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Marine and terrestrial nitrifying bacteria are sources of diverse bacteriohopanepolyols

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Abstract

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

KEYWORDS
bacteriohopanepolyols, biomarker, hopanoids, nitrifying bacteria, nitrite-oxidizing bacteria

1 | INTRODUCTION

Hopanoids are terpenoid lipids produced by aerobic and anaerobic bacteria (Fischer et al., 2005; Rohmer et al., 1984; 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 (Ourisson et al., 1987; Sáenz et al., 2012; Welander et al., 2009). They are commonly found as composite lipids called bacteriohopanepolyols (BHPs), consisting of hopanoid hydrocarbon skeletons bearing functionalized polar side chains (Ourisson & Rohmer, 1992; Rush et al., 2016; Talbot et al., 2007, 2008). Due to their characteristic distribution among bacteria and their structural diversity, both within the hopanoid skeleton and the polar side chain, hopanoids and BHPs are used as biomarkers for diverse clades of bacteria or distinct ecological niches (Ricci et al., 2013; Rush et al., 2016; Sáenz et al., 2011). Due to their diagenetic stability, hopanoids are important biomarkers for elucidating biogeochemical and microbial evolution from the early Proterozoic onwards (Briggs & Summons, 2014; Brocks & Banfield, 2009; Brocks & Pearson, 2005).

The 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 was later expanded considerably 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 (Figure 1; 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 (Figure 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 (Daims et al., 2016; Spieck & Bock, 2005). NOB of the genera *Nitrobacter* (Alphaproteobacteria) and *Candidatus* Nitrotoga (Betaproteobacteria) are found primarily in freshwater, wastewater, and soils (Alawi et al., 2007; Daims et al., 2016; Spieck & Bock, 2005). NOB of the genus *Nitrospira* (phylum Nitrospirae), on the contrary, inhabit both terrestrial and marine environments, including hydrothermal systems (Bayer et al., 2021; Daims et al., 2016; Spieck & Bock, 2005). Moreover, some *Nitrospira* spp. can perform both steps of nitrification (ammonia and nitrite oxidation, "comammox"; Daims et al., 2015; van Kessel et al., 2015), although this capacity appears to 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 (Füssel et al., 2017; Mincer et al., 2007; Pachiadaki et al., 2017; Santoro et al., 2010); however, there also are marine species of *Nitrobacter* (Ward et al., 1989; Ward & Carlucci, 1985). 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, Sass, et al., 2020; Spieck, Spohn, et al., 2020), 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 1) the widespread association of BHP production with the capacity for nitrite oxidation, and 2) the characterization of distinct distributions of these compounds and their carbon isotopic compositions 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 extend 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 | MATERIALS 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 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*

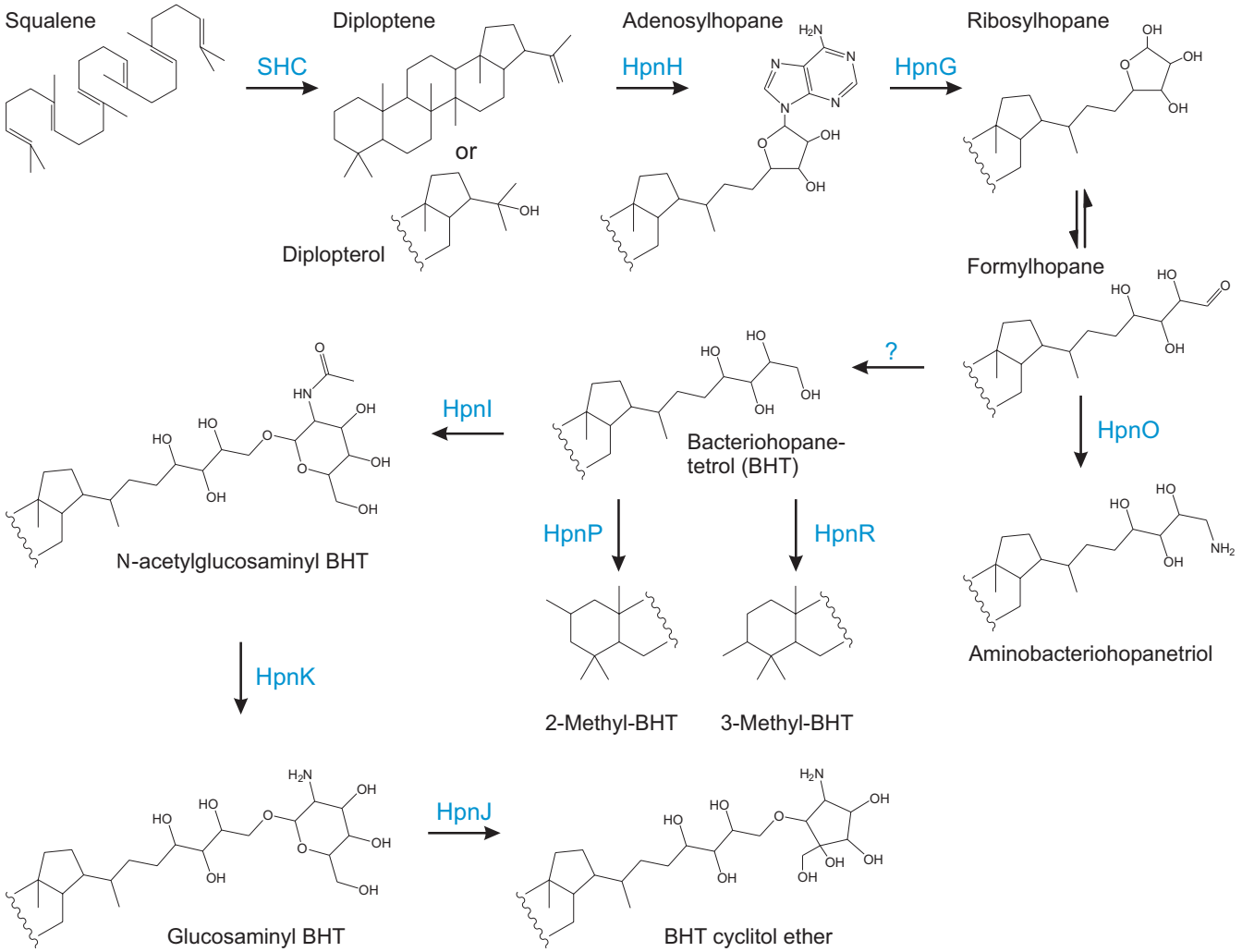


FIGURE 1 Pathways and mediating enzymes (blue) of bacteriohopanepolyol biosynthesis and structural modification (after Belin et al., 2018; Bradley et al., 2010; Liu et al., 2014; Schmerk et al., 2015; Sohlenkamp & Geiger, 2016; Welander et al., 2010, 2012). The order of methylhopanoid biosynthesis and 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

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 (Lücker et al., 2013; Watson & Waterbury, 1971) 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 the 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 spectrophotometric 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, and *N. vulgaris* AB1). However, some experiments were limited to specific strains due to distinct growth requirements (Table 1). Growth was assessed by monitoring nitrite consumption and/or optical density at 600 nm (OD_{600}), as described below. 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.

Nitrobacter vulgaris AB1 was grown in batch cultures under aerobic chemolithoautotrophic (mid-growth phase and early stationary phase), aerobic heterotrophic, anaerobic heterotrophic, and aerobic mixotrophic conditions. To test the influence of methionine and hydroxocobalamin (vitamin B12a), 7 mM $NaNO_2$ was added to the medium, with either 0.5 μM hydroxocobalamin or 0.5 μM methionine. To 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). Aerobic heterotrophic 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}. Anaerobic heterotrophic (nitrate-reducing) cultures were grown in a heterotrophic medium with 1 g L^{-1} $NaNO_3$ in serum bottles sealed gas-tight with 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 the heterotrophic medium described above with 7 mM $NaNO_2$ added. Growth of anaerobic heterotrophic and aerobic mixotrophic cultures was monitored by quantifying nitrite formation/consumption and OD_{600}. All cultures were agitated using an orbital shaker (150 rpm) during incubation.

Nitrospina gracilis, *N. marina* Nb-295, and *N. mobilis* Nb-231 were grown chemolithoautotrophically in triplicate batch cultures in natural seawater medium alone, as well as with either 0.5 μM methionine or 0.5 μM hydroxocobalamin added. 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 (in this case, 2 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 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 1290 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

Nitrobacter 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 Figure 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 *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 and *N. gracilis* Nb-211: 0.011 h^{-1}; *N. vulgaris* AB1: 0.013 h^{-1}; Table S2

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		✓									

and S3). Equilibrium conditions were reached within three to four 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). 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

Bacteriohopanepolyols 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). The mass spectrometer was calibrated before analysis, with a typical error in mass 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 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 two 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 the 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 the 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 Data 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 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. $\delta^{13}C_{CO_2}$ 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

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 of chemolithoautotrophically grown nitrite-oxidizing bacteria. Data for *N. vulgaris* are from Elling et al. (2020)

	$\delta^{13}C_{DIC}$ (‰)	$\delta^{13}C_{CO_2}$ (‰)	$\delta^{13}C_{bio}$ (‰)	$\delta^{13}C_{C16FA}$ (‰)	$\delta^{13}C_{C18FA}$ (‰)	$\delta^{13}C_{diplop.}$ (‰)
$\delta^{13}C$						
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

	$\varepsilon_{CO_2\text{-bio}}$ (‰)	$\varepsilon_{CO_2\text{-C16FA}}$ (‰)	$\varepsilon_{CO_2\text{-C18FA}}$ (‰)	$\varepsilon_{CO_2\text{-diplop.}}$ (‰)
ε relative to substrate				
N. gracilis	2.0 ± 0.3	−0.4 ± 0.3	–	7.4 ± 0.9
N. marina	1.8 ± 0.1	−3.5 ± 0.4	–	7.1 ± 0.2
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 relative to biomass			
N. gracilis	−2.4 ± 0.3	–	5.2 ± 0.9
N. marina	−5.3 ± 0.4	–	5.2 ± 0.2
N. mobilis	7.8 ± 0.3	5.1 ± 0.7	3.1 ± 1.8
N. vulgaris	–	6.4 ± 0.3	8.1 ± 0.4

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‰; and USGS41a: 36.55‰).

