Journal of the Marine Biological Association of the United Kingdom

cambridge.org/mbi

Antagonistic interactions among marine sedimentary bacteria in multispecies microcosms

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Research Article

Cite this article: Hook W, Plante C (2022). Antagonistic interactions among marine sedimentary bacteria in multispecies microcosms. *Journal of the Marine Biological Association of the United Kingdom* **102**, 196–208. https://doi.org/10.1017/S0025315422000376

Received: 27 August 2021
Revised: 31 January 2022
Accepted: 13 May 2022
First published online: 6 July 2022

Key words:
Antibiotic; antimicrobial; benthic; interference competition; intertidal

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Abstract

Antagonism among bacteria is widespread and plays an important role in structuring communities. Inhibitory compounds can confer competitive advantage, but energetic trade-offs can result in non-transitive (i.e. 'rock-paper-scissors') interactions, ultimately allowing co-existence and community stability. Competition in sedimentary habitats is especially keen given high densities and attachment to inorganic particles. Because measuring trade-offs between bacterial species is challenging, much of our understanding of competitive interactions is based on theoretical modelling and simplified *in vitro* experiments. Our objectives were to determine (1) if interference competition occurs in microcosms mimicking *in situ* conditions; (2) whether the presence of sediment influences antagonistic interactions; and (3) if more complex assemblages alleviate or synergize interactions. Four sedimentary isolates, including antibiotic-producing, resistant and susceptible strains were incubated in porewater microcosms in 1-, 2- and 3-species combinations, both with and without natural sediments. Microcosms were sampled over 72 h to generate growth curves using quantitative PCR. Multiple growth attributes (growth rate, maximum density, lag time) were used to assess effects of treatment (species combinations) and environment (sediment *vs* porewater alone). Antimicrobial producers were more effective at inhibiting target species in microcosms that included sediment, in agreement with theory. We observed growth inhibition by antimicrobial-producing bacteria in both 2- and 3-species microcosms. However, the expected protection of sensitive bacterial strains by resistant strains was observed in only one (of four) 3-species combinations, thus the 'rock-paper-scissors' prediction was not fully supported. These results reinforce the notion that interspecies interactions are context-dependent, reliant on environmental conditions and the species involved.

Introduction

Antibiotic-mediated antagonism among bacteria appears to be ubiquitous and ecologically important. The secretion of antimicrobials by bacteria as a competitive means either kills or inhibits sensitive strains, effectively reducing their fitness. In general, antibiotic production is thought to play a crucial role in determining bacterial dispersion patterns and structuring assemblages in natural habitats (Long & Azam, 2001; Czárán *et al.*, 2002; Rypien *et al.*, 2010; Ghoul & Mitri, 2016), which can have direct implications for the biogeochemical processes attributed to bacteria on a global scale (Azam, 1998; Simon *et al.*, 2002; Falkowski *et al.*, 2008; Louca *et al.*, 2016).

High proportions of bacteria associated with organic particulates and sediments in the marine environment produce antimicrobial compounds, ranging from 40.9–54.1% (Long & Azam, 2001; Grossart *et al.*, 2004). Competitive interactions between species in sediments are likely, given the ~10^9 cells ml^{-1} densities at which they cohabitate (Schmidt *et al.*, 1998; Torsvik *et al.*, 1998; Musat *et al.*, 2006). Bacterial abundances in seawater are typically orders of magnitude less, at ~10^6 cells ml^{-1} (DeLong *et al.*, 1993; Grossart *et al.*, 2001), which corresponds to an often lower frequency of antimicrobial-producers in the water column (Burgess *et al.*, 1999; Long & Azam, 2001; Hook & Plante, 2019). This disparity could be a reflection of the effectiveness of antibiotic use as a competitive strategy within these two environments, which vary in both bacterial density and lifestyle strategy. Extracellular secretions by free-living bacteria rapidly diffuse into the water column, thus potential competitors may not be near enough to be inhibited. Free-living bacteria are also typically motile, and thus have the capacity to move away from the unfavourable condition that antimicrobials present to sensitive strains (Grossart *et al.*, 2001). Sediment-associated bacteria, attached to sediment grains and organized in mixed-species biofilms (Decho, 1990; Costerton *et al.*, 1995), experience a much different competitive milieu than do free-living microbes. Use of antimicrobials is aided by the direct proximity and relative immobility of competitors. Surface-associated producers also benefit from diffusion of secreted antibiotics along the particle surface, and potential particle adsorption of the secreted molecules (Grossart *et al.*, 2004). So, while biofilms can be cooperative environments (Kato *et al.*, 2005; Narisawa *et al.*, 2008), they are also prone to intense competition.

In theory, antimicrobial-producing bacteria (P) will completely suppress or kill off susceptible bacteria (S) (Chao & Levin, 1981). This type of interference competition differs from the negative interactions among bacteria in scramble competition for resources such as space and nutrients (Ganter & Starmer, 1992). Due to metabolic costs associated with antibiotic production, resistant strains (R) are assumed to be competitively superior to producers (P). Similarly, S strains will outcompete R strains because they do not incur the metabolic cost of producing a resistance factor. This cyclic interaction can be referred to as a theoretical game of 'rock-paper-scissors', wherein P outcompetes S which outcompetes R which outcompetes P (Czárán *et al*., 2002; Kerr *et al*., 2002). In general, it is accepted that the interaction between two bacterial species is influenced by the addition of other species.

With interactions involving multiple bacterial competitors (≥3 species), sensitive bacteria have been found to coexist with antibiotic-producers *in vitro* in flow-cell biofilms (Rao *et al*., 2005; Narisawa *et al*., 2008; Kelsic *et al*., 2015). The metabolic costs associated with antibiotic production and resistance may result in trade-offs that facilitate the coexistence of competing species. Ecological trade-offs between competitive ability and resource acquisition or utilization are possible mechanisms to enable species coexistence. The inhibitory strength of the interaction is thought to be a determining factor in whether species coexist or one competitor dominates (Bohannan *et al*., 2002). These same ecological trade-offs also apply to free-living bacterial populations, but because these bacteria do not aggregate to form biofilms, they do not experience the same interactive dynamics as species within biofilms.

Because measuring trade-offs between competing species is challenging, much of the framework for our understanding of competitive bacterial interactions is based on theoretical modelling (Czárán *et al*., 2002; Kirkup & Riley, 2004; Kosakowski *et al*., 2018). In this study, bacteria isolated from intertidal sediments were employed in microcosm competition experiments that included antimicrobial-producing *Pseudoalteromonas* sp. and *Vibrio harveyi*, and non-producing species *Bacillus pumilus* and *Roseivivax* sp., each differing in their sensitivities to the producer strains. Quantitative PCR was used to obtain empirical measures of bacterial growth during monoculture, 2-species and 3-species growth experiments in microcosms with and without the sedimentary matrix. The objectives of this study were to determine (1) if interference competition is observed in microcosms mimicking *in situ* conditions; (2) whether the presence of sediment influences antagonistic interactions and antibiotic effectiveness; and (3) if more complex bacterial assemblages alleviate or synergize these interactions, particularly between antibiotic-producing and susceptible bacteria.

Materials and methods

Microcosm design

Intertidal sediments were collected from Breach Inlet, SC, USA (32°46′36″N 79°48′42″W) at low tide and vacuum-filtered to extract water. This porewater was autoclaved and filtered (0.2 µm), then stored at 4 °C. Sterility was checked by spread plating on half-strength (50%) marine agar 2216 (Difco Laboratories, Detroit, MI) and inoculating 50% marine broth 2216. The sediment was dried for at least 24 h at 60 °C and sieved to collect the 125–250 µm grain size fraction, which was previously determined to be the dominant size fraction at the collection site (Wilde & Plante, 2002). A series of three 121 °C 20-minute autoclave cycles at 24 h intervals was performed to ensure the absence of viable cells. Initially, sediment was spread in a 1-cm thick layer, covered and autoclaved. The second autoclave cycle was done in 50 g aliquots in sealed flasks. Sediment was further divided into 3 g portions into the 100-ml serum bottle microcosms for the final autoclave sterilization. Serum bottles designated for porewater treatments were also autoclaved. Microcosms were sealed by rubber stopper with a 10-cc syringe outlet filled with glass fibre to maintain sterility while allowing air exchange. Sediment was checked for sterility by adding to 2216 marine broth then monitoring for change in turbidity. After autoclaving, 9 ml of porewater were pipetted into each sterile serum bottle. All subsequent microcosm incubations and samplings were performed in a laminar-flow hood equipped with a 0.2–0.3 µm filter.

