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Fungi present distinguishable isotopic signals when grown on glycolytic versus tricarboxylic acid cycle intermediates

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Abstract. Microbial activity in soils controls both the size and turnover rates of large carbon (C) inventories stored in the subsurface, having important consequences for the partitioning of C between terrestrial and atmospheric reservoirs as well as the recycling of mineral nutrients such as nitrogen or phosphorus (often bound to the C) that support plant growth. Fungi are major decomposers of soil organic matter (SOM); however, uncertainty in the predominant C substrates that fuel respiration confound models of fungal production and SOM turnover. To further define the signals of microbial heterotrophic activity, we applied a dual hydrogen (H) and C stable isotope probing (SIP) approach on pure fungal cultures representing the phyla Ascomycetes, Basidiomycetes, and Zygomycetes growing on monomeric (glucose, succinate) or complex substrates (tannic acid, β-cyclodextrin). Our findings demonstrate that the investigated species incorporated only minor amounts of inorganic C (provided as bicarbonate) into their membrane lipids, amounting to < 3% of lipid-C, with no consistent patterns observed between species or growth substrates. The net incorporation of water-derived H (i.e., α_W) into lipids also did not differ significantly between incubations with monomeric versus complex substrates; however, growth on succinate solicited significantly higher α_W values than glucose or β-cyclodextrin. This finding suggests that ^{2}H-SIP assays have the potential to distinguish between microbial communities supported predominantly by substrates that are catabolized by the tricarboxylic acid cycle versus glycolytic pathway. Furthermore, the average α_W value of heterotrophic fungal incubations [0.69 ± 0.03 (SEM)] is consistent with that observed for bacterial heterotrophs, and may be applied for upscaling lipid-based estimates of fungal production in environmental assays.

Short Summary

Microbial production is a key parameter in estimations of organic matter cycling in environmental systems, and fungi play a major role as decomposers. In order to investigate fungal production and turnover times in soils, we incubated fungal pure cultures with isotopically labelled water and bicarbonate to investigate growth signals encoded into lipid biomarkers, which can be applied to improve flux estimates in environmental studies.

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1 Introduction

Soil organic matter (SOM) is the major reservoir of carbon (1580×10^{15} g C) in the biosphere, and active microbial populations act to redistribute this C to other reactive reservoirs, such as atmosphere (Carson et al., 2001; Grinhut et al., 2007). Major uncertainties in modeling C and climate dynamics stem from insufficient knowledge on the controls of SOM degradation and transformation (Ciais et al., 2014; Lindahl and Tunlid 2015). Saprotrophic soil fungi are one of the major decomposers in soils, who are known to degrade naturally occurring complex molecules such as lignin (Kirk & Farrell, 1987; Fioretto et al., 2005; Baldrian et al., 2011), cellulose (Šnajdr et al., 2011) and humic substances (Grinhut et al., 2007), but are also reported to compete for accessible plant photosynthate excreted by roots (De Boer et al., 2005; Högberg et al., 2001; Smith & Read, 2008). Despite the unique and important fungal niche in biogeochemical cycles, their contributions to SOM cycling remains poorly constrained (Frey 2019; Grinhut et al., 2007). Furthermore, heterotrophic organisms feeding on organic substrates to gain energy and build biomass are also known to fix a variable amount of inorganic C, in order to replenish intermediates in the tricarboxylic acid (TCA) cycle (Kornberg 1965). It has been suggested that 2 - 8% of the biomass C in heterotrophs originates from inorganic C incorporated through anaplerotic carboxylation reactions (Romanenko 1964; Roslev et al., 2004; Braun et al., 2021). Although, the awareness of these processes has existed for decades (Kornberg 1965; Sorotkin 1966), the relevance and the metabolic controls on heterotrophic inorganic C fixation remains poorly understood, partly due to the lack of reliable estimates for most organisms and habitats (Braun et al., 2021).

Advanced analytical techniques now allow linking microbial taxa to specific processes in environmental studies by measuring the incorporation of stable isotopes into biomarkers (Boschker et al., 1998; Dumont and Murrell, 2005; Kreuzer-Martin, 2007), such as fungal and bacterial membrane lipid fatty acids (Treonis et al., 2004; Willers et al., 2015) or other biomarkers (Boschker and Middelbourg, 2002). Previous studies have demonstrated that variability in the $^{2/1}H$ composition of microbial lipids is redundant with that of environmental water (Hoefs, 2018; Kopf et al., 2015), and stable isotope probing (SIP) assays applying enrichments in $^{2}H_2O$ have proven to be a useful tracer of microbial activity in a diverse range of environments (Fischer et al., 2013; Kellermann et al., 2012; Wegener et al., 2016; Wu et al., 2018). Large H-isotope fractionations, yielding $\delta^{2}H$-values between −400‰ and +200‰, have been observed during biosynthetic incorporation of water hydrogen (water-H) into individual compounds within a single cell or total biomass, which can be indicative of metabolic processes (Osborn et al., 2011; Sachse et al., 2012; Zhang et al., 2009). To fully exploit the potential of SIP experiments, a dual-SIP approach was developed to track total microbial production by adding heavy water ($^{2}H_2O$) together with ^{13}C-labeled inorganic C (IC), enabling simultaneous estimates of total and autotrophic metabolism, respectively (Wegener et al., 2012; Wu et al., 2020). Recently, Jabinski et al. (2024) validated an innovation of the dual-SIP assay by using rapid pyrolysis of fungal biomass to determine the stable C and H isotopic composition of fungal lipids, and demonstrated that water-H and IC assimilation signatures could successfully distinguish between fungal ecotypes growing on glucose or glutamic acid as the C source.

The aim of the current study was to further assess the controls on water-H and inorganic C incorporation into lipids and expand our knowledge for interpreting environmental signals by applying the dual-SIP assay on a broader range of pure fungal cultures

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and growth substrates, including labile monomers versus more complex, high molecular weight molecules. We hypothesized that I) the incorporation of inorganic C and water-H into the fungal fatty acid biomarker $C_{18:2}$ will be similar for fungal species growing on the same substrate, and II) that inorganic C and water-H incorporation will distinguish between growth on labile versus more complex C substrates.

