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The Role of Bacteria-Particle Interactions in Marine Snow Dynamics

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THE ROLE OF BACTERIA-PARTICLE INTERACTIONS IN MARINE SNOW DYNAMICS

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APPROVAL SHEET

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DEDICATION

To my family
To my ohana

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ABSTRACT

Marine snow aggregates are one of the primary vehicles for deep-sea carbon sequestration. Bacterial activity on marine snow affects both degradation and aggregation processes that determine the flux of carbon to depth, biogeochemical cycling, and microbial food webs. The microscale processes occurring on aggregates depend on specific interactions between bacteria and particles. In this thesis, I describe two such interactions which have larger scale implications on marine snow dynamics: (1) the effects of starvation on bacterial motility and colonization behaviors on marine aggregates and (2) interactions between bacteria and phytoplankton which could contribute to the production of TEP.

Current models describing bacterial colonization on particles do not account for changes in bacterial behavior due to starvation, which may happen between successful encounters with particles and is a possible condition in the oligotrophic ocean. In my first study, I examine the effects of starvation on the colonization and detachment of several bacterial isolates on particles. I also describe the changes in bacterial motility resulting from starvation. Laboratory experiments on model aggregates indicate that responses to starvation are strain-specific, and can result in lower short-term steady-state bacterial abundances on the aggregates. Bacterial detachment from aggregates was unchanged. Motility data indicate that two of three strains tested had reduced swimming velocities, resulting in diffusivities six times lower in starved treatments than in fed

treatments. This was corroborated by colonization data. Future models describing bacterial colonization should consider the shifting physiology and behavior of bacteria responding to starvation.

In my second study, I investigated the interactions between bacterial isolates and the marine diatom *Thalassiosira weissflogii* (TW) on the production and characteristics of TEP, a major component of marine snow. One of two bacterial isolates (either *Microscilla furvescens* or *Curacaobacter baltica*) was added to jars of TW and incubated on a rolling table for seven days. During the time course, each jar was sampled for TEP length, area, total TEP, and bacterial distribution among the free-living and TEP-associated fractions. The two strains of bacteria showed different responses. Jars inoculated with *Curacaobacter baltica* had a significantly higher fraction of total bacteria that were associated with TEP, although the number of bacteria per unit area of TEP was lower. These results suggest that the strain-specific interactions between bacteria, phytoplankton, and TEP could impact the population distributions of bacteria. Over seven days, jars inoculated with *Curacaobacter baltica* produced more TEP; TEP coverage was almost four times higher (~8% of the total filter area) in jars inoculated with *Curacaobacter baltica* than those inoculated with *Microscilla furvescens*.

Results from both studies stress the importance of strain-specific interactions in describing microscale processes. Integrating our understanding of responses of individual strains with information on the diversity and activities of bacterial communities on aggregates will better determine how these complex interactions may affect the fate of sinking aggregates and the solubilization of particles into dissolved organic matter.

CHAPTER 1

INTRODUCTION

The Role of Marine Aggregates

The ocean is the largest active reservoir in the global carbon cycle, and therefore could play a major role in mitigating the effects of global warming and increased anthropogenic emissions of CO_2 (Siegenthaler and Sarmiento, 1993). The sinking of particulate organic matter (POM) in the ocean, such as marine snow, is a key mechanism for transporting carbon to depth (Fowler and Knauer, 1986; Alldredge and Silver, 1988; Kiørboe, 2001). Marine snow is made of large, fractal aggregates (>500 μm) composed of phytoplankton aggregates, zooplankton fecal material, or other large organic structures, including mucous structures and secretions (Alldredge and Silver, 1988; Kiørboe et al. 2003). Marine snow constitutes up to 63% of total particulate organic carbon in some areas (Alldredge and Silver, 1988). Large fluxes of aggregates are typically coupled to primary productivity (Alldredge and Silver, 1988; Alldredge and Gottschalk, 1990; Kiørboe 2001) and are associated with the termination of phytoplankton blooms (Smetacek, 1985; Allredge, 1995; Logan et al. 1995).

Aggregation of phytoplankton depends on the abundance (Hill, 1992) and size spectrum of particles (Jackson 2001), the physical environment (McCave, 1984), and the stickiness of the particles (Kiørboe et al. 1996; Jackson 1995; Jackson 2001), which can depend on growth stage (Passow et al. 2002a), physiological condition of the phytoplankton (Hong et al. 1997) and the presence of exopolymeric secretions (EPS). One type of EPS is transparent exopolymer particles (TEP) (Passow and Alldredge,

1995), which are discrete particles that are considered a primary factor in determining particle stickiness (Passow et al. 1994b).

Organic macroaggregates are sites of elevated heterotrophic activity which undergo a succession of microbial colonizers that change the character of the particles as they are transported to depth (Alldredge and Silver, 1988). These microbial colonizers include bacteria (Smith et al. 1992; Kiørboe et al. 2002), protozoans, (Silver et al. 1984; Artolozaga et al. 1997; Kiørboe et al. 2003), and larger zooplankton (Bochdansky and Herndl, 1992; Steinberg et al. 1994; Dilling et al. 1998).

Colonization of Marine Aggregates by Heterotrophic Bacteria

Heterotrophic bacteria play an important role in the degradation and solubilization of POM (Azam et al. 1983) although particle-associated bacteria make up only a small portion (< 10%) of that total activity (Alldredge and Youngbluth, 1985; Karl et al. 1988). Aggregate-associated heterotrophic bacteria play an important role in the biogeochemical cycling of POM (Smith et al. 1992; Smith et al. 1995) because these bacteria release exoenzymes and solubilize particulates (Cho and Azam, 1988) faster than they can take up the dissolved products (Vetter et al. 1998).

Initial colonization of aggregates by bacteria, occurring on a time scale of minutes, can influence the subsequent population dynamics, diversity, and community development on aggregate surfaces (Lawrence et al. 1995). The early stages of bacterial colonization on aggregates have been modeled (Kiørboe et al. 2002), and these models can be used to predict the steady-state abundances of attached bacteria based on the ambient bacterial concentration, the size of the aggregate, bacterial motility, and the flow

environment (Kiørboe et al. 2002). Behaviors like chemotaxis (Fenchel, 2001), quorum sensing (Gram et al. 2002), and antagonism (Grossart et al. 2004) further complicate the colonization process. Colonizing bacteria have also been shown to detach from aggregates, even after relatively short residence times (Kiørboe et al. 2002), suggesting that, while an aggregate may be a rich source of nutrients, it is also a risky environment in terms of predation (Kiørboe et al. 2003).

Although many microscale processes involved in the bacterial colonization and degradation of particles have been described (Kiørboe et al. 2002, 2003; Grossart et al. 2003b), there are still many areas that remain understudied or unexamined, including the role of nutritional status on changing bacterial behavior and aggregate encounter. The complex interactions and subsequent responses of heterotrophic bacteria occurring on aggregates have implications in the flux of elements from particulate to dissolved phases, aggregation processes, and food web dynamics.

Heterotrophic Bacterial Interactions with Transparent Exopolymer Particles (TEP)

Microbial dynamics associated with TEP, an important component in phytoplankton aggregates, may also influence aggregate formation. Microbially-mediated aggregation depends on the nutrient environment (Mykelstad, 1977; Guerrini et al. 1998; Grossart, 1999), the distribution of heterotrophic bacteria between the particulate and dissolved fractions (Grossart et al. 2006b), grazing pressure (Caron, 1987), and production of enzymes (Martinez et al. 1996) and extracellular exudates.

Grossart et al. (2006b) reported that bacteria were required for the aggregation of the diatom *Thalassiosira weissflogii*, but not for *Navicula sp.*, which is a "stickier"

diatom. Bacteria may contribute to phytoplankton aggregation by producing TEP (Decho, 1990; Stoderegger and Herndl, 1999), that make phytoplankton cells stickier or that can aggregate with other TEP (Jackson, 2001). Bacteria have been shown to take up DOM and create extracellular exudates which contribute to particle formation (Paerl, 1978). In addition to producing TEP or TEP precursors, bacteria may also degrade, transform, and utilize TEP as an energy source (Passow, 2002b; Radic et al. 2006). Passow and Alldredge (1994) indicate that the variability in microbial colonization on TEP could be explained by variability in composition of TEP precursors. However, colonization patterns on other surfaces (aggregates, biofilms, and the like) suggest that interactions may be more complex, and could be strain-specific (Passow, 2002a). The strain-specific interactions between bacteria and TEP are not well-studied, but could have larger implications in aggregation dynamics because of the role of TEP as a biological glue and organic substrate.

Objectives

Heterotrophic bacteria clearly have a strong influence on the formation and fate of aggregates in the ocean. The microscale processes occurring on aggregates are determined by the strain-specific responses of the bacteria to the environment on and around the aggregates. The purpose of this thesis was to examine the specific responses of heterotrophic bacteria in a controlled, laboratory setting and to describe the possible ecological implications of these responses on the dynamics of marine aggregates. My specific research objectives were:

(1) To determine the species-specific effects of starvation on bacterial colonization on, and detachment from, aggregates. (2) To compare the motility of starved and fed bacteria. (3) To compare the effects of interactions between the bacteria and the diatom *Thalassiosira weissflogii* leading to differences in TEP abundance, size, and bacterial distributions, and examine the implications of these differences in bacterial population dynamics, grazing, and aggregate formation.

Thesis Organization

This thesis is organized into the following chapters:

Chapter 2: Starvation effects on aggregate colonization and motility of marine bacteria. In this chapter, I report the effects of starvation on the bacterial colonization of aggregates through changes in bacterial motility.

Chapter 3: Production of transparent exopolymer particles by the diatom *Thalassiosira weissflogii* under the influence of two different bacterial strains. In this chapter, I examine how species-specific interactions between marine snow bacterial isolates and the marine diatom *Thalassiosira weissflogii* may affect TEP quantity, characteristics, and bacterial distributions between free-living and TEP-associated fractions, and the ecological implications of those interactions.

Chapter 4: Summary and concluding remarks. I conclude with a discussion on how results from these studies further our understanding of the specific role of bacteria-particle interactions on microscale processes involved with the dynamics of marine snow.

CHAPTER 2

Starvation effects on aggregate colonization and motility of marine bacteria

ABSTRACT

Fluxes of particulate matter to depth and dynamics of dissolved organic matter in the water column are influenced by microbial processes associated with organic aggregates like marine snow. These microscale processes include the encounter between bacteria and aggregates, which has been previously modeled and tested with well-fed and actively growing bacteria. In this study, we investigated the effects of starvation on initial bacterial colonization of aggregates by measuring colonization and detachment of six isolates in different physiological states (fed vs. starved) using model aggregates. Because aggregate encounter depends on motility, the motility behaviors of fed and starved bacteria of three select strains were also compared using image analysis. All six fed isolates colonized faster and achieved significantly higher steady-state abundances on model aggregates than those that were starved. However, there was no difference in detachment rates between fed and starved bacteria. Three select strains had significantly lower average swimming speeds when starved. Diffusivities calculated from motilities of two starved isolates were more than six times lower than those of their fed counterparts. Our results show that starvation significantly affects bacterial behavior and bacteria-aggregate interactions, which may lead to differences in particulate and dissolved organic matter fluxes and cycling under different productivity regimes.

INTRODUCTION

Marine aggregates constitute up to 63% of the total particulate organic carbon in some parts of the ocean (Alldredge 1979), and could be important for carbon sequestration even in oligotrophic waters (Benitez-Nelson et al. 2001, Pilskaln et al. 2005). Marine aggregates are often sites of elevated heterotrophic activity (Alldredge & Gottschalk 1990, Silver et al. 1978, Caron et al. 1982, Artolozaga et al. 1997, Dilling et al. 1998), and colonization and subsequent solubilization of aggregates by bacteria can influence biogeochemical cycling of organic matter (Cho & Azam 1988, Smith et al. 1992). Aggregate-associated bacteria typically comprise a small portion of the total bacteria in the water column (Kirchman, 1993, and references therein). However, per unit volume, aggregates are highly enriched with bacteria relative to the ambient water (Simon et al. 2002). Three functional types of bacteria have been described based on their interactions with aggregates (Riemann et al. 2000, Kirchman 2002, Grossart et al. 2006b): (1) Free-living bacteria that tend not to attach to aggregates, (2) "particle-specialists" that specialize in colonizing particles and aggregates, and (3) "generalists" that can grow both in suspension and on particles. Particle-specialists are phylogenetically distinct from the others (DeLong et al. 1993, Grossart et al. 2006b), and their survival may depend on their ability to locate and colonize aggregates.

