
Article:
Almalki, W.J. orcid.org/0000-0002-6455-8746, Nwokeoji, A.O. and Vaidyanathan, S. orcid.org/0000-0003-4137-1230 (2024) Density and composition of cohabiting bacteria in Chlorella vulgaris CCAP 211/21A is influenced by changes in nutrient supply. SynBio, 2 (2). pp. 190-204. ISSN 2674-0583

https://doi.org/10.3390/synbio2020011

SynBio

MDPI

Article

Density and Composition of Cohabiting Bacteria in *Chlorella vulgaris* CCAP 211/21A Is Influenced by Changes in Nutrient Supply

Wasayf J. Almalki †, Alison O. Nwokeoji and Seetharaman Vaidyanathan *

Department of Chemical and Biological Engineering, The University of Sheffield, Sheffield S1 3JD, UK; almalki@uj.edu.sa (W.J.A.); obinnanwokeoji@gmail.com (A.O.N.)

* Correspondence: s.vaidyanathan@sheffield.ac.uk

† Current address: Faculty of Science, The University of Jeddah, Jeddah 21959-34, Saudi Arabia.

Abstract: Microalgae have considerable potential as a renewable feedstock for biochemical and bioethanol production that can be employed in processes associated with carbon capture. Large-scale microalgae cultivations are often non-axenic and are often cohabited by bacteria. A better understanding of the influence of cohabiting bacteria on microalgae productivity is required to develop sustainable synthetic co-culture processes at scale. Nutrient limitation is a frequently employed strategy in algal cultivations to accumulate energy reserves, such as lipids and carbohydrates. Here, a non-axenic culture of an estuarine green microalga, *Chlorella vulgaris* CCAP 211/21A, was studied under nutrient replete and deplete conditions to assess how changes in nutrient supply influenced the cohabiting bacterial population and its association with intracellular carbohydrate accumulations in the alga. Nutrient limitation resulted in a maximum carbohydrate yield of 47%, which was 74% higher than that in nutrient replete conditions. However, the latter condition elicited a 2-fold higher carbohydrate productivity. Three cohabiting bacterial isolates were cultivable from the three culture conditions tested. These isolates were identified using the 16S rRNA gene sequence to belong to *Halomonas* sp. and *Muricauda* sp. The composition of the bacterial population varied significantly between the growth conditions and time points. In all cases and at all time points, the dominant species was *Halomonas* isolates. Nutrient depletion resulted in an apparent loss of *Muricauda* sp. This finding demonstrates that nutrient supply can be used to control cohabiting bacterial populations in algal cultures, which will enable the development of synthetic co-culture strategies for improving algae productivity.

Keywords: co-cultures; renewable feedstock; carbohydrate content; nutrients limitation; consortia

Citation: Almalki, W.J.; Nwokeoji, A.O.; Vaidyanathan, S. Density and Composition of Cohabiting Bacteria in *Chlorella vulgaris* CCAP 211/21A Is Influenced by Changes in Nutrient Supply. *SynBio* **2024**, *2*, 190–204. https://doi.org/10.3390/synbio2020011

Academic Editor: Anwar Sunna

Received: 28 December 2023
Revised: 5 March 2024
Accepted: 11 May 2024
Published: 17 May 2024

1. Introduction

Algae have been considered as a potentially sustainable feedstock for bioethanol and biochemical production due to the conceptual promise of enabling production processes incorporating CO_2 capture. Algae are photosynthetic microorganisms that can capture solar energy and transform it into biochemical products such as carbohydrates, lipids, proteins, vitamins, and pigments that can be of economic value. Microalgae have long been considered to be a candidate for bioethanol production because of their relatively faster growth rates compared to most bio-based feedstock and the potential for sustainably sourcing nutrients from wastewaters for their cultivation. Although reaching practical economies of scale has been challenging, they offer a conceptually attractive framework for developing sustainable feedstock for industrial-scale productions. Despite the higher cost compared to the production of fossil fuels, bioethanol production is increasing worldwide [1–3]. Given the need to develop sustainable renewable energy sources to meet the ever-increasing demand, the geopolitical uncertainties concerning fuel supply and distributions, and the unsustainability of using fossil fuels to meet our demands, there is a continuing interest

in developing sustainable feedstock for bioethanol production. In addition to biodiesel, algae can be grown to accumulate fermentable carbohydrates that can then be fermented to bioethanol and biobutanol.

The possibility of sustainably cultivating algae as a feedstock for bioethanol production is attractive when considering alternatives for sourcing fermentable sugars. Some genera of microalgae such as *Chlorella*, *Dunaliella*, *Chlamydomonas*, and *Scenedesmus* can accumulate high amounts of carbohydrates (>40% of the dry weight). *Chlorella* species, especially *C. vulgaris*, can accumulate 37–55% of their dry weight as carbohydrates [4]. Algae accumulate different types of carbohydrates, for example, starch in green algae, glycogen in cyanobacteria, floridean starch in red algae, and chrysolaminarin in diatoms. The most common monomers in microalgae are glucose (dominant), xylose, mannose, and rhamnose [5], rendering carbohydrates derived from microalgae more suitable for fermentations. Macroalgae also accumulate complex polysaccharides such as laminarin, glucans, galactan, and cellulose. Nevertheless, these have compositions that are less favourable and less cost-effective for bioethanol production compared to microalgae-sourced carbohydrates [6]. Microalgae have received more attention because their cultivation processes can be more controlled. Typically, up to 60% of the dry biomass weight can be accumulated as carbohydrates, depending on the strain type, growth mode, and environmental conditions. Carbohydrates can be saccharified into fermentable sugars for bioethanol production, but this will need cost-effective pre-treatment.

One of the most common approaches to increasing carbohydrate accumulation in microalgae is nitrogen limitation, which routes the fixed carbon from Calvin's Cycle to produce lipids and carbohydrates instead of nitrogen-based products (proteins) [7]. In addition, many studies have pointed out that one approach to increasing algae productivity is to employ a co-culture system which enhances microalgae growth and metabolite production [8–11].

In the natural environment, microalgae cohabit with a wide range of different microorganisms. Synergistic relationships between microalgae and other microorganisms have a significant effect on natural ecosystems [11]. Microalgae can provide bacteria with dissolved oxygen during photosynthesis, improving bacterial respiration and increasing organic matter consumption [12]. Algae-associated bacteria can promote the growth of microalgae via commensalism and mutualism relationships and the secretion of some vitamins such as thiamine, biotin, and cobalamin [8,13,14]. Moreover, through remineralization, bacteria are able to reassimilate organic molecules in the decomposition process. Bacterial associations have been shown to be of value in increasing algal biomass productivity and growth rates [15,16]. Equally, they have also been shown to reduce algal growth, despite an increase in the accumulation of intracellular components, such as pigments [17].

Although co-cultured bacteria have been shown to have positive and negative influences on microalgae cultivations, and some of these are known to influence microalgal carbohydrate productivities [16,18–20], the field is still developing, requiring more detailed investigations on specific associations to develop conceptual frameworks and underlying principles that are useful in establishing strategies to improve productivity. A high-yielding strain of *Chlorella vulgaris* (CCAP 211/21A) that showed a high propensity to accumulate carbohydrates in preference to lipids was reported earlier [21]. Industrial-scale cultivations of microalgae, especially those in open pond systems, are likely to be non-axenic, which promotes the growth of cohabiting microbial species, on which very little information exists. The aim of this study was to further develop the understanding of this strain with respect to potential cohabiting bacteria. Towards this end, the behaviour of the bacterial population in a non-axenic culture of the algal strain was studied in conjunction with nutrient fluctuations that could lead to improved carbohydrate productivities. We believe this to be the first attempt of this sort, the findings of which will enable the development of a conceptual framework that can be used to establish co-cultures as a strategy to improve algal productivity.

2. Results

In this study, we examined a laboratory-grown, non-axenic culture of the halotolerant *Chlorella vulgaris* CCAP 211/21A. Cultivations were carried out under photoautotrophic mode in *f/2*, a commonly used maintenance/cultivation medium [22]. Two modifications of the media, where the nitrogen and phosphate concentrations were halved (*f/4*) or doubled (*2f*), were examined to study changes in the cohabiting bacteria associated with nutrient supply changes that elicit differential carbohydrate accumulations.

2.1. Changes in the Nitrogen and Phosphorus Supply Strongly Influence Algal Growth and Biomass Productivity in the Non-Axenic Culture

Over the course of the cultivation of non-axenic *C. vulgaris* under different nutrient concentrations, the continuous growth of microalgae was observed in all the tested conditions. Increasing the nutrient concentration increased the algal growth, as can be seen from all three measures of growth (cell counts, optical density, and dry cell weight) ($p < 0.001$ for ANOVA on median growth rates), with a doubling of all measures of biomass in the nutrient replete condition (*2f*) compared to the deplete condition (*f/4*) (Figure 1).

