Marine and terrestrial nitrifying bacteria are sources of diverse bacteriohopanepolyols

Felix J. Elling[1,*], Thomas W. Evans[2], Vinitra Nathan[1], Jordon D. Hemingway[1], Jenan J. Kharbush[1,3], Barbara Bayer[4], Eva Spieck[5], Fatima Husain[2], Roger E. Summons[2], Ann Pearson[1]

[1]Department of Earth and Planetary Sciences, Harvard University, Cambridge, MA 02138, USA

[2]Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]University of Michigan, Department of Earth and Environmental Science, Ann Arbor, MI 48109

[4]Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA, USA

[5]Department of Microbiology and Biotechnology, University of Hamburg, 22609 Hamburg, Germany

[*]Corresponding author: felix_elling@fas.harvard.edu

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PROF. ROGER EVERETT SUMMONS (Orcid ID : 0000-0002-7144-8537)

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Marine and terrestrial nitrifying bacteria are sources of diverse bacteriohopanepolyols

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[1]Department of Earth and Planetary Sciences, Harvard University, Cambridge, MA 02138, USA

[2]Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[3]University of Michigan, Department of Earth and Environmental Science, Ann Arbor, MI 48109

[4]Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA, USA

[5]Department of Microbiology and Biotechnology, University of Hamburg, 22609 Hamburg, Germany

[*]Corresponding author: felix_elling@fas.harvard.edu

Abstract

Hopanoid lipids, bacteriohopanols and bacteriohopanepolyols, are membrane components exclusive to bacteria. Together with their diagenetic derivatives, they are

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commonly used as biomarkers for specific bacterial groups or biogeochemical processes in the geologic record. However, the sources of hopanoids to marine and freshwater environments remain inadequately constrained. Recent marker gene studies suggest widespread potential for hopanoid biosynthesis in marine bacterioplankton, including nitrifying (i.e., ammonia- and nitrite-oxidizing) bacteria. To explore their hopanoid biosynthetic capacities, we studied the distribution of hopanoid biosynthetic genes in the genomes of cultivated and uncultivated ammonia-oxidizing (AOB), nitrite-oxidizing (NOB), and complete ammonia oxidizing (comammox) bacteria, finding that biosynthesis of diverse hopanoids is common among seven of the nine presently cultivated clades of nitrifying bacteria. Hopanoid biosynthesis genes are also conserved among the diverse lineages of bacterial nitrifiers detected in environmental metagenomes. We selected seven representative NOB isolated from marine, freshwater and engineered environments for phenotypic characterization. All tested NOB produced diverse types of hopanoids, with some NOB producing primarily diploptene and others producing primarily bacteriohopanepolyols. Relative and absolute abundances of hopanoids were distinct among the cultures and dependent on growth conditions, such as oxygen- and nitrite-limitation. Several novel nitrogen-containing bacteriohopanepolyols were tentatively identified, of which the so-called BHP-743.6 was present in all NOB. Distinct carbon isotopic signatures of biomass, hopanoids, and fatty acids in four tested NOB suggest operation of the reverse tricarboxylic acid cycle in Nitrospira spp. and Nitrospina gracilis and of the Calvin-Benson-Bassham cycle for carbon fixation in Nitrobacter vulgaris and Nitrococcus mobilis. We suggest that the contribution of hopanoids by NOB to environmental samples could be estimated by their carbon isotopic compositions. The ubiquity of nitrifying bacteria in the ocean today and the antiquity of this metabolic process suggest the potential for significant contributions to the geologic record of hopanoids.

1. Introduction

Hopanoids are terpenoid lipids produced by aerobic and anaerobic bacteria (Rohmer et al., 1984; Fischer et al., 2005; Talbot et al., 2008) and are ubiquitous in the modern environment and the geologic record (Ourisson & Albrecht, 1992). In living bacteria, hopanoids are involved in membrane homeostasis (e.g., Ourisson et al., 1987; Welander et al., 2009; Sáenz et al., 2012). They are commonly found as composite lipids called bacteriohopanepolyols (BHPs), consisting of hopanoid hydrocarbon skeletons bearing

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functionalized polar sidechains (Ourisson & Rohmer, 1992; Talbot et al., 2007, 2008; Rush et al., 2016). Due to their characteristic distribution among bacteria and their structural diversity, both within the hopanoid skeleton and the polar sidechain, hopanoids and BHPs are used as biomarkers for diverse clades of bacteria or distinct ecological niches (e.g., Sáenz et al., 2011; Ricci et al., 2013; Rush et al., 2016). Due to their diagenetic stability, hopanoids are important biomarkers for elucidating biogeochemical and microbial evolution from the early Proterozoic onwards (Brocks & Pearson, 2005; Brocks & Banfield, 2009; Briggs & Summons, 2014).

Application of hopanoid biomarkers relies on the genotypic and phenotypic characterization of their biosynthesis among extant bacteria (Newman et al., 2016). The known diversity of hopanoid producers initially was constrained by analysis of bacterial cultures (Rohmer et al., 1984; Talbot et al., 2008). The potential for wider taxonomic diversity expanded considerably, however, through genomic and metagenomic characterization of biosynthetic genes such as squalene-hopene cyclase (SHC), which catalyzes the first step in hopanoid biosynthesis forming diploptene/diplopterol (Fig. 1; e.g., Fischer et al., 2005; Pearson et al., 2007; Ricci et al., 2014). Such metagenomic surveys have outpaced phenotypic characterization, leaving many bacterial clades either unstudied or understudied. Additionally, the biosynthetic pathways leading from diploptene/diplopterol to the diverse array of BHPs remain only partially resolved (Fig. 1) and the phylogenetic distribution of known biosynthetic genes has not been studied systematically.

Recent lipidomic and metagenomic surveys of SHC gene homologs identified nitrite-oxidizing bacteria (NOB) of the genera Nitrospina and Nitrospira as potential sources of BHPs in the marine water column, particularly in suboxic settings (Kharbush et al., 2013, 2015, 2018). NOB are a polyphyletic group of bacteria spanning six clades across four phyla. NOB mediate the second step of nitrification, the oxidation of nitrite to nitrate, and show environmental niche speciation (Spieck & Bock, 2005; Daims et al., 2016). NOB of the genera Nitrobacter (Alphaproteobacteria) and Candidatus Nitrotoga (Betaproteobacteria) are found primarily in freshwater, wastewater and soils (Spieck & Bock, 2005; Alawi et al., 2007; Daims et al., 2016). NOB of the genus Nitrospira (phylum Nitrospirae), on the contrary, inhabit both terrestrial and marine environments, including hydrothermal systems (Spieck & Bock, 2005; Daims et al., 2016; Bayer et al., 2021). Moreover, some Nitrospira spp. are capable of performing both steps of nitrification (ammonia and nitrite oxidation, "comammox"; Daims et al., 2015; van Kessel et al., 2015), although this capacity appears to

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be limited to strains occurring in freshwater and wastewater environments (Palomo et al., 2018). In the modern ocean, Nitrospira spp., Nitrospinaceae (phylum Nitrospinae) and Nitrococcus spp. (Gammaproteobacteria) are the predominant NOB (Mincer et al., 2007; Santoro et al., 2010; Füssel et al., 2017; Pachiadaki et al., 2017); however, there also are marine species of Nitrobacter (Ward & Carlucci, 1985; Ward et al., 1989). Finally, five NOB from the phylum Chloroflexi (Ca. Nitrocaldera robusta, Ca. Nitrotheca patiens, and three Nitrolancea strains) have previously been cultivated from terrestrial hydrothermal springs and bioreactors (Sorokin et al., 2012; Spieck et al., 2020a, 2020b), but their environmental distribution remains largely unconstrained. Despite the ubiquity of NOB in terrestrial and marine ecosystems, their potential to produce hopanoids, including BHPs, has not been systematically assessed and their imprint on the geologic record of hopanoids remains unresolved.

Here we describe the use of BHPs as biomarkers for NOB, based on widespread association of BHP production with the capacity for nitrite oxidation, and on characterization of distinct distributions and carbon isotopic compositions of these compounds in seven marine and non-marine species from four genera of NOB: Nitrospina gracilis (marine), Nitrospira marina (marine), Nitrococcus mobilis (marine), Nitrobacter vulgaris (wastewater), Nitrospira moscoviensis (heating system), Nitrospira lenta (wastewater), and Nitrospira defluvii (wastewater). Through genomic analyses we demonstrate that BHP biosynthesis is common in cultivated and uncultivated NOB with exception of the Ca. Nitrotoga and Nitrolancea clades. We extended this genomic approach to other nitrifying bacteria and find hopanoid biosynthesis to be ubiquitous among ammonia oxidizing bacteria (AOB) and comammox bacteria. Coupled genomic-lipidomic-isotopic analysis of BHPs, as demonstrated here, provides valuable insights into the adaptation mechanisms of NOB as well as their roles in past ecosystems.

2. Material and Methods

2.1 Cultivation

2.1.1 Origin of cultures

The strains N. gracilis Nb-3/211, N. marina Nb-295, N. mobilis Nb-231, N. vulgaris AB1, N. moscoviensis M-1, N. lenta BS10, and N. defluvii A17 were obtained from the culture collection of Eva Spieck at the University of Hamburg. Strain N. gracilis Nb-211 was

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obtained through the culture collection of Alyson Santoro at the University of California, Santa Barbara. All combinations of strains and culture conditions are shown in Table 1.

2.1.2 Cultivation of nitrite-oxidizing bacteria for isotopic analysis

To generate sufficient biomass for carbon isotopic analysis, the four NOB species N. gracilis Nb-3/211, N. mobilis Nb-231, N. marina Nb-295, and N. vulgaris AB1 were grown autotrophically in 10 L glass bottles containing 6 L of medium. N. gracilis Nb-3/211, N. mobilis Nb-231, and N. marina Nb-295 were grown in a natural seawater medium (Watson & Waterbury, 1971; Lücker et al., 2013) using 30% ultra-pure water and 70% Gulf of Maine seawater (0.2 µm sterile-filtered; Bigelow National Center for Marine Algae and Microbiota, East Boothbay, ME, USA). N. vulgaris was grown in an artificial freshwater medium (Bock et al., 1983). After autoclaving, the media were left at room temperature for 1-3 days followed by pH adjustment to 7.5 using sterile-filtered 1 M NaOH or HCl. Before inoculation, 0.3 mM $NaNO_2$ (final concentration) was added from a sterile stock solution. Cultures were inoculated with 1% of mid-growth phase pre-cultures and incubated without stirring at 28 °C in the dark. After consumption of 0.3 mM nitrite, the cultures were continuously stirred (150 rpm). Nitrite was then fed incrementally due to sensitivity of some NOB to high nitrite concentrations during early growth (Spieck & Lipski, 2011). An additional 0.5 mM nitrite was added to each culture and replenished when 0.4 mM of the addition was consumed; this continued until a total of 2.3 mM nitrite had been consumed, after which a final increment of 5 mM nitrite was added. Growth was monitored by photometric quantification of nitrite (Strickland & Parsons, 1972). Cultures were harvested in early stationary phase after consumption of 7.3 mM nitrite.

2.1.3 Cultivation of nitrite-oxidizing bacteria for lipidomic analyses

To evaluate the effects of growth parameters on hopanoid composition, the seven NOB N. gracilis Nb-3/211, N. marina Nb-295, N. mobilis Nb-231, N. vulgaris AB1, N. moscoviensis M-1, N. lenta BS10, and N. defluvii A17 were grown with 150 mL medium in 250-mL Erlenmeyer flasks at 28 °C in the dark (37 °C for N. moscoviensis). Triplicate autotrophic cultures were grown on artificial freshwater or natural seawater media as described above. In addition, the influence of different growth conditions on hopanoid distributions was tested, using four NOB strains (N. gracilis Nb-3/211, N. marina Nb-295, N. mobilis Nb-231, N. vulgaris AB1). However, some experiments were limited to specific strains due to distinct growth requirements (Table 1). Growth was assessed by monitoring

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nitrite consumption or optical density at 600 nm (OD_{600}). Cultures were harvested at the beginning of stationary phase, defined as the first day after unchanged OD_{600} values or nitrite concentration. Mid-growth phase nitrite oxidation rates are given in Table S1.

