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ORIGINAL PAPER

Biological record of added manganese in seawater: a new efficient tool to mark in vivo growth lines in the oyster species *Crassostrea gigas*

Vincent Barbin · Karl Ramseyer · Mikael Elfman

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Abstract The biological response of increased manganese in seawater was tested experimentally with the oyster species *Crassostrea gigas* by adding, once per day, a fixed quantity of $MnCl_2$ to the container where the oysters were living. Uptake of Mn^{2+} in the shell was traced with cathodoluminescence and quantified with a high spatial resolution proton microprobe. The daily addition of $MnCl_2$ resulted in the visualization of distinct growth increments seen simultaneously in both the calcitic shell and the aragonitic ligament. A relation was observed between the addition of Mn^{2+} to the seawater and incorporation of Mn in the mineral part of the shell. Thus, addition of $MnCl_2$ to seawater is an efficient tool to mark in vivo growth increments in biomineralised carbonates.

Keywords Growth lines · Cathodoluminescence · PIXE · Biomineralisation · *Crassostrea gigas*

V. Barbin (✉)
IUFM of Rheims Champagne-Ardenne & URCA,
EA 3795, GEGENA, CREA,
2 esplanade Roland Garros, 51100 Rheims, France
e-mail: Vincent.barbin@univ-reims.fr

K. Ramseyer
Institute of Geological Sciences,
University of Bern, Baltzertstrasse 1+3,
3012 Bern, Switzerland

M. Elfman
Department of Nuclear Physics,
University of Lund, Sölvegatan 14,
Box 118, 221 00 Lund, Sweden

Introduction

Numerous studies (Rosenberg 1980; Barbin et al. 1991; Barbin 2000 and references therein; Rousseau et al. 2003; Cravo et al. 2004) have shown that during biomineralization, bivalve shells may take up trace elements. This incorporation depends not only on the concentration of these elements in water but also on ontogeny and environmental parameters, such as salinity, water temperature and nutrition. There are variations not only in the trace elemental take-up, but also the pattern of isotope variation through ontogeny may differ between seasonal banding and banding caused by secondary stressors such as salinity (Fred et al. 2000). Furthermore, biologically induced fractionation of $\delta^{40}Ca$ was observed in the aragonitic skeleton between cultured and open ocean tropical reef corals (Böhn et al. 2006). Thus, biomineralization depends on a multitude of environmental and ontogenic parameters which influence the uptake of trace elements and the fractionation of isotopes.

The growth of bivalve shells, i.e., biomineralization, (e.g., Lowenstam 1981; Dubois and Chen 1989; de Riclès and Livage 2004) takes place at the interface between the mantle of the animal and the inner shell surface, which is filled with an extrapallial fluid. The bicarbonate of the shell is derived from the external medium and metabolic CO_2. Carbonate saturation within the extrapallial fluid is attained either by a sufficient increase of the HCO_3^- concentration through transport by the outer mantle epithelium and/or by a sufficient decrease in the chelation of Ca^{2+} (Wilbur and Saleuddin 1983). Both processes highly correlate with the opening and closing of the mollusc valves which generates significant variations in the O_2, pH, calcium

and succinic acid concentrations in the extrapallial fluid (Lowenstam and Weiner 1989). Thus, environmental changes influence through the living conditions, i.e., ideal or stressful, the biochemistry of the bivalve shell.

Besides these external effects, chemical heterogeneity was observed between calcitic and/or aragonitic regions/layers of the oyster species *Crassostrea virginica* (Carriker et al. 1991) pointing to mineralogical/ontogenetic influences for trace element uptake during biomineralization. For example, manganese was found to be more concentrated in the prismatic than in the chalky and foliated microstructures in the calcitic part of the oyster shell (Carriker et al. 1991). Similarly, oysters have a higher concentration of manganese in the calcitic shell than in the aragonitic myostracum (Carriker et al. 1991).

