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Seasonal Variation in Population Density and Heterotrophic Activity of Attached and Free-Living Bacteria in Coastal Waters

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The abundance and heterotrophic activity of attached and free-living bacteria were examined seasonally in coastal water. Heterotrophic activity was determined by the uptake of [^{14}C]glucose. The density of attached bacteria was always minor, not showing a seasonal variation, whereas the free-living bacteria were more numerous and showed a marked seasonal variation, their density being higher under warmer conditions. The contribution of the attached bacteria to the total assimilation of [^{14}C]glucose (from 10 to 38%) was lower than that of the free-living bacteria, neither of them showing a seasonal variation. On a cellular basis, attached bacteria were more active, since they assimilated more [^{14}C]glucose and showed, under warmer conditions, a higher cellular volume (0.102 versus 0.047 μm^3). We consider that the factors responsible for these observations were the amount and quality of the particulate material, the different availability of organic matter for the two types of bacteria, and in a fundamental way, the variation in water temperature.

The importance of attached bacteria in aquatic systems, such as lakes, rivers, estuaries, and the ocean, has been the subject of much discussion. Various investigations regarding both the number and the activity of these microorganisms have produced differing results.

Reports as to the numerical percentage represented by attached bacteria show it to vary from less than 1% in the North Atlantic Ocean (23) to as much as 98% in the Humber Estuary, England (16), and many values can be found within these extremes (Table 1). The contribution made by bacteria associated with particulate matter to the total bacterial incorporation of dissolved organic carbon also varies greatly with different aquatic environments (Table 2). In spite of this uncertainty regarding the magnitude of contributions in number and activity of attached bacteria, investigators agree that on a cellular basis, attached bacteria are metabolically more active than free-living bacteria (19, 30).

In contrast to the abundant, and at times contradictory, information on the relative importance of attached bacteria in different aquatic systems, very few workers have studied the seasonal variation of the number and activity of attached and free-living bacteria (3, 29, 35). Moreover, the results obtained are sometimes contradictory (16, 43). We think that more information about seasonal variation of planktonic bacteria is needed. The objective of this study is to analyze the results of an investigation into seasonal variation in population density and heterotrophic activity of free-living and attached planktonic bacteria in coastal waters. The seasonal variation of other factors which might influence density and heterotrophic activity of bacteria, such as temperature, chlorophyll *a* (Chl*a*) phaeophytin, number of particles, and particulate organic carbon, were also examined.

MATERIALS AND METHODS

Sampling and physicochemical determinations. Water samples were taken at a station 500 m off the coast of Bilbao, Spain. A total of 17 water samples were collected, weekly and biweekly, during the period from 4 March 1985 to 3 September 1985. Samples were taken from a depth of approximately 0.5 m in polypropylene bottles cleaned with diluted acid and processed in the laboratory within 2 h of sampling. Subsamples of water were preserved in formaldehyde (final concentration, 2%) for microscopical observations. Water temperature was measured with a simple mercury thermometer. Chl*a* and phaeophytin were determined spectrophotometrically after acetone extraction with glass fiber filters (39). Particulate organic carbon (POC) was measured by the dichromate oxidation method of Strickland and Parsons (39). Detrital carbon was calculated by the method of Kirchman (29) by subtracting the phytoplankton carbon from POC concentrations; the phytoplankton carbon was estimated by multiplying Chl*a* concentrations by 50 (11).

Bacterial abundance and cell volumes. Bacterial abundance was measured by the acridine orange direct count method (22). Two 2.0-ml portions were stained with acridine orange (final concentration, 0.01% [wt/vol]) for 2 to 3 min and then filtered onto black-stained 0.2- and 3.0-μm-pore-size filters. The filters were previously stained with an irgalan black solution, 0.2% (wt/vol) in 2% acetic acid, for 24 h and rinsed in distilled water immediately before use. The 0.2-μm-pore-size filter was used to count free-living bacteria, and the 3.0-μm-pore-size filter was used to count particles and bacteria attached to particles. Approximately 200 attached and 500 free-living bacteria were counted in each sample. Cell volumes were estimated by measuring the shortest and longest axes of the bacteria. The bacteria were classified as either spheres (for cocci) or cylinders (for rods). Fuhrman (15) determined that cell volumes are best determined from epifluorescence preparations.

