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Trophic positioning and microphytobenthic carbon uptake of biofilm-dwelling meiofauna in a temperate river

NABIL MAJDI*,†, MICHÈLE TACKX*,† AND EVELYNE BUFFAN–DUBAU*,†
*Université de Toulouse, INP, UPS, EcoLab, Toulouse, France
†CNRS, EcoLab, Toulouse, France

SUMMARY

1. $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures combined with an *in situ* microphytobenthic ^{13}C labelling experiment were performed on epilithic biofilms of a large temperate river (the Garonne, France) to infer the trophic positioning of biofilm-dwelling meiofauna and their uptake of microphytobenthic carbon.
2. Chironomidae larvae and *Chromadorina* spp. nematodes rapidly incorporated freshly produced microphytobenthic carbon in contrast to Rhyacophilidae larvae and Naididae oligochaetes. Quantitatively, macrofaunal Chironomidae incorporated more microphytobenthic carbon per day than did meiofauna. Moreover, Chironomidae seemed more involved in the spatial export of microphytobenthic carbon than nematodes.
3. Rhyacophilidae larvae were predators feeding on large meiofauna (Naididae and Chironomidae) but not on nematodes. Naididae oligochaetes primarily gained their carbon from allochthonous and/or microbial-loop recycled sources.
4. A rapid and significant loss of labelled microphytobenthic carbon was observed. Feeding activity of biofilm-dwelling invertebrates seemed not to be primarily involved in this loss.

Keywords: carbon flows, food web, meiofauna, periphyton, stable isotopes

Introduction

The epilithic biofilm is a complex assemblage comprising microphytes, bacteria, meiofauna and macrofauna embedded in a mucous matrix of exopolymeric substances (EPS) together with entrapped allochthonous imports (e.g. Romaní *et al.*, 2004). This biofilm coats any hard submerged substrate and, when enough light is available, microphytobenthos (and their EPS exudates) contributes copiously to the organic content of biofilm (Azim & Asaeda, 2005). Epilithic biofilms contribute significantly to biogeochemical processes and sustain secondary production (e.g. Lock *et al.*, 1984; Pusch *et al.*, 1998; Battin *et al.*, 2003; Cardinale, 2011).

Meiofauna are small invertebrates that pass through a 500-μm mesh and are retained on a 40-μm mesh (Fenchel, 1978). Understanding their trophic role is a key issue to disentangle energy flows in freshwater food webs (Hildrew, 1992; Ward *et al.*, 1998; Reiss & Schmid-Araya, 2010). In freshwater sediments, meiofauna can specifically ingest microphytobenthos and smaller heterotrophic organisms (Borchardt & Bott, 1995; Traunspurger, Bergtold & Goedkoop, 1997; Reiss & Schmid-Araya, 2011). From *in situ* studies conducted in marine intertidal habitats, it is now well established that meiofauna can rapidly take up freshly photosynthetically fixed microphytobenthic carbon, as such improving its transfer rate to higher trophic levels (e.g. Montagna, 1984; Middelburg *et al.*, 2000; Moens *et al.*, 2002; Pinckney *et al.*, 2003). Generally, the quantitative *in situ* uptake of microphytobenthic carbon by meiofauna has received little attention in freshwater systems (Borchardt & Bott, 1995; Moens, Traunspurger & Bergtold, 2006), though the role of meiofauna as intermediates in stream food webs is increasingly recognised (e.g. Schmid & Schmid-Araya, 2002; Schmid-Araya *et al.*, 2002; Dineen & Robertson, 2010; Spieth *et al.*, 2011).

There are some indications that meiofauna can influence key processes in epilithic biofilms, such as oxygen turnover, secondary metabolites release and detachment

Correspondence: Nabil Majdi, Université Paul Sabatier, EcoLab, 118 route de Narbonne, 31062 Toulouse, France.
E-mail: nabil.majdi@univ-tlse3.fr

(Sabater *et al.*, 2003; Gaudes *et al.*, 2006; Mathieu *et al.*, 2007). Some recent studies have addressed the feeding habits of biofilm-dwelling meiofauna. For example, Kathol, Fischer & Weitere (2011) produced a budget of the importance of pelagic-benthic import through rotifer and ciliate filtration activity, while Majdi *et al.* (2012b) showed that *Chromadorina* spp. nematodes feed on biofilm diatoms non-selectively. However, most studies addressing grazing within epilithic biofilms remain focused on macrofauna (Hillebrand, 2009).

Stable isotope analysis (SIA) multi-approaches (i.e. trophic tracers in addition to natural isotopic signatures) can be used to unravel trophic processes in ecosystems (Boschker & Middelburg, 2002). SIA multi-approaches have often been successfully applied to examine *in situ* trophic linkages and carbon flows involving meio- and macrofauna in marine and brackish benthic systems (Middelburg *et al.*, 2000; Galvan, Fleeger & Fry, 2008; Pascal *et al.*, 2008a; Evrard *et al.*, 2010). SIA multi-approaches using addition of dissolved ^{13}C and/or ^{15}N as trophic tracers are also commonly used in freshwater benthic systems to disentangle a variety of trophic processes (e.g. Hall, 1995; Parkyn *et al.*, 2005; Cardinale, 2011). However, so far, no freshwater studies have applied SIA multi-approaches to examine trophic positioning and *in situ* grazing of meiofauna.

Using an *in situ* SIA multi-approach, our objectives are: (i) to specify the organisation of the biofilm food web by including meiofauna, (ii) to quantify the importance and rate of carbon transfer from benthic photosynthesis to both meio- and macrofauna inhabiting the biofilm. These objectives should contribute to the elucidation of the fate of biofilm microphytobenthic carbon.

