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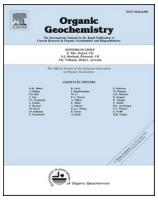

Bacterial physiology highlighted by the $\delta^{13}C$ fractionation of bacteriohopanetetrol isomers

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ABSTRACT

Lipid biomarkers, such as the various bacteriohopanetetrol (BHT) isomers studied here, are useful tools in tracing bacterially mediated nitrogen and carbon cycle processes affecting greenhouse gas emissions, including the anaerobic oxidation of ammonia. Three BHT isomers occur commonly in the environment. By gas chromatography, BHT-34*S* elutes first; it is produced by numerous bacteria. The two later eluting isomers are more constrained in their origin. The marine anammox bacteria '*Ca.* Scalindua' is the only known producer of a BHT isomer of unknown stereochemistry (BHT-*x*), making BHT-*x* a diagnostic biomarker in anoxic marine settings. The BHT-34*R* isomer is produced by three freshwater aerobic heterotrophic producers (*Frankia* spp., *Acetobacter pasteurianus*, and *Komagataeibacter xylinus*), a freshwater serine-cycle (Type II) methanotroph (*Methylocella palustris*), and the freshwater anammox '*Ca.* Brocadia', which makes the detection of freshwater anammox using BHT-34*R* more complicated. We investigated whether the source of BHT-34*R* in freshwater environments could be ascertained *via* its $\delta^{13}C$ value. We used conventional on-column gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (as opposed to high temperature GC-C-IRMS) to determine the $\delta^{13}C$ composition of acetylated BHT isomers in cultured bacteria and bacterial enrichments. We combined these with bulk biomass and substrate $\delta^{13}C$ compositions to establish carbon isotopic fractionation factors. The two anammox genera had large fractionation factors from dissolved inorganic carbon (DIC) to biomass ($\Delta^{13}C_{biomass\ -\ DIC}$ = –43.8 to –26.4 ‰) and to BHTs ($\Delta^{13}C_{BHT\ -\ DIC}$ = –53.8 to –38.2 ‰), which clearly distinguished them from the freshwater aerobic heterotrophic producers ($\Delta^{13}C_{biomass\ -\ substrate}$ = –2.3 to –0.1 ‰; $\Delta^{13}C_{BHT\ -\ substrate}$ = –12.8 to 5.2 ‰). *Methylocella* assimilated mainly carbon from DIC, rather than from methane, into its biomass and BHT, and previous work suggested this assimilation comes with relatively small fractionation. Thus, in peatlands, the BHT $\delta^{13}C$ values of *Methylocella* would not reflect the low $\delta^{13}C$ values of biogenic methane. Consequently, the presence of BHT-34*R* with low $\delta^{13}C$ values relates to '*Ca.* Brocadia' and presents a novel tool to trace anammox in freshwater environments.

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1. Introduction

Anaerobic ammonium oxidation by anammox bacteria plays a key role in the nitrogen cycle since it removes bioavailable nitrogen from anoxic settings (Strous et al., 1999). In modern anoxic marine settings, anammox bacteria are responsible for ~30 % of the loss of the bioavailable nitrogen (Ward, 2013). Anammox bacteria fix carbon in a chemoautotrophic fashion (Schouten et al., 2004); thus, they are potentially important in carbon storage in marine oxygen deficient zones (ODZ) (Lengger et al., 2019). While '*Candidatus* Scalindua' is the dominant genus of anammox bacteria in marine settings, six genera of anammox bacteria ('*Ca.* Brocadia', '*Ca.* Jettenia', '*Ca.* Kuenenia' and '*Ca.* Anammoxoglobus', '*Ca.* Anammoxibacter', *Ca.* Bathyanammoxibiaceae) are found in non-marine ecosystems, including wastewater treatment plants, peatlands, and freshwater lakes (Strous et al., 1999; Schmid et al., 2000; Kuypers et al., 2003; Kartal et al., 2007; Quan et al., 2008; Hu et al., 2011; Yoshinaga et al., 2011; Yang et al., 2017; Wang et al, 2019; Shi et al., 2021; Suarez et al., 2022; Zhao et al., 2022). Although anammox may play an important role in modern lacustrine and terrestrial nitrogen cycling (Schubert et al., 2006; Wang et al., 2012), the contribution of anammox in paleoenvironmental reconstructions remains unclear as there is currently a lack of recalcitrant lipid biomarkers for non-marine anammox.

Ladderanes are lipids unique to anammox bacteria (Sinninghe Damsté et al., 2002). However, their application in paleoreconstructions is limited by their susceptibility to degradation (Rush et al. 2011; Jaeschke et al. 2008). Evidence for anammox in the paleo-marine nitrogen cycle comes from the detection of a more recalcitrant lipid: a unique isomer of bacteriohopanetetrol (BHT; Fig. 1). While BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*S* (BHT-34*S*; Fig. 1a) is ubiquitous in the environment and not source-specific, a later-eluting (by gas chromatography, GC) isomer of BHT with unknown side-chain stereochemistry (referred to as BHT-*x*) has only one known producer: the marine anammox bacteria '*Candidatus* Scalindua' (Rush et al., 2014; Schwartz-Narbonne et al., 2020). BHT-*x* has been used as a biomarker for marine anammox in sapropel deposition of the Pliocene (Hemingway et al., 2018; Rush et al., 2019; Elling et al., 2021) and in Pleistocene sediments underlying the Alaskan ODZ (Zindorf et al., 2020). BHT-34*S* has been detected in marine Palaeogene cores dated to ca. 50 Ma (van Dongen et al., 2006), demonstrating the recalcitrance of BHTs over geological time frames.

