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Anti-bacterial activity in egg masses of *Melanochlamys diomedea* across habitats differing in sediment properties and bacterial load

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ABSTRACT: Sedimentary habitats show large variation in physical and ecological conditions that can influence microbial abundance and composition. Such variation can place resident organisms under different risks of microbial infestation and thereby select for different levels of anti-microbial defense. The egg masses deposited by a diversity of marine organisms in these environments may be especially vulnerable to microbial colonization. Previous studies have demonstrated interspecific variation in the level of anti-microbial activity in egg masses, but have not examined whether intraspecific variation among populations relates to variation in risks among habitats. We compared anti-bacterial activity in egg masses among populations of the opisthobranch mollusc *Melanochlamys diomedea* in relation to sediment features, including bacterial load, particle size, and organic content. Egg masses were extracted with ethyl acetate and methanol to derive fractions with relatively low and high polarity, respectively. The extracts were then tested in a bacterial growth assay for anti-microbial activity against the bacteria *Vibrio harveyi* and *Bacillus subtilis*. Sediment characteristics and bacterial load were all highly correlated and varied significantly among sites, with organic content appearing to be a stronger predictor of bacterial load than sediment size. Among populations, the level of anti-bacterial activity within egg masses also correlated as expected with these sediment characteristics and with bacterial load. These results support the hypothesis that anti-microbial defenses in egg masses are related to the environmental risks of microbial exposure. Thus, microbial interactions appear to drive an important component of the reproductive biology of sediment-dwelling organisms.

KEY WORDS: Egg mass · Anti-bacterial activity · Sediment · Bacterial load · Population · Benthic development · Organic content · Grain size

INTRODUCTION

Bacteria are a major cause of disease in aquatic organisms. In the marine environment, they are found in high concentrations on hard substrates (up to 10^8 cm^{-2}; Wahl 1995), in sediment (10^{11} g^{-1} dry mass; Schmidt et al. 1998), and in seawater (10^6 ml^{-1}; Zobell & Allen 1935). Marine sediments have especially high concentrations of bacteria compared with other marine substrates and may present a greater risk of infection for resident organisms. Variation in the bacterial load of sediment (10^6 to 10^{11} g^{-1} dry mass; Alongi 1988, Schmidt et al. 1998) can be influenced by a number of factors including sediment organic content, water content, and grain size and shape, which can all covary (Dale 1974). Sediments consisting of finer particles, for example, tend to have higher organic content and higher bacterial densities than those with coarser particles (Zobell 1946, Rublee & Dornseif 1978). If bacterial loads and sediment characteristics vary across habitats, and bacterial load correlates with the prevalence of infection, these conditions could select for different levels of defense against microbial infection.

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Given their higher and more variable bacterial densities (Kicklighter & Hay 2007, reviewed in García-Martínez et al. 2009), soft-sediment habitats can present special risks of microbial infection for organisms that deposit egg clutches. Furthermore, the composition of egg clutch structures can promote bacterial growth. For example, gelatinous egg masses, consisting primarily of nutrient-rich proteins and polysaccharides, provide a favorable substrate for colonization and proliferation of microorganisms (Benkendorff et al. 2001). As a result, the egg masses of many benthic marine invertebrates have evolved chemical defenses against microorganisms (Gil-Turnes et al. 1989, Benkendorff et al. 2001). Benkendorff et al. (2001) found that the egg masses of 79% of 39 species of molluscs and polychaetes displayed broad-spectrum anti-microbial activity against various marine and human pathogens. While anti-microbial activity within molluscan egg masses is widespread among species, it remains unclear whether such defenses can be adjusted in response to local conditions. Plasticity in the level of anti-microbial defenses in egg masses could evolve, for example, if the density or composition of microbes where an individual settles were variable and unpredictable.

Here, we examine variation among populations of an egg mass-depositing species in both microbial conditions and levels of the anti-microbial defense in their egg masses. A positive relationship between these measures would be consistent with the hypothesis that organisms adjust the level of protection in response to variation in the risk of microbial exposure. First, we determined whether sediment characteristics (organic content and particle size distributions) varied among egg-laying sites of a common intertidal cephalaspid snail, *Melanochlamys diomedea*. Second, we tested for covariation between bacterial load and these sediment characteristics. Third, we used laboratory assays of anti-bacterial activity to test for an association between anti-bacterial activity of egg masses and the bacterial load of these field sites. We predicted that if organisms can adjust their level of anti-bacterial defense, habitats with finer grain sizes, higher organic content, and higher bacterial densities would elicit greater anti-bacterial protection of egg masses.

MATERIALS AND METHODS

Study organism and field sites

Melanochlamys diomedea (Bergh) is a small (~1 cm length) cephalaspid gastropod that lives on tidal flats from Alaska to California (Strathmann 1987). The hermaphroditic adults of *M. diomedea* anchor a balloon-shaped gelatinous egg mass (1–2 cm) to the sediment by a tether composed of egg mass gel and sand (Castro & Podolsky 2012). Each egg mass holds thousands of encapsulated embryos that spiral through a gelatinous matrix composed of mucopolysaccharides and proteins.

The 6 sites used in this study — False Bay (FB), Garrison Bay (GB), Mitchell Bay (MB), Fourth of July Beach (FJ), and 2 locations in Argyle Lagoon (AL) that differed in sediment type (AL1 and AL2) — are located on San Juan Island, Washington, USA (Fig. 1) near the University of Washington's Friday Harbor Laboratories (FHL). These sites were chosen because they show obvious differences in hydrology and sediment characteristics and have large populations of *M. diomedea* (Castro & Podolsky 2012). FB is a large sand flat composed mostly of sand and shell hash that is relatively exposed and experiences fast tidal flow. FB egg masses were collected near the mouth of the bay in outer tide pools. FJ is an exposed broad beach with occasional wave action. Egg masses at FJ were

