Feeding of biofilm-dwelling nematodes examined using HPLC-analysis of gut pigment content

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Abstract The natural feeding behaviour of the nematodes *Chromadorina bioculata* (Schultze *in* Carus 1857) and *Chromadorina viridis* (Linstow 1876) was studied in situ, within epilithic biofilms of the Garonne River (France). Based on their feeding-type characteristics and population dynamics, it was hypothesised that these species feed selectively on microphytobenthos (MPB) within the biofilm, and that among MPB groups, diatoms are preferred. High-performance liquid chromatography (HPLC) was used for separation, identification and quantification of pigments both in nematode guts and in the biofilm. This is the first time that nematode gut pigment contents were examined under natural conditions. Diatoms dominated the MPB which also comprised cyanobacteria and green microalgae. The comparison between chlorophyll *a* content in nematode guts versus in the biofilm showed that *C. bioculata* and *C. viridis* fed opportunistically (non-selectively) on MPB within the biofilm. Only diatom biomarker pigments were found in nematode guts suggesting that they could preferentially fed on diatoms among MPB groups. However, the non-detection of biomarker pigments for other microphyte groups could be also linked to HPLC detection limits. It was estimated that *Chromadorina* nematodes daily ingested on average 0.03–0.67% of the MPB standing stock. This grazing covered only a small part of their energetic requirements, suggesting that besides MPB they probably also fed on other biofilm food sources. Some considerations on the applicability of the HPLC gut pigment analysis technique for the examination of nematode feeding are also presented.

Keywords Selectivity · Grazing · Diatoms · Periphyton · Meiofauna · Chromadorina

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Introduction

Meiofauna is extremely species rich and abundant in freshwater benthos, contributing substantially to secondary production, acting as food web intermediates and informing general ecological theories such as the metabolic theory of ecology (Schmid-Araya & Schmid, 2000; Schmid-Araya et al., 2002; Bergtold & Traunspurger, 2005; Stead et al., 2005; Reiss et al., 2010; Reiss & Schmid-Araya, 2010). Free-living nematodes are among the most important contributors

to meiofauna (Traunspurger, 2002). Nematodes feed on a variety of microorganisms including microphytes (Moens & Vincx, 1997; Höckelmann et al., 2004), protozoans (Hamels et al., 2001), fungi (Ruess et al., 2002) and bacteria (Traunspurger et al., 1997) and probably also on organic matter through enzyme-sharing interactions with bacteria (Riemann & Helmke, 2002).

In freshwater epilithic biofilms, microphytes, protozoans, fungi and bacteria are embedded in close connection within a three-dimensional mucous matrix of self-produced exo-polymeric substances (EPS; Flemming & Wingender, 2010). These biofilms offer a shelter and a rich variety of potential food items for nematodes (Höckelmann et al., 2004; Peters & Traunspurger, 2005). In return, nematode activity might influence key biofilm processes such as detachment, oxygen turnover and secondary metabolites release (Sabater et al., 2003; Gaudes et al., 2006; Mathieu et al., 2007). Biofilm biomass dynamics can, to a considerable extent, be modelled as a function of hydrodynamics and self-detachment (e.g. Boulêtreau et al., 2006). However, functional field studies assessing nematode feeding habits within these biofilms are lacking (Moens & Vincx, 1997), hampering an appropriate assessment of their trophic role within the mat and their potential feeding impact on biofilm biomass. This lack of in situ data is mostly due to the difficulty of measuring nematode feeding in such complex habitats: not only are epilithic biofilms composed of a complex organic matrix containing a variety of potential food sources for nematodes, but the mucous nature of the biofilm itself poses practical experimental problems.

The quantification of the chlorophyll *a*-equivalent (Chl *a*-eq, i.e. Chl *a* + phaeopigments) contained in guts allows to obtain in situ data on the grazing activity of post-mortem isolated taxa of animals. To date, this technique is routinely used with e.g. planktonic copepods: the quantitative measurement of their gut Chl *a*-eq content with regards to Chl *a* concentration in the surrounding habitat has allowed to investigate their selective grazing on phytoplankton, with a disproportion between gut Chl *a*-eq content and Chl *a* concentration indicating a selective grazing (e.g. Price, 1988; Gasparini et al., 1999; Irigoien et al., 2000; Tackx et al., 2003).

Gut pigment analyses using high-performance liquid chromatography (HPLC) can inform on feeding selectivity among various microphytic taxa by identifying and quantifying their biomarker pigments. This technique was applied with some meiobenthic groups: harpacticoid copepods (Buffan-Dubau et al., 1996; Buffan-Dubau & Carman, 2000) and chironomids (Goldfinch & Carman, 2000) in muddy salt marshes, but not with nematodes (Moens et al., 2006). Although both selective and non-selective feeding strategies were observed for free-living marine bacterial feeding or predaceous nematodes under laboratory conditions, nematode selectivity on microphytobenthos (MPB) in situ and in freshwater habitats is poorly documented (Moens & Vincx, 1997; Moens et al., 2006).

In order to determine ingestion rates from gut pigment contents, these have to be reported to gut passage times (GPT). However, information on nematode GPT and their dependence on environmental factors remain scarce and mainly restricted to bacterial-feeding nematodes (Moens et al., 1999, 2006). Thus, a careful approach is needed for determining ingestion rates from measurements of gut pigment contents by using literature GPT. Nonetheless, given our generally limited knowledge about the grazing rates of freshwater nematodes (Borchardt & Bott, 1995), even such estimations represent, at present, a significant advancement in the evaluation of their grazing pressure on MPB.

