Bacterial stimulation of lipid vesicle production in green microalga *Dunaliella tertiolecta*

by

Catherine C. Bannon

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

University of Alberta

Abstract

Algae and bacteria form complex interactions through the exchange of bioactive molecules and nutrients. A wide range of phenotypes, and often their very survival, are influenced by these chemically mediated relationships, shaping the ecology and diversity of marine communities. Here we sought to uncover the effect of various marine bacteria on lipid production and storage in the biofuel candidate, unicellular green alga *Dunaliella tertiolecta* (CCMP1320). The roseobacter *Phaeobacter italicus* R11 was found to increase *D. tertiolecta*'s lipid per biomass ~25%, with no effect on growth rate or photosynthetic health, during 16 d co-cultivation experiments. Although the amount of lipid produced increased, the fatty acid methyl esters composition was not altered by *P. italicus*. Imaging flow cytometry and fluorescence microscopy revealed that the increase in lipid per biomass coincided with the production of algal-derived, extracellular lipid vesicles. The lipid vesicles were not degraded but accumulated in abundance and could fuse, forming larger vesicles. This is the first description of bacterial-stimulated lipid vesicle production in green microalgae, and these findings could expedite algal lipid harvesting during biofuel production.

Preface

This thesis is an original work by Catherine Bannon. No part of this thesis has been previously published. In Chapter 2, the fatty acid analysis conducted was part of the collaborative work between the Case lab and Harynuk group at the University of Alberta. In order to complete the fatty acid analysis, Dr. Paulina de la Mata and Michael D. A. Sorochan optimized and performed the final fatty acid extraction and analysis using comprehensive two-dimensional gas chromatography time-of-flight. As such, it is their work presented under the heading 2.3.6 in the Material and Methods section of Chapter 2 after the lyophilizing of algal biomass.

Acknowledgements

I would like to take this opportunity to thank the people who made my time at the University of Alberta so rewarding. Firstly, I extend my gratitude to Dr. Rebecca Case for accepting me into her lab and fueling my interest in this field. Her guidance and enthusiasm for research throughout this project was central to its completion.

To Dr. Lisa Stein and Dr. Rolf Vinebrooke, for their support and mentorship that made a lasting impression on me and I hope, one day, I become a fraction of the supervisors they are.

Thank you to my peers; Yue Xu, Dr. Anna Bramucci, Nora Hussain, Phillip Sun, Morganne Wall and Carrie-Ann Adams for being a constant fountain of encouragement and inspiration. Each one of you have taught me so much and I will continue to look up to you as role models after my time at UofA.

My sincere appreciation to my partner Joceline Lebans for making Edmonton home and reminding me daily what the important parts of life are. Your calming reassurance, unwavering support and gentle reminders of selfcare were invaluable. I am honoured that you are my partner.

Finally, thank you to my family, near and far, for their assistance and support throughout my time in Edmonton. Seamus, Liam, Mom and Dad, you all inspire me daily and I am eternally grateful for each of you. To Livi and Cole, you two made all the difference. Thank you for your friendship and love that will stay strong long after our days of being neighbors. To the YEG Corcorans, thank you for the meals that filled our stomachs and our souls. To the amazing people I am lucky to have gotten to know, Hayley, Kathrin, Ryley, Miranda, Jeff and Yahweh, thank you for making the memories of Edmonton so, so sweet.

Table of Contents

List of Figures

List of Abbreviations

CFU	colony forming units
DGDG	digalactosydiacyglycerol
ER	endoplasmic reticulum
EPS	extracellular polymeric substances
EV	extracellular vesicle
FA	fatty acid
FAME	fatty acid methyl ester
GM	genetically modified
LD	lipid droplet
MA	marine agar
MGDG	monogalactosyldiacyglycerol
PE	phophatidylethanolamine
PC	phosphatidylcholine
PG	phosphatidylglycerol
PSII	photosystem II
SE	standard error
TAG	triacylglycerols
GCxGC TOFMS	two dimensional gas chromatography with time-of-flight mass spectroscopy

Chapter 1: Introduction

1.1 Microalgae

Microalgae are a diverse group of photosynthetic organisms that dominate primary production in all phototrophic aquatic ecosystems and produce almost half of Earth's atmospheric oxygen (Field, 1998). Extensive research has been performed to understand the crucial role microalgae play in the biogeochemical cycling of carbon, oxygen and sulfur (Falkowski, 1998; Simó, 2001).

The ecological role of these eukaryotes is shaped by their complex evolutionary history responsible for their diversity. Microalgae are the product of ancient primary endosymbiosis' of an early cyanobacteria into an Archaeplastida's ancestor, which happened over 1.6 billion years ago (Gould et al., 2008; Rockwell et al., 2014). Since then, additional secondary and tertiary endosymbiotic events, between green and red algae with the majority of eukaryotic supergroups, have generated a heterogeneous group of unicellular microorganisms (Bhattacharya et al., 2004). Microalgal groups have been classified by pigment composition, cell wall composition and ultrastructural features such as plastids or cilia (Simon et al., 2009). This systematic classification produced 9 divisions with the 6 larger groups of Phaeophyceae (brown algae), Pyrrophyceae (dinoflagellates), Chrysophyceae (golden-brown algae), Bacillariophyceae (diatoms), Chlorophyceae (green algae) and Rhodophyceae (red algae) (Tirichine and Bowler, 2011).

Due to the remarkable phylogenetic and phenotypic diversity of microalgae, effort has been made to define their physiological and metabolic processes for decades (Larkum, 2016; Liu and Benning, 2013; Singh et al., 2005). Important progress to explore mechanisms of their photosynthesis, nutrient assimilation, carotenoid biosynthesis and environmental stress tolerance in order to determine their phenotypic plasticity to upcoming climate change and trace their evolution has been performed (Beardall and Raven, 2004; Eberhard et al., 2008; Gong and Bassi, 2016; MacIntyre et al., 2002; Monteiro et al., 2012). Special attention has been given to microalgal lipid metabolism, which has exploitable characteristics in the biotechnology industry (Harwood and Guschina, 2009).

1.1.1 Microalgal lipids

Rapid advances in analytical methods and technology, such as transcriptomics and gas chromatography, have enabled the characterization of algal lipid metabolism and the identification of novel lipid classes (Danielewicz et al., 2011; Mendoza Guzmán et al., 2011; Rismani-Yazdi et al., 2011). Microalgal lipids fall under two categories: polar lipids and neutral lipids.

Phospholipids, glycolipids, sphingolipids and sterols are classified as polar lipids and are key components of cellular membranes with a central role to maintain membrane integrity. Phospholipids most commonly occur in extra-chloroplast membranes, except for phosphatidylglycerol (PG) which is found in the thylakoid membranes, and stabilizes the photosynthetic apparatus (Kumari et al., 2013). Structurally, they are comprised of a phosphate group that is bound to a hydrophilic, glycerol head which, in turn, is bound to two hydrophobic fatty acid tails (Figure 1.1a). The major phospholipids in microalgae are phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Yao et al., 2015).

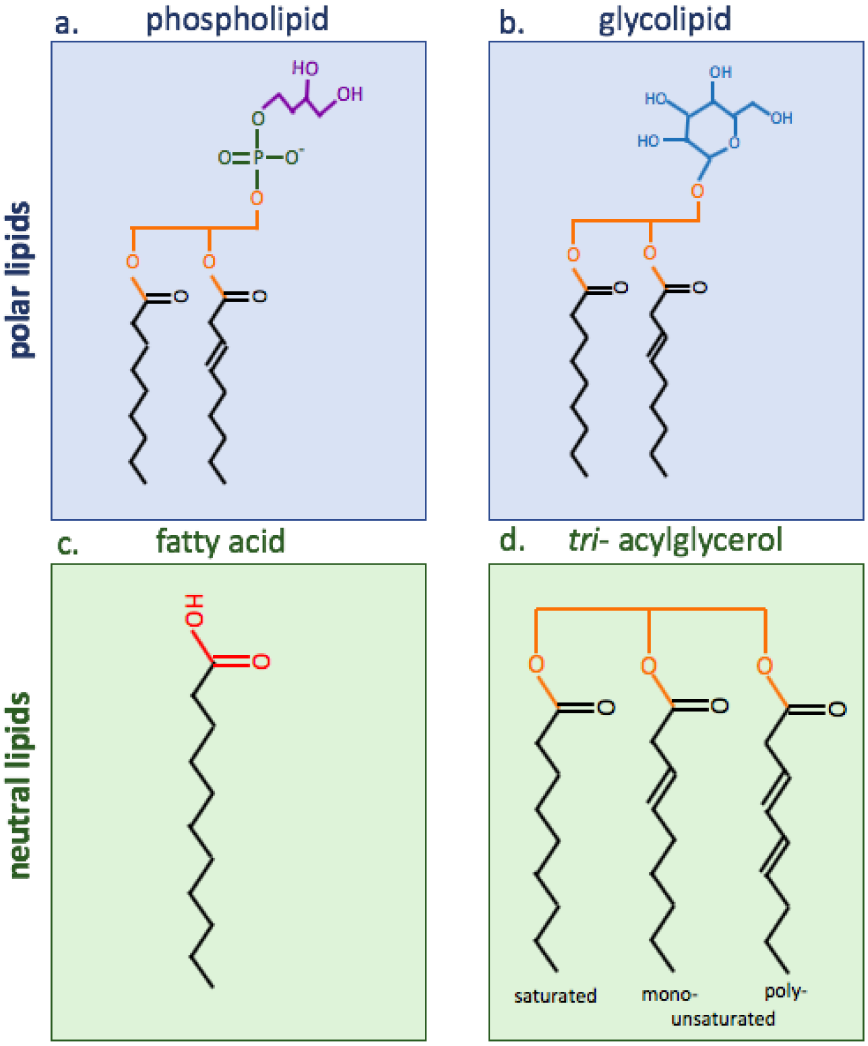


Figure 1.1. Skeletal formulas of microalgal lipids types. Lipids are first separated into polar lipids (a,b) and neutral lipids (c,d). Polar lipids are represented by (a) phosphatidylglycerol (PG) molecule with two fatty acid tails (black), a glycerol backbone (orange), a phosphate group (green) and the hydrophobic head (purple) and (b) monogalactosyldiacyglycerol (MGDG) molecule, with fatty acid tails, a glycerol backbone and a carbohydrate head group (blue). A free fatty acid (c) is shown with its saturated hydrocarbon tail (black) and carboxylic acid head group (red). Triacylglycerol molecule is comprised of a glycerol backbone and three fatty acid tails (black) in saturated, mono-unsaturated and poly-unsaturated forms from left to right.

Glycolipids are mainly located in the thylakoid membrane where they are essential structural components and imperative to the repair and assembly mechanisms of photosystem II (Mizusawa and Wada, 2012). The basic structure of glycolipids is a mono or oligosaccharide group bound to a glycerol backbone that has one or two fatty acid tails (Figure 1.1b). The major

glycolipids in microalgae are monogalactosyldiacyglycerol (MGDG) and digalactosydiacyglycerol (DGDG), but the proportion of these molecules present in a microalga is species dependent (Harwood and Guschina, 2009). Polar lipids also include chlorophyll pigments, which are essential for harvesting light energy. The majority of a microalgal cell's lipid production during favorable conditions is devoted to synthesizing these polar membrane lipids to sustain their reproduction and growth (Basova, 2005).

The second group of lipids in microalgae, neutral lipids (NL), include fatty acids and acylglycerols. These molecules are the foremost component of traditional transportation fuels (Sorigué et al., 2016). Generally, these molecules only constitute under 1.75% of total dry weight in algae species, with the exception of the micro green algae, *Botyococccus braunii,* where hydrocarbons can account for up to 8% of total dry weight (Qin, 2010).

Fatty acids (FA) differ in structure from hydrocarbons because of their carboxyl acid group on one end of a long, hydrocarbon tail (Figure 1.1c). Fatty acids are considered lipid building blocks, as they are the fundamental component for numerous types of lipids in microalgae (Hulatt et al., 2017; Jónasdóttir, 2019). They are present as 'tails' in phospholipids and glycolipids, as displayed in Figure 1. This neutral lipid is classified based on the number of carbons and double bonds. Saturated FA contain no double bonds, while unsaturated have one (mono unsaturated FA), or more than one (polyunsaturated FA) present (Figure 1.1d). Relative to higher land plants, microalgae have been noted to have a higher abundance of fatty acids that are 16 to 18 carbons in length (Thompson, 1996). Commercially, microalgae have received attention due to their ability to synthesize long chain poly-unsaturated fatty acids, which are essential for human brain and fetal development (Khozin-Goldberg et al., 2011).

Fatty acids can be found freely floating in the cytoplasm or bound in acylglycerols when two or three fatty acid 'tails' are attached to a central backbone through ester bonds (Figure 1.1d). Triacylglycerols (TAGs) are the most common neutral lipid synthesized in microalgae and can account for up to 60% of total dry weight during adverse conditions in some species (Dong et al., 2016). TAGs are the most efficient energy storage molecule and yield twice the energy per unit of mass than starch due to their reduced and anhydrous chemical structure (Berg et al., 2002). During unfavorable conditions, oleaginous microalgae will send stress signals that shut down membrane synthesis and re-direct metabolic energy to the accumulation of TAGs for energy reservoirs. The most prevalent environmental stresses microalgae experience in their natural environment are extreme salinity, pH, or temperature, intense light fluctuations and

nutrient limitations (Goold et al., 2015; Solovchenko, 2012). The most common stressor utilized in laboratory settings is nitrogen deprivation (Solovchenko, 2012). As a result of these stresses, microalgae will inhibit cellular division, increase expression of enzymes involved in the fatty acid cycle and begin degrading thylakoid membranes, in order to convert the polar membrane lipids into TAGs (Zienkiewicz et al., 2016). Only a few transcriptional regulations of TAG accumulation in microalgae have been identified, the most characterized being transcription factor NRR1 (nitrogen responsive regulator 1) which was up-regulated in *Chlamydomonas reinhardtii* during nitrogen deprivation (Boyle et al., 2012).

Characterization of TAG biosynthesis pathways in microalgae has proven to be complex partially due to the limited number of genomes partially annotated and the sheer diversity of those genomes, especially in comparison to the model plant genome of *Arabidopsis thaliana* (Zienkiewicz et al., 2016). Currently, two pathways of TAG synthesis have been suggested in microalgae, the Kennedy (glycerol phosphate) pathway and the monoacylglycerol pathway (Zienkiewicz et al., 2016). These pathways are believed to be localized in the ER and chloroplast, respectively. Although research into the genetic characterization of TAG accumulation in microalgae is ongoing, it is accepted that the metabolic shift in oleaginous microalgae during adverse conditions lead to the synthesis of organelles named lipid droplets (LD), that are the location of TAG storage.

The current model for LD formation in microalgae proposes the biogenesis of the organelle occurs in the endoplasmic reticulum and is supported by two facts; the enzymes required for TAG synthesis are located in the ER and LDs are usually surrounded by the ER (Farese and Walther, 2009) (Figure 2.2a).

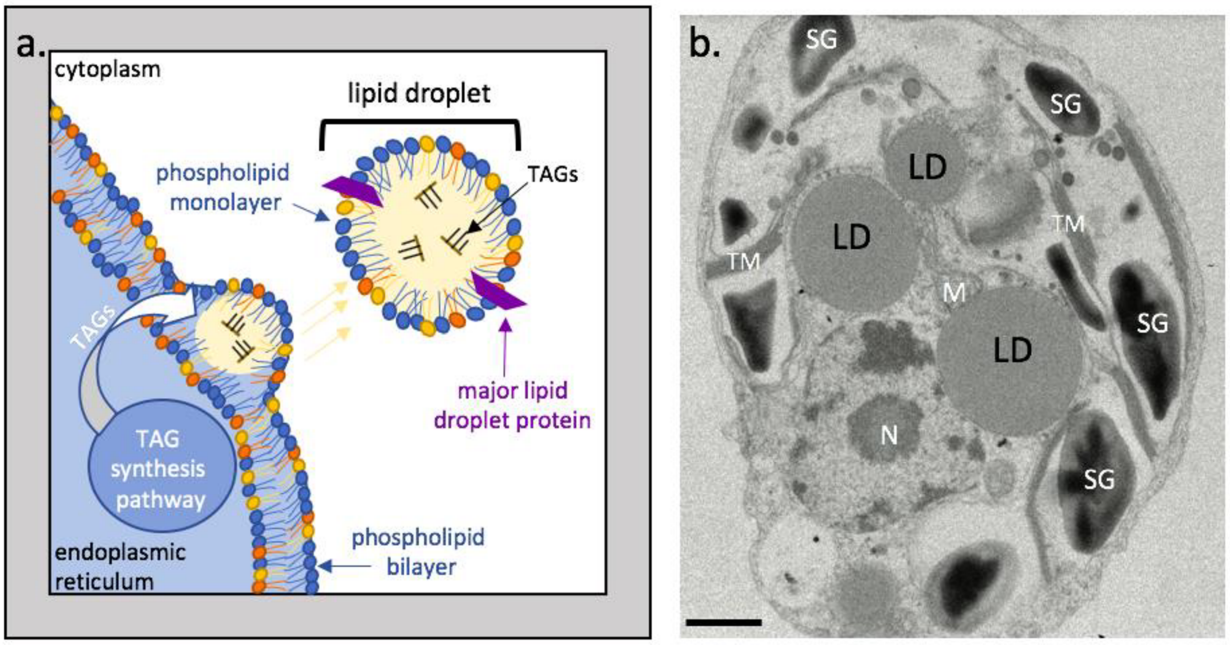


Figure 1.2. Depiction of lipid droplet biosynthesis and transmission election microscopy image of lipid droplets in green microalgae. (a.) Lipid droplet biosynthesis in microalgae is believed to begin in the endoplasmic reticulum (ER), where the triacylglycerol (TAG) synthesis pathway enzymes are found. Accumulation of TAGs will begin in-between the phospholipid bilayer in the ER, budding from the ER will occur once sufficient TAG and create a cytoplasmic lipid droplet (LD). A LD is sphere made from a monophospholipid layer shell, with major lipid droplet protein, and filled with TAGs. (b.) Lipid droplets imaged in green algae *Dunaliella tertiolecta* through transmission electron microscopy image. *LD* = lipid droplet, *N* = nucleus, *M* = mitochondria, *SG* = starch granule, *TM* = thylakoid membrane. Scale bar is 1 µm.

LDs, also referred to as oil bodies or lipid bodies, are spherical organelles that constitute a core of TAGs that is contained in a monolayer of polar lipids (Figure 1.2). Two proteins have been identified in microalgae LDs; major lipid droplet protein and lipid droplet surface protein (LDSP) (Davidi et al., 2014; Vieler et al., 2012). These proteins regulate LD size, stability and recruitment of additional proteins to the lipid droplet (Moellering and Benning, 2010). A single LD can range from nms to 10 µm in diameter depending on species and environmental conditions (Lin et al., 2012) (Figure 1.2b). It is recognized that LDs are used as a transient storage of carbon for microalgae and are produced during lipid accumulation as the site of TAG storage (Goold et al., 2015).

The role of TAG accumulation in microalgae is three-fold. Firstly, TAGs are the building blocks for photosynthetic membranes and, as such, storing a pool of TAG during stressful times allows for the quick synthesis of chloroplasts after the re-introduction of microalgae into amicable conditions. Secondly, the formation lipid bodies for TAG and secondary carotenoid storage has been demonstrated to protect cells against photodamage. Lastly, synthesizing TAG absorbs the excessive reactive oxygen species produced during photosynthesis and inhibits photo-oxidative injuries especially during periods when little to no photosynthetic energy is required for cell division (Solovchenko, 2012).

To meet the increasing need of alternative energy, researchers began to study microalgal TAG accumulation and lipid droplet synthesis in order to cultivate microalgae for the production of renewable energies called biofuels (Merchant et al., 2012).

1.2 Biofuels

Algae have a long history in the field of biotechnology with records beginning in the 1600s when *Porphya* was cultivated for food additives and agar production (Pulz and Gross, 2004). In recent years, this group has been exploited by cosmetic, pharmaceutical and medical industries because of their rich content of polyunsaturated fatty acids, colorful pigments, and antioxidants (Cheregi et al., 2019; Hopfner et al., 2014; Pulz and Gross, 2004; Spolaore et al., 2006). Microalgae have also been given considerable attention due to their potential as a renewable energy source.

Due to the increasing human population and standard of living, there is an unremitting demand for energy. Modern energy requirements are leading to a continuous depletion of finite fossil fuel reserves and this depletion is expected to substantially quicken over time (Mejriszan Toosi et al., 2018; Ok and Tang, 2012). To ensure future energy sustainability, investment and research must be funneled into green alternatives.

Biofuels are an environmentally friendly alternative to fossil fuels that approach carbon neutrality and are renewable (Garlapati et al., 2019; Lee, 2011; Raheem et al., 2018). Biogas, like biomethane or biohydrogen, can be produced through anaerobic microbial digestion or pyrolysis of agricultural and food waste (Asomaning et al., 2016; Weiland, 2010). The two main types of biofuel are bioethanol and biodiesel. Bioethanol is a sugar derived alcohol that is usually produced by the fermentation of plant biomass (Dave et al., 2019; Gray et al., 2006).

Bioethanol is blended into traditional gasoline to reduce polluting emissions by improving octane level (Balat et al., 2008; Fazal et al., 2018). Biodiesel is derived from natural oils through a process of transesterification which separates the hydrocarbons alkyl esters from glyceride backbone (Hoekman et al., 2012). This type of biofuel is non-toxic, biodegradable and a direct alternative to tradition fuel (Nicolò et al., 2017; Singh et al., 2011). The feedstock oil used for biodiesel can be extracted from terrestrial plant crops like soybean or palm, but also from oleaginous microalgae (Colling Klein et al., 2018; Hoekman et al., 2012). In order to understand the role microalgal lipids take in the current state of the industry, we must first acknowledge the development of the biofuel industries.

1.2.1 Timeline of biofuel development

The history of biofuels is commonly told in generations in order to follow the development, limitations and advantages of the field. The 1st generation of biofuels used common food crops, such as corn and sugar cane, for the production of bioethanol (Deneyer et al., 2018; Naik et al., 2010). The rise of this generation was paralleled by the “food versus fuel” debate, which condemned the biofuel industry for diverting resources such as arable land and fresh water from the food market to the energy market (Havlík et al., 2011). These limitations prompted the 2nd generation of biofuels, which extracted lignocellulosic biomass from non-food waste products such as soft wood lumber waste for bioethanol production (Robak and Balcerek, 2018). Lignocellulosic biomass can be converted into bioethanol and biofuel through a series of enzymatic treatment, pyrolysis or microbial bioconversion (Jönsson et al., 2013; Raud et al., 2019). The goal of both processes is to break the lignocellulosic biomass into individual parts of cellulose, hemicellulose and lignin (Jönsson et al., 2013). Once the monosaccharide sugars are separate from the lignin, they can be fermented and further used to produce bioethanol (Kumar et al., 2016).

Advance biofuels rose from the limitations of the 1st and 2nd generation’s terrestrial plant-based system, including massive land area requirements and small lipid yield. Advance biofuels focused on the potential of microalgae for the high-yield production of biodiesel (Nicolò et al., 2017; Singh et al., 2011). Microalgae have received attention from the biotechnology industry because of the physiochemical similarities between the structure of their fatty acids and traditional fossil fuels hydrocarbon in regards to carbon chain length and saturation (Hoekman et al., 2012; Sajjadi et al., 2018). Although the composition of fatty acids is species-specific,

microalgae generally have an increased amount of saturated and mono-unsaturated fatty acids versus land plants. Saturated and monounsaturated fatty acids are favored in biofuel production because they are less likely to be oxidized and, in turn, have higher stability which is a trait that is preferred in fuel sources (Arguelles et al., 2018; Puhan et al., 2010) Microalgae are suitable candidates for biofuel production due to their quick growth rates, high intracellular lipid content, and the potential to modify their metabolism through genetic engineering. They are also cultivable in salt water and therefore independent of arable land (Brennan and Owende, 2010; Chisti, 2007; Mofijur et al., 2019).

As previously described, TAG accumulation is stimulated in oleaginous microalgae as a response to various stresses. As such, researchers commonly induce TAG accumulation in laboratory setting through nitrogen starvation and the current strategies for doing this this reviewed by Singh et al, 2015. Unfortunately, the vast majority of techniques for inducing lipid accumulation detrimentally affect algal biomass and growth rate making the total production more energy-intensive relative to total overall energy yield. The low net yield impedes the ability to commercialize these systems, which dictates the industry's financial sustainability and, as such, environmental impact (Milano et al., 2016).

1.2.2 Current trends in microalgal biofuels

Production of advance generations biodiesel can be simplified into three steps: (1) cultivating the microalga strain to optimal lipid content and biomass, (2) harvesting the biomass from media, and (3) extracting the lipid content from the organisms before processing the lipids through trans-esterification to produce commercial biodiesel.

Cultivation is an energy-consuming step of biofuel production, and a current question in the advance generations biofuel industry is the ideal cultivation method for commercial production of microalgae. During cultivation, factors such as temperature, nutrient availability and light intensity have been shown to impact TAG yield. In a lab-based bioreactor, these parameters can be easily monitored and modified in order to maximize lipid yield. This level of control in laboratory-based systems, though essential for initial research, is energy-intensive and difficult to achieve in commercial-scale bioreactors (Khan et al., 2018).

Some parts of industry are moving towards outdoor open-pond cultivation systems with the intention to use natural light and heat to culture algae. Open-pond systems begin to

approach economic stability that is marketable against fossil fuels due to their low operation costs and requirement for energy (Costa et al., 2019; Jorquera et al., 2010). Nevertheless, these systems suffer from unpredictable weather, salinity imbalances, and contamination that negatively affect microalgal productivity (Zhu, 2015). It is suspected that these bioreactors will have to be polymicrobial in order to alleviate energy spent maintaining axenic commercial cultures at such a large volume (Naghavi and Sameipour, 2019). Researchers have also advanced the idea of offshore cultivation systems, which minimize the land and water requirements for cultivation (Novoveská et al., 2016).

Another developing subject in this biotechnological field is genetically modifying (GM) microalgae to optimize lipid productivity. Genes that control lipid metabolism, carbohydrate metabolism, stress tolerance and auto-flocculation have been of primary focus to benefit biofuel production (Shin et al., 2016; Trentacoste et al., 2013; Xue et al., 2015). Currently, the most common traits being modified in microalgae now are those involved in reactive oxygen species defence, inhibiting contamination, and increasing robustness, that allow microalgae to tolerate and maintain high biomass concentration (Gressel et al., 2014). The main concern of GM microalgae is the risk to public health and natural ecosystem when, inevitably, these organisms escape. Modified traits could give GM microalgae increased fitness over wildtype or native microalgae, therefore researchers suggest deleting genes needed to survive in nature, but not required for commercial cultivation in GM microalgae in order to prevent reproduction in natural ecosystems (Gressel et al., 2014). Released microalgae also have the potential to displace local microalgae and develop into harmful algal blooms that could produce toxins which disrupt the native ecosystem and cause contamination (Henley et al., 2013). Microalgae are the bottom of the aquatic food chain and the effect that GM microalgae would have on higher trophic levels has not be sufficiently studied (Henley et al., 2013). It is known that organisms in higher trophic levels rely on poly-unsaturated fatty acids for key stages of development and exposure to microalgae with altered lipid content, in particular increased saturated fatty acids, might have negative health impacts (Pettersen et al., 2010). There is also a possibility of implemented genetic elements spreading into the wild-type population that could disrupt local ecosystem.

Assessing the promise and the risk of GM microalgae will have to be specific to the microalgal species and their modified genes and traits (Beacham et al., 2017). Environmental risk assessments for GM microalgae will have to analyze the GM microalgal fitness as well as modes of competition between released microalgae and the native population, the stability of local community during disruptions and the potential for gene flow between populations

(Beacham et al., 2017). These assessments should be a priority in order to inform government on regulating GM microalgae and minimize the risks on human health and local environment associated with their commercialization.

The ideal microalgae for biofuel production would be grown outside with minimal nutrients, providing high lipid productivity and have production of other high-value molecules that would offset the price of the biofuel production pipeline. This microalga would be successfully cultivatable to high biomass and lipid concentration at low costs, with a simple biomass harvesting method, or used wet biomass, that optimizes net energy yield. In order to overcome this energy bottleneck within the biofuel industry, researchers have been examining microalgae in their natural habitat in which there is constant association with other microorganisms (Ramanan et al., 2016).

1.3 Algal-bacterial interactions

In nature, the presence and composition of microalgal communities are driven by resource availability and intra- and inter-species interactions (Amin et al., 2012). Microalgae constantly interact with marine bacteria within the phycosphere, which is the nutrient-rich microscale region surrounding the algal cell (Bell and Mitchell, 1972; Seymour et al., 2017). Marine bacteria, like the abundant roseobacter clade, are a major participant in biogeochemical cycles and preferentially co-occur with specific groups of microalgae (Buchan et al., 2005; Lima-Mendez et al., 2015; Martin-Platero et al., 2018; Wagner-Döbler and Biebl, 2006). Bacteria and algae exchange bioactives, such as indol-3-acetic acid (IAA), dimethulsulphoniopropionate (DMSP) and roseobacticides, which can affect the physiology of both partners (Amin et al., 2015; Bramucci and Case, 2019; Curson et al., 2011; Labeeuw et al., 2016; Seyedsayamdost et al., 2011). Most of the bioactives that mediate algal-bacterial interactions remain uncharacterized due to their small size, structural novelty and relatively low concentrations in the environment. We are only beginning to understand the association between these groups and their impact in structuring and supporting aquatic habitats.

In the ocean, there are many advantages for microorganisms to associate with each other. Bacteria consume dissolved oxygen and organic carbon produced by photosynthetic microalgae and in exchange provide accessible nitrogen, phosphorus and inorganic nutrients to their partners (Meersche et al., 2004; Mouget et al., 2006). Bacteria can produce antibiotics that

protect the algal host from unwanted bacterial colonization, ensuring that the mutualistic relationship remains exclusive (Rao et al., 2007). The coastal diatom *Pseudo-nitzschia multiseries* produces tryptophan for bacterial symbionts to use in the production of indole-3-acetic acid, a plant auxin that promotes growth in some microalgal species (Amin et al., 2015). In some cases, interactions are necessary for the survival of one or both members. Bacteria synthesize vitamin B12, which is essential for microalgal growth but many microalgae species are unable to independently synthesize it (Croft et al., 2005; Grant et al., 2014; Helliwell et al., 2014). Some bacteria can increase the binding affinity of algal siderophores to iron (Amin et al., 2009). Continued exploration of these mutualistic interactions will likely uncover novel exchanges and symbiotic relationships that could relieve nutrients addition required for biofuel production.

Antagonistic interactions between bacteria and algae can be just as advantageous for bacteria, as killing their algal host provides them with the host's nutrients. Algicides are molecular compounds secreted by bacteria in order to kill algal cells through the production of reactive oxygen species, hindering photosynthesis and causing degradation of the algal cell wall (Demuez et al., 2015; Jia et al., 2014; Mayali and Azam, 2004; Seyedsayamdost et al., 2011). These antagonistic interactions are species-specific and most of their underlying mechanisms remain unclear. Many of these algicidal compounds are released in response to chemical signals that indicate aging of algal host (Seyedsayamdost et al., 2011). Further research and careful exploitation of these antagonistic interactions between algae and bacteria could provide the biotechnology industry with a low-energy method to release lipids from algae biomass.

1.4 Role of algal-bacterial interactions in biofuels

Traditionally, bacteria in algal cultures have been considered a contamination, which affects nutrient availability and microalgal growth negatively (Liu et al., 2012; Scott et al., 2010). With emerging research on the potential of algal-bacterial interactions, the beneficial side of this partnership has been acknowledged (Ramanan et al., 2016; Wang et al., 2016). Bacterial and algal cocultures have augmented the commercial production of bio-products and relieved energy-intensive steps during harvesting and extraction of those products (Kouzuma and Watanabe, 2015). All stages in the biofuel pipeline can be enhanced by utilizing a beneficial bacteria consortium: improving algal growth, algal harvesting and lipid extraction from algal biomass (Figure 1.3).