Short-term colonization of aggregates by bacteria, occurring on a scale of minutes to a few hours, is governed by the rate at which bacteria encounter, attach to, and detach from the aggregates (Kiørboe et al. 2002). In still water, encounter rate depends on the

aggregate size, ambient concentration of bacteria, and diffusivity of the bacteria, which is in turn determined by motility of the bacteria. Bacterial motility can be described as 'random walk' where straight runs are interrupted by tumbles when the bacteria randomly reorient their swimming direction (Berg, 1983). Differences in bacterial diffusivity can be partly attributed to differences in run speeds, run duration, turn angles and turn frequency (Mitchell & Kogure 2006). Bacteria may also change their motility patterns in response to chemical stimuli (Mitchell 1991, Fenchel 2001, Thar & Kuhl 2003) or physiological stress (Malmcrona-Friberg et al. 1990, Wrangstadh et al. 1990, Stretton et al. 1997, Wei & Bauer 1998), and these responses can be heterogeneous even within a population (Stretton et al. 1997). A study by Wrangstadh et al. (1990) suggests that both adhesion and detachment of *Pseudomonas* sp. from surfaces could be affected by starvation in as little as three hours. After the onset of starvation, peripheral exopolymer substance (EPS) production prevented flagellar movement in bacteria by increasing the viscosity around the cell (Wrangstadh et al. 1990). Malmcrona-Friberg et al. (1990) showed the portion of motile cells decreased from 60% to nearly zero after 3 to 24 hours of starvation due to the loss of flagella. Wei and Bauer (1998) reported that prolonged starvation in the terrestrial bacterium *Rhizobium meliloti* resulted in a graded response, ranging from loss or modification of flagella to inactivation of the flagellar motor.

Kiørboe et al. (2002) estimated that the search time for a bacterium to encounter an aggregate in the upper ocean was 0.02 to 12 d, with a median of 0.4 d; thus, most bacteria should reach an aggregate within one day of continuous searching. However, this estimation assumes constant diffusivity and velocity for well-fed, exponentially-growing bacteria (Kiørboe et al. 2002). Because bacteria may change motility or

physiology within only a few hours of starvation, previous estimates of search time, encounter, and colonization rates may not apply to situations where bacteria may starve between successful encounters with aggregates, especially in nutrient-limiting environments.

The goal of this study was to investigate starvation effects on short-term bacterial colonization of aggregates and motility patterns. First, we compared the colonization and detachment rates of six strains of marine bacteria under fed vs. starved conditions using model aggregates (agar beads). To further describe the effects of starvation on colonization rates, we selected three of the bacterial strains and studied their motility patterns under different nutritional conditions.

MATERIALS AND METHODS

Bacterial Isolates

Bacteria strains HP1, HP5, HP11, HP66 were originally isolated from aggregates in the Wadden Sea, Germany (Grossart et al. 2004). Cultures of the isolates were maintained on solid media (2% marine broth agar). Additional strains (YR2, YR7) were isolated from the York River, Virginia, on solid media. Fed and starved bacteria were prepared by resuspending isolates in liquid media for 24 h at 22° C in the dark: For the fed treatment, bacteria were inoculated in 1% marine broth (MB); for the starved treatment, bacteria were inoculated in organic matter-free, nutrient-free water (NFW) prepared according to Kemp et al. (1990).

Bacterial Colonization and Detachment

We used model aggregates made of 2% agar (Fisherbrand) in artificial seawater according to Cronenberg (1994). The use of agar beads as an analog to marine aggregates is a proven and effective means for studying initial microbial colonization of aggregates (Kiørboe et al. 2002, 2003). Fed or starved bacteria were resuspended into experimental chambers with 500 ml of NFW at a concentration of 10^5 cells ml^{-1}. Negligible levels of MB from the fed treatment were introduced into the experimental chambers (less than 0.4% of the final experimental volume). As such, observed

differences between fed and starved bacteria could be attributed to the different physiological status of the bacteria rather than differences in the ambient environments.

For the colonization experiments, agar beads (4 mm in diameter) were suspended by glass needles in the experimental chambers for up to 150 minutes. At each sampling point, triplicate beads were removed and bacteria attached to the bead surfaces were enumerated using DAPI direct counts at 600× magnification. A 1-ml aliquot of the ambient bacteria was also counted at the start and end of the colonization experiment. The model of Kiørboe et al. (2002) was fitted to the data to estimate diffusivities (D_m) and detachment rates (δ_m):

Equation 1.

$$N_t = 4\pi a C D_m (1+\exp(-\delta_m t)\bullet\left(erfi\left[\left(\frac{D_m t}{a^2}\right)^{0.5}\right]\left(\frac{a^2\delta_m}{D_m}\right)^{0.5} -1\right)/\delta_m$$

where N_t is bacterial abundance on agar beads; a is radius of agar beads; C is ambient bacterial concentration; t is time; *erfi* is the imaginary error function for integrating a Gaussian distribution. D_m determines the rate at which the bacteria encounter an aggregate based on random-walk motility, whereas δ_m determines the rate at which recently attached bacteria detach from the aggregate. Because D_m and δ_m are not independent of each other in the model, we further define a colonization coefficient R_m as the numerical value of D_m/δ_m, which characterizes the net accumulation of bacteria on the aggregate, and allows us to compare the colonization rates between treatments.

At the end of the colonization experiments, remaining beads with attached bacteria were transferred to nutrient-free water, and the detachment of bacteria from the agar beads was monitored over a course of 60 to 100 min. An exponential decay function

was fitted to the remaining bacterial counts over time to estimate an empirically-derived detachment rate (δ_e) (Kiørboe et al. 2002).

Bacterial Motility and Image Analysis

Three Wadden Sea isolates (HP1, HP11, HP66) were selected for detailed motility observations. Starved and fed bacteria were resuspended in an observation chamber consisting of two rubber o-rings (1.9 cm in diameter) glued together and affixed to a microscope slide. The chamber was 0.5 cm deep and sealed with a cover slip. The bacteria were observed using dark-field microscopy and filmed at a 100× magnification. Twenty seconds of footage were recorded for each treatment. Bacterial swimming tracks were digitized using ImagePro 5 software. The digital field of view was approximately 900 μm × 600 μm. Forty to fifty projected 2-dimensional swimming tracks that were in focus were analyzed for run length at a 0.1s resolution and turn angle at a 0.5 to 0.7s resolution. Convective heating created a small net flow in the chambers, which was corrected for in the analysis using additional tracks of abiotic particles in the field of view. The average 3-dimensional speed of the bacterium was estimated as $(3/2)^{1/2}$ multiplied by the corrected 2-dimensional speed, and the empirically derived diffusivity (D_e) was calculated according to Kiørboe et al. (2002):

Equation 2.

$$D_e = \frac{u^2 \tau}{6(1-\alpha)}$$

where u is the swimming velocity; τ is the run length; and α is the mean cosine of the angles between two successive runs. Additionally, swimming speed data were used to generate frequency distributions for both starved and fed treatments. Bacterial diffusivity, swimming speed and turn angles between treatments were tested for

significant differences by Student's t-test. When data were not normally distributed, the Mann-Whitney test was used. Analyses were performed using MiniTab.

RESULTS

Colonization

All six strains of bacteria accumulated on agar spheres over time in a fashion similar to that previously described (Figure. 2.1). Ambient cell counts showed were not significantly different before and after the 150-minute colonization incubation (student's t-test, $p > 0.147$) and no dividing cells were observed. Bacterial abundances on the beads were normalized to ambient bacterial concentration to correct for slight differences in ambient bacterial concentrations within treatments. A two-way ANOVA was performed on rank-transformed, normalized abundances for each strain. For all six strains, there was a significant difference between fed and starved treatments ($p < 0.001$), a significant effect over time ($p < 0.001$), and a significant effect of treatment and time on the rank of the normalized abundance ($p < 0.001$).

Significant differences between normalized starved and fed bacterial abundances on beads were tested using Mann-Whitney test for each time point. For all strains tested, fed bacteria accumulated to higher overall abundances than starved bacteria. Normalized abundances of fed HP5 and YR3 bacteria were always significantly higher on beads than their starved bacterial abundances for all sampled time points (HP5, $p < 0.031$; YR3, $p < 0.011$). Three strains tested (HP1, HP11, YR7) had fed and starved normalized abundances that were not significantly different at time zero but diverged thereafter (HP1, $p = 0.163$; HP 11, $p = 0.186$; YR7, $p = 0.104$). Fed bacterial abundances for those three strains were significantly higher from 5 to 150 min (HP1, $p < 0.003$; HP 11, $p <$

0.026; YR7, $p < 0.005$). Normalized abundances for fed and starved HP66 were not statistically different for time 0 ($p = 0.427$) or 5 min ($p = 0.385$). However, fed HP66 accumulated to significantly higher levels after 10 – 150 min ($p < 0.031$).

The model of Kiørboe et al. (2002; Equation 1) fit significantly to the data ($p < 0.011$). The coefficient R_m averaged (± 1 s.d.) 9.1×10^{-3} (± 3.8×10^{-3}) for all starved bacteria and 24.9×10^{-3} (± 13.7×10^{-3}) for all fed bacteria (Table 2.1). R_m for fed Wadden sea isolates was 2.4 to 3.7 times greater than their starved counterparts. Fed York River isolates had an R_m 1.5 to 2 times higher than their starved counterparts. The model, run in Matlab, does not output measures of error for diffusivity or detachment.

Figure 2.1 Colonization of model aggregates (agar beads) by fed (closed circles) and starved (open circles) bacteria. Abundances of attached bacteria per aggregate are normalized to ambient bacteria counts. Error bars indicate the standard deviation of ten counts. Plotted lines represent fits of colonization model (Kiørboe et al. 2002) to the data. "HP" bacteria were originally isolated from the Wadden Sea, Germany; "YR" strains were isolated from the York River, VA, USA.