In a recent study conducted in the Garonne River, Majdi et al. (2011) found a coupling pattern between epilithic diatom biomass and the density of the two dominant biofilm-dwelling nematode species: *Chromadorina bioculata* (Schultze *in* Carus 1857) and *Chromadorina viridis* (Linstow 1876). According to their buccal morphology, both these species were classified as epistrate-feeders after Traunspurger (1997), and hence are expected to feed predominantly on microphytes (Traunspurger, 2000). In marine environments, a diatom-feeding behaviour is well-documented for Chromadoridae (i.e. the family including *Chromadorina* spp. nematodes), which puncture or crack diatom frustules to suck inner cellular contents (Tietjen & Lee, 1977; Jensen, 1982; Romeyn & Bouwman, 1983; Moens & Vincx, 1997). Examining the digestive physiology of C*hromadorina germanica* Bütschli 1874, Deutsch (1978) also suggested that it must have a fairly narrow diet primarily composed of diatoms. As stated above, river epilithic biofilms offer a vast variety of potential food items to the nematode community. Within this offer, MPB seem a likely preferred food source considering the above mentioned knowledge on the feeding behaviour

of the dominant nematode species (*Chromadorina* spp.). It can also be expected that epilithic diatoms are selected among the other microphyte groups available in the biofilm.

In this context, this study aims: (1) to test the hypothesis that biofilm-dwelling *C. bioculata* and *C. viridis* nematodes feed selectively on biofilm MPB under natural conditions and that diatoms are preferred among microphyte groups, (2) to estimate their grazing pressure on MPB biomass.

Methods

Study site and sampling

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. The Garonne is characterised by strong hydrodynamics (Chauvet & Décamps, 1989) displaying a pluvio-nival flow regime with relatively short flash-floods caused by heavy rainfall and a long and intense spring flood period due to snow-melt. The river bed consists mainly of cobbles and gravels, and between floods, a thick epilithic phototrophic biofilm typically coats the upper surfaces of cobbles. Sampling was undertaken at a cobble bar of the Garonne river situated 36 km upstream the city of Toulouse (01°17′53″E, 43°23′45″N). At this site, the residence time is too low for important phytoplankton development, and it is assumed that benthic biofilms provide most of the riverine primary production (Ameziane et al., 2003).

Epilithic biofilm samples were weekly collected on September and October 2008, January, March and September to November 2009. On each sampling occasion ($N = 23$), water temperature (T), dissolved oxygen concentration (O_2), conductivity, pH and flow velocity were recorded at 5 cm above the streambed using an automated YSI 6000 multi-parameter probe (YSI inc., Yellow springs, OH, USA) and a Flow-meter Flo-Mate 2000 (Flow-Tronic, Welkenraedt, Belgium). Twelve submerged cobbles covered by epilithic biofilm (diameter $\sim$ 10 cm) were collected and processed: (1) to determine nematode species assemblages, density and individual biomass, (2) to measure total epilithic dry mass (DM) and ash-free dry mass (AFDM), (3) to measure biofilm MPB pigment concentrations using HPLC-analysis, and (4) to estimate the relative contribution of the different MPB groups to total MPB biomass in terms of chlorophyll *a* (Chl *a*) using CHEMTAX version 1.95 software (Mackey et al., 1996). These procedures are detailed in Majdi et al. (2011).

For nematode gut pigment analysis, four more cobbles were collected on each sampling occasion. The biofilm covering cobbles was collected in the field by scraping-off the upper cobble surface with a scalpel and immediately immerged into liquid N_2. This instant freezing minimises nematode gut content egestion (Moens et al., 1999). Frozen biofilm samples were then stored at −80°C until nematode sorting for gut pigment analyses.

Nematode sorting for gut pigment analysis

A biofilm sample was allowed to thaw in a 5-l bucket with 100 ml tap water. Once defrosted, aggregates were crumbled with scissors. Then, a water jet was used to mix the biofilm suspension, in order to facilitate the separation of nematodes from heavier particles by decantation after Hodda & Abebe (2006). After 2 min of decantation, the supernatant containing nematodes and other light particles was poured through a 40-µm sieve to retain nematodes. The decantation operation was repeated four times. Then, undamaged nematodes were sorted from the bulk of gathered filtrate and isolated in small groups of 50 individuals under a stereomicroscope (9×–90×) while avoiding rare large suction-feeding nematodes. Each group was transferred with a 10 µl pipette to a petri dish containing a cold milliQ water rinsing bath. The operation was repeated until at least 400 nematodes lay in the rinsing bath. There, nematodes were thoroughly cleaned from any adherent particles, isolated by groups of 20 individuals, photographed and carefully pipetted in an eppendorf tube. All sorting operations were conducted under minimum light exposure and above a thin ice block to limit pigment photo- and/or thermo-degradation. At least a 400 nematode sample was prepared on each sampling occasion.

Extraction and HPLC-analysis of nematode gut pigment contents

Each sample of sorted nematodes was centrifuged (500 g, 5 min) to allow the settlement of a "nematode pellet". Excess water was removed by freeze-drying

and pigments were extracted from nematode samples in 200 µl of 98% cold-buffered methanol (with 2% of 1 M ammonium acetate) by sonicating for 90 s in an ultrasonic bath (Elmasonic S-10 series, IMLAB, Lille, France). Extraction was then allowed overnight at −20°C in the dark. The pigment extract so obtained was then filtered on a 0.2 µm PTFE syringe filter with very low dead volume < 10 µl (ReZist series Ø13 mm, Whatman inc., Florham Park, NJ, USA) and analysed using the method described for biofilm pigment analyses in Majdi et al. (2011). A high-performance liquid chromatograph (HPLC) consisting of a 100 µl loop auto-sampler and a quaternary solvent delivery system coupled to a diode array spectrophotometer (LC1200 series, Agilent Technologies inc., Santa Clara, CA, USA). The mobile phase was prepared and programmed according to the analytical gradient protocol described in Barlow et al. (1997). Pigment separation was performed through a C8, 5 µm column (MOS-2 HYPERSIL, Thermo Fisher Scientific inc., Waltham, MA, USA). The diode array detector was set at 440 nm to detect carotenoids, and at 665 nm to detect chlorophylls and phaeopigments (Wright et al., 1991). Data analysis was performed using ChemStation software (version A.10.02, Agilent Technologies inc.). Pigments were identified by comparing their retention time and absorption spectra with those of authentic standards (DHI LAB products, Hørsholm, Denmark), except for peridinin and diatoxanthin, which were obtained from the dinoflagellate species *Amphidinium carterae* Hubert 1967, CCAP strain 1102/3 (Culture Collection of Algae and Protozoa, Oban, UK). For pigment quantification, a response factor was calculated for each standard from the linear relationship between the concentration and the corresponding peak area on HPLC chromatograms. Pigments that were spectrally similar to, but did not have the same retention time as standards were designated 'like'-pigments. They were quantified using the response factor obtained from corresponding standards and summed to the value of the corresponding original pigment, e.g. Chlorophyll *a* (Chl *a*) quantification = Chl *a*-like$_1$ + Chl *a*-like$_2$ + Chl *a* (see Table 1).