Bacterial strains

Four bacterial strains isolated from Breach Inlet, SC and identified using 16S rDNA gene sequencing (Hook & Plante, 2019), were used in microcosm experiments. Two of these isolates, a *Vibrio harveyi* strain (GenBank MN066575) and *Pseudoalteromonas* sp. (MN066571), are known to produce antimicrobial compounds through the significant inhibition of other bacterial strains in disc-diffusion assays (Hook & Plante, 2019). A *Bacillus pumilus* strain (MN066572) and *Roseivivax* sp. (MN066577) were used as non-antimicrobial producing target strains. *Roseivivax* was susceptible to the antimicrobials produced by both *V. harveyi* and *Pseudoalteromonas* sp. Gu72 in Kirby–Bauer disc-diffusion assays, while *B. pumilus* was susceptible only to *Pseudoalteromonas* and maintained resistance to the compound(s) produced by *V. harveyi* (Hook & Plante, 2019).

Microcosm communities were constructed in both porewater-only and sediment + porewater systems. Treatments included negative controls, monocultures for each bacterial strain (hereafter referred to using respective genus name), 2-species and 3-species combined communities (Supplementary Figure S1). Negative controls consisted of sediment and/or porewater without bacterial inoculum. All treatments were performed in triplicate for a total of 78 microcosms.

Bacterial cell numbers for microcosm inoculation were determined by microscopy counts at known optical densities using SYBR Gold staining. Cell quantities were further confirmed through colony forming unit (CFU) counts of spread plates from inoculated microcosms in preliminary optimization trials. Bacterial monocultures were grown in half-strength marine broth (2216) to the individual optical densities corresponding to 10^8 cells ml^{-1}. Cells were centrifuged, washed and resuspended in seawater, then used to inoculate microcosms to a density of ~10^3 cells ml^{-1}.

Microcosm sampling and DNA extraction

Throughout the duration of the experiment, microcosms were incubated at 27 °C and continuously mixed on a shaker table to maintain a homogeneous environment. The time series for sampling bacteria from microcosms was determined from preliminary trials (data not shown), which employed spread plate CFUs and qPCR to follow bacterial growth through stationary phase. A range of sample volumes (250, 500 and 1000 µl) was tested with the DNA extraction protocol and the DNA was quantified using a NanoDrop 2000 system (Thermo Scientific; Asheville, NC, USA). It was determined that DNA of sufficient quantity and quality could be extracted from as small a volume as 250 µl for the downstream qPCR application. A 300-µl sample volume was used, as this allowed for the necessary sampling time points to be met without over-sampling from the microcosm itself (≤10% of the total volume). Microcosms were vortexed for 30 s and allowed to settle for 15 min prior to sampling.

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Samples were collected from three randomly chosen porewater microcosms and three randomly selected sediment + porewater microcosms prior to bacterial inoculum to quantify the baseline DNA already present in the constructed environment (from autoclaved sediment and/or porewater). At time 0 (immediately upon inoculation), three of each microcosm type (porewater and sediment + porewater) were sampled for each of the four bacterial strains (24 total). All microcosms were sampled at 6, 12, 24 and 72 h timepoints using sterile 1-ml pipettes. DNA extraction was performed immediately after each sampling.

DNA was extracted from microcosm porewater samples as described in Boström *et al.* (2004). Bacterial cells were recovered by centrifuging for 20 min at 16,000 × ***g*** and resuspended in lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl, pH 9.0). Lysozyme was added to a final concentration of 1 mg ml^{-1} and incubated at 37 °C for 30 min. Sodium dodecyl sulphate (SDS) (1% final conc.) and proteinase K (100 µg ml^{-1}, final conc.) were then added for incubation at 55 °C for ~12 h. DNA was precipitated with 0.1 volume of 3 M NaOAc and 0.6 volume isopropanol. Samples were mixed by inversion prior to a 1 h incubation at −20 °C. An ethanol dehydration series followed, with a 4 °C centrifugation at 20,800 × ***g*** for 20 min. DNA was washed twice with 70% cold EtOH. Samples were then vacufuged (Vacufuge plus; Eppendorf, Hamburg, Germany) for 10 min at room temperature to ensure alcohol removal before resuspending cells in 20 µl restriction-digested water (NlaIII; New England BioLab, Ipswich, MA, USA).

Primer optimization and quantitative PCR analysis

Species-specific primer sets were developed or determined from previous publications for each bacterial strain (Table 1). Template DNA was extracted from pure cultures at 10^8 cells ml^{-1}. Primers were initially assessed using standard PCR to ensure amplification of the intended bacterial target and negative amplification of non-intended species. Annealing temperature, primer concentration and amplification protocols for the selected primer pairs were also narrowed down using PCR. Final optimization for each of these factors was performed on the MyiQ Real Time PCR Detection System (BioRad Laboratories; Hercules, CA, USA) using a 10^3–10^8 cells ml^{-1} standard curve for each bacterial species and corresponding primer set. Reaction parameters were determined based on overall reaction efficiency from standard curve amplification. A two-step protocol was used for all primer sets. Melt-curve data were collected for each run to ensure amplification of a single product.

Quantitative PCR was conducted in 96-well plates with reactions consisting of 6.25 µl ABsolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific; Waltham, MA, USA), BSA (100 nM final conc.), 0.25 µl DNA template, the specified primer concentrations (Table 1), and restriction-digested water to bring the total reaction volume to 12.5 µl. Only one bacterial species and corresponding primer set was used per plate, each of which was film-sealed and centrifuged for 3 min at 2250 × ***g*** at room temperature prior to loading on the iCycler. Each plate run consisted of triplicate no-template control wells and 10^2–10^8 cells ml^{-1} dilution series. Included in duplicate for each run, along with the microcosm samples, were the pre-inoculum and time 0 h samples for the respective bacterial strain.

Data analysis

The cycle threshold (CT) value was collected for each sample and the initial cell quantities (starting quantities) were inferred from the amplification of the designated standard curve. These values were established with the iQ5 Optical System software (BioRad). The coefficient of variation (CV) was then calculated for each sample replicate ((SD/mean) × 100). Samples with a CV value ≥15% were re-run. Any quantifiable amount of DNA in the pre-inoculum samples (microcosm DNA baseline) was subtracted from the DNA copy number generated for each microcosm sample. This number was never measured at more than ~10^2 cells.

Growth curves generated from the qPCR gene copy number data over the collection time series were fitted to the Gompertz 3-parameter model using Sigmaplot (Systat Software, San Jose, CA). Data were transformed by taking the natural log of gene copy number/(gene copy number at time 0). The calculated parameters describe different phases of the bacterial growth curve, including exponential growth rate (μ_{max}), maximum achieved gene copy density (A) and lag time (λ) (Zwietering *et al.*, 1990).

The microcosm environment (sediment *vs* porewater) and bacterial treatment within each microcosm were both taken into account as having potential effects on bacterial interactions. To evaluate the effect of environment on bacterial growth, corresponding treatments from porewater and sediment were compared using *t*-test analyses. The effect of bacterial treatment (the species combinations in microcosms) on growth characteristics was assessed using one-way ANOVA with the post hoc Tukey pairwise multiple comparison correction. A central part of this analysis was the comparison of monoculture growth parameters to the 2- and 3-species treatments. All data were tested for equal variance (Levene's test) and normality of model residuals were checked using the Shapiro–Wilk test ($P > 0.05$). All statistical analyses were performed using Sigmaplot and were applied to each parameter: μ_{max}, A and λ. For differences between means we calculated Cohen's d as a measure of effect size.

