

NOTE

Strontium as a marker for estimation of microscopic growth rates in a bivalve

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ABSTRACT: Microscopic growth increments in bivalve shells were measured by marking the shells of the short-necked clam *Ruditapes philippinarum* with the strontium chloride (strontium marking method, SMM). The results were compared with those achieved by the fluorescent marking method (FMM) using calcein. Because strontium has a higher atomic mass than calcium, strontium-enriched areas in the shells are visible as a bright band in the back-scattered electron image under the scanning electron microscope (SEM). *R. philippinarum* were immersed in strontium chloride solutions of varying concentrations, and the effects of immersion time (17 or 24 h) and concentration (0.72, 1.44 or 2.88 g l⁻¹) were investigated. These shells exhibited clearly visible marks 19 or 20 d post-immersion. Using the SMM, a microscopic (single-digit μm) increment in shell growth could be detected under SEM with a magnification of at least 1200×. Using the FMM, growth increments of only tens of micrometers were detectable under fluorescence optical microscopy up to a magnification of 600×. The SMM is thus superior to previous methods for detecting microscopic increases in shell growth. Marking methods should ideally be based on long-lasting markers that are easily detected and involve simple methodologies. The SMM meets these requirements. It can be used to estimate growth in small bivalve specimens and growth rates in slow-growing species such as deep-sea and polar bivalves.

KEY WORDS: Strontium elemental marking method · Growth rate · Short-necked clam · Microscopic growth

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The growth rate of an organism provides basic ecological data. The growth rates of bivalves have been well studied, since many species are important for the fishing industry (e.g. Shaul & Goodwin 1982, Tanabe 1988, Tian & Shimizu 1997). Many estimation methods have been developed to detect increases in shell growth, including band-analysis (Stevenson & Dickie 1954, Thompson et al. 1980, Sasaki 1981, Richardson

1989), marking methods (mark-release-recapture) using labeling paint, tags, filed notches and fluorescent chemicals (Heald 1978, Jones et al. 1978, Ito 1985, Parsons et al. 1993, Kesler & Downing 1997, Kaehler & McQuaid 1999, McQuaid & Lindsay 2000, Sato-Okoshi & Okoshi 2002), and calculation from age determination using radium-228 chronology (Turekian et al. 1975). Among these, marking methods are efficient in estimating bivalve growth rates because they are inexpensive and relatively easily applied by untrained observers. However, it is difficult to detect microscopic growth increases in shells on a scale of less than tens of micrometers, since such increases must be measured with a sliding caliper or by optical microscopy. One unique marking method uses rust to mark the shell surfaces, making it possible to detect microscopic increases in shell growth of fewer than tens of micrometers with an electron-probe X-ray microanalyzer (Koshikawa et al. 1997). However, at least 14 d of immersion are needed for the rust to be retained by the shell surface.

Recently, artificial marks have been made on otoliths with strontium (Sr) to study the periodicity of otolith growth increments of less than tens of micrometers in fish (gobies) (Iglesias et al. 1997, Hernaman et al. 2000). Using scanning electron microscopy (SEM) Sr-enriched areas in the otolith are detected as bright bands in the back-scattered electron image.

To the best of our knowledge, the use of Sr chloride (SrCl₂) as a growth marker in clam shells has not been previously investigated. In the present study, we assessed the potential of Sr as a marker to estimate microscopic growth rates in bivalve shells. The Sr marking method (SMM) was applied to estimate the growth rate of the short-necked clam *Ruditapes philip-*

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pinarum. This paper describes the practical advantages of the SMM in detecting microscopic growth increments on a single-digit μm scale and compares the results with those of the fluorescent marking method (FMM) using calcein. Collection, immersion, shell preparation, Sr detection and growth rate measurements were as follows.

