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LETTER

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The second life of terrestrial and plastic carbon as nutritionally valuable food for aquatic consumers

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Abstract

Primary production is the basis for energy and biomolecule flow in food webs. Nutritional importance of terrestrial and plastic carbon via mixotrophic algae to upper trophic level is poorly studied. We explored this question by analysing the contribution of osmo- and phagomixotrophic species in boreal lakes and used ^{13}C-labelled materials and compound-specific isotopes to determine biochemical fate of carbon backbone of leaves, lignin–hemicellulose and polystyrene at four-trophic level experiment. Microbes prepared similar amounts of amino acids from leaves and lignin, but four times more membrane lipids from lignin than leaves, and much less from polystyrene. Mixotrophic algae (*Cryptomonas* sp.) upgraded simple fatty acids to essential omega-3 and omega-6 polyunsaturated fatty acids. Labelled amino and fatty acids became integral parts of cell membranes of zooplankton (*Daphnia magna*) and fish (*Danio rerio*). These results show that terrestrial and plastic carbon can provide backbones for essential biomolecules of mixotrophic algae and consumers at higher trophic levels.

KEYWORDS
allochthonous carbon, amino acids, carbon cycle, fatty acids, food web, mixoplankton, plastic

INTRODUCTION

The trophic use of carbon and dietary energy produced within (autochthonous) and outside (allochthonous) aquatic ecosystems is still poorly understood. Autotrophic primary production is the basal source of dietary energy and essential biomolecules in most ecosystems (Thornton, 2012). However, autotrophic primary production in terrestrial and aquatic environments is affected substantially by the availability of light and CO_2. Immobile phytoplankton species, such as diatoms, cannot actively move towards the surface, making them dependent on upwelling currents. Moreover, the high content of dissolved organic carbon and iron significantly reduces light availability in brown-water habitats (Ask et al., 2009). In addition to the well-examined phosphorous and/or nitrogen limitation for lake phytoplankton (Bergström & Karlsson, 2019), recent studies suggested that primary production during summer stratification may become carbon limited in both temperate and boreal lakes (Huotari et al., 2009; Kragh & Sand-Jensen, 2018). Mixotrophic algae cannot only perform photosynthesis but also uptake dissolved organic substances (osmotrophy) or organic particles (phagotrophy), and they can constitute a significant part of the plankton in marine and freshwater ecosystems (Flynn et al., 2019; Hartmann et al., 2012).

Limitations of inorganic carbon and light may be overcome by utilizing terrestrial organic carbon (Calderini et al., 2022; Tittel et al., 2009). In addition to highly labile terrestrial carbohydrates, freshwater ecosystems also receive potentially recalcitrant organic carbon sources,

for example lignocellulose, which forms 80 to 90% of terrestrial inputs to lakes and rivers (Brett et al., 2017; Jones, 1992). Conversely, we have recently revealed trophic uptake and conversion of plastic carbon to essential omega-3 (ω-3) and omega-6 (ω-6) polyunsaturated fatty acids (PUFA) (Taipale et al., 2019). This demonstrated trophic upgrading of microplastic (MP) carbon was an important finding since the persistence of MP in marine and freshwater ecosystems is an increasing environmental concern due to the predicted growth of slowly degrading plastic waste (Borrelle et al., 2020; Taipale et al., 2022; Vesamäki et al., 2022). Despite the slow process, direct utilization of some plastic blends and their leachates can support microbial biomass, complementing the trophic transfer of biomolecules (Sheridan et al., 2022; Taipale et al., 2019, 2022; Vesamäki et al., 2022; Yoshida et al., 2016).

However, microbial decomposition of recalcitrant organic carbon is generally slow and related to certain taxa including Actinobacteria and Proteobacteria (Bugg et al., 2011; Taipale et al., 2022; Vesamäki et al., 2022). These two phyla differ in their resistance to flagellate and zooplankton grazing, and thus, the microbial community structure can influence the trophic transfer of organic carbon (Taipale et al., 2012; Tarao et al., 2009). While it is now known that microbes can build new biomass from a variety of carbon sources, it is not known how much of the biomolecules prepared from labile leaves or recalcitrant polymers is transferred up to the food web (Hessen & Tranvik, 1998).

The importance of terrestrial carbon in freshwater ecosystems has been highly debated in the last decades using bulk stable isotope analysis or carbon isotope analysis of fatty acids and/or amino acids (Arsenault et al., 2022; Berggren et al., 2014; Brett et al., 2017; Cole et al., 2011; Liew et al., 2019; Taipale, Galloway, et al., 2016; Tanentzap et al., 2014). However, such studies have not shown the biochemical mechanism of terrestrial or plastic carbon transfer to higher trophic levels. Moreover, previous studies have not taken into account that mixotrophic algae could combine different organic carbon sources as an inseparable pool, as previously shown with microplastic carbon (Mitra et al., 2014; Taipale et al., 2019). Therefore, microbial degradation of terrestrial and microplastic carbon, together with dietary upgrading by mixotrophic algae, could be a key link in recycling carbon in aquatic food webs. Cryptophytes are a common mixotrophic phytoplankton group in temperate and boreal lakes (Lepistö & Rosenström, 1998) whose nutritional value for zooplankton (cladoceran) is superior to others (Peltomaa et al., 2017). Previous studies have shown that mixotrophic species can produce essential fatty acids from alternative carbon sources, for example glucose and microplastic (Piasecka et al., 2020; Taipale et al., 2019). However, in these experiments, light has been available, and thus, it is uncertain whether the actual biosynthesis of essential biomolecules occurs only under light (autotrophy). Based on the previous literature (Glibert & Legrand, 2006), it seems that almost all phytoplankton species are capable of osmotrophy and thus could utilize terrestrial dissolved organic carbon (DOC) (Tittel et al., 2009) or plastic leachates (Sheridan et al., 2022), and thus could integrate autochthonous, allochthonous and anthropogenic carbon sources in aquatic food webs (Calderini et al., 2022; Granéli et al., 1999; Selosse et al., 2017; Tittel et al., 2009).

Lake browning influences the trophic mode of phytoplankton, but the nutritional importance of terrestrial organic carbon for pelagic food chain remains controversial (Attermeyer et al., 2013; Guillemette et al., 2016; Kritzberg et al., 2004). This question can only be studied by tracking the flow of terrestrial carbon sources to biomolecules of different trophic levels. Therefore, we comprehensively assessed the contribution of osmo- and phagomixotrophic species in boreal lakes ($n = 11{,}000$ samples) based on prior taxonomic categories (Flynn et al., 2013; Glibert & Legrand, 2006). Thereafter, we studied the biochemical fate of ^{13}C-labelled leaves, lignin/hemicellulose and polystyrene in a four-trophic level experiment, tracking the C flow to different biomolecules by using stable isotope labelling. We analysed microbial utilization of carbon from different biodegradable sources (leaves, lignin and plastic), inspecting the ratio of carbon channelling to energy and biomass. Secondly, we used compound-specific stable isotope analysis to follow the flow of terrestrial and microplastic carbon to amino and fatty acids of mixotrophic algae (*Cryptomonas* sp.) and further to zooplankton (*Daphnia magna*) and fish (*Danio rerio*) to unravel the biochemical fate of these alternative carbon sources to consumers at higher trophic levels.