2 Methods

2.1 Cultivation & Harvesting

Fungal pure cultures of two Basidiomycetes (Paxillus involutus (PI, strain SB-22); Phanerodontia chrysosporium (PC, strain CCM8074)), two Zygomycetes (Mortierella (MO, strain RK-38); Umbelopsis (UM, strain RK-43)) and two Ascomycetes (Penicillium jancewskii (PJ, strain BCCO20_0265); Paecilomyces lilacinus (PL, strain DP-23)) were incubated at 25 °C in the dark in 500 mL Schott bottles containing 50 mL of a mineral media described previously (Bukovská et al 2018) with the vitamins left out, which was inoculated with approximately 10^6 spores or a hyphal block < 0.5 cm^3 (Basidiomycetes) recovered from a previous culture using the same cultivation medium solidified with agar (1.5%).

The growth medium contained per liter: 4 g organic C in various forms ($C_6H_{12}O_6$ glucose; $C_4H_6O_4$ succinic acid; $C_{42}H_{70}O_{35}$ β-Cyclodextrin or $C_{76}H_{52}O_{46}$ tannic acid), 0.01 g $FeSO_4$ * $7H_2O$, 2 g KH_2PO_4, 0.5 g $MgSO_4$ * $7H_2O$, 0.1 g NaCl, 0.1 g CaCl, 2.5 g $(NH_4)_2SO_4$, 0.45 g $NaHCO_3$ and 1 mL of a mixed solution (per liter: 0.5 g H_3BO_3, 0.04 g $CuSO_4$ * $5H_2O$, 0.1 g KI, 0.4 g $MnSO_4$ * $5H_2O$, 0.2 g $NaMoO_4$ * $2H_2O$, 0.4 g $ZnSO_4$ * $7H_2O$). The pH of the medium was adjusted to 4.5 before inoculation. Dual-SIP experiments were performed using ^{13}C-bicarbonate (^{13}C-DIC, $NaH^{13}CO_3$) and deuterated water (D_2O). Each fungal strain was grown in triplicate with non-labeled substrates (Treatment I), with δ^2H of the medium water adjusted to 100‰ and 10% of ^{13}C-DIC (Treatment II), 200‰ δ^2H and 10% ^{13}C -DIC (Treatment III), and 400‰ δ^2H and 10% ^{13}C -DIC (Treatment IV). The Schott bottles were closed with a rubber stopper in order to keep the labeled ^{13}C-DIC from outgassing, and ample headspace was provided to maintain oxic conditions throughout the growth experiment. Fungal growth was monitored via the accumulation of CO_2 in the headspace, and we aimed to harvest when CO_2 levels reached 10%; however, without preliminary knowledge of the fungal growth dynamics, some cultivations exceeded this level more quickly than they could be sampled and analyzed.

Mycelia were separated from the growth medium via filtration through 5 µm Isopore polycarbonate filters (47 mm diam, Merck catalogue number TMTP04700) using vacuum filtration device allowing to collect the cultivation medium into sterile 50 mL tube. Thereafter, the mycelium was washed with ample MilliQ water, transferred to pre-weighed, sterile 50 mL tubes, fresh weight of the biomass was recorded, and the samples were frozen at -80 °C until lyophilization. A subsample of the cultivation medium was also frozen at -80 °C and the rest used to determine pH post-incubation. After lyophilization, the dry weight of each sample was determined and stored at -20 °C until further analysis.

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2.2 Measurements

2.2.1 Headspace CO_2 concentration and isotope composition

Samples of headspace (0.3 mL) were collected weekly from each bottle into helium flushed 12 mL exetainer vials (Exetainer, Labco Limited, UK) and analyzed for their CO2 concentration and 13C/12C isotopic ratio using Gasbench II equipped with a single cryo-trap connected to Delta V Advantage isotopic ratio mass spectrometer (IRMS) via Conflow IV (Thermo Scientific, Bremen, Germany). Ambient air (with the CO2 concentration measured using LiCor 850 gas analyzer previously) was used as standard for CO2 concentration measurements, whereas laboratory cylinder with CO2 gas (δ13C = -2.86 ‰) was used as a standard for the isotopic composition of the C. The analytical error was below 1‰. Data were analyzed and exported using the Isodat 3.0 software.

2.2.2 Medium water (^2H_2O)

Liquid samples were transferred into 1.5 ml glass vials (32 x 11.6 mm, Fischer Scientific) and then measured using Triple Liquid Water Isotope Analyzer (Los Gatos Research), which is based on the principle of high-resolution laser absorption spectroscopy. Samples were dispensed into the instrument using an autosampler (PAL3 LSI, ABB company) and a 1.2 µL syringe (Hamilton). Samples were measured and evaluated against prepared laboratory standards of known isotopic composition. The isotopic ratios of these laboratory standards were verified by measuring against international standards (VSMOW2, SLAP2) made by the IAEA. For quality control purposes, the measurements of the samples were also interspersed with periodic measurements of the prepared verification samples with known isotopic composition. The final isotopic composition (δ^2H) was determined using LIMS software. Analytical error of δ^2H was <1.5‰.

Water sampled from incubations with tannic acid could not be measured using the laser, as described above, due to its high organic carbon content, and was rather measured via a GasBench II system (Thermo Scientific, Bremen, Germany; Application Note: 30049). Medium water samples (200 µL) were added with a platinum catalyst to a 12 mL exetainer vials (Exetainer, Labco Limited, UK). The headspace was flushed with 1% H_2 in He at approximately 100 mL min-1 with for 6 min. After an equilibration time of over 40 min, the samples were measured by purging the exetainer using a double-holed needle with helium into a 250 µL sample loop. The sample was then injected and separated via a Carboxen PLOT 1010 (0.53 mm ID; Supelco, Bellefonte, USA) held at 90 °C with a flow rate of 0.75 bar, and then introduced into the MAT253 Plus IRMS via a Conflo IV interface. Each sample was injected three times during one analysis. The isotopic composition was determined using Isodat 3.0 software against the corresponding H_2 working gas (-239‰ for δ^2H) and the values were corrected and normalized using international standards VSMOW2 (0‰ for δ^2H), SLAP2 (-427.5‰ for δ^2H), USGS53 (+40.2‰ for δ^2H) and GFLES-2 (159.9‰ for δ^2H). The analytical error was around 1‰.

