**Using *Bathymodiolus* tissue stable carbon, nitrogen and sulfur isotopes to infer
biogeochemical process at a cold seep in the South China Sea**

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26 **Abstract**

27 More than thirty sites of active and inactive methane seepage have been
28 discovered on the continental slope of the South China Sea over the past decade. Here
29 we use stable isotopes of carbon, nitrogen and sulfur in the tissue of two bathymodioline
30 mussel species with different chemotrophic symbionts (methanotrophs in
31 *Bathymodiolus platifrons* and sulfide-oxidizers in *B. aduloideis*) to gain insights into the
32 biogeochemical processes at an active site in 1120 m depth on the Formosa Ridge,
33 called Site F. Because mussels with methanotrophic symbionts acquire the isotope
34 signature of the used methane, the average $\delta^{13}\text{C}_{\text{tissue}}$ values of *B. platifrons* (-70.3‰ ;
35 $n=36$) indicates a biogenic methane source at Site F, consistent with the measured
36 carbon isotope signature of methane (-61.1‰ to -58.7‰) sampled 1.5 m above the
37 mussel beds. The only small offset between the $\delta^{13}\text{C}$ signatures of the ascending
38 methane and the authigenic carbonate at site F (as low as -55.3‰) suggests only minor
39 mixing of the pore water with marine bicarbonate, which in turn may be used as an
40 indicator for advective rather than diffusive seepage at this site. *Bathymodiolus*
41 *aduloideis* has much higher average $\delta^{13}\text{C}_{\text{tissue}}$ values of -34.4‰ ($n=9$), indicating
42 inorganic carbon (DIC) dissolved in epibenthic bottom water as its main carbon source.
43 The DIC was apparently marine bicarbonate with a small contribution of ^{13}C -depleted
44 carbon from locally oxidized methane. The $\delta^{34}\text{S}$ values of the two mussel species
45 indicate that they used two different sulfur sources. *Bathymodiolus platifrons* (average
46 $\delta^{34}\text{S} = +6.4 \pm 2.6\text{‰}$; $n=36$) used seawater sulfate mixed with isotopically light re-
47 oxidized sulfide from the sulfate-dependent anaerobic oxidation of methane (AOM),
48 while the sulfur source of *B. aduloideis* ($\delta^{34}\text{S} = -8.0 \pm 3.1\text{‰}$; $n=9$) was AOM-derived
49 sulfide used by its symbionts. $\delta^{15}\text{N}_{\text{tissue}}$ values differed between the mussels, with *B.*
50 *platifrons* having a wider range of on average slightly lower values (mean =

+0.5±0.7‰, n=36) than *B. aduloide*s (mean = +1.1±0.0‰). These values are significantly lower than δ¹⁵N values of South China Sea deep-sea sediments (+5‰ to +6‰), indicating that the organic nitrogen is of local origin, possibly resulting from the activity of autotrophic bacteria and due to assimilation of isotopically light nitrate or ammonium by the symbionts.

Key words: Stable isotopes; Hydrocarbon seep; Bathymodiolin mussel; South China Sea; Methane

1. Introduction

The leakage of methane-rich fluids from subsurface reservoirs to the seafloor at specific sites on continental slopes, termed cold seeps, sustains some of the richest ecosystems on the sea bed (e.g. Boetius and Wenzhöfer, 2013). Such ecosystems are characterized by dense accumulations of chemosynthetic organisms that can utilize the chemical energy contained in the seep fluids, such as methane and sulfide (e.g. Levin, 2005). Among the dominant animals are bathymodiolin mussels, which live in symbiosis with thiotrophic and/or methanotrophic symbionts and derive the vast majority of their nutrition from these symbionts (e.g. Paull et al., 1985; Childress et al., 1986; Cordes et al., 2009; Duperron, 2010). The carbon source for the methanotrophic and thiotrophic bacteria is methane and dissolved inorganic carbon (DIC), respectively (Fisher, 1990; Conway et al., 1994). Sulfur sources for seep mussels are dominated by either isotopically light hydrogen sulfide or isotopically heavy seawater sulfate, depending on the type of symbionts in their gills (Vetter and Fry, 1998; Yamanaka et al., 2000). Therefore, stable isotopes of these elements are particularly useful to decipher the nutrition and lifestyle in seep environments (e.g. Childress et al., 1986; Levin et al.,

76 2005; MacAvoy et al., 2008; Becker et al., 2010, 2013, 2014; Rodrigues et al., 2013).

