Marine Pollution Bulletin 62 (2011) 2293–2298

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Pore water transport of enterococci out of beach sediments

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ARTICLE INFO

Keywords:
Enterococci
Beach sand
Pore water
Sand cores
Columns

ABSTRACT

Enterococci are used to evaluate the safety of beach waters and studies have identified beach sands as a source of these bacteria. In order to study and quantify the release of microbes from beach sediments, flow column systems were built to evaluate flow of pore water out of beach sediments. Results show a peak in enterococci (average of 10% of the total microbes in core) released from the sand core within one pore water volume followed by a marked decline to below detection. These results indicate that few enterococci are easily removed and that factors other than simple pore water flow control the release of the majority of enterococci within beach sediments. A significantly larger quantity and release of enterococci were observed in cores collected after a significant rain event suggesting the influx of fresh water can alter the release pattern as compared to cores with no antecedent rainfall.

1. Introduction

The US Environmental Protection Agency (EPA) recommends enterococci to evaluate the safety of recreational marine beaches (US EPA, 1980). Enterococci are commonly found in sewage and their levels correlate with human health outcomes at sewage impacted beaches. Enterococci have also been shown to inhabit beach sediments and can contaminate water at beaches without a source of sewage contamination (Shibata et al., 2004; Whitman et al., 2003; Badgley et al., 2010; Wright et al., 2011). While sewage sources of enterococci are easier to identify and control, enterococci from beach sediments present a unique problem as they are more difficult to remediate. In 2009, over half of all closings nationwide were due to unknown sources of enterococci (Dorfman and Rosselot, 2010) and beach sediments can be potentially responsible a large fraction of these closures.

Multiple studies have evaluated the movement and filtration of microbes through porous media via the introduction of allochthonous microbes. These studies determined that factors such as motility of the bacteria (Camesano and Logan, 1998; Camper et al., 1993), saturation of the media (Schäfer et al., 1998), dissolved and sediment organic matter (Johnson and Logan, 1999; Morales et al., 2011), grain size, surface area and other abiotic and biotic parameters (Stevik et al., 2004) that affect the retention of bacteria. While these and other studies have analyzed the filtration of allochthonous microbes through porous media, few studies have evaluated the conveyance of indigenous microbes out of beach sediments through the effects of tidal induced pore water flow. An understanding of the processes by which indigenous microbes are released from beach sediments into the water column would assist in the control and prediction of microbial contamination in recreational waters.

The hypothesis driving this study is that pore water flow through sand interstitial spaces accounts for the majority of the enterococci released from beach sand. Thus this study set out to evaluate the fraction of enterococci released into the water column via flow induced by a head difference, as opposed to mechanical stirring of bottom sediment by waves. This study specifically looked at the contribution of interstitial water flowing out of naturally stratified sediments. *In situ* this flow would be driven by pressure differentials created from the rising and falling tides (Turner, 1993). Though not an original objective of the study, we were also able to observe the release of enterococci from beach sands following a significant rain event. Twelve states preemptively post beach advisories following significant rain events before levels of enterococci can be measured in the water. Theses closings were responsible for 21% of closings nationwide in 2009 (Dorfman and Rosselot, 2010). We were able to investigate the validity of this practice and explore differences in the levels and release of enterococci during and in the absence of antecedent rainfall.

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doi:10.1016/j.marpolbul.2011.08.049

2. Materials and methods

2.1. Site description

Sand for this study was obtained from Hobie Cat Beach (Miami, FL), a site which exceeds the EPA recommended enterococci counts 20% of the time (Dorfman and Rosselot, 2010) and whose source of enterococci has been well documented to be the sands within the intertidal zone (Shibata et al., 2004; Wright et al., 2011; Shah et al., 2011). The sand at this site has been well characterized with respect to grain size (average D_{50} = 0.39 ± 0.05 mm; D_{50} is the size at which 50% of the sand grains by mass are finer), volatile organic compounds (VOC) (VOC = 0.81% ± 0.61%), composition and levels of extracellular polymeric substances (EPS) (EPS = 6.89 ± 5.4 μg EPS/g dry sand) the quantifiable component of biofilms (Piggot and Klaus, 2010). At this beach, elevated levels of enterococci have been observed in shallow water near the shoreline during outgoing tides (Abdelzaher et al., 2010; Wright et al., 2011). These elevated levels could be attributed to the tidal flushing of enterococci out of beach sediments. We aimed to emulate this release with flow column experiments.

