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Original Contribution

Characteristics of Marine Aggregates in Shallow-water Ecosystems: Implications for Disease Ecology

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Abstract: Marine aggregates were evaluated for their potential role in the ecology of aquatic pathogens using underwater video surveys coupled with direct collection of aggregates in modified settling cones. Six locations, two each in New York, Connecticut, and Massachusetts, were surveyed over 8 months to explore differences in the characteristics of aggregates found in habitats populated by clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*). Microaggregate (<500 μm) concentrations were always greater than macroaggregate (>500 μm) concentrations, but peak concentrations of macroaggregates and microaggregates, mean size of particles, and volume fraction of aggregated material varied among the six shallow-water habitats. Concentrations (colony-forming units per ml) of total heterotrophic bacteria (THB) and total mesophilic pathogenic bacteria (MPB) from samples of aggregates were significantly different among the four locations bordering Long Island Sound (LIS). The highest concentrations and enrichment factors in aggregates were observed in August for THB and in June for MPB. Significant correlations were detected for salinity and the concentrations and enrichment factors of THB in aggregates and for the concentrations and percentages of MPB in seawater samples. Significant correlations were also detected for temperature and the concentrations of MPB in aggregates and the enrichment factors for THB and MPB (marginal significance). Bacterial species identified in association with aggregates included: *Vibrio cholerea, V. parahaemolyticus, V. vulnificus, V. alginolyticus, Aeromonas hydrophila, Pseudomonas aeruginosa*, *Escherichia coli*, and *Mycobacteria* sp. These results have important implications for the way in which aquatic pathogens are collected, quantified, and monitored for risk-based surveillance in shallow-water ecosystems.

Keywords: Marine snow, aquatic pathogens, mesophilic pathogenic bacteria, enrichment factors, risk-based surveillance, water quality

INTRODUCTION

Information on the basic ecology of marine diseases has increased over the past few decades as our appreciation for their impacts on individuals, populations, and ecosystems has grown (Harvell et al., 1999, 2002). In this research, we focus on diseases associated with suspension-feeding bivalves (e.g., clams and oysters), including bivalve pathogens and human pathogens acquired via consumption of contaminated bivalves. We hypothesize that one path for the

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transmission and accumulation of pathogens in bivalves involves the aggregated particles suspended in seawater. The role of aggregates (i.e., marine snow, flocs, organic detritus) as a link between aquatic pathogens and hosts, however, remains relatively unexplored. Only the hard clam pathogen, Quahog Parasite Unknown (QPX; Lyons et al., 2005), and the human pathogen, *Vibrio parahaemolyticus* (i.e., "sinking particles"; Venkateswaran et al., 1990), have been evaluated and documented in marine aggregates. The freshwater counterparts of marine aggregates, now called lake and river aggregates, have also been shown to contain pathogenic bacteria, including *Vibrio cholerae* (i.e., "particulates > 20 μm"; Colwell et al., 2003).

Marine aggregates have been difficult to characterize because they are destroyed by common sampling equipment (Riley, 1963; Alldredge and Silver, 1988; Simon et al., 2002). In the oceans, marine aggregates have been described and quantified by SCUBA divers, sediment traps, and underwater video cameras with specialized lighting systems (Alldredge and Silver, 1988; Jackson et al., 1997; Pilskaln et al., 1998). Aggregate concentrations have been shown to vary on a wide range of temporal and spatial scales, including daily, seasonally, interannually, between oceans, between latitudes, and from offshore waters to coastal zones (Kiørboe, 2001; Simon et al., 2002; Turner, 2002). These studies substantiate that the specific study site is critical in determining the distribution and characteristics of marine aggregates on a global scale. There are only a few studies that have addressed marine aggregates in shallow waters (Shanks, 2002; Newell et al., 2005).

The purpose of this study was to describe the characteristics of marine aggregates near shellfish beds to (1) explore the potential role of aggregates in the ecology of diseases associated with marine bivalve molluscs and (2) identify which pathogens may be associated with marine aggregates. These concepts have important implications for the way in which aquatic pathogens are collected, quantified, and monitored for water quality assessments and risk-based surveillance in coastal environments.

Methods

Field Sites

Six locations along the northeastern U.S. (New York, Connecticut, and Massachusetts) were chosen to explore differences in the characteristics of aggregates found in habitats populated by hard clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) (Fig. 1). Two locations were selected in each state, one location known to support oyster populations or culture sites, and the other known to support hard clam populations or culture sites. New York locations included Mattituck Inlet (A) and Hashamomuck Pond (B), both on the southern edge of Long Island Sound (LIS). Connecticut locations included Mystic River (C) and Stonington Harbor (D), both on the northern edge of LIS. The two locations in Massachusetts were Edgartown Great Pond on Martha's Vineyard (E) and Barnstable Harbor (F).

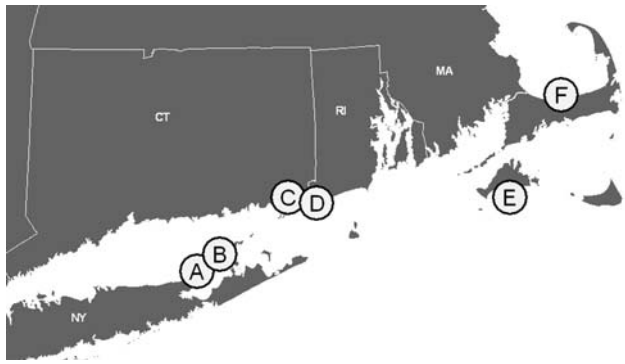

Figure 1. Six field sites were studied in three states. New York locations were Mattituck inlet (A, oyster habitat) and Hashamomuck Pond (B, clam habitat). Connecticut locations were Mystic Harbor (C, oyster habitat) and Stonington Harbor (D, clam habitat). Massachusetts locations were Edgartown Great Pond on Martha's Vineyard (E, oyster habitat) and Barnstable Harbor (F, clam habitat).

Field Sampling

At each of the six locations (A–F), three sites were sampled to describe the aggregates to which suspension-feeding bivalves would be exposed. The elaborate sampling methods developed for characterizing aggregates in deeper waters were too cumbersome to function in the shallow coastal ecosystems where clams and oysters thrive. Therefore, at each site aggregates were collected in modified settling cones as previously described (Lyons et al., 2005), while a minimum of 10 minutes of underwater video was filmed with a Sony TRV99 camera in an Ikelite underwater housing. The small camera was fitted with a light, a monofilament wire grid, and a dark gray backplate. The grid (1-cm^2 squares) served as a frame of reference and was secured halfway from the backplate to the edge of the housing (2 cm total distance), allowing calculation of the volume (cm^3) within each subunit of the grid. For each site, temperature and salinity were measured with a YSI meter. The clam habitats in CT and MA locations were sampled

once per month in April, May, June, July, August, September, and November of 2005. Logistics and inclement weather prevented sampling in October. The clam habitat in NY and the oyster habitats in NY, CT, and MA locations were sampled once per month in April, June, August, and November of 2005.