The nematode community was strongly dominated by *C. bioculata* and *C. viridis* (see results). The few other species isolated concomitantly were all deposit-feeders which have a minute unarmed buccal cavity allowing them only to swallow small preys such as bacteria (Moens et al., 2006). Therefore, the presence of potential MPB pigments in their guts was presumed to be minor. Hence, pigment concentrations measured from nematode extracts were reported to the expected proportion (number) of *C. bioculata* and *C. viridis* individuals extracted. To correct for possible nematode pigment which did not stem from the gut content, 415 nematodes were starved for 48 h in filtered (0.2 µm) river water to represent a nematode control sample analysed using the same HPLC protocol described above.

Nematode ingestion rates, production and energy requirements

Data on nematode gut passage times (GPT) are rare, but since their gut is completely emptied with each defecation (Duncan et al., 1974), and defecation intervals are very short (Avery & Thomas, 1997), GPT are likely to last only few minutes for most nematode species (Moens et al., 2006). GPT shorter than 2 min were reported for the bacterial-feeding *Caenorhabditis elegans* Maupas 1900 (Ghafouri & McGhee, 2007). Defecation intervals of < 4–43 min were observed in the marine Monhysterida *Daptonema* sp., and defecation intervals of 14–23 min were observed for the marine Chromadoridae *Spilophorella* sp. while feeding on diatoms (Moens et al., 1999, see discussion). Consequently, and knowing that *C. bioculata* was reported to be very active (Croll & Zullini, 1972), we used an average GPT of 14 min for all sampling occasions to estimate daily ingestion rates based on gut pigment content data. However, due to our uncertainty about the GPT of *Chromadorina* in field conditions, ingestion rates were calculated with an error interval using GPT five-fold shorter or longer than 14 min (i.e. 2.8–70 min).

Nematode wet weights were calculated from their body dimensions (length and width) after Andrássy (1956) and converted into carbon content assuming a dry/wet weight ratio of 0.25 (Warwick & Gee, 1984) and a carbon/dry weight ratio of 0.45 (Peters, 1983). Nematode production was calculated for each sampling after Plante & Downing (1989): $\mathrm{Log}(P) = 0.06 + 0.79 \times \mathrm{Log}(B) - 0.16 \times \mathrm{Log}(M_{\max}) + 0.05 \times T$, with mean nematode biomass (B, mgC m^{-2}), maximum individual biomass ($M_{\max}$, µgC ind^{-1}) and average surface water temperature (T). Nematode production was then expressed per day by dividing P by 365. This method was recently recognised to give

Table 1 Microphytobenthic pigments in biofilm and nematode extracts. Biofilm pigment concentrations are reported to corresponding biofilm dry mass (DM). Gut pigment contents are expressed per individual *Chromadorina* spp. Pigments are listed following their elution order. Probable pigment sources were compiled after Johansen et al. (1974), Jeffrey et al. (1997) and Majdi et al. (2011)

Peak #	Pigment	Biofilm (μg gDM^{-1})		Gut (pg ind^{-1})		Probable pigment source
		Mean	Range	Mean	Range	
1	Chlorophyll *c*[a]	67	6–158	0.25	0–1.21	Diatoms
2	Pheophorbide *a*	8	2–23	0.29	0.03–1.56	Chlorophyll *a* degradation
3	Pheophorbide *a*-like					
4	Fucoxanthin-like	290	26–704	1.16	0.05–2.74	Diatoms
5	Fucoxanthin					
6	Violaxanthin	7	2–20	Not detected		Green microalgae
7	Diadinoxanthin-like	41	3–128	0.03	0–0.18	Diatoms
8	Diadinoxanthin					
9	Zeaxanthin	5	1–16	Not detected		Cyanobacteria
10	Lutein	8	2–19	Not detected		Green microalgae
11	Chlorophyll *b*	11	2–23	Not detected		Green microalgae
12	Chlorophyll *a*-like$_1$[b]					
13	Chlorophyll *a*	709	72–1740	0.90	0.06–4.51	All microphytes
14	Chlorophyll *a*-like$_2$					
15	Pheophytin *a*	13	2–24	4.61	1.46–7.56	Chlorophyll *a* degradation
16	Carotenes ($\alpha + \beta$)	23	3–58	0.21	0–0.96	All microphytes

[a] Chlorophyll *c* = chlorophylls $c_1 + c_2$

[b] Chlorophyll *a*-like$_1$ = three chlorophyll *a* allomer compounds

the most reliable estimates of invertebrate production, partly because it takes into account the effect of temperature on invertebrate metabolism (Butkas et al., 2011). Further, nematode energetic requirements (in terms of carbon) were estimated from production assuming a 20% factor for energy conversion efficiency (Heip et al., 1990). Assuming an assimilation/ingestion efficiency of 25% (Herman & Vranken, 1988), assimilation rates of MPB were compared to energetic requirements, to infer the contribution of MPB to the diet of nematodes.

Statistical analyses

All data fulfilled normality assumptions (Kolmogorov–Smirnov test) and homogeneity of variances (Levene test). Hence they were not transformed. Correlations were examined by Pearson correlation coefficient. To disentangle the potential co-influence of correlated predictors, e.g. biofilm biomass, pigment concentrations, temperature and O_2 on gut Chl *a*-equivalents (Chl *a*-eq, i.e. Chl *a* + phaeopigments), a multiple regression was performed using stepwise forward selection. *F* to enter was set at 1 with a *P* value < 0.001. By comparing the statistical significance of predictors in a stepwise design, this procedure allowed selecting the most relevant predictor(s) which explained gut Chl *a*-eq variations. All tests were performed using Statistica software (version 8.0, Statsoft inc., Tulsa, OK, USA).

