UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

ÉTUDE DE L'IMPACT DE DIFFÉRENTES CARACTÉRISTIQUES DE LA LUMIÈRE SUR *PHAEODACTYLUM TRICORNUTUM*

INVESTIGATING DIFFERENT LIGHT CHARACTERISTICS IMPACT ON *PHAEODACTYLUM TRICORNUTUM*

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PREFACE

This Ph.D. has taught me many things but some of the important things that I have adopted in my life are:

1 Research is all about Isolation-Integration- Improvisation which one should use in their Ph.D. project but also for oneself.

2 Playfulness is an important element for research which one include in daily life can replace a lot of procrastination.

The thesis content and analysis resulting in this document are primarily the product of the intellectual efforts of Nikunj Sharma. But multiple co-authors participated in contributing towards the final publications for the following chapters and their contribution are explained in detail.

RÉSUMÉ

La disponibilité de la lumière et son impact sur la diatomée *Phaeodactylum tricornutum* est un facteur limitant et un thème essentiel à explorer. Un accès limité à la lumière et une dynamique de culture inefficace rendent les applications à base de diatomées et de microalgues infructueuses sur le plan commercial. Les différentes caractéristiques physiques de la lumière ont un impact régulateur sur les voies moléculaires; la perception de la lumière par *P. tricornutum* modifie la croissance globale et l'accumulation de biomolécules importantes pour l'industrie telles que les lipides, les acides gras, les pigments, etc. Une compréhension approfondie de ces processus moléculaires nécessite de comprendre comment la lumière modifie la machinerie cellulaire des diatomées et affecte la croissance et l'accumulation de différentes molécules de *P. tricornutum*. Par conséquent, l'objectif de cette thèse était d'analyser les caractéristiques fondamentales de la lumière (intensité lumineuse, qualité spectrale ou système lumineux biphasé/changement de la lumière) sur le type sauvage de la diatomée. Pour cela, nous avons effectué plusieurs réglages de lumière et paramètres de culture pour optimiser les conditions d'éclairage précises. L'objectif principal était de tester la qualité spectrale (jaune, rouge et blanc) de la lumière sur la croissance la quantité et le profil des lipides totaux chez le type sauvage dans des conditions autotrophes et mixotrophes. Deux sources de carbone différentes (glucose et glycine) ont été testées et il s'avère que les conditions mixotrophes sont meilleures pour la croissance et l'accumulation de lipides dans toutes les conditions d'éclairage par rapport aux conditions autotrophes. Les conditions de croissance en lumière rouge et dans deux conditions autotrophes et mixotrophes ont montré une plus importante accumulation de lipides par rapport aux deux lumières testées à savoir jaune et blanche. Ensuite une analyse de l'impact de la lumière rouge sur différentes phases de croissance telles que la phase d'acclimatation, la phase exponentielle, et la phase stationnaire a été effectuée. Les résultats ont montré que la substitution de la lumière rouge (RL) par la lumière blanche (WL) dans des conditions autotrophes, une condition de changement vers le rouge (RS), augmentait la biomasse et la teneur en lipides par rapport aux niveaux trouvés sous une exposition continue WL ou RL seule. Les résultats ont montré également une augmentation de la biomasse de l'ordre de 2 fois et de la teneur en lipides de 2,3 fois dans la condition RS par rapport à WL. Aucune différence significative n'a été observée concernant la taille ou la morphologie des gouttelettes lipidiques, mais une modification de la composition en acides gras (AG) a été observée. Plus précisément, les AG polyinsaturés ont augmenté, tandis que les AG monoinsaturés ont diminué chez *P. tricornutum* cultivé en RS par rapport à WL.

De plus, il était important de caractériser la composition spectrale précise et l'impact de l'intensité lumineuse sur la croissance de l'algue et la production des lipides. Pour cela, deux compositions différentes de spectres de lumière rouge ont été testées, à savoir rouge 1 (R1) 34 % > 600 nm > 66 % et rouge 2 (R2) 8 % > 600 nm > 92 % et deux intensités lumineuses; 65 µmol m^{-2} s^{-1}, correspondant à une faible luminosité (LL) et 145 µmol m^{-2} s^{-1}, correspondant à une lumière moyenne (ML). Les résultats ont indiqué que *Phaeodactylum tricornutum* utilisait la qualité spectrale R1 plus efficacement que

celle R2 pour la production de la biomasse et les lipides totaux. Ainsi les résultats ont montré que ML améliorait la croissance et l'accumulation de lipides par rapport à LL, lorsque la densité de flux de photons a augmenté dans R1 de 65 µmol m^{-2} s^{-1} à 145 µmol m^{-2} s^{-1}, l'accumulation de lipides a augmenté de 2,8 fois. Après optimisation des caractéristiques lumineuses à la qualité R1 et intensité lumineuse 145 µmol m^{-2} s^{-1}, les conditions d'éclairage continue en lumière rouge et le changement de la lumière rouge à 145 µmol m^{-2} s^{-1} à une lumière blanche ont été appliqués comme preuve de concept de l'utilisation de conditions d'éclairage variables et étudier l'effet sur la croissance et l'accumulation des lipides dans l'algue *P. tricornutum* hébergeant, le vecteur vide pPtGE30 (Ptev), et le transformant pPtGE30 contenant YFP la protéine fluorescente jaune (PtYFP). Nous avons remarqué que les algues (Ptev) dans les conditions de croissance RS avaient une augmentation significative de la croissance et de l'acide eicosapentaénoïque (EPA) par rapport aux autres cultures.

L'étude fournit une approche globale de l'utilisation de différentes caractéristiques lumineuses pour optimiser la croissance et la production de lipides. En comprenant comment ces facteurs affectent la croissance des diatomées, le métabolisme des lipides et des acides gras, des conditions de culture expérimentales pourraient être établies pour une culture à grande échelle pour obtenir un profil élevé de la biomasse, des lipides et des acides gras spécifiques pour les applications industrielles. En outre, cela démontre également l'importance des protéines fluorescentes qui peut être utilisé pour manipuler la croissance et le profil des acides gras chez *P. tricornutum*.

Mots-clés : *Phaeodactylum tricornutum*, lumière, acides gras, lipides, diatomées

ABSTRACT

Light availability and its impact on *Phaeodactylum tricornutum* is a limiting factor which makes it an essential theme to be explored. Ineffective light conditions and culture dynamics make diatoms and microalgae-based application commercially unsuccessful. Different light settings have a regulatory impact on molecular pathways, light sensing of *P. tricornutum* which changes the overall growth and accumulation of important biomolecules such as lipids, fatty acids etc. for industrial benefit. An understanding of these molecular processes requires understanding of how light changes the *P. tricornutum* growth and accumulation of different molecules by affecting the cellular machinery. Therefore, the objective of this study was to analyse the light settings (light intensity, spectral quality, or two-phase light system/light shift) on model organism *P.tricornutum.* For this we performed multiple culture and light settings to optimize the light conditions. The objective of the primary set of experiments was to test spectral quality (Yellow, Red, and White) on growth and total lipid on wild type in both autotrophic and mixotrophic conditions. We tested two different carbon sources (glucose and glycine) and found out mixotrophic conditions are slightly better for growth and lipid accumulation in all light conditions as compared to autotrophic conditions. The most suitable wavelength was red in both autotrophic and mixotrophic conditions as compared to yellow and white. We further analysed the impact of red light (RL) on different growth phases such as log, lag and stationary. Thus, results showed that substituting red (RL) for white light (WL) in autotrophic conditions, a condition called red to white shift (RS), increased biomass and lipid content compared to levels found under continuous WL or RL exposure alone. Results showed an increase by 2-fold biomass and 2.3-fold lipid content in RS as compared to WL. There was no concrete evidence obtained related to lipid droplet size or morphology, but the fatty acid (FA) composition was altered. Specifically, polyunsaturated FAs were increased, whereas monounsaturated FAs decreased in *P. tricornutum* grown in RS compared to WL.

Furthermore, we continued our investigation to find the appropriate spectral composition, and light intensity impact on growth and lipid on *P. tricornutum* harboring pPtGE30 empty vector (*Pt*ev). For that we used two different composition of red light spectra i.e., Red 1 (R1) 34% > 600 nm > 66% and Red 2 (R2) 8% > 600 > 92% and two light intensity; 65 µmol m^{-2} s^{-1} Low light (LL) and 145 µmol m^{-2} s^{-1} Medium light (ML). Results indicated that *Phaeodactylum tricornutum* grown in Red 1 (R1) which was more enriched in spectra over 600 nm showed higher growth and biomass as compared to Red 2 (R2). ML was improving growth and lipid accumulation as compared to LL. Furthermore, as the photon flux density was increased in R1 from 65 µmol m^{-2} s^{-1} to 145 µmol m^{-2} s^{-1}, the lipid accumulation increased by 2.8-fold times. From the studied light conditions, light settings with spectral quality R1 and light intensity 145 µmol m^{-2} $s^{-1.}$ was used further on microalgae *P. tricornutum* harboring pPtGE30 empty vector (*Pt*ev) and yellow fluorescent protein YFP transformant (*Pt*YFP) to modify growth and lipid. We noticed that

microalgae (*Pt*ev) in Rs culture condition had a significant increase in growth and fatty acid eicosapentaenoic acid (EPA) as compared to other cultures.
The study provides an approach of using different light settings to optimize growth and lipid production. By understanding how these factors affects the diatoms growth, lipid and fatty acids, experimental culture conditions could be established for large scale cultivation for high biomass, lipid and specific fatty acid profile for industrial applications Furthermore, there is a small possibility that the use of episomal vectors and fluorescent proteins might interfere with the growth rate and fatty acid profile of *P.tricornutum*.

Keywords: *Phaeodactylum tricornutum,* light, fatty acids, lipids, diatoms

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List of Abbreviations

NPQ- Non photochemical quenching
CCM- Carbon concentration mechanism
EPA - Eicosapentaenoic acid
DHA - Docosahexaenoic acid
PDC- Pyruvate dehydrogenase complex enzyme
PUFA- Polyunsaturated fatty acids
ACCase- Acetyl CoA carboxylase
TAG- Triacylglycerol
PC- Phosphatidylcholine
FCP- Fucoxanthin-chlorophyll a/c-binding proteins
HL- High light
LL- Low light
ML-Medium light
R1- 34% > 600 nm > 66%
R2- 8% > 600 nm > 92%
RS- Red to white light shift
RT1- Red light treatment 1
RT2- Red light treatment 2
RT3- Red light treatment 3

CHAPTER I

INTRODUCTION TO DIATOMS AND IMPACT OF LIGHT ON DIATOMS GROWTH AND LIPID

1.1 What are Diatoms?

Diatoms (Bacillariophyceae) are single cell microscopic organisms comprising approximately 100,000 species known for 20–40% of global carbon fixation (1, 2). It is also being estimated that diatoms alone produce 40% of the 45 to 50 billion metric tons of organic carbon in sea which is equivalent to all terrestrial rain forests combined (3, 4). They are key drivers of maintaining the aquatic system and various biogeochemical cycles in oceans (5). Diatoms are part of heterokontophyte, known as stramenopiles that are adaptable to wide range of environmental conditions ranging from tropical to polar ecosystems (1, 6, 7). Even though, there are common features between diatoms and green algae or plants they come along with distinct differences as well. The cellular features that makes them distinctive from green algae and plants are hydrated cell wall made of silicon dioxide, a chloroplasts surrounded by four layers instead of two and fucoxanthin chlorophyll proteins (8,9,10). Evolutionary lineage of diatoms is quite distinct from plants, green algae and red algae and some reports have suggested the possible evolutionary route through secondary endosymbiosis between a red alga and a heterotrophic flagellate (11, 12). Therefore, it led to accumulation of new set of genes and complex plastid that has four membranes instead of two. In the past 2–3 decades, the progress at the genomic and genetic level for the model systems in green algae *Chlamydomonas reinhardtii* and diatoms such as *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* to study photosynthesis, chloroplast biology, flagellar motility, and micronutrient homeostasis (13-16). Moreover, the ability of diatoms to do photosynthesis and produce high-valued carbohydrates, proteins, and fats, under varied conditions, is an interesting attribute for industrial applications. *P.tricornutum* is a good model organism to be used for industrial applications as its genome has been published and various genetic tools are available. The transformation techniques have been developed for ten diatom strains but the most studied and successful strains are *P. tricornutum* and *T. pseudonana.* It is known to accumulate high amount of omega fatty acids like EPA or very long chain fatty acids like arachidonic acid which are key ingredients in pharmaceutical and nutraceutical industries (17). Furthermore, it can be

used as biofertilizer or bio-stimulant to enhance crop productivity (18, 19). Diatoms are also beneficial for ecological monitoring which has potential to be more cost effective than the traditional methods (20).

The world population is constantly increasing so is the need for alternative energy and food source. As discussed above and in supplementary data (Appendix B), diatoms and microalga could have the potential to contribute in addressing the global challenges such as energy, climate change,food security, ecology, nutrition, etc. (21).

1.2 Exploitation of natural products from diatoms

Diatoms are of particular interest for the production of omega fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are essential for human health as we are not capable of *de novo* synthesis of omega fatty acids which are the necessary compounds that aids in prevention of cardiovascular and inflammatory diseases (22). Presently, most of the demands of EPA are met through consumption of wild fish or fish oil capsules which have raised economic, ethical and environmental concerns. Large scale cultivation of microalgae have the potential to provide the supply of sustainable source of omega fatty acids for human and animal nutrition. But there are still some economic challenges for large scale production of omega fatty acids from microalgae which requires optimization of cost-effective production. And there are some advantages of diatoms and microalgae over land plants as it needs no agricultural land which have it own sets of challenges such as reducing soil organic content worldwide (23). Therefore, we need alternative renewable source of raw material that can provide critical nutrients like omega fatty acids for human health or biofertilizer blend from microalgae that can nourish the soil.

The above mentioned commercial benefits have attracted the attention of both researchers, industries, and government agencies due to potential industrial applications and develop sustainable products to reduce the consistent damage to our environment along with sustainable economic upliftment (24). Furthermore, I added a detailed supplementary chapter on possible industrial applications of diatoms entitled “Diatoms Biotechnology:Various industrial applications for greener tomorrow” (Appendix B).

1.3 Lipids and fatty acids

Lipids is the name given to the heterogenous set of fat soluble compounds containing fats, oils, steroids, waxes and related compounds. Lipids and their derivatives have a chain of hydrocarbons containing carboxylic group which is hydrophilic in nature and aliphatic tail which is hydrophobic. These compounds are relatively insoluble in water and soluble in polar solvents. Lipids can be broadly divided into four groups; triglycerides, phospholipids, steroids, and waxes whose general functions are energy storage, making biological membranes, insulation, protection and acting as precursors for metabolic activities.

Fatty acids are the integral building blocks for lipids which are either saturated or unsaturated. The characterization of fatty acids is usually done in terms of carbon chain length and number of bonds which determines their properties in the cell. Thus, a fatty acid with 16 carbons and 1 double bond would be abbreviated as C16:1 known as palmitoleic acid. Fatty acids are broadly categorized into three kinds based on the number of bonds, fatty acid with no double bonds are saturated fatty acids, fatty acid with single double bond is monounsaturated fatty acid and more than one double bond is termed as polyunsaturated fatty acids.

Lipids and biomass composition in microalgae are affected by culture, light and stress conditions which has been reported by many studies (25-27). Nutrient starvation such as nitrogen and phosphorus can trigger the production of lipids in microalgae cells as reported in the following articles (28-31). The other important limiting factor is light which has been shown to have variable impacts on growth, total lipid content and accumulation of important fatty acid like EPA which is given high value in human nutrition (32-34).

Microalgae are one of the best organism for high production of omega fatty acid such as EPA. *Phaeodactylum tricornutum* is one of the ideal source for the production of omega fatty acids which exhibits potential value to investors and industries. At the present moment, they are being use for aquafeed whose demand has doubled in last decade and might become economically unstable (35). It has been reported that the fish meal supplemented with freeze dried biomass of *P. tricornutum* was effective to improve fish immune status or immunostimulating effects (36, 37). Therefore, it is possible to say that diatoms can be a potential sustainable source of omega fatty acids for humans, animal feed additives or can be used as biodiesel blend (38).

1.4 Fatty acid synthesis pathway in diatoms

In general diatoms have similar classes of lipids as plants and green algae, but there are few differences, diatoms contains the combination of medium chain and very long chain fatty acids like EPA and DHA which are usually absent in plants (11,39-41). Another lipid that is present in diatoms and absent in plants thylakoid membrane is phospholipid phosphatidylcholine (PC). One of the important difference that is relevant for industrial and human consumption is the presence of high amount of EPA and DHA in aquatic life such as phytoplanktons and fishes as compared to terrestrial plants (42). Considering the potential benefits and need of sustainable supply of omega fatty acids, we have given more attention towards the growth, total lipid and EPA in the thesis in general.

Centric *T. pseudonana* and the pennate *P. tricornutum* are the two most studied diatoms species that have been exploited for industrial uses considering the availability of omics and physiochemical data for these species. But our knowledge of lipid metabolism is still limited as compared to plants, animals and green microalgae (39). Thus, the present knowledge on diatoms lipid metabolism is derived from green microalgae, plants and genome predictions. Lipid metabolism in diatoms could be understood in two sections, a prokaryotic pathway found in membranes of plastids and a eukaryotic pathway found in the endoplasmic reticulum (ER) (43-45). Plastids are the primary site of fatty acid synthesis where acetyl-CoA is the precursor which is also found in mitochondria and peroxisomes. So, the pool of acetyl-CoA from mitochondria and peroxisomes needs to be transported to plastids (46, 47). Acetyl-CoA could be synthesized from different routes either through synthesis from free acetate or decarboxylation of pyruvate catalysed by pyruvate dehydrogenase complex (48). Based on homology with flowering plants, one can manipulate the total lipid accumulation in diatoms by regulating pyruvate dehydrogenase complex enzyme (PDC, Figure 1). The FA synthesis is multistep enzyme process that has been used to modify the lipid metabolism (46). Conversion of acetyl-CoA to malonyl-CoA is the initiation of FA synthesis catalysed by acetyl-CoA carboxylase (ACCase, Figure 1). The next steps of FA synthesis in plastids consist of a sequential condensation of two-carbon units, which is catalyzed by the enzymes of the FA synthase (FAS) complex. From here on, chain reactions sets up where each cycle results in the addition of 2 carbon units to the growing acyl chain, with malonyl-ACP as substrate for the condensation reactions. This step leads to the synthesis of C16:0 ACP which is synthesized by KAS (keto-acyl synthesis). The terminal step of the fatty acid synthesis is the hydrolysis of

acyl-acp protein to create the pool of free fatty acids in the endoplasmic reticulum which is considered to be the substrate of very long chain fatty acyl-Coa synthetases. There are missing loops about how these free fatty acids are transported to the membrane for the synthesis of long chain fatty acids. The mechanism is unknown for the transport of fatty acids from plastids to endoplasmic reticulum (46). Further fatty acids can be stored in form of triacylglycerol (TAGs) which is stored energy and can be formed by two pathways: an acyl-CoA dependent pathway and an acyl-CoA independent pathway (49-51). In acyl-coA dependent pathway, it is proposed that initiation happens with acylation of G-3-P which further goes through series of acylation steps to form lysophosphatidic acid. Phosphattidic acid may be de-phosphorylated by phosphatidic acid phosphatase (PAP) to produce DAG. In the final acylation step, acyl-CoA:DAG acyltransferase (DGAT) forms TAG from DAG by incorporating the third acyl-CoA (49, 50). In the acyl-CoA independent TAG biosynthesis pathway, the acyltransferase enzyme PDAT uses phosphatidylcholine (PC) as an acyl donor for the formation of TAG from DAG (52, 53). The overall pictorial representation of fatty acid synthesis is given in Figure 1.

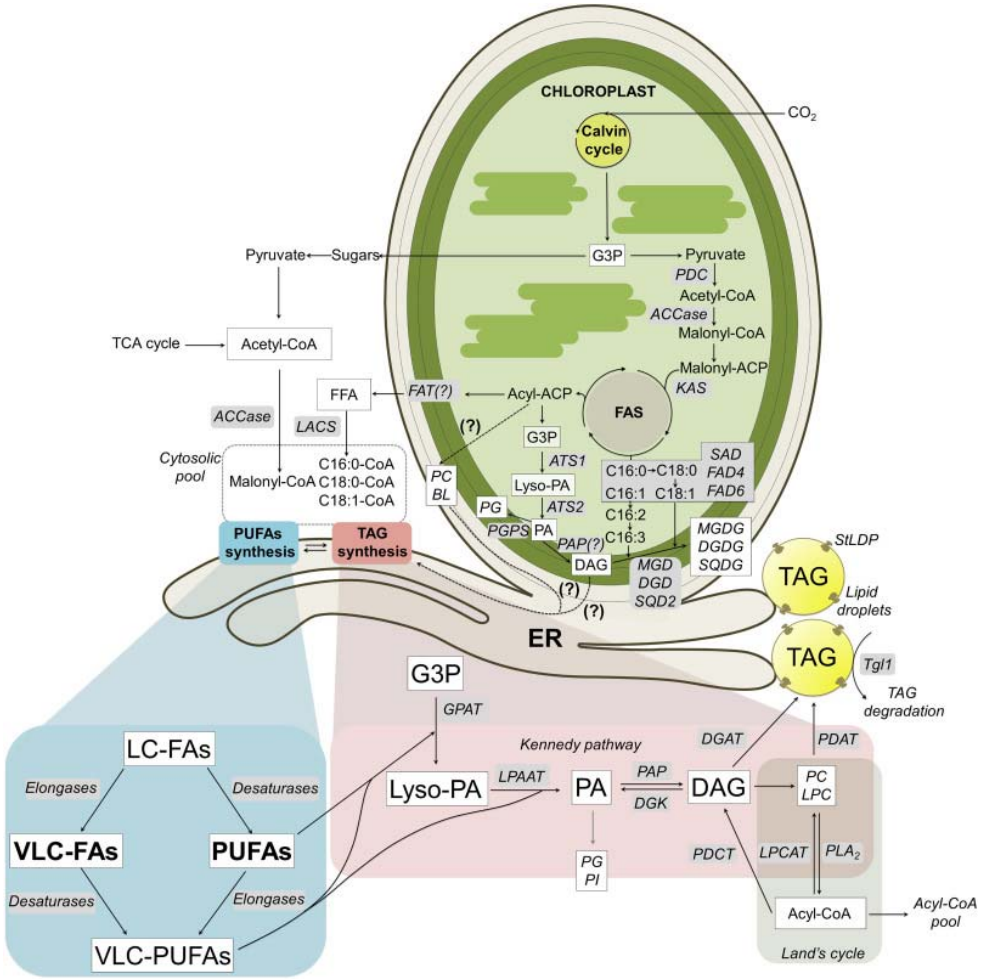

Figure 1- Schematic representation of fatty acid synthesis, very long chain fatty acid synthesis and Triacylglycerol formation (46) . ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; ATS1, plastidic glycerol-3-phosphate acyltransferase; ATS2, plastidic lysophosphatidic acid acyltransferase; BL, betaine lipids; CoA, coenzyme A; DAG, diacylglycerol; DGAT, acyl-CoA:diacylglycerol acyl transferase; DGDG; digalactosyldiacylglycerol; DGK, diacylglycerol kinase; FAT, fatty acyl-ACP thioesterase; FFAs, free fatty acids; KAS, 3-ketoacyl-ACP synthase; G3P, glycerol 3 phosphate; GPAT, acyl-CoA:glycerol-3-phosphate acyl transferase; LACS, long chain acyl-CoA synthetase; Lyso-PA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyl transferase; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; MGDG; monogalactosyldiacylglycerol; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDC, pyruvate dehydrogenase complex; PG, phosphatidylglycerol; PGPS, phosphatidylglycerol phosphate synthase; PLA_2,

phospholipase A_2, PUFA, polyunsaturated fatty acid; SQDG, sulfoquinovosyldiacylglycerol; StLDP, stramenopile-type lipid droplet protein; TAG, triacylglycerol; TCA, tricarboxylic acid.

Good number of research articles have explored the use of genetic transformation to overexpress different genes throughout the pathway. As such, overexpressing the malic acid enzyme can increase total lipid content 2.5 fold (54) and diacylglycerol acyltransferase (DGAT2) in *P. tricornutum* can stimulate more lipid bodies (55). Heterologous expressions of two thioesterases (a myristic acid biased thioesterase (C14-TE) from *Cinnamomum camphora* and a lauric acid biased thioesterase (C12-TE) from *Umbellularia californica*, causes increased accumulation of shorter chain length fatty acids in *P. tricornutum* (56).

Changes in the environmental parameters such as light and nutrient conditions impacts the accumulation and synthesis of fatty acids**.** Further, we discussed about few important genes like the overexpression of malic enzyme, DGAT2 that could impact the accumulation of more lipid. With more interest towards the production of single end product- EPA supplement, *P. tricornutum* is a suitable system as it contains few intermediates in the EPA biosynthetic pathway.

Diatoms require right physiological conditions for the effective accumulation of lipids, synthesis of membrane lipid as well as partitioning of different fatty acids into various lipids (44, 57). All the conditions such as pH, salinity, temperature, nutrition and light have an impact on the growth, lipid accumulation and even fatty acid composition (44). For instance, it is known that fatty acid like EPA and DHA are deposited in TAGs under genetic modification or stress conditions during stationary phase of diatom culture (57, 58). Research is still under progress for the impact of different carbon sources, nutrient deprivation on diatoms growth and lipid accumulation. The other side that requires attention is the impact of light on growth and lipid accumulation considering that ~5% of light is converted to biomass and should be explored using the available tools.

1.5 Lighting systems

Light is the main element for the process of photosynthesis and is also a limiting factor for photosynthetic organism growth. It is known that only a fraction of solar spectrum mostly blue (420-470 nm) and red wavelength (650-680); is used for the process of photosynthesis and the rest is either reflected or dissipated through heat (59, 60). All photosynthetic organisms use

different wavelength with distinct efficiency owing to the differences in pigments such as chlorophyll a, b, c, d and e, bacteriochlorophylls, pheophytin a and b and other kinds of pigment molecules such as carotenoid α and β, xanthophyll (61). Spectral quality, light intensity and light distribution are considered some of the important variables to increase the light to biomass conversion efficiency. And these are some of the common challenges observed in both open and closed scale cultivation system.

So far, the most common and effective reported light modes used for algae and diatoms cultivation are LED (light emitting diodes) and fluorescent bulbs and have provided good amount of data for the base reference (32, 62-64) and will be discussed more in detail in the following sections. The use of artificial light sources in large scale photobioreactors adds to the total cost of production which is not sustainable for commercial projects. The advantages of using artificial lightning system in photobioreactor system such as controllable culture environment and ability to easily manipulate the conditions is still not affordable to be applied at commercial scale successfully. There are multiple challenges of using photobioreactor such as coupling of light, hydrodynamics, mass transfer, and cell growth in efficient reactor. Initial capital requirement and the overall cost of operations are challenges (65, 66).

The alternative method is to use the available outdoor culture which is also not easy conditions for microalgae, considering the seasonal variations in lights and solar spectrum also contain other wavelength such as infrared and near infrared light that is not suitable for microalgae growth (66, 67). Therefore, cost effective distribution of light to large scale cultivation is still the missing link for algae biotechnology (67).

Another way to modulate the light environment is the use of light filters which can be used to transmit the needed spectra required for microalgae cultivation. There are few studies that have used light filters made out of cellophane, nanomaterials or tar colour additives to improve growth and biomass (65, 67, 68) and have reported the possible potential use in large scale open cultivation. A study analysed the use of light filters and observed no changes in the flask as compared to filters used in flat panel reactors, and rotating algal biofilm considering the possible reflection of light coming from the walls of flasks (67). Another route that has been adopted to optimize the spectral composition is the use of fluorescent proteins such as GFP and YFP which can capture the excess light in the cell for expanding the spectral coverage and photosynthetic efficiency (69).