Bacterial activity. We measured the relative heterotrophic activity of free-living and attached bacteria by the size fractionation of glucose uptake. Three subsamples were analyzed for each uptake measurement. In this study, the compound used was [U-^{14}C]glucose (250 mCi · mmol^{-1}, Radiochemical Centre, Amersham, England) at a concentration of 60 μg · liter^{-1}. This concentration was chosen as a result of a previous study (25) in which we used the kinetic

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TABLE 1. Percent of total bacterial counts attached to particles: summary of previous reports

Location	% Attached bacteria	Reference
Oceans		
Pacific	20–50	26
Pacific	33–50	38
North Atlantic	<1	23
Coastal North Atlantic	Very few	41
Coastal North Atlantic	15 (numbers)	14
Coastal North Atlantic	45 (biomass)	14
Off Long Island, N.Y.	Very few	13
Off Bilbao, Spain	1–22	Present study
Estuaries and salt marshes		
Newport River Estuary, N.C.	1–7	34
Estuary surface	Very few	7
Humber Estuary, England	56–94 (summer)	16
Humber Estuary, England	94–98 (winter)	16
Kiel Fjord, Federal Republic of Germany	2.9–8.1	46
Salt marsh, S.C.	18–75 (summer)	43
Salt marsh, S.C.	13–31 (winter)	43
Salt marsh, Palo Alto, Calif.	20–90	19
Freshwaters		
Bay of Quinte, Ontario, Canada	17.5	8
Lake Tanning, Denmark	73	37
Coastal ponds, northeast United States	<10	30
Lake Mendota, Wis.	<1–30	35
Freshwater pond	<10	29

approach method (44, 45). At this concentration, the bacteria are the principal organisms responsible for the substrate uptake (44, 45). The subsample volume was 25 ml, and the volume of the incubation flasks was 120 ml. The subsamples were incubated and shaken in the dark (to minimize uptake of respired $^{14}CO_2$) at the in situ temperature (± 1°C) for 1 h. After this incubation period, 0.2 ml of H_2SO_4 (2 N) was injected into the flasks, and the subsamples were further incubated for 1 h to trap the $^{14}CO_2$ released by using a filter paper impregnated with β-phenetilamine (21). After both incubation periods, the entire volume of each replicate was sequentially filtered through 3.0- and 0.2-μm-pore-size filters (Nuclepore Corp., Pleasanton, Calif.). The filters were rinsed three times with 25 ml of filtered seawater (0.2-μm-pore-size membrane filters), placed in Unisolve-1 (Hispanoland, Barcelona, Spain), and radioassayed by liquid scintillation counting. Quench curves were computed with the channel ratio method. Controls for abiotic adsorption were prepared in a similar manner, except that Lugol solution (Carlo Erba, Milan, Italy) was added.

RESULTS

The water temperature in the system studied does not follow a characteristic spring-summer-autumn-winter cycle. This fact has been analyzed in another study (25). The characteristic evolution of the temperature of this system results in the fact that two clearly different situations can be distinguished, i.e., a cold situation and a warm situation. This characteristic evolution of the temperature will be used throughout this study to discuss the results arising from cold and warm situations.

The results concerning the characterization of the two situations, such as temperature and concentrations of Chl*a*, phaeophytin, and POC, are shown in Table 3. The cold situation is characterized by low temperatures, with an average value of 12.3°C. Between May and June, there is an increase of 8.5°C, thus leading to the beginning of the warm situation, with an average temperature of 19.8°C. In the warm situation, the average concentrations of Chl*a*, phaeophytin, and POC are greater than in the cold situation, although the differences in these average values are not statistically significant (in all cases, $P > 0.05$, Student's *t* test).

Bacterial abundance. The results concerning bacterial abundance are shown in Fig. 1. The number of attached bacteria was less than the number of free-living bacteria. Attached bacteria represented between 1 and 22% of the total population. The number of free-living bacteria showed a clear seasonal variation, being less in the cold situation (average value, 3.2×10^5 cells · ml^{-1}) than in the warm situation (average value, 12.8×10^5 cells · ml^{-1}). The average values are significantly different ($P < 0.001$, Student's *t* test). In contrast, the number of attached bacteria did not show a seasonal variation but fluctuated irregularly throughout the period; the average value for the total study period is 4.9×10^4 cells · ml^{-1}.

