Abstract: Anaerobic ammonium oxidation (anammox) is a major process of bioavailable nitrogen removal from marine systems. Previously, a bacteriohopanetetrol (BHT) isomer, with unknown stereochemistry, eluting later than BHT using high performance liquid chromatography (HPLC), was detected in 'Ca. Scalindua profunda' and proposed as a biomarker for anammox in marine paleo-environments. However, the utility of this BHT isomer as an anammox biomarker is hindered by the fact that four other, non-anammox bacteria are also known to produce a late-eluting BHT stereoisomer. The stereochemistry in Acetobacter pasteurianus, Komagataeibacter xylinus and Frankia sp. was known to be 17β, 21β(H), 22R, 32R, 33R, 34R (BHT-34R). The stereochemistry of the late-eluting BHT in Methylocella palustris was unknown. To determine if marine anammox bacteria produce a unique BHT isomer, we studied the BHT distributions and stereochemistry of known BHT isomer producers and of previously unscreened marine ('Ca. Scalindua broadeae') and freshwater ('Ca. Brocadia sp.') anammox bacteria using HPLC and gas chromatographic (GC) analysis of acetylated BHTs and ultra high performance liquid chromatography (UHPLC)-high resolution mass spectrometry (HRMS) analysis of non-acetylated BHTs. The 34R stereochemistry was confirmed for the BHT isomers in Ca. Brocadia sp. and Methylocella palustris. However, 'Ca. Scalindua sp.' synthesise a stereochemically distinct BHT isomer, with still unconfirmed stereochemistry (BHT-x). Only GC analysis of acetylated BHT and UHPLC analysis of non-acetylated BHT distinguished between late-eluting BHT isomers. Acetylated BHT-x and BHT-34R co-elute by HPLC. As BHT-x is currently only known to be produced by 'Ca. Scalindua spp.', it may be a biomarker for marine anammox.
HIGHLIGHTS

• Five known bacterial genera produce late-eluting bacteriohopanetetrol (BHT) isomers
• Four genera, including ‘Ca. Brocadia’, synthesize BHT with 34R stereochemistry
• Only ‘Ca. Scalindua’ synthesizes a BHT with unknown stereochemistry: BHT-x
• BHT-34R and BHT-x are separated by GC (acetylated) and UHPLC (non-acetylated)
• Acetylated BHT-34R and BHT-x could not be resolved by conventional HPLC
A unique bacteriohopanetetrol stereoisomer of marine anammox

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is a major process of bioavailable nitrogen removal from marine systems. Previously, a bacteriohopanetetrol (BHT) isomer, with unknown stereochemistry, eluting later than BHT using high performance liquid chromatography (HPLC), was detected in ‘Ca. Scalindua profunda’ and proposed as a biomarker for anammox in marine paleo-environments. However, the utility of this BHT isomer as an ammonox biomarker is hindered by the fact that four other, non-anammox bacteria are also known to produce a late-eluting BHT stereoisomer. The stereochemistry in Acetobacter pasteurianus, Komagataeibacter xylinus and Frankia sp. was known to be 17β, 21β(H), 22R, 32R, 33R, 34R (BHT-34R). The stereochemistry of the late-eluting BHT in Methylocella palustris was unknown. To determine if marine anammox bacteria produce a unique BHT isomer, we studied the BHT distributions and stereochemistry of known BHT isomer producers and of previously unscreened marine (‘Ca. Scalindua brodeae’) and freshwater (‘Ca. Brocadia sp.’) anammox bacteria using HPLC and gas chromatographic (GC) analysis of acetylated BHTs and ultra high performance liquid chromatography (UHPLC)-high resolution mass spectrometry (HRMS) analysis of non-acetylated BHTs. The 34R stereochemistry was confirmed for the BHT isomers in Ca. Brocadia sp. and Methylocella palustris. However, ‘Ca. Scalindua sp.’ synthesise a stereochemically distinct BHT isomer, with still unconfirmed stereochemistry (BHT-x). Only GC analysis of acetylated BHT and UHPLC analysis of non-acetylated BHT distinguished between late-eluting BHT isomers. Acetylated BHT-x and BHT-34R co-elute by HPLC. As BHT-x is currently only known to be produced by ‘Ca. Scalindua spp.’, it may be a biomarker for marine anammox.
1. Introduction

In anoxic and low-oxygen marine systems, anaerobic ammonium oxidation (anammox) removes bioavailable nitrogen by converting ammonium and nitrite into dinitrogen gas (Strous et al., 1999). This limits the availability of a major nutrient for phytoplankton and thus may have pronounced effects on biogeochemical cycling in the ocean. Marine anammox is suggested to account for ca. 30% of the loss of bioavailable nitrogen from the global oceans today (Ward, 2013). Reconstructing the presence of anammox in paleo-environments is therefore particularly important for understanding the changes in the nitrogen cycle.

