Stable isotopic characterization of carbon, nitrogen and sulfur uptake of *Acharax japonica* from central Japan

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Abstract: A unique community of *Acharax japonica* has been found in the reductive bottom sediments in two natural seawater sinks, where a number of the clams can easily be collected and successfully maintained for over a year in laboratory conditions. Stable isotopic signature of their soft body parts shows typical range for chemosynthesis-based bivalves, indicating that the clams rely on sulfur-oxidizing bacteria in their gill. $\delta^{15}\text{N}$ values of substrate ammonia have been measured together with those of the clam soft body parts. These results confirm that the characteristic negative $\delta^{15}\text{N}$ values of the clam soft-body parts were caused by a large isotope fractionation during ammonia assimilation as previously documented for those from various locations. In addition, sulfur isotopic ratios of each organ show significant variation. Such trend is observed for some thiotrophic chemosynthesis-based bivalves inhabited on a cold-seep environment, suggesting that it is a common feature for thiotrophic bivalve communities depended on bacterial derived sulfide. Heterogeneity of sulfur isotopic signature among different organs from one specimen was considered due to primary heterogeneity of $\delta^{34}\text{S}$ values for source sulfide in their habitat. In addition a different turnover rate of the sulfur nutrition in each organ was conspicuous for the sulfur isotopic variation. This was confirmed by long-term maintenance using an external sulfur budget under laboratory conditions. The estimated turnover rates among three organs were the highest for gill (shorter than three months), the lowest for viscera (longer than a year), and intermediate for foot.

Key words: *Acharax japonica*, chemosynthesis-based animal, stable isotopes, acquisition of nutrients, laboratory culture

Introduction

Solemyid clams have been found in reductive marine sediments from various water depths between littoral and abyssal zones (>6,000 m), where hydrogen sulfide prevails.

The clams lack gut and nutritionally rely on sulfur-oxidizing bacteria in their gill, which provide organic substances derived from their chemosynthesis and sustain host lives (Nelson & Fisher 1995). The endosymbionts are considered as belonging to a group of gamma-Proteobacteria (Imhoff et al. 2003), which uses Calvin-Benson cycle for carbon fixation and are characterized by low carbon isotope ratio of their soft tissues ($\delta^{13}\text{C} \leq -30\text{‰}$, Van Dover & Fry 1989).

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A unique community of *Acharax japonica* has recently been found in the reductive bottom sediments in two natural seawater sinks in central Japan. Unlike the other known natural occurrences of solemyid clams, which inhabit the deep subsurface of marine sediment, it was very easy to collect the living specimens together with the associated sediments. This provided an opportunity to conduct a detailed isotopic analysis between the host bivalves and nutrient budgets, particularly carbon, nitrogen and sulfur in the sediments. The isotopic ratios of these three biologically essential elements clearly indicate their inorganic source and/or assimilation processes (Mizota & Yamanaka 2003). In particular, sulfur isotopic ratios of the bivalves harboring thioautotrophic symbionts are similar to those of their substrate sulfides. In addition, most bivalves harboring chemosynthetic endosymbionts are significantly depleted in ^{15}N relative to the common marine bivalves. Nevertheless, source and assimilation pathway of nitrogen for endosymbionts are still unknown. Therefore, such an analysis is a prerequisite for precise evaluation of the adaptation of the unique family to reduced environment, although very limited information is currently available, as summarized in Table 1 (Kulm et al. 1986, Conway et al. 1989, 1992, Suess et al. 1998).

The objective of the present study was to elucidate biological isotopic fractionation ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$) associated with carbon, nitrogen and sulfur uptake and turnover of the solemyid clams. *Acharax* specimens were maintained in a controlled aquarium to carry out feeding experiments over an extended period (360 days). Such experiments with chemosynthesis-based animals have been carried out using a deep-sea mussel, *Bathymodiolus* (Dattagupta et al. 2004). In this case, the specimens were transplanted among several habitats of the upper Louisiana slope. In the experiment, shift of carbon and nitrogen isotope ratios corresponding with the changes in nutrient sources was observed. Nevertheless, the cause of change in sulfur isotope ratios was still unclear. A principal problem of this experiment may be that the deep-sea mussel (*Bathymodiolus childressi*) harbors both types of the symbionts (methanotrophic and thioautotrophic; Brooks et al. 1987), and also ability of filter feeding is still active (Dattagupta et al. 2004). In this case, assessment of the contribution of sulfide-sulfur as nutrient is quite difficult because various plausible sources of sulfur exist. By contrast, our study may be a first case of an experiment performed in an aquarium at an off-shore laboratory. *A. japonica* harbors only sulfur-oxidizing bacteria and lacks feeding ability. The analytical data obtained were evaluated by comparison with those in the previous studies.