Table 1. Primers utilized for species-specific detection and quantification by qPCR

Bacterial Species	Primer	Primer Sequence (5′ – 3′)	Product Size (bp)	Primer Conc. (nM)	Melt Temp. (°C)	Cycles
Bacillus pumilus (MN066572)	314 F[a]	CCTACGGGAGGCAGCAG	176	50	60	40
	Bacillus R[b]	GACAACGCTTGCCACCTACGTAT		100		
Roseivivax sp. (MN066577)	1330 F	CGTCGGCTTGGTAGGCTT	130	50	58	42
	1400 R	AATACCGCATACGCCCTTCG		150		
Vibrio harveyi (MN066575)	V. harveyi F[c]	CGAGCGGAAACGAGTTATCTG	176	70	65	40
	V. harveyi R[c]	CTCACCAACTAGCTAATCCCACCTA				
Pseudoalteromonas sp. (MN066571)	790 F	GGGTCAACACTGACGCTCATGTA	70	100	60	40
	885 R	GTTGTCCGAAGACCCCAGC				

[a]314 F; universal 16S; [b]Bacillus R modified from Castiglioni *et al.* (2008); [c]V. harveyi F/R (Fukui & Sawabe, 2008).

https://doi.org/10.1017/S0025315422000376 Published online by Cambridge University Press

Results

Quantitative PCR

The four sets of species-specific 16S primers (Table 1) had linear ranges of detection spanning 5–6 orders of magnitude. Amplification of all samples was within the bounds of the 10^3–10^8 or 10^2–10^8 cell densities that made up the standard curve. Reaction efficiency ranged from 90–101% with regression coefficients between 0.965–0.999. Growth curves were constructed based on gene copy number and indicated that stationary phase was generally reached well within the 72-h incubation period (e.g. Figure 1). Curves were successfully fitted to the Gompertz model with an average regression coefficient of 0.942 for sediment samples and 0.960 for porewater.

Monocultures

Bacterial monocultures depict growth characteristics without the influence of interspecies interactions. Growth rate during the exponential phase differed significantly between sediment and porewater microcosms only for *Vibrio*, which demonstrated a higher μ_{max} in porewater ($P = 0.027$, $d = 2.775$, Figure 2A). In contrast, both target strains, *Bacillus* and *Roseivivax*, appeared to exhibit higher μ_{max} in sediment than in porewater (Figure 2A), although differences were not significant ($P = 0.100$ and 0.248, resp.). Overall, *Roseivivax* exhibited highest maximal growth rates (gene copy number h^{-1}) in both microcosm types, whereas *Vibrio* appeared to have the lowest μ_{max} (Figure 2A).

With respect to maximal densities (growth parameter, *A*), results also suggested superior growth in sediment for all strains

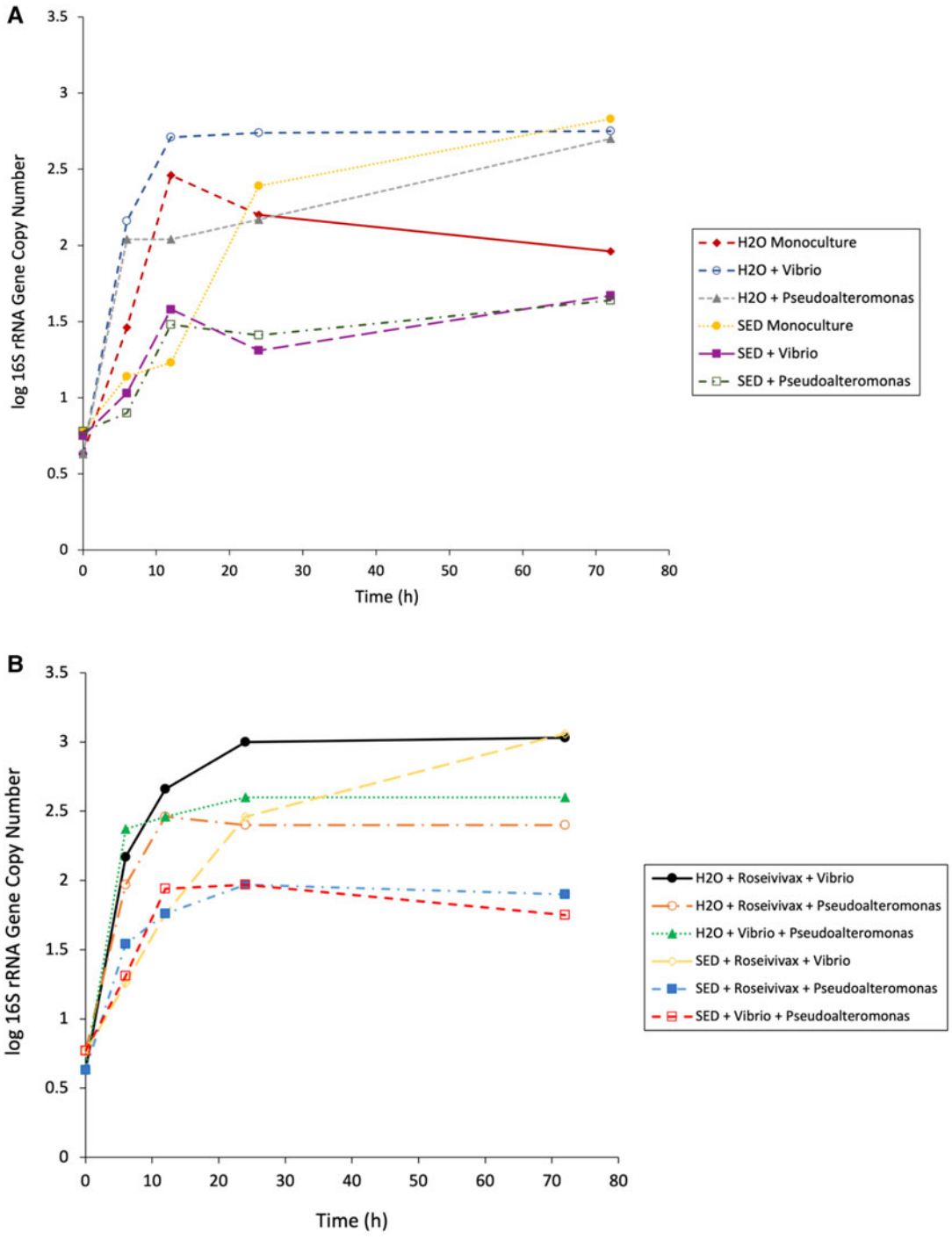

Fig. 1. Growth curves for *Bacillus* constructed using qPCR for (A) 2-species and (B) 3-species interactions in porewater (H_2O) and sediment (SED) microcosms.

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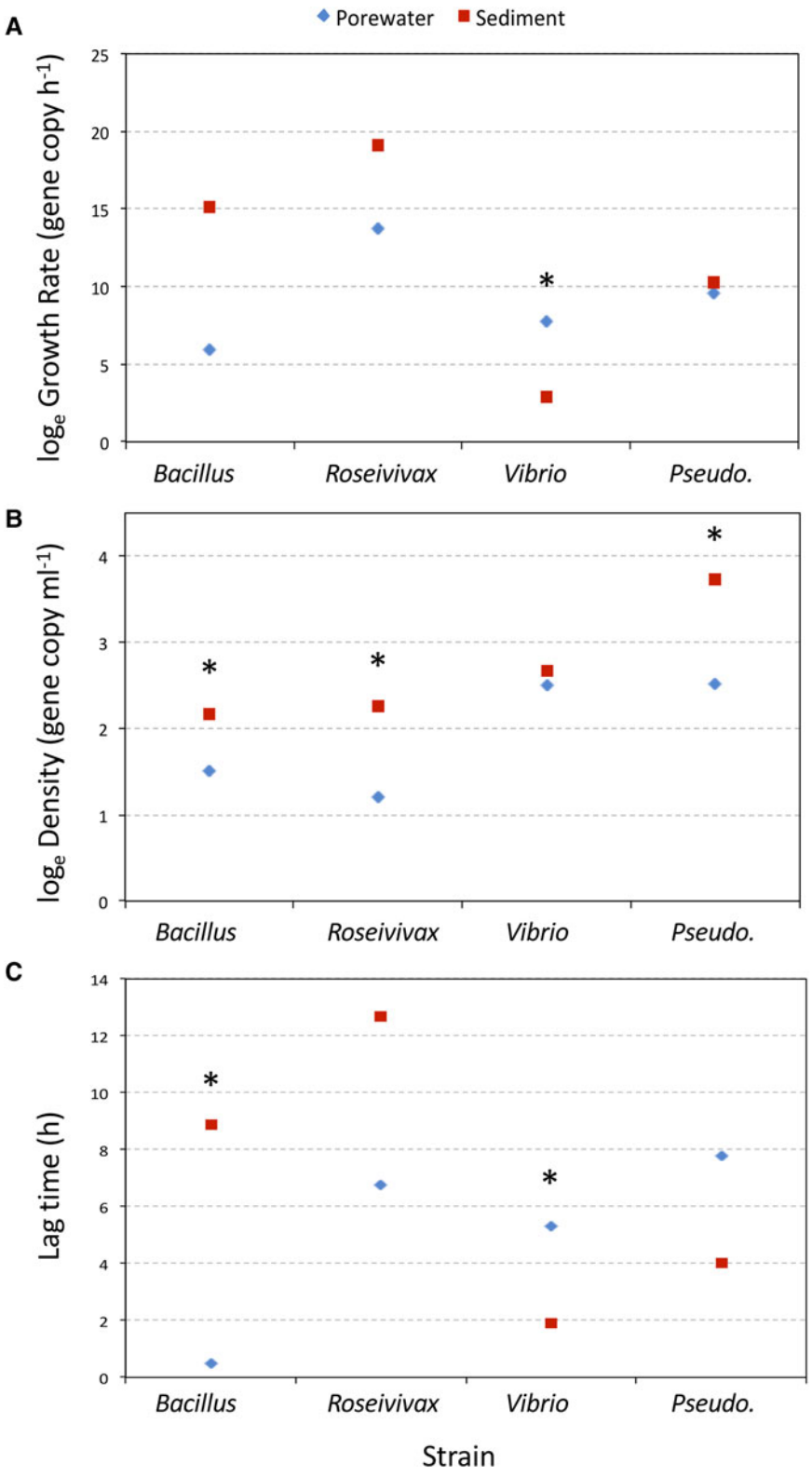