Anammox is performed by bacteria belonging to the Planctomycetes. Anammox was first recognized in an anaerobic waste water treatment system (Strous et al., 1999) and subsequently in the environment (Kuypers et al., 2003). Amongst the five currently known genera of anammox bacteria, four are primarily found in non-marine/freshwater environments: ‘Candidatus Brocadia’, ‘Ca. Jettenia’, ‘Ca. Kuenenia’ and ‘Ca. Anamnoxoglobus’ (Kartal et al., 2007; Kuypers et al., 2003; Quan et al., 2008; Schmid et al., 2000; Strous et al., 1999). ‘Ca. Scalindua’ genus is typically reported only in marine systems (Schmid et al., 2007; Woebken et al., 2007; Villanueva et al., 2014), although it was also reported to be the dominant genus in a rice paddy (Wang and Gu, 2013).

Ladderane lipids are also used as biomarkers for the detection of anammox. These highly specific lipids possess three or five concatenated cyclobutyl moieties and are synthesized exclusively by anammox bacteria (Sinninghe Damsté et al., 2002). However, the strained nature of the cyclobutyl moieties of these lipids means ladderanes are transformed relatively quickly during sediment burial (Jaeschke et al., 2008; Rush et al., 2012). The oldest detected ladderanes are from marine sediments of ca. 140,000 yr (Jaeschke et al., 2008). Therefore the presence of anammox in much older sediments cannot be evaluated using these biomarkers (Jaeschke et al., 2008; Rush and Sinninghe Damsté, 2017; Rush et al., 2012, 2019).

Bacteriohopanepolyols (BHPs) are lipids produced by both aerobic and anaerobic bacteria (Belin et al., 2018; Rohmer et al., 1984; Talbot et al., 2007a), including anammox bacteria (Sinninghe Damsté et al., 2004). BHPs have been found to be preserved for ~55 Myr (Talbot et al., 2016; van Dongen et al., 2006), making them viable biomarkers in many sedimentary records. Bacteriohopanetetrol (bacteriohopane-17β,21β(H), 22R, 32R, 33R, 34S, 35-tetrol; hence referred to as BHT-34S; Fig. 1a) is ubiquitous in the environment (Bisseret and
and has been shown to be produced by a diverse array of bacteria (Rohmer et al., 1984; Talbot et al., 2007a; Talbot and Farrimond, 2007 and references therein). Marine suspended particulate matter (SPM) from anoxic and low-oxygen water columns and marine sediments have also been found to contain a late-eluting BHT isomer (Berndmeyer et al., 2014, 2013; Blumenberg et al., 2010; Kharbush et al., 2013; Kusch et al., 2018; Rush et al., 2014; Sáenz et al., 2011; Wakeham et al., 2012). The fractional abundance of late-eluting ‘marine BHT isomer’ was proposed as a way to assess anoxia levels in marine environments, as the ratio of BHT isomer to BHT was found to correlate with anoxia in multiple marine environments (Sáenz et al., 2011).

Rush et al. (2014) identified BHT-34S, as well as a late-eluting stereoisomer of BHT, using high pressure liquid chromatography-atmospheric pressure chemical ionization/mass spectrometry (HPLC-APCI/MS analysis) (Talbot et al., 2007a) in an enrichment culture of the marine anammox species ‘Ca. Scalindua profunda’ (van de Vossenberg et al., 2008; 2013). Further evidence linking this ‘marine BHT isomer’ to ‘Ca. Scalindua spp.’ was found by correlating the concentration of the BHT isomer with that of ladderanes (Rush et al., 2014, 2019), with δ15N and genomic information (Matys et al., 2017), and with low δ13C values in the late-eluting BHT isomer from anoxic marine datasets (Lengger et al., 2019; Hemingway et al., 2018). It was, therefore, proposed that this late-eluting BHT isomer is a more appropriate biomarker for the presence of marine anammox in the deeper sedimentary record than ladderanes (Rush et al., 2014; 2019).

Some ambiguity remains in the use of late-eluting BHT isomer as a biomarker for marine anammox. To the best of our knowledge, a late-eluting BHT isomer has been reported in four other bacterial cultures (Rush et al., 2014): the aerobic, terrestrial nitrogen-fixing bacteria *Frankia* spp. (Rosa-Putra et al., 2001), the acetic acid bacteria *Acetobacter pasteurianus* and *Komagataeibacter xylinus* (formerly *Glucnaacetobacter xylinus* and *A. aceti* ssp. *xylinum*) (Peiseler and Rohmer, 1992), and the Type II methanotrophic bacterium *Methylocella palustris* (van Winden et al., 2012). Unlike ‘Ca. Scalindua profunda’, these four bacteria are all non-marine, aerobic bacteria. Thus, the presence of a late-eluting BHT isomer in anoxic, marine environments with no terrigenous contribution can be regarded as indicative of the presence of ‘Ca. Scalindua’ or some other, as yet unidentified, marine anammox genera (Rush et al., 2014). Low concentrations of a later-eluting BHT isomer also have been found in non-marine environments, though their source has not been determined (Talbot et al.,
2003), as well as in oxic marine environments, though these were associated with anoxic environments (Matys et al., 2017; Kusch et al., 2018). The stereochemistry of the late-eluting BHT isomer in *A. pasteurianus*, *K. xylinus* and *Frankia* spp. has been shown to be 17β, 21β(H), 22R, 32R, 33R, 34R (BHT-34R; Fig. 1b) (Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001). A number of early eluting BHT isomers also have been found, both in non-anammox bacterial cultures (Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001) and in marine sediments (Kusch et al., 2018). Studies of bacterial cultures with BHT isomers of known stereochemistry have found that, when measured as their acetylated derivatives by gas chromatography (GC) and HPLC, isomers with distinct ring stereochemistry elute before BHT-34S (Fig. 1c-e) (Talbot et al., 2007b, 2008).

