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Novel reverse radioisotope labelling experiment reveals carbon assimilation of marine calcifiers under ocean acidification conditions

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Abstract

1. Ocean acidification by anthropogenic carbon dioxide emissions is projected to depress metabolic and physiological activity in marine calcifiers. To evaluate the sensitivity of marine organisms against ocean acidification, the assimilation of nutrients into carbonate shells and soft tissues must be examined.
2. We designed a novel experimental protocol, reverse radioisotope labelling, to trace partitioning of nutrients within a single bivalve species under ocean acidification conditions. Injecting CO_2 gas, free from radiocarbon, can provide a large contrast between carbon dissolved in the water and the one assimilated from atmosphere. By culturing modern aquifer organisms in acidified seawater, we were able to determine differences in the relative contributions of the end members, dissolved inorganic carbon (DIC) in seawater and metabolic CO_2, to shell carbonate and soft tissues.
3. Under all pCO_2 conditions (463, 653, 872, 1,137 and 1,337 μatm), radiocarbon ($\Delta^{14}C$) values of the bivalve *Scapharca broughtonii* shell were significantly correlated with seawater DIC values; therefore, shell carbonate was derived principally from seawater DIC. The $\Delta^{14}C$ results together with stable carbon isotope ($\delta^{13}C$) data suggest that in *S. broughtonii* shell $\delta^{13}C$ may reflect the kinetics of isotopic equilibration as well as end-member contributions; thus, care must be taken when analysing end-member contributions by a previous method using $\delta^{13}C$. The insensitivity of *S. broughtonii* to perturbations in pCO_2 up to at least 1,337 μatm indicates that this species can withstand ocean acidification.

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[Correction added on 09 February 2021, after first online publication: The copyright line was changed.]

4. Usage of radioisotope to dope for tracer experiments requires strict rules to conduct any operations. Yet, reverse radioisotope labelling proposing in this study has a large advantage and is a powerful tool to understanding physiology of aquifer organisms that can be applicable to various organisms and culture experiments, such as temperature, salinity and acidification experiments, to improve understanding of the proportions of nutrients taken in by marine organisms under changing environments.

KEYWORDS

bivalve, calcifier, carbon assimilation, culture experiment, ocean acidification, radiocarbon, radioisotopes, reverse radioisotpe labelling

1 | INTRODUCTION

Anthropogenic activity has resulted in the release of colossal amounts of geologically trapped carbon dioxide (CO_2) into the atmosphere and consequent changes in global climate (IPCC, 2013). As the oceans absorb CO_2 to equilibrate this imbalance, seawater pH shifts to more acidic values and carbonate ion availability is reduced, a phenomenon known as ocean acidification (Feely et al., 2004; Orr et al., 2005). The relative amounts of dissolved inorganic carbon (DIC) species in seawater, namely, CO_2 (aq), H_2CO_3, HCO_3^- and CO_3^{2-}, at equilibrium are determined by the following reactions (Feely et al., 2004):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+. \quad (1)$$

A decrease in the $CaCO_3$ saturation state due to ocean acidification can depress metabolic and physiological activity in calcifiers (Fabry, Seibel, Feely, & Orr, 2008; Gazeau et al., 2013; Kroeker et al., 2013; Orr et al., 2005; Pörtner, 2008; Pörtner, Langenbuch, & Reipschläger, 2004; Wittmann & Pörtner, 2013) and lower calcification rates, and at high pCO_2 it can even cause net shell dissolution (Gazeau et al., 2013; Haynert, Schönfeld, Riebesell, & Polovodova, 2011). Calcifiers at embryonic and larval stages of life are more sensitive to high pCO_2 levels (Onitsuka, Kimura, Ono, Takami, & Nojiri, 2014; Suwa et al., 2010; Tanaka et al., 2014). Effects of increased pCO_2 can vary among species, growth stages and habitats (Fabry et al., 2008; Watson et al., 2012); some species exhibit unchanged or even increased calcification rates (Nishida et al., 2018; Ries, Cohen, & McCorkle, 2009; Thomsen, Casties, Pansch, Körtzinger, & Melzner, 2013). Expected carbon dioxide emissions (e.g. RCP4.5:538 ppm, RCP8.5:936 ppm in 2,100) are projected to have negative effects on over 50% of molluscan species (Wittmann & Pörtner, 2013). To evaluate acidification effects on molluscan calcification, metabolism and physiology, the assimilation of carbon into molluscan shells and soft tissues must be examined. The results are expected to enable fisheries to better assess their stocks under ocean acidification.

The main carbon sources for shell formation are DIC in ambient seawater and respiratory (metabolic) carbon (mainly CO_2) derived from metabolized food (Fritz & Poplawski, 1974; McConnaughey & Gillikin, 2008). Stable carbon isotope $\delta^{13}C$ analysis results indicate that 62%–98% of carbon in marine bivalve shells is sourced from seawater DIC (Gillikin, Lorrain, Bouillon, Willenz, & Dehairs, 2006; Gillikin, Lorrain, Meng, & Dehairs, 2007; McConnaughey, Burdett, Whelan, & Paull, 1997; McConnaughey & Gillikin, 2008; Mook & Vogel, 1968; Poulain et al., 2010; Zhao et al., 2018), although some estimates of the contribution of metabolic CO_2 to bivalve shells exceed 50% (Dillaman & Ford, 1982; Tanaka, Monaghan, & Rye, 1986). From $\delta^{13}C$ analysis of the Manila clam *Ruditapes philippinarum* Zhao et al. (2018) observed that in acidified seawater there was an increase in the metabolic carbon contribution to calcification and a decrease in the seawater DIC contribution; moreover, transgenerational exposure of experimental clams to elevated pCO_2 led to a larger metabolic contribution to shell calcification compared with a non-acclimated line of clams. These variations in carbon uptake may indicate that the degree of tolerance to ocean acidification depends on calcification physiology; thus, responses to ocean acidification must be evaluated in multiple species and at different life stages. In this study, to further understand calcification physiology and metabolism, we applied radiocarbon isotopic techniques to examine the effects of changing seawater carbonate chemistry on shell carbon sources of a marine calcifier.

