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Measurement and Significance of Specific Activity in the Heterotrophic Bacteria of Natural Waters

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It is now possible to obtain accurate total counts of the bacteria of natural waters with the use of acridine orange staining and epifluorescence microscopy. This approach can be coupled to highly sensitive measurements of heterotrophic activity using radioisotopes. To accomplish this, three variations of a "specific activity index" are suggested, based on different approaches to measuring heterotrophic activity with radiolabeled organic solutes. The denominator of each index is the direct count of bacteria from a given natural sample. Three numerators are presented, each of which has been shown to vary directly with heterotrophic bacterial activity: V_{max}, turnover rate, and direct uptake (at high substrate concentrations). Each approach is illustrated with data from estuarine and coastal waters of northeastern Massachusetts. The data show major differences in specific activity that accompany such habitat differences as distances within or offshore from an estuary and vertical location in the water column. These and other data suggest that specific activity is a valid indicator of the physiological state and metabolic role of the bacteria. Some evidence is presented in support of the hypothesis that the natural bacteria are adapted to conditions of nutrient starvation by becoming "dormant," existing for an unknown period of time in a reversible physiological state that reflects the availability of organic nutrients.

Bacteria in natural waters and sediments have long been considered important agents of organic matter decomposition and mineral regeneration, as well as food for higher organisms. Until fairly recently, however, information on their activities and numbers was severely limited by the available methodology. Numbers were measured by "viable counts" on agar media, and activity measurements consisted of laboratory tests of the ability of cultured forms to perform various biochemical or mineral transformations. Recent advances in fluorescence microscopy, staining, and filter technology have led to the acridine orange direct count (AODC), using Nuclepore filters, as described most recently by Hobbie et al. (13). For measuring the activity of the natural heterotrophic bacteria, methods based on the uptake of ^{14}C- and ^{3}H-labeled organic solutes have proven adequate even in the most oligotrophic waters (see 28 for a summary of these methods). The result is that there is now available both a sensitive measure of natural heterotrophic activity and an accurate method for counting the bacteria. Combining these two approaches is an obvious step, one that may yield new insights into the functioning of natural bacteria.

Several workers have already used this basic idea in their studies of heterotrophic activity in natural waters. Azam and Hodson (1) related glucose uptake to ATP-determined biomass in order to evaluate the relative uptake of a given size fraction as separated by Nuclepore filters. Their "relative metabolic activity" is dimensionless and compares the activity of a fraction to total activity within a natural water sample; also, biomass is calculated by ATP measurements, which are not specific for bacteria. The main thrust of this paper was to show that over 90% of heterotrophic activity in a variety of marine waters was by organisms passing through a 1-μm filter, obviously bacteria.

Fry and Ramsey (9) used an activity index based on uptake kinetics, V_{max} per bacterium, in their work on the heterotrophic activity of freshwater epiphytic bacteria. Their index is given in micrograms per liter per hour $\times 10^7$ and appears to be in error because of the inclusion of a volume dimension. It is also several orders of magnitude too high. In this work autoradiography was used to determine the proportion of total bacteria actually metabolizing the substrate used. This is also a valuable approach to heterotrophic activity, one that has been used by several workers to show that a variable proportion of the total number of bacteria (deter-

mined by direct count) is metabolizing a given substrate (7, 14, 22). It is a qualitative approach, however, and has many technical problems that relate to concentrations of substrate, time of exposure to film, and the counting of radiographic spots on filters.

The present paper speaks to the need for a quantitative expression of the relationship between heterotrophic activity and the bacteria responsible for that activity. I will present three variations of a "specific activity index," based on different approaches to measuring heterotrophic activity. I will then show some results which indicate the usefulness of this combined approach.

THEORETICAL CONSIDERATIONS

The concept of specific activity is used in enzymology to relate the amount or rate of enzyme activity to the more or less pure protein containing the enzyme. It seems appropriate to adopt this concept for relating heterotrophic activity to the organisms responsible for that activity (I am grateful to D. W. Schindler for this suggestion). Throughout the paper, the term specific activity index represents the use of the concept of specific activity in a quantitative relationship.

