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Assessing the reliability of fatty acid–specific stable isotope analysis for trophic studies

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Summary

1. Fatty acid–specific stable isotope analysis (FA-SIA) is expected to encompass most of the limits encountered when using more classical trophic markers such as bulk tissue stable isotope or fatty acid analyses. However, an essential premise for using FA-SIA for trophic studies is that the individual FA $\delta^{13}C$ values of the consumer reflect those of its diet. Field studies using FA-SIA have so far made this assumption, which is not necessarily supported by the rare experimental tests.

2. A feeding experiment was conducted on *Daphnia* to test whether the $\delta^{13}C$ values of individual fatty acids in *Daphnia* were actually related to those of its food.

3. Only the stable isotope composition of polyunsaturated (PUFA) and branched fatty acids (BrFA) was globally transmitted from the diet to *Daphnia* lipids, with however a significant isotope fractionation that varied depending on the considered diet source.

4. A model was constructed to evaluate how such variability may affect the reliability of FA-SIA to track the dietary sources of consumers' PUFA and BrFA in the field. Results suggest that provided the endmembers are substantially isotopically different, FA-SIA could provide valuable insights into the pathways conveying these particular FA to consumers in the field.

Key-words: compound-specific stable isotope analysis, feeding experiment, lipids, trophic fractionation

Introduction

Trophic markers, such as bulk tissue stable isotope analysis (SIA), or fatty acid trophic markers (FATM), have been widely employed to track the origins and document the pathways conveying nutrient or organic molecules sustaining the secondary production of food webs (Alfaro *et al.* 2006; Perga *et al.* 2006). Both methods are based on the two same premises: (i) potential basal sources have distinct signatures and (ii) the source signature is predictively transmitted from the food to the consumer. Both methods however encounter the same limits as these premises are not necessarily met. In aquatic food webs for instance, some fatty acids (FA) had been identified as specific of certain algal classes in laboratory experiments but further field studies tend to show that they can be shared by various distinct taxa, therefore limiting the source specificity of these FA as trophic biomarkers (Bec *et al.* 2010). There are also many examples of SIA in which the aquatic and terrestrial endmembers could not be discriminated from their $\delta^{13}C$ values (France 1996). Another issue concerns the adequate measurements of the endmembers. Phytoplankton stable isotope signature has rarely been measured directly (Grey, Jones, & Sleep 2000) as phytoplankton cells are difficult to isolate from other particles (bacteria, heterotrophic protists and detritus of different origins) that compose the bulk particulate organic matter. In addition, in SIA studies, phytoplankton is usually tacitly considered as a single, isotopically homogenous dietary source but the isotopic heterogeneity within the phytoplankton community was recently shown to be a potential source of overestimation of terrestrial C contribution to lake food webs (Perga, Kainz, & Mazumder 2008).

Many physiological processes were also shown to limit the condition of the predictive transmission of the source signature during trophic transitions. For SIA, trophic fractionation

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factors can be highly variable between species or dietary modes (Post 2002; McCutchan *et al*. 2003). Similarly, bioconversion of some FA from other FA precursors, as well as *de novo* biosynthesis, can impede the success of FATM approaches (Bec *et al*. 2003; Brett, Müller-Navarra, & Persson 2009).

Some of the biases related to the source specificity of the signature might be solved resorting to fatty acid–specific stable isotope analyses (FA-SIA; Pel, Floris, & Hoogveld 2004; Pel, Hoogveld, & Floris 2003). Indeed, two endmembers might share some of their marker FA: for instance, sea ice algae and pelagic diatoms, the two end-members of the study by Budge *et al*. (2008), shared high levels of the diatom FA biomarkers, i.e. 16:4(n-1) and 20:5(n-3), but these FA were shown to exhibit differing $\delta^{13}C$ values and then discriminating them. Therefore, compound-specific stable isotope analysis is expected to provide a greater specificity to biomarkers (Evershed *et al*. 2007) and to encompass the difficulty to isolate single members from the bulk particulate organic matter (Pel, Hoogveld, & Floris 2003).

However, the second essential premise for using FA-SIA as trophic biomarkers in the field is, as for classical SIA, that the isotope 'signal' is actually transmitted from the diet to the consumer, i.e. that the individual FA $\delta^{13}C$ values of the consumer reflect those of its diet. Field studies using FA-SIA on zooplankton have so far made this assumption (Budge *et al*. 2008). However, experimental tests of this essential assumption are still rare and restricted to a limited number of arthropod taxa (Chamberlain *et al*. 2004; Pond, Leakey, & Fallick 2006; Lau, Leung, & Dudgeon 2009). In addition, these rare experiments did not necessarily support this premise (Chamberlain *et al*. 2004; Pond, Leakey, & Fallick 2006; Lau, Leung, & Dudgeon 2009).

Actually, the FA $\delta^{13}C$ values of the consumer are expected to be related to those of its diet if consumer's FA are from dietary origin, i.e. if (i) the dietary FA are integrated within the consumer's lipids with no further modification of their C-chain (i.e. elongation or desaturation) and (ii) the consumer's FA are not the result of any *de novo* synthesis from non-lipid components. Although this assumption might not be validated for every FA and in all types of consumers, *Daphnia* might be an ideal candidate as: (i) earlier studies suggested that up to 98% of *Daphnia* FA were from dietary origins, with very limited *de novo* synthesis (Goulden & Place 1990), and (ii) *Daphnia* has very limited desaturase activities and might not be able to synthesize *de novo* (n-3) and (n-6) PUFA such as 18:2(n-6), 18:3(n-3) and 20:5(n-3) (von Elert 2002). For such reasons, it has been suggested that the $\delta^{13}C$ values of these essential FA should be transmitted unchanged from the diet to the consumer (Stott *et al*. 1997).