N. vulgaris AB1 was grown in batch cultures under chemolithoautotrophic (mid growth phase, early stationary phase), heterotrophic aerobic, heterotrophic anaerobic, and aerobic mixotrophic conditions. To test the influence of methionine and hydroxocobalamin (vitamin B12a), 7 mM $NaNO_2$ were added to the medium and either 0.5 µM hydroxocobalamin or 0.5 µM methionine were added. To additionally investigate the effect of light, autotrophic cultures amended with 0.5 µM hydroxocobalamin and 0.5 µM methionine were grown under 6 h light/18 h dark cycles (fluorescent cool-white lamps). Heterotrophic aerobic cultures were grown with added 1.5 g L^{-1} yeast extract (Difco), 1.5 g L^{-1} peptone (Difco), and 0.55 g L^{-1} sodium pyruvate. Growth of aerobic heterotrophic cultures was monitored by measuring OD_{600}. Heterotrophic anaerobic (nitrate-reducing) cultures were grown in heterotrophic medium with 1 g L^{-1} $NaNO_3$ in serum bottles gas-tight blue butyl rubber stoppers. Oxygen was purged from the medium and headspace using an ultrasonic bath and replaced with sterile N_2. Mixotrophic cultures were grown aerobically in heterotrophic medium with 7 mM $NaNO_2$ added. Growth of anaerobic heterotrophic and mixotrophic cultures was monitored by quantifying nitrite formation/consumption and OD_{600}. All cultures were agitated using an orbital shaker (150 rpm) during incubation.

N. gracilis, N. marina Nb-295 and N. mobilis Nb-231 were grown chemolithoautotrophically in triplicate batch cultures in natural seawater medium as well as with addition of either 0.5 µM methionine or 0.5 µM hydroxocobalamin. Additionally, N. marina Nb-295 was grown under mixotrophic conditions. For mixotrophic growth, 0.15 g L^{-1} peptone, 0.15 g L^{-1} yeast extract, 0.055 g L^{-1} sodium pyruvate, and 7 mM $NaNO_2$ were added to the medium. Additional cultures of N. marina and N. mobilis grown with 0.5 µM methionine and 0.5 µM hydroxocobalamin were harvested during late stationary phase, i.e., two weeks after nitrite depletion.

To investigate the ability for hopanoid methylases to use the cyanobacterial vitamin B_{12} variant pseudocobalamin, N. marina Nb-295, N. mobilis Nb-231 and N. vulgaris AB1 were grown chemolithoautotrophically in single batch cultures in artificial seawater or freshwater medium containing 100 nM pseudocobalamin. As pure pseudocobalamin was not commercially available, it was extracted from 4 g of commercial Spirulina dietary

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supplement as described by Heal et al. (2017) and purified using Thermo Scientific HyperSep C_{18} solid phase extraction cartridges, yielding a fraction containing 1.5 µg of hydroxopseudocobalamin with traces of cyanopseudocobalamin and other non-cobalamin solutes. Pseudocobalamin was quantified using an Agilent 1260 Infinity series high performance liquid chromatograph (HPLC) coupled to an Agilent 6410 triple-quadrupole mass spectrometer (MS). The MS was operated in multiple reaction monitoring mode, to monitor the parent ions and transitions of hydroxopseudocobalamin, adenosylpseudocobalamin, and cyanopseudocobalamin as described in Heal et al. (2017). The HPLC method was modified from Heal et al. (2017) by using a Phenomenex Kinetex C_{18} column (4.6 × 150 mm; 2.6 µm particle size), a flow rate of 1 mL min^{-1}, and 90% water/10% acetonitrile (v/v) as the initial solvent composition. Hydroxocobalamin, adenosylcobalamin, and cyanocobalamin standards were used to optimize ionization parameters and instrument performance. As no hydroxopseudocobalamin standard was available, concentrations were estimated using a hydroxocobalamin solution and assuming identical instrument response.

2.1.4 Nitrite- and oxygen-limited continuous cultures

N. vulgaris AB1, N. gracilis Nb-211, and N. mobilis Nb-231 were additionally grown in continuous culture under constant, substrate-limited conditions (chemostat). The experimental setup is illustrated in Fig. S1. The chemostat consisted of a gas-tight 2.2-L reactor vessel containing 2 L of culture, kept at a constant 28 °C in the dark and stirred at 150 rpm. For N. vulgaris AB1, the reactor was fed with freshwater medium containing 10 mM $NaNO_2$, 0.5 µM hydroxocobalamin, and 0.5 µM methionine. For N. gracilis Nb-211, and N. mobilis Nb-231, the reactor was fed with artificial seawater medium (Bayer et al., 2021) containing 2 mM $NaNO_2$ and 0.1 µM hydroxocobalamin. For nitrite-limited growth (i.e., oxygen-replete), the cultures were constantly aerated using sterile-filtered (0.2 µm pore size), humidified air delivered through aquarium pumps, resulting in full oxygen saturation (6.0 ± 0.2 ppm; Table S2) of the growth medium. For oxygen-limited growth (i.e., nitrite-replete), aeration of the reactor was stopped, and the headspace was flushed with sterile N_2 gas (0.2 µm pore size filter; 0.2 L min^{-1}). The feeding medium was flushed with air to allow full oxygen saturation (8.6 ± 0.2 ppm; Table S2), as the medium was the only source of dissolved oxygen to the reactor. The chemostat outflow was flushed with sterile-filtered N_2 gas (0.2 L min^{-1}) to inhibit growth in the collection bottle. For N. vulgaris, nitrite and oxygen concentrations were sampled through a sterile sampling port directly from the reactor and monitored continuously to assess performance of the chemostat. For N. gracilis Nb-211, and

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N. mobilis Nb-231, samples for nitrite concentration assays were taken from the chemostat outflow. Due to interference of high nitrite concentrations with the Winkler method—even after the addition of sodium azide—the oxygen concentration measurements under oxygen-limited conditions were considered unreliable. For all experiments, oxygen-limited conditions were indicated by strongly increased nitrite concentrations in the chemostat outflow. The cultures were maintained at constant growth rates (N. mobilis Nb-231, N. gracilis Nb-211: 0.011 h^{-1}; N. vulgaris AB1: 0.013 h^{-1}; Table S2, S3). Equilibrium conditions were reached within 3-4 turnovers of the culture volume, as indicated by constant nitrite concentrations in the reactor outflow. For all chemostat experiments, chemostat outflow was collected after equilibration in a glass bottle placed in an ice bath. Cells were harvested from the outflow using filtration as described below for batch cultures. For N. vulgaris, triplicate harvests of biomass (0.2-0.6 L) were spaced apart by at least one full turnover (2-3 days) of the chemostat vessel. For N. gracilis Nb-211 and N. mobilis Nb-231, biomass was sampled daily (~0.5 L) and combined to yield duplicate samples of 1 L for each species and condition. Cell densities were determined by epifluorescence microscopy of 2% formaldehyde-fixed samples using SYBR Green I staining (Lunau et al., 2005).

2.1.5 Harvesting and lipid extraction

Biomass was harvested by filtration through two stacked, combusted glass fiber filters (GF-75, 0.3 µm pore size, Advantec MFS, Dublin, CA, USA). Lipids were extracted using a four-step Bligh and Dyer extraction (Bligh & Dyer, 1959) using an ultrasonic bath and as modified by Sturt et al. (2004) and Sáenz (2010). The total lipid extracts (TLEs) were gently dried under N_2, reconstituted in dichloromethane:methanol 5:1, and stored at -20 °C.

2.2 Mass-spectrometric analysis of bacteriohopanepolyols

BHPs in dried TLE aliquots for all experiments were acetylated with 100 µL acetic anhydride and 100 µL pyridine at 50 °C for 1 h and then left overnight at room temperature (Spencer-Jones, 2015). After acetylation, samples were evaporated to dryness, reconstituted in 30:70 methanol/isopropanol, filtered through 0.45 µm pore size syringe-tip PTFE filters, and stored at -20 °C until analysis. Aliquots of the acetylated TLEs were injected into a coupled HPLC-MS system consisting of a 1200 series HPLC and a 6520 quadrupole time-of-flight mass spectrometer (QTOF-MS) equipped with an atmospheric-pressure chemical ionization interface operated in positive mode (all Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer was calibrated before analysis, with a typical error in mass

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accuracy of < 1 ppm, and two reference masses were constantly monitored. The ion source was set to the following parameters: gas temperature 325°C, vaporizer temperature 350°C, N_2 drying gas flow 6 L min^{-1}, N_2 nebulizer flow 40 L min^{-1}, capillary voltage 1200 V, corona needle current 4 µA, and fragmentor voltage 150 V (Matys et al., 2017). Analyses were performed in full-scan mode with a scan rate of 2 spectra per second over the m/z range of 200-1300 for MS^1 mode and 100-1300 for MS^2 mode. MS^2 precursor selection was performed in data-dependent mode targeting the two most abundant ions per MS^1 scan with an isolation width of 4 Da and active exclusion after 2 spectra over 0.4 min. BHPs were chromatographically separated using a Poroshell 120 EC-C_{18} column (2.1 × 150 mm, 2.7 µm particle size; Agilent Technologies) following the protocol of Matys et al. (2017). BHPs were identified by retention time, MS^2 fragment spectra, accurate molecular mass, and isotope pattern match of proposed sum formulas in full scan mode using Agilent MassHunter B.06.00 and Bruker DataAnalysis 4.4 software. Absolute quantification was achieved by normalizing hopanoid concentrations to consumed substrate (mmol NO_2^- oxidized). BHP concentrations were corrected for differences in relative response using authentic standards of diplopterol, 2-Me diplopterol, bacteriohopanetetrol (BHT), 2-Me BHT, and aminobacteriohopanetriol (BHaminotriol). Due to lack of standards for some compounds, the relative response of BHaminotriol was used for correction of other nitrogen-containing BHPs and the relative response of 2-Me BHT was used to correct abundances of 3-Me BHT. We therefore consider the reported relative abundances of BHPs to be semi-quantitative. The lower limit of detection was estimated at 13 pg on-column (relative abundance of <0.01% of total hopanoids), as determined by the lowest concentration of a BHP (monounsaturated BHT) detected in the culture extracts. Averages and 1σ standard deviations of hopanoid relative abundances and concentrations from triplicate cultures are given in Supplementary Datafile S2.

2.3 Isotopic analysis of dissolved inorganic carbon, bacteriohopanepolyols, biomass, and fatty acids

Isotopic analyses were performed only for chemolithoautotrophically-grown batch cultures of N. marina, N. gracilis, N. mobilis, and N. vulgaris as described in section 2.1.2. The primary goal of the isotopic analyses was to confirm operation of the predicted carbon fixation pathways and to characterize the isotopic offsets between biomass and lipids derived from the isoprenoid (hopanoids) and acetogenic (fatty acids) pathways. Headspace-free samples for stable carbon isotopic composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were

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collected immediately after inoculation by overflowing 30 mL glass bottles with culture and capping with Qorpak polycone caps. 20 µL of saturated $HgCl_2$ solution were added to inhibit microbial activity and samples were stored at 4 °C in the dark. Values of $\delta^{13}C_{DIC}$ were determined in triplicate at the University of Florida Light Stable Isotope Mass Spec Lab using a Thermo Finnigan DeltaPlus XL isotope ratio mass spectrometer (IRMS) coupled to a GasBench II interface. The long-term precision of samples and reference standard measurements was < 0.1‰. All results are reported in delta notation relative to VPDB. $\delta^{13}C_{CO2}$ was calculated from $\delta^{13}C_{DIC}$ after Mook et al. (1974), at 28 °C. During growth, increasing utilization of the dissolved inorganic carbon pool could have led to Rayleigh fractionation, which could have affected apparent ^{13}C fractionation values presented in Table 2. However, no significant difference in $\delta^{13}C_{DIC}$ was observed for samples collected from N. gracilis cultures at three growth phases: early growth phase (0.17 ± 0.44 ‰), mid-growth phase (0.36 ± 0.43 ‰), and stationary phase (-0.16 ± 0.64 ‰).

Biomass stable carbon isotopic composition was analyzed in triplicate using a ThermoScientific Flash EA coupled to a Delta V Plus IRMS. Filter samples were freeze-dried, decarbonated with 1 N HCl, and dried at 60 °C. Stable carbon isotopic compositions were peak-size-corrected and offset-corrected using laboratory and authentic reference standards (glutamic acid: -13.90‰ $\delta^{13}C$ VPDB; tyrosine: -24.90‰; USGS40: -26.39‰; USGS41a: 36.55‰).