Results

Biofilm microphytobenthos (MPB)

Along the study period, the biofilm DM averaged 328 g m^{-2} (ranging from 91–679 g m^{-2}), AFDM averaged 26.1 g m^{-2} (8.8–58 g m^{-2}) and Chl *a* averaged 235 mg m^{-2} (46–803 mg m^{-2}). The identified pigments from biofilm extracts are listed in Table 1 and examples of biofilm HPLC-chromatograms are shown in Fig. 1a, b. Among biomarker pigments, fucoxanthin and chlorophyll *c* (Chl *c*) were present in substantial concentrations (> 50 μg gDM^{-1}). They may originate from diatoms and other groups of

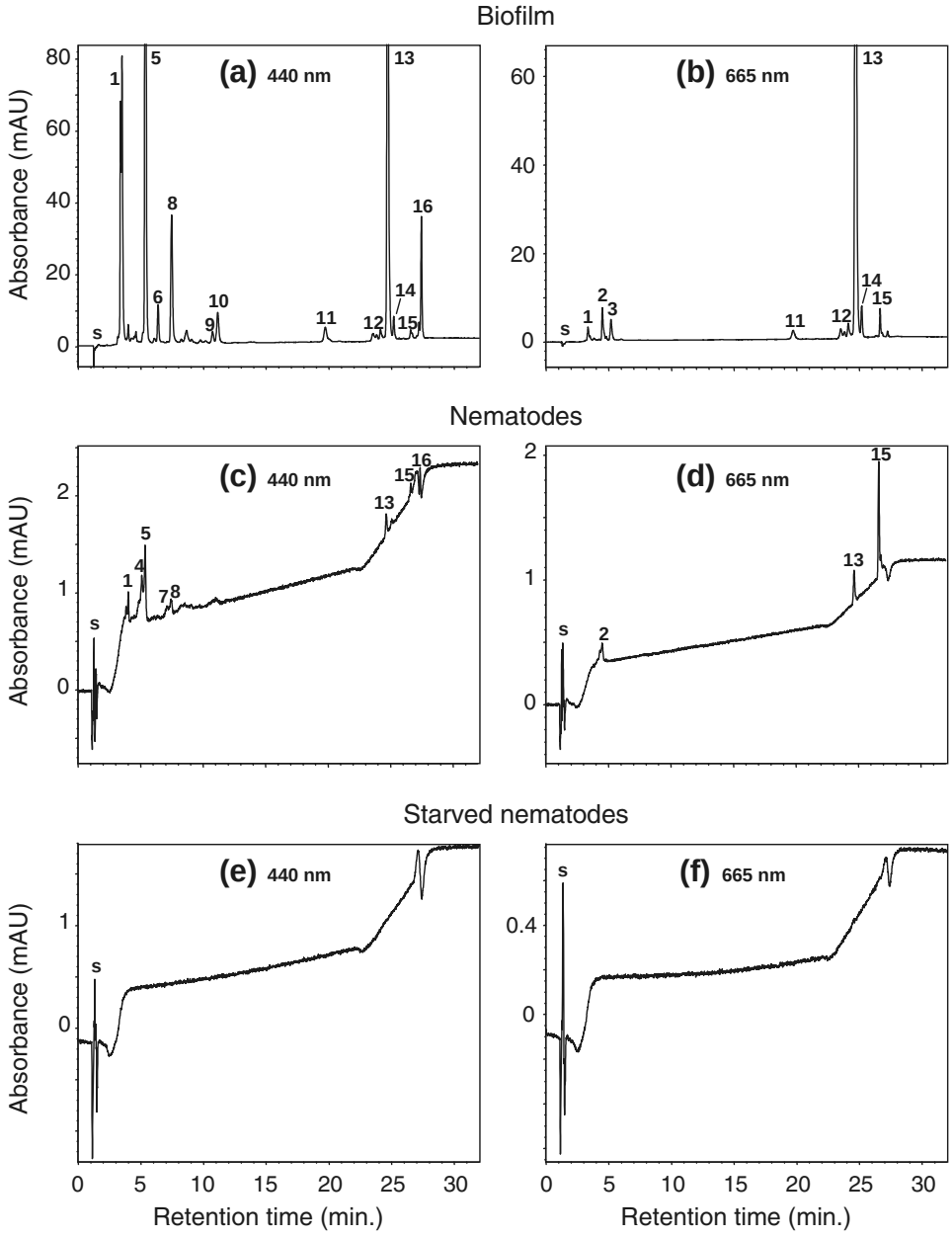

Fig. 1 Examples of absorbance HPLC-chromatograms measured at 440 and 665 nm of **a**, **b** biofilm extract, **c**, **d** extract of nematodes collected in the biofilm (comprising 400 individuals), **e**, **f** extract of nematodes starved for 48 h (comprising 415 individuals). For peak identification see Table 1. *S* solvent-front peak. Absorbance is expressed in milli arbitrary units (mAU)

chromophyte algae, e.g. prymnesiophytes and chrysophytes (Stauber & Jeffrey, 1988). However, typical biomarkers for prymnesiophytes and chrysophytes such as 19′-butanoyloxyfucoxanthin and 19′-hexanoyloxyfucoxanthin (Jeffrey et al., 1997) were not detected in the biofilm, indicating that fucoxanthin and Chl *c* mainly originated from diatoms. Likewise, diadinoxanthin which may be produced by diatoms, euglenophytes and dinoflagellates, was detected. However, neoxanthin which is a typical biomarker pigment for euglenophytes (Schagerl et al., 2003) as well as peridinin and diatoxanthin which are biomarker pigments for dinoflagellates (Johansen et al., 1974) were not detected, implying that diadinoxanthin also mainly originated from diatoms. Zeaxanthin was detected in the biofilm, and although it may be found as a minor pigment in green algae, it is primarily a product of cyanobacteria (Brotas & Plante-Cuny, 1998). Lastly, biomarker pigments chlorophyll *b* (Chl *b*) and lutein accounting for green algae and vascular plants were also detected. However, field and microscopic observations did not reveal the presence of macrophytes within the biofilm community. Furthermore, the biofilm Chl *a*/phaeopigments ratio averaged 36.5, indicating that the epilithic phototrophic community was in a viable state (Buffan-Dubau

et al., 1996) and that the potential contribution of fine particulate plant and/or macrophyte-derived detritus to the biofilm matrix was minute. Hence, green microalgae were likely the main source of lutein and Chl *b*.

