The potential of fatty acid isotopes to trace trophic transfer in aquatic food-webs

Running head: Fatty acid isotopes as dietary tracers

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Abstract

Compound-specific isotope analyses (CISA) of fatty acids (FA) constitute a promising tool for tracing energy flows in food-webs. However, past applications of FA-specific carbon isotope analyses have been restricted to a relatively coarse food-source separation and mainly quantified dietary contributions from different habitats. Our aim was to evaluate the potential of FA-CSIA to provide high-resolution data on within-system energy flows using algae and zooplankton as model organisms. First, we investigated the power of FA-CSIA to distinguish among four different algae groups, namely cyanobacteria, chlorophytes, haptophytes and diatoms. We found substantial within-group variation but also demonstrated that $\delta^{13}C$ of several FA (e.g. 18:3ω3 or 18:4ω3) differed among taxa resulting in group-specific isotopic fingerprints. Second, we assessed changes in FA isotope ratios with trophic transfer. Isotope fractionation was highly variable in daphnids and rotifers exposed to different food sources. Only $\delta^{13}C$ of nutritionally valuable poly-unsaturated FA remained relatively constant, highlighting their potential as dietary tracers. The variability in fractionation was partly driven by the identity of food sources. Such systematic effects likely reflect the impact of dietary quality on consumers' metabolism and suggest that FA isotopes could be useful nutritional indicators in the field. Overall, our results reveal that the variability of FA isotope ratios provide a substantial challenge but that FA-CSIA nevertheless have several promising applications in food-web ecology.

Introduction

Nutrient and energy flows between prey and consumers characterise food-web structure and therefore represent a key driver of ecosystem functioning and stability [7, 8]. In many microbial food-webs (e.g. plankton or soil communities) trophic structures are only rudimentarily resolved [9] and food sources are frequently aggregated into larger, taxonomically diverse groups [10]. The resulting blurred picture of these functionally important food-webs hampers the ability to understand internal dynamics and assess the vulnerability of microbial communities to global environmental change [9].

One factor restricting our ability to fully resolve the complexity of food webs is the shortage of quantitative tracers [14, 15]. Currently, the most commonly used tracing approach is the measurement of bulk carbon (C) and nitrogen (N) isotope ratios in consumers and their diets [16, 17]. Analyses of these two elements have proven to be powerful tools, for example, to assess the contributions of allochthonous and autochtonous food sources [16, 18]. However, bulk isotope analyses are restricted by a low amount of available markers limiting the number of food sources that can be considered in diet tracing [13, 15]. A promising approach to evaluate more complex food-webs is the analysis of isotopic ratios of specific compounds such as amino acids (AA) or fatty acids (FA). Compared to bulk approaches, compound-specific isotope analyses (CSIA) provide a larger number of markers increasing in principle their tracing power [24, 25].

CSIA of FA provide a number of additional advantages. For example, the existence of group-specific biomarker FA facilitates their measurement in mixed samples without physical separation [11, 26, 27]. Furthermore, several poly-unsaturated FA (PUFA) highlighted as potential biomarkers in CSIA [28, 29], represent essential resources that often limit consumers' productivity [30, 31]. Yet, similar to bulk isotope analyses, the successful application of CSIA is linked to a number of pre-requisites. One requirement is the adequate differentiation among food sources [32, 33]. The successful separation of possible diets

thereby depends on the establishment of source-specific tracer signals using either direct sampling or a reference data-based library approach. Library based approaches (e.g. applied in genetic tracing tools) require the availability of large reference data bases as well as small variation in tracers across space and time. In contrast, direct source sampling depends on the physical isolation of individual food sources which can be challenging in microbial food-webs. A second requirement for the application of FA-CSIA is the need to clearly understand the modification of tracer molecules with trophic transfer [34, 35]. Many FA [29, 36] and AA [37] show systematic differences in isotopic values between prey and their consumers. Only if such isotopic fractionation with trophic transfer is reliably quantified, biomolecules can be used to trace energy flows in food webs.

In aquatic systems, FA isotope analyses are commonly used to quantify consumers' reliance on food sources from different habitats [e.g. from ice and pelagic algae; 38, 39] or from allochthonous and autochthonous C sources [40, 41]. However, the potential use of FA isotopes to distinguish among multiple food sources within one ecosystem remains largely underexplored [26] despite indications that differences in isotopic ratios among algae groups can be substantial [1]. Moreover, trophic fractionation of FA isotopes has only been explored in a hand full of studies [28, 29, 36, 42] and little is known about the influence of consumer identity, or the food environment, on changes in isotopic ratios during trophic transfer. Consumers frequently alter FA through the elongation or desaturation of FA chains to satisfy physiological requirements [43] and such modifications likely represent important drivers of trophic fractionation [21, 44]. The degree to which FA are modified in consumers depends on food quantity and quality [45, 46] as well as consumers' capability to synthesize certain FA [47], turning these factors into potential determinants of isotopic fractionation.

Our aim in this study was to evaluate the potential of FA carbon isotope applications to trace energy and nutrient flows in plankton communities. Firstly, we assessed FA isotope ratios in polar and total lipids of 29 freshwater and marine algae strains to evaluate the

potential of using systematic differences across four major algal groups (chlorophytes, cyanobacteria, diatoms and haptophytes) as tool for source separation. Further, we evaluated isotopic fractionation with trophic transfer. In controlled feeding experiments, we reared the cladoceran *Daphnia magna* and the rotifer *Brachionus plicatilis* either on the chlorophyte *Chlamydomonas reinhardtii* or the cryptophyte *Rhodomonas salina* which differ in their FA profiles and thus food qualities. Together, these experiments allowed us to evaluate the strength and weaknesses of FA isotope-based approaches to trace nutrient and energy flows in aquatic food-webs.

Methods

Assessment of algae group differences

In total, 29 phytoplankton strains from four major taxonomic groups were cultured in strain-specific culture media under replete conditions. Strains included both freshwater and marine species (see Table 1 and Table S1) and comprised 12 chlorophytes, nine cyanobacteria, five diatoms and three haptophytes. All strains were grown in 2L batch mono-cultures at 18-20C˚ and a 13:11hrs day-night cycle. Light intensity ranged between 160-210 µmol m^{-2} s^{-1} due to slight alternation of culturing set-ups (no systematic variation across groups). All strains were acclimatised for three weeks to facilitate adaptation to local culture conditions. Duplicate cultures for each strain were set up. Samples for bulk isotope analysis and FA-CSIA were taken during exponential growth. *Spirulina major* and *Achnantes* sp. formed macromolecular agglomerations and were concentrated on 20 µm sieves, washed with deionised water, freeze-dried and stored at -80°C for further analysis. All other strains were filtered onto pre-combusted GF/F filters (Whatman, 0.47 µm nominal pore-size, 4 h at 450°C) and stored at -80°C.

