Canadian Journal of Microbiology
Revue canadienne de de microbiologie

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Journal:	*Canadian Journal of Microbiology*
Manuscript ID:	cjm-2014-0815.R2
Manuscript Type:	Article
Date Submitted by the Author:	08-Jun-2015
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Keyword:	bacteria, sediment bacteria, floc, wave action, bacterial mobilization

Influence of Wave Action on the Partitioning and Transport of Unattached and Floc-Associated Bacteria in Freshwater

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Running Title: Influence of wave action on bacteria-floc associations

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Abstract: The dynamic interaction of bacteria within bed and suspended sediment/floc in a wave dominated beach environment is assessed using a laboratory wave flume. The influence of shear stress (wave energy) on bacterial concentrations, and the partitioning and transport of unattached and floc-associated bacteria is investigated. The study showed that increasing wave energy (0.60 and 5.35 N/s) resulted in a 0.5 to 1.5 log increase in unattached cells of the test bacterium *Pseudomonas* sp. strain CTO7::*gfp-2* in the water column. There was a positive correlation between the bacterial concentrations in water and total suspended solids, with the latter increasing from near zero values up to 200 mg/L over the same wave energy increase. The median equivalent spherical diameter of flocs in suspension also increased by an order of magnitude in all experimental trials. Under both low (0.60 N/s) and high (5.35 N/s) energy regime, bacteria were shown to preferentially associate with flocs upon cessation of wave activity. The results suggest that collection of water samples during periods of low wave action for the purpose of monitoring the microbiological quality of water may underestimate bacterial concentrations, due in part to an inability to account for the effect of shear stress on the erosion and mobilization of bacteria from bed sediment to the water column. This highlights the need to develop a more comprehensive beach analysis strategy that not only addresses presently uncharacterized shores and sediments, but also recognizes the importance of eroded floc as a vector for the transport of bacteria in aquatic environments.

Key words: Bacteria, floc, sediment bacteria, wave action, bacterial mobilization

Introduction

Monitoring the microbiological quality of recreational water is vital for assessing the human health risk at public beaches. Pathogenic bacteria can be introduced to freshwater from a variety of point sources such as combined and sanitary sewer overflows (Perdek 2003) and nonpoint sources such as stormwater runoff and fecal droppings from wildlife (Kinzelman et al. 2004; Craun et al. 2005; Ji 2008). In order to characterize the public health risk associated with contaminated sands and sediments, public health units typically collect only whole water samples with the assumption that planktonic cells represent the total bacterial community present in beach environments. This practice may underestimate potential sedimentary sources of pathogens, which have been shown to be temporal sinks and sources of bacteria to the water column (Ishii et al. 2007), and the ecological importance of floc on the erosion, transport and delivery of bacteria in aquatic environments (Droppo et al. 2009; 2011).

Flocs found within the water column are composed of a viable and non-viable biological component, inorganic particles, and water. The development of these flocs is dependent on interacting biological, physical, and chemical properties including dissolved organic matter, surface charge, hydrophobicity, pH, redox potential, turbulence, and suspended solids (Droppo et al. 1997). One abiotic phenomenon, electrochemical flocculation, is influenced by the net surface charge of a particle, expressed as zeta potential, resulting in attraction or flocculation due to the reduction of the electrochemical double layer. This double layer is created when flocs imparting a net negative surface charge interact with positive cations in solution that electrically screen the surface charge

and allow for the association of negatively charged particles (including bacteria). Bioflocculation is influenced by the metabolic action of microorganisms leading to the production of extracellular polymeric substances (EPS) and the "sticking" together of flocs (Gerbersdorf and Wierpecht 2014). In reality, electrochemical and bioflocculation occur simultaneously, however, it is generally accepted that the latter dominates flocculation of particles in the freshwater environment (Droppo et al. 2009). The net effect of flocculation is to increase the downward flux of particles, thus facilitating the transport of bacteria from the overlying water column to the lake bed (Wu et al. 2009).

Concomitant with floc deposition on the bed sediment is the incorporation and persistence of the floc associated bacteria into the bed biofilm. Biofilms represent a mixed community of microorganisms bound to a surface and encased in an exopolymeric matrix (Cogan et al. 2011). Within a sand dominated bed such as a beach environment, a variety of niches that allow for biofilm assembly are available given the large surface area of sand grains, interstitial voids between grains, and microhabitats that are created by variable surface topography. Bacteria can exploit these niches for growth and survival (Bonilla et al. 2007), and protection from predation (Hartz et al. 2008). There is potential for sediment-associated biofilms to assimilate, and subsequently act as a source of bacteria to the water column through re-suspension caused by shear stress due to wave action, disturbance of sediments by swimmers (Brookes et al. 2004), or biofilm-to-planktonic yield (Bester et al. 2009; 2010; Ghadakpour et al. 2014).

Wave action has been suggested as a potential mechanism of bacterial re-suspension in water (McLellan and Salmore, 2003; Petersen et al. 2005) and foreshore sand (Ishii et al. 2007, Whitman et al. 2003). However few studies have attempted to

measure the contribution of wave energy to elevated levels of bacteria in water (Kinzelman et al. 2004), partly because the interpretation of data from field studies is difficult due to a large number of overlapping physicochemical, biotic and abiotic factors that influence bacterial mobilization, as well as growth and decay kinetics, as indicated by Dette et al. (2002).

The goal of the present study was to assess the effect of shear stress imparted by wave action on bacterial concentrations in sediments and the water column, as well as the partitioning and transport of unattached and floc-associated bacteria by utilizing a wave flume (mesocosm) and an environmental test bacterial strain.

Materials and methods

Flume design

A linear flume was used to subject a meso-scale beach model to waves of variable height (**Figure 1**). The system, described in detail by Droppo et al. (2007) measured 13 m in length, 0.3 m in width and 0.5 m in height. The test area was the wash zone, separated from the rest of the flume by a flexible non-permeable wave energy transmitting membrane (WETM). The WETM allowed energy (waves) rather than materials to pass through the propagation channel to the 1.6 m long wash zone; thus containing introduced test bacteria in a relatively small volume. The wash zone contained a constructed beach with a 1:10 slope and maximum water depth of 15 cm. One Hz sinusoidal waves of 2, 4 and 6 cm were generated at the end opposite of the wash zone using a wave paddle oscillator. Wave height was monitored using calibrated wave staffs (converts voltage to wave height) positioned on each side of the WETM.

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144 **Inoculum, beach sand, and water**

145 *Pseudomonas* sp. strain CTO7::*gfp-2* (DQ777633) was used to track bacterial transport in
146 all experiments. While other strains have been used in studies of bacterial sediment
147 interactions, we were interested in the ability of *Pseudomonas* strains to form aggregates
148 such as biofilms within various aquatic environments (see e.g., Tolker-Nielsen et al.
149 2000; Sauer et al. 2002, Purevdorj-Gage et al. 2005) where they create
150 microenvironments in which other strains, including pathogens, can persist and
151 proliferate, and thereby extend their habitat range. The inoculum was grown in 500 mL of
152 sterile 3 g/L tryptic soy broth (EMD Biosciences) for 15 h in a tabletop shaking incubator
153 (250 r/min) at 30°C. The stable and site-specific chromosomal insertion of green
154 fluorescent protein (GFP) was verified previously using PCR and growth curve analysis
155 (Bester et al. 2009; Wolfaardt et al. 2008). Previous studies also demonstrated the ability
156 of the strain to form biofilms on such surfaces as Plexiglas, borosilicate glass and silicon
157 (Kroukamp and Wolfaardt 2009; Bester et al. 2009; 2010). Beach sand was obtained from
158 the swash zone at the Sunnyside Beach of Lake Ontario, Toronto, Canada, and was used
159 without sterilization for experiments that examined the effect of shear stress on the
160 partitioning of unattached and floc-associated bacteria, as well as bacterial concentrations
161 in water. This sediment was selected since Sunnyside Beach has to be closed for up to
162 69% of the swimming season due to high levels of *E. coli* (City of Toronto 2009). Lake

Ontario water was used in the wash zone (experimental area), while dechlorinated tap water was used for wave propagation to the WETM from the wave paddle.

