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► To cite this version:

Rachel Schwartz-Narbonne, Philippe Schaeffer, Ellen Hopmans, Margot Schenese, E. Alex Charlton, et al.. A unique bacteriohopanetetrol stereoisomer of marine anammox. *Organic Geochemistry*, 2020, 143, pp.103994. 10.1016/j.orggeochem.2020.103994 . hal-02553771

HAL Id: hal-02553771

<https://hal.science/hal-02553771>

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A unique bacteriohopanetetrol stereoisomer of marine anammox

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ARTICLE INFO

Article history:

Received 26 October 2019

Received in revised form 21 February 2020

Accepted 21 February 2020

Available online 25 February 2020

Keywords:

Bacteriohopanepolyols

Bacteriohopanetetrols

Stereoisomers

Anammox

Biomarkers

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 when examined by 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 17 β , 21 β (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* spp.’) anammox bacteria, using HPLC and gas chromatography (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* spp.’ synthesises 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 when examined by HPLC. As BHT-x is currently only known to be produced by ‘*Ca. Scalindua* spp.’, it may be a biomarker for marine anammox.

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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

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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. Anammoxoglobus*’ (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-17 β ,21 β (H), 22R, 32R, 33R, 34S, 35-tetrol; hence referred to as BHT-34S; Fig. 1a) is ubiquitous in the environment (Bisseret and Rohmer, 1989; Talbot et al., 2003; Talbot and Farrimond, 2007) 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 $\delta^{15}\text{N}$ and with genomic information (Matys et al., 2017), and with low $\delta^{13}\text{C}$ 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 *Gluconacetobacter 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.,

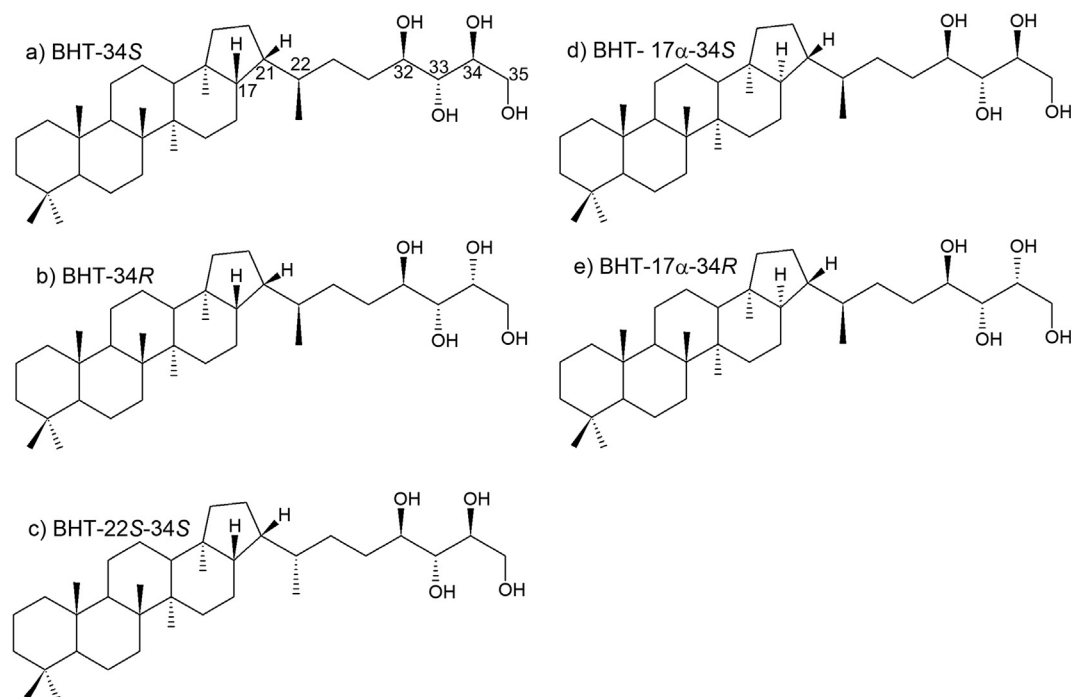


Fig. 1. Stereoisomers of BHT: a) 17 β , 21 β (H), 22R, 32R, 33R, 34S (BHT-34S) b) 17 β , 21 β (H), 22R, 32R, 33R, 34R (BHT-34R) c) 17 β , 21 β (H), 22S, 32R, 33R, 34S (BHT-22S-34S) d) 17 α , 21 β (H), 22R, 32R, 33R, 34S (BHT-17 α -34S) and e) BHT-17 α , 21 β (H), 22R, 32R, 33R, 34R (BHT-17 α -34R). Carbon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

2014). Low concentrations of a later-eluting BHT isomer also have been found in non-marine environments, though the 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 17 β , 21 β (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. 1 c-e) (Talbot et al., 2007b, 2008).

Here, we provide 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), FeSO₄, KH₂PO₄ and substrates of ammonium and nitrite at room temperature (20 °C). To maintain anoxic conditions, the bioreactor was continuously flushed with Ar/CO₂ (95/5% v/v) at a rate of 10 mL/min. The pH was controlled at 7.3 with 100 g L⁻¹ KHCO₃ (Speth et al., 2015; Russ et al., 2014; van de Vossenberg et al., 2008).

The enrichment cultures of fresh water '*Ca. Brocadia* sp.' were maintained in anoxic membrane bioreactors at Radboud University, Nijmegen, The Netherlands at 33 °C. Mineral medium containing trace element solution, FeSO₄, KHCO₃, CaCl₂, MgSO₄ and substrates NH₄⁺ and NO₂ 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 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 μ M (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 g for 10 min.

M. palustris strain K was cultivated in 20 mL of modified dilute nitrate mineral salts medium (Farhan Ul 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 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* 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 wet biomass of *K. xylinus* was used to prepare and isolate BHT-34R, to serve as an authentic standard, since the stereochemistry has previously been verified by NMR spectroscopy (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 dichloromethane/methanol (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 dichloromethane and methanol (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.

