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Lethal marine snow: Pathogen of bivalve mollusc concealed in marine aggregates

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Abstract

We evaluated marine aggregates as environmental reservoirs for a thraustochytrid pathogen, Quahog Parasite Unknown (QPX), of the northern quahog or hard clam, *Mercenaria mercenaria.* Positive results from in situ hybridization and denaturing gradient gel electrophoresis confirm the presence of QPX in marine aggregates collected from coastal embayments in Cape Cod, Massachusetts, where QPX outbreaks have occurred. In laboratory experiments, aggregates were observed and recorded by entering a quahog's pallial cavity, thereby delivering embedded particles from the water column to its benthic bivalve host. The occurrence of pathogen-laden aggregates in coastal areas experiencing repeated disease outbreaks suggests a means for the spread and survival of pathogens between epidemics and provides a specific target for environmental monitoring of those pathogens.

Marine aggregates (i.e., marine snow, flocs, organic detritus) ranging in size from a few microns to more than a centimeter are common in coastal environments where large populations of benthic, suspension-feeding invertebrates thrive. Aggregation of living and nonliving material is a natural process affected by a number of well-documented physical, chemical, and biological interactions (Alldredge and Silver 1988; Kiørboe 2001), and is recognized as an important mechanism for the transport of carbon, nutrients, and other materials to benthic ecosystems (Fowler and Knauer 1986; Alber and Valiela 1995). Although several studies have focused on the composition, formation, distribution, and fate of aggregates (for review, see Simon et al. 2002), none have addressed the role of aggregates in the transmission of diseases of marine animals. Here we provide evidence of marine aggregates facilitating disease transmission by serving as an environmental reservoir for a thraustochytrid pathogen of a bivalve mollusk.

Marine pathogens cause extensive ecological and economical damage (Harvell et al. 2002). To fully understand the impacts of marine diseases on organisms, populations, and ecosystems, it is essential to establish the modes of pathogen transmission (Harvell et al. 2004), including their reservoirs. Since the 1950s, northern quahogs, also known as hard clams and *Mercenaria mercenaria,* from the northeast coast of North America have suffered severe mortalities (30–100%) from a pathogen known as Quahog Parasite Unknown (QPX) (Whyte et al. 1994; Ragone-Calvo et al. 1998; Smolowitz et al. 1998). QPX infections typically occur in the quahog mantle, gill, and siphon tissues, indicating direct infection from seawater (Smolowitz et al. 1998). QPX is a small (4–25-μm), round, nonmotile thraustochytrid (Maas et al. 1999; Ragan et al 2000; Stokes et al. 2002). The thraustochytrids are a group of single-celled, fungal-like, marine protists associated with decaying vegetation, shells, and detritus (Ragukumar 2002). Because some nonpathogenic thraustochytrids may survive in marine aggregates, we investigated the possibility that QPX may also be located in aggregates. The presence of pathogen-laden aggregates in areas subjected to disease outbreaks would suggest a method for the spread and survival of pathogens between epidemics. Aggregates may then provide a specific target for environmental monitoring of those pathogens. The purpose of this article is to report the presence of a thraustochytrid pathogen (QPX) of an ecologically and economically important invertebrate species (*Mercenaria mercenaria*) concealed in marine snow.

Methods

Collection of marine aggregates—Marine aggregates were obtained from three locations in Barnstable Harbor, Massachusetts, in June, July, and August of 2004 and from two locations in Pleasant Bay, Massachusetts in July and August of 2004. Aggregates were collected once each month during low tides (average depth of water = 0.5–1 m), in sandy areas near quahog beds known to be infected with QPX. To obtain a sample, a three-pronged sheppard's hook was assembled and pushed into the sand. Polycarbonate Im-