There are more publications that have shown the impact of light intensity and spectral quality impact on microalgae growth and lipid content

(25-27, 32-34). It can be cautiously said that, optimizing the light settings can have an impact on diatoms industrial growth.

1.6 Impact of light on diatoms

Considering the wide range of light conditions that diatoms can survive; it is important to identify the needed light conditions to be applied for industrial applications. And we know that diatoms can harvest the photons from visible light in the range of 400–700 nm spectra, which is initiated by the antennae pigments and forming the complex network of signalling between pigments to reach the reaction center. Published literature reviews and research articles have provided the data on the different light set ups such as light intensity, spectral quality, two phase light system impact on biomass and bioactive molecule accumulation and using genetic engineering tools to study the impact of light on *P. tricornutum* physiology (70-73). Broadly, diatoms can respond to light in following two ways: 1) under low light conditions, diatoms try to capture the sufficient number of photons for the basic physiological activities 2) under high light conditions, diatoms try to avoid the excess number of photons and if not, it leads to photodamage (74). Although, there is ample data about the impact of light on plants and green algae, it is not transferable to diatoms considering the difference in the structure of chloroplast and composition of pigments where the research is still in development phase and is a debatable area (75). Land plants and green algae use LHCII containing chlorophyll a and b whereas diatoms use FCP protein complex containing chlorophyll a and c to harvest light (76). Diatom chloroplast is quite distinct from the plants and green algae, it consists of an envelope with four membrane, the thylakoids are arranged in stacks of three, there is no spatial segregation of the photosystems, and have peculiar protein and pigment organization (77, 78).

To obtain the best growth conditions, phototrophs have developed multiple processes such as thermal dissipation of excess light energy using non photochemical quenching, photoharvesting, short term physiological response which involves the regulation of three kinds of light harvesting proteins i) a set of lhcr protein ii) stress related lhcx proteins iii) large group of lhcf proteins (79-81). And these light harvesting proteins have variable response towards difference spectral quality for e.g. : Blue light has been majorly shown to activate the stress related pigments and it has been observed the *PtLhcx3 may* participate in photoprotection by elevating the non photochemical quenching which is more activated under blue light as compared to red and white light (82). On the other side, cultures grown in red light usually

have to modulate the process of light harvesting by adjusting the light antenne size or by change in thylakoid number which was higher in red light culture as compared to white light (83). Another study showed the red shifted form of FCP protein complex which is present in red light culture and absent in white light culture might enhance its light harvesting ability (81). This allows the cells to have enhanced absorption spectra in longer wavelength which might alter the energy transfer process between photosystem based reactions. The same study also suggested the possible quenching of PSII process in blue light cultures whereas absent in red light culture suggesting that energy was transferred to red shifted FCP form which is energetically bound to PSII reaction center.

There are multiple studies attempting to optimize the light conditions to have better light to biomass conversion efficiency to produce high biomass, lipids, etc., which includes studying microalgae and diatoms in variable light intensity, spectral quality, short term light acclimation as discussed in the text so far (59, 84-87).

A study reported comparable growth and photosynthesis rate under blue and red light acclimation but observed rapid changes during light shift after initial acclimation. There were severe changes in metabolic organisation during red to blue light shift such as increase in amino acid pool, protein synthesis, TCA cycle, inhibition of growth whereas there was a decrease in amino acid pool, accumulation of carbohydrates and no changes in growth during blue to red light shift. It was further noticed that there was increase in parts of carbohydrates at the cost of proteins during blue to red to white light shift whereas an increase in ratio of protein/carbohydrates during red to blue light shift. This can be explained with increase in carbon/nitrogen ratio (C/N) under red light culture as compared to blue light (88). The change in C/N ratio also leads to more accumulation of pyruvate in red to blue light shift as compared to blue to red to white light shift. Pyruvate is an important mediating molecule for *de novo* fatty acid synthesis, amino acid synthesis and glycolysis. More studies have reported the increase in biomass, total lipid or specific lipid fraction such as EPA under red light or combination of red and blue in *P. tricornutum* and other microalgae species (89-91). Therefore, it can be cautiously said that light profile can change growth patterns, lipid accumulation or specific fatty acid profile.

In addition to photosystems and light harvesting protein complexes, diatoms possess photoreceptors that are essentially light responsive such as crytochromes, phytochromes and aureochromes allowing the sensing of light intensity and wavelength. It is known that aureochrome are involved in various physiological processes such as detection of blue light, high light acclimation and cell cycle. Mann et al. (2020) observed an upregulation of genes

related to light reaction, glycolysis and fatty acid metabolism during red to blue light exposure (71). Furthermore, they observed this response was inhibited or downregulated when aureochromes was absent. Another study investigated cryptochrome-like proteins which are involved in DNA repair, Calvin cycle, the photosynthetic light reaction, the tetrapyrrole biosynthesis, and the carbohydrate metabolism which are more upregulated in blue light (70). The study reports the dynamic response of different photoreceptors like aureochromes, cryoptchromes in wild type and mutants in different wavelength. The literature on the impact of different wavelengths and light intensity demonstrates rapid and reversible changes, coordination between growth and lipid production and metabolic flexibility. Reviewing these events have opened many questions for the future such as the possible sensing of different spectral light quality or intensity by either of light harvesting complex/photoreceptors or collaboration between different sets of light harvesting pigments/photoreceptors to have wide range of adaptive states which requires more research.

These reports have provided a broad idea that specific combination of light spectra is more effective as compared to broad light spectrum. Therefore, increasing the light utilization efficiency can improve the overall diatom cell machinery for carbon fixation, photosynthesis, etc. Hence, optimizing the light conditions for specific application such as high biomass, lipid or omega fatty acid production could ensure a consistent output. We can use light intensity, spectral filtering, genetic engineering of light harvesting and photosynthetic related components and two-phase light system for increasing biomass and lipid productivity.

1.7 Impact of Spectral quality on Diatoms

Few studies have reported the impact of spectral quality on gene expression, pigments, fatty acid profile which are noticeable and should be explored extensively. Diatoms respond to different spectral quality in highly discriminatory way using variations in pigment profile or photoreceptors. The main pigments in diatoms are chl a, c which absorbs light in red (600-700 nm) and blue (400-500 nm) wavelength and fucoxanthin which absorbs light in green (500-570 nm) wavelength. Furthermore, there are conformations in fucoxanthin with specific absorption maxima which are developed in different spectral profiles such as 505 and 510 nm-$Fuco_{red}$), (488-492nm-$Fuco_{green}$) and (445-463nm -$Fuco_{blue}$). The differences in absorption properties reflects that it might affect the overall protein environment impacting the overall light harvesting process. For example, a study has reported the development of red shifted FCP

under red light, with prolonged fluorescence time under red light as compared to blue light. They further noticed the formation of large structural domains containing PSI-lhcr supercomplexes that are present in red light culture and absent in day light culture (81). Photoprotection and repair of PSII are the other side of the coin that are related to non photochemical quenching process which might leads to slow growth and inhibition of bioactive molecules. Studies have reported that non-photochemical quenching or photoprotections are activated or dependent on blue lights (73, 81).

Considering from the application point of view, a study observed the 8 different variations of red and blue monochromatic LED on growth and lipid profile of *P. tricornutum* and observed that highest biomass was obtained under white light. It was further observed that light settings enriched with red light spectra was conducive for total lipid production and light settings enriched with blue light was suitable for EPA production (89) . Another study reported, that high proportions of red light might be useful for high lipid production and high EPA production whereas blue light will be more conducive to saturated fatty acid production (90). Similar patterns for high biomass and growth trend was observed in Red :blue ratio (6 :1) in batch cultures compared to white light and other ratios of R :B (0 :1,6 :1,1 :1,1 :2,1 :0) (92).

Light shift is a cultivation process where culture is grown in one light condition with an intent to increase the biomass and then shifted to another light conditions to accumulate more lipids (84, 88, 93). Jungandreas et al. (2014) have reported the accumulation of proteins and lipids in red to blue light shift and accumulation of carbohydrate in blue light to red to white shift (88). And it was observed that acclimation to specific spectral quality could change the accumulation of metabolite profile such as TCA cycle intermediates, amino acids and intermediates of glycolysis (88). Yang et al. (2020) observed the light shift from the ratio of R:B (6:1) to (5:1) to be most suitable for biomass and fucoxanthin production in *P. tricornutum* (92). Another study observed that the ratio of 50:50 was better for effective biomass and lipid accumulation in *P. tricornutum* (94). Light shift is an effective method to be applied in large scale cultivation in photobioreactors. In addition to high lipid content in red light, another study reported the low level of non photochemical quenching in red light that means less energy dissipation which indicates the higher light use efficiency/photon or low energy loss (82). The results are non conclusive regarding the use of spectral quality and its impact on total lipid, increasing the fraction of specific fatty acid like EPA considering the different light setting and different composition of light spectra used in reported studies.

Another way to approach this is to look into the functions of the photoreceptors such as aureochromes and cryptochromes that senses the different spectral quality light and can

modulate the processes related to the photosynthesis, lipid and carbohydrate metabolism where they used light shift based experiments (70, 71).

1.8 Impact of light intensity on diatoms

There is ample literature on the impact of light on various physiochemical parameters in different microalgae species along with diatom *P. tricornutum*. Light intensity can affect the diatoms physiology and genetics at multiple levels. For example, diatoms have evolved to have defense mechanism under high light stress conditions such as non photochemical quenching (NPQ) where there are major changes in xanthophyll pigment pool or changes in transcript abundance of lhcx (stress related proteins) under high radiance or shift from low to high light (28, 29,95, 96). The defense mechanism also represents an energy loss to the cell. Furthermore, few studies have reported the degradation of D1 protein of PSII which is known as function of light intensity (79, 96, 97). The rate of NPQ is usually high under blue light as compared to red light.

It has been shown that light intensity can regulate the carbon reorientation which allows the storage of energy dense molecules like lipids. Heyderizadeh et al., (2017) showed that carbon limited environment and variable light intensity impacts the use of pyruvate/phophoenolpyruvate to produce organic acids in low light and lipids and proteins in high light. They reported low rate of mitosis, progressive enlargement in light harvesting complex which might require deriving the energy from nonphotosynthetic processes making it an energy expensive process for the culture under low light (30 $\mu mol\ m^{-2}\ s^{-1}$). Another way to understand the low growth rate in low light is the requirement of more energy for light capture to meet the energy allocation needed for carbon concentration mechanism (CCMs). Further comparing the ML (300 $\mu mol\ m^{-2}\ s^{-1}$) and HL (1000 $\mu mol\ m^{-2}\ s^{-1}$), they reported the maximum growth at ML and low growth in HL considering the stress generated by the excess photons at HL leads to energy dissipation. The same study noticed the most lipid in ML, proteins and carbohydrates in HL and LL respectively (98). Another study analysed the changes in gene expression and pigment accumulation at 150 $\mu mol\ m^{-2}\ s^{-1}$ (LL) and 750 $\mu mol\ m^{-2}\ s^{-1}$ (HL). They reported that HL inhibits the synthesis of pigments which impacts the absorption of excess excitation and activation of non-photochemical quenching. They also reported the low EPA at high irradiance (750 $\mu mol\ m^{-2}\ s^{-1}$) as compared low irradiance (150 $\mu mol\ m^{-2}\ s^{-1}$) (87). Few studies have confirmed the light variations can affect the lipid synthesis specially

EPA production needed for membrane synthesis and thylakoid structure maintenance (34, 40, 99).

1.9 Project Aim and Research Objectives

The overall aim of the project was to investigate the different light settings on the growth and biomass, lipid and fatty acid profile of wild type and transconjugant strains in *P. tricornutum.* The research objectives for the following PhD thesis were to design a way to add value in industrial application while keeping in mind to derive new knowledge that could be used for future PhD projects for both fundamental as well as industrial output. The objectives of the project broadly covers different light settings on wild type and transconjugant *P. tricornutum*.

Chapter II: The objective was to identify the suitable light conditions that enhances the total biomass, lipid and the general fatty acid composition. This chapter provides some insights on the importance of spectral wavelength. We tested how different light spectra in autotrophic and mixotrophic conditions affects the growth and lipid in *P. tricornutum*. Further we tested the light shift strategy which could also be called as two phase light system to enhance total biomass and lipid. Hence, the obtained data might provide us the tools to establish some cost-cutting ideas to be utilised in commercial applications.

Chapter III: The objective was to find the best light settings in terms of spectral quality, light intensity and light shift on transconjugant strains of *Phaeodactylum tricornutum*. We looked at the impact of different light conditions on transconjugant strains containing an episomal vector and reporter gene which has never been studied on model organism *P. tricornutum*. There are still many gaps between the academic research and its application for microalgae based commercial applications therefore investigating the possible strategies using genetic tools like episomal vectors and reporter genes for heterologous protein could provide some clues for further advancements.

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CHAPTER II

RED LIGHT VARIATION AN EFFECTIVE ALTERNATIVE TO REGULATE BIOMASS AND LIPID PROFILES IN *PHAEODACTYLUM TRICORNUTUM*

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The major and initial creation of conceptualization and methodology was done by N.S and I.D.-P. All the Co-Authors have contributed in the following sections.

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Methodology, N.S.; F.A.; F.M.-M.; H.G. and I.D.-P.
Validation, N.S.; G.F.; F.A.; M.C.; S.M.B.; F.M.-M.; H.G., and I.D.-P.
Formal analysis, N.S.; F.A.; F.M.-M. and I.D.-P.
Investigation, N.S.; G.F.; and F.A.
Writing-original draft preparation, N.S.; F.A.; F.M.-M. and I.D.-P.
Writing-review and editing, N.S.; G.F.; F.A.; M.C.; F.M.-M.; S.M.B.; H.G. and I.D.-P.
Supervision, H.G. and I.D.-P.
Project administration, I.D.-P.; funding acquisition, I.D.-P.
All authors have read and agreed to the published.

2.2 Full article in English: Red Light Variation an Effective Alternative to increase Biomass and Lipid Profiles in *Phaeodactylum tricornutum*

Abstract

Marine water diatom *Phaeodactylum tricornutum* is a photosynthetic organism that is known to respond to the changing light environment and adapt to different temperatures to prevent photoinhibition and maintain its metabolic functions. The objective of the present study was to test whether light shift in different growth phases changes the growth and lipid metabolism of *P. tricornutum*. Thus, we investigated red light (R) exposure in different growth phases to find the better light shift condition. The results showed that substituting red light by white light under autotrophic conditions, a condition called red to white shift (RS), increased biomass and lipid content compared to levels found under continuous W or R exposure alone. We observed an increase by 2-fold biomass and 2.3-fold lipid content in RS as compared to W. No significant change was observed in the morphology of lipid droplets, but the fatty acid (FA) composition was slightly altered. Specifically, polyunsaturated FAs were increased, whereas monounsaturated FAs decreased in *P. tricornutum* grown in RS compared to W control. Therefore, we propose that a light shift during the beginning of the stationary phase is a low-cost cultivation strategy to boost the total biomass and lipids in *P. tricornutum.*

Keywords: light shift; *Phaeodactylum tricornutum; biomass; microalgae; lipids; autotrophy; metabolites*

2.3 Introduction

Diatoms *Phaeodactylum tricornutum* have an important industrial potential for the production of biofuels, nutraceuticals, cosmetics, valuable lipids, and bioactive molecules. However, the commercial use of diatom biomass is dependent on input energy and nutrient cost. The large-scale cultivation for a single cell organism at the industrial scale is a challenge considering the variables involved (e.g., energy, nutrients, and contamination

issues). Diatoms are known to grow in both benthic and planktonic environment, making them capable of adapting in wide range of light spectra (1–3).

Light is the regulating parameter that controls the photosynthetic machinery of diatoms and regulates the range of light-induced physiological responses (3). The variation in light settings affects the growth rate, photosynthesis, lipid metabolism, which is crucial to enhance the quantity and quality of diatom biomass for the high production of desired bioactive molecules. Therefore, light should be provided with specific spectral components, intensity, and duration. Multiple ecophysiological studies have been published reporting on the distinct behaviors of different diatoms and algae in response to variations in light (4,5). It has been shown that light quality has an impact on chloroplast migration and on the light acclimation reactions of photosynthesis (6,7). Red light has been linked to stimulating growth, ethylene production, lipid accumulation, and increasing thylakoid stacking (6,7,8). López-Figueroa et al.(1991) have suggested the involvement of phytochromes in increasing the intracellular nitrate content on exposure to red light for short periods altering the overall cellular nitrate content (9). Recent discoveries in genomics have revealed exciting information on phytochromes, blue-light sensing cryptochromes, and aureochromes (10). In a study by Jungandreas et al.(2014) it has been reported that *P. tricornutum* cells acclimated to both red and blue light have comparable profiles, but drastic changes in metabolites like pyruvate, malate and citrate which might be involved reorganisation of intrernal carbon flux during light shift experiments (11). In addition, various interdisciplinary studies have shown that light perceived by the red-light receptor (phytochromes) can regulate nutrient metabolism, cellular events, and signaling cascades (12). These discoveries are still in progress and much remains unknown, but these studies can be explored for application-based diatom experiments such as developing low-cost cultivation technology or efficient bioreactors.

In both culture medium and natural water bodies, light (intensity, distribution, photoperiod) may vary and will not behave similar to the dissolved salts or nutrients in culture medium. Therefore, the light intensity and color will have different impacts based on the volume of culture, geometrical shape of the reactor, shaker speed, aeration, types

of culture media like mixotrophic or heterotrophic conditions (13). Even though mixotrophic conditions have received attention from various researchers to increase biomass and total lipid but it also adds to cost and possible contamination challenges. Considering all these factors and the available literature on the photosensory abilities of *P. tricornutum*, it is of paramount importance to create an effective light distribution system that can be used for commercial applications while reducing cost of input energy. Some studies have investigated the production of mutants of different antenna size to create an effective strain for efficient light utilization, but this process resulted in a reduced fitness of strains (14,15).

In this study, we aimed to assess if specific shifts in light color enhance the biomass and total lipid content. Thus, we conducted the prescreening of different light colors on *P. tricornutum* in autotrophic and mixotrophic. We have used glucose and glycine as carbon source for our mixotrophic conditions considering the ease of availability. Furthermore, we expanded our investigation on timing of red-light exposure and light shift impact on wild-type *P. tricornutum* growth by analyzing its biomass, lipid, and fatty acid composition. Our research has shown the importance of light color, light availability, and timing of different red light (R) exposures, which can be explored as a strategy to enhance the industrial production of wild-type *P. tricornutum* biomass and lipid content. This study provides an alternative strategy that can be useful for lab and large-scale cultivation.

2.4 Materials and Methods

2.4.1 Microalgae Strain and Culture Conditions

The inoculum culture for all experiments was prepared the same: all the experiments were conducted into 250 mL Erlenmeyer flasks containing 50 mL of liquid media with and an initial inoculum size of 0.2 OD. Axenic cultures of *P. tricornutum* (Culture Collection of Algae and Protozoa CCAP 1055/1) were obtained from Western University, Canada. *P. tricornutum* cells were grown and maintained in L1 media without silica pH 8 (Artificial Sea Water) as described in (16) in 250 mL Erlenmeyer flasks (50 mL of culture)

at 18 ± 1 °C. The experiments were conducted in the growth Chamber CMP6050 with light intensity 75 μE m^{-2} s^{-1} and a photoperiod of 16:8 h light/dark cycles. The light source was cool white light F54T5/841, which was kept 60 cm above the bottom of the culture flask. The light conditions were variable by ±1–2 μE m^{-2} s^{-1} in different corners of the rotational shaker. The humidity of the chamber was 50% with no additional air supply, and the rotary shaker speed was fixed at 130 rpm.

For experiments, the transmittance spectrum of studied light wavelengths has been measured and reported in Chapter 3, Figure 1. The light intensity was decreased by 10 μE m^{-2} s^{-1} using the red sheet. The growth of microalgae was measured by optical density at 680 nm every 48 h. The growth curve represented optical density, an indirect measurement of cell number plotted as a function of time and could be divided into four phases according to the slope representing the four stages lag, exponential, stationary, and death.

2.4.2 Experimental Setup for Autotrophic and Mixotrophic Culture under Different Light Colors

The study was divided into two series of experiments. The first set of preliminary experiments studied the impact of three light colors: Red (R), Yellow (Y), and White (W) in both mixotrophic and autotrophic conditions under the growth conditions explained above. All the experiments were conducted into 250 mL Erlenmeyer flasks containing 50 mL of liquid media with and an initial inoculum size of 0.2 OD. For mixotrophic cultivation, glucose or glycine was added to the L1 media at a concentration of 1%, which were labeled as 1 and 2, respectively. The experiment identifications are shown in Table 1. In this experiment, all the cultures were exposed to the conditions for the complete culture duration (i.e., from day 1 to day 10). We harvested the cultures at day 10 during the stationary phase.

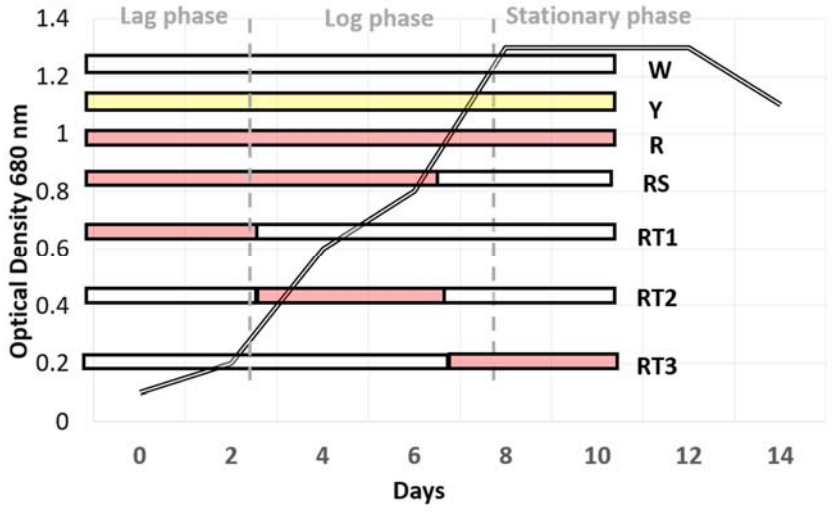
Table 1. Summary of *P. tricornutum* culture conditions and experiment identifications. *P. tricornutum* was cultured in L1 media, and all experiments were done in triplicate.

Light	Autotrophic	Mixotrophic	
		1% Glucose	1% Glycine
White (Control)	W	W1	W2
Red	R	R1	R2
Yellow	Y	Y1	Y2

In the second series of experiments, the microalgae culture in autotrophic growth conditions was exposed to a light shift from red light to white light in different growth phases (Table 2). For this, *P. tricornutum* culture was submitted to red light treatment during the specific growth phase i.e., lag, exponential, stationary, and then cultures were exposed to white light labeled as RT1, RT2, RT3, and RS (Table 2). All experiments were done in triplicate. All the inoculum for these experiments were grown in control white light.

Table 2. Overall experimental plan of four culture conditions (red light treatment 1 to 3 and RS). After the R exposure using cellophane sheets during the treatment time period as mentioned for (RT1–RT3, RS), the sheets were removed, and *P. tricornutum* culture was exposed to the full spectrum of white light. In RT1, the culture was exposed to red light for first 4 days and then exposed to white light. In RT2, the culture was exposed to white light during first 3 days; then, it was exposed to red light by wrapping the flask from the fourth to seventh day and then again exposing the flask to white light. In RT3, the culture was exposed to white light until the seventh day and after was given red light treatment. Similarly, in RS, the red light was given from the first to seventh day and then exposed to white light for 72 h before harvesting. Below, the graphical representation of the setup aligned with the growth curve.

Treatment	Description
RT1	Red light exposure only during the lag phase (1^{st}–4^{th} day)
RT2	Red light exposure only during the log phase (4^{th}–7^{th} day)
RT3	Red light exposure only during the stationary phase (7^{th}–10^{th} day)
RS	Red light until the end of the log phase (1^{st}–7^{th} day)



2.4.3 Cell Growth and Biomass Analysis

Wild-type *P. tricornutum* was cultured in 5 mL of L1 medium in a flat-bottomed sterile 6-well culture plate with a lid (Costar®, Cat no. 3516). The cultures were maintained with consistent shaking on an orbital shaker at 130 rpm. Cell growth was monitored by measuring the optical density at 680 nm using a Synergy H1 Microplate Reader, BioTek. All the conditions were studied in triplicate.

The biomass was estimated by dry weight by centrifuging of *P. tricornutum* cultures at 7000× *g* for 10 min on the 10th day. The supernatant was discarded, and the pellet was dried at 60 °C for biomass analysis until the constant mass was achieved.

2.4.4 Visualization of Lipid Bodies

Intracellular lipid bodies (LBs) were visualized using a modified Nile Red (9-diethylamino-5H-benzo[a]-phenoxazine-5-one) staining method (17,18). Briefly, 1 mL of the algal culture was centrifuged at 10,625× *g* for 10 min. Then, the cell pellet was resuspended in 500 µL of 20% dimethyl sulfoxide (DMSO) and vortexed for 1 min at room temperature. Cells were centrifuged at 10,625× *g* for 5 min, and the cell pellet was resuspended in 500 µL of water and vortexed before adding Nile red solution (0.5 mg mL^{-1} dissolved in acetone) and incubated for 5 min in dark at room temperature. Stained LBs imaging was performed under a confocal laser scanning microscope (Leica

SP8) using 40× magnification. The Nile red fluorescence was detected using a UV light source at excitation/emission wavelengths of 488 and (490–550) nm.

2.4.5 Quantification of Total Lipids

Total lipids were extracted using Bligh and Dyer method (19) with the following modifications. To a 5 mL Eppendorf tube containing a known amount of dry algal biomass, mixtures of methanol and chloroform were added in 2:1 ratio. The mixture was vortexed for 2 min and incubated at room temperature for 24 h, after which 1 mL of chloroform and 0.9 mL of water were added. The mixture was vortexed for 2 min, and the different layers were separated by centrifugation for 10 min at 300× *g*. The lower layer was evaporated, and the residue was dried at 80 °C for 30 min. The weight was calculated using a precision scale.

2.4.6 Nile Red Screening

In order to detect neutral lipids, Nile red assay (20,21) was done in 96-well plate. Briefly, 250 µL of culture was added with 15 µL of Nile red dissolved in acetone from the stock solution of 0.5 mg mL^{-1}. The plate was incubated in the dark at room temperature for 30 min. The fluorescence intensity was measured at an excitation of 530 nm and emission of 590 nm using a Synergy H1 Microplate Reader, BioTek.

2.4.7 Fatty Acid Profiling

Fatty acid methyl esters were prepared directly from the wet algal biomass. NaOH in methanol (1 mL of 0.5 N) was added to test tubes containing the biomass; then, the tubes were placed in a sonicating bath for 3 min, followed by heating at 90 °C for 10 min. The samples were allowed to cool, and 1 mL of 1.5% H_2SO_4 in methanol was added. Then, samples were heated again at 90 °C for 10 min. After cooling, 1 mL of water and 1 mL of hexane were added. Test tubes were vortexed for 2 min and then centrifuged at 1200× *g* for 5 min. The hexane layer containing Fatty acid methyl esters analysis (FAME)

was recovered and dried over anhydrous sodium sulfate. FAME were quantified using temperature-programmed gas liquid chromatography on a Scion 436 gas chromatograph fitted with a 30 m × 0.25 mm column coated with 50% cyanopropyl-methylpolysiloxane (DB-23) and linked to a computerized integration system (22). The fatty acid data were expressed as the mass percent of total fatty acid identified. Furthermore, the fatty acid profile was used to analyze the physical properties of biodiesel using biodiesel analyzer (21,23,24).

