MICROBIAL ECOLOGY

Microb Ecol (1999) 37:107–115

Growth of Amoebae and Flagellates on Bacteria Deposited on Filters

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Received: 3 August 1998; Accepted: 20 November 1998

Abstract

Artificial bacterial biofilms were formed by making microwave-irradiated, dual-radioisotope-labelled *Vibrio* bacteria adhere to 0.4 μm pore size filters with albumin. The rate of release of ^{3}H from thymidine label in these bacteria into the surrounding seawater when protozoa were incubated with the biofilm indicated the predator's grazing rate, and the rate of accumulation of ^{14}C in the predators from leucine label in the bacteria indicated the assimilation rate of the protozoa. The amoeba *Vanella septentrionalis* consumed about 60% of the available bacteria between the 5th and 15th days of incubation with a gross growth efficiency of 22 ± 6%, compared with about 75% consumption at 29 ± 8% efficiency for the surface-feeding flagellate *Caecitellus parvulus,* and about 55% consumption at 16 ± 5% efficiency for the suspension-feeding flagellate *Pteridomonas danica.* As a result of their grazing and metabolism these protozoa regenerated about 70–85% of the nutrients present in their food and released these nutrients in the immediate vicinity of the bacterial biofilm. The biomass of the amoeba *Vanella* was calculated to be 166 pg protein $cell^{-1}$ during maximum growth and 93 pg protein $cell^{-1}$ in the stationary phase.

Introduction

Any solid surface immersed in water attracts a deposit of organic molecules and is subject to rapid colonization by microorganisms that are able to attach and consequently exploit the nutrient-rich interface microenvironment. Generally, pioneering bacteria, such as *Vibrio* and *Pseudomonas,* harvest the organic material and initiate the structure named a microbial biofilm [2, 7, 13]. Bacterial biofilm development has been a subject of intensive investigation, and numerous studies have shown that bacterial activity can be stimulated by the presence of algae within complex biofilms [4, 14]. There is also a diverse community of protozoa—amoebae, foraminiferans, flagellates and ciliates—that inhabits and thrives on biofilms [10, 12, 15]. These microorganisms are important consumers of bacteria and microalgae; they disrupt the integrity of a biofilm and are presumably the major cause of its fragmentation and sloughing [11]. Additionally,

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metabolising protozoa release nutrients which can enhance bacterial growth.

There have been a number of qualitative descriptions of the impact of protozoa on biofilms, but few quantitative estimates of protozoan predation on bacteria on surfaces [1, 3, 5, 6, 8, 9, 21]. This is significantly different from the present understanding of the role of protozoa, both flagellates and ciliates, in the water column, which is based on numerous qualitative and quantitative studies [18]. The main problem that frustrates progress in studies of protozoan predation on attached bacteria is the absence of a test system and underdeveloped methodology. Generally, there is no problem with observing protozoan ingestion of bacterial prey; the problem emerges when one tries to quantify this process. Several model systems have been proposed to mimic and reproduce natural bacterial biofilms: suspended living or killed, fluorescently labeled bacteria were mechanically spread over the surface to form a uniform lawn [5, 17]; or suspended bacteria were used to fill a column of sand or glass beads [9, 21]; or a determined quantity of suspended bacteria was deposited by centrifuging onto the bottom of a test tube [24]; or bacteria were incubated in the vicinity of glass or chitin surfaces [3, 6] to facilitate natural bacterial attachment. Alternatively, suspended bacteria were used at such a high concentration that nonsuspension-feeding amoebae were ultimately able to grow [20]. The result of predation was quantified either by direct observation of bacterial ingestion into protozoan food vacuoles or by monitoring numbers of predator and/or prey cells during the grazing experiment. The design of these experiments imposed certain constraints, one of which was a general inability to determine gross growth efficiency of bacterivorous surface-dwelling protozoa, which is also a surrogate measure of nutrient regeneration rate. Quantitative centrifuging of bacteria allowed gross growth efficiency of surface-dwelling flagellates to be estimated, but the compact pellet was insufficient for growth of amoebae; furthermore, the numbers of firmly attached feeding amoebae could not be monitored in such a model system.

Therefore, we designed a simple model system using dual-radioactive-labeled bacteria, *Vibrio natriegens,* deposited on filters, to quantify growth and estimate gross growth efficiency of a bacterivorous amoeba, *Vanella septentrionalis,* and to compare its growth with the growth of surface-dwelling and suspension-feeding flagellates, *Caecitellus parvulus* and *Pteridomonas danica,* respectively.

