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FEATURE ARTICLE

Theory of island biogeography on a microscopic scale: organic aggregates as islands for aquatic pathogens

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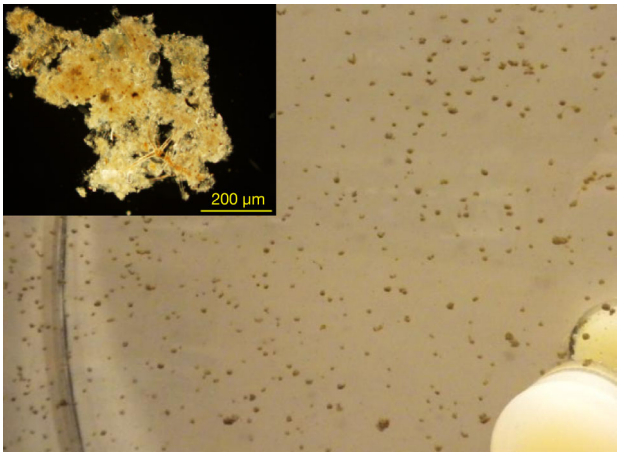
ABSTRACT: Four predictions of the MacArthur-Wilson theory of island biogeography were evaluated to assess the degree to which detrital-based organic aggregates (e.g. marine snow, organic detritus, and bioflocs) may provide a favorable microhabitat (i.e. an 'island') for bacteria in general, and specifically aquatic pathogens. We demonstrate the theory's relevance for microbial communities in aquatic environments by describing the community metabolic response and functional diversity of individual organic aggregates while documenting the persistence of potential pathogens and fecal indicator bacteria. Our results support the 4 predictions, including a significant species–area relationship, consistency of species richness at equilibrium, non-zero level of species turnover at equilibrium, and variance to mean ratios of less than 0.5 at equilibrium. The aggregate-associated microbial communities demonstrated significantly higher rates of metabolic response and functional diversity, and contained higher concentrations of culturable vibrios and fecal indicator bacteria compared to aggregate-free water, supporting the idea that organic aggregates are sites of favorable habitat surrounded by a less favorable matrix. These results substantiate that organic aggregates may be represented as microscopic islands. Using island biogeography theory to understand the microbial ecology of aquatic pathogens associated with organic aggregates is important with respect to environmental sampling of recreational waters and mathematical modeling of the transmission of waterborne diseases from aquatic reservoirs to humans.

KEY WORDS: Organic aggregates · Island biogeography · Functional diversity · Aquatic pathogens · *Vibrio* · Fecal indicator bacteria



Organic aggregate (light micrograph inset and dark spots in main photo) produced in 10 l rotating tanks function as islands for aquatic bacteria, including potential pathogens

Photo: M. M. Lyons

INTRODUCTION

The term 'organic aggregate' is a general expression that encompasses multiple kinds of aggregated material suspended in aquatic systems, including marine, lake and river snow, macro- and microaggregates, organic detritus, flocs and bioflocs (see Fig. 1A,C,D). Aggregation of living, dead, and inorganic particles in aquatic ecosystems is a natural process influenced by numerous biological, chemical, and physical interactions, and affects the net transport of carbon, nutrients, metals, and other materials from the water column to

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benthic habitats (Fowler & Knauer 1986). For decades the biology and ecology of organic aggregates has been studied in the context of oceanography (e.g. Alldredge 1979, Silver & Alldredge 1981, Alldredge & Cohen 1987, Lampitt et al. 1993, Azam & Long 2001), environmental microbiology (e.g. Riley 1963, Caron et al. 1986, Logan & Hunt 1987, Herndl 1988, Azam et al. 1994, Kiørboe 2003, Grossart et al. 2006, Yam & Tang 2007), the distribution of metals (e.g. Cowen & Silver 1984, Hebel et al. 1986), phytoplankton ecology (Riebesell 1991, Kaltenböck & Herndl 1992, Kiørboe & Hansen 1993, Silver et al. 1998, Thornton 2002), mesoplankton and benthic trophic interactions (e.g. Bochdansky & Herndl 1992, Alber & Valiela 1996, Shanks & Walters 1997, Kiørboe & Thygesen 2001), and carbon and nutrient cycling (e.g. Shanks & Trent 1979, Cho & Azam 1988, Alldredge & Gotschalk 1990, Brzezinski et al. 1997, Ploug et al. 1999, Alldredge 2000, Kiørboe 2001). Detailed reviews of the processes governing aggregation (Eisma et al. 1991, Jackson & Burd 1998, Burd & Jackson 2009) and the microbial ecology of aggregates (Alldredge & Silver 1988, Simon et al. 2002, Turner 2002) have summarized the breadth of information regarding these ubiquitous and dynamic conglomerations of living and nonliving particles.

Although the importance of marine, estuarine, and freshwater aggregates continues to be an area of active research (e.g. Guidi et al. 2008, Kach & Ward 2008, Ploug et al. 2008, Stemmann et al. 2008, Engel et al. 2009, Stevens et al. 2009, Ward & Kach 2009), the role of organic aggregates in the ecology of aquatic pathogens is only now beginning to be evaluated (Lyons et al. 2007, Lyons 2008). For example, aggregates have been shown to be an environmental reservoir for the eukaryotic hard clam pathogen, Quahog Parasite X (QPX; Lyons et al. 2005, Lyons 2008), and to contain *Vibrio parahaemolyticus* (Venkateswaran et al. 1990) and *V. cholerae* (Colwell et al. 2003). Elsewhere, Lyons et al. (2007) also found these 2 bacterial species in aggregates and expanded the list of potentially harmful, aggregate-associated bacteria to include: *V. vulnificus, V. alginolyticus, Escherichia coli*, *Enterococcus* sp., *Mycobacterium* sp., *Aeromonas hydrophila, Pseudomonas aeruginosa, Photobacterium damselae*, *Shigella sonnei, Stenotrophomonas maltophila,* and *Burkholderia cepacia.* These species include potential pathogens (and pathogen indicators) associated with sewage-related pollution events and natural aquatic microbial communities, yet little is known about the community ecology of aggregates with respect to these pathogens.

