

## Tracking carbon flow in a 2-week-old and 6-week-old stream biofilm food web

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### *Abstract*

We studied the carbon flow of an allochthonous carbon source (sucrose) in a biofilm food web using stable isotope analysis and lipid biomarkers. Initial biofilms were grown for 2 weeks in a third order stream in Thuringia, Germany, and then incubated in replicate flow channels in climate-controlled chambers. Either of two sucrose types, differing in their  $\delta^{13}\text{C}$  values, was added either immediately (2-week-old biofilm) or after a pre-incubation of 4 weeks (6-week-old biofilm). Although sucrose decrease rates were similar with both biofilms, 2-week-old biofilms showed a higher carbon uptake capacity  $\text{cell}^{-1}$ . The 2-week-old biofilm was characterized by low abundances of all trophic levels, which increased one to two orders of magnitude during sucrose consumption. The 6-week-old biofilm had higher abundances. Biofilm bacteria incorporated added sucrose carbon, but algae showed no significant carbon incorporation, although a part of this carbon should be mineralized to carbon dioxide by bacteria. Sucrose carbon was also incorporated into ciliates and possibly other protozoans. Grazing rates indicated that up to 23.3% of the sucrose carbon reached higher trophic levels in 2-week-old biofilms. Less sucrose carbon was transferred to higher trophic levels in 6-week-old biofilms, where similar carbon amounts might have been channelled via filter feeding from the water column to ciliates. Ciliate community composition seemed to be affected by highly abundant rotifers. Whereas total carbon flow in 2-week-old biofilms was controlled by bacteria capable of high carbon uptake rates, higher trophic levels were more important in 6-week-old biofilms.

Biofilms are biochemical hotspots in small streams. Stream biofilms consist of bacteria, algae, and protozoans (Lock et al. 1984) embedded in a matrix of extracellular polymeric substances (EPS). The majority of bacteria lives attached to the streambed (Geesey et al. 1978). Enzyme activities (Romani and Sabater 1999) and sugar assimilation rates (Fletcher 1986) are much higher in biofilms than in the water column which underlines the importance of biofilms for the molecular breakdown of organic matter.

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Flow velocity is an important factor influencing biofilm structure and function (Battin et al. 2003), e.g., sugar uptake is enhanced with increasing flow velocity (Kaplan and Newbold 2002). However, high discharge events can reset biofilm growth by abrading the biofilm which can inhibit the biofilm from reaching a mature status.

The close proximity of stream biofilm organisms suggests that autochthonous carbon sources are allocated immediately to the next trophic level creating a tight nutrient spiral in the biofilm. Primary producers can provide labile dissolved organic matter (DOM) as exudates. Allochthonous nutrients have to enter the biofilm via diffusion across the overlying laminar boundary layer, and the flux is regulated by the concentration gradient between biofilm and water column. Since the polysaccharide matrix can function as a nutrient storage, retention of DOM in small streams occurs mainly in biofilms (Fischer et al. 2002). Flagellates and ciliates are often the most abundant groups of protozoa in biofilms. Mixotrophic and heterotrophic flagellates feed mainly on bacteria (Fenchel 1982), whereas ciliates feed on bacteria, algae, flagellates, and smaller ciliates (Parry 2004). The trophic link from bacteria to flagellates and ciliates is an important energy link in planktonic food webs (Azam et al. 1983). However, the effect of protozoan grazing on biofilm food webs has rarely been investigated (Parry 2004). Laboratory experiments

demonstrate that an amoeba can reach a grazing rate in a biofilm of  $30,000 \text{ cells cm}^{-2} \text{ h}^{-1}$ , and ciliates reduce the thickness of a multispecies biofilm from  $500 \mu\text{m}$  to  $<200 \mu\text{m}$  (Huws et al. 2005). In contrast, low grazing pressures (0.02–1.67%) are estimated using feeding rates in riverbed sediments of the sixth-order River Spree in Germany (Gücker and Fischer 2003). However, these rates might be not representative for the in situ matter and energy flow in stream and riverine biofilms. Rotifers can feed on bacteria, flagellates, ciliates, and algae (Arndt 1993; Duggan 2001), but the role of small metazoans in stream food webs is generally unresolved (Schmid-Araya and Schmid 2000).

One method of tracking the carbon flow in aquatic food webs uses stable isotope tracers. With this technique the autotrophic pathway can be labeled using  $\text{NaH}^{13}\text{CO}_3$  to estimate the effect of terrestrial matter input into microbial food webs in lakes (Pace et al. 2004). A high proportion of bacterial carbon was found in invertebrates after the release of sodium acetate- $1\text{-}^{13}\text{C}$  ( $\text{CH}_3^{13}\text{COONa}$ ) into first-order streams (Hall Jr. and Meyer 1998). To differentiate between different groups of microorganisms, phospholipid fatty acids (PLFA) are widely used as microbial biomarkers in aquatic ecosystems (Napolitano 1999). In particular, these biomarkers are a prominent tool to elucidate between bacterial and algal biomass or aquatic and terrestrial primary production.

In this study, we elucidated stream microbial biofilm food webs by tracking the isotopic carbon isotope signature of an allochthonous carbon source in different trophic levels by analyzing PLFA representative for bacteria, algae, or protozoans. We expected a high carbon uptake rate by microorganisms occupying a young biofilm due to increasing microbial growth and low number of protozoan predators. When microbial growth reached a stationary phase and protozoans had more time to colonize and proliferate, bacterial carbon uptake should have decreased and a stronger link to higher trophic levels should have occurred in older biofilms. Sucrose with different  $\delta^{13}\text{C}$  values, derived from either C3 or C4 plants, was supplemented to 2-week-old and 6-week-old biofilms grown under high- and low-flow velocity conditions, respectively, in flow channels in climate-controlled chambers. The proportion of incorporated sucrose carbon of organism groups was determined by comparison of biomarker  $\delta^{13}\text{C}$  values between the C3 and C4 sucrose treatment.

