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Study of metal bioaccumulation by nuclear microprobe analysis of algae fossils and living algae cells

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Abstract

Microscopic ion-beam analysis of palaeo-algae fossils and living green algae cells have been performed to study the metal bioaccumulation processes. The algae fossils, both single cellular and multicellular, are from the late Neoproterozonic (570 million years ago) ocean and perfectly preserved within a phosphorite formation. The biosorption of the rare earth element ions Nd^{3+} by the green algae species *euglena gracilis* was investigated with a comparison between the normal cells and immobilized ones. The new Leipzig Nanoprobe, LIPSION, was used to produce a proton beam with 2 μm size and 0.5 nA beam current for this study. PIXE and RBS techniques were used for analysis and imaging. The observation of small metal rich spores (< 10 μm) surrounding both of the fossils and the living cells proved the existence of some specific receptor sites which bind metal carrier ligands at the microbic surface. The bioaccumulation efficiency of neodymium by the algae cells was 10 times higher for immobilized algae cells. It confirms the fact that the algae immobilization is an useful technique to improve its metal bioaccumulation.

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1. Introduction

It has been known for some time that heavy metal ions are accumulated by microorganisms, such as bacteria, fungi and algae. The biosorption of microorganisms is a valuable means to remove metal ions from water [1]. The metals of interest for biosorption can be divided basically in two groups. One group comprises toxic metals that must be extracted from effluents due to their toxic actions on the environment [2]. The second group is constituted by noble metals. In the case of noble metals, their recovery from the solutions is interesting because of their strategical value [3]. The phenomenon of metal bioaccumulation was used

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by paleogeological scientists to explain the origin and evolution of some mineral deposits [4].

A good understanding of the biosorption processes facilitates the application of the procedure in the area of wastewater treatment and in the mining industry. However, the metal transport mechanism by the microorganisms is still not clear. A number of controversial possibilities of the biosorption processes have been reported. Some suggested that the metal ion removal was being effected by a simple process of adsorption onto the surface of algae [5–7]. However, many other facts in contrast to the behavior of surface adsorption have been observed [8–10]. These observations led to the conclusion that the biological activity of algae cells played an important role in the metal bioaccumulation [11]. The absorption occurred through interaction of metal ions with some functional groups either intracellular or on the exterior wall of the cell [12].

In order to characterize the location of the binding sites and the mechanism of metal ion transportation, further experiments are needed. The nuclear microprobe provides an ideal means for locating the sites of metal accumulation in microorganisms. It has been used to study nickel microscopic distributions in leaves of hyperaccumulator plants [13,14]. Its ability of microscopic analysis was further demonstrated by the measurements of single phytoplankton cells [15] and single microfossils [16].

The goal of this work is to study the metal bioaccumulation by measurements of metal distribution patterns in algae with the nuclear microprobe. Both, algae fossils and living algae cells were investigated. The algae fossils have single cellular or multicellular structures. The living green algae cells were treated in different ways. Because the accumulative quantities of the interesting metals are relatively low and the distribution pattern is in size of a cell, a nuclear microprobe with high spatial resolution and high analytical sensitivity is necessary for the microscopic investigation of the metal bioaccumulation. The Leipzig nanoprobe, LIPSION, consisting of a 2-stage MARC microbeamline MPU-3 and a dedicated Singletron^{TM} manufactured by HVEE was used to produce submicron beams of high energy ions with high beam current and excellent energy stability. A detailed description of LIPSION can be found in another contribution to this conference [17].

2. Metal distribution in algae fossils

2.1. Sample description

The algae fossils were recently discovered in Southern China [18]. They lived in the late Neoproterozoic (570 million years ago) ocean and were perfectly preserved within a phosphorite mineral formation. The algal thalli range from undifferentiated single cellular species to diverse multicellular algae. The discoidal parenchymatous single cellular thalli are morphologically simple (bottom right of Fig. 1). Spicules and filaments surrounding the cellular periphery were exquisitely preserved. The multicellular algae fossils are characterized by tissue differentiation and distinct reproductive structures similar to the *corposporangia* and *spermatangia* of living algae. The sizes of both algae species range from 200 to 800 μm.

The fossil samples were prepared by cutting the phosphorite in slices with a thickness of about 100 μm. The slices were then glued on a piece of glass. The surface of the samples was ground and polished to form a final thickness of 40 μm. It was not necessary to cover the samples with a conductive layer since the phosphorite matrix has good conductivity.

