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Setting new standards: Multiphasic analysis of microplastic mineralization by fungi

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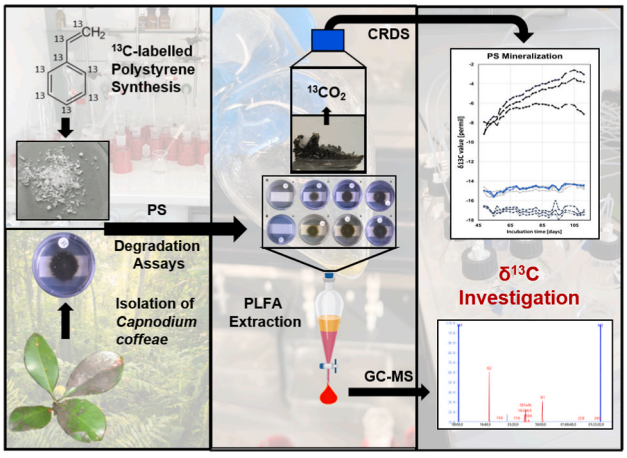
HIGHLIGHTS

- *Capnodium coffeae* develops larger colonies on MP-amended semi-solid medium.
- CRDS indicates slow but steady fungal ^{13}C-PS mineralization.
- PLFA stable isotope probing indicates minor PS-derived ^{13}C-assimilation.
- Generic workflow for identification of plastic biodegraders is presented.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic materials provide numerous benefits. However, properties such as durability and resistance to degradation that make plastic attractive for variable applications likewise foster accumulation in the environment. Fragmentation of plastics leads to the formation of potentially hazardous microplastic, of which a considerable amount derives from polystyrene. Here, we investigated the biodegradation of polystyrene by the tropical sooty mold fungus *Capnodium coffeae* in different experimental setups. Growth of *C. coffeae* was stimulated significantly when cultured in presence of plastic polymers rather than in its absence. Stable isotope tracing using ^{13}C-enriched polystyrene particles combined with cavity ring-down spectroscopy showed that the fungus mineralized polystyrene traces. However, phospholipid fatty acid stable isotope probing indicated only marginal assimilation of polystyrene-^{13}C by *C. coffeae* in liquid cultures. NMR spectroscopic analysis of residual styrene contents prior

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to and after incubation revealed negligible changes in concentration. Thus, this study suggests a plastiphilic life style of *C. coffeae* despite minor usage of plastic as a carbon source and the general capability of sooty mold fungi to stimulate polystyrene mineralization, and proposes new standards to identify and unambiguously demonstrate plastic degrading capabilities of microbes.

1. Introduction

The mass production of polystyrene (PS) started in the 1930s and paved the way for our present 'plasticized' world (Geyer, 2020; Pohlemann and Echte, 1981; Thompson et al., 2009). A century later, science has come up with a huge variety of cheap and diverse plastics for most aspects of modern life (Nielsen et al., 2020), of which PS accounts for about 6% of the European plastics market. Along with other types of plastics, PS is utilized in single-use applications, especially in packaging (Plasticeurope 2019). Improper disposal of plastics, particularly single-use products, further increase the occurrence and accumulation of this material in the environment (Schnurr et al., 2018). Plastics integrity is constantly challenged by environmental stresses (Mauel et al., 2022; Meides et al., 2021). Consequently, they are prone to break down into microplastics (MP, <5 mm), exponentially increasing the number of particles and plastic surface area in the environment (Cox et al., 2019; Kwon et al., 2017). Thus, among other things, MP formation facilitates the uptake of plastic particles into the biosphere while providing a myriad of artificial microhabitats for potentially harmful microorganisms (Gkoutselis et al., 2021, 2023; Zarus et al., 2021; Zettler et al., 2013), making MP pollution a serious threat to the environment and human health. MP is well known to shape microbial communities, to alter soil genetic functional potentials as well as, besides others, impacting gut microbiomes of soil dwelling invertebrates which *vice versa* influence MP mitigation in ecosystems (Hink et al., 2023; Holzinger et al., 2022; Rohrbach et al., 2022). Most recalcitrant fossil-based plastics have been reported to have lifespans exceeding that of humans, making PS waste a long-term environmental concern (Sonke et al., 2022). Weathering and UV-radiation have a large, measurable impact on the abiotic degradation of PS (Liu et al., 2020; Mauel et al., 2022; Meides et al., 2021). However, little is known about the potential of (micro) organisms to biodegrade PS (Ho et al., 2018).

Numerous studies have investigated the biodegradation potential of eukaryotic and prokaryotic microorganisms for different types of plastic (Gambarini et al., 2021; Sánchez, 2020). The most prominent bacterial candidate is *Ideonella sakaiensis,* which can efficiently degrade polyethylene terephthalate (PET) into its monomeric units (Yoshida et al., 2016). In addition, strains of the genera *Bacillus, Rhodococcus* and *Pseudomonas* can modify and partially degrade different types of plastics (Gambarini et al., 2021; Goudriaan et al., 2023; Jacquin et al., 2019). Similarly, it has been reported that various fungi can degrade plastics (Brunner et al., 2018; Ojha et al., 2017). The latter found that several fungi isolated from environmental plastics can degrade polyurethane (PU), while Álvarez-Barragán et al. (2016) demonstrated a complete invasion of PU foams when exposed to fungi of the genus *Cladosporium* for 12 days. In addition, other studies have reported that certain filamentous fungi and yeasts partially degrade polyethylene (PE) (Spina et al., 2021; Vaksmaa et al., 2023). Furthermore, modeling studies have hypothesized possible PE biodegradation pathways for fungal-mediated enzymes (Santacruz-Juárez et al., 2021). Accordingly, the fungal kingdom may represent a largely untapped reservoir of plastic biodegraders, including strains that can even depolymerize PS.

Fungi are particularly well suited to adopt a plastiphilic lifestyle and thus biodegrade plastic polymers due to their powerful digestive exoenzymes, strong biomass production and filamentous growth (Gkoutselis et al., 2021; Harms et al., 2011). Another advantage is their ability to secrete hydrophobins, which form chemical bonds with hydrophobic surfaces to enhance MP attachment and degradation (Ho et al., 2018; Puspitasari et al., 2021; Wösten and Scholtmeijer, 2015). Red-rot and white-rot fungi from Ascomycota and Basidiomycota have considerable degradation potential as they can utilize lignin, one of the most recalcitrant biopolymer on Earth (Bucher et al., 2004; Pointing et al., 2003). Several genera of Ascomycota are known for their ligninolytic capabilities and are therefore likely to be involved in plastic biodegradation (El-Sayed et al., 2021; Motta et al., 2009; Munir et al., 2018; Tian et al., 2017), including the ubiquitous genera *Aspergillus* (Hamed, 2013), *Penicillium* (Peterson et al., 2004) and *Trichoderma* (Ghorbani et al., 2015). In addition to woody habitats, plant leaves provide recalcitrant and hydrophobic substrates used by metabolically versatile fungi (Sridar et al., 2015; Veen et al., 2019). Therefore, the phyllosphere is a reservoir for potential xenobiotic biodegraders. Flessa et al. (2021) investigated mycobiomes of the phyllosphere from tropical and temperate regions and pointed out the dominance of sooty molds of the order Capnodiales (Dothideomycetes, Ascomycota) in epiphyllous communities (Flessa et al., 2021). We therefore hypothesized to find a promising candidate for plastic degradation among sooty mold fungi from the tropical phyllosphere.