Considering all sampling occasions, it was estimated that the total biofilm MPB biomass consisted on average of 82% diatoms, 17% green microalgae and 1% cyanobacteria. This dominance of diatoms was also underlined by significant positive correlations found between Chl *a* and diatom biomarker pigment concentrations in the biofilm (Pearson correlation, $N = 23$; Chl *a* and Chl *c*: $R = 0.98$, $P < 0.001$; Chl *a* and fucoxanthin: $R = 0.97$, $P < 0.001$; Chl *a* and diadinoxanthin: $R = 0.94$, $P < 0.001$). Biofilm Chl *a* concentration correlated also positively with AFDM and O_2 (Pearson correlation, $N = 23$; Chl *a* and AFDM: $R = 0.61$, $P < 0.001$; Chl *a* and O_2: $R = 0.47$, $P < 0.01$), whereas negatively with water temperature (Pearson correlation, $N = 23$, $R = -0.6$, $P < 0.001$, see Fig. 2a).

Nematode community

Over the study period, nematode density averaged (±SD) $2.8 \times 10^5 \pm 0.3 \times 10^5$ ind m^{-2} (ranging from $0.8–6.1 \times 10^5$ ind m^{-2}). Nematode individual biomass averaged 0.11 µgC ind^{-1} (0.08–0.14 µgC ind^{-1}). The total biomass of nematodes in the biofilm averaged 32.4 ± 4 mgC m^{-2} (9.4–78 mgC m^{-2}). The epistrate-feeding species *C. bioculata* and *C. viridis* dominated strongly, averaging 94.2% (75–100%) of nematode species inhabiting the biofilm (Fig. 2b). The other species contributing to nematode community were all deposit-feeders: *Eumonhystera dispar* (Bastian 1865), *Eumonhystera vulgaris* (de Man 1880), *Eumonhystera barbata* Andrássy 1981, *Monhystrella paramacrura* (Meyl 1954), *Plectus opisthocirculus* Andrássy 1952 and *Plectus aquatilis* Andrássy 1985. Large suction-feeding *Dorylaimus* cf *subtiliformis* (Andrássy 1959) were rarely encountered.

Gut pigment contents and feeding behaviour of nematodes

The identified pigments from nematode extracts are listed in Table 1 and examples of HPLC-chromatograms are shown in Fig. 1c, d. Neither MPB pigments nor nematode body constituent pigments were detected from the control sample conducted with starved nematodes (Fig. 1e, f). Thus, it was assumed that pigments detected in field nematode extracts stem

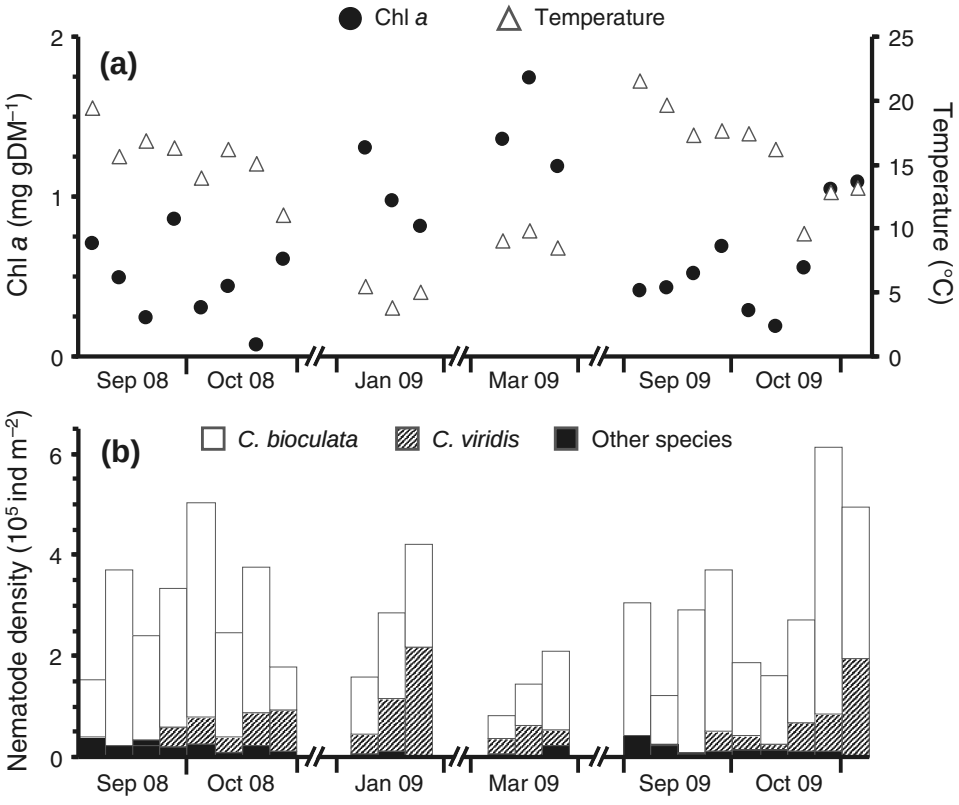

Fig. 2 Temporal dynamics ($N = 23$) of **a** water temperature and biofilm chlorophyll *a* concentration (Chl *a*), **b** nematode density in the biofilm with the relative proportion of *Chromadorina bioculata*, *Chromadorina viridis* and other nematode species

from their gut contents. Fucoxanthin was the major biomarker pigment observed in nematode extracts, indicating that nematodes fed on diatoms. This was corroborated by the presence of diadinoxanthin and Chl *c* in nematode extracts (Fig. 1c; Table 1). Biomarker pigments of cyanobacteria and green microalgae (e.g. zeaxanthin and Chl *b*) were not detected in nematode extracts (Table 1). The Chl *a*/phaeopigments ratio averaged 0.18 in nematode extracts. This value, which is very low compared to that found in biofilm extracts, reflects the Chl *a* breakdown during digestive processes of nematodes. Hence, to account for this degradation, the Chl *a*-equivalent (Chl *a*-eq) was quantified by summing Chl *a*, pheophorbide *a* and pheophytin *a*. Chl *a*-eq was considered as a proxy for total MPB biomass in nematode guts. It averaged (±SD) 5.8 ± 0.3 pg ind^{-1} (ranging from 2.6 to 9.1 pg ind^{-1}).