Transport of bacteria in beach sand

To assess bacterial transport through sand, the beach was formed using well-characterized, commercially available sand (Ottawa sand; Bell and Mackenzie Co. Ltd., Hamilton, Canada), consisting of 99.88% SiO_2, 0.015% Fe_2O_3, 0.050% Al_2O_3, 0.010% CaO, 0.003% MgO, 0.003% K_2O, 0.007% Na_2O, and 0.1% clay and silt. Screen analysis provided by the manufacturer stated that 62% of the particles passed through a 70-mesh sieve (particles smaller than 0.210 mm). These characteristics were chosen based on the average grain size of the Sunnyside Beach sand. Eighty L of Lake Ontario water was mixed with 10^9 cells (total viable count) of the test strain and carefully siphoned into the test area in order to cause minimum disruption of the sand profile. The flume was left with no wave action for 24 hours, after which sterile syringes were used to core 6 cm into the sediment along five transects (**Figure 2B**). Cores were divided into three 2 cm sections and labelled top (upper 2 cm of the sediment bed; sediment/water or air interface), middle and bottom (lower 2 cm of sediment). Viable cell counts of the sediment samples were then determined to assess microbial migration through sand.

Influence of shear on in-bed sediment bacterial distribution and erosion

Approximately 120 L of saturated beach sediment was homogenized with 10^9 cells (total cell count) of the test strain using a rotating mixer. The inoculated sediment was left to

stand for 72 hours at room temperature (22 ± 2°C) to allow for the test strain to become associated with the particles in the sediment. The sediment bacteria mixture was then re-homogenized and laid down in the wash zone of the flume to form the beach (Figure 1). Sand core samples were taken (see below) to verify that there was a uniform distribution of the test organism within the sediment.

Hamilton Harbour water was collected and stored at 4°C until use in the flume. The water was equalized to room temperature before it was added to the flume without sterilization. Once the sediment was laid down in the flume, 80 L of Hamilton Harbour water was carefully siphoned into the wash zone to minimize disturbance of inoculated sediment. Dechlorinated tap water was added to the propagation channel and the system was left for one hour.

To assess the effect of shear stress on bacterial concentrations in water, the flume was sequentially operated for one-hour intervals at 2, 4, and 6 cm wave heights. This procedure is analogous to an annular flume test where bed shear stress is increased sequentially to simulate a hydrograph. While this results in a cumulative effect, it does allow for the assessment of a dynamic storm event given that environmental conditions will always be changing. Experiments with consistent wave energy were also performed and are described below. As shear stress is difficult to determine in a wave-breaking environment, a wave energy flux (in Newton per second) was used to represent a measure of shear as described by Turker and Kabdash (2006). Wave heights of 2, 4, and 6 cm with a 15 cm water depth were equivalent to a wave energy flux of 0.60, 2.38, and 5.35 N/s, respectively. At the end of every wave height, sterile 10 mL syringes (BD Biosciences) with the front tip cut off were used to core, in duplicate, 6 cm deep into the sediment

along four transects (T1 – T4; **Figure 2A**). Triplicate water samples were collected in 15 mL polypropylene tubes (BD Biosciences) every 15 minutes in the wave-breaking zone to determine culturable cell counts. Duplicate 50 mL water samples were collected to determine suspended sediment concentration by filtration under vacuum through pre-dried and tared 0.45 μm filters (Millipore). Plankton chambers were filled after every wave height for visualization of flocs and determination of particle size distribution using a combination of microscopy, photography, and image analysis (Droppo et al. 1997). Four separate trials were conducted.

In order to verify that there was no significant growth of the test strain during the three hour time period of wave action, triplicate flasks were packed with inoculated sediment, and 50 mL of Hamilton Harbour water was added on top. The flask was left to sit for 1 hour to equilibrate and allow cells to move into the aqueous phase. Flasks were then placed in a benchtop shaker (200 rpm; 22°C) and shaken for 3 hours. Samples were taken every 30 minutes, which demonstrated a steady test strain count in water at around 5.2 log over 3 hours.

Comparison of low and high wave energy flux to evaluate partitioning of unattached and floc-associated bacteria

The beach was prepared as previously described, and in two separate experiments the flume was operated with 2 and 6 cm wave heights (0.60 and 5.35 N/s) to assess the effect of shear strength on the partitioning of unattached and floc-associated bacteria in the water column. For each of these wave heights, the initial one-hour equilibration period

was followed by 4 h of continuous wave activity followed by 2.5 h of no wave activity to investigate the influence of settling dynamics on the partitioning of bacteria. Sediment core samples were collected with sterile 10 mL syringes with the front tip cut off as described above, and an additional 50 mL water sample was collected for enumeration of unattached and floc-associated fractions. In addition to the analyses done in plankton chambers, a CILAS 930 particle size analyzer (CILAS, Orleans, France) was used for real-time measurements of median equivalent spherical diameter (d_{50}) in the 0.2 to 500 μm diameter range.

Enumeration of test strain

Core Samples

Each core sample was sectioned into 2 cm aliquots and serial dilutions prepared in 0.9% (m/v) sterile buffered saline followed by spread plating on 3 g/L tryptic soy broth with 1.5% (m/v) agar for routine enumeration of the test strain. After incubation at 30°C for 24 h, colonies were screened for gfp fluorescence using a fluorescence dissection microscope (Leica). Bacteria were removed from sand grains by vortexing 1 g of sand with 0.5 mL of 0.9% (m/v) sterile buffered saline for 30 seconds (corresponded to approximately 80% cell removal; data not shown).

Water Samples

To separate bacteria into unattached and floc-associated fractions, 50 mL water samples were passed through 5 μm cellulose acetate filters (Sterlitech). Cellulose acetate filters

were chosen because they offered low binding of microorganisms (BSA protein binding of 3.8 μg/cm^2). A previous study found that selective size filtration was useful for the estimation of particle-associated *E. coli* in river water (Alm et al. 2006). Bacteria in the filtrate were considered unattached, while bacteria that remained on the filter were considered floc-associated. Both fractions were subjected to ultrasonication (35 kHz) for 1 minute (optimal time that maximized floc break up and minimized cell death; data not shown). Samples were then diluted in 0.9% (m/v) sterile buffered saline, filtered through 0.45 μm polycarbonate filters (Pall Corporation), plated, and screened as described previously. Log_{10} transformations were applied to all bacterial counts to normalize data.

