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Bacterial Colonization and Ectoenzymatic Activity in Phytoplankton-Derived Model Particles. Part II. Cleavage and Uptake of Carbohydrates

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Abstract

The bacterial colonization and development of the ectoenzymatic glucosidase activity and glucose uptake were followed together with bacterial growth (measured as thymidine incorporation) in laboratory experiments, using phytoplankton-derived particles incubated in rolling tanks. Bacterial colonization of the particles was rapid. In the particles, bacterial turnover rates (production/biomass) were low (0.02 to 0.14 d^{-1}). In the ambient water, turnover rates increased from 0.1 d^{-1} to 23.3 d^{-1}, until the end of the experiment. In the control, lacking any particles, turnover of bacteria ranged from 0.3 to 7.6 d^{-1}. Similarly, glucose uptake rates, per bacterium, were 1 to 2 orders of magnitude lower for particle-attached bacteria than for their free-living counterparts. Generally, K_m values for glucosidase activity declined, over the incubation period, in particles and free-living bacteria until 168 h, and slightly increased, thereafter, to values of approximately 0.1 μM. Particle-attached bacteria exhibited significantly lower uptake rates of both thymidine and glucose, per bacterium, throughout the incubation. The per-cell ectoenzymatic activity was similar in particle-associated and free-living bacteria during the initial phase of the experiment, but was significantly higher after ≈200 h. Dissolved total (TCHO), as well as monomeric carbohydrates (MCHO), declined continuously in both particles and ambient water; they remained constant in the control; TCHO comprised about 50% of the dissolved organic carbon (DOC) in the particles. In ambient water TCHO contribution to DOC varied, with only one exception, between 25 and 45%; and in the control, between 20 and 50%. The shift detectable in the relation between ectoenzymatic activity and uptake of glucose between free-living and attached bacteria over the incubation period may reflect changes in the physiological status of the bacteria.

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Introduction

The ubiquitous existence of detrital particles, such as fecal pellets and amorphous marine aggregates in the ocean, has been well documented over the past 2 decades [1, 9, 11, 23, 31, 32]. It has been suggested that particles, such as marine snow, play a crucial role in the transfer of carbon from the surface layers to the deep sea, thereby dampening the increase of atmospheric carbon dioxide. Marine snow can originate from several planktonic components, such as phytoplankton, fecal pellets, and gelatinous zooplankton brought together by turbulence. The matrix of this marine snow consists of polysaccharides [19, 25, 27, 28, 29].

Similar to phytoplankton, bacteria are usually highly enriched in particles such as marine snow [7, 23, 34]. It has recently been shown that: 1) attached bacteria exhibit a high phylogenetic diversity, 2) these bacteria are distinctly different from their free-living counterparts, and 3) considerable fraction of free-living bacteria are present in marine snow, as well [8]. Moreover, it was found that particle-attached bacteria were phylogenetically different from free-living bacteria [5]. Although significant fluctuations were detected in the species composition of the free-living bacterial community along a salinity gradient, the attached bacterial community remained remarkably stable. Particles are colonized by bacteria of the ambient water, as indicated by the progressive increase in bacterial abundance in marine snow from its formation to the final decay [23]. It has been shown, however, that the same bacterial strain alters its physiology, depending on the availability of colonizable particles [37]. Thus, not only genotypic differences, but also phenotypic differences, are detectable in bacterial communities colonizing particles.

Particle-attached bacteria are frequently larger and exhibit lower turnover rates than free-living bacterioplankton [17]. Moreover, there is accumulating evidence that attached bacteria, despite their lower activity, exhibit higher extracellular enzymatic activity [15, 16, 17, 20, 34]. Thus, while free-living bacteria are characterized by a tight coupling between hydrolysis of molecules and subsequent uptake of the cleaved substrate, attached bacteria exhibit a loose hydrolysis-uptake coupling, i.e., they cleave relatively more substrates than they assimilate.

Although there are several reports describing the difference in ectoenzymatic activity and substrate uptake between free-living and attached bacteria, all of them were performed on natural marine snow [20, 23, 34] or on artificial inert substrates [14, 15]. Under natural conditions, however, the possible shift in the hydrolysis-uptake coupling from tight to loose can only be detected by following a distinct parcel of water for several weeks, beginning with the decay of a phytoplankton bloom when aggregation starts. This is only possible on certain occasions [23]. Using inert surfaces for colonization does not reflect natural conditions in particles highly enriched in both organic and inorganic nutrients [1, 19, 23].

The aim of this study was to follow the colonization of artificial, sterile phytoplankton-derived particles by the free-living bacterial consortia, thereby mimicking natural conditions as closely as possible. We hypothesized that free-living bacteria colonizing these particles respond with a subsequent alteration in their hydrolysis-uptake pattern. This alteration should be a smooth transition from a tight to a loose hydrolysis-uptake coupling, if free-living continuously attach to the marine snow. Although this study focuses on the hydrolytic activity of glucosidase and the carbohydrate dynamics, the relationship between aminopeptidase activity and amino acid uptake was investigated in a previous paper [36].