Video Analysis

To enumerate aggregates, digital still frames were isolated from analog video using frame-grabber software (Pinnacle Studio 9) and a Sony digital video cassette recorder (GV-D800). The general procedure was as follows: The first minute of each video was disgarded because it included the deployment of the camera into the water. After a random start, frames were systematically grabbed every 15 seconds for 8 minutes (Newell et al., 2005) for a total of 33 frames per video sample. The 33 images were imported into image analysis software (ImageJ), converted to 8-bit gray scale, and inverted to produce dark particles on a light background. If ambient lighting conditions were bright enough to generate images with dark particles on a light background, then the inversion step was omitted. The particles in each frame were numbered, counted, and sized using a binary threshold and an automated particle counter. Counts were then manually verified, frame-by-frame, against the original still photographs to confirm the presence of particles. Any "particles" that appeared in the exact position and shape in two or more consecutive frames (separated by 15 seconds) were identified as artifacts of the image analysis procedure and removed because they were not true particles suspended in the moving seawater. Artifacts included scratches or smudges on the housing or backplate, debris caught on the frame or stuck to the backplate, and shadows of the wire grid produced by ambient lighting conditions.

Mean particle sizes of aggregates were always less than 500 μm (see Results; Fig. 1); therefore, to enumerate the larger, but relatively rare, macroaggregates (aggregates > 500 μm), we manually determined the number of macroaggregates per unit volume of seawater. First, an average velocity (cm/second) was calculated for each video sample by tracking and timing particles across the grid. Then, the velocity was multiplied by the cross-sectional area of flow (2 cm × 1 cm) and the video sample time (up to 10 minutes) to calculate the volume observed per unit time. Aggregates larger than 500 μm were manually counted to determine a concentration (number per liter observed).

The variance-to-mean ratios (VMR, or coefficient of variation) of the concentration of macroaggregates (number per liter) and microaggregates (number per milliliter) were calculated as a measure of patchiness at each location. A VMR close to 1 indicated an even distribution of particles between the three video samples that were obtained within 100 m and 1 hour of each other at each location (A–F) on a given sampling day. As VMR increases, the estimate of patchiness also increases.

The volume fraction of aggregated material (in parts per million) was calculated for each video sample by multiplying the mean concentration of aggregates by the volume of an average ellipsoidal particle [$4/3\pi \times$ ½ long axis $\times$ (½ short axis)^2], and then dividing by the video sample volume.

Microbiological Analysis of Seawater and Aggregate Samples

To investigate some of the biological differences in marine aggregates, samples from the locations bordering LIS (Fig. 1A–D) were processed for bacterial composition. Aliquots (100 μl) of seawater and aggregate samples were serially diluted and used to inoculate two types of agar plates. Marine agar, a nonselective medium for growing heterotrophic marine bacteria, was used to quantify the total number of colony-forming units (CFUs) of heterotrophic bacteria. Marine agar plates were incubated for 48 hours at room temperature (21 ± 1°C). CHROMagar Vibrio® (CHROMagar Microbiology, Paris, France), a chromogenic medium selective for bacteria from the genus *Vibrio* (Hara-Kudo et al., 2001), was used to determine the number of CFUs of mesophilic bacteria, which includes several human pathogens. CHROMAgar Vibrio plates were incubated for 48 hours at 37°C to permit growth of the targeted human pathogens. Colonies grown on each plate were recounted after 4–7 days to include slow-growing bacterial species. The number of CFUs recorded from marine agar plates was used as a measure of the total heterotrophic bacteria (THB) in these samples. A representative number of colonies ($5 < n = \sqrt{N}$, where n is the number of biochemically characterized isolates and N is the number of colonies belonging to each morphotype) cultured on the CHROMAgar Vibrio plates were isolated and identified using a biochemical classification system (API 20E, Biomerieux). The number of CFUs recorded from the CHROMAgar Vibrio plates (that were subsequently identified as known pathogenic species) was used as a measure

Table 1. Qualitative Results from the Screening of a Subset of Environmental Samples of Marine Aggregates[a]

Location	State	Habitat	Month	*E. coli*	*Mycobacteria*	*A. minutum*
A	NY	Oyster	APR	+	−	−
C	CT	Oyster	APR	+	+	−
E	MA	Oyster	APR	+	−	−
B	NY	Clam	APR	−	+	−
D	CT	Clam	APR	+	+	−
F	MA	Clam	APR	−	−	−
A	NY	Oyster	AUG	+	−	−
C	CT	Oyster	AUG	−	+	−
E	MA	Oyster	AUG	+	+	−
B	NY	Clam	AUG	+	+	−
D	CT	Clam	AUG	+	−	−
F	MA	Clam	AUG	+	−	−
A	NY	Oyster	JUN	−	−	−
C	CT	Oyster	JUN	−	−	−
E	MA	Oyster	JUN	−	−	−
B	NY	Clam	JUN	+	−	+
D	CT	Clam	JUN	−	−	−
F	MA	Clam	JUN	+	+	−
A	NY	Oyster	NOV	+	+	−
C	CT	Oyster	NOV	−	−	−
E	MA	Oyster	NOV	+	−	−
B	NY	Clam	NOV	+	−	−
D	CT	Clam	NOV	+	−	−
F	MA	Clam	NOV	+	+	−

[a] Positive results were found using primer sets targeting the bacteria *Escherichia coli* and *Mycobacteria* sp. and the toxic dinoflagellate *Alexandrium minutum*.

of the total mesophilic pathogenic bacteria (MPB). In both cases (THB and MPB), the counts are minimum estimates because they represent only cultivable bacterial species. The numbers of CFUs for bacteria in marine aggregate samples were normalized by the concentration of macroaggregates (i.e., aggregates large enough to settle in the settling cones).

Concentrations of bacteria were determined as the number of CFUs from 1 ml of sample. Enrichment factors (EF; Caron et al., 1986) for THB and MPB were determined as a measure of the relative abundance of bacteria in marine aggregates compared to seawater. EFs were calculated by dividing the concentrations (CFUs per ml) of bacteria in aggregates by the concentrations of bacteria in seawater for each type of media. The percentage of MPB was determined by dividing the concentrations of bacteria from the CHROMAgar Vibrio plates by the concentrations from the marine agar plates and multiplying by 100. These calculations were used as relative estimates of potentially pathogenic bacteria in the environmental samples even though not all bacterial species will grow equally well on the two types of media. Nevertheless, because each environmental sample was plated on both media types, the relative differences among samples are comparable.