The aim of our experiments was to test: (1) whether oysters take up Mn^{2+} added to the seawater, and incorporate it during the biomineralization of their shells, and (2) whether this technique is applicable as a time-marker to unravel the growth dynamics of bivalve shells. *C. gigas*, a living oyster species, was taken for these experiments as the shell's extrapallial cavity is directly connected with the surrounding seawater. Thus, environmental parameters, i.e., the chemistry of the seawater, should directly influence the chemistry of the growing shell. Moreover, oysters add an incremental layer to the shell each day, which allows a clear timing and quantification of shell growth. The choice of manganese as tracing element is based on the fact that manganese is (1) naturally incorporated in bivalve shells during growth (Barbin 2000), and (2) easily detectable by cathodoluminescence at trace concentrations as low as 10 ppm (Barbin 1997; Barbin et al. 1998; El Ali et al. 1993; Götze 2002). Furthermore, the cathodoluminescence characteristics of manganese in the crystal structure of the $CaCO_3$ polymorphs calcite and aragonite are well known, and differ strongly, so that their visual distinction is easy (e.g., Marshall 1988).

Experimental growth conditions and sample preparation

The experiments were carried out at the Institut Français de Recherche pour l'Exploitation de la MER (IFREMER) in Tremblade-Ronce les bains. Living cultured oysters of the species *C. gigas* with a diploid (sample D12) or triploid (sample T31) chromosome set were placed in a container filled with natural seawater for 50 days. For the first 20 days, salinity and temperature were kept at a constant a 33.2 ± 0.4‰ and 21.5 ± 0.5°C, respectively (Table 1). During this period, $MnCl_2$ was added daily, at a fixed time in the morning, so that the concentrations in the seawater increased from a starting value of 0.04 mg/l on the seventh day to 1.11 mg/l on the tenth day. After a two-day break (11th and 12th day) addition of $MnCl_2$ was continued with concentrations in the seawater

Table 1 Physico-chemical conditions during the 50 days of culturing specimen D12 and T31 in natural seawater

Date	Day	Temperature (°C)	Salinity (‰)	$MnCl_2$ (mg/l)
10/08/94	1	21.5	32.6	0
11/08/94	2	21.2	32.6	0
12/08/94	3	21.0	32.8	0
13/08/94	4	21.3	33.0	0
14/08/94	5	21.4	33.2	0
15/08/94	6	21.7	33.2	0
16/08/94	7	21.9	33.4	0.045
17/08/94	8	21.7	33.5	0.11
18/08/94	9	21.7	33.6	0.22
19/08/94	10	21.4	33.4	1.11
20/08/94	11	21.6	33.7	0
21/08/94	12	22.2	33.5	0
22/08/94	13	22.4	33.2	2.20
23/08/94	14	22.3	33.2	4.45
24/08/94	15	21.1	33.2	11.10
25/08/94	16	21.2	33.8	11.10
26/08/94	17	20.7	33.0	22.20
27/08/94	18	21.7	33.1	0
28/08/94	19	21.8	33.0	0
29/08/94	20	20.7	33.5	0
30/08/94	21	21.1	33.4	0
31/08/94	22	22.0	33.6	0
01/09/94	23	21.0	33.7	0
02/09/94	24	19.3	33.5	0
03/09/94	25	19.7	33.2	0
04/09/94	26	20.3	33.4	0
05/09/94	27	20.3	33.2	0
06/09/94	28	20.8	33.6	0
07/09/94	29	21.1	32.8	0
08/09/94	30	19.8	32.5	0
09/09/94	31	19.3	32.3	0
10/09/94	32	18.6	32.6	0
11/09/94	33	19.9	33.2	0
12/09/94	34	20.2	32.7	0
13/09/94	35	20.2	32.2	0
14/09/94	36	18.4	32.7	0
15/09/94	37	17.9	32.5	0
16/09/94	38	17.8	31.3	0
17/09/94	39	17.0	32.0	0
18/09/94	40	16.2	32.2	0
19/09/94	41	16.3	32.6	0
20/09/94	42	17.4	31.8	0
21/09/94	43	16.4	32.0	0
22/09/94	44	16.3	32.4	0
23/09/94	45	16.7	32.0	0
24/09/94	46	16.7	31.8	0
25/09/94	47	16.8	31.4	0
26/09/94	48	18.7	30.9	0
27/09/94	49	17.8	31.7	0
28/09/94	50	17.5	30.8	0

increasing from 2.22 mg/l on the 13th day to 22.23 mg/l on the 17th day (Table 1). After this period, the oysters lived for an additional 33 days in the same tank filled with seawater but without the addition of $MnCl_2$. Afterwards, the shells were separated from the soft tissue, embedded in epoxy and cut in half through the ligament and the umbo for the preparation of 30 μm thick, polished thin sections.