Material and Methods

Preparation of Phytoplankton-Derived Particles

The diatom *Chaetoceros* sp. was grown in Guillard f/2 medium under continuous radiation of ≈100 $\mu E\ m^{-2}\ s^{-1}$ to stationary phase. Growth was determined by measuring the optical density at 550 nm, daily. Dense cultures in the stationary phase were transferred into rolling tanks [30], to enhance aggregation of the senescent cells. After ≈1 h, marine snow-type aggregates of ≈1 cm in dia were formed. These particles were allowed to settle, transferred into a 1-liter flask, and autoclaved. The dense particle suspension was used for further experimentation.

Experimental Set-up

Bacterial colonization of the lab-reared particles was followed by transferring filtered seawater, (0.8 μm pore size, 14 cm filter dia, polycarbonate, Millipore) collected about 1 km off Rovinj (Croatia; northern Adriatic Sea), into a 10-liter rolling tank containing 500 ml of the marine snow suspension. Another 10-liter rolling tank was filled with only 0.8 μm filtered seawater to serve as a control. The tanks were placed on a rolling table; the rotations were adjusted to minimize turbulence and to avoid settling of the particles. The rolling table was kept in dim light at ~in situ temperature (≈24°C). Once a day, between 80 and 180 ml of water (not containing any visible aggregates) were removed from each tank, to determine the microbial parameters described below. Additionally, 3 to 28 ml of particles were removed with a Pasteur pipet mounted on a 5-ml Socorex pipet. The size of the particles allowed sampling

without inclusion of ambient water. Throughout the text, unless stated otherwise, the particle volume refers to the particulate fraction of the particles and its porewater. After sampling, 0.8 μm filtered seawater from the same sampling site was added.

Parameters Measured

Bacterial abundance was determined from 5-ml water samples fixed with 2% formaldehyde (final conc). For enumeration of particle-associated bacteria, 10 μl of particles collected by pipets were transferred into 5 ml of 0.2 μm, double-filtered seawater, fixed with formaldehyde, sonicated (to disrupt the particle for a more even distribution of the bacteria on the filter), stained with a few drops of an 0.2 μm-filtered acridine orange solution, and filtered onto a black, 0.2 μm, polycarbonate filter (25 mm filter dia), Millipore [12, 23]. All samples were analyzed in duplicates. Bacterial enumeration was performed using a Leitz Laborlux S epifluorescence microscope; 30 fields, or at least 300 bacteria, were counted, per sample. Bacterial biovolume was determined from acridine orange preparations using a Nikon epifluorescence microscope and an image analyzing system consisting of a video camera (Hamamatsu 2400) and VIDS IV (Nikon) software. Cell size was calibrated with a stage micrometer at a magnification of 1250×. Length (L) and width (W) of about 200 randomly selected bacteria, per sample, were measured; the biovolume (V) was calculated according to the equation $V = (\pi/4)\ W^2 \times (L-W/3)$. Bacterial carbon biomass was calculated from the biovolume and the allometric relationship between biovolume and carbon content [21].

Bacterial production was determined using [^{3}H]thymidine incorporation into bacteria and the centrifugation technique [33]. For ambient water and the control, 1.7 ml of water and 300 μl of particles suspended in 1.4 ml ambient water, respectively, were used. Uptake kinetics for thymidine were determined at the beginning, the middle, and the end of the experiment, for bacteria of the ambient water, particles, and the control. Five different concentrations (1, 5, 10, 30, and 100 nM) of [^{3}H]thymidine (specific activity 85 Ci ml^{-1}) were used. Samples were incubated, in triplicate, with 2 blanks killed with 100% TCA, in the dark, at ambient temperature, for 30 min.

Glucose incorporation by bacteria was measured by adding ^{14}C-glucose (specific activity 340 mCi mmol^{-1}, mixed with unlabeled glucose, 389.6 nM final conc) to 30 ml of ambient water and the control. For particle-attached bacteria, 2 ml of marine snow were incubated (final conc 5845 nM glucose). Together with the sample, 1 formalin-killed blank (2% formaldehyde, v/v final concentration) was incubated in the dark, at in situ temperature, for 1 h. Isotope dilution was calculated by determining the monomeric carbohydrate concentration, as described below, and assuming that this pool is assimilated with the same efficiency as the added glucose. Glucose is, by far, the dominant carbohydrate species in the monomeric fraction of the carbohydrates [26].