Another late-eluting stereoisomer of BHT-34*S*, the 34*R* stereoisomer (BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*R*; BHT-34*R*; Fig. 1b), has only five known producers: the freshwater anammox genera '*Ca.* Brocadia'; the Type II aerobic acidic peatland methanotroph, *Methylocella palustris,* that uses the serine cycle; the aerobic nitrogen-fixer *Frankia* spp.; and the aerobic acetic acid producing bacteria *Acetobacter pasteurianus* and *Komagataeibacter xylinus* (Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001; van Winden et al., 2012; Rush et al., 2014; Schwartz-Narbonne et al., 2020). These two acetic acid bacteria produce relatively large abundances of another BHT isomer (i.e. BHT-17β(H), 21β(H), 22*S*, 32*R*, 33*R*, 34*S*; BHT-22*S*-34*S*; Fig. 1c) and two unsaturated BHT isomers (i.e. Δ^6-BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*S*; Δ^6-BHT-34*S*; Fig. 1d and Δ^6-BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*R*; $\triangle^6$-BHT-34*R* Fig. 1e) (Peiseler and Rohmer, 1992). Consequently, given the existence of multiple source organisms, the biological origin of BHT-34*R* observed in environments such as terrestrial soils (Pearson et al., 2009), freshwater lakes (Talbot et al., 2003; Matys et al., 2019) and saline and hypersaline lakes (Talbot et al., 2003; Blumenberg et al., 2013) cannot be determined without additional information beyond stereochemical structure.

The stable carbon isotopic composition (δ^{13}C) of lipid biomarkers can help to identify their bacterial source if a distinct δ^{13}C value results from either the carbon substrate or the biosynthetic isotopic fractionation between substrate and lipid. Ribulose monophosphate (RuMP)-pathway methanotrophs (also known as Type I methanotrophs) consume methane, which typically has a strongly ^{13}C-depleted composition, and consequently synthesize hopanoids with low δ^{13}C values (Summons et al., 1994; Jahnke et al., 1999; Cordova-Gonzalez et al., 2020; van Winden et al., 2020). These methanotrophs often produce amino-BHPs as well, which can be used to identify the bacterial producer (Rush et al., 2016). Consequently, ^{13}C-depleted bacteriohopanepolyols (BHPs) or their derivatives have been used to elucidate methane-cycle processes in diverse environments, including modern and paleo-peatlands (e.g. Inglis et al., 2019). Anammox bacteria use the acetyl coenzyme A pathway for CO_2 fixation (van de Vossenberg et al., 2013) and have low biomass and hopanoid δ^{13}C values (Schouten et al., 2004). Low δ^{13}C values of BHT-*x* were measured in Mediterranean Sea sapropel sediments using an analytical approach that first separated BHT isomers using a three-column semi-preparative UPLC system followed by high temperature (HT)-GC-combustion-isotope ratio mass

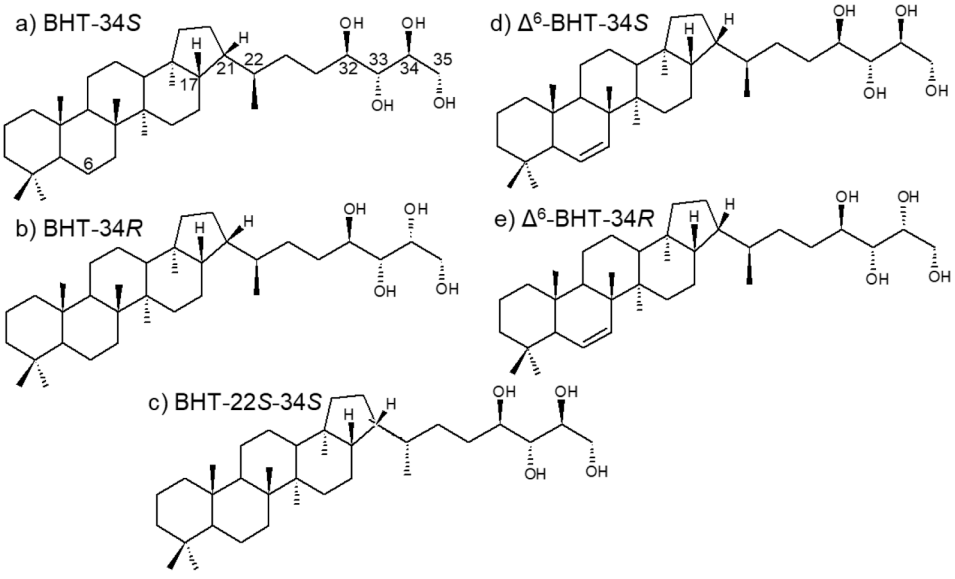

Fig. 1. Structure of bacteriohopanetetrols with known stereochemistry measured in this study. a) BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*S* (BHT-34*S*); b) BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*R* (BHT-34*R*); c) BHT-17β(H), 21β(H), 22*S*, 32*R*, 33*R*, 34*S* (BHT-34*S*-22*S*); d) Δ^6-BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*S* (Δ^6-BHT-34*S*); and e) Δ^6-BHT-17β(H), 21β(H), 22*R*, 32*R*, 33*R*, 34*R* (Δ^6-BHT-34R). Carbon numbering shown on a) BHT-34*S*.

spectrometry (C-IRMS) (Kusch et al., 2018; Hemingway et al., 2018; Elling et al., 2021). Furthermore, the $\delta^{13}C$ values of BHT-*x* have been used to quantify the contribution of anammox bacteria to organic carbon fixation in anoxic marine environments by HT-GC-C-IRMS (Lengger et al., 2019).