Study site and Methods

Collection of sample *Acharax japonica*

Living specimens of *Acharax japonica* were collected on 3rd March, 2003 (No. 2 sink), 1st October, 2003 (No. 1 sink)

and 24th September, 2004 (No. 2 sink) from two setting sinks at the Shimoda Marine Research Center, The University of Tsukuba, Shimoda, Shizuoka, central Japan (34°40'N, 138°56'E, Altitude: 4 m). Our measurements showed the No. 1 sink was approximately 1.1 m long, 4.2 m wide and 0.5 m high, and No. 2, 9 m, 2.9 m and 1.3 m, respectively. Fresh seawater was drawn continuously into the sinks from an intake 600 m southeast of the research center at a depth of 3 meters.

Solemyid clams in the larval stage in the sink sediments may be delivered by the fresh seawater accompanying the calcareous sediment. In addition, a large amount of organic materials including detritus of marine algae should accumulate in the sinks because large populations of *Ecklonia cava* and *Eisenia bicyclis* inhabit the surrounding area. Such organic materials would decay in the sediment, resulting in the anaerobic sediment interior. The prevailing conditions had been expected to be suitable for maintaining solemyid clams which harbor thiotrophic endosymbionts.

The settling sinks were filled with fine calcareous sediment (approximately 40 cm thick in No.1 sink and 80 cm thick in No. 2 sink). The sediment in the sinks has been removed every year at No. 1 and every 5 years at No. 2 sink. All solemyid clams were collected just before the dredges at both sinks. The clams were sieved from the sediment using a stainless steel sieve (mesh size: 1 mm). Upon recovery, the clams were immediately transferred to fresh seawater (15°C) and were maintained in an aquarium at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) (Yokosuka, Japan) until dissection. The associated sediment for sulfide and ammonia analyses was collected from the same sinks and each sample was preserved in an airtight container at 4°C. Extraction of sulfide and ammonia was performed within a couple of days of returning to the laboratory of Iwate University.

Feeding experiments of *Acharax japonica* in laboratory

Several living specimens of *Acharax japonica* were fed in a small aquarium installed at JAMSTEC for 116 and 360 days. A small vessel (15 cm wide, 10 cm deep and 10 cm high) was filled with the sediment (8 cm thick) collected at the clam habitats, and was placed in a seawater basin (35 cm wide, 24 cm high and 35 cm deep), which had a seawater filtering system. Artificial seawater (Rohto Chemicals, Co. Ltd., Osaka, Japan) was used for this experiment. Sodium sulfide solution (Wako Chemicals Co., Ltd., Osaka, Japan) was injected into the sediment using an electromagnetic metering pump. The final concentration of sulfide was maintained within a range from 0.1 to 0.59 mg-S/L under the controlled water temperature of 15°C. Samples of living specimen for isotope analysis were taken carefully to avoid disturbing the sediment.

Sample preparation for stable isotopic measurement

Depending on the sample numbers available for isotopic

analysis, one to three specimens were dissected into gill, foot and viscera under a binocular microscope. Each organ was centrifugally washed repeatedly with 0.1 M LiCl solution to eliminate seawater sulfates, and then freeze-dried. Carbon and nitrogen isotopic composition was determined using continuous flow-gas chromatography coupled with a stable mass spectrometer, DELTA plus (Thermo Electron Corp., USA) installed at the Prefectural University of Kumamoto, Tsukide, Kumamoto, Japan. Sulfur isotopic composition of the soft body part was determined using MAT 252 (Thermo Electron Corp., USA) installed at the Department of Geology, Tohoku University, Aoba-Ku, Sendai, Japan.

