Towards measuring growth rates of pathogens during infections by D$_2$O-labeling lipidomics

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Rationale: Microbial growth rate is an important physiological parameter that is challenging to measure in situ, partly because microbes grow slowly in many environments. Recently, it has been demonstrated that generation times of S. aureus in cystic fibrosis (CF) infections can be determined by D$_2$O-labeling of actively synthesized fatty acids. To improve species specificity and allow growth rate monitoring for a greater range of pathogens during the treatment of infections, it is desirable to accurately quantify trace incorporation of deuterium into phospholipids.

Methods: Lipid extracts of D$_2$O-treated E. coli cultures were measured on liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) instruments equipped with time-of-flight (TOF) and orbitrap mass analyzers, and used for comparison with the analysis of fatty acids by isotope ratio gas chromatography (GC)/MS. We then developed an approach to enable tracking of lipid labeling, by following the transition from stationary into exponential growth in pure cultures. Lastly, we applied D$_2$O-labeling lipidomics to clinical samples from CF patients with chronic lung infections.

Results: Lipidomics facilitates deuterium quantification in lipids at levels that are useful for many labeling applications (>0.03 at% D). In the E. coli cultures, labeling dynamics of phospholipids depend largely on their acyl chains and between phospholipids we notice differences that are not obvious from absolute concentrations alone. For example, cyclopropyl-containing lipids reflect the regulation of cyclopropane fatty acid synthase, which is predominantly expressed at the beginning of stationary phase. The deuterium incorporation into a lipid that is specific for S. aureus in CF sputum indicates an average generation time of the pathogen on the order of one cell doubling per day.

Conclusions: This study demonstrates how trace level measurement of stable isotopes in intact lipids can be used to quantify lipid metabolism in pure cultures and provides guidelines that enable growth rate measurements in microbiome samples after incubation with a low percentage of D$_2$O.

INTRODUCTION

Bacteria continually react to diverse stimuli, such as the availability of nutrients and electron acceptors, exposure to antimicrobial drugs or attack by the immune system. However, measuring microbial metabolites and growth rates within a complex environment still poses many technical challenges. Two recent advances in microbial ecology are beginning to make it possible to measure average growth rates in environmental samples. The first advance is based on metagenomic DNA sequencing and takes advantage of the observation that growing...
cells yield more sequencing reads at genomic regions near the origin of replication. This method is applicable to any microbial species in a microbiome as long as its assembled genome has a high sequence coverage. The second advance uses isotopic labeling, to identify active cells and to determine the biosynthesis rates of microbial lipid metabolites by mass spectrometry. Stable-isotope probing has a larger dynamic range than sequencing and can be used to quantify slow growth rates under environmental conditions. A limitation of stable-isotope probing, however, is the identification of metabolites that are diagnostic for a specific microorganism. It is therefore desirable to combine isotopic labeling with a method such as lipidomics, which can detect a large number of microbial metabolites.

Lipids have been used for decades in ecology as markers of microbial metabolism, where they reveal information about viable biomass, nutritional status or changes in the microbial community structure. Also, lipids can still be analyzed long after nucleic acids and peptides are degraded. In order to estimate the growth rates of microbes, the active production of strain- and genus-specific lipid metabolites can be measured with stable isotope labeling. With advances in soft ionization mass spectrometry we can now attempt to combine isotope quantification and lipidomics for the study of microbes in situ.

Soft ionization mass spectrometry detects thousands of lipids in environmental extracts and would in principle be well suited to quantify the biosynthesis of lipid biomarkers by itself. However, extraction yields and ionization efficiency vary widely between samples. This currently poses severe constraints on measuring lipid production rates and effectively limits estimating bacterial growth rates in natural environments.

Ratios of isotopes can be measured with high accuracy by mass spectrometry, partly because ratiometric readouts vary less than absolute ion intensities. For lipid biosynthesis, the incorporation of an isotope tracer such as 13C-labeled substrates can provide a robust way to quantify anabolic activity and lipid turnover. In microbiome samples bacteria differ widely in their ability to take up carbon sources and gases such as CO2, depending on their genetic capabilities and metabolic states. D2O is a non-discriminating tracer of de novo lipid biosynthesis and thus often better suited for microbiome studies. D2O-labeling has recently been used to estimate in situ growth rates of Staphylococcus aureus in chronically infected lungs. After labeling of expectorated sputum with D2O, the deuterium enrichment of anteiso fatty acids was quantified using gas chromatography/pyrolysis/isotope-ratio mass spectrometry (GC/P/IRMS) in order to estimate the growth rate of the pathogen. Many previous studies have been used to study lipid biosynthesis with D2O in vivo. Environmental samples, however, provide particular challenges. For example, microbes often grow slowly in situ and one can expect low rates of deuterium incorporation into lipids. Whether trace levels of incorporation can reliably be detected remains to be studied before deuterium incorporation can be used to determine lipid biosynthesis rates in situ by lipidomics.