Here, we provided further insight into these biomarkers. We examined the stereochemistry and distributions of the BHT isomers of unknown structure produced by two marine anammox organisms of the genus ‘*Ca. Scalindua*’—‘*Ca. Scalindua profunda*’ and ‘*Ca. Scalindua brodeae*’—as well as by *Methylocella palustris*. We also investigated an unscreened anammox enrichment culture of ‘*Ca. Brocadia* sp.’ for the presence of late-eluting BHT isomers and re-evaluated a culture of ‘*Ca. Kuenenia stuttgartiensis*’ to determine if trace quantities of late-eluting BHT isomer were present in this non-marine anammox bacterium.
2. Materials and methods

2.1. Bacterial cultures and enrichments

2.1.1. Anammox enrichments

The enrichment culture of ‘Ca. Scalindua brodeae’ was maintained in an anoxic sequencing batch bioreactor at Radboud University, Nijmegen, The Netherlands. ‘Ca. S. brodeae’ was grown with a sea salt medium containing 33 g L\(^{-1}\) sea salt (Red Sea Salt, Dabrowski Aquaria, Nijmegen, NL), FeSO\(_4\), KH\(_2\)PO\(_4\) and substrates of ammonium and nitrite at room temperature (20 °C). To maintain anoxic conditions, the bioreactor was continuously flushed with Ar/CO\(_2\) (95/5% v/v) at a rate of 10 mL/min. The pH was controlled at 7.3 with 100 g L\(^{-1}\) KHCO\(_3\) (Speth et al., 2015; Russ et al., 2014; van de Vossenberg et al., 2008).

The enrichment cultures of fresh water ‘Ca. Brocadia sp.’ was maintained in anoxic membrane bioreactors at Radboud University, Nijmegen, The Netherlands at 33 °C. Mineral medium containing trace element solution, FeSO\(_4\), KHCO\(_3\), CaCl\(_2\), MgSO\(_4\) and substrates NH\(_4^+\) and NO\(_2\) was supplied continuously. (Kartal et al., 2011, van de Graaf et al., 1995). Anoxic conditions and pH were maintained as described above.

All anammox cultures were harvested from the reactor and centrifuged (4000 x g, 20 min, 4°C) to obtain cell pellets, which were subsequently freeze-dried prior to analysis.

2.1.2. Other bacterial cultures

*Frankia* sp. strain Ea1-12 (DSM107422) (Fernandez et al., 1989) was cultivated in liquid medium containing ammonium, using a modification of a previously published method (Alloisio et al., 2010). The medium was modified to contain no vitamins and iron citrate was replaced by iron [III] chloride, at a final concentration of 20 μM (medium FBM). The cells were inoculated at an Optical Density (OD)600 of 0.1 and grown for 14 days in 200 mL until they reached an OD600 of 0.6. Cells were then harvested by centrifugation at 5000 x g for 10 min.

*M. palustri* strain K was cultivated in 20 mL of modified dilute nitrate mineral salts medium (Farham U1 Haque et al., 2019) in 120 mL serum vials to which 20% (v/v) methane in air was added. Vials were incubated at 25 °C with shaking (as described by Crombie and Murrell, 2014). Cultures were harvested at an OD (540 nm) of 0.12, pelleted by centrifugation at 10 000 x g, and then stored at 4 °C until analysis.
K. xylinus strain R-2277 (gift from Prof. M. Rohmer) was obtained as frozen cells in culture medium from an industrial culture (Hoffmann-La Roche, Basel), grown under confidential conditions. The cells were used to isolate a BHT isomer standard. This was an aliquot of the same sample of K. xylinus as originally studied by Peiseler and Rohmer (1992).

### 2.2. BHP extraction methods

Bacterial biomass was extracted following a modified Bligh & Dyer extraction (BDE) method (Cooke et al., 2008). Freeze-dried biomass (> 100 mg) in a 50 mL Teflon centrifuge tube was extracted using a monophasic mixture of water/methanol/chloroform (4 mL/10 mL/5 mL), sonicated for 15 min at 40 °C, followed by centrifugation at 4000 rpm for 5 min. The supernatant was transferred to a second centrifuge tube. The cellular residue pellet was re-extracted twice using the same methods to third and fourth centrifuge tubes. Chloroform (5 mL) and water (5 mL) were added to centrifuge tubes 2-4 to obtain a biphasic mixture. These were centrifuged at 4000 rpm for 5 min and the chloroform layers were removed, combined and taken to near dryness using a rotary evaporator. The BDE was transferred to vials using a solution of chloroform/methanol (2:1; v/v) and evaporated to dryness at 40 °C under a stream of N₂.