2.4.8 Statistical Analysis

All experiments were conducted in triplicate. Statistically significant differences were identified by one-way ANOVA performed on data with a 5% level of probability ($p < 0.05$) using GraphPad Prism software 8.1.2. followed by a pairwise mean comparison Tukey's test conducted where differences were detected.

2.5 Results and Discussion

2.5.1 Effect of Light Colors on Biomass and Lipid Yield in Mixotrophic and Autotrophic Conditions

For the mixotrophic conditions, two different carbon sources were used by supplementing the standard L1 media with 1% glucose (W1, Y1, and R1) or 1% glycine (W2, Y2, and R2). The highest biomass was achieved under the red light condition compared to white or yellow light (Figure 1A). Specifically, the level of biomass productivity obtained in autotrophic growth (R) was significantly higher by 1.16 and 1.05-fold compared to W and Y autotrophic conditions, respectively. Our results are in agreement with multiple studies that have reported an increased higher production rate of *Scenedesmus* sp. under red and blue light (25) and *Nannochloropsis* sp. under red and yellow light (26). No additional biomass was obtained in R1 or R2 conditions compared to R, suggesting that mixotrophic conditions are less interesting for an industrial application.

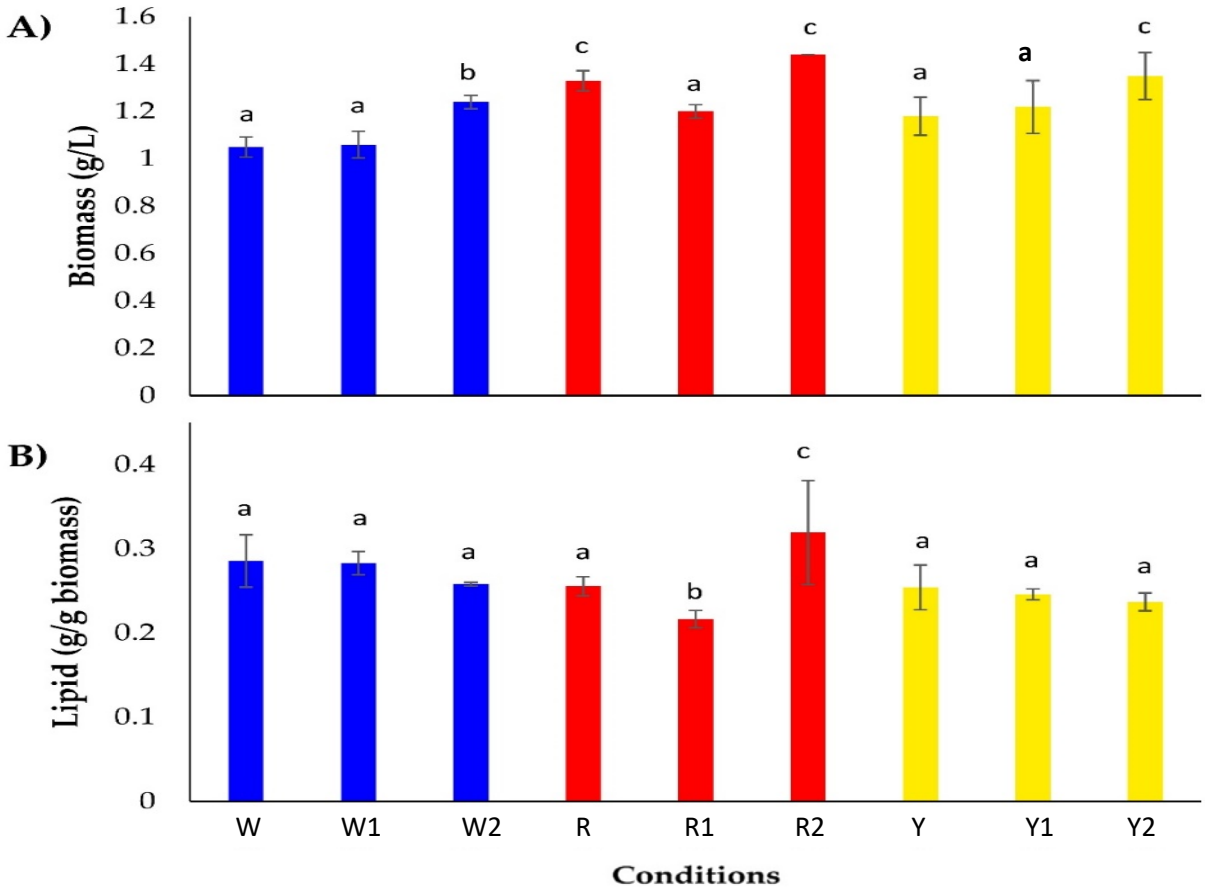


Figure 1. Analysis of *P. tricornutum* biomass (**A**) and lipid yield (**B**) in mixotrophic and autotrophic conditions under different colors of light. Abbreviations for the conditions are W: white, R: red, and Y: yellow, W1, R1 and Y1 refer to L1 media supplemented with 1% glucose, whereas W2, R2, and Y2 refer to the L1 media supplemented with 1% glycine. Each bar represents the average of three replicates, and error bars represent standard deviation. Means with different letters above the bars correspond to significant differences calculated using the multiple t-test. The columns identified with different letters (a–c) are significantly different using one-way analysis of variance (ANOVA) followed by a pairwise mean comparison Tukey's test where differences were detected ($n = 3$, $p < 0.05$).

The lipid production of *P. tricornutum* was significantly different in red light conditions where we observed red light culture supplemented with glycine showed maximum lipid production (Figure 1B). It was expected, as studies have reported an increase in biomass and lipids by multi-fold under mixotrophic conditions in different species (27,28). Furthermore, many studies have shown a positive trend of red light with

an increase in the specific growth rate and lipid production in *Chlorella, Botryococcus*, and *Nannochloropsis* (7,26,29). Another study showed a positive trend of the combined impact of red and blue light on biomass productivity and fucoxanthin production in *P. tricornutum* (30). However, contrasting results have been reported where they observed high productivity under yellow light as compared to red and blue light for *Chlamydomonas reinhardtii* (13). However, these experiments were conducted in turbidostat controlled lab-scale panel photobioreactors and reported that in mass culture, productivity and biomass specific light absorption are inversely correlated (13). Furthermore, in the same study, they have reported that the supplementation of blue light to yellow light is better for stable cultivation.

The growth of microalgae in mixotrophic conditions is an interesting choice for enhancing the productivity of particular metabolites, which will vary according to the product of interest wanted (31). However, there are cost-effective and economic advantages to growth in mixotrophy if the carbon source is derived from cheap waste resources such as agricultural and municipal wastewater that are rich in micronutrients and carbon sources (32). There is good amount of research conducted on the impact of varied carbon source such as glucose, fructose, and glycerol on enhancing the biomass of *P. tricornutum* (31,33). However, our results, combined with findings from several published studies, have shown the importance of the spectral quality or mixing of wavelengths on the biomass and lipid production in different microalgae species as discussed above (7,13,25,26). Therefore, after prescreening the different light settings (W, R and Y) in both autotrophic and mixotrophic conditions along with few literature evidence, we planned to continue to investigating the red light filter for further experimentations in autotrophic conditions. Mixotrophic conditions are interesting choice, but it comes with certain challenges such as cost and bacterial contamination which does not mean that it should not be studied. But further down this chapter, we are investigating different light settings in autotrophic conditions.

2.5.2 Growth Curve Analysis of P. tricornutum during Red to white Shift

Studies have reported that variation in light color and intensity leads to change growth and metabolic pathways (32,33). Photoautotrophic conditions create certain limitations when grown in bioreactors because self-shading by microalgal cells in high-density cultures limits the light available for growth, which can be confirmed in lab-scale experiments by observing the growth curve. Therefore, to reduce the impact of either loss of light as heat or self-shading, the strategy of light shifting from one spectrum to another can trigger different photoreceptors to activate different light-signaling pathways, allowing a better conversion of light energy (34,35,36). Therefore, we investigated the effect of red to white light shifts on *P. tricornutum* growth during different growth phases under batch cultivation for 11 days.

P. tricornutum grown in red light until the end of exponential phase (from day 1 to 7) and then shifted to white light (RS) showed the greatest growth compared to all other conditions tested (Figure 2, Appendix A–Figure A1).

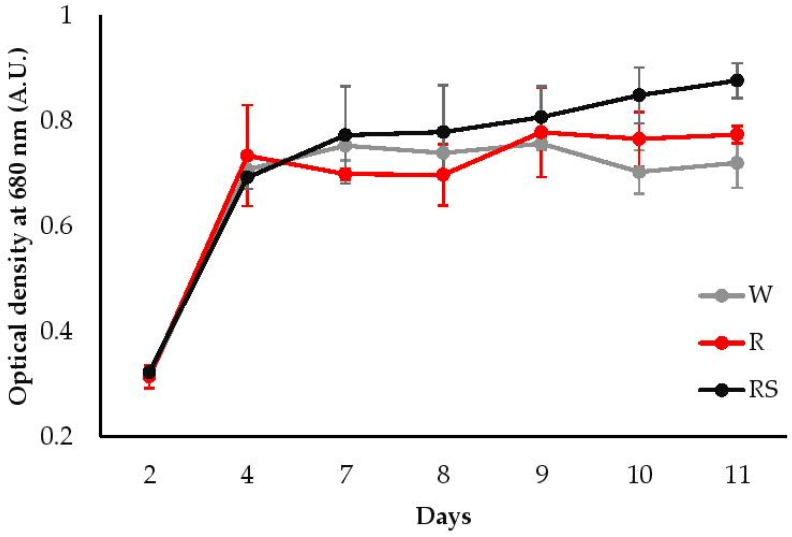


Figure 2. Growth curve of *P. tricornutum* in continuous white light (W), red light (R), and in red to white light shift (RS) (i.e., red light during the exponential phase and then white light during the stationary phase). Cells were grown on continuous shaking mode in 5 mL culture; each point represents the mean optical density at 680 nm of three independent experiments (±SD).

P. tricornutum cultured cells were adapting to each condition in a similar manner during the adaptation and exponential phases (Figure 2). It should be noted that there seems to be a crucial lag between day 4 and day 7 (exponential to stationary), where it is not clear whether cell growth continued before slowing down (Figure 2). Additionally, the slight difference between R and RS at day 7 is unexpected, since they were under the same conditions. It is possible that these small differences are due to replicate variation and growth chamber light variations. Indeed, when we checked the light intensity using the flux meter, we observed a variation in light intensity of 1–2 μE m^{-2} s^{-1} between the different corners of the growth chamber, which could explain the variation between R and RS at day 7 and the width of the error bars. However, differences in growth were observed at the end of the exponential phase and during the stationary phase, where RS clearly promoted growth compared to other treatments (Figure 2; Appendix A–Figure A1). Indeed, at day 11, the RS growth was significantly higher with 22% and 14% more cells than continuous W and R light, respectively (Figure 2). The cell growth observed under R and RS treatment can be explained, in part, by the presence of a wide range of photoreceptors with the ability to sense red/far-red wavelengths and their potential to prevent photoinhibition (30,32). Few studies have reported that combination of red and blue spectra or supplementing with different spectra light can change the growth or mass productivity in *P. tricornutum* and *Chlamydomonas reinhardtii.* It could also be said cautiously that different species might respond to different combination of spectra differently considering the differences in pigment profile and chloroplast structure. (13,30). Furthermore, few literature studies have reported that microalgae might modulate the use of light throught different photoreceptors like crytochromes, aureochromes or phytochromes to have a specific response which are not yet completely understood. (2,37,38).

The W or full spectrum (λ = 400–700 nm) tends to cause slow growth while reducing the overall time of stationary phase as compared to R (Figure 2). Similar results have been reported for *Chlorella* sp. (39) and *Scenedesmus* sp. (25). In all, the RS further increased growth, which can be attributed to the use of different photoreceptors.

Interestingly, the timing of light shift was important. Indeed, red to white light shifts during other growth phases (lag, exponential, and stationary phase (RT1, RT2, and RT3) were not efficient to increase biomass compared to RS (Appendix A–Figure A1). Future work should investigate other experimental strategies to study circadian rhythms and photosynthesis during light shift conditions.

2.5.3 Analysis of Total Biomass and Lipid Content under Red to white Light Shift Treatment

The biomass of *P. tricornutum* was significantly greater with RS treatment by 2-fold and 1.4-fold compared to W and R conditions, respectively (Figure 3A). This correlates with increased cell density (Figure 2). In addition, our results confirm observations from another study on the impact of red light where biomass increased by 40% under enriched red light (40). Similarly, another study reported better growth and higher fucoxanthin content in *P. tricornutum* under red and blue light (30). That study is similar to our treatment RS, where we have supplied a red wavelength for the first 7 days and then exposed the culture to the white light during the stationary phase. Furthermore, it has been reported that cultured *P. tricornutum* under R contains a greater number of thylakoids, where light-dependent reactions occur, compared to *P. tricornutum* cultured in W (41). Altogether, the data suggest that the use of red light and variations thereof improves the overall biomass of *P. tricornutum* at the laboratory scale, offering a promising first stage, which should be pursued at a larger scale to be considered a useful strategy for industrial applications.

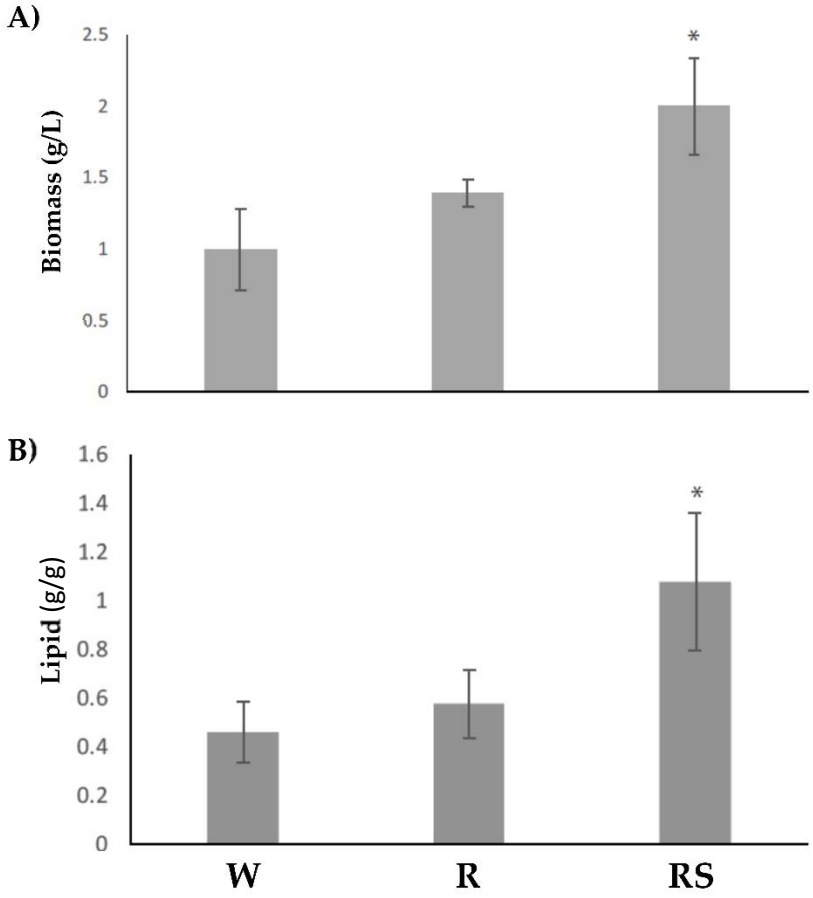


Figure 3. Analysis of *P. tricornutum* biomass (**A**) and total lipid accumulation (**B**) in autotrophic conditions under continuous white (W) light, red (R) light, and red with light shift (RS), which is red light during the exponential phase and then a shift to white light. The total lipids iExperiments were done in triplicate in 50 mL liquid culture, and cells were collected at day 10. Results are expressed as mean value ± standard deviation. An asterisk (*) indicates significant differences compared to the white light (W) used as control. Statistical analysis was done using one-way ANOVA.

Total lipids were also analyzed in the same conditions, using dry weight estimation. Nile red assay was used for rapid screening of lipid and confocal microscopy for visual analysis, as described previously (20). *P. tricornutum* produces lipids that are potentially significant for several industrial applications. It has been reported that the crude lipids extracted from *P. tricornutum* are around 321.89 mg/g dry weight (42) or 34% of dry weight (43) and are comparable to the lipid extracted in W in our study, which is 0.4 g/g dry weight in control white light. Furthermore, the lipid dry weight in RS was significantly increased to 2.3-fold and 1.8-fold as compared to W and R, respectively (Figure 3B). It is interesting to note the 1.8 fold increase in lipid from R to RS, which suggests that

shifting from red light to full spectrum during the stationary phase has an impact on lipid accumulation. It is known that the usual change in the lipid composition is influenced by the cellular C/N ratio, light intensity, nutrient depletion, and other possible inducible biotic/abiotic stress conditions (28–30). An increase in C/N ratio, lipid, and carbohydrate in red light acclimated *P. tricornutum* culture has been reported (11). The same study reported an increase in the lipid content from red light to blue light shift, whereas the reverse shift, from blue to red light, promoted an increase in carbohydrates. In addition, lipid accumulation under red light has been observed in *Botryococcus* sp. (7) and *Chlorella* sp. (29), which can be linked with effective nitrogen consumption rate and a doubling of cell numbers in red light, which aids in increasing the total lipid content. In addition, high light intensity has been shown to affect lipid biosynthesis in *Nannocloropsis gaditana,* which has been correlated with the regulation of cytosolic fatty acid synthase of type 1 (*FAS1*) and polyketide synthase (*PKS*) and the down-regulation of the chloroplast fatty acid synthase of type 2 (*FAS2*) (45). One of the studies has reported a significant increase in lipids in *P. tricornutum* at low light, which is in the range of (30 μmol photons m^{-2} s^{-1}) and medium light (300 μmol photons m^{-2} s^{-1}) (46). Therefore, lower intensities or light shift treatment may also affect the expression levels of the cytosolic and chloroplastic fatty acid synthases, which in turn would regulate lipid content. Various studies have shown the relation between wavelength, specific wavelength, and wavelength shift on the accumulation of lipids, which can be attributed to enzymes involved in carbon cycle and lipid synthesis (11,40,47). Therefore, it appears that the combined effect of red-light acclimation and lower light intensity, observed by the red sheet, which reduces light intensity by 10 μE m^{-2} s^{-1}, may be acting together to increase the total lipids in RS (Figure 3B).

2.5.4 Visual Analysis of P. tricornutum Cell Morphology and Lipid Droplets

We next examined *P. tricornutum* lipid droplets using confocal microscopy (Figure 4). Our initial expectation based on a gravimetric analysis of lipids was to observe a change in the lipid droplet size. The results showed that the selected light conditions were not responsible for any changes in the morphology, size, or number of lipid droplets

of *P. tricornutum* cultures grown in the studied light conditions (Figure 4a,b). The morphological changes in cells are important for microbes to respond to various environmental conditions such as the state of media, the temperature, or the salinity. Previous studies have reported the presence of three morphotypes in *P. tricornutum* culture: oval, fusiform, and triradiate (48). It is also known that light absorption is the function of the cell shape, size, and pigments (49). Microscopic analyses (Figure 4c,d) revealed a high occurrence of fusiform cells, a limited number of triradiate shapes, and no appearance of the oval form in all of our growth conditions. It has been reported that the fusiform type is dominant in suspension culture over the other forms (43,50), which is in agreement with our tested conditions. It has also been reported that the triradiate *P. tricornutum* cells are rarely found in laboratory growth conditions (1,43). However, our study detected the presence of a limited number of triradiate cells (Appendix–Figure A2). *P. tricornutum* can be grown in both silicon or silicon-free medium; we studied silicon-free media, as it showed better growth for our strain, and it has been reported that fusiform cells have a greater lipid percentage as compared to other forms (43,51).

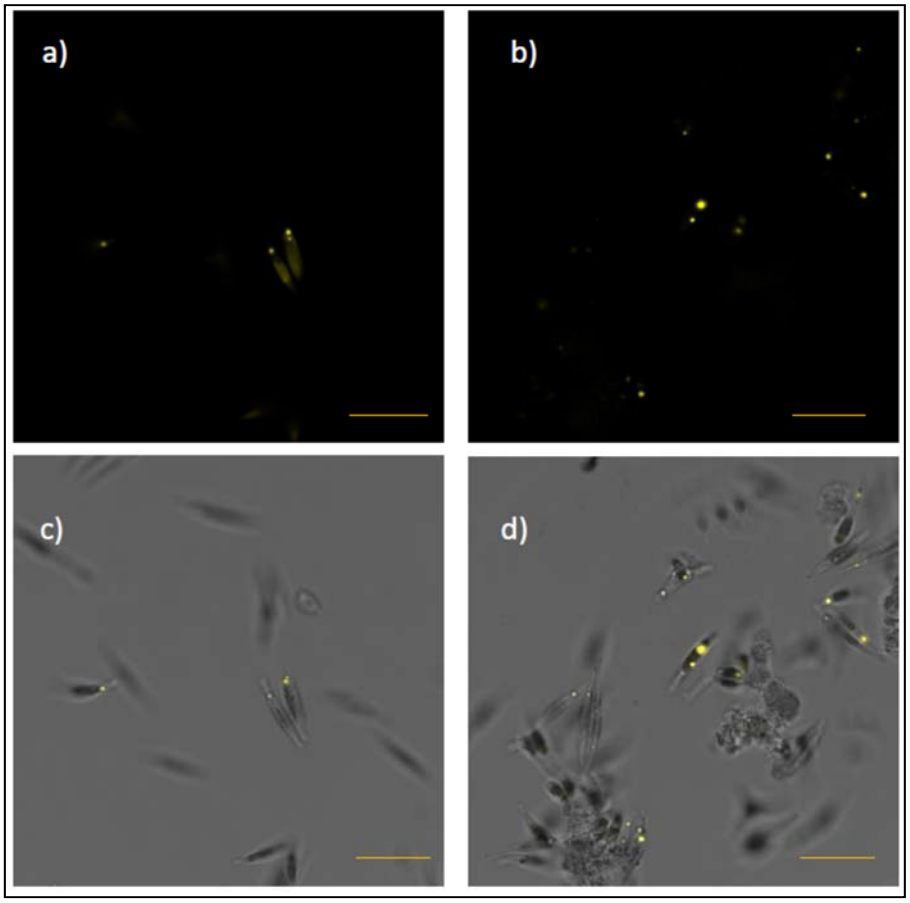


Figure 4. Microscopic fluorescent images (top) and transmitted with fluorescent overlay (bottom). Visualization of lipid droplets in *Phaeodactylum tricornutum* using Nile red staining. Each panel represents Nile red staining of lipid droplets in white light conditions (**a**,**c**) and in red light (**b**,**d**). Scale represents 100 nm.

2.5.5 Fatty Acid Methyl Esters Analysis (FAMEs)

Polyunsaturated fatty acids (PUFA) were present in greatest abundance, followed by monounsaturated fatty acids (MUFA) and lower amounts of saturated fatty acids (SFA), which constituted 41.86%, 31.80%, and 26.34%, respectively (Table 3). MUFA levels were lower in RS (28.91%) compared to W (31.80%) or R (31.85%) (Table 3). In addition, the *P. tricornutum* PUFA ratio of omega-3/omega-6 was lower in R and RS compared to control W (Table 3). The evidence suggests that a high ratio of dietary omega-3/omega-6 PUFAs reduces the risk of several diseases (52). Therefore, *P. tricornutum* cultured in W is a potentially more valuable source of functional food and animal feed components than *P. tricornutum* cultivated under R or RS conditions.

These results suggest that the RS condition regulated the fatty acid content in *P. tricornutum*, specifically lowering MUFAs and increasing PUFAs. It has been reported that stress conditions such as nitrogen depletion, high and low temperature, and continuous high light intensity slightly changes the fatty acid composition, specifically PUFA in *P. tricornutum* (53–55).

Table 3. Fatty acid (FA) composition (mass% of total FA identified) analyzed by GC with Flame-Ionization Detection (GC-FID) of *P. tricornutum* culture under different conditions (white light (W), red light (R), or red to white light shift (RS)). Data are expressed as the mean of three independent experiments.

Fatty acids	W	R	RS
Saturated fatty acids (SFA)	26.34	24.86	26.25
Monounsaturated fatty acids (MUFA)	31.80	31.85	28.91
Polyunsaturated fatty acids (PUFA)	41.86	43.29	44.83
Omega-3/Omega-6	6.46	5.68	5.77
Total	100	100	100

The distribution of fatty acids was characterized (Appendix A; Table A1), and various lipid classes of *P. tricornutum* are summarized in Table 4. The main lipid components were mid-chain FAs, 14:0, 16:0, 16:1n-7, and 20:5n-3, which together represented 76.46% of total fatty acids (Table 4). Our results are similar to those reported by (42). Palmitoleic acid (16:1n-7), which constituted 28.20% of the total FAs, was predominant in *P. tricornutum* control (W) but was reduced to 24.29% in RS condition (Appendix A; Table A1). Interestingly, hexadecatrienoic acid (16:3n-4) was increased in R and RS compared to W (Appendix A; Table A1). There was a comparable amount of the omega-3 fatty acid, eicosapentaenoic acid (20:5 n-3), in all light conditions and it constituted almost 22% of total acids, which was the second most predominant FA (Table 4). In other studies, it was the PUFA that was present in highest mass proportions (53–55). Humans do not produce sufficient long-chain omega-3 fatty acids to meet their physiological needs; therefore, it is essential to obtain them from external sources. Omega-3 fatty acids have been associated with proper fetal development, including neuronal, retinal, and immune function (56). They have also shown positive effects on

coronary disease and inflammation, and they have been approved for the treatment of patients with severe hypertriglyceridemia (33). These fatty acids are predominantly found in cold water fish, such as salmon. However, our work suggests that microalgae and diatoms can be considered as a sustainable and vegetarian source of omega-3 fatty acids.

Table 4. Fatty acid composition of total lipid in *P. tricornutum* cultured in different conditions such as full spectrum of white light (W), red light (R), or red to white shift (RS). Major fatty acids are identified as the mass percent of total fatty acids. Monounsaturated fatty acids are expressed as the sum of all fatty acids with that chain length. Data are the mean ± standard deviation (SD) of three independent experiments.

