest observed cell cycle (within the experimental lines) to those equal to double the longest observed cell cycle. At least 5 fixation times should be tested to estimate the proper time to fix a specific combination of each experimental line on each plate at every sampling time (Fig. 4).

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Article V.

Cultivation of the microalgae *Chlamydomonas reinhardtii* and *Desmodesmus quadricauda* in highly deuterated media: Balancing the light intensity

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Cultivation of the microalgae *Chlamydomonas reinhardtii* and *Desmodesmus quadricauda* in highly deuterated media: Balancing the light intensity

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The production of organic deuterated compounds in microalgal systems represents a cheaper and more versatile alternative to more complicated chemical synthesis. In the present study, we investigate the autotrophic growth of two microalgae, *Chlamydomonas reinhardtii* and *Desmodesmus quadricauda*, in medium containing high doses of deuterated water, D₂O. The growth of such cultures was evaluated in the context of the intensity of incident light, since light is a critical factor in the management of autotrophic algal cultures. Deuteration increases the light sensitivity of both model organisms, resulting in increased levels of singlet oxygen and poorer photosynthetic performance. Our results also show a slowdown in growth and cell division processes with increasing D₂O concentrations. At the same time, impaired cell division leads to cell enlargement and accumulation of highly deuterated compounds, especially energy-storing molecules. Thus, considering the specifics of highly deuterated cultures and using the growth conditions proposed in this study, it is possible to obtain highly deuterated algal biomass, which could be a valuable source of deuterated organic compounds.

KEYWORDS

microalgae, deuterium, light intensity, cell division, deuterated compounds, physical stress

Introduction

Algal biomass is a source of numerous valuable compounds such as lipids, carbohydrates or carotenoids that are used in biotechnology to produce biofuel (Brennan and Owende, 2010), biopolymers (Brennan and Owende, 2010; Madadi et al., 2021), animal feed (Becker, 2007) or dietary supplements (Eriksen, 2016; Deeba et al., 2020). The compounds can be further exploited by producing their stable-isotope-

labelled counterparts, which is possible by cultivating microalgae on labelled substrates (Gireesh et al., 2001; Bhosale et al., 2006; Saha et al., 2013; Zachleder et al., 2018). Due to the photoautotrophic nature of microalgae, cheap inorganic labelled substrates are sufficient to produce various labelled biomolecules with high added value, making production economically viable. One specific application of this approach is the production of highly deuterated biomolecules in microalgae. Deuterium is a stable isotope of hydrogen with an extra neutron in its nucleus. Compounds labelled with deuterium, along with other isotopically labelled molecules, are commonly used as tracers in ecology, proteomics, and metabolomics (Lehmann, 2016; Zachleder et al., 2018) or in pharmacology to produce deuterated drugs (Kushner et al., 1999; Cargnin et al., 2019). The production of deuterated biomolecules, such as fatty acids or pigments, using microalgae has been achieved in several microalgal species, including *Chlorella* spp. (Delente, 1987; Gireesh et al., 2001; Bhosale et al., 2006). The percentage of deuterium enrichment ranged from 25% in deuterated eicosapentaenoic acid, chlorophyll *b*, lutein, astaxanthin, or β -carotene produced by two biotechnologically important green algae *Haematococcus pluvialis* and *Phaeodactylum tricornutum* (Hattori et al., 1965; Crespi et al., 1968) up to 50% in chlorophyll *a* produced by cyanobacterium *Halomicronema hongdechloris* (Garg et al., 2017) and 58% in lutein produced by *Chlorella protothecoides* (Bhosale et al., 2006). The deuterated compounds produced by microalgae are more valuable than their protonated variants, but their production is fraught with difficulties. Replacement of protium (^1H) with deuterium (^2H or D) elicits a kinetic isotope effect (KIE). This is described as a change in the reaction rates of isotopically substituted atoms or molecules (Bigeleisen and Mayer, 1947). The KIE is a cause of discrimination between lighter and heavier isotopes, as observed in a variety of biochemical reactions, e.g., the well-documented discrimination of RubisCO regarding use of ^{14}C in carbon fixation (von Caemmerer et al., 1997; Kubásek et al., 2013). The isotopic exchange between hydrogen and deuterium exhibits the largest KIE among the stable isotopes of all bioelements, which is due to the large mass differences between hydrogen and deuterium (Wade, 1999). The strong KIE caused by deuterium causes numerous perturbations in cell physiology. The most prominent effects of deuteration are the triggering of a general stress response and the reduction of cell growth and rates of division as already published in several microalgae, including *Chlorella* spp. or *Chlamydomonas* sp. (Unno et al., 1987; Kollars and Geraets, 2008; Zachleder et al., 2021a; Kseliková et al., 2021). Therefore, understanding the stressful effect of deuterium in microalgae is a prerequisite for successful cultivation of microalgae on highly deuterated substrates. Describing the cellular response to deuterium-induced stress will enable an understanding of the consequences of KIE in complex biological systems. This knowledge could then be used to optimize culture

management of deuterated cultures. However, little attention was paid to specific changes in cell cycle progression of deuterated cultures so far. At the same time, necessary alterations of cultures conditions induced by deuterium treatment remains understudied as well.

Light is a critical abiotic factor for microalgal growth, especially in photoautotrophic cultures where microalgae rely on light as the sole source of energy for carbon fixation. Available light can be characterized from two main perspectives - by its quality and quantity. The quality of light describes its spectral composition, with photosynthetically active radiation (PAR) typically between 400 and 700 nm, corresponding to the absorption of the major plant/algal pigments. Light quality, particularly the proportion of red and blue light, affects growth and cell cycle progression (Oldenhof et al., 2004; Li et al., 2021) and could be used in biotechnology to promote the production of valuable compounds such as lipids (Shu et al., 2012; Chavez-Fuentes et al., 2018) or carotenoids (Katsuda et al., 2004; Fu et al., 2013; Lee et al., 2018) in microalgae. The amount of light is usually expressed as light intensity, although this term can correspond to several metrics with different definitions. In algal cultivation, it usually refers to photosynthetic photon flux density (PPFD) in $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, i.e., the amount of photosynthetically active photons incident on a surface per unit time. PPFD is a relevant way to express light intensity in algal cultures, since it represents only the photosynthetically active radiation available in the area of interest (the surface of the bioreactor, etc.). In general, increasing light intensity increases biomass productivity; however, excess light can cause stress and lead to photoinhibition, which limits further biomass production (Amini Khoeyi et al., 2011; Difusa et al., 2015; Zachleder et al., 2021a).

The present work describes the growth of the microalgae *Chlamydomonas reinhardtii* and *Desmodesmus quadricauda* exposed to light of different intensities and highly deuterated media. The effects of light intensity and deuterium content in culture medium are discussed individually and in combination. Particular attention is given to the characterization of cell growth and cell division, as these processes are sensitive to the two factors discussed. Both *C. reinhardtii* and *D. quadricauda* are well-established model organisms (Harris, 2001; Bisova and Zachleder, 2014) that can also be used in algal biotechnology thanks to their rapid growth and well-characterized biology. They both divide by multiple fission, a division pattern that allows the formation of more than two daughter cells within a cell cycle. The number of daughter cells formed is within a species-specific range and is largely influenced by external growth conditions, such as light intensity and temperature (Bisova and Zachleder, 2014; Zachleder et al., 2021a; Zachleder et al., 2021b). Consequently, microalgae that divide by multiple fission show considerable plasticity in response to changing environmental conditions, allowing for better monitoring and descriptions of microalgal responses to such treatments.

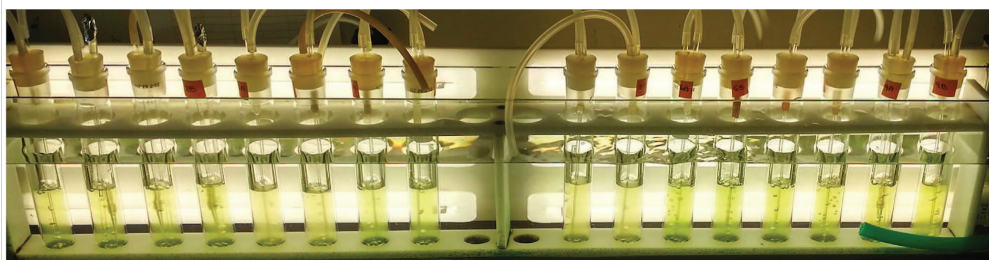


FIGURE 1
Cylindrical glass bioreactors in a temperature-controlled water bath illuminated by fluorescent tubes. Cultures are mixed by bubbling from the bottom of the bioreactors.

Materials and methods

Model organism and culture conditions

Two microalgae were used in this study: *Chlamydomonas reinhardtii* strain 21gr (CC-1690; Chlamydomonas Genetics Center, Duke University, Durham, NC, United States) and *Desmodesmus quadricauda* strain Greifswald/15 (CCALA strain number 463; CCALA, Czech Academy of Sciences, Třeboň, Czech Republic). Cultures were maintained in cylindrical glass bioreactors (inner diameter 30 mm, height 200 mm, effective volume 100 ml), placed in a temperature-controlled water bath ($30^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) and illuminated from one side with dimmable incandescent lamps (Dulux L55W/950 Daylight, Osram, Munich, Germany). Cultures were mixed by bubbling 2% CO_2 in air (v/v) through a thin glass tube from the bottom of the cylinder (Figure 1).

All experiments were performed with synchronous cultures, i.e., cultures, in which all cells are at approximately the same stage of their cell cycle. Such cultures were prepared using light-dark synchronization protocols (Hlavová et al., 2016). Briefly, cultures were inoculated into fresh medium from a plate and grown at 30°C and an incident light intensity of $500 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the surface of the cultivation vessel. The light-dark regime was set to 13 h of light and 11 h of darkness for *C. reinhardtii* and 15 h of light and 9 h darkness for *D. quadricauda* under these conditions. Cell density was maintained by dilution to 1×10^6 cells mL^{-1} at the beginning of each light cycle. Cultures were maintained under the same conditions for at least three consecutive cycles to obtain highly synchronous cultures. Synchronous cultures consisting of small daughter cells were centrifuged at $3000 \times g$ for 5 min and re-suspended in desired growth medium with/without D_2O to the initial cell density before the start of light period. Growth conditions during the

experiment were the same as described above for synchronization, except for the incident light intensity, which was set to $100/200/400 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ continuous light measured with a ULM-500 light meter equipped with a spherical microquantum sensor US-SQS/L (Walz, Effeltrich, Germany) placed in the centre of the cultivation vessel. The sensor provides a reliable measurement of the scattered light and the values obtained better represent the light available to the cultures in the cylindrical vessel. No dark periods were used during the course of the experiments. To prevent the light available to individual cells decreasing with increasing cell density, cultures were maintained in a semi-batch mode controlled by the optical density measured at 750 nm (OD_{750}), i.e., when cultures reached an OD_{750} of 0.4, they were diluted with fresh medium to $\text{OD}_{750} = 0.1$ to ensure an approximately constant supply of light to individual cells. The duration of the experiment (102 h) was set to follow a complete cell cycle in the slowest monitored culture (see below).