The radiogenic carbon isotope, ^{14}C, is produced in the upper atmosphere through interactions of cosmic rays with the atmosphere, and it enters the biogeochemical carbon cycle as $^{14}CO_2$. Precise calibration sequences have been established for worldwide conversion of 'conventional' radiocarbon ages to calendar ages (Reimer et al., 2013), and radiocarbon dating techniques are extensively used in archaeological and paleoclimate studies to determine late Quaternary ages (e.g. Arnold & Libby, 1949; Libby, 1955; Yokoyama et al., 2018). The extent of $^{14}C/^{12}C$ isotopic fractionation is approximately double that of the equivalent $^{13}C/^{12}C$ ratio. The stable carbon isotope ratio, $\delta^{13}C$, is defined as the permil (‰) deviation from the Vienna Pee Dee Belemnite (VPDB) standard:

$$\delta^{13}C = \left(\left({}^{13}C/{}^{12}C \right)_{sample} \Big/ \left({}^{13}C/{}^{12}C \right)_{VPDB} - 1 \right) \times 1{,}000. \quad (2)$$

$\Delta^{14}C$ is defined as the permil (‰) deviation of a sample from the ^{14}C activity of 19th-century wood and is calculated as follows (Stuiver & Polach, 1977):

$$\Delta^{14}C = \delta^{14}C - 2 \times \left(\delta^{13}C + 25\right)\left(1 + \left(\delta^{14}C/1{,}000\right)\right), \quad (3)$$

$$\delta^{14}C = \left(\left(A_s/A_{abs}\right) - 1\right) \times 1{,}000, \quad (4)$$

where A_s is measured sample activity and A_{abs} is absolute international standard activity. In the 1950s and early 1960s, atmospheric nuclear weapons testing produced large amounts of atmospheric ^{14}C (Grottoli & Eakin, 2007; Hua et al., 2009; Hua, Barbetti, & Rakowski, 2013), which approximately doubled the natural atmospheric ^{14}C value (Grottoli & Eakin, 2007; Hua et al., 2009, 2013). The transient ^{14}C enrichment of the biosphere and its subsequent dissipation, the bomb-^{14}C curve, provides a high-resolution isotopic chronometer for the last 60 years (Hirabayashi et al., 2017; Hua et al., 2009, 2013) that has been used extensively in environmental (Murphy, Davis, Long, Donahue, & Jull, 1989; Ota et al., 2019; Rethemeyer et al., 2004), forensic (Zoppi et al., 2004) and biogeochemical studies (Furla, Galgani, Durand, & Allemand, 2000; Ishikawa, Hyodo, & Tayasu, 2013; Ishikawa, Uchida, Shibata, & Tayasu, 2010; Ota et al., 2019; Stenström et al., 2010; Wong & Levinton, 2006). Ota et al. (2019) revealed past ocean mixing histories by using $\Delta^{14}C$ of abalone shells as a proxy that depends purely on past seawater radiocarbon variation and is unaffected by biological isotopic fractionation processes. Artificial radiocarbon tagging of biological molecules in laboratory experiments has been used to determine carbon assimilation and transport pathways (Furla et al., 2000; Wong & Levinton, 2006; Wyatt et al., 2019), and ^{14}C bomb-pulse dating has been applied to food web studies (Ishikawa et al., 2010, 2013; Larsen, Yokoyama, & Fernandes, 2018) and to the estimation of turnover times of human tissues and cells (Stenström et al., 2010).

Because of its half-life (5,730 years), there is no ^{14}C in fossil fuels, whereas the activity of the present-day atmosphere in terms of ^{14}C is almost 100% (Hua et al., 2009, 2013). In our acidification study of the marine bivalve *Scapharca broughtonii* (Bivalvia: Arcoidea: Arcidae; Nishida et al., 2018), the seawater pCO_2 in the experimental aquaria was maintained at different nominal values by injection of CO_2 gas derived from fossil fuels (i.e. with no ^{14}C content) in varying proportions (Figure 1a), the seawater ^{14}C values decreased correspondingly at high pCO_2. The organic plankton feed which was cultured in control seawater (Figure 1b) showed bomb carbon-enriched ^{14}C values similar to the value in the present atmosphere. As a result, seawater ^{14}C in the experimental aquaria (Figure 1c) was decoupled from current atmospheric values as well as from those of the plankton cultured in modern seawater that served as feed. This decoupling allowed the seawater and feed end-member contributions to ^{14}C values of *S. broughtonii* shell and tissue resulting from the different experimental pCO_2 concentrations to be differentiated. In this study, we therefore made use of '^{14}C-free carbon' to investigate calcification in *S. broughtonii*. We propose to call this novel technique 'reverse radioisotope labelling' to distinguish it from laboratory techniques that use radiocarbon tracers.

$\delta^{13}C$ values, which have been used previously to assess end-member contributions to biogenic carbonate (Gillikin, Hutchinson, & Kumai, 2009; Gillikin et al., 2006, 2007; McConnaughey et al., 1997; Tanaka et al., 1986; Zhao et al., 2018), reflect both biological and kinetic fractionation effects. In contrast, $\Delta^{14}C$ is a unique tool that depends purely on the carbon source and is unaffected by biological isotopic fractionation processes (Stuiver & Polach, 1977); thus, it should reflect only the actual contributions of seawater DIC and organic carbon to bivalve shell and tissue carbon. This study aimed to use $\Delta^{14}C$ analysis data together with $\delta^{13}C$ data to elucidate the effects of ocean acidification on carbon assimilation into the shell and tissues of a bivalve species cultured in the laboratory at different pCO_2 levels. Furthermore, we sought to determine (a) the relative

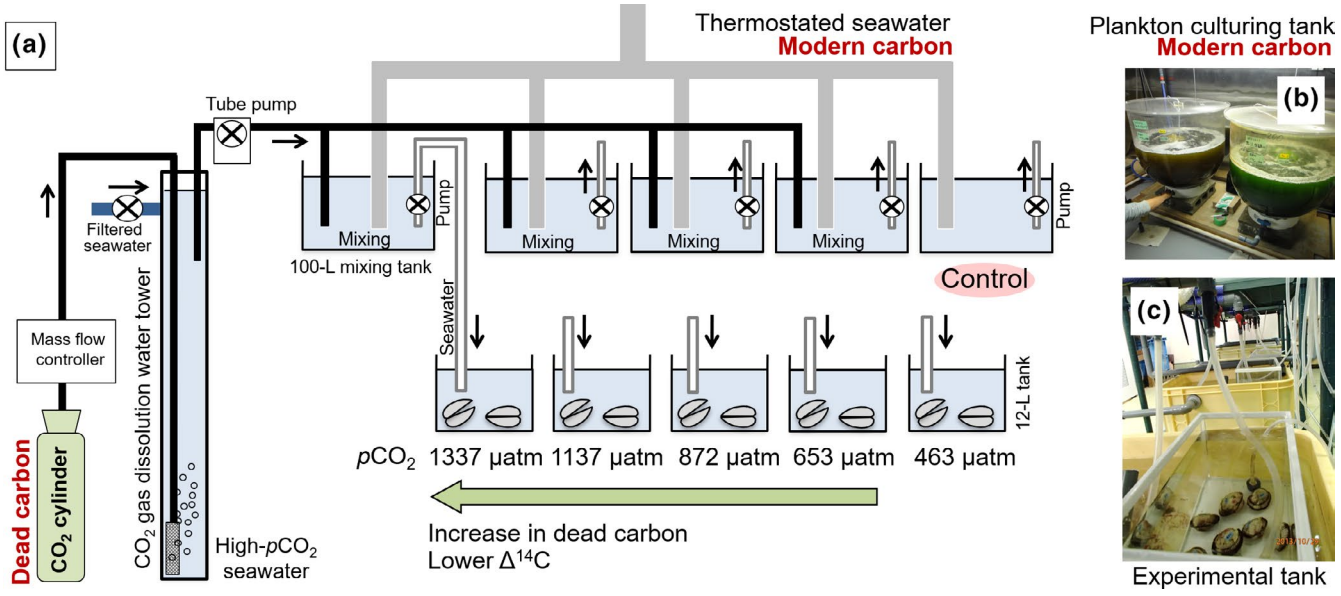

FIGURE 1 Schematic diagram of the experimental design used in this study (a) and photographs of the plankton culturing tanks (b) and the experimental tanks. The seawater pCO_2 in the experimental aquaria was maintained at different nominal values by injection of CO_2 gas free from radiocarbon

