frontiers in Marine Science

ORIGINAL RESEARCH
published: 10 March 2022
doi: 10.3389/fmars.2022.831286

Lipid Biomarker Patterns Reflect Nutritional Strategies of Seep-Dwelling Bivalves From the South China Sea

Hongxiang Guan[1,2], Dong Feng[2,3], Daniel Birgel[4]*, Steffen Kiel[5], Jörn Peckmann[4], Sanzhong Li[1,2] and Jun Tao[6]*

[1] Frontiers Science Center for Deep Ocean Multispheres and Earth System, Key Lab of Submarine Geosciences and Prospecting Techniques, Ministry of Education and College of Marine Geosciences, Ocean University of China, Qingdao, China, [2] Laboratory for Marine Mineral Resources, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China, [3] Shanghai Engineering Research Center of Hadal Science and Technology, College of Marine Sciences, Shanghai Ocean University, Shanghai, China, [4] Institute for Geology, Center for Earth System Research and Sustainability, Universität Hamburg, Hamburg, Germany, [5] Department of Paleobiology, Swedish Museum of Natural History, Stockholm, Sweden, [6] MLR Key Laboratory of Marine Mineral Resources, Guangzhou Marine Geological Survey, Guangzhou, China

OPEN ACCESS

Edited by:
Fengping Wang,
Shanghai Jiao Tong University, China

Reviewed by:
Wang Minxiao,
Institute of Oceanology (CAS), China
Xin Chen,
Shanghai Jiao Tong University, China
Solveig Irena Bühring,
University of Bremen, Germany

***Correspondence:**
Hongxiang Guan
guanhongxiang@ouc.edu.cn
Daniel Birgel
daniel.birgel@uni-hamburg.de

Specialty section:
This article was submitted to Marine Molecular Biology and Ecology, a section of the journal Frontiers in Marine Science

Received: *08 December 2021*
Accepted: *15 February 2022*
Published: *10 March 2022*

Citation:
Guan H, Feng D, Birgel D, Kiel S, Peckmann J, Li S and Tao J (2022) Lipid Biomarker Patterns Reflect Nutritional Strategies of Seep-Dwelling Bivalves From the South China Sea. Front. Mar. Sci. 9:831286. doi: 10.3389/fmars.2022.831286

Invertebrates living at methane seeps such as mussels and clams gain nutrition through symbiosis with chemosynthetic, chiefly methanotrophic and thiotrophic bacteria. Lipid biomarkers, including their compound-specific carbon stable isotope compositions, extracted from the host tissues are predestined for deciphering the various sources of diets and the associations among varying environments, endosymbionts, and hosts. Here, we investigated lipid inventories of soft tissues of two bathymodiolin mussel species hosting aerobic methanotrophic bacteria (*Gigantidas platifrons* from Site F and *Gigantidas haimaensis* from Haima seeps), one bathymodiolin mussel with thiotrophic bacteria (*Bathymodiolus aduloides* from Haima seeps), and one vesicomyid clam (*Archivesica marissinica* from Haima seeps) from the South China Sea. The gills of mussels hosting methanotrophic symbionts were found to contain high amounts of lipids of aerobic methanotrophic bacteria, such as the 4,4-dimethyl lanosterol, and other 4-methyl sterols, and the type I methanotroph-specific monounsaturated fatty acids (MUFAs) $C_{16:1\omega9}$ and $C_{16:1\omega8}$. Production of methyl-sterols is favored over fatty acids at low oxygen concentrations, as demonstrated in culture experiments with *Methylococcus capsulatus*. Since lesser fatty acids and abundant sterols are found in *G. haimaensis* compared to *G. platifrons*, *G. haimaensis* apparently lived at very low oxygen levels. Extremely high levels of MUFAs $C_{16:1\omega7}$ and $C_{18:1\omega7}$ were found in gill tissue of both *B. aduloides* and the vesicomyid clam *A. marissinica*. Given the absence of ω8 fatty acids, both *B. aduloides* and the vesicomyid clam contain thiotrophic bacteria only. The occurrence of ^{13}C-enriched 24-methylenecholesterol in *B. aduloides* indicates that the animal complemented its diet by filter-feeding (ca. 3% of the total sterol inventory) on photosynthetically derived carbon, whereas the majority of sterols are pointing to a diet relying on endosymbionts. Different types of 4-methyl

sterols were observed between the thiotroph-containing mussel and methanotroph-containing mussels, suggesting different biosynthetic steps are present from lanosterol to cholesterol between animal hosts and aerobic methanotrophs. Among the four bivalve species, specific lipid biomarker patterns diagnostic for either the symbionts or the hosts yielded similar $\delta^{13}C$ values in each species, indicating that the host obtained its nutrition either directly from the symbionts or derived at least most of its carbon in this way. The information derived from lipid biomarkers of bivalves and their corresponding symbionts in modern environments is vital to interpret data from the rock record, where most other methods to study microbial community composition are not applicable.

Keywords: chemosynthesis, symbiosis, aerobic methanotrophic bacteria, thiotrophic bacteria, lipid biomarkers, compound-specific carbon isotopes, South China Sea

INTRODUCTION

Chemosymbiotic invertebrates from hydrothermal vents and methane seeps, including mussels and clams, gain nutrition through chemosynthetic bacterial symbionts (Childress et al., 1986; Brooks et al., 1987; Fisher et al., 1987; Duperron et al., 2009, 2014). Such symbionts use either reduced sulfur compounds like hydrogen sulfide or methane as electron donors, with oxygen as electron acceptor, providing the invertebrate hosts with nutrition (Petersen and Dubilier, 2010). The host animal provides a stable environment, electron donors, and electron acceptors for the symbionts (Petersen and Dubilier, 2010).

The first report of mussels living in symbiosis with thiotrophic bacteria was from a hydrothermal vent site (Rau and Hedges, 1979), followed by reports from methane seeps, where mussels were found to harbor abundant intracellular methanotrophic bacteria in their gills (Childress et al., 1986). To date, more than 10 species of chemosymbiotic bathymodiolin mussels and clams have been reported from methane seep habitats and whale falls (Cordes et al., 2009; Faure et al., 2015; Duperron and Gros, 2016; Feng et al., 2018; Lan et al., 2019; Xu et al., 2019). It has been demonstrated that some bathymodiolin mussels host either thiotrophic (Cavanaugh, 1983; Distel et al., 1988) or aerobic methanotrophic bacteria, others harbor both types of symbionts, a phenomenon referred to as dual symbiosis (Childress et al., 1986; Fisher et al., 1993; Fiala-Médioni et al., 2002; Cordes et al., 2009; Xu et al., 2019). Putative chemosymbiotic invertebrates were also common at ancient seeps (Kiel, 2010; Kiel and Peckmann, 2019; Kiel et al., 2021).

The phylogeny of bacterial symbionts varies with chemosymbiotic invertebrate species, habitat characteristics, and locations (Nelson and Fisher, 2000; Thornhill et al., 2008; Lorion et al., 2013; Laming et al., 2015; Szafranski et al., 2015; Duperron and Gros, 2016), but can also vary with symbiont populations in response to environmental change (Kádár et al., 2005; Bettencourt et al., 2008). For example different clades of symbionts were found for *Escarpia laminata* from Alaminos Canyon and the Florida Escarpment, both Gulf of Mexico (Nelson and Fisher, 2000; Thornhill et al., 2008). Furthermore, gradual loss and re-establishment of symbiosis in the gills of *Bathymodiolus azoricus* was observed when keeping the host in sulfide-free or sulfide-containing seawater, respectively (Kádár et al., 2005; Bettencourt et al., 2008). Fiala-Médioni et al. (2002) suggested the existence of dual symbiosis, providing the host with greater environmental tolerance and increased niche space. Unfortunately, symbiotic bacteria from hydrothermal vents and hydrocarbon seeps have not been cultured successfully to date (Franke et al., 2021). Symbiotic bacteria and their symbiotic associations between hosts and symbionts are typically studied with electron microscopy, enzymatic analyses, 16*S* *r*RNA work, as well as lipid biomarkers and their carbon stable isotope compositions (Duperron et al., 2009; Petersen and Dubilier, 2010; Feng et al., 2015).

The analysis of lipid biomarkers is not only a powerful tool to identify bacterial signatures in symbiotic invertebrates, but also to quantify the abundance of bacterial symbionts. Typically, the most abundant lipids are fatty acids. The fatty acid inventory of aerobic methanotrophic bacteria is characterized by the monounsaturated fatty acid (MUFA) $C_{16:1\omega9}$, but especially by the group-specific MUFAs $C_{16:1\omega8}$, and $C_{18:1\omega8}$. In contrast, thiotrophic symbionts produce the ubiquitous MUFAs $C_{16:1\omega7}$ and $C_{18:1\omega7}$ (Jannasch, 1985; Nichols et al., 1985; Jahnke and Nichols, 1986; Bowman et al., 1991; Guezennec and Fiala-Medioni, 1996), which are common constituents of bacteria and eukaryotes, and are also produced or taken up by the host animals. Polyunsaturated fatty acids (PUFAs) *n*-C_{18}, *n*-C_{20}, and *n*-C_{22} are most likely taken up and stored by the host after heterotrophic consumption (Fullarton et al., 1995; Pond et al., 2000) rather than produced by endosymbiotic bacteria (Dunstan et al., 1993; Kawashima and Ohnishi, 2003). Besides fatty acids, chemosymbiotic mussels contain bacteriohopanepolyols (aminotriol and aminotetrol), and various 4,4-dimethyl sterols and 4-methyl sterols, exclusively derived from symbiotic aerobic methanotrophic bacteria (Fang et al., 1993; Jahnke et al., 1995; Pond et al., 1998; Riou et al., 2010; Kellermann et al., 2012).

Apart from the lipids of endosymbiotic bacteria, lipid biomarkers and compound-specific carbon stable isotope compositions of free-living aerobic methanotrophic bacteria have been studied intensively (Summons et al., 1994; Jahnke et al., 1999; Schouten et al., 2000; Talbot et al., 2001; Cordova-Gonzalez et al., 2020). In contrast to the ubiquitous occurrence of Type I and X methanotrophs in diverse natural environments, including the marine realm, type II methanotrophs seem to be confined to terrestrial habitats (Knief, 2015). The identification

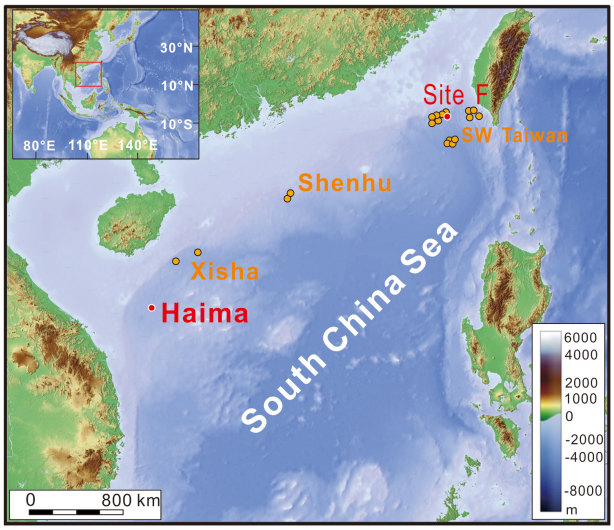

FIGURE 1 | Map showing the location of the sampling sites. Site F and Haima are active methane seeps (highlighted by red circles); orange circles indicate inactive methane seeps on the northern and western continental slope of the South China Sea.

of the different types of aerobic methanotrophs (Type I, X, and II) is commonly possible with biomarkers, since the different types of methanotrophs contain different biomarkers. For example, a high abundance of the MUFA $C_{16:1\omega8}$ was found in Type I and X methanotrophs, whereas MUFA $C_{18:1\omega8}$ tends to be predominant in Type II methanotrophs (Nichols et al., 1985; Bowman et al., 1991). On the other hand, 4-methyl steroids are highly specific biomarkers of Type I methanotrophs (e.g., Bouvier et al., 1976; Schouten et al., 2000). Information from lipid biomarkers can be complemented by their carbon stable isotope patterns, reflecting different carbon assimilation pathways of aerobic methanotrophs. Type-I and -X methanotrophs use the ribulose monophosphate (RuMP) pathway, whereas Type-II methanotrophs use the serine pathway (Anthony, 1982; Summons et al., 1994; Jahnke et al., 1999). The different assimilation pathways lead to distinct carbon isotopic fractionation (Jahnke et al., 1999; Cordova-Gonzalez et al., 2020). For example, Type I methanotrophs produce more highly ^{13}C-depleted lipids relative to Type II methanotrophs (Summons et al., 1994; Jahnke et al., 1999; Cordova-Gonzalez et al., 2020). Moreover, the δ^{13}C values of hopanoids and steroids synthesized by Type I methanotrophs are as much as 5–7‰ more negative than fatty acids, whereas in Type II methanotrophs, fatty acids are more depleted in ^{13}C than hopanoids (Summons et al., 1994; Jahnke et al., 1999). Therefore, lipid biomarkers alongside compound-specific carbon stable isotopes are primed to become part of the standard toolkit to discriminate the different types of symbionts and evaluate the nutritional status and associations between symbionts and hosts in chemosymbiosis.