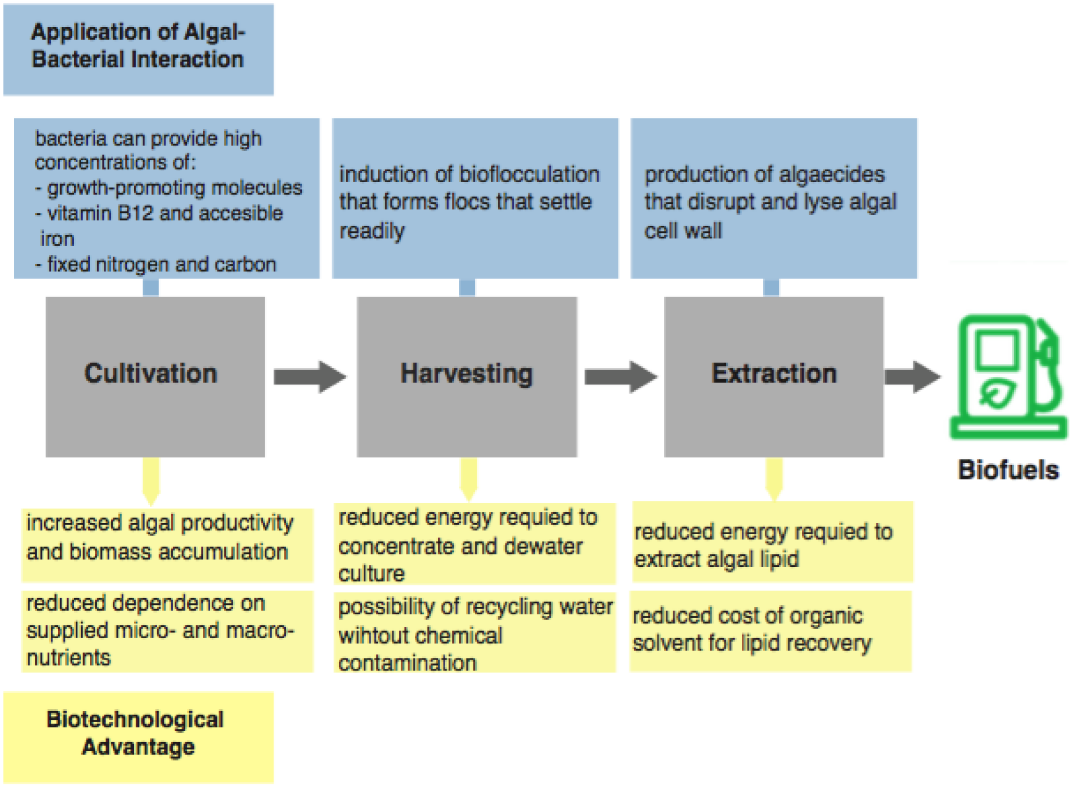


Figure 1.3. Summary of current research on the algal-bacterial interaction in biofuel production. Application of algal-bacterial interaction and their associated biotechnological advantage within the biofuel pipeline.

1.4.1 Cultivation

Bacteria play a crucial role in the accumulation of microalgae biomass by providing fixed nutrients, growth promoters and vitamins essential for algal health and productivity (Cho et al., 2017). Bacteria interact with the microalgae within the alga's phycosphere through the diffusions of bioactives and nutrients or via direct colonization on alga host (Case et al., 2011). The immediate proximity of interactions ensure that micro- and macro-nutrients are delivered to the alga in high concentration which, in turn, means a reduction of supplemented nutrients that could minimize cost of commercial cultivation (Cooper and Smith, 2015). For example,

exploiting a bacterium's ability to supply vitamin B12 eliminates the need to provide vitamin B12 in the growth media, reducing production costs (Croft et al., 2005). Bacteria can also stimulate growth in microalgae through growth-promoting compounds such as indole-3-acetic acid (Amin et al., 2015). A recent study demonstrated that polymicrobial cultures foster constant biomass productivity and yield over time (Cho et al., 2017). This suggests that communities of microorganisms follow the diversity-productivity paradigm; that increased diversity leads to increased stability of the microbial community (Beyter et al., 2016). Harnessing these natural interactions to provide nutrients to and promote the growth of biotechnologically valuable microalgae could further the industry by reducing energy requirements and cost of production.

1.4.2 Harvesting

Harvesting the microalgae biomass from the liquid medium accounts for the majority of the total energy required for biofuel production (Gallagher, 2011). Techniques such as centrifugation, filtration, and flocculation are currently being used. However, they are energy-intensive and must be further optimized for the biofuel industry to become sustainable (Barros et al., 2015). Currently, there are no commercialized examples of bacterial aided harvesting available. But, bacteria are able to promote aggregation of some microalgae, which concentrates the culture through settling to the bottom of reactor and therefore improves the efficiency of algal biomass harvesting (Wang et al, 2012). This aggregation is usually due to ionic interactions between positively charged functional groups on the surface of algal cells and negatively charged teichoic acids in gram-positive bacteria (Powell and Hill, 2013). In a pilot scale study, researchers also demonstrated the addition of bacteria that produce extracellular polysaccharide substances (EPS) stimulated 90% increase of total biomass to bio-flocculate (Ndikubwimana et al., 2016). EPS produced by the bacteria in this coculture encouraged cell-to-cell contact that promoted algal aggregation followed by sedimentation or flotation (Lee et al., 2013). Bioflocculation is an economically efficient way to harvest microalgal biomass through the direct, charge-dependent interactions between bacteria and microalgae.

1.4.3 Extraction

After the concentration of microalgal biomass, there must be a disruption of the microalgal cell walls in order to release the intracellular lipids. Once the lipids are extracted, they are separated from other cellular biomass and collected for further processing. Methods for the disruption of cell walls usually fall under three categories; mechanical, chemical or enzymatic. Mechanical extraction approaches include bead beating, ultrasonic-assisted extraction, electroporation and expeller press that physical break open the cells in order to release the intracellular lipids (Ranjith Kumar et al., 2015). These methods are advantageous as they do not contaminate the lipids with solvents or enzymes and do not depend on type of microalgal species (Ranjith Kumar et al., 2015). Chemical extractions utilize specific ratios of organic solvents, like methanol and chloroform, in order to collect lipids based on their differences in polarity from other cellular biomass (Mubarak et al., 2015; Ranjan et al., 2010). Although effective, the use of toxic organic solvents like chloroform on a large scale is unfavorable because of cost and potential health risks (Ranjith Kumar et al., 2015). The use of enzymes that degrade the cell walls, such as cellulase or lysozyme, have also been evaluated for lipid extraction (Taher et al., 2014). Although the enzymatic method use less energy than mechanical methods, they can be non-selective and degrade the other high-value products being harvested from the cell (Mubarak et al., 2015).

An alternative to these methods of extraction is the use of bacterial algaecides to stimulate microalgae autolysis. The majority of algaecide research has focused on bacteria's role in mediating harmful algal blooms (Kim et al., 2008; Masó and Garcés, 2006; Mayali and Azam, 2004; Su et al., 2007). These pathogenic interactions are species-specific and induce morphological changes that effectively degrade the alga cell wall, releasing the intracellular material that can be a nutrient source for the bacteria (Case et al., 2011; Seyedsayamdost et al., 2011). Bacterial algaecides could improve the extraction of lipids from algal cultures, reducing the energy expended during this stage of the process, but further research into the specificity and mode of action of these algaecides is required before they can be effectively implemented into the biofuel production pipeline (Demuez et al., 2015). An additional possibility for promoting lipid release in microalgae is bacterial-stimulated apoptosis (Bramucci and Case, 2019). Optimizing bacterial-mediated extraction requires balancing the culture density and algaecide concentration in order to optimize lipid yield, which may in turn be affected by the sensitivity of specific microalgae to specific algaecides.

1.4.4 Bacterial modification of algal lipids

A largely unrecognized aspect of biofuel production that could benefit from interactions between algae and bacteria is the potential of the latter to influence structure of accumulated algal lipids. One study determined that the bacteria *Phaeobacter inhibens* cause a 2.5-fold decrease in the alkenone containing lipid bodies in *Emiliania huxleyi* during co-cultivation (Segev et al., 2016). This was a significant finding for paleoceanography, as the ratio of long chain polyunsaturated alkenones of this microalga have been a key tool in determining historic sea surface temperature, but also has implications when discussing developments in microalgal biofuel production (Prahl and Wakeham, 1987). Bacteria could increase the yield of desired algal fatty acids by influencing the microalga's lipid metabolism. This would ultimately benefit the overall production of microalgal product if the interaction was controlled. Research on this topic is in its infancy, and no application within the biotechnology field has been studied.

1.5 *Phaeobacter italicus - Dunaliella tertiolecta* model

Promising algal-bacterial interactions for biofuel production have been identified through phenotypic screening for advantageous characteristics such as increased growth rate, biomass and/or lipid recovery and production (Le Chevanton et al., 2013). During her time in the Case lab, Dr. Leen Labeeuw screened different microalgae and bacteria for such phenotypes and discovered that the green alga *Dunaliella tertiolecta* (Family: Chlorophyceae), had significantly more lipid per biomass when co-cultivated with the marine bacterium *Phaeobacter italicus* strain R11 (Labeeuw, 2016). I have adopted this interaction as the focus of this thesis.

1.5.1 *Dunaliella tertiolecta*

The genus *Dunaliella* has been studied for its halotolerant characteristics that allow some species to persist at extreme salt concentrations (Borowitzka and Siva, 2007). *D. tertiolecta* is a marine, flagellated green alga that was first identified in 1959 by R.W. Butcher (Butcher, 1959). Cells are radially symmetric in shape and vary from 5-18 μm long. This species has a distinct cup-shaped chloroplast, which is filled with colorless starch granules (Figure 1.4).

Figure 1.4. Cellular morphology in *Dunaliella tertiolecta* (CCMP 1320) under light microscopy. Image was taken at 100X magnification and displayed in merged images of bright field and DIC.

No mechanisms of sexual reproduction or sporulation have been described for this species (Berges and Falkowski, 1998; Borowitzka and Siva, 2007). *D. tertiolecta* is used as a model for light-dependent apoptosis in unicellular eukaryotic photo-synthesizers (Segovia et al., 2003).

This microalgal species is a candidate for biofuel production because of its high lipid accumulation, ease of cultivability and lack of a rigid cell wall (Minowa et al., 1995; Tang et al., 2011). This organisms also has high content of beta-carotene, vitamin C and vitamin E, which is promising for the co-production of value-added molecules during cultivation (Abalde et al., 1991). The growth, lipid accumulation and photosynthetic health of *D. tertiolecta* has been broadly characterized under multiple nutrient, metal and growth conditions (López et al., 2015; Song et al., 2016). Additionally, using this specie as research organism benefits from previous descriptions of its metabolic pathways and stress induced carbon allocation (Chen et al., 2011; Rismani-Yazdi et al., 2011; Tan et al., 2016).

1.5.2 *Phaeobacter italicus* R11

Phaeobacter italicus R11 is a marine bacterium belonging to the monophyletic roseobacter clade (Class: Alphaproteobacteria)(Case et al., 2011; Fernandes et al., 2011). It was isolated from *Delisea pulchra* (Rhodophyta) in Botany Bay, New South Wales, Australia (Case et al., 2011). Roseobacters are a strictly marine group, encompass many microbial lifestyles from planktonic to biofilm forming and inhabit a diverse range of marine ecosystems from sea ice to the sea floor (Brinkhov et al., 2008). Organisms in this clade are known for aerobic anoxygenic photosynthesis and role in the biogeochemical cycle of sulfur (Wagner-Döbler and Biebl, 2006). This clade accounts for up to 25% of bacteria population in photic ocean which could explain their multiple descriptions of relationships with micro- and marco-algae (DeLong and Karl, 2005; Selje et al., 2004).

P. italicus has linked genetic traits to locate and approach algal surfaces through chemotaxis, characteristics commonly associated with bacteria that have symbiotic life history strategies (Fernandes et al., 2011). *P. italicus* is able to consume algal exudates and protect itself from reactive oxygen species (Fernandes et al., 2011). Reactive oxygen species are the primary algal defence mechanism against potential pathogens (Brinkhov et al., 2008). Studies have shown that the bacterium releases proteins into the extracellular space and analysis of the protein content, called exoproteomes, have shown that *P. italicus* releases RTX-like proteins and virulence genes (VarR) (Gardiner et al., 2017, 2015). These genes have been determined to control *P. italicus*' colonization of algal hosts and contribute to the bacterium's pathogenicity. *P. italicus* has previously reported temperature-dependent virulence against the red macroalgae *Delisea pulchra* and the bloom-forming haptophyte *Emiliania huxleyi* (Case et al., 2011; Mayers et al., 2016). *P. italicus* is an interesting candidate for phenotypic screening for interaction with *D. tertiolecta* because of its previously established relationships with micro and macroalgae.

1.6 Thesis objectives

In this thesis, I aim to further investigate the relationship between green alga *D. tertiolecta* and marine roseobacter *P. italicus* through a biofuel production lens, focusing on how *P. italicus* induces increases in lipid per biomass of the alga. I performed mono- and co-culture experiments with *D. tertiolecta* and *P. italicus* to monitor lipid accumulation along with photosynthetic health of microalgae in both conditions (Bramucci et al., 2015; Labeeuw, 2016).

Furthermore, I performed fatty acid analysis using comprehensive GCxGC mass spectroscopy on large-scale volumes of algal control and coculture to determine if *P. italicus* modifies the composition of algal fatty acids. Finally, I describe this interaction by the quantification and visualization of algal cells and lipid droplets.

Based on the findings from phenotypic screening of algal-bacterial interactions and current literature describing *D. tertiolecta*'s role in biofuel production, I hypothesize that marine bacterium *P. italicus* increases lipid production and storage in the lipid droplet organelles of candidate biofuel microalgae *D. tertiolecta*.

To answer this hypothesis, five experimental aims were established:

1. Determine if the algal pathogen, *P. italicus*, influences growth, PSII health or lipid accumulation of *D. tertiolecta* through co-cultivation experiments.
2. Determine if lipid accumulation coincides with nitrogen starvation.
3. Determine if *P. italicus* alters the composition of fatty acids in the alga.
4. Determine if *P. italicus* changes the lipid droplet morphology in the alga.
5. Quantify and visualize extracellular lipid vesicle production.

1.7 References

Abalde, J., Fabregas, J., Herrero, C., 1991. fi-Carotene, vitamin C and vitamin E content of the marine microalga *Dunaliella tertiolecta* cultured with different nitrogen sources. Bioresource Technology 38, 121–125.

Alvarez, H., Steinbüchel, A., 2002. Triacylglycerols in prokaryotic microorganisms. Applied Microbiology and Biotechnology 60, 367–376. https://doi.org/10.1007/s00253-002-1135-0

Amin, S., Green, D.H., Hart, M.C., Kupper, F.C., Sunda, W.G., Carrano, C.J., 2009. Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism. Proceedings of the National Academy of Sciences 106, 17071–17076. https://doi.org/10.1073/pnas.0905512106

Amin, S.A., Hmelo, L.R., van Tol, H.M., Durham, B.P., Carlson, L.T., Heal, K.R., Morales, R.L., Berthiaume, C.T., Parker, M.S., Djunaedi, B., Ingalls, A.E., Parsek, M.R., Moran, M.A., Armbrust, E.V., 2015. Interaction and signaling between a cosmopolitan phytoplankton and associated bacteria. Nature 522, 98–101. https://doi.org/10.1038/nature14488

Amin, S.A., Parker, M.S., Armbrust, E.V., 2012. Interactions between diatoms and bacteria. Microbiology and Molecular Biology Reviews 76, 667–684. https://doi.org/10.1128/MMBR.00007-12

Arguelles, E.DLR., Laurena, A.C., Monsalud, R.G., Martinez-Goss, M.R., 2018. Fatty acid profile and fuel-derived physico-chemical properties of biodiesel obtained from an indigenous green microalga, Desmodesmus sp. (I-AU1), as potential source of

renewable lipid and high quality biodiesel. J Appl Phycol 30, 411–419. https://doi.org/10.1007/s10811-017-1264-6
Asomaning, J., Omidghane, M., Chae, M., 2016. Thermal processing of algal biomass for biofuel production. Current Opinion in Green and Sustainable Chemistry 2, 1–5. https://doi.org/10.1016/j.cogsc.2016.08.005
Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H., Mekhilef, S., 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renewable and Sustainable Energy Reviews 16, 2070–2093. https://doi.org/10.1016/j.rser.2012.01.003
Balat, M., Balat, H., Öz, C., 2008. Progress in bioethanol processing. Progress in Energy and Combustion Science 34, 551–573. https://doi.org/10.1016/j.pecs.2007.11.001
Barros, A.I., Gonçalves, A.L., Simões, M., Pires, J.C.M., 2015. Harvesting techniques applied to microalgae: A review. Renewable and Sustainable Energy Reviews 41, 1489–1500. https://doi.org/10.1016/j.rser.2014.09.037
Basova, M.M., 2005. Fatty acid composition of lipids in microalgae. International Journal on Algae 7, 33–57. https://doi.org/10.1615/InterJAlgae.v7.i1.30
Beacham, T.A., Sweet, J.B., Allen, M.J., 2017. Large scale cultivation of genetically modified microalgae: A new era for environmental risk assessment. Algal Research 25, 90–100. https://doi.org/10.1016/j.algal.2017.04.028
Beardall, J., Raven, J.A., 2004. The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia 43, 26–40. https://doi.org/10.2216/i0031-8884-43-1-26.1
Bell, W., Mitchell, R., 1972. Chemotactic and growth responses of marine bacteria to algal extracellular products. The Biological Bulletin 143, 265–277. https://doi.org/10.2307/1540052
Berg, J., Tymoczko, J., Stryer, L., 2002. Triacylglycerols are highly concentrated energy stores, in: Biochemistry. 5th Edition. New York: W H Freeman;, p. Section 22.1.
Berges, J.A., Falkowski, P.G., 1998. Physiological stress and cell death in marine phytoplankton: Induction of proteases in response to nitrogen or light limitation. Limnology and Oceanography 43, 129–135. https://doi.org/10.4319/lo.1998.43.1.0129
Berthold, D.E., Shetty, K.G., Jayachandran, K., Laughinghouse, H.D., Gantar, M., 2019. Enhancing algal biomass and lipid production through bacterial co-culture. Biomass and Bioenergy 122, 280–289. https://doi.org/10.1016/j.biombioe.2019.01.033
Bertozzini, E., Galluzzi, L., Penna, A., Magnani, M., 2011. Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. Journal of Microbiological Methods 87, 17–23. https://doi.org/10.1016/j.mimet.2011.06.018
Beyter, D., Tang, P.-Z., Becker, S., Hoang, T., Bilgin, D., Lim, Y.W., Peterson, T.C., Mayfield, S., Haerizadeh, F., Shurin, J.B., Bafna, V., McBride, R., 2016. Diversity, productivity, and stability of an industrial microbial scosystem. Applied and Environmental Microbiology 82, 2494–2505. https://doi.org/10.1128/AEM.03965-15
Bhattacharya, D., Yoon, H.S., Hackett, J.D., 2004. Photosynthetic eukaryotes unite: endosymbiosis connects the dots. BioEssays 26, 50–60. https://doi.org/10.1002/bies.10376
Biller, S.J., Schubotz, F., Roggensack, S.E., Thompson, A.W., Summons, R.E., Chisholm, S.W., 2014. Bacterial vesicles in marine ecosystems. Science 343, 183–186. https://doi.org/10.1126/science.1243457
Blumberg, L.M., Klee, M.S., 2000. Optimal heating rate in gas chromatography. Journal of Microcolumn Separations 12, 508–514. https://doi.org/10.1002/1520-667X(2000)12:9<508::AID-MCS5>3.0.CO;2-Y

Borowitzka, M.A., Siva, C.J., 2007. The taxonomy of the genus *Dunaliella*(Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. Journal of Applied Phycology 19, 567–590. https://doi.org/10.1007/s10811-007-9171-x

Boyle, N.R., Page, M.D., Liu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S.J., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S.J., Gallaher, S.D., Johnson, S., Benning, C., Pellegrini, M., Grossman, A., Merchant, S.S., 2012. Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*. Journal of Biological Chemistry 287, 15811–15825. https://doi.org/10.1074/jbc.M111.334052

Bramucci, A.R., Case, R.J., 2019. *Phaeobacter inhibens* induces apoptosis-like programmed cell death in calcifying *Emiliania huxleyi*. Scientific Reports 9, 5215. https://doi.org/10.1038/s41598-018-36847-6

Bramucci, A.R., Labeeuw, L., Mayers, T.J., Saby, J.A., Case, R.J., 2015. A small volume bioassay to assess bacterial/phytoplankton co-culture using WATER-Pulse-Amplitude-Modulated (WATER-PAM) Fluorometry. Journal of Visual Experiments. https://doi.org/10.3791/52455

Bramucci, A.R., Labeeuw, L., Orata, F.D., Ryan, E.M., Malmstrom, R.R., Case, R.J., 2018. The bacterial symbiont *Phaeobacter inhibens* shapes the life history of its algal host *Emiliania huxleyi*. Frontiers in Marine Science 5. https://doi.org/10.3389/fmars.2018.00188

Brennan, L., Owende, P., 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14, 557–577. https://doi.org/10.1016/j.rser.2009.10.009

Brinkhov, T., Giebel, H.-A., Simon, M., 2008. Diversity, ecology, and genomics of the Roseobacter clade: a short overview. Archives of Microbiology 189, 531–539. https://doi.org/10.1007/s00203-008-0353-y

Buchan, A., Gonzalez, J.M., Moran, M.A., 2005. Overview of the marine Roseobacter lineage. Applied and Environmental Microbiology 71, 5665–5677. https://doi.org/10.1128/AEM.71.10.5665-5677.2005

Butcher, R.W., 1959. An introductory account of the smaller algae of British coastal waters. Part I: Introduction and Chlorophyceae, Fishery investigations.

Case, R.J., Longford, S.R., Campbell, A.H., Low, A., Tujula, N., Steinberg, P.D., Kjelleberg, S., 2011. Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. Environmental Microbiology 13, 529–537. https://doi.org/10.1111/j.1462-2920.2010.02356.x

Cavonius, L.R., Carlsson, N.-G., Undeland, I., 2014. Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. Analytical and Bioanalytical Chemistry 406, 7313–7322. https://doi.org/10.1007/s00216-014-8155-3

Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology 102, 1649–1655. https://doi.org/10.1016/j.biortech.2010.09.062

Cheregi, O., Ekendahl, S., Engelbrektsson, J., Strömberg, N., Godhe, A., Spetea, C., 2019. Microalgae biotechnology in Nordic countries – the potential of local strains. Physiologia Plantarum 166, 438–450. https://doi.org/10.1111/ppl.12951

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnology Advances 25, 294–306. https://doi.org/10.1016/j.biotechadv.2007.02.001

Cho, D.-H., Ramanan, R., Heo, J., Lee, J., Kim, B.-H., Oh, H.-M., Kim, H.-S., 2015. Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. Bioresource Technology 175, 578–585. https://doi.org/10.1016/j.biortech.2014.10.159

Cho, H.U., Kim, Y.M., Park, J.M., 2017. Enhanced microalgal biomass and lipid production from a consortium of indigenous microalgae and bacteria present in municipal wastewater

under gradually mixotrophic culture conditions. Bioresource Technology 228, 290–297. https://doi.org/10.1016/j.biortech.2016.12.094
Colling Klein, B., Bonomi, A., Maciel Filho, R., 2018. Integration of microalgae production with industrial biofuel facilities: A critical review. Renewable and Sustainable Energy Reviews 82, 1376–1392. https://doi.org/10.1016/j.rser.2017.04.063
Cooper, M.B., Smith, A.G., 2015. Exploring mutualistic interactions between microalgae and bacteria in the omics age. Current Opinion in Plant Biology 26, 147–153. https://doi.org/10.1016/j.pbi.2015.07.003
Costa, J.A.V., Freitas, B.C.B., Santos, T.D., Mitchell, B.G., Morais, M.G., 2019. Open pond systems for microalgal culture, in: Biofuels from Algae. Elsevier, pp. 199–223. https://doi.org/10.1016/B978-0-444-64192-2.00009-3
Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93. https://doi.org/10.1038/nature04056
Curson, A.R.J., Todd, J.D., Sullivan, M.J., Johnston, A.W.B., 2011. Catabolism of dimethylsulphoniopropionate: microorganisms, enzymes and genes. Nature Reviews Microbiology 9, 849–859. https://doi.org/10.1038/nrmicro2653
Danielewicz, M.A., Anderson, L.A., Franz, A.K., 2011. Triacylglycerol profiling of marine microalgae by mass spectrometry. Journal of Lipid Research 52, 2101–2108. https://doi.org/10.1194/jlr.D018408
Dave, N., Selvaraj, R., Varadavenkatesan, T., Vinayagam, R., 2019. A critical review on production of bioethanol from macroalgal biomass. Algal Research 42, 101606. https://doi.org/10.1016/j.algal.2019.101606
Davidi, L., Shimoni, E., Khozin-Goldberg, I., Zamir, A., Pick, U., 2014. Origin of carotene-rich plastoglobuli in *Dunaliella bardawil*. Plant Physiology 164, 2139–2156. https://doi.org/10.1104/pp.113.235119
de-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillos, J.J., 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Canadian Journal of Microbiology 48, 514–521. https://doi.org/10.1139/w02-051
DeLong, E.F., Karl, D.M., 2005. Genomic perspectives in microbial oceanography. Nature 437, 336–342. https://doi.org/10.1038/nature04157
Demuez, M., González-Fernández, C., Ballesteros, M., 2015. Algicidal microorganisms and secreted algicides: New tools to induce microalgal cell disruption. Biotechnology Advances 33, 1615–1625. https://doi.org/10.1016/j.biotechadv.2015.08.003
Deneyer, A., Ennaert, T., Sels, B.F., 2018. Straightforward sustainability assessment of sugar-derived molecules from first-generation biomass. Current Opinion in Green and Sustainable Chemistry 10, 11–20. https://doi.org/10.1016/j.cogsc.2018.02.003
Do Nascimento, M., Dublan, M. de los A., Ortiz-Marquez, J.C.F., Curatti, L., 2013. High lipid productivity of an Ankistrodesmus – Rhizobium artificial consortium. Bioresource Technology 146, 400–407. https://doi.org/10.1016/j.biortech.2013.07.085
Dong, T., Knoshaug, E.P., Pienkos, P.T., Laurens, L.M.L., 2016. Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. Applied Energy 177, 879–895. https://doi.org/10.1016/j.apenergy.2016.06.002
Durham, B.P., Sharma, S., Luo, H., Smith, C.B., Amin, S.A., Bender, S.J., Dearth, S.P., Van Mooy, B.A.S., Campagna, S.R., Kujawinski, E.B., Armbrust, E.V., Moran, M.A., 2015. Cryptic carbon and sulfur cycling between surface ocean plankton. Proceedings of the National Academy of Sciences 112, 453–457. https://doi.org/10.1073/pnas.1413137112

Eberhard, S., Finazzi, G., Wollman, F.-A., 2008. The dynamics of photosynthesis. Annual Review of Genetics 42, 463–515. https://doi.org/10.1146/annurev.genet.42.110807.091452
Falkowski, P.G., 1998. Biogeochemical controls and feedbacks on ocean primary production. Science 281, 200–206. https://doi.org/10.1126/science.281.5374.200
Falkowski, P.G., 1994. The role of phytoplankton photosynthesis in global biogeochemical cycles. Photosynthesis Research 39, 235–258.
Farese, R.V., Walther, T.C., 2009. Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 139, 855–860. https://doi.org/10.1016/j.cell.2009.11.005
Fazal, M.A., Suhaila, N.R., Haseeb, A.S.M.A., Rubaiee, S., Al-Zahrani, A., 2018. Influence of copper on the instability and corrosiveness of palm biodiesel and its blends: An assessment on biodiesel sustainability. Journal of Cleaner Production 171, 1407–1414. https://doi.org/10.1016/j.jclepro.2017.10.144
Fernandes, N., Case, R.J., Longford, S.R., Seyedsayamdost, M.R., Steinberg, P.D., Kjelleberg, S., Thomas, T., 2011. Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*. PLoS ONE 6, e27387. https://doi.org/10.1371/journal.pone.0027387
Field, C.B., 1998. Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281, 237–240. https://doi.org/10.1126/science.281.5374.237
Franz, A.K., Danielewicz, M.A., Wong, D.M., Anderson, L.A., Boothe, J.R., 2013. Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity. ACS Chemical Biology 8, 1053–1062. https://doi.org/10.1021/cb300573r
Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., González-Del-Valle, M., Vílchez, C., 2016. Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. Marine Drugs 14, 100–100. https://doi.org/10.3390/md14050100
Gallagher, B.J., 2011. The economics of producing biodiesel from algae. Renewable Energy 36, 158–162. https://doi.org/10.1016/j.renene.2010.06.016
Gardiner, M., Bournazos, A.M., Maturana-Martinez, C., Zhong, L., Egan, S., 2017. Exoproteome analysis of the seaweed pathogen *Nautella italica* R11 reveals temperature-dependent regulation of RTX-like proteins. Frontiers in Microbiology 8, 1203–1203. https://doi.org/10.3389/fmicb.2017.01203
Gardiner, M., Fernandes, N.D., Nowakowski, D., Raftery, M., Kjelleberg, S., Zhong, L., Thomas, T., Egan, S., 2015. VarR controls colonization and virulence in the marine macroalgal pathogen *Nautella italica* R11. Frontiers in Microbiology 6, 1130. https://doi.org/10.3389/fmicb.2015.01130
Garlapati, V.K., Tewari, S., Ganguly, R., 2019. Life cycle assessment of first-, second-generation, and microalgae biofuels, in: Advances in Feedstock Conversion Technologies for Alternative Fuels and Bioproducts. Elsevier, pp. 355–371. https://doi.org/10.1016/B978-0-12-817937-6.00019-9
Geng, H., Belas, R., 2010. Molecular mechanisms underlying roseobacter–phytoplankton symbioses. Current Opinion in Biotechnology 21, 332–338. https://doi.org/10.1016/j.copbio.2010.03.013
Gill, S., Catchpole, R., Forterre, P., 2019. Extracellular membrane vesicles in the three domains of life and beyond. FEMS Microbiology Reviews 43, 273–303. https://doi.org/10.1093/femsre/fuy042
Gong, M., Bassi, A., 2016. Carotenoids from microalgae: A review of recent developments. Biotechnology Advances 34, 1396–1412. https://doi.org/10.1016/j.biotechadv.2016.10.005
Goold, H., Beisson, F., Peltier, G., Li-Beisson, Y., 2015. Microalgal lipid droplets: composition, diversity, biogenesis and functions. Plant Cell Reports 34, 545–555. https://doi.org/10.1007/s00299-014-1711-7

Gould, S.B., Waller, R.F., Mcfadden, G.I., 2008. Plastid evolution. Annual Review of Plant Biology 59, 491–517. https://doi.org/10.1146/annurev.arplant.59.032607.092915
Grant, M.A., Kazamia, E., Cicuta, P., Smith, A.G., 2014. Direct exchange of vitamin B12 is demonstrated by modelling the growth dynamics of algal–bacterial cocultures. ISME Journal 8, 1418–1427. https://doi.org/10.1038/ismej.2014.9
Gray, K.A., Zhao, L., Emptage, M., 2006. Bioethanol. Current Opinion in Chemical Biology 10, 141–146. https://doi.org/10.1016/j.cbpa.2006.02.035
Greenspan, P., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. The Journal of Cell Biology 100, 965–973. https://doi.org/10.1083/jcb.100.3.965
Gressel, J., van der Vlugt, C.J.B., Bergmans, H.E.N., 2014. Cultivated microalgae spills: hard to predict/easier to mitigate risks. Trends in Biotechnology 32, 65–69. https://doi.org/10.1016/j.tibtech.2013.11.003
Harwood, J.L., Guschina, I.A., 2009. The versatility of algae and their lipid metabolism. Biochimie 91, 679–684. https://doi.org/10.1016/J.BIOCHI.2008.11.004
Havlík, P., Schneider, U.A., Schmid, E., Böttcher, H., Fritz, S., Skalský, R., Aoki, K., Cara, S.D., Kindermann, G., Kraxner, F., Leduc, S., McCallum, I., Mosnier, A., Sauer, T., Obersteiner, M., 2011. Global land-use implications of first and second generation biofuel targets. Energy Policy 39, 5690–5702. https://doi.org/10.1016/j.enpol.2010.03.030
Helliwell, K.E., Scaife, M.A., Sasso, S., Araujo, A.P.U., Purton, S., Smith, A.G., 2014. Unraveling vitamin B12-responsive gene regulation in algae. Plant Physiology 165, 388–97. https://doi.org/10.1104/pp.113.234369
Henley, W.J., Litaker, R.W., Novoveská, L., Duke, C.S., Quemada, H.D., Sayre, R.T., 2013. Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. Algal Research 2, 66–77. https://doi.org/10.1016/j.algal.2012.11.001
Hirose, M., Mukaida, F., Okada, S., Noguchi, T., 2013. Active hydrocarbon biosynthesis and accumulation in a green alga, *Botryococcus braunii* (Race A). Eukaryotic Cell 12, 1132–1141. https://doi.org/10.1128/EC.00088-13
Hoekman, S.K., Broch, A., Robbins, C., Ceniceros, E., Natarajan, M., 2012. Review of biodiesel composition, properties, and specifications. Renewable and Sustainable Energy Reviews 16, 143–169. https://doi.org/10.1016/j.rser.2011.07.143
Hopfner, U., Schenck, T.-L., Chávez, M.-N., Machens, H.-G., Bohne, A.-V., Nickelsen, J., Giunta, R.-E., Egaña, J.-T., 2014. Development of photosynthetic biomaterials for in vitro tissue engineering. Acta Biomaterialia 10, 2712–2717. https://doi.org/10.1016/j.actbio.2013.12.055
Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54, 621–639. https://doi.org/10.1111/j.1365-313X.2008.03492.x
Hulatt, C.J., Wijffels, R.H., Bolla, S., Kiron, V., 2017. Production of Fatty Acids and Protein by Nannochloropsis in Flat-Plate Photobioreactors. PLoS ONE 12, e0170440. https://doi.org/10.1371/journal.pone.0170440
Jia, W., Huang, X., Li, C., 2014. A preliminary study of the algicidal mechanism of bioactive metabolites of *Brevibacillus laterosporus* on *Oscillatoria* in prawn ponds. The Scientific World Journal 2014, 1–11. https://doi.org/10.1155/2014/869149
Jónasdóttir, S.H., 2019. Fatty acid profiles and production in marine phytoplankton. Marine Drugs 17. https://doi.org/10.3390/md17030151
Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnology for Biofuels 6, 16. https://doi.org/10.1186/1754-6834-6-16

Jorquera, O., Kiperstok, A., Sales, E.A., Embiruçu, M., Ghirardi, M.L., 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresource Technology 101, 1406–1413. https://doi.org/10.1016/j.biortech.2009.09.038

Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial Cell Factories 17. https://doi.org/10.1186/s12934-018-0879-x

Khozin-Goldberg, I., Iskandarov, U., Cohen, Z., 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Applied Microbiology and Biotechnology 91, 905–915. https://doi.org/10.1007/s00253-011-3441-x

Kim, M.-J., Jeong, S.-Y., Lee, S.-J., 2008. Isolation, identification, and algicidal activity of marine bacteria against *Cochlodinium polykrikoides*. Journal of Applied Phycology 20, 1069–1078. https://doi.org/10.1007/s10811-008-9312-x

Klee, M.S., Blumberg, L.M., 2002. Theoretical and practical aspects of fast gas chromatography and method translation. Journal of Chromatographic Science 40, 234–247. https://doi.org/10.1093/chromsci/40.5.234

Kouzuma, A., Watanabe, K., 2015. Exploring the potential of algae/bacteria interactions. Current Opinion in Biotechnology 33, 125–129. https://doi.org/10.1016/j.copbio.2015.02.007

Kulp, A., Kuehn, M.J., 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annual Review of Microbiology 64, 163–184. https://doi.org/10.1146/annurev.micro.091208.073413

Kumar, R., Tabatabaei, M., Karimi, K., Sárvári Horváth, I., 2016. Recent updates on lignocellulosic biomass derived ethanol - A review. Biofuel Research Journal 3, 347–356. https://doi.org/10.18331/BRJ2016.3.1.4

Kumari, P., Kumar, M., Reddy, C.R.K., Jha, B., 2013. Algal lipids, fatty acids and sterols, in: Functional Ingredients from Algae for Foods and Nutraceuticals. Elsevier, pp. 87–134. https://doi.org/10.1533/9780857098689.1.87

Labeeuw, L., 2016. A big role for small molecules in mediating Emiliania huxleyi – Roseobacter interactions. Department of Biological Sciences, University of Alberta Thesis.