contributions of carbon from ambient DIC and metabolic CO_2 to shell and tissues and (b) the effects of pH changes caused by ocean acidification on carbon incorporation by applying reverse radioisotope labelling to trace carbon partitioning in a single bivalve species. These data in combination with stable carbon isotopic data allow the biological contributions to shell and tissue to be parsed from a geochemical perspective to improve understanding of bivalve carbon assimilation and biomineralization. We propose the technique described here is also applicable to various organisms in aquifers. This powerful method has advantages including higher sensitivity than the $\delta^{13}C$ method (50–100 times more sensitive). In addition, the reverse radioisotope labelling method that uses fossil fuel-derived CO_2 gas can be performed in any laboratory environment, unlike radioisotope doping experiments that must be performed inside a designated radiation controlled area. This method is also more cost-effective compared to the existing ^{14}C-labelling experiment that requires expensive ^{14}C-labelled seawater and/or feed). Recent advances in radiocarbon measurements (e.g. Yokoyama et al., 2019) greatly improve the precision of radiocaron analysis while increasing the measurement thoughput and decreasing cost. Without these advances, the reverse labelling method would not be feasible.

2 | MATERIALS AND METHODS

2.1 | Sample collection and experimental design

Scapharca broughtonii is an aragonitic marine bivalve that is widely distributed in coastal regions of the northwestern Pacific, including the Sea of Japan, the East China Sea and the northern Philippine Sea (Evseev & Lutaenko, 1998; Habe, 1965; Matsukuma & Okutani, 2000), and it is also an economically important species in Asian aquaculture (Broom, 1985). It is a sedentary endobyssate species that digs shallow burrows in sandy mud or muddy bottoms at water depths of 5–50 m (Matsukuma & Okutani, 2000).

Specimens of *S. broughtonii* were cultured in a coarse-meshed cage half-buried in sediment at a water depth of 10 m in the Seto Inland Sea at the Kudamatsu Institute of Mariculture, Yamaguchi Prefecture, Japan. In Japanese *S. broughtonii*, shell growth slows markedly at maturity (age 2–4 years; Sasaki, 1997), so faster growing immature 1-year-old individuals were used in the experiment. In all, 64 living specimens were collected on 3 September 2013 and transferred the next day to the Demonstration Laboratory, Marine Ecology Research Institute (MERI), Kashiwazaki City, Niigata Prefecture, Japan, for the culturing experiments. No individuals died during the transfer. The specimens were kept in tanks maintained at approximately 23°C for 2 months for acclimatization to the new environment. An equal volume mixture of *Pavlova lutheri* ($3–5 \times 10^6$ cells/ml) and *Tetraselmis tetrathele* ($1–2 \times 10^6$ cells/ml) cultured in natural seawater was provided as phytoplankton feed (total volume 1.2 L) twice a day during the acclimatization and experimental period. Before the experiment, the part of the periostracum covering the external shell margin was removed so that any new growth would be covered by regenerated periostracum and shell growth during the experiment could be clearly observed.

The culture experiment (experiment 2 in Nishida et al., 2018) was conducted for 8 weeks, from 24 October to 18 December 2013. In this experiment, *S. broughtonii* specimens were cultured in seawater maintained at a different pCO_2 level by a high-precision pCO_2-controlling system (figure 1 in Nishida et al., 2018): four pCO_2-controlled treatments of 653 ± 87, 872 ± 100, 1,137 ± 140 and 1,337 ± 167 μatm (pH_{total} 7.85 ± 0.05, 7.74 ± 0.05, 7.64 ± 0.05 and 7.57 ± 0.04, respectively) and a control treatment, without CO_2 gas aeration (463 ± 68 μatm, pH_{total} 7.98 ± 0.05). We prepared high-pCO_2 seawater by bubbling carbon dioxide gas continuously from a compressed CO_2 gas cylinder through seawater in a closed CO_2 gas dissolution water tower (1.9 m high; Figure 1). High-pCO_2 seawater was continuously supplied to 100-L mixing tanks with a tube pump and mixed with thermoregulated seawater to control the targeted pCO_2. pCO_2-regulated seawater was supplied to the 12-L experimental aquaria. The water temperature and seawater pCO_2 in the all experimental tanks were real-time monitored and recorded at 2-hr intervals throughout the experiment by using a nondispersive infrared analyzer system (Model SCD-12; Kimoto Electric Co. Ltd.). Water in the system has been continuously supplied and the carbonate chemistry and flow speed have been precisely controlled throughout the experiment. As a result, little fluctuations of carbonate chemistry in the system are expected. Details of the experimental setup and seawater pH, carbonate chemistry and saturation states in each treatment are available in Nishida et al. (2018).

2.2 | Sample preparation

Four types of samples were prepared: (a) shell carbonate; (b) organic soft tissue (foot and mantle tissue); (c) organic plankton feed and (d) seawater. For radiocarbon analysis of shell carbonate, we carefully removed the periostracum from the shell surface and then immersed the shells in a solution of 1 mol/L hydrochloric acid for 30 min to remove any modern atmospheric ^{14}C contamination from the outer surface; this procedure reduced the sample dry weight by about 10%. Samples were then placed in an ultrasonic bath filled with purified MilliQ water for further cleaning. A dental drill operated at a low rotational speed was used to extract powdered shell samples from the cleaned samples. The samples were obtained from the external margin of the original shells so that only the growth increment during the experiment was sampled.

Tissue samples were extracted from the specimens and freeze-dried (FD-5N freeze dryer; EYELA CO., LTD) for a week. Foot (1.0–2.0 g) and mantle (0.05–0.13 g) tissues were separated from each specimen and treated separately. Samples of each plankton species, *P. lutheri* and *T. tetrathele*, as well as one of an equal volume mixture of the two species, were retrieved from their respective culture tanks and subjected to one of two different treatments. In treatment 1, plankton samples were passed through a MF-Millipore membrane filter with a pore size of 0.45 μm, and then the plankton-containing

filters were collected and dried in a desiccator containing a silica gel desiccant. In treatment 2, the collected samples were centrifuged and freeze-dried. Samples collected on 24 October and 11 December 2013 for $\delta^{13}C$ analysis, and on 24 January 2015 for $\Delta^{14}C$ analysis, were subjected to treatment 1. Samples collected on 11 December 2015 for $\Delta^{14}C$ analysis were subjected to treatment 2.