Free and intact polar lipid-bound fatty acids in the TLE were converted into 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 ($C_{16:0}$, $C_{19:0}$, and $C_{24:0}$ 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).

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*, and *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

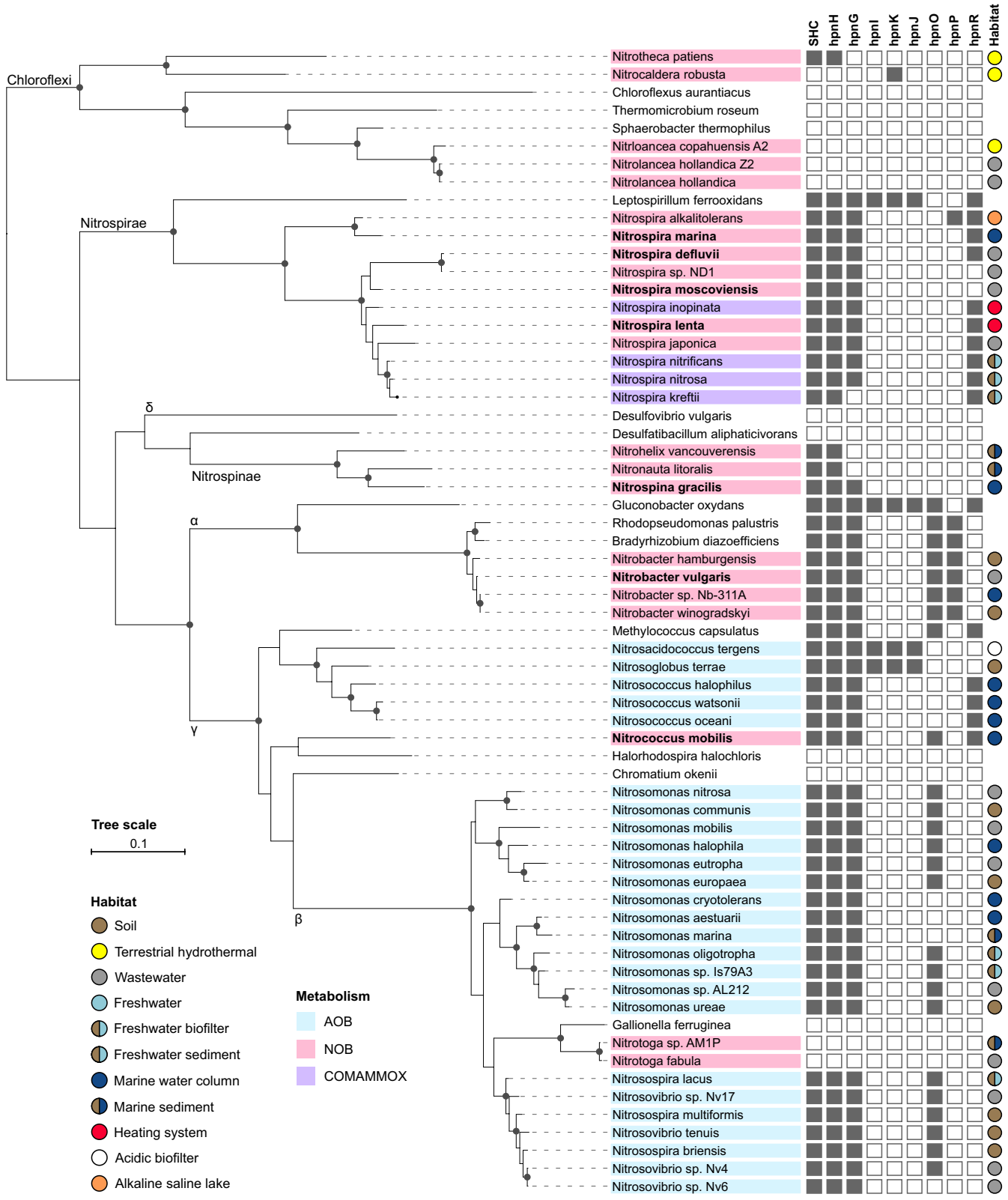


FIGURE 2 Distribution of hopanoid biosynthetic genes (gray squares: presence of gene; white squares: absence of gene; see also Table S1) in genomes of selected nitrite-oxidizing bacteria (pink; NOB), ammonia-oxidizing bacteria (cyan; AOB), complete ammonia oxidizers (purple; COMAMMOX), and closely related, non-nitrifying bacteria. Habitat is indicated by colored circles. For additional data from cultivated nitrifiers and environmental genomes, see Data 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

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 multidomain (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

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 (Figure 2; Data S1). Similarly, all sequenced genomes of cultivated AOB (e.g., *Nitrosomonas europaea* and *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 (Data 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; Belin et al., 2018; Sohlenkamp & Geiger, 2016), 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; Liu et al., 2014; Sato et al., 2020; Welander et al., 2012). 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*, and *Nitrosospira*), but not in the gammaproteobacterial AOB (*Nitrosococcus*, *Ca*. Nitrosoglobus, and *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.

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 (Figure 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* (Figure 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 (Figure 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 (Figures 4 and 5; Data S2).

Although each NOB produced a similar array of BHPs, their relative abundances were variable (Figures 4 and 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%; Figure 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 (Figure 4).

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

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

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

3.4 | Changes in hopanoid concentrations in nitrite-oxidizing bacteria

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 not only 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 (Figures 4 and 5). During chemolithoautotrophic growth, the highest concentrations were observed in *N. vulgaris* and *N. marina* (27 μg $mmol^{-1}$ NO_2^-), and the lowest concentrations were observed in *N. moscoviensis* (0.01 μg $mmol^{-1}$ NO_2^-).

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

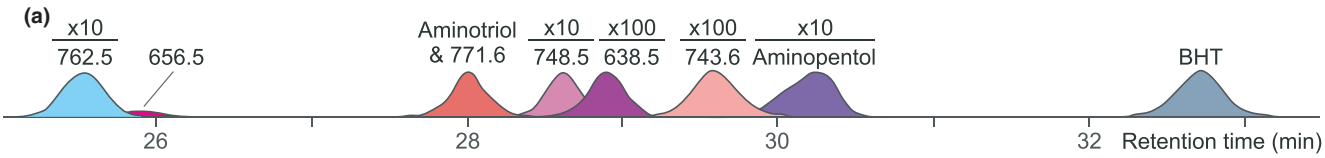
The biomass of all four tested, chemolithoautotrophically grown NOB was depleted in ^{13}C relative to CO_2, with *N. vulgaris* showing a more pronounced fractionation ($\varepsilon_{CO_2\text{-biomass}}$ = 23.2 ± 0.3‰) compared to *N. mobilis* (8.5 ± 0.3‰), *N. gracilis* (2.0 ± 0.3‰), and *N. marina* (1.8 ± 0.1‰; Table 2). Carbon isotopic compositions of fatty acids showed strong ^{13}C depletion relative to CO_2 in *N. mobilis* ($\varepsilon_{CO_2\text{-C16FA}}$ = 16.6 ± 0.1‰ and $\varepsilon_{CO_2\text{-C18FA}}$ = 13.8 ± 0.6‰) and *N. vulgaris* ($\varepsilon_{CO_2\text{-C18FA}}$ = 30.1 ± 0.3‰). Fractionation relative to CO_2 was small in *N. gracilis* ($\varepsilon_{CO_2\text{-C16FA}}$ = −0.4 ± 0.3‰) and *N. marina* ($\varepsilon_{CO_2\text{-C16FA}}$ = −3.5 ± 0.4‰). The hopanoid diploptene showed greater carbon isotopic fractionation relative to CO_2 ($\varepsilon_{CO_2\text{-diploptene}}$) 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 $\varepsilon_{CO_2\text{-diploptene}}$ 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

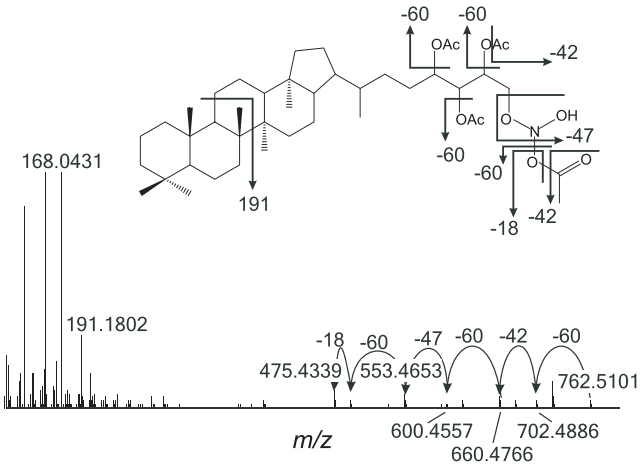
4.1 | Phenotypic and genotypic characterization of hopanoid production in nitrifying bacteria