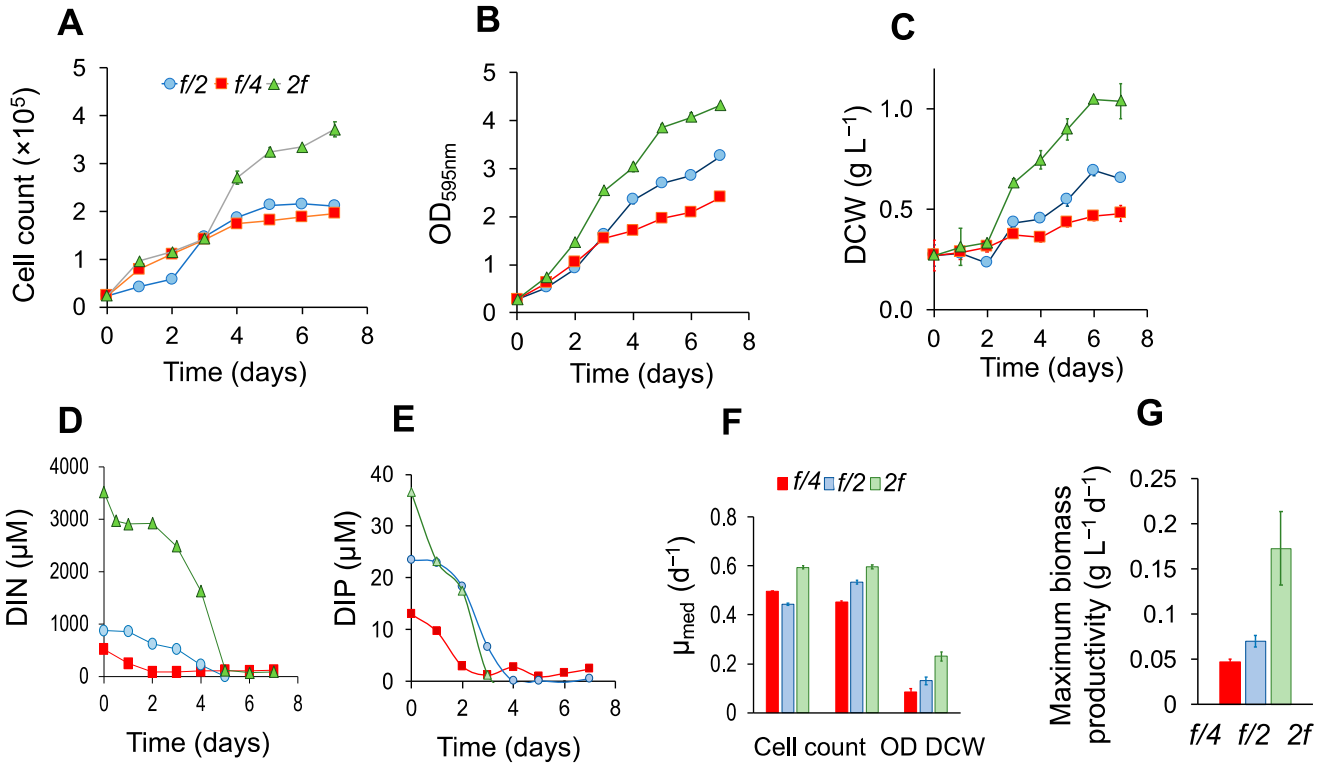


Figure 1. Growth characteristics of non-axenic *C. vulgaris* in *f/2* and modified *f/2* media cultured in batch mode for 7 days; (**A**) cell count, (**B**) optical density (OD_{595nm}), (**C**) dry cell weight of algae, (**D**) nitrate consumption over the cultivation period, (**E**) phosphate consumption over the cultivation period, (**F**) median specific growth rate of algae over the cultivation period, and (**G**) maximum biomass productivity.

Dissolved inorganic nitrate was consumed fully in two days under the nutrient-depleted conditions (*f/4*), whilst it took 5 days for total consumption in the replete conditions (*2f*) (Figure 1D). The median specific growth rate over 7 days of cultivation was also seen to be higher in the replete conditions (*2f*), as would be expected (Figure 1F). An 8-fold difference in nutrient supply resulted in a nearly 3-fold difference in median growth rates (DCW basis) ($p < 0.01$). Increasing the nutrient (N and P) supply 4-fold (*2f*) resulted in a 78% increase in the median growth rate (DCW basis). However, halving the nutrient supply (*f/4*) resulted in reducing the median growth rate (DCW basis) by 34% ($p < 0.05$)

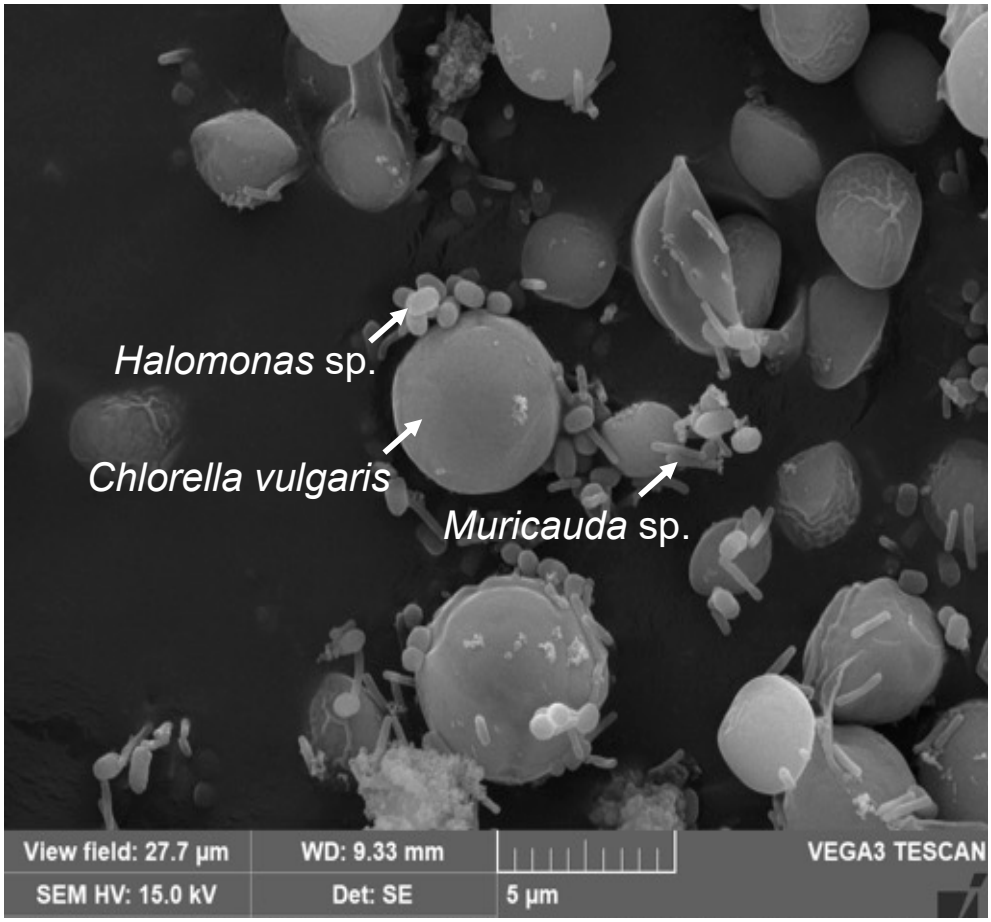
(Figure 1F). A similar change could also be seen in the maximum biomass productivity (Figure 1G). A similar result was reported by Meyers et al. [23], where a reduction in the maximum biomass productivity by 15% was reported between nutrient-replete and N-deplete conditions in a *Chlorella salina* cultivation. Differences in the cell count between media containing different nutrient concentrations were noticed, primarily after 24 h. In addition, the final dry cell weight increased in the medium that had high initial nitrate levels (*2f*), by 2-fold compared to the deplete condition (*f/4*) ($p < 0.05$). There was also a 2-fold increase in cell counts in the replete medium (*2f*) compared to the deplete medium (*f/4*) after 7 days of cultivation ($p < 0.01$).

