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Bacterial exudates as growth-promoting agents for the cultivation of commercially relevant marine microalgal strains

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Abstract

In laboratory and industrial cultivation of marine microalgae, it is customary to enrich cultures with macronutrients (N, P), chelated trace metals, and vitamins at $\sim 10^4\times$ concentrations found in nature to obtain high culture densities. Other naturally occurring growth-promoting compounds found in local seawater are not enriched and remain at environmental concentrations. Microalgae may thus be deprived of the mutualistic contributions of co-occurring microorganisms with which they have evolved complex chemical relationships. In the present study, we assess the direct (mixed bacteria–microalgae cultivation) and indirect (exposure to exudates only, without physical contact) effects of 10 bacterial strains on the growth of five marine microalgal strains used as feeds in marine aquaculture hatcheries. Bacterial strains were selected based upon previously reported growth-promoting characteristics in plants or microalgae, or known release of probiotics. Our experiments demonstrate superior stimulation of microalgal growth by bacterial exudates, and without the presence of the bacteria that produced these exudates. However, response to bacterial exudate enrichment was dependent upon the microalgae strain and bacterial pairing. Exudates from *Bacillus*, *Mesorhizobium*, and *Phaeobacter* strains were most effective,

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with 22%–69% increases in microalgal specific growth rate. Such findings indicate that bacterial exudates accelerate rate-limiting processes governing nutrient acquisition, assimilation, or anabolism, and possibly algal release of exopolymeric substances. Maximal cell density, however, remained constrained by macronutrient limitation. Scaled-up trials in an oyster hatchery confirmed the practical benefit of bacterial exudate culture medium enrichment and demonstrated the suitability of exudate-enriched microalgae to feed hatchery-reared bay scallops. This work presents a promising strategy to improve microalgal culture media formulations using bacterial exudate components as growth promoters, and is the first such study to identify specific pairings with relevance for aquaculture production.

KEYWORDS
bacteria, bivalves, co-cultivation, hatchery, microalgae

1 | INTRODUCTION

Microalgae are the subject of increasing attention as a sustainable and renewable bioresource in a wide range of industrial applications, including bio-energy, pharmaceuticals, nutraceuticals, and animal feed (Khan et al., 2018; Koller et al., 2014; Nagarajan et al., 2021). In aquaculture, microalgae—organisms rich in lipids, proteins, and various bioactive compounds (e.g., polyunsaturated fatty acids, pigments, and antioxidants)—are functional aquafeeds, increasingly alleviating dependence on fish meal and fish oil, with an improved nutritional content and quality and providing diverse growth performance enhancing properties (Beal et al., 2018; Eryalcin, 2018; Eryalçın, 2019; Malcata et al., 2019; Matassa et al., 2016; Michalak & Chojnacka, 2015; Nagappan et al., 2021; Yarnold et al., 2019). The relatively low biomass productivities and high operating costs of existing microalgal cultivation systems are important limitations to the economic viability of algae-based industries (Fernández et al., 2019; Oostlander et al., 2020). The inclusion of plant growth-promoting bacteria (PGPB) within microalgal culture has been well explored as an approach to boost productivity (Lian et al., 2018; Natrah et al., 2014; Ríos Castro et al., 2021). Numerous examples of synergistic microalgae–bacteria associations that can accelerate algal growth and improve other aspects of microalgal physiology and metabolism (e.g., cell size, pigment and lipid content, and fatty acid profile) have been reported (Gonzalez & Bashan, 2000; Hernandez et al., 2009; Kim et al., 2014; Lian et al., 2018; Park et al., 2017; Ukeles & Bishop, 1975), with some notable recent innovations based on this potential (Jeon et al., 2019; Toyama et al., 2018). The mechanisms underlying these microbial dynamics involve the exchange of a wide array of enzymes, metabolites, info-chemicals, and waste products that are known to also supply the metabolic needs of co-occurring organisms (Ramanan et al., 2016; Ryu et al., 2004; Seymour et al., 2017; Tsavkelova et al., 2006). These compounds include micronutrients, vitamin co-factors, siderophores, chelators, phytohormones, and antibiotics, which collectively can contribute to algal growth stimulation and pathogen resistance (Amavizca et al., 2017; Amin et al., 2009, 2015; Kazamia et al., 2012; Ramanan et al., 2016). The suite of compounds used in routine microalgae growth media formulations does not include compounds of bacterial origin, such as exudates, which we consider a fundamental missed opportunity. Although it is well known that bacterial products have a beneficial effect on algal

growth rates, compensating for uncharacterized chemical deficiencies is challenging because of our very limited understanding of the functions of these bacteria-derived compounds on algal physiology (Sharifi & Ryu, 2018). Recently, several studies in freshwater environments have evaluated microalgal culture responses following exposure to bacterially released compounds. Such studies have found promising effects on microalgal growth and biomass chemical composition (Amavizca et al., 2017; Cho et al., 2019; Peng et al., 2020). However, the relevance of bacterial remote effects (i.e., indirect effects occurring through exudates without physical contact between the algae and bacteria) for microalgal cultivation remains largely unexplored, representing an underutilized opportunity to enhance biomass yield in mass-production systems in a sustainable and cost-effective manner. In the present study, we selected five marine microalgal strains: three diatoms (*Chaetoceros calcitrans*, *Chaetoceros neogracile*, and *Thalassiosira pseudonana*) and two flagellates (*Tisochrysis lutea* and *Tetraselmis chuii*), of widespread use as feeds in aquaculture hatcheries, and tested the growth-promoting potential of 10 bacterial isolates with each microalgal strain (Borowitzka, 1997). To compare the respective roles of remote versus co-culture bacterial effects on microalgal culture growth characteristics, we performed a systematic, laboratory-scale screening consisting of pairwise culture media enrichment experiments with bacterially released compounds (remote effects) and bacterial co-cultivants (co-culture effects). A fraction of the bacterial strains included in this screening were selected based upon known or inferred plant-growth-promoting characteristics, combined with known tolerance of seawater (Table 1). Bacterial strains were selected from diverse genera, including *Azospirillum*, *A. halopraeferens*, *Mesorhizobium*, *M. sanjuanii* and *M. thiogangeticum*, *Bacillus*, *B. subtilis subsp. subtilis* and *B. altitudinis*, *Flavobacterium*, *F. nitratireducens*, and *Alteromonas*, *A. haloplanktis* (Gonzalez & Bashan, 2000; Hernandez et al., 2009; Le Chevanton et al., 2013; Wei et al., 2020). Probiotic bacterial strains (i.e., strains known to synthesize molecules with antibacterial or disease-preventive action of relevance within the aquaculture industry) and no apparent plant-growth-promoting properties from the genus *Bacillus*, *Phaeobacter*, *P. inhibens* and *P. gallaeciensis*, and *Paracoccus*, *P. zeaxanthinifaciens*, were also included (Table 1; Suva et al., 2016; Seyedsayamdost et al., 2011).