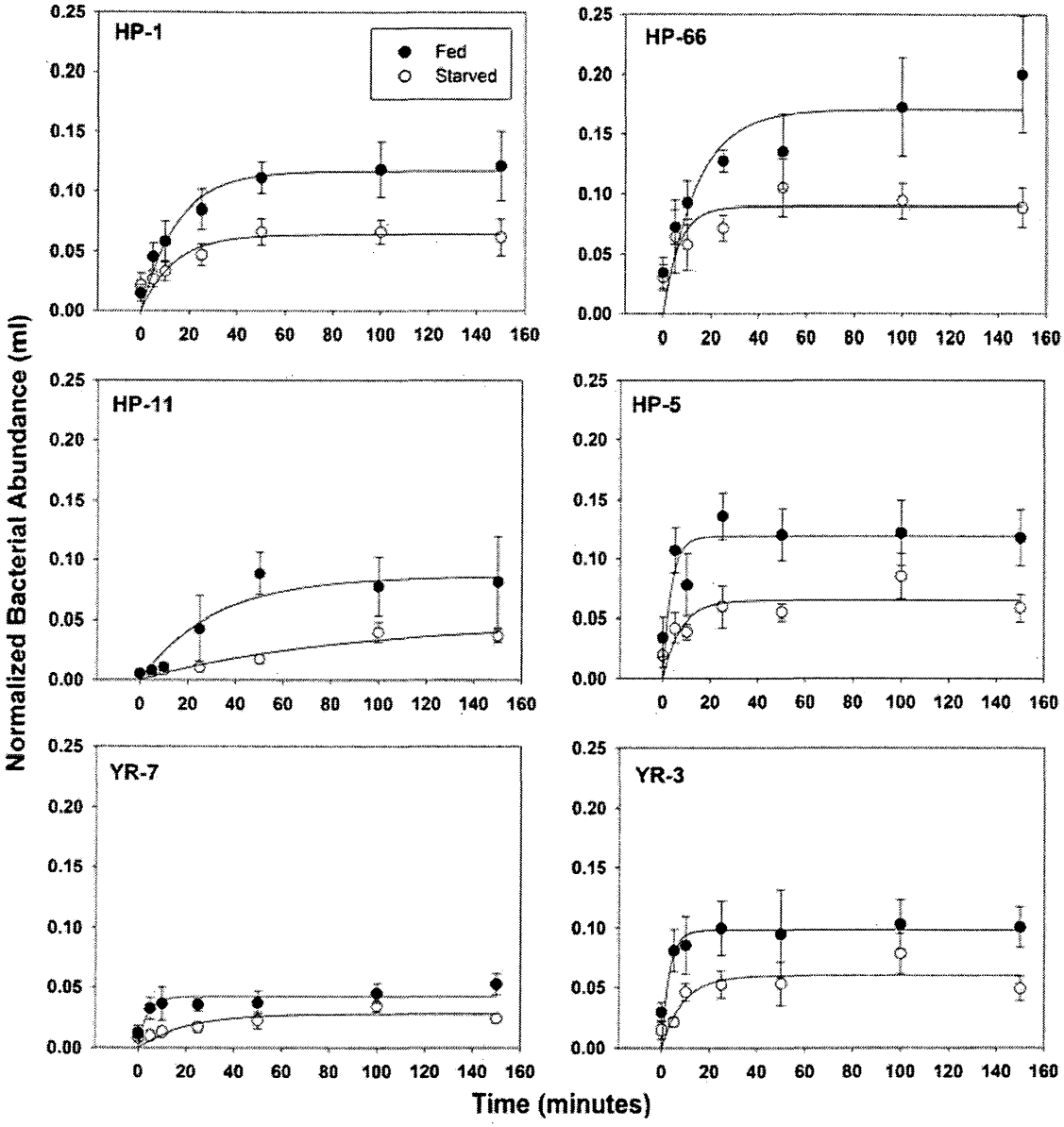

HP-1
Fed
Starved
HP-66
HP-11
HP-5
YR-7
YR-3
Normalized Bacterial Abundance (ml)
Time (minutes)
0.00
0.05
0.10
0.15
0.20
0.25
0
20
40
60
80
100
120
140
160

Table 2.1 Colonization coefficient (R_m) based on diffusivity (D_m) and detachment rate (δ_m) derived from model fits to the colonization experiment data using Equation 1.

	R_m	
Strain	Starved	Fed
HP1	0.0089	0.0289
HP5	0.0104	0.0248
HP11	0.0058	0.0214
HP66	0.0152	0.0482
YR3	0.0100	0.0192
YR7	0.0045	0.0067
Mean (± SD)	9.1 (± 3.8) x 10^{-3}	24.9 (± 13.7) x 10^{-3}

Detachment

Bacterial detachment from the agar beads followed an exponential decay function (Figure 2.2). Curve fits were significant ($p < 0.05$) except for HP5 and HP66 fed, and YR3 starved, where scatter was larger. Calculated detachment rates (δ_e) were on the order of 10^{-4} to 10^{-3} s^{-1} (Table 2.2). Raw data were natural log transformed to linearize the data. Linearized data were analyzed using a student's t-test to compare slopes; slopes were not statistically different between starved and fed treatments for any of the six strains tested ($p > 0.05$).

Figure 2.2 Detachment of fed (filled circles) and starved (open circles) bacteria from model aggregates (agar beads). Error bars indicate the standard deviations from ten bacteria counts. Plotted lines represent fits of exponential decay functions to the data. Note the change in y-axis for HP11.

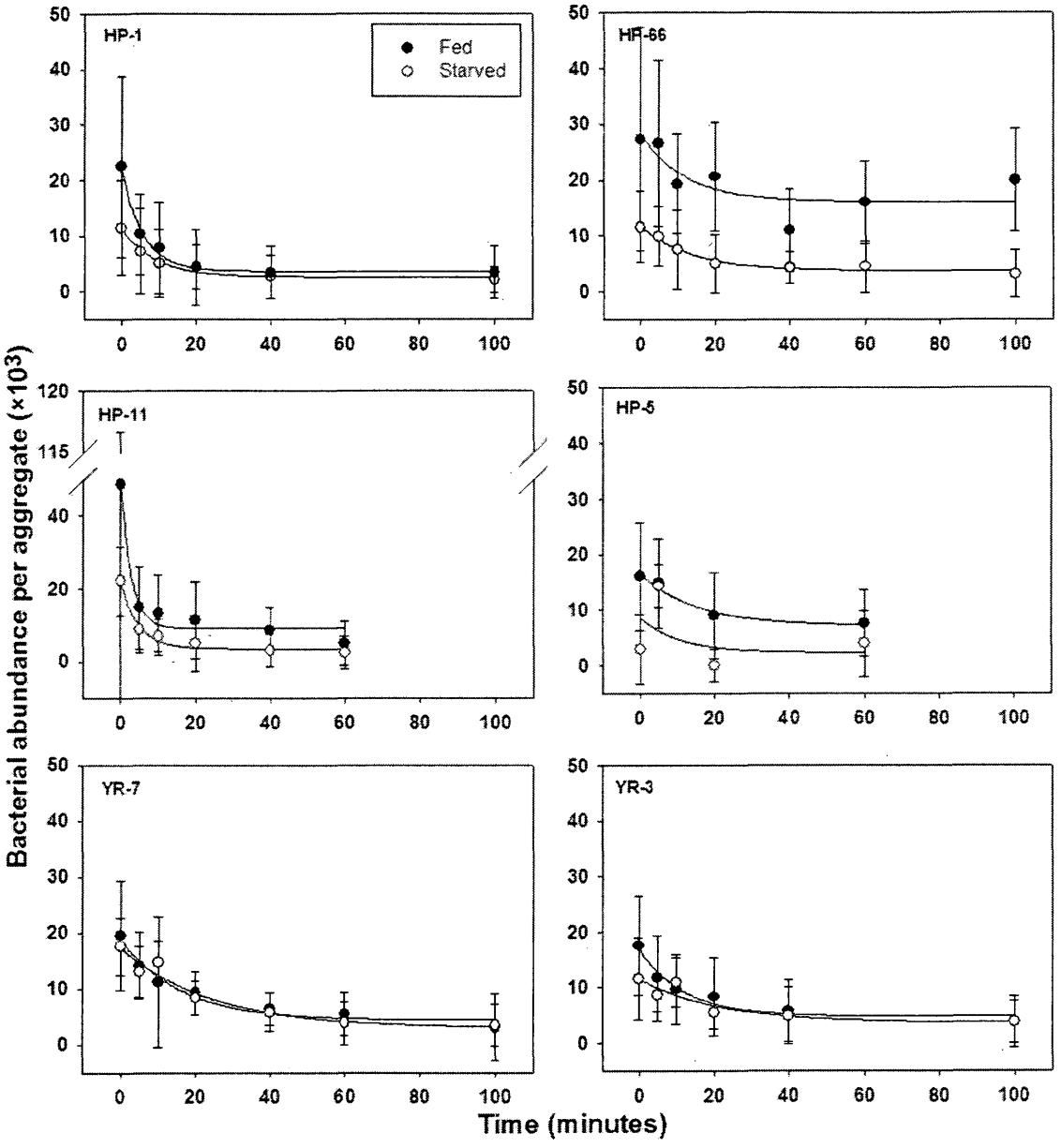

HP-1
Fed
Starved
HP-66
HP-11
HP-5
YR-7
YR-3
Bacterial abundance per aggregate (×10³)
Time (minutes)

Table 2.2 Empirically derived detachment rate (δ_e) based on detachment experiments.

	δ_e ($\times 10^{-3}$ s^{-1})	
Strain	Starved (± SD)	Fed (± SD)
HP1	1.88 (0.4)	3.01 (0.4)
HP5	1.39 (0.5)	1.10 (0.6)
HP11	3.55 (0.7)	5.81 (1.8)
HP66	1.23 (0.2)	1.38 (1.2)
YR3	0.79 (0.6)	1.38 (0.4)
YR7	0.69 (0.2)	1.05 (0.2)
Mean (SD)	1.59 (1.05)	2.29 (1.87)

Motility and Image Analyses

Motility data were collected for HP1, HP11, and HP66. Average diffusivities (D_e) calculated from motility data ranged from 0.20×10^{-5} to 4.23×10^{-5} cm^2s^{-1} (Table 3). Average D_e of fed bacteria was 6.9-7.7 times higher than that of starved bacteria for HP1 (t-test; $p < 0.0001$) and HP11 (t-test; $p = 0.008$). D_e of starved and fed HP66 were not significantly different (Mann-Whitney, $p = 0.61$). Swimming speeds varied by 2- to 3-

fold even within treatments (Table 2.3). The frequency distributions of swimming speeds between starved and fed treatments overlapped to different extents among the tested strains (Figure 2.3). About 70% of starved HP1 had average speeds between 10 and 20 $\mu m\ s^{-1}$, where nearly 88% of fed HP1 had average speeds between 30 – 60 $\mu m\ s^{-1}$. The average speed for starved HP1 (18.4 $\mu m\ s^{-1}$) was significantly lower than fed HP1 (37.8 $\mu m\ s^{-1}$; Mann-Whitney, $p < 0.0001$). For HP11, 86% starved and 22% fed bacteria had average speed between 20 and 40 $\mu m\ s^{-1}$. The average speed for starved HP11 (22.6 $\mu m\ s^{-1}$) was significantly lower than fed HP11 (45.6 $\mu m\ s^{-1}$; t-test, $p < 0.0001$). Turn angles were not statistically different between treatments for either strain (Mann-Whitney, $p = 0.21$ and 0.78 for HP1 and HP11, respectively).

Approximately 98% of the starved HP66 and 91% of the fed HP66 swam at speeds from 20 to 75 $\mu m\ s^{-1}$. A small percentage of fed HP66 had speeds reaching 88.6 $\mu m\ s^{-1}$. Despite the overlap, average speeds for starved HP66 were lower than fed HP66 (t-test, $p < 0.007$). The average turn angle was significantly higher for starved versus fed treatments (Mann-Whitney, $p = 0.007$) such that HP66 had an average α of 0.82 in the starved treatment and 0.73 in the fed treatment. Overall diffusivities for starved and fed HP66 bacteria were not statistically different (Mann-Whitney, $p = 0.62$).

The empirically derived D_e and δ_e were used to calculate the coefficient R_e, The calculated R_e was higher for the fed treatments in HP1 and HP11, but not HP66. R_e values for starved and fed HP1 were 1×10^{-3} and 5.1×10^{-3}, respectively. HP11 had an R_e of 1.7×10^{-3} in the starved treatment and a higher R_e in the fed treatment (7.3×10^{-3}). R_e for HP66 showed the reverse trend, where starved bacteria had a higher R_e (31.3×10^{-3}) compared to the fed bacteria (17.4×10^{-3}).

Figure 2.3 Frequency distributions of swimming speeds for fed (filled bars) and starved (unshaded bars) bacteria. Frequencies are expressed as percentages of the observed populations.