2.2. Measurements and results

A 200 μm object diaphragm and a 200 μm aperture diaphragm were selected to produce a focused proton beam with 2 μm size and 0.5 nA beam current. A pair of post scanning coils with 128 turns was used for a maximum scan size of 800 μm for the 2.25 MeV proton beam. PIXE and RBS techniques were used for the element analysis and for sample imaging. In order to reduce the intense X-ray counting rates caused by calcium in the matrix, a 16.5 mg/cm^2 aluminium filter and a 54 μm Mylar sheet were inserted in front of the X-ray detector. With the filter, the average X-ray

counting rate was below 1000 cps which is ideal for the two station list mode data acquisition.

The single cellular algae fossils were embeded in the phosphorite which has a high concentration of manganese in contrast with the low manganese concentration in the fossils. It formed very sharp boundaries between the mineral and the fossil in the element distribution map of manganese (top left of Fig. 1, the scan size of each map was 600 μm × 600 μm). Some other metal accumulative patterns were also presented in Fig. 1. It was obvious that the algal thallus absorbed a large amount of metals like barium, strontium, iron and calcium from its surroundings. The absorbed strontium seemed homogeneously distributed throughout the thallus. However, the barium ions penetrated the epidermic wall and were only preserved within the parenchyma of the thallus. There was a small amount of barium in the wall. Both the fossil and the mineral contained a high density

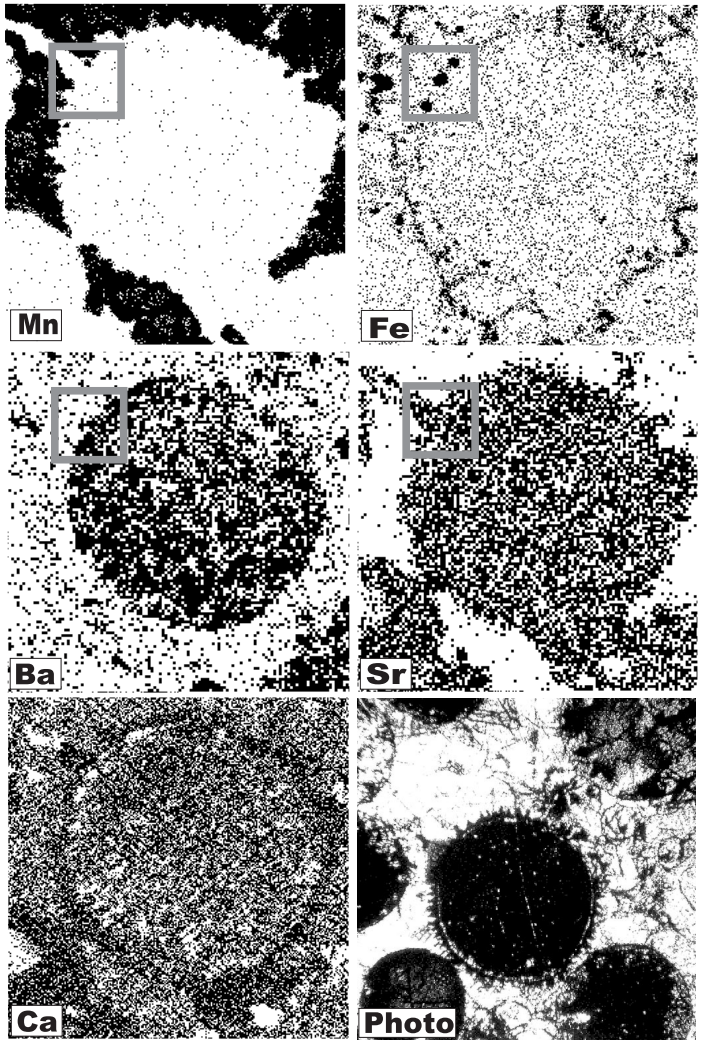

Fig. 1. Element distribution maps of a single cellular alga fossil in a 600 μm × 600 μm scan area. Three iron rich spores exist at the top left surface. A square in this region is marked for a zoom-in scanning area displayed in Fig. 2. The optical image of the thallus is presented in the bottom right picture.