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2.2.3 Carbon ($\delta^{13}C$) substrate analysis

Substrates (~100 µg) were weighed into tin capsules (8 * 5 mm, Sercon, Crewe, UK) and placed in a helium-flushed carousel autosampler, then introduced to an Elemental Analyzer IsoLink device (EA IsoLink CNSOH, Thermo Scientific, Bremen, Germany) equipped with a CHN/NC/N EA combustion/reduction reactor (Sercon, Crewe, UK) heated to 1020 °C. A pulse of oxygen was introduced to the reactor simultaneously with the sample. The sample gases were quantified via a thermal conductivity detector (TCD) and then introduced to a MAT 253 Plus isotope ratio mass spectrometer (IRMS; Thermo Scientific; Bremen, Germany) via the open split of a Conflo IV interface, with helium as the carrier gas. The isotopic composition was determined using Isodat 3.0 software against the corresponding CO2 working gas (-4.191‰ for δ13C), and the values were corrected for linearity and normalized to the VPDB scale using international reference material IAEA-600 (-27.771‰ for δ13C). The analytical error was <0.04‰.

2.2.3 Pyrolysis GC for lipid analysis

The pyrolysis unit Shimadzu 3030D (Shimadzu, Kyoto, Japan/ Frontier Laboratories, Fukushima, Japan) was installed on top of the GC Trace1310 gas chromatograph SSL injector (Thermo Scientific, Bremen, Germany) and the GC was equipped with an SLB-IL60 column (non-bonded; 1,12-Di(tripropylphosphonium)dodecane bis(trifluoromethanesulfonyl)imide phase, 30 m, 0.25 mm ID, 0.20 µm df, Supelco, Bellefonte, USA). The furnace temperature was 650 °C and the interface temperature was 370 °C. The injector temperature was 360 °C and the GC oven was held at 80 °C for 1 min then ramped to 175 °C at 15 °C min^{-1}, then ramped to 195 °C at 2 °C min^{-1}, then ramped to 300 °C at 10 °C min^{-1}, and finally held at 300 °C for 7 min. Helium was used as carrier gas with a constant flow of 1.5 mL min^{-1} with a split ratio of 40 and a split flow of 26.7 mL min^{-1}. The column flow was split via a multichannel device to acquire MS and isotopic data simultaneously from one injection. The GC-MS (ISQ QD; Thermo Scientific, Bremen, Germany) ion source was set to electron impact ionization mode (EI) at 70 eV and a scan range of m/z 50 – 500 with a scan time of 0.2 sec^{-1} was applied. Scan started after 8 min to avoid the solvent peak in the MS. Transfer line temperature was set to 300 °C and the Ion source was set to 250 °C.

The samples (freeze-dry biomass, 0.1 mg – 1.3 mg) were weighed into an ultra clean stainless steel Eco-Cup LF (Frontier Laboratories, Fukushima, Japan) which were burned with a torch before usage to ensure no contamination. FAMEs signals were acquired in the same run. Immediately prior to the measurement, 30 µL of trimethylsulfonium hydroxide (TMSH) was added on the sample to increase the volatization of the fatty acids and improve measurement sensitivity. Identification was performed using fragmentation patterns and the NIST 14 library.

Stable carbon and hydrogen isotope compositions of FAMEs were determined by splitting the flow from the GC column to a GC-Isolink II reactor, coupled to a MAT253 Plus IRMS via a Conflo IV interface. Values are expressed in standard delta notation ($\delta^{13}C$ and $\delta^{2}H$). MS information was simultaneously acquired by use of the multi-channel device described above. For conversion of FAMEs and ergosterol to CO_2, the combustion reactor (nickel oxide tube with CuO, NiO, and Pt wires) was set to 1000 °C. For conversion of FAMEs and ergosterol to H_2, the pyrolysis reactor (aluminum tube) was set to 1420 °C.

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FAMEs were identified by their retention times and fragmentation patterns. The isotopic composition was determined using Isodat 3.0 software against the corresponding CO_2 or H_2 working gas (-4.191‰ for $\delta^{13}C$, -239.5‰ for $\delta^{2}H$). Isotope corrections for instrument drifts, linearity, and normalization to the VPDB or VSMOW scales were performed according to the response of USGS70 (-30.53‰ for $\delta^{13}C$, -183.9‰ for $\delta^{2}H$) and USGS72 (-1.54‰ for $\delta^{13}C$, 348.3‰ for $\delta^{2}H$) reference standards. The analytical error was <0.5‰ and <10‰ for $\delta^{13}C$ and $\delta^{2}H$, respectively.

3 Results

3.1 Fungal growth and CO_2 production

All fungal species were pure cultures, which were incubated in a mineral medium with either glucose, succinate, β-cyclodextrin, or tannic acid serving as the sole organic C source. Growth was monitored by the evolution of CO_2 into the headspace, which ranged from 0.36% (no respiration of substrate) to a maximum of 35%, after incubation times ranging from 5 to 160 days (Fig. 1). The pH of the media in all incubations ranged from 2 to 5.5 at the time of harvest, with a general trend of decreasing pH with increasing CO_2; however, the trend was opposite when succinate was the carbon source, with pH increasing from 4 to 5.5. For samples that produced sufficient biomass, the dry biomass of harvested fungal hyphae ranged up to 250 mg, and at least 30 µg dry biomass was used to analyze fungal membrane fatty acids by Pyr-GC-IRMS. Only the Ascomycetes species PL and PJ grew sufficiently on each tested substrate to produce enough biomass for stable isotope analysis. Incubations of Zygomycetes species with glucose or succinate also yielded sufficient dry biomass, and only UM and not MO was able to grow on β-cyclodextrin; Zygomycetes species produced neither CO_2 nor biomass when incubated with tannic acid. The Basidiomycetes typically exhibited the slowest growth, and both species (PI and PC) only produced enough biomass when grown on glucose. The CO2 levels in Basidiomycetes incubations with succinate increased to a maximum of ~ 2%, but only PI yielded sufficient biomass for analysis. PC grew sufficiently on β-cyclodextrin, with CO_2 levels increasing to a maximum of 3%, while CO_2 remained < 0.6% in PI incubations.