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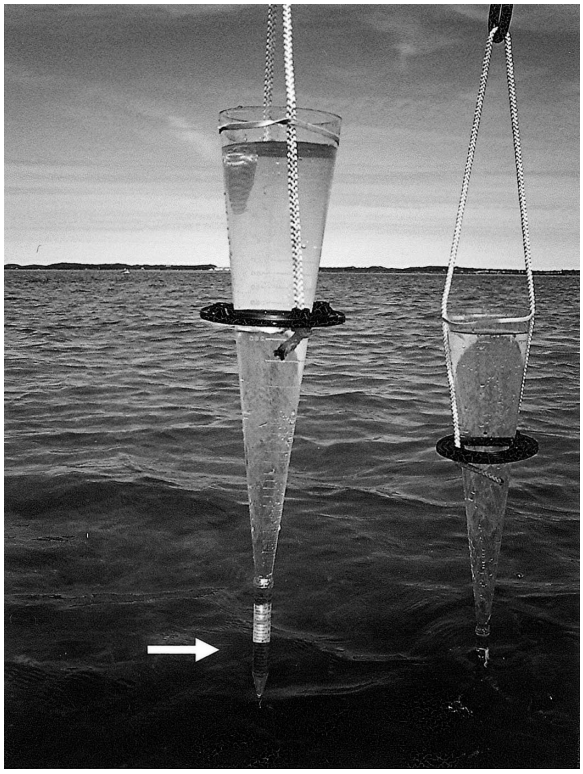

Fig. 1. Apparatus for collecting marine aggregates in shallow coastal habitats with soft substrates. A 15-mL Falcon tube (arrow) was secured to the bottom of a 1-liter settling cone, which was hung from a sheppard's hook using a ring-rope harness.

hoff settling cones were hung from each hook by use of a thick rubber band to keep the cone upright within its ring-rope harness. A 15-mL Falcon tube was secured to the bottom of each cone (Fig. 1). One liter of seawater was gently collected from just below the surface with a wide-mouth, tricorner beaker and transferred to the settling cone by tilting the cone and pouring as slow as was practical. The cone was then returned to the vertical position, and aggregated material was allowed to settle for 10 min. Triplicate samples were obtained for each location. Operationally defined ''aggregate-free'' seawater was removed by siphoning from the top of the cone. The last 15 mL (volume of the Falcon tube) was settled overnight in the refrigerator. The following day, the top 13 mL was removed with a pipette, and the final 2 mL was preserved in 6–8 mL of tissue storage buffer (0.25 mol L^{-1} ethylenediaminetetraacetic acid, 20% dimethyl sulfoxide, saturated NaCl) until analyzed by in situ hybridization (ISH; described below), or directly frozen (no tissue storage buffer) until analyzed by denaturing gel gradient electrophoresis (DGGE; described below).

Generation of QPX-enriched marine aggregates—QPX-enriched marine aggregates were generated in the laboratory by adding 8–10 mL of cultured QPX (culture maintained at Marine Biological Laboratory) to 1-liter jars of unfiltered seawater (22°C, salinity = 30, pH = 8.1) collected from Avery Point, Connecticut, in Long Island Sound, an area historically free of QPX mortalities. Jars were placed on a roller table (Shanks and Edmonson 1989) and rotated at room temperature for 2–4 days. After rolling, jars were removed from the roller table, and visible aggregates were allowed to settle for 10–15 min. Operationally defined aggregate-free seawater was siphoned out of the top of the jar, leaving ~150 aggregates suspended in a minimal amount of seawater (<2 mL total). Aggregates were combined, and aliquots were prepared for analysis as described below.

Analysis of marine aggregates for QPX—For ISH, aliquots of the aggregate mixture were dried according to a procedure for marine aggregate preparation for ISH (Grossart and Ploug 2001) and fixed according to a procedure for the detection of QPX in cell smears and paraffin-embedded bivalve tissue (Stokes et al. 2002). Aggregate aliquots were smeared onto Superfrost Plus slides, dried at 46°C for ~2 h, and fixed with fresh 8% paraformaldehyde overnight in a humid chamber. Paraformaldehyde was then decanted, and slides were rinsed three times with TE buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} ethylenediaminetetraacetic acid at pH 7.6), air dried, and stored frozen until analysis. Similar slides, made with pure QPX culture, were simultaneously prepared for visual comparison of ISH results. The ISH procedure used a cocktail of two DNA oligonucleotide probes (QPX641 and QPX1318) targeting the small subunit ribosomal RNA molecules (Stokes et al. 2002). The hybridization solutions contained 4 ng μL^{-1} of each digoxigenin-labeled probe except for a negative control that was incubated in hybridization buffer without probe. The hybridizations were followed by color development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. TE buffer and water rinses were used to stop the color development. Aggregate samples provided enough natural background color and contrast such that no counterstaining with Bismarck brown Y was necessary. Slides were cover-slipped with aqueous-based mounting solution, sealed with nail polish, and examined by using light microscopy (National DC3-163 digital microscope, 40× objective).