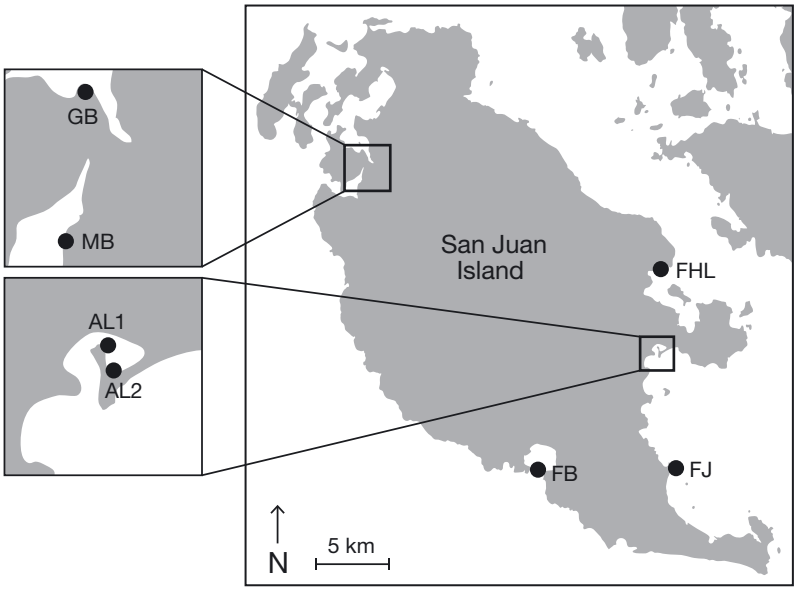

Fig. 1. Six field locations of populations of *Melanochlamys diomedea* used in this study: MB = Mitchell Bay, GB = Garrison Bay, FB = False Bay, AL1 = Argyle Lagoon 1 (proximal to tidal creek), AL2 = Argyle Lagoon 2 (distal to tidal creek), FJ = Fourth of July Beach. FHL = University of Washington's Friday Harbor Laboratories (48° 32′ 46″ N, 123° 0′ 46″ W)

collected from sand flats near an extensive eelgrass bed. AL is a saltwater lagoon fed by a tidal creek with sediment particles that are coarse in places and fine in others. AL1 is proximal to the tidal creek with a gravel and sand bed, while AL2 is further from the creek and included finer silt. GB and MB experience mild tidal flow and the sediment is composed of fine grain sizes.

Sediment characteristics

Sediment was collected at each site during the reproductive season (late spring to early summer) to estimate grain size distributions and organic content. For organic content and grain size, three 200 g samples of sediment were collected adjacent to egg masses at each site and transported on ice within 2 h of collection before they were dried at 65°C for 72 h. To measure the organic content of sediment at each site, 2 g of dried sediment from each of the 3 samples per site were placed in a combustion oven (Vulcan 3-550) at 500°C for 6 h. The remainder of the sample was placed in a plastic container and transported to the laboratory for grain size measurements.

Percent organic content was calculated by subtracting ashed mass from dry mass and dividing by dry mass. For grain size measurements, a RoTap (W.S. Tyler, RO-TAP RX-2918416) sediment sorter was used. Fifty grams of dried sediment from each site (n = 3) was massed and passed through a series of sieves of mesh sizes 2 mm, 1 mm, 500 µm, 250 µm, 125 µm, 62.5 µm, and 32 µm. The mass of sediment on each of the 8 fractions was divided by the total mass of the sample to give a percentage for each fraction. Sediment profiles as well as the percentage of the sample that was silt or clay (<62.5 µm; 'percent silt + clay') by dry mass were calculated for each site.

Bacterial counts

To estimate bacterial densities, each of six 2 g samples of sediment was collected to a depth of 1 cm adjacent to egg masses and placed into a separate sterile 15 ml centrifuge tube, put on ice before transport to the laboratory, and then fixed in 3.7% formalin in sterile seawater within 2 h of collection. Total bacterial counts in sediment were made with a modified version of the protocol of Hymel & Plante (1998). Fixed sediment samples were centrifuged at 5000 × *g* for 15 min (15°C), the supernatant was removed, the sample was mixed, and a subsample (0.5 g) was then resuspended in 5 ml Trizma buffer (0.05 mol l^{-1}, pH 8.10) with 100 µl dispersing agent (0.5% Triton-X 100). The resuspended subsample was sonicated for three 20 s bursts with a 3 mm microtip sonic probe at 90 W (Branson Digital Sonifier S-250D). Samples were sonicated over ice and allowed to cool for at least 1 min between bursts. After the final sonication, the sediment was vortexed briefly and allowed to settle for 20 s. For samples from FB, AL1, AL2, and FJ, 100 µl of supernatant was diluted in 900 µl of autoclaved, sterile seawater, stained with 100 µl SYBR Gold (Invitrogen, 5×10^{-4} final concentration) in the dark for 20 min, and then concentrated onto 0.2 µm black polycarbonate membrane filters (Millipore) backed by glass fiber filters (Gelman, Type A/E). Because their bacterial concentrations were higher, samples from GB and MB involved the same procedure except that 10 µl of supernatant was diluted in 990 µl of seawater and final values were adjusted accordingly.

Filters were placed on glass slides and examined under 1250× magnification (Nikon Fluorite oil immersion lens) with a standard blue filter combination (365 nm excitation filter, 400 nm barrier filter). Ten haphazardly selected fields were counted on each filter (>200 bacteria per slide), and replicate filters were averaged (n = 6). We define the 'bacterial load' of a sample as the total number of bacterial cells per gram of dry sediment. The remaining sediment from which the subsample had been taken was filtered onto pre-massed glass fiber filters and dried for 10 h at 60°C to measure dry masses of sediment per subsample.

Egg mass collection and processing

Egg masses were collected from the 6 study sites during spring low tides in April and May 2011. In the laboratory, tethers were cut from egg masses and any adhering sediment was removed by rinsing in sterile seawater before the egg masses were frozen at −80°C for lyophilization. Three aggregated samples that each provided 2 g dry mass (160–300 g wet mass) were collected from each site for assays of antibacterial activity.

Crude extracts of these samples were prepared using a protocol adapted from Benkendorff et al. (2001) and Harrison & Chan (1980). Frozen egg masses were lyophilized, pulverized with a sterile mortar and pestle, and placed into relatively nonpolar 100% ethyl acetate (EtOAc) at a 1:3 (mass:volume) ratio and allowed to extract overnight at −80°C.

The extract was removed from the egg masses by pipetting the liquid into a scintillation vial and drying in a rotary evaporator (Yamato Rotary Evaporator RE500). The same material was then re-extracted in relatively polar 100% methanol (MeOH) at the same ratio. The dried extracts were first re-dissolved to their natural concentrations, using dry-to-wet mass ratios for egg masses (see Table A1 in the Appendix for extract yields and dry-to-wet mass ratios). They were then tested against the target strains of marine bacteria at a dilution of 2% of their natural concentration, which provides a conservative estimate of absolute activity in this assay but maintains the ability to compare relative activity across extracts.