### 2.3. Preparation of acetylated BHT-34R and BHT-34S standards

A batch (not weighed) of a culture medium containing cells of K. xylinus was used to prepare and isolate BHT-34R, which could serve as an authentic standard since its stereochemistry has previously been verified by NMR (Peiseler and Rohmer, 1992). The total lipid extract (TLE) was recovered under magnetic stirring at 40 °C using 1 L acetone for 40 min and subsequently a mixture of DCM/MeOH (1:1 v/v; 1000 mL, x 3) for 40 min. At each step, the supernatant was recovered by filtration over celite after decanting, with the cells returned back for the next extraction step. At the last filtration step, the celite was rinsed with both DCM and MeOH (cf. Schaeffer et al., 2010). The extracts were combined, the solvent was removed under vacuum and BHPs were acetylated as described in section 2.5.1.

As K. xylinus strain R-2277 produces BHT-34R in minute amounts, but biosynthesizes Δ⁶-BHT-34R as a predominant BHT (Peiseler and Rohmer, 1992); catalytic hydrogenation of a chromatographic fraction enriched in Δ⁶-BHT-34R separated from the acetylated TLE from
K. xylinus (Supplementary Fig. S1a) was used to increase the yield of BHT-34R. It is worth noting that hydrogenation of the sterically-hindered \( \Delta^6 \) position could only be achieved under harsh conditions (Pd/C under pressure at 50 bars and 60 °C over 4 days), leading to a mixture of predominantly hydrogenated BHTs and minor amounts of unreacted \( \Delta^6 \)-BHTs (Supplementary Fig. S1b). Separation of the hydrogenated BHTs using silica gel column chromatography and thin layer chromatography (TLC) followed by C\(_{18}\) reverse phase HPLC yielded an isolate of BHT-34R with a purity of ca. 96% (Supplementary Fig. S1e).

An acetylated BHT-34S standard isolated from the bacterium Zymomonas mobilis in a previous study (cf. Schaeffer et al., 2010) was used as a standard for co-injection in GC-FID and GC-MS co-elution experiments. The stereochemical structure of the acetylated BHT-34R and BHT-34S side chains was confirmed using 1- and 2-dimensional \(^1\)H and \(^{13}\)C NMR experiments. Detailed separation methods and NMR data are reported in the supplementary material (Section 1 and Section 2).

### 2.4. Isolation of non-derivatised BHT-34S and late-eluting BHT isomers from anammox biomass

A multidimensional 3-step isolation procedure was employed to isolate BHT-34S and the late-eluting BHT stereoisomers with previously unconfirmed stereochemistries were isolated separately from both ‘Ca. Brocadia sp.’ and ‘Ca. Scalindua brodeae’ (detailed procedure in Supplementary Material Section 3). Although pure compounds were not obtained, fractions enriched in either BHT-34S or BHT isomers (enriched BHT fraction) with minimal interfering lipids recovered (Supplementary Material Section 3).

### 2.5. Analysis of acetylated BHTs

#### 2.5.1. Acetylation

‘Ca. Scalindua profunda’ and ‘Ca. Kuenenia stuttgartiensis’ BDE had been previously extracted, acetylated, and measured by HPLC-APCI-MS (Rush et al., 2014) and, following re-acetylation, was used in this study. Aliquots of BDEs of Ca. Scalindua profunda’, Frankia sp. strain Ea1-12, Methylocella palustris, partially purified ‘Ca. Brocadia sp.’, and partially purified ‘Ca. Scalindua brodeae and of the TLE of K. xylinus strain R-2277 were acetylated prior to GC-FID, GC-MS, and HPLC-MS analysis. Equal volumes of acetic anhydride and
pyridine were added to the extracts, and they were heated at 60 °C for 1 h. The excess of reagents was evaporated to dryness under a stream of N₂ or under vacuum.

2.5.2. GC-FID

Gas chromatography with flame ionisation detection (GC-FID) analyses of acetylated samples dissolved in ethyl acetate were carried out on a Agilent Technologies 7890A gas chromatograph equipped with an on-column injector, a flame ionization detector and a HP-5 fused silica capillary column (30 m x 0.32 mm; 0.25 μm film thickness) at the Université de Strasbourg-CNRS. H₂ was used as carrier gas (constant flow, 2.5 mL min⁻¹), and the oven was programmed as follows: 70 °C - 320 °C (10 °C min⁻¹), 60 min isothermal at 320 °C. Samples were injected individually and co-injected with acetylated BHT-34R or BHT-34S standard.

