2019

COCULTURE OF PROBIOTIC BACTERIA IN ALGAL FEEDSTOCKS FOR DISEASE MANAGEMENT IN BIVALVE HATCHERIES

Samuel Hughes
University of Rhode Island, shughes224@gmail.com

Follow this and additional works at: <https://digitalcommons.uri.edu/theses>

Recommended Citation

Hughes, Samuel, "COCULTURE OF PROBIOTIC BACTERIA IN ALGAL FEEDSTOCKS FOR DISEASE MANAGEMENT IN BIVALVE HATCHERIES" (2019). *Open Access Master's Theses*. Paper 1448.
<https://digitalcommons.uri.edu/theses/1448>

This Thesis is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Master's Theses by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

COCULTURE OF PROBIOTIC BACTERIA IN ALGAL FEEDSTOCKS FOR
DISEASE MANAGEMENT IN BIVALVE HATCHERIES

BY
SAMUEL HUGHES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOLOGICAL & ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

MASTER OF SCIENCE THESIS

OF

SAMUEL HUGHES

APPROVED:

Thesis Committee:

Major Professor Marta Gomez-Chiarri

David Nelson

David Rowley

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2019

ABSTRACT

Bivalve hatcheries include microalgae culture operations as a food source for stock, and these algae cultures harbor dynamic bacterial communities. Because algae from these cultures and their commensal microbiota are distributed to stock larval tanks daily upon feeding, the presence of pathogenic *Vibrio* spp. in hatchery microalgae cultures is a threat to stock health and survival. This study investigates the algal/bacterial and bacterial/bacterial interaction between four popular species of microalgae feedstock, a *Vibrio* sp. of known pathogenicity to bivalves (*V. coralliilyticus* RE22 [RE22]), a probiotic marine bacterium with demonstrated effectiveness in reducing larval shellfish mortality in culture operations (*Phaeobacter inhibens* S4 [S4]), and bacteria strains isolated directly from these algal cultures. Algal growth was unaffected by the addition of probiotics and/or RE22. Results showed that RE22 and S4 have different abilities to grow and persist in coculture, and that the four microalgae species studied have species-specific effects on the levels of RE22 and S4. For example, S4 titers were unaffected by coculture with the microalga *Pavlova pinguis*, yet significantly decreased more than 3- $\log_{10}$ when cocultured with *Tisochrysis lutea*. An antibiotic knockdown experiment and a zone of inhibition assay with commensal isolates suggest that RE22 and S4 do interact with commensal bacteria. Probiotics cocultured with algae and RE22 did not increase the effect of the algae on RE22 levels, and probiotics did not reach high enough levels in coculture to contribute to stock health via routine algae feeding protocols. For these reasons, benefits of supplementing probiotic to microalgal cultures cannot be claimed without further research and development. This research enhances our understanding of algal/bacterial interaction in shellfish hatcheries and informs methods of probiotic delivery to larvae in bivalve hatcheries.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my advisor, Dr. Marta Gomez-Chiarri. I have been continuously inspired by your infectious (hehe!) scientific enthusiasm and remarkable work ethic. You gave me the opportunity to run with my strengths, while helping me develop the abilities that needed work, both in science and in the world, and I am very appreciative.

Thank you to Dr. David Nelson and Dr. David Rowley, who were instrumental in their guidance on all aspects of this thesis, and for your contributions to my personal and professional development.

Thank you to the members of the Gomez-Chiarri lab and the Probiotics working group; I couldn't have completed this project without the instruction and feedback that I received from each of you.

Thank you to Janet Atoyan for your assistance with microscopy and sequencing, to Jan Rines for your assistance with microscopy, and to Gary Wikfors for your time and knowledge.

Finally, thank you to my family, for your unceasing support of my happiness and goals.

PREFACE

Manuscript format is in use for this thesis.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
PREFACE.....	iv
TABLE OF CONTENTS	v
MANUSCRIPT	1
REFERENCES.....	34
APPENDICES.....	40

MANUSCRIPT

To be submitted for publication in Aquaculture, Spring 2019

Coculture of Probiotic Bacteria in Algal Feedstocks for Disease Management in Bivalve Hatcheries

Samuel Hughes^a, Kathryn Brooks^a, Amanda Chesler^b,
David Rowley^c, David Nelson^d and Marta Gomez-Chiarri^a

^aDepartment of Fisheries, Animal and Veterinary Sciences, University of Rhode Island, 9 East Alumni Ave., Kingston, RI 02881, USA

^bAquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062, USA

^cDepartment of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 7 Greenhouse Rd., Kingston, RI 02881, USA

^dDepartment of Cell and Molecular Biology, University of Rhode Island, 120 Flagg Rd., Kingston, RI 02881, USA

Corresponding author:

Marta Gomez-Chiarri, gomezchi@uri.edu

Abstract

Bivalve hatcheries include microalgae culture operations as a food source for stock, and these algae cultures harbor dynamic bacterial communities. Because algae from these cultures and their commensal microbiota are distributed to stock larval tanks daily upon feeding, the presence of pathogenic *Vibrio* spp. in hatchery microalgae cultures is a threat to stock health and survival. This study investigates the algal/bacterial and bacterial/bacterial interaction between four popular species of microalgae feedstock, a *Vibrio* sp. of known pathogenicity to bivalves (*V. coralliilyticus* RE22 [RE22]), a probiotic marine bacterium with demonstrated effectiveness in reducing larval shellfish mortality in culture operations (*Phaeobacter inhibens* S4 [S4]), and bacteria strains isolated directly from these algal cultures. Algal growth was unaffected by the addition of probiotics and/or RE22. Results showed that RE22 and S4 have different abilities to grow and persist in coculture, and that the four microalgae species studied have species-specific effects on the levels of RE22 and S4. For example, S4 titers were unaffected by coculture with the microalga *Pavlova pinguis*, yet significantly decreased more than 3- $\log_{10}$ when cocultured with *Tisochrysis lutea*. An antibiotic knockdown experiment and a zone of inhibition assay with commensal isolates suggest that RE22 and S4 do interact with commensal bacteria. Probiotics cocultured with algae and RE22 did not increase the effect of the algae on RE22 levels, and probiotics did not reach high enough levels in coculture to contribute to stock health via routine algae feeding protocols. For these reasons, benefits of supplementing probiotic to microalgal cultures cannot be claimed without further research and development. This research enhances our understanding of algal/bacterial interaction in shellfish hatcheries and informs methods of probiotic delivery to larvae in bivalve hatcheries.

1. Introduction

Bivalve aquaculture is a major industry – in 2015, global production reached 16 million metric tons, with a value of over 17 billion USD (Food and Agriculture Organization of the United Nations, 2017). Domestic production of oysters, clams and mussels, the three primary groups of bivalves farmed in the United States, was valued at close to \$300 million in 2015 (NOAA Fisheries, 2017). While many different farming methods exist for bivalve culture, the industry relies heavily on hatchery production of seed, as it offers many advantages over the alternative of wild seed collection (Marshall et al., 2010). These benefits notwithstanding, hatcheries still represent the most volatile stage of bivalve production, as disease outbreaks are common and frequently devastating. Of particular concern are pathogenic bacteria of the genus *Vibrio* – among bacterial pathogens affecting reared larvae of bivalves, vibrios are considered to be the most significant, causing documented hatchery losses of larval stock of up to 59% (Elston et al., 2008; Rojas et al., 2016). The genus *Vibrio* contains many species that infect and cause disease in a high diversity of aquatic life (Chatterjee and Haldar, 2012), but certain species, such as *V. alginolyticus*, *V. coralliilyticus*, and *V. tubiashii*, cause the most mortalities in cultured larval bivalves (Beaz-Hidalgo et al., 2010; Dubert et al., 2017).

Currently, bivalve hatcheries rely on water treatment, biosecurity measures and antibiotics to prevent the introduction and/or spread of disease (Dubert et al., 2017; Shumway, 2011). While the first two strategies are largely prophylactic and without negative health, economic, or environmental side effects, the same cannot be said of antibiotics, for several reasons: agricultural usage of antibiotics is widely recognized as a human health concern due to the development and spread of bacterial resistance to the

drugs, the detection of antibiotic residues in aquacultural products is alarming to consumers, and antibiotics inherently disrupt the natural bacterial community in rearing tanks and on the animals themselves, leading to a potential increase in disease susceptibility (Defoirdt et al., 2011; Schmidt et al., 2017). For these reasons, alternative management strategies are in need.

Probiotics represent one such alternative. Defined as “Live, nonpathogenic microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (World Health Organization and Food and Agriculture Organization, 2006), probiotics have already been demonstrated as effective in mitigating disease in aquaculture (de Azevedo and Braga, 2012). *Phaeobacter inhibens* S4 (S4), a member of the *Roseobacter* clade of alpha-proteobacteria, is one such microorganism: hatchery-scale experiments have proven this probiotic to be effective at reducing larval oyster mortalities following a challenge with *Vibrio coralliilyticus* RE22 (RE22), as well as other pathogens (Karim et al., 2013; Sohn et al., 2016). In order to maintain larval protection, however, S4 must be supplied daily to stock tanks, a common requirement for realizing the benefits of probiotics (Karim et al., 2013; Kesarcodi-Watson et al., 2008; Verschuere et al., 2000).

In addition to use in bivalve stock tanks, probiotics could be used to prevent the growth of pathogenic organisms in algal cultures. Bivalves are filter feeding organisms at all life stages, with strict nutritional requirements and filtering capabilities at each stage, and the typical bivalve hatchery grows large quantities of multiple species of microalgae as feedstock (Helm et al., 2004). Microalgal monocultures, such as those found in bivalve

hatcheries, are supplied with high levels of nutrients and illumination for maximum algal production (Helm et al., 2004). Hatchery microalgal cultures contain an abundance of bacteria, with substantial variation in the bacterial community composition observed from one culturing system to another, between algae species, and over time (Nicolas et al., 2004), and these bacterial communities play a role in disease dynamics in hatcheries – pathogenic vibrios are often present in the communities, and bacteria in the microalgae cultures are introduced into bivalve stock tanks upon feeding (Dubert et al., 2017; Elston et al., 2008).

