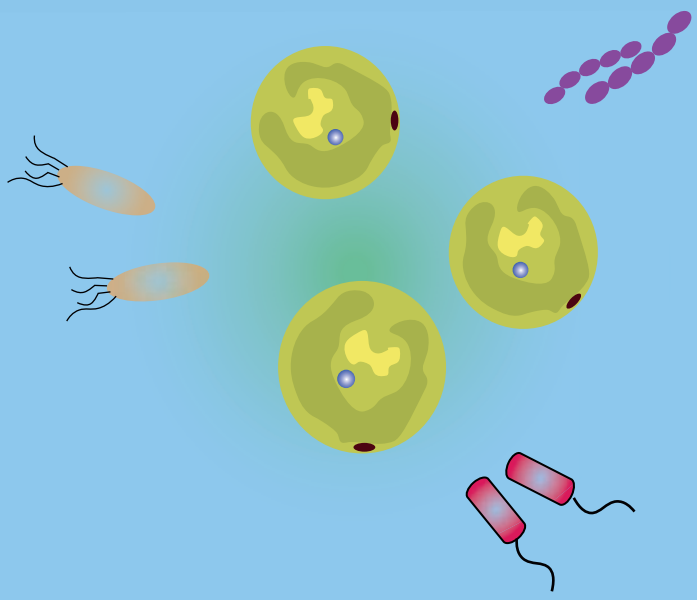
Algae-associated bacteria in photobioreactors



Jie Lian

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Thesis committee

Promotors

Prof. Dr Hauke Smidt

Personal chair, Laboratory of Microbiology

Wageningen University & Research

Prof. Dr Rene Wijffels

Professor of Bioprocess Engineering

Wageningen University & Research

Co-promotor

Dr Detmer Sipkema

Associate professor, Laboratory of Microbiology

Wageningen University & Research

Other members

Prof. Dr Eddy Smid, Wageningen University & Research

Dr Dedmer van de Waal, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen

Dr Alette Langenhoff, Wageningen University & Research

Dr David Green, The Scottish Association for Marine Science (SAMS), Oban, UK

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Algae-associated bacteria in photobioreactors

Jie Lian

Thesis

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Jie Lian

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Chapter 1

General introduction and thesis outline

1.1. Symbioses of algae and bacteria

Algae are prominent primary producers in aquatic ecosystems [1, 2]. Approximately 50% of global photosynthesis is attributed to algae that consequently play pivotal roles in the global carbon cycle and oxygen flux [3, 4]. They are the ancestors of land plants and can be dated back to 450~470 million years ago [5]. However, research on plant-microbe interactions has drawn much more attention than algae-microbe interactions. Plant-microbe interactions have been investigated with a major focus on plant pathogens, symbiotic nitrogen-fixing rhizobia and plant-associated arbuscular mycorrhizal fungi [6-8]. In recent years, it has been shown that multipartite interactions continuously take place in and on plants, and that plant-associated microorganisms provide a range of benefits for plant growth and health through influencing nutrient uptake, affecting plant-pathogen interactions and increasing the tolerance to biotic/abiotic stresses [9-12].

In plant biology, the most intensively studied biome is the rhizosphere. The term rhizosphere was first introduced in 1904 to describe the narrow region of soil which is subjected to the influence of plant roots [13, 14]. The microenvironment surrounding algal cells where algae and bacteria interact is analogous to the plant rhizosphere; thus Bell and Mitchell [15] coined the term phycosphere to fit an aquatic equivalent.

The long coevolutionary history of algae and bacteria has led to multifaceted and highly sophisticated interactions [16, 17]. Lucas [18] first pointed out that there are non-predatory relationships in water between algae and bacteria based on the release of metabolites, ranging from toxins to vitamins and hormones. Provasoli [19] went a step further to suggest that bacteria can enhance the growth of algae. Following Provasoli's review numerous papers provided ample evidence of algal-bacterial interactions [20-24]. These interactions span a wide range of ecological relationships from mutualism to commensalism and parasitism [25]. Mutualistic interactions between algae and bacteria are characterised by bacterial provision of growth-

limiting factors to algae in exchange for organic carbon. Two well-studied examples are that *Halomonas* sp. and *Mesorhizobium* sp. supply vitamin B_{12} to the marine red alga *Porphyridium purpureum* and the green alga *Chlamydomonas nivalis*, respectively [26, 27]. Another example is that the nitrogen fixing bacterium *Bacillus pumilus* ES4 provides nitrogen to the green microalga *Chlorella vulgaris* in oligotrophic environments [28]. Cho, Ramanan [29] noted that other bacteria in the phycosphere of *Chlorella vulgaris* might be commensals, residing in the algal sheath for carbon and shelter. Commensalism differs from mutualism in that only one partner directly benefits from the interaction. However, in most cases it is ambiguous whether mutualistic or commensal interactions prevail; or as Zapalski [30] pointed out, commensals can be regarded as non-interacting partners and it is difficult to prove absence of interaction.

In contrast to mutualism and commensalism, antagonism/parasitism has been rather well investigated [31-34]. Multiple bacteria can negatively affect algal growth, which has encouraged research into possible applications of these adverse effects to lyse microalgal and cyanobacterial cells that cause harmful algal blooms [35-37]. For instance, the filtrate of *Rhodococcus* sp. cultures showed cyanobactericidal activity on *Microcystis aeruginosa* and *Anabaena variabilis* [37], and *Brevibacterium* sp. was shown to produce algicidal compounds to kill the toxic dinoflagellate *Alexandrium tamarense* [38]. This research has seen an increased attention, because algal blooms occur more frequently in recent years [25, 39, 40]. Toxins produced by these harmful algae can accumulate throughout the food chain, which has great impact on aquatic ecology, public health and local economy [41-43].

1.2. Omics methods for studying host-microbe interactions

The specificity and extent of most algal-bacterial interactions still remains to be characterized [44]. Standard microbiology methods and biochemical analysis are still indispensable in deciphering algal-bacterial interactions (Figure 1.1). For instance, bacterial production of

micronutrients such as vitamin B_1 or B_{12} has been demonstrated in defined co-cultures of bacteria and algae with standard microbiological approaches [26, 45], and mutualistic/parasitic relationships between *Emiliana huxleyi* and *Phaeobacter gallaeciensis* were studied by detailed biochemical analysis [46]. More recently, the rapid advances in omics technologies offer alternative ways to study interactions between algae and bacteria [47].

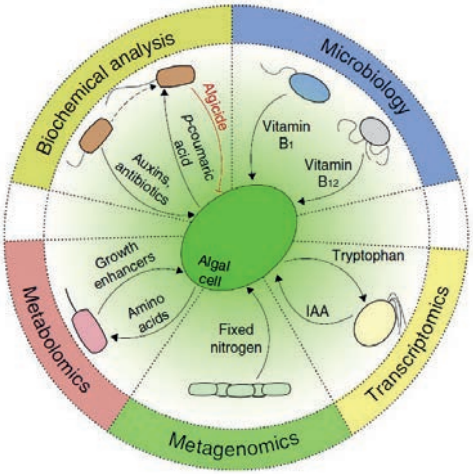

Figure 1.1. Different approaches used to characterize algal-bacterial interactions, including examples for their application. Adapted from Cooper and Smith [48].

Combining metagenome, transcriptome and metabolome data is powerful for identifying the major bacterial groups associated with algae and characterization of the pathways and molecules involved in trophic exchange or signalling processes (Figure 1.1). Together with mechanistic examinations of algal physiology and biochemistry [17], these omics-enabled analyses will allow unravelling how algae sense and cooperate with bacteria, and how interactions change in response to fluctuating conditions or chemical signals. At the moment, multi-omics approaches for the study of algae and their associated bacteria are in their initial stages, but a number of successful cases exist already. For example, a metagenomics approach was used to elucidate the range of algal-bacterial species engaged in nitrogen-based symbioses

in the Atlantic Ocean [49]. Furthermore, transcriptomic analysis of a co-culture of the diatom *Pseudonitzschia multiseries* and *Sulfitobacter* sp. SA11 revealed up-regulation of tryptophan biosynthesis genes in the alga and indole 3-acetic acid (IAA) production in the bacterium. The produced IAA, an auxin, was found to promote cell division in the diatom [16].

1.3. Microalgae production

Microalgal biomass is a promising source for chemicals, food and feed supplements, and biofuels [50, 51]. High areal yields, high oil content, low water consumption and the possibility of production on non-arable land make microalgae more compelling than many other crops [52, 53]. Currently, only algal specialties or high value products, for instance, unsaturated fatty acids and pigments, are commercially profitable [54, 55]. For commercial production of commodities from algal biomass the production costs should decrease to less than 1 €/kg dry weight [52, 56], while with state of the art technology the current cost can be around 3 €/kg as estimated in techno-economic models for a 100 ha facility [57].

A major parameter affecting microalgal production cost is photosynthetic efficiency [56, 57]. Photosynthetic efficiencies obtained under outdoor conditions (3%) are lower than values obtained under laboratory conditions (6%) [52]. At least in part this is caused by the continuously changing weather conditions occurring under outdoor conditions where critical cultivation parameters such as light intensity cannot be controlled and temperature can only partly be controlled.

1.3.1. Microalgae production systems

One of the key factors for commercially viable microalgal production is the design of a suitable reactor system. The open raceway pond, horizontal and vertical tubular photobioreactors, and flat panel photobioreactors are the most often used designs at a semi-industrial scale [58]. Each of the reactor designs has its own advantages and disadvantages, which are outlined below.

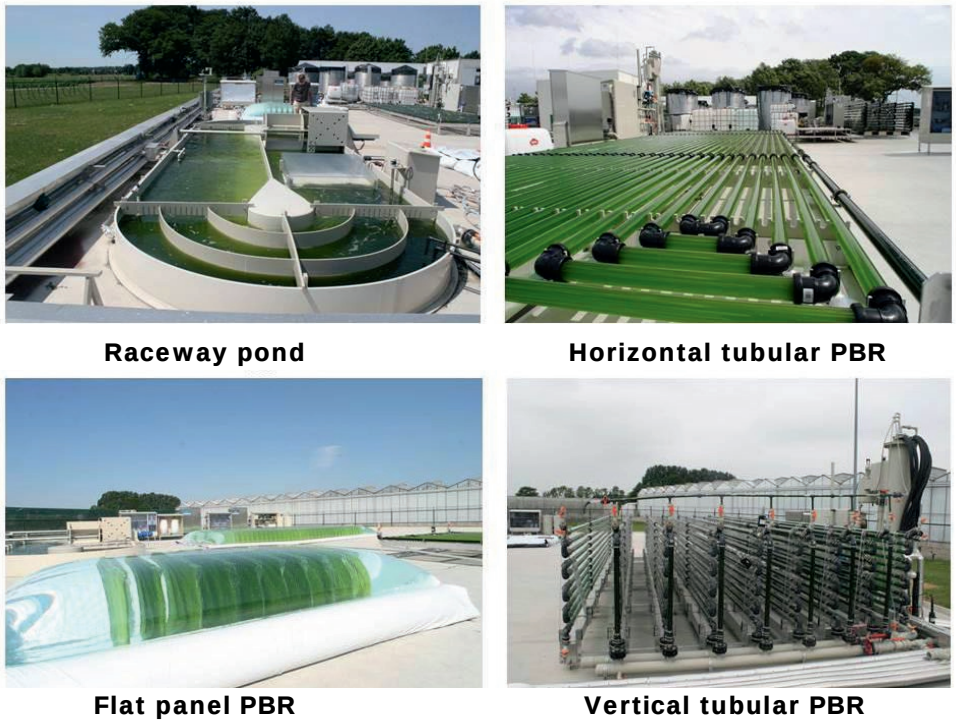

Figure 1.2. Overview of cultivation systems installed at the AlgaePARC pilot facility (Wageningen, the Netherlands). PBR: photobioreactor. Adapted from [59].

1.3.1.1. Open ponds

Open ponds (natural waters and artificial ponds or containers) are the most commonly used systems to grow algae at large scale [58, 60]. Open ponds are equipped with paddle wheels to mix algal cultures through long channels. One of the major advantages of open ponds is that they are cheaper and easier to build and operate than most closed photobioreactors [61, 62]. However, open systems are subject to a range of limitations such as poor light utilization by algal cells, water evaporation and diffusion of CO_2 to the atmosphere.

1.3.1.2. Closed tubular photobioreactors

In closed tubular photobioreactors algal cultures are confined in transparent glass- or plastic tubes and circulated by a centrifugal pump [63]. Tubular photobioreactors can be found as a single horizontal plane or multiple vertical planes (Figure 1.2). Typically, the liquid velocity in tubular systems is 0.3-0.6 m s^{-1}, while higher velocity (0.6-0.9 m s^{-1}) is used to prevent fouling. The degasser is an important part of any tubular system and is used to remove dissolved oxygen produced by photosynthesis. Accumulation of dissolved oxygen is known to negatively affect algal growth [64]. The closed tubular system is one of the most suitable photobioreactors for outdoor mass cultures because of its large illumination surface area [58]. Nevertheless, temperature is difficult to control in outdoor tubular photobioreactors, and long tubular systems are characterized by gradients of oxygen and CO_2 concentrations along the tube [65].

1.3.1.3. Flat panel photobioreactors

Flat panel photobioreactors are compact rectangular vertical vessels that are made of transparent materials: glass, plastic plates or plastic films [66]. Due to the design, mixing takes place along the vertical axis, whereas there is limited mixing capacity along the horizontal axis. Insufficient horizontal axis mixing makes the width of separate units of this system shorter than that of tubular systems, and as a result, more labour and infrastructure are required to construct and operate a commercial plant with flat panels in comparison to tubular systems [59]. A successful example of flat panels designs that have solved these issues is the ProviAPT system designed by Proviron (Proviron Holding NV, Belgium) (Figure 1.2).

1.3.2. Bacteria in algae production systems

In addition to above-mentioned factors, another important aspect of photobioreactor design for cultivation of microalgae is to reduce the risks of microbial contamination. Contamination by algal predators (ciliates and flagellates) and other fast growing heterotrophic bacteria have restricted the commercial production of algae [67, 68]. In contrast to closed bioreactors, open

ponds are exposed to their ambient environment, and thus are more prone to contamination in general [69]. Therefore, only a few of the algal species that can be grown under extreme conditions (high salinity, low pH, etc.) that hinder successful invasion of ambient microorganisms are suitable to be grown in open ponds [67]. Although it is a general consensus that open ponds are more easily contaminated than closed bioreactors, studies that simultaneously compare bacterial diversity and abundance in open ponds and enclosed bioreactors are limited in literature. On the other hand, for large-scale production of microalgae it is neither practical nor economical to completely sterilize the growth media. Therefore, it is inevitable that all large-scale microalgae production systems contain a number of non-target organisms [70, 71].

1.4. Targeted algal species and their associated bacteria

1.4.1. *Botryococcus braunii*

Botryococcus braunii (Chlorophyta) is of industrial interest for its ability to produce significant amounts of long-chain hydrocarbons (C_{30}-C_{40}) and exopolysaccharides [72-74]. *B. braunii* can be subclassified into four races depending on the types of hydrocarbons and exopolysaccharides produced [75, 76]. Another trait of *B. braunii* is that it secretes the majority of hydrocarbons and polysaccharides, facilitating the harvesting of these products. However, wild type strains of *B. braunii* are slow growers with a productivity of ~0.1-0.2 g L^{-1} d^{-1} [77, 78].

The majority of *B. braunii* cultures are not axenic [75, 79], but co-exist with various microbes [80, 81]. Earlier research has revealed the presence of *Pseudomonas* spp. and *Flavobacterium* spp. among other bacteria in *B. braunii* cultures [82]. Metagenomic profiling showed that *B. braunii*-associated bacteria include representatives of *Bradyrhizobium* and *Methylobacterium* (both members of the order *Rhizobiales*), *Dyadobacter*, *Achromobacter* and *Asticcacaulis* [81]. Furthermore, the addition of selected bacterial species (*Rhizobium* sp.) to axenic cultures

resulted in an increase in biomass productivity and hydrocarbon yield of *B. braunii* [80]. More recent growth experiments indicated that *B. braunii* Ba10 has a higher biomass (1.8-fold) and hydrocarbon (1.5-fold) yield in the presence of "*Candidatus* Phycosocius bacilliformis" [83]. However, the precise reasons for this increase remain unknown. Contrasting these reports, Gouveia *et al*. [84] found that biomass productivity and extracellular carbohydrate production of *B. braunii* were significantly enhanced after removal of its associated bacteria with UV-C, which indicates that bacteria can also be antagonistic to microalgae in this respect.

1.4.2. *Nannochloropsis*

Species of the genus *Nannochloropsis* (Ochrophyta) possess a high ability to produce triacylglycerols under nitrogen limitation. Therefore these oleaginous species are considered promising candidates for biofuel production [85]. Additionally, *Nannochloropsis* is an important source of the essential ω-3 LC-PUFA eicosapentaenoic acid (EPA; 20:5 5-3), which is an important ingredient in marine aquaculture nutrition [86].

In a small photobioreactor system bacterial counts were shown to outnumber *Nannochloropsis* cell counts by 10- to 100-fold [87]. Investigating *Nannochloropsis*-associated bacteria received attention because *Nannochloropsis* is widely used as nutritional source in fish hatcheries, and the bacterial community structure is a key factor affecting the survival of fish larvae [88, 89]. Nakase and Eguchi [90] applied direct viable counts and fluorescence *in situ* hybridization and revealed that the most abundant bacteria in a *Nannochloropsis* sp. culture were members of the *Alphaproteobacteria* and the *Cytophaga-Flavobacterium* cluster. In another bacterial community analysis of large-scale cultures of *Nannochloropsis salina*, the most abundant bacterial taxon was a member of *Deltaproteobacteria* [91]. In addition, a recent investigation of the bacterial communities in small, medium and large cultivation setups of *Nannochloropsis*

salina showed that more than 70% of all 16S ribosomal RNA gene sequences belonged to *Saprospirae, Cytophagia, Flavobacteria* and *Alphaproteobacteria* [69].

In addition to bacterial community profiling of *Nannochloropsis* cultures, specific bacterial interactions with *Nannochloropsis* have been found. For example, *Nannochloropsis oculata* was found to enhance the ability of a member of the *Roseobacter* clade to inhibit the fish pathogen *Vibrio anguillarum* [92]. In addition, bacterium HW001 (*Pseudomonadales*) was shown to cause aggregation of *Nannochloropsis oceanica* IMET1 cells, which provides a novel approach for the harvest of algal biomass [87].

1.5. Research aim and outline of the thesis

Understanding of interactions between algae and bacteria is important both for aquatic ecology and for biotechnological purposes as a result of a growing interest in exploiting algae as a biotechnological platform for production of high-value molecules. However, knowledge on algae-associated bacteria is still rather limited, especially in large-scale cultivation systems.

The aim of this thesis is therefore to improve our understanding of interactions between microalgae and bacteria in photobioreactors in order to improve microalgae cultivation at the large scale. Here we focussed on bacterial community composition dynamics of two common microalgal species, *Botryococcus braunii* and *Nannochloropsis* sp. In addition, this thesis explores how bacterial isolates affect the growth of *Nannochloropsis* sp., which may have potential biotechnological implications.

Chapter 2 reviews recent research progress on algal-bacterial interactions. We aim to summarize the general trend of bacterial community composition in association with different microalgal species and to understand physiological and molecular mechanisms behind beneficial and adverse algal-bacterial interactions. In addition, we discuss a wide range of examples on how principles of algal-bacterial interactions can be applied in algal biotechnology.

Chapter 3 investigates the bacterial community of 12 strains of *Botryococcus braunii* originating from six culture collections. In this chapter we aim to identify the bacterial core community of *B. braunii* strains. Furthermore, we try to correlate specific bacterial communities to the different races of *B. braunii*, and discuss the possible roles of the most abundant bacterial species in their interaction with *B. braunii*.

Chapter 4 compares the bacterial community of *Nannochloropsis* sp. CCAP211/78 cultivated in different types of reactors (lab scale *vs* pilot scale, indoor reactors *vs* outdoor reactors) and bacterial community dynamics during different algal growth stages, and delves into how prevailing environmental/chemical factors correlate to bacterial community composition and abundance.

Chapter 5 describes experimental approaches for the isolation of bacteria from two outdoor bioreactors and generation of an axenic culture of *Nannochloropsis* sp. CCAP211/78. In addition, experiments are designed to screen how *Nannochloropsis* sp. CCAP211/78 responds to the addition of isolated bacterial strains in 24-well microplates illuminated by a custom-made LED (light-emitting-diode) box.

Finally, **chapter 6** provides a general discussion by summarising the findings of this PhD thesis and discussing the potential of bacterial-algal co-cultures for further improvement of algal production and how we can better understand algal-bacterial interactions by new state of art technology.

Chapter 2

The effect of the algal microbiome on industrial production of microalgae

Jie Lian, Rene H. Wijffels, Hauke Smidt, Detmer Sipkema

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Abstract

Microbes are ubiquitously distributed and they are also present in algae production systems. The algal microbiome is a pivotal part of the alga holobiont and has a key role in modulating algal populations in nature. However, there is a lack of knowledge on the role of bacteria in artificial systems ranging from laboratory flasks to industrial ponds. Co-existing microorganisms, and predominantly bacteria, are often regarded as contaminants in algal research, but recent studies manifested that many algal symbionts not only promote algal growth but also offer advantages in downstream processing. Because of the high expectations for microalgae in a bio-based economy, better understanding of benefits and risks of algal-microbial associations are important for the algae industry. Reducing production cost may be through applying specific bacteria to enhance algae growth at large scale as well as through preventing the growth of a broad spectrum of algal pathogens. In this review, we highlight the latest studies of algae-microbial interactions and their underlying mechanisms, discuss advantages of large scale algal-bacterial co-cultivation and extend such knowledge to a broad range of biotechnological applications.

Keywords: microalgae-associated bacteria; algae-bacteria interaction; co-cultivation; algal biotechnology

2.1. Introduction

During the last forty years efforts have been undertaken to realize the high potential of algal products for industrial applications. Algae have been widely recognized for their capacity to produce polysaccharides, lipids, pigments and other valuable compounds in significant amounts [56]. Algae are used for producing healthy food and food supplements, and as an ingredient in aquaculture, animal feed and as soil bio-fertilizer [93, 94].

Most algae, if not all, live in symbiosis with multiple associated microorganisms throughout their lifespan [47]. In many cases, attempts to remove bacteria or fungi from microalgae have failed. Even in cases where such attempts were successful, microbiota-deprived algae usually exhibited poorer growth or aberrant phenotypes compared to the original strains, which indicates that the association between algae and other microorganisms is important for their existence [95].

Algae are known to release dissolved organic matter or signalling molecules to nurture specific bacterial communities in the phycosphere [96]. Close interactions in the phycosphere influence algal evolution and ecology in various ways. First of all, algae such as the diatoms *Phaeodactylum tricornutum* and *Thalassiasira pseudonana* have been shown to have acquired hundreds of genes predicted to be involved in nitrogen and organic carbon utilization, cell wall assembly, DNA recombination and the ornithine-urea cycle from co-occurring bacteria during more than 200 million years [97]. Secondly, bacteria synthesize important compounds for algal growth stimulation, spore germination, morphogenesis and pathogen resistance [16, 25, 96]. These compounds include micronutrients, siderophores, growth stimulants and antibiotics [46, 98-102]. In addition, symbiotic microorganisms help their algal hosts to cope with changing environmental conditions [24, 103].

On the other hand, many microbes have been reported to negatively affect algal growth [104, 105] and constitute big constraints for translating laboratory experiments to industrial practice. Unlike conventional microbial fermentation, large-scale algal cultivation is driven by light and mostly operated in fully exposed open ponds for microalgae and in open sea for macroalgae. However, open ponds are more susceptible to biological contaminations, such as viruses, predators/grazers, and parasites of various sources [106]. Therefore, stable production of algae in open systems is only possible when contaminants and infections are well studied so that monitoring and contingency measures can be implemented [107].

Apart from playing a role in enhancing microalgae production, associated bacteria can help the algae to perform more complex tasks with diverse applications. For instance, algae and bacteria cooperate in faster and more efficient removal of organic and inorganic waste and hazardous substances in wastewater treatment [108-110]. In turn, bacterial and viral pathogens are able to weaken or decompose the algal cell wall, which is a crucial step in algal-based extraction of chemicals and could also be explored to tackle frequently occurring harmful algae blooms at an early stage of the bloom [111, 112]. Furthermore, proteins or secondary metabolites of algicidal bacteria are potential biological agents in algal biomass harvest and cell disruption prior to biorefinery [113].

The aim of this review is to provide an overview of both beneficial and antagonistic algal-microbial interactions in natural and artificial systems, as well as to provide new perspectives about how to utilise such knowledge in algal biotechnology (Figure 2.1).

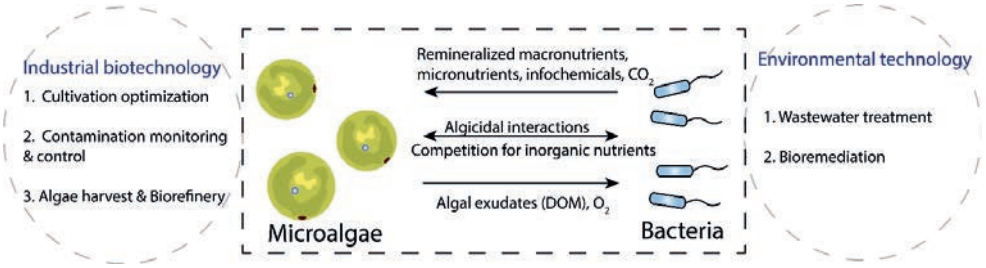

Figure 2.1. Potential applications of algal-bacterial interactions in industrial biotechnology and environmental biotechnology. DOM is dissolved organic matter.

2.2. Alga-associated bacteria in algae production systems

Although next generation sequencing (NGS) has led to an explosion of microbial diversity studies in microbial ecology research, only a limited number of studies have been published on NGS-based microbiota analysis in the context of microalgal production systems. In fact, most knowledge of alga-bacteria communities in applied settings come from wastewater treatment studies [114-116]. However, those systems are too different to microalgae production systems due to the presence of high concentrations of organic and inorganic material to expect a large overlap in microbial communities in wastewater treatment systems and algae production facilities. For that reason wastewater treatment with algae-bacteria consortia is treated separately in section 6. The molecular survey of bacterial diversity in three cultures (*Nannochloropsis salina* from a raceway pond and a closed photobioreactor respectively, and *Botryococcus braunii* from laboratory flasks) [69, 81, 91] and one biofilm sample from an outdoor photobioreactor (mixture of *Chlorella vulgaris* and *Scenedesmus obliquus*) [117] revealed that *Deltaproteobacteria* and *Gammaproteobacteria* in raceway pond and *Alphaproteobacteria* and *Bacteroidetes* in closed bioreactor were dominant in *N. salina* whereas *Gammaproteobacteria*, *Betaproteobacteria* and *Firmicutes* were the most prominent phyla in *B. braunii*. *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria* and *Gammaproteobacteri*a made up nearly three quarter of the biofilm bacterial community. Based

on this limited number of studies, *Proteobacteria*, and *Gammaproteobacteria* in particular, are found associated to cultured microalgae. *Cytophagales* and *Flavobacteriales* were the only two common bacterial orders among four studies. Several other taxa such as *Pseudomonadales*, *Burkholderiales*, *Caulobacterales* and *Rhodobacterales* were shared between either two studies. Our limited knowledge of bacterial communities associated to microalgae that is based on cultivation-independent studies currently prevents general statements about bacteria that are frequently found associated to microalgae, but finding correlations between algae and associated bacteria will be a good starting point for coming up with hypotheses on functional relationships. Therefore, more studies of bacterial communities found in microalgae bioreactors are urgently needed to obtain a clearer view on the species and genera that are commonly associated to algae.

2.3. Beneficial roles of bacteria

Although for most of the bacteria detected in microalgae production systems it is not known if/how they interact with the microalgae, recent observations have demonstrated that mutualistic algal-bacterial interactions are prevalent [2]. Multiple bacteria have been tested in co-cultivation to evaluate the effects on the growth of microalgae [104, 118, 119], or more specifically looked at the exchange of metabolites and how bacteria may lead to more robust algal cultures that can better withstand environmental perturbations.

Table 2.1. Impact of added bacteria on microalgae growth.

Microalga	Added bacteria	Effect	Methodology to prepare axenic algae	Reference
Chlorella vulgaris	*Bacillus pumilus*	Final cell density increased by 150% in N-free medium	Axenic but method not mentioned	[28]
Chlorella vulgaris	*Flavobacterium* sp., *Rhizobium* sp., *Hyphomonas* sp., *Sphingomonas* sp.	Cell density increased by more than 100%	Ultra-sonication, fluorescence activated cell sorter and micro-picking	[29]
Chlorella vulgaris	*Rhizobium* sp.	Cell count increased 72%, growth rate increased by 11%	Not axenic	[105]

Chlorella vulgaris	Multiple bacteria from tap water	Higher growth rate	Not axenic	[120]
Chlorella ellipsoidea	*Brevundimonas* sp.	Algal cell density increased three times after seven days	Serial streaking	[121]
Chlorella sorokiniana IAM C-212	*Microbacterium trichotecenolyticum*	Growth rate increased 16 %	Streptomycin, gentamicin, penicillin G, vancomycin and pimaricin	[20]
Dunaliella sp. SAG 19.3	*Alteromonas* sp. and *Muricauda* sp.	Biomass enhanced by 22%, 26%	Ampicillin, gentamicin, kanamycin, neomycin	[104]
Botryococcus braunii	BOTRYCO-2	Grow faster and biomass enhanced by 80%	Ampicillin	[83]
Lobomonas rostrata	*Mesorhizobium loti*	Providing vitamin B_{12}	Axenic but method not mentioned	[22]
Scrippsiella trochoidea	*Marinobacter* sp. strain DG879	Cell density increased over 6%	Streptomycin	[98]
Thalassiosira rotula	*Roseobacter* sp. and *Hyphomonas* sp.	Earlier start of growth, higher algal cell numbers	Axenic but method not mentioned	[122]
Phaeodactylum tricornutum Utex 646	*Alphaproteobacteriq* sp. strain 29	Cell density increased up to 55%	Axenic but method not mentioned	**[123]**

2.3.1. Alga-associated bacteria that enhance algal growth

Using either axenic or non-axenic algal cultures, a number of different bacteria ranging from specific isolates to microbial communities present in tap water have been evaluated for their effects on microalgae growth (Table 2.1). The best studied algae with respect to associated bacteria are members of the genus *Chlorella* (Table 2.1). Bacteria that have been shown to be beneficial to *Chlorella vulgaris* include members of the genera *Bacillus*, *Flavobacterium*, *Rhizobium*, *Hyphomonas* and *Sphingomonas*. *Bacillus pumilus* ES4 was shown to promote *Chlorella vulgaris* growth by providing fixed atmospheric nitrogen [28]. In another study when *Chlorella vulgaris* was cultivated with four different bacteria, maximum algal growth rate and final cell mass increased from 0.22 day^{-1} to 0.47 day^{-1} and from 1.3 g/L to 3.31 g/L, respectively (Table 2.1). This increased growth was furthermore accompanied by a slight rise in algal lipid content from 22.4% to 28% [29].