The bacteria can be represented in the relationship in several ways: numbers, volume, and biomass. Two approaches are available for measuring numbers: the direct count and the plate count on some selected medium. Plate counts occasionally correlate with heterotrophic activity (23), but it is well known that the plate count yields only a small fraction of the total number of bacteria present in a sample (15). There is certainly a use for plate counts, but the evidence clearly shows that the plate count does not measure either total numbers or living, active bacteria (6, 17). The direct count, on the other hand, is a rapid and simple method that yields a count of all bacteria present; it does not distinguish among living, dormant, and dead bacteria, and it does not readily yield information on the different sizes of the bacteria in a sample. However, in the opinion of this worker, the direct count using acridine orange and epifluorescence is much less equivocal than the plate count and will ultimately yield more information on the heterotrophic bacteria as it is more widely used.

Volume measurements are tedious and imprecise with the light microscope and more precise but also tedious and costly with the scanning electron microscope (4). Biomass estimations can be made from volume measurements, or from a new method using *Limulus* amebocyte lysate to measure bacterial lipopolysaccharide (25). This latter method is very promising but has the disadvantages of being costly and as yet not widely tested. The advantages seem to be with the direct count (AODC), which correlates well with the *Limulus* amebocyte lysate method. The counts from natural waters range from 10^8 to 10^{10} liter^{-1} (5, 10; unpublished data); hence, a unit of 10^9 bacteria liter^{-1} would appear to be the most useful for the denominator of the specific activity index.

The numerator of the index should be some measure that varies directly with heterotrophic bacterial activity and is sensitive to activity at natural substrate concentrations. Three indexes are proposed, based on different approaches to the application of radiolabeled organic solutes to natural waters (28).

(i) V_{max}—kinetic approach. The kinetic approach to measuring heterotrophic uptake of organic solutes uses a series of concentrations of the labeled solute in the microgram-per-liter range (29, 30). This approach generates V_{max}, an interpolated parameter that implies a full-capacity uptake rate for the substrate and population in question. If respired $^{14}CO_2$ is measured, the method gives V_{max} for total uptake of a solute. If not, V_{max} for assimilation is obtained. Values for V_{max} range considerably, from 10^{-4} to 10^{1} μg liter^{-1} h^{-1}. For a V_{max} specific activity index, a useful set of units would be 10^{-3} μg liter^{-1} h^{-1}, yielding the index: V_{max} (10^{-3} μg liter^{-1} h^{-1})/AODC (10^9 cells liter^{-1}), or 10^{-12} μg h^{-1} cell^{-1}.

(ii) Turnover rate—tracer approach. If labeled substrate is used at the tracer level, it is possible to obtain the natural turnover time for a substrate with a measurement at only one concentration (2, 26). The kinetic approach also yields the natural turnover time but requires more samples and is of limited use in oligotrophic waters. The tracer approach involves adding ^{3}H- or ^{14}C-labeled substrate at low concentrations (<1 μg of substrate liter^{-1}) and then measuring the fraction of added substrate that was assimilated or respired or both. It should be emphasized that this approach is valid only if added substrate is substantially lower in concentration than naturally occurring substrate. Gocke (10) has compared the turnover times obtained via the kinetic approach with those determined with a single low concentration. The results show good agreement between the two approaches in all but the most oligotrophic marine waters, the advantages of one or the other depending on a tradeoff of simplicity for the tracer approach versus the additional data provided by the kinetic approach, namely, V_{max} and ($K_t + S_n$) (28).

Natural turnover time is defined as the num-

ber of hours required for the existing heterotrophic population to take up and/or respire a quantity of substrate equal in concentration to the existing concentration. The more active the population, therefore, the shorter the natural turnover time will be; it is an inverse function of heterotrophic uptake and as such would be awkward to use in an activity index. The simplest device is to use the inverse of turnover time. This parameter has been called "relative uptake rate" (2) or "respiration rate" when only respired $^{14}CO_2$ was measured (26) and has units of h^{-1}. It is a measure of the percentage of available substrate taken up per unit of time. Odum (21) calls this measurement "turnover rate," a term that seems to be less confusing and therefore preferable. Since turnover is a function of uptake and substrate concentration, variations in both of these parameters will influence the measured turnover rate. However, uptake will vary over several orders of magnitude, quite comparable to the variation in V_{max} values, whereas substrate concentrations in natural waters typically show little or no variation (5, 31). Turnover rates range from high values of 1 h^{-1} or more in highly eutrophic, warm waters to 10^{-5} h^{-1} in extremely oligotrophic waters like the open oceans. For use in a specific activity index, a turnover rate of 10^3 h^{-1} is appropriate, giving an index: turnover rate (10^3 h^{-1})/AODC (10^9 cells $liter^{-1}$) or 10^{-6} h^{-1} $cell^{-1}$ liters.