Labeeuw, L., Khey, J., Bramucci, A.R., Atwal, H., de la Mata, A.P., Harynuk, J., Case, R.J., 2016. Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells. Frontiers in Microbiology 7, 828–828. https://doi.org/10.3389/fmicb.2016.00828

Lang, I., Hodac, L., Friedl, T., Feussner, I., 2011. Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biology 11, 124. https://doi.org/10.1186/1471-2229-11-124

Larkum, A.W., 2016. Photosynthesis and light harvesting in algae, in: Borowitzka, M.A., Beardall, J., Raven, J.A. (Eds.), The Physiology of Microalgae. Springer International Publishing, pp. 67–87. https://doi.org/10.1007/978-3-319-24945-2_3

Le Chevanton, M., Garnier, M., Bougaran, G., Schreiber, N., Lukomska, E., Bérard, J.-B., Fouilland, E., Bernard, O., Cadoret, J.-P., 2013. Screening and selection of growth-promoting bacteria for *Dunaliella* cultures. Algal Research 2, 212–222. https://doi.org/10.1016/j.algal.2013.05.003

Lee, C.-H., Chae, H.-S., Lee, S.-H., Kim, H.S., 2015. Growth characteristics and lipid content of three Korean isolates of *Botryococcus braunii* (Trebouxiophyceae). Journal of Ecology and Environment 38, 67–74. https://doi.org/10.5141/ecoenv.2015.007

Lee, D.H., 2011. Algal biodiesel economy and competition among bio-fuels. Bioresource Technology 102, 43–49. https://doi.org/10.1016/j.biortech.2010.06.034

Lee, J., Cho, D.-H., Ramanan, R., Kim, B.-H., Oh, H.-M., Kim, H.-S., 2013. Microalgae-associated bacteria play a key role in the flocculation of *Chlorella vulgaris*. Bioresource Technology 131, 195–201. https://doi.org/10.1016/j.biortech.2012.11.130

Lima-Mendez, G., Faust, K., Henry, N., Decelle, J., Colin, S., Carcillo, F., Chaffron, S., Ignacio-Espinosa, J.C., Roux, S., Vincent, F., Bittner, L., Darzi, Y., Wang, J., Audic, S., Berline, L., Bontempi, G., Cabello, A.M., Coppola, L., Cornejo-Castillo, F.M., d'Ovidio, F., De Meester, L., Ferrera, I., Garet-Delmas, M.-J., Guidi, L., Lara, E., Pesant, S., Royo-Llonch, M., Salazar, G., Sanchez, P., Sebastian, M., Souffreau, C., Dimier, C., Picheral, M., Searson, S., Kandels-Lewis, S., Tara Oceans coordinators, Gorsky, G., Not, F., Ogata, H., Speich, S., Stemmann, L., Weissenbach, J., Wincker, P., Acinas, S.G., Sunagawa, S., Bork, P., Sullivan, M.B., Karsenti, E., Bowler, C., de Vargas, C., Raes, J., 2015. Determinants of community structure in the global plankton interactome. Science 348, 1262073–1262073. https://doi.org/10.1126/science.1262073

Lin, I.-P., Jiang, P.-L., Chen, C.-S., Tzen, J.T.C., 2012. A unique caleosin serving as the major integral protein in oil bodies isolated from *Chlorella* sp. cells cultured with limited nitrogen. Plant Physiology and Biochemistry 61, 80–87. https://doi.org/10.1016/j.plaphy.2012.09.008

Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., Procházková, K., Adkins, I., Hejnová-Holubová, J., Sadílková, L., Morová, J., Šebo, P., 2010. RTX proteins: a highly diverse family secreted by a common mechanism. FEMS Microbiology Reviews 34, 1076–1112. https://doi.org/10.1111/j.1574-6976.2010.00231.x

Liu, B., Benning, C., 2013. Lipid metabolism in microalgae distinguishes itself. Current Opinion in Biotechnology 24, 300–309. https://doi.org/10.1016/j.copbio.2012.08.008

Liu, H., Zhou, Y., Xiao, W., Ji, L., Cao, X., Song, C., 2012. Shifting nutrient-mediated interactions between algae and bacteria in a microcosm: Evidence from alkaline phosphatase assay. Microbiological Research 167, 292–298. https://doi.org/10.1016/j.micres.2011.10.005

López, A., Rico, M., Santana-Casiano, J.M., González, A.G., González-Dávila, M., 2015. Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron. Environmental Science and Pollution Research 22, 14820–14828. https://doi.org/10.1007/s11356-015-4717-y

Lu, N., Wei, D., Jiang, X.-L., Chen, F., Yang, S.-T., 2012. Fatty acids profiling and biomarker identification in snow alga *Chlamydomonas Nivalis* by NaCl stress using GC/MS and multivariate statistical analysis. Analytical Letters 45, 1172–1183. https://doi.org/10.1080/00032719.2012.673094

MacIntyre, H.L., Kana, T.M., Anning, T., Geider, R.J., 2002. Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. Journal of Phycology 38, 17–38. https://doi.org/10.1046/j.1529-8817.2002.00094.x

Madureira, L.A.S., van Kreveld, S.A., Eglinton, G., Conte, M.H., Ganssen, G., van Hinte, J.E., Ottens, J.J., 1997. Late Quaternary high-resolution biomarker and other sedimentary climate proxies in a Northeast Atlantic Core. Paleoceanography 12, 255–269. https://doi.org/10.1029/96PA03120

Manning, A.J., Kuehn, M.J., 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiology 11, 258. https://doi.org/10.1186/1471-2180-11-258

Marlowe, I.T., Brassell, S.C., Eglinton, G., Green, J.C., 1990. Long-chain alkenones and alkyl alkenoates and the fossil coccolith record of marine sediments. Chemical Geology 88, 349–375. https://doi.org/10.1016/0009-2541(90)90098-R

Martens, T., Gram, L., Grossart, H.-P., Kessler, D., Müller, R., Simon, M., Wenzel, S.C., Brinkhoff, T., 2007. Bacteria of the roseobacter clade show potential for secondary

metabolite production. Microbial Ecology 54, 31–42. https://doi.org/10.1007/s00248-006-9165-2
Martin-Platero, A.M., Cleary, B., Kauffman, K., Preheim, S.P., McGillicuddy, D.J., Alm, E.J., Polz, M.F., 2018. High resolution time series reveals cohesive but short-lived communities in coastal plankton. Nature Communications 9. https://doi.org/10.1038/s41467-017-02571-4
Masó, M., Garcés, E., 2006. Harmful microalgae blooms (HAB); problematic and conditions that induce them. Marine Pollution Bulletin 53, 620–630. https://doi.org/10.1016/j.marpolbul.2006.08.006
Mayali, X., Azam, F., 2004. Algicidal bacteria in the sea and their impact on algal blooms. Journal of Eukaryotic Microbiology 51, 139–144. https://doi.org/10.1111/j.1550-7408.2004.tb00538.x
Mayers, T.J., Bramucci, A.R., Yakimovich, K.M., Case, R.J., 2016. A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*. Frontiers in Microbiology 7, 892. https://doi.org/10.3389/fmicb.2016.00892
Meersche, K.V. den, Middelburg, J.J., Soetaert, K., Rijswijk, P. van, Boschker, H.T.S., Heip, C.H.R., 2004. Carbon-nitrogen coupling and algal-bacterial interactions during an experimental bloom: Modeling a 13C tracer experiment. Limnology and Oceanography 49, 862–878. https://doi.org/10.4319/lo.2004.49.3.0862
Mejriszan Toosi, N., Esfahani, J.A., Reza Safaei, M., 2018. Investigation of energy consumption and renewable energy resources in top ten countries with most energy consumption. IOP Conference Series: Materials Science and Engineering 397, 012108. https://doi.org/10.1088/1757-899X/397/1/012108
Mendoza Guzmán, H., de la Jara Valido, A., Carmona Duarte, L., Freijanes Presmanes, K., 2011. Analysis of interspecific variation in relative fatty acid composition: use of flow cytometry to estimate unsaturation index and relative polyunsaturated fatty acid content in microalgae. Journal of Applied Phycology 23, 7–15. https://doi.org/10.1007/s10811-010-9526-6
Merchant, S.S., Kropat, J., Liu, B., Shaw, J., Warakanont, J., 2012. TAG, You're it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation. Current Opinion in Biotechnology 23, 352–363. https://doi.org/10.1016/j.copbio.2011.12.001
Milano, J., Ong, H.C., Masjuki, H.H., Chong, W.T., Lam, K., Loh, P.K., Vellayan, V., 2016. Microalgae biofuels as an alternative to fossil fuel for power generation. Renewable and Sustainable Energy Reviews 58, 180–197. https://doi.org/10.1016/j.rser.2015.12.150
Milici, M., Deng, Z.-L., Tomasch, J., Decelle, J., Wos-Oxley, M.L., Wang, H., Jáuregui, R., Plumeier, I., Giebel, H.-A., Badewien, T.H., Wurst, M., Pieper, D.H., Simon, M., Wagner-Döbler, I., 2016. Co-occurrence analysis of microbial taxa in the Atlantic Ocean reveals high connectivity in the free-living bacterioplankton. Frontiers in Microbiology 7. https://doi.org/10.3389/fmicb.2016.00649
Minowa, T., Yokoyama, S., Kishimoto, M., Okakura, T., 1995. Oil production from algal cells of *Dunaliella tertiolecta* by direct thermochemical liquefaction. Fuel 74, 1735–1738. https://doi.org/10.1016/0016-2361(95)80001-X
Mizusawa, N., Wada, H., 2012. The role of lipids in photosystem II. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1817, 194–208. https://doi.org/10.1016/j.bbabio.2011.04.008
Moellering, E.R., Benning, C., 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryotic Cell 9, 97–106. https://doi.org/10.1128/EC.00203-09
Mofijur, M., Rasul, M.G., Hassan, N.M.S., Nabi, M.N., 2019. Recent development in the production of third generation biodiesel from microalgae. Energy Procedia 156, 53–58. https://doi.org/10.1016/j.egypro.2018.11.088

Mondello, L., Casilli, A., Tranchida, P.Q., Dugo, P., Dugo, G., 2003. Detailed analysis and group-type separation of natural fats and oils using comprehensive two-dimensional gas chromatography. Journal of Chromatography A 1019, 187–196. https://doi.org/10.1016/S0021-9673(03)01130-0

Monteiro, C.M., Castro, P.M.L., Malcata, F.X., 2012. Metal uptake by microalgae: Underlying mechanisms and practical applications. Biotechnology Progress 28, 299–311. https://doi.org/10.1002/btpr.1504

Mouget, J.-L., Dakhama, A., Lavoie, M.C., Noüe, J., 2006. Algal growth enhancement by bacteria: Is consumption of photosynthetic oxygen involved? FEMS Microbiology Ecology 18, 35–43. https://doi.org/10.1111/j.1574-6941.1995.tb00159.x

Mubarak, M., Shaija, A., Suchithra, T.V., 2015. A review on the extraction of lipid from microalgae for biodiesel production. Algal Research 7, 117–123. https://doi.org/10.1016/j.algal.2014.10.008

Naghavi, N.S., Sameipour, F., 2019. Phototrophic microbial consortium: A technology for enhanced biofuel production, in: Rastegari, A.A., Yadav, A.N., Gupta, A. (Eds.), Prospects of Renewable Bioprocessing in Future Energy Systems. Springer International Publishing, Cham, pp. 185–200. https://doi.org/10.1007/978-3-030-14463-0_6

Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K., 2010. Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews 14, 578–597. https://doi.org/10.1016/j.rser.2009.10.003

Ndikubwimana, T., Zeng, X., Murwanashyaka, T., Manirafasha, E., He, N., Shao, W., Lu, Y., 2016. Harvesting of freshwater microalgae with microbial bioflocculant: a pilot-scale study. Biotechnology for Biofuels 9, 47. https://doi.org/10.1186/s13068-016-0458-5

Nguyen, H.M., Baudet, M., Cuiné, S., Adriano, J.-M., Barthe, D., Billon, E., Bruley, C., Beisson, F., Peltier, G., Ferro, M., Li-Beisson, Y., 2011. Proteomic profiling of oil bodies isolated from the unicellular green microalga *Chlamydomonas reinhardtii*: With focus on proteins involved in lipid metabolism. Proteomics 11, 4266–4273. https://doi.org/10.1002/pmic.201100114

Nicolò, M.S., Guglielmino, S.P.P., Solinas, V., Salis, A., 2017. Biodiesel from Microalgae, in: Lee, S.Y. (Ed.), Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals. Springer International Publishing, Cham, pp. 55–74. https://doi.org/10.1007/978-3-319-50436-0_210

Novoveská, L., Zapata, A.K.M., Zabolotney, J.B., Atwood, M.C., Sundstrom, E.R., 2016. Optimizing microalgae cultivation and wastewater treatment in large-scale offshore photobioreactors. Algal Research 18, 86–94. https://doi.org/10.1016/j.algal.2016.05.033

Ok, M.Ö., Tang, X., 2012. Depletion of fossil fuels and anthropogenic climate change—A review. Energy Policy 52, 797–809. https://doi.org/10.1016/j.enpol.2012.10.046

Orata, F.D., Xu, Y., Gladney, L.M., Rishishwar, L., Case, R.J., Boucher, Y., Jordan, I.K., Tarr, C.L., 2016. Characterization of clinical and environmental isolates of *Vibrio cidicii* sp. nov., a close relative of *Vibrio navarrensis*. International Journal of Systematic and Evolutionary Microbiology 66, 4148–4155. https://doi.org/10.1099/ijsem.0.001327

Padmaperuma, G., Kapoore, R.V., Gilmour, D.J., Vaidyanathan, S., 2018. Microbial consortia: a critical look at microalgae co-cultures for enhanced biomanufacturing. Critical Reviews in Biotechnology 38, 690–703. https://doi.org/10.1080/07388551.2017.1390728

Pettersen, A.K., Turchini, G.M., Jahangard, S., Ingram, B.A., Sherman, C.D.H., 2010. Effects of different dietary microalgae on survival, growth, settlement and fatty acid composition of blue mussel (Mytilus galloprovincialis) larvae. Aquaculture 309, 115–124. https://doi.org/10.1016/j.aquaculture.2010.09.024

Powell, R.J., Hill, R.T., 2013. Rapid aggregation of biofuel-producing algae by the bacterium Bacillus sp. Strain RP1137. Applied and Environmental Microbiology 79, 6093–6101. https://doi.org/10.1128/AEM.01496-13
Prahl, F.G., Wakeham, S.G., 1987. Calibration of unsaturation patterns in long-chain ketone compositions for palaeotemperature assessment. Nature 330, 367–369. https://doi.org/10.1038/330367a0
Puhan, S., Saravanan, N., Nagarajan, G., Vedaraman, N., 2010. Effect of biodiesel unsaturated fatty acid on combustion characteristics of a DI compression ignition engine. Biomass and Bioenergy 34, 1079–1088. https://doi.org/10.1016/j.biombioe.2010.02.017
Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Applied Microbiology and Biotechnology 65, 635–648. https://doi.org/10.1007/s00253-004-1647-x
Qin, J.G., 2010. Hydrocarbons from Algae, in: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 2817–2826. https://doi.org/10.1007/978-3-540-77587-4_209
Raheem, A., Prinsen, P., Vuppaladadiyam, A.K., Zhao, M., Luque, R., 2018. A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. Journal of Cleaner Production 181, 42–59. https://doi.org/10.1016/j.jclepro.2018.01.125
Ramanan, R., Kang, Z., Kim, B.-H.H., Cho, D.-H.H., Jin, L., Oh, H.-M.M., Kim, H.-S.S., 2015. Phycosphere bacterial diversity in green algae reveals an apparent similarity across habitats. Algal Research 8, 140–144. https://doi.org/10.1016/j.algal.2015.02.003
Ramanan, R., Kim, B.-H., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2016. Algae–bacteria interactions: Evolution, ecology and emerging applications. Biotechnology Advances 34, 14–29. https://doi.org/10.1016/j.biotechadv.2015.12.003
Ranjan, A., Patil, C., Moholkar, V.S., 2010. Mechanistic assessment of microalgal lipid extraction. Industrial & Engineering Chemistry Research 49, 2979–2985. https://doi.org/10.1021/ie9016557
Ranjith Kumar, R., Hanumantha Rao, P., Arumugam, M., 2015. Lipid extraction methods from microalgae: A comprehensive review. Frontiers in Energy Research 2. https://doi.org/10.3389/fenrg.2014.00061
Rao, D., Webb, J.S., Holmström, C., Case, R., Low, A., Steinberg, P., Kjelleberg, S., 2007. Low densities of epiphytic bacteria from the marine alga *Ulva australis* inhibit settlement of fouling organisms. Applied and Envriomental Microbiology 73, 7844–7852. https://doi.org/10.1128/AEM.01543-07
Raud, M., Kikas, T., Sippula, O., Shurpali, N.J., 2019. Potentials and challenges in lignocellulosic biofuel production technology. Renewable and Sustainable Energy Reviews 111, 44–56. https://doi.org/10.1016/j.rser.2019.05.020
Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of Cell Biology 17, 208–212. https://doi.org/10.1083/jcb.17.1.208
Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K., Peccia, J., 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. BMC Genomics 12. https://doi.org/10.1186/1471-2164-12-148
Robak, K., Balcerek, M., 2018. Review of second generation bioethanol production from residual biomass. Food Technology and Biotechnology 56, 174–187. https://doi.org/10.17113/ftb.56.02.18.5428
Rockwell, N.C., Lagarias, J.C., Bhattacharya, D., 2014. Primary endosymbiosis and the evolution of light and oxygen sensing in photosynthetic eukaryotes. Frontiers in Ecology and Evolution 2.

Ryckebosch, E., Muylaert, K., Foubert, I., 2012. Optimization of an analytical procedure for extraction of lipids from microalgae. Journal of the American Oil Chemists' Society 89, 189–198. https://doi.org/10.1007/s11746-011-1903-z

Sahu, A., Pancha, I., Jain, D., Paliwal, C., Ghosh, T., Patidar, S., Bhattacharya, S., Mishra, S., 2013. Fatty acids as biomarkers of microalgae. Phytochemistry 89, 53–58. https://doi.org/10.1016/j.phytochem.2013.02.001

Sajjadi, B., Chen, W.-Y., Raman, Abdul.Aziz.A., Ibrahim, S., 2018. Microalgae lipid and biomass for biofuel production: A comprehensive review on lipid enhancement strategies and their effects on fatty acid composition. Renewable and Sustainable Energy Reviews 97, 200–232. https://doi.org/10.1016/j.rser.2018.07.050

Schatz, D., Rosenwasser, S., Malitsky, S., Wolf, S.G., Feldmesser, E., Vardi, A., 2017. Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga. Nature Microbiology 2, 1485–1492. https://doi.org/10.1038/s41564-017-0024-3

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: High-efficiency microalgae for biodiesel production. BioEnergy Research 1, 20–43. https://doi.org/10.1007/s12155-008-9008-8

Schreiber, U., Schliwa, U., Bilger, W., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynthesis Research 10, 51–62. https://doi.org/10.1007/BF00024185

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., Smith, A.G., 2010. Biodiesel from algae: challenges and prospects. Current Opinion in Biotechnology 21, 277–286. https://doi.org/10.1016/j.copbio.2010.03.005

Segev, E., Castañeda, I.S., Sikes, E.L., Vlamakis, H., Kolter, R., 2016. Bacterial influence on alkenones in live microalgae. Journal of Phycology 52, 125–130. https://doi.org/10.1111/jpy.12370

Segovia, M., Haramaty, L., Berges, J.A., Falkowski, P.G., 2003. Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the evolution of apoptosis in higher plants and metazoans. Plant Physiology 132, 99–105. https://doi.org/10.1104/pp.102.017129

Selje, N., Simon, M., Brinkhoff, T., 2004. A newly discovered Roseobacter cluster in temperate and polar oceans. Nature 427, 445–448. https://doi.org/10.1038/nature02272

Seyedsayamdost, M.R., Case, R.J., Kolter, R., Clardy, J., 2011. The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. Nature Chemistry 3, 331–335. https://doi.org/10.1038/nchem.1002

Seymour, J.R., Amin, S.A., Raina, J.-B., Stocker, R., 2017. Zooming in on the phycosphere: the ecological interface for phytoplankton–bacteria relationships. Nature Microbiology 2, 17065. https://doi.org/10.1038/nmicrobiol.2017.65

Sharma, K.K., Schuhmann, H., Schenk, P.M., 2012. High lipid induction in microalgae for biodiesel production. Energies 5, 1532–1553. https://doi.org/10.3390/en5051532

Shin, S.-E., Lim, J.-M., Koh, H.G., Kim, E.K., Kang, N.K., Jeon, S., Kwon, S., Shin, W.-S., Lee, B., Hwangbo, K., Kim, J., Ye, S.H., Yun, J.-Y., Seo, H., Oh, H.-M., Kim, K.-J., Kim, J.-S., Jeong, W.-J., Chang, Y.K., Jeong, B., 2016. CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. Scientific Reports 6. https://doi.org/10.1038/srep27810

Simó, R., 2001. Production of atmospheric sulfur by oceanic plankton: biogeochemical, ecological and evolutionary links. Trends in Ecology & Evolution 16, 287–294. https://doi.org/10.1016/S0169-5347(01)02152-8

Simon, N., Cras, A.-L., Foulon, E., Lemée, R., 2009. Diversity and evolution of marine phytoplankton. Comptes Rendus Biologies 332, 159–170. https://doi.org/10.1016/j.crvi.2008.09.009

Singh, A., Nigam, P.S., Murphy, J.D., 2011. Renewable fuels from algae: An answer to debatable land based fuels. Bioresource Technology 102, 10–16. https://doi.org/10.1016/j.biortech.2010.06.032
Singh, P., Kumari, S., Guldhe, A., Misra, R., Rawat, I., Bux, F., 2015. Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. Renewable and Sustainable Energy Reviews 55, 1–16. https://doi.org/10.1016/j.rser.2015.11.001
Singh, S., Kate, B.N., Banerjee, U.C., 2005. Bioactive compounds from cyanobacteria and microalgae: An overview. Critical Reviews in Biotechnology 25, 73–95. https://doi.org/10.1080/07388550500248498
Solovchenko, A.E., 2012. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. Russian Journal of Plant Physiology 59, 167–176. https://doi.org/10.1134/S1021443712020161
Song, D., Xi, B., Sun, J., 2016. Characterization of the growth, chlorophyll content and lipid accumulation in a marine microalgae *Dunaliella tertiolecta* under different nitrogen to phosphorus ratios. Journal of Ocean University of China 15, 124–130. https://doi.org/10.1007/s11802-016-2797-z
Sorigué, D., Légeret, B., Cuiné, S., Morales, P., Mirabella, B., Guédeney, G., Li-Beisson, Y., Jetter, R., Peltier, G., Beisson, F., 2016. Microalgae synthesize hydrocarbons from long-chain fatty acids via a light-dependent pathway. Plant Physiology 171, 2393–2405. https://doi.org/10.1104/pp.16.00462
Sosik, H.M., Mitchell, B.G., 1994. Effects of temperature on growth, light absorption, and quantum yield in *Dunaliella Tertiolecta* (chlorophyceae). Journal of Phycology 30, 833–840. https://doi.org/10.1111/j.0022-3646.1994.00833.x
Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering 101, 87–96. https://doi.org/10.1263/jbb.101.87
Su, J.Q., Yang, X.R., Zheng, T.L., Tian, Y., Jiao, N.Z., Cai, L.Z., Hong, H.S., 2007. Isolation and characterization of a marine algicidal bacterium against the toxic dinoflagellate *Alexandrium tamarense*. Harmful Algae 6, 799–810. https://doi.org/10.1016/j.hal.2007.04.004
Taher, H., Al-Zuhair, S., Al-Marzouqi, A.H., Haik, Y., Farid, M., 2014. Effective extraction of microalgae lipids from wet biomass for biodiesel production. Biomass and Bioenergy 66, 159–167. https://doi.org/10.1016/j.biombioe.2014.02.034
Tan, K.W.M., Lin, H., Shen, H., Lee, Y.K., 2016. Nitrogen-induced metabolic changes and molecular determinants of carbon allocation in *Dunaliella tertiolecta*. Scientific Reports 6. https://doi.org/10.1038/srep37235
Tang, H., Abunasser, N., Garcia, M.E.D., Chen, M., Simon Ng, K.Y., Salley, S.O., 2011. Potential of microalgae oil from *Dunaliella tertiolecta* as a feedstock for biodiesel. Applied Energy 88, 3324–3330. https://doi.org/10.1016/j.apenergy.2010.09.013
Thompson, G.A., 1996. Lipids and membrane function in green algae. Biochimica et Biophysica Acta 1302, 7–45.
Tirichine, L., Bowler, C., 2011. Decoding algal genomes: tracing back the history of photosynthetic life on Earth: Decoding algal genomes. The Plant Journal 66, 45–57. https://doi.org/10.1111/j.1365-313X.2011.04540.x
Toyofuku, M., Morinaga, K., Hashimoto, Y., Uhl, J., Shimamura, H., Inaba, H., Schmitt-Kopplin, P., Eberl, L., Nomura, N., 2017. Membrane vesicle-mediated bacterial communication. The ISME Journal 11, 1504–1509. https://doi.org/10.1038/ismej.2017.13
Trentacoste, E.M., Shrestha, R.P., Smith, S.R., Glé, C., Hartmann, A.C., Hildebrand, M., Gerwick, W.H., 2013. Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. Proceedings of the National Academy of

Sciences of the United States of America 110, 19748–19753. https://doi.org/10.1073/pnas.1309299110
Ummalyma, S.B., Gnansounou, E., Sukumaran, R.K., Sindhu, R., Pandey, A., Sahoo, D., 2017. Bioflocculation: An alternative strategy for harvesting of microalgae- An overview. Bioresource Technology 242, 227–235. https://doi.org/10.1016/j.biortech.2017.02.097
van Kooten, O., Snel, J.F.H., 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynthesis Research 25, 147–150. https://doi.org/10.1007/BF00033156
Vieler, A., Brubaker, S.B., Vick, B., Benning, C., 2012. A lipid droplet protein of Nannochloropsis with functions partially analogous to plant oleosins. Plant Physiology 158, 1562–1569. https://doi.org/10.1104/pp.111.193029
Vinayak, V., Manoylov, K., Gateau, H., Blanckaert, V., Hérault, J., Pencréac'h, G., Marchand, J., Gordon, R., Schoefs, B., 2015. Diatom milking: A review and new approaches. Marine Drugs 13, 2629–2665. https://doi.org/10.3390/md13052629
Wagner-Döbler, I., Biebl, H., 2006. Environmental biology of the marine *Roseobacter* lineage. Annual Review of Microbiology 60, 255–280. https://doi.org/10.1146/annurev.micro.60.080805.142115
Wang, H., Hill, R.T., Zheng, T., Hu, X., Wang, B., 2016. Effects of bacterial communities on biofuel-producing microalgae: stimulation, inhibition and harvesting. Critical Reviews in Biotechnology 36, 341–352. https://doi.org/10.3109/07388551.2014.961402
Wang, R., Xue, S., Zhang, D., Zhang, Q., Wen, S., Kong, D., Yan, C., Cong, W., 2015. Construction and characteristics of artificial consortia of *Scenedesmus obliquus* -bacteria for *S. obliquus* growth and lipid production. Algal Research 12, 436–445. https://doi.org/10.1016/j.algal.2015.10.002
Weiland, P., 2010. Biogas production: current state and perspectives. Applied Microbiology and Biotechnology 85, 849–860. https://doi.org/10.1007/s00253-009-2246-7
Xue, J., Niu, Y.-F., Huang, T., Yang, W.-D., Liu, J.-S., Li, H.-Y., 2015. Genetic improvement of the microalga *Phaeodactylum tricornutum* for boosting neutral lipid accumulation. Metabolic Engineering 27, 1–9. https://doi.org/10.1016/j.ymben.2014.10.002
Yáñez-Mó, M., Siljander, P.R.-M., Andreu, Z., Bedina Zavec, A., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J.M., Ghobrial, I.M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N.H.H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Krämer-Albers, E.-M., Laitinen, S., Lässer, C., Lener, T., Ligeti, E., Linē, A., Lipps, G., Llorente, A., Lötvall, J., Manček-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E.N.M., Nyman, T.A., O'Driscoll, L., Olivan, M., Oliveira, C., Pállinger, É., del Portillo, H.A., Reventós, J., Rigau, M., Rohde, E., Sammar, M., Sánchez-Madrid, F., Santarém, N., Schallmoser, K., Stampe Ostenfeld, M., Stoorvogel, W., Stukelj, R., Van der Grein, S.G., Helena Vasconcelos, M., Wauben, M.H.M., De Wever, O., 2015. Biological properties of extracellular vesicles and their physiological functions. Journal of Extracellular Vesicles 4, 27066. https://doi.org/10.3402/jev.v4.27066
Yao, L., Gerde, J.A., Lee, S.-L., Wang, T., Harrata, K.A., 2015. Microalgae lipid characterization. Journal of Agricultural and Food Chemistry 63, 1773–1787. https://doi.org/10.1021/jf5050603
Zamith-Miranda, D., Nimrichter, L., Rodrigues, M.L., Nosanchuk, J.D., 2018. Fungal extracellular vesicles: modulating host–pathogen interactions by both the fungus and the host. Microbes and Infection 20, 501–504. https://doi.org/10.1016/j.micinf.2018.01.011
Zhu, L., 2015. Microalgal culture strategies for biofuel production: a review. Biofuels, Bioproducts and Biorefining 9, 801–814. https://doi.org/10.1002/bbb.1576

Zienkiewicz, K., Du, Z.-Y., Ma, W., Vollheyde, K., Benning, C., 2016. Stress-induced neutral lipid biosynthesis in microalgae — Molecular, cellular and physiological insights. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1861, 1269–1281. https://doi.org/10.1016/j.bbalip.2016.02.008

Zulu, N.N., Zienkiewicz, K., Vollheyde, K., Feussner, I., 2018. Current trends to comprehend lipid metabolism in diatoms. Progress in Lipid Research 70, 1–16. https://doi.org/10.1016/j.plipres.2018.03.001

Chapter 2: Bacterial stimulated production of lipid vesicles in green microalgae *Dunaliella tertiolecta*

2.1 Introduction

Phytoplankton drive marine primary production, fixing an estimated 40% of total global carbon annually, and are essential for the biogeochemical cycling of elements (Falkowski, 1994; Field, 1998; Simó, 2001). These microalgae are closely associated with specific bacterial groups, which uptake some of the output of their primary production as well as biogeochemical intermediates, directing the flow of global nutrient cycles and the structuring of microbial communities in the oceans (Durham et al., 2015; Lima-Mendez et al., 2015; Milici et al., 2016; Ramanan et al., 2015). These interactions encompass various relationships, from mutualism to competition and pathogenesis, which are mediated through the exchange of bioactives and nutrients (Amin et al., 2012; Bramucci et al., 2018; Bramucci and Case, 2019; Geng and Belas, 2010). However, algal-bacterial interactions are not only essential for modeling marine biogeochemical cycles, but also have potential in the microalgal biotechnology industry, as bacterial stimulation of their hosts metabolism could be useful in optimizing production of commercial metabolites such as lipids (Pulz and Gross, 2004; Spolaore et al., 2006).

