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Different Nitrogen Sources Fuel Symbiotic Mussels at Cold Seeps

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Cold seeps globally host dense unique oasis-type ecosystems, mainly fuelled by chemosynthetic microorganisms via reduced gases such as methane and hydrogen sulfide. However, the origin and pathway of nitrogen chemosynthesis in this widely distributed symbiont ecosystem remain poorly understood. Here, we explore biomarker methods (bulk stable isotope, amino acid (AA), fatty acid (FA) and compound-specific isotope analyses in gill tissues of mussels) to demonstrate the relative contributions of inorganic and organic nitrogen to symbiotic mussels at cold seeps in the South China Sea and their impact on the synthesis and metabolism of amino acids. *Gigantidas platifrons (G. platifrons)* symbioses with type II methanotrophs ***via*** the Serine pathway, and *Bathymodiolus aduloides (B. aduloides)* thrives with sulfur-oxidizing bacteria ***via*** the Calvin pathway, as revealed by bulk $\delta^{13}C$ and $\delta^{13}C$ of FAs. Based on the $\delta^{15}N$ values in gill tissues of mussels, organic nitrogen from sediment is estimated as the dominant nitrogen source for *B. aduloides* (97-98%), in contrast, NH_4^+ was the main nitrogen source for *G. platifrons.* Different dominant nitrogen sources result in the $\delta^{15}N$ of AAs in the gills of two mussel species having opposite trends, which might be related to synthesis and metabolism of AAs in symbiotic bacteria and host, respectively. Our findings reveal that the mechanism of nitrogen acquisition in cold seep systems is plastic and related to DIN sources/uptake and changing environmental conditions. These findings uncover novel biosynthesis of nitrogen in the deep sea, typically at cold seeps, and may have important implications for nitrogen biogeochemistry and deep-sea conservation.

Keywords: symbiotic mussels, cold seeps, nitrogen sources, biomarker methods, $\delta^{13}C$ of fatty acids, $\delta^{15}N$ of amino acids

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INTRODUCTION

Cold seeps are widespread seafloor ecosystems supported by the emission of reduced gases from seabed reservoirs (Ruff et al., 2015; Goffredi et al., 2020). Symbioses associated with bacteria have played a central role in shaping the ecosystem surrounding invertebrates in cold seeps (Rubin-Blum et al., 2019). Among the dominant animals in these ecosystems are symbiotic mussels, which live in symbiosis with thiotrophic and/or methanotrophic symbionts and derive the vast majority of their nutrition from these symbionts (Feng et al., 2015; Rubin-Blum et al., 2017). Since their first discovery, cold seeps have been recognized as important pathways of element cycling on Earth (Paull et al., 1984; Feng et al., 2018). Many studies on symbiotic relationships, especially related to

carbon chemosynthetic processes, have been conducted. These findings have led to a basic understanding of seep ecosystems in terms of biodiversity and community composition (Suess, 2014) and energy and nutritional carbon sources (Brooks et al., 1987; Orphan et al., 2001). However, much more work has been focused on the carbon-fuelled symbiont system between bacteria and mussels than the nitrogen-fuelled system (Macavoy et al., 2002; Raggi et al., 2013; Rubin-Blum et al., 2017). Evidence that methane and CO_2 are the main carbon sources to the symbiont ecosystem suggests that different carbon sources result in distinct $\delta^{13}C$ values in the gills of mussels (Brooks et al., 1987), yet the nitrogen cycles in this widely distributed symbiont ecosystem remain largely unexplored (Vokhshoori et al., 2021). The nitrogen cycles can not only reveal the synthesis and metabolism of amino acids (AAs) in symbiotic mussels but also help to elucidate the interactions between fatty acids (FAs) and AAs and to understand the adaption of mussels to extreme environments and their interactions in cold seep ecosystems. Thus, understanding the nitrogen cycles in symbiotic mussels is vital to shed light on deep sea conservation.

Biochemical tracers, such as bulk stable isotopes ($\delta^{13}C$ and $\delta^{15}N$), FAs, and compound-specific isotope analysis (CSIA), have been applied to bacteria and symbionts. Because of the limited kinetic isotope fractionation of carbon associated with methanotrophs, bulk $\delta^{13}C$ values have successfully indicated the various carbon source contributions (Orphan et al., 2001; Michaelis et al., 2002). The varied $\delta^{15}N$ values also suggest that symbionts are capable of using different nitrogen sources (Petersen et al., 2016). FA composition analysis demonstrated that type II methanotrophs were the predominant microorganism responsible for methane oxidation (Bull et al., 2000; Kellermann et al., 2012), especially in high-methane or low combined-nitrogen environments (Jahnke et al., 1999). The analysis of $\delta^{15}N$ in AAs, however, has rarely been applied to quantitatively estimate the nutritive nitrogen sources used by symbionts in cold seeps but has emerged as a powerful approach for tracing the origins and fate of nitrogen in ecological and biogeochemical studies (Ohkouchi et al., 2017). Moreover, the combination of multiple isotope analyses, including bulk stable isotopes and stable isotopes in FAs and AAs, can help to elucidate the interactions between AAs and FAs (Riou et al., 2010; Takano et al., 2018).

In this study, we explored the biomarker methods previously used to investigate the trophic interactions of fishes (Gerringer et al., 2017; Wang et al., 2019) to delineate the biogeochemistry and nitrogen dynamics of symbiotic mussels in the Formosa Ridge (Site F) of the South China Sea (SCS). Site F is located in the northeast region of the continental slope of the SCS. It is a NNW–SSE oriented ridge that is nearly 2 km in length and rises over 100 m above the surrounding seabed (**Figure 1A**) (Feng et al., 2015). At site F, *Gigantidas platifrons* and *Bathymodiolus aduloides* were the most common and abundant taxa (**Figure 1B**) and symbiosed with methanotrophs and sulfur-oxidizing bacteria in their gills, respectively (Feng et al., 2015; Xu et al., 2019). Therefore, a comparative study of the gills of *G. platifrons* and *B. aduloides* is conducted in this research.