K. xylinus strain R-2277 produces BHT-34R in minute amounts, but biosynthesizes Δ^6 -BHT-34R as a predominant BHT (Peiseler and Rohmer, 1992). Catalytic hydrogenation of a chromatographic fraction enriched in Δ^6 -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 Δ^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 Δ^6 -BHTs (Supplementary Fig. S1b). Separation of the hydrogenated BHTs using silica gel column chromatography and thin layer chromatography (TLC) followed by C₁₈ 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 ¹H and ¹³C NMR spectroscopy 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 which were isolated separately from both '*Ca. Brocadia sp.*' and '*Ca. Scalindua brodeae*' (detailed procedures are shown 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 were 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, were also 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 × 0.32 mm; 0.25 μm film thickness). H₂ was used as carrier gas (constant flow, 2.5 mL min⁻¹), and the oven was programmed as follows: 70–320 °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. 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 × 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), 70–320 °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. Separation was achieved on a Zorbax ODS column (4.6 mm × 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 (N₂) flow 6 L min⁻¹ and temperature 350 °C, capillary voltage -4 kV, corona 4 μA, scanning range *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) following previous methods ([Rush et al., 2019](#) modification of [Wörmer et al., 2013](#)). Briefly, a solvent gradient was run using an Acquity BEH C₁₈ column (Waters, 2.1 × 150 mm, 1.7 μm) at 30 °C. Solvent A was methanol/water/formic acid/14.8 M NH_{3aq} (85:15:0.12:0.04 [v/v/v/v]) and B was IPA/methanol/formic acid/14.8 M NH_{3aq} (50:50:0.12:0.04 [v/v/v/v]), with an initial percentage of 95% A for 3 min, decreased to 40% A at 12 min, then decreased to 0% A at 50 min and maintained until 80 min, with a flow rate of 0.2 mL min⁻¹. Positive ion electrospray ionisation (ESI) with a capillary temperature of 300 °C was used. ESI sheath gas (N₂) pressure was 40 arbitrary units and the auxiliary gas (N₂) 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 *m/z* 350–2000 (resolution 70,000 ppm at *m/z* 200) and then data-dependent tandem MS². Integrations were made on the summed 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-acetylated BHT. BDEs were injected for all samples except '*Ca. Scalindua profunda*': a) *Frankia* spp. Ea1-12, b) *Komagataeibacter xylinus*, c) *Methylocella palustris*, d) '*Ca. Brocadia sp.*', e) '*Ca. Scalindua brodeae*', and f) '*Ca. Kuenenia stuttgartiensis*'.

3. Results & discussion

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

Analyses of acetylated BDEs from *M. palustris*, *A. pasteurianus*, *K. xylinus*, *Frankia* spp., '*Ca. Brocadia sp.*', '*Ca. S. profunda*' and '*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 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-derivatised BHTs ([Rush et al., 2019](#); Fig. 2). All known non-marine producers of BHT isomers (*Frankia* sp., *K. xylinus*, *M. palustris*, and '*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 *A. pasteurianus*, *K. xylinus* and *Frankia* spp. were previously analysed by NMR spectroscopy and all were found to possess the BHT-34R stereochemistry ([Peiseler and Rohmer, 1992](#); [Rosa-Putra et al., 2001](#)). In contrast, the marine

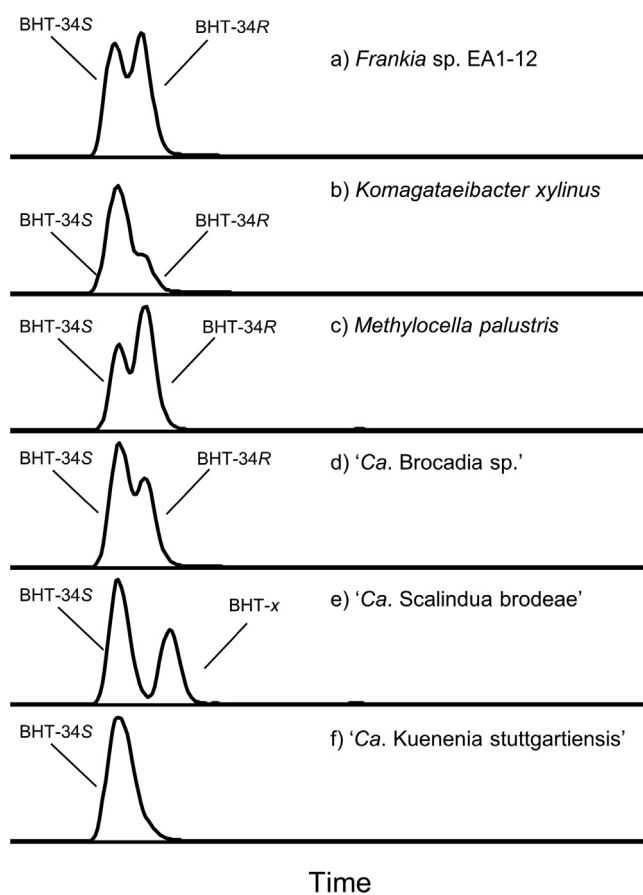


Fig. 2. UHPLC-HRMS combined mass chromatograms (within 3 ppm) of the $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ (m/z 547.472, 564.499, and 569.454, respectively) of non-acetylated BHT and isomers of a) *Frankia* sp. Ea1-12b) *Komagataeibacter xylinus*, c) *Methylocella palustris*, d) '*Ca. Brocadia* sp.', 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).

anammox species '*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. '*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 '*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 MS² spectra of BHT and late-eluting BHT isomers were too similar to allow discrimination between compounds with the different stereochemistries (Fig. 5).

3.1.2. Purification and NMR spectroscopy analyses of BHT isomers

The stereochemistries of the BHT isomers in *M. palustris*, '*Ca. Brocadia* sp.', '*Ca. S. profunda*' and '*Ca. S. brodeae*' were not assessed by NMR spectroscopy. We attempted to isolate the late-eluting BHT isomers of '*Ca. Scalindua brodeae*' and '*Ca. Brocadia* sp.' to allow for direct NMR spectroscopy measurements. However, as anammox bacteria are slow-growing, insufficient biomass was obtained to allow isolation of the late-eluting BHT isomers separated 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 allow isolation of 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 spectroscopy (Supplementary Material Table S2a-b and Fig. S2a-b). The ¹H and ¹³C chemical shifts of the side chains 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 ¹H and ¹³C chemical shifts of the side chains 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. 3e) 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. 3f). To verify that other species belonging to the genus *Ca. Scalindua* also contain

