Applied and Environmental Microbiology, Dec. 2010, p. 8222–8230 Vol. 76, No. 24
0099-2240/10/$12.00 doi:10.1128/AEM.01632-10

Development of a Fatty Acid and RNA Stable Isotope Probing-Based Method for Tracking Protist Grazing on Bacteria in Wastewater▽

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Received 8 July 2010/Accepted 15 October 2010

Removal of potential pathogenic bacteria, for example, during wastewater treatment, is effected by sorption, filtration, natural die-off, lysis by viruses, and grazing by protists, but the actual contribution of grazing has never been assessed quantitatively. A methodical approach for analyzing the grazing of protists on ^{13}C-labeled prey bacteria was developed which enables mass balances of the carbon turnover to be drawn, including yield estimation. Model experiments for validating the approach were performed in closed microcosms with the ciliate *Uronema* sp. and ^{13}C-labeled *Escherichia coli* as model prey. The transfer of bacterial ^{13}C into grazing protist biomass was investigated by fatty acid (FA) and RNA stable isotope probing (SIP). *Uronema* sp. showed ingestion rates of ~390 bacteria protist^{-1} h^{-1}, and the temporal patterns of ^{13}C assimilation from the prey bacteria to the protist FA were identified. Nine fatty acids specific for *Uronema* sp. were found (20:0, i20:0, 22:0, 24:0, 20:1ω9c, 20:1ω9t, 22:1ω9c, 22:1ω9t, and 24:1). Four of these fatty acids (22:0, 20:1ω9t, 22:1ω9c, and 22:1ω9t) were enriched very rapidly after 3 h, indicating grazing on bacteria without concomitant cell division. Other fatty acids (20:0, i20:0, and 20:1ω9c) were found to be indicative of growth with cell division. The fatty acids were found to be labeled with a percentage of labeled carbon (atoms percent [atom%]) up to 50. Eighteen percent of the *E. coli*-derived ^{13}C was incorporated into *Uronema* biomass, whereas 11% was mineralized. Around 5 mol bacterial carbon was necessary in order to produce 1 mol protist carbon ($y_{x/s} \approx 0.2$), and the temporal pattern of ^{13}C labeling of protist rRNA was also shown. A consumption of around 1,000 prey bacteria (~98 atom% ^{13}C) per protist cell appears to be sufficient to provide detectable amounts of label in the protist RNA. The large shift in the buoyant density fraction of ^{13}C-labeled protist RNA demonstrated a high incorporation of ^{13}C, and reverse transcription–real-time PCR (RT-qPCR) confirmed that protist rRNA increasingly dominated in the heavy RNA fraction.

Water is a scarce resource, and with an increasing world population of humans, agriculture will become the biggest freshwater consumer worldwide (60). Treated wastewater will be of increasing importance for irrigation in agriculture, particularly in countries with scarce water resources (3, 61). Therefore, there is an urgent need to establish cheap and safe techniques to treat wastewater for irrigation, e.g., by use of constructed wetlands (34).

The removal processes for potential pathogenic bacteria during wastewater treatment in such systems are considered to be a combination of adsorption, filtration, natural die-off, lysis by viruses, and grazing by protists. Physical, chemical, and biological mechanisms are involved in the removal process (18, 51, 54). The biological removal processes include predation by bacterivorous organisms, natural die-off, lysis by phages, and competition for limiting nutrients or trace elements (28, 50). Although it is widely applied in technical systems, there has been disagreement about the relative contributions of biological elimination by predation (14, 16, 17, 57). Grazing by protists is considered to contribute to bacterial elimination (27, 40), but, for example, the extent of pathogen elimination by protist grazing in wastewater systems has rarely been quantified, and the key organisms involved are poorly understood. Various methods have been used to determine protist grazing rates, e.g., optical density measurements (65), fluorescence *in situ* hybridization of prey bacteria in ciliate food vacuoles (20), visualization with high-speed cameras to evaluate the ingestion rate for small flagellates (7), or quantification of fluorescently labeled prey bacteria (26). While these techniques allow the grazing potential of the protists to be estimated, the elimination of the prey bacteria and identification of the feeding grazers in complex environmental systems have been revealed in only a few cases (23, 44).

Combining stable isotope probing (SIP) for various biomarkers with molecular biological techniques provides powerful tools for investigating the trophic interactions between microorganisms and the transfer of biomass carbon between trophic levels. Applying RNA-SIP, Lueders et al. (36) identified potential micropredators of ^{13}C-labeled *Escherichia coli* in soil and Frias-Lopez et al. (23) identified the protists actively grazing on marine picocyanobacteria. Other authors identified ciliate grazers on ammonia-oxidizing bacteria in activated sludge (44). While nucleic acid-based SIP applies only the phylogenetic marker information for a taxonomic affiliation of the organisms involved in the elimination of bacterial prey, no additional quantitative information on carbon fluxes between trophic levels can be obtained. Combining these techniques with use of the more sensitive but less specific biomarker fatty acids (FA-SIP) allows the quantitative description of carbon fluxes in complex environmental media, such as soils or marine systems (12, 25,

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▽ Published ahead of print on 29 October 2010.

32, 41). It thus appears that the combination of nucleic acid and FA-SIP opens up new possibilities for studying bacterial removal by protists, for example, in wastewater.

Therefore, the objective of this study was to develop a methodical approach for future application in complex environmental systems by tracing the elimination of ^{13}C-labeled bacterial prey during protist grazing. In a model experiment with defined conditions, the mineralization, metabolization, and incorporation of the bacterial carbon into the biomarkers (fatty acids and RNA) were analyzed, and the advantages and constraints of this approach were evaluated. A closed microcosm system was developed, and the ciliate *Uronema* sp. and ^{13}C-labeled *E. coli* cells as potential pathogenic model prey bacteria were applied in the experiment.