C:D	Relative Fatty Acids (%)		
	W	**R**	**RS**
Saturated fatty acids (SFAs)			
14:0	8.64 ± 0.17	7.54 ± 0.02	8.39 ± 0.55
16:0	15.18 ± 2.64	14.80 ± 1.80	15.24 ± 3.67
18:0	0.40 ± 0.10	0.39 ± 0.06	0.42 ± 0.13
20:0	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.02
22:0	0.16 ± 0.01	0.19 ± 0.01	0.20 ± 0.02
24:0	1.32 ± 0.11	1.30 ± 0.02	1.41 ± 0.03
Monounsaturated fatty acids (MUFAs)			
14:1	0.08 ± 0.02	0.09 ± 0.01	0.08 ± 0.01
16:1	30.11 ± 1.38	30.25 ± 1.74	27.27 ± 1.65
18:1	1.08 ± 0.46	1.07 ± 0.17	1.05 ± 0.39
20:1	0.04 ± 0.02	0.06 ± 0.02	0.08 ± 0.03
22:1	0.17 ± 0.04	0.12 ± 0.03	0.14 ± 0.04
24:1	0.17 ± 0.02	0.11 ± 0.03	0.16 ± 0.04
Polyunsaturated fatty acids (PUFAs)			
Omega-3			
18:3n-3	0.44 ± 0.06	0.36 ± 0.04	0.41 ± 0.04
18:4n-3	0.21 ± 0.06	0.21 ± 0.03	0.23 ± 0.05
20:3n-3	0.06 ± 0.03	0.05 ± 0.02	0.06 ± 0.02
20:4n-3	0.18 ± 0.01	0.21 ± 0.02	0.24 ± 0.04

C:D	Relative Fatty Acids (%)		
	W	R	RS
20:5n-3	22.53 ± 1.23	21.59 ± 1.98	22.15 ± 3.24
22:5n-3	0.19 ± 0.18	0.16 ± 0.04	0.18 ± 0.06
22:6n-3	2.23 ± 1.96	1.72 ± 0.08	1.96 ± 0.27
Omega-6			
16:2n-6	0.94 ± 0.20	1.13 ± 0.11	1.16 ± 0.19
16:3n-6	0.96 ± 0.22	0.60 ± 0.15	0.76 ± 0.25
18:2n-6	1.41 ± 0.25	1.51 ± 0.13	1.62 ± 0.021
18:3n-6	0.18 ± 0.11	0.23 ± 0.03	0.21 ± 0.08
20:2n-6	0.09 ± 0.03	0.10 ± 0.01	0.08 ± 0.03
20:3n-6	0.05 ± 0.02	0.08 ± 0.02	0.07 ± 0.01
20:4n-6	0.33 ± 0.8	0.62 ± 0.05	0.46 ± 0.08
22:4n-6	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
22:5n-6	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01

The structural features (chain length, unsaturation, and branching) of fatty acids determine the properties of algae-derived biodiesel, including ignition quality, heat of combustion, cold flow, oxidative stability, exhaust emissions, viscosity, and lubricity (57). Thus, the major fractions of fatty acid composition, which was accepted as input in the Biodiesel Analyzer, were studied for properties of biodiesel (23) (Table 5). For biofuel of an industrial quality, it needs to have similar physical properties as diesel, which can be used efficiently in combustion engines. According to a study, fatty acids with chain lengths ranging from C16 to C18 should be high in potential feedstock for suitable biodiesel production (58). It is suggested that FAME with a high percentage of monounsaturation (C16–C18) is the most desirable compromise between cold flow and oxidative stability (59). The W and R induced the production of monounsaturated fatty acids as compared to RS. The total percent of saturation and unsaturation—especially the amount of long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—will have an impact on the biodiesel fuel properties (60). The results showed that the cetane number (CN), which determines the ignition quality of the fuel, is higher in *P. tricornutum* cultured in R and RS as compared to W

(Table 5). It is known that the higher the CN, the better the ignition of the fuel and vice versa (57). Literature studies have also mentioned that increases in the degree of unsaturation lead to decreases in CN viscosity, which is a useful measure for monitoring oxidation progression. As reported, fuels with high viscosity meeting the international standards have better lubricating quality, which increases the life of the engine (61). In the same study, there is a relationship between hydrocarbon (HC) emission and viscosity. The HC emissions increase with decreasing viscosity (61). In our study, the viscosity is lower in all the conditions comparing the standard requirement of EN 14214:2008, but it meets the requirement of ASTM D6751.

Table 5. Values of physical properties of biodiesel related to the fatty acid compositions in each culture conditions (W, R, and RS) compared to the International Standard ASTM and EN14214.

Physical Properties	W	R	RS	EN 14214:2008	ASTM D6751
density	0.749	0.728	0.719	0.860–0.900	0.875–0.900
iodine value	122.5	118.4	121.6	< 120	-
cetane number	49.6	51.4	51.2	≥ 51	≥ 47
viscosity	2.7	2.6	2.6	3.5–5.0	1.9–6.0

Since this study presents the importance of light as a crucial factor for affecting biomass and lipid content in *P. tricornutum*, it supports its importance in metabolic processes of microalgae in the biotechnology field. However, the specific behavior of each species may be unique with respect to the varied light conditions, as supported by the following evidence studied in an interdisciplinary approach. For example, Orefice et al. (2016) showed that the fluctuation of red light on blue light spectral distribution affected the metabolic state of cells via increase in non-photochemical quenching and ultimately promoting an increase in carbohydrates, glycolipids, and saturated fatty acids in *Skeletonema marinoi* (62). In another study, it was observed that different doses of blue wavelengths cause significant alternations in the growth cycle of *Skeletonema marinoi* (63). The change in the growth pattern and biochemical composition can be referred to the ability of diatoms and microalgae to finely balance the light-harvesting and photoprotective capacity (64,65,66,67). The ability of diatoms to respond favorably to red

light is given to the presence of various photoreceptors such as phytochromes and exclusive aureochromes that are involved in the RL signaling process in *P. tricornutum* (68,69,70). In a different approach using spectroscopy, the occurrence of red-shifted fucoxanthin-chlorophyll protein (FCP) showing the fluorescence peak at 714 nm was observed (71). It is considered that *Phaeodactylum* red-shifted FCP is involved in enhancing the light-harvesting ability of cells by the prolonged excited state lifetimes and the absorption spectrum extended to wavelengths longer than 700 nm (71). In general, the clarity in the relationship between diatoms and light is still an open topic for researchers to study both fundamental as well as industrial applications.

Subtle changes in the fatty acid profile was observed under RS light shift condition which should be further investigated to have a potential application. Overall, it is interesting to note that changes in the specific wavelength during the stationary phase can impact the total accumulation of lipid and fatty acid composition.

2.6 Conclusions

In summary, the present study investigated the impact of red light (R) and a red to white shift to full spectrum (RS) on *P. tricornutum* cell growth, biomass, and lipid production. The introduction of the RS during the beginning of the stationary phase in the cultures of *P. tricornutum* had a positive impact on both biomass and lipid production. This is due to the favorable light pattern, as red light-acclimated cells during the lag and exponential phases provide better conditions for cellular and development processes and white light during the stationary phase helps in the accumulation of more lipids as compared to a single spectrum of light. It was also discovered that the specific light wavelength R and RS had a slight effect on the level of polyunsaturated fatty acids (PUFAs), specifically the omega-3 fatty acid eicosapentaenoic acid (20:5 n-3). Moreover, a light shift can be an effective system to reduce the negative effects of self-shading and the proper distribution of light in large-scale photobioreactors. However, more detailed studies on photosystems and metabolite distribution in time-scale patterns are necessary to bring more clarity about wavelength shifts and lipid accumulation. In addition, the

experiment is easily replicable and scalable in developing countries with fewer resources. This system has the potential to be used to enhance the productivity while reducing the loss of biomass because of potentially excessive heat generation and self-shading due to high-density cultures. The acclimation and light shift studies for different species of microalgae such as diatoms, in both autotrophic and mixotrophic conditions, will open new avenues for algal biotechnology to help produce a wide range of special metabolites.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

LB	Lipid bodies
W	White light
R	Red light
Y	Yellow light
RS	Red to white shift
RT1-RT	Red light treatment
SFA	Saturated fatty acid
PUFA	Polyunsaturated fatty acids
MUFA	Monounsaturated fatty acids
FAMEs	Fatty acid methyl ester

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Chapter III

Impact of Different Light Characteristics on the Growth and Lipid Content of Diatom *Phaeodactylum tricornutum* Transconjugant Strains

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3.1 Author Contribution

The major and initial creation of conceptualization and methodology was done by N.S and I.D.-P. All the Co-Authors have contributed in the following sections.

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Methodology, N.S.; F.A.; E.F.; N.M.; F.M.-M.; and I.D.-P.
Validation, N.S.; F.A.; E.F.; A.A.; N.M.; F.M.-M.; and I.D.-P.
Formal analysis, N.S.; F.A.; E.F.; A.A.; N.M.; F.M.-M.;and I.D.-P.
Investigation: N.S.; F.A.; E.F.; A.A.; N.M.
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Writing-review and editing- N.S.; F.A.; E.F.; A.A.; N.M.; F.M.-M.; and I.D.-P.
Supervision: I.D.-P.
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Full article: Analysis of different light characteristics on the growth and lipid content of diatom *Phaeodactylum tricornutum* transconjugant strains

3.2 Abstract

Light regulates important metabolic processes in microalgal cells, which can further impact the metabolism and the accumulation of biomolecules such as lipids, carbohydrates, proteins and other metabolites. Different light settings have been studied on various strains of the model diatom *Phaeodactylum tricornutum,* but not on transconjugant cells and information on wild-type strains is still limited. Therefore, we studied the impact of different light characteristics such as spectral quality, light intensity and light shift on growth, lipid and fatty acid composition on *P. tricornutum* cells to provide a alternative for industrial application. Initially, we tested the impact of spectral quality and light intensity on *P. tricornutum* transformed with an episomal vector (*Pt*ev), harboring the resistance gene S*h ble*. Results indicated that *Pt*ev cells accumulated more biomass and overall lipids in spectral quality Red 1 (R1: 34% > 600 nm > 66%) more effectively as compared to Red 2 (R2: 8% > 600 nm > 92%). It was also detected that cell granulosity was higher in R1 as compared to R2. Furthermore, by testing two light intensities 65 µmol m^{-2} s^{-1} and 145 µmol m^{-2} s^{-1} light,it was observed that 145 µmol $m^{-2}s^{-1}$

lead to an increase in growth trend, total biomass and lipid. Combining spectral qualities and light intensities, we show that as the photon flux density increased, the lipid accumulation raised by 2.8-fold. Studying the light intensity and spectral quality allowed us to optimize the light conditions to R1 spectral quality and light intensity 145 µmol m^{-2} s^{-1}. These initial results showed that red light R1 at 145 µmol m^{-2} s^{-1} was the best condition from the tested conditions for biomass and total lipids accumulation in *Pt*ev cells. Next, we further used the studied light conditions with a third light setting, *i.e.* light shift, where the cultures were shifted during the early stationary phase from R1 light setting to white light. We studied Red to white shift (Rs) to investigate how light condition variations impact on *P. tricornutum* transconjugants *Pt*ev and with an episomal vector containing reporter gene YFP (*Pt*YFP). We observed that Rs induced a subtle change in growth and fatty acid Eicosapentaenoic acid (EPA) in *Pt*ev as compared to *Pt*YFP. Altogether, the study shows that red to white shift of R1 at 145 µmol m^{-2} s^{-1} promoted biomass and total lipids accumulation in *Pt*ev and *Pt*YFP cells. . The study provides a approach to using different light settings with the aim to optimize growth and lipids, as well as to fatty acid production.

Keywords- *Phaeodactylum tricornutum*, light condition , episomal vector, diatoms, fatty acids, biomass

Abbreviations:

R1 - spectral quality Red 1 which is 34% > 600 nm > 66%

R2 - spectral quality Red 2 which is 8% > 600 nm > 92%

EPA – Eicosapentaenoic acid

FAME- Fatty acid methyl esters

SFA – Saturated Fatty Acids

MUFA – MonoUnsaturated Fatty Acids

PUFA – PolyUnsaturated Fatty Acids

GC-MS – Gas Chromatography-mass spectrometry

YFP- Yellow fluorescent protein

3.3 Introduction

Currently, there is a growing concern about the need of renewable feedstock for human fulfilment and the consequences of overconsumption by the ever-growing human population. Our society would highly benefit from including the principles of bioeconomy, such as using more renewable biomass and utilizing/recycling by-products, instead of non-renewable feedstock for non-essential uses (1). Microalgae and diatoms hold a big potential to contribute to solving problems related to the lack of renewable feedstock because of their possible usage in fossil fuel-based technologies (2-5). Moreover, the potential of using *Phaeodactylum tricornutum* species to produce a variety of industrially important bioproducts such as fatty acids-derived molecules, is gaining interest (6). Fatty acid derivatives, such as hydroxy fatty acids, fatty alcohols, fatty acid methyl/ethyl esters, and fatty alka(e)nes, have a wide range of industrial applications including bioplastics, cosmetics, pharmaceuticals, lubricants, and fuels (7).

Adaptability, maintenance, and regulation of biochemical and physiochemical processes under high stress conditions are key features of diatoms, which could be exploited for sustainable economy and human well-being. These characteristics give diatoms the plasticity to respond and survive in the changing ecosystem and to utilize the resource in the most efficient manner (8-10). Diatoms have the potential to adapt to different conditions such as variations in light intensity, spectral quality and light shift (11-13). It is well established that precise light condition has a significant and strong impact on the overall functioning of microalgae photosystem and nutrient cycle. For example, blue light controls the onset of cell division, while shifting the culture from red to blue light increases the production of proteins (10). Spectral quality also influence the light signalling pathways (10, 12, 14-18). Several studies have reported on the impact of different spectral light qualities on *P. tricornutum* growth, biomass, lipids and pigment accumulation (19-21). It was observed that, red and blue lights have an impact on accumulation of sterols (20), lipids (22) and pigments (23). The total amount of sterols was lowest in blue light and sterol glycosylation was affected with a 100-fold decrease in steryl glycosides under blue light and a 100-fold decrease in acylated steryl glycosides under red light (20). In addition to the spectral quality, and variations in light intensity affects the growth and metabolites production by manipulating biological processes such as membranes

remodelling, polyunsaturated fatty acids (PUFA) biosynthesis, rerouting, light-harvesting or photo-protection activity in *P. tricornutum* (23, 24).

More recently, metabolic engineering has also been used to investigate the potential of diatoms for industrial uses such as increasing the total biomass, lipid production etc. For example the biolistic transformation in *P. tricornutum* has been used to produce omega fatty acids, betulin and its precursors (25, 26). Also studies have the shown that overexpression of reporter genes such as GFP and YFP modulating the growth and biomass in *Phaeodactylum tricornutum* and *Rhodobacter sphaeroaides* respectively (27, 28). Furthermore, overexpression of a putative plastidial pyruvate transporter has been used to increase biomass, lipid content, and growth (29). For the production of proteins or metabolites of interest, metabolic engineering researchers exploit the potential of bacteria and yeasts, however these platforms do not always support the assembly of complex plant-derived metabolic pathways. Microalgae cells possess several advantages over other microorganisms; (*i*) unlike prokaryotic bacteria, microalgae are able to perform the post-translational modifications of recombinant proteins necessary to the native eukaryotic organism, (*ii*) as is the case in plants, recombinant protein expression in microalgae can be done through the nuclear, mitochondrial or chloroplastic genomes. The latter is well established and allows one to successfully bypass nuclear regulation mechanisms, (*iii*) microalgae biomass doubles in size every 48 hours allowing rapid large-scale production, (*iv*) autotrophic or heterotrophic growth conditions can be used, (*v*) cultivation in photobioreactors insure control of growth conditions and prevents the possible escape of transgenes into the wild. Thus, microalgae such as diatoms represent an ideal platform to support the production of complex plant metabolites (30). Nuclear transformation offers the advantages of post-translational modifications of recombinant proteins, the possibility of protein targeting and flexibility in regulatory expression (native or heterologous promoters and untranslated regions). However, the main disadvantage of nuclear transformation is the low expression levels of the genes of interest due to silencing and positions effects (random integration). The chloroplastic gene transformation offers higher levels of transgenic protein accumulation but is localized in the chloroplast, lacks post-translational modifications, and is limited in regulatory tools. Both, nuclear and chloroplastic transformation are also limited in the size of the transgenes to be inserted.

Recently, Slattery et al have used the robust episomal conjugative system to edit urease gene with 60% efficiency and stably transform eight genes involved in vanillin synthesis for 4 months with no rearrangement (31). To this date, no study has reported on the impact of the episomal vector and the presence of reporter gene on *P. tricornutum* growth and metabolism . Therefore, we have used the episomal based transformation to clone YFP as reporter gene to analyse its impact on growth, lipid, and fatty acid profile.

In the actual context, *P. tricornutum* holds great promise for the light-driven bioproduction of biofuels and high-value, industrially relevant biochemicals (6). They are the primary food and energy sources in many aquatic food systems and play an important ecological role in global carbon and silicon cycling. In this study, we aim to provide a strategy to study light-based experiments on bioengineered *P. tricornutum* to get a view of light intensity, light shift and spectral quality impact on biomass, lipid accumulation and fatty acid composition. Furthermore, we provide a possible alternative route to acknowledge the importance of culture conditions on diatoms-based compounds production both in *P. tricornutum* containing the resistant gene *Sh ble* in the episomal vector (*Pt*ev) and *P. tricornutum* YFP transconjugants harboring genes of interest fused with YFP fluorescent proteins (*Pt*YFP). We also analyzed the effect of light on YFP accumulation by studying YFP fluorescence levels, and on the lipid accumulation or fatty acid composition of *P. tricornutum*.

3.4 Methodology

3.4.1 Growth and culture conditions

Axenic *P. tricornutum* Bohlin, (Culture Collection of Algae and Protozoa CCAP 1055/1), was kindly provided by Prof. Bogumil Karas from Western University, Canada. Cultures were maintained in L1 media. Experiments for growth curve were done in 250 mL flask with 50 mL L1 media with an equal inoculum size of 0.2 OD at absorbance 680 nm (OD_{680nm}). *P. tricornutum* cells were grown and maintained in L1 media without silica at pH 8 (Artificial Sea Water) in a growth chamber at 18 ± 1.5 °C with cool white light, as standard condition, for 16:8 h light/dark photoperiod of cycles and shaking at 120 rpm.

3.4.2 Transconjugant strains generation, and selection

Recombinant plasmid named here YFP consist in the pPtGE30 plasmid (31) carrying genes of interest fused to yellow fluorescent protein (YFP) in N-terminal. The reporter genes are under the regulatory region that contains a strong constitutive promoter (40SRPS8) and a fucoxanthin-chloroplast protein complex A (FcpA) as terminator.

Saccharomyces cerevisiae VL6−48 (ATCC MYA-3666: MATα his3-Δ200 trp1-Δ1 ura3−52 lys2 ade2−1 met14 cir0) was used for the yeast assembly as described previously (30). Positive yeast strains containing His selection grew on minimal yeast media without histidine. A pool of the grown yeasts was harvested 5 days after assembly and total DNA was extracted as described previously (35)**.** Assembled plasmids was then amplified in chemicompetent *Escherichia coli* (Epi300, Epicenter) grown on Luria Broth (LB) media supplemented with appropriate antibiotic (chloramphenicol (25 $mg.L^{-1}$)) overnight at 37°C. Plasmids were then extracted from chloramphenicol-*E. coli* colonies that were tested by colony PCR using a miniprep kit allowing the extraction of large vectors (Biobasic EZ10 miniprep kit, NY, USA). Plasmids were verified by sequencing and then amplified in *E. coli* Epi 300 strain containing pTA-MOB plasmid to allow conjugation with wild type diatoms as described in the literature (30)**.** Transfer of plasmid DNA to *P. tricornutum* via conjugation from *E. coli* was performed as described (36). For this, 250 µL of wild type *P. tricornutum* culture were adjusted to a density of 10^8 $cells.mL^{-1}$, this density was obtained by plating 1 mL of wild type *P. tricornutum* on 1/2 × L1 1% agar plates and grown at 18 °C under cool fluorescent lights (75 µmol m^{-2} s^{-1} on a light/dark cycle of 16/8h for 4 days. Prior to transformation, 1 mL of L1 media was added to each agar plate, cells were scraped then harvested by pipetting in a sterile tube. Cells were then diluted and mounted in an improved Neubauer hemacytometer (BLAUBRAND®counting chamber, Sigma, USA) to be counted and then cell concentration was adjusted to 5.0 × 10^8 $cells.mL^{-1}$. A volume of 50 mL *E. coli* culture containing the assembled plasmid and pTA-MOB was grown at 37°C under agitation to OD_{600nm} of 0.9. Cells were then centrifuged at 3,000 g for 10 min and resuspended in 500 µL of SOC media. Conjugation was initiated by adding 200 µL of *P. tricornutum* to 200 µL of *E. coli* cells. The cell mixture was then plated on ½ L1, 5% LB, 1% agar plates, incubated at 30 °C for 90 min

in the dark and then transferred to 18 °C in the light and grown for 2 days. Two days later, 1 mL of L1 media was added to the plates to collect cells by scrapping and a volume of 200 µL of cells were plated on ½ L1, 1% agar plates supplemented with zeocin 50 µg.mL^{-1} for selection and incubated at 18 °C under light (75 µE m^{-2}s^{-1}). Two weeks later, colonies were collected and streaked again on ½ L1, 1% agar plates supplemented with zeocin 50 µg mL^{-1}. Positive colonies were screened by fluorescence under a fluorescent Stereo Microscope Leica M165 FC with GFP filter. YFP fluorescence from *P. tricornutum* transconjugants was assessed using the Synergy H1 Bio Tek microplate reader. A volume of 200 µL cell culture transconjugants was measured for fluorescence in black 96 well plate at Ex/Em wavelengths of 500/539 nm (n=3). Strains containing plasmid YFP (17,389 bp) and pPtGE30 (16,149 bp) are named here *Pt*YFP and *Pt*ev respectively

3.4.3 Flow cytometry and fluorescence-activated cell sorting (FACS)

The BD FACS Melody (BD Biosciences, La Jolla, CA, USA) equipped with blue (488 nm), red (640 nm) and violet (405 nm) lasers were used to sort *Pt*YFP cells according to YFP production. Prior to the first sort, selected cells were grown in L1 liquid medium supplemented with Zeocin (50 µg.mL^{-1}) and grown for 14 days. *P. tricornutum* cultures were washed in L1 medium, filtered on a 100 µm Nylon Net filter (Merck Millipore, Ireland) and diluted to an OD680 nm= 0.1 in L1 media prior to sorting.

Events were acquired at a fixed flow rate and at least 10,000 events were analysed. Cells were gated according to FSC-A (forward scatter area) and SSC-A (side scatter area) parameters and doublons were excluded according to further gating on homogeneous FSC-H (height) *vs*. FSC-W (width) and SSC-H *vs.* SSC-W populations. Chloroplast autofluorescence was measured on the PerCP channel (700/54 nm). Cells with high homogeneous levels of PerCP fluorescence were further gated, whereas cells with high non-specific autofluorescence were excluded based on their emission in 448/45 nm channel. YFP was further analysed on the 527/32 nm band-pass filter channel. Sorted cells (1.0×10^5 cells.mL^{-1}) were collected in 1.5 mL tube containing 500 µL of L1 media without antibiotics, centrifuged 10 min at 3,500 x g and 90-95% of the supernatant was removed and replaced by 500 µL of L1 media supplemented with Zeocin 50 µg.mL^{-1}, chloramphenicol 35 µg.mL^{-1} and ampicillin 100 µg.mL^{-1} to avoid contamination. This

was used as inoculum to L1 media 20 mL culture, supplemented with Zeocin 50 µg.mL^{-1}. *Pt*ev cells were used as negative control.

For the second round of sorting, 1st round-sorted cultures were incubated for 11 days and diluted in 1 mL to an OD_{680nm} of 0.1. The cultures were then grown for another 11 days, and sorted, following the same procedure. In this round, non-fluorescent (YFP$^-$) *Pt*YFP cells were sorted too. After the second sorting round, *Pt*YFP YFP$^+$ fluorescent and YFP$^-$ non-fluorescent cells were monitored for 11 and 23 days, respectively to monitor YFP production. Figures and statistics were analyzed using BD FlowJo version 10 software (BD Biosciences, La Jolla, CA, USA, 2020).

3.4.4 Light settings

Spectral Quality – We studied the different spectral quality impacts such as (Red 1 (R1), Red 2 (R2) Blue (B), Yellow (Y) and White (C)) on *P. tricornutum* which were provided using the different light or filters. The wavelength absorbed by the filters were obtained by measuring the spectra scanning between 400-700 nm using Synergy H1 Microplate Reader, BioTek (Supplementary Figure 1). Transmittance curve is calculated from the absorption curve. A = 2 - $\log_{10}$ %T (T= Transmittance) therefore, we can calculate the transmittance by undoing the log function(%T = antilog (2 – absorbance) . The methodology was adopted from the following article (37). We used here different filters (red, blue, yellow), compared to white light as control, to study the impact of different light spectra on growth and lipid. The following conditions were used to study the *P. tricornutum* cell growth trend and lipid accumulation.

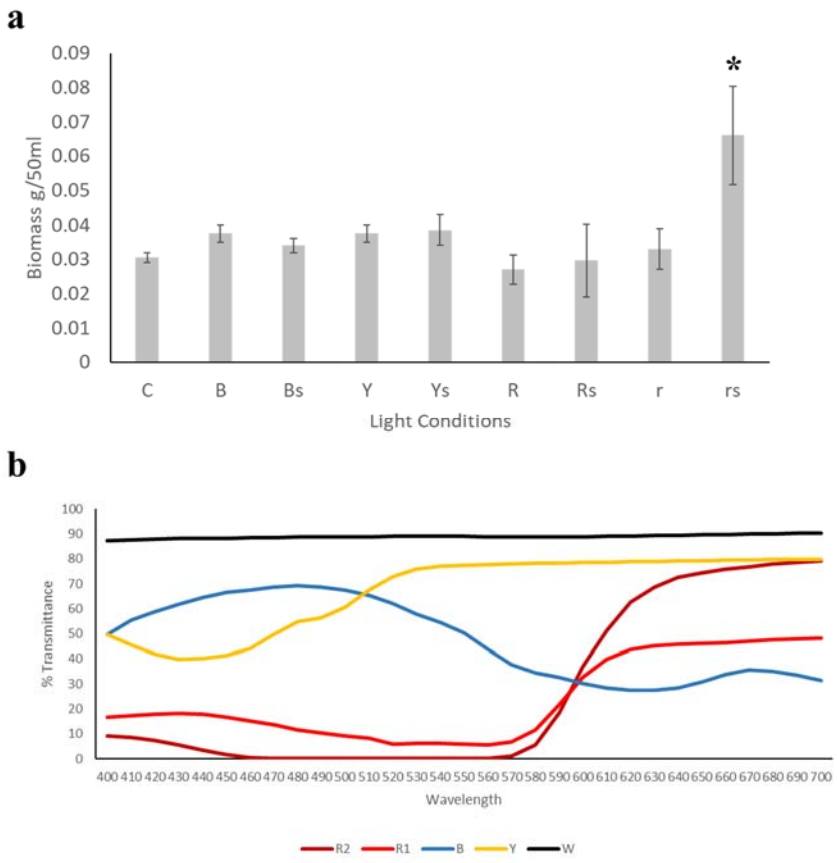

Figure 1. Analysis of *P. tricornutum (*Pt*ev*) biomass (a) and spectral transmittance used (b) under different colors of light. Abbreviations for the conditions are C: white, B: blue, Bs: blue shift, Y: yellow, Ys: yellow shift, R: red R2, Rs: red R2 shift, r: red R1, and rs: red R1 shift. Each bar represents the average of three replicates, and error bars represent standard deviation. * correspond to significant differences calculated using one-way analysis of variance (ANOVA) followed by a pairwise mean comparison Tukey's test where differences were detected ($n = 3$, $p < 0.05$).

Light intensity- The flux density of 65 µmol $m^{-2}s^{-1}$ Low light (LL) and 145 µmol m^{-2} s^{-1} Medium light (ML) was used in the present study for the effect on growth trend and lipid profile.