(iii) Direct uptake measurement. If the natural substrate concentration is measured independently, a direct calculation of v_n, the natural uptake velocity, can be made with either the kinetic approach or the tracer approach (28). This is probably the best heterotrophic activity measurement, but it is also the most difficult to obtain. If it were used in a specific activity index, the units would be the same as for the V_{max} index calculations.

However, if substrate is added in concentrations above 100 μg $liter^{-1}$, it is legitimate to ignore the unknown concentration of naturally occurring substrate in calculating subsequent uptake (28). This approach does not measure natural activity in the sense of in situ rates or substrate turnover because of the unnaturally high substrate concentration. Because this approach uses a single concentration and direct calculation, it is not concerned with the kinetics of uptake. It might be used, for example, in testing the response of a natural population to organic enrichment over a period of days (3). The heterotrophic activity is then expressed as a rate $v = [f \cdot (A)]/t$, where v = uptake and/or respiration of the substrate, f = the fraction of available labeled substrate taken up, A is the added substrate concentration, and t is the incubation time. Note that natural substrate concentration, S_n, is ignored in the calculation. In combination with bacteria numbers, this specific activity index would have the same units as the V_{max} index: 10^{-12} μg h^{-1} $cell^{-1}$.

One major factor in measuring and comparing values for heterotrophic activity is the influence of temperature on the measured values. In a very extensive study of the temperature effects on glucose uptake in marine waters, Takahashi and Ichimura (24) found a range of temperature sensitivity as measured by Arrhenius plots of log V_{max} versus the reciprocal of absolute temperature. Surface waters and mixed-water masses gave the highest activation energies, a mean of 21,900 ± 1,500 cal (91,600 ± 6,300 J). Lower mean values were found in deep, stable waters (10,600 ± 1,000 cal [44,300 ± 4,200 J]). Hamilton et al. (12) found values of 34,200 (143,000 J) and 26,800 cal (112,200 J) for the activation energies of V_{max} for glucose uptake in two cultured marine bacteria. In view of these data, it is necessary to control and state carefully the temperature under which activity measurements are made. Given, say, an activation energy of 21,900 cal (91,600 J), a 10°C difference in temperature would account for a fivefold variation in the specific activity index of a natural population by its effect on uptake rate. Clearly, seasonal variations in bacterial specific activity will be a function of temperature as well as other environmental or physiological factors.

MATERIALS AND METHODS

Bacteria were counted with the AODC method of Hobbie et al. (13), which uses Nuclepore filters counterstained with irgalan black. An AO Spencer Fluorestar epifluorescent microscope was fitted with a ruled ocular micrometer, and the bacteria in randomly selected fields were counted until at least 200 were accumulated in the tally. In our hands better results were obtained by increasing the staining time to 30 min and by using an irgalan black-counterstained and oiled (type A; refraction index, 1.51) membrane filter (Millipore Corp.) as backing for the Nuclepore filter.

Bacterial activity was measured with the use of ^{14}C-uniformly labeled organic solutes, employing the kinetic approach of Wright and Hobbie (most recently described in 27) and the tracer approach of Azam and Holm-Hansen (2) to measure turnover rates. Enrichment experiments (direct uptake approach) involved the addition of labeled and unlabeled substrate to natural water incubated in sterile biological oxygen demand bottles, under dark conditions. Plate counts were performed with 0.1-ml spread plates, using the medium of Murcheleno and Brown (18), diluting where necessary with sterile seawater.

RESULTS

V_{max}—kinetic approach. Figure 1 and Table

1 present data relating bacteria numbers and V_{max} specific activity for [^{14}C]glucose total uptake (assimilated plus respired) as measured at a 1-m depth along a transect from well up the Essex estuary (42° 39′ lat., 70° 45′ long.) to a station 14 km offshore in the Gulf of Maine. The transect involves a profound change in marine habitats, from a shallow, salt-marsh-bordered tidal river to offshore waters 46 m in depth. Changes in bacteria numbers and specific activity index suggest that the heterotrophic bacteria are not uniformly active and, in fact, reflect physiological differences that may well correspond with the habitat changes.