METHODS

Detailed description of methods is given in supplementary document (File S1).

Phytoplankton osmo- and phagomixotrophy

The data contain 7480 phytoplankton samples taken from 1382 lakes or larger lake basins in July–August between 1975 and 2019 from surface to 2-m depth. There are 1–34 samples from each lake, depending on the lake size and sampling frequency. As almost all phytoplankton species are suggested to be able to utilize dissolved or particulate organic compounds (Glibert & Legrand, 2006), they were categorized as osmomixotrophs (utilization of organic substrates) or phagomixotrophs (utilization of particulate organic substrates) according to their nutritional mode (Table S1). The raphidophyte genus *Gonyostomum*, which has numerous chloroplasts but may also cause cell lysis of other phytoplankton and utilize the released

nutrients by osmotrophy (Rengefors et al. 2008), was kept in a separate category. The lakes were grouped according to their concentrations of total phosphorus (TP) and dissolved organic carbon (DOC). The TP groups are <35 and ≥35 μg L^{-1}. DOC concentration was transformed from water colour determined based on a hexachloroplatinate scale (SFS-EN ISO 7887:en or harmonized results obtained by a corresponding standard method) using the formula (DOC = 0.0872 × water colour +3,55, r^2 = 0.86, n = 976) (Kortelainen, 1993). The DOC categories are <6, 6–12 and >12 mg L^{-1}. The phytoplankton, TP and water colour data were obtained from the open-access phytoplankton and water chemistry databases of the Finnish Environment Institute Syke (www.syke.fi/avointieto).

Mixotrophy experiment (Experiment I)

In this experiment, we studied if mixotrophic algae can biosynthesize amino and fatty acids in the dark from leaf ^{13}C-extract (tDOC + microbiome). A ^{13}C-labelled beech leave extract (DOC = 28.6 ± 0.5 mg C^{-1} L^{-1}) was prepared by incubating leaves in humic lake water for 1 month. Dark-cultured mixotrophic algae (*Cryptomonas* sp. CPCC 336) was split into 3 × 200 mL tissue tubes (Sarstedt), 100 mL for each. Then, a 10 mL of ^{13}C-extract (tDOC + microbiome) was added to algal bottles. Dark bottles were wrapped in foil and control did not have additions. Cultures were kept at 18 ± 1°C under 14 h:10 h light:dark cycle with the light intensity of 30–50 μmol m^{-2} s^{-1} for 48 h, which after population density was measured using a cell counter (Casy Model TT). Fatty acids were harvested after 5 days of incubation by filtering each sample through cellulose nitrate membrane filters (Whatman, pore size 3 μm) to harvest only algal cells. Filters were frozen at −80°C, freeze-dried and weighed.

Biodegradation and trophic transfer experiment (Experiment II)

Here, we compared the mineralization of ^{13}C-beech leaves (*Fagus sylvatica*; IsoLife), ^{13}C-lignin–hemicellulose (*Triticum aestivum*; Isolife, purity ~80%; van Erven et al., 2019), and ^{13}C-polystyrene (Sigma-Aldrich) and their biochemical upgrade via mixotrophic algae (*Cryptomonas* sp., CPCC 336) to zooplankton (*Daphnia magna*) up to the fish (*Danio rerio*) (Figure 1). We weighed 1000 mg of 13.4% ^{13}C-labelled beech leaves, 30 mg of 97% of ^{13}C-labelled lignin (including 15% hemicellulose) or 30 mg of 99% of ^{13}C-labelled polystyrene into 3000 mL Erlenmeyer flasks with foil stopper, which after 2.6 L of prefiltered (<20 μm) humic lake water (Lake Haukijärvi, DOC = 36.3 mg C L^{-1}, pH = 6.55) was added. Flasks (n = 3 for all treatments) were incubated for 14 days, shaking bottles once a day for 15 s at 18 ± 1°C, except polystyrene incubation lasted for 56 days. Additionally, polystyrene (1 mg) was incubated in a sterile algal medium (n = 4, 200 mL plastic bottles) for 28 days to determine whether polystyrene could decompose passively. The actual values of transferred biomolecules to upper trophic levels were divided by added amount of substrate as carbon making the different masses of substrates unimportant.

After incubations, mixotrophic algae (*Cryptomonas* sp. CPCC366, cell concentration 2.8 × 10^5; added amount 500 mL) was let to graze on bacteria and DOC for 5 days. During these 5 days, we used 14 h:10 h light:dark cycle with the light intensity of 30–50 μmol m^{-2} s^{-1}. After 5 days, *Daphnia magna* was introduced to the bottles (60

