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Elemental Imaging of the Rotula Bone of the Sea Urchin (*Strongylocentrotus intermedius*) using LA-ICP-MS and its Potential for Ecotoxicological Time-Scale Monitoring of Marine Environments

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Summary

Sea urchins do not actively travel long distances. Therefore, it is believed that their growth zones can be characterized to obtain information on marine environmental conditions. We analyzed trace elements in the rotula bones of Ezo-bahun-uni sea urchins (*Strongylocentrotus intermedius*) from brackish and salt water through two-dimensional elemental imaging using laser ablation inductively coupled plasma spectrometry (LA-ICP-MS). Before the LA-ICP-MS analysis, a conventional ICP-MS analysis was performed to determine the approximate elemental composition. The imaging results revealed different distribution patterns of Li, Sr, and Mn in the urchin rotula bones in brackish and salt water areas. Collectively, these findings support the use of sea urchins to monitor the inorganic composition of marine environments on an ecotoxicological time-scale.

Key words: Urchin, laser ablation inductively coupled plasma mass spectrometry, elemental imaging

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

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Introduction

The elemental composition and concentration and isotopic composition in the hard tissues of marine organisms can be used to assess various factors of marine environments [1]. It is well known that the otoliths of fish grow throughout their life and retain elements incorporated in their annual growth bands. Analysis of specific parts of the otoliths can provide retrospective information on past environmental conditions [2, 3]. Fish otoliths are useful to assess the environment over a wide area of ocean as fish migrate.

On the contrary, benthic organisms, such as shellfish [4] and

coral [5] are considered to be beneficial for understanding the environmental conditions of specific marine areas. Sea urchins are also known as a good biological model for studying the marine environment, especially for pollution research [6-8]. Ternengo et al. suggested that benthic organisms can become a bioindicator of environmental trace elements because of their ecological relevance, benthic and relatively sedimentary lifestyle, rapid response, and high sensitivity to many types of contaminants [6]. For example, green sea urchins accumulate certain metals and can be used to monitor Ag, Cu, Fe, Hg, Pb, and Zn pollution in mining sites [7].

In this study, we focused on the rotula bone of the mouth organ (Aristotle's lantern) of sea urchin (*Strongylocentrotus intermedius*). This is because the bone continues to grow, similar to an otolith of fish, showing annual growth bands [9]; therefore, the content of elements in the bone reflects the environmental conditions when the bone developed. High-sensitivity elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS) provides the contents of trace elements in biological samples, however, in-situ elemental analysis and reconstruction of mapping/imaging data is of great importance for precisely assessing the changes in elemental concentration over time, which can reflect the changes in marine environment. Hence, laser ablation ICP-MS (LA-ICP-MS) has been widely adopted to assess the bioaccumulation of metal elements in ecological and toxicological studies in humans, animals, and plants [10]. In the present study, we aimed to investigate the potential of the sea urchin as a bioindicator to chronologically monitor marine environments by comparing the elemental imaging data of its rotula bone from brackish and salt water using LA-ICP-MS.

Materials and Methods

Sample preparation for conventional ICP-MS

Ezo-bahun-uni sea urchins (*S. intermedius*) were purchased from two fishery cooperatives in Hokkaido, Japan. The first set of 10 sea urchin was collected from the brackish waters of Saroma, and the second set of 10 sea urchins was sourced from the salt waters of Yoichi. The 20 specimens in total (7-8 cm in length) were collected in June. Of the five rotula bones collected from each urchin (**Fig. 1a**), one was weighed and placed in a perfluoro-ethylene bottle containing 0.4 mL of 68% HNO_3 (ultrapure grade; Tama Chemicals Co., Kawasaki, Japan). The bones (n = 20) were immersed in HNO_3 overnight at 25 °C and then were added 0.2 mL of H_2O_2 (ultrapure grade; Tama Chemicals Co., Kawasaki, Japan). Microwave digestion was performed using Ethos Plus (Milestone General, Bergamo, Italy) under the following conditions; 250 W for 5 min, 0 W for 1 min, 250 W for 5 min, 400 W for 5 min, and 600W for 5 min. The volume of the digested sample was then adjusted to 1.0 mL with ultrapure water. The fixed-volume solution was diluted 25 times in 1.5% nitric acid and 20 ng/mL Sb was added as an internal standard. The containers for samples and all measured solutions were rinsed with ultrapure water (18.2 MΩ/cm) after pre-cleaning with nitric acid.

