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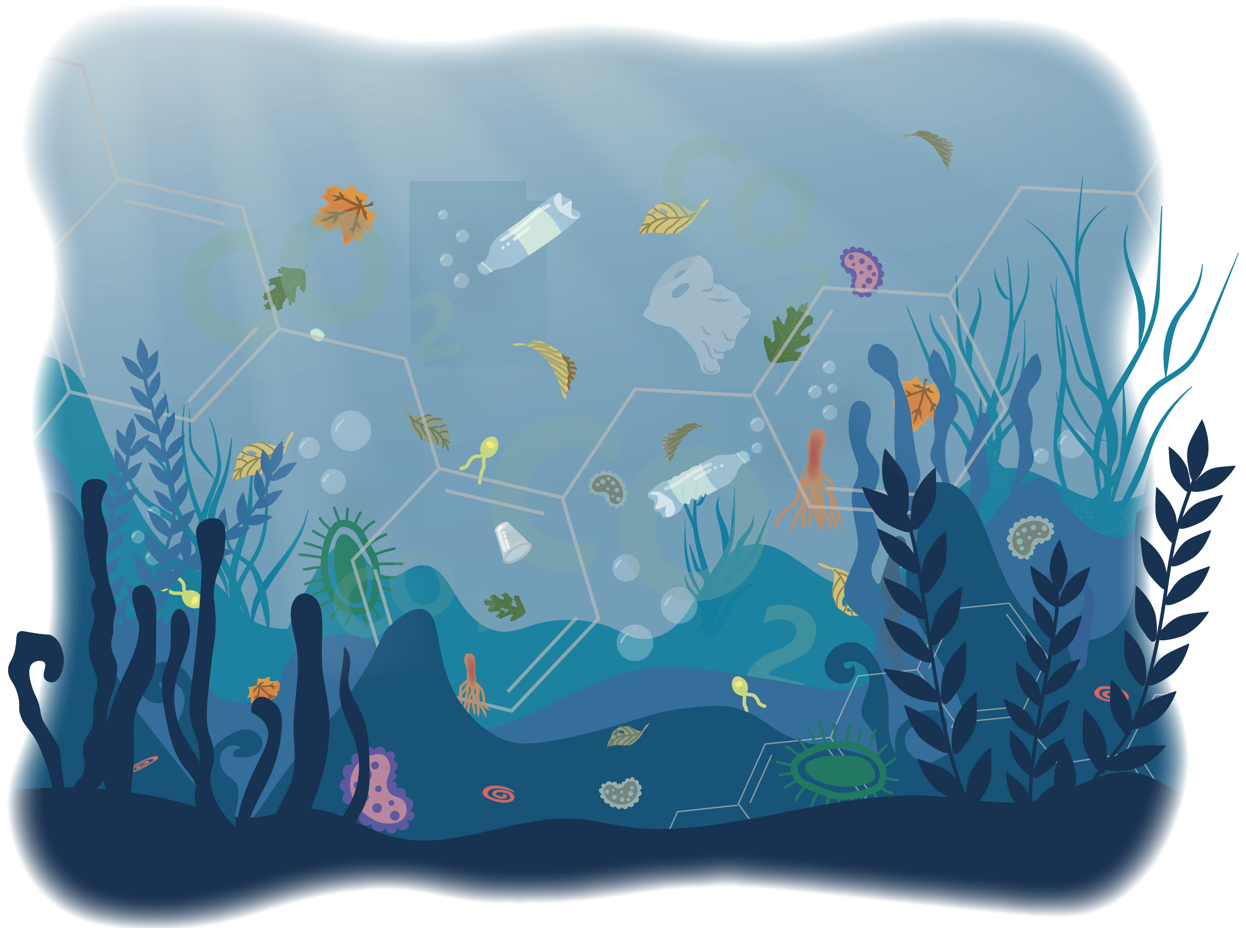
Jussi Vesamäki

Microbial Degradation of Terrestrial Organic Matter and Microplastics in Boreal Lakes



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS AND SCIENCE

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Jussi Vesamäki

Microbial Degradation of Terrestrial Organic Matter and Microplastics in Boreal Lakes

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Editors
Anssi Lensu
Department of Biological and Environmental Sciences, University of Jyväskylä
Ville Korkiakangas
Open Science Centre, University of Jyväskylä

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ABSTRACT

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Microbes are the main decomposers of organic matter in lakes, playing an important role as recyclers of elements and energy. Natural sources of organic matter include soil organic matter and plant litter, composed of natural polymers such as lignin and carbohydrates. Additionally, the increasing amount and high recalcitrance of plastics in the environment have become a concern worldwide. In this thesis, I study the microbial utilization of different substrates across the recalcitrance gradient (plant litter, hemicellulose, lignin, microplastics), carbon's biochemical fate, and seasonal variation. I also aim to identify the main microbial groups behind the decomposition. The biochemical fate of carbon was affected by the substrate's recalcitrance and chemical structure. The carbon from both recalcitrant and labile compounds was mainly respired to carbon dioxide, whereas a smaller proportion ended up in microbial biomass. However, microbial starvation led to the opposite result. Highly recalcitrant polystyrene carbon was more efficiently used for new biomass formation than carbon from labile leaves. Among environmental variables, particularly temperature controlled the decomposition rate and carbon cycling. The relative importance of assimilation in contrast to mineralization was higher in low temperatures. The effect of lake type on decomposition was unclear and contradictory. Labile carbon sources were utilized by a larger number of microbial groups than recalcitrant microplastics. Overall, natural substrates were efficiently utilized, and they subsidized microbial biomass production. In contrast, recalcitrant microplastics decomposed extremely slowly, suggesting their accumulation in lake ecosystems. The correlation of temperature with higher mineralization rates indicates that elevating temperature increases microbial mineralization of terrestrial polymers and thus further increases carbon emissions from lakes, whereas the proportion of terrestrial carbon bound to microbial biomass and aquatic ecosystems decreases.

Keywords: Lake; microbial decomposition; microplastic; mineralization; polymer; stable isotope; terrestrial organic matter.

Jussi Vesamäki, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

TIIVISTELMÄ

Vesamäki, Jussi
Terrestrisen orgaanisen aineksen ja mikromuovien mikrobiologinen hajotus boreaalisissa järvissä
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Mikrobit ovat pääasiallisia orgaanisen aineksen hajottajia järvissä, ollen tärkeässä roolissa aineiden ja energian kierron säätelijöinä. Luontaisia orgaanisen aineksen lähteitä ovat muun muassa maaperän orgaaninen aines ja karike, jotka muodostuvat luonnon polymeereistä kuten ligniinistä ja hiilihydraateista. Näiden lisäksi ympäristöön päätyvän muovijätteen määrä ja pysyvyys on herättänyt maailmanlaajuista huolta. Väitöskirjassani tutkin mikrobien kykyä hyödyntää hajoavuusasteeltaan erilaisia substraatteja (lehtikarike, ligniini, hemiselluloosa, mikromuovit), substraatin hiilen biokemiallista kohtaloa ja prosessien vuodenaikaisvaihtelua. Pyrin myös tunnistamaan hajotuksesta vastaavat olennaisimmat mikrobiryhmät. Hiilen kohtaloon vaikuttivat materiaalin hajoavuusaste ja kemiallinen rakenne. Substraatin hiili päätyi pääosin hiilidioksidiksi, kun taas pienempi osa hiilestä sitoutui biomassaan, joskin mikrobien nälkiintyminen ennen materiaalin lisäämistä aiheutti päinvastaisen tuloksen. Hankalasti hajotettavan polystyreenin hiili hyödynnettiin hieman tehokkaammin biomassan tuotantoon kuin lehtikarikkeen. Ympäristöolosuhteista erityisesti lämpötila sääteli hajotusprosessin nopeutta ja hiilen kiertoa. Biomassan tärkeys hiilen sijoituspaikkana suhteessa mineralisaatioon oli korkeampi matalissa lämpötiloissa. Järvityypin vaikutuksesta hajotukseen saatiin ristiriitaisia tuloksia. Helposti hajotettavia yhdisteitä hyödynsi laajempi kirjo eri mikrobiryhmiä kuin hankalasti hajotettavia mikromuoveja. Kaiken kaikkiaan luonnonmateriaalit hyödynnetään tehokkaasti ja ne tukevat mikrobibiomassan muodostamista. Sen sijaan äärimmäisen hidas mikromuovien hajotus järviekosysteemeissä johtanee niiden hiljattaiseen kertymiseen. Lämpötilan aiheuttama muutos substraatin hiilen kohtaloon viittaa siihen, että ilmastonmuutoksen myötä nousevat lämpötilat lisäävät mikrobihajotuksen seurauksena vapautuvan hiilen määrää, kun taas akvaattiseen ekosysteemiin jäävän hiilen osuus pienenee.

Avainsanat: Hajottajamikrobi; järvi; mikromuovi; mineralisaatio; polymeeri; terrestrinen orgaaninen aines; vakaa isotooppi.

Jussi Vesamäki, Jyväskylän yliopisto, Bio- ja ympäristötieteiden laitos PL 35, 40014 Jyväskylän yliopisto

Author's address Jussi Vesamäki
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
jussi.s.vesamaki@jyu.fi

Supervisors Associate professor Sami Taipale
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

University lecturer Riitta Nissinen
Department of Biology
FI-20014 University of Turku
Finland

Reviewers Leading researcher Hermanni Kaartokallio
Marine Research Centre,
Finnish Environment Institute
FI-00790 Helsinki
Finland

Senior researcher Nanna Hartmann
Department of Environmental Engineering
Technical University of Denmark
2800 Kgs. Lyngby
Denmark

Opponent Professor Andrew Tanentzap
Ecosystems and Global Change Group
School of the Environment
Trent University
Peterborough
Canada

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–III. The responsibilities and contributions of other authors have been explained in Table 1.

I Vesamäki J.S., Rigaud C., Litmanen J.J., Nissinen R., Taube R. & Taipale S.J. 2024. Recycled by leaf inhabitants: terrestrial bacteria drive the mineralization of organic matter in lake water. Submitted manuscript.

II Vesamäki J.S., Nissinen R., Kainz M.J., Pilecky M., Tiirola M. & Taipale S.J. 2022. Decomposition rate and biochemical fate of carbon from natural polymers and microplastics in boreal lakes. *Frontiers in Microbiology* 13, 1041242, doi.org/10.3389/fmicb.2022.1041242.

III Vesamäki J.S., Laine M.B., Nissinen R. & Taipale S.J. 2024. Plastic and terrestrial organic matter degradation by the humic lake microbiome continues throughout the seasons. Submitted manuscript.

TABLE 1 Contributions of all authors in the original papers. Author abbreviations: CR = Cyril Rigaud, JJL = Jaakko J. Litmanen, JSV = Jussi S. Vesamäki, MT = Marja Tiirola, MJK = Martin J. Kainz, MP = Matthias Pilecky, MBL = Miikka B. Laine, RN = Riitta Nissinen, RT = Robert Taube, SJT = Sami J. Taipale.

	I	II	III
Planning	SJT, JSV	SJT, JSV, RN	SJT, JSV, RN
Sampling in field	JSV, SJT	SJT	JSV
Carrying experiments	JSV	JSV	JSV
PLFA and sterol analyses	JSV, SJT	JSV	JSV, MBL
Greenhouse gas quantification	JSV	JSV	JSV
Stable isotope analyses	JSV	JSV, MJK, MK	JSV
Microbial community analyses	CR	JSV, RN, MT	JSV
QFASA	JJL, RT	-	-
Data analysis	JSV	JSV	JSV
First version of the manuscript	JSV	JSV	JSV
Manuscript revision	All	All	All
Correspondence	JSV	JSV	JSV

1 INTRODUCTION

1.1 Terrestrial carbon sources in boreal lake ecosystems

Phytoplankton and its exudates are the basis of lake food webs, offering an important source of carbon to aquatic consumers via binding atmospheric carbon photosynthetically into lake ecosystems (Wilken *et al.* 2018). In addition to the lake's primary production (often referred to as autochthonous carbon sources), lakes receive loads of terrestrial (known also as allochthonous) organic matter (t-OM) that subsidize aquatic carbon pools and food web. Allochthonous carbon enters the lake typically via soil organic matter (SOM) runoff or direct entry of plant litter (Attermeyer *et al.* 2013) (Fig. 1). SOM is produced in terrestrial ecosystems from recalcitrant residuals of decomposing plant litter, e.g., lignin and cellulose (Beyer 1996, Solomon *et al.* 2015, Danise *et al.* 2018). A remarkable proportion of SOM leaks into aquatic systems, where it contributes to aquatic carbon cycling (Solomon *et al.* 2015), emphasizing the importance of plant litter residuals to the aquatic carbon budget.

In addition to natural allochthonous carbon sources, varying concentrations (0.27–34000 particle m^{-3}) of microplastics (particle diameter < 5 mm) have been found in lakes worldwide, mostly as fibers (Dusaucy *et al.* 2021, Rebelein *et al.* 2021, Tanentzap *et al.* 2021). Plastic pollution has become a global concern due to continuously increasing plastic production and, consequently, plastic pollution. Particularly their ecotoxicology, the role as vectors of chemicals, bioaccumulation along the food web, and physical damage to aquatic organisms have gathered attention (Hartmann *et al.* 2017, Jovanović 2017, Guzzetti *et al.* 2018, Garcia *et al.* 2021). Plastic particles enter lake water via wind or water flow or directly via human impact and are concentrated in landfill and agricultural areas, snow dumping sites, harbors, and wastewater management plants (Uurasjärvi *et al.* 2020, Priya *et al.* 2022). After being introduced to the aquatic ecosystem, plastic particles can remain in the water column for a long time, accumulate organisms or sediments, and reduce the decomposition rate of organic matter (OM) (Dusaucy *et al.* 2021, D'Avignon *et al.* 2022, Welsh *et al.* 2022, Bertoli *et al.* 2023).

Despite their potentially harmful effects, plastic leachates can subsidize microbial biomass relatively more than t-OM and even plastic itself can be utilized as a carbon source (Sheridan *et al.* 2022, Taipale *et al.* 2023). Therefore, plastics represent a novel source of terrestrial organic matter for aquatic ecosystems. Although this point of view is recognized in riverine ecosystems (Hoellein *et al.* 2019, Vincent and Hoellein 2021), it has gathered less attention in freshwater lakes.

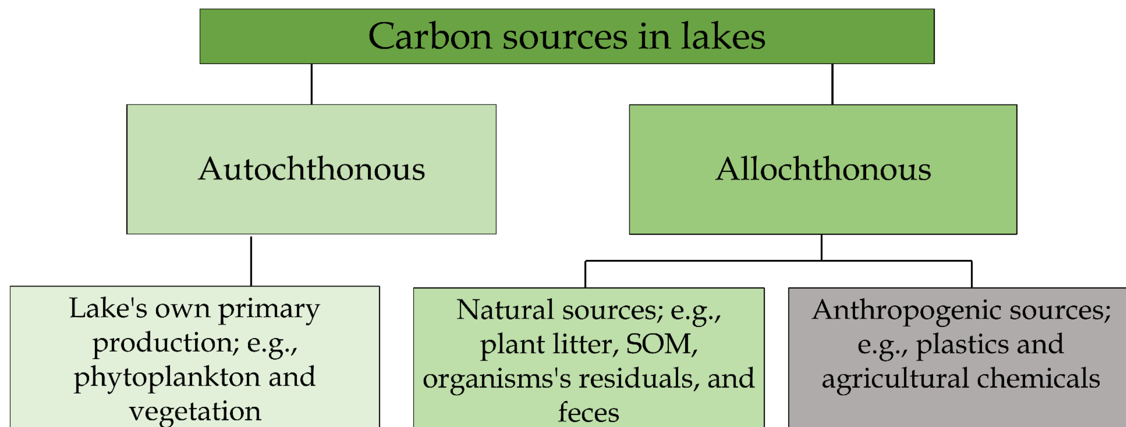

FIGURE 1 Classification of different carbon sources in lake ecosystems based on their origin. Together all these organic carbon sources from different origins (autochthonous, natural allochthonous, and synthetic allochthonous carbon sources) form a lake's carbon pool and play a role in aquatic carbon cycling.

1.2 Impacts of terrestrial organic matter on lakes

Being an important source of carbon, nutrients, and energy for aquatic organisms, t-OM subsidizes the aquatic food web and affects the carbon cycling in the whole lake ecosystem. Effects of t-OM input target abiotic conditions (water chemistry and light availability) and biotic factors. The input of t-OM affects water chemistry e.g. by lowering pH, increasing nutrient concentration and the amount of particulate and dissolved organic compounds, consequently increasing the brownish color of water and decreasing the light penetration into a lake (Hessen 1998, Tank *et al.* 2010, Toming *et al.* 2013). Biotic factors affected by t-OM input influence both primary production and consumption. These include e.g. algal blooms as a consequence of increased nutrient availability (Anderson *et al.* 2002) and changes in microbial community structure, microbial activity, and biomass production (Wardle 1993, Jansson *et al.* 2008, Attermeyer *et al.* 2013, Taube *et al.* 2018). The magnitude of the effects depends on lake morphometry, nutrient content, and water clarity. For instance, large clear water lakes rely on t-OM input less than small, shallow humic lakes, where the

significance of t-OM input increases since it covers a larger proportion of the whole lake carbon budget in relation to lake volume (Pace *et al.* 2007).

Typically t-OM input promotes heterotrophy and therein affects the carbon balance of the lake (Sobek *et al.* 2006, Jansson *et al.* 2008, Ask *et al.* 2009). If the heterotrophy exceeds primary production, the ecosystem turns into a net source of greenhouse gases, contributing the global climate change (Berggren *et al.* 2012). Indeed, humic lakes (defined often as DOC > 10 mg l^{-1}) (Seekell *et al.* 2015), that are rich in OM, are mainly heterotrophic (Sobek *et al.* 2006, Jansson *et al.* 2008, Ask *et al.* 2009). Humic lakes are common in the boreal zone and the most abundant lake type in Finland over clear water lakes (DOC < 10 mg l^{-1}) (Kortelainen 1993, Kortelainen 1999, Rantakari *et al.* 2004). They are characterized by brownish water, low pH, and high content of nitrogen, phosphorus, iron, and recalcitrant humic compounds originating from the t-OM (Kortelainen 1993, Nürnberg and Shaw 1998, Rantakari *et al.* 2004, Roth *et al.* 2014, Brett *et al.* 2017). Climate change is predicted to enhance the flow of organic matter from terrestrial ecosystems into lakes, causing brownification and increasing the proportion of humic lakes in relation to clear water lakes (Williamson *et al.* 2020, Blanchet *et al.* 2022), highlighting the importance of humic lake ecosystems in global carbon cycling. Additionally, fish from humic lakes are nutritionally poorer than clear lake fish, and thus, consequently, brownification has potential effects on human nutrition, health, and economy (Strandberg *et al.* 2016, Taipale *et al.* 2016a). Additionally, carbon cycle processes in humic lakes may indicate the future state of current clear water lakes, therein predicting the future and likely attracting more both scientific and economical interest as a dominant lake type.

1.3 Microbial decomposition of organic matter and polymers

1.3.1 Microbial decomposition process and the fate of carbon

Microbes are the major decomposers of plant litter over shredders in lakes (Raposeiro *et al.* 2017, DeGasparro *et al.* 2020), playing a significant role in transferring carbon from t-OM to upper trophic levels (Taipale *et al.* 2023). Particularly in humic lakes, the microbially transferred terrestrial carbon may form a large proportion of total carbon content at the upper trophic levels (Münster *et al.* 1999, Jonsson *et al.* 2001). In addition to the labile t-OM, the lake microbiome can bring carbon available for aquatic consumers from highly recalcitrant microplastic and polymers, linking terrestrial carbon from varying substrates into the aquatic food web (Taipale *et al.* 2023).

After entering the lake ecosystem, the substrate faces microbial colonization and decomposition (Simon *et al.* 2002, Krevš *et al.* 2017). Leachates and low molecular weight compounds can directly be taken up into microbial cells, whereas larger and potentially more recalcitrant molecules are digested by extracellular enzymatic degradation and by endocytosis (Sánchez 2020, Yuan *et al.* 2020, Liu *et al.* 2021, Priya *et al.* 2022). Produced oligo-, di- or monomers can be

further taken up into microbial cells where they are processed intracellularly and mineralized to CO_2 or CH_4 or used for anabolic processes (Fig. 2) (Yuan *et al.* 2020, Du *et al.* 2021, Liu *et al.* 2021, Priya *et al.* 2022, Sun *et al.* 2023). Mineralized carbon can be released into the surrounding water in an inorganic form, where it further can be returned to the atmosphere or bound via aquatic primary production. Via assimilation into the microbial cell and its structures, carbon can be linked to an aquatic food web (Attermeyer *et al.* 2013, Scharnweber *et al.* 2014, Taipale *et al.* 2023). Therein, microbes determine not only the decomposition rate of a given substrate but also the biogeochemical fate of its carbon, playing a key role in terrestrial carbon recycling in a lake ecosystem.

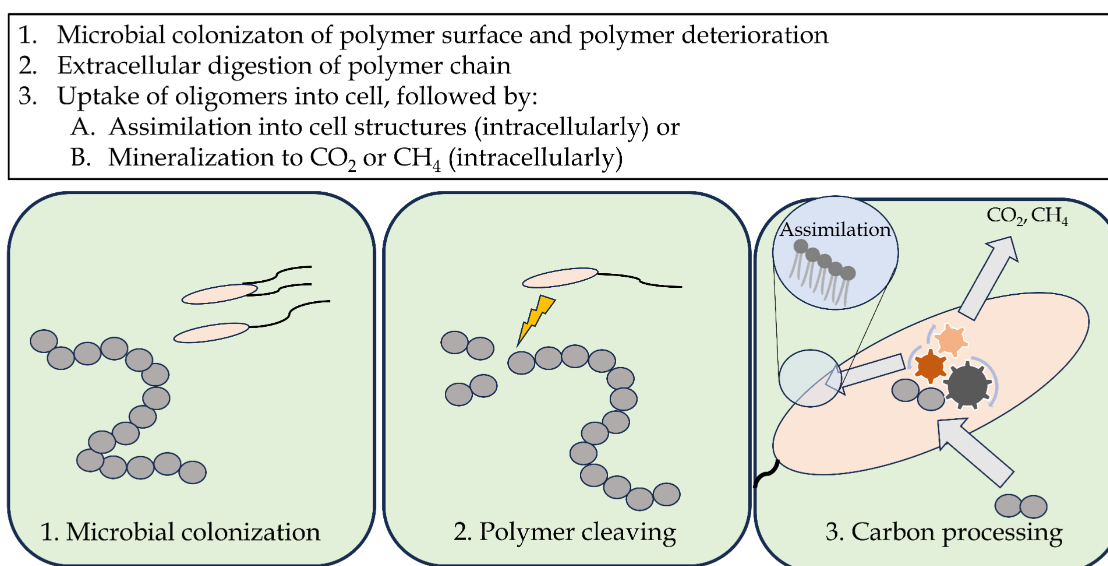

FIGURE 2 Schematic illustration of the polymer degradation process and carbon utilization. Step 1: Microbes colonize the substrate's surface. Step 2: Microbes secrete extracellular metabolites that catalyze polymer oxidation, hydrolysis, and the formation of oligo-, di-, and monomers. Step 3: Microbes take up small oligo-, di-, and monomers into the cell, where they further assimilate the carbon into the structural compounds of the cell (e.g., PLFAs, AAs, sterols) or mineralize the polymer carbon into CO_2 or CH_4.