Materials and methods. Study species: Specimens of the short-necked clam *Ruditapes philippinarum* were collected from the littoral at Nojima-koen, Yokohama, Kanagawa Prefecture, Japan ($35^{\circ} 19.4' \text{N}$, $139^{\circ} 39.2' \text{E}$) on 7 July 2001, and taken immediately to the Japan Marine Science and Technology Center (JAMSTEC). The shell length of all specimens was measured, and they were then placed in the sea off a pier fronting the JAMSTEC facilities.

Immersion: *Ruditapes philippinarum* shells were marked with SrCl_2 hexahydrate ($\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, Wako Pure Chemical Industries, Osaka, Japan). For comparison, they were also marked by immersion in the diluted fluorescent chemical calcein ($\text{C}_{30}\text{H}_{26}\text{N}_2\text{O}_{13} \cdot \text{HCl}$, Wako Pure Chemical Industries). Clams were placed in static aquaria and submitted to 6 treatments (3 different concentrations for 2 immersion periods of different lengths) with either SrCl_2 or calcein (Table 1). Immersion techniques were standardized among treatments.

SrCl_2 or calcein was dissolved in plastic aquaria containing 2 l of artificial seawater (Rohtemarine, Rei-Sea). Each aquarium was placed in an incubator at 25°C in the dark to prevent light degradation of the

fluorescent chemicals during the immersion period. The chemicals were dissolved at concentrations of 0.72, 1.44 and 2.88 g l^{-1} SrCl_2 and 0.3, 0.4 and 0.7 g l^{-1} calcein. During immersion, small compressors provided air to each aquarium. The clams remained in the aquaria for a treatment period of either 17 or 24 h. Except for the highest concentration, the concentrations and immersion periods for both chemicals were approximately the same as those used in previous studies for marking fish otoliths (Iglesias et al. 1997, Hernaman et al. 2000). After immersion, the clams were reared in ambient seawater (21.5 to 28.0°C) in front of the JAMSTEC pier (water depth approximately 1 to 3 m), and culled 19 to 20 d later. As a control, 10 non-marked specimens were also reared together with the chemically marked specimens. All specimens were retained by nylon nets ($60 \times 30 \times 30 \text{ cm}$).

Shell preparation: After culling, the shells were cleaned of adhering tissue and dried in an oven (60°C) for 24 h. A transverse section was cut across the longest shell axis, and the ventral part was divided into anterior and posterior sections (Fig. 1). Methacrylate-based resin (Technovit 7200 VLC, Heraeus Kulzer) was used as embedding medium. Each shell was first embedded in the resin. For SMM, the cross sections were ground with 600-grit sandpaper and then wet-polished using $9 \mu\text{m}$, $3 \mu\text{m}$ (METADI Diamond Suspension), and finally $0.05 \mu\text{m}$ (Masterprep) polishing suspension. For FMM, embedded shells were sliced into approximately $200 \mu\text{m}$ sections with a lapidary bandsaw (BS-300CP, Exakt Apparatebau). The sections were attached to glass slides and the facings wet-polished with a $9 \mu\text{m}$ polishing suspension (Metadi Diamond Suspension).

Detection of incorporated bands and analysis of Sr concentration in shells:

The embedded shells were carbon-coated using a vacuum-evaporator (JE-400, JEOL). This treatment produced bands of Sr-enriched areas in the shells that were detectable in a back-scattered electron image under SEM (SM-5800L, JEOL). Once the positions of these bands had been determined, the Sr concentrations in the bright band and background (i.e. outside the bright bands) areas were analyzed by spot-analysis using an energy-dispersive X-ray spectrometer (Oxford Lind ISIS 2001, Oxford Instruments) and a detector (Supermini Cup, JEOL). The electron beam was irradiated at an accelerating voltage of 25 kV and a lifetime of 100 s.