In this study, we conducted an experiment in which *Daphnia* sp. were fed three protist food sources: since two were pigmented protists (the diatom *Cyclotella* sp. and the flagellate *Rhodomonas lacustris*) and the other a heterotrophic protist (the ciliate *Cyclidium glaucoma*), these food sources exhibited very distinct FA and FA $\delta^{13}C$ compositions. The overarching aim of this paper was to test whether the $\delta^{13}C$ values of individual FA in *Daphnia* were actually related to those of its food and to critically consider the improvements and beneficial knowledge of FA-SIA for future food web studies.

Material and methods

NUTRITION EXPERIMENT

The pigmented flagellate (cryptophyte) *Rhodomonas lacustris* (12 μm long) and the centric diatom *Cyclotella* sp. (12 μm) were batch-cultivated in a modified Synura® medium (Vera *et al*. 2001). The ciliate *Cyclidium glaucoma* (18 μm long) was grown in the same medium enriched with milk powder (0·8 g L^{-1}).

Daphnia sp. was isolated from zooplankton samples collected in Lake Annecy (France), cultured in spring water, and fed every other day with a 50/50 mixture of freeze-dried fish foods (Tetramin® + Tetraphyll®) broken down into fine particles by ultrasound.

Each nutrition experiment was conducted in triplicates using the following protocol: 3×120 neonates of *Daphnia* sp. (< 12 h old) were placed in three glass tanks filled with 800 ml of spring water that was renewed every other day. The glass tanks were placed in a temperature-controlled chamber (20 °C) with a 12·12 h light/dark cycle. For the nutrition experiments, ciliate cells were separated from their medium by repeated centrifugations and resuspensions in fresh medium. Subsequently, the ciliate suspension was slowly filtered through a 5-μm membrane filter without vacuum, and retained cells were immediately resuspended from the filter into spring water. Food suspensions of the flagellate *Rhodomonas lacustris* and diatom *Cyclotella* sp. were obtained by centrifugation and resuspension of the cultured cells in spring water. Subsamples of the protists stock cultures were taken to estimate the number of cells in the food suspensions using a Sedgwick-Rafter chamber. Carbon concentrations offered to daphnids were estimated using the carbon conversion factors proposed by Menden-Deuer & Lessard (2000). The four food suspensions, i.e. the diatom *Cyclotella* sp., the flagellate *Rhodomonas lacustris* (1 mg C L^{-1}), the ciliate *Cyclidium glaucoma* (1 mg C L^{-1}) and the mixed diet composed of the flagellate *Rhodomonas lacustris* (0·5 mg C L^{-1}) and ciliate *Cyclidium glaucoma* (0·5 mg C L^{-1}) were added daily within the 10 days lasting experiment. Previous tests had shown that ciliates from the strain used in this experiment did not feed on the flagellate *R. lacustris* (unpublished results), hence limiting substantial changes in the ciliate FA content when present within the mixed diet as a result of predation on the flagellate. At the end of the experiment, daphnids were individually pipetted and placed on precombusted GF/A filters after being rinsed with clean spring water. Protists stock solutions were filtered on precombusted GF/C filters and stored at −60 °C before lipid analysis.

LIPID ANALYSIS

Lipids were extracted from triplicate samples in a mixture of chloroform/methanol (2:1, v/v; Folch, Les, & Stanley 1957). Fatty acid analyses were performed on total lipids (TL) extracted from protists and on neutral lipids (NL) and phospholipids (PL) from *Daphnia* sp. Lipid classes were separated by thin-layer chromatography on silica gel plates, with hexane/diethyl ether/methanol/acetic acid (90:20:3:2 v/v). Lipid classes were identified by comparison with commercial standards purchased from Sigma (Sigma-Aldrich, St Louis, Missouri, USA). Acylglycerols (mainly triacylglycerols and diacylglycerols) were grouped together with free fatty acids and sterols esters to constitute the neutral lipids. Fatty acid methyl esters (FAME) were prepared by hydrolysis and methylation. The lipid extract was

maintained at 90 °C for 40 min in sealed tubes containing hexane and 2 N methanolic sodium hydroxide. The tubes were then cooled, and 2 N methanolic sulphuric acid was added. After 20 min at 90 °C and a short centrifugation, the supernatant was transferred to another tube and dried under nitrogen and the FAME stored at −40 °C in hexane. FAME were analysed on a Chrompack CP 9001 gas chromatograph connected to a recording integrator. The GC was equipped with a Chrompack CP7747 capillary column (25 m × 0·32 mm i.d., film thickness: 0·20 μm). The oven temperature was programmed to increase from 160 to 240 °C at a rate of 2·5 °C min^{-1}. FAME were identified by comparison with known laboratory standards and commercial standards from Sigma (Sigma-Aldrich, St Louis, Missouri, USA) and Supelco (Bellefonte, Pennsylvania, USA). The concentration of total FA was estimated using a double internal standard (13:0 and 23:0), added as free fatty acids before derivatization. FA concentrations were expressed as per cent of total identified FA.