Results concerning the number of particles and related parameters are shown in Table 4. The number of particles varied between 7.9×10^3 and 39.5×10^3 particles · ml^{-1}, being somewhat higher in the warm situation (average value, 22.4×10^3 particles · ml^{-1}) than in the cold situation (average value, 15.4×10^3 particles · ml^{-1}), although the difference is not statistically significant. In particles on which bacteria were observed, the average number of bacteria fluctuated between 2.9 and 6.4 bacteria per particle. This average number of bacteria per colonized particle did not show a seasonal variation, and the average values were very similar (4.3 and 4.6 for the cold and warm situations, respectively). However, a seasonal variation was observed in the proportion of particles with attached bacteria. The average value in the cold situation (68.1%) was higher than that in the warm situation (52.0%), and this difference is

TABLE 2. Activity of attached and free-living bacteria: summary of previous reports

Location	Compound	% Uptake of compound by fraction: <3.0 μm	% Uptake of compound by fraction: >3.0 μm	Reference(s)
Oceans				
Mediterranean	Amino acids	62	38	42
Mediterranean	Glucose	72	28	42
Northeast Atlantic	Amino acids	53	47	42
Northeast Atlantic	Glucose	76	24	42
Gulf of California	Glucose	>90	<10	4
Gulf of California	Glucose	>90	<10	1
Off northwest Africa	Acetate	>90	<10	1
Saanich Inlet, British Columbia, Canada	Serine	>90	<10	1
Gulf of Marseilles, France	Glucose	55–95	5–45	10
Gulf of Marseilles, France	Protein hydrolysate	32–100	0–68	10
Kiel Bight	Glucose	>90	<10	24
Gulf Stream	Glucose	80	20	18
Off Long Island, N.Y.	Glucose	74[a]	26[b]	13
Off Bilbao, Spain	Glucose	62–90	10–38	Present study
Estuaries and salt marshes				
Newport River Estuary, N.C.	Amino acids	75–97	3–25	34
Humber Estuary, England	Glucose	1–44	56–99[c]	3, 16
Salt marsh estuary, Sapelo Island, Ga.	Glucose	20	80	18
Salt marsh estuary, Sapelo Island, Ga.	Glucose		Most[d]	17
Salt marsh, Palo Alto, Calif.	Tetrazolium salts	1–37	63–99[e]	19
Freshwaters				
Bay of Quinte, Ontario, Canada	Glucose	47[f]	17[g]	8
Clear Lake, Calif.	Glucose	72	28	32
Four New Zealand lakes	Glucose	61–89	11–39	32
Lake Kinneret, Israel	Glucose	>90	<10	5
Mirror Lake, N.H.	Glucose	70–87	13–30	27
Frome and Hook rivers, England	Glucose		50–72[h]	9
Four Danish lakes	Glucose	27–93[i]	7–73[f]	37
Lake Tahoe, Calif.	Glucose, acetate, glycine	67–88	12–33	33
Lake Rotongaio, New Zealand	Glucose, acetate, glycine	58–85	15–42	33
Five ponds and two marshes, Mass.	Glucose	26–100	0–74	30
Five ponds and two marshes, Mass.	Glutamate	77–91	9–23	30
Lake Mendota, Wis.	Acetate	10–85	15–90	35
Lake Mendota, Wis.	Sulfate	20–100	0–80	35
Freshwater pond	Glucose	57–96	4–43	29
Freshwater pond	Glutamate	82–96	4–18	29
Freshwater pond	Acetate	89–97	3–11	29

[a] Fraction, <2.0 μm.
[b] Fraction, >2.0 μm.
[c] Calculated by dividing the >3.0-μm-fraction V_{max} of $^{14}CO_2$ remineralization by the unfractionated V_{max}.
[d] Fraction, >10 μm.
[e] Percent of all the respiring cells which were attached to particles.
[f] Fraction, >1.0 μm.
[g] Fraction, >5.0 μm.
[h] Percent [^{3}H]glucose uptake by a >8.0-μm fraction.
[i] Fraction, <1.0 μm.

statistically significant ($P < 0.01$). Also we found a direct correlation between the number of particles and the number of attached bacteria ($P < 0.001$; Spearman coefficient of rank correlation, $r = 0.87$). Finally, with respect to detrital carbon per particle (Table 4) this parameter did not show a seasonal variation.