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

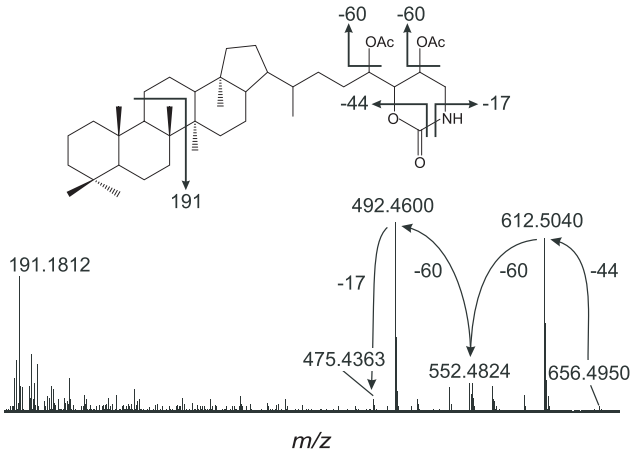
FIGURE 3 (a) Composite extracted ion chromatograms of novel BHPs (*m/z* $[M+H]^+$ 638.5, 656.6, 748.5, 743.6, 762.5, and 771.6) and previously characterized BHPs (aminotriol, aminopentol, and bacteriohopanetetrol) from *Nitrobacter vulgaris* and *Nitrococcus mobilis* (factor indicates magnification of 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 Supporting information



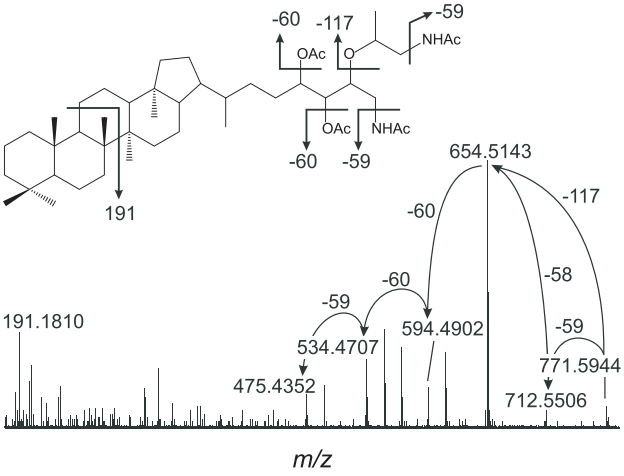
(b) MS^2 of *m/z* 762.5 ($C_{43}H_{72}NO_{10}^+$)



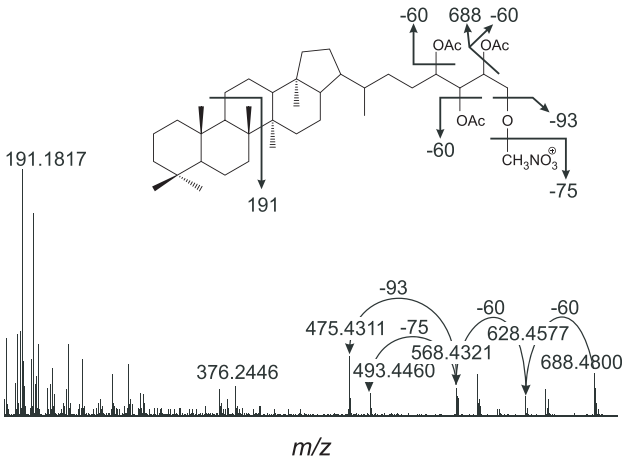
(c) MS^2 of *m/z* 656.5 ($C_{40}H_{66}NO_6^+$)



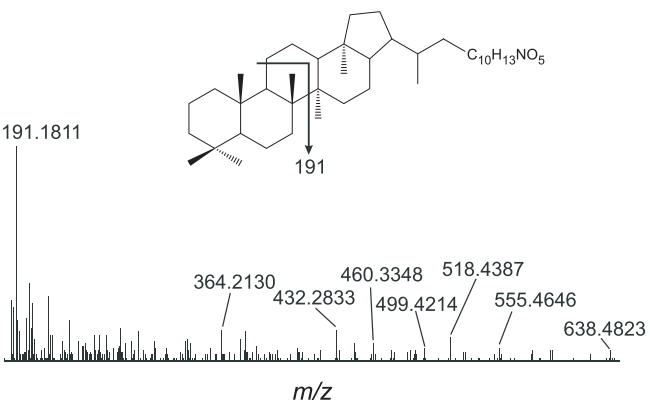
(d) MS^2 of *m/z* 771.6 ($C_{46}H_{79}N_2O_7^+$)



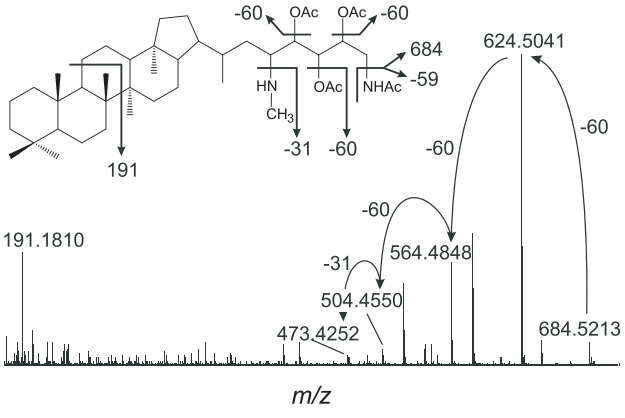
(e) MS^2 of *m/z* 688.5 from 748.5 ($C_{42}H_{70}NO_{10}^+$)



(f) MS^2 of *m/z* 638.5 ($C_{40}H_{64}NO_5^+$)



(g) MS^2 of *m/z* 684.5 from 743.6 ($C_{44}H_{75}N_2O_7^+$)



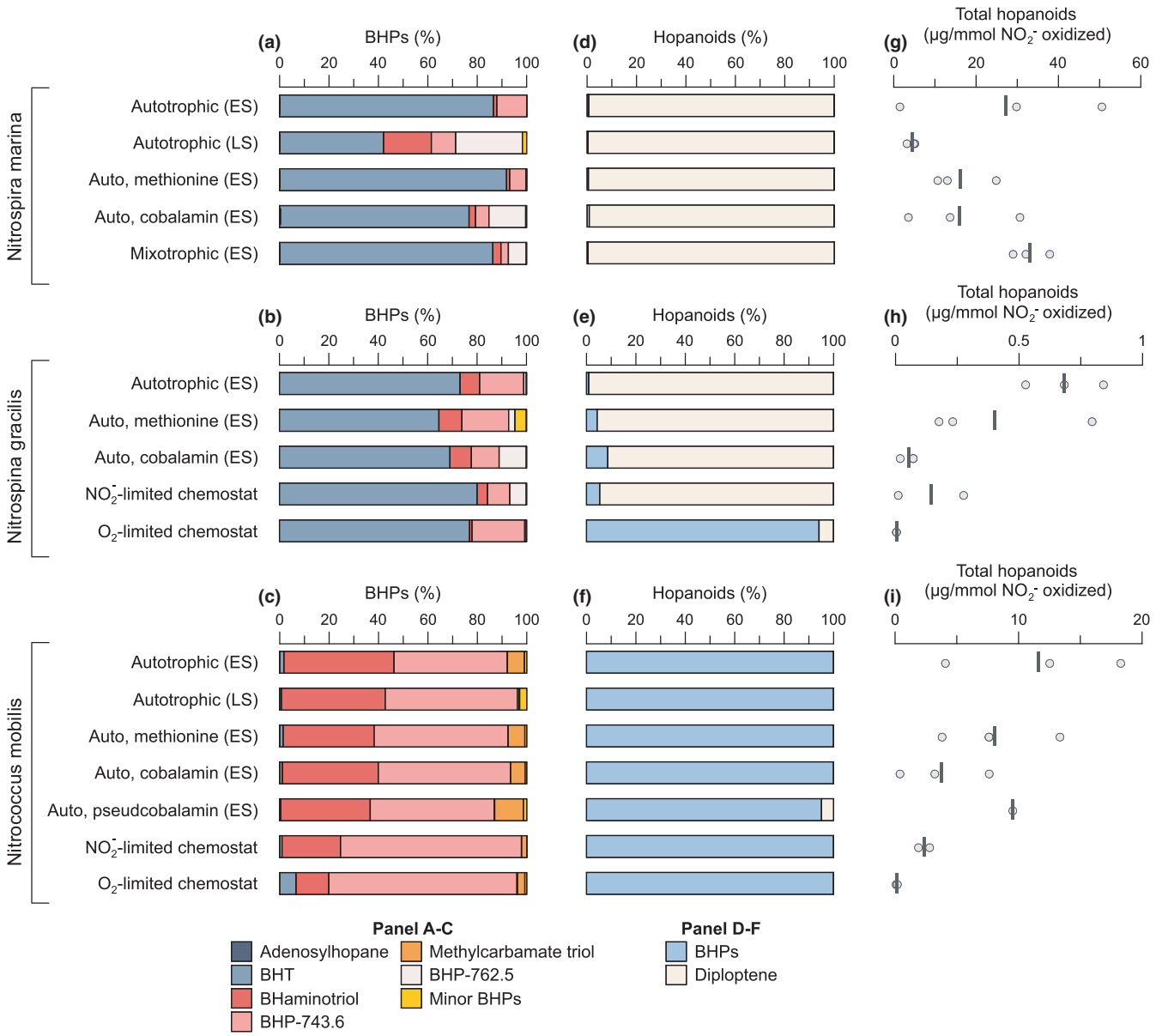


FIGURE 4 Relative abundance of hopanoids (averages of triplicate cultures, except for duplicate cultures for *N. mobilis* and *N. gracilis* chemostat experiments, and single culture for pseudocobalamin) in three marine species of nitrite-oxidizing bacteria under varying growth conditions: autotrophic (NO_2^- as electron donor; ES, early stationary phase; and 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}), and 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; n.a., not available). 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

nitrifying bacteria should be able to produce C_{30} hopanoids (e.g., diploptene and diplopterol; encoded by the *SHC* gene) and side-chain extended hopanoids (BHPs; Figure 2). Similarly, 29 of 84 surveyed incomplete environmental genomes and enrichment cultures of Nitrospirae, Nitrospinae, and *Nitrobacter* spp. possess at least one hopanoid biosynthetic gene (Data S1). Among nitrifying bacteria, hopanoid biosynthetic pathways are absent only in the genus *Ca.* Nitrotoga (Betaproteobacteria) and the Chloroflexi NOB (*Nitrolancea*, *Ca.* Nitrocaldera robusta). Although only two complete genomes for *Ca.* Nitrotoga and none for Chloroflexi NOB are currently available (Ishii et al., 2020; Kitzinger et al., 2018), hopanoid biosynthesis genes are also absent from five near-complete *Ca.* Nitrotoga genomes and six near-complete Chloroflexi NOB genomes (Data S1; Boddicker & Mosier, 2018; Sorokin et al., 2012; Spieck, Sass, et al., 2020; Spieck,