2.2. Three Cultivable Cohabiting Bacteria Could Be Isolated from C. vulgaris CCAP 211/21A

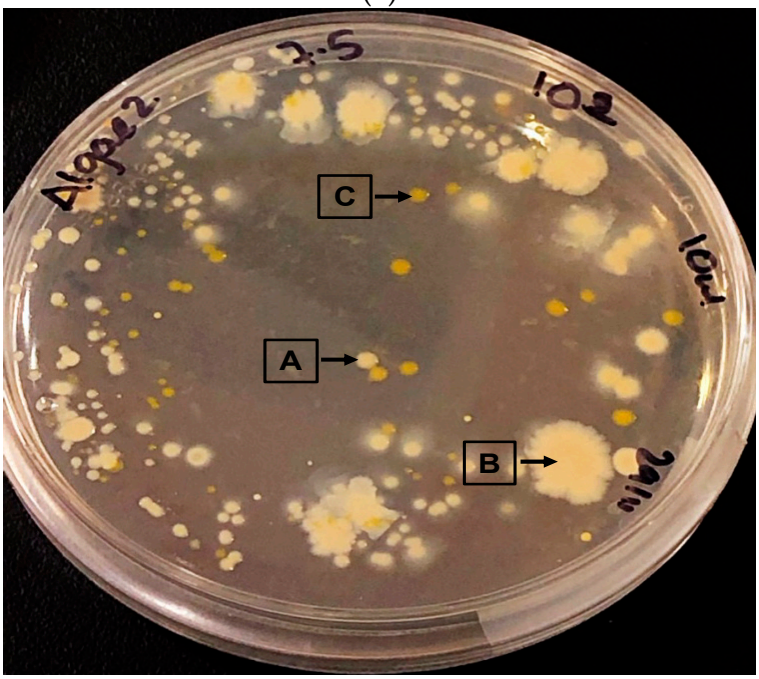
Three cohabiting bacterial isolates grew when the algal cultures were plated in R2A agar prepared with *f/2*, *f/4*, or *2f*. An SEM image of the algal cells and the two co-habiting bacteria is shown in Figure 2a. The colony morphology of these three isolates was, respectively, seen to be (a) small rounded, (b) large irregular, and (c) yellow rounded colonies (Figure 2b). All three isolates stained negative on Gram staining. The 16S rRNA molecular typing indicated two of the isolates (small and large colonies) to belong to *Halomonas* species (accession numbers OM666636.1 and OM665417.1), whilst the third yellow colony was indicated to belong to *Muricauda* sp. (accession number OM666632.1) (Figure 3). It can be seen from the literature (Table 1) that different bacterial species are associated with *Chlorella* sp. from different environments (freshwater and marine), demonstrating that the bacterial cohabitation of algae cultures is reasonably well established. *Halomonas* sp. is generally associated with *Dunaliella* cultivations [24], but has also been shown to be co-cultivated with *Chlorella* sp. [25]. *Muricauda* sp. has been isolated from algal cultivations and shown to be of utility in developing co-cultures with different microalgae species [26]. Bacteria of the classes Gama-proteobacteria (to which *Halomonas* belongs) and Flavobacteria (to which *Muricauda* belongs) have been associated with industrial algae cultivations [27], and also reported to be the dominant bacterial types associated with microalgae in aquatic karst ecosystems [28].

Table 1. Literature reports of cohabiting bacteria isolated from cultures of *Chlorella* sp. in freshwater and marine growth media before this study.

***Microalgae* sp.**	**Medium for Algal Growth**	**Bacterial Species Identified**	**Medium for Bacterial Growth**	**Reference**
Chlorella sp. P02 (freshwater)	BG-11	*Pseudomonads*, Brevundimonas, *Caulobacterales* and *Rhodospirillales*	BG11 solidified with 1.5% BactoAgar and 2% Gelzan	[29]
C. vulgaris (marine)	F/2	*Rhodovulum sulfidophilum*	Marine agar 2216	[30]
C. sorokiniana (freshwater)	BG-11	*Klebsiella pneumoniae* and *Acinetobacter calcoaceticus*	Nutrient broth (Hi Media)	[31]
C. sorokiniana (freshwater)	Fresh photoautotrophic medium	*Ralstonia pickettii*, *Sphingomonas* sp., *Microbacterium trichotecenolyticum* and *Micrococcus luteus*	Heterotrophic media	[32]
C. vulgaris (freshwater)	BG-11	*Stenotrophomona smaltophili*	-	[33]
C. vulgaris (freshwater)	BG11	*Flavobacterium*, *Hyphomonas*, *Rhizobium* and *Sphingomonas*	R2A, TSA, BG11 + glucose 100 ppm	[34]
C. ellipsoidea (marine)	Modified Bold's basal medium	*Brevundimonas* sp.	1% MBBM agar	[35]
C. vulgaris (freshwater)	BG11	*Rhizobium* sp.	LB agar	[36]
C. vulgaris (freshwater)	BG11	*Bacillus* sp.	LB agar	[37]



(**a**)



(**b**)

Figure 2. (**a**). An SEM image of non-axenic *C. vulgaris* culture, showing *Halomonas* (A,B) and *Muricauda* (C) cells in the co-culture, alongside *C. vulgaris* cells. (**b**). Three species of co-habiting bacteria isolated from *C. vulgaris* culture after 7 days of cultivation on *f/2* + R2A agar plate incubated at room temperature.

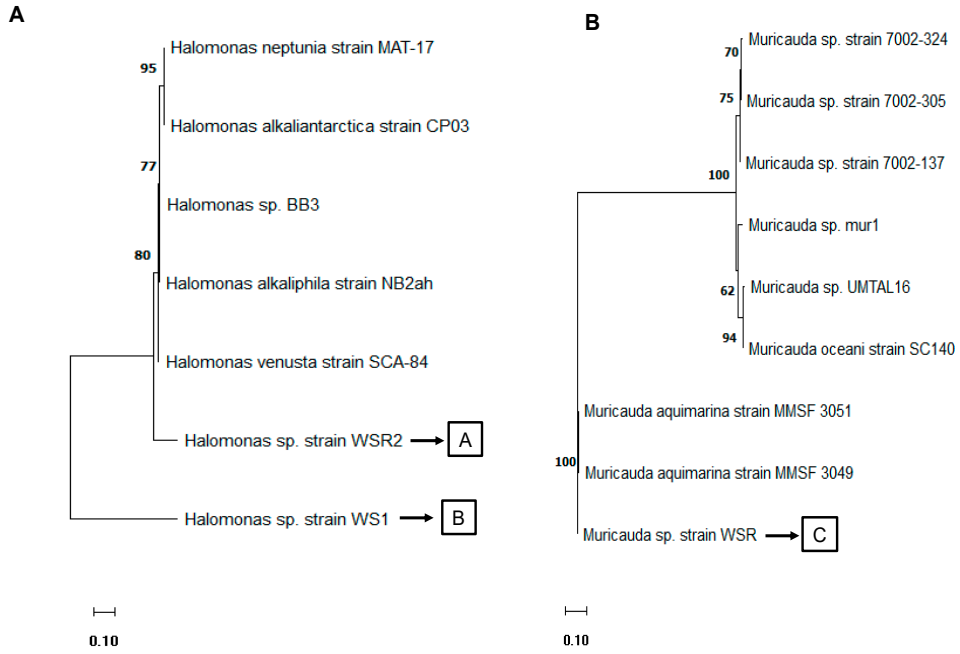


Figure 3. Phylogenetic tree of isolated cohabiting bacteria (**A**): *Halomonas* (A,B) and (**B**): *Muricauda* (C) from *C. vulgaris* culture and closely related species based on 16SrRNA sequencing, distance was estimated with Tamura-Nei model and branch support were assessed with 500 bootstraps using Mega 11.0 software.

2.3. Nutrient Supplementation Is Required to Enable Growth of Cohabiting Bacteria in Isolation

In order to understand the growth requirements of the cohabiting bacteria and how their needs were supplemented by *C. vulgaris*, we examined the growth of the bacterial isolates in defined media in isolation of the algae. The *f/2* medium used for the algae cultivation does not contain any organic carbon source, which would be required for the isolated bacteria to grow. In addition, a more easily assimilable nitrogen source is required. Considering these points, the bacterial growth was assessed in isolation as colony-forming units (CFU/mL) in agar supplemented with *f/2* media components, with the added supplementation of (a) glucose as the carbon source (*f/2* + G), (b) ammonium chloride as a simple N source, in addition (*f/2* + GN), (c) yeast extract as a complex N source, in addition (*f/2* + GNY), and (d) R2A, as a complex cultivation medium (*f/2* + R2A). In addition, growth was also characterised in *f/2* alone as a control, as well as R2A with and without salt (R2A + salt, R2A) for comparison.

The results are displayed in Figure 4. No growth was observed in the *f/2* media (control) because it does not contain the essential elements for bacterial growth. Although R2A medium is rich in nutrients for growth, there was no observable growth when it was tested for 5 days, due to lack of salt. However, the addition of salt to R2A resulted in noticeable growth for all three bacteria. This indicates that the isolated bacteria were halophilic, meaning they needed salt to grow, as well as enough nutrients. It is known that halophilic bacteria do not grow on media without the presence of salt (NaCl) [38].