Target bacteria

Anti-microbial activity in egg mass extracts was quantified against the target marine bacteria *Vibrio harveyi* BB7 and *Bacillus subtilis* ATTC19659. *V. harveyi* is a Gram-negative bacterium and a known pathogen to marine invertebrates and vertebrates (Austin & Zhang 2006). *B. subtilis* is a Gram-positive bacterium that has anti-microbial properties (Ivanova et al. 1999). Bacterial strains were cultured at 25°C on an oscillating plate at 75 rpm in autoclaved nutrient broth (Becton Dickinson BBL) made with natural seawater. For assays, cultures were grown overnight to log-phase and diluted to an optical density at 590 nm (OD_{590}) of 0.1 in a 2.5% NaCl, 10 mmol l^{-1} HEPES buffer (pH = 7.5). Bacterial concentrations at 0.1 OD_{590} were 2.99×10^7 cells ml^{-1} in *B. subtilis* and 3.85×10^9 cells ml^{-1} in *V. harveyi*. Bacteria were further diluted 10 000-fold in the same buffer before being added to the assay wells.

Anti-microbial assay

To test for anti-microbial activity of extracts, we used a 96-well microplate assay adapted from Morrow et al. (2011). The assay measured the growth of bacteria in the presence or absence of extract as judged by a change between the initial and final OD_{590} of the bacterial culture. Experimental wells of a ClearPro polypropylene 96-well microplate (Corning) were inoculated with 190 µl of nutrient broth (Becton Dickinson BBL), 26 µl of a 10^{-4} dilution of a 0.1 OD_{590} bacterial culture, and 4 µl of stock extract. On a given plate, either EtOAc or MeOH extracts from each of the 6 sites (3 independent extracts per site) were tested in triplicate against each bacterial strain. Each plate also included negative control wells containing nutrient broth, bacteria, 4 µl of the solvent for that plate (either EtOAc or MeOH) or 4 µl of HEPES buffer in place of extracts, as well as various positive (+ 4 µl spectinomycin, tetracyclin, ampicillin) and blank (nutrient broth + 26 µl HEPES buffer, 4 µl solvent or HEPES buffer) controls. Culture plates were sealed with a sterile plate cover and incubated at 25°C with shaking at 75 rpm. OD_{590} readings were taken on a 96-well microplate reader (Spectra Thermo, Tecan) initially when the bacterial culture was added and again at the exponential growth phase (22 h for *B. subtilis* and 7 h for *V. harveyi*) as determined from bacterial growth curves with solvent and no extract. An anti-bacterial activity index for the extract was calculated using the following equation:

$$\text{Activity index} = -\log[(E_e - E_i)/(C_e - C_i)] \quad (1)$$

where E_e, E_i, C_e, and C_i are OD_{590} readings for extract (E) and solvent control wells (C) at exponential (e) and initial (i) times, respectively (Morrow et al. 2011), using the average of the independent extracts (n = 3) from each site. Positive values reflect 'anti-bacterial' activity that reduced bacterial growth, while negative values reflect 'pro-bacterial' activity that promoted bacterial growth relative to the solvent control. Standardization of each plate by its negative solvent control allowed for comparison between plates.

Statistical analyses

Analyses were carried out using the software R v.2.15.2 (R Core Team 2012). For comparisons of percent organic content, an ANOVA was performed and Tukey multiple comparisons were used to test for differences between sites. For comparisons of bacterial load, percent silt + clay, and median grain size that did not meet the assumptions of ANOVA, we used a Kruskal-Wallis test and non-parametric multiple comparisons for relative contrast effects (package 'nparcomp'; Konietschke 2006). For testing relationships among bacterial load, organic content, and percent silt + clay, we used Pearson's correlations (package 'corr') and partial correlations (package 'ppcor'; Kim 2011). Values were log-transformed for correlation analyses. One-tailed tests were used because the variables were hypothesized to be positively correlated. Correlations between each of the sediment features (bacterial load, organic content, and percent silt + clay) and anti-bacterial activity were also tested using the 'corr' package. ANOVA was used for com-

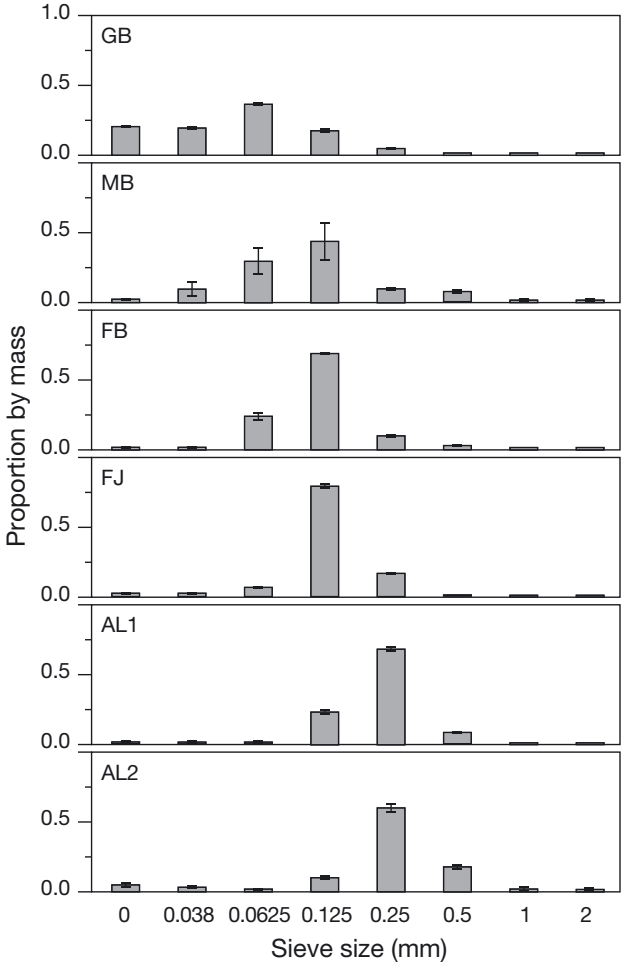

Fig. 2. Proportion (by dry mass) of sediment collected on each sieve size at each study site. Percent silt + clay is the sum of the 2 smallest sieve sizes (<62.5 µm). Error bars represent 1 SE. Site abbreviations as in Fig. 1