Trophic fractionation experiments

FA isotope ratios of two zooplankton species and their diets (two mono-algal diets in two experiments per consumer; Table 1) were measured in controlled feeding experiments to evaluate isotopic fractionation with trophic transfer. We selected the rotifer *B. plicatilis* and the cladoceran *D. magna* as representative example consumers from two important functional zooplankton groups. These consumers were reared on one low and one high FA quality diet. The chlorophyte *C. reihardtii*, which lacks highly unsaturated FA with >4 unsaturated C-bonds [48], and the cryptomonad *R. salina* with high concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [49] were used as high and low FA quality diet, respectively. Both algae were cultured in chemostats (1L volume, 0.3 daily dilution rates) on a nutrient replete medium to ensure a consistent food quality of zooplankton diets throughout the experiments. Experiments were conducted at a salinity of 6 ppt. Both the algae and zooplankton were acclimatised for >3 months to salinity conditions and zooplankton was transferred to experimental food treatments four days prior to the start of experiments. Experiments were set up in triplicate for a duration of 12 days to ensure that zooplankton biomass fully incorporated the new food source during incubations (a conservative estimate of population growth rate of 0.20 day^{-1} [50] leads to the replacement of >95% of body mass during the experimental period). Food and water were changed every second day to prevent changes in food quality between feeding events. Food was provided in excess (>3 mg C L^{-1}) and experimental vessels (6L for rotifers and 8L for daphnids; large vessels were required to satisfy high biomass needs for biochemical analyses) were gently aerated to maintain oxygen conditions and circulate food particles. Samples from food algae were collected on pre-combusted GF/F filters. Rotifer and daphnid cultures contained a mix of juvenile and adults. For daphnid samples, 40 adult individuals per sample were hand-picked and stored in Eppendorf tubes. Rotifer cultures were first settled in 2L sedimentation chambers to sediment detritus and feces. The top 1.8L were then sieved through 60 μm nets to remove food algae and collected on pre-combusted GF/F filters. All samples were stored at -80°C.

Zooplankton growth and production measurements

One aim was to relate trophic fractionation of FA $\delta^{13}C$ to food quality in feeding experiments. Under constant food quantity, growth and egg production of zooplankton are indicators of food quality [51]. We therefore measured consumers' growth and reproduction for all zooplankton-food combinations. Prior to these experiments, zooplankton species were maintained for two weeks under experimental food conditions to minimize maternal effects. Neonate daphnids (n = 10) from a second brood batch were placed in individual 40 mL containers. Individual rotifer neonates (n = 8) were placed in 1.5 mL Eppendorf tubes. Rotifer and daphnid experiments were maintained for six and 12 days, respectively. Every day, specimens were transferred to new experimental containers with excess food. For rotifers, neonates and the number of parthenogenetic eggs were recorded and experiments were kept on a shaking table. For daphnid experiments, neonate production and biomass gain were measured. Biomass gain was established by comparing the mass of neonates (3 x 6 neonates were pooled at the start of the experiment) with the mass of individual daphnids at the end of the experiment (all samples were dried for 24 hrs at 60°C). Neonates were counted daily and experiments were gently aerated to keep microalgae in suspension.

FA extraction and isotopic analyses

Bulk as well as FA-specific $\delta^{13}C$ values were measured in phytoplankton and consumer samples. For phytoplankton strains used for algae group separation, two samples per strain were analysed. For zooplankton, four replicates per food algae and three replicates per zooplankton species were analysed for each treatment. FA were extracted, purified and separated into lipid fractions following the protocol of Grosse, van Breugel and Boschker [52]. The separation of FA resulted in three fractions, one sub-sample in chloroform (containing neutral FA), one sub-sample in propanone (dominated by glycolipids) and one in methanol (containing polar phospholipids). Additionally, we split the samples before separation resulting in one sub-

sample containing all lipid groups. Because of high analytical costs, we measured only the subsample containing all lipids and the methanol sub-sample containing phospholipids, which are further referred to as total and polar lipid samples, respectively. After esterification, FA were measured as fatty acid methyl esters (FAME) with a Thermo GC Ultra gas chromatograph equipped with a polar column (70% Cyanopropyl Polysilphenylene-siloxane; TR-FAME®, 10m, 0.1 µm ID, 0.2 µm film, Thermo Fisher Scientific, Germany). The isotopic composition of individual FA was determined using GC-C-IRMS [53]. In short, an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Fisher Scientific, Germany) was connected via a combustion interface (Combustion III, Thermo Finnigan, Germany) to a gas chromatograph (GC-TRACE Ultra; Thermo Fisher Scientific). Sample injection was performed on a polar column (90% biscyanopropyl, 10% phenylcyanopropyl polysiloxane; RTX-2330, 30 m, 0.2 µm film, Restek, Germany). Reference gas calibration was implemented by combustion of a known isotopic composition of nonadecanoic acid methyl ester (-35.53‰). FA peak identification was conducted by comparison with known FAME reference standards. Three sets of peaks co-eluted across all samples: (*i*) 16:1ω7 and 16:1ω9, (*ii*) 18:1ω7 and 18:1ω9 and (*iii*) 20:3ω3, 20:4ω6 and 22:0. Because of the biochemically heterogeneous co-elution of (*iii*), we did not include this peak in the fractionation study. While (*i*) and (*iii*) co-eluted in all samples and joint isotope values for the respective sets of peaks are presented, (*ii*) could be separated in some samples. Consequently, we report for 18:1ω7 and 18:1ω9 results of separated peaks based on a sub-set of samples as well as a joint value based on all samples. The effect of methylation during the analytical procedure was accounted for by correcting FA $\delta^{13}C$ by the measured methanol $\delta^{13}C$ of -38.8‰ following Bec *et al.* [29]. FA concentrations are presented as % of total FA for samples from our controlled feeding experiments. Differences in FA concentrations between algae groups are reported elsewhere [54, 55] and are not presented in detail. Bulk stable isotopes were analysed at the University of California stable isotope facility (no pre-treatments) using a

PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Data analysis

Stable isotope data are expressed in the delta notation ($\delta^{13}C$) relative to Vienna Pee Dee Belemnite, the international standard for C. Trophic discrimination was calculated as the difference in δ (‰) of bulk or $\delta^{13}C$ FA values between consumer and diet (e.g. $\delta^{13}C_{consumer}$ - $\delta^{13}C_{diet}$) and denoted as Δ(‰). In analyses of phytoplankton, we aimed to identify FA that can be widely used for group differentiation and hence we only included FA in our analyses if they were found in >15% of algae strains in a group. For zooplankton experiments, only FA that occurred in two out of three replicates were included. Further, we standardized FA prior to statistical analyses, a technique frequently applied to explore the potential of CSIA of AA for carbon tracing [25, 56]. Standardisation was implemented by subtracting the $\delta^{13}C$ ratios of 16:0 measured in a sample from the $\delta^{13}C$ values of that specific FA. 16:0 is a common FA and was present in all 142 samples measured and as such suitable for standardisation. Standardisation was required because algae species were cultured on different media, which can lead to differences in absolute isotopic ratios of dissolved inorganic C and algae C [57]. Such media-dependent differences would obscure systematic differences in FA $\delta^{13}C$ driven by different FA synthesis pathways among algae groups and therefore had to be removed through standardisation (see discussion and Box 1 for a further details). As an alternative to 16:0 standardisation, we also evaluated a standardisation by the average FA $\delta^{13}C$ of all measured FA isotope values. Results were largely similar and displayed in Fig. S2.