Analytical methods

Polished thin sections from the shells were examined under a cold cathode luminescence instrument from OPEA (France) mounted on a petrographic microscope (Olympus, BX-50). Observations were carried out at 15–20 keV accelerating voltage and at an operating current of 250–400 μA. Images were collected with a Sony DXC-930-P digital camera in time integration mode. A good quality of the photomicrographs was obtained by further stacking of single images (Witkowski et al. 2000). In addition, a high sensitivity hot cathode CL-microscope was used at an electron energy of 30 keV and 0.4 $\mu A/mm^2$ beam current density (Ramseyer et al. 1989). The CL spectra between 400 and 700 nm (1.5 nm resolution) were recorded from an area of 75×75 μm using a GATAN® MonoCL3 system equipped with a 1,200 grating and a HAMAMATSU® R374 photomultiplier, attached to a Zeiss® EVO50 scanning electron microscope. The spectra are corrected for the spectral response of the grating and the photomultiplier.

Qualitative analyses of the Mn content were carried out with the Nuclear Microprobe (PIXE) at Lund University (Malmquist et al. 1993). The PIXE (Johansson and Cambell 1988) analyses were performed with a 2.55 MeV proton beam of 1.5 μm diameter. X-rays generated in the carbon coated samples were collected using a 50 mm^2 Si(Li) detector (KEVEX®), and processed with the CAMAC data acquisition and beam control system (Elfman et al. 1997, 1999). The Mn concentration is given as the net peak-intensity of the Mn-Kα line.

Results

After living under controlled conditions (Table 1), e.g., salinity, temperature, day-night cycling, the oyster shells revealed under cathodoluminescence an older sequence of 4 and a younger one of 5 barely visible to weakly and strongly orange/green luminescing wobbly laminae with peak emissions at 615/548 nm, separated by dark to non-luminescing layers, respectively (Fig. 1b). The thickness of these couples, containing each a luminescent laminae and a non-luminescent layer, is ≈24 μm at the region of the ligament/umbo, and decreases towards the ventral side irrespective of their di- or triploid chromosomal set (Table 2, Fig. 1b, d). Furthermore, the distance between the two sequences is ~3 times larger than the thickness of a couple in both sequences (Table 2, Fig. 1d). In addition, all orange luminescing laminae cross over in the umbonal region from the calcitic shell into the aragonitic ligament (Stenzel 1962) without any displacement but by changing the luminescence colour from orange to green (Fig. 1b). In the umbonal region these luminescent laminae have a mean thickness of ≈12.5 μm in the younger sequence, and 4.3 μm in the older sequence, whereas in the ligament, the values are clearly lower, i.e., ≈3.3 and 1.8 μm, respectively (Table 2, Fig. 1b).

Comparison with the experimental set-up clearly indicates a one-to-one relationship between the nine orange/green luminescing laminae and the 9-times daily addition of $MnCl_2$ to the seawater in the tank. But surprisingly, the oysters incorporate preferentially this manganese shortly after addition of the $MnCl_2$ to the seawater, as reflected by daily couples of a thin luminescing laminae and a thicker non-luminescing layer (Table 3, Fig. 1d). Similarly, the low CL-intensity in the older sequence of four laminae corresponds well with the lower concentration of $MnCl_2$ added in the early sequence rather than in the later one (Table 1). Also, the three times broader zone between the sequence of four and five luminescent laminae correlates well with a growth of three days including one day with, and 2 days without $MnCl_2$ addition (Table 1).

Likewise, cultured oysters from the natural environment are characterized by dull, diffuse orange or greenish luminescence banding parallel to growth in the calcitic shell or the aragonitic ligament, respectively (Fig. 1f). As in the case of this study the luminescence bands cross over without any displacement at the calcite - aragonite boundary between the shell and the ligament. In the case of a crossover between the prismatic and the chalky structure of the shell the orange luminescing growth bands expand within the chalky part (Barbin et al. 1991; Fig. 1f, h).