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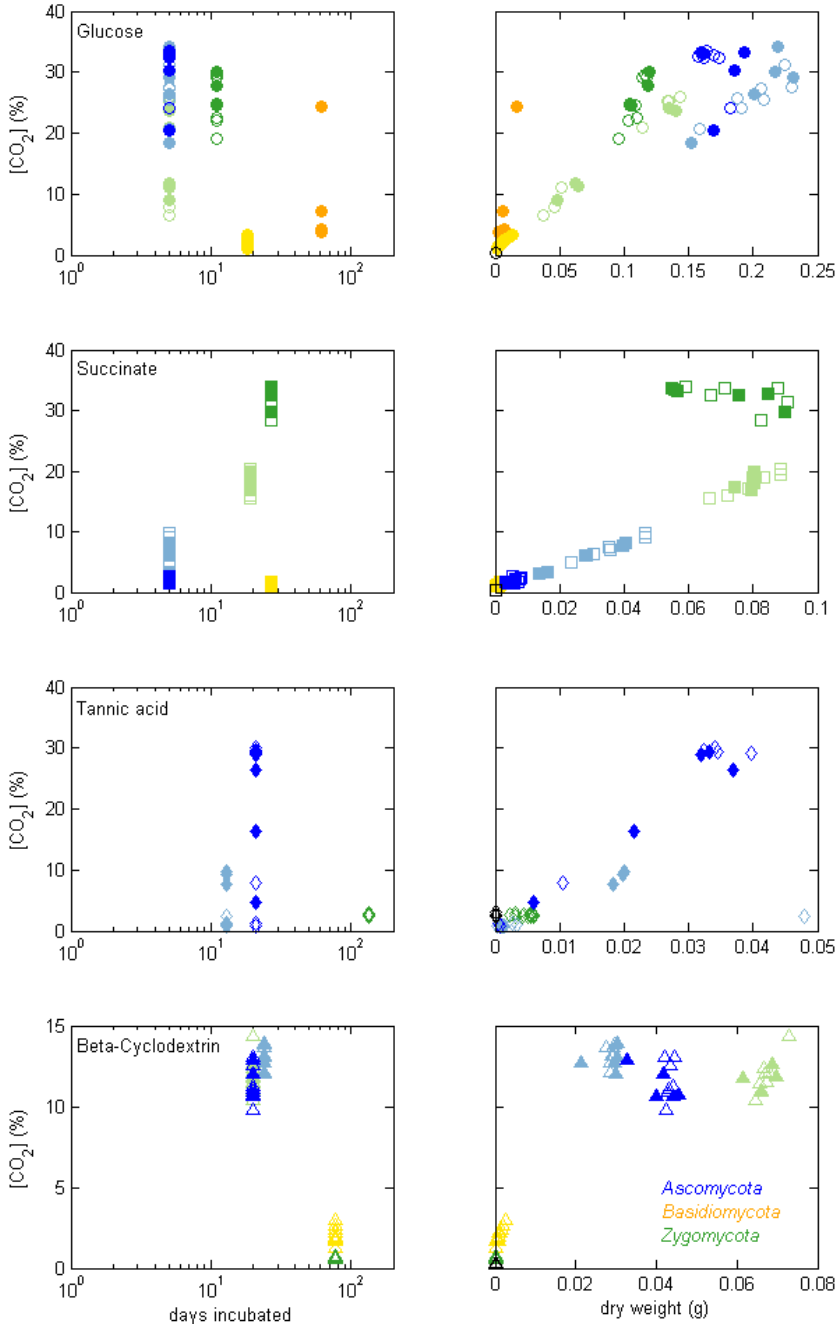

Figure 1. Growth of fungal species on each substrate as indicated by production of CO_2 versus days of incubation (left panels) or dry biomass (right panels). Filled symbols indicate samples for which the $C_{18:2}$ biomarker was measured by Pyr-GC-IRMS. Colors represent the Ascomycetes species PJ (dark blue) and PL (light blue), Zygomycetes species MO (dark green) and UM (light green), and Basidiomycetes species PC (orange) and PI (yellow). The symbols denote incubations with glucose (circles), succinate (squares), tannic acid (diamonds), or β-cyclodextrin (triangles).

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Fungal respiration of the growth substrates led to decreasing $\delta^{13}C$-CO_2 values as fungal biomass was produced, which followed a hyperbolic trend expected for the mixing of CO_2 from two different sources (Text S1; c.f., Kendall and Caldwell, 1998). The atom % ^{13}C in control incubations with no fungal inoculum was measured at the latest time of harvest of inoculated incubations and stayed below 0.4% except tannic acid which ranged from 2-3%; the δ13C values of the substrates were glucose = -26.5‰; succinate = -28.3‰, tannic acid = -27.4‰, β-cyclodextrin = -10.6‰. The mixing relationship was modeled using all CO_2 data, across all incubations, and integrated to approximate the mixing-weighted average $F^{13}C$ value of inorganic C for each incubation (cf., Text S1, Fig. S2), which was finally applied in the denominator of Eq. 1 to estimate the fraction of lipid-C derived from inorganic C. For incubations that produced sufficient fungal biomass for stable C isotopic analysis, the weighted average $\delta^{13}C$ values of inorganic C that were applied in Eq. 1 ranged from 200 to 1400 ‰ (i.e., ~ 1.3 to 2.6 AT% ^{13}C).

3.2 Stable isotopic composition of fungal lipids

3.2.1 Carbon isotopes

The $\delta^{13}C$ values of fungal biomarker $C_{18:2}$ was determined as described in section 2.2.4 and is reported in this section as standard delta values (‰). The inorganic C incorporation into the biomarker was calculated based on the following equation:

$$\%IC_{(assimilation)} = \frac{F^{13C}_{lipids\ labeling} - F^{13C}_{lipids\ control}}{F^{13C}_{DIC(medium)} - F^{13C}_{substrate}} \times 100\% \qquad \text{(Eq. 1)}$$

Equation 1: Inorganic carbon (IC) assimilation was calculated as the difference in the ^{13}C atom fraction (F^{13C}) of the lipids from the labeling experiment compared to the natural (control), relative to the difference between the mixing-weighted average F^{13C} of dissolved inorganic C (DIC, cf. Text S1) and the F^{13C} of the substrate. F was calculated as $F^{13}C = (R^{13C/12C})/(R^{13C/12C} + 1)$, where R is calculated from the $\delta^{13}C$ ratios as measured with the IRMS equipment using the reverse of the δ notations ($\delta^{13}C = ([^{13}C/^{12}C]_{sample}/[^{13}C/^{12}C]_{ref} - 1) * 1000$ (modified after Boschker & Middelburg 2002; Wegener et al., 2012).

