Natural ^{14}C abundances and stable isotopes suggest discrete uptake routes for carbon and nitrogen in cold seep animals

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Abstract

Cold seeps, where geofluids containing methane and other hydrocarbons originating from the subseafloor seeps through the sediment surface, play important roles in the elemental and energy flux between sediment and seawater. These seep sites often harbor communities of endemic animals supported by chemolithoautotrophic bacteria, either through symbiosis or feeding. Despite these animal communities being intensively studied since their discovery in the 1980's, the contribution of carbon from seep fluid to symbiotic microbes and subsequently host animals remains unclear. Here, we used natural-abundance radiocarbon to discern carbon sources: the ambient bottom water or the seeping geofluid. The ^{14}C concentrations were measured for vesicomyid clams, a parasitic calamyzine polychaete, and a siboglinid tubeworm species from four different cold seep sites around Japan. We found most vesicomyid clams exhibiting ^{14}C concentrations slightly lower than that of the ambient bottom water, suggesting up to 9% of C for chemolithoautotrophy originates from geofluid DIC. The different extent of fluid contribution across species may be explained by different routes to incorporate DIC and/or different DIC concentrations in the geofluid at each seep site. Stable nitrogen isotopic compositions further suggested N incorporation from geofluids in these clams, where the burrowing depth may be a key factor in determining their δ^{15}N values. The siboglinid tubeworm showed a clear dependency for geofluid DIC, with a contribution of >40%. Our results demonstrate the effectiveness of ^{14}C analyses for elucidating the nutritional ecology of cold seep animals and their symbionts, as was previously shown for hydrothermal vent ecosystems.

Keywords Nutrition, Stable carbon and nitrogen isotope ratios, Hydrocarbon seep, Chemosynthetic community, Chemosynthesis, Radiocarbon

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1 Introduction

Deep-sea benthic ecosystems mostly rely on organic matter derived from the ocean surface in the form of phytodetritus or 'marine snow' showering down to the deep for energy, due to a lack of photosynthetic primary production (Gage and Tyler 1991). Chemosynthetic communities on the deep-sea floor, such as those associated with hydrothermal vents or hydrocarbon seeps, are exceptions where primary production is enabled by microbes utilizing chemical energy from the oxidation of molecules contained in the geofluid (e.g., Fisher et al. 1989; Markert et al. 2007) to produce organic matter (OM). Many animals endemic to these ecosystems and living in close

proximity to geofluid flows are symbiotic with such bacteria and depend on them for nutrition (Dubilier et al. 2008). Due to the high productivity of OM in these chemosynthetic communities, they tend to exhibit total biomasses three to four orders of magnitude higher than the surrounding deep-sea floor sustained by phytodetritus (Levin 2005; Van Dover 2000).

The Japanese archipelago is located at boundaries of four plates including the Philippine Sea plate, the Pacific plate, the Eurasian (Amurian) plate, and the North American (Okhotsk) plate. Subduction of the Philippine plate and the Pacific plate beneath the Eurasian plate and the North American Plate, respectively, produce pore fluid seepage to the seafloor in the subducted accretionary prism (e.g., Boulègue et al. 1987, Gamo et al. 1992). These pore fluids contain high concentrations of hydrocarbons, such as methane, either of microbial origin (Toki et al. 2012; Riedinger et al. 2015) or thermogenic origin (Toki et al. 2014). There are numerous chemosynthetic communities around Japan associated with these hydrocarbon seeps from bathyal to hadal depths, especially in Japan Trench and Nankai Trough (Nakajima et al. 2014). These seeps are mainly dominated by vesicomyid clams and siboglinid tubeworms, but the species composition at each site is influenced by factors such as water depth and geographic location (Fujikura et al. 2000; Nakajima et al. 2014). Vesicomyid clams in the subfamily Pliocardiinae gain nutrition from thioautotrophic bacteria living in bacteriocytes within their gills (Arp et al. 1984; Krylova and Sahling 2010). The symbionts produce OM via the oxidation of hydrogen sulfide taken up by the clam's foot into the blood, using oxygen from the ambient seawater which is drawn in from the siphon and incorporated directly in the gill (Childress and Girguis 2011; Ip et al. 2021). Siboglinid tubeworms possess a unique organ filled with symbiotic thioautotrophic bacteria called the trophosome and have completely lost the gut as adults (Cavanaugh et al. 1981; Nussbaumer et al. 2006). Depending on the genus, tubeworms either conduct all gas exchanges through the plume (gill) or take up oxygen from the plume but sulfides from an extended posterior 'root' system (Childress and Girguis 2011; Dattagupta et al. 2006).

While energy sources, i.e., the coupling of reducing and oxidizing compounds in the pore fluid and bottom seawater, respectively, for chemoautotrophic carbon production has been a focus of study in chemosynthetic ecosystems, their carbon and nitrogen sources have rarely been investigated in detail. Symbiotic vesicomyid clams are thought to incorporate ammonium or nitrate for chemosynthesis from ambient seawater bathing their gills, which diffuses directly into the symbionts (Childress and Girguis 2011). The symbionts also mostly take up dissolved inorganic carbon (DIC) directly in the same way (Kochevar and Childress 1996), though the possibility that the host clam assists this by taking up DIC through the foot and transporting this through blood circulation to the symbionts has been suggested (Childress and Girguis 2011). For siboglinid tubeworms, some (e.g., *Riftia* and *Ridgeia*) are thought to take up DIC and ammonium/nitrate exclusively from the plume (Liao et al. 2014), while for others (e.g., *Lamellibrachia*) the capacity to do so from either the plume or 'root' remains little-studied. Stable isotope ratio of carbon ($\delta^{13}C$) can be used to estimate carbon sources of organisms (e.g., Nomaki et al. 2008, 2021). However, in the case of chemosynthetic communities, different pathways of chemosynthesis, such as reductive tricarboxylic acid cycle (rTCA), the Calvin–Benson–Bassham (CBB) cycle, and methane oxidation pathways (House et al. 2003; Pearson 2010), lead to varying $\delta^{13}C$ values of OM due to the different degrees of isotope fractionation from carbon dioxide to biomass (c.a. 2–13‰ for rTCA, c.a. 5–26‰ for CBB) or from methane to biomass (16–31‰ for aerobic methanotrophs, and 0–40‰ for anaerobic methanotrophs, Pearson 2010). This makes the interpretation of vent and seep animals' carbon sources challenging from analysis of $\delta^{13}C$ alone.