Materials and Methods

Cultures

A culture of the gymnamoeba *Vanella septentrionalis* Page 1980 (CCAP 1589/10) was purchased from the Culture Collection of Algae and Protozoa (Institute of Freshwater Ecology, Ambleside, LA22 0LP, U.K.). The flagellates *Caecitellus parvulus* (Griessmann, 1913) [15] and *Pteridomonas danica* (Patterson and Fenchel, 1985) were isolated from Southampton Water by Dr. S.M. Tong [19]. All microorganisms were grown, and the experiments done, in the dark at a temperature of 10°C.

Growth Experiments

The principal prey used to feed all protozoa studied in these experiments was *Vibrio natriegens.* It was maintained on marine agar plates (Difco) and was harvested at the stationary phase. Bacteria were suspended in freshly filtered (0.2 μm polycarbonate membrane filter, Whatman), aged seawater and the suspension was subsequently filtered through a 0.8 μm polycarbonate filter to retain or break clumps and reach a bacterial concentration of about 1–2 × 10^9 *Vibrio* ml^{-1} in the filtrate. A preliminary estimate of the bacterial concentration was made from the absorbance at 420 nm according to an empirical calibration of absorbance against numbers and biomass of bacteria. Replicated 50 μl subsamples were frozen for subsequent protein determination using the bicinchoninic acid method (BCA, Sigma), with bovine serum albumin as a standard.

The stock of suspended *Vibrio* was diluted with 0.2 μm-filtered seawater to reach a final bacterial concentration of 50–80 × 10^6 *Vibrio* ml^{-1}. The concentration of these suspended bacteria was measured more precisely using epifluorescence microscopy. To do this, a subsample of 50 μl was taken, fixed with 1% glutaraldehyde in a final volume of 1 ml made up with 0.2-μm-filtered seawater, stained with 0.1 mg ml^{-1} of DAPI [16], and retained on a black 0.2 μm polycarbonate filter. Subsequently, 400–600 cells were counted in 15–20 ocular fields. Five ml of the bacterial suspension were spiked with 7 μl of [methyl-^{3}H]thymidine, specific activity 48 Ci/mmol (Amersham International), and simultaneously with 10.5 μl of L-[^{14}C(U)]leucine, specific activity 311 Ci/mol (DuPont NEN) and incubated for 1 h. Then the specific activities of precursors were reduced 1000-fold compared to the original activities by addition of nonlabeled thymidine and leucine. After a subsequent 1-h incubation to saturate intracellular pools, label levels stabilized in these dual-radioactively labeled *Vibrio* (DRLV) cells [22]. Preliminary experiments showed that once live DRLV were deposited on filters, they started metabolizing macromolecules with which they had been pulse-chase labeled. The rate of this metabolism was sufficient to obscure the results of grazing of slowly growing protozoa. As a result we sought a way of preserving radioactive labels in bacteria. We decided to kill the DRLV cells for experimental use by a succession of 6–10 bursts of 10 s each in a microwave oven. Microwave irradiation was chosen because of its rapid action on bacterial cells with minimal changes of water properties, compared

with boiling. The latter inevitably results in precipitation of some seawater salts and the crystals interfere with the deposition of bacteria on filters.