The $\delta^{13}C$ values of fungal biomarkers $C_{18:2}$ (Table 1) produced under natural cultivation conditions with glucose (i.e., non-labeled; $AT\%_{DIC}$ ~ 1%) ranged from -24.1‰ to -21.2‰ across all strains (n = 6 species). As expected, $C_{18:2}$ harvested from the labeled incubations exhibited slightly higher $\delta^{13}C$ values (up to +11‰; PC grown on glucose) than the corresponding experiment amended with natural bicarbonate, likely owing to the incorporation of labeled inorganic C into the $C_{18:2}$ fatty acid.

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Table 1: $\delta^{13}C$ values of fungal biomarker $C_{18:2}$ harvested from incubations with non-labeled substrates (nat) or those amended with ^{13}C-labeled bicarbonate. Incorporation of inorganic C (%IC) was calculated based on Eq.1. Not all fungal species grew on all substrates, and some did not give enough biomass for analysis (n.d*) and therefore no inorganic C incorporation was calculated (n.d). Errors represent the standard deviation of replicate incubations.

Species	**Glucose $\delta^{13}C$ (‰)**		**IC (%)**	**Succinate $\delta^{13}C$ (‰)**		**IC (%)**	**Tannic acid $\delta^{13}C$ (‰)**		**IC (%)**	**β-cyclodextrin $\delta^{13}C$ (‰)**		**IC (%)**
	nat	+		nat	+		nat	+		nat	+	
Paxillus involutus (PI)	-21.5	-16.7	0.6 (±0.2)	n.d*	n.d*	n.d	n.d*	n.d*	n.d	n.d*	n.d*	n.d
Phanerodontia chrysosporium (PC)	-24.1	-15.9	0.9 (±0.3)	-27.3	-23.5	0.3 (±0.1)	n.d*	n.d*	n.d	n.d*	n.d*	n.d
Mortierella (MO)	-21.9	-20.7	0.5 (±0.1)	-31.7	-31.5	0.1 (±0.1)	n.d*	n.d*	n.d	n.d*	n.d*	n.d
Umbelopsis (UM)	-21.2	-21.1	0.1 (±0.0)	-30.6	-28.2	0.7 (±0.2)	n.d*	n.d*	n.d	-21.7	-18.1	0.8 (±0.3)
Penicillium jancewskii (PJ)	-23.2	-22.1	0.5 (±0.2)	-30.1	-27.8	0.2 (±0.0)	-25.9	-20.5	2.2 (±0.5)	-20.7	-20.1	0.1 (±0.2)
Paecilomyces lilacinus (PL)	-23.0	-23.5	n.d	-30.8	-29.9	0.1 (±0.0)	-25.4	-25.0	0.1 (±0.0)	-19.1	-17.6	0.7 (±0.4)

The estimated incorporation of IC into $C_{18:2}$ (%IC) typically ranged up to 1%; only PJ grown on tannic acid exhibited higher %IC values, which ranged up to 2.2% (Fig. 2). There were no general trends observed in %IC with other measured or estimated parameters, including CUE; however, for the two species that were able to grow on tannic acid, %IC was positively correlated with the amount of CO_2 and biomass produced during the incubation ($R^2 > 0.85$, $n = 5$, $p < 0.01$).

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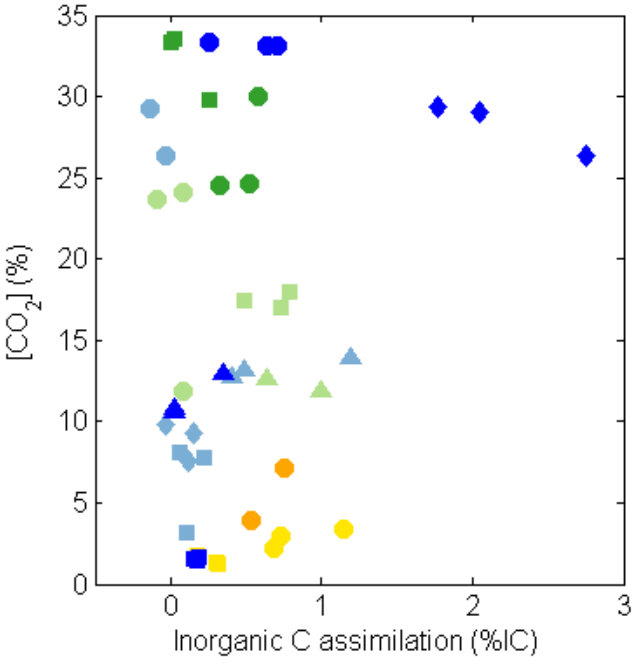

Figure 2. %IC values for fungal species respiring glucose (circles), succinate (squares), tannic acid (diamonds), or β-cyclodextrin (triangles). Colors represent fungal phyla as described in Fig. 1.

3.2.2 Water assimilation factor (α_W)

The "net" contribution of water hydrogen to lipid H is reported as the water hydrogen assimilation factor α_W (Kopf et al., 2015), and was estimated based on the slope of the linear regression line between H isotopic composition of lipid versus growth medium water (Fig. 3), which ranged from natural MilliQ (δ^2H = -45‰ ± 10‰) to the labeled treatments (65‰ ± 4‰; 166‰ ± 10‰; 368‰ ± 27‰). The α_W values for the fungal biomarker $C_{18:2}$ grown on glucose ranged from 0.37 ± 0.03 to 0.75 ± 0.06 with an average value of 0.60 ± 0.05 (n = 6 species; ±SEM). When grown on succinic acid, the α_W values for $C_{18:2}$ harvested from individual species ranged from 0.78 ± 0.01 to 0.96 ± 0.02 with an average value of 0.83 ± 0.04 (n = 4 species; ±SEM). When grown on tannic acid, the α_W values for $C_{18:2}$ harvested from individual species ranged from 0.74 ± 0.06 to 0.77 ± 0.03, and when grown on β-cyclodextrin the α_W values for $C_{18:2}$ ranged from 0.46 ± 0.03 to 0.68 ± 0.04 with an average value of 0.58 ± 0.06 (n = 4 species; ±SEM). The average α_W values for $C_{18:2}$ for all substrates and species was 0.67 ± 0.04 (±SEM).