Natural-abundance radiocarbon ($\Delta^{14}C$) has recently become an increasingly used tool for investigating carbon flows in marine ecosystems (Ishikawa et al. 2021). In deep-sea ecosystems, ingestion of freshly deposited organic matters derived from the photic zone (rich in ^{14}C) could be confirmed by analyzing ^{14}C concentrations in the gut contents, body tissue, plus surface sediments and sediment trap samples as potential food sources (Purinton et al. 2008). In chemosynthetic ecosystems such as hydrothermal vent ecosystems, $\Delta^{14}C$ analyses can clearly distinguish OM derived from surface primary production (c.a. 40‰) from chemosynthetic OM (below −800‰)—(Nomaki et al. 2019, 2024; Williams et al. 1981). Chemoautotrophic microbes at vents exhibit depleted ^{14}C concentrations because they utilize either ^{14}C-depleted bottom water (c.a. −200‰ in the Pacific Ocean, Stuiver et al. 1983) or ^{14}C-dead DIC from the vent fluid (−1000‰) as their inorganic carbon source (Williams et al. 1981). Hydrothermal vent animals living in close vicinity to vent orifices exhibit highly depleted $\Delta^{14}C$ values while those at periphery habitat tended to show $\Delta^{14}C$ values closer to that of the bottom water (Nomaki et al. 2024). An exception is that even at the periphery, *Lamellibrachia* tubeworms exhibited more depleted $\Delta^{14}C$ values than co-occurring vesicomyid clams, suggesting that the carbon source of *Lamellibrachia* endosymbionts includes vent fluid DIC taken up via roots of their hosts deeply embedded in the sediments (Nomaki et al.

2024). Similar to hydrothermal vent areas, $\Delta^{14}C$ values of pore fluid at cold seeps is also very low (down to −938‰: Masuzawa et al. 1995), making it possible to discriminate carbon sources for chemolithoautotrophic bacteria and their host animals between DIC in bottom water and pore fluid. To accurately interpret the results, however, it is important to make a clear distinction between these purely subduction-driven cold seeps (*sensu* Levin 2005) from 'hydrothermal seeps' fueled partly by hydrothermalism (Levin et al. 2012) or those supported by warm groundwater such as the Off Hatsushima seep in Sagami Bay, Japan (Tsunogai et al. 1996). In the present study, we focus on porewater-driven seeps and call them 'cold seeps' hereafter.

Here, we measured $\Delta^{14}C$, $\delta^{13}C$, and $\delta^{15}N$ values of siboglinid tubeworms, vesicomyid clams, and polychaetes living inside their mantle cavities collected from four different cold seep sites around Japan. Their $\Delta^{14}C$ values were compared with those of bottom water $\Delta^{14}C$ values to evaluate DIC sources of the symbiotic chemoautotrophic bacteria in these animals. Both $\delta^{13}C$ and $\delta^{15}N$ values were further evaluated to infer metabolism and ecology of animals.

2 Materials and Methods

2.1 Animal sample collections

Animal samples for isotopic measurements were collected between 1989 to 2004 using various research vessels and submersibles (Table 1), including vesicomyid clams, polychaetes living inside their mantle cavities, and

siboglinid tubeworms (for a list see Table 2 and Supplementary Table 1). We used samples from four different cold seep sites: landward slope of Japan Trench (39°06.4′ N, 143°53.6′ E, 5343–5345 m deep), Yukie Ridge in the Nankai Trough (33°50′ N, 137°54–55′ E, 1900–2200 m deep), Daisan Tenryu Submarine Canyon in the Nankai Trough (33°38.7′ N, 137°54.498′ E, 3851 m deep), and Kuroshima Knoll on the Ryukyu Arc (24°07–08′ N, 124°12–13′ E, 643–785 m deep) (Fig. 1). Animals were collected using either a suction sampler or a scoop sampler on the remotely operated vehicles (ROVs) *Hyper-Dolphin* and *Kaiko* or the human occupied vehicles (HOVs) *Shinkai 2000, Shinkai 6500,* and *Nautile*. Once recovered on-board, the animals were identified, dissected into different body parts in some cases, and then frozen in −80 °C freezer until use.

2.2 ^{14}C analyses of organisms

The frozen animal samples were freeze-dried and ground into fine powders. We placed 0.71–1.73 mg dry weight of samples in pre-combusted glass cups and then decalcified them with 0.1 M HCl, and completely dried again. The samples were graphitized by methods modified from Yokoyama et al. (2007, 2010). Briefly, dried samples were combusted in an evacuated quartz tube with copper oxide at 500 °C for 30 min and at 850 °C for 2 h. The CO_2 gas was cryogenically purified in a vacuum line and reduced to graphite with hydrogen and an iron catalyst at 630 °C for 6 h. We measured the $\Delta^{14}C$ values with a single-stage accelerator mass spectrometer (AMS) at the

Table 1 Investigated cold seep sites and sampling details. Natural-radiocarbon abundances ($D^{14}C$) of the bottom water were estimated from reported values

Cold seep site	Depth (m)	Latitude	Longitude	Research vessel	Cruise ID	Submersible	Dive no.	Animal sampling date	Bottom water DIC $\Delta^{14}C$ (‰)	Water sampling year
Landward slope, Japan Trench	5343–5345	39°06.39–43′ N	143°53.63–65′ E	*Kairei*	KR00-07 Leg 2	ROV *Kaiko*	10K183	11/14/2000	−210 to −200	1993[a]
Yukie Ridge, Nankai Trough	1900–2200	33°50′ N	137°54–55′ E	*Nadir*	KAIKO-Nankai	HOV *Nautile*	KN14	8/23/1989	−230 to −220	1994[b]
Yukie Ridge, Nankai Trough	2038	33°49.978′ N	137°54.852′ E	*Yokosuka*	YK99-09 Leg2	HOV *Shinkai 6500*	6K520	10/29/1999	−230 to −220	1994[b]
Yukie Ridge, Nankai Trough	2079	33°50.00′ N	137°55.08′ E	*Yokosuka*	YK99-09 Leg2	HOV *Shinkai 6500*	6K520	10/29/1999	−230 to −220	1994[b]
Daisan Tenryu Canyon, Nankai Trough	3784–3840	33°38.63–81′ N	137°54.40–48′ E	*Yokosuka*	YK00-06 Leg1	HOV *Shinkai 6500*	6K557	7/13/2000	−220 to −210	1994[b]
Kuroshima Knoll, Ryukyu Arc	643	24°07.80′ N	124°11.46′ E	*Natsushima*	NT04-03 Leg 2	ROV *Hyper-Dolphin*	HPD290	4/13/2004	−100 to −80	2019[c]
Kuroshima Knoll, Ryukyu Arc	785	24°07.68′ N	124°12.65′ E	*Natsushima*	NT97-14 Leg 5	HOV *Shinkai 2000*	2K981	10/8/1997	−140 to −135	2019[c]

[a] Key et al. (2002) (WOCE P10, Stn 74, 80)

[b] WOCE Atlas Volume 2: Pacific Ocean (https://doi.org/10.21976/C6WC77) (WOCE-P09)

[c] Ding et al. (2020) (Stn C5, C8)

Table 2 Cold seep animals measured in this study and their averaged isotopic compositions