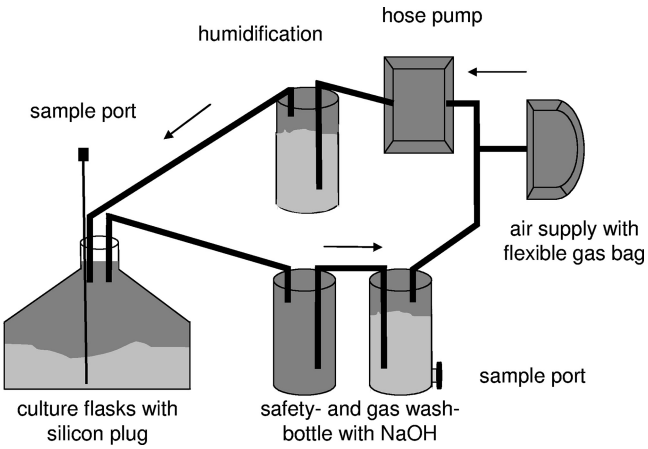

FIG. 1. Setup of the grazing experiment in a closed system with circulating and humidified airflow. Samples were taken at two septum ports without opening the system. The pressure loss caused by the absorption of the produced CO_2 in NaOH was compensated for by providing oxygen from a flexible gas bag, resulting in an oxygen-consumption-depending and self-dosing system.

MATERIALS AND METHODS

Chemicals. The chemicals and solvents were obtained in per analysis quality from Merck unless stated otherwise. D-[U-^{13}C]Glucose, with a chemical and isotope purity of >99%, was obtained from Chemotrade (Leipzig, Germany).

Isolation of protists and cultivation conditions of organisms. Water samples were taken from a pilot constructed wetland (CW) in Langenreichenbach (Saxony, Germany), which is supplied with raw domestic wastewater from a municipal sewage plant (for details, see reference 4). The CW achieves an *E. coli* reduction of 5 $\log_{10}$ units, with an *E. coli* inflow concentration of 10^7 CFU 100 ml^{-1} based on most-probable-number (MPN) determination (3). Two-liter water samples were taken with a peristaltic pump (80 ml min^{-1}) from various sampling tubes installed in the filter beds of the plant, transported to the laboratory, and processed within 4 h after sampling.

Protists were isolated by using stepwise dilution series of samples with Page's amoeba saline (PAS) (47) and addition of 5×10^7 ml^{-1} *E. coli* as a food source. Single ciliate cells were separated with a PatchMan micromanipulator (Eppendorf, Hamburg, Germany) and subsequently cultivated in PAS in 50-ml tissue culture flasks in the dark at 20°C; they were fed with 5×10^7 ml^{-1} food bacteria. Using standard DNA extraction, PCR amplification of the 18S rRNA gene with *Eukarya*-specific primers (9) and subsequent sequencing affiliated the isolate to *Uronema* sp.

For the grazing experiments, ^{13}C-labeled *Escherichia coli* strain RFM443 (49) was used as a model organism for potential pathogenic bacteria. *E. coli* cultures were grown in minimal medium with 2 g liter^{-1} ^{13}C-labeled D-glucose as a sole carbon source at 37°C (32). Early-stationary-phase cells were harvested by centrifugation and washed three times in phosphate-buffered saline (PBS) (pH 7), resuspended in PAS, and used immediately. Unlabeled cells for the control experiment were obtained by growth in the same medium but with unlabeled glucose. Bacterial cell numbers were measured with a Multisizer 3 Coulter counter (Beckmann Coulter, Krefeld, Germany) using a 20-μm capillary, and protists were measured using a 100-μm capillary. The isolated *Uronema* sp. organisms were precultured with 5×10^7 unlabeled *E. coli* cells ml^{-1} in stepwise-increasing cultivation volumes from 20 ml up to 1 liter. The ciliates were fed 24 h before the experiment started and cultured at 20°C in the dark to allow adaptation to the experimental conditions. This procedure kept the bacteria remaining after 24 h at a very low background level while at the same time avoiding starvation of the protists.

Experimental setup. A modified microcosm bioreactor system (32, 56) was built in order to measure the incorporation of bacterial carbon into protistan biomass during grazing experiments (Fig. 1). Briefly, the construction is a closed system with circulating airflow and CO_2 traps. All tubes are gas-proof Tygon R36003 (diameter, 15.9 mm; wall, 4.8 mm; Carl Roth, Karlsruhe, Germany) to minimize the gas loss of the system. Fernbach flasks (2.5 liters; Carl Roth, Karlsruhe, Germany) filled with 1 liter preculture of *Uronema* sp. were used as reactors in order to maximize the surface of the air-water interface. For the grazing experiment, 5.8×10^7 *E. coli* cells ml^{-1} (≈1.25 mmol C per treatment) were added to the vessels containing pregrown cells of the *Uronema* sp. The experiment was performed with unlabeled cells (control) and ^{13}C-labeled *E. coli* cells at 20°C in the dark, each in triplicate systems. The flasks were aerated with humidified air pumped by a multichannel peristaltic pump (flow rate, 2.8 liters h^{-1}). The CO_2 produced was collected in traps, each containing 50 ml of 0.1 M NaOH solution. The decrease in atmospheric pressure caused by adsorption of the CO_2 produced by respiration in the systems was compensated for by oxygen from a flexible gas bag (Tesseraux, Bürstadt, Germany), resulting in an O_2 consumption-depending and self-dosing system. Samples for cell counts, RNA, and lipid extractions were taken at t_0 and after 3, 6, 9, 12, 15, 24, and 50 h. A sample at 70 h was used for RNA analyses.

Analytical procedures. The amount and the isotopic composition of CO_2 were determined for samples collected after 12, 24, and 50 h according to the method of Miltner et al. (42) with some modifications. Briefly, 10-ml aliquots of the samples from the traps were titrated with 0.05 M HCl to pH 10.5 after precipitation with 2 ml of 1 M $BaCl_2$. The ^{13}C/^{12}C isotopic ratio of the CO_2 was determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) from the headspace of acidified 3-ml aliquots of the same solutions. GC-IRMS was conducted using a Finnigan MAT 252 isotope ratio mass spectrometer with a GC combustion interface (Finnigan, Bremen, Germany) coupled to an HP 6890 Plus gas chromatograph (Hewlett Packard, Wilmington, DE) as described previously (42).