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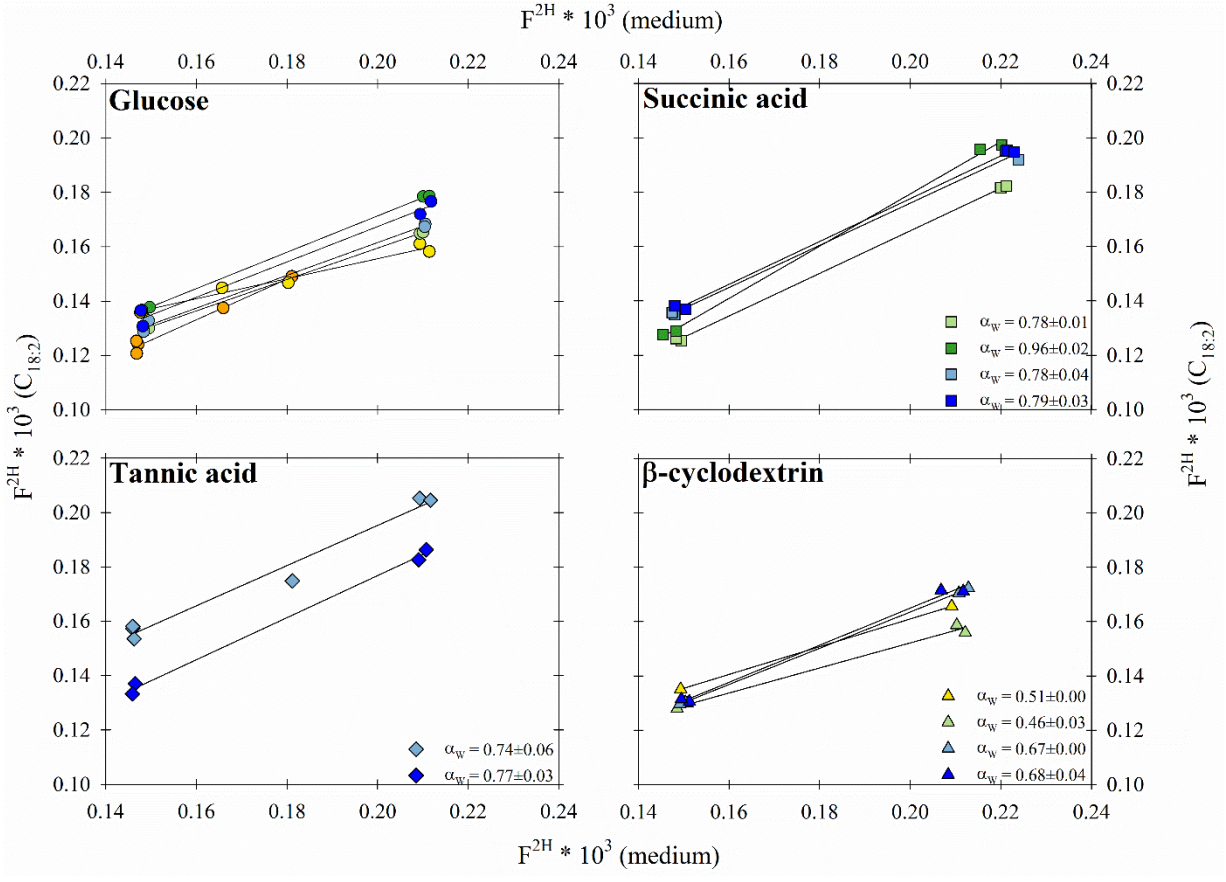

Figure 3. The water hydrogen assimilation factor (αW values) estimated as the slope of the fractional $^{2/1}H$ abundance (F^{2H}) in lipids (y-axis) versus medium water (x-axis). Data are shown for fungal biomarker $C_{18:2}$ produced during growth on the different *substrates (glucose, succinic acid, tannic acid and β-cyclodextrin) and harvested from the different fungal species [Paxillus involutus (PI), Phanerodontia chrysosporium (PC), Mortierella (MO), Umbelopsis (UM), Penicillium jancewskii (PJ) and Paecilomyces lilacinus (PL)]. R^2 values for all slopes were > 0.97.*

4 Discussion

4.1 Fungal growth dynamics

Collectively, the fungal incubation experiments included a total of six species representing three different phyla, and exhibited a large range in the relative amounts of CO_2 and biomass produced, with the estimated carbon use efficiency [CUE = biomass-C / (CO_2 + biomass-C)] ranging from 0.2 to 0.6 (Fig. 4). The incubations were initiated under atmospheric, oxic conditions, such that fungi were able to respire the substrate aerobically. While oxic conditions likely prevailed during most of the incubation period, it is probable that some incubations turned anoxic when CO_2 levels exceeded 21%, which occurred primarily in incubations with glucose or *Mortierella*. This was an unintended consequence of performing the incubations in closed bottles, which was required to prevent the escape of ^{13}C-labeled inorganic C. Nevertheless, such alteration between oxic and

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anoxic conditions is common in natural environments, and the measured inorganic C assimilation into fungal lipids was consistently low (<3%; Fig. 2), regardless the implied anoxia.