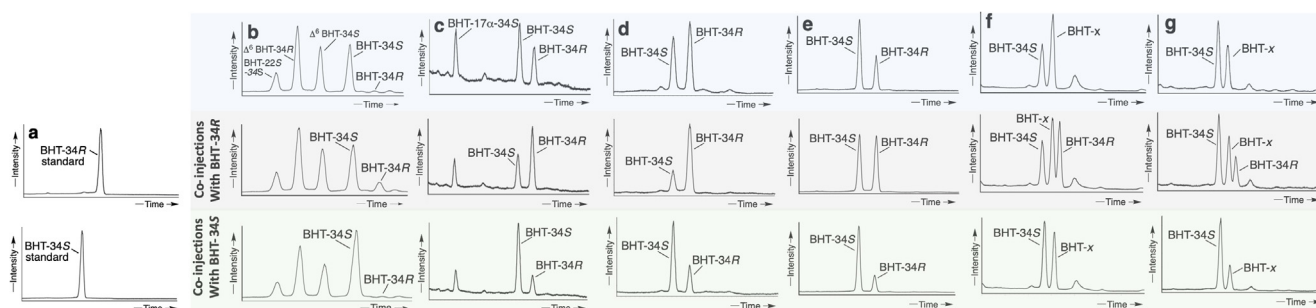


Fig. 3. Partial GC-FID chromatograms of (top: light blue) acetylated BDE, (middle: grey) acetylated BDE co-injected with BHT-34R standard and (bottom: light green) acetylated BDE co-injected with BHT-34S standard. a) BHT-34R and BHT-34S standards b) *Komagataeibacter xylinus* strain R-2277; c) *Frankia* 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

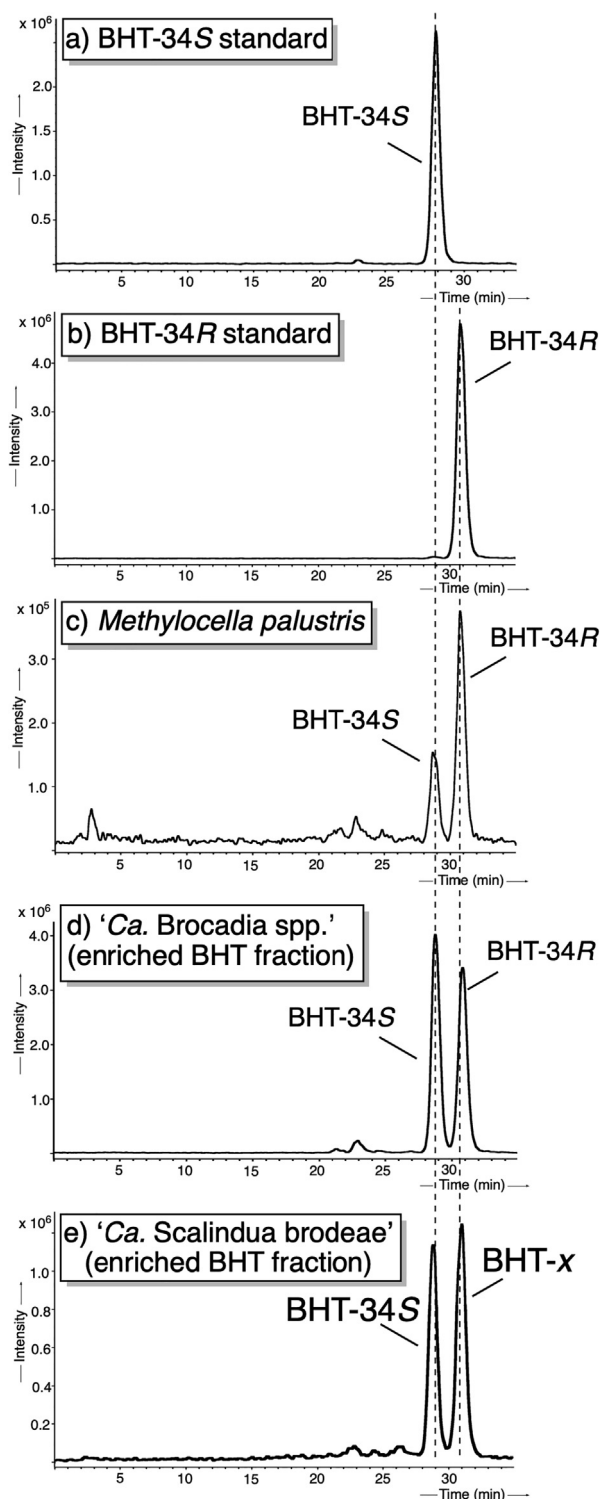


Fig. 4. HPLC-MS mass chromatograms (m/z 655) of acetylated a) BHT-34S standard; b) BHT-34R standard; c) BDE of *Methylocella palustris*; d) BDE '*Ca. Brocadia* sp.>'; e) BDE from '*Ca. Scalindua brodeae*'.

BHT-*x* (Rush et al., 2014), acetylated '*Ca. S. profunda*' TLE was also co-injected with authentic BHT standards (Fig. 3g). 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 examined by 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 17 α -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 for examination of 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 whether this method would allow acetylated BHT-34R and BHT-*x* to be distinguished. Previous HPLC studies of 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., chloroform/methanol 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 $\delta^{13}\text{C}$ values of BHT-34R. Anammox bacteria have lipids that are ^{13}C -depleted by up to 47‰ versus the CO_2 substrate (Schouten et al., 2004), resulting from their use of the acetyl coenzyme A pathway for carbon fixation (Strous et al., 2006). The $\delta^{13}\text{C}$ values of late-eluting BHT isomer from sediments taken from a Mediterranean sapropel and the Arabian Sea