To further assess the influence of bacterial exudates on microalgal physiology beyond growth and yield, we quantified the algal release of exopolymeric substances (EPS) following bacterial exudate enrichment in the culture media (Claquin et al., 2008; Passow, 2002a). Because microalgae modulate EPS biosynthesis machinery in part to adapt to environmental conditions, exposure to bacterial exudates likely influences algal EPS release; however, such remote bacterial effects on the EPS budget of microalgal cultures of relevance to commercial applications have not been well explored (Xiao & Zheng, 2016). Finally, we tested the scalability of our observations by selectively repeating the most promising bacterial–microalgae pairs from our laboratory-scale screening experiment in a larger photobioreactor characteristic of microalgae cultivation in aquaculture hatcheries. Our results provide the background to guide development of novel microalgal culturing enhancements strategies using bacterial exudates. Specifically, results from this study advance practical knowledge of the potential to use bacterial supplements in microalgal mass culture in three ways: (i) the scale of the screening (5 algal species individually paired with 10 bacterial isolates and bacterial exudate solutions); (ii) aquaculture use of marine microalgal species; and (iii) direct comparison of remote versus co-culture bacterial effects.

2 | MATERIALS AND METHODS

2.1 | Microalgae cultivation

Five bacteria-free microalgal strains (*T. lutea* [T-ISO], *T. chuii* [PLY429], *C. calcitrans* [Chaet call], *C. neogracile* [Chaet-G], and *T. pseudonana* [3H]) were obtained from the NOAA Milford Laboratory Microalgal Culture Collection, Northeast Fisheries Science Center, Milford, CT, USA. For initial culturing of the microalgae (starter cultures), 25-mL stock culture subsamples were transferred to 250-mL Erlenmeyer flasks filled with 100 mL 1-μm-filtered Milford Harbor seawater (salinity 26 ppt, pH 7.9), enriched with f/2 nutrients, and autoclave sterilized (Guillard, 1975;

TABLE 1 Phylogenetic description and isolate origin of the bacterial strains included in the laboratory screening of bacterial effects on microalgal culture development.

Class	Family	Genus	Species	Culture conditions (broth type, optimal temperature)	Isolate ID	References
Marine strains						
Alphaproteobacteria	Rhodobacteraceae	Paracoccus	*Paracoccus zeaxanthinifaciens*	Marine broth, 28°C	LMG 21293	Berry et al. (2003)
Alphaproteobacteria	Rhodobacteraceae	Phaeobacter	*Phaeobacter gallaeciensis*	Marine broth, 20°C	LMG 24391	Martens et al. (2006)
Alphaproteobacteria	Rhodobacteraceae	Phaeobacter	*Phaeobacter inhibens*	Marine broth, 25°C	LMG 22475	Martens et al. (2006); Ruiz-Ponte et al. (1998)
Gammaproteobacteria	Alteromonadaceae	Alteromonas	*Alteromonas haloplanktis*	Marine broth, 26°C	ATTC 23821	Reichelt and Baumann (1973)
Flavobacteriia	Flavobacteriaceae	Flavobacterium	*Flavobacterium nitratireducens*	Marine broth, 28°C	LMG 27772	Bhumika et al. (2013)
Non-marine strains						
Alphaproteobacteria	Rhodospirillaceae	Azospirillum	*Azospirillum halopraeferens*	Nutrient broth 30°C	LMG 7112	Reinhold et al. (1987)
Alphaproteobacteria	Phyllobacteriaceae	Mesorhizobium	*Mesorhizobium sanjuanii*	Yeast Mannitol broth, 28°C	LMG 30060	Sannazzaro et al. (2018)
Alphaproteobacteria	Phyllobacteriaceae	Mesorhizobium	*Mesorhizobium thiogangeticum*	Yeast Mannitol broth, 28°C	LMG 22697	Ghosh and Roy (2006)
Bacilli	Bacillaceae	Bacillus	*Bacillus subtilis sub sp. subtilis*	Tryptone broth, 28°C	LMG 27904	Ehrenberg (1835); Cohn (1872)
Bacilli	Bacillaceae	Bacillus	*Bacillus altitudinis*	Tryptone broth, 28°C	LGM 24750	Shivaji et al. (2006)

Note: Bacterial strains were sourced from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG—https://bccm.belspo.be/) or the American Type Culture Collection (ATCC).

Guillard & Ryther, 1962). Starter cultures were kept at 18.5°C under continuous illumination. Cultures were gently swirled daily and maintained by performing re-inoculations into fresh medium every 14 days. Prior to the start of the experiments, the absence of bacteria in starter cultures was confirmed by utilizing SYTO 9 (ThermoFisher) staining of culture aliquots that specifically binds to bacterial DNA. Stained bacterial cells were enumerated through flow cytometry (BD Accuri C6). Algal cultures were considered bacteria-free and adequate for experimental work if counts within the bacterial gate were below 100 flow-cytometer events per milliliter of sample analyzed (considered to be electronic noise events). Throughout the course of the experimental campaign, algal cultures used as inoculum were confirmed to be bacteria-free using inoculation of fluid thioglycollate, ThermoFisher, added to Milford Harbor seawater and steam-sterilized. Thioglycollate tubes were incubated for 8 days at 22°C before visual evaluation for the presence of possible aerobic and anaerobic bacterial and fungal contaminants.

Specific microalgal growth rates, μ (expressed in divisions per day, d^{-1}), are calculated across the linear part of the ln-transformed growth curve according to Equation (1) (Guillard, 1973).

$$\mu = \frac{3.322}{(t_t - t_1)} \log \frac{N_t}{N_1} \quad \text{(Eq1)}$$

With N_1, the starting microalgal cell density (in cells mL^{-1}) measured following inoculation at the start of the experiment (i.e., t_1, in days); N_t, the microalgal cell density at stationary phase, and t_t, the time required to reach stationary-phase cell density (Guillard, 1973). Microalgal cell densities were quantified immediately following culture subsample extraction using flow cytometry.