Seawater was collected from each experimental tank by filling a 200-ml glass bottle to the top to reduce any air–water exchange. Each sample was poisoned with saturated mercuric chloride solution for biological sterilization before the bottles were tightly crimped shut (Dickson, Sabine, & Christian, 2007).

2.3 | Radiocarbon (^{14}C) analysis of shell carbonate, seawater and organic materials

We obtained ^{14}C data from the samples by a four-step process: (a) CO_2 gas was produced by combustion or acid dissolution of the sample; (b) the gas was purified via a series of cryogenic traps in vacuum lines; (c) the purified gas was reduced to solid graphite and (d) the graphite was analysed by accelerator mass spectrometry (AMS). Three separate graphitization lines were used to perform steps 1–3. Shell carbonate samples with small masses (0.8–2.2 mg) were analysed in a specialized graphitization line by an AMS procedure capable of handling minute amounts of carbonate (Hirabayashi et al., 2017; Yokoyama, Koizumi, Matsuzaki, Miyairi, & Ohkouchi, 2010), whereas shell carbonate samples with larger masses (>3.0 mg) were processed in a standard graphitization line (Yokoyama, Miyairi, Matsuzaki, & Tsunomori, 2007). The organic samples (2.0–3.0 mg) were loaded into the standard graphitization line for cryogenic purification and reduction. Seawater samples were processed in the third graphitization line.

Standard reference materials—IAEA-C1, NIST HOxII and IAEA-C6 sucrose—were graphitized for data calibration. All solid carbon samples were pressed into aluminium cathodes and mounted on a target wheel to be analysed in sequence in the Single Stage Accelerator Mass Spectrometer at the Laboratory of Accelerator Mass Spectrometry, Atmosphere and Ocean Research Institute, The University of Tokyo, Japan (Yokoyama et al., 2019). All reported ^{14}C values were normalized using ^{13}C AMS data following Stuiver and Polach (1977).

2.4 | $\delta^{13}C$ analysis of soft tissue and plankton samples

Soft tissue (foot and mantle) and plankton samples (0.6–1.0 mg) were analysed for $\delta^{13}C$ in an elemental analyzer (Flash 2000) coupled via a CONFLO III interface to an isotope ratio mass spectrometer (Delta V Advantage; Thermo Electron Corporation). The $\delta^{13}C$ of organic matter relative to Vienna Peedee Belemnite (VPDB) was determined by adopting the consensus value of −19.6‰ for the alanine international reference standard relative to VPDB. A precision of better than 0.1‰ (1 *SD*) was obtained.

2.5 | Estimation of end-member contributions to the shell and tissue

To estimate the end-member contributions to shell and foot and mantle tissues quantitatively, we used mass balance equations adopted from Adkins, Byle, Curry, and Lutringer (2003) as follows:

$$\Delta^{14}C_{shell} = \left[R_{DIC} \times \Delta^{14}C_{DIC} + (100 - R_{DIC}) \times \Delta^{14}C_{plankton}\right] / 100, \quad (5)$$

$$\Delta^{14}C_{tissue} = \left[R_{DIC} \times \Delta^{14}C_{DIC} + (100 - R_{DIC}) \times \Delta^{14}C_{plankton}\right] / 100, \quad (6)$$

where R_{DIC} is the percentage of shell carbonate originating from ambient DIC. Equations 5 and 6 can be subsequently solved for R_{DIC}:

$$R_{DIC} = \left[\left(\Delta^{14}C_{shell} - \Delta^{14}C_{plankton}\right) \Big/ \left(\Delta^{14}C_{DIC} - \Delta^{14}C_{plankton}\right)\right] \times 100, \quad (7)$$

$$R_{DIC} = \left[\left(\Delta^{14}C_{tissue} - \Delta^{14}C_{plankton}\right) \Big/ \left(\Delta^{14}C_{DIC} - \Delta^{14}C_{plankton}\right)\right] \times 100. \quad (8)$$

The percentage of carbon from plankton feed in the shell and soft tissues, R_{feed}, was calculated as follows:

$$R_{feed} = 100 - R_{DIC}. \quad (9)$$

The percentage of metabolic carbon in shell carbonate (C_M) was estimated by using the two-endmember mixing equation proposed by McConnaughey et al. (1997):

$$C_M = 100 \times \left(\delta^{13}C_{shell} - \varepsilon_{ar-b} - \delta^{13}C_{DIC}\right) / \left(\delta^{13}C_R - \delta^{13}C_{DIC}\right), \quad (10)$$

where ε_{ar-b} is the enrichment factor between bicarbonate and aragonite (+2.7‰: Romanek, Grossman, & Morse, 1992) and $\delta^{13}C_R$ is the $\delta^{13}C$ value of respired carbon. Because $\delta^{13}C_R$ has not been previously measured in bivalves (McConnaughey & Gillikin, 2008), we approximated $\delta^{13}C_R$ using $\delta^{13}C$ values of soft tissues reported by McConnaughey et al. (1997) and Poulain et al. (2010) because no consensus has been reached with regard to which specific tissue is best for estimating C_M. The percentage contribution of ambient DIC to shell carbonate, R'_{DIC}, was calculated as follows:

$$R'_{DIC} = 100 - C_M. \quad (11)$$

3 | RESULTS

3.1 | Radiocarbon (^{14}C) data

Seawater $\Delta^{14}C_{DIC}$ was highly correlated with increased inputs of dead carbon with negligible ^{14}C ($\Delta^{14}C_{DIC}$ = 1,762.70 ± 97.56 (1/DIC) − 886.29 ± 48.28, R^2 = 0.98, p < 0.01; Figure 2) and showed a substantially large contrast among the experimental aquaria. The correlation between seawater $\delta^{13}C_{DIC}$ and added CO_2 gas was also high (Nishida et al., 2018; $\delta^{13}C_{DIC}$ = 71.60(1/DIC) − 37.34, R^2 = 0.98, p < 0.01). Thus, the experimental protocol was able to simulate the

uptake of CO_2 by the oceanic pool in response to anthropogenic perturbation of the atmosphere by fossil fuel combustion. $\Delta^{14}C_{shell}$ values ranged from −80.3‰ to +45.1‰, and the correlation between $\Delta^{14}C_{shell}$ and pH was significantly positive ($\Delta^{14}C_{shell}$ = 171.99 ± 32.36(pH) − 1,347.54 ± 251.30, R^2 = 0.53, p < 0.01, N = 28; Figure 3a). $\Delta^{14}C_{shell}$ also showed a positive linear correlation with $\Delta^{14}C_{DIC}$ ($\Delta^{14}C_{shell}$ = 0.98 ± 0.19$\Delta^{14}C_{DIC}$ + 0.47 ± 5.55, R^2 = 0.50, p < 0.01). The difference in $\Delta^{14}C$ between shell and seawater DIC, $\Delta^{14}C_{shell-DIC}$ (Figure 4), was not significantly correlated with pH (p > 0.05). In contrast, the difference in $\delta^{13}C$ between shell and seawater DIC, $\delta^{13}C_{shell-DIC}$, showed a significant positive correlation with pH ($\delta^{13}C_{shell-DIC}$ = −5.06 ± 0.83(pH) + 39.40 ± 6.46, R^2 = 0.67, p < 0.01; Figure 3; Nishida et al., 2018).