After cooling to 10°C, subsamples of 0.15–0.6 ml of these dead DRLV were made up to 1 ml with 0.2 μm-filtered seawater. These final volumes were filtered onto 4.2 cm^2 of transparent 0.4 μm Biopore [hydrophilic poly(vinylidene fluoride)] filters molded in inserts (Millipore) designed to fit into 6-well plates, and washed with 5 ml of 0.2 μm-filtered seawater. To achieve this filtration, the 24 mm diameter insert was placed on a wetted 25 mm diameter GF/B filter, supported on a wetted 47 mm diameter GF/A filter in a 47 mm filter holder (Millipore). Particular care was taken to prevent appearance of bubbles under the insert filter, which could interfere with the even deposition of bacteria on the filter. For better attachment of bacterial cells, each insert filter was first covered with 10 μg of bovine serum albumin dissolved in 0.2 ml of MQ-water, and subsequently dried at 30°C. Three minutes after filtration the inserts with deposited bacteria were placed in wells of sterile plates containing 2 ml of filtered seawater, according to the manufacturer's instructions, 3 ml more were poured carefully along the inside wall of the inserts, and finally 2–20 μl of a culture of protozoa, containing a few tens of amoebae or a few hundreds of flagellates, was inoculated inside each insert. Inserts with deposited bacteria but without added protozoa were used as controls. The growth of protozoa was monitored by removing subsamples of 100 μl of seawater from outside the insert at 15–50 h intervals during 18–25 days. The subsamples were mixed with 5 ml of scintillation cocktail and the amounts of the two radioisotopes assayed simultaneously using a liquid scintillation counter [22]. In parallel, the remaining DRLV bacterial stock was kept and subsamples of 0.15–0.6 ml were collected on glass-fiber filters (GF/F) during the first 3–5 days of the growth experiment for an additional check of label retention. Filters were washed with 5 ml of 0.2 μm-filtered seawater, placed in pony vials, filled with 5 ml of scintillation cocktail, left for solubilization of the biomass for 2–4 days, and radioassayed. At the end of an experiment the inserts were removed from wells, the water within each insert was filtered through its insert filter at −0.1 bar, and 5 ml of 0.2 μm-filtered seawater was passed through to wash both insert and filter. Each filter was then cut out and placed in a pony vial, which was filled with 5 ml of scintillation cocktail and radioassayed.

Additionally, the numbers of growing *Vanella* in the transparent inserts were counted under a stereo microscope at the time of subsampling, and the growth of flagellates was checked qualitatively.

We previously found that protozoa grazing on suspended DRLV rapidly release almost all ^3H label upon ingestion of the labeled bacteria [22]. Therefore, the rate of protozoan grazing could be determined as the difference between the amount of ^3H label in the particulate fraction in the control series without protozoa and in the experimental series with protozoa as a proportion of the amount of ^3H label in the former. The grazing (consumption) can thus be expressed as a fraction of total amount of DRLV, and, when the number and biomass of DRLV are known, it can be transformed into either numbers or biomass of consumed bacteria. By dividing the total number or biomass of consumed bacteria by the total number of amoebae at the end of the growth experiment, the number or biomass of bacteria required to produce an amoeba cell can be estimated. While the ^3H label from food was not retained by grazing protozoa, a proportion of the ^{14}C label from bacteria was assimilated in the predators. This contrast in retention allows the rate of assimilation (or gross growth efficiency) to be determined from the difference between the reduction of radioactivity of ^3H and ^{14}C labels in the particulate fraction in experiments with protozoa compared with controls without protozoa; this quantity can be expressed as a proportion of the reduction of the ^3H label, in order to present the assimilation as a fraction of the consumed prey. Using this fraction and an estimate of the biomass of bacteria required to produce one amoeba, the cell biomass (protein content) of amoebae can be estimated.

The growth rate of amoebae was estimated using an exponential approximation of the increase of protozoan numbers with time. Similarly, an exponential approximation was used to estimate the rate of ^3H label release in the water for comparison with the protozoan growth rates. A sigmoid approximation was used to compare the dynamics of ^3H release of the protozoa studied. All approximations closely describe the original values, with correlation coefficients r^2 of between 0.96 and 0.999.

Results

The Retention of Labels by Dual-Radioactive-Labeled Vibrio *(DRLV)*

Bacteria treated with brief bursts of microwaves retained almost all the ^3H label during the first 6–8 days and gradually released up to 10–20% of the initial amount of the ^3H label during the following 10 days (Fig. 1a), the proportion released depending on the number of deposited bacteria. Throughout the same experiments the amount of the ^{14}C label in the water did not exceed 1% of that originally present in the DRLV (Fig. 1e). The amount of each label in the particulate fraction at the end of the experiments was about 60 ± 8% of that at the beginning of the experiments. Therefore, about 40% of the original amount of the ^{14}C label and 20–30% of the ^3H label, taking account of the amount of the ^3H label found dissolved in the water (Fig. 1a), was not recovered.