Growth medium

C. reinhardtii was cultured in high salt (HS) medium (Sueoka, 1960) with modifications described by Hlavová et al. (2016); *D. quadricauda* was cultured in Šetlík's Simmer (ŠS) medium of half concentration as previously described (Hlavová et al., 2016). Both media do not contain any carbon source and therefore only support autotrophic growth. The media were prepared using distilled water or deuterated (heavy) water (deuterium oxide, D_2O ; 99.95 atom%, catalog number 300101500, Silantes, Munich, Germany). For the preparation of deuterated medium, all stock solutions were pre-dried to obtain the required amount of mineral salts, which were re-suspended in a mixture of normal and deuterated water to obtain complete mineral medium with 70% or 90% D_2O .

Evaluation of growth and division

Cultures were sampled every 6 h. Culture growth was measured as optical density at 750 nm (OD_{750}). For cell count analysis, 1 ml of the culture was fixed with 100 μ L of 2.5% glutaraldehyde and stored at 4°C until analysis. Cell numbers of *C. reinhardtii* were analyzed using a Beckman Coulter Multisizer 4 (Beckman Coulter Life Sciences, Brea, CA, United States) by diluting 50 μ L of the fixed cell suspension in 10 ml of 0.9% NaCl (w/v) electrolyte solution. Cells of *D. quadricauda* were counted manually using a Bürker chamber, as the presence of multicellular coenobia prevented accurate cell counting using the Multisizer 4. The same principle was applied to *C. reinhardtii* when cells formed clusters that prevented accurate machine counting. Cell division in synchronous culture was monitored by light microscopy, using fresh samples at regular intervals.

Fv/Fm measurement

The quantum yield of photosynthesis was measured as the F_v/F_m ratio of chlorophyll fluorescence. Aliquots of 2 ml were taken from the culture and darkened for 30 min in 10 mm $\times$ 10 mm plastic cuvettes at room temperature. The cell suspension was mixed gently by inverting the cuvettes several times before measurement. Quantum yield was measured using an Aqua-Pen-C 100 (Photon Systems Instruments, Drasov, Czech Republic) set according to the manufacturer's instructions.

Light and fluorescence microscopy

At each sampling time point, 1 ml of culture was centrifuged at 3000 $\times$ g for 3 min and frozen at -20°C until analysis. Freshly thawed pellets were stained with 5 $\mu\text{g ml}^{-1}$ of 4,6'-diamidino-2-phenylindole (DAPI) in S buffer (Kuroiwa and Suzuki, 1980) and incubated at room temperature for 20 min in the dark to visualize cell nuclei. The stained cells were observed using a microscope (model BX51, Olympus, Tokyo, Japan) equipped with a U-MWIBA2 filter block (excitation: 460–490 nm, emission: 510–550 nm). To visualize the starch granules, cells were stained with Lugol's solution (1 g I, 5 g KI, 100 ml H_2O) at a final concentration 0.5 μL Lugol's solution per 20 μL cell suspension directly on the slide. Photomicrographs were taken with a DP72 camera (Olympus, Tokyo, Japan).

Confocal Raman microscopy

Raman mapping was done using a confocal Raman microscope (WITec alpha300 RSA, Germany) equipped with a $\times 60$ water-immersion UPLANSApo objective (Olympus, Japan).

A 532 nm laser with a power of approximately 20 mW at the focal plane was used. To decrease the persistent fluorescence background of the impaired photosynthetic apparatus of the fixed cells, pigments (chlorophyll, carotenoids) were washed out by methanol for 30 s. After the methanol-washing, the cells were harvested by centrifugation (3000 $\times$ g, 30 s), the pellet was resuspended in deionized water and the cells were twice rinsed to remove traces of methanol. We have found that such a methanol-washing removes only pigments and neutral lipids leaving starch, crystalline guanine, polyphosphate, and remains of plastids in the cells. The 5 μL of the resulting cell suspension was mixed with 5 μL of 1% low-temperature-melting agarose and spread under a 20 mm diameter, 0.18 mm-thick quartz coverslip sealed with CoverGrip (Biotium, United States). Raman measurements were performed with a scanning step of 200 nm in both directions, voxel size 1 μm^3 , and an integration time of 0.07 s per voxel. On average, 5–10 cells of each strain from each cell culture were measured. Standards of pure chemical substances (deuterated starch, deuterated d5-guanine, polyphosphate) were measured in water suspension. Data was analyzed using WITec Project SIX Plus v6.0 software (WITec, Germany) to implement the following steps: cosmic ray removal, background subtraction, cropping of the spectral edges affected by detector margins, spectral unmixing with the True Component Analysis tool, and averaging of the mean spectra, summarizing multiple measurements in order to optimize the signal-to-noise ratio.

Estimation of oxidative stress

Oxidative stress in each experimental treatment was estimated using the commercial Singlet Oxygen Sensor Green probe (catalog number S36002, Thermo Fisher Scientific, Waltham, MA, United States), which measures the relative amount of singlet oxygen. For analysis, 100 μL of fresh culture was pipetted into a 96-well plate and combined with 5 μL of 0.1 mM Singlet Oxygen Sensor Green in 100% (v/v) methanol. The blank sample consisted of 100 μL of culture combined with 5 μL of 100% (v/v) methanol. The specific signal was measured using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland) equipped with an excitation filter of 485 nm and an emission filter of 535 nm. The fluorescence intensity of the samples was normalized against the blank value.

Statistics

All experiments were performed in three biological replicates ($n = 3$). Presented results are the average and standard deviations from all three replicates. MS Excel 2010 (Microsoft) with Real Statistics Resource Pack (<https://www.real-statistics.com/free-download/real-statistics-resource-pack/>, accessed on 5 April

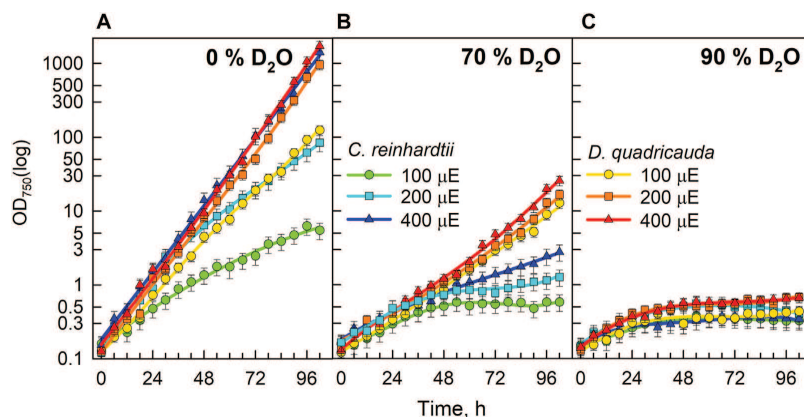


FIGURE 2

Growth of algal cultures measured as optical density at 750 nm (OD_{750}) in control culture in 0% D_2O (A), 70% D_2O (B) or 90% D_2O (C) at three light intensities 100, 200, and 400 $\mu mol\ m^{-2}\ s^{-1}$ extrapolated from semi-batch cultivation mode. Light intensities are referred to in μE (instead of $\mu mol\ photons\ m^{-2}\ s^{-1}$), in order to fit the legends. Note logarithmic scale on Y axis. Although the cultures were regularly diluted to ensure a constant light supply (semi-batch cultivation), the values obtained were recalculated using individual dilution factors. Therefore, the graphs represent a projection of growth in semi-batch cultivation mode to continuous growth. In this way, the graphs are easier to read and compare.

2022) was used to generate statistics. Comparison of mass, cell doubling times, and singlet oxygen levels between experimental treatments was performed using the two-way ANOVA test and Tukey's HSD test. A p-value < 0.05 was considered significant.

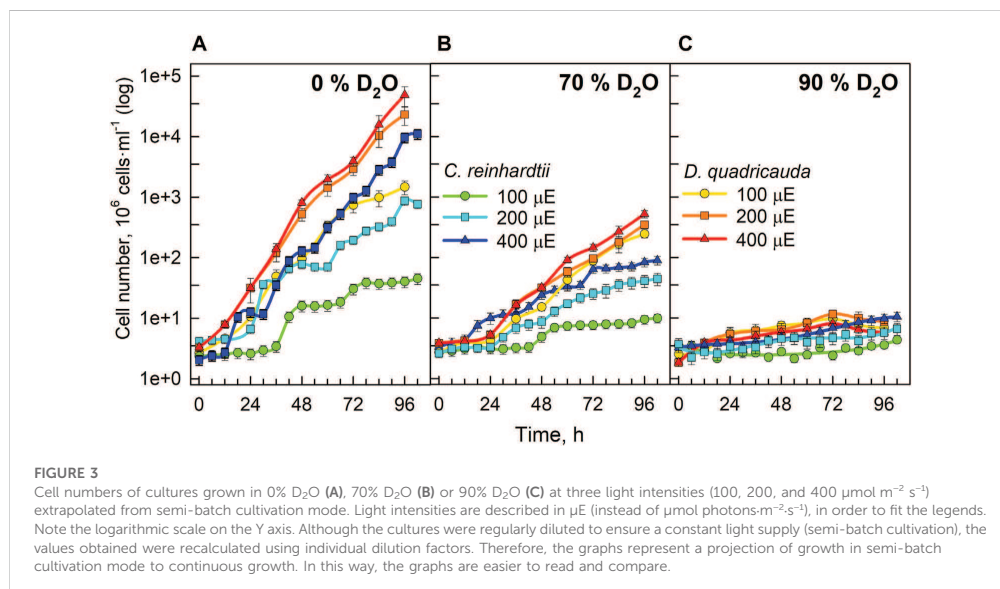
Results

Growth and division

The microalgae *Chlamydomonas reinhardtii* and *Desmodesmus quadricauda* were cultured in growth media containing 0%, 70% or 90% D_2O at incident light intensities of 100, 200, or 400 $\mu mol\ photons\ m^{-2}\ s^{-1}$. The cultivation conditions were chosen to cover as wide a range of conditions as possible. The two D_2O concentrations represent 1) a relatively high concentration with little effect on algal growth and division, and 2) a very high concentration with profound effects on algal physiology but still allowing some growth. Light intensities ranged from low to relatively high light levels: 1) the lowest light intensity represented the lowest light intensity that allowed some growth, 2) the highest was chosen so that the combined effect of stress from high light and high D_2O concentration did not result in immediate cell death. As measured by optical density at 750 nm (OD_{750}), the control culture of *D. quadricauda* in 0% D_2O grew comparably at both 200 $\mu mol\ photons\ m^{-2}\ s^{-1}$ and 400 $\mu mol\ photons\ m^{-2}\ s^{-1}$ but significantly slower at 100 $\mu mol\ photons\ m^{-2}\ s^{-1}$ (Figure 2A). The control culture of *C. reinhardtii*

at 400 $\mu mol\ photons\ m^{-2}\ s^{-1}$ grew comparably to the control culture of *D. quadricauda* at the same incident light intensity, whereas the culture of *C. reinhardtii* at 200 $\mu mol\ photons\ m^{-2}\ s^{-1}$ grew the same as that of *D. quadricauda* at 100 $\mu mol\ photons\ m^{-2}\ s^{-1}$. The culture of *C. reinhardtii* at 100 $\mu mol\ photons\ m^{-2}\ s^{-1}$ showed the slowest growth of all control cultures (Figure 2A). Cultures growing in medium containing 70% D_2O grew significantly slower than the controls at all light intensities tested (Figure 2B). Cultures of *D. quadricauda* in 70% D_2O showed comparable growth regardless of light intensity, but grew more slowly than any *D. quadricauda* culture in control medium (Figure 2, compare A and B). *C. reinhardtii* also grew more slowly in 70% D_2O than in any of the control cultures, but there was a clear effect of light intensity, with higher light intensity resulting in faster growth (Figure 2B). Cultures of *D. quadricauda* and *C. reinhardtii* in 90% D_2O grew significantly slower than in lower D_2O concentrations, reaching a maximum OD_{750} that was approximately twice the initial value for both *D. quadricauda* and *C. reinhardtii* (Figure 2C).