At each sampling date, 1 ml of particles was collected from the rolling tank, in duplicate, and stored at −20°C in the dark. The wet wt, dry wt, and the organic carbon and nitrogen contents were determined within 3 months after collecting the sample. Dry wt was determined after centrifuging the sample, discarding the supernatant, and replacing it with 0.2 μm-filtered, distilled water; this procedure was repeated 3 times. Thereafter, the particles were transferred into washed and weighed tin capsules, and dried at 60°C until constant weight was reached. After acidifying the sample with HCl to remove inorganic carbon, the carbon and nitrogen contents were determined using a Carlo Erba CHN NA1500 analyzer [23].

For dissolved organic carbon (DOC) of the ambient water, the control and the porewater of the particle, 1 to 5 ml were filtered through combusted (450°C for 4 h) Whatman GF/F filters (25 mm filter dia) and stored in combusted, sealed glass ampoules at −20°C until analysis. The samples were sparged with CO_2-free air and DOC content was determined using a Shimadzu TOC-5000. Standards were prepared with potassium hydrogen phthalate (Kanto Chemical Co Inc, Tokyo); a platinum catalyst on quartz was used [4].

Monomeric and total dissolved carbohydrates were determined in the ambient water, the porewater of marine snow, and in the control, using a modified MBTH-method [6, 18, 24]. For determination of total dissolved carbohydrates, samples were hydrolyzed under a nitrogen atmosphere with 6N HCl (final conc) in sealed ampules, at 110°C, for 22 h. Subsequently, the liquid was evaporated at 5°C, under vacuum (200 mtorr), overnight. The remaining powder was redissolved in 200 μl of 0.05N NaOH, sonicated, and brought up to the original sample volume with double-distilled water. The combination of a 6N HCl, instead of 0.1N HCl, for hydrolysis and freeze-drying, resulting in a significantly higher yield than using the original method [18]. Resulting absorbances of triplicate subsamples and 2 blanks were measured with a Hitachi U-2000 spectrophotometer. Glucose was used as a standard.

Bacterial ectoenzymatic alpha- and beta-glucosidase activity was determined using the fluorogenic substrate analogs 4-methylumbelliferyl-alpha-glucoside and 4-methylumbelliferyl-beta-D-glucoside (Sigma) [13, 23]. All measurements were performed in duplicates, using saturating substrate concentrations, as determined by kinetic studies. Enzyme kinetics were determined for particles, ambient water, and the control at the beginning, the middle, and the end of the experiment, using 8 different substrate concentrations (0.25, 1, 5, 10, 50, 100, 200, and 500 μM). Fluorescence of methylumbelliferone (MU) caused by the enzymatic cleavage was detected with a spectrofluorometer (JASCO 820-FP, Tokyo, Japan). Excitation wavelength was 360 nm; emission wavelength was 444 nm. Calibration of the fluorescent yield was performed for each measurement, using different 4-methylumbelliferone (MU) concentrations (Sigma). Parameters for enzyme kinetics (K_m and v_{max}) were calculated using non-linear regression analysis of a direct plot of the hydrolysis velocity versus substrate concentration, using SYSTAT.

Results

Particle Formation and Abundance

Particle formation occurred within 3 h after adding the autoclaved phytoplankton slurry to the rolling tanks. Particles

were round, with diameters ranging from 3 to 12 mm. Particle size and appearance remained fairly constant over the incubation period. The initial abundance of phytoplankton-derived particles was ≈150 l^{-1}.

Development of Bacterial Abundance and Activity

Bacteria rapidly colonized the phytoplankton-derived particles (Fig. 1a), reaching an abundance of 2×10^8 cells ml^{-1} after 3 h of incubation. Maximum abundance was reached 7 d (168 h) after starting the experiment. Bacterial abundance in ambient water also reached its maximum after 168 h (8.8×10^7 cells ml^{-1}, Fig. 1a). It declined thereafter, by more than 1 order of magnitude, approaching the bacterial abundance of the particle-free control (Fig. 1a). In the control, bacterial abundance varied by less than 1 order of magnitude. The appearance of heterotrophic flagellates (data not shown) caused a decline in bacterial abundance in the control from 48 h to 144 h, and from 168 h to 192 h in both the ambient water and the particles (Fig. 1a). In the initial phase of the experiment, bacterial abundance in particles was 3 orders of magnitude higher than in ambient water; in the later stage of the experiment (from 192 h onwards), bacterial abundance was 4–5 orders of magnitude higher in the particles. Generally, the volume of bacteria declined during the course of incubation (Fig. 1b). This tendency was most pronounced in bacteria of the ambient water. The ambient water bacterial community was dominated by large, rod-shaped bacteria at the beginning of the experiment. They gradually decreased in size during the experiment; at the end of the experiment, the biovolume was only one-third of the volume at the beginning. The volume of particle-attached bacteria was not significantly different (Wilcoxon, $P = 0.33$) from that of the bacterioplankton in the control.