Free and intact polar lipid-bound fatty acids in the TLE were converted to fatty acid methyl esters (FAMEs) following the protocol of Ichihara and Fukubayashi (2010) and were analyzed as described in (Tang et al., 2017). To monitor instrument performance, the fatty acid standards were analyzed in between every batch of three sample analyses. Due to insufficient chromatographic separation, $\delta^{13}C$ values of C_{16} fatty acids were determined as the integrated value of $C_{16:0}$+$C_{16:1\omega5}$+$C_{16:1\omega9}$ fatty acids for N. marina and $C_{16:0}$+$C_{16:1\omega9}$ fatty acids for N. mobilis. Similarly, $\delta^{13}C$ values of C_{18} fatty acids were determined as the integrated value of $C_{18:0}$+$C_{18\omega7}$ fatty acids for N. mobilis and $C_{18:0}$+$C_{18\omega1}$ fatty acids for N. vulgaris. The $\delta^{13}C$ values were corrected for (i) size-effects using dilution series of standards ($C_{16:0}$, $C_{19:0}$, $C_{24:0}$ fatty acids) of known isotopic composition and (ii) carbon derived from transesterification using the same standards prepared in parallel to the samples (Tang et al., 2017).

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The $\delta^{13}C$ values of diploptene were analyzed in triplicate from TLE aliquots, using the same instrumental setup and employing the corrections described above using an n-C_{38} alkane standard of known isotopic composition (obtained from Arndt Schimmelmann, Indiana University).

2.4 Homology detection and phylogenetic analysis of hopanoid and cobalamin biosynthesis genes

Hopanoid biosynthesis gene homologs were identified through sequence homology, conserved domains, and by clustering with reference sequences in phylogenetic trees. Genomes of NOB were searched for hopanoid biosynthesis protein homologs using blastp v2.7.1 (Altschul et al., 1990). Reference sequences (from Alicyclobacillus acidocaldarius, Burkholderia cenocepacia, Burkholderia pseudomallei, Koribacter versatilis, Methylococcus capsulatus, Nostoc punctiforme, Rhodopseudomonas palustris) were selected based on two criteria: (i) previously identified hopanoid biosynthesis gene homologs and (ii) production of the respective hopanoid confirmed through lipid analysis. Reference protein sequences were obtained through the UniProt database (http://www.uniprot.org) and were searched against all draft and finished NOB genomes in the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome), the NCBI non-redundant protein database, as well as environmental metagenome-assembled genomes (MAG) and single cell amplified genomes (SAG) of NOB in the Integrated Microbial Genomes & Metagenomes database of the Joint Genome Institute (http://img.jgi.doe.gov), using an expectation value cutoff of 10^{-5}. Complete and nearly complete 16S rRNA gene sequences of NOB and AOB were obtained through the NCBI and SILVA databases (Quast et al., 2013) or manually retrieved from genomes. For phylogenetic analyses, protein or nucleotide sequences were aligned using MAFFT v7.388 (Katoh & Standley, 2013) in multi-domain (G-INS-i) and single-domain (L-INS-i) modes, respectively, and manually refined. Maximum-likelihood phylogenies were generated using RAxML v7.4.2 (Stamatakis, 2014) using gamma distribution estimates and the Whelan & Goldman amino acid substitution model for proteins. Bootstrap support was calculated 500 times for each analysis. Phylogenetic trees were visualized using iTOL v3 (Letunic & Bork, 2016).

3. Results

3.1 Distribution of hopanoid biosynthetic genes in nitrifying bacteria

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Hopanoid biosynthetic gene homologs were detected in all sequenced cultures of the NOB/comammox families Nitrospiraceae and Nitrospinaceae, the genera Nitrococcus and Nitrobacter, as well as the Chloroflexi NOB Ca. Nitrotheca patiens and Ca. Nitrocaldera robusta (Fig. 2; Supplementary Datafile S1). Similarly, all sequenced genomes of cultivated AOB (e.g., Nitrosomonas europaea, Nitrosococcus oceani) encoded hopanoid biosynthetic genes. By contrast, no homologs were detected in the NOB genera Ca. Nitrotoga and Nitrolancea. The presence of hopanoid biosynthetic genes was not conserved among some closely related groups, such as in the phylum Nitrospirae, in which only two of four characterized genera (Nitrospira and Leptospirillum) contained hopanoid biosynthesis gene homologs. Only a subset of the studied environmental genomes (metagenome-assembled genomes and single cell genomes) contained hopanoid biosynthesis gene homologs (Supplementary Datafile S1). All hopanoid biosynthesis genes detected in environmental genomes were also found in cultivated representatives, implying that the biosynthetic capacity of the cultured species is generally representative for uncultured species of nitrifying bacteria.

Most NOB and AOB representatives that contain a putative gene for the initial step of hopanoid biosynthesis, the formation of diploptene/diplopterol by the enzyme squalene hopene cyclase (SHC; Sohlenkamp & Geiger, 2016; Belin et al., 2018), also contain hpnH and hpnG homologs. These genes encode enzymes for the biosynthesis of the polyfunctionalized hopanoids adenosylhopane and ribosylhopane, respectively (Bradley et al., 2010; Welander et al., 2012; Liu et al., 2014; Sato et al., 2020). Gene homologs for biosynthetic steps downstream of ribosylhopane and leading to the formation of N-acetylglucosaminyl BHT (hpnI), BHT cyclitol ether (hpnJ), and glucosaminyl BHT (hpnK) (Schmerk et al., 2015), were detected in only two AOB (Ca. Nitrosoglobus terrae and Ca. Nitrosacidococcus tergens). In addition, a single NOB (Ca. N. robusta) contained hpnK but not hpnI and hpnJ. The gene coding for the biosynthesis of BHaminotriol from ribosylhopane (hpnO) (Welander et al., 2012) was detected in Nitrococcus and Nitrobacter spp. as well as all betaproteobacterial AOB (Nitrosomonas, Nitrosovibrio, Nitrosospira), but not in the gammaproteobacterial AOB (Nitrosococcus, Ca. Nitrosoglobus, Ca. Nitrosacidococcus). Homologs of hpnO could not be unequivocally identified in Nitrospira spp., as multiple homologous amino acid aminotransferases were detected. However, these homologs shared low sequence similarity (≤30%) and did not cluster with any previously identified hpnO homologs in phylogenetic trees.

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Homologs coding for hopanoid A-ring methylases were found in almost all NOB/comammox and some AOB. Homologs of the hopanoid C-2 methylase hpnP (Welander et al., 2010) were found only in Nitrobacter spp. and a single Nitrospira sp., Ca. Nitrospira alkalitolerans (Fig. 2). By contrast, homologs coding for the C-3 methylase, hpnR (Welander & Summons, 2012), were more widespread and found in N. mobilis, all Nitrosococcus spp. and all Nitrospira spp. except for N. defluvii and N. lenta (Fig. 2).

3.2 Distribution of hopanoids in chemolithoautotrophically-grown nitrite-oxidizing bacteria

As a baseline experiment, we characterized hopanoid production in chemolithoautotrophic cultures. The seven studied NOB produced a diverse suite of 25 BHPs. Six novel BHPs with the ions of 762.5, 656.5, 771.6, 748.5, 638.5, and 743.6 Da were tentatively identified using MS^2 spectra and molecular formulas derived from accurate masses (Fig. 3; Table S4; interpretation of fragmentation patterns is provided in the supplementary information). Major BHPs common to all strains were adenosylhopane, BHT, BHaminotriol, and the novel BHP-743.6 and BHP-762.5, with minor amounts of other BHPs (Fig. 4 & 5; Supplementary Datafile S2).

Although each NOB produced a similar array of BHPs, their relative abundances were variable (Fig. 4 & 5). Each of the three non-marine Nitrospira spp. contained a different major BHP: adenosylhopane in N. defluvii (56%), BHP-743.6 in N. lenta (55%), and BHT in N. moscoviensis (54%). 35-Aminobacteriohopanepentol (BHaminopentol) was detected in significant amounts (4%) only in N. defluvii. N. marina contained predominantly BHT. Analysis of one additional large-volume culture (8 L) allowed detection of additional minor BHPs in N. marina–C_{16}, C_{18}, and C_{19} n-acylaminotriol BHPs that were not detected in any other NOB species. No methylated BHPs were detected in N. marina. The most abundant BHPs in N. gracilis were BHT (74%), BHP-743.6 (18%), and BHaminotriol (8%; Fig. 5). No methylated BHPs were detected in N. gracilis. In contrast, BHP-743.6 (45%) and BHaminotriol (44%) were the most abundant BHPs in N. mobilis, with minor contributions from BHT (2%) and other BHPs, including methylcarbamate triol (7%). N. mobilis contained trace amounts of 3-Me BHT (0.04%) but no other forms of 3-Me BHPs. N. vulgaris contained BHaminotriol (50%), BHP-743.6 (29%), adenosylhopane (9%), and BHT (2%). 2-Me BHT was detected in trace amounts in N. vulgaris (<0.1%) but no other forms of 2-Me BHPs were found in cultures grown chemolithoautotrophically (Fig. 4).

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412 The non-functionalized hopanoid diploptene was present in all cultures (Fig. 4 & 5).
413 Diploptene comprised 99% of total hopanoids (BHPs + diploptene) in N. marina, whereas
414 lower abundances were found in N. lenta (89%), N. moscoviensis (78%), N. gracilis (69%),
415 N. vulgaris (37%), and N. defluvii (11%). Diploptene was detected only in trace amounts in
416 N. mobilis. Trace amounts (0.5%) of 2-Me diploptene were detected in N. vulgaris, and 3-Me
417 diploptene was not detected in any strain under these conditions.

418 3.3 Effects of changes in culturing conditions on hopanoid distributions in nitrite-oxidizing
419 bacteria

420 Changes in growth conditions (Table 1) had no effect on hopanoid distribution in
421 batch cultures of N. marina and N. mobilis (Fig. 5), except for late stationary phase cultures,
422 where lower amounts of methylcarbamate triol were observed in N. mobilis and slightly
423 lower amounts of BHT and higher amounts of BHaminotriol were detected in N. marina.
424 Trace amounts of 3-methyl diploptene were observed in cultures of N. marina supplemented
425 with cobalamin. Chemostat cultures of N. mobilis grown under NO_2^- or O_2-limited conditions
426 had broadly similar hopanoid distributions. By contrast, BHP compositions of N. gracilis and
427 N. vulgaris changed notably with growth conditions. In N. gracilis, cultures supplemented
428 with methionine and cobalamin resulted in an increase of the BHP to diploptene ratio and
429 higher abundance of minor BHPs (Fig. 5). N. gracilis grown under NO_2^--limited conditions
430 produced predominantly diploptene (~95%) over BHPs but this ratio was reversed under O_2-
431 limitation (~94% BHPs). Similarly, N. vulgaris cultures supplemented with cobalamin
432 produced more BHPs relative to diploptene, as well as more 2-Me-BHPs, 2-Me-diploptene,
433 and BHaminotriol relative to the other BHPs (Fig. 4; Elling et al., 2020). These changes were
434 also observed for N. vulgaris grown mixotrophically, heterotrophically (anaerobic), and when
435 grown under NO_2^-/O_2-limited conditions in chemostats. Under heterotrophic aerobic
436 conditions, N. vulgaris showed lower relative abundance of total BHPs but the highest 2-Me-
437 BHP abundance of any tested condition. Under mixotrophic conditions and autotrophic
438 conditions with added pseudocobalamin, N. vulgaris produced higher amounts of
439 BHaminopentol (15% and 6%, respectively) compared to all other growth conditions (<2%).
440 Finally, autotrophic cultures of N. vulgaris grown without cobalamin and methionine and
441 harvested in mid-growth phase contained >90% adenosylhopane (Fig. 4).

442 3.4 Changes in hopanoid concentrations in nitrite-oxidizing bacteria

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Total hopanoid concentrations were normalized to substrate utilization (mmol NO_2^- oxidized) in lieu of cell counts, which enables comparison across species and between batch and chemostat experiments. This approach circumvents biases caused by varying cell size but also reflects the different energy requirements of the different carbon fixation pathways employed by NOB. Total hopanoid concentrations varied strongly across growth conditions and between species (Fig. 4 & 5). During chemolithoautotrophic growth, the highest concentrations were observed in N. vulgaris and N. marina (27 µg/mmol NO_2^-) and the lowest concentrations were observed in N. moscoviensis (0.01 µg/mmol NO_2^-).