Microalgal lipids fall under two categories, polar and neutral lipids. Polar lipids are the central component of cellular membranes and responsible for membrane integrity while neutral lipids are mainly considered energy storage molecules (Thompson, 1996). Triacylglycerols (TAG) are the most common neutral lipid in microalgae and specific species are able to accumulate up to 60% of their dry weight in TAGs (Dong et al., 2016). Microalgal lipid metabolism still remains poorly understood because of their enormous genetic diversity, which is extensive even within genera, as well as limited genome sequence availability and differences from model plant counterparts (Harwood and Guschina, 2009; Hu et al., 2008; Zulu et al., 2018). It is understood that microalgae synthesize TAGs through the Kennedy pathway and/or the monoacylglycerol pathway, and accumulate them in organelles called cytoplasmic lipid droplets (Farese and Walther, 2009; Goold et al., 2015; Zienkiewicz et al., 2016). Lipid droplet synthesis is stimulated by environmental stresses like extreme salinity, pH, temperature, intense light fluctuations and nutrient limitations (Solovchenko, 2012). In response to these stresses, microalgae will inhibit cellular division, increase TAG biosynthesis and begin degrading thylakoid membranes in order to convert the polar membrane lipids into TAGs (Zienkiewicz et

al., 2016). Understanding and controlling this stress response has been a target to increase lipid yield in biofuel-producing microalgae strains.

Microalgal TAGs are compositionally similar in carbon chain length and degree of saturation to traditional fossil fuels and as such are the focus of the advance generations of microalgal biofuel production (Atabani et al., 2012; Thompson, 1996). The common way to induce TAG accumulation in microalgae is through nitrogen limitation, however, this method can have detrimental effects on biomass and growth rates that impedes the ability to commercialize these systems (Singh et al., 2015). In order to overcome this energy bottleneck within the industry, attaining both high algal biomass and high lipid yield, researchers have been examining microalgae in their natural habitat in which they are a part of microbial consortia (Padmaperuma et al., 2018; Ramanan et al., 2016; Wang et al., 2016).

Polymicrobial cultures of microalgae and bacteria can be beneficial to biofuel production, as the latter feed on the waste products of the algae, removing autoinhibitory compounds (Fuentes et al., 2016; Padmaperuma et al., 2018). Bacteria can also play a crucial role in microalgae's productivity by providing bio-available nutrients, hormones and chemical defense against pathogens (Amin et al., 2009, 2015; Croft et al., 2005; Seyedsayamdost et al., 2011). The benefits of polymicrobial cultures are now being recognized at each stage of the biofuel production pipeline; cultivation, harvesting and lipid extraction (Berthold et al., 2019; Fuentes et al., 2016; Ramanan et al., 2016). During cultivation, bacteria can stimulate lipid productivity and enhance biomass by providing nutrients or growth promoting factors, which would optimize overall biofuel production yield (Berthold et al., 2019; Cho et al., 2015; de-Bashan et al., 2002; Wang et al., 2015). Harvesting the microalgal biomass from the liquid medium accounts for the majority of the total energy of production (Gallagher, 2011). Certain bacteria increase aggregation of algae which promotes culture concentration through settling (Powell and Hill, 2013). Bioflocculation and bio-aggregation are efficient ways to reduce energy required to harvest microalgal (Ndikubwimana et al., 2016; Ummalyma et al., 2017). Extracting lipids from microalgal cells traditionally relied on expensive chemicals or mechanical force to disrupt cell walls, releasing the intracellular lipids (Mubarak et al., 2015). Bacterial algicides could improve lipid extraction but the specificity and mechanisms of these processes still require research to be efficiently implemented (Demuez et al., 2015).

Here we explored the effect marine bacteria have on lipid production and localization on the biofuel candidate, green microalga *Dunaliella tertiolecta* (CCMP1320). To identify if

bacteria could stimulate lipid yield in this microalgal host, various bacterial strains were screened in a cocultivation assay with *D. tertiolecta* (CCMP 1320) using lipophilic dye Nile Red. The roseobacter *Phaeobacter italicus* increased lipid per biomass in stationary phase *D. tertiolecta* without affecting on photosynthetic health or biomass. This increase took the form of secreted, extracellular vesicles. It was hypothesized that the origin of the lipid vesicles were released algal lipid droplets. This is the first study, to our knowledge, that describes the production of lipid filled vesicle as a of result algal-bacterial interactions.

2.2 Importance

Interactions with bacteria can have a strong influence on algal metabolism and therefore need to be understood to gain a full picture of their physiology in natural and artificial aquatic ecosystems. Through the screening of such interactions, we have identified bacterial stimulation of extracellular lipid vesicle production in a unicellular green alga that is a candidate for biofuel production. This has implications in the biotechnology industry and microbial ecology. In industry, algal lipids are commercially cultivated as nutritional supplements and as feedstock for biofuel production. This research suggests using bacteria as the agents of extraction and stimulation during biofuel production, instead of toxic chemical or energy consuming methods. In microbial ecology, microalgal lipids are used as biomarkers for microalgal communities and as historic sea-surface temperature proxies in paleo-oceanography. These ecological techniques might require re-evaluation, in order for increased accuracy, since bacteria can affect the yield and localization of microalgal lipids.

2.3 Material and methods

2.3.1 Growth and maintenance of algal and bacterial strains

The chlorophyte *Dunaliella tertiolecta* CCMP 1320 was obtained from the Provasoli-Guillard National Centre for Marine Algae and Microbiota (NCMA) and maintained in L1-Si media made with artificial seawater (35 g/L of Instant Ocean, Blacksburg, VA, USA). *D. tertiolecta* was grown at 18 ℃ in a diurnal incubator (12:12 h dark:light cycle). *D. tertiolecta* and its medium were checked for bacterial contamination prior and throughout experimental use by growing 20 µL aliquots onto ½ marine agar (½ MA) (18.7 g Difco Marine Broth 2216 supplemented with 9 g NaCl and 15 g Difco agar in 1 L) and incubating at 28 ℃ for 24 h. Algal cultures were grown statically for 4 d prior to experimental (10^4 cells ml^{-1}).

The bacteria *Alteromonas macleodii* 9313c2, *Vibrio cidicii* 111013, *Phaeobacter inhibens* 2.10 and *P. italicus* R11, were maintained at 28 ℃ on ½ MA plates (½ Difco Marine Broth 2216) (Fernandes et al., 2011; Martens et al., 2007; Orata et al., 2016). Cells were grown in 5 mL ½ MA to stationary phase, at 28 ℃ for 24 h, before experiments.

2.3.2 Co-cultivation experiments

Algal-bacterial cocultivation experiments were performed in 48-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) as described in Bramucci *et al.* (Bramucci et al., 2015). Briefly, stationary phase bacterial cultures were washed twice by centrifugation and re-suspended in L1-Si medium made with artificial seawater before dilution to 10^4 colony-forming units (CFU) ml^{-1}. *D. tertiolecta* and bacteria were mixed in a 1:1 (v:v) ratio (10^4 cells ml^{-1} each) to start cocultures. Monoculture control of *D. tertiolecta* was inoculated in 1:1 (v:v) ratio with L1-Si made with artificial seawater. Mono- and co-cultures were aliquoted in 1 mL volumes into 48-wells plates and three independent wells were sampled at each time point. 48-wells plates were incubated in a diurnal incubator (12:12 h dark:light cycle) at 18 ℃.

At each time point, 20 µL of samples were plated on ½ MA and incubated for 24 h at 28 ℃ to confirm the absence of bacteria in the algal control and enumerate bacteria in coculture treatments.

2.3.3 Biomass and lipid measurement

Biomass (OD 680nm), chlorophyll *a* fluorescence (ex:445nm, em:680nm) and neutral lipid content were measured from 250 µL of three independent wells of mono- and co-cultures using a Synergy H1 microplate reader (Biotek, Winooski, VT, USA). Neutral lipid content was quantified using the Nile Red fluorometric test assay (Franz et al., 2013). Briefly, 25 µL of Nile Red (Sigma-Aldrich, St. Louis, MO, USA) (5 mg/100 mL of acetone) was added to 250 µL of cells and mixed well. Samples were incubated for 20 min at room temperature in the dark. Fluorescence was read at 590 nm emission after excitation at 530 nm. Sterile L1-Si medium made with artificial seawater was used as a blank. A standard curve was prepared from a stock solution of glyceryl trioleate (triolein) (Sigma-Aldrich, St. Louis, MO, USA) in the solvent isopropanol at 10 mg/mL (Fischer Scientific, Waltham, MA, USA) to quantify the neutral algal lipid content with triolein lipid equivalent (Bertozzini et al., 2011).

2.3.4 Nitrogen concentration analysis

Frozen samples of algal control and cocultures, from d 0, 4, 8, 12 and 16, were thawed prior to completing nitrate and nitrite analysis through Cayman's Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Michigan, USA). The assay used prepared Griess reagents and was performed in accordance to the manufacturer's instructions.

2.3.5 Pulse-amplitude-modulation (PAM) fluorometer

A PAM fluorometer (WATER-PAM, Waltz, Effeltrich, Germany) was used to measure *D. tertiolecta*'s photosynthetic potential quantum yield (F_v/F_m) (Schreiber et al., 1986). Triplicate samples were taken during the mid-point of the dark cycle and diluted, with sterile L1-Si medium, in the detection range of the PAM fluorometer. Each sample was processed after a 3 min dark adaptation followed by a saturating pulse. The potential quantum yield was calculated using $F_v/F_m=(F_m-F_0)/F_m$ (van Kooten and Snel, 1990).

2.3.6 Fatty Acid Analysis through GCxGC-TOFMS

Fatty acid analysis was performed in triplicate on *D. tertiolecta* alone and grown with *P. italicus*. Coculture was obtained as described previously with the exception that the volume was increased to 100 mL in an Erlenmeyer flask.

Samples were collected through suction filtration on pre-weighed Whatman ® glass fiber filters (0.22 µm, Grade GF/F) at 18 d. Each sample was rinsed three times with phosphate buffer solution (PBS, 1M) to remove salts for fatty acid extraction. Filters were placed in glass vial and stored in -80 °C overnight before being lyophilized for 24 h. After drying, filters were weighed and dried biomass was recorded.

The fatty acid extraction protocol was adapted from previous literature (Cavonius et al., 2014; Ryckebosch et al., 2012). All samples were extracted using equal amounts of methanol and chloroform, followed by sonication after each chemical addition. After addition of distilled water (3.5 mL), an extraction series of the bottom chloroform layer was performed. The samples were then dried (40 °C under nitrogen) and resuspended in cyclohexane prior to addition of toluene and 2% H_2SO_4. Samples were incubated for 1 h then dried in 5 mg of Na_2SO_4. Samples were extracted for a second time with a t-butyl methyl ether series before being dried (40 °C under nitrogen). Cyclohexane (250 µL) was added, and these samples, now containing the fatty acid methyl esters, were stored at at 4 °C until analysis.

Fatty acid methyl ester derivatives from the algal extracts were collected on a Leco Pegasus 4D GCxGC-TOFMS (Leco Instruments, St. Joseph, MI) with a cryogenic modulation system utilizing liquid nitrogen. The first dimension column is an Rxi-5SilMS (Chromatographic Specialties, Inc., Canada) with 30 m length 0.25 mm internal diameter and a 1.00 µm film thickness. The second dimension used was an Rtx-Wax (Chromatographic Specialties, Inc., Canada) with a 1.84 m length, 0.18 mm internal diameter, and a 0.18 µm film thickness. Ultra-pure helium (5.0 grade; Praxair Canada Inc., Edmonton) was used as the carrier gas with a speed-optimized (Blumberg and Klee, 2000) constant flow rate of 1.44 mL min^{-1}. The injection volume was 1 µL, run with a split ratio of 1:100 with a constant inlet temperature of 250 °C. The optimal heating rate was 3.50 C min^{-1} with an initial temperature hold at 80 °C for 4 min and a final temperature hold of 230 °C for 10 min (Klee and Blumberg, 2002). The secondary oven was held at +5 °C relative to the primary oven, and modulator +15 °C relative to the secondary oven. The modulation period (Pm) was 2.50 s, with a hot pulse duration of 0.60 s, and a cool time of 0.65 s. The transfer line temperature was maintained at 250 °C.

Data was analyzed using ChromaTOF (v.4.43; Leco). Reference peaks were identified in a certified reference material containing 37 fatty acid methyl esters (Supelco 37 Component FAME Mix, Millipore Sigma, Canada) or were identified by analyzing their retention in the first and second dimensions relative to known components (Mondello et al., 2003). Once the peaks were identified, they were searched for in other samples using the ChromatTOF Calibration function with first and second retention time deviations of 5 s and 0.5 s respectively, and a minimum mass spectral match threshold of 700 (1000 maximum). Each sample was normalized with dried biomass before calculations of percent fatty acid of total fatty acids were performed.

2.3.7 Fluorescence microscopy

During experiments, cells were fixed in 0.15% glutaraldyhyde for 10 min in the dark for brightfield and epifluorescence microscopy. Images were obtained using Zeiss Axio Scope A1 (Zeiss, Oberkochen, Germany), equipped with an Optronics digital camera and PictureFrame Software Ver 2.3. Epifluorescence microscopy was used to image cells and lipid vesicles with Nile Red fluorescence dye (Sigma-Aldrich, St. Louis, MO, United States) and nucleic acids with SYBR-Green 1 (Thermo Fischer Scientific, Waltham, MA, United States) according to the manufacturer's instruction (25 ℃, 10 min incubation). Images were acquired simultaneously with three different channels; 1. differential interference contract (DIC) channel, 2. Nile Red fluorescence (red/orange - ex: 530nm, em: 590nm) and 3. SYBR-Green 1 (green – ex: 497 nm, em: 520 nm) and overlaid using Zen 2 Blue Edition software.

2.3.8 Transmission electron microscopy

TEM was performed on *D. tertiolecta* alone and cocultured with *P. italicus.* Samples were concentrated and resuspended in fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M PBS) after the spent medium was removed before being stored overnight at 4 ℃. Samples were pelleted, washed three times with 0.1 M PBS buffer then post-fixed in 1% osmium tetroxide (OsO_4 in 0.1 M PBS buffer, pH 7.2) for 2 h. Three buffer washes were performed with 0.1 M PBS buffer before samples were dehydrated through a graded ethanol series three times each (50%, 70% 90% and 100% ethanol). Samples were then incubated for 2 h in a new 1:1 Ethanol:SPURR mix then transferred into 100% SPURR mix to incubate for 12

h. Sample was transferred to BEEM capsules (Ted Pella, Redding, CA, United States) in SPURR resin and then embedded. Molds were incubated in 75 ℃ oven for 16 h then cooled to room temperature. Sectioning was performed with a diamond blade on Ultracut E. Ultramicrotome (Leica Microsystems, Concord, ON, Canada). Sections (0.6 μm thick) were stained with uranyl acetate (4% in dH_2O) (Fisher Scientific, Hampton, NH, United States) and lead citrate stains (Reynolds, 1963). After staining, images were taken on a Morgagni 268 transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at 80 kV with a Gatan Orius CCD Camera (Gatan Inc., Pleasanton, CA, United States).

2.3.9 ImageStream flow cytometry

Samples for flow cytometry were fixed as described for fluorescent microscopy and then flash frozen in liquid nitrogen and stored at -80 ° C. Flow cytometry was performed using an Amnis ImageStream multi-spectral imaging flow cytometer (model mk1) (Amnis Corporation, Seattle, WA, United States) to enumerate algal cells and lipid-filled vesicles and confirm lipid vesicles did not contain DNA. Samples were stained with SYBR-Green 1 and Nile Red, as previously described for fluorescent microscopy, and 1,000 algal cells were acquired for each sample of mono- and co-cultures at 0, 4, 8, 12 and 16 d, in triplicate. To correct spectral overlay, a compensation matrix (Nile Red, SYBR-Green, Unstained cells with no brightfield) was applied to all data. Analyses were completed with fully compensated, focused, single objects as duplets were excluded. Objects were visually inspected to include in population enumeration. Data were processed using ImageStream IDEAS software (Amnis Corporation, Seattle, WA, United States).

2.3.10 Statistical Analysis

A Shapiro-Wilk test was performed on each independent variable to determine normality (p-value > 0.05). Homogeneity of Variance (p-value < 0.05) was confirmed using a Levene's test. Statistical significance (p-value < 0.05) was determined by One-Way ANOVA on parametric data and Kruskal-Wallis rank sum test on non-parametric data. For Figure 2.1, a one-tailed, two sample t-test between *P. italicus* and *D. tertiolecta* control was performed. Details on all data variables can be found in associated figure legend.

2.4 Results

2.4.1 Changes in the lipid production of *D. tertiolecta* during co-cultivation with various bacteria

The effect of marine bacteria on the production and storage of lipids of the green microalgae *D. tertiolecta* were tested by comparing lipid accumulation when grown alone and with four bacteria; *Alteromonas macleodii* 9313c2*, Vibrio cidicii* CIP 111013*, Phaeobacter inhibens* 2.10 and *P. italicus* R11. Lipids were quantified with lipophilic dye Nile Red using a standard curve to measure triolein equivalent lipid units (Bertozzini et al., 2011; Greenspan, 1985). As the Nile Red fluorescence of bacteria cultured alone was below the detection limit, the fluorescence reading was representative of microalgal lipids. Since lipid quantity is positively correlated with biomass, total lipids were normalized with biomass and lipid per biomass (OD 680 nm) was compared across cocultures and algal controls after 20 d growth (Figure 2.1).

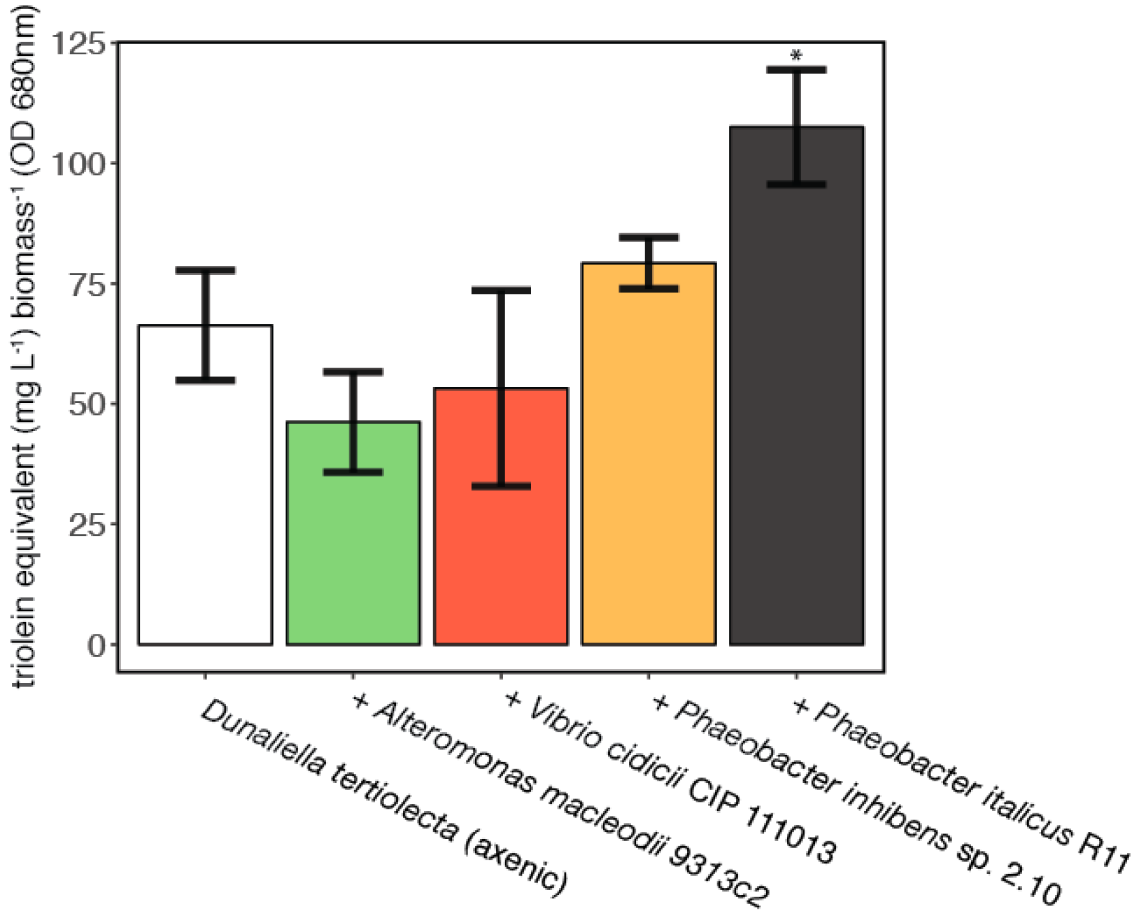


Figure 2.1. Effect of co-cultivation with various bacteria on the lipid content of *Dunaliella tertiolecta* (CCMP 1320). *D. tertiolecta* was grown axenically and in coculture with an *A. macleodii* 9313c2 (green), *V. cidicii* CIP 111013 (red), *P. inhibens* 2.10 (orange) and *P italicus* (black). Lipid concentration in triolein equivalent units (Nile Red fluorescence), normalized with biomass (OD680) was measured during late stationary phase (20 d) in triplicate. There was only a statistical significant increase in lipid per biomass when *D. tertiolecta* was co-cultivated with *P. italicus* compared to *D. tertiolecta* grown axenically (One-tailed T-test, p-value = 0.034 marked with a *). Error bars are ± 1 SE.

D. tertiolecta grown axenically (control) reached 70 mg L^{-1} triolein equivalent lipid units per biomass (OD 680). The alga was unaffected by *A. macleodii* 9313c2, *V. cidicii* CIP 111013 or *P. inhibens* 2.10. However, lipid per biomass significantly increased when *D. tertiolecta* was grown with *P. italicus* (p-value = 0.034) (Figure 2.1).

2.4.2 Co-cultivation of *P. italicus* and *D. tertiolecta* has minimal effects on microalgal growth, photosynthetic health and biomass accumulation

To investigate the physiological effects cultivation with *P. italicus* had on *D. tertiolecta* over time, we compared the chl *a* fluorescence, PSII yield and algal biomass of the alga in a coculture with those of an algal monoculture (Figure 2.2).

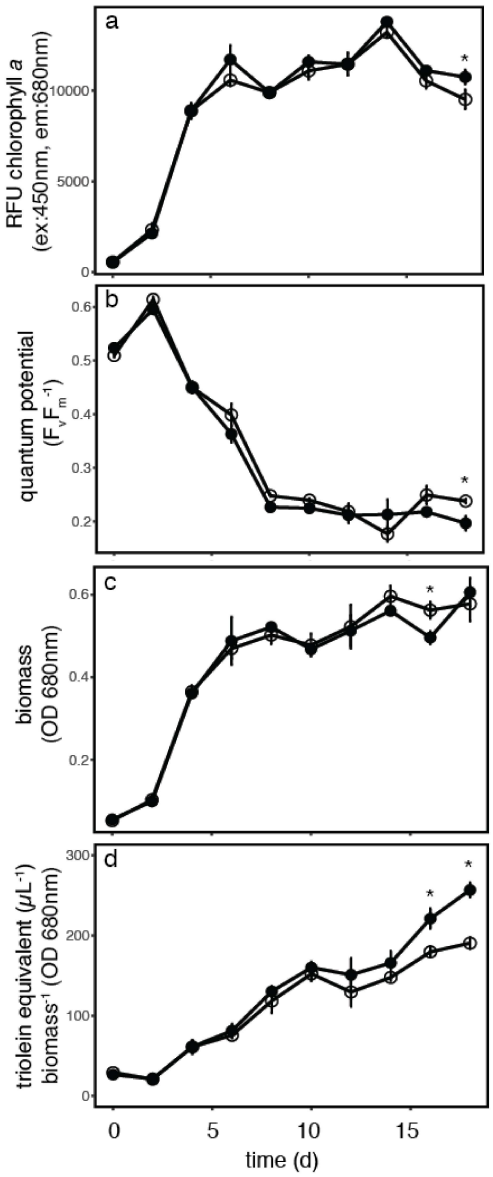


Figure 2.2. Physiological effects of co-cultivation with *Phaeobacter italicus* R11 on *Dunaliella tertiolecta* (CCMP 1320). Algae were grown in monoculture (white circles) and in coculture with *P. italicus* (black circles). The relative (a) chlorophyll *a* fluorescence, (b) potential quantum yield, (c) biomass (OD 680) and (d) lipid concentration in equivalent triolein units (Nile Red fluorescence) normalized with biomass (OD680) were measured. Mono- and co-culture were sampled in replicates every 2 d for 18 d. Cultures were significantly different from each other ($p<0.05$) when marked with a (*), as determined with Kruskal-Wallis rank sum test on non-parametric data. Error bars are ± 1 SE.

Chlorophyll *a* fluorescence is a relative proxy for microalgal density and was unaffected by *P. italicus* until 18 d (late stationary phase) when chl *a* was higher in the coculture than the algal control (Figure 2.2a). Potential quantum yield is a dimensionless expression of photosynthetic health and represents how effective photosystem II (PSII) is at pumping electrons down the election transport chain (Schreiber et al., 1986). It is calculated as $(F_m-F_o)/F_m$ where F_m represents maximum algal fluorescence and F_o is initial algal fluorescence. The control and coculture did not differ in photosynthetic health until 18 d, where the control was healthier than the coculture, although the decline in photosynthetic health was small compared the decline associated with the entrance into stationary phase from 2 to 8 d (Figure 2.2b). It is possible that lower photosynthetic health coupled with higher chl *a* fluorescence could be a physiological response to synthesis of additional chlorophyll pigments, compensating for overall lower functionality of PSII. Biomass did not differ between cultures with the exception of 16 d, when the coculture had less biomass than the control (Figure 2.2c). Through direct plating of coculture on media, it was determined that the bacterial population in coculture grew from 5 x 10^3 CFU per mL^{-1} on 0 d to a maximum of 1.00 x 10^5 CFU per mL^{-1} on 12 d (Figure 2.3). The bacterium completed exponential phase by 4 d and maintained its population density of 1 x 10^5 CFU per mL^{-1} throughout stationary phase. This maximum population density was maintained regardless of initial bacterial population inoculum.

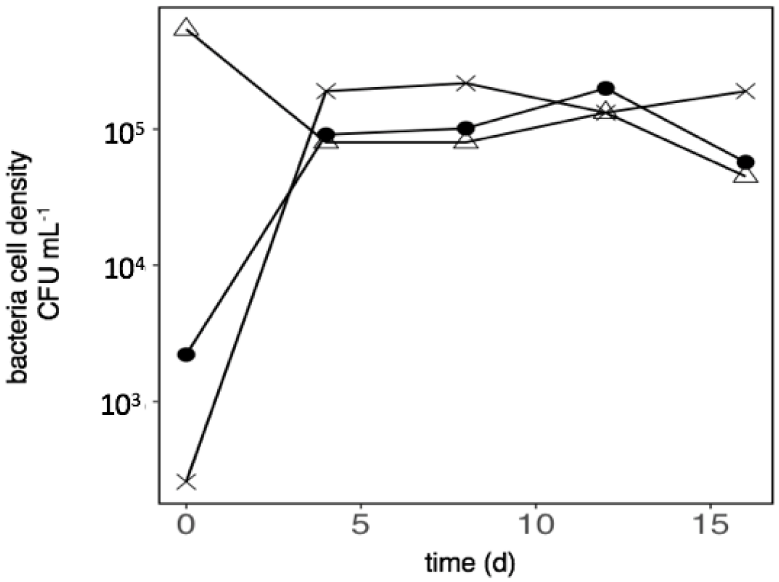


Figure 2.3. Effect of *Phaeobacter italicus* R11 inoculum concentration on population density at stationary phase when grown with *Dunaliella tertiolecta*. *P. italicus* was inoculated in a *D. tertiolecta* (CCMP 1320) monoculture (10^4 cells mL^{-1}) at concentrations of 10^6 CFU mL^{-1} (triangles), 5 x 10^3 CFU mL^{-1} (black circles) and 10^2 CFU mL^{-1} (cross). Cocultures were grown in L1-Si Instant Ocean media and bacterial counts were performed on each sample day.

2.4.3 *P. italicus* stimulates non-specific fatty acid accumulation in stationary phase *D. tertiolecta*

Monitoring lipid per biomass over time was performed to determine if the lipid increase of *D. tertiolecta* stimulated by *P. italicus* was continuous throughout algal growth or unique to the stationary phase (Figure 2.2d). During exponential phase (0 d – 4 d) and early stationary phase (4 d – 10 d) there was no difference in lipid per biomass of algal control and coculture. The coculture had higher lipid per biomass than algal control in late stationary phase with this trend beginning on 12 d and becoming statistically significant on 16 d. As the Nile Red fluorescence of *P. italicus* cultured alone was under the detection limit, this indicated that the algal lipid content per biomass in late stationary phase was increased ~25% due to the bacterial presence.

Comprehensive two-dimensional gas chromatography was performed to further investigate the effect *P. italicus* had on *D. tertiolecta*'s lipid metabolism. In order to determine if the bacterium was stimulating the general accumulation of lipids or specific fatty acid metabolites, we compared the composition of fatty acids is both cultures during late stationary phase. No significant difference was found in the proportions of different fatty acids synthesized (Figure 2.4).

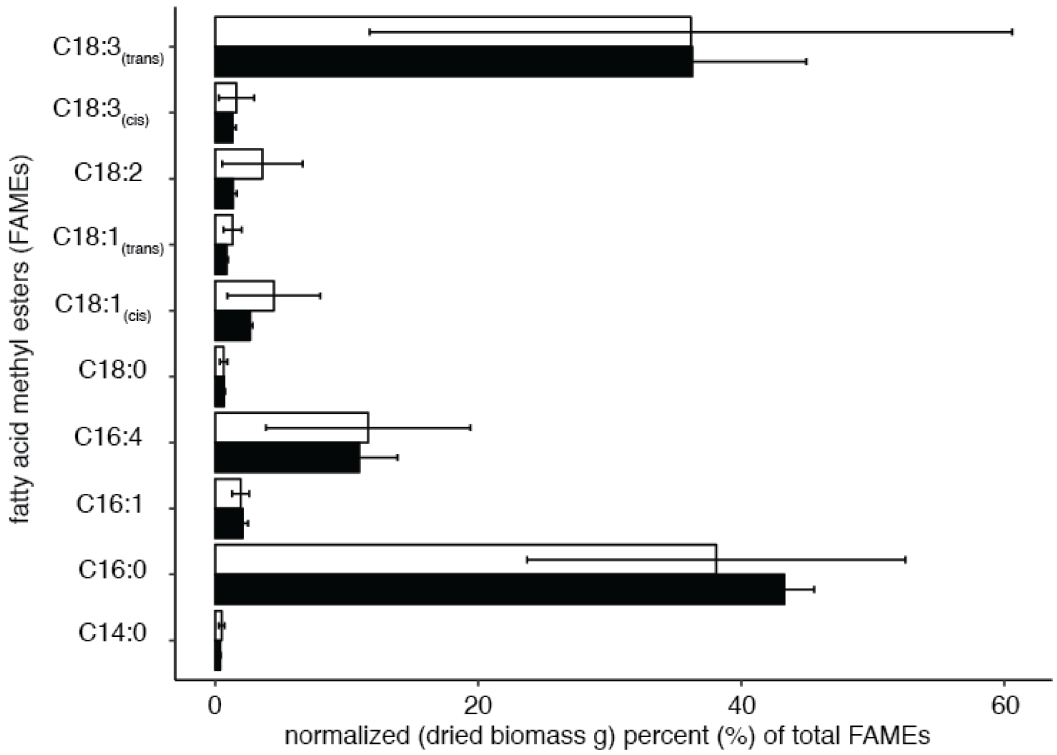


Figure 2.4. Fatty acid composition of *Dunaliella tertiolecta* (CCMP 1320) in mono- and co-culture with *Phaeobacter italicus* R11. Triplicate 100 mL cultures of *D. tertiolecta* (CCMP 1320) in mono- (white bars) and co-culture with *P. italicus* (black bars) where grown for 18 d before biomass (g) was collected. Fatty acids were extracted and analyzed through GCxGC-TOFMS. Percent of total peak areas of fatty acid methyl esters where normalized to dry weight of biomass of each replicate. No significant differences were found when non-parametric Kruskal-Wallis rank sum test was run. Error bars are ± 1 SE.