Methods

Site description

With a total length of 647 km and a drainage basin of 57 000 km^2, the Garonne is the largest river of south-western France, displaying alternate cobble bars in channels up to the seventh-order. The study site was on one of these cobble bars 36 km upstream of the city of Toulouse, where the Garonne is sixth-order (lat 01°17′53″E, long 43°23′45″N; elevation: 175 m asl, Fig. 1a). The dynamics of epilithic phototrophic biofilm with its bacterial, microphytobenthic and meiofaunal components has already been described at this site (Lyautey *et al.*, 2005; Boulêtreau *et al.*, 2006; Leflaive *et al.*, 2008; Majdi *et al.*, 2011, 2012a). In this stretch of the Garonne, the residence time of water is too low to allow substantial phytoplankton development, and it is assumed that benthic biofilms provide most of the riverine primary production (Ameziane, Dauta & Le Cohu, 2003). On the basis of these data, the study site was located along a longitudinal transect 45 m from the bank, so that depth remained between 40 and 50 cm (i.e. the depth where the phototrophic biofilm typically develops; Ameziane *et al.*, 2002).

Growth of the epilithic biofilm and natural $\delta^{13}C$ and $\delta^{15}N$ signatures

On 20 July 2009, eight 400-cm^2; ceramic tiles were placed on the riverbed at the chosen site (Fig. 1b). Biofilm was allowed to colonise tiles for 2 months, a sufficient exposure period for the establishment of mature biofilm communities in temperate rivers (Pusch *et al.*, 1998; Norf, Arndt & Weitere, 2009).

On 20 September 2009, four 50-cm^2 biofilm samples were collected (by scraping with a scalpel and a toothbrush) from four of the eight biofilm-colonised tiles to measure the natural $\delta^{13}C$ and $\delta^{15}N$ signatures of the biofilm and its associated invertebrates.

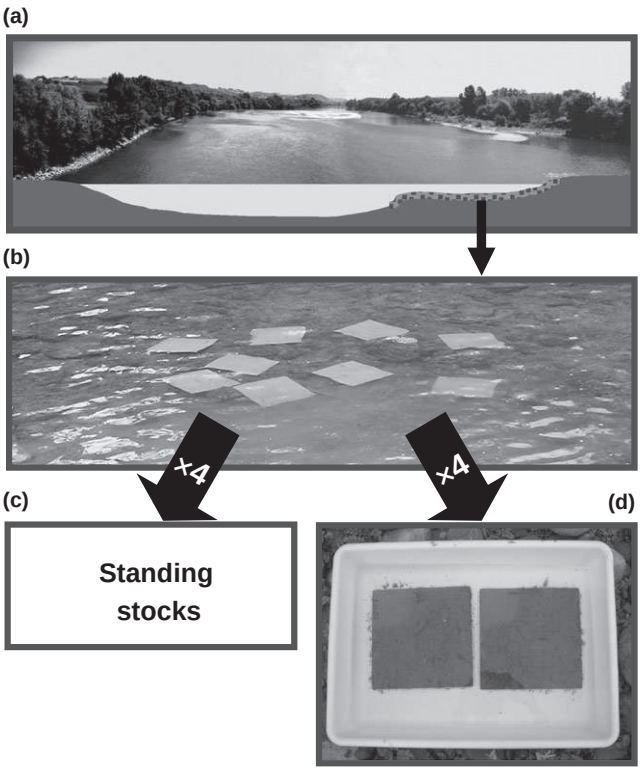

Fig. 1 Experimental design: (a) Cross-section view of the Garonne River at the study site. (b) Ceramic tiles lying on the streambed before colonisation by biofilm (20 July 2009). After 2 months of colonisation: (c) biofilm was gathered from four tiles to estimate initial standing stocks, and (d) biofilm from four other tiles was labelled with $NaH^{13}CO_3$ solution for 3 h.

A total of twenty leaves (mainly *Populus* sp. and *Alnus glutinosa* (L.) Gaertn.) were hand-collected underwater from natural accumulations in small depositional zones between the tiles to determine the isotopic signature of leaf litter. Four replicates of five leaves each were carefully rinsed with milliQ water to remove epibionts and stored (−20 °C) for further measurement of their natural $\delta^{13}C$ and $\delta^{15}N$ signatures.

Labelling experiment

Just after the collection of biofilm samples to measure natural $\delta^{13}C$ and $\delta^{15}N$ signatures, the same four sampled tiles were placed in two rectangular basins (Fig. 1d) filled with 1.5 L of low mineralised water (<25 mg L^{-1} dry residue; Mont-Roucous, Lacaune-les-bains, France), at ambient river water pH (7.1) and containing 160 mg L^{-1} $NaH^{13}CO_3$ (>99% ^{13}C; Sigma-Aldrich, Buchs, Switzerland). Photo-incorporation of ^{13}C by microphytobenthos was favoured by leaving the tiles exposed to sunlight from 11 am to 2 pm at ambient river water temperature (17 °C). At the end of this labelling period (t = 3 h), four 50-cm^2 biofilm samples (one from each tile) were collected by scraping with a scalpel and a toothbrush. The tiles were then replaced in the river at the colonisation site. Four additional 50-cm^2 biofilm samples (one from each tile) were collected on each of the following 3 days (at t = 24, 48 and 72 h). Attention was paid (i) to minimise any detachment of biofilm during the removal and replacement of the tiles by gentle handling and (ii) to always gather biofilm surfaces from non-previously scrubbed locations of the tile. All biofilm samples were preserved in 100 mL formaldehyde solution (4%) immediately after collection. No corrections were applied for any carbon added through the formaldehyde preservation.