Based on the observation that *G. platifrons* and *B. aduloides* have obviously different biological composition characteristics, such as bulk stable isotopes and CSIA, we tested the hypothesis that *G. platifrons* and *B. aduloides* have different nitrogen-fuelled systems—fuelled by inorganic and organic nitrogen-influenced by the surrounding environment, in turn resulting in different symbiotic bacteria (Feng et al., 2015). We identified the type of methanotrophs and carbon biogeochemical cycles in mussels based on the bulk $\delta^{13}C$ and $\delta^{13}C$ of FAs. We also estimated the relative contributions of different nitrogen sources from $\delta^{15}N$ values. To clarify the synthesis of AAs, we measured the $\delta^{15}N$ of AAs and described the conversion between AAs and FAs. This multifaceted study provides a broad perspective for carbon and nitrogen transformations in symbiotic mussels in cold seeps and lays the foundation for their origin and adaptation to extreme environments.

METHODS

Study Site and Mussels

As described in other studies (Feng et al., 2015; Sun et al., 2017), our study site, called Site F (Formosa Ridge), is located at 22°06.922′N and 119°17.130′E at a depth of 1120 m. It is an oriented ridge above the surrounding seabed and has active methane seepage on the northeastern corner of the continental slope of the South China Sea (**Figure 1A**). Two mussel species were collected using the manned submersible Jiaolong during the Dayang-31 expedition in July 2013 (**Figure 1B**). The mussels were identified as *Gigantidas platifrons* and *Bathymodiolus aduloides* by the National Center for Biotechnology Information's gene sequence database at http://www.ncbi.nlm.nih.gov and http://www.marinespecies.org/aphia.php?p=taxdetails&id=1346726 (Wong et al., 2015; Xu et al., 2019). *G. platifrons* was numerically dominant and occurred mostly on carbonate pavement (**Figure 1C**), while *B. aduloides* was rare and occurred mainly on muddy sediment (**Figure 1D**). *G. platifrons* and *B. aduloides* harboured methanotrophic and thiotrophic symbionts, respectively. All mussels were placed in the sample baskets of the submersible. Once the deck of the ship returned, twelve individuals of *G. platifrons* and five individuals of *B. aduloides* were dissected into the mantle, gill, viscera, and foot tissue. All samples were stored at -20°C and transferred to the laboratory. In this study, the gill tissues were used to conduct the following experiments.

To compare the bulk isotopic contents in the symbiotic mussels at cold seep, the shallow-water mussels *M. edulis* and *M. coruscus* were bought from Sanggou Bay (in July 2014, 37°08′N and 122°30′E) and the aquaculture areas of Zhoushan (in June 2015, 30°32′N and 121°25′E), respectively. The species names were confirmed based on their morphological description.

Abbreviations: AA, amino acid; Ala, alanine; Arg, arginine; Asn, asparaginate; Asp, aspartic acid; CSIA, compound-specific isotope analysis; FA,fatty acid; FAME, fatty acid methyl ester; Glu,glutamic; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; OM, organic matter; Phe, phenylalanine; POM, particulate organic matter; PUFA, polyunsaturated fatty acid; SCS,South China Sea, Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

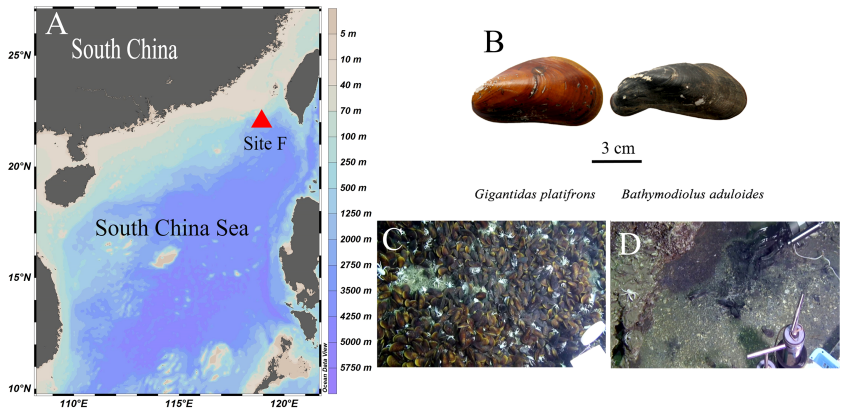

FIGURE 1 | Study site and mussel beds. **(A)** Site F location; **(B)** shells of the two mussels; **(C)** Gigantidas platifrons lives on carbonate pavement; **(D)** Bathymodiolus aduloides lives on muddy sediment; **(B–D)** were cited from Feng et al.[4]

Stable Isotope Analysis

All gill tissues were lyophilized in a freeze-dryer (LOC-1; Christ, Germany) and powdered using a mortar and pestle. Dried powdered samples (~0.2 mg for $\delta^{13}C$, ~1 mg for $\delta^{15}N$, three replicates) were weighed into tin cups, and stable isotopes ($\delta^{13}C$, $\delta^{15}N$) were measured using an isotope ratio mass spectrometer (Finnegan Delta plus XP; Thermo, Germany). The results were normalized to the Vienna Pee Dee Belemnite standard (V-PDB) for $\delta^{13}C$ and to atmospheric N (N_2) for $\delta^{15}N$. The stable isotope ratios are expressed in δ notation of units per mille as follows (Bond and Hobson, 2012):

$$\delta X(‰) = \left(\left(R_{sample} / R_{standard}\right) - 1\right) \times 1000 \quad (1)$$

where $X = {}^{13}C$ *or* ${}^{15}N$ and $R = {}^{13}C/{}^{12}C$ *or* ${}^{15}N/{}^{14}N$. The precision of the stable isotope analyses was ±0.1‰.