In this study we apply stable-isotope probing with D2O and measure deuterium incorporation by MS-based lipidomics. This approach can be used to obtain labeling rates for individual intact lipids in environmental samples, where microorganisms typically grow slowly. We begin by characterizing several technical aspects regarding quantifying low levels of deuterium labeling. We then refine our application of D2O-labeling lipidomics by tracking of lipid labeling in E. coli during the stationary-to-log phase transition. This reveals lipids that have distinct labeling dynamics that are not obvious from measuring absolute analyte concentrations alone. Lastly, we test D2O-labeling lipidomics in a clinical context with the aim of measuring the growth of S. aureus in cystic fibrosis lung infections. In sum this study establishes principles for how growth rates of microbes in situ can be estimated by stable isotope probing lipidomics.

2 | EXPERIMENTAL

2.1 | Deuterium-enriched growth medium

M9 minimal medium was prepared with 3.8 μM thiamine pyrophosphate and glucose (22.2mM) or sodium acetate (15mM). All media were sterilized by filtration (0.2 μm). D content was adjusted by isotope dilution (measured by weight) of D2O (D, 99.9 at%; Cambridge Isotope Laboratories) with natural abundance water (MilliQ, EMD Millipore) of known isotopic composition. The D content (fractional D abundance, 2D/WATER[corresponding to δD values from −124 to +287‰]) was measured on a DLT-100 liquid water isotope analyzer (Los Gatos Research). Samples were analyzed in three technical replicates, each comprising 10–12 injections. Samples with D abundances close to natural abundance were calibrated against standards ranging from 0.0136% to 0.0200% 2D/WATER (corresponding to δD values from −124 to +287‰). These were in turn calibrated against the VSMOW, GISP, and SLAP international standards. More enriched samples were measured against working standards made in-house, ranging from 0.050% to 0.150% 2D/WATER. The presence of doubly substituted species (D-O-D) was not taken into consideration due to fast equilibration of water molecules below 0.0150%. Samples beyond this scale were no longer in the linear response range of the instrument, and we calculated 2D/WATER based on the gravimetric preparation of the medium. Their 2D values were confirmed by water isotope analysis after dilution with natural abundance water of known isotopic composition.

2.2 | E. coli cultures

Escherichia coli K-12 (FRAG1) was streaked on LB agar plates for single colonies and used to inoculate 6 mL precultures of M9 minimal medium with glucose as carbon source. All cultures were incubated at 37°C and shaking at 250 rpm (Innova 44 shaker, New Brunswick Scientific). Cultures were checked by phase contrast microscopy (Axio Scope.A1, Zeiss). Optical density (OD) was measured at 600 nm wavelength on a DU 800 spectrophotometer (Beckman Coulter).

To investigate the detection limits of 2D/LIPID and the comparison of LC/MS with GC/P/IRMS, precultures were grown for 20h at natural D abundance. Volumes of 75 μL were used to inoculate 150 mL medium in 1 L Erlenmeyer flasks. For analysis by GC/P/IRMS, the medium had a 2D/WATER in the range from 0.0142% to 0.0202% (δD =90 to +300‰). For analysis using LC/MS the medium had a D content of 0.0142% to 4% 2D/WATER. Cultures were harvested at
OD_{600} 0.2–0.3 by chilling 50 mL culture in ice and centrifugation at 4°C for 20 min at 5000 g. Cell pellets were frozen in liquid nitrogen and stored at −20°C.

For monitoring change in $^2 F_{\text{LIPID}}$ during stationary phase exit, two precultures (0.0142% and 4% $^2 F_{\text{WATER}}$) were centrifuged at 15°C for 10 min at 15,000 g. The pellets were resuspended in prewarmed medium (0.0142% or 4% $^2 F_{\text{WATER}}$) and used to inoculate 200 mL M9 glucose medium at an initial OD_{600} of 0.1. This yielded four combinations ($\mu$L, uL, IU, IL) of unlabeled/labeled inoculum ($u$, I) in unlabeled/labeled growth medium ($U$, L). Aliquots were incubated and sampled as described above (20 min: 40 mL, 50 min: 30 mL, 100 min: 30 mL and 150 min: 30 mL). At each time point, the OD_{600} was recorded and the protein content of the bacterial culture was measured via BCA protein assay (Thermo Scientific) from a cell pellet (2 mL aliquot, centrifuged at 4°C for 2 min at 16,900 g). Maximum OD_{600} was 0.8 for cultures uL, uL and 1.2–1.3 for cultures IU, IL. Before the BCA assay cells were chemically lysed (BugBuster, EMD Chemicals). Absorbance was recorded at 562 nm on a plate reader (Synergy 4, BioTek).

### 2.3 Liquid chromatography/mass spectrometry (LC/MS)

For LC/MS analysis lipids were extracted based on the procedure by Matyash et al.\textsuperscript{29} Cell pellets were resuspended in 0.1% ammonium acetate to a protein concentration of 200 mg/mL (20 OD_{600}/mL). Then 100 μL of this suspension were added to 1.5 mL methanol, followed by 5 mL methyl tert-butyl ether (MTBE) and a mix of standards containing PE(17:0/17:0), PG(17:0/17:0) and 14:1(3)–15:1 cardiolipin was added (Avanti Polar Lipids). After incubation in an ultrasonic bath for 1 h, lipids (in the top phase) were extracted by adding 1.25 mL water and re-extracted by addition of 2 mL MTBE/methanol/water (10:3:2.5). Samples were dried under N₂ at −20°C and dissolved in 1 mL methanol/dichloromethane (9:1) for analysis by LC/MS.