FATTY ACID–SPECIFIC STABLE ISOTOPE ANALYSES

Isotopic analyses of individual FAME were carried out under a continuous helium flow using an HP 5890 gas chromatograph coupled with a CuO furnace (850 °C) and a cryogenic trap (−100 °C) coupled with a VG Optima mass spectrometer, monitoring continuously ion currents at m/z = 44, 45 and 46. The gas chromatograph was installed with a BPX70 column (60 m length, 0·32 mm internal diameter, 0·5-μm-film thickness). Oven temperature rose from 50 to 260 °C at 3 °C and held for 40 min. All $\delta^{13}C$ values were calibrated against CO_2 standards previously calibrated against Pee Dee Belemnite standard. A mix of 4 FAME (C12:0, C14:0, C16:0 and C18:0), purity of which had been previously checked by GC and individual $\delta^{13}C$ measured by EA-IRMS, was used as reference material. Analytical precision, determined from consecutive runs of the reference material over the whole range of linearity of the isotopic source, was $< 0{\cdot}3‰$. Each sample was run three times, and values were averaged. FAME $\delta^{13}C$ were corrected for the methyl group addition during methylation according to the formula (1)

$$\delta^{13}C_{FA} = \left((n+1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}\right)/n \qquad \text{eqn 1}$$

where $\delta^{13}C_{FAME}$ and $\delta^{13}C_{MeOH}$ are the $\delta^{13}C$ values of the measured FAME and methanol used during methylation, respectively. $\delta^{13}C_{FA}$ represents the $\delta^{13}C$ of the given FA prior to methylation, and n is the number of carbon atoms in the (non-methylated) FA. For this study, the $\delta^{13}C$ of the methanol used for the preparation of FAME was −45·3‰.

DATA ANALYSES

Results for per cent composition of all detected FA are summarized in Appendix 1. In the scope of this paper though, we focused only on the results for the most abundant FA, for which quantities and $\delta^{13}C$ values could be measured simultaneously in diets and *Daphnia* lipids. These FA were namely three-saturated FA (SAFA: C14:0, C16:0 and C18:0), two mono-unsaturated FA [MUFA: C16:1(n-7) and C18:1 (n-7)], three polyunsaturated FA [PUFA: C18:3(n-3), C18:4(n-3) and C20:5(n-3)] and the sum of branched FA (BrFA). FA compositions of food sources or *Daphnia* lipids were compared using pairwise comparisons with Bonferroni-adjusted significance levels.

Changes in the $\delta^{13}C$ value of a given FA between diet and *Daphnia* lipids ($\Delta^{13}CFA_{i,j}$) were calculated for each individual FA_i, for each individual diet *j*, as the differences in the $\delta^{13}C$ values of FA_i between *Daphnia* ($\delta^{13}CFA.Daphnia_{i,j}$) and its diet *j* ($\delta^{13}CFA_{i,j}$) (2):

$$\Delta^{13}CFA_{i,j} = \delta^{13}CFA.Daphnia_{i,j} - \delta^{13}CFA_{i,j}. \qquad \text{eqn 2}$$

Results

FATTY ACID COMPOSITIONS

The three diet sources exhibited significant differences in their per cent composition of the nine selected FA (Fig. 1). Yet, the relationships in FA patterns between *Daphnia* and their dietary source depended on the considered FA. *Daphnia* lipid contents in 16:0 and 18:0 were rather independent from the contents of these FA in the dietary source. Indeed, the diatom food source had significantly higher amounts of 16:0 and 18:0 than the two other diet sources. *Daphnia* fed diatom had yet rather similar amounts of 16:0 and 18:0 as in the other treatments. For the seven other FA though, *Daphnia* FA composition exhibited a marked dietary influence. In addition, *Daphnia* fed on the mixed diet (flagellate + ciliate) showed a composition in those FA that was intermediate between those of *Daphnia* fed each of these individual food sources. The adequacy between diet and *Daphnia* FA composition was yet lower for MUFA and 14:0 than for BrFA and PUFA. Hence, *Daphnia* fed a food source in which MUFA or 14:0 was particularly abundant [16:1(n-7) and 14:0 in diatoms, 18:1(n-7) in ciliates] would exhibit higher abundances of this given FA in its NL or PL. However, *Daphnia* could still exhibit substantial amounts of these FA in their lipids even when supplied at very low amounts by the diet [for instance, 14:0, 16:1(n-7) and 18:1(n-7) in flagellates]. In contrast, BrFA were present in *Daphnia* lipids only when supplied by the diet (the ciliate *C. glaucoma*). The PUFA composition of the dietary source was also clearly reflected in that of *Daphnia* NL, but the adequacy of PUFA patterns between dietary and *Daphnia* PL was somewhat lower. For instance, although 20:5(n-3) was *c.* 15 times less abundant in ciliates than in flagellates and diatoms, amounts of this FA in *Daphnia* PL were similar in all the treatments.

STABLE CARBON ISOTOPE RATIOS OF INDIVIDUAL FA

Average $\delta^{13}C$ value of FA in the flagellate *R. lacustris* was −32·9‰ (SD = 2·8‰), with a minimal value of −35·8‰ for 22:6(n-3) and a maximal value of −29·2‰ for 18:0 (Fig. 2a, Appendix 2). The average $\delta^{13}C$ value of FA in the diatom *Cyclotella sp.* was relatively similar to that of the flagellate *R. lacustris* (−31·8‰; SD = 1·4‰), with a minimal value of −33·2‰ for 18:4(n-3) and a maximal value of −27·9‰ for 18:0. FA $\delta^{13}C$ values for the ciliate *C. glaucoma* were much less depleted, with an average value of −22·3‰ (SD = 1·6‰) and values were ranging from −25·6‰ [18:2(n-6)] to −20·1‰ (14:0). The intermolecular variability in FA $\delta^{13}C$ values within a single food source ranged from 4·8‰ (ciliate) up to 6·6‰ (flagellate).