The application of BHT isomer $\delta^{13}C$ values is currently limited because the stable carbon isotopic fractionations from the substrate to biomass and the substrate to BHT isomers are not yet fully elucidated for all source organisms. Fractionation factors from the substrate to bulk biomass are known for some aerobic heterotrophic bacteria (Blair et al., 1985; Hayes, 1993; Teece et al., 1999; Cowie et al., 2009), but not for acetic acid-producing aerobic heterotrophic bacteria, nor for *Frankia*. To the best of our knowledge, no fractionation factors from the substrate to BHTs are known for aerobic heterotrophs. ^{13}C fractionations from substrate to biomass and from substrate to hopanoid (measured after side-chain cleavage) have been determined for some serine cycle (Type II) methanotrophic bacteria (Jahnke et al., 1999; Templeton et al., 2006), but not for *Methylocella* spp. ^{13}C fractionations from the substrate to biomass and from the substrate to hopanoid (hop-17(21)-ene) are known for '*Ca.* Brocadia' (Schouten et al., 2004) but only estimated for '*Ca.* Scalindua' based on other lipid classes (Lengger et al., 2019).

In this study, we elucidate the ^{13}C fractionation factors of BHT-34*S*, BHT-34*R*, and BHT-*x* of known source bacteria by measuring the $\delta^{13}C$ composition of the substrate, biomass, and BHTs. This work will strengthen confidence in the application of BHT-34*R* and BHT-*x* as biomarkers for carbon and nitrogen cycle processes in past and recent environmental settings.

2. Materials and methods

2.1. Bacterial cultures and enrichments

The enrichment culture of '*Ca.* Scalindua brodae' was maintained and harvested in a sequencing batch reactor. The enrichment culture of '*Ca.* Brocadia sp.' was maintained and harvested in a membrane bioreactor under previously reported conditions (Schwartz-Narbonne et al., 2020). Dissolved inorganic carbon (DIC) was sampled following the methods reported by Schouten et al. (2004).

Frankia soli strain Ea1-12 (DSM107422) was cultured and harvested as described previously (Schwartz-Narbonne et al., 2020). Propionate was supplied as substrate at 5 mM. An aliquot of propionate was reserved for isotopic analysis.

M. palustris strain K did not grow in sufficient quantities for bulk and compound-specific isotopic analysis. Instead, a substitute *Methylocella* species (*M. tundrae* T4) was grown in 250 mL DNMS medium (with lanthanum included in the trace elements) as previously described (Crombie, 2021), supplemented with 1 mL L^{-1} of vitamin solution (Dedysh et al., 2000) in 2 L sealed bottles fitted with rubber septa. The headspace was sampled immediately after preparation for isotopic analysis of carbon dioxide and methane.

An aliquot of cultured cells from *K. xylinus* strain R-2277 (gift from Prof. M. Rohmer) from the same sample studied by Peiseler and Rohmer (1992) and Schwartz-Narbonne et al. (2020) was obtained. Two additional strains of this species (TISTR 107 and ATCC 53524) were cultured on glucose as the sole carbon source, as previously described by Mikkelsen and Gidley (2011), which was supplied in excess. An aliquot of the glucose used in the Hestrin–Schramm growth medium was reserved to determine its isotopic composition.

After harvesting, all bacterial cultures and enrichments were freeze-dried and aliquoted for bulk isotopic analysis and for lipid extraction. All isotopic analyses were performed on bacterial samples from single cultures.

2.2. Lipid extraction and preparation

Freeze-dried biomass was extracted via a modified Bligh & Dyer extraction procedure as described previously (Schwartz-Narbonne et al., 2020). In brief, >100 mg of biomass was extracted in glass centrifuge tubes using a monophasic mixture of water/methanol/chloroform (4 mL/10 mL/5 mL), sonication for 15 min, and centrifugation at 4000 RPM for 5 min. The supernatant was transferred to a second centrifuge tube. This extraction was repeated twice more on the cellular residue pellet with supernatant transferred to a third and fourth centrifuge tubes. Chloroform (5 mL) and water (5 mL) were added to centrifuge tubes 2–4 to obtain a biphasic mixture. After centrifugation (4000 RPM, 5 min), the chloroform layer was removed, combined, and taken to near dryness using a rotary evaporator. The solvent extract was transferred to vials in chloroform/methanol (2:1; v/v) and evaporated to dryness at 40 °C under a stream of N_2. Extracts were acetylated with a mixture of acetic anhydride and pyridine (1/1, v/v) at 60 °C for 1 h. The derivatized solvent extract was purified on a silica-gel short column to remove the most polar material. The nonpolar fraction (F1) containing the derivatized BHTs was obtained by elution with a mixture of cyclohexane/ethyl acetate 4:1 v/v (4 dead volumes). The most polar part of the extract (F2), not further investigated in the present study, was obtained by eluting with a mixture of dichloromethane/methanol 1:1 v/v (2 dead volumes).