Molecular Analysis of Aggregate Samples

To examine a subset of the marine aggregate samples (n = 24) for several microorganisms, a master screening plate of DNA was designed (see Results; Table 1). DNA was extracted from samples of marine aggregates (i.e., aggregates settled out of 2 liters of seawater) from NY, CT, and MA using QIAamp-DNA Stool Mini Kits (Qiagen, Valencia, CA), following the manufacturer's protocol for DNA isolation of pathogen DNA. In the final step, DNA was eluted with 200 µl of AE buffer for 5 minutes as recommended in Audemard et al. (2004). The DNA was screened for protists and bacteria using primer sets available from other publications (see references below). Screening for protists in-

cluded species-specific primers for *Cryptosporidium parvum* (Godiwala et al., 2006), *Giardia lambia* (Guy et al., 2003), *Perkinsus marinus* (S. Roberts, unpublished), Quahog Parasite Unknown (QPX; Lyons et al., 2006), *Alexandrium minutum* (Galluzzi et al., 2004), and *Aureococcus anophagefferens* (Doblin et al., 2004). Screening for bacteria included general primers for *Escherichia coli*, genus-specific primers for *Mycobacteria* sp. (De La Torre et al., 2001), and species-specific primers for *Vibrio parahaemolyticus* (Myers et al., 2003) and *Vibrio vulnificus* (Panicker et al., 2004). The PCR assays were performed in a final volume of 25 µl with the concentrations of components as GoTaq Green Master Mix (Promega, Madison, WI) 1× and primers 0.4 µM. Reactions had a final dNTP and $MgCl_2$ concentration of 200 µM and 1.5 µM, respectively. Parameters for all PCR reactions were as follows: initial denaturation of the template DNA at 94°C for 2 minutes, followed by 40 cycles of (1) 94°C for 1 minute (denaturation of dsDNA), (2) 50°C for 2 minutes (annealing of primers), (3) 72°C for 2 minutes (extension), followed by 72°C for 8 minutes.

Statistical Analysis

From the underwater videos, mean and standard deviation ($n = 3$ sites per location, 6 locations) of the concentrations, sizes, and volume fraction of aggregates were determined. When the data were not normally distributed (Anderson–Darling test, $p < 0.05$), data were transformed using Johnson transformations (Minitab 15). Means of the transformed data were compared using two-way analysis of variance (ANOVA) by location and month and one-way ANOVA for the type of bivalve habitat (clam vs. oyster). Means were also evaluated as a function of temperature, salinity, and velocity using nonparametric Spearman's rank correlation procedure. For each location bordering LIS (i.e., those where samples were also processed for microbiological analysis), geometric means for the concentrations of total heterotrophic bacteria (THB) and mesophilic pathogenic bacteria (MPB), enrichment factors for THB and MPB, and percentage MPB in seawater and aggregates were also calculated ($n = 3$ sites per location, 4 locations). When the data were not normally distributed (Anderson–Darling test, $p < 0.05$), data were transformed using Johnson transformations (Minitab 15). Means of the transformed data were evaluated using two-way ANOVA by location and month and one-way ANOVA for type of bivalve habitat. Means were also evaluated as a function of temperature, salinity, and velocity using Spearman's rank correlation procedure. Results were considered significant when the calculated p values were less than or equal to $\alpha = 0.05$. Marginally significant results (defined as $0.05 < p < 0.1$) were also reported. When applicable, post-hoc tests for transformed data were evaluated via Tukey HSD (when equal variances were verified with Levene's test, $p > 0.05$) or Dunnett's T3 (when equal variances were not detected, $p < 0.05$). Minitab and SPSS software were used for the statistical analysis.

Results

Clam Habitat vs. Oyster Habitat

Locations with predominantly oyster habitat (locations A, C, E) and predominantly clam habitat (locations B, D, F) had similar measurements (no significant differences via one-way ANOVA) for the physical characteristics of aggregates, including concentration of macroaggregates or microaggregates, size, and volume fraction of aggregated material. Locations with clam habitat tended to have higher salinities than locations with oyster habitat ($p < 0.001$).

Environmental Variables

All samples were collected in waters with a depth of 4 m or less. Temperatures ranged from 7.8°C in November to 28.4°C in August. Salinities ranged from 18.6 to 30.8 PSU. No significant correlations were found between temperature or salinity and the physical characteristics of aggregates, including concentration of macroaggregates or microaggregates, patchiness of macroaggregates or microaggregates, size, and volume fraction of aggregated material (data not shown). Velocities ranged from 0.3 to 8.6 cm/second. Higher velocities were correlated with lower concentrations of macroaggregates ($r = -0.36$, $p = 0.049$). The highest velocities (6.4 ± 2.5 cm/second) were measured at a clam habitat in MA (location F).

Characteristics of Aggregates

Concentrations

Concentrations of macroaggregates were correlated (marginal significance) with concentrations of microaggregates ($r = 0.36$, $p = 0.053$), but maximum concentrations of both macroaggregates and microaggregates varied within and among the six shallow-water habitats (Fig. 2). For the

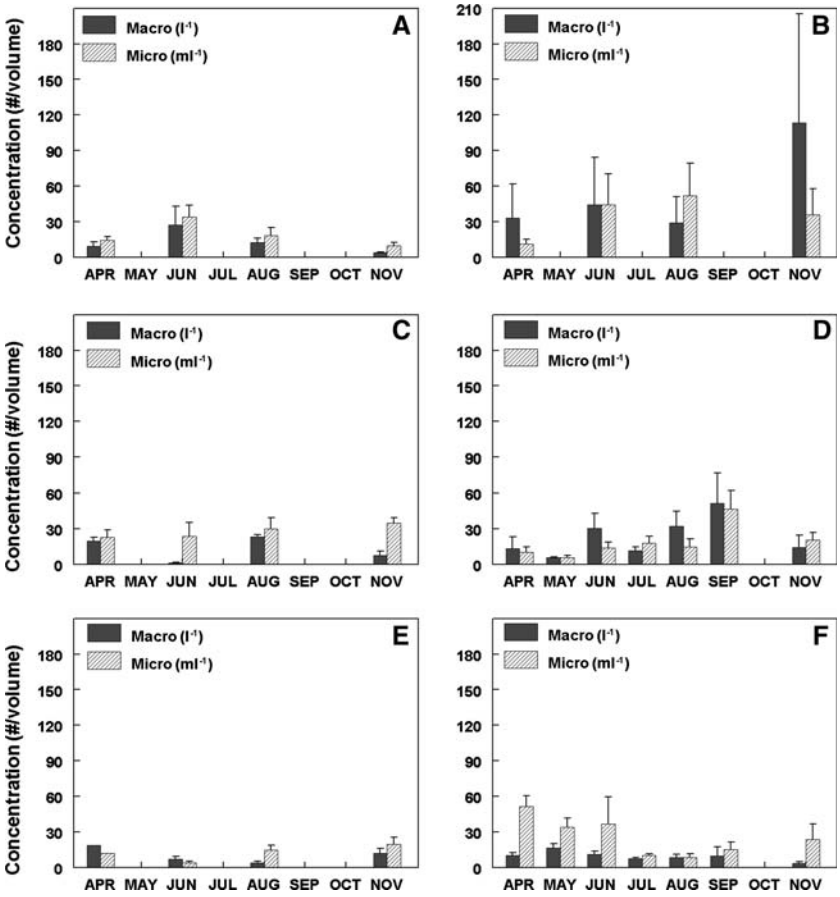

Figure 2. Peak concentrations of macroaggregates (aggregates > 500 μm; number per liter) and microaggregates (aggregates < 500 μm; number per milliliter) varied for each of the six nearshore habitats. Top graphs are from New York, middle graphs are from Connecticut, and bottom graphs are from Massachusetts. Left graphs (**A, C, E**) are oyster habitats and right graphs (**B, D, F**) are clam habitats. Microaggregate concentrations (striped bars; mean ± SD, $n = 3$) were always greater (note difference in units) than macroaggregate concentrations (dark bars; mean ± SD, $n = 3$), but no consistent seasonal patterns were detected, even with more frequent sampling at the CT clam and MA clam habitats (locations D and F).