Consistently, FA of *Daphnia* fed ciliates were enriched in ^{13}C by 6–7‰ compared with those of *Daphnia* fed the autotrophic protists (Fig. 2b,c). In addition, FA $\delta^{13}C$ values of *Daphnia* fed the mixed diet ranged between those

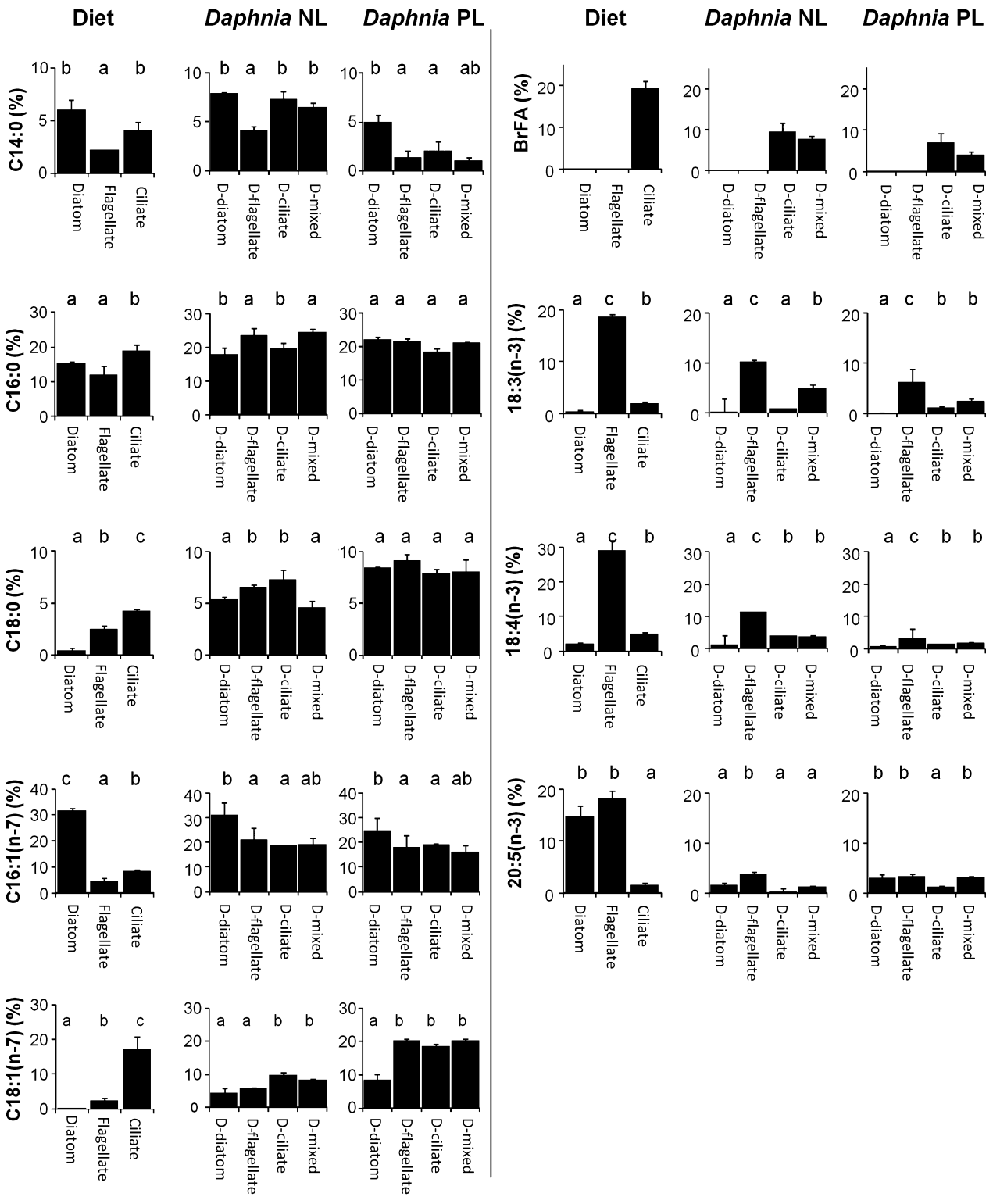

Fig. 1. Error plots (average ± standard deviation) of some fatty acids (FA) contents for the food sources (Diatom: *Cyclotella* sp., Flagellate: *Rhodomonas lacustris*, Ciliate: *Cyclidium glaucoma*) and neutral lipids (NL) and phospholipids (PL) of *Daphnia* fed these food sources in the nutrition experiment [D-Diatom: *Daphnia* fed the diatom *Cyclotella* sp., D-Flagellate: *Daphnia* fed the flagellate *R. lacustris*; D-Ciliate: *Daphnia* fed the ciliate *C. glaucoma*; and D-mixed: *Daphnia* fed the mixed diet (50% Flagellate; 50% Ciliate)].

of *Daphnia* fed to two individual food sources (Fig. 2b,c). However, looking closer to the data, isotope differences for individual FA, $\Delta^{13}CFA_{i,j}$, were highly variable between diets and FA (Fig. 3). For instance, $\Delta^{13}C18:1(n\text{-}7)$ ranged from −5‰ (for PL of *Daphnia* fed ciliates) up to +1‰ (for PL of *Daphnia* fed diatoms). $\Delta^{13}CFA_{i,j}$ was also