Statistical comparisons among algae groups were based on ANOVA followed by Tukey post-hoc tests. Homogeneity of variance between groups was assessed prior to analyses and if required logarithmic or square root transformation were applied. If homoscedasticity could not be achieved, Kruskal-Wallis followed by pairwise Mann-Whitney-U-Test were performed. Results of zooplankton trophic fractionation experiments were assessed with

Student's t-tests after transformations to reach normality and homoscedasticity when necessary. In order to assess the impact of diet on isotopic fractionation in consumers we accounted for consumer and FA identity by applying a nested ANOVA with consumer and FA identity as nested (random) factors. All statistical tests were implemented using the software R 3.5.2 [58].

Results

Separation of algae groups by their FA isotope values

Mean differences in stable isotope values of bulk C and 16:0, the most abundant FA across all samples, revealed that 16:0 was generally depleted by 8-18‰ compared to bulk $\delta^{13}C$ (Fig. 1). Despite substantial variation within algae groups, the difference between 16:0 and bulk isotopic values in chlorophytes was significantly smaller than in haptophytes (Tukey post-hoc test; $p = 0.02$) and in cyanobacteria ($p = 0.03$) in total lipid samples (Fig. 1A). Further, the average differences in isotopic values between 16:0 and all other FA differed among groups (Fig. 1B) with cyanobacteria FA showing a significantly higher similarity with $\delta^{13}C$ values of 16:0 than diatom (Tukey post-hoc test; $p < 0.01$) and chlorophyte FA ($p < 0.01$). FA $\delta^{13}C$ of polar lipids generally reflected the patterns found in total lipids (Fig. S1).

Standardised $\delta^{13}C$ of individual FA displayed a w-shaped pattern when listed based on their C-chain length and degree of unsaturation (Fig. 2A-B, Fig. S2). Generally, 14:0, 14:1 and 16:0 were relatively enriched in ^{13}C while desaturation led to ^{13}C depletion. Elongation and the enzymatic step producing 18:1 FA resulted in higher $\delta^{13}C$ values, but desaturation again lowered isotopic ratios. Elongation to 20-C PUFA led to isotopic enrichment and intermediate $\delta^{13}C$ values and consequently, desaturation and elongation seemed to shape FA $\delta^{13}C$ profiles across groups.

A number of FA showed differences in isotopic ratios between certain algae groups (Fig. 2). For example, diatoms showed significantly lower $\delta^{13}C$ values for 18:2ω6 in the total lipids (Tukey post-hoc test; $p < 0.001$) and haptophytes significantly higher $\delta^{13}C$ values for 18:4ω3 in polar lipids (pairwise-Wilcoxon tests; $p < 0.04$) than all other algae groups (Fig. 2B). However, many PUFA were not consistently present across all groups and therefore, we concentrated our comparison on saturated, mono-unsaturated and di-unsaturated FA occurring in most samples. A screening of potential tracers revealed that differences between $\delta^{13}C$ of 16:0 and 18:2ω6 as well as the difference between 18:1ω7/9 and 18:2ω6 resulted in reasonable well separation of the four algae groups (Fig. 2C). Nonetheless, variation within groups was still evident.

Food identity impacts on consumers' growth, FA composition and isotope ratios

The two algae species, *C. reinhardtii* and *R. salina*, used as food in controlled feeding experiments differed substantially in their FA composition (Fig. 3A). *C. reinhardtii* was characterised by higher relative concentrations of saturated FA (e.g. 16:0) and mono-unsaturated FA (e.g. 18:1ω7 and 18:1ω9) but contained no long-chained (>18 C atoms) PUFA. In contrast, *R. salina* contained substantial quantities of the nutritionally valuable EPA and DHA.

Dietary differences were transmitted to FA profiles of daphnids and rotifers (Fig. 3B-C, Fig. S3). While rotifers had more compound-rich FA profiles than daphnids, both showed higher 16:0 (T-test; $p < 0.01$) and lower EPA concentrations ($p < 0.001$) when feeding on *C. reinhardtii*. Also 18:4ω3 and DHA were significantly lower in rotifers (T-test; $p < 0.01$) and entirely absent in *D. magna* with *C. reinhardtii* as food source. The contribution of the polar lipid fraction to total lipids increased significantly in rotifers when feeding on *C. reinhardtii* (T-test; $p = 0.02$), but an opposite pattern emerged in *D. magna* with higher relative polar contributions in the *R. salina* treatment (Fig. S4).

The FA differences in food algae translated to a slightly higher food quality of *R. salina* compared to *C. reinhardtii* diets (Fig. S5). Both food types supported high reproduction rates in rotifers and daphnids. Though, cumulative egg production per female after 6 days in rotifers (T-test; $p = 0.04$) as well as the number of neonates of daphnids in the first brood (T-test; $p = 0.02$) were significantly higher after feeding on *R. salina*. Biomass increases of daphnids during the experiment did not significantly differ between food treatments (t-test; $p = 0.79$; Fig. S5B).

Fractionation between the $\delta^{13}C$ of FA in algae and zooplankton was highly variable and there were no clear differences between treatments and consumer species across all FA (Fig. 4A). When average FA fractionation was considered, only the treatments of *B. plicatilis* and *D. magna* feeding on *C. reinhardtii* differed significantly in trophic fractionation (Tukey post-hoc test; $p = 0.01$). Isotopic fractionation of individual FA commonly ranged between -2 and 5‰ and many FA (e.g. 16:1ω13, 18:1ω7/ω9 and 18:3ω3) showed both positive and negative fractionation across treatments. However, 18:4ω3, EPA and DHA displayed little isotopic fractionation whenever present in both prey and consumer species (Fig. 4B-C).

Finally, we assessed patterns in standardised $\delta^{13}C$ values of individual FA in zooplankton. These analyses were complementary to assessments of isotopic fractionation because they were not limited to FA present in both food and consumers and thereby allowed more detailed analyses of $\delta^{13}C$ in e.g. 14:0, EPA and DHA. Saturated and mono-unsaturated FA in the total lipid fraction of consumers displayed a consistent pattern in their $\delta^{13}C$ values in response to different food sources (Fig. 5A). $\delta^{13}C$ values of these FA were significantly higher in the *R. salina* treatment compared to the *C. reinhardtii* treatment when FA and consumer identity were taken into account (nested ANOVA with consumer and FA identity as random factors; $p < 0.001$). Moreover, isotope values of EPA (Fig. 5B) and 14:0 (Fig. S6) showed distinct differences between food treatments and consumers (nested ANOVA performed for each FA

separately; 14:0 showed differences between food treatments as well as between consumers, $p < 0.01$; EPA showed differences between food treatments with a $p < 0.01$ and marginally non-significant differences between consumers with a $p = 0.10$). Hence, the isotopic fingerprints of consumers were significantly influenced by the food type they consumed.