Acid volatile sulfide (AVS), which represents a mixture of dissolved sulfides (H_2S , HS^- , S^{2-}) in pore-water and amorphous iron sulfide (FeS), was liberated by anaerobic acidification in 1 N HCl of sediments during active distillation (pure nitrogen gas) of ca. 5 cm³ core fraction obtained from nearby habitat of the clams. Collection of liberated H_2S in traps containing cadmium acetate solution (2.5%) was made, resulting in the precipitation as a form of cadmium sulfide (CdS). The yellow CdS precipitate was oxidized with a few drops of hydrogen peroxide solution (34.5%). The first samples collected on March, 2003, were not determined AVS content and its sulfur isotope ratio. Instead, the concentration of total sulfides, which represent all forms of sulfide, was estimated by the following procedure. The sediment samples were first dialyzed against the distilled water to eliminate pore-water sulfate ion. During the process dissolved sulfides might be lost. Sulfide-sulfur in the pore water could be ignored, since almost sulfides are in solid form. The total sulfides were converted into sulfate by treatment with hot hydrogen peroxide solution. These resulting sulfates were recovered as BaSO_4 by adding BaCl_2 solution. Both reagent sulfide- and sulfate-sulfur used for the feeding experiment were similarly recovered as BaSO_4 . The BaSO_4 sample was then mixed with excess $\text{V}_2\text{O}_5\text{-SiO}_2$ and finally converted into SO_2 using a vacuum line, as described by Yanagisawa & Sakai (1983). The evolved SO_2 sample was subjected to measurement of $^{34}\text{S}/^{32}\text{S}$ ratios using a VG SIRA 10 mass spectrometer.

Ammonium in the sediment under the clam habitat was extracted by shaking 2 M KCl solution (100 g of wet sediment with 200 ml of the solution). Ammonium in the extract was recovered by the steam distillation technique using a gas-tight container (semi-micro Kjeldahl apparatus) and finally converted into a water insoluble form of ammonium tetra-phenyl-borate, as described by Sakata (2001). Another aliquot of the extract was used for quantitative estimation of ammonium-nitrogen. Care was taken with quantitative recovery ($99 \pm 1\%$) and consistency of $\delta^{15}\text{N}$ value ($\pm 0.1\%$) of the reagent grade ammonium chloride. $\delta^{15}\text{N}$ value of the recovered samples of the ammonium tetra-phenyl-borate was determined by DELTA plus a mass spectrometer by a continuous-flow mode. All the values were shown as $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ notations, per mil variation

relative to V-PDB, V-CDT and air dinitrogen, respectively. The analytical precision for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ measurements was better than $\pm 0.2\%$ during the present analysis.

Results and Discussion

Stable isotopic signatures of naturally occurring *Acharax japonica* in the sinks

Table 1 summarizes stable isotopic compositions for carbon, sulfur and nitrogen of the soft tissues from *Acharax japonica*, together with those obtained from several species of the Solemyid clams in previous studies. Sulfur and nitrogen isotopic compositions of substrate sulfide and ammonium in the relevant sediments are also included. Living specimens of *A. japonica* inhabited the black sandy sediments. Concentration of sulfide-sulfur in the black sediments was 0.14 and 0.25 mg/kg dry sediment, respectively. Dead clam shells were observed in grey sediments, where sulfide content (0.07 mg/kg) was lower than that in the black sediments (Table 1).

Carbon isotopic compositions ($\delta^{13}\text{C}$ values) of soft body parts obtained from natural *Acharax* specimens in the sinks ranged from -32.3 to -28.2% . The values were comparable to those in previous studies (Table 1). These lower $\delta^{13}\text{C}$ values indicated the existence of a carbon fixation pathway involving the Calvin-Benson cycle (Nelson & Fisher 1995) and confirmed the previous studies for the *Acharax* symbionts, which belong to gamma-Proteobacteria (Imhoff et al. 2003).