Species	Cold seep site	Water depth (m)	Numbers of specimens	δ¹³C (‰ VPDB)	δ¹⁵N (‰ AIR)	Δ¹⁴C (‰)	Δ¹⁴C_animal-bottom water	Contribution of fluid C (%)
Abyssogena phaseoliformis	Landward slope, Japan Trench	5343–5345	4	−36.1 ± 0.2	−4.7 ± 0.6	−275 ± 9.1	−70 ± 9.1	8.8 ± 1.1
Nautiliniella calyptogenicola	Landward slope, Japan Trench	5343–5345	4	−36.3 ± 0.3	−1.8 ± 0.6	−271 ± 1.3	−66 ± 1.3	8.3 ± 0.2
Archivesica tsubasa	Yukie Ridge, Nankai Trough	1900–2200	4	−37.3 ± 1.1	−6.2 ± 2.1	−237 ± 5.6	−12 ± 5.6	1.6 ± 0.7
Calyptogena fausta	Yukie Ridge, Nankai Trough	1900–2200	3	−39.4 ± 0.2	1.1 ± 0.3	−247 ± 3.3	−22 ± 3.3	2.9 ± 0.4
Archivesica mag-nocultellus	Yukie Ridge, Nankai Trough	2038	4	−37.7 ± 0.3	4.2 ± 1.8	−245 ± 3.3	−20 ± 3.3	2.6 ± 0.4
Lamellibrachia columna	Yukie Ridge, Nankai Trough	2079	4	−32.8 ± 9.3	−0.4 ± 1.0	−424 ± 100.7	−199 ± 100.7	25.7 ± 13.0
Ectenagena laubieri	Daisan Tenryu Canyon, Nankai Trough	3784–3840	4	−36.9 ± 0.2	−2.2 ± 2.0	−243 ± 4.5	−28 ± 4.5	3.6 ± 0.6
Archivesica tsubasa	Daisan Tenryu Canyon, Nankai Trough	3784–3840	4	−37.6 ± 0.1	−2.1 ± 2.8	−245 ± 1.0	−30 ± 1.0	3.8 ± 0.1
Abyssogena kaikoi	Daisan Tenryu Canyon, Nankai Trough	3784–3840	4	−37.5 ± 0.8	−2.0 ± 2.8	−242 ± 5.3	−27 ± 5.3	3.4 ± 0.7
Akebiconcha kawamurai	Kuroshima Knoll, Ryukyu Arc	643	4	−34.1 ± 0.6	6.8 ± 1.1	−96 ± 2.2	−6 ± 2.2	0.7 ± 0.2
Pliocardia kuro-shimana	Kuroshima Knoll, Ryukyu Arc	785	4	−32.5 ± 0.4	7.4 ± 0.3	−169 ± 2.4	−31 ± 2.4	3.6 ± 0.3

Atmosphere and Ocean Research Institute, the University of Tokyo (Chiba, Japan; AMS lab code YAUT; Yokoyama et al. 2019). Blank tests for sample preparations and processing found no contamination of modern carbon (Yamane et al. 2019; Yokoyama et al. 2022).

Radiocarbon data reported here are corrected for radioactive decay between 1950 and the year when the samples were measured. The equation used is as follows:

$$\Delta^{14}\text{C} = \left\{ \frac{\left(^{14}\text{C}/^{12}\text{C}\right)_{\text{sample}}}{\left(^{14}\text{C}/^{12}\text{C}\right)_{\text{OX-1}} e^{\frac{y-1950}{8267}}} - 1 \right\} \quad (1)$$

The Δ¹⁴C value of the international standard (Ox-1: oxalic acid-1) takes into account of radioactive decay since 1950 (Stuiver and Polach 1977).

Radiocarbon measurements were conducted with 6 to 9 sets of 6 min measurements. Numbers of measurements depended on the samples, since we aimed to collect more than 20,000 counts to achieve accurate measurements. We also took into account all possible mass fractionations by measuring δ¹³C values using the AMS instead of an offline isotope ratio mass spectrometer (IRMS). We used four international standards, namely IAEA-C1, IAEA-C3, IAEA-C6, and NIST SRM 4990C, with each standard having different radiocarbon concentrations.

Our measured values were calibrated with the calibration curve obtained from the measurements of these standards.

2.3 Analyses of total organic carbon (TOC), total nitrogen (TN) contents, and their stable isotopic compositions

The freeze-dried and powdered samples (111 to 213 μg dry weight) were placed into pre-cleaned silver capsules. The samples were decalcified with 0.1 M HCl and dried on a hot plate at 60 °C, and the capsules were sealed using pre-cleaned forceps. Stable carbon and nitrogen isotopic composition were determined using an isotope ratio mass spectrometer coupled to an elemental analyzer (Flash EEA 1112-DELTA V Advantage ConFloIV System, Thermo Fisher Scientific). Isotope ratios were conventionally expressed as δ values in ‰ (Coplen 2011) relative to carbon and nitrogen international standards (Vienna PeeDee Belemnite and atmospheric N₂, respectively). After measuring every 10 to 15 samples, isotopic compositions of internal working standards (Glycine: −33.8‰, Alanine: −19.6‰, Histidine: −10.7‰ for δ¹³C measurements, and Alanine: 1.58, 9.97, and 20.6‰ for δ¹⁵N measurements) were measured and analytical errors for δ¹³C and δ¹⁵N working standards were both < ±0.15‰.

2.4 ^{14}C abundances of bottom water DIC

We estimated Δ^{14}C values of ambient bottom water at each sampling site from previously reported values (Table 1). At both Yukie Ridge and Daisan Tenryu Submarine Canyon at Nankai Trough, the P09 line data obtained during the World Ocean Circulation Experiment (WOCE) sampling campaign in 1994, in close proximity to the animal collection sites (Fig. 1), were used to estimate Δ^{14}C values at 1900 to 2200 m depth (−230 to −220‰: Yukie Ridge) and 3850 m (−230 to −210‰: Daisan Tenryu Submarine Canyon). The P10 stations data of the WOCE sampling campaign in 1993, which were hundreds of kms south from the landward slope of

Japan Trench site, were used to estimate bottom water Δ^{14}C values (Fig. 1). Despite the distance, since Δ^{14}C values deeper than 3000 m were almost constant across latitudes along the P10 line (Key et al. 2002), we consider our estimated range (−209 to −199‰) to be not far off from ambient bottom water value at the time of the animal sampling event at Japan Trench.