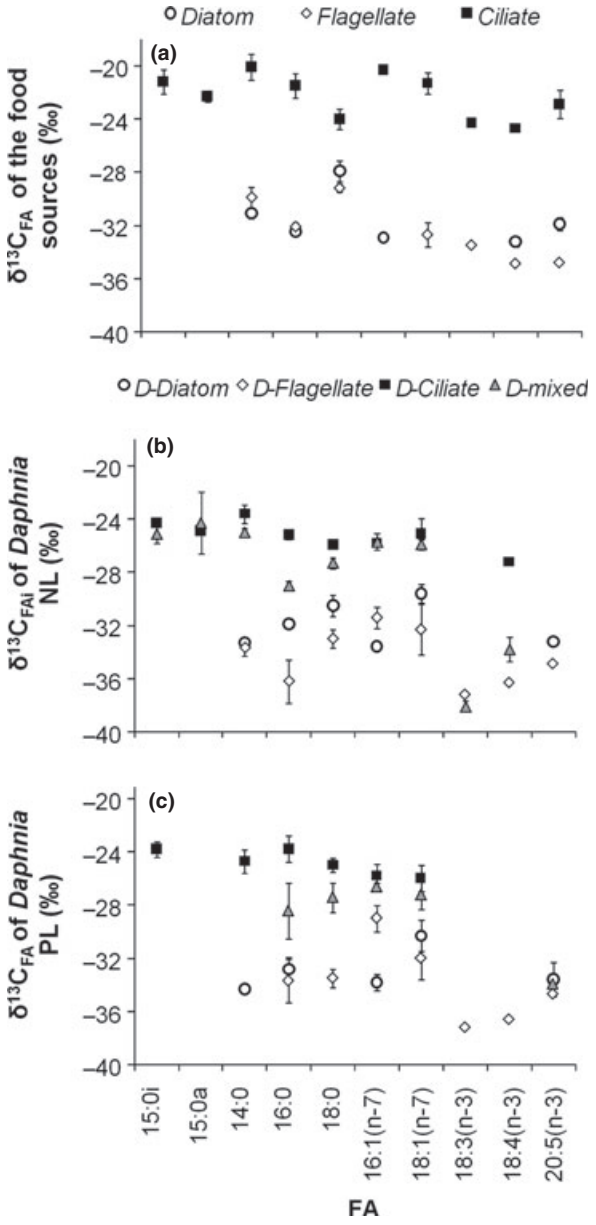

Fig. 2. $\delta^{13}C$ values (average ± standard deviation) for individual fatty acids (FA) for (a) the three diet sources, (b) neutral lipids (NL) and (c) phospholipids (PL) of *Daphnia* fed the three individual and the mixed diet sources.

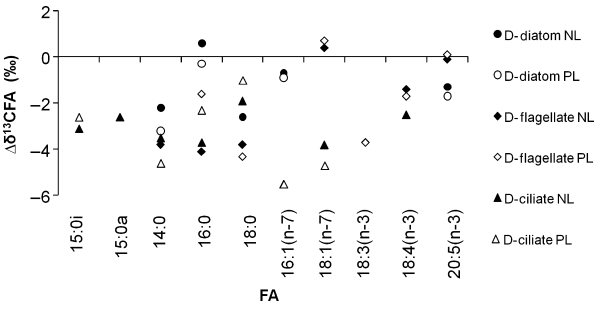

Fig. 3. Differences in fatty acids (FA) $\delta^{13}C$ values between diet and *Daphnia*'s individual neutral lipids (NL) and phospholipids (PL) FA for the three diet sources.

highly variable between FA of *Daphnia* fed a single food source. For instance, for *Daphnia* fed flagellates, $\Delta^{13}CFA_{i,j}$ could range from −4‰ (14:0, 16:0 and 18:0) to +1‰ [18:1(n-7)]. $\Delta^{13}CFA_{i,j}$ values were more similar between NL and PL of *Daphnia* fed a single diet source.

Discussion

With the increased use of SIA on bulk material to document trophic interactions within terrestrial and aquatic food webs, a number of confounding factors that might complicate data interpretation, such as isotope routing and turnover, or isotope heterogeneity of the endmembers, have been highlighted. Recent advances in gas chromatography–combustion–isotope ratio monitoring mass spectrometry now allow the measurements of the stable isotope compositions of specific compounds such as fatty acids, and the increased availability of such measurements is expected to help encompassing most of the problems that could be encountered when working with bulk material (Pel, Hoogveld, & Floris 2003). However, experimental studies are still required to document which information compound-specific stable isotope analyses could be expected to provide.

FA ORIGINS IN *DAPHNIA* LIPIDS

Not much is known about lipid metabolism and pathways in *Daphnia* (Brett, Müller-Navarra, & Persson 2009). In their seminal study, Goulden & Place (1990) measured fatty acid accumulation and synthesis rates of *Daphnia pulex* and *D. magna* when fed isotopically labelled chlorophycea *Ankistrodesmus falcatus*. According to their results, in their experimental context, at least 98% of the total lipids, and virtually all of the storage lipids, were derived from the diet. They suggested that storage lipid accumulation rates in daphniids were strongly regulated by the availability of dietary lipids. Overall, our feeding experiment is in line with Goulden & Place's (1990) conclusions as NL FA composition was shown to depend more on the diet than PL. Such results suggest that NL might be more suitable for FATM approaches in the field than PL or even total lipids.

