Non-equilibrium processes structuring benthic bacterial communities following deposit feeding by a sea cucumber

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ABSTRACT: Non-equilibrium models of community structure focus on the role of disturbance, recovery, and succession. We examined the disturbance effect of *Isostichopus badionotus* (Selenka, 1867) deposit feeding on the physical habitat and benthic bacterial communities. Physical sediment characteristics were compared between ingesta and egesta, and bacterial community structure was compared among ingesta, egesta, fore-, mid-, and hind-gut samples using epifluorescence microscopy and DGGE analysis of 16S rDNA. When compared with ambient sediments, fresh egesta were significantly higher in organic content and algal pigments, suggesting that these animals fed selectively. In contrast, total and metabolically active bacterial densities were significantly reduced by 33 and 60%, respectively, as sediment passed through the gut, producing disturbance patches of reduced abundance. We also compared different modes of bacterial recovery by experimentally isolating egesta from underlying sediment or overlying waters. Numerical recovery occurred between 1 and 6 h, depending on which mode of recovery (migration, recruitment, or regrowth) was precluded. In contrast to previous studies performed in intertidal settings, all forms of recolonization were significant, in the following order of importance: migration ≈ recruitment > regrowth. Species richness did not change significantly through time under any recolonization regime nor was species turnover evident; thus, bacterial communities in this subtidal habitat appear to be founder-controlled. Yet the mode of recovery did influence community structure, as reflected by a significant treatment effect. The pool of potential recruits can be restricted by the mode of recolonization; thus, patchiness in community structure and high species diversity can result even in a competitive lottery situation.

KEY WORDS: *Isostichopus badionotus* · Subtidal · Disturbance · Recolonization · Succession · Caribbean · Panama

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INTRODUCTION

Bacteria in marine sediments are primary remineralizers of detrital material in nearshore habitats. Diagenetic models demonstrate that changes in dominant bacterial metabolic pathways can alter rates of organic carbon uptake and utilization by up to 20% (Wirtz 2003). However, such models have been difficult to generate due to limited characterization of bacterial assemblages in marine sediments (Boudreau 2005). Consequently, these models have been vague generalizations, at best indicating hotspots of chemical conversion. A better understanding of the processes governing bacterial community composition would more clearly elucidate metabolic variations in diverse habitats.

Deposit-feeding invertebrates are often biomass dominants in the marine benthos and play a vital role

in structuring soft-bottom habitats (Lopez & Levinton 1987). The response of sedimentary bacterial communities to deposit feeding is highly variable. Ingestion by macrofauna can temporarily reduce bacterial biomass (Moriarty et al. 1985, Findlay et al. 1990) and alter species composition (Plante & Wilde 2004, Grossi et al. 2006) in egesta. Conversely, bacterial growth and activity can be accelerated in the guts and fecal casts of some deposit feeders (Plante et al. 1989, da Silva et al. 2006). Resident gut microbial assemblages in deposit feeders have been described across phyla, including Annelida (e.g. Grossi et al. 2006), Arthropoda (Lau et al. 2002), and Echinodermata (De Ridder & Jangoux 1985, da Silva et al. 2006). These assemblages may provide source populations to maintain high bacterial diversity in marine sediments (Plante et al. 1989).

Interactions between bacteria and deposit feeders historically have been viewed as predator–prey associations. However, there is little evidence that deposit feeders selectively ingest bacteria, and energetic gains from bacterivory are uncertain (Kemp 1987, van Oevelen et al. 2006). Several previous studies have instead characterized deposit feeding as a disturbance effect on bacterial communities (Findlay et al. 1990, Plante & Wilde 2004, Grossi et al. 2006). White & Pickett (1985) define a disturbance as 'a discrete event in time that disrupts ecosystem, community, or population structure or changes resources, substrate availability, or the physical environment.' Deposit feeding creates 2 types of disturbances: patches where surficial sediment is altered due to feeding activity, and deposited egesta. Deposit feeder egesta, which represent disturbed patches unlike the surrounding sediment, are the focus of our study. Most deposit feeders ingest bulk quantities of sediment, altering entire bacterial assemblages incidentally through gut passage. Additionally, deposit feeding may alter sediment sorting, grain size distribution, water content, and chemical gradients within sediments (Aller & Dodge 1974, Wild et al. 2005).

Possible mechanisms for bacterial re-establishment after deposit feeding include (1) regrowth from bacteria either surviving gut passage or introduced from the gut itself, (2) immigration by bacteria from adjacent sediments, or (3) recruitment by colonizing bacteria in the overlying waters (Plante & Wilde 2004). Subsequently, competition may help structure bacterial communities during succession. If members of a community have unequal competitive capabilities, a dominance-controlled successional pattern should lead to higher abundances of certain taxa over relatively short time scales (Jackson et al. 2001). If no members of the community have a strong competitive advantage, then initial colonization determines the community structure. This type of founder-controlled succession results in a random distribution of community members and no successional pattern emerges over time (Yodzis 1986).

Plante & Wilde (2004) suggested that intertidal bacterial communities are founder-controlled because bacterial assemblages in egesta did not become more similar to assemblages in surrounding sediments over time. Notably, this finding was limited by the time constraints of their intertidal study (~3 h of aerial exposure), so the bacterial communities likely were sampled while in pre-climax seral stages. In addition to the mechanisms of regrowth and immigration, the analysis of subtidal deposit feeding allows the evaluation of recruitment from the water column as a possible mechanism of recolonization. The deposit feeder employed in our study is an aspidochirote holothuroid that is found in mixed mangrove sand flats and seagrass beds throughout the Caribbean. The egesta are encased in a fragile, peritrophic membrane that can persist up to 26 h (Sloan & von Bodungen 1980), allowing a more complete evaluation of bacterial community change through time.