Sample processing for stable isotope analysis (SIA)

All biofilm samples were thoroughly homogenised and poured through stacked 500-, 40- and 25-µm mesh sieves. The resulting filtrate was then filtered on 1.2-µm glass fibre filter (GF/C; Whatman, Clifton, NJ, U.S.A.). Macro- and meiobenthic metazoans were gathered from the >500 and 40- to 500-µm biofilm fractions respectively and sorted to the lowest practical taxonomic level under a stereomicroscope (9–90×). From each biofilm sample, two 80-µL aliquots of biofilm filtration residues were collected, one from the 25-µm sieve fraction (representing the 25- to 40-µm biofilm fraction) and the other from the GF/C filter (representing the 1.2- to 25-µm biofilm fraction). These aliquots were transferred into tin cups and prepared for SIA as described below.

Nematodes were not identified to the species level. However, at the time and site of the study, most biofilm-dwelling nematodes (>95%) belonged to two species from the genus *Chromadorina* (Majdi *et al.*, 2011). Hence, we assumed that nematode SIA results should depict mainly the feeding habits of *Chromadorina* spp. Oligochaetes were strongly dominated by Naididae (>85%). Naididae were selectively isolated for SIA since they were easily distinguishable from the Lumbricidae and Tubificidae that comprised the rest of the oligochaete assemblage. Chironomidae larvae were pooled without further taxonomic distinction. However, a size distinction was made between meio- and macrofaunal Chironomidae. Psychomyiidae and Rhyacophilidae larvae (Trichoptera) comprised the rest of the biofilm-dwelling macrofauna. Psychomyiidae were not found in sufficient numbers in each sample, so only Rhyacophilidae were isolated for SIA. From each biofilm sample, 500 *Chromadorina* nematodes, 30 Naididae oligochaetes, 30 meio- and 10 macrofaunal Chironomidae and one Ryacophilidae were isolated, checked for body integrity, thoroughly washed in two successive milliQ water baths to remove any adherent particles, transferred to cleaned pre-weighed tin cups (one cup for each taxon), dried at 55 °C overnight, pinched closed, weighed and stored (−20 °C) until SIA.

Leaf litter samples were freeze-dried and ground to a homogeneous powder prior to encapsulation in tin cups for SIA.

Isotopic analyses

Samples were analysed for organic carbon (C) and nitrogen (N) content and isotopic composition using a FLASH EA-1112 elemental analyser coupled to a DELTA V Advantage mass spectrometer (both Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). C and N percentage contributions to organic dry weight were measured for each sample. $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios were expressed with the standard ‰ unit notation: $\delta X(‰) = ([R_{sample}/R_{standard}]-1) \times 1000$, where R is either the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ standardised according to Vienna Pee Dee Belemnite (VPDB) for C, and to atmospheric N_2 for N. Measurement reproducibility was <0.15 ‰ for both C and N. The incorporation of ^{13}C label was defined as excess ^{13}C.

Specific uptake was calculated as $\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{control}$, with $\delta^{13}C$ expressed relatively to VPDB. Total uptake (I) was quantified in mg ^{13}C m^{-2} with I = excess ^{13}C (E) × organic C content, according to standing stocks. E is the difference between the ^{13}C fraction of the control

($F_{control}$) and the sample (F_{sample}), where $F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R + 1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as $R = (\delta^{13}C/1000 + 1) \times R_{VPDB}$, with $R_{VPDB} = 0.0112372$. For invertebrates, daily total microphytobenthic carbon assimilated was calculated as the product of I with the initial (t = 3 h) proportion of ^{13}C to microphytobenthic carbon stock of the pooled 1.2- to 25-μm and 25- to 40-μm biofilm fractions, assuming equal uptake of labelled versus non-labelled carbon.

Standing stocks

On 20 September 2009, at the beginning of the labelling experiment, the biofilm from four additional replicate tiles (Fig. 1c) was removed by scraping, and each sample was thoroughly homogenised and suspended in 200 mL formaldehyde solution (4%) for the following analyses.

Four 20-mL subsamples obtained from the four homogenised biofilm suspensions were used for biofilm biomass determination. Each subsample was dried overnight at 55 °C, weighed for its dry mass (DM) and then combusted for 8 h at 450 °C to determine its ash-free dry mass (AFDM).

Four 500-μL subsamples from the four homogenised biofilm suspensions were gently sonicated for 15 min at 35 kHz in an ultrasonic bath (Transsonic T460; Elma, South Orange, NJ, U.S.A.) and vortexed for 15 min to disaggregate bacterial aggregates (Garabétian, Petit & Lavandier, 1999). Then, the density of bacteria was determined following a DAPI-staining method (Porter & Feig, 1980). Bacterial counting was carried out using a Leitz Dialux microscope (1250×) fitted for epifluorescence: HBO 100 W mercury light source (Osram, Winterthur, Switzerland), with an excitation filter for 270 and 450 nm, a barrier filter of 410 nm and a 515-nm cut-off filter. Bacterial biomass was assumed to be 20 fgC cell^{-1} after Lee & Fuhrman (1987).

The remaining four replicates of 179.5-mL biofilm suspensions were size-fractionated by sieving as described above. Since microphytes were rarely encountered in >40-μm fractions, we focused on 1.2- to 25- and 25- to 40-μm biofilm fractions to determine biofilm microphytobenthos (MPB) density and biomass. Microphytes were enumerated using a Malassez counting chamber under a Nikon Optiphot-2 microscope (50–600×). Diatoms were identified to genus level. For each replicate, 50 cells of each MPB group (i.e. diatoms, green algae and cyanobacteria) were measured to calculate their cell biovolumes after Hillebrand *et al.* (1999). Then, cell carbon content was calculated from biovolume after Menden-Deuer & Lessard (2000).

The density of meio- and macrobenthic invertebrates was determined by counting four replicates of the 40–500 and >500 μm fractions, respectively. Their biomasses were measured from DM and Carbon (C)/DM values obtained after SIA.