1.3.2 Plant litter and its compounds as a carbon source

Among different plant litter types (e.g., leaves, twigs, bark), leaves are one of the most studied sources of t-OM in aquatic systems. Leaves contain a varying mixture of polymers and biomolecules. Rapidly after leaves enter lake water, leaching of water-dissolving compounds starts. Leachates cover approximately 7 % of leaf mass and can directly be utilized by aquatic microorganisms, whereas the remaining proportion of leaf mass is exposed to decomposition by aquatic organisms, particularly microbes (Attermeyer *et al.* 2013). Leaf chemistry has direct effects on its decomposition since decomposers favor nutrient-rich leaves. Particularly C:N ratio, lignin, and nitrogen content are considered to affect the leaf litter decomposition process (Cepáková and Frouz 2015). Moreover, leaf

chemistry affects the microbiome living in and on the leaf. For instance, C:N:P stoichiometry influences directly the dynamics of bacteria and fungi – changing C:N:P during the decomposition process leads to changes in the ratio of bacteria in relation to fungi (Danger *et al.* 2016).

The chemical composition of leaves varies highly between species not only in their carbon, nitrogen, and phosphorus content but also the concentrations of lignin, cellulose, and hemicellulose, which together form a majority of leaf biomass (Lynd *et al.* 2002, Hättenschwiler *et al.* 2008, Brett *et al.* 2017). Across different plant litter tissues, cellulose is the main component of plant tissue matrix, ranging from 35 to 50 % of dry weight, and is followed by contents of hemicellulose (from 20 to 35 % of dry weight) and lignin (from 5 to 30 % of dry weight) (Lynd *et al.* 2002). In leaves, the proportion of cellulose is lower, covering 10 ± 3 % of leaf biomass among broadleaf tree species (Kern *et al.* 2022), whereas lignin covers 22 ± 14 % of leaf litter dry mass (Rahman *et al.* 2013), forming a significant part of total plant litter mass, although highly varying between tree species. Particularly lignin and cellulose are important to an aquatic ecosystem, since they are the main precursors of humus formation (Danise *et al.* 2018), and thus play a significant role in affecting water chemistry and its humic content.

Lignin is highly recalcitrant against degradation and is slowly mineralized in humic waters (Vähätalo *et al.* 1999). Its degradation is affected by photodegradation, nitrogen concentration, and labile organic carbon source availability (Klotzbücher *et al.* 2011, Rahman *et al.* 2013). Therein, lignin from plant litter with high nitrogen content is more likely depolymerized and used as a carbon source. In addition to abiotic degradation, lignin faces biotic depolymerization by microbes and invertebrates (Rahman *et al.* 2013). Decomposition is mainly initialized by white-rot fungi belonging to Basidiomycota, but also some bacterial taxa, e.g., Spirochetes and *Clostridium* sp., have been associated with lignin decomposition (Rahman *et al.* 2013, Song *et al.* 2019). Contradictory observations have been made in wetlands, where increasing fungal biomass was linked to decreased decomposition of lignin and cellulose (Zhang *et al.* 2018).

As a labile carbon source, hemicellulose is consumed faster than cellulose and lignin (Ágoston-Szabó and Dinka 2008). Bacteria dominate both in cellulose and hemicellulose decomposition over fungi (Torres *et al.* 2014). Indeed, several bacterial genera have been identified with an enzymatic potential to decompose cellulose and hemicellulose (López-Mondéjar *et al.* 2016). In contrast to lignin decomposers, cellulose, and hemicellulose decomposers are considered less substrate specific, and they are proposed to utilize a wide range of polysaccharides as their carbon source (Eichlerová *et al.* 2015, López-Mondéjar *et al.* 2016).

1.3.3 Microplastic degradation

Polyethylene (PE) is one of the most common microplastic types found in lakes, followed by polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) (Uurasjärvi *et al.* 2020, Dusaucy *et al.* 2021). The degradation process of plastics is

typically initialized by photodegradation, and further accelerated by physical degradation mechanisms and biological degradation (Chamas *et al.* 2020, Ali *et al.* 2021, Priya *et al.* 2022). Both plastic's physical properties and chemical composition affect their toxicity (Monikh *et al.* 2022) and decomposition process (Debroas *et al.* 2017). Heteroatomic microplastics, like nylon and polyvinyl, are more prone to degradation due to their reactive groups and more diverse structure (Debroas *et al.* 2017). For instance, freshwater fungi growing on plastic particles were able to degrade aromatic and nitrogen-involving polyurethane but not aliphatic polyethylene (Brunner *et al.* 2018). Microbes play a key role not only by detoxifying plastic additives (Taipale *et al.* 2019) but also by catalyzing the decomposition process via exoenzyme production (Priya *et al.* 2022). Indeed, across various ecosystems, several bacterial (e.g., *Ideonella sakaiensis*, *Bacillus cereus*, and *Bacillus gottheilii*) and fungal species (e.g., *Aspergillus cereus*, *A. sydowii,* and *Rhodotorula mugilacinosa*) have been identified as microplastic decomposers (Yoshida *et al.* 2016, Auta *et al.* 2017, Sangale *et al.* 2019, Vaksmaa *et al.* 2023). Although the potential of fungi to degrade microplastics has been recognized (Sangale *et al.* 2019, Sánchez 2020) the role of fungi in the decomposition of microplastics in freshwaters is still much less known than the role of bacteria which is partly explained by the lack of fungal sequence data in databases.

1.3.4 Organic matter decomposers and the community origin

Linking a microbial taxon to a specific function sheds light on the role of microbes in a given environment. However, due to limitations in sequence databases and interactions between microbial groups, identification of the taxon-specific functions is challenging. Even at the kingdom level, the separation of roles can be difficult due to cross-kingdom interactions. For instance, bacteria are known to dominate over fungi during litter decomposition (Mille-Lindblom and Tranvik 2003). However, it has been suggested that bacteria benefit the production of fungal enzymes and can utilize byproducts of the decomposition, whereas their contribution to the decomposition itself may be lower in comparison to fungi (Cline and Zak 2015, Purahong *et al.* 2016, Zhan *et al.* 2021). Bacteria are also known to restrict both the growth of fungi and their enzymatic production, therein controlling the amount of low molecular weight molecules that are produced as a byproduct of the leaf decomposition (Mille-Lindblom and Tranvik 2003). Thus, although not necessarily driving the decomposition themselves, they play a major role as a controller of the decomposition process. This affects the carbon and nutrient cycling and aquatic food webs in lakes by controlling decomposition rates of OM and by lowering the nutritional value of biofilms, since fungi are nutritionally more valuable for aquatic consumers than bacteria and preferred by aquatic invertebrates (Bärlocher and Kendrick 1974, Danger *et al.* 2016).

1.4 Environmental factors affecting decomposition and carbon cycling

Environmental conditions determine the microbial community composition in lake waters, therein directly affecting the decomposition processes. For instance, in acidic waters, the fungal contribution to microbial community structure increases (Wurzbacher *et al.* 2010, Taube *et al.* 2018). Particularly in wetland lakes that are rich in organic matter content and have low pH, fungi are abundant (Wurzbacher *et al.* 2010). In addition to abiotic conditions, terrestrial microbes are an important link between terrestrial and aquatic ecosystems: as a vector of terrestrial microbes, t-OM entering the lake strengthens the connection of terrestrial and aquatic ecosystems to each other, linking a terrestrial microbial community to an integral part of the aquatic system. Indeed, a previous study indicated that the terrestrial microbiome of leaves was mainly responsible for leaf decomposition in streams (Jackrel *et al.* 2019), suggesting the importance of the terrestrial microbiome to the decomposition of OM in aquatic systems. The role and contribution of these microbial communities from aquatic versus terrestrial origin to the decomposition of t-OM and carbon cycling, however, has been understudied in lentic freshwater. In this thesis, the study I separates the roles of aquatic and terrestrial microbiomes during the early-stage decomposition process of leaves.

In addition to locally occurring environmental conditions, temporal changes also affect the decomposition processes and carbon cycling in aquatic systems. Seasonal changes alter the environmental conditions of the lake, affecting the whole ecosystem processes, including the decomposition of t-OM. In spring and summer, elevating temperature leads to a fast flux of carbon, nutrients, and energy, whereas in autumn and winter, decreasing temperature inhibits microbial activity. Although changing temperature is the driving force, changing nutrient and oxygen concentrations, light availability, and pH also affect the microbial activity and community structures, and consequently, the decomposition processes in lakes (Xie 2006, Shang *et al.* 2022). For instance, during winter, under-ice microbial decomposition of OM consumes oxygen, accumulating dissolved inorganic carbon and turning conditions more anaerobic and potentially inducing methanogenesis (Kirillin *et al.* 2012, Babanazarova *et al.* 2013). In summer, elevating temperature again increases both primary production and microbial processes.

The addition of t-OM varies seasonally as well. In autumn, huge input of leaf litter enters lake ecosystems as a result of leaf fall, offering new carbon, nutrient, and energy sources for aquatic consumers and microbes. Leaves are consumed at varying rates in different seasons. In a previous study, the microbial decomposition rate of litter was approximately 3-fold slower in winter than in summer although the mean water temperature was even 5-fold lower in winter than in summer (van Dokkum *et al.* 2002). This suggests that microbial activity is

not linearly decreased by temperature, and microbes are able to utilize labile OM sources in winter.

1.5 Advantages of SIA in tracing recalcitrant polymer decomposition

When measuring highly recalcitrant polymer degradation, traditional quantification via mass loss requires a long incubation time before changes in mass are detectable. Recalcitrant material degradation has been studied visually e.g. by size-exclusive microscopy that images changes in the surface structure. If exposed to decomposition, damage on the surface of a substrate should be detected. Imaging methods are commonly less quantitative, and thus a weak tool to determine decomposition rates. The use of stable isotope analysis (SIA) encounters both of these problems since the decomposition can be studied at the atomic level and the transfer of atoms from the substrate can be quantified (Balzer *et al.* 1997, Boschker and Middelburg 2002, Wilhelm *et al.* 2019, Twining *et al.* 2020, Taipale *et al.* 2022). For instance, ^{13}C-labelled microplastics have successfully been used to study degradation in mealworms and freshwaters (Yang *et al.* 2015, Taipale *et al.* 2019, 2023). Notably, stable isotope analysis can be used to track the biochemical pathways of carbon (Yang *et al.* 2015, Twining *et al.* 2020, Taipale *et al.* 2022), allowing the quantitative carbon cycle analysis and providing more information about the decomposition process. In addition, by combining stable isotope analysis with biomarker analysis, the identification of decomposers becomes possible (Boschker and Middelburg 2002, Twining *et al.* 2020).

2 THE AIMS OF THE THESIS

Substrate recalcitrance is defined as its resistance against decomposition: the slower the rate, the higher the substrate recalcitrance. In this thesis, 10 substrates across the recalcitrance gradient are studied, including plant litter of five plant species, hemicellulose, lignin, polystyrene, polypropylene, and polyethylene. The main aim of the thesis is to trace the endpoint of carbon from different substrates across recalcitrance gradient (labile plant litter, natural polymers, different types of microplastics) during the microbial decomposition process (Fig. 3). Decomposition rates, the biochemical fate of carbon, and decomposer communities were compared in lakes with varying environmental variables. Seasonal variation and its effects on decomposition rate and carbon cycling were studied to evaluate the microbial processes more comprehensively in changing conditions. Additionally, the aim was to identify the most important microbial decomposers by combining results from compound-specific isotope analysis (CSIA) of microbial phospholipid fatty acids (PLFAs) with microbial community analysis. The following study questions relating to substrate carbon cycling and decomposer taxa were asked:

1) Can freshwater microbes degrade plastics?
2) What was the main endpoint of carbon from different substrates across the recalcitrance gradient?
3) Do differences in leaf chemistry alter the biochemical fate of leaf carbon?
4) How do seasonal changes affect microbial carbon uptake and cycling from labile t-OM and microplastics?
5) Is the CSIA of PLFAs suitable for studying microbial decomposition processes and decomposer communities?
6) What are the most important microbial decomposer taxa of different substrates across the recalcitrance gradient?
7) Do the plant litter and microplastic decomposer communities change seasonally?

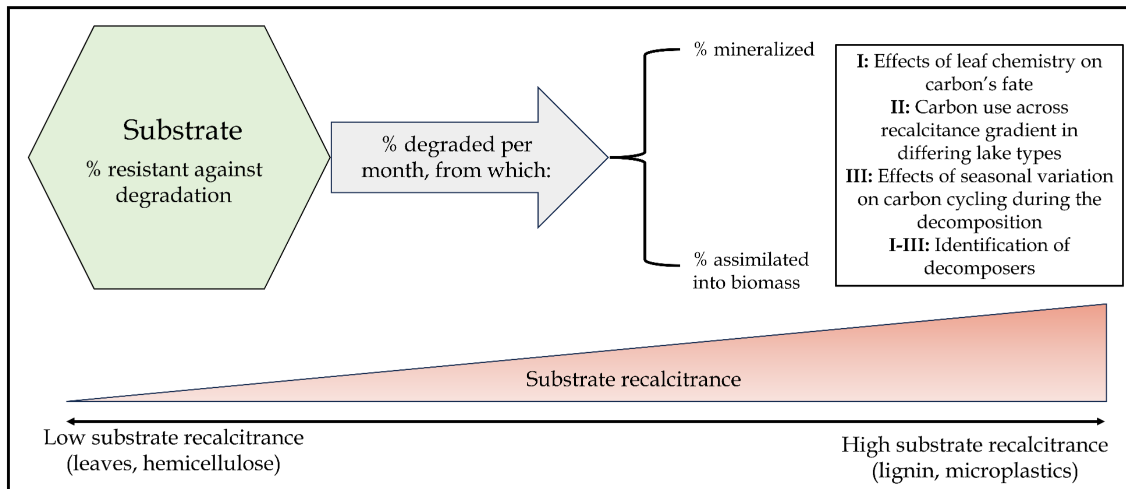

FIGURE 3 Graphical illustration of the main aims of this thesis.

3 MATERIALS AND METHODS

3.1 Study regions and experimental setups

To examine the effect of OM input on microbial community in lake water and the roles of aquatic versus terrestrial microbiomes in carbon cycling, unprocessed and autoclaved lake waters with different leaf species were incubated (I). Lake water and leaves were collected from lake Tuomiojärvi and its shoreline (WGS84: 62°25′50″N, 25°74′31″E; Jyväskylä, Finland) in October 2020. Collected waters were filtrated through a 3 µm pore size filter to remove bacterivores. Half of the collected lake water was autoclaved to separately examine the role of aquatic and terrestrial microbiomes. Autoclaved lake water had a slightly higher content of dissolved nitrogen, whereas pH and the concentrations of DOC and TIC did not differ between two lake waters. 300 ml of autoclaved or unprocessed lake water was added to 540 ml gastight glass bottles and alder, birch, or aspen leaves were added to bottles with autoclaved or unprocessed lake water. Control treatments (autoclaved and unprocessed lake water) had no leaf addition. Bottles were incubated at 17 °C in darkness for 21 days. Four replicates were made for leaves in unprocessed lake water and three replicates were made for controls and autoclaved lake water treatments.

Biochemical fate of carbon from substrates along recalcitrant gradient were studied by comparing the decomposition processes in clear and humic lake water (II). Lake waters were collected from lake Nimetön (WGS84: 61°22′82″N, 25°19′26″E; Evo, Finland) and lake Vesijärvi (WGS84: 61°22′82″N, 25°19′26″E; Lahti, Finland) in July 2020 for microplastic and lignin-hemicellulose treatments and in September 2020 for leaf treatments. Collected waters were filtrated through a 3 µm pore size filter to remove bacterivores and preincubated at 18°C for 3 weeks before the start of the experiment to let microbes consume most of the easily available carbon sources. After the preincubation, 300 ml of water was poured into a 540 ml glass bottle and 4 mg C of ^{13}C-substrate (PE (Polyethylene-$^{13}C2$, 99 atom% ^{13}C, Sigma-Aldrich, United States); PP (Polypropylene-1-^{13}C, 99 atom% ^{13}C, Sigma-Aldrich, United States); PS (Polystyrene-α-^{13}C, 99 atom% ^{13}C,

Sigma-Aldrich, United States); lignin-hemicellulose (U-^{13}C lignin organosolv from wheat (*Triticum aestivum*), 97 atom% ^{13}C, IsoLife bv, Netherlands), leaves (P-^{13}C Beech leaf (*Fagus sylvatica*), 13.4 atom% ^{13}C, IsoLife bv, Netherlands) was added. Used lignin-hemicellulose was composed of approximately 80 % of lignin and 20 % of carbohydrates, including mostly hemicellulose (van Erven *et al.* 2017). Control treatments had no substrate addition. Lake waters with added substrates were incubated at 18°C in closed glass bottles for three (leaves), or six (lignin-hemicellulose, microplastics, and controls without any substrate addition) week(s). Bottles were daily shaken during the experiment. Four replicates were made for each treatment.

To further expand the understanding considering microbial carbon utilization, seasonal variation of two plastic materials and plant litter was compared between four seasons in three humic lake waters (III). Lake waters were collected from three highly humic lakes called lake Haukijärvi (WGS84: 61°22′29″N, 25°13′79″E), lake Majajärvi (WGS84: 61°21′49″N, 25°13′68″E), and lake Nimetön (WGS84: 61°22′82″N, 25°19′26″E) in Evo (Hämeenlinna, Finland) in July 2021, October 2021, January 2022, and May 2022. Collected waters were filtrated through a 3 µm pore size filter to remove bacterivores after which 150 ml of lake water was poured into a 240 ml glass bottle and 2 mg C of ^{13}C-PE (Polyethylene-$^{13}C2$, 99 atom% ^{13}C, Sigma-Aldrich, USA), ^{13}C-PS (Polystyrene-α-^{13}C, 99 atom% ^{13}C, Sigma-Aldrich, USA), or ^{13}C-plant litter (*Typha latifolia*, 5.6 atom% ^{13}C, IsoLife bv, Netherlands) was added into lake water. Four experiments (one for each season) were conducted in three different humic lake water all of which had four treatments (PE, PS, and plant litter addition, and control without any substrate addition). Four replicates were made for each treatment. Bottles were incubated for four weeks at either 21 °C (summer), 8°C (autumn), 2 °C (winter), or 15°C (spring) and shaken daily. The total number of bottles was thus 48 per season (three lakes, four treatments, four replicates), and the total number of samples from all experiments was 192.

3.2 Lake water chemistry

Lake water pH was measured with PHM220 Lab pH Meter, MeterLab™. The device was calibrated using standard solutions at pH 4 and pH 7. DOC concentration in lake waters was measured by a Shimadzu TOC-V cph total organic carbon analyzer. For analysis, a 20 ml subsample of water was filtered (Sartorius 0.45 µm pore size) and 80 µl of 2 M HCl was added. A standard curve with known concentrations of carbon and nitrogen diluted with deionized H_2O was created for the quantification of DOC and DN.

The ascorbic acid method for quantification of phosphorus concentrations was performed according to the standard method (SFS 3026). 500 µl of 4M H_2SO_4 was added to a 50 ml filtrated lake water sample (Sartorius 0.45 µm pore size). Dissolved phosphorus was measured spectrometrically at 880 nm (Ordior UV-1800 Spectrophotometer, Shimadzu). DIC concentration was measured similarly

to the amount of CO_2 and is described in the next section and papers I–III. All measured water parameters of studied lake waters have been presented in Table 2.

TABLE 2 Averages and standard deviations of lake water parameters from studied lakes (below the lake name, sampling time has been addressed). For lakes Haukijärvi, Majajärvi, and Nimetön, only summer values are presented (III). n.d. = not determined.

	Tuomiojärvi 10/2020	Vesijärvi 7/2020	Haukijärvi 7/2021	Majajärvi 7/2021	Nimetön 7/2020 7/2021
DOC (mg l^{-1})	7.8 ± 0.1	5.2 ± 0.1	18.7 ± 1.1	24.7 ± 0.6	22.6 ± 0.19 26.0 ± 0.4
DIC (mg l^{-1})	0.8 ± 0.4	6.3 ± 0.2	3.0 ± 0.2	1.9 ± 0.4	1.8 ± 0.1 1.7 ± 0.1
DN (µg l^{-1})	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.3 0.7 ± 0.1
DP (µg l^{-1})	n.d.	6.7 ± n.d.	29.9 ± n.d.	31.9 ± n.d	27.8 ± n.d. 28.6 ± n.d.
pH	7.6 ± 0.0	7.8 ± 0.0	6.6 ± 0.3	5.8 ± 0.4	6.4 ± 0.1 5.6 ± 0.4

3.3 Microbial respiration and greenhouse gas production

Gas samples were collected from the air phase of the bottle to follow CO_2 and CH_4 production in bottles (Fig. 4). 5 ml of gas sample was transferred into an air-free Exetainer® tube after which the amount of CO_2 and CH_4 was determined by an Agilent 7890B gas chromatograph (Agilent Technologies, Palo Alto, CA, USA). Dissolved inorganic carbon (DIC) was analyzed by taking 5 ml of water into a He-flushed Exetainer® tube with 200 µl of 85 % H_3PO_4 (Taipale and Sonninen 2009). Water samples were mixed by a vortex and 5 ml of the gas phase was taken from the Exetainer® tube into a new tube. The gaseous DIC samples were further processed and analyzed identically to air phase samples. After conversion of the measured partial pressure to mass, concentrations were calculated by multiplying the mass of CO_2, DIC, and CH_4 with the phase volume (I–III). More detailed descriptions can be found in studies I-III.

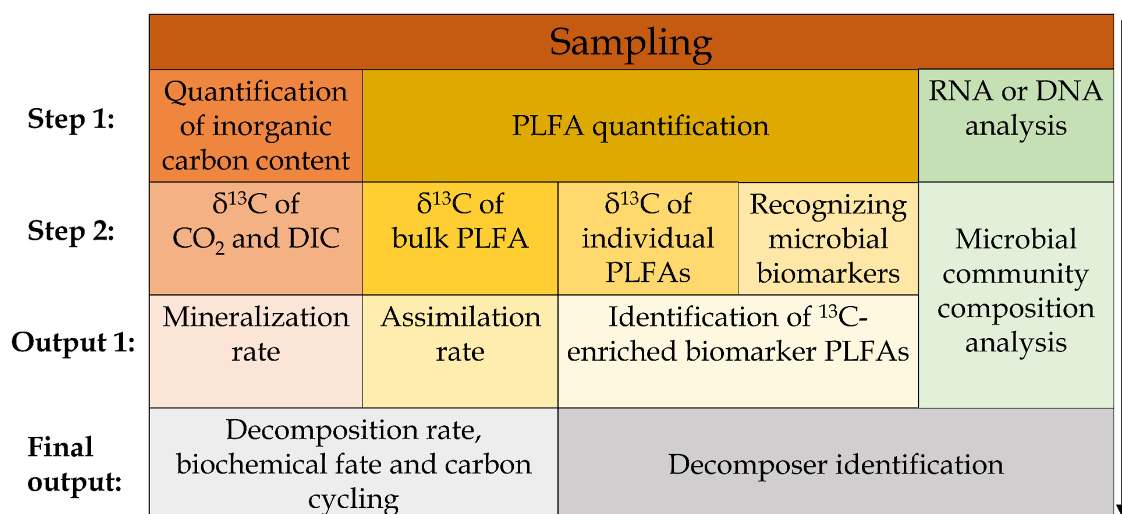

FIGURE 4 Presentation of main methods and steps used to answer the study questions of this thesis.

3.4 Quantifying biomass by PLFA and sterol analyses

Phospholipid fatty acids (PLFAs) and sterols can be used as microbial biomass indicators and biomarkers (Weete *et al.* 2010, Willers *et al.* 2015; Fig. 4). Based on PLFAs, the mass of selected microbial biomarkers is multiplied with a correction factor. In this thesis, the used correction factor was 25 since 4 % of microbial biomass is composed of PLFAs (Taipale *et al.* 2015). For fungi, ergosterol content correlates with fungal biomass and can thus be used to estimate total fungal biomass separately from bacteria (Djajakirana *et al.* 1996, Stahl and Parkin 1996). Thus, sterol profiles were determined to evaluate the participation of fungal taxa based on biomarker sterols (I), whereas PLFAs were used as biomarkers for both bacteria and fungi (I-III).