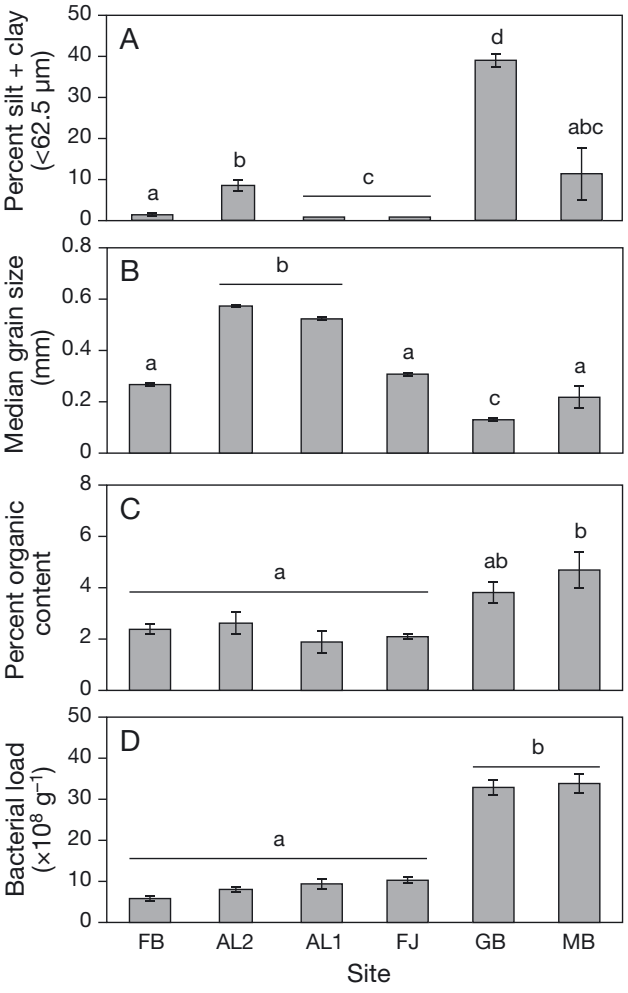

Fig. 3. Sediment characteristics (A: percent silt + clay, B: median grain size, C: percent organic content, D: bacterial load) at the 6 field sites. The sites are arranged from smallest to largest bacterial load. Error bars represent 1 SE. Site abbreviations as in Fig. 1. Bars with different lowercase letters are sites that were significantly different in percent organic content by Tukey multiple comparisons tests and in percent silt + clay, median grain size, and bacterial load by non-parametric multiple comparisons

parisons of anti-bacterial activity and extract yield among sites. The 'potency' of extracts, defined as the activity per unit gram of extract, was examined by dividing anti-bacterial activity by the extract yield and testing for correlations with the sediment features also using the 'corr' package.

RESULTS

Sediment characteristics and bacterial load

The 6 sites on San Juan Island displayed wide variation in sediment grain size profiles and organic content (Figs. 2 & 3). Percent silt + clay (Kruskal-Wallis: $\chi^2 = 30.3$, df = 5, $p < 0.001$) and median grain size ($\chi^2 = 16.2$, df = 5, $p < 0.01$) differed significantly among sites. GB had the finest distribution of grain sizes and the lowest modal size. FJ, FB, and MB had the same modal sizes and included increasingly finer sediments, respectively. The 2 AL sites had the largest modal sizes and the coarsest sediments. GB had by far the largest and significantly highest percent silt + clay at 39% (Tukey's HSD; Fig. 3A), while the other sites ranged from 11.2% to 0.49% (Fig. 3A). GB also had significantly lower median grain size (non-parametric Tukey; Fig. 3B) than the other sites. MB had the second highest average percent silt + clay but was not significantly different from the other sites. The 2 AL sites had the largest median grain sizes and were significantly different from FB, FJ, and MB.

The percent organic content of dry sediment was significantly different among sites, ranging from 1.82% to 4.65% (ANOVA: $F_{5,12}$ = 6.44, p < 0.01; Fig. 3C). MB had the highest organic content, followed by GB; MB was significantly greater than the remaining 4 sites, but GB was not different from either group (Tukey's HSD; Fig. 3C). The bacterial load of sediment (bacteria g^{-1} dry sediment) differed significantly among sites (Kruskal-Wallis: χ^2 = 29.7, df = 5, p < 0.001) (Fig. 3D). GB and MB had significantly higher bacterial loads than FB, FJ, AL1, and AL2 (non-parametric Tukey).