Analysis of plastics biodegradation is generally highly prone to bias, as widely used indices such as gravimetric index and molecular weight, do not account for residual monomers or leaching additives (Ho et al., 2018; Reddy et al., 2009). However, as these can serve as an easier to use energy source for the plastic degrader in question (Stiborova et al., 2015), analytical results can be compromised (Gu, 2017). Consequently, there is an urgent need for accurate, sensitive and efficient laboratory workflows as well as analytical pipelines for investigating plastic biodegradation *in vitro*. An efficient and accurate approach to study and document mineralization of plastics can be achieved by monitoring the $\delta^{13}C$ values to determine plastic derived CO_2 evolution after ^{13}C-labelling of the test substrate (Goudriaan et al., 2023; Zumstein et al., 2018). In this way, the loss of MP and nanoplastic (NP) resulting from fragmentation rather than mineralization is eliminated. In the absence of other substrates, mineralization of plastics only affects the $\delta^{13}C$ value, minimizing disruptive effects that can also lead to plastic loss (Zumstein et al., 2018). The $\delta^{13}C$ values are generally accepted for describing the $^{13}C/^{12}C$-ratio (Gleixner et al., 1993). Two techniques dominate the measurement of $\delta^{13}C$, namely isotope ratio mass spectrometry (IRMS) and the more recent cavity ring-down spectroscopy (CRDS) (Craig, 1957; Meepho et al., 2021). To our knowledge, these techniques have never been used in series to study the biodegradation of PS by fungi, and are generally rarely used in plastic degradation studies (Nelson et al., 2022; Sander et al., 2019).

Our objective was to isolate and identify a promising fungus from tropical phyllosphere communities that degrades plastic and to investigate its PS degradation potential based on growth behavior, mineralization, and assimilation. We hypothesized that the candidate fungus, identified as *Capnodium coffeae*, Pat. (Capnodiaceae, Dothideomycetes) would be able to (1) grow on plastic as its sole carbon source, (2) bind PS and mineralize it to CO_2 and (3) assimilate PS-derived carbon. To test our hypotheses, we performed a sole carbon assay using plate-cultures and different MP types as well as a comprehensive degradation assay using ^{13}C-labeled PS in liquid culture (bioreactors). Growth behavior was observed by macroscopic imaging. In addition, adhesion and morphology of the strain were determined using scanning electron microscopy (SEM). *Capnodium coffeae*-mediated degradation and mineralization were documented using CRDS, and PS assimilation was determined using phospholipid fatty acid-based stable isotope probing (PLFA SIP). Based on our results and a sophisticated study design, we demonstrate plastiphily and *C. coffeae*-mediated biodegradation of PS

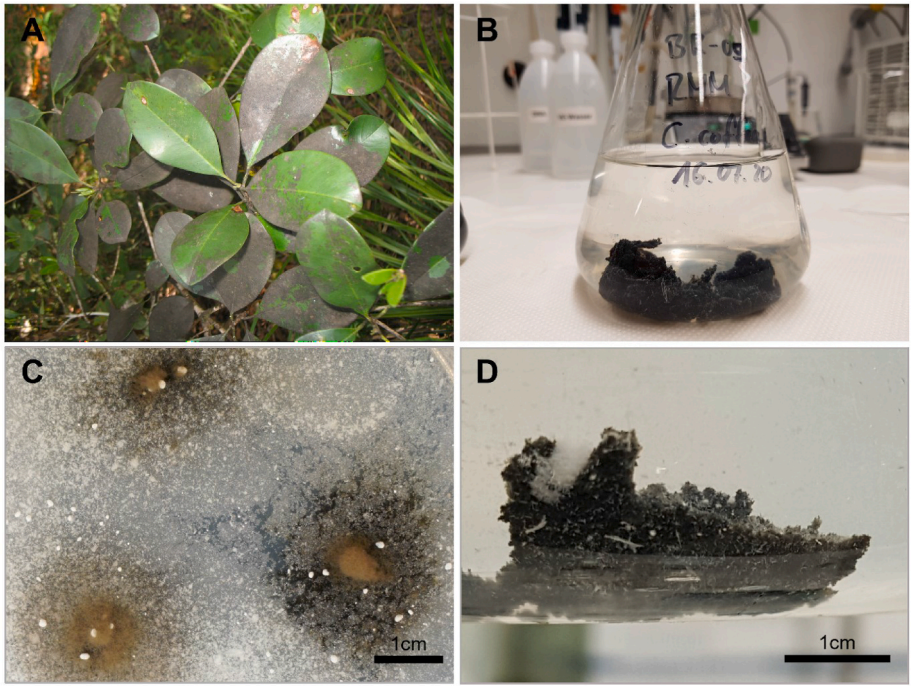

Fig. 1. ***Capnodium coffeae*** **in nature and culture A** Leaves of *Dillenia* sp. showing heavy sooty mold infestations. **B** Fresh mycelium of *C. coffeae* in liquid acclimatization medium (RMM). **C** Colonies of *C. coffeae* on PS-amended agar plates. **D** Extensive attachment of PS MP particles to mycelium in RMM bioreactors.

and propose a fundamental experimental and analytical workflow for the study of plastic degradation by fungi *in vitro*.

2. Materials and methods

2.1. Sampling and fungal isolation

In October 2006, leaves of trees of the genus *Dillenia* (Dilleniaceae) that exhibited sooty mold-like infestations were sampled in the Serra de Itabaiana National Park (Sergipe State, Brazil; Fig. 1A). Leaves were plucked with sterilized tweezers and collected in pre-labeled envelopes. In the laboratory, hyphal structures were scraped from the leaf surface, aseptically plated on potato glucose extract agar (PDA, Carl Roth, Karlsruhe, Germany) and incubated in the dark at ~25 °C for two weeks. The resulting colonies were grouped by morphotype and sub-cultured on PDA plates until axenic working cultures were obtained (Fig. 1). For long-term preservation of the strains, mycelial plugs were excised from the growth front of 7-day old PDA (PDA, Carl Roth, Karlsruhe, Germany) pure cultures of the fungi using a flamed scalpel and submerged in cryotubes containing 30% glycerol. All cryo-cultures were stored at −80 °C until further processing. The original leaf sample was deposited in the herbarium of the "Botanische Staatssammlung München (M)" under accession number M-0129165.