From these tests we conclude that the microwave irradiation of dual-radioactive-labeled *Vibrio* resulted in adequate, predictable, and reproducible retention of both labels within bacterial cells, especially during the first half of the growth experiments when the protozoa were growing rapidly. This therefore made it possible to use microwave-killed DRLV deposited on filters as a tool to study the longer-term growth

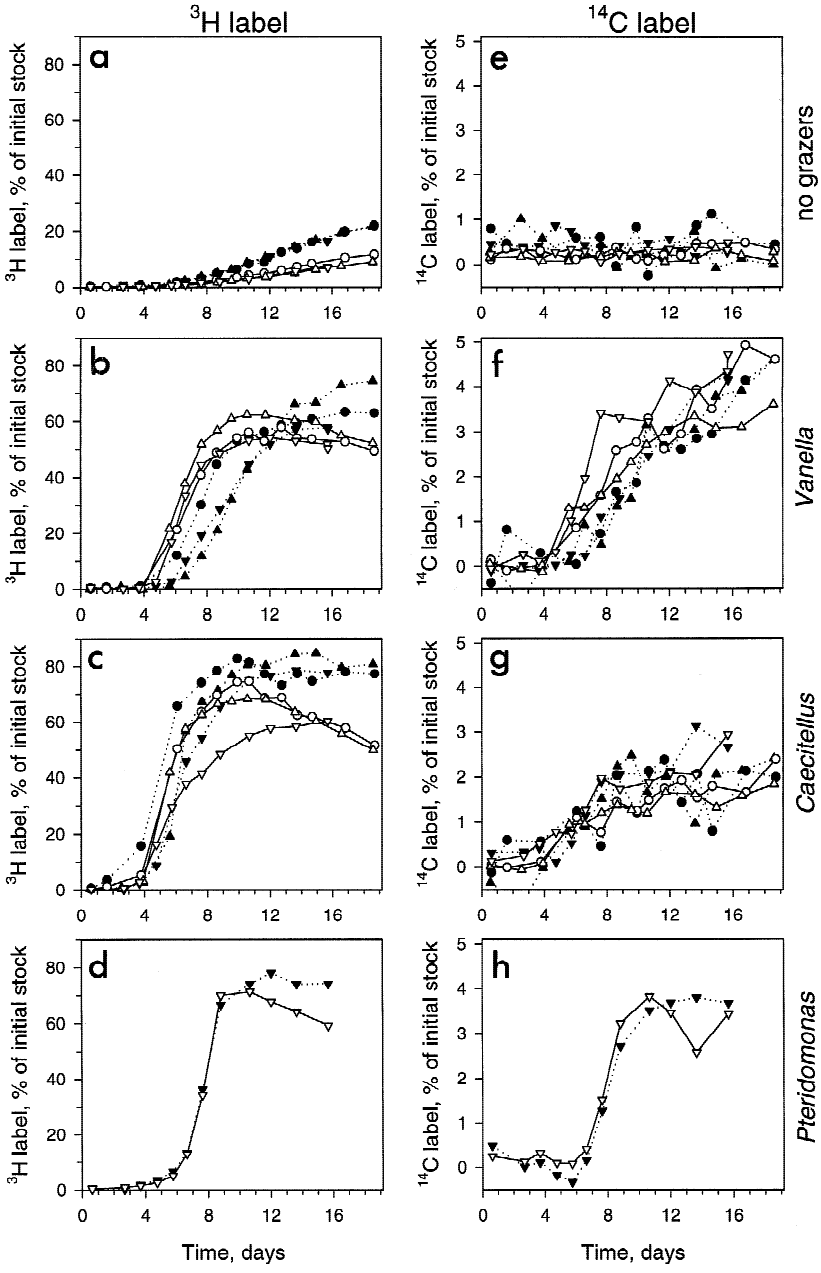

Fig. 1. ^{3}H and ^{14}C label release in the water in various incubation experiments, expressed as a percentage of the amount initially present in the stock of microwave-treated DRLV deposited on insert filters. In control experiments (a,e) bacteria were incubated without protozoan predators; in other experiments the bacteria were incubated with the amoeba *Vanella* (b,f), or the flagellates *Caecitellus* (c,g) or *Pteridomonas* (d,h). In most cases three experimental series (circles, triangles, up and triangles down), each with two different amounts of bacteria, 0.3 μg (filled symbols, dotted lines) and 0.9 μg (open symbols, solid lines) of bacterial protein cm^{-2}, were monitored. One series with two different amounts of bacteria is shown for *Pteridomonas.* The values in b–d and f–h are corrected for the release of ^{3}H label by DRLV in the control experiments.

of surface-dwelling protozoa, tracing radioactivity in both particulate and dissolved fractions.