## Methods

*Experimental setup*—For initial biofilm growth, 360 unglazed clay tiles ( $5 \times 5 \text{ cm}$ ) were exposed for 14 d in a third-order gravel stream, Ilm, in Thuringia, Germany, during a snowmelt period in March 2005 (2-week-old biofilm), and in March 2006 after abating of the high discharge due to the snowmelt (6-week-old biofilm). Water temperature varied between  $1.0^\circ\text{C}$  and  $1.5^\circ\text{C}$  in March 2005 and between  $5.0^\circ\text{C}$  and  $5.9^\circ\text{C}$  in March 2006. Average sunshine period was  $5.2 \text{ h d}^{-1}$ , with a light intensity of  $\sim 102 \mu\text{mol m}^{-2} \text{ s}^{-1}$  photosynthetic active radiation. Clay

tiles were placed in a flat frame that was fixed on a paving stone to prevent washout by the current. During exposition of the clay tiles, the stream's flow velocity averaged  $0.7 \text{ m s}^{-1}$  in March 2005 and  $0.3 \text{ m s}^{-1}$  in March 2006. For harvesting, frames were placed into plastic vats filled with ambient stream water. Additionally, 200 L of stream water was poured into plastic cans. After transport to the laboratory, clay tiles were immediately transferred to six flow channels ( $160 \text{ cm} \times 10 \text{ cm} \times 18 \text{ cm}$ ; length  $\times$  width  $\times$  height) in a climate chamber. Each channel received 48 clay tiles and either 30 L of stream water for biofilms of the 2-week experiment (spring 2005), or 10 L for biofilms of the 6-week experiment (spring 2006). The latter were allowed to grow for additional 4 weeks under controlled conditions in the climate chamber to avoid potential abrasion of biofilms in the stream due to potential high discharge events. To achieve a flow velocity of  $0.7 \text{ m s}^{-1}$  for 2-week-old biofilm experiment (spring 2005), channels were positioned with a slight inclination, and water was circulated by using a rotary pump ( $1,700 \text{ L h}^{-1}$ ) connected to PVC tubings (diameter:  $2.54 \text{ cm}$ ; length:  $2 \text{ m}$ ). To achieve a flow velocity of  $0.3 \text{ m s}^{-1}$  for 6-week-old biofilm experiment (spring 2006), water was circulated by using a less powerful rotary pump ( $900 \text{ L h}^{-1}$ ). To minimize differences in biofilm development in 6-week-old biofilms, all channels were connected to one water reservoir. After 4 weeks of pre-incubation, the water flow was separated resulting in six water circuits. Channel water was exchanged with fresh stream water after 2 and 4 weeks. The climate chamber had a temperature of  $13^\circ\text{C}$ . Flow channels were exposed to light sources (Phillips SON-T AGRO 400) centered on the channels with a 12-h light–dark cycle. The photosynthetic active radiation was  $\sim 100 \mu\text{mol m}^{-2} \text{ s}^{-1}$  above the water surface during the light period.

To track the carbon flow, sucrose ( $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ ) was used as a preferable available carbon source. In the Ilm stream water, sucrose was the most abundant free sugar (Grossart, pers. comm.). Sucrose derived from either C3 or C4 plants (denoted as C3 sucrose and C4 sucrose; C3 sucrose: Haushaltszucker, Südzucker AG; C4 sucrose: C&H Sugar) was added to the flow channels, each sucrose type to three channels (further denoted as C3 and C4 treatments). The final concentration of sucrose was  $\sim 10 \text{ mg C L}^{-1}$ . The experiment was stopped when sucrose was consumed in all flow channels.

Water samples of 1 mL were taken by sterile syringes from each channel five times per day (every 4 h, and one 8-h interval during night). Concentrations of sucrose were determined using a high pressure liquid chromatograph (HPLC) connected to a refractive index detector (System Gold, Beckman Coulter; Küsel and Drake, 1995). Physical and chemical parameters of the water were determined immediately before sucrose addition and at the end of the experiment. Concentrations of dissolved organic carbon (DOC), ammonia ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), and orthophosphate ( $\text{PO}_4^{3-}$ ) were determined on filtered water ( $0.45\text{-}\mu\text{m}$  filter; Rotilabo, Germany). Concentrations of  $\text{PO}_4^{3-}$  were determined using the ascorbic acid method (Clesceri 1998), and concentrations of  $\text{NO}_3^-$  were determined using the ultraviolet spectrophotometric screening method (Clesceri

1998) using a DR 4000 photometer (Hach).  $\text{NH}_4^+$  was measured with a Metron ISE ammonium sensor (Metrohm). DOC was measured using a DIMATOC 100 infrared spectrometer (Dimatec Analysentechnik GmbH).

**Microbial abundances**—To determine abundances of bacteria, flagellates, ciliates, and chlorophyll *a* (Chl *a*) content, six samples of 2-week-old biofilms (spring 2005) were analyzed from each flow channel before sucrose addition and three samples of 2-week-old biofilms at the end of the experiment. The high number of samples was due to the heterogeneous biomass distribution in 2-week-old biofilms. From 6-week-old biofilms (spring 2006), two samples from each flow channel were obtained before sucrose addition and at the end of the experiment. For each sample, the biofilm from one tile was scraped off into filter-sterilized tap water using a sterilized glass slide. Formaldehyde was added to yield a final concentration of 4% to enumerate bacteria and flagellates. Bouin's solution was added to yield a final concentration of 23% in order to enumerate ciliates. Numbers of bacteria and flagellates were determined using staining with 4',6-diamidino-2-phenylindoldihydrochloride (DAPI) (Porter and Feig 1980) following a mild sonication (40% sonication strength; Sonopuls HD2200, Bandelin) for 10 min to disrupt the biofilm matrix. Samples for ciliate enumeration were shaken by hand, and 15 mL were transferred into sedimentation chambers and counted after 24 h. Chl *a* contents were determined spectrophotometrically (Uvikon 931, Kontron Instruments) following an acetone extraction (Clesceri 1998). For 6-week-old biofilms, the abundances of rotifers and algae were determined additionally after staining with Bouin's solution. According to morphological characteristics of the algae, diatoms, non-filamentous, and filamentous green algae were enumerated.