2.2. Field sampling

Beach sand sampling occurred from July 2009 to January 2010. Samples were collected from both the "dry" sand above the mean high tide line and from "wet" sand below the wrack line in the swash zone. Samples were collected using a series of four cores, a primary experimental core and three background cores. All cores were made of polyvinylchloride (PVC). The experimental core was placed first and driven into the ground after which three smaller cores were each placed 120° apart, immediately adjacent to the experimental core (Supplementary Fig. S-3). The experimental core was used for the flow column and the three smaller cores were used to evaluate the pre-experimental microbial content of the sand. The primary experimental core measured 10.14 cm in diameter and 16 cm in length. The background cores were 2.54 cm in diameter and 16 cm in length. All of the PVC cores were sanitized with 75% isopropyl alcohol prior to sampling. The cores were collected by removing sediment around the cores and by sliding a sterile stainless steel plate under the cores to prevent sediment loss when they were removed. The cores were then capped and immediately transported back to the laboratory. All microbial testing, was performed within 3 h of sample collection.

2.3. Flow column systems

Two separate flow column systems were utilized to evaluate flow and to quantify the release of enterococci as water flows through beach pore water. The first system was designed to measure the release of enterococci by water moving up through the beach sand. The second system was designed to measure the release of bacteria by water moving downward through the beach sand, looking for differences between upward and downward flow. Pore water velocities mimicked the average spectrum of natural pore water outflow from sediments which range up to over 40 cm/h (Precht and Huettel, 2004). Velocities exceeding these levels were also tested to accentuate any trends. Both systems incorporated the same beach sand sampling technique, same microbe analysis techniques and same methods for documenting the physico-chemical characteristics of the sand.

The flow-column system for upward flow consisted of a sanitized, "U" shaped, 10.14 cm diameter PVC pipe where the sand core was placed on the right side and aseptic marine water was added to the left-hand side of the "U" maintaining a positive and constant head for flow through the experimental core (Supplementary Fig. S-1). The outflow overflowed into a collection chamber where water volume was measured and water samples were collected into pre-sterilized 10 mL test tubes. Water volume versus time was recorded in order to determine flow rate.

The flow column system for downward flow consisted of a sanitized 30 cm long, 10.14 cm diameter PVC pipe which served as a reservoir where aseptic marine water was added and kept at a constant head above the core (Supplementary Fig. S-2). The bottom of the reservoir was attached to the top of the experimental sand core. A sample collection funnel was attached to the bottom of the experimental sand core and samples were collected in the same manner as for the upward flow system.

2.4. Laboratory procedures for microbial analysis

Water samples were analyzed for enterococci within 1 h of sample collection using the EPA standard membrane filtration method based on the utilization of mEI agar (Method 1600, US EPA, 1997). Sediment samples from the background and experimental cores were analyzed in triplicate, within 3 h from the time of sampling. Enterococci were extracted from sediment samples by shaking 10 g of sand in 100 mL of PBS for 2 min. This method was utilized in past studies of Hobie Beach (Shibata et al., 2004; Treatment 1 in Boehm et al., 2009) and produced the highest possible extraction yield using simple extraction assays typically used for recreational beach studies. All results were expressed in CFUs (Colony Forming Units) per 100 mL or CFUs per gram of dry sand.

2.5. Mass balance

Total enterococci in the sand core was computed as the sum of the number of CFU's removed from the core (dislodgeable bacteria) during the flow column experiments plus the number of bacteria in the core after the flow through experiment. This number was compared to a theoretical initial level which was computed by multiplying the dry weight of the sand in the core by the average level of enterococci within the three background cores.

2.6. Physical characterization of water and sediment

Sediment and water samples were both analyzed for physical characteristics. Excess water from the flow column systems (water not used for enterococci analysis) was tested for pH, temperature and salinity, using a multi parameter probe (YSI 650MDS) and for turbidity (Turner Designs Model 40 Nephelometer). Sediment samples from the experimental cores were analyzed for grain size, water content, porosity, hydraulic conductivity and volatile organic compounds fraction using standardized methods. These parameters sought to identify possible characteristics in the sediments that could be responsible for changes in release or levels of dislodgeable enterococci. Grain size analysis was conducted using the sieve method (Syvitski, 1991) and expressed as D_{50} (the grain size at which 50% of the grains are finer) and as UC (the uniformity coefficient, D_{60}/D_{10}). Water content and VOC were measured using gravimetric methods and porosity was determined using a volumetric method. Hydraulic conductivity was calculated from the mean flow rate (measured during the experiment), the static head measurements and by invoking Darcy's Law. See Supplementary material for more details about the measurement methods.