Since Goulden & Place's (1990) study however, only one similar labelling experiment has been performed (Bychek *et al.* 2005). Most of our knowledge on *Daphnia* lipids was obtained through supplementation experiments (von Elert 2002) or controlled feeding experiments (Bec *et al.* 2003; as we did herein), in which changes in *Daphnia* fatty acid compositions in relation to dietary fatty acids were monitored. Feeding experiment studies have shown that diet has a very strong impact on the FA composition of some zooplankton (and in particular *Daphnia* spp.; reviewed in Brett, Müller-Navarra, & Persson 2009). Supplementation experiments identified (n-3) PUFA as essential fatty acids as for most animals (von Elert 2002; Ravet, Brett, & Muller-Navarra 2003). Studies on *Daphnia* lipids have focused on the physiological role of these (n-3) PUFA on *Daphnia* growth and fecundity (Weers & Gulati 1997; Ravet, Brett, & Muller-Navarra 2003), on the impact of environmental factors on their

accumulation in *Daphnia*, such as temperature (Masclaux *et al.* 2009) and, more generally, on (n-3) PUFA pathways within aquatic food webs (Perga, Bec, & Anneville 2009). The physiological roles of the other FA that can be synthesized *de novo* have been much less documented. In addition, so far, poor efforts have been dedicated to the study of synthesis and conversion pathways in *Daphnia* and zooplankton in general (Brett, Müller-Navarra, & Persson 2009).

Our results are actually consistent with previous studies (Weers, Siewertsen, & Gulati 1997; Brett *et al.* 2006), showing that *Daphnia* FA composition varied generally according to the diet they were fed, although amongst the nine FA on which we focused, not all were from dietary origins (i.e. FA directly transmitted from the food to the consumer's lipids with no further changes in the C backbone). Typically, amounts of C16:0 and C18:0 in *Daphnia* NL varied between 15–20% and 3–6%, respectively, but such differences were not dependent on the amounts of these same FA in *Daphnia* diet sources. Furthermore, proportions of 16:0 and C18:0 were constant in *Daphnia* PL (20% and 7%, respectively), whatever were the amounts of these FA in the dietary source, confirming that their amounts in *Daphnia* tissues, and especially in cell membrane, are regulated by active physiological processes, consistently with Bychek *et al.*'s (2005) observations. 14:0 and the MUFA 16:1(n-7) and 18:1(n-7) were more likely at least partially from dietary origins as *Daphnia* that were fed with diets rich in these FA exhibited the highest proportions of these same FA in their own lipids. However, differences in the amounts of these FA were not as large in *Daphnia* as in their respective diet sources, implying that differential accumulation, *de novo* synthesis or bioconversion occur when these lipids are supplied in limited amounts by the diet (Sargent and Henderson 1986). For instance, the flagellate *R. lacustris* was rich in 16:1(n-7) but very depleted in 18:1(n-7) compared with the other food sources; however, amounts of 18:1(n-7) in *Daphnia* fed the flagellate were relatively high as this FA might arise from the elongation of the abundant dietary 16:1(n-7). At last, BrFA and PUFA were clearly of dietary origins because dietary differences in the amounts of these FA were clearly reflected in *Daphnia* PL and NL. BrFA could not be detected in *Daphnia* fed diet sources that had not such FA, clearly highlighting that *Daphnia* cannot synthesize *de novo* these FA typical for heterotrophic microbes (Ederington, McManus, & Harvey 1995). Differences in the contents of (n-3) PUFA in *Daphnia* lipids, and especially in NL, were related to those between diets. The ciliate diet source was distinct from the other diet sources because of its relative depletion (about 10 times lower) in FA from the (n-3) series. Nevertheless, lipids, and especially PL, of *Daphnia* fed ciliates exhibited amounts of 20:5(n-3) that were not that much lower than lipids of *Daphnia* fed the other diet sources. 18:3(n-3) has been documented as a potential precursor for 20:5(n-3) (von Elert 2002; Bec *et al.* 2003) but as the ciliate food source was also poor in this shorter PUFA, it is more likely that these relatively high amounts of 20:5(n-3) arise from preferential retention of this peculiar FA (Kainz *et al.* 2009).

RELATIONSHIPS OF FA δ^{13}C VALUES BETWEEN DIET AND *DAPHNIA*

Our estimates of changes in δ^{13}C between dietary and *Daphnia* FA might be affected by some experimental biases related to FA turnover in *Daphnia*. Indeed, phytoplankton cultured in batch conditions might undergo significant and relatively quick changes in their δ^{13}C and FA δ^{13}C values, because chemical conditions in batch cultures, especially pH and nutrients, can change over the course of the experiment (van Dongen, Schouten, & Damste 2002; Pond, Leakey, & Fallick 2006). The FA δ^{13}C values of the food sources, which were actually measured after 10 days, could differ from those at the beginning of the feeding experiment by several per mil. Depending on the turnover of FA in *Daphnia*, *Daphnia* FA δ^{13}C values could reflect those of their diet over a longer-integrated time period. As a result, the apparent difference in the FA δ^{13}C values between *Daphnia* and its diet could be, at least partly, the consequence of these differences in FA turnover rates between the protists and their consumers. However, *Daphnia* grown under experimental conditions with food supplied at optimal rates will increase its mass by a factor 10 in 6 days (Brett, Müller-Navarra, & Persson 2009), which, in our context, guarantees a full FA turnover over the duration of the experiment. In addition, earlier feeding trials using ^{14}C-labelled diets showed that lipid, and especially FA, turnovers in *Daphnia* NL and PL are very quick and that *Daphnia* FA C-isotope values reach equilibrium with those of its food within 24H (Farkas, Kariko, & Csengeri 1981; Goulden & Place 1990; Bychek *et al.* 2005). Hence, such experimental biases are very likely to have only minor effects on our results.