Ciliate grazing pressures were calculated following Gücker and Fischer (2003). The microbial biomasses needed for these calculations were obtained from the abundance using conversion factors. Bacterial biomass was calculated using the conversion factor of  $2.2 \times 10^{-13}$  g C  $\mu\text{m}^{-3}$ , assuming a mean cell volume of  $0.1 \mu\text{m}^3$  (Romani and Sabater 2000). Ciliate biomass was calculated using the conversion factor of  $1.1 \times 10^{-11}$  g C  $\mu\text{m}^{-3}$  (Turley et al. 1986). Mean cell volume was estimated by calculating the mean length and width of ciliates from the samples and assuming an ellipsoid body shape.

**Biomarker analysis**—For lipid extraction, biofilms from 20 clay tiles from each flow channel were scraped off into 50 mL of  $0.05 \text{ mol L}^{-1}$  phosphate buffer from 2-week-old biofilms at the end of the experiment (spring 2005). Samples were not pooled. From 6-week-old biofilms (spring 2006), biofilms from ten tiles per channel were scraped off, and samples of the same treatment (C3 or C4 sucrose, respectively) were pooled. Then 125 mL of methanol ( $\text{CH}_3\text{OH}$ ) and 62.5 mL of chloroform ( $\text{CHCl}_3$ ) were added to the biofilm suspension, and lipids were extracted by vigorous shaking for 3 h. The chloroform phase, which held the lipids, was separated using solid-phase extraction columns into neutral lipids, glycolipids,

and phospholipids by eluting with chloroform, acetone, and methanol, respectively (Zelles and Bai 1993). Mild alkaline methanolysis was applied to the phospholipid fraction in order to obtain phospholipid fatty acid methyl esters (FAME). A freshly prepared potassium hydroxide solution (0.055 g of potassium hydroxide [KOH] dissolved in 5 mL of  $\text{CH}_3\text{OH}$ ) was added after concentration of the phospholipid fraction, followed by an incubation for 20 min at  $38^\circ\text{C}$ . Chloroform was added, and the FAME-holding chloroform phase was separated by shaking and centrifugation (Bligh and Dyer 1959; Kramer and Gleixner 2006).

Isotope ratios ( $\delta^{13}\text{C}$  values) were determined by gas chromatography:mass spectrometry-combustion-isotope ratio mass spectrometry (GC/MS-C-IRMS) (Gleixner et al. 1999). Identification of FAME's was performed with the Wiley 6.0 MS library and by comparison with reference spectra recorded from FAME standards. Isotope data were evaluated using ISODAT NT 2.0 software and were expressed as  $\delta^{13}\text{C}$  values in per mil [‰] relative to the international reference standard V-PDB (Vienna PeeDee Belemnite, International Atomic Energy Agency, Vienna).

**Statistical analyses**—Pearson correlation was used to test for correlations between abundances, Chl *a* concentrations, and nutrient concentrations in all six flow channels. *T*-tests were used to test for differences between mean abundances determined at the start and end of the experiment. The analyses were done using SigmaStat for Windows version 2.03 (SPSS, Inc.). Parameter values are given as range (lower mean to higher mean) for channel pairs, and as mean  $\pm$  standard deviation (SD) for larger channel numbers. Rates of sucrose decrease were determined by linear regression analysis of the linear part of sucrose concentration data. To test for differences in sucrose decrease rates in the 2-week-old biofilm experiment, sucrose concentrations were transformed into percent values, the starting concentrations being 100%. These values were used as dependent variable in an ANCOVA with time as a covariate to test for differences between channels with SPSS version 13.

## Results

**Microbial abundances and biomass**—Mean abundances of biofilm bacteria, heterotrophic nanoflagellates, and ciliates increased 10-fold, 19-fold, and 192-fold, respectively, during 7 d of incubation in 2-week-old biofilms (Table 1). The Chl *a* content increased 8-fold. Ciliate and bacterial abundances were significantly positively correlated ( $p < 0.05$ ; Pearson Correlation). Bacterial biomass was  $3\text{--}11 \text{ ng C cm}^{-2}$  at the start, and  $48\text{--}148 \text{ ng C cm}^{-2}$  at the end of incubation. Ciliate biomass was  $16\text{--}63 \text{ ng C cm}^{-2}$  at the start, and  $1,821\text{--}26,925 \text{ ng C cm}^{-2}$  at the end of incubation. Mean ciliate biovolume was  $71,994 \mu\text{m}^3$ . Mean grazing pressures were 4.3% before sucrose addition and 18% at the end of incubation in the 2-week-old biofilms. Rotifers were virtually absent ( $<0.1 \text{ cells cm}^{-2}$ ) in all samples. Bacterial and ciliate abundances were higher in 2-week-old biofilms with fast sucrose decrease rates

Table 1. Abundances of bacteria, heterotrophic flagellates, ciliates, rotifers, and algae and mean values of Chl *a* in 2-week-old biofilms and 6-week-old biofilms. Experiments lasted 7 d (2-week-old biofilms) and 5 d (6-week-old biofilms), respectively.