2.7. Data analysis

All statistical analysis was performed using Microsoft Excel, XL STAT (Addinsoft USA, New York, NY). Data was tested for normality. Correlations for parametric data were acquired using Pearson's

Test (r). Correlations for non-parametric data were acquired using Spearman's Rank Order Test (r_s). An alpha of 0.05 was used for all tests, both parametric and non-parametric. Correlations were analyzed using data from all cores except rain cores ($N = 13$). The "rain cores" ($N = 2$) were analyzed separately and compared to the rest of the trials using a single variable ANOVA.

3. Results and discussion

No rainfall was measured within 24 h prior to the collection of any set of sand cores with the exception of the two cores called "rain cores" which were collected during a period of heavy rainfall. Results from the "rain cores" were very different than the results from the remaining cores and for this reason the "rain cores" results are discussed separately at the end of this section.

The primary objective of this study was to understand and quantify the release of enterococci from beach sediments through the action of pore water flow. Working with the assumption that the enterococci primarily reside within the pore water or can be easily dislodged from the sand grains, we hypothesized that the majority of the microbes would be released via conveyance by pore water and that the percentage of microbes released would increase with increasing flow velocity. The results presented here indicate that the enterococci released from natural beach sands by means of conveyance make up a minority (10%) of the total bacteria contained within the core. After a hydrostatic pressure driven flow passed through the core, 90% of the bacteria, on average, remained within the sediment. The relatively small fraction of enterococci that washed off the core, or dislodgeable bacteria, were either residing in the pore water or were more susceptible to hydrodynamic forces than the remaining bacteria which were not removed. Our original hypothesis was thus negated as results showed that only 10% of the bacteria were removed through hydrostatic pressure induced flow. These results are valid for all cores collected after periods of no rainfall. No significant differences were seen between cores collected from "dry" sand as opposed to "wet" sand in the intertidal zone.

3.1. Upward flow experiments

Results from the upward flow experiments ($N = 9$) show that the vast majority of dislodgeable bacteria were removed within the first pore water volume (measured to be approximately 400 mL) (Fig. 1). In the majority of trials, over half of the microbes that were removed from the core were released within half a pore water volume. The number of microbes released decline sharply as the amount of eluent approached one pore water volume. Samples collected after one pore water volume contained less than 10 CFU/100 mL, if detectable, and only represented less than 2%, on average, of the total dislodgeable bacteria removed during the experiment.

3.2. Downward flow experiments

The results of the downward flow experiments ($N = 4$) closely mimicked those of the upward flow experiments. The majority of the bacteria that were removed were dislodged at the initiation of flow in the experiment. Almost all of these bacteria were removed within one pore water volume of water flow through the experiment core. The mean trends of both upward flow and downward flow align closely as seen in Fig. 1.

3.3. Comparison to previous flow experiments

Studies have been successful in using flow columns to model release of nutrients (Spagnoli and Bergamini, 1997) and chemicals (Wirtz, 2003) out of sediment but very few have evaluated the release of bacteria into beach waters. One prior study has evaluated the transport of enterococci out of beach sands (Yamahara et al., 2007). In both our flow experiments and those of Yamahara et al., the majority of enterococci were released at the beginning of flow out of the sand. A major difference observed between the two studies was the volume of eluent required to release the dislodgeable bacteria. In the Yamahara et al. study, enterococci were released through four pore water volumes as opposed to our study where enterococci were released in one pore water volume. The reason for the difference could be attributed to the method of sample collection. Our study relied on taking cores where the natural strata of the sediment were kept intact whereas the Yamahara et al. study packed sand into a chromatography column. The sands used in each study were also very different. The Yamahara et al. study was conducted using beach sands from Monterey Bay, California which are medium to course grained and primarily composed of quartz, feldspar, and granitic rock fragments (Cambellick and Osborne, 1977). Our study used South Florida beach sands which are fine to coarse grained and composed primarily of calcium carbonate and quartz (Piggot and Klaus, 2010). The higher calcium carbonate composition of the South Florida beach sand could contribute to its higher retention of enterococci. Calcium carbonate sand grains are composed of fibrous pins and have pits where enterococci can evade hydrological forces. These pits could prevent the conveyance of enterococci out of the sediments. Other compositions and grain sizes of sand could also have different release characteristics for enterococci.