Although the composition of the commensal microbial communities varies considerably from one tank to another, and over time (Nicolas et al., 2004; Wang et al., 2016), certain clades of copiotrophic bacteria, (well-adapted to high nutrient concentrations), and microalgal-symbiotic heterotrophic bacteria have been found to dominate these environments, specifically members of the *Roseobacter* clade and *Cytophaga-Flavobacterium-Bacteroides* group (Nicolas et al., 2004). It is well-established that bacteria influence the growth characteristics of microalgae, and in turn microalgae influence the bacterial community (Cooper and Smith, 2015; Fuentes et al., 2016; Kazamia et al., 2012). Studies of the physiology and ecological roles of roseobacters, and *P. inhibens* S4 specifically, suggest that S4 is an excellent probiotic candidate for coculture with microalgae (Geng and Belas, 2010; Rooney-Varga et al., 2005; Zhao et al., 2016). Closely-related bacteria, *i.e.* other members of the *Roseobacter* clade, have been found to consistently associate with microalgae in nature (Amin et al., 2012; Geng and Belas, 2010), as well as in culture (Nicolas et al., 2004; Sandaa et al., 2003). These associations are believed to be due at least in part to the ability of most roseobacters to

metabolize an organosulfur byproduct of microalgae, dimethylsulfoniopropionate (DMSP) (Burkhardt et al., 2017; Cui et al., 2015); preliminary *in silico* data suggests that S4 does possess this capability (Cui et al., 2015; Genbank accession number NZ_LOHU000000000). Furthermore, S4 produces the antibiotic tropodithietic acid (TDA) and is an excellent biofilm former, characteristics that may provide a sufficient competitive edge for growth and persistence in microalgal coculture (Zhao et al., 2016). As for the ability of S4 to inhibit vibrios in such a setting, one study has demonstrated that additions of *P. inhibens* cause a substantial drop in *V. anguillarum* in a non-axenic culture of the microalgae *Tetraselmis suecica* (Grotkjær et al., 2016), a finding consistent with previous research on the closely-related *Phaeobacter gallaeciensis* and axenic microalgal cultures (D'Alvise et al., 2012).

The objectives of this research were to a) determine the abilities of the probiotic *Phaeobacter inhibens* S4 and the pathogen *Vibrio coralliilyticus* RE22 to grow and persist in microalgae cultures, and b) determine the effect of probiotic coculture on microalgal growth, abundance of *V. coralliilyticus* RE22 added to the cultures, and titers of commensal bacteria. If probiotics can be successfully grown in microalgae cultures, the health benefits of S4 on hatchery stock could be provided with less operational expense – not only would there be no need for daily handing and delivery of probiotic, but bivalve stock are already fed multiple times per day, and the probiotic would be delivered in conjunction with routine feedings. Additionally, cocultured probiotics may succeed in reducing or eliminating the establishment and growth of vibrios in these algae cultures, a potential cause for algal mortality and a recognized entry route of the pathogen into bivalve tanks (Elston et al., 2008).

2. Materials and methods

2.1. Design of coculture experiments

Four different species of microalgae were investigated, based on their current, widespread use as feedstock in bivalve hatcheries: the diatom *Chaetoceros neogracile* Chaet B, the chlorophyte *Tetraselmis chui* PLY-429, and the haptophytes *Tisochrysis lutea* T-Iso and *Pavlova pinguis* CCMP-609 (Bendif et al., 2013; Milke et al., 2008; Napolitano et al., 1990; Wikfors et al., 1996). Microalgal culture stocks were obtained from the Aquaculture Breeding Center at the Virginia Institute of Marine Sciences.

The experimental design consisted of 100 mL algal cultures grown at 22°C with constant illumination (see details below). Predetermined quantities of bacteria and algae were introduced into sterile, fertilized seawater for all coculture and control flasks, and samples were drawn at multiple time points to track: a) the levels of algae using cell counts, and b) the levels of each bacterial strain (probiotic, pathogen, and total bacterial counts) in the cultures using selective media. The treatments tested were: algae (all four strains, see Table 1) co-incubated with a) probiotic S4; b) pathogen RE22; and c) both S4 and RE22. Controls included each algae species alone, S4 only, RE22 only, and S4 and RE22. Each treatment was performed in triplicate and each experiment was performed at least twice.

2.2. Algal cultures

Culture flasks were soaked for 24 hours in Citranox acid detergent (source) and scrubbed thoroughly prior to experiments to remove biofilms. All materials were autoclaved for sterility, except for those that cannot withstand the autoclave: these were bleach-sterilized, rinsed with 0.22 μm filter-sterilized sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), and then rinsed again thoroughly with sterile type-2 deionized water. Instant Ocean® artificial seawater was autoclaved and f/2 fertilizer (Guillard, 1975) and sodium metasilicate (for diatom cultures) were 0.22 μm filter-sterilized. Microalgal cultures were grown up to mid-exponential phase in 1 L flasks containing sterile seawater fertilized with f/2 formula (Fritz Industries, complete algal growth media), with sodium metasilicate (40 mg/L) added to *C. neogracile* cultures, as diatoms require supplemental silicate for growth (Andersen, 2005). Flasks were lightly aerated with 0.3 μm filtered air. Just prior to the beginning of each experiment, a sample of algal culture (200 μL) was taken, fixed with 10% Lugol's iodine solution, and counted with an improved Neubauer counting chamber on a Nikon Eclipse 50i compound microscope using the 40X objective (400X total magnification) and brightfield condenser setting. This measurement of cell density was used to calculate the volume needed for each flask to achieve the target starting density, typically $\sim 10^5$ cells/mL.

2.3. Bacterial cultures

The bacterial strains used in this experiment were selected for antibiotic resistance to facilitate enumeration of each strain from coculture samples using selective media (Table 1). Both strains are resistant to the antibiotic streptomycin (Sm) by spontaneous mutation from the parent strains S4 and RE22. S4 has been engineered for chloramphenicol (Cm)

resistance as well; the plasmid pRhokHi-2-OFP was transferred from *E. coli* Sm10 by conjugation, and the resulting strain named WZ02 (Zhao et al., 2016).

Table 1. Bacteria and microalgae strains used in this study.

Strain	Description	Resistance	Reference/source
<i>Phaeobacter inhibens</i> WZ02	S4Sm (pRhokHi-2-ofp); constitutive expression of orange fluorescent protein	Sm ^r Cm ^r Km ^r	Zhao et al., 2016
<i>Vibrio coralliilyticus</i> RE22Sm	Spontaneous Sm ^r mutant of RE22	Sm ^r	Zhao et al., 2016
<i>Chaetoceros neogracile</i> Chaet B	Originally isolated in Boothbay Maine, USA. Date unknown	n/a	(Milford Culture Collection, n.d.)
<i>Pavlova pinguis</i> CCMP-609	Originally isolated at 34 deg N 65 deg W (just north of Bermuda), 1980	n/a	(Milke et al., 2008)
<i>Tetraselmis chui</i> PLY-429	Originally isolated in Plymouth, UK, 1988	n/a	(Wikfors et al., 1996)
<i>Tisochrysis lutea</i> T-Iso	Originally isolated at the Centre Oceanologique du Pacifique in Taravao, Tahiti, 1977	n/a	(Bendif et al., 2013)

Bacteria were prepared from glycerol freezer stocks and grown up in broth culture containing the antibiotics to which each strain is resistant. Briefly, bacterial freezer stocks were streaked on mYP30 media plates (30 g Instant Ocean salt, 1 g yeast extract, 5 g peptone, filled to 1000 mL type-2 deionized water, pH of 7.5-7.6, and 16 g Bacto agar) for WZ02 (hereafter S4), and Difco TCBS media (thiosulfate-citrate-bile salts-sucrose agar) for RE22sm (hereafter RE22), containing the antibiotic(s) to which each strain is

resistant: Sm (200 µg/mL) for RE22, and Sm (200 µg/mL) & Cm (5 µg/mL) for S4.

Inoculated media plates were then incubated at 28°C until colonies appeared (about 48 hours for S4 and 24 hours for RE22). Following incubation, colonies were picked and

used to inoculate conical tubes

containing 10 mL YP30 broth with the

corresponding antibiotic(s) at the

same concentrations as the media

plates. Broth cultures were shaken

and incubated at 150 rpm and 28°C

for 24 hours for RE22, and 48 hours

for S4. Bacteria were then washed by

three cycles of centrifugation, decanting of supernatant, and resuspension in sterile

seawater. Dilutions to starting concentrations were based on measurement of the optical

density at 600 nm (OD₆₀₀) and calculation of concentration (in colony forming units,

CFU, per mL) based on growth curves (CFU versus optical density at 600 nm) (Zhao et

al. 2016).

Fig. 1. Experimental flasks in incubator.

2.4. Coculture of bacterial strains with microalgae

Microalgal cultures were established by adding a volume of algae from stock cultures to

achieve a starting concentration of 10⁵ cells/ml for *C. neogracile*, *P. pinguis* and *Ti. lutea*,

and 10⁴ cells/ml for *Te. chui*, to 100 mL of artificial, autoclaved seawater (28 psu),

fertilized with f/2 nutrient formula in 125 mL Erlenmeyer flasks (Fig. 1). Bacteria (S4

and RE22) were added from stock cultures to achieve a starting density of 10⁵ CFU/mL.

S4 was added at the beginning of each experiment, while RE22 was added after 24 hours,

since prior research has shown that pre-colonization with S4 inhibits RE22 growth more effectively than simultaneous coculture (Zhao et al., 2016) (Fig. 2). Algal-bacteria cocultures were grown at 22°C under constant illumination (two 2600-lumen T12 bulbs at a color temperature of 4100K) and aerated lightly with 0.3 µm filtered air. Experiments were run for 10-18 days without media exchange, in order to capture any interactions or growth changes that occur over all algal growth stages. One 200 µL sample was taken from each flask at each timepoint (typically day 1, 2, 4, 7, and 10) using sterile technique in a laminar flow hood.