FA Analysis

FAs were extracted using a modified Folch method (Folch et al., 1957; Wang et al., 2019). Approximately 100 mg of the gill tissues was extracted by dichloromethane/methanol (2:1 v/v, including 0.01% BHT) and then centrifuged (3000 rpm, 10 min). The upper organic solvent layer was transferred to a flask using a pipette and evaporated to dryness under a stream of N_2 at room temperature. The dried FAs were transesterified to FA methyl esters (FAMEs) in a mixture of methanol (containing 5% HCl) and n-hexane at 50 for 12 h.

The FAMEs were analysed using gas chromatography (7890A GC; Agilent, United States) equipped with a DB-FFAP capillary column (30 m in length, 0.25 mm i.d., 0.25 mm film thickness; Agilent, United States). As an internal recovery standard, 21:0 was added to the samples, and 19:0 methyl ester was added as an internal quantification standard. The injector and detector temperatures were both 250°C. Injections (1 mL) were made at 60°C, and the temperature was increased to 170°C at a rate of 30°C/min. The temperature was held constant for 5 min, increased to 220°C at 1°C/min and held at this temperature for 10 min. Nitrogen (N_2) was used as the carrier gas at a flow rate of 1 mL/min. FAMEs were identified by comparing retention times with those of commercial standards (37 Component FAME Mix; Sigma, United States). The individual FAs were expressed as percentages of the total FA content based on peak areas. The FA recoveries in the analysis were >85%. If more than one sample was analysed, the data were reported as the mean ± standard deviation (SD).

Compound-Specific ^{13}C FA Analysis

To investigate the synthetic pathways of FAs from carbon sources, the $\delta^{13}C$ values of FAs in *B. aduloides* and *G. platifrons* were measured. The compound-specific ^{13}C FA values were analysed using a Trace Ultra GC coupled to a Delta V mass spectrometer *via* a GC combustion interface using helium (He) as the carrier gas (Thermo Scientific, Germany). The FAMEs were separated on a DB-5MS column (50 m, 0.32 mm i.d., 0.25 mm film thickness). The $\delta^{13}C$ values of the FAMEs were calibrated by analysing an 18:0 FAME certified standard (certified: -23.24‰, measured: -23.34‰ ± 0.1‰) supplied by Indiana University, and the analytical precision was ≤0.3‰ (Wang et al., 2019). All samples were analysed in triplicate.

To obtain isotope data for the FAs, FAME isotopes were corrected for the isotopes of the methyl moiety originating from methanol using the following formula:

$$\delta^{13}C_{FA} = \left[\left(n+1\right) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}\right] / n \quad (2)$$

where $\delta^{13}C_{FA}$ is the isotope of the FA, $\delta^{13}C_{FAME}$ is the isotope of the FAME, $\delta^{13}C_{MeOH}$ is the isotope of the methanol derivatization reagent (-37.4 ± 0.1‰), and n is the number of C atoms in the FAs.

AA Analysis

AAs were extracted from all the samples *via* HCl hydrolysis. Briefly, 1 mg of gill tissues was hydrolysed with 6 M HCl at 110°C for 20 h in precombusted sealed glass ampoules. After hydrolysis, the solution was neutralized to pH 8.5 with NaOH (Fitznar et al., 1999). The AAs were determined by high-performance liquid chromatography equipped with a Phenomenex™ Hyperclone column (5 μm particle diameter, BDS C18, 250 mm length, 4 mm inner diameter) (Wu et al., 2007). Eluent A was 125 mM sodium acetate with 2% methanol adjusted to pH 6.8 with acetic acid, and eluent B was 100% methanol. Gradient elution was used to separate all AAs, and external standards of the AAs (Sigma-Aldrich, Merck, USA) were used for calibration. The relative standard deviation triplicate analysis of the individual AAs was <3.5%.

Compound-Specific ^{15}N AA Analysis

The δ^{15}N of AAs was measured by coupling instrumental analysis (ion chromatography and Precon-GC-IRMS) and oxidation-reduction of AAs (Zhang et al., 2021; Wang et al., 2022). Briefly, AAs were separated using an IC system (Dionex, ICS-5000$^+$, Thermo Scientific, USA) equipped with an autosampler (Dionex, AS-AP) with an adjustable injection volume and coupled to an automated fraction collector (Dionex, UltiMate 3000). For the separation of AAs, the IC was equipped with an IC column (Dionex™ AminoPac™PA10, Thermo Scientific, USA), and a solvent ramp programme was used. At the end of each sample, 1 M sodium acetate was used to wash the column to remove any residue (Zhang et al., 2021). IC procedural blanks were collected after AA fractions, and were re-injected in IC system to ensure that all AAs has been collected completely. Meanwhile, the solvent blanks were also collected between Phe and Glu. These blanks were used to correct the isotopic interference from any nitrogenous compounds introduced by the analytical procedure up to this point that could be potentially converted to nitrite by NaClO [more details showed in Zhang et al. (2021)] Purified AAs were oxidized to NO_2^- by Strecker degradation (Schonberg and Moubacher, 1952; Zhang and Altabet, 2008). The next step was the conversion of NO_2^- to N_2O; here, $NH_2OH{\bullet}HCl$ was used as an alternative reductant (Bothner-By and Friedman, 1952; Liu et al., 2014). The stable nitrogen isotopes of the produced N_2O were analysed by a Precon-GC-IRMS (Trace GC 2000, Delta-V advantage, Thermo Scientific, USA) equipped with an autosampler (Precon Automated, Thermo Scientific, USA). Standard AAs (Merck, USA; Glu, USGS40, Gly, USGS64, the Reston Stable Isotope Laboratory of the U.S. Geological Survey) were treated with the same protocol as the samples and were used to calibrate the δ^{15}N value of the AAs. The results from this method were compared with the general GC-IRMS analysis with two lab standards (McCarthy Lab AA mixture and cyanobacteria) and only 0.6‰ deviations were found (Zhang et al., 2021).