A fundamental part of disease risk is exposure. Accordingly, to understand the disease risk presented by aggregate-associated pathogens, it is important to understand the processes that determine the number and diversity of species associated with an aggregate or collection of aggregates. In this study, we adopt the theory of island biogeography (MacArthur & Wilson 1963, 1967) as a conceptual framework for understanding the accumulation of bacterial species on aggregates. Originally, island biogeography theory was developed to explain the composition of biological communities found on oceanic islands. The theory predicts a dynamic equilibrium between colonization of new species and extinction of resident species in which the total number of species (i.e. species richness) is an increasing function of island size and a decreasing function of the distance to a source of potential colonizers (e.g. continental mainland). Previous applications include the species richness of birds on California's Channel Islands (Diamond 1969, Jones & Diamond 1976) and of ants within the Malaysian Archipelago (Wilson 1959), re-colonization of islands following volcanic eruptions (Krakatau Islands; Bush & Whittaker 1991, Thornton 1996), and intentional depopulations (mangrove islands in Florida Keys; Simberloff & Wilson 1970). More recently the theory of island biogeography has been extended to other ecosystems with high-quality habitat surrounded by less-suitable habitat, such as mountains bounded by deserts (Lomolino et al. 1989, Kebede et al. 2007), and ecological reserves encircled by urban environments (Fore & Guttman 1999, Ohmura et al. 2006). In contrast with the many applications of island biogeography to macro-organisms, the theory has seldom been tested on a smaller scale for microbial communities (for exceptions see Kinkel et al. 1987, Bell et al. 2005, Reche et al. 2005), and to our knowledge, not in the context of disease ecology.

Here, we argue the theory's relevance for microbial communities in aquatic environments by describing the community metabolic response and functional diversity of individual organic aggregates while monitoring the persistence of potential pathogens and fecal indicator bacteria. Four predictions of island biogeography theory were evaluated including (1) species–area relationship, (2) consistency of the number of species, (3) degree of species turnover, and (4) variance to mean ratio of species diversity at equilibrium (Gilbert 1980, Brown & Dinsmore 1988). Other predictions of the theory, including the distance from a source and colonization of new (e.g. sterilized) islands, are the focus of ongoing experiments not described in this paper. Our long-term objective is to apply this community-level approach to the study of waterborne diseases and illnesses by evaluating the degree to which detrital-based organic aggregates may provide a favorable micro-habitat (i.e. an 'island') for aquatic pathogens. If so, aggregates may facilitate persistence, prevalence, and dispersal of aquatic pathogens in

nature. Furthermore, an improved understanding of the processes involved will inform and influence environmental sampling and mathematical modeling of aquatic pathogens.

MATERIALS AND METHODS

Source water. Surface water (15 l, salinity 15 psu) was collected from Knitting Mill Creek (KMC), Virginia, USA (36.89° N, 76.29° W). The creek is a small branch of the Lafayette River, a tributary of the Elizabeth River, which ultimately empties into the mouth of the Chesapeake Bay. Water was collected in a sterile container from an area near (~1 m) a storm-water drainage pipe.

Generation of aggregates. Aggregates (Fig. 1A, C,D) were generated in a rotating 10 l cylindrical tank (Fig. 1B, 40 cm diameter, 8 cm width) modified from Shanks & Edmonson (1989). The tank was filled with source water (well mixed) and rolled at 1.5 rpm for 35 d at room temperature.

Aggregate sampling. On Days 1, 2, 7, 14, and 35 the tank was temporarily removed from the rolling table and placed horizontally on a calibrated white background for photography (see 'Materials and methods: Image analysis') and sample collection. Individual aggregates (n = 6) of similar size (2 to 4 mm long axis) were collected, one at a time, through the tank's center port with minimal surrounding water (<0.1 ml) using sterile 1 ml disposable pipettes. Individual aggregates were then transferred to labeled 15 ml centrifuge tubes in which each aggregate was diluted to 10 ml with sterile (autoclaved and 0.2 µm filtered) KMC water. This dilution was used for both the community composition analysis and the culturable vibrios assay (see sections 'Microbial community composition' and 'Concentration of culturable vibrios (TCBS)'). A total of 30 samples (i.e. 5 d × 6 aggregates per day) were used for all statistical analyses of aggregate data . Additional aggregates (n = 3) were collected to evaluate the concentration of *Escherichia coli* in the aggregate-associated microbial communities (total n = 15).

Water sampling. Water samples with no aggregates visible to the naked eye (i.e. operationally defined 'aggregate-free' water) were collected from the center port of the tank using sterile 10 ml disposable pipettes. The starting water (Day 0 for both aggregate and aggregate-free samples) included 1 sample based on 3 replicates, whereas on all other days, 6 samples (10 ml each) were collected for determination of community composition and concentration of culturable vibrios. These water