Calcein was detected by examining the sectioned shells under fluorescence opti-

Table 1. *Ruditapes philippinarum*. No. of individuals, shell length, chemical concentrations, immersion period, and number of days reared post-treatment for the Sr marking method (SMM) and the fluorescent marking method (FMM)

	No. individuals	Shell length (mm)	Conc. (g l^{-1})	Immersion period (h)	No. days post-treatment
Strontium chloride					
(SMM)	3	26.1–27.5	0.72 ^a	17	19
	3	21.7–28.0	0.72	24	20
	3	22.9–25.4	1.44 ^b	17	19
	3	21.0–22.2	1.44	24	20
	3	23.9–29.5	2.88 ^c	17	19
	3	23.8–25.3	2.88	24	20
Calcein					
(FMM)	3	25.0–26.9	0.3	17	19
	3	21.7–30.4	0.3	24	20
	3	22.3–28.1	0.4	17	19
	3	25.7–25.8	0.4	24	20
	3	22.9–24.6	0.7	17	19
	3	22.7–26.3	0.7	24	20

^a30× Sr concentration in natural seawater
^b60× Sr concentration in natural seawater
^c120× Sr concentrations in natural seawater

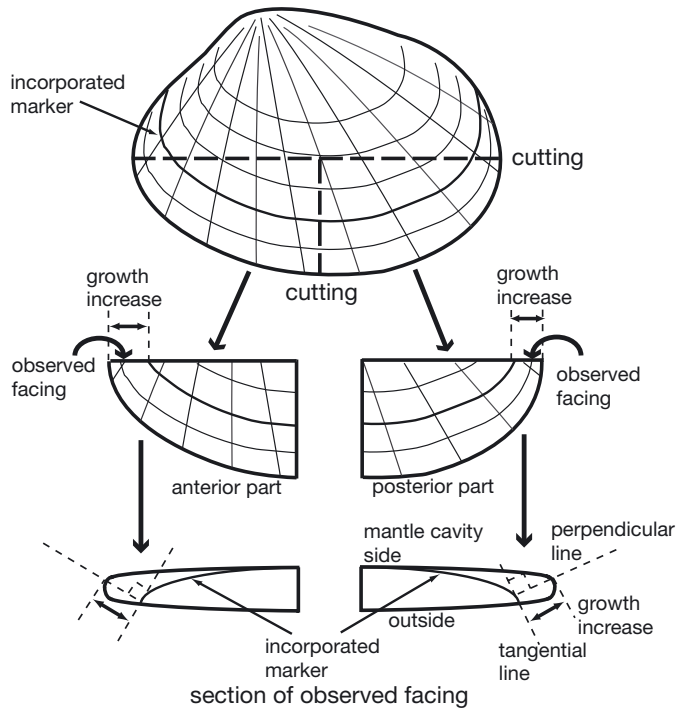


Fig. 1. Shell sectioning for detection of Sr-enriched and fluorescent areas. Shells were prepared to provide transverse section across longest shell axis; ventral portion was divided into anterior and posterior sections. Sr-enriched or fluorescent areas were examined at anterior and posterior margins

cal microscopy (Optiphot, Nikon) fitted with an ultraviolet (UV) light source (HB-10101AF, Nikon) and a fluorescence filter (V-1A, Nikon). In the ultra violet excitation wavelength, calcein appeared as a bright lime-green band in the sectioned shells.

Growth rate: Once the position of a bright band (SrCl_2) or a fluorescent band (calcein) had been determined, the growth increment between the band and the shell margin was measured under SEM for the

SMM or under optical microscopy for the FMM. Growth increments were measured as follows (Fig. 1):

(1) A tangential line was drawn close to an incorporated band on the sectioned shell; (2) a perpendicular line was drawn from a tangential line to the shell margin; (3) the maximum length of the perpendicular line from the tangential line to the shell margin was regarded as the growth increment of the anterior or posterior part of the sectioned shell; (4) the total growth increment equalled the sum of both the anterior and posterior growth increments.

Growth rate was calculated using the formula growth rate ($\mu\text{m d}^{-1}$) = L/D , where L is the total growth increase and D is the number of days post-treatment.

Results. Sr and calcein markings were both successful, but to different degrees.