Figure 2 and Table 2 present total glucose V_{max} specific activity and bacteria counts from a vertical profile taken at the 14-km-offshore station 9 days after the horizontal series. Again, major changes in specific activity accompany habitat differences, this time vertical location in the water column.

Turnover rate—tracer approach. The simplicity of this approach facilitates the collection of much more data in a given period of time. Our practice has been to add [^{14}C]glutamate at a concentration of 0.3 μg liter^{-1} to a single sample and a blank (to correct for background and isotope adsorption) and measure assimilation only. Figure 3 represents one set of a monthly monitoring series sampling from two fixed points 3.5 km apart in the Essex estuary during a complete flood tide. The salinity of the upriver station (Fig. 3A) at high tide overlaps the salinity of the downriver station (Fig. 3B) at low tide;

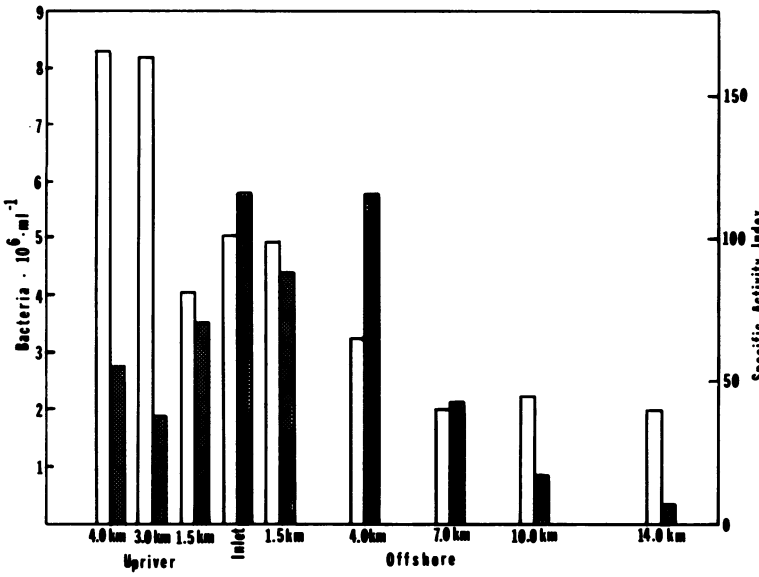

FIG. 1. *Bacteria numbers (clear) and V_{max} specific activity index (10^{-12} micrograms per cell per hour) for glucose uptake (stippled) at 1-m depth along a transect from within the Essex estuary to a station 14 km offshore, 4 August 1976.*

TABLE 1. *Data from horizontal profile from 1-m depth along a transect from within the Essex estuary to a station 14 km offshore, 4 August 1976*[a]

Station location and depth	Salinity (‰)	Temp (°C)	Secchi depth (m)	AODC ($\times 10^6$ ml^{-1})	V_{max} glucose (μg liter^{-1} h^{-1})	V_{max} sp act index (μg cell^{-1} h^{-1} 10^{-12})	r[b]
14 km out; 46 m	33.3	17.0	9	2.02	0.016	8.0	0.90
10 km out; 34 m	33.3	17.2	9.5	2.26	0.040	18	0.86
7 km out; 36 m	32.3	16.9	6.5	2.01	0.22	110	0.90
4 km out; 28 m	32.0	17.3	4.5	3.27	0.16	50	0.99
1.5 km out; 7 m	32.0	17.3	4.5	4.94	0.75	150	0.99
Estuary mouth; 8 m	32.0	18.5	2.5	5.07	1.31	262	0.96
1.5 km in; 7 m	31.6	20.3	1.5	4.05	0.96	240	0.99
3 km in; 3 m	29.8	20.7	2.5	8.21	0.87	160	0.95
4 km in; 2 m	28.0	20.9	1	8.29	0.81	98	0.87

[a] Incubation temperature, 18°C.
[b] Correlation coefficient for linearity of V_{max} calculation.