77 At cold seeps, one of the main biogeochemical process is the anaerobic oxidation
78 of methane (AOM) via sulfate reduction (Boetius et al., 2000). During this process,
79 consumption of methane and sulfate results in the accumulation of ^{13}C -depleted DIC
80 and ^{34}S -depleted sulfide because the involved microbes preferentially use the lighter
81 isotopes for their metabolism (cf. Peckmann and Thiel, 2004; Joye et al., 2004; 2010).
82 Deciphering the variability in the stable carbon isotopes ($\delta^{13}\text{C}$) of pore water DIC is
83 critical to define the spatial extent of AOM and seepage activities (e.g. Aloisi et al.,
84 2004). Such processes regulate the transmission of potent greenhouse gas methane
85 from sediments to the world's ocean (Marlow et al., 2014; Yoshinaga et al., 2014). In
86 addition, sulfide produced during sulfate reduction can be oxidized back to sulfate as
87 revealed by large variation in the sulfur isotopic composition of sulfide and sulfate at
88 seeps, thus providing an essential backdrop for interpreting thiotrophic and
89 methanotrophic symbiosis among the seep-dwelling macrofauna (Aharon and Fu, 2003;
90 Joye et al., 2004; Bowles et al., 2011). Taken together, tissue isotopes of seep mussels
91 provide a significant natural system for studying coupled carbon-sulfur cycles and their
92 geochemical fingerprints. In addition, tissue isotopes of nitrogen can be utilized to
93 delineate food webs and decipher the nitrogen turnover at seep sites (Levin, 2005;
94 MacAvoy et al., 2008).

95 In the past decade, over 30 cold seeps have been discovered on the northern
96 continental slope of the South China Sea (SCS), including Site F (Fig. 1). A common
97 feature of these sites is that they are presently not active and have apparently been
98 inactive since at least 11.5 ka BP (Suess, 2005; Tong et al., 2013; Han et al., 2014). Our
99 study site, Site F (named Formosa Ridge by Liu et al. 2008) is the only one known
100 with active gas seepage (Feng and Chen, in press). However, a challenging issue that

101 remains is determining the temporal variability of seepage activity. Here, we
102 investigated stable isotopes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$) in tissues of seep mussels collected
103 at Site F with the aim to 1) better understand the main biogeochemical processes, 2)
104 reconstruct the composition of fluids and the mode of seepage, and 3) get insights into
105 sulfate and sulfide cycling at this seep site.

106

107 **2. Materials and methods**

108 *2.1. Geological background of the sample materials*

109 Formosa Ridge is located on the northeastern corner of the continental slope of the
110 South China Sea (Fig. 1). It is a NNW-SSE oriented ridge that is nearly 2 km in length
111 and rises over 100 m above the surrounding seabed. A first survey of the area found an
112 active gas plume on top of the ridge, indicating active methane seepage (Liu et al.,
113 2008). Our study site, called here Site F, spans an area of about 50 m $\times$ 100 m, located
114 at 22°06.922'N; 119°17.130'E in 1120 m depth. During the Dayang-31 expedition in
115 2013, a multidisciplinary team of researchers worked to explore and sample this seep
116 community using the manned deep submergence vehicle (DSV) Jiaolong (Feng and
117 Chen, in press). The ridge crest is characterized by large cold-seep carbonate blocks
118 and pavements, and numerous densely packed mussel beds. Ongoing seepage was
119 evident from the gas bubbles, fracturing of the carbonate pavements and mussels
120 colonizing the cracks in the pavements.

121 Two mussel species were the most common and abundant symbiotic taxa, and
122 were identified as *Bathymodiolus platifrons* and *B. aduloides* (cf. Sun et al., submitted
123 for publication). The identification was made by amplifying the cytochrome oxidase 1
124 gene and NADH dehydrogenase 4 gene fragments from the adductor muscle and a
125 BLAST search against the US National Center for Biotechnology Information's gene

sequence database at <http://www.ncbi.nlm.nih.gov>; (J. Sun, pers. comm. 2015). Among the two mussels, *Bathymodiolus platifrons* was numerically dominant and occurred mostly on carbonate pavements. A population of this species living at cold seeps in Sagami Bay, Japan, harbors exclusively methanotrophic symbionts in its gills (Barry et al. 2002) and this type of symbiotic relationship is also assumed for the specimens at Site F. The other mussel species, *B. aduloides*, was rare and occurred mainly on muddy sediment. A specimen of this species collected from a vent site in the Okinawa Trough harbored exclusively thiotrophic symbionts (Fujiwara et al., 2000; Lorion et al. 2013) and this type of symbiotic relationship is also assumed for the specimens at Site F. In addition to the bathymodiolin mussels, a white sponge, a spider crab, the squat lobster *Shinkaia crosnieri*, the gastropods *Bathymacrea nipponica* and *Provanna* sp., the thyasirid bivalve *Conchocele* sp. (empty shells only), and the commensal scale worm *Branchipolynoe pettibonae* were found in the mantle cavity of *B. platifrons*, but no tubeworms were observed during the dives.

2.2. Animal and fluid sampling procedures

Mussels were collected by the manipulator arm using a hand-net mounted on the front of the DSV, from mussel aggregations on rock outcrops (*B. platifrons*) and on the seafloor (*B. aduloides*) in different assemblages (Fig. 2). Specimens were placed in a sample basket during recovery of the submarine. The specimens of *B. platifrons* measured 7.5 cm to 11.0 cm in length (8.5 cm on average; n=12); the specimens of *B. aduloides* were relatively uniform in length (~ 10.5 cm; n=3). Seawater samples for dissolved methane and CO₂ analyses were collected about 1.5 m above the seafloor at two different mussel beds using a pressure-maintaining device (250 ml). Water samples were withdrawn immediately after collection and injected into pre-evacuated

151 glass bottles (500 ml). The bottles were stored upside down for onshore analysis.