Since the OD_{750} reflects the growth of the culture, the increase in OD_{750} could be caused by an increase in cell number, an increase in cell volume, or their combination. The changes in cell numbers of *D. quadricauda* and *C. reinhardtii* cultures grown in different D_2O concentrations and incident light intensities are shown in Figure 3. Cell numbers in the control cultures of *D. quadricauda* increased exponentially at both 400 $\mu mol\ photons\ m^{-2}\ s^{-1}$ and 200 $\mu mol\ photons\ m^{-2}\ s^{-1}$; at 100 $\mu mol\ photons\ m^{-2}\ s^{-1}$,



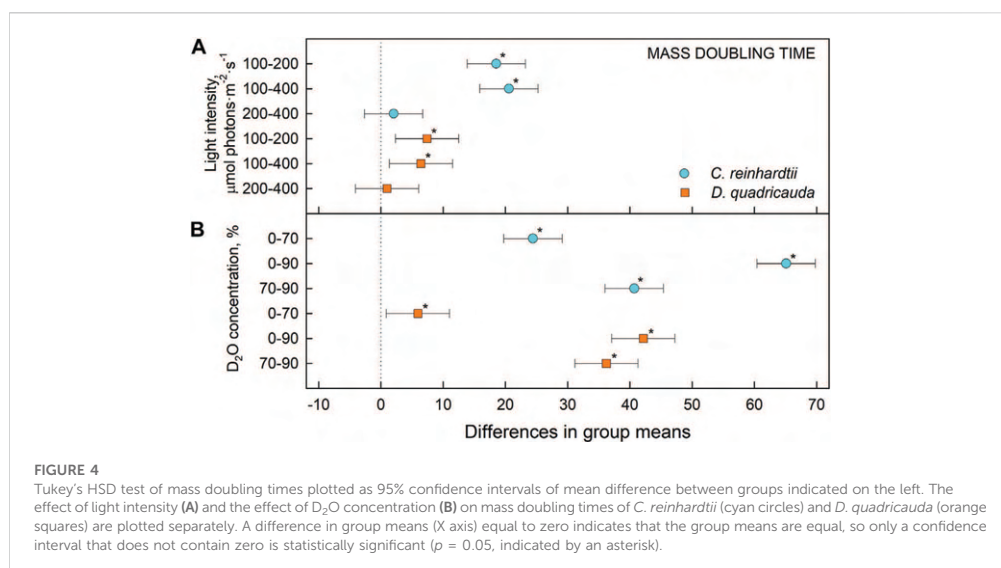
the increase in cell number was similar but slightly slower, reflecting the trends observed for OD₇₅₀ (Figure 3A). Cell numbers in *C. reinhardtii* control cultures showed a clear response to incident light intensity, with higher light intensities producing more cells. The synchrony of the cultures and the sampling interval also allowed individual divisions to be seen as a sharp increase in cell numbers during the experiment (Figure 3A). The control culture of *C. reinhardtii* at 100 μmol photons·m⁻²·s⁻¹ performed two complete cell divisions, the first after approximately 35 h, and the second after 70 h. The same culture at 200 μmol photons·m⁻²·s⁻¹ performed a total of 3 cell cycles, each about 27 h apart. At the highest light intensity, the first two divisions were about 18 h apart. Subsequent divisions were more difficult to distinguish because the degree of synchrony in the continuously illuminated culture decreases with time (Figure 3A). Cultures of *D. quadricauda* grown in 70% D₂O achieved significantly lower cell numbers than in the control culture regardless of incident light intensity. Cultures of *C. reinhardtii* in 70% D₂O at 400 μmol photons·m⁻²·s⁻¹ and 200 μmol photons·m⁻²·s⁻¹ attained cell numbers comparable to those of the control culture at 100 μmol photons·m⁻²·s⁻¹. From the course of cell number increase, we can conclude that *C. reinhardtii* in 70% D₂O completed the first cell cycle after approximately 48 h at 100 μmol photons·m⁻²·s⁻¹, after 35 h at 200 μmol photons·m⁻²·s⁻¹ and after 23 h at 400 μmol photons·m⁻²·s⁻¹ (Figure 3B). The course of subsequent cell divisions was more difficult to determine (see above). All

cultures grown in 90% D₂O barely increased their cell number during the experiment and reached a cell number about twice as high as the initial value (Figure 3C).

To quantify culture growth, we calculated doubling times for each experimental treatment based on OD₇₅₀ (Figure 2) and cell numbers (Figure 3), expressing doubling times for mass and cell number, respectively (Table 1). The general trend of faster culture growth, both in terms of mass and cell numbers, at higher light intensities (seen as a decrease in doubling times) was disrupted at the highest D₂O concentration, as increasing the light intensity above 200 μmol photons·m⁻²·s⁻¹ did not result in a significant decrease in doubling times for either *D. quadricauda* or *C. reinhardtii*. On the other hand, an increase in doubling times, both in terms of mass and cell numbers, was observed with increasing D₂O concentrations at all light intensities tested. Two-way analysis of variance (ANOVA) was performed to analyze the effects of light intensity and D₂O concentration on mass and cell number doubling times for both *D. quadricauda* and *C. reinhardtii*. It revealed that there was a statistically significant interaction between the effects of light intensity and D₂O concentration on mass doubling time for *C. reinhardtii* [F (4, 18) = 5.531, $p = 0.004$] as well as for *D. quadricauda* [F (4, 18) = 4.695, $p = 0.009$]. A simple main effects analysis showed that both light intensity and D₂O concentration had a statistically significant effect on mass doubling times in *C. reinhardtii* (both with $p < 0.001$) and *D. quadricauda* ($p = 0.003$ and $p < 0.001$, respectively). In addition, a two-way ANOVA revealed a statistically significant interaction between the effects of light

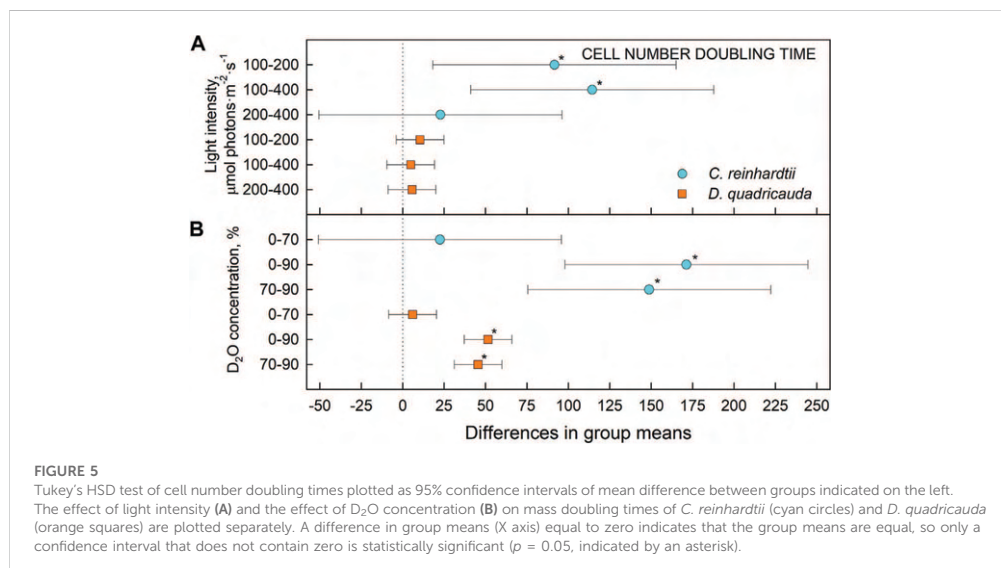
TABLE 1 Mass and cell doubling times (DT) of *C. reinhardtii* and *D. quadricauda* in different combinations of D₂O and light intensities. Values are given as mean $\pm$ standard deviation ($n = 3$).

D ₂ O conc. (%)	Light intensity ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	<i>C. reinhardtii</i>		<i>D. quadricauda</i>	
		Mass DT (h)	Cell number DT (h)	Mass DT (h)	Cell number DT (h)
0	100	20.23 $\pm$ 1.37	13.60 $\pm$ 0.92	10.27 $\pm$ 0.06	10.60 $\pm$ 0.43
	200	10.83 $\pm$ 0.14	13.56 $\pm$ 0.43	7.90 $\pm$ 0.01	7.58 $\pm$ 0.31
	400	7.77 $\pm$ 0.01	8.23 $\pm$ 0.20	7.43 $\pm$ 0.01	6.98 $\pm$ 0.27
70	100	54.77 $\pm$ 1.91	55.94 $\pm$ 8.50	15.27 $\pm$ 0.85	14.77 $\pm$ 0.53
	200	32.16 $\pm$ 3.62	25.27 $\pm$ 2.18	14.75 $\pm$ 0.03	14.79 $\pm$ 0.48
	400	25.15 $\pm$ 0.18	21.59 $\pm$ 1.25	13.35 $\pm$ 0.41	13.59 $\pm$ 0.77
90	100	94.44 $\pm$ 2.87	365.61 $\pm$ 148.63	61.95 $\pm$ 1.39	72.57 $\pm$ 22.76
	200	68.87 $\pm$ 1.25	121.51 $\pm$ 2.30	42.65 $\pm$ 2.06	44.17 $\pm$ 7.96
	400	72.84 $\pm$ 7.91	62.12 $\pm$ 11.65	47.45 $\pm$ 9.99	62.81 $\pm$ 16.52



intensity and D₂O concentration on cell number doubling time in *C. reinhardtii* [$F(4, 18) = 6.145$, $p = 0.003$], but not in *D. quadricauda* [$F(4, 18) = 1.369$, $p = 0.284$]. A simple main effects analysis showed that both light intensity and D₂O concentration had a statistically significant effect on *C. reinhardtii* mass doubling times ($p = 0.002$ and $p < 0.001$, respectively). To evaluate the differences between each experimental treatment in terms of mass and cell doubling times, Tukey's HSD test for multiple comparisons was performed for all light intensities and D₂O concentrations tested. The results, presented as 95% confidence intervals of difference between group means, are

summarized in Figure 4 for mass doubling times and in Figure 5 for cell number doubling times. Tukey's HSD test confirmed that increasing light intensity from 200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ –400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ had no significant effect on growth rate (mass doubling time) in *D. quadricauda* and *C. reinhardtii* (Figure 4A). D₂O concentration significantly affected mass doubling time in all combinations tested, although the difference between 0% D₂O and 70% D₂O was the least significant in *D. quadricauda* (Figure 4B). In terms of cell number doubling time, only the light intensity of 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in *C. reinhardtii* showed a significant