concentration of macroaggregates, a two-way ANOVA detected no significant effects of the variables location (i.e., 6 locations, A–F) and month (i.e., month of sampling), nor significant interactions between location and month. For the concentration of microaggregates, marginally significant effects of location ($p = 0.08$) but not month ($p = 0.79$) were detected. A significant interaction between location and month was detected ($p = 0.013$). Overall, microaggregate concentrations (number per milliliter) were always greater than macroaggregate concentrations (number per liter), but no consistent seasonal patterns were detected, even with more frequent sampling at the clam habitats in CT and MA (locations D and F). The concentration of macroaggregates was inversely correlated with mean velocity ($r = -0.36$, $p = 0.049$).

Patchiness

The two-way ANOVAs of VMR data (estimates patchiness for macroaggregates and microaggregates) detected significant effects of the variable location, but not the variable month on both macroaggregate ($p = 0.002$) and microaggregate ($p = 0.028$) concentrations. The clam habitat in NY (location B) consistently had the highest estimates of patchiness, while the oyster habitat in MA (location E) usually had the least (Fig. 3). The patchiness of macroaggregates was significantly correlated with the patchiness of microaggregates ($r = 0.47$, $p = 0.009$).

Size

Mean aggregate size was always less than 500 μm but was highly variable among the three sites within a location (see error bars in Fig. 4). For the mean length of long axis (i.e., variable "long"), significant effects of location ($p < 0.001$), month (0.002), and a significant interaction of location and month ($p = 0.001$) were detected. The largest particles (i.e., longest) were detected at the clam habitat in MA, whereas the smallest were detected at clam habitats in NY and CT. Aggregates observed later in the study (November) were larger than those sampled earlier in the study (April and May), but the largest aggregates were found in July. For the mean length of short axis (i.e., variable "short"), no significant effects were detected for location, month, or an interaction between location and month.

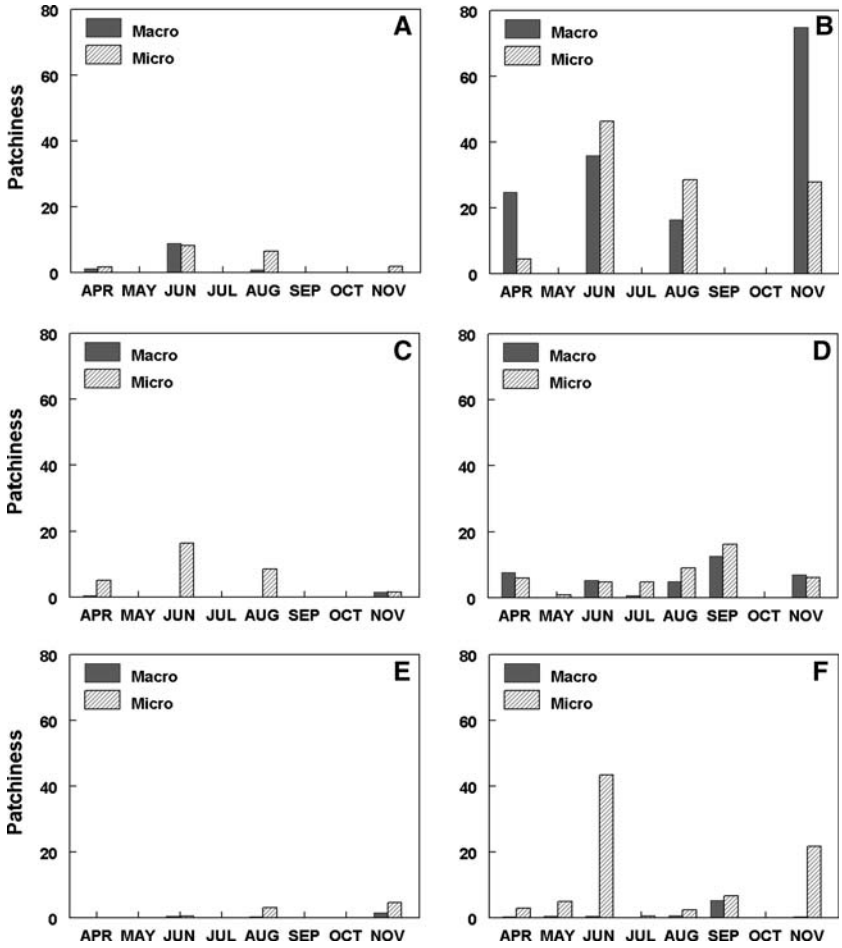

Figure 3. Patchiness (variance-to-mean ratio; VMR) of macroaggregates and microaggregates between the three sites at each location. Top graphs are from New York, middle graphs are from Connecticut, and bottom graphs are from Massachusetts. Left graphs (**A, C, E**) are oyster habitats and right graphs (**B, D, F**) are clam habitats. The NY clam habitat (location B) consistently had the highest estimates of patchiness, whereas the MA oyster habitat (location E) had the lowest.

Volume Fraction

Trends in the volume fraction of aggregated material also varied at each of the six shallow-water habitats (data for four of the six locations in Fig. 5). Marginally significant effects of location ($p = 0.059$), but not month ($p = 0.36$) were detected; however, a significant interaction between location and month was detected ($p = 0.014$). Volume fraction was not correlated with velocity, temperature, or salinity (data not shown).

Microbial Analysis of Environmental Samples

Microbiology

Total heterotrophic bacteria (THB) and total mesophilic pathogenic bacteria (MPB) were enumerated for samples of both marine aggregates and seawater from the four LIS locations. The MPB species identified included *Vibrio cholerea, V. parahaemolyticus, V. vulnificus, V. alginolyticus, Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. Also identified, but not used in the total counts due to their scarcity, were *Shigella sonnei, Stenotrophomonas maltophila, Photobacterium damsela*, and *Burkholderia cepacia*.