Total nucleic acids were recovered from marine aggregates by using the hot detergent/bead beating method of Kuske et al. (1998) as described in Gast et al. (2004). Polymerase chain reaction (PCR) products for DGGE were generated by using the eukaryote-specific 18S ribosomal GC-clamped primer 960FGC (Gast et al. 2004) and the QPX-specific primer QPXR2 (Stokes et al. 2002) following touchdown PCR and precipitation conditions as previously described (Gast et al. 2004). Perpendicular gel analysis indicated that a denaturing gradient of 45–75% was most useful for the 960FGC/QPXR2 product (~200 base pairs in length). Five microliters of sample were loaded per lane, along with a sample of PCR product generated from the QPX organism in culture. The gel was run overnight at 100 V, stained with ethidium bromide, and photographed. Bands corresponding to material from field samples (marked with an ''o'' in Fig. 3) were recovered from the gel, reamplified by using the non-GC-clamped 960F (Gast et al. 2004) and QPXR2 primers, and sequenced by using the 960F primer and ABI BigDye terminators (PE Applied Biosystems) on an ABI 377 DNA sequencer to assess their taxonomic affiliation.

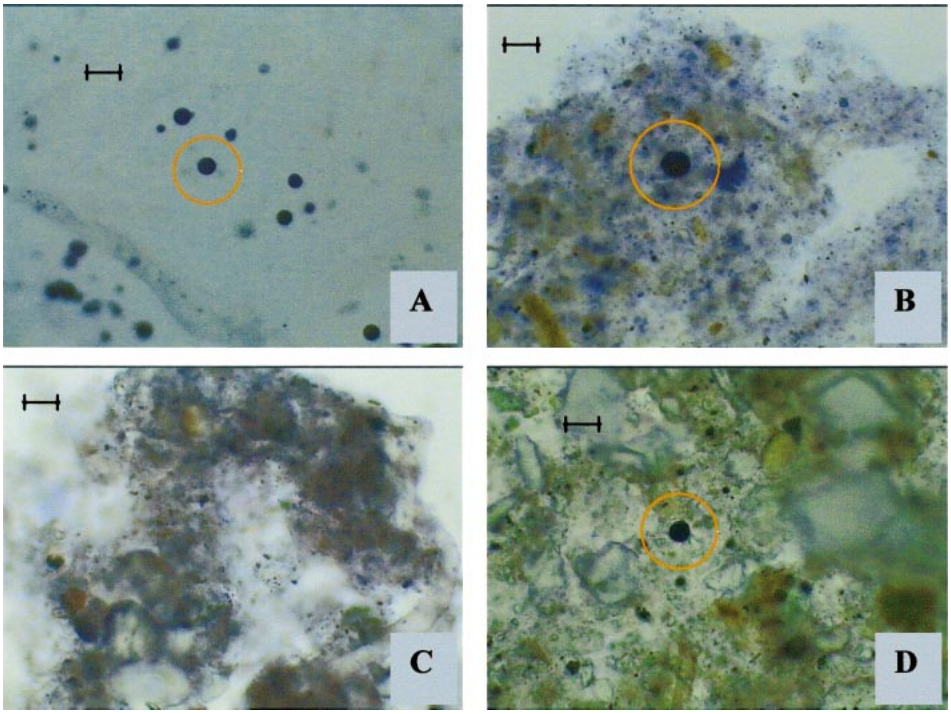

Fig. 2. Examples of in situ hybridization results for Quahog Parasite Unknown (QPX). (A) Positive results for QPX culture. (B) Positive results for laboratory-generated aggregates made with seawater and QPX culture (positive control). (C) Negative results for laboratory-generated aggregates made with seawater with no culture added (negative control; no dark round spheres present). (D) Positive results for natural aggregates collected near quahog beds infected with QPX. Each darkly stained sphere within a red circle is one QPX thallus. Scale bars, 25 μm.