Our central objective was to investigate the effects of deposit feeding by *Isostichopus badionotus* (Selenka) on the physico-chemical habitat and sedimentary bacterial community structure. Three environmental variables, the total organic matter, grain size distribution, and algal pigments, were compared between ambient sediment and fresh egesta. We used microscopy and molecular techniques (PCR-DGGE) to compare bacterial numbers and community structure in ingesta vs. egesta. Finally, we performed experiments to ascertain modes of recolonization and identify whether the community structuring was dominance- or founder-controlled.

MATERIALS AND METHODS

Study site and organism

Sampling was conducted during 2 consecutive summers, in 2005 and 2006, at Starfish Cove (9° 24.293′ N, 82° 19.519′ W) off Isla Colon, Bocas del Toro Province, Panama. The perimeter of this site transitions from a narrow, sandy beach to mangrove habitat with some freshwater input from a lagoon ~150 m behind the mangroves. The region is domi-

nated by *Thalassia* meadow to the low tide line and mangrove edge. The sampling area was delimited by natural barriers (beach shoreline and mangrove prop roots) and encompassed ~3.6 ha. This site provided a large area where experimental treatments were manipulated within a reasonable depth (<5 m) utilizing snorkeling gear. Mean *Isostichopus badionotus* densities, determined along 6 underwater transects (1 m × 60 m), were 0.017 and 0.025 ind. m^{-2} in 2005 and 2006, respectively.

Physical habitat disturbance

To evaluate the effect of deposit feeding on the physical environment, 3 sediment qualities were compared between fresh egesta and ambient sediment. Actively feeding *Isostichopus badionotus* were flagged using weighted buoys and observed every 10 min until the next defecation. Animals were continually monitored, with the first deposited egesta reserved for microbiological studies, while subsequent deposits were collected for sediment characterization. For analyses of organic matter and grain size, ~2 ml of fresh egesta along with a feeding sample of similar volume were collected using a modified bulb pipet. Feeding samples consisted of undisturbed sediment (<2 cm depth) taken within 1 cm of the sea cucumber's extended buccal podia. Samples for plant pigment analysis were collected using the same methods; however, the egesta were sampled at 3 time points (0, 4, and 24 h), thereby reducing the volume of sediment available to be collected for each individual time point. All egesta and ambient sediment samples were held on ice in microcentrifuge tubes until laboratory processing.

Total organic matter (TOM) was estimated using the loss-on-ignition method. Field samples were dried at 60°C for 48 h, then heated in a muffle furnace to 500°C for 6 h. TOM was calculated as the proportion of dry mass lost to combustion. Grain size analysis was performed on paired egesta and feeding samples by separating size classes (63, 90, 125, 180, and 1000 μm sieve mesh), with the final silt–clay fraction captured on a glass fiber filter (7 μm). The fractions were kept overnight at 60°C to obtain dry weights (DW). Cumulative size frequency curves were generated (Buchanan & Kain 1971), and median grain size, Φ quartile deviations (indicates the spread of Φ units between the first and third quartile diameters) and Φ quartile skewness (indicates the tendency of Φ values to be spread to 1 side of the median) were calculated.

Samples for pigment analysis were extracted in the dark overnight (24 h) at 4°C, using 90% acetone. Absorbance peaks (750, 665, 663, 645, and 630 nm) were measured using a Spectronic Genesys 20 spectrophotometer (Thermo Scientific), and the samples were dried 48 h at 60°C to express pigment concentrations per sediment DW (g).

Disturbance and bacterial recolonization

Actively feeding *Isostichopus badionotus* were flagged and monitored until egesta were produced. A total of 22 ind. were left undisturbed, while 20 others were manipulated such that the posterior portion of their body was atop a 0.125 m^2 plexiglass sheet at the time of defecation. Consequently, fecal mounds either remained in direct contact with the natural substratum during the course of sampling 'natural egesta' (NAT), allowing bacterial succession to progress through regrowth, immigration, or recruitment. Or mounds were isolated from the underlying sediment, denoted 'plexiglass-isolated egesta' (PLEX), blocking immigration from underlying sediments as a mode of recolonization. To minimize disturbance of the egesta, the individuals were removed from the plexiglass after defecation. Egesta were flagged to mark their locations because some fecal coils deteriorated and lost organization over the sampling period. Egesta were sampled immediately at $t = 0$, then again at 1, 2, 4, 6, and 24 h post-defecation. Efforts were made to randomize sampling with regard to position in the fecal mound. However, due to the uncertain orientation of fecal coils (relative to position in the gut) and overlapping of coils, no strict method for randomized sampling was utilized. For each *I. badionotus* mound sampled, a corresponding sample of ambient sediment was also collected.

To distinguish between regrowth and recruitment from overlying seawater, 10 *Isostichopus badionotus* were placed into either alcohol-washed aquaria or alcohol-washed buckets containing 0.2 μm filter-sterilized seawater until defecation. These 'tank-isolated egesta' (ISO) were effectively segregated from bacteria in both underlying sediment and the water column; the only mode of recolonization was regrowth from bacteria that survived gut transit or those that were introduced from the gut. Similar to the PLEX treatments, sea cucumbers were removed from containers immediately after defecation. Sampling followed the same method and time intervals as in the NAT and PLEX treatments. From 5 sacrificed sea cucumbers the entire gut was removed, and

samples were taken from the fore-, mid-, and hindgut regions.