We studied seep bivalves collected from two active seep sites of the South China Sea (**Figures 1**, **2**). The mussel *Gigantidas platifrons* was collected at Site F seep (also called Formosa ridge) at 1120 m water depth (Feng et al., 2015). One of the mussels and a clam retrieved from the Haima seep at 1390 m water depth were identified as new species and described as *G. haimaensis* and *A. marissinica* (Liang et al., 2017). The other mussel, *Bathymodiolus aduloides,* was collected at the Haima seep at 1390 m water depth. Transmission electron microscopy and 16S *r*RNA-encoding gene sequence analyses indicated that *G. platifrons* and *G. haimaensis* contain mainly aerobic methanotrophic bacteria as symbionts in their gills (Barry et al., 2002; Xu et al., 2019), whereas *B. aduloides* and *A. marissinica* were reported to live in symbiosis with thiotrophic bacteria (Feng et al., 2018; Lan et al., 2019). In this study, lipid biomarkers and compound-specific carbon stable isotope compositions were used to examine carbon assimilation pathways, nutritional status, and different host–symbiont associations in various environments, hosts, and symbionts of the four species of seep-dwelling bivalves from the South China Sea.

MATERIALS AND METHODS

Sample Collection and Storage

Specimens of *G. platifrons* were collected in May 2018 using the ROV *ROPOS* during a cruise with the Research Vessel "*Tan Kah Kee*" to Site F. Specimens of *G. haimaensis*, *A. marissinica*, and *B. aduloides* were retrieved in April 2018 and May 2019 from the Haima seep using the ROV *Haima* (**Table 1**). At the sampling sites, dense *G. platifrons*, *G. haimaensis*, and *A. marissinica* clusters were observed, respectively, covering large areas of the seabed. Several *B. aduloides* were scattered among carbonate blocks. Bottom water temperatures at both Site F and Haima seep were 3.0 to 3.5°C. After sampling, bivalves were stored onboard at −80°C and shipped to the home laboratory and kept at −25°C. The bivalves were dissected into gill, foot, and remaining tissue, immediately freeze-dried, ground, homogenized and kept at −25°C until further analysis.

Lipid Extraction and Analysis

Lipids were extracted from the gill and foot tissues of *G. platifrons*, *G. haimaensis*, *A. marissinica*, and *B. aduloides*. The homogenate was extracted with the solvents dichloromethane (3×), methanol:dichloromethane (1:1, v:v) (3×), and methanol (3×) using an ultrasonic bath to obtain the total lipid extracts (TLE). An aliquot of the TLE was saponified after addition of internal standards (19-methylarachidic acid, and tridecyl alcohol) with 6% KOH (w/v) in methanol (2 h at 70°C). Unfortunately, the concentration of the 19-methylarachidic acid standard in the fatty acid fraction was too low in comparison with the target compounds, resulting in a potential error in the quantification of fatty acids. The absolute contents of fatty acids should consequently not be taken at face value. Therefore, the relative portions of carboxylic acids and alcohols of the overall compound inventory of the respective fractions are provided in **Tables 2**, **3** in addition to absolute contents. The neutral fractions were extracted from the saponified TLE with *n*-hexane. To obtain carboxylic acids, the residuals were treated with 10% HCl to pH = 2 and extracted using *n*-hexane. It needs to be stressed

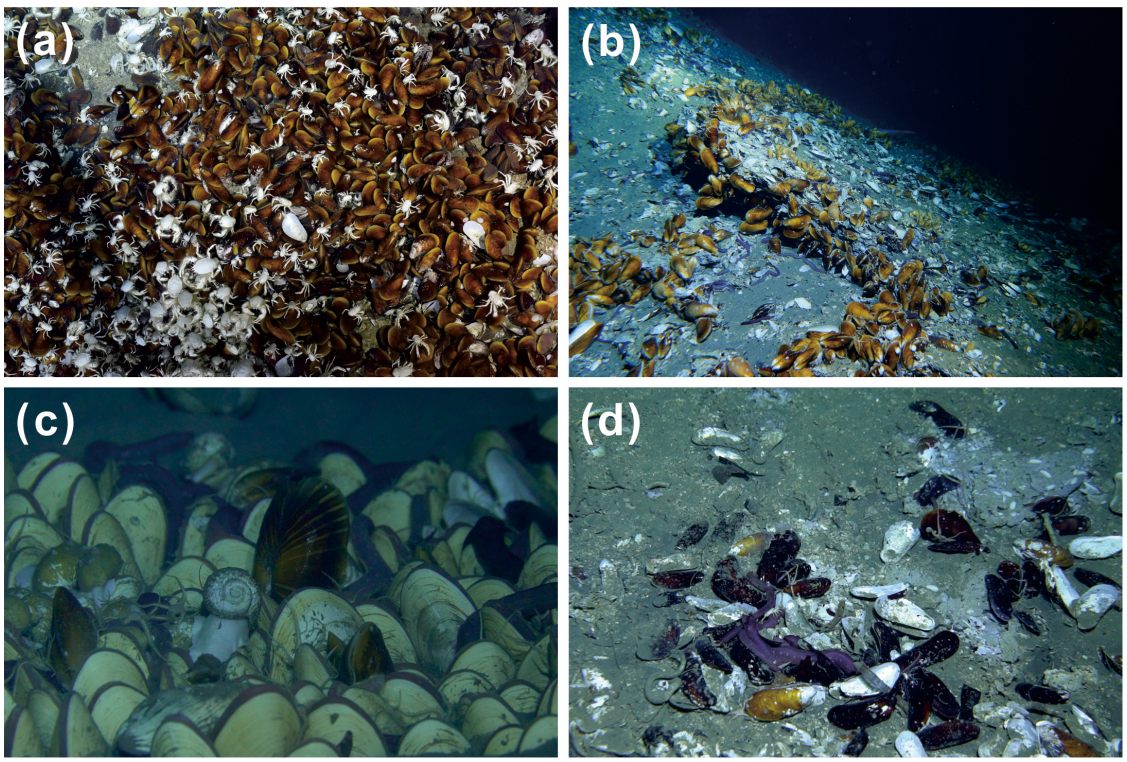

FIGURE 2 | Seafloor images from Site F **(a)** and Haima **(b–d)** methane seeps and the four species of bivalves *Gigantidas platifrons* **(a)**, *Gigantidas haimaensis* **(b)**, *Archivesica marissinica* **(c)**, and *Bathymodiolus aduloides* **(d)**.

TABLE 1 | Information on the four studied seep bivalves.

Host species	Sampling location	Habitat	Gammaproteobacterial endosymbionts	Associated Epsilonproteobacterial bacteria	References for symbionts
Gigantidas platifrons	South China Sea/Sagami Bay/Okinawa Trough	Hydrothermal vents/methane seeps	Methanotrophic bacteria	Yes	Duperron et al., 2009; Feng et al., 2015; Assié et al., 2016
Gigantidas haimaensis	South China Sea	Methane seeps	Methanotrophic bacteria	Yes	Xu et al., 2019
Archivesica marissinica	South China Sea	Methane seeps	Thiotrophic bacteria	n.d.	Lan et al., 2019 and this study
Bathymodiolus aduloides	South China Sea	Methane seeps	Thiotrophic bacteria	n.d.	Feng et al., 2018 and this study

n.d., not detected.

that bacteriohopanepolyols (BHPs) are commonly present in aerobic methanotrophic bacteria; however, we decided to focus on fatty acids and sterols and did not analyze BHPs in this study. The neutral fractions were silylated with bis(trimethylsilyl) trifluoroacetamide (BSTFA) for 3 h at 70°C, and the acid fractions were methylated with 14% BF_3-methanol in a screwcap vial (2 h at 60°C). To determine double bond positions of monounsaturated fatty acids, fatty acid methyl esters in *n*-hexane (50 μl) were treated with dimethyl disulfide (DMDS, 100 μ1) and iodine solution (500 μl; 6% w/v in diethyl ether), filled with argon and stored at 50°C for 48 h. After cooling, iodine was removed by shaking with drops of 5% (w:v) aqueous sodium thiosulfate (Nichols et al., 1986; Guan et al., 2021). The organic phase was removed and the aqueous phase was extracted with *n*-hexane to obtain the DMDS adducts. All fractions were analyzed with a Thermo Electron Trace GC–MS equipped with a 60-m DB-5 MS fused silica capillary column (0.32 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.2 ml/min. Compound-specific carbon stable isotope analysis was performed on a GV Isoprime system interfaced to a Hewlett-Packard 6890 gas chromatograph at the Guangzhou Institute of Geochemistry, Chinese Academy of Sciences (GIG, CAS). The same chromatographic column was used for GC-MS analysis. The following GC temperature program was applied for GC-MS and GC-IRMS: initial temperature was held at 60°C for 2 min, from 60°C to 160°C at a ramp of 10°C/min; from 160

TABLE 2 | Contents and isotopic compositions of fatty acids (FAs) from bivalve tissues.