Concentrations

The concentrations of bacteria (CFUs per ml) fluctuated throughout the sampling period, but were frequently higher in marine aggregate samples for both (THB and MPB (Fig. 5). For concentrations of THB from marine aggregate samples, significant effects of location ($p < 0.001$), month ($p < 0.001$), and an interaction of location and month ($p = 0.020$) were detected. For seawater samples, significant effects of location ($p = 0.001$), month ($p < 0.001$), and an interaction of location and month ($p = 0.010$) on THB concentrations were found. In both cases samples from the oyster habitat in CT (location C) had the highest concentrations of THB in aggregates and seawater, whereas samples from the clam habitat in NY (location B) had the least. Samples collected in August had the highest concentrations of THB in marine aggregates and seawater,

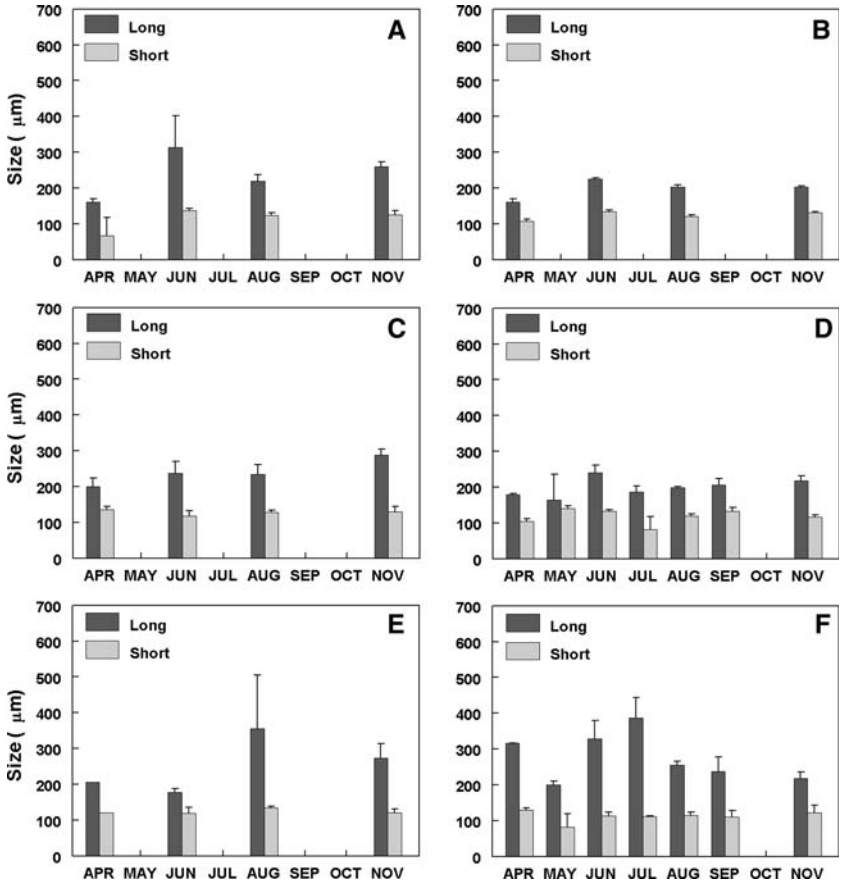

Figure 4. Mean sizes (long and short axes) of aggregates in six shallow-water locations. Top graphs are from New York, middle graphs are from Connecticut, and bottom graphs are from Massachusetts. Left graphs (**A, C, E**) are oyster habitats and right graphs (**B, D, F**) are clam habitats. Mean aggregate size was always less than 500 µm but was highly variable (mean ± SD, $n = 3$).

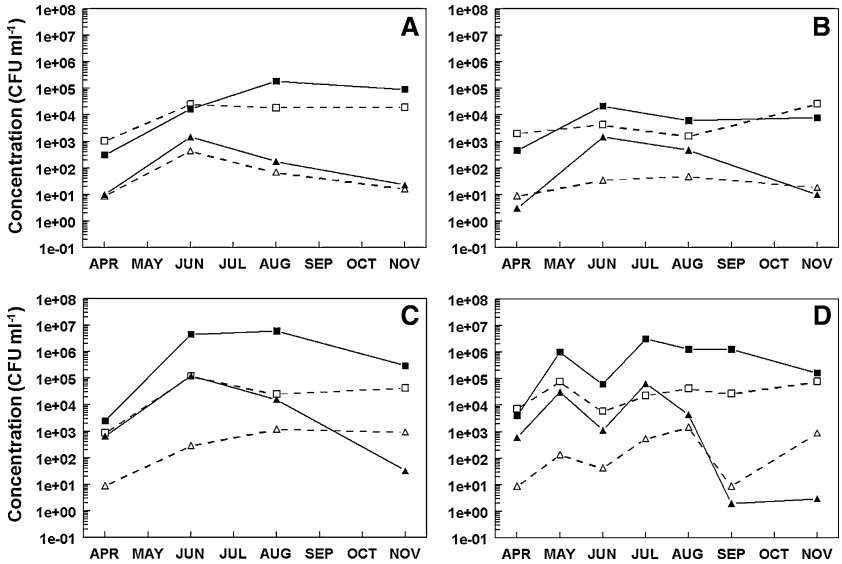

Figure 5. Bacterial concentrations in Long Island Sound samples for locations in New York (A: oyster habitat; B: clam habitat) and Connecticut (C: oyster habitat; D: clam habitat). Geometric means of the concentration of total heterotrophic bacteria (THB, squares) and mesophilic pathogenic bacteria (MPB, triangles) were frequently higher in samples of marine aggregates (solid lines, filled shapes) than in the corresponding seawater samples (dashed lines, unfilled shapes). The amount of enrichment varied throughout the sampling period.

whereas samples collected in April had the lowest concentrations of THB. For concentrations of MPB from marine aggregates, significant effects of location ($p < 0.001$), month ($p < 0.001$), and an interaction of location and month ($p = 0.013$) were also detected. Samples collected from the oyster habitat in CT had the highest concentration of MPB, while samples taken from the oyster habitat in NY had the lowest. The highest concentrations of MPB in marine aggregates were detected in June samples, whereas the lowest concentrations were observed in samples collected in November. For concentrations of MPB from seawater samples, significant effects of location ($p = 0.001$), month ($p < 0.001$), and an interaction of location and month ($p = 0.010$) were found. As with THB, samples from the oyster