2.3. Stable isotope analysis

All biomass from bacterial cultures (*F. soli* strain Ea1-12, *M. tundrae* T4, and *K. xylinus* strain R-2277, strain TISTR 107, and strain ATCC 53524) and bacterial enrichments ('*Ca.* Scalindua brodae' and '*Ca.* Brocadia sp.') was freeze-dried and analysed as solids for $\delta^{13}C$ analysis. Most bacterial substrates (DIC, glucose and propionate) were similarly analysed as solids; the only exception was for *M. tundrae*, where headspace carbon dioxide and methane were analysed as gases. Solid analysis was performed on a Thermo Scientific Flash EA Delta V Plus isotope ratio monitoring mass spectrometer (EA-IRMS). Replicate measurements of each sample were performed at least in duplicate. Standard deviation on replicate measurements varied from <0.1 ‰ to ±0.2 ‰. Inter-culture variability was additionally considered by analysing the bulk biomass $\delta^{13}C$ values of *M. tundrae* from three cultures grown under identical conditions; the standard deviation was ±0.2 ‰.

Headspace gas from *M. tundrae* was analysed by Elemtex via GC-C-IRMS. 250 μL of the headspace gas was injected splitless into the gas chromatograph. Gases were separated by 45 °C isothermal over a GS-Carbonplot column (30 m, I.D. 0.530 mm; film thickness 3 μm). Methane was passed through an oxidising furnace at 900 °C packed with Cu/CuO. Water generated upon oxidation of methane was removed with a Nafion water trap before lipid analysis by the isotope ratio mass spectrometer Thermo Delta V. $\delta^{13}C$ CO_2 was converted to $\delta^{13}C$ DIC assuming an offset of 7.4 ‰ at 30 °C (Mook et al., 1974).

The $\delta^{13}C$ values of BHT-34*S* (22*R* and 22*S*), BHT-34*R*, BHT-*x*, BHT-22*S*-34*S*, Δ^6-BHT-34*S*, and Δ^6-BHT-34*R* were determined by on-column GC-C-IRMS as shown below and Fig. 2). In contrast to the GC-C-IRMS methods reported in the literature for $\delta^{13}C$ measurements of acetylated BHT, which involve high temperature conditions (e.g., Hemingway et al., 2018; Lengger et al., 2019; Elling et al., 2021), we used a conventional GC-C-IRMS; our method will therefore be more accessible to a range of laboratories as it does not require specialist instrumentation. On-column injection was selected to reduce mass discrimination, as BHT is a large molecule. GC-C-IRMS measurements of individual BHTs were carried out at Strasbourg University on a Delta V Plus mass spectrometer (Thermo Scientific) coupled to a Trace GC Ultra gas chromatograph equipped with a TriPlus autosampler, an on-column injector, and an Agilent HP5-MS column (30 m × 0.25 mm i.d.; 0.1 μm film thickness), and connected to a ConFlow IV interface system and a GC Isolink II conversion unit, comprising a combustion oven at 1000 °C for $\delta^{13}C$ measurements. Helium was used at carrier gas at a constant flow rate of 1.1 mL/min. The temperature program was: 80–320 °C (10 °C/min) – isothermal at 320 °C (40 min). Each analysis was repeated a