Abundances and Chl <i>a</i>	Unit	2-week-old biofilm				6-week-old biofilm				
		Start†	End all	End fast†	End interm.‡	End slow‡	Start†	End		
bacteria	(10 <sup>6</sup> cells cm <sup>-2</sup> )	0.33±0.20	4.06±0.53	**	5.04-6.75	3.29-3.39	2.19-3.68	13.50±3.17	10.91±2.14	ns
flagellates	(10 <sup>2</sup> cells cm <sup>-2</sup> )	0.51±0.10	9.93±0.44	**	7.98-16.74	5.26-11.63	6.44-12.76	6.05±1.78	8.33±1.38	*
ciliates	(cells cm <sup>-2</sup> )	0.07±0.04	13.46±11.51	*	16.94-34.96	8.52-9.56	2.33-8.43	28.35±11.32	69.62±40.73	*
rotifers	(ind. cm <sup>-2</sup> )	n.a.§	n.a.		n.a.	n.a.	n.a.	79.64±35.70	115.8±92.48	ns
Chl <i>a</i>	(µg cm <sup>-2</sup> )	0.007±0.005	0.056±0.018	***	0.025-0.068	0.045-0.063	0.061-0.075	0.058±0.024	0.040±0.015	ns
diatoms	(10 <sup>4</sup> cells cm <sup>-2</sup> )	n.d.¶	n.d.		n.d.	n.d.	n.d.	8.15±2.45	7.20±4.89	ns
non-filamentous green algae	(10 <sup>4</sup> cells cm <sup>-2</sup> )	n.d.	n.d.		n.d.	n.d.	n.d.	15.43±8.73	51.38±25.58	**
filamentous green algae	(10 <sup>4</sup> cells cm <sup>-2</sup> )	n.d.	n.d.		n.d.	n.d.	n.d.	0.59±0.27	0.48±0.28	ns¶¶

\* Significant difference between start and end value ( $p < 0.05$ ).

\*\* Significant difference between start and end value ( $p < 0.01$ ).

\*\*\* Significant difference between start and end value ( $p < 0.001$ ).

† Moment of sucrose addition.

‡ End values of 2-week-old biofilms with fast, intermediate, or slow sucrose decrease rates.

§ n.a., not abundant. Rotifers were not abundant ( $< 0.1$  ind. cm<sup>-2</sup>) in all samples.

¶ n.d., not determined.

¶¶ ns, no significant difference between start and end value ( $p > 0.05$ ).

compared with 2-week-old biofilms with intermediate or slow rates (Table 1). The highest ciliate increase occurred in those biofilms with the highest bacterial abundances suggesting that ciliates might have responded to the high amount of bacteria as food source.

In 6-week-old biofilms, Chl *a* and abundances of bacteria, heterotrophic flagellates, and ciliates prior to sucrose amendment were one to two orders of magnitude higher than those in 2-week-old biofilms (Table 1). Mean abundances of flagellates, ciliates, and non-filamentous green algae were higher after sucrose depletion ( $p < 0.05$ , *t*-test), whereas Chl *a* and abundances of bacteria, rotifers, diatoms, and filamentous green algae were not different ( $p > 0.05$ , *t*-test) (Table 1). Large individuals of *Vorticella* spp. were the dominating ciliates in all samples. Prior to sucrose amendment, bacterial abundances were negatively correlated with ciliate ( $p < 0.05$ , Pearson correlation) and rotifer abundances ( $p < 0.05$ , Pearson correlation). Ciliates and rotifer abundances were positively correlated ( $p < 0.05$ , Pearson correlation). After sucrose depletion, diatoms, non-filamentous, and filamentous green algae were positively correlated with each other ( $p < 0.05$ , Pearson correlation). Bacterial and ciliate biomasses were 183–374 ng C cm<sup>-2</sup> and 85,574–260,314 ng C cm<sup>-2</sup>, respectively, at the start, and 195–315 ng C cm<sup>-2</sup> and 183,033–762,638 ng C cm<sup>-2</sup>, respectively, at the end of sucrose consumption. Grazing pressures from ciliates on bacteria were 8.1% before sucrose amendment and 26.4% at the end of incubation in 6-week-old biofilms. Mean ciliate biovolume approximated 530,396 µm<sup>3</sup>, which was about one order of magnitude higher than in 2-week-old biofilms. The ratio of bacterial to ciliate biomass in 6-week-old biofilms was 67 times and 15 times lower than in 2-week-old biofilms at the start and end of incubation, respectively. The ratio of bacterial biomass to Chl *a* in 6-week-old biofilms was 4.9 times and 3.8 times higher at the start and at the end of incubation, respectively, than in 2-week-old biofilms. Hence, 6-week-old biofilms were more heterotrophic compared to 2-week-old biofilms.

**Sucrose consumption dynamics**—Concentrations of sucrose measured in the water phase of the flow channels with 2-week-old biofilms declined linearly immediately after amendment. The time needed for complete consumption of sucrose in the six flow channels varied between 55 h and 168 h (Fig. 1A,B). This variation was independent of the type of sucrose added. C3 sucrose decrease rates were ~0.189 mg C L<sup>-1</sup> h<sup>-1</sup>, 0.107 mg C L<sup>-1</sup> h<sup>-1</sup>, and 0.057 mg C L<sup>-1</sup> h<sup>-1</sup>, and C4 sucrose decrease rates were ~0.173 mg C L<sup>-1</sup> h<sup>-1</sup>, ~0.120 mg C L<sup>-1</sup> h<sup>-1</sup>, and ~0.067 mg C L<sup>-1</sup> h<sup>-1</sup>. Two-week-old biofilms showed a much higher variation in sucrose decrease rates than did 6-week-old biofilms (Fig. 1), which might be due to differences in bacterial abundances, originating from the spatial heterogeneity in young biofilms. Sucrose decrease rates were significantly different ( $p < 0.05$ , ANCOVA), except for the two fastest and the two slowest decrease rates. Because those represent pairs of one C3 and one C4 channel, these channels were grouped for further analyses. Considering the small difference in the decrease rates of the