Discussion

Stable isotope analyses of specific dietary compounds represent a promising tool to reveal carbon and nutrient flows in food-webs [11, 25, 29, 59, 60]. Our assessment of FA $\delta^{13}C$ values and their potential to trace trophic transfer revealed significant algae group-specific differences in FA isotopes, which may be used to establish unique signals for different food resources. However, isotopic fractionation of FA in consumers was highly variable, complicating the use of FA isotopes for dietary tracing. Only long-chain PUFA and 18:4ω3 showed consistently low fractionation between trophic levels in accordance with earlier zooplankton studies [28, 29] but in contrast to assessments in fish [36]. Consequently, a better understanding of the factors driving the variability of isotopic fractionation is essential to capitalise fully on FA $\delta^{13}C$ as trophic markers. Our findings of clear food-dependent differences in FA $\delta^{13}C$ of consumers represents a first step towards this goal and additionally suggest that FA fractionation in consumers may indicate food quality and consumers' physiological responses to their diets.

Algae source separation

We found clear differences in FA $\delta^{13}C$ values of key food sources at the base of aquatic food-webs. Differentiation among food items is a crucial first step in tracing energy flows across trophic levels. Generally, this can be achieved by sampling dietary sources directly or by inferring dietary signals from a reference library, a principle applied in genetic dietary tracing approaches [32, 61, 62] and in fatty acid source-tracking [63, 64]. Library approaches are linked to a number of advantages (e.g. low costs and wide applicability), but require

consistent source signals across ecosystems. Despite the group-specific FA-isotope fingerprints we discovered in this study, FA-CSIA alone are likely not powerful enough to support library-based source separation. A major obstacle for the use of literature values (e.g. these generated in this study) to infer in-*situ* food sources is the high variability of isotopic values within algae groups (Fig. 2). A library approach would rely on mean literature values and our analysis shows that within-group taxonomic identity can lead to large deviations from group means. Such deviations from literature group means are likely to be further increased by changes in growth environments in the target system [65-67]. Consequently, in the context of library approaches, group-differences in FA $\delta^{13}C$ seem mostly suitable to complement other methods such as FA source-tracking [63, 64] or AA isotope based library approaches [68].

Nevertheless, FA isotope based approaches relying on direct source sampling have a large potential to assess trophic interactions [11, 69, 70]. This is reflected by large differences in standardised FA $\delta^{13}C$ we found among specific algae species (e.g. 18:2ω6 $\delta^{13}C$ values of -8.1 for *Chaetocerus pluvialis* and 6.8 for *Dunaliella salina* or 20:5ω3 $\delta^{13}C$ values of -7.1 for *Emiliania huxleyi* and 0.7 for *Tetraselmis suecica*). This potential of FA-CISA can be capitalised upon by either relying on group-specific biomarkers (such as 16:1ω7 and 16:4ω1 for diatoms [12, 26]; Box 1, Option A) or by applying a multi-tracer approach (see Box 1, Option B). Both methods are linked to specific advantages and disadvantages, and method choice will depend on the aim and community characteristics of target systems.

In contrast to the use of group-specific biomarkers, a multi-tracer approach requires the physical separation of different food sources. Physical separation of different algae groups can be accomplished based on sedimentation, floatation and size fractionation techniques [1, 2]. Manual separation techniques are, however, work-intensive and restricted to certain groups (e.g. autotrophic flagellates are very difficult to separate). Technical advances such as flow

cytometers with a sorting function could be used to substantially simplify and expand separation processes. Automated separation of different algae groups based on their size and pigment content [71] could facilitate the generation of group or possibly even species-specific diet signals, which would help to utilise the full potential of FA $\delta^{13}C$ differences among taxa recorded in our study.

Trophic fractionation of FA isotopes

A low or predictable trophic fractionation is an important characteristic of reliable trophic markers [29, 36]. In principle, fractionation is rooted in the influence of molecular mass on the biochemical reactivity of molecules. Higher $\delta^{13}C$ values are thereby assumed to increase reactivity [36, 72]. However, FA metabolism is determined by a number of different processes, which can either lead to a ^{13}C enrichment or depletion with trophic transfer (Fig. 6; see also Fig. S6 for compilation of literature values). Our controlled feeding experiments showed that differences in FA isotope ratios between consumers and their prey were low and relatively constant for 18:4ω3, EPA and DHA. This consistently low fractionation, which accords with earlier studies on daphnids [28, 29; see also Fig. S7], indicates that these compounds show low fractionation during their uptake (high absorption efficiencies) and before their incorporation in consumer tissue (e.g. little use for respiration; Fig. 6). Other FA, however, displayed more variation in isotopic fractionation in consumers. Similar variation in isotopic fractionation of many FA has been reported in earlier studies [29, 36, 42] providing a substantial challenge for the use of these FA in dietary tracing approaches [44]. The uncertainty resulting from variable fractionation might be compensated by large isotopic differences in target systems. Ultimately, we need to improve our understanding of the factors driving $\delta^{13}C$ changes with trophic transfer to increase the reliability of our predictions.

While food-source dependent trophic fractionation is a disadvantage for tracing energy flows [34], patterns in fractionation of FA isotopes may provide valuable information on

consumers' diet quality [44]. We found that standardised FA isotope ratios in consumers showed clear differences as response to changes in food sources (Fig. 5). Such systematic differences may be either explained by (*i*) the isotopic depletion of 14:0 and monounsaturated FA in green-algae-consuming zooplankton or (*ii*) an enrichment of 16:0, which was used to standardise these FA. Such an enrichment in 16:0 could for example emerge from a relative surplus of 16:0 in respective food treatments and reduced absorption rates (Fig. 6). Moreover, EPA showed lower $\delta^{13}C$ values in consumers when absent in their diets. Whether such patterns in FA isotope values in consumers can be systematically linked to surplus or limitation of specific dietary FA is still unclear and needs to be evaluated in future studies. Nevertheless, the consistent FA $\delta^{13}C$ patterns in consumers fed with different food sources could provide valuable information on dietary quality and corresponding responses of consumers in their physiology.