Sr marking: Clear bright bands were visible on *Ruditapes philippinarum* shells after most immersion treatments, except at a concentration of 1.44 g l^{-1} for 24 h. The Sr-enriched bands tended to become more distinct with longer immersion periods and at higher SrCl_2 concentrations (Fig. 2). The band produced after 17 h immersion in solution with a SrCl_2 concentration of 0.72 g l^{-1} was faint (Fig. 2A). Although no clams died during any 17 h immersion period, 5 of 9 specimens immersed in SrCl_2 solution for 24 h died after being returned to the sea post-immersion. Of 10 control specimens (non-marked) 2 died during rearing in the sea fronting the JAMSTEC pier.

Sr concentration: Energy-dispersive X-ray spectrometer analysis confirmed that the bright bands in shells treated with SrCl_2 were due to greatly enhanced levels of the chemical. The Sr concentration in background areas in parts of the shell not treated with SrCl_2 showed characteristic X-ray counts of approximately 100 counts per 100 s (Fig. 3). However, the Sr concentrations in the bright bands in all treatments were notably higher than those in the background areas, indicating that Sr had accumulated in the bright bands.

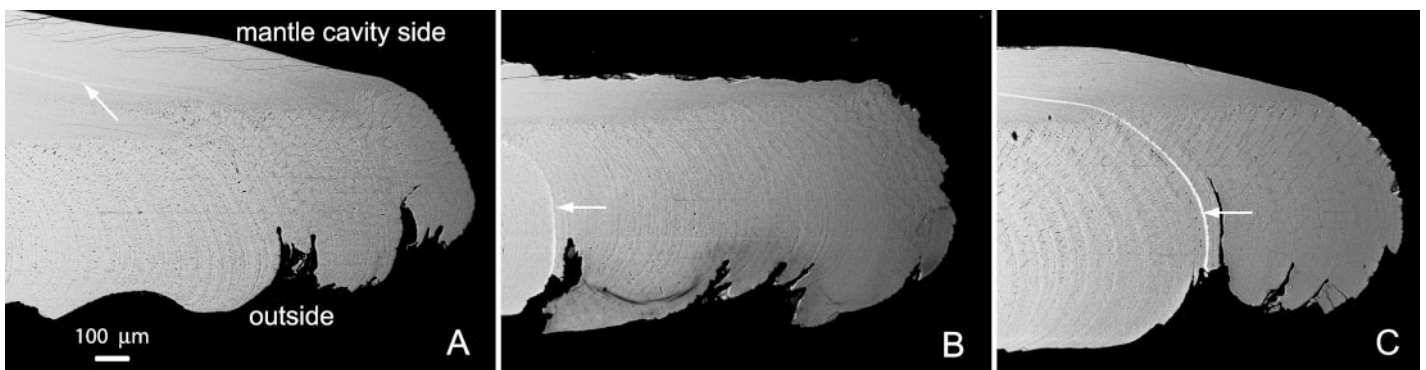


Fig. 2. *Ruditapes philippinarum*. Photomicrographs of back-scattered electron images (SEM) of anterior portions of shell sections after Sr marking. Arrows indicate bright Sr-enriched bands. (A) SrCl_2 conc. = 0.72 g l^{-1} , immersion period = 17 h; (B) SrCl_2 conc. = 2.88 g l^{-1} , immersion period = 17 h; (C) SrCl_2 conc. = 2.88 g l^{-1} , immersion period = 24 h