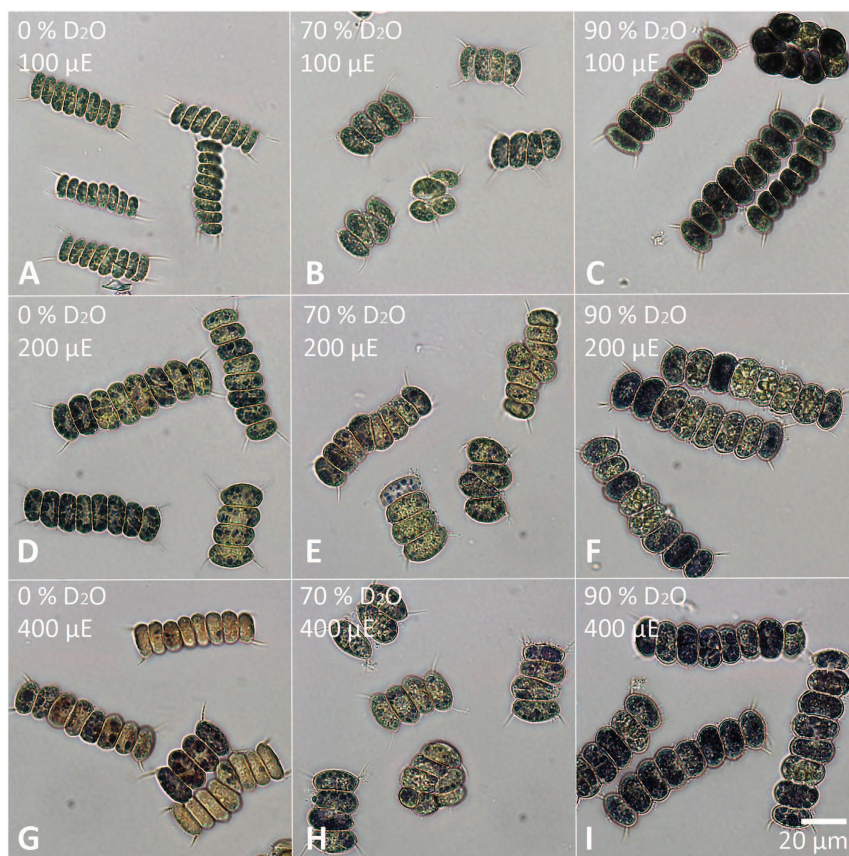


difference compared with the other tested light intensities (Figure 5A). Similarly, the only concentration of D₂O that showed a significant difference compared to the other tested variants was 90% D₂O (Figure 5B). The highest D₂O concentration proved to be significantly different for both *C. reinhardtii* and *D. quadricauda*.

Morphology of the cells

The cells of each experimental culture were sampled after 48 h and examined under a light microscope to assess their morphology as well as the presence of starch (Figures 6, 7). Furthermore, the cells were stained with DAPI to analyze the morphology and number of their nuclei (Figures 8, 9). The Lugol's staining showed presence of starch in the cells of all experimental cultures, with cultures in 90% D₂O tending to accumulate more visible starch granules (Figures 6C, F, I and 7C, F, I). The morphology of the cells reflected their cell cycle progression. At the time the photographs were taken, all control cultures of *D. quadricauda* had divided at least once, forming mostly eight-celled coenobia (Figures 6A, D, G) with two nuclei visible in most cells, although four nuclei were occasionally present at the highest light intensity (Figures 8A, D, G). In 70% D₂O, *D. quadricauda* cells also divided at least once at all incident light intensities, but they formed a mixture of eight and four-celled coenobia at 200 and 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and exclusively four-celled coenobia at 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$

(Figures 6B, E, H). The quality of the nuclear staining appeared to be slightly lower, but at the lowest light intensity, most cells clearly had two nuclei as in the control cultures (Figure 8B). At 200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, DAPI stained mostly one or two nuclei within the cells and many nucleoids within the chloroplast of the cells' (Figures 8E, H). The cells of *D. quadricauda* in 90% D₂O apparently did not divide during the first 48 h of cultivation, regardless of light intensity. Consequently, they showed increased starch accumulation (Figures 6C, F, I). Their nuclei were only weakly stained with DAPI. We observed dying cells with fragmented chloroplasts that showed little to no auto-fluorescence of chlorophyll (Figures 8C, F, I). *C. reinhardtii* cells from the control culture showed normal morphology with intermittent starch content at all light intensities. At the time of observation (48 h), they all underwent at least 1 cell division, mainly giving rise to four daughter cells (Figures 7A,D,G) with a single nucleus and multiple nucleoids (Figures 9A,D,G). *C. reinhardtii* cells grown in 70% D₂O also completed at least 1 cell division in 48 h and produced four daughter cells. The cells cultured at 200 and 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ formed multicellular structures resulting from successive rounds of cell division without hatching daughter cells (Figures 7B, E, H, 9E). The cells were generally enlarged, with the signal of a single nucleus being somewhat diffuse, especially in larger cells (Figures 9B, E, H). All *C. reinhardtii* cultures in 70% D₂O contained starch, as shown by staining (Figures 7B, E, H). *C. reinhardtii* cells in 90% D₂O were enlarged but showed no signs of cell

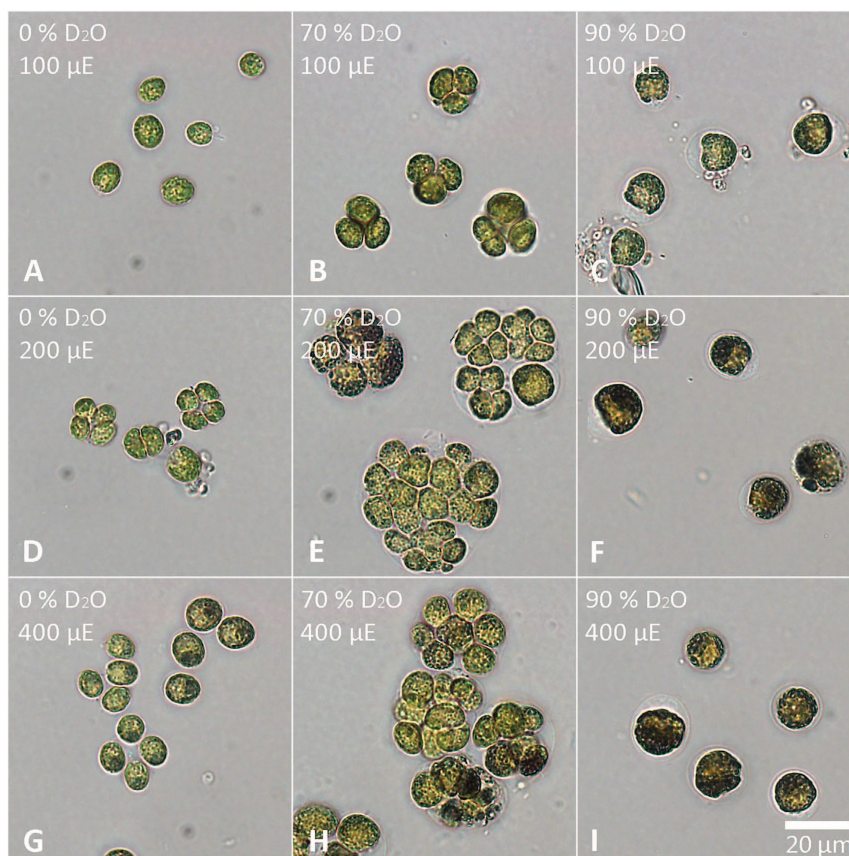
**FIGURE 6**

Staining of *D. quadricauda* with Lugol's solution. Cells grown in 0 (A,D,G), 70 (B,E,H) or 90% (C,F,I) D₂O (from left to right) at an incident light intensity of 100 (A,B,C), 200 (D,E,F) or 400 (G,H,I) $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (denoted as μE in the figure; from top to bottom) were stained after 48 h of cultivation. The starch granules appear dark purple to black.

division at the time of observation (48 h). Cells typically contained a single nucleus, which emitted a slightly diffuse signal, and multiple nucleoids (Figures 9C, F, I). The intensity of staining with Lugol's solution indicated a high starch content (Figures 7C, F, I).

The cells that grew in 70% D₂O increased their cell number by 2-fold to more than 200-fold during the experiment (Figure 3B) and therefore clearly had to contain deuterated biomolecules. For the cultures that grew in 90% D₂O and did not divide, the situation was not clear (Figure 3C). Therefore, the presence of deuterated

compounds in the cells was checked under the confocal Raman microscope. Raman microscopy was able to distinguish not only the individual biomolecules but also their deuteration. The high similarity of the Raman spectrum of starch in the cells transferred to 90% D₂O medium with the reference spectrum of highly deuterated starch indicated a high degree of deuteration (Figure 10). The most important Raman marker of starch deuteration is the intensity ratio of the broad bands of C-D and C-H stretching vibrations centered at 2173 and 2911 cm^{-1} , respectively. According to this ratio, a large fraction of hydrogens