Estimation of invertebrate carbon budgets

Daily production (P, mgC m^{-2} day^{-1}) was calculated from invertebrate taxon biomasses using Plante & Downing's regression (1989), assuming an average water temperature of 17 °C. We assumed net production efficiencies (NPE = P/Assimilation) of 0.6 for nematodes, 0.55 for predators and 0.4 for other taxa (Smock & Roeding, 1986; Herman & Vranken, 1988). Hence, the daily assimilation demand (in terms of C) of each invertebrate taxon was estimated from P and NPE. Daily assimilation demand was compared to the total microphytobenthic carbon (MPBC) assimilated daily, to budget the contribution of MPBC to consumer's daily assimilation demand. For predators, we estimated the number of potential preys needed daily to fulfil their daily assimilation demand.

Data analyses

The organisation of the biofilm food web was assessed by plotting natural $\delta^{13}C$ versus natural $\delta^{15}N$ isotopic signatures, with trophic levels being identified using $\delta^{15}N$ and food sources using $\delta^{13}C$ (Peterson & Fry, 1987). Natural $\delta^{13}C$ and $\delta^{15}N$ signatures of food sources were compared using one-way ANOVA, after assessing variance homogeneity using Levene's test. The expected trophic enrichment factor during food assimilation was assumed to be +0.5‰ for $\delta^{13}C$ and +2.2‰ for $\delta^{15}N$ (McCutchan *et al.*, 2003). Therefore, the isotopic signature of the probable food was estimated by removing 0.5‰ for C and 2.2‰ for N from the mean isotopic values of the consumers. Differences between specific label uptake dynamics were analysed by two-way ANOVA with meiofaunal taxa and post-labelling times as factors, after assessing variance homogeneity using Levene's test. Tukey's HSD test was performed for *a posteriori* pairwise comparisons. All statistical analyses, as well as total ^{13}C uptake dynamics fitting, were performed with STATISTICA software (version 8.0; Statsoft inc., Tulsa, OK, U.S.A.).

Results

Standing stocks

On 20 September 2009, the biofilm averaged (±SD) 129 ± 6 DM m^{-2} and 16.1 ± 1.5 gAFDM m^{-2}. Thus, or-

ganic content contributed 12% of the total biofilm dry mass. Biofilm C/AFDM ratio was assumed to be 0.45 (Whittaker & Likens, 1973), allowing the total biofilm organic C stock to be estimated at 7.2 gC m^{-2}. Bacterial density in the biofilm averaged 9.6 ± 1.1 × 10^{12} cells m^{-2}, or 193 mgC m^{-2} (i.e. 2.7% of the biofilm organic C stock).

Green algae and diatoms contributed equally to biomass in the 25- to 40-μm biofilm fraction (Table 1). Encountered diatom genera were mostly *Diatoma* spp., *Melosira* spp., *Cymbella* spp., *Amphora* spp. and *Gyrosigma* spp. In the 1.2- to 25-μm biofilm fraction, cyanobacteria were numerically dominant, but diatoms represented most of the MPB biomass (Table 1). Most abundant diatom genera were *Achnantidium* spp. and *Cyclotella* spp. Taken together, MPBC in both biofilm fractions (1165 mgC m^{-2}) contributed 16.2% of the estimated biofilm organic C stock.

Meiofauna and especially nematodes dominated the invertebrate community in terms of density (Table 1). Rotifers were also abundant but had a very small individual biomass (~15 ngC ind^{-1}). Hence, for practical reasons (>1000 individuals per sample needed for SIA measurements), they are not considered further. Macrofauna and especially Chironomidae larvae dominated in terms of biomass (Table 1). Taken together, invertebrate C (164.6 mgC m^{-2}) contributed 2.3% of the biofilm organic C stock.

Natural $\delta^{13}C$ and $\delta^{15}N$ signatures

The $\delta^{13}C$ versus $\delta^{15}N$ signatures of leaves, biofilm fractions and invertebrates are shown in Fig. 2. From their low $\delta^{15}N$ values, leaves and both biofilm fractions can be considered as basal food sources (Peterson & Fry, 1987). Their $\delta^{13}C$ signatures differed significantly (ANOVA, $F_{2,11}$ = 66.6, P < 0.001), leaves having the most negative $\delta^{13}C$ values.

The observed trophic enrichment factor (Fig. 2) suggested that: (i) *Chromadorina* nematodes, Naididae oligochaetes and Chironomidae larvae depended on basal food sources: *Chromadorina* fed on the 1.2- to 25-μm biofilm fraction. No clear food source was identified according to

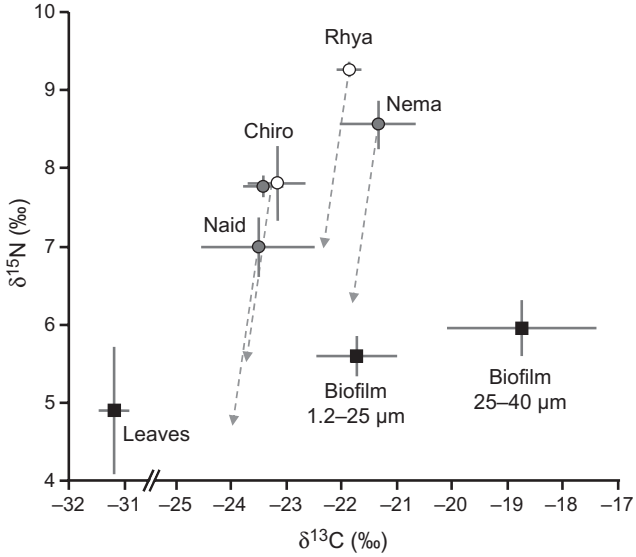

Fig. 2 Mean (n = 4, ±SD) $\delta^{13}C$ and $\delta^{15}N$ natural signatures of basal resources (black squares), meiofauna (grey circles) and macrofauna (white circles) gathered from the epilithic biofilm of the Garonne River. The expected trophic enrichment factors between consumers and their probable diet are shown by dashed arrows. (Nema) *Chromadorina* nematodes, (Chiro) Chironomidae larvae, (Naid) Naididae oligochaetes, (Rhya) Rhyacophilidae larvae.