Saturating concentrations for thymidine uptake measurements were 30 nM in the control and 100 nM in the ambient water and particle-attached bacteria. Bacterial turnover (i.e., bacterial carbon production measured via thymidine incor-

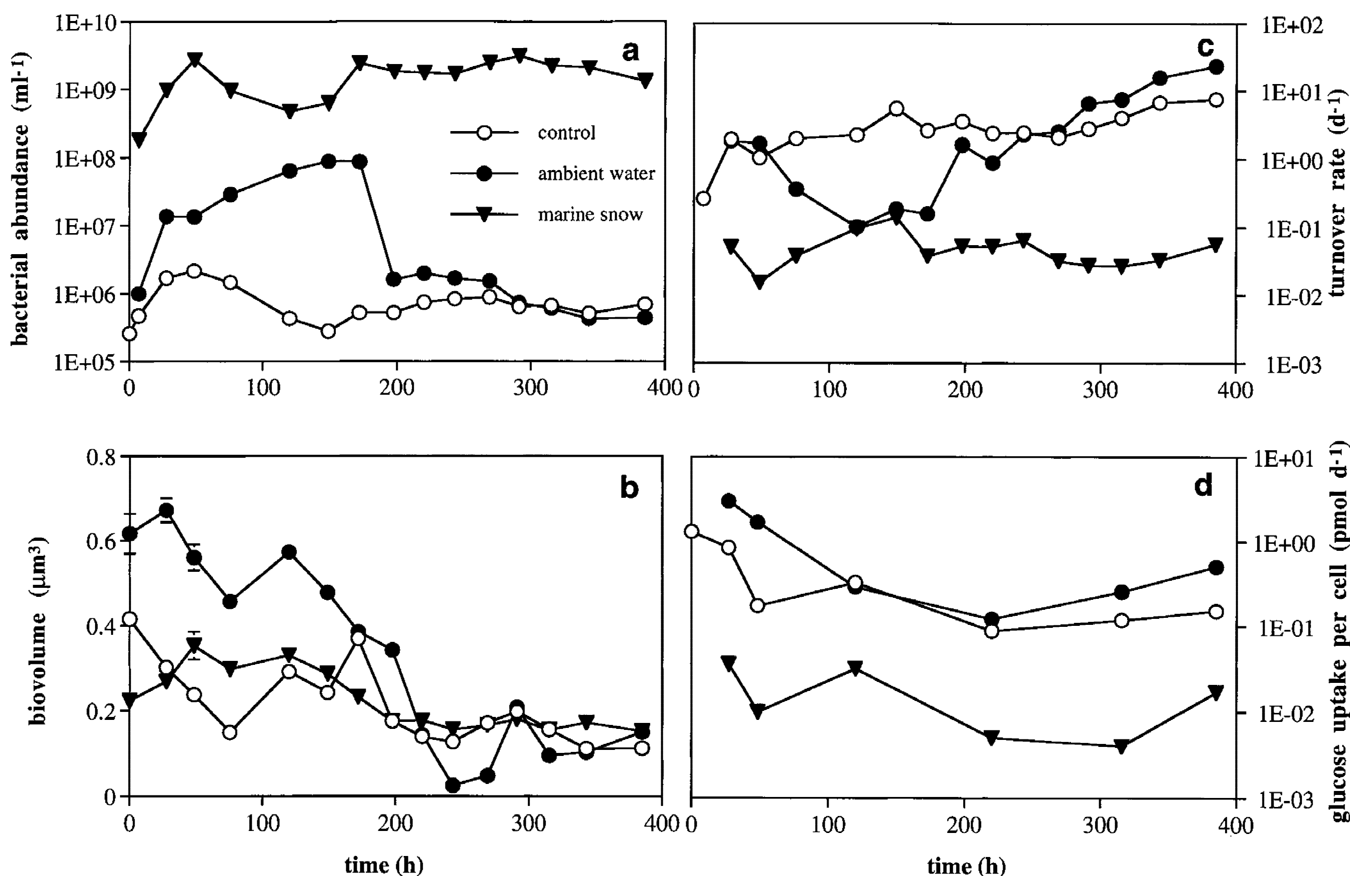

Fig. 1. Time course of **(a)** bacterial abundance, **(b)** bacterial biovolume, **(c)** turnover rate (bacterial production/bacterial biomass), and **(d)** glucose uptake in artificial marine snow, ambient water, and in the control (to which no phytoplankton-derived particles have been added).

poration/bacterial carbon biomass) was lowest in the particles (Fig. 1c), and ranged from 0.02 to 0.14 d^{-1}. The turnover rate of ambient water bacteria increased from 0.1 d^{-1} to 23.3 d^{-1}, until the end of the experiment (Fig. 1c). In the control, turnover of bacteria ranged from 0.3 to 7.6 d^{-1} (Fig. 1c). Thus, the largest variations in bacterial turnover rates were detected for free-living bacteria in ambient water (Fig. 1c). As with thymidine incorporation, glucose uptake, per bacterium, was similar for free-living bacteria of the control and the ambient water (Fig. 1d); particle-attached bacteria, however, exhibited glucose uptake rates, per bacterium, which were 1 to 2 orders of magnitude lower than their free-living counterparts.