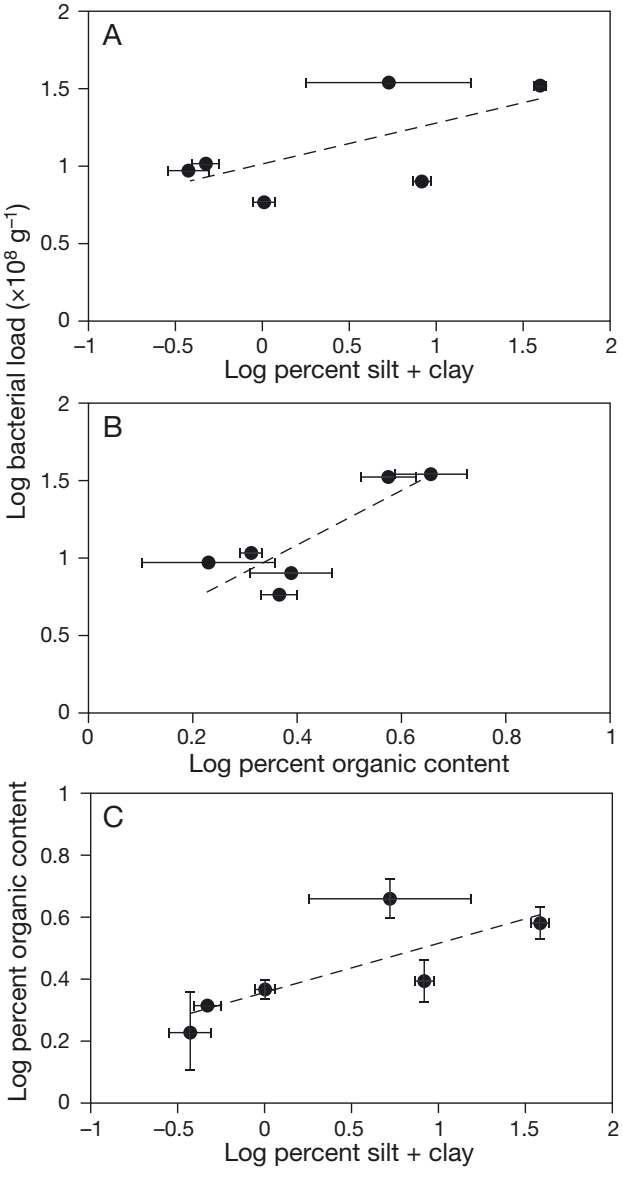

Fig. 4. Covariation (means ± 1 SE) among (A) sediment bacterial load and percent silt + clay, (B) bacterial load and percent organic content, and (C) percent organic content and percent silt + clay at 6 field sites. Each point represents the average for the site (left to right): (A,C) AL1, FJ, FB, MB, AL2, GB; (B) AL1, FJ, FB, AL2, GB, MB. Dashed lines are best fit from ordinary least squares regression. Error bars may be smaller than the symbols for bacterial load

Bacterial load was positively correlated with organic content (Pearson's r = 0.85, df = 4, p < 0.02) and showed a strong positive trend with percent silt + clay (r = 0.71, df = 4, p = 0.06) (Fig. 4A,B). Organic content and percent silt + clay were also positively correlated (r = 0.86, df = 4, p < 0.05; Fig. 4C). Although partial correlations of variable pairs holding the third variable constant were not significant, the partial correlation coefficient (pr) was notably higher between bacterial load and organic carbon content (pr = 0.68) than between bacterial load and grain size (percent silt + clay) (pr = –0.07; Fig. 5).

Anti-bacterial activity

The level of anti-bacterial activity in egg mass extracts varied among sites (Fig. 6). Activity against *Bacillus subtilis* was significantly different across sites only in the MeOH fraction (ANOVA: $F_{5,12}$ = 6.11, p < 0.01; EtOAc fraction ANOVA: $F_{5,12}$ = 1.67, p = 0.22; Fig. 6B), with Site AL1 significantly different from FJ and GB. Activity against *Vibrio harveyi* was significantly different across sites only in the EtOAc fraction (ANOVA: $F_{5,12}$ = 3.81, p = 0.03; MeOH fraction ANOVA: $F_{5,12}$ = 0.65, p = 0.67; Fig. 6C), with MB greater than FB. Average extract yield varied between sites within both the EtOAc (ANOVA: $F_{5,12}$ = 7.29, p < 0.01) and MeOH (ANOVA: $F_{5,12}$ = 10.6, p < 0.01) fractions (Table A1 in the Appendix). However, differences in yield were not associated with and therefore did not fully explain differences in anti-bacterial activity among sites. For instance, FJ and

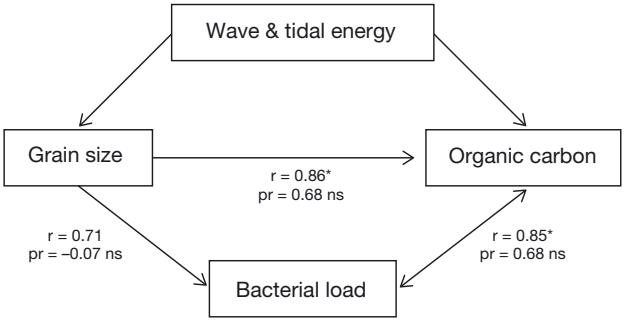

Fig. 5. Functional relationships expected among bacterial load, wave energy, grain size, and organic content in intertidal environments (adapted from Dale 1974). Values beside lines are correlation coefficients (r) and partial correlation coefficients (pr) calculated for sediment among the 6 habitats surveyed in the present study. *Significant (p < 0.05). ns = not significant

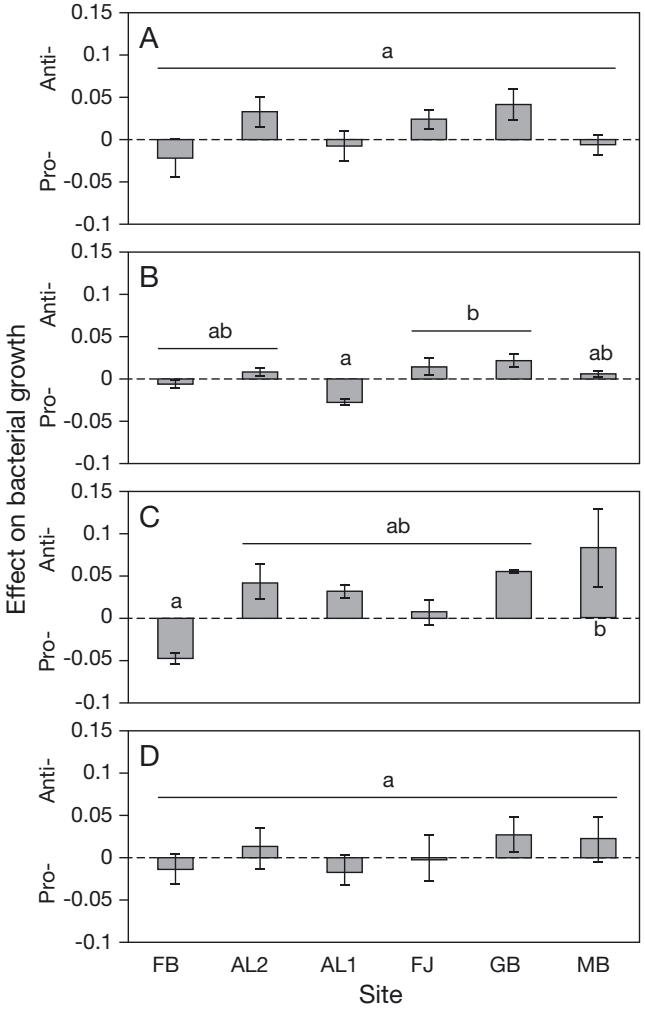

Fig. 6. Anti-bacterial activity against (A,B) *Bacillus subtilis* and (C,D) *Vibrio harveyi* for different sites, shown separately for (A,C) non-polar ethyl acetate extracts and (B,D) polar methanol extracts. Positive values indicate anti-bacterial activity and negative values indicate pro-bacterial activity. Error bars represent 1 SE. Site abbreviations as in Fig. 1. Bars with different lowercase letters are sites that were significantly different by Tukey HSD

GB had the highest yields in the EtOAc extracts and were significantly different from AL1 and AL2 (Tukey's HSD), but these patterns were not evident in activity against either *B. subtilis* or *V. harveyi*.