	Gigantidas platifrons				*Gigantidas haimaensis*				*Archivesica marissinica*				*Bathymodiolus aduloides*			
	Gill		Foot		Gill		Foot		Gill		Foot		Gill		Foot	
	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)
Fatty acids																
$nC_{14:0}$	0.03 (<1)	n.d.	0.01 (<1)	n.d.	0.01 (<1)	n.d.	0.01 (<1)	n.d.	19.71 (9)	−45	0.13 (2)	−44	0.37 (1)	n.d.	<0.01	−44
$nC_{15:0}$	0.03 (<1)	n.d.	0.01 (<1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 (<1)	n.d.
$nC_{16:1\omega9}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06 (1)	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{16:1\omega8}$	5.64 (12)	−75[a]	n.d.	n.d.	0.93 (9)	−72[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{16:1\omega7c}$	11.17 (24)	n.d.	0.71 (8)	−70	0.94 (9)	n.d.	0.49 (5)	−65	92.81 (43)	−41	0.76 (10)	−43	13.08 (30)	−44	0.77 (4)	−46
$nC_{16:1\omega7t}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.62 (4)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{16:1\omega5}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.99 (<1)	n.d.	n.d.	n.d.	0.62 (1)	−50	0.04 (<1)	n.d.
$nC_{16:2}$	4.19 (9)	n.d.	0.03 (<1)	n.d.	0.06 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{16:0}$	7.71 (16)	−74	1.54 (17)	−64	1.30 (12)	−67	1.62 (16)	−63	15.74 (7)	−40	0.88 (12)	−41	4.44 (10)	−41	2.63 (14)	−42
$nC_{17:0}$	0.14 (<1)	n.d.	0.15 (2)	−68	0.09 (1)	−68	0.06 (1)	−66	n.d.	n.d.	n.d.	n.d.	0.27 (1)	−40	0.20 (1)	−52
$nC_{18:3}$	1.79 (4)	n.d.	0.39 (4)	−59	0.05 (<1)	−58	0.15 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	1.42 (3)	n.d.	1.07 (6)	n.d.
$nC_{18:4}$	1.73 (4)	n.d.	0.31 (3)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.19 (7)	−44	1.48 (8)	n.d.
$nC_{18:2}$	0.15 (<1)	n.d.	0.04 (<1)	n.d.	0.04 (<1)	n.d.	0.03 (<1)	n.d.	n.d.	n.d.	n.d.	n.d.	0.52 (1)	n.d.	0.17 (1)	n.d.
$nC_{18:2}$	0.62 (1)	n.d.	0.02 (<1)	n.d.	0.02 (<1)	n.d.	0.02 (<1)	n.d.	3.52 (2)	n.d.	0.10 (1)	−50	0.57 (1)	n.d.	0.19 (1)	n.d.
$nC_{18:1\omega9}$	n.d.	n.d.	n.d.	n.d.	0.04 (<1)	n.d.	0.04 (<1)	n.d.	n.d.	n.d.	n.d.	n.d.	0.34 (1)	n.d.	0.22 (1)	n.d.
$nC_{18:1\omega8}$	1.21 (3)	n.d.	n.d.	n.d.	0.08 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{18:1\omega7c}$	3.09 (7)	−72	0.72 (8)	−62	0.46 (4)	−65	0.51 (5)	−56.	4.30 (2)	n.d.	0.16 (2)	−45	3.12 (7)	−37	1.11 (6)	−46
$nC_{18:1\omega7t}$[b]	1.59 (3)	n.d.	0.11 (1)	n.d.	0.06 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{18:1\omega5}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.44 (1)	−39	0.45 (2)	−46
$nC_{18:0}$	1.25 (3)	−75	0.93 (10)	−67	0.53 (5)	−64	0.56 (5)	−61	5.34 (2)	−41	0.37 (5)	−37	1.40 (3)	−40	1.38 (7)	−40
$nC_{19:1\omega7}$	n.d.	n.d.	n.d.	n.d.	0.08 (1)	n.d.	0.06 (1)	n.d.	2.79 (1)	n.d.	0.10 (1)	−45	n.d.	n.d.	n.d.	n.d.
$nC_{20:4}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.31 (3)	n.d.	1.30 (7)	n.d.
$nC_{20:3}$	0.12 (<1)	n.d.	0.09 (1)	n.d.	0.06 (1)	n.d.	0.18 (2)	−54	n.d.	n.d.	n.d.	n.d.	0.23 (1)	n.d.	0.39 (2)	n.d.
$nC_{20:3}$	0.90 (2)	n.d.	0.39 (4)	−71	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.52 (1)	n.d.	0.39 (2)	n.d.
$nC_{20:2}$	1.37 (3)	−75	0.80 (9)	−71	0.70 (7)	−64	n.d.	−61	28.09 (13)	−41	0.14 (2)	−37	2.17 (5)	n.d.	1.61 (8)	n.d.
$nC_{20:2}$	1.35 (3)	n.d.	0.44 (5)	−76	0.40 (4)	n.d.	0.38 (4)	n.d.	9.79 (5)	−51	0.90 (12)	−47	3.96 (9)	−42	1.74 (9)	−43
$nC_{20:2}$	0.45 (1)	n.d.	0.06 (1)	−71	n.d.	n.d.	n.d.	n.d.	2.47 (1)	n.d.	n.d.	n.d.	0.16 (<1)	n.d.	0.03 (<1)	n.d.
$nC_{20:1\omega9}$	0.30 (1)	n.d.	0.42 (5)	n.d.	0.36 (3)	n.d.	0.47 (5)	−72	n.d.	n.d.	n.d.	n.d.	0.59 (1)	−36	0.67 (4)	−37
$nC_{20:1\omega7}$	1.73 (4)	−68	0.49 (5)	−68	1.09 (10)	−61	0.89 (9)	−61	18.82 (9)	−40	1.13 (16)	−44	3.03 (7)	−44	1.26 (7)	−46
$nC_{22:4}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.03 (<1)	n.d.	0.08 (<1)	n.d.

(Continued)

TABLE 2 | (Continued)

	Gigantidas platifrons				*Gigantidas haimaensis*				*Archivesica marissinica*				*Bathymodiolus aduloides*			
	Gill		Foot		Gill		Foot		Gill		Foot		Gill		Foot	
	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)	Content (mg/g dw), %	$\delta^{13}C$ (‰)
$nC_{22:3}$	0.04 (<1)	n.d.	0.13 (1)	n.d.	0.05 (<1)	n.d.	0.10 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	0.06 (<1)	n.d.	0.13 (1)	n.d.
$nC_{22:3}$	0.07 (<1)	n.d.	0.08 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04 (<1)	n.d.	0.05 (<1)	n.d.
$nC_{22:2}$	0.11 (<1)	n.d.	0.16 (2)	n.d.	0.06 (1)	n.d.	0.25 (2)	n.d.	n.d.	n.d.	0.20 (3)	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{22:2}$	0.47 (1)	n.d.	1.13 (12)	−69	2.46 (23)	−66	2.82 (27)	−65	n.d.	n.d.	0.59 (8)	−48	1.32 (3)	−44	1.40 (7)	−47
$nC_{22:1\omega9}$	n.d.	n.d.	n.d.	n.d.	0.77 (7)	n.d.	1.58 (15)	n.d.	n.d.	n.d.	0.35 (5)	n.d.	0.06 (<1)	n.d.	0.15 (1)	n.d.
$nC_{22:1\omega7}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.33 (2)	n.d.	1.42 (19)	−44	0.08 (<1)	n.d.	0.04 (<1)	n.d.
$nC_{22:0}$	n.d.	n.d.	n.d.	n.d.	0.03 (<1)	n.d.	0.07 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sum	47.24		9.16		10.59		10.24		215.30		7.29		42.56		18.76	
Average $\delta^{13}C$ value		−73		−68		−62		−61		−43		−44		−41		−43

[a] $C_{16:1\omega8}$ + $C_{16:1\omega7}$.
[b] $C_{18:1\omega7t}$ co-eluted with $C_{18:2}$.
n.d., not detected.

to 320°C at a ramp of 1°C/min, and then held at 320°C for 30 min. The analytes were identified by their retention times and comparison with published mass spectra. The carbon isotopic composition is expressed in the standard δ notation in per mil (‰) relative to Vienna-PeeDee Belemnite (V-PDB) standard. The fatty acid methyl esters (FAMEs) and trimethylsiloxyl (TMS) derivatives were corrected for the addition of carbon during the derivatization. The fatty acids were measured as FAMEs and were corrected for the addition of an extra carbon due to the derivatization according to the following equation:

$$\delta^{13}C_{FA} = [(C_{n+1})\delta^{13}C_{FAME} - \delta^{13}C_{MEOH}]/C_n$$

The $\delta^{13}C_{FA}$ indicates the $\delta^{13}C$ value of the fatty acid, C_n represents the number of carbons in the fatty acid, $\delta^{13}C_{FAME}$ is the $\delta^{13}C$ value of the FAME, and $\delta^{13}C_{MEOH}$ is the $\delta^{13}C$ value of the methanol used for the methylation reaction.

The sterols or *n*-alcohols were measured as TMS-derivatives and were corrected for the addition of three extra carbons according to the following equation:

$$\delta^{13}C_{alcohol} = [(C_{n+3})\delta^{13}C_{TMS-derivatives} - 3\delta^{13}C_{TMS}]/C_n$$

The $\delta^{13}C_{alcohol}$ and C_n denotes the $\delta^{13}C$ value and the number of carbons in the target biomarker, respectively; $\delta^{13}C_{TMS}$ is the $\delta^{13}C$ value of the TMS in BSTFA, and $\delta^{13}C_{TMS-derivatives}$ is the $\delta^{13}C$ value for the TMS-derivative.

Each sample was measured at least in duplicate and the average values were used. Measurements were calibrated by several pulses of carbon dioxide with known $\delta^{13}C$ value before and after each run. An *n*-alkane mixture (C_{12} to C_{35}) of known isotopic composition was used to check instrument precision. All measured fractions and their compounds were diluted to concentrations of about 20 μg/ml. The injection volume was 1 to 2 μl. Generally, $\delta^{13}C$ values of target compounds were only collected when peak heights were higher than one third of the reference carbon dioxide peaks (see **Supplementary Figure S1**). The standard deviation of the compound-specific carbon isotope measurement was <0.8‰.

RESULTS

Fatty Acids

Generally, the net contents of fatty acids were much higher in gill than in foot tissues of *G. platifrons, A. marissinica*, and *B. aduloides*. Only for *G. haimaensis*, the contents of fatty acids were similar in gill and foot tissues. In foot tissues, PUFAs C_{18}, C_{20}, and C_{22} were the most abundant fatty acids of *G. platifrons*, *G. haimaensis*, and *B. aduloides* with contents varying from 4 to 10 mg/g dry weight (dw). In contrast, both gill and foot tissues of *A. marissinica* were dominated by MUFAs. Among the MUFAs, $C_{16:1\omega7}$ was the most abundant fatty acid, corresponding to 24% of total fatty acids in the gill tissues of *G. platifrons*, 30% of total fatty acids in *B. aduloides*, and 43% of total fatty acids in *A. marissinica*. However, PUFA $C_{22:2}$,

TABLE 3 | Contents and isotopic compositions of sterols, hopanoid, and *n*-alcohols from bivalve tissues.

	Gigantidas platifrons				***Gigantidas haimaensis***				***Archivesica marissinica***				***Bathymodiolus aduloides***			
	Gill		**Foot**		**Gill**		**Foot**		**Gill**		**Foot**		**Gill**		**Foot**	
	Content (mg/g dw), (%)	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**	**Content (mg/g dw), (%)**	**$\delta^{13}C$ (‰)**
Sterols																
Cholesterol (I)	1.15 (44)	−78	0.44 (54)	−78	12.76 (21)	−74	11.64 (48)	−75	0.01 (17)	n.d.	0.02 (4)	n.d.	0.16 (46)	−40	0.21 (53)	−41
Cholestanol (II)	0.05 (2)	−81	0.24 (29)	−81	3.19 (5)	−75	2.39 (10)	n.d.	<0.01	n.d.	<0.01	n.d.	0.02 (6)	−46	0.05 (13)	−45
Cholesta-5,24-*dien*-3β-ol (III)	0.32 (12)	−84	n.d.	n.d.	9.47 (16)	−76	4.83 (20)	−78	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24-Methylenecholesterol (IV)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 (3)	−32	0.01 (3)	−30
cholest-7-*en*-3β-ol (V)	0.13 (5)	−80	n.d.	n.d.	7.16 (12)	−78	3.39 (14)	−76	n.d.	n.d.	n.d.	n.d.	0.01 (3)	−40	0.01 (3)	−40
4α-Methylcholesta-8(14),24-*dien*-3β-ol (VI)	0.41 (16)	−79	n.d.	n.d.	8.91 (15)	−80	0.55 (2)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4α-Methylcholesta-8(14)-*en*-3β-ol (VII)	0.04 (2)	n.d.	0.14 (17)	−79	2.48 (4)	n.d.	0.78 (3)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lanost-8(9)-*en*-3β-ol (VIII)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.07 (20)	−45	0.07 (18)	−45
Lanosterol (IX)	0.38 (15)	−82	n.d.	n.d.	15.20 (25)	−76	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06 (17)	−43	0.02 (5)	−42
Nor-lanosterol (X)	0.11 (4)	−83	n.d.	n.d.	0.78 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Stigmast-5-en-3β-ol (XI)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<0.01	n.d.	0.01 (2)	n.d.	n.d.	n.d.	n.d.	n.d.
Sum	2.57		0.82		59.94		23.58		0.01		0.03		0.33		0.39	
Average $\delta^{13}C$ value		−81		−79		−77		−76						−41		−41
$\Delta\delta^{13}C_{sterols-FAs}$		−8.0		−11		−13		−13						0		2
Hopanoid																
Diplopterol	0.02 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n-Alcohols																
$nC_{22:0}$	n.d.	n.d.	n.d.	n.d.	0.31 (1)	n.d.	0.33 (1)	n.d.	0.03 (50)	n.d.	0.40 (77)	n.d.	0.02 (6)	−35	0.03 (8)	−38
$nC_{28:0}$	n.d.	n.d.	n.d.	n.d.	0.19 (<1)	n.d.	0.13 (1)	n.d.	0.01 (17)	n.d.	0.07 (13)	n.d.	n.d.	n.d.	n.d.	n.d.
$nC_{30:0}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.32 (1)	n.d.	0.01 (17)	n.d.	0.02 (4)	n.d.	n.d.	n.d.	n.d.	n.d.
Sum	n.d.				0.50		0.77		0.06		0.52		0.02		0.03	

n.d., not detected.