Similar to *Chlorella*, also for other green algae, such as those belonging to the genera *Dunaliella*, *Botryococcus* and *Lobomonas* beneficial effects were observed when adding specific bacterial partners to axenic cultures (Table 2.1). Biomass accumulation of *Botryococcus braunii* was almost doubled compared with that of axenic cultures [83]. Similarly, biomass production of *Dunaliella* sp. SAG 19.3 increased by 22% and 26% when co-cultivated with *Alteromonas* sp. or *Muricauda* sp., respectively [104]. Furthermore, it could be shown that the vitamin B_{12} synthesizing bacterium *Mesorhizobium loti* is indispensable for the survival of *Lobomonas rostrata* under conditions where the alga is cultivated without exogenous vitamin B_{12} [22]. Two diatoms and one dinoflagellate were all observed to benefit from co-existing bacteria (Table 2.1), as indicated by either higher cell numbers or a faster growth rate of the algae. The strongest stimulation of growth was reported for *Phaeodactylum tricornutum* in the presence of the *Alphaproteobacterium* strain 29, as demonstrated by a 55% rise in cell density [123].

2.3.2. Microbial associated salinity acclimation and thermal tolerance

Salinity is the major environmental factor that determines the distribution and performance of marine algae [124, 125]. Interestingly, in addition to their more direct ecophysiological roles, bacteria can also present a gene reservoir for algal evolution towards adaptation to different environmental conditions via horizontal gene transfer. The green alga *Picochlorum* sp. SENEW3 has a wide salt tolerance from at least 0.35% to 10.8% [126]. Compared to its less halotolerant sisters, the genome of the salt-tolerant strain was found to contain a suite of additional functional genes, twenty-four of which were derived from bacterial sources and were functional in response to salt stress [127]. Although not a microalga, it is interesting to note that the transition of the brown macroalga *Ectocarpus* sp. strain 371 from seawater to freshwater medium greatly depended on the associated bacterial community. Strain 371 is a small filamentous brown alga with broad range salinity tolerance that is mediated by adjusting cell wall structure and metabolism [128-130]. Cultures deprived of associated microbes were unable

to survive a salinity change, while this capability could be restored by restoring their microbiota [103].

Temperature is another important factor affecting growth and survival of algae [124]. This is relevant as industrially grown algal strains in shallow production ponds or flat panel bioreactors are exposed to considerable temperature fluctuations. The unicellular microalga *Chlamydomanas reinhardtii* grows best at a temperature between 20-32 °C [131]. The direct transfer of *C. reinhardtii* from an optimum (25°C) to a rather high temperature (45°C) results in chlorosis and cell death, which are caused by the repression of cobalamin-independent methionine synthase during heat stress. Through adding exogenous cobalamin or co-cultures of the alga with a cobalamin-producing bacterium (*Sinorhizobium meliloti*), cobalamin-dependent methionine synthase mediated methionine biosynthesis could be re-activated, thereby preventing death of algal cell [24].

Hence, a better understanding of adaptation and acclimation of both host and microbial symbionts to environmental changes may provide leads to improve robustness of large-scale cultivation of algae where environmental conditions cannot be as tightly controlled as in laboratory-based experiments.

2.3.3. Nutrient provision

Algae mainly need CO_2 and inorganic sources of nitrogen and phosphate for growth along with some micronutrients and cofactors [132]. Since fertilizer-grade nutrient input accounts for a major proportion of cost in algal cultivation, recycling or provision of these nutrients via bacteria may eventually make large-scale algal biomass production more economically viable [133].

2.3.3.1. Macro-nutrients

CO_2 is often the limiting substrate in large-scale algal ponds because gas transfer efficiency is limited from ambient air [134]. The main strategy to boost low CO_2 concentrations in algal cultures is to use CO_2-enriched gases, but additional supply of CO_2 comes with a significant cost [133]. Bacterial degradation of organic compounds released by algae contributes an additional source of CO_2 for algal growth, especially during CO_2 limiting conditions as this CO_2 can be fixed again by algae [135, 136]. This is exemplified with the case of a *Chlorella* sp. where carbon limitation was overcome when heterotrophic bacteria from a domestic wastewater treatment reactor were added to the algae culture and increased productivity of algal biomass by respectively 4.8 and 3.4 fold in two independent experiments [137].

Nitrogen fixing bacteria reduce atmospheric dinitrogen to ammonium that is the major preferred nitrogen source for algae growth [132]. For example, *Bacillus pumilus* ES4 is a plant-growth promoting bacterium that fixes nitrogen to enhance growth of *Chlorella vulgaris* [28]. Symbiotic nitrogen fixers are also present in coral holobionts, where they co-occur with *Symbiodinium* that is the most commonly coral-associated dinoflagellate genus [138]. Studies have revealed a strong positive correlation between the cell density of *Symbiodinium* and the number of nitrogen fixation gene copies from nitrogen-fixing bacteria, which partly demonstrate how corals and their dinoflagellate partners could survive in low-nutrient conditions [139]. The filamentous cyanobacteria *Richelia intracellularis* and *Calothrix rhizosoleniae* are close partners with diatoms living in the oligotrophic open ocean [140]. Higher growth rates were observed for diatoms with cyanobacteria as compared to diatoms without their nitrogen-fixing cyanobacterial partners. Moreover, using single cell resolution analyses it was shown that the N_2 fixation rates of cyanobacteria increased by 171-420 fold in symbiotic heterocystous cells associated with the corresponding diatoms as compared to free-living cyanobacteria [141].

Phosphorus is an essential nutrient for algal growth. In most cases algae can only take up inorganic phosphorus (P_i) derived from hydrolysis of organic phosphorus (P_o) [142]. Bacteria are the main agents involved in decomposing and mineralizing P_o through the secretion of phosphatases [143], and P_o from deteriorating algal cells can then be recycled to optimise algal yield on phosphate added. This process has been shown to occur with *Gordonia* sp. txj1302RI and *Burkholderia* sp. txj1302Y4, which degraded dissolved P_o to provide *Microcystis aeruginosa* with P_i needed for its growth in eutrophic lakes with abundant P_o but limited P_i [144].

2.3.3.2. Vitamins, phytohormones, iron-siderophore and antibiotics

Bacteria are not only capable of minimizing the requirement for external CO_2 and major essential nutrients (N, P) for algae cultivation through regeneration or fixation [139], but also provide algal hosts with vitamins [22, 26], phytohormones [16, 96, 145, 146], siderophores [98] and antibiotics [147]. The heterotrophic bacterium *Dinoroseobacter shibae* $DFL12^T$ has been demonstrated to provide growth-limiting vitamins B_1 and B_{12} to its dinoflagellate host. Based on a survey of 326 algal species, it was shown that vitamin B_{12} is required by more than half of the algal species [26]. Epiphytic bacteria on seaweed (*Bacteroidetes* strain YM2-23) produce the compound thallusin, which is essential for inducing growth, development and morphogenesis of *Monostroma oxyspermum* and other *Ulva* species [148, 149]. *Sulfitobacter* sp. SA11 promotes diatom cell division via synthesis of the hormone indole-3-acetic acid [16]. A *Marinobacter* sp. that lives in close association with *Scrippsiella trochoidea* is able to produce an unusual siderophore that promotes algal assimilation of iron [98]. The marine bacterium *Phaeobacter gallaeciensis* produces growth hormones (phenylacetic acid) and a broad spectrum antibiotic (tropodithietic acid) against pathogenic bacteria while the algal host (*Emiliania huxleyi*) provides fixed carbon in exchange [46].

Growing a particular strain of microalgae in an appropriate medium or adjusting media recipes for different algal growth-stages remains a complicated task. In practice, most investigators tend to use a medium that works for their algae, but might not necessarily be the best one [150]. Understanding the symbiosis between microalgae and bacteria could lead to identification of missing medium components that could possibly be provided by co-cultivation with bacteria.

2.4. Harmful microbes in algal mass culture

One of the major risks of large-scale intensive algae production is the emergence of viruses, parasites and bacterial pathogens [151]. Despite current advances in long-term algae cultivation systems and farm management, it is neither cost-effective nor achievable to completely avoid undesired contaminants at industrial scale [48]. An increasing number of pathogens and parasites have been discovered in recent years, and undoubtedly, this number will continue to grow as investment increases in algal farming [152, 153].

As with terrestrial plants, algae are susceptible to infection by a wide range of viruses, bacteria, protists and fungi (Figure 2.2)[106]. Oceanic algae are likely living with a multitude of viruses, however, only few algal viruses have been reported and characterized so far [154]. For example, the large double-stranded DNA coccolithovirus (EhV, *Phycodnaviridae*) is able to terminate *Emiliania huxleyi* blooms [111, 154, 155]. Algae are also adversely affected by a wide range of bacteria, however, underlying mechanisms remain underexplored. Algae-associated bacteria belonging to the families *Rhodobacteraceae*, *Saprospiraceae* and *Flavobacteriaceae* have been implicated in bleaching of the seaweed *Delisea pulchra* [156]. Gram negative bacteria such as members of the genera *Alteromonas*, *Cytophaga*, *Flavobacterium*, *Pseudomonas*, *Saprospira*, *Vibrio* and *Pseudoalteromonas* are mainly responsible for rot symptoms [157] and galls on seaweeds [158]. Furthermore, *Microbacterium* sp. LB1 was shown to be responsible for algal

cell lysis and damaged laboratory cultures of the green alga *Choricistis minor*, leading to dry weight reduction of 34% after 120 h of cultivation [32].

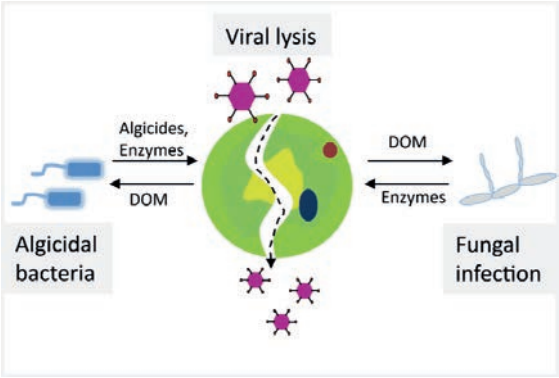

Figure 2.2. Illustration of antagonistic interactions between microalgae and microbes. DOM is dissolved organic matter.

Eukaryotic pathogens are prevalent but poorly understood, mostly because the strategies for detection, isolation and cultivation remain problematic [159]. A newly isolated algae-lytic protist, *Pseudobodo* sp. KD51 the 18S rRNA gene of which shares 99% similarity with that of *Pseudobodo tremulans*, was shown to cause more than 50% decrease in chlorophyll content of *Chlorella vulgaris* after inoculation within three days. In addition to inhibition of *Chlorella vulgaris*, *Pseudobodo* sp. KD51 displayed a wide predatory spectrum and negatively affected the growth of *Dunaliella salina, Platymonas subcordiformis* and the cyanobacterium *Microcystis aeruginosa* [112]. Rotifer grazers and ciliates prey on algal cells and can greatly decrease algal cell densities [160, 161]. Fungi are known to parasitize microalgae and often caused lethal epidemics in algal cultures in which infection rates can reach 100% [152]. So far, chytrid fungi have been reported to infect microalgae cultures of *Scenedesmus* [162], *Chlamydomonas* [163] and *Haematococcus pluvialis* [152].

2.4.1. Identification and monitoring

Algal biomass losses due to contaminants such as chytrid parasites can be rapid [162]. Therefore, fast and cost-effective methods to identify and control potentially harmful organisms in algal production systems are necessary. However, microbial community composition in algal cultures is complex and dynamic. The composition may vary with location, cultivation cycle stage or method and season [162]. Owing to the development of next generation sequencing methods, microbial identification can be carried out in a faster and less labour intensive way [164] and had been shown to effectively identify specific contaminants in algae cultivation reactors [165] or toxic algal species [166]. When pond or photobioreactor performance is abnormal, a retrospective analysis of the archived samples could reveal harmful contaminants and inappropriate operation strategies. Knowledge from long-term operation allows for identifying the most common and prevalent contaminants and this also gives operators predictive ability to some extent [106]. Systematic analysis and characterization of contaminants can be used for the development of specific probes, primers or other biomarkers for rapid monitoring of algae production systems. For instance, before initiating large-scale algae production, bacteria in algal inoculation stocks and the surrounding environments (water, soil, etc.) of the algae farm should be assayed for the presence of biological risks. A specific microbial pathogen library can be established and molecular tools can then be used to track harmful organisms of interest, and improving cultivation management.

2.4.2. Contamination and disease control

There is an increasing focus on preventing contamination to decrease major productivity losses in established systems [167]. Early detection and quantification of contaminants of algal cultures enables a fast response to infections. To protect algal cells from various contaminants, conventional methods such as physical filtration [106], applying decreased or elevated pH and temperatures [168] and chemical agents [169] are neither effective nor economical in algal industry, and hence new and efficient methods to combat contaminations are urgently needed.

Phaeobacter inhibens reciprocally exchanges beneficial molecules with the microalga *Emiliania huxleyi*. Among these molecules is the antibiotic tropodithietic acid thought to kill other bacteria [170]. In addition, a large screening of microbes indigenous to algae-cultivation systems has led to the discovery of an anti-fungal protein produced by the bacterium *Streptomyces* sp. strain AP77. This protein has been used to cure red rot disease of *Porphyra* spp. seaweeds caused by *Pythium porphyrae* [171]. Hence it is proposed that bacterial metabolites or bacteria that produce antimicrobial compounds could be supplied to bulk algal cultures in order to cost-effectively achieve more robust cultures that are less prone to harmful invaders.

2.5. Downstream processing of algal biomass using symbionts

Traditional mechanical or chemical pre-treatment methods that are used to harvest algal biomass and disrupt algal cells require a large energy input and are cost intensive [172]. To this end, algae-associated microbes offer several new alternatives for microalgae harvest and cell wall disruption.

Harvesting algal biomass is one of most important economic factors in producing compounds with microalgae [151]. Harvesting algal cells is different from harvesting seeds of oil-bearing plants, and oil extraction processes based on dry algal biomass are unlikely to be economical because of the high energy inputs needed to obtain dry algal biomass [151, 173]. Currently, up to 50% of total cost of biodiesel production is spent on harvesting because of the high energy input and/or the addition of expensive chemicals. Energy-intensive processes such as centrifugation are possible for high-value products but are too costly for biofuel applications. In addition, other methods such as extensive use of chemical flocculants can be applied to aid in the harvesting process, but could only be cost effective when the required amount is small

[151]. Therefore, development of economic and high efficiency harvesting techniques is important for alga bulk products, such as biofuels [174].

Bacteria can play an important role in microalgae aggregation [175, 176]. Diatom-attached bacteria are capable of increasing diatom aggregate formation leading to the settling of photosynthetically active *Thalassiosira weissflogii*, while free-living bacteria are not involved in this process [177]. In another study, mass cultures of *Nannochloropsis* were observed to form aggregates that consisted of algal cells, bacteria and debris that together resulted in a complex structure [178]. Wang et al. isolated a novel bacterium HW001 from Permian groundwater and demonstrated that this strain is able to stimulate aggregation of both *Nannochloropsis oceanica* IMET1 and other potential biofuel-producing green microalgae, diatoms and cyanobacteria [87]. In addition, two potent bioflocculants have been discovered from culture supernatant of *Burkholderia cepacia* [179] and *Bacillus licheniformis* CGMCC 2876 [180]. High flocculation efficiency of *Desmodesmus brasiliensis* (>98 %) was achieved at pilot-scale treatment with poly-γ-glutamic acid, a bioflocculant produced by *Bacillus licheniformis* CGMCC 2876 [179].

Besides bacteria, a number of filamentous fungal strains have also been reported to promote flocculation of microalgae [181-183]. Muradov et al. tested the fungal species (*Aspergillus fumigatus*) in co-culture with freshwater and seawater algal species and showed up to 90% flocculation after 24h of cultivation, while no aggregates were formed in the absence of the fungus. Furthermore, algal-fungal co-pelletization improved oil extraction efficiency because fungal secreted hydrolytic enzymes disrupted the thick cell walls of *Tetraselmis suecica* [184]. The same was seen between *Aspergillus lentulus* FJ172995 and *Chroococcus* sp., where algal and fungal cells formed a pellet, and nearly 100% of biomass settled down within 6 h at an optimized fungal/algal ratio of 1:3 [185].

2.6. Algae-bacteria based wastewater treatment

High biomass production costs obstruct the economic feasibility and competitiveness of algal biofuels [186]. The application of a combination of algae cultivation and wastewater treatment could provide a win-win solution to this problem [151, 187]. Wastewater from municipal sources, pig production, aquaculture and dairy cattle farming is rich in nutrients such as nitrates, ammonia and phosphates, which can be used for algae cultivation [132]. Mixed algal-bacterial populations in wastewater can not only perform more diverse tasks than single strains but are also better equipped to tolerate environmental fluctuations and pathogen invasions [136]. Moreover, the combination of algae and bacteria improves water treatment efficiency, and simultaneously the harvested algal biomass as by-product has been considered a promising source for feeds, biofuels and fertilizer [187, 188].

2.6.1. Carbon, nitrogen and phosphate removal

Algae produce oxygen during photosynthesis that is used by bacteria to mineralize organic matter [189]. Carbon dioxide released by bacteria during mineralization can in turn be utilised by algae [190]. Concurrently, abundant compounds in wastewater, such as ammonium and phosphate are eliminated by algal uptake [191]. Su et al. noted that the synergistic cooperation between photosynthetic organisms, including algae and cyanobacteria, and activated sludge bacteria enhanced organic carbon removal efficiencies [108]. More than 91.2% of chemical oxygen demand was removed, and the highest total nitrogen and phosphorus removal rates were 91.0 ± 7.0% and 93.5 ± 2.5%, respectively. *Chlorella sorokiniana* [192] *and Euglena viridis* [193] were also shown to enhance removal of carbon, nitrogen and phosphorous from piggery waste water when mixed with bacteria from activated sludge.

2.6.2. Removal of heavy metals and toxic organic compounds

In addition to enhanced removal of excessive nutrients, algal-bacterial consortia were also shown to be capable of removing heavy metals and toxic organic compounds from wastewater

[190]. Algal cells not only provide stable habitats for the bacteria but also concentrate pollutants to enhance bioavailability for bacterial degradation [194]. Algal-bacterial consortia successfully achieved higher biodegradation or removal rates of pollutants than single species [109].

Heavy metals belong to an important group of contaminants that pose global environmental risks [195]. Co-cultures of bacteria and algae were capable of removing 80% of the copper and 100% of the cadmium from wastewater in a continuous flow-through column [136]. In addition, a biofilm with immobilised algae (*Ulothrix* sp.) and bacteria in a photo-rotating biological contactor removed 20-50% of a large variety of metals (Cu>Ni>Mn>Zn>Sb>Se>Co>Al) within a ten-week period [196].

Polycyclic aromatic hydrocarbons are ubiquitous pollutants in various niches that might cast high risks on human and animal health [197]. A co-culture of the alga *Chlorella sorokiniana* and *Pseudomonas migulae* demonstrated higher phenanthrene degradation rates than most of the values reported in literature [198]. Luo et al established a consortium consisting of microalgae (*Selenastrum capricornutum*) and a bacterium (*Mycobacterium* sp. strain A1-PYR) that achieved faster degradation of pyrene than the systems that used algae or bacteria alone [109]. The same result was obtained by a synthetic consortium combining *Synechocystis* sp. and pyrene-degrading bacteria (*Pseudomonas* sp. and *Bacillus* sp.). The combination increased both algal growth and degradation of the polycyclic aromatic hydrocarbon [199].

Given the abovementioned advantages, integration of algae and bacteria has a large potential for wastewater treatment, especially under aerobic conditions. Oxygen produced by algae in the system can reduce the aeration demand in conventional activated sludge systems, which accounts for nearly 50% of the total energy input of the water treatment plants [200]. In addition,

removing nutrients from wastewater with a combination of algae and bacteria can increase the removal efficiency, system robustness and application potential of the sludge.

2.7. Outlook

Unravelling the complex biological mechanisms of algal-microbial interactions represents a largely understudied realm to improve production of high-value products and biofuels through large-scale cultivation of microalgae. Protective bacteria could inhibit growth of bacterial or fungal contaminants, which cause fouling or negatively affect algal growth. Macro fertilizers and expensive micronutrients supplied by bacterial metabolism can reduce the need for external input. Some bacteria are able to enhance synthesis of desired algal metabolites, for instance, lipids. However, currently our knowledge on algae bacteria interactions is too scattered to identify generalities with respect to bacterial species that are suitable for co-culture with microalgae. Alga species-specific knowledge would logically be first developed for industrial working horse species, such as *Arthrospira* spp., *Chlorella* spp., *Scenedesmus* spp., *Nannochloropsis* spp. and *Botryococcus* spp.[201]. In addition, the desired microbial community in algae cultures may depend on the required product specifications (biofuel, feed and food, fine chemicals) and harvesting methods applied.

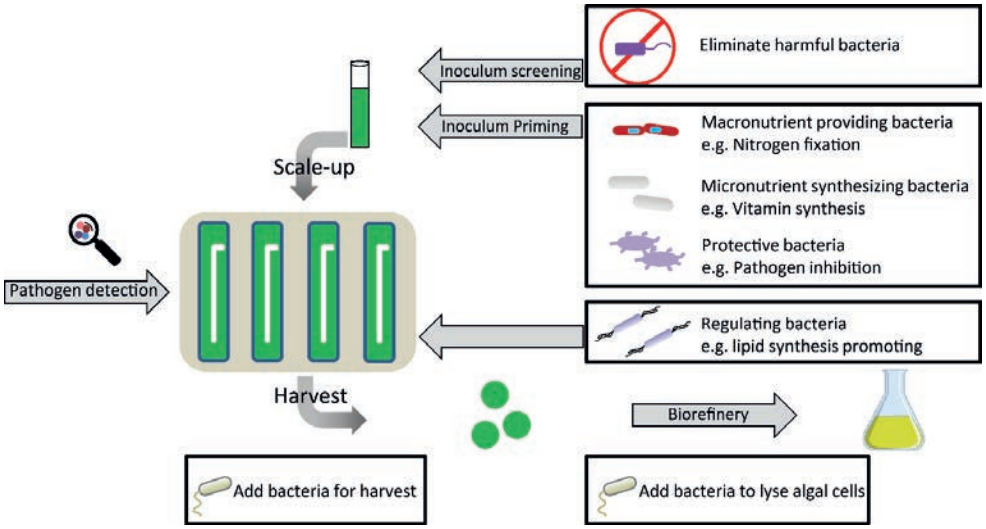

Figure 2.3. Potential integration strategies for including microbial community management into photobioreactor operations.

Further insights into evolution and establishment of mutualistic interactions allow for developing more resilient synthetic co-cultures (Figure. 2.3). Real time monitoring techniques are important to maintain stable and healthy mixed cultures in outdoor ponds exposed to changing weather and ubiquitous invaders. The main challenges for the application of bacteria in algal cultivation are to steer the bacterial community to its desired composition and how to maintain this balance during different modes of operation, different reactor types and fluctuations in outdoor conditions. The establishment and maintenance of optimized algae-bacterial co-cultures may require bioreactor operation management strategies that are extended beyond the performance of microalgae in the system, but consider and value the community present as a whole.

Chapter 3

Associated bacteria of *Botryococcus braunii* (Chlorophyta)

Joao D. Gouveia, Jie Lian, Georg Steinert, Hauke Smidt, Detmer Sipkema, Rene H. Wijffels, Maria J. Barbosa

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Abstract

Botryococcus braunii (Chlorophyta) is a green microalga known for producing hydrocarbons and exopolysaccharides. Improving the biomass productivity of *B. braunii* and hence, the productivity of the hydrocarbons and of the exopolysaccharides, will make *B. braunii* more attractive for industries. Microalgae usually cohabit with bacteria which leads to the formation of species-specific communities with environmental and biological advantages. Bacteria have been found and identified with a few *B. braunii* strains, but little is known about the bacterial community across the different strains. A better knowledge of the bacterial community of *B. braunii* will help to optimize the biomass productivity, hydrocarbons and exopolysaccharide accumulation. To better understand the bacterial community diversity of *B. braunii*, we screened 12 strains from culture collections. Using 16S rRNA gene analysis by MiSeq we described the bacterial diversity across twelve *B. braunii* strains and identified possible shared communities. We found three bacterial families common to all strains: *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae*. Additionally, the results also suggest that each strain has its own specific bacteria that may be the result of long-term isolated culture.

Keywords: *Botryococcus braunii*; associated bacteria; algal–bacterial interactions; 16S rRNA sequencing

3.1. Introduction

In recent decades many studies have focused on the physiology and cultivation process of several microalgae with potential for large scale production [202-206]. One microalga of interest for large scale cultivation is *B. braunii* because it can produce and secrete long chain hydrocarbons and exopolysaccharides (EPS) [73, 75, 79]. Hydrocarbons are naturally occurring compounds consisting entirely of hydrogen and carbon, and are one of the most important energy resources [207]. *B. braunii* is differentiated into different races (race A, B, L and S) depending on the type of hydrocarbons secreted [75, 76]. Race A strains synthesize odd-numbered alkadienes and trienes (C_{25} to C_{31}), race B strains synthesize isoprenoid type compounds termed botryococcenes (C_{30} to C_{37}), and methylated squalenes (C_{31} to C_{34}), race L strains synthesize lycopadiene (C_{40}), and race S strains synthesize C_{18} epoxy-*n*-alkanes and C_{20} saturated *n*-alkanes [75-77, 79]. EPS can have a range of applications, for example it can be applied as stabilisers and gelling agents in food products. In addition, it has applications in the pharmaceutical and cosmeceutical industries [208-210]. *B. braunii* comprises of a variety of strains from diverse parts of the world. The strains can differ in the hydrocarbon and exopolysaccharide content [72, 74, 77, 79, 211-214].

Bacteria can grow in close proximity to the microalgal cells due to the presence of EPS substances secreted by the microalgae [15]. The presence of bacteria within, or close to this EPS layer can lead to mutually beneficial interactions as well as interactions that are antagonistic in nature. Beneficial interactions for microalgae normally provide environmental advantages, such as nutrient exchange and community resilience to invasion by other species [215-218]. Antagonistic interactions will usually result in inhibition of the microalgal growth, either causing cell lysis, or directly competing for nutrients [48, 146, 219]. Studies investigating interactions of microalgae with bacteria show how important these interactions can be for the cultivation process [27, 105, 220, 221]. Understanding the interactions of microalgae and

bacteria, and how it can enhance the cultivation for industrial process, could lead to increased biomass productivity.

So far the bacterial community of *B. braunii* species is described in only a few studies. The earliest work is from Chirac and colleagues who described the presence of *Pseudomonas* sp. and *Flavobacterium* sp. in two strains of *B. braunii* [222]. Rivas and colleagues identified in the *B. braunii* UTEX strain the presence of *Pseudomonas* sp. and *Rhizobium* sp. [80]. One study using the *B. braunii* Ba10 strain showed the presence of rod shaped bacteria in the rim of the colony aggregations and proposed it is as growth promoting bacteria closely related to *Hyphomonadaceae* spp. [83]. One important finding was that *B. braunii* is a vitamin B_{12} autotroph, so it does not depend on bacteria for the synthesis of this important metabolite [223]. A more recent study using a *B. braunii* (race B) strain, revealed the presence of several Rhizobiales such as *Bradyrhizobium*, and the presence of *Bacteroidetes* sp [81]. So far, all studies have focused on only a few strains making it difficult to have a good overview of what bacterial community dominates *B. braunii*.

In this study we looked at twelve strains of *B. braunii* obtained from several culture collections to investigate the bacterial community composition that is associated with *B. braunii*.

3.2. Materials and methods

3.2.1. Strain collections and media preparation

Twelve *B. braunii* strains were obtained from culture collections (Table 3.1) and transferred to Erlenmeyer flasks with modified Chu 13 medium [224] without citric acid or vitamins, with the following composition: 1200 mg L^{-1} KNO_3, 200 mg L^{-1} $MgSO_4.2H_20$, 108 mg L^{-1} $CaCl_2.2H_2O$, 104.8 mg L^{-1} K_2HPO_4, 20 mg L^{-1} Fe-Na_2EDTA, 9.4 µg L^{-1} Na_2O_4Se, 2.86 mg L^{-1} H_3BO_3, 1.8 mg L^{-1} $MnSO_4.4H_2O$, 220 µg L^{-1} $ZnSO_4.7H_2O$, 90 µg L^{-1} $CoSO_4.7H_2O$, 80 µg L^{-1} $CuSO_4.5H_2O$, 60 µg L^{-1} $Na_2MoO_4.2H_2O$, 10 µl L^{-1} H_2SO_4. The final pH was adjusted to pH 7.2 with NaOH

and $NaHCO_3$ was added to a final concentration of 5 mM. The 12 strains were grown in Infors HT Multriton incubators in 250 mL conical flasks and a volume of 150 mL. The temperature was set at 23°C, with 2.5 % CO_2 enriched air and shaking at 90 rpm. Illumination was provided by Phillips lamps FL-Tube L 36W/77, with 150 μmol photon m^{-2} sec^{-1}, and a light:dark photoperiod of 18:6 h. Flasks were inoculated with *B. braunii* growing in the active growing phase, such that the initial absorbance at 680 nm was 0.2. The Erlenmeyer flasks were capped with aeraseal sterile film (Alphalabs). Samples were taken at day 1, 4, 8 and 11, for 16S rRNA gene analyses.

Table 3.1. Information of the culture collections providers of *Botryococcus braunii* strains and location of origin.

Culture collection	***Botryococcus braunii* Strain (our abbreviation)**	**Race**	**Location**	**Isolation, date of isolation**	**Reference**
Berkeley	Showa	Race B	culturing tanks, Berkley	by unknown, 1980	[225]
Scandinavian Culture Collection of Algae and Protozoa (SCCAP)	K1489	Race A	Belgium, Nieuwoort	by G. Hansen, 2008	No reference
UTEX Culture Collection of Algae	UTEX LB572 (UTEX)	Race A	Cambridge, England	by M. R. Droop, 1950	[77]
Culture Collection of Autotrophic Organisms (CCALA) check	CCALA778 (CCALA)	unknown	Serra da Estrela (Barragem da Erva da Fome) Portugal	by Santos, 1997	No reference
Culture Collection of Algae and Protozoa (CCAP)	CCAP807/2 (CCAP)	Race A	Grasmere, Cumbria, England	by Jaworski, 1984	[226]
	AC755	Race A	Lingoult-Morvan, France	by Pierre Metzger, 1981	[227]
	AC759	Race B	Ayame, Ivory Coast	by Pierre Metzger, 1984	[212]
	AC760	Race B	Kossou, Ivory Coast	by Pierre Metzger, 1984	[212]
ALGOBANK-CAEN	AC761	Race B	Paquemar, Martinique, France	by Pierre Metzger, 1983	[227]
	AC765	Race L	Kossou, Ivory Coast	by Pierre Metzger, 1984	[212]
	AC767	Race L	Songkla Nakarin, Thailand	by Pierre Metzger, 1985	[228]
	AC768	Race L	Yamoussoukro, Ivory Coast	by Pierre Metzger, 1984	[228]

3.2.2. DNA extraction

On sampling days, 5 mL of fresh culture was harvested with sterilized membrane filters (0.2 μm, Millipore) using a vacuum apparatus. The filters were cryopreserved in -80 °C until further processing. DNA was extracted from the cryopreserved filters that were cut into small pieces with a sterile scissor. Filter pieces were transferred to a 2 mL sterilized tube with zirconia/silica beads (Biospecs), and 1 mL S.T.A.R buffer (Roche, USA) was added. Cells were homogenized for two rounds of 45 seconds, at the speed of 5500 rpm with Precellys (Bertin Technologies). Then DNA was extracted using the Maxwell 16 Tissue LEV Total RNA purification kit (Promega, USA) with aid of the Maxwell 16 instrument (Promega, USA). The purity and quantity of DNA was examined by electrophoresis on a 1% agarose gel and measured with a Nanodrop (ND1000, Thermo Fisher Scientific Inc., Wilmington). The extracted DNA was stored at -20 ℃ until further use.