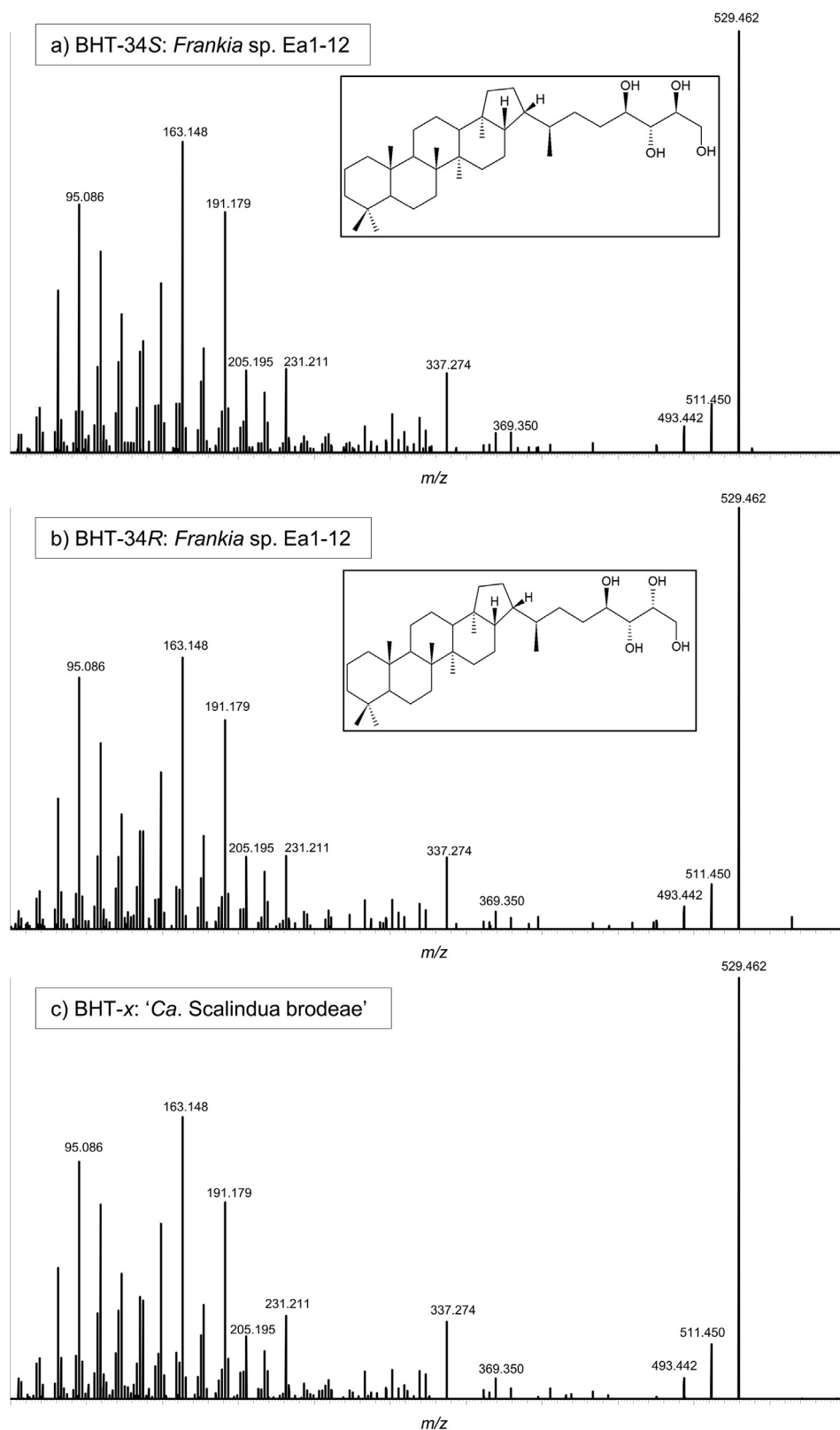


Fig. 5. Averaged ($n = 3$) ESI-HRMS² spectra of non-acetylated a) BHT-34S, b) BHT-34R and c) BHT-x from *m/z* 564.499 (ammoniated adduct of BHT; $[M+NH_4]^+$) of *Frankia* sp. a, b) and '*Ca. Scalindua brodeae*' c). Likely fragmentation patterns have been shown by Rush et al. (2019).

oxygen minimum zone were ¹³C-depleted, and had lower $\delta^{13}C$ 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, $\delta^{13}C$ analyses, as well as stereoisomer identification, should be

conducted (Hemingway et al., 2018; Lengger et al., 2019). BHT-34R produced by the methanotrophic bacterium *M. palustris* may also have a low $\delta^{13}\text{C}$ value, but Type II methanotrophs do not have consistently low lipid $\delta^{13}\text{C}$ 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.

4. 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. $\delta^{13}\text{C}$ 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.

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.

Acknowledgments

Funding was obtained through Natural Environment Research Council (NERC), United Kingdom project ANAMMARKS (NE/N011112/1) awarded to DR, SIAM, Netherlands 024002002 awarded to MJ and an EAOG, Netherlands Research Award to RSN. SKL was supported by Rubicon fellowship nr. 825.14.014 from the Netherlands Organization for Scientific Research (NWO), Netherlands. Further support came from the European Research Council (ERC), European Union under the European Union's Horizon 2020 research and innovation program (grant agreement no. 694569 – MICROLIPIDS) to JSSD. We also acknowledge support through The Leverhulme Trust, United Kingdom (Grant RPG2016-050) to JCM. The authors declare no competing financial or non-financial interests. We thank the Newcastle and NIOZ technical staff (David Earley; Paul Donohoe; Denise van der Slikke-Dorhout). We thank Prof. M. Rohmer for providing *Komagataeibacter xylinus* and Mrs Pascale Fournier (Université Lyon) for growth of

the *Frankia* sp. isolate. Dr. Sémeril (Université de Strasbourg) is greatly acknowledged for his help in the experiments of hydrogenation under pressure, of unsaturated BHTs. We thank Katinka van de Pas-Schoonen for running the Brocadia bioreactor. We thank reviewers Dr Kusch and Dr Hemingway for their substantive comments which improved the manuscript, as well as the editors of Organic Geochemistry.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.orggeochem.2020.103994>.