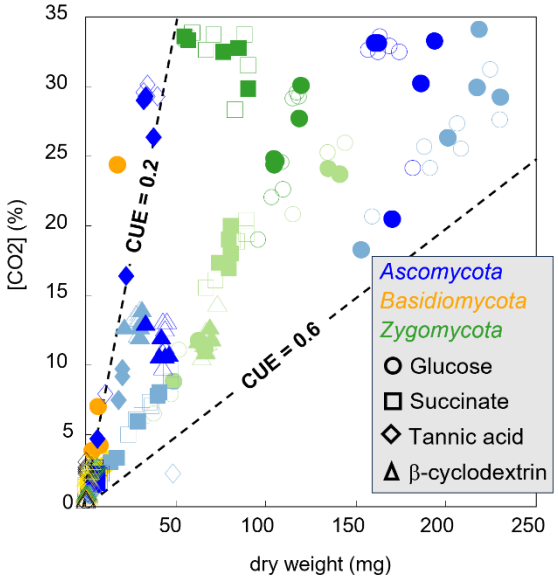

Figure 4. Carbon use efficiency of fungal species from three phyla growing on monomers or complex substrates. Lines were calculated assuming that fungal biomass was 44% C (w/w). Colors and symbols are redundant with Fig. 1.

4.2 Fungal IC assimilation into lipids

A fundamental process in nature and basis for ecological food webs is the fixation of inorganic C via photosynthesis and chemosynthesis by autotrophic organisms. Inorganic C assimilation by heterotrophic organisms also plays an important role in ensuring the provision of energy and to replenish intermediates in the TCA cycle that have been released for biosynthesis (Kornberg 1965). Therefore, inorganic C assimilation is a measure of both anabolic processes and the catabolic status of the cell, influenced by assimilation, biosynthesis, anaplerotic reactions and redox balancing reactions (Braun et al., 2021; Erb 2011). Previous reports on the by-fixation of inorganic C (%IC) via anaplerotic pathways into heterotrophic biomass varied between 1 - 8% (Dijkhuizen & Harder, 1985; Feisthauer et al., 2008; Romanenko 1964; Roslev et al., 2004), whereas for fungi it was previously reported to amount to roughly 1% (Sorokin 1961; Schinner & Concin, 1981; Schinner et al., 1982), and was recently shown to vary between 2 – 12% for Ascomycetes when grown on glucose or glutamic acid (Jabinski et al., 2024). Our results demonstrate a low range in %IC for all different substrates and species tested in this study (0 - 3%), with the Ascomycetes (0 - 2%) assimilating relatively less inorganic C then previously reported species (4.6% ± 1.6%; Jabinski et al., 2024). The highest incorporation was 2.8% by *Penicillium jancewskii* (PJ) when grown on tannic acid (Table 1; Fig. 3);

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notably, this experiment yielded high production of CO_2 and biomass, suggesting that increased assimilation of inorganic C may promote the ability to respire the complex substrate. The high CO_2 levels also suggest that the incubations of PJ with tannic acid may have turned anoxic, which may also explain the higher inorganic C incorporation in these incubations.

4.3 Water hydrogen derived Lipid synthesis

As demonstrated previously, the regression slope between hydrogen isotopic composition of water medium and microbial lipids (i.e., α_W) varies with the type of metabolism (Zhang et al., 2009; Valentine, 2009; Wijker et al., 2019; Jabinski et al., 2024). For fatty acid biosynthesis, H incorporation is suggested to be a function of transporters and electron acceptors (NADPH and NADH), with contributions accounting for around half of all lipid hydrogen. The remaining comprises equal contributions of H obtained directly from environmental water or acetyl-CoA (Valentine, 2009; Zhang et al., 2009; Caro et al., 2023). The consensus from previous studies that investigated the lipids of heterotrophic bacteria is that microbial heterotrophs exhibit α_W values ranging from 0 to 1.2, with a mean of 0.71±0.17 (e.g., Caro et al., 2023). Jabinski et al. (2024) demonstrated that five species of heterotrophic Ascomycetes exhibit similar α_W values (0.62 ± 0.04) for the fungal biomarker $C_{18:2}$ during growth on glucose. In the current study, α_W values for the fungal biomarker $C_{18:2}$ during growth on glucose (0.60 ± 0.05) were agreeable with Jabinski et al. (2024), but more variable, likely owing to the broader phylogenetic coverage of the current study. However, significant differences in α_W values were observed both between and within the different phyla and substrates tested.

Ascomycetes species exhibited the most consistent α_W values when grown on each of the four different substrates [0.63 ± 0.03 (Glu); 0.78 ± 0.01 (SA); 0.76 ± 0.02 (TA); 0.67 ± 0.01 (BC)]. Basidiomycetes only produced enough biomass for isotopic analysis when grown on glucose and β-cyclodextrin, and showed high variability in α_W between species. For example, during growth on glucose, *P. involutus* exhibited much higher α_W values than *P. chrysosporium* (0.75 ± 0.06 versus 0.37 ± 0.03, respectively), and both of these values were beyond the more confined range of α_W values determined for Zygomycetes and Ascomycetes species. Growth on β-cyclodextrin, which consists of seven glucanopyranose units ($C_6H_{12}O_6$), exhibited similar α_W values (0.58 ± 0.06) as growth on glucose (0.60 ± 0.05), suggesting that the catabolism of glucose subunits via glycolysis overprints signals of water-H incorporation that may derive during degradation of β-cyclodextrin. Succinate yielded significantly higher α_W values (0.83 ± 0.05), which was more similar to that reported previously for glutamic acid (0.90 ± 0.07; Jabinski et al., 2024). A one-way analysis of variance (ANOVA; Holm-Sidak method; SigmaPlot v11) confirmed the significant difference between glucose and glutamic acid ($p < 0.001$), glutamic acid and β-cyclodextrin ($p < 0.001$), succinate and glucose ($p < 0.003$) and succinate and β-cyclodextrin ($p < 0.005$). It also confirmed that there was no significant difference between the other substrate combinations ($p > 0.005$). Notably, glutamic acid and succinate are thought to be introduced into the TCA cycle through coupled metabolites, where succinate is a direct metabolite inside the TCA cycle and glutamic acid is converted to α-ketoglutarate intermediate by transamination before entering the TCA cycle, which is only 2 steps from succinate (Cooper et al., 2014). Also, being acids, these substrates may have a greater capacity than saccharides to exchange H with ambient water at experimental pH (typically $2 < pH < 5.2$), especially glutamic acid, which also comprises an amino moiety. Tannic acid (0.76 ± 0.02) yielded no significant differences ($p > 0.005$) from the other substrates, and is reported to

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be degraded to different subunits including gallic acid and glucose (Banerjee and Mahapatra, 2012; Lekha and Lonsane, 1997 and references within). Aromatic degradation pathways employed by fungi generate intermediates that go through the β-ketoadipate pathway (Mäkelä et al., 2015) before entering the TCA cycle as a succinyl-CoA metabolite (Lekha and Lonsane, 1997). The α_W values induced by degradation of TA suggest that it integrates both the low α_W signature of glycolysis and high α_W signature of the TCA cycle (Fig. 5).