Statistical Analysis

SPSS 23.0, Primer 7.0 and MATLAB were used to perform data analysis. By SPSS 23.0, the data were tested for normality by using Shapiro–Wilk test and homogeneity of variance using Levene's test. Cross validation using a discriminant analysis was applied to test the efficiency of FA and AA data, and linear regression was used to examine the relationship between N content and δ^{15}N of AAs in SPSS 23.0. A cluster analysis (K-Means) based on FAs was performed using Primer 7.0. Based on the coefficients in the linear combinations of variables making up the principal components (PCs), the dominant parameters were chosen to characterize the different mussel groups. To illustrate the reliability of the data, the Spearman correlation coefficient and the p value (two-tailed) were used to evaluate the significance of the correlation.

As described in previous studies, NH_4^+ was the main inorganic nitrogen source to *G. platifrons* and methane-oxidizing bacteria (Sun et al., 2017), and particulate organic matter (POM) only supply 5% of food sources to *G. platifrons* by filter-feeding (Wang, 2018). Thus, we assume that NH_4^+-derived nitrogen is considered the sole inorganic nitrogen source in the mussel gill of *G. platifrons* and contribute 95% of nitrogen, thereby particulate organic matter (POM)-derived nitrogen contributing 5% by the filter feeding of *G. platifrons*. A simple two-source mixing model in MATLAB was used to calculate the $\delta^{15}N_{ammonium}$ values in the surrounding environment. The following equations were used for this calculation:

$$f_{ammonium} + f_{POM} = 1 \tag{3}$$

$$\begin{aligned}&\left(\delta^{15}N_{ammonium} + \Delta^{15}N_{ammonium}\right)\times f_{ammonium}\\&+\left(\delta^{15}N_{OM} + \Delta^{15}N_{POM}\right)\times f_{POM} = \delta^{15}N_{gill}\end{aligned} \tag{4}$$

where $f_{ammonium}$ is the relative contribution of NH_4^+ (95%), f_{OM} is the relative contribution of OM (5%), $\delta^{15}N_{ammonium}$ is the δ^{15}N of NH_4^+, $\Delta^{15}N_{ammonium}$ is the ^{15}N enrichment of ammonium assimilation, $\delta^{15}N_{POM}$ is the δ^{15}N of POM, $\Delta^{15}N_{POM}$ is the δ^{15}N enrichment of POM assimilation, and $\delta^{15}N_{gill}$ is the δ^{15}N of mussel gill.

The $\Delta^{15}N_{ammonium}$ of ammonium assimilation is supposed as -4‰ when ambient ammonium is low (<1 mM) at the cold seeps (Liao et al., 2014). In site F, the concentration of NH_4^+ in sediment is about 10 mg/kg (Jing et al., 2020), which is lower than 1 mM. Thus, the $\Delta^{15}N_{ammonium}$ is estimated as -4‰ in this study. As measured in previous study, the δ^{15}N of sediment at site F was 3.7‰ (Jing et al., 2020). Based on the δ^{15}N relationship between the bulk sediment and organic nitrogen in the SCS, we estimated that $\delta^{15}N_{POM}$ was 3.7‰ (Kienast et al., 2005). In symbiotic organisms, the host ingested particulate detrital or living nitrogen sources (PON), there was no δ^{15}N enrichment observed (Ferrier-Pages and Leal, 2019), in turn $\Delta^{15}N_{OM}$ is supposed as zero. By the two-source mixing model, the δ^{15}N of NH_4^+ was then calculated (4.5 ± 0.4‰). Furthermore, we applied $\delta^{15}N_{ammonium}$ value to calculate the relative contributions of inorganic and organic nitrogen sources in *B. aduloides*. There are no genomics studies of *B. aduloides* so far. Thus, the relative contributions of inorganic nitrogen (including nitrate and ammonium) are unknown. We

assume that nitrate, ammonium and POM jointly determine the $\delta^{15}N_{gill}$ of *B. aduloides*. The following equations were used for this calculation:

$$f_{ammonium} + f_{nitrate} + f_{POM} = 1 \quad (5)$$

$$\begin{aligned}&\left(\delta^{15}N_{ammonium} - 4‰\right) \times ‰\, f_{ammonium} \\ &+\left(\delta^{15}N_{nitrate} + \Delta^{15}N_{nitrate}\right) \times f_{nitrate} \\ &+\delta^{15}N_{OM} \times f_{POM} = \delta^{15}N_{gill}\end{aligned} \quad (6)$$

where $f_{nitrate}$ is the relative contribution of nitrate, $\delta^{15}N_{nitrate}$ is the $\delta^{15}N$ of nitrate, $\Delta^{15}N_{nitrate}$ is the $\delta^{15}N$ enrichment of nitrate assimilation.

RESULTS

To investigate the symbiotic relationship of cold seep mussels with their symbiotic bacteria and the turnover of organic and inorganic nitrogen between the mussels and the surrounding environment, we collected 47 samples (gill tissues) from cold seeps and shallow water and analysed them for FAs, AAs, bulk $\delta^{13}C$ and $\delta^{15}N$, $\delta^{13}C$ of FAs, and $\delta^{15}N$ of AAs (**Supplementary Data**).

FA Biomarkers

In the principal component analysis (PCA) of FAs, all gill tissues of mussels were separated into four groups (**Figure 2**). PC1 (79.2%) distinguished mussels into cold seep areas and shallow water areas. 16:1n-7, 18:1n-7, 20:4n-6, 20:5n-3, and 22:6n-3 were the main loading factors contributing to PC1. PC2 (5.3%) also separated the mussels, and the main loading factors were 18:1n-7 and 20:4n-6. The PCA results revealed that symbiotic mussels and filter-feeding mussels had different FA components (**Supplementary Figures 1, 2**). The filter-feeding mussels (*Mytilus edulis*, n=15; *M. coruscus*, n=15) had higher n-3 polyunsaturated fatty acids (n-3 PUFAs) than symbiotic mussels in cold seeps. *B. aduloides* (n=5) harbouring sulfur-oxidizing bacteria had a low n-3 PUFA percentage, and *G. platifrons* (n=12), which thrived with methanotrophs, was not detected **(Supplementary Figure 1)**. In contrast, *B. aduloides* and *G. platifrons* had higher 16:1n-7 and 18:1n-7 levels than filter-feeding mussels **(Supplementary Figure 2)**. Moreover, 18:1n-7 was not detected in *M. edulis* and *M. coruscus*.