3.2.3. 16S rRNA gene amplification and Miseq sequencing

Amplicons from the V1-V2 region of 16S rRNA genes were generated by a two-step PCR strategy consisting of a forward primer (27F-DegS = 5'GTTYGATYMTGGCTCAG 3' where M = A or C; R = A or G; W = A or T; Y = C or T) and an equimolar mixture of reverse primers (338R I = 5'GCWGCCTCCCGTAGGAGT 3' and II = 5' GCWGCCACCCGTAGGTGT 3' where M = A or C; R = A or G; W = A or T; Y = C or T). Eighteen bp Universal Tags 1 and 2 (Unitag1 = GAGCCGTAGCCAGTCTGC; Unitag2 = GCCGTGACCGTGACATCG) were appended at the 5' end of the forward and reverse primer, respectively [229-231]. The first PCR mix (50 μL) contained 10 μL 5× HF buffer (Thermo ScientificTM, the Netherlands), 1 μL dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo ScientificTM), 1 μM of 27F-DegS forward primer, 1 μM of 338R I and II reverse primers, 1 μL template DNA and 32.5 μL nuclease free water.

Amplification included an initial denaturation at 98°C for 30 sec; 25 cycles of denaturation at 98°C for 10 sec; annealing at 56°C for 20 sec and elongation at 72°C for 20 sec; and a final extension at 72°C for 10 min. The PCR product size was examined by 1 % gel electrophoresis. The second PCR mix (100 µL) contained 62 µL nuclease free water, 5 µL of PCR1 product, 20 µL 5× HF buffer, 2 µL dNTP Mix, 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer equivalent to the Unitag1 and Unitag2 sequences respectively, each appended with an 8 nt sample specific barcode. Amplification included an initial denaturation at 98°C for 30 sec; 5 cycles of denaturation at 98°C for 10 sec, annealing at 52°C for 20 sec and elongation at 72°C for 20 sec; and a final extension at 72°C for 10 min. The concentration of PCR products was quantified with a Qubit Fluorometer (Life Technologies, Darmstadt, Germany) in combination with the dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). Purified products were then pooled in equimolar amounts of 100 ng μL^{-1} and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany).

3.2.4. Processing MiSeq data

Data was processed using the Quantitative Insights into Microbial Ecology (QIIME) 1.8.0. In short, paired-end libraries were filtered to contain only read pairs perfectly matching barcodes. Low quality or ambiguous reads were removed and then chimeric reads were removed and checked. Sequences with less than 0.1 % were discarded. Remaining filtered sequences were assigned into Operational Taxonomy Units (OTUs) at 97% threshold using an open reference method and a customized SILVA 16S rRNA gene reference [232]. Seven samples from day 4 were removed from the results due to contamination during the PCR steps: AC755, AC759, AC760, AC767, AC768, CCAP and UTEX572. The 16S rRNA gene dataset obtained in this study is deposited in the Sequence Read Archieve (SRA), NCBI with accession number SRP102970.

3.2.5. Microbial community analysis

For the interpretation of the microbial community data on family level, the Operational Taxonomic Unit (OTU) abundance table was converted to relative abundance and visualized as heatmaps using JColorGrid [233]. Ordination analyses to estimate the relationship of the *B. braunii* strains based on dissimilarity of the microbial community compositions among the individual samples was performed for, a) all strains of *B. braunii* used in this study, b) all strains received from ALGOBANK-CAEN culture collection. For both analysis a standardized 97% OTU table (*decostand* function, *method* = *hellinger*) and the nMDS function metaMDS (*distance* = Bray-Curtis) from the vegan package in R was used (R version 3.0.2) [234, 235]. Betadispersion and a permutation test were performed to test homogeneity dispersion within a group of samples. Adonis from the vegan package in R (v.3.0.2) was used to test significant differences in bacterial community between strains. Hierarchical clustering analysis was performed using hclust function in R using method = average.

3.3. Results

Figure 3.1 shows the bacterial families with a relative abundance above 1 % and a total of four bacterial phyla associated with *B. braunii* strains. The four phyla found associated with *B. braunii* are the *Bacteroidetes*, *Gemmatimonadetes*, *Planctomycetes* and *Proteobacteria*. *Proteobacteria* is the predominant bacterial phylum and representatives of this taxon are found in all 12 strains. *Bacteroidetes* is found in all strains with exception to strains AC761, AC768 and CCAP. *Gemmatimonadetes* is found only in the CAEN culture (with AC prefix) strains with exception to AC755. *Planctomycetes* is found in AC760, CCALA, K1489, Showa and UTEX strains. Three families are found across all 12 *B. braunii* strains and all are *Proteobacteria*.

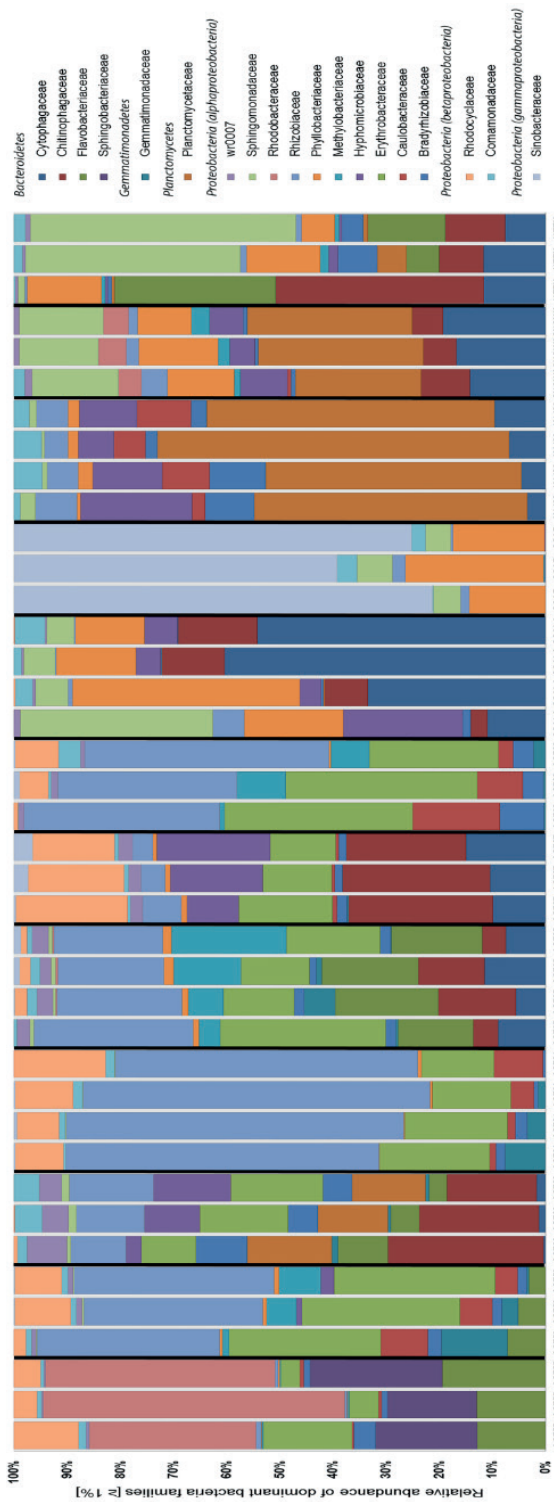

Figure 3.1. Relative abundance of bacterial families in 12 *B. braunii* strains. Strain abbreviations are used as explained in Table 1. Each bar displays the bacterial family relative abundance above 1 %. Strains are labelled below with sample day within square brackets. Bacterial families are organized according to the phyla (in italics) they belong to.

These are the *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae*. *Rhizobiaceae* is represented by 1 to 59 % of the bacterial reads. *Bradyrhizobiaceae* was found within the 1 to 8 % range. *Comamonadaceae* was found between 1 and 5 %. Two families of bacteria are only found in the strains obtained from the CAEN culture collection: *Erythrobacteraceae* with bacterial reads ranging from 1 to 29 % and *Rhodocyclaceae* with 1 to 18 %.

Some families of bacteria are particularly dominant in specific strains. *Sinobacteraceae* is dominant in CCAP with relative abundances ranging from 59 to 78 %. *Planctomycetaceae* is dominant in K1489 strain with relative abundances between 46 and 51 %. *Rhizobiaceae* is dominant in AC761 with relative abundances between 55 and 64 %. Other families of bacteria become dominant as the cultures become older. *Rhodobacteraceae* is present in AC755 strain with relative abundances ranging from 28 % at day 1 to 40 % at day 11. *Sphingomonadaceae* is present in UTEX with 10 % at day 1 and increases its presence to 47 % at day 11. *Chytophagaceae* is dominant in CCALA strain with relative abundance ranging from 10 % at day 1 to 52 % at day 11.

Because we found three common families across all strains, we wanted to investigate in more detail the bacterial composition in these selected families and see if we could identify an unique microorganism present in all strains. Therefore we zoomed in and looked at the Operational Taxonomy Units (OTUs) distribution belonging to the three families: *Rhizobiaceae, Bradyrhizobiaceae and Comamonadaceae*. In addition, we picked the OTUs found only in the strains obtained from the CAEN culture collection which belong to two families: *Erythrobacteraceae* and *Rhodocyclaceae*. The most abundant OTUs were selected and a total of 28 OTUs were investigated. From Figure 3.2 it is clear that there is not an OTU that is found across all strains but rather each family comprises of several different OTUs. The second important observation is that CCAP strain has no representative OTUs for *Bradyrhizobiaceae* and *Rhizobiaceae* in the most abundant OTUs. The most represented family taxon is

Rhizobiaceae with 12 OTUs. From the three families found in the 12 strains, OTU 233 assigned to the genus *Rhizobium* has the highest OTU frequency abundance with 10 % and is present in 7 out of 12 strains. The OTUs 143, 88 and 131 assigned to the genus *Shinella* are present in 9 out of 12 strains. The OTUs 477, 475 and 484 assigned to the genus *Bosea* cover 11 out of 12 strains. From the two families found only in the cultures originating from the CAEN culture collection, OTUs 333 and 539 are found in all seven CAEN strains with an assigned genus *Porphyrobacter* and *Methyloversatilis*, respectively.

The most abundant OTUs (as listed in Figure 3.2) were subjected to a Blast search against the NCBI database to infer their nearest neighbours (Table 3.2). OTUs 88, 115, 143 and 233 are similar in their nearest neighbours with four different *Rhizobium* spp. as candidates. Similar blast results are seen also for OTUs 566 and 567 with the nearest neighbours being *Hydrogenophaga* spp. The OTUs 819 and 832 with *Dyadobacter* spp. as nearest neighbour dominate CCALA bacterial community. Some OTUs show different species as closest neighbours such as OTUs 45 and 69 with *Frigidibacter albus*, *Paracoccus sediminis* and *Nioella nitratireducens* as neighbours. The OTU 415 with high abundance in K1489 belonging to *Planctomycetaceae*, has as closest neighbours uncultured bacterium and third closest neighbour uncultured *Planctomyces* spp. with the latter showing 87 % identity. The OTU 333 present only in the strains from CAEN culture collection, has 100 % identity with *Sphingomonas* as closest two neighbours, and third neighbour, also with 100 %, identity being *Porphyrobacter*.

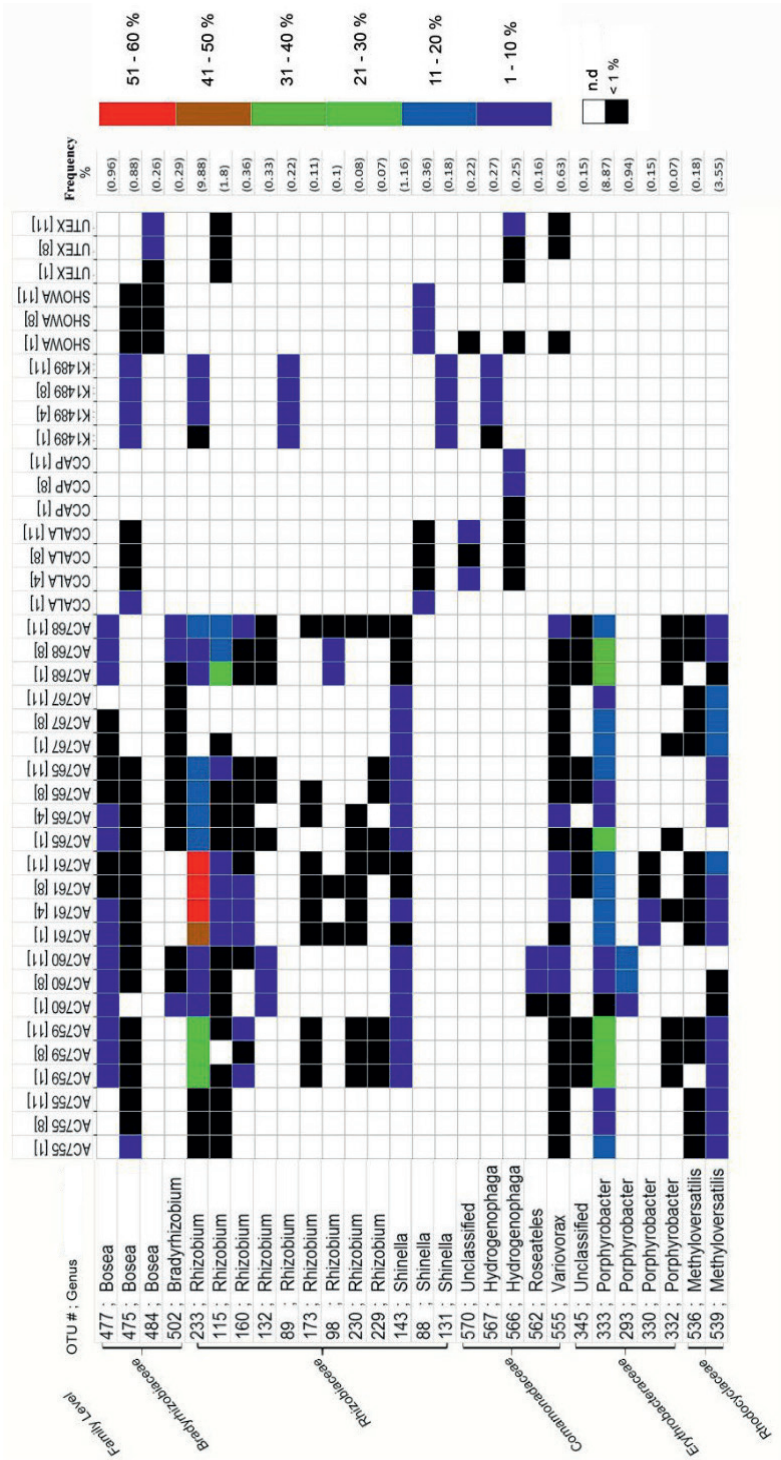

Figure 3.2. Heatmap of most abundant 16S rRNA gene OTUs belonging to the families *Rhizobiaceae, Bradyrhizobiaceae, Comamonadaceae, Erythrobacteraceae* and *Rhodocyclaceae*. The label on the right shows the colour code for the relative abundance. Frequency (average relative abundance) of each OTU is shown in percentage on the right between brackets. The label on the left shows the family level and OTU number followed by genus. n.d means no reads detected.

Table 3.2. NCBI database blast of OTUs for selected families. Closest first three neighbours with highest identity match and with a minimum of 85 % coverage for each OTU. NCBI blast on the 11th February 2016, except the OTU 662 which the blast search from 30th August 2016 and OTU 63 and 415 on February 2017.

OTU	nearest neighbour1	Genbank acc.	nearest neighbour2	Genbank acc.	nearest neighbour3	Genbank acc.
475	*Hyphomicrobium nitrativorans* (100)	NR_121713.1	*Hyphomicrobium nitrativorans* (100)	NR_118448.1	*Bosea lathyri* (100)	NR_108515.1
477	*Bradyrhizobium lupini* (100)	NR_134836.1	*Bradyrhizobium lupini* (100)	NR_044869.2	*Rhodopseudomonas palustris* (100)	NR_103926.1
484	*Bosea robiniae* (100)	NR_108516.1	*Bradyrhizobium lupini* (99)	NR_134836.1	*Bradyrhizobium ottawaense* (99)	NR_133988.1
502	*Bradyrhizobium daqingense* (100)	NR_118648.1	*Bradyrhizobium lablabi* (100)	NR_117513.1	*Beijerinckia doebereinerae* (100)	NR_116304.1
88	*Rhizobium rhizoryzae* (100)	NR_133844.1	*Rhizobium flavum* (100)	NR_133843.1	*Rhizobium azibense* (100)	NR_133841.1
115	*Rhizobium rhizoryzae* (100)	NR_133844.1	*Rhizobium flavum* (100)	NR_133843.1	*Rhizobium azibense* (100)	NR_133841.1
143	*Rhizobium rhizoryzae* (100)	NR_133844.1	*Rhizobium flavum* (100)	NR_133843.1	*Rhizobium azibense* (100)	NR_133841.1
233	*Rhizobium paranaense* (100)	NR_134152.1	*Rhizobium rhizoryzae* (100)	NR_133844.1	*Rhizobium flavum* (100)	NR_133843.1
555	*Variovorax guangxiensis* (100)	NR_134828.1	*Variovorax paradoxus* (100)	NR_074654.1	*Variovorax boronicumulans* (100)	NR_114214.1
566	*Hydrogenophaga flava* (100)	NR_114133.1	Hydrogenophaga bisanensis (100)	NR_044268.1	Hydrogenophaga defluvii (100)	NR_029024.1
567	*Hydrogenophaga flava* (100)	NR_114133.1	Hydrogenophaga bisanensis (100)	NR_044268.1	Hydrogenophaga defluvii (100)	NR_029024.1
333	*Sphingomonas gei* (100)	NR_134812.1	*Sphingomonas ginsengisoli* (100)	NR_132664.1	*Porphyrobacter colymbi* (100)	NR_114328.1
539	Uncultured bacterium (100)	KY606782.1	*Methyloversatilis discipulorum* (71)	KY284088.1	*Methyloversatilis discipulorum* (71)	KY284080.1
63	*Thioclava sp.* (100)	CP019437.1	*Rhodobacter* sp. (100)	KY608089.1	Uncultured *Rhodobacter* sp. (100)	KY606875.1
819	*Dyadobacter jiangsuensis* (100)	NR_134721.1	*Dyadobacter fermentans* (100)	NR_074368.1	*Dyadobacter tibetensis* (88)	NR_109648.1
832	*Dyadobacter jiangsuensis* (100)	NR_134721.1	*Dyadobacter fermentans* (100)	NR_074368.1	*Dyadobacter tibetensis* (88)	NR_109648.1
415	Uncultured bacterium (100)	KT769749.1	Uncultured bacterium (91)	KT724695.1	Uncultured *Planctomyces* sp. (87)	JX576019.1
45	*Frigidibacter albus* (100)	NR_134731.1	*Paracoccus sediminis* (96)	NR_134122.1	*Nioella nitratireducens* (94)	NR_134776.1
69	*Frigidibacter albus* (100)	NR_134731.1	*Paracoccus sediminis* (100)	NR_134122.1	*Nioella nitratireducens* (97)	NR_134776.1
302	*Sphingorhabdus arenilitoris* (100)	NR_134184.1	*Sphingopyxis italica* (100)	NR_108877.1	*Parasphingopyxis lamellibrachiae* (100)	NR_113006.1

310	*Sphingomonas yantingensis* (100)	NR_133866.1	*Sphingomonas canadensis* (100)	NR_108892.1	*Blastomonas natatoria* (100)	NR_113794.1
355	*Blastomonas natatoria* (100)	NR_113794.1	*Sphingomonas ursincola* (100	NR_040825.1	*Blastomonas natatoria* (100)	NR_040824.1

Non-metric multidimensional scaling ordination was performed for the 12 strains to determine the bacterial community dissimilarities (Figure 3.3A). *B. braunii* strains from the CAEN culture collection cluster together when compared to the other strains indicating these strains are similar to each other in bacterial community composition. This is supported by hierarchical cluster analysis showing CAEN strains in their own cluster (Supplementary Figure 3.1). The strains K1489, UTEX, CCAP, CCALA and Showa represent separate clusters. The homogeneity of dispersion within each strain with 1000 permutations show no significant difference (F=0.323). Using adonis to test for bacterial community similarities between all strains, the results show that the bacterial communities are significantly different (DF = 11, Residuals = 28, $R^2 = 0.921$, P = 0.001). Figure 3.3B zooms in to the CAEN culture collection strains. Races A, B and L are subdivisions of *B. braunii* according to the type of hydrocarbons produced. No clustering by type of hydrocarbons produced was seen by the distribution of the race B and race L strains which are found mixed, namely race B AC759 and AC761 with race L AC765 and AC768. Similarly, the bacterial community between CAEN strains are significantly different (DF = 6, Residuals = 16, R2 = 0.904, P = 0.001).

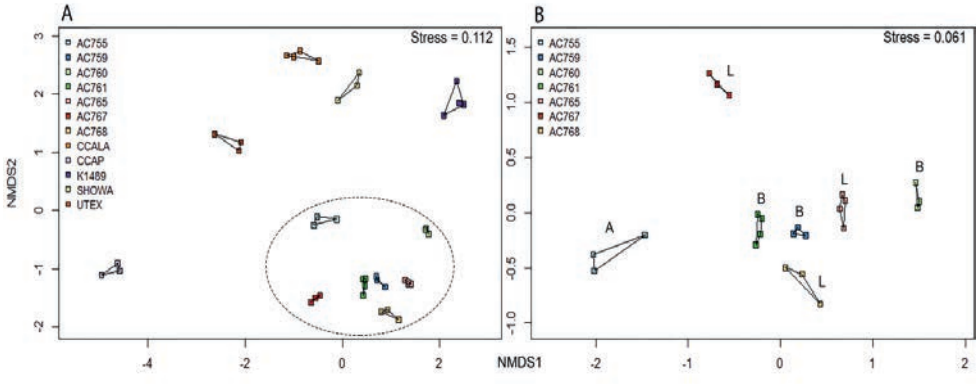

Figure 3.3. Non-metric multidimensional scaling (nMDS) ordination (based on Bray-Curtis distance matrix) of 16S rRNA gene sequences of 12 *B. braunii* strains. A) ordination of all strains with CAEN cultures clustering together (within the ellipse dotted line); B) ordination of the CAEN culture collection strains only. Capital letters in plot B refer to the race subclassification based on the type of hydrocarbons produced.

3.4. Discussion

It is evident that *B. braunii* possesses a highly diverse bacterial community as seen by the range of bacterial phyla and families present in all the strains used in this study (Figure 3.1 (for a more comprehensive list see Supplementary Figure 3.2)).

From the bacterial community analysis (Figure 3.3A, B), it appears that each *B. braunii* strain has a specific bacterial community and no OTU is shared between all strains. The strains from the CAEN culture collection cluster together while *B. braunii* strains from other culture collections appear as separate groups. This implies that the culture collection from which the strain was obtained could potentially have an effect. With this study we are not able to really deduce the potential impact of the culture collection on the bacterial community because the experimental design was not set-up to do so. The presence of weak (within a culture collection) and strong (between culture collections) migration barriers may explain the bacterial profiles as obtained in our study and they may be a result of historical contingencies [236] rather than pointing towards highly specific interactions for a large number of OTUs. OTUs 539 and 333

are only found with the CAEN cultures and contributes towards these strains clustering in close proximity. OTU 333 is especially high in relative abundance and contributes to the distinctive clustering of the CAEN culture collection strains. The remaining strains also contain their specific OTUs that contribute towards their own clustering: OTU 819 and 832 with CCALA, OTU 310 with UTEX and K1489 with OTU 415. The bacterial community between three race B and three race L are mixed together (Figure 3.3B). Therefore, no correlation was found between bacterial community and the type of hydrocarbons produced between the two races. Similar observations were made in another study using six strains of *B. braunii* in which the authors did not find a correlation between the bacteria and type of hydrocarbon produced [237].

Three bacterial families were found to be present with all twelve strains of *B. braunii*: *Bradyrhizobiaceae*, *Rhizobiaceae*, and *Comamonadaceae*. Two families were found abundantly only in the strains from the CAEN culture collection: *Erythrobacteraceae* and *Rhodocyclaceae*. The OTUs 88, 115, 143 and 233 blast hits show these are related to *Rhizobium* spp. (Table 3.2). *Rhizobium* spp. are known to form nodules in the roots of several plants within the family of legumes and are best known for nitrogen fixation. Nitrogen fixing bacteria were investigated in association with microalgae and it has been shown that they can enhance microalgae growth [28]. *Rhizobium* spp. associated with *B. braunii* could have a similar role. Rivas *et al*. [80] also found a *Rhizobium* sp. associated with *B. braunii* in particular UTEX LB572, and Kim *et al*. [105] showed the presence of *Rhizobium* sp. with *B. braunii* 572. Sambles *et al*. [81] identified *Rhizobium* sp. closely associated with *B. braunii* after submitting the cultures through a wash step and antibiotic treatment. Recent studies also shows *Rhizobium* spp. present with *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus* spp. [105]. *Rhizobium* spp. seem important to *B. braunii* strains as it appears in all 12 strains with more prominence in the CAEN cultures and K1489 with three to four OTUs (Figure 3.2). For the

remaining strains CCALA, CCAP, Showa and UTEX, *Rhizobium* spp. is represented only with one OTU.

OTU 475 from *Bradyrhizobiaceae* family shows 100 % similarity with the species *Hyphomicrobium nitrativorans* as the two closest neighbours and is present in 10 out of 12 *B. braunii* strains. *H. nitrativorans* is a known denitrifier isolated from a seawater treatment facility [238]. Denitrification is the process of reducing nitrate into a variety of gaseous compounds with the final being dinitrogen. Because denitrification mainly occurs in the absence of oxygen it is unlikely that this is happening within our cultures that are well oxygenated. The 3rd closest neighbour for OTU 475 is *Bosea lathyri* and is associated with root nodules legumes [239].

OTUs 555, 566 and 567 from *Comamonadaceae* family, appeared in seven out of twelve strains. The three closest neighbours of OTU 555 were *Variovorax* spp. and for OTUs 566 and 567 these were *Hydrogenophaga* spp., *Variovorax* and *Hydrogenophaga* spp. are not known for being symbionts but may be able to support ecosystems by their ability to degrade toxic compounds and assist in nutrient recycling, therefore potentially producing benefits to other microorganisms [240, 241]. *Comamonadaceae* also appeared as one of the main bacteria families associated with cultivation of microalgae in bioreactors using a mix of fresh water and municipal water as part of a water treatment strategy [242].

Erythrobacteraceae and *Rhodocyclaceae* were only found in the strains from CAEN culture collection. OTU 333 (*Erythrobacteraceae*) first two closest neighbours are from *Sphingomonas* spp., and third closest neighbour is *Porphyrobacter* spp. isolated from water in a swimming pool. Most *Porphyrobacter* spp. isolated originate from aquatic environments [243] and are associated with fresh water sediments [244]. *Porphyrobacter* spp. have also been associated with other microalgae such as *Tetraselmis suecica* [118]. OTU 539 (*Rhodocyclaceae*) second

and third closest neighbour is *Methyloversatilis discipulorum* which is a bacteria found in biofilms formation in engineered freshwater installations [245]. It is not clear why OTU 333 and 539 are specifically found only in the strains originating from the CAEN culture collection, but it could be an introduced species during handling. None the less, these two OTUs are present in high relative abundance (Figure 3.2), and would be interesting to know if they have a positive or negative influence on the growth of the CAEN strains. It would be interesting to confirm such statement by attempting the removal of these OTUs and investigate the biomass growth.

Sinobacteraceae is dominant in CCAP (Figure 3.1). This family was proposed in 2008 with the characterization of a bacteria from a polluted soil in Chi [246]. A recent bacteria related to hydrocarbon degradation shows similarities with *Sinobacteraceae* [247]. OTU 63 is highly abundant in CCAP and could have a negative impact in the cultivation of CCAP strain by reducing its hydrocarbon content.

The *Bactoroidetes* family *Cytophagaceae* dominates the culture CCALA at later stages of growth (Figure 3.1). *Cytophagaceae* has also been found present in laboratory scale photobioreactor cultivation using wastewater for production of microalgae biomass [242]. The two OTUs that dominate the bacterial community in CCALA are OTU 819 and OTU 832. The Blast search on NCBI database approximates these two OTUs as *Dyadobacter* spp. which have also been found co-habiting with *Chlorella* spp. [248].

Planctomycetaceae dominates the bacterial community in K1489 strain (Figure 3.1) with one OTU 415. This family can be found in freshwater biofilms and also strongly associated with macroalga [249, 250]. Species in this family could possibly be involved in metallic-oxide formation and be co-players in sulphate-reduction with the latter also involving a sulphur-reducing bacteria [251].

Rhodobacteraceae is present with up to 55 % of bacterial relative abundance in AC755. Members of this family have been also isolated from other microalgae, namely *Chlorella pyrenoidosa* and *Scenedesmus obliquus* [252]. The OTUs 45 and 69 blast searches in NCBI database show the closest neighbours to be *Frigidibacter albus*, *Paracoccus sediminis* and *Nioella nitratireducens* (Table 3.2). All three neighbours were isolated from water environments [253, 254].

Sphingomonadaceae is mostly found in freshwater and marine sediments [255]. OTUs 302, 310 and 355 from this family were found in 6 out of 12 strains above 1 % relative abundance. OTU 310 is only found in the UTEX strain with *Sphingomonas* spp. as the two closest neighbours. *Sphingomonas* spp. are shown to co-habit with other microalgae such as *Chlorella sorokiniana* and *C. vulgaris* [20, 218]. *Sphingomonas* spp. have been shown to be able to degrade polycyclic aromatic hydrocarbons [256] and could possibly be degrading the hydrocarbons secreted by *B. braunii* as its carbon source.

Another characteristic of many bacteria is the ability to produce EPS such as species from the *Rhizobiaceae* and *Bradyrhizobiaceae* family [257-259]. This characteristic could play a role on the colony aggregation of *B. brauniii* as EPS is known to be essential for biofilm formation [260]. Therefore it would be interesting in the future to study this possible relationship as *B. braunii* is a colony forming organism. Such studies could involve the introduction of bacteria associated with colony formation such as *Terramonas ferruginea* as it has been associated with inducing flocculation in *C. vulgaris* cultures [261].

With the present high microbial diversity, *B. braunii* shows qualities in resilience towards microbial activity, probably due to its colonial morphology and protective phycosphere made of hydrocarbons and EPS [262]. A number of microbes are potentially beneficial such as *Rhizobium* spp. which have been shown to have a positive effect on the biomass productivities

of *B. braunii* UTEX [80], and *Hydrogenophaga* with the ability to degrade toxic compounds [241]. There are also microbes that may cause detrimental effects on hydrocarbon productivities of *B. braunii* such as *Sphingomonas* spp. (OTU 310) with its ability to degrade hydrocarbons [256]. The removal of such detrimental microbes could enhance cultivation allowing more nitrogen available for biomass production and increase hydrocarbon accumulation of *B. braunii* as well as EPS production at larger industrial scale.

3.5. Conclusion

B. braunii can host a diverse microbial community and it is likely that some form of interaction is taking place with the members from the *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae* family, which all belong to the phylum *Proteobacteria*. There is not a specific bacterial community correlated to the different types of hydrocarbons produced by race B and L and mostly likely also not race A. *B. braunii* has many strains and each seems to have its own species-specific bacterial community. With a diverse microbial community present, it is also likely that some bacteria are having antagonistic effects on *B. braunii* such as competition with nutrients and degradation of hydrocarbons. *Botryococcus* is a microalga of high scientific interest and it is important to understand better the associated bacteria. *Botryococcus*-associated bacteria are hard to get rid of (Gouveia, J. unpublished data) and therefore it is important to start mass cultivation without those bacteria that are most harmful to the process.