**FIGURE 7**

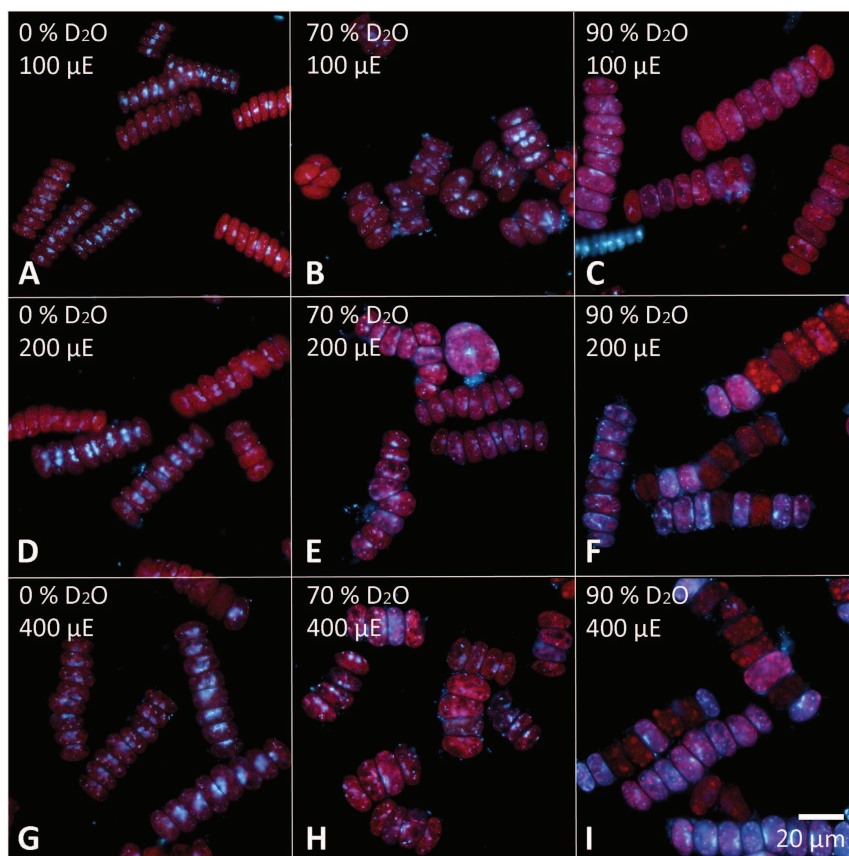
Staining of *C. reinhardtii* with Lugol's solution. Cells grown in 0 (A,D,G), 70 (B,E,H) or 90% (C,F,I) D₂O (from left to right) at an incident light intensity of 100 (A,B,C), 200 (D,E,F) or 400 (G,H,I) $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (denoted as μE in the figure; from top to bottom) were stained after 48 h of cultivation. The starch granules appear dark purple to black.

covalently bonded to carbon is replaced by deuterium. A small band at 2930 cm^{-1} indicated a small contribution from C-H groups, but the reference spectrum of highly deuterated starch also shows a small contribution from hydrogen bonds. The broad bands centered at 3400 cm^{-1} belong to O-H stretching vibrations of hydroxyl groups of starch, which are accessible to the solvent and can be rapidly exchanged by protium/deuterium depending on the nature of the solvent. Close examination of the individual cells showed that the entire cell biomass was deuterated (Figures 11D1–3) with clearly deuterated starch (Figures 11B1–3) and guanine crystals

(Figures 11C1–3). The cells also contained polyphosphates as another form of energy reserve (Figures 11E1–3). The Raman spectra of each component can be found in Supplementary Figure S1.

Cellular stress

To measure the cellular stress caused by the experimental treatment, measurements of chlorophyll fluorescence were used to estimate the stress acting on the photosynthetic machinery

**FIGURE 8**

Cells of *D. quadricauda* stained with DAPI to visualize the nuclei. Cells cultured in 0 (A,D,G), 70 (B,E,H) or 90% (C,F,I) D₂O (from left to right) at an incident light intensity of 100 (A,B,C), 200 (D,E,F) or 400 (G,H,I) $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (denoted as μE in the figure; from top to bottom) were stained after 48 h of cultivation.

(Figure 12). All control cultures, except *C. reinhardtii* at $400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, showed a decrease in F_v/F_m during the first 12 hours. Thereafter, F_v/F_m recovered and fluctuated between 0.58 and 0.7 throughout the experiment. In *C. reinhardtii* at $400 \mu\text{mol m}^{-2}\text{s}^{-1}$, F_v/F_m dropped to 0.4 during the first 24 h and then recovered to approximately 0.5 until the end of the experiment (Figure 12A). Cultures grown in 70% D₂O showed a drop in F_v/F_m similar to control cultures at the beginning of the experiment. However, complete recovery of F_v/F_m was observed in both *C. reinhardtii* and *D. quadricauda* only in cultures grown at the lowest light intensity. Cultures grown in 70% D₂O at light intensities of 200 and $400 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ recovered only partially, with the highest F_v/F_m observed in *D.*

quadricauda at $200 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. Moreover, it was evident that with increasing light intensity, the observed F_v/F_m values decreased in both *D. quadricauda* and *C. reinhardtii* (Figure 12B). This trend was even more pronounced in 90% D₂O, where light intensities of 400 and $200 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ caused a dramatic decrease in F_v/F_m , indicating severe stress in both *D. quadricauda* and *C. reinhardtii*. At a light intensity of $100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, both organisms maintained F_v/F_m at approximately 0.7 for the first 48 h, but then F_v/F_m decreased to 0.25 and 0.35 for *D. quadricauda* and *C. reinhardtii*, respectively (Figure 12C).

The relative levels of singlet oxygen were used to estimate oxidative stress caused by the experimental treatment. All

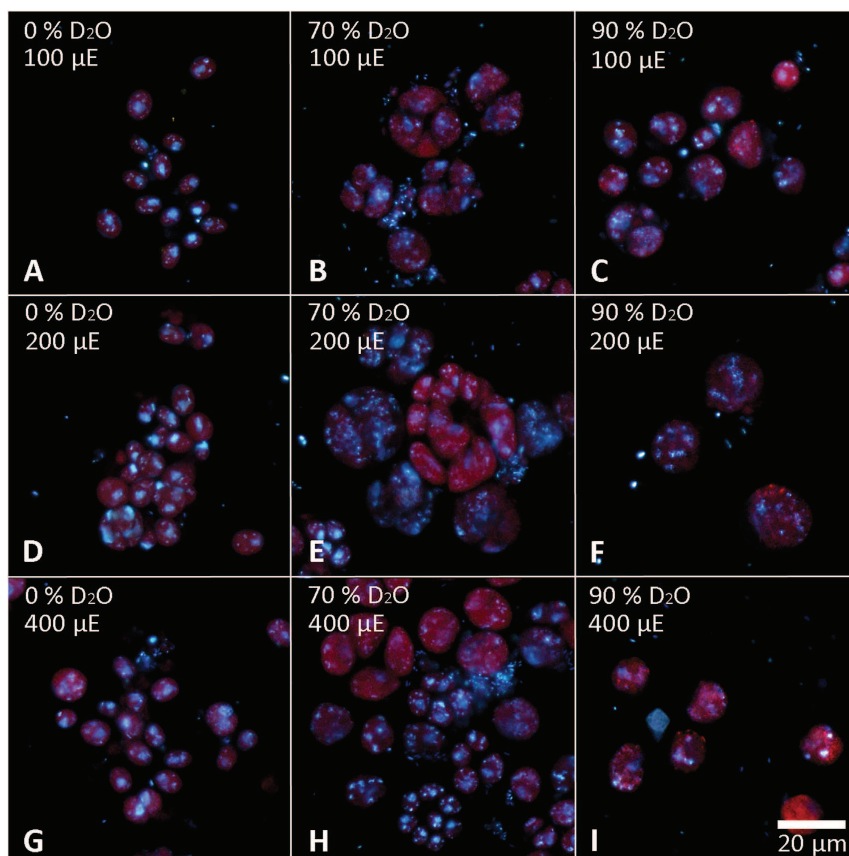


FIGURE 9

Cells of *C. reinhardtii* stained with DAPI to visualize the nuclei. Cells cultured in 0 (A,D,G), 70 (B,E,H) or 90% (C,F,I) D₂O (from left to right) at an incident light intensity of 100 (A,B,C), 200 (D,E,F) or 400 (G,H,I) $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (denoted as μE in the figure; from top to bottom) were stained after 48 h of cultivation.

cultures were sampled after 24, 48, 72, and 96 h of cultivation, and singlet oxygen levels were determined and compared between experimental treatments in *D. quadricauda* (Figure 13A) and *C. reinhardtii* (Figure 13B). While it is clear that singlet oxygen levels in a culture varied over the course of the experiment, there was also a trend toward higher singlet oxygen levels at higher D₂O concentrations, particularly in *C. reinhardtii* (Figure 13B). A two-way ANOVA revealed that there was no statistically significant interaction between the effects of light intensity and D₂O concentration on singlet oxygen levels in either *C. reinhardtii* [$F(4,27) = 1.053$, $p = 0.399$] or *D. quadricauda* [$F(4,27) = 1.978$, $p = 0.126$]. However,

a simple main effects analysis showed that both light intensity and D₂O concentration individually had a statistically significant effect on singlet oxygen levels in *C. reinhardtii* (both with $p < 0.001$) as well as in *D. quadricauda* ($p = 0.040$ and $p = 0.003$, respectively). The differences between individual light intensities and D₂O concentrations were evaluated using Tukey's HSD test, and the 95% confidence intervals of the mean differences between groups are shown in Figure 14. The differences in singlet oxygen levels between all tested light intensities in *C. reinhardtii* were statistically significant, but only the difference between the lowest and highest light intensity in *D. quadricauda* was significant

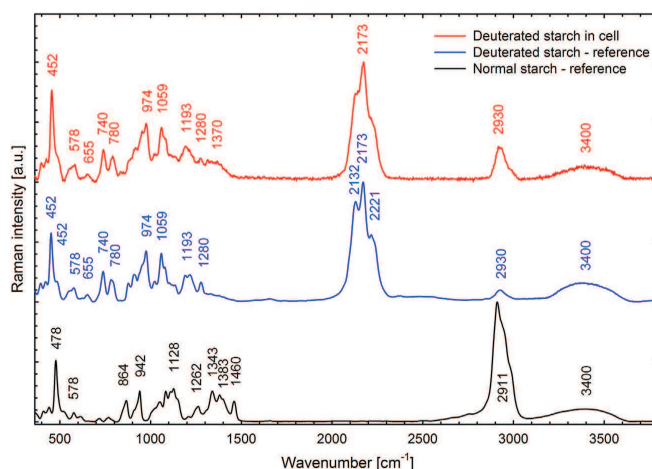


FIGURE 10

Raman spectrum of deuterated starch found in *C. reinhardtii* cells cultivated in the 90% D₂O medium for 4 days (red line) compared with a reference spectrum of highly deuterated starch extracted from the *C. reinhardtii* culture cultivated in 99% D₂O (blue line) and with a normal starch (black line). For all three specimens, the cells or starch granules were dispersed in H₂O. Spectral contribution of H₂O was subtracted and the background was corrected.

