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Impact of Phycosphere-Isolated Marine Bacteria on Nutritional Value, Growth, and Nutrient Uptake of Co-Cultured *Chaetoceros calcitrans*

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Abstract: Microalgae offer distinct advantages as a nutritional source for aquaculture and as a means of wastewater bioremediation. Studying the phycosphere bacteria and understanding their complex interactions is essential to optimizing high-quality biomass growth. This study aimed to isolate, characterize, and identify bacteria from the phycosphere of marine microalgae and to determine their potential to enhance growth, metabolism, and bioremediation capabilities of *Chaetoceros calcitrans* in stress nutrient-poor media simulating aquaculture wastewater enriched with nitrate, nitrite, or phosphorus. Bacterial characterization included tests for auxin and siderophore production, biofilm formation, amylase activity, phosphate solubilization, mobility, and antagonism evaluation. When *Alteromonas macleodii*, *Bacillus cereus*, and *Marinobacter* sp. were selected and then enriched (10^7 CFU/mL) in co-culture with *C. calcitrans*, growth levels significantly increased in four of six Synthetic Aquaculture Wastewater (SAW) media. Pigment levels were higher in five of six SAW media, and lipid levels were higher in SAW rich in nitrite (SAWni50) and phosphorus (SAWpho50). In addition, *C. calcitrans* with or without the bacterial consortium demonstrated excellent phosphorus bioremediation, achieving 67.6% average removal in SAWpho50. Nitrate and nitrite assimilation rates were approximately 10% in SAWna and SAWni50. This study marks the inaugural identification of these bacteria as microalga growth-promoting bacteria (MGPB) for enhancing growth and lipid and pigment production in *C. calcitrans*, and it also documents a maximum of 69.13% phosphorus removal.

Keywords: bacterial enrichment; phycoremediation; marine microalgae; wastewater; aquaculture

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1. Introduction

Microalgae are crucial for their high nutritional value and are especially important as feed for larvae in aquaculture [1]. Microalgae are also known for their phycoremediation action, defined as the use of algae to treat waste or wastewater [2]; they assimilate the key components of aquaculture effluents—nitrogen (N) and phosphorus (P)—as evidenced by numerous studies [3–5]. Therefore, microalgal production in aquaculture wastewater could generate economically valuable biomass and reduce contamination simultaneously.

Microalgae such as *Chaetoceros calcitrans* and *Isochrysis galbana* are commonly used species in the larviculture of marine fish, shrimp, prawns, and oysters [6]. Specifically, *Chaetoceros calcitrans* is a single-celled marine diatom of the *Chaetocerotaceae* family, with high-quality Eicosapentaenoic acid (EPA) lipid composition, demonstrating remarkable bioremediation capabilities [5,7,8].

Microalgae are surrounded by a bacterial phycosphere that is often understudied. The "phycosphere" refers to the microenvironment surrounding a microalga, comprising the algal cell surface and the immediate surrounding medium [9]. The phycosphere of the microalgae hosts diverse bacteria, including microalga growth-promoting bacteria (MGPB) [10]. These bacteria enhance microalgal growth and health by producing auxin molecules, a hormone that has been demonstrated to have a positive impact on microalgal development [11,12], and siderophores, crucial for improving microalgal growth, metabolism, and stress resistance [13]. Bacteria can also provide or help to assimilate several essential nutrients to nourish the microalgae, especially phosphorus and nitrogen [14], as well as sources of carbon like starch [15], frequently found as waste in aquaculture ponds, deriving from uneaten or poorly assimilated feed. The microalgal phycosphere is a complex community of interacting bacteria [16]. Biofilm formation and bacterial mobility are crucial for defending against threats, adapting to environmental changes, and facilitating nutrient uptake [17].

Recent studies have demonstrated the usefulness of cultivating bacteria from the microalgal phycosphere for growth [18], metabolism [19], and bioremediation [20]. Hence, the search for bacteria from the microalgal phycosphere that can enhance microalgal capabilities is a highly promising avenue of research. Therefore, the objectives of this study were (a) to isolate and characterize culturable bacteria associated with marine microalgae and design a biocompatible bacterial consortium with *C. calcitrans*; (b) to formulate a Synthetic Aquaculture Wastewater (SAW) to cultivate *C. calcitrans* in a stressful medium akin to aquaculture effluents; and (c) to determine in vitro the effects of this consortium on the growth and composition of the microalgae and on their bioremediation capacity.

2. Materials and Methods

2.1. Isolation and Identification of Phycosphere Bacteria

Triplicates of 100 µL aliquots from exponential phase cultures of *Chaetoceros calcitrans* and *Isochrysis galbana* were spread onto plates containing marine agar 2216 [21] to obtain the culturable fraction of the marine bacteria. The plates were incubated for 24 h at 25 °C and then examined for bacterial growth. Morphologically different, grown colonies were observed and selected using a stereoscope (Olympus VMT-C011, 1×, 4× Mod 296709, Hicksville, NY, USA). Twelve purified bacterial strains were evaluated in the present study. Bacteria were grown in marine broth 2216, and the total genomic DNA of each bacterium was extracted using a Promega kit (Wizard Genomic DNA Purification Kit), according to the manufacturer's instructions. Migration of the different bacterial DNAs on a 1.5% agarose gel was used to check the purity of the DNAs. Genome sequencing was performed using the Illumina Miniseq platform (300 cycles, 2 × 150 bp), and the genomic DNA libraries were prepared using the Nextera®XT Library at the Microbial Genomics and Molecular Diagnostics Laboratory of the Centro de Investigación en Alimentación y Desarrollo (CIAD, México). A bioinformatic analysis of the genomes was performed using the anvio6VM.v1.0.0.aov software taught at the CIAD. The full genome DNA sequences were cleaned (Fastx-trimmer), assembled (Pear), annotated (Prokka), and then analyzed (NCBI). Criteria for identification included an e-value under e-35 and sequence similarity over 98% to sequences deposited in GenBank at the time of analysis (March 2023).