This indicated that the increased lipid per biomass stimulated by the bacterium was a general increase of fatty acid synthesis and not specific fatty acids. The most common fatty acids for the class *Chlorophyceae*, C16:0 and C18:3, were present in both samples along with

favourable fatty acids for biofuel feedstock (C16:1, C18:1 and C14:0) (Lang et al., 2011; Schenk et al., 2008).

Since nitrogen starvation is a common method to stimulate lipid accumulation in microalgae, a nitrite/nitrate analysis was performed to establish if the increased algal lipid accumulation in the coculture was due to nitrogen competition with *P. italicus*. The nitrogen concentration (nitrate and nitrite) of mono- and co-cultures was measured over time with a colorimetric assay (Figure 2.5).

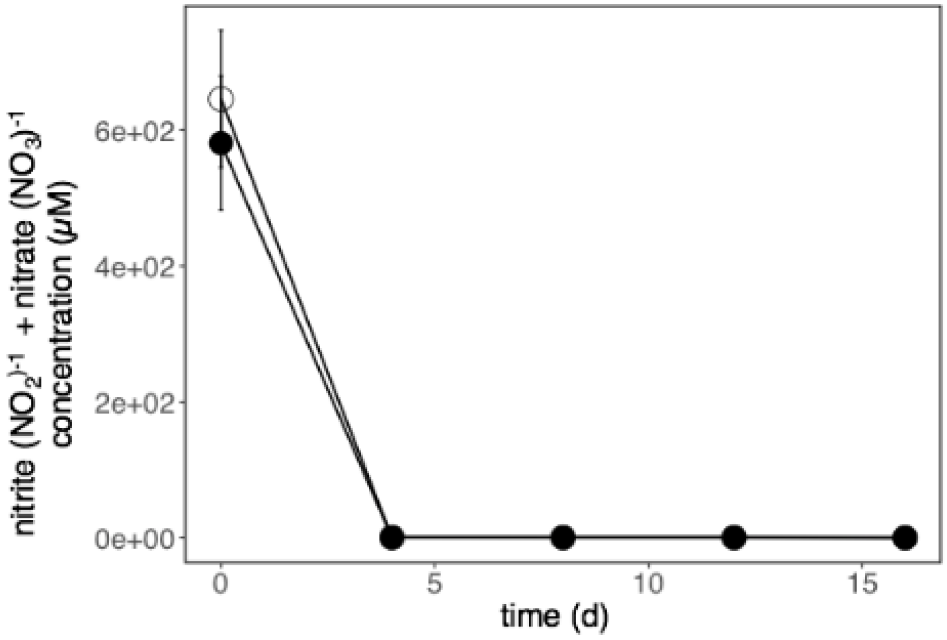


Figure 2.5. Nitrogen concentration in cultures of *Dunaliella tertiolecta* (CCMP 1320) grown alone and with *Phaeobacter italicus* R11. Control cultures of *D. tertiolecta* (CCMP 1320) (white) and cocultures of *D. tertiolecta* (CCMP 1320) and *P. italicus* (black) grown in L1-Si Instant Ocean media were assessed for total nitrate and nitrite concentration (μM) over time. No significant differences were found when non-parametric Kruskal-Wallis rank sum test was run. Error bars are ± 1 SE.

The nitrate and nitrite concentration of mono- and co-culture rapidly declined within the first days of growth and fell below the detection limit by 4 d. This steep decline was expected as it coincided with the exponential phase of growth in microalgae where nutrients would be rapidly consumed to support cellular reproduction. Nitrogen concentration remained below detection limit from 4 d into stationary growth phase. The nitrogen concentration did not significantly differ

between the algal control and coculture throughout the experiment. As increased lipid accumulation was only exhibited in coculture during late stationary phase (12 d – 18 d), it cannot be attributed to nitrogen starvation as both were under equal nitrogen limitation by 4 d. Furthermore, *D. tertiolecta* has been shown to prefer nitrate as a nitrogen source, the nitrogen source of the L1-Si medium, while *P. italicus*' genome indicates the bacterium does not have genes which are necessary for utilizing nitrate as nitrogen source (genome absent of EC 1.7.7.2, EC. 1.9.6.1, EC 1.7.1.1, EC 1.7.1.2, EC 1.7.1.3, EC 1.7.99.4) (Chen et al., 2011). Therefore, it is unlikely that there was competition for nitrogen, but rather that the bacteria and algae use different nitrogen sources, which they consume rapidly (<4 d). Both the control and coculture were likely under nitrogen limitation during early stationary phase (4 d – 10 d), and so the increased lipid accumulation detected in late stationary phase in the coculture is not due to nitrogen limitation but the presence and interaction with *P. italicus* itself.

2.4.4 Co-cultivation produces extracellular lipid vesicles

When lipid accumulates within algal cells, it is stored in spherical organelles called lipid droplets (Farese and Walther, 2009; Goold et al., 2015). To visualize lipid droplets within *D. tertiolecta* grown with and without *P. italicus,* epifluorescent microscopy was used with fluorescent stains for dsDNA (SYBR-Green) and lipids (Nile Red) (Figure 2.6).

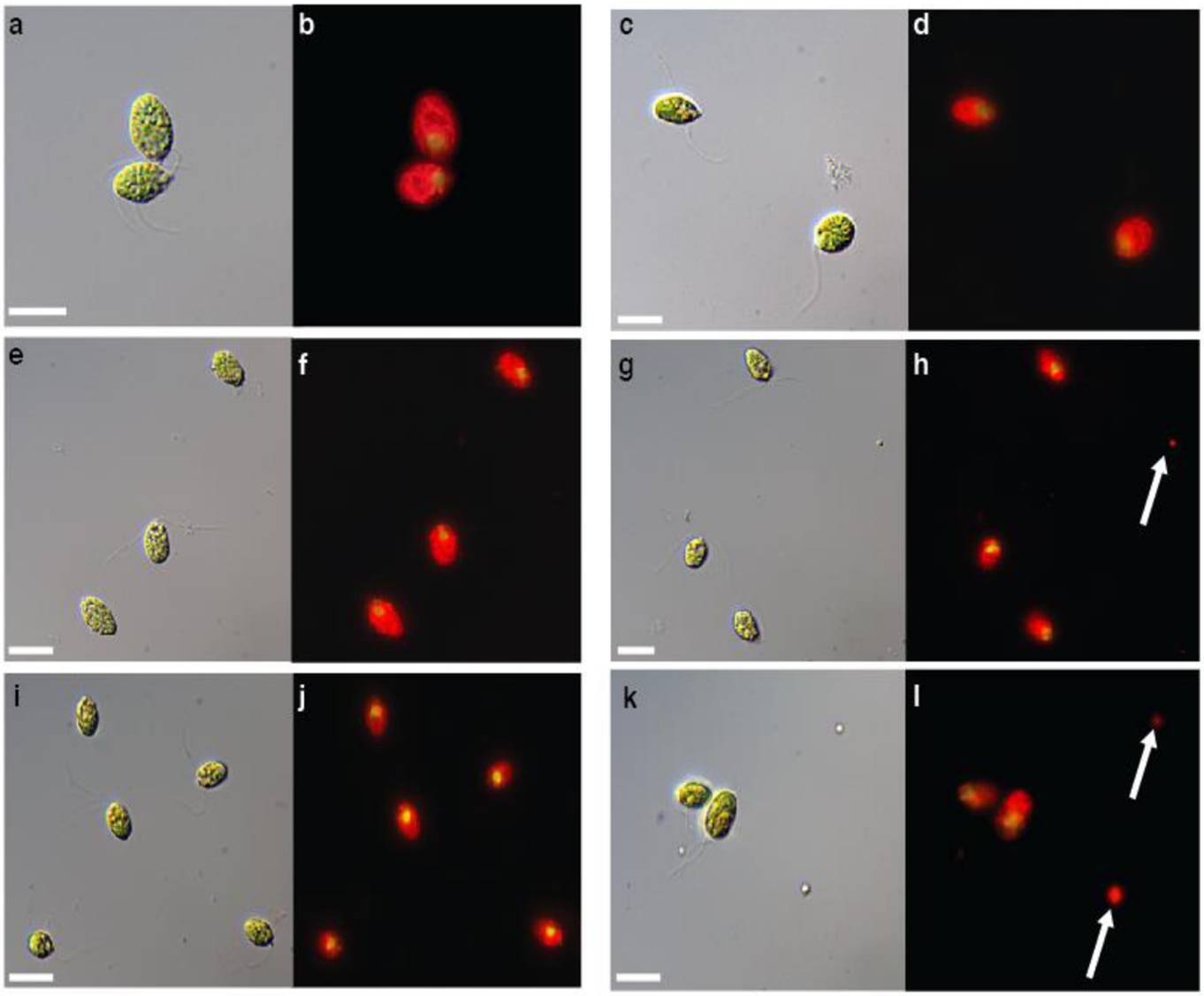
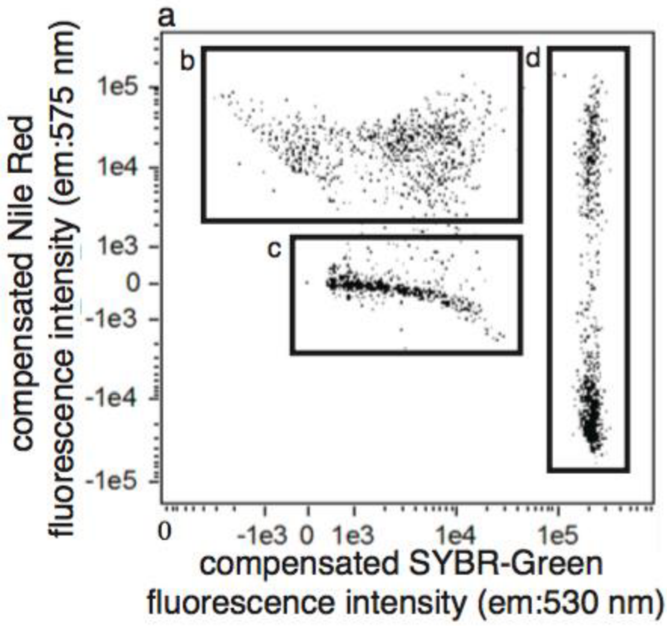


Figure 2.6. Production of extra-cellular lipid vesicles by *Dunaliella tertiolecta* (CCMP 1320) cocultured with *Phaeobacter italicus* R11. Differential interference contrast images (gray background) presented next to two overlaid fluorescent channels images taken simultaneously (black background). Fluorescent channels detected SYBR-Green 1 (DNA) (green - em: 520 nm) and Nile Red (lipid) (red/orange – em: 595 nm). Representative images of the algal controls at 4 d (a, b), 12 d (e, f) and 16 d (i, j) are presented with representative images of cocultures at 4 d (c, d), 12 d (g, h) and 16 d (k, l). Extracellular vesicles (white arrow) were visible at 12 d in co-cultivated samples and not in the control *D. tertiolecta*. Scale bars are 10 µm.

During exponential growth, algal cells in mono- and co-culture appeared identical (Figure 2.6a, b, c, d). Each cell had a nucleus and no lipid droplets. In early stationary phase (12 d) lipid

droplets accumulation was evident, consistent with nutrient limitation during stationary phase (Figure 2.6e, f). Extracellular vesicles, unattached to either algae or bacteria, were also present in coculture, staining with Nile Red but not with SYBR-Green (Figure 2.6g, h). It is unlikely that the lipid stained extracellular vesicles, referred to as lipid vesicles henceforth, were bacteria, as SYBR-Green fluorescence (DNA stain) was absent. The lipid-filled vesicles were spherical in shape, ranged from 1-3 μm in diameter. On 16 d, lipid vesicles were still absent in the control (Figure 2.6i, j) but increasing in numbers in coculture (Figure 2.6k, l).

As microscopy is 2D, it could miss DNA stained vesicles or misidentify bacterial cells attached to lipid vesicles. Therefore, fluorescent image flow cytometry with SYBR-Green and Nile Red fluorescent staining was performed for robust identification and comparison of *P. italicus* (DNA stained), *D. tertiolecta* (DNA and lipid stained) and extracellular lipid vesicles (lipid stained) populations (Figure 2.7).



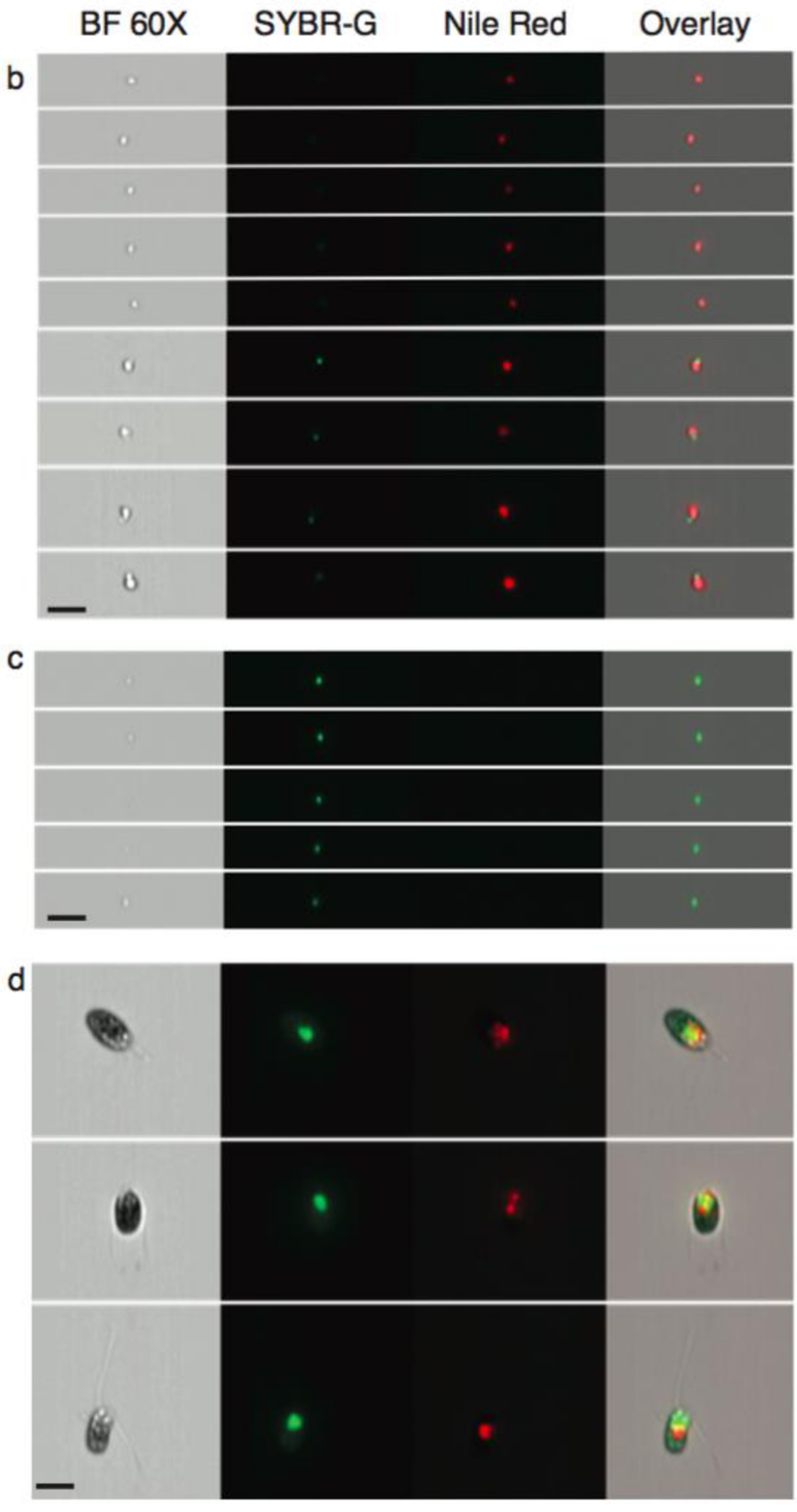


Figure 2.7. Imaging and enumeration of lipid vesicles using ImageStream flow cytometry of cocultured *Dunaliella tertiolecta* (CCMP 1320) and *Phaeobacter italicus R11*. Coculture samples were stained with Nile Red (lipid, em: 575 nm) and SYBR-Green (DNA, em: 520 nm). Different populations found in one representative sample of coculture at 12 d is shown were sorted using flow cytometry (a). Each of these populations was visualized after laser compensation and include lipid vesicles with high lipid intensity with and without bacteria attached (b); bacteria cells with DNA and no lipids (c); and *D. tertiolecta* cells with DNA (nucleus) and lipid (intracellular lipid droplets) (d). Intensity of SYBR-Green stain was increased to visualize bacteria. Image composites of representative objects in populations are displayed with in brightfield 60X magnification, with SYBR-Green fluorescence and Nile Red fluorescence overlaid on the image. Scale bar is 10 µm.

Three distinct populations could be identified after compensated flow-cytometric analysis dependent on lipid and nucleic acid content. Bacteria formed a distinct population and stained exclusively with SYBR-Green, indicating no bacterial lipid accumulation had occurred (Figure 2.7c). The lipid vesicle population stained intensely with Nile Red (Figure 2.7b). Some of the objects in the lipid vesicle population fall on the similar SYBR-Green axis as bacteria. Upon close inspection of these objects, it was determined that there was no true SYBR-Green fluorescence within the lipid vesicles and their position on the plot was because of two reasons. First, the lipid vesicles stained so intensely with Nile Red that it was read as SYBR-Green even with proper compensation, due to the inherent emission and excitement spectral overlap of these two stains. Second, for objects in the lipid vesicle population that fell higher on the SYBR-Green axis, brightfield images clearly depict separate bacterial cells (DNA stained) attached to lipid vesicles (lipid stained). Therefore, the DNA fluorescence attributed to some of this population is not within the lipid vesicle, but from the bacterium attached to it. Algal cells with no lipid droplets stained with SYBR-Green, forming a separate population from algal cells with lipid droplets and creating a bi-modal distribution of *D. tertiolecta* (Figure 2.7d). This distribution of the algal population on the lipid axis suggests that lipid accumulation could be a bi-modal phenotype in microalgae.

The lipid vesicles seen during epifluorescent and flow cytometric imaging were lipid-filled, spherical in shape and ranged from 1-3 μm in diameter. This description matches that of intracellular microalgal lipid droplets that form distinct spheres filled with lipids (Goold et al., 2015). Due to the morphological similarities between the extracellular lipid vesicles present in coculture and intracellular lipid droplets, it was hypothesized that *P. italicus* stimulated the release of *D. tertiolecta's* intracellular lipid droplets. Transmission electron microscopy was used to visualize lipid droplet localization in *D. tertiolecta* to gain insight on how *P. italicus* stimulates lipid vesicle release (Figure 2.8).

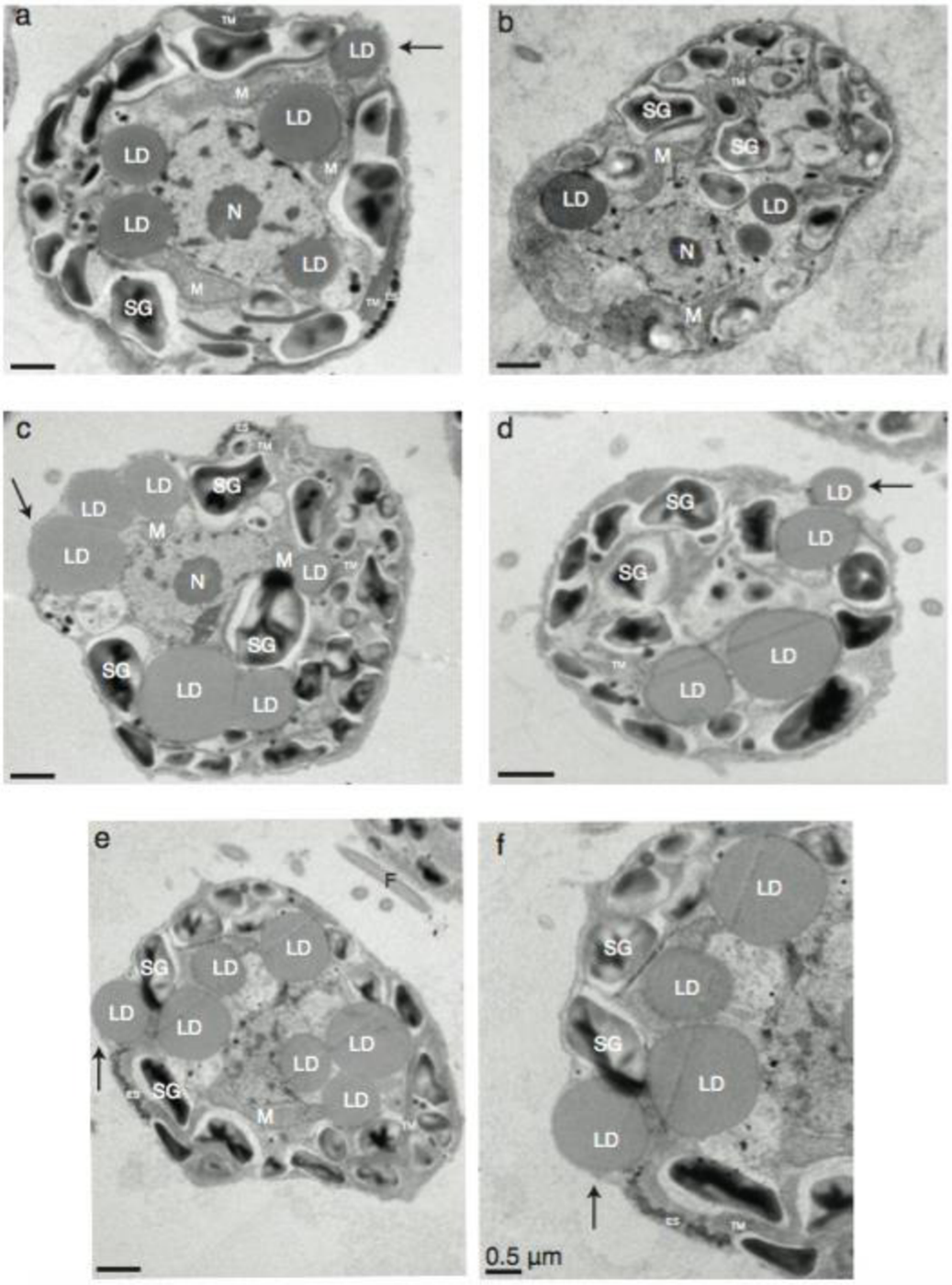


Figure 2.8. Transmission electron microscopy (TEM) of *Dunaliella tertiolecta* (CCMP 1320) to capture release of lipid vesicles in cells cultivated with and without *Phaeobacter italicus* R11. TEM images of *D. tertiolecta* monoculture (a, b) and in coculture with *P. italicus* (c, d, e, f). Samples were fixed at 12 and 14 d (stationary phase) and stained with 1% osmium tetroxide then embedded in SPURR resin. *D. tertiolecta* grown with *P. italicus* presented differences in localization of lipid bodies close to the cell wall (black arrow). LD: lipid droplets, SG: starch granule, ES: eye spot, N: nucleus, M: mitochondria, F: flagella, TM: thylakoid membrane. Scale bars (a-e) are 1 µm if not otherwise labelled.

Cultures of algal cells grown axenically revealed intracellular lipid droplet accumulation from 12-14 d (Figure 2.8a, b). In the control monoculture of algae, lipid droplets were occasionally located at the perimeter of the cell but still visibly surrounded by cell membrane and cell wall (black arrow) (Figure 2.7a). Algal cells grown with *P. italicus* also accumulated lipid droplets found at the perimeter of the algal cell (Figure 2.8c, d, e, f). Unlike control, these lipid droplets were found with thin visible cell membrane or cell wall surrounding them relative to algal control (black arrow) (Figure 2.8c, d, e, f).

2.4.5 Lipid vesicles dynamics in *D. tertiolecta-P. italicus* cocultures

Populations of algae and lipid vesicles were enumerated throughout the experiment with ImageStream flow cytometry to establish the abundance of lipid vesicles in the system and gain insight on the dynamics of these populations over time (Figure 2.9).

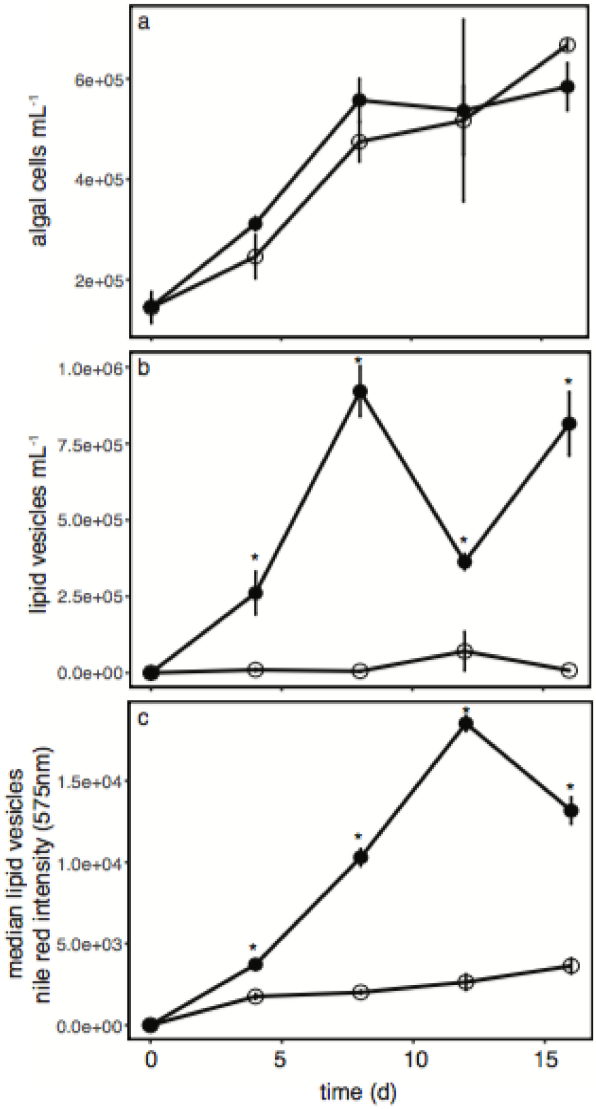


Figure 2.9. *Phaeobacter italicus* R11 stimulates vesicle formation in *Dunaliella tertiolecta* (CCMP 1320) but does not influence cell density over time. ImageStream flow cytometry was used to determined (a) algal cells and (b) lipid vesicles density, (c) lipid content in lipid vesicles in mono- and co- cultures. Error bars of triplicated data points are ± 1 SE. Mono- (white circles) and co-culture (black circles) were significantly different from each other ($p<0.05$) when marked with a (*) determined by a One-Way Analysis of Variance of parametric

The population of algal cells in both algal control and coculture increased during exponential phase and into early stationary phase (4 d - 8 d) before maintaining a density of 6 x 10^5 cells per mL in exponential phase (Figure 2.9a). No difference in cell numbers was detected between algal control and coculture throughout the experiment, indicating that *P. italicus* had no detectable effect on the growth of *D. tertiolecta*. Lipid vesicles per mL were enumerated over time in both algal control and coculture (Figure 2.9b).

In agreement with epifluorescence microscopy, lipid vesicles were only found in coculture where their concentration ranged from 2.5x10^5 to 1x10^6 lipid vesicles per mL from 8 d onwards. There was little variation between triplicates indicating their production in coculture is

tightly regulated. Number of vesicles peaked at 8 d, then dropped at 12 d before returning to their maximum concentration on 16 d. The decrease of lipid vesicle concentration at 12 d corresponds with a peak of median Nile Red fluorescence (Figure 2.9c). This increase in fluorescence, or total lipid, shows that while the number of lipid vesicles decreases, their lipid content increases, which could be explained by vesicle fusion.

2.5 Discussion

2.5.1 *P. italicus* and *D. tertiolecta* have a commensal interaction

P. italicus is an opportunistic pathogen to macro- and micro-algae (Case et al., 2011; Mayers et al., 2016). It can cause bleaching in older S type (scale-bearing) and C (coccolith-bearing) type cells of the microalga *Emiliania huxleyi* at 18 °C and rapid bleaching from temperature-enhanced virulence at 25 °C (Mayers et al., 2016). Through the analysis of *P. italicus*' exoproteome, it was determined that 30% of the proteins were affected by temperature, including amino acid and carbohydrate transporters that would aid in the consumption of any released nutrients following the host's death (Gardiner et al., 2017). Gardiner *et al*, (2015) also discovered temperature-regulated expression of novel RTX-like proteins, which are secreted virulence factors in other bacteria (Linhartová et al., 2010). Another virulence factor employed by *P. italicus* is a unique quorum sensing *luxR*-like regulators, *varR,* that is essential for the surface colonization, and subsequent infection, of macroalgae (Gardiner et al., 2015). *P. italicus* is also known to have cytolytic toxins that are known virulence factors in multiple gram-negative bacteria and cause host cell lysis (Fernandes et al., 2011).

In this study, *P. italicus* was not pathogenic to *D. tertiolecta* during co-cultivation at 18 °C, as PSII function (Figure 2.2) and cell density (Figure 2.8) during co-cultivation did not differ from the control. Furthermore, there was no loss of *D. tertiolecta* nuclear integrity during fluorescent microscopy or flow cytometry, hallmarks of apoptotic like- programmed cell death has observed in *P. italicus'* host (Segovia et al., 2003). This study was performed at 18 °C, a temperature at which *P. italicus* is not usually virulent. Previous studies of *P. italicus'* temperature-dependent virulence, pathogenesis was in some part due to the host's depleted defenses due to aging or environmental stress (Case et al., 2011). It has been shown that *D. tertiolecta* cultures are still viable in high temperature (28 °C) therefore it might not vulnerable to the attacks of *P. italicus* at these elevated temperatures (Sosik and Mitchell, 1994).

P. italicus population density increased when cocultured with *D. tertiolecta* (Figure 2.3), and so, presumably, there was exchange nutrients as the medium used had no carbon source. *P. italicus* would have to grow from organic carbon exudates, or lipid vesicles, from the photosynthetic *D. tertiolecta.* Since the population density of the alga, biomass and PSII function was unaffected by *P. italicus*, their relationship can be considered commensalism. The only observable effect that *P. italicus* had on *D. tertiolecta* was stimulation of lipid vesicles

production. However, the purpose of lipid vesicles is unknown and the role of vesicles in host physiology needs further elucidated to determine if this interaction should be considered symbiotic or parasitic. This work does demonstrate that *P. italicus* occupies a flexible niche extending beyond pathogenicity, and that its niche is host dependent.

2.5.2 *P. italicus-D. tertiolecta* interaction has potential application in biofuel

D. tertiolecta is a biofuel candidate because of its high lipid yield and fatty acid composition favorable to biodiesel production (Song et al., 2016; Tang et al., 2011). This species also lacks a rigid cell wall, which facilitates lipid extraction and genetic manipulation, and is tolerant to high salt concentrations, which can protect outdoor bioreactors to undesirable contamination and allow cultivation in salt water (Rismani-Yazdi et al., 2011). The interaction of *D. tertiolecta* with *P. italicus* described in this study has two potential applications in the biofuel industry.