At Kuroshima Knoll, we referred Δ^{14}C values from published data obtained off Taiwan in 2019, which is 200 to 300 km south/southwest of the animal collection sites (C05 and C08 of Ding et al. 2020). The Kuroshio current is running through these stations of Ding et al. (2020)
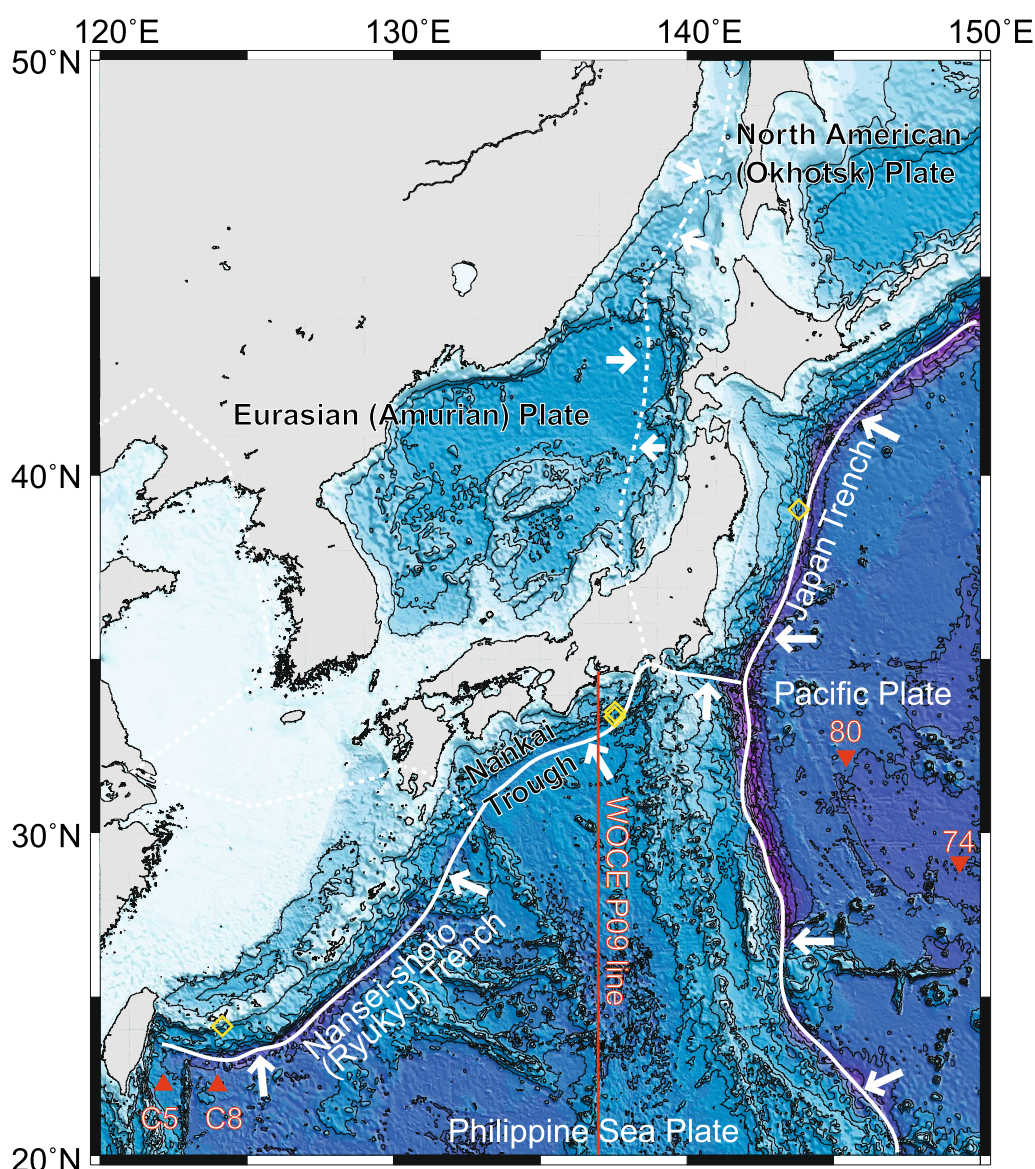

Fig. 1 Map of the relevant cold seep sites (open yellow diamonds) and the site where natural-abundance radiocarbon (D^{14}C) data of dissolved inorganic carbon in the seawater were taken from in this study (red lines: WOCE P09 lines, triangles: Ding et al. 2020, C5 and C8; reversed triangle: WOCE P10, 74 and 80)

and also around Kuroshima Knoll, thus it is reasonable to postulate that those stations exhibit similar $\Delta^{14}C$ profiles to those at Kuroshima Knoll. As $\Delta^{14}C$ profiles of these stations (Ding et al. 2020) as well as other areas where Kuroshio current runs through (Lan et al. 2023) showed sharp decreases in $\Delta^{14}C$ values from 500 to 1000 m deep where our sampling stations fall into (643 and 785 m), a small change in the water mass may cause some 10 s of ‰ of $\Delta^{14}C$ values in this case. Also, the atmospheric ^{14}C concentrations in northern hemisphere is decreasing with time due to the anthropogenic emission of fossil fuel (Graven et al. 2022), from around 80 to 90‰ during 1997 to 2004 when animals were collected at Kuroshima Knoll to almost 0‰ in 2019 when the $\Delta^{14}C$ profiles in

Ding et al. (2020) were obtained. The changes in surface water DIC $\Delta^{14}C$ values have been reported off California, from 35‰ in 2004 to −6‰ in 2022, where $\Delta^{14}C$ values of atmospheric CO_2 decreased from 60‰ to −5‰ during the same period (Hauksson et al. 2023). We note that the surface water DIC $\Delta^{14}C$ values near Kuroshima Knoll may also have changed to a similar extent, though the subsurface DIC $\Delta^{14}C$ values at depths over 500 m are expected to be more stable as shown at Nankai Trough area between 1994 and 2022 (Lan et al. 2024). As such, our estimates of the $\Delta^{14}C$ values of the ambient bottom water at Kuroshima Knoll must be treated with caution.

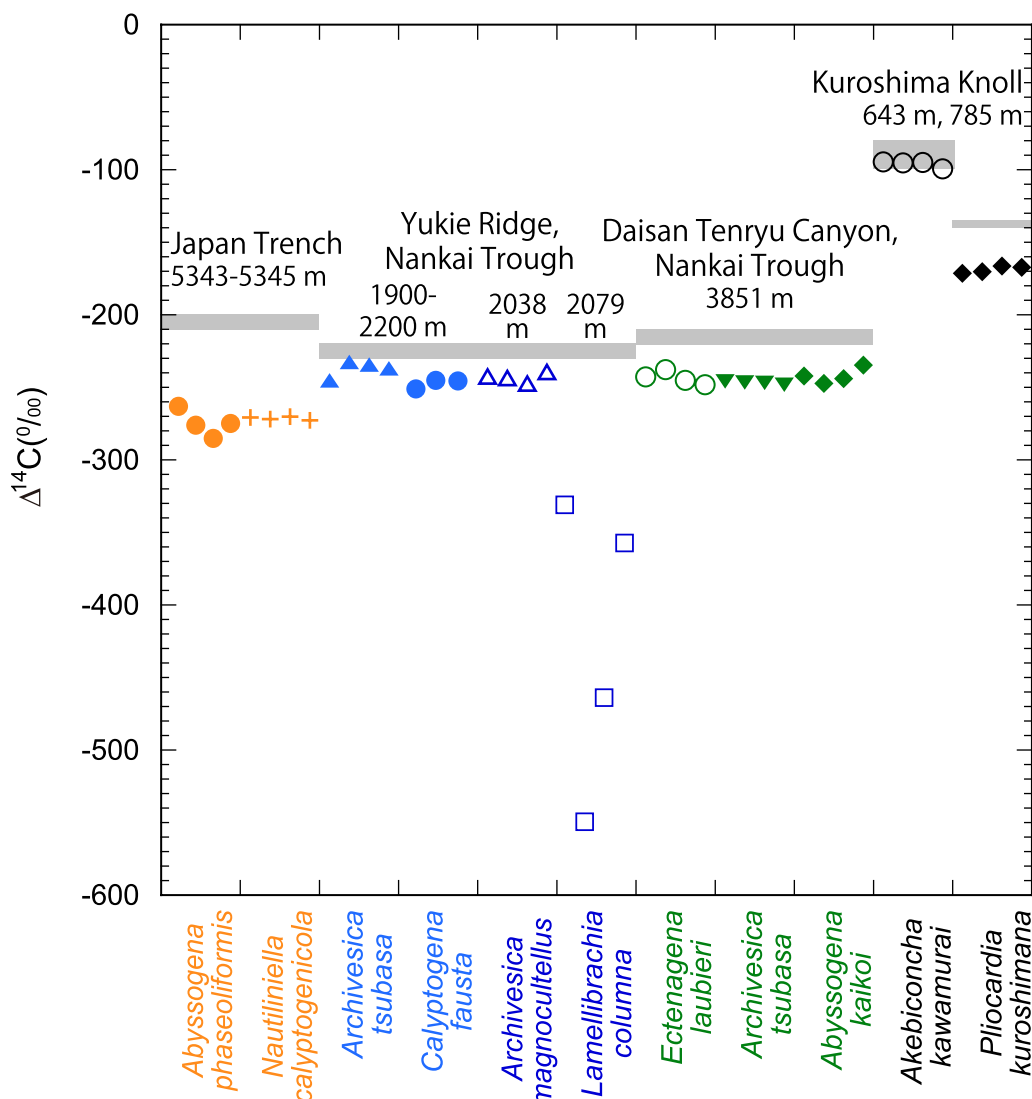Based on the measured $\Delta^{14}C$ values of animals (Table 2 and Supplementary Table 1) and above mentioned