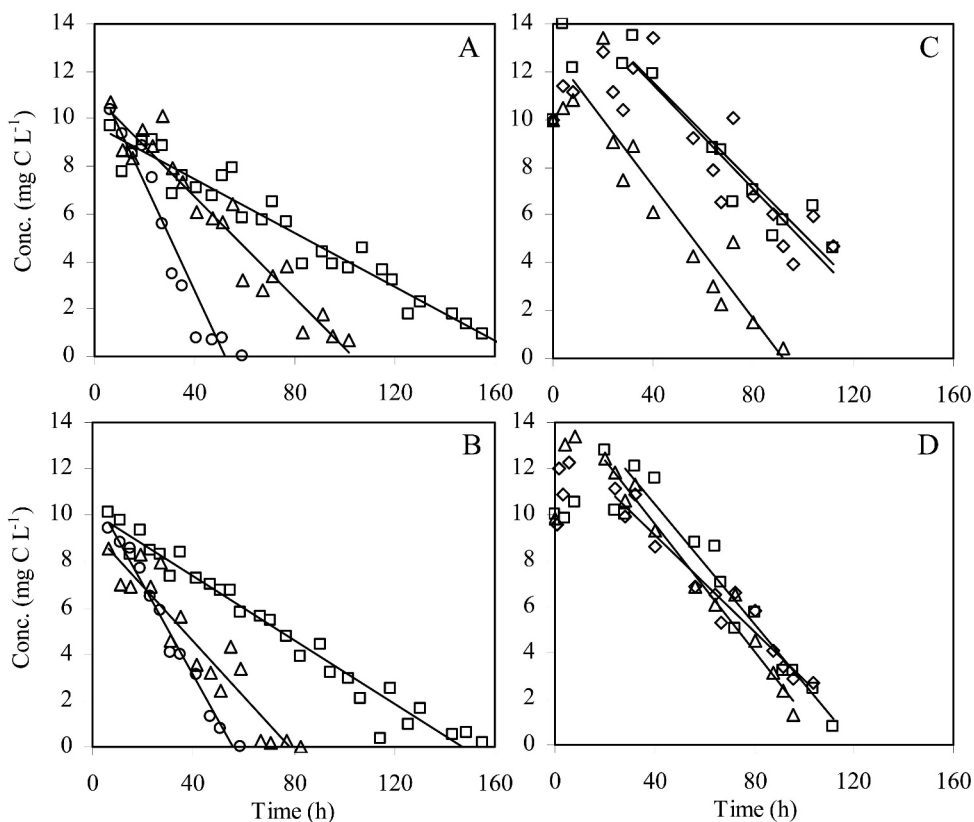


Fig. 1. Decrease of sucrose derived from either (A, C) C3 or (B, D) C4 plants in flow channels with (A, B) 2-week-old biofilms or (C, D) 6-week-old biofilms. Different symbols represent the biofilms defined (post hoc) as slow ( $\square$ ), intermediate ( $\Delta$ ) and fast ( $O$ ) sucrose decrease rates, respectively (see text for details).

remaining two channels, these were grouped as well. The uptake rates of 2-week-old and 6-week-old biofilms did not differ ( $p > 0.05$ ,  $t$ -test). Concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and  $\text{PO}_4^{3-}$  decreased during incubation (Table 2). Assuming linear decrease in dissolved inorganic nitrogen (DIN) over time, N-uptake rate was directly proportional to C-uptake rates (measured from sucrose consumption;  $C = 1.6 \text{ N} + 0.2$ ;  $R^2 = 99.4$ ). In all channels, concentrations of dissolved organic carbon (DOC) were 4-fold higher at the end of incubation than DOC concentrations prior to sucrose amendment (Table 2).

A lag phase of  $\sim 8$ – $32$  h was observed in 6-week-old biofilms before sucrose concentrations decreased linearly (Fig. 1C,D). C3 sucrose decrease rates were  $\sim 0.106$  mg C

$\text{L}^{-1} \text{ h}^{-1}$ ,  $\sim 0.109$  mg C  $\text{L}^{-1} \text{ h}^{-1}$ , and  $\sim 0.118$  mg C  $\text{L}^{-1} \text{ h}^{-1}$ , and C4 sucrose decrease rates were  $\sim 0.100$  mg C  $\text{L}^{-1} \text{ h}^{-1}$ ,  $\sim 0.130$  mg C  $\text{L}^{-1} \text{ h}^{-1}$ , and  $\sim 0.133$  mg C  $\text{L}^{-1} \text{ h}^{-1}$ . The decrease rates did not differ between C3 and C4 treatments ( $p > 0.05$ ,  $t$ -test). Concentrations of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  decreased during incubation, but  $\text{NH}_4^+$  concentrations (Table 2) did not. Concentrations of DOC at the beginning of incubation were higher compared to the other experiment, and increased about three-fold during incubation (Table 2).

*Tracking the carbon flow by lipid biomarkers and stable isotopes*—The  $\delta^{13}\text{C}$  values for C3 and C4 sucrose were  $-24.01\text{‰}$  and  $-11.36\text{‰}$ , respectively. From 19

Table 2. Mean concentrations of nutrients in the flow channels. Start concentrations were representative for all channels. End concentrations presented mean concentrations  $\pm$  SD of samples from all channels. Experiments lasted 7 d (2-week-old biofilms) and 5 d (6-week-old biofilms), respectively.