Acknowledgments

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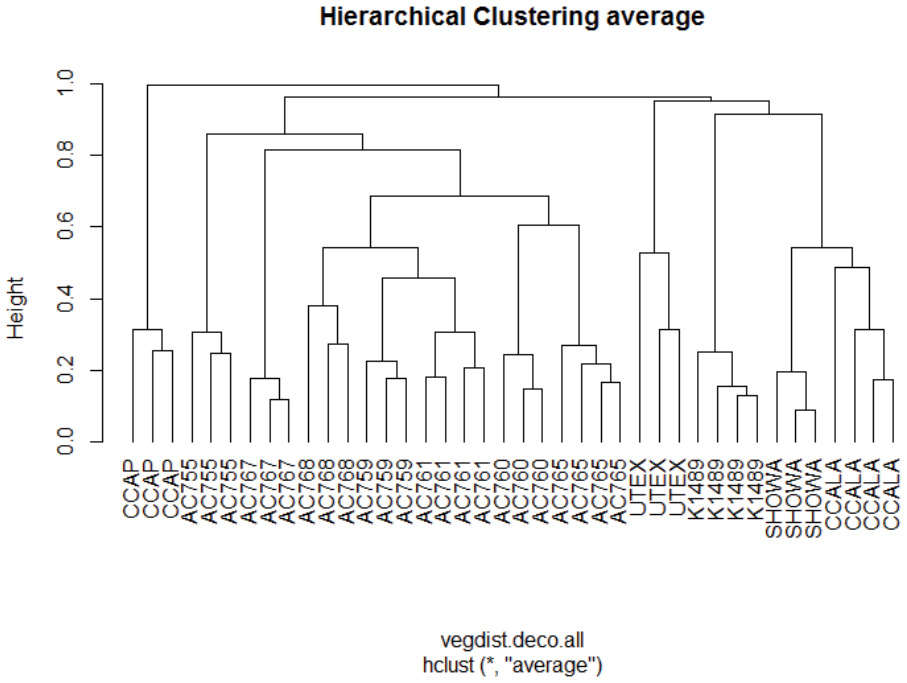
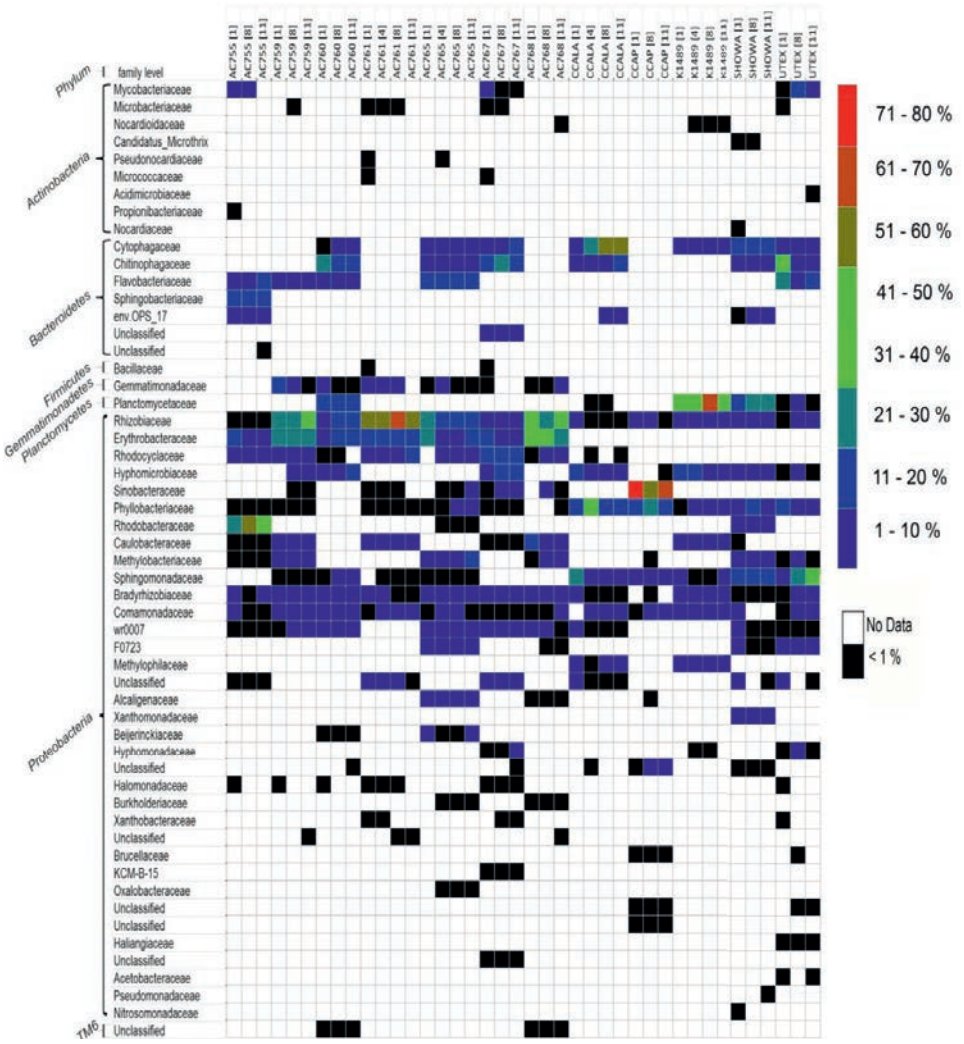
Supplementary Information



Supplementary Figure 3.1. Hierarchical clustering of *B. braunii* strains. Strains from CAEN culture collection (AC prefix) cluster together in comparison to the other strains.



Supplementary Figure 3.2. Family taxa relative abundance heatmap of 12 *B. braunii* strains. On the left, is the family taxa classification. On the right, the colour coded label describes the relative abundance in percentage.

Chapter 4

Bacterial diversity in different outdoor pilot plant photobioreactor types for production of the microalga *Nannochloropsis* sp. CCAP211/78

Jie Lian, Georg Steinert, Jeroen de Vree, Sven Meijer, Christa Heryanto,

Rouke Bosma, René H. Wijffels, Maria Barbosa, Hauke Smidt, Detmer Sipkema

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Abstract

A range of microalgae production systems are scaled up towards pilot and demonstration plants. As large-scale outdoor production cannot be done in complete containment, cultures are (more) open for bacteria, which may affect the productivity and stability of the production process. We investigated the bacterial diversity in two indoor reactors and four pilot-scale outdoor reactors for production of *Nannochloropsis* sp. CCAP211/78 spanning four months of operation from July to October. Illumina sequencing of 16S rRNA gene amplicons demonstrated that a wide variety of bacteria were present in all reactor types, with predominance of *Bacteroidetes* and *Alphaproteobacteria*. Our results showed that bacterial communities were significantly different in all reactor types (except a horizontal tubular reactor and a vertical tubular reactor) and also between runs in each reactor. Bacteria common to the majority of samples included one member each of the *Saprospiraceae* family and of the *NS11-12_marine group* (both *Bacteroidetes*). Hierarchical clustering analysis revealed two phases during the cultivation period separated by a major shift in bacterial community composition in the horizontal tubular reactor, the vertical tubular reactor and the raceway pond. The bacterial classes *Alphaproteobacteria, Sphingobacteriia, Deltaproteobacteria* and *Flavobacteriia* contributed most to the difference between the two phases, with a stark decrease of the *Saprospiraceae* and *NS11-12_marine group* that initially dominated the bacterial communities. Furthermore, we observed a less consistent pattern of bacterial taxa appearing in different reactors and runs, most of which belonging to the classes *Deltaproteobacteria* and *Flavobacteriia*. In addition, canonical correspondence analysis showed that the nitrate concentration in the reactor significantly correlated with bacterial community composition in most reactor types except the plastic flat panel reactor, where algal biomass productivity was the key factor associated with changes in bacterial community structure. This study contributes to our understanding of bacterial diversity and composition in different types of outdoor reactors exposed to a range of

dynamic biotic and abiotic factors. Differences in bacterial community composition between reactor types should be given more attention in order to improve the stability and success rate for large scale outdoor cultivation of microalgae.

Keywords: *Nannochloropsis*, outdoor reactors, algae-bacterial interactions, bacterial community composition

4.1. Introduction

Microalgae are one of the most promising feedstocks for production of food, feed, biofuels and other valuable chemicals [56, 263]. Algal cultivation does not necessarily compete for arable land and needs much less water to produce the same amount of biofuel compared to oil crops [56, 264]. Nevertheless, although algae have many appealing advantages as alternative cell factories, algal bulk products are still far away from large-scale application in industry due to high production costs [265].

Scale up of algae cultivation is carried out in different systems, but most commonly in shallow open ponds or in enclosed plastic tubular photobioreactors [266]. Despite the fact that a good number of systems has been proposed and tested, the industry is far from settled on a single approach. The high performance of algal strains in the laboratory can hardly be accomplished in large-scale outdoor cultivation systems because of varying ambient conditions, including physicochemical and biological factors [267]. Both open and closed outdoor algae production systems cannot easily be operated strictly axenically and are thus prone to microbial contamination. This is a substantial discrepancy compared to laboratory-based studies where whole reactors can be autoclaved. Therefore, in pilot-scale operation, bacteria present in photobioreactors cannot be ignored as is often the case for laboratory-based studies. However, relatively little is known about the bacteria present in algal photobioreactors and about their effects on algal cultivation [153, 268].

An increasing number of bacteria has been reported to be detrimental to microalgae and can cause mass algal cell destruction. Harmful impacts may be imposed through direct algal-bacterial cell contact, such as for the lytic bacteria *Saprospira* sp. (SS98-5) [269], *Pseudoalteromonas* sp. (J18/M01) [31] and *Microbacterium* sp. LB1 [32]. In addition, the synthesis of extracellular algicidal compounds may kill the algal host. For instance,

Streptomyces malaysiensis O4-6 was shown to release compound NIG355 capable of killing nearly 80% of *Phaeocystis globosa* in 24 h [33]. Nevertheless, recent studies have revealed that mutualistic relationships between algae and bacteria may even occur more commonly than antagonistic interactions [2, 40]. Associated bacteria benefit algal growth in mainly three ways. First of all, bacteria are key players in decomposing and mineralizing algal waste components, recycling carbon and phosphorus and making them again available for the algae [137, 144]. Secondly, bacteria can benefit algae through synthesizing a wide range of molecules ranging from vitamins [22, 26], phytohormones [16, 270], to siderophores [98, 271], which can stimulate algal growth. Lastly, some bacteria are able to kill algicidal bacteria by secreting antimicrobial compounds, such as tropodithietic acid in exchange for organic carbon [147].

Knowledge of bacterial communities in outdoor microalgae production systems is currently nearly non-existent. However, the impact of bacteria already presents in the microalgae inoculum, as well as temporal variation of bacterial communities due to variation of environmental parameters that are inevitably occurring in outdoor reactors are likely to be important for robust operation of these production systems. Therefore, we conducted a longitudinal study to investigate the composition and dynamics of bacterial communities within two indoor microalgae inoculum-production systems (i.e., a sterile-operated flat panel bioreactor and a non-sterile tubular indoor bioreactor) and four outdoor pilot-scale systems (i.e., an open raceway pond, a horizontal tubular bioreactor, a vertical tubular bioreactor, and a plastic flat panel reactor) during the production of the microalga *Nannochloropsis* sp. CCAP211/78 (the algal production data have been published by Vree *et al*. [272]) to assess the impact of non-sterile outdoor photobioreactor operation. One hundred and twenty-eight samples were collected from indoor and outdoor bioreactors over a period of four months. Bacterial 16S rRNA gene fragments were amplified and sequenced to determine the composition of associated bacterial communities.

4.2. Materials and Methods

4.2.1. *Algal cultivation and sampling procedures*

Nannochloropsis sp. CCAP211/78 was cultivated in 250 mL Erlenmeyer flasks, followed by cultivation in a flat panel reactor (FP, 4.5 L) and a horizontal tubular indoor reactor (TI, 280 L) located in a greenhouse at AlgaePARC (Wageningen, the Netherlands). The biomass harvested from TI was used to inoculate three pilot-scale outdoor reactors within one week: a horizontal tubular reactor (HT, 560 L), a vertical tubular reactor (VT, 1060 L) and a raceway pond (RP, 4730 L). An outdoor plastic flat panel reactor (PP, 60 L per panel) was inoculated with biomass directly harvested from the indoor FP. Samples were taken at AlgaePARC every Monday, Wednesday and Friday morning from July 3rd - October 16th, 2013. Detailed sample information can be found in Supplementary Figure S4.1. In addition, details of the production process of *Nannochloropsis* sp. CCAP211/78 [272] and a detailed description of outdoor reactors [273] were published previously. Liquid samples of 5 mL from each of the reactors were filtered through a sterile polycarbonate filter membrane (0.2 μm, Millipore) with a vacuum pump. Filter membranes were then rolled up, placed in 2 ml Eppendorf tubes and stored at -80 ˚C until further processing. Data generated in this study were derived from two separate reactor runs performed during the above-mentioned period, with the first and second runs being designated TI1, HT1, VT1, RP1, PP1 and FP2, TI2, HT2, VT2, RP2, PP2, respectively.

4.2.2. *DNA extraction, PCR amplification and sequencing*

To isolate total DNA, frozen filters were cut into small pieces with sterile scissors. DNA was extracted from these pieces using the FastDNA SPIN kit for soil (MP Biomedicals, USA) with the aid of a Precellys bead beater (Bertin Technologies, France) for two rounds of 45 s at a speed of 5500 rpm. DNA size and quantity were examined by electrophoresis on a 1% agarose

gel and measured spectrophotometrically with a Nanodrop (ND1000, Thermo Scientific, Wilmington, USA). The extracted DNA was stored at -20℃ until further use.

Amplicons from the V1-V2 region of bacterial 16S rRNA genes were generated using a two-step PCR strategy. Forward primer 27F-DegS (5'-GTTYGATYMTGGCTCAG-3'), and an equimolar mixture of reverse primers 338R I (5'-GCWGCCTCCCGTAGGAGT-3') and II (5'-GCWGCCACCCGTAGGTGT-3') were appended at the 5′ end with 18 bp universal tags (Unitag1: GAGCCGTAGCCAGTCTGC and Unitag2: GCCGTGACCGTGACATCG for the forward and reverse primers, respectively). PCR was conducted in a 50 µl reaction volume containing 1 µl DNA template, 10 µl 5× HF buffer (Thermo Scientific, The Netherlands), 1 µl dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 µM of each primer, 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, The Netherlands) and 32.5 µl nuclease free water (Qiagen, Germany). The PCR profile included the following steps: Pre-denaturation at 98°C for 30 s, followed by 25 cycles of denaturation (10 s at 98°C), annealing (20 s at 56°C), extension (20 s at 72°C), and a final elongation (10 min at 72°C). The PCR product size was examined by 1% agarose gel electrophoresis. The second PCR was conducted in a 100 µl reaction volume containing 5 µl of the first PCR product, 20 µl 5× HF buffer (Thermo Scientific), 2 µl dNTP Mix (10 mM; Promega), 500 nM forward and reverse primer (equivalent to the Unitag1 and Unitag2 sequences, respectively) that were each appended with an 8 nt sample-specific barcode [231], 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and 62 µl nuclease free water (Qiagen). The PCR conditions were pre-denaturation at 98°C for 30 s, followed by 5 cycles of denaturation (10 s at 98°C), annealing (20 s at 52°C), extension (20 s at 72°C), and a final elongation (10 min at 72°C). The PCR product was examined by gel electrophoresis and purified with the DNA HighPrep kit (Magbio Genomics, Rockville, MD, USA). The concentration of PCR products was quantified with a Qubit Fluorometer (Life Technologies, Darmstadt, Germany) in combination with the

dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). Purified products were then pooled in equimolar amounts (200 ng μl^{-1}) and sequenced on a MiSeq platform (GATC-Biotech, currently part of Eurofins Genomics Germany GmbH, Konstanz, Germany).

4.2.3. *Processing of MiSeq data*

Illumina sequencing data was processed using the NG-Tax pipeline [274]. In short, paired-end reads of 2 x 100 nucleotides were combined and filtered to retain only read pairs with perfectly matching primers and barcodes. Demultiplexing, Operational Taxonomic Unit (OTU) picking, chimera removal and taxonomic assignment were performed within one single step in NG-Tax. Filtered sequences were ranked per sample by abundance and unique OTUs (at a 100% identity level) were added to an initial OTU table for that sample starting from the most abundant sequence until the abundance was lower than 0.1%. The final OTU table was created by clustering the reads that were initially discarded to the OTUs from the initial OTU table with a threshold of 99% similarity. Taxonomic assignment was done using the UCLUST algorithm [275] and a customized SILVA SSU Ref 111 database [232]. Samples with less than 1000 reads (Bacterial 16S rRNA gene reads plus chloroplast 16S rRNA gene reads) were removed, and all chloroplast 16S rRNA reads were removed from the dataset. The number of retained reads for each sample was calculated again, and samples with less than 100 bacterial 16S rRNA gene reads were removed as well.

4.2.4. *Statistical analysis*

All statistical tests were performed in R (v.3.1.2) [235]. First, the OTU table was standardized by a square root transformation using the *decostand* function (method = “hellinger”) from the vegan package [234]. Transformed data was subsequently used to calculate alpha-diversity indices (Shannon diversity and Richness). Pairwise comparison of alpha-diversity between the different reactors within each run was calculated using Wilcoxon rank sum test and Benjamini-

Hochberg *p*-value adjustment as implemented in the "STATs" package [235, 276]. For further multivariate analyses the *vegdist* function from the vegan package was used to create a Bray-Curtis dissimilarity matrix of the standardised OTU table. Hierarchical clustering of all samples based on the Bray-Curtis dissimilarity matrix was performed using the "average" method. Then, a non-metric multidimensional (nMDS) scaling plot was generated using the *metaMDS* function based on pairwise Bray-Curtis distances. Overall differences in bacterial communities between reactors were assessed statistically with PERMANOVA (adonis) from the vegan package. PERMANOVA was also performed to test whether bacterial communities between reactor types are significantly different. Detrended Correspondence Analysis (DCA) was performed and Canonical Correspondence Analysis (CCA) was the best constrained ordination model for the bacterial communities. Significance of the environmental factors was tested by the *envfit* function with 999 Monte Carlo permutations. The overall significance of CCA and of each axis were tested by analysis of variance (ANOVA) permutation tests. Pearson's correlation analysis between each pair of parameters measured in this study was done using *rcorr* function in "Hmisc" package. The OTU heatmap was created with the "pheatmap" package.

4.3. Results

4.3.1 *Bacterial community profiles*

Bacterial community composition dynamics in six different photobioreactors in which *Nannochloropsis* sp. CCAP211/78 was grown, was investigated with Illumina MiSeq amplicon sequencing of the V1-V2 region of bacterial 16S rRNA genes. After removing twenty-one low-quality samples, we retained 3,574,708 high-quality sequences with an average of 33,408 reads per sample. These sequences represented 1,217 operational taxonomic units (OTUs). A total of 2,703,376 reads (75.6% of all retained reads, 237 OTUs) were derived from *Nannochloropsis* chloroplasts. After removal of chloroplast OTUs from the dataset, 980 bacterial OTUs were

used for bacterial diversity analyses. This final dataset of 16S rRNA gene reads from all reactors represented 13 phyla, with only a small fraction of sequences that could not be classified at the phylum level (2.39%) (Figure 4.1A). *Bacteroidetes* and *Proteobacteria* were on average the most predominant phyla in all reactors (44.0% ± 5.1% and 43.8% ± 6.8%, respectively). The raceway pond (RP) had the highest relative abundance of *Actinobacteria* (11.5%) and *Verrucomicrobia* (7.5%). The highest proportion of *Planctomycetes* was 3.7% in the outdoor flat panel (PP), and *Verrucomicrobia* and *Planctomycetes* were present in all reactors except the indoor flat panel (FP). The other eight phyla together only contributed to a minor part of total bacterial reads in all reactors, which was approximately 1.2% in the vertical tubular reactor (VT) and less than 1% in other reactors. We then assessed the most abundant bacterial taxa across all samples at the family level (Figure 4.1B). The *Rhodobacteraceae* (phylum *Proteobacteria*) were highly predominant in all reactors and were the most abundant family in FP, RP and PP with relative abundances of 40.5, 22.6 and 19.5%, respectively. The second most predominant family in FP was *Flammeovirgaceae* (phylum *Bacteroidetes*), constituting nearly 30% of the bacterial reads. However, the *Flammeovirgaceae* were absent or present at only low relative abundance in the other reactors. In contrast, two other families within the *Bacteroidetes* not detected in FP were present at high relative abundance in the other reactors: *Flavobacteriaceae* with relative abundances between 5.6% and 17.4%, and *Saprospiraceae* between 3.9% and 22.5% (Figure 4.1B). Some bacterial families were only predominant in specific reactors. For instance, *Microbacteriaceae* (phylum *Actinobacteria*) and the *NS11-12_marine_group* (phylum *Bacteroidetes*) were predominant in both TI and RP.

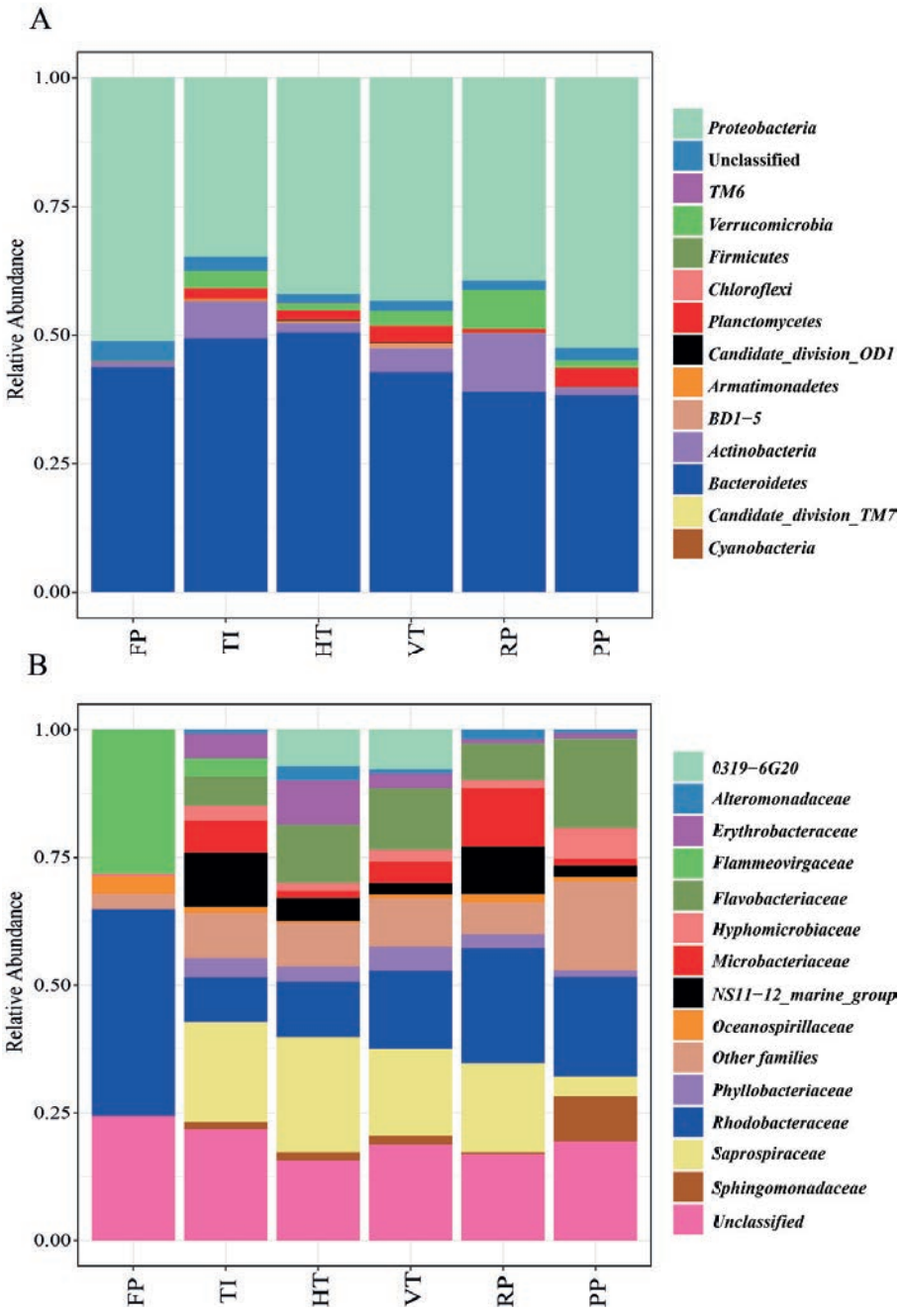


Figure 4.1. Relative abundance of (A) bacterial phyla and (B) families in different reactors. FP = Flat panel reactor, TI = Tubular indoor reactor, HT = Horizontal tubular reactor, VT = Vertical tubular reactor, RP = Raceway pond, PP = Plastic flat panel reactor.

At the OTU level, members of the genera *Rhodobacter* (OTU538) and *Ekhidna* (OTU1117) had the highest relative abundance in FP (Figure S4.4). OTUs from unidentified genera from the *Saprospiraceae* (OTU1261) and the *NS11-12_marine_group* (OTU1092) predominated all other reactors. Other OTUs had a more incidental occurrence and were present at high relative abundance only in certain reactors or individual runs, such as OTU249 (*Devosia*) and OTU288 (*Paracoccus*) in PP, OTU398 (*Erythrobacter*) in HT, and OTU863 (*Microbacteriaceae*) in RP (Figure S4.4).

4.3.2 *Bacterial diversity in indoor and outdoor reactors*

The bacterial communities present in the autoclaved indoor reactor FP already were characterized by a considerable alpha-diversity (Figure 4.2). Generally in the larger non-sterilely operated indoor reactor (TI) and outdoor reactors (HT, VT, PP, RP) Shannon diversity and OTU richness were not significantly different from FP. In addition, Shannon diversity and OTU richness were not significantly different between different outdoor reactor types (Figure 4.2, Table S4.1). Both alpha-diversity indices were significantly higher in VT and PP for the second run in the year (run2) than for the first run (run1).

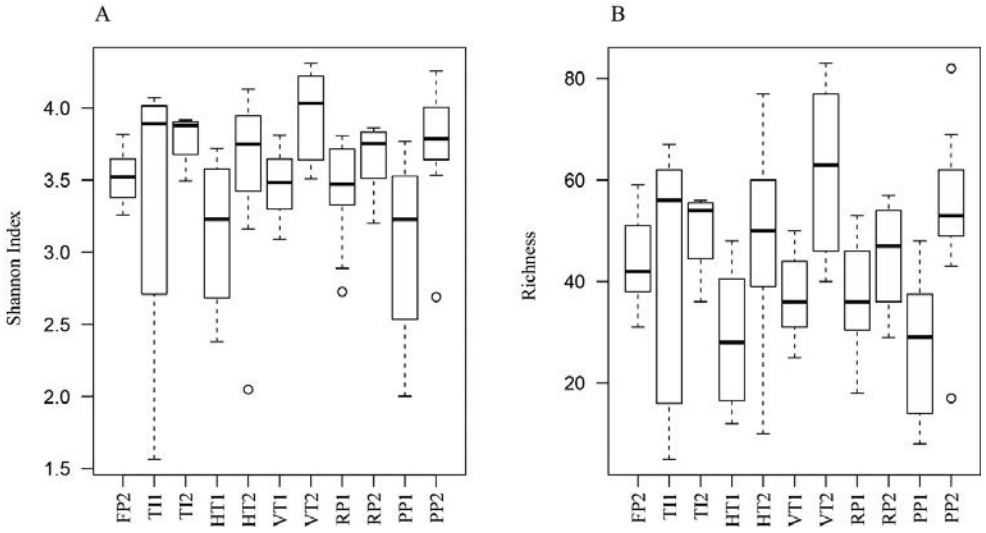


Figure 4.2. Box-plot of (A) Shannon diversity indices and (B) Observed OTU richness for each of the reactors for the two runs. Upper and lower lines correspond to the maximum and minimum of the distribution. The upper and lower limits of the boxes are third and first quartile. Horizontal black thick lines are the median values. Outliers are displayed as open circles.

The reactor type had a significant impact on the beta-diversity of the bacterial communities present in the reactors (Adonis test, $p = 0.001$). Pairwise comparisons of bacterial communities between reactor types revealed that bacterial communities in all reactors significantly differed from each other except HT and VT (Table S4.2A). Bacterial communities within the same reactor type were different in different runs (Table S4.2B) for all reactor types.

4.3.3 *Temporal fluctuations of bacterial communities*

Although bacterial communities in TI and PP reactors were initially similar to those in FP from which they were inoculated, the communities changed as the cultivation continued (Figure 4.3). Likewise, the other three outdoor reactor types (HT, VT, RP) initially clustered close to TI from which they were inoculated, but at a later stage became more dissimilar to the community in TI with especially rapid community changes near the end of a run in HT, VT and RP (Figure 4.3). Hierarchical clustering of bacterial community composition clearly showed temporal differences in profiles in HT, VT and RP1 where samples early in the runs were clustered in group 2 and all samples later in the runs in group 3 (Figure S4.2). No different phases were identified in RP2 as only five samples passed sequencing quality thresholds.

The twenty-one OTUs that contributed most to the dissimilarity between bacterial community profiles in the starting phase and end phase of the runs in HT, VT and RP1 (group 2 and 3 in Figure S4.2) were identified. In total, these twenty-one OTUs contributed more than 28% to the between-group dissimilarity. OTU1261 (family *Saprospiraceae*) had highest contribution (3.67%) to the dissimilarity between the two phases of cultivation and decreased dramatically in HT, VT as well as in RP1 during the run. Other predominant OTUs that nearly disappeare

Table 4.1. Similarity percentages (SIMPER) of bacterial OTUs contributing most to the dissimilarity between bacterial communities at the start phase and end phase of runs in HT, VT and RP. Increased or decreased average relative abundance of OTUs by more than 5% points between two phases in HT, VT and RP is shown in this table. Average: Species contribution to average between-group dissimilarity. Cumsum: cumulative contribution. Av.Group2 and Av.Group3 indicate average abundance of OTUs in those groups. The *p* value is indicated by "ns, not significant"; "*, <0.05"; "**, <0.01"; "***, <0.001".

Taxonomy	OTU ID	Average (%)	Cumsum (%)	Av.Group2 (%)	Av.Group3 (%)	*p*	HT1 (%)	HT2 (%)	VT1 (%)	VT2 (%)	RP1 (%)
Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Saprospiraceae; g	OTU1261	3.67	4.02	45.95	8.04	***	-26.1	-41.9	-24.8	-31.2	-7.7
Proteobacteria; Deltaproteobacteria; Myxococcales; 0319-6G20; g	OTU422	2.33	6.57	0	26.79	***		26.1		23.7	
Bacteroidetes; Sphingobacteriia; Sphingobacteriales; NS11-12_marine_group; g	OTU1092	1.98	8.74	23.51	2.35	***	-9.8				-8.2
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Gilvibacter	OTU1010	1.53	10.42	0.22	15.89	***	17.3				
Planctomycetes; Phycisphaerae; Phycisphaerales; f; g	OTU798	1.26	11.80	0	13.69	***			9.8		
Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; g	OTU101	1.12	13.03	12.68	5.00	*				-10.0	
Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; g	OTU858	1.11	14.24	12.67	1.65	*					
Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseovarius	OTU49	1.03	15.37	11.30	8.53	ns			5.1		
Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Maritimibacter	OTU66	1.00	16.47	11.50	0.90	ns					
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Erythrobacteraceae; Erythrobacter	OTU375	0.99	17.56	0.90	9.62	***	16.9				
Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Candidatus_Aquiluna	OTU875	0.98	18.63	11.01	0	***					
Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Saprospiraceae; g	OTU1223	0.97	19.69	5.91	7.60	**	8.3				7.3
Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; g	OTU86	0.94	20.72	3.17	10.07	***		6.6			
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; g	OTU1001	0.93	21.74	10.70	2.49	ns					
Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; g	OTU335	0.89	22.72	11.49	3.64	**					
Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Sphingobacteriaceae; g	OTU1131	0.86	23.66	6.30	6.90	*					
Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; DS001	OTU866	0.85	24.59	2.34	8.97	***					
Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; g	OTU1027	0.81	25.49	2.59	8.26	***					
Bacteroidetes; Flavobacteria; Flavobacteriales; f; g	OTU973	0.78	26.34	0.95	7.58	***	5.0		6.1		
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingopyxis	OTU411	0.77	27.19	8.45	0.58	ns					
Bacteroidetes; Sphingobacteriia; Sphingobacteriales; f; g	OTU1272	0.76	28.02	8.22	0	**					

in the end phase were OTU1092 (*NS11-12_marine_group*) in HT1 and RP1 and OTU101 (*Rhodobacteraceae*) in VT2. Eight OTUs were strongly increased in the late phase of the runs. These mostly varied by reactor type and run but many belonged to the classes *Alphaproteobacteria* and *Flavobacteriia* (Table 4.1).

