Culturing protocol and maintenance for
living calcareous plankton
~ Preliminary results of the culturing experiment ~

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We started the cultivation experiment of marine calcareous plankton (planktonic foraminifera and coccolithophorids) at the Mutsu Institute for Oceanography (MIO). Living samples are collected from the Tsugaru Strait, Northern part of Japan every month by plankton towing and water sampling. We have equipped the incubators and have created protocols for continuous culturing of living specimen. For 4 months, we have succeeded in long-time cultivation of planktonic foraminifera in the culture vessels and have observed chamber formation, feeding process, and gametogenesis.

Keywords: planktonic foraminifera, coccolithophorids, culture, ecology, gametogenesis

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1. Introduction

Planktonic foraminifera and coccolithophores, a group of free-floating marine protista secreted calcareous hard skeletons. Their morphology and chemistry are sensitive to changes ocean surface environmental changes. Their shells settle and deposit quickly in the sediment after they died, therefore they are widely used for past-environmental investigations. In spite of its importance, the ecology of them was not well understood. Especially the study of ecology of living cold water species are less. We then started the collection of calcareous plankton from the seawater and cultivation in the laboratory. In general, plankton are living from surface to great depth in their life, therefore it is not feasible to observe *in situ* life styles. However we should emphasize that culture in the laboratory is still only method to come close to their ecology. The aims of this program are 1) to clarify the ecology of calcareous plankton from the laboratory experiment, 2) to reveal the relationships between shell morphology and environments, and 3) to evaluate of the new tracer for paleoceanography from chemical analysis of the shells.

Living specimens were collected in every month from surface water in the Tsugaru Strait, northernmost part of Honshu, Japan (41°28.8′N, 141°14.2′E, Fig. 1). This area is locate in the mixing zone of the Tsugaru warm current and cold Oyashio component water, therefore it is suitable for investigation of the ecology of cold water species, as well as the warm water ones. As the first step, it is needed to establish the culturing protocol for keeping calcareous plankton alive in culture vessels. We noted here the culture methods and showed preliminary results.

2. Equipments and preparations for cultivation

2.1. Equipments for collection

1. Plankton net and sample bottles
   45cm NORPAC flame with 1 m length plankton net. Mesh sizes are used in 100 μm (XX13).
2. Plastic Bottles (for seawater sampling)
   Cleaned high density polyethylene (HDPE) bottles are better because the background concentration of trace metal contamination in HDPE is lower. We used 9 litter Nargen bottles.
3. Thermometer and Salinometer
   For the purpose of determination of culturing environment, basic environmental information are needed. *In situ* temperature and salinity (Conductivity) are measured by CT sensor. CT sensor we used is compact-CT (Alec electronics, co., ltd.), which is equipped thermistor and inductive cell. Accuracies are ±0.05°C for temperature and ±0.05mS/cm for conductivity, respectively. Depth sensor is also needed if available. We put it at the mouth of the flame and drift with plankton net. After coming back to laboratory, we connect it to desktop PC and read data from memory pack.
4. Cool box and cooler packs
5. GPS (Global Positioning System) sensor
   GPS sensor is needed to note sampling location and revisit.

2.2. Items for culture (Fig. 2)

1. Culture vessels (glass vials)
   For the observation of living specimen, we usually use inverted microscope. Therefore bottom glass of vessels should be homogeneous and flat. We are using a circular cylinder which is custom-made glass vials (7cm height, 2.5cm diameter, ca. 20ml). Height and diameter of culture vessel should be adjusted to observing environment in each laboratory. Living planktonic foraminifera often float in the culture vessel, so low-height flat glass petri dishes are unsuitable.
2. Water bath
   Low height plastic container is better.
3. Water circular with heater and thermostat
   We are using water circular with thermostat named...
“Thermal Robo” (AsOne). This water circular can set temperature in ± 0.1°C accuracy.

(4) Chiller (cooler)
Chiller is throw-in type cooling coil unit. This should be put into water bath with water circular.

(5) Fluorescent light
For symbionts-bearing species, illumination is required because almost symbionts need light for photosynthesis. We put a illumination light (10 W) on the culture bath, and turn on/off with 12 hour cycle using automatic timer. It should be careful with the distance from the light to water surface because the heat of the light would affect the temperature of culture vessels.

(6) Incubator
Multi-tire incubator (MTI-202B, EYELA) is used for cultivation of coccolithophorids and diatoms.

3. Collection and culture method
Collection and culture methods for planktonic foraminifera were presented by Hemleben1 and Anderson2 in detail. In this study, we revised their methods based on our objectives. We note here the revised methods and some tips for collecting flesher samples.

Starting before the cultivation, all glassware and plasticware for the isolation of living specimens should be sterilized by autoclave at 121°C, 15 min under pressure before using. Disinfected glass and plasticwares are stocked in sterilized stainless box at the dried area.

3.1. Collection of planktonic foraminifera
In general, SCUBA collection is common and best method for the study of planktonic foraminiferal culture (e.g. Bé et al., 1977), however the plankton towing is also useful if there are less damages to the living specimens. The boat for our sample collection is “Wakashio” (weight: 2.8 ton), which belongs to fishery association of Ohata town (Fig. 3). Sampling point is located at off Shimokita peninsula (Fig. 1), at a distance of 5 mile from MIO (Sekinehama) and water depth is ca.230m.
A place which enables us to come back within 30 min. to the lab. To collect the samples which held damage to a minimum, we conduct following procedure.
1) The plankton net is deployed from a gently drifting boat. The plankton net should be lowered on the windward.