Qualitative PIXE analyses of the Mn concentration were carried out along two profiles, crossing the younger sequence of five laminae (i.e., days 13–17, Table 1) close to the umbo and in the ligament, respectively. The results shown in Fig. 2 clearly indicate five well-defined peaks in both profiles with slightly lower intensities in the aragonitic ligament than

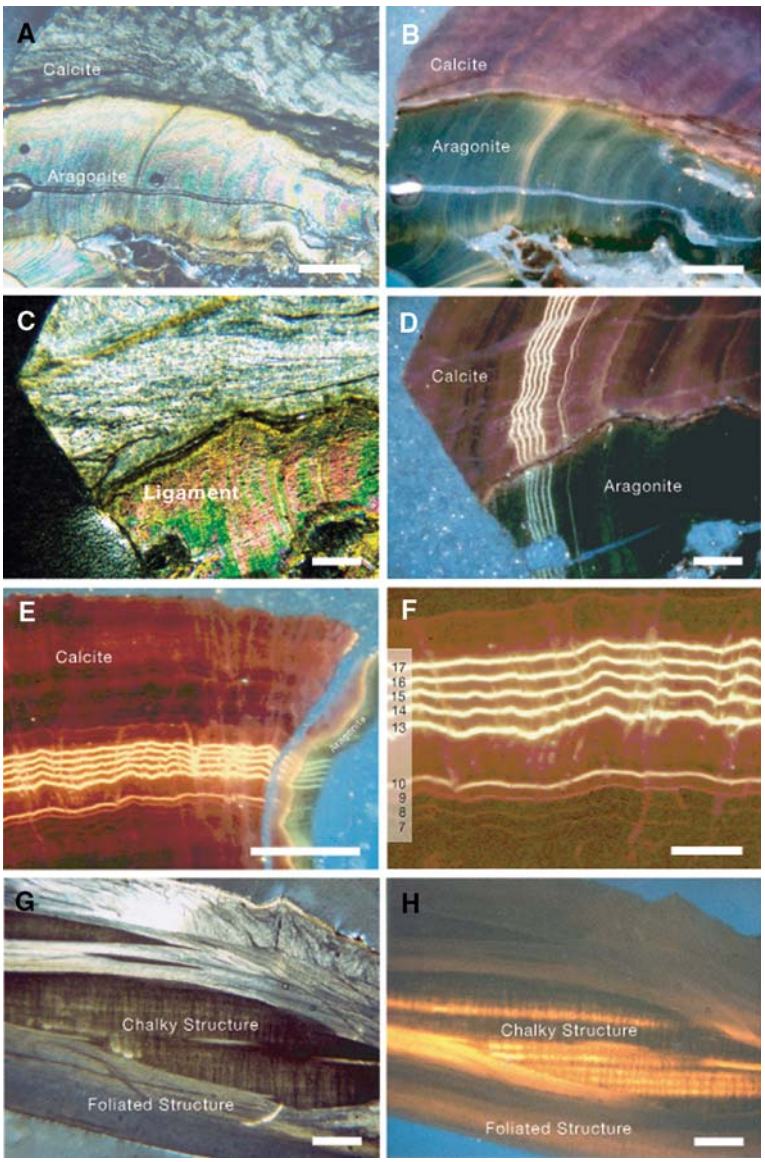

Fig. 1 Natural and experimentally generated growth bands in *Crassostrea gigas*. **a** Crossed-polarized light microphotograph of the region ligament-umbo from a cultured specimen in non-modified conditions. *Scale bar* is 500 μm. **b** Same field of view as **a**, but under cathodoluminescence. Note the fine laminae and broader bands of *orange* and *green* luminescence in the calcitic shell and aragonitic ligament, respectively. *Scale bar* is 500 μm. **c** Crossed-polarized light microphotograph of the region ligament-umbo in specimen D12 (for experimental conditions see Table 1). *Scale bar* is 200 μm. **d** Same field of view as **c**, but under cathodoluminescence. Note the younger sequence of five bright luminescing laminae and the third and fourth laminae of the older sequence which correspond with the 5-day and 4-day sequence of daily $MnCl_2$ addition to the seawater, respectively. In addition, the difference in the Cl-colour between the aragonitic ligament (i.e., *green*) and the calcitic shell (i.e., *orange*) is clearly visible. *Scale bar* is 200 μm. **e** Cathodoluminescence microphotograph of the region ligament-umbo in specimen T31 (for experimental conditions see Table 1). *Scale bar* is 500 μm. **f** Detail of the calcitic shell of specimen T31 shown in **e**. Note the five brightly luminescing bands which correspond with the second sequence of artificial Mn^{2+} addition from day 13 to day 17 and the four well to weakly visible laminae which correspond with the first sequence of artificial Mn^{2+} addition of day 7 to day 10 (Table 1). The gap between the two sequences equals the 2 days without any addition of manganese. *Scale bar* is 100 μm. **g** Crossed-polarized light microphotograph of the chalky and foliated structures in the calcitic shell of specimen T31 (for experimental conditions see Table 1). *Scale bar* is 500 μm. **h** Same field of view as **g**, but under cathodoluminescence. Note that the orange luminescing growth lines are expanded in the chalky part compared with the foliated part. *Scale bar* is 500 μm