2.2. Molecular identification

The most common fungal morphotype (strain) was selected for molecular analysis and various plastic biodegradation assays. To identify the fungal isolate, DNA extraction was performed using the Fungal Genomic DNA Kit (BioVision, Milpitas, United States) according to the manufacturer's instructions. Briefly, three mycelial colonies from 14-day pure PDA cultures were transferred to a 2-mL Eppendorf tube, crushed in liquid nitrogen, and chemically lysed, precipitated, washed, and eluted using the reagents and buffers provided with the kit. DNA content and quality were recorded and assessed using NanoDrop® ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA). PCR for amplification of ITS1 and ITS2 regions was performed using 6.5 μL GoTaq® Colourless Master Mix (Promega, Fitchburg, United States), 5.5 μL sterile PCR grade water, 1 μL of each primer [10 μM] (ITS1F and ITS4) and 1 μL of diluted DNA extract as template (100-fold dilution with ddH_2O of original extract obtained with the Fungal Genomic DNA Kit). PCR was performed in a thermal cycler (BioRad, Hercules, United States) with the following cycling conditions: Initial denaturation at 95 °C for 3 min, followed by 33 cycles at 94 °C for 27 s (denaturation), 57 °C for 1 min (annealing), 72 °C for 1:30 min (elongation), and final elongation at 72 °C for 7 min. Amplicons were checked for quality using agarose gel electrophoresis (0.8% agarose, 0.5x TBE buffer, 80 mV), purified with a clean-up kit (CleanPCR, CleanNA Waddinxveen, Netherlands) and the DNA concentration was adjusted to 5 ng/μL Tris-HCl buffer (pH 8, 10 mM). PCR amplicons were sequenced using ITS primers (10 μM) at Eurofins Genomic Germany using Sanger sequencing technology. Sequences were assembled and analyzed using the CLC Sequence Viewer 8.0.0 software and compared to Standard Nucleotide BLAST (GenBank, NCBI nucleotide database) query results to determine similarity to reported sequences from fungal species. Sequence data were deposited in the GenBank database (GenBank Acc. No.: HE584822).

2.3. Culture media

For biomass production, the fungus was grown in Czapek Dox broth (CZB) at 23 °C with shaking at 120 rpm in the dark (Paterson and Bridge, 1994; Wiyakrutta et al., 2004). After 120 d of incubation, mycelia were rinsed twice with sterile double-distilled water to wash off the CZB medium. The mycelia were then aseptically transferred to freshly prepared rich mineral medium (RMM) (Álvarez-Barragán et al., 2016) and incubated for two weeks for vitalization prior to plastic mineralization incubations (see "Degradation assay and ^{13}C evolution measurement" below). RMM contained all macronutrients and inorganic ions relevant to fungal metabolism and growth, but no carbon source to starve the mycelia.

2.4. Growth assay

Growth assay biomaterial was cultured on PDA agar plates (Carl Roth, Karlsruhe, Germany). To evaluate the suitability of MP as a substrate and to investigate the growth behavior of the fungus in the presence of different MP types as the sole carbon source, 1 mm^3 of fresh mycelial mats from 14-day-old PDA cultures (Carl Roth, Karlsruhe, Germany) cultures were centrally inoculated onto fungal minimal medium (FMM; [g/L] K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 0.5; $MgSO_4$, 0.5; agar, 15) supplemented with 2 g/L pristine MP beads of different polymer types, and without MP (control). The polymers tested were PE [Lupolen® 1800P LyondellBassell, Rotterdam, Netherlands], polypropylene (PP) [Moplen® HP526J, LyondellBassell, Rotterdam, Netherlands], polyamide (PA) [Ultramid® A27 E, BASF, Ludwigshafen, Germany], PS [Styrolution PS 158 N/L, INEOS Styrolution®, Hamburg, Germany], PET [CleanPET® WF, Veolia, Hamburg, Germany] and polyvinyl chloride (PVC) [PVC S3268, Vinnolit, Ismaning Germany]. Polymers were provided and analyzed by commercial manufacturers as well as the CRC 1357 Microplastic, and milled using liquid nitrogen in a cryomill (Retsch GmbH, Haan, Germany) to obtain particles of around 100 μm. Plates were sealed with Parafilm® to prevent desiccation and contamination and incubated for 100 days at 15 °C in the dark. All experiments were performed in triplicates. Cultures were photographed every two weeks using a Nokia Lumia 1320 camera (Nokia, Espoo, Finland) on a stationary tripod (Supplementary Fig. S1B). Images were loaded into ImageJ 1.48v, and the area of fungal colonies was measured to characterize the polymer-specific differential growth behavior of the strain, as previously established (Reeslev and Kjoller, 1995). Statistical analyses regarding the growth assay were calculated applying SigmaPlot 13.0 (Systat Software Inc. San Jose, CA, USA) using ANOVA on ranks and Fisher-LSD method.

2.5. Synthesis of partially ^{13}C enriched PS

Partially enriched ^{13}C PS was synthesized by free radical polymerization of a mixture of styrene and uniformly ^{13}C labeled styrene (Supplementary Fig. S3). The obtained polymer with a nominal ^{13}C enrichment degree of 8 atom-% was dissolved in toluene, precipitated in methanol and dried under vacuum to remove residual monomers [for details see supplementary information and (Mauel et al., 2022)]. The residual styrene content was determined to 0.30 ‰ ± 0.10 ‰ and the polymer had a number averaged molecular weight (M_N) and weight averaged molecular weight (M_W) of 65 000 g/mol and 175 000 g/mol, respectively, and a dispersity (Đ = Mw/Mn) of 2.7, as calculated with the software EMPOWER 3 (Waters, Milford, MA, USA).

2.6. Nuclear magnetic resonance (NMR) spectroscopy

Liquid-state ^1H NMR spectroscopic experiments were performed using a Bruker Avance-III HD spectrometer (Bruker, Massachusetts, USA) equipped with a helium-cooled TCI-CryoProbe operating at a B_0 field of 16.4 T ($\nu 0 = 700.2$ MHz). The polymer samples were dissolved in deuterated tetrahydrofurane (THF-d8) after freeze-drying. For quantitative 1H measurements, single pulse excitation (SP) with a recycle delay of 1 s was used. The pulse length was 3.0 μs, corresponding to a tip angle of 30°. The spectra were referenced to tetramethylsilane (TMS).

2.7. Gel permeation chromatography

The molecular weight distribution of the synthesized ^{12}C-PS and partially ^{13}C-enriched PS was measured by gel permeation chromatography (GPC) using a Waters GPC kit equipped with a 515 HPLC pump, a 2707 Autosampler, a 2414 Refractive Index Detector and a 2998 Photodiode Array Detector (Waters GmbH, Eschborn, Germany). One guard column (ResiPore Guard, 5 × 0.75 cm, particle size 3 μm) and two separation columns (ResiPore Guard, 30 × 0.75 cm, particle size 3 μm) were used. Analysis was performed at 30 °C with THF as mobile phase and 1,2-dichlorobenzene as internal standard (IS). The molecular weights of the samples were referenced to narrowly distributed PS standards. The samples were prepared by dissolving 2 mg thereof in 2 mL THF followed by filtration with 0.2 μm syringe filters.