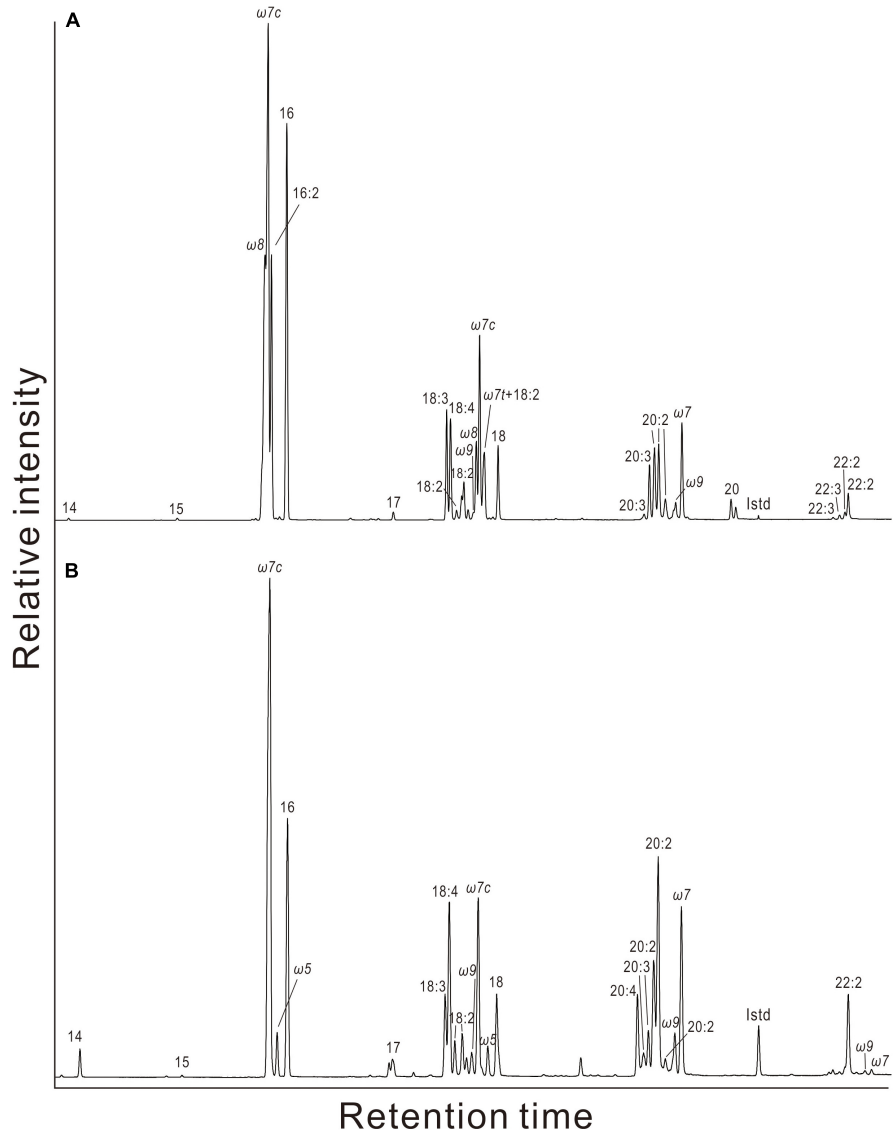

FIGURE 3 | Partial gas chromatograms of fatty acids in gill tissues of **(A)** *Gigantidas platifrons* and **(B)** *Bathymodiolus aduloides*. The ω indicates the double bond position of monounsaturated fatty acids (MUFAs), the suffix c is for *cis* and t for *trans*, referring to stereoisomers of MUFAs. Double bond positions of polyunsaturated fatty acids (PUFAs) have not been determined.

accounting for 23% of total fatty acids, dominated in the gill tissue of *G. haimaensis*. The fatty acids $C_{16:1\omega8}$ (12% and 9% of total fatty acids, respectively) and $C_{18:1\omega8}$ (3% and 1% of total fatty acids, respectively) were only observed in the gill tissues of *G. platifrons* and *G. haimaensis*. Generally, the fatty acids $C_{16:1\omega7}$, $C_{18:1\omega7}$, and $C_{20:1\omega7}$ were among the most abundant compounds of total fatty acids in gill and foot tissues of the four species, making up 32% and 22% for *G. platifrons*, 24% and 19% for *G. haimaensis*, and 58% and 28% for *A. marissinica*, and 44% and 17% for *B. aduloides*, respectively, with significantly higher relative abundances in gill tissues than in foot tissues. All relative percentages are referring to total fatty acids. Furthermore, $C_{16:1\omega5}$ was found in the gill tissue of *A. marissinica* in trace amounts (<1% of total fatty acids), while $C_{16:1\omega5}$ and $C_{18:1\omega5}$ were present in both gill and foot tissues of *B. aduloides* (**Figures 3**, **4**).

The fatty acids from the gill and foot tissues of *G. platifrons* and *G. haimaensis* were strongly depleted in ^{13}C, with $\delta^{13}C$ values ranging from −75‰ to −59‰ and −72‰ to −54‰, respectively. Compared to *G. platifrons* and *G. haimaensis*, *A. marissinica* and *B. aduloides* yielded moderately negative $\delta^{13}C$ values for fatty acids from −51‰ to −37‰ and −52‰ to −36‰, respectively (**Table 2**).

Lipid Biomarkers in the Alcohol Fractions

Various sterols were found in the gill and foot tissues of *G. platifrons*, *G. haimaensis*, *A. marissinica*, and *B. aduloides* (**Table 3** and **Figure 5**). Even though the inventory of sterols

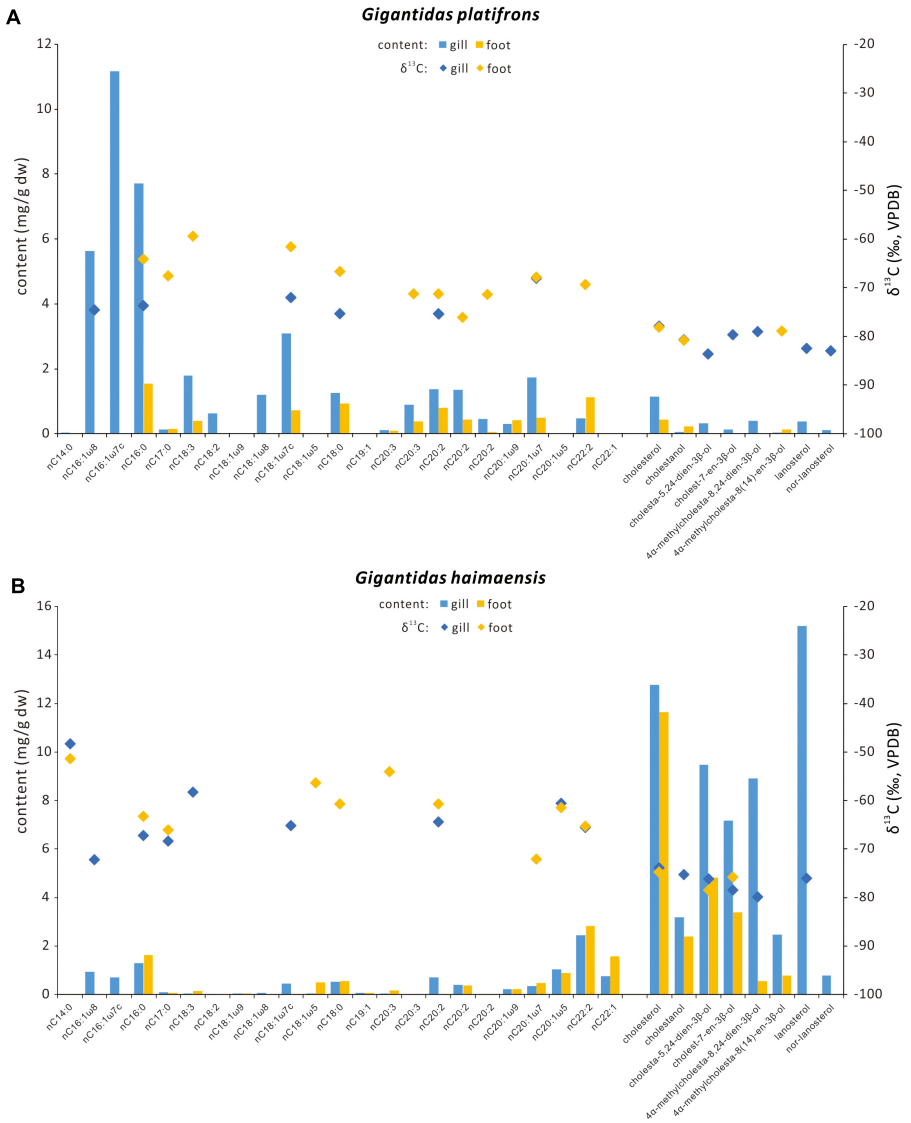

FIGURE 4 | Contents and carbon isotopic compositions of selected fatty acids and sterols for **(A)** *Gigantidas platifrons* and **(B)** *Gigantidas haimaensis*. Blue bars = contents of gill tissues; orange bars = contents of foot tissues; blue diamonds = δ^{13}C values of biomarkers from gill tissues; orange diamonds = δ^{13}C values of biomarkers from foot tissues.

was generally similar for *G. platifrons* and *G. haimaensis*, the contents of individual compounds varied significantly between the two species and types of tissues (**Figure 4**). The total content of sterols in the gill tissue of *G. haimaensis* was 60 mg/g dw, whereas the gill tissue content of sterols of *G. platifrons* was very low (2.6 mg/g dw). In *B. aduloides*, the sterol contents were even below 2 mg/g dw. Lanosterol (IX, see **Figure 6** for structure) and cholesterol (I) were the most abundant sterols in the gill tissue of both *G. haimaensis* (16 mg/g dw) and *G. platifrons* (1.1 mg/g dw), representing 25% and 44% of the total alcohols, respectively. In *B. aduloides*, cholesterol (I) was the most abundant sterol, accounting for 46% and 53% of the total alcohols in gill and foot tissues, respectively, followed by lanost-8(9)-*en*-3β-ol (VIII), lanosterol, cholestanol, 24-methylenecholesterol (IV), and cholest-7-*en*-3β-ol (**Table 3** and **Figure 7**). In the foot tissue, cholesterol (I) was most abundant in *G. platifrons* and *G. haimaensis* (0.4 mg/g dw and 12 mg/g dw, respectively), but was slightly more abundant in the gills (1.1 mg/g dw and 13 mg/g dw, respectively). Lanosterol, though, was not found in any of the foot tissue samples.