Visualization of bacteria associated with sand grains and eroded floc

To visualize biofilm development on sand grains, biofilms were cultivated in a continuous flow cell made from Plexiglas (dimensions of 30 mm × 6 mm × 60 mm). Sediment from Sunnyside Beach was placed in the flow cell, which was irrigated with Lake Ontario water supplemented with 0.3 g/L tryptic soy broth. To prevent movement of sand grains into waste and medium reservoirs, small-pore mesh was glued at connection ports. The flow cell was aseptically inoculated upstream using a sterile needle and syringe with 100 μL (10^6 cells) of a culture of *Pseudomonas* sp. strain CTO7::*gfp-2* that was previously cultured in a shaking incubator (0.3 g/L tryptic soy broth, 30°C, 250 r/min). The inoculated bacteria were allowed to adhere for 0.5 h under quiescent conditions, where after a medium flow rate of 5 mL/h was initiated with a Watson-Marlow 205S peristaltic pump. Biofilms were allowed to develop for 72 hours, then visualized using an LSM 510 confocal laser scanning microscope (CLSM; Carl Zeiss,

Ontario, Canada). Excitation with a 488 nm Ar laser line (15 % output) and emission with a band pass filter setting of 500-550 nm were used to visualize the test strain.

Results

Movement of bacteria through sand

The test strain was present throughout the flume beach after adding Hamilton Harbour water containing the test strain, and left for 24 hours without wave action. The highest concentration of bacteria was found at the water line (shoreline, transect 3; see **Figure 2B**), while the presence of bacteria along transects 4 and 5, which lay above the water line and water table, respectively, indicated that cells moved along a wet to dry gradient (beyond the shoreline) potentially by capillary action (**Figure 3A**). The distribution of cells was highly variable for all transects. The 1 to 3 log increase in bacterial levels (~8.3 x 10^3 cells /mL wet weight) in sediment can be attributed to the growth and association of the test organism during the initial 72 hour incubation period.

Sand biofilms

Visualization of unstained floc samples with CLSM, and TSA plates with a fluorescence dissection microscope containing indigenous microbial communities of Lake Ontario water and Sunnyside Beach sand, confirmed the absence of auto-fluorescence and thus the usefulness of the GFP-tagged test strain (data not shown). **Figure 3B** shows a Sunnyside Beach 72-hour sand biofilm formed by the test strain and indigenous bacteria within the continuous flow cell and CLSM imaging.

Effect of shear on bacterial concentrations in water

There appeared to be a strong correlation between wave energy, total suspended solids (TSS), floc median equivalent spherical diameter (d_{50}) and numbers of the test strain (**Figure 4, Table 3**). Numbers of the test strain increased between 0.5 log (Figures 4a, b, d) and 1.5 log (**Figure 4C**) with increasing wave energy flux. Total suspended solids concentration also increased over the same wave energy flux range reaching approximately 200 mg/L in all cases except for trial 4 (**Figure 4D**; ~120 mg/L). The d_{50} of flocs in suspension also increased by an order of magnitude in all trials with increasing wave energy. The increase in floc size may have been due to flocculation in the water column, and/or the re-suspension of larger particles with increasing wave energy. It should be noted that for trials 1 (**Figure 4A**) and 4 (**Figure 4D**) the d_{50} decreased at the highest wave energy flux, which may be reflective of higher turbulence resulting in floc breakage. The breaking of flocs at high wave energy may also be attributed to the composition of the sediment, as it was collected at different times as the project proceeded and thus may have varied in the composition of the cohesive fraction (silts and clays).

Effect of increasing shear on bacterial distribution in the sediment bed

The redistribution and winnowing of the test bacterial strain from the sediment bed was seen along three out of four beach transects. Transects corresponding to the wave breaking zone and swash zone show that increasing wave energy flux generally led to the

loss of the test organism from the bed sediment (**Figure 5A,B,C**) at the higher wave energy flux values. This effect was most prominent for the top core section (top 2 cm of the sediment bed), which correlates with visual observations that confirm this section as the most dynamic. Transect 1, which corresponded to the wave-breaking zone, showed a decrease in the numbers of test organism with increasing wave energy for the bottom, middle and top cores. The transect that corresponded to the far upshore region of the beach (T4) did not come into contact with the water table or swash and consequently did not have erosion of the test strain from the top core section. The bottom section of this core did however show a decrease in the numbers of test organism during wave events.

Partitioning of unattached and floc-associated bacteria under low (0.60 N/s) and high (5.38 N/s) wave energy flux

When water samples were partitioned into unattached and floc-associated fractions using selective size filtration, it was found that the viable cell count for both phases did not change substantially during the 4 hours of wave activity at 0.60 N/s (Table 1). It is interesting to note, however, that there was a higher concentration of the test organism attached to surfaces, with cell counts being one to two orders of magnitude greater than for the unattached phase per 50 ml of sample. When waves were turned off, there was an order of magnitude increase in the number of floc-associated cells per mg of floc material, even though the suspended sediment concentration was reduced by approximately 20% after just 30 minutes of settling. The median equivalent spherical diameter gradually decreased from 17 to 5 μm over the period of wave activity, which indicated that larger flocs were settling out of the water column under this condition. This

is substantiated by the gradual reduction in TSS during the wave period (Table 1). After an additional 2.5 hours of quiescent settling, the floc size further decreased to 2.6 µm. It is unlikely that floc breakage was occurring during the quiescent settling period.

Similar to what was observed for a wave energy flux of 0.60 N/s, the viable unattached and floc-associated cell counts did not vary significantly during the 4 hours of wave activity at 5.35 N/s (Table 2). The total suspended solids concentration decreased by roughly half when waves were shut off, and the number of floc-associated cells per mg of floc material also increased by an order of magnitude. In contrast to the 0.60 N/s shear, the median floc equivalent spherical diameter reduced initially, but then remained consistent at around 10 µm for the duration of wave activity. During the quiescent period, floc size gradually decreased, but only down to 7.75 µm in size. It is likely that higher wave energy flux prevented larger flocs from settling to the sediment bed and resulted in an equilibrium floc size carrying capacity of around 10 µm. This equilibrium floc size was maintained even though there was a gradual increase in TSS during the wave period (Table 2). Bacteria that are preferentially attached to larger flocs are removed from the water column by the downward flux of these particles.

Discussion

The effect of wave energy flux on bacterial distribution between the sediment and aqueous phases highlights the relevance of microbial behaviour and/or dynamics to public health, as it appears probable that beach water samples collected at times with little to no wave action may underestimate the bacterial health risk later in the same day when there are indeed stronger waves and disturbance of sediments by swimmers. Once re-

suspended, bacteria may become further mobilized by general water flow and wind-generated waves, leading to an increased potential for human ingestion (Droppo et al. 2009; Plach et al. 2011). The re-suspension of bacteria imparted by wave action (where diurnal variation in waves at a beach may render a morning sample irrelevant to afternoon conditions), together with the delay imparted by current methods used for microbial sample analysis, pose a challenge for public health units. Predictive models may therefore be an appropriate method for assessing the bacterial health risk during times of turbulence and in storm events. Kinzelman et al. (2004) found wave height to be the best predictor of *E. coli* concentration at beaches, and were able to derive a formula to predict daily *E. coli* counts. Our observed relationship between wave action and re-suspension of viable bacteria colonized in sediments, substantiate (reported) field studies that have listed wave action as a potential mechanism of bacterial re-suspension in surface waters (Hartz et al. 2008; McLellan and Salmore 2003). The correlation also addresses the influence of shear force on bacterial transport in freshwater systems (Yamahara et al. 2007).