2.8. Degradation assay and ^{13}C evolution measurement

After acclimatization of the fungus in RMM, the mycelia were removed from the flasks, weighed under aseptic conditions, and used in the RMM bioreactors for the biodegradation experiments. For this purpose, 150 mg of fresh mycelia were incubated with either 100 mg of 8-atom% isotopically labeled (^{13}C-PS) or unlabeled (^{12}C-PS) in 100 mL RMM in 500 mL flasks (Supplementary Fig. S1A). Mycelia without PS and $^{13/12}C$-PS without mycelia served as first and second negative control, respectively (Supplementary Fig. S1A). All incubations except the second negative controls (one replicate for each type of PS) were performed in triplicates. All flasks were sealed with lids containing two valves for 1/8″ plastic tubings (UGT, Müncheberg, Germany). A circulating pump A0702 (Picarro, Santa Clara, CA, USA) was also connected. The bioreactors were pre-incubated for 45 days at room temperature (22−24 °C) on a rotary shaker at 100 rpm. Then, headspace gas was measured in 60-min intervals with an intervening purge step every 48 h over a period of 60 consecutive days (main incubation). In addition, a purge step (5 min with ambient air) was performed between measurements. Valve opening was controlled automatically by connecting the bioreactors to a multiplexer (eosMX; eosense, Dartmouth, Canada). Gas samples were measured using a g2201-i CRDS spectrometer (Picarro, Santa Clara, CA, USA). Raw data were analyzed using eosAnalyze-AC_v3.9.2_A25 software (eosense, Dartmouth, Canada).

2.9. Phospholipid fatty acid stable isotope probing

Capnodium coffeae grown with PS was further incubated for 5 months with a one-time supplementation of 1 g/L sucrose. Further, PLFA was extracted from the fungal biomass as previously described with minor modifications (Andrino et al., 2021a, 2021b; Frostegård et al., 1991). PLFAs are components of the cell membrane and must therefore be exchanged during cell maintenance (Coronado et al., 2007; Joergensen and Wichern, 2008). Consequently, PLFA-SIP also occurs under maintenance conditions without growth of the organisms, making incorporation more sensitive than other types of SIP (Neufeld et al., 2007). Approximately 100 mg (liquid medium) or 1.5 g (solid medium) of the fresh sample was mixed with 18 mL freshly prepared Bligh & Dyer (B&D) solution and 500 μL of IS 1 (Supplementary Table S1) (Bligh and Dyer, 1959). After mixing the samples horizontally at 225 rpm for 1 h, they were shaken in an ultrasonic bath for 15 min and mixed further. The samples were then centrifuged at 3000 g and 7 °C for 12 min, and the supernatant was transferred to a separatory funnel. A second extraction was performed using 6 mL B&D. To the supernatant 6 mL of $CHCl_3$ and 6 mL of citrate-buffer were added, shaken by hand for 1 min and left to sediment for 2 h prior to separation. A second liquid-liquid extraction was performed with 6 mL $CHCl_3$ and incubated overnight. In addition, a serial dilution of a multi-standard (Supplementary Table S1) was prepared by mixing 500 μL of IS1 (Supplementary Table S1) with a serial dilution of 50–1000 μL of a PLFA reference collection. These samples were dried under a constant N_2 flow. The $CHCl_3$ extracts were evaporated and transferred to a silica gel column. Three fractions were separated: Neutral lipid fatty acids with 5 mL $CHCl_3$; glycolipids with 20 mL acetone and PLFA with 40 mL methanol. The extracts were evaporated using a rotary evaporator, and the residual sample was transferred to glass vials and dried under N_2. To derivatize the fatty acids, 0.5 mL of a 0.5 M NaOH in MeOH solution was added, mixed and incubated at 100 °C for 10 min. After cooling to room temperature, 0.75 mL of a 12.5 M BF_3 solution was added and the samples were incubated at 80 °C for 15 min. The toxicity of resulting hydrofluoric acid was

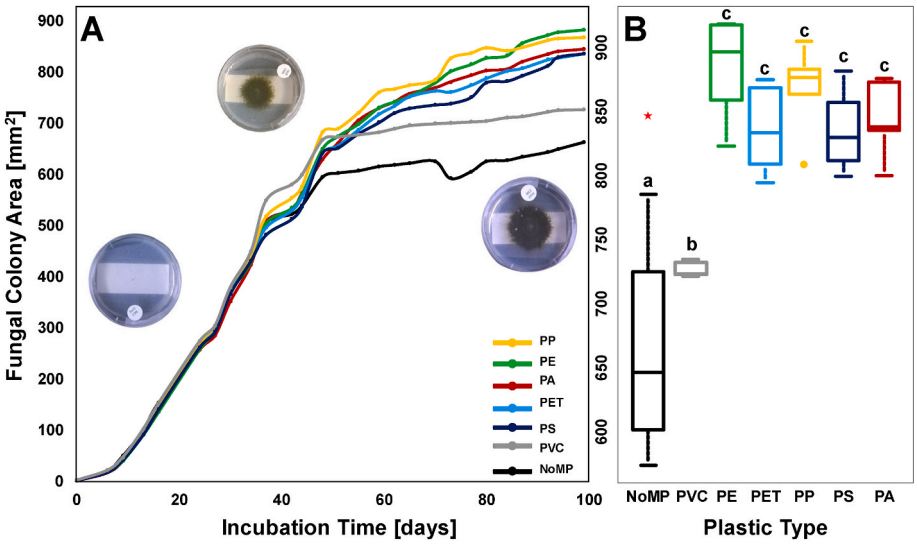

Fig. 2. Growth assay on semi-solid medium A Mean area (5 replicates) of *C. coffeae* colonies on agar plates containing semi-solid growth medium and different types of plastic. **B** Box plot of colony areas of the last measured time point after 99 days. The outlier marked as red star was excluded from box plot generation and down-stream calculations. Lower case letters indicate significant differences between different treatments (ANOVA, Fisher-LSD $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

minimized through precipitation by adding 1 mL of saturated NaCl solution. Then, 2 mL hexane was added and shaken for 3 min twice for extraction. The extracts were transferred into new vials and dried under gentle N_2 stream. After drying, 30 μL IS 2 (Supplementary Table S1) and 170 μL of toluene were added and the samples were mixed in an ultrasonic bath. Gas chromatography (GC) measurements of fatty acid methyl esters (FAMES) were performed using a HP5 column (60 m × 0.25 mm x 0.32 μm, Agilent Technologies, Santa Clara, CA, USA) in a Varian 450GC-220 Iontrap-MS (Varian Inc., Palo Alto, CA, USA) with a flow rate of 2 mL/min and a temperature gradient (Supplementary Fig. S2). For IS and multi-standard composition see supplementary data (Supplementary Table S1). Since we were only interested in the $\delta^{13}C$ value, it was not necessary to include IS recovery rates in the calculations (Boschker and Middelburg, 2002; Dungait et al., 2011).