152 Once onboard the ship, twelve individuals of *B. platifrons* and three individuals of
153 *B. aduloides* were sampled for stable isotope analyses by dissecting a piece of mantle,
154 gill, and foot tissue, respectively. Tissues from other species other than mussel were
155 also collected for isotope analyses. The tissue samples were rinsed with deionized
156 water to remove any residual seawater and stored at -20°C for further analyses.

157

158 2.3. Analytical procedures

159 Mussel tissue pretreatment and measurement of isotopes followed the procedures
160 described in previous studies (Becker et al., 2010; Rodrigues et al., 2013). Briefly, all
161 samples were freeze-dried, homogenized, and acidified to remove any inorganic
162 carbonate. Samples were redried and analyzed for stable carbon, nitrogen, and sulfur
163 isotopic compositions at the Stable Isotope Research Facility (SIRF) at Indiana
164 University and Oxy-Anion Stable Isotope Consortium (OASIC) at Louisiana State
165 University. The $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values of tissues were measured via CO_2 , N_2 , and
166 SO_2 generated by a Costech Elemental Analyzer (EC4010) coupled with a Thermo
167 Finnigan Delta V Advantage in a continuous flow mode. Values are reported in permil
168 (‰) using the δ notation, relative to the standards Vienna Pee Dee Belemnite (V-PDB)
169 for carbon, air N_2 for nitrogen, and the Vienna-Canyon Diablo Triolite (V-CDT) for
170 sulfur. The precision for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ determinations are $\pm 0.2\text{‰}$, $\pm 0.4\text{‰}$, and
171 $\pm 0.3\text{‰}$, respectively.

172 Standard procedures were used to collect gases dissolved in the water samples (e.g.
173 Pohlman et al., 2008). Water samples were poisoned with HgCl_2 and the glass vials
174 were capped with gas-tight butyl rubber stoppers. The gas samples were used to
175 measure the $\delta^{13}\text{C}$ signatures of CH_4 and CO_2 . Measurements were carried out using an

176 Isoprime isotope ratio mass spectrometer (Isoprime 100) at Tongji University. The
177 precision for $\delta^{13}\text{C}$ determinations was 0.05‰.

178

179 3. Results

180 Isotopic composition of mussel tissues are listed in Table 1 and summarized in
181 Table 2 and Figs. S1 and 3. $\delta^{13}\text{C}$ values differed significantly between *B. platifrons* and
182 *B. aduloides*, with *B. platifrons* being more negative (mean = -70.3‰ , sd = 3.4‰ ,
183 n=36) than *B. aduloides* (mean = -34.4‰ , sd = 1.8‰ , n=9). In *B. platifrons*, mantle
184 $\delta^{13}\text{C}$ values were on average $\sim 1.9\text{‰}$ lower than those of the gills and $\sim 4.8\text{‰}$ lower
185 than those of the foot. No clear pattern among $\delta^{13}\text{C}$ values between mantle, gill, and
186 foot were observed in *B. aduloides*.

187 Tissue $\delta^{15}\text{N}$ values in *B. platifrons* varied notably from -1.2‰ to $+1.3\text{‰}$ (mean =
188 $+0.5\text{‰}$, sd = 0.7‰ , n=36); while tissue $\delta^{15}\text{N}$ values in *B. aduloides* were from $+1.1\text{‰}$
189 to $+1.2\text{‰}$. The ranges in $\delta^{15}\text{N}$ values between mantle, gill, and foot were as low 0.1‰
190 in *B. aduloides* and as large as 1.4‰ in *B. platifrons*, but no clear trend in the variation
191 of $\delta^{15}\text{N}$ values between mantle, gill, and foot was seen in *B. platifrons*.

192 Bulk tissue $\delta^{34}\text{S}$ values varied substantially among the two mussels: between
193 $+1.4\text{‰}$ and $+10.5\text{‰}$ (mean = $+6.4\text{‰}$, sd = 2.6‰ , n=36) in *B. platifrons*, and between $-$
194 12.9‰ and -4.1‰ (mean = -8.0‰ , sd = 3.1‰ , n=9) in *B. aduloides*. In *B. platifrons*,
195 foot $\delta^{34}\text{S}$ values were on average $\sim 2.7\text{‰}$ higher than those for the mantle and the gill.
196 In *B. aduloides*, mantle $\delta^{34}\text{S}$ values were on average $\sim 5.8\text{‰}$ lower than those for the
197 gill and the foot.

198 Methane from the two water samples had $\delta^{13}\text{C}$ values of -58.7‰ and -61.1‰ , and
199 both samples showed the same $\delta^{13}\text{C}_{\text{CO}_2}$ value of -9.2‰ (Table 3). The isotope
200 signatures of a few specimens of the associated fauna are shown in Table 4. Most

201 notably are (i) the very negative $\delta^{13}\text{C}$ value of the sponge (as low as -63.4‰), the large
202 variation of $\delta^{34}\text{S}$ values between the two specimens of *S. crosnieri*, and (iii) the
203 uniform $\delta^{15}\text{N}$ values (1.1‰ to 1.2‰) among all species.