Fig. 2. Introductions of algae and S4 to experimental flasks took place at the beginning of each experiment, while RE22 was added after 24 hours. Sampling was typically conducted on day 1, 2, 4, 7 and 11.

2.5. Algal cell counts

One aliquot (90 µL) was taken from each sample obtained from flasks containing algae, fixed with 10 µL Lugol's iodine, and the microalgal cell density was then determined with a Neubauer improved counting chamber on a Nikon Eclipse 50i compound microscope using the 40X objective (400X total magnification) and brightfield condenser setting. Results are expressed in cells per mL of culture.

2.6. Bacterial cell counts

Planktonic bacterial density (CFU/mL) was determined via serial dilutions and plate-counts of samples, using the spot-plating method as described by Miles & Misra, 1938; since 10 µL was selected for the spotting volume, the lower detection limit for all bacteria in these experiments is 10² CFU/mL. Total culturable bacteria were enumerated by growth on mYP30 agar plates, while the two bacterial strains of interest, S4 and RE22, were each enumerated via selective agar media plates: mYP30 + Sm (200 µg/mL) + Cm (5 µg/mL) was used to enumerate S4 colonies, and thiosulfate-citrate-bile salts-sucrose agar (TCBS; Difco) + Sm (200 µg/mL) was used to enumerate RE22. Cycloheximide (40 µg/mL) was also added to the S4-selective media plates to prevent the growth of microalgae on the agar plates.

2.7. Effect of knockdown of the commensal bacteria in algal cultures on S4 cell counts

A coculture experiment was designed to knock down the commensal bacteria population, in order to determine whether commensal bacteria may affect the growth of S4 when co-incubated with algae. To this end, antibiotics (streptomycin, 200 µg/mL, and chloramphenicol, 5 µg/mL) were added directly to treatment flasks at the beginning of the experiment, and probiotic (S4) growth curves were compared with control cocultures to which no antibiotics were added.

2.8. Zone of inhibition assay

In an effort to determine if commensal bacteria associated with *C. neogracile*, *P. pinguis*, and *Te. chui* could be affecting levels of S4 or RE22 in microalgal coculture via direct inhibition (*Ti. lutea* was not available at the time of the assay), culturable bacteria were isolated from algae cultures and a zone of inhibition assay was conducted to identify isolates with inhibitory effects on S4 and RE22. The assay was conducted as described by Karim et al. (2013) with minor changes. Briefly, samples were collected from each of the three algae cultures, serially-diluted, and 10 μ L volumes of each dilution were then spotted on mYP30 media and incubated for 72 h. All morphologically-distinct colonies from each algae sample were picked and grown up overnight in mYP30 broth at 150 rpm and 28°C; 5 μ L of each of these overnight cultures was then spotted onto an RE22 lawn prepared on mYP30 media, and allowed to incubate at 28°C for 48h. S4 was selected as a positive control because it has known inhibitory effects against RE22 (Karim et al., 2013). Following incubation, zones of inhibition were identified and measured: the values reported are the average of two measurements (perpendicular to each other) of the difference in diameter between the zone of inhibited growth of RE22 and the colony size of each isolate. Results are expressed in mm.

2.9. Microscopy

Since another *P. inhibens* strain has been found to attach to microalgae cells (Bramucci et al., 2018; Mayers et al., 2016), this study sought to determine if any such interactions occur between *P. inhibens* S4 and the microalgae species investigated. To this end, *P. inhibens* strain WZ02 (Table 1) was selected for its constitutive expression of orange fluorescent protein (OFP), which can be visualized against a large background bacterial community via epifluorescent microscopy (Zhao et al., 2016). Samples were taken on

days 5 and 9 of each coculture experiment except for *Ti. lutea*, and visualized without fixation within one hour from sample collection on a Zeiss Axioimager M2 Imaging System, utilizing ZEN 2011 software; micrographs were captured with a Zeiss Axioacam HRc high resolution camera. Observations and micrographs were taken with a 10X, 40X, and 100X oil immersion objective (total magnification with 100X objective = 1000X) and the following settings: transmitted light brightfield, phase contrast, and epifluorescent observation with a Rhodamine filter set for visualization of OFP-expressing S4.

2.10. Statistical analysis

All analyses were conducted with Graphpad PRISM 6.0 (GraphPad Software, n.d.). Nonlinear regression and the extra sum-of-squares *F*-test were used to determine whether growth curves differed significantly between treatments in all experiments. All data were $\log_{10}$ transformed prior to curve-fitting. Two different models were used:

(a) algae and total bacteria, which showed traditional logistic growth curves, were modeled with a logistic function:

$$N = NM * N0 / ((NM - N0) * \exp(-k * x) + N0)$$

Where:

$N0$ is the starting population

NM is the maximum population

and K is the rate constant.

(b) all other bacterial growth curves were modeled with a quadratic (second-degree polynomial) function:

$$Y = B0 + B1 * X + B2 * X^2$$

Where:

B_0 is a constant

and B_1 and B_2 are coefficients to the first and second order terms, respectively.

An initial F -test was applied to all treatments in each experiment to test whether one curve adequately fits all data sets; a p -value of <0.05 was chosen to identify data sets that are better represented by more than one curve. Following the initial F -test of all treatments (except algae growth curves, none of which differed significantly), biologically-relevant pairwise comparisons were tested as well: (1) bacteria only (RE22 or S4) vs. the same strain grown in coculture with algae; (2) bacteria only (RE22 or S4) vs. both strains (RE22 & S4) incubated together; and (3) RE22 and S4 incubated together vs. RE22 & S4 incubated together with algae. A p -value of $<0.05 / 3 = 0.0167$ was used to identify data sets better represented by two curves than one, to account for multiple comparisons.

3. Results

3.1. Effect of bacterial additions on microalgal growth

This study sought to determine the effect of S4 and/or RE22 additions on microalgal growth. Growth curves of all four microalgae species were unaffected by bacterial (*P. inhibens* S4 and/or *V. coralliilyticus* RE22) additions (Fig. 3; global F -test p -values for all four species ranged from 0.828 to 0.9981; Supplementary Table S1).

Fig. 3. Effect of bacterial additions (probiotic S4 and pathogen RE22) on growth of the algae (A) *C. neogracile*, (B) *P. pinguis*, (C) *Te. chui*, and (D) *Ti. lutea*. Values are shown as means $\pm$ SD (n=3) of a representative experiment. Global *F*-tests on each set of curves revealed no significant differences between treatments (Supplementary table S1).

3.2. Dynamics of pathogen RE22 in coculture with microalgal strains

This study sought to determine the dynamics of the pathogen RE22 in coculture with microalgae. The levels of RE22 in fertilized seawater increased from 10^5 to 10^6 CFU/mL during the first day of incubation, and then levels were maintained at 10^6 CFU/mL for the length of the experiment (Fig. 4). As previously reported (Zhao et al., 2016), the

presence of S4 significantly affected the levels of planktonic RE22 in fertilized seawater, stabilizing at a titer of $\sim 10^5$ CFU/mL within the first 24 h of addition of RE22 to media containing a 24 h culture of S4, approximately 1 log lower than the RE22-only control ($p < 0.0017$, Appendix Table S2). Coculture with microalgae (all species) resulted in a significant decline in RE22 titers compared with RE22 in media alone, although the magnitude of decline varied between algal species: coculture with *C. neogracile* caused a decline of 2 log₁₀ to a stabilized planktonic cell density of $\sim 2 \times 10^4$ CFU/mL ($p < 0.0001$) (Fig. 4A, Appendix Table S2), while coculture with *P. pinguis*, *Te. chui*, and *Ti. lutea* caused a significant decline of 3-4 log₁₀ to a planktonic cell density of 10^2 - 10^3 CFU/mL ($p < 0.0001$ for all) (Fig. 4B-D, Appendix Table S2). No significant differences were found between growth curves of RE22 co-incubated with algae alone vs. RE22 co-incubated with algae and S4 (p -values ranged from 0.2507 to 0.8983) (Fig. 4, Appendix Table S2).

Fig. 4. Growth curves of *V. coralliilyticus* RE22 in coculture with probiotic S4 and (A) *C. neogracile*, (B) *P. pinguis*, (C) *Te. chui*, and (D) *Ti. lutea*. Values are shown as means $\pm$ SD ($n=3$) of a representative experiment. Global F -tests revealed significant differences between treatments for each set of curves ($p < 0.05$) (Supplementary table S2). Different letters indicate significantly different curves based on pairwise comparisons ($p < 0.0167$).

3.3. Dynamics of probiotic S4 in coculture with microalgal strains

This study sought to determine the dynamics of the probiotic S4 in coculture with microalgae. S4 showed a decline of 0.5-1 $\log_{10}$ of planktonic CFU/mL in the first 24h of incubation in fertilized seawater (control), followed by a more gradual, steady decline to 2×10^4 - 2×10^5 CFU/mL after 12 days (Fig. 5). The addition of RE22 (final concentration

10⁵ CFU/mL) to 24 h cultures of S4 led to a significant increase in planktonic S4 CFU/mL compared to S4 alone in 2 out of 4 experiments ($p<0.0043$) (Fig. 5A, 5C, Appendix Table S3). The effect of coculture with algae on S4 CFU/mL was found to be species-specific: no significant difference was observed for *C. neogracile* and *P. pinguis*, while coculture with *Te. chui* and *Ti. lutea* caused a significant decline in S4 CFU/ml beginning in the first 1-2 days of each experiment ($p<0.0001$) (Fig. 5C, 5D, Appendix Table S3). The extent of this decline differed between the two species: coculture with *Te. chui* caused an average 1 log₁₀ decline in S4 levels over the course of the experiment, decreasing to a final level of 2x10⁴ CFU/mL after 12 days (Fig. 5C), while coculture with *Ti. lutea* caused a much more substantial decline in S4 levels, dropping to 3x10² CFU/mL (Fig. 5D). Additionally, *Te. chui* was the only algae species for which RE22 addition significantly affected S4 levels in coculture, causing an average 0.5 log₁₀ increase in S4 CFU/mL in the final two sampling timepoints (days 8 and 12; $p=0.0154$; Fig. 5C).