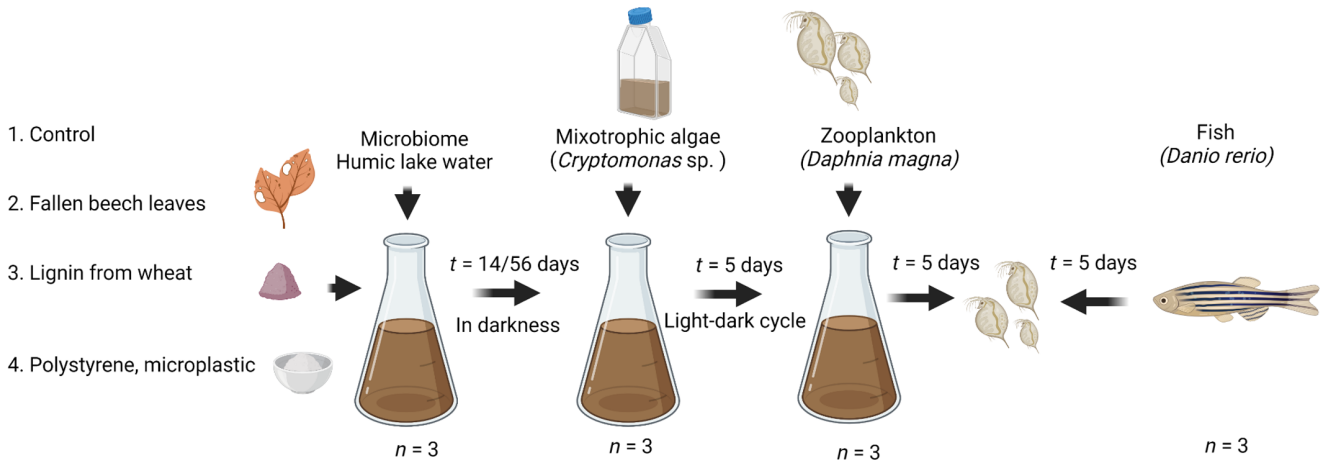

FIGURE 1 Setup of the four-trophic level experiment. The experiment included control (no addition), 13.5% ^{13}C-labelled beech leaves (*Fagus sylvatica*), 97% ^{13}C-labelled lignin–hemicellulose extracted from wheat (*Triticum aestivum*, ~80% lignin, 13% hemicellulose), and 99% ^{13}C-labelled polystyrene (microplastic). Incubation time in humic lake water was 14 days in the control, leaf and lignin experiment, but 56 days for polystyrene, which after mixotrophic algae (*Cryptomonas* sp.) was introduced to the bottles. In the next step, herbivorous zooplankton (*Daphnia magna*) consumed microbes, mixotrophic algae and particles for 5 days which after they were used as the diet to zebrafish (*Danio rerio*) during a 5-day experiment.

individuals per bottle) for consumption of mixotrophic algae, heteronanoflagellates (HNF) and microbiomes. The concentration of (mixotrophic) algae was measured before, and after adding zooplankton to the bottles using a flow cytometer (Figure S2). In the final step, cultured *Daphnia magna* were placed into 1.5 mL Eppendorf® tubes and stored at −80°C until used to feed zebrafish (*Danio rerio*). Three fish per treatment (altogether 12 individuals) were placed individually in one-litre rearing tanks in a zebrafish rack (Figure 1). The feeding experiment lasted for 5 days and during that time fish were fed twice a day only with *Daphnia* (no additional food was offered). During feeding, water flow to the rearing tanks was stopped for 1 h to prevent flushing the *Daphnia* out of the tank. Water temperature was kept at 28°C and the light regime at 14 h light: 10 h dark.

To further evaluate ^{13}C-diet in the gut influence on carbon isotope values of amino acids and phospholipid fatty acids, *Daphnia magna* and zebrafish were fed with ^{13}C-labelled diets. The gut content influence was determined by comparing control individuals with ^{13}C-diet-fed individuals (See detailed description in File S1, Table S2).

During the experiment, DIC samples for concentration and stable isotopes were taken three times a week. A 100 mL of water sample was filtered (0.2 µm cellulose nitrate filter, Whatman) for PLFA analysis after the initial incubation and mixotrophy stage. Correspondingly, we used 20–40 mL for filtering DNA/RNA samples through 0.2 µm filter papers (Pall Corporation, 25 mm PES). PLFA filter papers were freeze-dried and stored at −80°C, whereas DNA/RNA samples were stored in 800 µL of DNA/RNA-Shield (Zymo) at −80°C.

Analysis of the concentration and δ^{13}C of DIC

An Agilent 7890B GC (Agilent Technologies) was used for the analysis of DIC/ CO_2. Isotopic enrichment (Δ) of δ^{13}C was calculated between treatments and control (only lake water), and thus, positive values (‰) show mineralization of ^{13}C-polyethylene into carbon dioxide. For the calculation of mineralization of added substrates, we used previously published equations (Taipale et al., 2022; Supplemental document).

Microbial community analysis

To study the prokaryotic microbial community structure, RNA was extracted from the previously collected samples ($n = 3$ for each treatment) using glass-bead (⌀ 0.1 mm, 0.6 w:vol) homogenization and Chemagic™ 360 and the Chemagic™ Viral DNA/RNA 300 Kit H96 following the manufacturer's instructions (PerkinElmer). The RNA was treated with DNAse and reverse transcribed to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The effectiveness of the DNase treatment was validated by including negative RT samples. cDNA samples were stored at −20°C.

The target region of the bacterial 16S SSU rRNA was amplified using the earth microbiome primer pair 515F–806R and the sequencing was done using the Ion Torrent PGM (Thermo Fisher) (File S1). The sequences (average 357,000 per sample) were analysed using the CLC Genomics Workbench software (Qiagen). The primers were trimmed, and the short sequences were discarded (<150 bp). After the sequences were trimmed to the same length (average length = 242 bp), the OTU (Operational Taxonomic Unit) clustering was performed using the SILVA 16S v132 database with a similarity percentage of 97%, which provided 17,529 OTUs.

Lipid extraction, fractionation and derivatization

Lipids were extracted from the filters, zooplankton, and fish according to Folch et al. (1957). Samples were fractionated and methylated as Taipale et al. (2021). Fatty acid methyl esters were identified and quantified as previously published (Taipale, Hiltunen, et al., 2016). A complete description of the protocol can be seen in File S1.

Carbon isotope measurements from fatty acids

The δ^{13}C values of FAs were determined using a GC-C TA III connected to an Isotope Ratio Mass Spectrometer (IRMS, DELTAPLUSXP, Thermo Co.) at the inter-university centre for aquatic ecosystem research WasserCluster Lunz—Biological Station (Austria). Fatty acids were separated using a 60 m DB-23 column (0.25 × 0.15 mm) and then oxidized to carbon dioxide in an oxidation reactor at a temperature of 940°C with the reduction reactor kept at 630°C. We used a previously published temperature program (Taipale et al., 2022), and samples were run against an internal standard, 1, 2-Dinonadecanoyl-sn-Glycero-3-Phosphatidylcholine (Larodan, δ^{13}C = −28.43‰), which was used for drift and linear correction. The δ^{13}C value of FA was calculated from the δ^{13}C value of FAME by correcting the methyl group. Isotopic enrichment (Δ) of δ^{13}C was calculated between treatments and control, and thus, positive values (‰) indicate utilization of ^{13}C-substrates in fatty acid synthesis. For the calculation of assimilation carbon from added substrates to microbial biomass, we used previously published equations (Taipale et al., 2022).

Amino acid analysis

Freeze-dried filter papers or 2–5 mg of grounded zooplankton and fish were weighed in glass tubes, and proteins were hydrolysed with 1 mL of 6 M HCl at 110°C

for 20 h for AAs analysis. An internal standard (30 μg of Norvaline) was added to each sample. We prepared amino acid propyl chloroformates using the protocol of EZ: faast kit (Phenomenex). Propyl chloroformates were diluted (50–300 μL, depending on expected sample AA content) to drive elution (80 iso-octane, 20% chloroform, vol/vol) and run with a parallel run of GC–MSD/IRMS system and a using ZB-AAA column (9.5 m × 0.25 μm × 0.25 mm). The GC-MSD/IRMS system included Agilent 7890B GC, Agilent 5977B MSD, Elemental GC5 combustion interface and Isoprime precisION. We used four-point calibration curves derived from the standard mixture (Sigma-Aldrich AAS-18), and samples were recovery corrected by internal standard (norvaline). We were able to analyse seven EAAs (valine, leucine, isoleucine, threonine, phenylalanine, lysine and histidine) and six non-EAAs (alanine, glycine, proline, aspartic acid, glutamic acid and tyrosine).