Fatty acid (FA) analysis of total lipids from bacteria and protist cells was used to determine the ^{13}C incorporation into protist biomass, calculating the amount of labeled bacterial ^{13}C incorporated into protist biomass by multiplying the amount of label in specific protistan FA with the approximate abundance of protistan FA. For the extraction and analysis of lipids, triplicates of 60-ml samples were filtered on 0.2-μm Anodisc 47 filter membranes (Whatman, Dassel, Germany) and extracted using a chloroform-methanol-buffer system (5) as modified by White and Ringelberg (58). The separated phase containing the lipid fraction was dried under a gentle stream of nitrogen and transesterified to fatty acid methyl esters (FAMEs) by a mild alkaline methanolysis (29). The dried total lipid fatty acid methyl esters (TLFAME) were dissolved in *n*-hexane containing 59 pmol μl^{-1} 21:0 FAME as an internal standard. For the identification and quantification of the TLFAME, a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass spectrum analyzer (Agilent, Palo Alto, CA) was used. The carboxylic acid fractions were separated on an HP5-5MSI column (30 m by 0.25 mm by 0.25 μm) by a splitless injection of 1-μl sample with the following temperature program: initial temperature of 50°C for 1 min, heat at 50°C min^{-1} to 170°C, heat at 4°C min^{-1} to 300°C, and then heat at 40°C min^{-1} to 320°C and hold for 10 min.

The fatty acids were designated according to the A:BωC system, where A is the number of carbon atoms, B is the number of double bonds, and C is the distance of the closest double bond (unsaturation) from the aliphatic end (ω nomenclature) of the molecule. The prefixes i (iso) and a (anteiso) refer to methyl branching, whereas "cy" assigns a cyclopropyl fatty acid. Absolute and relative amounts of fatty acids in the samples were determined according to the concentrations of the added internal FAME standard. Since the isotope composition of the highly enriched ^{13}C-labeled fatty acids could not be analyzed accurately by using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), mass spectrometric analysis was used for the determination of isotope enrichment into fatty acids, as follows (10): the ^{13}C incorporation into fatty acids causes the appearance of a series of isotopomers beside the natural molecular

ion (M^+) (1, 22). According to the distribution of these isotopomers, the percentage of labeled carbon (atoms percent [atom%]) in the fatty acids can be described by

$$\text{atom\%} = \Sigma(\text{ratio of } ^{13}\text{C isotopes incorporated}) \times (\text{frequency of respective isotopomer}) \quad (1)$$

The factors of the equation can be calculated by

$$\text{ratio of } ^{13}\text{C isotopes incorporated} = \frac{J}{N} \quad (2)$$

and

$$\text{frequency of respective isotopomer} = \frac{A_{M+J}}{A_T} \quad (3)$$

where J is the number of ^{13}C isotopes, N the number of carbon atoms in the TLFA, and A_{M+J} is the abundance of the respective isotopomer. A_T is the total abundance of all isotopomers and can be described by

$$A_T = \sum_{J=1}^{N} A_{M+J} \quad (4)$$

Hence, the determination of atom% ^{13}C in the carbons of fatty acids can be described by

$$\text{atom\%} = \sum_{J=1}^{N} \frac{J}{N} \times \frac{A_{M+J}}{A_T} \quad (5)$$

RNA extraction and density gradient centrifugation. Ten ml of each reactor sample was filtered on a 0.2-μm cellulose acetate membrane filter (Whatmann, Dassel, Germany). Total RNA was extracted with Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions with some modifications. Briefly, the filters were cut into small pieces and transferred with 1 ml Trizol into lysic matrix b (Fast RNA; Bio 101 System, La Jolla, CA). The mixture was homogenized with a FastPrep cell disrupter (FP120; Bio101 Systems, La Jolla, CA) at speed 6 for 20 s and subsequently cooled on ice. The following steps were performed according to the manufacturer's protocol. The extracted RNA was precipitated with isopropanol overnight at −20°C. After washing with 70% ethyl alcohol (EtOH), the pellet was dried and resuspended in 30 μl RNase-free water. RNA extracts were further digested with RNA-*free* DNase (Ambion-Applied Biosystem, Darmstadt, Germany) prior to quantification using RiboGreen nucleic acid quantification dye (Invitrogen, Karlsruhe, Germany). Density gradient centrifugation was performed in 5-ml tubes in an Optima ultracentrifuge (Beckmann, Germany) using a near-vertical rotor (NVT 65.2; Beckmann, Germany). Centrifugation was performed at 20°C and 39,000 rpm for 65 h in cesium trifluoroacetate (CsTFA) gradients as described previously (38, 39) with some modifications. Briefly, the centrifugation media were prepared by mixing 4.65 ml CsTFA (2 g ml^{-1}), up to 850 μl of gradient buffer (GB) (0.1 M Tris-HCl, pH 8, 0.1 M KCl, 1 mM EDTA) and 175 μl formamide. Five hundred nanograms of extracted RNA was used for gradient centrifugation (RNA volume was subtracted from the GB volume). Gradient layers were harvested in 12 equal fractions according to the protocol of Lueders et al. (37), and the density of a small aliquot (60 μl) was measured with an AR200 refractometer (Reichert, New York). RNA was subsequently precipitated with isopropanol, washed twice with 70% ethanol, resuspended in diethyl pyrocarbonate (DEPC)-treated water, and subsequently stored (−80°C) for further investigation. The amount of RNA from each density fraction was determined using the RiboGreen assay.

Quantitative PCR. Four μl of RNA from each fraction was reverse transcribed by random priming using the RevertAid H Minus cDNA synthesis kit (Fermentas) for first-strand cDNA synthesis. This cDNA was quantified by reverse transcription–real-time PCR (RT-qPCR) with RT^2 PCR master mix SYBR green (SuperArray) in an iCycler iQ system (Bio-Rad, München, Germany) and standardized to relative ng μl^{-1} units using a dilution series of agarose gel-purified M13-PCR products of cloned 16S and 18S rRNA from *E. coli* and *Uronema* sp. Bacterial 16S rRNA was quantified using the primers F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 342R (5′-CTGCTGCSYCCCGTAG-3′) (35), whereas eukaryotic 18S rRNA was quantified with 18S-F (5′-AGGGTTCGATTCCGGAG-3′) and 18S-R (5′-GGAGCTGGAATTACCGC-3′) (21). Thermal cycling was an initial denaturation step for 15 min at 95°C, followed by 35 amplification cycles (40 s at 94°C, 50 s at 53°C for bacteria or 54.5°C for protists, and 50 s at 72°C), and a terminal extension step (5 min at 72°C). Melting curves were recorded after each run between 60°C and 94.5°C in order to discriminate between specific signals and signals from primer dimers. The efficiency of each PCR was checked with dilution series of the RNA used and was always in a similar range around 85%.