LC/MS data were collected on an Acquity I-Class UPLC coupled to a Xevo G2-S TOF mass spectrometer (Waters). Intact polar lipids were separated on an Acquity UPLC CSH C18 column (2.1 mm × 100 mm, 1.7 μm; Waters) at 55°C following a protocol established by Waters Corporation and adapted in our laboratory.\textsuperscript{30} Samples were run in three randomized instrument replicates (injection volume 5 μL). LC/TOF-MS data was collected in positive and negative mode (scan time 0.3 s; alternating MS and MS$^E$ scans; resolving power ~30,000 FWHM) using electrospray ionization (ESI) with a desolvation temperature of 550°C and source temperature of 120°C.

Lipids were identified by the mass-to-charge ratio (m/z) of their molecular ion, their fragmentation products in positive and negative mode, and comparison with representative standards. Note that the assignment of sn-1 and sn-2 fatty acyl positions is tentative.\textsuperscript{11} Lipids are named based on LIPIDMAPS classification.\textsuperscript{32} Table S1 (supporting information) provides a summary of ions and retention times used for quantification. The monoisotopic intensity alone yields incorrect values for absolute lipid concentrations in labeling experiments, where some of the signal has shifted to higher masses. So we quantified the absolute concentrations of labeled lipids by comparing the sum of all intensities of its isotopologues with those of an internal standard (Figure S7, supporting information). For each analyte, MS spectra were inspected for potential spectral overlap of co-eluting compounds. A window of retention time (~0.5 min; ~50 scans) was chosen and consistently used for peak integration across different samples and replicate runs. Peaks in extracted ion chromatograms were integrated using the software MAVEN.\textsuperscript{33} Up to eight mass isotopomers were integrated per analyte (4% D₂O labeling). Mass isotopomers with small signal intensities (~2 times the signal intensity of the M⁹ isotopomer) were excluded. Subsequent analysis was done in R.\textsuperscript{34} Models of isotopic distribution patterns were calculated using the R package Isopat.\textsuperscript{35}

In order to calculate the generation time, we fit our data to an exponential growth model. We consider that the number of lipids increases linearly with the number of cells. The number of lipid molecules at time $t$ are given by:

$$N(t) = N_0 \times 2^{t/GT} + N_i,$$

where $N_0$ is the initial number of exponentially reproduced molecules, GT is the generation time and $N_i$ the number of ‘inactive’ molecules at time 0. Time course data of the fractional abundance of D in the lipids ($^2 F_{\text{LIPID}}$) was fitted using the equation:

$$^2 F_{\text{LIPID}} = \max \times \left( \left(2^{t/GT} - 1\right) / \left(2^{t/GT} + N_i/N_0\right) \right),$$

where max is the saturation level of labeling.

For direct infusions a stock solution of PE(18:0/18:1) in chloroform (10 g/L; Avanti Polar Lipids) was diluted to 1 μmol/L in 4:1 acetonitrile/water containing 10 mM ammonium formate and 0.1% formic acid. H₂O (0.015 at% D) or D₂O (99.9 at% D) was used for the water portion. PE(18:0/18:1) was initially dissolved in acetonitrile, later ammonium formate dissolved in water, and 0.1% formic acid was added. Direct infusion data was collected for 4 min at a flow rate of 0.4 mL/min. ESI and detector settings were as in LC/MS lipidomics experiments. First the H₂O sample was measured, followed by washing the pumps and measuring the D₂O sample.

Orbitrap measurements were performed at Thermo Fisher Scientific (San Jose, CA, USA) using a Q Exactive Plus mass spectrometer operating in full MS scan mode at a resolution of 35,000 (FWHM at m/z 200). The m/z range was set from 150 to 2000 for negative ion mode and from 150 to 1200 for positive ion mode. The automatic gain control target value was set at 10⁶ and the maximum injection time was set at 50 ms. Chromatography was performed on a Vanquish UPLC system (Thermo Fisher Scientific), but otherwise unchanged. ESI was performed using a heated-ESI (HESI) ion source (spray voltage: 3.00 kV; sheath gas: 40; aux gas: 10; sweep gas: 1; capillary temperature: 300°C; S-Lens RF Level: 45; probe heater temperature: 325°C).

### 2.4 CF sputum and S. aureus

Sputum collection was approved by the Institutional Review Board at the Children’s Hospital Los Angeles (IRB# CCI-13-00211). All patients were recruited from the Children’s Hospital Los Angeles and informed consent or assent was obtained from all study participants or from a parent or legal guardian. Within 10 min of expectoration, sputum...
samples were incubated at 37°C for 1 h with an equivalent weight of prewarmed PBS (phosphate-buffered saline) solution containing 5% (w/w) D₂O as described previously. Information about microbial community composition and fatty acid analysis of sputum samples is published elsewhere.

Samples used for LC/MS were from Patient 1 (2nd hospitalization, day 5; method development), Patient 2 (day 2; growth rates). S. aureus negative controls: Patient 5 (day 1), Patient 7 (days 18 and 19).

Lipid extracts from S. aureus (grown in LB medium) were prepared and analyzed by LC/MS as for E. coli. For sputum, 10 mg of lyophilized material was extracted by the same method. The lipid extract was dissolved in 100 μL methanol and 0.2 μL was injected for routine lipidomic profiling. Detection of anteiso-containing phospholipids in sputum was performed by injecting 5 μL lipid extract and restricting flow into the ESI source to a retention time window of 5–8 min.