3.4. Physical analyses

Hydraulic conductivities ranged from 0.004 to 0.4 cm/s which were within previously reported ranges for fine to coarse sand (Das, 1985). Markedly lower percentages of the total enterococci were removed in the cores with lower hydraulic conductivities. A significant, positive linear correlation ($r = 0.57$, $p = 0.04$) can be seen between the total percentage of bacteria removed and the hydraulic conductivity of the sand in the experimental core (Fig. 2). There were no significant correlations seen with grain size (D_{50} mean = 0.35 mm: $r = 0.17$, $p = 0.25$), depth-averaged velocity (the velocity through the sand core as if the particles were not there) (mean = 0.015 cm/s: $r_s = 0.4$, $p = 0.17$) or porosity (mean = 0.46: $r = -0.45$, $p = 0.14$), all of which are associated with hydraulic conductivity. There were also no significant correlations seen with pH (mean = 7.2: $r_s = -0.08$, $p = 0.84$), salinity (mean = 32 ppt: $r = 0.04$, $p = 0.9$), VOC (mean = 0.2%: $r = -0.04$, $p = 0.91$) or turbidity (mean = 4.4 NTU: $r_s = 0.02$, $p = 0.98$) of the seawater eluent collected during the experiment (additional values for physical parameters located in Tables S-1 and S-2, for microbial parameters in Table S-3).

No trend was observed between the percentage of enterococci removed versus flow rate of the water (mean = 1.2 mL/s: $r_s = 0.4$, $p = 0.17$) or depth-averaged velocity of the water (see above). Furthermore, this result did not change based on the direction of flow, as the release pattern was the same for both the up-flow and down-flow columns (Fig. 1). This means that the percentage of enterococci removed from the sediment is independent of the velocities tested (11–145 cm/h) and vertical direction of flow.

We hypothesize that enterococci release is dependent upon the amount of biofilms present in the sand. Biofilms contain extracellular polymeric substances (usually consisting primarily of polysaccharides) which provide a stable niche for bacteria because they are adhered to sand grains and resistant to shear flows. It is known that enterococci produce biofilms (Mohamed and Huang, 2007) and that there are indeed biofilms present within the sand of Hobie Cat Beach (Piggot and Klaus, 2010). The fraction (90%)