Unlike carbon isotopic compositions, sulfur isotopic compositions ($\delta^{34}\text{S}$) of the soft body parts recovered from natural sediments varied somewhat, from -26 to -10% for samples from No. 2 sink, and from -25 to -6.3% for those from No. 1 sink (Table 1). Observed low $\delta^{34}\text{S}$ values indicate direct reliance on the thioautotrophic endosymbionts, because the $\delta^{34}\text{S}$ values of sulfides in the associated sediments show similar range from -22 to -6% . The $\delta^{34}\text{S}$ values of each organ varied significantly and the difference of $\delta^{34}\text{S}$ values between the organs reached over 10% in a specimen (the samples collected on 24th September, 2004; Table 1). The observed trend of variation in $\delta^{34}\text{S}$ values of each organ was comparable for the two other specimens collected at the same time on 24th September, 2004 (Table 1), while the difference of these $\delta^{34}\text{S}$ values between the individuals was negligible (less than 1% for each organ; Table 1). It suggests that there is a common trend that controls the isotopic compositions of each organ. Such variation of $\delta^{34}\text{S}$ values among organs has been reported for one *Solemya borealis* specimen (Conway et al. 1992, Table 1) and *Calymptogena soyocae* (Mizota & Maki 1998). It has been more commonly observed in chemosymbiotic invertebrates from cold seeps (Mizota & Yamanaka 2003). At cold seeps where hydrogen sulfide formed by bacterial reduction of seawater sulfate prevails, it was distinguished by significantly low $\delta^{34}\text{S}$ values less than 0% (Thode 1991). The

Table 1. Stable isotopic signatures of the soft body parts of present and previously reported Solemyid bivalves and of the associated sediments of the habitat.

Species	Location	Growth condition before analysis	Soft body part				Associated sediment			Reference
			Organ	$\delta^{13}\text{C}$ (‰)	$\delta^{34}\text{S}$ (‰)	$\delta^{15}\text{N}$ (‰)	Sulfide-sulfur Content ⁸⁾	$\delta^{34}\text{S}$ (‰)	Ammonical nitrogen Content ⁸⁾	
<i>Acharax japonica</i>	1)	Natural, collected on 3rd March 2003. Three composite samples	Gill	-32.2	-10.0	-10.9	TS=0.14	-21.8	4	This study
			Foot	-31.0	-26.2	-9.7	AVS=ND	ND	+12.0	
			Viscera	-32.2	-19.0	-11.2				
		Fed under lab condition for 116 days. Composite sample of three individuals. Substrate sulfide $\delta^{34}\text{S} = -3.5$ (‰), sulfate $\delta^{34}\text{S} = +0.1$ (‰)	Gill	ND	+5.5	ND				
			Foot	ND	-2.8	ND				
			Viscera	ND	-19.7	ND				
		<i>ditto</i> , for 360 days 1 individual	Gill	ND	-19.0	ND				
			Foot	ND	+9.7	ND				
			Viscera	ND	-17.2	ND				
	2)	Natural, collected on 1st October, 2003. Two individuals	Gill	-30.8, -30.8	-17.2*	-9.3, -8.5	TS=0.25	-5.9	10.1	+0.8
			Foot	-28.2, -28.4	-24.9*	-8.1, -7.7	AVS=0.03	-16.7		
			Viscera	-29.9, -30.3	-6.3*	-9.1, -8.6				
	2)	Natural, collected on 24th September, 2004. Three individuals	Gill	-32.2, -32.0, -31.4	+0.1, +0.6**	-10.3, -9.3, -8.7	TS=ND	ND	16	+4.3
			Foot	-31.1, -30.0, -28.5	-7.3, -6.8**	-9.3, -8.3, -8.0	AVS=0.014	-8.7		
			Viscera	-32.6, -30.8, -30.0	-0.3, +0.5**	-9.9, -9.3, -8.7				
	3)	Natural	Periostracum	-31.0	ND	ND	ND	ND	ND	Kulm et al. (1985)
	4)	Natural	Gill	-33.9~-33.2	-28.2	-9.8~-8.7	ND	ND	ND	Conway et al. (1989)
<i>Solemya borealis</i>	5)	Natural	Foot	-32.1~-31.5	-31.1	-8.6~-7.7	ND	ND	ND	Conway et al. (1989)
			Gill	-32.8~-32.4	-26.7	-4.6~+0.4	ND	ND	ND	
	6)	Natural	Foot	-31.3~-30.9	-29.2	-4.6~+4.4	ND	ND	ND	Conway et al. (1992)
Solemyid specimen	7)	Natural	Gill	-34.6	-15.7	-9.7	ca. 10	-25.1	ca. 10	
			Foot	-32.0	-32.6	-8.6	ND	ND	ND	Suess et al. (1998)
			Diverse	-35.4~-29.6	ND	ND	ND	ND	ND	

ND=not determined.

