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Improved Method of Enumeration of Attached Bacteria for Study of Fluctuation in the Abundance of Attached and Free-Living Bacteria in Response to Diel Variation in Seawater Turbidity†

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Sample preparation for enumerating attached bacteria in turbid seawater by epifluorescence microscopy was improved by treating samples with a surfactant (Tween 80) followed by sonication. With optimal treatment with Tween 80 (final concentration, 10 ppm [10 μg/ml]) and sonication, as many as 10 times more attached bacteria were enumerated from turbid seawater relative to the number enumerated from an untreated control. Dispersion of bacteria by sonication alone resulted in the enumeration of only 42 to 72% of the attached bacteria. By this technique, fluctuations in the number of attached and free-living bacteria were determined in water from Aransas Pass, Tex., where surface sediments are resuspended on a regular basis by tidal currents. The abundance of attached bacteria increased in proportion to the seawater turbidity that resulted from sediment resuspension. The variation in abundance of free-living bacteria was not directly related to seawater turbidity. However, the magnitude of fluctuation in the abundance of free-living bacteria was related to the extent of turbidity variation during diurnal tides.

Recently, there have been many reports about the ecological significance of attached bacteria in various aquatic systems (1, 2, 7, 10, 12, 17, 19). While epifluorescence microscopic techniques (9) have been considered an accurate tool for counting numbers of natural bacteria, a reliable method for enumerating attached bacteria has not been developed. Bent and Goulder (2) counted attached bacterial cells on the upper surfaces of particles and then doubled the counts to allow for the bacteria on the hidden surfaces of the particles. Kirchman and Mitchell (12) enumerated attached bacteria by counting the number of bacteria per particle and counting the number of particles per field. Physical dispersion techniques such as homogenization (4, 15) or sonication (5) were applied to sediment samples for enumerating bacteria more accurately, and these physical treatments yielded higher numbers of bacteria than the numbers obtained from untreated controls (15). However, Cammen and Walker (3) reported that blending at high speed to break up particles failed to increase significantly the total number of bacterial cells from water samples containing suspended matter. Using surface-active agents, Jones and Jannasch (11) and Scheraga et al. (18) obtained more CFU per unit of sediment than they did in an untreated control. Velji and Albright (20) used a combination of chemical and physical treatment to enumerate attached bacteria more accurately. They added PP_i as a deflocculating agent before they applied sonication to the samples.

In this study, sample preparation was improved for the accurate estimation of attached bacterial numbers by pretreating samples with a surface-active agent (Tween 80) followed by sonification. By this technique, the fluctuation in abundance of attached and free-living bacteria was observed during diurnal tides in a shallow bay where surface sediments are resuspended periodically by tidal currents.

MATERIALS AND METHODS

Sampling site. Seawater was sampled at Aransas Pass, Tex., which connects both Corpus Christi Bay and Redfish Bay to the Gulf of Mexico (Fig. 1). For laboratory simulation of turbid water, seawater and sediment samples were collected from Redfish Bay.

Treatment of samples for microscopic observation. Bacteria in natural seawater samples were fixed with a glutaraldehyde solution buffered with cacodylic acid (final concentrations, 2% glutaraldehyde, 0.1 M cacodylate [pH 7.5]). Fixed bacteria samples were diluted at least 10-fold with particlefree distilled water, depending on the concentration of suspended matter in seawater. Tween 80 (final concentration, 10 ppm [10 μg/ml]) was added to the diluted seawater, mixed well, and then allowed to stand for 1 to 2 h before sonication was applied. This procedure allowed time for the Tween 80 to penetrate the detritus. It also allowed for the larger particles to settle to the bottom, thus facilitating their sonication. Ultrasonic waves (10 W for 30 s) were generated with a half-wave step horn titanium probe (diameter, 1.3 cm) connected to the Ultratip Labsonic System (Lab-Line Instruments Inc., Melrose Park, Ill.). During sonication, the tip of the probe was dipped just below the surface of diluted seawater samples so that most particles were exposed to the ultrasonic energy. For homogenization, samples were blended (Waring blender 5011G) at 20,000 rpm for a total of 5 min. To minimize heating, samples were chilled after each minute of homogenization.