204

205 **4. Discussion**

206 *4.1. Constraints on fluid sources using $\delta^{13}\text{C}$ values*

207 Our study clearly shows than the methane at Site F is of biogenic origin. First,
208 tissue $\delta^{13}\text{C}$ values in *B. platifrons*, a species known to harbor exclusively
209 methanotrophic bacterial endosymbionts (Barry et al., 2002), should be similar to the
210 isotopic composition of its carbon source, because the isotopic fractionation by
211 methanotrophic bacterial endosymbionts is small (cf. Fisher, 1990). Accordingly, tissue
212 $\delta^{13}\text{C}$ values as low as -77.6‰ in *B. platifrons* from Site F indicate that biogenic
213 methane is the dominant carbon source fuelling the system. Second, methane dissolved
214 in seawater above the mussel beds at Site F had $\delta^{13}\text{C}$ values of -58.7‰ and -61.1‰
215 (Table 3), which is within the range of biogenic methane (Whiticar et al., 1986). The
216 two seawater samples were collected at about 1.5 m above the mussel beds and thus
217 the methane dissolved in it is most likely the residual of the seeping methane from the
218 subsurface, which has partially been oxidized under oxic and/or anoxic conditions. Due
219 to a fractionation of 0-10‰ during methane oxidation (cf. Alperin et al., 1988; Martens
220 et al., 1999), the residual methane from the water column should have slightly heavier
221 $\delta^{13}\text{C}$ values compared to the ‘original’ methane from the subsurface. Thus, the $\delta^{13}\text{C}$
222 value of methane within the mussel beds should have been more negative than that of
223 the methane sampled 1.5 m above the mussel bed, which in turn should explain the
224 more negative $\delta^{13}\text{C}_{\text{tissue}}$ values of *B. platifrons* compared to the sampled methane.

225 These data allow inferences about the geochemical conditions in the subsurface,

226 by combining the $\delta^{13}\text{C}_{\text{carbonate}}$ and $\delta^{13}\text{C}_{\text{tissue}}$ values to calculate the degree of mixing
 227 between different carbon sources in the DIC of the pore fluids at the zone of AOM and
 228 carbonate precipitation. Feng and Chen (in press) reported $\delta^{13}\text{C}_{\text{carbonate}}$ values from –
 229 55.3‰ to –34.3‰ for the carbonates at Site F. Assuming that (i) the $\delta^{13}\text{C}$ value of the
 230 authigenic carbonates reflects that of the DIC (e.g. Peckmann and Thiel, 2004;
 231 Peckmann et al., 2009; Feng et al., 2014), (ii) the $\delta^{13}\text{C}$ values of the tissue of *B.*
 232 *platifrons* reflect those of the ascending methane, and (iii) the $\delta^{13}\text{C}$ signature of
 233 seawater DIC = –0.2‰ (cf. Chou et al., 2007), then a simple mass balance calculation
 234 suggests that about 69% of the DIC of the pore fluids at the zone of AOM at site F is
 235 derived from methane and the rest from seawater $[(-48.5‰ - (-0.2‰))/(-70.3‰) \times$
 236 $100\%]$, with –48.5‰ being the average $\delta^{13}\text{C}_{\text{carbonate}}$ value of the authigenic carbonates
 237 collected at Site F (n=47; Feng and Chen, in press); see also Fig. 4. It should to be
 238 noted that the use of local carbonates as an indication of the DIC pool may cause some
 239 uncertainties. Romanek et al. (1992) found that there is a small carbon isotope
 240 fractionation between carbonate and bicarbonate during the precipitation of calcium
 241 carbonate minerals. Using the average aragonite-bicarbonate enrichment factor of 2.7‰
 242 at 10–40°C, it is suggested that the $\delta^{13}\text{C}$ value of the DIC pool is about –51.2‰ $[-48.5‰$
 243 $- 2.7‰ = -51.2‰]$ (cf. Romanek et al., 1992). Thus, the contribution of DIC from
 244 methane to the pore fluids might be underestimated, but probably by no more than 5%.

245 *Bathymodiolus aduloide*s harbors thiotrophic endosymbionts and therefore utilized
 246 DIC rather than methane as a carbon source (cf. Becker et al., 2010). The tissue $\delta^{13}\text{C}$
 247 values ($-34.4 \pm 1.8‰$) in *B. aduloide*s in this study were similar to those of other
 248 chemosymbiotic bivalves with thiotrophic endosymbionts at cold seeps (e.g. Kennicutt
 249 et al., 1992; Fiala-Médioni et al., 1993) and at hydrothermal vents (e.g. Childress et al.,
 250 1993; Fisher et al., 1988), indicating that chemosymbiotic bivalves with thiotrophic

251 endosymbionts use analogous inorganic carbon sources at different locations and that
252 this source shows little isotopic variation (Becker et al., 2010). This carbon source is
253 likely DIC in epibenthic bottom water taken up by the bivalves (Becker et al., 2010).
254 Because thiotrophic bacteria fractionate carbonate isotopes by $\sim -25\text{‰}$ (Ruby et al.,
255 1987), the $\delta^{13}\text{C}_{\text{tissue}}$ values of *B. aduloides* indicate that DIC in this epibenthic bottom
256 water had a $\delta^{13}\text{C}$ signature of around -9.4‰ ($-34.4\text{‰} - (-25.0\text{‰})$). This value agrees
257 well with the measured $\delta^{13}\text{C}$ values of CO_2 dissolved in seawater above the mussel
258 beds, which had $\delta^{13}\text{C}_{\text{CO}_2}$ values of about -9.2‰ (Table 3).