The $\delta^{13}C$ value of norvaline (internal standard) and picoline (derivatization reagent) was run with EA-SIRMS system (DeltaPlus Advantage Thermo Finnigan), which was used to calculate the $\delta^{13}C$ value of individual AA. The $\delta^{13}C$ value of AA was calculated from the $\delta^{13}C$ value of amino acid propyl chloroformates by correcting the propyl group.

Flow cytometry

A 1 mL of lake water was stored in 100 μL of 1.6% paraformaldehyde solution at −80°C to measure algal cell density in bottles. Cell density was measured from 300 μL of sample water by Guava® easyCyteTM HT flow cytometer (Luminex®). Forward and side scatters (FSC and SSC, respectively) at the red-blue fluorescence wavelength (ex = 488 nm, em = 500 nm) were used for fluorescence detection. The main parameter for counting events was sided scatter, and the second parameter was red fluorescence. Channels were deployed in the logarithmic mode and used threshold level for red fluorescence was 10. The sample was acquired for 3 min or until 10,000 events were detected.

Statistical analyses

Due to the unequal variances, $\log_{10}$ transformed data of osmomixotrophic, phagomixotrophic and *Gonyostomum semen* were tested using the non-parametric Welch ANOVA and Dunnet's T3 as a post hoc test. The comparison was made between two total phosphorus categories and three DOC categories. The comparison was conducted using IBM SPSS (version 28.0.1.1; IBM 2021) software.

Rest of statistical analysis was done using software Primer 7.0.21 (Primer-E). We tested for differences in the average cell density, concentration of CO_2, PLFA biomass and ^{13}C-enrichment of fatty acids among treatments with three univariate analyses of variance (ANOVA), that is one ANOVA for each of the three response variables. The ANOVA was implemented with permutational multivariate analysis of variance (PERMANOVA, main and pairwise tests; Anderson, 2001) after calculating Euclidean distances for each variable in turn. For a single variable and Euclidean distances, PERMANOVA gives the same test statistic (F-ratio) as traditional ANOVA (Anderson, 2001), but has the added advantage of calculating *p*-values via permutation, rather than assuming a distribution for the test statistic. Monte Carlo simulations were used for *p*-values due to the low number of replicates (Anderson & Robinson, 2003).

The comparison of $\delta^{13}C$ values and the contribution of monounsaturated fatty acids (14:0, 16:0, 18:0), monounsaturated fatty acids (16:1ω7, 18:1ω7 and 18:1ω9), ALA and SDA, and EPA and DHA in mixotrophic algae in experiment 1 was tested using multivariate analysis by PERMANOVA (Primer 7) analysis and Bray–Curtis similarity as a resemblance matrix.

Most common OTUs (117) with >0.5% average sequence abundance in the sequenced samples (control, beech leaves, lignin and polystyrene, $n = 3$ for each) were standardized (sample values were divided by the total for that sample), and the contribution of each OTU as % was used for statistical analysis. The comparison of microbial community structure among treatments was studied by using non-metric multidimensional scaling (nMDS) (Clarke, 1993), which for Bray–Curtis similarity matrix of % of OTU was used in Primer (Clarke & Gorley, 2015). The Spearman correlations were calculated between nMDS1 and OTU%, and nMDS2 and OTU%. The goodness of fit of the nMDS was measured using Kruskal's stress; low stress scores (<0.2) indicate that the nMDS is an adequate representation of the dissimilarity matrix (Clarke, 1993). Hierarchical cluster analysis was used to create similarity groups in nMDS.

RESULTS

Phytoplankton osmo- and phagomixotrophy in boreal lakes

The contribution of osmomixotrophic algae, phagomixotrophic algae or *Gonyostomum semen* did not differ between oligo-mesotrophic ($TP > 35\ \mu g\ P\ L^{-1}$) and eutrophic lakes ($TP \geq 35\ \mu g\ P\ L^{-1}$) (Welch ANOVA, $F_{2,\ 3563.433} = 60.5$, $p > 0.07$). The contribution of osmomixotrophs was greatest in the lakes with highest DOC ($DOC > 12\ mg\ C\ L^{-1}$) and then in lakes with medium DOC ($DOC = 6–12\ \mu g\ C\ L^{-1}$) and lowest in low DOC lakes ($DOC < 6\ \mu g\ C\ L^{-1}$, Welch ANOVA: $F_{2,\ 3563.433} = 60.5$, $p < 0.01$, Figure S1). In contrast, the contribution of phagomixotrophic algae (Welch ANOVA: $F_{2,\ 3821.281} = 392.8$, $p < 0.01$) and *Gonyostomum semen* (Welch ANOVA: $F_{2,\ 853.492} = 190.5$, $p < 0.01$) was lowest in the low DOC lakes and highest in high DOC lakes (Figure S1).

Mixotrophy experiment (Experiment I)

After 48 h, the cell density of *Cryptomonas* increased in the control and beech-leaf treatment with light (PERMANOVA: Pseudo-$F_{3,11}$ = 15.6, P(MC) = 0.001), but not in total darkness for 48 h (Pairwise PERMANOVA: t = 0.90, P(perm) = 0.498, p = 0.38, Figure S2a, Table S4). However, in both light and dark treatments, saturated fatty acids (SFA), 16 and 18 monosaturated fatty acids (16 MUFA), and linoleic acid (LIN), alpha-linolenic acid (ALA) and stearidonic acid (SDA) became equally ^{13}C-labelled from leave carbon sources (PERMANOVA: Pseudo-$F_{3,11}$ = 10–76.1, P(MC) < 0.002, Figure S2b). The corresponding origin of terrestrial carbon (from beech leaves) in fatty acids varied from 1 to 29% among fatty acids and did not differ statistically between light and dark treatments (PERMANOVA: Pseudo-$F_{1,5}$ = 3.0–6.0, P(MC) > 0.06, Figure S2c, Table S4).

Biodegradation of substrates (Experiment II)

After 14 days for leaves and lignin and 56 days for PS-MP, we quantified the mineralization and assimilation of substrate carbon into microbial biomass (Figure 1). Microbial respiration was highest in the leaf, followed by lignin treatment, whereas control and polystyrene treatments had equally low CO_2 production (PERMANOVA: Pseudo-$F_{3,11}$ = 470, P(MC) = 0.0001, Figure 2a). Similarly, respired CO_2 quickly became ^{13}C-enriched (PERMANOVA: Pseudo-$F_{3,11}$ = 694, P(MC) = 0.0001, Figure 2b, Table S3), whereas ^{13}C-labelling was slower in the polystyrene treatment ($\Delta_{\delta 13C}$ = 20 ± 18‰). Moreover, when polystyrene was incubated in a sterile algal medium for 28 days, ^{13}C-labelling of CO_2 was one-tenth of this ($\Delta_{\delta 13C}$ = 2.2 ± 0.9‰). Microbial biomass was higher in leaf treatment than in any other treatments based on bacterial phospholipid fatty acids (PERMANOVA: Pseudo-$F_{3,11}$ = 15.0, P(MC) = 0.002, Figure 2c). Moreover, high bacterial biomass multiplied biomass of mixotrophic algae in leaf treatment (PERMANOVA: Pseudo-$F_{3,11}$ = 29.7, P(MC) = 0.001, Figure S3).