$\Delta^{14}C$ values of foot and mantle tissues ranged from +11.6‰ to +46.9‰ and from +6.0‰ to +50.0‰, respectively, but they were not significantly correlated (p > 0.05) with pH (Figure 3a). In the plankton feed collected on 11 December 2015, $\Delta^{14}C$ was +10.5 ± 2.7‰ (M ± SD) in *P. lutheri* and +6.0 ± 3.1‰ in *T. tetrathele*. Soft tissue $\delta^{13}C$, which ranged from −21.1 to −18.9‰ in foot tissue and from −20.5‰ to −18.8‰ in mantle tissue, was also not significantly correlated with pH (p > 0.05; Figure 3b). The $\delta^{13}C$ was −23.8‰ in *P. lutheri*, −24.0‰ and −21.9‰ in *T. tetrathele*, and −23.5‰ and −22.1‰ in the equal mixture of the two species.

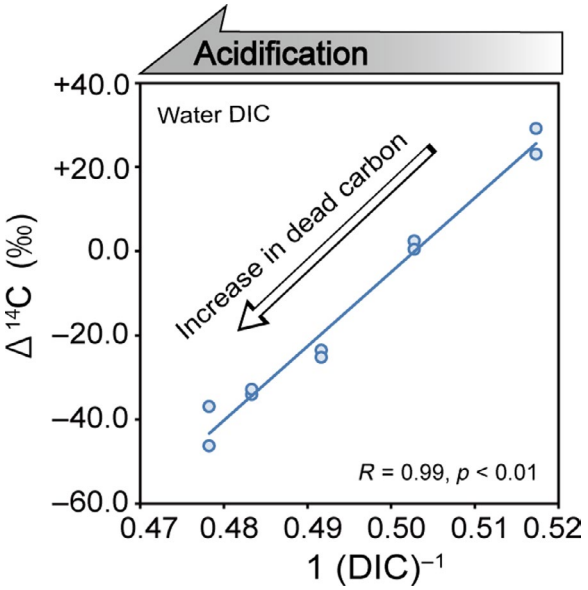

FIGURE 2 Relationship between $\Delta^{14}C$ and DIC^{-1} showing a significant positive correlation (R = 0.99, p < 0.01)

3.2 | End-member contributions to $\Delta^{14}C$ of shell and tissues

End-member contributions (R_{DIC} and R_{feed}) to $\Delta^{14}C_{shell}$ calculated by using the $\Delta^{14}C$ for each pCO_2 treatment with Equations 5 and 9 are shown in Table 1. R_{DIC} values were decoupled from changes in pH; they were 98%–100% in all pCO_2 treatments except that at 653 µatm (Table 1). The low values at 653 µatm, 87.5% (77.4%) calculated by using $\Delta^{14}C$ of *P. lutheri* (*T. tetrathele*), can be dismissed as a consequence of the similar $\Delta^{14}C$ values of plankton and seawater DIC at that pCO_2 level, which caused the R_{DIC} estimates to have large spread (Table 1). Excluding that pCO_2 level, carbon for shell calcification in *S. broughtonii* was derived mainly from seawater DIC (97%–100%), and the contribution of metabolic carbon to shell carbonate was less than 3% (Table 1). R'_{DIC} calculated with Equations 10 and 11 by using $\delta^{13}C$ ranged from 80.6% to 88.1%, from 81.2% to 88.5%, when the $\delta^{13}C$ values of mantle and foot, respectively,

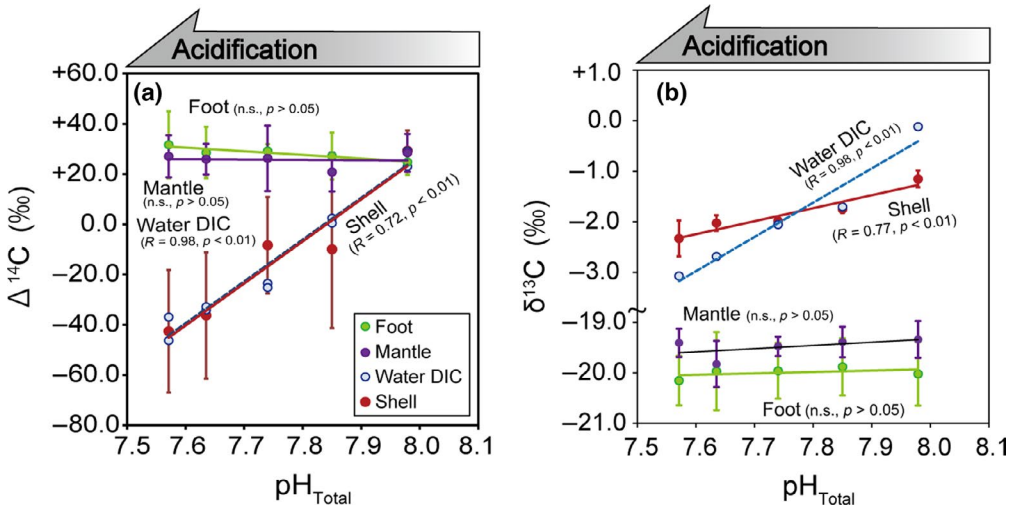

FIGURE 3 Effects of pH on $\Delta^{14}C$ and $\delta^{13}C$ values of aragonite shells, soft tissue (foot and mantle) and water DIC in a rearing experiment conducted at 25°C. (a) $\Delta^{14}C$ versus pH for shell (N = 28), water DIC (N = 10), foot tissue (N = 27) and mantle tissue (N = 25). (b) $\delta^{13}C$ versus pH ($\delta^{13}C$ shell and water DIC data are from Nishida et al., 2018): shell (N = 20), aqueous DIC (N = 5), foot tissue (N = 36), mantle tissue (N = 36). The mean value is shown for each pH treatment, and the error bars represent the standard deviations. Analytical precision was <0.05‰, <0.1‰ and <0.1‰ (1 SD) for $\delta^{13}C$ of shell, soft tissue and water DIC, respectively