Samples for PLFA and sterol analyses were collected by filtrating sample water through a preweighed filter (Whatman™ cellulose nitrate filters, pore size 0.2 µm, diameter 47 mm). Lipids from freeze-dried filter papers were extracted according to Folch's method (Folch *et al.* 1957) using PLFA 19:0 (1,2-Dinonadecanoyl-sn-Glycero-3-phosphocholine) and 5-α-cholestane as internal standards. Extracted lipids were fractionated into neutral lipids (including sterols), glycolipids (including pigments), and polar lipids (including PLFAs), using solid phase extraction (SPE) columns (Bond Elut Silica cartridge). Neutral, glycol, and polar lipids were eluted with 8 ml of chloroform, acetone, and methanol, respectively.

Sterol fraction was treated with N,O-bis[trimethylsilyltrifluoro-acetamide] (BSTFA) with 1 % (w) trimethylchlorosilane (TMCS) and pyridine, and produced trimethylsilyl (TMS) derivatives of sterols were analyzed with a gas chromatograph equipped with a mass detector (GC-MS; Shimadzu)(I).

Calibration curves were created for individual sterols to quantify their concentration in samples. Standard solutions of plant sterol mixture from Larodan (Solna, Sweden; including β-sitosterol, stigmasterol, campesterol, brassicasterol), and fucosterol and cholesterol from Sigma-Aldrich were used as standards at four concentrations. Pearson correlation coefficient was confirmed to be >0.99 for each sterol-specific calibration curve. 5-α-cholestane (0.5044 mg/ml; Sigma-Aldrich) as an internal standard to calculate the recovery percentage for each sample.

PLFA fraction was methylated and analyzed with a gas chromatogram connected to a mass spectrometer (GC-MS; Shimadzu) (I-III). The FAMEs were identified by using specific target ions and their retention times (Taipale *et al.* 2016b). We applied four-point calibration curves based on a known standard solution of a FAME standard mixture (GLC standard mixture 566c, Nu-Chek Prep, Elysian, MN, USA) to calculate the concentrations for the individual FAMEs in the samples. The quantifications were made with GCMS solution software (v4.42, Shimadzu, Japan) and the Pearson correlation value of the calibration curves for each FAME in the standard mixture was >0.99. Recovery of PLFAs was corrected based on the internal standard and the amount of PLFAs in a sample was calculated as mg l^{-1} of carbon.

3.5 Stable isotope analyses

After the quantification of CO_2 and DIC in sample tubes, $\delta^{13}C$ values of CO_2 and DIC were analyzed using an Isoprime TraceGas pre-concentrator unit connected to an Isoprime IRMS (Isoprime100 IRMS, Elementar UK Ltd., Cheadle, UK) at the University of Jyväskylä, Finland. $\delta^{13}C$ values were drift corrected and two-point calibrated based on external standards.

The $\delta^{13}C$ of the bulk PLFA sample was measured from the subsample of the original PLFA sample. A subsample was collected after fractionation and transferred to a preweighed tin cup. After the subsample eluent had evaporated, the sample was measured with a Thermo Finnigan DELTAplusAdvantage CF-IRMS at the University of Jyväskylä, Finland. $\delta^{13}C$ values were drift-corrected based on external standards. Based on quantified $\delta^{13}C$ values of the bulk PLFA sample, we calculated the assimilation rate for each substrate, as described in Section 3.6.

CSIA of PLFAs was used to investigate the ^{13}C transfer into specific PLFAs, that can be used as biomarkers and associated with microbial groups, providing direct insights into the identities of active decomposers and substrate carbon utilizers (Boschker and Middelburg 2002). After quantifying PLFA content in samples on GC–MS, samples were evaporated under nitrogen flow and dissolved in 70 µl. Then, $\delta^{13}C$-values of individual PLFAs were analyzed using a GC-C TA III connected to an Isotope Ratio Mass Spectrometer (IRMS, DELTAPLUSXP, Thermo Co.) at the WasserCluster Lunz -Biological Station (Donau-Universität Krems, Austria) according to the protocol described by

Taipale *et al.* (2019) (II) or with a 5977B GC/MSD (Agilent) coupled with the Isoprime PrecisION (Elementar) isotope ratio mass spectrometer at the University of Jyväskylä (III). Samples were run against an internal standard 1,2-Dinonadecanoyl-sn-Glycero-3-Phosphatidylcholine (Larodan, $\delta^{13}C$ = –28.43‰) (II) or an external standard F8.3 (III) which were used for drift correction. Data was corrected by the $\delta^{13}C$-value of methanol used for transesterification (Twining *et al.* 2020). Only peaks whose height (nA) was > 0.015 were included in further analysis (III).

3.6 Calculations of decomposition rate, fate of carbon, and BGE

Measured and corrected $\delta^{13}C$ values of CO_2, DIC, bulk PLFA sample, and specific PLFAs were converted into atomic percentages (*AP*) (Fry 2006):

$$AP\ (\%) = \frac{(\delta^{13}\mathrm{C} + 1000)}{\left(\delta^{13}\mathrm{C} + 1000 + \frac{1000}{Rstd}\right)} * 100,$$

where $\delta^{13}C$ is the measured $\delta^{13}C$ value of a gas or bulk PLFA sample and R_{std} is 0.01118 (VPDB). Further, the transfer of ^{13}C from the substrate to CO_2 or PLFAs was determined as a difference between the *AP* value of the sample and the mean *AP* of the control:

$$\Delta\mathrm{AP_{PLFAs}}\ \text{or}\ \Delta\mathrm{AP_{CO2}} = \mathrm{AP_{sample}} - \mathrm{AP_{control}}.$$

Daily assimilation and mineralization rates were calculated as:

$$\text{Mineralization day}^{-1}(\%)\ = \frac{m_{\mathrm{CO2}} * \Delta\mathrm{AP_{CO2}}}{m_{\mathrm{added13C}} * t_{\mathrm{experiment}}} * 100\ \text{and}$$

$$\text{Assimilation day}^{-1}(\%)\ = \frac{m_{\mathrm{biomass}} * \Delta\mathrm{AP_{PLFAs}}}{m_{\mathrm{added13C}} * t_{\mathrm{experiment}}} * 100,$$

where m_{CO2} is the mass of carbon dioxide in the bottle (mg), $m_{biomass}$ is the microbial biomass (mg) in the bottle, $m_{added13C}$ is the mass (mg) of added ^{13}C-carbon, and $t_{experiment}$ is the duration of the incubation period (days). The decomposition rate of a substrate was calculated as the sum of mineralization and biomass assimilation rates:

$$\text{Decomposition day}^{-1}\ (\%) = \text{Mineralization day}^{-1} + \text{Assimilation day}^{-1}.$$

The utilization of the substrate carbon via anabolic processes (new microbial biomass formation) versus energy-producing pathways (respiration) was studied as a relation of assimilated carbon to total decomposition, often referred

to as bacterial growth efficiency (BGE) (del Giorgio and Cole 1998). BGE was calculated:

$$\text{BGE (\%)} = \frac{\text{Assimilation day}^{-1}}{\text{Decomposition day}^{-1}}.$$

3.7 Quantitative fatty acid analysis (QFASA)

Instead of assessing fungi:bacteria (F:B) ratio by single PLFAs, a PLFA-based mixing model has been proposed as an optional method to estimate the microbiome composition more comprehensively (Taube *et al.* 2018). Mixing model-based estimation requires a reference dataset (library) constructed from monoculture isolation experiments and sample data. In contrast to a Bayesian mixing model-based prey composition estimation method FASTAR (Galloway *et al.* 2015) that has been used to quantify fungal contribution to lake water microbiota (Taube *et al.* 2018, 2019), the numerical optimization mixing model-based QFASA (Iverson *et al.* 2004) has been shown to produce more accurate results compared to FASTAR (Guerrero and Rogers 2020, Litmanen *et al.* 2020). Thus, QFASA analysis was conducted to estimate the proportional contributions of bacterial and fungal groups (I).

The used library was applied from the same reference dataset that was used in a previous study examining the potential of using a Bayesian mixing model in the quantification of fungi from environmental samples (Taube *et al.* 2019). The library was analyzed by SIMPER in Primer 7 -software to identify the most suitable biomarkers for bacteria and fungi. Based on SIMPER analysis, we identified 18:2ω6, 18:1ω9, 18:3ω3, 18:3ω6, 16:0, and 18:0 as characteristic PLFAs to fungi, whereas PLFAs 18:1ω7, 16:1ω7, a15:0, sum of 16:1 (non7), i15:0, 14:0, and a17:0 were characteristic to bacteria. Together these PLFAs contributed >98 % of all differences between bacterial and fungal PLFA profiles. In addition, PLFA i14:0 was included in the analysis since it was found only in bacteria but not in fungi. Collected PLFA data was processed before estimation, and only PLFAs with a proportion >0.5 % were included in the estimation.

3.8 Microbial community analyses

Water for microbial community samples was filtrated through a filter with a pore size of 0.2 µm (Supor®0.2µm/25 mm, PES, Pall Corporation). Filters were immediately transferred into a bashing bead lysis tube with 800 µl of DNA/RNA Shield™ and stored at –80 °C. RNA (for bacterial communities) and DNA (for fungal communities) were extracted using a Chemagic™ 360 and the Chemagic™ Viral DNA/RNA 300 Kit H96 following the manufacturer's instructions (PerkinElmer, Waltham, MA, USA). RNA was treated with DNAse

and reverse transcribed to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The target region of the bacterial 16S SSU rRNA was amplified with polymerase chain reaction (PCR) using the primer pair 515F–806R (Caporaso *et al.* 2011, Parada *et al.* 2016), to which were added the M13 linker (to the forward primer 515F) and the P1 adapter (to the reverse primer 806R). The target region of the fungal internal transcribed spacer (ITS) was amplified with primers ITS7 and ITS4 similar to the PCR reaction with bacterial 16S sequences but using the genomic DNA as a template. Amplified sequences were tagged with the second PCR using forward primers with M13-tailed Ion Torrent™ barcodes. After the barcoding step, each sample was purified using the SparQ PureMag Beads (Quantabio, Beverly, MA, USA). Samples concentrations were measured using a Qubit fluorometer (Invitrogen/Thermo Fisher Scientific). Then, 10 ng of each sample was pooled together, and the pool was purified again. The pool quality and molarity were checked using a TapeStation 2200 and the High Sensitivity D1000 ScreenTape and reagents (Agilent). The sequencing was performed with the Ion Torrent Personal Genome Machine (Thermo Fisher Scientific) using the Ion PGM Hi-Q View OT2 400 kit, the Ion PGM Hi-Q View Sequencing kit (quality control included), and the Ion 318v2 chip. The sequences were then analyzed using the CLC Genomics Workbench software (CLC Bio, Qiagen). The primers were trimmed, and the short sequences were discarded. After the sequences were trimmed to the same length, the OTU clustering was performed using the SILVA 16S v132 database with a similarity percentage of 97% (I) or 99% (II, III).

3.9 Statistical analyses

Statistical analyses were conducted using the software Primer 7 (Primer-E). Differences in single variables (concentration of CO_2, CH_4, DIC, TIC, biomass, mineralization rate, assimilation rate, decomposition rate, bacterial growth efficiency (BGE), leaf mass loss, and the comparison of $\delta^{13}C$ values of each PLFA) were tested separately with univariate analysis of variance (ANOVA) applying permutational multivariate analysis of variance (PERMANOVA) on Euclidean distance matrices for single variables as described previously (Taipale *et al.* 2023).

OTUs with relative abundance > 0.5% of all detected 16S rRNA sequence reads were included for further analysis and the contribution of each OTU as % was used for statistical analysis. Differences in environmental parameters (III), PLFA profiles (I), and microbial community structures (I-III) were tested with permutational multivariate analysis of variance (PERMANOVA) after the square-root transformation of data and calculation of the Euclidean (environmental parameters) or Bray-Curtis (PLFA profiles, microbial communities) similarity matrices. Analysis of similarity percentages (SIMPER) was further conducted to identify microbial classes that increased their proportion as a consequence of substrate addition (I, III). Non-metric multidimensional analysis (nMDS) with hierarchical cluster analysis were

combined to analyze and visualize the clustering of treatments based on microbial community data and environmental variables (Clarke 1993). Monte Carlo's simulations were used for p-values as suggested for data with a low number of replicates (Anderson and Robinson 2003). Additionally, regression analyses were conducted to detect Pearson correlations between the relative abundance of microbial genera and the decomposition rate of PS (III). The used confidence level for all tests was 95 %.

4 RESULTS AND DISCUSSION

4.1 Recycling of labile and recalcitrant carbon in boreal lakes

During the decomposition processes, most of the decomposed plant litter and plastic carbon was respired (except in II) and released to the surrounding water column and the atmosphere, where it can again be reused by photosynthetic organisms, supporting findings of another study (Taipale *et al.* 2023). However, microbial starvation led to an opposite carbon distribution; when lake water was preincubated before starting experiments, carbon from both labile and recalcitrant polymers was mostly assimilated into biomass whereas a smaller part was used as an energy source (II). Although mineralization rates varied seasonally, substrate carbon was mainly respired in all studied lake waters throughout all seasons, whereas a minor part was assimilated into biomass (III).

In contrast to seasonally varying mineralization, the assimilation rate of plant litter and PS was equal throughout all seasons, suggesting that it is not as sensitive to changing environmental conditions as microbial respiration. From microbial biomass, carbon can further be utilized by aquatic consumers and nutritionally upgraded via the food web (Taipale *et al.* 2023), emphasizing the importance of the microbial link as a link between terrestrial ecosystems and aquatic food web. Moreover, decreasing temperature was shown to decrease the substrate mineralization, suggesting the higher importance of microbial assimilation in low temperatures. The support of recalcitrant polymer carbon to aquatic food web is higher in low temperatures, thus, in winter seasons and potentially in subarctic and arctic freshwaters. However, decomposition rates and carbon utilization pathways of carbon from studied substrates were influenced by environmental factors, substrate chemistry, and the experimental preparation.

4.1.1 Effects of experimental preparation on decomposition

The effects of experimental preparation are seen by comparing decomposition rates and carbon utilization pathways between studies (I–III). For instance, dry

leaf powder was decomposed and mineralized faster than wet leaves (II, III). Although decomposed and mineralized at a faster rate, powdered broadleaf cattail carbon was assimilated at a slower rate into biomass than carbon from deciduous tree leaves. Thus, results suggested that differences in moisture content and particle size affected plant litter decomposition processes, likewise in a previous study (Bani *et al.* 2018).

In one of the conducted studies, lake water was preincubated for three weeks in the laboratory in order to let microbes use labile carbon sources before substrate addition so that microbes would be more sensitive and "hungry" to new labile carbon sources (II). Indeed, the highest decomposition rate among studied plant litter types was observed when lake water was preincubated (beech leaves k = 0.75 ± 0.38 % per day). In addition to enhanced decomposition, lake water preincubation affected the biochemical fate of leaf litter. Without preincubation, the plant litter and microplastic carbon were mostly respired (I, III). After preincubation, the major proportion of carbon from all studied substrates (leaves, hemicellulose, lignin, PS, PP, PE) ended up in microbial biomass, whereas a minor proportion was respired (II). Unfortunately, due to different volumes of filtrated water for PLFA analysis, the variation in assimilation rates within treatments was high. Nevertheless, since the biochemical fate and decomposition rate of plant litter carbon in this study differ greatly in contrast to other studies (I, III), it seems likely that microbial starvation led to more efficient substrate utilization, exposing the true potential of microbial decomposition capacity.

4.1.2 Response of microbial biomass to substrate addition

The input of terrestrial plant litter into lake water enhanced microbial respiration and biomass production (I–III), confirming the current view that terrestrial leaves are an important source of carbon and energy for aquatic ecosystems under phytoplankton deficiency (Carpenter *et al.* 2005, Attermeyer *et al.* 2013, Scharnweber *et al.* 2014). Although not necessarily increasing microbial biomass itself, the support of recalcitrant polymers to microbiome can be seen as the amount of assimilated carbon during the decomposition; carbon from all studied substrates was shown to become assimilated into microbial biomass at varying rates across the recalcitrance gradient, and thus all substrates supported microbial biomass. However, although the formation of new microbial biomass formation was commonly accelerated as a consequence of plant litter addition, supporting the difference in comparison to the control microbiome was not always statistically significant: e.g., in Lake Majajärvi, other available carbon sources caused the diminished supporting effect of added leaves into lake water as a consequence of high DOC impulse in autumn, that has provided other labile carbon sources for lake microbiome (III). Thus, when other labile carbon sources such as increased SOM or leaf input (Singh *et al.* 2014, Kim *et al.* 2017) appears, the microbial response is not as strong as under carbon deficiency. Thus, leaf input is particularly important for microbes in lake waters under labile carbon deficiency, whereas its importance decreases when other resources are available.

This finding is also supported by the distinctively high decomposition rate of beech leaves that were consumed fast after the preincubation the lake water, making microbes to “starve” and more aggressively attach the added leaf material (II).

4.1.3 Decomposition rates and biochemical fate of carbon

Natural substrates were utilized faster than synthetic polymers (Table 3, Fig. 5). All studied plant litter materials (alder, aspen, birch, beech, and broadleaf cattail) were efficiently utilized by the freshwater microbiome, as shown in high daily decomposition rates ranging from 0.16 % to 0.75 % (at the temperature range of 15–18 °C) (Fig. 5). Hemicellulose was utilized at the fastest rate, reaching a daily decomposition rate of 2.33 ± 1.22 %. In contrast, lignin was more resistant to degradation, as observed previously in soils (Torres *et al.* 2014), and thus it likely contributes more to humus formation and sedimentation in freshwaters than labile hemicellulose. Microplastics decomposed extremely slowly, the decomposition process lasting from hundreds to tens of thousands of years. Due to the long-lasting decomposition process, they are likely eaten by aquatic organisms (Driscoll *et al.* 2021, Tanentzap *et al.* 2021) or end up to sediments where they contribute to lake’s carbon storage and are exposed to degradation of sediment microbes and benthic invertebrates (Bellasi *et al.* 2020, Dong *et al.* 2020). Notably, decomposition rates are very lake- and substrate-specific: decomposition rates and substrate carbon utilization pathways were affected by environmental factors and substrate recalcitrance. Recalcitrant polystyrene carbon was more efficiently utilized for biomass than labile litter carbon in humic lake waters (II, III), supporting another study (Taipale *et al.* 2023). However, due to the high variation of assimilation rates (II), this was not observed for all substrates across the recalcitrance gradient.

TABLE 3 Daily mean decomposition rates (%) of studied substrates across the temperature gradient (°C) in studied lake waters.

Substrate	2 °C	8 °C	15 °C	17 °C	18 °C	21 °C
Alder	-	-	-	0.27	-	-
Aspen	-	-	-	0.16	-	-
Beech	-	-	-	-	0.75	-
Birch	-	-	-	0.18	-	-
Cattail	0.20	0.28	0.4	-	-	0.77
Hemicellulose	-	-	-	-	2.33	-
Lignin	-	-	-	-	0.33	-
PS	0.000130	0.000197	0.000210	-	0.000404	0.000658
PP	-	-	-	-	0.000004	-
PE	0.000022	0.000032	0.000018	-	0.000197	0.000036

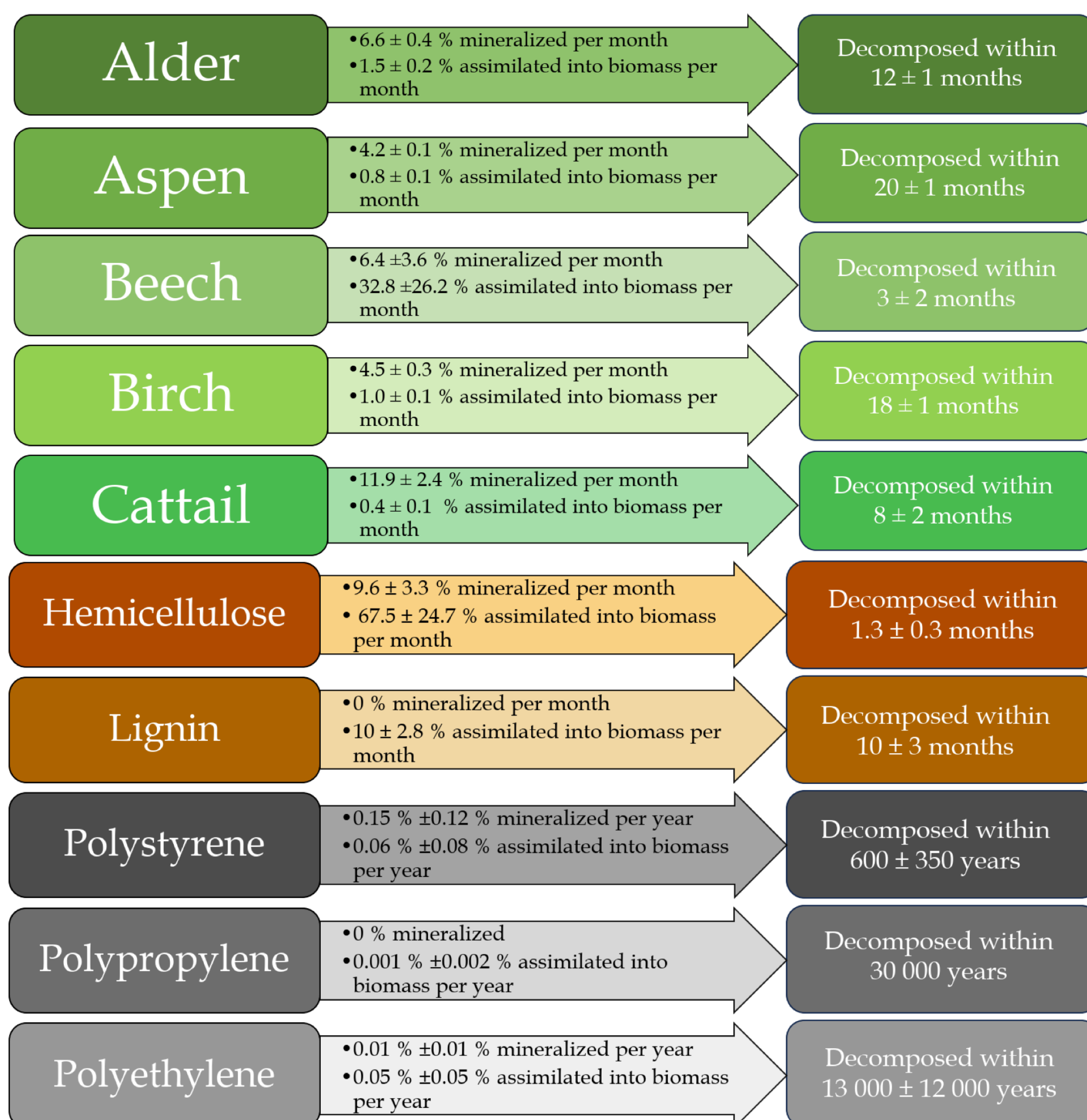

FIGURE 5 Averages and standard deviations decomposition, mineralization, and assimilation rates of five types of plant litters (temperature 15–17 °C), hemicellulose, lignin, PS, PP, and PE in boreal lakes (temperature 18–21 °C) in boreal lake waters. Note that estimated total decomposition times are calculated in more or less optimal temperatures (>15 °C) without any seasonal variation for better visualization of differences in decomposition processes between studied substrates. Thus, the real decomposition times are higher under natural conditions.