Fig. 5. Growth curves of *P. inhibens* S4 in coculture with pathogen RE22 and (A) *C. neogracile*, (B) *P. pinguis*, (C) *Te. chui*, and (D) *Ti. lutea*. Values are shown as means $\pm$ SD (n=3) of a representative experiment. Global *F*-tests on each set of curves revealed significant differences between treatments when co-incubated with all algae species but *P. pinguis* ($p < 0.05$) (Supplementary table S3). Different letters indicate significantly different curves based on pairwise comparisons ($p < 0.0167$).

3.4. Effect of probiotic and pathogen additions on commensal bacterial density

This study sought to determine if S4 and/or RE22 additions affected total culturable bacterial density in microalgae cultures. Total culturable bacterial density in cultures of all four microalgae species was unaffected by additions of S4 and RE22 (Fig. 6; global *F*-test *p*-values for all four species ranged from 0.2726 to 0.8508).

Fig. 6. Abundance of total culturable bacteria in flasks inoculated with (A) *C. neogracile*, (B) *P. pinguis*, (C) *Te. chui*, (D) *Ti. lutea*, and RE22 and S4. Values are shown as means $\pm$ SD (n=3) of a representative experiment. Global *F*-tests on each set of curves revealed no significant differences between treatments ($p>0.05$) (Supplementary table S5).

Fig. 7. Density of *P. inhibens* S4 in coculture with *C. neogracile*, with and without added antibiotics. Values are shown as means $\pm$ SD ($n=3$). A global F -test revealed significant differences between treatments ($p<0.05$) (Supplementary table S4). Different letters indicate significantly different curves based on pairwise comparisons ($p<0.0167$).

3.5. Dynamics of S4 in coculture with antibiotic-treated microalgae

This study sought to determine if knockdown of the commensal bacteria in algae cultures affects the dynamics of S4 in coculture with algae. Addition of streptomycin (200 $\mu\text{g/mL}$) and chloramphenicol (5 $\mu\text{g/mL}$) to *C. neogracile* cultures did not eliminate the commensal bacterial population, but did reduce total culturable bacteria by 2.5 $\log_{10}$ on day 4 and 1.5 $\log_{10}$ on day 8 (compared to control cultures to which no antibiotics were added) (Appendix, Fig. S2). Antibiotic additions had minimal effect on S4 density on day 4, but on day 8 S4 was significantly higher in coculture with algae to which antibiotics were added, compared to both controls (S4 only with antibiotics, and S4 and algae without antibiotics; $p=0.0057$ and 0.0178, respectively [Appendix, table S4]) (Fig. 7). An increase in planktonic S4 CFU density of $\sim 0.5 \log_{10}$ was observed compared to the S4-only with antibiotics control, and an increase of $\sim 1 \log_{10}$ was observed compared

to the S4 and algae without antibiotics control. The antibiotics had no effect on algae cell density, based on a preliminary trial (Appendix, Table S6).

3.6. Isolation of commensal bacteria able to inhibit the growth of RE22 *in vitro*

In an effort to determine if commensal bacteria could be causing the observed decline of RE22 (and to a lesser extent, S4) in microalgal coculture, isolates cultured from algal cultures were tested for inhibitory activity against RE22 and S4 in a zone of inhibition assay. A total of 40 bacterial isolates were tested: 14 from *C. neogracile*, 15 from *P. pinguis*, and 11 from *Te. chui* (*Ti. lutea* was not available at the time of the assay). None of the 40 bacterial isolates inhibited S4 in a zone of inhibition assay. Seven of these isolates inhibited RE22 to varying degrees (Table 2); six of these strains came from a culture of *C. neogracile*, and one came from a culture of *Te. chui*. S4 was selected as a positive control for its known inhibitory effects against RE22 (Karim et al., 2013); RE22 growth was inhibited to the same degree as the control S4 by one isolate from *C. neogracile*, while the remaining isolates all had smaller zones of inhibition.

Table 2. Inhibition of RE22 growth by bacterial isolates from algal cultures. A total of 40 isolates (11 from *T. chui*, 14 from *C. neogracile*, and 15 from *P. pinguis*) were tested. S4 was selected as a positive control for its known inhibitory effects against RE22. 7/40 isolates inhibited RE22 growth. None of the isolates from algal cultures showed higher inhibitory activity than S4.

Source	Isolate	ZOI Avg (mm) $\pm$ SD
<i>C. neogracile</i>	E	1.3 $\pm$ 1.5
<i>C. neogracile</i>	S	1.7 $\pm$ 0.8
<i>C. neogracile</i>	Q	0.3 $\pm$ 0.6
<i>C. neogracile</i>	R	1.0 $\pm$ 0.9
<i>C. neogracile</i>	J	1.3 $\pm$ 1.3
<i>C. neogracile</i>	D	0.8 $\pm$ 0.3
<i>T. chui</i>	TD	0.8 $\pm$ 0.8
Control	S4	1.7 $\pm$ 0.3

3.7. Association of S4 with algal cells

Fluorescent imaging conducted on samples of each treatment throughout all experiments (except for *Ti. lutea*, which was not investigated) revealed no physical associations between S4 and microalgae cells of any of the three species (*C. neogracile*, *P. pinguis* and *Te. chui*). Therefore, the methods used here for measuring bacterial levels in media mainly reflect planktonic cells. S4 was observed to exist planktonically as both single cells and in rosette structures (multicellular aggregations) (Bruhn et al., 2007) (Fig. 8) on both sampling timepoints (d 5 and 9). Qualitative observations did not identify any variation in the proportion of S4 existing as single cells vs. rosettes across timepoints, treatments, or co-incubated algae species.

Fig. 8. Planktonic S4 in algal coculture existed as both single cells (A, top two cells) and rosettes (A, bottom cell grouping, and B).

4. Discussion

This study explores the incorporation of probiotics into algae cultures in bivalve hatcheries for improved disease management in bivalve hatcheries. Results revealed no effect of probiotic or pathogen coculture on the growth of microalgae, reaffirming a previous report on RE22 – algal interactions (Elston et al., 2008). Coculture of bacteria with microalgae revealed species-specific effects on both RE22 and S4 dynamics in algal coculture, and these dynamics differed between the two bacterial strains, particularly when cocultured with *P. pinguis* and *Te. chui*. Results from the antibiotic knockdown experiment and zone of inhibition assay with commensal isolates suggest that RE22 and S4 interact with commensal bacteria in algal coculture, but direct inhibition of RE22 and S4 by commensal bacteria is unlikely to be the only driver of their observed dynamics. While S4 inhibits RE22 *in vitro* (Karim et al. 2013, Zhao et al. 2016), the same effect was not observed when these two species were cocultured with algae, probably due to the effect of the algal species on RE22. This research sheds new light on microbial-algal interactions and provides practical applications for bivalve hatcheries.

RE22 maintained a titer in media alone at a density of 10^6 CFU/ml. Previous work has identified *Vibrio* spp. titers of 10^4 - 10^5 CFU/mL in surface seawater (Elston et al., 2008) and titers of up to $2\text{-}3 \times 10^9$ CFU/ml in nutrient-rich growth media under controlled laboratory conditions (Zhao et al., 2016). When added to media pre-colonized by S4, RE22 titers dropped approximately 1 $\log_{10}$, exhibiting the probiotic effect *in vitro* and corroborating prior observations (Zhao et al., 2016).

S4 also maintained a high titer in media alone, and declined gradually. Research has found roseobacters can be abundant in natural seawater, especially among algal blooms: the relative abundance of roseobacters among the marine bacterial community can reach 30% (Buchan et al., 2005). S4 dynamics in media alone were similar to RE22, except titers of S4 were slightly lower, and decreased slightly over time; this may be due to nutrient depletion or settlement of planktonic cells onto surfaces (Bruhn et al., 2007; Kolter et al., 1993). When cocultured with RE22, S4 titers increased significantly in 2 out of 4 experiments. Previous work did not detect such an effect (Zhao et al., 2016), although experimental conditions (*e.g.* media used) were different. This observed increase in some of the experiments may be due to increased nutrient availability following killing of RE22 cells by S4, resulting in increased growth; a study of nutrient limitation of S4 in this media could provide greater insight. A second explanation could be an effect of RE22 on the “swim or stick” phenotype of S4: studies have found that environmental and chemical cues control the tendency of some roseobacters to synthesize flagella and enter media as motile, single cells, or to shed their flagella and aggregate in biofilms and rosettes (Belas et al., 2009; Sule and Belas, 2013). Since the methods here only account for colony-forming units of planktonic or particle (algae)- associated cells (algae in cultures were also plated), a change in S4 phenotype towards the motile life stage would be detected as an increase in S4 titer.

In most cases, dynamics of RE22 and S4 differed between bacteria-only controls and coculture with algae; for RE22, titers declined in coculture with all four algal species, compared to the RE22-only control. *C. neogracile* supported and maintained the highest levels of RE22, while coculture with the other three algae species caused a steady decline

in RE22 levels as time progressed. The results reported here corroborate previous research which found that *Te. chui* and a close relative of *Ti. lutea*, *Isochrysis galbana*, inhibit the growth of vibrios in coculture (Giménez Papiol et al., 2018; Molina-Cárdenas et al., 2014). The species-specific nature of these interactions has great relevance to bivalve aquaculture, since abundance of pathogenic vibrios in algal feedstocks is directly linked to stock exposure to these pathogens (Elston et al., 2008). Possible mechanisms of this species-specific inhibition of RE22 include algal production of antimicrobials (Austin et al., 1992) and interference in bacterial quorum sensing (Natrash et al., 2011), as well as inhibition by commensal bacteria specific to each algae culture; the antibiotic knockdown experiment and zone of inhibition assay (discussed below) were designed to probe this possibility. Furthermore, relationships between microalgae and bacteria can be dynamic: in one case, vibrios were found to proliferate in dense cultures of the microalgae *Nannochloropsis oculata*, but drop to undetectable levels in lower density cultures (D'Alvise et al., 2012). More research is needed to explore the dynamics of other pathogenic vibrio species/strains found in microalgae cultures, since members of the *Vibrio* clade show variation in their ability to grow and persist in coculture with algae (Molina-Cárdenas et al., 2014).