Samples were collected for both quantitative (microscopic cell counts) and qualitative (PCR-DGGE) analysis for all time points within each treatment (NAT, PLEX, or ISO), but due to incomplete sampling, only egesta from 15 ind. in the NAT treatment and 11 in each of the PLEX and ISO treatments were used for molecular analysis. Incomplete sampling was primarily due to destruction of egesta, e.g. storms buried egesta or recreational snorkelers moved buoys. Samples from gut dissections were collected for quantitative and qualitative community analysis, but due to limited time for processing, assays for active bacterial counts could not be performed.

Community analysis

Total bacterial cell abundances were determined using the fluorescent nucleic acid stain SYBR Gold (Molecular Probes). The metabolically active fraction of bacteria was identified using the fluorogenic redox dye 5-cyano-2, 3-ditoyltetrazolium chloride (CTC; Polysciences).

Immediately upon return to the laboratory, sample slurries were exposed to a short burst of sonication (20 s) with a 3 mm sonic probe (Branson Ultrasonifier 250, power output 65 W) to dislodge attached bacterial cells from sediment. Samples were then allowed to settle briefly (<5 min), and the 2 ml of overlying liquid was removed and used for the CTC reduction assay and SYBR Gold staining. Within 4 to 8 h of sampling, 200 µl CTC (25 mM) was added to the aliquot and incubated for 3 h in the dark with constant agitation (200 rpm). Samples were then fixed in glutaraldehyde (2 % final concentration) and held at 4°C until SYBR Gold staining.

Fixed samples were centrifuged (5000 × g, 15 min, 15°C), then resuspended in 2 ml Trizma buffer (0.05 M, pH 8.1) with Triton X-100 (10 µl ml^{-1}). Samples were then sonicated as described above, stained with SYBR Gold (50 ppm final concentration) for 20 min, then concentrated onto 0.22 µm polycarbonate black membranes. SYBR-staining bacteria were counted using a wide-blue filter set (excitation 450 to 490 nm, 510 nm cut-off; emission 520 nm). Active bacteria were counted using a rhodamine (green) filter set (excitation 510 to 560 nm, 580 nm cut-off; emission 605 nm). A minimum of 20 grids or 200 total cells were counted, and total bacteria were taken as the sum of CTC and SYBR Gold stained cells due to

interference of CTC with other nucleic acid stains (Cook & Garland 1997). The remaining sediment was dried overnight at 60°C and weighed to normalize to DW.

Bacteria in sediments were physically lysed using bead-mill homogenization, then extracted as described by Plante & Wilde (2004). The Wizard PCR DNA purification system (Promega) was used for final purification, following manufacturer's instructions. PCR was performed using the primer set 314F-GC and 517R for amplification of the V3 region of the 16S rRNA gene. PCR conditions followed those described by Plante & Wilde (2004), with the exceptions that GoTaq Green Master Mix (Promega) was employed and combined with 2.5 µg acetylated bovine serum albumin. DNA was quantified on agarose gels using either Hyperladder II molecular mass ladder (Bioline USA) or EZ load molecular mass ruler (Bio-Rad Laboratories).

Denaturing gradient gel electrophoresis (DGGE) employed the D-Code™ Mutation Detection System (Bio-Rad Laboratories). An 8 % polyacrylamide gel with a 40 to 60 % gradient of denaturant was used. Sample volumes were varied so that 300 ng of DNA was loaded for each sample, and the gel was run at 60°C for either 70 V for 14.2 h or 100 V for 10 h. Gels were stained with 1X SYBR Gold (Molecular Probes) for 30 min and visualized in a Model 1000 VersaDoc imaging system (Bio-Rad Laboratories). In addition to experimental samples on each gel, at least 1 lane contained a laboratory reference standard consisting of 5 bacterial isolates (amplified as previously described) to allow normalization for cross-gel comparisons.

Data analysis

Paired comparisons between TOM, pigment concentrations, Φ quartile deviations, and proportions of silt−clay (<63 µm) were made using t-tests generated through JMP statistical software (SAS Institute). Median grain size and Φ quartile skewness data could not be transformed to meet the assumption of normality; therefore, comparisons used a paired Wilcoxon signed ranks test. Time-series chlorophyll a (chl a) and pheophytin a (pheo a) concentrations in egesta were analyzed using repeated-measures analysis of variance ANOVA.

Total and active bacterial densities were tested for normality (Shapiro-Wilk W-test) and heteroscedasticity (Levene's test) and subsequently log-transformed as required. Differences between sediment

and fresh egesta were evaluated using paired *t*-tests. Total count data from gut regions (fore-, mid-, and hindgut) could not be transformed to meet the assumption of normality for parametric tests and were therefore analyzed using Wilcoxon's signed ranks test. Because bacterial recovery counts were part of a time series, ANOVA for repeated measures was used to assess the effects of time (0, 1, 2, 4, 6, and 24 h) and sample type (NAT, PLEX, and ISO). When main effects were found to be significant, pairwise multiple comparisons were made using Tukey's HSD correction.

