

## Effects of Inorganic Particles on Metabolism by a Periphytic Marine Bacterium

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Measurements were made of adsorption of a periphytic marine bacterium, glucose, and glutamic acid to inorganic particles in seawater and defined bacterial growth medium. Measurements of the metabolism of bacteria were made in the presence and absence of particles by microcalorimetry and radiorespirometry. It was found that hydroxyapatite adsorbs glutamic acid, but not glucose, from the experimental medium. It was also found that hydroxyapatite adsorbs essentially all of the bacteria from the medium when the bacterial concentration is approximately  $6 \times 10^7$  bacteria per ml. If the bacterial concentration is approximately  $6 \times 10^7$ , then only a small fraction of cells become attached. It was therefore possible to select bacterial concentrations and organic nutrients so that bacterial attachment, organic nutrient adsorption, or both would occur in different experiments. In this experimental system the metabolism by attached and nonattached bacteria of adsorbing and nonadsorbing organic nutrients was measured. The results show that bacterial activity in this model system was not enhanced by the particles, regardless of whether the bacteria, the organic nutrient, or both were associated with the surface. In fact, the respiratory activity of the attached bacteria was diminished in comparison with that of free bacteria.

Bacteria attach to solid surfaces in aquatic environments (5, 6, 28, 31, 32) and in laboratory culture (9, 10, 14, 23). Little is known about the activity and growth of bacteria on surfaces in natural waters or in laboratory culture.

There is some evidence that, in nutrient-poor growth media, inorganic surfaces at surface area/volume ratios of 2 to 200 cm<sup>2</sup>/ml enhance bacterial growth and respiration (20, 34, 36). There are several potential interactions which may explain these results. The most commonly accepted explanation is that the organic nutrients used by the bacteria are adsorbed from the solution onto the surface and are thereby concentrated. Due to this increased concentration, the nutrients are thought to be more easily assimilated by the bacteria (7, 8, 22, 30, 36). Other explanations consistent with the experimental data are that the bacteria are more efficient at scavenging nutrients from solution or in their metabolic processes when they are attached to a surface. This increased entrapment efficiency may be due to secretion of extracellular polymers for attachment which also serve to entrap nutrients (11, 26). Still another explanation, hypothesized by Kriss (25), is that refractory organic material in seawater is rendered biodegradable by interaction with the surface. It is important to know more about this surface

effect if one wishes to understand the factors controlling microbial activity at interfaces in natural waters.

There is currently a variance of opinion concerning the relative importance of attached and free-living bacteria to heterotrophic processes in aquatic environments. Some studies carried out in the field have concluded that the activity of attached bacteria constitutes the majority of heterotrophic activity (17, 24), whereas others conclude that free-living bacteria account for the majority of this process (1, 21). In regard to the specific activity of attached bacteria, some data indicate higher levels for attached bacteria (21), whereas other data show reduced activity of attached cells in comparison with that of free-living cells (2). Studies carried out in situ are difficult to interpret because of the presence of a multitude of organic nutrients and a lack of knowledge about what factors are controlling microbial activity. In addition, the presence of both organic and inorganic particles complicates the picture.

In the present study we have tested the supposition that adsorption of organic nutrients or bacteria (or both) to an inorganic surface results in enhanced metabolic activity by the bacteria in medium with low organic nutrient concentrations.

## MATERIALS AND METHODS

**Bacterial strain.** A bacterial strain was selected which attached to surfaces exposed to seawater, grew easily in laboratory culture media, and could utilize a variety of organic substrates. A collection of periphytic marine bacteria isolated from metal and glass surfaces exposed to Biscayne Bay seawater was available (12). A bacterial strain was selected from among these isolates on the basis of being representative of a large group of isolates depicted by a computer-generated dendrogram. The organism was identified on the basis of API 20E (Analytical Profile Index; Analytab Products, Plainview, N.J.) as *Vibrio alginolyticus*.

The culture was maintained on tryptic soy agar slants. The stock culture was routinely tested for purity by streaking on tryptic soy agar plates and was periodically tested on API 20E to ascertain that no detectable biochemical changes had occurred. The characteristics of the strain never exhibited any variation as determined by API 20E.