First, the co-cultivation of these microorganisms increase lipid yield by ~25% in late stationary phase. This increase, stimulated by *P. italicus,* is of general lipid metabolism that include fatty acids favorable for biofuel production (C16:0, C16:1, and C14:0) (Figure 2.4). The increase of lipid yield, with no effect on biomass accumulation, is an attractive characteristic in biofuel producing microalgae as it increases overall biofuel production. Secondly, this interaction produces lipid vesicles that are already separate from the algal biomass, which could reduce the energy required during harvesting and efficiency during transesterification. Harvesting accounts for the majority of energy used during biofuel production, which leads to an energy imbalance impeding commercialization of the industry (Gallagher, 2011). Lipid vesicles are found in the extracellular space in high abundance (Figure 2.9), and could be concentrated through chemical flocculation or filtration based on size, buoyancy or hydrophobicity differences from algal cells. The presence of *P. italicus* does not affect the growth or health (Figure 2.2, Figure 2.9) of *D. tertiolecta* and therefore the same culture can be used for subsequent generations of lipid vesicle harvesting, a process similar to ‘milking’ diatom cultures (Vinayak et al., 2015).

2.5.3 *P. italicus* alters *D. tertiolecta’s* lipid production

Lipid accumulation in microalgae can be influenced by various interaction occurring in polymicrobial communities. If a microalga is in a mutualistic relationship with a bacterium, lipid

accumulation could be delayed due to a prolonged exponential phase. Potentially, this indicates a mutualistic interaction between these microorganisms which most likely alleviated nutrient stress and, as such, reduced lipid accumulation. Pathogenic interactions would limit algal biomass through algal cell lysis or stimulating programmed cell-death, and thus impeding algal lipid accumulation. Bacteria can also compete with the alga for nutrients, thus stimulating stress that leads to lipid accumulation. A parasitic bacteria might consume host's lipids resulting in decreased lipid accumulation, or stimulate lipid accumulation by depleting the host of essential nutrients.

Relationships between microalgae and bacteria that promote biomass accumulation, stimulate growth or increase lipid yield have been previously described (Berthold et al., 2019; Fuentes et al., 2016; Ramanan et al., 2016). An increase of chl *a* and *b*, lutein and violoaxanthin pigments, lipid content and an increase from four to eight fatty acids was detected in *Chlorella vulgaris* and *Chlorella sorokiniana* when cultivated with *Azospirillum brasilense* (de-Bashan et al., 2002). Although *Azospirillum brasilense* is known to promote growth in other microalgae, interactions between the microorganisms that lead to these increases were not described (de-Bashan et al., 2002). Another example of algal-bacterial interactions that affects lipid production is between an oleaginous green microalgae strain, *Ankistrodesmus* sp. strain SP2-15, and the bacterium *Rhizobium* strain 10II (Do Nascimento et al., 2013). The cocultivation of these two microorganisms resulted in an increase of up to 30% in lipid accumulation as a possible result of bacterial production of indol-3-acetic acid and/or vitamin B12 (Do Nascimento et al., 2013). Although studies report increases in lipid accumulation, it is uncommon that the cocultures are imaged in order to describe lipid droplet morphology, which are the location of neutral lipid accumulation. One study did detect a 2.5 fold reduction of lipid droplets in haptophyte *E. huxleyi* when cocultured with another roseobacter, *P. inhibens* 2.10 (Segev et al., 2016). Quantification of algae containing lipid droplets was performed with algal control and coculture at two separate time points through counting algae during fluorescent imaging using Nile Red dye (Segev et al., 2016).

Here we have shown that *P. italicus* stimulates lipid accumulation in stationary growth phase of *D. tertiolecta* (Figure 2.2). The lipid accumulation during co-cultivation corresponds to the presence of extracellular lipid vesicles (Figure 2.9). The novelty of this study is not the lipid accumulation of a green microalgae as a result of bacterial interaction, but the production of extracellular lipid vesicles.

2.5.4 *D. tertiolecta* produces extracellular lipid vesicles

In this model, we demonstrated that the lipid per biomass ratio was increased due to a production of lipid vesicles that were released into the extracellular space. These vesicles ranged from 1-3 μm in diameter, were spherical in shape and stained intensely with Nile Red dye (Figure 2.6). Lipid vesicles were abundant in the system, maintaining a 1:1 ratio of lipid vesicles to algae during stationary phase of growth (Figure 2.9).

It is highly unlikely that the lipid vesicles described were lipid-filled *P. italicus,* as they did not display DNA fluorescence during epifluorescence imaging (Figure 2.6) or ImageStream flow cytometry independent of attached bacterial cells (Figure 2.7). The lipid vesicles observed ranged from 1-3 μm, which is up to six times the size of *P. italicus* bacterium (0.5 μm). Furthermore, TAG accumulation in bacteria is an uncommon characteristic shared by select strains in the actinomycetes group and has never been observed in the *Roseobacter* clade (Alvarez and Steinbüchel, 2002). The lack of lipid accumulating phenotype in the *Roseobacter* clade and absence of DNA content in vesicles, we concluded that the Nile red staining population was composed of algal-derived vesicles and were not bacteria or bacteria-derived.

There are multiple types of extracellular vesicles (EV) that are usually categorized by size and cellular origin (Zamith-Miranda et al., 2018). For example, bacteria create extracellular vesicles by budding off their cell membrane (Kulp and Kuehn, 2010). The resulting vesicles can contribute to quorum sensing, cellular defence and horizontal gene transfer processes (Manning and Kuehn, 2011; Toyofuku et al., 2017). *Prochlorococcus* is an abundant cyanobacteria that was discovered to release lipid vesicles (<200 nm) that contain protein, DNA and RNA (Biller et al., 2014). These vesicles were able to sustain the bacterial growth and possibly serve as a gene reservoir in the open ocean (Biller et al., 2014). There is a gap in knowledge for extracellular vesicles production in eukaryotic microorganisms, especially in marine microalgae (Yáñez-Mó et al., 2015). In fungus and yeast, it has been confirmed that EVs are used for communication and to modulate host-pathogen interactions (Zamith-Miranda et al., 2018). The only characterized study of eukaryotic microalgal EV production is on haptophyte *Emiliania huxleyi* (Schatz et al., 2017). When *E. huxleyi* is infected by viruses, it will produce info-chemicals that stimulate EV production and increase virion uptake in non-infected cells (Schatz et al., 2017). The EVs are exploited by the viruses in the system to prolong their life and increase infectivity (Schatz et al., 2017). Currently, there are no published evidence of

extracellular vesicles in green microalgae as a phenotypic characteristic in result of a viral infection or bacterial interaction (Gill et al., 2019).

Lipid vesicles produced during co-cultivation of *P. italicus* and *D. tertiolecta* were larger than eukaryotic EVs studied (>500 nm) and were morphologically similar to algal lipid droplets. During co-cultivation, lipid droplets of *D. tertiolecta* were positioned on the perimeter of the cell with only thin membrane separating the lipid droplet and extracellular space (Figure 2.8). This could be a snapshot of lipid droplets extrusion into the extracellular space stimulated by *P. italicus*. The colonial green algae *Botryococcus braunii* is the only microalga known to accumulate lipids in the immediate environment, but this is a unique growth characteristic has never been attributed to bacterial interaction, and the lipids are not fully released into the extracellular space (Hirose et al., 2013; Lee et al., 2015). If the lipid vesicles are excreted lipid droplets, this process did not decrease microalgal photosynthetic health and therefore it is unlikely that the release was a consequence of algal cellular lysis via bacterial algicides. *P. italicus* has the ability to synthesize indol-3-acetic-acid, which is a plant auxin with growth-promoting properties in some microalgal species (Amin et al., 2015; Fernandes et al., 2011). This bioactive has also been demonstrated to show increased cell membrane permeability in some cell types of haptophyte *Emiliania huxleyi* (Labeeuw et al., 2016). Perhaps this attribute, along with *D. tertiolecta*'s lack of rigid cell wall, facilitated the release of the lipid droplets. However, organelle trafficking is regulated by the cell and this mechanism could therefore not be completely passive. The function and mechanism of production and/or release of the lipid droplets are still unclear, and further investigation into the lipid vesicles proteome or the systems metabolome would be insightful.

2.5.5 Conclusion

Cocultivation of *D. tertiolecta* and *P. italicus* produced extracellular lipid vesicles and increased lipid per biomass of coculture by ~25% in stationary phase without changing their fatty acids makeup. This, to our knowledge, is the first example of bacterial stimulation of lipid vesicle release in eukaryotic microalgae. Although the production of lipid vesicles from a green microalgae is a gateway to many questions in marine microbial ecology, the most obvious application is in biofuel production as a way to expedite lipid harvesting.

2.6 References

Alvarez H, Steinbüchel A (2002) Triacylglycerols in prokaryotic microorganisms. Applied Microbiology and Biotechnology 60:367–376 . doi: 10.1007/s00253-002-1135-0

Amin S, Green DH, Hart MC, Kupper FC, Sunda WG, Carrano CJ (2009) Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism. Proceedings of the National Academy of Sciences 106:17071–17076 . doi: 10.1073/pnas.0905512106

Amin SA, Hmelo LR, van Tol HM, Durham BP, Carlson LT, Heal KR, Morales RL, Berthiaume CT, Parker MS, Djunaedi B, Ingalls AE, Parsek MR, Moran MA, Armbrust EV (2015) Interaction and signaling between a cosmopolitan phytoplankton and associated bacteria. Nature 522:98–101 . doi: 10.1038/nature14488

Amin SA, Parker MS, Armbrust EV (2012) Interactions between diatoms and bacteria. Microbiology and Molecular Biology Reviews 76:667–684 . doi: 10.1128/MMBR.00007-12

Atabani AE, Silitonga AS, Badruddin IA, Mahlia TMI, Masjuki HH, Mekhilef S (2012) A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renewable and Sustainable Energy Reviews 16:2070–2093 . doi: 10.1016/j.rser.2012.01.003

Berthold DE, Shetty KG, Jayachandran K, Laughinghouse HD, Gantar M (2019) Enhancing algal biomass and lipid production through bacterial co-culture. Biomass and Bioenergy 122:280–289 . doi: 10.1016/j.biombioe.2019.01.033

Bertozzini E, Galluzzi L, Penna A, Magnani M (2011) Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. Journal of Microbiological Methods 87:17–23 . doi: 10.1016/j.mimet.2011.06.018

Biller SJ, Schubotz F, Roggensack SE, Thompson AW, Summons RE, Chisholm SW (2014) Bacterial vesicles in marine ecosystems. Science 343:183–186 . doi: 10.1126/science.1243457

Blumberg LM, Klee MS (2000) Optimal heating rate in gas chromatography. Journal of Microcolumn Separations 12:508–514 . doi: 10.1002/1520-667X(2000)12:9<508::AID-MCS5>3.0.CO;2-Y

Bramucci AR, Case RJ (2019) *Phaeobacter inhibens* induces apoptosis-like programmed cell death in calcifying *Emiliania huxleyi*. Scientific Reports 9:5215 . doi: 10.1038/s41598-018-36847-6

Bramucci AR, Labeeuw L, Mayers TJ, Saby JA, Case RJ (2015) A small volume bioassay to assess bacterial/phytoplankton co-culture using WATER-Pulse-Amplitude-Modulated (WATER-PAM) Fluorometry. Journal of Visual Experiments. doi: 10.3791/52455

Bramucci AR, Labeeuw L, Orata FD, Ryan EM, Malmstrom RR, Case RJ (2018) The bacterial symbiont *Phaeobacter inhibens* shapes the life history of its algal host *Emiliania huxleyi*. Frontiers in Marine Science 5: . doi: 10.3389/fmars.2018.00188

Case RJ, Longford SR, Campbell AH, Low A, Tujula N, Steinberg PD, Kjelleberg S (2011) Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. Environmental Microbiology 13:529–537 . doi: 10.1111/j.1462-2920.2010.02356.x

Cavonius LR, Carlsson N-G, Undeland I (2014) Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. Analytical and Bioanalytical Chemistry 406:7313–7322 . doi: 10.1007/s00216-014-8155-3

Chen M, Tang H, Ma H, Holland TC, Ng KYS, Salley SO (2011) Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology 102:1649–1655 . doi: 10.1016/j.biortech.2010.09.062

Cho D-H, Ramanan R, Heo J, Lee J, Kim B-H, Oh H-M, Kim H-S (2015) Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. Bioresource Technology 175:578–585 . doi: 10.1016/j.biortech.2014.10.159

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438:90–93 . doi: 10.1038/nature04056

de-Bashan LE, Bashan Y, Moreno M, Lebsky VK, Bustillos JJ (2002) Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Canadian Journal of Microbiology 48:514–521 . doi: 10.1139/w02-051

Demuez M, González-Fernández C, Ballesteros M (2015) Algicidal microorganisms and secreted algicides: New tools to induce microalgal cell disruption. Biotechnology Advances 33:1615–1625 . doi: 10.1016/j.biotechadv.2015.08.003

Do Nascimento M, Dublan M de los A, Ortiz-Marquez JCF, Curatti L (2013) High lipid productivity of an Ankistrodesmus – Rhizobium artificial consortium. Bioresource Technology 146:400–407 . doi: 10.1016/j.biortech.2013.07.085

Dong T, Knoshaug EP, Pienkos PT, Laurens LML (2016) Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. Applied Energy 177:879–895 . doi: 10.1016/j.apenergy.2016.06.002

Durham BP, Sharma S, Luo H, Smith CB, Amin SA, Bender SJ, Dearth SP, Van Mooy BAS, Campagna SR, Kujawinski EB, Armbrust EV, Moran MA (2015) Cryptic carbon and sulfur cycling between surface ocean plankton. Proceedings of the National Academy of Sciences 112:453–457 . doi: 10.1073/pnas.1413137112

Falkowski PG (1994) The role of phytoplankton photosynthesis in global biogeochemical cycles. Photosynthesis Research 39:235–258

Farese RV, Walther TC (2009) Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 139:855–860 . doi: 10.1016/j.cell.2009.11.005

Fernandes N, Case RJ, Longford SR, Seyedsayamdost MR, Steinberg PD, Kjelleberg S, Thomas T (2011) Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*. PLoS ONE 6:e27387 . doi: 10.1371/journal.pone.0027387

Field CB (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281:237–240 . doi: 10.1126/science.281.5374.237

Franz AK, Danielewicz MA, Wong DM, Anderson LA, Boothe JR (2013) Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity. ACS Chemical Biology 8:1053–1062 . doi: 10.1021/cb300573r

Fuentes JL, Garbayo I, Cuaresma M, Montero Z, González-Del-Valle M, Vílchez C (2016) Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. Marine Drugs 14:100–100 . doi: 10.3390/md14050100

Gallagher BJ (2011) The economics of producing biodiesel from algae. Renewable Energy 36:158–162 . doi: 10.1016/j.renene.2010.06.016

Gardiner M, Bournazos AM, Maturana-Martinez C, Zhong L, Egan S (2017) Exoproteome analysis of the seaweed pathogen *Nautella italica* R11 reveals temperature-dependent regulation of RTX-like proteins. Frontiers in Microbiology 8:1203–1203 . doi: 10.3389/fmicb.2017.01203

Gardiner M, Fernandes ND, Nowakowski D, Raftery M, Kjelleberg S, Zhong L, Thomas T, Egan S (2015) VarR controls colonization and virulence in the marine macroalgal pathogen *Nautella italica* R11. Frontiers in Microbiology 6:1130 . doi: 10.3389/fmicb.2015.01130

Geng H, Belas R (2010) Molecular mechanisms underlying roseobacter–phytoplankton symbioses. Current Opinion in Biotechnology 21:332–338 . doi: 10.1016/j.copbio.2010.03.013
Gill S, Catchpole R, Forterre P (2019) Extracellular membrane vesicles in the three domains of life and beyond. FEMS Microbiology Reviews 43:273–303 . doi: 10.1093/femsre/fuy042
Goold H, Beisson F, Peltier G, Li-Beisson Y (2015) Microalgal lipid droplets: composition, diversity, biogenesis and functions. Plant Cell Reports 34:545–555 . doi: 10.1007/s00299-014-1711-7
Greenspan P (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. The Journal of Cell Biology 100:965–973 . doi: 10.1083/jcb.100.3.965
Harwood JL, Guschina IA (2009) The versatility of algae and their lipid metabolism. Biochimie 91:679–684 . doi: 10.1016/J.BIOCHI.2008.11.004
Hirose M, Mukaida F, Okada S, Noguchi T (2013) Active hydrocarbon biosynthesis and accumulation in a green alga, *Botryococcus braunii* (Race A). Eukaryotic Cell 12:1132–1141 . doi: 10.1128/EC.00088-13
Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54:621–639 . doi: 10.1111/j.1365-313X.2008.03492.x
Klee MS, Blumberg LM (2002) Theoretical and practical aspects of fast gas chromatography and method translation. Journal of Chromatographic Science 40:234–247 . doi: 10.1093/chromsci/40.5.234
Kulp A, Kuehn MJ (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annual Review of Microbiology 64:163–184 . doi: 10.1146/annurev.micro.091208.073413
Labeeuw L, Khey J, Bramucci AR, Atwal H, de la Mata AP, Harynuk J, Case RJ (2016) Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells. Frontiers in Microbiology 7:828–828 . doi: 10.3389/fmicb.2016.00828
Lang I, Hodac L, Friedl T, Feussner I (2011) Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biology 11:124 . doi: 10.1186/1471-2229-11-124
Lee C-H, Chae H-S, Lee S-H, Kim HS (2015) Growth characteristics and lipid content of three Korean isolates of *Botryococcus braunii* (Trebouxiophyceae). Journal of Ecology and Environment 38:67–74 . doi: 10.5141/ecoenv.2015.007
Lima-Mendez G, Faust K, Henry N, Decelle J, Colin S, Carcillo F, Chaffron S, Ignacio-Espinosa JC, Roux S, Vincent F, Bittner L, Darzi Y, Wang J, Audic S, Berline L, Bontempi G, Cabello AM, Coppola L, Cornejo-Castillo FM, d'Ovidio F, De Meester L, Ferrera I, Garet-Delmas M-J, Guidi L, Lara E, Pesant S, Royo-Llonch M, Salazar G, Sanchez P, Sebastian M, Souffreau C, Dimier C, Picheral M, Searson S, Kandels-Lewis S, Tara Oceans coordinators, Gorsky G, Not F, Ogata H, Speich S, Stemmann L, Weissenbach J, Wincker P, Acinas SG, Sunagawa S, Bork P, Sullivan MB, Karsenti E, Bowler C, de Vargas C, Raes J (2015) Determinants of community structure in the global plankton interactome. Science 348:1262073–1262073 . doi: 10.1126/science.1262073
Linhartová I, Bumba L, Mašín J, Basler M, Osička R, Kamanová J, Procházková K, Adkins I, Hejnová-Holubová J, Sadílková L, Morová J, Šebo P (2010) RTX proteins: a highly diverse family secreted by a common mechanism. FEMS Microbiology Reviews 34:1076–1112 . doi: 10.1111/j.1574-6976.2010.00231.x
Manning AJ, Kuehn MJ (2011) Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiology 11:258 . doi: 10.1186/1471-2180-11-258

Martens T, Gram L, Grossart H-P, Kessler D, Müller R, Simon M, Wenzel SC, Brinkhoff T (2007) Bacteria of the Roseobacter Clade Show Potential for Secondary Metabolite Production. Microbial Ecology 54:31–42 . doi: 10.1007/s00248-006-9165-2

Mayers TJ, Bramucci AR, Yakimovich KM, Case RJ (2016) A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*. Frontiers in Microbiology 7:892 . doi: 10.3389/fmicb.2016.00892

Milici M, Deng Z-L, Tomasch J, Decelle J, Wos-Oxley ML, Wang H, Jáuregui R, Plumeier I, Giebel H-A, Badewien TH, Wurst M, Pieper DH, Simon M, Wagner-Döbler I (2016) Co-occurrence analysis of microbial taxa in the Atlantic Ocean reveals high connectivity in the free-living bacterioplankton. Frontiers in Microbiology 7: . doi: 10.3389/fmicb.2016.00649

Mondello L, Casilli A, Tranchida PQ, Dugo P, Dugo G (2003) Detailed analysis and group-type separation of natural fats and oils using comprehensive two-dimensional gas chromatography. Journal of Chromatography A 1019:187–196 . doi: 10.1016/S0021-9673(03)01130-0

Mubarak M, Shaija A, Suchithra TV (2015) A review on the extraction of lipid from microalgae for biodiesel production. Algal Research 7:117–123 . doi: 10.1016/j.algal.2014.10.008

Ndikubwimana T, Zeng X, Murwanashyaka T, Manirafasha E, He N, Shao W, Lu Y (2016) Harvesting of freshwater microalgae with microbial bioflocculant: a pilot-scale study. Biotechnology for Biofuels 9:47 . doi: 10.1186/s13068-016-0458-5

Orata FD, Xu Y, Gladney LM, Rishishwar L, Case RJ, Boucher Y, Jordan IK, Tarr CL (2016) Characterization of clinical and environmental isolates of *Vibrio cidicii* sp. nov., a close relative of *Vibrio navarrensis*. International Journal of Systematic and Evolutionary Microbiology 66:4148–4155 . doi: 10.1099/ijsem.0.001327

Padmaperuma G, Kapoore RV, Gilmour DJ, Vaidyanathan S (2018) Microbial consortia: a critical look at microalgae co-cultures for enhanced biomanufacturing. Critical Reviews in Biotechnology 38:690–703 . doi: 10.1080/07388551.2017.1390728

Powell RJ, Hill RT (2013) Rapid aggregation of biofuel-producing algae by the bacterium Bacillus sp. Strain RP1137. Applied and Environmental Microbiology 79:6093–6101 . doi: 10.1128/AEM.01496-13

Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Applied Microbiology and Biotechnology 65:635–648 . doi: 10.1007/s00253-004-1647-x

Ramanan R, Kang Z, Kim B-HH, Cho D-HH, Jin L, Oh H-MM, Kim H-SS (2015) Phycosphere bacterial diversity in green algae reveals an apparent similarity across habitats. Algal Research 8:140–144 . doi: 10.1016/j.algal.2015.02.003

Ramanan R, Kim B-H, Cho D-H, Oh H-M, Kim H-S (2016) Algae–bacteria interactions: Evolution, ecology and emerging applications. Biotechnology Advances 34:14–29 . doi: 10.1016/j.biotechadv.2015.12.003

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of Cell Biology 17:208–212 . doi: 10.1083/jcb.17.1.208

Ryckebosch E, Muylaert K, Foubert I (2012) Optimization of an analytical procedure for extraction of lipids from microalgae. Journal of the American Oil Chemists' Society 89:189–198 . doi: 10.1007/s11746-011-1903-z

Schatz D, Rosenwasser S, Malitsky S, Wolf SG, Feldmesser E, Vardi A (2017) Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga. Nature Microbiology 2:1485–1492 . doi: 10.1038/s41564-017-0024-3

Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C, Kruse O, Hankamer B (2008) Second generation biofuels: High-efficiency microalgae for biodiesel production. BioEnergy Research 1:20–43 . doi: 10.1007/s12155-008-9008-8

Schreiber U, Schliwa U, Bilger W (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynthesis Research 10:51–62 . doi: 10.1007/BF00024185
Segev E, Castañeda IS, Sikes EL, Vlamakis H, Kolter R (2016) Bacterial influence on alkenones in live microalgae. Journal of Phycology 52:125–130 . doi: 10.1111/jpy.12370
Segovia M, Haramaty L, Berges JA, Falkowski PG (2003) Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the evolution of apoptosis in higher plants and metazoans. Plant Physiology 132:99–105 . doi: 10.1104/pp.102.017129
Seyedsayamdost MR, Case RJ, Kolter R, Clardy J (2011) The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. Nature Chemistry 3:331–335 . doi: 10.1038/nchem.1002
Simó R (2001) Production of atmospheric sulfur by oceanic plankton: biogeochemical, ecological and evolutionary links. Trends in Ecology & Evolution 16:287–294 . doi: 10.1016/S0169-5347(01)02152-8
Singh P, Kumari S, Guldhe A, Misra R, Rawat I, Bux F (2015) Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. Renewable and Sustainable Energy Reviews 55:1–16 . doi: 10.1016/j.rser.2015.11.001
Solovchenko AE (2012) Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. Russian Journal of Plant Physiology 59:167–176 . doi: 10.1134/S1021443712020161
Song D, Xi B, Sun J (2016) Characterization of the growth, chlorophyll content and lipid accumulation in a marine microalgae *Dunaliella tertiolecta* under different nitrogen to phosphorus ratios. Journal of Ocean University of China 15:124–130 . doi: 10.1007/s11802-016-2797-z
Sosik HM, Mitchell BG (1994) Effects of temperature on growth, light absorption, and quantum yield in *Dunaliella Tertiolecta* (chlorophyceae). Journal of Phycology 30:833–840 . doi: 10.1111/j.0022-3646.1994.00833.x
Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. Journal of Bioscience and Bioengineering 101:87–96 . doi: 10.1263/jbb.101.87
Tang H, Abunasser N, Garcia MED, Chen M, Simon Ng KY, Salley SO (2011) Potential of microalgae oil from *Dunaliella tertiolecta* as a feedstock for biodiesel. Applied Energy 88:3324–3330 . doi: 10.1016/j.apenergy.2010.09.013
Thompson GA (1996) Lipids and membrane function in green algae. Biochimica et Biophysica Acta 1302:7–45
Toyofuku M, Morinaga K, Hashimoto Y, Uhl J, Shimamura H, Inaba H, Schmitt-Kopplin P, Eberl L, Nomura N (2017) Membrane vesicle-mediated bacterial communication. The ISME Journal 11:1504–1509 . doi: 10.1038/ismej.2017.13
Ummalyma SB, Gnansounou E, Sukumaran RK, Sindhu R, Pandey A, Sahoo D (2017) Bioflocculation: An alternative strategy for harvesting of microalgae- An overview. Bioresource Technology 242:227–235 . doi: 10.1016/j.biortech.2017.02.097
van Kooten O, Snel JFH (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynthesis Research 25:147–150 . doi: 10.1007/BF00033156
Vinayak V, Manoylov K, Gateau H, Blanckaert V, Hérault J, Pencréac'h G, Marchand J, Gordon R, Schoefs B (2015) Diatom milking: A review and new approaches. Marine Drugs 13:2629–2665 . doi: 10.3390/md13052629
Wang R, Xue S, Zhang D, Zhang Q, Wen S, Kong D, Yan C, Cong W (2015) Construction and characteristics of artificial consortia of *Scenedesmus obliquus* -bacteria for *S. obliquus* growth and lipid production. Algal Research 12:436–445 . doi: 10.1016/j.algal.2015.10.002
Yáñez-Mó M, Siljander PR-M, Andreu Z, Bedina Zavec A, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM,

Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NHH, Hendrix A, Kierulf P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers E-M, Laitinen S, Lässer C, Lener T, Ligeti E, Linē A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-'t Hoen ENM, Nyman TA, O'Driscoll L, Olivan M, Oliveira C, Pállinger É, del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, Sánchez-Madrid F, Santarém N, Schallmoser K, Stampe Ostenfeld M, Stoorvogel W, Stukelj R, Van der Grein SG, Helena Vasconcelos M, Wauben MHM, De Wever O (2015) Biological properties of extracellular vesicles and their physiological functions. Journal of Extracellular Vesicles 4:27066 . doi: 10.3402/jev.v4.27066

Zamith-Miranda D, Nimrichter L, Rodrigues ML, Nosanchuk JD (2018) Fungal extracellular vesicles: modulating host–pathogen interactions by both the fungus and the host. Microbes and Infection 20:501–504 . doi: 10.1016/j.micinf.2018.01.011

Zienkiewicz K, Du Z-Y, Ma W, Vollheyde K, Benning C (2016) Stress-induced neutral lipid biosynthesis in microalgae — Molecular, cellular and physiological insights. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1861:1269–1281 . doi: 10.1016/j.bbalip.2016.02.008

Zulu NN, Zienkiewicz K, Vollheyde K, Feussner I (2018) Current trends to comprehend lipid metabolism in diatoms. Progress in Lipid Research 70:1–16 . doi: 10.1016/j.plipres.2018.03.001

Chapter 3: Discussion

3.1 General synthesis of results

The experiments presented in this thesis were performed to address the following hypothesis: *Phaeobacter italicus* R11 influences the production and storage of lipids in biofuel producing microalgae *Dunaliella tertiolecta* (Figure 3.1). Previous research has revealed bacterial influence on the lipid metabolism and yield of microalgae but this is the first study to demonstrate bacterial stimulated lipid vesicles production (de-Bashan et al., 2002; Do Nascimento et al., 2013; Segev et al., 2016).

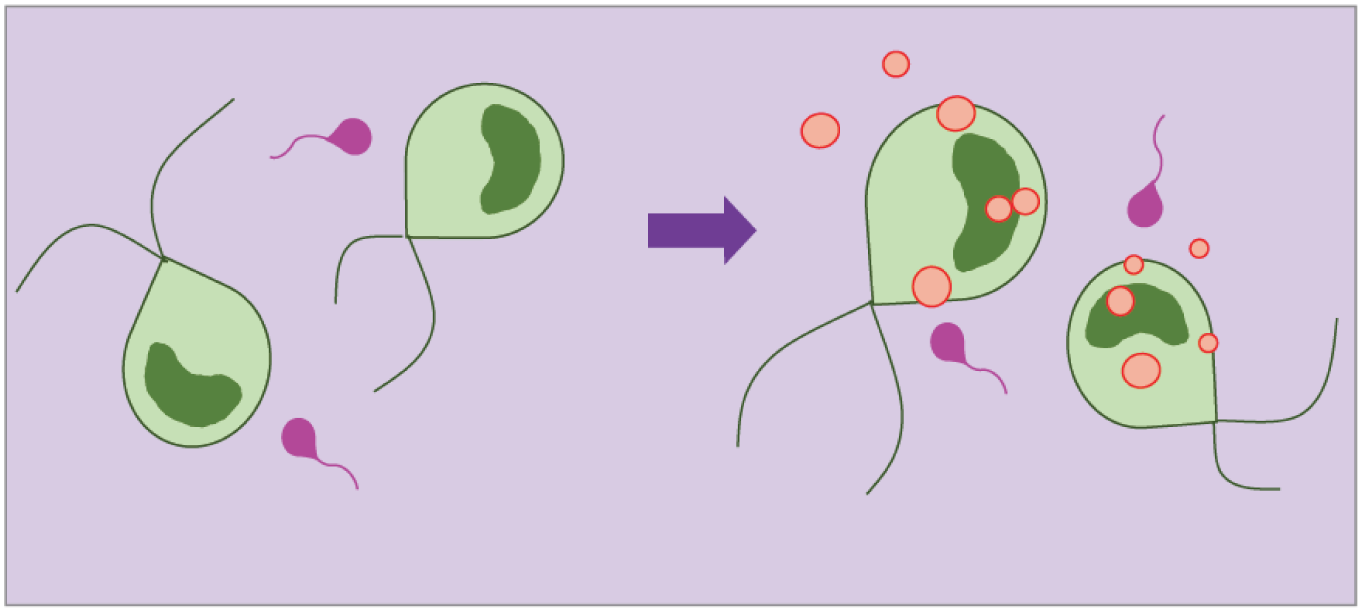


Figure 3.1. Summary of algal-bacterial interaction presented in thesis. Depiction of *Phaeobacter italicus*'s influence on the production and storage of lipids in microalgae *Dunaliella tertiolecta* (CCMP 1320).

Physiology was monitored through coculturing experiments, it was determined that the bacterium increased lipid per biomass of alga by ~25% in late stationary phase. Subsequent experiments determined there was no change in fatty acid composition between mono- and co-culture. The bacterial stimulated lipid increase was most likely a result of lipid vesicles present in the extracellular space exclusively during co-cultivation. The lipid vesicles were 1-3 μm in diameter, spherical in shape and reached concentrations of 1 x 10^6 per mL in coculture (Chapter 2). Lipid vesicles were not degraded over time and fused or aggregated forming larger vesicles

(Chapter 2, Figure 2.9). This response was species specific and is, to our knowledge, the first description of an algal-bacterial interaction that produces lipid vesicles. We suggest that the vesicles were algal derived lipid droplets, because of localization of these organelles at the perimeter of microalgal cell, with very thin membrane separating the lipid droplet and the extracellular space, when they are grown with the bacterium. Furthermore, the extracellular lipid vesicles are similar in size and shape to algal lipid droplets. Although lipid droplet production in a common characteristic in oleaginous microalgal species there is no research suggesting that microalgae can actively release their lipid bodies into the extracellular space (Goold et al., 2015). The cellular mechanism of lipid droplet release to produce the extracellular lipid vesicles in this study remains unknown.

During fluorescence flow cytometry sampling, bacteria were imaged individually and seen 'attached' to lipid vesicles. Lipid vesicles were abundant and dynamic over time, but the bacterial population was maintained at ~$1x10^5$ CFU per mL in stationary phase, even at differing initial population densities (Chapter 2, Figure 2.3). The only observed increase of bacterial density was during exponential phase until carrying capacity was reached and maintained. The increase of bacteria population (0 d - 4 d) did not overlap with the production of lipid vesicles later in the experiment (10 d - 16 d). If bacteria were consuming the lipid vesicle's contents as a nutrient source, there would be an expected increase of bacteria population density over time. There was no carbon supplement in the medium used, therefore bacterial growth had to be sustained by the algal produced dissolved organic carbon.