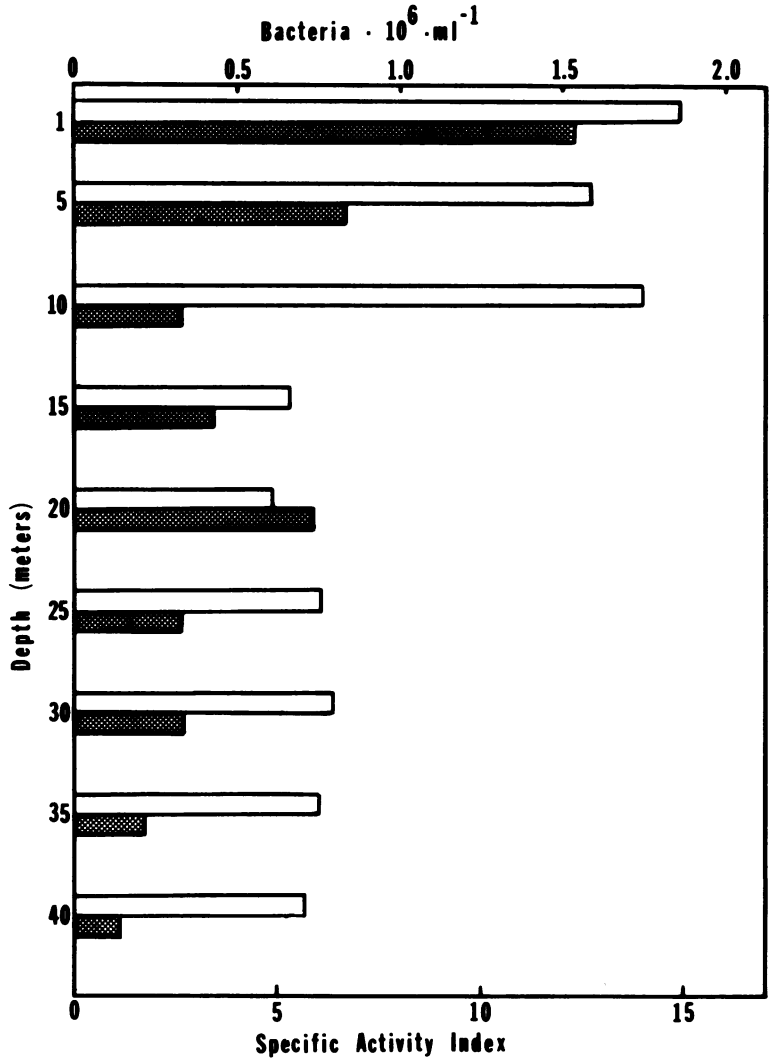

FIG. 2. *Bacteria numbers (clear) and V_{max} specific activity index (10^{-12} micrograms per cell per hour) for glucose uptake (stippled) from a vertical profile at a station 14 km offshore in the Gulf of Maine, 13 August 1976.*

TABLE 2. *Data from a vertical profile at a station 14 km off Cape Ann in the Gulf of Maine, 13 August 1976*[a]

Depth (m)	Salinity (‰)	Temp (°C)	AODC ($\times 10^6$ ml^{-1})	Plate count (CFU ml^{-1})	V_{max} glucose (μg $liter^{-1}$ h^{-1})	V_{max} sp act index (μg $cell^{-1}$ h^{-1} 10^{-12})	r[c]
1	31.9	18.6	1.87	6,120	0.053	28	0.99
5	33.7	16.2	1.59	2,140	0.018	11.3	0.97
10	33.8	14.8	1.75	2,240	0.006	3.4	0.91
15	34.0	10.0	0.66	1,120	0.0038	5.7	0.98
20	34.0	9.4	0.61	540	0.0017	2.8	0.91
25	34.1	9.2	0.76	330	0.0026	3.4	0.88
30	34.1	9.0	0.79	220	0.0027	3.4	0.96
35	34.1	8.8	0.76	170	0.0028	3.7	0.97
40	34.2	8.7	0.72	240	0.0040	5.5	0.91

[a] Incubation temperature for glucose uptake, 13°C.
[b] CFU, Colony-forming units.
[c] Correlation coefficient for linearity of V_{max} calculation.