2.2. Qualitative Characterization of Phycosphere Bacterial Strains

2.2.1. Auxin Production

The initial selection was designed to identify bacteria capable of stimulating the growth of marine microalgae. A test on the auxin production of each bacterium was conducted adapting the method of Ref. [22]. A 50 mL flask containing 25 mL of 2216 marine broth (50% *w*/*v*), 200 µg/mL L-tryptophan serving as a precursor for auxin biosynthesis, and 100 µL of bacterial inoculum (OD $0.4_{\lambda=540nm}$) was prepared. After four days of incubation at 30 °C with constant agitation (125 rpm), Salkowski's reagent determined auxin concentration in the supernatant. Microplates were incubated in the dark for 30 min, with a color change

from pink to red indicating auxin production, measured at 530 nm absorbance. A standard curve using 50% (*w*/*v*) 2216 marine broth covered auxin concentrations ranging from 0.78 to 50 µg/mL.

2.2.2. Siderophore Production

Siderophore production was evaluated with adaptations to a liquid medium using spectrophotometric methods, following the procedure outlined by Ref. [23]. In a 50 mL flask, 25 mL of 10% (*w*/*v*) 2216 marine broth and 100 µL of bacterial inoculum (OD $0.4_{\lambda=540nm}$) were combined. The flasks were then incubated for four days at 30 °C with constant agitation at 125 rpm. After incubation, 100 µL samples of the supernatants were transferred to a microplate, adding 200 µL of CAS solution [24]. Plates were then incubated for four hours at room temperature. Finally, absorbance was measured at 630 nm, and the percentage of siderophore units was calculated using the formula:

$$\% \text{ Siderophore units} = (AR - AS)/AR \times 100 \quad (1)$$

where AR = absorbance of reference (blank) and AS = absorbance of the sample.

2.2.3. Amylase Activity and Phosphate Solubilization

The second selection was about bacterial strains with properties for aquaculture wastewater treatment, such as the metabolism of carbohydrates and Ca-P solubilization. For that, extracellular amylase activity and phosphate solubilization of the 12 bacterial strains were assessed following the protocols outlined by Refs. [25,26]. For amylase activity, bacterial suspensions (OD $0.4_{\lambda=540nm}$) were inoculated onto starch-enriched plates and incubated for 48–72 h at 30 °C. The presence of starch solubilization halos was confirmed by adding 20% Lugol's solution. Following Ref. [27]'s recommendations, plates inoculated with bacterial suspensions (OD $0.4_{\lambda=540nm}$) were incubated at 30 °C for five days to observe the presence or absence of phosphate solubilization halos, using hydroxyapatite and calcium phosphate.

2.2.4. Specific Biofilm Formation

The specific biofilm formation (SBF) index was assessed according to Ref. [28] with some adaptations. The bacterial inoculum (OD $0.4_{\lambda=540nm}$) was added to 20 mL of 2216 marine broth at 50% (*w*/*v*) and incubated at 30 °C for 16 h at 125 rpm. Then, sterile microplates were filled with 250 µL of these bacterial cultures and incubated at 30 °C without shaking for 24 h. Simultaneously, another microplate was filled with the different samples and read at 600 nm to measure the cell growth of the bacterial suspension culture (G). At the end of the incubation periods, 50 µL of 3% (*w*/*v*) crystal violet was added. Plates were left to stand for 15 min, rinsed four times in water, and dried for at least four hours. Optical density measurement was performed at 540 nm and the specific biofilm formation (SBF) index was calculated using the following formula:

$$SBF = (AB - CW)/G, \quad (2)$$

where SBF = specific biofilm formation index, AB = $O.D._{\lambda=540nm}$ of the bacteria adhered to the microplate well wall, CW = $O.D._{\lambda=540nm}$ of the colored control wells containing bacteria-free medium only, and G = $O.D._{\lambda=600nm}$ of the cell growth from bacterial suspension culture.

2.2.5. Bacterial Motility

Bacterial swimming motility assays were conducted using Petri dishes containing 2216 marine broth with agar concentrations of 0.5% (*w*/*v*) [29]. Bacterial biomass was inoculated at the center and up to half the depth of the agar. Plates were then incubated at 30 °C for 24 h for all assessments. The diameters of the bacterial halos formed were ultimately measured.

2.3. Antagonism Assay

A volume of 10 µL of the bacterial suspensions (OD $0.4_{\lambda=540nm}$) of the possible antagonistic bacteria was dropped onto Petri dishes containing 2216 marine agar and left in the laminar flow hood for 20 min. The plates were later incubated for 24 h at 30 °C. Once bacterial growth was observed, the plates were refrigerated (4 °C) for 4–8 h to enable exudates to diffuse onto the agar. At the end of the cooling period, 5 µL of the potential antagonist strain suspension (OD $0.4_{\lambda=540nm}$) was dropped onto the sides of the previously inoculated colony. The plates were then incubated at 30 °C for 24 h. After this period, the presence or absence of halos attributed to growth inhibition or competitive exclusion effect was observed.