Table 1 Invertebrates and microphytobenthos (MPB) mean density (±SD, n = 4), carbon (C) to dry mass (DM) content, biomass, contribution to biofilm organic C stock and natural $\delta^{13}C$ and $\delta^{15}N$ signatures (±SD, n = 4) in the epilithic biofilm of the Garonne River on 20 September 2009

Biofilm invertebrates and MPB	Density (ind m^{-2}) or (cell m^{-2})	C/DM (%)	Biomass (mgC m^{-2})	C stock (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Macrofauna (>500 μm)						
Chironomidae larvae	3257 ± 98	42.9	125.1	1.74	−23.39 ± 0.5	7.80 ± 0.5
Rhyacophilidae larvae	230 ± 13	43.1	14.9	0.21	−21.65 ± 0.2	9.25 ± 0.1
Meiofauna (40–500 μm)						
Chromadorina nematodes	369954 ± 32790	45.3	14.2	0.20	−21.22 ± 0.7	8.56 ± 0.3
Chironomidae larvae	11642 ± 1834	38.4	7.2	0.10	−23.36 ± 0.4	7.77 ± 0.1
Naididae oligochaetes	3631 ± 436	38.5	3.5	0.05	−23.18 ± 1.0	6.99 ± 0.4
Biofilm fraction (25–40 μm)						
Diatoms	0.28 × 10^9 ± 46 × 10^6	–	138.7	1.93	−19.22 ± 1.3	5.96 ± 0.4
Green algae	0.15 × 10^9 ± 38 × 10^5	–	123.7	1.72		
Biofilm fraction (1.2–25 μm)						
Diatoms	11.18 × 10^9 ± 1.6 × 10^9	–	804.2	11.17		
Green algae	2.24 × 10^9 ± 0.5 × 10^9	–	68.0	0.94	−21.73 ± 0.7	5.60 ± 0.3
Cyanobacteria	44.72 × 10^9 ± 3.3 × 10^9	–	30.3	0.42		

the trophic enrichment factor of Naididae and Chironomidae. (ii) Rhyacophilidae trichopterans were predators, feeding at least on Naididae.

Labelling experiment

Invertebrate-specific uptake ($\Delta\delta^{13}C$). Invertebrate-specific uptake showed taxa and time effects (two-way ANOVA taxa and time as factors, $F_{12,77} = 2.1$, $P < 0.05$). Significant $\Delta\delta^{13}C$ differences were detected at 24 and at 48 h between a group comprising *Chromadorina* and Chironomidae versus a group comprising Naididae and Rhyacophilidae (Fig. 3, ANOVA taxa as factor, $F_{4,15} = 17.3$, $P < 0.001$ and HSD test). On the one hand, the $\Delta\delta^{13}C$ of *Chromadorina* increased significantly between 3 and 24 h. The $\Delta\delta^{13}C$ temporal dynamics of meio- and macrofaunal Chironomidae was similar to that of *Chromadorina*, except that it decreased significantly at 72 h. On the other hand, the $\Delta\delta^{13}C$ temporal dynamics of Naididae and Rhyacophilidae did not show significant changes after labelling (ANOVA time as factor, $F_{3,19} = 13.3$, $P < 0.001$ and HSD test).

Total ^{13}C uptake (I). *I* evolution in biofilm fractions is shown in Fig. 4. After labelling ($t = 3$ h), MPB from the 1.2- to 25-μm biofilm fraction had incorporated on average (±SD) 41.1 ± 5.3 $mg^{13}C\ m^{-2}$. MPB from the 25- to 40-μm biofilm fraction had incorporated on average 5.8 ± 1 $mg^{13}C\ m^{-2}$. Thus, at the end of the labelling period ($t = 3$ h), 4% of the MPBC stock was ^{13}C-labelled in pooled biofilm fractions. Subsequently *I* decreased exponentially in both biofilm fractions ($n = 16$; 1.2–25 μm: $R = -0.89$, $P < 0.001$; 25–40 μm: $R = -0.85$, $P < 0.001$) with similar attenuation coefficients (–0.026 h^{-1}; Fig. 4), corresponding to a ^{13}C half-life period of 1.1 day (loss of the half initial excess ^{13}C = ln 2/attenuation coefficient).

I evolution in invertebrates is shown in Fig. 5. Macrofaunal Chironomidae incorporated the largest amount of label: with *I* peaking at 1.36 ± 0.23 $mg^{13}C\ m^{-2}$ at $t = 48$ h, whereas Naididae were minor contributors to total invertebrate *I*. Second-order polynomial regressions (bell-shaped curves) best fitted *I* evolution after labelling for *Chromadorina* ($n = 16$, $R = 0.69$, $P < 0.001$), meiofaunal