Parameter	Unit	2-week-old biofilm		6-week-old biofilm	
		Start	End	Start	End
$\text{NO}_3^-$	( $\mu\text{mol L}^{-1}$ )	129.0	$56.4 \pm 45.6$	132.3	$39.4 \pm 22.4$
$\text{NH}_4^+$	( $\mu\text{mol L}^{-1}$ )	8.3	$2.5 \pm 1.2$	4.4	$4.2 \pm 3.5$
$\text{PO}_4^{3-}$	( $\mu\text{mol L}^{-1}$ )	1.6	$< 0.5$	1.5	$0.5 \pm 0.7$
DOC	(mg C $\text{L}^{-1}$ )	0.8	$3.4 \pm 0.4$	1.9	$6.6 \pm 0.8$

Table 3. Fatty acid biomarkers determined in all replicate samples of 2-week-old and 6-week-old biofilms and their mean  $\delta^{13}\text{C}$  values of C3 and C4 sucrose treatments.

Fatty acid	Biomarker	Mean $\delta^{13}\text{C}$ value (‰)				Reference <sup>a</sup>
		2-week-old biofilm		6-week-old biofilm		
		C3	C4	C3	C4	
i14:0	cytophaga, gram <sup>+</sup>	-29.43±1.61	-24.52±1.54	d. <sup>b</sup>	d.	B
i15:0	cytophaga, gram <sup>+</sup>	-27.81±3.01	-19.90±0.31	n.d. <sup>c</sup>	n.d.	B
a15:0	cytophaga, gram <sup>+</sup>	-29.56±1.13	-22.39±1.49	-26.87	-21.07	B
i16:0	cytophaga, gram <sup>+</sup>	n.d.	n.d.	-27.03	-22.08	A
16:1(n-5)	general bacteria	n.d.	n.d.	-24.84	-23.67	E
i17:0	general bacteria	n.d.	n.d.	-24.17	-21.02	A
a17:0	general bacteria	n.d.	n.d.	-23.98	-21.21	A
17:1(n-8)	general bacteria	n.d.	n.d.	d.	d.	A
17:1(n-6)	gram <sup>-</sup>	n.d.	n.d.	d.	d.	C
18:1(n-7)	gram <sup>-</sup>	-33.32±1.12	-30.51±0.82	d.	d.	B
16:2(n-4)	diatoms and green algae	n.d.	n.d.	-30.36	-30.73	H
16:3(n-3)	diatoms and green algae	n.d.	n.d.	-33.63	-32.98	H
18:1(n-9)	algae, also some in bacteria	-30.47±0.70	-29.54±1.00	-31.28	-30.45	G,H
18:3(n-3)	green algae, cyanobacteria	n.d.	n.d.	-33.65	-33.94	D
20:5(n-3)	diatoms	n.d.	n.d.	-32.38	-32.19	F
18:2(n-7)	<i>Tetrahymena pyriformis</i>	-33.05±3.18	-27.14±2.40	n.i. <sup>d</sup>	n.i.	G
20:4(n-6)	protozoa in general	n.d.	n.d.	-33.27	-32.21	A,G

<sup>a</sup> (A) Sargent et al. 1987, (B) Ratledge and Wilkinson 1988, (C) White 1988, (D) Ahlgren et al. 1992, (E) Tunlid and White 1992, (F) Viso and Marty 1993, (G) Desvillettes et al. 1997, (H) Napolitano 1999.

<sup>b</sup> (d.: determined in some, but not in all replicate samples.

<sup>c</sup> (n.d., not determined.

<sup>d</sup> (n.i., not identified; a peak was detected in all samples, but it could not be clearly assigned to 18:2(n-6) or 18:2(n-7).

PLFAs detected in 2-week-old biofilms, 6 were used as biomarkers (Table 3): 3 biomarkers for cytophaga/flavobacteria and gram positive bacteria; 1 biomarker for gram negative proteobacteria; 1 biomarker for ciliates; and 1 biomarker for algae. All bacterial biomarkers expressed a difference in the  $\delta^{13}\text{C}$  values between C3 and C4 sucrose treatments (Table 3). C3 samples were always more depleted than the respective C4 samples. The mean differences were 4.91‰, 7.91‰, and 7.17‰ for i14:0, i15:0, and i16:0, respectively. Mean difference for the algal biomarker was 0.93‰ and 5.91‰ for the ciliate biomarker. These differences were calculated by subtracting the  $\delta^{13}\text{C}$  value of a biomarker of the C4 treatment from the  $\delta^{13}\text{C}$  value of this biomarker of the respective C3 treatment. The mean difference in bacterial  $\delta^{13}\text{C}$  values averaged 22.1–62.5% of the difference observed in sucrose values.

The difference of the  $\delta^{13}\text{C}$  values between C3 and C4 sucrose treatments was lower in three of the four bacterial biomarkers in those channels with fast compared to intermediate or slow sucrose decrease rates (Fig. 2). The difference in  $\delta^{13}\text{C}$  values of the biomarker indicative for ciliates, 18:2(n-7), approximated (96.4%) the difference of the two sucroses in the biofilms with fast decrease rates, indicating that ciliates fed solely on labelled bacteria. However, this difference was much lower in biofilms with intermediate (17.6%) and slow (26.2%) sucrose decrease rates.

From 33 PLFAs detected in 6-week-old biofilms, 15 were representative for biomarkers, and 11 of them could be identified in all samples (Table 3). Nine biomarkers for

bacteria (5 in all samples), 5 biomarkers for algae and 1 for protozoa were detected. In addition, a peak was detected for an 18:2 fatty acid, but it was not distinguishable between 18:2(n-6) and 18:2(n-7). All biomarkers detected in the 2-week-old biofilm were also present in the 6-week-old except for i15:0. The difference in isotope ratios between the C3 and C4 treatment ranged between 1.2‰ and 5.8‰ for bacterial biomarkers (Fig. 3). Algal biomarkers displayed almost no sucrose carbon uptake; all differences were close to zero (Fig. 3). The protozoan biomarker had a small difference of 1.1‰ between the C3 and C4 treatment. Additionally, the ratio of 16:1(n-7):16:0 was calculated to evaluate the relationship between diatom and flagellate abundances (Viso and Marty 1993; Reuss and Poulsen 2002), with a high value (>1) indicating the presence of diatoms. The ratio of 16:1(n-7):16:0 was 1:1 in six, and 1.2:2.3 in 2-week-old biofilms.