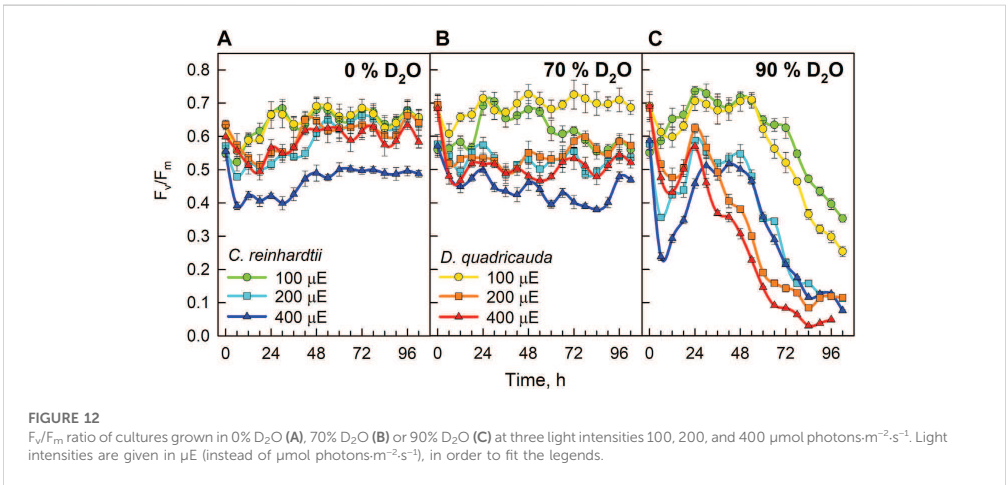
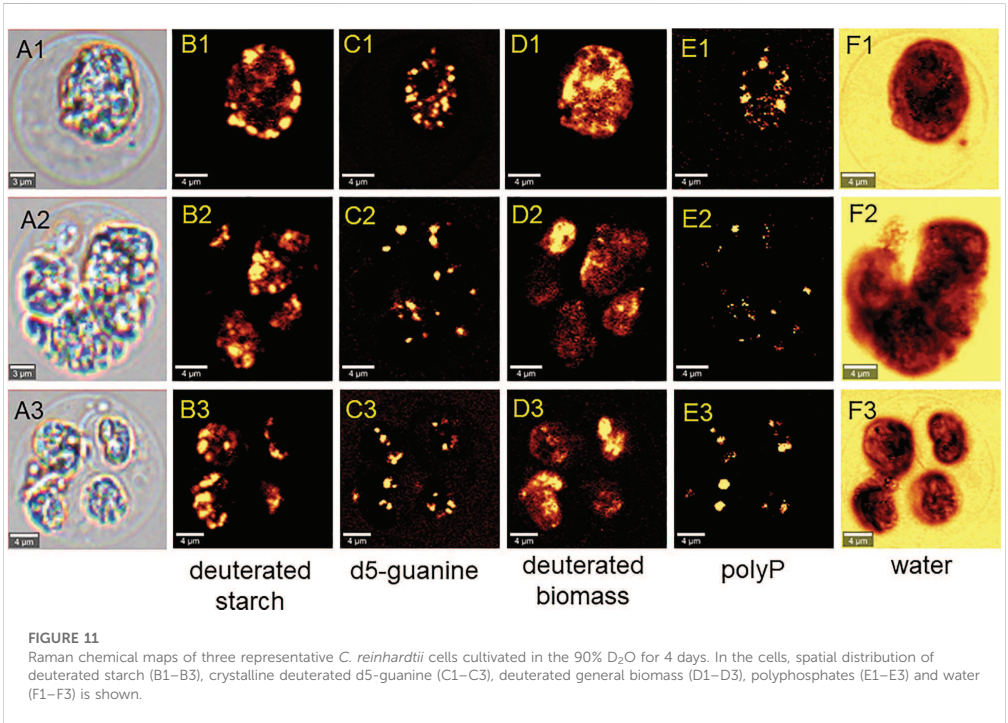
(Figure 14A). The differences in singlet oxygen levels between all tested D₂O concentrations were statistically significant in both *C. reinhardtii* and *D. quadricauda*, except for the comparison of 0% D₂O and 70% D₂O, which showed no statistically significant difference in singlet oxygen levels in *D. quadricauda* (Figure 14B).

Discussion

Growth and division

Light intensity is a crucial factor affecting biomass yield in microalgal cultivation. However, biomass yield can also be affected by other factors, such as nutrient availability, temperature or CO₂ levels (Lee et al., 2015). Therefore, it is important to identify the saturating light intensity for each organism in a given cultivation setup. Monitoring the optical density of cultures (OD₇₅₀) in different combinations of deuterated media and incident light intensities led to the estimation of the saturating light intensity in such cultures. Comparing the performance of the control cultures of *D. quadricauda* and *C. reinhardtii* at different light intensities (Figure 2A; Table 1), it was clear that the *D. quadricauda* cultures in this cultivation setup were saturated at 200 μmol photons·m⁻²·s⁻¹, as a further increase in light intensity did not significantly improve culture growth. *C. reinhardtii*, on the other

hand, was saturated at incident light intensities of 400 μmol photons·m⁻²·s⁻¹ or higher (Figure 2A; Table 1). Data from higher light intensities are required to confirm that 400 μmol photons·m⁻²·s⁻¹ is sufficient to fully saturate such a culture. These results are consistent with the published results of Bialevich et al. (2022), who showed a saturation light intensity of 500 μmol photons·m⁻²·s⁻¹ or more for *C. reinhardtii* and 200 μmol photons·m⁻²·s⁻¹ for *D. quadricauda* in the same cultivation setup. Cultures of *D. quadricauda* in 70% D₂O were saturated even at the lowest incident light intensity tested (Figure 2B; Table 1), indicating that D₂O is the main stressor affecting cell physiology regardless of light intensity. *C. reinhardtii* in 70% D₂O showed increasing performance with increasing light intensity, confirming that saturating light intensity was generally higher than in *D. quadricauda*, even in deuterated medium (Figure 2B; Table 1). Growth of cultures in 90% D₂O was affected primarily by the presence of deuterium, which greatly retarded growth regardless of incident light intensity (Figures 2C, 4, 5; Table 1). A reduction in saturating light intensity or higher sensitivity to light under suboptimal stress conditions has been previously described for salinity or high temperature stress (León and Galván, 1999; Possmayer et al., 2011; Annan, 2014). However, the apparent growth of the culture could be caused (at least in the short term) by two interrelated cellular processes: growth in size and cell division. While the increase in optical density could be caused by either of these factors, the increase in cell number reflects only the ability



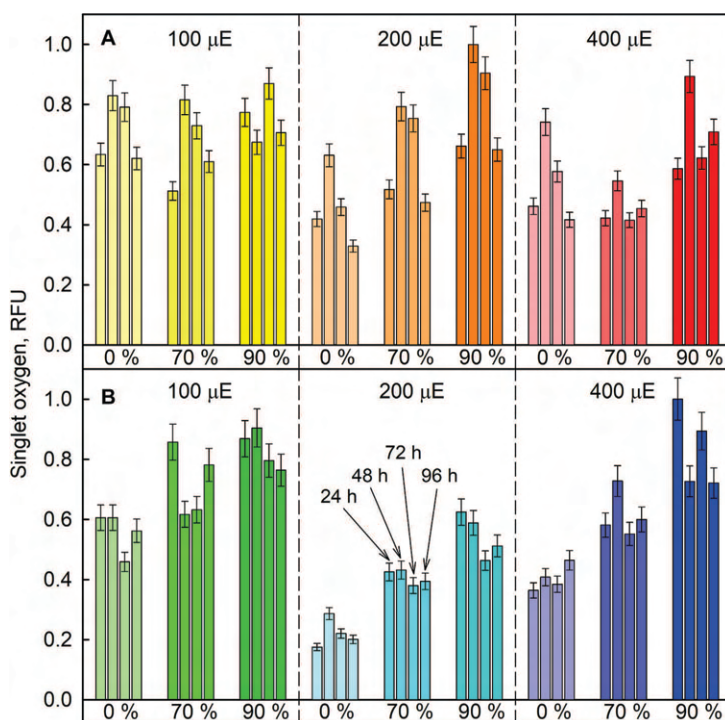


FIGURE 13

Relative amount of singlet oxygen in cultures grown at different incident light intensities (100, 200, and 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and at different D₂O concentrations (0%, 70% and 90%, indicated as percentages under the bars) in *D. quadricauda* (A) and *C. reinhardtii* (B). Singlet oxygen levels were determined spectrophotometrically and fluorescence was normalized to the maximum value. Individual bars within one group (same color) represent individual measurements after 24, 48, 72, and 96 h of cultivation (as indicated in panel B). Light intensities are given in μE (instead of $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), in order to fit the legends.

of cells to divide. The increase in cell number of cells grown in 0% D₂O largely reflected their increase in optical density, indicating normal progression of the cell cycle in which the increase in cell volume is followed by cell division (Figure 3A; Table 1). Cells in 70% D₂O were generally capable of cell division, as evidenced by the increase in cell number (Figure 3B), although the time required for cell division was longer compared with the control culture at the same light intensity (Figure 3B; Table 1). In addition, the number of daughter cells formed within 1 cell cycle tended to be lower in 70% D₂O for both *D. quadricauda* and *C. reinhardtii* (compare 0% D₂O and 70% D₂O in Figures 6, 7). A reduced number of daughter cells is a typical sign of suboptimal growth conditions or stress in algae dividing by multiple fission and has already been described for *C. reinhardtii* and *Parachlorella kessleri* grown in medium containing D₂O (Zachleder et al., 2021a; Kseliková et al.,

2021). Cultures grown in 90% D₂O showed barely any increase in cell number regardless of light intensity (Figures 3C, 5; Table 1). Therefore, cell division was severely delayed in both *D. quadricauda* and *C. reinhardtii* in 90% D₂O. These results are consistent with previously published results in *C. reinhardtii* (Kseliková et al., 2021), *Parachlorella kessleri* (Zachleder et al., 2021a; Zachleder et al., 2021b) or *Chlorella ellipsoidea* (Unno et al., 1992). Interestingly, Tukey's HSD test revealed that while both mass and cell number doubling times were affected by deuterium treatment (Figures 4, 5), the difference between 0% D₂O and 70% D₂O was statistically significant only with respect to cell number doubling time. This clearly indicates differing sensitivities of cell growth (reflected in mass doubling time) and cell division (reflected in cell number doubling time) to deuterium treatment, especially at intermediate D₂O concentrations. At the same time, a

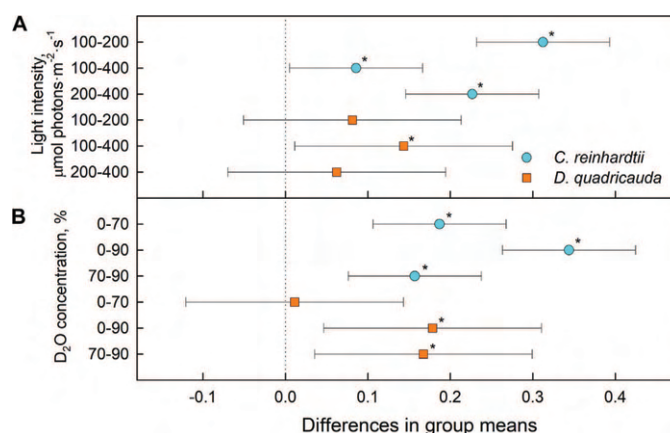


FIGURE 14

Tukey's HSD test of singlet oxygen levels plotted as 95% confidence intervals of mean differences between groups indicated on the left. The effect of light intensity (A) and the effect of D₂O concentration (B) on singlet oxygen levels in *C. reinhardtii* (cyan circles) and *D. quadricauda* (orange squares) are shown separately. A difference in group means (X axis) equal to zero indicates that the group means are equal, so only a confidence interval that does not contain zero is statistically significant ($p = 0.05$, indicated by an asterisk).

statistically significant difference in mass and cell number doubling time between 70 % and 90% D₂O proves a concentration-dependent delay in growth (and division) in both *C. reinhardtii* and *D. quadricauda*. Therefore, careful selection of the appropriate D₂O concentration is of utmost importance if algal cultures are to be used for the production of deuterated compounds, especially with respect to production goals. No less attention should be paid to the choice of organism, as our data show that *C. reinhardtii* is more sensitive to deuterium for all monitored parameters (Table 1; Figures 4, 5, 12).