Calculations and statistics. Except where stated otherwise, all analyses were performed in triplicate. The results are shown as means with standard deviations.

The isotopic data of the TLFAME were used to calculate the amount of theoretically completely ^{13}C-labeled FA. Isotopic fractionation during sample preparation was assumed to be negligible compared to the high ^{13}C enrichment in the samples. The methylation of FA results in the introduction of one additional carbon atom at natural abundance. In our calculations, however, the additional carbon was automatically accounted for because we calculated the absolute amount of ^{13}C present in the TLFAME of a given sample, which then was represented by concentrations of theoretically completely ^{13}C-labeled FA.

Measured CO_2 values were corrected as follows: since for each measurement 80 ml of medium was removed from the Fernbach flasks, the measured values were linearly scaled up to the initial volume of 1 liter, taking into account all distinct time points.

Protist ingestion rates I (bacterial cells $protist^{-1}$ $hour^{-1}$) were estimated with the equation (30) $I = (\mu_{\text{bacteria control}} - \mu_{\text{bacteria experiment}}) * F_{m\ \text{prey}}/F_{m\ \text{predator}}$, where F_m is the mean concentration of the cells (bacterial or protist ml^{-1}). F_m was calculated for the time of an exponential increase according to the following method: $F_m = (F_t - F_0)/(\ln F_t - \ln F_0)$, where F_0 and F_t refer to the concentrations of the cell at the beginning and at the end of the time interval, respectively. The growth rate μ was calculated by the equation $\mu = (\ln F_t - \ln F_0)/\Delta t$, where F_0 and F_t denote the concentrations of protist at the beginning and at the end of the time interval (Δt) of exponential increase (62). In the predator-free control, there was no significant decrease in bacterial cell numbers observed for the first 12 h, so we decided to use the value 0 for the term $\mu_{\text{bacterial control}}$. Thus, in the equation, only the start and end of the bacterial growth and the ratio of prey to predators were relevant.

Different conversion factors were used to convert biovolumes into the corresponding carbon masses, covering a representative range of data for bacteria (240 ± 70 fg C μm^{-3}) (24) and protists (200 ± 50 fg C μm^{-3}) (15). Biovolumes of *E. coli* cells were determined to be 1.5 to 1.8 μm^3, and those of *Uronema* sp. were determined to be 540 to 830 μm^3. The biovolume of protists was estimated from microscopy (Axioskop; Zeiss, Germany) measurements of linear cell dimensions using AxioVision 3.1 imaging software and simple geometric models as described by Børsheim and Bratbak (11). Biovolumes of bacteria were estimated in the same manner using models described by Bratbak (13).

RESULTS

Ingestion rates, population dynamics, and CO_2 production. A closed microcosm bioreactor system was established to operate the grazing experiments with unlabeled and ^{13}C-labeled *E. coli* cells, each in triplicate, at the one-liter scale (Fig. 1). After 12 h, *Uronema* sp. showed ingestion rates of 379 ± 4 bacteria $protist^{-1}$ h^{-1} with unlabeled and 392 ± 76 with ^{13}C-labeled prey bacteria. Abundances of *E. coli* cells increased during the first 3 h, indicating an initial stimulation effect, followed by a reduction of 3 orders of magnitude toward the end in both grazing experiments. The *E. coli* control cultures without protists did not show any bacterial growth (data not shown). The number of protists in the ^{12}C setup increased to $2.0 \times 10^4 \pm 1.0 \times 10^3$ cells ml^{-1} in the first 24 h and then to $6.5 \times 10^4 \pm 6.5 \times 10^3$ cells ml^{-1} after 50 h (Fig. 2, ^{12}C), whereas abundances of protists fed with ^{13}C-labeled bacteria did not change as much within the first 24 h (Fig. 2, ^{13}C) and reached a maximum value of $2.5 \times 10^4 \pm 3.3 \times 10^3$ at 50 h. Repeated cultures showed similar trends but with various final numbers of protists. The differences were also reflected in the CO_2 production over time (Fig. 3), with a 2-fold higher production in the ^{12}C experiment than in the ^{13}C experiment. This indicates that turnover-rate-decreasing effects of ^{13}C-labeled

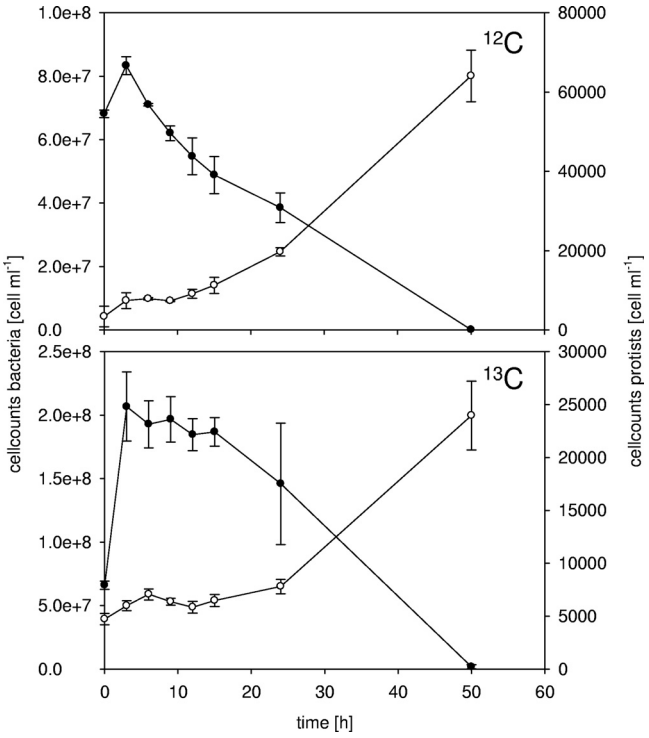

FIG. 2. Triplicate cell counts of ^{12}C and ^{13}C experiments. *E. coli* cell numbers (●) are shown on the left axis, and *Uronema* sp. counts (○) are shown on the right axis. Error bars indicate the standard deviations among triplicate results.

substrates can be expected in such experiments, as has already been reported for some experiments with low-molecular-weight substrates (22). However, these effects need to be analyzed in more detail in future experiments. At the end of the experiment, 16.6% of all produced CO_2 was ^{13}C labeled. This represents 10.7% of the applied label (i.e., labeled bacterial carbon) or about 6.0×10^9 *E. coli* cells.