Litter chemistry is known to affect decomposition rate (Muto *et al.* 2011, Krevš *et al.* 2017) this was supported by the finding that nitrogen-rich alder leaves were utilized faster (daily carbon uptake rate k = 0.27 ± 0.02 %) than birch and aspen leaves (k = 0.18 ± 0.01 % and k = 0.16 ± 0.01 %, respectively) that have lower nitrogen concentration (Muto *et al.* 2011, Lotfiomran *et al.* 2016). In contrast to the importance of nitrogen on microbial decomposition, phosphorus is not as important (Mooshammer *et al.* 2012), and was relatively equal between leaf species (I, Muto *et al.* 2011). Thus, the faster decomposition rate of alder leaves

was associated with high N content, which also supported earlier studies that have found the high nitrogen content to accelerate the decomposition process (Muto *et al.* 2011, Mooshammer *et al.* 2012, Bani *et al.* 2018). Although leaf chemistry was confirmed to affect the decomposition rate, it did not affect carbon's fate; majority of plant litter carbon was mineralized (I, III) except under microbial starvation (II). Thus, independent of plant litter chemistry, microbes process leaf carbon by the same carbon pathways, using the majority of leaf carbon in energy-producing pathways and a minority for biomass building (I). In addition to CO_2, concentrations of CH_4 were shown to increase after leaf input (I). Particularly the addition of birch leaves led to higher CH_4 levels than the addition of alder or aspen leaves (I). Thus, the quality of organic matter, which is strongly affected by surrounding vegetation and climate (Tank *et al.* 2010, Kothawala *et al.* 2014), can potentially affect the molecular composition of carbon emissions from the lake ecosystems, but field experiments are needed to confirm this at the ecosystem level. Moreover, the rapid mineralization rate of terrestrial OM entering the lake ecosystem increases the release of greenhouse gases from the lake water, which could potentially turn the lake ecosystem temporarily into a net source of greenhouse gases and facilitate net heterotrophy after an annual leaf fall (Berggren *et al.* 2012). However, this is dependent on the autotrophy of a lake and its seasonal changes (Berggren *et al.* 2012, Laas *et al.* 2012).

Lignin and hemicellulose, which were introduced into lake waters as a mixture, were shown to be biodegradable at a relatively fast rate. Lignin carbon was only assimilated into biomass and not respired (Fig. 5) although slow lignin mineralization in freshwaters has been reported previously (Vähätalo *et al.* 1999, Taipale *et al.* 2023). The daily decomposition (thus, assimilation) rate of lignin was 0.33 ± 0.26 %. Low or absent mineralization suggests the high permanence of lignin subunits in aquatic system, where it can become a part of larger humic particles, affect water chemistry, and contribute to lake's carbon storages (Danise *et al.* 2018). Since labile carbon source co-occurring with lignin is known to accelerate lignin degradation (Klotzbücher *et al.* 2011), the observed lignin degradation rates are likely higher than without the presence of hemicellulose. The degradation-promoting effect of hemicellulose is likely explained by the positive response in microbial biomass since high microbial biomass is known to promote lignin carbon assimilation as well (Torres *et al.* 2014). In contrast to lignin carbon, hemicellulose carbon was both respired and assimilated into biomass. The daily decomposition rate of hemicellulose reached the rate of 2.33 ± 1.22 %, indicating faster utilization than any other of studied substrates (I-III). The assimilation and mineralization rates of hemicellulose carbon were 2.02 ± 1.23 % and 0.31 ± 0.08 % per day, respectively. As discussed in a previous section, results indicated higher plant litter carbon assimilation capacity under starvation for lake water microbiomes. Although microbial starvation is suggested to overestimate the microbial biomass as a carbon endpoint, results suggest the effective microbial utilization of carbon from hemicellulose and lignin. Via microbial decomposition, natural polymer carbon is effectively linked to an aquatic food web where it contributes to the whole aquatic carbon budget (Taipale *et al.* 2023).

In contrast to naturally produced polymers, synthetic plastics (PE, PP, and PS) were decomposed very slowly, with total decomposition time ranging from hundreds to tens of thousands of years. Different plastic types, however, were decomposed at varying rates. Aromatic and heteroatomic structures are known to enhance plastic degradation rate (Debroas *et al.* 2017). Indeed, aromatic PS was degraded faster than aliphatic PE and PP (II, III). PS carbon is more likely mineralized than used for biomass (III), but assimilation into biomass may become more important fate if microbes have consumed other carbon sources, as discussed above concerning plant litter decomposition (II). However, the importance of microbial biomass as a biochemical endpoint of PS carbon was observed also by Taipale *et al.* (2023) who found that PS carbon was mainly used for biomass building. However, they did not use closed bottles, which likely produced an underestimation of the mineralization rate. Nevertheless, in all these studies, varying proportions of PS carbon were shown to be assimilated into microbial biomass, thus revealing that even highly recalcitrant, synthetic polymer carbon supports microbial biomass. Notably, the assimilation rate was equal throughout seasons, suggesting that microbial assimilation of PS carbon is not influenced by changing environmental conditions. However, as a consequence of decreased microbial respiration and carbon mineralization in low temperatures, a relatively higher proportion of carbon ended up in microbial biomass. Therein, the importance of recalcitrant polymer carbon to aquatic food web is relatively higher in low temperatures and potentially in subarctic and arctic freshwaters.

As predicted, aliphatic PE and PP are highly resistant to degradation due to their simple and chemically strong structure, which are formed only by carbon and hydrogen atoms and lack functional groups. PP carbon was decomposed extremely slowly at the rate of 0.0000043 ± 0.0000062 % per day, from which most of the carbon was assimilated into biomass at the rate of 0.0000034 ± 0.0000056 % per day and mineralized at the average rate of 0.0000009 ± 0.0000012 % per day (II), showing extremely high resistance against microbial degradation (Fig. 5).

PE was decomposed at the rate of 0.000025 ± 0.000022 per day at a temperature of 21 °C (Table 3). PE carbon was assimilated into biomass at the daily rate of 0.0000002 ± 0.0000004 % and mineralized at the rate of 0.0000249 ± 0.0000217 % per day (III). However, after preincubation of lake water, degradation was faster at the temperature of 18 °C than observed at the higher temperature of 21 °C (III), and reached a daily rate of 0.00020 ± 0.00025 %, thus highly varying (II). Faster decomposition is partly explained by a preincubation of lake water, during which lake microbes utilized natural carbon sources to make them starve before the experiment began. This suggests that freshwater microbes are capable of using aliphatic microplastic carbon as their carbon sources, but if other more easily degradable sources are available, they utilize these at first.

Overall, degradation rates of aliphatic microplastics were much lower rates than measured previously (II, III; Taipale et al. 2019, 2022). This difference is potentially caused by the lack of initial chemical reaction, caused for example by UV-radiation that is known to accelerate the microplastic degradation by

oxidizing polymer and therein making it more prone to microbial digestion (Chamas *et al.* 2020, Ali *et al.* 2021, Priya *et al.* 2022). Indeed, results suggest that the used PE differed by its chemical structure before starting experiments. Two different batches of ^{13}C-PE (from the same manufacturer) were used for the summer and autumn seasons and winter and spring seasons, respectively (III). In the winter and spring seasons, the proportion of assimilated carbon from all decomposed PE carbon was higher than in summer and autumn, suggesting that these two batches differed chemically. Thus, ordered ^{13}C-PE may differ by its chemical structure (that is, oxidized or unoxidized polymer chain) between batches, resulting diverging decomposition rates and even biochemical distribution.

4.1.4 Effects of environmental factors on substrate degradation

The differences in decomposition processes between clear and humic lake waters were studied (II). In both lake waters, decomposed substrate carbon was mainly used in microbial biomass (Table 4, Fig. 6). However, carbon assimilation rates varied highly within treatments, and therein, the mineralization of substrate carbon was considered to be a better indicator in examining differences in decomposition processes between the two lake types. Plant litter, hemicellulose, and PS carbon were mineralized at a faster rate in humic than in the clear lake water, whereas lignin and PE were mineralized at the equal rate and PP was mineralized faster in clear lake water (Fig. 6). Faster mineralization of plant litter in humic lakes is indirectly supported by the higher mineralization rate of broadleaf cattail litter (III) in contrast to alder, aspen, and birch leaves (I), although this comparison includes other variables as well. Results suggest that higher DOC and nutrient content of humic lakes may enhance particularly the labile substrate mineralization. This might be explained by other covariation factors such as lake nutrient concentration (III, Fernandes *et al.* 2012, Duarte *et al.* 2016). Overall, the effect of lake type that was considered as differing DOC content (II) was a weak predictor for decomposition rate and was dependent of the studied substrate.

TABLE 4 Daily mean assimilation rates (%) of studied substrates across the temperature gradient (°C) in studied lake waters.

Substrate	2 °C	8 °C	15 °C	17 °C	18 °C	21 °C
Alder	-	-	-	0.05	-	-
Aspen	-	-	-	0.03	-	-
Beech	-	-	-	-	0.53	-
Birch	-	-	-	0.03	-	-
Cattail	0.02	0.01	0.01	-	-	0.01
Hemicellulose	-	-	-	-	2.02	-
Lignin	-	-	-	-	33	-
PS	0.000224	0.000236	0.000170	-	0.000351	0.000029
PP	-	-	-	-	0.000003	-
PE	0.000013	0.000003	0.000038	-	0.000196	0.000002

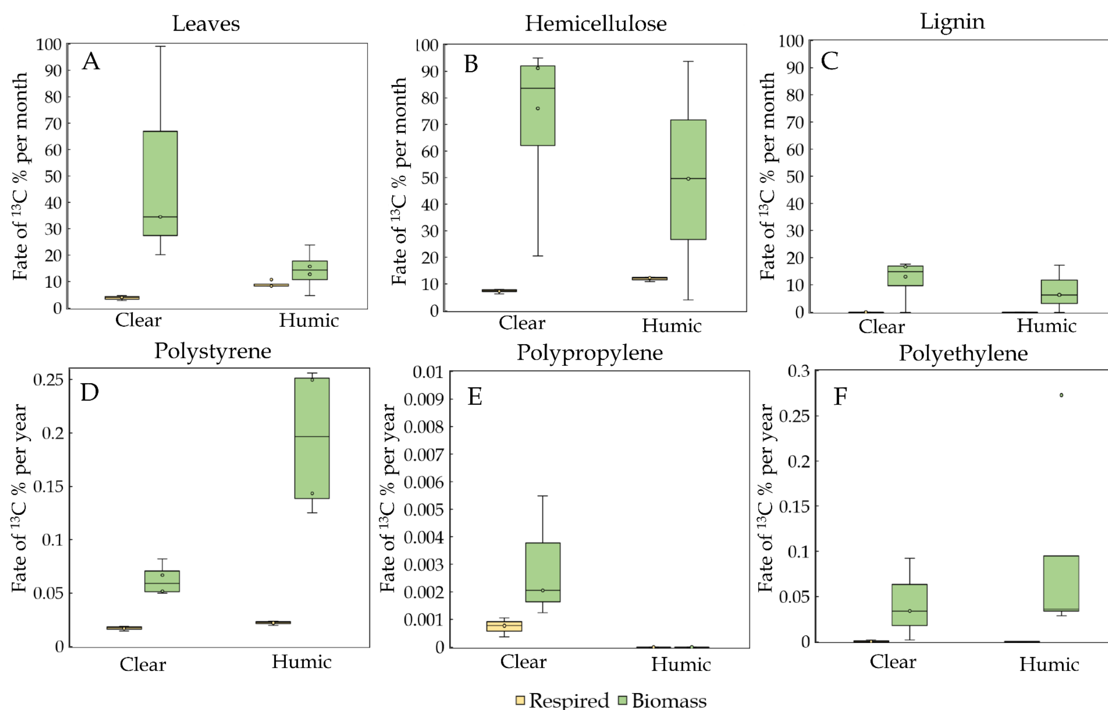

FIGURE 6 Biochemical fates (assimilation or mineralization rate per time unit) of carbon from A) leaves, B) hemicellulose, C) lignin, D) polystyrene, E) polypropylene, and F) polyethylene in clear and humic lake waters.

Seasonal variation of plant litter, PS, and PE carbon utilization and environmental factors affecting the decomposition were examined (III). The effect of seasonal variation on PE decomposition was not observed, due to extremely low degradation rate (Fig. 7A). In contrast, plant litter and PS decomposition rate were strongly affected by seasonal variation (Fig. 7B and 7C). The seasonal effect on decomposition rate was relatively similar between labile plant litter and recalcitrant microplastic: plant litter and PS decomposition rates were 5- and 4-fold higher, respectively, in summer than in winter (III). Temperature is known to regulate mineralization (Hall *et al.* 2008, Gudasz *et al.* 2010) and it was the most important environmental variable determining the decomposition rate of PS and plant litter (III). Degradation rates of plant litter and PS increased slowly across the temperature gradient until 15 °C, after which the rate accelerated. At the temperature of 21 °C, the carbon uptake rate from PS in humic lake waters reached 0.0066 % per day, which was the highest microplastic carbon assimilation rate observed in conducted studies (Table 4). Plant litter and PS decomposition did not follow linear regression along temperature gradient, and seasonal fluctuation in nutrient concentrations between spring and summer were concluded to affect the decomposition as well, supported by other studies (Grasset *et al.* 2017, Wang *et al.* 2019, DeGasparro *et al.* 2020, Yindong *et al.* 2021). Notably, decomposition did not stop even in winter at the temperature of 2 °C, revealing that decomposition processes of even highly recalcitrant polymers are continuous throughout the year, although the rate slows down, particularly below 15 °C.

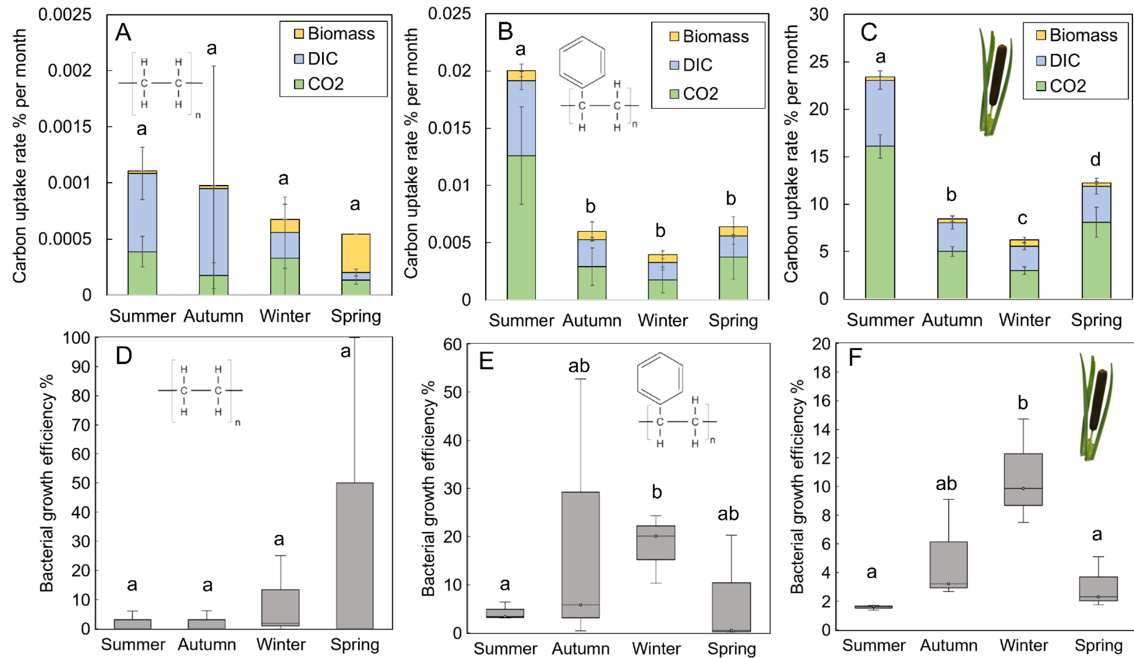

FIGURE 7 Seasonal variation in decomposition rates and carbon end points (biomass assimilation vs. respiration) was shown to be strong and systematic for polystyrene and plant litter, but not for polyethylene which was extremely slowly decomposed in all seasons. Seasonal variation of biochemical fate of carbon from A) PE, B) PS, and C) plant litter. Bacterial growth efficiency that was used to examine substrate carbon's relative use in a biomass versus as an energy source, did not vary seasonally in D) PE treatments, but showed seasonal variation in the E) PS and F) plant litter treatments.

In all seasons, litter and microplastic carbon was mostly mineralized (Fig. 7A–C). Interestingly, however, assimilation rates of PS and plant litter carbon were equal throughout seasons. The effects of seasonally changing environmental conditions affected only microbial activity and mineralization of carbon from plant litter and PS (III). In low temperatures, however, a decreasing proportion of mineralized carbon led to a relatively higher importance of biomass as an endpoint of substrate carbon in PS and plant litter treatments, but not in the PE treatment (Fig. 7D–F; Table 5). Thus, a higher proportion of substrate carbon is bound to the aquatic food web in low temperatures. Subsequently in subarctic and arctic lakes, the supporting effect of terrestrial carbon may be more powerful than in boreal lakes. On the other hand, plastics' slow decomposition in lower temperatures suggests that their permanence in fragile arctic and subarctic ecosystems, where microplastic pollution has recently been observed (Bergmann *et al.* 2022, Citterich *et al.* 2023), is even higher than in southern ecosystems. Moreover, warming climate may have cascading effects on the carbon balance of boreal lakes by inducing the release of CO_2 via increased microbial respiration (Berggren *et al.* 2012). Additionally, since temperature was recognized as the most important environmental factor affecting the microbial decomposition of plant litter and microplastics, it could be used to predict changing decomposition pathways and carbon cycling processes in aquatic ecosystems.

TABLE 5 Daily mean mineralization rates (%) of studied substrates across the temperature gradient (°C) in studied lake waters.

Substrate	2 °C	8 °C	15 °C	17 °C	18 °C	21 °C
Alder	-	-	-	0.22	-	-
Aspen	-	-	-	0.14	-	-
Beech	-	-	-	-	0.22	-
Birch	-	-	-	0.15	-	-
Cattail	0.18	0.26	0.39	-	-	0.76
Hemicellulose	-	-	-	-	0.31	-
Lignin	-	-	-	-	0	-
PS	0.000107	0.000131	0.000184	-	0.000054	0.000629
PP	-	-	-	-	0.000001	-
PE	0.000018	0.000007	0.000004	-	0.000001	0.000025

4.2 Microbial decomposers across the substrate recalcitrance gradient

Although bacteria are generally considered to be more efficient in competing due to their better substrate utilization, faster population growth, and biomass production (Mille-Lindblom and Tranvik 2003), there has been debate about whether they actually participate in the decomposition or only assimilate leachates and byproducts of fungal decomposition (Purahong *et al.* 2016, Zhan *et al.* 2021). To shed light on this problem in aquatic ecosystems, the CSIA of PLFAs was combined with microbial community analysis to explore the active microbial groups driving the substrate decomposition. All substrates were mainly decomposed by bacteria (I-III). Labile plant litter and hemicellulose carbon are utilized by a high number of different microbial taxa, supporting previous studies (Eichlerová *et al.* 2015, López-Mondéjar *et al.* 2016). In contrast, highly recalcitrant microplastic decomposition was limited to Proteobacteria and potentially Planctomycetes (II, III). Thus, results suggest that highly recalcitrant polymers can be degraded only by limited number of microbial taxa.

4.2.1 Origin of plant litter decomposers

Terrestrial microbiome recycled carbon from terrestrial organic matter and shaped the direction of microbial community succession in lake ecosystems, revealing the importance of terrestrial microbiome in aquatic carbon cycling that has been understudied in lentic freshwaters (I). Terrestrial microbes become an integral part of the aquatic microbiome not only on leaves (Jackrel *et al.* 2019) but in the surrounding lake water as well (I). Free-living microbes utilizing DOM are further eaten by filter-feeders (Taipale *et al.* 2014, Brett *et al.* 2017, Tang *et al.* 2019), and therein integrated into the aquatic food web. Although the terrestrial microbiome (i.e., leaf microbiome) shaped the succession of the microbial communities after introducing leaves into lake water (Fig. 8A and 8B), the aquatic

microbiome affected the carbon cycling processes, contradicting the view that leaf carbon is recycled by leaf associated microbiome only (Attermeyer *et al.* 2013). Nevertheless, terrestrial microbiome was responsible of majority of carbon recycling, supporting the current view that terrestrial microbiome is more important from the point of view of decomposition (Attermeyer *et al.* 2013, Hayer *et al.* 2022). The contribution of aquatic microbiome to carbon cycling was shown as faster assimilation and mineralization rates and higher microbial biomass production in contrast to treatments without aquatic microbiome (I).

To examine the origin of different microbiomes more closely, leaf microbiomes were divided into epiphytic (surface-living) and endophytic (living inside the leaf tissue) microbiomes. Epiphytic and endophytic bacteria contributed equally to bacterial community development. Fungal communities were more similar to epiphytic than endophytic fungal communities at the end of the experiment, suggesting that epiphytic fungi shape the fungal community succession more than endophytes (Fig. 9A), although endophytic fungi often are latent saprotrophs, and their location inside the plant material is thought to favor their role as decomposers (Osono 2006, Saikkonen *et al.* 2015). Among fungi, the separation between lake water and leaf communities was not clear, challenging the evaluation of the contribution of terrestrial and aquatic fungal communities to the final community composition after the incubation period. Additionally, a high proportion of uncultured fungi was detected (Fig. 9B). The lack of fungal sequence information in current databases has been recognized but the problem remains, challenging the identification of ecologically significant fungal taxa. Particularly freshwater fungi are still a highly unknown group and need more research to build a more comprehensive picture about the identity and roles of aquatic fungi.

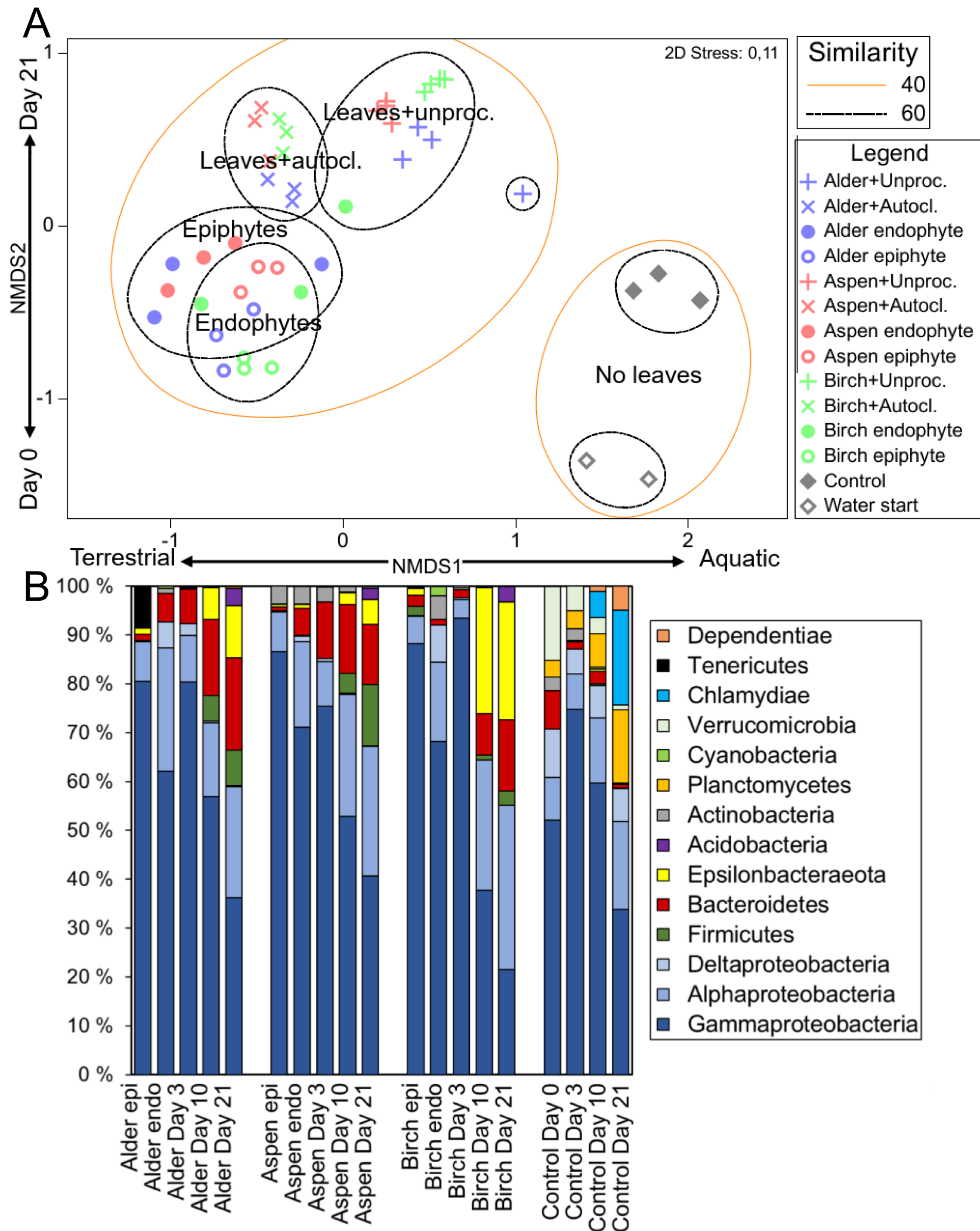

FIGURE 8 Bacterial community succession in lake water with different leaf species additions. A) Non-metric multidimensional scaling plots of Bray-Curtis similarity of bacterial OTU data (>0.5 % of all sequences) at the genus level. Autocl. = autoclaved lake water, and Unproc. = unprocessed lake water. B) Bacterial community succession in unprocessed lake water with leaf addition (alder, aspen, or birch) or without any leaf addition (control) during the 21 days incubation period, shown as averages of bacterial phyla (Proteobacteria divided into Alpha-, Delta-, Gammaproteobacteria). Epi = epiphytic community, and endo = endophytic community.