As for S4, coculture with two of the four algal species caused a decline in S4 compared to the S4-only control, while the other two species had no effect. Furthermore, the two species that inhibited S4 did so to different degrees – *Ti. lutea* caused nearly a 2 log₁₀ greater decline than *Te. chui*. In addition to the potential mechanisms driving bacterial dynamics in coculture as discussed above for RE22, variable production of DMSP between algae species may also contribute to the species-specific effect on S4 growth.

The common natural association between roseobacters and microalgae is believed to be due at least in part to the relatively unique capability of most roseobacters to metabolize DMSP, an organosulfur byproduct of microalgal metabolism which is abundant among dense algae growth (Burkhardt et al., 2017; Cui et al., 2015); preliminary *in silico* data of the draft genome (Genbank accession number NZ_LOHU000000000) by our team suggests that S4 does possess this capability.

Interestingly, S4 did not cause a decline in RE22 when cocultured with algae, even though the effect was observed in bacteria-only controls (as expected) (Zhao et al., 2016). Possible explanations for this observation include: a) RE22 is already declining on its own in coculture with three out of four algal species, obscuring the effect of S4 (*C. neogracile* being the exception, which cannot be explained by RE22-algal dynamics); b) titers of S4 are lower in algal coculture than the bacteria-only controls, so a lesser inhibitory effect (if any) is to be expected (Karim et al., 2013); and/or c) the environmental changes (both physical and chemical) caused by the presence and growth of microalgae alters the phenotype of S4 in such a way that it does not inhibit RE22, such as decreased production of TDA (Bruhn et al., 2007). Although no effect was observed here, it has been demonstrated that additions of *P. inhibens* DSM17395 cause a substantial drop in *V. anguillarum* in a non-axenic culture of the microalgae *Tetraselmis suecica* (Grotkjær et al., 2016), a finding that was further supported by research on the closely-related *P. gallaeciensis* and axenic microalgal cultures (D'Alvise et al., 2012). Interrelated species effects have been observed as well – coculture with *Nannochloropsis* was found to enhance the antibiotic effect of a roseobacter against *V. anguillarum* (Sharifah and Eguchi, 2011).

Even though S4 did not affect RE22 in coculture with any of the four algal species tested, RE22 additions did result in significantly higher S4 levels when cocultured with *Te. chui* (not observed with the other three algae species). This increase due to RE22 addition was also observed in the bacteria-only coculture controls, and may be due to the same factors hypothesized above: increased nutrient availability following killing of RE22 cells by S4, and an effect of RE22 on the “swim or stick” phenotype of S4.

An antibiotic knockdown experiment and a zone of inhibition assay with commensal isolates were carried out in an effort to determine if (and to what degree) commensal bacteria affect the growth dynamics of RE22 and S4 in coculture, and if the observed declines of RE22 and S4 in coculture with some algal species could be attributed to direct antagonism by the commensal bacteria. The antibiotic experiment was conducted with *C. neogracile* only, and revealed minor inhibition of S4 by commensal bacteria; commensal bacteria of other algal strains may affect S4 differently. By day eight, S4-only controls increased 0.5 log₁₀ with antibiotic additions, and S4 co-incubated with algae and antibiotics increased 1 log₁₀, compared to coculture with algae and no antibiotics, which leaves a 0.5 log₁₀ increase that may be explained by the knocked-down bacterial community. A possible explanation for the surprising increase in planktonic S4 CFU/mL following antibiotic additions (to which it is resistant) may be due to a phenotypic change triggered by the antibiotics associated with less aggregation into rosettes or settlement onto surfaces. Although the 0.5 log₁₀ CFU/mL increase due to the knocked-down bacterial community is slight (but statistically significant), the antibiotic treatment most probably merely disrupted the commensal bacterial community, and did not eliminate it

completely. Future experiments with axenic algae cultures will shed more light on this interaction; previous work has found that competition for limited space and nutrients can be intense in microbial communities, such as those associated with microalgae cultures, and these communities can prevent the establishment of strains that would otherwise grow alone in media or with algae (Behringer et al., 2018; Wietz et al., 2013).

A zone of inhibition assay was conducted to further explore the nature of this observed interaction between S4 and commensal bacteria, and to shed light on the species-specific effect of RE22 in algal coculture. It was expected that if the assay discovered inhibitory bacterial isolates with inhibitory activity against RE22 and/or S4, they would be from the algal cultures in which RE22 and S4 showed the greatest decline. While no bacterial isolates from algal cultures inhibited S4, we were surprised to find that six out of the seven isolates that are inhibitory towards RE22 *in vitro* came from *C. neogracile* – the algae culture that inhibits RE22 growth the least (of the four studied). Furthermore, none of the bacteria isolated from *P. pinguis* inhibited RE22, even though RE22 dropped over 3 log₁₀ in coculture with this species. As seen here and previously, bacterial inhibition *in vitro* does not always correspond to inhibition *in vivo* (Gram et al., 2001). Possible explanations include a) phenotypic differences between isolates grown on agar media vs. algae culture, where differences in nutrient availability and cell density may play a role in expression of inhibitory cell products and/or mechanisms (Gram et al., 2001; Kinnula et al., 2017); b) complex interactions between inhibitory isolates and algae and/or commensal bacteria, such as competition and quorum sensing interference (Natrah et al., 2014; Wietz et al., 2013); and c) the possibility that inhibition still occurs in coculture, but the signal is lost among other factors of higher impact affecting RE22 growth or the

inhibitory activity is due to commensal bacteria that do not grow on the media used for isolations. These findings taken together suggest that direct inhibition of RE22 by culturable commensal bacteria may not be the primary factor contributing to the observed decline in RE22 when cocultured with the algae species investigated here. Furthermore, the slight inhibition of S4 growth that may be attributable to interaction with commensal bacteria appears not to be due to direct (antagonistic) inhibition by culturable strains, since no S4-inhibitory isolates were discovered.

Fig. 9. Summary diagram of observed effects of algal coculture on planktonic abundance of probiotic S4 and pathogen RE22. Coculture with algae inhibited growth of S4 and RE22 in most cases (red arrows); arrow thickness signifies the observed magnitude of inhibition. Results of the antibiotic knockdown experiment suggest that commensal bacteria do play a role in probiotic dynamics in algal coculture. Experiments with axenic algal cultures can shed more light on the mechanisms underlying these interactions (Fig. S4).

This research informs the practical application of probiotic usage in bivalve hatcheries. While S4 did persist in coculture with all four algae species, albeit to varying degrees, the levels never reached the high density required for supplying this probiotic to stock tanks at an effective dose. Previous research found that a minimum S4 density of 10^4 CFU/mL is required to confer a health benefit on stock (Karim et al., 2013), and the dilution factor of feeding has been estimated at 1000X (Elston et al., 2008); these values taken together suggest that for microalgae cultures to serve as an effective incubator and delivery vehicle of S4 to stock tanks, the probiotic would have to grow to a density of $\sim 10^7$ CFU/mL. The highest densities attained after early timepoints were in coculture with *P. pinguis* and *Te. chui*, where S4 levels remained in the range of 10^4 - 10^5 CFU/mL, 2-3 $\log_{10}$ below the required density. Importantly, this study has revealed that for maximum delivery of S4 to stock tanks, S4 must be mixed with algal feedstocks *just prior* to feeding, versus allowing a period of co-incubation, during which time S4 titers are likely to drop (differences in environmental conditions and media composition from those used here may affect S4 dynamics (Zech et al., 2013)).

Further implications of this research for bivalve hatcheries include the observed resilience of feedstock microalgae to bacterial antagonism, and the interspecific variation in their effect on the pathogen RE22. Since many bacteria have been found to parasitize or inhibit the growth of microalgae (Ramanan et al., 2016), the lack of antagonism of algae by S4 is encouraging for future applications of this or related probiotics in microalgae coculture. Regarding the species-specific effect of algae on RE22, hatchery managers seeking to minimize vibrio introductions to stock tanks are advised to closely monitor for contamination algal feedstocks that support higher levels of pathogenic

vibrios, such as *C. neogracile*, which this study has shown to support relatively high levels of RE22.

The results presented here emphasize the complex algal/bacterial interactions that dictate the growth and/or decline of aquaculturally-important microbes in bivalve feedstock cultures. More research is needed to shed light on the drivers of these bacterial dynamics in order to develop more effective probiotic-based disease management strategies.

Ethics statement: Not applicable.

Conflict of interest: The authors declare that they have no conflicts of interest.

Acknowledgements:

We would like to thank all members of the Gomez-Chiarri lab and the Probiotics Working Group at the University of Rhode Island; Janet Atoyan, University of Rhode Island Genomics and Sequencing Center; Gary Wikfors, NOAA Milford Laboratory; and Jan Rines, University of Rhode Island Graduate School of Oceanography.

This research was supported by the United States Department of Agriculture (USDA) – NIFA Northeast Aquaculture Regional Center (NRAC) grant award number 28598-Z5659005 and by the Department of Commerce/NOAA Saltonstall-Kennedy grant award number NA18NMF4270193.

This material is based upon work conducted at a Rhode Island NSF EPSCoR research facility, the Genomics and Sequencing Center, supported in part by the National Science Foundation EPSCoR Cooperative Agreements 0554548, EPS-1004057, and OIA-1655221.

References

- Amavizca, E., Bashan, Y., Ryu, C.-M., Farag, M.A., Bebout, B.M., de-Bashan, L.E., 2017. Enhanced performance of the microalga *Chlorella sorokiniana* remotely induced by the plant growth-promoting bacteria *Azospirillum brasilense* and *Bacillus pumilus*. *Scientific Reports* 7. <https://doi.org/10.1038/srep41310>
- Amin, S.A., Parker, M.S., Armbrust, E.V., 2012. Interactions between diatoms and bacteria. *Microbiology and Molecular Biology Reviews* 76, 667–684. <https://doi.org/10.1128/MMBR.00007-12>
- Andersen, R.A. (Ed.), 2005. *Algal culturing techniques*. Elsevier/Academic Press, Burlington, Mass.