**Minimal salts medium (M9).** Buffer solution was prepared by dissolving dibasic sodium phosphate (0.05 M), monobasic potassium phosphate (0.02 M), ammonium chloride (0.02 M), and sodium chloride (0.09 M) in distilled water. This solution was sterilized by autoclaving. After cooling, 10 ml of a 4% (wt/vol) solution of magnesium sulfate per liter and 2.0 ml of a 1% (wt/vol) calcium chloride solution per liter were added aseptically. These solutions had been sterilized separately by autoclaving. The final concentrations were 0.002 M magnesium sulfate and 0.0002 M calcium chloride. The organic nutrient was dissolved in deionized water, filter sterilized (0.1  $\mu$ m filter; Gelman Sciences, Inc., Ann Arbor, Mich.), and added aseptically to make the desired concentration.

**Seawater.** Seawater for adsorption experiments was prepared by filtering Gulfstream seawater (0.45- $\mu$ m filter; Millipore Corp., Bedford, Mass.) followed by UV irradiation in a 4-liter reactor with a 450-W mercury vapor lamp (Ace Glass) for 24 h. The solution was filter sterilized (0.1- $\mu$ m filter; Gelman).

**Particles.** Hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.) was prepared by rinsing the particles in distilled water and decanting the fine particles until the supernatant was clear after a settling time of 10 min. The particles were sterilized by autoclaving.

**Bacterial suspension.** Bacteria were grown in liquid medium for 18 h (ca. 9 h in the stationary phase) and harvested by centrifugation at  $1,200 \times g$  for 20 to 30 min. The cells were washed twice in liquid medium without organic nutrient and suspended to the desired final absorbance measured in a Bausch & Lomb Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) at 520 nm.

**Organic nutrients.** Organic nutrients were prepared in millimolar concentration ranges in distilled water and then diluted volumetrically 1:1,000 in medium by using a 100- $\mu$ l Oxford pipette and a 100-ml volumetric flask. This procedure yielded organic nutrients at micromolar concentrations which were then filter sterilized.

**Medium preexposed to particles.** The medium for measurements of the activity of free cells was prepared by preexposure to the particles so that any removal of inorganic nutrients by adsorption or other changes in the medium would be the same in experiments with

and without particles. The medium was added to particles at the same weight/volume ratio used in the calorimetry and adsorption experiments. The mixture was shaken and allowed to equilibrate for 2 to 3 h. This time was shown to be more than enough for the adsorption of organic nutrients to reach equilibrium, and it was assumed that the inorganic nutrients would adsorb in equivalent times. The particles were allowed to settle, and the supernatant was removed with a Pasteur pipette. The supernatant was filter sterilized and transferred aseptically into a sterile bottle.

**Calorimetry procedures.** The calorimetric apparatus (LKB Instruments Inc., Rockville, Md.) and the procedures used in this study have been described previously in detail (15, 16).

Bacterial cell suspensions and solutions prepared as described above were placed in the appropriate chamber of the calorimeter with a sterile 5-cm<sup>3</sup> syringe. A stable baseline was achieved in 1 to 2 h, and then the reactants were mixed. The data were collected on a Hewlett Packard model 7101B strip chart recorder or on a Heath-Schlumberger model Eu-205-11 recorder. After an experiment the reaction cells were flushed with water to remove particles and solutions, rinsed with 0.5 N NaOH, 0.1 N HCl, and distilled water, soaked for 20 min in 70% ethanol, rinsed in acetone, and dried with air or nitrogen.

**Organic nutrient adsorption.** Solutions of [ $U$ -<sup>14</sup>C]glucose and [ $U$ -<sup>14</sup>C]glutamic acid were procured from New England Nuclear Corp., Boston, Mass. The specific activity of the glucose was 14 mCi/mmol, and the specific activity of glutamic acid was 292 mCi/mmol. These solutions were supplied in ethanol. The ethanol was removed by evaporation (50°C), and the isotopes were dissolved in sterile medium before use. Solutions of carrier were prepared, and the radiolabel was spiked into the solution to give 5,000 to 10,000 cpm and to bring the final organic nutrient concentration to the desired value. Adsorption experiments were carried out by placing 20 ml of medium containing radioactive organic in a sterile, acid-washed (chromic acid), 125-ml Erlenmeyer flask with a ground glass stopper. Samples (100  $\mu$ l) were placed in 5 ml of scintillation fluid (Aquasint; ICN Pharmaceuticals Inc.) in a vial. Two replicates of each sample were taken. The particles were then placed into the solution, and the mixture was shaken on a rotary shaker with sufficient force to keep the particles in suspension. When a sample was to be taken, 1 ml of the suspension was withdrawn with an Oxford pipette with a sterile tip. The sample was placed in a conical 15-ml centrifuge tube and centrifuged in a clinical centrifuge. The supernatant (100  $\mu$ l) was sampled for counting. Counting was done in a Packard Tri-Carb liquid scintillation counter (model 3375). The raw counts were corrected for background counts by subtraction.