Cell morphology

Cell growth in microalgae is associated with the accumulation of energy-storing molecules that are catabolized later in the cell cycle to support cell division processes (Zachleder et al., 2016; Torres-Romero et al., 2019; Ivanov et al., 2021). Both microalgae tested store energy for cell division mainly in the form of starch (Figures 6, 7, 11), no lipids were detected (data not shown). Since cell division is impaired in deuterated medium, both *D. quadricauda* (Figure 6) and *C. reinhardtii* (Figure 7) cells were noticeably enlarged. In addition, cells in deuterated medium, especially in 90% D₂O, contained significant amount of starch granules, as shown by Lugol's staining (Figures 6C, F, I, 7C, F, I) as well as by confocal Raman microscopy (Figures 11B1–3). The same trend was observed in other microalgae after treatment with deuterium (Zachleder et al., 2021a; Kseliková et al., 2021), supraoptimal temperature (Zachleder et al.,

2019; Zachleder et al., 2021b) or nutrient starvation (Jerez et al., 2015). All of the above treatments have one thing in common—they impair/delay cell division, so starch accumulation is in fact a consequence of cell cycle arrest/slowdown. To further investigate cell cycle impairment in deuterated cultures, we performed DAPI staining to assess the ability of cells to divide their nuclei, as DNA replication and nuclear division are among the most sensitive processes of cell cycle. Both *D. quadricauda* and *C. reinhardtii* grown in 70% D₂O divided their nuclei (Figures 8B, E, H, 9B, E, H), whereas in 90% D₂O nuclei division was less common (Figures 8C, F, I, 9C, F, I). However, nuclear division, although negatively affected, is not the only obstacle to complete the cell cycle in highly deuterated cultures. The presence of large binuclear cells without any sign of protoplast fission (e.g., Figures 9C,F,I) suggests that other division-related processes may be the bottleneck for complete cell division in highly deuterated cultures. In addition, cells in deuterated cultures of *C. reinhardtii* showed evidence of defective cell hatching after division, as documented by the appearance of multicellular division clusters or palmelloids that remained intact for extended periods of time (Figures 7, 9). The formation of palmelloids is a mechanism for coping with stress in *C. reinhardtii* (Khona et al., 2016; de Carpentier et al., 2019), further highlighting the effect of deuterium as a stress-inducing agent. Also noteworthy is the morphology of chloroplasts in 90% D₂O, as seen under fluorescence microscopy (Figures 8C, F, I, 9C, F, I). Compared with control cells or even cells in 70% D₂O, cells in 90% D₂O showed uneven chlorophyll auto-fluorescence, as if their chloroplasts were fragmented. Structural changes in chloroplasts in response to various environmental stresses have been described previously in

algae (Poppe et al., 2002; Zuppini et al., 2007). They are also directly related to oxidative stress in chloroplasts (Woodson, 2022), as has also been observed in deuterated cultures (see below).

Cellular stress

The different physicochemical properties of deuterium oxide compared to normal water include not only differences in boiling and freezing points, but also in density and viscosity (Yang, 2016), and affect the function of deuterium oxide as a solvent in biological systems. The larger mass of deuterium leads to the formation of stronger bonds and increases the dissociation and activation energy of bonds. In addition to increasing the energy demand of cells, deuterium also decreases the energy pool (Vasilescu and Katona, 1986) by affecting ATP synthase (Kotyk et al., 1990; Olgun, 2007), the mitochondrial respiratory chain (Salomonsson et al., 2008), and photosynthesis (de Kouchkovsky et al., 1982). Consequently, the presence of deuterium affects overall metabolism at multiple levels. The algal cultures coped well with 70% D₂O, which allowed division into 2 to more than 200 daughter cells over 96 h, depending on light intensity (Figure 3B). The daughter cells formed contained biomass deuterated up to 70% confirming the resistance of algal cells to lower deuterium concentrations (Bhosale et al., 2006; Garg et al., 2017). In 90% D₂O, both biomass and energy compounds were deuterated (Figures 11B1–D3), although (almost) no cell division occurred (Figure 3C). Given the widespread effect of deuterium on general metabolism, there is likely not a single causative process behind the observed deuterium effect. Nevertheless, it may be interesting to narrow down the differences in response to 70% and 90% D₂O by combining transcriptomics gene expression analyses guiding metabolomics. Irrespective of the mechanism, deuteration induced a complex stress response in the microalgae tested. Therefore, we chose to measure the F_v/F_m ratio, which represents the maximum potential quantum efficiency of photosystem II, when all reaction centers are open (Figure 12). F_v/F_m is a widely used indicator of stress affecting photosystem II. Under stress conditions, photoprotective mechanisms are active, thus effectively reducing the maximum efficiency of photosystem II. The majority of the cultures, including the control cultures, showed a light-dependent decrease in the F_v/F_m ratio at the beginning of cultivation. This could be due to the initial adaptation of the diluted synchronous culture to the sudden incidence of light immediately after dark treatment. Such a drop is not always the rule for synchronous cultures under similar culture conditions. The control cells eventually adapted to the new conditions and recovered from the drop. Deuterium treatment decreased F_v/F_m values as expected (Figure 12). In addition, the extent of the F_v/F_m decrease was largely influenced by light intensity, with higher light intensity generally further decreasing F_v/F_m values (Figures 12B,C). Moreover, F_v/F_m values appeared to be stabilized in 90% D₂O for about 48 h (24 h at the highest light intensity), and then dropped rapidly, reflecting the overall decreasing fitness of cells under harsh stress conditions (Figure 12C). However, higher stress levels,

determined by F_v/F_m measurements in deuterated cultures at higher light intensities, did not result in poorer culture performance, as higher light intensities resulted in better culture growth, both in terms of cell mass and cell numbers, for both organisms tested under control conditions and for *C. reinhardtii* even in 70% D₂O (Figures 2, 3). Because the maximum potential quantum efficiency of photosystem II was reduced in deuterated cultures, we wanted to determine whether excess light could cause oxidative stress in cells. In situations where the absorbed light exceeds the capacity of photosynthesis, singlet oxygen could be generated via the formation of triplet chlorophyll (Figure 13). Singlet oxygen is normally detoxified by the antioxidant machinery of cells, otherwise it triggers the upregulation of genes involved in the response to photo-oxidative stress (Krieger-Liszka, 2005). In addition, deuterium has been found to increase the lifetime of singlet oxygen due to solvent isotope effects (Kajiwara and Kearns, 1973). In our data set, singlet oxygen generally increased with increasing D₂O concentration, except for *D. quadricauda* grown in 0% D₂O and 70% D₂O, where no statistically significant differences in singlet oxygen levels were observed (Figures 13, 14B). The effect of light intensity on singlet oxygen level was significantly different in *C. reinhardtii* at all intensities tested (Figure 14A), but only the lowest and highest light intensities resulted in a statistically significant difference in *D. quadricauda* (Figure 14A). Higher levels of singlet oxygen could contribute to overall lower fitness of cells grown in D₂O by causing photo-oxidative damage or by playing a role in chloroplast degradation (Fischer et al., 2006; Erickson et al., 2015; Woodson, 2022).

Conclusion

In the present work, we investigated the effects of light intensity and deuterated water in the culture medium on growth and division of microalgae *Desmodesmus quadricauda* and *Chlamydomonas reinhardtii*. Our results show a significant slowdown in culture growth in deuterated medium, with division processes being more affected than cell growth. Impaired cell division processes in highly deuterated medium lead to enlargement of cells and the accumulation of energy-storing molecules, such as starch, that would normally be consumed to support cell division processes. By combining growth in deuterated medium with the use of different light intensities for cultivation, we were able to determine the saturating light intensity for both organisms under all conditions tested. The saturating light intensity of the deuterated cultures was significantly lower because the stress caused by deuteration is enhanced by strong light, as evidenced by an increase in singlet oxygen, which can contribute to photooxidative damage, and an upregulation of photoprotection mechanisms and subsequent decrease in the F_v/F_m ratio. Increased photosensitivity associated with deterioration of photosynthetic performance at high deuterium concentrations is apparently part of the general stress response induced by deuterium and its strong KIE. Although such findings may discourage further attempts to

culture microalgae in deuterated medium, it should be noted that the D₂O concentrations used in this study were very high to elucidate a response of model organisms to extreme environmental conditions and to set the limits of possible use of deuterium in microalgal biotechnology. A higher concentration of deuterium in the growth medium led to a rapid and high degree of labelling, but also negatively affected the growth of the culture. On the other hand, a lower deuterium concentration, which had less impact on cell cycle progression, may be more than sufficient to produce deuterated compounds with a satisfactory degree of labelling. Moreover, the type of deuterated compound required plays a crucial role in the design of experiments, as the production of some compounds is not linked to the ability of cells to complete their cell cycle or may even be enhanced when cell cycle progression is blocked, particularly energy-storing molecules such as starch, lipids, or polyphosphates (Yao et al., 2012; Zachleder and Brányiková, 2014; Vitova et al., 2015; Moudříková et al., 2016). Moreover, different organisms show differing sensitivities to deuterium, as shown by our data, where *C. reinhardtii* was more sensitive to deuterium for all monitored parameters. Therefore, we believe that proper culture management can exploit the potential of microalgae in the production of deuterated biomolecules. Although the D₂O used to produce the growth media is expensive (currently over 1000 EUR/litre), deuterated molecules are of even higher value and their biological production is a viable process that is cheaper than chemical synthesis (Zachleder et al., 2018). Moreover, D₂O used in the growth media might be partly re-used in further rounds of cultivation if properly purified. We have shown that cultures grown in 70% D₂O in the growth medium can maintain adequate growth and cell fitness while producing deuterated biomolecules. If the goal is to obtain biomolecules with a higher degree of deuteration, another option might be to transfer synchronous cultures of microalgae in the early stages of their cell cycle to highly deuterated medium. These cells would grow larger and produce energy-storing molecules. If an appropriate cell density is used as an inoculum, the yield of highly deuterated compounds would overcome both the lower biomass yield and the cost of the two-step cultivation process. However, other factors affecting algal productivity (temperature, medium composition, CO₂ concentration, etc.) may still prove critical to the successful management of deuterated cultures.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

VK: methodology, validation, visualization, formal analysis, investigation, data curation, and writing—original draft

preparation; KH: methodology, investigation, data curation, and writing—review and editing; PM: methodology, investigation, visualization, data curation, and writing—review and editing; VZ: conceptualization, methodology, validation, visualization, data curation, and writing—review and editing; KB: conceptualization, methodology, formal analysis, investigation, data curation, visualization, supervision, project administration, funding acquisition, and writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fbioe.2022.960862/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Raman spectra of biomolecules identified in *C. reinhardtii* cells cultivated in the 90% D₂O for 4 days. All the biomolecules containing C-H groups (starch, crystalline guanine, and general biomass covering protein and lipids from plastids) were synthesized *de novo* because they show high degree of deuteration. As anionic polyphosphate does not contain any hydrogen bond, cultivation in D₂O has no effect on its Raman spectrum. Since pigments were washed out by methanol on the specimen preparation, their Raman spectra were not detected.