in the calcitic shell. The intensities of the five peaks show a general increase from day 13 to 17, which is in accordance with the increase of the $MnCl_2$ added to the seawater in the tank. In the calcitic part of the shell the uptake of the manganese at day 10 could also be observed. Moreover, the distance between the peaks is

Table 2 Thickness of orange (calcite) and green (aragonite) luminescing laminae and dark/non-luminescing bands

Sample	Mineralogy	Younger sequence Laminae 1–5				Intermediate zone		Older sequence Laminae 1–4			
		Laminae (μm)	*n*	Couple (μm)	*n*	(μm)	*n*	Laminae (μm)	*n*	Couple (μm)	*n*
T31	Calcite	12.3 ± 3.0	20	23.6 ± 3.6	16	71.4 ± 5.9	3	4.4 ± 1.8	9	20.6 ± 2.0	9
	Aragonite	3.8 ± 1.0	5	19.5 ± 3.2	5	66.0	1			23.4 ± 0.8	2
D12	Calcite	13.0 ± 2.3	15	24.5 ± 3.5	12	83.2 ± 7.3	3	4.2 ± 1.7	6	21.2 ± 3.5	6
	Aragonite	2.9 ± 0.8	10	24.8 ± 2.8	8	84.0 ± 1.7	2	1.8 ± 0.8	2	24.0	1

Table 3 Percent of orange (calcite) and green (aragonite) luminescing laminae of daily couple

Sample	Mineralogy	Younger sequence Laminae 1–5 %Laminae of couple	Older sequence Laminae 1–4 %Laminae of couple
T31	Calcite	52 ± 21	21 ± 11
	Aragonite	20 ± 8	
D12	Calcite	53 ± 17	20 ± 11
	Aragonite	12 ± 5	8

for the calcitic shell 24.2 ± 1.3 μm and for the aragonitic ligament 23.6 ± 3.2 μm. These values are in excellent agreement with measurements obtained from cathodoluminescence microphotographs, where the couple thickness is ≈24 μm (Table 2).

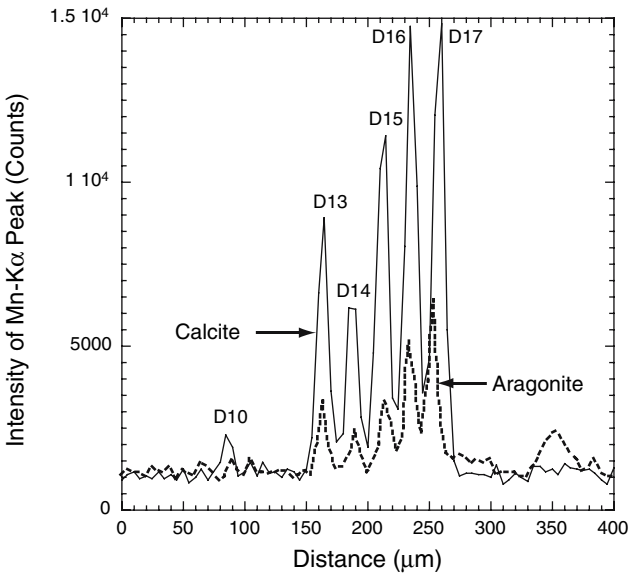

Fig. 2 Qualitative PIXE analyses of two profiles through the calcitic shell close to the umbo and the aragonitic ligament of sample D12 (Fig. 1**c**/**d**). The labelled peaks correspond to days 10–17 when increasing amounts of $MnCl_2$ were added to the seawater in the tank (for more details see Table 1)

Discussion

The experiments clearly demonstrate that addition of manganese to seawater in which oysters of the species *C. gigas* are living at fixed environmental conditions, e.g., temperature, salinity and day–night cycle, causes preferential uptake of this dissolved manganese into the oyster shell after its addition. Neither the mineralogy, aragonitic or calcitic, nor environmental conditions, e.g., salinity, temperature, day–night cycle, seem to play the primary control of this uptake as long as the oysters grow. It is the concentration of Mn^{2+} in the seawater which controls this uptake. These results support earlier studies by Carriker et al. (1980, 1991), who observed in the oyster species *C. virginica* a higher manganese concentration in all microstructural parts of oyster shells fed with a higher daily ratio of algae containing manganese. But only the shell material showed this difference, not the soft tissue of the oyster.