Both treated and untreated samples were stained with 0.01 mM 4′,6-diamidino-2-phenylindole (DAPI) for 20 min. Samples were then filtered through 0.2-μm-pore-size membrane filters (Nuclepore Corp., Pleasanton, Calif.) which were already stained with 0.2% irgalan black (dissolved in 2.0% acetic acid). Bacterial cells retained on the membrane filter were counted by epifluorescence microscopy (9) with a microscope (Zeiss Universal; Carl Zeiss, Oberkochen, Fed-

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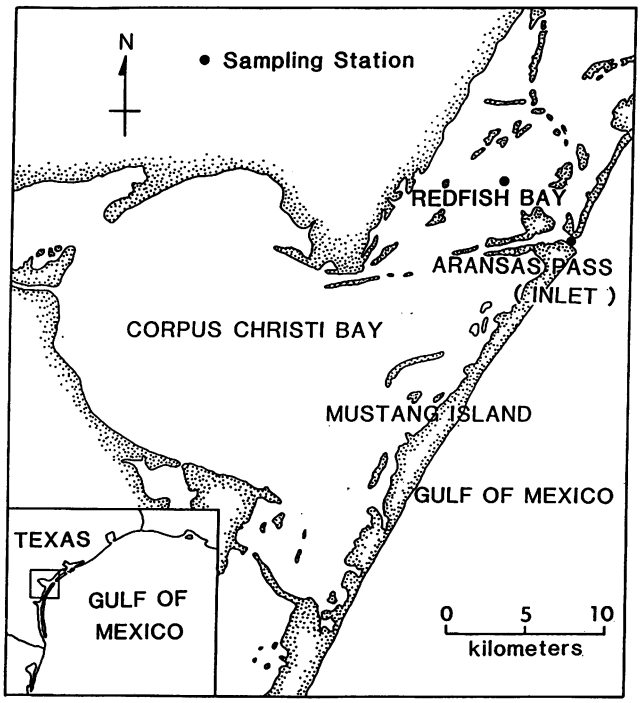

FIG. 1. Locations of the sampling stations used in this study.

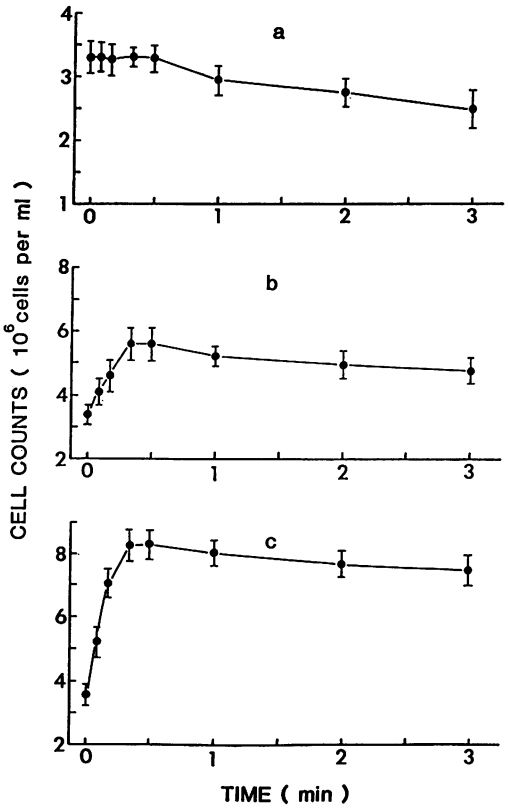

FIG. 2. Effect of sonication period on estimation of total bacterial numbers. Mean values ± standard deviations from 15 microscopic observations are shown. All seawater samples were pretreated with Tween 80 followed by sonication. Bacterial cell numbers were counted by epifluorescence microscopy. (a) Natural free-living bacteria were obtained by filtering seawater through 3.0-μm-pore-size filters (Nuclepore). (b) The unfiltered seawater sample was relatively clear (turbidity, 2.2 JTU) with a low suspended sediment load. (c) The unfiltered seawater sample was relatively turbid (turbidity, 6.5 JTU) and contained a relatively high suspended sediment load.

eral Republic of Germany) fitted with a 50-W direct current HBO mercury light source and G365 excitation, FT395 beam splitter and refractor, LP 420 barrier filters (Zeiss, filter set number 487902), and a ×63 oil objective (Plan-Neofluor).