Fig. 2 Natural-abundance radiocarbon (D^{14}C) of animals collected from cold seep sites around Japan. The gray bars indicate the estimated range of natural-abundance radiocarbon ($\Delta^{14}C$) values of bottom water dissolved inorganic carbon

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estimations of bottom water DIC Δ^{14}C values, we calculated the contributions of seep fluid DIC to the chemoautotrophic productions in the endosymbionts of each animal using the following equation:

$$\text{Contribution of geofluid C} = \left(\Delta^{14}\text{C}_{\text{animal}} - -\Delta^{14}\text{C}_{\text{bw}}\right) \Big/ \left(\Delta^{14}\text{C}_{\text{sf}} - -\Delta^{14}\text{C}_{\text{bw}}\right) \quad (2)$$

where Δ^{14}C $_{\text{animal}}$ is the averaged Δ^{14}C value for each animal species (Table 2), Δ^{14}C$_{\text{bw}}$ is a median of the estimated bottom water DIC value range (Table 2), and Δ^{14}C$_{\text{sf}}$ is −1000‰ assuming DIC in seepage fluid contained DIC originated from old (i.e., older than 50,000 years) organic matter preserved in the sediments. Although reported Δ^{14}C values of pore fluid DIC at seep sites are limited, Masuzawa et al. (1995) found that the seep fluid collected in vesicomyid clam colonies indeed exhibited decreasing Δ^{14}C profiles with increasing sediment depths and showed highly depleted values (−938 ± 20‰ and −938 ± 4‰) at 18 cm and 36 cm depths in the sediments.

3 Results
3.1 Natural-abundance radiocarbon
Natural-abundance radiocarbon concentrations (Δ^{14}C) of vesicomyid clams ranged from −275 ± 9‰ (*Abyssogena phaseoliformis* at landward slope of Japan Trench) to −96 ± 2‰ (*Akebiconcha kawamurai* at Kuroshima Knoll), and varying with the sampling locality (Fig. 2). Clams from the same sampling locality exhibited almost identical Δ^{14}C values: −247 ± 3‰ (*Calyptogena fausta*), −245 ± 3‰ (*Archivesica magnocultellus*), and −237 ± 6‰ (*Archivesica tsubasa*) at Yukie Ridge; −245 ± 1‰ (*Archivesica tsubasa*), −243 ± 5‰ (*Ectenagena laubieri*), and −242 ± 5‰ (*Abyssogena kaikoi*) at Daisan Tenryu Canyon, which were 10 to 20‰ (Yukie Ridge) and ca. 30 ‰ (Daisan Tenryu Canyon) lower than ambient bottom water values (Fig. 2 Table 2). At Kuroshima Knoll, *Akebiconcha kawamurai* collected at 643 m water depth exhibited higher Δ^{14}C values (−96 ± 2‰) than *Pliocardia kuroshimana* collected at 785 m water depth (−169 ± 2‰). Those values were mostly the same (*Akebiconcha kawamurai* at Kuroshima Knoll) to ca. 30‰ lower than those of the estimated Δ^{14}C values of ambient bottom water (Fig. 2., Table 1). *Abyssogena phaseoliformis* from Japan Trench exhibited even lowered Δ^{14}C values, ca. 70‰ lower than ambient bottom water values.

Nautiliniella calyptogenicola, a calamyzine (Chrysopetalidae: Calamyzinae) annelid worm living inside the mantle cavity of *Abyssogena phaseoliformis* (Miura and Laubier 1989), exhibited Δ^{14}C values of −271 ± 1‰, which was almost identical to the host clam values (−275 ± 9‰). For the siboglinid tubeworm *Lamellibrachia columna*

('*Lamellibrachia* sp. L2' of Kojima et al. (2001), shown to be conspecific with *L. columna* in McCowin et al. (2019)) exhibited greatly varying Δ^{14}C values among individuals, from −548 to −329‰ (average −424 ± 101‰), corresponding on average 200‰ lower than those of ambient bottom water (Table 2).

3.2 Stable carbon and nitrogen isotopic compositions
Stable carbon isotopic compositions (δ^{13}C) of cold seep vesicomyid calms differed slightly among sites. At Yukie Ridge and Daisan Tenryu Submarine Canyon in the Nankai Trough, the δ^{13}C values of clams were between −39.4 ± 0.2‰ and −36.9 ± 0.2‰ (Fig. 3). The δ^{13}C value of *Abyssogena phaseoliformis* collected from the landward slope of Japan Trench (−36.1 ± 0.2‰) were slightly higher than those of the Nankai Trough clams. At Kuroshima Knoll, *Akebiconcha kawamurai* (−34.1 ± 0.6‰) and *Pliocardia kuroshimana* (−32.5 ± 0.4‰) displayed even higher δ^{13}C values.

The δ^{13}C value of the calamyzine polychaete *Nautiliniella calyptogenicola* (−36.3 ± 0.3‰) was almost identical to its host clam *Abyssogena phaseoliformis* (−36.1 ± 0.2‰). The δ^{13}C values of the tubeworm *Lamellibrachia columna* varied greatly among individuals (−44.0 to −23.7‰). These trends in δ^{13}C values were similar to those observed in Δ^{14}C values.

Stable nitrogen isotopic compositions (δ^{15}N) varied greatly across species, even from within the same cold seep site (Fig. 3). At Yukie Ridge, *Archivesica tsubasa* exhibited very low δ^{15}N values (−6.2 ± 2.1‰), ~10‰ lower than those of *Archivesica magnocultellus* (4.2 ± 1.8‰). At Daisan Tenryu Canyon, the δ^{15}N values were highly variable across individuals (−5.2 to 1.8), although the interspecific variations were small (*Ectenagena laubieri*: −2.2 ± 2.0‰, *Archivesica tsubasa*: −2.1 ± 2.8‰, *Abyssogena kaikoi*: −2.0 ± 2.8‰). Intraspecific variations were generally low for *Abyssogena phaseoliformis* (−4.7 ± 0.6‰) at the landward slope of Japan Trench, as well as *Akebiconcha kawamurai* (6.8 ± 1.1‰) and *Pliocardia kuroshimana* (7.4 ± 0.3‰) from Kuroshima Knoll.