Bulk $\delta^{13}C$ and $\delta^{15}N$ of Gill Tissues in Mussels

The bulk stable isotopes of mussels in cold seeps and shallow water differed (**Figure 3**). *G. platifrons* had more negative $\delta^{13}C$ (-69.8 ± 1.7‰) and $\delta^{15}N$ (0.7 ± 0.5‰) values than other mussel species, which was similar with symbiotic mussels in the cold seeps (Vokhshoori et al., 2021). The $\delta^{13}C$ and $\delta^{15}N$ values of *B. aduloides* were -34.7 ± 2.0‰ and 3.6 ± 0.3‰, respectively. *M. edulis* and *M. coruscus* had similar $\delta^{13}C$ (-20.7 ± 0.8‰, -21.2 ± 0.5‰; $P<0.05$) values; however, the $\delta^{15}N$ values significantly differed (9.7 ± 0.3‰, 2.8 ± 1.0‰; $P<0.01$).

$\delta^{13}C$ of FAs

δB. *aduloides* (-49.2 to -38.5‰) had more positive $\delta^{13}C$ of FAs than *G. platifrons* (-78.4 to -70.4‰). Considering the carbon content of FAs, the average $\delta^{13}C$ values of FAs in *B. aduloides* and *G. platifrons* were -41.3 ± 0.6‰ and -76.8 ± 0.4‰, respectively (**Figure 4**). However, the $\delta^{13}C$ of 18:0 and 18:1n-7 in *G. platifrons* was more positive than other FAs, which was inconsistent with the results for *B. aduloides* (**Figure 4** and **Supplementary Figures 3, 4**).

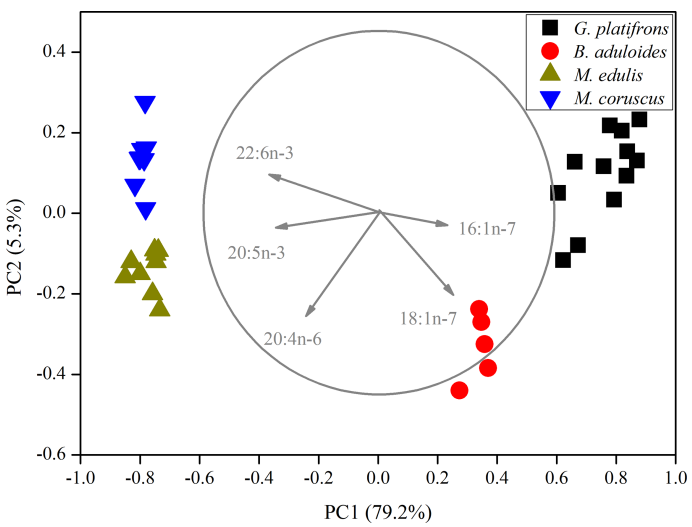

FIGURE 2 | Principal component analysis of mussels based on fatty acids. The length of vectors represents their contributions to axis.

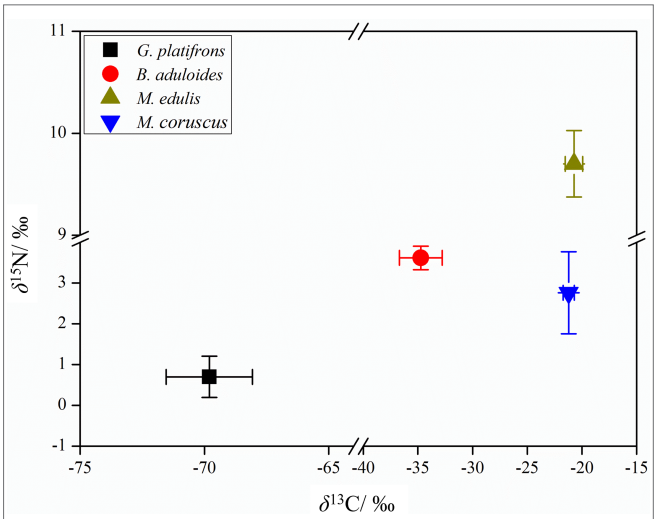

FIGURE 3 | Carbon and nitrogen stable isotope ratios (‰) of symbiotic and filter-feeding mussels.

δ15N of AAs

To characterize the turnover of organic and inorganic nitrogen in cold seep mussels, the $\delta^{15}N$ values of AAs in *B. aduloides* and *G. platifrons* were used to trace the nitrogen flow between the symbiont and host. In *G. platifrons*, the N content was positively related to the $\delta^{15}N$ of AAs ($y=0.25x+6.52$, $R^2 = 0.67$, $p<0.05$) (**Figure 5**). In contrast, the N content in *B. aduloides* was negatively related to the $\delta^{15}N$ of AAs ($y=-0.35x+12.36$, $R^2 = 0.64$, $p<0.05$), which has not been reported to our knowledge. Glycine (Gly) and threonine (Thr) were also measured in this study (**Supplementary Figures 5, 6**) but these results are not shown in **Figure 5** due to the Gly cleavage system (Yamaguchi et al., 2017).