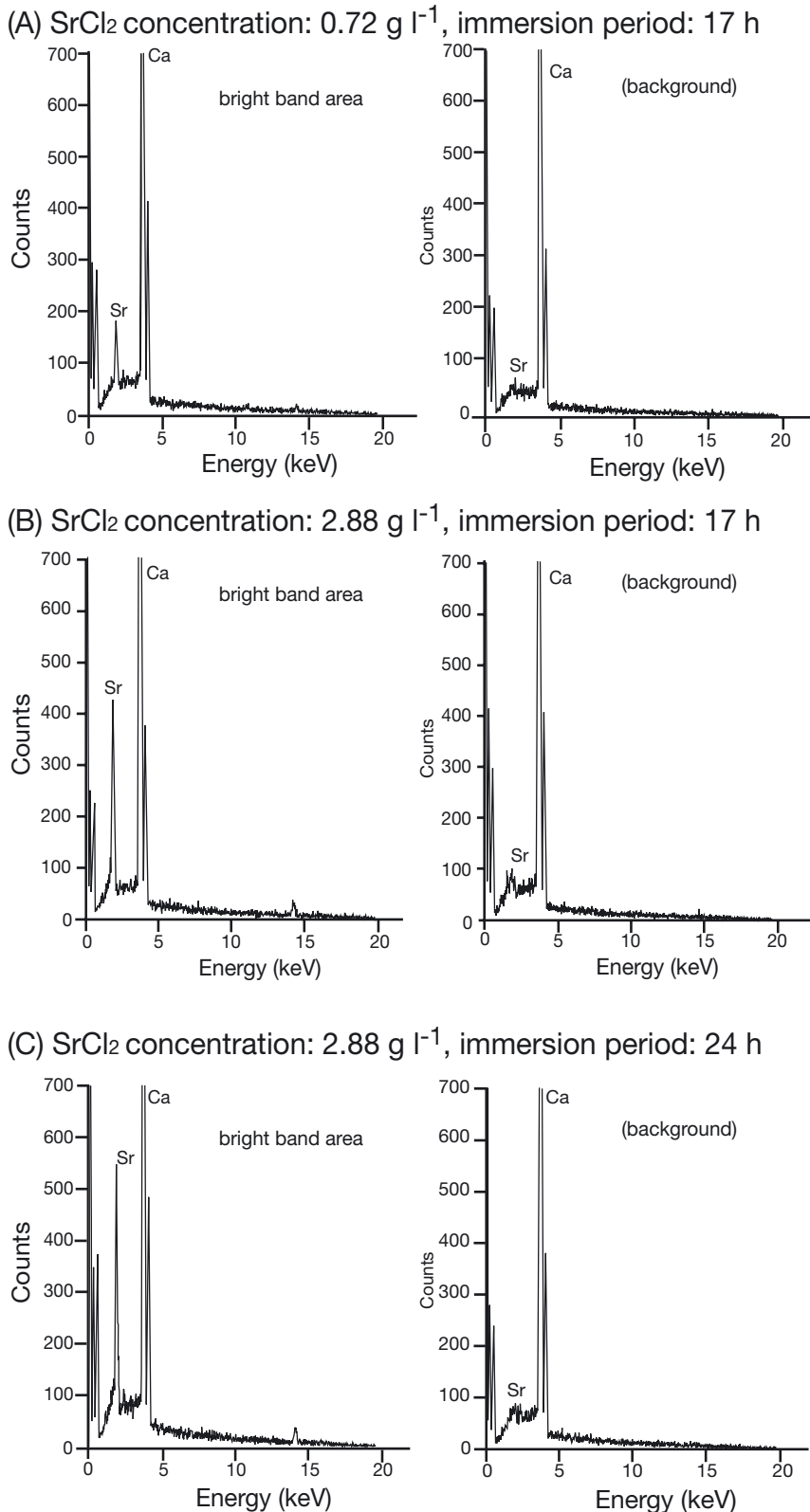


Fig. 3. *Ruditapes philippinarum*. Sr concentration (cps) determined by energy-dispersive X-ray spectrometry of bright band and background band areas in shell sections subjected to 3 different treatments. The Sr concentration in bright-band area is notably higher than that in background area

The Sr concentrations in the bright bands increased with increasing SrCl_2 concentration of the immersion solution (Fig. 3).