The δ^{15}N value of the calamyzine worm *Nautiliniella calyptogenicola* was −1.8 ± 0.6‰, ~3 ‰ higher than its host clam *Abyssogena phaseoliformis*. The siboglinid tubeworm *Lamellibrachia columna* exhibited intermediate δ^{15}N values (−0.4 ± 1.0‰) among the measured cold seep animals, again with a relatively small intraspecific variation.

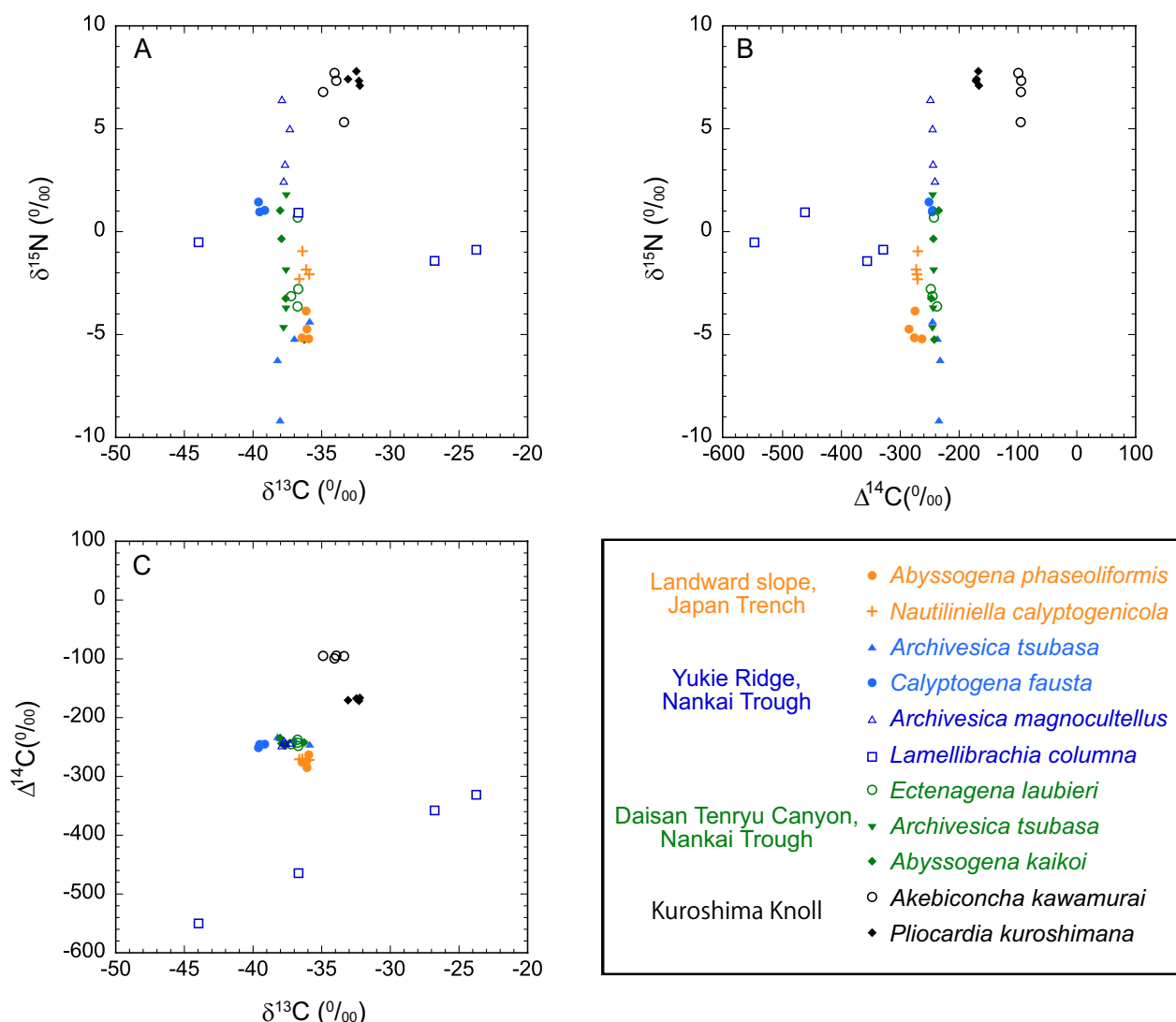**Fig. 3** Plots showing **A** stable carbon isotopic compositions ($\delta^{13}C$) versus stable nitrogen isotopic compositions ($\delta^{15}N$), **B** $\delta^{15}N$ versus natural-abundance radiocarbon ($\Delta^{14}C$), and **C** $\delta^{13}C$ versus $\Delta^{14}C$, of animals collected from cold seep sites around Japan.

4 Discussion

4.1 C sources of hydrocarbon seep clams revealed by $\Delta^{14}C$ values

The natural-abundance radiocarbon concentrations in the cold seep vesicomyid clams were generally similar or slightly lower than those of bottom water concentrations. Symbiotic vesicomyid clams burrow into the sediment so they can reach the sulfide-rich layer with their foot to take up hydrogen sulfide, which they then circulate to the gill endosymbionts via the hemolymph (Zal et al. 2000). As their symbionts inhabit cells on the gill surface in contact with bottom seawater in the mantle cavity drawn in through the siphon, the symbionts can take up oxygen and CO_2 directly with the aid of carbonic anhydrases (Kochevar and Childress 1996). Our results in general

agree with this, given the similar $\Delta^{14}C$ values between animals and ambient bottom water $\Delta^{14}C$ values. However, there were minor discrepancies between animal and bottom water $\Delta^{14}C$ values except in *Akebiconcha kawamurai*, typically 20 to 30‰ and up to 70‰ which was observed in *Abyssogena phaseoliformis* (Fig. 2). Those small discrepancies can be attributed to the uptake of geofluid-originated CO_2 which is depleted in ^{14}C (Masuzawa et al. 1995), either from their foot or from their siphon. Since vesicomyid clams require oxygen for both respiration and oxidation of hydrogen sulfide, the water they circulate to the gill and mantle cavity is oxygen-rich ambient bottom water and unlikely to contain a high proportion of geofluid containing ^{14}C-depleted CO_2 and other reduced compounds. Therefore, it seems likely that

vesicomyid clam take up some DIC through the foot and transport it through blood circulation to the symbionts, a possibility that has been suggested previously (Childress and Girguis 2011). The largest discrepancy between clam and ambient bottom water $\Delta^{14}C$ values was found in *Abyssogena phaseoliformis* collected from the landward side of the Japan Trench (Fig. 2). This may be caused by a greater dependency on foot-derived CO_2 or higher CO_2 fluxes through subsurface fluids at this site. Further geochemical studies across different seepage sites will shed more light on these discrepancies of the dependency on geofluid derived CO_2 among sites and species.