DGGE gels were analyzed using Quantity One software (Bio-Rad Laboratories). Band number (species richness, *S*) was compared among experimental treatments and times using repeated measures ANOVA. Similarity indices were then calculated using band position via the method of the Dice coefficient, from which Bray-Curtis similarity matrices were generated for each gel using Primer5 software (Plymouth Marine Laboratory). Similar cross-gel analyses were made using a subset of the total bands in each lane; only the bands that migrated within the top and bottom reference bands of the laboratory standards were used. Non-metric multidimensional scaling (nMDS) ordination was used to visualize the similarity of DGGE fingerprint patterns among treatment and time points for individual gels and cross-gel comparisons. Additionally, analysis of similarity (ANOSIM; Primer5) was used to compare community composition between time points and treatments. One-way ANOSIM as described by Clarke & Green (1988) was performed on each individual gel similarity matrix, and a nested 2-way ANOSIM for the global, cross-gel similarity matrix was employed. Pairwise comparisons among treatment types and time series within each treatment were also performed. Clarke & Gorley (2001) interpret values of R > 0.75 as well separated, values > 0.5 as overlapping but different, values > 0.25 as barely separable, and values < 0.25 as indistinguishable.

RESULTS

Physical habitat disturbance

Sea cucumber egesta had significantly higher TOM (mean ± standard error of the mean [SEM]: 6.0 ± 0.3%) than surrounding sediments (4.1 ± 0.4%; p < 0.001); however, no difference was found in either median grain size (243 vs. 256 µm; p = 0.829) or percentage of silt−clay (3.9 vs. 4.9%; p = 0.144).

Cumulative size distributions were similar, and egesta and sediment were similarly sorted (1.07 vs. 0.99 Φ quartile deviation; p = 0.359), consisting of moderately to poorly sorted grains. Most samples were characterized as medium or fine sand regardless of sample type, and fecal and sediment samples were similarly skewed (−0.22 vs. −0.16; p = 0.388) toward larger size classes.

There was no difference in chl *a* concentration between ambient sediment and fresh egesta (p = 0.718; Fig. 1a), but there were significantly higher concentrations of pheo *a* in fresh egesta compared to sediment (p = 0.001; Fig. 1b). In addition, pheo *a* concentration was lower (p = 0.042) in 24 h old egesta than in fresh or 4 h aged egesta.

Bacterial community disturbance

The total number (±1 SEM) of bacteria in fresh *Isostichopus badionotus* egesta ($1.90 \times 10^{10} \pm 0.30 \times 10^{10}$ cells [g DW]$^{-1}$) was significantly lower than in
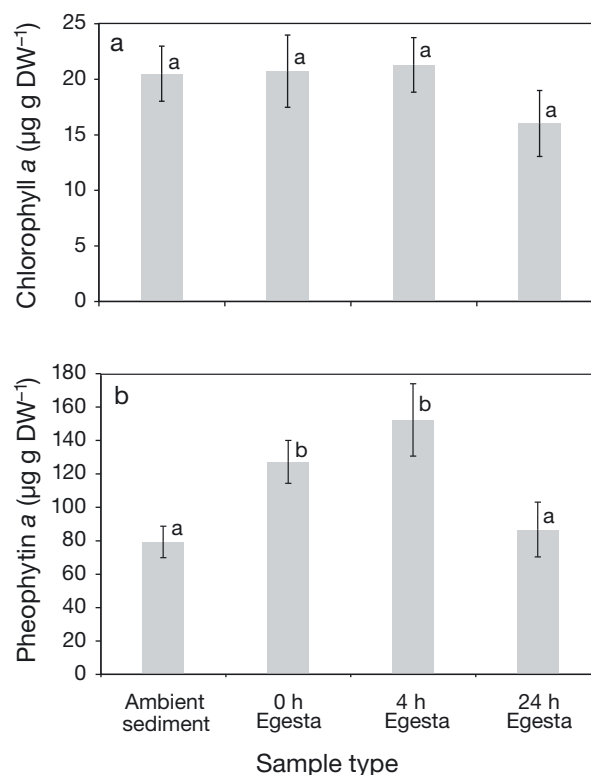

Fig. 1. *Isostichopus badionotus*. (a) Chlorophyll *a* and (b) pheophytin *a* concentration in ambient sediment and egesta at 0, 4 and 24 h after deposition. Means ± SEM, n = 20. Different letters above each column indicate significant differences (α = 0.05)

the surrounding sediment ($2.82 \times 10^{10} \pm 0.31 \times 10^{10}$ cells [g DW]$^{-1}$; p = 0.003). Similarly, active bacterial cell numbers were reduced in egesta compared to sediment ($0.72 \times 10^9 \pm 0.08 \times 10^9$ vs. $1.82 \times 10^9 \pm 0.18 \times 10^9$ cells [g DW]$^{-1}$; p < 0.001).

The average number of phylotypes detected per sample on DGGE gels was 39.3, ranging between 28 and 51 bands per lane. Comparison of DGGE fingerprints of paired sediment and fresh egesta showed that although qualitative distinctions were observed, these differences typically were not consistent among replicates. ANOSIM indicated significant variation among sample pairs (R = 0.653; p = 0.001) but no separation of communities based on sample type (ambient sediment vs. egesta; R = −0.114; p = 0.954). Community fingerprints of paired samples were generally more similar to each other than to other samples of the same type, as shown in the tighter clustering of sediment–egesta pairs (Fig. 2). Furthermore, in the cross-gel analysis, no clustering of egesta or ambient sediment was observed (data not shown).