Finally, we want to address potential benefits of joint analyses of FA $\delta^{13}C$ in polar and neutral lipids. While we found some differences in $\delta^{13}C$ of FA between total (neutral + polar) and polar lipids, recorded patterns were largely similar among lipid groups. Nevertheless, neutral and polar lipids can show substantial differences in turn-over times, especially when food is available in excess and consumers rapidly build up reserves as neutral FA [73]. Hence, dietary shifts to new and abundant food sources can lead to faster isotopic changes in neutral than in polar lipids. This implies that after such diet shifts, the analysis of isotope ratios in total instead of neutral FA may lead to misinterpretations of consumers feeding behaviour. However, the slower turn-over of polar lipids [74] also creates the chance to use polar lipid fractions as dietary archives providing exciting opportunities to reconstruct past feeding histories of consumers. Differences between FA $\delta^{13}C$ ratios in polar and neutral lipids have previously been used to infer changes in feeding behaviour of fish [73], similar to applications that used variable isotope turnover rates of amino acids to establish migration patterns [75]. Our experiments demonstrated that FA $\delta^{13}C$ in different lipid fractions are largely similar

when zooplankton is feeding on one resource. Consequently, differences between isotope ratios of polar and neutral FA can indeed indicate changes in feeding behaviour, opening up new opportunities using polar lipid fractions as dietary archives in plankton food-web studies.

Outlook

FA-CSIA represent a valuable methodological approach that is increasingly applied in studies focusing on e.g. food-webs in aquatic systems [76, 77], terrestrial consumers [78] or the reconstruction of past human nutrition [79]. Nevertheless, there are clear caveats linked to the use of this method, such as variable trophic fractionation and high within source variation highlighted in our study. Essential premises for FA $\delta^{13}C$ based applications are that differences among dietary sources are large enough to offset these caveats and that researchers respect the same guidelines that have been established for bulk isotope analyses [13, 15]. When such principles are considered, advantages such as the group-specificity of marker FA or the use of polar FA as dietary archives provides exciting tools to trace nutrient and energy flows within complex food-webs.

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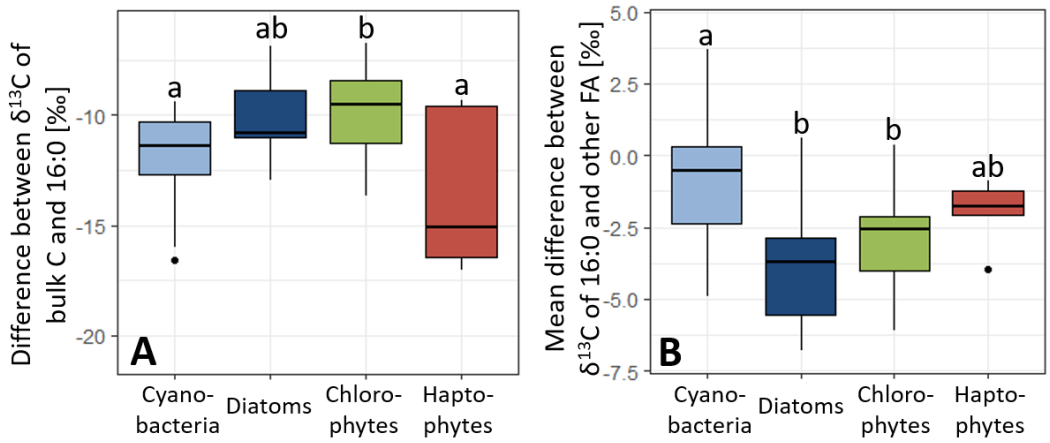
Table 1: Overview of different monocultures and zooplankton experiments established to test the potential of compound-specific isotope analyses of FA to improve energy tracing approaches in aquatic food webs. Two strains of *Emilia huxleyi* were cultured, one calcifying and one non-calcifying. Also for *Synchocystis* two strains were cultured, one isolated from the Baltic Sea and one from Lake Nakuru (Kenya). Zooplankton experiments included two experiments with each consumer, one with *Chlamydomonas reinhardtii* and one with *Rhodomonas salina* as diet.

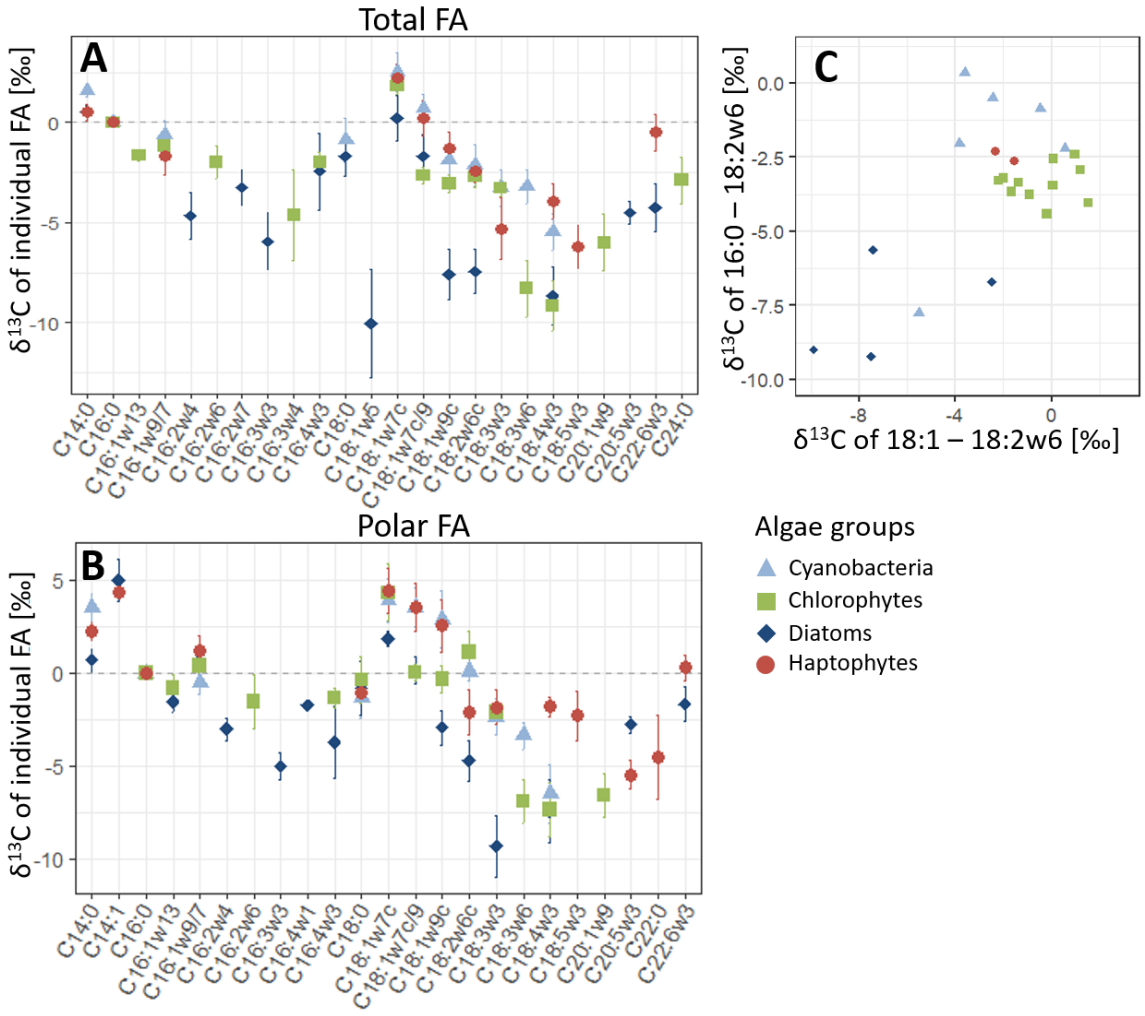
Algae monocultures	
Chlorophyceae	*Chlamydomonas reinhardtii*
	Chlorella emersonii
	Chlorella vulgaris
	Choricystis minor
	Dunaliella tertiolecta
	Dunaliella salina
	Haematococcus pluvialis
	Monoraphidium sp.
	Pseudokirchneriella subcapitata
	Scenedemus sp.
	Selenastrum capricornutum
	Tetraselmis suecica
Diatoms	*Achnantes sp.*
	Chaetoceros peruvianus
	Navicula pelliculosa
	Thalassiosira weissflogii
	Phaeodactylum tricornutum
Cyanobacteria	*Anabaena variabilis*
	Anabaena sp.
	Anabanopsis elenkinii
	Leptolyngbya sp.
	Nodularia balthica
	Spirulina major
	Synechococcus sp.
	Synechocystis strain a
	Synechocystis strain b
Hapto-phytes	*Emilia huxleyi* (calcifying)
	Emilia huxleyi (naked)
	Gephyrocapsa oceanica