4.2 Variations in $\delta^{15}N$ values and potential N sources of vesicomyid clams

Some vesicomyid clam species, namely *Archivesica tsubasa*, *Abyssogena kaikoi*, *Archivesica magnocultellus*, and *Ectenagena laubieri* exhibited large (4.0 to 6.4‰) variations in their $\delta^{15}N$ values among individuals, whereas *Abyssogena phaseoliformis*, *Calyptogena fausta*, and *Pliocardia kuroshimana* exhibited similar $\delta^{15}N$ values across individuals (differences of 0.4 to 1.3‰) (Fig. 3). Animals endemic to deep-sea chemosynthetic ecosystems have been reported to exhibit a wide variety of $\delta^{15}N$ values, potentially reflecting different inorganic nitrogen

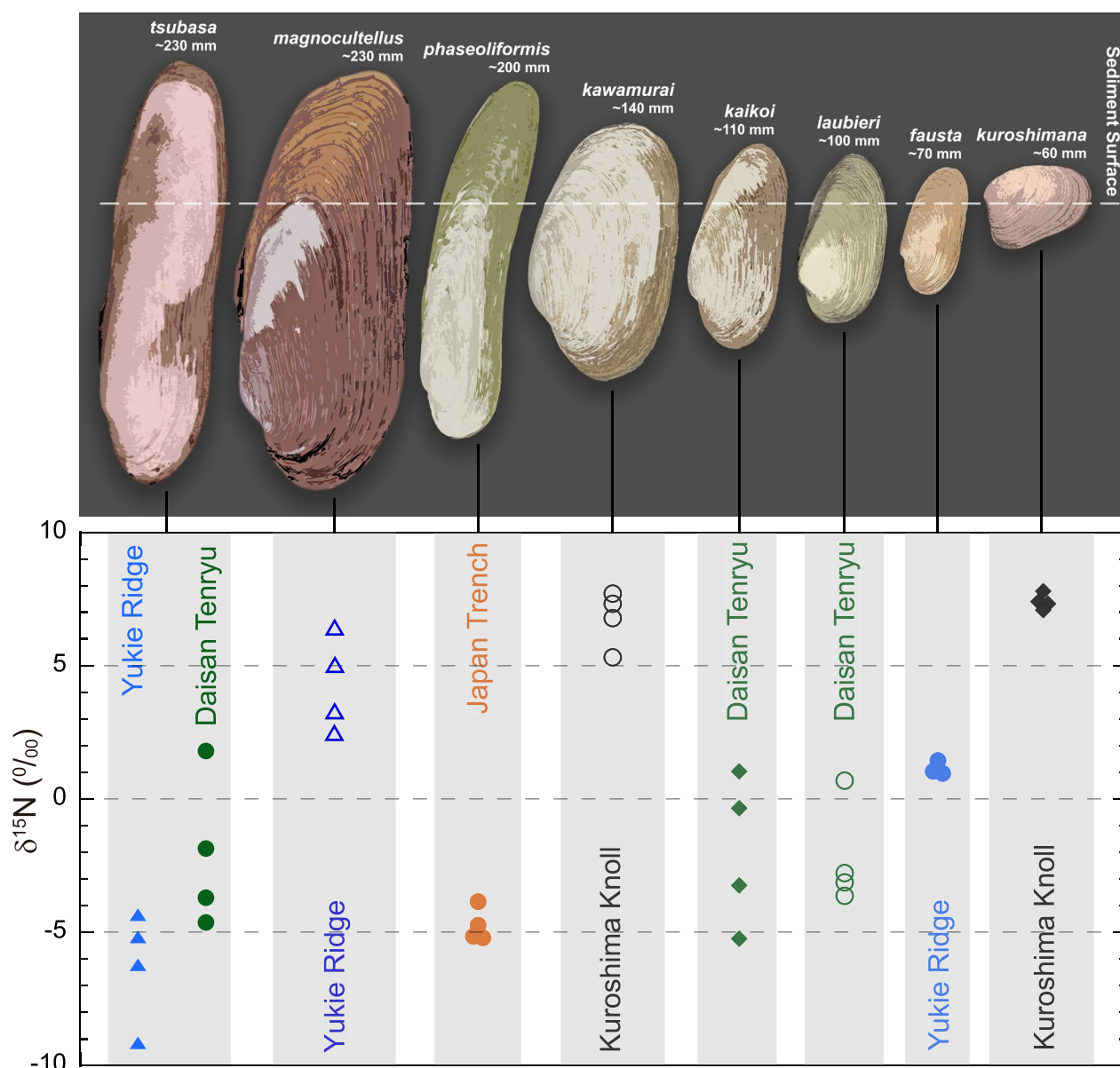**Fig. 4** Stable nitrogen isotopic compositions ($\delta^{15}N$) of vesicomyid clams and their maximum sizes (shell length), reflecting their maximum burrowing depth in sediments

sources assimilated by chemoautotrophic bacteria (Van Dover 2002). It has also been reported that the isotope effects between microbial biomass and inorganic N source tended to be large when environmental inorganic N sources were high (Liao et al. 2014; Sigman and Fripiat 2019). At a hydrothermal vent field in the Okinawa Trough, endemic animals living in close proximity to the vent orifice tended to have lower $\delta^{15}N$ values where the NH_4^+ concentration was high (Nomaki et al. 2024). As pliocardiine vesicomyid clams rely almost entirely on their endosymbiotic microbes for nutrition (Ip et al. 2021), $\delta^{15}N$ values of the host clams are related to those of their symbionts (He et al. 2023; Ishikawa et al. 2024), which is in turn affected by the NO_3^- or NH_4^+ concentrations in the environment.

Previously, inorganic nitrogen sources for vesicomyid clam symbionts such as NH_4^+ and NO_3^- were thought to be drawn in through the gill from bottom water like O_2 and CO_2 (Childress and Girguis 2011). Since NH_4^+ concentrations in the bottom water at vesicomyid clam colonies were very low (Ohnishi et al. 2018, E. Tasumi, unpublished data) as is typical for the deep-sea bottom water, the available inorganic nitrogen sources for vesicomyid clam in the bottom water would appear to be NO_3^-. The high variability of $\delta^{15}N$ values seen in some species of vesicomyids analyzed herein (Fig. 4), however, can be explained by the hypothesis that their inorganic nitrogen source is not solely the bottom water but also the sediment porewater.

It is notable that the two largest (Fujikura et al. 2000; Fujikura et al. 2008) species (*Archivesica magnocultellus, Archivesica tsubasa*) exhibited large intraspecific $\delta^{15}N$ variations, whereas the two smallest species (*Calyptogena fausta, Pliocardia kuroshimana*) exhibited small $\delta^{15}N$ intraspecific variations (Fig. 4). Vesicomyid clam species with large body size and shell length can burrow deeper into the sediment, where the NH_4^+ concentration is higher (e.g., Toki et al. 2014). The NH_4^+ concentration in the sediment at Oomine Ridge, Nankai Trough, which is less than 100 km west from Yukie Ridge and Daisan Tenryu Canyon, showed sharp increase from sediment surface (0 to 17 μM) to 5 or 10 cm depths in sediments (up to 300 μM) (Toki et al. 2014). In the case of small individuals of the same species, however, they can likely only reach the shallower part of the sediment (e.g., shallower than 5 cm), where NH_4^+ concentration is low. Since the availability of NH_4^+ induces different isotopic fractionation (Sigman and Fripiat 2019), the differences in $\delta^{15}N$ values of the same species of vesicomyid clam potentially reflect their burrowing depths. Two size factors may be at play here, including the maximum body size which dictates how deep a clam species can reach (i.e., the range of NH_4^+ concentrations), and

the individual body size of the specimen analyzed (i.e., how deep the exact individual burrowed). In the case of a large species, while large individuals can burrow deeper into sediments, smaller individuals would reside at relatively shallower depths, and thus measuring $\delta^{15}N$ values across a size range may lead to a large intraspecific variation. The possible range of burrowing depth depends on the maximum body size of the species (the larger the species, the larger the difference between juveniles and adults). This may be one reason contributing to the overall pattern seen where large-bodied species tend to exhibit larger variability. Conversely, two smallest species can only burrow in a narrow depth range in the sediment across growth, resulting distributions at similar lower NH_4^+ concentrations.