The Release of Radioactive Labels by Grazing Protozoa

The dynamics of both ^{3}H and ^{14}C labels in the presence of protozoa were corrected for bacterial release of ^{3}H label in the control series according to the proportion of ungrazed bacteria remaining in protozoan cultures. The resulting dynamics clearly differed from those in the controls where protozoa were absent (Figs. 1b–d and f–h, cf. Figs. 1a and e). After an initial lag phase there was a 60–80% release of ^{3}H label into the water between the fourth and tenth days of the growth experiments in all protozoan cultures. Later, the radioactivity in the water either remained at the same high level, in experiments involving the lower initial concentration of DRLV, or somewhat decreased in experiments where the higher initial concentration of DRLV was used (Figs. 1b–d).

The dynamics of the ^{14}C label in the dissolved fraction diverged from that of the ^{3}H label. First, the concentration of

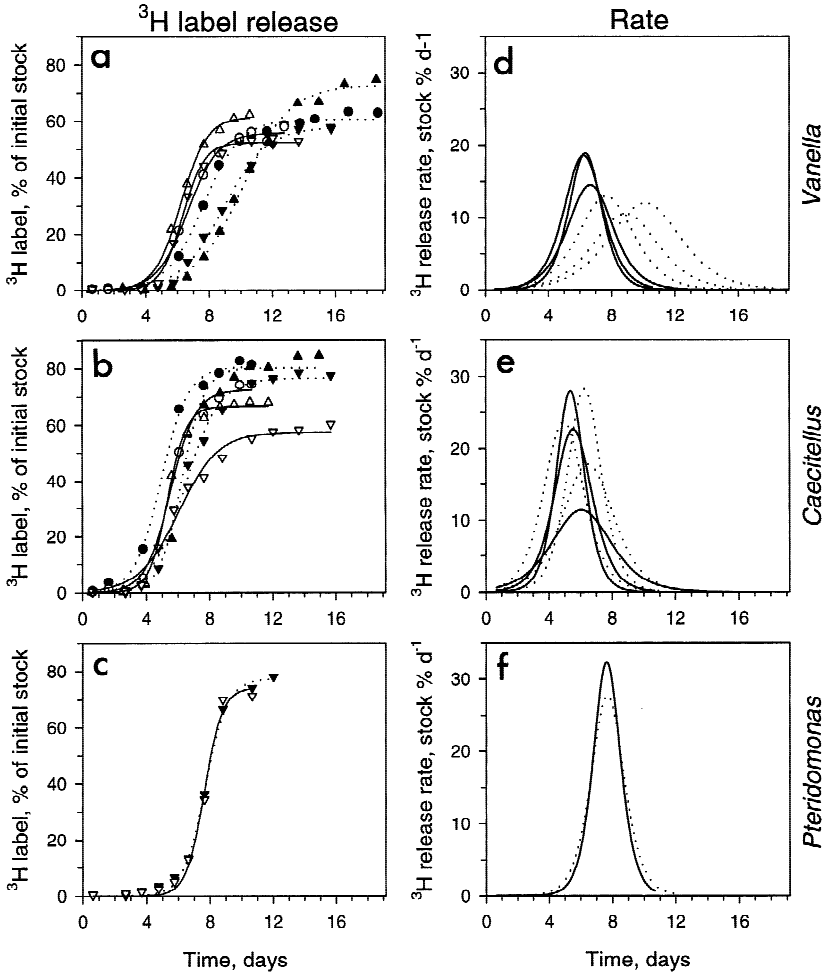

Fig. 2. ^{3}H release in experiments shown in Fig. 1 is modeled by sigmoid approximations based on data for *Vanella* (a), *Caecitellus* (b), and *Pteridomonas* (c). Rates of ^{3}H release derived from these sigmoid approximations for each experiment are shown in d–f.

^{14}C label in the water never exceeded 3–5% of the total initial radioactivity of deposited DRLV. Second, the concentration of ^{14}C label increased gradually in *Vanella* and *Caecitellus* cultures (Figs. 1f,g), and only the dynamics of ^{14}C in the *Pteridomonas* culture resembled the dynamics of dissolved ^{3}H label (Fig. 1h, cf. Fig. 1d).