Gut Chl *a*-eq and gut pheophytin *a* correlated positively with biofilm Chl *c*, fucoxanthin, diadinoxanthin, Chl *a*, AFDM and dissolved oxygen (O_2) (Table 2), whereas negatively with water temperature (*T*). Gut Chl *a* correlated positively with biofilm Chl *c*, fucoxanthin, AFDM and O_2, whereas negatively with T. Gut pheoporbide *a* correlated positively with O_2, whereas negatively with T. Gut diadinoxanthin correlated positively with O_2. Lastly, gut Chl *c* correlated negatively with *T*. Conductivity, pH, streambed flow velocity, biofilm DM, pheophytin *a*, pheophorbide *a* and α,β-carotenes concentrations were not presented in Table 2, since they did not show any significant correlation with gut pigment contents.

Results from the stepwise multiple regression analysis indicated that among the predictors which were correlated with gut Chl *a*-eq variations (Table 2), only Chl *a* concentration in the biofilm was significantly selected ($F = 34$, $P < 0.001$). This was expected since all of these predictors were also correlated with biofilm Chl *a* concentration (see above). The relationship between nematode gut Chl *a*-eq and biofilm Chl *a* concentration (Fig. 3) was rectilinear ($N = 31$, $R^2 = 0.54$, $P < 0.001$), showing that nematodes ingested MPB (in terms of Chl *a*-eq) proportionally to MPB availability in the biofilm (in terms of Chl *a* concentration).

Grazing pressure and energy requirements covered by MPB ingestion

Assuming GPT of 2.8, 14, and 70 min (see "Methods" section), the *C. bioculata* and *C. viridis* population grazed a mean (min–max) of 875 (271–3023), 175 (54–605) and 35 (11–120) μgChl *a*-eq m^{-2} day^{-1}, respectively. Compared to biofilm Chl *a* standing stocks, this means that they daily ingested 0.67 (0.04–1.87), 0.13 (0.01–0.37) and 0.03 (0.002–0.07) % of biofilm MPB biomass (in terms of Chl *a*), respectively. Assuming a carbon (C)/Chl *a* ratio of 17.2, estimated from biofilm-microphyte biovolume measurements at the study site (Leflaive et al., 2008), the MPB C ingested yearly averaged 5.5, 1.1 and 0.2 gC m^{-2} year^{-1}, respectively.

Yearly production of *C. bioculata* and *C. viridis* was 1.4 gC m^{-2} year^{-1}. However, daily production

Table 2 Pearson correlations ($N = 31$) between nematode gut pigment concentration and biofilm habitat characteristics

Gut pigments (pg ind^{-1})	Biofilm pigments (μg gDM^{-1})				AFDM (g m^{-2})	*T* (°C)	O_2 (mg l^{-1})
	Chl *c*	Fuco	Diad	Chl *a*			
Chl *c*	ns	ns	ns	ns	ns	−	ns
Fuco	ns	ns	ns	ns	ns	ns	ns
Diad	ns	ns	ns	ns	ns	ns	+
Car	ns	ns	ns	ns	ns	ns	ns
Chl *a*	+	+	ns	ns	+ +	− −	+ +
Pheob *a*	ns	ns	ns	ns	ns	−	+ +
Pheot *a*	+ + +	+	+ + +	+ + +	+ +	− − −	+ + +
Chl *a*-eq	+ + +	+ + +	+ + +	+ + +	+ +	− − −	+ +

Chl c Chlorophyll *c*, *Fuco* fucoxanthin, *Diad* diadinoxanthin, *Car* α,β-carotenes, *Chl a* chlorophyll *a*, *Pheob a* pheophorbide *a*, *Pheo a* pheophytin *a*, *Chl a-eq* chlorophyll *a*-equivalents, *AFDM* ash-free dry mass, *T* water temperature, O_2 dissolved oxygen. Pearson correlation abbreviations: not significant (ns), significantly negative at $P < 0.05$ (−), $P < 0.01$ (− −) and $P < 0.001$ (− − −); significantly positive at $P < 0.05$ (+), $P < 0.01$ (+ +) and $P < 0.001$ (+ + +)

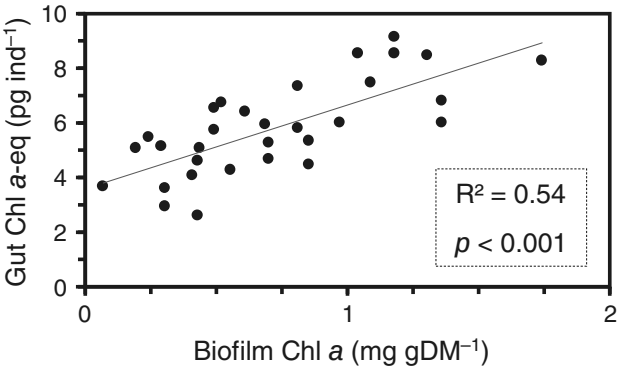

Fig. 3 Linear correlation ($N = 31$) between individual gut content of *Chromadorina bioculata* and *Chromadorina viridis* in chlorophyll *a*-equivalent (Chl *a*-eq) and the biofilm chlorophyll a concentration (Chl *a*)

fluctuated substantially: 1–9 mgC m^{-2} day^{-1} (Fig. 4). When production was expressed in terms of carbon requirements, *C. bioculata* and *C. viridis* needed to assimilate yearly 7.2 gC m^{-2} $year^{-1}$ to fulfil 100% of their requirements. Always assuming GPT of 2.8, 14 and 70 min, the MPB C assimilated (25% of ingestion, see methods) yearly covered on average 1, 5 and 27% of their requirements, respectively. But this fluctuated from 0.1 to 100% depending on the sampling date and on the GPT assumed (Fig. 4).