259

260 4.2. Constraints on seepage activity from tissue $\delta^{13}\text{C}$ values

261 A set of observations indicates that the mode of seepage at Site F was advective
262 rather than diffusive. The carbon source was biogenic methane as indicated by the
263 extremely low $\delta^{13}\text{C}$ values of both the methane sampled from the water column above
264 the seep site and the tissue of *B. platifrons*. The occurrence of thiotrophic symbionts-
265 harboring species *B. aduloides* indicates that hydrogen sulfide was available at this site
266 (Yamanaka et al., 2000). At cold seeps, the main horizon for the production of
267 hydrogen sulfide – the so called sulfate methane transition zone (SMTZ) – is controlled
268 largely by the flux of methane (Borowski et al., 1996). Only advective transport of
269 methane could produce enough hydrogen sulfide to sustain the mussels because sulfate
270 reduction at seeps is closely coupled to anaerobic oxidation of methane (Boetius et al.,
271 2000; Lichtschlag et al., 2010). In addition, the SMTZ tends to be situated closer to the
272 sediment-water interface at sites of advective seepage than at sites of diffusive seepage
273 (Borowski et al., 1996; Luff and Wallmann, 2003). Similarly, the extremely low $\delta^{13}\text{C}$
274 values of the Site F authigenic carbonates (as low as -55.3‰ ; Feng and Chen, in press)
275 is an additional argument in favor of this interpretation. Luff and Wallmann (2003)

276 suggested that the high methane flux and efficient methane oxidation results in low
277 $\delta^{13}\text{C}_{\text{carbonate}}$ values.

278

279 4.3. Tissue $\delta^{34}\text{S}$ values and sulfur cycling

280 Tissue $\delta^{34}\text{S}$ values of mussels with different symbionts may be used to infer the
281 sulfur isotopic composition of their sulfur sources and thereby also the processes that
282 affect the sulfur isotopic composition of these sources. This should be possible because
283 there is only negligible fractionation between sulfide and sulfate or organic sulfur
284 during sulfide oxidation by chemoautolithotrophic bacteria (Canfield, 2001), and
285 because there appears to be only little fractionation during uptake and incorporation of
286 sulfur isotopes into the tissue of the mussels (Vetter and Fry 1998). Animals with
287 sulfide-oxidizing symbionts typically record the $\delta^{34}\text{S}$ signature of the sulfide used by
288 their symbionts, while taxa with methanotrophic symbionts (and asymbiotic species)
289 record the $\delta^{34}\text{S}$ signature of the ambient water that they take up (Vetter and Fry 1998).
290 The isotopic signatures of these sulfur sources at methane seeps, in turn, are influenced
291 by various biogeochemical and mixing processes. The majority of sulfide at seeps is
292 produced via microbial sulfate reduction using methane (Boetius et al., 2000), which
293 can lead to a sulfide pool with $\delta^{34}\text{S}$ values as much as 50‰ lower than those of
294 seawater sulfate (Chambers and Trudinger, 1978). A large part of this sulfide is
295 reoxidized back to sulfate in the subsurface sediment, resulting in porewater sulfate
296 that is depleted in ^{34}S (cf. Aharon and Fu, 2003; Lichtschlag et al. 2010) compared to
297 seawater sulfate with its $\delta^{34}\text{S}$ value of +21‰ (Rees et al. 1978).

298 *Bathymodiolus aduloides*, the species with sulfide-oxidizing symbionts, at our
299 study site had low and variable $\delta^{34}\text{S}$ values ranging from -12.9‰ to -4.1‰, indicating
300 assimilation of hydrogen sulfide derived from sulfate-dependent AOM. Even lower

301 values (-15.4‰ to -12.4‰) were reported from a population of *B. aduloidea* at cold
 302 seeps off of Kikaijima Island in Japanese waters (Yamanaka et al., 2000). These low
 303 values were suggested to have resulted from the unique habitat of these animals, which
 304 live buried in sand where there is less mixing of the sulfate from reoxidized AOM
 305 sulfide with sulfate from seawater (Yamanaka et al., 2000). Indeed, it has been
 306 suggested that the distribution of the chemosynthetic biota at seeps is controlled by the
 307 flux of sulfide from the underlying sediment, for example *Beggiatoa* mats and
 308 bathymodiolin mussels being associated with slow and fast fluxes, respectively
 309 (Sahling et al. 2002; Aharon and Fu, 2003). This interpretation and the occurrence of
 310 mussels at Site F is in agreement with our assumption based on tissue $\delta^{13}\text{C}$ values (see
 311 section 5.2) that the mode of seepage at our study site was advective rather than
 312 diffusive.