We used a sigmoid model to simulate ^{3}H label release, including its saturation but excluding the final decrease of the concentration of dissolved ^{3}H label (Figs. 2a–c). This model is adequate ($r^2 > 0.99$) to describe the observed dynamics but does not reveal their nature. However, the bell-shaped curves of the rate of ^{3}H release clearly show the maximum rates (Figs. 2d–f). Using this simplification, it is interesting that *Vanella* reached its maximum ingestion rate earlier than *Pteridomonas* when the amoebae grazed on higher bacterial amounts, but later than *Pteridomonas* when the amoebae grazed on lower bacterial amounts. The amount of deposited bacteria did not affect the maximum rate of ^{3}H release by these flagellates, but *Vanella* grazed quicker and more uniformly on larger amounts of deposited bacteria.

This observation is supported for the amoebae using data on growth in numbers of *Vanella* on different amounts of deposited *Vibrio* (Fig. 2d). When the amount of deposited bacteria was high, 1.4–4.3 μg protein cm^{-2}, the growth of *Vanella* was more uniform compared with growth on low bacterial amounts of 0.3–0.9 μg protein cm^{-2}. The difference in the initial amount of prey stock slightly affected the rate of growth of amoeba cell numbers, which was 0.2 ± 0.035 d^{-1}, $n = 4$, and 0.16 ± 0.025 d^{-1}, $n = 6$, respectively. These estimates of *Vanella* growth rate are about one-third of the estimates of the rate of ^{3}H label release. The maximum rate of the latter was reached earlier than the corresponding maximum rate of growth in numbers of *Vanella,* which occurred after 12–14 d with higher amounts of bacteria and after 9–15 d with lower amounts of bacteria (data not shown).

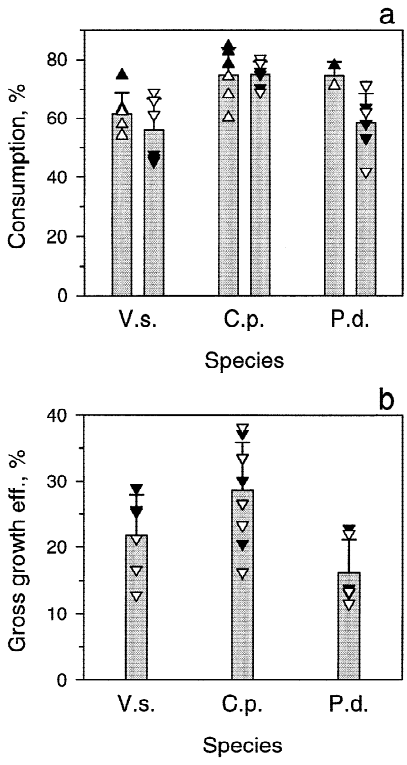

Fig. 3. (a) Comparison of the total consumption of deposited DRLV by the protozoan species: *Vanella* (V.s.), *Caecitellus* (C.p.), and *Pteridomonas* (P.d.) estimated by the release of ^{3}H label in the water (left column in each pair, triangles up) and by the amount of ^{3}H label in the particulate fraction at the end of experiments (right column in each pair, triangles down). (b) Comparison of the gross growth efficiency of the same three species estimated from the difference in the amounts of ^{3}H and ^{14}C label in the particulate fraction at the end of the growth experiments. The symbols show individual estimates derived from the low (filled triangles) and high (open triangles) initial amounts of deposited bacteria. The columns represent the average values, and error bars indicate one standard deviation of these averages.

Comparison of DRLV Consumption and Growth Efficiency of the Protozoa Studied

The total amount of DRLV consumed during the growth of batch cultures of the protozoa studied was estimated in two different ways: from the total release of ^{3}H label in the water (Figs. 1b–d) and from the difference in the amount of ^{3}H label retained in the particulate fraction in the presence and in the absence of protozoa at the end of the experiments. The two estimates agreed fairly well (Fig. 3a), apart from a somewhat higher consumption of *Pteridomonas* estimated by ^{3}H release. However, this might be a result of making fewer measurements with dissolved labels than with particulate ones in this case, and the value of consumption for *Pteridomonas* derived from particulate labels is therefore regarded as more meaningful. In these experiments *Caecitellus* was the most efficient predator, able to consume about 75% of the prey, compared with 55–60% consumed by the other protozoa studied. The gross growth efficiency, estimated as the difference between the relative retention of ^{3}H and ^{14}C labels in the particulate fraction (Fig. 3b), was quite similar for the three protozoans. It was somewhat higher for *Caecitellus* at an average of 29 ± 8%, compared with 22 ± 6% for *Vanella* and only 16 ± 5% for *Pteridomonas.*

Using the estimates of consumption and efficiency of growth, and knowing the original amount of bacterial protein and the numbers of *Vanella* that grew in the batch culture, we could estimate the biomass of amoebae. It was 166 ± 100 pg and 93 ± 45 pg protein cell^{-1} (n = 6) at the time of maximum growth and at the end of the experiments, respectively.