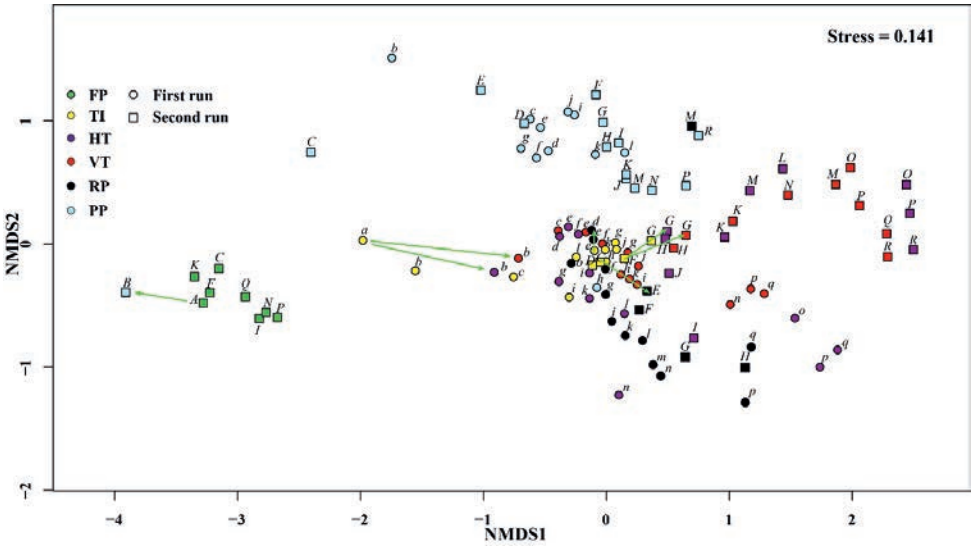


Figure 4.3. Non-metric multidimensional scaling (nMDS) of Bray-Curtis distances based on normalised relative abundance of OTUs in bacterial communities in six reactor types (different colours). Each reactor was run twice (except FP), and samples from the first run are indicated by circles and the second run by squares. Inoculation is indicated by green arrows. First run samples are sequentially labelled with lower case letters (a-q), and second run samples are sequentially labelled with upper case letters (A-R). Same letters indicate that samples were taken at the same day. FP = Flat panel reactor, TI =Tubular indoor reactor, HT = Horizontal tubular reactor, VT = Vertical tubular reactor, PP = Plastic flat panel reactor, RP = Raceway pond.

4.3.4 *Environmental drivers of Nannochloropsis-associated bacterial community development*

To identify the main driver(s) underlying temporal changes in bacterial community composition in different reactors as well as differences in bacterial community composition between

different reactor types, the correlation of temperature (Temp), pH, nitrate concentration (NO_3^-), photon flux density (PFD), and algal biomass productivity (PRO, defined as volumetric productivity: g L^{-1} d^{-1}) with bacterial community structure was investigated. Overall, only approximately 4.8 % of the compositional variation could be explained by the first axis and 3.5 % by the second axis of the CCA using the parameters evaluated in this study (Figure 4.4A). The bacterial community in PP correlated best with PRO and Temp, while NO_3^-, PFD and pH correlated best with bacterial community composition in all samples of FP and part of the samples in TI, HT, VT and RP (Figure 4.4A). Another part of the HT and VT samples were correlated with lower values of Temp, PFD and PRO. From the parameters we measured, Temp, PFD and PRO were positively correlated with each other (Figure 4.4B). By contrast, NO_3^- was negatively correlated with PRO and PFD. This trend also corresponded with the observation that the first run in the outdoor reactors was characterized by higher PRO than the second run, which likely resulted from higher Temp and PFD in the first run (Figure S4.5).

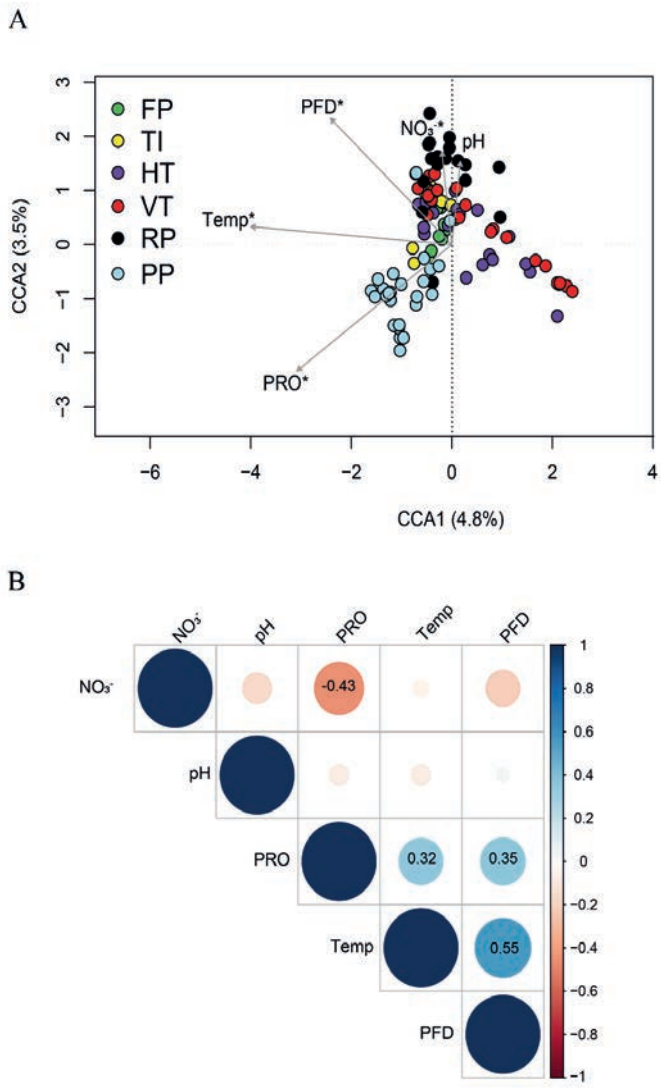


Figure 4.4. (A) Canonical correspondance analysis (CCA) showing correlation between bacterial communities (response variables) and environmental factors (explanatory variables). The percentage of variation in the bacterial community explained by each axis is indicated in parentheses after the axis label. The environmental factors with * significantly contribute to explaining the observed variation in bacterial community composition ($p < 0.05$). **(B)** Pearson correlation analysis between environmental factors. Only correlation coefficients with $p < 0.05$ are indicated. The factors included are Temp (temperature), pH, NO_3^- (nitrate concentration), PFD (photon flux density), PRO (algal biomass productivity).

4.4. Discussion

Bacteria in association with microalgae have rarely been investigated in large microalgae culture systems and the studies that did, only assessed one type of outdoor reactor. For instance, bacterial communities were analysed before in a 300 L outdoor flat panel reactor with *Tetraselmis suecica* [118], in a 550 L outdoor tank with *Nannochloropsis salina* [91], in a 200 L polyethylene flat panel reactor with *Nannochloropsis salina* [69] and in a 1600 L enclosed membrane reactor with *Desmodesmus* and *Scenedesmus* for treatment of domestic waste water [162].

4.4.1 *Differences between reactors*

We compared for the first time the bacterial communities of four pilot-scale outdoor photobioreactors operated under identical climatological conditions for the production of *Nannochloropsis* sp. CCAP211/78. We found bacterial communities were significantly different between FT, TI and the outdoor reactors (Figure 4.1A and Table S4.2). This result was in accordance with a previous study that showed that the bacterial community of *Nannochloropsis salina* differed between small indoor reactors (Volume: 5 mL-4 L), medium indoor reactors (Volume: 20-60 L) and a large outdoor reactor (Volume: 200 L) [69]. Fulbright *et al*. [69] also reported that OTU richness increased as the size of the reactors increased. However, neither OTU richness nor Shannon index was significantly different between reactor types in this study (Figure 4.2, Table S4.2A). This finding reveals that *Nannochloropsis* cultures in FP were already colonised by diverse bacteria before being harvested to inoculate other reactors. We observed a close association between inoculum samples in one reactor and receiver samples in the other reactor (Figure 4.3), which corroborates that the initial bacterial community composition in each reactor is largely determined by the bacterial community in precultures from which the subsequent reactors was inoculated.

Despite differences in bacterial community composition between reactor types, the most abundant bacterial phyla in all reactors were similar, with predominance of *Proteobacteria* (predominantly *Alphaproteobacteria*) and *Bacteroidetes* (Figure 4.1). In previous studies, *Alphaproteobacteria* and *Bacteroidetes* were shown to be the most abundant phyla in *Nannochloropsis* cultures [69, 87, 277]. Several bacterial families found in this study are similar to those found in the cultures of *N. salina*, which include members of the families *Saprospiraceae*, *Phyllobacteriaceae*, *Hyphomonadaceae*, *Rhodobacteraceae* and *Alteromonadaceae* [69, 278]. The occurrence of the same taxa associated with *Nannochloropsis* species in different environments and locations suggests that these bacteria may have specific interactions with *Nannochloropsis* [87, 279]. For example, members of the *Phyllobacteriaceae* have been shown to enhance the growth of algae through vitamin supplementation [22] and nitrogen fixation [105].

At the OTU level, the FP bacterial community was predominated by a few highly abundant OTUs, whereas more diverse OTUs were found in the larger reactors (Figure S4.4). Three predominant representatives in FP (*Rhodobacter*_OTU538, *Ekhidna*_OTU1117 and *Balneola*_OTU835) have previously been found either in cultures of *Nannochloropsis oculata* [92], *Ectocarpus* sp. [103] or *Emiliania huxleyi* [280]. Yet their roles in algal cultures have not been characterized. The most abundant taxon (OTU1261) in TI and all outdoor reactors belongs to the *Saprospiraceae* (Figure S4.4). The best hit returned by a Blast search against the NCBI nr/nt database is *Phaeodactylibacter xiamenensis* (100% identity), which was isolated from a culture of the marine diatom *Phaeodactylum tricornutum* [281]. Although two *Saprospiraceae*-related OTUs were also observed in the inflowing seawater (Table S4.4), these were different from the most abundant OTU (OTU1261) and represented only minor fractions of the bacteria found in the different reactors. In addition, OTU1261 is not closely related to the lytic bacterium *Saprospira* sp. (92% identity) that was reported to kill and lyse the cells of the diatom

Chaetoceros ceratosporum [269]. A bacterium belonging to the *Saprospiraceae* family was previously found to be most abundant on average (comprising 34.7% ± 14.3% of bacterial communities) in large-scale cultures of *Nannochloropsis salina* in a closed polyethylene growth panel (0.05 m wide × 0.28 m high × 17.3 m long) located outdoor in a water basin [69]. Although no correlation was observed between relative abundance of *Saprospiraceae* and *N. salina growth* performance [69], its ubiquitous predominance in all mass culture systems both in a previous study and this study suggests that there are important interactions between members of this family and *Nannochloropsis* or at least a commensal relationship. Another OTU common to TI and outdoor reactor samples was classified as a member of the *NS11-12_marine group* (*Bacteroidetes*) that has been mainly detected in marine environments [282]. However, as the unresolved taxonomy indicates, we still know little of their ecological roles. Some genera with random occurrence in certain reactors or runs, such as *Devosia* (OTU249), *Paracoccus* (OTU288) and *Erythrobacter* (OTU398) (Figure S4), have been frequently found to associate with either seaweeds [283-285] or microalgae [286]. *Devosia* sp. was inferred to play a role in nitrogen fixation as an epiphytic bacterium associated with the macroalga *Cladophora glomerata* [287], and may have a similar interaction with *Nannochloropsis.* *Paracoccus* as well as *Erythrobacter* were reported to be diatom-associated and found to be resistant to polyunsaturated aldehydes released by diatom cells upon disruptions by grazers, suggesting co-evolution of resistance to toxic molecules in diatoms and their associated bacteria [96].

The longitudinal sampling strategy helped us examine the influence of biotic and abiotic factors on the structure of *Nannochloropsis* sp. CCAP211/78-associated bacterial communities. Temperature, salinity and nutrient concentration (nitrate) are the most important factors structuring bacterial communities in aquatic environments, such as in estuaries and coastal seawater [288-290]. Salinity was not measured in this study because the fluctuation in salinity

is negligible in our experimental setup. To this end, it should be noted that salinity fluctuation of RP due to evaporation or rain was adjusted by daily addition of fresh water or sodium chloride. Temperature and nitrate can directly affect bacterial growth, but also influence algal growth [291, 292], which would in turn affect bacterial growth. Similarly, light intensity and pH can affect the growth of both algae and bacteria [293, 294]. Correspondence canonical analysis revealed that nitrate is a primary factor that drives variation in bacterial community composition in all reactor types except PP. Nitrate is a key chemical that influences microbial communities through its effects on nutrient utilization and growth [295, 296]. Many bacteria can utilize nitrate and even compete with algae when nitrate concentration is low [96, 297, 298]. Besides, nitrate was actively consumed by *Nannochloropsis*, which was demonstrated by the negative correlation between algae biomass productivity and nitrate concentrations (Figure 4B). These results indicate that the bacterial community is at least partly structured by the availability of nitrate but also by the growth of algae. On the other hand, the distinct bacterial community composition in PP could be explained by the highest biomass productivity of *Nannochloropsis*. It is likely that algal physiology and metabolites released by microalgae could substantially contribute to the distinctness of bacterial communities [272]. It should be noted that the biggest part of bacterial community variation cannot be explained by the monitored factors included in the CCA (Figure 4.4A). The omission of some important environmental factors, such as phosphorus concentration and dissolved organic matter, could be a reason. These environmental factors were previously shown to affect microbial community composition in marine waters [299, 300] and should be measured in future studies. In addition, stochastic effects related to microorganisms entering the reactors from the outside could contribute to the different changes of bacterial community composition in different systems. For instance, bacteria may enter reactors through the addition of seawater for the daily dilution of algal biomass, which is

supported by the observation that a range of bacteria are shared between seawater samples and microalgal cultures (Table S4.3).

4.4.2 *Differences between runs*

Bacterial community composition was significantly different between runs. Presumably, one factor governing this difference relates directly to inoculation. Specifically the bacterial community of the inoculum used for the first run was different from the inoculum for the second run (Figure 4.3). More importantly, since the first run spanned the period from July till August and the second run spanned the period from August till October, temperature and light intensity differed between both runs, which may directly or indirectly change the bacterial community. These discrepancies between two runs might also be linked to the observation that both alpha-diversity indices were seemingly higher for the second run than for the first run for all outdoor reactor types. In marine environments, the maximum OTU richness and evenness were found in a temperature range from 15 °C to 20 °C, with lower diversity both above and below those temperatures [301]. All our outdoor reactors were operated at an average temperature >20 °C (except HT2) and the temperature was at least 2 °C higher during the first run than during the second run (Figure S4.5). Therefore, the higher temperature in the first run may have led to the reduction of both alpha-diversity indices. Furthermore, the higher algal biomass productivity of the first run might have resulted in higher concentrations of extracellular organic compounds, which favour the growth and dominance of fast growing copiotrophic bacteria and thus lowering OTU richness and diversity. In addition, this observation can be supported by independent studies that have found a decrease of OTU richness and/or Shannon diversity during algal blooms [302-304].

4.4.3 *Bacterial community dynamics within runs*

Bacterial community composition also varied within runs in all reactor types from the start of monitoring to the end (Figure 4.3). The FP reactor showed least variation, whereas this variation was more apparent in outdoor reactors. Presumably, this variation was caused by the inherently more variable environmental conditions (temperature, for instance) that were not as well controlled as in the indoor reactors. We identified a substantial number of the OTUs that increased pronouncedly in relative abundance near the end of the cultivation in outdoor runs in HT, VT and RP. These OTUs were annotated as members of the *Flavobacteriaceae* (2 OTUs) and *Rhodobacteraceae* (3 OTUs). These two families were also shown to be dominant in the stationary phase of batch cultures of *Nannochloropsis salina* [279], as well as in algal blooms [305] and in a range of algal production systems in general [117, 162, 306]. Bacteria belonging to the *Flavobacteriaceae* are fast-growing specialists observed during algae blooms and specialize in the degradation of algal-derived complex organic matter [304, 307]. Members of the *Rhodobacteraceae* are often most abundant in bacterial communities that are closely associated with marine algae, including natural phytoplankton blooms and algal cultures [308, 309]. The frequent occurrence of *Flavobacteriaceae* and *Rhodobacteraceae* from independent studies emphasizes the specialized fitness of these taxa for thriving in algal cultures [279]. Three OTUs showed a strong decrease in relative abundance at the end of cultivation including the most prevalent OTU (*Saprospiraceae*_OTU1261). It has been shown that the growth phase and physiological state of algal cultures could serve as selective factors affecting bacterial composition and governing bacterial community structure [306]. As the growth of *Nannochloropsis* sp. CCAP211/78 in outdoor reactors at the end phase of cultivation is often associated with fouling and contamination as indicated by Vree *at al*. [272], the observed shift of the predominant bacterial taxa in relative abundance near the end phase could potentially be a first indicator of culture instability [310]. Nevertheless, mechanistic insights are needed to

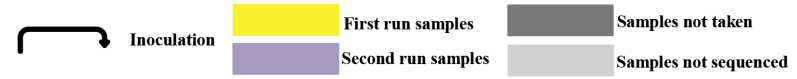
understand the observed correlations of certain bacteria with the growth performance of *Nannochloropsis* sp. CCAP211/78.

4.5. Conclusions

16S rRNA gene amplicon sequencing enabled us to gain detailed insights into composition and dynamics of bacterial communities of *Nannochloropsis* sp. CCAP211/78 cultures grown under a range of environmental conditions and different pilot-scale photobioreactor types. We showed changes in bacterial community composition during the successional scaling up process of algal cultivation from a small indoor reactor to large outdoor reactors. Each reactor type had a significantly different bacterial community composition except HT and VT. Bacterial community composition also significantly differed between runs of each reactor type. The inoculum source played a critical role in determining the initial bacterial community composition of each reactor type, whereas the physio-chemical factors affected later development of bacterial community composition. Nitrate concentration was the main abiotic factor that could be identified in this study regulating diversity and composition of the bacterial community in all reactors except PP where algal biomass productivity had a significant impact on shaping community structure. Although interactions between the bacterial community and biotic and abiotic factors across different reactors were explored in our study, a large fraction of the observed variation in community structure could not be explained by the variables we measured. We also identified a number of bacterial species with large changes in their relative abundance between the start and end of the cultivation of *Nannochloropsis* sp. CCAP211/78 and they may serve as a potential indicator of microalgal culture stability

Supplementary Information

A



Sampling Date	FP	TI	HT	VT	RP	PP	Labels
1-Jul-2013							
3-Jul-2013							a
5-Jul-2013							b
8-Jul-2013							c
10-Jul-2013							d
12-Jul-2013							e
15-Jul-2013							f
17-Jul-2013							g
19-Jul-2013							h
22-Jul-2013							i
24-Jul-2013							j
26-Jul-2013							k
29-Jul-2013							l
31-Jul-2013							m
2-Aug-2013							n
5-Aug-2013							o
7-Aug-2013							p
9-Aug-2013							q
12-Aug-2013							
14-Aug-2013							
16-Aug-2013							A
19-Aug-2013							
21-Aug-2013							B
23-Aug-2013							C
26-Aug-2013							D
28-Aug-2013							E
30-Aug-2013							F
2-Sep-2013							G
4-Sep-2013							H
6-Sep-2013							I
9-Sep-2013							J
12-Sep-2013							K
14-Sep-2013							L
16-Sep-2013							M
19-Sep-2013							N
23-Sep-2013							O
26-Sep-2013							P
30-Sep-2013							Q
3-Oct-2013							R

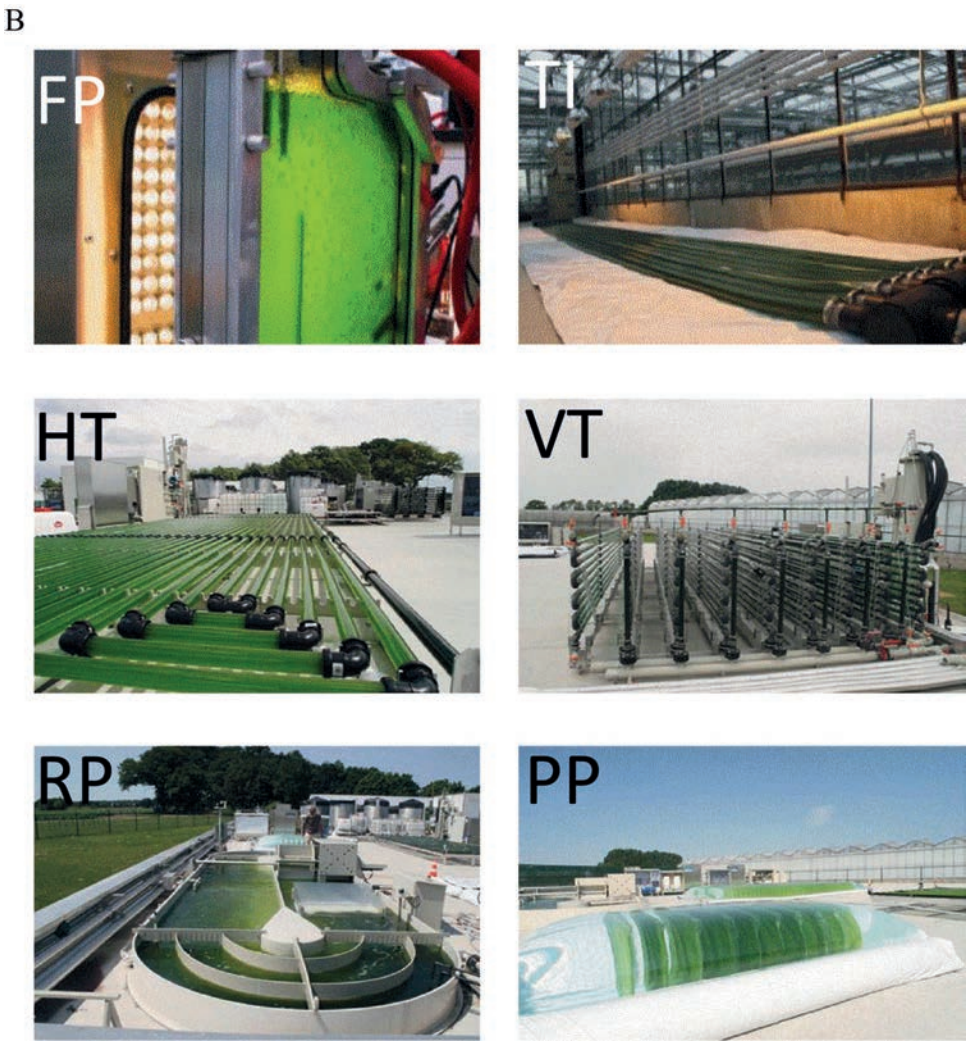


Figure S4.1. (A) Sampling and inoculating information for six reactors of two separate runs. (B) Overview of cultivation systems in this study. FP = Flat panel reactor, TI = Tubular indoor reactor, HT = Horizontal tubular reactor, VT = Vertical tubular reactor, RP = Raceway pond, PP = Plastic flat panel reactor. Pictures were taken at AlgaePARC, Wageningen, the Netherlands.

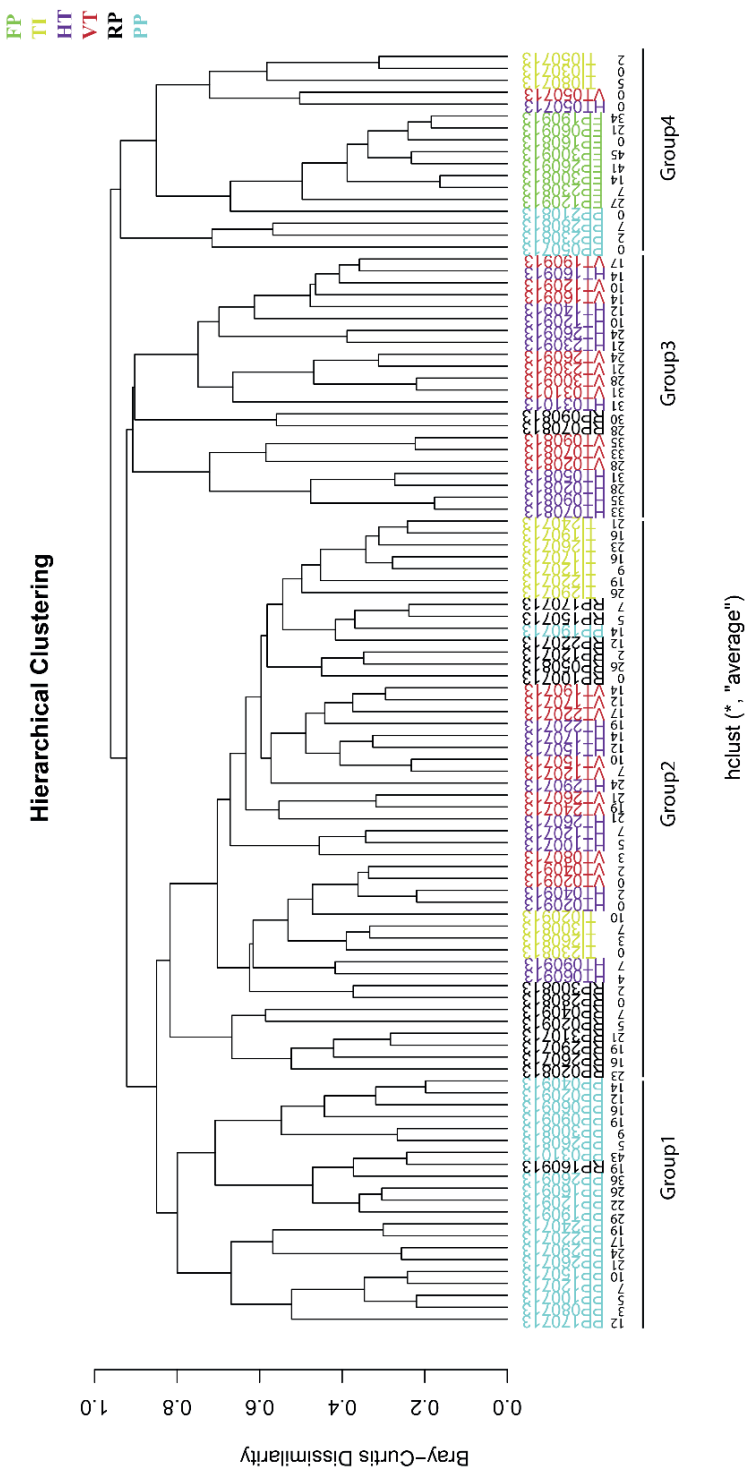


Figure S4.2. Clustering analysis of samples of the six reactors calculated by the average clustering method (UPGMA). Clustering was done using the OTU distance matrix based on Bray-Curtis (dis)similarities. Numbers below sample name represent the number of cultivation days after inoculation.

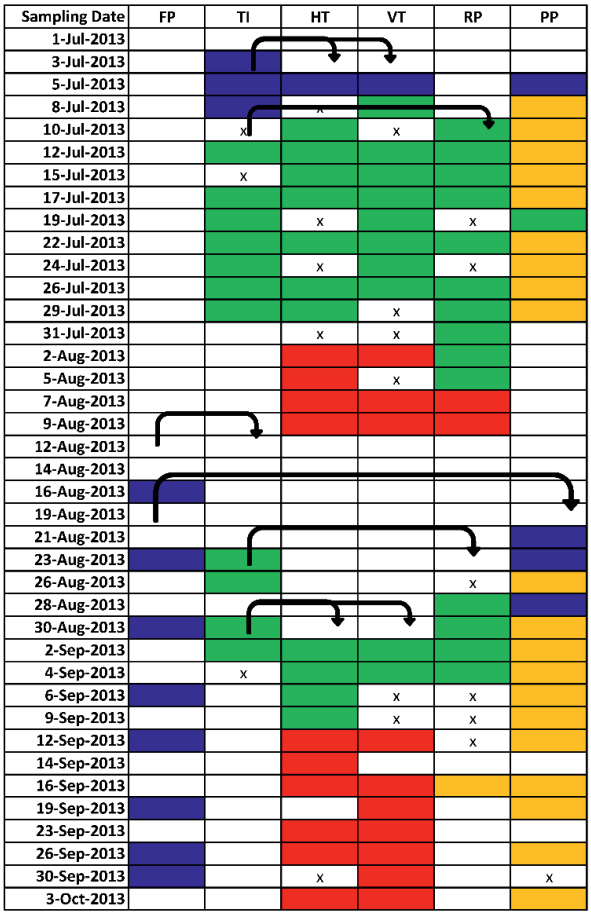


Figure S4.3. Samples marked with different colours according to the clustering analysis in Figure S2**.** Low-quality samples indicated with x were removed before analysis.

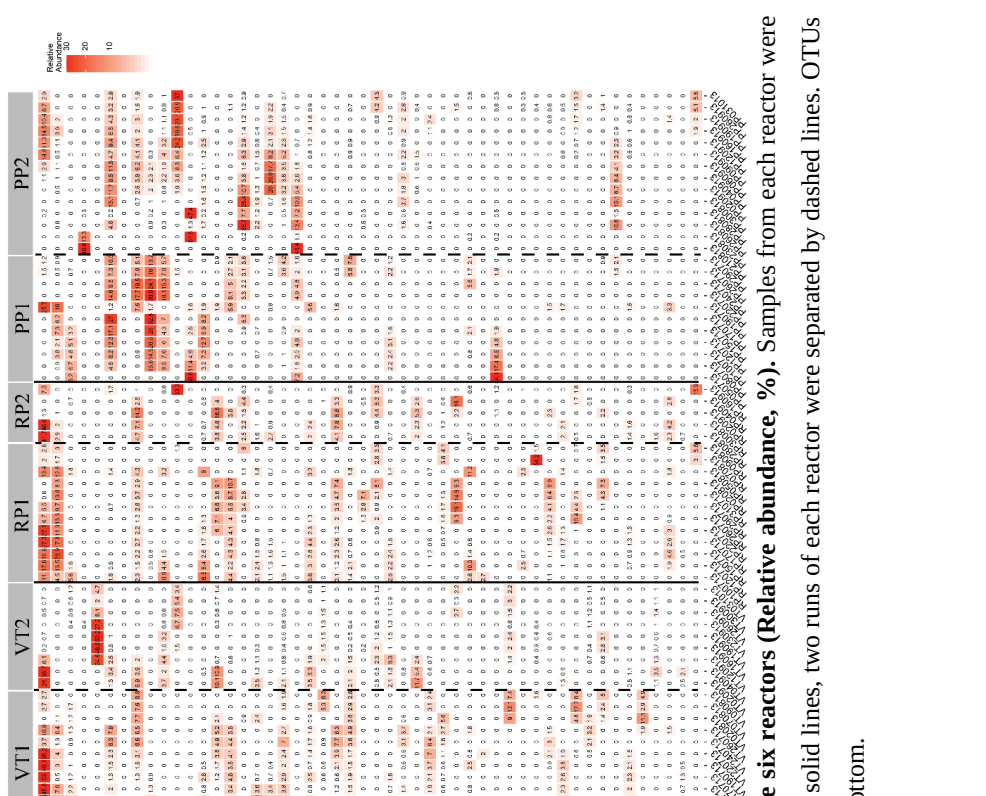


Figure S4.4. Heatmap of the most abundant bacterial OTUs in the six reactors (Relative abundance, %). Samples from each reactor were ordered sequentially by sampling time. Each reactor was separated by solid lines, two runs of each reactor were separated by dashed lines. OTUs are ordered from most abundant ones at top to less abundant ones at bottom.