313 *Bathymodiolus platifrons* with methanotrophic symbionts had high $\delta^{34}\text{S}$ values
 314 ($+6.4 \pm 2.6\text{‰}$), indicating the use of seawater sulfate ($\delta^{34}\text{S} = +21\text{‰}$; Rees et al. 1978)
 315 mixed with ^{34}S -depleted sulfur from the AOM reaction. A populations of *B. platifrons*
 316 from seeps off of Hatsushima Island in Sagami Bay, Japan, had a $\delta^{34}\text{S}$ signature close
 317 to that of seawater sulfate ($+17.8\text{‰}$ to $+18.5\text{‰}$; Yamanaka et al., 2000), indicating the
 318 assimilation of seawater sulfate that had not been isotopically modified by biological
 319 sulfate reduction. Unlike *B. platifrons* from the Hatsushima Island seep site, tissue $\delta^{34}\text{S}$
 320 values of *B. platifrons* from Site F were highly variable among individuals of the same
 321 species (Fig. 3). This variation might reflect high variability in the sulfate pool on a
 322 very small spatial scale (cf., Rodrigues et al., 2013), a pattern that is consistent with the
 323 cycling of sulfate between chemosymbiotic mussels and sediment microbial consortia
 324 as suggested recently by Becker et al. (2013, 2014). Alternatively, the large variation of
 325 tissue $\delta^{34}\text{S}$ values in *B. platifrons* could indicate that the degree of mixing between

326 reoxidized AOM derived sulfide and seawater sulfate changes with time.

327

328 4.4. Control on the tissue $\delta^{15}\text{N}$ values at seeps

329 The range of $\delta^{15}\text{N}$ values ($\delta^{15}\text{N} = -1.2\text{‰}$ to $+1.3\text{‰}$) in the two mussel species
330 from Site F is similar to that observed in bathymodiolin mussels from the Gulf of
331 Cadiz (Rodrigues et al., 2013). Even lower tissue $\delta^{15}\text{N}$ values (-23.7‰) were found in
332 bathymodiolin mussels from the Gulf of Mexico continental slope (Becker et al., 2010).
333 Becker et al. (2010) proposed that elevated concentrations of ammonium derived from
334 brines allows for a high degree of isotope fractionation during uptake and/or
335 assimilation by the mussels or their symbionts.

336 Tissue $\delta^{15}\text{N}$ values of the Site F mussels were significantly lower than $\delta^{15}\text{N}$ values
337 of the SCS deep-sea sediments ($\delta^{15}\text{N} = +5\text{‰}$ to $+6\text{‰}$; Gaye et al., 2009). As suggested
338 by many others, low $\delta^{15}\text{N}$ values indicate that organic nitrogen is of local origin (e.g.
339 Becker et al., 2010; Rodrigues et al., 2013), presumably resulting from the activity of
340 autotrophic bacteria (Levin, 2005; MacAvoy et al., 2008), and are possibly due to
341 assimilation of isotopically light nitrate or ammonium by the symbionts (Rodrigues et
342 al., 2013). The ranges of tissue $\delta^{15}\text{N}$ values in *B. platifrons* and in *B. aduloides* were
343 2.5‰ and 0.1‰ , respectively. The variations in nitrogen isotopic composition of
344 mussel tissues may reflect (i) differences in the geochemical environment at very small
345 scales (cf. Joye et al., 2004, 2010), (ii) resource partitioning among the two species (cf.
346 Becker et al. 2010), or (iii) differences in isotope fractionation among their symbionts.
347 The isotope compositions of the occurring organic nitrogen sources such as ammonium
348 and nitrate will need to be analyzed in the future if isotope trends are to be used as
349 proxies of nitrogen turnover at seep sites (Hoch et al., 1994; Lee and Childress, 1994;
350 Levin, 2005; Granger et al., 2008; MacAvoy et al., 2008).

351

352 4.5. Variation among tissue isotopes in the bathymodiolins and associated taxa

353 Too few specimens of the associated taxa were available to assess the entire food
354 web at Site F, but the available data provide some insight. Most species have lower
355 $\delta^{13}\text{C}$ signatures than expected from a nutrition based on photosynthetic food or sulfur-
356 oxidizing bacteria alone, and indicate methane-derived carbon as an important carbon
357 source. This fact supports our conclusions that methane-derived carbon plays a major
358 role in the ecosystem at Site F. Notable is the very negative $\delta^{13}\text{C}$ signature of the
359 sponge (-63.4‰), indicating that its diet is based largely on methane. Methanotrophic
360 sponges are known from the Gulf of Mexico (Vacelet et al., 1995, 1996). An isotope
361 study of seep fauna from New Zealand indicated that the sponge *Pseudosuberites* sp.
362 derives a similar proportion of its diet from methane-based carbon (Thurber et al., 2010)
363 as the specimen reported here from the South China Sea. The large difference between
364 the $\delta^{34}\text{S}$ values of the two specimens of *S. crosnieri* (-4.6‰ in the small specimens vs.
365 $+14.5\text{‰}$ in the large one) may reflect variations in the sulfur pool as seen in *B.*
366 *platifrons*, which *S. crosnieri* is always associated (Fig. 2A). Also specimens of *S.*
367 *crosnieri* collected at various vent sites in the Okinawa Trough show a wide range of
368 $\delta^{34}\text{S}$ values (from about -3‰ to $+8\text{‰}$; cf. Yamanaka et al., 2015). This finding
369 supports our conclusion from the variability of sulfur isotopes in *B. platifrons* that the
370 isotopic signature of the sulfate pool at Site F is highly variable even on small spatial
371 scales.