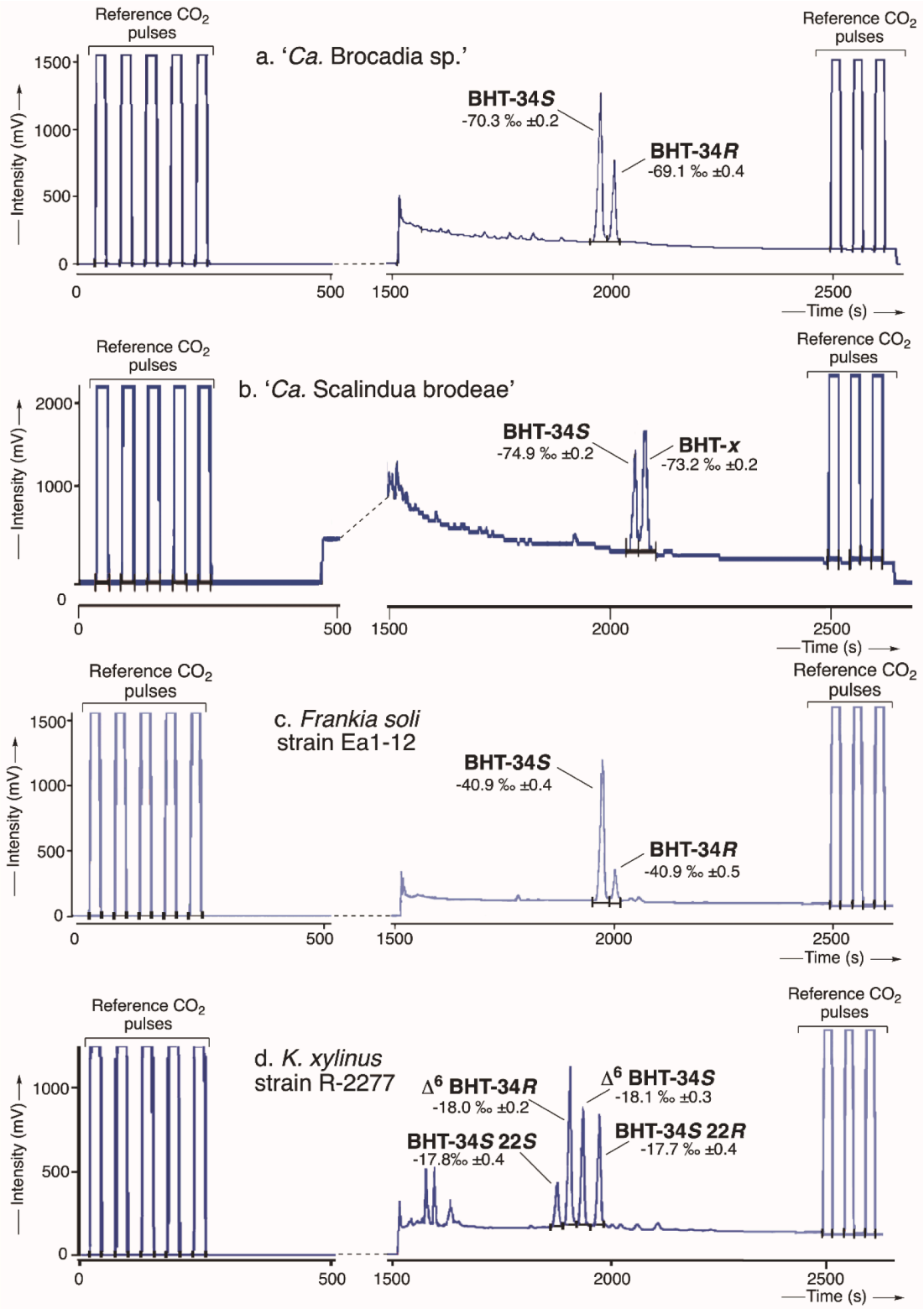

Fig. 2. GC-C-IRMS chromatogram of BHTs extracted from enrichment cultures of a) '*Ca.* Brocadia'; b) '*Ca.* Scalindua'; c) *Frankia soli* strain Ea1-12; d) *Komagataeibacter xylinus* strain R-2277. BHTs were analyzed as acetate derivatives. Samples were analysed in different analytical runs with column trims in between, resulting in some retention time shifts.

minimum of 3 times. Before and after each triplicate measurement, the carbon isotopic composition of a certified *n*-alkane mixture (Type A5; Arndt Schimmelmann, Biogeochemical Laboratories, Indiana University, USA) was measured and used for calibration. The stability and calibration of the measurements were checked using pulses of reference CO_2 with a known isotopic composition prior to (5 pulses) and after (3 pulses) each GC run (Fig. 2). The data were analysed using Isodat 3.0 software.

2.4. $\delta^{13}C$ correction for acetylated BHTs

To determine the $\delta^{13}C$ values of acetylated BHTs, a correction is needed for the 8 carbon atoms added through the acetylation. Since the $\delta^{13}C$ isotopic composition of the acetic anhydride used was known ($\delta^{13}C$ = -48.96 ‰), the following equation was used to calculate the $\delta^{13}C$ value of underivatized BHTs (cf. Rieley, 1994):

$$43 \times \delta\ ^{13}C_{BHTOAc} = 35 \times \delta\ ^{13}C_{BHTunderiv} + 8 \times \delta^{13}C_{Ac2O}$$

It is noteworthy that in theory, acetylation is expected to result in a $^{12}C/^{13}C$ isotopic fractionation (where the reaction proceeds faster with ^{12}C), the effect of which would be maximized when acetic anhydride is present in a large excess (Rieley, 1994). This is the case in the present situation, where a large excess of reagent is used. This "theoretical" effect has been measured in different situations using ribose and 3 different molar concentrations of acetic anhydride (Table 1). Acetylation reactions were carried out at room temperature overnight. In the case of the experiment involving 1 equivalent of Ac_2O (Table 1), the crude mixture obtained after acetylation has been purified by column chromatography over silica gel using a mixture of CH_2Cl_2/ethyl acetate (9:1, v/v, 1 dead volume) to recover pure tetraacetylated ribose. For the 2 other experiments, tetraacetylated ribose was analyzed without prior purification. The results indicate that the $\delta^{13}C$ values of tetraacetylated ribose under these different conditions as measured by GC-C-IRMS are identical (in the 0.1 ‰ range for the average $\delta^{13}C$ values measured for tetraacetylated ribose in each of the 3 experiments) (Table 1), and we therefore consider that corrected values of acetylated BHTs can be used with confidence.