Little growth was noticed in *f/2* + G and *f/2* + GN, which contained lower amounts of nitrogen and carbon sources. However, growth in *f/2* + GNY was better than that in *f/2* + G and *f/2* + GN (as can be seen from the maximum specific growth rates, $p < 0.001$ for the two *Halomonas* sp., and $p < 0.05$ for *Maricauda* sp.) because it contained a richer source of carbon, which is important for bacterial growth. The maximum specific growth rates of the *Halomonas* sp. WSR2 were comparable in *f/2* + R2A, *f/2* + GNY, and R2A + salt ($p > 0.05$). However, *Halomonas* sp. WS1 and *Maricauda* sp. WSR showed a higher growth rate in *f/2* + R2A compared to *f/2* + GNY and R2A + salt ($p < 0.05$ for *Halomonas* sp. WS1 and $p < 0.001$ for *Maricauda* sp. WSR). The growth profile also indicated a slower growth

of *Maricauda* sp. WSR in R2A + salt (Figure 4C), suggesting that *f/2* + R2A is a more suitable optimal medium to cultivate all three species. This medium contains sufficient concentrations of N and P to sustain the growth of bacteria and contain salt. All three bacterial species appeared to synchronise their growth to coincide with algal growth, as these growth rates are quite low for bacteria. This was also observed to be the case in earlier reports, e.g., [26,29]. Cohabiting bacteria rely on carbohydrate exudates from the algal culture to survive and may have evolved to grow on scant resources at slow rates.

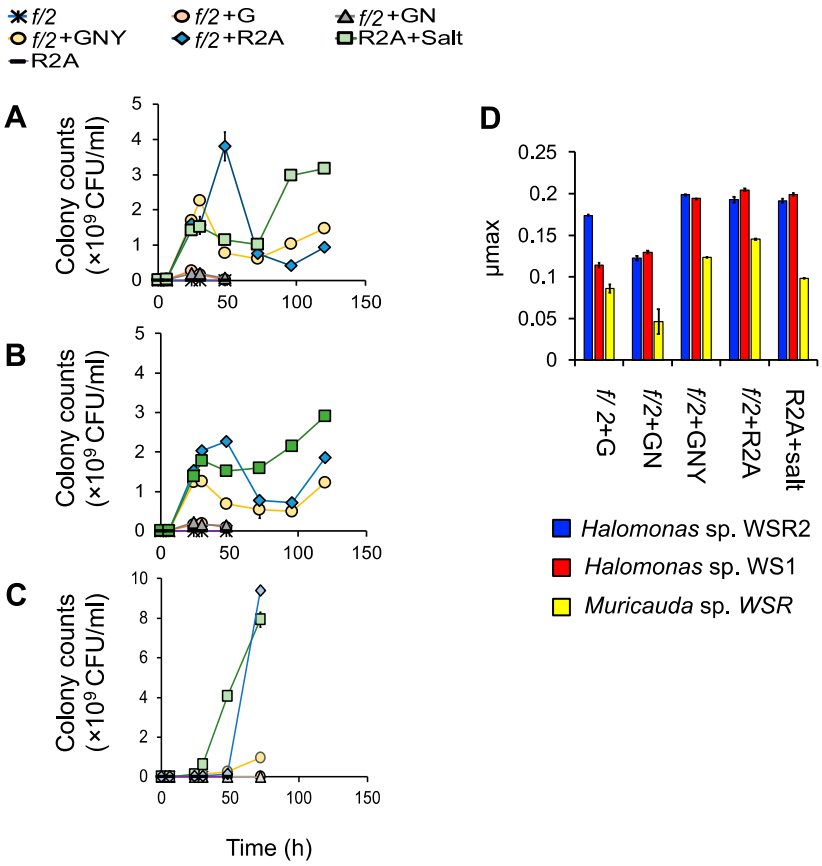


Figure 4. Growth rate by colonies count of bacterial isolates that grow in different media for optimal growth (**A**) *Halomonas* sp. WSR2, (**B**) *Halomonas* sp. WS1, (**C**) *Muricuda* sp. WSR, and (**D**) µmax of the growth rate of all three species.

2.4. Changes in Nutrient Supply Increase Cohabiting Bacterial Load and Minimise Population Diversity

The distributions of the three bacterial isolates in the three nutrient conditions (*f/4*, *f/2*, and *2f*) tested are shown in Figure 5 for three time points (days 0, 3, and 7) of algal cultivation. *Halomonas* sp. WSR2 (small colonies) can be seen to dominate the bacterial population under all conditions. *Muricauda* sp. WSR (yellow colonies) appeared on all days in *f/2* but appeared only on day 7 in the deplete (*f/4*) and day 3 in the replete (*2f*) cultures. This species appeared to be sensitive to changes in the nutrient supply, as movement to both replete (*2f*) and deplete conditions (*f/4*) from the control condition (*f/2*) resulted in lower cell counts or complete absence. *Halomonas* sp. WS1 (large colonies) showed a similar trend of reduced numbers in replete (*2f*) and deplete (*f/4*) conditions compared to *f/2*. *Halomonas* sp. WSR2 (small colonies), on the other hand, showed an increase in cell counts in both deplete (*f/4*) and replete (*2f*) conditions compared to the control (*f/2*). Environmental fluctuations are known to influence composition and behaviour in microbial communities [39], and changes in the bacterial composition of algae–bacterial co-cultures exposed to different nutrient regimes are known to occur [29]. *Muricauda* is sensitive to changes in nutrient levels, as has been noted before in algal co-cultures inhabited by this

bacterial species [40–42]. *Halomonas* is known to dominate among bacteria cohabiting algal cultures [41,43].

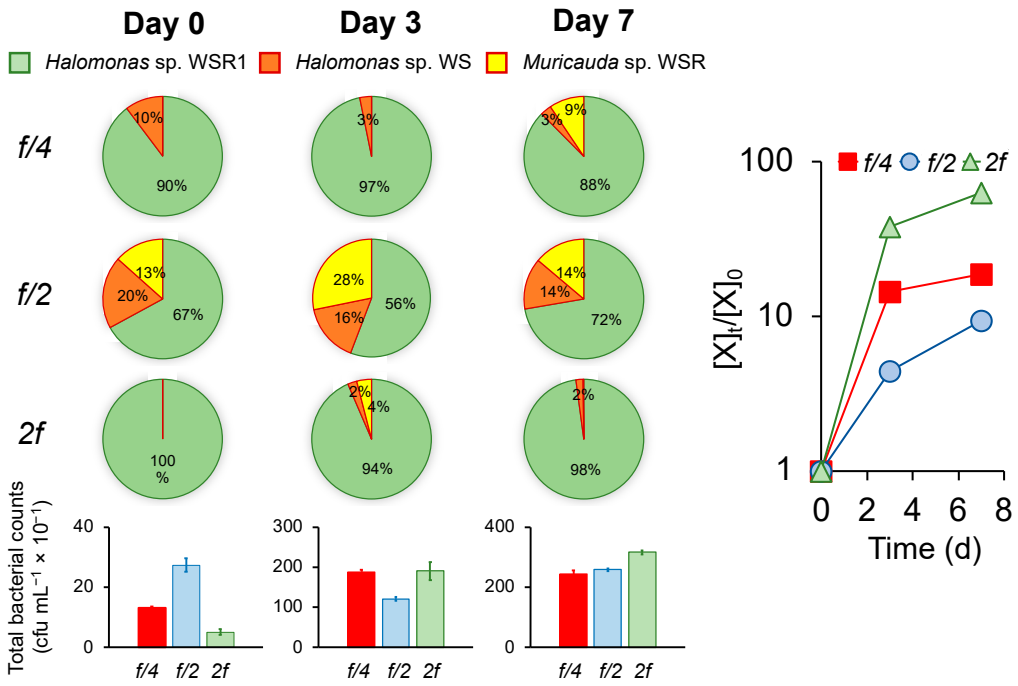


Figure 5. Distribution of three species of bacteria at three isolation points during microalgae cultivation in *f/2*, *f/4*, and *2f* media. Nutrient depletion increases carbohydrate yields but repletion increases productivity with an apparent loss of a bacterial isolate from the cohabiting population.

The increase in the bacterial load over the algal cultivation relative to the start ($[X]_t/[X]_0$) was minimal in *f/2*, whilst the two extreme conditions resulted in a greater increase, seen to be greater under replete compared to deplete conditions ($p < 0.001$ for day 3 and $p < 0.01$ for day 7). This is also reflected in the total counts on day 3 for the three conditions (ANOVA, $p < 0.001$). Both the type and concentration of the nitrogen source have been shown to influence the bacterial load in co-cultures [44]. Changes in the nutrient supply appear to significantly perturb the cohabiting bacterial composition of the algal culture (ANOVA, $p < 0.001$). However, interestingly, the total bacterial counts increased when the nutrient supply regime was changed. A more uniform bacterial composition could be seen throughout the *f/2*-grown cultures compared to the *f/4* and *2f* cultures, and the increase in the total bacterial load was also higher in *f/4* and *2f* compared to *f/2*.