**Bacterial attachment.** Bacterial attachment to hydroxyapatite was assayed by a modified version of the technique of Clark et al. (4). The assay involves incubating radiolabeled bacteria with the solid of interest and measuring the decrease in radioactivity in the solution as the bacteria become adsorbed to the surface. Bacteria were grown to an optical density of 0.2 absorbance units (log phase,  $\sim 2 \times 10^8$ /ml) in M9 and then harvested by centrifugation ( $12,000 \times g$  for 20 min). The cells were washed once with the medium

and then suspended in medium containing 5  $\mu\text{M}$  [ $U\text{-}^{14}\text{C}$ ]glucose. The bacteria were incubated for 1 h. After this time, plate counts were made, and the bacterial suspension was diluted with medium to make suspensions with various concentrations of bacteria. Samples (1 ml) of the suspensions were added to test tubes (15 ml; 16-mm outer diameter) containing 0.125 g of hydroxyapatite powder. The tubes were incubated at room temperature on an angle on a rotary shaker (Lab-Line Junior Orbit Shaker; 200 to 250 rpm) for 18 h. After the incubation period the tubes were removed from the shaker, and the particles were allowed to settle. A 0.1-ml sample of the supernatant was filtered through a 0.45- $\mu\text{m}$  Millipore filter. The filter was rinsed three times with 0.1-ml samples of medium, and the radioactivity retained by the filter was assayed. Radioactivity in 0.1 ml of the total slurry was also determined. These values were compared with values similarly obtained from control tubes without particles to determine the decrease in counts in the solution due to adsorption of the bacteria to the particles.

**Radiorespirometry.** Radiorespirometric measurements were made with glucose and glutamic acid as the sole carbon sources in the presence and absence of hydroxyapatite particles. Respirometry flasks (10 ml) with center wells (Kontes) were used for this experiment. Two different procedures were used for these experiments, depending on whether metabolism by attached bacteria or bacterial metabolism of organic nutrients preexposed to particles was to be examined.

The metabolism of organic nutrients preexposed (but not necessarily adsorbed) to hydroxyapatite was measured by the following procedure. Medium containing 6.7  $\mu\text{M}$  organic nutrient was exposed to the particles (0.125 g/ml) for 1 h on a rotary shaker. After this time 0.33 ml of bacterial suspension was added. The final concentration of organic carbon source was 5  $\mu\text{M}$ , and the final concentration of bacteria was  $6 \times 10^7/\text{ml}$ . Sodium hydroxide (0.1 ml of 10%, wt/vol) was added to the center well, and the serum caps were sealed. Flasks with and without particles were prepared in duplicate. The experiment was carried on for 1 h, 0.3 ml of 85% phosphoric acid was added, and the flasks were replaced on the shaker for 0.5 h. This concentration of acid dissolves all hydroxyapatite so that it can not retain any carbon dioxide. The contents of the center well were removed and placed in a 10-ml scintillation vial containing 5 ml of Aquascint. The well was rinsed 3 times with 0.1 ml of distilled water, and the rinse water was placed into the vial. The culture solution (0.1 ml) was also placed in a scintillation vial. The vials were assayed for radioactivity as described above.

Metabolism by attached bacteria was measured by the following procedure. Bacteria attached to hydroxyapatite were prepared by the procedure described above for the attachment assay, except that the bacteria were not radiolabeled. Bacteria in flasks without particles were also prepared as a control. A 1:500 dilution of the washed culture was used for the experiment; this yielded a suspension of  $6.2 \times 10^5 \pm 0.8 \times 10^5$  bacteria per ml as determined by plate count. At this concentration 85 to 87% of the bacteria became attached to the surface (see below). After exposure to the particles, [ $U\text{-}^{14}\text{C}$ ]glucose or [ $U\text{-}^{14}\text{C}$ ]glutamic acid was added to make the desired final concentration. The experiment was carried on for 24 h, and the

contents of the NaOH trap and the solution were harvested and assayed for radioactivity as described above.