Previous studies showing the persistence and/or growth of fecal indicators and pathogens in beach sand suggested that an evaluation of bacteria in beach sand and sediments in conjunction with a water sampling regimen contributes to a more successful water quality monitoring program (Lee et al. 2006; Scopel et al. 2006). Our results in Figure 5 showing no erosion of the test strain from the section of the core that did not come into contact with swash, while the bottom section of this core indeed showed a decrease in the numbers during wave events, suggest that cells were being drawn from this area of the beach as backwash moved down the shoreline. Droppo et al. (2007)

394 indicated that the cyclic shear stress resulting from wave action, referred to as
395 ‘‘pumping’’, may be accentuated by the presence of gas in pore spaces, which is a
396 probable explanation for the decreased cell numbers in the bottom core section of the far
397 upshore region.

398 Monitoring programs in the United States generally rely on one sample collected
399 at the shoreline (Scopel et al. 2006), while federal guidelines in Canada suggest that
400 sediment samples should be collected when there is evidence that bathing beaches could
401 be the source of waterborne disease (Health and Welfare Canada 1992). Amending these
402 guidelines to include a sampling regime that involves the routine examination of
403 sedimentary components, shoreline and near shore water, as well as regions up-shore of
404 the beach (i.e. considering the ecology of the related microorganisms) could be an
405 effective strategy for improving health risk assessment at public beaches.

406 The positive correlation ($r > or = 0.80$ in all replicate trails) between the
407 concentration of bacteria in the water column and TSS supports a suggestion by Droppo
408 et al. (2011) that turbidity may be an indicator of the microbiological quality of water.
409 Using samples collected from water and lake-bottom sediments along with additional
410 environmental data, Francy and Darner (1999) found that turbidity, antecedent rainfall,
411 volumes of wastewater-treatment plant overflows and metered outfalls, and wave height,
412 were statistically related to levels of *E. coli* at three public bathing beaches along Lake
413 Erie. However, Kinzelman et al. (2004) found that turbidity was not predictive of *E. coli*
414 levels, suggesting that specific environmental conditions (local phenomena) may
415 influence the predictive capabilities of the relationship.

With the wave flume, sand grains are too large to remain in suspension at the energy regimes studied, however, the flocculated cohesive fraction (clays, silts, and organic matter) found within the biofilms forming in the interstitial voids of the sand grains may be re-suspended and subsequently transport floc-associated bacteria within the water column. Planktonic cells may also be released by erosion or dissociation from flocs in suspension (Ghadakpour et al. 2014). The possible long-range transport of mobilized microbial cohesive flocs is related to their high water content and low density (often close to that of water). Typical quiescent settling velocities of flocs range from 0.1 to 4 mm s^{-1} (Droppo et al. 1997), suggesting that settling in a turbulent environment will be even less. This was substantiated by the very slow rate of reduction in TSS during wave periods. It is also probable that sand biofilms will form loosely associated micro-colonies with relatively little cohesive sediment, which will be removed with increased shear as floc (a phenomenon known as sloughing) (Stoodley et al. 2001; Ghadakpour et al. 2014).

During the post wave period, larger particles settled towards the bed while cells demonstrated an affinity for the smaller flocs remaining in suspension. This higher number of cells associated with suspended flocs is likely due to a combination of physical, chemical, and biological mechanisms, where cells actively attach to the floc material when the kinetic energy of the system is reduced, and there is decreased turbulence (time 330 to 450 minutes). Gerba and McLeod (1976) have shown that bacteria preferentially attach to particles as they represent a source of protection from environmental stress (e.g. energy conditions) and a source of food (i.e. DOC and POC). Laboratory mesocosm experiments described by Garcia-Armisen and Servais (2009)

found that water samples containing greater than 50 mg/L of suspended matter had a relatively constant settling rate of particle-associated *E. coli*. In contrast, unattached *E. coli* did not settle. This trend was observed in the unattached data of the current study when waves were shut off. Lawrence et al. (1987) demonstrated that *Pseudomonas fluorescens* cells were able to swim up-stream using flagellar-driven motility near (2 μm) the surface of a slide culture chamber where the bulk liquid flow velocity was 200 μm/s compared to 10 cm/s in the bulk phase. While the flow conditions were different, such a result may suggest that the decrease in turbulence in the wave flume could allow for similar flagellar-driven motility and thus possible preferential attachment to flocs.

Further, electrochemical conditions could also contribute to the observed increased attachment of cells to the suspended flocs. The surface charge of particles is known to influence particle-particle interactions. Reduction of the electrochemical double layer through interactions between negative particles and positive cations can result in attraction and/or flocculation, thus affecting the number of cells associated with settling particles (Ongerth and Pecoraro 1996). In general, freshwater particles have a zeta potential between -15 to -30 mV (Ongerth and Pecoraro 1996). The zeta potential of the test strain used in this study was determined to be -35 mV, therefore, aggregation of small particles through the reduction of the electrochemical double layer may have provided new niches for the attached cells, and contributed to the increased number of cells observed under quiescent conditions.

A recent study using the test strain found that the average per cell CO_2 production rate (measure of metabolic activity) in biofilms formed by the test strain was significantly higher for the cells in the outer regions at and near the biofilm-liquid interface than the

462 cells positioned in the deeper regions. It was shown that when the shear susceptible cells
463 at the outer layers were removed, the newly exposed cells rapidly increased metabolic
464 activity in response to the higher nutrient and oxygen concentrations (Bester et al. 2010).
465 It is possible that bacteria in floc and biofilms partially depend on shear forces (such as
466 those related to wave energy) to maintain an optimum aggregate size to derive maximum
467 benefit (e.g. synergistic metabolism of complex nutrients, protection against
468 antimicrobial agents), while also maintaining flux of nutrients in – and metabolites out –
469 of the aggregates and thereby allowing the majority of the cells to remain active (Plach et
470 al. 2011).

471 In order to summarize the dynamic interaction of the physical environment with
472 the microbial community, a conceptual model for a wave environment has been modified
473 from the river scenario of Droppo et al. (2011) (**Figure 6)**. In this model, bacterial cell
474 erosion occurs when the bed shear stress (imparted by waves) is greater than the critical
475 bed shear stress (energy at which bed sediment mobilizes (red decision box)). Eroded
476 cells may be either associated with flocs (silts, clays and viable and non-viable biological
477 material) or present in their planktonic phase. If the fluid shear imparted by waves is
478 greater than the suspended floc shear strength, which is the force that must be applied to
479 break up the floc, then floc-associated cells will dissociate (green decision box). It is the
480 dissociated cells (and those linked with smaller flocs) along with the planktonic phase
481 cells that remain in suspension due to turbulence or natural buoyancy. Bacteria attached
482 to larger flocs are removed from the water column with the downward flux of larger
483 particles. Planktonic bacteria may undergo passive reattachment via the physical
484 processes of flocculation or scavenging during settling, or active reattachment given the

485 flocs representing a desirable surface to colonize (i.e. floc may represent a more effective
486 source of organic matter for consumption/energy). This concentration effect of planktonic
487 bacteria was particularly observed in experiments when turbulence was removed from the
488 system. As flocs settle, if the bed shear is not greater than the floc shear strength, then
489 larger flocs are deposited on the sediment bed and this leads to consolidation and
490 incorporation in biofilms (blue decision box). Alternatively, if the bed shear is greater
491 than the floc shear strength, flocs will break up with cells and sediment being transported
492 further within the system. This conceptual model highlights the transient nature of floc
493 transport in freshwater systems and demonstrates the dynamic nature of cell-floc
494 associations. Considering the benefits for cells to be incorporated in microbial flocs, it
495 also highlights the potential importance of floc as a vector for bacterial transport in lake
496 systems.