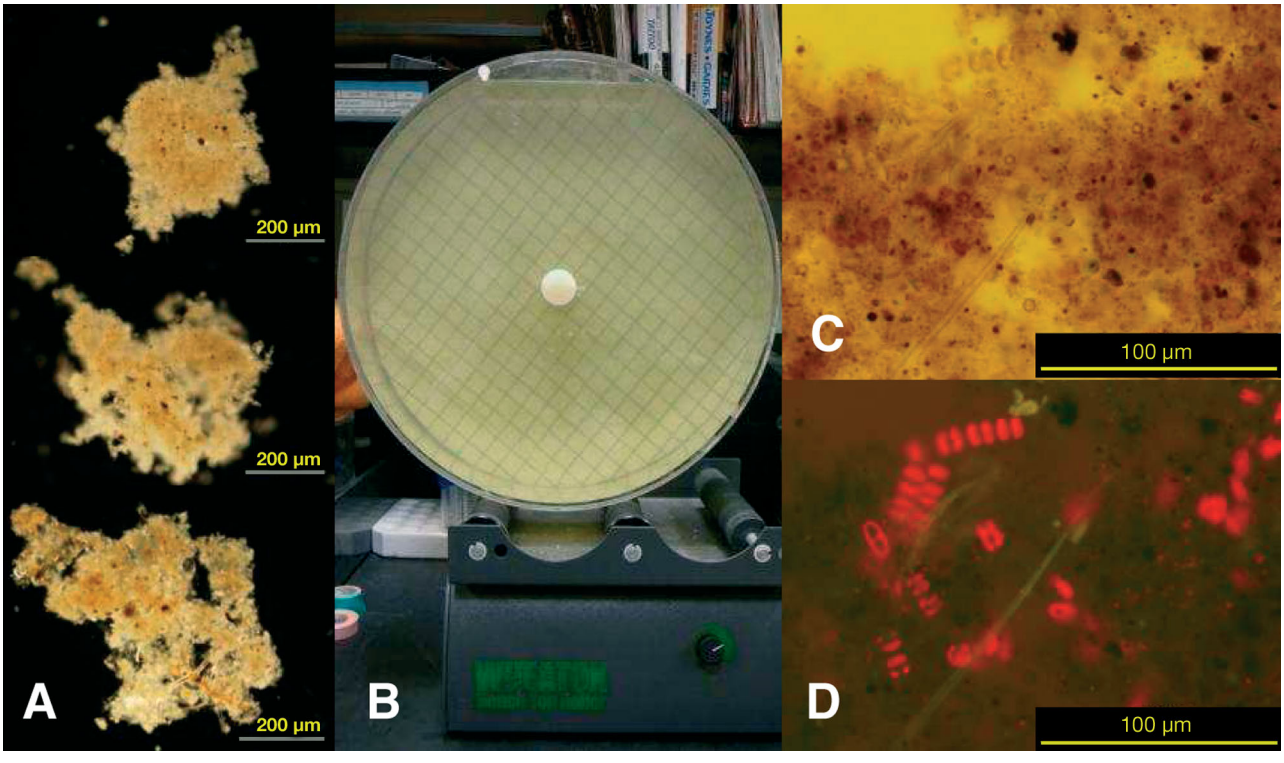

Fig. 1. (A) Concurrently created aggregates typically differing in size, shape, and complexity. (B) Aggregates were made from natural waters in large cylindrical tanks (diameter = 40 cm) that were rotated (1 to 2 rpm for 1 to 35 d) on bench top roller tables (Shanks & Edmonson, 1989). Resulting aggregates contained a variety of plankton as seen with a microscope under (C) transmitted light and (D) with epifluorescent blue-light excitation (same field as C)

samples were processed without dilution. A total of 31 water samples (5 d × 6 samples per day, plus 1 initial water sample) were collected and used for statistical analysis with the exception of the assessment of community metabolic response and functional diversity for which 28 samples were used (due to loss of 3 samples during processing). Additional 10 ml samples (n = 3) were collected on each sampling day to evaluate the concentration of *Escherichia coli* in the aggregate-free microbial communities (total n = 18). After sampling was completed, a similar volume of sterile KMC water (~90 ml) was added back to the tank to maintain a constant volume. Although this additional water diluted the microbial community in the 10 l tank, the effect is likely to be small given the size of the tank relative to the amount of the water added (i.e. < 1 % per time point).

Image analysis. With the tank in the horizontal position, individual aggregates were photographed and analyzed using previously developed image analysis procedures for marine aggregates (Lyons et al. 2007). Digital color photographs were uploaded to an image analysis program (ImageJ; available at http://rsbweb.nih.gov) and converted to 8-bit gray scale. Aggregates were identified, numbered, counted, and sized using a binary threshold and an automated particle counter. Results were manually verified against the original photographs (Fig. 2A–D). Several metrics of size, including long and short axes, surface area, and perimeter were recorded. Volumes of individual aggregates were calculated using the long and short axes of each aggregate and the equation for volume (V) of an ellipsoid ($V = 4/3\ \pi ab^2$; a: ½ long axis, b: ½ short axis).

Microbial community composition. Biolog EcoPlate™ microplates (Biolog, Inc.) were used to characterize and compare the aggregate-associated and