Enumeration of attached and free-living bacteria. At least 15 fields of view and 300 cells were counted per slide. Total numbers of bacteria (free-living plus originally attached cells) were counted in treated samples. The number of free-living bacteria was enumerated in untreated samples. Untreated samples were diluted so that particles covered no more than 25% of the microscopic field, even when samples were from very turbid seawater. The number of attached bacteria was obtained by subtracting the number of free-living bacteria from the total number of bacteria. The number of free-living bacteria was derived from the free-living bacteria count adjusted to include those bacteria that were either hidden from view by large detritus or that settled onto detritus during filtration. A correction factor was derived from each microscopic field that was covered by detritus in untreated samples, assuming an even distribution of free-living bacteria over the filter surface [corrected free-living bacterial number of the microscopic field = (free-living bacteria enumerated from the microscopic field/the ratio of the microscopic field area not covered by detritus to the total microscopic field area)].

Effect of simulated resuspension of sediment on bacterial abundance in the water column. Seawater and surface sediment (upper 0.5 cm of sediments) samples were taken from Redfish Bay. Seawater was filtered through 3.0-μm-pore-size filters (Nuclepore) to remove detritus and most of the attached bacteria. Surface sediments were added to the filtered seawater (final concentration, 40 mg [dry weight]/liter) in a 4-liter beaker and were suspended evenly by rapid stirring to simulate naturally occurring turbid seawater. Prior to sampling, stirring was stopped. The surface water cleared as suspended matter gradually settled. Samples for determination of turbidity and numbers of bacteria were collected from 1 cm below the surface of the water every 2 h.

Turbidity measurement. The turbidity of seawater was measured with a turbidimeter (model 2100A; Hach Chemical Co., Loveland, Colo.). Turbidity standards supplied by the manufacturer were used to calibrate the turbidimeter in Jackson turbidity units (JTU).

Current measurement. The current velocity in Aransas Pass was measured with a digital flow meter (model 2030; General Oceanics, Miami Fla.) calibrated with a remote recording current meter (Endeco model 110; Environmental Devices Corp., Marion, Mass.).

RESULTS

Turbidity of seawater and concentration of suspended matter. The turbidity of seawater in the study area correlated linearly with the dry weight concentration of suspended matter between 1 and 20 JTU (W. B. Yoon, Ph.D. thesis, University of Texas at Austin, 1986). Seawater of 10 JTU contained approximately 40 mg of suspended matter per liter.

Effect of sonication time on enumeration of attached bacteria after treatment with Tween 80. Control experiments

TABLE 1. Effect of Tween 80 concentration on direct counts of bacterial numbers

Tween 80 concn (mg/liter)	Bacterial counts[a]			
	Total[b]	Percentage of control	Attached[c]	% of control
0	4.67 ± 0.24	100	1.35	100
2.5	5.07 ± 0.32	108	1.75	129
5	5.28 ± 0.33	113	1.96	145
10	5.68 ± 0.28	122	2.36	175
25	5.50 ± 0.24	118	2.18	161
50	5.19 ± 0.26	111	1.87	139
100	5.17 ± 0.32	109	1.85	137

[a] Direct counts were made by DAPI epifluorescence. Seawater was taken from Aransas Pass on 25 July 1985 (turbidity, 4.0 JTU). All samples were fixed with glutaraldehyde, treated with Tween 80, and sonicated for 30 s prior to DAPI staining and enumeration. Unit of bacterial number is 10^6 cells per ml.

[b] Total bacteria were estimated by observing 15 microscopic fields. Means and 95% confidence intervals are given.

[c] Number of attached bacteria = total number of bacteria − number of free-living bacteria.

showed that counts of naturally occurring free-living bacteria were not changed by the addition of 10 ppm of Tween 80 followed by sonication for 30 s (Fig. 2a). We assumed, therefore, that counts of total bacteria in natural seawater were not affected by 30 s of sonication in the presence of Tween 80.

Counts of total bacteria in natural seawater samples increased in proportion to the time of sonication for periods up to 20 s (Fig. 2b and c). The increase in total bacterial counts resulted from a more accurate estimation of attached bacteria in the treated samples. Between sonication times of 20 and 30 s, there was no significant change in total bacterial counts. Enumerated bacteria gradually decreased when the period of sonication exceeded 30 s. A 20-s treatment by this procedure increased the bacterial counts in relatively clear (2.2 JTU) and in relatively turbid (6.5 JTU) seawater by 9- and 10.5-fold, respectively (Fig. 2b and c).