2.2 | Bacterial strains and bacterial exudate solutions

Ten bacterial strains with known or inferred salt tolerance, previously reported or inferred plant growth-promoting or microalgae culture performance enhancing qualities, or probiotic qualities (e.g., production of phytohormones, release of antibiotic and antioxidant molecules, stimulation of EPS production), were selected to perform a screening for beneficial effects on marine microalgal culture performance (Table 1). Bacterial strains were sourced from the Belgian Coordinated Collections of Microorganisms Bacteria Collection (BCCM/LMG) or the American Type Culture Collection (ATCC). Taxonomic affiliation, origins, and optimal growth conditions of the isolates are given in Table 1. Bacterial strains included in the experimental screening are phylogenetically diverse, with two gram-positive strains from the *Bacillus* genus and eight gram-negative strains belonging to the genera *Azospirillum*, *Paracoccus*, *Phaeobacter*, *Mesorhizobium*, *Alteromona*s, and *Flavobacterium*. Selected isolates are of marine (*Paracoccus zeaxanthinifaciens*, *Phaeobacter gallaeciensis*, *Phaeobacter inhibens*, *Alteromonas haloplanktis*, and *Flavobacterium nitratireducens*) or terrestrial (*Azospirillum halopraeferens*, *M. sanjuanii*, *Mesorhizobium thiogangeticum*, *B. subtilis subsp. subtilis*, and *Bacillus altitudinis*) origins. The non-marine strains of *Mesorhizobium* and *Bacillus* are salt tolerant to moderately halophilic, thus allowing cultivation in an intermediate-salinity seawater based medium (Laranjo & Oliveira, 2011; Sannazzaro et al., 2018; Yue et al., 2019).

Bacterial isolates were obtained as freeze-dried powders in vacuum-sealed ampoules. Cells were rehydrated on agar, subcultured three times prior to use in experimental work, and finally cultured in bacterial broth. Bacterial strains were cultured on four different agar compositions followed by their respective bacterial broths (i.e., marine broth [BD Difco 279110]/agar [BD Difco 212185], nutrient broth [BD Difco 234000]/agar [BD Difco 213000], tryptic soy broth [BD Difco 211825]/agar [BD Difco 236950], and yeast mannitol broth [SigmaAldrich Y3377]/agar [SigmaAldrich Y3252]) at the optimal growth temperature for each bacterium (Table 1). To start the experiment, bacterial strains were precultured for 24 h in 15-mL glass culture tubes (20 × 150-mm) partially filled with 8 mL sterilized bacterial broth at 27°C, except for *P. gallaeciensis*, which was incubated at 19°C. Following this incubation period, bacterial counts reached stationary phase as determined by daily counts of flow-cytometer events within the

bacterial gate, ranging 2–7 × 10^9 cells/mL. The cultures were subsequently used for bacteria–microalgae co-culture experiments or for the preparation of bacterial exudate to add to the microalgal culture media. For the latter, bacterial cells were separated from the spent culture medium by centrifugation at 3000×*g* at 20°C for 15 min. The supernatant was subsequently passed through a 0.25-μm filter. The absence of bacterial cells in the filtered supernatant was verified through PicoGreen (ThermoFisher) staining of an aliquot followed by flow cytometric analysis for detection of any stained particles. On confirmation of bacterial absence, bacterial exudate solutions were used for microalgal culture enrichment experiments or stored at 10°C pending use.

2.3 | Experimental design and culture conditions

Co-culture and remote bacterial effects upon microalgal growth were evaluated by performing parallel microalgae cultivation experiments consisting of: (i) control treatments (standard microalgal culture media formulations); (ii) mixed bacteria–microalgae cultures (co-culture bacterial effects); and (iii) microalgal cultures supplemented with bacterial exudate additives (remote bacterial effects). Microalgal growth characteristics (maximum specific growth rate, time required to reach stationary phase, and final cell density) were quantified and compared between treatments.

Experiments were set up as batch cultures in 30-mL glass 20 × 150-mm culture tubes with screw caps, filled with 9 mL culture medium, and inoculated with 1 mL microalgal starter culture. The surface-to-volume ratio of the partially filled tube, coupled with daily mixing by hand, allows for sufficient gas exchange until maximum cell density is approached. The culture medium consisted of Milford Harbor seawater (salinity 26 ppt, pH 7.9) enriched with f/2 nutrients, 0.25-μm filtered, and autoclave sterilized. Starting algal biomass in control, co-culture, and exudate-enriched cultures fluctuated between 2 and 5 × 10^5 cells mL^{-1}, depending on the microalgal strain. Following inoculation, a bacterial culture (1 mL) or bacterial exudate solution (1 mL) subsample was added aseptically to each microalgal culture. Mixed bacteria–microalgae experiments were inoculated at an initial ratio of 10 bacterial cells per microalgal cell following guidelines from Le Chevanton et al. (2013) and Amavizca et al. (2017). The volumetric dosage of the exudate solution treatments was designed to match the dosage of bacterial culture volume added to the mixed bacteria–microalgae cultures. Control treatments were supplemented with 1 mL of autoclaved, deionized water to account for the dilution of f/2 nutrients with respect to the other treatments. Procedural control experiments consisting of microalgal cultures supplemented with 1 mL of sterilized bacterial broth were also included in this screening experimental design to assess the possible effects of bacterial broth constituents on microalgal growth. In summary, the screening experiment of bacterial effects on microalgal culture performance consisted of control cultures, 10 bacterial-co-cultivation treatments, 10 bacterial exudate media additive treatments, and three bacterial broth media additive treatments, each performed on five microalgal species, in triplicate.

In all experiments, microalgal cultures were grown at 18.5°C, under constant illumination. The artificial light source consisted of 1.2-m fluorescent light tube (T8 35 Watt, 4100K, GE Ecolux with Starcoat F32T8 SPP4) located 30 cm from the culture tubes, illuminating one side of the cultures at a PAR light intensity of 140 μmol photons m^{-2} s^{-1}, measured at the surface of the culture tubes with a Licor Inc., Quantum/Radiometer/Photometer (Model LI-185B). Culture tube caps were left loose to allow for gas exchange, and each tube was swirled daily to remobilize algal cells and promote gas exchange. The culture pH was between 7.9 and 8.0. Microalgal culture development was monitored daily throughout the course of the experiment by aseptically extracting a 200-μL culture subsample followed by the enumeration of microalgal cells using flow cytometry. The experiments were terminated after confirmation that the microalgae had reached stationary phase for two consecutive sampling points. Experiments typically lasted 8 days. Prior to termination of the cultures, a 1-mL culture subsample was extracted to quantify microalgal-produced transparent exopolymeric particles (TEPs) abundance at stationary phase. For bacteria–microalgae co-culture experiments, the bacterial population was quantified using flow cytometry with SYTO 9 at the start and termination of the experiment.