Light shift- The light shift is a strategy where we shift the cultures from one light condition to another condition after a certain period of time. As such, the cultures were grown under red light until they reached an OD_{680mn} of 0.6 absorbance units (*i.e.* 5-6 days) and then cultures were shifted from red light to white light to growth until collection. The data of red to white shift was compared to data from cultures grown in continuous red

light. We performed a pre-screen for all the spectral quality (R1, R2, B, Y and C) and the respective light shift (R1s, R2s, Bs, Ys) to decide on the best light conditions to be used for further optimization (Supplementary Figure 1)

3.5 Lipid analysis

Total lipid were extracted using Bligh and Dyer method with slight modifications (38). Briefly, to a 5 mL Eppendorf tube containing a known amount of dry algal biomass, mixtures of methanol and chloroform were added in 2:1 ratio. The mixture was vortexed for 2 min and incubated at room temperature for 24 h, after which 1 mL of chloroform and 0.9 mL of water were added. The mixture was vortexed for 2 min again, and the different layers were separated by centrifugation for 10 min at 300×*g*. The lower layer was evaporated, and the residue was dried at 80 °C for 30 min. The weight was calculated using a precision scale.

3.6 Neutral Lipid detection by flow cytometry

Equal volumes of cultures sampled on day 6 and 8 were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and 10% of DMSO was added. Tubes were vortexed for 30 seconds and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded, and the pellet was mixed with 1 mL of autoclaved milli-Q water. Cultures were further aliquoted to 250 µL in 96 well black plate and 10 µL of Nile red (9-(diethylamino)-5h-benzo[a]phenoxazinone) (0.1 mg mL^{-1} dissolved in acetone) was added. Plates were incubated for 10 min in the dark. The accumulation of intracellular lipid was measured on an FC500 MPL cytometer (Beckman Coulter, Brea, CA, USA) equipped with Air-cooled Argon ion laser, 488 nm and a red solid-state laser, 631 nm. The fluorescence signals of both the control and stained cells were acquired to gate for Nile-Red^{+} cells in the 620 nm/20 FL3 channel. Data was analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA). Other variables like cell size, granulosity and chlorophyll were also measured from different channels.

3.7 GC-MS analysis of fatty acids

Fatty acid methyl esters were prepared directly from the wet algal biomass. NaOH in methanol (1 mL of 0.5 N) was added to test tubes containing the biomass; then, the tubes were placed in a sonicating bath for 3 min, followed by heating at 90 °C for 10 min. The samples were allowed to cool, and 1 mL of 1.5% H_2SO_4 in methanol was added. Then,

samples were heated again at 90 °C for 10 min. After cooling, 1 mL of water and 1 mL of hexane were added. Test tubes were vortexed for 2 min and then centrifuged at 1200×*g* for 5 min. The hexane layer containing Fatty acid methyl esters analysis (FAME) was recovered and dried over anhydrous sodium sulphate. FAME was quantified using temperature-programmed gas liquid chromatography on a Scion 436 gas chromatograph fitted with a 30 m × 0.25 mm column coated with 50% cyanopropyl-methylpolysiloxane (DB-23) and linked to a computerized integration system (39). The fatty acid data were expressed as the mass percent of total fatty acid identified as described previously (22).

3.8 Statistics

All experiments were conducted in triplicate. Statistically significant differences (SD) were identified paired t-test and two-way ANOVA performed on data with a 5% level of probability using Graph Pad Prism software 9.2.0. We have used * to annotate the statistical significance which are as following: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.00001$.

4 Results

4.1 Effect of light characteristics on cell growth and lipid accumulation of *P. tricornutum* transconjugant cells

Diatoms *P. tricornutum* are popular microalgae for the production of biofuels and lipids. In a previous work, we studied the effect of light on *P. tricornutum* in autotrophic and mixotrophic conditions to promote biomass and lipids accumulation. It was observed that red light and red to white shift (*i.e.* substituting red light by white light), impacted biomass yield and lipid content of wild type *P. tricornutum* (22). With the development of efficient conjugation-based transformation system allowing the introduction of stable episomes (31), *P. tricornutum* has become an ideal platform for metabolic engineering applications such as the production of biofuels and lipids. However, the impact of the presence of an episome and high levels of heterologous proteins on *P. tricornutum* growth or lipid accumulation has not been measured. Similarly, the impact of different light characteristics on *P. tricornutum* transconjugant cells containing an episomal vector has not been investigated.

We first evaluated the effect of different light characteristics (*i.e.* spectral quality, light intensity, and light shift) on *P. tricornutum* transconjugant cells containing an episomal vector (*Ptev*). We confirmed our prior analysis by testing the spectral quality of Red (R), Blue (B), Yellow (Y), White light (C) on biomass (Fig. S1b). Similar to results obtain with *P. tricornutum* wild type cells (22), the only characteristic that significantly impacted biomass accumulation of *Pt*ev transconjugant cells was red to white shift which causing > 1.5 fold increase (Fig. 1a).

To strengthen our knowledge on the effects of the different compositions of red light spectra, we used two types of red (R) filter, *i.e.* R1 with 66 % of transmittance of over 600 nm and in R2 with transmittance spectra of 90 % in over 600 nm (Fig. 1b). When comparing the two R1 and R2 spectra qualities on *Pt*ev biomass and growth, we observed R1 light condition resulted in a more effective biomass accumulation and growth as compared to R2 (Fig. 2a, Fig. 3a). Then, we evaluated the impact of R1 and R2 on the lipid content of *Pt*ev using gravimetric analysis (38). The average lipid content in R1 was 1.6-fold higher as compared to R2, but the difference was not statistically significant (Fig.

2b). Since R1 significantly increased *Pt*ev biomass and impacted lipid accumulation, R1 was selected for the subsequent experiments.

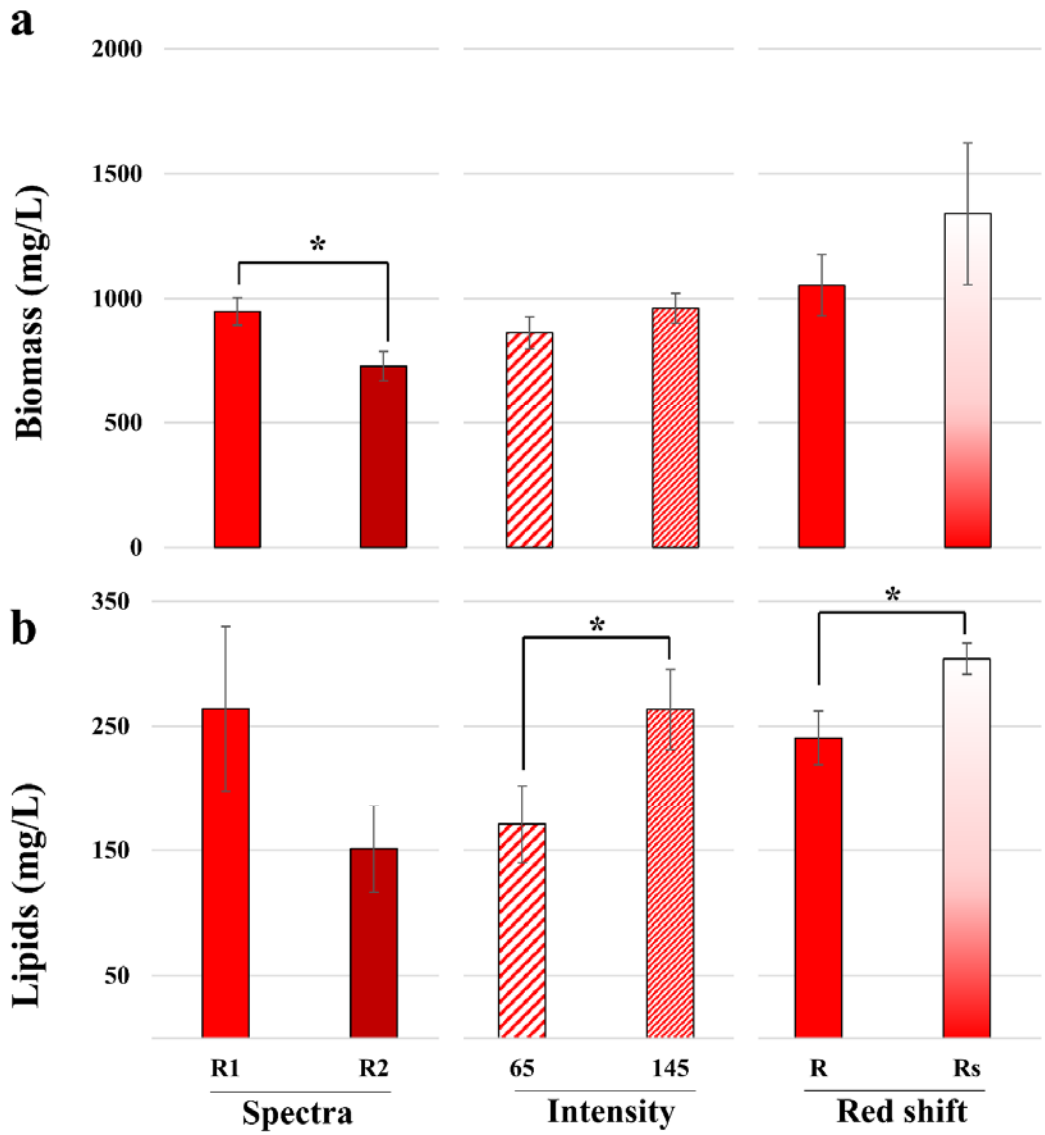

Fig. 2. Effect of different light characteristics on *P. tricornutum* cells growth and lipid accumulation. The effect on (a) biomass - dry weight (mg/L) and on (b) lipids accumulation as measured by the Bligh and Dyer method was determined. The different light characteristics includes spectra: shows the effect of the exposure to spectra quality R1 (34% > 600 nm > 66%) and R2 (8% > 600 nm > 92%), intensity: shows the effect of the exposure to light intensities of 65 and 145 $\mu mol.m^{-2}.s^{-1}$ and shift: with the light conditions of continuous red light (RL) and red to white shift (Rs). All data points are presented as a mean of triplicates with standard deviation (n = 3). * statistically different using two-way Anova, $p < 0.05$.

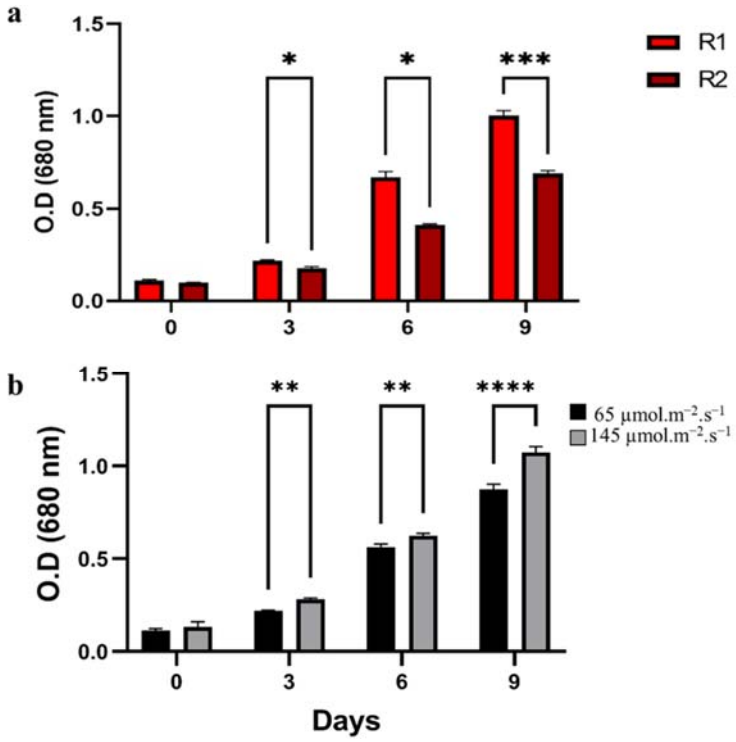

Figure 3. Analysis of the growth of *P. tricornutum* transconjugant cells containing an empty episomal vector (*Pt*ev) following (a) the effect of two different spectra quality where R1 (34% > 600 nm > 66%) and R2 (8% > 600 nm > 92%) and (b) the exposure to two different light intensities of 65 and 145 µmol.m^{-2}.s^{-1}. Each bar represents the average of three replicates, and error bars represent standard deviation. Statistically significant differences were identified using a paired t-test and two-way ANOVA performed on data with a 5% level of probability. We have used * to annotate the statistical significance which are as following: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.00001$.

Next, we investigated the impact of two light intensities, (*i.e.* 65 µmol m^{-2} s^{-1} and 145 µmol m^{-2} s^{-1}) of R1 filter on *Pt*ev cells by monitoring the growth curve. The results showed that during the lag phase, cell growth did not differ with respect to the initial light intensity, consistently with the fact that this is an adaptation phase. However, the growth rate, as measured at OD_{680nm}, significantly increased at 145 µmol m^{-2} s^{-1} as compared to 65 µmol m^{-2} s^{-1} until the end of the exponential phase (Fig. 3b). However, we calculated the dry weight biomass of *Pt*ev and noted that there was no difference between the light intensities (Fig. 2a). Furthere, we observed that lipid content for spectra R1 at 65 µmol m^{-2} s^{-1} and 145 µmol m^{-2} s^{-1}, we observed that R1 spectral quality provides a significant increase in lipid accumulation at 145 µmol m^{-2} s^{-1} as compared to 65 µmol m^{-2} s^{-1} (Fig. 4). Thus, we proceeded with R1 at 145 µmol.m^{-2}.s^{-1} for our next study.

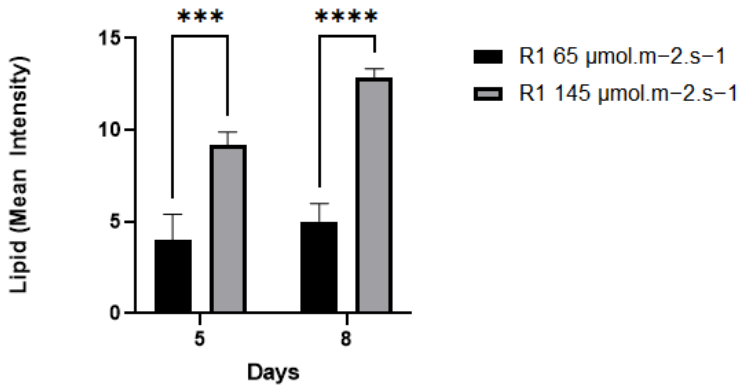

Figure 4 : Analysis of lipid content for *Pt*ev under two red light intensity 65 µmol m^{-2} s^{-1} and 145 µmol m^{-2} s^{-1} on day 5 and day 8. We have used * to annotate the statistical significance which are as following: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.00001$.

We further investigated two shortlisted conditions red light spectra (R) and red to white shift (Rs) (from red to white light spectra) on *Pt*ev cells based on previous results obtained with *P. tricornutum* wild type cells (22). Results showed that *Pt*ev biomass accumulates in both, R and Rs conditions, however the accumulation of lipids was significantly higher in Rs (Fig.2b).

4.2 Effect of red and red to white shift on cell growth, lipid analysis in different *P. tricornutum* transconjugants

P. tricornutum transconjugant cells represent an interesting biotechnological alternative for the production of various biomolecules such as lipids however the impact of the episome and the expression of its inserted genes on *P. tricornutum* transconjugant is not known. As such, we investigated the effect of the above selected Rs condition on *Pt*ev and on *P. tricornutum* transconjugant cells containing the same episomal vector but to which the yellow fluorescent protein (YFP) reporter gene under the control of a strong constitutive promoter was added. Thus, in addition to have to replicate an episome like *Pt*ev cells, *Pt*YFP cells express and accumulate recombinant proteins. In order to select cells with high levels of heterologous recombinant protein YFP, the *Pt*YFP transconjugant cells used in this study were obtained after repetitive sorting rounds of FACS that ensured the enrichment of the YFP fluorescence as described in materials and method section.

*Pt*YFP cells used in this study were from the 2nd sorting round of selection and displayed 76.88% of fluorescent cells (Supplementary Fig. 5, middle panels).

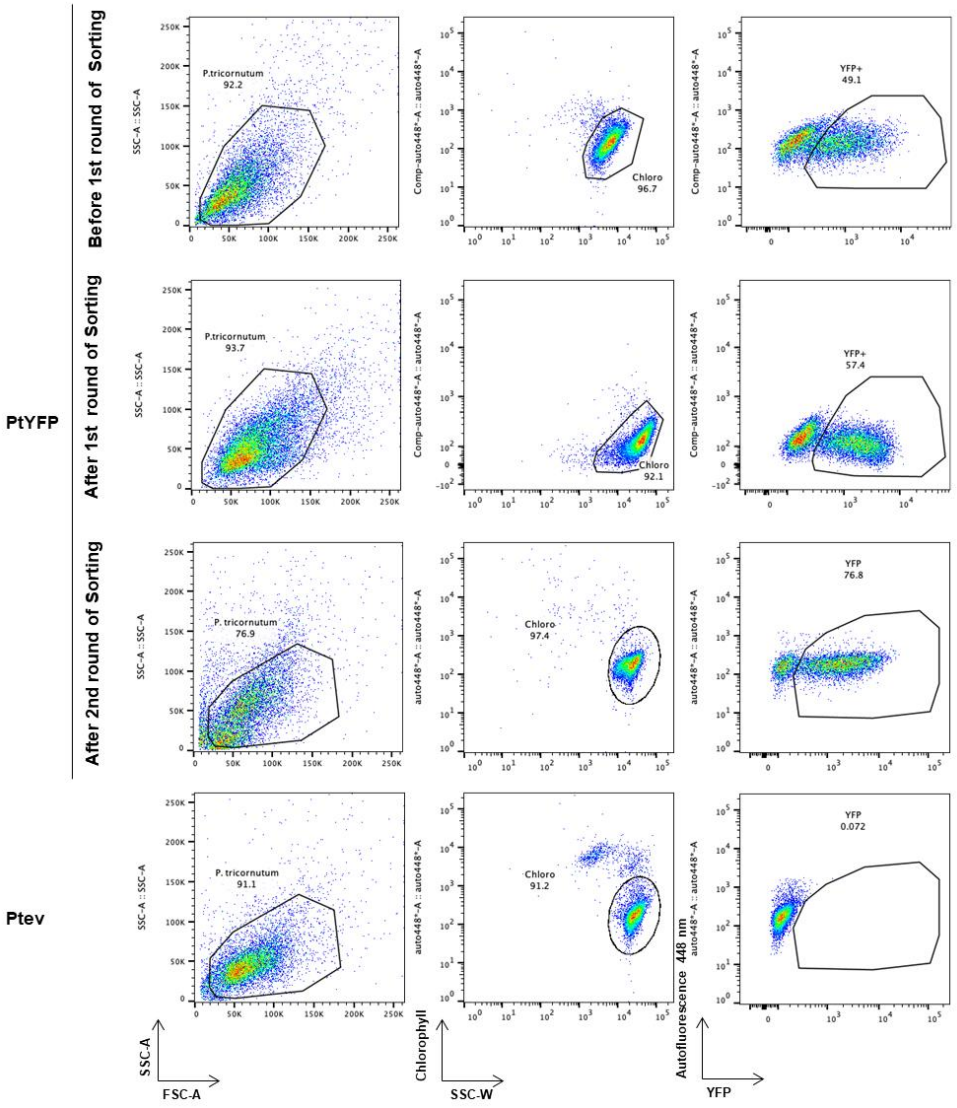

Figure 5: FACS analysis of *P. tricornutum* transconjugant cells containing an empty episomal vector (Ptev) and an episomal vector expressing the reporter gene YFP (PtYFP) and cell sorting.

In the above-mentioned results, we further investigated the effect of the two shortlisted conditions R and Rs on the growth of *Pt*ev and *Pt*YFP transconjugant cells. It was observed that the growth trend in transconjugant with the reporter gene (*Pt*YFP) was slow as compared to without reporter gene (*Pt*ev). The growth for *Pt*YFPR was significantly slower as compared to *Pt*evR and *Pt*evRs for days 3,4,5,6,7,8,9,11 (Fig. 6a). After day 5,

culture were shifted to white light (Rs,) and it was observed that after the light shift, *Pt*evRs growth was significantly higher as compared to *Pt*YFPR on day 6,7,8, and 11. Lipid accumulation at day 6 and 8 was then measured by Nile red staining analysis using a flow cytometer and dry weight estimation using Bligh and Dyer method (Fig. 6b,c). As previously noted, lipid accumulated in higher amounts when *Pt*ev was submitted to Rs (Fig. 2b; Fig. 6b, c). Interestingly, there was more lipids per cell in *Pt*YFP as compared to *Pt*ev for all conditions at both day 6 and 8, although results were only significant when comparing *Pt*evR with *Pt*YFP-R and *Pt*YFP-Rs (Fig. 6b). Even though, the data was bit different when compared with dry weight lipid on day 8, the data showed that Rs was effective and significantly increased the total lipid in both *Pt*ev and *Pt*YFP (Fig. 6c).

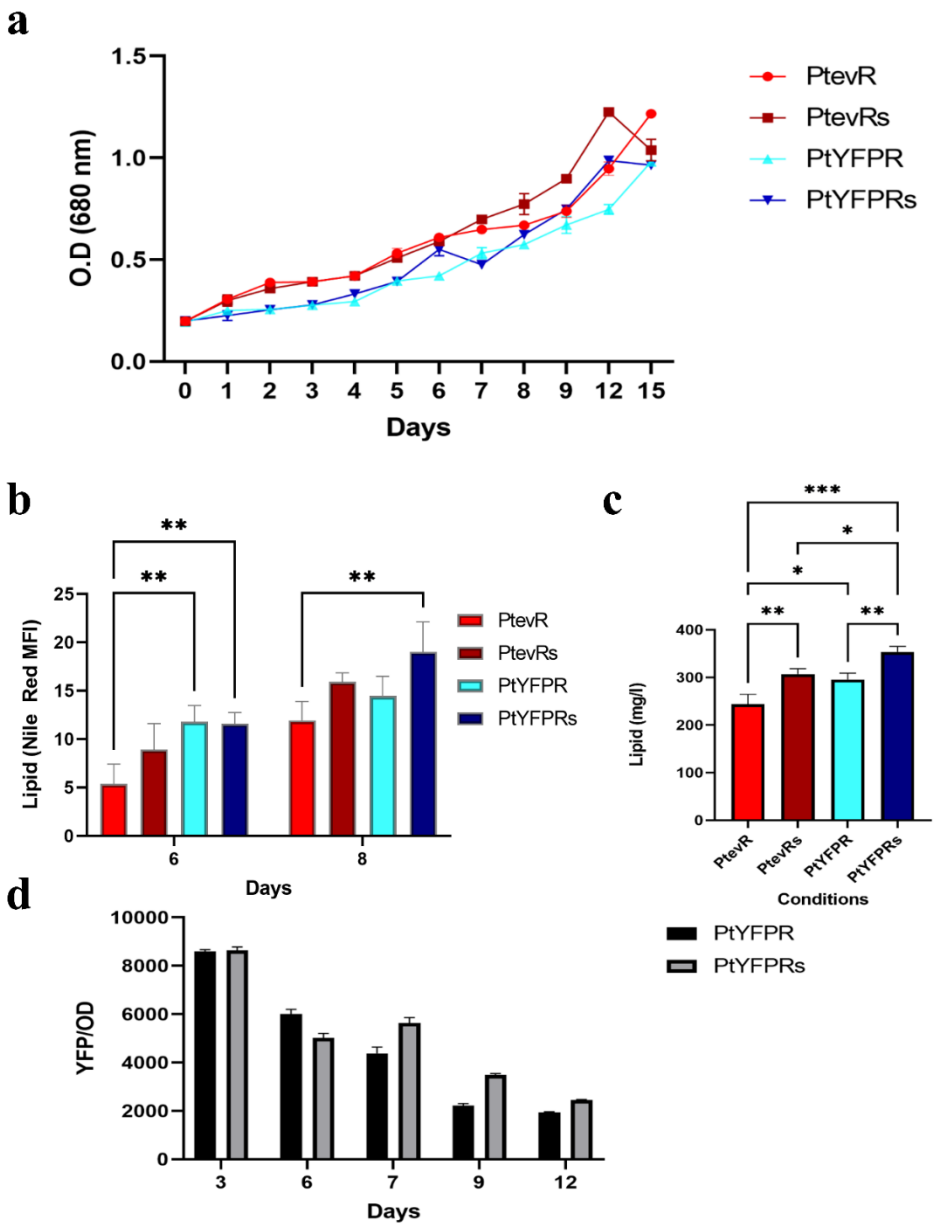

Fig. 6. Effect of two different light conditions Red light (R1) and Red to white shift (Rs) on *P. tricornutum* transconjugant cells containing an empty episomal vector (*Pt*ev) and an episomal vector harboring the Yellow fluorescent Protein reporter gene (*Pt*YFP). (a) Growth curve over 15 days of *P. tricornutum* transconjugant cells *Pt*ev and *Pt*YFP. (b) Lipid estimation by Nile Red staining and measured by flow cytometer (Mean fluorescence intensity; MFI). (c) Dry weight lipid estimation using gravimetric method on day 8. (d) Ratio of the fluorescence YFP over the number of cells for *Pt*YFP growth in R and Rs conditions.

We also observed the impact of R and Rs on total YFP fluorescence of *Pt*YFP-R and *Pt*YFP-Rs. Results showed a max fluorescence intensity at day 3 for the two recombinant

strains (Fig. 6d). The difference in fluoroscence coming out of the transconjugants cultures could be related to difference in protein accumulation or rearranged episomes which has been discussed in discussion. Interestingly, we observed more granulosity in R1 as compared to R2 (Fig. 7).

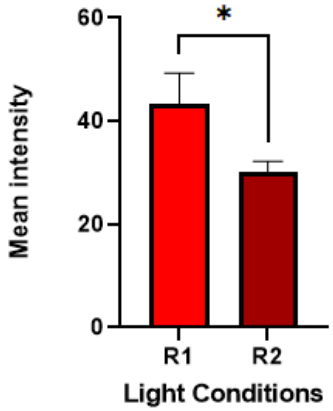

Figure 7- Mean intensity of cell granulosity calculated using FACS for *Pt*ev in R1 and R2. * $p \leq 0.05$.

4.3 Fatty Acid composition of *Pt*ev and *Pt*YFP in Red Light and Red to white shift

The fatty acid (FA) composition of *Pt*ev and *Pt*YFP cells grown under R and Rs condition showed no significant differences (Table 1 and 2). The FA composition (% of total FA) for *Pt*ev and *Pt*YFP in R and Rs condition are similar and showed a highest abundance (44.66-50.30 %) of polyunsaturated fatty acids (PUFA) (Table 1), whereas monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) are in same abundance level (around 25% each). These results are comparable to those previously described for *P. tricornutum* wild type (22).

Table 1: Fatty acid (FA) composition (mass % of total FA identified) analysed by gas chromatography (GC) with Flame-Ionization Detection (GC-FID) from *Pt*ev and *Pt*YFP biomass under two light conditions (R and Rs) on Day 9. (n=3)

Fatty acids	***Pt*evR**	***Pt*evRs**	***Pt*YFPR**	***Pt*YFPRs**
SFA	24.2±3.23	24±1.04	27.55±4.2	27.05±4.25
MUFA	27.69±1.33	25.7±1.13	27.79±2.93	27.84±3.03
PUFA	48.11±7.63	50.30±2.21	44.66±8.63	45.11±9.29

Total	100.00	100.00	100.00	100.00

The exact fatty acids identified from *Pt*ev and *Pt*YFP are summarized in Table 2. The main FA components for all samples analyzed in this study were mid- and long-chain FAs, 14:0, 16:0, 16:1n-7, and 20:5n-3. Palmitoleic acid (16:1n-7) was predominant, compared to the other FAs, in all samples under R and Rs light conditions tested. The only FA that was significantly different was eicosapentaenoic acid (EPA), with *Pt*evRs displaying the maximum amount of EPA which was approximately 23% and 17% more as compared to *Pt*YFPR and *Pt*YFPRs respectively (Two way ANOVA, $P<0.05$).