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1 **A unique bacteriohopanetetrol stereoisomer of marine anammox**

2

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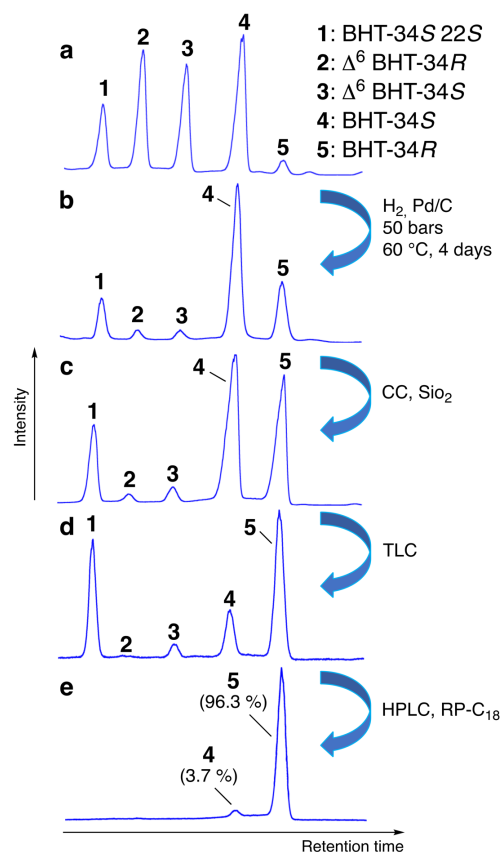
29 **Supplementary Material**

30 **Section 1. Preparation of acetylated BHT-34R and BHT-34S standards**

31 **- Isolation of BHT-34R**

32 An aliquot (233 mg) of the acetylated lipid extract from *Komagataeibacter xylinus* was
33 dissolved in a minimum amount of dichloromethane and fractionated by column
34 chromatography (CC) over silica gel to yield 3 fractions (2 column dead volumes for each
35 fraction), eluting with a mixture of cyclohexane/ethyl acetate (4:1, v/v), followed by pure ethyl
36 acetate and finally dichloromethane/methanol (1:1, v/v). The second fraction (95 mg) was
37 shown by GC to be considerably enriched in BHT-17 β , 21 β (H), 22*S*, 32*R*, 33*R*, 34*S* (BHT-
38 22*S*-34*S*) and BHT-17 β , 21 β (H), 22*R*, 32*R*, 33*R*, 34*S* (BHT-34*S*) (Fig. S1a; 14 % and 34 % of
39 summed BHTs, respectively), BHT-34*R* (Fig. SI 1-1b; 3%), together with the Δ^6 homologues
40 of BHT-34*S* (23 %) and BHT-34*R* (26 %) and minor amounts of 3 β -Me BHTs (Peiseler and
41 Rohmer, 1992). This separation scheme was repeated several times, starting from aliquots of
42 acetylated extracts from *K. xylinus*, leading to fractions enriched in BHTs, which were finally
43 combined (*ca.* 404 mg in total). Since this fraction contained BHT-34*R* in minor abundance
44 but Δ^6 BHT-34*R* as a predominant compound, the fraction was hydrogenated in order to obtain
45 higher amounts of BHT-34*R*. Several attempts using PtO₂ in ethyl acetate/acetic acid (1:1 v/v,
46 reflux, 2 h) and Pd/C (reflux, 8 h) were unsuccessful and catalytic hydrogenation of the
47 sterically-hindered Δ^6 position could only be achieved in ethyl acetate using Pd/C under
48 pressure (50 bars) at 60 °C for 4 days, leading to a mixture of hydrogenated BHTs comprising
49 BHT-34*S* 22*S* (12 %), BHT-34*S* 22*R* (57 %), BHT-34*R* (25 %) and unreacted Δ^6 -BHTs (6 %)
50 (Fig. S1b). The hydrogenated fraction was then purified by column chromatography on silica
51 gel eluting with a mixture of cyclohexane/ethyl acetate (6:1, v/v; 0.5 dead volume elution after
52 a 1.5 dead volume pre-elution), yielding a BHT fraction enriched in BHT-34*R* (51 mg; Fig.
53 S1c). This fraction was further purified by TLC (Merck Silica gel 60 F₂₅₄, 20 x 20 cm, 0.5 mm
54 thickness) using a mixture of cyclohexane/ethyl acetate (3:1, v/v) as developer, yielding a
55 subfraction (24.5 mg; R_f = 0.51 – 0.55), in which BHT-34*R* was the predominant compound
56 (47 % of the total fraction; Fig. S1d). Part of this fraction was subjected to reversed-phase
57 HPLC separation using a Waters 590 pump, a U6K injector, a differential refractometer
58 detector and an Agilent Zorbax Extend C₁₈ analytical column (2.1 x 250 mm, 5 μ m). MeOH
59 was used as the mobile phase (0.5 mL min⁻¹). Finally, BHT-34*R* (2 mg) was isolated with a
60 purity of *ca.* 96 % as determined by GC (Fig. S1e).

61



62

63 Figure S1. Partial gas chromatograms (GC-FID; for GC conditions, see § 2.5.2.) showing the
 64 BHT distributions at each purification step followed to isolate the BHT-34R standard from
 65 *Komagataeibacter xylinus*: a) the second-eluted fraction using silica gel column
 66 chromatography (elution using pure ethyl acetate) from the acetylated TLE; b) the second-
 67 eluted fraction of the acetylated TLE after hydrogenation (Pd/C, 60 °C, 50 bars, 4 days); c) the
 68 BHT fraction recovered after column chromatography of hydrogenated BHTs (elution with
 69 cyclohexane/ethyl acetate, 6:1, v/v); d) the subsequent TLC fractionation; and e) the isolated
 70 BHT-34R after reverse-phase HPLC fractionation.

71

72 The stereochemistry of the side-chain of acetylated BHT-34R standard was determined using
 73 1- and 2-dimensional ¹H and ¹³C NMR. The NMR spectra of BHT-34R standard were recorded
 74 on a Bruker Avance I 500 MHz spectrometer (500 MHz for ¹H; 125 MHz for ¹³C). The
 75 chemical shifts are reported in ppm relative to tetramethylsilane with the solvent used as
 76 internal standard (CDCl₃: $\delta^1\text{H}$ 7.26 ppm; $\delta^{13}\text{C}$ 77.0 ppm).

77

78

79 - **BHT-34S preparation**

80 A standard of BHT-34S was previously isolated from *Zymomonas mobilis* (cf. Schaeffer et al.,
81 2010) and was used for NMR studies and co-elution experiments. The same NMR analytical
82 conditions as those described for BHT-34R were used (see above).