Calcein marking: Clams immersed in calcein solution had clearly visible fluorescent growth bands in their shells at all concentrations and exposures (e.g. Fig. 4); 2 specimens died after 24 h immersion in solution at a calcein concentration of 0.3 g l^{-1} . The fluorescent bands appeared more distinct after longer immersion and at higher calcein concentrations.

Higher magnification: The maximum magnification under the fluorescent optical microscope was $600\times$ (eyepiece $15\times$, objective lens $40\times$) and the fluorescent bands in the shells could be detected at this magnification (Fig. 5B). In the back-scattered electron image under SEM, however, the Sr-enriched bands were detectable at a magnification of $1200\times$ (Fig. 5A). The boundary between Sr-enriched and non-enriched areas appeared more distinct than that between fluorescent and non-fluorescent areas.

Growth rate measured using SMM: Under SEM, the microscopic growth rate could be estimated in single-digit μm based on the growth increment between an Sr-enriched band and the shell edge. At 19 or 20 d post-immersion, growth rates of *Ruditapes philippinarum* ranged from 13.5 to $116.8 \mu\text{m d}^{-1}$; they tended to decrease with increasing shell length (Fig. 6).

Discussion. SrCl_2 was found to be a useful growth marker in the short-necked clam *Ruditapes philippinarum*. After incorporation into the shell, Sr-enriched areas were visible as bright bands in the back-scattered electron image (SEM), and were easily distinguishable from the non-Sr-enriched (background) areas (Fig. 2). Energy-dispersive X-ray spectrometric analysis of the clam shells showed that the bright bands were attributable to high SrCl_2 concentrations. The distinct and narrow bright bands incorporated into the growing margins of the shells at the time of Sr administration were successfully detected.

The SMM (1) allows detection of microscopic growth increases, and (2) requires only shorter post-immersion removal periods. Although the FMM method (Parsons et al. 1993, Kaehler & McQuaid 1999, Sato-Okoshi & Okoshi 2002) has similar advantages, it can generally only measure shell growth increments in tens of micrometers at magnifications between 400 and 600 \times (magnification of 600 \times in the present study [Fig. 5]; magnification of 400 \times for the postlarval giant scallop *Placopecten magellanicus* marked with Alizarin red dye and using compound microscopy in Parsons et al. 1993). Using the SMM, smaller shell growth increments can be detected with the SEM at a magnification of at least 1200 \times (Fig. 5).

Marking methods previously used to estimate bivalves growth rates required post-immersion periods after marker administration ranging from 4 wk to a few years (Shimizu et al. 1988, Parsons et al. 1993, Kesler & Downing 1997, Isono et al. 1998, McQuaid & Lindsay 2000, Sato-Okoshi & Okoshi 2002), whereas the SMM can estimate growth rate at least 19 to 20 d after post-immersion of the Sr marker because it is able to detect smaller microscopic-level shell growth increments.

The growth rates of *Ruditapes philippinarum* estimated with the SMM were similar to those reported in other studies, i.e. estimated growth rates of 13.5 to 116.8 $\mu\text{m d}^{-1}$ in the present study (Fig. 6) compared to approximately 10 to 100 $\mu\text{m d}^{-1}$ from July to August (same season as in the present study) using the FMM (Shimizu et al. 1998). This indicates that immersion in SrCl_2 solution did not impede the