Effect of Tween 80 on enumeration of attached bacteria. As the concentration of added Tween 80 was increased to 10 ppm, total bacterial counts increased (Table 1). When the concentration of Tween 80 exceeded 25 ppm, cell counts were reduced. The optimum concentration of Tween 80 was 10 ppm. Treatment both with Tween 80 and by sonication always increased counts of attached bacteria relative to the counts of samples treated by sonication alone, regardless of sample turbidity (Table 2). Sonication without Tween 80 resulted in the counting of only 42 to 76% of the attached bacterial population.

Comparison of sonication and homogenization of samples treated with Tween 80. Homogenization of samples from Aransas Pass (4.6 JTU) pretreated with Tween 80 increased the bacterial counts by a factor of 1.8 compared with the bacterial counts in those samples treated by homogenization only. Sonication with Tween 80 increased bacterial counts by a factor of 1.6 over the counts in those samples treated by sonication alone. For samples that were either pretreated with Tween 80 or not treated, there was no significant difference in the numbers of total bacterial cells between sonicated and homogenized samples ($P > 0.05$) (data not shown).

Fluctuations of attached and free-living bacteria during diurnal tides. Fluctuations in the abundance of attached and free-living bacteria were observed at three distinct stages of the tidal cycle at the Aransas Pass sampling station: when the tidal range was at a maximum, at a minimum, and halfway between these two extremes. During the study period, the tide level at the sampling station was primarily controlled by the astronomical tide.

Both variation in seawater turbidity and fluctuation in the abundance of attached bacteria during diurnal tides at the study area were related to the extent of tidal range (Fig. 3). When the tidal range declined from 0.95 to 0.52 m and then to 0.15 m, variation in seawater turbidity decreased from 0.9 to 6.8 JTU, to 1.0 to 4.4 JTU, and 1.1 to 2.2 JTU, respectively. Accordingly, fluctuation in the abundance of attached bacteria was reduced from 0.4×10^6 to 4.6×10^6 to 0.2×10^6 to 3.0×10^6 and 0.4×10^6 to 1.3×10^6 cells per ml, respectively.

The concentration of free-living bacteria was usually higher than that of attached bacteria, except when the turbidity of seawater was greater than 6 JTU (Fig. 3a through c). In general, the abundance of attached bacteria increased proportionally as the turbidity of seawater increased (Fig. 4b), while the abundance of free-living bacteria did not (Fig. 4a). The most turbid water during a diurnal tide was observed when the current flowed with the maximum velocity from the bay toward the Gulf of Mexico. The least turbid water during a diurnal tide was generally observed at or near the slack time between high and low tides (Fig. 3a and b).

TABLE 2. Effect of Tween 80 treatment on direct counts of bacterial numbers from seawaters with different turbidities

Turbidity (JTU)[a]	Bacterial no. (10^6 cells/ml)[b]					% Increase in attached bacteria after Tween 80 treatment
	Free-living[c]	Sonicated[d]		Tween 80 and sonicated[e]		
		Total[f]	Attached[g]	Total[f]	Attached[g]	
2.5	2.95 ± 0.25	4.57 ± 0.29	1.62	5.15 ± 0.17	2.17	34
3.2	4.42 ± 0.43	5.87 ± 0.23	1.45	6.90 ± 0.34	2.48	71
4.0	2.61 ± 0.28	3.48 ± 0.26	0.87	3.75 ± 0.23	1.14	31
4.4	4.14 ± 0.27	6.08 ± 0.39	1.94	7.10 ± 0.27	2.96	53
6.8	2.80 ± 0.20	3.66 ± 0.30	0.86	4.83 ± 0.31	2.03	136

[a] Seawater samples were collected at Aransas Pass on 6 July 1985, except for those with turbidities of 4.0 and 6.8 JTU, which were collected at the same station on 2 July 1985.

[b] Obtained from direct counts by DAPI epifluorescence. Means from the observation of 15 microscopic fields and 95% confidence intervals are given for total and free-living bacterial numbers.

[c] Free-living bacterial numbers were determined from untreated seawater samples as described in the text.

[d] Direct bacterial counts from samples that were treated by sonication for 30 s.

[e] Direct bacterial counts from samples that were pretreated with Tween 80 (10 ppm) followed by sonication for 30 s.

[f] Total bacterial numbers from treated seawater samples.

[g] Numbers of attached bacteria were calculated by subtracting the number of free-living bacteria from the number of total bacteria in a treated sample.