of calcium, but more calcium was on the epidermic wall than inside the thallus. The iron distribution map in Fig. 1 showed an interesting pattern. The spiculate membrane of the discoidal thallus was rich with iron in forms of cystic spores. There were three iron rich spores on the upper left surface of the thallus. In order to have a close look of their location, we made another zoom-in-scan of 120 μm × 120 μm which is marked in Fig. 1. The corresponding element distribution maps in the zoom-in area are displayed in Fig. 2. It is evident by closely looking at the element maps that the iron rich cystic spores are located at the wall of the algal thallus. The iron transport in certain bacteria is well understood [19,20]. Some selective carrier ligands excreted from the cells scavenged the metal ions and formed extremely stable complexes with Fe^{3+}. These ligands bound to specific receptor sites at the cell surface. The observation of iron rich spores on the microfossil surface proved the existence of Fe^{3+} bound ligands in the cell membrane. The cystic spores containing high metal concentration were also found in the multicellular algae fossils.

3. Metal ion biosorption of living algae cells

3.1. Sample preparation

It is clear that the transport of elements by living organisms has influenced the established ecosystem of the earth surface for some billion years. Among the 92 elements in the periodic table, 30 have been found to be involved in the natural biosorption. However, following the industrialization, more elements, especially the rare earth elements, have participated in the ecologic circulation. In order to gain better understanding of the rare earth metal bioaccumulation, we selected the uptake of trivalent neodymium ions by the green algae species *euglena gracilis* 277 as our object of

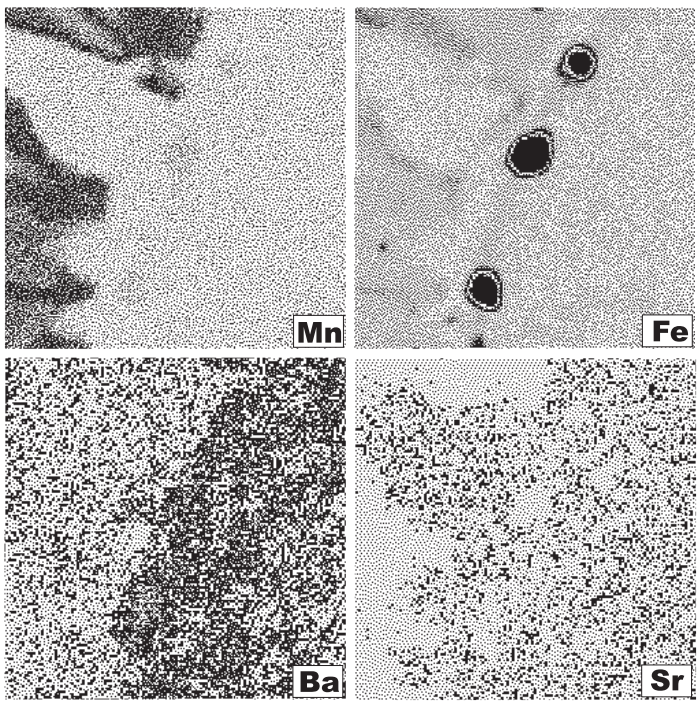

Fig. 2. The element distribution maps in the top left square of Fig. 1. The scan size is 120 μm × 120 μm. Iron rich spores are located at the thallus membrane.

investigation. The immobilization technique has been used to increase the efficiency of the metal bioaccumulation [10]. Thus, for comparison, some samples of the *gracilis* cells were immobilized before biosorption.

Cultivation. An inoculum of the *gracilis* cells was added to a suitable nutrients medium and agitated for three periods of 12 h illumination and 12 h darkness at room temperature. The cultured cells were harvested by centrifugation, washed three times with deionized water, dialyzed against the water and lyophilized for storage.

Immobilization. 20 ml of solution containing 25% glutaraldehyde ($CHO(CH_2)_3CHO$) was added to 2 g of the centrifuged cells. The mixture was then extensively agitated for 10 min. The immobilized *gracilis* cells thus formed were washed, centrifuged and stored for neodymium biosorption.

Biosorption. The *gracilis* cells were placed directly in contact with solutions containing 50 mg/l neodymium ions. After 30 min biosorption, the biomass was removed by centrifugation, washed and suspended in deionized water.

Freezedrying. A droplet of the cell suspention was put onto a very thin nylon foil which was attached to a stainless steel frame. The production of the thin foil was described in another contribution to this conference [21]. After 1 h drying in fresh air, the cells were killed and freezedried.