The carbohydrate yield in the algal cells was highest (47% DW) on day 3 of the nutrient deplete condition (*f/4*), compared to a highest yield of 27% DW observed in the replete conditions (*2f*), which has been demonstrated in algal monocultures [23]. A number of other studies [4,7,45,46] have indicated that *C. vulgaris* (freshwater) accumulate a high carbohydrates content (42.3–50% DW) under nitrogen depletion. In our study, an 8-fold decrease in nitrogen (N) and phosphorus (P) supply resulted in a 75% increase in the carbohydrate yield ($p < 0.01$). However, when considering the maximum carbohydrate productivity, halving the N and P supply (*f/4*) resulted in reducing this productivity to 75% of the control (*f/2*), whereas a 4-fold increase in the N and P supply (*2f*) led to a 1.6-fold increase in the carbohydrate productivity ($p < 0.05$). The maximum carbohydrate productivity was reduced by 53% in the deplete (*f/4*) compared to the replete (*2f*) conditions. Yield and productivity were inversely proportional in this case, given the strong influence of nutrient depletion on biomass productivity. Interestingly, earlier reports on algal monocultures, e.g., [23], have reported increases in carbohydrate productivity and yield under nitrogen depletion conditions compared to replete conditions.

A statistically significant difference in the bacterial composition was detected for the three conditions on ANOVA ($p < 0.001$) at day 3, when the carbohydrate yield and productivity were at their highest (Figure 6). Both the depletion ($p < 0.001$) and repletion ($p < 0.05$) of nutrients resulted in a statistically significant increase in the total bacterial load at this time point. As noted earlier, *Halomonas* sp. WSR2 dominated the bacterial population (97%), with a much smaller contribution (3% of the population) by *Halomonas* sp. WS1. and no detection of *Muricauda* sp. WSR in the nutrient replete condition (*2f*), which showed the highest carbohydrate productivity, although this species could be detected under the other two conditions.

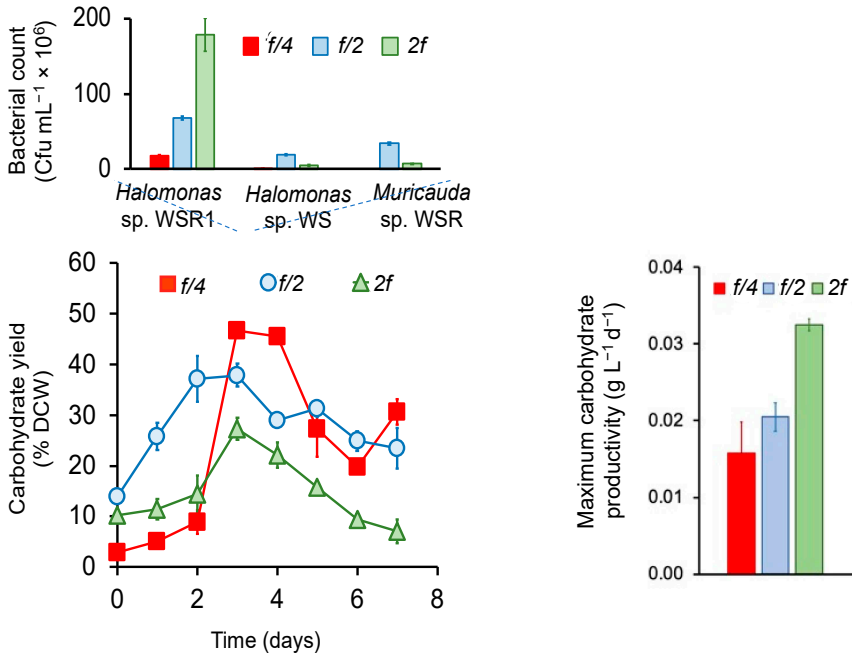


Figure 6. Time profile of carbohydrate yield (%DCW) of *C. vulgaris* in *f/2*, *f/4*, and *2f* media. The cohabiting bacterial distribution in the three media at a time point when the maximum yield was observed is plotted on top, as well as the maximum carbohydrate productivity at this time point, plotted alongside.

3. Discussion

In this study, we report that a non-axenic culture of *C. vulgaris*, which was found to have three cohabiting bacteria, responded strongly to changes in nitrogen and phosphorus supply with respect to its growth and biomass productivity. Previous studies have shown that nitrogen and phosphorus are essential for the growth of microalgae, and their optimal supply is vital to maintain a high cell density and elevated growth rate. Nitrogen plays an essential role, being an inherent component of proteins and the photosynthetic machinery. Phosphorus is incorporated in nucleic acids and is a critical component of cellular energy currency. It is well known that nitrogen deficiency can inhibit growth and cell division in microalgae. N-deficiency negatively affects algal growth, biomass concentration, and chlorophyll synthesis. A lack of nitrogen decreases the photosynthetic rate and diverts electron flow to producing sugars and lipids in microalgae [47,48]. P-deficiency likewise influences biomass growth and productivity. Phosphate is also known to influence biomass productivity in N-depleted cultures of *Chlorella* sp., with higher phosphate levels leading to higher productivities than lower phosphate levels [49]. An optimal level of phosphate can lead to a higher biomass productivity even under nitrogen depletion [50]. P limitation has been shown to influence biomass productivity to a lesser degree than *p*-depletion in freshwater *Chlorella* cultivations [51]. The absence of nitrogen or phosphorus (15–20% of the replete conditions) significantly reduced biomass productivity. In contrast, phosphorus limitation alone reduced biomass productivity to 92% of that observed under replete

conditions in a freshwater *C. vulgaris* cultivation. The observations in our study mostly mirrored these observations from earlier investigations, albeit in apparently axenic cultures.

The three cohabiting bacterial candidates were isolated, identified, and characterised within this study. These were found to have synergistic effects with respect to algal growth. Synergistic bacterial associations with microalgae are known to exist, where vitamins from bacteria are provided for the algae in exchange for organic carbon [13,14]. An increased bacterial load over the cultivation period in co-cultures has been frequently observed [34,44]. Changes in community compositions in bacteria associated with microalgae in response to nitrogen fluctuations in the environment are known [52], as well as designed nitrogen source variations affecting the bacterial composition in algae–bacterial consortia [53]. We show in this study that a shift in nutrient supply (both nitrogen depletion and repletion) reduces the diversity of the cohabiting bacterial population, despite increased bacterial growth in the co-cultures. This is interesting, as it provides evidence for using nutrient stress as a lever to control the bacterial population and its influence on the behaviour of the co-cultured algae.

In addition, changes in nitrogen supply have been shown to result in changes in the influence of bacteria on algal productivity in co-cultures [54]. The positive influence of bacteria such as *Azospirillum brasilense* [18,19] and *Bacillus pumilus* [19] in enhancing carbohydrate accumulations in *Chlorella sorokiniana* have been reported, as well as the effect of a combination of selected bacterial strains on enhancing the biochemical composition and biomass productivities in *Muriellopsis* sp. [16]. The enhancement of carbohydrates in biomass by 55% has also been reported in microalgae–bacteria consortia under low nitrate concentrations [20].

In this investigation, we highlighted the positive role of cohabiting bacteria in combination with nutrient alterations in enhancing carbohydrate accumulations in microalgae. We also showed that not all cohabiting bacteria behave in a similar manner and that there is merit in characterising the cohabiting bacterial population and studying its effect in co-cultures as an enabler to increase productivities.

4. Materials and Methods

4.1. Microalgae and Culture Condition

Chlorella vulgaris CCAP 211/21A was sourced from the Culture Collection of Algae and Protozoa (CCAP, Oban, Bodmin, UK). The microalgae were cultivated in sterile *f/2* medium, which consisted of (M): $NaNO_3$ 0.882; $NaH_2PO_4.H_2O$ 0.0362; trace metal stock: $FeCl_3.6H_2O$ 0.011; $Na_2EDTA.2H_2O$ 0.011; $CuSO_4.5H_2O$ 0.3×10^{-5}; $Na_2MoO_4.2H_2O$ 0.9×10^{-5}; $ZnSO_4.7H_2O$ 0.8×10^{-5}; $CoCl_2.6H_2O$ 0.4×10^{-4}; $MnCl_2.4H_2O$ 0.3×10^{-2}; vitamins: Thiamine HCl (B_1) 0.2×10^{-5}; Biotin 0.2×10^{-7}; Cyanocobalamin (B_{12}) 0.3×10^{-7}. An inoculum maintained in *f/2* medium was used to seed the culture with a starting OD_{595} of 0.25. The cultivations were carried out in 1 L Duran bottles with continuous stirring (with the help of a magnetic stirrer) and aeration (0.04% CO_2), with the intermittent use of 5% CO_2 for 1 h daily. The culture was irradiated with continuous illumination at 200 µmol photons/m^2/s of light (LED fluorescent lamp). Two additional modifications of the *f/2* medium were employed, one in which both the nitrate and phosphorus concentrations were halved (*f/4*) to simulate nutrient-limited conditions and the other in which both the concentrations were quadrupled (*2f*) to simulate nutrient replete conditions.

4.2. Isolation of Cohabiting Bacteria from Algae Culture

The bacterial population in algal cultivation media (*f/2* and modified *f/2* media) was detected by taking 100 µL of the algal culture at three different time points (the day of inoculation, third, and seventh day of cultivation), then diluting for inoculation on plates. R2A agar mixed with the respective algae culture media (*f/2*, *f/4*, or *2f*) was used to cultivate the bacteria. The agar plates were incubated at room temperature for 7 days. The colonies that formed were counted then sub-cultured to obtain pure isolates for further identification and characterisation.