3.2 Biotechnological application of research

The potential role of algal-bacterial interactions was previously summarized, but the model described in this thesis has additional implications that could alleviate the energy and cost required for biofuel production (Chapter 1) (Figure 3.2).

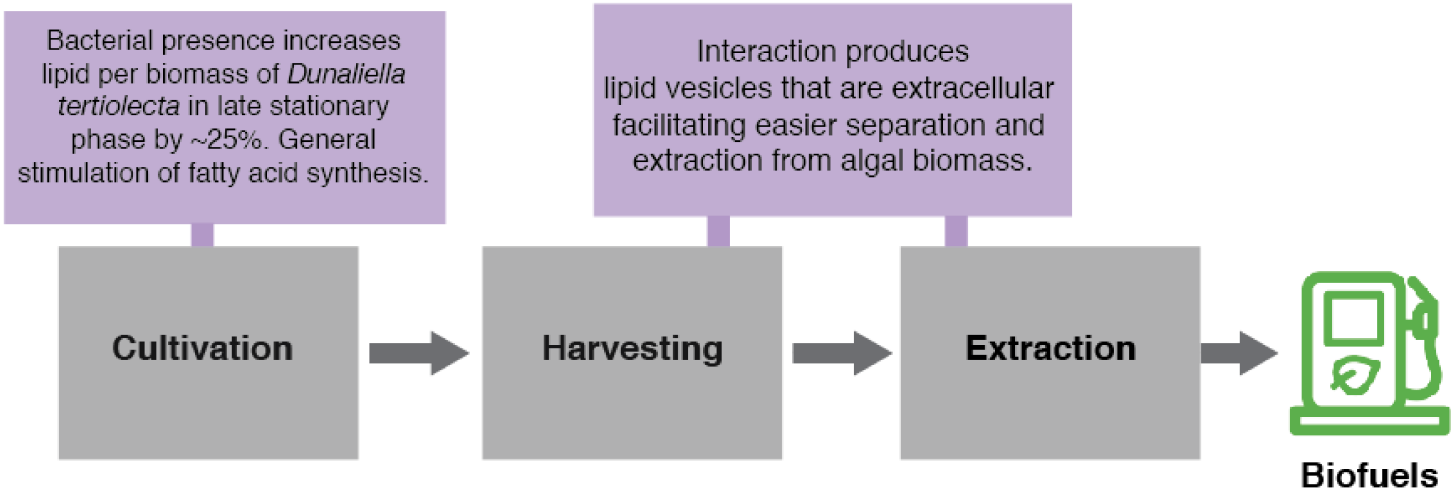


Figure 3.2. Role of *Dunaliella tertiolecta-Phaeobacter italicus* R11 model in biofuel production. Summary of presented *D. tertiolecta-P. italicus* model's associated biotechnological advantage within the biofuel production pipeline of cultivation, harvesting and extraction.

During co-cultivation, bacterial presence increases lipid per biomass by ~25%, which is comparable to the increases obtained by the simultaneous deprivation of nitrogen and phosphorus in some biofuel producing microalgae (Sharma et al., 2012). This increase was a general increase of fatty acid synthesis, which includes biofuel fatty acid feedstock already found in green microalgal species such as palmitic acid (C16:0). This increase would benefit overall biofuel yield and make the commercial production of microalgal biofuel more economically feasible.

The second application of this system can be put somewhere in-between harvesting and extraction on the biofuel production pipeline. This interaction produced algal-derived lipid vesicles that are already separated from the algal cell biomass. The lipids are already in the extracellular space and could be collected because of their inherent differences in buoyancy and size from the algal cells. The lipid vesicles could potentially be concentrated through a chemical flocculant or size-specific filtration.

Since this interaction had no effect on the algal health, the culture can then be reused for subsequent generations of lipid vesicle harvesting. This method of preserving algal biomass for following generations is referred to as 'milking' in the biofuel industry (Vinayak et al., 2015). This term is in reference to 'milking a cow' and the concept is focused on preserving the original

algal culture during lipid extraction. Milking is most commonly performed in diatoms where the physical force applied at each frustule forces lipid droplets into the extracellular space, but the diatom is unharmed and used for additional culture cycles (Vinayak et al., 2015). It is possible that this model can be a form of 'milking' microalgae as lipid vesicles are already released without compromising health and viability of *D. tertiolecta* cultures.

Overall, this thesis presents research with potential application in biofuel field but additional studies will have to be performed in order to ensure this response is maintained at commercial-sized cultivation.

3.3 Impact of research on microbial ecology

This thesis reveals that algal-bacterial interactions can influence the production and storage of lipids in green microalgae and therefore, relationships between microorganisms must be considered when using microalgal lipids as proxies. In addition to their role in the biotechnological industry, algal lipids are used as proxies in paleo-oceanography and marine ecology (Kumari et al., 2013; Sahu et al., 2013). Fatty acids are taxon-specific biomarkers for microalgal communities in marine systems (Sahu et al., 2013) and some can also be used as indicators of stressful environments for algae (Lu et al., 2012). In paleoceanography, microbial lipids are used as fossils for past microalgal communities due to their chemical stability over long periods of time (Madureira et al., 1997). Furthermore, the ratio of saturated and unsaturated alkenones found in *Emiliania huxleyi* is used to model historic sea-water temperature (Marlowe et al., 1990). Research has already demonstrated that bacteria affect the haptophytes alkenone production, causing a 4 °C temperature change from the model (Segev et al., 2016).

The intricate relationships between algae and bacteria will be significant when discussing global, and ocean, warming (Beardall and Raven, 2004; Case et al., 2011). The virulence of some bacterial pathogens, like *P. italicus*, is induced by temperature increases and their impact on algal populations will be more pronounced with the increasing ocean warming (Mayers et al., 2016). We don't know how the majority of marine microorganisms will react individually to upcoming environmental pressures much less how inter-species interactions will change. Warming will also impact the biofuel industry which is moving towards poly-microbial, open pond systems. These bioreactors are small water bodies that will be susceptible to climate

impacted interactions. Microbial relationships are an essential part of the ocean's stability and cannot be ignored during discussions of climate change.

3.4 Future directions

Further research is required to define the origin of lipid vesicles and the mechanism of release from the green microalgae in this study. To begin, it would be advantageous to isolate the lipid vesicle from algal biomass through size-dependent filtration or centrifugation separation. The isolation of lipid vesicle would allow proteomic, metabolomic and transcriptomic analysis, which would give insight of the vesicle's origin and possible function. In particular, during proteomic studies, I would look for the presence of major lipid droplet protein which is a central protein in the development of microalgal lipid droplets (Nguyen et al., 2011). This would be able to confirm that lipid vesicles were in fact, released lipid droplets. A transcriptomic study could be completed in order to determine genes involved with mechanism of release and the alga's metabolic response to *P. italicus*. This research would be optimized when the complete sequencing of *D. tertiolecta* genome is available, which does not exist at this time.

A metabolomic study of the three treatments; the bacteria, the algae and the coculture, would be essential in identifying any bioactive molecule that are exchanged in order to elicit the response of algal lipid vesicle production. This study would also allow to determine if there were bioactive molecules, such as indol-3-acetic acid, that played a role in the interaction.

Further studies could test the breadth of this interaction. Cocultures of *P. italicus* and other species of green microalgae could be cultivated and imaged for lipid vesicle presence. The bacterium *Phaeobacter gallaeciensis*, which is the closest genetic relative to *P. italicus*, could be cocultured with *D. tertiolecta* and lipid vesicle production could be investigated (Fernandes et al., 2011). These studies could determine if this response is specific to the microalgae, the bacterium or unique to the relationship between them. It would also determine if this interaction could be stimulated in other biofuel producing microalgae, expanding its practicality in the field.

3.5 Conclusions

In this thesis a novel interaction between a green alga, *D. tertiolecta,* and a marine bacterium, *P. italicus*, that produced algal-derived lipid vesicles in the extracellular space was studied. Algal lipids, which were the focus of this study, have an important role in the biotechnological field as well as in marine microbial ecology. We have demonstrated that bacteria can influence the production and location of algal lipids therefore microbial interactions must be considered in fields that use algal lipids as agents. This is the first description, to our knowledge, of extracellular vesicle production in green microalgae and the first study to show the extracellular vesicle production that is stimulated by an algal-bacterial interaction.

3.6 References

Beardall J, Raven JA (2004) The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia 43:26–40 . doi: 10.2216/i0031-8884-43-1-26.1

Case RJ, Longford SR, Campbell AH, Low A, Tujula N, Steinberg PD, Kjelleberg S (2011) Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. Environmental Microbiology 13:529–537 . doi: 10.1111/j.1462-2920.2010.02356.x

de-Bashan LE, Bashan Y, Moreno M, Lebsky VK, Bustillos JJ (2002) Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. Canadian Journal of Microbiology 48:514–521 . doi: 10.1139/w02-051

Do Nascimento M, Dublan M de los A, Ortiz-Marquez JCF, Curatti L (2013) High lipid productivity of an Ankistrodesmus – Rhizobium artificial consortium. Bioresource Technology 146:400–407 . doi: 10.1016/j.biortech.2013.07.085

Fernandes N, Case RJ, Longford SR, Seyedsayamdost MR, Steinberg PD, Kjelleberg S, Thomas T (2011) Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*. PLoS ONE 6:e27387 . doi: 10.1371/journal.pone.0027387

Goold H, Beisson F, Peltier G, Li-Beisson Y (2015) Microalgal lipid droplets: composition, diversity, biogenesis and functions. Plant Cell Reports 34:545–555 . doi: 10.1007/s00299-014-1711-7

Kumari P, Kumar M, Reddy CRK, Jha B (2013) Algal lipids, fatty acids and sterols. In: Functional Ingredients from Algae for Foods and Nutraceuticals. Elsevier, pp 87–134

Lu N, Wei D, Jiang X-L, Chen F, Yang S-T (2012) Fatty acids profiling and biomarker identification in snow alga *Chlamydomonas Nivalis* by NaCl stress using GC/MS and multivariate statistical analysis. Analytical Letters 45:1172–1183 . doi: 10.1080/00032719.2012.673094

Madureira LAS, van Kreveld SA, Eglinton G, Conte MH, Ganssen G, van Hinte JE, Ottens JJ (1997) Late Quaternary high-resolution biomarker and other sedimentary climate proxies in a Northeast Atlantic Core. Paleoceanography 12:255–269 . doi: 10.1029/96PA03120

Marlowe IT, Brassell SC, Eglinton G, Green JC (1990) Long-chain alkenones and alkyl alkenoates and the fossil coccolith record of marine sediments. Chemical Geology 88:349–375 . doi: 10.1016/0009-2541(90)90098-R

Mayers TJ, Bramucci AR, Yakimovich KM, Case RJ (2016) A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*. Frontiers in Microbiology 7:892 . doi: 10.3389/fmicb.2016.00892

Nguyen HM, Baudet M, Cuiné S, Adriano J-M, Barthe D, Billon E, Bruley C, Beisson F, Peltier G, Ferro M, Li-Beisson Y (2011) Proteomic profiling of oil bodies isolated from the unicellular green microalga *Chlamydomonas reinhardtii*: With focus on proteins involved in lipid metabolism. Proteomics 11:4266–4273 . doi: 10.1002/pmic.201100114

Sahu A, Pancha I, Jain D, Paliwal C, Ghosh T, Patidar S, Bhattacharya S, Mishra S (2013) Fatty acids as biomarkers of microalgae. Phytochemistry 89:53–58 . doi: 10.1016/j.phytochem.2013.02.001

Segev E, Castañeda IS, Sikes EL, Vlamakis H, Kolter R (2016) Bacterial influence on alkenones in live microalgae. Journal of Phycology 52:125–130 . doi: 10.1111/jpy.12370

Sharma KK, Schuhmann H, Schenk PM (2012) High lipid induction in microalgae for biodiesel production. Energies 5:1532–1553 . doi: 10.3390/en5051532

Vinayak V, Manoylov K, Gateau H, Blanckaert V, Hérault J, Pencréac'h G, Marchand J, Gordon R, Schoefs B (2015) Diatom milking: A review and new approaches. Marine Drugs 13:2629–2665 . doi: 10.3390/md130526

Work Cited

Abalde, J., Fabregas, J., Herrero, C., 1991. fi-Carotene, vitamin C and vitamin E content of the marine microalga *Dunaliella tertiolecta* cultured with different nitrogen sources. Bioresource Technology 38, 121–125.

Alvarez, H., Steinbüchel, A., 2002. Triacylglycerols in prokaryotic microorganisms. Applied Microbiology and Biotechnology 60, 367–376. https://doi.org/10.1007/s00253-002-1135-0

Amin, S., Green, D.H., Hart, M.C., Kupper, F.C., Sunda, W.G., Carrano, C.J., 2009. Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism. Proceedings of the National Academy of Sciences 106, 17071–17076. https://doi.org/10.1073/pnas.0905512106

Amin, S.A., Hmelo, L.R., van Tol, H.M., Durham, B.P., Carlson, L.T., Heal, K.R., Morales, R.L., Berthiaume, C.T., Parker, M.S., Djunaedi, B., Ingalls, A.E., Parsek, M.R., Moran, M.A., Armbrust, E.V., 2015. Interaction and signaling between a cosmopolitan phytoplankton and associated bacteria. Nature 522, 98–101. https://doi.org/10.1038/nature14488

Amin, S.A., Parker, M.S., Armbrust, E.V., 2012. Interactions between diatoms and bacteria. Microbiology and Molecular Biology Reviews 76, 667–684. https://doi.org/10.1128/MMBR.00007-12

Arguelles, E.DLR., Laurena, A.C., Monsalud, R.G., Martinez-Goss, M.R., 2018. Fatty acid profile and fuel-derived physico-chemical properties of biodiesel obtained from an indigenous green microalga, Desmodesmus sp. (I-AU1), as potential source of renewable lipid and high quality biodiesel. J Appl Phycol 30, 411–419. https://doi.org/10.1007/s10811-017-1264-6

Asomaning, J., Omidghane, M., Chae, M., 2016. Thermal processing of algal biomass for biofuel production. Current Opinion in Green and Sustainable Chemistry 2, 1–5. https://doi.org/10.1016/j.cogsc.2016.08.005

Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H., Mekhilef, S., 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renewable and Sustainable Energy Reviews 16, 2070–2093. https://doi.org/10.1016/j.rser.2012.01.003

Balat, M., Balat, H., Öz, C., 2008. Progress in bioethanol processing. Progress in Energy and Combustion Science 34, 551–573. https://doi.org/10.1016/j.pecs.2007.11.001

Barros, A.I., Gonçalves, A.L., Simões, M., Pires, J.C.M., 2015. Harvesting techniques applied to microalgae: A review. Renewable and Sustainable Energy Reviews 41, 1489–1500. https://doi.org/10.1016/j.rser.2014.09.037

Basova, M.M., 2005. Fatty acid composition of lipids in microalgae. International Journal on Algae 7, 33–57. https://doi.org/10.1615/InterJAlgae.v7.i1.30

Beacham, T.A., Sweet, J.B., Allen, M.J., 2017. Large scale cultivation of genetically modified microalgae: A new era for environmental risk assessment. Algal Research 25, 90–100. https://doi.org/10.1016/j.algal.2017.04.028

Beardall, J., Raven, J.A., 2004. The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia 43, 26–40. https://doi.org/10.2216/i0031-8884-43-1-26.1

Bell, W., Mitchell, R., 1972. Chemotactic and growth responses of marine bacteria to algal extracellular products. The Biological Bulletin 143, 265–277. https://doi.org/10.2307/1540052

Berg, J., Tymoczko, J., Stryer, L., 2002. Triacylglycerols are highly concentrated energy stores, in: Biochemistry. 5th Edition. New York: W H Freeman;, p. Section 22.1.

Berges, J.A., Falkowski, P.G., 1998. Physiological stress and cell death in marine phytoplankton: Induction of proteases in response to nitrogen or light limitation. Limnology and Oceanography 43, 129–135. https://doi.org/10.4319/lo.1998.43.1.0129
Berthold, D.E., Shetty, K.G., Jayachandran, K., Laughinghouse, H.D., Gantar, M., 2019. Enhancing algal biomass and lipid production through bacterial co-culture. Biomass and Bioenergy 122, 280–289. https://doi.org/10.1016/j.biombioe.2019.01.033
Bertozzini, E., Galluzzi, L., Penna, A., Magnani, M., 2011. Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. Journal of Microbiological Methods 87, 17–23. https://doi.org/10.1016/j.mimet.2011.06.018
Beyter, D., Tang, P.-Z., Becker, S., Hoang, T., Bilgin, D., Lim, Y.W., Peterson, T.C., Mayfield, S., Haerizadeh, F., Shurin, J.B., Bafna, V., McBride, R., 2016. Diversity, productivity, and stability of an industrial microbial scosystem. Applied and Environmental Microbiology 82, 2494–2505. https://doi.org/10.1128/AEM.03965-15
Bhattacharya, D., Yoon, H.S., Hackett, J.D., 2004. Photosynthetic eukaryotes unite: endosymbiosis connects the dots. BioEssays 26, 50–60. https://doi.org/10.1002/bies.10376
Biller, S.J., Schubotz, F., Roggensack, S.E., Thompson, A.W., Summons, R.E., Chisholm, S.W., 2014. Bacterial vesicles in marine ecosystems. Science 343, 183–186. https://doi.org/10.1126/science.1243457
Blumberg, L.M., Klee, M.S., 2000. Optimal heating rate in gas chromatography. Journal of Microcolumn Separations 12, 508–514. https://doi.org/10.1002/1520-667X(2000)12:9<508::AID-MCS5>3.0.CO;2-Y
Borowitzka, M.A., Siva, C.J., 2007. The taxonomy of the genus *Dunaliella*(Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. Journal of Applied Phycology 19, 567–590. https://doi.org/10.1007/s10811-007-9171-x
Boyle, N.R., Page, M.D., Liu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S.J., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S.J., Gallaher, S.D., Johnson, S., Benning, C., Pellegrini, M., Grossman, A., Merchant, S.S., 2012. Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*. Journal of Biological Chemistry 287, 15811–15825. https://doi.org/10.1074/jbc.M111.334052
Bramucci, A.R., Case, R.J., 2019. *Phaeobacter inhibens* induces apoptosis-like programmed cell death in calcifying *Emiliania huxleyi*. Scientific Reports 9, 5215. https://doi.org/10.1038/s41598-018-36847-6
Bramucci, A.R., Labeeuw, L., Mayers, T.J., Saby, J.A., Case, R.J., 2015. A small volume bioassay to assess bacterial/phytoplankton co-culture using WATER-Pulse-Amplitude-Modulated (WATER-PAM) Fluorometry. Journal of Visual Experiments. https://doi.org/10.3791/52455
Bramucci, A.R., Labeeuw, L., Orata, F.D., Ryan, E.M., Malmstrom, R.R., Case, R.J., 2018. The bacterial symbiont *Phaeobacter inhibens* shapes the life history of its algal host *Emiliania huxleyi*. Frontiers in Marine Science 5. https://doi.org/10.3389/fmars.2018.00188
Brennan, L., Owende, P., 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14, 557–577. https://doi.org/10.1016/j.rser.2009.10.009
Brinkhov, T., Giebel, H.-A., Simon, M., 2008. Diversity, ecology, and genomics of the Roseobacter clade: a short overview. Archives of Microbiology 189, 531–539. https://doi.org/10.1007/s00203-008-0353-y
Buchan, A., Gonzalez, J.M., Moran, M.A., 2005. Overview of the marine Roseobacter lineage. Applied and Environmental Microbiology 71, 5665–5677. https://doi.org/10.1128/AEM.71.10.5665-5677.2005

Butcher, R.W., 1959. An introductory account of the smaller algae of British coastal waters. Part I: Introduction and Chlorophyceae, Fishery investigations.
Case, R.J., Longford, S.R., Campbell, A.H., Low, A., Tujula, N., Steinberg, P.D., Kjelleberg, S., 2011. Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. Environmental Microbiology 13, 529–537. https://doi.org/10.1111/j.1462-2920.2010.02356.x
Cavonius, L.R., Carlsson, N.-G., Undeland, I., 2014. Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. Analytical and Bioanalytical Chemistry 406, 7313–7322. https://doi.org/10.1007/s00216-014-8155-3
Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology 102, 1649–1655. https://doi.org/10.1016/j.biortech.2010.09.062
Cheregi, O., Ekendahl, S., Engelbrektsson, J., Strömberg, N., Godhe, A., Spetea, C., 2019. Microalgae biotechnology in Nordic countries – the potential of local strains. Physiologia Plantarum 166, 438–450. https://doi.org/10.1111/ppl.12951
Chisti, Y., 2007. Biodiesel from microalgae. Biotechnology Advances 25, 294–306. https://doi.org/10.1016/j.biotechadv.2007.02.001
Cho, D.-H., Ramanan, R., Heo, J., Lee, J., Kim, B.-H., Oh, H.-M., Kim, H.-S., 2015. Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. Bioresource Technology 175, 578–585. https://doi.org/10.1016/j.biortech.2014.10.159
Cho, H.U., Kim, Y.M., Park, J.M., 2017. Enhanced microalgal biomass and lipid production from a consortium of indigenous microalgae and bacteria present in municipal wastewater under gradually mixotrophic culture conditions. Bioresource Technology 228, 290–297. https://doi.org/10.1016/j.biortech.2016.12.094
Colling Klein, B., Bonomi, A., Maciel Filho, R., 2018. Integration of microalgae production with industrial biofuel facilities: A critical review. Renewable and Sustainable Energy Reviews 82, 1376–1392. https://doi.org/10.1016/j.rser.2017.04.063
Cooper, M.B., Smith, A.G., 2015. Exploring mutualistic interactions between microalgae and bacteria in the omics age. Current Opinion in Plant Biology 26, 147–153. https://doi.org/10.1016/j.pbi.2015.07.003
Costa, J.A.V., Freitas, B.C.B., Santos, T.D., Mitchell, B.G., Morais, M.G., 2019. Open pond systems for microalgal culture, in: Biofuels from Algae. Elsevier, pp. 199–223. https://doi.org/10.1016/B978-0-444-64192-2.00009-3
Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93. https://doi.org/10.1038/nature04056
Curson, A.R.J., Todd, J.D., Sullivan, M.J., Johnston, A.W.B., 2011. Catabolism of dimethylsulphoniopropionate: microorganisms, enzymes and genes. Nature Reviews Microbiology 9, 849–859. https://doi.org/10.1038/nrmicro2653
Danielewicz, M.A., Anderson, L.A., Franz, A.K., 2011. Triacylglycerol profiling of marine microalgae by mass spectrometry. Journal of Lipid Research 52, 2101–2108. https://doi.org/10.1194/jlr.D018408
Dave, N., Selvaraj, R., Varadavenkatesan, T., Vinayagam, R., 2019. A critical review on production of bioethanol from macroalgal biomass. Algal Research 42, 101606. https://doi.org/10.1016/j.algal.2019.101606
Davidi, L., Shimoni, E., Khozin-Goldberg, I., Zamir, A., Pick, U., 2014. Origin of carotene-rich plastoglobuli in *Dunaliella bardawil*. Plant Physiology 164, 2139–2156. https://doi.org/10.1104/pp.113.235119
de-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillos, J.J., 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting

bacterium *Azospirillum brasilense*. Canadian Journal of Microbiology 48, 514–521. https://doi.org/10.1139/w02-051
DeLong, E.F., Karl, D.M., 2005. Genomic perspectives in microbial oceanography. Nature 437, 336–342. https://doi.org/10.1038/nature04157
Demuez, M., González-Fernández, C., Ballesteros, M., 2015. Algicidal microorganisms and secreted algicides: New tools to induce microalgal cell disruption. Biotechnology Advances 33, 1615–1625. https://doi.org/10.1016/j.biotechadv.2015.08.003
Deneyer, A., Ennaert, T., Sels, B.F., 2018. Straightforward sustainability assessment of sugar-derived molecules from first-generation biomass. Current Opinion in Green and Sustainable Chemistry 10, 11–20. https://doi.org/10.1016/j.cogsc.2018.02.003
Do Nascimento, M., Dublan, M. de los A., Ortiz-Marquez, J.C.F., Curatti, L., 2013. High lipid productivity of an Ankistrodesmus – Rhizobium artificial consortium. Bioresource Technology 146, 400–407. https://doi.org/10.1016/j.biortech.2013.07.085
Dong, T., Knoshaug, E.P., Pienkos, P.T., Laurens, L.M.L., 2016. Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. Applied Energy 177, 879–895. https://doi.org/10.1016/j.apenergy.2016.06.002
Durham, B.P., Sharma, S., Luo, H., Smith, C.B., Amin, S.A., Bender, S.J., Dearth, S.P., Van Mooy, B.A.S., Campagna, S.R., Kujawinski, E.B., Armbrust, E.V., Moran, M.A., 2015. Cryptic carbon and sulfur cycling between surface ocean plankton. Proceedings of the National Academy of Sciences 112, 453–457. https://doi.org/10.1073/pnas.1413137112
Eberhard, S., Finazzi, G., Wollman, F.-A., 2008. The dynamics of photosynthesis. Annual Review of Genetics 42, 463–515. https://doi.org/10.1146/annurev.genet.42.110807.091452
Falkowski, P.G., 1998. Biogeochemical controls and feedbacks on ocean primary production. Science 281, 200–206. https://doi.org/10.1126/science.281.5374.200
Falkowski, P.G., 1994. The role of phytoplankton photosynthesis in global biogeochemical cycles. Photosynthesis Research 39, 235–258.
Farese, R.V., Walther, T.C., 2009. Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 139, 855–860. https://doi.org/10.1016/j.cell.2009.11.005
Fazal, M.A., Suhaila, N.R., Haseeb, A.S.M.A., Rubaiee, S., Al-Zahrani, A., 2018. Influence of copper on the instability and corrosiveness of palm biodiesel and its blends: An assessment on biodiesel sustainability. Journal of Cleaner Production 171, 1407–1414. https://doi.org/10.1016/j.jclepro.2017.10.144
Fernandes, N., Case, R.J., Longford, S.R., Seyedsayamdost, M.R., Steinberg, P.D., Kjelleberg, S., Thomas, T., 2011. Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*. PLoS ONE 6, e27387. https://doi.org/10.1371/journal.pone.0027387
Field, C.B., 1998. Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 281, 237–240. https://doi.org/10.1126/science.281.5374.237
Franz, A.K., Danielewicz, M.A., Wong, D.M., Anderson, L.A., Boothe, J.R., 2013. Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity. ACS Chemical Biology 8, 1053–1062. https://doi.org/10.1021/cb300573r
Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., González-Del-Valle, M., Vílchez, C., 2016. Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. Marine Drugs 14, 100–100. https://doi.org/10.3390/md14050100
Gallagher, B.J., 2011. The economics of producing biodiesel from algae. Renewable Energy 36, 158–162. https://doi.org/10.1016/j.renene.2010.06.016
Gardiner, M., Bournazos, A.M., Maturana-Martinez, C., Zhong, L., Egan, S., 2017. Exoproteome analysis of the seaweed pathogen *Nautella italica* R11 reveals temperature-dependent regulation of RTX-like proteins. Frontiers in Microbiology 8, 1203–1203. https://doi.org/10.3389/fmicb.2017.01203

Gardiner, M., Fernandes, N.D., Nowakowski, D., Raftery, M., Kjelleberg, S., Zhong, L., Thomas, T., Egan, S., 2015. VarR controls colonization and virulence in the marine macroalgal pathogen *Nautella italica* R11. Frontiers in Microbiology 6, 1130. https://doi.org/10.3389/fmicb.2015.01130
Garlapati, V.K., Tewari, S., Ganguly, R., 2019. Life cycle assessment of first-, second-generation, and microalgae biofuels, in: Advances in Feedstock Conversion Technologies for Alternative Fuels and Bioproducts. Elsevier, pp. 355–371. https://doi.org/10.1016/B978-0-12-817937-6.00019-9
Geng, H., Belas, R., 2010. Molecular mechanisms underlying roseobacter–phytoplankton symbioses. Current Opinion in Biotechnology 21, 332–338. https://doi.org/10.1016/j.copbio.2010.03.013
Gill, S., Catchpole, R., Forterre, P., 2019. Extracellular membrane vesicles in the three domains of life and beyond. FEMS Microbiology Reviews 43, 273–303. https://doi.org/10.1093/femsre/fuy042
Gong, M., Bassi, A., 2016. Carotenoids from microalgae: A review of recent developments. Biotechnology Advances 34, 1396–1412. https://doi.org/10.1016/j.biotechadv.2016.10.005
Goold, H., Beisson, F., Peltier, G., Li-Beisson, Y., 2015. Microalgal lipid droplets: composition, diversity, biogenesis and functions. Plant Cell Reports 34, 545–555. https://doi.org/10.1007/s00299-014-1711-7
Gould, S.B., Waller, R.F., Mcfadden, G.I., 2008. Plastid evolution. Annual Review of Plant Biology 59, 491–517. https://doi.org/10.1146/annurev.arplant.59.032607.092915
Grant, M.A., Kazamia, E., Cicuta, P., Smith, A.G., 2014. Direct exchange of vitamin B12 is demonstrated by modelling the growth dynamics of algal–bacterial cocultures. ISME Journal 8, 1418–1427. https://doi.org/10.1038/ismej.2014.9
Gray, K.A., Zhao, L., Emptage, M., 2006. Bioethanol. Current Opinion in Chemical Biology 10, 141–146. https://doi.org/10.1016/j.cbpa.2006.02.035
Greenspan, P., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. The Journal of Cell Biology 100, 965–973. https://doi.org/10.1083/jcb.100.3.965
Gressel, J., van der Vlugt, C.J.B., Bergmans, H.E.N., 2014. Cultivated microalgae spills: hard to predict/easier to mitigate risks. Trends in Biotechnology 32, 65–69. https://doi.org/10.1016/j.tibtech.2013.11.003
Harwood, J.L., Guschina, I.A., 2009. The versatility of algae and their lipid metabolism. Biochimie 91, 679–684. https://doi.org/10.1016/J.BIOCHI.2008.11.004
Havlík, P., Schneider, U.A., Schmid, E., Böttcher, H., Fritz, S., Skalský, R., Aoki, K., Cara, S.D., Kindermann, G., Kraxner, F., Leduc, S., McCallum, I., Mosnier, A., Sauer, T., Obersteiner, M., 2011. Global land-use implications of first and second generation biofuel targets. Energy Policy 39, 5690–5702. https://doi.org/10.1016/j.enpol.2010.03.030
Helliwell, K.E., Scaife, M.A., Sasso, S., Araujo, A.P.U., Purton, S., Smith, A.G., 2014. Unraveling vitamin B12-responsive gene regulation in algae. Plant Physiology 165, 388–97. https://doi.org/10.1104/pp.113.234369
Henley, W.J., Litaker, R.W., Novoveská, L., Duke, C.S., Quemada, H.D., Sayre, R.T., 2013. Initial risk assessment of genetically modified (GM) microalgae for commodity-scale biofuel cultivation. Algal Research 2, 66–77. https://doi.org/10.1016/j.algal.2012.11.001
Hirose, M., Mukaida, F., Okada, S., Noguchi, T., 2013. Active hydrocarbon biosynthesis and accumulation in a green alga, *Botryococcus braunii* (Race A). Eukaryotic Cell 12, 1132–1141. https://doi.org/10.1128/EC.00088-13
Hoekman, S.K., Broch, A., Robbins, C., Ceniceros, E., Natarajan, M., 2012. Review of biodiesel composition, properties, and specifications. Renewable and Sustainable Energy Reviews 16, 143–169. https://doi.org/10.1016/j.rser.2011.07.143

Hopfner, U., Schenck, T.-L., Chávez, M.-N., Machens, H.-G., Bohne, A.-V., Nickelsen, J., Giunta, R.-E., Egaña, J.-T., 2014. Development of photosynthetic biomaterials for in vitro tissue engineering. Acta Biomaterialia 10, 2712–2717. https://doi.org/10.1016/j.actbio.2013.12.055

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54, 621–639. https://doi.org/10.1111/j.1365-313X.2008.03492.x

Hulatt, C.J., Wijffels, R.H., Bolla, S., Kiron, V., 2017. Production of Fatty Acids and Protein by Nannochloropsis in Flat-Plate Photobioreactors. PLoS ONE 12, e0170440. https://doi.org/10.1371/journal.pone.0170440

Jia, W., Huang, X., Li, C., 2014. A preliminary study of the algicidal mechanism of bioactive metabolites of *Brevibacillus laterosporus* on *Oscillatoria* in prawn ponds. The Scientific World Journal 2014, 1–11. https://doi.org/10.1155/2014/869149

Jónasdóttir, S.H., 2019. Fatty acid profiles and production in marine phytoplankton. Marine Drugs 17. https://doi.org/10.3390/md17030151

Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnology for Biofuels 6, 16. https://doi.org/10.1186/1754-6834-6-16

Jorquera, O., Kiperstok, A., Sales, E.A., Embiruçu, M., Ghirardi, M.L., 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresource Technology 101, 1406–1413. https://doi.org/10.1016/j.biortech.2009.09.038

Khan, M.I., Shin, J.H., Kim, J.D., 2018. The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial Cell Factories 17. https://doi.org/10.1186/s12934-018-0879-x

Khozin-Goldberg, I., Iskandarov, U., Cohen, Z., 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Applied Microbiology and Biotechnology 91, 905–915. https://doi.org/10.1007/s00253-011-3441-x

Kim, M.-J., Jeong, S.-Y., Lee, S.-J., 2008. Isolation, identification, and algicidal activity of marine bacteria against *Cochlodinium polykrikoides*. Journal of Applied Phycology 20, 1069–1078. https://doi.org/10.1007/s10811-008-9312-x

Klee, M.S., Blumberg, L.M., 2002. Theoretical and practical aspects of fast gas chromatography and method translation. Journal of Chromatographic Science 40, 234–247. https://doi.org/10.1093/chromsci/40.5.234

Kouzuma, A., Watanabe, K., 2015. Exploring the potential of algae/bacteria interactions. Current Opinion in Biotechnology 33, 125–129. https://doi.org/10.1016/j.copbio.2015.02.007

Kulp, A., Kuehn, M.J., 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annual Review of Microbiology 64, 163–184. https://doi.org/10.1146/annurev.micro.091208.073413

Kumar, R., Tabatabaei, M., Karimi, K., Sárvári Horváth, I., 2016. Recent updates on lignocellulosic biomass derived ethanol - A review. Biofuel Research Journal 3, 347–356. https://doi.org/10.18331/BRJ2016.3.1.4

Kumari, P., Kumar, M., Reddy, C.R.K., Jha, B., 2013. Algal lipids, fatty acids and sterols, in: Functional Ingredients from Algae for Foods and Nutraceuticals. Elsevier, pp. 87–134. https://doi.org/10.1533/9780857098689.1.87

Labeeuw, L., 2016. A big role for small molecules in mediating Emiliania huxleyi – Roseobacter interactions. Department of Biological Sciences, University of Alberta Thesis.