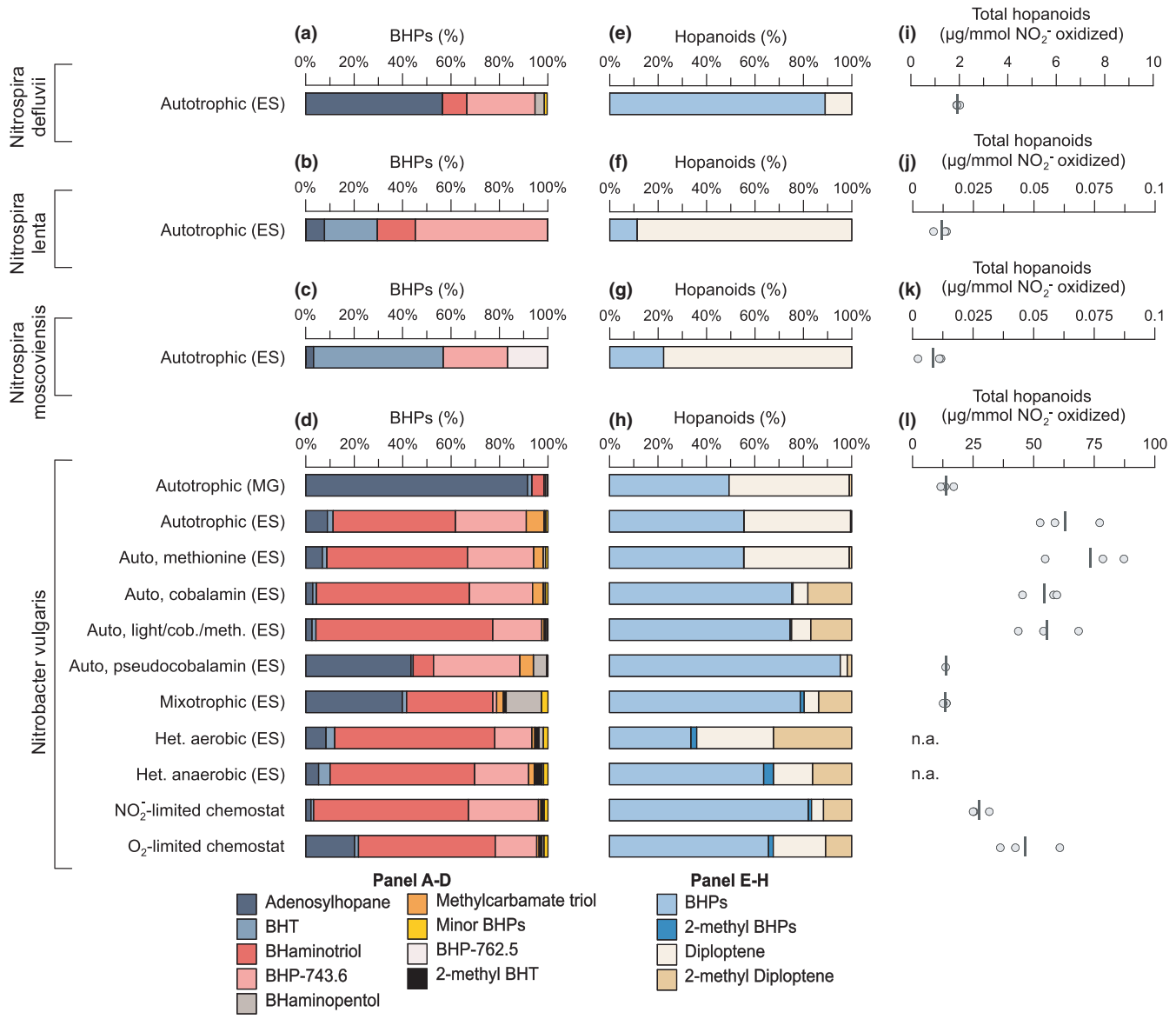


FIGURE 5 Relative abundance of hopanoids (averages of triplicate cultures, except for single cultures for pseudocobalamin) in four freshwater/wastewater species of nitrite-oxidizing bacteria under varying growth conditions: autotrophic (NO_2^- as electron donor; MG, mid-growth phase; and ES, early stationary phase), autotrophic + methionine (ES), autotrophic + cobalamin (ES), autotrophic + cobalamin + methionine + 6h/18h light/dark cycles (ES), autotrophic + pseudocobalamin, mixotrophic (NO_2^- + complex organics; ES), 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}), and 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; and 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

Spohn, et al., 2020). This suggests that *Ca.* Nitrotoga and Chloroflexi NOB likely do not produce hopanoids.

The occurrence of hopanoid biosynthetic genes may allow prediction of the types of hopanoids produced by nitrifying bacteria (Bradley et al., 2010; Liu et al., 2014; Schmerk et al., 2015; Welander et al., 2010, 2012; Welander & Summons, 2012). Our analyses show good agreement between genomic predictions and observations in culture. All seven NOB species investigated here, as well as the previously studied NOB species *Nitrospira defluvii* (Lücker et al., 2010) and the AOB *Nitrosomonas europaea* (Seemann et al., 1999), contained the *SHC* gene and produced at least C_{30} hopanoids (Figure 2). Similarly, *hpnG* and *hpnH* gene homologs were present in most *SHC*-positive nitrifier genomes, and their products adenosylhopane and ribosylhopane/ribonylhopane also were detected in all seven tested

NOB strains (Figures 4 and 5, Data S2). However, ribosylhopane, ribonylhopane, and adenosylhopane were present only in small amounts in the seven NOB (with the exception of *N. defluvii*). In contrast, adenosylhopane was abundant in *N. vulgaris* in mid-growth phase (>90%) and early stationary phase (up to 40%). The low abundance of these BHPs under most conditions, with high abundance during mid-growth phase, likely reflects their role as central intermediates in the biosynthesis of downstream BHPs (Bodlenner et al., 2015; Bradley et al., 2010; Welander et al., 2012). Consequently, downstream BHPs such as BHT and BHaminotriol were the most abundant BHPs in the studied NOB strains.

While the enzyme responsible for BHT formation remains to be identified, biosynthesis of BHaminotriol is known to be facilitated by the aminotransferase HpnO using ribonylhopane or ribosylhopane as the precursor (Welander et al., 2012). The *hpnO* gene is found in NOB of the genera *Nitrobacter* and *Nitrococcus* as well as many AOB. Exceptions are gammaproteobacterial AOB (*Nitrosococcus* spp., *Ca*. Nitrosoglobus terrae, and *Ca*. Nitrosacidococcus tergens) and several *Nitrosovibrio* and *Nitrosomonas* species. BHaminotriol was observed previously in the AOB *Nitrosomonas europaea* (Seemann et al., 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 (Figure 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 was detected in the seven tested NOB. However, we identified a *hpnK* homolog in the incomplete MAG of the thermophilic Chloroflexi NOB *Ca*. N. robusta, which lacks all other hopanoid biosynthetic genes. Although this homolog 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, and 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, Bischoff, et al., 2016; Talbot, McClymont, et al., 2016). 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. Presence of both *hpnR* and *hpnP* in *Ca*. N. alkalitolerans (Figure 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 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 *Nitrospira* species. Similarly, hopanoid biosynthetic capacity differs between the two studied genera of the phylum Nitrospirae, *Nitrospira* and *Leptospirillum* (Figure 2). Furthermore, each studied *Nitrospira* species contained a different dominant BHP (Figures 4 and 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 (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 (Figure 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 side-chain backbone and another as part of an aminopropyl group bound to the side-chain 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 one methylamino group, both directly bound to the backbone of the side chain. 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; Figure 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 the 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 (Bradley et al., 2017; Sáenz et al., 2012; Welander et al., 2009, 2012; Wu et al., 2015). Here, we specifically explored the effects of metabolism (autotrophy, mixotrophy, heterotrophy, NO_2^- and O_2^- limitation, and light) and the cofactors 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 cofactor for the biosynthesis of BHT cyclitol ether, which is mediated by HpnJ (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 (Figure 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 a cofactor by their HpnP—only slightly stimulated 2-methylhopanoid production in *N. vulgaris*. We suggest that this lack of 2-methylhopanoid production is caused by cofactor specificity of HpnP, potentially resulting from substitutions in the cobalamin-binding domains of alphaproteobacterial versus cyanobacterial HpnP (Figure 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; Elling et al., 2020; Heal et al., 2017).