Total bacterial cells in gut compartments differed significantly (p = 0.046), with foregut densities ($1.64 \times 10^{11} \pm 0.93 \times 10^{11}$ cells [g DW]$^{-1}$) higher than in the midgut ($0.76 \times 10^{11} \pm 0.46 \times 10^{11}$ cells [g DW]$^{-1}$) and hindgut ($0.75 \times 10^{11} \pm 0.45 \times 10^{11}$ cells g^{-1} [g DW]$^{-1}$). DGGE fingerprints from bacterial communities within gut compartments indicated complex communities present in all gut regions. However, consistent clustering by gut region was not evident (R = −0.01, p = 0.528), although midgut samples did appear to cluster more tightly than fore- or hindgut samples (Fig. 3).
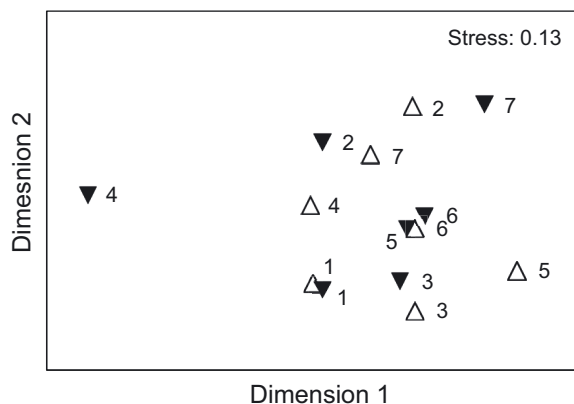

Fig. 2. *Isostichopus badionotus*. nMDS plot showing similarity of DGGE community fingerprints of 7 pairs (same numbers) of (▼) ambient sediment and (△) fresh egesta from a single gel analysis
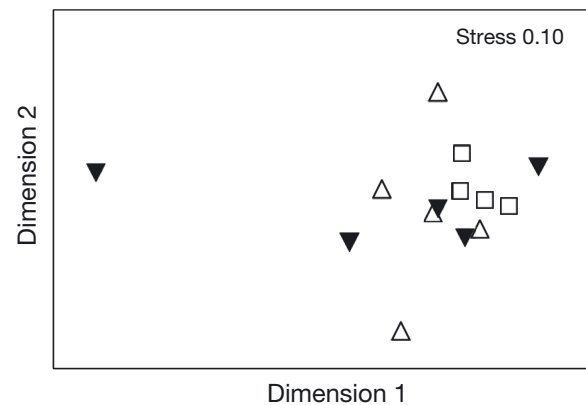

Fig. 3. *Isostichopus badionotus*. nMDS plot of DGGE profiles of gut sections: (△) foregut, (□) midgut, and (▼) hindgut

Bacterial community recovery

Total bacterial counts in each treatment differed significantly among the 6 time points (p = 0.003; Fig. 4). Total bacterial recovery in PLEX treatments was no different than in the NAT or ISO treatments (p = 0.591 and 0.094, respectively); however, recovery in ISO treatments was significantly slower than in NAT treatments (p = 0.028). The interaction of treatment and time did not contribute to the variation in bacterial abundance (p = 0.093). When all treatments were pooled, bacterial abundance in fresh egesta (t = 0 h) was lower than in ambient sediment (p = 0.003).

Numerical recovery of total bacteria in NAT treatments required between 1 and 4 h (Fig. 4a). No difference in bacterial counts between the 1, 2, 4, 6, or 24 h samples or ambient sediment was detected; however, fresh egesta had lower densities than at 6 and 24 h (p < 0.001 for both comparisons). Recovery in the PLEX treatment was not as rapid as in NAT treatments but exhibited a significant increase in bacteria over the time series (p = 0.020; Fig. 4a). Pairwise comparisons indicated an increase in total bacteria after the 4 h time point, with densities in 6 and 24 h egesta significantly higher than in fresh egesta and egesta aged 1 and 2 h (p < 0.001 for all such comparisons). In the ISO treatment, no difference in bacterial abundance was found between time points (p = 0.162; Fig. 4), masked partly by high variability in the 6 and 24 h samples. Pairwise comparisons with ambient sediment revealed a progressive recovery: 0, 1, 2, and 4 h egesta had significantly lower bacterial abundance than sediment (p < 0.040 for all comparisons), whereas no difference was seen between sediment and 6 or 24 h egesta.
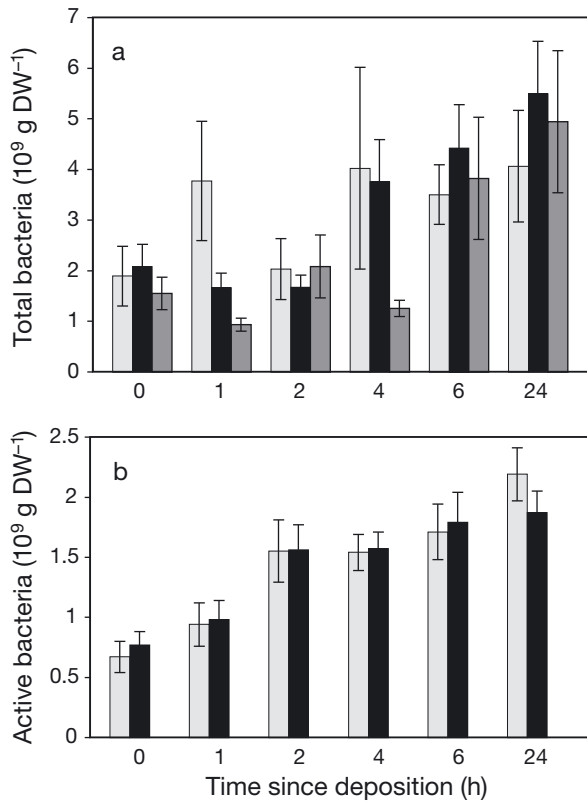
Fig. 4. (a) Total bacterial cell densities for recovery in natural (NAT; light grey), plexiglass-isolated (PLEX; black) and tank-isolated egesta (ISO; dark grey) treatments. (b) Active bacterial densities in NAT (light grey) and PLEX (black) treatments. Means ± SEM, n = 15, 11, and 11 for NAT, PLEX, and ISO, respectively