Anti-bacterial activity was positively related to each of the 3 sediment characteristics across sites in both the EtOAc and MeOH fractions (Fig. 7). Against *V. harveyi*, it was also positively correlated with bacterial load in both extract fractions (EtOAc: r = 0.80, df = 4, p = 0.03, Fig. 7A; MeOH: r = 0.83, df = 4, p < 0.05, Fig. 7B) and with organic content (r = 0.87, df = 4, p = 0.01) and with percent silt + clay (r = 0.93, df = 4, p < 0.01) in the MeOH fraction (while in EtOAc — organic content: r = 0.69, df = 4, p = 0.06; percent silt + clay: r = 0.49, df = 4, p = 0.16). Against *B. subtilis*, activity showed a similar positive trend with bacterial load and the other sediment features, but the correlations were not significant (Table A2 in the Appendix). MB and GB, which had the highest bacterial load, organic content, and percent silt + clay (Fig. 3), also had the highest anti-bacterial activity against *V. harveyi* in EtOAc extracts (Fig. 7A,C,E). Sites FB, AL1 and FJ, which had the lowest bacterial loads, organic content, and percent silt + clay, also had the lowest anti-bacterial activities against both *V. harveyi* and *B. subtilis* in both EtOAc and MeOH fractions (Fig. 7A–F). The potency of the anti-bacterial extracts was not correlated with bacterial load or with the other sediment features for either bacterium (Table A2).

DISCUSSION

A set of interrelated features of sediment can contribute to risks of microbial infestation for organisms dwelling in soft-sediment habitats. In the present study, field sites for populations of *Melanochlamys diomedea* differed significantly in the small-particle content of sediment, sediment organic content, bacterial load, and level of anti-bacterial defense found in egg masses. Furthermore, all of these measures positively covaried across sites. Although past studies have detected covariation among sediment characteristics and bacterial densities in soft-sediment habitats (Dale 1974, summarized in Rublee 1981), the present study is the first to demonstrate a relationship between these environmental factors and the level of anti-bacterial defense exhibited by resident organisms.

Because organic content, grain size, and bacterial load were highly correlated, it is unclear whether one or a combination of these features is responsible for variation in anti-bacterial defenses. We hypothesize that physical characteristics drive variation in bacterial communities, and that the resulting variation in either overall bacterial load or a particular component of the bacterial community induces changes in anti-bacterial defense. Below we discuss evidence for these relationships.

Relationship between sediment properties and bacterial load

Marine sediments exhibit variation in bacterial densities (10^6 to 10^{11} g^{-1} dry sediment; Alongi 1988,

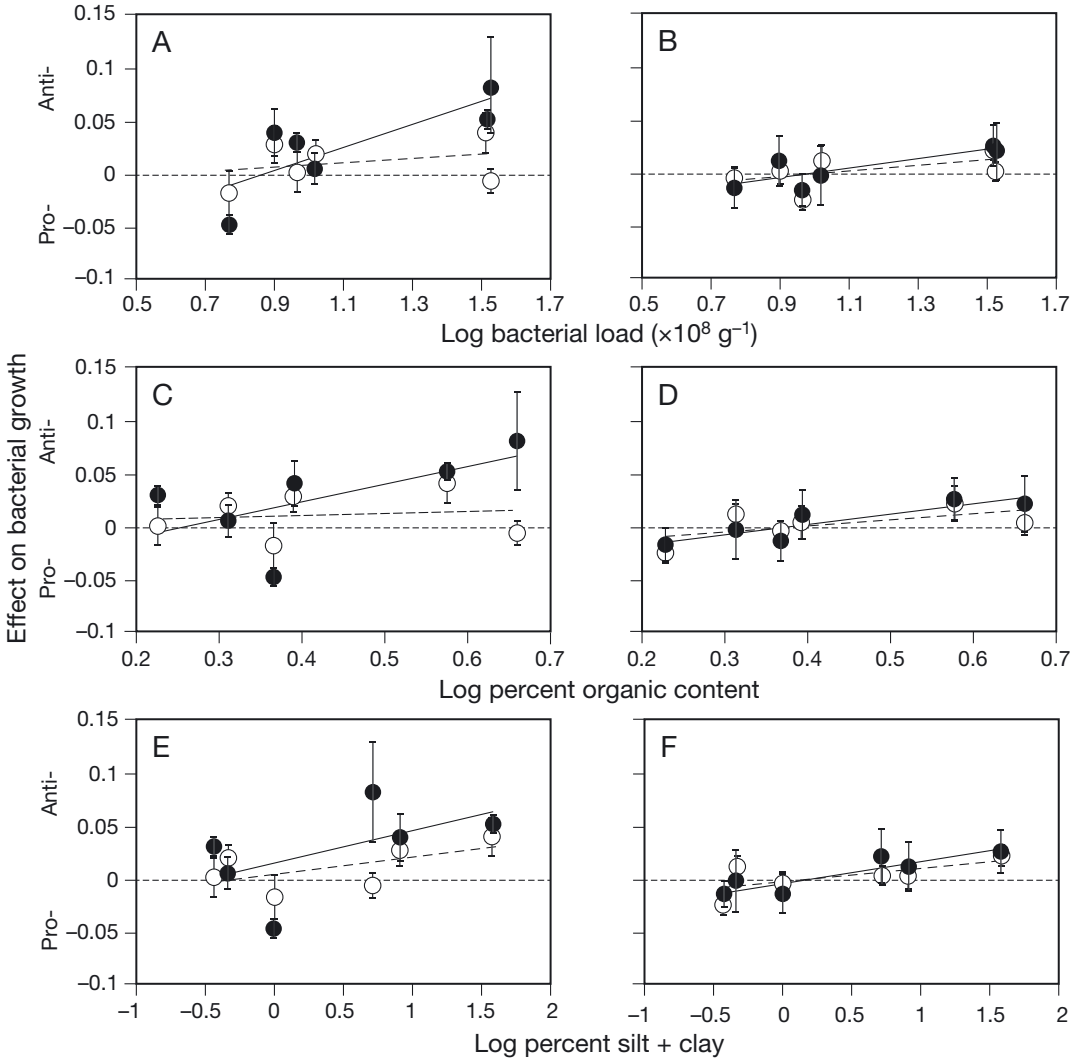

Fig. 7. Anti-bacterial activity against *Bacillus subtilis* (open circles and dashed regression line) and *Vibrio harveyi* (closed circles and solid regression line) of (A,C,E) ethyl acetate extracts and (B,D,F) methanol extracts of egg masses in terms of (A,B) sediment bacterial load, (C,D) percent organic content, and (E,F) percent silt + clay at each field site. Positive values indicate anti-bacterial activity and negative values indicate pro-bacterial activity. The order of sites from left to right is (A,B) FB, AL2, AL1, FJ, GB, MB; (C,D) AL1, FJ, FB, AL2, GB, MB; (E,F) FJ, FB, AL1, AL2, MB, GB (site abbreviations as in Fig. 1). Error bars represent 1 SE