Furthermore, the intensity of the cathodoluminescence increases with manganese concentration in both the calcitic shell and the aragonitic ligament. This is supported by PIXE analyses, which show a general increase of the Mn-Kα peak intensity in the shell and the ligament in layers grown during increased Mn^{2+} concentration in the water. Thus, oysters incorporate manganese during biomineralization in their hard part, i.e., the shell and the ligament, depending primarily on its concentration in the water. This direct relation between manganese in the water and the shell is not so surprising, as the extrapallial cavity, where the shell growth is located, is directly connected with the surrounding water.

Similarly, a study of trace elemental partitioning between soft tissue and shell in the gastropod *Patella aspera* shows that the manganese concentration is related, and higher in the shell than in the soft tissue (Cravo et al. 2004). Higher manganese concentrations in the shells than in the soft tissues were also reported by Carriker et al. (1980) for oyster genera *C. virginica* and by Szefer et al. (2002) for the mussel *Mytilus edulis*. Cravo et al. (2004) state that these findings

''suggest that the mantle tissue takes up manganese directly from seawater for incorporation into the shell, with no involvement of the remaining soft tissue, and that this rate of uptake and accumulation is greater than that into the remaining soft tissues directly''.

A concentration as low as 0.04 mg/l $MnCl_2$ (≈0.017 mg/l Mn^{2+}) or about an 85 times increase above the mean seawater value (0.2 ppb, Nordstrom et al. 1979) is barely visible, but detectable by cathodoluminescence in both the calcitic shell and the aragonitic ligament (Fig. 1b, d). The experimental concentration range is clearly orders of magnitude higher than in ordinary seawater, but the fact that oysters are living in a littoral environment, where manganese might be introduced by fluvial input, diffusion from underlying sediment with anoxic pore water, during high energy events (e.g. storms, hurricanes) or from nearby hydrothermal sources, may cause higher than mean manganese concentrations in natural seawater. It was shown by Waldichuk (1974) that contamination of the marine water by sewage or fresh water increases the availability of Mn^{2+} which is then preferentially incorporated into the shell. This is confirmed by Cravo et al. (2004) who state that the bioavailability of manganese is higher in a contaminated estuarine site than in a clean coastal marine one. Such higher values were detected by cathodoluminescence in the aragonitic myostracum of oysters living in the Leucate pond in Eastern France (Barbin et al. 1991) and the cultured oyster shown in Fig. 1g, h.

Moreover, the preferred uptake of manganese from seawater is an excellent time-marker to constrain the growth dynamics of bivalve shells in a sub-daily time frame throughout all different shell structures (e.g., prismatic, chalky) and carbonate modifications (e.g., calcite, aragonite) without any visible impact on the growth dynamics. This latter fact is very important, as the proposed technique does not influence biomineralization and, thus, neither the studied objects.

Conclusions

Oysters of the species *C. gigas* with a di- or triploid chromosome set, living under controlled experimental conditions, e.g., temperature, salinity and day–night cycle, clearly demonstrate their ability to incorporate manganese during biomineralization of their carbonaceous (aragonitic or calcitic) shell. If manganese is added to their seawater, they will preferentially incorporate this manganese, forming thin laminae of higher concentrations throughout the whole growth surface irrespective of mineralogy (calcitic or aragonitic) or shell structure. The fixed environmental conditions used in the experiments demonstrate that the uptake of manganese is primarily controlled by the manganese concentration in the seawater. Thus, the distribution of manganese in oysters is a sensitive biological detecting system of increased manganese in seawater and/or environmental parameters. Moreover, this technique, i.e. adding manganese, is a very sensitive tool to study the growth dynamics of biomineralized material without having any impact on the growth itself.

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