83

84

85 Section 2. Partial ^1H and ^{13}C NMR data for acetylated BHT-34R and BHT-34S

86 - NMR data for acetylated BHT-34R

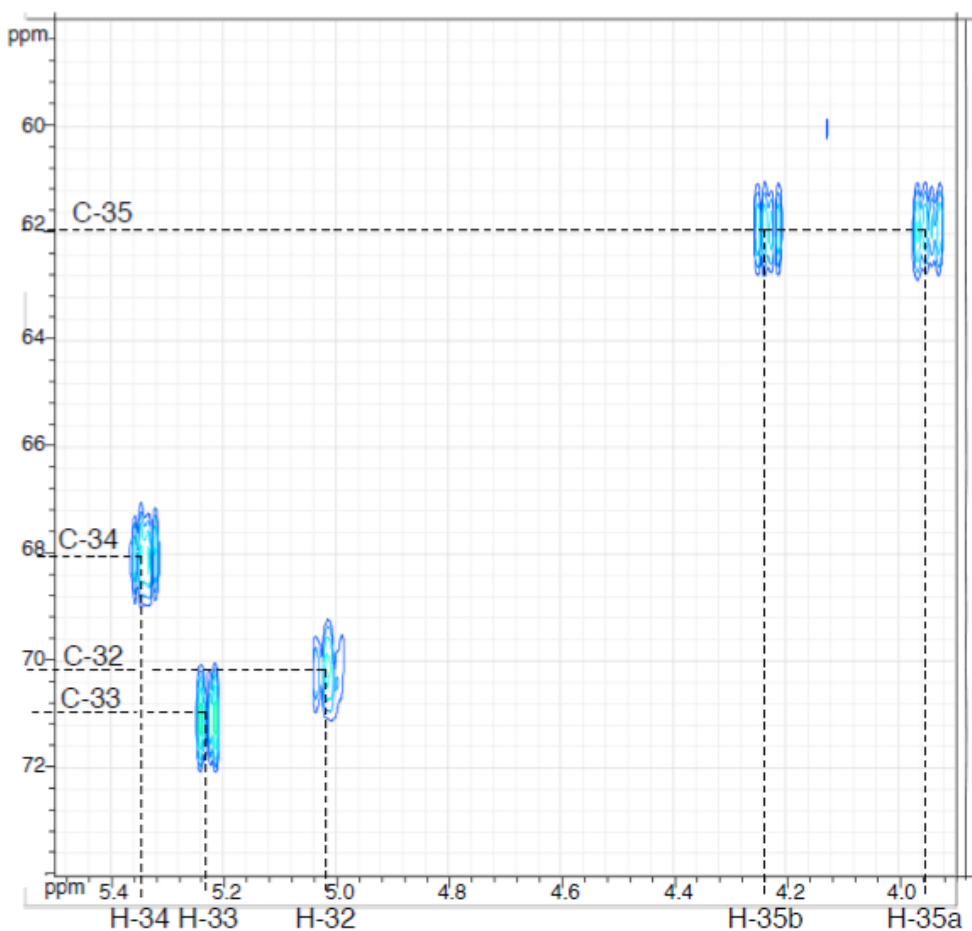
87

88 Table S2a: ^1H and ^{13}C values (ppm) determined for acetylated C-32 to C-35 for
89 BHT-34R.

	Bisseret & Rohmer (1989)		Peiseler and Rohmer (1992)		Our work	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
C-32	5.02	68.2 ^a	5.02	68.3 ^a	5.01	70.4
C-33	5.24	70.3 ^a	5.23	70.5 ^a	5.23	71.2
C-34	5.34	71.1 ^a	5.34	71.3 ^a	5.34	68.2
C-35	3.96 - 4.24	62.0	3.96 - 4.24	62.1	3.95 - 4.23	62.1

90 ^aThe assignments of these three C-signals were inverted in these studies due to the absence of two
91 dimensional NMR data at that time.

92



93

94 Fig. S2a: Partial HSQC spectrum of acetylated BHT-34R (this work).

95

96

- **NMR data for acetylated BHT-34S**

97

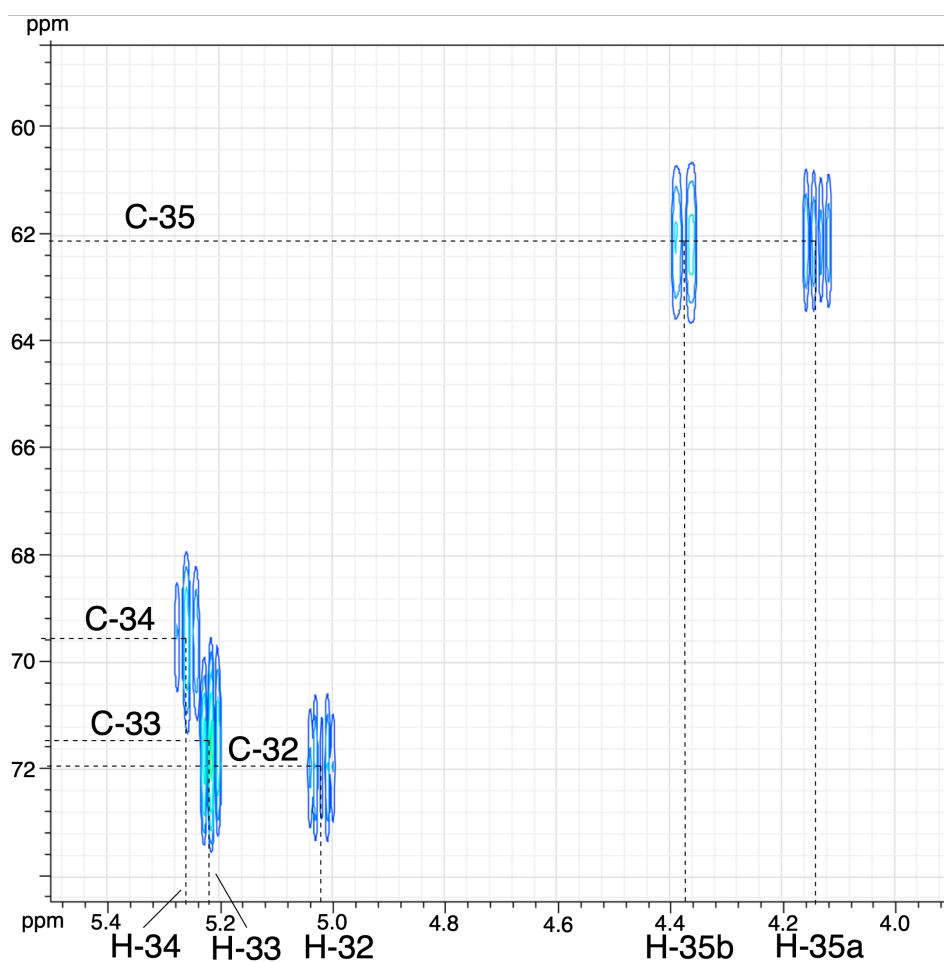
Table S2b: ^1H and ^{13}C values (ppm) determined for C-32 to C-35 for acetylated BHT-34S.