2.9.1. Scanning electron microscopy

Fungal mycelia were collected directly from bioreactors with and without PS after 160 days of co-incubation and dried at 70 °C. The dried samples were coated with a gold layer (≈10 nm) using an S150A Sputter Coater (Edwards, Irvine, CA, USA). The prepared samples were examined using a Quanta 200 electron microscope (FEI, Hillsboro, OR, USA) with a 10 kV electron beam and different magnifications.

3. Results

3.1. Molecular and ecological characterization

The adaxial surface of the sampled *Dillenia* leaves was strongly infested by a dark green to black coating of sooty to velvety appearance (Fig. 1A). The abaxial leaf surface showed no sooty mold symptoms. Filamentous structures scraped from the leaf surface and inoculated in PDA standard medium revealed predominantly a distinct morphotype (Fig. 1B). The fungal strain was characterized by sequencing of the ITS gene. BLAST queries of the ITS sequence in the GenBank and UNITE databases unambiguously returned *Capnodium coffeae* as the best match, with 100% sequence similarity in both databases. *Capnodium coffeae* is a typical representative of the family Capnodiaceae (Capnodiales, Dothideomycetes, Ascomycota) and the paraphyletic group of sooty molds, which exhibit strongly melanized mycelia, show slow superficial growth of dense hyphal networks and have a characteristic epiphytic, i.e. epiphyllous lifestyle (Abdollahzadeh et al., 2020). *Capnodium coffeae* is considered saprotrophic on sugary exudates and nutrients that accumulate on various natural surfaces (Abdollahzadeh et al., 2020).

3.2. Differential growth behavior

During the first 40 days of the applied growth assay on MP-amended agar plates, the extension of colonies was similar in all treatment groups. After 40 days of incubation, colonies in the MP-supplemented samples, started to grow larger than in the control samples (Fig. 2). After 50 days, colonies grown on PVC-supplemented medium began to show weaker colony extension compared to the other treatments. After 100 days, a strong difference in growth was observed between MP-treatments and the controls. PE and PP had the strongest effect on colony area in this study. PA, PS, and PET stimulated colony extension in a similar way. PVC effects were less prominent. Such data suggests plastiphilicity and eventually a degradation potential of *C. coffeae* for all tested plastics. To efficiently use resources, PS was selected as a representative target plastic for further analysis due to its growth-stimulating effect, its aromatic chemical structures, better enzymatic accessibility in contrast to PE or PP, which is beneficial for biodegradation (Bergbreiter, 1994; Echte et al., 1981; Natta and Corradini, 1967). Partially enriched ^{13}C-PS was synthesized and used as substrate for incubation of *C. coffeae* in the ^{13}C-PS mineralization assay described below.

3.3. SEM of PS incubations

Observation of the mycelium from the incubations of the mineralization study showed that the fungal mycelium adheres to and accumulates PS with its thallus (Fig. 3). This biophysical interception of PS material even at the macroscopic level forms the basis for the direct proximity of plastic material and fungal biomass and suggests a plastiphilic lifestyle.

3.4. ^{13}C-PS mineralization

Since we hypothesized that *C. coffeae* is capable to metabolize PS, we conducted an experiment to investigate the evolution of $^{13}CO_2$ from ^{13}C-PS in liquid medium. CRDS measurements of incubations containing *C. coffeae* and ^{13}C-PS showed a 6 ‰ $\delta^{13}C$ increase in CO_2 and consequently an accumulation of $^{13}CO_2$ over a period of about 50 days (Fig. 4). Considering that ^{13}C-labeled PS was the sole carbon source, the

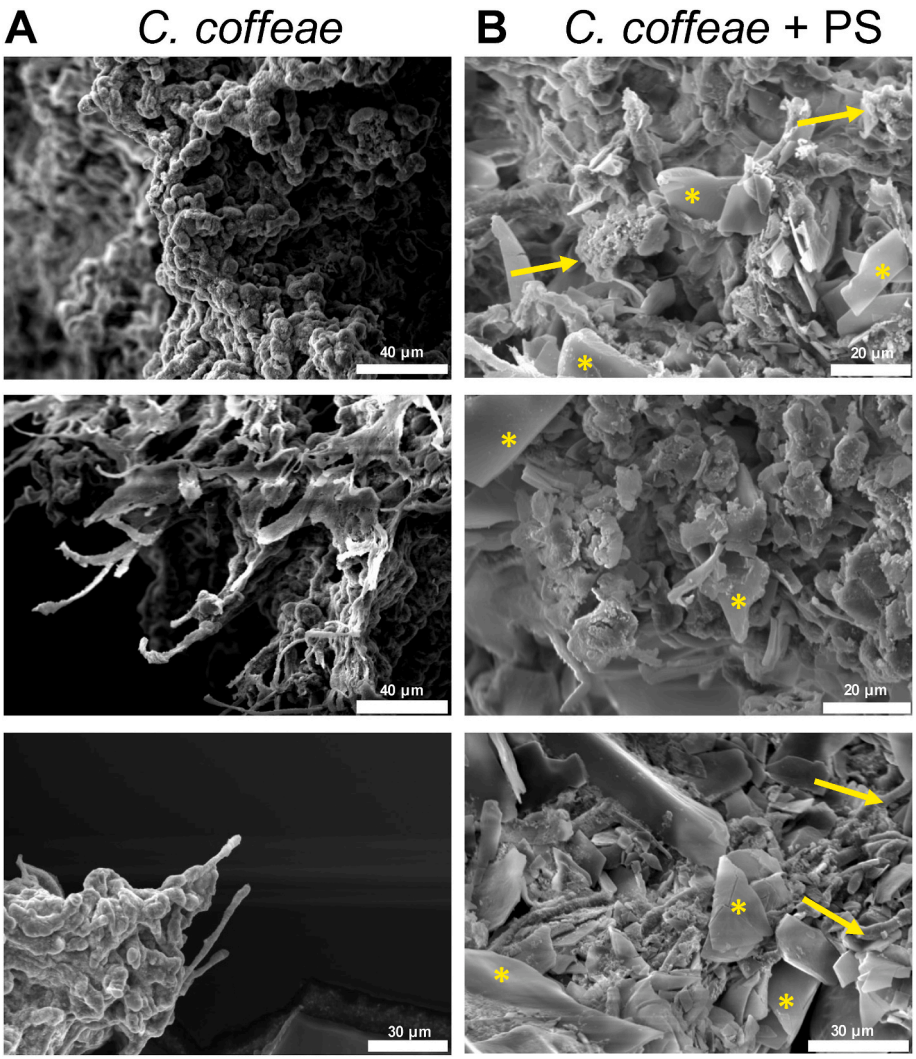

Fig. 3. SEM of *C. coffeae* within the incubations Fungal biomass treated in the same manner as in the incubations for 5 months prior to SEM imaging. **A** Hyphae of *C. coffeae* forming a dense mycelium. **B** PS material attached to the fungal hyphae forming a conglomerate. Yellow arrows point on exemplary hyphae and asterisks indicate PS material, respectively. Scale bars are given in the partial images. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CRDS measurements thus provided direct evidence of mineralization of PS or PS-derived styrene molecules. $^{13}CO_2$ evolution of treatments supplemented with ^{13}C-PS reached a plateau after 105 days, except for one replicate where the plateau occurred after 65 days. In comparison, δ^{13}C levels did not increase in the controls supplemented with ^{12}C-PS, ^{13}C-PS alone, or with the fungus alone (Fig. 4). All incubations were characterized by a constant CO_2 concentration comparable to ambient CO_2 levels (Supplementary Fig. S5), suggesting little to no growth during the incubation.