497 Future work utilizing a flow cell /shear cell to examine flocculation and break-up
498 could provide insight to the potential release of floc-associated *Pseudomonas* sp. strain
499 CTO7::*gfp-2* under conditions of increasing shear. Differentially-labeled test strains may
500 also be useful to assess the degree of mixing of sediment-associated and suspended
501 bacteria with wave action.

502

503 **Acknowledgements**

504 This research was supported by contributions from the National Science and
505 Engineering Research Council of Canada (NSERC), Ryerson University, Environment
506 Canada and the Canada Research Chairs Program. Technical assistance was provided by

Christina Jaskot, Brian Trapp, Evan Ronan, Claire Alexia Lane, Kristen King, and Katrina Bernal. Karen Quinto assisted with the drafting of Figure 6.

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633

634 **Table 1** Partitioning of free-floating and floc-associated cells before, during and after
635 operating the flume at 0.60 N/s.

Wave Energy Flux (N/s)	Time (min)	Median Floc ESD[1] (μm)	Total Suspended Solids (mg/L)	Free-Floating Cells/ 50 mL	Floc-Associated Cells/ 50 mL	%Cells in floc	Floc-Associated Cells (CFU/ mg floc)
0	30	nd[3]	10 ± 1	$6.20x10^2$	$1.29x10^4$	95	$2.58x10^4$
0	60	14.75	13 ± 2	$1.35x10^3$	$1.02x10^4$	88	$1.57x10^4$
0.60	90	17.07	56 ± 2	$4.30x10^3$	$2.71x10^5$	98	$9.68x10^4$
0.60	120	15.09	67 ± 1	$3.80x10^3$	$5.40x10^4$	93	$1.61x10^4$
0.60	150	13.56	61 ± 2	$1.80x10^3$	$8.10x10^4$	98	$2.66x10^4$
0.60	180	12.42	56 ± 1	$2.10x10^3$	$5.50x10^4$	96	$1.96x10^4$
0.60	210	11.33	55 ± 2	$2.40x10^3$	$7.80x10^4$	97	$2.84x10^4$
0.60	240	8.56	44 ± 2	$1.90x10^3$	$9.30x10^4$	98	$4.23x10^4$
0.60	270	7.93	36 ± 1	$3.50x10^3$	$9.00x10^4$	96	$5.00x10^4$
0.60	300	5.36	20 ± 1	$2.40x10^3$	$6.60x10^4$	96	$6.60x10^4$
0	330	4.63	16 ± 2	$1.50x10^3$	$1.14x10^5$	99	$1.43x10^5$
0	360	3.24	8 ± 1	$2.00x10^3$	$1.51x10^5$	99	$3.78x10^5$
0	390	3.05	16 ± 9	$1.20x10^3$	$5.90x10^4$	98	$7.38x10^4$
0	420	3.11	23 ± 4	$6.90x10^3$	$2.28x10^5$	97	$1.98x10^5$
0	450	2.66	21 ± 7	$3.80x10^3$	$1.31x10^5$	97	$1.25x10^5$

636 1) ESD: equivalent spherical diameter
637 2) n=2 for total suspended solids (± = standard deviation)
638 3) nd: no data

639
640

641

642 **Table 2** Partitioning of free-floating and floc-associated cells before, during and after
643 operating flume at 5.35 N/s.

Wave Energy Flux (N/s)	Time (min)	Median Floc ESD (μm)	Total Suspended Solids (mg/L)	Free-Floating Cells/ 50 mL	Floc-Associated Cells/ 50 mL	%Cells in floc	Floc-Associated Cells (CFU/ mg floc)
0	30	Nd	2 ± 1	$7.70x10^{1}$	$2.70x10^{3}$	97	$2.70x10^{4}$
0	60	15.19	2 ± 1	$2.30x10^{1}$	$3.60x10^{2}$	94	$3.60x10^{3}$
5.35	90	11.99	117 ± 3	$1.60x10^{2}$	$1.40x10^{4}$	99	$2.39x10^{3}$
5.35	120	11.03	130 ± 4	$1.00x10^{2}$	$1.10x10^{4}$	99	$1.69x10^{3}$
5.35	150	10.53	132 ± 7	$4.00x10^{1}$	$1.80x10^{4}$	99.9	$2.73x10^{3}$
5.35	180	10.37	125 ± 1	$1.60x10^{2}$	$1.70x10^{4}$	99	$2.72x10^{3}$
5.35	210	10.83	143 ± 1	$1.10x10^{2}$	$1.10x10^{4}$	99	$1.54x10^{3}$
5.35	240	10.76	146 ± 3	$6.00x10^{1}$	$3.60x10^{4}$	99.9	$4.93x10^{3}$
5.35	270	10.86	145 ± 1	$2.00x10^{1}$	$9.00x10^{3}$	99.9	$1.24x10^{3}$
5.35	300	10.50	153 ± 1	$8.00x10^{1}$	$1.00x10^{4}$	99	$1.31x10^{3}$
0	330	10.21	120 ± 8	$1.50x10^{1}$	$5.00x10^{4}$	99.9	$8.33x10^{3}$
0	360	9.43	92 ± 2	$1.60x10^{2}$	$4.70x10^{5}$	99.9	$1.02x10^{5}$
0	390	8.97	78 ± 8	$1.10x10^{2}$	$8.80x10^{5}$	99.9	$2.26x10^{5}$
0	420	8.26	55 ± 11	$1.70x10^{2}$	$4.50x10^{5}$	99.9	$1.64x10^{5}$
0	450	7.75	42 ± 6	$1.20x10^{2}$	$6.50x10^{5}$	99.9	$3.10x10^{5}$

644 1) ESD: equivalent spherical diameter
645 2) n=2 for total suspended solids (standard deviation)
646 3) nd: no data

647

Table 3 Pearson correlation table of wave energy to numbers of the test strain and total suspended solids (n = minimum 3 measurements)

Wave Energy (N/s)	CFU r	TSS r
0	0.15	0.80
	0.03	0
	--	--
	0.32	0.17
0.60	0.77	0.89
	0.97	0.49
	0.04	0.89
	0.91	0.93
2.38	0.61	0.97
	0.85	0.97
	0.00	0.60
	0.02	0.17
5.35	0.91	0.65
	0.11	0.74
	0.62	0.69
	0.91	0.78

Titles and legends to figures

Figure 1: Schematic of wave flume. WETM: wave energy transmitting membrane. Not to scale.

Figure 2 A) Schematic of core sampling strategy used in shear experiments. Transect 1 (T1) refers to below water line, and was roughly the wave breaking zone. T2 refers to the swash zone; the area where the shoreline moves back and forth as waves meet the shore. T3 is at the air-water-sediment interface, which was the furthest area where water travelled up the beach and varied with wave energy, as higher wave energies pushed water further up the beach. T4 was the far upshore region of the beach beyond the furthest point of wave movement. **B)** Schematic of core sampling strategy to study transport of the test strain in sand. T1 and T2 were below the water line. T3 was at the water line, and T4 and T5 were above the water line. All transects were separated by a distance of approximately 25 cm. WETM: wave energy transmitting membrane.