Conventional ICP-MS analysis

The elemental concentrations in each diluted solution were determined using an inductively coupled plasma mass spectrometer

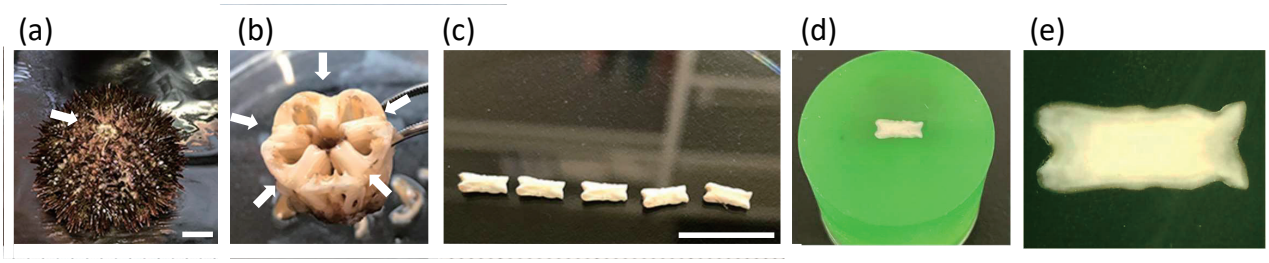

Fig. 1. **Pretreatment of rotula bones for LA-IC-MS analysis**
(a) the mouth organ "Aristotle's lantern" was harvested from each sea urchin (white arrow, scale bar = 1 cm) ;
(b) five rotula bones (white arrows) were collected from the upper side of the mouth organ of each sea urchin ;
(c) the five rotula bones were dried, and one was selected (n = 10) and digested for conventional ICP-MS analysis (scale bar = 1 cm) ;
(d) a second rotula bone (n = 3) was embedded in a resin mold and polished using diamond slurry ;
(e) by placing the polished surface facing the laser source in the LA sample chamber, LA-ICP-MS analysis was performed.

(ICP-MS; Agilent 8800; Agilent Technologies, Tokyo, Japan). ICP-MS were calculated using 0, 0.4, 4.0, and 40.0 ng/mL of the multi-element standard solutions XSTC-1 and XSTC-13 (SPEX Industries Inc., NJ, USA) diluted with 0.5% nitric acid. The concentration of 45 elements (Ag, Al, As, Ba, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ho, In, K, La, Li, Lu, Mg, Mn, Na, Nd, Ni, Pb, Pr, Rb, Sc, Se, Sm, Sr, Tb, Th, Tl, Tm, U, V, Y, Yb, and Zn) was measured using a standard calibration method with the signal intensity of Sb as the internal standard. The elemental concentrations in each tested rotula bone were determined using the obtained mean elemental contents and the dry weight of the digested bone.

Statistical analysis

The element concentrations in rotula bone of urchins from brackish (n = 10) and salt (n = 10) waters, determined using conventional ICP-MS analysis, were compared using the unpaired Student t-test in Microsoft Excel for Microsoft 365 MSO (16.0.14228.20216). The level of significance was set at $p < 0.05$.

Sample prepatation for LA-ICP-MS

The rotula bones (**Fig. 1b-d**) of three urchins from each sampling area were imaged using LA-ICP-MS. One bone per urchin was embedded in epoxy resin (Technovit 4071; Kulzer, Wehrheim, Germany), and the epiphyseal side of the bone was polished using diamond pads (Cameo® Disk Platinum Type 1 and Type 3; Leco Corporation, St Joseph, MI, USA). Next, the bones were ultrasonically cleaned and polished on a polishing cloth using diamond slurry (6 μm and 9 μm in diameter) (Maruto Co., Tokyo, Japan). Following a second wash with ultrapure water, the bones were observed under a stereomicroscope to estimate the age of the urchin from the ring pattern on the polished surface as described by Watanabe and Takada [11].

LA-ICP-MS analysis

Elemental imaging was performed via multiple-line profiling LA-ICP-MS analysis with the parameters listed in **Table 1**. The signal intensity (count per second) obtained by ICP-MS was reconstructed using iQuant2 (Ver.2018 Apr) [12] to generate elemental images. The range of the color bar was adjusted for each element to get better visualization.