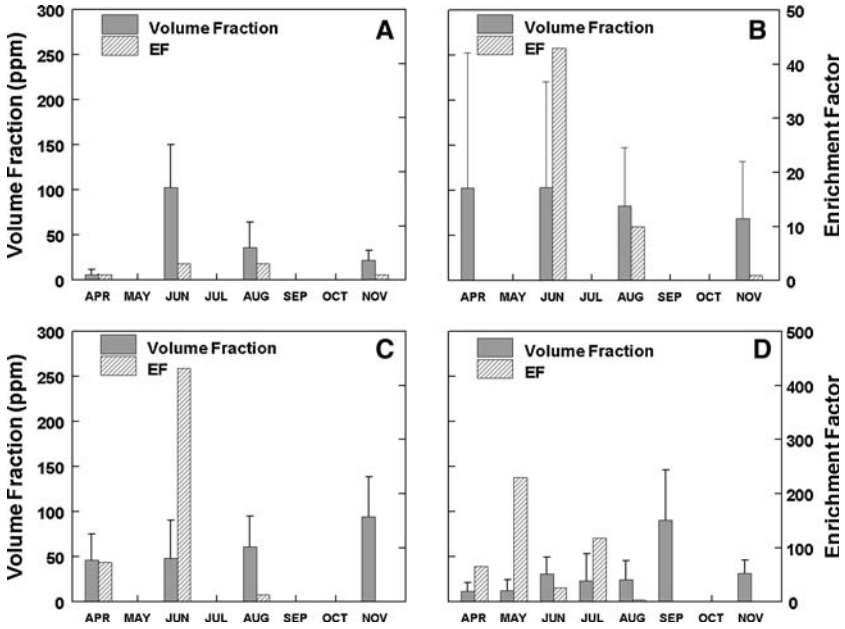

Figure 6. Trends in the volume fraction of aggregated material (dark bars; mean ± SD, n = 3) were specific to each coastal habitat. Top graphs are from New York, bottom graphs are from Connecticut (note difference in scale on the right y axis). Left graphs (**A, C**) are from oyster habitats, while right graphs (**B, D**) are from clam habitats. Enrichment Factors (EF) of pathogenic bacteria were highest in June. Suspension-feeding bivalves would have maximum exposure to aquatic pathogens (via marine aggregates) when a high-volume fraction of aggregated material overlaps with a high enrichment factor of pathogenic bacteria (e.g., CT oyster habitat in June and NY clam habitat in June).

habitat in CT had the highest concentration of MPB in seawater, whereas samples from the clam habitat in NY had the lowest. In addition, samples collected during August had the highest concentrations of MPB in seawater, whereas samples taken in April had the lowest concentrations of MPB.

For THB, concentrations from seawater samples were not correlated with temperature or salinity. Concentrations of THB from marine aggregate samples were also not correlated with temperature, but were correlated with salinity (r = 0.67, $p < 0.005$). For MPB, concentrations from seawater samples were marginally correlated with temperature (r = 0.46, p = 0.075) and significantly correlated with salinity (r = 0.65, p = 0.007), whereas concentrations of MPB from marine aggregate samples were correlated only with temperature (r = 0.65, p = 0.006) and not salinity (p = 0.21). Concentrations of THB from marine aggregate samples were correlated with mean size (variable long) of aggregates (r = 0.58, p = 0.018), but the concentrations of MPB were not (p = 0.21). Concentrations of MPB in marine aggregates were also correlated with patchiness of microaggregates (r = 0.54, p = 0.031). Concentrations of THB and MPB were not correlated with volume fraction of aggregated material (data not shown).

Enrichment

Enrichment factors (EFs) varied throughout the sampling period. For THB, a two-way ANOVA detected significant effects of location (p = 0.001) and month ($p < 0.001$), but not an interaction of location and month (p = 0.16). Overall, the oyster habitat in CT had significantly higher EFs than the other three locations. The EFs observed in June and August were significantly higher than the those observed in April. The maximum EF (349.1) for THB was observed in samples from August collected at the oyster habitat in CT. EFs for MPB were approximately an order of magnitude higher in CT locations than in NY locations (see difference in scale in Fig. 5). For MPB, significant effects of location (p = 0.041), month ($p < 0.001$), and an interaction of location and month (p = 0.004) on EFs were detected. EFs for MPB were highest at the oyster habitat in CT and lowest at the clam habitat in CT, and highest in samples collected in June and lowest in sample collected in November. The maximum EF (531.2) for MPB occurred in June at the oyster habitat in CT. EFs were graphed alongside volume fraction (Fig. 6) to pinpoint when peaks in EF corresponded to high levels of volume fraction of aggregated material (e.g., June at the clam habitat in NY and June at the oyster habitat in CT).

For THB, EFs were correlated with temperature (r = 0.58, p = 0.019) and salinity (r = 0.83, $p < 0.001$), whereas for MPB, EFs were only marginally correlated with temperature (r = 0.43, p = 0.099) and not correlated with salinity (p = 0.66). Enrichment for MPB was correlated with the patchiness of microaggregates (r = 0.65, p = 0.007), but enrichment for THB was not (p = 0.14). No other significant correlations were detected between the EFs of THB or MPB and the concentrations of aggregates, mean sizes of aggregates, or volume fraction (data not shown).

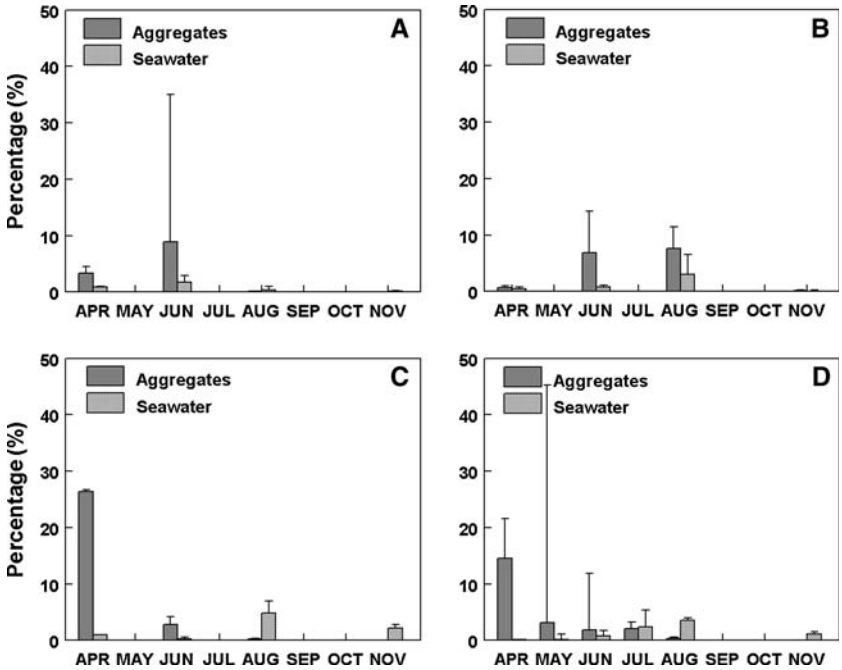

Figure 7. Percentage of mesophilic pathogenic bacteria (MPB) in samples of marine aggregates (dark bars) and seawater (striped bars) for locations in New York (A: oyster habitat; B: clam habitat) and Connecticut (C: oyster habitat; D: clam habitat).

Enrichment for THB was also not correlated with enrichment for MPB bacteria ($p = 0.18$).