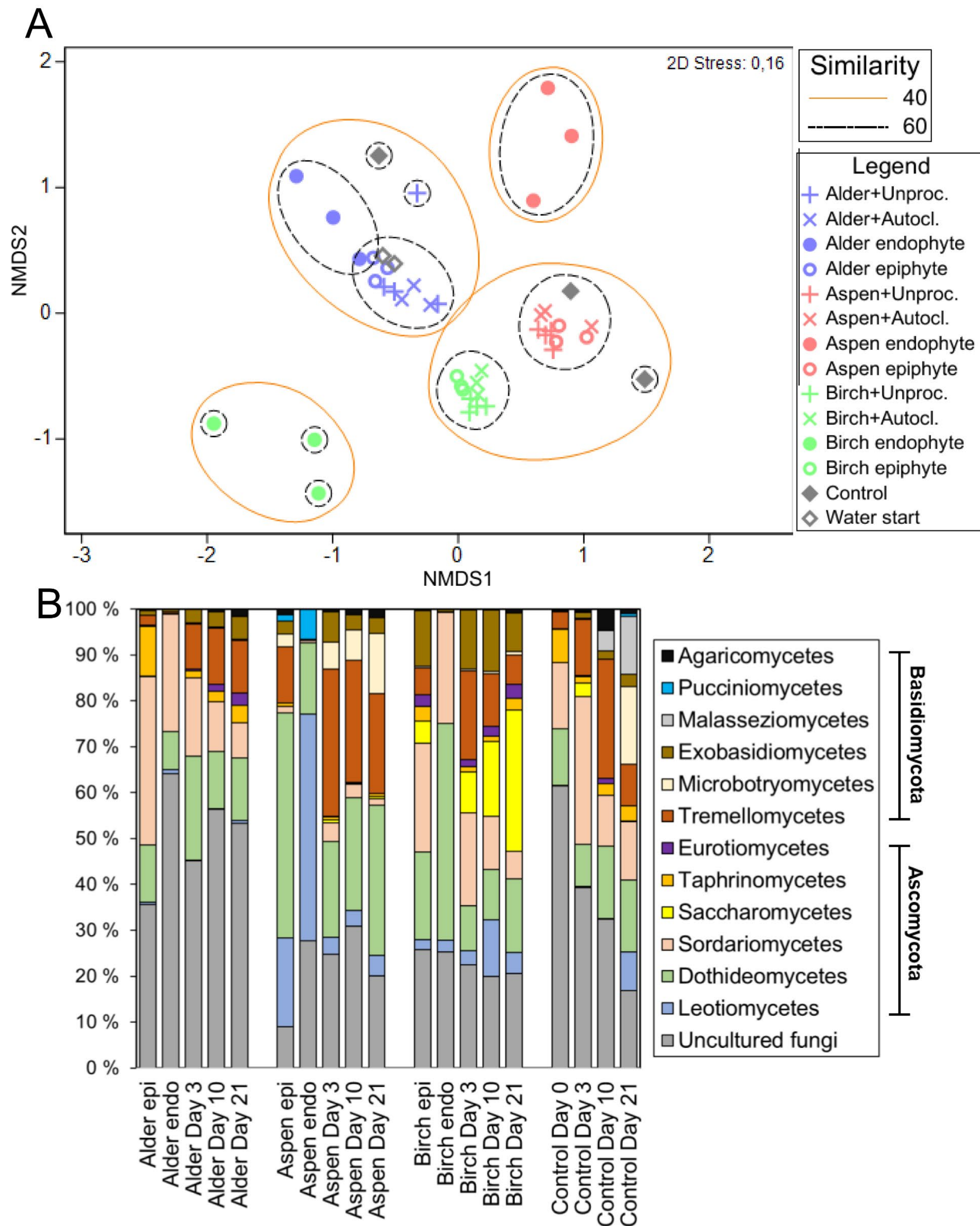

FIGURE 9 Fungal community succession in lake water with different leaf species additions. A) Non-metric multidimensional scaling plots of Bray-Curtis similarity of fungal OTU data (>0.5 % of all sequences) at the genus level. Autocl. = autoclaved lake water, and Unproc. = unprocessed lake water. B) Fungal community succession in unprocessed lake water with leaf addition (alder, aspen, or birch) or without any leaf addition (control) during the 21 days of incubation, shown as averages of fungal classes. Epi = epiphytic community, and endo = endophytic community.

4.2.2 Plant litter and plant compounds

Terrestrial microbiome, particularly bacteria, initializes the decomposition of plant litter in lake ecosystems (I; Fig. 8A, 10A). Notably, however, the contribution of fungi to microbial biomass and the fungal biomass itself increased after leaf addition into lake water (I; Fig. 10A–B). Although low in biomass, fungal participation in plant litter decomposition may be significant due to the secretion of exoenzymes (Purahong *et al.* 2016, Zhan *et al.* 2021). Fungal enzyme production benefits bacteria, that can utilize low molecular weight compounds that are released via enzymatic cleaving of chemical bonds (Purahong *et al.* 2016). CSIA of PLFAs supported the view of bacteria-driven decomposition process, since the ^{13}C-enrichment in bacterial biomarker PLFAs was shown, whereas fungal biomarkers were undetected or less enriched with ^{13}C (II, III; Fig. 11A and 11B). All five studied plant litter species were decomposed mainly by bacteria, suggesting that plant litter decomposition is initialized by bacteria despite leaf species or its chemical composition. Although the CSIA suggested that fungi did not uptake the plant litter carbon, it cannot be excluded if they used other pathways, such as the amino acid cycle, in binding carbon into their biomass.

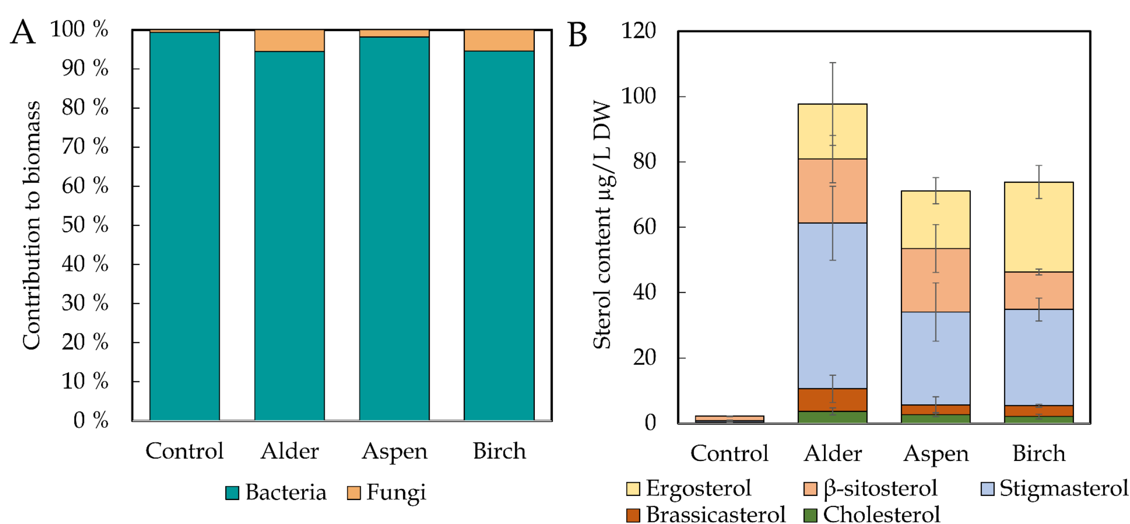

FIGURE 10 A) Fungal contribution was shown to increase slightly in each leaf litter treatment, but bacteria dominated over fungi by contributing over 90 % of total microbial biomass. B) An increase in fungal biomass was detected also as the increasing amount of sterols after introducing leaves into the lake water (adapted from I).

Various bacterial taxa participated in the decomposition of plant litter, as shown by a high ^{13}C-enrichment of several biomarker PLFAs (II, III; Fig. 11A and 11B). Strong seasonal fluctuation in microbial community structures suggested that plant litter decomposers vary seasonally (III). Interestingly, however, the same biomarker PLFAs were enriched in all seasons, suggesting that decomposer genera belong to the same higher taxonomic unit in all seasons (Fig. 11B). Microbial taxa that were linked to plant litter decomposition belonged to several

microbial classes, but particularly Proteobacteria and Verrucomicrobia were recognized as plant litter decomposers (I-III). Other proposed decomposers include bacteria from Alpha-, Delta-, and Gammaproteobacteria, Bacteroidia, Verrucomicrobia, Actinobacteria, Fibrobacteria, and vadinHA49 (I-III; Table 6). A wide range of different bacterial classes initializing the decomposition is explained by the labile composition of plant litter: leaching carbohydrates and low molecular weight compounds are utilized efficiently by all bacterial taxa, although probably favoring fast-growing, opportunistic bacteria.

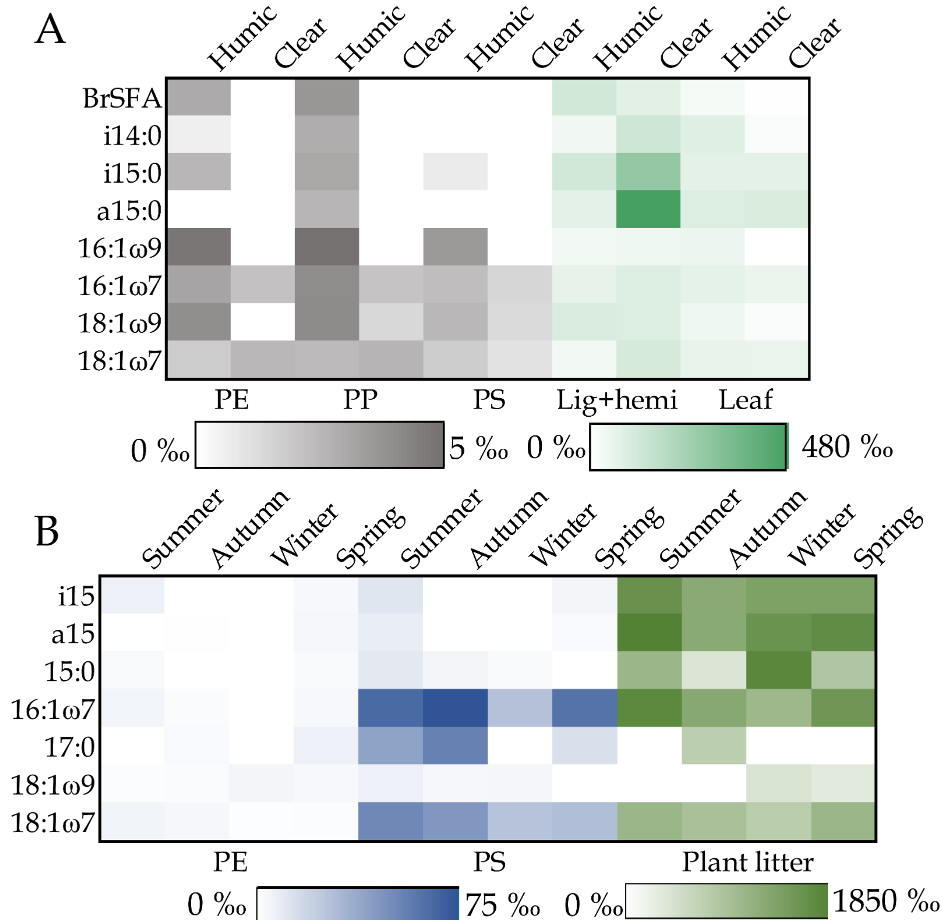

FIGURE 11 A) Heatmap showing ^{13}C-enrichment of microbial PLFAs in humic and clear lake waters with PE. PP, PS, lignin-hemicellulose, or leaf addition. Data is square-root transformed for better visualization of differences. In humic lake waters with plastic additions, a wider range of PLFAs was ^{13}C-enriched than in clear lake waters, whereas in natural substrate treatments (lignin-hemicellulose and leaves) ^{13}C-enrihment of microbial PLFAs was rather similar between two lake types. B) Heatmap showing ^{13}C-enrichment of microbial PLFAs in humic lake waters with PE, PS, (blue) or plant litter (green) addition in four seasons. Plant litter treatments reveal a wider range of ^{13}C-enriched PLFAs in contrast to microplastic treatments in all seasons. Notably, in each substrate treatment, the same PLFAs showed ^{13}C-enriched in all seasons.

Despite the recalcitrant structure of lignin, a relatively high number of microbial taxa has been linked to lignin degradation. Li *et al.* (2020) found that aquatic

bacteria (e.g., Actinobacteria, Proteobacteria, and Planctomycetes) degrade lignin-derived polymers in freshwaters. Lignin and hemicellulose were studied as a mixture of these polymers, and thus, unfortunately, the CSIA results and changes in microbial community composition are mostly caused by the more labile substrate, i.e., hemicellulose (II). Therein, lignin decomposers cannot be identified. However, the lack of ^{13}C-enrichment of fungal biomarker PLFAs suggests that bacteria rather than fungi were driving the lignin degradation (Fig. 10A).

In contrast to the weak identification of lignin decomposers, CSIA indicated that hemicellulose decomposition was driven by several bacterial taxa (Fig. 11A, Table 6), whereas in the clear lake water, PLFAs i15 and a15, commonly found in Acidobacteria, Bacteroides, Actinobacteria, and Verrucomicrobia (Ismaeil *et al.* 2018, Oshkin *et al.* 2019, Sheu *et al.* 2019, Taipale *et al.* 2022) but more commonly used as biomarkers of gram-positive bacteria (Willers *et al.* 2015), had the highest levels of ^{13}C-enrichment. Therein, likewise with plant litter, results suggest that microbial utilization of labile hemicellulose-derived carbon is not driven by any specific taxa but is rather started by the bacteria that are already present when the substrate is introduced into a given environment.

TABLE 6 Suggested substrate decomposer taxa of studied substrates in boreal lake waters.

	Taxon	Reference
Alder	Several bacteria; particularly Alpha- and Gammaproteobacteria	I
Aspen	Several bacteria; particularly Alpha- and Gammaproteobacteria	I
Beech	Several bacteria; particularly Proteobacteria and Bacteroidota	II
Birch	Several bacteria; particularly Alpha- and Gammaproteobacteria	I
Cattail	Actinobacteria, Bacteroidota, Fibrobacteria, Proteobacteria, Verrucomicrobia, VadinHA49	III
Hemicellulose	Several bacteria	II
Lignin	Not identified	II
PS	Alpha- and Gammaproteobacteria (Burkholderiaceae), Verrucomicrobia	II, III
PP	Planctomycetes	II
PE	Planctomycetes	II

4.2.3 Microplastics

Bacteria drove the microplastic decomposition process in all studied lakes and seasons (II, III), regulating carbon uptake from microplastics and thus determining their biochemical fate. In contrast to plant litter, microplastics were utilized very slowly by a narrower range of microbial taxa (III; Table 6). Throughout all seasons, PS decomposition was initialized by Alpha- and Gammaproteobacteria, that has been reported to involve numerous potential candidates of microplastic degraders in other environments as well (Sekiguchi *et al.* 2011, Roager and Sonnenschein 2019). PS decomposing Proteobacteria varied seasonally at the genus level (III). Several identified PS decomposer genera belong to the family Burkholderiaceae (Gammaproteobacteria), suggesting the

potential of this family as an aromatic microplastic decomposer in lentic freshwaters (II, III), which is also supported by their ability to degrade aromatic polymers (Pérez-Pantoja *et al.* 2012). In addition, Verrucomicrobia and Planctomycetes, that has been associated with PS degradation in another study (Taipale *et al.* 2023), participated in the PS decomposition. Notably under microbial starvation, a more diverse range of bacterial biomarker PLFAs showed ^{13}C-enrichment, suggesting that starvation also promoted the participation of other microbial groups in the decomposition process.

In addition to bacteria, fungi are considered to be potential microplastic decomposers due to their secretion of extracellular enzymes and hydrophobins that can initialize the decomposition reaction in polymer bonds (Sánchez 2020). However, results did not indicate that fungi would have participated in the decomposition of microplastics as shown by a low amount of fungal biomarker PLFAs and their weak ^{13}C-enrichment (II, III). PLFA 18:1ω9 was the only biomarker PLFA that is commonly found in fungi; however, it is also commonly found in members of Planctomycetes. The lack of other fungal biomarker PLFAs suggests that the ^{13}C-enrichment of PLFA 18:1ω9 is rather linked to Planctomycetes. This was supported by the ^{13}C-enrichment of PLFA 16:1ω9 (II), which is also found among Planctomycetes (Elshahed *et al.* 2007). Indeed, Planctomycetes were considered to participate in the degradation of PE and PP both in clear and humic lakes (II; Table 6). However, the decomposition rate of these plastics is so slow that the Planctomycetes-driven plastic degradation seems to be highly inefficient.

4.3 Evaluation of methods and notes for future research

Labile and recalcitrant substrate degradation are studied differently. Due to high recalcitrance, measurements such as mass loss after incubation in a given environment, increased respiration, and/or biomass production are weakly applicable for measuring the decomposition rate and process of recalcitrant materials. Quantifying the decomposition of recalcitrant polymers by stable isotope analysis provided good results and revealed differences between different microplastic types. For labile substrates, more traditional methods are sufficient and less expensive. However, in order to study carbon cycling more closely, stable isotope analysis can be recommended based on the results of this thesis.

Worth noticing is the impact of temperature on plant litter and PS decomposition rates and the relatively low deviation of their decomposition rates among humic lakes (II, III). This suggests that the microbial degradation rate of a given substrate is equal between humic lakes with close DOC values and can be well predicted. In contrast, the substrate recalcitrance affects its degradation rate. Moreover, humic lake microbes seem to efficiently utilize aromatic microplastics as their carbon source, as previously suggested for PE (Taipale *et al.* 2019). Since the assimilation rate of plant litter and PS carbon was also equal

throughout all seasons (within substrates), measuring the mineralization alone could provide a good proxy for the total decomposition rate of labile and recalcitrant carbon sources in humic lakes. Due to this low variation under standardized conditions, I encourage researchers to develop a model that predicts the behaviour and decomposition process of substrates across the recalcitrance gradient. Particularly temperature, DOC content, litter nitrogen content, and mineralization rate seem to be useful variables to produce a proxy of the decomposition rate of labile and recalcitrant carbon sources and consequently, over both short and long periods.

Microbial taxa living on plastics have often been associated directly with decomposition, without actual examination of their active functionality. CSIA of PLFAs provided information about active microbial groups responsible for carbon uptake and assimilation into cell structures (II, III). When combined with microbial community analysis, the method was successfully used to identify potential decomposers of studied substrates more accurately. However, the resolution of the identification methods still needs development. Particularly at the lower taxonomic levels the microbial plant litter decomposers are likely lake-specific, but often belong to the same bacterial classes (I-III). Microbial communities vary between lakes, lake types, and seasons (I–III). Thus, if comparisons between different lake microbiomes are made the number of environmental variables must be minimized, and the conclusions should be made with proper caution.

5 CONCLUSIONS

Increasing loadings of terrestrial organic matter, increasing plastic pollution, and rapidly changing environmental conditions raise interest in the decomposition processes of different types of terrestrial substrates in the aquatic environment. The microbial decomposition process greatly affects the biochemical utilization pathways of the substrate carbon, playing an important role in aquatic carbon cycling. Understanding the role of terrestrial carbon is essential for effective lake management and conservation, especially in the context of land-use changes and climate change, which can alter the quantity and quality of terrestrial carbon inputs to lakes. Therefore, the succession of microbial communities on leaves, fungi-bacteria interactions, and their abundance during the organic matter and polymer decomposition processes are important factors affecting the bottom-up regulation of the aquatic food web.

Although the decomposition rate of substrates and the amount of carbon uptake varied across the recalcitrance gradient as expected, the percentual distribution was similar between labile plant litter and microplastics. Commonly, carbon from both labile plant litter and highly recalcitrant microplastics was mainly mineralized to CO_2 and thus respired by the lake microbiome, whereas a smaller proportion is bound to microbial biomass. However, the changing environmental conditions may affect the carbon's biochemical fate. Seasonal changes, particularly temperature, altered the total decomposition rate of microplastics and plant litter. Interestingly, assimilation rates were equal throughout all seasons, whereas mineralization varied seasonally. In lower temperatures, microbial respiration and substrate mineralization decreased, increasing the proportional importance of biomass as a carbon's endpoint. Microbial starvation may also favor the utilization of substrate carbon as a structural component rather than in energy-producing pathways. Moisture, structure, and high nitrogen content of plant litter were associated with increased decomposition, assimilation, and mineralization rates. However, although differing in rates, leaf chemistry did not affect the distribution of carbon: the same proportions of decomposed leaf carbon were respired and bound to biomass

among different leaf species. The lake DOC and nutrient content had a poor effect on the decomposition rates and processes of studied substrates.

Compound-specific isotope analysis of phospholipid fatty acids combined with microbial community analysis provided insights into microbial groups responsible for substrate decomposition. Bacteria initialized the decomposition process of all studied substrates, as shown by ^{13}C-enrichment of bacterial biomarkers and the lack of ^{13}C-enriched fungal biomarkers. Bacteria utilized labile hemicellulose and plant litter carbon effectively in all lakes. Labile carbon sources were utilized by various microbial taxa, and they supported microbial communities by increasing microbial biomass. On the contrary, recalcitrant carbon sources were more taxon-specific, allowing better exclusion and identification of decomposer taxa and suggesting the high potential of the compound-specific isotope analysis in tracing the active decomposers behind the highly recalcitrant microplastic degradation. Microbial taxa associated with plant litter and microplastic decomposition varied seasonally at the genus level but belonged to a limited number of phyla in all seasons, suggesting that decomposition is initiated by members belonging to these specific phyla. Particularly Proteobacteria were often associated with polystyrene degradation.

