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NOTES

Enumeration of Particle-Bound and Unattached Respiring Bacteria in the Salt Marsh Environment

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Proportions of respiring bacteria determined with a 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride dye-epifluorescent technique were significantly elevated in the 300-μm surface layer of a salt marsh estuary. Almost all the detectably respiring bacteria in the particle-laden surface layer and a significant proportion in subsurface waters were attached to particles.

Three current methods for enumerating respiring bacteria combine acridine orange epifluorescence (6) with procedures for counting cells involved in the uptake of specific substances, i.e., nalidix acid (8), [^{3}H]glucose (10), and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (13). The INT method involves counting cells with intracellular deposits of INT-formazan and is simpler and less time consuming than [^{3}H]glucose autoradiography and does not require a long incubation period with unnaturally high levels of labeled nutrients. However, the appropriateness of this method for use with particle-bound bacteria is unknown as it is questionable whether INT-formazan deposits may be distinguished from adjacent particles when transmitted light is used. This study assesses the suitability of the INT technique for particle-bound bacteria and examines levels of respiring bacteria determined by the INT procedure in the particle-rich, 300-μm surface layer and subsurface waters of a Palo Alto, Calif., salt marsh.

Surface microlayer and subsurface waters of a major tidal creek in the salt marsh were sampled every 15 min over a portion of the outgoing tide. The screen-dip method described by Garrett (3) was used to sample the surface layer, and corresponding subsurface samples were collected at a depth of 0.2 m. Each 10-ml sample was immediately incubated at an ambient temperature in the dark for 20 min with 1 ml of 0.2% (wt/vol) INT (18377; Sigma), followed by fixation with 100 μl of 37% formaldehyde and storage at 4°C. The appropriateness of the 20-min incubation time was checked by examining incubation times of 10 min and 2 h. No significant differences in numbers of INT-precipitating bacteria were found, confirming similar findings by Zimmermann et al. (13), who found no change in response after the first 2 min.

Numbers of unattached and particle-bound bacteria were determined by acridine orange epifluorescence (6). Cell size distribution was determined by visual measurements with a calibrated ocular micrometer. Numbers of respiring bacteria were assayed by the procedure of Zimmermann et al. (13) which matches INT precipitate observed under bright-field illumination with cells stained with acridine orange detected under reflective fluorescence illumination.

As the INT technique is acknowledged to be a function of "respiratory intensity" (13), cells respiring at a level below detection would not be accounted for. Consequently, our data are likely to underestimate the respiring population. Compared with the activities measured by ^{14}C-heterotrophic uptake or microautoradiography (7, 10), we seemed to be detecting a smaller fraction of the active population. Nonetheless, the advantages offered by the INT method make it quite useful for examining relative differences in bacterial populations such as those found with the particle-bound and unattached cells in the salt marsh.

Certain modifications of the previously described INT procedure (13) facilitated the counting of respiring bacteria attached to particles. A magnification of 1,250× instead of 1,600× was used on our Olympus Vannox microscope for all counts. The greater depth of focus was helpful in viewing cells attached to large particles. The intensity of the bright-field image was attenuated to 50% with neutral density filters until a mixed bright-field-fluorescent image was obtained. In this manner, fluorescent light, mixed

light, or bright-field images could be obtained by increasing or decreasing the intensity of the transmission beam lamp.

Nuclepore polycarbonate filters with 0.05-μm-pore diameters yielded less interference than 0.1-μm filters because of filter structure. The flow-through rate, however, was reduced by a factor of 18. It was further observed that the 0.08-μm filters maintained the same flow-through rate as the 0.1-μm filters, in spite of a smaller pore diameter, and were therefore more suitable for the identification of INT-formazan deposits.

Particle-bound bacteria were easily distinguished under reflective fluorescence because of their contrasting color and their size and morphology. Examples of particle-bound and free bacteria are shown in Fig. 1. With bright-field illumination, the INT-formazan deposits appeared as dark dots and were distinguished from particles, which were identified by outlines. As black dots caused by crystalline material of high optical density were also observed, it was necessary to first identify each cell with reflective fluorescence and then switch to bright-field illumination. As only a limited number of particle-bound cells were located in the same focal plane, frequent refocusing was required to enumerate all respiring cells in one field.

Approximately 16% of the bacteria in our samples were smaller than 0.4 μm. The metabolically active cells in this size fraction may not have been assayed with the INT procedure as the size of the INT deposits would have been too small to be observable. The remaining 84% of ≥0.4-μm bacteria were accountable by the INT procedure. Indeed, more than 50% were >0.8 μm and were very easily assayed by the method. However, the INT procedure may not be suitable for offshore and open ocean environments, where more than 50% of the bacteria are <0.3 μm and up to 80% are <0.5 μm in size (2, 12).