3. Results and discussion

3.1. General $\delta^{13}C$ patterns of substrate, biomass, and BHTs

Peak separation was achieved for all saturated and unsaturated BHTs (Fig. 2). GC-C-IRMS was used to measure the $\delta^{13}C$ values of BHT-34*S* and BHT-34*R* in the anammox enrichment of '*Ca.* Brocadia sp.' (Fig. 2a), BHT-34*S* and BHT-*x* in the marine anammox enrichment of '*Ca.* Scalindua brodae' (Fig. 2b), and BHT-34*S* and BHT-22*S*-34*S* in the culture of *Frankia soli* strain Ea1-12 (DSM107422) (Fig. 2c). The BHT-34*R* peak of *K. xylinus* was too small for determination of the ^{13}C carbon isotopic composition. However, the $\delta^{13}C$ values of Δ^6-BHT-34*R*, Δ^6-BHT-34*S*, BHT-22*S*-34*S*, and BHT-34*S* were measured in the cultures of all three strains of *K. xylinus* (Fig. 2d). *Methylocella tundrae* only produces BHT-34*S*, and only it was measured (Table 2). The standard deviation of BHTs within one bacterial culture ranged from <0.1 ‰ to ±1.2 ‰ (Table 2). A pattern of $\delta^{13}C$ substrate ≥ $\delta^{13}C$ biomass ≥ $\delta^{13}C$ BHTs held for all samples in this study (Table 2).

To the best of our knowledge, this is the first time that carbon isotopic compositions have been measured for any saturated and unsaturated bacteriohopanepolyols within a single bacterial culture. The respective BHTs of each studied enrichment and culture had similar $\delta^{13}C$ values, which suggests similar metabolic pathways for their synthesis. Consequently, we assumed that the isotopic composition of a measured BHT-34*S* is comparable with that of other saturated or unsaturated BHTs produced by one bacterial culture. We made use of this assumption to compare fractionation factors of different bacterial species, as all bacteria analysed contained measurable quantities of BHT-34*S*, even when they had minor amounts (*K. xylinus*), or no (*M. tundrae* and '*Ca.* Scalindua') BHT-34*R*.

Table 1
Experimental conditions used to acetylate ribose, and the resulting $\delta^{13}C$ values of tetraacetylated ribose as measured by GC-C-IRMS. Ac_2O = acetic anhydride.

Ribose (mg)	Ribose (mmol)	Ac_2O (mg)	Ac_2O (mmol)	Ac_2O equivalents[a]	$\delta^{13}C$ (‰)[b]
15	0.1	41	0.4	1	−46.81 ± 0.1
15	0.1	205	2.0	5	−46.85 ± 0.7
3	0.02	820	8.0	100	−46.76 ± 0.1

[a] 1 equivalent unit corresponds to the amount of Ac_2O needed to acetylate the 4 hydroxy groups from ribose; [b] $\delta^{13}C$ is average ± standard deviation of triplicate measurements of tetraacetylated ribose.

3.2. Aerobic heterotrophic freshwater bacteria

For aerobic heterotrophic freshwater bacteria consuming multicarbon substrates, the fractionation from substrate to bulk biomass ($\Delta^{13}C_{biomass\ -\ substrate}$) is typically small (Hayes, 1993), varying from –0.6 to 0 ‰ for bacteria consuming glucose (Blair et al., 1985; Cowie et al., 2009) and +1.5 ‰ for bacteria consuming lactate (Teece et al., 1999). Here, *Frankia soli* strain Ea1-12 (DSM107422) and both measured subspecies of *K. xylinus* had a fractionation –0.1 ‰ to –2.3 ‰, consistent with the expected small fractionation. BHTs were significantly more depleted in ^{13}C, such that $\Delta^{13}C_{BHT\text{-}34S\ -\ substrate}$ ranged from –5.7 to –5.2 ‰ for glucose-consuming *K. xylinus* and was –12.8 ‰ for propionate-consuming *Frankia soli* strain Ea1-12 (DSM107422) (Table 3). BHTs are synthesized via pyruvate (Rohmer et al., 1996), and only minor fractionation occurs during metabolism from glucose to pyruvate in aerobic heterotrophs (DeNiro and Epstein, 1977). However, the metabolism of propionate to pyruvate likely occurs either via the methyl citrate cycle, or via the methylmalonyl-CoA pathway, rather than the glycolytic pathway used for glucose (Stowers et al., 1986). These different metabolic reactions may introduce distinct isotopic fractionations to the BHTs.