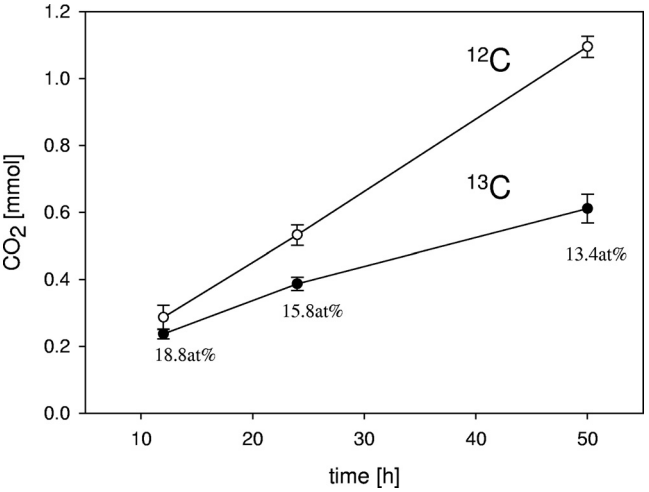

FIG. 3. CO_2 production in mmol during protist grazing on *E. coli*. Error bars indicate the standard deviations among triplicate results. ^{13}C enrichment of CO_2 is given in atoms percent (at%).

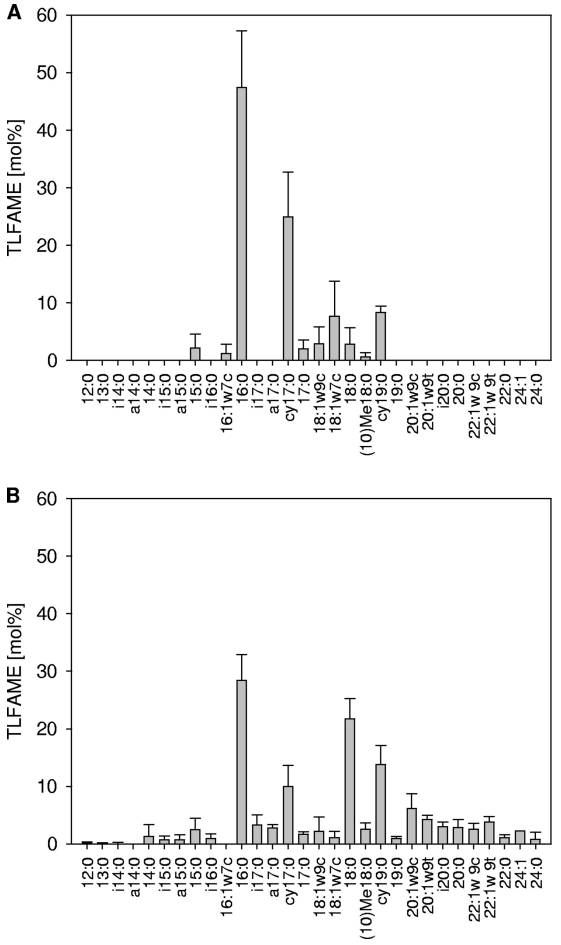

FIG. 4. Total fatty acid profile extracted from *E. coli* RFM 443. (A) Pure culture of early stationary phase. (B) Microcosms before the start of the grazing experiment and the inoculation of *E. coli* into the microcosms. Standard deviations are indicated by error bars.

Fatty acid SIP. The FA profile of *E. coli* RFM 443 is dominated by four typical FA (54), i.e., 16:0, cy17:0, 18:1ω7c, and cy19:0, which represented 88.5% of the total FA (Fig. 4A). Some unsaturated (16:1ω7c and 18:1ω9c) and saturated (15:0, 17:0, and 18:0) FA were found at levels below 10%. Because *E. coli* cells could not be completely separated from *Uronema* sp. cells, the characteristic protist FA was determined by excluding all bacterial FA (Fig. 4A) from the mixed-culture FA profile. The FA composition extracted from the microcosms before the start of the experiment is shown in Fig. 4B. This sample contains mainly protist cells, with some remaining prey bacteria in the culture medium and in the food vacuoles.

After subtraction of the bacterial FA profile, nine FA (20:0, i20:0, 22:0, 24:0, 20:1ω9c, 20:1ω9t, 22:1ω9c, 22:1ω9t, and 24:1) can be considered to represent *Uronema* sp. FA. After the inoculation of *E. coli* cells into the microcosms, the total amounts of FA reached a maximum at 15 h and decreased thereafter due to the decreasing of bacterial cell numbers by grazing (Fig. 5A). The fatty acid profile of the mixed culture was dominated during the entire experiment by the FA 16:0, cy17:0, and cy19:0 (Fig. 5A), which add up to 83% of the total amount. The fatty acids assigned to *Uronema* sp. (20:0, i20:0,