3.2. Measurements and results

The same experimental setup as before was used except the thickness of the aluminium absorption filter was reduced to 5.1 mg/cm^2, less than one third of the original one. The reason for that was because the *gracilis* cells were so small (< 10 μm) that they could be considered a thin target. The 2.25 MeV protons could fully penetrate the sample. Most of the *gracilis* cells were connected in chains, but there were some single isolated cells. 47 ordinary and 35 immobilized *gracilis* cells have been measured. The PIXE and RBS spectra of the cells were extracted from the raw scanning data according to their shapes. The intensity of neodymium L-X-rays was used to measure its concentration and to produce the element

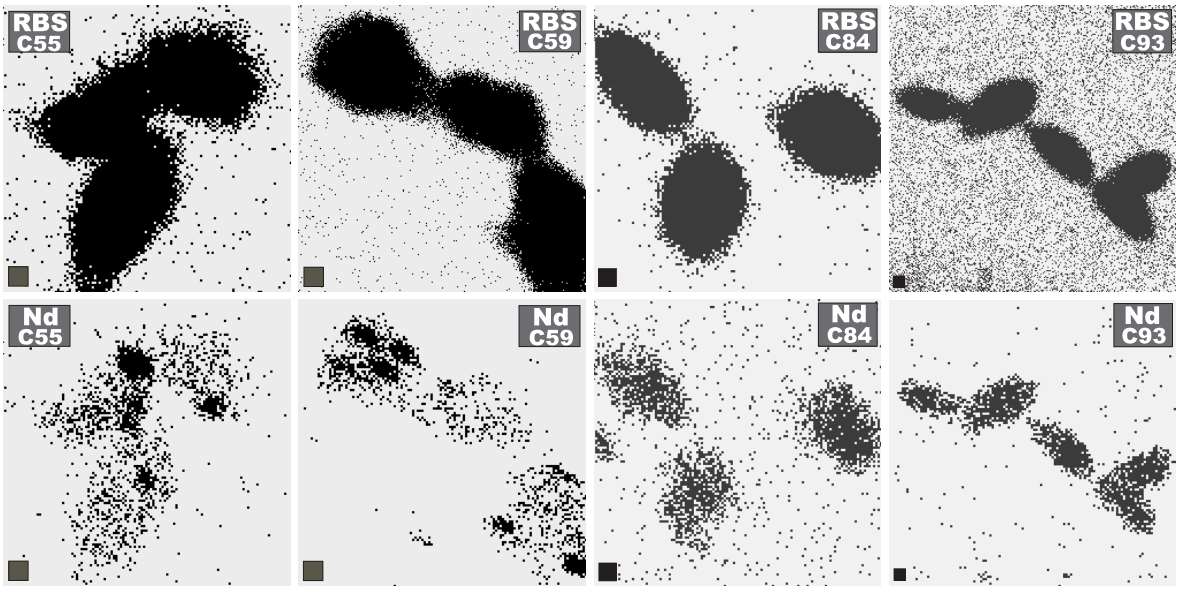

Fig. 3. Four examples of the Nd distribution patterns in the *gracilis* cells. Top row: RBS patterns. Bottom row: Nd PIXE maps. Sample C55 and C59 are ordinary cells, but sample C84 and C93 have been immobilized. A 2 μm × 2 μm square scale is attached at the bottom left corner of each map.

distribution map of the cells. The relative biomass of the cells could be estimated by their potassium contents. The semi-quantitative analytical program TTSPM [22] was used to calculate the concentration ratio of neodymium to potassium. The RBS yields in the cells were much higher than that of PIXE. Therefore, it was favourable for cell imaging.

Fig. 3 shows four examples of the neodymium distribution patterns in *gracilis* cells. Samples C55 and C59 are ordinary cells, but samples C84 and C93 have been immobilized before putting them into the neodymium solution. The distinct difference of the Nd maps between the two groups shows that the ordinary *gracilis* preserved less neodymium inside the cells but kept a high density of the metal in some cystic spores on their surface. We did not find neodymium rich spores in the immobilized *gracilis* cells. However, these cells could absorb much more neodymium which was homogenously distributed inside the cells. The concentration ratios of Nd^{3+} to K^{+} were 0.018 for ordinary cells and 0.26 for immobilized cells. It is apparent that the metal bioaccumulation of immobilized *gracilis* cells was ten times more efficient than that of ordinary ones.