2.5 Calculation of D content in lipids (²F_LIPID)

The mass spectra of a labeled and unlabeled lipid were compared to determine the fractional abundance of D (²F_LIPID). We calculated the molecular weights of the two isotopologue distributions and divided their difference by the number of C-bound hydrogen atoms. Note that we did not calculate the molecular weights using accurate masses. At 30,000 mass resolving power each signal M1, M2, etc., contains isotopologues which differ slightly in mass. Measured masses have additional experimental uncertainties. For simplicity, we rather used the fact that each isotopologue must have gained a certain number of neutrons; for example, M8 has gained a total of 8 neutrons from ¹³C, ²H, etc. We used the isotopologue distribution to calculate by how many neutrons the distribution had shifted with respect to the monoisotopic mass M0. This approach eliminates inaccuracies. It also avoids the complicating fact that the mass difference between a D atom and ¹H is not exactly 1.

2.6 GC/pyrolysis/isotope-ratio mass spectrometry

20 mg of frozen and lyophilized cell pellet was transesterified and extracted in hexane/anhydrous methanol/acetyl chloride at 100°C for 10 min. The extract was concentrated under N₂. Fatty acid methyl esters (FAMEs) were first analyzed by gas chromatography/mass spectrometry (GC/MS) on a Thermo-Scientific Trace/DSQ with a ZB-5 ms column (30 m x 0.25 mm, film thickness 0.25 μm) and PTV injector operated in splitless mode. Peaks were identified by comparison of mass spectra and retention times with authentic standards and library data.

The δD of FAMEs was measured by gas chromatography/pyrolysis/isotope-ratio mass spectrometry (GC/P/IRMS) on a Thermo-Scientific DELTAplusXP with methane of known isotopic composition as the calibration standard. Chromatographic conditions were identical as for regular GC/MS, and peaks were identified by retention order and relative height. Samples were analyzed in triplicate. All data were corrected for methyl H originating from methanol by analyzing the dimethyl derivative of a phthalic acid standard, for which the δD value of ring H is known. For comparison with LC/MS, δD values were converted into fractional abundances (²F).

3 RESULTS

When cells grow after addition of heavy water, the newly synthesized biomass will contain more deuterium (D). This also means that each lipid pool will be a mixture of molecules that vary more in their D abundance. The introduced heterogeneity causes broadening of chromatographic peaks, which could skew the isotope ratio observed by LC/MS as ionization efficiency varies over time. In order to evaluate how this affects isotope quantification by lipidomics, we grew an E. coli culture in 4% D₂O (fractional D-abundance, ²F_WATER) and measured lipids after chromatographic separation using an electrospray ionization time-of-flight (ESI-TOF) mass spectrometer.

E. coli has a comparatively simple lipid composition and its lipid metabolism has been studied for decades. The bacterium therefore provides a solid model system to develop and test methods for stable isotope labeling lipidomics. E. coli lipid extracts contain mainly phosphatidylethanolamines (PE), phosphatidylglycerol (PG) and cardiolipins (CL). When fully labeled in 4% D₂O, the molecular ions from PE and PG lipids extend over a range of 8 m/z. As expected, labeling causes a shift in retention time (Figure 1A). Strongly deuterated molecules elute earlier than more depleted ones (Figure 1B). The maximum shift in retention time is about half of the chromatographic peak width, which indicates that all molecular species have overlapping elution profiles (Figure 1C). Notably, a much larger shift in retention time occurs in GC and this can lead to artefacts when quantifying D/H ratios. We expect that the relatively small shifts observed in LC typically do not alter isotope ratios, as long as a moderate amount of labeling is used. Nevertheless, when integrating the mass spectrum a sufficiently wide retention time window should be chosen to minimize potential artefacts due to early elution of D-enriched lipids.

The quantification of D in intact lipids is complicated by ¹³C, which is naturally present in lipids at about 1.1%. Mass gained by ¹³C or D cannot be distinguished by current TOF mass analyzers (resolving power ~30,000). Resolving the minute mass difference (~3 mDa) is possible for small lipids (~<600 Da) by Orbitrap MS, but this approach is currently not ideal for LC/MS due to the long scan times (~1 s). So we need a procedure to determine the gain of isotopic label indirectly by comparing lipid extracts from bacterial cultures grown with and without label. Methods for evaluating biosynthesis using stable isotopes based on the analysis of isotopomer distributions have been previously developed. However, their application in microbial ecology is limited by low isotopic enrichment, among other factors. For a more robust readout we here use the average molecular weights of the two mass distributions and calculated their difference, ΔMW, interpreted as the mass gain by D incorporation (Figure 2).