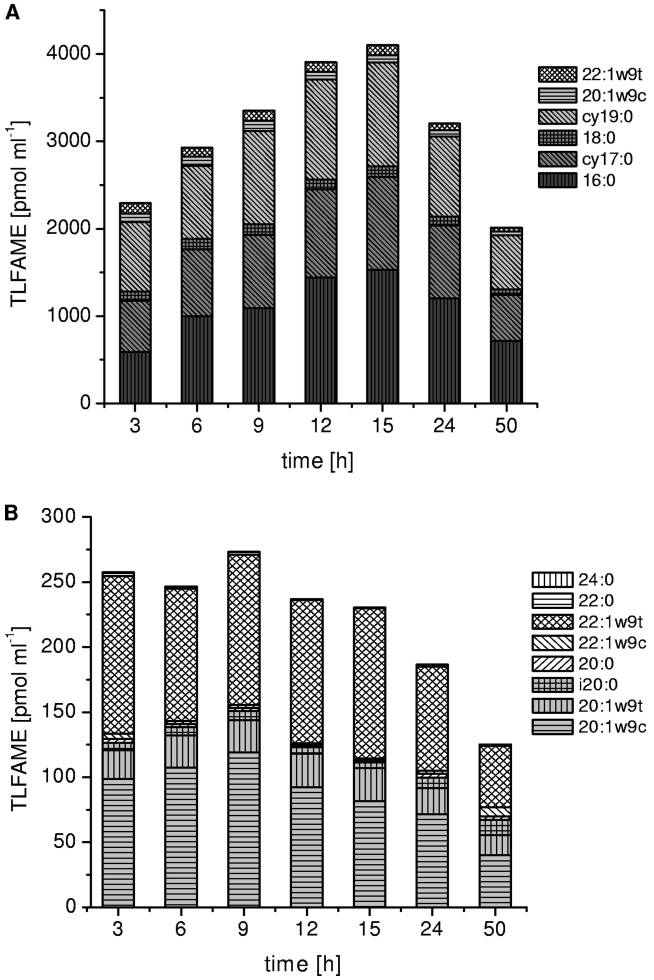

FIG. 5. (A) Dynamics of TLFAME extracted from ^{13}C-labeled-*E. coli*-fed microcosms sampled between 3 h and 50 h. All fatty acids with more than 3% abundance are presented. (B) Dynamics of *Uronema* sp. TLFAME extracted from ^{13}C-labeled-*E. coli*-fed microcosms between 3 h and 50 h.

22:0, and 24:0 and the unsaturated fatty acids 20:1ω9c, 20:1ω9t, 22:1ω9c, and 22:1ω9t) represented <10% of the total amount (Fig. 5B). The signal of FA 24:1 and in most cases also 24:0 was too small for analysis during the experiment. The FA composition remained stable during the experiment, with only slight changes in relative abundances.

In addition, FA analysis of total lipids from bacteria and protists was used to determine incorporation of ^{13}C into protist biomass. After 3 h, the fatty acids of *Uronema* sp. already showed a ^{13}C enrichment of 15 to 60 atom% (Table 1), although protist cell numbers did not change significantly. This is a clear indication that the carbon from *E. coli* was assimilated before the protists started cell division. The data show that the incorporation of ^{13}C starts immediately as a result of grazing whereas actual growth together with cell proliferation was delayed. While all protist FA were ^{13}C labeled within 3 h, only one group (20:0, i20:0, and 20:1ω9c) became additionally enriched as soon as protist cell numbers significantly increased, i.e., after 24 h (Table 1). Only two protistan FA (terminal-branched i20:0 and unsaturated 22:1ω9c) showed an increase in the total amount toward the end of the experiment (from 3.0 to 11.6 and 4.0 to 6.74 pmol/ml, respectively).

Overall, there was average ^{13}C enrichment in the *Uronema* sp. FA of 51 ± 11 atom% due to the incorporation of ^{13}C derived from *E. coli* by grazing. Assuming that the *Uronema*-specific FA in general account for approximately 0.5 to 1% (6, 41, 55) and that the total lipid fatty acid methyl esters (TLFAME) represent 10% of the protist biomass, roughly 9 to 18% of the inoculated bacterial carbon was actually incorporated by the protists. This corresponds to 5.5×10^9 to 1.1×10^{10} of the provided *E. coli* cells. Alternatively, calculating the increase in protistan biomass via biovolume measurements and cell counts, it was shown that 17.9% ± 3.6% of the inoculated bacterial carbon was finally incorporated into protists. This amount equals $1.0 \times 10^{10} \pm 0.3 \times 10^{10}$ *E. coli* cells. These transfer rates are in a range similar to those found by other authors (37, 59). Overall, 29% of the *E. coli*-derived carbon was mineralized or incorporated into protist biomass.

RNA stable isotope probing. RNA served as the second biomarker for tracing the transfer of bacterial carbon into actively grazing protist cells. For the organism-specific quantification of ^{13}C-enriched RNA molecules, we extracted total RNA from samples taken after 6, 12, 24, 50, and 70 h and separated ^{13}C-enriched (i.e., heavy) from ^{12}C-unlabeled (i.e., light) RNA by equilibrium density gradient centrifugation (37). The total amounts of RNA, as well as *Bacteria*- and *Eukarya*-specific rRNA, within all gradient fractions were quantified. ^{12}C-labeled *E. coli* RNA alone was found to show a band at a buoyant density (BD) of 1.79 g ml^{-1} (data not shown), whereas the maximum BD of mixed-culture ^{12}C-labeled RNA was at 1.81 to 1.82 g ml^{-1} (Fig. 6A).

During the first 24 h, RNA from ^{13}C-fed microcosms formed broad peaks between BD of 1.79 and 1.84 g ml^{-1} (Fig. 6A),

TABLE 1. ^{13}C enrichment of TLFAME derived from *Uronema* sp.

Time (h)	^{13}C enrichment of fatty acid (atom%)						
	20:0	Iso 20:0	20:1ω9c	20:1ω9t	22:0	22:1ω9c	22:1ω9t
0	6.0 ± 3.2	7.7 ± 1.9	7.3 ± 1.5	8.2 ± 3.3	7.9 ± 4.5	6.9 ± 3.7	6.5 ± 3.7
3	15.7 ± 3.7	31.1 ± 4.7	41.5 ± 4.1	47.9 ± 3.4	18.6 ± 9.3	53.6 ± 4.3	57.5 ± 11.6
6	15.3 ± 5.0	31.2 ± 4.9	48.4 ± 2.8	34.1 ± 18.6	20.7 ± 9.4	42.4 ± 4.6	51.3 ± 4.5
9	17.5 ± 2.2	37.7 ± 7.7	42.7 ± 2.7	47.7 ± 3.0	22.6 ± 5.2	54.4 ± 4.6	52.7 ± 4.2
12	18.1 ± 8.8	32.1 ± 3.5	42.6 ± 2.8	47.5 ± 3.1	26.8 ± 15.6	53.8 ± 4.8	70.8 ± 3.5
15	20.2 ± 4.1	39.7 ± 7.2	42.5 ± 2.9	48.4 ± 3.3	26.3 ± 6.2	54.0 ± 4.5	56.2 ± 6.8
24	18.3 ± 4.8	44.5 ± 4.6	41.5 ± 2.5	47.1 ± 4.0	14.6 ± 5.8	52.5 ± 5.3	59.8 ± 9.9
50	43.6 ± 3.4	53.8 ± 3.1	50.6 ± 6.5	46.0 ± 8.0	23.2 ± 4.4	55.5 ± 6.2	57.5 ± 8.5