2.4 | Scale-up microalgal cultivation trial

The reproducibility of bacterial remote effects on microalgal growth observed in the initial lab screening experiments was assessed in a large-scale microalgal cultivation system at the NOAA Milford Laboratory. One single microalgae–bacterial exudate pairing demonstrating beneficial effects on algal growth in the small-scale laboratory screening was evaluated in a scaled-up experimental trial. *C. neogracile* cultures were grown in a system used to cultivate this species in a small shellfish hatchery (carboys), with the culture medium enriched with exudates from a *B. subtilis subsp. subtilis* culture. The selection of *C. neogracile* as a model microalgae species for the scale-up trial was based on its widespread cultivation for use as algal feed in the rearing of larval and juvenile aquatic organisms (Coutteau & Sorgeloos, 1992; Lebeau & Robert, 2003).

The bacterial exudate solution was prepared following a 24-h incubation of *B. subtilis subsp. subtilis* cultures at 27°C in loosely-sealed, 500-mL Erlenmeyer flasks followed by centrifugation and 0.25-μm filtering of the supernatant as applied in the initial screening experiment (exudate solution at 90.1 mL L^{-1} dosage). Photobioreactors were set up in batch mode and consisted of 19-L carboys, with a screw-on plastic top containing two holes serving as gas inlet and outlet. Air supplemented with 0.2% CO_2 was continuously introduced at the bottom of the carboy at a flow rate of 1.5 L min^{-1} through a silicone tube that was inserted through one of the holes in the cap. Photobioreactors were kept at 18.5°C, and one side of the carboy was continuously illuminated with fluorescent lighting at a PAR light intensity of 140 μmol photons m^{-2} s^{-1}, as measured at the surface of the carboy. The total culture volume in each reactor was 16 L, consisting of autoclave-sterilized Milford Harbor seawater, enriched with f/2 nutrients, 1 L algal inoculum (1.6×10^6 cells mL), and 1.45 L *B. subtilis subsp. subtilis* exudate solution, matching the dosage applied in the initial screening experiment. The experiment was set up in triplicate with three control and three experimental photobioreactors. The CO_2/air mixture ratio was increased gradually during algal culture development to adjust the desired culture pH to 8.2, and cultures were manually swirled to resuspend any settling cells. In the first 2 weeks after inoculation, aliquots were aseptically harvested every 24 h for microalgal cell density determination by flow cytometry. Possible contamination of bacterial cells was monitored by flow cytometric analysis after staining with SYTO 9 at the beginning of the experiment, during the growth phase, and at the stationary phase. Photobioreactors were maintained for 12 weeks under continuous irradiance. After termination, the control and bacterial-exudate-enriched microalgal cultures were used as live feed for Northern bay scallop, *Argopecten irradians irradains*, cultivation. Early stage larvae (D-stage, 75-μm) were reared in 400-L conical tanks for a 9-day period and fed twice daily a mix of exudate-enriched and conventional microalgae. This was not a formal experiment, but production of seed scallops for a field deployment.

2.5 | Transparent exopolymer particle quantification

The abundance of TEPs in microalgal cultures and bacterial exudate solutions were determined according to the dye-binding assay technique developed by Passow and Alldredge (1995). Samples were filtered (<130 mbar) onto 0.4-μm pore-size polycarbonate filters (nucleopore track-etched membranes, Whatman). For each sample, filters were prepared in triplicate. The volume of algal culture sample filtered was 0.5 mL. Particles on the filter were stained for approximately 4 s with 1 mL of a 0.02% aqueous solution of alcian blue (8GX; SigmaAldrich) dissolved in 0.06% acetic acid (0.2-μm-filtered, pH = 2.5). Stained filters were rinsed gently with 2 mL ammonium formate solution to remove excess dye, transferred into centrifuge tubes, and stored at −20°C for subsequent colorimetric determination. An ammonium formate (0.47 M) rinse solution (2 mL per sample) was chosen to avoid cell damage or cell bursting, in the case of microalgal culture samples, from osmotic imbalance during the excess stain removal step. The alcian blue stain was extracted from the filters in 80% sulfuric acid (3 h incubation period) and analyzed for UV absorption at 787 nm using a spectrophotometer (Cytation 3 Cell Imaging Multi-Mode Reader, BioTek Instruments).

Given that alcian blue binds to acidic and sulfated polysaccharides, this method is semi-quantitative when the chemical composition of TEP is unknown (Passow, 2002b). Methods described in Bittar et al. (2018) were followed for the calibration of TEP measurements using a commercially available polysaccharide xanthan gum powder as reference material (Bittar et al., 2018). The concentration of TEP was determined in units of mass xanthan (*X*) gum equivalents per volume sampled, or μg X_{eq}. L^{-1}. The abundance of TEP in microalgal cultures enriched with bacterial exudate medium additives were quantified at stationary phase for all biological replicates ($n = 3$) and are presented as means with associated 95% confidence intervals.

2.6 | Statistics

Each microalgal culture experiment was performed in triplicate. Specific growth rates are presented as means with associated 95% confidence intervals. Bacterial effects on microalgae culture growth and TEP production were compared using one-way analysis of variance (ANOVA; $p < 0.05$) using the R software.

3 | RESULTS

3.1 | Effect of bacterial co-cultivation on microalgal growth

The individual effects of 10 bacterial strains on 5 microalgae species, inoculated at a starting ratio of 10 bacterial cells per microalgal cell, were compared with bacteria-free microalgal cultures at laboratory scale. Microalgal growth rates (divisions day^{-1}) are presented in Table 2. Bacterial broth constituents (i.e., marine, tryptone, and nutrient broth) added to bacteria-free microalgae cultures at a volumetric dosage equivalent to treatments investigating bacterial effects had no statistically significant effect on microalgae growth rate except for *T. chuii* cultures, wherein a 7% increase in algal growth rate was observed in culture media supplemented with tryptone broth (Table 2). The microalgal culture response to bacterial co-cultivants was dependent on the bacterial strain considered. Individual microalgae species grew either faster or slower in co-culture with selected bacteria (Table 2). Three bacterial strains—the marine bacterium *P. gallaeciensis* (in association with *C. calcitrans* and *C. neogracile*), the marine bacterium *F. nitratireducens* (in association with *C. neogracile* and *T. chui*), and the non-marine bacterium *B. subtilis subsp. subtilis* (in association with *C. calcitrans*, *C. neogracile*, and *T. chui*)—were found to improve microalgal culture performance. Amplification of the specific growth rate in the presence of bacterial co-cultivants ranged between 10% for *P. gallaeciensis* up to 20% for *F. nitratireducens* and *B. subtilis subsp. subtilis*. Of the 50 bacteria–microalgae pairs examined in this screening, 28 combinations resulted in a statistically significant decrease of up to 41% (i.e., *C. neogracile* co-cultured with *M. sanjuanii*) in algal division rate. Co-cultivation of diatom species with *A. halopraeferens*, *B. altitudinis*, *P. zeaxanthinifaciens*, or bacterial strains of the Genus *Mesorhizobium* resulted in marked decreases in specific growth rate, ranging between 10% and 40%. Across marine microalgal species examined, inclusion of *P. gallaeciensis*, *F. nitratireducens*, and *A. haloplanktis* co-cultivants had generally no detrimental effect on microalgal culture performance. No positive microbial interactions were observed in terms of algal growth rate increase for cultures of *T. pseudonana* and *T. lutea*. *M. thiogangeticum* and *P. zeaxanthinifaciens* co-cultivants inhibited the flagellate species tested in this study compared with bacteria-free cultures. Following 7 days of mixed bacteria–microalgae culture, cultures were visibly opaque, especially in the case of *T. lutea* and *T. pseudonana*, with a bacteria to microalgae cell ratio ranging between ~100 and 750 depending on the microalgal–bacteria pair considered. In the case of flagellate species, culture opacity was also accompanied by cell clumping and poor culture stability. Following a 10-day culture period, most co-culture experiments did not reach stationary phase.