Overall, the thesis shows that lake microbes are able to utilize varying substrates as their carbon source and that the decomposition continues throughout all seasons. Additionally, results emphasize that multiple factors affect the decomposition process of a given substrate and its carbon recycling rates. These factors include e.g., substrate’s chemical structure, biotic factors such as microbial activity, and abiotic factors such as temperature. The percentual, but not the quantitative, biochemical fate of different terrestrial carbon sources was found to be similar between substrates across the recalcitrance gradient. However, changing environmental conditions may affect also the percentual distribution of substrate carbon in aquatic ecosystems. In future research, I recommend to examine more closely different plastic and biodegradable plastic types, since their relatively similar, well-known, and simple chemical structure allows the more accurate identification and control of the chemical factors affecting their carbon recycling. The long-term following of the decomposition processes and the changing microbial community still need further research to achieve a full understanding of the power of microbes as controllers of element recycling from varying terrestrial substrates in aquatic environments.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Mikrobien rooli terrestrisen orgaanisen aineksen ja mikromuovien hajotuksen ja hiilen kierron säätelijänä boreaalisissa järviekosysteemeissä

Maa- alias terrestrisestä ekosysteemistä peräisin oleva orgaaninen aines on tärkeä hiilen, ravinteiden ja energian lähde järviekosysteemille. Valuma-alueelta vesiin päätyvän humusaineen lisäksi kasvien tuottama karike on yksi tärkeimmistä orgaanisen aineksen lähteistä. Karike sisältää muiden muassa vesiliukoisia yhdisteitä, helposti hajotettavia sokereita, selluloosaa, hemiselluloosaa sekä hankalasti hajotettavia polymeerejä kuten ligniiniä. Luonnollisten hiilen lähteiden lisäksi ihmisen toiminnan seurauksena ympäristöön päätyy kasvavia määriä muovijätettä. Mikromuoveista (halkaisija < 5 mm) on tullut maailmanlaajuinen huolenaihe niiden lisääntyvän määrän, eliöihin kertymisen ja mahdollisen ympäristömyrkkyjä kerryttävän vaikutuksen takia. Mikromuovien hajoaminen on lisäksi äärimäisen hidasta, johtuen vahvoista sidoksista polymeerirakenteessa ja reaktiivisten sivuryhmien vähäisyydestä.

Mikrobit ovat tärkeimpiä orgaanisen aineksen hajottajaeliöitä järvissä. Hajottajamikrobit lisäävät toiminnallaan hiilen ja ravinteiden saatavuutta akvaattisille kuluttajille ollen näin ollen tärkeässä roolissa terrestrisen hiilen kierrossa akvaattisessa ekosysteemissä. Mikrobien on havaittu hajottavan jopa äärimmäisen vahvarakenteisia mikromuoveja lukuisissa erilaisissa ympäristöissä. Erityisesti humusjärvissä tapahtuvat hajotusprosessit ovat kiinnostava tutkimuskohde, sillä polyetyleenin hajoamisen on havaittu aiemmin olevan nopeampaa humusjärvessä kirkasvetiseen järviveteen verrattuna viitaten humusjärvien mikrobiston tehokkaampaan kykyyn hyödyntää hankalasti hajotettavaa muovia hiilen lähteenään. Lisäksi boreaalisen vyöhykkeen järvistä valtaosa on humuspitoisia järviä, ja näiden määrän ennustetaan kasvavan ilmastonmuutoksen aiheuttaman vesien ruskettumisen myötä. Humusjärvet vapauttavat enemmän hiiltä kuin sitovat ja ovat näin ollen hiilen lähteitä globaalissa hiilen kierrossa.

Mikrobien käyttäessä hajotettavasta substraatista peräisin olevaa hiiltä osana uuden biomassan muodostamista hiili sitoutuu osaksi mikrobibiomassaa ja akvaattista ravintoverkkoa. Sen sijaan mikrobien käyttäessä pilkottavan aineksen kemiallisten sidosten pilkkomisesta vapautuvaa energiaa, hiili vapautuu soluhengityksen kautta ympäristöön päätyen joko karbonaatiksi veteen tai hiilidioksidiksi ilmakehään. Näin ollen mikrobit ovat avainasemassa hiilen biokohtalon määrittelijänä.

Väitöskirjassani tutkin mikrobien kontrolloimaa erilaisten substraattien hajoamisprosessia akvaattisessa ympäristössä. Erityisesti tarkastelin hiilen biokemiallista kohtaloa ympäristössä, ja pyrin määrittämään tähän vaikuttavia ympäristötekijöitä sekä tunnistamaan hajotuksesta vastaavat mikrobiryhmät yhdistämällä yhdistespesifiä isotooppianalytiikkaa ja mikrobiyhteisöanalyysia. Tutkittavat materiaalit valittiin kattamaan laaja kirjo hajotettavuusasteeltaan poikkeavia materiaaleja: viisi eri kasvilajista peräisin olevaa kariketyyppiä, ligniini, hemiselluloosa, ja kolme kemialliselta rakenteeltaan erilaista mikromuovilaatua (poly-

styreeni, polypropyleeni, polyetyleeni). Väitöskirjan tavoitteena on lisätä tietämystä eri materiaalien hajoamisprosesseista ja näistä peräisin olevan hiilen kierrosta järviekosysteemeissä.

Eri materiaalien hajotusnopeudet vaihtelivat odotetusti, karikkeen ja luonnonpolymeerien hajotessa mikromuoveja nopeammin. Hajotettavasta materiaalista riippumatta mikrobit hyödynsivät materiaalin hiilen energiaa tuottavia aineenvaihduntaprosesseja käyttäen. Näin ollen prosentuaalisesti suurin osa hajotetusta substraatista käytettiin soluhengityksessä ja havaittiin vapautuvana hiilidioksidina, kun taas pienempi osa käytettiin rakenteellisesti uuden mikrobibiomassan muodostamiseen. Tähän hajotetun substraatin hiilen biokemialliseen kohtaloon vaikuttivat kuitenkin ympäristöolot. Kun mikrobien annettiin kuluttaa muut hiilen lähteet ja näin ollen ”nälkiintyä” ennen tutkittavan materiaalin lisäämistä, biomassaan sidottavan hiilen osuus ylitti hengitettävän substraattihiilen määrän. Lisäksi lehtien, polystyreenin ja polypropyleenin hajotusnopeudet erosivat kirkasvetisen ja humuspitoisen järviveden kesken.

Vuodenajoittain vaihtelevista ympäristömuuttujista lämpötilalla oli suurin vaikutus hiilen kiertoon. Kesällä tapahtuvaan hajotukseen verrattuna sekä karikkeen että mikromuovin hajotusnopeus laski talvella alenneen mineralisaation seurauksena, kun taas substraatin hiilen hyödyntäminen biomassan muodostuksessa pysyi tasaisena ympäri vuoden. Näin ollen hiilen tärkeys biomassan muodostamisessa on suhteellisesti tärkeämpi matalissa lämpötiloissa. Tulokset viittaavat siihen, että maaperäekosysteemeistä peräisin olevat materiaalit ovat talvella ja mahdollisesti myös subarktisissa ja arktisissa vesiekosysteemeissä erityisen tärkeitä hiilen lähteitä mikrobeille.

Vaikka lehtien lisääminen järviveteen lisäsi sekä bakteerien että sienten biomassaa, ei sienille tyypillisten biomarkkerien leimaantumista havaittu. Tämä viittaa siihen, että bakteerit vastasivat kaikkien materiaalien hajotuksesta hajotusprosessien alkuvaiheessa. Laaja kirjo bakteereja yhdistettiin helposti hajotettaviin materiaaleihin (lehdet, hemiselluloosa), kun taas mikromuovien hajottajiksi tunnistettiin vain muutamia bakteeriryhmiä. Tämä viittaa siihen, että tiettyihin ryhmiin, erityisesti Proteobakteereihin, kuuluvat mikrobisuvut ovat muita ryhmiä tehokkaampia hyödyntämään hankalasti hajotettavia polymeerejä hiilen lähteenään.

Vaikka erilaisten materiaalien hajotusnopeudet voivat vaihdella suuresti, havaittiin niiden sisältämän hiilen biokemiallisen kohtalon olevan samankaltainen samankaltaisissa olosuhteissa. Hiilen biokohtalo lieneekin ympäristötekijöiden säätelemää, kun itse hajotusnopeuteen vaikuttaa myös materiaalin kemiallinen laatu. Tulevissa tutkimuksissa olisi suotavaa tarkastella monipuolisemmin samankaltaisia materiaaleja, kuten mikromuoveja ja biohajoavia muoveja, joiden rakenne on helpommin mallinnettavissa ja näin ollen hajotusprosessiin ja alkuaineiden kiertoon vaikuttavat kemialliset tekijät tarkasti määritettävissä. Myös hajotusprosessien pitkäaikaistarkastelu ja mikrobiyhteisön muutokset kaipaavat lisätutkimuksia.

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ORIGINAL PAPERS

I

RECYCLED BY LEAF INHABITANTS: TERRESTRIAL BACTERIA DRIVE THE MINERALIZATION OF ORGANIC MATTER IN LAKE WATER

by

Jussi S. Vesamäki, Cyril Rigaud, Jaakko J. Litmanen, Riitta Nissinen, Robert Taube & Sami J. Taipale 2024

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II

DECOMPOSITION RATE AND BIOCHEMICAL FATE OF CARBON FROM NATURAL POLYMERS AND MICROPLASTICS IN BOREAL LAKES

by

Jussi S. Vesamäki, Riitta Nissinen, Martin J. Kainz, Matthias Pilecky, Marja Tiirola & Sami J. Taipale 2022

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Tony Gutierrez,
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REVIEWED BY
Xiaobo Liu,
Nanjing University of Science and Technology, China
Marika Kokko,
Tampere University,
Finland

*CORRESPONDENCE
Jussi S. Vesamäki
jussi.s.vesamaki@jyu.fi

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Decomposition rate and biochemical fate of carbon from natural polymers and microplastics in boreal lakes

Jussi S. Vesamäki[1]*, Riitta Nissinen[1], Martin J. Kainz[2], Matthias Pilecky[2], Marja Tiirola[1] and Sami J. Taipale[1]

[1]Department of Biological and Environmental Science, Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland, [2]WasserCluster Lunz—Biological Station, Donau-Universität Krems, Lunz am See, Austria

Microbial mineralization of organic compounds is essential for carbon recycling in food webs. Microbes can decompose terrestrial recalcitrant and semi-recalcitrant polymers such as lignin and cellulose, which are precursors for humus formation. In addition to naturally occurring recalcitrant substrates, microplastics have been found in various aquatic environments. However, microbial utilization of lignin, hemicellulose, and microplastics as carbon sources in freshwaters and their biochemical fate and mineralization rate in freshwaters is poorly understood. To fill this knowledge gap, we investigated the biochemical fate and mineralization rates of several natural and synthetic polymer-derived carbon in clear and humic lake waters. We used stable isotope analysis to unravel the decomposition processes of different ^{13}C-labeled substrates [polyethylene, polypropylene, polystyrene, lignin/hemicellulose, and leaves (*Fagus sylvatica*)]. We also used compound-specific isotope analysis and molecular biology to identify microbes associated with used substrates. Leaves and hemicellulose were rapidly decomposed compared to microplastics which were degraded slowly or below detection level. Furthermore, aromatic polystyrene was decomposed faster than aliphatic polyethylene and polypropylene. The major biochemical fate of decomposed substrate carbon was in microbial biomass. Bacteria were the main decomposers of all studied substrates, whereas fungal contribution was poor. Bacteria from the family Burkholderiaceae were identified as potential leaf and polystyrene decomposers, whereas polypropylene and polyethylene were not decomposed.

KEYWORDS

decomposition, microplastic, polymer, mineralization, Burkholderiaceae

Introduction

Microbial decomposition of organic compounds is essential for carbon recycling in food webs. In addition to autochthonous carbon sources, freshwaters receive terrestrial loadings whose microbial decomposition processes are poorly understood. Terrestrial-derived organic carbon includes labile carbon, such as carbohydrates, polysaccharides, and

cellulose, which are quickly utilized by microbes. In addition to these, terrestrial loadings also include more recalcitrant polymers, e.g., lignin and cellulose, both precursors for humus formation (Danise et al., 2018) and are found in high concentrations in boreal humic, brown-water lakes (Hessen and Tranvik, 1998). Clear-water lakes [defined often as DOC <10 mg/L (Kortelainen, 1993)] typically are rich in labile organic carbon sources, which are utilized efficiently by microbes (Bręk-Laitinen et al., 2012), whereas humic lakes have more recalcitrant carbon compounds (Hessen and Tranvik, 1998). It is thus plausible to assume that humic freshwater microbes are highly adapted to utilize diverse carbon sources and have developed a vast arsenal of biochemical weathering agents to use recalcitrant carbon sources. To date, microbial decomposition of lignin has been studied in soils even at the molecular level [e.g., (Pathan et al., 2017; Bhatnagar et al., 2018)] and sediments [e.g., (Song et al., 2019; Yang et al., 2021)], but their decomposition and the role of different microbial groups in the decomposition process in freshwaters are weakly understood.

In addition to natural polymers, microplastics have been found in several environments worldwide (Shahul Hamid et al., 2018), including lentic (Di Pippo et al., 2020; Uurasjärvi et al., 2020) and lotic (Crew et al., 2020; Mora-Teddy and Matthaei, 2020) freshwaters. Microplastic pollution has become an increasing environmental concern worldwide due to increasing plastic pollution in oceans (Jambeck et al., 2015) and freshwaters (Hurley et al., 2018). However, their mineralization rate and biochemical fate in freshwater systems remain poorly investigated (Anderson et al., 2016; Taipale et al., 2019). Until now, microplastic degradation studies have focused on microbes colonizing the surface of microplastic (e.g., Debroas et al., 2017) and pure culture testing (e.g., Yoshida et al., 2016). Currently used methods include imaging methods such as scanning electron microscopy (SEM) imaging, which does not give any information about the biochemical fate of plastic-derived carbon.

Bacterial diversity increases with microplastic surface roughness but does not vary among different plastic types (Di Pippo et al., 2020). Although particle size has not been found to significantly affect bacterial community composition between smaller and larger microplastic particles (Frère et al., 2018), bacterial diversity has been higher on larger mesoplastic particles than on microplastics and lowest on PS particles (Debroas et al., 2017). However, plastic's physical properties and chemical composition play an important role in the decomposition process. Microbes can attach more easily to heteroatomic microplastics (Debroas et al., 2017) like nylon and polyvinyl. For example, freshwater fungi that grew on plastic particles were found to be able to degrade aromatic and nitrogen-involving polyurethane but not aliphatic polyethylene (Brunner et al., 2018). In contrast to microplastics, natural carbon sources can involve a diverse mix of easily utilizable carbon sources such as carbohydrates and heteroatomic compounds and more recalcitrant carbon sources such as benzene rings. For example, lignin and recalcitrant humic substances are mineralized slowly in humic waters (Vähätalo et al., 1999) whereas microbial glucose mineralization is fast (Schneckenberger et al., 2008).

Microbes can assimilate polymer carbon by extracellular enzymatic degradation or by endocytosis (Sánchez, 2020; Yuan et al., 2020; Liu L. et al., 2021). In addition, microbes can break chemical bonds of a polymer by enzymatic hydrolysis, and these oligo-, di- or monomers can be further processed intracellularly and mineralized to CO_2 or CH_4 or used for anabolic processes (Yuan et al., 2020; Du et al., 2021; Liu X. et al., 2021). Both mineralization rate and the biochemical fate of polymer carbon into inorganic carbon *via* respiration (CO_2) and microbial biomass can be studied using ^{13}C-labelled substrates and stable isotope analysis (Steffens et al., 2015; Yang et al., 2015; Taipale et al., 2019). Measuring the assimilation of ^{13}C into phospholipid fatty acid (PLFA) biomarkers by compound-specific isotope analysis (CSIA) enables the tracking of the carbon cycle and decomposition pathways (Twining et al., 2020). Furthermore, PLFA-biomarkers and CSIA can be combined with microbial community analysis to identify microbial taxa responsible for decomposition (Taipale et al., 2019). However, only a few studies (e.g., Yang et al., 2015; Taipale et al., 2019; Liu L. et al., 2021) have used ^{13}C-labeled microplastics to study microbe-driven plastic degradation, despite its high sensitivity and usefulness as a tool to study the decomposition of highly recalcitrant and slowly degraded materials.

In this study, we compared decomposition rate and pathways, and the biochemical fate of carbon from different natural polymers and microplastics using ^{13}C-labeled materials to gain a deeper understanding of microbial decomposition processes in freshwaters. We selected deciduous tree leaves (*Fagus sylvatica*) and lignin-hemicellulose for natural carbon sources and polyethylene (PE), polypropylene (PP), and polystyrene (PS) for microplastics. We compared their decomposition rates and biochemical pathways in clear-water lake water containing labile organic carbon (Bręk-Laitinen et al., 2012) and humic lake water, where microplastic mineralization was observed in a previous study (Taipale et al., 2019). The following hypotheses were tested: (1) decomposition rate is faster in humic lake water than in clear lake water for microplastic polymers and lignin, whereas decomposition rate was assumed to be similar for leaves in both lake types, (2) decomposition is faster for aromatic polystyrene than for aliphatic polyethylene or polypropylene, (3) carbon from decomposed substrate is mainly utilized to build biomass whereas smaller proportion is respired, and (4) bacteria are primary decomposers for easily degradable carbon sources (lignin-hemicellulose and leaves), whereas fungi play a more important role in the decomposition of plastics.

Materials and methods

Sampling sites, experiment preparation, and experimental setup

Waters were collected from highly humic lake Nimetön [Evo, Finland 61°22′81″N, 25°19′23″E; DOC = 22.60 ± 0.88 mg/L] and from clear water lake Vesijärvi (Lahti, Finland, (61°02′42″N,

25°35′10″E; DOC = 5.19 ± 0.05 mg/L) in July 2020 for microplastic and lignin-hemicellulose treatments and in September 2020 for leaf treatments. Collected waters were filtrated through a 3 μM pore size filter to remove bacterivores and preincubated at 18°C for 3 weeks before the start of the experiment to let microbes consume most of the easily available carbon sources. After the preincubation, 300 mL of water was poured into a 540 mL glass bottle and 4 mg C of ^{13}C-substrate (PE (Poly(ethylene-$^{13}C_2$) Sigma-Aldrich, 99 atom% ^{13}C, United States); PP (Poly(propylene-1-^{13}C) Sigma-Aldrich, 99 atom% ^{13}C, United States); PS (Poly(styrene-α-^{13}C) Sigma-Aldrich, 99 atom% ^{13}C, United States); lignin-hemicellulose (U-13C lignin organosolv from wheat (*Triticum aestivum*), IsoLife bv 97 atom% ^{13}C, Netherlands); NLD Hygroscopic); leaves (P-^{13}C Beech leaf (*Fagus sylvatica*) 13.4 atom% ^{13}C, IsoLife bv, Netherlands) was added. Used lignin-hemicellulose was composed approximately 80% of lignin and 20% of carbohydrates, including mostly hemicellulose (van Erven et al., 2017). Control treatments had no substrate addition. Lake waters with added substrates were incubated at 17–18°C in closed glass bottles for three (leaves), or six (lignin-hemicellulose, microplastics, and controls without any substrate addition) week(s) (Figure 1). Bottles were daily shaken during the experiment. Four replicates were made for each treatment.

Mineralization

Gas samples were taken weekly from the air phase of a bottle. 5 mL of gas sample was transferred into an air-free Exetainer® tube. At the end of the experiment, ^{13}C-DIC was analyzed by taking 5 mL of water into a He-flushed Exetainer® tube and adding 200 μL of 85% H_3PO_4 (Taipale and Sonninen, 2009). Water samples were mixed by a vortex and 5 mL of the gas phase was taken from the Exetainer® tube into a new tube. The gaseous DIC samples were further processed and analyzed identically to CO_2 samples. The amount of CO_2 in the sample was determined by an Agilent 7890B gas chromatograph (Agilent Technologies, Palo Alto, CA, United States). After the quantification of CO_2 in sample tubes, $\delta^{13}C$ values of CO_2 and DIC were analyzed using an Isoprime TraceGas pre-concentrator unit connected to an Isoprime IRMS (Isoprime100 IRMS, Elementar UK Ltd., Cheadle, UK) at the University of Jyväskylä, Finland. $\delta^{13}C$ values were drift corrected and two-point calibrated based on external standards. Mineralization rate calculations are presented in Supplementary material.

Analysis of ^{13}C-PLFAs by GC–MS, *CF*-IRMS, and compound-specific isotopes

At the end of the experiment, water was filtered through a preweighted filter (Whatman™ cellulose nitrate filters, pore size 0.2 μM, diameter 47 mm) and stored at −80°C. After that, filters were freeze-dried, weighed, and transferred into a Kimax® tube. 3 mL of chloroform-methanol (2:1) and 750 μL distilled water and internal standards C19:0 and C23:0 (0.4997 mg/mL and 0.5044 mg/mL, respectively) were added into a sample tube. Tubes were sonicated for 10 min and then vortexed and centrifuged (3,000 rpm for 3 min). The lower phase was transferred into a new Kimax® tube. The sample was evaporated under nitrogen flow which after it was dissolved in 300 μL of $CHCl_3$.

Extracted fatty acids were fractionated by using a Bond Elut Silica cartridge. At first, the cartridge was activated by 6 mL of $CHCl_3$-MeOH (1:1) mixture after which sample was added to the cartridge. The neutral lipid fraction was eluted with 8 mL of chloroform, and glycolipids were eluted with 8 mL of acetone, after which the fraction was discarded. Phospholipid fatty acids were eluted with 8 mL of methanol.

The PLFA fraction was evaporated to emptiness under nitrogen (N_2) flow, which after it was dissolved to 1 mL of chloroform. 300 μL subsample was transferred into a pre-weighed tin cup and evaporated. The rest of the sample was stored at −20°C to wait for further processing. Chloroform was evaporated and the tin cup was weighted. $\delta^{13}C$ of the PLFA sample was measured with a Thermo Finnigan DELTA^{plus}Advantage *CF*-IRMS at the University of Jyväskylä, Finland. Based on quantified $\delta^{13}C$ values of bulk PLFA sample, we calculated bacterial growth efficiency (BGE) as described in del Giorgio and Cole (1998) and the total decomposition rate for each substrate.

The rest of the divided PLFA fraction (700 μL) was evaporated under nitrogen flow after which 1 mL of hexane and 2 mL of 1% H_2SO_4 were added. Tubes were flushed under nitrogen flow for 5 s and incubated at 90°C for 90 min. After incubation, 1.5 mL of H_2O and 4 mL of hexane were added. Tubes were vortexed and centrifuged at 3,000 rpm for 3 min. The upper phase was transferred into a new Kimax® tube. The collected phase was evaporated under nitrogen flow. The sample was dissolved by 500 μL of hexane and transferred into a small vial. The sample was still concentrated before analysis by evaporating it and dissolving it in 100 μL of hexane.