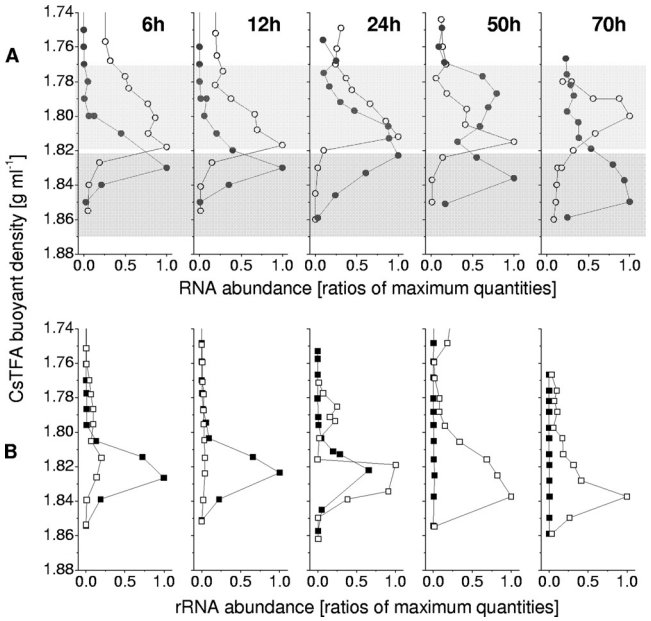

FIG. 6. CsTFA density gradient centrifugation of RNA extracted after various times of grazing on ^{13}C-labeled and unlabeled *E. coli*. (A) Total RNA within gradient fractions was quantified fluorometrically (^{12}C-labeled RNA [○] and ^{13}C-labeled RNA [●]). The ^{12}C area (i.e., light RNA) is indicated in light gray; the ^{13}C area (i.e., heavy RNA) is indicated in dark gray. (B) The domain-specific ^{13}C rRNA template distribution within gradient fractions was determined with quantitative RT-qPCR. Shown are the distributions of *E. coli* 16S rRNA (■) and *Uronema* sp. 18S rRNA (□).

representing a mixture of light and heavy RNA. After 6 h, a first clear enrichment in the RNA was detected, which corresponds to a consumption of around 996 bacterial cells per protist cell. After 6 and 12 h of grazing, heavy ^{13}C-labeled RNA was present at a BD of 1.826 g ml^{-1} (Fig. 6A). Quantification of bacterial and eukaryotic small subunit (SSU) rRNA in these samples by RT-qPCR showed that *E. coli* rRNA was predominating in the heavy fractions whereas only a small amount of *Uronema* sp. rRNA was labeled (Fig. 6B). The increase of eukaryotic rRNA in the heavy fractions at 24 h suggests an incorporation of ^{13}C derived from the *E. coli* cells into protistan biomass by the grazing activity of *Uronema* sp. Only a small peak of light rRNA from *Uronema* sp. was observed, indicating the presence of certain inactive protist cells. After 24 h, heavy RNA fractions consisted mainly of protistan rRNA, indicating a high degree of labeling within the ciliate population. Determination of RNA after 50 h resulted in two distinct RNA peaks in the light and heavy gradient fractions, which, however, were not reflected by RT-qPCR of 18S rRNA. At the end of the experiment at 70 h, fluorometric analysis showed only one clear broad peak at a BD of 1.825 to 1.85 g ml^{-1}, which was correlated to nearly the same distribution of protistan rRNA. Overall, the data show that in the beginning of the grazing experiment, the extracted RNA contained a mixture of ^{12}C-labeled RNA from *Uronema* sp. and ^{13}C-labeled RNA from *E. coli*, but after grazing more than 70 h, the RNA consisted mostly of ^{13}C-labeled protist RNA.

DISCUSSION

Ingestion or grazing rates are often hardly comparable between studies due to different methods applied (7, 20, 26). Many factors, e.g., medium composition or the food source, can influence the measurement, whereas the prey concentration in particular affects the particle-specific "efficiency of ingestion" (8, 31). The developed model microcosm approach enabled tracing quantitatively the incorporation of ^{13}C-labeled biomass carbon from fully ^{13}C-labeled *E. coli* cells into protist biomolecules by fatty acid stable isotope probing (FA-SIP) and by RNA-SIP. The amount of labeled ^{13}C derived from *E. coli* being mineralized to CO_2 accounted for 10.7% of the applied label, whereas 17.9% was incorporated into protist biomass. The average ^{13}C enrichment in the FA of *Uronema* sp. of 51 atom% corresponds to a consumption of roughly 9 to 18% of the labeled bacterial carbon. Data for bacterial carbon incorporation into protist biomass from other experiments are slightly higher and ranged between 12 and 60% (41, 52, 65). Similar to other reports, an enrichment of feces pellets was observed by microscopy but was not quantified and may account for the missing ^{13}C equivalents.

In the present study, the prey abundances initially increased during the first 3 h. Bacterivorous protists are known to excrete organic compounds or waste products (53), and in fact, fecal pellets in the ciliate culture used for the grazing experiment, which presumably served as an initial carbon source for bacterial growth, were noticed. However, the differences in the absolute bacterial abundances between the ^{12}C experiment and the ^{13}C experiment could be due to turnover-rate-decreasing effects of ^{13}C-labeled substrates (22) or may simply be due to a slight variation in the time points of harvesting the bacterial precultures. The observed ingestion rates of ≈400 bacteria protist^{-1} h^{-1} resulted in the elimination of prey bacteria from 5.8×10^7 ml^{-1} in the beginning to 7.5×10^4 ml^{-1} after 50 h. Many authors suggest measuring the ingestion rates in the first 3 h or only in short-time experiments (26), but the extrapolation from the first few hours to longer periods or later time points is generally not justified (48). Thus, the observed ingestion rates over 12 h of *Uronema* sp. (379 [^{12}C] and 392 [^{13}C] bacteria protist^{-1} h^{-1}) are below the range reported by Zubkov and Sleigh (66) of about 500 to 1,700 bacteria protist^{-1} h^{-1} obtained in small-scale and short-term experiments.

Dynamics of fatty acid composition of *Uronema* sp. One principal obstacle for the FA analysis of bacterivorous protists is the difficulty in obtaining axenic, bacterium-free protist cultures and bacterium-free FA extracts, respectively. Neither separation of *Uronema* sp. cells by filtration or centrifugation nor single-cell picking with the micromanipulator resulted in axenic cultures. This is a general problem, since separation of, e.g., flagellates from bacteria by filtration or centrifugation may still result in a bacterial FA background of up to 6% (55).