Fig. 2. Growth parameter comparisons of all bacterial strains comparing mean (A) growth rate μ_{max}, (B) maximum cell density (*A*) and (C) lag time (λ). Asterisks (*) indicate significant difference ($P < 0.05$; *t*-test) between sediment and porewater microcosms. Error bars have been excluded for clarity; data variability can be found in Supplementary Table S1.

except *Vibrio*. *Bacillus*, *Roseivivax* and *Pseudoalteromonas* strains attained significantly greater densities in sediment than in porewater systems ($P = 0.019$, 0.048, 0.037, and $d = 3.115$, 2.295, 2.518, respectively) whereas *Vibrio* attained nearly the same density in both microcosm types ($P = 0.514$, Figure 2B). Among the four strains, *Pseudoalteromonas* reached the highest density in both microcosm types. *Vibrio*, *Bacillus* and *Roseivivax* grew to similar, lesser densities in sediment, but *Roseivivax* achieved somewhat lower densities in porewater microcosms (Figure 2B).

Vibrio reached exponential growth significantly quicker in microcosms with sediment than in porewater-only microcosms ($P = 0.050$, $d = 2.243$, Figure 2C). The opposite was seen with the target species, *Bacillus*, which emerged from lag phase sooner in porewater ($P = 0.021$, $d = 3.016$, Figure 2C). *Pseudoalteromonas* and *Roseivivax* displayed a similar lag time regardless of environment (Figure 2C). Lag times were comparable among all species except *Bacillus* in the porewater-only microcosms, which reached exponential growth sooner (Figure 2C).

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Two-species microcosms

Some evidence for depressed maximal growth rate (μ_{max}) was observed for both target species when cultured with antimicrobial producers. *Bacillus* exhibited a significant decrease in μ_{max} when cultured with *Pseudoalteromonas* in sediment ($P = 0.042$, $d = 4.200$, Figure 3A). Likewise, culture with *Vibrio* seemed to reduce μ_{max} for *Bacillus* in sediment (Figure 3A), although this difference was not significant ($P = 0.144$). In contrast, no difference in μ_{max} relative to monocultures was evident for *Bacillus* when cultured with *Pseudoalteromonas* or *Vibrio* in porewater-only microcosms ($P = 0.992$, 0.990, respectively, Figure 3A). The second target strain, *Roseivivax*, showed lower relative μ_{max} when cultured with *Vibrio* in both sediment and porewater (Figure 4A), although these differences were not significant ($P = 0.175$, 0.122, respectively). Relative μ_{max} was unchanged when *Roseivivax* was co-cultured with *Pseudoalteromonas* compared with monoculture growth. However, μ_{max} was significantly greater for *Roseivivax* when cultured with *Pseudoalteromonas* in sediment compared with the same combination in porewater ($P = 0.007$, $d = 4.201$, Figure 4A). As expected, neither producer strain showed reduction in relative μ_{max} when grown with targets in co-culture relative to growth in monoculture

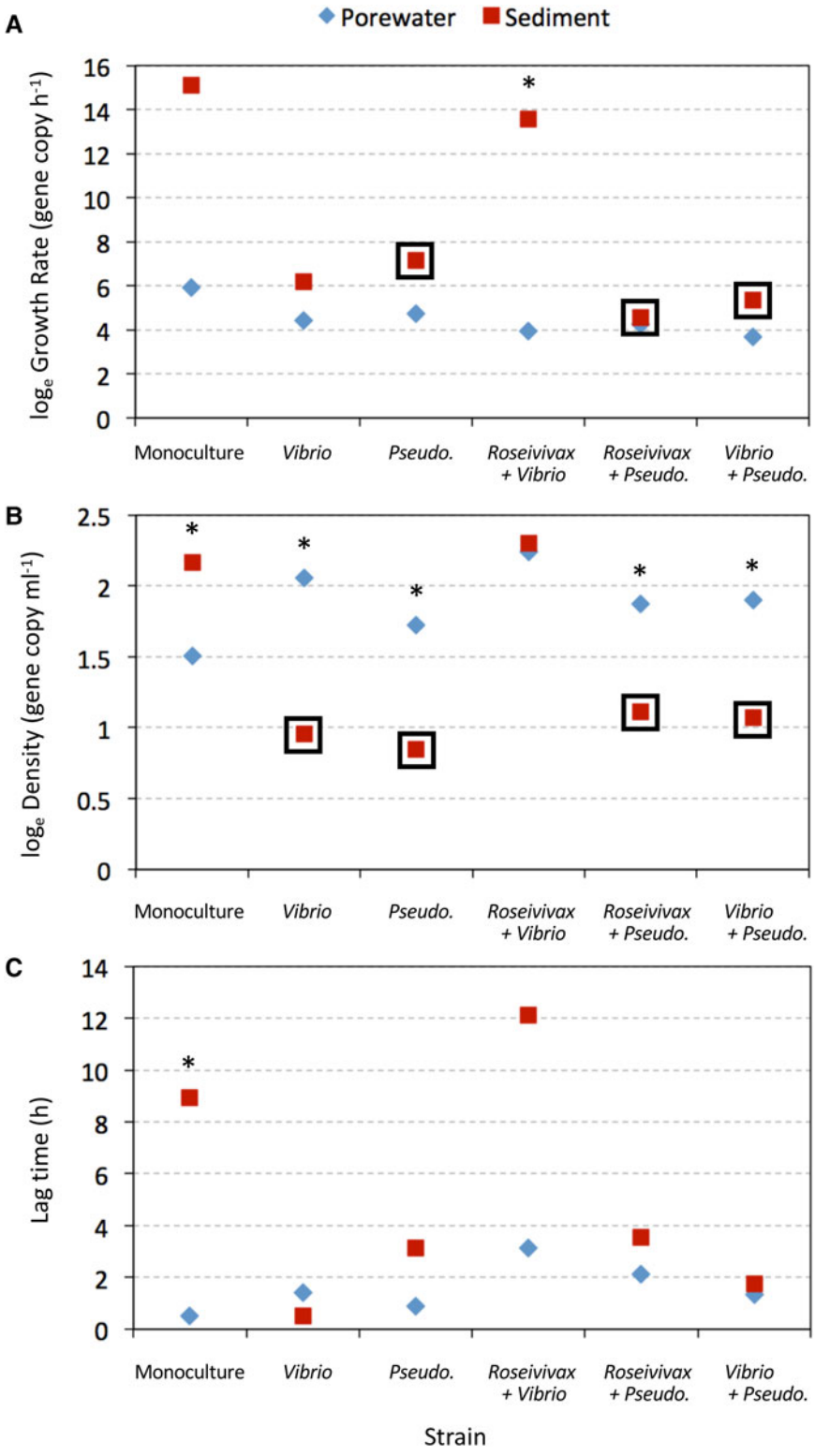

Fig. 3. Growth parameters for *Bacillus* comparing mean (A) growth rate μ_{max}, (B) maximum cell density (*A*) and (C) lag time (λ). Asterisks (*) indicate significant difference ($P < 0.05$; *t*-test) between sediment and porewater microcosms. Boxes around data points denote significant difference between combined cultures and monoculture ($P < 0.05$; one-way ANOVA) from the same microcosm type. Error bars have been excluded for clarity; data variability can be found in Supplementary Table S1.