372 In *B. platifrons*, the $\delta^{13}\text{C}$ values of the mantle tissue are consistently lower than
373 those of the gill and the foot (Table 1, Fig. S1). The same trend has also been reported
374 by Childress et al. (1986) from seep-inhabiting mussels from the Gulf of Mexico and
375 by Yamanaka et al. (2015) from vent-inhabiting mussels from the Okinawa Trough.

376 Also $\delta^{34}\text{S}$ values vary between tissue types, though not as consistent as the $\delta^{13}\text{C}$ values.
377 The mechanisms leading to these differences among different body parts (mantle, gill,
378 and foot) are unknown. It is speculated that carbon isotope fractionation may occur
379 during the translocation of organic carbon derived from methane from the gills to other
380 tissues throughout the animal, or incorporation of other carbon sources during the
381 process (cf. Childress et al., 1986; Barry et al., 2002). However, this association has
382 not yet been conclusively established.

383

384 **5. Conclusions**

385 We used tissue stable isotopes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$) of two bathymodiolin mussel
386 species – *Bathymodiolus platifrons* with methanotrophic symbionts and *B. aduloide*s
387 with thiotrophic symbionts – collected from Site F of the South China Sea to constrain
388 fluid flow and biogeochemical processes. Biogenic methane is the main carbon source
389 fuelling the ecosystem as revealed by the presence of extremely low tissue $\delta^{13}\text{C}$ values
390 (average: -70.3‰) in the dominant symbiotic species *B. platifrons*. The average $\delta^{13}\text{C}$
391 values of authigenic carbonates at Site F (-48.5‰) – an indicator of the carbon isotopic
392 composition of dissolved inorganic carbon in pore water – suggest that mainly ($\sim 69\%$)
393 DIC from the seeping methane was incorporated into this carbonate. This rather small
394 offset between the $\delta^{13}\text{C}$ values of the methane and the carbonates, together with the
395 presence of a thiotrophic epifaunal species (*B. aduloide*s) indicates that seepage at Site
396 F is likely advective rather than diffusive. The significant difference in $\delta^{34}\text{S}$ values
397 between the two mussel species indicates high variability in the inorganic sulfur pools
398 at this site on a very small spatial scale. Our $\delta^{15}\text{N}$ data suggest that the co-occurring
399 bathymodiolin mussels either partition nitrogen sources or that their symbionts
400 fractionate nitrogen isotopes in different ways. Overall, this study confirms that tissue

isotopes of seep mussels are promising indicator of fluid sources, seepage activity,
sulfate and sulfide coupling, and nitrogen cycling at seeps.

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618 **Figure captions**

619 **Figure 1.** Image of the northern South China Sea showing the locations of all seep
 620 sites. Site F, the mussel sampling site in this study, is highlighted by a yellow dot
 621 (left). Bathymetry map on the right was modified from Feng and Chen (in press),
 622 the study area is delimited by the black box.

624 **Figure 2.** The Site F mussel beds. (A) Abundant living *Bathymodiolus platifrons*
 625 mussels of relatively uniform size on a carbonate pavement. (B) Live and dead

626 mussels on the sediment. (C) A small mussel bed of living *Bathymodiolus*
 627 *aduloides* mussels, partially covered by bacterial mats. (D) Shells of the two
 628 investigated mussel species. Images (A), (B), and (C) are about 2-3 m across.

629
 630 **Figure 3.** Plots of $\delta^{15}\text{N}$ vs. $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ vs. $\delta^{13}\text{C}$ values for *Bathymodiolus platifrons*
 631 and *B. aduloides* from Site F in the South China Sea.

632
 633 **Figure 4.** Schematic diagram of the microbially mediated carbon and sulfur cycles
 634 associated with chemosynthetic communities at Site F in the South China Sea
 635 (modified from Lichtschlag et al., 2010). The $\delta^{13}\text{C}_{\text{DIC}}$ value of the pore water is
 636 estimated from $\delta^{13}\text{C}$ values of authigenic carbonate from the same site (Feng and
 637 Chen, in press). $\delta^{34}\text{C}_{\text{SO}_4}$ and $\delta^{13}\text{C}_{\text{DIC}}$ of the bottom seawater are from published
 638 sources (Rees et al. 1978; Chou et al., 2007). Intensities of fluid flows are
 639 represented by triangles (red for methane and blue for sulfate). Arrows denote
 640 biological or chemical turnovers. AOM, anaerobic oxidation of methane; SR,
 641 sulfate reduction. See text for details.

642 643 **Table captions**

644 **Table 1.** Tissue $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values of *Bathymodiolus platifrons* and *B.*
 645 *aduloides* from Site F in the South China Sea.

646
 647 **Table 2.** Means and standard deviations for tissue $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values of
 648 *Bathymodiolus platifrons* and *B. aduloides* from Site F in the South China Sea.

649
 650 **Table 3.** $\delta^{13}\text{C}$ values of CH_4 and CO_2 dissolved in seawater about 1.5 m above the

651 mussel bed from Site F of the South China Sea.

652

653 **Table 4.** Tissue $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values of the associated taxa from Site F in the
654 South China Sea.

655

656 **Supplementary material**

657 **Figure S1.** Tissue-specific stable isotope (C, N, and S) values for *Bathymodiolus*
658 *platifrons* and *B. aduloides*.