98

	Bisseret & Rohmer (1989)		Our work	
	^1H	^{13}C	^1H	^{13}C
C-32	5.03	69.4 ^a	5.02	71.9 ^a
C-33	5.23	71.5	5.22	71.6
C-34	5.27	71.9 ^a	5.26	69.5 ^a
C-35	4.15- 4.39	62.0	4.14 - 4.39	62.1

^a The assignments of these two C-signals were inverted in the studies from Bisseret and Rohmer (1989) due to the absence of two dimensional NMR data at that time.

99



100

101 Fig. S2b: Partial HSQC spectrum of acetylated BHT-34S (this work).

102

103 **Section 3. Isolation of non-derivatised BHT-34S and late-eluting BHT isomers from**
104 **anamnox biomass**

105 A multidimensional 3-step isolation procedure was employed. BHT-34S and the late-eluting
106 BHT stereoisomers with previously unconfirmed stereochemistries were isolated separately
107 from both '*Ca. Brocadia sp.*' and '*Ca. Scalindua brodeae*'. This was achieved using an Agilent
108 Technologies (Santa Clara, CA, USA) 1260 Infinity II HPLC system equipped with a fraction
109 collector (Foxy R1, Teledyne Isco, Lincoln, NE, USA). A LiChrospher DIOL 100 A column
110 (10 x 250 mm, 5 μ m particles; Alltech, Deerfield, IL, USA) was used to separate BHTs from
111 the bulk of the extracted lipids. The mobile phase was (A) hexane/isopropanol (IPA) (80:20
112 [v/v]) and (B) IPA:water (90:10 [v/v]) at 3 mL min⁻¹, with a gradient of 10% B to 30% B from
113 0 to 15 min, followed by an increase to 65% B in 1 min. Total run time was 30 min, after which
114 the system was re-equilibrated to 10% B. The injection volume was 200 μ L containing > 1 mg
115 BDE. The column effluent was collected in 1 min fractions. Fractions were screened for
116 presence of BHT using flow injection analysis on an Agilent Technologies 1100 series HPLC
117 with a Thermo Scientific LTQ XL ion trap with electrospray ionisation (cf. Moore et al. 2013).
118 A solvent flow of 0.2 mL min⁻¹ was used, with 80% solvent A (hexane/IPA/formic
119 acid/ammonia (79 : 20 : 0.12 : 0.04 [v/v/v/v])) and 20% solvent B (IPA/water/formic
120 acid/ammonia (88 : 10 : 0.12 : 0.04 [v/v/v/v])). The separation process was repeated on the
121 pooled fractions containing BHT (isomer) using a diol column with smaller dimensions (4.6 x
122 250 mm) with the same gradient at 1 mL min⁻¹, and 1 min fractions, as a smaller column allows
123 better separation but is unable to be loaded with the large quantities of material used in the
124 earlier stages. Fractions were checked using the method described.

125

126 To separate BHT-34S from the late-eluting BHT isomers of '*Ca. Brocadia sp.*' and '*Ca.*
127 *Scalindua brodeae*', an XBridge C₁₈ column (4.6 x 250 mm, 3.5 μ m; Waters Corporation,
128 Milford, MA, USA) was used. BHTs were eluted using (A) methanol/water (85:15 [v/v]) and
129 (B) methanol/IPA (50:50 [v/v]) at 1 mL min⁻¹. The gradient was as follows: 5% B for the first
130 3 min, followed by an increase to 60% B at 12 min, to 90% B at 40 min, after which the column
131 was washed with 100% B for 20 min and subsequently re-equilibrated at 5% B for 10 min. The
132 injection volume was 100 μ L containing > 1 mg BDE. The column effluent was collected in
133 0.25 min fractions and screened for the presence of BHT and/or BHT isomer using UHPLC-
134 HRMS, as described in section 2.6 of main manuscript. Separation of BHT and the late-eluting
135 isomers was not complete. A heart-cutting strategy was therefore applied for this final stage of
136 purification using the C₁₈ column. Fractions containing a mixture with a majority of BHT-34S

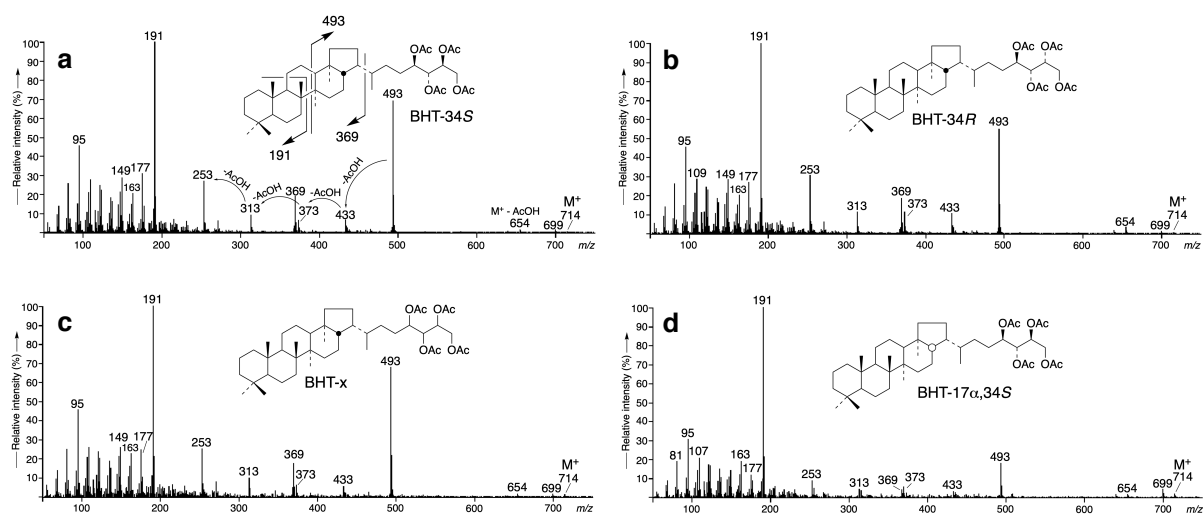
137 were combined and re-injected. The same was done for fractions containing a mixture with a
138 majority of BHT isomer. While pure lipid fractions were not obtained, the fractions enriched
139 in either BHT-34S or BHT isomer (enriched BHT fraction), included only minimal amounts
140 of other lipids.