The CTC assay proved unreliable during the second sampling season, beyond the July 7, 2006, sampling date. The majority of the ISO treatments fell after this date; thus, enumeration of active bacteria was limited to NAT and PLEX treatments. Repeated measures ANOVA of active bacteria in egesta indicated significant variation among time points (p = 0.007; Fig. 4b), although neither the treatment effect (p = 0.631) nor the treatment × time interaction was significant (p = 0.238). Progressive recovery was observed as soon as 1 h after defecation in NAT treatments (Fig. 4b). Fresh egesta had fewer active bacterial cells than all later (≥2 h) times or ambient sediment (p < 0.001 for all comparisons). Complete recovery had not occurred within 1 h of deposition (p = 0.102), but both 2 and 4 h time points were statistically indistinguishable from the 1 h samples (adjusted p > 0.100 for each comparison). In PLEX treatments, recovery followed a similar progression (Fig. 4b). Although some recovery was observed in the first hour, there was no statistical difference

between fresh egesta and egesta 1 h after deposition (adjusted p = 0.236). Pairwise comparisons with ambient sediment revealed significantly fewer active bacteria only in egesta at 0 and 1 h (p < 0.001), indicating recovery by 2 h after deposition.

DGGE band number did not vary among treatment (p = 0.968), time (p = 0.695), or the treatment × time interaction (p = 0.405). Variation in banding pattern between replicates at a given time point was generally as high as variation among time points, thus global R statistics (and associated p-values) for each gel indicated no observable pattern for recovery. Comparison of each full time series to another series on an individual gel (i.e. ambient sediment and egesta at t = 0, 1, 2, 4, 6, and 24 h from 1 sea cucumber to the same series from a different sea cucumber) indicated complete separation of communities in 12 of the 17 gels with replicate time series (data not shown). Since only 2 time series could be run on any single gel, efforts to compare different treatments on individual gels were limited.

In cross-gel ANOSIM comparisons, bacterial community fingerprints could be separated based on treatment (R = 0.998, p = 0.001; Fig. 5a) but not
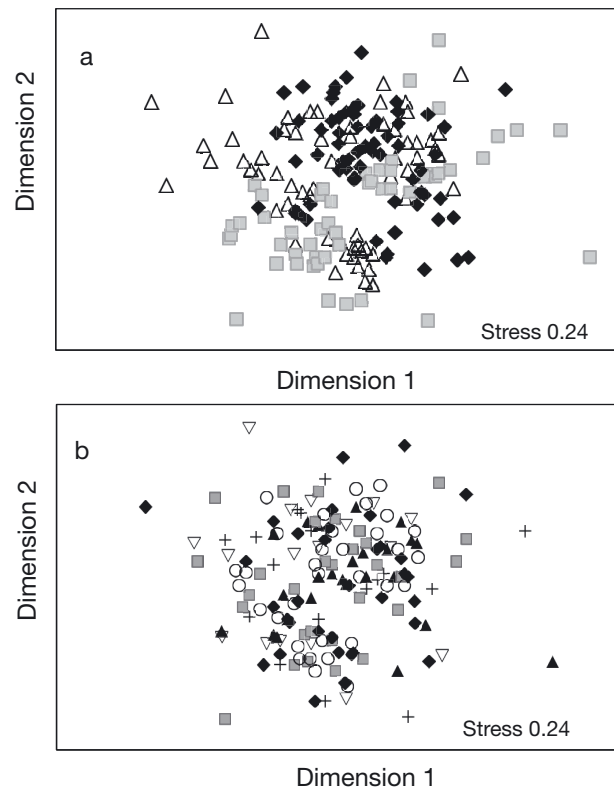

Fig. 5. Cross-gel nMDS plot for (a) treatments (△ NAT, ◆ PLEX, and ▪ ISO) and (b) time points (▲ 0, ▪ 1, ◆ 2, ○ 4, × 6, + 24 h egesta, and ▽ sediment at 0 h)

based on time (R = −0.064, p = 1.000; Fig. 5b). All treatments were well separated from one another: NAT vs. PLEX (R = 1.000, p = 0.002), NAT vs. ISO (R = 1.000, p = 0.002), and PLEX vs. ISO (R = 0.997, p = 0.003).