For future application using complex environmental samples, the area conversion of the measured FA peaks with subsequent subtraction as described by Mauclaire et al. (41) cannot be applied. If there are more than two different origins for the very common 16:0 and 18:0 FA, there is no way to use the scaling method of this model. Therefore, all bacterial FA from the profile obtained from the protist-bacterium mixed culture were excluded, and calculations were related only to the de-

fined specific protist FA. Using this approach, we determined nine FA to be specific for *Uronema* sp. Of these, however, only two monounsaturated FA (20:1ω9c and 22:1ω9t) were continuously present with more than 5 mol%. The data indicated that dominant fatty acids are not always adequate as marker FA in mixed communities and are sufficient only to estimate biomass, while less-abundant FA may be more promising as specific markers (63).

The various dynamics of the protist FA during the experiment can be explained by the initial increase in the protist FA, which was based not on higher protist cell numbers but on cell growth before cell division. Between 3 and 50 h, the *Uronema*-specific FA showed a differential enrichment. The FA 20:1ω9t, 22:0, 22:1ω9c, and 22:1ω9t had already reached maximum ^{13}C enrichments after 3 h of incubation with the labeled prey bacteria, thus being indicative for a quick response to the provided food source, whereas the FA 20:0, i20:0, and to some extent also 20:1ω9c became additionally enriched as soon as protist cell numbers significantly increased. Therefore, one set of FA can be used as an indicator for grazing activity and growth (without cell division), and a second set of FA (20:0, i20:0, and 20:1ω9c) is indicative for growth and cell division of *Uronema*.

However, due to limited knowledge of protist FA data, the application for field or microcosm studies requires careful consideration; nevertheless, a clear enrichment in FA already after 3 h but with differences for specific FAs could be detected. In contrast to findings of other studies on heterotrophic protists (2, 12, 19, 41, 64), no polyunsaturated fatty acids were detected which may be specific for the applied organism. In analyzing the fatty acid profiles of 18 *E. coli*-fed protists isolated from the same constructed wetland, polyunsaturated fatty acids were found in only a very few strains (unpublished data). However, FA compositions also depend on the food source or the composition of the medium provided. The kinetics of ^{13}C enrichment in a bacterivorous protist may also change with the nature of the food source and the feeding modes of the predator. Moodley et al. (43) fed foraminifers with ^{13}C-labeled *Chlorella* and detected the first significant ^{13}C enrichment only after 12 h. However, comparative studies over a range of protistan taxa are necessary to elucidate this aspect.

RNA gradient fractions. In addition to the fatty acids, the incorporation of carbon derived from ^{13}C-labeled *E. coli* into the 18S rRNA molecules of actively grazing *Uronema* sp. was traced. RNA-SIP instead of DNA-SIP was applied in this experiment, since RNA-SIP identifies carbon incorporation by active grazing protists irrespective of cell division (46, 59). Most of the previous RNA-SIP studies have focused only on the incorporation of ^{13}C from a labeled substrate into the nucleic acids (44, 45, 59). Only a few studies have analyzed the dynamics and the fate of fully stable-isotope-labeled biomass carbon (23, 32, 33, 36).

Quantitative RT-qPCR of the gradient fractions already showed a low ^{13}C enrichment of protistan rRNA after 6 h, which corresponds to a consumption of around 996 bacterial cells per protist cell. At 24 h, most of the fluorometrically detected heavy RNA is 18S rRNA, and after 50 and 70 h, nearly all is. This shows a clear transfer of ^{13}C originating from *E. coli* to the *Uronema* sp. cells. Although the amount of ^{13}C amended to the microcosms in the present study was only 15 μg ^{13}C per liter (1.15 μmol ^{13}C ml^{-1}), the ^{13}C enrichment of protist RNA resulted in the banding of heavy RNA at a BD of 1.836 g ml^{-1} (ranging from 1.82 to 1.85 g ml^{-1}), which is similar to the values observed in other feeding experiments (23). The shift of 0.021 g ml^{-1} thus allowed the separation of unlabeled and labeled RNA.

With the defined model experiment, the strengths and weaknesses of the applied approach and methods for application in complex environmental systems were evaluated. In particular, (i) the temporal patterns of ^{13}C assimilation from the prey bacteria to the protist FA, (ii) some FA being indicative for grazing activity and some for growth with cell division, (iii) the temporal pattern in ^{13}C labeling of the protist rRNA, and (iv) the amount of carbon being incorporated and mineralized could be shown. In future experiments, the transfer of the label into the dissolved and particulate organic carbon fractions should be focused. To extend the approach to complex environments, it is necessary to consider that some turnover-decreasing effects are caused by the application of ^{13}C-labeled prey organisms, but an overall consumption of roughly 1,000 prey bacteria per protist cell appears to be sufficient to provide sufficient amounts of label at least in the RNA of *Uronema* sp. The numbers of *E. coli* cells eliminated indicated that around 5 mol bacterial carbon is necessary in order to produce 1 mol protist carbon ($y_{x/s} \approx 0.2$). However, in more complex systems, a slower enrichment of RNA, e.g., after 24 to 48 h, can be expected, which certainly depends on the stable isotope enrichment of prey and the substrates used. In general, for optimal sensitivity of the label transfer, a maximum enrichment of the prey bacteria should be aimed for. In systems with complex microbial communities, the added labeled bacteria constitute just a fraction of total bacteria and thus can act only as a tracer for identifying specific predators without the option of full mass balances. Based on the enrichment of the fatty acids in the present experiment, the fraction of labeled bacteria within the overall amount of prey should not be less than 2% to 5%. Overall, the present study demonstrates that the use of the model microcosm combined with activity-related RNA- and fatty acid SIP is a promising approach for investigating and balancing the mineralization and flux of bacterial carbon through microbial trophic levels.

ACKNOWLEDGMENTS

This work was funded by the Helmholtz Centre for Environmental Research-UFZ.

We thank Matthias Gehre and Usula Günther for supporting quantitative isotope analysis, Petra Bombach for help with fatty acid analysis, and Verena Jaschik for supporting the RNA extraction. In addition, we thank reviewer no. 2 for fruitful suggestions that improved the manuscript substantially.

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