141

142

143 **Section 4. GC-MS spectra (EI, 70 eV) of acetylated BHT**

144



145

146 Figure S4. GC-MS spectra (EI, 70 eV) of acetylated a) BHT-34S from *Komagataeibacter*

147 *xylinus*, b) BHT-34R, from *Komagataeibacter xylinus* c) BHT-x from ‘*Ca. Scalindua*

148 *profunda*’, and d) 17 α -BHT-34S from *Frankia* sp. strain Ea1-12.

149

150 **Section 5. Distributions of late-eluting BHT isomer(s) in bacterial cultures and marine**
151 **environments**

152 Previous work has used the fractional abundance of late-eluting marine BHT isomer (BHT
153 isomer ratio; late-eluting BHT isomer over total BHTs; Equation 1; Sáenz et al., 2011) to assess
154 anoxia levels and anammox contributions (e.g. Rush et al., 2014; Sáenz et al., 2011).

155

156 Equation 1: BHT isomer ratio = late-eluting BHT isomer / (BHT-34S + late-eluting BHT
157 isomer)

158

159 However, the fractional abundance of BHT isomer(s) varies with species and with growth
160 conditions (Table S5). ‘*Ca. Scalindua profunda*’ has been found to produce more BHT-*x*
161 relative to BHT-34S at a higher growth temperature (0.55 vs 0.64 at 15 °C and 23 °C,
162 respectively) (Rush et al., 2014). The relative proportions of BHT-34S and BHT-34R in *A.*
163 *pasteurianus* varied slightly with the length of culture growth (0.18 at 24 h vs. 0.25 at 96 h)
164 (Peiseler and Rohmer, 1992). Comparing the two fermentative bacteria *K. xylinus* and *A.*
165 *pasteurianus*, the latter produced more BHT-34R relative to BHT-34S than the former (0.10
166 vs. 0.18-0.25) (Peiseler and Rohmer, 1992). In most cases, the ratio of late-eluting marine BHT
167 isomer reported in environmental samples is less than that found in ‘*Ca. Scalindua spp.*’
168 cultures (Table S5). However, higher relative proportions of late-eluting marine BHT isomer
169 were observed in samples from the Humboldt Current System (Matys et al., 2017). These
170 samples may have been influenced by the species present, the temperature of the system, the
171 extraction method, the instrumentation method used for analysis, or a combination of these
172 different parameters.

173

175 Table SI 5. BHT isomer ratio in bacterial and environmental samples

Sample	BHT isomer ratio*	Method
<u>Species</u>		
' <i>Ca. Scalindua profunda</i> ', 15 °C	0.55 ^a	acetylated BHPs by HPLC- APCI-MS
' <i>Ca. Scalindua profunda</i> ', 23 °C	0.64 ^a	acetylated BHPs by HPLC- APCI-MS
' <i>Ca. Scalindua brodeae</i> ', 20 °C	0.37 ^b	non-acetylated BHPs by UHPLC-HRMS
' <i>Ca. Brocadia sp.</i> ', 33 °C	0.41 ^b	non-acetylated BHPs by UHPLC-HRMS
<i>Frankia</i> strain Ea1-12	0.55 ^b	non-acetylated BHPs by UHPLC-HRMS
<i>K. xylinus</i> strain R-2277	0.20 ^b ; 0.10 ^c	non-acetylated BHPs by UHPLC-HRMS; acetylated BHPs by HPLC-refractive index detector
<i>M. palustris</i> strain K	0.64 ^b	non-acetylated BHPs by UHPLC-HRMS
<i>A. pasteurianus</i>	0.18-0.25 ^c	acetylated BHPs by HPLC- refractive index detector
<u>Marine sample</u>		
Arabian Sea, sediment cores (Lengger et al., 2019)	0.4-0.7 ^d	acetylated BHPs by high temperature GC-MS
Humboldt Current System OMZ, SPM [¶] ? (Matys et al., 2017)	0.00-0.73 ^e	acetylated BHPs by HPLC- APCI-MS
Humboldt Current System OMZ, surface sediment (Matys et al., 2017)	0.55-0.69 ^e	acetylated BHPs by HPLC- APCI-MS

Gulfo Dulce surface sediments, (Rush et al., 2014)	0-0.16 ^a	acetylated BHPs by HPLC-APCI-MS
S5 sapropel record from site 64PE406 (R/V <i>Pelagia</i>) in the Levantine Basin (Rush et al., 2019)	0.12-0.58 ^f	non-acetylated BHPs by UHPLC-HRMS
Pliocene sapropel (2.67Ma) from the Levantine Basin (ODP Leg 160 Site 967) (Rush et al., 2019)	0.22-0.89 ^f	non-acetylated BHPs by UHPLC-HRMS
Arabian Sea; SPM (Saenz et al., 2011)	~0.0-~0.2 ^g	acetylated BHPs by HPLC-APCI-MS
Arabian Sea; sediment and interfacial material (Saenz et al., 2011)	0.22-0.30 ^g	acetylated BHPs by HPLC-APCI-MS
Cariaco Basin; SPM (Saenz et al., 2011)	~0.0-~0.5 ^g	acetylated BHPs by HPLC-APCI-MS
Peru Margin; SPM (Saenz et al., 2011)	~0.0-~0.6 ^g	acetylated BHPs by HPLC-APCI-MS

176 *BHT isomer ratio calculated by: late-eluting BHT isomer / (BHT-34S + late-eluting BHT
177 isomer)

178 [¶]SPM: suspended particulate matter

179 ^a Rush et al. (2014)

180 ^b This study

181 ^c Peiseler and Rohmer (1992)

182 ^d Lengger et al. (2019)

183 ^e Matys et al. (2017)

184 ^f Rush et al. (2019)

185 ^g Sáenz et al. (2011)

186

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