Table 2: Major fatty acid composition of total lipid in *Pt*ev and *Pt*YFP Red light (R) and Red to white shift (Rs). Data are the mean ± standard deviation (SD) of three replicates (n=3)

Fatty acid name	**Fatty acid formula**	***Pt*evR**	***Pt*evRs**	***Pt*YFPR**	***Pt*YFPRs**
Myristic acid	14:0	7.16 ± 0.03	7.38 ± 0.17	7.53 ± 0.20	7.45 ± 0.33
Palmitic acid	16:0	15.73 ± 2.74	15.24 ± 0.69	18.03 ± 3.32	17.73 ± 3.24
Palmitoleic acid	16:1n-7	23.63 ± 0.32	21.01 ± 0.35	22.97 ± 0.47	21.57 ± 0.84
Stearic acid	18:0	0.58 ± 0.3	0.55 ± 0.12	1.17 ± 0.50	1.07 ± 0.55
Oleic acid	18:1n-9	0.42 ± 0.14	0.58 ± 0.35	0.89 ± 0.37	1.36 ± 0.86
Eicosapentaenoic acid*	20:5n-3	20.99 ± 4.31	24.23 ± 0.58	18.5 ± 3.6	20.04 ± 4.52
Docosahexaenoic acid	22:6n-3	1.98 ± 0.17	2.36 ± 0.06	1.40 ± 0.20	1.75 ± 0.35

Analysis of fatty acid profile of transconjugants *Pt*ev and *Pt*YFP in Red and Red to white shift conditions. We have used * to annotate the statistical significance which are as following: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.00001$. The data for fatty acid Eicosapentaenoic acid was significant as following- Ptev vs PtevRs (**), PtevRs vs PtYFP (****), PtevRs vs PtYFPRs (***).

5. Discussion

Diatoms are affected by different conditions and light is one of the important factors. Growth and biomass tends to increase with precise light and culture conditions whereas lipids tends to increase under stress conditions. Moreover, the photosynthetic apparatus in diatoms are distinctly different as compared to green algae and plants which provides us an alternative route to produce biomass for industrial use. Recent advances in both physiochemical and synthethic biology, many reseachers are investigating *Phaeodactlyum tricornutum* for there potential to produce lipids and fatty acid production. This study investigated the impact of light settings on transconjugants *Pt*ev and *Pt*YFP for growth, lipid and fatty acid composition. An analysis on transconjugants showed that light settings can have subtle impact on growth, lipid and fatty acid profile

We showed that there was significant increase in both growth and harvested biomass for *Ptev* in R1 as compared to R2 (Fig 1, Fig. 2). Previously, Valle et al and Yi et al and co-authors showed that optimal red:blue ratio can improve light harvesting efficiency (19, 23).

We also observed cell granuolosity which is affected by the cell morphology and chemical composition such as starch and lipid (40). And we noticed a significant increase in granulosity in R1 as compared to R2 which charaterizes the optical complexity of the cell which is dependent on particulate matter inside the cell (Fig. 7) (41). There are few plausible explanation such as change in thylakoid structure in different light conditions (42) and change in curvature of endoplasmic reticulum during lipid droplet expansion (43). Although detail mechanism is still unclear and needs more verifications. Importance of the quantity of red light spectra on growth and fucoxanthin accumulation (44) and lipid synthesis (45, 46) has been reported and is in support with our results. R1 was chosen for our next experiments.

Considering the light intensity, it was observed that tested light conditions LL and ML have shown no difference in the total biomass but a significant increase in total lipid in ML (Fig 2-4). Even though high growth rate was observed for ML during the exponential phase, it was expected to have high biomass which was not observed (Fig. 2a). Although, there are few studies reported on faster growth trend of *P. tricornutum* in medium light

intensity, as compared to low and high light intensity (23, 47, 48). It has been shown the non photochemical energy intensity is higher in ML (300 µmol $m^{-2} s^{-1}$) as compared to LL (30 µmol $m^{-2} s^{-1}$) which might be one of the reason for decrease in total biomass in our experiment (47). Therefore, part of incoming light energy may be getting dissipated as heat energy when cultures are grown in 145 µmol $m^{-2} s^{-1}$. Alternatively it can also be said that light use efficiency is higher under LL (65 µmol $m^{-2} s^{-1}$) which has been mentioned by Li et al (48). These studies and ours suggest that light intensity between 120 and 220 µmol $m^{-2} s^{-1}$ is an suitable choice for various industrial applications, subject to further optimization based on bioreactor-based observations. Observing that light intensity can have change the total lipid, we tested the impact of R1 spectral quality at 65 µmol $m^{-2} s^{-1}$ and 145 µmol $m^{-2} s^{-1}$ and observed that, as we increase the photon flux density to 145 µmol $m^{-2} s^{-1}$ the total lipid increases by 2.5 folds (Figure 4). There is one study reporting a drop in fucoxanthin, chlorophyll *a* and beta-cryptoxanthin content under 255 µmol $m^{-2} s^{-1}$ red light as compared to 128 µmol $m^{-2} s^{-1}$ red light (23). Thus, light intensity of a specific light quality can change the compositions of metabolites such as pigments and lipids.

Here, the light characteristics promoting lipid accumulation [spectral quality - R1 and light intensity-145 µmol $m^{-2} s^{-1}$ with red to white shift, was selected to study the effect on *P. tricornutum* transconjugants (*Pt*ev and *Pt*YFP), the latter accumulating reporter fluorescent proteins. fluorescent proteins is known to have a noticeable impact on various cellular events (27, 49, 50). Studies has been published to use GFP and YFP to enhance the overall spectral coverage and increase the light harvesting efficiency in *P. tricornutum* and *Rhodobacter sphaeroides* respectively. Considering that only 5% of of the total photons is converted to biomass in diatoms, so it is needed to manipulate the spectal composition to reduce the possible dissipation of excess light by using fluorescent proteins (27, 28).Also, there is one study that we came across that have studied the impact of culture conditions on episomal vector along with reporter gene mVenus for the production of heterologous protein (51). Here, we assessed if the episomal vector along with reporter gene YFP could have an impact on growth, biomass, lipid and fatty acid profiles.

We did cell corting and by the 2rd round of sorting, 76 % of the cells were positive and used for further analysis (Figure 5). This suggests that heterologous protein production

such as YFP by extrachromosomal expression can be stably enriched in *P. tricornutum* transconjugants (52).

The growth of the *Pt*YFP transconjugants was significantly slower as compared to *Pt*ev in both R and Rs. This could be discussed in two different context i.e., replication of episomal vector and fluoroscence of YFP. The size of episomal vector and fluorescence of YFP could negatively affect *P. tricornutum* by extra energy consumption or dissipation and therefore leading to slow cell division rate (Fig. 6). It has been reported that rate of replication in episomal vector is comparable to native nuclear *P. tricornutum* and have reported the stability of episomes for 4 weeks but no enzymatic activity for the heterologous protein. (30). A study have reported the comparable growth between transgenic controls, wild type and episome containing heterologous gene with a reporter gene mVenus which is a fluorescent protein (51). The same study observed impact of continuous light or light/dark on culture and found no change in the mVenus fluorescence. Also, it is known that YFP emit yellow fluorescence which is not the suitable wavelength to be absorbed by accessory pigment fucoxanthin and reduces the overall photosynthesis efficiency. (53). The fluorescent proteins are not just sensititve to light conditions but conditions such as pH, chloride ion concetration, pressure and temperature which can affect the expression of fluorescent proteins (54-56). Therefore, there is a small possibility that the change in light conditions might affect the overall accumulation of protein or total fluorescence inside the cells by modulating the intracellular spectral composition (Fig. 6(d)).

Diatoms induce the synthesis of lipid droplets under various stress and culture conditions like light (57, 58) and nitrogen limitation (59). Here we showed that *Pt*YFP has slower growth in both Red light and Red to white shift as compared to *Pt*ev but have shown significant increase in lipid accumulation on Day 6 and Day 8 in both light conditions (Fig. 6). And the similar trend of increase in growth and lipid in red to white shift was observed in wild type *P. tricornutum* (22). Furthermore, we have reported a decrease in total polyunsaturated fatty acids in YFP transconjugants along with significant decrease in EPA, which is one of the major polyunsaturated fatty acids in *P. tricornutum* (Table 1). Generally, the increase in unsaturated fatty acids or specifically EPA is known to have an important function in the structure of the thylakoid membrane and regulating

photosynthesis such as high pool of EPA in glycerolipids like sulfoquinovosyl diacylglycerides associated with crucial activities like photosynthesis, energy transduction and plastid membranes (60-62). Red light spectra was shown to increase specific fatty acids such as hexadecatrienoic acid (16:3) and eicosapentaenoic acid (EPA) (20:5), which might be due to induction phospholipase activity which might induce the recycling of plastidial membrane free fatty acids (63).

This study have provided an alternative approach of studying light on transconjugants growth, lipids and fatty acid composition which are few in number in published literature. And the data obtained from this study have opened new questions regarding the use of combination of different light conditions, use of episomal vectors and reporter genes to study diatoms for industrial applications. Although we could not come to a distinct conclusion about the precise light charaterisitics that could increase the overall growth, lipid and the specific fatty acid profile in transcojugants. The data obtained from the study shows the small differences between *P. tricornutum*, transconjugants, carrying, or not , a reporter fluorescent protein.

6. Conclusion

Light conditions (light intensity, spectral quality and light shift) can affect the growth, lipid, specific fatty acid and possibly the accumulation of heterologous protein accumulation. We performed physiochemical studies to analyse the impact of spectral quality (R1 and R2), light intensity (LL & ML) on *Pt*ev to to select R1 and ML as the suitable light characteristics for our further studies. However, analysing more spectral compositions along with different light intensity is definitely needed to further expand the database. Using R1 and ML, we tested red to white shift impact on transconjugants (*Ptev* and *PtYFP*). Red to white shift have shown increasing trend for both growth and lipid in transconjugants. And in general the growth of *Pt*ev was more as compared to *Pt*YFP. It was also observed that there could be an impact of episomal vector or reporter gene on growth, biomass and fatty acid EPA. So there can be some unknown challenges of using episomal vector and fluorescent proteins as the data could be misleading or inconsistent. Therefore, it needs further investigations on the mode of transformation considering the spatial dynamics of episomal vector, localisation of the proteins and how different light or culture conditions could impact the protein production.

Acnowledgments

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Authors contributions

N.S., F.M. and I.D-P. contributed to the study conception and design. Methodology, material preparation, data collection and analysis were performed by N.S., E.F., F.A., N.M., A.A. and F.M. The first draft of the manuscript was written by N.S. E.F., F.A., N.M., A.A. and F.M. edited previous versions of the manuscript. I.D-P. supervised the project and secure funding. All authors read and approved the final manuscript.

Statement of competing interest

The authors have no competing or financial interests to declare that are relevant to the content of this article.

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CHAPTER IV

CONCLUSION AND FUTURE PERSPECTIVES

4.1 Conclusion and future perspectives

P. tricornutum is a complex and unique organism to study for the production of specialised metabolites but is also considered as a potent host for industrial use as discussed throughout the thesis. It is known for its complex evolutionary path containing genes from red and green algal sources (1). Such diversity in genome structure along with unique chloroplast structure and FCP protein complex have provided diatoms with adaptation mechanism and metabolic profile which can be exploited to produce valuable compounds (2). Moreover, the availability of multiple omics-based resources and physiochemical data makes it a suitable model organism.

The broader objective of the study was to investigate the impact of different light settings; light intensity, spectral quality and light shift or two phase light system on both wild type and transconjugants (*Pt*ev *and Pt*YFP) for better growth, high lipid and to manipulate the composition of fatty acids. *P. tricornutum* is known to adapt to wide range of light conditions ranging from UV to visible spectrum (3-7). And diatoms can naturally synthesize variety of fatty acids of different lengths and saturation (8-10). Chapter I discusses the detailed general introduction of the thesis to build of the knowledge base and make the readers aware about different key points that are discussed in this thesis. Chapter II investigates the impact of different light spectra and culture conditions on growth, biomass, lipid and fatty acid profil of wild type *P. tricornutum* which was published in Applied Science. Chapter III provides study on different light settings (light intensity, spectral quality and light shift) on transconjugant strains of *P. tricornutum* which was published in American Journal of Plant Science.

Among different light-based published studies, considering the differences in the experimental design, it might be challenging to have a firm conclusion but the trend in the data can be discussed with respect to growth, lipid, fatty acids which we have tried to comprehend in our Chapter II. These trends therefore can be used to assist in further optimizing light settings for the specific production of fatty acid, increasing the total lipid or production of heterologous protein.

The project has investigated and shortlisted few light settings that needs further optimization to be used for large scale project for better growth, total lipid, and specific fatty acid profile such as EPA. For example, in Chapter II, we did the prescreening test and found out that mixotrophic conditions are somewhat better than autotrophic conditions in all three tested light quality that was provided by different light filters (white, red and yellow). We didn't investigated the mixotrophic conditions in detail considering that it adds to cost of production and have challenges with bacterial contamination. Therefore, to narrow down the focus of the thesis, I pursued my investigation towards light conditions. So further, we investigated the impact of light shift during different growth phases on wild type *P. tricornutum.* Few studies have reported the importance of two phase light system or light shift and spectral quality impact on *P. tricornutum* (3, 11). Also, multiple studies have reported the importance of red light acclimation or in general the red light intensity on *P. tricornutum* growth, carbon flux, bioactive molecule accumulation (3, 12-15).

Considering, the available literature showing the possible positive impact of red light spectra, we observed the importance of red light spectra exposure provided by red light filter on different growth phases (log, lag and stationary) and observed that red to white shift during the stationary phase is more effective for growth and lipid as compared to red to white shift during lag and log phase (Figure 1, Chapter II). Thus, identifying the relationship between growth phases and light exposure is one of the key principles to understand the growth pattern, biomass and total lipid and the impact these light conditions will have on overall economic feasibility of the project.

P. tricornutum was selected in this study because of most literature available on the impact of light on various factors relevant to industrial applications such as high production of lipid, pigments, fatty acids along with few omics based resources. And there are few studies providing the evidence of studied three light settings (spectral quality, light intensity and light shift) on growth, lipid and fatty acid profile in *P. tricornutum***.** These three light settings could be considered as a limiting factor in determining the fate of *P. tricornutum* growth, lipid and overall fatty acid profile. For example, we observed that spectral quality R1 which had spectra of over 600 nm ~ 34% was more effective for both growth and lipid as compared to R2 which had spectra of over 600 nm ~ 90% (Chapter III). Literature studies have provided an evidence of importance of red light spectra and intensity impact on *P. tricornutum* growth, sterol, fucoxanthin content, change in ultrastructure of chloroplast and composition of photosystem 1 (10, 14, 16, 17)**.** Two studies have investigated the impact of spectral quality on gene regulation with respect to photoreceptors such as aureochromes and cryptochromes which is another route to modulate the light (18, 19). So far, we have found out that stationary phase is an important transitionary phase that can be used in light shift for increasing the biomass and lipid as shown in Chapter II which was studied in wild type. R1 spectra is more effective for growth and lipid as compared to R2 (Chapter III) which was investigated in transconjugant strain (*Ptev*). There is definitely more room for improvement for the experimental design and the use of different materials such as monochromatic LEDs or industrial scale light filters which can improve the quality of the data.

The other important parameter that we studied was light intensity which is known to affect the overall cell growth and total lipid accumulation. In Chapter III, we have tested the impact of low light and medium light intensity and observed that medium light intensity was more effective for higher growth and total lipid. We further tested if the same trend is followed when different light intensity is applied with R1 spectra, and it was observed that R1 at ML had 2.8-fold more lipid as compared to R1 at LL intensity. In addition to spectral quality and light intensity, timing of the precise light exposure

according to the growth phases have an impact on overall growth and total lipid accumulation.

External light conditions like light intensity and spectral quality have an impact on overall cell growth, lipid and fatty acid compositions as discussed in chapter 2 and 3 in wild type and transconjugant (*Ptev*) respectively. Further, in Chapter III we have studied transconjugants (*Pt*ev and *Pt*YFP) under red light and red to white shift. We noticed that *Pt*YFP strain growth was slower as compared to *Pt*ev and observed a significant increased in total lipid on day 6 and day 8. The slow growth in *Pt*YFP has been discussed with two plausible explanations. One is the use episomal vector along with reporter gene in transconjugants might delay the onset of cell division considering the increase in plasmid size. Another minor reason could be the accumulated emitted fluorescence from YFP inside the cell might interfere with some of metabolic process related to cell division or photosynthesis. There are two interesting studies showing the utilization of fluorescent proteins on enhancing the overall spectral coverage (20, 21). Therefore, this remains an open question for future investigations about the cell division and how the episomal vector and fluorescent proteins might impact the growth and heterologous protein accumulation.

Lipid components within the diatom cell can vary in carbon chain length, number of double bonds, and positioning of double bonds on the chain. Variations in these components are necessary factors for different commercial applications. Depending on the composition,lipid can be used for cosmetics, biodiesel production, antibacterial properties, etc. The impact of different light characteristics on *P. tricornutum* total lipid content was studied using spectrophotometer analysis, flow cytometer and dry weight estimation (Bligh and dyer) as discussed in Chapter II and III. We found out that red to white shift increased the total lipid by 2.3 fold as compared to white light in wild type as reported in chapter II.

We reported the increase in total lipid but decrease in the specific fatty acid EPA in *Pt*YFP transformant in both red and red to white shift. EPA is considered to have an important role in regulating the cell membrane. The idea that EPA from the cell membrane migrates to the neutral lipid under stress conditions and is an important fatty acid involved in photosynthesis has been proposed by few (22-26). The stress conditions could be

stimulated internally from the episomal vector or the studied external light condition (red light and red to white shift). This might be one of the reasons for the increase in total lipid content in *Pt*YFP which requires further investigations. So far, we have noted that red to white shift have increased the total lipid content in wild type as discussed in chapter II and *Ptev* as disccused in Chapter III. Similar trend was observed for *Pt*YFP transconjuants which had more lipid in red to white shift as compared to red light. Therefore, it could be assumed that stress conditions are crucial to stimulate lipid productions which might affect the strains differently as observed in the thesis. And these stress conditions could be provided in form of change in spectral quality or light shift during stationary phase as it is an important phase of lipid accumulation or change in light intensity. Through this research, we could identify few key points about light settings which can be applied to the industries to produce valuable specific fatty acid such as EPA in addition to increasing the biomass and total lipid.

The purpose of the project was to identify the light settings for enhanced growth, total lipid and manipulating fatty acid profile in both wild type and transconjugants. Through this research, we have identified two possible business applications; bioenergy and nutraceuticals (omega fatty acids). Overall, growth conditions in terms of nutrition and light characteristics are the critical components influencing growth, fatty acid profile, total lipid accumulation in a diatom cell. These factors are also critical for the industrial applications.

While this study sheds some light on the impact of different light settings on wild type and transconjugant strains of *Phaeodactylum tricornutm,* there is still a lot to learn and investigate in this area and possibly in multiple diatom species. And there are few directions for the future research with potential industrial applications. One of the important area to investigate more in detail would be the use of episomal vectors with different fluorescent proteins to enhance th light conversion efficiency, modualte lipid metabolism, increase the growth rate and total biomass. This could also involve studying the expression of genes related to photosynthesis, lipid metabolism, pigment profile,

circadian rhythm. Furthermore, there is also requirement of using the omics based tools to generate data as more data will allow us to understand the mechanism better.

Another important area of research would be to investigate the diaomts under two variables such as light and carbon dioxide or investigating the symbiotic relationships between diatoms and other microrganism for the production important bioacitve molecules. Research on developing the tools like cultivation strategies, strain development ,modulating the fatty acid profile will improve the productivity and minimize the environmental impacts.

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APPENDIX A

SUPPLEMENTARY DATA FOR CHAPTER II

Our study showed that red light in autotrophic condition proved to be an affordable and effective option to increase *P. tricornutum* biomass and lipid accumulation. Thus, in the second series of experiments, we further explored the effect of red light on *P. tricornutum* biomass and lipid accumulation in autotrophic conditions. Several studies had reported that change in light settings can change the growth patterns, lipid accumulation and metabolic pathways [31,56].

Light shift from one spectrum to another will change the light ambience and culture environment, which can trigger different photoreceptors to activate signaling pathways allowing a better conversion of light energy. However, the exact growth phase on which to exert a light shift is unknown. Thus, we investigated the effect of red to white shift on *P. tricornutum* growth during different growth phases under batch cultivation for 10 days (Appendix A–Figure A1). Specifically, the effect on growth was studied using optical density for six conditions. The impact of red to white shift to a full spectrum during different growth phases i.e., lag, exponential, and stationary are labeled as RT1, RT2, RT3; RS (Table 2), along with continuous red light (R), was investigated. RT2 and RT3 were found to be less effective at promoting growth, whereas RS was significantly better to promote *P. tricornutum* growth (Appendix A–Figure A1).

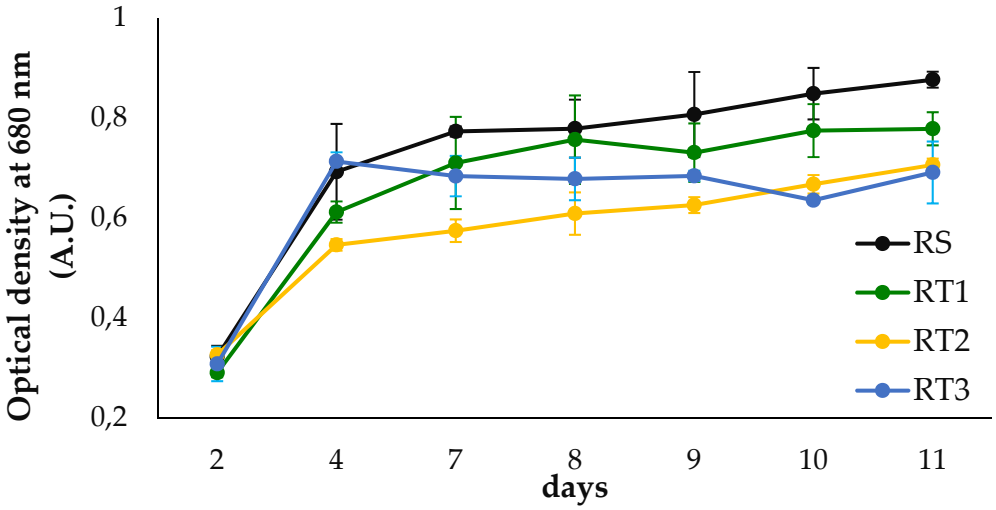

Figure A1. Analysis of *P. tricornutum* growth determined using optical density at 680 nm in different light conditions: red light during the exponential phase and then shifting in the stationary phase (RS), red light exposure during the lag phase (RT1), red light during the exponential phase (RT2), and red light during the stationary phase (RT3).

The red to white shift treatment during the specific growth phases—lag, exponential, and stationary phase (RT1, RT2, and RT3)—was not an efficient method to increase growth (Figure A1). Thus, these treatments are not interesting for our investigation, since we aimed to enhance the overall biomass and total lipid count. Similarly as observed in Figure 2, a lag is observed between day 4 and day 7 (exponential to stationary) where it is not clear whether cell growth continued before slowing down (Figure A1). However, RT1, RT2, and RT3 could be interesting parameters to consider for studying *bonafide* metabolic pathways in diatoms once there is more information on the characterization of these photoreceptors such as phytochromes.

Cell morphology is sometimes indicative of changes in response to stress. Three morphotypes in *P. tricornutum* culture have been reported: oval, fusiform and triradiate [48]. Microscopic analyses (Figure 4c,d) revealed a high occurrence of fusiform cells, a limited number of triradiate-shaped cells, and no appearance of the oval form in all of our growth conditions (Appendix A–Figure A2).

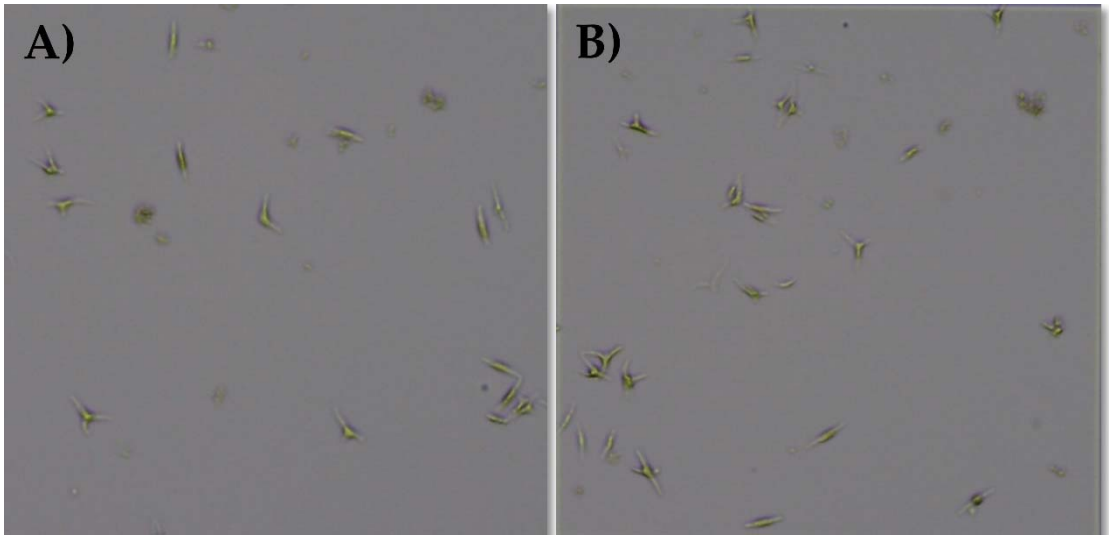

Figure A2. Light microscope images of Phaeodactylum tricornutum in different light conditions (A) in white light and (B) in red light.

Table A1. GC analysis of fatty acid compositions in each culture condition (W, R, and RS). Results (mass% of identified fatty acids) are expressed as mean ± SD of three replicates for each culture conditions. Highlighted means ± SD are most abundant FAs.