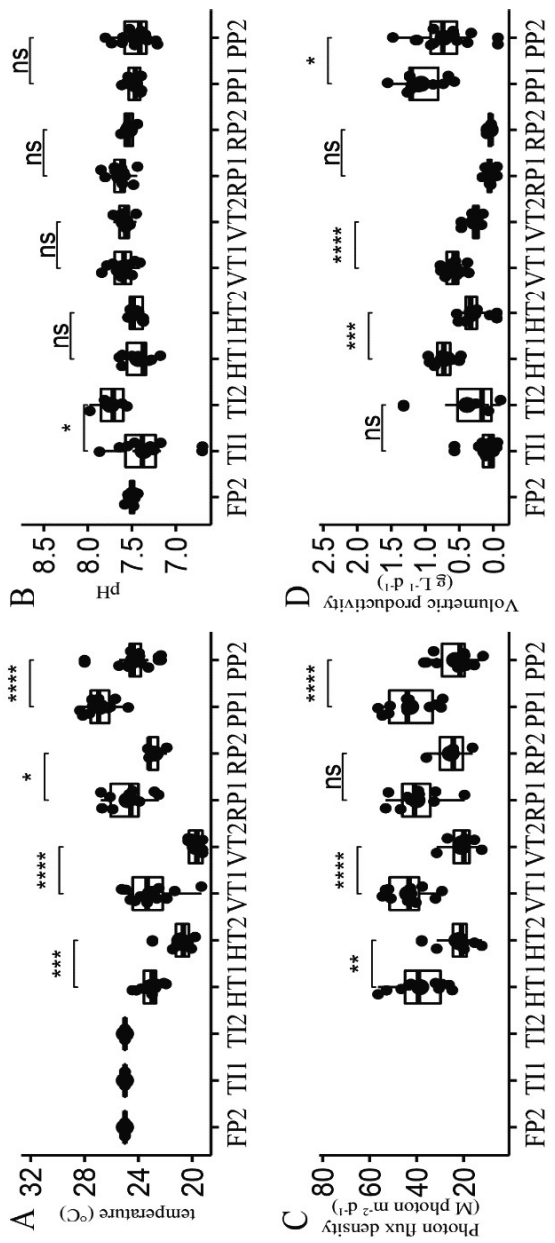


Figure S4.5. Environmental and chemical factors measured in this study. The difference between runs within each reactor type was tested with a t test. *p* value is indicated by "ns, not significant"; "*, <0.05"; "**, <0.01"; "***, <0.001"; "****, <0.0001".

Table S4.1. Pair-wise comparison of Shannon index (A) and richness (B) for different runs in each reactor calculated according to the Wilcoxon test (*p* adjustment method: Benjamini-Hochberg), Bold values indicate $p < 0.05$.

(A) Shannon Index

	FP2	TI1	TI2	HT1	HT2	VT1	VT2	RP1	RP2	PP1
TI1	0.4604									
TI2	0.2000	0.9774								
HT1	0.2230	0.2488	0.0659							
HT2	0.3868	0.9774	0.8416	0.0809						
VT1	0.8112	0.3681	0.1020	0.2230	0.2331					
VT2	**0.0379**	0.2230	0.3101	**0.0084**	0.2230	**0.0144**				
RP1	0.8416	0.3681	0.0809	0.1823	0.3101	0.9774	**0.0338**			
RP2	0.4604	0.7426	0.3101	0.1182	0.8447	0.3101	0.2230	0.3101		
PP1	0.2230	0.2214	0.0627	0.9774	0.1020	0.2448	**0.0110**	0.2230	0.1988	
PP2	0.0731	0.8442	0.9774	**0.0062**	0.7640	**0.0280**	0.4199	0.0659	0.4596	**0.0062**

(B) Observed OTUs

	FP2	TI1	TI2	HT1	HT2	VT1	VT2	RP1	RP2	PP1
TI1	0.6734									
TI2	0.3790	0.8120								
HT1	0.0733	0.2577	0.0785							
HT2	0.5545	0.9107	1.0000	0.0822						
VT1	0.2577	0.3484	0.1175	0.2466	0.1210					
VT2	0.1175	0.2265	0.3196	**0.0128**	0.2905	**0.0180**				
RP1	0.4326	0.3388	0.1175	0.1712	0.1959	0.8984	**0.0317**			
RP2	0.9590	0.6734	0.7149	0.1175	0.7149	0.2905	0.2095	0.3196		
PP1	0.0785	0.1959	0.0785	0.9107	0.0785	0.2916	**0.0128**	0.2265	0.1403	
PP2	0.1210	0.6734	0.9107	**0.0091**	0.6734	**0.0128**	0.4838	**0.0317**	0.3559	**0.0091**

Table S4.2. Pairwise comparison of bacterial communities based on Bray-Curtis (dis)similarity at OTU level in different reactor types (PERMANOVA) (A) between pairs of reactors and **(B)** between different runs within each reactor. Bold values indicate p <0.05.

(A)					
	FP	**TI**	**HT**	**VT**	**RP**
FP					
TI	**0.001**				
HT	**0.001**	**0.001**			
VT	**0.001**	**0.002**	0.177		
RP	**0.001**	**0.001**	**0.001**	**0.001**	
PP	**0.001**	**0.001**	**0.001**	**0.002**	**0.001**

(B)	
TI1-TI2	**0.007**
HT1-HT2	**0.001**
VT1-VT2	**0.001**
RP1-RP2	**0.04**
PP1-PP2	**0.001**

Table S4.3. Bacterial OTUs shared between samples of seawater and reactor samples.

OTUs	Bacterial taxonomy	FP	TI	HD	VD	RP	PP
889	*Actinobacteria; Actinobacteria; Corynebacteriales; Nocardiaceae; Rhodococcus*		1				
880	*Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; SV1-8*					1	
1040	*Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; g*						1
933	*Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; NS3a_marine_group*					1	
936	*Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; NS3a_marine_group*					1	
1162	*Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Ulvibacter*					1	
926	*Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Winogradskyella*					1	1
1295	*Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Saprospiraceae; g*		1	1			
1298	*Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Saprospiraceae; g*				1	1	
582	*Planctomycetes; Planctomycetacia; Planctomycetales; f; g*					1	
312	*Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae; g*				1		
18	*Proteobacteria; Alphaproteobacteria; DB1-14; f; g*				1		
34	*Proteobacteria; Alphaproteobacteria; OCS116_clade; f; g*					1	
86	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; g*			1	1	1	1
103	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; g*			1		1	1
125	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; g*						1
162	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; g*			1	1		
231	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Labrenzia*	1	1	1	1	1	
309	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Phaeobacter*			1			1
217	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseobacter_clade_DC5-80-3_lineage*					1	
145	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseobacter_clade_OCT_lineage*		1				
49	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseovarius*		1	1	1	1	1
109	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseovarius*		1	1	1		1
105	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Sulfitobacter*						1
122	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Sulfitobacter*						1
144	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Sulfitobacter*			1	1		1
28	*Proteobacteria; Alphaproteobacteria; Rhodospirillales; f; g*		1				
594	*Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Defluviicoccus*					1	
504	*Proteobacteria; Alphaproteobacteria; SAR11_clade; Surface_1; g*					1	
25	*Proteobacteria; Alphaproteobacteria; Sneathiellales; Sneathiellaceae; Sneathiella*		1		1	1	1
375	*Proteobacteria; Alphaproteobacteria; Sphingomonadales; Erythrobacteraceae; Erythrobacter*			1	1	1	
410	*Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingopyxis*		1	1	1		
411	*Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingopyxis*		1	1	1	1	1
721	*Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Neptuniibacter_*		1	1	1	1	1
787	*Proteobacteria; Gammaproteobacteria; Order_Incertae_Sedis; Family_Incertae_Sedis; g*		1				
	Sum	**1**	**12**	**13**	**14**	**19**	**14**
	Percentage of reads of OTUs shared with seawater	**0.9%**	**3.3%**	**8.9%**	**5.2%**	**11.4%**	**15.9%**

Chapter 5

Different co-occurring bacteria enhance or decrease the growth of the microalga *Nannochloropsis* sp. CCAP211/78

Jie Lian, Patrick Schimmel, Selene Sanchez-Garcia, Rene H. Wijffels, Hauke Smidt, Detmer Sipkema

Manuscript in preparation

Summary

Marine photosynthetic microalgae are ubiquitously associated with bacteria in nature. However, the influence of these bacteria on algal cultures in bioreactors is still largely unknown. In this study, eighteen different bacterial strains were isolated from cultures of *Nannochloropsis* sp. CCAP211/78 in two outdoor pilot-scale tubular photobioreactors. The majority of isolates was affiliated with the classes *Alphaproteobacteria* and *Flavobacteriia*. To assess the impact of the eighteen strains on the growth of *Nannochloropsis* sp. CCAP211/78, 24-well plates coupled with custom-made LED boxes were used to simultaneously compare replicate axenic microalgal cultures with addition of individual bacterial isolates. Co-culturing of *Nannochloropsis* sp. CCAP211/78 with these strains demonstrated distinct responses, which shows that the technique we developed is an efficient method for screening the influence of harmful/beneficial bacteria. One strain of *Maritalea porphyrae* (DMSP31) and one strain of *Labrenzia aggregata* (YP26) significantly enhanced microalgal growth with a 14% and 12% increase of the chlorophyll concentration, respectively, whereas flavobacterial strain YP206 greatly inhibited the growth of the microalga with 28% reduction of the chlorophyll concentration. Our study suggests that algal production systems represent a “natural” source to isolate and study microorganisms that can either benefit or harm algal cultures. Thus, the addition of specific bacteria may be used to maximise production of microalgae biomass.

Keywords: Algae-bacteria interaction, algal biotechnology, bacterial isolation, co-cultivation

5.1. Background

Microalgae show great potential in producing numerous sustainable bioproducts as alternatives to fossil feedstocks [56, 57, 311]. A long-neglected aspect in algal biomass production is the role of bacteria that are co-occurring in algae cultivation systems [29, 312]. Algal cultures are axenic in only a few applications, whereas all microalgae mass production systems inevitably contain a number of non-target organisms (contaminants), including bacteria [70, 266]. Bacteria are introduced in algae cultivation systems as algae stocks used as starter cultures are often not axenic [118, 312, 313]. On the other hand, bacterial contaminants may enter cultivation systems through multiple operation processes, such as the supplementation of unsterilized medium or simply as airborne invaders in open algal cultures.

Microalgae-bacteria interactions are prevalent in natural aquatic environments, where microalgae release exudates into the phycosphere, the region immediately surrounding individual cells. Chemotaxis drives multiple bacteria to the phycosphere [314], and metabolites are readily exchanged between algae and bacteria [2]. Although the phycosphere represents only a tiny area that can be as small as 1 μm surrounding the algal cell, it represents the hotspot for most of the algal-bacterial interactions that can profoundly affect the productivity and stability of aquatic ecosystems [2, 96].

Recent research on algal-bacterial interactions has usually been centred around the competitive or antagonistic aspects, which often involve competition for nutrients [277, 315, 316] or algicidal activities [2, 317]. For instance, in a microcosm experiment it was found that bacteria were more efficient than algae in the uptake of phosphorus [315]. The advantage for bacteria is especially evident under phosphorus-limiting conditions [318]. Apart from competing for nutrients with algae, some bacteria are known to inhibit algal cell division [319] or cause algal cell lysis via secretion of algicidal compounds [33, 35, 46].

In contrast to early views that bacteria mostly affect microalgae negatively, it has been demonstrated that mutualistic relationships between microalgae and bacteria are also prevalent, or even more common than antagonistic interactions [2, 71]. Proof has been found from frequent observations that the absence of bacteria in algal cultures negatively affects algal physiology and growth [221, 320]. In exchange for dissolved organic matter from microalgae, bacteria fix nitrogen [141, 321] and synthesize a wide range of molecules, including vitamins [22, 24], the growth-promoting hormone indole-3-acetic acid [16, 270] and the siderophore vibrioferrin [271, 322]. Such division of labour and close cooperation enable the holobiont to better adapt to and grow in changing aquatic environments, which has also triggered a growing interest for applications in industrial settings [323-325].

Contrary to extensive tests of effects of environmental and chemical factors (insolation, temperature, pH, nutrients, etc.) on algal growth in industrial photobioreactors, only a few studies have considered the effects of biotic factors such as associated bacteria. In order to assess the effects of co-occurring bacteria on microalgae in algal cultivation systems, we isolated and characterized bacteria from two pilot-scale outdoor tubular photobioreactors. Subsequently, a 24-well plate-based co-cultivation device was used to evaluate algal growth with addition of the isolated bacterial strains to axenic microalgae. Effects of bacteria on microalgae were further tested on a double-layer agar plate to verify algal-bacterial interactions.

5.2. Experimental Procedures

5.2.1 *Algal cultivation*

Nannochloropsis sp. CCAP211/78 cultures used for bacterial isolation were obtained from one horizontal and one vertical tubular photobioreactor at AlgaePARC, Wageningen, the Netherlands. *Nannochloropsis* sp. CCAP 211/78 was cultivated in seawater (Eastern Scheldt, the Netherlands) enriched with a nutrient stock solution resulting in the following final

concentrations (in mM); $NaNO_3$, 25; KH_2PO_4, 1.7; Na_2EDTA, 0.56; $Fe_2SO4{\cdot}7H_2O$, 0.11; $MnCl_2{\cdot}2H_2O$, 0.01; $ZnSO_4{\cdot}7H_2O$, $2.3{\cdot}10^{-3}$; $Co(NO_3)_2{\cdot}6H_2O$, $0.24{\cdot}10^{-3}$; $CuSO_4{\cdot}5H_2O$, $0.1{\cdot}10^{-3}$; $Na_2MoO_4{\cdot}2H_2O$, $1.1{\cdot}10^{-3}$. For the cultivation in outdoor photobioreactors, seawater was chemically sterilized by using sodium hypochlorite. Active chlorite was deactivated by filtration over activated carbon, followed by filtration across a filter with a pore size of 1 µm. Subsequently, the nutrient stock solution was added through a sterile filter (0.45 µm). The detailed description of the cultivation process was given by de Vree, Bosma [265].

For the co-cultivation experiment, the non-axenic pre-cultures of *Nannochloropsis* sp. CCAP211/78 (100 mL liquid volume in 250 mL Erlenmeyer flasks) were maintained in autoclaved seawater supplemented with HEPES (20 mM) and Na_2EDTA (5 mM). The nutrient stock solution with the same final concentrations as above was added to the autoclaved seawater through a syringe filter (0.2 µm). We refer to this medium as enriched seawater medium (ESW medium). The Erlenmeyer flask cultures of *Nannochloropsis* were capped with aeraseal sterile film (Alphalabs) and placed in an orbital shaker incubator (Sanyo), shaken at 120 rpm, illuminated with continuous light of 50 µmol photons m^{-2} s^{-1} at 25 °C, and the headspace was enriched with 2 % CO_2.

5.2.2 *Generation of axenic algal cultures*

Axenic cultures of *Nannochloropsis* sp. CCAP211/78 were prepared using a treatment with an antibiotics cocktail consisting of Streptomycin (50 µg/mL), Gentamycin (67 µg/mL), Ciprofloxacin (20 µg/mL), Ampicillin (100 µg/mL) and Chloramphenicol (2.2 µg/mL). Specifically, 2 mL exponentially growing non-axenic *Nannochloropsis* sp. CCAP211/78 were taken from a 250 mL flask, washed twice in 2 mL sterile ESW medium and concentrated by centrifugation at 8000 g for 3 min and transferred into a six-well microplate. After adding the abovementioned antibiotics cocktail, the plate was incubated at 25 °C and illuminated with a

16/8 h light/dark cycle with a light intensity of 50 µmol photons m^{-2} s^{-1}. Every two days, the cultures in the well plate were washed as mentioned before, fresh antibiotics solution was added and the cultures were incubated as described above. This procedure was repeated four more times.

Axenicity of *Nannochloropsis* sp. CCAP211/78 was confirmed via inoculating ESW-YP agar (sterile ESW supplemented with 1 g/L of yeast extract, 1 g/L of peptone and 15 g/L of agar) with 100 µL of antibiotics-treated algal cultures. Furthermore, algal cultures were incubated for 10 min with 1 µg/ml 4',6-diamidin-2-phenylindol (DAPI, dissolved in phosphate buffer). Stained samples were inspected with a fluorescence microscope (Olympus). The obtained axenic *Nannochloropsis* sp. culture was maintained in 250 mL flasks in the orbital shaker incubator (Sanyo) according to the same method as described for the non-axenic culture.

5.2.3 *DNA isolation and 16S rRNA gene profiling of bacteria*

Four samples of 5 mL from two outdoor photobioreactors were vacuum-filtered onto a cellulose nitrate membrane filter (0.2µm, Millipore). To isolate the genomic DNA, filters were cut in half using sterile scissors and DNA was extracted from half a filter using the FastDNA SPIN kit for soil (MP Biomedicals) with the aid of a Precellys bead beater (Bertin Technologies) with two rounds of bead beating for 45 s at speed of 5500 rpm.

Amplicons of the 16S ribosomal RNA (rRNA) gene were generated from the extracted DNA with a two-step PCR reaction carried out in a BIOKÉ SensoQuest Labcycler 48. During the first step of 16S rRNA gene PCR, a gene amplicon of approximately 311 bp comprising the V1 and V2 regions was generated using degenerate primers 27F-DegS [326] and a mixture of 338R-I and 338R-II [327]. The forward primer was used with Unitag1 attached to the 5' end of the primer and Unitag2 was attached to the 5' end of the reverse primer to facilitate the second step of the PCR (Supplementary Table S5.1). The first PCR reaction (50 µl) contained 10 µl 5×

HF buffer (Thermo Scientific, the Netherlands), 1 µl dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 500 nM of Unitag1-27F-DegS forward primer, 500 nM of Unitag2-338R I and II reverse primer and 1 µl template DNA. The PCR was performed using the following conditions: an initial denaturation at 98°C for 30 s, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 20 s, elongation at 72°C for 20 s, and a final extension at 72°C for 10 min. Subsequently, the first PCR product was used as template in a second PCR in order to add sample-specific barcodes (8 nucleotides). The second PCR reaction (100 µl) contained 20 µl 5× HF buffer, 2 µl dNTP Mix, 2 U of Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 500 nM of a forward and reverse primer equivalent to the Unitag1 and Unitag2 sequences, respectively, that were each appended with an 8 nt sample specific barcode (Table S5.1) [328]. The second PCR was performed using the following conditions: an initial denaturation at 98°C for 30 s, followed by 5 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 20 s, elongation at 72°C for 20 s, and a final extension at 72°C for 10 min. The barcoded PCR products from the second PCR were purified using the High-Prep PCR purification kit (MOBIO) according to the manufacturer's protocols. The concentration of purified DNA was measured using a Qubit 2.0 Fluorometer, according to manufacturer's instructions for the Qubit dsDNA BR assay (INVITROGEN). Then, the second step PCR products were pooled in an equimolar concentration and again purified using the High Prep PCR purification Kit. The purified PCR products (final concentration: 200 ng/µl) were sequenced at GATC Biotech Europe (Konstanz, Germany, now part of Eurofins Genomics Germany GmbH) using the Illumina MiSeq Genome Sequencer platform. Nucleotide sequences of all samples were deposited at NCBI GenBank under Bio Project ID number PRJNA488170 with accession number: SRR7760408.

Illumina sequencing data was processed and analyzed using the NG-Tax pipeline [329] as previously described by Dat *et al*. [330]. Briefly, paired-end libraries were combined, and only read pairs with matching primers and barcodes were retained. Both forward and reverse reads were trimmed to 100 bp and concatenated to yield sequences of 200 bp that were used for subsequent sequence data processing. Demultiplexing, OTU picking, chimera removal and taxonomic assignment were performed within one single step. Reads were ranked per sample by abundance, and sequences (at a 100% identity level) were added to an initial OTU table starting from the most abundant sequence until the abundance was lower than 0.1% per sample. The final OTU table was created by clustering the reads that were initially discarded (as they represented OTUs <0.1% of the relative abundance) with the OTUs from the initial OTU table allowing a single mismatch. Taxonomic assignment was done utilizing the UCLUST algorithm algorithm [331] and the SILVA 111_SSU Ref database [332, 333].

5.2.4 *Bacterial isolation and identification*

Cryopreserved (15% glycerol) algal cultures from two outdoor reactors (horizontal tubular bioreactor and vertical tubular bioreactor) at AlgaePARC and stored at -80 °C were used as inoculum for bacterial isolation. The description of bioreactors and algal cultivation process was given by de Vree, Bosma [265]. Aliquots of cryopreserved cultures were diluted (10^4 fold) and plated on ESW agar (1.5% agar) supplemented with one of the following carbon sources: 2 g/L glucose (ESW-GLU); 2 g/L propionate (ESW-PRO); 2 g/L casamino acids (ESW-CAS); 2 g/L *Nannochloropsis* extract (ESW-ALG; 2 g freeze-dried *Nannochloropsis* cells from AlgaePARC suspended in 10 mL ESW, French-pressed twice at 110 MPa and centrifuged at 8000 g for 5 min. Subsequently, all the resulting supernatant was filter-sterilised (0.2 μm) and added to 1 L of autoclaved ESW medium); 2 g/L succinate (ESW-SUC); 1 g/L yeast extract and 1 g/L peptone (ESW-YP); 2 g/L palmitate (ESW-PAL) or 0.6 mM dimethylsulfoniopropionate (ChemCruz, Dallas, TX) (ESW-DMSP). Plates were maintained in

the dark at room temperature. Single colonies were picked and streaked until pure cultures were obtained. All the pure bacterial strains were maintained in ESW-YP medium. For bacterial identification, the 16S rRNA gene was amplified with universal primers 27F and 1492R (Table S5.1). The PCR reaction (50 µl) contained 10 µl 5× HF buffer (Thermo Scientific), 1 µl dNTP Mix (10 mM; Promega), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 1 µM of 27F primer, 1 µM of 1492R primer and 1 µl bacterial culture. The PCR protocol consisted of a pre-denaturation step (10 min at 98 °C) followed by 30 cycles of denaturation (30 s at 98 °C), annealing (40 s at 60 °C) and elongation steps (1.5 min at 72 °C) with a final elongation step at 72°C for 10 min. The purified amplicons (High-Prep PCR purification kit, MOBIO) were Sanger sequenced with primer 806R (Table S5.1) by GATC Biotech Europe (Konstanz, Germany). The sequences were clustered into contigs with a cut off of 99% similarity using ContigExpress (Invitrogen). One representative isolate from each contig was selected (Table 5.1) and analysed with the BLASTn tool from NCBI (http://www.ncbi.nlm.nih.gov/) to determine its phylogenetic affiliation. All sequences of selected isolates were deposited at NCBI GenBank under accession numbers as listed in Table 5.1.

5.2.5 *Co-culturing of algae with bacteria in microplates*

For co-cultivation in 24-well microplates, both exponentially growing axenic and non-axenic cultures of *Nannochloropsis* sp. CCAP211/78 grown in flasks were diluted with ESW medium supplemented with 5 mM $NaHCO_3$ to a fluorescence intensity of ~5000 (Excitation: 450 nm, Emission: 685 nm). Single bacterial strains, separately grown in ESW-YP broth, were washed twice in sterile ESW medium and concentrated by centrifugation (8000 g for 5 min), then re-suspended in ESW medium and diluted to an OD600 of 0.2. Subsequently, 1 mL of axenic *Nannochloropsis* and 50 µL of bacterial strain suspension were inoculated in 24-well plates. The control cultures (either 1 mL of axenic *Nannochloropsis* sp. CCAP211/78 or 1 mL of non-

axenic *Nannochloropsis* sp. CCAP211/78) were supplemented with 50 µL ESW medium instead of diluted bacteria. All treatments included three replicates and were randomly allocated into different wells of the microplate. The microplates were incubated in a custom-made LED box with one LED for each well and continuously illuminated at a light intensity of 71.1 ± 6.2 µmol photons m^{-2} s^{-1} (Figure 5.1). The LED box was then placed in a shaking incubator (Innova, New Brunswick), agitated at 180 rpm/min and incubated at a temperature of 23 ± 1°C. Fluorescence intensity (Excitation: 450 nm, Emission: 685 nm) of co-cultures was measured at the same time every day with a plate reader in the endpoint mode (BioTek Synergy). Fluorescence intensity was measured from the bottom at 8 mm read height and 100 ms delays after plate movement. We determined that the relative fluorescence intensity was linearly correlated to cell counts of *Nannochloropsis* (Beckman-Coulter, Multisizer3) (Pearson's r = 0.98, $p < 0.0001$) (Figure S5.1). Data from different treatments was compared using a t-test and the *p* value was adjusted with the "Holm" method [334].

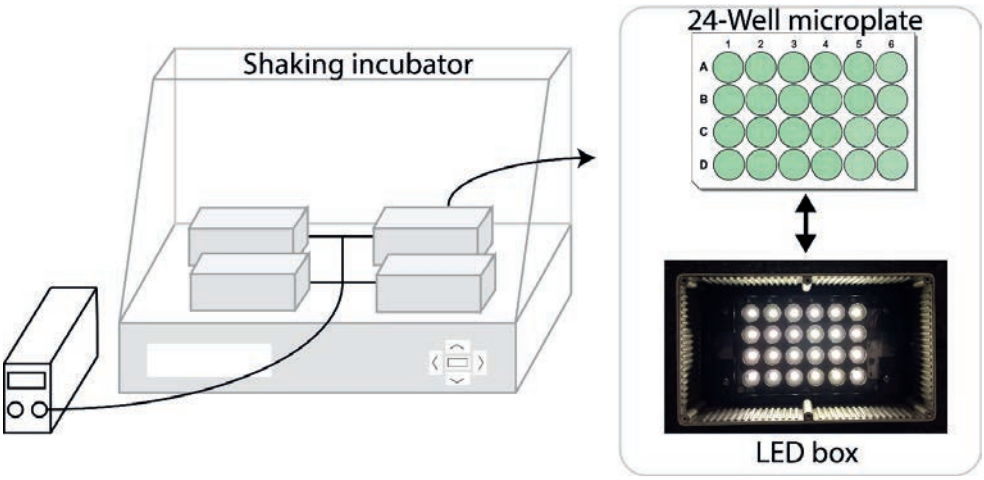


Figure 5.1. Diagram illustrating the 24-well plate coupled with LED box for co-cultivation in a shaking incubator.

5.2.6 *Co-culturing of algae with bacteria on agar plates*

For mixed cultivation of algae and bacteria on agar plates, an ESW plate (2% agar, diameter Petri dish 94 mm diameter) was overlaid with 5 mL 0.5% ESW agar (top agar) containing axenic *Nannochloropsis* cells (~10^5 mL^{-1}). After solidification of the top agar, 15 µL of bacterial culture was dropped onto the surface of the top agar. The plates were incubated for one week at a light intensity of 50 µmol photons m^{-2} s^{-1} with a 16:8 h light/dark cycle at 25 °C.

5.3. Results

5.3.1 Bacterial isolation and identification

Eighteen bacterial strains were isolated from four outdoor photobioreactor cultures of *Nannochloropsis* sp. CCAP211/78, of which three were obtained from a horizontal tubular photobioreactor and one from a vertical tubular photobioreactor, respectively, at AlgaePARC. Of the 18 isolates, 11 belong to the class *Alphaproteobacteria* and five to the *Flavobacteriia*. In addition, single isolates were obtained from the classes *Cytophagia* and *Saprospiria* (Table 5.1). At the family level, isolates were mainly classified into three families: *Hyphomicrobiaceae*, *Rhodobacteraceae* and *Flavobacteriaceae*. When Sanger-sequenced 16S rRNA genes of the bacterial strains were compared to the 138 operational taxonomic units (OTUs) present in the four original bioreactor cultures, 14 out of 18 bacterial strains had an identical match with OTUs encountered in the reactors, while four isolates had not (Table 5.1).

Table 5.1. Bacterial strains isolated from *Nannochloropsis* cultures.

Strain	Accession Number (bacterial isolate)	Class	Family	Blast result*	Identity [%]	Accession Number (Genbank best hit)	OTUs in bioreactors**	Identity [%]
GLU107	MH843917	*Alphaproteobacteria*	*Erythrobacteraceae*	*Porphyrobacter sanguineus*	100	LC349792	OTU247	100
PRO103	MH843918	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Algimonas arctica*	98	NR_137369	OTU321	100
DMSP31	MH843919	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Maritalea porphyrae*	99	AB583776	OTU327	100
DMSP20	MH843920	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Maritalea* sp.	99	AB758563	OTU331	100
PRO34	MH843921	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Maritalea* sp.	96	KP301112	OTU343	100

YP210	MH843922	*Alphaproteobacteria*	*Phyllobacteriaceae*	*Pseudohoeflea suaedae*	100	LT600545	OTU490	100
YP18	MH843923	*Alphaproteobacteria*	*Rhodobacteraceae*	*Celeribacter* sp.	100	MF045112	OTU582	100
YP26	MH843924	*Alphaproteobacteria*	*Rhodobacteraceae*	*Labrenzia aggregata*	100	MG273739	OTU247	100
YP29	MH843925	*Alphaproteobacteria*	*Rhodobacteraceae*	*Roseovarius mucosus*	99	CP020474	OTU585/709	100
YP202	MH843926	*Alphaproteobacteria*	*Rhodobacteraceae*	*Sulfitobacter* sp.	99	KY272045	OTU143/289	100
PAL103	MH843927	*Alphaproteobacteria*	*Sphingomonadaceae*	*Sphingorhabdus* sp.	99	KT325114	OTU259	98
DMSP2-Y	MH843928	*Cytophagia*	*Cytophagaceae*	*Emticicia* sp.	99	KP265953	OTU574	100
YP206	MH843929	*Flavobacteriia*	*Flavobacteriaceae*	*Aquaticitalea lipolytica*	99	NR_149769	OTU532/533	94
ALG110	MH843930	*Flavobacteriia*	*Flavobacteriaceae*	*Arenibacter* sp.	98	JX529985	OTU582	100
PAL10	MH843931	*Flavobacteriia*	*Flavobacteriaceae*	*Cellulophaga lytica*	100	MG456766	OTU519	96
PAL110	MH843932	*Flavobacteriia*	*Flavobacteriaceae*	*Maribacter* sp.	99	KT731371	OTU525	96
SUC105	MH843933	*Flavobacteriia*	*Flavobacteriaceae*	*Muricauda* sp.	99	KJ188010	OTU512	100
PRO13	MH843934	*Saprospiria*	*Saprospiraceae*	*Phaeodactylibacter xiamenensis*	99	NR_134132	OTU579	100

*The best hit (highest percent identity) in Genbank.