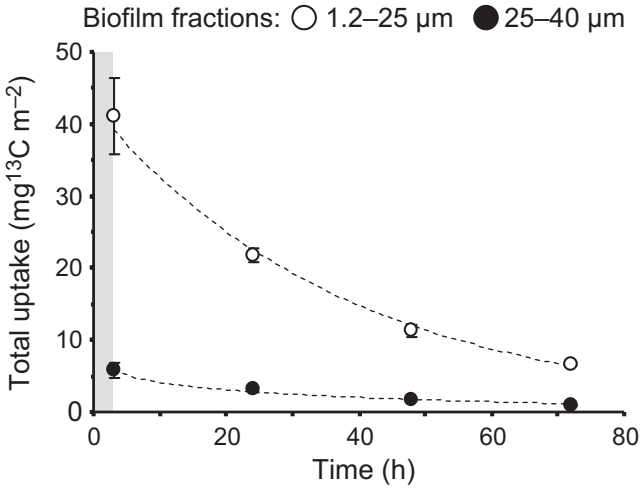

Fig. 4 Evolution of total ^{13}C uptake (*I*) in the 1.2- to 25-μm and 25- to 40-μm biofilm fractions during the post-labelling period ($t = 3$–72 h). Values are means ($n = 4$, ±SD). Exponential fits: $y = 42.47^{-0.026x}$ (1.2–25 μm fraction) and $y = 6.14^{-0.026x}$ (25–40 μm fraction). The shaded area shows the period of microphytobenthos labelling using $NaH^{13}CO3$ ($t = 0$–3 h).

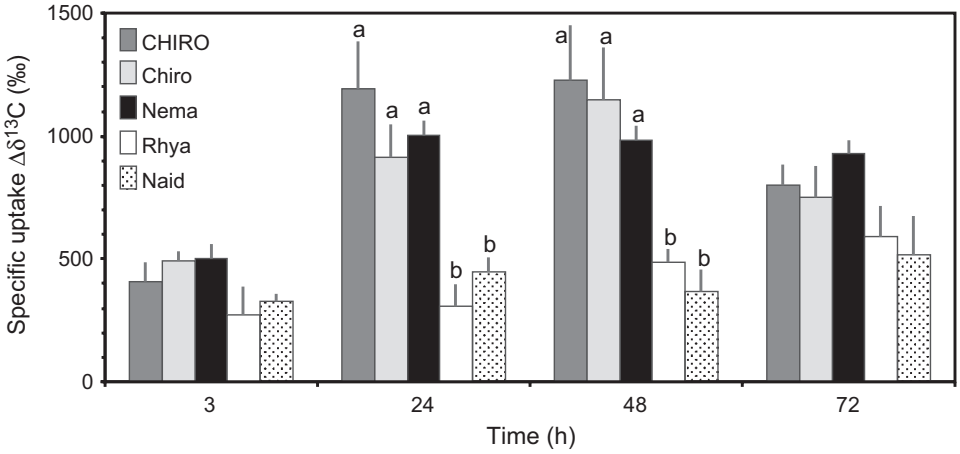

Fig. 3 Specific ^{13}C uptake ($\Delta\delta^{13}C$) by biofilm-dwelling invertebrates during the post-labelling period ($t = 3$–72 h). Values are means ($n = 4$, +SD). (CHIRO) macrofaunal-sized Chironomidae larvae, (chiro) meiofaunal-sized Chironomidae larvae, (Nema) *Chromadorina* nematodes, (Rhya) Rhyacophilidae larvae, (Naid) Naididae oligochaetes. Different letters above bars show significant $\Delta\delta^{13}C$ differences between invertebrate taxa at a given time.

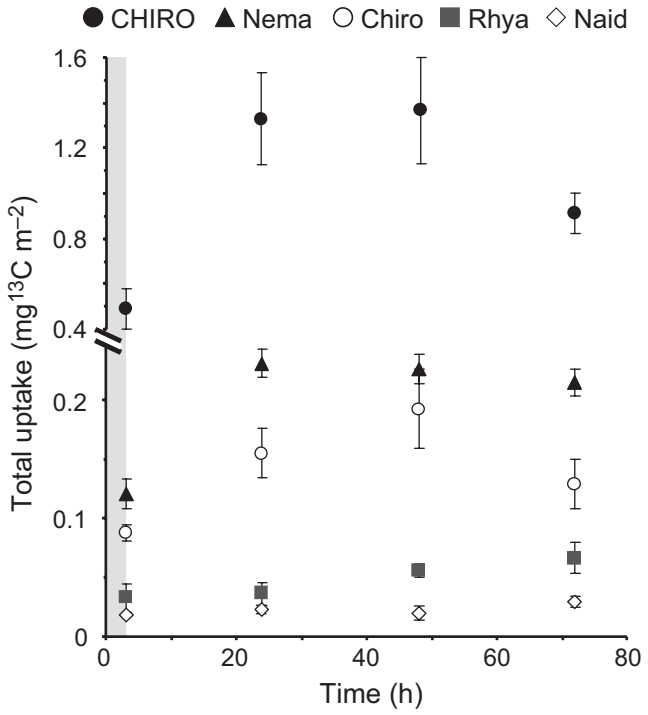

Fig. 5 Evolution of total ^{13}C uptake (I) by biofilm-dwelling invertebrates during the post-labelling period (t = 3–72 h). Values are means (n = 4, ±SD). (CHIRO) macrofaunal-sized chironomidae larvae, (Nema) *Chromadorina* nematodes, (chiro) meiofaunal-sized chironomidae larvae, (Rhya) Rhyacophilidae larvae, (Naid) Naididae oligochaetes. The shaded area shows the period of microphytobenthos labelling using $NaH^{13}CO3$ (t = 0–3 h).

Chironomidae (n = 16, R = 0.54, $P < 0.05$) and macrofaunal Chironomidae (n = 16, R = 0.52, $P < 0.05$). Linear regressions best fitted I evolution for Naididae (n = 16, R^2 = 0.31, $P < 0.05$) and Rhyacophilidae (n = 16, R^2 = 0.54, $P < 0.001$).