Mineralization rate was slightly higher in lignin–hemicellulose than in leaf treatment (PERMANOVA: Pseudo-$F_{2,8}$ = 33.5, P(MC) = 0.002, Figure 2d) in whereas microbes assimilated 26 times higher rate carbon from lignin–hemicellulose than from leaves (PERMANOVA: Pseudo-$F_{3,11}$ = 138.7, P(MC) = 0.007) in their biomass. Mineralization and accumulation of PS carbon were much lower than from lignin or leaves. Microbes used

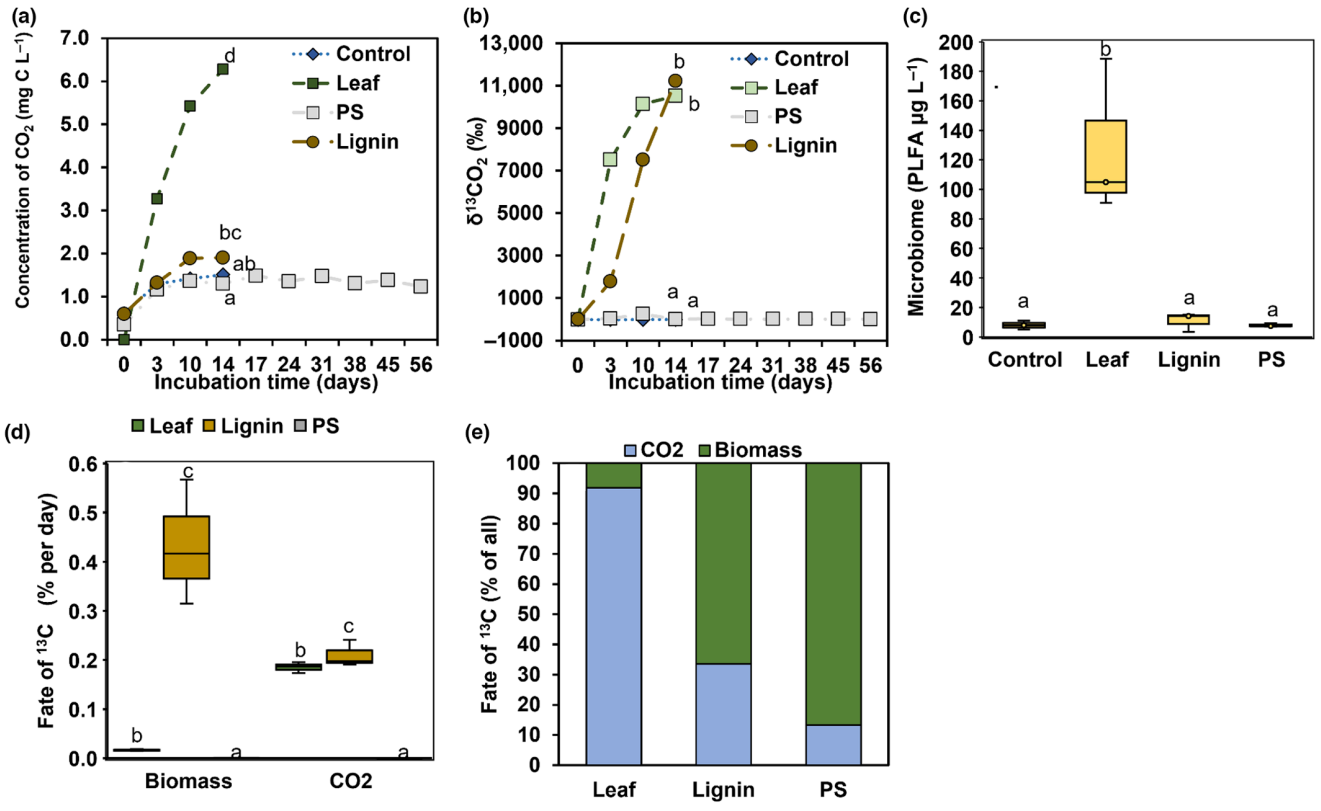

FIGURE 2 Microbial decomposition of leaves, lignin and polystyrene. (a) The CO_2 concentration during the incubations in control, beach leaves (leaf), lignin–hemicellulose (lignin) and polystyrene (PS) treatments. (b) Carbon isotope value (‰) of respired CO_2 in control and treatments. (c) Microbial biomass as PLFA in control and treatments. (d) The fate of carbon from ^{13}C-polymers into carbon dioxide (CO_2) and microbial biomass (PLFA) % per day and (e) normalized between carbon dioxide (CO_2) and microbial biomass. Different letters indicate statistically significant differences between treatments in the order d > c > b > a, p < 0.05.

organic carbon in leaves for energy production (CO_2 respiration; 92 ± 1.0% of all carbon; Figure 2d), and only a small contribution was used for biomass (8 ± 1.0%), whereas polystyrene was mainly used for building new biomass (87 ± 13%) and only a small fraction was used for energy (13 ± 13%). Most organic carbon in lignin–hemicellulose was used for biomass (66 ± 9%).

Response of microbial community structure to substrates

Sequencing of the 16S rRNA gene revealed that the most abundant bacteria belonged to Proteobacteria phyla in all treatments (Figure 3, Table S4). Microbial communities differed among treatments, and the microbial community in leaf treatment had the highest distance from microbial communities of other treatments. Uncultured Burkholderiaceae strains (Betaproteobacteria), *Limnohabitans* sp. (Gammaproteobacteria) and *Geobacter* sp. (Deltaproteobacteria) were characteristic bacteria in leaf samples, whereas Verrumicrobiaceae and Planctomycetes were more common in polystyrene than in any other treatment. *Leptothrix* sp. (also belongs to Burkholderiaceae) was more abundant in lignin than any other treatments (Figure 3). Sequencing did not indicate chemoautotrophic organisms like sulphur-oxidizing or ammonia-oxidizing (*Nitrosomonas*) bacteria but a low contribution (<2.0% of all OUT) of nitrating-oxidizing bacteria *Nitrospira* was found in PS treatment.