Table 1 summarizes data for detectable respiring and total bacterial counts for 10 salt marsh surface layer samples and 10 subsurface samples. These data show significantly higher concentrations of bacteria in the surface layer than in the subsurface waters and support our previous work, in which the INT dye procedure had not been used (5). As illustrated in Table 1, total counts of bacteria were observed to be five times higher in surface waters than in subsurface waters. Furthermore, numbers of detectable respiring bacteria were 16 times higher in surface layer samples, which suggests that there are factors at the surface which have a selective influence on respiring cells.

One such factor may be the presence of high numbers of particles in the surface layer. The presence of sedimentary particles has been reported to increase bacterial survival two to three orders of magnitude above pure seawater systems (11). As seen in Table 1, the great majority of bacteria in the surface layer microenvironment were particle bound (93%), in contrast to the subsurface environment, which consisted primarily of unattached cells, only ~22% of which were particle bound.

Figure 2 depicts the percentages of particle-bound and detectable respiring bacteria in 10 subsurface samples. Data for two of the surface microlayer samples are also presented for comparison. These results illustrate that samples with high percentages of particle-bound cells generally correlate with high percentages of detectable respiring cells.

Upon closer examination of the population of respiring bacteria as determined by INT, it can be seen in Table 1 that a small percentage of the total population in the surface layer was detectably respiring (16%), and virtually all of these bacteria were attached to particles (99.4%). Similarly, in the subsurface water, the majority (62.5%) of the respiring cells assayed were also particle bound. Assuming that the cell size distributions of the attached and unattached populations are the same, this indicates that a greater proportion of the particle-bound cells are detectably respiring, as compared with those unattached, even though the subsurface population is largely comprised of unattached cells (78.3%). These findings concur with both Hanson and Wiebe (4) and Kirchman and Mitchell (D. L. Kirchman and R. Mitchell, Abstr. Annu. Meet. Am. Soc. Limnol. Oceanogr., 1979) who, with ^{14}C-heterotrophic uptake techniques, reported most of the heterotrophic activity in salt marsh waters to be particulate associated.

This may not be the case in other marine environments. For example, Hanson and Wiebe (4) reported that although more than 80% of the heterotrophic activity was found in the >3-μm fraction for salt marsh samples, that same size fraction constituted only 20% of the activity in samples taken from the Gulf Stream. Azam and Hodson (1), in a detailed study, reported that about 90% of the heterotrophic activity in their offshore and open ocean samples was associated with the <1-μm fraction, which they considered to be free-living organisms. The relatively high activity of the small cell size fraction in these marine environments can be understood in part because of the sparsity of particle-bound cells (2) and because of a higher proportion of very small cells in these systems.

Our salt marsh samples contained many chains of rod-shaped bacteria generally associated with the particulate matter (Fig. 1). In