TABLE 2 Microalgal specific growth rates, in divisions per day, for bacteria-free cultures (top); and microalgal cultures exposed to bacterial broth, bacterial exudate (remote effect), and living bacteria (co-culture effects) culture medium additives (bottom).

	Chaetoceros calcitrans		*Chaetoceros neogracile*		*Thalassiosira pseudonana*		*Tetraselmis chuii*		*Tisochrysis lutea*	
Control (axenic)	1.26 ± 0.02		1.21 ± 0.02		1.00 ± 0.02		1.03 ± 0.02		0.93 ± 0.03	
Marine broth	1.28 ± 0.03		1.22 ± 0.02		0.98 ± 0.01		1.01 ± 0.03		0.91 ± 0.03	
Tryptone broth	1.24 ± 0.02		1.25 ± 0.02		1.04 ± 0.01		1.10 ± 0.02		0.98 ± 0.02	
Nutrient broth	1.25 ± 0.01		1.18 ± 0.01		1.03 ± 0.03		1.01 ± 0.01		0.97 ± 0.01	
	Bacterial exudates	Living bacteria	Bacterial exudates	Living bacteria	Bacterial exudates	Living bacteria	Bacterial exudates	Living bacteria	Bacterial exudates	Living bacteria
Paracoccus zeaxanthinifaciens	1.63 ± 0.04	0.94 ± 0.02	1.67 ± 0.02	0.83 ± 0.02	1.38 ± 0.05	0.73 ± 0.05	1.28 ± 0.02	0.99 ± 0.02	1.08 ± 0.03	0.88 ± 0.03
Phaeobacter gallaeciensis	1.71 ± 0.04	1.38 ± 0.01	1.30 ± 0.02	1.35 ± 0.01	1.31 ± 0.01	0.87 ± 0.06	1.43 ± 0.02	1.01 ± 0.03	1.14 ± 0.02	0.98 ± 0.04
Phaeobacter inhibens	1.44 ± 0.04	1.17 ± 0.03	1.42 ± 0.03	0.89 ± 0.03	1.19 ± 0.02	0.91 ± 0.02	1.35 ± 0.03	0.98 ± 0.03	0.94 ± 0.04	0.75 ± 0.03
Altermonas haloplanktis	1.63 ± 0.03	1.27 ± 0.03	1.29 ± 0.03	1.25 ± 0.03	1.44 ± 0.03	0.94 ± 0.03	1.19 ± 0.03	0.99 ± 0.05	1.22 ± 0.03	0.87 ± 0.04
Flavobacterium nitratireducens	1.23 ± 0.03	1.24 ± 0.03	1.33 ± 0.03	1.29 ± 0.03	1.14 ± 0.03	0.97 ± 0.03	1.25 ± 0.03	1.25 ± 0.03	0.98 ± 0.06	1.02 ± 0.06
Azospirillum halopraeferens	1.25 ± 0.03	1.13 ± 0.03	1.34 ± 0.02	0.89 ± 0.03	0.98 ± 0.05	0.75 ± 0.08	0.93 ± 0.03	0.98 ± 0.03	0.89 ± 0.03	0.78 ± 0.01
Mesorhizobium sanjuanii	1.44 ± 0.05	0.76 ± 0.02	1.43 ± 0.02	0.72 ± 0.02	0.90 ± 0.03	0.67 ± 0.05	1.75 ± 0.05	1.05 ± 0.02	1.09 ± 0.02	0.72 ± 0.02
Mesorhizobium thiogangeticum	1.85 ± 0.05	1.15 ± 0.03	1.16 ± 0.04	0.76 ± 0.01	0.92 ± 0.02	0.62 ± 0.03	1.52 ± 0.02	0.94 ± 0.03	1.07 ± 0.04	0.64 ± 0.03
Bacillus subtilis subsp. subtilis	1.65 ± 0.04	1.33 ± 0.03	1.59 ± 0.01	1.29 ± 0.01	1.27 ± 0.01	0.72 ± 0.05	1.69 ± 0.04	1.23 ± 0.03	1.37 ± 0.05	0.90 ± 0.04
Bacillus altitudinis	1.18 ± 0.01	0.99 ± 0.03	1.18 ± 0.01	0.87 ± 0.01	0.89 ± 0.01	0.77 ± 0.03	1.12 ± 0.01	0.91 ± 0.01	1.11 ± 0.02	0.93 ± 0.03

Note: Green and red shadings represent a statistically significant increase and decrease, respectively, in algal growth rate relative to the control (i.e., bacteria-free, no exudate additives) culture. Yellow shadings highlight treatments with no detectable effect on algal growth rate relative to the control. Bacteria–microalgae cultures were characterized by an initial bacteria-to-microalgal cell ratio of 10. Specific growth rates are reported as mean division rates ($n = 3$) with 95% confidence intervals.