The fractional abundance of D in a lipid (²F_LIPID) is then calculated by dividing ΔMW by the number of C-bound hydrogen atoms. Using this method, glycerophospholipids produced by E. coli grown in 4% D₂O yield a ²F_LIPID value of about 2.5%. Values lower than 4% are expected because hydrogen atoms from the unlabeled carbon source are incorporated into the lipids and biosynthetic enzymes favor ¹H over D due to kinetic isotope effects. Note that this calculation assumes that N- and O-bound hydrogen atoms equilibrate fully with water during extraction and chromatography. Additionally, the natural level of D, which is about 0.015%, is neglected for the purpose of this study.
In order to evaluate the utility of D$_2$O-labeling lipidomics for estimating bacterial growth rates, we grew *E. coli* cultures in glucose minimal medium ranging from 0.0156% (natural abundance) to 4% 2$F_\text{WATER}$ and quantified the glycerophospholipids PE and PG on a Waters Xevo QTOF instrument. The minimal medium supports fast growth and was chosen to minimize processes that can interfere with label incorporation into newly made lipids (e.g., fatty acid uptake and recycling). $2F_\text{LIPID}$ increases linearly ($R^2 > 0.99$) with $2F_\text{WATER}$ (Figure 3A). Analyzing the same samples on a Q Exactive Plus Orbitrap operated at $R = 35,000$ yields nearly identical slopes (Figure 3B). Assuming that 0.03% $2F_\text{LIPID}$ is in the linear range of D$_2$O-labeling lipidomics, we suggest that incubating cells for 15 min with 5% 2$F_\text{WATER}$ will enable quantification of lipid biosynthesis from microbes growing at one doubling per day (Figure 3C). These boundary conditions indicate that D$_2$O-labeling lipidomics can be developed further into a method to estimate microbial growth rates in environmental samples. An important consideration for microcosm incubations is that two separate populations of molecules co-occur after labeling, an unlabeled pool and a labeled pool that is enriched in D. High labeling strength would create molecules that occur far away from the monoisotopic mass in the spectrum and become difficult to quantify as the isotopic label gets distributed over a wide m/z range. Ionization conditions can affect isotopologue distributions and thus alter isotope ratios. We varied the injected sample amount, ionization mode, capillary voltage, desolvation temperature and desolvation gas flow without noticing changes. For instance, PE(16:0/16:1) had a $2F_\text{LIPID}$ value of 2.549 ± 0.003 (1σ) in positive ionization mode and 2.567 ± 0.019 in negative mode (lower precision.
due to increased background noise). Less abundant analytes have greater standard deviations. PE(16:0/16:0), which was 10-times less abundant, had a $^2F_{\text{LIPID}}$ value of 2.432 ± 0.078 (positive mode). These trials show that the D abundance of lipids can be measured reproducibly. However, the most intense signals must be within the linear range of the mass analyzer and the detector must not be in dead time on a TOF instrument. Also, isotopeologue patterns that are affected by co-eluting compounds have to be excluded. In our ultra-performance liquid chromatography (UPLC) setup this was the case for some cardiolipins (m/z > 1200). In principle, fragmentation of phospholipids in negative mode can be used to also quantify D in fatty acid fragments. However, we found that lipids with a similar acyl chain composition, e.g. containing C18:0 or C18:1, often yielded overlapping MS$^5$ fragmentation spectra. We therefore here focus on quantifying D in intact lipids. By using a LC column (‘charged surface hybrid’) that separates lipids based on a combination of charge and hydrophobicity, intact lipids of similar m/z were generally well separated by chromatography.

For the calculation of $^2F_{\text{LIPID}}$ we assume that C-bound hydrogens do not exchange with solvent water during sample preparation and electrospray ionization, while N- and O-bound hydrogens fully equilibrate. If this is not the case, we would obtain inaccurate $^2F_{\text{LIPID}}$ values.$^{46,47}$ To test for H/D exchange we compare UPLC/ESI-TOF with GC/P/IRMS, which quantifies near-natural isotopic composition of fatty acids.$^{48}$ The two methods are distinct in many ways, but they should yield a similar linear relationship between $^2F_{\text{WATER}}$ and $^2F_{\text{LIPID}}$.$^{28,47}$ For lipidomics, we determine an average slope for intact lipids produced by E. coli in glucose minimal medium of 0.577 ± 0.003 (Figure S1, supporting information; $^2F_{\text{WATER}}$ between 0.125 and 4%). Slightly higher slopes have been reported previously for E. coli fatty acids using GC/P/IRMS (0.65 ± 0.04 for C16:0, 0.60 ± 0.02 for C16:1 and 0.63 ± 0.03 for C18:1).$^{38}$ Growth on acetate raises $^2F$ in E. coli fatty acids analyzed by GC/P/IRMS, and it does so also for intact lipids measured by LC/MS (Figure S1, supporting information). Overall, we obtain comparable slopes by lipidomics and GC/P/IRMS.