3.5. *^{13}C incorporation into the fungal biomass*

PLFA-based SIP was applied to detect possible incorporation of PS-derived ^{13}C-atoms into the fungal biomass. PLFA SIP analyses showed minor ^{13}C enrichment in fungal PLFAs (Fig. 5), which was consistent with the *C. coffeae* dependent $^{13}CO_2$ evolution in ^{13}C-PS treatments (Fig. 4). ^{13}C was neither enriched in fungal PLFAs in control incubations with ^{12}C-PS nor in the absence of PS, nor during incubation of *C. coffeae* in solidified medium containing ^{13}C-PS (Supplementary Tables S3 and S4).

To determine the extent of residual styrene monomers to the observed increase of $^{13}CO_2$ quantities, liquid-state ^{1}H NMR spectroscopy was performed. The NMR spectroscopic analysis revealed that the PS substrate contained roughly 0.30 ‰ ± 0.10 ‰ residual styrene (Supplementary Fig. S4 and Supplementary Table S2) matching contents known from industrial PS sources (Garrigós et al., 2004; Newell, 1951). Incubation with or without *C. coffeae* did not change styrene contents substantially (0.38‰ ± 0.10‰). This indicates that the observed PS mineralization is not exclusively due to mineralization of residual styrene, but also due to the depolymerization of PS.

4. Discussion

We determined moderate *C. coffeae*-mediated PS mineralization by tracking δ^{13}C values in laboratory incubations, which goes beyond previous studies that have usually determined mass loss as an indication of plastic degradation (Ho et al., 2018; Reddy et al., 2009; Timmins et al., 2021). However, mass loss can also be due to the loss of particles following plastic fragmentation or leaching of additives and its degradation products (Ghatge et al., 2020; Sánchez, 2020). The determination of mass loss alone is therefore not necessarily indicative for complete plastic degradation. To circumvent these uncertainties of gravimetric analysis, isotopic tracing is an effective way to assess the degradability of plastics not only in complex matrices as suggested in previous studies

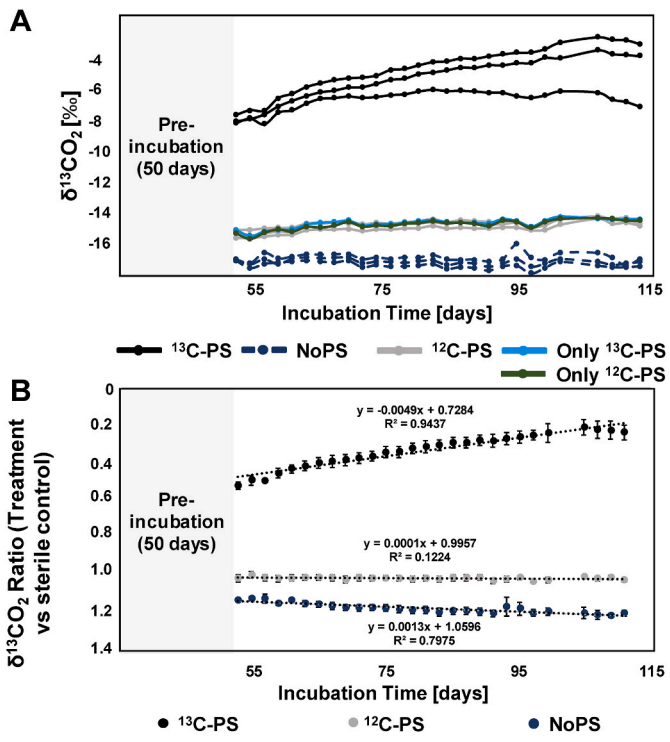

Fig.4. ^{13}CO2 evolution measured by CRDS. Changes in δ^{13}C level in the headspace during incubation of *C. coffeae*, as determined by CRDS. **A** Direct δ^{13}C values were shown for each treatment. **B** Median changes after subtraction of only PS controls. Standard deviations were calculated excluding the mentioned outlier with limited PS-mineralization activities. $^{13/12}$C-PS: Incubations with *C. coffeae* and labeled or unlabeled PS; No PS: Incubations without PS, but with mycelium; Only $^{12/13}$C-PS: Second negative controls with PS without fungal biomass.

(Danso et al., 2019; Sander et al., 2019) but also in pure fungal cultures. Thus, we developed a workflow to identify plastic polymer degrading and mineralizing capabilities of new microorganisms.

The proposed workflow starts with the selection of a suitable habitat for potentially plastic-biodegrading organisms. The phyllosphere of tropical leaves turned out to be a suitable habitat since their hydrophobic recalcitrant surface is comparable to plastic polymer structures (Sridar et al., 2015). After isolation of microbial strains or consortia, we recommend an initial analysis with simple and relatively quick low-cost tests, using different types of MP. *C. coffeae* coped with each type of plastic tested, but other candidates might be more selective. Therefore, this should be done prior to synthesizing costly isotopic-labeled plastic substrates. Combining observations from macroscopic and microscopic analyses (Figs. 1 and 3), it was found that *C. coffeae* is predestined for effective biomodification of MP, as it not only grows on MP-supplemented semi-solid medium, but also filters PS particles from liquid cultures (Fig. 1D). Electron microscopy revealed close connections between fungal structures and plastic particles. Thus, *C. coffeae* fulfilled several requirements like a plastiphilic life style to qualify for the next steps of the workflow.

Mineralization of ^{13}C-PS was detected after ~50 days of pre-incubation, and decreased after 100 days, as shown by the δ^{13}C values of headspace CO_2 (Fig. 4). We assume that PS-derived carbon mineralization and assimilation did not maintain *C. coffeae* in the long-term (>100 days), as we detected only minor incorporation of PS-derived ^{13}C in the PLFA of *C. coffeae*. Initial CO_2-producing activity of *C. coffeae* was attributed to the usage of carbon storage compounds remaining after the starvation step prior to inoculation, and eventually autophagy until the metabolism was changed to attack the PS. Indeed, *C. coffeae* contains ultrastructural features that resemble intracellular carbon storage granules (Kim, 2016), and filamentous fungi are well known to adapt their metabolisms during starvation (Nitsche et al., 2012), like increased expression of genes known for autophagy and extracellular polymer breakdown (Nitsche et al., 2012). Laccase and oxidase encoding genes were likewise induced during starvation. Such enzymes are associated with PS degradation, indicating that PS main- and side chain cleavage might have occurred (Mohanan et al., 2020; Zhang et al., 2022).