Schmidt et al. 1998), which could be influenced by several environmental factors including sediment organic content, water content, grain size, and grain shape (Dale 1974; our Fig. 5). Our measures of bacterial load, organic content, and percent silt + clay are in the same range as those reported in past site comparisons of sediment bacterial load (Cammen 1982, Luna et al. 2002) and of covariation in sediment characteristics (Dale 1974, DeFlaun & Mayer 1983). We found the highest bacterial loads in habitats such as GB and MB that have muddy substrates with smaller grain sizes representative of lower-energy habitats. In contrast, sites FB and FJ are relatively high-energy habitats with sandy sediments (Fig. 3). As expected, both sediment organic content and percent silt + clay were positively correlated with bacterial load. In turn, these high bacterial loads in GB and MB could increase the exposure of resident organisms to detrimental bacteria.

Because sediment characteristics and hydrodynamic regime covary strongly, it has remained unclear which sediment features drive variation in bacterial load. Particle size is thought to play a major role because it determines surface area for microbial colonization (Dale 1974, DeFlaun & Mayer 1983), but the presence of organic material can enhance microbial metabolism and promote production (Dale 1974, Rublee & Dornseif 1978, Fischer et al. 2002). The rela-

tionship between organic content and bacterial load is also confounded by bacterial contributions to the total organic carbon of sediment (0.2–9.5%; reviewed in Danovaro & Fabiano 1995). Although our results do not definitively separate contributions of these correlated variables, we found that organic content had a stronger partial correlation with bacterial load among habitats than did sediment size (Figs. 5 & 6).

Relationship between microbial load and anti-microbial activity

Variation in microbial abundance is thought to drive variation in the production of anti-microbial defenses (Dube et al. 2002, Woodhams et al. 2006). Organisms living in or on sediments are therefore predicted to have relatively high levels of chemical defense against predators and microbes compared with those on hard substrates (Kicklighter & Hay 2007). In our study system, for example, the microbial load of sediment that egg masses are in contact with could influence bacterial concentrations in egg masses. High concentrations of surface fouling by bacteria, microalgae, and protists have been associated with increased rates of embryonic mortality (Biermann et al. 1992, Przeslawski & Benkendorff 2005, Peters et al. 2012), although at more moderate levels, fouling microalgae can benefit embryos by enhancing oxygen availability (Fernandes & Podolsky 2011). Anti-microbial defenses in sites with high bacterial loads, such as GB and MB, could be especially important under environmental conditions that promote microbial growth. The reproductive period of *M. diomedea*, for example, peaks in the late spring and summer when water temperatures within tidal pools are high (Podolsky 2003) and bacterial abundance would likely increase (Dale 1974, DeFlaun & Mayer 1983). During this period, GB and MB were highest in both bacterial load and level of anti-bacterial activity in egg masses, against both *Vibrio harveyi* and *Bacillus subtilis.* We found a similar correspondence between bacterial load and anti-bacterial activity in GB for egg masses of another cephalaspid, *Haminoea vesicula* (S. C. Smoot et al. unpubl. data). Conversely, increased levels of organic content in sediment, as observed in GB and MB, could change properties of the sediment (e.g. pH) that could counteract the potency of anti-microbials (Inderjit & Callaway 2003). Future studies should examine temporal variation over the reproductive period season in the relationship between bacterial load and anti-bacterial activity.

In addition to bacterial concentration, variation in bacterial species composition could also contribute to variation in the level of anti-microbial activity among sites. For example, pathogenic bacteria such as *V. harveyi*, which commonly cause disease in molluscs, crustaceans, and fish (Austin & Zhang 2006), could vary in prevalence as a function of physical or biotic conditions. Molecular methods including fluorescent *in situ* hybridization (FISH), community fingerprinting (e.g. denaturing gradient gel electrophoresis [DGGE]), and sequencing will be needed to begin characterizing microbial species composition and quantifying particular pathogens among populations or experimental conditions.

Studies investigating spatial variation in anti-microbial defenses are essential to understanding their function and evolution. Between-population variation in anti-microbial defense is widespread among sponges (Rohde et al. 2012), corals (Dube et al. 2002), amphibians (Tennessen et al. 2009), and birds (Ruuskanen et al. 2011). Despite the taxonomic diversity among these studies, relatively few have examined the role of environmental variation in driving anti-microbial defenses. Where variation in activity among sites was not apparent — such as the EtOAc extracts against *B. subtilis* and the MeOH extracts against *V. harveyi* — we still found variation within sites. Future studies should account for this level of variability when designing experiments to understand the spatial pattern of variation in anti-microbial defenses in the field.

Possible adjustment of anti-bacterial defenses to local conditions

The nature and source of compounds involved in anti-bacterial activity, and therefore the mechanism by which they could be adjusted, are uncertain. It is possible that anti-microbial peptides (AMPs) (reviewed in Rollins-Smith & Woodhams 2012) or other secondary metabolites are adjusted by adults, by embryos, or by associated microbiota. However, the higher levels of anti-microbial activity we observed in the more lipophilic EtOAc extracts argue against AMPs (Rollins-Smith & Woodhams 2012) and in favor of more non-polar compounds. Benkendorff et al. (2005) observed similarly higher anti-microbial activity from unsaturated fatty acids and sterols in lipophilic extracts of gelatinous egg masses. The fact that extract potency did not vary among sites and was not correlated with sediment properties suggests that an increase in production, rather than a change in com-

position of anti-microbial compounds, is a more likely explanation for variation in defense among sites.