Percent Pathogenic Bacteria

The percentage of mesophilic pathogenic bacteria in marine aggregates was calculated for all samples (Fig. 7). Results of a two-way ANOVA indicated that there were no significant effects of location ($p = 0.19$), but significant effects of month ($p < 0.001$) and a significant interaction between location and month ($p < 0.001$) on the percentage of MPB were detected. Aggregate samples collected in April had the highest percentages of MPB, whereas samples from November had the least. For seawater samples, significant effects of location ($p = 0.009$), month ($p < 0.001$), and an interaction of location and month ($p < 0.001$) were found. Seawater samples collected from the oyster habitat in CT had the highest percentage of MPB, while samples collected from the oyster habitat in NY had the least. The seawater samples collected in August had the highest percentage of MPB, while the samples collected in November had the lowest. Percentages of MPB in marine aggregate samples were not correlated with temperature ($p = 0.49$) or salinity ($p = 0.23$). Percentages of MPB in seawater samples were also not correlated with temperature ($p = 0.11$) but were correlated with salinity ($r = 0.62$, $p = 0.011$). The percentages of MPB in aggregates and seawater were not correlated with concentrations of macroaggregates or microaggregates, patchiness of macroaggregates or microaggregates, mean sizes of aggregates, or volume fraction of aggregated material (data not shown).

Molecular Biology

Screening the extracted DNA from a small subset of our marine aggregate samples yielded several positive results for *E. coli*, and *Mycobacteria* sp., and one positive result for *Alexandrium minutum* (Table 1). Positive results for *E. coli* in marine aggregates were most common in samples collected in August and November, whereas positive results for *Mycobacteria* spp. in marine aggregates were most common in samples collected in April and August. Positive results for both *E. coli* and *Mycobacteria* sp. were slightly more common in marine aggregates collected from clam habitats than oyster habitats (Table 1).

Discussion

Implications for Disease Ecology

Marine aggregates are ubiquitous (Simon et al., 2002), so their presence will overlap with a range of aquatic hosts and pathogens. Their importance in a specific host-pathogen-environment relationship will depend on several factors. For hosts, contact with pathogen-enriched marine aggregates affects exposure by increasing the size of the inoculums. This applies to both suspension-feeding animals that filter large volumes of water and to sessile animals (e.g.,

corals) that may be inundated with sinking aggregates (Wolanski et al., 2003). For facultative pathogens, the ability to survive and persist in marine aggregates increases the probability of survival between hosts. Life history traits of pathogens expected to be found in marine aggregates include (1) flagellated stages, because colonization of marine aggregates is governed by motility of organisms (Kiørboe et al., 2004); (2) saprophytic stages, because marine aggregates are small-scale patches of increased nutrients, biomass, and productivity (Alldredge, 2000); and (3) cyst- and spore-forming stages, because marine aggregates provide microhabitats of nutrient-replete conditions in otherwise nutrient-depleted waters (Brzezinski et al., 1997). Cysts of dinoflagellates (Faust et al., 1996) and resting spores of diatoms (Brzezinski et al., 1997) have already been documented in marine aggregates. In addition, pathogens that survive passage through digestive systems are likely to be found in aggregates because fecal pellets are often a component of marine aggregates (Turner, 2002). Finally, aggregates introduce a dynamic layer of complexity to the intricate ecological relationships that exist between hosts and pathogens. Our research supports the observation that the specific study site is critical in determining the characteristics of aggregates and provides a starting point for risk-based surveillance of aquatic pathogens found to be associated with marine aggregates.

Aggregate Concentrations and Patchiness

The study of marine aggregates is particularly important for disease research regarding bivalve mollusks because of the bivalve's mode of feeding. Aggregate concentrations can reflect the amount and quality of food available to suspension-feeders. On the other hand, for aggregates enriched with pathogens, aggregate concentrations affect the level of exposure of the host bivalve to the aquatic pathogens. The mean concentration of macroaggregates in our nearshore environments ranged from 1.5 ± 1 per liter in a clam habitat in CT to 113 ± 92 per liter in a clam habitat in NY. These concentrations are comparable to those (5–370 per liter) documented by Shanks (2002) working in slightly deeper (6 m) coastal waters. They are also similar to those reported for offshore environments, which averaged 100 macroaggregates per liter (Simon et al., 2002), but ranged from less than 1 per liter off the coast of California (Alldredge, 1979) to 5.3×10^3 per liter in the North Sea (Riebesell, 1991). The concentration of microaggregates in our nearshore environments varied from 4.3×10^3 per liter in an oyster habitat in MA to 5.2×10^4 per liter in a clam habitat of MA, a much narrower range of abundance when compared to oceanic environments which range from 2.0×10^3 (Passow and Alldredge, 1994) to 3.8×10^8 per liter (Mari and Burd, 1998). The higher concentrations reported for oceanic samples are most likely due to the inclusion of transparent exopolymer particles (TEP), which require specialized collection and staining techniques to be quantified (Alldredge et al., 1993).

Most previous studies have not evaluated the concentrations of macroaggregates and microaggregates simultaneously, but those that have (Kiørboe et al., 1998; Grossart et al., 1998) reported that microaggregates were more abundant than macroaggregates. Our work supports this observation, as the concentration of microaggregates within each of our habitats was found to be orders of magnitude greater than that of the macroaggregates. This finding was expected because in shallow, tide-driven habitats higher hydrodynamic forcing would discourage the formation of larger aggregates (Simon et al., 2002), and it was further supported by our results that showed that the concentration of the larger aggregates (macroaggregates) was inversely correlated to mean velocity. Quantifying aggregate concentrations revealed that in some situations the concentrations of aggregates varied more within a location (i.e., larger differences among three replicate sites on the same day) than across locations (i.e., differences among locations in the three states), indicating a high level of patchiness in some habitats. These differences suggest that the mechanisms of formation and degradation of marine aggregates in shallow-water ecosystems may occur on relatively short time (<1 hour) and space (<100 m) scales, and as a result several sites may need to be sampled to describe the marine aggregates within an area of interest. For any location, patchiness will be an important factor to determine because it provides information on the number of sites needed to adequately describe the marine aggregates at a location. For example, in our research the clam habitat at the NY location (with high measures of patchiness) would require more replicate sites to characterize aggregates than the oyster habitat at the MA location (consistently low measures of patchiness).

Aggregate Sizes and Volume Fraction

Our analysis found that total mean aggregate size (microaggregates and macroaggregates) for each shallow-water environment was always less than 500 μm. This corroborates

the observation that particles in the microaggregate size class dominate over the relatively rare macroaggregates. Our hypothesis is that these aggregates (both microaggregates and macroaggregates) contribute to the accumulation of bacteria in bivalves. Since individual bacterial cells (≤ 1 μm) are generally smaller than the minimum particle sizes (2–4 μm) efficiently retained by suspension-feeding bivalves (Riisgård, 1988), single cells of bacteria suspended in seawater should not be effectively accumulated in clams and oysters. Yet bacteria, including human pathogens, are accumulated by both clams and oysters. In fact, Kach and Ward (in review) demonstrated that 0.5- and 1-μm polystyrene beads were ingested at a significantly higher rate by four species of bivalves when the beads were incorporated into marine aggregates. It is therefore our contention that the bacteria embedded in marine aggregates (on the order of several 100 μm) would all be captured (i.e., collected) by suspension-feeding bivalves because aggregates are larger than the minimum particle sizes retained by the gills of clams and oysters. Consequently, bacteria-enriched marine aggregates would facilitate and accelerate the accumulation of bacteria in bivalves and should be monitored for potential pathogens.