Video-endoscope techniques—Endoscopy was performed according to methods described previously (Ward et al. 1991). The endoscope (Olympus K-17 series), with 1.7-mm-diameter optical insertion tube, was connected to an optical zoom-adapter and attached to a color, charge-coupled device camera (Cohu, Inc.). The resolution of the video endoscope was approximately 3 μm at a maximum magnification of ~150×. Video signals were recorded on an 8-mm video cassette recorder (Sony Hi8). Marine aggregates were generated as described above but with the incorporation of 10-μm yellow-green fluorescent beads (instead of pathogens) as tracers for pathogens, which were fed to the bivalves. The optical insertion tube of the endoscope was maneuvered above the incurrent siphon of a quahog that was partially buried in a container of sand submerged in a small, aerated aquarium.

Results

We compared ISH results for pure QPX culture (dark spheres in Fig. 2A) to ISH results for aggregates generated in the laboratory with (positive control; dark sphere in Fig. 2B) and without QPX culture (negative control; no dark spheres in Fig. 2C). These laboratory-generated, artificially enriched aggregates were used as visual comparisons for evaluating the occurrence of QPX in natural marine aggregate samples. Positive ISH results revealed QPX thalli embedded in natural marine aggregates (Fig. 2D) from all samples collected near quahog beds known to be infected with QPX.

To confirm our discovery, representative aggregate samples were also processed for DGGE. These aggregate samples indicated the presence of QPX-specific DNA fragments (Fig. 3). Bands (accession numbers DQ083533 to DQ08538) were recovered from the gel and sequenced. All bands yielded sequences that were confirmed by Blast analysis and sequence alignment as having significant similarity to QPX ribosomal sequences from GenBank (Fig. 4). The bands at positions other than the QPX control band in aggregate samples still have significant similarity to the QPX sequence (Fig. 4). The presence of these additional bands can result for several reasons. DGGE is able to separate fragments with a single base difference, so the multiple bands in the natural samples may represent either natural strain variation or variability within the ribosomal repeat unit. When multiple sequences that are very similar are the target for amplification, they can form heteroduplexes during the repeated rounds of PCR (Kanagawa 2003). These heteroduplexes occur as multiple bands in the same sample on DGGE, but they show very little sequence variation. We were unable to confirm whether multiple strains, repeat unit variation, or PCR error was the source of the additional bands in the natural samples.

Chimeric sequences are another artifact that can occur in the PCR amplification. These occur through incompletely extended products acting as primers or through template switching during the extension phase (Kanagawa 2003). It

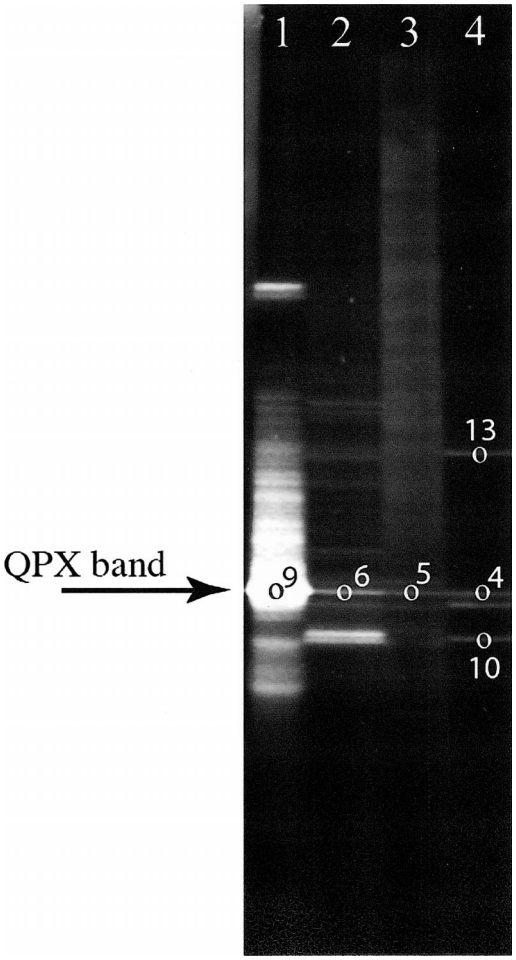

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) results for marine aggregates. Lane 1, QPX culture; lanes 2–4, aggregate samples from Barnstable Harbor. The band indicated by the arrow corresponds to the QPX culture–positive control band. Bands marked with an ''o'' were recovered from the gel, reamplified, and confirmed by Blast analysis and sequence alignment as being QPX. Bands are identified as 102004.4, 102004.5, 102004.6, 102004.9, 102004.10, and 102004.13.

seems unlikely that chimeric molecules would be generated on such a small fragment, especially as our extension times are long enough to ensure full-length amplification and our amplification cycle number is low, at least for the first two rounds of amplification. Unfortunately, it would likely be very difficult to identify chimeric sequences if they did occur because the target sequences are so similar to each other.