3.3. Anammox bacteria

Previous work has found low $\Delta^{13}C_{biomass\ -\ DIC}$ for anammox bacteria (*Ca.* 'Brocadia anammoxidans' strain Delft' $\Delta^{13}C_{biomass\ -\ DIC}$ = –29.4 to –23.3 ‰) and suggested that anammox bacteria fix carbon by the acetyl coenzyme A pathway (Schouten et al., 2004), which was subsequently confirmed by genomic and proteomic analysis by van de Vossenberg et al. (2013). As expected, we found low $\Delta^{13}C_{biomass\ -\ DIC}$ for both species: '*Ca.* Brocadia sp.' ($\Delta^{13}C_{biomass\ -\ DIC}$ = –26.4 ‰) and '*Ca.* Scalindua brodae' ($\Delta^{13}C_{biomass\ -\ DIC}$ = –43.8 ‰) (Table 3). Carbon isotopic fractionation for anammox hopanoids was previously investigated for *Candidatus* 'Anammoxoglobus propionicus' ($\Delta^{13}C_{hopanoid\ -\ DIC}$ = –36.9 ‰) and *Ca.* 'Brocadia anammoxidans' strain Delft ($\Delta^{13}C_{hopanoid\ -\ DIC}$ = –44.4 to –45.4 ‰) (Schouten et al., 2004; Kartal et al., 2007). These are in the same range as the values found for '*Ca.* Brocadia sp.' ($\Delta^{13}C_{BHT\text{-}34S\ -\ DIC}$ = –38.2 ‰) and '*Ca.* Scalindua brodae' ($\Delta^{13}C_{BHT\text{-}34S\ -\ DIC}$ = –53.8 ‰) measured in this study (Table 3). The cause of the larger fractionations from substrate to biomass, and from substrate to BHTs, for '*Ca.* Scalindua' compared to '*Ca* Brocadia' is presently unknown, as, to the best of our knowledge, there are no published differences in their ACS/CODH pathway. Both genera have similar carbon isotopic offsets between their lipids and bulk biomass ($\Delta^{13}C_{BHT\text{-}34S\ -\ biomass}$ = –11.8 and –9.9 ‰). Our results will contribute to more accurate modelling of the contribution of marine anammox to fixed organic carbon in marine settings: Lengger et al. (2019) relied on the fractionation factor of '*Ca.* Brocadia' to estimate the contribution of anammox biomass to the surface sediment organic matter in the Arabian Sea oxygen minimum zone, because the carbon isotopic fractionation factor of the dominant marine anammox genus ('*Ca.* Scalindua') was unknown at the time. Applying the carbon isotopic fractionation factor determined for '*Ca.* Scalindua (this study), to their data, however, marine anammox biomass in the Arabian Sea would have a $\delta^{13}C$ value of approx. 35.6 ± 6 ‰, resulting in a downwards revision of said contribution (from a Bayesian mean of 17 % to 10%).

Table 2
$\delta^{13}C$ values (‰) of biomass, BHTs, and substrates for bacterial and environmental samples. $\delta^{13}C$ is average ± standard deviation of replicate measurements. Biomass and substrate were measured at least in duplicate; BHTs were measured at least three times. The carbon dioxide substrate *of M. tundrae* was measured in the gas phase and corrected to DIC assuming an offset of 7.4 ‰ (Mook et al., 1974).

Bacteria	Bulk Biomass	BHT- 22*S*-34*S*	Δ^6-BHT-34*R*	Δ^6-BHT-34*S*	BHT-34*S*	BHT-34*R*	BHT-*x*	Substrate	Substrate
K. xylinus strain R-2277	–15.1 ± 0.1	–17.8 ± 0.4	–18.0 ± 0.2	–18.1 ± 0.3	–17.7 ± 0.4	–	–	N/A[1]	N/A[1]
K. xylinus strain TISTR 107	–13.3 ± 0.1	–17.5 ± 0.9	–17.3 ± 0.3	–17.4 ± 0.4	–17.6 ± 0.4	–	–	glucose	–11.9 ± 0.1
K. xylinus strain ATCC 53524	–14.2 ± 0.1	–16.6 ± 0.2	–16.5 ± 0.2	–16.6 ± 0.4	–17.1 ± 0.4	–	–	glucose	–11.9 ± 0.1
M. tundrae	–35.8 ± 0.2	–	–	–	–35.7 ± 0.2	–	–	CH_4	+66 ± 2
								DIC	–28.2 ± 0.2
'*Ca.* Brocadia sp.'	–58.5 ± 0.2	–	–	–	–70.3 ± 0.2	–69.1 ± 0.4	–	DIC	–32.1 ± 0.1
'*Ca.* Scalindua brodae'	–65.0 ± 0.2	–	–	–	–74.9 ± 0.2	–	–73.2 ± 0.2	DIC	–21.1 ± 0.2
Frankia soli strain Ea1-12	–28.2 ± 0.1	–	–	–	–40.9 ± 0.4	–40.9 ± 0.5	–	propionate	–28.1 ± 0.1

[1] Not analyzed

Table 3
Fractionation factors ($\Delta^{13}C$, ‰) between biomass, BHT-34*S* and substrate for measured bacteria. The $\delta^{13}C$ value for the substrate was not available for *K. xylinus* strain R-2277. The relative portion of methane and carbon dioxide used by *M. tundrae* to synthesize bulk biomass and hopanoid lipids is unknown, so the range of possible values was calculated by assuming 100 % utilization of each substrate.

Species	Substrate	$\Delta^{13}C_{biomass\ -\ substrate}$	$\Delta^{13}C_{BHT\text{–}34S\ -\ substrate}$	$\Delta^{13}C_{BHT\text{–}34S\ -\ biomass}$
K. xylinus strain R-2277	N/A[1]	–	–	–2.6
K. xylinus strain TISTR 107	glucose	–1.4	–5.7	–4.3
K. xylinus strain ATCC 53524	glucose	–2.3	–5.2	–2.9
M. tundrae	100% CH_4 assumed	–102.1	–102.0	0.1
	100% DIC assumed	–7.5	–7.5	0.1
'*Ca.* Brocadia sp.'	DIC	–26.4	–38.2	–11.8
'*Ca.* Scalindua brodae'	DIC	–43.8	–53.8	–9.9
Frankia soli strain Ea1-12	propionate	–0.1	–12.8	–12.7