**The best hit of photobioreactor OTUs.

The cultivable bacteria isolated in this study accounted for approximately 11% of the total OTUs (14 of 124) present in the original photobioreactor samples, which represented nearly 7% of the total number of reads (11,820 of 152,260) in the bioreactor samples. Thus, a substantial fraction of bacteria in algal cultures remained uncultured. We observed 16 OTUs with high relative abundance (≥5%) in our algal cultures (Table S5.2), of which four (OTU533, 579, 327, 331) were successfully cultured. It is noticeable that although *Gammaproteobacteria* was one of the most abundant classes in two of four bioreactor cultures based on cultivation-independent assessment of bacterial diversity, no strains belonging to this class were recovered (Table S5.2 and Figure S5.2).

5.3.2. Effect of bacterial isolates on the growth of algae

To examine potential interactions between *Nannochloropsis* and the bacterial isolates, the bacterial isolates were re-introduced to axenic microalgae. All the cultures except the ones supplemented with strain YP206 had a similar growth pattern, that is, after rapid growth for nearly five days the stationary phase was reached, which continued until the end of the experiment at day 11 (Figure S5.3). No significant difference was found in relative fluorescence

between axenic and non-axenic control cultures of *Nannochloropsis*. Addition of bacteria to the axenic *Nannochloropsis* sp. cultures mostly resulted in a slight decrease of the maximal fluorescent intensity reached at the stationary phase (Figure 5.2). For strain YP206 (*Flavobacteriia*), *Nannochloropsis* growth was strongly inhibited, leading to a reduction of more than 28% in fluorescence intensity. In contrast, two bacterial strains (DMSP31, YP26) resulted in significantly better growth of the algae and the intensity of algal fluorescence increased by 12 – 14% compared to the axenic control (Figure 5.2).

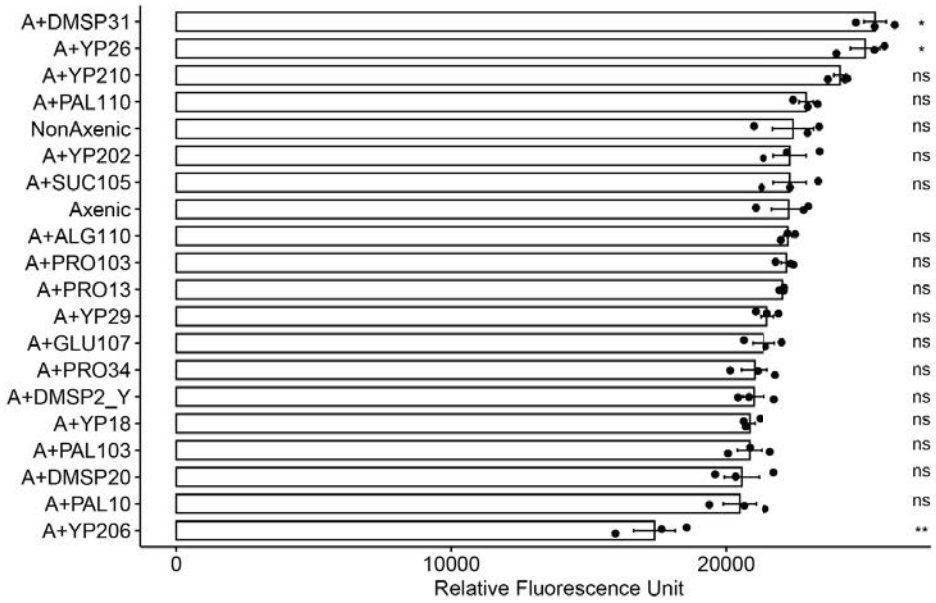


Figure 5.2. Relative Fluorescence (~ algal biomass) of *Nannochloropsis* sp. CCAP211/78 co-cultured with individual bacterial strains. Relative Fluorescence Unit (RFU) for *Nannochloropsis* sp. CCAP211/78 was calculated as maximal fluorescent intensity and compared to RFU of the axenic culture. Error bars represent standard deviation. Results of the statistical analysis are indicated by NS (*p*.adjust > 0.05), * (*p*.adjust <=0.05), and ** (*p*.adjust <= 0.01), respectively. The statistical results of pair-wise comparison against non-axenic culture (not shown) are the same as for the comparison to the axenic culture.

To further confirm the effect of the addition of bacteria on algal growth we observed in liquid cultures we added individual fresh cultures of the bacterial strains to the top-agar that contained

axenic *Nannochloropsis* cells on double-layer agar plates. When co-cultured with YP26, growth of *Nannochloropsis* was strongly stimulated, indicated by the greenest algal lawns after one-week of incubation (Figure 5.3). Interestingly, two other bacteria (ALG110, PRO34) slightly enhanced the algal growth on solid medium (Figure 5.3), whereas this beneficial effect was absent in liquid culture. Two agar-degrading bacteria (PAL10 and PAL110) formed much bigger colonies than other strains, and thus promoted the algal growth on a much bigger surface on the solid medium (Figure 5.3).

Figure 5.3. Co-cultivation of *Nannochloropsis* sp. CCAP211/78 and bacterial strains on double-layer agar plates after seven days. ESW (Enriched natural seawater medium) and ESW-YP (ESW medium with peptone and yeast extract) were used as controls. The labelled names referred to the added bacteria. YP26 was added on three different plates as replicates indicated by arrows.

5.4. Discussion

Bacterial isolation was carried out from four samples taken from two outdoor photobioreactors of AlgaePARC where bacterial strains were present in microalgae cultures that were not

supplemented with external organic carbon. Therefore, we suspected that heterotrophic co-occurring bacteria depended on the organic carbon released by microalgae, which suggests at least a commensal relationship between *Nannochloropsis* and bacteria. In total, 18 bacterial strains were isolated from the outdoor photobioreactor samples. These 18 isolates constituted approximately ten percent of the whole bacterial community, whereas nearly 90% remained uncultured under the conditions applied in our experiment. Hence it cannot be excluded that even more potent candidates with respect to an algae growth-promoting effect remain to be discovered from the yet uncultured fraction. The bacteria in our culture collection were mostly classified as *Alphaproteobacteria* and *Flavobacteriia* (Figure S5.2). It has also been corroborated by global surveys that phytoplankton-associated bacterial communities are often restricted to only a few bacterial classes including *Alphaproteobacteria* (*Rhodobacteraceae*), *Gammaproteobacteria* (*Alteromonadaceae*) and *Flavobacteriia* (*Flavobacteraceae*) [96, 304, 335, 336]. These apparently widespread patterns imply that the lifestyle of some bacteria within these groups is substantially related to that of algae. Likewise algae can either benefit or suffer from the bacterial partners [2]. Bacteria reported to be beneficial to microalgae are mostly *Alphaproteobacteria*, and algal growth-promoting bacteria belonging to *Alphaproteobacteria* are quite diverse, including members of *Rhizobium* [105], *Brevundimonas* [121], *Mesorhizobium loti* [22], and *Hyphomonas* [122]. Within *Alphaproteobacteria*, bacteria from the family *Rhodobacteraceae* are frequently associated with algae, of which the most studied ones are *Phaeobacter gallaeciensis* [46], *Dinoroseobacter shibae* [337], *Sulfitobacter* sp. [16] and *Ruegeria pomeroyi* [17].

Labrenzia aggregata (YP26) was the only isolate in our experiment that exhibited significant growth enhancement both in liquid and solid media (Figure 5.2, Figure 5.3 and Figure S5.3). Members of the genus *Labrenzia* have been isolated from a wide range of habitats and found to be frequently associated with other marine organisms [338]. These organisms include

invertebrates such as molluscs, corals and sponges, and a wide variety of photosynthetic partners including seaweeds, diatoms, dinoflagellates, green and red algae [339]. *Labrenzia aggregata* has also been isolated previously from *Nannochloropsis oculata* and *Nannochloropsis gaditana* [340]. A recent study revealed that *Labrenzia* sp. increased the biomass accumulation of the marine microalga *Isochrysis galbana* by 72% and the growth rate by 18% [341]. On the other hand, it has been reported that a bacterial isolate (KD531) with 100% similarity to the partial 16S rRNA gene of our *Labrenzia aggregata* isolate had an algicidal effect on *Chlorella vulgaris* [342]. The addition of bacterial lysate of KD531 to *Chlorella vulgaris* cultures caused nearly 20% reduction in biomass dry weight and nearly 60% reduction in lipid content. The contradiction between these and our observations may be due to strain-specific differences between isolates of *L. aggregata,* and/or different interactions of the bacterium with different algal hosts. Prior research has shown that some bacteria that are mutualistic to their native algal partner can be parasitic to foreign algae, which hints at co-adaption and evolution of algae and their associated microbiome [119]. In addition, we added live bacteria rather than a bacterial lysate, which may lead to a different effect. *Nannochloropsis* sp. also appeared to grow faster and denser on a solid growth medium in the presence of *Labrenzia aggregata*. To our knowledge, this is the first time that one bacterium has been shown to strongly promote the growth of microalgae on solid agar. Agar plates have been the most commonly used method to study algal-bacterial interactions [27, 34]. For example, the vitamin B_{12}-dependent microalga *Lobomonas rostrata* could grow on agar plates only when vitamin B_{12} or a vitamin B_{12}-synthesizing bacterium (*Mesorhizobium loti*) was added [27]. Therefore, it is tempting to speculate that the growth promotion observed here for *Labrenzia aggregata* could be related to inorganic nutrient exchange or algal acquisition of growth factors released by bacteria.

Although the growth increase of *Nannochloropsis* in the presence of *Maritalea porphyrae* (DMSP31) was significant in liquid cultures, this beneficial effect disappeared on the agar plate. This discrepancy between two screening methods corroborated that algae-bacteria interactions are complex and may vary under different culture conditions. Therefore, preliminary screening results should be confirmed by other methods such as flask cultures or bioreactors before claims regarding beneficial effects of bacteria on large-scale algal growth can be made. It is interesting to note that *Maritalea porphyrae* (DMSP31) and *Pseudohoeflea suaedae* (YP210), the latter of which caused a numerical, but non-significant increase in algal growth in the plate assay, have been previously isolated from the thalli of the red alga *Pyropia yezoensis* [343] and root of the halophyte *Suaeda maritima* [344], respectively. However, experimental evidence showed that these bacteria exhibited no apparent morphogenesis effects on the red alga [345], and therefore the nature of a symbiotic relationship -if any- with the phototroph remains unknown. The genus *Pseudohoeflea* (reclassification of *Hoeflea*) to which YP210 belongs, has not yet been well characterized [346], however, it has been speculated that the genus *Pseudohoeflea* may resemble some members of the marine *Roseobacter* clade in phytoplankton colonization by the production of secondary metabolites that inhibit the growth of competing bacteria (antibiotics) and promote growth of phytoplankton (auxins) [46, 346].

Three out of five *Flavobacteriia* strains (ALG110, SUC105, PAL110) showed no significant effect on the growth of *Nannochloropsis* cultures, despite the fact that members of the *Flavobacteriia* have repeatedly been reported to have antagonistic relationships with algae. For instance, *Kordia algicida* was shown to excrete an extracellular protease to lyse algal cells to acquire their dissolved organic carbon [317], and *Croceibacter atlanticus* was observed to release an unidentified molecule to arrest diatom cell division and increase secretion of organic carbon [319]. On the other hand, the greatest reduction in chlorophyll content in the stationary phase compared to the controls was observed in the presence of strain YP206 from the

Flavobacteriaceae family (Table 5.1 and Figure 5.2). The closest relative of strain YP206 is *Aquaticitalea lipolytica* (99% identity of the 16S rRNA gene) that was isolated from Antarctic seawater and known to hydrolyse lipids [347]. However, when YP206 was co-cultured on agar plates with *Nannochloropsis*, the growth inhibition observed in liquid culture was not observed (Figure 5.2 and Figure 5.3). Although mechanistic insight requires future research, one can speculate that the incubation time (seven days) used in the agar plate experiments described here was too short or that the algal density was still too low on the agar plate for the bacterial inhibition to take place, as some algicidal bacteria have been shown to only kill senesced algal cells in the stationary phase or decline phase [46, 337]. This has previously been explained by competition for limiting nutrients such as nitrogen [348] and phosphorus [315, 349]. However, that is not likely to be the case for our experiments as nitrogen and phosphorus concentrations added would support much higher algae concentrations than those present in the stationary phase, and for nitrogen it was confirmed in the stationary phase that it was not depleted (data not shown). Alternatively, release of toxic compounds by bacteria could contribute to the inhibitory effects observed at stationary phase [350, 351]. Many bacteria belonging to the family *Flavobacteriaceae* are able to glide on solid surfaces and decompose agar [352]. PAL10 and PAL110 displayed these features and formed larger and concave colonies on the agar surface (Figure 5.3). Although both strains showed no significant effects on algal growth in liquid co-culture, they slightly enhanced the growth of *Nannochloropsis* sp. in the agar-plate assay (Figure 5.3). A possible explanation for the growth promotion on solid media could be that *Nannochloropsis* cells consumed the by-products from the agar degradation by the bacteria. For instance, *Cellulophaga lytica* has previously been shown to synthesize different kinds of agarases [353], and the enzymatic hydrolysis of agar yields monomeric sugars, such as D-galactose, 3,6-anhydro-L-galactose, and L-galactose-6-sulfate [354]. Research has shown that

supplementation with galactose increases the growth rate of *Nannochloropsis salina* by nearly 10 % [355].

PRO13 (*Saprospiria*) and DMSP2-Y (*Cytophagia*) were the only two isolates not belonging to *Alphaproteobacteria* or *Flavobacteriia*. Both PRO13 and DMSP2-Y had no significant effect on the growth of *Nannochloropsis* sp. A previous study has found that the family *Saprospiraceae* was the most prevalent taxon, and also the most abundant one in industrial cultures of *Nannochloropsis salina* [69]. The 16S rRNA gene of strain PRO13 was identical to OTU579 found in the outdoor photobioreactors, particularly in sample HD0105 where this bacterium made up nearly 25% of the whole bacterial community (Table S5.2). In spite of this strikingly high relative abundance, co-culturing with strain PRO13 had no significant effect on the growth of *Nannochloropsis* sp. neither in liquid co-cultures nor on agar plates (Figure 5.2 and Figure 5.3). Similarly, the study by Fulbright, Robbins-Pianka [69] reported that there was no correlation between the abundance of *Saprospiraceae* and growth of *N. salina*. However, the prevalence of this bacterium suggests it may have other functions in algal cultures, and the lytic capability of members of this bacterial family may relate to degrading cell debris for nutrient recycling [69]. DMSP2-Y is closely related to *Emticicia* sp., and species from the genus *Emticicia* have been recorded to live with *Chlorella vulgaris* [248] and the macroalga *Cladophora glomerata* [287]. *Emticicia* sp. was found to slightly reduce the growth rate of axenic *Chlorella vulgaris* in co-cultivation, but the co-culture revealed prolonged stationary phase [356].

5.5. Conclusion

In this study, we isolated 18 bacterial strains from two outdoor photobioreactors for cultivation of microalgae. Two strains assigned to *Maritalea porphyrae* and *Labrenzia aggregata,* respectively, significantly promoted growth of *Nannochloropsis* sp. CCAP211/78 in liquid

cultures in well plates (14% and 12% increase of chlorophyll in stationary phase compared to the controls, respectively), and the *Labrenzia aggregata* strain also notably increased growth of the alga on agar plates. In addition, one strain most closely related to *Aquaticitalea lipolytica* significantly reduced the chlorophyll content with 28% compared to the axenic and non-axenic controls. Our results suggest that some bacteria from algal production systems may have a pronounced impact on algal growth under controlled laboratory conditions, an effect that should be verified for larger-scale algae cultures. Our results indicated that in the practice of improving the production of microalgae, the bacterial community in algal inocula should be considered. If harmful bacteria are present, the inoculum should be replaced by an inoculum where these bacteria are absent to increase the cultivation success. Perhaps even more interesting, beneficial bacterial strains may be supplemented as a new means to improve algal productivity and culture stability.

Acknowledgements

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Supplementary Information

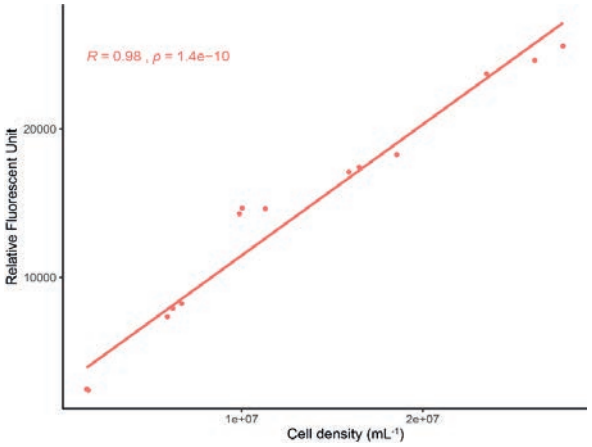

Figure S5.1. Linear correlation between the cell density of *Nannochloropsis* sp. CCAP211/78 as determined by direct cell counting and the corresponding relative fluorescence. Samples shown here were taken from a pilot experiment that was carried out in a microplate under the same conditions as for the co-cultivation experiments.

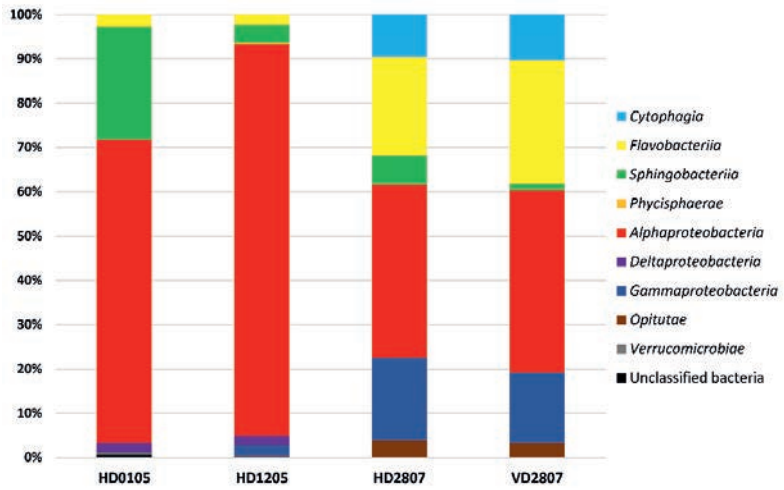

Figure S5.2. Relative abundance of bacteria (class level) in samples from different photobioreactors. HD and VD refer to horizontal tubular bioreactor and vertical tubular bioreactor, respectively. The number in the sample name refers to the sampling time point (day-month) in 2015. The total number of bacterial 16S rRNA gene reads for each sample was HD0105 (16,033), HD1205 (132,458), HD2807 (9,672) and VD2807 (4,762), respectively.

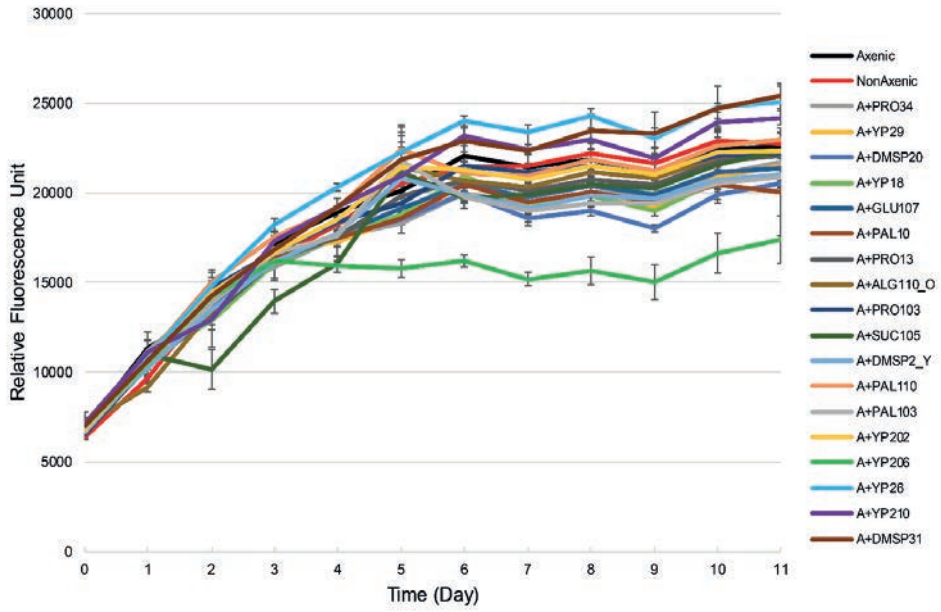

Figure S5.3. Co-culturing of *Nannochloropsis* sp. CCAP211/78 with individual bacterial strains. For each group, relative fluorescence was calculated as the mean of triplicate samples.

Table S5.1. Oligonucleotides used in this study.

Primer Name	Oligonucleotide sequence (5´-3´)	Reference
27F-DegS-Fwd	GTTYGATYMTGGCTCAG	[229]
338R-I-rev	GCWGCCTCCCGTAGGAGT	[230]
338R-II-rev	GCWGCCACCCGTAGGTGT	[230]
Unitag1	GAGCCGTAGCCAGTCTGC	[231]
Unitag2	GCCGTGACCGTGACATCG	[231]
27F	AGAGTTTGATCMTGGCTCAG	[357]
1492R	CGGYTACCTTGTTACGACTT	[357]
806R	GGACTACNVGGGTWTCTAAT	[358]

Table S5.2. Composition of OTUs in four outdoor photobioreactor cultures of *Nannochloropsis*. The OTUs highlighted in green are identical to 16S rRNA gene sequences from bacterial isolates, the ones highlighted in yellow are the closest match (94% ~ 98% identity) to the corresponding 16S rRNA gene sequence of bacterial isolates. OTUs with relative abundance of more than 5 % in a reactor sample are highlighted in red.

OTU ID	HD0105 (%)	HD1205 (%)	HD2807 (%)	VD2807 (%)	Taxonomy
539	0.0	0.0	1.7	2.3	*Bacteroidetes;Cytophagia;Cytophagales*;f;g
540	0.0	0.0	2.1	3.3	*Bacteroidetes;Cytophagia;Cytophagales*;f;g
581	0.0	0.0	2.0	2.6	*Bacteroidetes;Cytophagia;Cytophagales*;f;g
537	0.0	0.0	3.6	2.0	*Bacteroidetes;Cytophagia;Cytophagales;Flammeovirgaceae*;g
525	0.0	0.8	0.0	0.0	*Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae;Fluviicola*
494	0.0	0.0	0.0	0.5	*Bacteroidetes;Flavobacteria;Flavobacteriales*;f;g
497	0.0	0.0	2.3	3.2	*Bacteroidetes;Flavobacteria;Flavobacteriales*;f;g
498	0.0	0.0	0.0	1.0	*Bacteroidetes;Flavobacteria;Flavobacteriales*;f;g
499	0.0	0.0	5.5	10.2	*Bacteroidetes;Flavobacteria;Flavobacteriales*;f;g
509	0.5	0.0	0.0	0.0	*Bacteroidetes;Flavobacteria;Flavobacteriales*;f;g
519	0.0	0.0	1.4	1.4	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Arenibacter*
532	0.0	0.0	0.7	0.8	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae*;g
533	2.2	0.0	6.4	6.7	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae*;g
570	0.0	0.8	0.0	0.0	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Lutibacter*
512	0.0	0.4	0.8	0.0	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Muricauda*
520	0.0	0.0	4.3	3.7	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Muricauda*
531	0.0	0.4	0.0	0.0	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Muricauda*
560	0.0	0.0	0.6	0.0	*Bacteroidetes;Flavobacteriia;Flavobacteriales;Flavobacteriaceae;Tenacibaculum*
577	0.0	1.2	0.0	0.0	*Bacteroidetes;Sphingobacteriia;Sphingobacteriales*;f;g
579	24.7	0.0	6.4	1.4	*Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Saprospiraceae*;g
580	0.4	2.3	0.0	0.0	*Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Saprospiraceae*;g
578	0.4	0.5	0.0	0.0	*Bacteroidetes;Sphingobacteriia;Sphingobacteriales;Saprospiraceae;Lewinella*
712	0.6	0.0	0.0	0.0	BD1-5;c;o;f;g
482	0.0	0.4	0.0	0.0	*Planctomycetes;Phycisphaerae;Phycisphaerales;Phycisphaeraceae;SM1A02*
259	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae*;g
307	1.9	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae*;g
321	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae;Hellea*
260	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae;Hyphomonas*
109	0.0	11.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
110	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
111	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
112	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
113	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
583	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
592	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;DB1-14*;f;g
125	1.1	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;OCS116_clade*;f;g
279	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;OCS116_clade*;f;g
489	0.0	0.0	0.0	0.4	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Aurantimonadaceae;Martelella*
255	0.0	0.0	1.3	3.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;Devosia*
277	0.0	0.0	0.0	0.6	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;Devosia*

330	1.0	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;Maritalea*
271	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae*;g
603	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae*;g
265	3.7	7.9	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Hoeflea*
267	0.0	0.7	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Hoeflea*
272	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Hoeflea*
273	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Hoeflea*
490	0.0	0.0	0.5	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Hoeflea*
591	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Hoeflea*
299	0.0	0.0	0.7	0.0	*Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Nitratireductor*
266	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales*;f;g
327	6.5	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales*;f;g
331	5.2	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales*;f;g
343	0.6	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales*;f;g
605	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales*;f;g
606	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales*;f;g
41	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
65	1.8	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
81	20.9	0.0	0.0	4.1	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
143	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
144	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
145	0.0	4.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
146	0.0	0.7	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
147	2.5	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
158	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
161	1.5	4.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
162	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
168	0.8	0.0	6.4	3.4	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
172	0.0	0.9	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
205	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
206	0.0	0.7	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
208	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
211	0.0	11.9	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
212	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
213	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
214	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
290	0.0	0.0	2.7	12.6	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
582	0.9	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
586	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
587	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
709	0.0	0.0	0.6	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae*;g
247	1.6	0.0	0.6	1.8	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Labrenzia*
51	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Loktanella*
141	0.0	0.0	0.9	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Maritimibacter*
151	2.5	0.0	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Oceanicola*
289	0.0	0.6	0.9	4.4	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Phaeobacter*
282	0.0	0.0	0.9	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Rhodobacter*
40	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
43	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*

45	0.0	0.8	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
46	12.4	6.8	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
48	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
174	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
177	0.0	0.2	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
182	0.0	12.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
185	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
188	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
201	0.0	0.5	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_AS-21_lineage*
175	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_NAC11-7_lineage*
187	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_NAC11-7_lineage*
202	0.0	0.4	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_NAC11-7_lineage*
142	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_OCT_lineage*
157	1.2	1.7	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_OCT_lineage*
196	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseobacter_clade_OCT_lineage*
118	0.0	0.3	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
128	0.0	0.0	2.9	1.6	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
129	0.0	0.0	0.4	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
130	0.0	0.0	0.5	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
131	0.0	0.0	1.3	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
132	2.4	0.0	15.9	7.2	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
140	0.0	0.0	1.3	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
585	0.0	0.0	0.9	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Roseovarius*
159	0.0	2.9	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae;Sulfitobacter*
234	0.0	0.6	0.0	0.0	*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae;Thalassospira*
235	0.0	0.0	0.0	1.6	*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Rhodospirillaceae;Thalassospira*
1	0.4	0.0	0.0	0.0	*Proteobacteria;Deltaproteobacteria;Bdellovibrionales*f;g
320	0.8	0.0	0.0	0.0	*Proteobacteria;Deltaproteobacteria;Desulfuromonadales*f;g
351	1.0	0.0	0.0	0.0	*Proteobacteria;Deltaproteobacteria;Desulfuromonadales;GR-WP33-58*;g
323	0.0	2.2	0.0	0.0	*Proteobacteria;Deltaproteobacteria;Syntrophobacterales*;f;g
358	0.0	0.0	0.0	1.6	*Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Alteromonas*
365	0.0	0.0	1.9	5.2	*Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Alteromonas*
376	0.0	0.0	0.7	1.4	*Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Alteromonas*
469	0.0	2.1	0.0	1.9	*Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;Marinobacter*
379	0.0	0.3	0.0	0.0	*Proteobacteria;Gammaproteobacteria;Alteromonadales;Alteromonadaceae;OM60(NOR5)_clade*
405	0.0	0.0	0.0	1.7	*Proteobacteria;Gammaproteobacteria;Alteromonadales;Idiomarinaceae;Pseudidiomarina*
425	0.0	0.0	0.7	0.0	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Marinobacterium*
426	0.0	0.0	0.4	0.0	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Neptuniibacter_*
427	0.0	0.0	0.5	0.0	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Neptuniibacter_*
428	0.0	0.0	1.1	0.0	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Neptuniibacter_*
429	0.0	0.0	0.6	0.0	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Neptuniibacter_*
430	0.0	0.0	11.9	2.3	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Neptuniibacter_*
590	0.0	0.0	0.5	0.0	*Proteobacteria;Gammaproteobacteria;Oceanospirillales;Oceanospirillaceae;Neptuniibacter_*
480	0.0	0.0	0.0	1.5	*Proteobacteria;Gammaproteobacteria;Thiotrichales;Piscirickettsiaceae;Methylophaga*
348	0.0	0.3	0.0	0.0	*Verrucomicrobia;Opitutae;Puniceicoccales*;f;g
347	0.0	0.0	3.8	3.3	*Verrucomicrobia;Opitutae;Puniceicoccales;Puniceicoccaceae;Pelagicoccus*
322	0.5	0.0	0.0	0.0	*Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae*;g
574	0.0	0.5	1.8	0.0	Unclassified
711	0.0	0.2	0.0	1.3	Unclassified

Chapter 6

General discussion

6.1. Overview of the thesis

This thesis investigated the bacterial community composition in cultures of 12 strains of *Botryococcus braunii* obtained from six culture collections and the bacterial community composition of cultures of *Nannochloropsis* sp. CCAP211/78 maintained in different reactor types using 16S rRNA gene amplicon sequencing on an Illumina MiSeq platform. This was complemented by a co-cultivation study in microplates using axenic *Nannochloropsis* sp. CCAP211/78 and cultivable bacterial strains isolated from two outdoor reactors. In addition, a literature review was done to summarise the current knowledge on algal-bacterial interactions and biotechnological applications. A brief overview of results obtained from each chapter is presented in Figure 6.1, which will be further elaborated in the following sections.

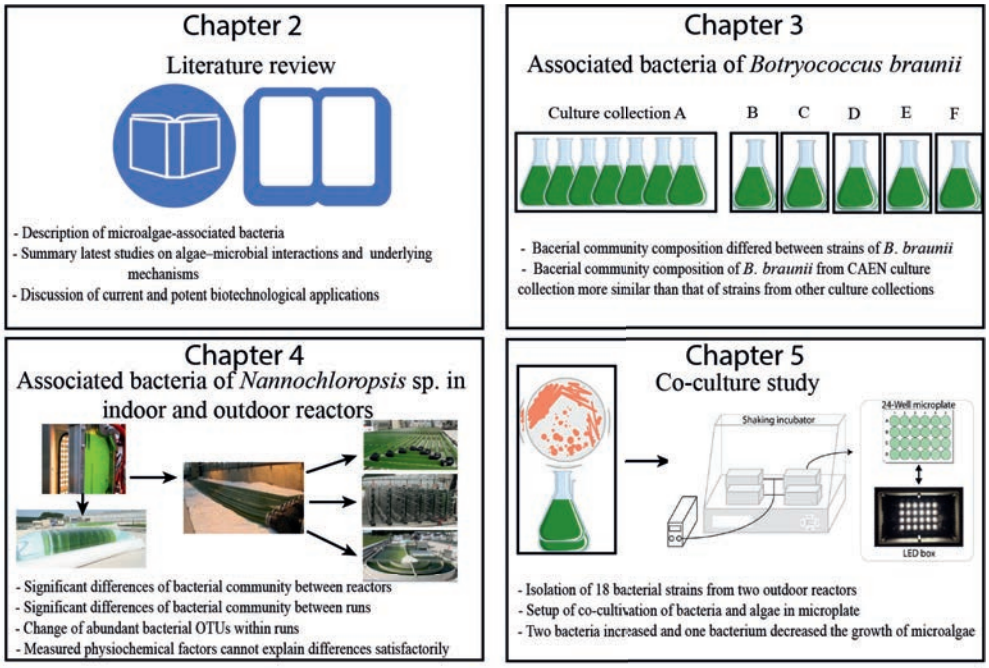

Figure 6.1. A brief overview of results obtained in Chapters 2 - 5.