Bacterial activity. The absolute incorporation values (assimilation plus respiration) of [^{14}C]glucose by the bacterial population cannot be analyzed, as we did not measure the natural substrate concentration. We have analyzed the relative assimilation and respiration values of the population over a period of time (Table 5). The relative assimilation of the population, and consequently, the respiration did not show a seasonal variation but fluctuated irregularly in the two situations. The range of values is wide, from 41.8 to 83.2%, in the case of assimilation. The average percentages

TABLE 3. Parameters concerning the characterization of the cold and warm situations[a]

Situation (date)	No. of samples	Temp (°C)	Chl*a* (μg · liter^{-1})	Phaeophytin (μg · liter^{-1})	POC (μg · liter^{-1})
Cold (3/4/85–5/16/85)	8	12.3 ± 1.1	1.35 ± 0.76	1.11 ± 0.60	369.7 ± 202.5
Warm (6/6/85–9/3/85)	9	19.8 ± 1.3	2.16 ± 0.63	1.71 ± 0.96	485.1 ± 120.8

[a] Each value is the arithmetic mean ± the standard deviation.

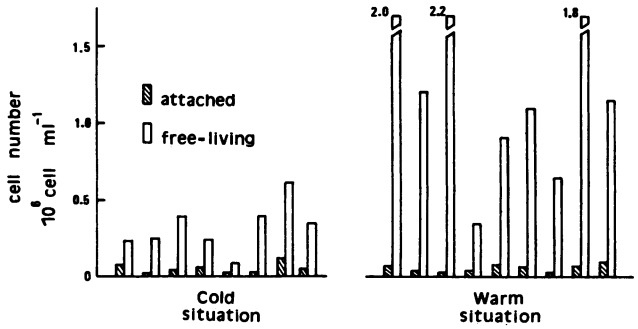

FIG. 1. Attached and free-living bacterial abundance in the cold and warm situations.

of assimilation and respiration for the total study period are 68.3 and 31.7%, respectively.

The results of the relative assimilations of attached and free-living fractions are shown in Table 5. The contribution of attached bacteria to the assimilation of [^{14}C]glucose was minor in all cases. Attached bacteria assimilated between 10 and 38% of the total amount assimilated by the population. On the other hand, we did not find seasonal variations in relative assimilations of the attached fraction (retained by a 3.0-μm-pore-size filter) or free-living fraction (which passed through a 3.0-μm-pore-size filter); the average values for the cold and warm situations are not significantly different ($P > 0.05$ in all cases).

Metabolic state of attached and free-living bacteria. The metabolic states of the attached and free-living bacteria were evaluated by dividing the value of [^{14}C]glucose assimilated by each fraction between the number of attached and free-living bacteria. The values obtained cannot be analyzed in their absolute form, as the natural substrate concentration was not measured, but values for the two types of bacteria in the two situations can be comparatively analyzed. We assume, like Kirchman and Mitchell (30), that the specific activity of the radiolabeled compound was the same in the microenvironments around attached and free-living bacterial cells.

The metabolic state or activity per cell was clearly different in attached and free-living bacteria. Assimilation by attached bacteria was always (with only one exception) greater than that by free-living bacteria (Fig. 2). The ratio of attached to free-living bacterial assimilation of [^{14}C]glucose per cell was significantly greater than 1 ($P < 0.01$, Student's t test). Moreover, we found a seasonal variation in the ratio of attached bacteria-to-free-living-bacteria assimilation of [^{14}C]glucose per cell. The average value was significantly greater in the warm situation than in the cold situation, with mean values of 1.88 for the cold and 7.43 for the warm situations ($P < 0.01$, Student's t test).