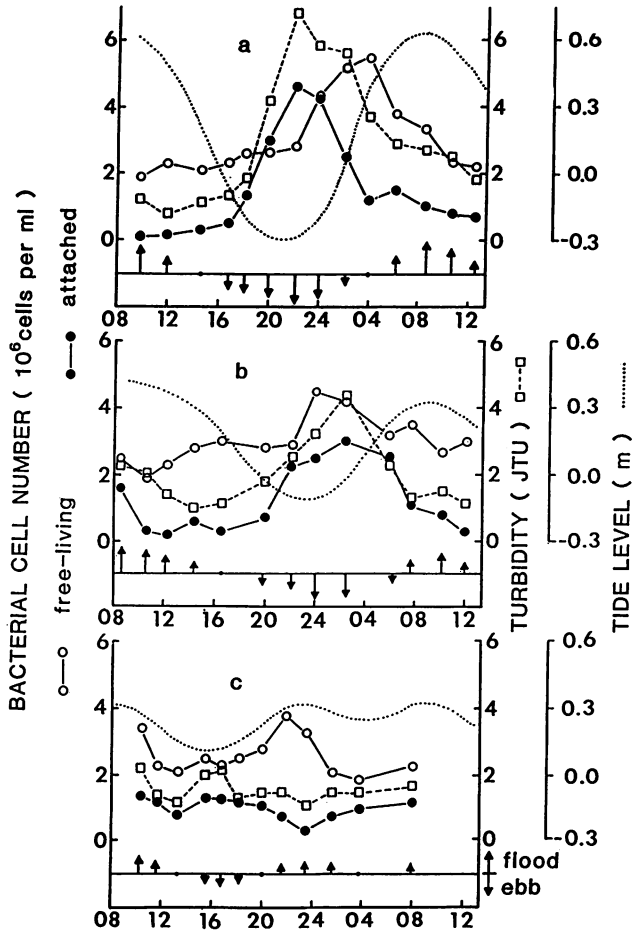

FIG. 3. Variation in the abundance of bacteria and the turbidity of seawater during diurnal tide at Aransas Pass (inlet). The lengths of the arrows indicate the relative velocity of the tidal current. (a) Samples taken on 2 and 3 July 1985, when the tidal range was relatively large (0.95 m). (b) Samples taken on 6 and 7 July 1985, when the tidal range was intermediate (0.52 m). (c) Samples taken on 9 and 10 July 1985, when the tidal range was small (0.15 m).

When bay water flowed to the Gulf of Mexico, both the turbidity and the density of attached bacteria increased as the current speed increased (Fig. 5a). When the gulf water flowed into the bay system, both the turbidity and the concentration of attached bacteria were affected to a lesser extent by current velocity (Fig. 5b).

Although the fluctuation in the number of free-living bacteria did not correlate directly with seawater turbidity, the variability of free-living bacterial numbers increased as the range of turbidity variation increased during diurnal tides. When the fluctuation in seawater turbidity was small (from 1.1 to 2.2 JTU), the abundance of free-living bacteria varied from 1.9×10^6 to 3.8×10^6 cells per ml (Fig. 3c). When the fluctuation of turbidity was in a medium range (from 1.0 to 4.4 JTU), the range of variation in the abundance of free-living bacteria was from 1.9×10^6 to 4.5×10^6 cells per ml (Fig. 3b). When the variation of turbidity was large (0.9 to 6.8 JTU), the abundance of the free-living population varied from 1.9×10^6 to 5.4×10^6 cells per ml (Fig. 3a). The numbers of free-living bacteria decreased as gulf seawater invaded the bay system.

Simulation of transient sediment resuspension in the laboratory also showed a strong relationship between the density of attached bacteria and turbidity (Fig. 6a). The density of free-living bacteria was not significantly changed ($P > 0.05$) by resuspended sediment. Both the abundance of attached bacteria and the turbidity decreased rapidly during the first 2 h because of sedimentation of relatively large particles. For seawater with both suspended sediment and seawater without sediment (control), the abundance of free-living bacteria gradually increased as time elapsed (Fig. 6a and b). The abundance of free-living bacteria at the initial time was significantly different ($P < 0.01$) from that after 17 h for both types of seawater samples.