3.5 Carbon isotopic composition of biomass, fatty acids, and hopanoids in nitrite-oxidizing bacteria

The biomass of all four tested, chemolithoautotrophically-grown NOB was depleted in ^{13}C relative to CO_2, with N. vulgaris showing a more pronounced fractionation ($\varepsilon_{CO2\text{-}biomass}$ = 23.2 ± 0.3‰) compared to N. mobilis (8.5 ± 0.3‰), N. gracilis (2.0 ± 0.3‰), and N. marina (1.8 ± 0.1‰; Table 2). Carbon isotopic compositions of fatty acids showed strong ^{13}C-depletion relative to CO_2 in N. mobilis ($\varepsilon_{CO2\text{-}C16FA}$ = 16.6 ± 0.1‰, $\varepsilon_{CO2\text{-}C18FA}$ = 13.8 ± 0.6‰) and N. vulgaris ($\varepsilon_{CO2\text{-}C18FA}$ = 30.1 ± 0.3‰). Fractionation relative to CO_2 was small in N. gracilis ($\varepsilon_{CO2\text{-}C16FA}$ = -0.4 ± 0.3‰) and N. marina ($\varepsilon_{CO2\text{-}C16FA}$ = -3.5 ± 0.4‰). The hopanoid diploptene showed greater carbon isotopic fractionation relative to CO_2 ($\varepsilon_{CO2\text{-}diploptene}$) compared to biomass and fatty acids in N. gracilis (7.4 ± 0.9‰), N. marina (7.1 ± 0.2‰), and N. vulgaris (31.9 ± 0.4‰). The $\varepsilon_{CO2\text{-}diploptene}$ value was similar to those of the fatty acids in N. mobilis (11.7 ± 1.7‰). C_{18} fatty acids were depleted relative to biomass ($\varepsilon_{bio\text{-}C18FA}$) in all strains (Table 2). In contrast, C_{16} fatty acids ($\varepsilon_{bio\text{-}C16FA}$) were depleted relative to biomass in N. mobilis (7.8 ± 0.3‰) but enriched in N. gracilis (-2.4 ± 0.3‰) and N. marina (-5.3 ± 0.4‰).

4. Discussion

4.1 Phenotypic and genotypic characterization of hopanoid production in nitrifying bacteria

To assess the hopanoid biosynthetic capacity of nitrifying bacteria, we surveyed the genomes of ammonia-oxidizing bacteria, comammox, and nitrite-oxidizing bacteria, and found that most cultivated nitrifying bacteria should be able to produce C_{30} hopanoids (e.g., diploptene, diplopterol; encoded by the SHC gene) and side-chain extended hopanoids (BHPs; Fig. 2). Similarly, 29 out of 84 surveyed incomplete environmental genomes and enrichment cultures of Nitrospirae, Nitrospinae, and Nitrobacter spp. possess at least one

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475 hopanoid biosynthetic gene (Supplementary Datafile S1). Among nitrifying bacteria,
476 hopanoid biosynthetic pathways are absent only in the genera Ca. Nitrotoga
477 (Betaproteobacteria) and the Chloroflexi NOB (Nitrolancea, Ca. Nitrotheca patiens, and Ca.
478 Nitrocaldera). Although only two complete genomes for Ca. Nitrotoga and none for
479 Chloroflexi NOB are currently available (Kitzinger et al., 2018; Ishii et al., 2020), hopanoid
480 biosynthesis genes are also absent from five near-complete Ca. Nitrotoga genomes and six
481 near-complete Chloroflexi NOB genomes (Supplementary Datafile S1; Sorokin et al., 2012;
482 Boddicker & Mosier, 2018; Spieck et al., 2020a, 2020b). This suggests that Ca. Nitrotoga
483 and Chloroflexi NOB likely do not produce hopanoids.

484 The occurrence of hopanoid biosynthetic genes may allow prediction of the types of
485 hopanoids produced by nitrifying bacteria (Bradley et al., 2010; Welander et al., 2010, 2012;
486 Welander & Summons, 2012; Liu et al., 2014; Schmerk et al., 2015). Our analyses show
487 good agreement between genomic predictions and observations in culture. All seven NOB
488 species investigated here, as well as the previously studied NOB species Nitrospira defluvii
489 (Lücker et al., 2010) and the AOB Nitrosomonas europaea (Seemann et al., 1999) contained
490 the SHC gene and produced at least C_{30} hopanoids (Fig. 2). Similarly, hpnG and hpnH gene
491 homologs were present in most SHC-positive nitrifier genomes, and their products
492 adenosylhopane and ribosylhopane/ribonylhopane also were detected in all seven tested NOB
493 strains (Fig. 4 & 5, Supplementary Datafile S2). However, ribosylhopane, ribonylhopane, and
494 adenosylhopane were present only in small amounts in the seven NOB (with exception of N.
495 defluvii). In contrast, adenosylhopane was abundant in N. vulgaris in mid-growth phase
496 (>90%) and early stationary phase (up to 40%). The low abundance of these BHPs under
497 most conditions, with high abundance during mid-growth phase, likely reflects their role as
498 central intermediates in the biosynthesis of downstream BHPs (Bradley et al., 2010;
499 Welander et al., 2012; Bodlenner et al., 2015). Consequently, downstream BHPs such as
500 BHT and BHaminotriol were the most abundant BHPs in the studied NOB strains.

501 While the enzyme responsible for BHT formation remains to be identified, biosynthesis
502 of BHaminotriol is known to be facilitated by the aminotransferase HpnO using
503 ribonylhopane or ribosylhopane as the precursor (Welander et al., 2012). The hpnO gene is
504 found in NOB of the genera Nitrobacter and Nitrococcus as well as many AOB. Exceptions
505 are gammaproteobacterial AOB (Nitrosococcus spp., Ca. Nitrosoglobus terrae, Ca.
506 Nitrosacidococcus tergens), and several Nitrosovibrio and Nitrosomonas species.
507 BHaminotriol was observed previously in the AOB Nitrosomonas europaea (Seemann et al.,

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1999), which contains the hpnO gene. Surprisingly, we did not find hpnO homologs in any of the closed or incomplete Nitrospira genomes or in the ~99% complete genome of N. gracilis, even though the tested species contained abundant BHaminotriol (Fig. 5). We hypothesize that BHaminotriol is produced by a different aminotransferase in these species and that absence of hpnO can thus not be used to infer the absence of BHaminotriol production. Downstream of BHT, the hpnI, hpnJ, and hpnK genes encode biosynthesis of N-acetylglucosaminyl BHT, glucosaminyl BHT, and BHT cyclitol ether (Schmerk et al., 2015). The whole pathway consisting of hpnI, hpnJ, and hpnK is present in only two nitrifying bacteria, the AOB Ca. Nitrosoglobus terrae and Ca. Nitrosacidococcus tergens. None of the respective BHPs were detected in the seven tested NOB. However, we identified a hpnK homologue in the incomplete MAG of the thermophilic Chloroflexi NOB Ca. N. robusta, which lacks all other hopanoid biosynthetic genes. Although this homologue could represent contamination of the MAG, no indication of contamination was found (based on contig length, position on the contig, phylogeny of neighboring genes, tetranucleotide frequency, GC content) suggesting that the rest of the biosynthetic pathway may not have been recovered during sequencing and/or assembly. Based on these results, biosynthesis of acetylglucosaminyl, glucosaminyl, and cyclitol BHTs seems to be rare among nitrifying bacteria. Schmerk et al. (2015) previously demonstrated that these BHPs enhance tolerance to low pH in Burkholderia cenocepacia. Likewise, cyclitol BHTs and their diagenetic derivatives are abundant in modern and ancient acidic peats (Talbot et al., 2016b, 2016a). As the hpnIJK genes occur only in the acid-tolerant AOB Ca. N. terrae (Hayatsu et al., 2017) and Ca. N. tergens (Picone et al., 2020) but not in neutrophilic AOB, it appears likely that the respective BHPs are involved in acclimatization to low pH.

Hopanoids can be further modified by methylation at C-2 and C-3 of the A ring, mediated by the HpnP and HpnR enzymes, respectively (Welander et al., 2010; Welander & Summons, 2012). Among nitrifying bacteria, the hpnP gene is common only in Nitrobacter species and consequently, 2-methylhopanoids were detected in N. vulgaris. The only other nitrifier carrying hpnP is Ca. Nitrospira alkalitolerans, an alkalitolerant freshwater NOB. The hpnP sequence of Ca. N. alkalitolerans falls into a cluster shared only with Nitrospirae and Verrucomicrobia sequences from metagenomes. The hpnR gene is found in both AOB (Nitrosococcus spp.) and NOB (N. mobilis and most Nitrospira species) and trace amounts of 3-methylhopanoids were detected in N. mobilis and N. marina. Absence of hpnR homologs in some Nitrospira spp. could result from incomplete coverage of the draft genome sequences.

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Presence of both hpnR and hpnP in Ca. N. alkalitolerans (Fig. S2) suggests a capacity to produce hopanoids methylated at both C-2 and C-3, which has so far only been reported from the acidobacterium Ca. Koribacter versatilis (Sinninghe Damsté et al., 2017).

While the major hopanoids found in the investigated NOB were congruent with predictions based on genotypes, the cultures also contained a wide range of other minor hopanoids, such as BHaminopentol, bacteriohopanepentol, and methylcarbamate triol, as well as novel BHPs. The biosynthetic pathways for these and other BHPs found in environmental samples remain unknown. Given the abundance of amino-BHPs in the tested NOB cultures, we hypothesize that HpnO and related aminotransferases could promiscuously aminate a range of precursors in addition to ribonylhopane, leading to the formation of a wide range of BHPs with terminal amino groups such as aminotetrol, aminopentol, aminohexol and the novel compounds BHP-743.6 and BHP-771.6, as discussed in the next section.

There seems to be no specific pattern linking nitrification to a specific repertoire of hopanoid biosynthetic genes or to similar abundances of specific hopanoids. This is possibly related to the polyphyly of nitrifiers, as their repertoire of hopanoid biosynthesis genes does not seem to universally follow phylogeny. For example, while all Nitrobacter spp. share the same repertoire of hopanoid biosynthesis genes including the hpnP gene, the hpnR gene is present in some but not all Nitrospira species. Similarly, hopanoid biosynthetic capacity differs between the two studied genera of the phylum Nitrospirae, Nitrospira and Leptospirillum (Fig. 2). Further, each studied Nitrospira species contained a different dominant BHP (Fig. 4 & 5). Therefore, we conclude that hopanoid biosynthetic capacity and its expression are not necessarily governed by either the primary metabolism or phylogeny but could also reflect other factors such as adaptations to specific habitats and environmental conditions, as suggested earlier (e.g., Doughty et al., 2009; Ricci et al., 2014).

4.2 Biosynthesis and physiological significance of novel BHPs in nitrite-oxidizing bacteria

We tentatively identified several novel nitrogen-containing BHPs in nitrite-oxidizing bacteria (Fig. 3). Two of these novel BHPs putatively contained two amino groups (BHP-771.6 and BHP-743.6). BHP-771.6 apparently contains one amino group connected to the C_5 sidechain backbone and another as part of an aminopropyl group bound to the sidechain backbone via an ether bond. The incorporation of aminopropanol is unprecedented for BHPs, but it is a common metabolite in bacteria, known to be involved, for instance, in the biosynthesis of cobalamin (Warren et al., 2002). BHP-743.6 contains one amino group and

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one methylamino group, both directly bound to the backbone of the sidechain. We speculate that these amino groups could be derived from either a novel aminotransferase or via sequential amination by a single aminotransferase, followed by methylation. No additional candidate aminotransferases were detected other than HpnO within the cluster of hopanoid biosynthesis genes in N. vulgaris. This suggests that HpnO could mediate sequential amination to form BHP-743.6 from a ribonylhopane precursor. Other candidates include a large number of aminotransferases outside the hopanoid biosynthetic gene cluster of N. vulgaris. The scattered distribution of hopanoid biosynthetic genes in the other NOB complicates the identification of candidate genes. For BHP-656.5, the structural similarity to BHaminotriol suggests that it could be derived from this compound through an unknown cyclase.

The occurrence of two presumably nitrated BHPs (BHP-762.5 and BHP-748.5; Fig. 3) is unprecedented, and their biosynthetic origin is elusive. Comparison with fatty acids offers clues regarding the origin and function of nitrated BHPs. Nitro-fatty acids are common in eukaryotes, where they are involved in oxidative stress response, post-translational modification of proteins, and cell signaling (Schopfer & Khoo, 2019). Nitrated fatty acids are thought to be formed through reaction of unsaturated fatty acids with reactive nitrogen species such as $\cdot NO$, $\cdot NO_2$, and $ONOO^-$ (Schopfer & Khoo, 2019). Analogously, reactive nitrogen species formed during nitrite oxidation or assimilatory nitrate reduction could react with unsaturated BHPs to form nitrated BHPs. Further research is needed to determine whether nitrated BHPs are inadvertent byproducts of oxidative stress and/or whether they serve physiological functions.