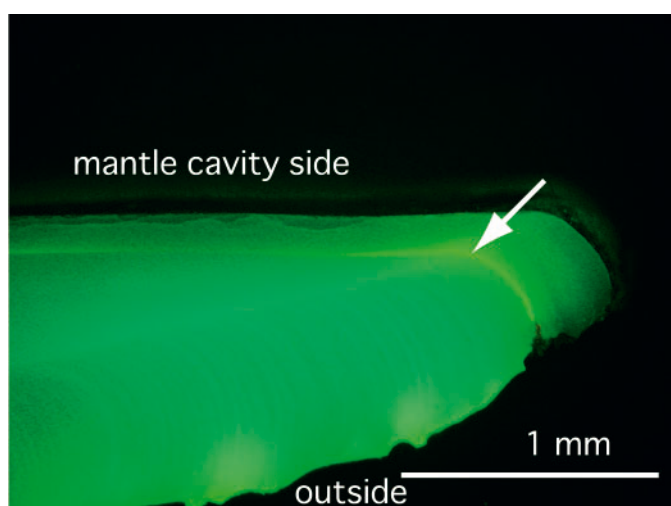


Fig. 4. *Ruditapes philippinarum*. Photomicrograph of shell section after fluorescent marking method (FMM) with calcein. Arrow indicates bright lime-green bands in calcein-enriched area. Photomicrograph was taken under fluorescent optical microscope. Calcein conc. = 0.7 g l^{-1} , immersion period = 24 h

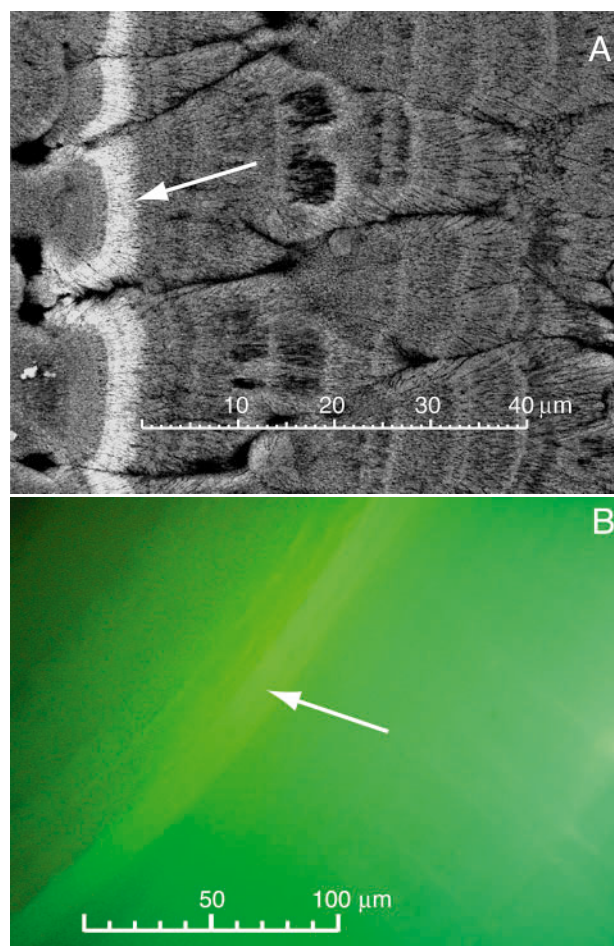


Fig. 5. *Ruditapes philippinarum*. Higher magnification of shell sections. (A) Back-scattered electron image (SEM; original magnification 1200 \times) of shell section marked with Sr; arrow shows Sr-enriched band; this specimen was treated with SrCl_2 concentration of 2.88 g l^{-1} for immersion period of 17 h. (B) Fluorescence optical microscopic image (original magnification 600 \times) of shell section marked with calcein; arrow shows calcein-fluorescent band; this specimen was treated with SrCl_2 concentration of 0.72 g l^{-1} for immersion period of 24 h

growth of *R. philippinarum* even though the experimental clams were immersed in seawater containing high concentrations of dissolved SrCl_2 and accumulated high levels of Sr in their shells. All specimens remained alive during the 17 h immersion period, but 5 of 9 died after 24 h immersion. Of 10 control specimens (non-marked), 2 also died. Whether immersion in SrCl_2 or calcein was fatally toxic to *R. philippinarum* could not be determined because the sample number was too small. The rearing conditions (in the sea in front of the JAMSTEC pier) may have been unsuitable for *R. philippinarum*. To avoid injurious effects, the immersion period should be as short as possible and the SrCl_2 concentration as low as