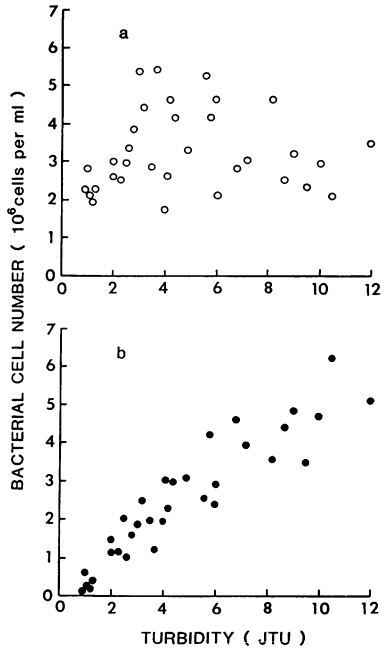

FIG. 4. Relationship of seawater turbidity to numbers of free-living bacteria (a) and to numbers of attached bacteria (b) in seawater of Aransas Pass.

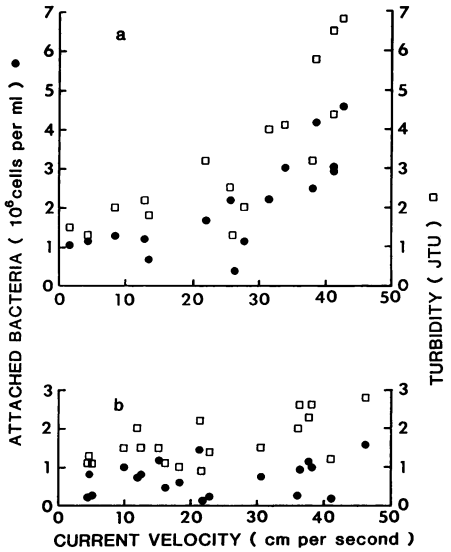

FIG. 5. Relationship of current velocity to seawater turbidity or to abundance of attached bacteria in seawater at Aransas Pass when bay water flowed to the Gulf of Mexico (a) and when the gulf water flowed into the bay (b).

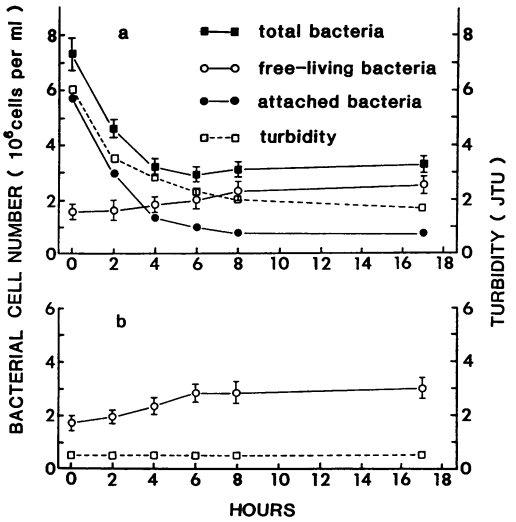

FIG. 6. Laboratory simulation of transient resuspension of sediment. Seawater and sediment samples were taken from Redfish Bay. (a) Changes in turbidity and density of attached and free-living bacteria in the water column were observed as suspended sediment settled. Time zero was when the mixing of sediment with filtered seawater (pore size, 3.0 μm) was stopped. (b) The control consisted of only the filtered seawater (pore size, 3.0 μm), without added sediment.

DISCUSSION

Most suspended matter in seawater at the study area consisted of aggregations of detritus and fine inorganic particles; such aggregations are generally observed in estuarine environments (21). During sonication, organic and inorganic aggregates were either broken into very fine particles or dispersed. Simultaneously, attached bacteria were also scattered, facilitating counting by epifluoresence microscopy. Since control experiments showed that sonication did not destroy glutaraldehyde-fixed free-living bacteria, it was assumed that glutaraldehyde-fixed attached bacteria were also stable to the sonication that was applied (Fig. 2a).

The surface-active agent Tween 80 was used for the first time in this study to improve direct counts of attached bacteria in seawater samples. When suspended matter in seawater was temporarily dispersed by gentle shaking, homogenization, or sonication, small particles rapidly reaggregated, presumably because of electrical double-layer effects (14). Tween 80 prevented the reaggregation of fine particles, including bacteria, possibly by depression of surface tension (11). Tween 80 is also known to enhance detachment of bacterial cells from particles in sediment samples, thereby increasing the CFU from sediment samples (18). The combination of direct detachment of attached bacteria and stable dispersion of aggregates contributed to increased attached bacterial counts. Velji and Albright (20) successfully used PP_i as a deflocculating agent. Since we did not use PP_i, we cannot comment on the relative merits of PP_i and of Tween 80 and sonication treatment.