Figure 3A). Tracking the movement of *Pseudomonas* sp. strain CTO7::*gfp-2* from the water into the sand with sand cores taken along five beach transects (see Figure 2B). Transects 1 and 2 were below the water line. Transect 3 was at the water line, and transects 4 and 5 were above the water line and water table. All transects were separated by a distance of approximately 25 cm. **B)** CLSM image of a 72 hour sand biofilm formed in interstitial voids, showing that the biofilms contain both indigenous bacteria (bright fluorescence) and the green test strain.

677

678 **Figure 4 A-D.** Cumulative effect of increasing wave energy flux on total suspended
679 solids (TSS) and levels of *Pseudomonas* sp. strain CTO7::*gfp-2* in water. A, B, C, D
680 represent four trials. The d_{50} value for each wave energy flux is reported above the solid
681 black line. Counts of test strain and total suspended solids represent average values (n=3
682 and n=2, respectively).

683

684 **Figure 5.** Enumeration of *Pseudomonas* sp. strain CTO7::*gfp-2* from sand cores taken
685 along four beach transects (trial 1;). Transect 1 (T1) refers to below water line, and was
686 roughly the wave breaking zone. B) Transect 2 (T2) refers to the swash zone, which was
687 the area where the shoreline moves back and forth as waves meet the shore. C) Transect 3
688 (T3) characterized the air-water-sediment interface, and was the furthest area where water
689 travelled up the beach when waves were run. The exact location of transect 3 varied with
690 wave energy, as higher wave energies pushed water further up the beach. Bottom, middle
691 and top refer to the enumeration of the test organism from 6 cm, 4cm and 2 cm below the
692 surface of the sediment bed. D) Transect 4 was the far upshore region of the beach
693 beyond the furthest point of wave movement. Counts of the test organism represent
694 average (n=2).

695

696 **Figure 6.** Conceptual model of sediment-microbial dynamics in freshwater beach
697 systems influenced by wave energy.

Figure 1

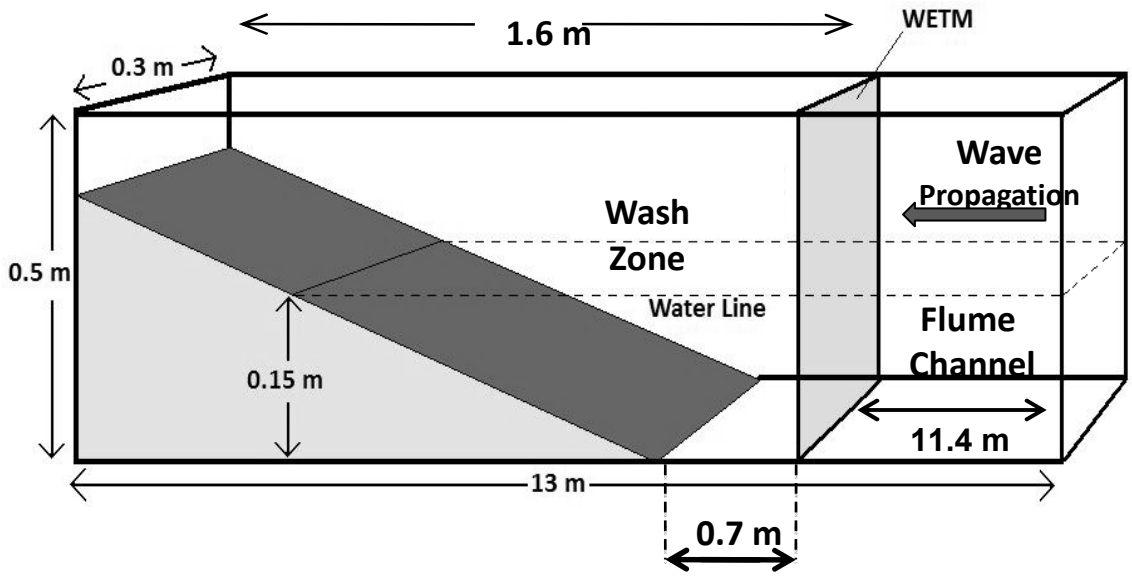


Figure 2

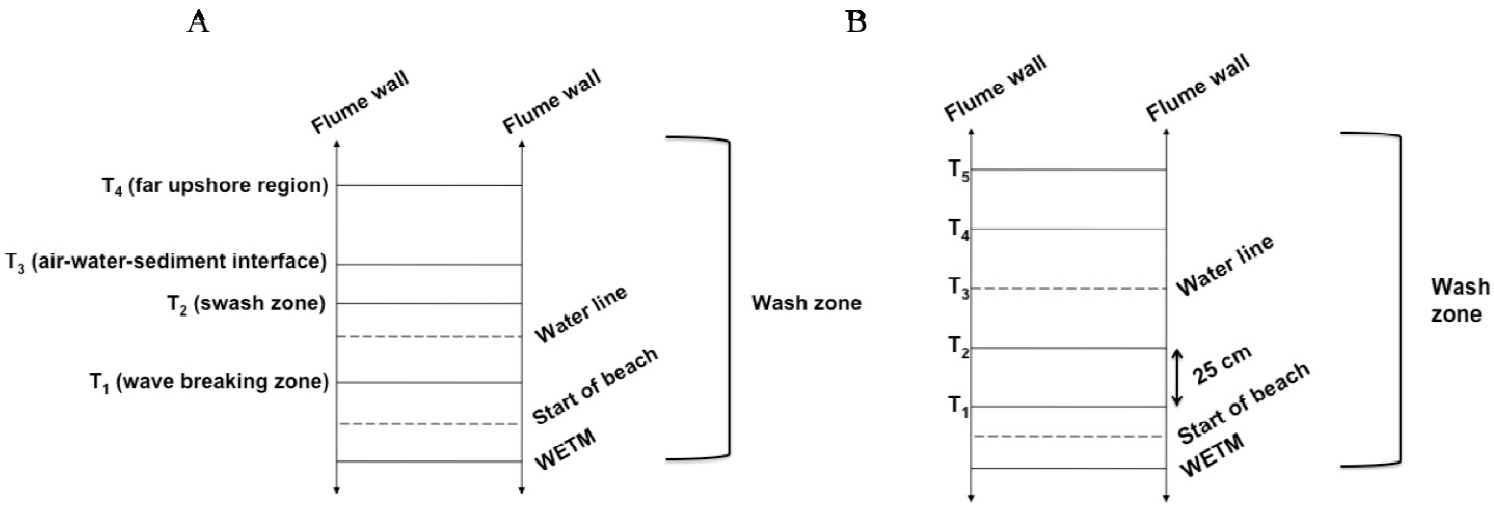


Figure 3 A)