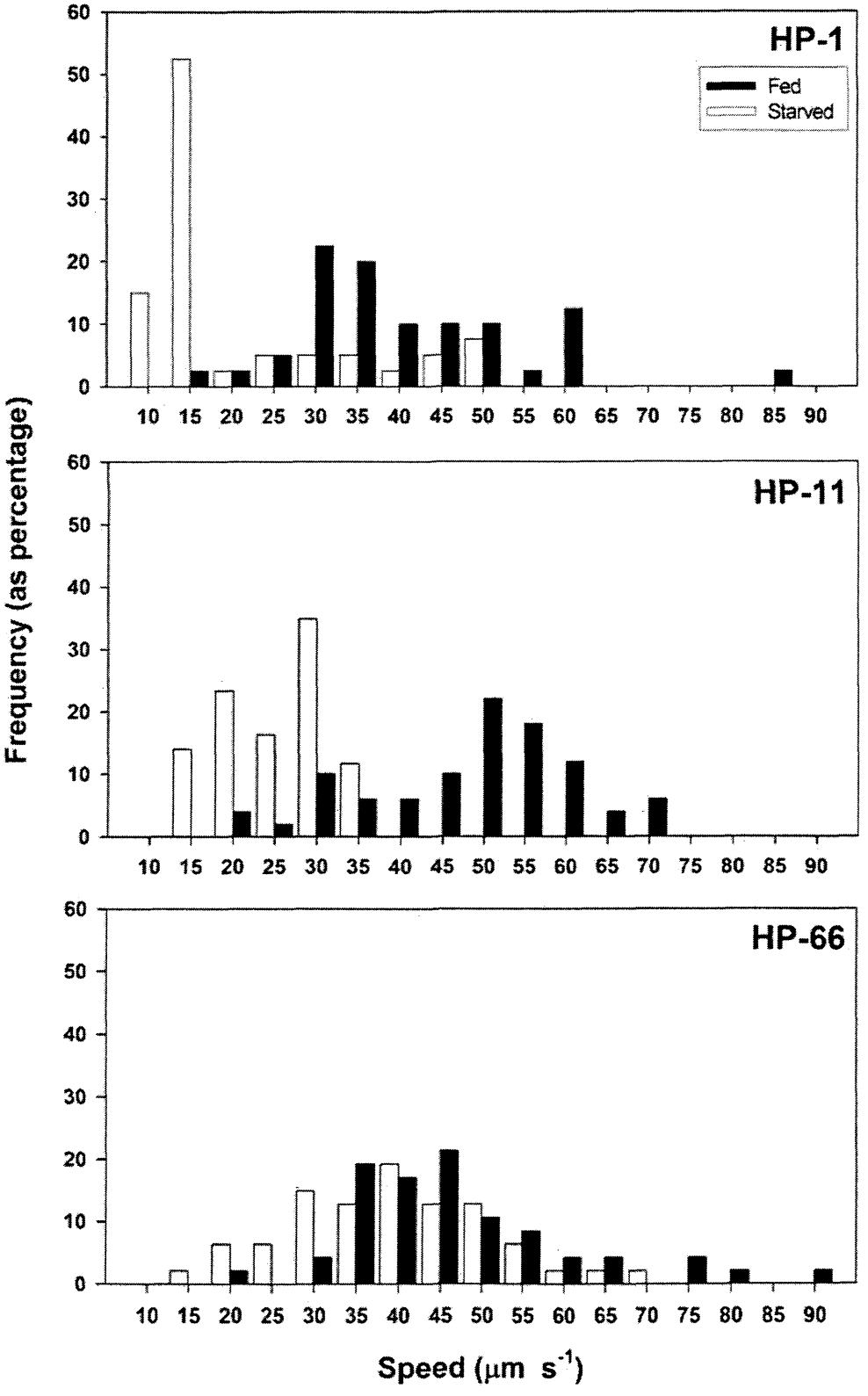

HP-1
Fed
Starved
HP-11
HP-66
Frequency (as percentage)
Speed (μm s-1)
0
10
20
30
40
50
60
10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90

Table 2.3 Motility parameters for starved and fed bacteria based on image analysis of swimming tracks. Numbers are average values for 40 to 50 tracks (*n*) per treatment. Diffusivities (D_e) are calculated using Equation 2. α is the cosine of turn angles of the swimming tracks.

Strain	Treatment	D_e ($\times 10^{-5}$ $cm^2 s^{-1}$)	Swimming speed ($\mu m\ s^{-1}$)				α	*n*
			Mean (± SD)	Median	Minimum	Maximum		
HP1	Starved	0.20	18.37 (12.3)	11.50	8.02	48.00	0.79	40
HP1	Fed	1.54	37.83 (13.6)	34.53	14.81	83.16	0.67	40
HP11	Starved	0.61	22.56 (5.9)	22.58	12.24	31.93	0.84	43
HP11	Fed	4.23	45.55 (12.6)	46.88	15.93	68.35	0.83	50
HP66	Starved	3.85	37.49 (12.2)	37.42	9.27	66.14	0.82	47
HP66	Fed	2.35	44.52 (13.8)	42.31	19.69	88.55	0.73	47

Table 2.4 Colonization coefficient (R_e) based on empirically derived diffusivity (D_e) and detachment rate (δ_e).

	R_e	
Strain	Starved	Fed
HP1	0.0010	0.0051
HP11	0.0017	0.0073
HP66	0.0313	0.0174
Mean (± SD)	0.0113 (± 0.0173)	0.0099 (± 0.0066)

DISCUSSION

Previous experimental and modeling studies of aggregate-bacteria interactions have not adequately considered the effects of starvation on bacteria, and are largely biased toward actively growing bacteria in non-limiting nutrient environments. However, studies using metabolic dye or micro-autoradiography typically reveal a high percentage of inactive bacteria across different aquatic systems (Smith & del Giorgio 2003), and a recent study showed that marine snow bacteria can up- and down-regulate their enzyme activity while on and off aggregate surface in as little as 2 h (Grossart et al. 2007). Other studies have also shown clear effects of starvation on bacterial physiology and behavior (Kjelleberg et al. 1984, Wrangstadh et al. 1986, 1990), and that these effects could occur within the time required for a bacterium to encounter an aggregate in the upper ocean

(Malmcrona-Friberg et al. 1990, Wrangstadh et al. 1990, Wei & Bauer, 1998). Much of the world's surface ocean is characterized by low nutrients and primary production, and aggregate distribution is highly patchy such that starvation (short to long term) is an important consideration for understanding bacteria-aggregate interactions.

In this study, starvation effects were evident within 24 h, resulting in 40 to 70% reduction in R_m such that starved bacteria colonized the model aggregates at a much lower rate, and accumulated to significantly lower steady-state abundance on the model aggregates. Differences in the colonization behaviors between fed and starved bacteria were corroborated by independently observed bacterial motility, in which starvation resulted in nearly 50% reduction in average swimming speeds for two of the three strains. Empirically derived diffusivities (D_e) and detachment rates (δ_e) were similar to those reported in other studies (Kiørboe et al. 2002, Kiørboe et al. 2003, Grossart et al. 2006b). Starvation had no effect on detachment rates. This indicates that, although starvation may affect motility, it may not change cell surface structure and bacterial attachment to particles. Another possible explanation is that the starved bacteria could have been able to derive some organics from the agar bead after colonization and could have returned to a similar physiological state as fed bacteria.

Despite the limited effect of starvation on detachment, detachment is still an important factor in understanding microbial dynamics on marine snow. Although aggregates represent a source of nutrients for marine snow bacteria, they are also risky environments where bacteria could be exposed to high grazing pressure (Caron 1987, Kiørboe et al. 2003). In a recent mesocosm study, Tang et al. (2006) showed that strong grazing pressure from flagellated protozoa could limit the residence time of bacteria on

aggregate surfaces, calculated as the steady-state abundance of bacteria divided by the colonization rate, to 21 min or less. The duration of attachment to aggregates may be a trade-off between the risk of predation and the need for nutrients.

The motility response of HP1, HP11 and HP66 to starvation varied even within the populations. Heterogeneous physiological responses to starvation have been observed by others. For example, Wei & Bauer (1998) reported that after 8 h of starvation, while the majority of the bacterium *Rhizobium meliloti* had shortened or lost flagella, some maintained at least two full-length flagella. Similarly, Stretton et al. (1997) showed that after 24 h of starvation, the cell morphology of *Vibrio* sp. S14 varied from coccoid to rod shaped cells with or without flagella. Heterogeneity in flagellation and cell morphology may also account for the variations in motility in our experiments.

Despite the variation, starved HP1 and HP11 typically moved slower, which would lead to lower encounter rate with aggregates, consistent with the observed lower colonization rates in our experiments. Analyses of the swimming tracks also showed that differences in diffusivities between fed and starved treatments were mainly driven by differences in swimming speeds and not turn angles.

Not all of the strains were affected by starvation in the same way. For HP66, colonization data show that fed and starved treatments were not significantly different for the first several minutes, consistent with the observations that fed and starved HP66 had similar swimming speeds and turn angles. Nevertheless, fed HP66 did accumulate to a significantly higher abundance than starved HP66 on the model aggregates. This observation could not be explained by a difference in their detachment rate (δ_e), but rather may be a result of their different chemotactic response to the model aggregates or

ability to attach upon encounter. These factors could not be adequately accounted for by the colonization model or motility observations.

In this study we define a coefficient R (= D/δ) to characterize the overall colonization process. Comparisons between starved and fed treatments were similar for both R_m (model) and R_e (empirical) such that the R values were consistently higher for fed bacteria (except for HP66). However, the calculated R_e tend to be smaller than R_m within treatments. A number of factors may contribute to a discrepancy between R_e and R_m: (1) Empirically derived diffusivities (D_e) were based on observed motility of the bacteria in suspension, but not all motile bacteria that encounter an aggregate will necessarily attach, in which case D_e would overestimate the true diffusivity. (2) On the other hand, D_e assumes that the presence of aggregates would not affect the motility pattern of the bacteria. Many bacteria, however, exhibit chemotactic response to aggregates (Kiørboe et al. 2002, Grossart et al. 2007). Although we used plain agar beads to minimize this effect, it is still possible that the bacteria were chemically attracted to the agar beads, resulting in a higher diffusivity than D_e. (3) Lastly, attached marine snow bacteria may produce signaling molecules (Gram et al. 2002) that facilitate or inhibit the subsequent arrival of other bacteria (Grossart et al. 2003b). These cell-cell interactions could not be revealed by motility observations.

Microbial dynamics on marine snow are complex and involve processes leading to encounter and the development of microbial populations on the aggregates, and fate of microbes either through death, grazing, or detachment. Marine snow bacteria play a critical role in solubilizing marine aggregates and mediating organic matter fluxes between particulate and dissolved phases (Smith et al. 1992). Understanding how

bacteria influence microscale processes in biogeochemical cycling requires determining not only the mechanisms by which bacteria colonize aggregates, but also potential shifts in behavior and responses to starvation, which is a likely condition given rapidly changing nutrient conditions and the patchy distribution of aggregates in the open ocean. Changes in physiology due to starvation should be incorporated into models examining microscale processes occurring on marine snow aggregates, as these processes have important implications for the fluxes of organic matter on a global scale.

CHAPTER 3

Production of transparent exopolymer particles by the diatom *Thalassiosira weissflogii* under the influence of two different bacterial strains

ABSTRACT

Transparent exopolymer particles (TEP), produced by phytoplankton and bacteria, play an important role in aggregate formation. We performed laboratory experiments in which one of two strains of marine snow bacteria (either *Microscilla furvescens* or *Curacaobacter baltica*) were added to jars containing an axenic culture of the marine diatom *Thalassiosira weissflogii*. During the time series, we observed changes in total- and TEP-associated- bacterial abundance and characteristics of TEP produced in the system. The two systems showed different responses. The jars inoculated with *Microscilla furvescens* had no increases in overall TEP abundance and no change in the area of TEP. *M. furvescens* had higher densities on TEP, although a lower fraction of the total bacteria was TEP associated. On the other hand, jars inoculated with *Curacaobacter baltica* had a significantly higher fraction of total bacteria that were TEP associated, although the bacteria colonized TEP at lower densities. We also observed increases in median TEP area, and total TEP increased (mean ± 1 SD) from 1.6 (± 0.2)% of the total filter area on day 0 to 4.4 (± 0.7)% on day 7. Total TEP area in jars inoculated with *M. furvescens* remained at 1.2 (± 0.3)% of the filter area throughout the time course. The amount and character of the TEP produced as well as the distribution of bacteria have broader implications in microbial food web dynamics and the sedimentation of phytoplankton blooms.

INTRODUCTION

A key mechanism for carbon sequestration in the ocean is aggregation and sinking of phytoplankton. The termination of diatom blooms is marked with increased flocculation, which may be aided by the production of phytoplankton exudates (Passow et al. 1994). One type of exudate is an exopolymeric secretion called transparent exopolymer particles (TEP), which are operationally defined as discrete organic particles made of acidic polysaccharides that can be visualized using alcian blue (Alldredge et al. 1993). TEP act as 'biological glues' in aggregate formation (Alldredge et al. 1993; Logan et al. 1995). As such, TEP are important components of marine snow (Alldredge et al. 1993), thereby influencing sedimentation processes (Wells and Goldberg, 1993) by increasing aggregation. Engel et al. (2004) reported that TEP dominated polysaccharide-aggregation dynamics in a bloom of *Emiliana huxleyi*; TEP concentrations increased overall particle concentration, which lead to increased sedimentation.

Although phytoplankton are typically considered the major producer of TEP, both laboratory and field studies have shown that bacteria also produce TEP (Passow, 2002a). Stoderegger and Herndl (1999) estimated that, in the North Sea, 4-5% of total abundances (by mass) of exopolymeric particles were derived from bacteria. The production of high molecular weight polysaccharides derived from capsular coatings shed by bacteria could be an important component in the cycling of dissolved organic carbon (DOC) due to the sheer abundance of bacteria in the ocean (Stoderegger and Herndl, 1999).