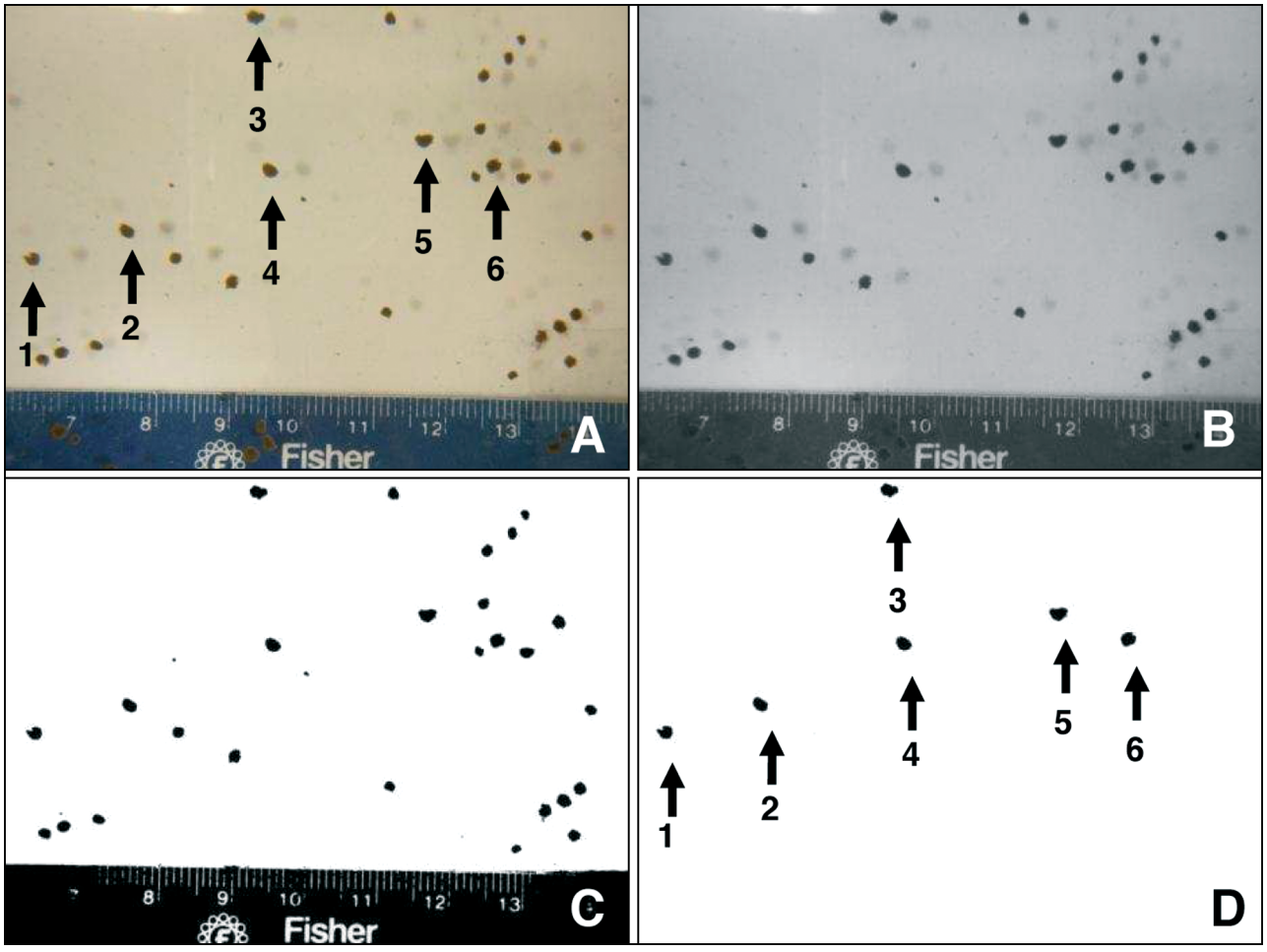

Fig. 2. Individual aggregates were measured (e.g. long axis, surface area, volume) via image analysis prior to being collected with sterile 1 ml disposable pipettes. Selected aggregates (1 to 6) were identified on digital color photographs (A) before being converted to an 8-bit gray-scale (B). A binary threshold was used to produce dark particles on a light background (C; notice shadows, located to the right of aggregates [in A,B], were eliminated by this procedure). An automatic particle analyzer (ImageJ) was used to determine the size of selected aggregates (D), the locations of which were then verified against the original photographs (Lyons et al. 2007)

aggregate-free heterotrophic microbial communities. EcoPlate™ microplates consist of 3 replicate sets of 31 carbon substrates (see Table 1), predominantly amino acids, carbohydrates, and carboxylic acids, individually arrayed in a 96-well format. Each well also contains a minimal growth medium and tetrazolium violet dye. The redox dye turns purple in the presence of electron transfer, indicating utilization of the substrate by inoculated microbes (Bochner 1989). A control well contains no sole-carbon substrate, thus any color development indicates utilization of carbon sources inherent in the inoculated water or storage polymers of microbes. EcoPlate™ microplates have been shown to be effective at discriminating among aquatic heterotrophic microbial communities (Choi & Dobbs 1999). Preliminary experiments (data not shown) revealed that pooling aggregates prior to dilution yielded consistent results among replicate wells, but that diluting a single aggregate to the necessary volume required to fill all 96 wells of an EcoPlate™ did not (presumably because excessive dilution reduced heterotrophic expression of the microbial community below threshold levels). Consequently, individual aggregates were only diluted enough to fill one set of 32 wells per plate. Samples of aggregates (diluted) and aggregate-free water (undiluted) were vigorously shaken and vortexed (30 s) before 150 µl aliquots were inoculated into each well. Optical density (λ = 590 nm) of each well was determined immediately (time = 0 h) and after 3 d (time = 72 h) of dark incubation at room temperature (20°C) with a BioTek plate reader (model ELX800, BioTek). Bacterial concentrations of the aggregates and aggregate-free water were not determined. Christian & Lind (2006) showed no correlation, after 72 h incubation, between optical density of wells and bacterial concentration of the starting inoculum (i.e. if the community can utilize a substrate, it will have done so by then). Average well color development (AWCD, measure of average community metabolic response) was calculated in accordance with Garland & Mills (1991) after subtracting the starting values of each EcoPlate™ microplate from its 72 h readings (ΔAWCD) to account for intrinsic differences in the absorbance of the carbon substrates (Insam & Goberna 2004). The average (n = 6 per time point) ΔAWCD (i.e. difference between times 0 and 72 h) was used to assess and compare community metabolic response and functional diversity of microbial communities in aggregates and aggregate-free water samples. The number of substrates utilized by the microbial community of the sample (i.e. a measure of functional diversity, Zak et al. 1994) was determined by comparing the change in well color development (adjusted for the control well) to a threshold value (0.250 optical density). Readings greater than the threshold were counted as substrates used by the community in the sample, whereas readings less than or equal to the threshold value were scored as substrates not used. Species turnover was assessed by comparing the specific substrates used by the aggregate-associated communities at each of 4 consecutive time points (Days 0, 1, 2, 7) when no significant differences were detected in the number of substrates used (i.e. equilibrium, see section 'Results'). To do so, ΔAWCD values of the 6 aggregates at each time point were averaged, then compared to the threshold (as described above) to determine if the substrate was utilized or not utilized. Finally, variance to mean ratios of the functional diversity (i.e. number of substrates used) of aggregate-associated microbial communities were calculated for each set of 6 aggregates collected at each of the 4 time points.

Concentration of culturable vibrios (TCBS). Triplicate 100 µl aliquots of the 10 ml dilutions of individual aggregates (n = 6) and the 10 ml samples of undiluted aggregate-free water (n = 6), were spread-plated onto individual thiosulfate citrate bile salts sucrose (TCBS) agar plates. Plates were incubated overnight at 35°C, after which colony forming units (CFUs) of sucrose-fermenting vibrios (i.e. yellow colonies) and total vibrios (i.e. yellow and green colonies) were counted and recorded. Sucrose-fermenting species of the genus *Vibrio* comprise several potential pathogens including, *V. cholerae, V. alginolyticus, V. harveyi, V. cincinnatiensis, V. fluvialis, V. furnissi,* and *V. metschnikovii.*

Concentration of fecal indicator bacteria (*Escherichia coli*). Triplicate samples of aggregates (diluted 1:999) and aggregate-free water (diluted 1:9) were analyzed for *E. coli* using Colilert-18 (IDEXX Laboratories) to enumerate most probable number (MPN) values for *E. coli* in the aggregate-associated and aggregate-free microbial communities.

Statistical analysis. We used Minitab® for all statistical tests. When exploring a functional or predictive relationship between variables, we used regression analysis (general linear model, GLM), whereas when estimating their degree of association, we used correlation analysis (Sokal & Rohlf 1981). We analyzed aggregate sizes using 1-way ANOVA (factor was Day). We tested average community metabolic response, functional diversity, and bacterial concentrations using 2-way ANOVA (with interaction); factors were Day (0, 1, 2, 7, 14, and 35) and Sample type (aggregates and water). Counts of bacteria (vibrios and *E. coli*) were not normally distributed and were log-transformed before ANOVA. All counts <1 colony forming unit (CFU) ml^{-1} or <1 most probable number (MPN) ml^{-1} were assigned a value of 0.1 prior to transformation. Results were considered significant when the calculated p-value was less than or equal to $\alpha = 0.05$.