Other sterols, such as *nor*-lanosterol (X), cholestanol (II), cholesta-5,24-*dien*-3β-ol (III), cholest-7-*en*-3β-ol (V), 4α-methylcholesta-8(14),24-*dien*-3β-ol (VI), and 4α-methylcholesta-8(14)-*en*-3β-ol (VII), were as well more prominent in the gill tissues than in foot tissues of *G. platifrons* and *G. haimaensis*. In *B. aduloides*, the composition and contents of sterols in the foot tissue are similar to gill tissue. The single

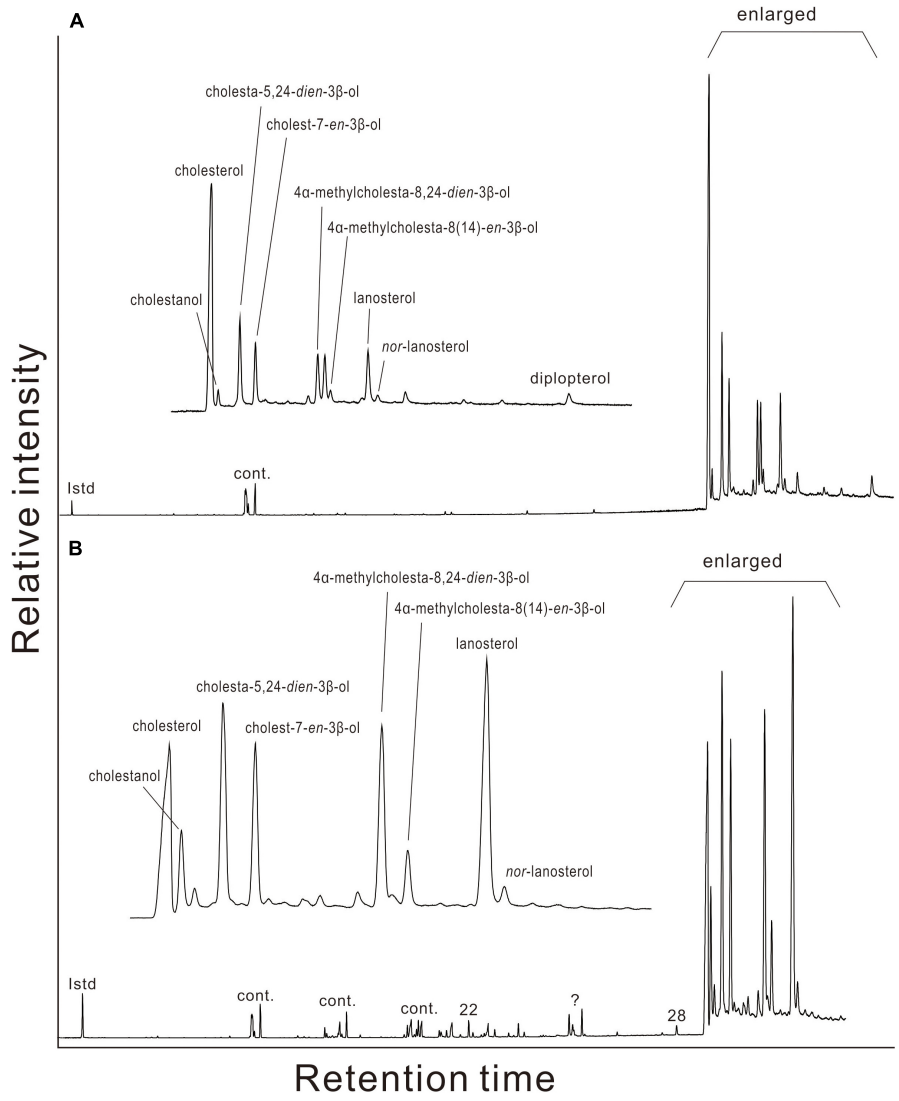

FIGURE 5 | Gas chromatograms of alcohols in gill tissues of **(A)** *Gigantidas platifrons* and **(B)** *Gigantidas haimaensis*. Roman numerals indicate *n*-alcohols; ? denotes presently unknown; Cont. = contaminant.

hopanoid, found only in traces in the gills of *G. platifrons*, was diplopterol (**Figure 5**). Minute amounts of cholesterol (I), cholestanol (II), and stigmast-5-en-3β-ol (XI) were found in *A. marissinica*. The total sterols accounted for 17% and 6% of all alcohols in the gill and foot tissues, respectively, with relatively higher contents in the foot than in the gill.

The *n*-alcohols $C_{22:0}$, $C_{28:0}$, and $C_{30:0}$ were found in gill and foot tissues of *G. haimaensis*, *A. marissinica*, and *B. aduloides*. The gill and foot tissues revealed similar ^{13}C depletion for sterols, with $\delta^{13}C$ values varying from −84‰ to −78‰ and −80‰ to −74‰ for *G. platifrons* and *G. haimaensis*, respectively. For *B. aduloides*, cholesterol, cholestanol, cholest-7-*en*-3β-ol, and the 4,4-dimethyl sterols (lanosterol and lanost-8(9)-*en*-3β-ol) in both gill and foot tissues yielded $\delta^{13}C$ values from −46‰ to −40‰, whereas the $\delta^{13}C$ values of 24-methylenecholesterol in gill and foot tissues ranged from −32‰ to −30‰.

DISCUSSION

Lipid Biomarker Signatures of Aerobic Methanotrophic Symbionts and Carbon Assimilation of *Gigantidas platifrons* and *Gigantidas haimaensis*

Both *G. platifrons* (Site F) and *G. haimaensis* (Haima seeps) were referred to as *B. platifrons* previously (Feng et al., 2015, 2018) and were reported to host methanotrophs (Duperron et al., 2009; Feng et al., 2015; Assié et al., 2016; Xu et al., 2019). Xu et al. (2019) analyzed the microbial 16S *r*RNA genes in gill tissue of *G. haimaensis* collected from the Haima seep site, and found that the bacteria were dominated by three phylotypes of symbiotic Gammaproteobacteria and three phylotypes of symbiotic Epsilonproteobacteria. The most

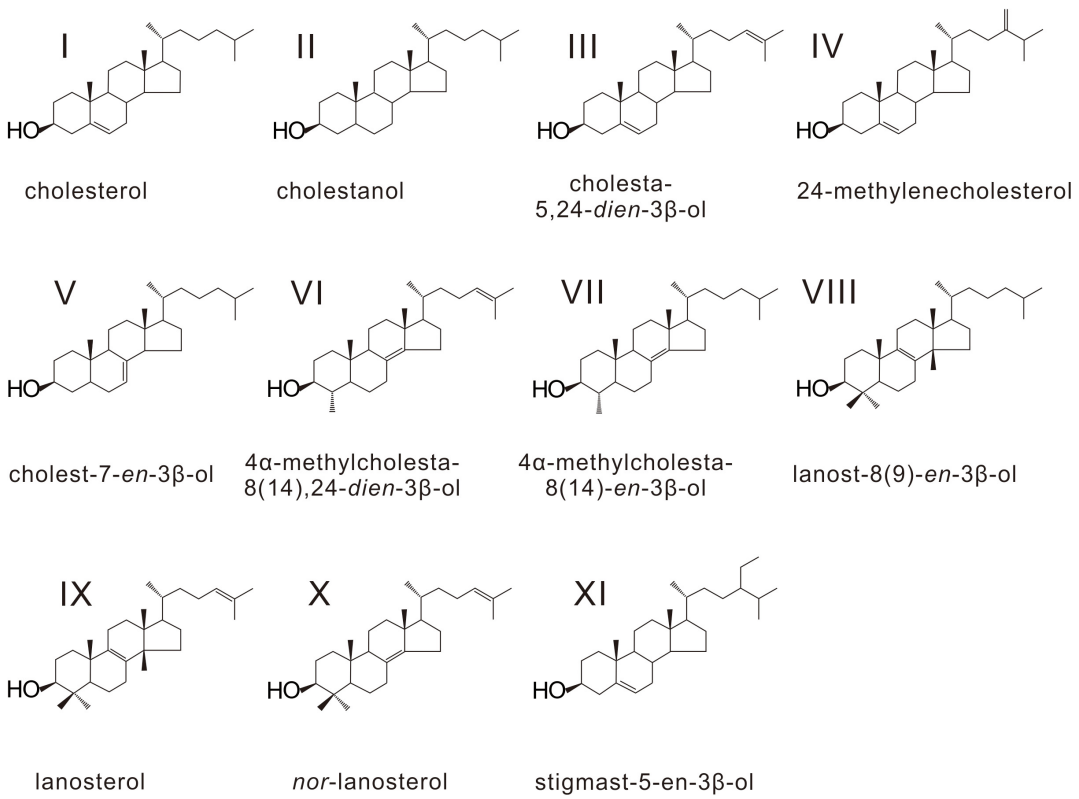

FIGURE 6 | Structures of sterols found in soft tissues of seep bivalves in the South China Sea.

abundant gammaproteobacterial phylotype of *G. haimaensis* clustered close to methanotrophic Gammaproteobacteria of other *Gigantidas* species and were assigned to Type I aerobic methanotrophic bacteria (Xu et al., 2019). Given Type II methanotrophs have never been reported in the marine realm (Knief, 2015), the symbionts harbored in *G. platifrons* are likely to be Type I methanotrophs too. Type I methanotrophs are the most prominent symbionts in the gills of both *G. platifrons* and *G. haimaensis*, which is supported by characteristic fatty acid inventories, including *cis* and *trans* isomers of $C_{16:1}$ with double bonds at ω6, ω7, ω8, and ω9 (Bowman et al., 1993) and a predominance of $C_{16:1\omega8}$ fatty acid over $C_{18:1\omega8}$ fatty acid (cf. Nichols et al., 1985; Bowman et al., 1991; Niemann et al., 2006; Riou et al., 2010).

In addition to fatty acids, 4,4-dimethyl sterols (lanosterol and *nor*-lanosterol), 4-methyl sterols (4α-methylcholesta-8(14),24-*dien*-3β-ol and 4α-methylcholesta-8(14)-*en*-3β-ol), and desmethyl sterols (cholesterol, cholestanol, cholesta-5,24-*dien*-3β-ol, and cholest-7-*en*-3β-ol) were observed in soft tissues of *G. platifrons* and *G. haimaensis*. The absolute and relative contents of sterols are generally different, for example lanosterol accounted for 15% and 25% of the total alcohols in gills of *G. platifrons* and *G. haimaensis*, respectively. However, the overall inventory of sterols of *G. platifrons* and *G. haimaensis* is similar (**Table 3**). Cholesterol is the most abundant sterol in the gill of *G. platifrons* (44%), whereas in *G. haimaensis* lanosterol dominates (25%; **Figure 4**). Cholesterol and other desmethyl sterols are common in eukaryotes, but lanosterol-like 4,4-dimethyl sterols and 4-methyl sterols are only present in free-living (Jahnke and Nichols, 1986; Cordova-Gonzalez et al., 2020) and endosymbiotic aerobic methanotrophs among bacteria (Jahnke et al., 1995; Kellermann et al., 2012). Both 4,4-dimethyl sterols and 4-methyl sterols are demethylation products of lanosterol in aerobic methanotrophic bacteria (*Methylococcus capsulatus*; Bouvier et al., 1976; Summons et al., 1994). Based on similar ^{13}C-depletions and the co-occurrence of 4-methyl sterol intermediates and desmethylated cholesterol in soft tissues other than gills, cholesterol was supposed to be synthesized by the animal host using bacterial 4-methyl sterols as diet (Jahnke et al., 1995). For *G. platifrons* and *G. haimaensis*, 4-methyl sterols and cholesterol occurred in both gill and foot tissues and yielded similar $\delta^{13}C$ values, indicating that 4-methyl sterols are most likely biosynthetic intermediates and represent metabolic precursors in cholesterol biosynthesis. The high abundance of 4,4-dimethyl sterols and 4-methyl sterols agrees with the presence of Type I methanotrophs, which are the only bacteria known to synthesize these source-specific compounds in noteworthy amounts (Bird et al., 1971; Schouten et al., 2000; Wei et al., 2016; Cordova-Gonzalez et al., 2020). In culture, *Methylococcus capsulatus* biosynthesizes fatty acids with significant ^{13}C-depletion compared to methane, with Δ values ranging from −25‰ to −17‰ (Jahnke et al., 1999), whereas the