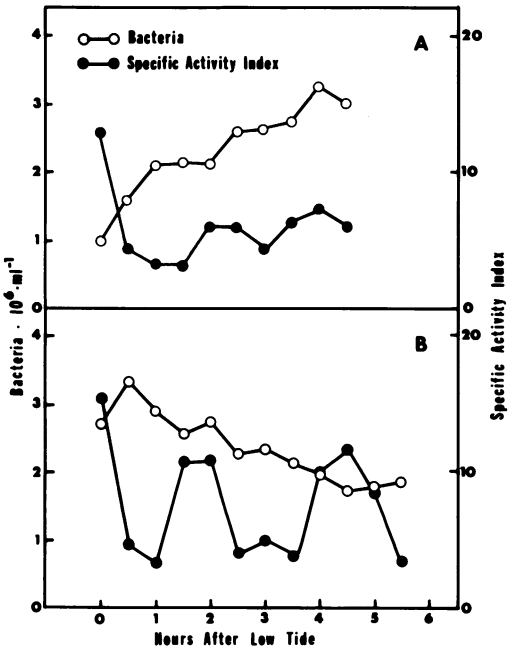

FIG. 3. *Bacteria numbers (clear) and turnover rate specific activity index (10^{-6} per hour per cell liters) for glutamate uptake (solid) of surface waters taken from fixed points in the Essex estuary during flood tide. (A) Data from a station 4 km upriver, 14 November 1977: incubation temp, 5°C; natural temperature range, 5 to 7°C; salinity range, 4.7 to 28.8‰. (B) Data from station 1.5 km upriver, 15 November 1977: incubation temperature, 7°C; natural temperature range, 5 to 8°C; salinity range, 27.6 to 30.0‰.*

other studies of the horizontal tidal range of this river have shown that the low tide-high tide excursion of water from the downriver station does in fact carry it to the upriver station. Figure 3 indicates a midestuary peak in bacteria numbers, with a somewhat erratic pattern of specific activity. Figure 4 shows results from an entirely different kind of estuary, the Merrimack River. It is a much larger river system, heavily polluted, and lacks the extensive salt marshes that characterize the Essex estuary. The data indicate highest specific activity and numbers in the freshwater portion of the estuary, a distinctively different pattern from the Essex, as shown in Fig. 1 and 3. Moving downriver, brackish conditions mark a drastic drop in specific activity, whereas numbers drop only slightly. Another way of using this approach is shown in Fig. 5, where both numbers and specific activity from the same run are fractionated with the use of Nuclepore filters. The polluted freshwater bacteria are much larger in size than those in saline waters, probably explaining some of the great difference in specific activity.

Direct uptake measurement. Figure 6 shows the results of incubating replicate winter samples of Essex estuary water at 2°C (the natural temperature) and 15°C for 48 h, enriched with 100 or 300 μg of glucose liter^{-1}. The pattern in the 2°C sample is a substantial increase in specific activity with little increase in numbers; with the higher incubation temperature, specific activity increases more rapidly and then declines due to exhaustion of labeled substrate, whereas numbers increase fourfold.

In another enrichment experiment, several concentrations of ^{14}C-labeled glucose were used over 48 h with a midsummer water sample from the inlet of Essex Bay. Results (Table 3) indicate the following: (i) a threefold increase in direct counts; (ii) exponential increase in the use of glucose, with an increase in specific activity of two orders of magnitude; (iii) no significant influence of varying glucose concentration on direct counts; (iv) "viable count" increases that indicate definite dependence on glucose concentration as well as a pattern of exponential increase. These data, and those of Fig. 5, clearly show pronounced increases in specific activity (and "viability") without large increases in numbers of bacteria. The higher values for activity index from the summer sample enriched with glucose are similar in magnitude to V_{max}-per-cell values reported by Hamilton et al. (12) for several strains of chemostat-isolated and -grown marine bacteria. It seems likely that towards the end of the incubation time the enriched population was largely made up of active cells at close to maximum metabolic activity for the temperature conditions.

DISCUSSION

The main purpose of the data presented is to indicate the usefulness of looking at heterotrophic activity with a quantitative convention such as the specific activity index. There are some problems with this approach: sizes of bacteria vary, temperature differences pose problems of comparison, and three different indexes are offered which are not interconvertible. Yet the data show substantial variations in bacterial activity, variations which indicate some interesting patterns. For example, one of the most intriguing questions about the coastal bacteria is: are they largely a population that emerges from estuarine inlets and gradually dies out as it is mixed with increasingly nutrient-poor water, or are they an indigenous population directly reflecting the nutrient quality of the water in which they are entrained? For that matter, are