The interactions between bacteria and phytoplankton can ultimately influence aggregation dynamics and sedimentation of particles (Grossart et al. 2006a). Grossart et al. (2006a) showed continuous TEP formation and enhanced aggregation of the diatom *Thalassiosira rotula* in the presence of bacteria. Guerrini et al. (1998) reported that, in a phosphorous-limited environment, bacteria could stimulate more polysaccharide production in the diatom *Cylindrotheca fusiformus*, since bacteria are more efficient at assimilating any available phosphorous. Passow et al. (1994) suggest that bacteria colonizing TEP could supply nutrients for phytoplankton growth. Additionally bacteria could contribute polysaccharides to the overall TEP pool (Grossart et al. 2006a).

TEP also provide a substrate for bacteria attachment and are utilized by bacteria (Passow, 2002b), although the importance of TEP mineralization by bacteria is still controversial (Mari and Kiørboe, 1996). Passow and Alldredge (1994) found that bacterial abundance on TEP varied inversely with TEP size, such that small TEP particles (<10 μm) had more attached bacteria per unit area than did larger particles (>100 μm). On the other hand, Mari and Kiørboe (1996) found that bacterial abundance on TEP did not correlate with surface area or volume, but scaled with the radius of the particles raised to a power of 1.5, indicating that TEP must be fractal.

Bacterial colonization and subsequent usage of TEP likely depends on the composition of TEP, which can be highly variable based on its origin. The partitioning of carbon that is assimilated into phytoplankton or released as carbohydrates can depend on the nutrient status of the medium in which the phytoplankton grows (Myklestad and Haug, 1972). An additional source of variability in the composition of TEP comes from its ability to scavenge nitrogen-rich particles from the water column, including low

molecular weight amino acids (Schuster et al. 1995; Decho, 1990). Although bacteria use TEP as an organic substrate, TEP-associated bacteria can be grazed upon by protozoans (Tranvik et al., 1993) and other organisms (Passow and Alldredge, 1999; Ling and Alldredge, 2003).

The goal of this study was to compare the effects of two strains of bacteria on the production of TEP by the marine diatom *Thalassiosira weissflogii*. To accomplish this, we measured the length, area, and amount of TEP, as well as bacterial distributions and densities associated with TEP during a 7-day incubation period. We discuss the species-specific interactions between bacteria and this diatom leading to differences in TEP abundance, size, and bacterial distributions, and the implications of these differences for bacterial population dynamics, grazing, and aggregate formation.

MATERIALS AND METHODS

Experimental Set Up

One-liter axenic cultures of *Thalassosira weissflogii* (TW) cultures were grown in f/2 medium supplied with silica. Cultures were maintained at 20° C in continuous light for 25 days on an orbital shaker and grown to a concentration of ~2 x 10^5 cells ml^{-1}. TW cells were resuspended into 10 acid-washed, sterile 940 ml glass jars filled with 0.2 μm-filtered, sterile Instant Ocean ™ artificial seawater at a concentration of 3 x 10^4 cells ml^{-1}. Each jar was inoculated with a monoculture of bacteria. Two time-series experiments were run a week apart. Seed cultures originated from the same culture, and were staggered a week apart when aging. Therefore, the phytoplankton used in each experiment was grown under identical conditions for the same duration.

Cultures of *Curacaobacter baltica* (HP1) and *Microscilla furvescens* (HP11), isolated from aggregates in the Wadden Sea (Germany) (Grossart et al., 2004) were used in this study. Isolates were grown in marine broth made from artificial seawater. Cultured bacteria were inoculated into each of the jars of phytoplankton at a concentration of 1 x 10^4 cells ml^{-1}. TW jars inoculated with HP1 will be referred to as Experiment A; jars inoculated with HP11 will be referred to as Experiment B.

Sampling Regime

Jars were sampled for phytoplankton counts, total bacteria counts, TEP-associated bacteria counts, TEP abundance and TEP size distribution on days 0, 4, and 7. Once jars were inoculated with phytoplankton and bacteria, three jars were randomly selected for

sampling on day 0. The remaining jars were placed on a rolling table in continuous light, rolling at a speed of 2.2 rpm, and sampled as described below. *Thalassiosira weissflogii* is considered a low-flocculent species on its own (Kiørboe and Hansen, 1993; Logan et al. 1994b), although it has been used to generate aggregates in the laboratory (Grossart et al., 2006b). During this incubation, no phytoplankton aggregates formed.

Sterile double-plungered syringes were used to gently collect TEP samples. Samples were transferred to a filtration tower using a wide-bore pipet. Two-milliliter samples were filtered at low pressure (< 50 mm Hg) onto 0.2-μm pore size black polycarbonate filters until nearly dry and stained with 100 μl of freshly prepared, 0.2 μm-filtered 0.03% alcian blue stain for 10 seconds. Samples were rinsed with sterilized milli-Q water to prevent precipitation of salts, and no support filter was used. The filtered samples were then transferred to prefrosted Cytoclear slides and embedded with immersion oil. TEP were counted and measured from 20 randomly selected grids at a magnification of 200X using image analysis software (Image Pro v. 5.0) to obtain TEP size distributions. A total of 150-450 TEP per slide were measured. The vast majority of TEP observed were small, ovate particles. TEP were grouped into geometric size classes based on maximal length. The maximal and minimum length axes were used to calculate the elliptical areas of the TEP. The size spectrum from 20 grids was also used to calculate the total amount of TEP on each slide.

Two additional one-milliliter samples were filtered onto 0.2-μm pore size black polycarbonate filters, double stained with 4'-6-Diamidino-2-phenylindole (DAPI), followed by Alcian Blue. Filters were rinsed with sterilized milli-Q water when stained with Alcian Blue and transferred onto Cytoclear slides (Logan et al., 1994a). Double-

stained slides were used to enumerate bacteria associated with TEP. First, TEP were observed with light microscopy and were manually, digitally outlined using Image Pro software. The resulting digital outline was then superimposed onto the same field of view under UV illumination and the bacteria were counted. Bacteria falling within or on the boundary of the digital outline were counted as "TEP-associated bacteria". About 10 TEP per slide were analyzed this way. The average number of bacteria per unit area of TEP was multiplied by the total cumulative area of TEP per unit volume to calculate the number of TEP-associated bacteria per unit volume.

One milliliter aliquots were collected from each jar and diluted by 10-fold with sterile artificial seawater. One milliliter of the diluted aliquots were filtered onto 0.2-μm pore size black polycarbonate filters for DAPI direct count of total bacteria (Porter and Feig, 1980). Samples were kept in the freezer until they were counted. An additional aliquot from each jar was preserved using Lugol's solution, and settled in a Sedgwick-Rafter counter for phytoplankton counts. Ten counts were made for each jar.

Data Analysis

Two-way ANOVA was used to compare phytoplankton concentration, total area of TEP, total bacteria, percentage of the total bacteria associated with TEP, and densities of bacteria on TEP. When data did not have equal variances, data were rank-transformed (as indicated in the results). TEP length and area measurements were binned into frequency tables; these tables were analyzed in SAS using the Cochran-Mantel-Haenszel test. In this case, we used the test to assign row-mean scores to TEP lengths or area distributions to test changes through time, while controlling for strain.

RESULTS

TEP Length and Area

The concentration of *Thalassiosira weissflogii* was not significantly different on day 0 (Student's t-test, $p = 0.242$, $n = 60$). Experiment A had an average concentration (± 1 SD) of 3.12 (± 0.64) x 10^4 cells ml^{-1} and Experiment B had an average concentration of 2.52 (± 0.34) x 10^4 cells ml^{-1} on day 0. Between days 0 and 7, the concentration of TW increased significantly faster in Experiment A than in B (Two-way ANOVA, $p < 0.001$, $n = 180$) (Figure 3.1). By day 7, Experiment A had an average concentration (± 1 SD) of 4.77 (± 0.79) x 10^4 cells ml^{-1} where Experiment B had an average concentration of 3.54 (± 0.73) x 10^4 cells ml^{-1}. There was a significant interaction between treatment and time ($p < 0.001$).

A total of 3860 and 2088 TEP were measured in Experiments A and B, respectively. Size frequency distributions of the individual lengths and areas of TEP over time were analyzed using the Cochran-Mantel-Haenszel (CMH) Test. There was no significant change in TEP lengths between Experiments A and B ($p = 0.423$). Mean maximal lengths had a great deal of variability; the majority of particles were between 4 – 16 μm in both experiments, although there were a few particles whose lengths exceeded 64 μm.

Frequency distributions of TEP area increased significantly over time between experiments (CMH test, $p = 0.033$) (Figure 3.2). Although there was considerable

variability, Experiment A produced TEP with larger individual areas over time. Most particles had individual areas between the 8-16 μm^2 and 16-32 μm^2 size classes, although the distribution for day 0 in Experiment B did had particularly large TEP which inflated the mean area for that time point. Approximately 40 - 55% of the total number of particles sampled on a given day fell between the 8-16 μm^2 and 32 μm^2 size classes. While TEP in Experiment B maintained the same approximate average area and median area, particles in Experiment A were increasing in area, as seen in shifts in both the means and medians (Table 3.1).

Over the time course, Experiment A had more TEP, as measured by the area of coverage on a filter per volume filtered (Two-way ANOVA, $n = 16$, $p < 0.001$). There was a significant interaction between time and treatment (Two-way ANOVA, $n = 16$, $p = 0.003$). By the end of the time course, the total area covered by TEP was 3.5 times higher in Experiment A than in B (Figure 3.3). The total area of TEP ($\pm$ 1 SD) in Experiment A was 8.28 ($\pm$ 1.25) mm^2 TEP ml^{-1}, representing 4.39 ($\pm$ 0.66)% of the total filter area. Throughout the time course, Experiment B maintained a low total-TEP area of 2.31 ($\pm$ 0.63) mm^2 TEP ml^{-1}, equivalent to 1.22 ($\pm$ 0.33)% of the total filter area.

Table 3.1 Statistics summary for the area of individual TEP.

exp	strain	day	mean (μm^2)	SD	median (μm^2)	*n*
A	HP 1	0	53.56	103.11	23.72	1242
A	HP 1	4	84.23	228.51	20.70	1348
A	HP 1	7	145.63	415.40	30.30	1270
B	HP 11	0	92.41	380.98	16.38	536
B	HP 11	4	46.44	107.25	19.18	1151
B	HP 11	7	43.02	90.11	20.15	401

Figure 3.1 Average concentration of *Thalassiosira weissflogii*. Filled circles represent the average phytoplankton concentration in Experiment A (phytoplankton + HP 1 bacteria) and open circles show Experiment B (phytoplankton + HP11 bacteria) over the course of one week. Error bars indicate standard deviation of the mean of three replicates.