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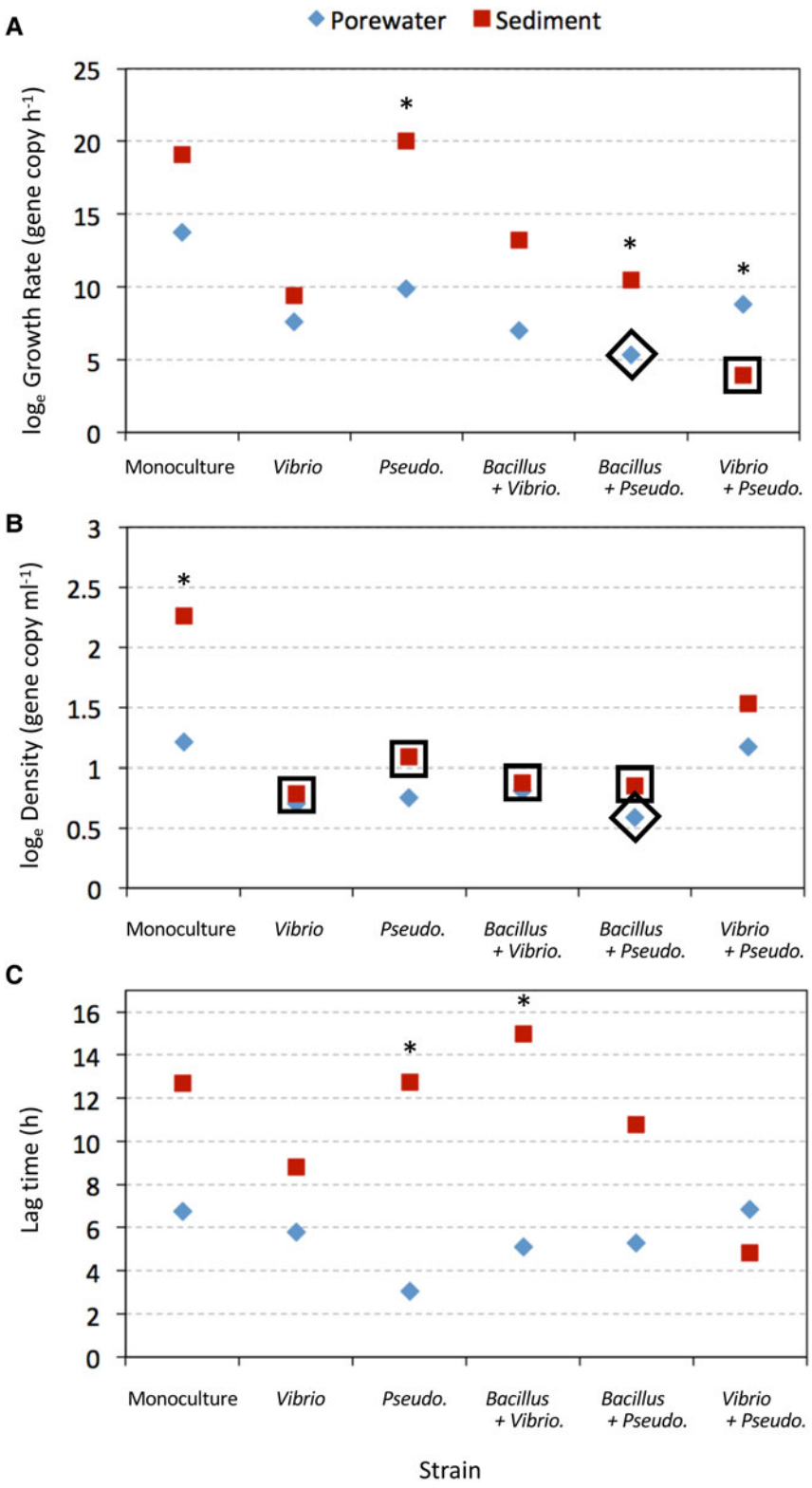

Fig. 4. Growth parameters for *Roseivivax* comparing mean (A) growth rate μ_{max}, (B) maximum cell density (*A*) and (C) lag time (λ). Asterisks (*) indicate significant difference ($P < 0.05$; *t*-test) between sediment and porewater microcosms. Boxes around data points denote significant difference between combined cultures and monoculture ($P < 0.05$; one-way ANOVA) from the same microcosm type. Error bars have been excluded for clarity; data variability can be found in Supplementary Table S1.

(Figures 5A & 6A). However, somewhat surprisingly, μ_{max} values for *Vibrio* showed significant increases when cultured with either *Bacillus* or *Roseivivax* in sediment microcosms ($P = 0.019$, 0.023 and $d = 4.699$, 5.179, respectively, Figure 5A).

Maximal densities (*A*) of target strains achieved in co-culture also demonstrated inhibition by antimicrobial producers. Final densities were depressed relative to monoculture microcosms when *Bacillus* was grown with either *Vibrio* or *Pseudoalteromonas* in sediment ($P < 0.001$, $d = 4.883$ and 5.936, respectively, Figure 3B). Notably, no such reduction in *A* was observed when co-cultured in porewater microcosms (Figure 3B). In fact, maximal densities for *Bacillus* grown in porewater significantly exceeded densities obtained in sediment when co-cultured with either producer (+*Vibrio*, $P = 0.002$, $d = 6.155$; +*Pseudoalteromonas*, $P = 0.004$, $d = 4.877$, Figure 3B). Likewise, *Roseivivax* showed reduced maximal densities in sediment for both 2-way interactions with antimicrobial producers *Vibrio* ($P = 0.006$, $d = 3.369$) and *Pseudoalteromonas* ($P = 0.027$, $d = 2.426$), whereas depression of growth was not significant in the porewater microcosms (Figure 4B). Maximal densities were unchanged relative to

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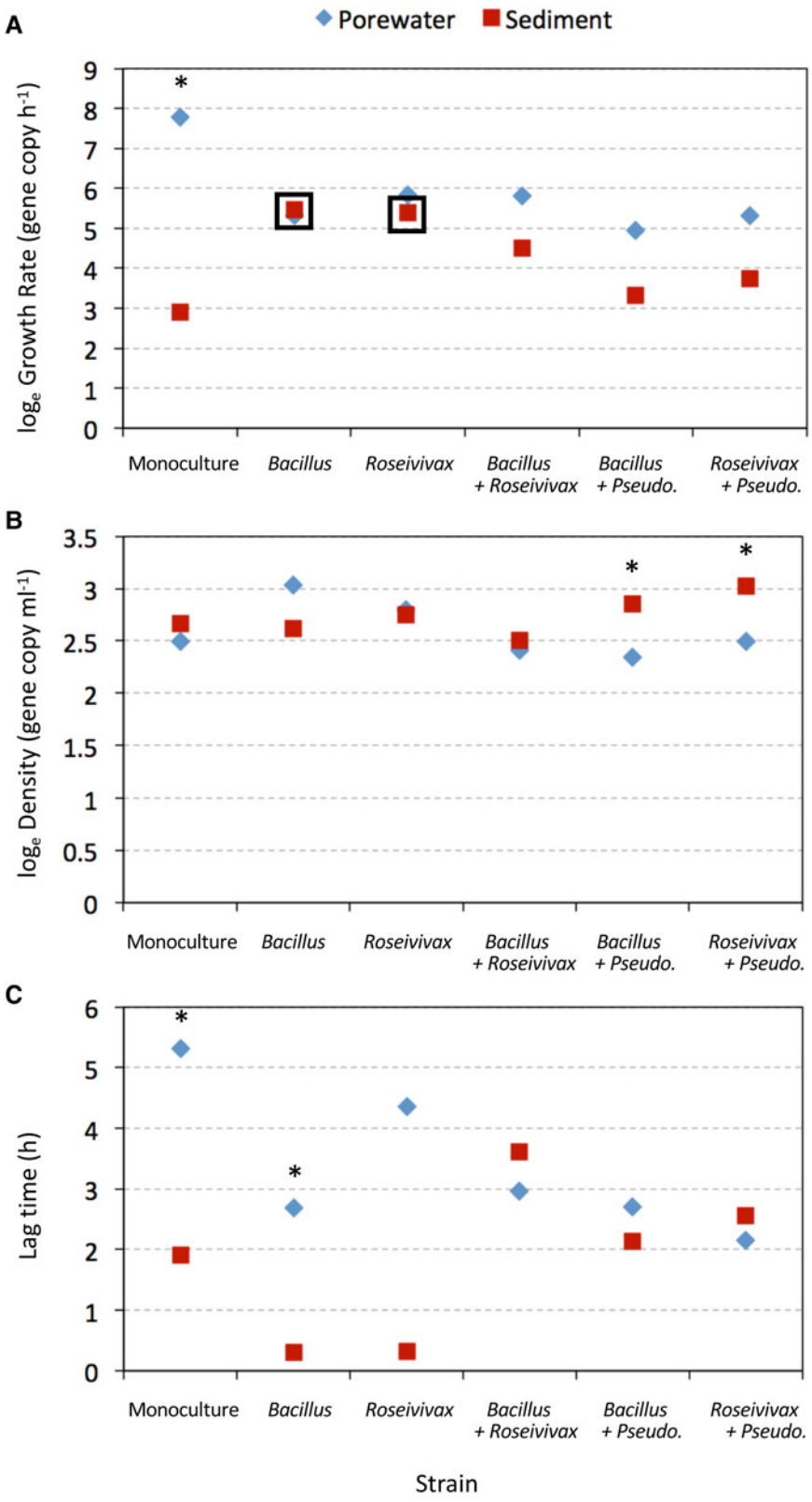