4.3. Identification of Isolated Bacteria

The colony morphology of the bacterial isolates was studied, in addition to Gram staining and molecular typing.

4.4. 16S rRNA Molecular Identification of Isolated Bacteria

Colonies PCR 16S rRNA genes were carried out for isolated bacteria using 27F (5′ AGAGTTTGATCMTGGCTCAG-3′) as the forward primer and 1492R (5′-GGTT ACCTTGTTACGACTT-3′) as a reverse primer. A Phusion polymerase kit (NEB, Biolabs, Ipswich, MA, USA) was used to perform amplification of the target templates following the PCR kit manufacturer's guidelines. The total volume of PCR for all samples was 50 µL: 25 µL master mix, 2.5 µL each of forward and reverse primers, 1 µL of 20 of 20 ng/µL gDNA, and made up to 50 µL with nuclease-free water. The PCR was carried out in a thermocycler (Applied Biosystems, Waltham, MA, USA), applying the following conditions: an initial denaturing step at 98 °C for 30 s followed by 30 cycles of the denaturing step at 98 °C for 1 min, annealing at 58 °C for 10 s, and elongation at 55 °C for 1 min. Lastly, final elongation was performed at 72 °C for 5 min. The PCR products were cleaned up using a PCR clean up kit, and the products were analysed by a spectrophotometer (Thermo Scientific Nanodrop, 2000) to determine their concentration and were investigated by gel electrophoresis (MupidTM-one, Advance Co. Ltd., London, UK) using 1% agarose gel containing an aliquot of Gel Red (6 µL/100 mL) (Insight Biotechnology Ltd., London, UK). Moreover, the DNA concentration was determined by a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the absorbance ratio was at A260 nm. In total, 1 µL of bacterial DNA was used to measure the DNA quantity.

The PCR amplification products were purified using the QIAquick® PCR Purification Kit according to the manufacturer's instructions and analysed on 1% agarose gel (Figure 7). The concentration of the purified PCR products was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The primers used for DNA sequencing, designed for the molecular identification of the strains, included the Forward Primer and Reverse Primers used for DNA amplification. Primers were prepared at concentrations (10 µM each), and sequencing was performed using the Sanger method. The resulting sequences were analysed with sequencing analysis software (Sequencing Analysis Software, Thermo Fisher Scientific) for base calling. Quality trimming of the sequence data was conducted to remove low-quality bases from both ends of the sequence reads. The trimmed sequences were then aligned to reference sequences in the GenBank database using a standard nucleotide BLAST search to confirm the identity of the amplified fragments.

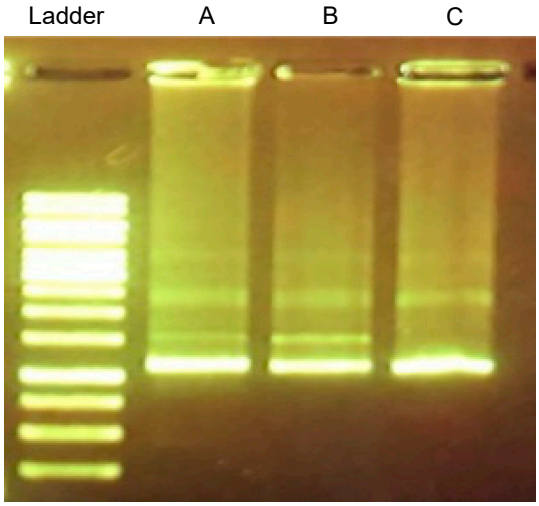


Figure 7. Agarose gel electrophoresis of microbial genomic DNA of *Halomonas* sp. WSR2 (A), *Halomonas* sp. WS1 (B), and *Muricuda* sp. WSR (C).

4.5. Experimentation of Media Composition for Growth of Cohabiting Bacteria in Isolation

The growth of the isolates' bacteria was monitored in different media that contained different sources of essential nutrients (with respect to C and N, primarily): *f/2*, R2A, *f/2* + Glucose (*f/2* + G), *f/2* + Glucose + Ammonium chloride (*f/2* + GN), *f/2* + G + Ammonium chloride + Yeast extract (*f/2* + GNY), R2A + salt, and *f/2* + R2A. The cultivations were carried out in broth for 72 h in a shaking tray at 150 rpm and room temperature 20 ± 1 °C. The samples were taken daily to observe the growth by CFU.

4.6. Analytical Methods

4.6.1. Microalgal Growth

Microalgal growth was measured using three approaches. The optical density (OD_{595nm}) of *C. vulgaris* was measured daily for 7 days using a UV-Vis spectrophotometer (SEPECTROstar NANO). The microalgal cell count was measured using a Haemocytometer. In addition, the dry cell weight (DCW) was measured by sampling 5 mL of culture, removing the culture medium using centrifugation, and freeze-drying the pellet after one wash.

The specific growth rate of the algae was calculated using the following Formula (1):

$$\mu = (\text{In } N_t - \text{In } N_0)/(t - t_0) \quad (1)$$

N_t is the algal biomass, OD, or cell count at the beginning of the exponential growth, and N_0 is the corresponding value at different time (t) intervals.

4.6.2. Dissolved Inorganic Nitrate and Phosphate

Dissolved inorganic nitrogen (DIN) was measured using the method of Collos et al. [55]. Briefly, 5 mL of algae was centrifuged at 4000 rpm for 10 min, 2 mL of the algal suspension was transferred into a glass cuvette, and the optical density was measured directly at 220 nm using a UV-Vis spectrophotometer (SEPECTROstar NANO, BMG Labtech, Ortenberg, Germany).

Dissolved inorganic phosphate (DIP) was measured using the method of Strickland and Parsons [56]. Briefly, 100 µL of mixed reagent (ammonium molybdate 1 mL, sulphuric acid 2.5 mL, ascorbic acid 1 mL, and potassium antimolnyl tartrate 0.5 mL) was added to 1 mL of culture filtered samples. The absorbance of the mixture was then measured after half an hour at 885 nm, and the phosphate concentrations were inferred from a standard graph.

4.6.3. Carbohydrate Assay

Carbohydrates in the microalgal cells were estimated by using the anthrone method, as a part of a combined biochemical assay [57]. Briefly, the pellets were destroyed by bead-beating for 30 min to release the biochemical components outside the cells. In total, 100 µL of supernatant was taken, then, 400 µL of pre-chilled 75% H_2SO_4 and 800 µL of anthrone reagent (25 mg of anthrone, 500 µL of ethanol, and 12 mL of 75% H_2SO_4) were added to the samples. The samples were thoroughly mixed and incubated at 100 °C for 15 min and measured at 578 nm. Glucose was used to create a standard curve to estimate the carbohydrate concentration in the algae cells.

4.7. Scanning Electron Microscopy (SEM)

The samples were processed using an electron microscope unit, as detailed elsewhere [58]. The samples were fixed on a glass slide with glutaraldehyde (1.5%) and kept at 4 °C for 24 h. Then, the samples were washed with ultra-pure water and dehydrated by 25–100% Ethanol. The fixed samples were coated with gold and observed under SEM (TESCAN Vega 3 LMU, Tescan UK, Cambridge, UK). The Scanning Electron Microscope had an accelerating voltage of 15 kV.

4.8. Statistical Analysis

All algal and bacterial experiments were carried out in triplicates, and so were biological replicates. Data are shown as mean and standard error about the mean. A one-way analysis of variance (ANOVA) test was applied to assess the statistical significance of the

influence of conditions on the various outcomes. In some instances, a two-way ANOVA was carried out when two factors were considered. A *t*-test with unequal variance was employed to assess thr statistical significance for comparing two sets of data. Where *p* values are reported, these are for the *t*-test, except in cases where ANOVA is mentioned.

5. Conclusions

In summary, three species of cohabiting bacteria (belonging to *Halomonas* and *Muricauda* sp.) were detected in a *C. vulgaris* non-axenic cultivation. *Halomonas* sp. WSR2 was the dominant species under different nutrient conditions. The bacterial population increased over time with the microalgae, indicating a synergistic relationship with the algae. The nutrient availability in the culture not only affected the algal growth, but also influenced the cohabiting bacterial load. Three bacterial isolates were detected at all isolation points in *f/2*, but only one of those was dominant when the nutrient levels were increased (*2f*) or decreased (*f/4*). Nutrient depletion resulted in increased carbohydrate yields, but repletion increased productivities, with an apparent loss of *Muricauda* sp. This study provides evidence for using nutrient stress as a lever to control the bacterial population and its influence on the behaviour of the co-cultured algae. Further understanding with respect to the influence of the cohabiting bacteria on the algal cultures will enable appropriate applications of this approach to be developed.