RESULTS

Aggregate size

Aggregates ranged in size (long axis) from 1.9 to 3.7 mm with surface areas from 2.6 to 8.6 mm^2 and volumes of 0.024 to 0.137 ml. The sets of 6 aggregates collected on each of the 5 sampling days were not significantly different in size (i.e. no relationship between sample day and size, n = 30) as measured by long axis (p = 0.095), area (p = 0.149), or volume (p = 0.903). Over the small range of aggregate sizes evaluated, surface area was weakly (r^2 = 0.160), but significantly (p = 0.016, n = 30) related to average community metabolic response (ΔAWCD) and functional diversity (number of substrates used; r^2 = 0.101, p = 0.049, n = 30) (Fig. 3A).

Average community metabolic response

A 2-way ANOVA detected highly significant effects of Day (p < 0.001, n = 58), Sample type (p < 0.001, n = 58), and a significant interaction (p = 0.009, n = 58). For all sampling days, the average community metabolic response, measured as the change in the average well color development (ΔAWCD), was significantly greater in aggregates compared to water (see asterisks in Fig. 4A), indicating a greater utilization of more substrates

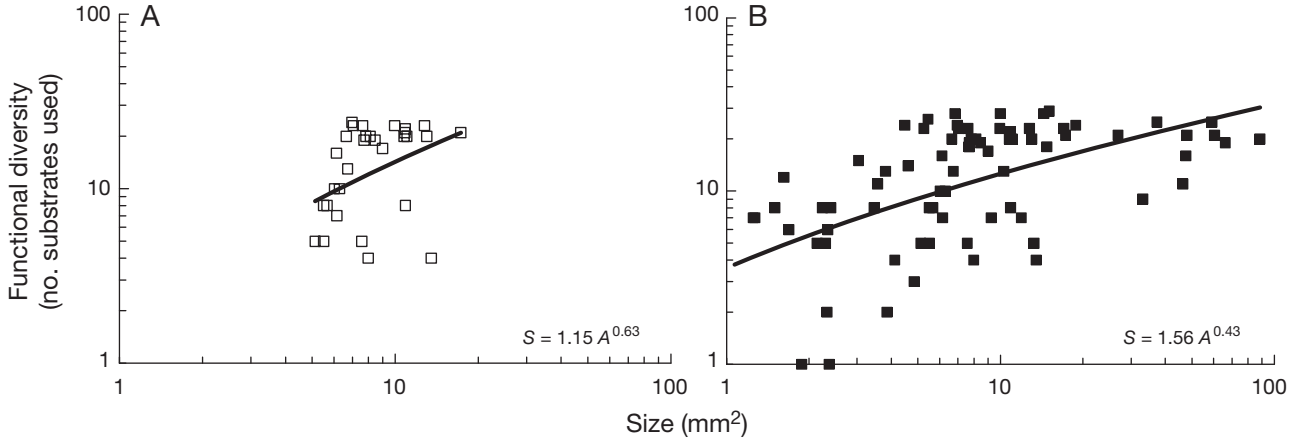

Fig. 3. Functional diversity (number of substrates used), a proxy for number of species (*S*), was significantly related to the size (area; *A* in equations) for (A) aggregates surveyed in this experiment (n = 30) and for (B) all aggregates evaluated to date (n = 79) (see 'Discussion: Species–area relationship' for details)

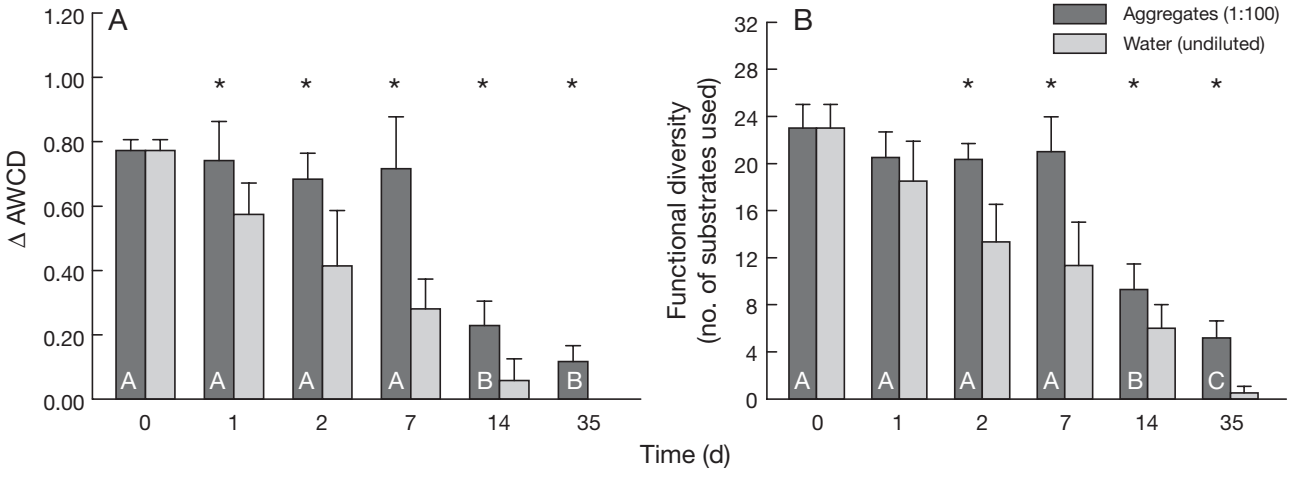

Fig. 4. (A) Community metabolic response, measured by ΔAWCD (i.e. change in the average well color development), and (B) functional diversity, measured by the number of substrates used, remained relatively constant in aggregates (darker bars) for at least 1 wk (i.e. no significant differences for Days 0, 1, 2, 7) as compared to operationally defined 'aggregate-free' water (lighter bars) which steadily declined in both cases. Letters on darker bars: significantly different values among aggregates; ⋆: significantly different concentrations between the aggregate-associated and the aggregate-free (water) microbial communities sampled at that time point. Significance was determined without factoring in the dilution of the aggregates (~2 orders of magnitude)

by microbes associated with aggregates. The metabolic response steadily declined in the water samples over the duration of the experiment, whereas the response in aggregates was maintained for at least 1 wk (i.e. no significant differences among Days 0, 1, 2, 7) before declining on Days 14 and 35 (see letters on dark bars in Fig. 4A). The largest difference in response between the 2 types of microbial communities occurred on Day 7. Metabolic response was significantly related to all metrics of size including long axis ($r^2 = 0.134$, $p = 0.027$, $n = 30$), surface area ($r^2 = 0.160$, $p = 0.016$, $n = 30$), and volume ($r^2 = 0.146$, $p = 0.021$, $n = 30$).