Discussion

In the control experiments where no protozoa were added to the labeled bacteria, about 40% of the original amount of the ^{14}C label and 20–30% of the ^{3}H label, taking into account the amount of the ^{3}H label found dissolved in the water (Fig. 1a), was not recovered. There are two likely reasons for this loss. First, some of the labeled bacteria could be retained on the walls of the inserts and therefore were not present on the radioassayed insert filters. Second, some *Vibrio* cells may not be killed by microwaves, but only damaged, and after a period of rehabilitation of about 6–8 d they could start metabolizing labeled macromolecules, with the consequence that the concentration of the ^{3}H label in the water gradually increased. At the same time, missing ^{14}C-labeled macromolecules were probably completely metabolized, and the major part of the ^{14}C label would be converted to $^{14}CO_2$ under the conditions of the experiment. The water/air equilibration of CO_2 will result in its release into the atmosphere, and this could explain the irreversible loss of the ^{14}C label from the dissolved fraction. By contrast, ^{3}H-labeled metabolic products, possibly including ^3H_2O, accumulated in the water.

In the experiments where bacteria were grazed, the final gradual decrease of the radioactivity due to the ^{3}H label at the higher initial concentration of prey, compared with the steady level after grazing on the lower amount of bacteria, could be due to a disappearance of ^{3}H label incorporated in small volatile molecules as a result of avid, wasteful protozoan feeding on plentiful bacteria. Alternatively, some small labeled molecules could have been reincorporated by scav-

enging, ungrazed bacteria. Correspondingly, it is most probable that bacterial macromolecules labeled with ^{14}C were metabolized by protozoa into $^{14}CO_2$ that escaped into the atmosphere. Because this release into the atmosphere was not traced in these experiments, the use of dissolved ^{14}C label for tracing protozoan grazing has limited value, while the ^{3}H label dissolved in the water can give an instant estimate of the rate of protozoan grazing.

The real mechanism of the release of ^{3}H label is complex and involves at least two interrelated processes: ingestion and digestion of bacteria by protozoa; and subsequent protozoan metabolism and growth, which is eventually limited by the amount of unconsumed prey. A variety of simplifying models can be employed to simulate the dynamics of ^{3}H label release. The initial increase of the amount of ^{3}H label in the water can be adequately approximated by an exponential function with an r^2 value of between 0.93 and 0.99. Because protozoa multiply exponentially when bacteria are in abundance, the rate of exponential release of ^{3}H label, 0.82 ± 0.18 d^{-1}, $n = 6$, for *Caecitellus,* 0.69 ± 0.04 d^{-1}, $n = 2$, for *Pteridomonas,* and 0.47 ± 0.13 d^{-1}, $n = 6$, for *Vanella,* could be related with the specific growth rate of these protozoa. The estimated growth rate of *Caecitellus,* fed on live *Vibrio* prey deposited by centrifugation in a previous study, was similar at 0.81 ± 0.27 d^{-1}, while the growth rate of *Pteridomonas,* fed on suspended *Vibrio* in that earlier study, was almost twice that found here at 1.28 ± 0.27 d^{-1} [24]. The latter difference probably results because the stalked *Pteridomonas* is less well suited to feeding on deposited than on suspended bacteria; it was actually surprising that these ''suspension-feeding'' flagellates managed to ingest deposited DRLV at all.

The reason why *Vanella* consumes bacteria more quickly at higher concentrations is probably the result of contrasting locomotion of flagellates and amoebae. The former are more mobile and can move faster over the bacterial ''lawn'' and consequently are less dependent on the local abundance of bacteria.