## Discussion

Stable isotope analysis of fatty acid biomarkers allowed us to trace added sucrose carbon in a biofilm food web and to estimate the proportion originating from sucrose versus other carbon sources. However, the scarce knowledge of biomarkers for protozoans limited the use of this method in determining the carbon flow in microbial biofilm food webs. Microscopic enumerations gave a more complete picture of the different trophic levels and enabled also the calculation of grazing pressures. The combination of both tools may give valid estimates of the carbon flow in situ.

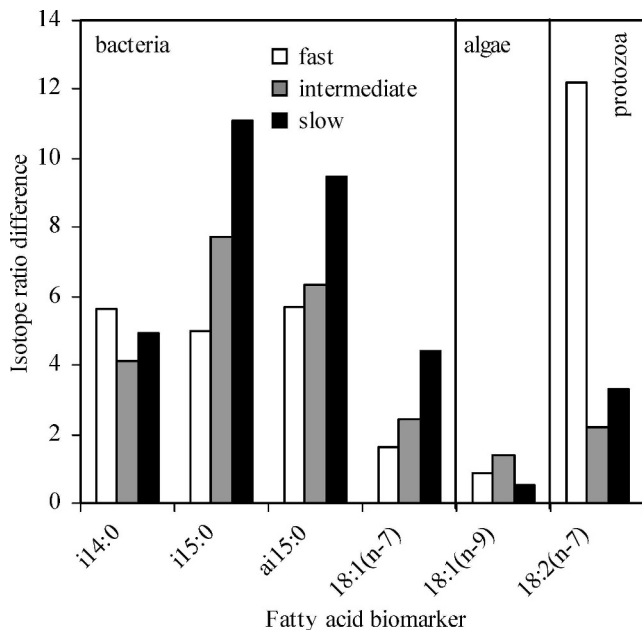


Fig. 2. Isotope ratio differences between biomarkers of 2-week-old biofilms obtained from C3 and C4 treatments of flow channels with similar sucrose decrease rates. Sucrose derived from either C3 or C4 plants was added to flow channels, each sucrose type to three channels (C3 and C4 treatments).

*Stream biofilm population structure*—The 2-week-old biofilm could be representative for initial stream biofilms which develop during periods of fluctuating discharge regimes and cold temperatures. The mean initial abundances of bacteria, flagellates, and ciliates and the Chl *a* content of these biofilms were one to several orders of magnitude lower compared to the 6-week-old biofilms and stream biofilms investigated in other studies (e.g., Bott and Kaplan 1989; Hunt and Parry 1998; Romani and Sabater 2000; Tank and Dodds 2003; Fukuda et al. 2004). In this experiment, clay tiles for initial growth were exposed when the water temperature in the Ilm was only  $\sim 1.0$ – $1.5^\circ\text{C}$ . All abundances increased to values similar to the low range of those observed in Japanese streams (Fukuda et al. 2004) after 1 week of incubation at  $13^\circ\text{C}$  and amendment of a carbon source, suggesting that the low water temperatures and the low availability of allochthonous carbon in the Ilm may be responsible for the low initial abundances. The 6-week-old biofilms could be representative for biofilms that develop under more continuous low discharge regimes. Higher microbial abundances, no increase in bacterial abundances despite sucrose addition, and the appearance of rotifers displayed their more mature state compared to 2-week-old biofilms. The shift in the biomass ratio between ciliates and bacteria and the abundant occurrence of rotifers suggested an increased grazing pressure from higher trophic levels. Neither Chl *a* nor any algal abundances were correlated to potential predator abundances. Therefore, the low nutrient concentrations or the low temperature might be responsible for the relatively low Chl *a* content compared with 2-week-old biofilms.

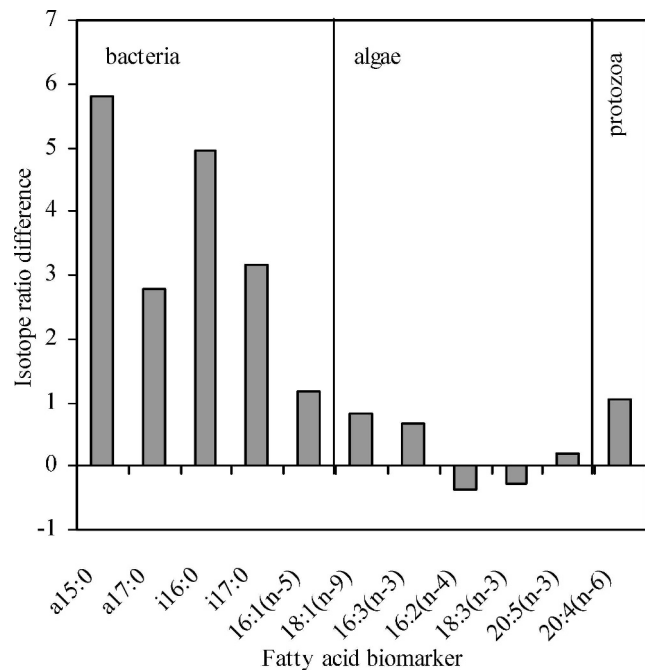


Fig. 3. Isotope ratio differences between biomarkers of 6-week-old biofilms obtained from C3 and C4 treatments. Sucrose derived from either C3 or C4 plants was added to flow channels, each sucrose type to three channels (C3 and C4 treatments).