- Austin, B., Baudet, E., Stobie, M., 1992. Inhibition of bacterial fish pathogens by *Tetrasetelmis suecica*. *Journal of Fish Diseases* 15, 55–61.
<https://doi.org/10.1111/j.1365-2761.1992.tb00636.x>
- Beaz-Hidalgo, R., Balboa, S., Romalde, J.L., Figueras, M.J., 2010. Diversity and pathogenicity of *Vibrio* species in cultured bivalve molluscs. *Environmental Microbiology Reports* 2, 34–43. <https://doi.org/10.1111/j.1758-2229.2010.00135.x>
- Behringer, G., Ochsenkühn, M.A., Fei, C., Fanning, J., Koester, J.A., Amin, S.A., 2018. Bacterial communities of diatoms display strong conservation across strains and time. *Frontiers in Microbiology* 9. <https://doi.org/10.3389/fmicb.2018.00659>
- Belas, R., Horikawa, E., Aizawa, S.-I., Suvanasuthi, R., 2009. Genetic Determinants of *Silicibacter* sp. TM1040 Motility. *Journal of Bacteriology* 191, 4502–4512.
<https://doi.org/10.1128/JB.00429-09>
- Bendif, E.M., Probert, I., Schroeder, D.C., de Vargas, C., 2013. On the description of *Tisochrysis lutea* gen. nov. sp. nov. and *Isochrysis nuda* sp. nov. in the Isochrysidales, and the transfer of Dicrateria to the Prymnesiales (Haptophyta). *Journal of Applied Phycology* 25, 1763–1776. <https://doi.org/10.1007/s10811-013-0037-0>
- Bigelow National Center for Marine Algae and Microbiota, n.d. Algae Culture Collection.
- Bramucci, A.R., Labeeuw, L., Orata, F.D., Ryan, E.M., Malmstrom, R.R., Case, R.J., 2018. The Bacterial Symbiont *Phaeobacter inhibens* Shapes the Life History of Its Algal Host *Emiliania huxleyi*. *Frontiers in Marine Science* 5.
<https://doi.org/10.3389/fmars.2018.00188>
- Bruhn, J.B., Gram, L., Belas, R., 2007. Production of Antibacterial Compounds and Biofilm Formation by *Roseobacter* Species Are Influenced by Culture Conditions. *Applied and Environmental Microbiology* 73, 442–450.
<https://doi.org/10.1128/AEM.02238-06>
- Buchan, A., Gonzalez, J.M., Moran, M.A., 2005. Overview of the Marine *Roseobacter* Lineage. *Applied and Environmental Microbiology* 71, 5665–5677.
<https://doi.org/10.1128/AEM.71.10.5665-5677.2005>
- Burkhardt, I., Lauterbach, L., Brock, N.L., Dickschat, J.S., 2017. Chemical differentiation of three DMSP lyases from the marine *Roseobacter* group. *Organic & Biomolecular Chemistry* 15, 4432–4439. <https://doi.org/10.1039/C7OB00913E>
- Carney, L.T., Reinsch, S.S., Lane, P.D., Solberg, O.D., Jansen, L.S., Williams, K.P., Trent, J.D., Lane, T.W., 2014. Microbiome analysis of a microalgal mass culture growing in municipal wastewater in a prototype OMEGA photobioreactor. *Algal Research* 4, 52–61. <https://doi.org/10.1016/j.algal.2013.11.006>
- Chatterjee, S., Haldar, S., 2012. *Vibrio* Related Diseases in Aquaculture and Development of Rapid and Accurate Identification Methods. *Journal of Marine Science: Research & Development* s1. <https://doi.org/10.4172/2155-9910.S1-002>
- Cooper, M.B., Smith, A.G., 2015. Exploring mutualistic interactions between microalgae and bacteria in the omics age. *Current Opinion in Plant Biology* 26, 147–153.
<https://doi.org/10.1016/j.pbi.2015.07.003>
- Cui, Y., Suzuki, S., Omori, Y., Wong, S.-K., Ijichi, M., Kaneko, R., Kameyama, S., Tanimoto, H., Hamasaki, K., 2015. Abundance and distribution of dimethylsulfoniopropionate degradation genes and the corresponding bacterial community structure at dimethyl sulfide hot spots in the tropical and subtropical

- Pacific ocean. *Applied and Environmental Microbiology* 81, 4184–4194.
<https://doi.org/10.1128/AEM.03873-14>
- D’Alvise, P.W., Lillebø, S., Prol-Garcia, M.J., Wergeland, H.I., Nielsen, K.F., Bergh, Ø., Gram, L., 2012. *Phaeobacter gallaeciensis* reduces *Vibrio anguillarum* in cultures of microalgae and rotifers, and prevents vibriosis in cod larvae. *PLoS ONE* 7, e43996. <https://doi.org/10.1371/journal.pone.0043996>
- de Azevedo, R.V., Braga, L.G.T., 2012. Use of Probiotics in Aquaculture, in: Rigobelo, E. (Ed.), *Probiotic in Animals*. InTech, pp. 103–118.
<https://doi.org/10.5772/50056>
- Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* 14, 251–258.
<https://doi.org/10.1016/j.mib.2011.03.004>
- Dubert, J., Barja, J.L., Romalde, J.L., 2017. New insights into pathogenic vibrios affecting bivalves in hatcheries: present and future prospects. *Frontiers in Microbiology* 8. <https://doi.org/10.3389/fmicb.2017.00762>
- Elston, R., Hasegawa, H., Humphrey, K., Polyak, I., Häse, C., 2008. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. *Diseases of Aquatic Organisms* 82, 119–134.
<https://doi.org/10.3354/dao01982>
- Food and Agriculture Organization of the United Nations, 2017. Fisheries and aquaculture statistics 2015. *FAO Statistics* 107.
- Fuentes, J., Garbayo, I., Cuaresma, M., Montero, Z., González-del-Valle, M., Vílchez, C., 2016. Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. *Marine Drugs* 14, 100.
<https://doi.org/10.3390/md14050100>
- Geng, H., Belas, R., 2010. Molecular mechanisms underlying *Roseobacter*–phytoplankton symbioses. *Current Opinion in Biotechnology* 21, 332–338.
<https://doi.org/10.1016/j.copbio.2010.03.013>
- Giménez Papiol, G., Roque, A., Estévez, A., 2018. Variations in Bacterial Community of Rearing Water and Gut of Common Dentex, *Dentex dentex* (Linnaeus 1758), Larvae Using Three Microalgae Management Approaches. *Journal of the World Aquaculture Society* 49, 953–963. <https://doi.org/10.1111/jwas.12437>
- Goecke, F., Thiel, V., Wiese, J., Labes, A., Imhoff, J.F., 2013. Algae as an important environment for bacteria–phylogenetic relationships among new bacterial species isolated from algae. *Phycologia* 52, 14–24.
- Gram, L., Løvold, T., Nielsen, J., Melchiorson, J., Spanggaard, B., 2001. In vitro antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis 11.
- GraphPad Software, n.d. Prism 6.0h for Mac. La Jolla, California.
- Grotkjær, T., Bentzon-Tilia, M., D’Alvise, P., Dierckens, K., Bossier, P., Gram, L., 2016. *Phaeobacter inhibens* as probiotic bacteria in non-axenic *Artemia* and algae cultures. *Aquaculture* 462, 64–69.
<https://doi.org/10.1016/j.aquaculture.2016.05.001>
- Helm, M.M., Bourne, N., Lovatelli, A., 2004. Hatchery culture of bivalves: a practical manual.
- Karim, M., Zhao, W., Rowley, D., Nelson, D., Gomez-Chiarri, M., 2013. Probiotic strains for shellfish aquaculture: protection of eastern oyster, *Crassostrea*

- virginica*, larvae and juveniles against bacterial challenge. *Journal of Shellfish Research* 32, 401–408. <https://doi.org/10.2983/035.032.0220>
- Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G., 2012. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation: Algal-bacterial interactions for delivery of vitamin B12. *Environmental Microbiology* 14, 1466–1476. <https://doi.org/10.1111/j.1462-2920.2012.02733.x>
- Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2008. Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. *Aquaculture* 274, 1–14. <https://doi.org/10.1016/j.aquaculture.2007.11.019>
- Kinnula, H., Mappes, J., Valkonen, J.K., Pulkkinen, K., Sundberg, L.-R., 2017. Higher resource level promotes virulence in an environmentally transmitted bacterial fish pathogen. *Evolutionary Applications* 10, 462–470. <https://doi.org/10.1111/eva.12466>
- Kolter, R., Siegele, D.A., Tormo, A., 1993. The stationary phase of the bacterial life cycle. *Annu. Rev. Microbiol.* 47, 855–874.
- Krohn-Molt, I., Alawi, M., Förstner, K.U., Wiegandt, A., Burkhardt, L., Indenbirken, D., Thieß, M., Grundhoff, A., Kehr, J., Tholey, A., Streit, W.R., 2017. Insights into microalga and bacteria interactions of selected phycosphere biofilms using metagenomic, transcriptomic, and proteomic approaches. *Frontiers in Microbiology* 8. <https://doi.org/10.3389/fmicb.2017.01941>
- Marshall, R., McKinley, S., Pearce, C.M., 2010. Effects of nutrition on larval growth and survival in bivalves. *Reviews in Aquaculture* 2, 33–55. <https://doi.org/10.1111/j.1753-5131.2010.01022.x>
- Mayers, T.J., Bramucci, A.R., Yakimovich, K.M., Case, R.J., 2016. A Bacterial Pathogen Displaying Temperature-Enhanced Virulence of the Microalga *Emiliania huxleyi*. *Frontiers in Microbiology* 7. <https://doi.org/10.3389/fmicb.2016.00892>
- Miles, A.A., Misra, S.S., 1938. The estimation of the bactericidal power of the blood. *J Hyg* 38, 732–749.
- Milke, L.M., Bricelj, V.M., Parrish, C.C., 2008. Biochemical characterization and nutritional value of three *Pavlova* spp. in unialgal and mixed diets with *Chaetoceros muelleri* for postlarval sea scallops, *Placochelys magellanicus*. *Aquaculture* 276, 130–142. <https://doi.org/10.1016/j.aquaculture.2008.01.040>
- Moejes, F., Succurro, A., Popa, O., Maguire, J., Ebenhö, O., 2017. Dynamics of the bacterial community associated with *Phaeodactylum tricornutum* cultures. *Processes* 5, 77. <https://doi.org/10.3390/pr5040077>
- Molina-Cárdenas, C.A., Sánchez-Saavedra, M. del P., Lizárraga-Partida, M.L., 2014. Inhibition of pathogenic *Vibrio* by the microalgae *Isochrysis galbana*. *Journal of Applied Phycology* 26, 2347–2355. <https://doi.org/10.1007/s10811-014-0270-1>
- Napolitano, G.E., Ackman, R.G., Ratnayake, W.M.N., 1990. Fatty Acid Composition of Three Cultured Algal Species (*Isochrysis galbana*, *Chaetoceros gracilis* and *Chaetoceros calcitrans*) Used as Food for Bivalve Larvae. *Journal of the World Aquaculture Society* 21, 122–130. <https://doi.org/10.1111/j.1749-7345.1990.tb00532.x>
- Natrah, F.M.I., Bossier, P., Sorgeloos, P., Yusoff, F.M., Defoirdt, T., 2014. Significance of microalgal-bacterial interactions for aquaculture. *Reviews in Aquaculture* 6, 48–61. <https://doi.org/10.1111/raq.12024>