2.4. Design of Synthetic Aquaculture Wastewater

The 6 tested SAWs were composed of a base medium, Instant Ocean®, which simulates seawater composition and is devoid of nitrogen or phosphorus sources. Instant Ocean® has been employed in various studies [30,31]. Vital ingredients such as silicate, vitamins, or metals found in the F/2 medium, were supplemented for the optimal growth of *C. calcitrans*. The nitrogen or phosphorus content in the SAWna50, SAWni50, and SAWpho50 media was equivalent to a concentration of 50 mg/L Similarly, in the SAWna100, SAWni100, and SAWpho100 media, the N or P content corresponded to a concentration of 100 mg/L (Table 1).

Table 1. Composition of designed Synthetic Aquaculture Wastewater (SAW) (mg/L).

	SAWna		**SAWni**		**SAWpho**	
	SAWna50	**SAWna100**	**SAWni50**	**SAWni100**	**SAWpho50**	**SAWpho100**
$NaNO_3$	303.41	606.81	/	/	37.50	37.50
$NaNO_2$	/	/	246.29	492.58	/	/
NaH_2PO_4 H_2O	1.25	1.25	1.25	1.25	222.72	445.43
N_aSiO_3 $9H_2O$	15.00	15.00	15.00	15.00	15.00	15.00
Metals	3.90	3.90	3.90	3.90	3.90	3.90
Vitamins	3.34×10^{-5}	3.34×10^{-5}	3.34×10^{-5}	3.34×10^{-5}	3.34×10^{-5}	3.34×10^{-5}
Instant Ocean	35.79	35.79	35.79	35.79	35.79	35.79

(50): 50 mg/L; (100): 100 mg/L. na: nitrate; ni: nitrite; pho: phosphorous.

2.5. Co-Culture of Chaetoceros Calcitrans with the Bacterial Consortium in SAW

The microalga *Chaetoceros calcitrans* (CIB 36, from the microalgae collection of CIBNOR-Centro de Investigaciones Biológicas del Noroeste S.C., México) were maintained in flasks containing F/2 and F media [32,33]. Before the bacterial enrichment, *C. calcitrans* cells were washed twice (5000 g, 5 min) to reduce the naturally associated bacteria with 0.85% (*w*/*v*) saline solution. Bacterial consortium enrichment in all SAW media was assessed at the beginning of the microalgal cultures using 1×10^7 CFU/mL. Each treatment was run in quadruplicate. For eight days, bacteria-enriched cultures of *C. calcitrans* (100 mL, SAW media), with an initial cell density of 8.5×10^5 cells/mL, were kept at constant temperature (25 ± 2 °C) with orbital shaking at 125 rpm, light intensity (60 lux), and a light/dark cycle of 12 h/12 h. Aseptic 1 mL samples were withdrawn for cell counting in a Neubauer chamber under an Olympus BX41 microscope at 40× magnification, and the number of microalgal cells was calculated using the formula:

$$C = (N) \cdot (10^4) \cdot (dil) \quad (3)$$

where C = concentration of microalgae in cells/mL, N = number of cells counted, and dil = dilution [34].

Additionally, the lipid, carbohydrate, and pigment (chlorophyll and carotenoid) content of *C. calcitrans* was assessed following the methodology of Refs. [35–37], respectively,

by collecting samples of each treatment at the beginning and end of the experiment. Finally, the capacity of *C. calcitrans* to assimilate nitrite, nitrate, and phosphorus in various SAW media was also evaluated following the methodology of Refs. [34,38], respectively.

2.6. Statistical Analysis

Regarding the auxin and amylase assays, the non-parametric Mood's median test was used to compare the medians, followed by the Dunn post hoc test. A binary logistic regression was conducted to evaluate the siderophore content, involving an analysis of variance using the Wald test followed by a Tukey post hoc analysis. A binary logistic regression model was also used to evaluate antagonism between bacteria. For the parametric results, the normality of the data was verified using the Shapiro–Wilk test, and the homogeneity of variances was assessed with Levene's test. Biofilm formation among strains was compared using a one-way analysis of variance (ANOVA), followed by Duncan's post hoc test. MANOVA was also performed on the biofilm data to assess whether the factors had significant effects on multiple dependent variables simultaneously. For mobility experiments, ANOVA was used with Tukey's post hoc test. ANOVA and Tukey tests were also used to examine the growth, metabolic rates, and nutrient assimilation of *C. calcitrans* in co-culture with bacteria on a specific day. Additionally, microalgal growth was assessed using a linear model with repeated measures over time. For this analysis, the eight-day culture period was evaluated as a single unit to determine whether any significant differences in treatments existed. All tests were performed at a 95% confidence level.

3. Results

3.1. Identification of Phycosphere-Isolated Bacterial Strains and Selection of the Bacterial Consortium

Twelve bacterial strains were isolated from the marine microalgae *Chaetoceros calcitrans* and *Isochrysis galbana*. Three strains were identified as *Marinobacter* sp., and sequence similarity ranged from 98.84% to 100%. Most of the bacteria were rod-shaped and Gram-negative, except *Bacillus cereus* (40g1), which was Gram-positive and round-shaped (Table 2). Five did not match the NCBI database.

Table 2. Identification of seven bacterial strains isolated from the phycosphere of marine microalgae through similarity searches based on complete DNA sequences.