Labeeuw, L., Khey, J., Bramucci, A.R., Atwal, H., de la Mata, A.P., Harynuk, J., Case, R.J., 2016. Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells. Frontiers in Microbiology 7, 828–828. https://doi.org/10.3389/fmicb.2016.00828

Lang, I., Hodac, L., Friedl, T., Feussner, I., 2011. Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC Plant Biology 11, 124. https://doi.org/10.1186/1471-2229-11-124

Larkum, A.W., 2016. Photosynthesis and light harvesting in algae, in: Borowitzka, M.A., Beardall, J., Raven, J.A. (Eds.), The Physiology of Microalgae. Springer International Publishing, pp. 67–87. https://doi.org/10.1007/978-3-319-24945-2_3

Le Chevanton, M., Garnier, M., Bougaran, G., Schreiber, N., Lukomska, E., Bérard, J.-B., Fouilland, E., Bernard, O., Cadoret, J.-P., 2013. Screening and selection of growth-promoting bacteria for *Dunaliella* cultures. Algal Research 2, 212–222. https://doi.org/10.1016/j.algal.2013.05.003

Lee, C.-H., Chae, H.-S., Lee, S.-H., Kim, H.S., 2015. Growth characteristics and lipid content of three Korean isolates of *Botryococcus braunii* (Trebouxiophyceae). Journal of Ecology and Environment 38, 67–74. https://doi.org/10.5141/ecoenv.2015.007

Lee, D.H., 2011. Algal biodiesel economy and competition among bio-fuels. Bioresource Technology 102, 43–49. https://doi.org/10.1016/j.biortech.2010.06.034

Lee, J., Cho, D.-H., Ramanan, R., Kim, B.-H., Oh, H.-M., Kim, H.-S., 2013. Microalgae-associated bacteria play a key role in the flocculation of *Chlorella vulgaris*. Bioresource Technology 131, 195–201. https://doi.org/10.1016/j.biortech.2012.11.130

Lima-Mendez, G., Faust, K., Henry, N., Decelle, J., Colin, S., Carcillo, F., Chaffron, S., Ignacio-Espinosa, J.C., Roux, S., Vincent, F., Bittner, L., Darzi, Y., Wang, J., Audic, S., Berline, L., Bontempi, G., Cabello, A.M., Coppola, L., Cornejo-Castillo, F.M., d'Ovidio, F., De Meester, L., Ferrera, I., Garet-Delmas, M.-J., Guidi, L., Lara, E., Pesant, S., Royo-Llonch, M., Salazar, G., Sanchez, P., Sebastian, M., Souffreau, C., Dimier, C., Picheral, M., Searson, S., Kandels-Lewis, S., Tara Oceans coordinators, Gorsky, G., Not, F., Ogata, H., Speich, S., Stemmann, L., Weissenbach, J., Wincker, P., Acinas, S.G., Sunagawa, S., Bork, P., Sullivan, M.B., Karsenti, E., Bowler, C., de Vargas, C., Raes, J., 2015. Determinants of community structure in the global plankton interactome. Science 348, 1262073–1262073. https://doi.org/10.1126/science.1262073

Lin, I.-P., Jiang, P.-L., Chen, C.-S., Tzen, J.T.C., 2012. A unique caleosin serving as the major integral protein in oil bodies isolated from *Chlorella* sp. cells cultured with limited nitrogen. Plant Physiology and Biochemistry 61, 80–87. https://doi.org/10.1016/j.plaphy.2012.09.008

Linhartová, I., Bumba, L., Mašín, J., Basler, M., Osička, R., Kamanová, J., Procházková, K., Adkins, I., Hejnová-Holubová, J., Sadílková, L., Morová, J., Šebo, P., 2010. RTX proteins: a highly diverse family secreted by a common mechanism. FEMS Microbiology Reviews 34, 1076–1112. https://doi.org/10.1111/j.1574-6976.2010.00231.x

Liu, B., Benning, C., 2013. Lipid metabolism in microalgae distinguishes itself. Current Opinion in Biotechnology 24, 300–309. https://doi.org/10.1016/j.copbio.2012.08.008

Liu, H., Zhou, Y., Xiao, W., Ji, L., Cao, X., Song, C., 2012. Shifting nutrient-mediated interactions between algae and bacteria in a microcosm: Evidence from alkaline phosphatase assay. Microbiological Research 167, 292–298. https://doi.org/10.1016/j.micres.2011.10.005

López, A., Rico, M., Santana-Casiano, J.M., González, A.G., González-Dávila, M., 2015. Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron. Environmental Science and Pollution Research 22, 14820–14828. https://doi.org/10.1007/s11356-015-4717-y

Lu, N., Wei, D., Jiang, X.-L., Chen, F., Yang, S.-T., 2012. Fatty acids profiling and biomarker identification in snow alga *Chlamydomonas Nivalis* by NaCl stress using GC/MS and multivariate statistical analysis. Analytical Letters 45, 1172–1183. https://doi.org/10.1080/00032719.2012.673094

MacIntyre, H.L., Kana, T.M., Anning, T., Geider, R.J., 2002. Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. Journal of Phycology 38, 17–38. https://doi.org/10.1046/j.1529-8817.2002.00094.x

Madureira, L.A.S., van Kreveld, S.A., Eglinton, G., Conte, M.H., Ganssen, G., van Hinte, J.E., Ottens, J.J., 1997. Late Quaternary high-resolution biomarker and other sedimentary climate proxies in a Northeast Atlantic Core. Paleoceanography 12, 255–269. https://doi.org/10.1029/96PA03120

Manning, A.J., Kuehn, M.J., 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiology 11, 258. https://doi.org/10.1186/1471-2180-11-258

Marlowe, I.T., Brassell, S.C., Eglinton, G., Green, J.C., 1990. Long-chain alkenones and alkyl alkenoates and the fossil coccolith record of marine sediments. Chemical Geology 88, 349–375. https://doi.org/10.1016/0009-2541(90)90098-R

Martens, T., Gram, L., Grossart, H.-P., Kessler, D., Müller, R., Simon, M., Wenzel, S.C., Brinkhoff, T., 2007. Bacteria of the roseobacter clade show potential for secondary metabolite production. Microbial Ecology 54, 31–42. https://doi.org/10.1007/s00248-006-9165-2

Martin-Platero, A.M., Cleary, B., Kauffman, K., Preheim, S.P., McGillicuddy, D.J., Alm, E.J., Polz, M.F., 2018. High resolution time series reveals cohesive but short-lived communities in coastal plankton. Nature Communications 9. https://doi.org/10.1038/s41467-017-02571-4

Masó, M., Garcés, E., 2006. Harmful microalgae blooms (HAB); problematic and conditions that induce them. Marine Pollution Bulletin 53, 620–630. https://doi.org/10.1016/j.marpolbul.2006.08.006

Mayali, X., Azam, F., 2004. Algicidal bacteria in the sea and their impact on algal blooms. Journal of Eukaryotic Microbiology 51, 139–144. https://doi.org/10.1111/j.1550-7408.2004.tb00538.x

Mayers, T.J., Bramucci, A.R., Yakimovich, K.M., Case, R.J., 2016. A bacterial pathogen displaying temperature-enhanced virulence of the microalga *Emiliania huxleyi*. Frontiers in Microbiology 7, 892. https://doi.org/10.3389/fmicb.2016.00892

Meersche, K.V. den, Middelburg, J.J., Soetaert, K., Rijswijk, P. van, Boschker, H.T.S., Heip, C.H.R., 2004. Carbon-nitrogen coupling and algal-bacterial interactions during an experimental bloom: Modeling a 13C tracer experiment. Limnology and Oceanography 49, 862–878. https://doi.org/10.4319/lo.2004.49.3.0862

Mejriszan Toosi, N., Esfahani, J.A., Reza Safaei, M., 2018. Investigation of energy consumption and renewable energy resources in top ten countries with most energy consumption. IOP Conference Series: Materials Science and Engineering 397, 012108. https://doi.org/10.1088/1757-899X/397/1/012108

Mendoza Guzmán, H., de la Jara Valido, A., Carmona Duarte, L., Freijanes Presmanes, K., 2011. Analysis of interspecific variation in relative fatty acid composition: use of flow cytometry to estimate unsaturation index and relative polyunsaturated fatty acid content in microalgae. Journal of Applied Phycology 23, 7–15. https://doi.org/10.1007/s10811-010-9526-6

Merchant, S.S., Kropat, J., Liu, B., Shaw, J., Warakanont, J., 2012. TAG, You're it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation. Current Opinion in Biotechnology 23, 352–363. https://doi.org/10.1016/j.copbio.2011.12.001

Milano, J., Ong, H.C., Masjuki, H.H., Chong, W.T., Lam, K., Loh, P.K., Vellayan, V., 2016. Microalgae biofuels as an alternative to fossil fuel for power generation. Renewable and Sustainable Energy Reviews 58, 180–197. https://doi.org/10.1016/j.rser.2015.12.150
Milici, M., Deng, Z.-L., Tomasch, J., Decelle, J., Wos-Oxley, M.L., Wang, H., Jáuregui, R., Plumeier, I., Giebel, H.-A., Badewien, T.H., Wurst, M., Pieper, D.H., Simon, M., Wagner-Döbler, I., 2016. Co-occurrence analysis of microbial taxa in the Atlantic Ocean reveals high connectivity in the free-living bacterioplankton. Frontiers in Microbiology 7. https://doi.org/10.3389/fmicb.2016.00649
Minowa, T., Yokoyama, S., Kishimoto, M., Okakura, T., 1995. Oil production from algal cells of *Dunaliella tertiolecta* by direct thermochemical liquefaction. Fuel 74, 1735–1738. https://doi.org/10.1016/0016-2361(95)80001-X
Mizusawa, N., Wada, H., 2012. The role of lipids in photosystem II. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1817, 194–208. https://doi.org/10.1016/j.bbabio.2011.04.008
Moellering, E.R., Benning, C., 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryotic Cell 9, 97–106. https://doi.org/10.1128/EC.00203-09
Mofijur, M., Rasul, M.G., Hassan, N.M.S., Nabi, M.N., 2019. Recent development in the production of third generation biodiesel from microalgae. Energy Procedia 156, 53–58. https://doi.org/10.1016/j.egypro.2018.11.088
Mondello, L., Casilli, A., Tranchida, P.Q., Dugo, P., Dugo, G., 2003. Detailed analysis and group-type separation of natural fats and oils using comprehensive two-dimensional gas chromatography. Journal of Chromatography A 1019, 187–196. https://doi.org/10.1016/S0021-9673(03)01130-0
Monteiro, C.M., Castro, P.M.L., Malcata, F.X., 2012. Metal uptake by microalgae: Underlying mechanisms and practical applications. Biotechnology Progress 28, 299–311. https://doi.org/10.1002/btpr.1504
Mouget, J.-L., Dakhama, A., Lavoie, M.C., Noüe, J., 2006. Algal growth enhancement by bacteria: Is consumption of photosynthetic oxygen involved? FEMS Microbiology Ecology 18, 35–43. https://doi.org/10.1111/j.1574-6941.1995.tb00159.x
Mubarak, M., Shaija, A., Suchithra, T.V., 2015. A review on the extraction of lipid from microalgae for biodiesel production. Algal Research 7, 117–123. https://doi.org/10.1016/j.algal.2014.10.008
Naghavi, N.S., Sameipour, F., 2019. Phototrophic microbial consortium: A technology for enhanced biofuel production, in: Rastegari, A.A., Yadav, A.N., Gupta, A. (Eds.), Prospects of Renewable Bioprocessing in Future Energy Systems. Springer International Publishing, Cham, pp. 185–200. https://doi.org/10.1007/978-3-030-14463-0_6
Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K., 2010. Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews 14, 578–597. https://doi.org/10.1016/j.rser.2009.10.003
Ndikubwimana, T., Zeng, X., Murwanashyaka, T., Manirafasha, E., He, N., Shao, W., Lu, Y., 2016. Harvesting of freshwater microalgae with microbial bioflocculant: a pilot-scale study. Biotechnology for Biofuels 9, 47. https://doi.org/10.1186/s13068-016-0458-5
Nguyen, H.M., Baudet, M., Cuiné, S., Adriano, J.-M., Barthe, D., Billon, E., Bruley, C., Beisson, F., Peltier, G., Ferro, M., Li-Beisson, Y., 2011. Proteomic profiling of oil bodies isolated from the unicellular green microalga *Chlamydomonas reinhardtii*: With focus on proteins involved in lipid metabolism. Proteomics 11, 4266–4273. https://doi.org/10.1002/pmic.201100114
Nicolò, M.S., Guglielmino, S.P.P., Solinas, V., Salis, A., 2017. Biodiesel from Microalgae, in: Lee, S.Y. (Ed.), Consequences of Microbial Interactions with Hydrocarbons, Oils, and

Lipids: Production of Fuels and Chemicals. Springer International Publishing, Cham, pp. 55–74. https://doi.org/10.1007/978-3-319-50436-0_210
Novoveská, L., Zapata, A.K.M., Zabolotney, J.B., Atwood, M.C., Sundstrom, E.R., 2016. Optimizing microalgae cultivation and wastewater treatment in large-scale offshore photobioreactors. Algal Research 18, 86–94. https://doi.org/10.1016/j.algal.2016.05.033
Ok, M.Ö., Tang, X., 2012. Depletion of fossil fuels and anthropogenic climate change—A review. Energy Policy 52, 797–809. https://doi.org/10.1016/j.enpol.2012.10.046
Orata, F.D., Xu, Y., Gladney, L.M., Rishishwar, L., Case, R.J., Boucher, Y., Jordan, I.K., Tarr, C.L., 2016. Characterization of clinical and environmental isolates of *Vibrio cidicii* sp. nov., a close relative of *Vibrio navarrensis*. International Journal of Systematic and Evolutionary Microbiology 66, 4148–4155. https://doi.org/10.1099/ijsem.0.001327
Padmaperuma, G., Kapoore, R.V., Gilmour, D.J., Vaidyanathan, S., 2018. Microbial consortia: a critical look at microalgae co-cultures for enhanced biomanufacturing. Critical Reviews in Biotechnology 38, 690–703. https://doi.org/10.1080/07388551.2017.1390728
Pettersen, A.K., Turchini, G.M., Jahangard, S., Ingram, B.A., Sherman, C.D.H., 2010. Effects of different dietary microalgae on survival, growth, settlement and fatty acid composition of blue mussel (Mytilus galloprovincialis) larvae. Aquaculture 309, 115–124. https://doi.org/10.1016/j.aquaculture.2010.09.024
Powell, R.J., Hill, R.T., 2013. Rapid aggregation of biofuel-producing algae by the bacterium Bacillus sp. Strain RP1137. Applied and Environmental Microbiology 79, 6093–6101. https://doi.org/10.1128/AEM.01496-13
Prahl, F.G., Wakeham, S.G., 1987. Calibration of unsaturation patterns in long-chain ketone compositions for palaeotemperature assessment. Nature 330, 367–369. https://doi.org/10.1038/330367a0
Puhan, S., Saravanan, N., Nagarajan, G., Vedaraman, N., 2010. Effect of biodiesel unsaturated fatty acid on combustion characteristics of a DI compression ignition engine. Biomass and Bioenergy 34, 1079–1088. https://doi.org/10.1016/j.biombioe.2010.02.017
Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Applied Microbiology and Biotechnology 65, 635–648. https://doi.org/10.1007/s00253-004-1647-x
Qin, J.G., 2010. Hydrocarbons from Algae, in: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 2817–2826. https://doi.org/10.1007/978-3-540-77587-4_209
Raheem, A., Prinsen, P., Vuppaladadiyam, A.K., Zhao, M., Luque, R., 2018. A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. Journal of Cleaner Production 181, 42–59. https://doi.org/10.1016/j.jclepro.2018.01.125
Ramanan, R., Kang, Z., Kim, B.-H.H., Cho, D.-H.H., Jin, L., Oh, H.-M.M., Kim, H.-S.S., 2015. Phycosphere bacterial diversity in green algae reveals an apparent similarity across habitats. Algal Research 8, 140–144. https://doi.org/10.1016/j.algal.2015.02.003
Ramanan, R., Kim, B.-H., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2016. Algae–bacteria interactions: Evolution, ecology and emerging applications. Biotechnology Advances 34, 14–29. https://doi.org/10.1016/j.biotechadv.2015.12.003
Ranjan, A., Patil, C., Moholkar, V.S., 2010. Mechanistic assessment of microalgal lipid extraction. Industrial & Engineering Chemistry Research 49, 2979–2985. https://doi.org/10.1021/ie9016557
Ranjith Kumar, R., Hanumantha Rao, P., Arumugam, M., 2015. Lipid extraction methods from microalgae: A comprehensive review. Frontiers in Energy Research 2. https://doi.org/10.3389/fenrg.2014.00061
Rao, D., Webb, J.S., Holmström, C., Case, R., Low, A., Steinberg, P., Kjelleberg, S., 2007. Low densities of epiphytic bacteria from the marine alga *Ulva australis* inhibit settlement of

fouling organisms. Applied and Envriomental Microbiology 73, 7844–7852. https://doi.org/10.1128/AEM.01543-07
Raud, M., Kikas, T., Sippula, O., Shurpali, N.J., 2019. Potentials and challenges in lignocellulosic biofuel production technology. Renewable and Sustainable Energy Reviews 111, 44–56. https://doi.org/10.1016/j.rser.2019.05.020
Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of Cell Biology 17, 208–212. https://doi.org/10.1083/jcb.17.1.208
Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K., Peccia, J., 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. BMC Genomics 12. https://doi.org/10.1186/1471-2164-12-148
Robak, K., Balcerek, M., 2018. Review of second generation bioethanol production from residual biomass. Food Technology and Biotechnology 56, 174–187. https://doi.org/10.17113/ftb.56.02.18.5428
Rockwell, N.C., Lagarias, J.C., Bhattacharya, D., 2014. Primary endosymbiosis and the evolution of light and oxygen sensing in photosynthetic eukaryotes. Frontiers in Ecology and Evolution 2.
Ryckebosch, E., Muylaert, K., Foubert, I., 2012. Optimization of an analytical procedure for extraction of lipids from microalgae. Journal of the American Oil Chemists' Society 89, 189–198. https://doi.org/10.1007/s11746-011-1903-z
Sahu, A., Pancha, I., Jain, D., Paliwal, C., Ghosh, T., Patidar, S., Bhattacharya, S., Mishra, S., 2013. Fatty acids as biomarkers of microalgae. Phytochemistry 89, 53–58. https://doi.org/10.1016/j.phytochem.2013.02.001
Sajjadi, B., Chen, W.-Y., Raman, Abdul.Aziz.A., Ibrahim, S., 2018. Microalgae lipid and biomass for biofuel production: A comprehensive review on lipid enhancement strategies and their effects on fatty acid composition. Renewable and Sustainable Energy Reviews 97, 200–232. https://doi.org/10.1016/j.rser.2018.07.050
Schatz, D., Rosenwasser, S., Malitsky, S., Wolf, S.G., Feldmesser, E., Vardi, A., 2017. Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga. Nature Microbiology 2, 1485–1492. https://doi.org/10.1038/s41564-017-0024-3
Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: High-efficiency microalgae for biodiesel production. BioEnergy Research 1, 20–43. https://doi.org/10.1007/s12155-008-9008-8
Schreiber, U., Schliwa, U., Bilger, W., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynthesis Research 10, 51–62. https://doi.org/10.1007/BF00024185
Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., Smith, A.G., 2010. Biodiesel from algae: challenges and prospects. Current Opinion in Biotechnology 21, 277–286. https://doi.org/10.1016/j.copbio.2010.03.005
Segev, E., Castañeda, I.S., Sikes, E.L., Vlamakis, H., Kolter, R., 2016. Bacterial influence on alkenones in live microalgae. Journal of Phycology 52, 125–130. https://doi.org/10.1111/jpy.12370
Segovia, M., Haramaty, L., Berges, J.A., Falkowski, P.G., 2003. Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the evolution of apoptosis in higher plants and metazoans. Plant Physiology 132, 99–105. https://doi.org/10.1104/pp.102.017129
Selje, N., Simon, M., Brinkhoff, T., 2004. A newly discovered Roseobacter cluster in temperate and polar oceans. Nature 427, 445–448. https://doi.org/10.1038/nature02272

Seyedsayamdost, M.R., Case, R.J., Kolter, R., Clardy, J., 2011. The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. Nature Chemistry 3, 331–335. https://doi.org/10.1038/nchem.1002

Seymour, J.R., Amin, S.A., Raina, J.-B., Stocker, R., 2017. Zooming in on the phycosphere: the ecological interface for phytoplankton–bacteria relationships. Nature Microbiology 2, 17065. https://doi.org/10.1038/nmicrobiol.2017.65

Sharma, K.K., Schuhmann, H., Schenk, P.M., 2012. High lipid induction in microalgae for biodiesel production. Energies 5, 1532–1553. https://doi.org/10.3390/en5051532

Shin, S.-E., Lim, J.-M., Koh, H.G., Kim, E.K., Kang, N.K., Jeon, S., Kwon, S., Shin, W.-S., Lee, B., Hwangbo, K., Kim, J., Ye, S.H., Yun, J.-Y., Seo, H., Oh, H.-M., Kim, K.-J., Kim, J.-S., Jeong, W.-J., Chang, Y.K., Jeong, B., 2016. CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. Scientific Reports 6. https://doi.org/10.1038/srep27810

Simó, R., 2001. Production of atmospheric sulfur by oceanic plankton: biogeochemical, ecological and evolutionary links. Trends in Ecology & Evolution 16, 287–294. https://doi.org/10.1016/S0169-5347(01)02152-8

Simon, N., Cras, A.-L., Foulon, E., Lemée, R., 2009. Diversity and evolution of marine phytoplankton. Comptes Rendus Biologies 332, 159–170. https://doi.org/10.1016/j.crvi.2008.09.009

Singh, A., Nigam, P.S., Murphy, J.D., 2011. Renewable fuels from algae: An answer to debatable land based fuels. Bioresource Technology 102, 10–16. https://doi.org/10.1016/j.biortech.2010.06.032

Singh, P., Kumari, S., Guldhe, A., Misra, R., Rawat, I., Bux, F., 2015. Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. Renewable and Sustainable Energy Reviews 55, 1–16. https://doi.org/10.1016/j.rser.2015.11.001

Singh, S., Kate, B.N., Banerjee, U.C., 2005. Bioactive compounds from cyanobacteria and microalgae: An overview. Critical Reviews in Biotechnology 25, 73–95. https://doi.org/10.1080/07388550500248498

Solovchenko, A.E., 2012. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. Russian Journal of Plant Physiology 59, 167–176. https://doi.org/10.1134/S1021443712020161

Song, D., Xi, B., Sun, J., 2016. Characterization of the growth, chlorophyll content and lipid accumulation in a marine microalgae *Dunaliella tertiolecta* under different nitrogen to phosphorus ratios. Journal of Ocean University of China 15, 124–130. https://doi.org/10.1007/s11802-016-2797-z

Sorigué, D., Légeret, B., Cuiné, S., Morales, P., Mirabella, B., Guédeney, G., Li-Beisson, Y., Jetter, R., Peltier, G., Beisson, F., 2016. Microalgae synthesize hydrocarbons from long-chain fatty acids via a light-dependent pathway. Plant Physiology 171, 2393–2405. https://doi.org/10.1104/pp.16.00462

Sosik, H.M., Mitchell, B.G., 1994. Effects of temperature on growth, light absorption, and quantum yield in *Dunaliella Tertiolecta* (chlorophyceae). Journal of Phycology 30, 833–840. https://doi.org/10.1111/j.0022-3646.1994.00833.x

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering 101, 87–96. https://doi.org/10.1263/jbb.101.87

Su, J.Q., Yang, X.R., Zheng, T.L., Tian, Y., Jiao, N.Z., Cai, L.Z., Hong, H.S., 2007. Isolation and characterization of a marine algicidal bacterium against the toxic dinoflagellate *Alexandrium tamarense*. Harmful Algae 6, 799–810. https://doi.org/10.1016/j.hal.2007.04.004

Taher, H., Al-Zuhair, S., Al-Marzouqi, A.H., Haik, Y., Farid, M., 2014. Effective extraction of microalgae lipids from wet biomass for biodiesel production. Biomass and Bioenergy 66, 159–167. https://doi.org/10.1016/j.biombioe.2014.02.034

Tan, K.W.M., Lin, H., Shen, H., Lee, Y.K., 2016. Nitrogen-induced metabolic changes and molecular determinants of carbon allocation in *Dunaliella tertiolecta*. Scientific Reports 6. https://doi.org/10.1038/srep37235

Tang, H., Abunasser, N., Garcia, M.E.D., Chen, M., Simon Ng, K.Y., Salley, S.O., 2011. Potential of microalgae oil from *Dunaliella tertiolecta* as a feedstock for biodiesel. Applied Energy 88, 3324–3330. https://doi.org/10.1016/j.apenergy.2010.09.013

Thompson, G.A., 1996. Lipids and membrane function in green algae. Biochimica et Biophysica Acta 1302, 7–45.

Tirichine, L., Bowler, C., 2011. Decoding algal genomes: tracing back the history of photosynthetic life on Earth: Decoding algal genomes. The Plant Journal 66, 45–57. https://doi.org/10.1111/j.1365-313X.2011.04540.x

Toyofuku, M., Morinaga, K., Hashimoto, Y., Uhl, J., Shimamura, H., Inaba, H., Schmitt-Kopplin, P., Eberl, L., Nomura, N., 2017. Membrane vesicle-mediated bacterial communication. The ISME Journal 11, 1504–1509. https://doi.org/10.1038/ismej.2017.13

Trentacoste, E.M., Shrestha, R.P., Smith, S.R., Glé, C., Hartmann, A.C., Hildebrand, M., Gerwick, W.H., 2013. Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. Proceedings of the National Academy of Sciences of the United States of America 110, 19748–19753. https://doi.org/10.1073/pnas.1309299110

Ummalyma, S.B., Gnansounou, E., Sukumaran, R.K., Sindhu, R., Pandey, A., Sahoo, D., 2017. Bioflocculation: An alternative strategy for harvesting of microalgae- An overview. Bioresource Technology 242, 227–235. https://doi.org/10.1016/j.biortech.2017.02.097

van Kooten, O., Snel, J.F.H., 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynthesis Research 25, 147–150. https://doi.org/10.1007/BF00033156

Vieler, A., Brubaker, S.B., Vick, B., Benning, C., 2012. A lipid droplet protein of Nannochloropsis with functions partially analogous to plant oleosins. Plant Physiology 158, 1562–1569. https://doi.org/10.1104/pp.111.193029

Vinayak, V., Manoylov, K., Gateau, H., Blanckaert, V., Hérault, J., Pencréac'h, G., Marchand, J., Gordon, R., Schoefs, B., 2015. Diatom milking: A review and new approaches. Marine Drugs 13, 2629–2665. https://doi.org/10.3390/md13052629

Wagner-Döbler, I., Biebl, H., 2006. Environmental biology of the marine *Roseobacter* lineage. Annual Review of Microbiology 60, 255–280. https://doi.org/10.1146/annurev.micro.60.080805.142115

Wang, H., Hill, R.T., Zheng, T., Hu, X., Wang, B., 2016. Effects of bacterial communities on biofuel-producing microalgae: stimulation, inhibition and harvesting. Critical Reviews in Biotechnology 36, 341–352. https://doi.org/10.3109/07388551.2014.961402

Wang, R., Xue, S., Zhang, D., Zhang, Q., Wen, S., Kong, D., Yan, C., Cong, W., 2015. Construction and characteristics of artificial consortia of *Scenedesmus obliquus* -bacteria for *S. obliquus* growth and lipid production. Algal Research 12, 436–445. https://doi.org/10.1016/j.algal.2015.10.002

Weiland, P., 2010. Biogas production: current state and perspectives. Applied Microbiology and Biotechnology 85, 849–860. https://doi.org/10.1007/s00253-009-2246-7

Xue, J., Niu, Y.-F., Huang, T., Yang, W.-D., Liu, J.-S., Li, H.-Y., 2015. Genetic improvement of the microalga *Phaeodactylum tricornutum* for boosting neutral lipid accumulation. Metabolic Engineering 27, 1–9. https://doi.org/10.1016/j.ymben.2014.10.002

Yáñez-Mó, M., Siljander, P.R.-M., Andreu, Z., Bedina Zavec, A., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais,

S., Falcon-Perez, J.M., Ghobrial, I.M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N.H.H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Krämer-Albers, E.-M., Laitinen, S., Lässer, C., Lener, T., Ligeti, E., Linē, A., Lipps, G., Llorente, A., Lötvall, J., Manček-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E.N.M., Nyman, T.A., O'Driscoll, L., Olivan, M., Oliveira, C., Pállinger, É., del Portillo, H.A., Reventós, J., Rigau, M., Rohde, E., Sammar, M., Sánchez-Madrid, F., Santarém, N., Schallmoser, K., Stampe Ostenfeld, M., Stoorvogel, W., Stukelj, R., Van der Grein, S.G., Helena Vasconcelos, M., Wauben, M.H.M., De Wever, O., 2015. Biological properties of extracellular vesicles and their physiological functions. Journal of Extracellular Vesicles 4, 27066. https://doi.org/10.3402/jev.v4.27066

Yao, L., Gerde, J.A., Lee, S.-L., Wang, T., Harrata, K.A., 2015. Microalgae lipid characterization. Journal of Agricultural and Food Chemistry 63, 1773–1787. https://doi.org/10.1021/jf5050603

Zamith-Miranda, D., Nimrichter, L., Rodrigues, M.L., Nosanchuk, J.D., 2018. Fungal extracellular vesicles: modulating host–pathogen interactions by both the fungus and the host. Microbes and Infection 20, 501–504. https://doi.org/10.1016/j.micinf.2018.01.011

Zhu, L., 2015. Microalgal culture strategies for biofuel production: a review. Biofuels, Bioproducts and Biorefining 9, 801–814. https://doi.org/10.1002/bbb.1576

Zienkiewicz, K., Du, Z.-Y., Ma, W., Vollheyde, K., Benning, C., 2016. Stress-induced neutral lipid biosynthesis in microalgae — Molecular, cellular and physiological insights. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1861, 1269–1281. https://doi.org/10.1016/j.bbalip.2016.02.008

Zulu, N.N., Zienkiewicz, K., Vollheyde, K., Feussner, I., 2018. Current trends to comprehend lipid metabolism in diatoms. Progress in Lipid Research 70, 1–16. https://doi.org/10.1016/j.plipres.2018.03.001