The apparent outlier in our data was *Abyssogena phaseoliformis* which has a large maximum shell length (202 mm: Okutani et al. 2009) but showing low intraspecific variability in $\delta^{15}N$ values. This was also the only species for which shell lengths of the analyzed specimens was available (93.8 to 121.0 mm; Supplementary Table 1). There appeared to be no clear trend in this species between shell length and $\delta^{15}N$ values, though we note the size range of these specimens was small.

It was unfortunately not possible to measure individual sizes of other species used in this study (only pieces of dissected tissue were available in most cases), the relationships between body size (availability of inorganic nitrogen source in sediments) and their $\delta^{15}N$ values would be a hypothesis warranting future study. Measuring the shell length of individuals clams and their burrowing depth in situ is now within reach using acoustic imaging devices (Mizuno et al. 2022). Investigating the $\delta^{15}N$ values of clams with such detailed in situ data, together with measuring pore water NH_4^+ profiles in vesicomyid clam colony, will be able to provide key evidences required to test this hypothesis.

4.3 Ecology of the calamyzine polychaete and the siboglinid tubeworm

The calamyzine worm *Nautiliniella calyptogenicola* was first reported from the mantle cavity of the vesicomyid clam *Abyssogena phaseoliformis* (Miura and Laubier 1989), and recently also discovered from *Abyssogena mariana*, implying its capacity to colonize multiple hosts in the genus *Abyssogena* (Jimi et al. 2022). Although assumed to be a parasite (Miura and Laubier 1989), its actual relationships with the host have not been closely examined to date. Calamyzine worms are either free-living or symbiotic/parasitic with molluscs or other annelids (Rouse et al. 2022). Using worms collected from *Abyssogena phaseoliformis* in Japan Trench, we found that *N. calyptogenicola* exhibited almost identical $\Delta^{14}C$ and $\delta^{13}C$

values with the host clam, while its $\delta^{15}N$ value was 2.9‰ higher the host, on average. The observed ~3.0‰ increase in $\delta^{15}N$ value relative to the host clam agrees well with the trophic level increment between consumer and prey organisms (Minagawa and Wada 1984). Together with the identical $\Delta^{14}C$ and $\delta^{13}C$ values between them, these serve as strong evidence that *Nautiliniella calyptogenicola* is indeed parasitic on its vesicomyid host.

The siboglinid tubeworm *Lamellibrachia columna* was the only animal exhibiting clearly ^{14}C-depleted tissue relative to the bottom water, indicating OM production using DIC from the seeping fluid. Unlike the giant tubeworm *Riftia pachyptila* for which physiology and metabolism is best known among siboglinid tubeworms (Childress and Girguis 2011), how exactly *Lamellibrachia* incorporates DIC has remained unclear. As tubeworms host symbionts in the completely internal trophosome, the host animal must supply all resources required by the endosymbiont via blood. *Rifita* and some other genera carry out all gas exchange exclusively across the plume (Childress and Fisher 1992), including taking in CO_2 from the plume and transporting it to the symbionts (Williams et al. 1981). *Lamellibrachia*, however, is capable of gas exchange at both plume and the posteriorly extended 'root' which reaches deep into the sediment or fissures to take up sulfides (Cordes et al. 2005; Dattagupta et al. 2006; Li et al. 2019). Recently, a study of *Lamellibrachia columna* (*Lamellibrachia* sp. L1 in Kojima (2000)) at a hydrothermal vent in the Okinawa Trough using $\Delta^{14}C$ revealed much depleted ^{14}C values compared to the bottom water, suggesting the tubeworm also utilizes DIC from the vent fluid deep inside the sediment (Nomaki et al. 2024). Here, we found cold seep individuals of *L. columna* displayed a very variable range of $\Delta^{14}C$ values (-548 to -329‰). This could indicate one of two scenarios: one where the tubeworm flexibly takes in CO_2 from both the plume and the root, depending on the environmental condition at its microhabitat; and another where it only uses CO_2 diffused in from the root but different intrusion depth of the root leads to variable porewater contribution. Since porewater rises from below, a shallower extension into the sediment would mean more bottom water at that sediment layer, leading to less depleted $\Delta^{14}C$ values. This latter scenario is more consistent with previous results from hydrothermal vents where little variability was seen (Nomaki et al. 2024), suggesting that root intrusion (and potentially body size) has a strong effect on how much carbon from seep porewater is fixed by the symbionts. As for vesicomyid clams, body size and in situ distributions in the sediments would provide detailed insights on the controlling factors of carbon and nitrogen isotopic compositions, which reflect the uptake mechanisms.

5 Conclusions

We applied the analyses of natural-abundance radiocarbon to cold seep ecosystems for the first time, in order to elucidate the carbon sources of animals inhabiting these chemosynthetic ecosystems in terms of geofluid vs ambient bottom water. Previously, the stable isotope ratio of carbon ($\delta^{13}C$) has been widely used to estimate carbon sources of seep animals, but differing pathways of chemosynthesis with varying degrees of isotope fractionation made these inconclusive. Based on the measurements of natural radiocarbon (^{14}C) abundances, we found a minor contribution (0 to 9%) of carbon from geofluids in vesicomyid clams, previously thought to only use carbon from the ambient seawater. Furthermore, our stable nitrogen isotope ($\delta^{15}N$) analyses suggested that the nitrogen source of vesicomyid clams also contains the geofluid, which further pointed toward to a novel hypothesis where the $\delta^{15}N$ values may reflect burrowing depths with varying NH_4^+ concentrations. In the siboglinid tubeworm *Lamellibrachia* we also found a strong carbon contribution from the geofluid but with high variability among individuals, likely reflecting flexibilities in uptake strategies or different intrusion depths of the 'root'. Cold seeps are widespread systems in the ocean floor that play key roles in the elemental and energy flux between sediment and seawater. Our results show that radiocarbon is a useful probe for pinpointing nutritional sources for seep organisms, opening a new and additional route to understanding these fluxes. These promising results from radiocarbon analyses follow previous successes at hydrothermal vent ecosystems (Nomaki et al. 2019, 2024), and further confirm its usefulness across different chemosynthetic systems.

Supplementary Information

Author contributions
HN and CC conceived and designed the study. SK collected and prepared the animal samples. YY and YM measured the natural-abundance radiocarbon. HN and CC drafted the manuscript, all authors revised the manuscript critically and approved its submission.

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Availability of data and materials

The datasets supporting the conclusions of this article is(are) included within the article and its additional files.

Declarations

Competing interests

The authors declare that there is no competing interest regarding this study.

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