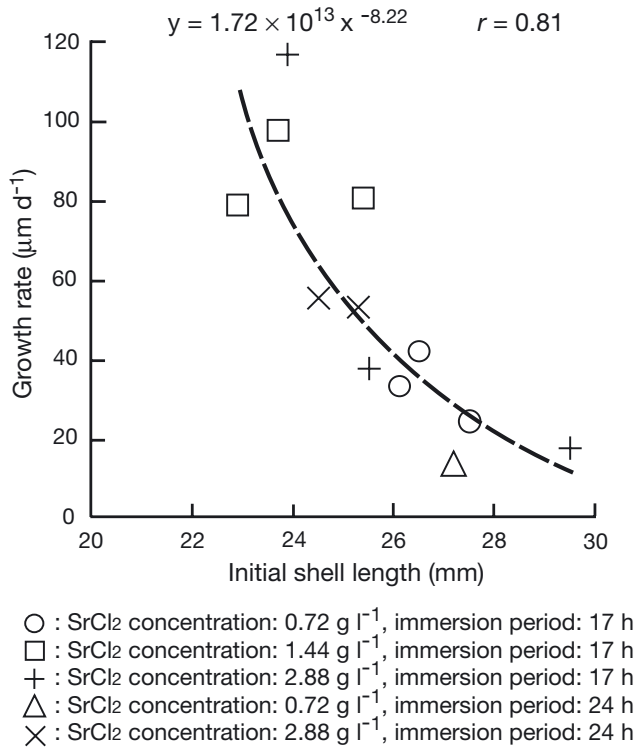


Fig. 6. *Ruditapes philippinarum*. Growth rate estimated by Sr marking method (SMM). After immersion in SrCl₂ in laboratory, the clams were reared in the sea for 19 or 20 d in July 2001

possible. The present experiment indicates that when the SMM is applied to *R. philippinarum* at the same element concentrations and water temperature as in the present study, an immersion period of 17 h (or probably less) is sufficient. Optimal marking conditions, including the minimum chemical concentration and immersion period, will be estimated in a future study.

Marking methods for growth-rate estimations in bivalves should involve long-lasting markers, easy detection, and simple methodologies. Classic marking methods using paint, bonded labels, and tagged labels may damage the marked specimens while exposing them to air, and such markers occasionally peel off the shells. The rust-marking method, while not harmful (Koshikawa et al. 1997), requires at least 14 d to mark shell surfaces. Microscopy employs a variety of fluorescent chemicals that are not easily distinguishable from the very similar natural autofluorescence (Day et al. 1995), and it is also difficult to detect the boundary between fluorescent and non-fluorescent areas, especially at higher magnifications (Fig. 5). The SMM eliminates these problems; however, it involves time-consuming shell preparation, and therefore is not suitable for the analysis of large numbers of samples. Despite this disadvantage, the SMM is simple and can efficiently detect microscopic growth increases (Table 2). Consequently, it can be used to estimate growth rates in small specimens and in slow-growing species such as deep-sea and polar bivalves. In addition, the method is useful for detecting growth rates over short experimental periods: by multiple marking of clam shells using the SMM, it is possible to estimate daily, weekly, monthly, tidal and seasonal growth rate variations in bivalves.

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Table 2. *Ruditapes philippinarum*. Comparison of advantageous characteristics among 4 marking methodologies for growth rate estimation of bivalves. Marker detection: ease of distinguishing between marked and background areas; +++: very good; ++: fairly good; +: poor

Method		Advantages				
Marking	Detection	Detection of microscopic growth increments	Marker longevity	Marking period	Marker detection	Shell preparation
Strontium chloride	Scanning electron microscopy	+++	+++	++	+++	+
Paint labels, tags, notches	Slide caliper	+	+	+++	+++	+++
Fluorescent chemicals	Optical microscopy	++	++	++	++	++
Rust	Electron-probe X-ray microanalyzer	+++	+++	+	+++	+

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