The additional bands in the QPX culture lane could also arise owing to repeat unit variability, but we have not determined whether this is the case. We believe that much of the banding pattern here occurs owing to PCR artifact and overloading of the sample on the gel. Dilution of the sample has, of course, reduced the number of additional bands that are visible, as has reduction of the number of amplification cycles.

Observations from the video endoscope revealed that all aggregates smaller than the diameter of the siphon entered the quahog's pallial cavity, thereby delivering embedded particles from the water column to the tissues of the quahog.

Discussion

Aggregates are ubiquitous in the marine environment (Alldredge and Silver 1988), but they are often overlooked in field studies because they are destroyed by traditional sampling equipment such as plankton nets and other techniques that homogenize bulk water samples before analysis. Although the preferred method for aggregate collection is via scuba divers with wide-bore syringes (Alldredge 1979), the shallow depth of water in tidally driven embayments prohibits the use of this method. Instead, the use of the settling cones with attached Falcon tubes provides a quick and reliable method to acquire sufficient material for testing for the presence or absence of pathogens. Although our method generally undersamples the microaggregates (aggregates <500 μm) that do not settle as quickly as do the marine-snow–size aggregates (aggregates >500 μm), it also limits sampling of individually suspended cells that settle slower than do the larger aggregates. In addition, it is the larger, more rapidly sinking aggregates that the benthic, suspension-feeding quahogs are more likely to encounter.

Marine aggregates are a relatively unexplored link between waterborne pathogens and their benthic hosts. To our knowledge, these results are the first documentation of QPX in the environment outside of quahogs, as well as the first report of a protistan (thraustochytrid) pathogen embedded in marine aggregates. The thraustochytrids are important ecological group in coastal environments, but comparatively little is known about their taxonomy and phylogeny (Ragukumar 2002). Thus, the specificity of the amplification primers needs to be addressed. The FA2/RA3 and QPXF/QPXR2 primers were designed and tested by other researchers (Mo et al. 2002; Stokes et al. 2002). We re-examined their specificity relative to sequences in current databases through Blast (Altschul et al. 1997) searches of GenBank and Check Probe at the Ribosomal Database Project (Cole et al. 2005). Our searches found no strong matches for the QPX-specific primers other than the QPX organism, but the primers that were supposed to be thraustochytrid-specific did match a significant range of other organisms. It is likely that the first amplification would cross-react with an unknown organism in the environment, but the second and third rounds of amplification are more specific. To date, all of the bands that we have recovered and sequenced from marine aggregate samples represent QPX-like organisms.

We focused on a thraustochytrid pathogen, but the concept of pathogens persisting in and being transported by marine aggregates applies to other pathogens, including those infective to humans and those entering the marine ecosystem from the terrestrial environment. Aggregates have been reported to harbor human bacterial pathogens such as *Vibrio parahaemolyticus* (found in ''sinking particles'') (Venkateswaran et al. 1990) and *Vibrio cholerae* (found in ''particulates >20 μm'') (Colwell et al. 2003). Aggregates are microscale ecosystems with relatively higher levels of productivity and biomass than that of the surrounding sea-

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T. sp. C9G       ............G......A................................T.............
T. pachydermum   ......--..........GA................................T.............
QPX Stokes       CTTACCAGGTCCAGACATAGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCA
QPX Ragan        CTTACCAGGTCCAGACATAGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCA
QPX Maas         CTTACCAGGTCCAGACATAGGAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCA
102004-4         -.........T.....................................................................
102004-5         ---------------------------------------------------------------------------
102004-6         -------.........................................................................
102004-9         -...............................................................................
102004-10        ................................................................................
102004-13        ................................................................................


T. sp. C9G       ...................TT...A.................................G..........
T. pachydermum   ................................................................................
QPX Stokes       TGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTACTAAA
QPX Ragan        TGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTACTAAA
QPX Maas         TGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTACTAAA
102004-4         ................................................................................
102004-5         ---------.......................................................................
102004-6         ................................................................................
102004-9         ................................................................................
102004-10        ..............................W.................................................
102004-13        ..............................W.................................................