Trophic transfer of terrestrial and anthropogenic carbon in biomolecules to upper trophic levels (Experiment II)

Bulk carbon isotopes were not measured from seston due to the free ^{13}C particles disturbing measurements. Bulk carbon isotope measurement showed that *Daphnia* became ^{13}C-enriched in all treatments in relation to control group after 5 days of feeding on the microbiome and mixotrophic algae (Figure S4). This ^{13}C-labelling was similar also after 6 months incubation of substrates when a five-day experiment with *Daphnia* was repeated (Figure S4). Bulk carbon isotope measurement showed that ^{13}C-labelling of zebrafish was not as strong as *Daphnia* (e.g. ~6300‰ (*Daphnia*) vs. ~250‰ (Zebrafish) in lignin–hemicellulose treatment, Figure S4).

The ^{13}C-labelling of amino acids and fatty acids of the microbiome and mixotrophic algae were transferred via *Daphnia* up to fish even though ^{13}C-labelling was relatively low in polystyrene treatment (Figures S5, S6). In contrast, bacterial PLFAs did not become ^{13}C-labelled from polystyrene without lake microbial inoculum in algal sterile medium ($\Delta_{\delta 13C} = -2.3 \pm 0.4$‰). However, treatments differed in which amino or fatty acids were most ^{13}C-labelled (Figures S5, S6). For example, histidine and aspartic acid were only strongly ^{13}C-labelled amino acids in the polystyrene experiment (Figure S5d). Amino and fatty acids of mixotrophic algae (*Cryptomonas* sp.) also became ^{13}C-enriched and synthesized long-chain PUFA, EPA and DHA, from a ^{13}C-labelled backbone (Figure S6).

Our calculation based on bulk carbon isotopes showed that 27–72% of carbon in *Daphnia* originated from terrestrial leaves, whereas corresponding values were 6–10% and 0.1–0.2% for lignin–hemicellulose and polystyrene treatments, respectively (Figure S4b). After feeding zebrafish for 5 days with *Daphnia*, 0.9 ± 0.3% of bulk carbon originated from leaves. Corresponding percentages for lignin and polystyrene were 0.3 ± 0.15% and 0.001 ± 0.001%, respectively. We took into account the influence of gut content for assimilation percentages of amino acids and fatty acids in *Daphnia* and zebrafish. Diet influence in the gut was 5.52 ± 1.82% and 0.23 ± 0.96% from the total $\delta^{13}C$ value of diet for *Daphnia*, but negative for zebrafish (Table S2). Leaf's carbon consisted of up to 94% of single fatty acid of microbes, whereas corresponding values were 12.7% and 0.23% for lignin and polystyrene treatment. The contribution of the carbon backbone of leaf, lignin and polystyrene in amino acids and fatty acids was similar in fish and in *Daphnia*, however, in which leaf contributed three times more in single fatty acids than in any amino acid (Figure 4).

Our calculation of synthesized amino acids from leaf, lignin–hemicellulose and polystyrene by microbes showed similar synthesized amounts from leaf and lignin–hemicellulose. In contrast, mixotrophic algae prepared two times more amino acids from lignin–hemicellulose than from leaf carbon backbone (Figure 5). Moreover, the transfer of amino acids from the mixotrophic algae to *Daphnia* and from *Daphnia* to fish was four times higher in lignin–hemicellulose treatment than from leaf treatment. In terms of fatty acids, microbes and mixotrophic algae prepared two times more fatty acids from lignin–hemicellulose than from leaves. *Daphnia* and zebrafish contained four and 10 times more fatty acids prepared from lignin than leaf carbon backbone, respectively.

DISCUSSION

Microbes synthesized essential and non-essential amino acids and simple fatty acids from the ^{13}C-backbone of leaves, lignin and polystyrene. Our experimental microbiome also contained some heteronanoflagellates (HNF) that prepared essential fatty acids (LIN and ALA) and SDA from added substrates as previously found with polyethylene (Taipale et al., 2019). Therefore, it seems that HNF and protozoans, together with mixotrophic algae, are key links in the nutritional upgrading of the microbiome to upper trophic levels (Hiltunen et al., 2017;

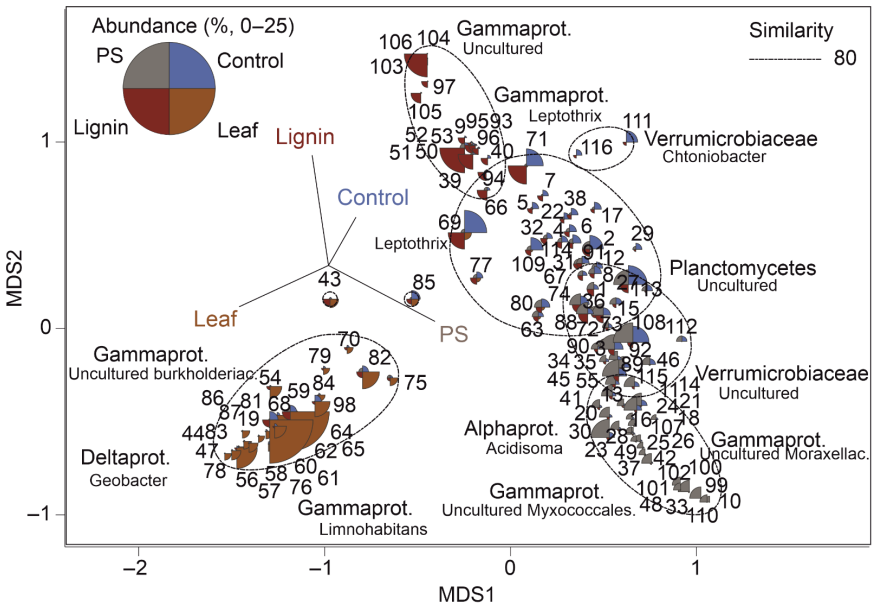

FIGURE 3 Response of microbial community structure to carbon input from leaf, lignin and polystyrene. (a) Non-metric multidimensional scaling plots of Bray–Curtis similarity of standardized and square root transformed OTU data (>0.5% of all rRNA sequences) at phylum and genera level (except Proteobacteria at class level) using averages of each treatment ($n=3$): control, leaf, lignin and polystyrene. Circles cite the 80% similarity according to the cluster analysis. The size of the bubble refers to the abundance of OTU. Numbers references OTU number (Table S1). Stress for 2 D MDS was 0.1.

Klein Breteler et al., 1999; Park et al., 2003). The added mixotrophic algae (*Cryptomonas* sp.) synthesized long-chain PUFA, EPA and DHA, from a ^{13}C of leaf, lignin or PS. The contribution of terrestrial origin of amino acids was higher for mixotrophic algae than in the microbiome, suggesting that algae used CO_2 directly to photosynthesis or used leaves extract (DOC) directly (osmotrophy) for preparing these amino acids. This coincides with the previous finding that terrestrial DOC can be converted into the biomass of mixotrophic algae (Tittel et al., 2009).