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 (Figure 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 2 weeks after nitrite consumption did not contain increased quantities of 3-methylhopanoids. 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* (Figures 4 and 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 mid-plane of the lipid bilayer and reducing membrane permeability (Caron et al., 2014; Mangiarotti et al., 2019; Poger & Mark, 2013). 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 *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 ($p < .05$, 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, suggest distinct regulation mechanisms across NOB clades. Still, our results imply that changes in 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 (Berndmeyer et al., 2013; Blumenberg et al., 2013; Wakeham et al., 2007, 2012; Zhu et al., 2011). 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, Bischoff, et al., 2016). Partial loss of labile functional groups such as nitro groups may result in loss of specificity, but the putative, more stable 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 (Rush et al., 2016; Talbot et al., 2014). 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‰; Birgel et al., 2006; Thiel et al., 2003), they may have contributed to the deposition of many records that contain less ^{13}C-depleted hopanoids (~−30 to −40‰; 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 the presence of planktonic nitrogen-fixing cyanobacteria and methanotrophic bacteria, respectively (Cao et al., 2009; Kasprak et al., 2015; Kuypers et al., 2004; Summons et al., 1999), although other sources of these compounds have been recognized (Rashby et al., 2007; Summons et al., 1999; 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) on specific environmental conditions or physiological stresses promoting methylhopanoid production. On geologic timescales, these processes are controlled by nutrient cycles (Fe, P) and ocean oxygenation (Falkowski, 1998; Tyrrell, 1999; Van Cappellen & Ingall, 1996). 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

(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 (Figure S2), suggesting a different phylogenetic origin of *hpnP* 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 (Figure 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 (Elling et al., 2020; Ricci et al., 2014, 2015), 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 the 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 (Close et al., 2013; Lengger et al., 2019; Richardson & Jackson, 2007). 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 (Berg et al., 2010; Preuß et al., 1989), as previously shown for *Nitrospira defluvii* (Lücker et al., 2010). Enrichment in ^{13}C in C_{16} fatty acids relative to biomass and diplopteene 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 *N. vulgaris* is indicative of the Calvin–Benson–Bassham (CBB) cycle for carbon fixation (Berg et al., 2010; Sirevåg et al., 1977). Similarly, all known AOB use the CBB cycle (Data S1; Koops & Pommerening-Röser, 2015; Sakata et al., 2008). 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 ($\varepsilon_{CO_2\text{-biomass}}$ = 20–30‰; McNevin et al., 2007; Quandt et al., 1977). Given that *Nitrospina* and *Nitrospira* spp. are the dominant NOB in most of the modern ocean (Mincer et al., 2007; Pachiadaki et al., 2017; Santoro et al., 2010), 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 (Füssel et al., 2017; Lücker et al., 2010). 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, the 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. However, 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 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, and 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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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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REFERENCES

Alawi, M., Lipski, A., Sanders, T., Pfeiffer, E. M., & Spieck, E. (2007). Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. *The ISME Journal*, *1*, 256–264. https://doi.org/10.1038/ismej.2007.34

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bayer, B., Saito, M. A., McIlvin, M. R., Lücker, S., Moran, D. M., Lankiewicz, T. S., Dupont, C. L., & Santoro, A. E. (2021). Metabolic versatility of the nitrite-oxidizing bacterium *Nitrospira marina* and its proteomic response to oxygen-limited conditions. *The ISME Journal*, *15*, 1025–1039. https://doi.org/10.1038/s41396-020-00828-3

Belin, B. J., Busset, N., Giraud, E., Molinaro, A., Silipo, A., & Newman, D. K. (2018). Hopanoid lipids: From membranes to plant–bacteria interactions. *Nature Reviews Microbiology*, *16*, 304–315. https://doi.org/10.1038/nrmicro.2017.173

Berg, I. A., Kockelkorn, D., Ramos-Vera, W. H., Say, R. F., Zarzycki, J., Hügler, M., Alber, B. E., & Fuchs, G. (2010). Autotrophic carbon fixation in archaea. *Nature Reviews Microbiology*, *8*, 447–460. https://doi.org/10.1038/nrmicro2365

Berndmeyer, C., Thiel, V., Schmale, O., & Blumenberg, M. (2013). Biomarkers for aerobic methanotrophy in the water column of the stratified Gotland Deep (Baltic Sea). *Organic Geochemistry*, *55*, 103–111. https://doi.org/10.1016/j.orggeochem.2012.11.010

Berry, A. M., Harriott, O. T., Moreau, R. A., Osman, S. F., Benson, D. R., & Jones, A. D. (1993). Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 6091–6094. https://doi.org/10.1073/pnas.90.13.6091

Birgel, D., Peckmann, J., Klautzsch, S., Thiel, V., & Reitner, J. (2006). Anaerobic and aerobic oxidation of methane at late cretaceous seeps in the Western Interior Seaway, USA. *Geomicrobiology Journal*, *23*, 565–577. https://doi.org/10.1080/01490450600897369

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Biochemistry and Cell Biology*, *37*, 911–917.

Blumenberg, M., Berndmeyer, C., Moros, M., Muschalla, M., Schmale, O., & Thiel, V. (2013). Bacteriohopanepolyols record stratification, nitrogen fixation and other biogeochemical perturbations in Holocene sediments of the central Baltic Sea. *Biogeosciences*, *10*, 2725–2735. https://doi.org/10.5194/bg-10-2725-2013

Bock, E., Sundermeyer-Klinger, H., & Stackebrandt, E. (1983). New facultative lithoautotrophic nitrite-oxidizing bacteria. *Archives of Microbiology*, *136*, 281–284. https://doi.org/10.1007/BF00425217

Boddicker, A. M., & Mosier, A. C. (2018). Genomic profiling of four cultivated *Candidatus* Nitrotoga spp. predicts broad metabolic potential and environmental distribution. *The ISME Journal*, *12*, 2864–2882. https://doi.org/10.1038/s41396-018-0240-8

Bodlenner, A., Liu, W., Hirsch, G., Schaeffer, P., Blumenberg, M., Lendt, R., Tritsch, D., Michaelis, W., & Rohmer, M. (2015). C_{35} hopanoid side chain biosynthesis: Reduction of ribosylhopane into bacteriohopanetetrol by a cell-free system derived from *Methylobacterium organophilum*. *ChemBioChem*, *16*, 1764–1770.

Boenigk, J., Stadler, P., Wiedlroither, A., & Hahn, M. W. (2004). Strain-specific differences in the grazing sensitivities of closely related ultramicrobacteria affiliated with the *Polynucleobacter* cluster. *Applied and Environmental Microbiology*, *70*, 5787–5793.

Bradley, A. S., Pearson, A., Sáenz, J. P., & Marx, C. J. (2010). Adenosylhopane: The first intermediate in hopanoid side chain biosynthesis. *Organic Geochemistry*, *41*, 1075–1081. https://doi.org/10.1016/j.orggeochem.2010.07.003

Bradley, A. S., Swanson, P. K., Muller, E. E. L., Bringel, F., Caroll, S. M., Pearson, A., Vuilleumier, S., & Marx, C. J. (2017). Hopanoid-free *Methylobacterium extorquens* DM4 overproduces carotenoids and has widespread growth impairment. *PLoS One*, *12*, e0173323. https://doi.org/10.1371/journal.pone.0173323

Briggs, D. E. G., & Summons, R. E. (2014). Ancient biomolecules: Their origins, fossilization, and role in revealing the history of life. *BioEssays*, *36*, 482–490. https://doi.org/10.1002/bies.201400010

Brocks, J. J., & Banfield, J. (2009). Unravelling ancient microbial history with community proteogenomics and lipid geochemistry. *Nature Reviews. Microbiology*, *7*, 601–609.

Brocks, J. J., & Pearson, A. (2005). Building the biomarker tree of life. *Reviews in Mineralogy and Geochemistry*, *59*, 233–258. https://doi.org/10.2138/rmg.2005.59.10

Cao, C., Love, G. D., Hays, L. E., Wang, W., Shen, S., & Summons, R. E. (2009). Biogeochemical evidence for euxinic oceans and ecological disturbance presaging the end-Permian mass extinction event. *Earth and Planetary Science Letters*, *281*, 188–201. https://doi.org/10.1016/j.epsl.2009.02.012

Caron, B., Mark, A. E., & Poger, D. (2014). Some like it hot: The effect of sterols and hopanoids on lipid ordering at high temperature. *The Journal of Physical Chemistry Letters*, *5*, 3953–3957. https://doi.org/10.1021/jz5020778

Close, H. G., Shah, S. R., Ingalls, A. E., Diefendorf, A. F., Brodie, E. L., Hansman, R. L., Freeman, K. H., Aluwihare, L. I., & Pearson, A. (2013). Export of submicron particulate organic matter to mesopelagic depth in an oligotrophic gyre. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 12565–12570. https://doi.org/10.1073/pnas.1217514110

Collister, J. W., Summons, R. E., Lichtfouse, E., & Hayes, J. M. (1992). An isotopic biogeochemical study of the Green River oil shale. *Organic Geochemistry*, *19*, 265–276. https://doi.org/10.1016/0146-6380(92)90042-V

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R. H., Von, B. M., Rattei, T., Bendinger, B., Nielsen, P. H., & Wagner, M. (2015). Complete nitrification by *Nitrospira* bacteria. *Nature*, *528*, 504–509. https://doi.org/10.1038/nature16461

Daims, H., Lücker, S., & Wagner, M. (2016). A new perspective on microbes formerly known as nitrite-oxidizing bacteria. *Trends in Microbiology*, *24*, 699–712. https://doi.org/10.1016/j.tim.2016.05.004

Doughty, D. M., Hunter, R. C., Summons, R. E., & Newman, D. K. (2009). 2-Methylhopanoids are maximally produced in akinetes of *Nostoc punctiforme*: Geobiological implications. *Geobiology*, *7*, 524–532.

Doxey, A. C., Kurtz, D. A., Lynch, M. D., Sauder, L. A., & Neufeld, J. D. (2015). Aquatic metagenomes implicate Thaumarchaeota in global cobalamin production. *The ISME Journal*, *9*, 461–471. https://doi.org/10.1038/ismej.2014.142

Elling, F. J., Hemingway, J. D., Evans, T. W., Kharbush, J. J., Spieck, E., Summons, R. E., & Pearson, A. (2020). Vitamin B_{12}-dependent biosynthesis ties amplified 2-methylhopanoid production during oceanic anoxic events to nitrification. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 32996–33004.