Fig. 5. Growth parameters for *Vibrio* comparing mean (A) growth rate μ_{max}, (B) maximum cell density (*A*) and (C) lag time (λ). Asterisks (*) indicate significant difference ($P < 0.05$; *t*-test) between sediment and porewater microcosms. Boxes around data points denote significant difference between combined cultures and monoculture ($P < 0.05$; one-way ANOVA) from the same microcosm type. Error bars have been excluded for clarity; data variability can be found in Supplementary Table S1.

monoculture growth for both producers when cultured with target species (Figures 5B, 6B).

When lag times for growth in monoculture microcosms were compared with lags observed in 2-species combinations, no significant differences were noted for any of the four bacterial strains in either sediment or porewater microcosms ($P > 0.285$ for all such comparisons). However, *Vibrio* did demonstrate a significantly shorter lag when cultured with either target strain in sediment *vs* the corresponding lags in porewater (Figure 5C). On the other hand, the target *Roseivivax* showed a relatively longer lag in sediment *vs* porewater microcosms when cultured with *Pseudoalteromonas* ($P = 0.003$, $d = 3.108$, Figure 4C).

Three-species microcosms

Growth parameters for more complex microcosm communities (3-species) were compared with 2-way interactions. Three-species interactions appeared to have mixed effects on

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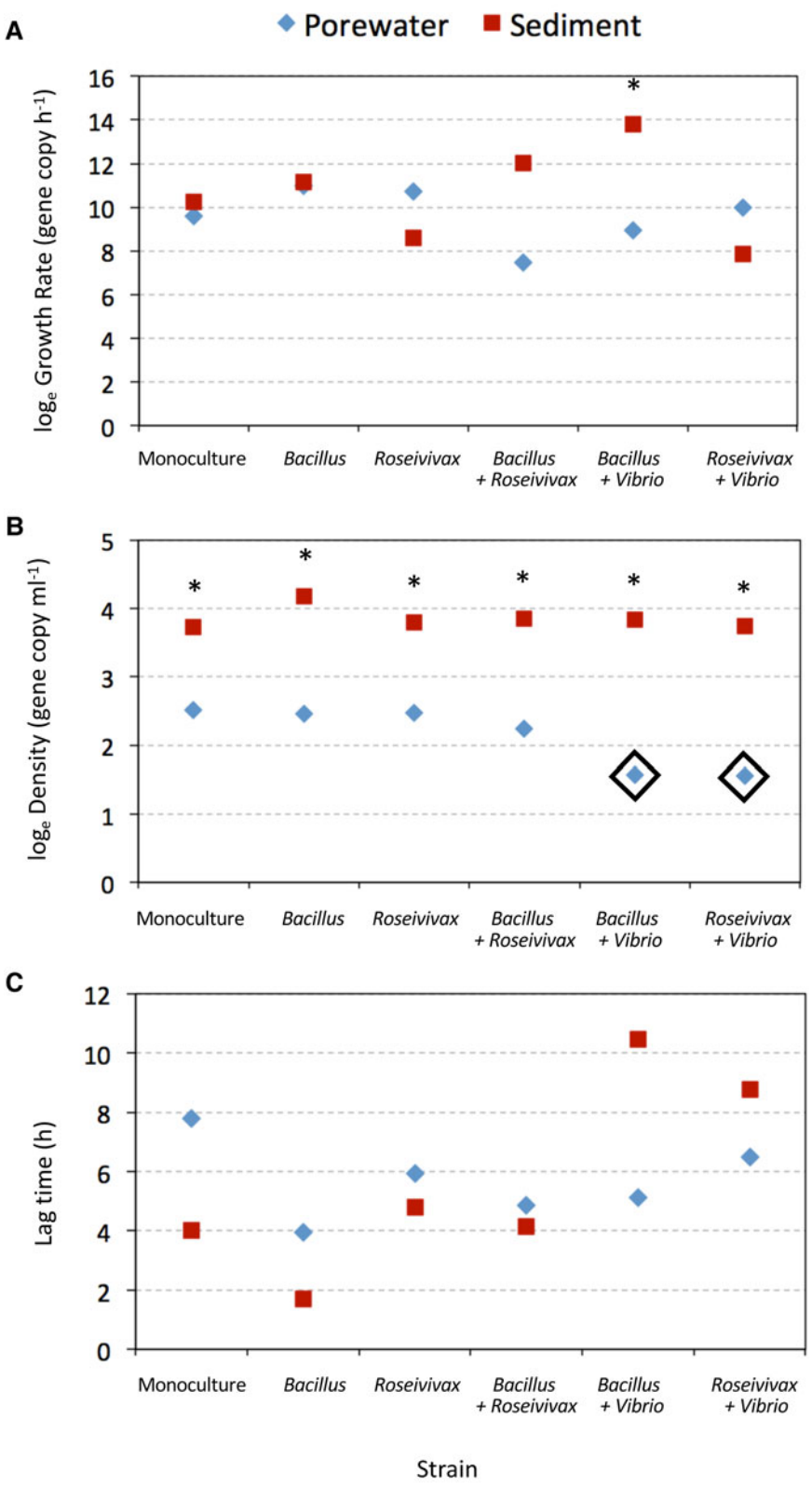

Fig. 6. Growth parameters for *Pseudoalteromonas* comparing mean (A) growth rate μ_{max}, (B) maximum cell density (*A*) and (C) lag time (λ). Asterisks (*) indicate significant difference ($P < 0.05$; *t*-test) between sediment and porewater microcosms. Boxes around data points denote significant difference between combined cultures and monoculture ($P < 0.05$; one-way ANOVA) from the same microcosm type. Error bars have been excluded for clarity; data variability can be found in Supplementary Table S1.

growth parameters of target species. Growth rates for *Bacillus* in sediment decreased slightly with the addition of a third species, with the exception of *Roseivivax* + *Vibrio* wherein μ_{max} was higher ($P = 0.014$, $d = 2.251$, Figure 3A). Growth rate in this combination was also significantly higher than the other 3-species treatments (*vs* *Vibrio* + *Pseudoalteromonas*, $P = 0.020$, $d = 3.059$; *vs* *Roseivivax* + *Pseudoalteromonas*, $P = 0.034$, $d = 2.938$). No real difference in *Bacillus* growth rate among different 3-species combinations was observed in porewater (Figure 3A). *Roseivivax* had the slowest growth rate when cultured with both antimicrobial producers in sediment (Figure 4A). This was also the only occurrence in which a treatment resulted in a higher μ_{max} in porewater, and this difference was significant ($P = 0.006$, $d = 4.451$). *Roseivivax* growth appeared to be more negatively affected by three-way interactions that included *Pseudoalteromonas* than when cultured one-on-one (with *Pseudoalteromonas vs Vibrio* + *Pseudoalteromonas*, $P = 0.010$, $d = 7.424$). *Vibrio* growth rates were lower for 3-species interactions compared with 2-species

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cultures in sediment, although μ_{max} values were still higher than in monoculture (Figure 5A). This was not the case in porewater, wherein the rate of growth was similar for 2- and 3-species interactions (Figure 5A). *Pseudoalteromonas* μ_{max} was suppressed in porewater and generally increased in sediment across three-way interactions, with the exception of the *Roseivivax* + *Vibrio* treatment, in which the lowest μ_{max} overall was observed (Figure 6A).