PLFAs were analyzed by combined gas chromatography and mass spectrometer (GC–MS). The length of a column (DB-23) was 30 metres and the diameter was 250 μM. The column film was 0.25 μM thick. The splitless mode was used for the mass spectrum. The injection temperature was 260°C. Total helium flow was 47.4 mL/min. The initial temperature of gas chromatography was 60°C and it was held for 1 min, after which the temperature was raised to 130°C and further to 180°C, and further to 220°C. The running time was 47 min per sample. Four different concentrations of the GLC Reference standard (Nu-Chek Prep Inc.) were prepared and analyzed to create a standard curve. Fatty acids were identified and integrated with GC Solution Postrun -software (Shimadzu). Based on the standard curve and recovery of internal standard, the amount of fatty acids in a sample was calculated as mg/g of carbon. After running PLFA samples on GC–MS, samples were evaporated under nitrogen flow and dissolved in 70 μL. Samples were sent to Austria where they were analyzed by

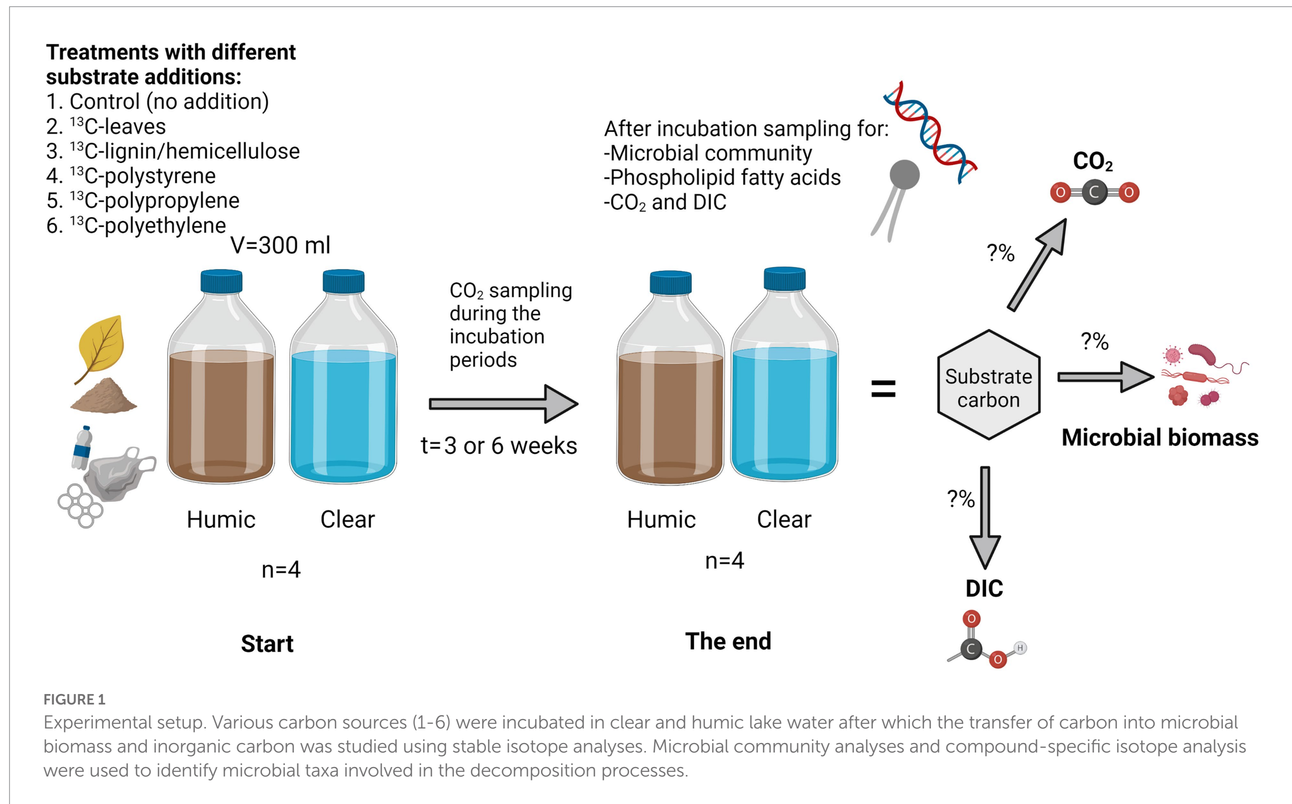

FIGURE 1
Experimental setup. Various carbon sources (1-6) were incubated in clear and humic lake water after which the transfer of carbon into microbial biomass and inorganic carbon was studied using stable isotope analyses. Microbial community analyses and compound-specific isotope analysis were used to identify microbial taxa involved in the decomposition processes.

using compound-specific isotope analysis (CSIA). The CSIA run was performed as in Taipale et al. (2019). Assimilation calculations are described in the Supplementary material.

Decomposition rate and biochemical fate calculations

The decomposition rate of each substrate per year was calculated as a sum of the total mineralization rate per year and the assimilation rate per year. To convert this to months, the decomposition rate per year was divided by 12. Moreover, to determine total decomposition time as years or as months, 100 was divided by the percentual decomposition rate per year or month.

Proportional biochemical fate of decomposed substrate carbon in CO2, DIC, or biomass was calculated by dividing the mineralization or assimilation rates by total decomposition rate and multiplying it by 100 to get percent values.

DNA and RNA analysis, and sequence data processing

At the end of the experiments, a 10–30 mL subsample of water was filtered through a pore size of 0.2 µM (Supor® 0.2 µM/25 mm, PES, Pall Corporation), and the filter was transferred into a bashing bead lysis tube (ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm), Nordic BioSite, United States) with 800 µL of DNA/RNA Shield™ (Zymo Research, United States). Samples were homogenized by bead-beating at 5.5 m/s for 40 s (Bead Ruptor Elite 24 bead mill homogenizer, Omni International Inc., United States).

DNA and RNA were simultaneously extracted by Chemagic™ 360 (PerkinElmer Inc., United States). For bacterial community analysis, cDNA was synthesized from RNA by using Maxima First-Strand Synthesis Kit with dsDNase (Thermo Fischer Scientific). Genomic DNA was eliminated by adding 0.5 µL of 10x dsDNase buffer and dsDNase and 1.5 µL of H_2O and 2.5 µL of the sample. Samples were incubated at 37°C for 30 min after which it was incubated at 65°C for 5 min. During the last incubation, 0.5 µL of random hexamer primers and dNTPs and 1.5 µL of H_2O were added. Samples were cool down to 4°C for 1 min after which 2 µL of 5x reverse transcriptase buffer and 0.5 µL of reverse transcriptase were added. Samples were incubated at 25°C for 10 min after which the temperature was raised to 50°C for 30 min, then heated to 85°C for 5 min and cooled down to 4°C.

PCR reactions were prepared by mixing 12.5 µL Maxima SYBRGreen/Fluorescein qPCR (2X) Master mix (Thermo Fisher Scientific, Lithuania. Polymerase: Hot Start Taq DNA polymerase), 2.5 µL of bovine serum album (BSA, 1 mg/mL), 1 µL of primers (10 µM P1-ITS4 and M13-ITS7 (Mäki et al., 2016) (Merck, CCTCTCTATGGGCAGTCGG-TGATCCTCCGCTTATTGATA TGC and TGTAAAACGACGGCCAGTG-TGARTCATCGAAT CTTTG, respectively) for fungi and P1-806R and M13-515FY (Merck, CCTCTCTATGGGCAGTCGG-TGATGGACTACNVG

GGTWTCTAAT and TGTAAAACGACGGCCAGTG-TGYCAG CMGCCGCGGTAA, respectively) for bacteria) and 2 µL of extracted DNA or synthesized cDNA, which was used as a template for fungi and bacteria, respectively. PCR-grade H_2O was added up to the total volume of 25 µL.

For the 16S rRNA gene, the PCR reaction was initialized with heating at 95°C for 3 min, followed by the denaturation phase at 95°C for 45 s, annealing at 50°C for 1 min, and elongation phase at 72°C for 90 s. Phases from denaturation to elongation were repeated 34 times after which the reaction was kept at 72°C for 10 min before cooling down to 4°C. For ITS, the PCR reaction was initialized with heating at 95°C for 3 min, followed by the denaturation phase at 95°C for 30 s, annealing at 55°C for 30 s, and the elongation phase at 72°C for 45 s. Phases from denaturation to elongation were repeated 35 times after which the reaction was kept at 72°C for 7 min before cooling down to 10°C. The size of the PCR amplicons was checked by running 5 µL of amplicons on 1% agarose gel (120 V for 45 min).

Sample barcoding was performed as PCR described above but using 1 µL of PCR amplicons from the first reaction as a template and with barcoded fusion primer IonA-M13F and P1-806R or IonA-M13F and P1-ITS4. The second PCR was started with heating samples up to 95°C for 3 min, followed by 10 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s and elongation at 72°C for 1 min. After 10 cycles, the temperature was kept at 72°C for 5 min before cooling down to 4°C. After the reaction, barcoded PCR amplicons were run on 1% agarose gel (120 V for 45 min). Barcoded PCR amplicon size was checked by agarose gel electrophoresis. Samples with low DNA content were purified and concentrated with SparQ Quanta purification beads (SparQ PureMag Beads, Quantabio) according to the manufacturer's protocol. Amplicons were quantified with Qubit (Qubit Fluorometric Quantification, Thermo Fischer Scientific) and pooled equimolarly. Pooled library was purified by SparQ Quanta using 1.3x ratio (v/v), quantified by 2200 TapeStation system (Agilent Technologies, United States), and sequenced by Ion Torrent Personal Genome Machine (PGM) (Life Technologies, United States) using IonPGM Hi-Q View OT2 400 kit and Sequencing kit with a 318 IonChip (Thermo Fisher Scientific). Sequences were deposited to the NCBI Sequence Read Archive as project PRJNA810989.

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The sequence data from the IonTorrent server was processed by the CLC Microbial genomics module (CLC Genomic Workbench 12 with microbial genomics module, Qiagen, Denmark). 16S sequences were filtered based on the presence of both forward and reverse primers and ITS sequences based on the presence of the forward primer. In addition, sequences shorter than 250 bp and longer than 450 were discarded. The trimmed data was further subsampled using thresholds to 20000 and 18000 reads for 16S and ITS sequences, respectively. The minimum occurrence of 1 was set for OTUs before clustering and the creation of new OTUs with 80% taxonomic similarity was allowed. For the reference-based OTU clustering, the SILVA 16S v132 database at 99% resolution sequences and UNITE v7.2 database at 99% resolution were used for bacterial and fungal sequences, respectively. OTUs whose appearance was less than 1% were discarded from further data processing. "N/A" OTUs were checked to be fungi by aligning sequences against the RDP Classifier database (RDP Naive Bayesian rRNA Classifier Version 2.11, September 2015; UNITE fungal ITS trainset 07-04-2014) with an 80% confidence threshold.

Statistical testing

PERMANOVA for microbial community and CSIA data was carried out in PRIMER7. Data were square root transformed and Bray–Curtis similarity was used to create resemblance tables. Non-metric multidimensional scaling (NMDS) analysis was performed to unravel microbial genera correlating with $\delta^{13}C$-values of PLFAs. Differences in mineralization rates, biomass assimilation, degradation rate, and water quality parameters were conducted by pair-wise PERMANOVA in PRIMER7. The used confidence level for all tests was 95%.

Results

Mineralization and assimilation rates of substrates and biochemical fate

Mineralization of ^{13}C-labeled substrates into the gas phase, indicative of microbial respiration, was measured during the experiments and calculated by subtracting the average $\delta^{13}C$ value of the control group from the $\delta^{13}C$ of each treatment: $Mineralization_{gas} = \delta^{13}C_{control} - \delta^{13}C_{treatment}$. Total ^{13}C-labelled leaves and hemicellulose were rapidly mineralized in both humic and clear lake water, whereas recalcitrant lignin and microplastics were mineralized slowly or not at all. In humic lake water, mineralization of carbon, especially from leaves and hemicellulose, increased rapidly during the first week, after which the isotopic signal reached a steady-state (Figures 2A,B). In clear lake water, the mineralization of these natural substrates was not as rapid, and the ^{13}C-enrichment increased during the whole experiment. Among plastic treatments, only the polystyrene carbon was mineralized during the experiment (Figure 2C).

Total mineralization rates were calculated at the end of the experiment. Leaves were mineralized at two times faster rate in humic (9.0 ± 1.2% per month) than in clear-lake water (4.0 ± 0.8% per month), and with statistically significant

difference (pair-wise PERMANOVA: $p = 0.028$, $t = 6.8505$, Figure 3A). Hemicellulose was effectively mineralized during the experiment at the rate of 11.9 ± 0.9% and 7.3 ± 0.8% per month in humic and clear lake water, respectively (Figure 3B), whereas lignin from the added lignin-hemicellulose substrate was not mineralized at all (Figure 3C). Overall, microplastics were mineralized slowly or not at all (humic PS: 0.22 ± 0.02 ‰ per year; clear PS: 0.17 ± 0.02 ‰ per year; humic PP: not mineralized; clear PP 0.006 ± 0.004 ‰ per year; humic PE: 0.003 ± 0.006 ‰ per year; clear PE: 0.005 ± 0.009 ‰ per year; Figures 3D–F). Nevertheless, the mineralization rate of PS was significantly faster in humic lake water than in clear lake water ($p = 0.026$; $t = 3.8699$).

The assimilation rate of ^{13}C-labeled substrate carbon into microbial biomass was quantified by measuring the ^{13}C-enrichment and concentration of microbial PLFAs. Microbes utilized carbon from leaves at the rate of 14 ± 8% and 51 ± 42% per month in humic and clear lake water, respectively (Figure 3A). In addition, bacterial growth efficiency (BGE; (Steffens et al., 2015); Supplementary Figure S1) was significantly higher in clear than in humic lake water for leaves ($p = 0.035$, $t = 2.920$). Microbes utilized carbon from lignin at the rate of 8 ± 9% and 12 ± 8% per month in humic and clear lake waters, respectively. PS carbon was assimilated at the rate of 0.2 ± 0.1% and 0.1 ± 0.01% per year in humic and clear lake waters (Figure 3D) and the BGE of PS was significantly higher in humic than in clear lake water for ($p = 0.032$, $t = 4.697$). In humic lake water, PP carbon was not assimilated at all, whereas microbes assimilated PP carbon at the rate of 0.003 ± 0.002% per year in clear lake water. Microbes assimilated PE carbon at the rate of 0.1 ± 0.1% and 0.04 ± 0.05% per year in humic and clear lake waters, respectively (Figure 3F).

The biochemical fate of substrate-derived carbon was calculated by combining stable isotope measurements of microbial biomass, CO_2, and DIC, and calculating percentual proportions of decomposed substrate carbon in each end-product. In all treatments, substrate carbon was assimilated into microbial biomass rather than respired. However, in humic lake water, only 58 ± 14% of decomposed ^{13}C-leaves, and 65 ± 33% of ^{13}C-hemicellulose were assimilated into microbial biomass, whereas in clear lake water, 91 ± 4% of decomposed ^{13}C-leaves and 88 ± 8% of ^{13}C-hemicellulose were assimilated into biomass (Figure 4A). In contrast, all the decomposed lignin carbon ended up in microbial biomass in both lake waters. ^{13}C-microplastics were also mainly assimilated into biomass (PS humic 89 ± 4%; PS clear 78 ± 3%; PP humic not decomposed; PP clear 74 ± 17%; PE humic 99.9 ± 0.2%; PE clear 82 ± 31%; Figure 4B).

Decomposition rates of natural substrates did not differ between studied lake waters, whereas PS and PE were decomposed faster in humic lake water (Table 1). Leaves were decomposed within 5 ± 2 months in humic and 3 ± 2 months in clear lake water. Hemicellulose was decomposed at the same speed as leaves from 1 to 6 months and from 1 to 2 months in humic and clear lake water, respectively, whereas lignin from hemicellulose was decomposed within 1 or 2 years or was not decomposed at all during the experiment. PS was decomposed within 500 ± 150 years in humic and 1,300 ± 250 years in clear lake water. PP was not decomposed at all in humic lake water and in clear lake water its decomposition rate was defined to be higher than 10,000 years. In contrast, PE was decomposed within 2,350 ± 1,350 years in humic lake water whereas in clear lake water PE decomposition rate is at least 1,100 years but varies up to over 10,000 years, similarly to PP decomposition.

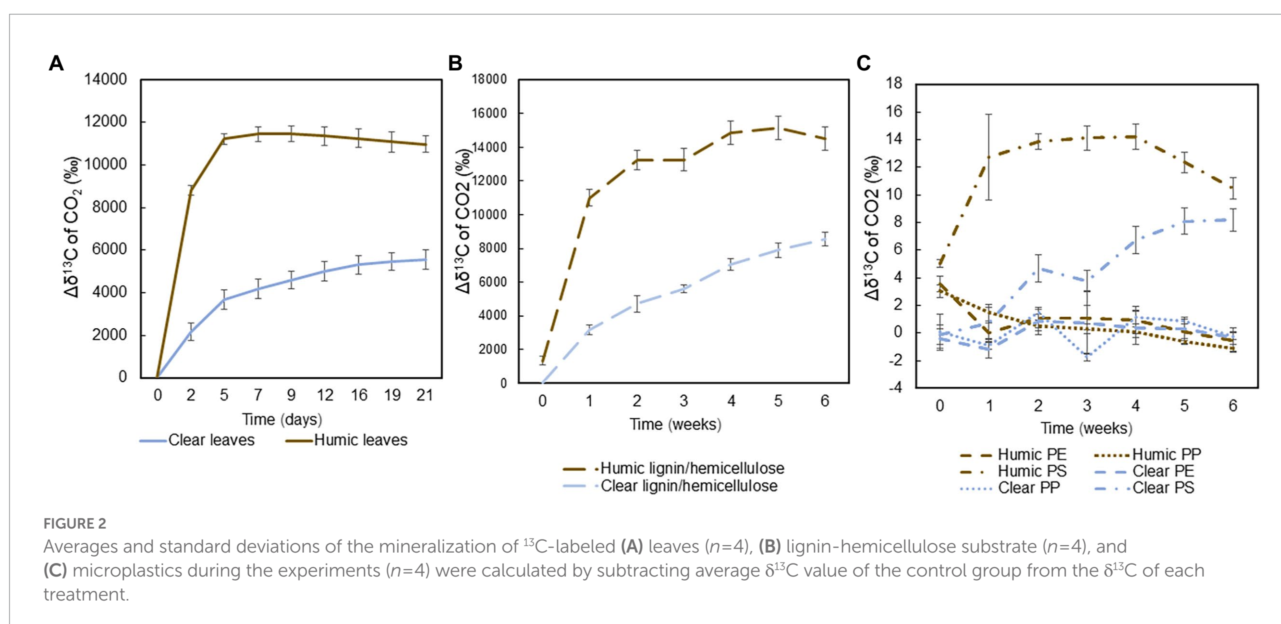

FIGURE 2
Averages and standard deviations of the mineralization of ^{13}C-labeled **(A)** leaves (n=4), **(B)** lignin-hemicellulose substrate (n=4), and **(C)** microplastics during the experiments (n=4) were calculated by subtracting average $\delta^{13}C$ value of the control group from the $\delta^{13}C$ of each treatment.

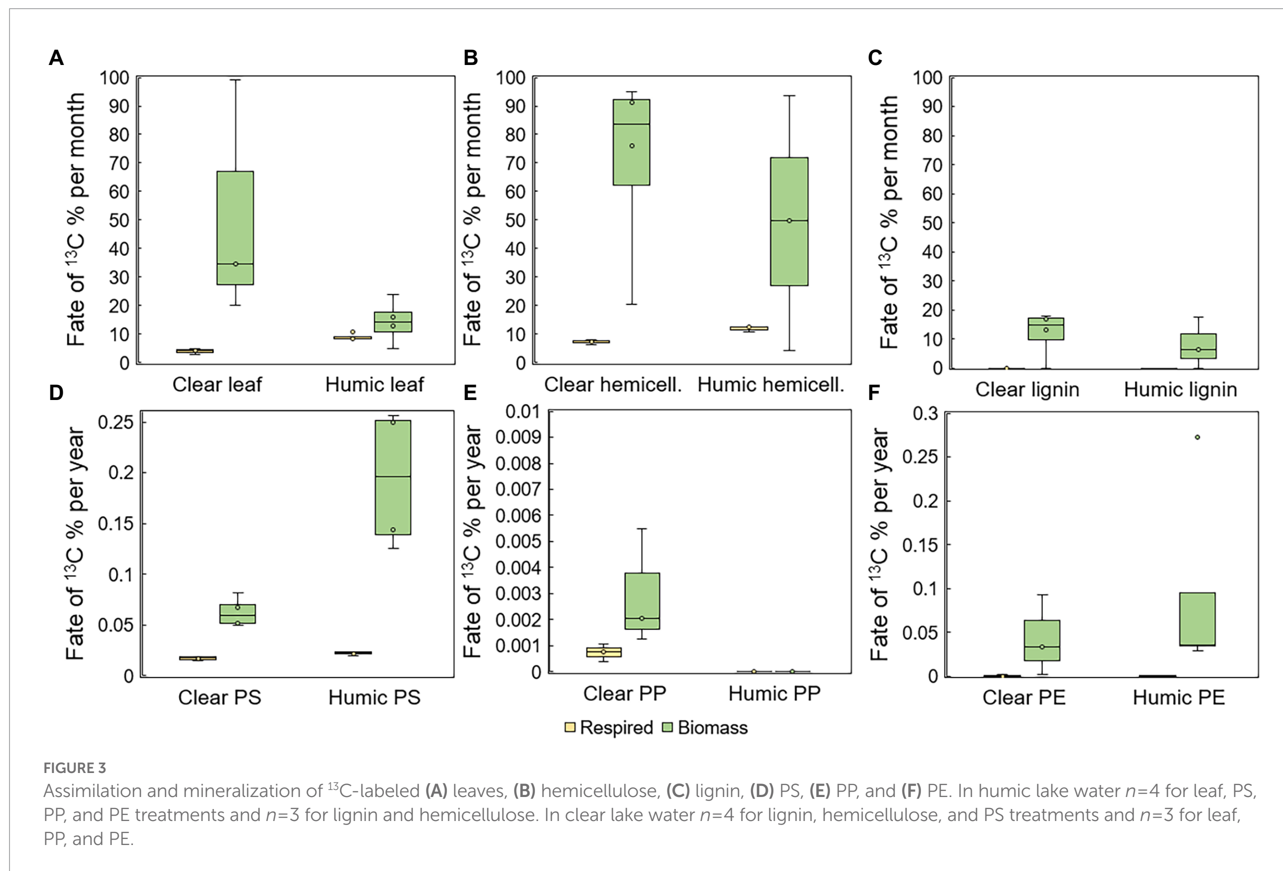

FIGURE 3
Assimilation and mineralization of ^{13}C-labeled **(A)** leaves, **(B)** hemicellulose, **(C)** lignin, **(D)** PS, **(E)** PP, and **(F)** PE. In humic lake water $n=4$ for leaf, PS, PP, and PE treatments and $n=3$ for lignin and hemicellulose. In clear lake water $n=4$ for lignin, hemicellulose, and PS treatments and $n=3$ for leaf, PP, and PE.

Identification of substrate decomposers by combining community data and CSIA

Differences between $\delta^{13}C$-values of treatments and controls were used to identify the most efficient decomposers of each substrate (Figures 5A–D). In addition, to identify decomposers more accurately, assimilated ^{13}C from the added substrate to each PLFAs was calculated as $\Delta\delta^{13}C = \delta^{13}C\text{-PLFA}_{treatment} - \delta^{13}C\text{-PLFA}_{control}$ and $\Delta\delta^{13}C$-results were combined with microbial community data in NMDS ordination analysis (Figure 6). In clear lake water leaf treatment, BrSFAs, i15:0, a15:0, 16:1ω7, 18:1ω9, and 18:1ω7 had higher δ13C-values in comparison to control ($p=0.033$, $t=2.22$; $p=0.031$, $t=5.54$; $p=0.034$, $t=5.59$; $p=0.026$, $t=10.86$; $p=0.034$, $t=15{,}48$; $p=0.027$, $t=4.63$). In humic lake water leaf treatment, all detected PLFAs had higher $\delta^{13}C$-values than the control ($p<0.05$). NMDS ordination analysis of leaf samples showed that especially $\Delta\delta^{13}C$ values of 16:1ω7, i14:0, 18:1ω9, BrSFA, 16:1ω9, and 18:1ω7 correlated (Pearson correlation) with humic lake water samples. Several bacterial genera correlated with humic lake water samples and were identified to belong to Alpha-, Delta-, and Gammaproteobacteria, Bacteroidetes, Planctomycetes, Verrucomicrobia, and Chloroflexi. $\Delta\delta^{13}C$ values of 16:1ω7, which is characteristic for Alpha- and Gammaproteobacteria and major fatty acid of *Arcicella* sp. (Bacteroidetes) (Kämpfer et al., 2009; Hahn et al., 2010; Taipale et al., 2019), correlated with five genera from the family Burkholderiaceae, and *Arcicella* sp. (Bacteroidetes). In addition, the involvement of two uncultured Planctomycetes in the decomposition process of leaves in humic lake water was exposed by the correlation of high $\Delta\delta^{13}C$ values of 18:1ω9 with Planctomycetes OTUs. Only one fungal genus *Rhodotorula* sp. (Basidiomycota) correlated with $\Delta\delta^{13}C$ values of 18:1ω9, characteristic to fungi Planctomycetes (Willers et al., 2015; Taipale et al., 2019).