Table 1. | Instrumentation and operational settings

Laser (New Wave Research NWR213)		ICP-MS (Agilent 8800)	
Wavelength	213 nm	RF incident power	1600 W
Pulse energy	10%	Plasma gas flow rate	15.0 L min^{-1}
Fluence	4.0 J cm^{-2}	He carrier gas flow rate	0.80 L min^{-1}
Repetition rate	10 Hz	Ar carrier gas flow rate	0.95 L min^{-1}
Spot size	50 × 50 μm (square)	Monitored isotopes	^{7}Li, ^{31}P, ^{43}Ca, ^{52}Cr, ^{55}Mn, ^{66}Zn, ^{88}Sr,
Scan speed	35 μm s^{-1}		^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm
Stabilizer	not used	Data acquisition mode	Time-resolved analysis
		Dwell time	^{7}Li, ^{52}Cr ^{88}Sr, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm : 0.05 sec
			^{66}Zn: 0.05 sec
			^{55}Mn: 0.01 sec
			^{31}P, ^{43}Ca: 0.0005 sec

Results and Discussion

Data obtained from conventional ICP-MS analysis (mean ± S.D. [μg/g]) showed that Mn (Saroma: 4.15 ± 2.62, Yoichi: 0.77 ± 0.60) and La (Saroma: 0.01 ± 0.005, Yoichi: 0.005 ± 0.002) concentrations were significantly higher in the Saroma brackish water group than in the Yoichi salt water group ($p < 0.05$). Conversely, Li (Saroma: 1.49 ± 0.39, Yoichi: 2.16 ± 0.64) and Sr (Saroma: 796.1 ± 186.9, Yoichi: 1047.0 ± 275.0) concentrations were significantly higher in the salt water group than in the brackish water group ($p < 0.05$). The strata around Saroma are known to be rich in Mn [13], and it is possible that Mn was brought in from river water. For Li and Sr, it was assumed that their concentrations were lower in Saloma because they were diluted in fresh water compared to seawater. The other trace elements measured did not show sufficient concentrations for comparison. Subsequently, we performed LA-ICP-MS analysis of aforesaid four elements that exhibited the differences between the two areas in ICP-MS analysis mainly, in addition to Ca as a major component of bone tissue.

Sea urchin age was estimated by measuring the number of growth band on the sampled bones (**Fig. 2**). Approximately 5 bands were clearly observed were observed only in the brackish water group, indicating that the Saroma sea urchins were 5-6 years old. On the contrary, clear growth bands were not observed in salt water sea urchin samples. Narvaez et al. [14] reported that the absence of distinct growth bands in sea urchins was dependent on their growth environment (e.g., food availability); the sea urchin selected for the present study may have been similarly affected by food availability in their habitat.

The LA-ICP-MS analysis (**Fig. 3**) showed that Ca, a major component of bone, was homogeneously distributed throughout the bone, whereas Li, Sr, and Mn were distributed in a ring along the growth bands. The integrated signal intensity from LA-ICP-MS did not always match the concentration results from the conventional ICP-MS, which may be due to heterogeneous distribution in the bone. Although the mechanisms underlying the uptake of these elements and their ring-shaped accumulation in the rotula bones of sea urchin remain unknown, these phenomena exhibit a certain periodicity. The cyclic repeated pattern of these elements in sea urchin bones may be influenced by the seasonal changes of the salt water environment, as observed in fish [2,3].

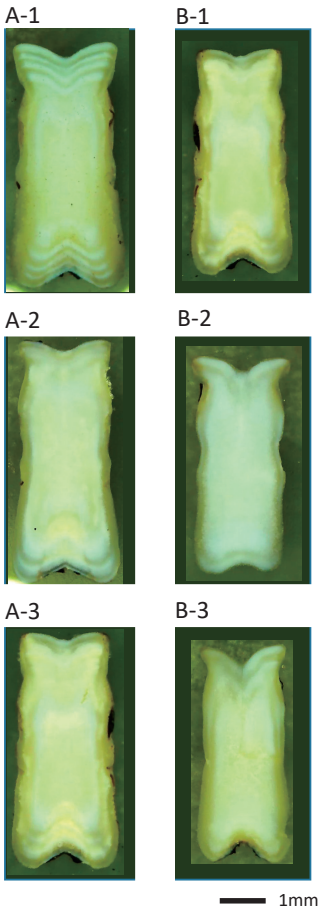

Fig. 2. **Observation image by optical microscope rotula bone of *Strongylocentrotus intermedius***
A-1, -2, and -3 are samples from Saroma (brackish water); B-1, -2, and -3 are samples from Yoichi (salt water). Scale bar = 1mm.

Elemental imaging of sea urchin rotula bones offers the possibility to monitor the marine environment chronologically on an ecotoxicological time scale. The longevity of *S. intermedius* is estimated to be 6-10 years [15]. Our findings indicate that this method is particularly effective on specimens from brackish waters where there are seasonal variations in elemental composition exists. The analysis of sea urchins by LA-ICP-MS is also useful to assess contamination by toxic metals and radioisotopes, and to assess the bioavailability of inorganic nutrients in the marine environments over time.