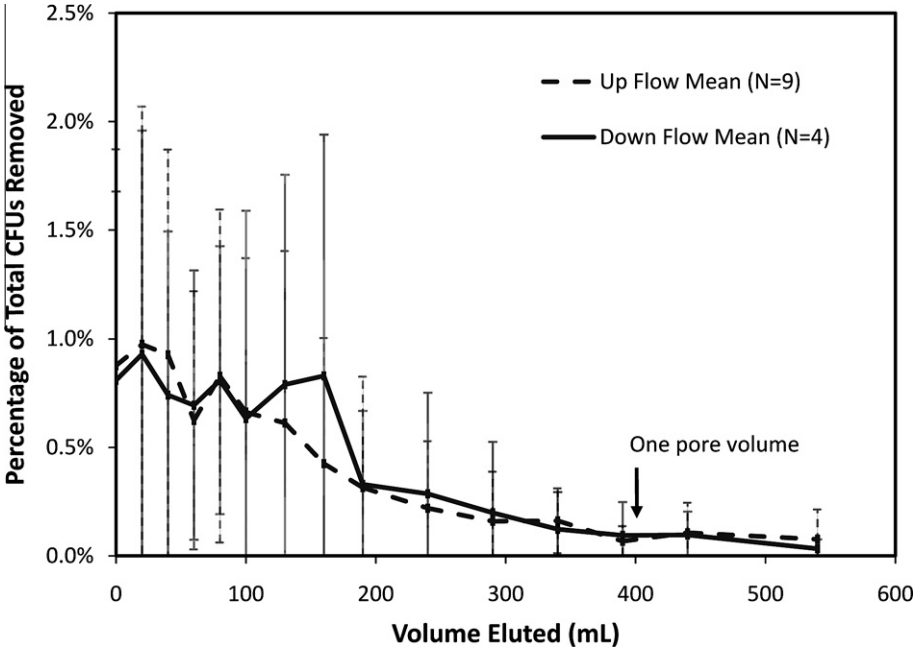

Fig. 1. Percentage of enterococci CFUs removed versus volume eluted. Bacteria removed were standardized to percentage of the total bacteria present in the core. On average, 98% of the dislodgeable CFUs were released within the first pore water volume (400 mL as indicated by the arrow). The same trend was observed in both the upward flow experiments and the downward flow experiments. Vertical error bars correspond to the standard deviation of results with the vertical solid lines corresponding to the upward flow experiments and the vertical dashed lines corresponding to the downward flow experiments.

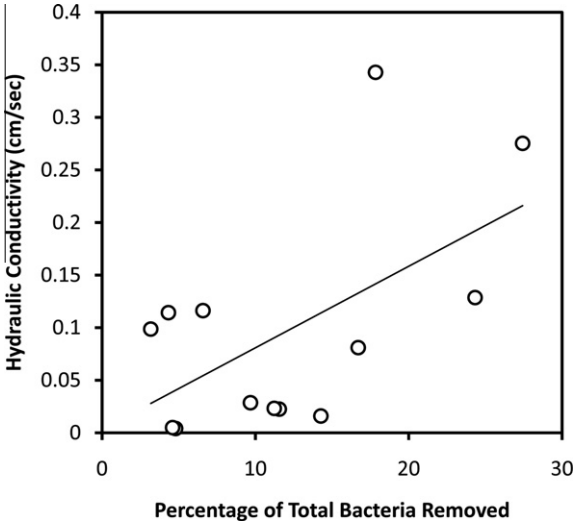

Fig. 2. Hydraulic conductivity versus percentage of total enterococci removed. A significant correlation was seen between percentage of bacteria removed from the core and hydraulic conductivity ($r = 0.57$, $p = 0.04$).

of the enterococci that were not removed from the experimental core by hydrostatic pressure driven flows could represent the approximate percentage of enterococci contained within biofilms.

The positive linear correlation between percentage of CFUs released and hydraulic conductivity (Fig. 2) could also point to biofilms as an important parameter. Biofilms can interconnect sand grains to one another, stabilizing the sediments and obstructing the flow of water through beach sediment (Yallop et al., 2000). This has been shown to reduce the hydraulic conductivity and can explain the correlation between percentage of bacteria removed and hydraulic conductivity (Kim et al., 2008). In sediments containing higher quantities of biofilms there is a larger biomass of bacteria contained within those biofilms (Yallop et al., 2000). Thus

Table 1
Selected results from the rain cores ($N = 2$) compared to the mean of all other experimental cores ($N = 13$) samples.

Parameter	Mean of rain cores	Mean of other cores
CFU/g dry sand before	340	3.5
CFUs washed off cores	166,400	659
% removed from cores	43	11

the dislodgeable enterococci would make up a smaller percentage of the total enterococci within the core. Therefore a core with high biofilm content would display a low hydraulic conductivity and a low percentage of dislodgeable bacteria whereas a core with low biofilm content would produce a higher hydraulic conductivities and a higher percentage of dislodgeable bacteria.

3.5. Rain core

On 17 December 2009 the two experimental cores were sampled after 2.5 h of rainfall totaling 3.45 cm. The results from these cores were significantly different from the rest of the cores sampled on days without antecedent rainfall (Table 1). Both average concentrations of enterococci in the rain cores prior to (340 CFU/g dry sand) and after experimentation (130 CFU/g dry sand) were two orders of magnitude higher than the average of all other experimental cores (3.5 CFU/g dry sand). The enterococci levels in the eluent of the rain cores peaked at 1.7×10^5 CFU/100 mL, within the same order of magnitude as raw sewage (10^5–10^6 CFU/100 mL, Fujioka et al., 1999). Aside from having higher levels of enterococci present in the experimental cores, a larger percent of the bacteria present in the experimental core were released from the rain core over the average of all other cores (43% of total CFU released versus 10% average released from other cores). These results were also significant using a single variable ANOVA ($p < 0.01$).