**Carbon assimilation.** Incorporation of carbon from glucose or glutamic acid into cell material was measured by the following procedure. Attached bacteria and control flasks were prepared as described above. After the addition of the radioactive substrate, the cultures were incubated for 24 h and then fixed with 4% glutaraldehyde (final concentration) for 15 min. A 0.1-ml sample of the culture was added to 5 ml of Aquascint in a 10-ml scintillation flask; another sample (0.1 ml) was filtered through a 0.45- $\mu\text{m}$  Millipore HA filter, and the filtered material was rinsed with three 0.1-ml samples of medium. The filter was placed into scintillation fluid and dissolved by shaking. Radioactivity was assayed as described above. Comparison of total counts in unfiltered samples from flasks with and without particles showed no significant quenching due to the particles.

## RESULTS

**Adsorption of glutamic acid and glucose onto particles.** No adsorption of glucose onto hydroxyapatite could be detected. The adsorption isotherm of glutamic acid onto hydroxyapatite was found to be described acceptably by a linear function over the concentration range examined (Fig. 1). The adsorption of glutamic acid (5  $\mu\text{M}$ ) to hydroxyapatite in M9 and seawater was 193 and 533  $\text{nmol}/\text{m}^2$ , respectively.

**Bacterial attachment.** The attachment of the bacteria to hydroxyapatite was fit to the following equation by linear regression:  $C/Q = 1/KN + C/N$ , where  $C$  is the equilibrium concentration of bacteria in solution after adsorption,  $Q$  is the number of bacteria associated with the surface, and  $N$  and  $K$  are constants. A plot of  $Q$  versus  $C$  for adsorption of *V. alginolyticus* to hydroxyapatite is shown in Fig. 2. The linear fit of the data according to this equation has a correlation coefficient of 0.996. The error of the fit is 0.137, so the the number of bacteria associated with the surface under these fixed conditions can be predicted from the fit to within  $1.4 \times 10^4$  bacteria. This equation has been used by other workers to fit bacterial attachment data and has been found to be satisfactory (4, 13).

**Metabolism of organic nutrients in solution and on surfaces.** Experiments were performed in the LKB calorimetry system and by radiorespirometry. Heat production and respiration by *V. alginolyticus* at a concentration of  $6 \times 10^7/\text{ml}$  and organic substrate concentration of 5  $\mu\text{M}$  were not significantly affected by the presence of hydroxyapatite particles in the system (Table 1). Metabolic heat production did not significantly differ in experiments with and without particles regardless of whether the organic carbon source adsorbed to the particles (as was the case with glutamic acid) or did not (as was the case with glucose). The heat of metabolism was

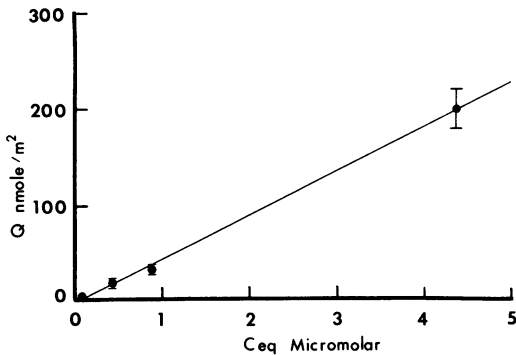


FIG. 1. Adsorption of glutamic acid onto hydroxyapatite in minimal growth medium as a function of equilibrium concentration of glutamic acid.

also unaffected by the presence of quartz particles. Radiorespirometry carried out with the same concentrations of bacteria and organic nutrient also showed that the particles did not significantly affect the metabolism of the bacteria (Table 1).

**Metabolism by attached bacteria.** Partitioning of the bacteria between the hydroxyapatite surface and the solution was 85% or more only at concentrations below  $10^6$ /ml (Fig. 2). The concentration of bacteria in experiments measuring the metabolism of attached bacteria, therefore, had to be on the order of  $10^5$  bacteria per ml. The rate of heat production by this number of starved bacteria cannot be detected by the calorimetry system used in this study. Consequently no calorimetric data were obtained on attached bacteria.