Isolate ID	Microalgae	Gram Staining	Morphological Characteristics	Closest Match Strain NCBI	Accession Number	Sequence Identity (%)
40pB	*I. galbana*	Gram-negative	Rod-shaped	*Marinobacter* sp.	CP014754.1	99.54
40pA	*I. galbana*	Gram-negative	Rod-shaped	*Marinobacter* sp.	CP014754.1	100.00
40g1	*I. galbana*	Gram-positive	Round-shaped	*Bacillus cereus*	CP053997.1	99.52
38	*I. galbana*	Gram-negative	Rod-shaped	*Marinobacter alkaliphilus*	CP115811.1	98.84
37r	*I. galbana*	Gram-negative	Rod-shaped	*Cetobacterium somerae*	MN646996.1	99.43
28	*C. calcitrans*	Gram-negative	Rod-shaped	*Alteromonas macleodii*	CP098772.1	99.35
29	*C. calcitrans*	Gram-negative	Rod-shaped	*Algoriphagus* sp.	CP048415.1	100.00

Auxin production was detected in 6 of the 12 bacteria (29, 37r, 40pA, 40pB, 41, and 42a), with 40pA producing the highest auxin concentration at 5.03 µg/mL (Table 3). Siderophore production was present in all bacteria but did not exceed 13%. Bacterium 40g1 was the only organism capable of solubilizing calcium phosphate. None of the bacteria showed the ability to solubilize hydroxyapatite. All bacteria except bacteria 29, 37b, and 37r demonstrated amylase solubilization capabilities.

Table 3. Evaluated parameters to select phycosphere-isolated consortium bacteria.

Experiments	Control	Strains											
		27a	28	29	37b	37r	38	40g1	40pA	40pB	41	42a	42b
Auxin production (µg/mL)	0.00 ± 0.09 ^{f}	0.00 ± 0.25 ^{f}	0.00 ± 0.40 ^{f}	2.43 ± 0.28 ^{c}	0.00 ± 0.34 ^{f}	1.61 ± 0.48 ^{d}	0.00 ± 0.35 ^{f}	0.00 ± 0.26 ^{f}	5.03 ± 0.38 ^{a}	4.85 ± 0.29 ^{a}	0.15 ± 0.79 ^{e}	4.17 ± 0.46 ^{b}	0.00 ± 0.45 ^{f}
Siderophore production (%)	0.00 ± 4.71 ^{g}	6.23 ± 1.64 ^{cd}	10.44 ± 0.46 ^{b}	5.56 ± 1.15 ^{d}	1.10 ± 1.63 ^{f}	10.26 ± 1.70 ^{ab}	9.40 ± 1.28 ^{b}	10.60 ± 1.56 ^{ab}	7.29 ± 0.46 ^{c}	12.54 ± 0.97 ^{a}	11.06 ± 2.08 ^{ab}	2.36 ± 1.89 ^{ef}	3.11 ± 0.18 ^{e}
Phosphorus solubilization test (cm)	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}
Amylolytic activity (cm)	0.00 ± 0.00 ^{h}	1.85 ± 0.07 ^{d}	2.55 ± 0.07 ^{ab}	0.1 ± 0.00 ^{g}	0.10 ± 0.00 ^{g}	0.10 ± 0.00 ^{g}	2.50 ± 0.00 ^{b}	1.90 ± 0.00 ^{de}	2.50 ± 0.00 ^{b}	2.60 ± 0.00 ^{a}	0.50 ± 0.00 ^{f}	2.50 ± 0.00 ^{b}	2.40 ± 0.14 ^{bc}
Biofilm formation test (SBF)	0.00 ± 0.00 ^{b}	5.87 ± 0.52 ^{e}	10.96 ± 0.89 ^{b}	1.37 ± 0.02 ^{h}	1.66 ± 0.14 ^{h}	2.63 ± 0.12 ^{gh}	4.74 ± 0.23 ^{f}	9.16 ± 0.14 ^{c}	18.81 ± 0.67 ^{a}	9.86 ± 0.95 ^{bc}	3.19 ± 0.23 ^{g}	2.59 ± 0.09 ^{gh}	7.37 ± 0.04 ^{d}
Swimming motility test (cm)	0.00 ± 0.00 ^{i}	0.75 ± 0.07 ^{fg}	5.65 ± 0.21 ^{b}	0.35 ± 0.07 ^{gh}	0.20 ± 0.00 ^{h}	0.35 ± 0.07 ^{gh}	1.15 ± 0.07 ^{ef}	8.50 ± 0.00 ^{a}	2.30 ± 0.14 ^{c}	1.85 ± 0.21 ^{d}	0.90 ± 0.00 ^{ef}	1.20 ± 0.00 ^{e}	2.00 ± 0.00 ^{cd}
Antagonism test	/	++	++	++	++	++	++	++	+	+	+	+	+

Means with different letters in the rows indicate significant differences ($p < 0.05$). The + symbol indicates the strength of the antagonistic effects, from the lowest (+) to the strongest (+++).

All bacteria formed biofilms; bacteria 40pA, 28, 40pB, and 40g1 had the highest SBF index values (18.81, 10.96, 9.86, and 9.16, respectively), with significant differences ($p < 0.05$).

Only two bacteria (28 and 40g1) exhibited notable mobility and none showed growth-inhibitory actions against one another in the antagonism experiment. Although all bacteria, to a greater or lesser extent, showed competitive exclusion behavior towards other bacteria strains. The most exclusionary competition against other bacteria is indicated by +++ in Table 3.

In conclusion, the bacteria identified as *Marinobacter* sp. (40pB), *Bacillus cereus* (40g1), and *Alteromonas macleodii* (28) were selected for their MGPB potential and their ability for bioremediation.