4.3 Factors influencing hopanoid distributions in nitrite-oxidizing bacteria

Due to the role of hopanoids in membrane organization and fluidity, bacteria adapt their hopanoid composition in response to growth phases and environmental stresses (e.g., Welander et al., 2009, 2012; Sáenz et al., 2012; Wu et al., 2015; Bradley et al., 2017). Here, we specifically explored the effects of metabolism (autotrophy, mixotrophy, heterotrophy, NO_2^- and O_2 limitation, light) and the co-factors cobalamin (vitamin B_{12}) and methionine on the production of hopanoids. Cobalamin and methionine are presumably involved in several steps of hopanoid biosynthesis. Methionine and cobalamin are cofactors for the methylases HpnP and HpnR (Welander et al., 2010; Welander & Summons, 2012), and methionine is a predicted co-factor for the biosynthesis of BHT cyclitol ether, which is mediated by HpnJ

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(Welander et al., 2012). Because the tested NOB do not contain hpnJ gene homologs, we expected no production of cyclitol ethers. However, four of the seven tested NOB cannot synthesize cobalamin and of these non-producers, only two can synthesize methionine in the absence of cobalamin (Table S5). Therefore, we hypothesized that addition of cobalamin could influence abundances of methylhopanoids.

The hpnP-encoding strain N. vulgaris contained only trace amounts of 2-methylhopanoids (predominantly 2-methyl diploptene) when grown autotrophically on a defined mineral medium without cobalamin (Fig. 4; Elling et al., 2020). Addition of cobalamin to the mineral medium or growth on complex medium (containing yeast extract as a cobalamin source) resulted in up to 32% of 2-methylhopanoids relative to total hopanoids. Further modification of growth conditions of cobalamin-replete cultures, such as heterotrophic growth or nitrite- or oxygen-limitation, resulted in comparatively small changes in 2-methylhopanoid abundance. These results imply that 2-methylhopanoid abundance in N. vulgaris is primarily dependent on "true" cobalamin availability and only secondarily dependent on the tested growth conditions. Supplementation with pseudocobalamin—the form of vitamin B_{12} produced by cyanobacteria and likely used as co-factor by their HpnP—only slightly stimulated 2-methylhopanoid production in N. vulgaris. We suggest that this lack of 2-methylhopanoid production is caused by co-factor specificity of HpnP, potentially resulting from substitutions in the cobalamin-binding domains of alphaproteobacterial versus cyanobacterial HpnP (Fig. S3). Specifically, this result further supports the notion that ammonia-oxidizing archaea are the most likely source of cobalamin to NOB, rather than cyanobacteria (Doxey et al., 2015; Heal et al., 2017; Elling et al., 2020).

In the hpnR-encoding NOB, N. mobilis and N. marina, 3-methylhopanoids were found only in trace amounts when grown on a natural seawater medium that contains only trace levels of cobalamin. Addition of cobalamin, pseudocobalamin (only tested for N. mobilis), or methionine, or mixotrophic growth on complex organics (which supply methionine and cobalamin) did not stimulate production of 3-methylhopanoids (Fig. 5). Similarly, 3-methylhopanoids were not detected in the cobalamin-prototroph N. moscoviensis. Previously, Welander et al. (2012) suggested that starvation could trigger production of 3-methylhopanoids, based on accumulation of 3-methylhopanoids and increased survival during late stationary phase in wild-type versus hpnR deletion mutants of Methylococcus capsulatus. Still, cultures of N. marina and N. mobilis harvested in late stationary phase two weeks after nitrite consumption did not contain increased quantities of 3-methylhopanoids.

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Thus, 3-methylhopanoid production in NOB is dependent on factors that remain to be identified.

Variations in the composition and concentration of other BHPs in response to growth conditions seem to be species-specific. For instance, N. gracilis produced almost exclusively BHPs (94% of total hopanoids, i.e., BHPs plus diploptene and 2-methyl diploptene) when grown under oxygen-limited conditions compared to nitrite-limited growth (5% BHPs), but this was not observed in N. mobilis or N. vulgaris (Fig. 4 & 5). Higher abundance of BHPs under oxygen-limited conditions in N. gracilis could be related to the upregulation of HpnH (mediating the first step in BHP biosynthesis from diploptene), which was observed during oxygen-limited growth of N. marina (Bayer et al., 2021). It has been suggested that BHPs and diploptene have contrasting effects on membrane physiology, with BHPs integrating into the membrane perpendicular to the membrane surface, resulting in enhanced membrane lipid ordering, and diploptene localizing to the hydrophobic midplane of the lipid bilayer and reducing membrane permeability (Poger & Mark, 2013; Caron et al., 2014; Mangiarotti et al., 2019). This hopanoid-mediated regulation of membrane physiology appears to be less important under oxygen-limited conditions. Total hopanoid concentrations were lowest under oxygen-limited conditions for both N. mobilis and N. gracilis; similar changes were not observed in N. vulgaris. A causal link between hopanoid abundance and microaerobic conditions may be a higher efficiency of carbon fixation under oxygen-limitation, a mechanism that may also extend to other NOB such as Nitrospira spp. The carbon fixation pathways in N. gracilis and N. mobilis–the rTCA and the CBB cycle, respectively–contain oxygen-sensitive enzymes (Erb, 2011). A significantly higher cell yield ($p < 0.05$, two-tailed t-test) was observed for oxygen-limited growth over nitrite-limited growth in N. gracilis and N. mobilis (Table S3). Hopanoids, and in particular diploptene, could thus serve as modulators of membrane O_2 permeability in N. mobilis and N. gracilis, as previously suggested for nitrogen-fixing bacteria (Berry et al., 1993). A role of hopanoids in controlling oxygen permeability is a plausible explanation for both higher relative abundance of BHPs over diploptene in N. gracilis and lower total hopanoid concentrations under oxygen-limited conditions in N. gracilis and N. mobilis. Differences in BHP over diploptene abundance in N. gracilis and N. mobilis as well as lack of a similar response in hopanoid composition in N. vulgaris (cell yield was not determined), which also uses the CBB cycle, suggests distinct regulation mechanisms across NOB clades. Still, our results imply that changes in

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biogeochemical parameters such as oxygenation could significantly alter the hopanoid signal in environmental samples and the geological record.

4.4 Hopanoids as biomarkers for nitrifying bacteria and implications for past environments

The sources of hopanoids in modern and past environments remain inadequately characterized, especially for the marine realm. Our results confirm previous suggestions that Nitrospinae and Nitrospirae may contribute to hopanoid production in the ocean (Kharbush et al., 2013, 2018; Mueller et al., 2020) and expand the range of producers to include most terrestrial and marine nitrifying bacteria: Nitrobacter and Nitrococcus spp., all known AOB, and comammox bacteria. Because concentrations of total hopanoids varied up to 10-fold between growth conditions and several orders of magnitude between species, we suggest that both community composition and growth conditions could modulate the relative contribution of NOB to the geological record of hopanoids. The major hopanoids of nitrifying bacteria are diplopterol/diploptene, adenosylhopane, BHaminotriol, and BHT. However, these hopanoids are non-specific as they are produced by a wide range of bacteria and thus are ubiquitous in the ocean across the oxic-anoxic continuum, in marine sediments, freshwater, and soils (Wakeham et al., 2007, 2012; Zhu et al., 2011; Berndmeyer et al., 2013; Blumenberg et al., 2013). Novel hopanoids such as BHP-743.6 (tentatively a methylamino aminotriol BHP) could potentially serve as biomarkers specific for NOB. However, further studies are needed to confirm its specificity and diagenetic stability. Anoxic conditions, such as during oceanic anoxic events or in peats, and low maturity of organic matter could help preserve BHP-743.6 as previously shown for other BHPs (van Dongen et al., 2006; Talbot et al., 2016a). Partial loss of labile functional groups such as nitro-groups may result in loss of specificity, but the putative C-N-C bond could help preserve the signature functional group of BHP-743.6 even if the molecule were to be partially degraded.

Along with common hopanoids, some nitrifying bacteria may contribute to the production of widely used biomarkers, such as BHaminopentol. In soil and freshwater environments, BHaminopentol has commonly been interpreted as a biomarker for aerobic methanotrophic bacteria (Talbot et al., 2014; Rush et al., 2016). Production of this compound by N. vulgaris and Nitrospira defluvii suggests nitrifier sources in these environments in addition to methanotrophic bacteria. Although Nitrobacter and Nitrospira spp. are unlikely sources of highly ^{13}C-depleted hopanoids attributed to methanotrophs (Collister et al., 1992; <-50‰; Thiel et al., 2003; Birgel et al., 2006), they may have contributed to deposition of

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many records that contain less ^{13}C-depleted hopanoids (~ -30 to -40‰; e.g., Talbot et al., 2014) previously attributed primarily to methanotrophs. It is possible that additional nitrifying bacteria not examined here may also produce BHaminopentol. Since N. vulgaris produced high quantities of BHaminopentol only under one growth condition (mixotrophy), further investigation is needed to constrain the impact on the applicability of BHaminopentol as a biomarker for methanotrophs.

The 2- and 3-methylhopanoids found in the marine geologic record were previously thought to indicate presence of planktonic nitrogen-fixing cyanobacteria and methanotrophic bacteria, respectively (Summons et al., 1999; Kuypers et al., 2004; Cao et al., 2009; Kasprak et al., 2015), although other sources of these compounds have been recognized (Summons et al., 1999; Rashby et al., 2007; Welander et al., 2010; Welander & Summons, 2012). Based on our culture experiments and bioinformatic analyses, we propose nitrifying bacteria as additional sources of these compounds. Because nitrifying bacteria depend on remineralized nitrogen for energy, the abundance of nitrifying bacteria and their imprint on the sedimentary record of methylhopanoids would have depended on (i) overall productivity (ii) on the relative flux of nitrogen through nitrification versus anaerobic nitrogen loss processes, and (iii) specific environmental conditions or physiological stresses promoting methylhopanoid production. On geologic timescales, these processes are controlled by nutrient cycles (Fe, P) and ocean oxygenation (Van Cappellen & Ingall, 1996; Falkowski, 1998; Tyrrell, 1999). In the geologic record, widespread ocean deoxygenation, for example during Cretaceous Oceanic Anoxic Event 2, was associated with enhanced nitrification rates and deposition of 2- and 3-methylhopanoids (e.g., Kuypers et al., 2004; Naafs et al., 2019). Based on our culture experiments, we hypothesize that the proliferation of nitrifying bacteria due to intensified nitrification may have contributed to methylhopanoid deposition during these events. Specifically, marine AOB such as Nitrosococcus and NOB such as Nitrococcus and Nitrospira could have contributed to 3-methylhopanoid deposition, although the specific conditions triggering production of 3-methyl hopanoids in these bacteria and thus their imprint on sedimentary records remain to be resolved. Similarly, marine Nitrobacter ecotypes may have contributed 2-methylhopanoids to the geological record of these events as suggested previously (Elling et al., 2020). Furthermore, detection of a HpnP sequence among Nitrospira spp. suggests that they are potential additional sources of 2-methylhopanoids. Remarkably, this and related sequences are basally related to HpnP sequences from all known 2-methylhopanoid producers (Fig. S2), suggesting a different phylogenetic origin of HpnP

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than previously acknowledged (i.e., outside alphaproteobacteria and cyanobacteria; Ricci et al., 2014, 2015) and the existence of further undiscovered clades of 2-methylhopanoid producers (Fig. S2). Importantly, contributions of nitrifying bacteria to methylhopanoid deposition do not rule out contributions from cyanobacteria and methanotrophs. However, since 2-methylhopanoid biosynthesis is generally absent from contemporary marine cyanobacteria (Ricci et al., 2014, 2015; Elling et al., 2020), it seems plausible that 2-methylhopanoid deposition during ocean anoxic events could reflect the proliferation of marine Nitrobacter ecotypes. Although cyanobacterial contributions to the 2-methylhopanoid signal cannot be excluded, we urge caution when interpreting 2-methylhopanoid deposition as evidence for proliferation of nitrogen-fixing cyanobacteria.