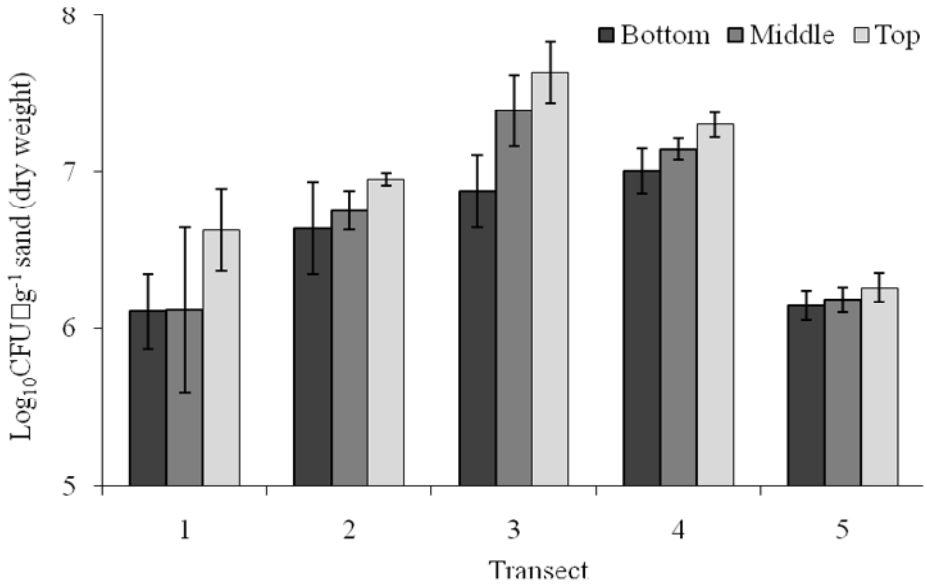


Figure 3 B)

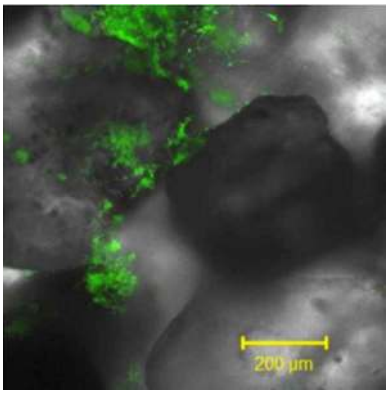


Figure 4

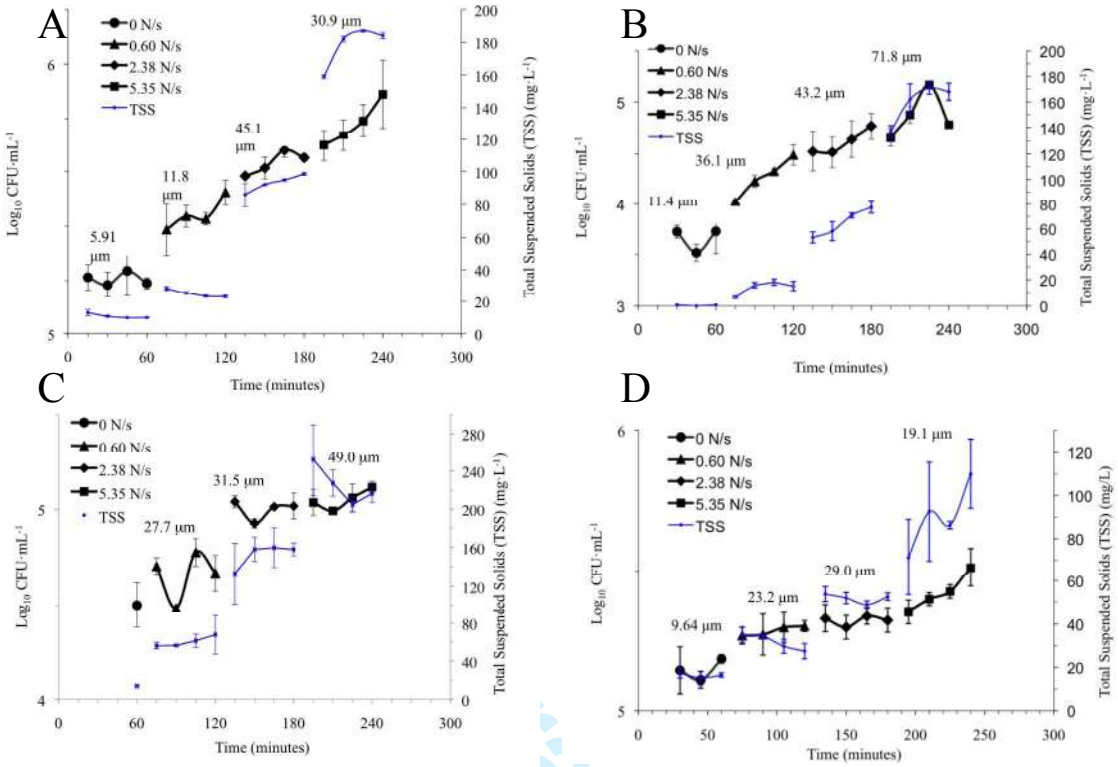
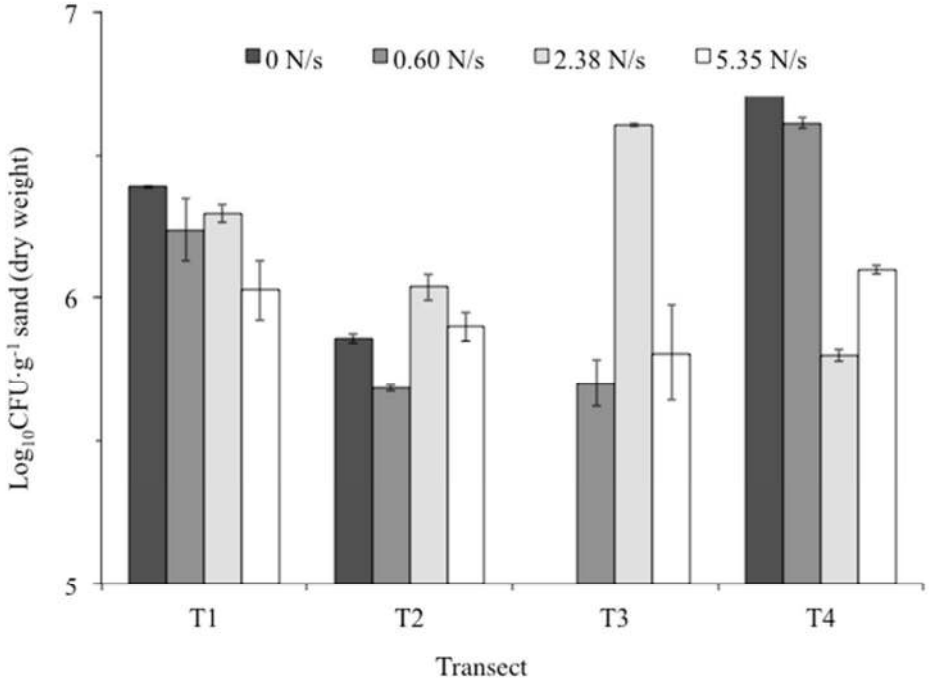
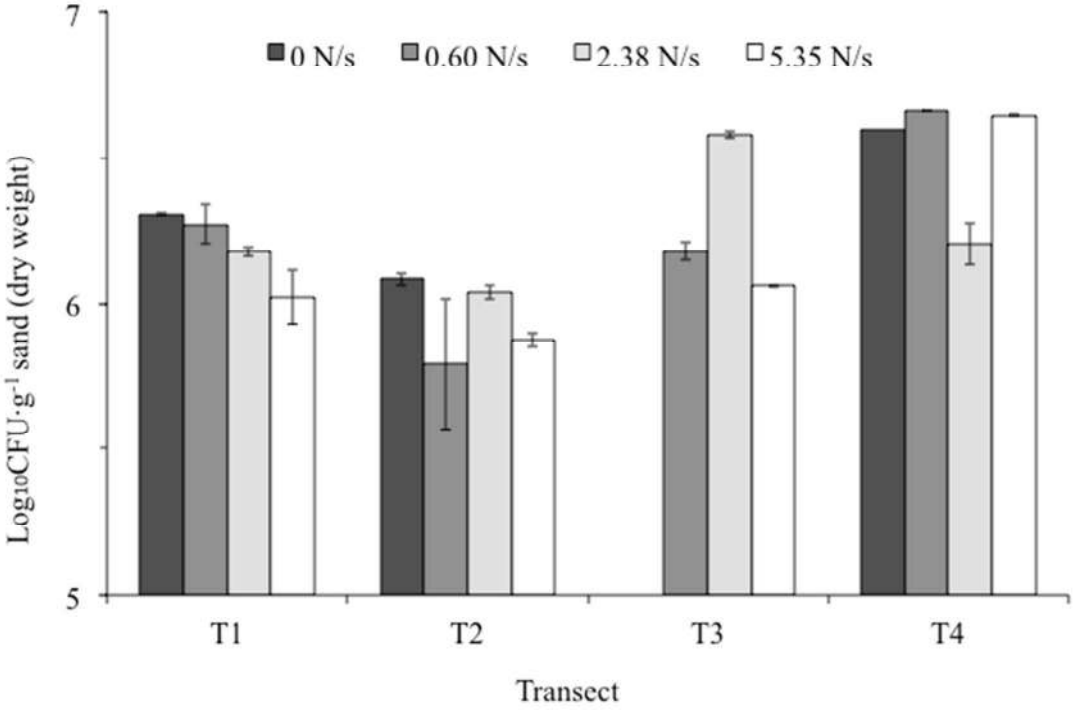

