Culture experiments to better understand biomineralization under varying geochemical conditions
--Zombie factory in Japan: the first trial on Scandinavian foraminifera--

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Ocean acidification is a consequence of ongoing global climate change, and it may have severe impacts on calcifying organisms. This process may be amplified in coastal regions where erosion, land run-off and eutrophication contribute in lowering the pH.
The aim of our study is to define how bottom-dwelling foraminifera will respond to pH changes in the Skagerrak/Baltic Sea region in addition to other environmental stressors such as temperature and salinity changes.
First results demonstrate variations in shell preservation of the living foraminiferal fauna. The more marine foraminifera have pristine shells, while the low salinity Baltic ones are more or less dissolved and only inner organic linings are still visible. However, these “zombie” foraminifera are still alive, as determined from the CellTracker Green labelling. The dissolution can be linked to the lower pH in the Baltic.
In order to investigate the zombie foraminifera further, we set up culture experiments on healthy foraminifera under controlled geochemical parameters. We discovered that lowering the pH is not sufficient to create zombie foraminifera and that abrasion between foraminifera and sediment is probably involved in the shell loss. Those results highlight the multiple factors affecting the balance of benthic ecosystems subjected to environmental stressors.

Keywords: benthic foraminifera, ocean acidification, laboratory experiment

Approach to comprehensive analyses of molecular phylogeny, morphometrics, and geochemistry of planktonic foraminifera

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Planktonic foraminifers have been widely used for the studies of paleontology and paleoceanography. Stable oxygen and carbon isotope ratios of planktonic foraminiferal shells are the major proxies for paleo-environment, as these isotopes are affected by inhabiting water temperature and the chemical components. Most of previous studies have been conducted with the traditional species concept based on the morphological differences of their calcareous shells. However, molecular phylogenetic studies have unveiled the presences of multiple cryptic species in a single morphospecies of planktonic foraminifers. Such a high diversity of planktonic foraminifers suggests that the current paleoceanographic proxies are underestimated due to the mixed information of multiple biological species. Therefore, it is the urgent task to re-assess the ecological and geochemical characters at each biological species. For this purpose, we need to work out the implementation method, which combines the multiple analyses: DNA, morphological, and geochemical analyses, for a single individual. The DNA extraction method by using the buffer based on the guanidium isothiocyanate now enables us to preserve the calcareous shells after the extraction. By using this method, we can detect the morphological and geochemical characters on a same individual, which is identified by the molecular technique. However, the thermal and chemical reactions of this DNA extraction method to the calcareous shells are still unknown. In this study, we test whether or not the method of the molecular experiment physically and chemically damage the calcareous shells. We collected the living specimens of planktonic foraminifers and divided them into three experiment sets. In the first set, the specimens were applied to the DNA extraction with incubation process at 70 °C for 40 minutes as usual. In the second set, the time for incubation was three times longer than the first one. Through the comparison between these two patterns, the effect of the incubation time to the calcareous shells can be detected. We also prepared the specimens just collected from the sea-water but without the process for the DNA extraction, as a control. The densities of the calcareous shells were measured by the micro-focus CT scanning, and then their stable oxygen and carbon isotopes were analyzed one by one. The comparisons of physical and geochemical characters of the planktonic foraminiferal shells showed that the heat and chemical treatments concerning the DNA extraction never changed the shell component. Thus, we succeed to establish the experimental methods, which investigate the morphological and geochemical features for each biological species from single individual.

Keywords: planktonic foraminifers, Molecular phylogeny, micro-focus CT scan, Stable isotopes