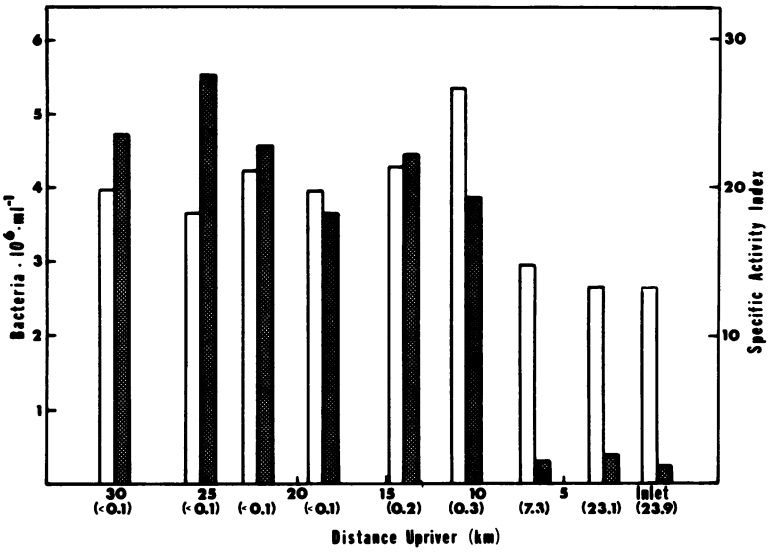

FIG. 4. *Bacteria numbers (clear) and turnover rate specific activity index (10^{-6} per hour per cell liters) for glutamate uptake (stippled) from the Merrimack River estuary, 1-m depth, 21 November 1977. Values in parentheses: salinity in parts per thousand. Temperature range, 6.2 to 8°C; incubation temperature, 7°C.*

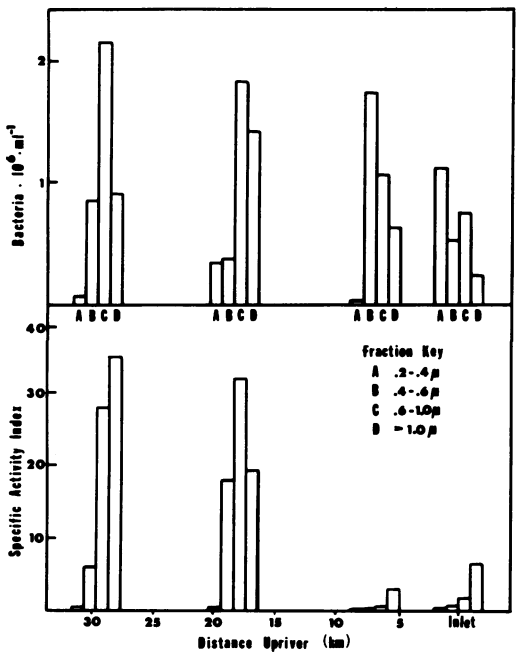

FIG. 5. *Bacteria numbers and turnover rate specific activity index (10^{-6} per hour per cell liters) from several stations along the Merrimack River estuary. Numbers and heterotrophic activity were fractioned using Nuclepore filters of different pore sizes. Environmental data and stations as for Fig. 4.*

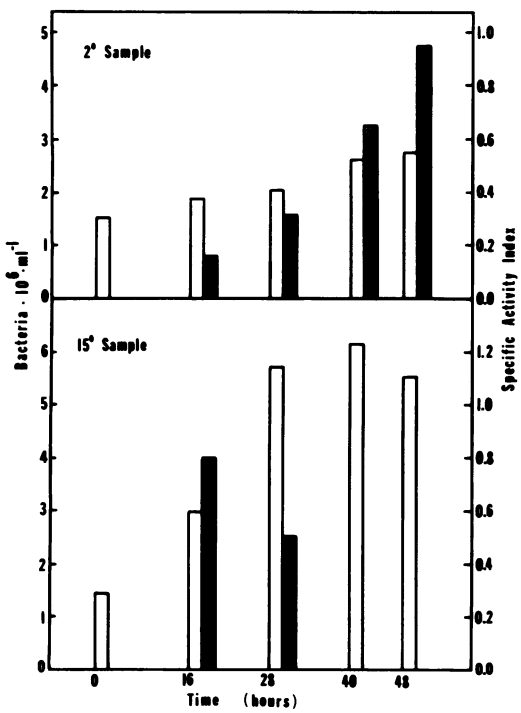

FIG. 6. *Bacteria numbers (clear) and direct uptake specific activity index (10^{-12} micrograms per cell per hour) for glucose uptake (stippled) in Essex estuary water enriched with 100 μg (2°C incubation) or 300 μg (15°C incubation) of glucose liter^{-1}.*

the estuarine bacteria largely the result of admixture from the salt marsh and estuarine sediments?