Elling, F. J., Hemingway, J. D., Kharbush, J. J., Becker, K. W., Polik, C. A., & Pearson, A. (2021). Linking diatom-diazotroph symbioses to nitrogen cycle perturbations and deep-water anoxia: Insights from Mediterranean sapropel events. *Earth and Planetary Science Letters*, *571*, 117110. https://doi.org/10.1016/j.epsl.2021.117110

Erb, T. J. (2011). Carboxylases in natural and synthetic microbial pathways. *Applied and Environmental Microbiology*, *77*, 8466–8477. https://doi.org/10.1128/AEM.05702-11

Falkowski, P. G., Barber, R. T., & Smetacek, V. (1998). Biogeochemical controls and feedbacks on ocean primary production. *Science*, *281*, 200–206. https://doi.org/10.1126/science.281.5374.200

Fischer, W. W., Summons, R. E., & Pearson, A. (2005). Targeted genomic detection of biosynthetic pathways: Anaerobic production of hopanoid biomarkers by a common sedimentary microbe. *Geobiology*, *3*, 33–40. https://doi.org/10.1111/j.1472-4669.2005.00041.x

Füssel, J., Lücker, S., Yilmaz, P., Nowka, B., van Kessel, M. A. H. J., Bourceau, P., Hach, P. F., Littmann, S., Berg, J., Spieck, E., Daims, H., Kuypers, M. M. M., & Lam, P. (2017). Adaptability as the key to success for the ubiquitous marine nitrite oxidizer *Nitrococcus*. *Science Advances*, *3*, e1700807.

Hayatsu, M., Tago, K., Uchiyama, I., Toyoda, A., Wang, Y., Shimomura, Y., Okubo, T., Kurisu, F., Hirono, Y., Nonaka, K., Akiyama, H., Itoh, T., & Takami, H. (2017). An acid-tolerant ammonia-oxidizing γ-proteobacterium from soil. *The ISME Journal*, *11*, 1130–1141. https://doi.org/10.1038/ismej.2016.191

Heal, K. R., Qin, W., Ribalet, F., Bertagnolli, A. D., Coyote-Maestas, W., Hmelo, L. R., Moffett, J. W., Devol, A. H., Armbrust, E. V., Stahl, D. A., & Ingalls, A. E. (2017). Two distinct pools of B_{12} analogs reveal community interdependencies in the ocean. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, 364–369.

Ichihara, K., & Fukubayashi, Y. (2010). Preparation of fatty acid methyl esters for gas-liquid chromatography. *Journal of Lipid Research*, *51*, 635–640. https://doi.org/10.1194/jlr.D001065

Ishii, K., Fujitani, H., Sekiguchi, Y., & Tsuneda, S. (2020). Physiological and genomic characterization of a new '*Candidatus* Nitrotoga' isolate. *Environmental Microbiology*, *22*, 2365–2382.

Kasprak, A. H., Sepúlveda, J., Price-Waldman, R., Williford, K. H., Schoepfer, S. D., Haggart, J. W., Ward, P. D., Summons, R. E., & Whiteside, J. H. (2015). Episodic photic zone euxinia in the northeastern Panthalassic Ocean during the end-Triassic extinction. *Geology*, *43*, 307–310. https://doi.org/10.1130/G36371.1

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*, 772–780. https://doi.org/10.1093/molbev/mst010

Kharbush, J. J., Kejriwal, K., & Aluwihare, L. I. (2015). Distribution and abundance of hopanoid producers in low-oxygen environments of the Eastern Pacific Ocean. *Microbial Ecology*, 401–408.

Kharbush, J. J., Thompson, L. R., Haroon, M. F., Knight, R., & Aluwihare, L. I. (2018). Hopanoid-producing bacteria in the Red Sea include the major marine nitrite oxidizers. *FEMS Microbiology Ecology*, *94*, fiy063. https://doi.org/10.1093/femsec/fiy063

Kharbush, J. J., Ugalde, J. A., Hogle, S. L., Allen, E. E., & Aluwihare, L. I. (2013). Composite bacterial hopanoids and their microbial producers across oxygen gradients in the water column of the California current. *Applied and Environmental Microbiology*, *79*, 7491–7501. https://doi.org/10.1128/AEM.02367-13

Kitzinger, K., Koch, H., Lücker, S., Sedlacek, C. J., Herbold, C., Schwarz, J., Daebeler, A., Mueller, A. J., Lukumbuzya, M., Romano, S., Leisch, N., Karst, S. M., Kirkegaard, R., Albertsen, M., Nielsen, P. H., Wagner, M., & Daims, H. (2018). Characterization of the first "*Candidatus* Nitrotoga" isolate reveals metabolic versatility and separate evolution of widespread nitrite-oxidizing bacteria. *MBio 9*, e01186-18.

Koops, H.-P., & Pommerening-Röser, A. (2015). The lithoautotrophic ammonia-oxidizing bacteria. In W. B. Whitman (Ed.), *Bergey's manual of systematics of archaea and bacteria* (pp. 1–17). Jonh Wiley & Sons Inc.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G., & Wagner, M. (2006). The lithoautotrophic ammonia-oxidizing bacteria. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The prokaryotes* (pp. 778–811). Springer New York.

Kuypers, M. M. M., Blokker, P., Erbacher, J., Kinkel, H., Pancost, R. D., Schouten, S., & Sinninghe Damsté, J. S. (2001). Massive expansion of marine archaea during a mid-cretaceous oceanic anoxic event. *Science*, *293*, 92–95. https://doi.org/10.1126/science.1058424

Kuypers, M. M. M., Blokker, P., Hopmans, E. C., Kinkel, H., Pancost, R. D., Schouten, S., & Sinninghe Damsté, J. S. (2002). Archaeal remains dominate marine organic matter from the early Albian oceanic anoxic event 1b. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *185*, 211–234. https://doi.org/10.1016/S0031-0182(02)00301-2

Kuypers, M. M. M., van Breugel, Y., Schouten, S., Erba, E., & Sinninghe Damsté, J. S. (2004). N_2-fixing cyanobacteria supplied nutrient N for Cretaceous oceanic anoxic events. *Geology*, *32*, 853. https://doi.org/10.1130/G20458.1

Lengger, S. K., Rush, D., Mayser, J. P., Blewett, J., Schwartz-Narbonne, R., Talbot, H. M., Middelburg, J. J., Jetten, M. S. M., Schouten, S., Damsté, J. S. S., & Pancost, R. D. (2019). Dark carbon fixation in the Arabian Sea oxygen minimum zone contributes to sedimentary organic carbon (SOM). *Global Biogeochemical Cycles*, *33*, 1715–1732. https://doi.org/10.1029/2019GB006282

Letunic, I., & Bork, P. (2016). Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research*, *44*, W242–W245. https://doi.org/10.1093/nar/gkw290

Liu, W., Sakr, E., Schaeffer, P., Talbot, H. M., Donisi, J., Härtner, T., Kannenberg, E., Takano, E., & Rohmer, M. (2014). Ribosylhopane, a novel bacterial hopanoid, as precursor of C_{35} bacteriohopanepolyols in *Streptomyces coelicolor* A3(2). *ChemBioChem*, *15*, 2156–2161.

Lücker, S., Nowka, B., Rattei, T., Spieck, E., & Daims, H. (2013). The genome of *Nitrospina gracilis* illuminates the metabolism and evolution of the major marine nitrite oxidizer. *Frontiers in Microbiology*, *4*, 27.

Lücker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., Rattei, T., Damsté, J. S. S., Spieck, E., Le Paslier, D., & Daims, H. (2010). A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 13479–13484. https://doi.org/10.1073/pnas.1003860107

Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., & Simon, M. (2005). An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environmental Microbiology*, *7*, 961–968. https://doi.org/10.1111/j.1462-2920.2005.00767.x

Mangiarotti, A., Genovese, D. M., Naumann, C. A., Monti, M. R., & Wilke, N. (2019). Hopanoids, like sterols, modulate dynamics, compaction, phase segregation and permeability of membranes. *Biochimica Et Biophysica Acta (BBA) – Biomembranes*, *1861*, 183060.

Matys, E. D., Sepúlveda, J., Pantoja, S., Lange, C. B., Caniupán, M., Lamy, F., & Summons, R. E. (2017). Bacteriohopanepolyols along redox gradients in the Humboldt Current System off northern Chile. *Geobiology*, *15*, 844–857. https://doi.org/10.1111/gbi.12250

McNevin, D. B., Badger, M. R., Whitney, S. M., von Caemmerer, S., Tcherkez, G. G. B., & Farquhar, G. D. (2007). Differences in carbon isotope discrimination of three variants of D-ribulose-1,5-bisphosphate carboxylase/oxygenase reflect differences in their catalytic mechanisms. *Journal of Biological Chemistry*, *282*, 36068–36076. https://doi.org/10.1074/jbc.M706274200

Menzel, D., Hopmans, E. C., Schouten, S., & Sinninghe Damsté, J. S. (2006). Membrane tetraether lipids of planktonic Crenarchaeota in Pliocene sapropels of the eastern Mediterranean Sea. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *239*, 1–15. https://doi.org/10.1016/j.palaeo.2006.01.002

Mincer, T. J., Church, M. J., Taylor, L. T., Preston, C., Karl, D. M., & DeLong, E. F. (2007). Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre. *Environmental Microbiology*, *9*, 1162–1175. https://doi.org/10.1111/j.1462-2920.2007.01239.x

Mook W. G., Bommerson J. C., & Staverman W. H. (1974). Carbon isotope fractionation between dissolved bicarbonate and gaseous carbon dioxide. *Earth and Planetary Science Letters*, *22*, 169–176. http://dx.doi.org/10.1016/0012-821x(74)90078-8

Mueller, A. J., Jung, M.-Y., Strachan, C. R., Herbold, C. W., Kirkegaard, R. H., Wagner, M., & Daims, H. (2020). Genomic and kinetic analysis of novel Nitrospinae enriched by cell sorting. *The ISME Journal*, *15*, 732–745.