Another hypothesis is that our findings are based on mineralization of residual styrene monomers in the polymer matrix being more readily available substrates and causing the shift in the δ^{13}C value in CO_2. However, this explanation is unlikely, as the degradation of residual styrene would lead to a large and rapid shift in δ^{13}C values within days, instead of the observed very slow and gradual increase (Braun-Lüllemann et al., 1997; Cox et al., 1993). Additionally, ^{1}H NMR spectroscopy after incubation in the presence and absence of *C. coffeae* showed no measurable changes of styrene contents, further supporting the conclusion that *C. coffeae* is capable of depolymerizing PS and simultaneously mineralizing styrene at a low rate.

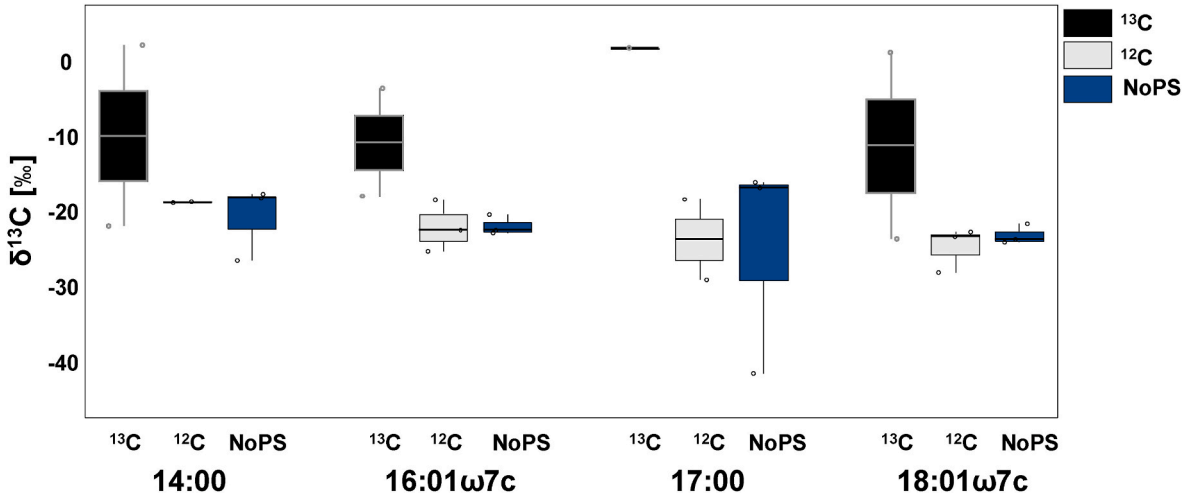

Fig. 5. ^{13}C-Enrichment of fungal PLFA ^{13}C-Enrichment of fungal PLFAs at the end of the ^{13}C-PS mineralization assay. Boxplots were generated based on 3 replicates, except for ^{13}C samples in which one mineralization-deficient replicate was excluded. Lacking boxes indicate that only one δ^{13}C-value could be acquired. PLFA fatty acid nomenclature: X:YωZc X: Chain length; Y: Number of double-bonds; Z: Position of double-bonds within the C-backbone; c: cis-Conformation of the double-bond. For complete PLFA SIP data sets, please refer to Supplementary Tables S3 and S4.

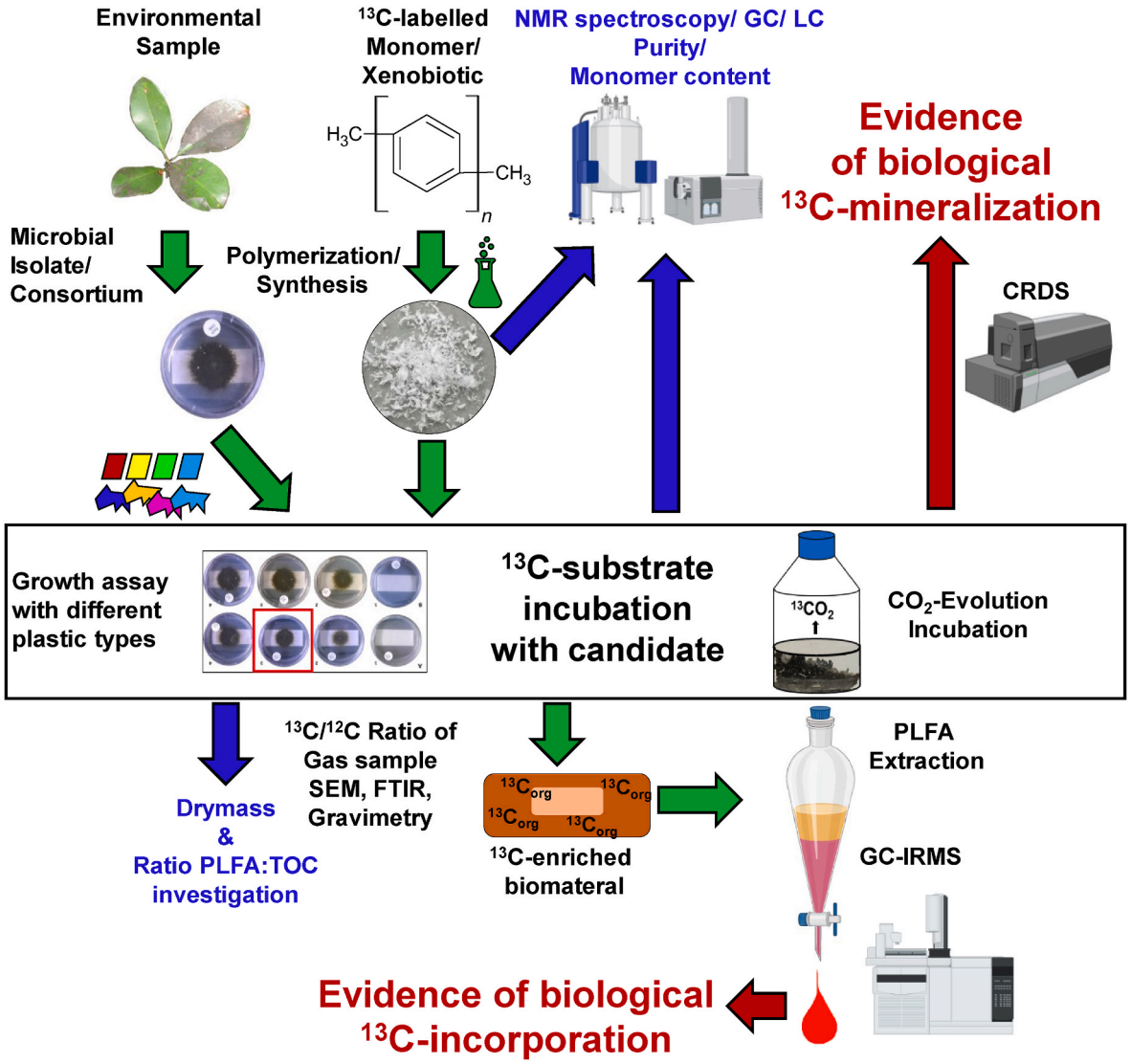

Fig. 6. Proposed workflow for microbial polymer biodegradation studies Green arrows indicate general workflow, red arrows main results, and blue arrows evaluation of potential disturbance factors; NMR spectroscopy: Nuclear magnetic resonance spectroscopy; GC: Gas chromatography; LC: Liquid chromatography; PLFA: Phospholipid fatty acid; CRDS: Cavity ring-down spectroscopy; IRMS: Isotopic ratio mass spectrometry; SEM: Scanning electron microscopy; FTIR: Fourier-transform infrared spectroscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