DISCUSSION

Characteristics of Carbon Sources and FAs in Gill Tissues of Symbiotic Mussels

G. platifrons mainly use methane-based energy sources as their carbon sources (Orphan et al., 2001; Michaelis et al., 2002), and in support of this, the $\delta^{13}C$ of *G. platifrons* is consistent with the $\delta^{13}C$ of methane (Michaelis et al., 2002; Feng et al., 2015). In contrast, the $\delta^{13}C$ values of *B. aduloides* indicate a -25‰ fractionation from the measured $\delta^{13}C$ of CO_2 in seawater, which reveals that sulfur-oxidizing bacteria utilize the Calvin cycle for carbon dioxide fixation (Ponnudurai et al., 2017). Meanwhile. this species was consistent with the chemosymbiotic species from the Gulf of Cadiz, which inferred a thiotrophic mode of nutrition (Rodrigues et al., 2013). Moreover, the low content of n-3 PUFAs and high contents of 16:1n-7 and 18:1n-7 in symbiotic mussels also demonstrate that energy derived from photosynthesis has an extremely low impact on the symbiotic mussels (Ackman, 1989). The high contents of 16:1n-7 and 18:1n-7 in the mussels indicate that *G. platifrons* and *B. aduloides* symbiose with type II methanotrophs (Serine pathway) and sulfur-oxidizing bacteria, respectively **(Supplementary Figure 2)**, which is consistent with the genome analysis (Sun et al., 2017). Type II methanotrophs are favoured in high-methane environments and utilize methane as the sole carbon and energy source (Jahnke et al., 1999). This is also the reason why *G. platifrons* occurs mostly on carbonate pavement, where methane is abundant. In the muddy sediment of cold seeps, sulfate reduction is achieved by sedimentary microbial mats with high metabolic rates (Tobler et al., 2016). Thus, sulfur-oxidizing bacteria provide *B. aduloides* with nutrition through the fixation of CO_2 into biomass using produced sulfide compounds as an energy source (Volland et al., 2018).

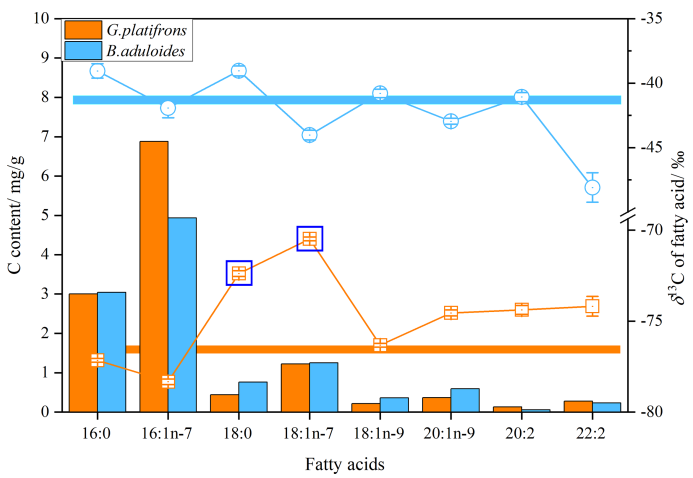

FIGURE 4 | $\delta^{13}C$ and carbon content of fatty acids in symbiotic mussels. Histograms represent the carbon content of fatty acids in tissues (dry weight), points represent the $\delta^{13}C$ of a single fatty acid, and straight lines represent the average $\delta^{13}C$ of fatty acids

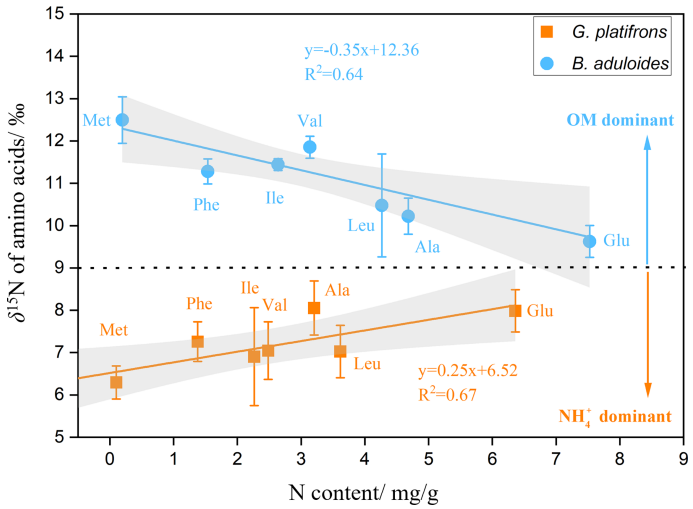

FIGURE 5 | Nitrogen content and $\delta^{15}N$ of amino acids in symbiotic mussels. OM, organic matter; above the black dotted line represents OM as the main nitrogen source for mussels; below the black dotted line represents nitrate as the main nitrogen source for mussels; the grey area represents the 95% confidence interval

These factors result in *B. aduloides* living mainly on muddy sediment.

The bulk $\delta^{13}C$ and $\delta^{13}C$ of FAs differed substantially between the two symbiotic mussels, which is consistent with the assimilation of different carbon sources, as discussed above. Compared with the bulk $\delta^{13}C$, the average $\delta^{13}C$ values of FAs were depleted by approximately 7–9‰ in *B. aduloides* and 2–7‰ in *G. platifrons*, suggesting *de novo* synthesis from dietary carbon sources (Deniro and Epstein, 1977). However, the different carbon isotope fractionation of $\delta^{13}C$ in mussels might be a result of the energy supply, with ^{12}C being the more favourable carbon source when energy is limited (Hugler and Sievert, 2011). In carbonate pavement, the abundant methane supplies enough energy to the type II methanotrophs symbiosing with *G. platifrons*, even though Serine pathway organisms operate in a less energetically favourable way (Anthony, 1982; Jahnke et al., 1999). Different from type II methanotrophs, sulfur-oxidizing bacteria use reduced sulfide from the anaerobic oxidation of methane as the energy source (Feng et al., 2015; Tobler et al., 2016). Instead of methane, *B. aduloides* use seawater CO_2 fixed *via* sulfur-oxidizing bacteria, which agrees well with the measured $\delta^{13}C$ values of CO_2 dissolved in seawater above the mussel beds (Feng et al., 2015). Thus, different carbon sources result to various carbon isotope fractionations. In addition, in both specimens, the differences in the $\delta^{13}C$ of FAs are also indicative of different biochemical pathways (Macavoy et al., 2002; Riou et al., 2010). In *B. aduloides*, unsaturated FAs are depleted relative to saturated FAs, indicating *de novo* transformations (Fang et al., 1993). In contrast, 18:1n-7 in *G. platifrons* shows more positive $\delta^{13}C$ values than 18:0, which provides additional evidence for a biochemical pathway involving FAs in the symbiosis of type II methanotrophs and mussels. A previous study suggested that host cells can assimilate AAs directly into FAs (Riou et al., 2010). Thus, the positive $\delta^{13}C$ of 18:1n-7 in type II methanotrophs demonstrates that this process might occur in bacteria (Bull et al., 2000; Takano et al., 2018).