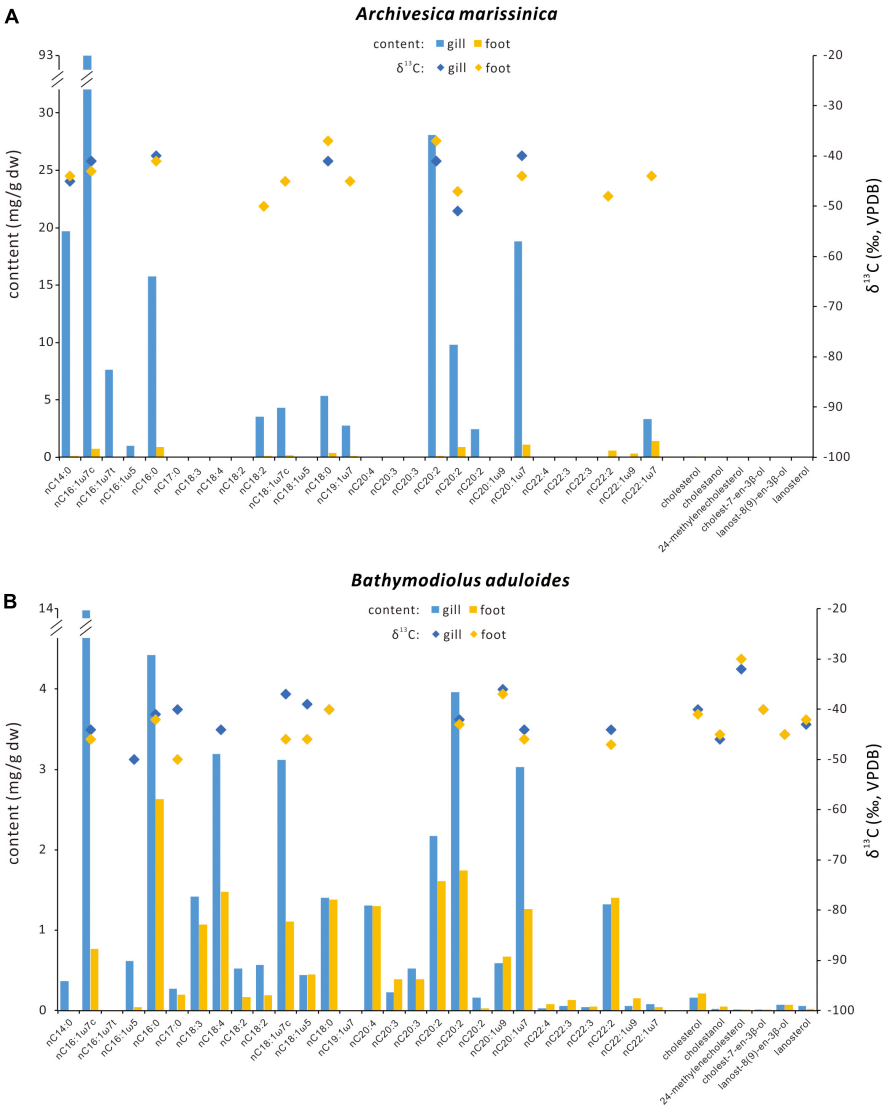

FIGURE 7 | Contents and carbon isotopic compositions of selected fatty acids and sterols for **(A)** *Archivesica marissinica* and **(B)** *Bathymodiolus aduloides*. Blue bars = contents of gill tissues; orange bars = contents of foot tissues; blue diamonds = $\delta^{13}C$ values of biomarkers from gill tissues; orange diamonds = $\delta^{13}C$ values of biomarkers from foot tissues.

fatty acids in the gills of type I methanotroph-containing mussels were more ^{13}C-depleted by ca. −16‰ (Jahnke et al., 1995). At site F and Haima seeps, available $\delta^{13}C$ values of methane approximately correspond to −60‰ (Feng et al., 2015; Fang et al., 2019). In the gills of *G. platifrons* and *G. haimaensis*, the symbiont-specific $C_{16:1\omega8}$ fatty acid and most fatty acids yielded $\delta^{13}C$ values from −75‰ to −72‰ and −75‰ to −65‰, respectively, which is consistent with fractionation patterns of fatty acids in type I/X methanotrophs and mussels that live in symbiosis with type I methanotrophs (Jahnke et al., 1995; Kellermann et al., 2012). Further, both 4,4-dimethyl sterols and 4-methyl sterols were more ^{13}C-depleted than fatty acids, with an average $\Delta\delta^{13}C_{sterols-fatty\ acids}$ between −13‰ and −8‰, similar to values reported from laboratory cultures (Summons et al., 1994; Jahnke et al., 1999; Cordova-Gonzalez et al., 2020). As for fatty acids, the isotopic pattern of sterols confirms a predominance of Type I and/or X methanotrophs (cf. Cordova-Gonzalez et al., 2020).

Although $\delta^{13}C$ values of fatty acids and sterols were uniform for *G. platifrons* and *G. haimaensis*, contents of the various lipids varied in the two mussels. While the same fatty acid inventory was found in gill and foot tissues of both mussels, contents of fatty acids were much higher in *G. platifrons* (see **Figure 4**), whereas both 4,4-dimethyl and 4-methyl sterols were more abundant in *G. haimaensis*. The strong variation in the membrane lipid composition, especially the ratio of fatty acids over sterols, is most likely caused by variation of oxygen levels in the environment. Such an effect was shown by

Jahnke and Nichols (1986) for *Methylococcus capsulatus* grown under varying oxygen tensions, revealing that sterols (4-methyl and 4,4-dimethyl sterols) and phospholipids were most abundant in cells grown with 0.5 and 1.1% oxygen. As oxygen was reduced, the amount of MUFA $C_{16:1}$ decreased but the 4,4-dimethyl sterols were predominantly produced at very low oxygen tensions (0.1%). Except for type X methanotrophs, oxygen level affecting membrane lipid composition was also reported for *Methylobacterium organophilum* (Type II methanotroph; Patt and Handon, 1978). Bathymodiolin mussels acquire symbionts horizontally and may take up various types and different amounts of symbionts in response to varying environmental conditions (Won et al., 2003; Kádár et al., 2005; Franke et al., 2021), which may also affect the biosynthesis of membrane lipids. In the present study, *G. platifrons* yielded significantly higher contents of MUFA $C_{16:1}$ than *G. haimaensis*, which may reflect a higher oxygen level for *G. platifrons*. Although no oxygen concentrations have been reported for the study sites, applying these observations to the biomarker patterns in *G. haimaensis* suggests that *G. haimaensis* most likely lived at a low level of dissolved oxygen.

Apart from the symbiont-specific fatty acids, the MUFAs C_{20} and C_{22}, as well as PUFAs C_{20} and C_{22} of the animal host (Conway and McDowell-Capuzzo, 1991; Pond et al., 1998) were present in both *G. platifrons* and *G. haimaensis*. In chemotrophic symbioses, some bivalves are highly dependent on the metabolic intermediates transferred by the symbionts, which leads to a similar range of $\delta^{13}C$ values for the lipids produced by hosts and symbionts (Jahnke et al., 1995; Pond et al., 1998; Kellermann et al., 2012). With respect to the seep-dwelling bivalves from the South China Sea, most symbiont- and host-specific fatty acids of *G. platifrons* and *G. haimaensis* yielded $\delta^{13}C$ values from −75‰ to −68‰ and −72‰ to −60‰, respectively, indicating that the host obtained most of its nutrition either directly from the symbionts or consumes symbiont-derived carbon.

Nutritional Signatures in *Archivesica marissinica* and *Bathymodiolus aduloides*

Membrane lipids of free-living sulfide-oxidizing bacteria are usually characterized by prominent ω7 fatty acids with either $C_{16:1\omega7}$ or $C_{18:1\omega7}$ predominating (Jannasch, 1985; Guezennec and Fiala-Medioni, 1996; Arning et al., 2008). These compounds were also found to be abundant in thiotrophic bacteria living in mussels (Pond et al., 1998; Kellermann et al., 2012) and mussels cultured in the presence of hydrogen sulfide (Riou et al., 2010). The high abundance of MUFAs $C_{16:1\omega7}$ and $C_{18:1\omega7}$ and the absence of 4-methyl sterols in *A. marissinica* (yielding cholesterol, cholestanol, and stigmast-5-en-3β-ol instead) agrees with thiotrophic bacteria as only endosymbionts.

For *B. aduloides*, in addition to the abundant MUFAs $C_{16:1\omega7}$ and $C_{18:1\omega7}$, a range of sterols, including cholesterol, cholestanol, 24-methylenecholesterol, cholest-7-*en*-3β-ol, lanost-8(9)-*en*-3β-ol, and lanosterol, were found in both gill and foot tissues. It has been demonstrated that some mussels are capable of synthesizing sterols (Teshima and Kanazawa, 1974; Teshima and Patterson, 1981). Sterols in soft tissues of symbiont-containing mussels may originate from its symbionts, diets from ingested plant and algal material, or synthesis by the animal host. For *B. aduloides*, a derivation of sterols except for 24-methylenecholesterol from filter-feeding is unlikely, because sterols synthesized by plants and algae are characterized by high abundance of sterols alkylated at C-24 containing either a methyl group or ethyl group (Jarzebski and Popov, 1985; Volkman, 2003, 2005). Furthermore, such photosynthetically derived carbon reveals $\delta^{13}C$ values around −30‰ to −20‰ (Conway and McDowell-Capuzzo, 1991; Kellermann et al., 2012), which is inconsistent with the carbon stable isotopic pattern of the *B. aduloides* sterols (from −46‰ to −40‰). Feng et al. (2018) reported $\delta^{13}C_{biomass}$ values (−37‰ to −33‰) in soft tissues of *B. aduloides* from Site F of the South China Sea and concluded that the mussels host thiotrophic bacteria. However, the thiotrophic symbionts are not a potential source candidate for sterols, since no sulfide-oxidizing bacteria have been shown to synthesize sterols to date (McCaffrey et al., 1989; Conway and McDowell-Capuzzo, 1991). Given the absence of ω8 fatty acids, a derivation of 4,4-dimethyl sterols (lanosterol and lanost-8(9)-*en*-3β-ol) from aerobic methanotrophic bacteria is highly unlikely as well.

Apart from the bacterial domain, eukaryotes including animals are known to produce lanosterol. Using ^{14}C-labeling in cultures revealed that the oyster *Crassostrea virginica* incorporated acetate to form squalene, 4,4-dimethylsterols, and 4-methylsterols, which can also convert lanosterol to cholesterol (Teshima and Patterson, 1981). These results suggest that the lanosterol in *B. aduloides* was most likely synthesized by the animal host. However, the lanost-8(9)-*en*-3β-ol as an intermediate has never been reported in aerobic methanotrophs and symbiont-containing invertebrates before, but it was found to co-occur with lanosterol in hyperlipidemic serum and rat livers (Gray et al., 1969; Scallen et al., 1971). As one of the intermediates in the conversion from lanosterol to cholesterol, the production of lanost-8(9)-*en*-3β-ol may occur as the first step when the side chain of lanosterol is reduced (Gray et al., 1969; Scallen et al., 1971). Although uncertainties are present, the absence of lanost-8(9)-*en*-3β-ol in aerobic methanotrophs is most likely caused by differences in biosynthetic steps from lanosterol to cholesterol between aerobic methanotrophs and animals.

In addition to nutrients supplied by the endosymbionts, *B. aduloides* complemented its diet by filter-feeding on photosynthetically derived carbon, as indicated by the presence of ^{13}C-enriched 24-methylenecholesterol ($\delta^{13}C$ values from −32‰ to −30‰) in both gill and foot tissues. Since consumers are generally ^{13}C-enriched by only 0.4‰ to 1‰ compared to their diet (McCutchan et al., 2003), these $\delta^{13}C$ values suggest higher plants or marine phytoplankton as sources (Goericke et al., 1994). Similar observations were made for the Pacific-Antarctic Ridge and the Gulf of Mexico. At the Pacific-Antarctic Ridge, a chemosynthesis-based community was observed to progressively shift its nutritional lifestyle to filter-feeding when diffuse hydrothermal venting vanished (Stecher et al., 2002). Reduction in symbiont abundances and changes in energy sources induced by decrease of hydrogen sulfide

concentration were also reflected by very low contents of symbiont-specific lipids and relatively ^{13}C-enriched cholesterol (−22‰) in *Bathymodiolus* cf. *thermophilus* from seeps of the Gulf of Mexico (Kellermann et al., 2012). Although both *A. marissinica and B. aduloides* contain thiotrophic bacteria, there are major differences when it comes to their symbionts. *A. marissinica* transmits the symbionts from parent to offspring (Ohishi et al., 2016; Lan et al., 2019), whereas *B. aduloides* acquires symbionts from the ambient environments for each new generation (Franke et al., 2021). Such differences in symbiont clades and amounts may lead to variations in the biomarker inventories of both hosts and symbionts. The above findings including our study confirm that lipid biomarkers and their isotope signatures are excellent tracers to decode varying environmental conditions and carbon and energy pathways in chemosynthesis-based ecosystems.