- Natrah, F.M.I., Kenmegne, M.M., Wiyoto, W., Sorgeloos, P., Bossier, P., Defoirdt, T., 2011. Effects of micro-algae commonly used in aquaculture on acyl-homoserine lactone quorum sensing. *Aquaculture* 317, 53–57. <https://doi.org/10.1016/j.aquaculture.2011.04.038>
- Nicolas, J.L., Corre, S., Cochard, J.C., 2004. Bacterial population association with phytoplankton cultured in a bivalve hatchery. *Microbial Ecology* 48, 400–413. <https://doi.org/10.1007/s00248-003-2031-6>
- Nicolas, J.L., Robic, E., Ansquer, D., 1989. Bacterial flora associated with a trophic chain consisting of microalgae, rotifers and turbot larvae: influence of bacteria on larval survival. *Aquaculture* 83, 237–248. [https://doi.org/10.1016/0044-8486\(89\)90036-7](https://doi.org/10.1016/0044-8486(89)90036-7)
- NOAA Fisheries [WWW Document], 2017. URL <https://www.fisheries.noaa.gov/insight/understanding-shellfish-aquaculture> (accessed 6.4.18).
- Powell, R.J., Hill, R.T., 2013. Rapid aggregation of biofuel-producing algae by the bacterium *Bacillus* sp. Strain RP1137. *Applied and Environmental Microbiology* 79, 6093–6101. <https://doi.org/10.1128/AEM.01496-13>
- Ramanan, R., Kim, B.-H., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2016. Algae–bacteria interactions: evolution, ecology and emerging applications. *Biotechnology Advances* 34, 14–29. <https://doi.org/10.1016/j.biotechadv.2015.12.003>
- Rojas, R., Miranda, C.D., Santander, J., Romero, J., 2016. First report of *Vibrio tubiashii* associated with a massive larval mortality event in a commercial hatchery of scallop *Argopecten purpuratus* in Chile. *Frontiers in Microbiology* 7. <https://doi.org/10.3389/fmicb.2016.01473>
- Rooney-Varga, J.N., Giewat, M.W., Savin, M.C., Sood, S., LeGresley, M., Martin, J.L., 2005. Links between phytoplankton and bacterial community dynamics in a coastal marine environment. *Microbial Ecology* 49, 163–175.
- Sandaa, R.-A., Magnesen, T., Torkildsen, L., Bergh, Ø., 2003. Characterisation of the bacterial community associated with early stages of great scallop (*Pecten maximus*), using denaturing gradient gel electrophoresis (DGGE). *Systematic and Applied Microbiology* 26, 302–311. <https://doi.org/10.1078/072320203322346164>
- Sapp, M., Schwaderer, A.S., Wiltshire, K.H., Hoppe, H.-G., Gerdt, G., Wichels, A., 2007. Species-specific bacterial communities in the phycosphere of microalgae? *Microbial Ecology* 53, 683–699. <https://doi.org/10.1007/s00248-006-9162-5>
- Schmidt, V., Gomez-Chiarri, M., Roy, C., Smith, K., Amaral-Zettler, L., 2017. Subtle microbiome manipulation using probiotics reduces antibiotic-associated mortality in fish. *mSystems* 2, e00133–17.
- Sharifah, E.N., Eguchi, M., 2011. The phytoplankton *Nannochloropsis oculata* enhances the ability of Roseobacter clade bacteria to inhibit the growth of the fish pathogen *Vibrio anguillarum*. *PLoS ONE* 6, e26756. <https://doi.org/10.1371/journal.pone.0026756>
- Shumway, S. (Ed.), 2011. Shellfish aquaculture and the environment. Wiley-Blackwell, Ames, Iowa.
- Sohn, S., Lundgren, K.M., Tammi, K., Karim, M., Smolowitz, R., Nelson, D.R., Rowley, D.C., Gómez-Chiarri, M., 2016. Probiotic strains for disease management in hatchery larviculture of the eastern oyster *Crassostrea virginica*. *Journal of Shellfish Research* 35, 307–317. <https://doi.org/10.2983/035.035.0205>

- Sule, P., Belas, R., 2013. A novel inducer of *Roseobacter* motility is also a disruptor of algal symbiosis. *Journal of Bacteriology* 195, 637–646.
<https://doi.org/10.1128/JB.01777-12>
- Univ. of Rhode Island, n.d. *Phaeobacter inhibens* S4Sm. NCBI Genome Assembly and Annotation report RefSeq: NZ_LOHU000000000.1.
- Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* 64, 655–671. <https://doi.org/10.1128/MMBR.64.4.655-671.2000>
- Wang, H., Hill, R.T., Zheng, T., Hu, X., Wang, B., 2016. Effects of bacterial communities on biofuel-producing microalgae: stimulation, inhibition and harvesting. *Critical Reviews in Biotechnology* 36, 341–352.
<https://doi.org/10.3109/07388551.2014.961402>
- Wietz, M., Duncan, K., Patin, N.V., Jensen, P.R., 2013. Antagonistic Interactions Mediated by Marine Bacteria: The Role of Small Molecules. *Journal of Chemical Ecology* 39, 879–891. <https://doi.org/10.1007/s10886-013-0316-x>
- Wikfors, G.H., Patterson, G.W., Ghosh, P., Lewin, R.A., Smith, B.C., Alix, J.H., 1996. Growth of post-set oysters, *Crassostrea virginica*, on high-lipid strains of algal flagellates *Tetraselmis* spp. *Aquaculture* 143, 411–419.
- World Health Organization, Food and Agriculture Organization, 2006. Probiotics in food Health and nutritional properties and guidelines for evaluation. Rome.
- Zech, H., Hensler, M., Koßmehl, S., Drüppel, K., Wöhlbrand, L., Trautwein, K., Hulsch, R., Maschmann, U., Colby, T., Schmidt, J., Reinhardt, R., Schmidt-Hohagen, K., Schomburg, D., Rabus, R., 2013. Adaptation of *Phaeobacter inhibens* DSM 17395 to growth with complex nutrients. *PROTEOMICS* n/a-n/a.
<https://doi.org/10.1002/pmic.201200513>
- Zhao, W., Dao, C., Karim, M., Gomez-Chiarri, M., Rowley, D., Nelson, D.R., 2016. Contributions of tropodithietic acid and biofilm formation to the probiotic activity of *Phaeobacter inhibens*. *BMC Microbiology* 16. <https://doi.org/10.1186/s12866-015-0617-z>

Appendix A: all F-test results.

Table S1: Comparison of algal growth curves

	P-value	F (DFn, DFd)
<i>C. neogracile</i> global	0.9289	0.4026 (9,60)
<i>C. neogracile</i> pairwise comparisons		
algae only vs algae & S4	0.9159	0.1698 (3,30)
algae only vs algae & RE22	0.7463	0.4110 (3,30)
algae only vs algae, S4 & RE22	0.4888	0.8282 (3,30)
<i>P. pinguis</i> global	0.8288	0.5527 (9,52)
<i>P. pinguis</i> pairwise comparisons		
algae only vs algae & S4	0.7397	0.4207 (3,26)
algae only vs algae & RE22	0.5802	0.6665 (3,26)
algae only vs algae, S4 & RE22	0.3285	1.202 (3,26)
<i>Te. chui</i> global	0.9981	0.1424 (9,50)
<i>Te. chui</i> pairwise comparisons		
algae only vs algae & S4	0.8891	0.2091 (3,24)
algae only vs algae & RE22	0.8734	0.2316 (3,24)
algae only vs algae, S4 & RE22	0.8315	0.2909 (3,24)
<i>Ti. lutea</i> global	0.9323	0.3918 (9,42)
<i>Ti. lutea</i> pairwise comparisons		
algae only vs algae & S4	0.3982	1.048 (3,16)
algae only vs algae & RE22	0.5629	0.7049 (3,16)
algae only vs algae, S4 & RE22	0.7085	0.4682 (3,16)