3.2. Growth of Chaetoceros Calcitrans with Bacterial Consortium in SAW

The final cell density observed in *C. calcitrans* co-cultures using the SAWna medium ranged between 4×10^6 and 7×10^6 cells/mL (Figure 1A). No significant differences were observed in microalgal growth between days 0 and 5. On day 6, *C. calcitrans* cultures not enriched with bacteria showed lower growth with 4.32×10^6 cells/mL in the SAWna100 medium ($p < 0.05$). The bacterial consortium significantly enhanced the growth of *C. calcitrans* during the seventh and eighth days of cultivation compared to non-enriched cultures ($p < 0.05$). The statistical analysis results using the linear model with repeated measurements over time demonstrate that, when considering the eight days of co-culture as a single unit, the growth curves of microalgal cultures without bacterial enrichment were statistically lower than those corresponding to microalgae associated with bacteria ($p < 0.05$).

In SAWni media, microalgae reached a final cell density between 4×10^6 and 6×10^6 cells/mL (Figure 1B). From the third day onwards, the growth of *C. calcitrans* cultured in the SAWni50 medium exhibited statistically higher levels in the bacterial consortium's presence than other evaluated media ($p < 0.05$), except on days 5 and 6, where no statistical differences were observed ($p > 0.05$). The results of the statistical analysis using the linear model with repeated measurements over time confirmed these findings, indicating that when considering the eight days of co-culture as a single unit, the growth of *C. calcitrans* in the SAWni50 medium in the presence of the bacterial consortium was statistically higher than the remaining growth curves ($p < 0.05$).

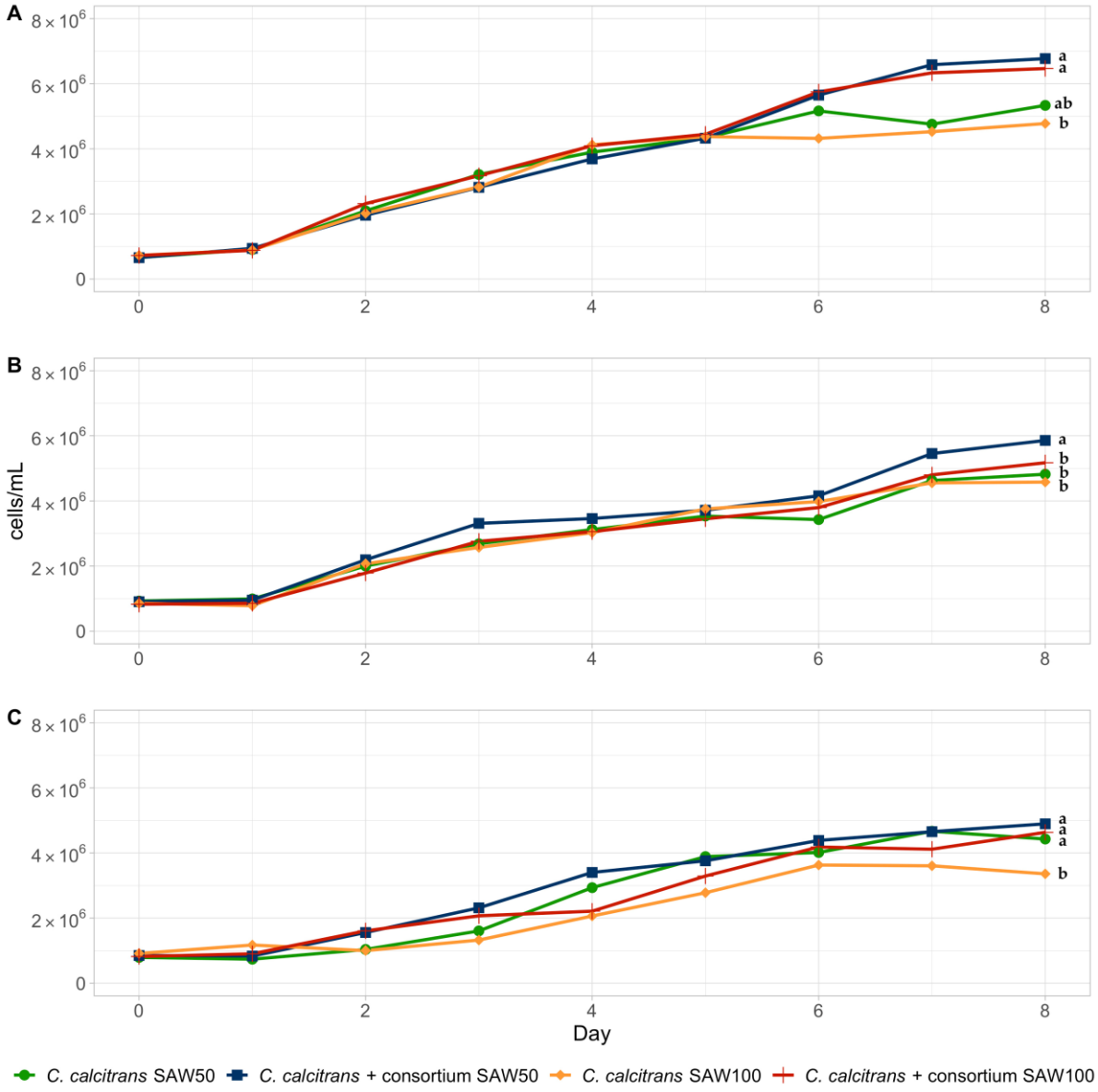

Figure 1. Growth of *C. calcitrans* with or without the bacterial consortium (cells/mL; Mean ± standard error; $n = 4$) in media: (**A**) SAWna, (**B**) SAWni, and (**C**) SAWpho.