Zooplankton experiments	
Zooplankton consumer	**Algae food treatment**
Brachionus plicatilis	*Chlamydomonas reinhardtii*
	Rhodomonas salina
Daphnia magna	*Chlamydomonas reinhardtii*
	Rhodomonas salina



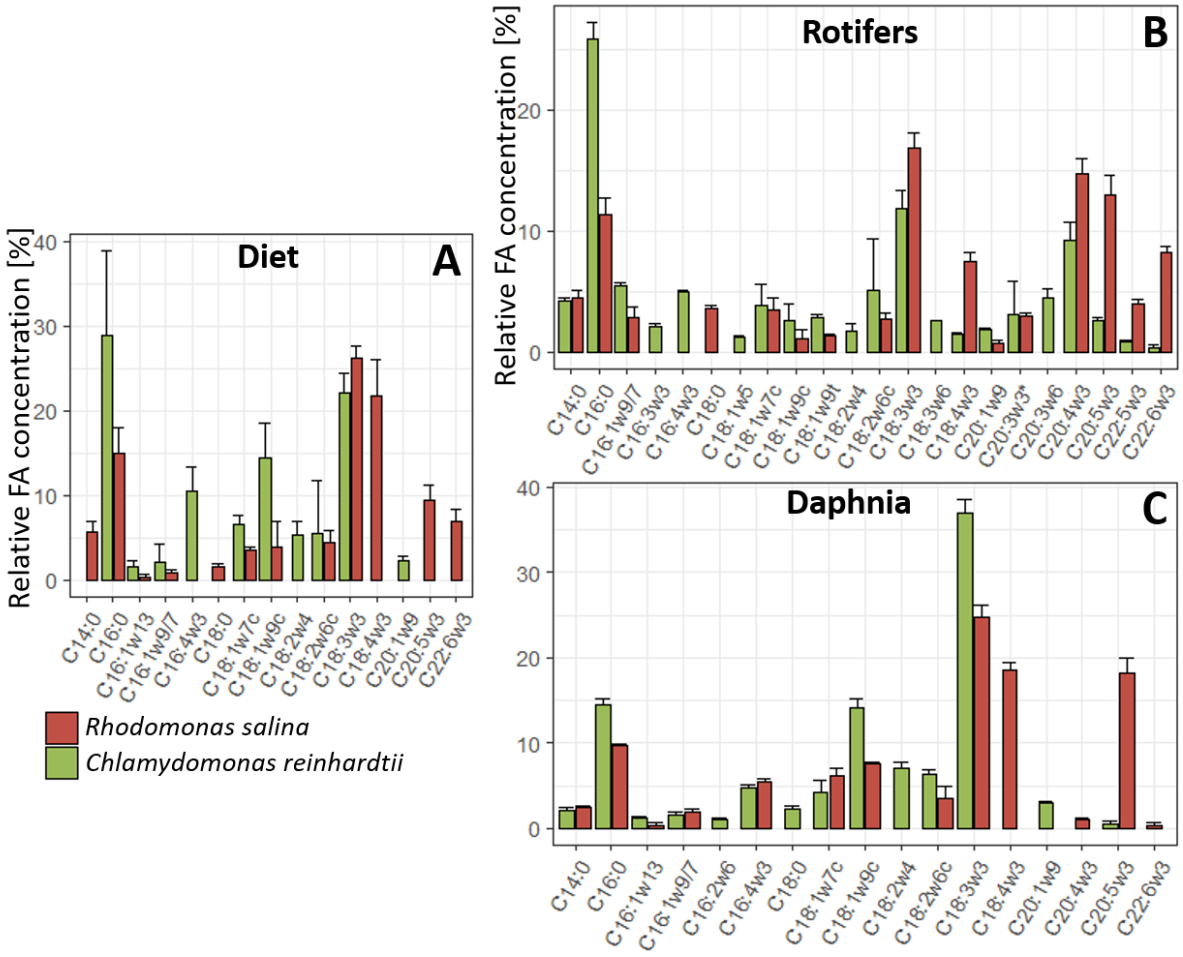
643

644 Fig. 1: Boxplots of the differences in $\delta^{13}C$ values between C16:0 and bulk C (**A**), and between
645 C16:0 and other FA (**B**) in total lipids of four major algae groups. Bulk C constitutes an
646 integrated isotope value of all C-compounds of an alga. In total, 29 species (59 samples) were
647 analysed. Lowercase letters denote significant differences across algae groups as those with
648 the same letters are not statistically different from each other.



649

650 Fig. 2: FA $\delta^{13}C$ fingerprints of four major algae groups. Displayed are the standardised $\delta^{13}C$
651 values of individual FA in total (**A**) and polar (**B**) lipids of all FA that were present in at least
652 15% of all samples from a specific algae group. 16:1ω7 and 16:1ω9 co-eluted in all and
653 18:1ω7 and 18:1ω9 co-eluted in some samples and are hence displayed together. Individual
654 values of the latter FA are restricted to samples where peaks could be separated. (**C**)
655 Differences between $\delta^{13}C$ of 16:0 and 18:2ω6 as well as between, 18:1ω7/9 and 18:2ω6 of
656 total lipids were used to differentiate between algae groups. One outlier (*Dunaliella*
657 *tertiolecta*, a green algae) falls outside the plotted range and is not displayed (values: x=8.7,
658 y=3.5). Error bars represent standard errors of the mean.