Bacillus reached higher maximum densities (*A*) in 3-species interactions relative to cultures with either antimicrobial producer alone in sediment, with the *Roseivivax* + *Vibrio* treatment being the only one resulting in a significantly higher *A* ($P < 0.001$ for both, $d = 7.410$ and 10.062, respectively, Figure 3B). There was no difference among porewater treatments, but the maximum densities were significantly higher than most sediment treatments (Figure 3B). With *Roseivivax*, *A* was higher with *Pseudoalteromonas* + *Vibrio* than all other treatments (2- and 3-species) for porewater and sediment (Figure 4). All other 3-species interactions did not differ from what was observed with either antimicrobial-producer one-on-one. Across 3-species treatments, *Vibrio* achieved a slightly higher maximum density in sediment, but lower in porewater than in 2-species interactions, although differences were not significant (Figure 5B). *Pseudoalteromonas* densities were significantly lower than 2-species cultures in porewater within *Bacillus* + *Vibrio* (*vs Bacillus*, $P = 0.016$, $d = 2.820$) and *Roseivivax* + *Vibrio* (*vs Roseivivax*, $P = 0.013$, $d = 4.610$) treatments (Figure 6B). All porewater densities were significantly lower than in sediment for 3-species interactions as well ($P = 0.010$, 0.002, 0.002 and $d =$ 3.775, 5.820, 6.261, Figure 6B).

No significant difference in lag times was observed in 3-species interactions relative to monocultures, as was the case with 2-species cultures. The addition of species had a mixed effect on λ for targets *Bacillus* (Figure 3C) and *Roseivivax* (Figure 4C). In sediment, *Vibrio* began exponential growth much later in the *Bacillus* + *Roseivivax* treatment than with *Bacillus* ($P = 0.006$, $d = 4.133$) or *Roseivivax* ($P = 0.007$, $d = 4.108$) added individually, while λ in porewater was the same across all treatments ($P > 0.676$, Figure 5C). *Pseudoalteromonas* had comparable lag times in sediment and porewater among 3-way interactions, which corresponds to no real difference from 2-species interactions (Figure 6C). The exception was with *Bacillus* co-culture in sediment having shorter lag times than the *Bacillus* + *Vibrio* treatment ($P = 0.032$, $d = 3.315$, Figure 6C).

Discussion

All four bacterial strains grew well as monocultures, in both microcosms with just porewater and in microcosms that included sediment. Although *V. harveyi* appeared to grow slightly better in porewater-only microcosms than in those with sediment, the other three strains grew better with sediment added, as evident from both growth rate (μ_{max}) and maximal population density (*A*). The relatively higher nutrient levels (Grossart *et al.*, 2004) and diffusional advantages (Vetter *et al.*, 1998) associated with a particulate matrix likely contributed to the support of greater bacterial abundances. In sediment microcosms, growth rates were consistent with a potential 'rock-paper-scissors' dynamic in that the sensitive strain (*Roseivivax*) had the highest rate, followed by the resistant strain (*Bacillus*), followed by the two producers. Similarly, in porewater microcosms the sensitive strain had the highest growth rate; however, the growth rate of the resistant strain was slightly lower than that of both producers.

One objective of the current study was to determine whether the interference competition indicated by common antimicrobial-screening methods would likewise be observed in microcosms modelling *in situ* conditions. Antimicrobial production and the competitive relationships among these bacteria were previously determined using a Kirby–Bauer disc-diffusion assay (Hook & Plante, 2019). *Roseivivax*, susceptible to antimicrobial compounds from both *Vibrio* and *Pseudoalteromonas* in disc-diffusion assays, was indeed inhibited by both producers in sediment microcosms, especially with respect to maximal densities. Both producers inhibited *Bacillus* maximal density in sediment microcosms, despite *Bacillus* demonstrating resistance to *Vibrio* in disc assays. Growth rate of *Bacillus* was likewise inhibited when co-cultured with *Pseudoalteromonas*, whereas μ_{max} was not significantly reduced by *Vibrio*. This discrepancy regarding inhibition by *Vibrio* could possibly be due to a greater resistance by target bacteria on diffusion plates compared with more realistic settings. For example, Prosecka *et al.* (2009) noted it took a 10–25× higher concentration of the toxin methyl viologen in disc-diffusion assays to achieve the same level of growth inhibition against resistant cyanobacteria strains than was observed in liquid culture.

Another possibility for greater inhibition in microcosms than in diffusion plates is that of resource competition. Nutrients for growth in microcosms were provided only through natural sediment and/or porewater, thus available resources were much less than levels present in nutrient-agar plates used in disc-diffusion assays. Resource competition favours rapid acquisition of nutrients, therefore under these microcosm conditions rapid growth rate and a lower yield would be favoured (Frank, 2010). One way to distinguish resource from interference competition is by examining distinct growth parameters, which together give a complete picture of growth progression. That *Vibrio* growth rate (but not maximal density) increased when grown in combination with *Bacillus* (and *Roseivivax*) provides support for the notion that resource competition was also relevant in microcosms. Additionally, *Bacillus* maximal densities were negatively affected by *Vibrio* interaction, while there was no significant effect on μ_{max} or λ. It is possible that the extra energy devoted to rapid resource acquisition resulted in the reduction of net yield (density) (Frank, 2010). In any case, the disc-diffusion assay appears to be a rather conservative means to detect antibiotic production and patterns of resistance/susceptibility in terms of relevance in nature.

As expected, *Vibrio* and *Pseudoalteromonas* maximal densities were unaffected by interactions with either target species. A competitive advantage among the species used in two-species microcosms does appear to exist in favour of the antimicrobial producers. While this may be due to a combination of interference and resource competition, it is apparent that the capacity of the antimicrobial producers to affect the growth of target species is greater than the inverse. In our experimental bacterial communities, antimicrobial producers were generally more effective at inhibiting target species in microcosms that included sediment. This was reflected by both reduced growth rates and densities of *Bacillus* and *Roseivivax* in sediment when co-cultured with antimicrobial producers, in contrast to the lack of significant inhibition in corresponding porewater treatments. Enclosure in pore spaces among sand grains can lead to profit from extracellular molecules, e.g. antimicrobials, since the probability that their effect will return to the individual bacterium is enhanced (Plante *et al.*, 1990; Vetter *et al.*, 1998). In contrast, free-living microbes do not benefit from surface adsorption and would be subject to rapid diffusional loss of secretions (Jumars *et al.*, 1993; Simon *et al.*, 2002; Greig & Travisano, 2008).

The formation of biofilms in sediment systems is another major distinction between planktonic and attached bacterial lifestyles. Antimicrobial production has been correlated with enhanced success in acquiring particle space and biofilm formation (Bruhn *et al.*, 2005; Gram *et al.*, 2010; Cude *et al.*, 2012). Furthermore, Rao *et al.* (2005) demonstrated that antimicrobial use conferred a competitive advantage during growth within biofilms on glass flow cells. In certain species, it has even been shown

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that when attached to agar particles bacteria produce antibacterial compounds, but as free-living cells no production occurs (Yan *et al*., 2002). On the other hand, bacterial encasement in biofilms may confer more resistance to antibiotics and other potential antimicrobials (Costerton *et al*., 1995; Høiby *et al*., 2010; Verderosa *et al*., 2019), but screening for antibiotic susceptibility and resistance in lab-based studies is typically done outside of the biofilm matrix. Disc-diffusion assays allow for the direct effects of an isolates' production capabilities towards a single bacterial species to be measured, but may not reflect the relationships *in situ*, within biofilm community dynamics. Many bacterial species have been shown to attach to surfaces, such as glass flow cells and agar particles, within only a few hours (∼2 h) after inoculation (Marshall, 1992; Grossart *et al*., 2003; Rao *et al*., 2005). In order to obtain an accurate sample of all bacteria from sediment microcosms in our study, vortexing was necessary to dislodge the attached bacterial cells. As a result, stable biofilms were unlikely to have developed over the duration of the experiment. Mature biofilms may take 72 h to form, without disturbance (Rao *et al*., 2005). While the microcosms used in this study allowed for comparison of antagonism between sediment and porewater environments, the competition taking place is likely a reflection of interactions occurring during the attachment and space acquisition stage of biofilm formation.