Microalgae in SAWpho media reached a final cell density ranging between 3×10^6 and 5×10^6 cells/mL (Figure 1C). The growth of *C. calcitrans* varied over the eight-day assessment period without continuous significant differences between days, except for cultures without bacterial enrichment in the SAWpho100 medium. From the fifth day onwards, significantly lower growth was observed than the rest of the cultures. The statistical analysis results using the linear model with repeated measurements over time corroborated these findings, demonstrating that, when considering the eight days of co-culture as a single unit, the growth of cultures without bacterial enrichment in the SAWpho100 medium was statistically lower than the other treatments.

3.3. Composition of Chaetoceros Calcitrans with Bacterial Consortium in SAW

The metabolic composition analyses of *C. calcitrans* in the different SAW media indicated that the levels of chlorophyll a, chlorophyll b, and carotenoids were statistically higher ($p < 0.05$) when the bacterial consortium was present. The only exception was in the SAWni media, where there was no significant difference in chlorophyll b content, regardless

of the bacterial presence ($p > 0.05$). Moreover, lipid content exhibited a notable increase in the SAWni50 and SAWphos50 media, with elevated levels observed in the presence of the bacterial consortium. Additionally, carbohydrate content was slightly lower in the SAWni50 medium in the presence of the bacteria (Table 4).

Table 4. Content of lipids, carbohydrates, and pigments in *C. calcitrans* cultures with or without the bacterial consortium in SAW media (mean ± standard error; $n = 4$).

	Content	*C. calcitrans* Day 0	*C. calcitrans* Day 8	*C. calcitrans* + consortium Day 8
SAWna50	Lipids (µg/mL)	0.00 ± 0.00 ^{b}	304.74 ± 52.98 ^{a}	239.03 ± 61.52 ^{a}
	Carbohydrates (µg/mL)	0.00 ± 0.00 ^{b}	4.96 ± 0.54 ^{a}	4.87 ± 0.24 ^{a}
	Chlorophyll a (µg/mL)	80.09 ± 5.27 ^{c}	170.99 ± 9.52 ^{b}	305.83 ± 9.44 ^{a}
	Chlorophyll b (µg/mL)	164.72 ± 4.95 ^{c}	189.83 ± 9.38 ^{b}	270.87 ± 29.63 ^{a}
	Carotenoids (mg/mL)	1.04 ± 0.09 ^{c}	2.19 ± 0.18 ^{b}	3.64 ± 0.39 ^{a}
SAWna100	Lipids (µg/mL)	0.00 ± 0.00 ^{b}	231.65 ± 32.31 ^{a}	313.35 ± 63.98 ^{a}
	Carbohydrates (µg/mL)	0.00 ± 0.00 ^{b}	5.16 ± 1.02 ^{a}	5.34 ± 0.91 ^{a}
	Chlorophyll a (µg/mL)	80.09 ± 5.27 ^{c}	170.67 ± 13.74 ^{b}	297.04 ± 18.50 ^{a}
	Chlorophyll b (µg/mL)	164.72 ± 4.95 ^{c}	185.99 ± 6.63 ^{b}	278.57 ± 25.53 ^{a}
	Carotenoids (mg/mL)	1.04 ± 0.09 ^{c}	2.23 ± 0.21 ^{b}	3.66 ± 0.19 ^{a}
SAWni50	Lipids (µg/mL)	0.00 ± 0.00 ^{c}	304.74 ± 52.81 ^{b}	840.10 ± 69.01 ^{a}
	Carbohydrates (µg/mL)	0.00 ± 0.00 ^{c}	5.35 ± 0.65 ^{a}	2.51 ± 1.08 ^{b}
	Chlorophyll a (µg/mL)	94.07 ± 9.91 ^{c}	134.09 ± 12.81 ^{b}	186.50 ± 20.06 ^{a}
	Chlorophyll b (µg/mL)	185.70 ± 27.06 ^{a}	184.01 ± 20.65 ^{a}	211.08 ± 13.00 ^{a}
	Carotenoids (mg/mL)	1.28 ± 0.20 ^{c}	1.68 ± 0.12 ^{b}	2.39 ± 0.23 ^{a}
SAWni100	Lipids (µg/mL)	0.00 ± 0.00 ^{b}	233.70 ± 49.93 ^{a}	334.43 ± 26.63 ^{a}
	Carbohydrates (µg/mL)	0.00 ± 0.00 ^{b}	3.55 ± 0.94 ^{a}	3.25 ± 0.38 ^{a}
	Chlorophyll a (µg/mL)	94.07 ± 9.91 ^{c}	135.71 ± 2.50 ^{b}	197.07 ± 10.50 ^{a}
	Chlorophyll b (µg/mL)	185.70 ± 27.06 ^{a}	185.70 ± 12.33 ^{a}	211.27 ± 16.54 ^{a}
	Carotenoids (mg/mL)	1.28 ± 0.20 ^{c}	1.61 ± 0.16 ^{b}	2.42 ± 0.25 ^{a}
SAWpho50	Lipids (µg/mL)	0.00 ± 0.00 ^{c}	172.20 ± 8.70 ^{b}	315.57 ± 55.46 ^{a}
	Carbohydrates (µg/mL)	0.00 ± 0.00 ^{b}	1.56 ± 0.64 ^{a}	2.54 ± 0.55 ^{a}
	Chlorophyll a (µg/mL)	102.51 ± 21.50 ^{c}	139.77 ± 12.12 ^{b}	198.34 ± 15.93 ^{a}
	Chlorophyll b (µg/mL)	218.58 ± 24.26 ^{b}	233.13 ± 22.64 ^{b}	317.96 ± 31.10 ^{a}
	Carotenoids (mg/mL)	1.47 ± 0.26 ^{c}	2.79 ± 0.22 ^{b}	4.04 ± 0.25 ^{a}
SAWpho100	Lipids (µg/mL)	0.00 ± 0.00 ^{b}	268.96 ± 63.20 ^{a}	328.96 ± 63.73 ^{a}
	Carbohydrates (µg/mL)	0.00 ± 0.00 ^{b}	0.44 ± 0.58 ^{a}	1.11 ± 0.47 ^{a}
	Chlorophyll a (µg/mL)	102.51 ± 21.50 ^{b}	110.64 ± 1.65 ^{b}	135.01 ± 14.46 ^{a}
	Chlorophyll b (µg/mL)	218.58 ± 24.26 ^{b}	202.38 ± 7.61 ^{b}	240.78 ± 24.78 ^{a}
	Carotenoids (mg/mL)	1.47 ± 0.26 ^{c}	2.06 ± 0.09 ^{b}	2.69 ± 0.28 ^{a}