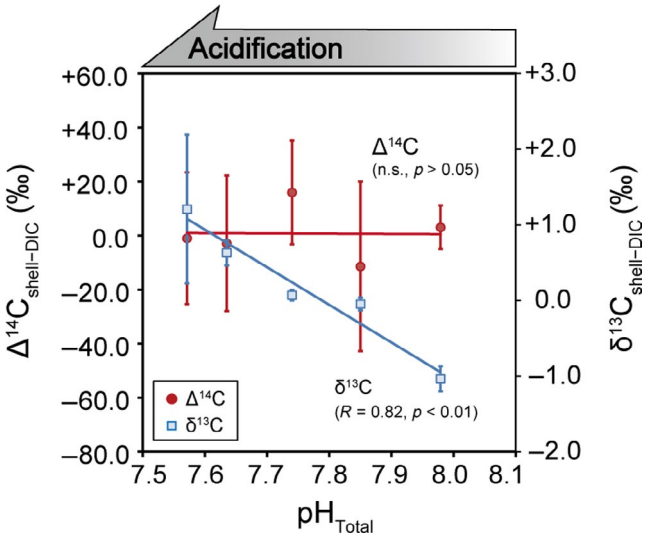

FIGURE 4 Comparison of differences in $\Delta^{14}C$ and $\delta^{13}C$ between the shell and water DIC ($\Delta^{14}C_{shell-DIC}$ and $\delta^{13}C_{shell-DIC}$, respectively) with pH. Values of $\delta^{13}C$ for the shell and water DIC are from Nishida et al. (2018). Error bars represent standard deviations of $\Delta^{14}C_{shell-DIC}$ and $\delta^{13}C_{shell-DIC}$

possibly because of the similar $\Delta^{14}C$ values of seawater DIC and soft tissues at that pCO_2 level.

4 | DISCUSSION

4.1 | Responses of shells and soft tissues to acidified seawater inferred by using radiocarbon

The mass balance equations using measured $\Delta^{14}C$ values indicated that *S. broughtonii* incorporated the same fraction of seawater DIC despite decreasing pH (and increasing pCO_2), and shell calcification neither decreased nor increased its reliance on organic nutrients. Less than 10% of shell carbonate originated from metabolic carbon, in agreement with previous studies of marine bivalves (Gillikin et al., 2006; McConnaughey et al., 1997). This result is in contrast to *R. philippinarum*, which shows a decline in the fraction of seawater DIC (and an increase in the fraction of metabolic DIC) contributing to shell carbonate with increasing pCO_2 (Zhao et al., 2018). The different responses of shell growth to low pH between *R. philippinarum* (Zhao et al., 2018) and *S. broughtonii* (Nishida et al., 2018) might reflect

TABLE 1 End-member contributions to $\Delta^{14}C$ of *Scapharca broughtonii* shells at five pH treatments. R_{DIC} and R_{feed} indicate the percentage contributions of DIC in seawater and feed, respectively, to $\Delta^{14}C_{shell}$. All *R* values are averages ± 1 *SE*. R_{DIC} and R_{feed} values were determined by calculating regression lines between shell $\Delta^{14}C$ and the $\Delta^{14}C$ values of two species of plankton (*Pavlova lutheri* and *Tetraselmis tetrathele*)

		R_{DIC} calculated by $\Delta^{14}C$		R_{feed} calculated by $\Delta^{14}C$	
pH	pCO_2 (µatm)	*P. lutheri*	*T. tetrathele*	*P. lutheri*	*T. tetrathele*
7.98	463 ± 68	100.0 ± 26.2	100.0 ± 19.9	0.0 ± 26.2	0.0 ± 19.9
7.85	653 ± 87	87.5 ± 155.5	77.4 ± 281.7	12.5 ± 155.5	22.6 ± 281.7
7.74	872 ± 100	97.8 ± 28.6	97.4 ± 33.5	2.2 ± 28.6	2.6 ± 33.5
7.64	1,137 ± 140	99.7 ± 25.9	99.7 ± 28.5	0.3 ± 25.9	0.3 ± 28.5
7.57	1,337 ± 167	100.0 ± 19.6	100.0 ± 21.1	0.0 ± 19.6	0.0 ± 21.1

TABLE 2 End-member contributions to $\delta^{13}C$ of *Scapharca broughtonii* shells at five pH treatments. R'_{DIC} indicates percentage contributions of DIC in seawater to $\delta^{13}C_{shell}$. All R' values are averages ± 1 *SE*. $\delta^{13}C$ values of mantle and foot tissues were used as $\delta^{13}C_R$ values, which have not been analysed in molluscs

		R'_{DIC} calculated by $\delta^{13}C$	
pH	pCO_2 (µatm)	$\delta^{13}C_R = \delta^{13}C_{mantle}$	$\delta^{13}C_R = \delta^{13}C_{foot}$
7.98	463 ± 68	80.6 ± 0.4	81.2 ± 0.4
7.85	653 ± 87	84.5 ± 0.2	84.9 ± 0.2
7.74	872 ± 100	84.9 ± 0.2	85.3 ± 0.2
7.64	1,137 ± 140	88.1 ± 0.5	88.2 ± 0.4
7.57	1,337 ± 167	88.0 ± 1.1	88.5 ± 1.0

were used to calculate $\delta^{13}C_R$, and it showed an increasing trend with decreasing pH (Table 2). R_{feed} (Equation 9) was 100.0% at 653, 872, 1,137 and 1,337 µatm, but it was low at 463 µatm (Table 3), a difference in calcification physiology between the two species. The R'_{DIC} values estimated by using $\delta^{13}C$ values with Equations 10 and 11 showed an increasing reliance of shell calcification on seawater with decreasing pH, and they were 10%–21% lower than the R_{DIC} values calculated using $\Delta^{14}C$ (Tables 1 and 2). However, because the end-member calculation using $\delta^{13}C$ includes isotopic fractionation effects such as the kinetic effect (discussed in the next paragraph), care must be taken when using $\delta^{13}C$ for end-member calculations. In addition, the mass balance calculation results support the reliability of $\Delta^{14}C$ in *S. broughtonii* as a paleoenvironmental proxy for seawater DIC because $\Delta^{14}C_{shell}$ was only marginally reliant on metabolic carbon despite perturbations in pH and pCO_2. This knowledge of the extent of reliability of a DIC proxy obtained experimentally under present-day conditions can be used to clarify and calibrate seawater DIC as a paleoenvironmental proxy. However, because the contribution of seawater DIC to shell calcification changed in *R. philippinarum* with changing pH (Zhao et al., 2018), the use of bivalves living in an environment undergoing

TABLE 3 End-member contributions to soft tissue $\Delta^{14}C$ of *Scapharca broughtonii* at five pH treatments. R_{feed} indicates percentage contributions of DIC in seawater to $\delta^{13}C_{shell}$. All *R* values are averages ± 1 *SE*