CONCLUSION

Four species of seep bivalves collected from Site F (*Gigantidas platifrons*) and Haima seeps (*Gigantidas haimaensis*, *Bathymodiolus aduloides*, and *Archivesica marissinica*) in the South China Sea were analyzed for lipid biomarkers and compound-specific carbon stable isotope patterns. *Gigantidas platifrons* and *Gigantidas haimaensis* were demonstrated to live in symbioses with methanotrophic bacterial symbionts as revealed by abundant ^{13}C-depleted lanosterol, *nor*-lanosterol, 4-methylsterols, and monounsaturated fatty acids $C_{16:1\omega9}$ and $C_{16:1\omega8}$ extracted from gill tissues. The relatively higher abundance of $C_{16:1\omega8}$ relative to $C_{18:1\omega8}$ and the occurrence of 4,4-dimethylsterols and 4-methylsterols agree with an assignment to type I methanotrophs. The much higher sterol contents and the less abundant fatty acids in *G. haimaensis* compared to *G. platifrons* probably reflect low oxygen levels during the growth of *G. haimaensis*. The gill tissues of both *B. aduloides* and *A. marissinica* contained significant amounts of $C_{16:1\omega7}$ and $C_{18:1\omega7}$ fatty acids, and their nutritional status was found to be different from *G. platifrons* and *G. haimaensis*. *A. marissinica* apparently contains only thiotrophic bacteria, indicated by the lack of ω8 fatty acids, 4,4-dimethylsterols, and 4-methylsterols. Based on the biomarker patterns, *B. aduloides* is also suggested to contain thiotrophic bacteria, but the animal host complemented its diet through filter-feeding as revealed by relatively ^{13}C-enriched 24-methylenecholesterol. The different types of 4-methyl sterols between the thiotroph-containing mussel (*B. aduloides*) and the methanotroph-containing mussels (*G. platifrons* and *G. haimaensis*) are likely caused by different biosynthetic steps from lanosterol to cholesterol between animal hosts and aerobic methanotrophs. Furthermore, the host-specific polyunsaturated fatty acids C_{20} and C_{22} in all four bivalve species yielded δ^{13}C values close to those of symbiont-specific fatty acids, suggesting that the hosts acquired most of their nutrition from the symbionts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because invertebrate studies at seeps are common.

AUTHOR CONTRIBUTIONS

HG, DF, and JT collected the samples. HG did the experiments. HG, DB, and JP analyzed the biomarker data. All authors contributed to the preparation of the manuscript.

FUNDING

This research was supported by NSF of China (Grant Number 91958105) and the Shandong Special Fund of Pilot National Laboratory for Marine Science and Technology (Qingdao; Grant Number 2021QNLM020002).

ACKNOWLEDGMENTS

We express our sincere appreciation to the crews of the *Haima*, *ROPOS*, *Haiyang-06*, and "*Tan Kah Kee*" Cruises for their professionalism in sampling. We thank S. Gao (GIG, CAS) for sample preparation and technical assistance. The manuscript benefited from insightful comments of the reviewers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.831286/full#supplementary-material

REFERENCES

Anthony, C. (1982). *The Biochemistry of Methylotrophs*. London: Academic Press.

Arning, E. T., Birgel, D., Schulz-Vogt, H. N., Holmkvist, L., Jørgensen, B. B., Larson, A., et al. (2008). Lipid biomarker patterns of phosphogenic sediments from upwelling regions. *Geomicrobiol. J.* 25, 69–82.

Assié, A., Borowski, C., van der Heijden, K., Raggi, L., Geier, B., Leisch, N., et al. (2016). A specific and widespread association between deep-sea Bathymodiolus mussels and a novel family of Epsilonproteobacteria. *Environ. Microbiol. Rep.* 8, 805–813. doi: 10.1111/1758-2229.12442

Barry, J. P., Buck, K. R., Kochevar, R. K., Nelson, D. C., Fujiwara, Y., Goffredi, S. K., et al. (2002). Methane-based symbiosis in a mussel, *Bathymodiolus platifrons*, from cold seeps in Sagami Bay, Japan. *Invertebrate Biol.* 121, 47–54. doi: 10.1111/j.1744-7410.2002.tb00128.x

Bettencourt, R., Dando, P., Rosa, D., Riou, V., Colaço, A., Sarrazin, J., et al. (2008). Changes of gill and hemocyte-related bio-indicators during long term

maintenance of the vent mussel *Bathymodiolus azoricus* held in aquaria at atmospheric pressure. *Comparat. Biochem. Physiol. Part A* 150, 1–7. doi: 10.1016/j.cbpa.2008.02.020

Bird, C. W., Lynch, J. M., Pirt, S. J., and Reid, W. W. (1971). Steroids and squalene in *Methylococcus capsulatus* grown on methane. *Nature* 230, 473–474. doi: 10.1038/230473a0

Bouvier, P., Rohmer, M., Benveniste, P., and Ourisson, G. (1976). Δ8(14)-Steroids in the bacterium *Methylococcus capsulatus*. *Biochem. J.* 159, 267–271. doi: 10.1042/bj1590267

Bowman, J. P., Skerrat, J. H., Nichols, P. D., and Sly, L. I. (1991). Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. *FEMS Microbiol. Lett.* 85, 15–21.

Bowman, J. P., Sly, L. I., Nichols, P. D., and Hayward, A. C. (1993). Revised taxonomy of the methanotrophs: description of *Methylobacter gen*. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int. J. Systematic Evol. Microbiol.* 43, 735–753.

Brooks, J. M., Kennicutt, M. C., Fisher, C. R., Macko, S. A., Cole, K., Childress, J. J., et al. (1987). Deep-sea hydrocarbon seep communities: evidence for energy and nutritional carbon sources. *Science* 238, 1138–1142. doi: 10.1126/science.238.4830.1138

Cavanaugh, C. M. (1983). Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats. *Nature* 302, 58–61. doi: 10.1016/j.scitotenv.2021.150054

Childress, J. J., Fisher, C. R., Brooks, J. M., Kennicutt, M. C., Bidigare, R., and Anderson, A. E. (1986). A methanotrophic marine molluscan (Bivalvia: Mytilidae) symbiosis: mussels fueled by gas. *Science* 233, 1306–1308. doi: 10.1126/science.233.4770.1306

Conway, N., and McDowell-Capuzzo, J. (1991). Incorporation and utilization of bacterial lipids in the *Solemya velum* symbiosis. *Mar. Biol.* 108, 277–291.

Cordes, E. E., Bergquist, D. C., and Fisher, C. R. (2009). Macro-ecology of Gulf of Mexico cold seeps. *Annual Rev. Mar. Sci.* 1, 143–168. doi: 10.1146/annurev.marine.010908.163912

Cordova-Gonzalez, A., Birgel, D., Kappler, A., and Peckmann, J. (2020). Carbon stable isotope patterns of cyclic terpenoids: a comparison of cultured alkaliphilic aerobic methanotrophic bacteria and methane-seep environments. *Organ. Geochem.* 139:103940.

Distel, D., Lane, D., Olsen, G., Giovannoni, S., Pace, B., Pace, N., et al. (1988). Sulfur-oxidizing bacterial endosymbionts–analysis of phylogeny and specificity by 16S ribosomal RNA sequences. *J. Bacteriol.* 170, 2506–2510.

Dunstan, G. A., Volkman, J. K., and Barrett, S. M. (1993). The effect of lyophilization on the solvent extraction of lipid classes, fatty acids and sterols from the oyster *Crassostrea gigas*. *Lipids* 28, 937–944. doi: 10.1007/bf02537504

Duperron, S., and Gros, O. (2016). *Colwellia* and sulfur-oxidizing bacteria: an unusual dual symbiosis in a Terua mussel (Mytilidae: Bathymodiolinae) from whale falls in the Antilles arc. *Deep-Sea Res. Part I* 115, 112–122. doi: 10.1016/j.dsr.2016.05.012

Duperron, S., Gaudron, S. M., Lemaitre, N., and Bayon, G. (2014). A microbiological and biogeochemical investigation of the cold seep tubeworm *Escarpia southwardae* (Annelida: Siboglinidae): symbiosis and trace element composition of the tube. *Deep-Sea Res. Part I* 90, 105–114.

Duperron, S., Lorion, J., Samadi, S., Gros, O., and Gaill, F. (2009). Symbioses between deep-sea mussels (Mytilidae: Bathymodiolinae) and chemosynthetic bacteria: diversity, function and evolution. *C. R. Biol.* 332, 298–310. doi: 10.1016/j.crvi.2008.08.003

Fang, J., Abrajano, T. A., Comet, P. A., Brooks, L. M., Sassen, R., and MacDonald, I. R. (1993). Gulf of Mexico hydrocarbon seep communities XI. Carbon isotopic fractionation during fatty acid biosynthesis of seep organisms and its implication for chemosynthetic processes. *Chem. Geol.* 109, 271–279.

Fang, Y., Wei, J., Lu, H., Liang, J., Lu, J., Fu, J., et al. (2019). Chemical and structural characteristics of gas hydrates from the Haima cold seeps in the Qiongdongnan Basin of the South China Sea. *J. Asian Earth Sci.* 182:103924. doi: 10.1016/j.jseaes.2019.103924

Faure, B., Schaeffer, S. W., and Fisher, C. R. (2015). Species distribution and population connectivity of deep-sea mussels at hydrocarbon seeps in the Gulf of Mexico. *PLoS One* 10:e0118460. doi: 10.1371/journal.pone.0118460

Feng, D., Cheng, M., Kiel, S., Qiu, J.-W., Yang, Q., Zhou, H., et al. (2015). Using *Bathymodiolus* tissue stable carbon, nitrogen and sulfur isotopes to infer biogeochemical process at a cold seep in the South China Sea. *Deep-Sea Res. Part I* 104, 52–59. doi: 10.1016/j.dsr.2015.06.011

Feng, D., Peckmann, J., Li, N., Kiel, S., Qiu, J.-W., Liang, Q., et al. (2018). The stable isotope fingerprint of chemosymbiosis in the shell organic matrix of seep-dwelling bivalves. *Chem. Geol.* 479, 241–250. doi: 10.1016/j.chemgeo.2018.01.015

Fiala-Médioni, A., McKiness, Z. P., Dando, P., Boulegue, J., Mariotti, A., Alayse-Danet, A. M., et al. (2002). Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis. *Mar. Biol.* 141, 1035–1043. doi: 10.1007/s00227-002-0903-9

Fisher, C. R., Brooks, J. M., Vodenichar, J. S., Zande, J. M., Childress, J. J., and Burke, R. A. Jr. (1993). The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. *Mar. Ecol.* 14, 277–289.