A study on chemical composition of living acantharian (Radiolaria) shell

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Radiolaria is one of marine zooplanktons having skeletons/shell of opal (SiO_2+nH_2O) and/or celestite ($SrSO_4$), ranging from Cambrian to Recent. Living Radiolaria is divided into 5 Orders, Collodaria, Nassellaria, Spumellaria, Acantharia, and Taxopodia. The first three orders, have siliceous skeletons and then become to be microfossils in pelagic sediments. While Acantharia has a skeleton consisting of celestite, and thereby this order group cannot become fossils. In this study, we have conducted radiolarian culturing in laboratory at Ehime University for direct observation of their skeletal growth system. The living radiolarian specimens were obtained from surface waters of warm Kuroshio Current near off Kashiwajima Island, Kochi Prefecture, Shikoku Island, Japan. The detection of additional growth on skeletons was used a fluorescent compound following the method by Ogane et al. (2009, 2010). Through this direct-observation study, a possibility that acantharian shells contain siliceous compound was suggested. We therefore focus on to present the data of living Acantharia obtained in the poster.
Living acantharian cells were collected from surface waters (ca. <5-m depth) on 12the July, 30thh November,2015 and 11th January, 2016 by using a plankton net (85 micron meters mesh). We picked up individual cells from sample materials at Kashiwajima, and then performed on culturing experiments for radiolarian cells at a laboratory of Ehime University. The samples were incubated under an artificial day-night cycle (12hours day/12hours night) with white and blue LED lights at 27dgree Celsius.
After 24hours when we left radiolarian cells within incubators, we added a solution including fluorescent compound called HCK-123 into culturing dishes (final concentration ca. 0.5-1 micron mole). After 24-30 hours culturing, we mounted slides for observation with a confocal laser scanning microscope (Carl Zeiss LSM510) at Department of Biology, Faculty of Science of Ehime University. Some fluorescent compounds such as PDMPO and HCK-123 have been used one of the tracers for biological silicification (ex. Shimizu et al., 2001; Desclés et al., 2007; Ogane et al. 2010), which can stained newly formed siliceous skeletons of microplanktons because these agents are incorporated in siliceous depositions during biogenic mineralization. Therefore we can detect Silicon distributions in additional parts in siliceous shells. We also conducted FE-SEM and WDS analyses to check Si-distribution in the acantharian shells.
[Results]
Four specimens of Acantharia, identified, *Acanthometra muelleri*, *Amphilonche complanata, Acanthostaurus conacanthus* and *Acanthometron pellucidum*, among culturing radiolarian specimens clearly emitted fluorescence using by a fluorescent compound HCK-123 labeling. Each specimen shows fluorescence emission at the following parts of living cells: surface of spines and central soft parts for *A. muelleri*, proximal parts of spines and centrals for *A. complanata*, proximal surface parts of spines for *A. conacanthus*, and broken spines including its tip parts for *A. pellucidum.* WDS analysis was also performed on *A. complanata* shell for distribution mapping of Sr, S, O and Si contents. Sr, S and O, which are considered as major compositions of acantharian shells ($SrSO_4$), occurred from whole skeletal part. Si signals were also detected from a tip and surface of spines. Considering these results from our culturing experimental works and WDS analyses on living Acantharia above-mentioned, it is revealed that living acantharian cells contain Silica, in

particular on an active growth part of skeletons. This fact suggests that SiO_2 is one of the important components on skeletal forming of Acantharia.

Keywords: Radiolaria, Acantharia, skeletal composition

Effects of ocean acidification on shell and somatic growth, and stable isotopes of shell carbonate of two species of abalones

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Ocean acidification is now one of the important issue to appreciate the impact on marine calcifiers (IPCC, 2013), and potentially affects their survival, calcification, growth, physiology and development. To evaluate the effects of elevated CO_2 levels on shell and somatic growth, and stable isotope compositions of two species of abalones (*Haliotis discus discus*, *Haliotis gigantea*), we conducted culture experiments at three treatment levels of seawater pCO_2 (400, 750, and 1200 µatm), at approximately 23 °C.

The effects of seawater pH on calcification (shell width, shell weight) was non-significant in both species. On the other hand, the positive relationships between pH and wet weight of soft tissue of two species were observed. Their adjusted wet weight of soft tissue at 1200 µatm was significantly greater than that at 400 µatm. These results suggest that elevated pCO_2 affected their metabolism (e.g. higher metabolic rates to maintain homeostasis).

Stable oxygen isotope compositions of outer (calcite) and inner (aragonite) shell layers of two species showed non-significant relationships with pH. The negative correlations between carbon isotope compositions and pH of both layers appeared in both species, and the slopes of these relationships of shells were lower than that of dissolved inorganic carbon (DIC) of seawater. We estimated the equilibrium values of carbon isotope compositions at each pCO_2 treatment, and the difference between the carbon isotope compositions of shell and equilibrium values showed gradual increases in shell carbon isotope compositions with decreasing pH. Thus, the pCO_2-induced change in metabolism of abalones might appear in carbon isotope compositions of shells as the metabolic effect.

Keywords: ocean acidification, culture experiment, stable isotopes, abalone, metabolism, biomineralization