Means with different letters in the rows indicate significant differences ($p < 0.05$).

3.4. Bioremediation of Chaetoceros calcitrans with Bacterial Consortium in SAW

Significant nitrate assimilation of 11.79% was observed at the end of the culture period in the SAWna100 when *C. calcitrans* were cultured alone ($p < 0.05$) (Table 5). In the SAWni50 with or without the bacterial consortium, a significant reduction in nitrites by the microalgae was observed at the end of the bioassay (10.04% and 9.68%, respectively). In the SAWpho50 media, phosphorus assimilation rates were 66.14% and 69.13%, respectively, without and with the bacterial consortium, indicating significant assimilation in both scenarios ($p < 0.05$). Phosphorus assimilation rates in the SAWpho100 media were 28.70% and 31.44% under the same conditions, demonstrating significant assimilation ($p < 0.05$). Overall, phosphorus assimilation in the SAWpho media showed no statistically significant difference with or without the bacterial consortium ($p > 0.05$).

Table 5. Removal of nitrate, nitrite, and phosphorus of *C. calcitrans* with or without the bacterial consortium in SAW media (mean ± standard error; $n = 4$).

Treatments		Initial Content (mg/L)	Final Content (mg/L)	Removal (%)
SAWna	50	220.92 ± 26.13 [a]	199.74 ± 8.69 [a]	9.58
	100	456.77 ± 13.51 [a]	402.91 ± 16.88 [b]	11.79
SAWna + consortium	50	220.92 ± 26.13 [a]	226.43 ± 37.18 [a]	/
	100	456.77 ± 13.51 [a]	424.00 ± 53.73 [a]	7.17
SAWni	50	199.72 ± 3.50 [a]	180.39 ± 10.85 [b]	9.68
	100	288.54 ± 35.00 [a]	300.40 ± 28.51 [a]	/
SAWni + consortium	50	199.72 ± 3.50 [a]	179.66 ± 11.17 [b]	10.04
	100	288.54 ± 35.00 [a]	308.54 ± 14.71 [a]	/
SAWpho	50	51.16 ± 2.87 [a]	17.32 ± 1.97 [b]	66.14
	100	94.06 ± 8.47 [a]	67.06 ± 6.22 [b]	28.70
SAWpho + consortium	50	51.16 ± 2.87 [a]	15.79 ± 1.03 [b]	69.13
	100	94.06 ± 8.47 [a]	64.48 ± 4.93 [b]	31.44

Means with different letters in the row indicate significant differences ($p < 0.05$).

4. Discussion

The bacteria associated with the phycosphere of microalgae and the specific exchanges they carry out among themselves are gradually being studied and considered for biotechnological purposes [16,39]. Characterizing the 12 bacterial strains isolated from the phycosphere of marine microalgae focused on microalga growth-promoting bacteria (MGPB) and the potential of the bacteria to assist microalgae in treating aquaculture wastewater. Antagonism tests showed no inhibition among the 12 bacterial strains, but some competitive exclusion was observed and factored into the consortium design. Three bacterial culturable strains were therefore selected to enrich *C. calcitrans* cultures: *Marinobacter* sp. (40pB), *Bacillus cereus* (40g1), and *Alteromonas macleodii* (28), species known to be commonly associated with microalgae [18,40,41].

Previous research has shown that microalgal growth can increase by 29% when certain bacteria are present [42]. For example, a study by Ref. [43] found that *Bacillus* sp. bacteria significantly boosted the growth of *C. calcitrans* by around 38%. Similar results were observed in this study, as the bacterial consortium significantly increased microalgal biomass in most SAW formulations evaluated. Thus, the consortium composed of *Alteromonas macleodii*, *Marinobacter* sp. and *Bacillus cereus* was considered a group of MGPB. The absence of a discernible increase in growth in SAWpho50 could be attributed to the fact that the microalgae were already attaining their maximal growth capacity within this medium under the specified conditions and nutrient availability. While using the SAWpho100 medium, bacteria had a growth-promoting effect, as the microalgae without bacterial enrichment showed less growth than the microalgae in the SAWpho50 medium. This is likely attributable to a toxic concentration of 100 mg/L for *C. calcitrans*. Although there is no specific data on phosphorus toxicity for *C. calcitrans*, toxicity levels may vary widely among different microalgal species [44,45]. Similarly, in the SAWni100 medium, the bacterial consortium's lack of activity may be due to the high nitrite concentrations, which could be toxic to one or more of the bacteria in the consortium [46].