Our lake survey showed osmomixotrophic species to be more common than phagomixotrophic species in all type of DOC lakes. A previous study in temperate lakes showed an increase in mixotrophic species (chrysophytes, cryptophytes, dinoflagellaes and raphidophytes) along a DOC gradient (Senar et al., 2021). Therefore, it was surprising that in our study, phagomixotrophic species (cryptophytes, dinoflagellates, prymnesiophytes and certain species of golden algae) did not increase along a DOC gradient but were highest in low DOC lakes. However, we did not include *Gonyostomum semen*, which has been thought to use tricocyst to lyse cells and then use osmotrophy to take in the released nutrients (Guillard, 1975). This species benefits most from lake browning (Lebret et al., 2018; Lepistö et al., 1994), but due to its large size, zooplankton may have difficulties to feed on it (Johansson et al., 2013). Altogether, our results suggest that phagomixotrophy can be a more profitable trophic strategy in oligotrophy, as previously reported in marine environments (Fonseca et al., 2022; Granéli et al., 1999; Hartmann et al., 2012; Troost et al., 2005).

In our experiment, we used *Cryptomonas* sp. species which is common phytoplankton in boreal lakes. Meanwhile, our results showed that this phagomixotrophic alga could not grow in total darkness but could synthesize non-essential as well as essential fatty acids from terrestrial carbon (leaves) in total darkness. Since light and carbon can limit phytoplankton production in northern lakes (Bergström & Karlsson, 2019), it is beneficial for cryptophytes and other mixotrophic species to complete their autotrophic biomolecule production by heterotrophic processes (López et al., 2019). Our result suggests that most of the easily usable terrestrial carbon is respired by the microbes and does not become an integrated part of the microbial biomass. This is in accordance with the previous finding that only 7% of the carbon uptake from leaves enters the microbial food web (Attermeyer et al., 2013). The biomass of bacteria and mixotrophic algae was highest in leaf treatment; however, the added amount of leaf was 33 times higher in relation to added amount of lignin–hemicellulose or polystyrene, and thus, results of microbial biomass are not really comparable. Actually, calculation of accumulation rate was 26 times higher in lignin–hemicellulose than in leaf treatment. Previous experiments in the terrestrial ecosystem have shown that decomposers of lignin require a labile carbon source to facilitate mineralization (Klotzbücher et al., 2011). Therefore, the abundance of labile algal and terrestrial sources may promote the utilization of recalcitrant lignin and plastic in lakes (sensu 'priming') (Guenet et al., 2010). Since our lignin contained hemicellulose part, it is possible that more easily degradable hemicellulose supported also

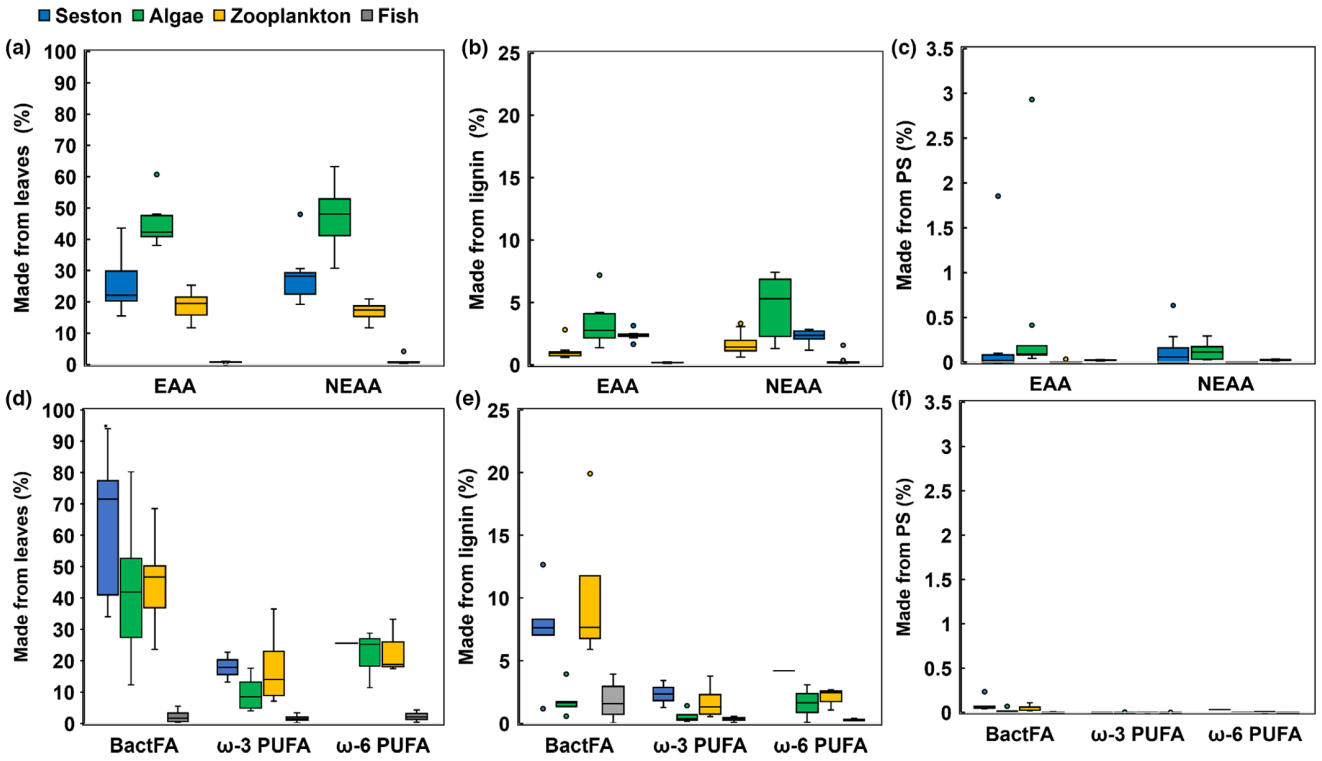

FIGURE 4 Utilization of carbon from leaves, lignin/hemicellulose, and polystyrene (PS) for amino acids and fatty acids by microbes and mixotrophic algae and their contribution to zooplankton and fish. (a–f) The contribution of beach leaves (leaf), lignin-hemicellulose, and polystyrene (PS) of all carbon in essential amino acids (EAA), non-essential amino acids (NEAA), bacterial fatty acids (BactFA), ω-3 and ω-6 polyunsaturated fatty acids in microbes (microbiome), mixotrophic algae (*Cryptomonas* sp.), zooplankton (*Daphnia magna*) and fish (*Danio rerio*).

biodegradation of more recalcitrant lignin (van Erven et al., 2019; Vesamäki et al., 2022). Our results showed that easily degradable organic compounds are mainly used for energy, whereas more recalcitrant materials are preferably used as a carbon backbone for the synthesis of new biomolecules.