Although highly variable between FA and diets, FA in *Daphnia* lipids were generally ^{13}C-depleted compared with their counterpart in the corresponding diet, although previous results on *Collembola* and shrimps showed that these differences, usually large, can be either positive or negative (Chamberlain *et al.* 2004, 2006a,b; Lau, Leung, & Dudgeon 2009).

Large isotope differences between the diets' and the consumers' lipids are expected for FA that might be synthesized *de novo* or result from the elongation or desaturation of FA precursors. Indeed, *de novo* synthesis from non-lipid dietary constituents involves the enzymatic oxidation of pyruvate to acetyl-coenzyme A during which significant selection of the lighter pyruvate occurs. Such processes result in FA ^{13}C-depleted compared with other non-lipid constituents (De Niro & Epstein 1977) and, potentially, with the diet FA. Because 14:0, 16:0, 18:0, 16:1(n-7) and 18:1(n-7) might arise at least partly from *de novo* synthesis, they could be expected as more ^{13}C-depleted in *Daphnia* lipids when a higher proportion had to be synthesized *de novo*. However, no relationships between Δ^{13}C and FA dietary concentrations were detected for these FA. Such results might suggest that *de novo* synthesis proceeds from different precursors depending on the dietary composition. As elongase and desaturase enzymes preferentially use the lighter precursor, FA derived from elongation and/or desaturation processes in the con-

sumer lipids were also expected to exhibit lower $\delta^{13}C$ values than the dietary FA precursors (Monson & Hayes 1982). According to the mass–balance rule, the remaining pool of precursor FA in the consumer should be enriched in ^{13}C compared with the dietary ones. As mentioned previously, 18:1(n-7) in *Daphnia* fed *Cyclotella* sp. might result from the elongation of 16:1(n-7) that was abundant in this dietary source, but contrary to our expectations, 18:1(n-7) $\delta^{13}C$ value was higher than that of 16:1(n-7) in both the diet and the consumer. Such results imply that FA synthesis and conversion pathways are complex and that further researches are deserved to interpret $\delta^{13}C$ patterns in *Daphnia* non-essential FA.

Significant differences in the isotope values between diet and *Daphnia* lipids were also observed for FA that are only from dietary origins, such as BrFA and PUFA. For such FA that are transmitted directly from the diet to the consumers, these isotopic differences can be considered as the result of trophic fractionation processes. The assumption that the metabolism of dietary FA, and especially essential FA, might not be associated with significant isotope fractionation is based on the results of a single feeding experiment that showed a close similarity of 18:2(n-6) $\delta^{13}C$ values between the diet and bones of pigs (Stott *et al.* 1997). However, our results show that significant trophic fractionation may occur even for FA that are directly assimilated from the diet with no biosynthetic inputs from the consumer. Such a fractionation could result from assimilation, lighter compounds being preferentially assimilated (Chamberlain *et al.* 2006b). Fractionation processes could also occur during the FA integration within the consumer's lipids. FA in organisms are usually present as esterified to a glycerol backbone under the form of triacylglycerol or phospholipids rather than under their free form. Although such FA from dietary origins do not undergo strong changes in their C-backbone structure, their integration within the consumer's lipids involves several hydrolysis and esterification steps. Our results could suggest that the involved enzymes might catalyse these processes with significant isotope fractionation rates.

Although the analytical limits of the method did not allow to obtain $\delta^{13}C$ values for PUFA for *Daphnia* fed all the dietary sources, results on 18:4(n-3) and 20:5(n-3) show that the isotope fractionation could vary over a 1–2‰ range even between two different food sources. Such variability in trophic fractionation factors for a single FA had been observed in previous experiments on *Collembola* (Chamberlain *et al.* 2004) and shrimps (Lau, Leung, & Dudgeon 2009). Similarly, FA $\delta^{13}C$ values of zooplankton and fish could not be directly related to those of particulate organic matter in a marine field study conducted in the Pacific Ocean, off the West Coast of Vancouver Island (Veefkind 2003). It is difficult, in our experimental context, to provide any potential explanation for such patterns but it highlights the need for further experiments on that specific point. The variability in trophic fractionation factors might constitute a major fence to the use of FA-SIA to track FA origins in the field.

APPLICATIONS TO FIELD STUDIES

The use of FA-SIA in the field is expected to allow tracking the origins of dietary FA, i.e. estimating the contribution of different food sources to these FA in consumers' tissues. For FA that were identified in this study from dietary origins (PUFA and BrFA), we backcalculated the contributions of each individual diet sources to these dietary FA in the lipids of *Daphnia* fed the mixed diet (50% flagellate, 50% ciliate). The contribution of the ciliate *C. glaucoma* ($\alpha FAi_{Ciliate.Daphnia}$) and of the flagellate *R. lacustris* ($1 - \alpha FAi_{Ciliate.Daphnia}$) to *Daphnia* dietary FAi was estimated resorting to a simple two-source mixing model. The isotope compositions of FAi in the individual dietary sources ($\delta^{13}CFAi_{Ciliate}$ and $\delta^{13}CFAi_{Flagellate}$) were used as endmembers. Trophic fractionation values were, for each FAi, averaged ($\pm$SD) between PL and NL and the diet sources ($\Delta^{13}CFAi$) as it is usually impossible to determine trophic fractionation values for all potential endmembers in the field (Table 1). Hence, from the $\delta^{13}C$ values of FAi in *Daphnia* fed the mixed diet ($\delta^{13}CFAi_{Daphnia}$), the contribution of the ciliate *C. glaucoma* ($\alpha_{Ciliate.Daphnia}$) to FAi in *Daphnia* lipids was estimated as follows:

$$\alpha FAi_{Ciliate.Daphnia} = \frac{\delta^{13}CFAi_{Daphnia} - \delta^{13}CFAi_{Flagellate} - \Delta^{13}CFAi}{\delta^{13}CFAi_{Ciliate} - \delta^{13}CFAi_{Flagellate}}. \quad \text{eqn 3}$$