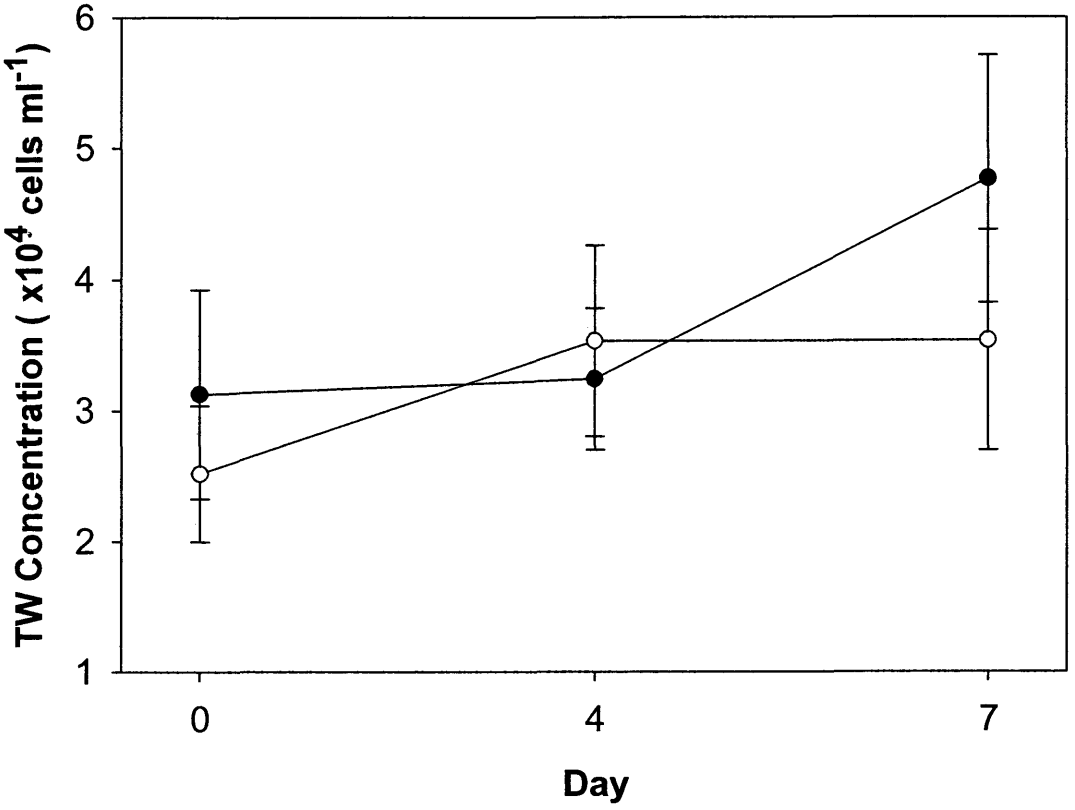

TW Concentration (x10^4 cells ml^-1)
6
5
4
3
2
1
0
4
7
Day

Figure 3.2 Frequency distribution, expressed as a percentage, of area of individual TEP in (A) Experiment A and (B) Experiment B. Unfilled bars represent day 0, hatched bars are day 4, and shaded bars are day 7. The total number of particles (n) measured on a given day are also provided. There was a significant change in the distribution over time (Cochran Mantel Haenszel Test, $p = 0.033$).

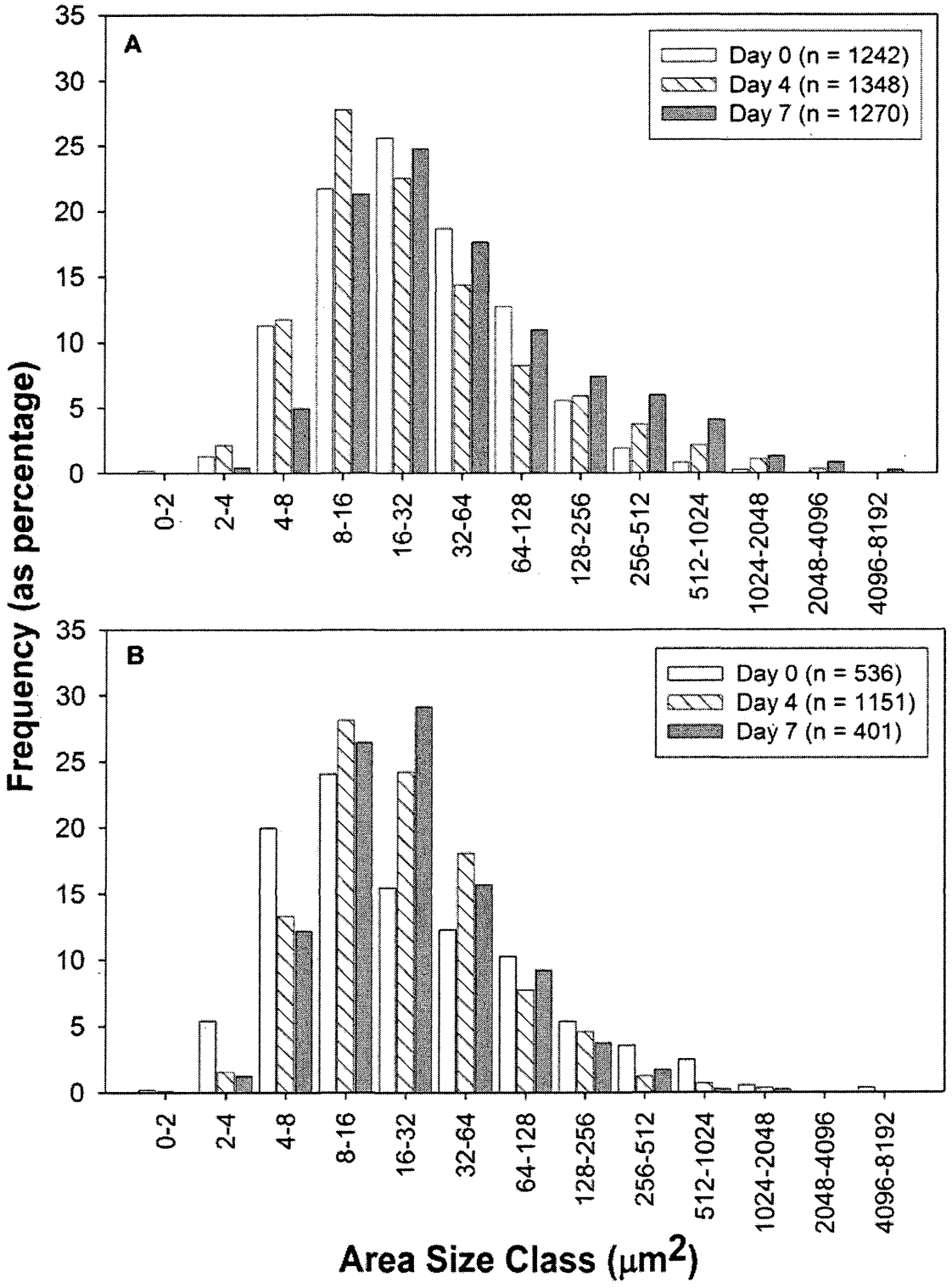
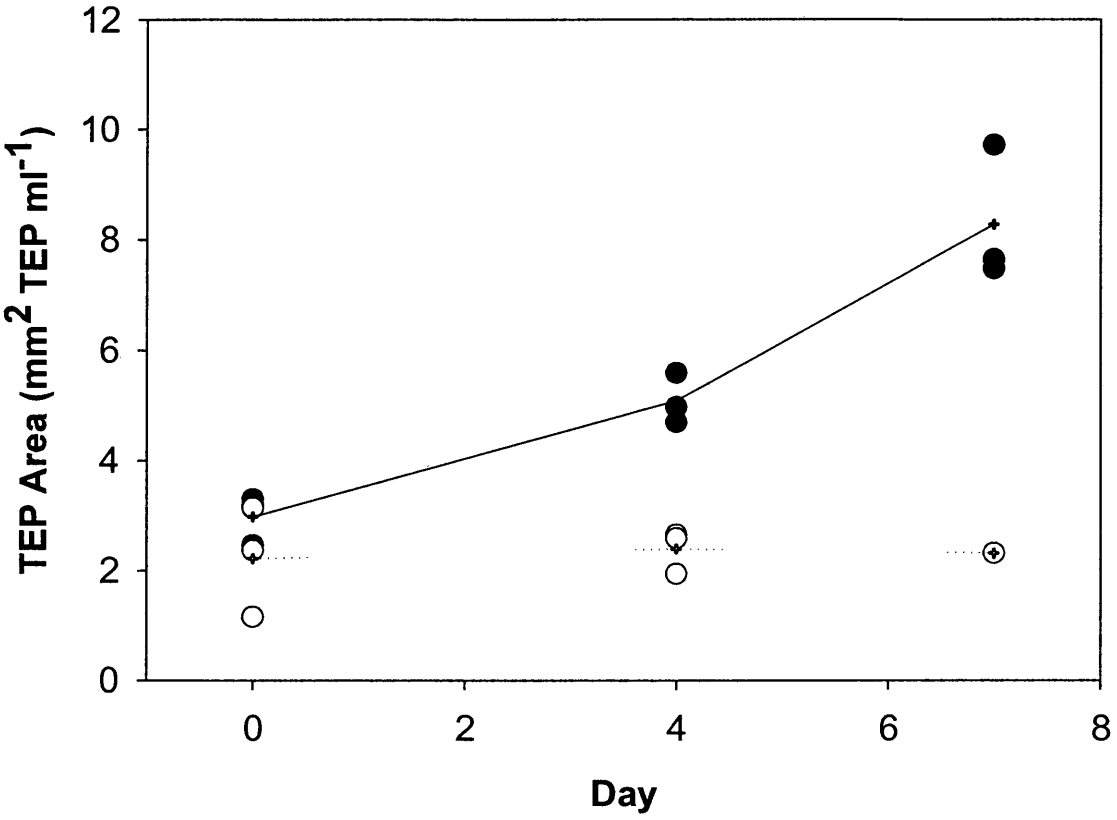

A
Day 0 (n = 1242)
Day 4 (n = 1348)
Day 7 (n = 1270)
B
Day 0 (n = 536)
Day 4 (n = 1151)
Day 7 (n = 401)
Frequency (as percentage)
0
5
10
15
20
25
30
35
0-2
2-4
4-8
8-16
16-32
32-64
64-128
128-256
256-512
512-1024
1024-2048
2048-4096
4096-8192
Area Size Class (μm2)

Figure 3.3 Total area of TEP per volume (mm^2 TEP ml^{-1}). Triplicate jars were sampled for TEP size; the circles indicate the cumulative area of TEP per milliliter for each jar. Filled circles are for Experiment A, open circles are for Experiment B. The small crosses indicate the average calculated from the pooled raw data from all three replicates for each experiment sampled each day.


12
10
8
6
4
2
0
TEP Area (mm2 TEP ml-1)
0
2
4
6
8
Day

Bacteria Colonizing TEP

Total bacterial abundances increased in both experiments during the time course. Experiment B, inoculated with HP11 bacteria, had more variance in bacterial counts, but still had significantly higher overall abundances than Experiment A (rank transformed Two-way ANOVA, $n = 180$) ($p < 0.001$). Mean concentrations in Experiment B started at 3.01×10^5 cells ml^{-1} on day 0, and rose to 3.37×10^6 cells ml^{-1} by day 7. In Experiment A, mean bacterial concentrations increased from 2.88×10^5 cells ml^{-1} on day 0 to 1.94×10^6 cells ml^{-1} on day 7 (Figure 3.4).

The percentage of total bacteria that was associated with TEP was significantly different over time and between strains (two-way ANOVA, $n = 18$, $p < 0.03$). In Experiment B, the percentage of bacteria associated with TEP was less than 4% of total bacteria. Experiment A showed increases in the fraction of bacteria associated with TEP; on day 0, 2% of HP1 were associated with TEP, increasing to about 8% on days 4 and 7 (Figure 3.5). Post-hoc analyses show that the percentages of bacteria associated with TEP were not significantly different on day 0 and 4 ($p > 0.24$) but were different on day 7 ($p = 0.05$).

Bacterial densities associated with TEP varied a great deal, as some particles were not colonized by any bacteria, while others had more than 1×10^5 cells per mm^2 TEP (Figure 3.6). The average density (± SD) of HP11 bacteria in Experiment B was 4.5 (± 4.0) $\times 10^4$ cells per mm^2 TEP. Experiment A had an average density of 2.34 (± 1.8) $\times 10^4$ cells per mm^2 TEP. Rank-transformed two-way ANOVA ($n = 182$) showed that Experiment B had higher densities of bacteria per area TEP than Experiment A (p =

0.001). Post-hoc pair-wise analyses on ranks show that densities were the same between treatments on day 0 ($p = 0.386$), but different on days 4 and 7 ($p < 0.013$).

Figure 3.4 Box plots of total bacteria sampled each day collected from pooled, triplicate jars, each with ten counts for Experiment A (grey boxes) and Experiment B (unshaded boxes). Means are plotted as filled circles in each box. Median values are represented by lines in each box. The lower and upper boundaries represent the 25^{th} and 75^{th} percentiles. Whiskers represent 10^{th} and 90^{th} percentiles. Experiment B (HP11) had significantly higher bacterial concentrations on day 4 and day 7 (rank transform ANOVA, $p < 0.001$).