Cell volume is also a relative index of metabolic activity. We did not measure the cell volume in all the samples, but we did measure a high number of attached and free-living cells in each of the two situations. In the cold situation, 609 free-living and 299 attached bacteria were measured; the mean cell volumes were 0.0403 μm^3 for free-living and 0.0417 μm^3 for attached bacteria. In the warm situation, 688 free-living and 344 attached bacteria were measured; the mean cell volumes were 0.0472 μm^3 for free-living and 0.1017 μm^3 for attached bacteria. In all cases, the majority of cells showed the cocci shape. Owing to measuring techniques, in all cases the variation coefficients were very high (as much as 96%). Our results (they may not be representative) indicate that attached bacteria are not always larger than free-living bacteria but that there is a clear difference, depending on the system conditions. The biovolumes of free-living and attached bacteria were very similar in the cold situation, while in the warm situation the biovolume of free-living bacteria remained the same and that of attached bacteria was multiplied by a factor somewhat higher than 2. This increase in the biovolume of attached bacteria is statistically significant ($P < 0.01$).

DISCUSSION

The results we obtained indicate that attached bacteria were always less numerous than free-living bacteria and that their contribution to the assimilation of an organic compound was also less. However, on a cellular basis, attached bacteria were metabolically more active than were free-living bacteria. On the other hand, our results also reveal the existence of very important differences between the cold and warm situations. Whereas the number of attached bacteria did not vary from one situation to another, the number of free-living bacteria was much higher in the warm situation. Also, whereas the assimilation per attached bacterium increased from the cold to the warm situation, the assimilation per free-living bacterium decreased.

The majority of previous studies on attached and free-living bacteria have focused on the comparative analysis of very different ecological systems or habitats. This has led to the identification of several regulating factors of greater or lesser importance for the attachment phenomenon. The more important factors are the number and quality of particles, the concentration of dissolved organic matter, and grazing by consumers. We think that a seasonal study would allow us to introduce another factor, water temperature, which is especially important, at least in some systems, and which should always be taken into account. The relative importance of these factors with reference to the results that we found will be discussed below.

In our case, the number of particles is one of the most important determining factors in regulating the number of attached bacteria. Although neither of the two parameters (particle numbers and attached bacteria) showed a clear seasonal variation, there was a close correlation between them ($P < 0.001$; Spearman coefficient of rank correlation, $r = 0.87$). Although Bent and Goulder (3) found a seasonal variation in the number of attached bacteria that we did not find, they nevertheless found that the determining factor, perhaps the main one, for attachment in the Humber Estary,

TABLE 4. Parameters concerning the particulate material in the cold and warm situations[a]

Situation (date)	No. of samples	No. of particles (10^3/ml)	Avg no. of cells/particle	% Particles with cells	Detrital carbon/particle (μg of C · particle^{-1})
Cold (3/4/85–5/16/85)	8	15.4 ± 10.4	4.30 ± 0.97	68.1 ± 11.4	0.031 ± 0.022
Warm (6/6/85–9/3/85)	9	22.4 ± 8.3	4.56 ± 0.78	52.0 ± 6.7	0.024 ± 0.015

[a] Each value is the arithmetic mean ± the standard deviation.

TABLE 5. [^{14}C]glucose uptake (assimilation and respiration) for the total population and contribution of the attached and free-living bacterial fractions to the total assimilation in the cold and warm situations

Situation (date)	No. of samples	% ± SD of [^{14}C]glucose:			
		Uptake		Assimilation	
		Assimilation	Respiration	Attached fraction	Free-living fraction
Cold (3/4/85–5/16/85)	8	71.2 ± 7.5	28.8 ± 7.5	18.2 ± 6.6	81.8 ± 6.6
Warm (6/6/85–9/3/85)	9	65.7 ± 11.2	34.3 ± 11.2	23.1 ± 8.0	76.9 ± 8.0

England, is the number of particles. With respect to the quality of the particles as a regulating factor for the attachment phenomenon, Pedrós-Alió and Brock (35) indicated that attachment can be useful because organic matter adsorbs to particles, creating a local accumulation of possible nutrients from which cells can benefit (2, 31, 40). Also, particles colonized by bacteria are frequently organic in appearance rather than inorganic (33, 41). Our results in this respect indicate that detrital carbon per particle was lower in the warm situation than in the cold one (mean values), and this could explain why there is not a greater proportion of particles with attached bacteria during the warm situation than during the cold situation. In this way, as the quality of particles in the warm situation is not greater, this means that the number of particles would be the main regulating factor if we were to take only these two factors into account.