		Mantle		Foot	
		R_{feed} calculated by $\Delta^{14}C$		R_{feed} calculated by $\Delta^{14}C$	
pH	pCO_2 (μatm)	*P. lutheri*	*T. tetrathele*	*P. lutheri*	*T. tetrathele*
7.98	463 ± 68	−11.9 ± 11.6	−14.9 ± 14.6	6.8 ± 17.1	8.5 ± 21.5
7.85	653 ± 87	100.0 ± 83.6	100.0 ± 46.3	100.0 ± 62.9	100.0 ± 34.8
7.74	872 ± 100	100.0 ± 4.0	100.0 ± 3.5	100.0 ± 18.9	100.0 ± 16.7
7.64	1,137 ± 140	100.0 ± 10.4	100.0 ± 9.5	100.0 ± 6.2	100.0 ± 5.7
7.57	1,337 ± 167	100.0 ± 11.3	100.0 ± 10.4	100.0 ± 7.8	100.0 ± 7.2

severe pH changes as a $\Delta^{14}C_{DIC}$ proxy must take account of species-specific differences. The $\Delta^{14}C$ values of soft tissues of *S. broughtonii* at 653–1,337 μatm, calculated with Equation 9, were clearly derived from the $\Delta^{14}C$ values of the plankton feed, and they were not significantly linked to the ambient DIC value, despite the variation among individual bivalves. The $\delta^{13}C$ of soft tissues of juvenile *R. philippinarum* individuals reared for 6 months in the laboratory also did not show a statistically significant correlation with pH (Zhao et al., 2018). Thus, the contribution of seawater DIC to soft tissues might be limited in marine molluscs. Further studies are required to determine how long the residence time of the feed in an animal's soft tissues is.

4.2 | Inferring bivalve calcification physiology in acidified seawater by multi-isotopic analyses

The use of $\Delta^{14}C$ data in concert with stable isotope ($\delta^{13}C$ and $\delta^{18}O$) data allows isotopic fractionation effects (i.e. biological and kinetic fractionation effects) other than those caused by actual changes in the end-member contributions to be isolated, and yields insights into calcification and the physiology of marine calcifiers. By comparing the $\Delta^{14}C$ results for *S. broughtonii* with stable isotopic data (Nishida et al., 2018), we may be able to infer the carbonate chemistry in the extrapallial fluid (EPF) and the calcification physiology of this species. In molluscs, the mantle plays a key role in extracellular shell synthesis by supplying inorganic ions and organic matrix to the extrapallial cavity for the periostracum and the shell (Petit, Davis, Jones, & Hagler, 1980). Molecular CO_2 diffusing across biological membranes may be mostly used for calcification in the EPF (McConnaughey & Gillikin, 2008), where it reacts with H_2O (hydration) or OH^- (hydroxylation) to produce HCO_3^- as follows:

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+, \quad (12)$$

$$CO_2 + OH \rightarrow HCO_3^-. \quad (13)$$

Two enzymes, in particular, are key to shell calcification (Le Roy, Jackson, Marie, Ramos-Silva, & Marin, 2014; McConnaughey & Gillikin, 2008; Spalding, Finnegan, & Fischer, 2017). Carbonic anhydrase (CA) catalyses the reversible CO_2 hydration reaction in the EPF by regulating its carbonate chemistry constant (Le Roy et al., 2014; Paneth & O'Leary, 1985; Wilbur & Jodrey, 1955). In addition, Ca^{2+} ATPase acts as an ion pump, removing H^+ from, and adding Ca^{2+} to, the EPF (Le Roy et al., 2014; Spalding et al., 2017). This proton extraction, by increasing the EPF alkalinity, promotes CO_2 ionization in the EPF and the production of HCO_3^- for calcification.

Shell $\delta^{13}C$ and $\delta^{18}O$ values depend on both environmental (shell $\delta^{13}C$, temperature and $\delta^{13}C$ of seawater DIC; shell $\delta^{18}O$, temperature, $\delta^{18}O$ of seawater and ambient DIC) and internal (shell $\delta^{13}C$, kinetic and metabolic isotope effects; shell $\delta^{18}O$, the kinetic isotope effect) factors (Gillikin et al., 2006, 2007; Goodwin, Schone, & Dettman, 2003; Jones & Quitmyer, 1996; Klein, Lohmann, & Thayer, 1996; McConnaughey & Gillikin, 2008; Nishida et al., 2015, 2018). Although shell $\delta^{13}C$ has been used to estimate the contribution of seawater DIC and metabolic carbon to calcification in marine calcifiers, to analyse end-member contributions with $\delta^{13}C$, a kinetic isotope effect should be considered, especially in a changing pH environment. Kinetic isotope effects occur because molecules enriched in heavier ^{13}C and ^{18}O isotopes slow CO_2 hydration and hydroxylation reactions at isotopic disequilibrium (McConnaughey et al., 1997). Moreover, the relative proportions of HCO_3^- produced by these two reactions depend on the pH of the calcification fluid (Johnson, 1982; McConnaughey, 2003; Rollion-Bard, Chaussidon, & France-Lanord, 2003, 2011). The rate of hydroxylation increases with pH (McConnaughey & Gillikin, 2008), and the increase in this rate in the EPF at high pH leads to DIC that is more depleted in ^{13}C and ^{18}O (McConnaughey, 2003). By comparing $\delta^{13}C_{shell}$ and $\delta^{18}O_{shell}$ with isotopic equilibrium values, Nishida et al. (2018) used 'kinetic models' of isotopic disequilibrium to interpret isotopic fractionation of *S. broughtonii* shells reared in different pCO_2 treatments. In *S. broughtonii*, the correlations of both $\delta^{13}C_{shell}$ and $\delta^{18}O_{shell}$ with pH are significantly negative, and both approach isotopic equilibrium at lower pH (Nishida et al., 2018). The dependence of these different results on pH might be due to internally (via a kinetic isotope effect) or externally (by the influx of seawater DIC) controlled isotopic variabilities (Nishida et al., 2018). However, because the contributions of ambient DIC and metabolic carbon were constant in all pCO_2 treatments according to our $\Delta^{14}C$ results, changes in the influx of seawater and respiratory DIC to the EPF might have little impact on shell isotopic compositions. In addition, because marine molluscs have lower internal pCO_2 levels than terrestrial molluscs (Adkins et al., 2003; McConnaughey & Gillikin, 2008), the metabolic isotope effect on $\delta^{13}C_{shell}$ of marine molluscs might be small, as the results of our $\Delta^{14}C$ mass balance calculations showed (Table 1). Therefore, the pH-regulated shifts in $\delta^{13}C_{shell}$ and $\delta^{18}O_{shell}$ in our experimental

specimens might both be due to a kinetic isotope effect on shell isotopic composition via CO_2 hydration and hydroxylation reactions in the EPF.