Bacterial alpha-glucosidase activity fluctuated from 9.3 to 543 amol bacterium^{-1} d^{-1} (Fig. 2a); beta-glucosidase activity ranged from 16.9 to 542 amol bacterium^{-1} d^{-1} (Fig. 2b). During the initial phase of the experiment, ectoenzymatic activity fluctuated in all 3 treatments. After ≈200 h, ectoenzymatic activity increased in the particles and the ambient water. Alpha-glucosidase activity, per bacterium, was significantly higher in particle-attached bacteria than in ambient water (Fig. 2a, Wilcoxon, $P = 0.011$); no such difference was detectable for beta-glucosidase (Fig. 2b, Wilcoxon, $P = 0.173$). This pattern was caused by the erratic fluctuation during the first half of the experiment. During the second half, ectoenzymatic activity per cell, was always higher in particles than in ambient water (Fig. 2a and b). The ratio alpha-:beta-glucosidase was consistently higher in particles and the control, compared to the ambient water (Fig. 2c). In the ambient water, beta-glucosidase always dominated over alpha-glucosidase; in particles, alpha- and beta-glucosidase activity was about the same (as indicated by the ratio ≈1); and in the control, alpha-glucosidase activity tended to dominate (Fig. 2c). The K_m values in particle-attached bacteria remained comparatively stable over the incubation period, ranging from 0.055 μM to 1.011 μM (mean = 0.322, SD ± 0.338) for alpha-glucosidase and from 0.039 to 1.983 μM (mean = 0.470, SD ± 0.675) for beta-glucosidase. In both the ambient water and the control, the K_m values declined over the incubation period (Fig. 3).

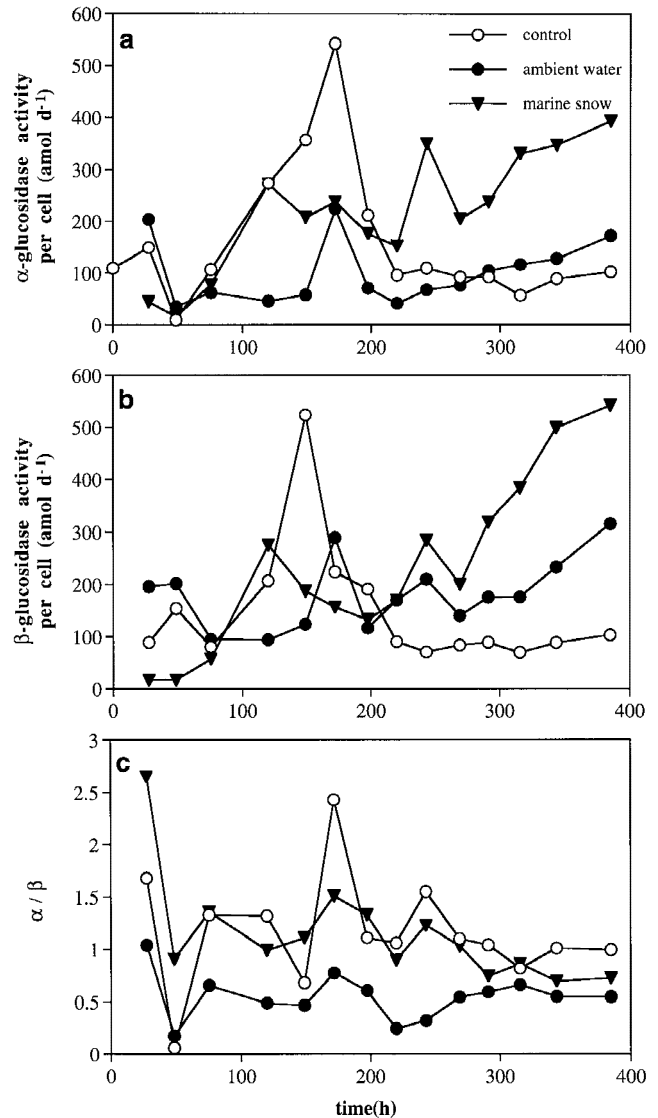

Fig. 2. Development of **(a)** alpha-glucosidase activity per cell, **(b)** beta-glucosidase activity per cell, and **(c)** the ratio between alpha- and beta-glucosidase activity in marine snow, ambient water, and the control. Ectoenzyme activities are expressed as a mol cell d^{-1} (amol = 10^{-18} mol).