Relative Contributions of Inorganic and Organic Nitrogen in *B. aduloides*

Bulk nitrogen isotopes, similar to bulk carbon isotopes, reflect the nitrogen diet sources of mussels. For example, the high $\delta^{15}N$ values of *M. edulis* illustrate their trophic interaction with POM in Sanggou Bay (Mahmood et al., 2016), while the low $\delta^{15}N$ values of *M. coruscus* suggest that their diets different with *M. edulis*. In a previous study, the lower $\delta^{15}N$ values of phytoplankton suggested that the primary producer in the East China Sea depend mainly on atmospheric nitrogen fixation (Minagawa and Wada, 1984). Thus, the low $\delta^{15}N$ values of *M. coruscus* might be caused by atmospheric nitrogen indirectly. In extreme environments, these aspects have not been sufficiently explored; however, it is clear that the symbiotic mussels in cold seeps must use alternative nitrogen sources, such as ammonium, nitrate or organic nitrogen, in the sediment (Petersen et al., 2016; Ferrier-Pagès and Leal, 2018), which are rarely quantitatively estimated.

In order to estimate the various sources of nitrogen to *B. aduloides*, we have to delineate the contribution of ammonia, nitrate and organic nitrogen. $\delta^{15}N_{inorg}$ in the SCS had a range of 3.1-4.8‰, and with the $\delta^{15}N$ of N_{inorg} generally being significantly isotopically lighter than bulk sedimentary $\delta^{15}N$ (3.7‰ in this study) (Kienast et al., 2005; Jing et al., 2020). Thus, the $\delta^{15}N_{nitrate}$ in this study was assumed between 3.1and 3.7‰. The average values of $\delta^{15}N_{nitrate}$ were used in this study (3.4 ± 0.3‰). During assimilation of nitrate by microbes, $\Delta^{15}N_{nitrate}$ is supposed as 5‰ when nitrate is not limiting (Liao et al., 2014). In the three-source mixing model, the relative contributions of NH_4^+, NO_3^-, and OM cannot be calculated directly. Thus, we calculated the ranges of inorganic nitrogen contributions when we assumed NH_4^+or NO_3^- had the contribution to *B. aduloides*. When the NH_4^+ was the inorganic nitrogen source to *B. aduloides*, the OM had a relative contribution of 97%. Otherwise, the NO_3^- was the inorganic nitrogen source to *B. aduloides*, the OM had a relative contribution of 98%. In other words, POM was the main nitrogen

source to *B. aduloides* (97-98%). As suggested in another study, filter feeding selectively on a nitrogen-rich suspension might be an important component of the nutritional requirements of *B. childressi*, which is consistent with our results (Pile and Young, 1999). This observation is also consistent with the regional differences, in which *G. platifrons* lives mainly on carbonate pavement and *B. aduloides* occurs mainly on muddy sediment. Moreover, these results illustrated that the limited sulfide might not satisfy their energy needs for the synthesis of all types of matter, and filter feeding OM from the surrounding environment might play a more prominent role in *B. aduloides* (Ponnudurai et al., 2020).

Synthesis and Metabolism of AAs in Symbiotic Mussels Using δ^{15}N of AAs

Based on the different contributions of inorganic and organic nitrogen to the symbiotic mussels, more details about the synthesis and metabolism of AAs can be revealed. The large ^{15}N depletion of Gly relative to other AAs is caused by the Gly cleavage system in organisms, which favours ^{15}N degradation and causes ^{14}N depletion in the remaining Gly molecules (Yamaguchi et al., 2017). Thr is also impacted by the Gly cleavage system because of irreversible transamination reactions (Mcmahon and Mccarthy, 2016). The peculiar fractionation patterns of Thr and Gly are completely different from those of other AAs, routinely exhibiting significant depletion of ^{15}N (Mccarthy et al., 2013; Mcmahon and Mccarthy, 2016). The degradation pattern of "heavy" AAs might produce metabolic fuel for *de novo* FA biosynthesis (Berg et al., 2002). Thus, the products of Thr and Gly degradation might result in positive $\boldsymbol{\delta}^{13}$C values in 18:1n-7 in *G. platifrons*. However, the degradation pathways of nitrogen in AAs are independent of the synthesis of carbon in FAs. The complex carbon flows in symbiotic mussels might cause the differences in the δ^{13}C of FAs between *G. platifrons* and *B. aduloides* (Riou et al., 2010).