In clear lake water lignin-hemicellulose treatment PLFAs i14:0, a15:0, 16:1ω7, and 18:1ω7 had higher $\delta^{13}C$-values than control ($p=0.041$, $t=3.26$; $p=0.041$, $t=2.01$; $p=0.026$, $t=3.32$; $p=0.029$, $t=4.00$). In contrast, only $\delta^{13}C$-values of i14:0, i15:0, and a15:0 differed from control in humic lake water lignin-hemicellulose treatment ($p=0.025$, $t=2.74$; $p=0.034$, $t=4.96$; $p=0.023$, $t=5.64$). Only bacterial taxa correlated towards high $\Delta\delta^{13}C$ values in lignin-hemicellulose treatment. These bacterial genera belonged to Bacteroidetes, Planctomycetes, Alphaproteobacteria, and Verrucomicrobia.

In humic PS treatment, 16:1ω9 was the only PLFA whose $\delta^{13}C$-values differed from the control ($p=0.031$, $t=2.68$). In contrast, $\delta^{13}C$-values of i14:0, i15:0, 16:1ω9, 16:1ω7, 18:1ω9, and 18:1ω7 in clear lake water PS treatment differed from control ($p=00.27$, $t=4.27$; $p=0.019$, $t=3.77$; $p=0.047$, $t=3.05$; $p=0.025$, $t=6.95$; $p=0.043$, $t=t=3.07$; $p=0.031$, $t=7.89$, respectively). The highest $\delta^{13}C$-values were observed in 16:1ω7 and 18:1ω7, characteristic to Alpha- and Gammaproteobacteria (Taipale et al., 2019). NMDS ordination analysis for lake waters with PS addition

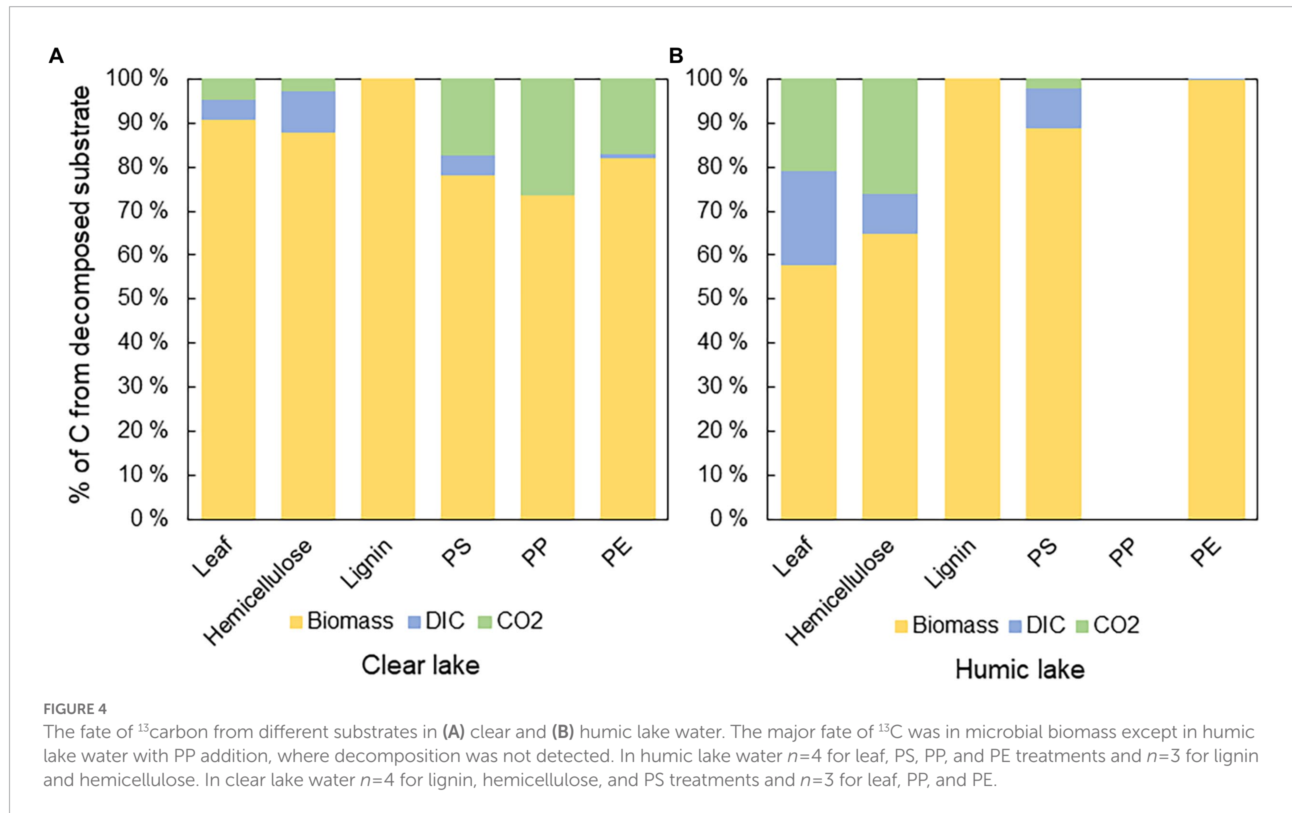

FIGURE 4
The fate of ^{13}carbon from different substrates in **(A)** clear and **(B)** humic lake water. The major fate of ^{13}C was in microbial biomass except in humic lake water with PP addition, where decomposition was not detected. In humic lake water *n*=4 for leaf, PS, PP, and PE treatments and *n*=3 for lignin and hemicellulose. In clear lake water *n*=4 for lignin, hemicellulose, and PS treatments and *n*=3 for leaf, PP, and PE.

TABLE 1 Decomposition rates and main decomposers of different substrates in humic and clear lake water.

Substrate	Decomposition rate as years or months (humic)	Decomposition rate as years or months (clear)	Main decomposers
Leaf	5 ± 2 months	3 ± 2 months	Bacteria (Burkholderiaceae and *Arcicella* sp.)
Lignin-hemicellulose	See separately below	See separately below	Bacteria
Hemicellulose	From 1 to 6 months	From 1 to 2 months	Not separated from lignin-hemicellulose
Lignin	1 year or not degraded at all	2 ± 1 or not degraded at all	Not separated from lignin-hemicellulose
PS	500 ± 150 years	1,300 ± 250 years	Alpha- and Gammaproteobacteria; potentially family Burkholderiaceae
PP	Not degraded	>10,000 years	Planctomycetes
PE	2,350 ± 1,350 years	From 1,100 to >10,000 years	Planctomycetes

showed that high $\Delta\delta^{13}$C values of 16:1ω7 and 18:1ω7 correlated highly (>0.9) with seven genera from class Alphaproteobacteria, identified as *Elstera* sp., *Novosphingobium* sp., AT-s3-44 (Sneathiellaceae), uncultured Rhodospirillales, *Hirschia* sp., *Reyranella* sp., and *Bosea* sp., and six genera belonging to Gammaproteobacteria, identified as *Malikia* sp., *Pelomonas* sp., *Hydrogenophaga* sp., *Hydrocarboniphaga* sp., uncultured Burkholderiaceae, and *Polynucleobacter* sp. Among Gammaproteobacteria, five of six potential PS decomposers belong to the family Burkholderiaceae. Moreover, highly positive regression ($R^2=0.986$) was exposed between the amount of detected *Hydrocarboniphaga* sp. (Gammaproteobacteria) sequences and mineralization rate in clear lake water (Supplementary Figure S3).

In clear PE and PP treatments, only i14:0 had significantly higher δ^{13}C-values in comparison to control ($p=0.026$, $t=5.38$; $p=0.027$, $t=4.03$, respectively). In humic PE and PP treatments, 16:1ω9 and 18:1ω9 had higher δ^{13}C-values in comparison to control (PE: $p=0.026$, $t=3.01$; $p=0.03$, $t=2.74$; PP: $p=0.032$, $t=4.04$; $p=0.03$, $t=3.65$, respectively) 16:1ω9 is typical to Spumella-like flagellates (Taipale et al., 2019) and 18:1ω9 is characteristic to Planctomycetes and fungi (Willers et al., 2015; Taipale et al., 2019), suggesting that these microbial groups can decompose aliphatic microplastics in humic lakes. NMDS ordination analysis showed that these PLFAs correlated with two and three fungal OTUs from phylum Basidiomycota in PP and PE treatments, respectively. Furthermore, high $\Delta\delta^{13}$C values of 18:1ω9 correlated with *Singulisphaera* sp., I-8 (Phycisphaeraceae), and an uncultured

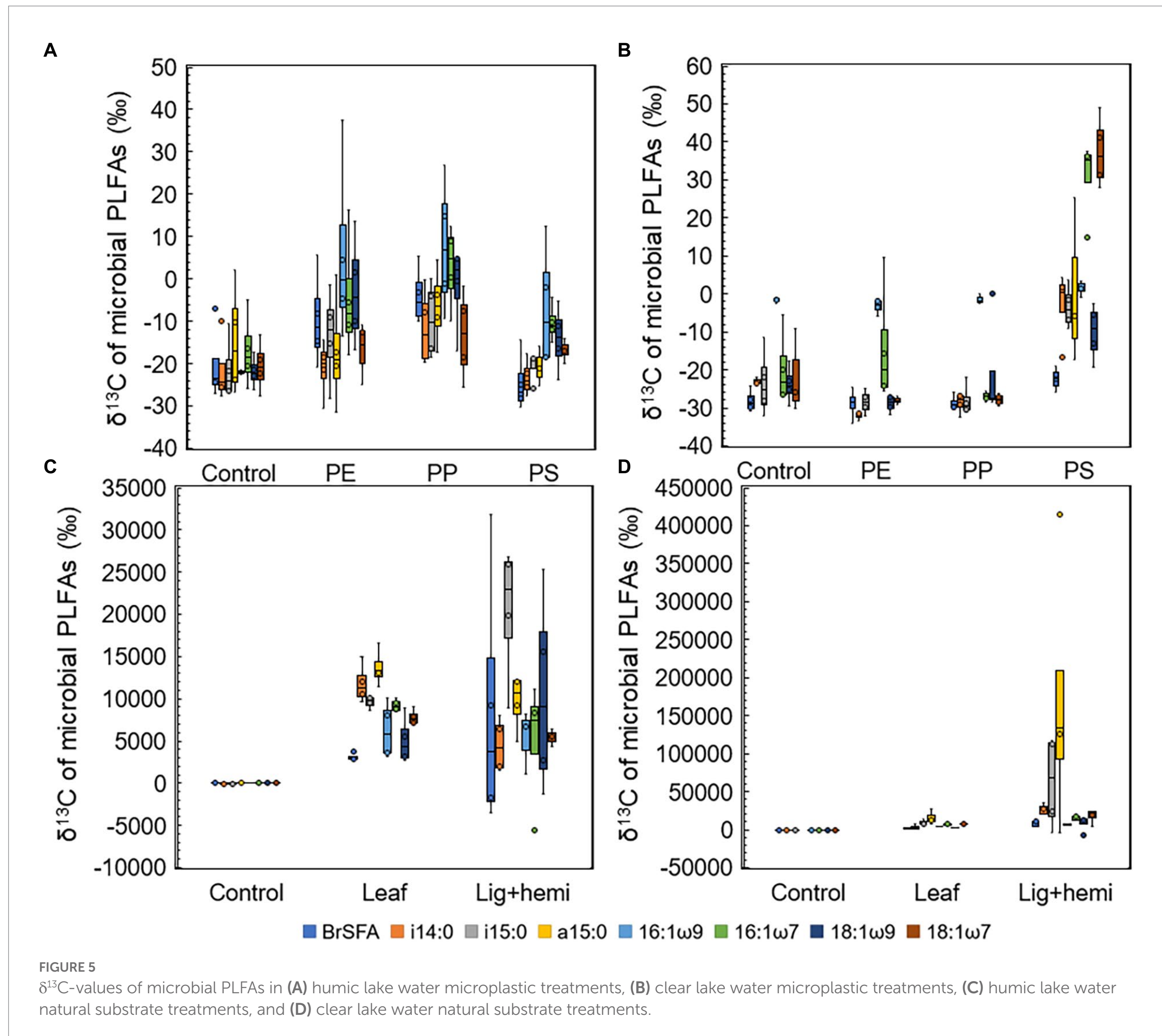

FIGURE 5
δ^{13}C-values of microbial PLFAs in **(A)** humic lake water microplastic treatments, **(B)** clear lake water microplastic treatments, **(C)** humic lake water natural substrate treatments, and **(D)** clear lake water natural substrate treatments.

BD7-11 in the PP treatment and with uncultured Pirellulaceae, *Singulisphaera* sp., I-8 (Phycisphaeraceae), and uncultured BD7-11 in PE treatment.

Discussion

Mineralization and assimilation rates are dependent on substrate recalcitrance

Leaves, hemicellulose, and PS were mineralized faster in humic than in clear lake water, whereas other substrates were mineralized in both studied lake waters with similar rates. Environmental parameters shape aquatic microbial community structure and affect microbial ability to utilize different carbon sources (Wang et al., 2018; Yang et al., 2020; Meng et al., 2022). For example in humic lakes, heterotrophic microbial lifestyle dominates over autotrophy (Jansson et al., 2007), which likely increases competition over carbon sources and favors microbes that can effectively utilize carbon sources when they enter a lake ecosystem. Thus, humic lake water microbes have adapted to utilize easy carbon sources fast after they enter lake water. In contrast, clear lakes have naturally more labile organic carbon sources (Taipale and Sonninen, 2009), which could explain why the mineralization rates of the added easily degradable substrates were slower in clear lake water. Nevertheless, in both studied lake waters, high mineralization rates of leaves and hemicellulose indicated that easily degradable carbon sources are rapidly utilized by freshwater microbes and respired. In addition, also PS carbon can be mineralized although the rate is very low.

Polymers containing aromatic rings are highly recalcitrant and common in humic substances and thus commonly present in humic aquatic ecosystems. Since humic lakes are typically poor in labile carbon sources (Hessen and Tranvik, 1998), we hypothesized that humic lake water microbes have adapted to utilize recalcitrant aromatic compounds. Against our hypothesis, decomposition

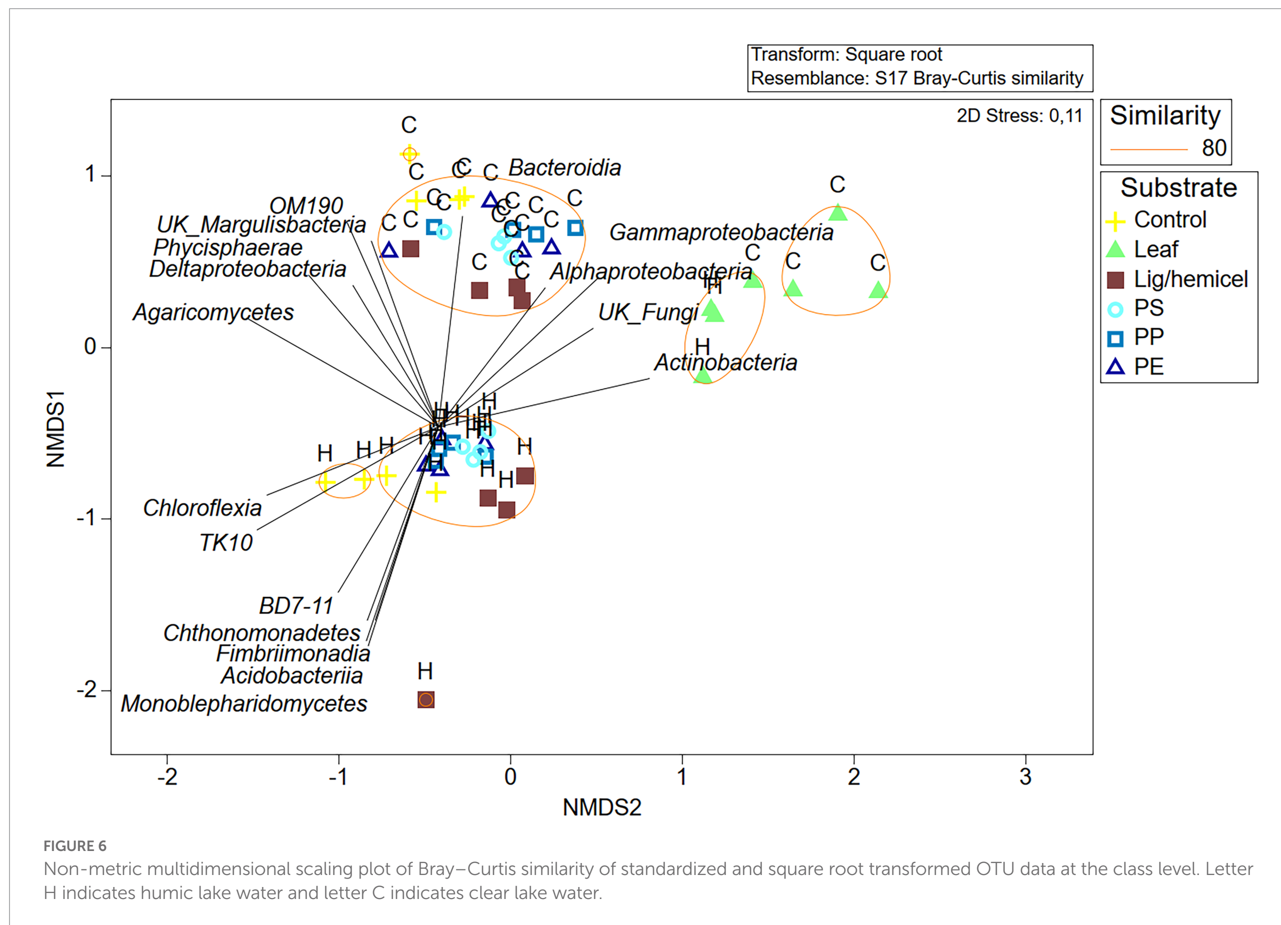

FIGURE 6
Non-metric multidimensional scaling plot of Bray–Curtis similarity of standardized and square root transformed OTU data at the class level. Letter H indicates humic lake water and letter C indicates clear lake water.

rates of PE and PS did not differ significantly in clear lake water. However, the decomposition rate of aromatic PS was faster than the decomposition of aliphatic PE and PP in humic lake water. Unlike PE and PP, PS is denser than water, which allows it to submerge (Anderson et al., 2016) and microbes to attach to its surface more easily. In nature, polystyrene can end up in sediment (Anderson et al., 2016), where also anaerobic microbes could utilize it as a carbon source. Decomposition rates of microplastic carbon in nature can be higher than what our results suggest since photodegradation increases plastic degradation in water (Tian et al., 2019) together with thermo-oxidation (Anderson et al., 2016).

Our results show that natural carbon substrates – even lignin – are utilized faster than synthetic polymer carbon in both lake water types. The mineralization rates of studied substrates indicated that humic lake water microbes can be more efficient to mineralize both easier and more recalcitrant carbon sources than clear lake water microbes are. However, only PS carbon was assimilated faster into microbial biomass in humic than in clear lake water, whereas other substrates were equally assimilated into microbial PLFAs in clear and humic lake waters. In addition, the assimilation rate of PS carbon was not significantly different in comparison to PE. It seems that PS is converted more efficiently to CO_2 than PE, whereas PS and PE carbon are preferably and equally well utilized in a structural cell component. However, in this study, we measured only ^{13}C assimilation into PLFAs. Since some amino acids, e.g., phenylalanine and tyrosine, contain aromatic rings, one could assume that polymers containing aromatic rings are processed rather *via* the amino acid cycle due to the similar structures of these compounds. Thus, other assimilation pathways such as the amino acid cycle should be considered in future research as well.

The major biochemical fate of decomposed substrate carbon is microbial biomass

The chemical structure and recalcitrance of a carbon substrate play an important role in decomposition and affect its biochemical fate in nature. In accordance with our hypothesis, the major fate of all studied substrates' carbon that was decomposed was in microbial biomass, whereas mineralization was found to play only a minor role in the decomposition process of microplastics and lignin. Similar results were found for PE decomposition by the freshwater microbial community (Taipale et al., 2022) but not for PS

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decomposition by mealworms which used only minor amounts for biomass formation (Yang et al., 2015). Although the mineralization of substrate carbon covers only a small proportion of all carbon fate, measurement of δ^{13}C-values from gas could be used as an indicator for decomposition, since it is relatively easy, fast, and cheap to measure in comparison to CSIA. Nevertheless, the measurement of both mineralization and assimilation is necessary when the aim is to quantify decomposition rates.

Microbes behind the decomposition process

Bacteria are known to dominate the early-stage decomposition of plant litter due to their faster growth rates and better competition compared to fungi (Ágoston-Szabó et al., 2006) Similarly, our results showed that bacteria were the main decomposers of leaves at the early-stage decomposition process. Several PLFAs had high δ^{13}C values, but especially *Arcicella* sp. (Bacteroidetes) and five genera from the family Burkholderiaceae were identified as the major decomposer of leaves (Table 1). Moreover, bacteria are more efficient to decompose cellulose and lignin-hemicellulose than fungi in semiarid soils (Torres et al., 2014). Our results suggested that carbon from lignin-hemicellulose was assimilated mainly by bacteria also in aquatic systems. However, it should be noted that since hemicellulose is a more easily degradable substrate than lignin, it is likely that the δ^{13}C signal originated mainly from hemicellulose and only a small proportion originated from lignin, whose carbon was also shown to be assimilated into microbial biomass. Therefore, the importance of fungi in the decomposition of lignin itself cannot be excluded.

In clear lake water PS treatment, δ^{13}C values of several PLFAs differed from control and therefore indicated that several microbial groups are capable of decomposing PS. Our results suggested that Gammaproteobacteria, especially the family Burkholderiaceae, play potentially an important role in the decomposition process of polystyrene. Burkholderiaceae are known to be able to degrade aromatic compounds (Pérez-Pantoja et al., 2012) and a member of Burkholderiaceae, *Ideonella sakaiensis*, has been found to decompose polyethylene terephthalate (PET), which contains aromatic and heteroatomic structures (Yoshida et al., 2016). In humic lake water, 16:1ω9 was the only fatty acid whose δ^{13}C values were significantly different in comparison to control in humic lake water. 16:1ω9 is characteristic to eukaryotic Spumella-like flagellates, which were found to participate in PE decomposition in humic lake water (Taipale et al., 2019). Thus, our results suggest that Spumella-like flagellates could participate also in the decomposition process of PS, although it cannot be confirmed since other Eukaryota except fungi were not studied. Despite of that fungi have been recognized as potential plastic decomposers (Sánchez, 2020), fungal participation in the decomposition of PS particles was not observed in our study.

In humic lake water PE and PP treatments, δ^{13}C values of PLFAs 16:1ω9 which is characteristic to Spumella-like flagellates, and 18:1ω9, typical to fungi and Planctomycetes, differed from control. The absence of fungal biomarker 18:2ω6 suggests that Planctomycetes have a more likely ability to assimilate PE and PP carbon in comparison to fungi. Thus, against our hypothesis, fungi were not contributing to the decomposition of microplastics. However, decomposition rates of PP and PE were extremely slow in contrast to earlier studies (Taipale et al., 2019, 2022), and the assimilation of PP and PE carbon into PLFAs was likely inhibited by the presence of other easier carbon sources in lake water. Therefore, it is plausible that several freshwater bacteria can assimilate aliphatic microplastic carbon, but the high recalcitrance of microplastics and the access to easier natural carbon sources make it an unfavorable carbon source and it is therefore slowly decomposed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SRX14310857- SRX14310911.

Author contributions

ST designed the research. JV performed the research and wrote the manuscript. RN helped with bioinformatics. MT contributed microbial community analyses. MK and MP contributed and performed the CSIA. All authors discussed the results and commented on the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1041242/full#supplementary-material

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III

PLASTIC AND TERRESTRIAL ORGANIC MATTER DEGRADATION BY HUMIC LAKE MICROBIOME CONTINUES THROUGHOUT THE SEASONS

by

Jussi S. Vesamäki, Miikka B. Laine, Riitta Nissinen & Sami J. Taipale 2024

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