T. sp. C9G       ............GA....TC......
T. pachydermum   .....A.AT...AA....TT......
QPX Stokes       TAGTACTGCTTTTCGCAAGAAAGGTA
QPX Ragan        TAGTACTGCTTTTCGCAAGAAAGGTA
QPX Maas         TAGTACTGCTTTTCGCAAGAAAGGTA
102004-4         ..........................
102004-5         ..........................
102004-6         ..........................
102004-9         ..........................
102004-10        ..........Y...............
102004-13        ..........Y...............
```

Fig. 4. Alignment of sequences from aggregate bands with a portion of the QPX and thraustochytrid ribosomal genes from GenBank. The fragment corresponds to positions 1164–1331 in the QPX sequence. *Thraustochytrium* sp C9G (AF474172) is a thraustochytrid sequence recovered from a clam. *Thraustochytrium pachydermum* (AB022113) and *Thraustochytrium* sp. C9G are the most similar sequences to QPX. QPX Stokes (AY052644), QPX Ragan (AF261664), and QPX Maas (AF155209) represent sequences for the QPX organism isolated independently and from different locations. ''–'' indicate gaps; periods, the same base as the QPX sequence; and letters, substitutions.

water (Alldredge and Silver 1988; Simon et al. 2002), and would provide a means for pathogen survival and transport between epidemics by serving as environmental reservoirs. Pathogens with reservoirs in the environment have the potential to infect and kill all of their hosts because the pathogens would not be limited by the density of the host species (Harvell et al. 2004). Pathogens that exploit aggregates as reservoirs will be difficult to control because marine aggregates can not be eliminated from the ecosystem, but the marine aggregates may provide an opportunity for targeted surveillance of those embedded pathogens.

Examples of marine diseases with vectored transmission are scarce compared with terrestrial diseases that rely on flying insect vectors for dispersal (Harvell et al. 2004). We hypothesize that marine aggregates also function as vectors (i.e., vehicles of transmission) linking pathogens in seawater to their benthic hosts. QPX is a nonmotile, internal parasite that requires a means of transport to its quahog host. Observations by means of video endoscopy revealed that all aggregates, smaller than the diameter of the siphon, are drawn into the pallial cavity of the quahog. Suspension-feeding bivalves preferentially ingest and reject particles acquired from filtering seawater (Ward and Shumway 2004). For quahogs, those particles (including aggregates) that are not ingested are collected at the base of the incurrent siphon until periodically expelled as pseudofeces (Grizzle et al. 2001). Because the base of the incurrent siphon is also a location of localized inflammatory nodules in quahogs infected with QPX (Smolowitz et al. 1998), we propose that marine aggregates facilitate direct infection of this tissue by delivering pathogens to this site.

To date, no studies have evaluated the role of marine aggregates in the uptake of pathogens by suspension-feeding invertebrates, but a few studies have examined the role of marine aggregates as a food resource. In laboratory experiments, Alber and Valiela (1995, 1996) examined incorporation of nitrogen by bivalves fed aggregates produced from organic matter released by marine macrophytes. They found that scallops and mussels could gain some organic matter when in the form of aggregates and suggested that this might be an important pathway in the detrital food web. In field surveys, Graf et al. (1982) reported increases in glycogen and lipid resources of bivalves after the settling of aggregates formed after the spring phytoplankton bloom. These studies support our contention that suspension-feeding bivalves come in contact with and process marine aggregates and, in doing so, could be infected with embedded pathogens. Other marine invertebrates that use organic aggregates as a food resource (e.g., zooplankton) (Dilling et al. 1998

and references therein) may also be susceptible to infections via this process.

As reports of marine disease epidemics continue to increase (Harvell et al. 2002, 2004; Ward and Lafferty 2004), the need for comprehensive surveillance programs will also increase. Many current surveillance programs monitor pathogens in seawater, sediments, and animals but fail to evaluate the role of marine aggregates. We believe that one path for the transmission of diseases to benthic, suspension-feeders flows through aggregated material. We used bivalves as our model organisms because they are both ecologically and economically important, but the concepts reported here apply to other marine animals and demonstrate the need to evaluate the role of marine aggregates as reservoirs and vectors of disease.

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