DOC and Carbohydrate Dynamics in Particles and Ambient Water

The DOC concentrations in the porewater of the particles were, on average, twice as high as in the ambient water. They ranged between 12 mg C l^{-1} at the beginning and 5.17 mg C l^{-1} toward the end of the experiment (Fig. 4a). The DOC in the control remained fairly constant—usually below 1 mg C l^{-1}. The only exceptions were at 240 and 264 h, when DOC increased in the control, particle, and ambient water. This probably reflected unknown sources of contamination (Fig. 4a).

Dissolved total (TCHO), as well as monomeric carbohydrates (MCHO), declined continuously in both particles and ambient water, and remained constant in the control (Fig. 4b and c). The decline in MCHO was most pronounced in the initial phase of the experiment; it remained fairly constant thereafter (Fig. 4c). TCHO comprised about 50% of

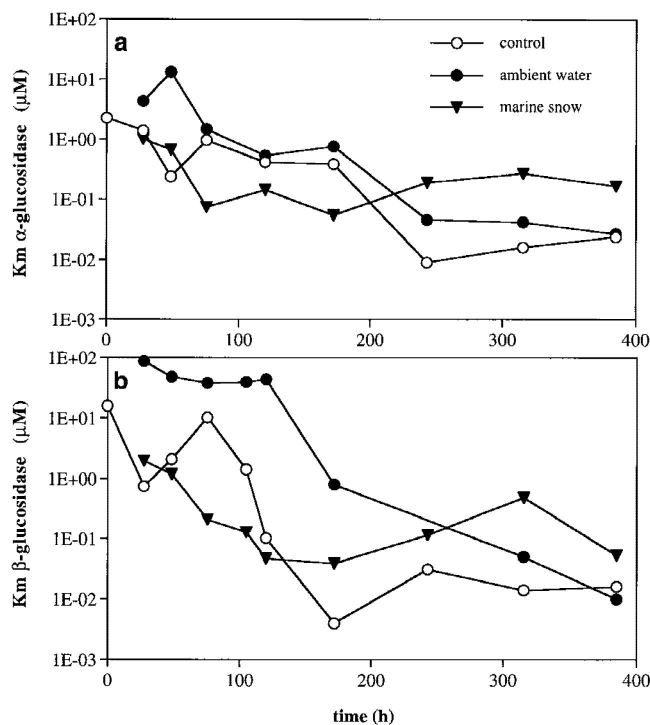

Fig. 3. Development of the K_m values for **(a)** alpha-glucosidase and **(b)** beta-glucosidase activity over the incubation period.

the DOC in the particles. In ambient water, TCHO contribution to DOC varied, with only one exception, between 25 and 45%. In the control it varied between 20 and 50%.

The C:N ratio of the particle declined over the incubation period from ≈8 to ≈6, reflecting a relative enrichment in nitrogen as the particles aged (Fig. 4d). Bacterial carbon contributed to the particulate organic carbon (POC) fraction, on average, 1.8% (SD = 0.84, $n = 14$). To the nitrogen pool, however, bacterial nitrogen contribution was, on average, 22.4% (SD = 10.6, $n = 14$).

Discussion

The alteration of the bacterial community following attachment to particles can only be studied under conditions where the colonization pattern can be followed over time. Although this could, at least to some extent, also be done in situ, laboratory experiments allow work under better-defined conditions. Our phytoplankton-derived particles were meant to provide a model substrate (both in terms of colonizable surface and metabolizable substances) for bacteria, to study the potential metabolic shifts in the bacterial community after attachment. Free-living bacteria exhibited higher turnover rates (measured by thymidine incorporation) than attached bacteria. Also, glucose uptake on a per-cell basis was significantly higher (Wilcoxon, $P = 0.028$) in the free-living than in the attached bacterial community (Fig. 5). Ectoenzymatic activity of particle-attached bacteria was, on a per-cell basis, not significantly different from that of the free-living bacterial consortia. Thus, there is a detectable shift in the relation between ectoenzymatic activity and uptake of substrate (in our case, thymidine and glucose) between free-living and attached bacteria.

These differences in the relation between metabolic and ectoenzymatic activity have been observed by several authors [15, 17, 20, 23, 34]. They have been interpreted as loose hydrolysis–uptake coupling for attached bacteria, and a tighter coupling for free-living bacteria [2, 3, 15]. Some of the studies report higher ectoenzymatic activity on a per-cell basis in particle-attached, compared to free-living bacteria [20, 22, 34, 35]. In other studies [23, 35], no significant difference was found. In our study, only alpha-glucosidase activity was significantly higher (Wilcoxon, $P = 0.011$). Significantly lower turnover and glucose uptake rates for particle-attached bacteria were consistently detectable. The lower turnover rates (determined by thymidine incorporation) for particle-associated bacteria detected in this study agree with earlier findings [23]. Lower thymidine incorporation rates, however, have to be considered with caution since it is well known that at least some anaerobic bacteria do not take up thymidine, and it is likely that anaerobic bacteria developed in the aggregate during our experiment. When comparing the development of bacterial abundance (Fig. 1a) with the turnover rates shown in Fig. 1c, it becomes evident that bacterial abundance increased more rapidly than indicated by the turnover rates. This could be caused by the development of an anaerobic community unable to incorporate thymidine or by the attachment of free-living bacteria to the particles.