Functional diversity

Functional diversity (i.e. number of substrates used) varied from 0 to 24 (mean ± SD: 13 ± 8) out of 31 substrates available. A 2-way ANOVA determined highly significant effects of Day ($p < 0.001$, $n = 58$), Sample type ($p < 0.001$, $n = 58$), and a significant interaction ($p = 0.004$, $n = 58$). For Day 1 (after 24 h in the rolling tank), there was no significant difference between the functional diversity of aggregate-associated and aggregate-free microbial communities ($p = 0.251$, $n = 58$), but for all other time points, the functional diversity of the aggregate-associated microbial communities was significantly greater than that of its aggregate-free counterpart (Day 2: $p = 0.002$, Day 7: $p = 0.001$, Day 14: $p = 0.020$, Day 35: $p = 0.001$; see asterisks in Fig. 4B). Similar to results for community metabolic response detailed above, functional diversity of the microbial community in the water steadily declined from Day 0 to Day 35, whereas the functional diversity of the microbial communities in aggregates was maintained for at least 1 wk (no significant differences among Days 0, 1, 2, and 7, representing an equilibrium period for the aggregate-associated microbial community), before declining on Days 14 and 35. The largest difference in diversity between the aggregate-associated and aggregate-free microbial communities was on Day 7. For all samples (water and aggregates) the functional diversity of the microbial community was highly correlated with the average community metabolic response ($r = 0.98$, $p < 0.001$, $n = 58$). Functional diversity of the aggregate-associated communities was also significantly related to long axis ($r^2 = 0.105$, $p = 0.045$) and surface area ($r^2 = 0.101$, $p = 0.049$), but not volume ($p = 0.066$; $n = 30$ in all cases).

Species turnover

Substrate utilization was analyzed for the 4 consecutive time points, i.e. Days 0, 1, 2, and 7, when no significant differences in the functional diversity of aggregates were detected (Table 1). A total of 17 of the 31 carbon substrates were used at all 4 time points, whereas only 1 (2-hydroxy benzoic acid) of the 31 substrates was not used at all. Against this 'background' signal of relatively constant number of species (functional diversity being a proxy for species number), there was temporal variation in utilization of the

Table 1. Variation over time in the utilization of specific substrates by the aggregate-associated communities supports the concept of species turnover (+ = utilized, 0 = not utilized). The proxy for number of species (functional diversity) remained relatively constant across the same 4 time points.

Specific substrate	Substrate type	Day 0	Day 1	Day 2	Day 7
Putrescine	Amine	+	+	+	+
Phenylethylamine	Amine	+	0	0	+
L-arginine	Amino acid	+	+	+	+
L-asparagine	Amino acid	+	+	+	+
L-phenylalanine	Amino acid	+	0	0	0
L-serine	Amino acid	+	+	+	+
L-threonine	Amino acid	+	+	+	+
Glycyl-L-glutamic acid	Amino acid	+	+	+	+
D-cellobiose	Carbohydrate	+	+	+	+
α-D-lactose	Carbohydrate	+	+	+	+
β-methyl-D-lucoside	Carbohydrate	+	+	+	+
D-xylose	Carbohydrate	+	+	+	0
I-erythritol	Carbohydrate	+	+	+	+
D-mannitol	Carbohydrate	0	+	+	+
N-acetyl-D-glucosamine	Carbohydrate	+	+	+	+
Glucose-1-phosphate	Carbohydrate	+	0	0	0
D,L-α-glycerol phosphate	Carbohydrate	+	+	+	+
D-galacturonic acid-γ-lactone	Carbohydrate	+	+	0	0
Pyruvic acid methyl ester	Carboxylic acid	0	+	+	+
Glucosaminic acid	Carboxylic acid	+	0	0	0
D-galacturonic acid	Carboxylic acid	0	+	+	+
γ-hydroxybutyric acid	Carboxylic acid	+	+	+	0
α-ketobutyric acid	Carboxylic acid	+	0	0	+
D-malic acid	Carboxylic acid	+	0	0	0
Itaconic acid	Carboxylic acid	+	+	+	+
2-hydroxy benzoic acid	Carboxylic acid/ phenol	0	0	0	0
4-hydroxy benzoic acid	Carboxylic acid/ phenol	+	0	0	0
Tween 40	Polymer	+	+	+	+
Tween 80	Polymer	+	+	+	+
α-cyclodextrin	Polymer	+	+	+	+
Glycogen	Polymer	+	+	+	+

remaining 13 substrates, evidence of species turnover in the aggregate-associated microbial communities. No consistent utilization pattern among the 6 substrate types was discernable.

Variance to mean ratio (VMR) of functional diversity

VMR values of the functional diversity for each set of 6 aggregates collected at each time point in the equilibrium period (i.e. Days 0 to 7 when no differences in functional diversity were detected) were all under the minimum value of 0.5 (Day 1 = 0.23, Day 2 = 0.09, Day 7 = 0.42) predicted by the theory of island biogeography, supporting the notion that a non-random process generated the patterns observed.

Concentration of culturable vibrios

A 2-way ANOVA showed highly significant effects of Day ($p < 0.001$, n = 61), Sample type (aggregates > water; $p < 0.001$, n = 61), and a significant interaction ($p = 0.027$, n = 61) for sucrose-fermenting vibrios. For total vibrios, there were significant effects of Day ($p < 0.001$, n = 61) and Sample type (aggregates > water, $p < 0.001$, n = 61), but the interaction was not significant ($p = 0.074$, n = 61). For both types of culturable vibrios, the highest concentrations occurred in aggregates sampled on Day 2 (Fig. 5). Culturable vibrios were detected in at least some aggregates sampled up to 35 d, whereas no culturable vibrios were detected in any water samples after 2 d. For the aggregate-associated microbial communities, functional diversity was significantly correlated with the concentration of both total vibrios ($r = 0.46$, $p = 0.011$, n = 30) and sucrose-fermenting vibrios ($r = 0.37$, $p = 0.047$, n = 30), whereas community metabolic response (ΔAWCD) was only correlated with the concentration of total vibrios ($r = 0.44$, $p = 0.015$, n = 30) and not sucrose-positive vibrios ($r = 0.35$, $p = 0.058$, n = 30).