Discussion

The nematodes *C. bioculata* and *C. viridis* strongly dominated the biofilm-dwelling nematode community at the study site. Widespread in European freshwater periphytic habitats (Decraemer & Smol, 2006), these two species show a typical epilithic lifestyle with their ability to attach themselves to hard substrates with sticky silks produced by their caudal glands (Meschkat, 1934; Croll & Zullini, 1972; Decraemer & Smol, 2006). Both species are described as epistrate-feeders expected to feed predominantly on MPB, although feeding on bacteria or on unicellular heterotrophic eukaryotes is not excluded (Traunspurger, 2000).

Gut Chl *a*-eq content and non-selective feeding on MPB

Our study confirms that biofilm-dwelling *C. bioculata* and *C. viridis* fed on MPB under natural conditions, as Chl *a*-eq was found in their guts. However, our results also show that their gut Chl *a*-eq content was rectilinearly correlated with biofilm Chl *a* concentration, implying that their grazing on MPB was proportional to MPB availability in the biofilm. Some previous laboratory studies highlighted such proportional feeding responses to prey density with bacterial-feeding nematodes (e.g. Nicholas et al., 1973; Schiemer, 1983; Moens & Vincx, 2000), predaceous nematodes (e.g. Bilgrami et al., 1984; Bilgrami & Gaugler, 2005) and marine algal-feeding nematodes (Montagna et al., 1995; Pascal et al., 2008). Nevertheless, to the best of our knowledge, this is the first time that such proportionality was observed for nematodes feeding under natural conditions. A linear relationship between ingestion and prey concentration reveals that either preys are taken up non-selectively, proportionally to their abundance in the medium (e.g. Gasparini et al., 1999), or that the prey abundance is below the critical

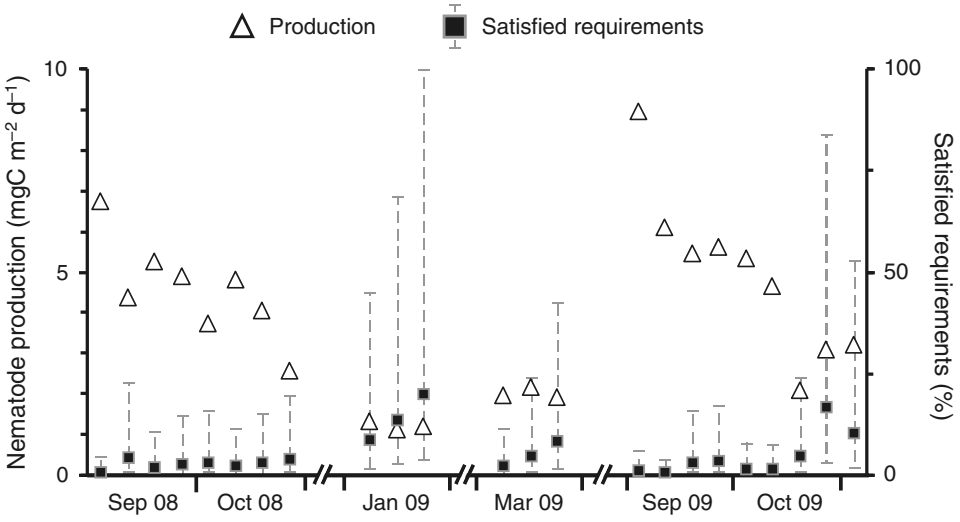

Fig. 4 Temporal dynamics ($N = 23$) of the daily production of *Chromadorina bioculata* and *Chromadorina viridis*, and the proportion of their energetic requirements satisfied by MPB consumption. The use of gut passage times (GPT) of 2.8, 14, and 70 min is depicted by *upper interval*, *black square* and *lower interval*, respectively

concentration at which ingestion is saturated (i.e. type II and III functional responses: Holling, 1959). Only a minor fraction of the biofilm MPB biomass (in terms of Chl *a*) was consumed by nematodes. Hence, it seems unlikely that biofilm-dwelling *Chromadorina* nematodes were capable of selecting MPB, but did not arrive at their ingestion saturation given the high MPB availability encountered. While a strong competition with other biofilm inhabitants (e.g. rotifers, insect larvae) could perhaps explain such a situation, we find rather likely that the linear relationship between nematode gut Chl *a*-eq content and biofilm Chl *a* concentration reflected a non-selective feeding on biofilm MPB.

River epilithic biofilms are structurally complex assemblages where distribution of organisms can be very patchy, constrained by environmental biotic and abiotic disturbances (e.g. Murga et al., 1995; Lyautey et al., 2005; Leflaive et al., 2008; Majdi et al., 2011). For instance, in the Garonne River, the observed negative correlation between temperature and biofilm MPB biomass is likely linked to a temperature-dependent bacterial degradation of the biofilm inducing its self-detachment from the cobbles occurring during summer–autumn low-flow periods (Lyautey et al., 2005; Boulêtreau et al., 2006). Hence, to overcome biofilm biotic composition fluctuations, biofilm-dwelling nematodes likely have an interest to adopt a non-selective, opportunistic feeding behaviour in response to available food, as observed by many estuarine nematodes (Moens & Vincx, 1997).

Gut biomarker pigments and nematode feeding on diatoms

A non-selective nematode feeding on MPB in general does not necessarily exclude that a potential selectivity occurred for (a) specific group(s) of microphytes among MPB. Only diatom biomarker pigments were found in nematode gut extracts. This could perhaps suggest that they mainly ingested diatoms. This result would not be surprising, since diatoms strongly dominated the biofilm MPB community throughout the sampling occasions. It is also well known that diatoms are a high-quality food resource often used by benthic invertebrates—including marine nematodes—probably because of their high content of polyunsaturated fatty acids (e.g. Phillips, 1984; Goedkoop & Johnson, 1996; Buffan-Dubau & Carman, 2000). Besides, marine nematodes can also feed on green algae and cyanobacteria (e.g. Tietjen & Lee, 1973; Evrard et al., 2010). In our study, no biomarker pigments for green algae (e.g., lutein) or cyanobacteria (zeaxanthin) were detected in nematode extracts.