The concentration of Tween 80 was critical for the optimal enumeration of attached bacterial cells (Table 1). During filtration, high concentrations (>25 ppm) of the surfactant washed the irgalan black dye from filters, resulting in less counts of bacteria because of a brightly fluorescent background; this problem may be reduced with the use of commercial black epifluorescence filters. Also, fragile bacteria may be destroyed by a Tween 80 concentration of greater than 25 ppm.

Even after sonication with the surfactant, some attached bacteria are not counted if they are attached to the bottom sides of relatively large inorganic particles which are not dispersed or fragmented by this technique. This problem increases when samples contain many inorganic particles. However, after sonication of surfactant-treated samples, few bacteria were usually observed to be associated with the visible surfaces of the remaining large particles.

In this study area, the turbidity of seawater increased as the current velocity increased during the ebb tide (Fig. 5a). The maximum turbidity during a diurnal tide was always observed when the current speed was maximum during the ebb tide. This indicates that seawater turbidity in this area depends on the extent of sediment resuspension by tidal currents.

A linear relationship between the concentration of suspended matter and the numbers of attached bacteria was observed in the Humber Estuary, England (17), and also in the Oosterschelde Basin, The Netherlands (13). The turbidity of seawater in Aransas Pass was proportional to the concentration of suspended matter up to the highest turbidity observed, which corresponded to 40 mg of suspended matter per liter (Yoon, Ph.D. thesis). While similar numbers of attached bacteria were observed in this study and in the Humber Estuary, the concentration of suspended matter in Aransas Pass seawater was 1 order of magnitude less than that in Humber Estuary seawater.

Wilson and Stevenson (22) reported that tidal action in North Inlet Marsh, S.C., had very little influence on the overall density of the bacterial population in the water column, particularly on the free-living population. For samples from Aransas Pass, the abundance of free-living bacteria was also not directly related to the turbidity of the seawater. However, the variability of the free-living bacterial numbers was positively related to the range of turbidity during diurnal tides (Fig. 3a through c). During diurnal tides, when sediment resuspension was obvious, the overall pattern of free-living bacterial numbers was similar to that of seawater turbidity, although these two parameters did not correlate strongly (Fig. 3a and b). Therefore, we presume that the abundance of free-living bacteria is both indirectly and directly related to sediment resuspension. The observed increases in numbers of free-living bacteria may be due to a combination of factors, including (i) growth in response to the injection of dissolved organic nutrients from enriched pore waters into the water column during resuspension of sediments (8), (ii) release of attached bacteria from particles as surface sediments mix with overlying waters, and (iii) the possible reduction of the impact of grazing during sediment resuspension.

Results of the simulation experiment in the laboratory showed no increase in free-living bacteria in the water column for 17 h after the transient sediment resuspension (Fig. 6a and b). The laboratory simulation experiment showed that the abundance of attached bacteria is related to seawater turbidity, but the abundance of free-living bacteria is not related to seawater turbidity. This result is consistent with our observations of Aransas Pass seawater (Fig. 4a and b). The gentle, transient resuspension of sediments in the laboratory, however, does not explain the observed variability of the free-living bacterial population in natural seawater. This may reflect an imperfect modeling of the energy in the natural system. For example, it is possible that bacteria that are loosely associated with suspended particles in the natural

environment may be released by repeated turbulence resulting from currents or continuous wave actions, or continuous resuspension of sediments may cause physicochemical changes (e.g., the concentration of suspended matter, heavy metals, and organic and inorganic nutrients) in the water column which may alter the metabolism of bacteria or bacteriovores, resulting in changes in the concentration of free-living bacteria.

At flood tide, the inflow of seawater from the Gulf of Mexico resulted in a rapid decrease in the numbers of free-living bacteria in the water column at Aransas Pass. Since gulf water has lower concentrations of suspended matter and bacteria (16), and presumably contains lower numbers of grazers than bay water does, increased rates of grazing or sorption of free-living bacteria onto particles were probably insignificant; and this decrease in number resulted primarily from dilution. Dilution of the bacterial population by ocean water at high tide has been observed in Mission Bay, Calif. (23), and at salt marsh creeks in South Carolina (6).

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