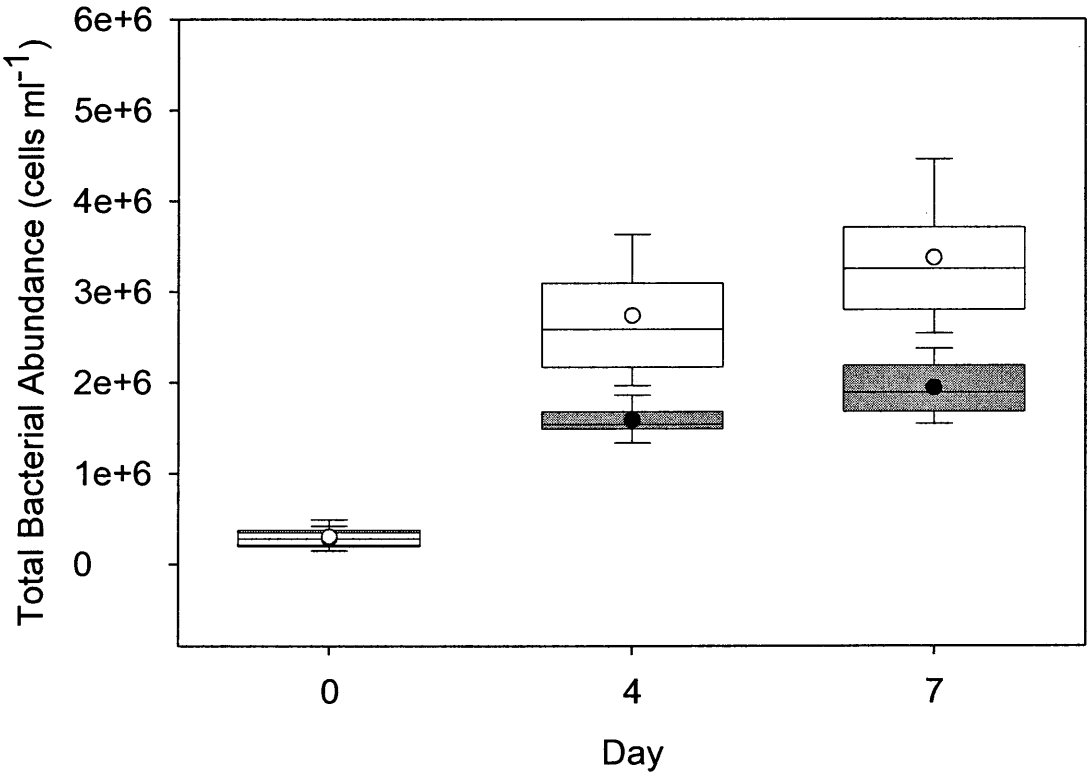

Total Bacterial Abundance (cells ml-1)
6e+6
5e+6
4e+6
3e+6
2e+6
1e+6
0
0
4
7
Day

Figure 3.5 Percentages of total bacteria that were TEP-associated in Experiment A (grey boxes) and B (unshaded boxes). Averages are represented by filled circles in Experiment A and open circles in Experiment B. Medians are plotted as lines in boxes. TEP-associated bacteria were significantly higher in Experiment A between day 4 and 7 (two-way ANOVA, $p < 0.03$).

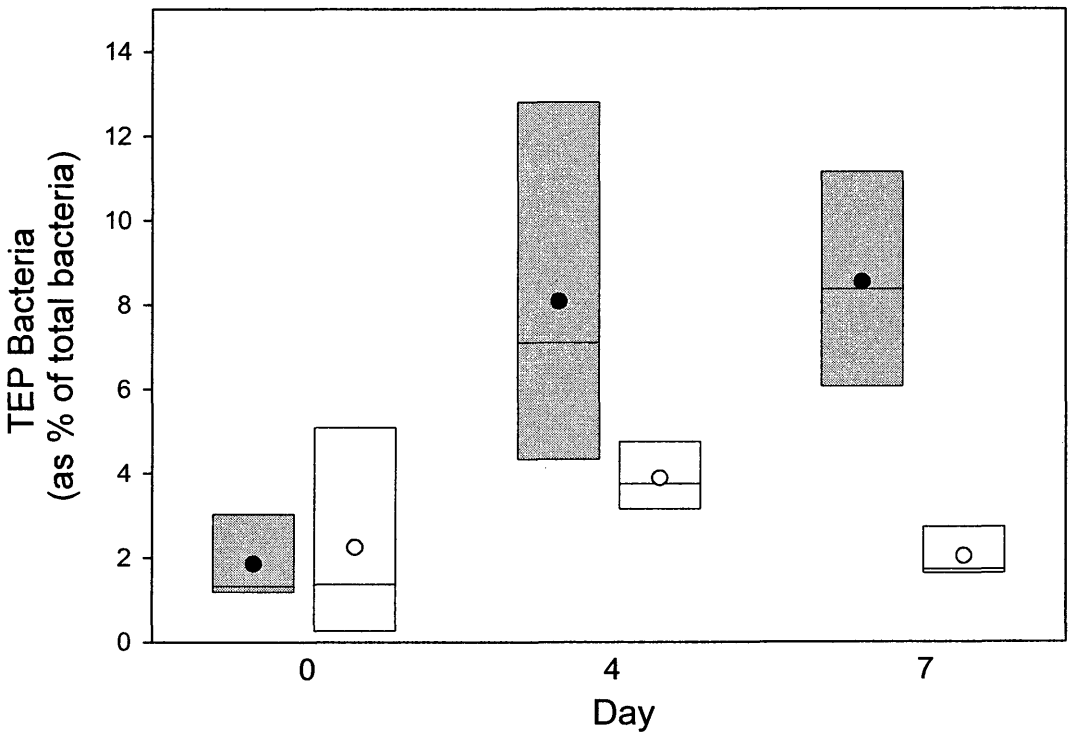
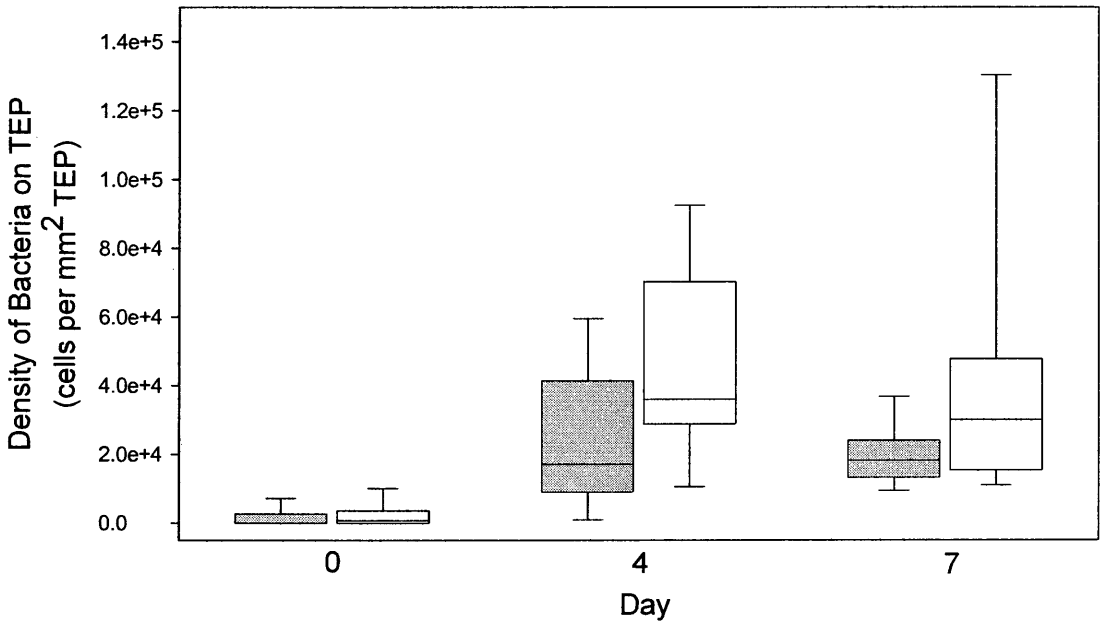

TEP Bacteria
(as % of total bacteria)
14
12
10
8
6
4
2
0
0
4
7
Day

Figure 3.6 Densities of bacteria associated with TEP. Box plots show the densities of bacteria associated with TEP in Experiment A (grey boxes) and Experiment B (unshaded boxes). Whiskers represent the 10^{th} and 90^{th} percentiles.


Density of Bacteria on TEP
(cells per mm2 TEP)
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
0
4
7
Day

DISCUSSION

TEP Production and Characteristics

TEP have been recognized as an important factor in aggregation dynamics (Hill, 1992; Logan et al. 1995) because they increase the number and size of particles, while also contributing to particle stickiness (Jackson, 1995). Jackson (1995) also discussed the need for aggregation models to include different phytoplankton morphologies, TEP-particle interactions, and the dispersion of TEP particles. Current theories of TEP dynamics, however, have not sufficiently considered the species-specific interactions between bacteria and phytoplankton and the resultant effects on TEP production and characteristics.

In this study, the addition of two different strains of bacteria to *T.weissflogii* cultures resulted in different TEP production and characteristics over the time course. In Experiment B, length, area, and total area remained relatively unchanged during the incubation. Bacterial abundances and TEP characteristics in Experiment A, on the other hand, seem to suggest that bacteria-phytoplankton interactions contribute more strongly to TEP formation. Total TEP area normalized to phytoplankton abundance was more than 2.3 – 2.6 times higher in Experiment A than in B on days 4 and 7. Such a difference suggests that the bacteria in Experiment A must be contributing TEP to the system, or the diatom was producing more TEP under the influence of HP1. Other studies have shown that some bacteria are capable of contributing to the TEP pool (Decho, 1990; Stoderegger

and Herndl, 1998, 1999; Passow, 2002b). Laboratory experiments using natural populations of marine bacteria showed that treatments without antibiotics produced significantly higher quantities of TEP when compared to bacteria treated with antibiotics (Passow, 2002b). Stoderegger and Herndl (1998) used radio-labeled DOM to demonstrate that capsular material released into the water by bacteria was equivalent to ~25% of carbon respired. Stoderegger and Herndl estimate that 17 – 33% of bacterial capsular material coagulates to form exopolymers which can also coagulate quickly, especially under turbulent conditions (Stoderegger and Herndl, 1999).

Although individual TEP length did not change over the time course, individual area and total area of TEP was significantly higher in Experiment A than in B. Our results suggest that the amount of TEP and TEP characteristics produced by diatoms vary under the influence of different bacterial strains. Potential mechanisms for increased TEP production could include bacterial stimulation of exudates production in phytoplankton, or secretion of TEP by bacteria.

The different TEP abundances and dimensions resulting from interactions between bacteria and phytoplankton could indicate different strategies by the bacteria to reduce predation by specific types of grazers. TEP concentration was experimentally shown to have effects on the structure of protozoan community structure and, therefore, control on the microbial food web (Mari et al., 2004b). Mari and Rassoulzadegan (2004a) demonstrated that increased TEP production changed the prey size spectrum for the ciliate *Strombidium sulcatum*, leading to a decline in population because particles became too large for ingestion. In Experiment A, HP1 bacteria could interact with phytoplankton to enhance TEP production as a strategy for protection from grazers which

specialize in selecting small particles and free-living bacteria. HP11 bacteria and phytoplankton maintain low, steady levels of TEP, lowering the potential for aggregation, and therefore, lowering the chance of being preyed upon by aggregate- or substrate-associated ciliates and flagellates.

Alternatively, differences in TEP characteristics could result from specific bacteria-phytoplankton interactions causing the release of DOM and TEP precursors. Bidle and Azam (1999) reported that the exoenzyme activities of bacteria colonizing diatoms resulted in the dissolution of diatom frustules. Some bacterial extracellular products isolated and identified in the laboratory have been shown to cause the lysis of algal cells (Lee et al. 2000). Although we did not test for these products, it is possible that specific interactions between bacteria and phytoplankton could also be contributing to the pool of TEP precursors.

Bacterial Distributions

Our experiments showed that interactions between HP1 and HP11 with TW resulted in different bacterial densities on the surface of TEP. Dense populations of HP11 could change the distribution of hydrolytic enzyme activity on the particle. If bacteria colonizing TEP also utilize it as organic substrate, it would be an advantageous strategy for the bacteria to colonize TEP at some critical threshold density to maximize enzymatic hydrolysis without breaking down too much of the matrix, destroying the substrate, and diluting the utilizable organic matter from TEP. This enzyme activity influences the flux of materials to the dissolved fraction, which can be substrates for both bacteria and phytoplankton. Although this was not measured in these experiments, an increase in the concentration of exoenzymes could have resulted in the solubilization of

more TEP in Experiment B. This may explain the lower total area of TEP measured in Experiment B, although many other factors could also be involved.