Because MPB became progressively less labelled, the temporal attenuation of available ^{13}C was implemented for the following calculation: between t = 3–24 h, daily invertebrate ^{13}C uptake rates were estimated at 1460, 117, 193, 8 and 5 $\mu g^{13}C\ m^{-2}\ day^{-1}$ for macro- and meiofaunal Chironomidae, *Chromadorina*, Naididae and Rhyacophilidae, respectively. Daily total MPBC uptake rates were calculated from these ^{13}C uptake rates (Table 2). It was estimated that the MPBC from the pooled 1.2- to 25- and 25- to 40-μm biofilm fractions contributed 309, 99, 104 and 13% to the daily assimilation demand of macro- and meiofaunal-sized Chironomidae, *Chromadorina* and Naididae, respectively (Table 2). As predators, Rhyacophilidae needed to assimilate 6.7 $\mu gC\ ind^{-1}$ daily, corresponding to the assimilation of, for example, six Naididae individuals.

After labelling (t = 3 h), 98% of the label was stocked in the pooled 1.2- to 25- and 25- to 40-μm biofilm fractions and 2% in invertebrates (Fig. 6). At t = 72 h, 16% of the initial amount of label was still stocked in the pooled biofilm fractions and 3% in invertebrates. However, 81% of the label was found neither in biofilm fractions nor in invertebrates.

Table 2 Biofilm-dwelling invertebrate daily production (P), daily assimilation demand (DAD), daily microphytobenthic carbon (MPBC) assimilation rates and contribution of assimilated MPBC to DAD in the epilithic biofilm of the Garonne River on 20 September 2009

Biofilm-dwelling invertebrates	P (mgC m^{-2} day^{-1})	DAD (mgC m^{-2} day^{-1})	MPBC assimilation (mgC m^{-2} day^{-1})	DAD fulfilled (%)
Macrofauna (>500 μm)				
Chironomidae larvae	4.73	11.82	36.48	309
Rhyacophilidae larvae	0.85	1.54	–	–
Meiofauna (40–500 μm)				
Chromadorina nematodes	2.79	4.65	4.82	104
Chironomidae larvae	1.18	2.95	2.93	99
Naididae oligochaetes	0.59	1.48	0.19	13

Discussion

In our study, microphytobenthos (MPB) represented 16% of biofilm organic content, which is in agreement with values commonly found in these habitats (Azim & Asaeda, 2005). Most of this biomass consisted of diatoms, which can exude high amounts of exopolymeric substances (EPS) for adhesion and protection functions (e.g. Winsborough, 2000). Our study did not disentangle the portion of microphytobenthic carbon (MPBC) incorporated by direct grazing on MPB cells from that incorporated indirectly by MPB–EPS consumption. However, on the basis of contrasting label uptake dynamics, our results allowed to distinguish between *Chromadorina* nematodes and Chironomidae larvae, which seemed to use this freshly produced MPBC, from Naididae oligochaetes and Rhyacophilidae larvae, which used MPBC via recycling by the microbial-loop or through predation, respectively.

From natural $\delta^{13}C$ and $\delta^{15}N$ signatures, we deduced that biofilm-dwelling *Chromadorina* spp. nematodes fed on the 1.2- to 25-μm biofilm fraction, which contained high amounts of small diatoms. This result makes sense, since biomarker pigments of diatoms were found in guts of *Chromadorina* inhabiting epilithic biofilms of the Garonne River (Majdi *et al*., 2012b). Moreover, marine Chromadoridae nematodes (comprising *Chromadorina*) feed commonly on diatoms by piercing and/or cracking frustules to suck out cellular contents (e.g. Tietjen & Lee, 1973; Romeyn & Bouwman, 1983; Moens & Vincx, 1997). In this

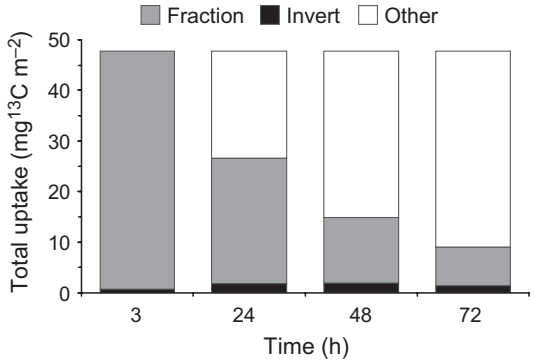

Fig. 6 Evolution of ^{13}C stocks among main biofilm compartments during the post-labelling period (t = 3–72 h). (Fraction) ^{13}C incorporated in pooled 1.2–25 and 25–40 μm biofilm fractions (mean, n = 8), (Invert) ^{13}C incorporated by biofilm-dwelling invertebrates (mean, n = 20), (Other) missing ^{13}C relative to post-labelling (t = 3 h) ^{13}C stocks.

study, *Chromadorina* nematodes rapidly incorporated MPBC, thus realising a rapid transfer of freshly photosynthesised C through the food web by feeding on MPB cells and possibly on their EPS exudates. A similar process was also reported for nematodes inhabiting intertidal sediments (Montagna, 1984; Middelburg *et al.*, 2000; Moens *et al.*, 2002). Considering only direct grazing on MPB cells, *Chromadorina* would be expected to assimilate daily 0.4% of biofilm MPB cell C stocks. This would correspond to a daily ingestion of about 1.6% of biofilm MPB cell C stocks, since nematode ingestion approximates four times assimilation (Herman & Vranken, 1988). This estimation fits well with grazing pressures commonly reported for marine nematodes (e.g. Moens *et al.*, 2002; Pascal *et al.*, 2008b). In superficial sediments of a third-order stream, using fluorescently labelled diatoms (FLD), Borchardt & Bott (1995) found negligible algivory by nematodes. However, these authors specify that, with FLD, only diatoms ingested whole are detected. In our study, the assimilation demand of *Chromadorina* was fully met (104%) by MPBC sources. Majdi *et al.* (2012b) estimate at the same date and site, that MPB cell-content ingestion contributes only 1–23% to *Chromadorina*'s demand. *Chromadorina* nematodes can agglutinate surrounding detritus using mucus silks exuded by their caudal glands (Meschkat, 1934). Bacterial colonisation and growth is generally promoted on these mucus silks (Moens *et al.*, 2005). Riemann & Helmke (2002) propose that bacterial external enzymatic activity initiates the decomposition of complex molecules associated with these agglutinations, so that resulting simple molecules can in turn be easily ingested and incorporated by nematodes. In this context, we suggest that within epilithic biofilms of the Garonne River, *Chromadorina* nematodes feed to a considerable extent on EPS exuded by MPB through such 'gardening' interactions with bacteria and to a lesser extent can graze directly on MPB cells.