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5. Conclusion and future prospects

In the early days of deuterium research in biology, microalgae were among the first organisms cultured in D₂O (Blake et al., 1961; Chorney et al., 1960; Crespi et al., 1960; Katz, 1960; Mandeville et al., 1964). Their potential to produce highly deuterated organic compounds such as pigments (Bhosale et al., 2006; Gireesh et al., 2001; Saha et al., 2013), carbohydrates (Kselíková et al., 2022a; Liu et al., 2019), amino acids (Blake et al., 1961) or fatty acids (Graff, 1970; Saha et al., 2013) has been demonstrated in the past. Apart from the more obvious use of deuterated compounds as tracers in climatology, metabolomics, ecology, etc. (Araguas-Araguas et al., 2000; Brook et al., 2017; Kopf et al., 2015; Werner and Rossmann, 2015); their unique properties could be exploited, such as the enhanced antioxidant properties of deuterated PUFAs (Firsov et al., 2022; Hill et al., 2011), or they could be used to produce deuterated drugs (Gant, 2014; Kushner et al., 1999). This dissertation, consisting of five published articles (four first author, one co-author), contributes to the current state of knowledge by describing the growth of model microalgae in media enriched with high concentrations of D₂O and the response to such treatment, with particular emphasis on cell cycle progression in treated cells. In addition, our results contribute to the understanding of cell cycle regulation in algae dividing by multiple fission.

Our experiments were initially aimed at describing the growth of the model microalgae *C. reinhardtii*, *P. kessleri* and *D. quadricauda* in highly deuterated media under different culture conditions. Based on our long experience with cultivation of microalgae in our laboratory, we chose to test two factors that most affect growth rates during autotrophic cultivation – light intensity (Article II. and Article V.) and temperature (Article III.). The reason for selecting these parameters is the well-established relationship between light intensity and growth, where light intensity is a key factor affecting growth rates (Bialevich et al., 2022; Carvalho et al., 2011; Vítová et al., 2011b), and the influence of cultivation temperature on all metabolic processes in the cell (Lürling et al., 2013; Vítová et al., 2011a; Zachleder et al., 2019b), especially considering the effect of deuteration on the reaction rates of biochemical reactions within the cell (Pirali et al., 2019). We tested light intensities from 150 to 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Article II.), or 100 to 400 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Article V.), for all three model species mentioned above. In all cases, saturation light intensity (manifested by the fastest growth rate) was reduced in deuterated media,

although the extent was slightly different for each model species, reflecting their optimal light intensity in nondeuterated media as well as their sensitivity to deuteration (Kselíková et al., 2022a; Zachleder et al., 2021a). The effect was directly proportional to the extent of deuteration. The increased photosensitivity was also reflected in the F_v/F_m values, the maximum quantum yield of the photosystem II, which is considered a sensitive indicator of photosynthetic performance and also reflects the stress acting on the cell. In all species tested, deuteration significantly lowered F_v/F_m values, although the decrease was greater at higher light intensities (Kselíková et al., 2022a; Zachleder et al., 2021a). Similar experiments were performed to determine the optimal temperature for cultivation of deuterated microalgae (Article III.). Three temperatures in the physiological range of 30 to 39 °C were tested for *P. kessleri* (Zachleder et al., 2021b). We specifically wanted to compare our standard cultivation temperature for this organism, which is 30 °C in our cultivation setup, to higher temperatures because deuterium is known to slow the rate of chemical reactions (Pirali et al., 2019), whereas a higher temperature could offset this effect. Surprisingly, our assumption only partially proved true, as increasing the temperature to 35 °C improved the growth of the culture in terms of mass, but not in terms of cell number (Zachleder et al., 2021b). This suggests a differential sensitivity of growth- and division-related processes to deuteration and/or temperature. Temperature of 39 °C, tolerated in non-deuterated cultures although stressful, caused immense stress in combination with deuteration and resulted in cell death (Zachleder et al., 2021b). The results summarized in Article II., Article III. and Article V. mainly fulfill objectives 1 and 2 of this work.

The most striking effect of deuteration on microalgae is the slowing of growth and delayed or blocked cell division (Chorney et al., 1960; Crespi et al., 1960; Kselíková et al., 2019; Mosin and Ignatov, 2015). The selection of microalgae that divide by multiple fission allowed us to assess the extent to which growth- and division-related processes are affected by deuteration. To this end, we developed a modified protocol to assess the achievement of the commitment point by microalgae at different deuterium concentrations (Article IV.). Our results with model microalgae *C. reinhardtii* (Article I., Article V.), *P. kessleri* (Article II., Article III.) and *D. quadricauda* (Article V.) show a clear and separate effect of deuterium on cell growth and cell division. Cell division processes were more affected by deuteration than cell growth, as evidenced by a greater increase

in cell number doubling times than mass doubling times (Kselíková et al., 2022a; Zachleder et al., 2021a; Zachleder et al., 2021b). However, growth is also affected by deuteration, as shown by the prolongation of the pre-commitment period in deuterated cultures (Kselíková et al., 2021). A detailed study of cell cycle progression in deuterated *C. reinhardtii* (Article I.) revealed further details about the effects of deuterium on cell cycle progression. Due to slowing down the growth, deuterium delays the attainment of all CPs in *C. reinhardtii*, but the first CP is affected the most. The prolonged cell cycle of deuterated cells and their lower ability to divide are reflected in their lower CDK activity (Kselíková et al., 2021). Moreover, switching from deuterated to normal medium in conjunction with culture darkening does not improve the ability of *C. reinhardtii* to divide cells; surprisingly, performance might even deteriorate, depending on the timing of the switch and the deuterium concentration (Kselíková et al., 2021). This shows a "reinterpretation" of CP attainment upon shift, contradicting the role of the sizer in cell cycle regulation of *C. reinhardtii*, similar to *D. quadricauda* in shifts between different temperatures (Kselíková et al., 2021; Zachleder et al., 2019b). Results summarized in Article I. Article II, Article III. and Article V. are assigned to aims 2 and 3 of this thesis.

Deuteration triggers a complex response in the affected microalgal cells. Apart from changes in the cell cycle, we observed a massive stress response in all tested species exposed to deuteration, which is reflected in a decrease in photosynthetic efficiency expressed by the F_v/F_m ratio (Kselíková et al., 2022a; Zachleder et al., 2021a; Zachleder et al., 2021b), increased relative levels of reactive oxygen species (Kselíková et al., 2022a), occurrence of palmelloids, a stress-induced structures, in *C. reinhardtii* (Kselíková et al., 2021), or, especially at the highest deuterium concentrations tested, cell death (Zachleder et al., 2021a; Zachleder et al., 2021b). However, we have also shown that stress is partially alleviated by acclimation to deuteration, especially at moderate deuterium concentrations and otherwise optimal growth conditions (Kselíková et al., 2022a; Zachleder et al., 2021a; Zachleder et al., 2021b). It is known that stress (and the consequent delay in cell division) stimulates the production of energy-storing molecules in microalgae (Ivanov et al., 2021; Rugnini et al., 2020; Vítová et al., 2015). We have demonstrated increased starch and neutral lipid content in deuterated microalgae compared to non-deuterated microalgae (Kselíková et al., 2022a; Kselíková et al., 2021; Zachleder et al., 2021a; Zachleder

et al., 2021b). The extent of deuterium labelling is linearly correlated with the amount of deuterium in the growth medium (Apt and Behrens, 1999), but varies among organic compounds, reflecting their biosynthetic pathways and hydrogen isotope fractionation (Zhang and Sachs, 2007). To prove our concept, we used Raman microscopy to evaluate the deuteration of selected biomolecules, including starch. Raman spectra of starch in *C. reinhardtii* grown in 90% D₂O revealed the presence of deuterated starch and guanine crystals (Kselíková et al., 2022a). At this point, it is fair to mention that deuterated cultures generally have lower biomass yield. However, our other results suggest that this could be partially overcome by acclimation to deuterium at moderate concentrations sufficient to ensure production of deuterated compounds or by opting for a two-stage cultivation system in which cells are grown to a higher cell density before transfer to deuterated media. Although both options would increase production costs, their use should be justified by the market value of the deuterated compounds produced.

In conclusion, our results on the deuteration of the model microalgae *C. reinhardtii*, *D. quadricauda* and *P. kessleri* can be summarized as follows: 1) deuteration lowers the saturation light intensity for autotrophic cultivation; 2) increasing the cultivation temperature could improve biomass yield but not the ability of cells to divide; 3) deuteration slows the growth of microalgae in a concentration-dependent manner; 4) deuteration affects both growth- and division- related processes; 5) attainment of the first CP in deuterated cultures is more delayed than attainment of other CPs; 6) CP attainment is not controlled by cell size (in *C. reinhardtii*); 7) deuteration affects the pattern of CDK activity; 8) switching from deuterated to non-deuterated media (depending on the duration of treatment and timing of the switch) improves or worsens the ability of cells to divide; 9) deuteration induces stress in treated microalgae; 10) deuterium-induced stress can be partially alleviated by acclimation; 11) deuteration induces (over)accumulation of energy-storing molecules such as starch and neutral lipids; 12) biomass of cells treated with high concentrations of deuterium is highly deuterated.

Although we have provided solid evidence for the potential of microalgae to produce deuterated compounds and described their cell cycle in a highly deuterated environment, several questions remain. It would be useful to analyze the genes and biochemical pathways that are up- or down-regulated

in deuterated media. Such knowledge would provide a deeper understanding of cellular processes following deuteration and allow these pathways to be targeted to improve yields of highly deuterated compounds. This effort would be easiest in *C. reinhardtii*, with a known genome and established molecular toolbox (Kselíková et al., 2022b). An alternative approach would be to use our findings to establish the production of deuterated compounds in other biotechnologically relevant microalgae such as *Chlorella* spp., *Haematococcus* sp. or *Nannochloropsis* spp.

6. References

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7. Curriculum vitae

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Cultivation of microalgae in deuterated media (poster)

PUBLICATIONS:

- **Kselíková, V.**, Vítová, M. and Bišová, K. (2019). Deuterium and its impact on living organisms. *Folia Microbiologica* 64, 673–681.
- **Kselíková, V.**, Vyhnánek, T., Hanáček, P., Martinek, P. (2020). Grain hardness in triticale: a physical and molecular evaluation. *Czech Journal of Genetics and Plant Breeding* 56, 102-110.
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