Two recent papers by Novitsky and Morita (19, 20) throw some light on these questions. The authors describe the transition of a laboratory population of a *Vibrio* species during star-

vation and subsequent reintroduction into nutrient-rich culture. Both size and endogenous respiration are greatly reduced during starvation but are restored when nutrients are once again provided. From the present study, Fig. 6 and Table 3 show a pronounced increase in specific activity by the natural bacteria in response to enrichment. In some enrichment experiments, there has also been a measurable shift from predominantly coccoid to larger rod-shaped bacteria during the incubation. Figures 1 and 2 suggest the reverse process—as bacteria encounter changes in the nutrient content of marine waters, moving from richer to poorer nutrient environments, heterotrophic activity per cell declines rapidly. It appears, then, that although bacteria numbers may be related in general to the nutrient quality of the water (or, more precisely, the water in which they originate), specific activity is a better indicator of the physiological state and metabolic role of the bacteria in direct response to organic nutrients. For example, Fig. 4 and 5 show a highly active pollution-related population of large freshwater bacteria which, when diluted with seawater, changes or disappears and is replaced with a much-lower-activity marine population dominated by the smaller cells (0.2 to 0.6 μm). In the transition, total numbers do not change very much.

The specific activity index does not, of course, distinguish between active and inactive bacteria. As yet there are no unequivocal methods for accomplishing this. A few studies using labeled organic solutes and autoradiography have shown a range of percent active bacteria usually over 30% and as high as 100% (6, 11). These measurements have used unnaturally high concentrations of substrate and hence are difficult to interpret. Other methods for detecting the "active" bacteria have been suggested based on autoradiography with ^{33}P (7) or on the presence of a functioning electron transport system (16). These methods also indicate a variable but high proportion of active bacteria, by comparison with direct counts. The problem with all of these methods is, of course, quantification. How active are the active bacteria?

The data presented in this paper support the hypothesis expressed by several workers (20, 23a) that the marine bacteria are adapted to conditions of nutrient starvation by becoming relatively inactive, or "dormant," existing for perhaps weeks or months in a reversible physiological condition that reflects the availability of organic nutrients. More measurements of specific activity, perhaps in conjunction with autoradiographic data, should help to explain how important and widespread this phenomenon is.

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I am grateful to Rebecca Buck for technical assistance in many phases of this work.

TABLE 3. *Enrichment of seawater with varying concentrations of labeled glucose, Essex inlet, 9 August 1976*[a]

Sample	AODC ($\times 10^6$ ml^{-1})	Plate count (CFU ml^{-1} 10^{-4})[b]	Cumulative amt used (μg liter^{-1})	Mean 12-h rate (μg liter^{-1} h^{-1})	Direct uptake sp act index (μg cell^{-1} h^{-1} 10^{-12})
Control (no added substrate)					
0 h	2.78	1.33			
12 h	2.94				
24 h	3.26	5.20			
36 h	6.14				
48 h	9.31	21.1			
Glucose, 250 μg/liter added					
0 h	2.78	1.33			
12 h	3.06		1.5	0.13	42.4
24 h	3.72	6.26	15.8	1.2	323
36 h	6.29		239	18.6	2,957
48 h	9.44	32.6	243	0.40	42
					.4
Glucose, 500 μg/liter added					
0 h	2.78	1.33			
12 h	2.66		2.1	0.18	67.7
24 h	3.25	4.84	18.1	1.5	462
36 h	6.00		505	40.6	6,767
48 h	8.82	43.0	434		
Glucose, 1,000 μg/liter added					
0 h	2.78	1.33			
12 h	2.81		3.8	0.31	110
24 h	3.66	6.90	26.3	1.9	5.9
36 h	7.29		716	57.4	7,874
48 h	9.87	69.2	956	20.1	2,036

[a] Salinity, 32.1‰; incubation temperature, 17°C.
[b] CFU, Colony-forming units.

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