3.2 | Promotion of algal growth by bacterial exudates

The effect of bacterial exudate medium additives on microalgal growth rate, examined at laboratory scale, is summarized in Table 2. Microalgal cultures reached stationary phase 3–5 days following inoculation, depending on microalgae strain and the bacterial exudate enrichment. Inclusion of bacterial exudates in culture media had a generally positive effect on algal growth rate. Microalgal culture response to bacterial exudates was species-dependent, both in terms of alga and bacterial exudate. Of the 50 bacterial exudate–microalgal pairs examined in the screening experiment, 12 combinations showed negative or no effects on microalgae culture development, whereas other combinations resulted in a 10%–69% increase in culture performance. Growth-stimulating effects of bacterial exudates were observed in all microalgal species examined with bacterial exudates originating from the *Bacillus*, *Mesorhizobium*, and *Phaeobacter* genera inducing the highest algal growth rate amplification. *T. chui* cultures had the most pronounced culture response to bacterial exudate exposure, with half of the bacterial exudate treatments resulting in a 30%–69% increase in algal growth rate. For the diatom species examined, exposure to bacterial exudates from *P. zeaxanthinifaciens*, *P. gallaeciensis*, *P. inhibens*, *A. haloplanktis*, and *B. subtilis subsp. subtilis* exudates increased algal growth rates 14%–36% for *C. calcitrans*, 6%–38% for *C. neogracile*, and 14%–43% for *T. pseudonana*, respectively. *T. lutea* was relatively less responsive to bacterial exudate culture medium additives, with algal yield amplifications ranging from no effect to a 20% increase, except for remote effects from *A. haloplanktis* and *B. subtilis subsp. subtilis*, resulting in an algal growth rate increase of 31% and 47%, respectively. Across microalgae species examined, culture media enrichments with exudates from *B. subtilis subsp. subtilis* increased algal growth rate 26%–63%. This bacterial exudate treatment, therefore, was selected for trials in a scaled-up microalgal cultivation system.

3.3 | Transparent exopolymer particle production in response to bacterial exudate culture media enrichments

Microalgal cell-normalized average abundance of TEPs at stationary phase for control and bacterial-exudate-enriched cultures are portrayed in Figure 1. Algal TEP abundance in the control cultures ranged between 1.79 and 2.04×10^{-4} μg X_{eq} cell^{-1} for diatoms, with *T. pseudonana* showing the highest algal TEP production. TEP secretion by flagellate species was lower compared with the diatom species by a factor of 3 for *T. chui*, and 5 for *T. lutea*. Addition of bacterial broth additives to microalgal cultures had no statistically significant effect on algal TEP secretion. TEP abundances in the bacterial exudate solutions were below the detection limit for the 10 bacterial strains examined in this study. Bacterial exudate enrichments generally resulted in increased algal-derived TEP (Figure 1). The effect of bacterial exudates on the algal culture TEP budget was particularly pronounced in cultures of *T. pseudonana*, *C. neogracile*, and *T. chui*, with TEP increases of 5.1, 6.0, and 5.9, respectively, relative to the control. For microalgal cultures in the Genus *Chaetoceros*, algal TEP abundances increased the most in response to *Bacillus* and *Phaeobacter* exudates, with 1.9 and 5.9× increases, respectively. For *T. pseudonana*, the highest increase in TEP was observed with exudates originating from *P. gallaeciensis* (5.1× increase) and *M. thiogangeticum* (4.5× increase). *F. nitratireducens* and *M. sanjuanii* exudates resulted in the highest TEP increase for *T. chuii*, exceeding a 5× increase in TEP relative to the control. Bacterial exudates from *Phaeobacter* species had the most significant effect on TEP production in cultures of *T. lutea*, resulting in a ~3.5× increase relative to the control.

3.4 | Scale-up microalgal cultivation trial

Enrichment of the microalgal culture medium with *B. subtilis subsp. subtilis* exudates had a positive effect on the culture performance of *C. neogracile* when grown in 17-L, aerated, CO_2 supplemented, and continuously illuminated carboys (Figure 2). In the first 3 days, bacterial exudate enrichment resulted in an 81% algal growth enhancement

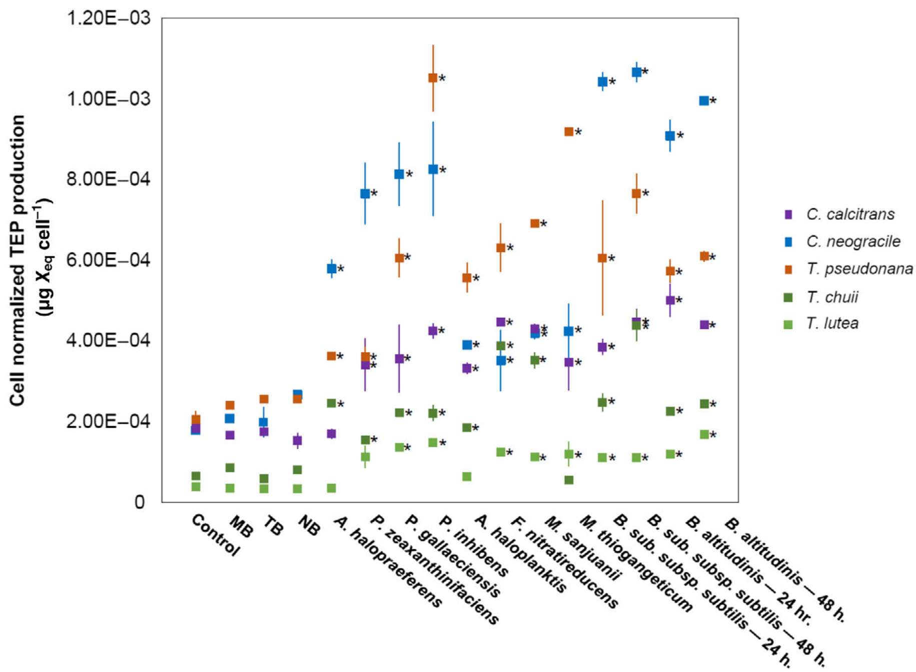

FIGURE 1 Cell-normalized average abundance of transparent exopolymer particles (TEP) at stationary phase, expressed in μg xanthan (*X*) gum equivalent per cell, from marine microalgae cultures without (i.e., control) and with bacterial exudate culture media enrichments. Vertical lines represent the 95% confidence interval based on triplicate algae culture experiments. MB, marine bacterial broth; NB, nutrient bacterial broth; TB, tryptone bacterial broth. Asterisks denote statistical significance.

relative to the control carboys. Bacterial exudate-containing carboys did not show an initial lag phase; whereas a 3-day lag phase was observed in the control carboys. Between days 3 and 5, growth paused during a pH incursion (pH of 6.92) following accidental excessive inflow of CO_2 supplemented air to the culture vessels. Microalgal culture pH stabilized at a pH of 7.99 on day 5, and remained stable at this level until the end of the experiment. A 34% algal growth amplification, relative to the control, was recorded for the treatment carboys when averaged over the first 10 days. Over the course of the 13-day exponential growth phase, a specific growth rate of 0.19 (±0.0054) divisions day^{-1} was observed in the control carboys, with 0.23 (±0.0068) divisions day^{-1}, for the treatment carboys, resulting in a 25% algal division rate amplification in the presence of bacterial exudate additives. Stationary-phase cell densities in both the control and treatment carboys converged to an equivalent final cell density of $6.32 \pm 0.08 \times 10^6$ algal cell mL^{-1}. *C. neogracile* cultures remained stable for 4 weeks following culture initiation and were used as live feed for the culture of *Argopecten irradians irradians* larvae (Northern bay scallop). This was not a formal experiment, but rather simple production of seed scallops for field deployment. Scallops developed and grew normally on the mixed diet incorporating exudate-enriched *C. neogracile*, and the expected yield of seed scallops was obtained.