A
0 N/s
0.60 N/s
2.38 N/s
5.35 N/s
TSS
5.91 µm
11.8 µm
45.1 µm
30.9 µm
Log10 CFU·mL-1
Total Suspended Solids (TSS) (mg·L-1)
Time (minutes)
B
0 N/s
0.60 N/s
2.38 N/s
5.35 N/s
TSS
11.4 µm
36.1 µm
43.2 µm
71.8 µm
Log10 CFU·mL-1
Total Suspended solids (TSS) (mg·L-1)
Time (minutes)
C
0 N/s
0.60 N/s
2.38 N/s
5.35 N/s
TSS
27.7 µm
31.5 µm
49.0 µm
Log10 CFU·mL-1
Total Suspended Solids (TSS) (mg·L-1)
Time (minutes)
D
0 N/s
0.60 N/s
2.38 N/s
5.35 N/s
TSS
9.64 µm
23.2 µm
29.0 µm
19.1 µm
Log10 CFU·mL-1
Total Suspended Solids (TSS) (mg/L)
Time (minutes)

Figure 5

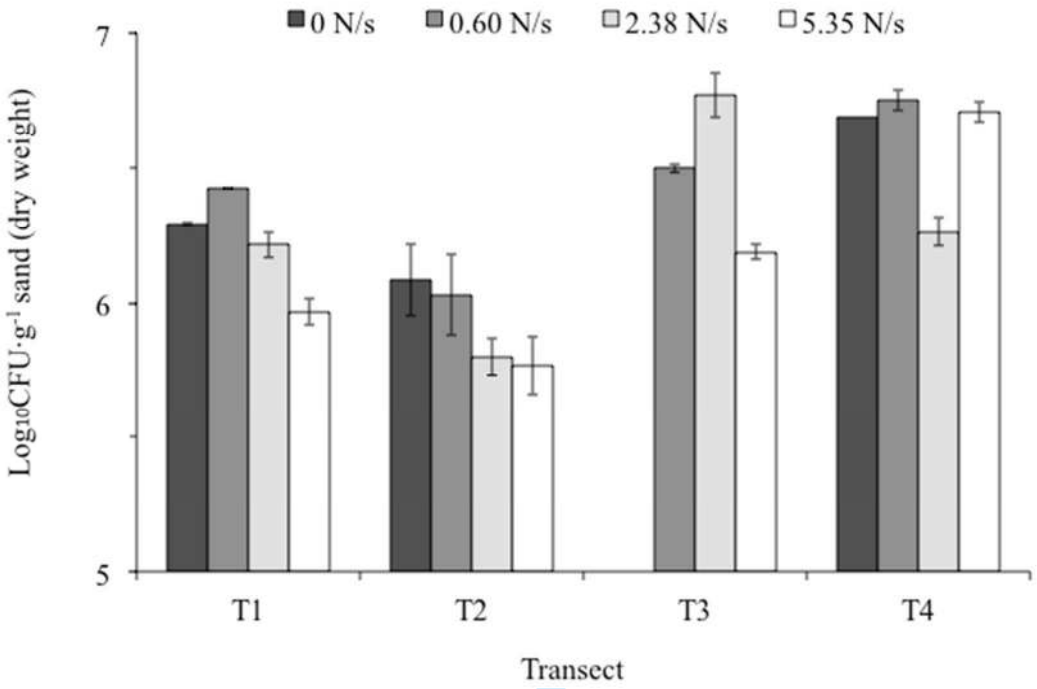
A) Bottom



B) Middle



C) Top


0 N/s
0.60 N/s
2.38 N/s
5.35 N/s
7
6
5
Log10CFU·g-1 sand (dry weight)
T1
T2
T3
T4
Transect

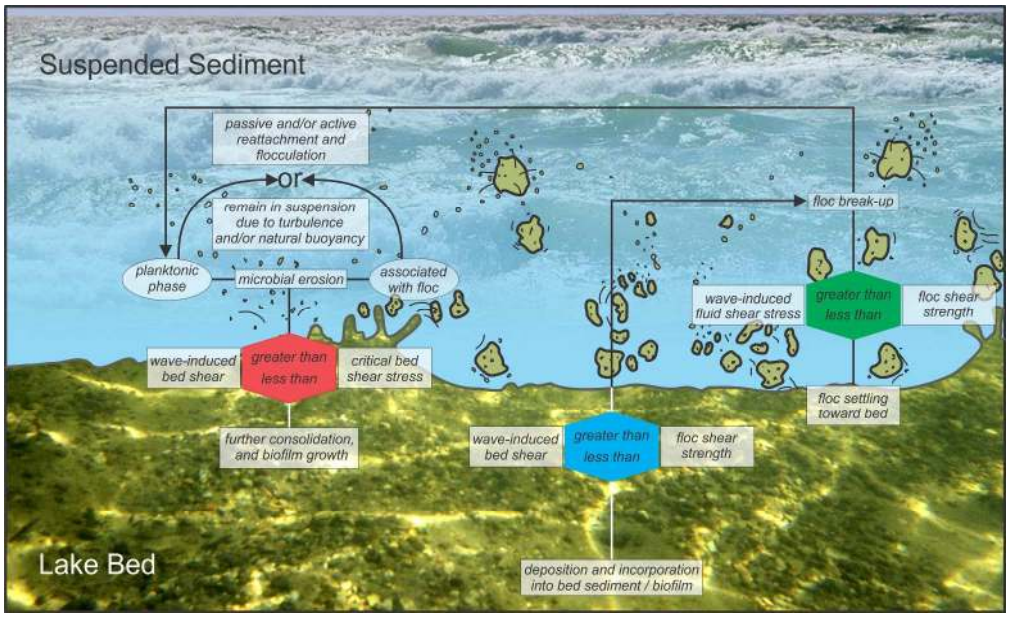


Figure 6. Conceptual model of sediment-microbial dynamics in freshwater beach systems influenced by wave energy.
249x151mm (300 x 300 DPI)