Even though our results showed differences in microbial community structure among different substrates, these changes were relatively small and did not seem to influence carbon transfer to upper trophic levels as seen similar ^{13}C-labelling of fatty acids in microbes, algae and zooplankton. Our results showed that ^{13}C-labelled amino acids and fatty acids were transferred via *Daphnia* up to fish even though ^{13}C-labelling of fish was not so strong due to the short feeding period (5 days). Our result showed higher ^{13}C-labelling of some fatty acids than any amino acids. This was because certain bacterial fatty acids became heavily ^{13}C-labelled, while all amino acids are present in all micro-organisms. However, the contribution of leaf, lignin or PS carbon backbone in essential amino acids and fatty acids in zooplankton or fish was equal. This suggests consumer's equal assimilation of essential amino acids and fatty acids, which could have implications when calculating the origin of the consumer's diet using amino or fatty acid carbon isotopes.

Finally, we calculated the quantity of synthesized amino acids and fatty acids from leaf, lignin and polystyrene carbon sources by microbes and their transfer up to the fish (Figure 5). Surprisingly, even though the lake microbiome produced an equal amount of amino acids from leaves and lignin–hemicellulose, the transfer of amino acids from the microbiome to upper trophic levels was three times higher in the lignin–hemicellulose than in leaf treatment. Moreover, two times more fatty acids were synthesized per carbon from lignin–hemicellulose than from leaves resulting also higher content of fatty acids at all four trophic levels. Essential fatty acids were highly retained in organisms at each trophic level up to zooplankton in all treatments, as found in lake studies (Kainz et al., 2004), whereas lower retention was found for essential amino acids. This suggests that dietary essential amino acids were sufficiently available as zooplankton can efficiently retain amino acids from diets (Burian et al., 2018).

Ecological studies often aim at identifying interactions between terrestrial and aquatic ecosystems and allochthonous and autochthonous carbon as competitive resources. However, our study shows that terrestrial and anthropogenic carbon emerged as an inseparable part of autochthonous carbon in amino and fatty acids in HNF and mixotrophic algae. Therefore, the mixotrophic algal isotope signal in lakes contains already terrestrial influence making it difficult to define the origin of carbon in nature. Moreover, it seems that the fate of carbon may

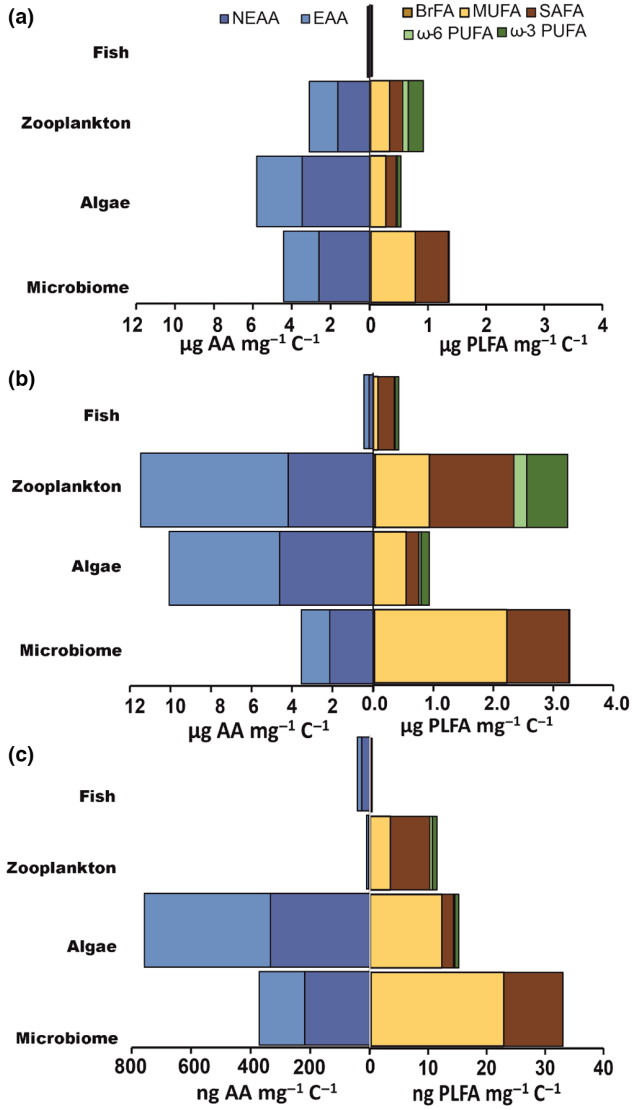

FIGURE 5 Synthesis and trophic transfer of essential fatty acids and amino acids in the four-trophic level experiment from terrestrial and plastic polymers (per µg AA or PLFA mg^{-1} C^{-1}). The produced and transferred amount of essential and non-essential fatty acids (blue) and, branched fatty acids (BrFA), monounsaturated fatty acids (MUFA), saturated fatty acids (SAFA), linoleic acid (LIN; 18:2w6) and long-chain ω-6 PUFA (LIN, ARA and DPA) and ω-3 PUFA (ALA, SDA, EPA and DHA) per mg of carbon of leaf (a), lignin/hemicellulose (b) and polystyrene (c).

vary constantly based on the availability of the overall quality of different organic carbon sources. Altogether, our experiments reveal that alternative carbon sources may complete the production of essential biomolecules and thus fuel consumers at various levels of the aquatic food web. This bottom-up process can ultimately affect species interaction, such as predator–prey relationships (Perga et al., 2008), by providing additional carbon that can increase consumer density beyond levels supported by primary production (Nakano & Murakami, 2001; Pace et al., 2004). By adding terrestrial and anthropogenic carbon sources to food-web models, we could predict the effects of these unusual carbon sources on species interaction and, consequently on population dynamics and ecosystem resilience.

AUTHOR CONTRIBUTIONS

S.J.T., C.R., and M.T. designed the study. S.J.T. and M.C. cultured phytoplankton and *Daphnia*. S.U-H. precultured fish (*Danio rerio*) and carried out the fish experiment. C.R. carried out experiments and analysed RNA from samples. S.J.T. analysed fatty acids and amino acids and run $\delta^{13}C$ from amino acids. M.P. and M.K. analysed $\delta^{13}C$ values of fatty acids. J.S.V. analysed DIC samples and run flow-cytometer analyses. K.V. analysed phytoplankton data of Finnish boreal lakes. S.J.T. wrote the initial draft of the paper. All authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Phytoplankton data are available at open-access interfaces for environmental data at the Finnish Environmental Centre (www.syke.fi), and fatty acid and amino acid data are available in Zenodo (https://doi.org/10.5281/zenodo.7856301).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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