One possible reason for the lower turnover rates for particle-associated bacteria could be the larger capsular envelope surrounding the cell [10]. This capsular layer could reduce the diffusion of molecules to the cell, and, consequently, retard uptake. This would lead to lower metabolic rates. No difference in turnover rates, however, were found between bacteria attached to surfaces and their free-living counterparts, although exopolysaccharide production was several-fold higher for attached bacteria [37]. Considering that 70% of the particle-attached bacteria have a capsular envelope of at least the size of the actual cell diameter [10], and that a significant portion of the ectoenzymes is embed-

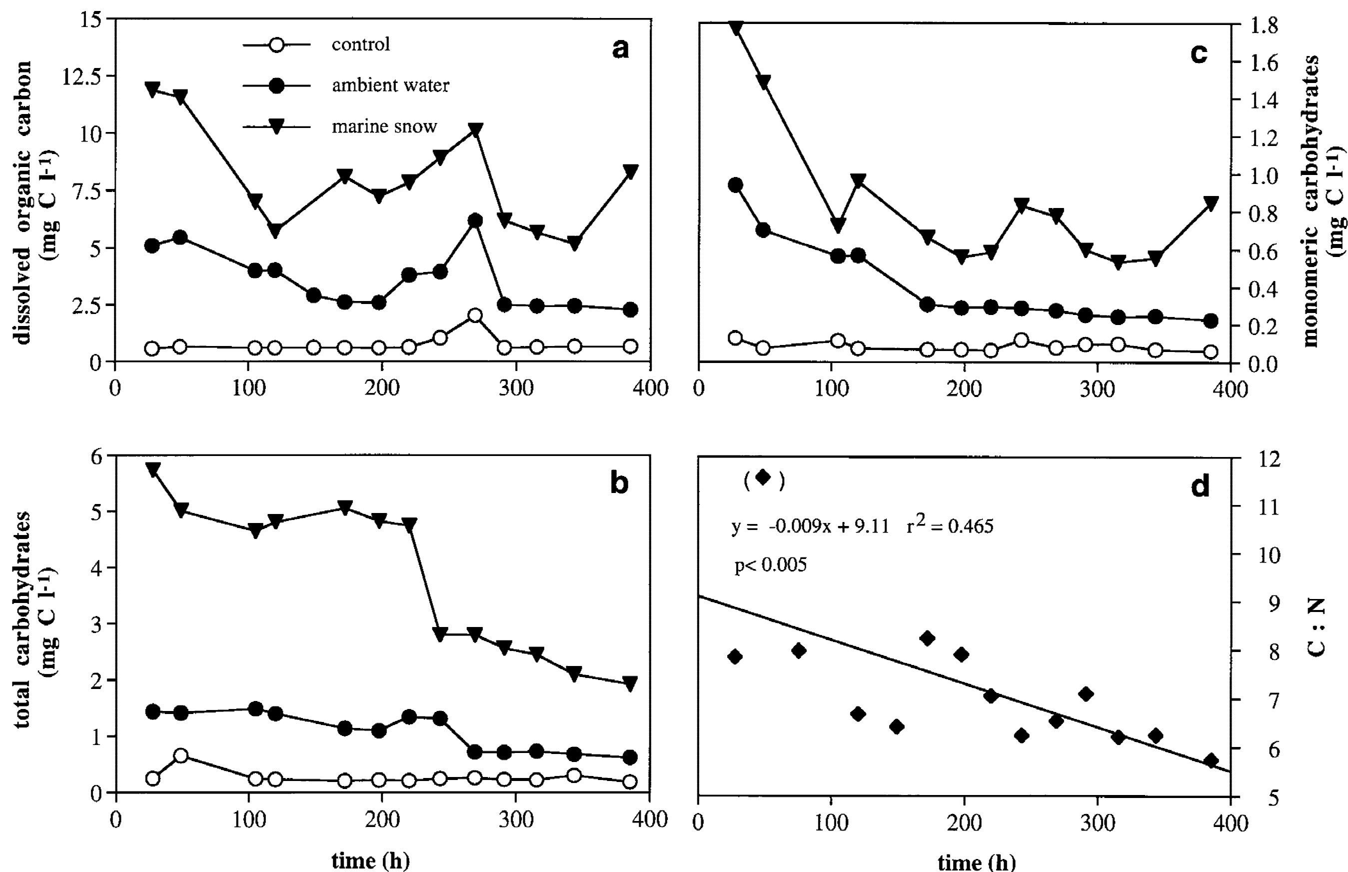

Fig. 4. Time course of **(a)** dissolved organic carbon, **(b)** total dissolved carbohydrates, **(c)** dissolved monomeric carbohydrates, and **(d)** the C:N ratio of the particulate fraction of phytoplankton-derived particles.