659

660 **HIGHLIGHTS:**

- 661 ● *B. platifrons* (methanotrophic) and *B. aduloides* (thiotrophic) co-occur at the seep.
- 662 ● Biogenic methane is the dominant carbon source fuelling the seep system.
- 663 ● Tissue $\delta^{13}\text{C}$ of mussels are useful to assess fluid intensities at cold seeps.
- 664 ● Tissue $\delta^{34}\text{S}$ of mussels suggests complex sulfate and sulfide coupling at cold seeps.

665

Table 1

Species	Sample ID	Sample Type	$\delta^{13}\text{C}_{\text{tissue}}$ (‰ vs. V-PDB)	$\delta^{15}\text{N}_{\text{tissue}}$ (‰ vs. air)	$\delta^{34}\text{S}_{\text{tissue}}$ (‰ vs. V-CTD)
<i>B. platifrons</i>	P1	Mantle	-76.4	0.2	2.1
		Gill	-75.2	-0.2	7.8
		Foot	-73.4	0.3	8.2
	P2	Mantle	-77.6	-0.1	5.8
		Gill	-76.4	1.0	6.2
		Foot	-72.8	1.1	10.3
	P3	Mantle	-74.3	-1.2	5.6
		Gill	-69.3	-0.7	8.2
		Foot	-67.7	-0.2	10.5
	P4	Mantle	-70.1	0.3	5.6
		Gill	-69.6	0.4	6.7
		Foot	-65.2	0.7	9.3
	P5	Mantle	-71.2	-0.2	4.4
		Gill	-68.3	0.5	6.9
		Foot	-64.7	0.8	9.4
	P6	Mantle	-70.7	0.5	6.1
		Gill	-68.2	0.5	6.8
		Foot	-66.2	1.0	9.4
	P7	Mantle	-71.5	-0.6	6.6
		Gill	-68.0	-0.9	10.4
		Foot	-66.0	0.5	10.1
	P8	Mantle	-70.3	0.3	1.4
		Gill	-69.0	0.0	6.8
		Foot	-66.1	0.9	7.4
	P9	Mantle	-72.9	0.7	6.3
		Gill	-72.4	0.9	1.6
		Foot	-68.5	1.3	7.6
	P10	Mantle	-74.9	1.3	3.6
		Gill	-73.5	1.2	1.7
		Foot	-69.6	1.2	8.9
	P11	Mantle	-70.0	1.1	4.0
		Gill	-68.1	1.1	4.6
		Foot	-66.2	1.1	7.1
	P12	Mantle	-71.1	1.1	4.9
		Gill	-70.4	1.0	3.9
		Foot	-66.5	1.2	5.7
<i>B. aduloides</i>	A1	Mantle	-37.4	1.2	-12.9
		Gill	-37.4	1.1	-6.1
		Foot	-33.4	1.1	-4.1
	A2	Mantle	-33.0	1.1	-11.0
		Gill	-33.4	1.1	-5.0
		Foot	-33.4	1.1	-6.2
	A3	Mantle	-34.2	1.2	-11.6
		Gill	-33.5	1.2	-7.1
		Foot	-33.6	1.2	-7.9

Table 2

Species	N	$\delta^{13}\text{C}_{\text{tissue}}$ (‰ vs. V-PDB)		$\delta^{15}\text{N}_{\text{tissue}}$ (‰ vs. air)		$\delta^{34}\text{S}_{\text{tissue}}$ (‰ vs. V-CTD)	
		mean±sd	min	max	mean±sd	min	max
<i>B. platifrons</i>	12	-70.3±3.4	-77.6	-64.7	0.5±0.7	-1.2	1.3
<i>B. aduloides</i>	3	-34.4±1.8	-37.4	-33.0	1.1±0.0	1.1	1.2
						-12.9	-4.1

Table 3

Sample number	$\delta^{13}\text{C}_{\text{CH}_4}$ (‰ vs. V-PDB)	$\delta^{13}\text{C}_{\text{CO}_2}$ (‰ vs. V-PDB)
56-1	-58.73	-9.20
56-2	-61.14	-9.24

Table 4

Species	$\delta^{13}\text{C}_{\text{tissue}}$ (‰ vs. V-PDB)	$\delta^{15}\text{N}_{\text{tissue}}$ (‰ vs. air)	$\delta^{34}\text{S}_{\text{tissue}}$ (‰ vs. V-CTD)
White sponge attached to rock	-63.4	1.1	6.4
Spider crab	-37.5	1.2	2.3
<i>Shinkaia crosnieri</i> #1 (large specimen)	-43.6	1.1	14.5
<i>Shinkaia crosnieri</i> #2 (small specimen)	-40.4	1.2	-4.6
<i>Bathymacrea nipponica</i> #1	-55.8	1.2	-5.0
<i>Bathymacrea nipponica</i> #2	-44.1	1.1	3.4
<i>Bathymacrea nipponica</i> #3	-55.5	1.2	1.9
<i>Provanna</i> sp. (3 pooled specimens)	-42.3	1.1	n.d.
<i>Branchiopolynoe pettibonae</i>	-58.9	1.1	-9.4

n.d., not detected

Figure 1

Figure 2

Figure 3

Figure 4