An imprint of nitrifying bacteria on the marine sedimentary record of hopanoids requires efficient export of these small cells from the lower euphotic zone. The export mechanisms of nitrifier biomass in the ocean remain uncharacterized but export efficiency may be appreciably lower than that of surface phytoplankton due to small cell size and consequently lower grazing efficiency and lack of passive sinking (Boenigk et al., 2004; Close et al., 2013). Nevertheless, several lines of evidence suggest that the export efficiency of small cells may be higher than canonically recognized (Richardson & Jackson, 2007; Close et al., 2013; Lengger et al., 2019). This is supported by the observation of archaeal biomarker accumulation during anoxic events in the Plio-Pleistocene Mediterranean Sea (Menzel et al., 2006; Polik et al., 2018) and during Cretaceous oceanic anoxic event 1b in the Atlantic Ocean (Kuypers, 2001; Kuypers et al., 2002). Based on their shared depth habitat and similar or larger cell size (Koops et al., 2006; Mincer et al., 2007; Santoro et al., 2010; Spieck & Lipski, 2011), it appears likely that biomass of other nitrifying bacteria such as NOB and AOB would have been exported with similar efficiency.

Contributions of nitrifying bacteria to hopanoid production may be identified through their distinct carbon isotopic compositions, determined here for chemolithoautotrophically-grown cultures (Table 2). The low carbon isotopic fractionation of lipids and biomass relative to dissolved inorganic carbon found in N. gracilis and N. marina is consistent with the operation of the reverse tricarboxylic acid (rTCA) cycle for carbon fixation (Preuß et al., 1989; Berg et al., 2010), as previously shown for Nitrospira defluvii (Lücker et al., 2010). Enrichment in ^{13}C in C_{16} fatty acids relative to biomass and diploptene in N. gracilis and N. marina reflects fractionation during acetyl-CoA generation in the rTCA cycle (Sirevåg et al., 1977; van der Meer et al., 1998; Williams et al., 2006). The higher fractionation observed in

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N. vulgaris is indicative of the Calvin-Benson-Bassham (CBB) cycle for carbon fixation (Sirevåg et al., 1977; Berg et al., 2010). Similarly, all known AOB use the CBB cycle (Supplementary Datafile S1; Sakata et al., 2008; Koops & Pommerening-Röser, 2015). Through mass balance, it may be possible to distinguish or quantify contributions of Nitrospina and Nitrospira spp. from those of autotrophs using the CBB cycle such as Nitrobacter spp., cyanobacteria, and AOB ($\varepsilon_{CO2\text{-}biomass}$ = 20-30‰; Quandt et al., 1977; McNevin et al., 2007). Given that Nitrospina and Nitrospira spp. are the dominant NOB in most of the modern ocean (e.g., Mincer et al., 2007; Santoro et al., 2010; Pachiadaki et al., 2017), a significant contribution to hopanoid deposition should be detectable through the carbon isotopic composition of hopanoids in suspended particulate matter and surface sediments. To similarly recognize this signature in geological samples, knowledge of the dissolved inorganic carbon isotopic composition, ideally at the habitat depth of NOB, would be needed. Dissolved inorganic carbon isotopic composition could be constrained through the carbon isotopic composition of lipids from ammonia-oxidizing archaea living in the same ecological niche and depth habitat (Elling et al., 2021).

Peculiar fractionation systematics indicate that N. mobilis uses carbon fixation pathways distinct from the other tested NOB. It has previously been suggested based on genomic data that N. mobilis uses the CBB cycle for carbon fixation (Lücker et al., 2010; Füssel et al., 2017). Yet, the ^{13}C-fractionation into biomass and lipids is much smaller than expected for the CBB cycle. It remains to be tested whether this fractionation pattern results from specific growth conditions or novel mechanisms of carbon fixation in N. mobilis.

5. Conclusions

The detection of hopanoids in nitrifying bacteria expands the known diversity of hopanoid producers and further ties hopanoid production to the nitrogen cycle. Specifically, potential for production of 2- and 3-methylhopanoids by NOB and AOB suggests that source assignments of these biomarkers in geological records and modern environments need to be broadened, although the specific conditions under which production of methylhopanoids is stimulated in NOB (and by extension, AOB) remain unclear. Due to the ubiquity of nitrifying bacteria in terrestrial, freshwater, brackish, and marine environments, it appears likely that they contributed to the geologic record of hopanoids, especially in times of intensified marine nitrogen cycling such as during oceanic anoxic events. The novel nitrogen-containing BHPs tentatively identified here could potentially serve as biomarkers for nitrite-oxidizing bacteria. Their physiological role remains elusive but a potential involvement in regulation of cellular

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processes such as oxidative stress response, alternative respiratory pathways, and protein modification warrants further attention. Further experiments on the effect of additional growth conditions (e.g., temperature, pH, salinity) on hopanoid distributions as well as analyses of AOB and currently uncultivated clades of NOB are needed to comprehensively assess the potential of hopanoids as biomarkers for nitrifying bacteria in the geologic past.

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Figure captions

Fig. 1. Pathways and mediating enzymes (blue) of bacteriohopanepolyol biosynthesis and structural modification (after Welander et al., 2010; Bradley et al., 2010; Welander et al., 2012; Liu et al., 2014; Schmerk et al., 2015; Sohlenkamp and Geiger, 2016; Belin et al.,

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2018). The order of methylhopanoid biosynthesis and the enzymes responsible for BHT formation and the pathways leading to other bacteriohopanepolyols (such as aminobacteriohopanetetrol and aminobacteriohopanepentol) are not known. Note that methylation at C-2 (HpnP) or C-3 (HpnR) can occur not only in BHT but also in other hopanoids.

Fig. 2. Distribution of hopanoid biosynthetic genes (grey squares: presence of gene; white squares: absence of gene; see also Table S1) in genomes of selected nitrite-oxidizing bacteria (pink; NOB), ammonia-oxidizing bacteria (cyan; AOB), complete ammonia-oxidizers (purple; COMAMMOX), and closely related, non-nitrifying bacteria. Habitat is indicated by colored circles. For additional data from cultivated nitrifiers and environmental genomes, see Supplementary Datafile S1. Species analyzed for BHP content in this study are highlighted in bold. The tree represents the 16S rRNA gene phylogeny of nitrifiers and closely related organisms, with phyla and proteobacterial classes (α, β, γ, δ) indicated along the branches. Circles indicate branches with >90% support based on 500 bootstrap analyses. The scale bar represents 0.1 substitutions per nucleotide.

Fig. 3. (A) Composite extracted ion chromatograms of novel BHPs (m/z $[M+H]^+$ 638.5, 656.6, 748.5, 743.6, 762.5, 771.6) and previously characterized BHPs (aminotriol, aminopentol, bacteriohopanetetrol) from Nitrobacter vulgaris and Nitrococcus mobilis (factor indicates magnification of small peaks). (**B-G**) MS^2 fragmentation spectra and tentative structural identification of novel BHPs. Positions of functional groups along the extended hopanoid backbone are speculative. Note that consecutive losses of -42 (CH_2CO), and -18 (H_2O) can be generated through cleavage of any acetylated hydroxyl group but could also be generated through cleavage of acetylated nitro groups. Accurate masses and proposed sum formulas of major fragment ions are shown in Table S4. Interpretation of fragmentation patterns is provided in the supplementary information.

Fig. 4. Relative abundance of hopanoids (averages of triplicate cultures, except for single cultures for pseudocobalamin) in four freshwater/wastewater species of nitrite-oxidizing bacteria under varying growth conditions: autotrophic (NO_2^- as electron donor; MG: mid-growth phase, ES: early stationary phase), autotrophic + methionine (ES), autotrophic + cobalamin (ES), autotrophic + cobalamin + methionine + 6h/18h light/dark cycles (ES), autotrophic + pseudocobalamin, mixotrophic (NO_2^- + complex organics; ES),

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heterotrophic aerobic (complex organics but no NO_2^-; ES), heterotrophic anaerobic (complex organics but N_2 headspace; ES), NO_2^--limited chemostat (growth rate 0.013 h^{-1}), O_2-limited chemostat (0.013 h^{-1}). Relative abundances of major bacteriohopanepolyols (BHPs) are shown in panels **A-D** (BHT: bacteriohopanetetrol; BHaminotriol: 35-aminobacteriohopanetriol; BHaminopentol: aminobacteriohopanepentol). Abundances of total BHPs relative to 2-methyl BHPs and the non-functionalized hopanoids diploptene and 2-methyl diploptene are shown in panels **E-H** (data for panel H from Elling et al., 2020). Abundances of total hopanoids (BHPs + 2-methyl BHPs + diploptene + 2-methyl diploptene) are shown in panels **I-L** (vertical line: average; n.a., not available). Distribution of minor BHPs (< 2% relative abundance) shown in Table S2.

Fig. 5. Relative abundance of hopanoids (averages of triplicate cultures, except for duplicate cultures for N. mobilis and N. gracilis chemostat experiments, single culture for pseudocobalamin) in three marine species of nitrite-oxidizing bacteria under varying growth conditions: autotrophic (NO_2^- as electron donor; ES: early stationary phase, LS: late stationary phase), autotrophic + methionine (ES), autotrophic + cobalamin (ES), mixotrophic (NO_2^- + complex organics; ES), autotrophic + pseudocobalamin, NO_2^--limited chemostat (growth rate 0.011 h^{-1}), O_2-limited chemostat (0.011 h^{-1}). Relative abundances of major bacteriohopanepolyols (BHPs) are shown in panels **A-C** (BHT: bacteriohopanetetrol; BHaminotriol: 35-aminobacteriohopanetriol). Abundances of total BHPs relative to the non-functionalized hopanoids diploptene and 2-methyl diploptene are shown in panels **D-F**. Abundances of total hopanoids (BHPs + diploptene) are shown in panels **G-I** (vertical line: average). Distribution of minor BHPs (< 2% relative abundance) shown in Table S2. Please note that 2-methyl derivatives of BHT, BHPs, and diploptene were not detected in any of the strains shown.

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Table 1. Growth conditions of cultures used in this study (medium type: AFW, artificial freshwater; ASW, artificial seawater; NSW, natural seawater).

	Medium type	Autotrophic, mid growth	Autotrophic, early stationary	Autotrophic, late stationary	Autotrophic + methionine	Autotrophic + cobalamin	Autotrophic + methionine + cobalamin + light	Mixotrophic	Heterotrophic aerobic	Heterotrophic anaerobic	Nitrite-limited chemostat + methionine + cobalamin	O_2-limited chemostat + methionine + cobalamin
Nitrospina gracilis Nb-3/211	NSW		✓		✓	✓						
Nitrospina gracilis Nb-211	ASW										✓	✓
Nitrospira marina Nb-295	NSW		✓	✓	✓	✓		✓				
Nitrococcus mobilis Nb-231	NSW		✓	✓	✓	✓						
Nitrococcus mobilis Nb-231	ASW										✓	✓
Nitrobacter vulgaris AB1	AFW	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓
Nitrospira defluvii A17	AFW		✓									
Nitrospira lenta BS10	AFW		✓									
Nitrospira moscoviensis M-1	AFW		✓									

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Table 2. Stable carbon isotopic composition ($\delta^{13}C$) and carbon isotopic fractionation (ε) of dissolved inorganic carbon (DIC), CO_2 (calculated from DIC after Mook et al. (1974), at 28 °C), biomass (bio), summed C_{16} fatty acids (FA; Nitrospina gracilis: $C_{16\omega9}$; Nitrospira marina: $C_{16:0}$+$C_{16:1\omega5}$+$C_{16:1\omega9}$; Nitrococcus mobilis: $C_{16:0}$+$C_{16:1\omega9}$), summed C_{18} fatty acids (Nitrococcus mobilis: $C_{18:0}$+$C_{18\omega7}$, Nitrobacter vulgaris: $C_{18:0}$+$C_{18\omega1}$), and the hopanoid diploptene in four species chemolithoautotrophically grown nitrite-oxidizing bacteria. Data for N. vulgaris are from Elling et al. (2020).