The respiratory metabolism of glucose and glutamic acid by bacteria attached to hydroxyapatite surfaces was found to be inhibited in comparison with that of bacteria free in solution (Fig. 3 and 4). In addition, the assimilation of

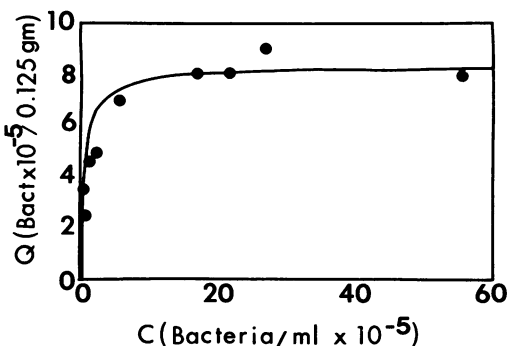


FIG. 2. Adsorption of *V. alginolyticus* to hydroxyapatite in minimal medium as a function of equilibrium concentration of bacteria.

TABLE 1. Effect of hydroxyapatite on the metabolism of *V. alginolyticus*

Conditions	Calorimetry (mcal/ $\mu$ mol)	Respirometry (nmol respired/ $\mu$ mol)
Glucose, no particles	$220 \pm 22^a$	$460 \pm 22$
Glucose, particles	$180 \pm 22$ (0.23) <sup>b</sup>	$480 \pm 22$ (0.52)
Glutamic acid, no particles	$120 \pm 22$	$440 \pm 44$
Glutamic acid, particles	$140 \pm 44$ (0.68)	$480 \pm 22$ (0.58)

<sup>a</sup> Data are given as means  $\pm$  standard errors.

<sup>b</sup> Numbers within parentheses are Student's *t* test probabilities of difference (*P* values) from experiments without particles.

carbon from glucose was shown to be inhibited (Fig. 5). The assimilation of glutamic acid by attached bacteria was not determined because of the high number of counts retained by the particle controls due to glutamic acid adsorption. As calculated from the slope of the lines in Fig. 3, the carbon dioxide production from glucose by unattached bacteria was 4 times higher than that from attached cells. The assimilation of carbon from glucose by unattached cells was 8 times higher than that from attached cells (Fig. 5). The ratio of assimilation to respiration was 1:1 for free cells and 1:2 for attached cells. This means that the attached cells have a decreased metabolic efficiency. The respiration of glutamic acid by unattached bacteria was 7 times higher than that from attached cells, indicating a decreased respiratory activity.

Long-term respiration experiments with the attached bacteria showed that the organisms were indeed active and that they could continue to mineralize the substrates at a low rate until a significant fraction of either substrate was consumed (Fig. 6). The data show that the rate of respiratory metabolism by the attached bacteria was lower than that of bacteria in suspension.

## DISCUSSION

The results of the present study do not support the supposition that the activity of marine bacteria is stimulated by the presence of inorganic surfaces. Bacterial activity was not enhanced regardless of whether the bacteria, the organic nutrient, or both were associated with the surface. In fact, the activity of the attached bacteria was diminished in comparison with that of free bacteria.

There are some important differences between the experimental systems employed in this study and those used by some of the workers who initially observed the surface effect and who

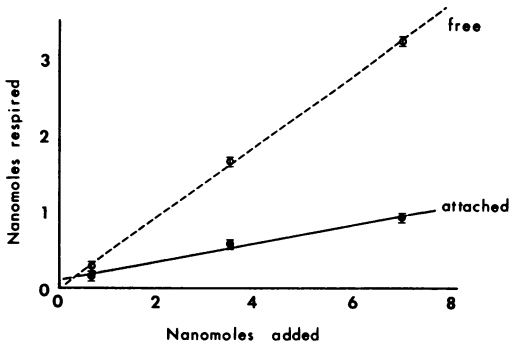


FIG. 3. Respiratory metabolism of glucose by *V. alginolyticus* in suspension compared with those attached to hydroxyapatite particles.

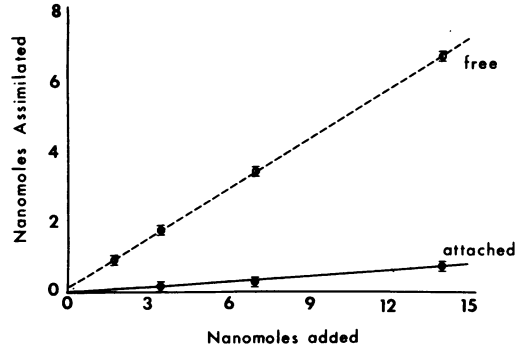


FIG. 5. Assimilation of glucose by *V. alginolyticus* in suspension compared with those attached to hydroxyapatite surfaces.