Author Contributions: Conceptualization, W.J.A. and S.V.; methodology, W.J.A.; formal analysis, W.J.A.; investigation, W.J.A., A.O.N. and S.V.; resources, S.V.; data curation, S.V.; writing—original draft preparation, W.J.A.; writing—review and editing, A.O.N. and S.V.; supervision, S.V.; project administration, S.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no direct external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: W.J.A. acknowledges financial support by the Embassy of Saudi Arabia for pursuing a PhD at the University of Sheffield. This work was carried out as a part of the PhD. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Quintana, N.; Van der Kooy, F.; Van de Rhee, M.D.; Voshol, G.P.; Verpoorte, R. Renewable energy from Cyanobacteria: Energy production optimization by metabolic pathway engineering. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 471–490. [CrossRef]
2. Chen, C.Y.; Zhao, X.Q.; Yen, H.W.; Ho, S.H.; Cheng, C.L.; Lee, D.J.; Bai, F.W.; Chang, J.S. Microalgae-based carbohydrates for biofuel production. *Biochem. Eng. J.* **2013**, *78*, 1–10. [CrossRef]
3. Yuan, H.; Zhang, X.; Jiang, Z.; Wang, X.; Wang, Y.; Cao, L.; Zhang, X. Effect of light spectra on microalgal biofilm: Cell growth, photosynthetic property, and main organic composition. *Renew. Energy* **2020**, *157*, 83–89. [CrossRef]
4. Ho, S.H.; Huang, S.W.; Chen, C.Y.; Hasunuma, T.; Kondo, A.; Chang, J.S. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresour. Technol.* **2013**, *135*, 191–198. [CrossRef] [PubMed]
5. Markou, G.; Angelidaki, I.; Georgakakis, D. Microalgal carbohydrates: An overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 631–645. [CrossRef] [PubMed]
6. Kawai, S.; Murata, K. Biofuel production based on carbohydrates from both brown and red macroalgae: Recent developments in key biotechnologies. *Int. J. Mol. Sci.* **2016**, *17*, 145. [CrossRef] [PubMed]
7. de Farias Silva, C.E.; Sforza, E. Carbohydrate productivity in continuous reactor under nitrogen limitation: Effect of light and residence time on nutrient uptake in *Chlorella vulgaris*. *Process Biochem.* **2016**, *51*, 2112–2118. [CrossRef]
8. Padmaperuma, G.; Kapoore, R.V.; Gilmour, D.J.; Vaidyanathan, S. Microbial consortia: A critical look at microalgae co-cultures for enhanced biomanufacturing. *Crit. Rev. Biotechnol.* **2018**, *38*, 690–703. [CrossRef] [PubMed]

9. Kapoore, R.V.; Padmaperuma, G.; Maneein, S.; Vaidyanathan, S. Co-culturing microbial consortia: Approaches for applications in biomanufacturing and bioprocessing. *Crit. Rev. Biotechnol.* **2021**, *42*, 46–72. [CrossRef]
10. González-González, L.M.; de-Bashan, L.E. Toward the Enhancement of Microalgal Metabolite Production through Microalgae–Bacteria Consortia. *Biology* **2021**, *10*, 282. [CrossRef]
11. Yee, C.S.; Okomoda, V.T.; Hashim, F.; Waiho, K.; Abdullah, S.R.S.; Alamanjo, C.; Hasan, H.A.; Mustafa, E.M.; Kasan, N.A. Marine microalgae co-cultured with floc-forming bacterium: Insight into growth and lipid productivity. *Peer J.* **2021**, *9*, e11217. [CrossRef] [PubMed]
12. Commault, A.S.; Laczka, O.; Siboni, N.; Tamburic, B.; Crosswell, J.R.; Seymour, J.R.; Ralph, P.J. Electricity and biomass production in a bacteria-*Chlorella* based microbial fuel cell treating wastewater. *J. Power Sources* **2017**, *356*, 299–309. [CrossRef]
13. Croft, M.T.; Lawrence, A.D.; Raux-Deery, E.; Warren, M.J.; Smith, A.G. Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. *Nature* **2005**, *438*, 90–93. [CrossRef] [PubMed]
14. Kazamia, E.; Czesnick, H.; Nguyen, T.T.V.; Croft, M.T.; Sherwood, E.; Sasso, S.; Hodson, S.J.; Warren, M.J.; Smith, A.G. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. *Environ. Microbiol.* **2012**, *14*, 1466–1476. [CrossRef] [PubMed]
15. Gonçalves, A.L.; Pires, J.C.; Simões, M. Biotechnological potential of Synechocystis salina co-cultures with selected microalgae and cyanobacteria: Nutrients removal, biomass and lipid production. *Bioresour. Technol.* **2016**, *200*, 279–286. [CrossRef]
16. Marticorena, P.; Gonzalez, L.; Riquelme, C.; Silva Aciares, F. Effects of beneficial bacteria on biomass, photosynthetic parameters and cell composition of the microalga *Muriellopsis* sp. adapted to grow in seawater. *Aquac. Res.* **2020**, *51*, 3609–3622. [CrossRef]
17. Huo, S.; Kong, M.; Zhu, F.; Qian, J.; Huang, D.; Chen, P.; Ruan, R. Co-culture of *Chlorella* and wastewater-borne bacteria in vinegar production wastewater: Enhancement of nutrients removal and influence of algal biomass generation. *Algal Res.* **2020**, *45*, 101744. [CrossRef]
18. Lopez, B.R.; Palacios, O.A.; Bashan, Y.; Hernández-Sandoval, F.E.; de-Bashan, L.E. Riboflavin and lumichrome exuded by the bacterium *Azospirillum brasilense* promote growth and changes in metabolites in *Chlorella sorokiniana* under autotrophic conditions. *Algal Res.* **2019**, *44*, 101696. [CrossRef]
19. Amavizca, E.; Bashan, Y.; Ryu, C.M.; Farag, M.A.; Bebout, B.M.; de-Bashan, L.E. Enhanced performance of the microalga *Chlorella sorokiniana* remotely induced by the plant growth-promoting bacteria *Azospirillum brasilense* and *Bacillus pumilus*. *Sci. Rep.* **2017**, *7*, 1–11.
20. Biswas, T.; Bhushan, S.; Prajapati, S.K.; Chaudhuri, S.R. An eco-friendly strategy for dairy wastewater remediation with high lipid microalgae-bacterial biomass production. *J. Environ. Manag.* **2021**, *286*, 112196. [CrossRef]
21. Slocombe, S.P.; Huete-Ortega, M.; Kapoore, R.V.; Okurowska, K.; Mair, A.; Day, J.G.; Stanley, M.S.; Vaidyanathan, S. Enabling large-scale production of algal oil in continuous output mode. *Iscience* **2021**, *24*, 102743. [CrossRef]
22. Guillard, R.R. Culture of phytoplankton for feeding marine invertebrates. In *Culture of Marine Invertebrate Animals*; Springer: Boston, MA, USA, 1975; pp. 29–60.
23. Mayers, J.J.; Vaiciulyte, S.; Malmhäll-Bah, E.; Alcaide-Sancho, J.; Ewald, S.; Godhe, A.; Ekendahl, S.; Albers, E. Identifying a marine microalgae with high carbohydrate productivities under stress and potential for efficient flocculation. *Algal Res.* **2018**, *31*, 430–442. [CrossRef]
24. Keshtacher-Liebso, E.; Hadar, Y.; Chen, Y. Oligotrophic bacteria enhance algal growth under iron-deficient conditions. *Appl. Environ. Microbiol.* **1995**, *61*, 2439–2441. [CrossRef]
25. Zhang, Q.; Zhang, C.; Zhu, Y.; Yuan, C.; Zhao, T. Effect of bacteria-to-algae volume ratio on treatment performance and microbial community of a novel heterotrophic nitrification-aerobic denitrification bacteria-*Chlorella* symbiotic system. *Bioresour. Technol.* **2021**, *342*, 126025. [CrossRef]
26. Han, J.; Zhang, L.; Wang, S.; Yang, G.; Zhao, L.; Pan, K. Co-culturing bacteria and microalgae in organic carbon containing medium. *J. Biol. Res.-Thessalon.* **2016**, *23*, 8. [CrossRef] [PubMed]
27. Fulbright, S.P.; Robbins-Pianka, A.; Berg-Lyons, D.; Knight, R.; Reardon, K.F.; Chisholm, S.T. Bacterial community changes in an industrial algae production system. *Algal Res.* **2018**, *31*, 147–156. [CrossRef] [PubMed]
28. Yan, Z.; Li, W.; Shen, T.; Wang, X.; Zhu, M.; Yu, L. Aquatic microalgal and bacterial communities in a karst wetland watershed. *J. Hydrol.* **2020**, *591*, 125573. [CrossRef]
29. Tait, K.; White, D.A.; Kimmance, S.A.; Tarran, G.; Rooks, P.; Jones, M.; Llewellyn, C.A. Characterisation of bacteria from the cultures of a *Chlorella* strain isolated from textile wastewater and their growth enhancing effects on the axenic cultures of *Chlorella vulgaris* in low nutrient media. *Algal Res.* **2019**, *44*, 101666. [CrossRef]
30. Zhu, J.; Hong, P.; Wang, S.; Hu, Z.; Wang, H. *Phycocomes zhengii* gen. nov., sp. nov., a marine bacterium of the family Rhodobacteraceae isolated from the phycosphere of *Chlorella Vulgaris*. *Int. J. Syst. Evol. Microbiol.* **2019**, *69*, 535–541.
31. Makut, B.B.; Das, D.; Goswami, G. Production of microbial biomass feedstock via co-cultivation of microalgae-bacteria consortium coupled with effective wastewater treatment: A sustainable approach. *Algal Res.* **2019**, *37*, 228–239. [CrossRef]
32. Watanabe, K.; Takihana, N.; Aoyagi, H.; Hanada, S.; Watanabe, Y.; Ohmura, N.; Saiki, H.; Tanaka, H. Symbiotic association in *Chlorella* culture. *FEMS Microbiol. Ecol.* **2005**, *51*, 187–196. [CrossRef] [PubMed]
33. Xue, L.; Shang, H.; Ma, P.; Wang, X.; He, X.; Niu, J.; Wu, J. Analysis of growth and lipid production characteristics of *Chlorella vulgaris* in artificially constructed consortia with symbiotic bacteria. *J. Basic Microbiol.* **2018**, *58*, 358–367. [CrossRef] [PubMed]
34. Cho, D.H.; Ramanan, R.; Heo, J.; Lee, J.; Kim, B.H.; Oh, H.M.; Kim, H.S. Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. *Bioresour. Technol.* **2015**, *175*, 578–585. [CrossRef] [PubMed]