Concentration of fecal indicator bacteria (*Escherichia coli*)

A 2-way ANOVA indicated highly significant effects of Day ($p < 0.001$, n = 36), Sample type (aggregates > water; $p < 0.001$, n = 36), and a significant interaction ($p < 0.001$, n = 36) for concentrations of the fecal indicator bacteria, *E. coli*. Subsequently, using 1-way ANOVA on samples of aggregates only, concentrations on Days 1, 2, and 7 were significantly greater than the starting concentration (Day 0) and the concentrations on Days 14 and 35 (see letters above lines in Fig. 6).

DISCUSSION

The advantages of applying the theory of island biogeography to organic aggregates are plentiful. For example, aggregates (i.e. 'islands') are easily produced in the laboratory from a variety of environmental waters with relatively simple equipment, making aggregates conducive to island biogeography studies.

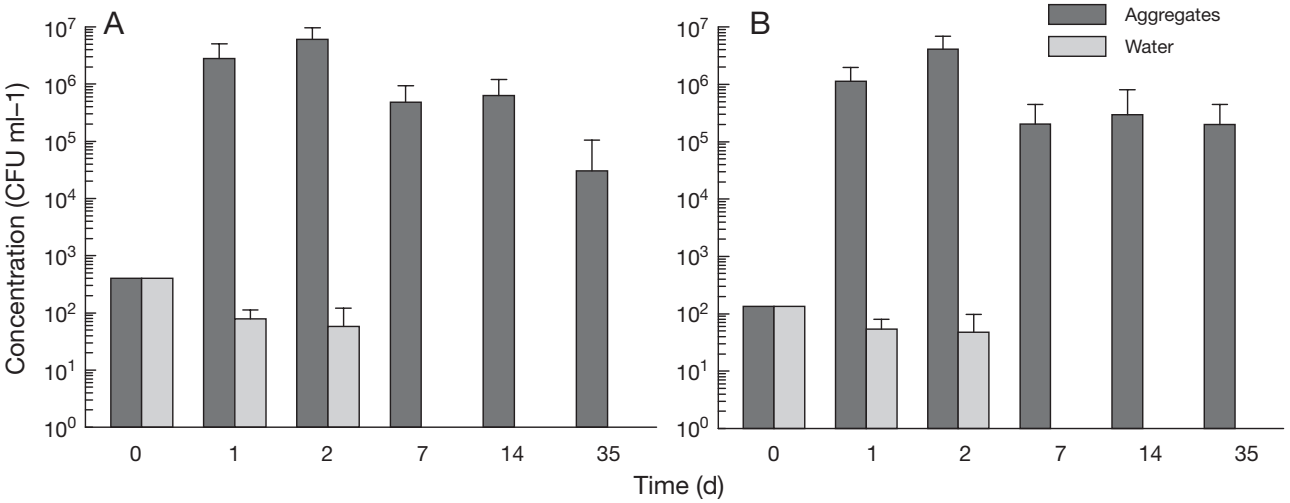

Fig. 5. Concentrations of vibrios (mean ± 1 SD) in aggregate-associated microbial communities (darker bars, n = 6 at each time point) and aggregate-free microbial communities (lighter bars, n = 6 at each time point) for both (A) total culturable vibrios and (B) sucrose-fermenting culturable vibrios. Concentrations of both microbe types increased in the first 2 d in aggregates, but not in water samples. Culturable vibrios were detected in at least some aggregates sampled up to 35 d, whereas none were detected in water samples after 2 d, suggesting the role of organic aggregates in the persistence of culturable vibrios in aquatic environments

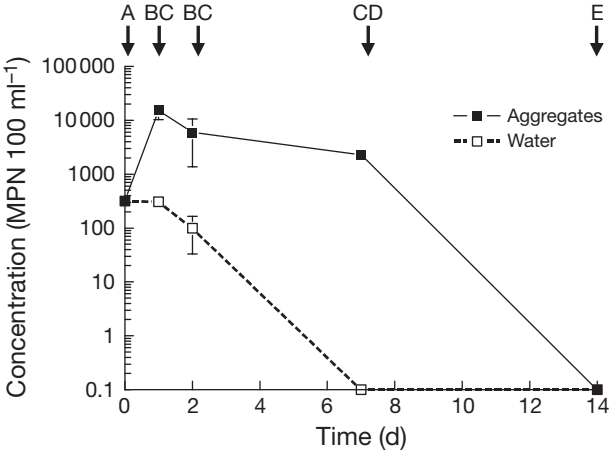

Fig. 6. Concentrations of *Escherichia coli* (mean ±1 SD) in samples of aggregates compared to aggregate-free water. No signals (recorded as <1 MPN, i.e. most probable number) were detected in either type of sample on Days 14 or 35 (Day 35 not shown). Letters above the lines denote significantly different concentrations among the samples of aggregates, demonstrating that concentrations of *E. coli* on Days 1, 2, and 7 were significantly higher than the starting concentration (Day 0). Given that positive results were detected in samples collected up to Day 7 for aggregates, but not for water, these results suggest a role for organic aggregates in the persistence of *E. coli* in aquatic environments

In addition, a large number of aggregates can be generated, yielding a greater number of replicates than is typically available with other island biogeography studies. Furthermore, since individual aggregate-islands can be isolated, the entire island can be sampled to determine the number of species, compared to studies on oceanic islands (as archetypal examples) that rely on subsamples to infer species numbers. Finally, the microbial community of aggregate-islands can be manipulated, observed, and analyzed on substantially shorter time scales than corresponding studies of birds, mammals, or insects on islands. We are using island biogeography theory to better understand an important issue in applied ecology, that is, the transmission of waterborne diseases. The theory of island biogeography predictions that were evaluated in our research included species consistency, non-zero species turnover rates, species-area relationship, and variance to mean ratio of species number.

Species consistency and non-zero level of species turnover

Island biogeography predicts that the number of species on an island tends toward equilibrium because of a balance between immigration and extinction. We used functional diversity (i.e. number of substrates utilized) as a proxy for species diversity to evaluate the temporal consistency of species number and found no significant differences in the aggregate-associated communities on Days 1, 2, and 7. Analysis of the specific substrates utilized during this time period indicated a non-zero turnover of species (i.e. there were changes in species composition without changes in the number of species). The consistency of species number, coupled with non-zero turnover rates, supports the premise that a dynamic equilibrium within the aggregate-associated microbial community was reached within 1 d and was maintained for at least 1 wk. Whether or not an individual aggregate could remain intact and in suspension for 1 wk in nature, is not known, but would most likely depend on the site- and time-specific hydrodynamics of the aquatic system in question. In addition, any period of dynamic equilibrium might be expected to vary with source water and season, but these variables were outside the scope of this experiment.