Common Name	C:D *n-x*	W	R	RS	Common Name	C:D *n-x*	W	R	RS
Myristic acid	14:0	8.64 ± 0.17	7.54 ± 0.02	8.39 ± 0.55	Linoleic acid	18:2n-6	1.41 ± 0.25	1.51 ± 0.13	1.62 ± 0.21
	14:1n-9	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00		18:2n-4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	14:1n-7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	γ-Linolenic acid	18:3n-6	0.18 ± 0.11	0.23 ± 0.0	0.21 ± 0.08
Myristoleic acid	14:1n-5	0.08 ± 0.02	0.09 ± 0.01	0.08 ± 0.01		18:3n-4	0.01 ± 0.01	0.05 ± 0.02	0.06 ± 0.03
	i-15:0	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	α-Linoleic acid	18:3n-3	0.44 ± 0.06	0.36 ± 0.04	0.41 ± 0.04
	ai-15:0	0.01 ± 0.01	0.00± 0.00	0.00 ± 0.00		18:3n-1	0.21 ± 0.06	0.60 ± 0.07	0.64 ± 0.14
	15:0	0.28 ± 0.07	0.26 ± 0.01	0.25 ± 0.03	Stearidonic acid	18:4n-3	0.21 ± 0.06	0.21 ± 0.03	0.23 ± 0.05
	i-16:0	0.12 ± 0.05	0.13 ± 0.02	0.12 ± 0.02		18:4n-1	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Palmitic acid	16:0	15.18 ± 2.64	14.80 ± 1.80	15.24 ± 3.67	Arachidic acid	20:0	0.00 ± 0.00	0.08 ± 0.01	0.08 ± 0.02
	16:1n-11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		20:1n-11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Hypogeic acid	16:1n-9	1.79 ± 0.42	2.99 ± 0.07	2.78 ± 0.51	Gondoic acid	20:1n-9	0.03 ± 0.01	0.04 ± 0.01	0.00 ± 0.00
Palmitoleic acid	16:1n-7	28.20 ± 0.95	27.17 ± 1.59	24.39 ± 1.07	Paullinic acid	20:1n-7	0.01 ± 0.01	0.02 ± 0.01	0.04 ± 0.02
	16:1n-5	0.12 ± 0.01	0.09 ± 0.08	0.10 ± 0.07		20:2n-9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	17:1(a)	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	Eicosadienoic acid	20:2n-6	0.09 ± 0.03	0.10 ± 0.01	0.08 ± 0.03
	i-17:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Dihomo-γ-linolenic acid	20:3n-6	0.05 ± 0.02	0.08 ± 0.02	0.07 ± 0.01
	16:2n-6	0.94 ± 0.20	1.13 ± 0.11	1.16 ± 0.19	Arachidonic acid	20:4n-6	0.33 ± 0.05	0.62 ± 0.05	0.46 ± 0.08
	ai-17:0	0.06 ± 0.00	0.09 ± 0.01	0.07 ± 0.04	Eicosatrienoic acid	20:3n-3	0.06 ± 0.03	0.05 ± 0.02	0.06 ± 0.02

Common Name	C:D *n-x*	W	R	RS	Common Name	C:D *n-x*	W	R	RS
	17:1(b)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Eicosatetraenoic acid	20:4n-3	0.18 ± 0.01	0.21 ± 0.02	0.24 ± 0.04
Hexadecadienoic acid	16:2n-4	3.53 ± 0.62	3.63 ± 0.27	3.85 ± 0.30	Eicosapentaenoic acid	20:5n-3	22.53 ± 1.23	21.59 ± 1.98	22.15 ± 3.24
Hexadecatrienoic acid	16:3n-6	0.96 ± 0.22	0.60 ± 0.15	0.76 ± 0.25	Behenic acid	22:0	0.16 ± 0.01	0.19 ± 0.01	0.20 ± 0.02
	17:0	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01		22:1n-11	0.08 ± 0.02	0.06 ± 0.01	0.07 ± 0.02
Hexadecatrienoic acid	16:3n-4	6.64 ± 1.30	9.47 ± 0.83	9.67 ± 1.64	Erucic acid	22:1n-9	0.09 ± 0.02	0.06 ± 0.01	0.07 ± 0.01
	17:1	0.08 ± 0.03	0.11 ± 0.01	0.09 ± 0.03		22:1n-7	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01
	16:4n-3	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	Docosadienoic acid	22:2n-6	0.11 ± 0.04	0.08 ± 0.01	0.09 ± 0.03
	16:4n-1	1.35 ± 0.29	0.71 ± 0.04	0.73 ± 0.07		21:5n-3	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
Stearic acid	18:0	0.40 ± 0.10	0.39 ± 0.06	0.42 ± 0.13		23:0	0.05 ± 0.02	0.02 ± 0.01	0.05 ± 0.01
	18:1n-13	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	Adrenic acid	22:4n-6	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
	18:1n-11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Osbond acid	22:5n-6	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Oleic acid	18:1n-9	0.75 ± 0.42	0.71 ± 0.13	0.66 ± 0.34		22:4n-3	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.02
Vaccenic acid	18:1n-7	0.21 ± 0.01	0.23 ± 0.03	0.23 ± 0.03	Docosapentaenoic acid	22:5n-3	0.19 ± 0.04	0.00 ± 0.00	0.18 ± 0.06
	18:1n-5	0.12 ± 0.03	0.13 ± 0.01	0.16 ± 0.02	Lignoceric acid	24:0	1.32 ± 0.11	1.30 ± 0.02	1.41 ± 0.03
	18:2d5.11	0.04± 0.02	0.06 ± 0.01	0.09 ± 0.02	Docosahexanoic acid	22:6n-3	2.23 ± 0.27	1.72 ± 0.08	1.96 ± 0.27
Rumenic acid	18:2n-7	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	Nervonic acid	24:1n-9	0.17 ± 0.01	0.11 ± 0.03	0.16 ± 0.04

APPENDIX B

PUBLISHED REVIEW – DIATOMS BIOTECHNOLOGY: VARIOUS INDUSTRIAL APPLICATIONS FOR A GREENER TOMORROW

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Keywords: diatoms, microalgae biotechnology, metabolic engineering, metabarcoding, sustainable economy, biofuel, lipids

Abstract

The benefits of the complex microscopic and industrially important group of microalgae such as diatoms is not hidden and have lately surprised the scientific community with their industrial potential. The ability to survive in harsh conditions and the presence of different pore structures and defined cell walls have made diatoms ideal cell machinery to produce a variety of industrial products. The prospect of using a diatom cell for industrial application has increased significantly in synch with the advances in microscopy, metabarcoding, analytical and genetic tools. Furthermore, it is well noted that the approach of industry and academia to the use of genetic tools has changed significantly,

resulting in a well-defined characterization of various molecular components of diatoms. It is possible to conduct the primary culturing, harvesting, and further downstream processing of diatom culture in a cost-effective manner. Diatoms hold all the qualities to become the alternative raw material for pharmaceutical, nanotechnology, and energy sources leading to a sustainable economy. In this review, an attempt has been made to gather important progress in the different industrial applications of diatoms such as biotechnology, biomedical, nanotechnology, and environmental technologies.

1 Introduction

The global trend of economy and society is shifting towards building a greener and more sustainable society to combat climate and health issues. This is a critical issue, which is being approached with various interdisciplinary strategies to produce a wide range of sustainable products. For instance, biotechnology research has invested a significant number of resources, time, and money in studying microorganisms to exploit them for human consumption in multiple ways. Furthermore, the decades of research and improvisation in cultivation strategies, extraction, and harvesting protocols strongly support a good return on investment in industrial applications of microbes. A pinch of soil and a drop of water contain a diversity of microbes that controls major biogeochemical cycles and subsequently have the potential of producing an abundance of sustainable products. Since the beginning of this century, a high amount of research work has been published on industrial applications of microbes such as bacteria, yeast, and microalgae (Figure 1). But limited attention has been paid to diatoms which have the potential of becoming a robust sustainable industry because diatoms can continuously grow with an average annual yield of 132 MT dry diatoms ha^{-1} over almost 5 years (1).

Diatoms are dynamic microorganisms with rich diversity and detailed membrane design. They are the most dominating phytoplankton with an overall number of around 200,000 species having complex variability in dimensions and shapes (2-4). Diatoms' distinctive characteristic compared to the phytoplankton community is their silica cell wall, known as a frustule. This innate ability to uptake silicon from the environment has

made them an interesting community of microbes since 19th century. Few studies have stated the role of frustule biosilicate as pH buffering material which facilitates shifting of bicarbonate to CO_2 dissolved in cell fluids (the latter is readily metabolized by diatoms) (5).

The access to advanced microscopes and modern genetic tools enabled us to study the detailed frustule structure and validate metabolic pathways involved in absorption, transportation, and polymerization of silicon and other biomolecules like lipids (6, 7). Furthermore, this advanced knowledge of metabolic pathways and validation of diatom structure can be applied to produce a wide range of renewable products such as optoelectronics, biofuels, nutritional supplements, ecology tools, *etc.* (8).

Other common factors that have shaped the evolution of diatoms are their ability to adapt and grow in various natural resources; fresh and marine water, wastewater, rivers, and oceans. Their abundance and adaptability in a wide range of climate and geographical areas make them suitable for different applications (9). It was reported that diatoms are responsible to produce yearly, 40% of the organic carbon and 20% of oxygen (10-12). Besides, these photoautotrophic organisms are involved in biogeochemical cycles, which play a significant role in global carbon fixation, carbon sequestration, and silicon cycle. They are also suitable candidates to capture nitrogen and carbon from various sources, which can be exploited by waste management and the biofuel industry to create carbon-neutral fuels (13). Furthermore, these algae are used to produce nutraceutical compounds, such as vegetarian proteins, omega, and other essential fatty acids for pharmaceutical industries (14, 15).

Multiple epidemiological, clinical, and pre-clinical studies have shown that omega fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are useful in slowing down age-related diseases such as cardiovascular diseases and cancer (16-19). The development of diatoms strains rich in omega fatty acids can replace the dependence on fish as a source of omega oils and reduce the problems associated with seasonal variations and ocean pollution which might affect the biochemical composition

of fish oil (20). Also, various marine diatoms are considered for the commercial production of antioxidant pigments such as fucoxanthin and other carotenoids. It has been reported that these pigments exhibit various protective effects such as strong antioxidant activities (21).

Thus, the flexible and complex nature of diatoms offers immense possibilities to develop a wide range of sustainable products and contributes to carbon neutrality. Because of its dimensions, pore distributions, and geometries, it is studied to develop tools for nanotechnology and biomedical industry such as nanofabrication techniques, chemo and biosensing, particle sorting, and control of particles in micro- and nano-fluidics (22). Silica and biosilica can be used to develop advanced nanomaterial for electronic and optical technologies which can be employed for ultra-sensitive detection of biological compounds (23).

Recent accomplishment in diatoms metabarcoding, a reference database of the global population of diatoms has advanced its use extensively in studying ecological problems such as climate change, acidification, and eutrophication (24). Because of its robust nature and potential to inhabit different photic regions, from the equator to the poles, diatoms offer the potential to develop tools and products for all geographical regions (25). The technological and infrastructure advancements of diatoms-based applications are at a new level. Besides, it requires different kinds of optimization either in laboratory or large-scale research such as energy utilization for different steps, financial modeling, and collaborating with different industries to make diatom-based products commercially successful. However, the standardization at various levels such as optimization of culture conditions, genetic tools, genome and transcriptome sequencing make diatoms based products commercially viable.

Therefore, this review aims to provide a better understanding of the potential of diatoms research at a laboratory scale. We have tried to provide comprehensive information on a variety of diatoms applications such as energy, biomedical products, environment monitoring which are being investigated at different levels. All these

applications have the potential to contribute towards a greener tomorrow. The purpose of the research is to increase the sustainable economy while reducing the dependence on non-renewable resources. Therefore, recovering and producing various sustainable products like biofuels, feed, bioactive molecules and services like environment monitoring embedded in diatoms is a promising opportunity to be seized as shown in Figure 2.

2 Biofuel Industry

Fast globalization and industrialization have impacted the ecosystem widely but shutting or slowing down the globalization is not the solution. At the moment, almost 95% of all the transportation industry is based on a non-renewable source of energy (26). Therefore, developing sustainable and carbon-neutral fuels could reduce the existing dependence on fossil fuels and contribute to bringing back harmony in nature without disrupting the existing economic development. Few economic aspects of biofuel production from microalgae such as biodiesel productivity, land use, and oil yield support the use of microalgae for commercial production as compared to corn and other food crops. The oil yield for microalgae with high oil content is almost 15-fold more as compared to corn. Whereas, the land use for corn and maize is 66-fold more as compared to microalgae (27).

The microalgae such as diatoms are the promising feedstock to replace non-renewable sources of energy. It has been proven by geochemists that algal lipids are the major feedstocks of petroleum and these lipids act as the biomarker remaining stable for millions of years (27). The main biomarker for the diatoms is the ratio of C28 and C29 steranes and highly branched isoprenoid alkenes which are found in high-quality oil fields around the globe (28).

Moreover, targeting the diatom lipids by manipulating and optimizing the growth and culture conditions such as light, stress, and nutrients can provide an interesting alternative to help meet the existing demands of commercial production of biofuel. Knowing the potential of diatoms to accumulate high lipids and varied compositions of

fatty acids, diatoms are an underexploited area of the biofuel industry. The most predominant saturated and unsaturated fatty acids in diatom species are 14:0; 16:0, 16:1, 16:2, 16:3, 18:1, 18:2, 18:3, 20:4 and 20:5 (29, 30). Various reports have been published on different species of diatoms regarding the lipid yield and triacylglycerol accumulation (TAG) under different treatments as shown in Table 1.

It is possible to improve the quality of biodiesel by optimizing the content of different fatty acids that impacts biodiesel properties; cetane number, level of emissions, cold flow, oxidative stability, viscosity, and lubricity (31). Fatty acids with chain lengths from C16 to C18 should contribute the maximum amount in the final product (32). Some researchers have reported that a high percentage of mono-unsaturation is also desirable for biodiesel (33). Thus, optimizing the fatty acid profile along with increased biomass will significantly enhance their economic value.

Statistical analyses predicted that 100 mt/ha/year biomass of diatoms is required for commercial biofuel production (34). Over ten years, productivity range was observed to be between 29-142 mt/ha/year (35, 36) these values motivate the researchers and industry experts to study diatom cell in-depth for the biofuel industry in both lab-scale and large scale.

Furthermore, the availability of advanced genetic tools can help to achieve the missing targets in developing diatoms cells as biofuel machinery (37, 38). Based on theoretical calculations about the land area, lipid production, and photosynthetic energy conversion, the biofuel demand of the complete USA population could be met using only 5% of USA land (39). Although various other factors that define the efficacy of biodiesel such as engine performance, that is based on (cylinder pressure, brake mean effective pressure, frictional mean effective pressure, power, torque, brake specific fuel combustion, brake thermal efficiency). The statistical data supports the use of microalgae-based biofuel but there are various limitations at a technological level for large-scale implementation of this project. Therefore, one of the alternatives is to use the blended form of biodiesel. It would be more efficient to make a blended version of petro-diesel

and microalgae/diatoms based fuel for large-scale operation. The comparative studies of blended (20% microalgae fuel plus 80% petrodiesel) and 100% petrodiesel have no major performance variations. Furthermore, it was reported that there was a reduction in the CO, unburnt HC, and smoke emissions in blended form as compared to pure diesel (40).

3 Biomedical Industry

3.1 Drug Delivery Systems

The cost required to bring a new drug to the market has been estimated by the Tufts Centre for the Study of Drug Development at approximately 2.6 billion dollars (41). In addition, the current drug delivery systems have limited solubility, poor bio-distribution, lack of selectivity, premature degradation, and unfavorable pharmacokinetics (42). Therefore, these limitations have motivated the research and development of alternative drug delivery systems to improve the performance of existing drugs (i.e. increasing bioavailability), while reducing undesirable effects. There is no doubt that existing biomedical technologies have increased the life span but the human society wants to improvise the quality of life further by adopting environment friendly methods. Therefore, we should speed up the process and conduct in-depth research on using diatom frustules, even other bio-inspired alternatives for biomedical applications.

Among the available drug delivery tools (liposomes, nanogels, carbon nanotubes), the intricate frustule characteristics of diatoms such as specific surface area, thermal stability, biocompatibility, and alterable surface chemistry, have attracted attention for its use in drug and gene delivery. It took million years of evolution for diatoms to manufacture this level of complex and delicate structure to protect from the unwanted conditions like high temperature and variable light fluctuations. 3-D section analyses of diatom frustules have shown the availability of multiple pore patterns that range from nanometer to micrometer (43-45). These characteristics are sufficient to explore alternative and low-priced silica-based materials for the biomedical industry (22, 46). Diatoms' frustule structure changes its homogenous nature, space, and intricate nature

according to various environmental factors and silicon uptake efficiency (6). This ability can be used to change the frustule shape and pore size, which has multiple applications in the biomedical and nanotechnology industry. The process of biosilification in diatoms is quite complex, it includes the role of silicic acid transporters, transportation of silica, and polymerization of silica monomers among other processes that have been extensively explained (6, 47). Moreover, a detailed investigation is being conducted to make the natural 3D porous structure an efficient substitute for delivery systems attributed to its chemical and mechanical features. For instance, some diatom species such as *Coscinodiscus concinnus sp* (48), *Thalassiosira weissflogii sp*. (42) are potential drug carriers candidates due to their amorphous nature and morphology. Additionally, various studies have shown that diatoms microcapsules are effective carriers for poorly soluble and water-soluble drugs, which can be applied in both oral and implant applications (43, 49).

The defined structural architecture of diatoms, such as pore volume and controllable particle size, allows the synthesis of biomolecules at the micro- to nano-scale (50-52). The growth of fibroblast and osteoblast has been observed on functionalized frustules supporting the idea of using biosilica from diatoms as smart support for cell growth (43). Regarding modified diatoms, Losic et al. (2010) have designed the magnetically guided drug carrier via a functional surface of diatoms with dopamine-modified iron oxide. This modification has shown the capability of sustained release of poorly soluble drugs for 2 weeks, presenting an enhanced performance for drug delivery (51). Moreover, genetically modified biosilica has been used to selectively deliver anticancer drugs to tumor sites (53). Overall, these findings have opened the doors to novel drug delivery systems using renewable material. Therefore, all properties of diatoms such as uniform pore structure, chemically inert and biocompatible, non-toxic, easy to transport, filtration efficiency, and specific drug delivery make it a potential model for drug delivery tools (54-57).

Although the use of silica for food and agriculture has been approved by the FDA and is also labeled/classified as non-carcinogenic by International Agency for Research

on Cancer This could be a big step towards accelerating its use at the biomedical level. It is not yet approved for biomedicine as it requires long-term evidence (46). All the biomedical inventions are scrutinized by multiple stakeholders like research leaders, public authorities such as provincial and federal government, before they reach the stage of commercial distribution. It is understandable considering that it will be used directly in the human body. Therefore, an innovative and different approach is required to bring in the academic researchers and bio-entrepreneurs to speed up the innovation rate in biomedical industry without harming the screening process set by public health authorities. The collaboration between entrepreneurs and researchers will allow thorough evaluation of the market for new inventions, manufacturing, investment, and globalization of the product. It seems plausible considering the rapid advancements in the biomedical infrastructure around the world. This has been demonstrated by the quick inventions in response to COVID-19 and should be adopted to be applied in other biotech based industries (58). The simultaneous advancements in the use of silica-based support system for drug delivery along with the change in infrastructure in pharmaceutical industries and hospitals to deliver these technologies to the users is possible in the near future. The other requirement is to join the gap of vast and complex scientific information and knowledge between entrepreneurs and academic researchers.

3.2 Analytical Tools

The controlled production of nanostructured silica is possible through chemical and mechanical treatment for a wide range of applications. This nanopore structure has a huge potential to attach the desired biomolecule (enzymes, DNA, antibodies) and develop label-free analytical tools or enhance the catalytic properties. It has also been shown that enzymes and DNA (oligonucleotides) can be conjugated to silica (50, 59). The encapsulation of enzymes in diatom biosilica exhibits improved enzymatic properties as compared to other immobilization technologies (60).

Additionally, luminescent nano- and micro-particles have gained the attention of the interdisciplinary scientific community (biology, chemistry and physics). Current available

fluorescent labeling agents are quantum dots, lanthanide-doped compounds, and organic fluorophore-tagged nanobeads, which offer good optical properties and a broad excitation spectrum. However, these agents have limitations in properties such as photobleaching and biocompatibility. For instance, De Stefano et al., studied diatoms' potential to incorporate fluorophores with increased stability used to study the molecular event of antibody-antigen identification. Moreover, molecular recognition between antibody and antigen was observed in relation to the change in the photoluminescence spectrum of diatoms. Concluding that diatom's frustules, due to their high sensitivity, low-cost, and availability are ideal alternative candidates for lab-on-particle applications (61, 62).

There is no concrete evidence of diatoms' presence in land animal bodies. Although, various studies showed the presence of diatoms in the internal organs and circulatory system of alive or dead animals in an aquatic environment (63-68). The siliceous cell wall of this organism is resistant to degradation even under high acidic conditions for a long period (64). The investigation on the occurrence of these organisms inside dead bodies of aquatic environment that died from different causalities opened up a new possibility of forensic analysis through the examination of diatoms called 'diatom axiom' or 'diatom test'(63). The diatom test is based on the hypothesis that the microalgae will not enter the systemic circulation and reach other internal organs and tissues such as bone marrow unless the circulation is functional. A forensic examiner can determine whether the individual was alive when it was entering the water by checking the presence of diatoms in various organs and tissues (68). In addition, since diatoms are highly sensitive to environmental conditions different water bodies have different diatom species abundance, which allows forensics to identify the drowning site (69). Despite being a distinguishable method, the diatom test has limitations also. One of the major issues is the occurrence of diatoms in a drowning medium. The absence or low presence of diatoms in a water body can lead to a false positive or negative result. The presence of diatoms in different layers (water base, deeper, and surface) of the water body also can be varied (68). Rapid death is another situation where the diatom test can be wrong. Instant death when an animal or human enters the water body for various reasons such as cold shock and cardiac diseases will give a negative result in the diatom test (70). The use of alcohol or drugs is another

factor that can mislead in the diatom test (71). Recent advances in DNA Barcoding and pyrosequencing opened the possibility of increasing the accuracy of the diatom test by checking the presence of plankton specific genes (e.g. Rubisco gene) in animal tissue (72).

3.3 Biosensors and Nanomaterials

The advances in biotechnological tools have made it effective to characterize the frustules of diatoms for the fabrication of optoelectronics. The uptake of various elements such as zinc and germanium by diatom like *Stephanodiscus hantzschii*, *Thalassiosira pseudonana etc* to change the pore size, shape, and other characteristics which are being studied for a variety of functions such as paleolimnological indicator and photonic device application (73, 74). It has been reported a relationship between the amount of Zn/Si (zinc/silicon) and free zinc ions which can be used as a proxy of paleolimnological indicators (73). The studies have raised intriguing questions about the uptake and the process of various elements which need detailed validations. Although, they have reported that they could only detect Zn and Fe as chemical elements. The analysis of various trace elements could be used as an environmental indicator which indeed will reduce the total workload needed to monitor large water bodies (75).

The complex nanobiochemical machinery of diatoms can be exploited to fabricate a wide range of nanostructures with diverse optical and electronic properties (76). The ability to manufacture different pore size nanostructure molecules has inspired many research groups and industries to use diatoms in biosensing (77). The incorporation of chemical elements such as germanium significantly affects the structure and size of frustule pores. A study tested the possibility of using Si- Germanium composite material in living diatoms in a two-stage photobioreactor cultivation process which reduced the pore size without disturbing the morphology (76). Another study reported that insertion of germanium in *Nitzschia frustulum* induces the nanocomb structure with blue photoluminescence (74). These nanostructure materials exhibit optical properties suitable for use in semiconductors and optoelectronics. Manufacturing of these materials combined with the silica frustule will improve the overall durability and range of

applications in nanotechnology industries. These lab-scale scientific discoveries have shown that it is possible to create advanced nanomaterials in living diatoms.

3.4 Nanoparticles

The development of well-defined, advanced, and eco-friendly nanoparticles has attracted the attention of many researchers in the area of nanotechnology and its applications. Nanoparticles can be applied to study antimicrobial activity, catalyst, and filtering waste and chemical compounds. Biosynthesis of metallic nanoparticles in photoautotrophic organisms has gained the attention of nanotechnology researchers. Various approaches such as the sol-gel process, atomic layer deposition, chemical bath deposition, and inkjet printing process, have been used to modify the chemical composition of frustules. In this regard, an inexpensive chemical deposition technique was tested to deposit cadmium sulfide (CdS) on the surface on *Pinnularia sp.* without changing its morphology, since CdS has a wide range of applications in photodetectors and solar cells (78).

Recently it has been reported that diatoms can biosynthesize the nanoparticles such as gold and silver which has shown strong cytotoxicity against harmful microorganisms. Additionally, a highly ductile and malleable metal platinum (Pt) has been introduced in presence of dihydrogen hexachloroplatinate (IV) hexahydrate (DHH) in the living diatom *Melosira nummuloides*, without interfering the native morphology (79). This is due to platinum's excellent resistance to corrosion and stability at high temperatures, hence having application in a broad spectrum of industries, besides biomedicine. Other various examples of the on-going investigation of diatoms silica-based materials and their applications in biomedicine are shown in table 2.

We have discussed the major application of diatoms for established industries such as biofuels, nanomaterials, and biomedicine. However, diatoms also have other fascinating applications in environment monitoring, animal feed, and aquaculture,

which indeed have huge potential considering climate change and devastating impacts of globalization on ecology and environment.

4 Environmental Technologies

4.1 River Ecology

Environment monitoring is an important aspect that is considered a necessity to deal with irregular changes or disturbances in our ecosystem. Therefore, researchers are developing tools using biotechnology and informatics to monitor the environment cost-effectively. Water resources are always under the influence of damaging anthropogenic pressures such as plastic waste and industrial sewage, which ultimately change or disturb the biogeochemical cycles and biodiversity. Besides, water is a universal solvent that holds the industries and economies together.

It is a well-established fact that diatoms hold the primary role in maintaining the aquatic ecosystem. Therefore, biodiversity assessment of diatom species in an environmental sample is one of the well-known strategies for biomonitoring. Presently, morphological assessment of the diatoms using microscopy is largely used which is time-consuming and requires special expertise (80). However, environmental metabarcoding has opened a quick way of analyzing the microbial DNA diversity in a natural environment such as flora and fauna (81, 82). The metabarcoding approach is based on DNA sequencing a specific region (barcode) of the whole DNA extracted from an environmental sample (eDNA). For example, the sequencing data obtained from diatom metabarcoding are then used to assign precise taxonomic identification of the diatoms present in the eDNA sample, which are further compared with the conventional morphological database to confirm the efficacy of metabarcoding results. Diatoms metabarcoding tool has been optimized significantly to quantify the diversity of diatoms at the genus and species level (83, 84).

Currently, this approach is still in development, since various questions have been raised especially when deciding which are the most suitable barcodes. The barcodes that had been used are the ribosomal small subunit, cytochrome c, and the internal transcribed spacer region combined with the 5.8S rRNA gene (85, 86).

Another main issue is processing the sequencing output data through computing. This method must be consistent with government policies for environmental regulation. For instance, MOTHUR is a comprehensive and efficient platform to study microbial diversity, but there are other bioinformatics software such as R, QIIME2 (87), LotuS (88), and PIPITS (89) that can be used to process a larger amount of data.

Additionally, various other research studies have supported the use of the diatoms metabarcoding approach as an alternative strategy to monitor river ecology on a timely basis. The results provide an estimated number of abundant and scarce species in samples obtained from different locations. Also, they give great insights into the fundamental status of the aquatic ecosystem (80). For instance, detailed evidence has been published by the Environmental Agency of the United Kingdom using diatoms indexes for river classification (83). A similar study on detailed information on diatom biodiversity using metabarcoding has been conducted using environmental samples from Mayotte Island, France (84). Moreover, a recently published work studied the impact of treated effluents on benthic diatom communities that showed a systematic change in diatom community composition (90). Concluding that detailed information about diatom diversity will give in-depth insights into climate change, micropollutants, and other organic pollutants, to study the disturbing effects of anthropogenic pressure on rivers. The use of metabarcoding for analyzing biodiversity is rapidly increasing and has been adopted by academic institutes and various companies/industries like Spygen (Canada), Naturemetrics (United Kingdom), IGAtech (Italy), Sinsoma (Austria), to name few. This particular strategy has been adopted by public authorities as well and has shown the potential to be used as an additional screening tool to replace the existing methods, which require excessive infrastructure and human resources. It is indeed possible to make it a

primary and permanent tool for river monitoring with advancements in sequencing, big data science, and artificial intelligence tools.