attributed it to increased availability of organic nutrients due to their adsorption on surfaces. In the now classic work by Zobell (36), respiration and plate count measurements showed that bacteria multiply in seawater stored in clean glass bottles and that this effect is related to the glass surface area exposed to the seawater. This effect has been observed by other workers (34). In natural seawater a large proportion of the total organic carbon is uncharacterized material (35) that is thought to be relatively resistant to biodegradation (33). Some of these materials may be expected to adsorb to surfaces like glass. For example, fulvic acid adsorbs to quartz and calcite (3). As is the case with oil droplets, the availability of these organic compounds to microorganisms could be limited by the exposed surface area of the organic material (27, 29). The exposed surface area of the organic material could be increased by adsorption onto glass. In cases where such refractory organic material is the organic nutrient, relatively low surface areas, as were used by Zobell, and relatively inert surfaces, such as glass, could reasonably be

expected to exert an influence on microbial growth.

The results of the present study concerning the metabolic activity of attached bacteria are consistent with the observations of Hattori and Furasaka (18, 19), who found that oxygen consumption by *Escherichia coli* and *Azotobacter agile* was inhibited when the cells were attached to an anion-exchange resin. These workers attributed the effect to calculated pH differences between the surface microenvironment and the bulk solution. The results of the present study may also be interpreted as being due to differences in conditions between the surface and the solution. Differences in conditions at the interface could be due to many factors, including the availability of inorganic nutrients, pH, availability of oxygen, and the proximity of the bacteria to one another. Attachment to a solid surface may alter certain attributes of the bacteria, such as motility and exposed cell surface area, which would be decreased. On the basis of the available data it is not possible to conclude which of these factors, if any, is responsible for the

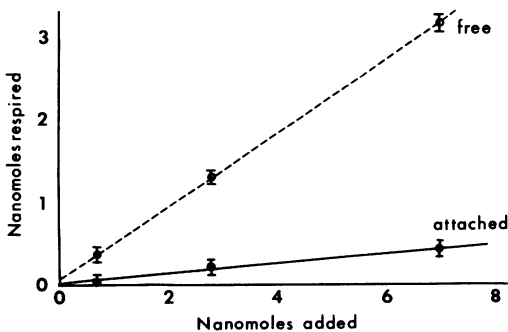


FIG. 4. Respiratory metabolism of glutamic acid by *V. alginolyticus* in suspension compared with those attached to hydroxyapatite particles.

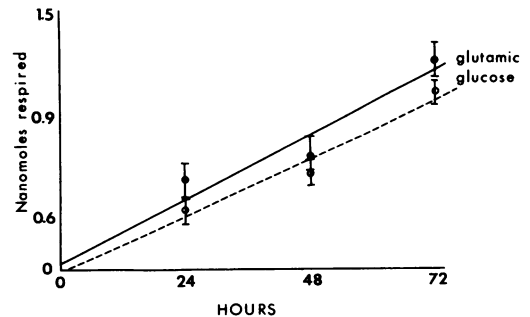


FIG. 6. Respiratory metabolism of glucose and glutamic acid by *V. alginolyticus* attached to hydroxyapatite particles as a function of time. The organic nutrient concentration was 5  $\mu$ M.

diminished activity of bacteria attached to surfaces in the present study. The adsorption of the organic nutrient on the surface does not alleviate the inhibition observed, suggesting that it is not due to a decrease or increase in the concentration of the carbon source at the surface. One would not expect any decrease in available phosphate or calcium on the surface of hydroxyapatite, a calcium phosphate mineral. In addition, since adsorption of glutamic acid does not alleviate the inhibition of respiration and is a nitrogen source which adsorbs to the surface, one would not expect that nitrogen availability is the controlling factor. If the observed decrease in bacterial activity at the interface is due to changed growth conditions there, then the most likely factors appear to be oxygen availability, trace metal availability, or some parameter such as pH.

Possible crowding of the bacteria on a surface could lead to competition for essential compounds such as oxygen and lead to reduced respiratory activity as was observed in this study. The approximate surface coverage in these experiments calculated from surface area of the particles, surface area of a bacterium, and the number of attached bacteria was quite low (<0.01%), so this type of inhibition is unlikely.

We feel that further studies under well-defined conditions employing a variety of surfaces, bacteria, organic nutrients, and media will aid in further clarification of the critical factors controlling the activity of bacteria at solid-liquid interfaces in natural waters.

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