659

660 Fig. 3: Relative FA concentrations in diet and consumers of controlled feeding experiments.

661 Presented are relative contribution to total lipids in two different food algae (**A**) fed to

662 *Brachionus plicatilis* (**B**) and *Daphnia magna* (**C**). Only FA with relative concentrations of

663 >1% are displayed. 16:1ω7 and 16:1ω9 as well as 20:3ω3, 20:4ω6 and 22:0 co-eluted and

664 joint quantifications for these FA are provided. Error bars represent standard deviations.

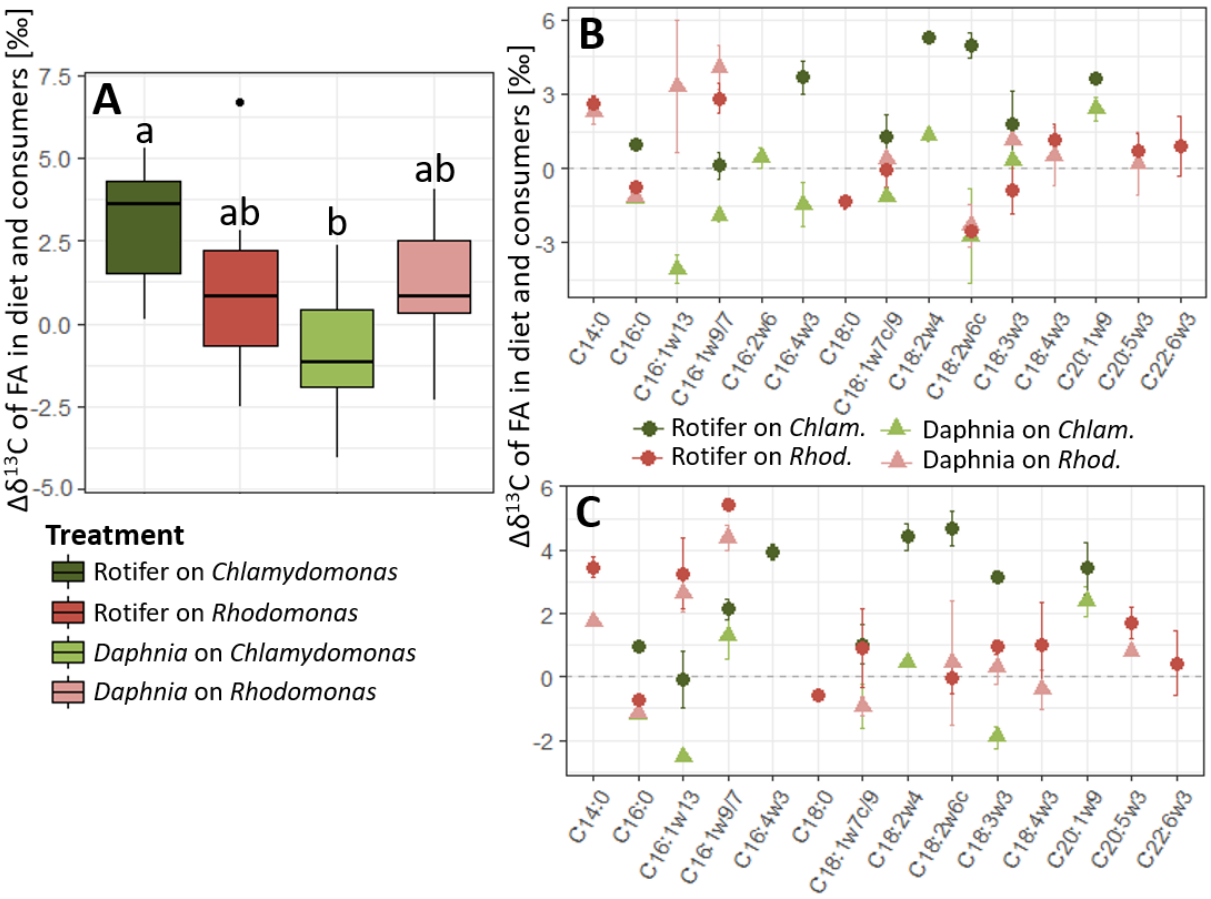
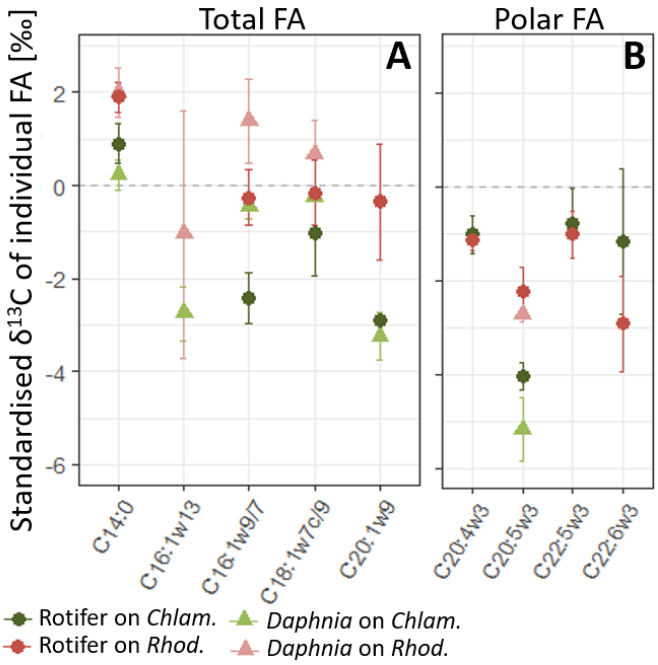
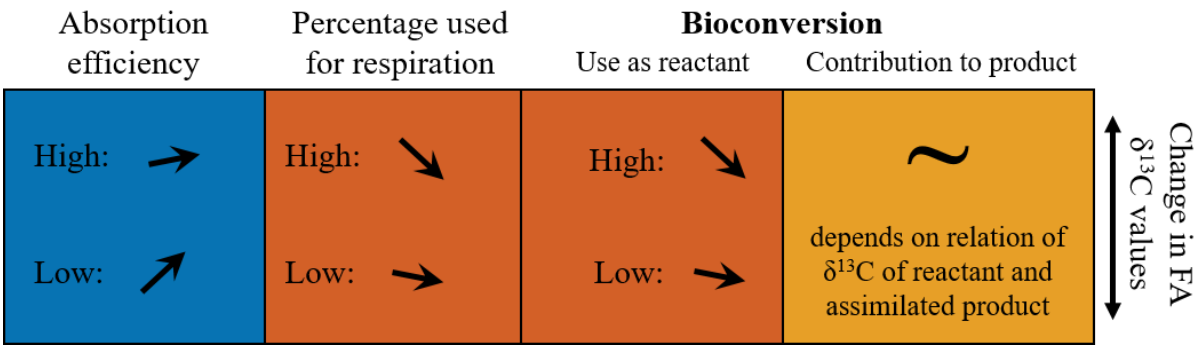


Fig. 4: Trophic fractionation in FA $\delta^{13}C$ measured in controlled feeding experiments. (**A**) The boxplots of isotopic fractionation values (difference between isotopic values of FA in consumer and diet) in total lipids of *Daphnia magma* and *Brachionus plicatilis* in the two dietary treatments. Only FA which were present in prey and consumers were included. Letters denote significant differences between treatments as in Fig. 1. Fractionation for individual FA (points represent means, error bars standard errors of the mean) in total and polar lipid fractions are displayed in (**B**) and (**C**), respectively. 16:1ω7 and 16:1ω9, 18:1ω7 and 18:1ω9 co-eluted and integrated isotopic values for these FA are provided. FA with relative concentrations <1% are not displayed.