1) Shimoda, central Japan, two No. 2 sink in the present study

2) Shimoda, central Japan, two No. 1 sink in the present study

3) Oregon subduction zone

4) Masachussets, USA: site 1

5) Masachussets, USA: site 2

6) Masachussets, USA

7) Aleutian subduction zone

8) mg/Kg dry sediment.

*) Data from composite sample of two individuals

**) Data from the two larger individuals

variation may primarily reflect the assimilation of hydrogen sulfide-sulfur, which has heterogeneous sulfur isotope ratios in the sediments during growth. Highly variable $\delta^{34}\text{S}$ values of sulfide were shown in different sediment samples collected within a narrow region of reductive sediments, reflecting the differences in micro-environment during microbial reduction of seawater sulfate (Masuzawa et al. 1995). The apparent difference of sulfur isotope ratios among organs should be reflected in a different turnover rate of each element in each organ. The turnover rate of sulfur in each organ is discussed based on the data of long-term maintenance in the next section.

Relative to the common marine clam (e.g., Kwak & Zedler 1997, Page & Lastra 2003), all the soft tissues examined in this study were characterized by fairly low $\delta^{15}\text{N}$ values, ranging from -10.9 to -7.7‰ (Table 1). Similar lower values are commonly observed for other samples of Solemyid clams (down to -9.8‰) (Mizota & Yamanaka 2003). Gill endosymbionts incorporate inorganic nitrogen in their habitat (Nelson & Fisher 1995). The observed nitrogen isotopic fractionation between soft tissues and ammonia in the relevant sediment was 10 to 23‰. Such a significantly high, negative fractionation of nitrogen has been shown in assimilation processes of nitrogen from ammonia using glutamine synthetase (fractionation: 8 to 13‰) (Yoneyama et al. 1993) or glutamine dehydrogenase (fractionation: 2 to 10‰) in *Solemya reidi* and *Riftia pachyptila* (Lee & Childress 1994). Therefore, a similar process utilizing ammonia as a source of nitrogen nutrition might exist in *A. japonica*.

Change in sulfur isotope ratios of *A. japonica* soft tissues during long-term maintenance

Several live specimens of *A. japonica* were maintained under a continuous supply of sodium sulfide ($\delta^{34}\text{S} = -3.5\text{‰}$) with matrix artificial sulfates ($\delta^{34}\text{S} = +0.1\text{‰}$). $\delta^{34}\text{S}$ values of gill and foot tissues of clams maintained for 116 and 360 days were changed from their original isotopic signatures, according to the environmental sulfur sources, whereas viscera keep their original isotopic signatures (Table 1). As shown in Fig. 1, the gill and foot tissues maintained for 116 days were enriched in ^{34}S , with gill tissues showing a figure 9‰ higher than that for sodium sulfide. This may be due to evaporation of a part of the sodium sulfide as a volatile sulfide during the feeding experiment, the leftover dissolved sulfide consequently enriched in ^{34}S . The $\delta^{34}\text{S}$ value of gill tissues maintained for 360 days, however, fell below the original value, while that of foot maintained for 360 days increased continuously. The low value of gills maintained 360 days may be due to assimilate sulfide-sulfur derived from sulfate-reducing bacteria rather than sodium sulfide, because the medium in the aquarium was filled with original sediment collected together with the clam. The sediment contains easily decomposable organic matter, so bacterial sulfate-reducing activity may still be present. In addition,

Fig. 1. Temporal changes in the sulfur isotope ratios of each tissue during the maintenance in laboratory environment.

tion, the $\delta^{34}\text{S}$ value of sulfate ion dissolved in aquarium water ($\delta^{34}\text{S}$ value $= +0.1\text{‰}$) was much lower than that of common seawater sulfate-sulfur. Therefore, the $\delta^{34}\text{S}$ value of the formed sulfide became lower than that of sulfide occurring in natural conditions. These results imply that the turnover rate of gill tissue is estimated to be shorter than three months and is higher relative to that of other tissues, while that of viscera is estimated to be far longer than a year. Nevertheless, the detailed mechanism of sulfur incorporation remains unclear.

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