2.5.3. GC-MS

Gas chromatography-mass spectrometry (GC-MS) analyses of acetylated BHTs were carried out using a Thermo Trace gas chromatograph (Thermo Scientific) coupled to a Thermo Scientific TSQ Quantum mass spectrometer equipped with a programmed temperature vaporizing (PTV) injector at the Université de Strasbourg-CNRS. The temperature of the source was set at 220 °C. The mass spectrometer was operated in electron impact (EI) mode at 70 eV and scanning m/z 50 to 850. Gas chromatographic separations were performed using a HP5-MS column (30 m x 0.25 mm; 0.1 μm film thickness) with He as carrier gas (constant flow rate of 1.2 mL min⁻¹). The oven temperature was programmed as follows: 70 °C (1 min), 70 °C-320 °C (10 °C min⁻¹), 40 min isothermal at 320 °C.

2.5.4. HPLC-MS

High performance liquid chromatography- mass spectrometry (HPLC-MS) analyses of BHTs were performed using an auto-injector-equipped HP 1100 series HPLC interfaced to a Bruker Esquire 3000+ ion trap mass spectrometer and Chemstation chromatography manager software at the Université de Strasbourg-CNRS. Separation was achieved on a Zorbax ODS column (4.6 mm x 250 mm, 5 μm) maintained at 30 °C. The injection volume was 10 μL. Compounds were eluted isocratically using a mixture of methanol/isopropanol 95:5 v/v as the mobile phase, with a flow rate of 1.0 mL min⁻¹. Detection was achieved using a positive ion atmospheric pressure chemical ionization (APCI). Conditions for APCI-MS analyses were:
nebulizer pressure 43.5 psi, vaporizer temperature 420 °C, drying gas (N\textsubscript{2}) flow 6 L min\textsuperscript{-1} and temperature 350 °C, capillary voltage -4 kV, corona 4 µA, scanning range \textit{m/z} 300-800.

2.6. UHPLC-HRMS measurement of non-derivatised BHTs

Non-derivatised samples were analysed by ultra high performance liquid chromatography - high resolution mass spectrometry (UHPLC-HRMS) using a Q Exactive Orbitrap MS system (Thermo Scientific) at NIOZ Royal Netherlands Institute for Sea Research, following previous methods (Rush et al., 2019 modification of Wörmer et al., 2013). Briefly, a solvent gradient was run using an Acquity BEH C\textsubscript{18} column (Waters, 2.1x150 mm, 1.7µm) at 30 °C. Solvent A was methanol/water/formic acid/14.8 M NH\textsubscript{3}aq (85:15:0.12:0.04 [v/v/v/v]) and B was IPA/methanol/formic acid/14.8 M NH\textsubscript{3}aq (50:50:0.12:0.04 [v/v/v/v]), with an initial percentage of 95 % A for 3 min, decrease to 40 % A at 12 min, then decrease to 0 % A at 50 min and maintained until 80 min, with a flow rate of 0.2 mL min\textsuperscript{-1}. Positive ion electrospray ionisation (ESI) with a capillary temperature of 300 °C was used. ESI sheath gas (N\textsubscript{2}) pressure was 40 arbitrary units and the auxiliary gas (N\textsubscript{2}) pressure was 10 arbitrary units. The spray voltage was 4.5 kV, the probe heater temperature was 50 °C and the S-lens voltage was 70 V. Target lipids were analysed following previously described methods and parameters (Besseling et al., 2018; Rush et al., 2019; Wörmer et al., 2013) using a mass range of \textit{m/z} 350–2000 (resolution 70,000 ppm at \textit{m/z} 200) and then data-dependent tandem MS\textsuperscript{2}. Integrations were made on the summed mass chromatograms (within 3 ppm) of the [M+H]\textsuperscript{+}, [M+NH\textsubscript{4}]\textsuperscript{+}, and [M+Na]\textsuperscript{+} (\textit{m/z} 547.472, 564.499, and 569.454, respectively) of non-acetylated BHT. BDEs were injected for all samples except ‘Ca. Scalindua profunda’: a) \textit{Frankia} spp. Ea1-12, b) \textit{Komagataeibacter xylinus}, c) \textit{Methylocella palustris}, d) ‘Ca. Brocadia sp.’, e) ‘Ca. Scalindua brodae’, and f) ‘Ca. Kuenenia stuttgartiensis’. 
3. Results & Discussion

3.1. Chromatographic separation and identification of late-eluting BHT isomers

Analyses of acetylated BDEs from \textit{M. palustris}, \textit{A. pasteurianus}, \textit{K. xylinus}, \textit{Frankia} spp., ‘\textit{Ca. Brocadia} sp.’, ‘\textit{Ca. S. profunda}’ and ‘\textit{Ca. S. brodeae}’ revealed that all contained a BHT isomer that eluted after BHT-34S when measured using HPLC and GC, and non-acetylated BDE measured by UHPLC (Figs. 2-4; Lengger et al., 2019; Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001; Rush et al., 2014, 2019; van Winden et al., 2012). Since it is likely that identifying the stereochemistry of these isomers may allow for better application of late-eluting BHT isomers as biomarkers for marine anammox, further characterisation of them was undertaken as detailed below.