676

677 Fig. 5: Patterns in isotopic values of individual FA in consumers fed with either a
678 *Chlamydomonas reinhardtii* (green symbols) or *Rhodomonas salina* (red symbols). Presented
679 are isotopic values of saturated and mono-unsaturated FA in total lipids (**A**), which display
680 systematic differences between diets (i.e. isotopic enrichment when feeding on *R. salina*
681 compared to *C. reinhardtii*). EPA in highly unsaturated long-chain FA of the polar lipid
682 fraction (**B**) showed the same pattern and additionally revealed a relative enrichment of
683 rotifers compared to daphnids. All values were standardized by $\delta^{13}C$ of 16:0. 16:1ω7 and
684 16:1ω9, 18:1ω7 and 18:1ω9 co-eluted and joint isotopic values for these FA are provided.



685

686 Fig. 6: Fractionation of FA isotopes with trophic transfer is the combined result of a
687 number of processes such as absorption, respiration and bioconversion in consumers. Arrows
688 indicate changes in FA ^{13}C values under high and low process activity, assuming a higher
689 activity of ^{13}C rich molecules [36, 72]. Based on this assumption, absorption results in ^{13}C
690 enrichment, while the use of a FA for respiration decreases $\delta^{13}C$ values and the consequences
691 of bioconversion are case specific. The efficiency (absorption) and the relative quantitative
692 importance of metabolic processes (i.e. biomass fraction respired/ bioconverted) determine
693 the magnitude of isotopic fractionation.

694

Box 1: **Fatty acids (FA) $\delta^{13}C$ in diet tracing: Direct source sampling approaches**

Different algae taxa that co-occur in a system can show systematic differences in their bulk $\delta^{13}C$ values [light blue area in figure below; 1, 2], caused by e.g. differences in carbon concentration mechanisms [3, 4] and C availability [5, 6]. In our study, differences among bulk $\delta^{13}C$ of algae were caused by different growth media and hence we standardised $\delta^{13}C$ FA values to cancel out these media effects. In general, isotopic values of bulk C is transmitted to $\delta^{13}C$ of total FA (second blue layer, Fig. below), which represent weighted means of individual FA $\delta^{13}C$ values. Differences in e.g. energy storage mechanisms (storage of depleted ^{13}C lipids vs. relatively enriched starch) among algae groups can determine bulk – total FA $\delta^{13}C$ relationships (e.g. Fig. 1).

There are two main applications of FA $\delta^{13}C$ for tracing trophic interactions when signals of food sources are established through direct sampling. **Option A** is the inference of group-specific isotope values based on a specific FA (see [11, 12] or below for an example of 18:5ω3 as tracer for haptophytes). This has the advantage that plankton communities can be sampled as a whole and no physical source isolation is necessary. However, tracing approaches are limited to a low number of food sources because only C isotopes are available as tracer [13]. **Option B** represents a multi-tracer approach. Instead of group-specific marker FA, common FA are used. If these FA $\delta^{13}C$ differ among food sources, e.g. because of group-specific FA synthesis pathways, multiple food sources can be identified based on their unique FA $\delta^{13}C$ fingerprints (i.e. group-specific $\Delta\delta^{13}C$ among different FA). Such characteristic $\Delta\delta^{13}C$ among algae groups can easily be explored through standardisation as has been done for AA [19, 20] or FA in our study. Major requirements for this approach are (*i*) the need to isolate and analyse different food sources in order to attain group-specific FA isotope values and (*ii*) a low or predictable isotopic fractionation between consumers and their prey.

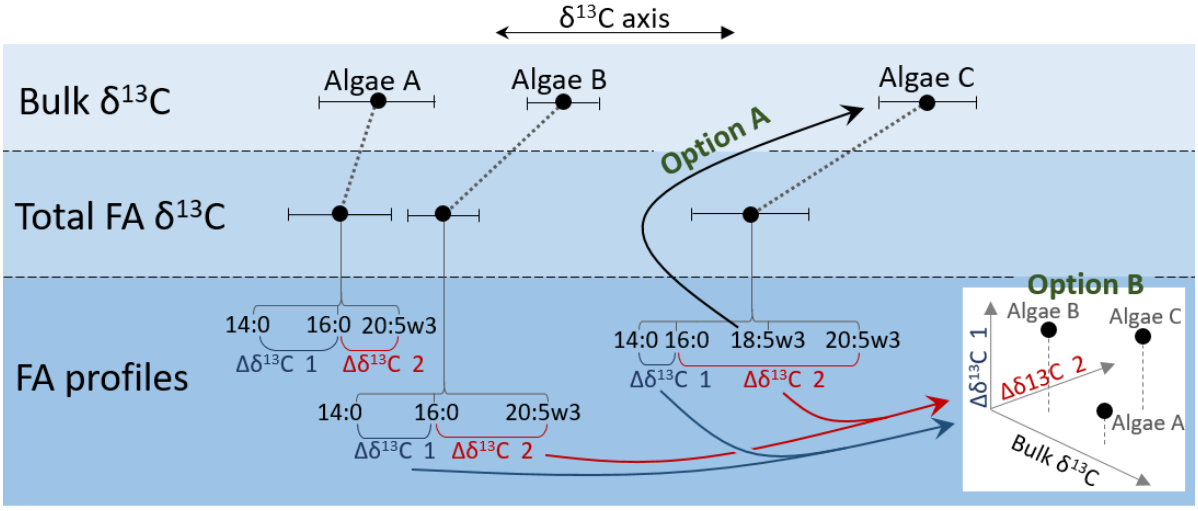


Fig. Box 1: Bulk isotope ratios are transmitted to total FA $\delta^{13}C$ values and $\delta^{13}C$ of individual FA, which are also shaped by enzymatic processes such as elongation and unsaturation [21-23]. $\delta^{13}C$ of individual FA can then be used to infer bulk $\delta^{13}C$ or to create a multi-dimensional isotopic space that allows to differentiate among multiple food sources. Separation of multiple sources using different $\Delta\delta^{13}C$ (red and blue) and bulk $\delta^{13}C$ in a multi-dimensional isotopic niche space is visualised on the bottom right.