Like nematodes, Chironomidae larvae rapidly incorporated freshly produced MPBC. Contrary to what was observed for nematodes, the ^{13}C incorporated by Chironomidae decreased significantly 3 days after labelling. This could reflect rapid population turnover processes (e.g. migration, emergence and removal by predation). We therefore suggest that Chironomidae could represent more important vectors for biofilm MPBC spatial export than nematodes (at a scale of 400 cm^2). Quantitatively, the daily MPBC incorporated by pooled meio- and macrofaunal Chironomidae represented 3.3% of biofilm MPB cell C stocks (considering only direct grazing on cells). However, their ingestion of MPBC must be substantially higher than this assimilation value (Rasmussen, 1984). From our estimates, the assimilation of MPBC greatly exceeded (309%) the demand of macrofaunal Chironomidae, while it fulfilled (99%) the demand of meiofaunal Chironomidae. The feeding activity and energetic needs of Chironomidae larvae can be much greater in later larval instars (Berg, 1995), and we probably underestimated the energetic demand of macrofaunal Chironomidae. In addition, it was somewhat surprising that both meio- and macrofaunal Chironomidae showed similar $\delta^{13}C$ and $\delta^{15}N$ signatures, which were slightly biased towards allochthonous signatures. A possible explanation is that Chironomidae larvae have a rather broad and flexible diet, including MPB as well as fine particulate organic matter (FPOM). Their diet also fluctuates with species and larval development (Berg, 1995; Schmid & Schmid-Araya, 2002). In this context, and since our study concerned pooled Chironomidae species, extrapolation of our findings for Chironomidae larvae must be done with caution and considered as a preliminary estimation.

Naididae oligochaetes feed on various food items, but particularly on FPOM (including bacteria) and diatoms (Learner, Lochhead & Hughes, 1978). Naididae can heavily colonise leaf packs placed in the Garonne River, which are pools of FPOM through the abundance of litter-processing microorganisms and/or the entrapment of fine suspended particles (Chauvet, Giani & Gessner, 1993). In our experiment, $\delta^{13}C$ and $\delta^{15}N$ signatures suggested that Naididae could use a mixture of biofilm fractions and leaf litter. Although we observed entire diatom frustules in Naididae guts, they only gained slight ^{13}C enrichment during the post-labelling period. This strengthens the conclusion that allochthonous C and/or microbial-loop

recycled MPBC contribute predominantly to the diet of Naididae.

The $\delta^{13}C$ and $\delta^{15}N$ signature of Rhyacophilidae larvae suggested that they were mainly predators feeding at least on Naididae oligochaetes and probably also on Chironomidae. This result corresponds to the well-known predatory habits of Rhyacophilidae larvae (Wiggins, 2004). We found no evidence of any predation on nematodes, probably because they were too small to be successfully handled.

After 3 days, on average, 81% of the label initially fixed by MPB photosynthesis could be detected neither in invertebrates nor in biofilm fractions. The ^{13}C half-life loss period ($\tau_{½}$) was 1.1 day in biofilm fractions. In comparison, from the top 5 mm of intertidal sediments of the Scheldt estuary, Middelburg *et al.* (2000) reported slower label losses for diatoms ($\tau_{½}$ = 1.9 day) and for the total carbon pool ($\tau_{½}$ = 2.5 day). To explain their observed MPBC loss, the latter authors pointed to processes such as resuspension, respiration and mixing to deeper sediment layers. For instance, respiration contributes 40% of the MPB ^{13}C loss in intertidal flats after 3 days. While respiration is indeed an important C loss pathway occurring also in the biofilm, river epilithic biofilm habitats differ strongly from intertidal sediments, and some specific hypotheses can be proposed to explain the rapid label loss observed.

1. A major part of the C initially fixed by photosynthesis may be rapidly exuded by diatoms as EPS, which are mostly low-molecular-weight compounds being preferentially and quickly assimilated by bacteria (e.g. Romaní & Sabater, 1999). This C pathway could substantially contribute to the observed label loss, since it was not accounted for in our experimental setup (the <1.2-μm biofilm fraction comprising bacteria and EPS was not analysed).

2. The biofilm may be detached from its substrate by flow constraints (e.g. Biggs & Close, 1989). However, this is not likely, since during our 3-day experimental time-window, the streambed flow velocity at the study site remained low (~10 cm s^{-1}), with discharge of the Garonne River ranging between 30–37 m^3 s^{-1} (Majdi *et al.*, 2011). Boulêtreau *et al.* (2006) showed, at the same site of the Garonne River, that self-detachment of the biofilm from its substratum occurs during extended low-water periods with high temperatures, presumably because of bacterial growth destabilising senescent algal layers. This self-detachment of free-floating biofilm fractions could partly account for the observed label loss.

3. Finally, it is plausible that during our experiment, highly mobile grazers (e.g. fishes, Van Dam *et al.*, 2002) might have grazed and exported some label away from our labelled biofilms.

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