Naafs, B. D. A., Monteiro, F. M., Pearson, A., Higgins, M. B., Pancost, R. D., & Ridgwell, A. (2019). Fundamentally different global marine nitrogen cycling in response to severe ocean deoxygenation. *Proceedings of the National Academy of Sciences of the United States of America*, *116*, 24979–24984. https://doi.org/10.1073/pnas.1905553116

Newman, D. K., Neubauer, C., Ricci, J. N., Wu, C.-H., & Pearson, A. (2016). Cellular and molecular biological approaches to interpreting ancient biomarkers. *Annual Review of Earth and Planetary Sciences*, *44*, 493–522. https://doi.org/10.1146/annurev-earth-050212-123958

Ourisson, G., & Albrecht, P. (1992). Hopanoids. 1. Geohopanoids: The most abundant natural products on Earth? *Accounts of Chemical Research*, *25*, 398–402.

Ourisson, G., & Rohmer, M. (1992). Hopanoids. 2. Biohopanoids: A novel class of bacterial lipids. *Accounts of Chemical Research*, *25*, 403–408. https://doi.org/10.1021/ar00021a004

Ourisson, G., Rohmer, M., & Poralla, K. (1987). Prokaryotic hopanoids and other polyterpenoid sterol surrogates. *Annual Review of Microbiology*, *41*, 301–333. https://doi.org/10.1146/annurev.mi.41.100187.001505

Pachiadaki, M. G., Sintes, E., Bergauer, K., Brown, J. M., Record, N. R., Swan, B. K., Mathyer, M. E., Hallam, S. J., Lopez-Garcia, P., Takaki, Y., Nunoura, T., Woyke, T., Herndl, G. J., & Stepanauskas, R. (2017). Major role of nitrite-oxidizing bacteria in dark ocean carbon fixation. *Science*, *358*, 1046–1051. https://doi.org/10.1126/science.aan8260

Palomo, A., Pedersen, A. G., Fowler, S. J., Dechesne, A., Sicheritz-Pontén, T., & Smets, B. F. (2018). Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*. *The ISME Journal*, *12*, 1779. https://doi.org/10.1038/s41396-018-0083-3

Pearson, A., Flood Page, S. R., Jorgenson, T. L., Fischer, W. W., & Higgins, M. B. (2007). Novel hopanoid cyclases from the environment. *Environmental Microbiology*, *9*, 2175–2188. https://doi.org/10.1111/j.1462-2920.2007.01331.x

Picone, N., Pol, A., Mesman, R., van Kessel, M. A. H. J., Cremers, G., van Gelder, A. H., van Alen, T. A., Jetten, M. S. M., Lücker, S., & Op den Camp, H. J. M. (2020). Ammonia oxidation at pH 2.5 by a new gammaproteobacterial ammonia-oxidizing bacterium. *The ISME Journal*, *15*, 1150–1164.

Poger, D., & Mark, A. E. (2013). The relative effect of sterols and hopanoids on lipid bilayers: When comparable is not identical. *The Journal of Physical Chemistry B*, *117*, 16129–16140. https://doi.org/10.1021/jp409748d

Polik, C. A., Elling, F. J., & Pearson, A. (2018). Impacts of paleoecology on the TEX_{86} sea surface temperature proxy in the pliocene-pleistocene Mediterranean Sea. *Paleoceanography and Paleoclimatology*, *33*, 1472–1489.

Preuß, A., Schauder, R., Fuchs, G., & Stichler, W. (1989). Carbon isotope fractionation by autotrophic bacteria with three different CO_2 fixation pathways. *Zeitschrift für Naturforschung*, *44*, 397–402.

Quandt, L., Gottschalk, G., Ziegler, H., & Stichler, W. (1977). Isotope discrimination by photosynthetic bacteria. *FEMS Microbiology Letters*, *1*, 125–128. https://doi.org/10.1111/j.1574-6968.1977.tb00596.x

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*, D590–D596.

Rashby, S. E., Sessions, A. L., Summons, R. E., & Newman, D. K. (2007). Biosynthesis of 2-methylbacteriohopanepolyols by an anoxygenic phototroph. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 15099–15104. https://doi.org/10.1073/pnas.0704912104

Ricci, J. N., Coleman, M. L., Welander, P. V., Sessions, A. L., Summons, R. E., Spear, J. R., & Newman, D. K. (2013). Diverse capacity for 2-methylhopanoid production correlates with a specific ecological niche. *The ISME Journal*, *8*, 1–10.

Ricci, J. N., Coleman, M. L., Welander, P. V., Sessions, A. L., Summons, R. E., Spear, J. R., & Newman, D. K. (2014). Diverse capacity for 2-methylhopanoid production correlates with a specific ecological niche. *The ISME Journal*, *8*, 675–684. https://doi.org/10.1038/ismej.2013.191

Ricci, J. N., Michel, A. J., & Newman, D. K. (2015). Phylogenetic analysis of HpnP reveals the origin of 2-methylhopanoid production in Alphaproteobacteria. *Geobiology*, *13*, 267–277. https://doi.org/10.1111/gbi.12129

Richardson, T. L., & Jackson, G. A. (2007). Small phytoplankton and carbon export from the surface ocean. *Science*, *315*, 838–840. https://doi.org/10.1126/science.1133471

Rohmer, M., Bouvier-Nave, P., & Ourisson, G. (1984). Distribution of hopanoid triterpenes in prokaryotes. *Microbiology*, *130*, 1137–1150. https://doi.org/10.1099/00221287-130-5-1137

Rush, D., Osborne, K. A., Birgel, D., Kappler, A., Hirayama, H., Peckmann, J., Poulton, S. W., Nickel, J. C., Mangelsdorf, K., Kalyuzhnaya, M., Sidgwick, F. R., & Talbot, H. M. (2016). The bacteriohopanepolyol inventory of novel aerobic methane oxidising bacteria reveals new biomarker signatures of aerobic methanotrophy in marine systems. *PLoS One*, *11*, 1–27. https://doi.org/10.1371/journal.pone.0165635

Sáenz, J. P. (2010). *Exploring the distribution and physiological roles of bacterial membrane lipids in the marine environment* (PhD Thesis).

Sáenz, J. P., Sezgin, E., Schwille, P., & Simons, K. (2012). Functional convergence of hopanoids and sterols in membrane ordering. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 14236–14240. https://doi.org/10.1073/pnas.1212141109

Sáenz, J. P., Wakeham, S. G., Eglinton, T. I., & Summons, R. E. (2011). New constraints on the provenance of hopanoids in the marine geologic record: Bacteriohopanepolyols in marine suboxic and anoxic environments. *Organic Geochemistry*, *42*, 1351–1362. https://doi.org/10.1016/j.orggeochem.2011.08.016

Sakata, S., Hayes, J. M., Rohmer, M., Hooper, A. B., & Seemann, M. (2008). Stable carbon-isotopic compositions of lipids isolated from the ammonia-oxidizing chemoautotroph *Nitrosomonas europaea*. *Organic Geochemistry, Stable Isotopes in Biogeosciences (II)*, *39*, 1725–1734. https://doi.org/10.1016/j.orggeochem.2008.08.005

Santoro, A. E., Casciotti, K. L., & Francis, C. A. (2010). Activity, abundance and diversity of nitrifying archaea and bacteria in the central California Current. *Environmental Microbiology*, *12*, 1989–2006. https://doi.org/10.1111/j.1462-2920.2010.02205.x

Sato, S., Kudo, F., Rohmer, M., & Eguchi, T. (2020). Characterization of radical SAM adenosylhopane synthase, HpnH, which catalyzes the 5′-deoxyadenosyl radical addition to diploptene in the biosynthesis of C_{35} bacteriohopanepolyols. *Angewandte Chemie International Edition*, *59*, 237–241.

Schmerk, C. L., Welander, P. V., Hamad, M. A., Bain, K. L., Bernards, M. A., Summons, R. E., & Valvano, M. A. (2015). Elucidation of the *Burkholderia cenocepacia* hopanoid biosynthesis pathway uncovers functions for conserved proteins in hopanoid-producing bacteria. *Environmental Microbiology*, *17*, 735–750.

Schopfer, F. J., & Khoo, N. K. H. (2019). Nitro-fatty acid logistics: Formation, biodistribution, signaling, and pharmacology. *Trends in Endocrinology & Metabolism*, *30*, 505–519. https://doi.org/10.1016/j.tem.2019.04.009

Seemann, M., Bisseret, P., Tritz, J.-P., Hooper, A. B., & Rohmer, M. (1999). Novel bacterial triterpenoids of the hopane series from *Nitrosomonas europaea* and their significance for the formation of the C_{35} bacteriohopane skeleton. *Tetrahedron Letters*, *40*, 1681–1684. https://doi.org/10.1016/S0040-4039(99)00064-7

Sinninghe Damsté, J. S., Rijpstra, W. I. C., Dedysh, S. N., Foesel, B. U., & Villanueva, L. (2017). Pheno- and genotyping of hopanoid production in Acidobacteria. *Frontiers in Microbiology*, *8*, 968.

Sirevåg, R., Buchanan, B. B., Berry, J. A., & Troughton, J. H. (1977). Mechanisms of CO_2 fixation in bacterial photosynthesis studied by the carbon isotope fractionation technique. *Archives of Microbiology*, *112*, 35–38.

Sohlenkamp, C., & Geiger, O. (2016). Bacterial membrane lipids: Diversity in structures and pathways. *FEMS Microbiology Reviews*, *40*, 133–159. https://doi.org/10.1093/femsre/fuv008

Sorokin, D. Y., Lücker, S., Vejmelkova, D., Kostrikina, N. A., Kleerebezem, R., Rijpstra, W. I. C., Damsté, J. S. S., Le, P. D., Muyzer, G., Wagner, M., van Loosdrecht, M. C. M., & Daims, H. (2012). Nitrification expanded: Discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. *The ISME Journal*, *6*, 2245–2256. https://doi.org/10.1038/ismej.2012.70

Spencer-Jones, C. L. (2015). *Novel concepts derived from microbial biomarkers in the Congo System: Implications for continental methane cycling* (PhD Thesis).