Conclusion

We used LA-ICP-MS to determine the distribution of elements in the rotula bones of *S. intermedius* collected from brackish water and seawater. Elemental imaging of sea urchin rotula bones suggested that they could be used to monitor the marine environment over time. In this study, the number and size of the sea urchin used were limited, and future analyses of more sea urchin in the same region using this methodology may provide additional information on the ecotoxicological time-scale changes in the marine environment.

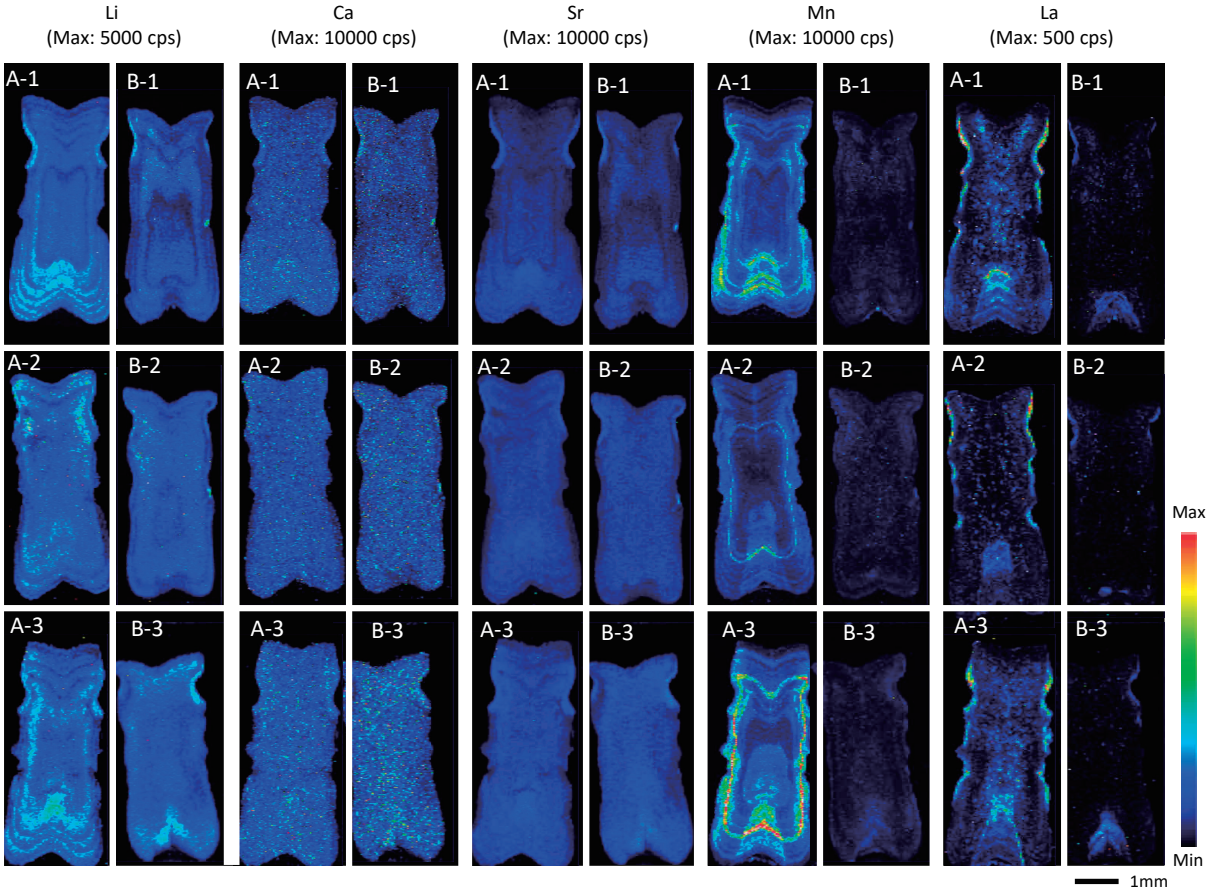

Fig. 3. **Distribution of inorganic elements in the rotula bones of *Strongylocentrotus intermedius***
each sea urchin sample was analyzed simultaneously using LA-ICP-MS. A-1, -2, and -3 are samples from Saroma (brackish water); B-1, -2, and -3 are samples from Yoichi (salt water). cps, count per second.

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