4.2 Phytoremediation

Besides the monitoring of river quality, water treatment is one of the major concerns for many countries around the world. In fact, human consumption has undoubtedly increased in the last few decades, subsequently, incrementing waste products presence in aquatic communities (91). Globally, almost 80% of the wastewater generated worldwide is discharged on rivers creating health and environmental hazards. The rise of nutrient accumulation in the aquatic system needs to be neutralized to maintain the balance in the environment. Increasing of pollution is disturbing the basic biogeochemical cycles, killing fish, depleting the dissolved oxygen, and producing different toxins, i.e. neurotoxins (92). Hence, there is an urgent need to explore new ways and upscale the existing systems to test reports and mitigate pollution from rivers and lakes worldwide.

The use of microalgae for wastewater treatment has been a subject of research for a long period which could be applied in collaboration with small- and large-scale industries. The excess of industrial waste discharged in the aquatic system can be used as nutrient supply by diatoms. Different kinds of wastewater such as brewery (93), aquaculture (94) and textile (95) have been studied for phytoremediation capability and have shown interesting results. The published studies have established that diatoms and microalgae can treat the wastewater to an extent, therefore, it would be less damaging to treat the wastewater with microalgae/diatoms before discharging in water bodies. In addition, use the harvested biomass for different industrial products such as biofuel. It is safe to assume that it is possible to develop small scale business in collaboration with restaurants, breweries, textile industries, to name a few, to treat wastewater and use the biomass for the production of valuable products such as fertilizers (96).

Heavy metal pollution is one of the major challenges which comes from the industries working with chemicals and dyes. Diatoms species are desirable organisms to

study heavy metal pollution because of the simplicity of metal exposure, absorption, and detoxification of metal ions by single cells. This is a unique detoxification process of diatoms and microalgae due to metal-binding peptides known as phytochelatins (PCs) that protect photosynthetic organisms from heavy metals (97). Some intracellular PCs have been characterized in cultures of *P. tricornutum* exposed to different metals such as Cd, Pb, or Zn. Besides, they are used widely in waste degradation considering the unique structure of diatoms and their ability to respond to the changing environment (98).

A study published in 2015 have reported a novel diatom *Bacillariophyta sp*. (BD1IITG) from petroleum biorefinery wastewater that can degrade phenol in a concentration range of 50-250 mg/L in Fog's media (99). Another example of the degradation of toxic molecules like phenylalanine hydroxylase into less toxic compounds using simple enzymatic oxidation has been identified in diatoms during the metabolism of phenanthrene and pyrene (100). These results are relevant considering that around 7 billion kg of phenol is produced for oil refining, pesticide production and to use in the pharmaceutical industry. Traditional phenol removal techniques involve several steps including the generation of by-products, which increments the cost of the treatment (101). However, there are very few reports available on exploiting the potential of diatoms in biodegrading waste materials. It is interesting to note that the studies have shown interesting results but the field of algae biotechnology requires more entrepreneurs to join the pieces of industrial and academic research to build a successful circular economy. Furthermore, there are some upcoming and growing ventures and companies in microalgae working in diverse applications and producing valuable products such as healthcare, animal feed, water management, chocolates, etc (Table 3).

5 Diatoms As Nutraceuticals And Feeds

Multiple epidemiological and clinical trials have shown the health benefits of omega fatty acids from fish oils and algae extracts (17-19, 102). Besides, there are few publications on cardio-protective and cognitive performance of omega fatty acids which

have led to commercial production of infant foods, infant formula, fortified snack bars, and other dairy products supplemented with omega fatty acids (102, 103).

Diatoms have an immense nutritional value that can be used to produce novel compounds such as antioxidants, vitamins, animal feed, and vegetarian protein supplements. Several photosynthetic pigments have been identified in diatoms including carotenoids such as fucoxanthin (104). Additionally, *Nitzschia laevis*, *Nitzschia inconspicia*, *Navicula saprophila* and *Phaeodactylum tricornutum* extracts have a noticeable amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that can be used as a nutritional feed in human diet and animal feed (14, 15, 105-107).

Moreover, diatoms are known to have diverse defense mechanisms in form of chemical substances for them to be protected against pathogens. For instance, *P. tricornutum* has a high amount of omega-7 monounsaturated fatty acids such as palmitoleic acid (C16:1) and other bioactive compounds that are active against gram-positive pathogens (108). Furthermore, the EPA-rich marine diatom, *Odontella aurita*, used as a dietary supplement has shown antioxidant effects in rats (109). *O. aurita* has been approved to be commercialized as food in France by following EC regulation 258/97 in 2002 (110, 111).

Increasing the content of these bioactive molecules in diatoms has attracted a large amount of research. Some studies have managed to enhance the production of flavonoid and polyphenol content by culture modifications, for instance, cultivation temperature and nutrient supplementation in *Amphora sp*. (112). The general tendency when changing the culture temperature is an increase in lipid content in most species, while the chemical composition varied between species (113). For example, the total amount of saturated and monounsaturated fatty acids increases with temperature in *Rhodomonas sp*. (NT15) and *Cryptomonas sp*. (CRFI01). Whereas there was a comparative decrease in polyunsaturated fatty acids in both *Rhodomonas sp.* (NT15) and *Cryptomonas sp*. (CRFI01) (113).

6 Genetic Engineering Of Diatoms

The debate on using genetically modified microalgae and diatoms is on-going. However, it is a more controlled alternative for the production of recombinant proteins or any precursor molecules, considering the use of bioreactors for their production. The employment of genetic engineering tools in diatoms, to produce or increase the yield of compounds, allows the companies to optimize their use in the applications mentioned above. Therefore, genetic engineering is a promising method and an important branch to be used in the diatoms industry to further enhance the economic value of diatoms. However, it comes with two big challenges, firstly, to redesign the natural metabolic pathways in order to increase the production of desired endogenous compounds, and secondly, producing new heterologous compounds.

In the last 20 years, several projects have shown that these challenges can be solved at lab scale, by optimization of transformation methods, utilization of different gene promoters, expression of recombinant proteins, gene silencing, and genome editing methods; such as targeted mutagenesis techniques using meganucleases, gene knockouts, TALENS, and CRISPR/Cas9. Marketable bioproducts like lipids, pigments, nanomaterials, food supplements, fuel, syntheses of chemicals, drugs, and metabolites have been produced in *P. tricornutum, T. pseudonana*, and other diatoms species. While most of these analyses are related to lipid production for biofuel or bioenergy purposes, other studies showed that diatoms are biological factories that can generate a wide range of products from food to pharmaceutics biomaterial industry (22, 114-117). In addition, there are few companies such as Algenol biofuels, Synthetic Genomics,which have reported the use of genetically modified microalgae for the production of biofuels.

Moreover, the approach of synthetic biology along with high throughput sequencing technologies open the doors to understanding the whole genome, the proteins that it encodes, and the regulatory elements of the cell during cellular growth and division (118, 119). Several sequencing projects have been performed in *P. tricornutum* and *T. pseudonana* strains (120-123), generating the transcriptomic and proteomic data sets that make possible precise reconstructions of metabolic networks (124, 125).

Recently, the Synthetic Diatoms Project website has been launched as a platform to provide information to grow, transform, edit, and analyze *P. tricornutum* and *T. pseudonana* (https://www.syntheticdiatoms.org/). These projects have been used as a springboard to facilitate genome annotation for other diatoms species: *T. oceanica, T. weissflogii, Fragilariopsis cylindrus, Pseudo-nitzschia multiseries*, *Pseudo-nitzschia multistriata, Seminavis robusta, Fistulifera solaris, Cyclotella cryptica* (Table 4).

Diatoms are a robust model for genome editing and cell transformation. Optimized methods of DNA delivery have been developed using biolistic or via electroporation. In both techniques, the transgenes are randomly integrated into the genome, with multiple integration events, variable transgene copy numbers, and chromosomal positions. The biolistic gene transfer method affects genome integrity due to the break and repair of the DNA double-strand by non-homologous end joining (NHEJ) (126). However, this method is needed if the aim is to transform the chloroplast genome. An alternative transformation technique is the extrachromosomal-based expression approach that depends on vectors containing a yeast-derived sequence, which can be delivered through bacterial conjugation using E. coli (127).

An important element for genetic engineering is the promoter. The most commonly used are the light-regulated promoters of the fucoxanthin chlorophyll a/c-binding protein genes fcpA/B/C/D (LHCF) (128, 129). Alternatively, the elongation factor 2 (EF2) promoter sequence is a constitutive promoter (130). Recently, the most abundant secreted protein in *P. tricornutum* was identified, named "highly abundant secreted protein 1" (HASP1), and the activities of its promoter and the signal peptide were characterized using green fluorescent protein (GFP) as a reporter (131). A couple of inducible promoters have been reported: like nitrate reductase (NR) and alkaline phosphatase gene promoters in *P. tricornutum*, which are induced under nitrogen or phosphate starvation respectively (115, 132) and glutamine synthetase gene promoter, induced by a blue light pulse (133, 134). In addition, promoter regions containing diatom-infecting viruses (DIVs) mediated a significantly higher level expression of the reporter gene in cells in the stationary phase compared to the exponential phase of growth (135). Other elements

needed for genetic engineering are reporter genes and selection markers. Among reporter genes, beta-glucuronidase *uidA* (GUS), fluorescent proteins like GFP/YFP/CFP, chloramphenicol acetyltransferase conferring resistance to chloramphenicol (CAT) and Luciferase (LUC) are the most employed, other reporter proteins are listed in Table 5. The classic selection markers in diatoms are genes that confer resistance to zeocin, phleomycin, and nourseothricin, as shown in Table 5are the most used. An alternative to using selective markers is the use of auxotrophic strains, such as uracil, histidine, and tryptophan auxotrophs (136, 137). Moreover, it is considered that the urease gene, either in an inactive or edited form, is an interesting tool for the selection of *P. tricornutum* and *T. pseudonana* strains (115, 138, 139). An endogenous selectable marker in diatoms was generated by point mutations at a conserved residue Gly290 to Ser/Arg in the phytoene desaturase (PDS1) gene, which confers resistance to the herbicide norflurazon (140).

Concerning heterologous recombinant protein expression, diatom gene codon optimization is required for optimal expression; to avoid silencing expression and better protein translation. Although it has not been reported in diatoms, different projects which were done in green algae, have shown that including introns in the expression cassette can increase transcript abundance (141-143) . In addition, 5'-UTR and 3′-UTR of nitrate reductase (NR) allow the control of timing and level of transgene expression in *C. fusiformis* (144). Down-regulation of gene expression can be achieved through silencing by expressing antisense repeat sequences of target genes (Table 5).

Industrial processes using diatoms are cost-effective and have performed well in large-scale cultures (145). This is supported by the plasticity to adapt to extreme environmental conditions of diatoms, making them great candidates for sustainable biofactories (146-148). Altogether, these developments in metabolic pathways and synthesis of heterologous compounds represent promising insights for the improvement of yield, quality of products, and sustainability in the use of diatoms as cell factories.

7 Conclusion And Future Perspectives

The documented studies stated the astounding nature and possible all-round use of diatoms. This is one of the approaches to increase human consumption of renewable products and contributes towards reducing carbon emissions. Although the commercial application of diatoms still needs improvements, it is indeed a crucial research area for human wellbeing. For example, developments in diatoms research can lead to innovative products in domains of drug delivery, sensing, and detection parts to build complex biomedical devices and nanoparticles for waste degradation. Moreover, recent advancements in sequencing technology and processing large biological datasets have made it possible to label and store the global biodiversity of diatoms in all geographical locations.

One of the major challenges in diatom-based industries is scaling up the process for large-scale manufacturing which is dependent on many micro and macro factors such as cultivation, harvesting, drying, genetic modification, lack of genomic, proteomic, and metabolic information, etc. However, it is possible to overcome these challenges in near future with advancements in genetic tools, bioreactors, and other infrastructure changes. In general, there are many challenges in bio-based industries at different levels; academic/industrial research, infrastructure, policies, education, and information gaps. The advancements in academic research and discoveries are consistent considering the publications but it requires support from other domains such as the development of infrastructure, reducing the knowledge gaps between scientific researcher and entrepreneurs, changes in the policies at both national and international level. And to conclude, the recent research phenomenon blasted in the last decade, which is diatoms' industrial potential, still leaves many unsolved questions. Major questions will involve studying the extent of genetic or artificial manipulation without compromising its intact structure and delicate silica pattern. The unfolding of various missing links in genetic engineering, cultivation, and harvesting will make it possible to replicate complex plant pathways in diatoms. These tools have opened the door to study diatoms for eco-friendly processes.

8 Author Contributions

N.S. and I.D-P. conceived, designed, and led the study. N.S., D.P.S., A.M.D-G., E.F. and A.M. collected and analyzed the data, prepared the figures and table. N.S., D.P.S., A.M.D-G., E.F., A.M., F.M-M., H.G. and I.D-P. authored and reviewed the drafts of the manuscript, and approved the final manuscript.

9 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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Tables

Table 1. Lipid content and productivities of different microalgae diatom species (- : no data).

Microalgae	Culture condition	Lipid (% dry weight)	Lipid productivity mg L^{-1} day^{-1}	TAG productivity µmol L^{-1} day^{-1}	% of TAG	References
***Thalassiosira weissflogii* P09**	-	29.94 ± 1.17	7.27 ± 0.28	-	51.0 ± 3.2	(148)
	Nitrogen limitation	-	-	19 (+20%)	-	(148)
***Thalassiosira weissflogii* CCMP 1010**	-	38.84 ± 0.78	4.87 ± 0.10	-	53.0 ± 1.9	(148)
***Thalassiosira pseudonana* CCMP 1335**	-	29.33 ± 1.17	1.72 ± 0.07	-	19.0 ± 0.9	(148)
	High CO_2 20,000 ppm	-	-	45.5 ± 26 (Exponential) (+285%)	-	(149)
***Cyclotella cryptica* CCMP 331**	-	41.97 ± 1.26	2.98 ± 0.09	-	55.0 ± 2.1	(148)
	Nitrogen limitation	-	-	45 (+20%)		(148)https://doi.org/10.1186/s13068-
***Phaeodactylum tricornutum* CCMP 632**	-	9.32 ± 0.28	2.09 ± 0.06	-	19.0 ± 0.6	(148)
	Tn19745_1 strain + nitrogen limitation	-	-	-	45-fold increase	(150)
	Dark	+2.3-fold	-	-	-	(151)
	High CO_2	-	-	75.7 ± 9 (Stationary) (+50%)	-	(149)
***Navicula pelliculosa* (marine)**	High CO_2	-	-	158.4 ± 29 (Stationary) (+35%)	-	(149)

Table 2. Biomedical applications of diatom silica-based materials using different diatom species.

Application	Organism	Reference
Specific nanoporous biosilica delivery system of chemotherapeutic drug, consisting in the attachment of antibodies and hydrophobic drug molecules, without using cross-linking, to the diatoms biosilica.	*T. pseudonana*	(53)
Modified frustule with self-assembled antibacterial aromatic amino acid conjugates Tyr-Zn^{II} as a zinc carrier for its controlled release to bacteria and inhibiting the bacterial growth.	*N. palea*	(152)
Genetically modified frustule with chimeric fusion proteins: diatom-derived silica targeting peptide Sil3T8 and a small synthetic antibody derivative to detect *Bacillus anthracis*	*T. pseudonana*	(153)
Rapid and selective detection of typhoid using cross-linked amine-functionalized diatom photoluminescent biosensor.	*Amphora* sp.	(154)
Nano composite of nanoporous diatom-ZrO_2 selective and highly sensitive sensor for non-enzymatic detection of methyl parathion.	*P. tricornutum*	(155)
Biomaterial for negative electrode composed by a 3D-structured diatom biosilica for lithium-ion batteries, showing increased charge capacity compared to graphite.	*P. trainorii*	(156)
Improved capacitor performance of in-situ coating of FeOx on live diatoms as a potential material for super capacitor electrodes.	*P. tricornutum*	(157)

Table 3. Different industries producing variety of products from microalgae and diatoms around the world.

Company	Products/Services	Country	Wesbite
Algae Biotechnologia	Wastewater treatment, Animal nutrition, Carbon dioxide fixation, biofuels, human health	Brazil	http://www.algae.com.br/site/pt/
Algae Farm	Omega3, Diatom, Water treatment & reuse, Nutraceuticals, Cosmeceuticals, Algae based solar fuels cell, Die sensitized solar panel, Bioplastics	Canada	https://www.algaefarm.us/
Algorigin	Nutritional supplements	Switzerland	https://algorigin.com/en/
Algaetoomega	Omega 3, Astaxanthin, Animal feed	United States	https://algae2omega.com/
Algae control Canada	Pond and lake water management	Canada	https://www.algaecontrol.ca/
The algae factory	Chocolate	Netherland	http://thealgaefactory.com/
Algae Health	Antioxidants	United States	https://www.algaehealthsciences.com/
Swedish Algae Factory	Personal Care products	Sweden	https://swedishalgaefactory.com/
Sabrtech	Recombinant Proteins, fuel, nutraceuticals, aquaculture etc	Canada	https://www.sabrtech.ca/
Pondtech	Astaxanthin, Aquaculture	Canada	https://www.pondtech.com/

Table 4. Sequence Database of different diatoms species**.**

Species	Genome Database
Phaeodactylum tricornutum **CCAP 1055/1**	http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index
Thalassiosira pseudonana **CCMP 1335**	https://genome.jgi.doe.gov/Thaps3/Thaps3.home.html
Thalassiosira oceanica **CCMP 1005**	https://genome.jgi.doe.gov/Thaoce1/Thaoce1. info.html
Thalassiosira weissflogii **CCMP1030**	https://genome.jgi.doe.gov/portal/
Fragilariopsis cylindrus **CCMP 1102**	https://genome.jgi.doe.gov/Fracy1/Fracy1.info.html
Pseudo-nitzschia multiseries **CLN-47**	https://genome.jgi.doe.gov/Psemu1/P semu1.home.html
Pseudo-nitzschia multistriata **B856**	http://apollo.tgac.ac.uk/Pseudo-nitzschia_multistriata_V1_ 4_browser/sequences
Seminavis robusta D6	https://genome.jgi.doe.gov/portal/Semrobnscriptome/Semrobnscriptome.info.html
Fistulifera solaris **JPCC DA058**	https://trace.ddbj.nig.ac.jp/DRASearch/submission?acc5DRA002403
Cyclotella cryptica **CCMP332**	http://genomes.mcdb.ucla.edu/Cyclotella/download.html

Table 5. Diatoms Genetic Engineering.

Species/strain	Genetic & Molecular tools				
	Transformation methods &Target compartment	**Promoters: (S) strong, (I) inducible & (H) heterologous**	**Reporters (R) & resistance (Re) genes**	**Expression of recombinant proteins**	**Genome Editing Methods & Gene Silencing**
Phaeodactylum tricornutum CCAP 1055/1	Biolistic (147) Electroporation (158) Conjugation (126) Nuclear and chloroplast transformation (159)	**(S):** Lhcf (Fcp), light responsive (127), (EF-1a, 40SRPS8, g-Tubulin, RBCMT (131) and EF2 (129), h4 (132), HASP1 (133). **(I):** rbcL (159), NR, low NO3 induce (160), V-ATPase C, AP1 low P induce (161) Fbp1, Fld, Isi1 iron-responsive (162) ca1, ca2 CO2-responsive (163, 164), U6, RNA polymerase III transcribed (165) **(H):** CdP1, ClP1, ClP2, TnP1, TnP2 (134), CMV, RSV-LTR, PCMV, CaMV35S (166)	**(R):** GUS, GFP (167), YFP, CFP (126) cat (127), LUC (147), Aequorin (168) **(Re):** Zeocin and Phleomycin/sh ble, Nourseothricin/nat, Blasticidin-S/bsr, Streptothricin/sat, Neomycin/nptII (127)	Expression of Acyl-ACP thioesterases, increased accumulation of shorter chain (169). Malic enzyme (170). G6PD (171), enhanced lipid productivity. Heterologous biosynthesis of the MIAs by CrGES expression under phototrophic conditions (115), Vanillin production (131). PHBs for Bioplastics production (172). Human IgGαHBsAg:(173) and IgG1/kappa Ab CL4mAb: antibody to hepatitis B virus surface protein against the nucleoprotein of Marburg virus(174). Over expression of DXS increased fucoxanthin synthesis(175).	Targeted mutagenesis methods: meganucleases, gene knockouts, TALENS, and CRISPR/Cas9 (144). Development of auxotrophic strains of *P. tricornutum* by CRISPR/Cas9 (136). A lipid producing strain through the disruption of the UDP-glucose pyrophosphorylase gene (150).

Species/strain	Genetic & Molecular tools				
	Transformation methods &Target compartment	**Promoters: (S) strong, (I) inducible & (H) heterologous**	**Reporters (R) & resistance (Re) genes**	**Expression of recombinant proteins**	**Genome Editing Methods & Gene Silencing**
Thalassiosira pseudonana CCMP 1335	Biolistic (176) Electroporation (177) Conjugation (126)	**(S):** Lhcf9 **(I):** nr (161) SIT1, Si-starvation inducible(178),Thaps3_9619, Si-starvation inducible (179), U6, RNA polymerase III transcribed (138)	**(R):** YFP (126) **(Re):** sh ble, nat (176)	Overexpression a multiple plasmids can be cotransformed; cloning multiple genes of interest Secretion of recombinant proteins has been shown. Localization of SiMat1-GFP (180). Expression of the protective HsIbpA DR2 antigen for the production of a vaccine against bovine respiratory disease (178), scFvTNT scFv and sdAbEA1 to detected *Bacillus anthracis* (181).	Targeted mutagenesis methods: meganucleases, TALENS, and CRISPR/Cas9 (138). Gene silencing and gene knockouts are well established (182).
Thalassiosira weissflogii (CCMP1030)	Biolistics (147)	**(S):** Lhcf2 (147)	**(R):** GUS(147)		
Pseudo-nitzschia multistriata B856	Biolistics (183)	**(S):** h4 (183)	**(Re):** sh ble (183)		
Pseudo-nitzschia arenysensis B858	Biolistics (183)		**(R):** GUS, GFP (183)		
Fistulifera solaris JPCC DA058	Biolistics (184)	**(S):** Lhcf2 and h4 **(H):** RSV and CaMV35S (184)	**(R):** GFP **(Re):** nptII,(184)	Overexpression of the endogenous *GK* improve lipid productivity (184)	

Species/strain	Genetic & Molecular tools				
	Transformation methods &Target compartment	**Promoters: (S) strong, (I) inducible & (H) heterologous**	**Reporters (R) & resistance (Re) genes**	**Expression of recombinant proteins**	**Genome Editing Methods & Gene Silencing**
Cylindrotheca fusiformis CCAP 1017/2 –CYL	Biolistics (141)	**(I):** nr (141)	**(R):** GFP **(Re):** sh ble (141)		
Navicula saprophila NAVICI	Biolistics (185)	**(S):** ACCase (185)	**(Re):** nptII (185)		
Chaetoceros gracilis UTEX LB2658	Biolistics (186)	**(S):** Lhcr5 **(I):** nr (186)	**(R):** GFP, LUC **(Re):** nat (186)		

Abbreviations: 1-Deoxy-D-xylulose 5-phosphate synthase (DXS), 40S ribosomal protein S8 (40SRPS8), Acetyl-CoA carboxylase (ACCase), Acyl–acyl carrier protein thioesterases (Acyl-ACP thioesterases), Alkaline phosphatase 1 (AP1), Blasticidin-S resistance gene (bsr), Carbonic anhydrase 1 (Ca1), Cauliflower mosaic virus 35S (CaMV35S), Chaetoceros debilis-infecting DNA virus (CdP), Chaetoceros lorenzianus-infecting DNA virus (ClP), Chloramphenicol acetyl transferase conferring resistance to chloramphenicol (cat), Clustered regularly interspaced short palindromic repeats (CRISPR), Cyan fluorescent protein gene (CFP), Cytomegalovirus (CMV), Diatom light-regulated promoters of the fucoxanthin chlorophyll a/c-binding protein genes Lhcf (Fcp), Elongation factor 1 alpha (EF-1α) and 2 (EF2), Ferrichrome binding protein1 (Fbp1), Flavodoxin (Fld), *Catharanthus_roseus* geraniol synthase (CrGES), Glucose-6-phosphate dehydrogenase (G6PD), Glycerol kinase (GK), Green fluorescent protein gene (GFP), Highly abundant secreted protein 1 (HASP1), Histone H4 (h4), Human IgGαHBsAg: antibody against hepatitis B virus surface IgG1/kappa Ab CL4mAb, IbpA DR2 antigen from *Histophilus somni* (HsIbpA DR2), Iron-starvation-induced gene 1 (Isi1), Monoterpenoid indole alkaloids (MIAs), Neomycin phosphotransferase II (nptII), Nitrate reductase (NR), Nourseothricin acetyl transferase (nat), Phosphate (P), Polyhydroxybutyrate (PHBs), Promoter sequences of the cytomegalovirus (PCMV), PSII reaction center core 2 quinones are associated with D1 (psba), Red algal-like LHCRs (Lhcr5), Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit N-methyltransferase I (RBCMT), *Rous sarcoma* virus long terminal repeat (RSV-LTR), Rubisco large subunit (rbcL), Silica Matrix protein (SiMat1), Silicon transporter (SIT1), Single chain antibodies (scFvTNT), Single domain antibodies (sdAbEA1), Small nuclear RNA of the U6 complex (U6), *Streptoalloteichus hindustanus* bleomycin resistance gene (sh ble), *Thalassionema nitzschioides*-infecting DNA virus (TnP), Transcription activator-like effector nucleases (TALENs), Treptothricin acetyl transferase (sat), Triacylglycerol (TAG), Tubulin gamma chain (g-Tubulin), uidA b-glucuronidase (GUS)-encoding gene, Vacuolar H+-ATPase (V-ATPase C), Yellow fluorescent protein gene (YFP).

Figures captions

Figure 1: Approximate number of research articles indexed in Scopus database (September 14th, 2020) in the area of industrial application of different microbes (bacteria, yeast, algae and diatoms).

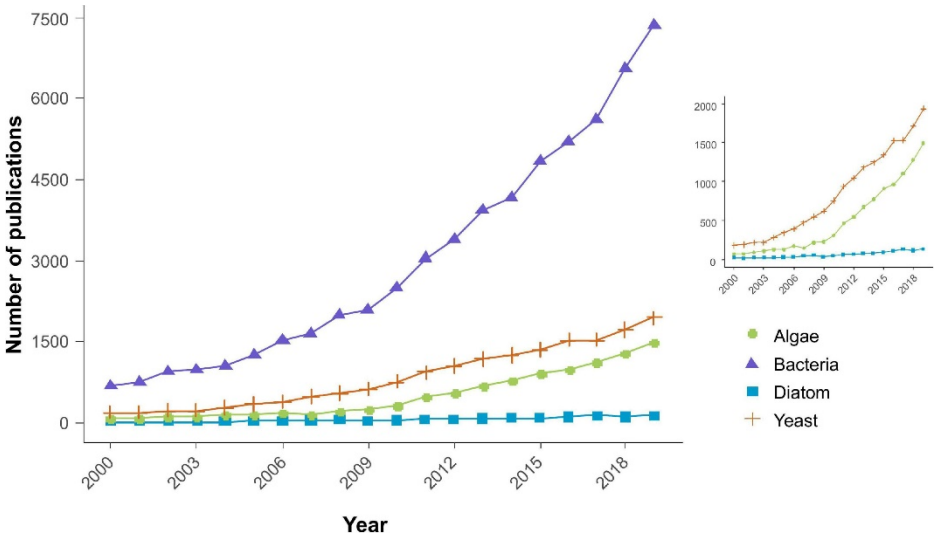

Figure 2: Scheme of the different uses of diatoms for green industry.