At the population level, the distributions of the two strains of bacteria are different. A significantly higher overall percentage of HP1 was associated with TEP. This suggests that HP1 may be better able to exploit nutrient resources associated with TEP, while at the same time being more vulnerable to grazer mortality associated with TEP. These different distribution patterns could reflect different strategies between the two bacterial populations for exploiting TEP resources while minimizing TEP-related grazing mortality.

The density of both strains of bacteria and the fraction of total bacteria associated with TEP remained relatively unchanged between day 4 and 7. This suggests that the majority of the colonization occurred between days 0- 4, after which the population reached a steady state. Passow (2002b) reports unpublished data from experiments (conducted by Passow and Azam), which indicated that newly-formed laboratory-generated TEP were colonized quickly (<16 h) and bacterial density remained constant thereafter. TEP may provide bacteria with an energy source although the degree of utilization and the amount of degradation varies (Mari and Kiørboe, 1996; Passow et al., 2001; Passow, 2002b).

Initial analyses on densities of bacteria associated with TEP on day 4 and day 7 suggest that HP11 colonized at higher densities than HP1. According to the model published by Kiørboe et al. (2002), colonization of bacteria on aggregates is determined in part by encounter rate, ambient bacterial concentration, and aggregate size. The model can also be generally applied to TEP as a substrate for colonization, in lieu of aggregates.

Results from Chapter 2 indicate that the net colonization (i.e., colonization that also accounts for detachment) on particles and motility behaviors between HP1 and HP11 are similar; therefore it is unlikely that differences in density can be attributed to motility and resultant encounter rates. If the size of the substrate was primarily controlling the differences in density, Experiment A would have had higher densities because the observed TEP area was greater. However, this was not the case.

The average densities of TEP-associated bacteria on day 4 and 7 were normalized to ambient bacterial concentration on those days. The normalized abundances were virtually identical for Experiments A and B (0.013 ml mm^2 TEP^{-1} in Experiment A, and 0.015 ml mm^2 TEP^{-1} in Experiment B). Therefore, differences in densities could be explained by the higher ambient concentration of bacteria in Experiment B.

The Kiørboe et al. (2002) model describes initial colonization, which occurs on a time scale of minutes (< 150 minutes). The density of bacteria on aggregates in the 2002 study reached steady-state abundances quickly. This was also observed during the colonization experiments in the previous chapter. Because this study was conducted on a time scale of days, differences in density could also be driven by other factors not described by the model. Bacteria associated with aggregates can grow quickly on time scales that exceed the initial period of colonization (Kiørboe et al. 2003); the dense populations of HP11 associated with TEP may be the result of rapid, post-colonization growth, suggesting that HP11 can utilize TEP as a nutrient source better than HP1. Some marine snow bacteria are also known to exhibit chemosensing behaviors which can increase colonization on aggregates (Kiørboe et al. 2002, Grossart et al. 2007). The high

densities of bacteria associated with TEP could result from a stronger chemotactic response on the part of HP11 compared to that of HP1.

Substrate-associated life can make bacteria susceptible to predation by surface-associated protozoans and some mesozooplankton. Protozoans may also consume TEP directly (Tranvik et al. 1993). TEP-dominated microaggregates, which concentrate TEP as well as bacteria and other nanoparticles, can be grazed upon by *Euphausia pacifica* (Passow and Alldredge, 1999). Ling and Alldredge (2003) determined that the copepod *Calanus pacificus* consumed TEP, and suggested that zooplankton grazing on TEP is a possible "short cut" in the microbial loop.

Species-specific Interactions

Other laboratory and field observations have shown that the production of TEP depends on the species of phytoplankton (Kiørboe and Hansen, 1993; Passow, 2002a). The chemical composition and degradability of TEP can, in turn, affect utilization by bacteria (Mari and Kiørboe, 1996; Zhou et al. 1998). TEP are readily colonized by bacteria, but colonization may depend on the age (Mari et al. 2001), and size of TEP (Mari and Kiørboe, 1996), as well as grazing pressure (Mari et al. 2004b). At the species level, associations between phytoplankton and bacteria can result in different responses (Grossart et al. 2006a), even when bacteria are interacting with the same species of phytoplankton (this study). These relationships are often complex, but studying them on a species-level allows us to explain possible effects seen at multi-species, ecologically relevant levels. Species-specific interactions between bacteria and phytoplankton have not been studied extensively, although it is clear that the relationship between bacteria,

phytoplankton, and TEP production could play a role in microbial food web dynamics, aggregation dynamics, and the sedimentation of diatom blooms.

CHAPTER 4

SUMMARY AND CONCLUDING REMARKS

The role of bacteria in marine snow dynamics

Bacteria and marine aggregates interact through many complex, microscale processes which determine their biogeochemical fate as they sink through the water column. Many of the processes that are mediated by bacteria have been well described, including colonization, detachment (Kiørboe et al. 2002), enzyme activity (Grossart et al. 2007), and production of component particles like TEP (Passow, 2002a). Improving our understanding of some of these complex processes requires examining individual shifts in behavior and bacterial interactions with aggregate components.

In this thesis, I conducted two studies showing different, strain-specific responses of heterotrophic bacteria interacting with aggregates or components of aggregates. The major findings of the first study are:

(1) Starvation effects were observed in heterotrophic bacteria in less than 24 hours, resulting in lower steady-state abundances associated with aggregates. There was no effect of starvation on detachment from aggregates.

(2) Motility responses to starvation were varied. Two strains of bacteria displayed reduced swimming speeds which subsequently affected encounter opportunities between bacteria and aggregates, reducing overall colonization. One strain of bacteria showed no changes in motility.

These results suggest that bacteria that are starved may have reductions in motility which decrease their chances of encounter with marine snow aggregates. But is this a

likely situation in a natural environment, given the distribution of marine snow aggregates? A review by Alldredge and Silver (1988) reported marine snow abundances between 1-10 aggregates per liter in near-surface waters across sites in the Atlantic as well as off the coast of California. These values are close to the values in the north Pacific gyre (Pilskaln et al. 2005), which ranged from 6 – 13 aggregates per liter. However, Wells and Shanks (1987) reported marine snow abundances which were orders of magnitude higher off the coast of North Carolina, nearly 500 aggregates per liter. In a hypothetical, 1 m^3 volume of water, monodispersed marine snow aggregates would be 5.5 cm apart from one another at the gyre concentration (6 aggregates per liter), and only 1.26 cm apart from on another at the coastal concentration (500 aggregates per liter.)

If two HP1 bacteria (one starved and one fed) were swimming in the hypothetical volume of water containing marine snow, my motility data suggests that the starved bacterium would be able to travel a straight-line distance of 1.73 m in one day, and the fed bacterium could swim twice that distance. However, bacterial swimming is characterized by series of runs, interspersed by tumbles which change the direction of swimming (Berg, 1983). The diffusivities calculated from my motility data reflect a more realistic search area for each bacterium. In one day, a fed HP1 bacterium would cover an area of 1.33 cm^2, and a starved HP1 bacterium would be able to search an area of 0.17 cm^2.

Based on these rough estimates, a fed bacterium may encounter an aggregate in an environment with coastal concentrations of marine snow, but not gyre concentrations. A starved bacterium has such low diffusivity, it would not cover enough area in a day to encounter a particle in water at either concentration. Although the monodisperse

condition is far from what is observed in-situ, these calculations suggest that, within a day's time (equivalent to the starvation time in this study) diffusivities decreased and resulted in much smaller search areas under starvation. Even at typical densities of marine snow, a starved bacterium would not encounter an aggregate. Starvation can clearly change and adversely affect the behaviors of marine snow bacteria searching for a nutrient source.

Starvation occurs when bacteria are in areas where the availability of energy-yielding substrates is suboptimal (Morita, 1982; Kjelleberg, 1993). The primary means of measuring bacterial responses to substrate availability is to measure bacterial production of cell biomass (Kjelleberg, 1993). However, production rates can vary on different time and space scales (Sherr et al. 2001) and growth may become unbalanced; the rate of DNA synthesis, measured by thymidine incorporation, does not equal the rate of protein synthesis, as measured by leucine incorporation (Kjelleberg, 1993; Ducklow, 2000). Bacterial productivity can be relatively low (0.79 $\mu g\ C\ l^{-1}d^{-1}$ – 6.19 $\mu g\ C\ l^{-1}d^{-1}$) in oligotrophic regions, like the Sargasso Sea (Fuhrman et al. 1989); but starvation does not necessarily mean that production will be zero. Kjelleberg (1993) suggests these measurements may be dominated by a few species in a diverse population of bacteria, so while some bacteria in the population better adapted to utilizing the organic matter available, there are others that are starving. Considering that most of the world's ocean is nutrient limited and have patchy distributions of aggregates, starvation is a likely condition among bacteria.

My results from the study indicate that future models about microscale processes occurring on aggregates should incorporate changes in bacterial physiology and behavior due to starvation, as these changes are strain-specific.

Additional findings from the second study in this thesis suggest that:

(3) The amount and character of TEP produced by diatoms can vary under the influence of different bacterial strains.

(4) Strains of bacteria exhibit different distributions between TEP-associated and free-living fractions and can reach different steady state densities on TEP.

Results from this study indicate that bacteria-phytoplankton interactions depend on the bacteria involved and that the resulting net TEP production could potentially affect aggregation dynamics. The density and distribution of bacteria may also be strain-specific and may represent a trade off between metabolic demands and protection from predation. Dense populations of bacteria may produce areas of concentrated exoenzyme activity, creating more DOM that can be effectively utilized by both bacteria and phytoplankton. Dense populations can also create microzones in which oxygen may be limited. However, high-density colonization on substrates also increases bacterial susceptibility to predation and viral infection.

Both studies detailed in this thesis describe the tradeoffs of substrate-associated life as a balance between the need to maximize nutrient uptake and utilization while minimizing predation. The development of bacterial populations on aggregates or on TEP depends on colonization and detachment on shorter time scales, and growth and predation on longer time scales. The strategies of bacteria operating in this balance are not as direct as it may seem. Bacteria do not "decide" to leave and detach from

aggregates, and bacteria do not "decide" to produce more TEP, although both behaviors have been observed and may be important aspects of success for marine snow bacteria.

In fact, the behaviors that make bacteria successful in a stressful environment could have both proximal and ultimate causes. For example, grazing pressure by bacterivorous protists has been shown to cause shifts in bacterial community composition, as well as changes in bacterial morphologies (Jürgens et al. 1999; Hahn and Höfle, 2001, and references therein). Although the majority of these studies have been conducted on free-living bacterial populations, it is likely that similar mechanisms occur on aggregates as well, whereby grazing pressure selects for certain bacterial genotypes that minimize losses to predation, including the production of antagonistic chemicals, or the growth of grazing-resistant filamentous structures (Jürgens et al. 1999). Some bacteria are also known to possess chemosensory behaviors, such that they can detect low concentrations of DOC from lysed cells (Fenchel, 2002). These behaviors are associated more with bacteria that are looking for concentrated, point sources of organics (Fenchel, 2002), but could also signal the presence of predators, in theory. It is already well-established that bacteria can use chemical signals, like acylated hemoserine lactones, to communicate with one another via quorum sensing if a critical density of bacteria is present (Gram et al. 2002).

Future research could include the effects of grazer populations on bacterial community composition associated with aggregates, as well as interactions between chemosensory behaviors, grazing pressure, and other anti-grazer behaviors, like the production of EPS. These mechanisms are not well-studied, and could play a role in the

dynamics on marine snow, as well as the fluxes of particulate and dissolved organic matter.

While field studies describe the net effects of bacterial communities acting upon aggregates, the results of my thesis indicate that interactions of individual strains are also important. Bacteria may change their physiology or behavior when interacting with other particles like TEP and phytoplankton. Although complex inter- and intraspecific interactions may also occur, describing strain-specific responses can help us to piece together microscale processes influencing aggregation dynamics as well as processes that maintain bacterial diversity on aggregates. Combining our understanding of individual bacterial responses and changes in behavior with more information on community composition and interspecific behaviors will allow us to more effectively describe the dynamics and fate of marine snow aggregates in the ocean.

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