![FIGURE 3](image)

**FIGURE 3** Isotope ratios by lipidomics are suitable for measuring microbial growth rates in situ. (A) Overnight cultures of E. coli were grown in M9-glucose medium containing varying amounts of D$_2$O and lipid extracts analyzed by LC/MS. The measured deuterium content of PE(16:0/16:0(Cp)) is shown. Enrichments with $^2F_{\text{LIPID}}$ > 0.03% were within the linear range. (B) Comparison of a Xevo G2 TOF instrument and a Orbitrap Q Exactive plus (resolving power 35,000 at m/z 200) using the same samples as in (A). $^2F_{\text{LIPID}}$ values for PE(16:0/16:0(Cp)) are plotted. The linear regression has a slope of 0.976 and $R^2$ of 0.999. (C) Estimate of how long D$_2$O incubation would need to be performed for 12.5 min or 1.1 h, respectively, to reach an enrichment of 0.03% over natural $^2F_{\text{LIPID}}$. We here estimate the enrichment at time $t$ using the following equation:$^{2}F_{\text{LIPID}}(t) = 2^t F_{\text{NEW LIPID}} *(1 - 2^{t/T})$ [Color figure can be viewed at wileyonlinelibrary.com]
In order to further constrain H/D exchange, we dissolved PE(18:0/18:1) in acetonitrile, added H2O or D2O (sold as 99.9 at% D), and recorded mass spectra by direct infusion. The addition of D2O shifts the mass spectrum by four units in positive ionization mode, as expected for an analyte that has four non-C-bound hydrogens (Figure 4). The ΔMW of 3.86 suggests that the four exchangeable hydrogens in PE(18:0/18:1) [M + H]+ either have not fully equilibrated with the solvent, or they have and there is an isotope effect of up to 3.5% between water and hydrogens in the amino and hydroxyl groups. A theoretical spectrum that assumes four positions in the ion to have equal probability for D in the four exchangeable sites closely matches the measured spectrum. Importantly, D2O addition does not yield a detectable signal beyond a shift of the unlabeled distribution by four mass units. Such isotopologues would occur if the exchange of C-bound hydrogens occurs at high rates during ESI. Absence of these signals indicates that C-bound hydrogens exchanged at least 2000-fold slower than non-C-bound hydrogens, which is in line with prior assessments of C-bound hydrogen exchange. Together these tests imply that for lipids labeled well above natural D-abundance, no relevant artifacts of 2F LIPID values due to exchange of C-bound hydrogen are likely in UPLC/ESI-TOF.

With the addition of small quantities of D2O to pure cultures we have an opportunity to measure lipid isotope ratios and absolute concentrations simultaneously and compare the two quantifications side by side. Our test case here is the lipid metabolism of E. coli during the transition from stationary phase into exponential growth. Cells from two stationary phase precultures (u: 'unlabeled' and l: 'labeled' in 4% 2F WATER) were used to inoculate two cultures each of unlabeled (U) or labeled (L) medium (Figure 5). Four growth cultures (uU, IU, uL, lL) were sampled to determine optical density (OD600), as well as bulk protein and lipid concentrations. During the 160 min of labeling, cells divided three to four times (Figure S2, supporting information).

In these tests, the stationary phase E. coli cultures contain a high proportion of cyclopropane fatty acids (CFAs), a lipid modification commonly observed in slow growing bacteria. Greater than 25 mol% of PE and PG phospholipids contain at least one acyl chain with a cyclopropyl ring. When cells resume growth, abundance of CFAs

![Figure 5](https://example.com/figure5.png)

**FIGURE 5** Lipid production in E. coli during stationary phase exit was followed by absolute concentrations and deuterium incorporation. (A) Four cultures were followed by lipidomics. Labeling scheme: l = labeled (4% D2O; dark blue) stationary preculture, u = unlabeled (light blue) stationary preculture, L = labeled (4% D2O) growth culture, U = unlabeled growth culture. (B) Relative abundance of PE and PG lipids. Cyclopropane fatty acyls (Cp) are more abundant in stationary phase compared to growth phase (data from uL culture). (C) Quantification of lipid classes (data from uU culture). Throughout the outgrowth the proportion of PE (grey) was 70–78 mol% and PG (blue) 19–24 mol%. CL (orange) is synthesized from two molecules of PG and often increased in stationary phase. In this time course CL was 3.5 mol% in the inoculum and 1.5–2.5 mol% during outgrowth. (D) Time course for PE and PG lipids with 0 (green), 1 (brown) or 2 cyclopropyl rings (violet) in cultures uL and lU. Size of the data points represents deuterium abundance in the lipid (2F LIPID) [Color figure can be viewed at wileyonlinelibrary.com]
decreases to about 12 mol% (Figures 5B and 5C). The formation of CFAs in *E. coli* is a post-synthetic modification of the unsaturated phospholipids that occurs predominantly as cultures enter the stationary phase. CFA synthase has an unusual regulation that involves enzyme instability as well as transcription of the *cfa* gene from two distinct promoters.\textsuperscript{52,53} This means that, although CFA synthase is synthesized at basal levels throughout the growth curve, a transient spike in activity occurs during the log-to-stationary phase transition. In agreement with this regulation, CFAs largely dilute out during stationary-to-log phase transition. Using D\textsubscript{2}O-labeling lipidomics we detect small levels of production of CFA lipids as well as D incorporation, which shows that CFA lipids were actively made during stationary phase exit (Figure 5D).

Untargeted labeling reveals striking differences between phospholipids. Here we describe the *ul* scenario in detail. Some D\textsubscript{2}O-labeling patterns fit an exponential growth model (Figure 6). Other lipids, in particular CFA-containing lipids, were inconsistent with simple exponential de novo production. For them the growth model needs to be extended. We include a parameter that accounts for lipid biomass in the inoculum that is inactive, i.e. not exponentially reproduced during the stationary-to-log phase transition (see section 2.3 for details).