Volume fraction of aggregated material was calculated as a product of mean concentration and mean size to evaluate trends among habitats. Volume fraction of aggregates varied among the six locations at different times of the year, indicating that the measurements of concentrations and sizes were specific to each location and that the relationship is complex. Volume fraction is of interest because mesophilic pathogenic bacteria (which includes several human and animal pathogens) were often enriched in marine aggregates relative to seawater. Therefore, larger and more numerous aggregates would increase encounter rates between aquatic pathogens and the bivalves. When a high-volume fraction of aggregated material overlaps with a high enrichment factor of pathogenic bacteria (e.g., CT oyster habitat in June) or a high percentage of pathogenic bacteria in aggregates (e.g., NY clam habitat in June), maximum exposure of aquatic pathogens via marine aggregates would occur. Whether this period translates into an outbreak of disease will be the next step to evaluate.

Microbial Composition of Marine Aggregates

The presence of pathogen-laden aggregates in environments prone to disease outbreaks would suggest a means for the spread and survival of pathogens, and would provide a target for environmental surveillance of those pathogens. Collection of aggregates in settling cones provided sufficient material to test for the presence or absence of pathogens. Once it is established that a particular pathogen is associated with marine aggregates, more specific techniques will be required to evaluate the distribution of pathogenic species within individual aggregates.

The biological composition of marine aggregates is a reflection of geographic location, depth, community structure, seasonality, and mechanism of formation (Kiørboe, 2001; Turner, 2002). Our microbiological analysis found that both total heterotrophic bacteria and total mesophilic pathogenic bacteria were enriched in marine aggregates relative to the surrounding seawater. This supports the earlier work that documented *Vibrio parahaemolyticus* (Venkateswaran et al. 1990) and *V. cholera* (Colwell et al., 2003) in aggregate-like material and expands the list to include *V. vulnificus, V. alginolticus, Aeromonas hydrophilia, Pseudomonas aeruginosa*, and occasionally *Shigella sonnei, Strenotrophomonas maltophilia, Photobacterium damsela*, and *Burkholderia cepacia*.

The DNA screening of a small subset of our marine aggregate samples yielded positive results for *E. coli*, *Mycobacteria* sp., and *Alexandrium minutum*. The molecular analysis did not detect some of the pathogens (e.g., *V. vulnificus*) that were detected via culturing. This result warrants further exploration as it may be due to the distribution of specific pathogenic species in individual marine aggregates. Variations on this scale were not targeted in our research. In addition, although the list of aquatic pathogens identified in our research included both human and animal pathogens, the virulence of these pathogens was not assessed.

Enrichment factors (EFs) for bacteria (<1 to 5700), phytoplankton (7 to 12,000), protists (1 to 8600), and metazoans (<1 to 765) have been documented previously (references in Simon et al., 2002), but our research is the first to measure EFs for a suite of aquatic pathogens. The range of EFs for aquatic pathogens (<1 to 531) was narrower and at the lower end of the range for nonpathogenic bacteria previously reported. This may be a reflection of the detection methods used in the different studies. Our calculations for EF were based on culturing, which should be considered a conservative method because many marine bacteria, including members of the genus *Vibrio*, exist in noncultivable states (Louis et al., 2003). In addition, bacterial pathogens that do not grow on the selective media plates used in our analysis would not be considered in our

counts of mesophilic pathogenic bacteria; therefore, our counts, EFs, and percentages are conservative estimates. Nevertheless, culturing has the advantage of including only viable bacteria in the determination of EFs and percent pathogenic bacteria in marine aggregates.

Environmental Factors

In this exploratory field study, the characteristics of marine aggregates, including concentrations, patchiness, mean sizes, and volume fraction, varied across six shallow-water ecosystems, including salt ponds, harbors, and estuaries. Lack of overriding trends between aggregate characteristics and season, location, temperature, and salinity suggests that the "population" of marine aggregates is not only dynamic but also unique and specific to each shallow-water habitat. In addition, our results suggest that physical variables (e.g., currents, tides, or winds) and biological processes (e.g., productivity or zooplankton feeding) other than those measured in this study may be important in determining the physical characteristics of marine aggregates.

Environmental factors were important in influencing the biological characteristics of marine aggregates. Significant correlations were detected for salinity and the concentrations of THB in marine aggregate samples, enrichment factors for THB in marine aggregates, concentration of MPB in seawater samples, and percentages of MPB in seawater samples. These findings agree with previous work that has shown the distribution of aquatic pathogens and diseases correlates with salinity. For example, Louis et al. (2003) and Huq et al. (2005) both demonstrated that the occurrence of the human pathogen *Vibrio cholerae* was linked to environmental factors, including salinity (although working in environments with lower salinities than investigated in our research). For bivalve pathogens, Ragone-Calvo et al. (1998) observed that the prevalence of the hard clam pathogen QPX was associated with salinity, and Goedken et al. (2005) reported effects of salinity on the oyster parasite *Perkinsus marinus*. Significant correlations were also detected for temperature and the concentrations of MPB in marine aggregate samples and enrichment factors for THB and MPB (marginal significance) in marine aggregates. More intensive field studies, including more frequent sampling over smaller distances, will be necessary to further describe the physical and biological characteristics of marine aggregates in any system where aggregates have been identified as an important component in the ecology of a particular aquatic pathogen.

Conclusions

As reports of marine disease epidemics continue to increase (Ward and Lafferty, 2004), the need for comprehensive, risk-based surveillance programs will also increase. Many current surveillance programs monitor pathogens in seawater, sediments, and animals but fail to examine pathogens in marine aggregates. This field study coupled underwater video surveys with direct sampling of aggregated material for the purpose of evaluating characteristics of potentially pathogen-laden marine aggregates in shallow-water ecosystems. The general design of these surveys will be applicable to any water-borne pathogen of interest. Our characterization of marine aggregates has important implications in disease ecology because the size of the marine aggregates and their EFs affect the size of the inoculum to the host. In addition, measures of patchiness and volume fraction of aggregated material affect the encounter rates between embedded pathogens and suspension-feeding hosts. Furthermore, our research has demonstrated that (1) peaks in concentrations and sizes of marine aggregates varied among shallow-water habitats, (2) marine aggregates were frequently enriched with total heterotrophic bacteria and mesophilic pathogenic bacteria relative to seawater, and (3) several important pathogens were associated with marine aggregates. Finally, we used marine bivalve mollusks as our model organisms because they are both economically and ecologically important, but the concepts reported here pertain to other aquatic animals and illustrate the need to evaluate the role of aggregates as potential reservoirs of other aquatic pathogens.

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