DISCUSSION

Higher TOM levels in egesta than in surrounding sediments suggest selective feeding on organic-rich sediments, which corroborates previous reports regarding feeding in *Isostichopus badionotus* (Hammond 1982a,b, Conde et al. 1991). The higher TOM in egesta is likely a result of our inability to adequately mimic sea cucumber feeding when we sampled surface sediments. Since no difference could be detected between the percentage of silt–clay or grain size distribution between ambient sediment and egesta, feeding patch selection is evidently not based upon these granulometric variables, confirming results from Hammond (1982b). Likewise, selection did not appear to be based on microalgal biomass because chl *a* concentrations were statistically indistinguishable between sediment and fresh egesta. Similarly, no shift in chl *a* concentration in egesta was observed in related holothuroids (*Holothuria* spp.; Uthicke & Karez 1999). Our findings contradict an earlier study with *I. badionotus* that showed elevated total plant pigment concentrations (chl *a* and pheophytin) within guts and egesta (Hammond 1983), although this discrepancy may be due to the pooling of total pigments in that study. Our results of ~80 µg of pheo *a* per g sediment DW appear to be higher than most prior records for tropical sediments (0 to 53 µg g^{-1} sediment; Hammond 1983, Cartaxana et al. 2003) but perhaps not unreasonable considering the decaying biomass available from local seagrass and mangrove litter. The relatively high concentration of pheo *a* in fresh and 4 h old egesta may be due to the degradation of chl *a* under anoxic gut conditions (Plante & Jumars 1992, Sun et al. 1993). After egestion into a more oxic environment, the pheophytin would then break down further into pheophorbides and other colorless pigments, potentially explaining the lower concentrations found in 24 h egesta.

Total and active bacterial densities in sediments were high but within published ranges for similar habitats (10^8 to 10^{11} cells (g DW)$^{-1}$; Cammen 1982, Alongi et al. 1989, Alongi 2005). The active fraction of bacteria was generally 3 to 9% (Fig. 4), which is on the low end of typical ranges for shallow sediments

(e.g. 4 to 30%; van Duyl et al. 1999). Several investigators have questioned the utility of the CTC method for enumerating active bacteria, usually citing concerns about CTC toxicity (Ullrich et al. 1996) or even cell disintegration as formazan particles form within cells (Gasol & Aristegui 2007). However, other studies have detected no toxicity at low CTC concentrations (<5 mM, like those used here; Sherr et al. 1999), and numerous studies have demonstrated good correlations between CTC reduction and measures of bacterial viability, productivity, and activity measured by other means (e.g. Sherr et al. 1999, Gasol & Aristegui 2007). Moreover, the toxicity or disintegration effects appear to occur posterior to CTC uptake and formazan particle formation; thus, the use of epifluorescence microscopy is not compromised (Gasol & Aristegui 2007).

The high bacterial numbers found in foregut sediments (>2 × 10^{11} cell g^{-1}) were supporting the assertion that sea cucumbers may be selectively feeding. Moriarty (1982) reported selective feeding by *Holothuria atra* and *Stichopus chloronotus* on bacteria and nitrogen-rich detrital matter. Cammen (1982) suggested that increased organic content of bulk sediments was linked to increased bacterial abundance. If these sea cucumbers were selecting high organic content patches, then this would support the observation of high bacterial densities in foregut samples. Our results suggest that the feeding behavior of *Isostichopus badionotus* may be more discriminatory than previously thought (Hammond 1982a,b, 1983).

Bacterial numbers in egesta were significantly lower than in sediments, reduced by 33% for total bacteria and 60% for the active fraction. The reduction of bacterial numbers from foregut to hindgut also indicated digestion of bacteria. Moriarty (1982) observed similar efficiencies (30 to 40%) of bacterial digestion in 2 species of mobile, surface-feeding holothuroids. These rates fall into the general range, albeit near the low end, of digestive removal of total bacteria by a wide taxonomic range of deposit feeders (30 to 90%; Cammen 1980, Grossmann & Reichardt 1991, Plante 2010).

In contrast, bacterial species richness, as estimated by DGGE bands, remained high after gut passage. Furthermore, no consistent pattern of holothuroid-induced changes in bacterial composition in egesta was found, e.g. by introduction of specific types from the gut, preferential removal of susceptible types, or differential proliferation. Paired samples (ambient sediment and fresh egesta) from the same experimental unit (sea cucumber) tended to be more similar

than replicate samples of the same type associated with different animals. This effect likely resulted from heterogeneity in the bacterial community composition within ambient sediments, as has been previously demonstrated at similar spatial scales (Hewson & Fuhrman 2006). Because deposit feeding in *Isostichopus badionotus* appears to have a relatively small impact on the bacterial composition, bacterial communities in egesta may simply be more similar to those in nearby sediments (ingesta). Although Hammond (1982a) reported 2 to 10 h gut residence times in this same species, field observations suggest that the 2 h estimate is a better approximation for our study site. All sea cucumbers were checked periodically (every 2 h) during the course of field experiments (8 to 10 h) and were observed to defecate multiple times throughout the day. Decreased residence time within the gut may not allow adequate time or resource availability for the propagation of bacterial dominants seen in other deposit feeders (Plante et al. 1989, Amon & Herndl 1991). Furthermore, faster processing of sediments may decrease the efficiency of digestion (Taghon & Jumars 1984), allowing even bacterial species that are more susceptible to digestive lysis to pass through the gut relatively unscathed.