Competitive dynamics involving antibiotic-producing, resistant and susceptible species of bacteria are thought to be an integral mechanism maintaining diversity in sedimentary bacterial communities (Long & Azam, 2001). Although antibiotic-sensitive bacteria have been found to coexist with antibiotic producers, little is known about the interactions leading up to this coexistence (Ghoul & Mitri, 2016). Application of the 'rock-paper-scissor' model of antagonism (Czárán *et al*., 2002; Kerr *et al*., 2002; Kelsic *et al*., 2015) in sediments is confounded by the heterogeneity of communities and the existence of multiple and diverse interaction types within biofilms (Mullis *et al*., 2019).

The sediment-based interaction involving *Bacillus* (R) + *Roseivivax* (S) + *Vibrio* (P) resulted in *Bacillus* growth rates (μ_{max}) that were significantly higher than all other species combinations and also maximal densities that were significantly higher than other 3-species treatments. Because the interaction in the *Bacillus* + *Vibrio* microcosms did not result in similar increased growth rate and density, and in fact depressed them, it can be inferred that the addition of *Roseivivax* allowed for more successful growth of *Bacillus*. Interestingly, a similar 'rescue' was not seen when *Pseudoalteromonas* was the antimicrobial producer. These results suggest the presence of the susceptible species aided in growth of the resistant strain. However, it should be noted that *Bacillus* was originally designated as resistant (R) based on disc-diffusion assays, whereas growth patterns observed in microcosms suggest it is negatively influenced by *Vibrio*. As discussed above, this could be a result of resource competition or due to greater detection sensitivity in microcosm assays. Therefore, this situation might exhibit a rescue of a sensitive strain by a second sensitive strain, as opposed to the hypothesized scenario of P, R and S.

For *Roseivivax*, the inhibitory effect on growth rate when co-cultured with *Vibrio* was reduced slightly with the addition of *Bacillus*, but final density remained the same, and overall effects were minimal. Although relatively few in number, prior studies have demonstrated that resistant bacteria aid in the coexistence of antimicrobial-producing and susceptible strains in simulation models (Zapién-Campos *et al*., 2015), in spot-lawn assays (Gallardo-Navarro & Santillán, 2019) and within biofilms (Narisawa *et al*., 2008). More specifically, the survival of sensitive bacteria in biofilms was achieved by shielding themselves from the producer and the compound-containing medium by incorporating a layer of resistant bacteria between them (Narisawa *et al*., 2008), which would support the notion of a cooperative dynamic between the resistant and susceptible bacterial strains. Burmølle *et al*. (2006) showed that biofilms consisting of more than two species acquired greater tolerance to antibiotics than those consisting of only one species. This balance between both antagonistic and cooperative relationships is considered to be essential for the stable coexistence of mixed species (Kato *et al*., 2005; Haruta & Yamamoto, 2018). Our finding that *Bacillus* did not protect the sensitive strain *Roseivivax* from antimicrobial producers appears to contradict these prior studies, as well as the theoretical rock-paper-scissors scenario. One potential explanation for the discrepancy is the aforementioned designation of *Bacillus* as a resistant strain *vs* a sensitive strain. If *Bacillus* does not actually inactivate or exclude antimicrobials, or does not do so in a microcosm setting, then the rock-paper-scissors dynamic is not predicted. Resource competition is a second factor that differs between microcosms and both *in vitro* work and simulation models. Significant resource competition could mask changes in interference competition, whereas the effect of resistant bacteria on antimicrobial production in diffusion assays (or in simulation models) is isolated and emphasized. Finally, as our study was performed in batch culture and focused on initial bacterial encounter and growth characteristics, rather than long-term patterns of survival and coexistence, our results for *Roseivivax* are not necessarily a direct contradiction.

In 3-species interactions involving *Vibrio* and *Pseudoalteromonas*, both producers were less successful in porewater, while densities in sediment were unaffected, or even slightly elevated. These findings suggest that negative effects of multispecies interactions, e.g. higher resource competition, are experienced more by antimicrobial-producers in the unstructured 'porewater' microcosms, as they lack many of the aforementioned benefits associated with antimicrobial use in biofilm formation. Antibiotic production can confer a competitive advantage within biofilms by deterring other potential colonizers as well (Tait & Sutherland, 2002; Rao *et al*., 2005). Long & Azam (2001) suggested that antimicrobial production is more commonly used by particle specialists for this reason, and because the ability of particle-attached bacteria to chemically inhibit free-living bacteria is greater than the ability of free-living bacteria to chemically inhibit particle-attached bacteria.

Our study includes several limitations that warrant mention. Although we endeavoured to study bacterial antagonisms in a more realistic manner than prior *in vitro* methods such as diffusion plates, our microcosms also fell short of replicating *in situ* conditions. Both biological (e.g. lack of predators) and physical aspects of the natural environment were necessarily simplified. One noteworthy aspect was the constant mixing on shaker tables and periodic vortexing (prior to sampling) of our microcosms. Because our bacteria were isolated from a beach habitat, the gentle stirring of the shaker table should not have caused uncharacteristic disturbance within our microcosms; however, the relatively intense vortexing may have unnaturally disrupted biofilms and other aspects of the sedimentary matrix. As discussed above, prior research has shown that biofilm formation can influence the production (Rao *et al*., 2005) and effectiveness (Costerton *et al*., 1995) of antimicrobial compounds. In addition, our experiments were performed in batch culture, therefore nutrient dynamics did not replicate the natural scenario. Nutrient concentrations would have declined in microcosms, resulting in elevated intensity of competition relative to *in situ* conditions. Because our experiments included only one sediment type from a semi-protected beach, generalizations should be made with caution. Coarser sediments such as those from an exposed, high-energy beach likely would show results closer to those of our porewater-

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only microcosms due to higher sediment permeability (Shepherd, 1989). On the other hand, our results indicating a relatively strong influence of antimicrobial compounds are likely conservative compared with expectations of similar studies using muddy sediments. Higher competition and greater influence of antimicrobial compounds would be predicted in fine sediments due to greater diffusional constraints (Plante *et al.*, 1990; Vetter *et al.*, 1998) and higher bacterial abundances (DeFlaun & Mayer, 1983).

In summary, we conducted multi-species microcosm experiments which provided new insights into interference competition among sedimentary bacteria. We found that sensitivity to antimicrobials was markedly higher in the more natural microcosms than in classic disc-diffusion assays. We also observed that antimicrobial producers were generally more effective at inhibiting target species in microcosms that included sediment, in agreement with the theory that predicts allelopathic interference competition to occur more readily in a spatially structured habitat than in well-mixed environments (Iwasa & Nakamaru, 1998; Greig & Travisano, 2008). We observed growth inhibition by antimicrobial-producing bacteria in both 2- and 3-species microcosms. However, the expected protection of sensitive bacterial strains by resistant strains was observed in only one (of four) 3-species combinations, thus the rock-paper-scissors prediction was not fully supported. These results reinforce the notion that interspecies interactions are context-dependent (Ghoul & Mitri, 2016; Haruta & Yamamoto, 2018), critically dependent on environmental conditions, the species/strains involved, time course of observation, among other factors. Interference competition has the potential to affect bacterial population sizes, shape species composition and functional diversity, and thereby impact the biogeochemical processes taking place in sediments. Furthermore, the prevalence of antimicrobial production suggests that intertidal sediments may be a potential source for novel natural products.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315422000376

Acknowledgements. We thank Ms Tricia Roth for technical advice and assistance. Grice Marine Laboratory contribution #580.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

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https://doi.org/10.1017/S0025315422000376 Published online by Cambridge University Press