Spieck, E., & Bock, E. (2005). The lithoautotrophic nitrite-oxidizing bacteria. In W. B. Whitman, F. Rainey, P. Kämpfer, M. Trujillo, J. Chun, P. DeVos, B. Hedlund, & S. Dedysh (Eds.), *Bergey's manual of systematics of archaea and bacteria* (pp. 1–10). John Wiley & Sons Ltd.

Spieck, E., & Lipski, A. (2011). Cultivation, growth physiology, and chemotaxonomy of nitrite-oxidizing bacteria. In M. G. Klotz (Ed.), *Research on nitrification and related processes, part A, Methods in enzymology* (pp. 109–130). Elsevier.

Spieck, E., Sass, K., Keuter, S., Hirschmann, S., Spohn, M., Indenbirken, D., Kop, L. F. M., Lücker, S., & Giaveno, A. (2020) Defining culture conditions for the hidden nitrite-oxidizing bacterium Nitrolancea. *Frontiers in Microbiology 11*, 1522.

Spieck, E., Spohn, M., Wendt, K., Bock, E., Shively, J., Frank, J., Indenbirken, D., Alawi, M., Lücker, S., & Hüpeden, J. (2020). Extremophilic nitrite-oxidizing *Chloroflexi* from Yellowstone hot springs. *The ISME Journal*, *14*, 364–379. https://doi.org/10.1038/s41396-019-0530-9

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Strickland, J. D. H., & Parsons, T. R. (1972). Ammonium and nitrite. In J. C. Stevenson, J. Watson, J. M. Reinhart, & D. G. Cook (Eds.), *A practical handbook of seawater analysis* (pp. 77–80). Fisheries Research Board of Canada.

Sturt, H. F., Summons, R. E., Smith, K., Elvert, M., & Hinrichs, K.-U. (2004). Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry – New biomarkers for biogeochemistry and microbial ecology. *Rapid Communications in Mass Spectrometry*, *18*, 617–628. https://doi.org/10.1002/rcm.1378

Summons, R. E., Jahnke, L. L., Hope, J. M., & Logan, G. A. (1999). 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. *Nature*, *400*, 554–557. https://doi.org/10.1038/23005

Talbot, H. M., Bischoff, J., Inglis, G. N., Collinson, M. E., & Pancost, R. D. (2016). Polyfunctionalised bio- and geohopanoids in the Eocene Cobham Lignite. *Organic Geochemistry*, *96*, 77–92. https://doi.org/10.1016/j.orggeochem.2016.03.006

Talbot, H. M., Handley, L., Spencer-Jones, C. L., Dinga, B. J., Schefuß, E., Mann, P. J., Poulsen, J. R., Spencer, R. G. M., Wabakanghanzi, J. N., & Wagner, T. (2014). Variability in aerobic methane oxidation over the past 1.2 Myrs recorded in microbial biomarker signatures from Congo fan sediments. *Geochimica et Cosmochimica Acta*, *133*, 387–401. https://doi.org/10.1016/j.gca.2014.02.035

Talbot, H. M., McClymont, E. L., Inglis, G. N., Evershed, R. P., & Pancost, R. D. (2016). Origin and preservation of bacteriohopanepolyol signatures in Sphagnum peat from Bissendorfer Moor (Germany). *Organic Geochemistry*, *97*, 95–110. https://doi.org/10.1016/j.orggeochem.2016.04.011

Talbot, H. M., Rohmer, M., & Farrimond, P. (2007). Structural characterisation of unsaturated bacterial hopanoids by atmospheric pressure chemical ionisation liquid chromatography/ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry*, *21*, 1613–1622. https://doi.org/10.1002/rcm.2997

Talbot, H. M., Summons, R. E., Jahnke, L. L., Cockell, C. S., Rohmer, M., & Farrimond, P. (2008). Cyanobacterial bacteriohopanepolyol signatures from cultures and natural environmental settings. *Organic Geochemistry*, *39*, 232–263. https://doi.org/10.1016/j.orggeochem.2007.08.006

Tang, T., Mohr, W., Sattin, S. R., Rogers, D. R., Girguis, P. R., & Pearson, A. (2017). Geochemically distinct carbon isotope distributions in *Allochromatium vinosum* DSM 180^T grown photoautotrophically and photoheterotrophically. *Geobiology*, *15*, 324–339.

Thiel, V., Blumenberg, M., Pape, T., Seifert, R., & Michaelis, W. (2003). Unexpected occurrence of hopanoids at gas seeps in the Black Sea. *Organic Geochemistry*, *34*, 81–87. https://doi.org/10.1016/S0146-6380(02)00191-2

Tyrrell, T. (1999). The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature*, *400*, 525–531. https://doi.org/10.1038/22941

Van Cappellen, P., & Ingall, E. D. (1996). Redox stabilization of the atmosphere and oceans by phosphorus-limited marine productivity. *Science*, *271*, 493–496. https://doi.org/10.1126/science.271.5248.493

van der Meer, M. T. J., Schouten, S., & Sinninghe Damsté, J. S. (1998). The effect of the reversed tricarboxylic acid cycle on the ^{13}C contents of bacterial lipids. *Geochimica et Cosmochimica Acta*, *28*, 527–533.

van Dongen, B. E., Talbot, H. M., Schouten, S., Pearson, P. N., & Pancost, R. D. (2006). Well preserved Palaeogene and Cretaceous biomarkers from the Kilwa area, Tanzania. *Organic Geochemistry*, *37*, 539–557. https://doi.org/10.1016/j.orggeochem.2006.01.003

van Kessel, M. A. H. J., Speth, D. R., Albertsen, M., Nielsen, P. H., Op den Camp, H. J. M., Kartal, B., Jetten, M. S. M., & Lücker, S. (2015). Complete nitrification by a single microorganism. *Nature*, *528*, 555–559. https://doi.org/10.1038/nature16459

Wakeham, S. G., Amann, R., Freeman, K. H., Hopmans, E. C., Jørgensen, B. B., Putnam, I. F., Schouten, S., Sinninghe Damsté, J. S., Talbot, H. M., & Woebken, D. (2007). Microbial ecology of the stratified water column of the Black Sea as revealed by a comprehensive biomarker study. *Organic Geochemistry*, *38*, 2070–2097. https://doi.org/10.1016/j.orggeochem.2007.08.003

Wakeham, S. G., Turich, C., Schubotz, F., Podlaska, A., Li, X. N., Varela, R., Astor, Y., Sáenz, J. P., Rush, D., Sinninghe Damsté, J. S., Summons, R. E., Scranton, M. I., Taylor, G. T., & Hinrichs, K.-U. (2012). Biomarkers, chemistry and microbiology show chemoautotrophy in a multilayer chemocline in the Cariaco Basin. *Deep Sea Research Part I: Oceanographic Research Papers*, *63*, 133–156. https://doi.org/10.1016/j.dsr.2012.01.005

Ward, B. B., & Carlucci, A. F. (1985). Marine ammonia- and nitrite-oxidizing bacteria: Serological diversity determined by immunofluorescence in culture and in the environment. *Applied and Environmental Microbiology*, *50*, 8.

Ward, B. B., Glover, H. E., & Lipschultz, F. (1989). Chemoautotrophic activity and nitrification in the oxygen minimum zone off Peru. *Deep Sea Research Part A. Oceanographic Research Papers*, *36*, 1031–1051. https://doi.org/10.1016/0198-0149(89)90076-9

Warren, M. J., Raux, E., Schubert, H. L., & Escalante-Semerena, J. C. (2002). The biosynthesis of adenosylcobalamin (vitamin B_{12}). *Natural Product Reports*, *19*, 390–412. https://doi.org/10.1039/b108967f

Watson, S. W., & Waterbury, J. B. (1971). Characteristics of two marine nitrite oxidizing bacteria, *Nitrospina gracilis* nov. gen. nov. sp. and *Nitrococcus mobilis* nov. gen. nov. sp. *Archiv für Mikrobiologie*, *77*, 203–230. https://doi.org/10.1007/BF00408114

Welander, P. V., Coleman, M. L., Sessions, A. L., Summons, R. E., & Newman, D. K. (2010). Identification of a methylase required for 2-methylhopanoid production and implications for the interpretation of sedimentary hopanes. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 8537–8542. https://doi.org/10.1073/pnas.0912949107

Welander, P. V., Doughty, D. M., Wu, C.-H., Mehay, S., Summons, R. E., & Newman, D. K. (2012). Identification and characterization of *Rhodopseudomonas palustris* TIE-1 hopanoid biosynthesis mutants. *Geobiology*, *10*, 163–177. https://doi.org/10.1111/j.1472-4669.2011.00314.x

Welander, P. V., Hunter, R. C., Zhang, L., Sessions, A. L., Summons, R. E., & Newman, D. K. (2009). Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1. *Journal of Bacteriology*, *191*, 6145–6156.

Welander, P. V., & Summons, R. E. (2012). Discovery, taxonomic distribution, and phenotypic characterization of a gene required for 3-methylhopanoid production. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 12905–12910. https://doi.org/10.1073/pnas.1208255109

Williams, T. J., Zhang, C. L., Scott, J. H., & Bazylinski, D. A. (2006). Evidence for autotrophy via the reverse tricarboxylic acid cycle in the marine magnetotactic coccus strain MC-1. *Applied and Environmental Microbiology*, *72*, 1322–1329. https://doi.org/10.1128/AEM.72.2.1322-1329.2006

Wu, C.-H., Bialecka-Fornal, M., & Newman, D. K. (2015). Methylation at the C-2 position of hopanoids increases rigidity in native bacterial membranes. *eLife*, *4*, e05663.

Zhu, C., Talbot, H. M., Wagner, T., Pan, J.-M., & Pancost, R. D. (2011). Distribution of hopanoids along a land to sea transect: Implications for microbial ecology and the use of hopanoids in environmental studies. *Limnology and Oceanography*, *56*, 1850–1865. https://doi.org/10.4319/lo.2011.56.5.1850

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