To gain more insights into the ^{13}C-flow, PLFA SIP was included into our workflow as a valid method for incorporation studies, as previously reported (Ho et al., 2019). PLFA analysis showed high technical variability in regard of potential assimilation of PS-derived ^{13}C into fungal PLFA. Based on previous findings, we assume that only certain fatty acids are enriched (Watzinger and Hood-Nowotny, 2019). Two out of three replicates from the PS mineralization assay showed slight ^{13}C-enrichment in PLFAs, suggesting that only little assimilation of PS carbon occurred (Fig. 5 and Supplementary Table S3 and, S4).

To qualify the low ^{13}C-enrichment in PLFAs, we estimated the detection limits of the applied PLFA analysis relative to the $^{13}CO_2$ evolution observed. The microbial assimilation efficiency ranges typically between >10% and <50% (Pomeroy and Wiebe, 1988). Hypothetically, *C. coffeae* was assumed to assimilate one C-atom per CO_2-molecule released. To make a rough estimate, the amount of PS degraded was calculated according to a previously reported study (Zumstein et al., 2018). Accordingly, ≈0.01 μmol PS was degraded during each incubation, which is in the same range as reported recently for the yeast *Rhodotorula mucilaginosa* during biodegradation of pristine PE, in contrast to much higher rates with UV-treated PE (Vaksmaa et al., 2023). This underlines the importance of ageing and weathering for environmental plastic biodegradation (Karkanorachaki et al., 2023; Vaksmaa et al., 2023). Assuming that the total amount of organic biomass was 20 mg, each incubation contained approximately 0.67 mmol of organic carbon. The resulting change in the ratio, neglecting the unknown proportion of PLFA on the total biomass, would be in the range of 2–3 ‰ in δ^{13}C value, which is close to the detection limit of our method, giving yield to high variations (Supplementary Table S3). Nanoscale secondary ion mass spectrometry (NanoSIMS) was reported to be a valid method besides PLFA SIP analyses and could be considered a more costly alternative in case a single cell-scale high spatial resolution of isotope distribution is warranted (Vaksmaa et al., 2023). Future assays should consider the use of higher ^{13}C-labeled substrates beyond 8-atom% for test candidates with slow substrate degradation rates. Further explanations for the low assimilation of PS-carbon come from fungal culture extracts that are able to degrade and mineralize polymers independently from assimilation into biomass and mycelial growth (Ntougias et al., 2015). Exo-enzymes have evolved to extracellularly disintegrate polymers into smaller compounds that can be taken up by the cell (Gopinath et al., 2005; Shankar Naik et al., 2019).

Mineralization can occur entirely extracellularly without integrating C-atoms into the living biomass as previously postulated for the extracellular degradation of styrene monomers mediated by extracellular enzymes (Braun-Lüllemann et al., 1997). Former studies likewise showed extracellular activity and corresponding mineralization based on isotopic-labeled biopolymer degradation by fungi (Liers et al., 2006). Such data demonstrates that plastiphily and a slow mineralization of PS does not necessarily indicate a strong assimilation of PS carbon and suggests a primarily co-metabolic degradation of PS by *C. coffeae*.

Capnodium coffeae is a microorganism that inhabits the phyllosphere in the tropics (Flessa et al., 2021). As surfaces of perennial leaves are coated with hydrophobic waxes and polymers (Holder, 2007; Sridar et al., 2015), it is likely that *C. coffeae* has hyphal structures that are able to adhere strongly to these surfaces as a site for potential degradation (Mathur et al., 2011). It is therefore rational to assume that *C. coffeae* has evolved ecological properties that allow the fungus to attach to hydrophobic polymers (Flessa et al., 2021). Although not commonly known from nature, *C. coffeae* may also possess the enzymatic capability to penetrate the hydrophobic surface after attachment. These traits are indeed advantageous for the degradation of plastic (Atanasova et al., 2021), and may partly explain the PS mineralization potential of *C. coffeae* as shown in this study. Enzymes with high potential for plastic degradation, in particular include manganese peroxidase, lignin peroxidase, versatile peroxidases, laccases and cutinases (Ali et al., 2021; Baker et al., 2012). However, the exact mechanism for PS degradation and the enzymes involved remains to be determined, for instance via transcriptomics.

5. Conclusion

This study provides a holistic analysis workflow for the identification of new plastic degrading microorganisms emphasizing fungi, starting with the selection of environmental samples that exhibit certain characteristics that may promote the evolution of specific adaptions for polymer biodegradation (Fig. 6). After isolates or microbial consortia have been obtained, growth assays are used to quickly prescreen for promising candidates. ^{13}C-labeled substrates with the desired chemical properties are synthesized and thoroughly characterized. The ^{13}C-substrates are then fed to the microbial candidates and the headspace is measured using CRDS or any other appropriate method like the costlier GC-IRMS. This provides information on biomineralization rates. PLFAs are then extracted from the microbial biomass and their ^{13}C-contents is determined using GC-IRMS to investigate whether ^{13}C-atoms ultimately end up in the microbial biomass that would thrive on target substrates. The analysis of ^{13}C-enrichments in target organisms specific PLFAs is highly recommended to circumvent biases due to ^{13}C-labeled polymer contamination of biomass. Mineralization rates, in combination with dry biomass and the ratio of PLFA to total organic carbon in the biomass, can provide information on the assimilation potentials. Depending on the specific resources of a particular study project, this workflow can be extended by using SEM and infrared spectroscopy of the substrates after incubation to further investigate biomodification of polymer surfaces. The major advantage of this workflow is the minimization of potential biases when it comes to identification of plastic degrading microbes. The resulting findings are crucial for improving future studies.

In conclusion, our workflow starting from general screening assays, synthesis of ^{13}C substrates, ^{13}C-tracing, SIP studies, complemented by NMR spectroscopic analysis, could set a new standard approach to evaluate plastic degradation in pure culture, and possibly in complex microbial communities for any isotopically labeled compound.

CRediT authorship contribution statement

Stephan Rohrbach: Conceptualization, Methodology, Writing - original draft. **Gerasimos Gkoutselis:** Conceptualization, Methodology, Writing - original draft. **Anika Mauel:** Writing - review & editing. **Nihal Telli:** Methodology. **Jürgen Senker:** Writing - review & editing. **Adrian Ho:** Writing - review & editing. **Gerhard Rambold:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Marcus A. Horn:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.141025.

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