		$\delta^{13}C_{DIC}$ (‰)	$\delta^{13}C_{CO2}$ (‰)	$\delta^{13}C_{bio}$ (‰)	$\delta^{13}C_{C16FA}$ (‰)	$\delta^{13}C_{C18FA}$ (‰)	$\delta^{13}C_{diplop.}$ (‰)
$\delta^{13}C$	N. gracilis	-0.5 ± 0.2	-8.9 ± 0.2	-10.9 ± 0.2	-8.5 ± 0.2	-	-16.1 ± 0.9
	N. marina	2.1 ± 0.1	-6.5 ± 0.1	-8.3 ± 0.1	-3.0 ± 0.4	-	-13.5 ± 0.1
	N. mobilis	1.7 ± 0.1	-6.7 ± 0.1	-15.1 ± 0.3	-22.9 ± 0.1	-20.2 ± 0.6	-18.2 ± 1.7
	N. vulgaris	-5.6 ± 0.2	-14 ± 0.2	-36.4 ± 0.2	-	-42.8 ± 0.2	-44.5 ± 0.3
				$\varepsilon_{CO2\text{-}bio}$ (‰)	**$\varepsilon_{CO2\text{-}C16FA}$ (‰)**	**$\varepsilon_{CO2\text{-}C18FA}$ (‰)**	**$\varepsilon_{CO2\text{-}diplop.}$ (‰)**
ε	N. gracilis			2.0 ± 0.3	-0.4 ± 0.3	-	7.4 ± 0.9
Relative	N. marina			1.8 ± 0.1	-3.5 ± 0.4	-	7.1 ± 0.2
to substrate	N. mobilis			8.5 ± 0.3	16.6 ± 0.1	13.8 ± 0.6	11.7 ± 1.7
	N. vulgaris			23.2 ± 0.3	-	30.1 ± 0.3	31.9 ± 0.4
					$\varepsilon_{bio\text{-}C16FA}$ (‰)	**$\varepsilon_{bio\text{-}C18FA}$ (‰)**	**$\varepsilon_{bio\text{-}diplop.}$ (‰)**
ε	N. gracilis				-2.4 ± 0.3	-	5.2 ± 0.9
Relative	N. marina				-5.3 ± 0.4	-	5.2 ± 0.2
to biomass	N. mobilis				7.8 ± 0.3	5.1 ± 0.7	3.1 ± 1.8

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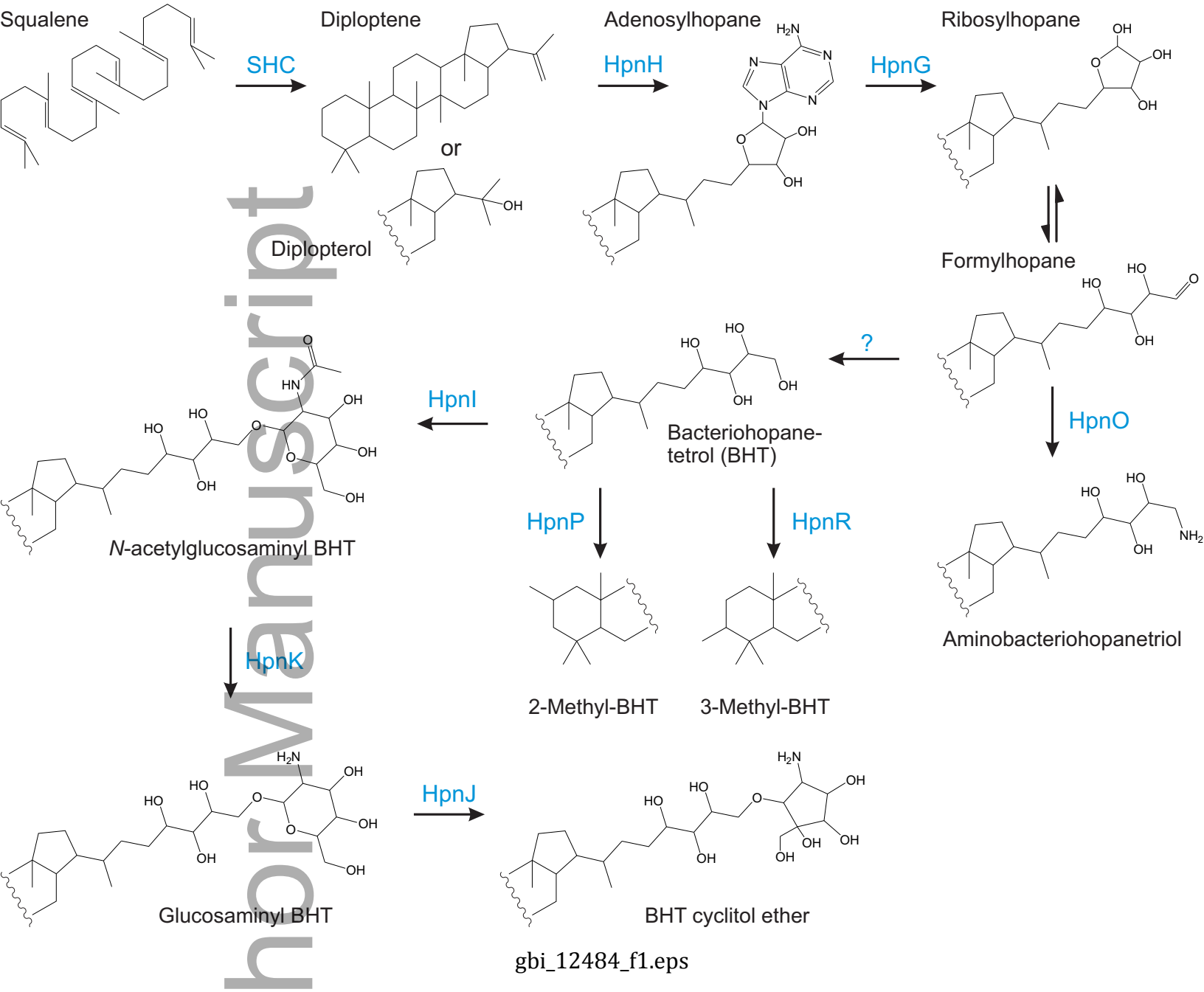

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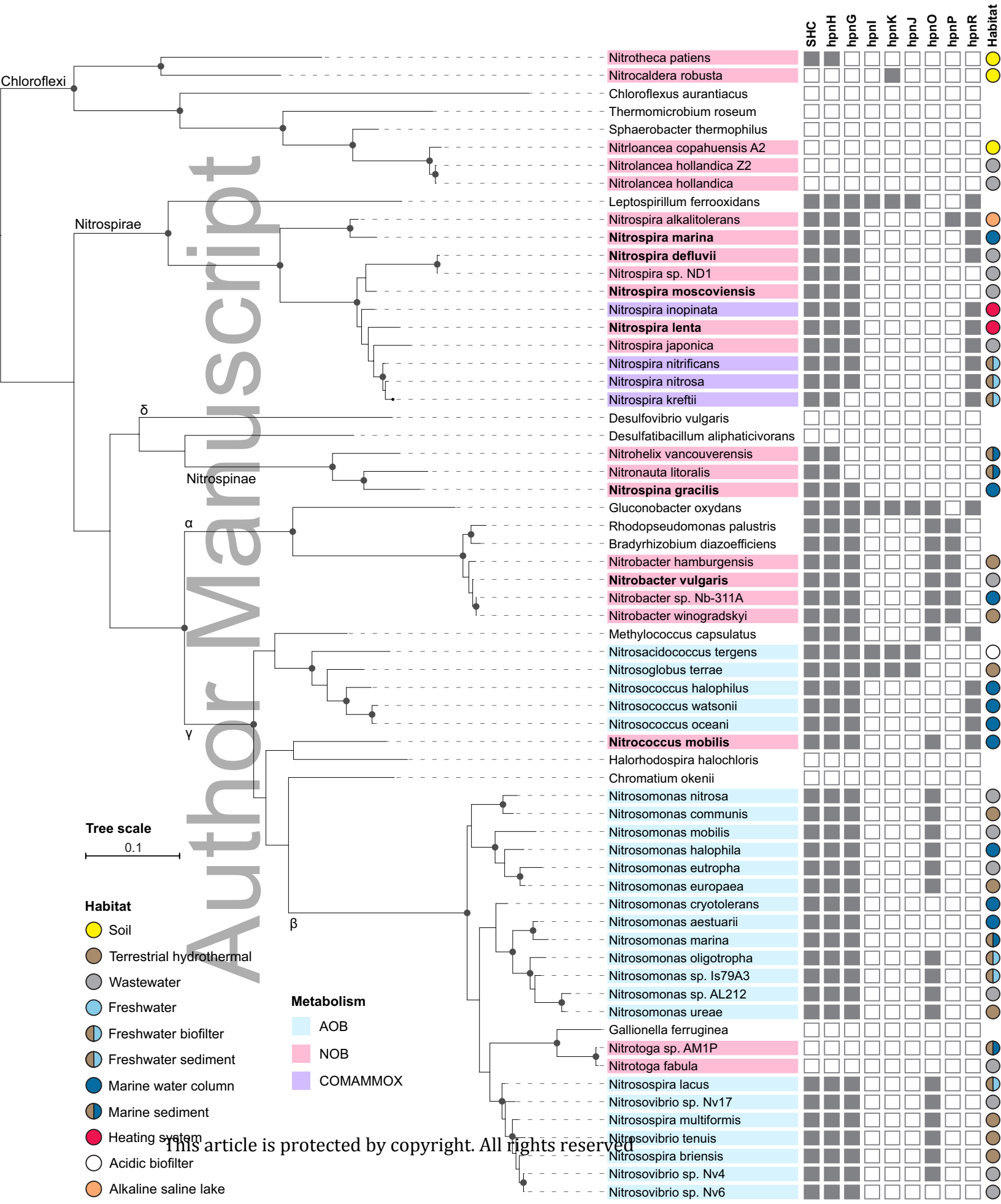

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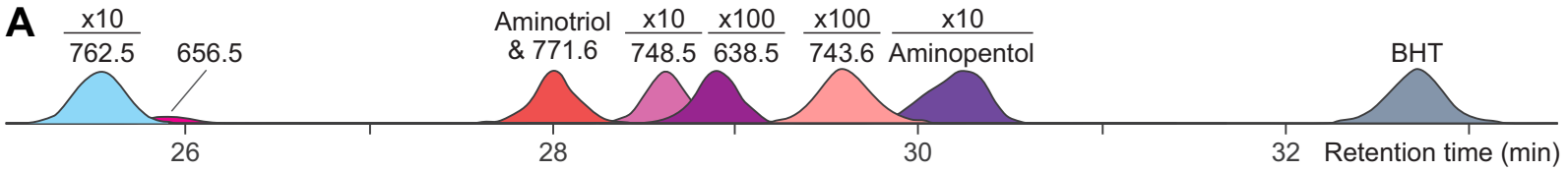