ded in this capsule, it might well be that these ectoenzymes cleave substrates further away from the actual cell than do free-living bacteria which have a much thinner capsular envelope [10]. Ectoenzymatic cleavage of particle-attached bacteria might result in a less efficient uptake of the hydrolysis products. This scenario would reflect the loose hydrolysis-uptake coupling of DOM described for attached bacteria [2, 3, 15]. Clearly, further investigations are necessary to decipher the potential differences in the hydrolysis-uptake coupling between free-living and attached bacteria. Of particular relevance, in this respect, is to determine how many bacteria in the community are actually able to express a particular ectoenzyme in our study of glucosidase. We presently have no means to specifically enumerate glucosidase-expressing bacteria in samples. There is evidence, however, that not all aerobic bacteria express the same amount of glucosidase; and that bacteria are common in marine snow exhibiting high levels of either alpha- or beta-glucosidase, but never both (Arrieta and Herndl, unpublished data). Thus, the pattern we observed in this study could also be a reflection of species shifts in the communities, although this does not alter the general conclusion that there is detectable uncoupling between hydrolysis rate and uptake of glucose in particle-attached bacteria.

The K_m values for particle-attached bacteria remained fairly constant compared to the values of free-living bacteria of the ambient water and the control (Fig. 2c). Alpha glucosidase K_m values for particle-attached bacteria ranged from 55 to 1011 nM (mean = 322, SD ± 338); for free-living bacteria of the ambient water, from 27 to 13250 nM (mean = 2553, SD ± 4550); and for control, from 9 to 2260 nM (mean = 632, SD ± 768; Fig. 2c). Generally, the K_m values for beta-glucosidase were similar to alpha-glucosidase. There was a general trend for K_m values to decrease from the beginning to the end of the experiment. The most pronounced decline in K_m was observed for free-living bacteria of the ambient water (Fig. 4). It is also reflected by the general decline in monomeric carbohydrate concentrations. It seems that either the ectoenzyme affinity to the substrate increased over time or that end-product inhibition declined

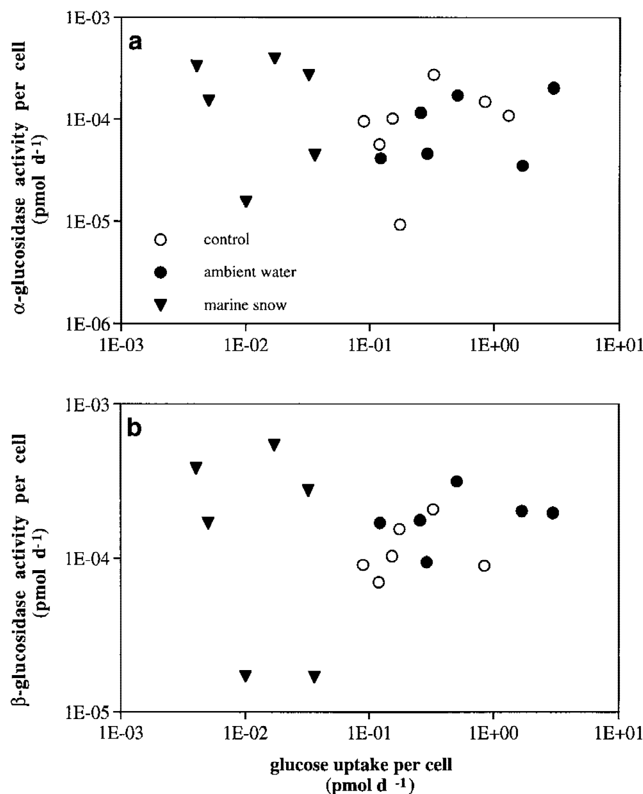

Fig. 5. Relation between glucose uptake per cell and **(a)** alpha-glucosidase and **(b)** beta-glucosidase activity per cell in particle-associated and free-living bacteria of the ambient water and the control.

during the course of the experiment as monomers became more depleted.

In summary, we have shown that particles are efficiently colonized by bacteria originating from ambient water; during the course of colonization, the metabolic characteristics of the particle-attached bacteria gradually change. Despite the high nutrient concentration in particles, attached bacteria exhibit lower glucose uptake rates compared to the free-living bacteria. Although in particle-attached bacteria, the uptake rates of glucose are lower, the ectoenzymatic activity remained at a level comparable to that of free-living bacteria. This distinct pattern might be caused by differences in the capsular envelope in particle-attached bacteria. We are currently investigating the role of the capsular envelope in the expression of ectoenzymes for free-living versus attached bacteria.

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