4. Discussion and conclusion

The transport of metal ions both intercellular and extracellular is governed by a combination of biological, chemical, kinetic and thermodynamic processes [8]. The cell membrane is the initial barrier for metal ion uptake, but some specific channels and pumps in the membrane provide pathways for metal ions in or out. In the biological process, the metal ions can be carried and transferred by many different organic ligands, e.g., humic acids. The carrier ligands are often bound in some functional groups at the cell surface or inside the cells. Metabolic activity of living cells is responsible for the synthesis of the carrier ligands and the ion channels and pumps. Some metals are essential for microbial growth and division, but an excess of these elements can be toxic. Their equilibrium is established by the cellular immune system. Excessive metal ions can be excreted by metabolism of the microorganisms. However, the breakdown of the immune system occurs when their metabolic activities are stopped by the immobilization treatment. The metal accumulation in the algae cells is thus out of control. The bioaccumulation efficiency of the rare earth metal neodymium by the green algae species *euglena gracilis* can be ten times higher if the algae activity is stopped by immobilization.

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References

[1] J.M. Wood, H.K. Wang, Environ. Sci. Technol. 18 (1984) 106.
[2] P.O. Harris, G.J. Ramelow, Environ. Sci. Technol. 24 (1990) 220.
[3] D.W. Darnall, B. Greene, Environ. Sci. Technol. 20 (1986) 206.
[4] Z. Dai, C. Ren, Q. Zhao, P. Wang, F. Yang, Nucl. Instr. and Meth. B 104 (1995) 619.
[5] M. Tsezos, B. Volesky, Biotechnol. Bioeng. 23 (1981) 583.
[6] A. Nakajima, T. Horikoshi, T. Sakaguchi, J. Appl. Microbiol. Biotechnol. 16 (1982) 88.
[7] A.B. Norberg, H. Persson, Biotechnol. Bioeng. 26 (1984) 239.
[8] H. Siger, Metal Ions in Biological Systems, vol. 18, Marcel Dekker, New York, 1984, p. 223.
[9] B. Yang, I. Fang, M. Hao, Science Bulletin (in Chinese, abstract in English), 43 (1998) 7.
[10] A.C.A. Costa, S.G.F. Leite, Biotechnol. Lett. 13 (1991) 559.
[11] M. Hosea, B. Greene, Inorganica Chimica Acta 123 (1986) 161.
[12] R.H. Crist, K. Oherholser, N. Shank, M. Nguegen, Environ. Sci. Technol. 15 (1981) 1212.

[13] W. Przybylowicz, C. Pineda, V. Prozesky, J. Mesjasz-Przybylowicz, Nucl. Instr. and Meth. B 104 (1995) 176.
[14] U. Krämer, G. Grime, J. Smith, C. Hawes, A. Baker, Nucl. Instr. and Meth. B 130 (1997) 346.
[15] J. Pallon, M. Elfman, P. Kristiansson, K. Malmqvist, E.Granéli, A. Sellborn, in: Proceedings of Sixth International Conference on Nucl. Microprobe Technol. & Appl., Spier Estate, S. Africa, 11–16 October 1998.
[16] J. Zhu, C. Yang, R. Lu, K. Sheng, Y. Wang, N. Homman, K. Malmqvist, Nucl. Instr. and Meth. B 104 (1995) 402.
[17] T. Butz, R.-H. Flagmeyer, J. Heitmann, D.N. Jamieson, G.J.F. Legge, D. Lehmann, U. Reibetanz, T. Reinert, A. Saint, D. Spemann, R. Szymanski, W. Tröger, J. Vogt, J. Zhu, Nucl. Instr. and Meth. 161–163 (2000) 323.
[18] S. Xiao, Y. Zhang, A.H. Knoll, Nature 391 (1998) 553.
[19] J. Neilands, Chem. Scripta 21 (1983) 161.
[20] U. Förstner, G. Wittman, Metal Pollution in the Aquatic Environment, Springer, New York, 1979.
[21] J. Wang, P. Guo, X. Li, J. Zhu, T. Reinert, J. Heitmann, D. Spemann, J. Vogt, R. Flagmeyer, T. Butz, Nucl. Instr. and Meth. 161–163 (2000) 830.
[22] X. Wu, J. Zhu, R. Lu, F. Yang, Nucl. Instr. and Meth. B 104 (1995) 196.