[1] Not analyzed

3.4. Serine cycle (Type II) methanotrophic bacteria

Methanotrophic bacteria convert methane to carbon dioxide for energy and assimilate carbon into biomass via a range of pathways. Acidophilic and intra-aerobic methanotrophs use the Calvin cycle for carbon dioxide fixation (Khadem et al., 2011; Kool et al., 2012; Rasigraf et al., 2014). RuMP-pathway methanotrophs (Type I) build biomass principally from methane carbon (Summons et al., 1994), whereas serine cycle methanotrophs (Type II), such as *Methylocella*, incorporate carbon from both methane and carbon dioxide in their biomass. However, the exact proportion of methane *vs.* carbon dioxide used for biomass in *Methylocella* and other serine-cycle methanotrophs is not fully elucidated.

Methylocella is an acidophilic peatland methanotroph commonly found associated with sphagnum peats (Dedysh et al., 2000). As a facultative methanotroph, *Methylocella* is capable of oxidizing multi-carbon substrates for energy (Dedysh et al., 2005). However, as the focus of this work was to determine whether the isotopic signature of methane is transferred into the biomass and lipids of *Methylocella*, the catabolism of carbon substrates other than methane and carbon dioxide was not investigated.

To constrain the carbon source of *Methylocella* biomass and hopanoids, the substrate gases added to the growth experiment had distinct $\delta^{13}C$ values: $\delta^{13}C$ methane = +66 ‰ and $\delta^{13}C$ carbon dioxide = –35.6 ‰ (an offset of 7.4 ‰ between CO_2 and DIC was assumed (Mook et al., 1974) yielding DIC of –28.2 ‰.) BHT-34*S* and bulk biomass had the same $\delta^{13}C$ values (Table 3). The fractionation factors were calculated assuming either 100 % methane carbon incorporation into biomass and BHT-34*S* ($\Delta^{13}C_{biomass\ -\ methane}$ = –102‰) or 100 % carbon dioxide incorporation (as DIC) into biomass and BHT–34S ($\Delta^{13}C_{biomass\ -\ DIC}$ = –7.5 ‰) (Table 3). The fractionation factors calculated assuming *Methylocella* use solely methane as carbon source are outside the range of previously reported values for serine-cycle methanotrophs (i.e. $\Delta^{13}C_{biomass\ -\ methane}$ = –11.0 ‰ to +7.6 ‰ and $\Delta^{13}C_{hopanoids\ -\ methane}$ = –11.9 to +10.4 ‰ (Jahnke et al., 1999; Templeton et al., 2006). In contrast, if carbon dioxide is the sole carbon source for *Methylocella* biomass and hopanoids, the fractionation factors calculated here are similar to previously measured values, which range from $\Delta^{13}C_{biomass\ -\ DIC}$ = –11.4 to +5.4 ‰ and $\Delta^{13}C_{hopanoids\ -\ DIC}$ = –14.5 to +7.8 ‰ (Jahnke et al., 1999; Templeton et al., 2006). A mass-balance calculation suggests that the maximum incorporation of methane consistent with these fractionation factors would be approx. 5 %. Our results, therefore, suggest that the majority of carbon used by *Methylocella* to build biomass and hopanoids is DIC-derived rather than methane-derived, such that *Methylocella* incorporate proportionally less methane-derived carbon as biomass than the 40 to 70 % previously suggested (cf. Jahnke et al., 1999; Yang et al., 2013). The difference between our results and previous work may result in part from an inability to use $\delta^{13}C$ values to distinguish methane-derived carbon directly incorporated into biomass carbon, which would be classified as "methane-derived biomass", from carbon dioxide incorporated into biomass when the source of that carbon dioxide was originally methane that was catabolized for energy. Future work using a flow-through system, where methane catabolized to carbon dioxide for energy is not combined with carbon dioxide provided as a substrate, would allow these to be distinguished. Alternatively, differences in methanotrophic metabolism may be responsible for the variation in substrate consumption. For example, despite both being serine-cycle methanotrophs, *Methylosinus* and *Methylocella* have some differences in their metabolic pathways (Chen et al., 2010; Yang et al., 2013). Furthermore, in the atypical anaerobic methanotroph '*Ca.* Methylomirabilis oxyfera', which uses neither the serine nor the RuMP pathway, but the Calvin-Benson-Bassham cycle instead, the majority of the carbon incorporated into biomass and hopanoids is derived from DIC rather than methane (Kool et al., 2014; Rasigraf et al., 2014). Further

work investigating the catabolism and metabolism of *Methylocella* and other serine-cycle methanotrophs is needed.

4. Conclusions

This study examined the ^{13}C isotopic composition of substrate, biomass, and BHTs of bacteria known to produce BHT isomers, including BHT-34*S* and BHT-*x*. Carbon isotopic fractionation factors were calculated for two aerobic heterotrophs, two genera of anammox bacteria, and a serine-cycle methanotroph. Our results better constrain the use of carbon isotopic fractionation in modelling and mass-balance calculations. Finally, this work suggests that '*Ca.* Brocadia' is the only known bacterial producer of BHT-34*R* that strongly fractionates against ^{13}C. Measuring the δ13C value of BHT-34*R* in environmental samples may enable the identification of freshwater anammox in modern and paleorecords.

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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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