However, there is a phenomenon in our system which we have not observed in previous reports on this subject and which is difficult to explain if we consider only the two regulating factors mentioned above, i.e., the number and quality of the particles. This phenomenon is the very important numerical increase in free-living bacteria that is produced in passing from the cold to the warm situation, an increase that is not accompanied by an increase in the activity of the free-living bacterial fraction. To understand this phenomenon and its implications regarding the free-living–attached bacteria relationship in this system, we think that there are two very important regulating factors that should be taken into account, i.e., the concentration of dissolved organic matter and the water temperature.

We did not quantify the dissolved organic matter in a direct manner, although an approximation could be reached by Chl*a* and phaeophytin values, given the scarce or null external contributions to this system. These results indicate that appreciable differences between the cold and warm situations did not exist, or at least there was only a slightly greater availability in the warm situation. With regard to the water temperature, the differences are considerable and clearly distinguish the two situations. The influence of temperature on heterotrophic bacterial activity is well known. Moreover, in this system and in others, a clear exponential relation of the Arrhenius model type between temperature and heterotrophic bacterial activity (6, 25) has been established. We think that the effect of temperature in our study should be understood as a trigger effect of bacterial activity. This means that in the cold situation, the low temperatures impede a high degree of heterotrophic activity both of attached bacteria, with a greater availability of nutrients, and of free-living bacteria, with less availability of nutrients. In contrast, in the warm situation, the high temperatures cause the changes that are produced both in attached and free-living bacteria. If this hypothesis is true, we should immediately think of different strategies for attached and free-living bacteria found in the warm situation. Our results show that the free-living bacteria reacted to warm conditions by increasing in number but not in cellular volume, while attached bacteria reacted basically by increasing their cellular volume but only slightly increasing their number. These different strategies would result mainly from the different nutritive resources of the attached and free-living bacteria in the warm situation. The undoubtedly greater availability of organic matter to attached bacteria would cause their increase in volume, while the lesser availability of nutrients to free-living bacteria would lead to a faster rate of reproduction, even at the cost of maintaining a lower cellular level of metabolic activity than they do in the cold situation. This means that the energy increase resulting from the higher temperatures in the warmer situation is used differently, i.e., an increase in cellular activity or an increase in the number of cells, depending on the vital strategy of the attached or free-living bacteria, respectively. However, we need more experience to confirm these ideas.

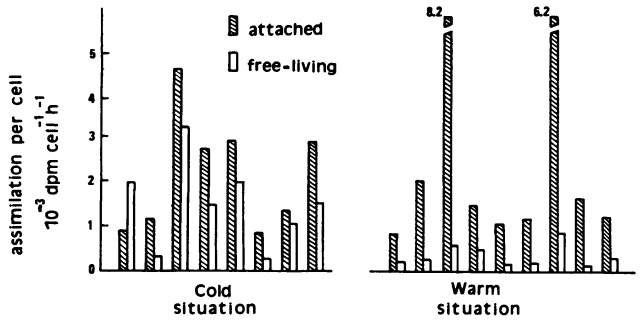

FIG. 2. Assimilation per cell for attached and free-living bacteria in the cold and warm situations.

On the other hand, we find it difficult not to consider a numerical increase of attached bacteria even if it were not as large as that of the free-living bacteria. In this respect, we must consider that our results could underestimate the number of attached bacteria in the warm situation, inasmuch as the division of an attached bacterium could give rise to one attached and another free-living cell. In this sense, we should not forget the experiments of Helmstetter and Cummings (20) to obtain synchronous cultures from cells which split apart from those that are attached to a solid substrate. If this is true in our case, then it should be taken into account that a fraction of the free-living bacteria found in the warm situation originated from the reporduction of attached bacteria.

Finally, we should not forget the grazing pressure by the consumers as a regulating factor of the importance of attached bacteria in an aquatic system. This study has not quantified the importance of grazing, although it seems clear that bacteria attached to particles experience a much greater

grazing pressure than do free-living bacteria (12, 38), even though some zooplankton can feed on free-living bacteria (28, 36). It should also be taken into account that the importance of zooplankton feeding will vary seasonally within a system, depending on the abundance and species composition of zooplankton.

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