However, this non-detection of green algal and cyanobacterial biomarker pigments could be due to the detection limit of the HPLC device. Indeed, in biofilm chromatograms, the average ratio of fucoxanthin/lutein peak areas was 49, and fucoxanthin/zeaxanthin was 106. In nematode chromatograms, the peak area of fucoxanthin averaged 3 mV s. Hence, assuming a grazing over MPB groups proportional to their availability in the biofilm, the peak area of lutein and zeaxanthin would have been 0.06 and 0.03 mV s, respectively, which is below the detection limit (0.1 mV s) of the HPLC device used.

Grazing pressure

Even using the shortest GPT considered (i.e. 2.8 min), it was estimated that *C. bioculata* and *C. viridis* nematodes exerted a rather small grazing pressure on biofilm MPB standing stocks (0.67%). Comparable low nematode grazing pressures are reported from various marine and brackish habitats (Epstein & Shiaris, 1992; Nozais et al., 2001; Moens et al., 2002; Rzeznik-Orignac et al., 2003; Pascal et al., 2008). In superficial sediments of a third-order stream, Borchardt & Bott (1995) find a negligible algivory of nematodes using fluorescently labelled diatoms. However, only swallowed whole diatoms are detected with this technique, so that the grazing of nematodes such as Chromadoridae, which suck out inner frustule contents, was probably underestimated by these authors. Our estimates also emphasised that nematode grazing pressure fluctuated with temporal constraints, as observed from other meiobenthic organisms (Buffan-Dubau & Carman, 2000; Goldfinch & Carman, 2000). River epilithic biofilms show high turnover rates, especially under grazing pressure (Lamberti & Resh, 1983). Hence, the low-estimated nematode grazing pressure suggests that, although rotifers and Chironomidae larvae are also abundant in the biofilm at the study site (Majdi et al., in press), the MPB biomass was probably more than sufficient to supply all biofilm-dwelling meiobenthic consumers. This also supports the hypothesis that direct top-down control of MPB biomass by meiofaunal grazing is not a primary regulating

mechanism. Indeed, biofilm-dwelling meiofauna likely play a secondary role by modifying the potential bottom-up controls of MPB through, e.g. bioturbation, which leads to alterations in the light environment and the enhancement of solute transport rates within the mat (Pinckney et al., 2003; Mathieu et al., 2007).

Contribution of MPB to *Chromadorina*'s diet

Although it can highly fluctuate depending on GPT, on production efficiency and on MPB availability, the energetic requirements of *C. bioculata* and *C. viridis* satisfied by grazing on MPB remained globally rather low (5% assuming a GPT of 14 min) compared to values reported in literature for marine nematodes (50%, Van Oevelen et al., 2006; 15%, Pascal et al., 2008). Hence, to fulfil 100% of their food requirements, *C. bioculata* and *C. viridis* probably depended on other food sources than MPB cell contents. Meschkat (1934) observed that freshwater Chromadoridae can collect and agglutinate detritus using their sticky silks to form a kind of pellet around their tail. This behaviour was also observed during our study with living specimens. Riemann & Schrage (1978) suggested that these detritus agglutinations, being crowded by bacteria, may contribute to nematode diet. In a more recent study, Riemann & Helmke (2002) pointed out that within these agglutinations, bacterial external enzymatic activity can contribute to cleave refractory polysaccharides, so that resulting sugars can easily be assimilated by nematodes. Considering the large proportion of EPS exuded by MPB and bacteria within the biofilm matrix (Nielsen et al., 1997), and the typical detritus-agglutinating behaviour of Chromadoridae nematodes described above, it can be speculated that organic matter uptake through "gardening" interactions with bacteria might contribute substantially to the diet of biofilm-dwelling *C. bioculata* and *C. viridis*.

Methodological considerations

Through its first application to nematodes, the HPLC-analysis of gut pigment contents revealed useful to examine their grazing behaviour and pressure on the MPB community as a whole and on diatoms in particular. The main advantage of this technique is that it gives ingestion data under natural conditions without utilisation of artificial markers and that it is applicable to organisms—in our case nematodes—embedded in complex matrices such as epilithic biofilms. However, three shortcomings have to be acknowledged concerning this HPLC-approach:

(1) Based on our experience, the HPLC detection of non-dominant microphyte biomarker pigments in guts of *Chromadorina*-sized nematodes (dry weight $\sim$0.2 µg ind^{-1}) would require sorting at least 1,300 individuals. Besides the fact that this would be extremely time consuming, isolating such a large number of nematodes would increase the risk of contamination and pigment degradation. As a comparison, Buffan-Dubau et al. (1996) recommend a minimum of 400 individuals of the meiobenthic harpacticoid *Canuella perplexa* T. & A. Scott 1893 (dry weight 2–10 µg ind^{-1}), to analyse gut pigments in detail. Hence, the detection of biomarker carotenoids for non-dominant microphyte groups may be practically restricted to larger algal-feeding nematode taxa (e.g. Dorylaimidae), if one wants to sort a reasonable number of nematodes.

(2) The analysis was applied to the entire natural nematode community and therefore the relevance of drawing conclusions from gut content data depends mainly on the complexity of the species assemblage occurring at the time of sampling. Hence, to overcome possible bias due to species specific diet, we recommend that this technique should either be restricted to the examination of nematode communities strongly dominated by a few species—as was the case in our study—or be applied to nematodes sorted to the best taxonomic level.

(3) With this technique only feeding on MPB cells is considered. Hence, potential feeding on heterotrophic preys (e.g. bacteria) and/or on EPS was not detected, while these latter resources likely contributed considerably to the diet of *C. bioculata* and *C. viridis* inhabiting epilithic biofilms of the Garonne River.

Conclusion

Our results showed that biofilm-dwelling *Chromadorina* spp. nematodes fed on MPB within epilithic

biofilms of the Garonne River, and that this feeding was non-selective. Only diatom biomarker pigments were found in their guts, however, a potential additional feeding on green algae and cyanobacteria can not be completely excluded. Our estimates of their ingestion rates emphasised a low grazing pressure on biofilm MPB cells and suggested that these nematodes used additional food sources (e.g. bacteria, EPS), which were not detected by means of HPLC gut pigment analysis. Thus, this aspect should be investigated in future studies.

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