3.1.1. Distribution of BHT isomer in bacterial cultures reveals unique marine anammox biomarker

The BHT distributions in these cultures were measured using a recently developed UHPLC-HRMS method for the analysis of non-derivatized BHTs (Rush et al., 2019; Fig. 2). All known non-marine producers of BHT isomers (\textit{Frankia} sp., \textit{K. xylinus}, \textit{M. palustris}, and ‘\textit{Ca. Brocadia} sp.’) were shown to produce a late-eluting BHT isomer with the same retention time. The isolated late-eluting BHT isomers synthesised by \textit{A. pasteurianus}, \textit{K. xylinus} and \textit{Frankia} spp. were previously analysed by NMR and all found to possess the BHT-34R stereochemistry (Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001). In contrast, the marine anammox species ‘\textit{Ca. Scalindua} brodeae’ produced a late-eluting BHT isomer with a distinct retention time, which eluted after both BHT-34S and BHT-34R. We provisionally named this isomer “BHT-x” to reflect the distinct retention time, likely indicating a distinct stereochemistry. ‘\textit{Ca. S. profunda}’ BDE was not measured using the UHPLC-HRMS method, as the available BDE had been acetylated during a previous study (Rush et al., 2014). Furthermore, the freshwater anammox species ‘\textit{Ca. Kuenenia} stuttgartiensis’ did contain BHT-34S, but did not contain any of the other late-eluting BHT isomers – i.e. neither BHT-34R nor BHT-x – (Fig. 2f), confirming the previous observation by Rush et al. (2014).

The MS\textsuperscript{2} spectra of BHT and late-eluting BHT isomers were too similar to discriminate between the different stereochemistries (Fig. 5).

3.1.2. Purification and NMR analyses of BHT isomers

The stereochemistries of the BHT isomers in \textit{M. palustris}, ‘\textit{Ca. Brocadia} sp.’, ‘\textit{Ca. S. profunda}’ and ‘\textit{Ca. S. brodeae}’ were not assessed by NMR. We attempted to isolate the late-
eluting BHT isomers of ‘Ca. Scalindua brodeae’ and ‘Ca. Brocadia sp.’ to allow for direct
NMR measurement. However, as anammox bacteria are slow-growing, insufficient biomass
was obtained to isolate the late-eluting BHT isomers separate from BHT-34S, and only an
enriched BHT fraction was obtained from these two bacteria (Supplementary Material
Section 3). Unfortunately, the amount of the *M. palustris* biomass was also insufficient to
isolate the late-eluting BHT isomer, and thus acetylated *M. palustris* BDE was used only in
GC and HPLC analyses. It was decided to compare the chromatographic behaviour of the
various isomers with confirmed standards of BHT-34S and BHT-34R. A specific protocol
was used for isolating and preparing BHT-34R for use in this study (Supplementary Material
Section 1), whereas a standard of acetylated BHT-34S previously isolated from *Zymomonas
mobilis* was available (cf. Schaeffer et al., 2010). The stereochemistries of these standards
were confirmed by NMR (Supplementary Material Table S2a-b and Fig. S2a-b). The $^1$H and
$^{13}$C chemical shifts of the side chain of BHT-34R and BHT-34S standards were generally in
agreement with those published for synthetic BHT-34R and BHT-34S (Bisseret and Rohmer,
1989) and with BHT-34R isolated from *K. xylinus* (Peiseler and Rohmer, 1992; cf.
Supplementary Material Section 2 for the $^1$H and $^{13}$C chemical shifts of the side chain from
BHT-34R and BHT-34S).

### 3.1.3. GC-FID analysis of acetylated BHT isomers by co-injections

The identity of the BHT isomers present in the bacterial cultures and enrichments was
evaluated using GC-FID analysis of acetylated BDEs (*Frankia* sp. strain Ea1-12, *K. xylinus
and *M. palustris*), or enriched BHT fractions (‘Ca. Scalindua brodeae’ and ‘Ca. Brocadia
sp.’) (Fig. 3). All samples contained BHT-34S, as confirmed by GC-FID co-injection with an
authentic standard (Fig. 3). Co-injections of the authentic acetylated BHT-34R standard with
acetylated extracts from *Frankia* sp. strain Ea1-12 (Fig. 3c), *M. palustris* (Fig. 3d), and the
anammox bacterium ‘Ca. Brocadia sp.’ (Fig. 4e) confirmed the BHT-34R stereochemistry of
the late-eluting BHT isomer of these cultures. The late-eluting BHT isomer (BHT-x) from
‘Ca. S. brodeae’ did not co-elute with BHT-34R (Fig. 4f). To verify that other species
belonging to the genus *Ca. Scalindua* also contain BHT-x (Rush et al. 2014), acetylated ‘Ca.
S. profunda’ TLE was also co-injected with authentic BHT standards (Fig. 4g). Both ‘Ca.
Scalindua’ species were found to produce the BHT-x isomer. Of the known bacterial
producers of BHT isomers, BHT-x is only synthesized by marine anammox bacteria,
suggesting that it can be applied as a biomarker for paleo-marine anammox studies.
3.2. Implications for the analysis of BHT isomers in cultures and sediments