If anti-microbial compounds are produced by mothers or embryos, then differences among populations in microbial defenses could arise from fixed genetic differences or from plasticity in the production of anti-bacterial compounds. Although the population structure of *M. diomedea* at this scale is unknown, it has a larval duration of at least 40 d (Strathmann 1987, Mach & Podolsky 2005), and the sites used in the present study have been shown by simulations of larval dispersal to be highly connected (Engie & Klinger 2007). Thus, local populations are likely to experience strong gene flow, and differences in anti-bacterial activity that we measured among habitats are more likely a result of plastic responses than of strong selection, especially given that adults are not likely to have been retained at their natal sites (Castro & Podolsky 2012).

The finding in other systems that anti-bacterial activity was higher earlier in development has suggested that the source of these compounds was maternal rather than embryonic (Kamiya et al. 1984, Benkendorff 1999). However, an alternative source of anti-microbial compounds is bacteria associated with the egg mass. Bacteria identified within freshly laid egg masses and the alimentary canal of cephalopods (Biggs & Epel 1991, Barbieri et al. 1997), as well as within freshly laid egg masses and the reproductive gland of adults of the nudibranch *Dendrodoris nigra* (Klussmann-Kolb & Brodie 1999), are thought to be the source of anti-microbial activity in those species. The production of anti-microbials by associated bacteria is common to reproductive material in several animal groups, including molluscs (Biggs & Epel 1991, Barbieri et al. 1997), arthropods (Gil-Turnes et al. 1989), amphibians (Brucker et al. 2008), and birds (Soler et al. 2010). In some cases, protective bacteria are introduced directly by the adult (e.g. in the externally brooding shrimp, *Palaemon macrodactylus*; Gil-Turnes et al. 1989). In most egg masses, anti-microbial activity tends to decline rather than increase during development (Kamiya et al. 1984, Benkendorff 1999), suggesting that horizontal transmission of protective bacteria is unlikely to be the source of defense. In other cases, maternally derived compounds influence the colonization of specific beneficial bacteria that provide protection to early embryos in hydra (Fraune et al. 2010), crustaceans (Gil-Turnes et al. 1989), and salamanders (Banning et al. 2008). Additionally, bacteria can upregulate their own anti-bacterial production under more competitive conditions (reviewed in Cornforth & Foster 2013), such as the higher bacterial load observed at MB and GB in the present study. If mothers are the source of such compounds, through the transmission of either metabolites or bacterial symbionts, then our results suggest that the bacterial challenge faced by the mother is critical for determining the level of defense. This hypothesis could be tested by separately manipulating maternal and embryonic microbial exposures.

The widespread distribution of anti-bacterial compounds in the egg masses of cephalopods and gastropods (Benkendorff et al. 2001, Ramasamy & Murugan 2005, Benkendorff 2010) suggests that they are important for the protection of sessile early life-history stages. Such defenses have been observed both in leathery egg capsules and gelatinous egg masses (Benkendorff et al. 2001). We have shown here that sediment has properties that can vary substantially across sites and that are associated with changes in the bacteria to which egg masses are exposed (Dale 1974, Cammen 1982). Further examination of the role of ecological factors using field techniques in determining the level of anti-bacterial defense will help to elucidate the specific defense mechanisms of organisms such as molluscs that lack an innate immune system.

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Appendix. Dry-to-wet mass ratios, extract yields, and correlations between anti-bacterial activity and sediment properties of *Melanochlamys diomedea* egg masses

Table A1. Average (±SE) dry-to-wet mass ratios and natural concentrations of *Melanochlamys diomedea* egg masses collected from field locations. Site abbreviations as in Fig. 1. EtOAc: ethyl acetate; MeOH: methanol

Site	Dry:wet ratio	Concentration of extract, mg extract ml^{-1} wet egg mass	
		EtOAc	MeOH
FB	$2.37 \times 10^{-2} \pm 2.57 \times 10^{-3}$	2.32 ± 0.12	3.03 ± 0.13
AL2	$1.66 \times 10^{-2} \pm 6.45 \times 10^{-4}$	1.53 ± 0.08	4.76 ± 0.65
AL1	$1.80 \times 10^{-2} \pm 8.48 \times 10^{-4}$	1.70 ± 0.17	3.95 ± 0.03
FJ	$3.21 \times 10^{-2} \pm 5.01 \times 10^{-4}$	3.67 ± 0.49	6.68 ± 0.76
MB	$2.02 \times 10^{-2} \pm 2.24 \times 10^{-4}$	2.25 ± 0.21	3.99 ± 0.72
GB	$3.34 \times 10^{-2} \pm 4.38 \times 10^{-4}$	4.05 ± 0.75	8.12 ± 0.76

Table A2. Correlations between anti-bacterial activity and sediment properties (df = 4 for all tests). Activity: absolute value; Potency: activity g^{-1} extract; Bs: *Bacillus subtilis*; Vh: *Vibrio harveyi*; EtOAc: ethyl acetate; MeOH: methanol

Anti-bacterial	Bacteria	Fraction	Characteristic	r	p
Activity	Bs	EtOAc	Bacterial load	0.25	0.32
Activity	Bs	EtOAc	Organic content	0.07	0.45
Activity	Bs	EtOAc	Percent silt + clay	0.65	0.08
Activity	Bs	MeOH	Bacterial load	0.52	0.15
Activity	Bs	MeOH	Organic content	0.49	0.16
Activity	Bs	MeOH	Percent silt + clay	0.67	0.07
Activity	Vh	EtOAc	Organic content	0.69	0.06
Activity	Vh	EtOAc	Percent silt + clay	0.49	0.16
Potency	Bs	EtOAc	Bacterial load	0.03	0.48
Potency	Bs	EtOAc	Organic content	−0.49	0.84
Potency	Bs	EtOAc	Percent silt + clay	−0.33	0.74
Potency	Bs	MeOH	Bacterial load	0.49	0.16
Potency	Bs	MeOH	Organic content	0.14	0.39
Potency	Bs	MeOH	Percent silt + clay	−0.20	0.65
Potency	Vh	EtOAc	Bacterial load	0.43	0.20
Potency	Vh	EtOAc	Organic content	0.20	0.35
Potency	Vh	EtOAc	Percent silt + clay	−0.03	0.53
Potency	Vh	MeOH	Bacterial load	0.71	0.06
Potency	Vh	MeOH	Organic content	0.43	0.20
Potency	Vh	MeOH	Percent silt + clay	−0.05	0.53

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