35. Park, Y.; Je, K.W.; Lee, K.; Jung, S.E.; Choi, T.J. Growth promotion of *Chlorella ellipsoidea* by co-inoculation with *Brevundimonas* sp. isolated from the microalga. *Hydrobiologia* **2008**, *598*, 219–228. [CrossRef]
36. Ferro, L.; Colombo, M.; Posadas, E.; Funk, C.; Muñoz, R. Elucidating the symbiotic interactions between a locally isolated microalga *Chlorella vulgaris* and its co-occurring bacterium *Rhizobium* sp. in synthetic municipal wastewater. *J. Appl. Phycol.* **2019**, *31*, 2299–2310. [CrossRef]
37. Xu, M.; Xue, Z.; Sun, S.; Zhao, C.; Liu, J.; Liu, J.; Zhao, Y. Co-culturing microalgae with endophytic bacteria increases nutrient removal efficiency for biogas purification. *Bioresour. Technol.* **2020**, *314*, 123766. [CrossRef] [PubMed]
38. Irshad, A.; Ahmad, I.; Kim, S.B. Culturable diversity of halophilic bacteria in foreshore soils. *Braz. J. Microbiol.* **2014**, *45*, 563–572. [CrossRef] [PubMed]
39. Nguyen, J.; Lara-Gutiérrez, J.; Stocker, R. Environmental fluctuations and their effects on microbial communities, populations and individuals. *FEMS Microbiol. Rev.* **2021**, *45*, fuaa068. [CrossRef]
40. Wu, Z.; Lee, W.H.; Liu, Z.; Lin, S.; Lam, P.K. Microbiome associated with *Gambierdiscus balechii* cultures under different toxicity conditions. *Front. Mar. Sci.* **2022**, *9*, 760553. [CrossRef]
41. Ma, Y.; Ding, W.; Wang, Y.; Chen, P.; Zhou, H.; Li, X.; Huang, Y.; Nan, P. Environmental factors shape the culturable bacterial diversity in deep-sea water and surface sediments from the Mariana Trench. *bioRxiv* **2022**. [CrossRef]
42. Brauer, V.S.; Stomp, M.; Bouvier, T.; Fouilland, E.; Leboulanger, C.; Confurius-Guns, V.; Weissing, F.J.; Stal, L.; Huisman, J. Competition and facilitation between the marine nitrogen-fixing cyanobacterium *Cyanothece* and its associated bacterial community. *Front. Microbiol.* **2015**, *5*, 795. [CrossRef] [PubMed]
43. Subasankari, K.; Thanappan, V.; Jeyapragash, D.; Anantharaman, P.; Sarangi, R.K. Growth promoting studies on co-culturing *Nannochloropsis oceanica* with *Halomonas aquamarina* actively enhance the algal biomass and lipid production. *Biocatal. Agric. Biotechnol.* **2020**, *29*, 101790.
44. Perera, I.A.; Abinandan, S.; Subashchandrabose, S.R.; Venkateswarlu, K.; Naidu, R.; Megharaj, M. Impact of nitrate and ammonium concentrations on co-culturing of *Tetradesmus obliquus* IS2 with *Variovorax paradoxus* IS1 as revealed by phenotypic responses. *Microb. Ecol.* **2022**, *83*, 951–959. [CrossRef] [PubMed]
45. Dragone, G.; Fernandes, B.D.; Abreu, A.P.; Vicente, A.A.; Teixeira, J.A. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Appl. Energy* **2011**, *88*, 3331–3335. [CrossRef]
46. Nordin, N.; Yusof, N.; Maeda, T.; Mustapha, N.A.; Yusoff, M.Z.M.; Khairuddin, R.F.R. Mechanism of carbon partitioning towards starch and triacylglycerol in *Chlorella vulgaris* under nitrogen stress through whole-transcriptome analysis. *Biomass Bioenergy* **2020**, *138*, 105600. [CrossRef]
47. Li, X.; Li, W.; Zhai, J.; Wei, H.; Wang, Q. Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. *Bioresour. Technol.* **2019**, *273*, 368–376. [CrossRef] [PubMed]
48. Cho, S.H.; Kim, J.; Han, J.; Lee, D.; Kim, H.J.; Kim, Y.T.; Cheng, X.; Xu, Y.; Lee, J.; Kwon, E.E. Bioalcohol production from acidogenic products via a two-step process: A case study of butyric acid to butanol. *Appl. Energy* **2019**, *252*, 113482. [CrossRef]
49. Zhu, S.; Wang, Y.; Xu, J.; Shang, C.; Wang, Z.; Xu, J.; Yuan, Z. Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella* sp. under nitrogen depletion. *Bioresour. Technol.* **2015**, *198*, 165–171. [CrossRef]
50. Nayak, M.; Suh, W.I.; Cho, J.M.; Kim, H.S.; Lee, B.; Chang, Y.K. Strategic implementation of phosphorus repletion strategy in continuous two-stage cultivation of *Chlorella* sp. HS2: Evaluation for biofuel applications. *J. Environ. Manag.* **2020**, *271*, 111041. [CrossRef]
51. Shen, X.F.; Chu, F.F.; Lam, P.K.; Zeng, R.J. Biosynthesis of high yield fatty acids from *Chlorella vulgaris* NIES-227 under nitrogen starvation stress during heterotrophic cultivation. *Water Res.* **2015**, *81*, 294–300. [CrossRef]
52. Wang, M.; Shi, L.D.; Wang, Y.; Hu, S.Q.; Tao, X.M.; Tian, G.M. The evolution of bacterial community structure and function in microalgal-bacterial consortia with inorganic nitrogen fluctuations in piggery digestate. *J. Clean. Prod.* **2021**, *315*, 128120. [CrossRef]
53. Poddar, N.; Sen, R.; Martin, G.J. Glycerol and nitrate utilisation by marine microalgae *Nannochloropsis salina* and *Chlorella* sp.; associated bacteria during mixotrophic and heterotrophic growth. *Algal Res.* **2018**, *33*, 298–309. [CrossRef]
54. Kim, H.J.; Choi, Y.K.; Jeon, H.J.; Bhatia, S.K.; Kim, Y.H.; Kim, Y.G.; Choi, K.Y.; Kim, H.J.; Lee, S.H.; Lee, Y.K.; et al. Growth promotion of *Chlorella vulgaris* by modification of nitrogen source composition with symbiotic bacteria, *Microbacterium* sp. HJ1. *Biomass Bioenergy* **2015**, *74*, 213–219. [CrossRef]
55. Collos, Y. A linear model of external interactions during uptake of different forms of inorganic nitrogen by microalgae. *J. Plank. Res.* **1989**, *11*, 521–533. [CrossRef]
56. Strickland, J.D.H.; and Parsons, T.R. Determination of dissolved oxygen. in A Practical Handbook of Seawater Analysis. *Fish. Res. Board Can. Bull.* **1968**, *167*, 71–75.
57. Chen, Y.; Vaidyanathan, S. A simple, reproducible and sensitive spectrophotometric method to estimate microalgal lipids. *Anal. Chim. Acta* **2012**, *724*, 67–72. [CrossRef]
58. Guo, Z.; Tong, Y.W. The interactions between *Chlorella vulgaris* and algal symbiotic bacteria under photoautotrophic and photoheterotrophic conditions. *J. Appl. Phycol.* **2014**, *26*, 1483–1492. [CrossRef]