*Tracking the carbon flow using stable isotopic signatures*—Fatty acid biomarkers are widely used to differentiate between algal and bacterial biomass in aquatic ecosystems (Napolitano 1999). Hence there are plenty of algal and bacterial biomarkers described (e.g., Sargent et al. 1987; Desvilettes et al. 1997; Boschker et al. 2005). In 2-week-old biofilms the low amount of biomass used for extraction might have been responsible for the low number of biomarkers determined, because numerous additional small peaks were visible that were below the detection limit (100 mV) of the GC:MS. More than twice as many biomarkers were detected in 6-week-old biofilms because a higher amount of biomass for extraction was obtained due to higher abundances and pooling of the samples. Knowledge of protozoan biomarkers is scarce (Desvilettes et al. 1997), and protozoans are often ignored in studies on trophic interactions. In 2-week-old biofilms, a ciliate-specific biomarker could be used. Ciliates can contain cilienic acid (18:2[n-7]), which was detected in *Tetrahymena pyriformis* (Desvilettes et al. 1997). *T. pyriformis* is a 40–60  $\mu\text{m}$ -long ciliate feeding mainly on bacteria, but also flagellates, viruses, and detritus (Foissner et al. 1994). This ciliate is abundant in many limnic ecosystems and also in the Ilm stream (Risse-Buhl, U., pers. comm.). Since the 18:2 fatty acid detected from the 6-week-old biofilm could not be clearly assigned to 18:2(n-7), the protozoan marker 20:4(n-6) (Sargent et al. 1987, Desvilettes et al. 1997) was used. Ciliates contain high proportions of fatty acids of the n-6 series. Since ciliates represented the bulk part of protozoan biomass, this protozoan biomarker should be representative.

In both experiments, the isotopic ratio difference in all bacterial biomarkers demonstrated the incorporation of sucrose carbon in bacteria. Despite the lower abundance of bacteria in 2-week-old biofilms, the mean sucrose decrease rates and the N-uptake rates were similar between 2-week-old and 6-week-old biofilms. Since the amount of water and thus the total amount of sucrose per channel was three times higher in channels with 2-week-old biofilms than in the channels with 6-week-old biofilms, the theoretical carbon uptake capacity per cell was about one order of magnitude higher in 2-week-old biofilms compared with 6-week-old biofilms. Differences in the ciliate biomarker displayed that sucrose was channelled up the food web in 2-week-old biofilms, whereas in 6-week-old biofilms ciliates incorporated carbon mainly from other sources. In channels with 2-week-old biofilms, the ingested bacterial biomass per hour by ciliates was equivalent to 13.2–23.3% of the sucrose decrease per hour. Hence, about one-fifth of the added sucrose could be channelled into higher trophic levels of the microbial loop. A part of the labelled carbon might be excreted by the bacteria to produce EPS or DOC, which would explain the increase in DOC over time. No sucrose carbon was detected in the algal biomarkers. One biomarker indicative for algae (18:1[n-9]) can also be found in some bacteria. However, it is usually not the dominant isomer (Napolitano 1999), and a strong input from the labelled bacterial carbon pool would have caused a difference in the 18:1(n-9) biomarker. Consequently, algae appeared to use carbon dioxide (CO<sub>2</sub>) mainly from the water column and not respired CO<sub>2</sub> produced by heterotrophic biofilm organisms. On the other hand, algal exudates might have provided an additional carbon source for bacteria.

*Stream biofilm food web analysis*—Sucrose was consumed by both biofilms and incorporated into bacteria and, to a lesser extent, into protozoa. In 2-week-old biofilms, sucrose as relatively labile food source was quickly incorporated. Still growing, young biofilms should have been dominated by organisms adapted to fast carbon turnover. Indeed, bacterial abundances increased by one order of magnitude during the experiment. However, 6-week-old biofilms showed a lag phase prior to sucrose decrease, and sucrose carbon uptake capacity per cell was lower than in 2-week-old biofilms. Assuming that labelling of the bacterial carbon pool was similar in all channels of the 2-week-old biofilm experiment in the presence of supplemental sucrose, the lower differences in  $\delta^{13}\text{C}$  values at the end of incubation in channels with higher sucrose decrease rates (Fig. 2) suggested that 33–50% of this bacterial carbon pool was turned over within 4–5 d after sucrose depletion. The higher proportion of Chl *a* in 2-week-old biofilms compared with 6-week-old biofilms and the high bacterial carbon turnover suggested that labile algal exudates are important carbon sources in 2-week-old biofilms without supplemental allochthonous carbon. In contrast, the relatively lower proportion of Chl *a* in 6-week-old biofilms indicated that algal exudates are less important, and the overall lower isotope ratio differences seemed to be due to other unlabeled carbon sources. The majority

of ciliates in these biofilms were *Vorticella* sp., which feed mainly on planktonic bacteria, suggesting that ciliates in 2-week-old biofilms and 6-week-old biofilms might have relied on different carbon sources. Grazing pressures predicated that ciliates fed on bacteria were equivalent to one-fourth of the bacterial production in these biofilms. Therefore, ciliate grazing on planktonic bacteria represented an important link to channel organic matter and energy from the water column into the biofilm without potential diffusion limitations across the laminar boundary layer. Rotifers reached high abundances only in 6-week-old biofilms, where ciliates smaller than rotifers were absent, indicating that small ciliates were consumed by rotifers. Negative correlations suggested that biofilm bacteria were controlled by rotifers in a top-down manner. Consequently, rotifers might have controlled ciliate size by predation as well as ciliate feeding behavior by competition for biofilm bacteria. In 2-week-old biofilms, ciliates seemed to be controlled by their food source bacteria in a bottom-up manner. Considering the high biomass of ciliates, they might be responsible for the retention of carbon assimilated by biofilm bacteria. Thus, as bacteria capable of high carbon uptake rates governed carbon flow in 2-week-old biofilms, higher trophic levels were more important in 6-week-old biofilms.

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