Species–area relationship

Island biogeography predicts that the relationship between the number of species (i.e. species richness, S) on an island and its area (A) is a power function: $S = C \times A^z$ (Fig. 3), where the constants C and z vary with taxonomic group, geography, and degree of isolation. Over the small size range of aggregates targeted in the present study (~2 to 4 mm long), the species–area relationship was confirmed with a weak but significant relationship between size (in area) and functional diversity. Prior experiments by our group (M. M. Lyons & F. C. Dobbs unpubl.), using the same methods, but with other sources of water over a wider range of aggregate sizes, support a more robust relationship (comparing Fig 3A to 3B). The species–area relationship documented in the present study supports other reports on the abundances of bacteria, flagellates, and ciliates scaling with the size of aggregates (Kiørboe 2003) and the existence of steep microbial species–area relationships (Bell et al. 2005).

Variance to mean ratio (VMR) at equilibrium

The 6 aggregates collected at each time point represent 6 islands that were similar in terms of source water, age, and size. In each set, the VMR of functional diversity was well below the 0.5 minimum predicted by the theory of island biogeography (MacArthur & Wilson 1963, Brown & Dinsmore 1988). In general, a VMR near 1.0 suggests that the number of resident species is due to a random process, whereas the further the VMR

is away from 1, the more likely the process governing the number of species is not random (i.e. either a patchy distribution if VMR > 1 or a patterned distribution if VMR < 1). In the case of island biogeography, the processes resulting in a patterned distribution are expected to be the balance of immigration and extinction rates.

Aggregates as islands — application in disease ecology

Although the details of the theory of island biogeography have been debated for some time (reviewed by Whittaker 2000), overall, the results of the present study support the idea that the theory applies to organic aggregates, which we have shown function as islands for bacteria. While arguing the relevance of the theory for microbial communities in aquatic environments, we have also demonstrated an elevated metabolic response and functional diversity of bacteria on individual organic aggregates compared to the surrounding water, supporting the concept that organic aggregates are favorable habitats surrounded by less favorable habitat. This result quantifies research describing organic aggregates as 'physically distinct benthic-like habitats' (Silver et al. 1978). Focusing on aggregates as islands is important in their context as potential reservoirs and vectors for aquatic pathogens (Lyons et al. 2005, Lyons 2008).

In aquatic ecosystems in general, the adhesion of bacteria to both biotic (e.g. phytoplankton, macrophytes, zooplankton, benthic invertebrates, pelagic vertebrates) and abiotic (e.g. clay particles, sediment grains, boat hulls, piers) surfaces is considered a protective mechanism for enhanced survival (Davies et al. 1995, Colwell et al. 1996, Lipp et al. 2002, Fries et al. 2006, 2007). Likewise, it has been proposed that aggregates might enhance the persistence of aquatic pathogens by providing a 'protective refuge' (Decho 2000) or a 'resource-rich microhabitat' (Cottingham et al. 2003), but prior to research presented herein, little data was available to support these ideas. We have demonstrated the relevance of the theory of island biogeography for microbial communities and simultaneously showed persistence of potential bacterial pathogens and fecal indicator bacteria in aggregates. For sewage-associated bacteria and naturally occurring pathogens such as vibrios, aggregates should enhance persistence, much as has been reported for bacteria associated with living plankton (Lipp et al. 2003, Cottingham et al. 2003, Signoretto et al. 2004). Indeed, we propose that when a planktonic organism dies and becomes part of a detrital aggregate, it brings with it a microbial community that persists even after the plankton has died. The same scenario would be expected for pathogens attached to sediment particles (Noble et al. 2006, Hartz et al. 2008) when they are resuspended and incorporated into aggregates.

An important application of our results is the decision framework that water quality managers use to make determinations about beach and shellfish bed closures. Specifically, our study shows that there is considerable variation in bacterial abundance among aggregates of the same age and similar size (Figs. 4 & 5). Since aggregates are typically present in densities on the order of 1×10^0 to 1×10^3 per liter (see references in Simon et al. 2002), variation in water samples of <1 l could be too high to reliably measure bacterial concentrations because the presence or absence of a single aggregate in an environmental water sample could drastically alter the measure of bacterial concentrations. In general, aggregates harbor bacteria at concentrations much higher than the surrounding waters with enrichment factors as high as 5700 per aggregate for non-pathogenic bacteria (Turley & Mackie 1994). Enrichment factors for potentially pathogenic species were generally less than 500 (Lyons et al. 2007), but still significantly elevated compared to bulk water concentrations. Attachment to aggregates may ameliorate some of the effects of environmental stressors (e.g. sunlight, changes in temperature, salinity, pH, and competition, and lack of nutrients; Sinton 2005), an idea that is supported by the results of the present study which show that *Vibrio* spp. and *Escherichia coli* associated with organic aggregates persist longer than their counterparts in aggregate-free water.

Aggregates also present a complication for modeling the transmission of waterborne diseases and illnesses from aquatic reservoirs to human hosts. At present, models do not consider the protection potentially provided to microorganisms by aggregates. We have shown that culturable vibrios proliferate in aggregates and decline in adjacent, aggregate-free water. Were these differences in population dynamics to be modeled, the presence or absence of aggregates would have profound effects on the model's predictions (McCallum et al. 2001, Eisenberg et al. 2002). This possibility is especially topical for models of *Vibrio cholerae* transmission (Koelle et al. 2005, Hartley et al. 2006, Pascual et al. 2006), given the well-recognized role of the aquatic reservoir in cholera dynamics (Codeço 2001). We therefore recommend that aggregates be incorporated into models of waterborne diseases and illnesses, similar to the way they have been added to models of carbon cycling and food webs (Azam & Long 2001, Burd & Jackson 2009).

In summary, we have provided evidence that organic aggregates function as islands for microbial communities. Aggregate-associated communities have

elevated levels of metabolic response and functional diversity compared to their aggregate-free counterparts. We have also demonstrated higher concentrations and greater persistence of culturable *Escherichia coli* and vibrios in aggregate-associated microbial communities and suggest that these findings have applications to the field of disease ecology.

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