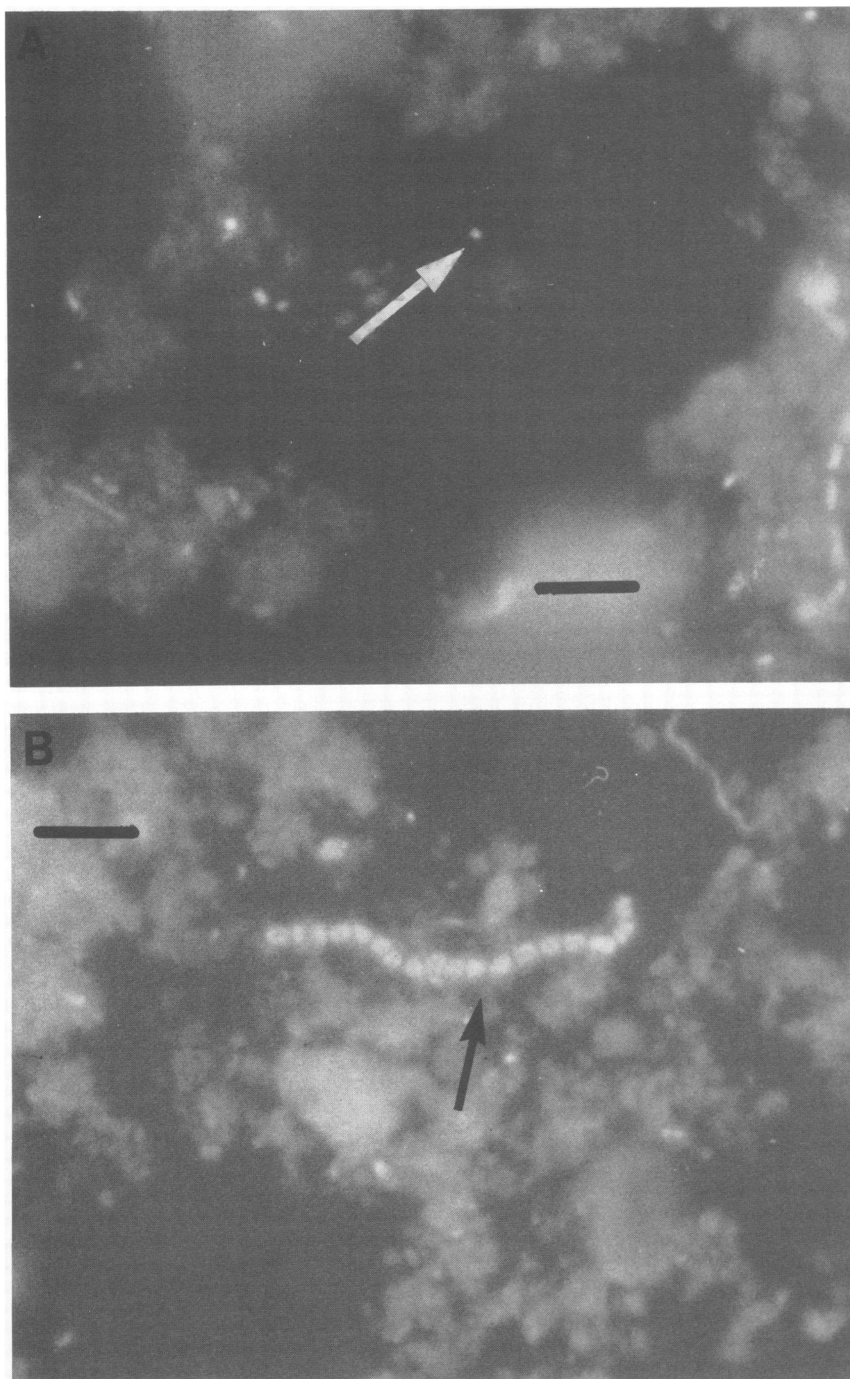

FIG. 1. *Reflective fluorescence photomicrographs of acridine orange-stained bacteria in a salt marsh microlayer sample. (A) Unattached cell (arrow) and particle-bound bacterial cells. (B) Chain of large bacteria (arrow) associated with particulate matter. Bars are 10 μm long.*

TABLE 1. *Bacteria in surface and subsurface waters of a Palo Alto salt marsh*

Sample	Total bacteria[a]		Respiring bacteria[b]	
	Total no. (per ml)[c]	% Particle bound	Total no. (per ml)[c]	% Particle bound
Surface (S_1) (n = 10)	(2.53 ± 0.96) × 10^7	93.0	(3.42 ± 1.51) × 10^6 (16.0%)	99.4
Subsurface (S_2) (n = 10)	(5.37 ± 0.99) × 10^6	21.7	(2.37 ± 0.80) × 10^5 (5.14%)	62.5

[a] Determined by acridine orange epifluorescence. Counts on individual samples were made to ± 10% at the 90% confidence level. The surface concentration factor (S_1/S_2) was 4.95.
[b] Determined by acridine orange-INT dye procedure. Counts on individual samples were made to ±10% at the 90% confidence level. Numbers in parentheses represent the percentage of total bacteria. The surface concentration factor (S_1/S_2) was 16.2.
[c] Data are presented as the mean ± standard deviation for the entire sample population.

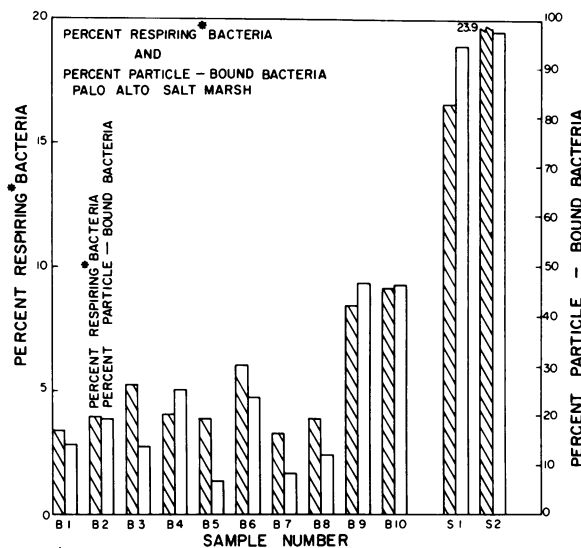

FIG. 2. *Percentages of respiring and particle-bound bacteria for 10 subsurface samples and two surface layer samples from a Palo Alto salt marsh. *Respiring is defined as metabolically active as measured by the INT procedure.*

many instances, we found all cells in the chain to be respiring, similar to the results reported by Zimmerman et al. (13). In addition, significant numbers of red and yellow fluorescing cells, believed to be more active than green fluorescing cells (6), were also particle associated.

In conclusion, the INT technique can be used successfully for examining bacteria in natural waters containing large quantities of particulate matter. Our data indicate that a much greater proportion of particle-bound bacteria are respiring at a detectable level than planktonic cells.

In addition, it appears that the salt marsh surface layer, which contains a higher percentage of these particle-bound cells, also has a higher proportion of respiring bacteria, in spite of the reportedly detrimental effects of ultraviolet radiation (9). The effect of particle-laden waters on total heterotrophic respiration is clearly significant and is worthy of further investigation.

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