Together, our incubation experiments suggest that α_W values determined for the fungal biomarker $C_{18:2}$ could not distinguish between fungal growth on relatively labile monomers (i.e., glucose and succinate; requiring as few as 5 days of cultivation) versus larger, less-labile substrates (i.e., β-cyclodextrin and tannic acid; requiring 20 to 183 days of cultivation). However, with the exception of Basidiomycetes, α_W values of fungal lipid biomarkers may be indicative of fungi employing primarily glycolytic or TCA pathways. Environmental assays that quantify fungal lipid production via the incorporation of ambient water-H (i.e., the lipid-SIP approach) may upscale to total production estimates by applying our calculated mean α_W value of 0.69 ± 0.03 [n = 27; ± (SEM)], which is consistent with the α_W value of 0.71 recommended for soil microbial communities (Caro et al., 2023).

4.4 Dual-SIP approach

Dual-SIP experiments with ^2H_2O and ^{13}C-dissolved IC previously highlighted the potential to track microbial activity and distinguish heterotopic vs autotrophic metabolic modes within environmental settings and pure cultures (Kellerman et al., 2012, 2016; Wegener et al., 2012; Huguet et al., 2017; Wu et al., 2018, 2020). This approach was also previously applied to investigate fungal pure cultures (Jabinski et al., 2024), in which the plot of assimilation of inorganic C versus water-H into the fungal biomarker $C_{18:2}$ could distinguish five Ascomycetes species growing on glucose or glutamic acid, with α_W values explaining most of the variability. While calculated IC:α_W are useful to distinguish autotrophic from heterotrophic growth, all calculated values in this study remained near zero, with %IC ranging up to 3% and α_W values ranging from 0.6 to 0.8. This pure culture study therefore suggests that fungal assimilation of inorganic C is low and less insightful than the more distinguishable α_W values for identifying fungal phylotypes or ecotypes in environmental assays.

5 Conclusion

The purpose of this work was to apply the dual-SIP assay on pure fungal cultures to define the effect of different carbon substrates on incorporation of water-H and inorganic C into their membrane lipids. Although heterotrophic C fixation by microbes may range up to 8% of biomass C, inorganic C assimilation into the fungal biomarker $C_{18:2}$ harvested from six species representing Ascomycetes, Basidiomycetes, and Zygomycetes did not vary consistently between species or substrate, and remained below 3%. However, *Penicillium jancewskii*, the species that was most successful at respiring tannic acid, also exhibited the highest %IC value of all incubations (Fig. 1; Fig. 5), suggesting that fungal degradation of similarly complex substrates may rely in part on the assimilation of inorganic C (e.g., via anaplerotic reactions).

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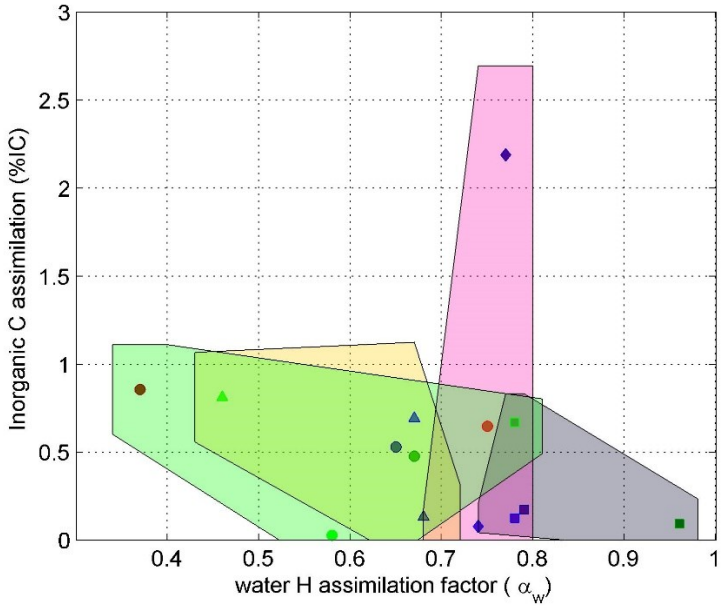

Figure 5. %IC and α_W value scatter plot for $C_{18:2}$ identifying the grouping of glucose (circles, green shape), succinic acid (squares, grey shape), tannic acid (diamonds, pink shape) and β-cyclodextrin (triangles, yellow shape) incubations with Ascomycetes, Basidiomycetes, and Zygomycetes. Refer to Fig. 1. Caption for further details.

In contrast to %IC, we conclude that substrates that activated the glycolysis pathway yielded significantly lower α_W values than those catabolized as TCA intermediates. The expanded dataset provided by this study indicates that inorganic C assimilation by heterotrophic fungi accounts for < 3% of lipid carbon, and fungal production estimated by ^{2}H-lipid SIP experiments can be adjusted by an average α_W value of 0.69, to provide a more accurate estimate of total lipid production. Furthermore, determination of α_W values in environmental ^{2}H-SIP assays may be useful to identify the prevalence of fungal ecotypes that rely on C substrates fueling glycolysis (e.g., leaf litter) versus those that are fed primarily by TCA intermediates (e.g., root exudate).

Data availability

Data presented in the figures and tables can be obtained by contacting the corresponding author and will be made available on the Fractome Database (https://fractome.caltech.edu/).

Author contribution

Stanislav Jabinski, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing

Vítězslav Kučera, Investigation, Methodology, Resources, Writing – review and editing

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Marek Kopáček, Formal analysis, Methodology, Resources, Validation,
Jan Jansa, Conceptualization, Formal analysis, Methodology, Resources, Validation, Writing – review and editing
Travis B. Meador, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing

Competing interests

The authors declare that they have no conflict of interest.

Disclaimer

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