Table S2: Comparison of RE22 growth curves

	P-value	F (DFn, DFd)
<i>C. neogracile</i> global	< 0.0001	40.92 (9,45)
<i>C. neogracile</i> pairwise comparisons		
RE22 only vs RE22 & algae	< 0.0001	47.37 (3,22)
RE22 only vs RE22 & S4	0.0017	7.038 (3,22)
RE22 & algae vs RE22, algae & S4	0.2507	1.463 (3,23)
<i>P. pinguis</i> global	< 0.0001	36.81 (9,38)
<i>P. pinguis</i> pairwise comparisons		
RE22 only vs RE22 & algae	< 0.0001	64.15 (3,19)
RE22 only vs RE22 & S4	< 0.0001	17.66 (3,20)
RE22 & algae vs RE22, algae & S4	0.2707	1.416 (3,18)
<i>Te. chui</i> global	< 0.0001	28.54 (9,37)
<i>Te. chui</i> pairwise comparisons		
RE22 only vs RE22 & algae	< 0.0001	55.36 (3,19)
RE22 only vs RE22 & S4	0.0005	9.648 (3,18)
RE22 & algae vs RE22, algae & S4	0.8983	0.1953 (3,19)
<i>Ti. lutea</i> global	< 0.0001	83.22 (9,40)
<i>Ti. lutea</i> pairwise comparisons		
RE22 only vs RE22 & algae	< 0.0001	302.6 (3,20)
RE22 only vs RE22 & S4	<0.0001	28.97 (3,20)
RE22 & algae vs RE22, algae & S4	0.6354	0.5793 (3,20)

Table S3: Comparison of S4 growth curves

	P-value	F (DFn, DFd)
<i>C. neogracile</i> global	< 0.0001	7.181 (9,58)
<i>C. neogracile</i> pairwise comparisons		
S4 only vs S4 & algae	0.0845	2.449 (3,28)
S4 only vs S4 & RE22	0.0043	5.443 (3,29)
S4 & algae vs S4, algae & RE22	0.9406	0.1314 (3,29)
<i>P. pinguis</i> global	0.1496	1.570 (9,51)
<i>P. pinguis</i> pairwise comparisons		
S4 only vs S4 & algae	0.0588	2.818 (3,26)
S4 only vs S4 & RE22	0.5291	0.7564 (3,25)
S4 & algae vs S4, algae & RE22	0.988	0.04275 (3,26)
<i>Te. chui</i> global	< 0.0001	29.58 (9,59)
<i>Te. chui</i> pairwise comparisons		
S4 only vs S4 & algae	< 0.0001	29.73 (3,30)
S4 only vs S4 & RE22	0.0009	7.308 (3,29)
S4 & algae vs S4, algae & RE22	0.0154	4.072 (3,30)
<i>Ti. lutea</i> global	< 0.0001	38.82 (9,52)
<i>Ti. lutea</i> pairwise comparisons		
S4 only vs S4 & algae	< 0.0001	123.5 (3,26)
S4 only vs S4 & RE22	0.9039	0.1876 (3,26)
S4 & algae vs S4, algae & RE22	0.3942	1.033 (3,26)

Table S4: Antibiotic experiment

	P-value	F (DFn, DFd)
<i>C. neogracile</i> global	0.0111	3.194 (9,24)
<i>C. neogracile</i> pairwise comparisons		
S4 only -Ab vs S4 only +Ab	0.0435	3.679 (3,12)
S4 only -Ab vs S4 & algae -Ab	0.9644	0.08949 (3,12)
S4 only +Ab vs S4 & algae +Ab	0.0057	6.980 (3,12)
S4 & algae -Ab vs S4 & algae +Ab	0.0178	4.996 (3,12)

Table S5: Comparison of total culturable bacteria (mYP30) growth curves

	P-value	F (DFn, DFd)
<i>C. neogracile</i> global	0.2726	1.270 (9,59)
<i>C. neogracile</i> pairwise comparisons		
algae only vs algae & S4	0.4183	0.9743 (3,29)
algae only vs algae & RE22	0.6431	0.5638 (3,30)
algae only vs algae, S4 & RE22	0.0271	3.511 (3,30)
<i>P. pinguis</i> global	0.8448	0.5317 (9,52)
<i>P. pinguis</i> pairwise comparisons		
algae only vs algae & S4	0.6329	0.5807 (3,26)
algae only vs algae & RE22	0.2587	1.423 (3,26)
algae only vs algae, S4 & RE22	0.7024	0.4750 (3,26)
<i>Te. chui</i> global	0.5791	0.8447 (9,52)
<i>Te. chui</i> pairwise comparisons		
algae only vs algae & S4	0.1383	2.003 (3,26)
algae only vs algae & RE22	0.1541	1.902 (3,26)
algae only vs algae, S4 & RE22	0.9252	0.1554 (3,26)
<i>Ti. lutea</i> global	0.8508	0.5218 (9,44)
<i>Ti. lutea</i> pairwise comparisons		
algae only vs algae & S4	0.1947	1.740 (3,18)
algae only vs algae & RE22	0.5778	0.6762 (3,18)
algae only vs algae, S4 & RE22	0.4713	0.8773 (3,18)

Appendix B, Fig S1. S4 growth curves with different starting concentrations.

Appendix C, Fig. S2. Effect of antibiotics on the density of commensal bacteria during coculture with antibiotics.

Effect of streptomycin (200 µg/mL) and chloramphenicol (5 µg/mL) coculture on total planktonic culturable bacteria.

Appendix D, Table S6. Effect of antibiotics on growth of *C. neogracile*

	Count 1	Count 2
No antibiotics added:		
Flask 1	356	328
Flask 2	492	396
Flask 3	272	300
Antibiotics added		
Flask 1	320	336
Flask 2	264	296
Flask 3	364	380

Streptomycin (200 µg/mL) and chloramphenicol (5 µg/mL) were added to flasks inoculated with *C. neogracile* (starting concentration 10^5 cells/mL), and allowed to incubate for five days. Samples were then drawn, fixed with Lugol's iodine, and algae cells were counted (numbers represent number of cells per square on a hemocytometer).

Appendix E: Coculture of *Bacillus pumilus* RI0695 with microalgae and RE22.

Introduction

Bacillus pumilus RI06-95, a firmicute, is a probiotic marine bacterium with demonstrated effectiveness in reducing larval shellfish mortality in culture operations (Karim et al., 2013). Unlike the roseobacters, *Bacillus* spp., and Gram-positive bacteria in general, have not been found to widely associate with microalgae. Many studies of the microbiomes of microalgae, both in natural settings and in culture, have not turned up any close relatives of *B. pumilus* RI06-95 at all (Behringer et al., 2018; Goecke et al., 2013; Nicolas et al., 1989; Rooney-Varga et al., 2005), while the rest have found firmicutes, or even more distantly-related actinobacteria (high C+G Gram positive bacteria), only in a small proportion of microalgal populations or cultures sampled, and at low levels compared to the dominant clades (Carney et al., 2014; Krohn-Molt et al., 2017; Moejes et al., 2017; Sapp et al., 2007). Although *Bacillus* spp. have not been found to reach high abundances in such settings, ecological interactions with microalgae have been discovered: *Bacillus* spp. induce flocculation of certain microalgae species (Powell and Hill, 2013), and one study showed that *B. pumilus* significantly enhanced growth of the microalgae *Chlorella sorokiniana* (Amavizca et al., 2017); however, a lack of experimental controls for CO₂ production as a growth stimulator means that a species-specific effect should not be assumed. Nevertheless, *Bacillus* spp. are proven to be effective probiotics in aquaculture (Karim et al., 2013; Kesarcodi-Watson et al., 2008), and the possibility of coculture with microalgae is worth investigating.

Methods

RI0695SmRif (hereafter 'RI'), the strain of *B. pumilus* developed for this study from the parent strain RI0695 (Karim et al., 2013), was selected via spontaneous mutation for resistance to rifampicin (Rif) and streptomycin (Sm). Bacterial cultures were prepared as described for S4, except the antibiotics and their concentrations were different: RI was grown up in the presence of Rif (100 µg/mL) and Sm (16 µg/mL), and selectively plated on mYP30 + Rif (100 µg/mL) & Sm (16 µg/mL).

This experiment sought to determine the ability of RI to grow in media and in coculture with *C. neogracile*, and whether it has an effect on titers of RE22 in algal coculture. Treatments were: *C. neogracile* co-incubated with a) probiotic RI and b) RI and RE22, and controls were: a) algae only and b) RI only.

Results/Discussion

Fig. S3. Growth curves of *B. pumilus* RI in coculture with *C. neogracile* and pathogen RE22. Values are shown as means $\pm$ SD (n=3). A global *F*-test revealed no significant differences between treatments ($p < 0.05$) (Supplementary table S4).

RI titers declined rapidly in both the fertilized seawater control treatment as well as in coculture with *C. neogracile* (Fig. S3). No significant differences in growth curves were observed between treatments (Global *F*-test: $p = 0.1105$, Table S7); in all treatments, RI titers stabilized at $\sim 10^3$ CFU/mL by day 2, and declined marginally ($< 0.2 \log_{10}$) for the remaining 6 days of the experiment. Neither RE22 titers nor total culturable bacterial density were affected by RI additions in coculture with *C. neogracile* (Fig. S3). Since RI declined rapidly in media alone, its effect on other algae species was not investigated.

Table S7: Comparison of RI growth curves

	P-value	F (DFn, DFd)
<i>C. neogracile</i> global	0.1105	1.924 (6,29)
<i>C. neogracile</i> pairwise comparisons		
RI only vs RI & algae	0.0381	3.427 (3,19)
RI only vs RI, algae & S4	0.0369	3.460 (3,19)
RI & algae vs RI, algae & S4	0.9999	0.001950 (3,20)

Fig. S3. Effect of RI on density of RE22 (A) and total culturable bacteria (B) in coculture with *C. neogracile*. Values are shown as means $\pm$ SD (n=3). *F*-tests on each pair of curves revealed no significant differences between treatments (A: $p=0.3879$; B: $p=0.9224$).

Appendix F: Coculture of *V. coralliilyticus* RE22 with axenic *Ti. lutea*.

Fig. S4. Growth curves of *V. coralliilyticus* RE22 in coculture with with axenic and bacterized *Ti. lutea*. Values are shown as means $\pm$ SD (n=3). Quadratic regression followed by a global *F*-test revealed significant differences between treatments ($p < 0.05$). Different letters indicate significantly different curves based on pairwise comparisons ($p < 0.0167$).

An additional coculture experiment was conducted to determine the effect of algal commensal bacteria on the inhibition of RE22 *in vivo*. Results suggest that the decline of RE22 in coculture with *Ti. lutea* is due to commensal bacteria, and not *Ti. lutea*.