It is also interesting that, in comparison to the flagellates, the relatively big *Vanella* cells were separating their grazing and subsequent multiplication in time, presumably avidly consuming food while it was available and starting their division only when they were unable to accomodate any more food. Consequently, they were growing relatively faster in biomass and slower in numbers in batch cultures with limited food supply. Other bacterivorous protozoa such as ciliates tend to have a similar strategy; for example, *Uronema* cells stuffed themselves with 60–80 food vacuoles, each containing about 15 bacterial cells, before they ceased their grazing, almost stopped locomotion, and proceeded with successive stages of food metabolism which resulted in one or two rapid divisions [22].

The observed sequence of growth efficiencies found in these experiments corresponds with the behavior of the predators, since *Caecitellus* is an active flagellate adapted to surface feeding, *Vanella* is a less active surface feeder, and *Pteridomonas* is a suspension-feeding flagellate. The efficiencies found are at the low end of the range reported for these and other flagellates [24] and are characteristic of an advanced stationary stage of protozoan batch cultures, as studied in the present case; see Fig. 2. Consequently, these protozoa regenerated about 70–85% of nutrients, presumably including the nitrogen stored in the protein of DRLV, as traced from the release of label from ^{14}C-leucine. This is potentially a very high rate of regeneration, which can significantly enhance bacterial growth in the immediate vicinity of feeding protozoa.

The estimates of protein biomass of *Vanella* in the range 93–166 pg protein $cell^{-1}$ may be compared with the biomass of *Uronema* at the stationary phase, which was about 500 pg protein $cell^{-1}$ [22], while the biomass of *Caecitellus* was only 3 pg protein $cell^{-1}$ [24]. To our knowledge there have been no published estimates of the protein content of small amoebae, but the mean cell volume of the closely related *Vanella caledonica* grown at 10°C was estimated to be 430 μm^3 for cells preserved with Lugol's iodine [5], which is equivalent to about 230 pg protein $cell^{-1}$ if we use a conversion factor determined for preserved flagellates [23]. The latter estimate is not substantially different from our estimate of the cell biomass of *Vanella septentrionalis.*

The mechanism of grazing by *Vanella* on deposited bacteria is admirably adapted for this purpose. An amoeba clings to the surface, slowly advancing and using its lobopodia first to detach deposited bacteria, then to elevate the hyaline margin of the cell, and finally to ingest the prey into the posterior granulated part of the cell. Certainly this efficient mechanism of detaching bacteria required additional energy investment compared with the avid engulfing of bacteria into a pouch-like structure by *Caecitellus.* The latter has a certain advantage in feeding on loosely attached bacteria, as used in the present study, but would be less efficient at grazing on more firmly anchored bacteria.

The observed grazing of *Pteridomonas* on deposited DRLV is rather surprising, because these suspension-feeding flagellates were the least efficient grazers on bacteria deposited on Nuclepore or Anopore (aluminium oxide) filters (our unpublished data). There is little doubt that DRLV

were not resuspended in the water; otherwise, neither *Caecitellus* nor *Vanella* would grow. It is more likely that DRLV that were initially well attached could be loosened in time by the active solvent action of seawater upon the albumen by which the bacteria adhered to the filters; this detachment of bacteria may explain the delayed growth of *Pteridomonas* (Figs. 2c,f), and it also illustrates the plasticity of flagellate feeding behavior.

In conclusion, the results of the present study suggest that surface-dwelling protozoa, both flagellates and amoebae, can have a similar, substantial impact upon deposited bacteria, consuming 55–75% of the bacteria in batch culture experiments with an estimated gross growth efficiency of 15–30% by the advanced stationary phase, and therefore recycling 70–85% of nutrients ingested with their food. The pattern of growth of bacterivorous protozoa can be followed by monitoring the release of ^{3}H label (originally incorporated in macromolecules of ^{3}H-thymidine-labeled prey bacteria) into the water. Specialized surface-dwelling protozoa, especially small flagellates such as *Caecitellus,* quickly consume deposited bacterial stock. However, flagellates which are primarily suspension-feeding, such as *Pteridomonas,* are also able to consume loosely attached bacteria. Therefore, the described simple experimental system to study grazing of surface-dwelling protozoa proved to be helpful in quantifying the growth and grazing of protozoa and demonstrated the importance of protozoa in the general dynamics of bacterial biofilms. The importance of the predatory role of protozoa, both amoebae and flagellates, in natural biofilms deserves further close quantitative investigation.

Acknowledgment

This work was supported by grant number GST/02/1554 from the Natural Environment Research Council.

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