In acidified seawater, $\delta^{13}C_{shell}$ and $\delta^{18}O_{shell}$ in *S. broughtonii* approach isotopic equilibrium via a kinetic process, which might be due to (a) relatively less CO_2 hydroxylation and more hydration or (b) more complete isotopic re-equilibration within the EPF due to CA activity. In our acidification experiment, both shell growth and somatic growth in the experimental specimens were independent of ambient pH (Nishida et al., 2018). (a) Relatively less CO_2 hydroxylation and more hydration may largely occur by fluid exchange around the periostracum (relatively less use of molecular CO_2). It may be unlikely, however, that the calcification physiology of *S. broughtonii* relies on directly derived ambient DIC because (a) DIC exchange across the periostracum may be hard to regulate and (b) the energetic cost of maintaining the carbonate chemistry in the EPF might be higher by this process. Therefore, isotopic re-equilibration is a more likely candidate. *S. broughtonii* may diminish the effects of the decreasing EPF pH and the aragonite saturation state on its growth by using molecular CO_2 to regulate the pH of the EPF homeostatically. Thus, it may exert tight control over shell calcification by biological isotopic fractionation. The CA enzyme may play an important role in chemical and isotopic equilibration of the EPF. Rapid ^{18}O equilibration in the presence of CA, observed in a barium carbonate precipitation experiment, is attributable to catalysis of CO_2 hydration by CA (Uchikawa & Zeebe, 2012). Moreover, CA promotes $\delta^{18}O$ isotopic equilibrium in synthetic calcite (Thomsen et al., 2013). Although our knowledge of how CA affects $\delta^{13}C$ kinetics is limited, Chen, Gagnon, and Adkins (2018) have proposed a kinetic biomineralization model that considers both $\delta^{13}C$ and $\delta^{18}O$. According to this model, the CA effect on the kinetics of isotope equilibration in the calcification fluid is greater for $\delta^{18}O$ than for $\delta^{13}C$; therefore, the slope of the $\delta^{18}O$–$\delta^{13}C$ relationship is steeper when CA is present in the calcification fluid than when it is not (Chen et al., 2018). The steep slope of the $\delta^{18}O$–$\delta^{13}C$ relationship for our experimental specimens (Nishida et al., 2018) may imply that a kinetic process together with CA activity might appear in *S. broughtonii* with changing pH.

4.3 | Application of bomb-pulse radiocarbon principles to experimental biology

We have successfully determined the end-member contributions to bivalve calcification under changing pH by reverse radioisotope labelling. The $\delta^{13}C_{shell}$ data also indicate that shell calcification in *S. broughtonii* depends primarily on DIC (Table 1), but shell $\delta^{13}C$ approaches apparent isotopic equilibrium in acidified waters (Nishida et al., 2018) and therefore reflects the kinetics of isotopic equilibration with water via hydration and hydroxylation reactions in the extrapallial fluid. Because kinetic fractionation effects and changing end-member contributions cannot be separated by using $\delta^{13}C$ data alone, $\Delta^{14}C$ data are useful for corroborating $\delta^{13}C$ data and determining the magnitude of fractionation effects. Use of $\delta^{13}C$ and $\Delta^{14}C$ data in combination thus ensures the reliability of the estimates of both effects. In addition, the dependence of *R′* estimation based on $\delta^{13}C$ on which soft tissues are used to determine $\delta^{13}C_R$ values (Poulain et al., 2010) complicates the analysis of end-member contributions from $\delta^{13}C$ values. Thus, the use of a multi-isotopic dataset allows a more accurate analysis of carbon assimilation and of the magnitude of kinetic isotope effects in relation to pH changes. Widespread use of such multi-isotopic data will allow high-resolution calibration of isotopic proxies in biogenic and synthetic carbonates that exhibit non-equilibrium behaviour.

To estimate end-member contributions more precisely with $\Delta^{14}C$, a culture experiment should be designed to provide a large difference between end-member signals. Because air with the current atmospheric pCO_2 level was used in the plankton culturing aquaria in our experiment, the end-member contributions at 653 μatm showed a large scatter. Better resolution of the contribution differences (i.e. lower $\Delta^{14}C$ values of plankton samples compared with those of seawater DIC) could be obtained by aerating the plankton tanks with both air and fossil fuel-derived CO_2 gas. Reverse radioisotope labelling is a powerful tool to understanding physiology of aquifer organisms that can be applicable to other organisms and culture experiments at various temperatures and salinities to improve understanding of the proportions of carbon taken in by marine organisms under changing environments.

5 | CONCLUSIONS

We demonstrated that a novel reverse radioisotope labelling experiment can reveal details of biology of marine organisms including bivalves. The plankton diet was the principal carbon source for both mantle and foot tissues, whereas the ambient DIC was essential for shell calcification by radiocarbon analyses. Experimental $\Delta^{14}C$ data revealed that the ambient DIC contribution to bivalve shell carbonate fell in the range 97%–100% at 463, 872, 1,137 and 1,337 μatm. With increasing ambient pCO_2, *S. broughtonii* did not shift to derive more shell carbon from the plankton feed. The insensitivity of *S. broughtonii* to perturbations in pCO_2, at least up to 1,337 μatm, indicates that this species is able to withstand ocean acidification, and end-member calibration by $\Delta^{14}C$ will be an invaluable tool in environmental studies for examining the sensitivity of marine organisms against ocean acidification. The high degree of correlation between $\Delta^{14}C_{shell}$ and $\Delta^{14}C_{DIC}$ was unaffected by changing pCO_2 conditions. The lack of a pH effect allows the use of $\Delta^{14}C$ isotopic data derived from *S. broughtonii* to estimate ambient DIC and suggests its suitability as a geochemical proxy of paleoenvironmental DIC.

Multi-isotopic data ($\Delta^{14}C$, $\delta^{13}C$ and $\delta^{18}O$) analyses for this species show that the stable isotopic signature of an *S. broughtonii* shell reflects the kinetics of isotopic equilibration with water via hydration and hydroxylation reactions in the EPF with changes in pCO_2, and this fact must be taken into consideration in analysing

end-member contributions. The use of $\Delta^{14}C$ removes any kinetic or metabolic fractionation effects and allows the contributions of end members to be isolated. Comparison of the $\delta^{13}C$ dataset with the $\Delta^{14}C$ dataset can reveal the extent to which isotopic fractionation effects unrelated to end-member contributions affect $\delta^{13}C$ values. As we demonstrated in the present study, $\Delta^{14}C$ differences are more than 50 times that of the $\delta^{13}C$-based experiment (see Figure 3). Thus, using reverse radioisotope labelling together with conventional $\delta^{13}C$ isotopic analysis can contribute to a better understanding of calcification mechanisms and of the physiology of calcifiers.

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AUTHORS' CONTRIBUTIONS

All authors contributed to the idea and discussion of the paper; K.N., Y.Y., A.S., M.H. and Y.N. designed the experiment, and K.N., Y.C.C., A.S., M.H., Y.Y. and M.S. performed the culture experiment and sampling; Y.C.C., Y.M., S.H. and Y.Y. analysed radio and stable carbon isotopes; K.N., Y.C.C. and Y.Y. mainly drafted the paper and managed the data. The corresponding authors (K.N. and Y.Y.) contributed equally to this work.

DATA AVAILABILITY STATEMENT

All analysed data are available by DRYAD entry: https://doi.org/10.5061/dryad.g4f4qrfmv (Nishida et al., 2020).

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