The nutritional composition of microalgae can increase in the presence of certain bacteria. When *C. calcitrans* was cultured in the SAW media, the concentration of pigment, including chlorophyll a, chlorophyll b, and carotenoids, increased in the presence of the bacterial consortium in almost all media. This was also demonstrated in the study [47], where the co-immobilization of *Azospirillum* and *Chlorella* enhanced carotenoid production. Pigments are essential for microalgae under light or nutrient-stress conditions due to their antioxidant properties, especially carotenoids [48]. Thus, the bacterial consortium helped the microalgae adapt to environmental stress. The bacterial enrichment also increased lipids in both SAWni50 and SAWpho50 media. An increase in lipids in microalgae may indicate a stress response such as nutrient limitation, which is beneficial, as it indicates the metabolic adaptation of microalgae to store energy reserves in response to extreme conditions [49].

An increase in lipids in *Chlorella vulgaris* was demonstrated in the study [50] in the presence of the bacterium *Stenotrophomonas maltophilia*.

Nitrogen is essential for microalgae's metabolism and cellular structure, constituting between 7% and 12% of their total biomass [51]. The present study revealed that *Chaetoceros calcitrans* assimilated approximately 9.51% of nitrate across different SAWna media formulations. In another study, *C. calcitrans* assimilated a percentage of nitrate/nitrite, around 55.29%, under diverse culture conditions [7]. Research by ref. [52] showed lower assimilation rates, with *Chaetoceros muelleri* assimilating 1.71 mg/L/day of nitrate from an initial concentration of 27.030 mg/L, whereas in this study, *C. calcitrans* in SAWna50 medium with an initial concentration of 50 mg/L assimilated an average of 37.52 mg/L over eight days.

Microalgae typically prefer ammonium as their primary source of N, but under nutrient limitation conditions, they can assimilate nitrite [53]. In the case of SAWni50, *C. calcitrans* assimilated around 9.86% of the nitrite available. There is limited literature on nitrite assimilation by *C. calcitrans*, but, for comparison, *Chlorella vulgaris* has been reported to assimilate nitrite at a rate of 2.93 mg/L/day starting with 405 mg/L of nitrite [44]. In this study, *C. calcitrans* absorbed an average of 19.70 mg/L over eight days in the SAWni50 medium, indicating a comparable nitrite uptake efficiency. In the SAWni100 medium, the microalgae did not uptake nitrite, suggesting that the nitrite concentration of 100 mg/L may be toxic to *C. calcitrans*. Although there is no defined maximum toxicity threshold for this microalga in the scientific literature, studies indicate that *Chlorella vulgaris* shows signs of toxicity at concentrations exceeding 81 mg/L of nitrite [44].

Phosphorus is an essential but relatively scarce element in the biosphere [54], hence the importance of its uptake. It is abundant in aquaculture effluents, contributing to environmental issues like eutrophication when released into natural habitats [55]. Phosphorus is essential for microalgal cell composition, including phospholipid membranes, ATP energy production, and nucleic acid synthesis [56]. In this study, using the SAWpho50 medium, *C. calcitrans* assimilated 67.7% of P and 30% when cultivated in the SAWpho100. When bacteria were present in co-cultures, slightly higher P assimilation was noted but not significantly different from assimilation without bacteria. Ref. [7] reported *C. calcitrans* assimilating only 15.87% of phosphate/phosphorus under their culture conditions. For other microalgae like *Scenedesmus* sp., a study showed similar phosphorus assimilation of 65% in domestic wastewater [57].

The microalgae likely benefitted from the bacterial consortium, leading to faster assimilation of phosphorous, nitrite, and nitrate. Under these culture conditions, it is plausible that *C. calcitrans* reached its maximum nutrient absorption capacity by the eighth day. This could explain the apparent lack of effect observed concerning the bacterial consortium. Further experiments would be needed to validate this hypothesis. Furthermore, microalgae can accumulate phosphorus in their cells, even when not actively used. This phenomenon, designated as "luxury P uptake" [58], could account for the greater phosphorus absorption observed compared to nitrogen.

5. Conclusions

The designed bacterial consortium composed of *Alteromonas macleodii*, *Bacillus cereus*, and *Marinobacter* sp. co-cultured with *Chaetoceros calcitrans* has shown promising results. These bacteria have proven to be effective MGPB (microalga growth-promoting bacteria), enhancing both the growth and nutritional quality of *C. calcitrans* when subjected to stress conditions in some of the synthetic SAW media. This improvement renders *C. calcitrans* a valuable and sought-after resource for various industries, notably the aquaculture and biofuel sectors, seeking cost-effective and eco-friendly biotechnological tools.

Furthermore, the phosphorus assimilation by *Chaetoceros calcitrans* has been poorly documented until now. *C. calcitrans* has demonstrated a notable capacity for phosphorus assimilation, which is important given that phosphorus can be an environmental contaminant in some cases and a depleting resource in others. Therefore, *C. calcitrans* can

offer innovative environmental solutions, while the bacterial consortium could optimize microalgal production.

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