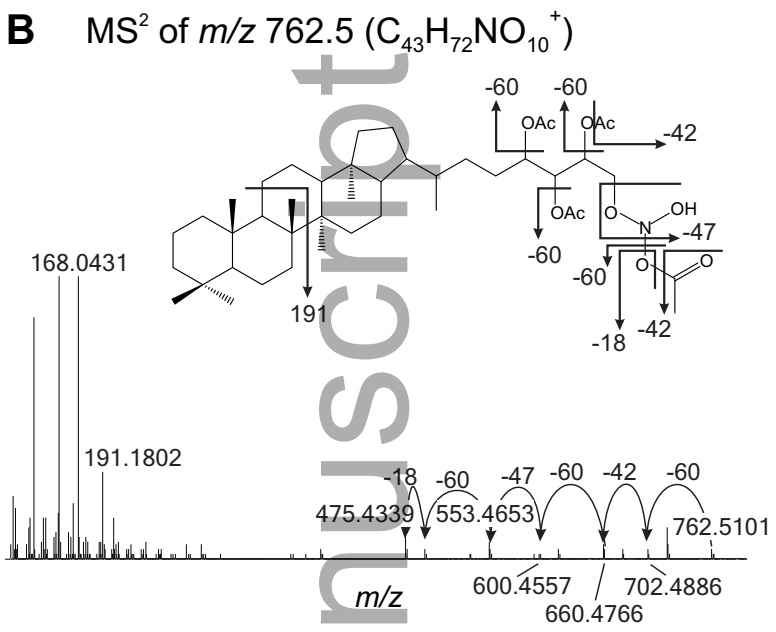

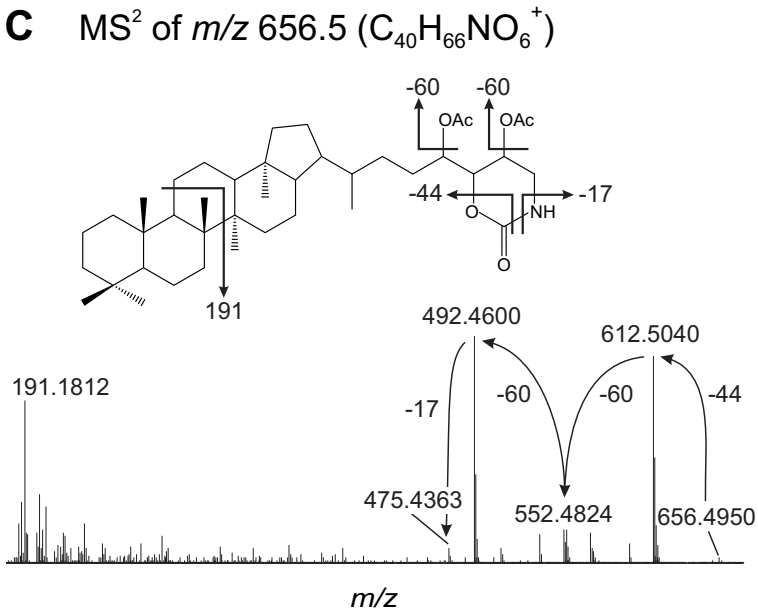

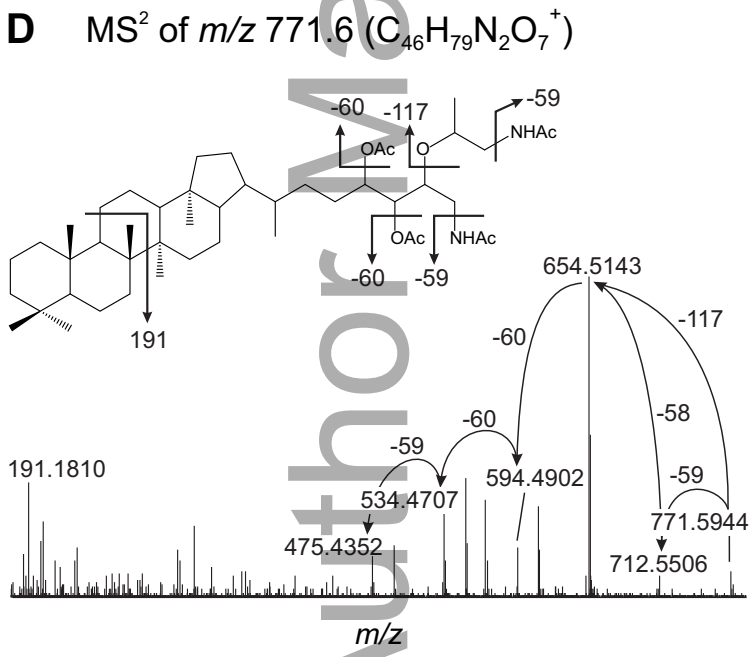

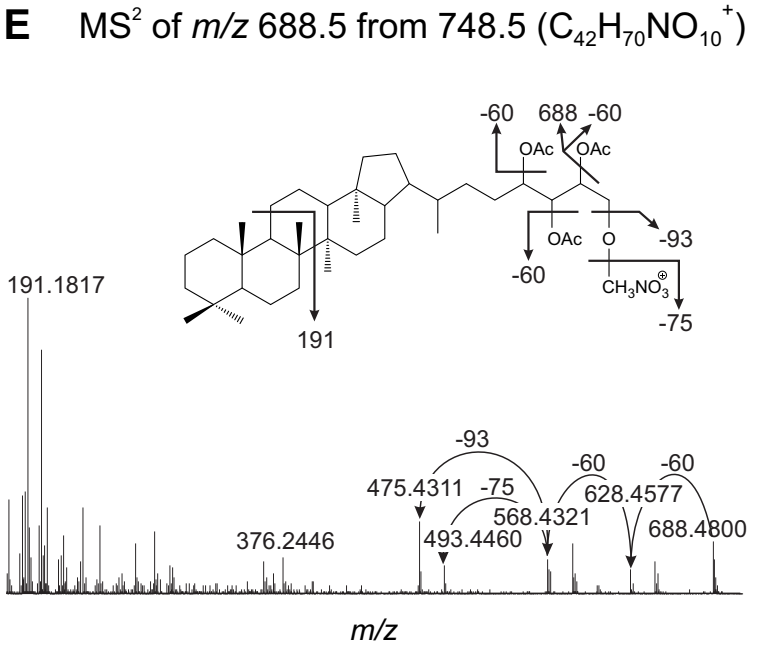

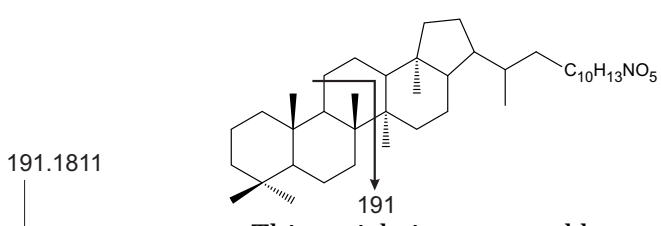

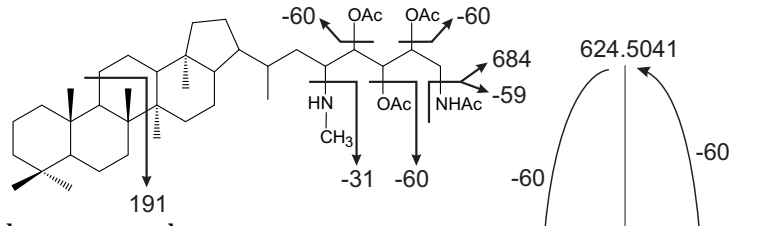

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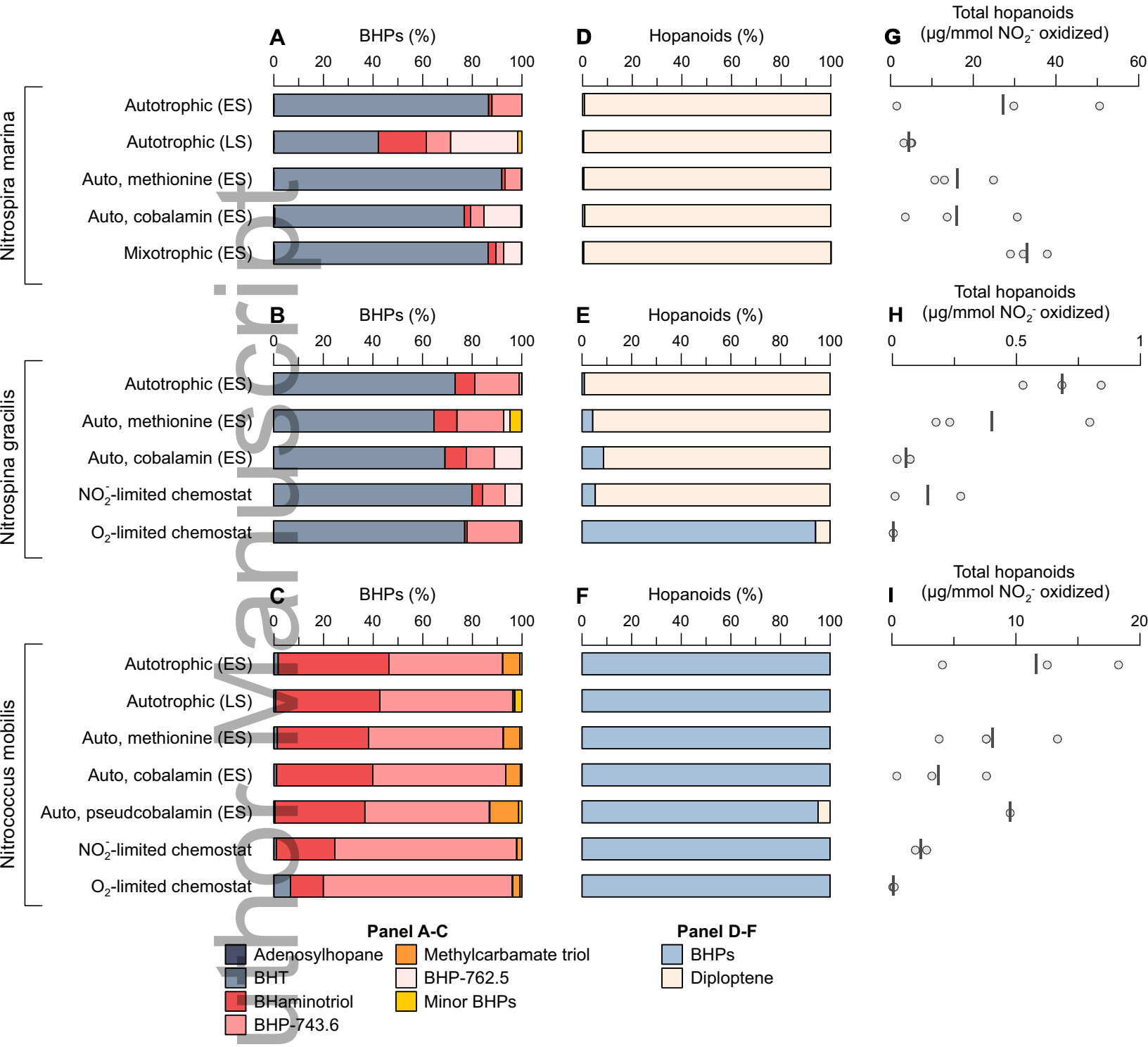

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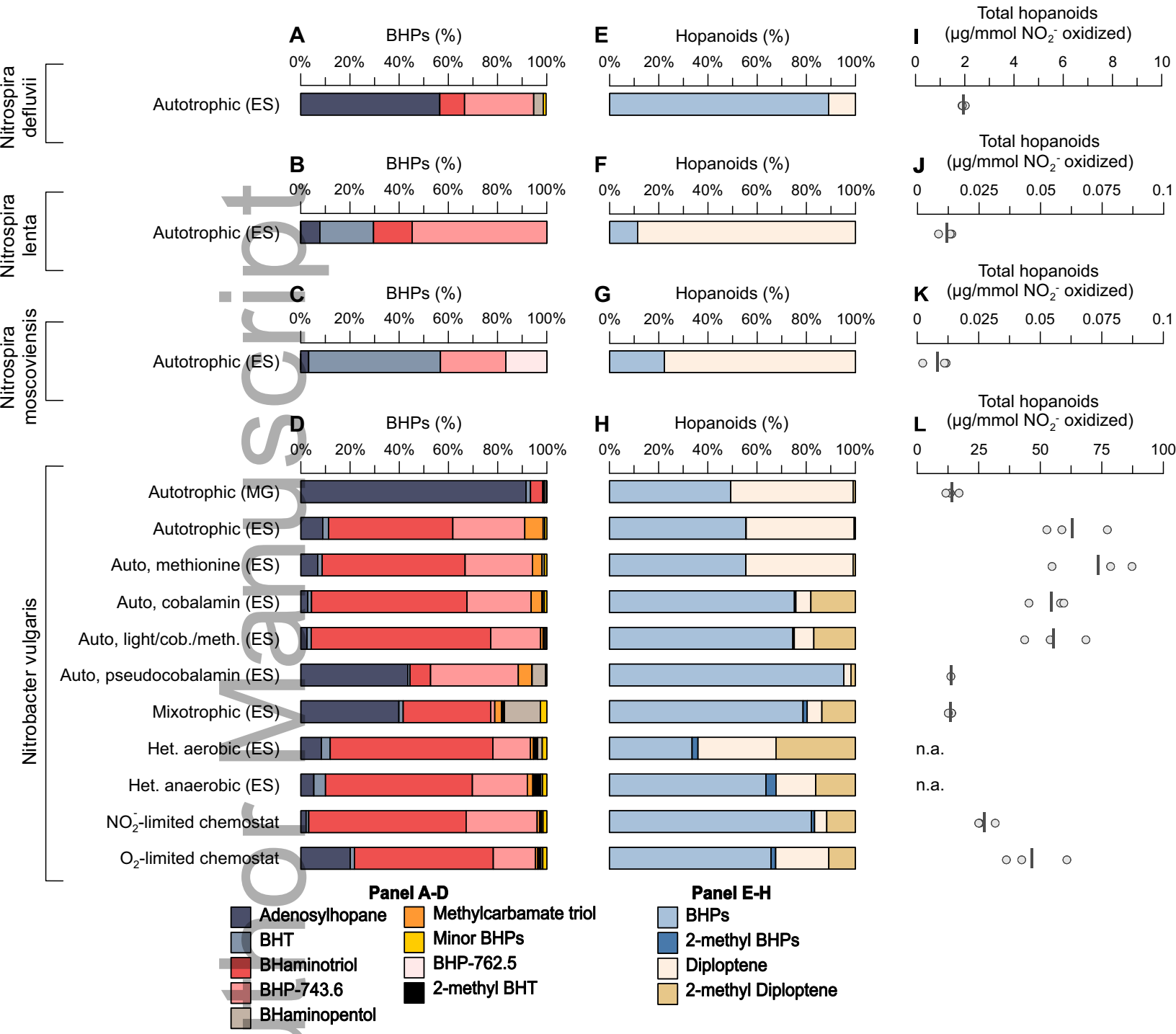

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