The isotopic labeling patterns of *E. coli* phospholipids are dominated by their two fatty acyl chains, as they contain most of the C-bound hydrogens. A major trend we notice is that lipids that contain unsaturated fatty acids label rapidly, while fully saturated lipids incorporate label more steadily (Figure 6; see also Figures S3 and S4, supporting information). In *E. coli*, unsaturated fatty acids are made during de novo fatty acid biosynthesis and not generated by modification of saturated fatty acids or phospholipids.\textsuperscript{41} The faster labeling of unsaturated lipids we observe thus likely reflects that the unlabeled inoculum contained little unsaturated phospholipids, because most were converted into CFAs during stationary phase. A second common trend is that most CFA-containing lipids show slow initial increase in \( ^2F_{\text{LIPID}} \) and often do not reach full saturation levels. This reflects the fact that CFA lipids are only produced in small quantities after inoculation and hence a large proportion of unlabeled material is carried over from the stationary phase. As CFA formation is a post-synthetic modification, labeling of CFA lipids additionally depends on the prior labeling of the precursor pool.

Interestingly, the two common trends we observe, namely slower labeling of saturated lipids compared to unsaturated lipids and slow and incomplete labeling of CFA-containing lipids, do not apply to all phospholipids. For example, PE(16:0/18:1) and PG(16:0/18:1) have distinct labeling patterns (Figure S3, supporting information). Generally, the labeling of PE lipids that have one unsaturated and one saturated straight chain fatty acyl reveal a significantly larger proportion of unlabeled lipid compared to their PG analogs. Distinct labeling dynamics also occur for some CFA lipids. While most CFA lipid pools label slowly and do not reach high labeling, production of PG(14:0/16:0(Cp)) is stimulated so that it gains label rapidly and to high levels (Figure S3, supporting information). This lipid occurs only in trace amounts in stationary phase, as does its precursor PG(14:0/16:1). Therefore, the material produced during outgrowth of the cultures is highly labeled and dominates the PG(14:0/16:0(Cp)) pool. Overall,

\[ \text{FIGURE 6} \] During stationary phase exit lipids have distinct labeling dynamics, which are dominated by the fatty acyl chains. The deuterium abundance (\( ^2F_{\text{LIPID}} \)) in three representative lipids is shown (data from culture *ul*). Lines represent the best fit of a growth model that estimates generation time (GT) and the ratio of inactive to active unlabeled material at time 0 (see section 2.3 for details). The insets show absolute concentrations for comparison. See Figures S3 and S4 (supporting information) for a comprehensive overview of lipids [Color figure can be viewed at wileyonlinelibrary.com]
these tests indicate that D₂O addition allows a readout of how much of the material has been newly synthesized even for minority components, whose absolute concentrations can be challenging to quantify in complex lipid extracts.

The results so far indicate that lipidomics can be used to measure bacterial lipid biosynthesis in pure cultures. If D₂O-labeling lipidomics could quantify microbial growth reliably in situ, this might for example enable the use of microcosm incubations to test how different drugs impact the microbial community of individual patients. A disease context that is well suited to assess the practicability of D₂O-labeling lipidomics for complex samples are lung infections in cystic fibrosis (CF) patients. These chronic lung infections contain heterogeneous mixtures of human biomass and microorganisms. Some of the bacteria in CF lungs become pathogenic and tend to develop drug-resistant phenotypes. In previous work on D₂O-labeled expectorated CF sputum we have examined the growth of S. aureus via D/H ratios of anteiso-fatty acids, which are abundant fatty acids of this pathogen. It is important, however, that anteiso-fatty acids are produced also by other bacterial species. In the context of CF sputum, Prevotella melaninigenica and Stenotrophomonas maltephilia are relevant sources of anteiso-C₁⁵:₀ and anteiso-C₁⁷:₀ fatty acids in some CF patients. Certain phospholipids, specifically those that contain anteiso-fatty acyls, may therefore be more specific markers of S. aureus in CF infections and could be used to assess activity of the pathogen by lipidomics. To evaluate this hypothesis, we analyzed samples that had been collected and characterized as part of a longitudinal study of CF patients undergoing pulmonary exacerbations.

A lipid that appears well suited to monitor the growth of S. aureus is PG(a-C₁⁵:₀/a-C₁⁷:₀). A signal for this compound was detected in lipid extracts of S. aureus. PG(a-C₁⁵:₀/a-C₁⁷:₀) was assigned based on the m/z of the molecular ion in positive and negative ionization mode as well as MS/MS fragmentation spectra (note that anteiso and straight chain fatty acyls cannot be distinguished by LC/MS/MS data alone; however, S. aureus produces only trace amounts of iso-C₁⁵:₀ and iso-C₁⁷:₀). Subsequently, MS and MS/MS signals from this lipid were also detected in expectorated sputum from several CF patients with S. aureus infection (Figure 7). Two patients whose lung infections did not contain S. aureus showed no signal corresponding to PG(a-C₁⁵:₀/a-C₁⁷:₀). Based on these observations PG(a-C₁⁵:₀/a-C₁⁷:₀) in CF sputum appears to be a specific marker for S. aureus in CF sputum.