Fisher, C. R., Childress, J. J., Oremland, R. S., and Bidigare, R. R. (1987). The importance of methane and thiosulfate in the metabolism of the bacterial symbionts of two deep-sea mussels. *Mar. Biol.* 96, 59–71. doi: 10.1007/bf00394838

Franke, M., Geier, B., Hammel, J. U., Dubilier, N., and Leisch, N. (2021). Coming together—symbiont acquisition and early development in deep-sea bathymodioline mussels. *Proc. R. Soc. B* 288:20211044. doi: 10.1098/rspb.2021.1044

Fullarton, J. G., Dando, P. R., Sargent, J. G., Southward, A. J., and Southward, E. C. (1995). Fatty acids of hydrothermal vent *Ridgeia piscesae* and inshore bivalves containing symbiotic bacteria. *J. Mar. Biol. Assoc. U K.* 75, 455–468. doi: 10.1017/s0025315400018300

Goericke, R., Montoya, J. P., and Fry, B. (1994). "Physiology of isotopic fractionation in algae and cyanobacteria," in *Stable Isotopes in Ecology and Environmental Science*, eds K. Lajtha and R. H. Michener (Oxford: Blackwell). doi: 10.1073/pnas.70.10.2727

Gray, M. F., Morrison, A., Farish, E., Lawrie, T. D. V., and Brooks, C. J. W. (1969). Characterization of 5α-lanost-8(9)-en-3β-ol in hyperlipaemic serum. *Biochim. Biophys. Acta* 187, 163–165. doi: 10.1016/0005-2760(69)90147-7

Guan, H., Birgel, D., Feng, D., Peckmann, J., Liu, L., Liu, L., et al. (2021). Lipids and their $\delta^{13}C$ values reveal carbon assimilation and cycling in the methane-seep tubeworm *Paraescarpia echinospica* from the South China Sea. *Deep–Sea Res. I* 174:103556. doi: 10.1016/j.dsr.2021.103556

Guezennec, J., and Fiala-Medioni, A. (1996). Bacterial abundance and diversity in the Barbados Trench determined by phospholipid analysis. *FEMS Microbiol. Ecol.* 19, 83–93. doi: 10.1111/j.1574-6941.1996.tb00201.x

Jahnke, L. L., and Nichols, P. D. (1986). Methyl sterol and cyclopropane fatty acid composition of *Methylococcus capsulatus* grown at low oxygen tensions. *J. Bacteriol.* 167, 238–242. doi: 10.1128/jb.167.1.238-242.1986

Jahnke, L. L., Summons, R. E., Dowling, L. M., and Zahiralis, K. D. (1995). Identification of methanotrophic lipid biomarkers in cold seep mussel gills: chemical and isotopic analysis. *Appl. Environ. Microbiol.* 61, 576–582. doi: 10.1128/aem.61.2.576-582.1995

Jahnke, L. L., Summons, R. E., Hope, J. M., and Des Marais, D. J. (1999). Carbon isotopic fractionation in lipids from methanotrophic bactria II: the effects of physiology and environmental parameters on the biosynthesis and isotopic signatures of biomarkers. *Geochimica Cosmochimica Acta* 63, 79–93. doi: 10.1016/s0016-7037(98)00270-1

Jannasch, H. W. (1985). The chemosynthetic support of life and the microbial diversity at deep-sea hydrothermal vents. *Proc. R. Soc. London* 225, 277–297.

Jarzebski, A., and Popov, S. (1985). Comparison of sterol composition of some Baltic Sea and Black Sea mussels. *Mar. Biol.* 86, 159–161.

Kádár, E., Bettencourt, R., Costa, V., Serrão Santos, R., Lododa-Cunha, A., and Dando, P. R. (2005). Experimentally induced endosymbiont loss and re-acquirement in the hydrothermal vent bivalve *Bathymodiolus azoricus*. *J. Exp. Mar. Biol. Ecol.* 318, 99–110.

Kawashima, H., and Ohnishi, M. (2003). Fatty acid compositions of various tissue lipids in the marine bivalves, *Megangulus venulosus* and *Megangulus zyonoensis*, from coastal waters of Hokkaido, Northern Japan. *J. Oleo Sci.* 52, 309–315.

Kellermann, M. Y., Schubotz, F., Elvert, M., Lipp, J. S., Birgel, D., Prieto-Mollar, X., et al. (2012). Symbiont–host relationships in chemosynthetic mussels: a comprehensive lipid biomarker study. *Organ. Geochem.* 43, 112–124.

Kiel, S. (2010). "The fossil record of vent and seep mollusks," in *The Vent and Seep Biota. Topics in Geobiology*, ed. S. Kiel (Heidelberg: Springer), 255–278. doi: 10.1098/rspb.2014.2908

Kiel, S., and Peckmann, J. (2019). Resource partitioning among brachiopods and bivalves at ancient hydrocarbon seeps: a hypothesis. *PLoS One* 14:e0221887. doi: 10.1371/journal.pone.0221887

Kiel, S., Birgel, D., Lu, Y., Wienholz, D., and Peckmann, J. (2021). A thyasirid-dominated methane-seep deposit from Montañita, southwestern Ecuador, from the Oligocene-Miocene boundary. *Palaeogeography Palaeoclimatol. Palaeoecol.* 575:110477.

Knief, C. (2015). Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on pmoA as molecular marker. *Front. Microbiol.* 6:1346. doi: 10.3389/fmicb.2015.01346

Laming, S. R., Szafranski, K. M., Rodrigues, C. F., Gaudron, S. M., Cunha, M. R., Hilário, A., et al. (2015). Fickle or faithful: the roles of host and environmental context in determining symbiont composition in two bathymodioline mussels. *PLoS One* 10:e0144307. doi: 10.1371/journal.pone.0144307

Lan, Y., Sun, J., Zhang, W., Xu, T., Zhang, Y., Chen, C., et al. (2019). Host–symbiont interactions in deep-sea chemosymbiotic vesicomyid clams: insights from transcriptome sequencing. *Front. Mar. Sci.* 6:680. doi: 10.3389/fmars.2019.00680

Liang, Q., Hu, Y., Feng, D., Peckmann, J., Chen, L., Yang, S., et al. (2017). Authigenic carbonates from newly discovered active cold seeps on the northwestern slope of the South China Sea: constraints on fluid sources, formation environments, and seepage dynamics. *Deep-Sea Res. Part I* 124, 31–41. doi: 10.1016/j.dsr.2017.04.015

Lorion, J., Kiel, S., Faure, B. M., Kawato, M., Ho, S. Y. W., Marshall, B. A., et al. (2013). Adaptive radiation of chemosymbiotic deep-sea mussels. *Proc. R. Soc. B* 280:20131243. doi: 10.1098/rspb.2013.1243

McCaffrey, M. A., Farrington, J. W., and Repeta, D. (1989). Geochemical implications of the lipid composition of *Thioploca spp*. from the Peru upwelling region–15°S. *Organ. Geochem.* 14, 61–68.

McCutchan, J. H., Lewis, W. M., Kendall, C., and McGrath, C. C. (2003). Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* 102, 378–390. doi: 10.1034/j.1600-0706.2003.12098.x

Nelson, K., and Fisher, C. R. (2000). Absence of cospeciation in deep-sea vestimentiferan tube worms and their bacterial endosymbionts. *Symbiosis* 28, 1–15. doi: 10.1002/jmor.1054

Nichols, P. D., Guckert, J. B., and White, D. C. (1986). Determination of monounsaturated fatty acid double-bond position and geometry for microbial monocultures and complex consortia by capillary GC–MS of their dimethyl disulphide adducts. *J. Microbiol. Methods* 5, 49–55.

Nichols, P. D., Smith, G. A., Antworth, C. P., Hanson, R. S., and White, D. C. (1985). Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for methane-oxidizing bacteria. *FEMS Microbiol. Ecol.* 31, 327–335.

Niemann, H., Lösekann, T., de Beer, D., Elvert, M., Nadalig, T., Knittel, K., et al. (2006). Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* 443, 854–858. doi: 10.1038/nature05227

Ohishi, K., Yamamoto, M., Tame, A., Kusaka, C., Nagai, Y., Sugimura, M., et al. (2016). Long-term cultivation of the deep-sea clam Calyptogena okutanii: changes in the abundance of chemoautotrophic symbiont, elemental sulfur, and mucus. *Biol. Bull.* 230, 257–267. doi: 10.1086/BBLv230n3p257

Patt, T. E., and Handon, R. S. (1978). Intracytoplasmic membrane, phospholipid, and sterol content of *Methylobacterium organophilum* cells grown under different conditions. *J. Bacteriol.* 134, 636–644.

Petersen, J. M., and Dubilier, N. (2010). "Symbiotic methane oxidizers," in *Handbook of Hydrocarbon and Lipid Microbiology*, ed. K. N. Timmis (Berlin and Heidelberg: Springer-Verlag).

Pond, D. W., Bell, M. V., Dixon, D. R., Fallick, A. E., Segonzac, M., and Sargent, J. R. (1998). Stable-carbon-isotope composition of fatty acids in hydrothermal vent mussels containing methanotrophic and thiotrophic bacterial endosymbionts. *Appl. Environ. Microbiol.* 64, 370–375. doi: 10.1128/AEM.64.1.370-375.1998

Pond, D. W., Gebruk, A., Southward, E. C., Southward, A. J., Fallick, A. E., Bell, M. V., et al. (2000). Unusual fatty acid composition of storage lipids in the bresiloid shrimp, *Rimicaris exoculata*, couples the photic zone with MAR hydrothermal vent sites. *Mar. Ecol. Prog. Series* 198, 171–179.

Rau, G. H., and Hedges, J. I. (1979). Carbon-13 depletion in a hydrothermal vent mussel: suggestion of a chemosynthetic food source. *Science* 203, 648–649. doi: 10.1126/science.203.4381.648

Riou, V., Bouillon, S., Serrão Santos, R., Dehairs, F., and Colaco, A. (2010). Tracing carbon assimilation in endosymbiotic deep-sea hydrothermal vent mytilid fatty acids by ^{13}C-fingerprinting. *Biogeosci. Discussions* 7, 3453–3475.

Scallen, T. J., Dhar, A. K., and Loughran, E. D. (1971). Isolation and characterization of C-4 methyl intermediates in cholesterol biosynthesis after treatment of rat liver in vitro with cholestan-3β,5α,6β-triol. *J. Biol. Chem.* 246, 3168–3174.

Schouten, S., Bowman, J. P., Irene, W., Rijpstra, C., and Sinninghe-Damsté, J. S. (2000). Sterols in a psychrophilic methanotroph, *Methylosphaera hansonii*. *FEMS Microbiol. Lett.* 186, 193–195. doi: 10.1111/j.1574-6968.2000.tb09103.x

Stecher, J., Türkay, M., and Borowski, C. (2002). Faunal assemblages on the Pacific–Antarctic ridge near the foundation seamount chain (37 30'S, 110 30'W). *Cahiers Biol. Mar.* 43, 271–274.

Summons, R. E., Jahnke, L. L., and Rocsandic, T. (1994). Carbon isotopic fractionation in lipids from methanotrophic bacteria: relevance for interpretation of the geochemical record of biomarkers. *Geochimica Cosmochimica Acta* 58, 2853–2863. doi: 10.1016/0016-7037(94)90119-8

Szafranski, K. M., Piquet, B., Shillito, B., Lallier, F. H., and Duperron, S. (2015). Relative abundances of methane- and sulfur-oxidizing symbionts in gills of the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* under pressure. *Deep-Sea Res. Part I* 101, 7–13. doi: 10.1016/j.dsr.2015.03.003

Talbot, H. M., Watson, D. F., Murrel, J. C., Carter, J. F., and Farrimond, P. (2001). Analysis of intact bacteriohopanepolyols from methanotrophic bacteria by reversed-phase high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. A* 921, 175–185. doi: 10.1016/s0021-9673(01)00871-8

Teshima, S.-I., and Kanazawa, A. (1974). Biosynthesis of sterols in abalone, *Haliotis gurneri*, and mussel, *Mytilus edulis*. *Comp. Biochem. Physiol. Part B: Comp. Biochem.* 47B, 555–561. doi: 10.1016/0305-0491(74)90004-2

Teshima, S.-I., and Patterson, S. W. (1981). Sterol biosynthesis in the oyster *Crassostrea virginica*. *Lipids* 16, 234–239.

Thornhill, D. J., Wiley, A. A., Campbell, A. L., Bartol, F. F., Teske, A., and Halanych, K. M. (2008). Endosymbionts of *Siboglinum fiordicum* and the phylogeny of bacterial endosymbionts in Siboglinidae (Annelida). *Biol. Bull.* 214, 135–144. doi: 10.2307/25066670

Volkman, J. K. (2003). Sterols in microorganisms. *Appl. Microbiol. Biotechnol.* 60, 495–506.

Volkman, J. K. (2005). Sterols and other triterpenoids: source specificity and evolution of biosynthetic pathways. *Organ. Geochem.* 36, 139–159.

Wei, J. H., Yin, X. C., and Welander, P. V. (2016). Sterol synthesis in diverse bacteria. *Front. Microbiol.* 7:990. doi: 10.3389/fmicb.2016.00990

Won, Y.-J., Hallam, S. J., O'Mullan, G. D., Pan, I. L., Buck, K. R., and Vrijenhoek, R. C. (2003). Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiolus*. *Appl. Environ. Microbiol.* 69, 6785–6792. doi: 10.1128/AEM.69.11.6785-6792.2003

Xu, T., Feng, D., Tao, J., and Qiu, J.-W. (2019). A new species of deep-sea mussel (Bivalvia: Mytilidae: Gigantidas) from the South China Sea: morphology, phylogenetic position, and gill-associated microbes. *Deep-Sea Res. Part I* 146, 79–90.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.