The isomer elution order of non-acetylated BHTs when measured by UHPLC-HRMS was different to that of the acetylated compounds on GC-FID or GC-MS. Non-acetylated BHT-x eluted last on UHPLC, while eluting before BHT-34R on GC. BHT-x displays the same mass spectrum when analysed by GC-MS as BHT-34S (cf. BHT (BHT-34S) and BHT’ (i.e. BHT-x) in Lengger et al., 2019). GC-MS spectra of BHT-34S, BHT-34R, BHT-x, and 17α-BHT-34S can be found in Supplementary Material Section 4. The similarity in mass spectra combined with distinct retention times by two chromatographic techniques support the identification of BHT-x as an isomer of BHT-34S. HPLC-APCI-MS analysis of acetylated BDE using a single column is a well-established method to examine BHPs (e.g. Talbot et al., 2007a, b). However, while the single-column HPLC-APCI-MS method developed in the present study for analysis of acetylated BHTs does separate BHT-34S from its late-eluting isomers, it does not provide distinct retention times for BHT-34R and BHT-x (Fig. 4). As acetylated BHT analysis by HPLC-APCI-MS does not differentiate between late-eluting BHT isomers, BHT isomer studies should be performed by GC on acetylated BHTs or by UHPLC on non-acetylated BHTs. It should be noted that the triple column method for UHPLC analysis of acetylated BHPs (Kusch et al., 2018) was not tested in this study, and future work should investigate if this method can distinguish acetylated BHT-34R and BHT-x. Previous HPLC studies on acetylated late-eluting BHT isomers assumed all the isomers were the same, and any late-eluting BHT isomer was designated as “BHT II” (e.g. Sáenz et al., 2011; Matys et al., 2017, 2019; Kusch et al., 2018). As ‘Ca. Scalindua sp.’ synthesizes BHT-x and currently is the only known marine bacterial source of any late-eluting BHT isomer, it is likely correct that these late-eluting BHT isomers isolated from anoxic marine systems had the same stereochemistry. However, the stereochemistry of a late-eluting BHT isomer in oxic and anoxic lacustrine systems was likewise identified and considered to be BHT II (e.g. Matys et al., 2019; Talbot et al., 2003). A late-eluting BHT isomer has also been found in oxic or seasonally anoxic marine settings (Kusch et al., 2018, 2019; Matys et al., 2019). In some of these cases, identification of the stereochemistry of the BHT isomer may reveal that it was not BHT-x, and thus not produced by ‘Ca. Scalindua sp.’, partially explaining its presence in oxic and/or non-marine environments. Furthermore, the ratio of late-eluting BHT isomer to total BHTs (BHT isomer ratio; Sáenz et al., 2011), derived from an acetylated culture analysed by HPLC with refractive index detection (ratio = 0.10; Peiseler and Rohmer, 1992) is different from an aliquot of the same non-acetylated culture, analysed by UHPLC (ratio = 0.20; this study) (Supplementary Material Section 5). Since the samples
were extracted using similar, though not identical methods, (i.e., CHCl₃/MeOH extraction vs. BDE), it seems more likely that instrumental differences caused the variation in observed BHT isomer ratio. We therefore advise against direct comparisons of BHT isomer fractional abundance performed using samples measured under different analytical conditions or instrumentation, without further investigation of which produces comparable BHT isomer ratios.

As multiple bacterial genera produce BHT-34R, additional measurements are required to elucidate the source of this biomarker in the environment. One potential method to differentiate these sources is by measuring the δ¹³C values of BHT-34R. Anammox bacteria have lipids that are ¹³C-depleted by up to 47 ‰ versus the CO₂ substrate (Schouten et al., 2004), resulting from their use of the acetyl coenzyme A pathway for carbon fixation (Strous et al., 2006). The δ¹³C values of late-eluting BHT isomer from sediments taken from a Mediterranean sapropel and the Arabian Sea oxygen minimum zone were ¹³C-depleted, and had lower δ¹³C values than those of BHT-34S in the same samples, suggesting a marine anammox source of late-eluting BHT isomer in both cases (Hemingway et al., 2018; Lengger et al., 2019). The additional application of compound specific carbon isotopic analysis to non-marine samples could differentiate between BHT-34R produced by anammox and by some other bacterial sources; if possible, δ¹³C analyses as well as stereoisomer identification should be conducted (Hemingway et al., 2018; Lengger et al., 2019). BHT-34R produced by the methanotroph *M. palustris* may also have a low δ¹³C value, but Type II methotrophs have do not have consistently low lipid δ¹³C values (Kool et al, 2014). Future work is required to differentiate the carbon isotopic composition of BHT-34R produced by ‘*Ca. Brocadia* sp.’ and *M. palustris*, as well as the other bacterial producers of this isomer.