In addition to Gly and Thr, the δ^{15}N values of AAs can illustrate the conversion between AAs. The opposite linear relationships between δ^{15}N values of AAs and nitrogen content of AAs might indicate the different synthesis and degradation of AAs in two symbiotic mussels. δIn a previous study, the nitrogen contents of AAs in bacteria show increases in the abundance of less energetically costly AAs (Akashi and Gojobori, 2002). Thus, Glu is the less energetically costly AA and have high content, Met, Phe is the highest energetic cost for biosynthesis and have low content, and other AAs (including Ile, Leu, Val, Ala) occupy the middle position (Yamaguchi et al., 2017). In *G. platifrons*, NH_4^+ was used as the inorganic nitrogen to *de novo* Glu synthesis. Glu, which is typically the most abundant AA in organisms, is often considered a "Glu pool" to form other AAs (Bender, 2012). In *G. platifrons*, both transamination and deamination favour the lighter stable isotope (^{14}N) and leave the residual "Glu pool" ^{15}N-enriched (Macko et al., 1986; Mcmahon and Mccarthy, 2016). Compared with that of Glu, the biosynthesis of other AAs requires more energy and favours lighter nitrogen (Wagner, 2005; Yamaguchi et al., 2017). Thus, the δ^{15}N values of AAs in *G. platifrons* reflected the order of the energy cost required to form different AAs. A previous study showed that the order of the energy cost was Ile>Leu> Val> Ala. The different order in *G. platifrons* (Ile>Val>Ala>Leu) may be caused by the mixture of *de novo* AA synthesis from inorganic nitrogen coupled with AA incorporation from OM (Ohkouchi et al., 2017). Met is considered as the high energetic cost for biosynthesis, it may be reasonable for the microbes to use incorporated Met directly *via* salvage incorporation and not to degrade it (Yamaguchi et al., 2017). Thus, Met had lowest δ^{15}N values in *G. platifrons*.

In contrast, *B. aduloides* has a negative correlation between N content and δ^{15}N values of AAs, which is consistent with the assimilation of different nitrogen sources. The filter feeding of *B. aduloides* might play a prominent role in its ingestion of POM from the surrounding sediment (97-98% from surrounding POM) (Ponnudurai et al., 2020). *B. aduloides* assimilates OM and degrades it into small molecules such as free AAs (Hoppe et al., 2002) and then transports the Glu to symbiotic bacteria (Hosie et al., 2002; Ponnudurai et al., 2020). Bacteria can use Glu by metabolizing it as carbon and nitrogen sources for resynthesis of AAs or by salvage incorporation (Davies and Humphrey, 1978; Ponnudurai et al., 2020). This process results in the remaining AAs being theoretically enriched in ^{15}N relative to the reacting AAs (Chikaraishi et al., 2007). However, the ^{14}N of metabolized AAs is not excreted as a waste product but is recycled by the symbiont and as resynthesized AAs (Ferrier-Pagès and Leal, 2018). Of the AAs, the Glu in the "Glu pool" was most affected in terms of its ^{14}N levels, as it incurred the lowest energy cost and was most abundant. Compared with Glu, Met is the most expensive AAs in terms of consumed moles of ATP per molecule (Kaleta et al., 2013). The symbiotic mussels use incorporated Met directly *via* salvage incorporation and not to degrade it as discussed above (Yamaguchi et al., 2017). Meanwhile, Met is rarely affected by the ^{14}N of metabolized AAs, and retain the highest δ^{15}N values in AAs.

Such kind of knowledge is the first time to be explored in seep ecosystem (**Figure 6**), it will better constrain the synthesize and metabolism of key elements governing the carbon, nitrogen and other element cycles in extreme environment. These studies will propose relationships between groups of fatty acids and amino acids, which allow testable predictions about biogeochemical processing in symbiotic mussels. An enhanced understanding of genomes and metabolic processes of microbials involved in those organic matter production and consumption in symbiotic mussels will contribute a significant relevance to this topic.

CONCLUSION

The biomarker evidence from this study supports the hypothesis that inorganic and organic nitrogen make different contributions to the nitrogen sources of *G. platifrons* and *B. aduloides*, respectively (**Figure 6**). We demonstrate that methane and CO_2 from the surrounding environment are the main carbon sources for *G. platifrons* and *B. aduloides* and impact the δ^{13}C values of FAs in these mussels. Their unique symbiotic relationships lead to obvious bacterial FA signatures in the mussels, which can represent methanotrophic and thiotrophic symbionts, respectively (Bull et al., 2000; Kellermann et al., 2012). The

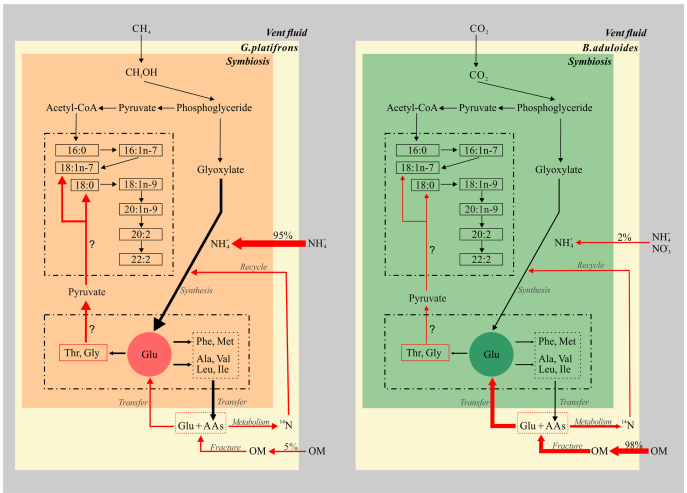

FIGURE 6 | Conceptual model of fatty acids and amino acids in symbiotic mussels. The left part represents the symbiont of *Gigantidas platifrons*, and the right part represents the symbiont of *Bathymodiolus aduloides*; OM, organic matter; the thickness of arrows represents the relative contributions of inorganic and organic nitrogen sources.

differences in AA $\delta^{15}N$ values, which showed opposite trends in the symbiotic mussels, might provide some evidence that the symbiotic mussels use different nitrogen sources. Inorganic nitrogen is the dominant nitrogen source for *G. platifrons* and OM is the dominant nitrogen source for *B. aduloides*, which are consistent with the nitrogen contributions from inorganic nitrogen and OM. The simple mixing model presented here provides baseline information for future studies aiming to quantify the different contributions of dietary sources in unique oasis-type ecosystems on the seafloor and to reveal the carbon and nitrogen flows in symbionts that are impacted by their surrounding environment in cold seeps.

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 author.

AUTHOR CONTRIBUTIONS

FW and YW contributed for conceptualization, and writing original draft preparation. DF contributed for sample preparation, data management, and draft modification. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.869226/full#supplementary-material

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