Numerical recovery of total and active bacterial densities occurred quickly (1 to 4 h) in NAT treatments. Recovery was more gradual in PLEX (≥4 h) and ISO treatments (≥6 h), suggesting that immigration and recruitment play dominant roles in bacterial recolonization but also that recovery can occur through regrowth alone. Recovery by all 3 general mechanisms (recruitment, migration, and regrowth) in subtidal patches is in contrast to microbial recolonization in intertidal habitats. Plante et al. (2010) showed that migration dominated the recolonization of fecal patches of the hemichordate *Balanoglossus aurantiacus* during low tide, accounting for >90% of recovery. Subsequent studies in similar systems demonstrated that after tidal immersion, sediment resuspension (recruitment) accounted for the majority of recovery by benthic bacteria (Plante 2010) and microalgae (Plante et al. 2011). Recruitment to subtidal egesta of *Isostichopus badionotus* could be derived from water-column bacteria; however, the more likely source is particulate matter settling onto the PLEX and NAT treatments over time. Flocculent material was abundant in suspension and on the seafloor at our study site. Especially after frequent rainfall events, organic flocs were abundant and appeared to move from the mangrove swamp onto the adjacent study area.

Perhaps most surprising was the significant recovery noted in the ISO treatment, suggesting rapid regrowth of bacteria. This contrasts with earlier studies in which growth was insignificant in the recolonization of fecal patches of *Balanoglossus aurantiacus* (Plante et al. 2010) and *Arenicola marina* (Plante 2010). Two likely contributors to this discrepancy are bacterial inhibitors and temperature. Hemichordates and some polychaetes, including the arenicolids ('lugworms'), are known to produce haloaromatic metabolites (Woodin et al. 1987), which have been documented to inhibit bacteria (King 1986). In contrast, holothuroids apparently do not produce haloaromatic or other inhibitory compounds (Woodin et al. 1987, Plante & Stinson 2003). Warmer water temperatures than in prior recolonization studies in temperate waters (Maine, USA, Plante 2010; South Carolina, USA, Plante et al. 2010) may also have accounted for the more rapid growth in the present study. Bacterial growth rates exhibit Q_{10} ranges of 2 to 4 over the temperature ranges (18 to 30°C; Hobbie & Cole 1984, White et al. 1991) observed in the 3 relevant studies. One caveat regarding the ISO treatment is that some free-living bacteria may have been introduced into the sterile environment while waiting for the sea cucumbers to produce egesta. In this treatment, there was no way to prevent bacterial introduction via the sea cucumber's cuticle or sediment grains attached to the tube feet.

Mode of recovery influenced community structure, as demonstrated by the clear distinction among the NAT, PLEX, and ISO treatments by ANOSIM and NMDS; the composition of bacterial communities was altered when immigration or recruitment was blocked. However, decreases in species richness through time were not observed in our cross-gel DGGE analyses, nor were successional changes detected over 24 h in any treatment. Although stress values for NMDS analyses were fairly high (~0.24; Fig. 5), they did not exceed the 'rule of thumb' threshold (>0.3) for unacceptable representation (Clarke & Warwick 2001). In addition, stress increases both with number of samples and with number of variables, both of which were high in our analyses. It is important to note that DGGE banding patterns are sensitive to slight differences in the gel gradient and other run conditions, making cross-gel comparisons difficult. However, numerous studies have employed reference standards to enable meaningful cross-gel comparisons of bacterial communities (Neufeld & Mohn 2005 and references therein). Tourlomousis et al. (2010) address this challenge, making specific recommendations that reference

standards include a sufficient (≥5) number of bands, that they span the entire gel, and that standardized, reproducible methodologies be used for all samples. Accordingly, our reference sample consisted of 5 bacterial isolates, and cross-gel comparisons employed only those bands running between the top and bottom of these 5 reference bands. In addition, electrophoresis conditions (time, voltage, etc.) were identical for all inter-gel comparisons. Additional limitations of DGGE and other fingerprinting techniques, namely the inability to detect rare species and lack of quantification, can be overcome in future studies with use of newer pyrosequencing techniques.

In dominance-controlled communities, predictable species sequences are seen, with early ruderal species replaced by superior competitors. In contrast, in founder-controlled communities, most species are good colonists and essentially equivalent competitors, so succession is not observed and species richness is maintained at a high level (Yodzis 1978). In our study, community structure differences under the disparate recolonization regimes, combined with a lack of structured seral changes, indicate that bacterial communities were founder controlled. Observations of high bacterial diversity in our study and in sediments in general (Hewson & Fuhrman 2006) also support a lack of competitive exclusion and the founder-control model.

Non-equilibrium models emphasize the important role of disturbance in generating species diversity by dampening competitive dominance and creating spatial heterogeneity. Under the assumption of dominance control, disturbances enhance diversity at landscape scales because the community is a mosaic of patches in different stages of succession. Our study shows that *Isostichopus badionotus* feeding disturbed the benthic bacterial community; however, these disturbances were not so intense as to eliminate major bacterial taxa. This type of low intensity, high frequency disturbance can also maintain maximal diversity within the scope of non-equilibrium theories. In founder-controlled communities, high diversity is expected at both patch and landscape scales because all species are potential colonizers of disturbed patches, with community composition determined largely by chance (i.e. 'competitive lottery'). However, as shown in our study, the pool of potential recruits can be restricted by the recolonization mode. For instance, when waters are cold, microbial growth may be an insignificant mode of colonization, in which case more passive processes, such as recruitment by sediment transport, may dom-

inate. Other factors that could influence the relative importance of regrowth, recruitment, or migration include patch size, water flow rate, and chemotactic cues. Therefore, even in a competitive lottery situation, patchiness in community structure can result, further contributing to diversity.

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