These estimates were compared with the contribution of the ciliate diet source to the same dietary FAi in the mixed diet ($\alpha FAi_{Ciliate.mixed\ diet}$), based on the content of the two individual food source in FAi ($\alpha FAi_{Ciliate}$ and $\alpha FAi_{Flagellate}$) (4)

$$\alpha FAi_{Ciliate.mixed\ diet} = \frac{\alpha FAi_{Ciliate}}{\alpha FAi_{Ciliate} + \alpha CAi_{Flagellate}} = \alpha FAi_{Ciliate.Daphnia}. \quad \text{eqn 4}$$

Under the assumption that, as non-selective filter-feeders, *Daphnia* grazed and assimilated both protists in the mixed diet with identical efficiencies (Burns 1968), the contribution of ciliates to FAi to the mixed diet and to *Daphnia* lipids could be considered as similar.

In the mixed diet, the ciliate *C. glaucoma* provided 100% of the BrFA C15:0i and C15:0a, but contributed only poorly to the PUFA of the mixed diet [9%, 14% and 7% of 18:3(n-3),

Table 1. Estimated trophic fractionation values of dietary FA (PUFA and BrFA) in *Daphnia* lipids, averaged over NL and PL and food sources

Dietary FAi	$\Delta^{13}CFAi$ (‰)	95% confidence interval (‰)
15:0i	−2·9	[−3·3; −2·4]
15:0a	−2·6	[−3·2; −2·0]
18:3(n-3)	−3·7	[−5·3; −2·1]
18:4(n-3)	−1·9	[−2·8; −1·0]
20:5(n-3)	−0·8	[−2·3; 0·8]

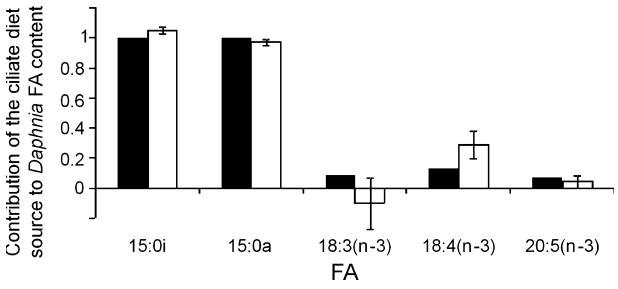

Fig. 4. Contribution of the ciliate *Cyclidium glaucoma* to the fatty acids (FA) content of *Daphnia* fed the mixed diet (50% flagellate and 50% ciliate); closed bars, calculated from the diet sources FA contents; open bars, backcalculated from the two-source mixing model (average ± confidence interval).

18:4(n-3) and 20:5(n-3), respectively, Fig. 4]. Consistently, the two-source mixing model detected that the ciliate *C. glaucoma* provided BrFA but contributed only poorly to the (n-3) PUFA in *Daphnia*'s lipids, which were rather provided by the flagellate food source.

Our results suggest that the stable isotope composition of PUFA and BrFA is globally transmitted from the diet to *Daphnia* lipids, with however a significant isotope fractionation (−4 to −1‰). Isotope fractionation factors for PUFA and BrFA varied depending on the considered diet source. Future studies should investigate which factors are driving such variability, to increase the predictability of trophic fractionation factors. More interestingly, it should be investigated whether isotopic fractionation operates at a molecular level during FA hydrolysis and esterification steps and – if it actually occurs – whether it depends on the physiological importance of the given FA for the consumer.

However, the variability in isotope fractionation observed at the individual FA level is finally quite comparable to that observed for discrimination factors at the bulk level between *Daphnia* and its diet (Power, Guiguer, & Barton 2003). Hence, in spite of such a variability observed for PUFA and BrFA trophic fractionation factors, FA-SIA might still be useful in the field, provided the potential dietary sources exhibit high differences in their isotope composition (*c.* 10‰ in our study). This is under such a premise that Chamberlain *et al.* (2006a) studied *Collembola* trophic preferences, using C3- and C4-derived organic matter as endmembers. Provided the endmembers are substantially isotopically different, FA-SIA could provide valuable insights into the pathways conveying certain FA, and especially essential FA, to consumers. Information potentially provided by FA-SIA are however not equivalent but complementary to those provided by SIA. While SIA would allow assessing the contribution of a dietary source to the consumer's C biomass (i.e. secondary production), FA-SIA would decipher which of the dietary sources are the major providers of some FA (and especially essential FA). Hence, the combined use of SIA and FA-SIA would be useful to document an uncoupling between essential compounds and major organic matter transfers, as it was shown in heterogeneous environments (Koussoroplis *et al.* 2010).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Percent composition in their main FA of the three individual diet sources and of the NL and PL of *Daphnia* fed the individual and mixed diets. Br = Branched FA; ND = not detected.

Appendix S2. FA $\delta^{13}C$ values (in ‰, average ± standard deviation) of the three individual diet sources and of the NL and PL of *Daphnia* fed the individual and mixed diets. N.D. stands for not detected. For some FA, amounts were too limited to provide reliable $\delta^{13}C$ values (B.L. stands for below detection limits).

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