4 | DISCUSSION

This study reports the first systematic comparison of remote and co-culture effects of 10 bacterial strains on the growth of multiple marine microalgal species used in aquaculture. This laboratory screening revealed accelerated growth rates in all microalgal strains tested when standard seawater enrichment f/2 was supplemented with exudates from at least some of the tested bacteria. Enrichment of the algal culture media with bacterial exudates was

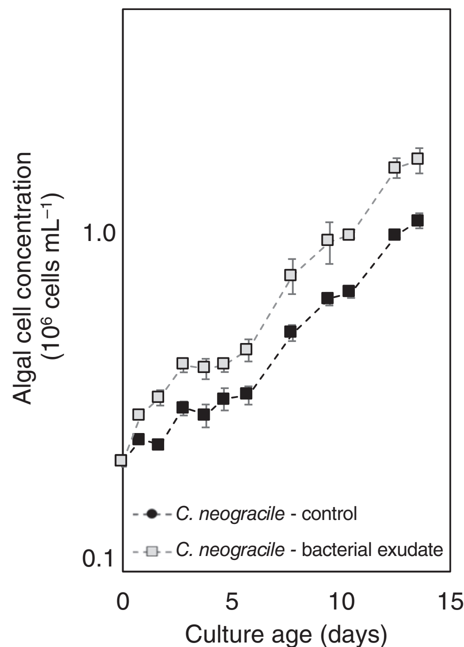

FIGURE 2 Growth curves for cultures of *C. neogracile* grown in f/2 medium in the presence (gray square symbols) and absence (black squares symbols) of bacterial exudate culture medium additives. The bacterial exudate treatment included exogenous *B. subtilis subsp. subtilis* exudate solution added at 90.1 mL L^{-1} to the microalgae culture. Error bars are 95% confidence intervals based on three biological replicates.

markedly more effective at enhancing algal growth compared with actual co-cultivation, with the response being dependent on the specific microalga and bacterial species/exudate combination. Although most bacterial exudate additives had a beneficial effect on microalgal culture performance, exposure to exudates from *Bacillus*, *Mesorhizobium*, and *Phaeobacter* strains resulted in the highest growth rate amplification, ranging from 22% to 69%. A limited number of bacteria–microalgae combinations, when grown in co-cultivation, also increased microalgal growth rate, but the scale of the co-culture bacterial effect (10%–20% increases) was distinctly lower than the exudate effects and highly species-dependent. Our observations demonstrate that, at laboratory and intermediate commercial scale, the inclusion of bacterial exudates in microalgal production systems could be an effective strategy to enhance microalgal culture productivity. A scale-up experiment confirmed the benefit of bacterial exudate culture medium enrichments for stimulating microalgae growth. Exudate-enriched microalgae were fed to hatchery-reared bay scallops confirming that the exudate-enriched algae were acceptable as feed, with larval performance equal to standard production methods. Together, these findings identify a promising role for bacterial exudate enrichment of microalgal culture media in industrial settings.

4.1 | Co-culture bacterial effects on microalgae growth

Three bacteria, including the marine bacterium *P. gallaeciensis* (in association with *C. calcitrans* and *C. neogracile*), the marine bacterium *F. nitratireducens* (in association with *T. lutea* and *T. chui*), and the non-marine bacterium *Bacillus subtilis subsp. subtilis* (in association with *T. chui*), were found to accelerate algal growth. Members of the *Cytophaga-Flavobacterium-Bacteroidetes* group, including *F. nitratireducens*, are abundant in natural aquatic ecosystems and commonly found in the phycosphere of commercial microalgae cultivation systems (Lakaniemi et al., 2012; Nicolas

et al., 2004; Sapp et al., 2007; Schäfer et al., 2002). Similar enhancement of microalgal growth was observed for the pairing of marine bacterial isolates belonging to the *Flavobacteriaceae* family with *Dunaliella* sp. and *Chaetoceros gracilis* (Hirayama, 1996; Le Chevanton et al., 2013). Given the ubiquity of bacteria belonging to *Flavobacterium* in the marine environment and the microbiota of microalgae cultivation systems, it is likely that *Flavobacterium* spp. have evolved to develop mutualistic relationships with a variety of marine microalgal species, resulting in the observed beneficial effect of *F. nitratireducens* symbionts on the growth of *T. lutea* and *T. chui* cultures in our study.

Bacteria belonging to *Bacillus* are ubiquitous in the environment and exhibit well-characterized plant growth-promoting and probiotic activities (Sivasakthi et al., 2014). Plant growth-promoting characteristics of *Bacillus* spp. have been shown to extend to algal physiology, both in microalgae–bacteria co-cultures and through remote bacterial exudate exposure of microalgae cultures (Amavizca et al., 2017; Hernandez et al., 2009). In our study, beneficial influence of *Bacillus* spp. on microalgae physiology was observed only between *B. subtilis subsp. subtilis* and *T. chui* co-cultures.

Members of the *Phaeobacter* Genus are of increasing interest as potent and multifunctional probiotic bacteria in a variety of aquaculture applications, for example, live feed cultivation and larval development, attributable to production and secretion of the potent broad spectrum antibiotics tropodithietic acid (TDA) (D'Alvise et al., 2012; Prado et al., 2009; Seyedsayamdost et al., 2011). For *Phaeobacter* spp. the production of these functional molecules in co-culture (i.e., microalgae) is known to be dependent on the phase of phytoplankton growth (i.e., *Jekyll and Hyde* effect) and on the phytoplankton genetics (Ahern et al., 2021; Seyedsayamdost et al., 2011). To our knowledge, this study presents the first examination of *Phaeobacter* spp. as potential microalgal growth-promoting bacterial co-cultivant. Our experiments show that, in addition to a variety of previously reported biocontrol functions (Prado et al., 2009), *P. gallaeciensis* in association with marine diatoms commonly cultivated for aquaculture live feed (*C. calcitrans* and *C. neogracile*) exhibits microalgal growth-promoting functions.