6.2. Microalgae-associated bacteria

Numerous studies over the past 100 years have shown that bacteria are involved in complex interactions with microalgae [96]. Particularly the rapid and ongoing developments in next-generation sequencing technologies have led to an increase of research on the bacterial communities associated with microalgae [71]. However, as microalgae are an extremely diverse group of eukaryotic organisms and comprise of tens of thousands of species, only a few model algal species or algae of biotechnological or ecological interest have been surveyed for their associated bacterial communities to date [69, 118, 252, 359, 360].

Among all the microalgae, diatoms are the best studied taxa with respect to their associated bacteria [306, 361]. A molecular survey of bacterial diversity from cultures of six diatom genera (*Ditylum*, *Thalassiosira*, *Asterionella*, *Chaetoceros*, *Leptocylindrus*, and *Coscinodiscus*) revealed distinct bacterial phylotypes associated with each species. However, *Alphaproteobacteria* (including the genera *Sulfitobacter*, *Roseobacter*, *Ruegeria*, and *Erythrobacter*), *Bacteroidetes* and to a lesser extent *Betaproteobacteria* were among the most prominent taxa across all diatoms examined [361]. In a number of other studies it was shown that with respect to the bacterial communities of diatom cultures the *Proteobacteria* and *Bacteroidetes* are the main bacterial phyla associated with diatoms. At the genus level, *Sulfitobacter*, *Roseobacter*, *Alteromonas* and *Flavobacterium* have been repeatedly found to be associated with diatoms [16, 306, 359, 360, 362, 363].

For this thesis, we observed that even at the strain level *B. braunii* cultures were associated with distinct bacterial communities. Interestingly, seven *B. braunii* strains obtained from the CAEN culture collection harbored more similar bacterial communities as compared to strains from other culture collections (**Chapter 3,** Figure 3.3A), suggesting that observed differences are at least in part due to different maintenance approaches exerted by those culture collections. Our

results are consistent with a previous study that showed that the bacterial communities of different strains of the diatom species *Thalassiosira rotula* were significantly different [359]. Sapp *et al.* [363] also demonstrated that diatom cultures exhibited differences in bacterial community composition over time after isolation, suggesting that changes in environmental conditions may be important drivers of bacterial community composition.

In addition, we examined similarities and differences in bacterial community composition between *Botryococcus braunii* (**Chapter 3**) and *Nannochloropsis* sp. CCAP211/78 (**Chapter 4**). To this end, we compared the cultures of 12 strains of *B. braunii* with *Nannochloropsis* sp. CCAP211/78 cultured in a flat panel reactor (FP), as only for this reactor, strain CCAP211/78 was grown in a small volume with sterilized medium, similar to conditions of *B. braunii* cultures. Nevertheless, it should be noted that the growth medium for both microalgae was different – a fact that is ignored here, but which should receive more attention in future studies. At the phylum level, *Proteobacteria* and *Bacteroidetes* were the most predominant bacterial taxa with both *Nannochloropsis* sp. CCAP211/78 and *B. braunii*. However, at family level, distinct predominant families were found to be associated with *Nannochloropsis* sp. CCAP211/78 and *B. braunii*. *Rhizobiaceae*, *Erythrobacteraceae* and *Cytophagaceae* were the most abundant families in cultures of *B. braunii* (**Chapter 3**, Figure 3.1), whereas *Rhodobacteraceae*, *Flammeovirgaceae* and *Oceanospirillaceae* predominated the bacterial community of *Nannochloropsis* sp. CCAP211/78 cultures in FP (**Chapter 4**, Figure S4.4).

There is an ongoing debate ongoing about whether the associations of bacteria with algae are species-specific. Jasti *et al.* [364] observed that *Alexandrium* strains isolated from the Gulf of Maine shared many bacterial phylotypes with other *Alexandrium* cultures, as based on denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments, regardless of geographic origin, whereas bacteria associated with *Alexandrium* were different from those found associated with other phytoplankton species isolated from the same habitat. Likewise,

another study demonstrated that two axenic marine diatoms (*Thalassiosira rotula* and *Skeletonema costatum*) incubated with the same natural bacterial assemblages exhibited very different bacterial community composition and were dominated by distinct phylogenetic groups [306]. Our results generally reinforce the notion that different microalgae harbour specific bacterial communities as we observed highly different bacterial communities associated with *Nannochloropsis* sp. (mainly *Rhodobacteraceae*, *Flammeovirgaceae* and *Oceanospirillaceae*), and *B. braunii* (mainly *Rhizobiaceae*, *Erythrobacteraceae* and *Cytophagaceae*), which also differed from bacterial communities previously reported to be associated with different diatom species (mainly *Rhodobacteraceae*, *Flavobacteraceae* and *Alteromonadaceae*). Some bacterial taxa, such as the family *Rhodobacteraceae* are found together with *Nannochloropsis* sp., diatoms [96, 306, 319, 359] and many surveyed phytoplankton samples [96, 304, 336, 365], but conservation is in general only observed at the family level. Overall, it seems that the composition of the bacterial communities is species-specific for microalgae.

One hypothesis to explain the existence of species-specific interactions between algae and bacteria is the niche hypothesis: if one algal species provides a defined and stable niche for a long period of time, the same well-adapted bacterial species will outcompete others when present [366]. Species-specific association of bacteria with algae may depend on various factors such as algal exudates [361, 367], surface structure and cellular components of algae [364, 368], cellular storage products and antibiotic production by algae [364]. Among these factors, algal exudates may play the most important role in structuring the bacterial community [359]. This assumption is supported by studies that several microalgal cultures secrete distinct exudates especially with regard to polysaccharides [369, 370]. Therefore, microalgae that share similarities in the composition of exudates might also be associated with similar bacterial communities [359]. In this context it should be noted that in our experiments we did not find correlations between bacterial community composition and the type of hydrocarbons produced

by different strains of, although *B. braunii* can be subclassified to different races (races A, B, and L) based on the type of hydrocarbons produced (**Chapter 3**). A future study to further investigate the effects of other microalgal exudates, e.g. carbohydrates and amino acids, on the composition and function of algal-associated bacteria would be of interest.

6.3. Reactor types and bacterial community composition

We surveyed bacterial community composition of *Nannochloropsis* sp. taken from different reactor types, ranging from a small laboratory reactor to large-scale outdoor reactors (**Chapter 4**). At the phylum level, generally a similar bacterial community composition was observed between each reactor type. However, at the OTU level we observed that each reactor type contained significantly different bacterial communities except a horizontal tubular reactor (HT) and a vertical tubular reactor (VT).

Reactor types varied in a number of physicochemical factors, which may be responsible for the observed differences in bacterial community composition between reactors. Our assumption is supported by a previous study showing that bacterial community composition of *Nannochloropsis salina* was significantly different between small indoor reactors (Volume: 5 mL-4 L), medium indoor reactors (Volume: 20-60 L) and a large outdoor reactor (Volume: 200 L) [69]. Differences in numerous environmental factors and aspects of reactor configuration (for instance, temperature, the ratio of surface area to volume and light intensity) during cultivation at small, medium, and large scales might affect bacterial populations and cause distinct bacterial communities to dominate different reactor systems [69]. HT and VT were not different in bacterial community composition, which may be due to their comparable configuration, which was indeed reflected by the small variation of physicochemical factors between these two reactors (**Chapter 4**). However, none of the physicochemical factors measured in our experiment alone could satisfactorily explain the shifts in the associated

bacterial communities in different reactors (**Chapter 4,** Figure 4.4A). The distinct bacterial communities in different reactor types are probably determined by the combination of environmental variables and reactor configuration/operation, which makes it more difficult to exactly pinpoint the factors responsible for bacterial community structuring. Furthermore, in principle we only have data from a handful of reactors and two reactor runs, which means that the number of independent experimental units is very small. The latter may lead to a rather big impact of stochastic effects during the reactor operation that may affect the bacterial community development. Altogether, at this point it is challenging to discern in a statistically sound way the impact of environmental factors. Nevertheless, additional research that includes more controlled experiments where environmental conditions are varied needs to be done, and multiple reactors from different locations over multiple runs can be monitored and compared.

6.4. Applying defined co-cultures to understand algal-bacterial interactions

Instead of ignoring them or treating bacteria as contaminants as a whole, investigating their interactions with microalgae could enable exploration of the concept of "synthetic ecology" as a microalgae (co-)cultivation technique [371, 372].

Co-cultivation is not a novel idea in biotechnology [372-374], although much of the focus has been on bacterial interactions [375]. In traditional microbially-based processes such as anaerobic digestion, fermentation, and bioremediation, the most efficient microbial assemblages are often selected and subsequently carefully maintained. With the exception of wastewater treatment with microalgae and bacteria, this approach is not common in algal biotechnology, however, the awareness of harnessing algae-bacteria interactions to enhance productivity and increase the financial and environmental benefits of cultivating algae has been accepted by the research community in recent years [372]. A number of examples have been reviewed in **chapter 2**. In **chapter 5**, by applying a co-culture method we showed that two

bacteria could significantly enhance the growth of a microalga. However, as the bacterial strains tested in co-culture only accounted for 10% of the bacterial community in terms of relative abundance in the algal cultures, many more potent bacterial candidates remain to be isolated and tested for their potential to affect the growth of microalgae.

In addition to co-culturing microalgae with one bacterium, a synthetic community approach could be used to select and test different defined microbial assemblages for their potential to increase productivity as well as resistance to contamination. To my knowledge, there are no studies related to this concept. To this end, random combinations of isolated bacterial strains could be grown with algae in the microplate system we developed, and the co-cultures with highest productivity could be selected and tested for large-scale cultivation. However, it can be anticipated that this approach will also face a range of challenges such as how much complexity of such engineered consortia is required and how the stability of synthetic communities can be maintained. Considering the highly complex and dynamic nature of bacterial communities observed in algal cultures, these natural communities may well harbour functional redundancy, and experiments will have to show to what extent such redundancy can be reduced to simplify communities, or whether functional redundancy is required to ensure culture stability when facing dynamic environmental conditions in large scale outdoor production systems.

6.5. New opportunities for the deployment of algal-bacterial interactions

As mentioned in Chapter 1 and Chapter 2, competition and antagonistic interactions are prevalent relationships between phytoplankton and bacteria [2, 96, 376]. However, our aim was to explore mutualistic interactions between algae and bacteria for applications in algal biotechnology. Thus, in this chapter we focus more on the beneficial effects of bacteria on algae. One of the most widely studied mutualistic interactions is the obligate relationship between vitamin-synthesizing bacteria and algal species that cannot synthesize several of the vitamins

that are essential for their growth [2]. For example, ~50% of 326 algal species examined in a study required exogenous supply of vitamins B_1, B_7 or B_{12} [26], whereas bacteria can synthesize these vitamins to sustain algal growth in exchange for organic carbon [17, 22, 26, 27]. Another common obligate mutualism involves nitrogen-fixing cyanobacteria and microalgae, where the cyanobacteria provide fixed nitrogen to the algae in exchange for amino acids and organic carbon [141, 321, 377]. Many marine bacteria, such as *Marinobacter* species, can alleviate iron limitation of diatoms, dinoflagellates and coccolithophores by excreting siderophores that have exceptionally high iron affinity [378]. In turn, algae release dissolved organic carbon to sustain bacterial growth [98]. In another example, *Sulfitobacter* sp. SA11 was shown to use diatom-derived tryptophan to produce the hormone indole-3-acetic acid that promotes the cell division of the diatom *Pseudo-nitzschia multiseries* [16]. Interestingly, the molecular exchanges in a range of algal-bacterial interactions bear resemblance to interactions that dominate the rhizosphere of plants [2]. Therefore, the methods and knowledge on plant-microbe interactions might guide and accelerate the research on algal-bacterial interactions.

Although some bacterial isolates have been shown to exert a positive effect on the growth of microalgae in laboratory Erlenmeyer flasks [83, 105, 120, 123, 270], it does not mean that under conditions in a large-scale bioreactor these bacteria continue to exert the same effect. Therefore, bacteria that are beneficial to microalgae in flasks must be tested in large-scale cultures. For instance, once we have gained mechanistic insight into the abovementioned synthetic communities, the co-cultures with higher productivity could be transferred to larger-scale reactors for further validation. If such co-cultures of higher productivity were to be employed successfully at large scale, a lower production cost of algal biomass would be achieved [71, 372]. Nevertheless, scientists should further seek potential applications for algae-bacteria co-culturing technology. For instance, would co-cultures of microalgae and bacteria have the possibility to produce high added value molecules more cost-effective than other conventional

production methods? A case in point is that an algae-bacterial co-culture was found more efficient in H_2 production than algae and bacteria separately [379]. Or would such co-culture allow production of molecules that could otherwise not be produced? Such a molecule could be the potent broad-spectrum antibiotic tropodithietic acid that can only be produced by co-cultivation of *Emiliania huxleyi* and *Phaeobacter gallaeciensis* [46].

On the other hand, advantages of algal-bacterial interactions through co-cultivation could be decreased contamination. When microalgae are co-cultured with symbiotic bacteria, the invasion by other bacteria may be decreased because invading bacteria would be less likely to establish in an already occupied niche [27]. Furthermore, studies of antagonistic or algicidal bacteria are also useful because of the potential application in mitigating harmful algal blooms, and more relevantly, in preventing the growth of algicidal bacteria when algae are grown at large scale for aquaculture or other valuable products [380]. Related to the latter, Ganuza *et al.* [381] investigated the possibility to alleviate bacterial infection of the green alga *Chlorella* by the parasitic bacterium *Vampirovibrio* through decreasing pH of the medium for 15 min in the presence of acetate. The treatment was successful in prolonging algal cultivation, and the algicidal bacterium appeared to be unable to build up immunity to this treatment. Bagwell *et al.* [382] also investigated the possibility of preventing *Vampirovibrio* infection of *Chlorella* cultures. In this case, the infection was prevented by inducing the production of bioactive small peptides and glycosides by *Chlorella* under iron limitation.

6.6. Novel methodologies to study algae-associated bacteria and algae-bacterial interactions

6.6.1. New primer set to characterize algae-associated bacteria

A common approach to characterize the composition of algae-associated bacterial communities comprises PCR amplification and sequencing of 16S rRNA genes using a universal bacterial

primer set [383]. Routine sampling strategies for studying algal microbiomes do not often separate algal and bacterial cells, which results in the combination of bacterial and algal DNA in the samples. However, as algal plastid genomes encode 16S rRNA closely related to that of cyanobacteria, sequencing data sets frequently comprise more than 75% plastid sequences (**Chapter 4**). As a result, bacterial diversity may be largely underrepresented due to the decreased sequencing depth.

Thomas *et al.* [384] evaluated several primer sets to amplify a 450 bp fragment spanning the V3-V4 region while minimizing the amplification of plastid sequences. The new primer set NOCHL was shown to have lower coverage for plastids and cover a significantly higher bacterial richness than other tested primer sets. This primer set is a potent alternative to study bacterial communities in environments where plastid contamination can be an issue such as terrestrial plants and microalgae.

6.6.2. Mass spectrometry imaging

Information obtained from (meta)genomic/transcriptomic and proteomic approaches is a prerequisite for a systems-level understanding of symbiont physiology [385], but is not sufficient to provide direct evidence of potential metabolic signatures because microbial interactions often involve a dynamic metabolic crosstalk and chemical communication [386, 387]. Therefore, a metabolomics approach has been developed as a powerful tool to unravel the means and consequences of microbial interaction at the metabolic level. Recent advances in mass spectrometry imaging (MSI) technologies allow the localization of specific metabolites of various dynamic processes and biotic interactions at the microscale level and, consequently, monitoring of metabolic changes at high spatiotemporal resolution [388]. MSI has been widely used to study the metabolic footprints of host-associated bacteria [385], bacterial population dynamics [389] and to detect pathogens [390]. Likewise, MSI is applicable to study algal-

bacterial interactions. For instance, targeted metabolic profiling could facilitate the quantification of known compounds (for instance, vitamins and auxins) exchanged between microalgae and bacteria, and untargeted metabolic profiling would enable the identification of novel molecules mediating algae-bacterial interactions.

A more recent study investigated metabolic alteration (mainly lipids) during virus infection (EhV) of the microalga *Emiliania huxleyi*. A culture of *E. huxleyi* was mixed with infectious EhV virions in agarose, and the mixture was then poured into a petri dish. When the plaques were formed, one plaque sample was blotted onto a filter and analysed directly using MALDI-MS or Flow-probe-MS. This experimental approach needs much smaller samples (one plaque or colony) as compared to conventional metabolomic approaches that require bulk liquid cultures (from milli-liters to liters) [388].

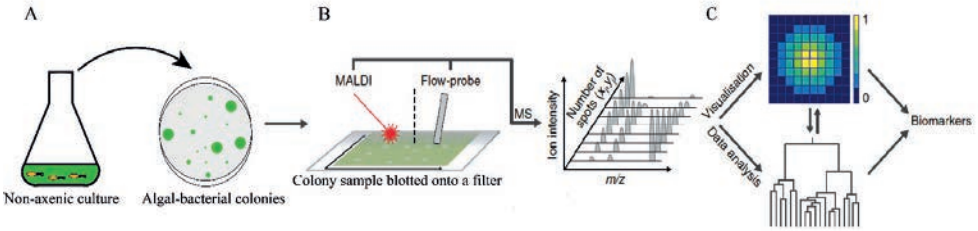

Figure 6.2. Overview of the workflow of MSI analysis. **A**, For the agar plate assay, a non-axenic algal culture can be diluted and mixed in agarose, and poured into a Petri dish, which is then incubated until the formation of colonies. **B**, A colony sample is blotted onto a filter. MSI techniques can be used to analyse colony samples: MALDI-MS or Flow-probe-MS. **C**, The collected spectra can be used for visualization of specific metabolic biomarkers for algal-bacterial interactions, as well as for untargeted data analysis using spatially aware unsupervised clustering (Flow-probe-MS) and colocalization (MALDI-MS). **B** and **C** were adopted from [388]

Inspired by this study, we propose here a comparable experimental approach to investigate metabolomes of algae-bacteria interactions (Figure 6.2). Non-axenic algal cultures or field samples can be directly used, for example by first separating algal and bacterial cells through

filtration or cell sorting, and then mixing fractions again at the desired ratio (algae: bacteria = 1:1) using bead- or microdroplet based approaches. The droplets are then spread on an agar plate and incubated under desired conditions until the formation of colonies. Theoretically, the algal colonies would contain on average one bacterial species, and the metabolomes of algae and specific bacteria can be analysed following the methods in Figure 2B and C. In addition to abovementioned samples, the samples of double-agar plate assays (**Chapter 5**, Figure 5.2) can be directly analysed following the same protocol as well. Compared to the double-agar plate assay used in **Chapter 5**, the sample preparation method (Figure 6.2A) skips the laborious work of bacterial isolation, and co-culturing algae and bacteria together on agar plates may increase the possibility of testing otherwise uncultivable bacteria. Furthermore, differently sized colonies of microalgae on the agar plate may be an indicator that co-existing bacteria have growth promoting/inhibiting ability, which can be directly picked for MSI analysis. With these advantages, this method combines screening of algae associated bacteria and metabolomes together, which stands for an efficient and fast way to studying metabolic shifts during algal-bacterial interactions.

6.6.3. Microfluidics and Raman-activated microbial cell sorting

Marine bacteria have evolved strategies to exploit exudates from living and dead microalgae. To forage such resources bacteria are guided by a chemoattractant leaking from algae [391]. This process is called chemotaxis and is a prerequisite for bacteria to sense and interact with microalgae [314, 392]. The advancement made in the fields of microfluidics enables the interrogation of the chemically mediated interactions between microalgae and bacteria.

As proposed by Lambert *et al.* [393], the ISCA (in situ chemotaxis assay) device is a robust device that can be used to study algae-bacterial interactions (Figure 6.3A). Algal cultures or any molecules produced by microalgae can be loaded into wells, and then the device can be

deployed in the ocean or photobioreactor, for instance. Upon deployment, chemicals in the well produce transient nutrient pulses, attracting chemotactic bacteria to swimming into the well of the device. After collection, those samples can be enumerated by flow cytometry, identified by sequencing and used for bacterial isolation.

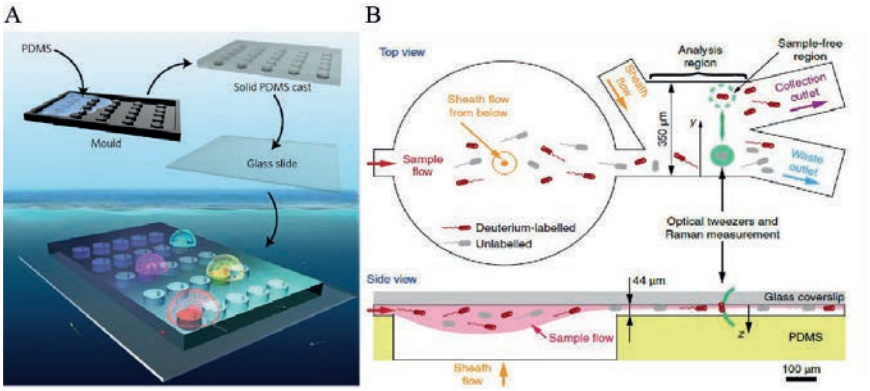

Figure 6.3. Fabrication of the in situ chemotaxis assay (ISCA) and laboratory tests. (A) Polydimethylsiloxane (PDMS) is cast onto a 3D printed mould and cured overnight. The solid PDMS, containing multiple wells, is then excised and plasma-bonded onto a glass slide (100 mm × 76 mm × 1 mm). Each well has an independent connection to the external environment via a port, through which chemicals can diffuse and microorganisms enter. Adapted from [393]. **(B)** Adapted from [394]

The captured bacteria from the ISCA device can be further subjected to an optofluidic platform for Raman-activated microbial cell sorting (RACS) (Figure 6.3B). Unlike previous RACS approaches, this method is applicable to a wide range of cells not containing compounds that enhance measurement sensitivity (for example, carotenoids and chlorophyll). Furthermore, this approach detects all metabolically active cells with no need for cell fixation and is thus a non-destructive method. For example, this approach could be used for cells that are active when incubated in the presence of a specific substrate that do not need to be labelled themselves. The RACS can analyse up to 200-500 cells h^{-1} and is thus suitable for function-based cell culturing and metagenomics, or as a front end to standard single-cell genomics platforms [394]. This

method is appropriate for analysing algae-associated bacteria and isolating active bacterial representatives for further growth essay in the co-culture platform we developed (**Chapter 5**, Figure 5.1). In combination with MSI, these methods represent powerful tools to identify algae-associated bacteria and disentangle the mechanisms behind the interactions.

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Thesis summary

Summary

Research described in this thesis aimed to investigate the microalgal microbial ecology by integrating both cultivation-dependent and cultivation-independent approaches. In addition, we examined effects of bacteria on growth of microalgae by developing a coculture system. Understanding the bacterial community composition and functional associations between microalgae and bacteria will provide important insights for both aquatic ecology and algal biotechnology.

Microalgae are living with diverse microbial communities and interactions between microalgae and bacteria are prevalent and strongly influence carbon and nutrient cycling in aquatic ecosystems. A brief overview of the current knowledge of algae-bacteria interactions and current research methodologies was given in **chapter 1**. This chapter furthermore introduced the microalgae production facilities at AlgaePARC, and highlighted two research objects (*Nannochloropsis* sp. and *Botryococcus braunii*), and current knowledge on their associated bacteria.

In **chapter 2** we comprehensively reviewed the recent research progress on algae-bacteria interactions and summarized the current knowledge on functional aspects of algae-associated microbiomes. In addition, we discussed the applications of knowledge on algae-bacteria interactions in algal biomass production and various other related biotechnological innovations. Finally, we concluded that principles of algae-bacteria interactions can be integrated into many aspects of the algal production chain, which may help push the limit of the algal industry.

In **chapter 3**, we studied the bacterial community associated with 12 strains of *B. braunii*. The changes of bacterial community composition were monitored over a period of 12 days. It was clear from this study that *B. braunii* hosts a wide variety of bacterial species, among which the bacterial families *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae* were found in all

12 strains. These families that all belong to the phylum *Proteobacteria* could have important interactions with *B. braunii*. Additionally, although each strain displayed a different bacterial community composition, all the strains from the CAEN culture collection had more similar bacterial communities, suggesting that the algae culture collection could have an influence on the bacterial community composition. Several bacterial genera were shown to be exclusively abundant in CAEN strains including *Rhizobium* spp. and *Porphyrobacter* spp.

In **chapter 4**, we compared bacterial community composition in cultures of *Nannochloropsis* sp. CCAP211/78 grown simultaneously in four outdoor large-scale bioreactors. We observed that the bacterial community composition significantly differed between all reactor types except for horizontal and vertical tubular bioreactors. Although the bacterial communities varied within each bioreactor at different cultivation stages, we found that a member of the family *Saprospiraceae* was the most abundant taxon (OUT_1261) in all large non-sterilized bioreactors. This indicates that *Saprospiraceae* may play important roles in *Nannochloropsis* cultures.

In **chapter 5**, we isolated bacteria from one horizontal tubular bioreactor and one vertical tubular bioreactor. Among all the isolates eighteen bacteria were phylogenetically classified as different species/strains based on their 16S rRNA gene sequence. Axenic cultures of *Nannochloropsis* sp. CCAP211/78 were successfully obtained by treatment with antibiotics. A microplate-based assay system coupled with a custom-made LED box was developed to assess the growth of axenic *Nannochloropsis* sp. CCAP211/78 with addition of isolated bacterial strains. Out of 18 strains, one *Maritalea porphyrae* strain (DMSP31) and one *Labrenzia aggregata* strain (YP26) were highlighted as having a significantly positive effect on microalga growth, whereas one flavobacterial strain (YP206) was shown to reduce this growth. YP26 was also found to substantially enhance the growth of *Nannochloropsis* sp CCAP211/78 using the

double-agar plate assay, which indicates that YP26 has strong positive impacts on *Nannochloropsis* sp CCAP211/78.

Finally, **chapter 6** elaborated on the implications of findings from the research described in this thesis. Bacterial community composition of *Nannochloropsis* sp CCAP211/7 and *B. braunii* was compared, and the results generally supported that bacterial communities are algal species specific. Additionally, I suggested that the combination of environmental variables and reactor configuration/operation (in addition to stochastic effects) may be responsible for the differences in bacterial community composition observed between different reactor types and between runs. Further exploration and exploitation of algae-bacteria interactions is needed. Therefore, I proposed several new techniques, including using a new primer set to better characterize bacteria living with microalgae, using advanced mass spectrometry to tap into the metabolic changes of microalgae when they interact with bacteria, and using microfluidics coupled with Raman-based cell sorting to isolate bacteria of interest. Future research should consider these approaches in combination with coculture experiments in order to gain better understanding on bacterial community composition and functional interactions between microalgae and bacteria.

Appendices

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About the author

Jie Lian was born on the 16th of June 1989 in Wuxi, Jiangsu Province, China. After completing his primary and secondary education at Wuxi in 2007, he continued his study with BSc in Biotechnology at Jiangsu University, Zhenjiang. His BSc thesis investigated the effect of salicylic acid on the germination of *Oryza sativa*. In 2011, he moved to Xiamen for the MSc education in Aquatic biology. Under the supervision of Dr Jun Zhang at Xiamen University, his research focused on unravelling the molecular mechanism of motility in cyanobacteria *Synechococcus* sp. and *Microcystis aeruginosa*. After obtaining his MSc degree in 2014, he moved to the Netherlands and started his PhD at the Laboratory of Microbiology supervised by Detmer Sipkema, as well as Prof. Hauke Smidt and Prof. René Wijffels (Bioprocess Engineering Group). His PhD Research, which is described in this book, investigated the interactions between microalgae and associated bacteria, and its ecological and biotechnological implications.

List of publications

Lian, J., Wijffels, R.H., Smidt, H., and Sipkema, D. (2018) The effect of the algal microbiome on industrial production of microalgae, Microbial biotechnology 11: 806-818.

Gouveia, J.D.[*], **Lian, J.**[*], Steinert, G., Smidt, H., Sipkema, D., Wijffels, R.H., Barbosa, M.J. 2019. Associated bacteria of *Botryococcus braunii* (Chlorophyta) PeerJ 7:e6610 https://doi.org/10.7717/peerj.6610

Lian, J., Steinert, G, de Vree, J.H, Meijer S., Heryanto, C., Bosma, R., Wijffels, R.H., Barbosa, M.J., Smidt, H., and Sipkema, D. Bacterial diversity in outdoor pilot plant production of the microalga *Nannochloropsis* sp. CCAP211/78. **Manuscript in preparation**.

Lian, J., Schimmel, P., Sanchez-Garcia, S., Wijffels, R.H., Smidt, H., Sipkema, D. Enhanced or decreased growth of *Nannochloropsis* sp. CCAP211/78 cultivated with diverse bacteria. **Manuscript submitted**.

Lian, J., Nijsse, B., Koehorst, J.J., Wijffels, R.H., Smidt, H., Sipkema, D. Dual RNA-Seq unveils interactions between *Nannochloropsis* sp. CCAP211/78 and two associated bacteria. **Manuscript in preparation**.

Other publications:

Lian, J. [*]**,** Zheng, X. [*], Chen, Y., Zhuo, X., He, C, Shi, Q., Zheng, Q., Lin, TH., Sun, J., Guo, WD., Cai, R., Jiao, N. Microbial transformation of distinct exogenous substrates into analogous refractory compounds. **Manuscript Submitted**.

[*] These authors contributed equally.

Overview of completed training activities

Discipline specific activities

Courses

SIAM Metagenomics Course, Nijmegen, NL	2016
BluePharmTrain Summer school: Statistic analysis using R, Wageningen, NL	2016
Big data course, Wageningen, NL	2017
Advance course in Bioprocess design, Wageningen, NL	2018

Meetings

KNVM Spring Meeting, Arnhem, NL	2017
New Approaches and Concepts in Microbiology, Heidelberg, DE	2017
SAME15, Zagreb, HR	2017
Prospects and challenges for the development of algal biotechnology, Bielefeld, DE	2017
Microbiology centennial conference, Wageningen, NL	2017
KNVM Microbial Ecology symposium, Wageningen, NL	2017
Young algaeneers symposium, Oban, UK	2018
Wageningen meets Ghent symposium, Ghent, BE	2018
XMAS, Xiamen, CN	2019

General courses

VLAG PhD week, Baarlo, NL	2015
Project and Time management, Wageningen, NL	2015
Scientific Writing, Wageningen, NL	2016
Reviewing a scientific paper, Wageningen, NL	2017
Adobe Illustrator for scientists, Wageningen, NL	2018
Writing grant proposal, Wageningen, NL	2018

Optional

Preparing project proposal	2014
Molecular Ecology group meeting and Marine Microbiology group meeting	2014-2018
PhD meeting in Microbiology	2014-2018
Microbiology PhD trip	2017

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