Microbial lipid metabolites make up only a minute fraction of the total lipid content of CF sputum. The high sensitivity of ESI-MS allows detection of trace components; however, we had to use concentrated lipid extracts to obtain sufficiently high signal intensities for the target analyte. In order to minimize contamination of the mass spectrometer, we only collected MS data at retention times that are needed to detect abundant phospholipids of S. aureus (5-8 min.). Labeling of CF sputum at 4%² F for 1 h resulted in 0.05±0.04 at% D enrichment of PG(a-C₁⁵:₀/a-C₁⁷:₀). This value can be used to estimate that the average generation time of S. aureus was approximately one cell doubling per day. This estimation is based on a previously established procedure that takes into account diffusion of the label, cell maintenance and other factors. For comparison, the generation time estimated by the D/H ratio of anteiso-C₁⁵:₀ fatty acid in this sample

![Figure 7](https://example.com/figure7.png)
was 3.3 days. The slower estimate based on GC/IRMS could for example be caused by contributions of anteiso-C15:0 from other sources or variability in the production rates of anteiso-C15:0 containing phospholipids in S. aureus. In summary, these initial tests indicate that it is possible to measure the activity of microbial pathogens in situ by D₂O-labeling lipidomics. Its main benefits are that LC/MS has increased species specificity, requires smaller sample amounts and is faster than alternative MS methods. Furthermore, D₂O-lipidomics can be performed on instrumentation that is available in many biomedical laboratories.

The example of CF sputum illustrates the opportunities and challenges of accurate quantification of isotopes in intact phospholipids extracted from microcosm incubations. A critical parameter for D₂O-labeling lipidomics is labeling strength. Very high concentrations of D₂O (e.g., 20 to ~100%) are tolerated by microorganisms and can be used to monitor biosynthesis. For LC/MS high labeling strengths are not desirable because they cause broad isotopic distributions. Quantification would become especially difficult when only a small proportion of the lipid is newly produced. In such a scenario, the labeled lipid would have a broad mass distribution far from the monoisotopic mass and potentially even overlap with other compounds.

Interestingly, the ratio M1/M0 increases approximately linearly with $^{2}$F_{LIPID} (Figures S5 and S6, supporting information). M1/M0 is a simple readout of isotopic label incorporation in environmental samples and we encourage its use when analyzing microcosm incubations. When we assume an excess of unlabeled over labeled lipid, as it is the case for many environmental incubations, we anticipate an optimal labeling strength that causes the greatest change in the M1/M0 ratio. This is achieved when the ΔMW of the newly made lipid is about +1.5 Da. Overall, a concentration of 2–3% $^{2}$F_{WATER} seems most suited for environmental microcosm incubations. The optimal value will depend on the complexity of the lipid sample, i.e. whether D incorporation can be assessed from isotopologue distributions or M1/M0 ratio. Another consideration is that the fraction of D that enters the lipid varies with microbial metabolism. We estimate that the combination of D₂O-labeling and lipidomics as used here can roughly quantify growth rates greater than one cell doubling per day after labeling for 15 min.

4 | CONCLUSIONS

The combination of D₂O-labeling and lipidomics allows a robust isotope ratio measurement, which reveals dynamic aspects of biosynthesis not accessible from absolute concentrations alone. The technology is also sufficiently sensitive to be adapted for environmental samples. Based on this study we encourage the development of LC/MS assays for the analysis of microbial growth in microbiome samples. Routine methods to measure bacterial growth in clinical samples are important to better understand microbial physiology in infections and improve diagnostics.

D₂O-lipidomics can in principle track lipid biosynthesis for many lipids in the same way as we have done here for 27 abundant glycerophospholipids in E. coli. As lipid extracts from tissues or environmental samples are much more complex, initial chemical fractionation of lipids could be used to make data analysis more tractable. Isotope ratios should be little affected by chemical separation and thus D₂O-labeling lipidomics can be optimized to a specific ecosystem. The readout that D₂O-labeling lipidomics enables can be applied to the study of microbial growth rates in clinical samples. It can, for example, also be applied to differentiate biologically active from inactive biomass, necromass, and contaminants. In this study we focused on label incorporation into phospholipids, a class of lipids found in almost all microorganisms. Additional lipid types are produced by some bacteria. Haptonoids are well-studied biomarkers in microbial ecology and would be suited to complement and validate information obtained from phospholipids in environmental samples.

The proof-of-concept demonstration for quantifying microbial growth in CF sputum indicated that quantifying D-label in microcosm incubations remains a profound technological challenge. The ΔMW of ~0.05 Da we observed in sputum for PG(a-C15:0/a-C17:0) is in the linear range of detection (Figure 3A) and suggests an average growth rate of S. aureus on the order of one cell doubling per day, a result similar to the value obtained by GC/IRMS. How accurate growth rate estimates are based on small ΔMW values remains to be established. This current study demonstrated that LC/MS offers significant advantages over previous methods. D₂O-labeling lipidomics can quantify D in a large diversity of lipids, thus increasing specificity of the lipid biomarker. Furthermore, the methods can be implemented in many bioanalytical laboratories by using existing MS instrumentation. Currently, differences in the lipid composition between microbes are already used to identify strains by chemotaxonomy. By combining large-scale lipid detection with the quantification of isotopic labeling, new applications might become possible. These include identifying microbial adaptations to drugs, determining instantaneous microbial growth rates and forecasting composition of microbial community composition after exposure to a stressor.

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