‘*Ca. Scalindua brodeae*’ and ‘*Ca. Brocadia* sp.’ are both anaerobic anammox bacteria and evolved from a common ancestor (Strous et al., 2006). However, ‘*Ca. Brocadia* sp.’ synthesizes the same BHT isomer (BHT-34R) as non-marine, aerobic bacteria. Another freshwater species of anammox bacteria, ‘*Ca. Kuenenia stuttgartiensis*’ does not produce significant quantities of any BHT isomer other than the BHT-34S. Currently, we cannot identify either genetic or environmental factors common to BHT isomer production in all bacteria. Future studies should focus on the gene(s) responsible for hopanoid biosynthesis. However, as species of marine genus ‘*Ca. Scalindua*’ are so far the only identified producers
of BHT-x, it appears that BHT-x can be confidently applied as a biomarker for marine anammox in the sedimentary record.

5. Conclusions

Five non-marine bacteria (Frankia spp., A. pasteurianus, K. xylinus, M. palustris and ‘Ca. Brocadia sp.’) were shown to synthesize BHT-34R. While this bacterial lipid is not specific to a class of organism, its use along with other evidence (e.g. $\delta^{13}$C values of BHT-34R) may be suggestive of its producers in the environment, including the non-marine anammox bacteria ‘Ca. Brocadia sp.’. ‘Ca. Scalindua profunda’ and ‘Ca. Scalindua brodeae’ are the only known producers of BHT-x (a BHT with an unidentified side chain stereochemistry), making this isomer a valuable biomarker for marine anammox. BHT-34S eluted before BHT-34R and BHT-x by all methods tested. However, BHT-34R eluted before BHT-x by UHPLC analysis of non-acetylated BHPs, after BHT-x by GC analysis of acetylated BHPs, and co-eluted with BHT-x by HPLC analysis of acetylated BHPs. The choice of chromatography and derivatization methods should be carefully considered in future BHT isomer studies, so marine anammox can be distinguished from non-marine inputs.

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Supplementary Material

Section 1: Preparation of acetylated BHT-34R and BHT-34S standards
Section 2: Partial $^1$H and $^{13}$C NMR data for acetylated BHT-34R and BHT-34S
Section 3: Isolation of non-derivatised BHT-34S and late-eluting BHT isomers from anammox biomass
Section 4: GC-MS spectra (EI, 70 eV) of acetylated BHT isomers
Section 5: Distributions of late-eluting BHT isomer(s) in bacterial cultures and marine environments
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Figures

Figure 1.
Figure 2.

a) Frankia sp. EA1-12
b) Komagataeae bacter xylinum
c) Methylbacteriella palustris
d) ‘Ca. Brocadia sp.’
e) ‘Ca. Scalindua brodeae’
f) ‘Ca. Kuenenia stuttgartiensis’
Figure 3.
Figure 4.
Figure Captions

Figure 1. Stereoisomers of BHT; a) 17β, 21β(H), 22R, 32R, 33R, 34S (BHT-34S) b) 17β, 21β(H), 22R, 32R, 33R, 34R (BHT-34R) c) 17β, 21β(H), 22S, 32R, 33R, 34S (BHT-22S-34S) d) 17α, 21β(H), 22R, 32R, 33R, 34S (BHT-17α-34S) and e) BHT-17α, 21β(H), 22R, 32R, 33R, 34R (BHT-17α-34R). Carbon numbering shown on a) BHT-34S.

Figure 2. UHPLC-HRMS combined mass chromatograms (within 3 ppm) of the [M+H]+, [M+NH₄]+, and [M+Na]+ (m/z 547.472, 564.499, and 569.454, respectively) of non-
acytelated BHT and isomers of a) Frankia sp. Ea1-12 b) Komagataeibacter xylinus, c) Methylocella palustris, d) ‘Ca. Brocadia sp.’, e) ‘Ca. Scalindua brodeae’ and f) ‘Ca. Kuenenia stuttgartiensis’. BHT isomers were identified based on relative retention times, in comparison to previously published stereochemical identification (Peiseler and Rohmer, 1992; Rosa-Putra et al., 2001).

Figure 3. Partial GC-FID chromatograms of (top: light blue) acetylated BDE, (middle: grey) acetylated BDE co-injected with BHT-34R standard and (bottom: light green) acetylated BDE co-injected with BHT-34S standard. a) BHT-34R and BHT-34S standards b) Komagataeibacter xylinus strain R-2277; c) Frankia sp. strain Ea1-12; d) Methylocella palustris; e) ‘Ca. Brocadia sp.’ (enriched BHT fraction); f) ‘Ca. Scalindua profunda’; g) ‘Ca. Scalindua brodeae’ (enriched BHT fraction).

Figure 4. HPLC-MS mass chromatograms (m/z 655) of acetylated a) BHT-34S standard; b) BHT-34R standard; c) BDE of Methylocella palustris; d) BDE ‘Ca. Brocadia sp.’; e) BDE from ‘Ca. Scalindua brodeae’.

Figure 5. Averaged (n = 3) ESI-HRMS² spectra of non-acetylated a) BHT-34S, b) BHT-34R and c) BHT-x from m/z 564.499 (ammoniated adduct of BHT; [M + NH₄]⁺) of Frankia sp. a, b) and ‘Ca. Scalindua brodeae’ c). Likely fragmentation patterns have been shown in Rush et al. (2019).