The doubling time under optimal laboratory conditions for enterococci is approximately 40 min (Leboeuf et al., 2000) but can take up to 10 h in environmental conditions (Hartz et al., 2008). Even if one were to assume that the rain created optimal laboratory conditions for the growth of enterococci; during the duration of the rain storm the enterococci population in the sand could, at a maximum, double about 4 times (2.5 h = 150 min of rain/40 min doubling time = 3.75 times the original population would double). Using the average for the other cores (3.5 CFU/g), this growth would still leave the population of enterococci at less than an eighth of the total CFUs per gram of dry sand ($3.5 \times 2^4 = 56$ CFU/g) obtained on average in the rain cores (340 CFU/g). Assuming that we can compare the prior average sand conditions to that rain core, this means that there are CFUs that were measured in the rain core that were either undetectable prior to the rain event or CFUs that were not present in the core prior to the rain event, or both. If the bacteria were not in the core prior to the rain event, it is possible that the inflated number of enterococci is caused by runoff from higher up on the beach transporting the microbes down to the swash zone (Auer and Niehaus, 1993; Wright et al., 2011). This is supported by the fact that the percentage of the total enterococci removed during experimentation from the rain core was much higher than average. This means that more of the enterococci were dislodgeable in the rain cores than in the other experimental cores. The larger amount of pore water within the rain cores was not enough to explain this increase in percentage suggesting it was not more pore water, just more contaminated pore water being released during the rain core experiments.

The higher percentage of dislodgeable bacteria could also be attributed to biofilms breaking down as the rain changes the osmolarity of the pore water. The biofilms within the sand are normally exposed to marine waters from tides and sea spray. The rapid influx of freshwater during a rain event structurally weakens or breaks down marine biofilms causing them to dissociate from their substrate (Kierek and Watnick, 2003). This would increase the percentage of dislodgeable bacteria within the sand.

Another possible explanation is that the enterococci measured in the rain core were previously non-culturable during antecedent dry conditions. Upon wetting of the sand, the previous non-culturable enterococci could then enter a culturable state. Enterococci enter a highly resistant starvation phase when exposed to stressful environmental conditions (Barcina et al., 1997). This phase, while not a spore, is less metabolically active than the normal longitudinal growth phase of enterococci. Because of this "stressed" enterococci do not show up on the conventional membrane filtration method for quantifying enterococci (del Mar Lleo et al., 2007; Oliver, 2005). The rain could potentially change the microbe's environment in a way that would cause the enterococci to switch from the viable but not culturable phase to the normal longitudinal growth phase. Once in the longitudinal growth phase, the membrane filtration technique would be able to quantify more of the bacteria in the experimental core. This would explain the inflated count of both the total enterococci (before) and the non-dislodgeable enterococci (after experimentation) in the rain cores.

4. Future work

A direct quantification of the amount of EPS needs to be conducted in unison with pore water flow experiments in order to more credibly conclude that biofilms do indeed determine the percentage of dislodgeable bacteria and effect hydraulic conductivity. Tests should also be conducted to determine if marine biofilms are structurally damaged or broken down by fresh water. Future work should also focus on evaluating the viable but non-culturable state of enterococci, possibly using microscopy and PCR methodologies. This would be especially relevant in looking at the differences in enterococci levels in the sand post rainfall versus antecedent dry conditions.

The identification of sediment parameters was purely intended to generate hypotheses, as only one beach was evaluated in this study. Sediments from other beaches should be investigated looking specifically at differences in grain size, sediment composition, VOC and EPS levels compared with the percentage of enterococci released. The method of sample collection should also be evaluated to determine if different release patterns are observed for sand collected via coring methods versus composited samples packed into columns.

Forces other than vertical flow should be modeled and tested for their effect on transporting microbes out of beach sediments. Shear forces such as those caused by the stirring action of waves against the shore should be examined in an attempt to more fully understand the mechanisms by which microbes are removed from the beach sand.

Acknowledgments

We thank all the assistance from Ashish Shah and Eduardo Estevez. We also thank Dr. Don Olson and Dr. Jim Klaus for their helpful ideas and suggestions. Funding for this project was received through the NSF REU program and through the National Science Foundation (NSF) and the National Institute of Environmental Health Sciences (NIEHS) Oceans and Human Health Center at the University of Miami Rosenstiel School [NSF 0CE0432368/0911373] and [NIEHS P50 ES12736].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.marpolbul.2011.08.049.

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