Although the natural occurrence of bacteria, at the genus or species level, in microalgal cultivation systems formed the basis for the selection process of some isolates used in our experimental screening, few microalgae–bacteria pairs examined in this study had synergistic outcomes in terms of algal growth rate amplification. The observed negative effects of bacterial symbionts in microalgae cultivation likely result from a competitive advantage of bacteria in bacteria–microalgae co-culture conditions, given comparatively faster growth rates resulting in bacteria outcompeting microalgae for nutrients (Azam & Malfatti, 2007; Meseck et al., 2007). Additionally, the proliferation of the bacterial population and associated release of secondary metabolites in the culture vessel contributes to decreased light penetration, with debilitating effects for phototrophic microalgae. Collectively, our results demonstrate that, although beneficial microalgae–bacteria associations for microalgae cultivation could be identified, in nutrient-replete culture conditions characteristic for commercial microalgae production, bacterial co-cultivants generally exhibit a competitive interaction with the cultivated microalgae, a consequence of resource utilization and light attenuation within the culture vessel.

4.2 | Remote bacterial effects on microalgal growth

Culture media enrichments with bacterial exudates resulted in microalgal growth stimulation across the five marine species examined, with exudates originating from *Bacillus*, *Mesorhizobium*, and *Phaeobacter* strains inducing the highest growth rate stimulation. Such findings support the significant bioactivity of bacterially released compounds on microalgal performance, without requiring the physical presence of bacteria within the culture system. Production of exudate compounds (vitamins, phytohormones, iron-siderophore, and antibiotics) by microorganisms commonly occurs as part of normal metabolism, and plays a critical role in the biology and ecological competence of bacteria (Lemfack et al., 2014; Schmidt et al., 2015; Sharifi & Ryu, 2018). Over 300 bacteria-derived organic compounds have been identified whose biological function and effect on algal physiology remains largely unknown, in part because of the overwhelming chemical variability of these compounds and limited knowledge on how microalgae may be

affected by these stimuli (Kai et al., 2009; Kai & Piechulla, 2009; Ramanan et al., 2016). The observed growth-promoting effects of bacterial exudates, originating from phylogenetically diverse bacteria, on the culture of a variety of marine microalgal species implies the ubiquitous presence of uncharacterized, growth-stimulating substances in bacterially released metabolites, which may include partially-digested organic nutrients used to grow the bacteria. Enhanced culture growth following culture media enrichment with bacterial exudates was recorded at laboratory scale, and one of the investigated bacterial exudate–algal interaction was repeated in a photobioreactor microalgal cultivation assembly, confirming the reproducibility and scalability of our observations. The absence of a lag phase at the onset of microalgal growth observed in the bacterial exudate-enriched photobioreactors in our scale-up experiment points to the possible role of these additives in the reduction in the time required to upscale mass cultures and lower the risk of failures following initial inoculation (Lavens & Sorgeloos, 1996).

No statistically significant difference was observed in the microalgal final cell density, implying that the bacterial exudate additives act on a rate-limiting step in cell division rather than as an additional source of substrate for mixotrophic microalgal metabolism. Given the macronutrient- and vitamin-replete culture conditions in our experiments (f/2 nutrients enriched seawater), hypothesized mechanisms behind the microalgal growth stimulation by bacterial exudates include the acceleration of some rate-limiting processes governing nutrient acquisition, assimilation, or anabolism through the action of bacteria released phytohormones, iron-chelators, and/or siderophores, and as yet other uncharacterized growth-promoting factors (Amin et al., 2009, 2012; Ramanan et al., 2016; Tsavkelova et al., 2006). Despite incomplete knowledge of the biological function of bacterial exudates, our study experimentally demonstrates that remote bacteria–microalgae relationships can be exploited to optimize microalgal cultivation. Culture media enrichments with bacterial exudates as a growth-promoting strategy for microalgal cultivation have numerous practical advantages, as opposed to bacterial symbionts, including (i) convenience of implementation, (ii) negligible bio-security risks, and (iii) operational/management simplicity. Further studies are required to ensure process stability and quality control for the implementation of bacterial exudates in microalgal cultivation (e.g., standardization of exudate production, effect of seawater composition, etc.). Collectively, our observations have important potential practical implications for bioprocesses in aquaculture and other biotechnology sectors.

4.3 | Stimulation of algal transparent exopolymer particles synthesis by bacterial exudates

We measured the abundance of TEPs because previous work indicated that the presence of bacteria in association with microalgae may not only modulate algal growth but also influence algal secretion of extracellular polymeric substances (EPS) (Bruckner et al., 2011; Grossart & Simon, 2007; Xiao & Zheng, 2016). In natural aquatic environments, exopolysaccharides are ubiquitous and important constituents of TEP (Aluwihare et al., 1997). Most microorganisms, including marine microalgae, produce EPS to fulfill a variety of physiological requirements (Claquin et al., 2008; Passow, 2002a). Biological processes associated with EPS production involve the transformation of organic matter, complexation of dissolved metals, nutrient assimilation, biofilm formation, flocculation, and protection against environmental stressors (Passow, 2002a; Salim et al., 2014; Xiao & Zheng, 2016; Yang et al., 2010). Across microalgal species examined, *Bacillus* and *Mesorhizobium* bacterial exudate treatments resulted in the highest increases in specific growth rates, coinciding with relatively higher algal cell-normalized TEP production. These saccharides act as organic ligands, enhancing iron solubility, mainly as colloidal iron, resulting in the formation of highly bioavailable organic associations for phytoplankton (Hassler et al., 2011). The paired increased microalgal culture productivity and algal EPS production observed in our experiments in the presence of bacterial exudate culture media enrichments points to the potential role of bacterial exudate additives on algal EPS production regulation, iron bioavailability, and algal growth.

5 | CONCLUSION

This study provides a proof of concept of the potential role of bacteria-derived compounds as growth-promoting factors for microalgae cultivation of relevance to biotechnological and aquaculture applications. Through a controlled laboratory investigation of algal–bacterial interactions, we demonstrate that exposure of microalgae to bacterial exudates is significantly more effective at enhancing culture productivity than the co-cultivation of bacteria from which the exudates were sourced. Bacterial exudates interact with marine microalgae in ways that can be beneficial, increasing algal growth rates, stimulating algal release of exopolymeric substances, promoting culture stability, and shortening the culture lag phase following inoculation. The observed enhanced microalgae culture performance in response to bacterial exudates originating from phylogenetically diverse bacterial strains implies that growth-stimulating substances are widely present in bacterially released metabolites. Revisiting culture media formulations to include bacterial exudates represents a promising and widely applicable strategy to enhance algal growth rates in microalgal production systems.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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