2) Less than approximately 5 min. is better for towing because the excessive aggregation of plankton would weaken the planktonic foraminifera.

3) After towing, only collect suspended materials and water in the collection bottle. Never collect the remnant materials on the side of plankton net because attached specimens would be damaged.

4) When the collection is finished, sample bottles are stocked in cooler box and brought back to the laboratory immediately.

5) When reaching the laboratory, sample bottles should be placed quietly for several minutes. This process allows the settling down of the planktonic foraminifera to the bottom of sample bottle. In this time, collected natural seawater is filtrated by 0.2 μm nuclopore filter and is put into culture vessels. Filtrated seawater should be poured 80% of culture vessel. The supernatant of sampling bottles should be dumped because almost planktonic foraminifera is always sitting on the bottom of the bottle. The remnant of the samples should be diluted immediately with filtrated seawater to prevent aggregation of particles.

6) Pick up living planktonic foraminifera by pasteur pipette. This transfer process should be done within 2 hours. Picked foraminifera passed more than 3 hours are almost damaged and weakened, therefore it is unsuitable for cultivation experiment. One picked planktonic foraminifer should be put into a culture vessel. This is important for easily identification between culture vessel and cultured specimen and for prevent cannibalization between planktonic foraminifera. The mouth of the culture vessel should be wrapped tightly by labofilm to restrict vaporization of seawater.

7) Culture vessels are stocked in the temperature-controlled water bath (or incubator).

3.2. Water sampling and isolation technique of coccolithophorids

Coccolithophorids are collected from surface water. We collected water sample using a bucket and put into 9 litter HDPE bottles. At the Tsugaru Starit, the isolation of coccolithophorids from huge amount of diatoms are important because the growing of coccolithophorids are disturbed with that of diatoms. At first, collected seawater is filtrated using 20 μm nylon or stainless mesh and is removed large-sized zooplankton and phytoplankton. Pre-filtrated seawater is weakly filtrated using 0.8 μm membrane filter immediately. At this point, concentrated liquid should be remained in the funnell (ca. ~ 50ml). Concentrated seawater is centrifuged by the gravity separator. Rotation speed of gravity separator should be set on ca. 3 min./300 rpm. After the centrifuge, suck up liquid of 2 ~ 3ml immediately from the bottom of the glass cell quietly and put into petri dish. Looking for a single cell of coccolithophorid under the inverted microscope and transfer the single cell into cultivation medium in the culture vessel. The medium we used is f50, diluted from Guillard’s f/2 marine water enrichment solution. GeO₂ powder is added to the medium to restrict the growing of diatoms. Repeat this picking process and stock samples in the incubator.

4. RESULTS

4.1. Assemblage of planktonic foraminifera

Since now, we collected following planktonic foraminifera (Fig. 4):

- Neogloboquadrina pachyderma (Ehrenberg) sinistral and dextral coiling
- Globogerina bulloides d’Orbigny
- Globigerina quinqueloba (Natland)

The former Neogloboquadrina is non-spinose species and the latter Globigerina are spinose one.

It is known that these planktonic foraminifera live in temperate to cold water in the world ocean. Above species are main component of planktonic foraminifera in the Tsugaru Strait and appear through the year. In March, the most dominant species was N. pachyderma right coiling. In this month, spring bloom was beginning but remained the water structure of winter season. In April and May, the season of spring bloom was characterized the dominant of G. quinqueloba. In June, N. pachyderma right coiling was dominant. G. bulloides seldom occurred except for March. At this time, it is difficult to interpret a causal relationship between assemblages, water temperature and salinity because these results are snapshot of each seasons. But it would likely that there is no relationship between them at this time (Table 1).
Fig. 4 Living calcareous plankton collected from the Tsugaru Strait.
a–c: planktonic foraminifera, d–g: coccolithophorids
a: Neogloboquadrina pachyderma, x200
b: Globigerina bulloides, x100
c: Globigerina quinqueloba, x100
d: Emiliania huxleyi, x9,000
e: Gephyrocapsa oceanica, x7,500
f: Coccolithus pelagicus, x5,500
g: Braarudosphaera bigelowii, x5,000
4.2. Feedings and growth

It is considered that planktonic foraminifera are generally omnivorous. But juvenile and some non-spinose planktonic foraminifera consume algal prey (Anderson et al., 1979; Lee et al, 1991). Just after living samples collected, N. pachyderma right coiling were almost attaching algae onto their surface shell. We never observed catching zooplankton of neogloboquadrinids just after collection since now. It is considered that probably they might be preying phytoplankton in the natural condition. In our laboratory, we routinely supply diatoms for feed (Thalassiosira pacifica and Coscinodiscus sp. etc.) which was cultured in the laboratory. As the need arises, each strains are added into culture vessels. Many N. pachyderma were successfully growing up and created chambers in culture vessels. On the other hand, in general, spinose species is animal prey (e.g. Anderson and Bé, 1976; Bé et al., 1977). Therefore we give Artemia salina (nauplii) that just after hatching to G. bulloides. Artemia is sucked up by Pasteur pipette and released just over the G. bulloides. Healthy G. bulloides can capture Artemia easily because surface of their spines is sticky. In this procedure, all G. bulloides successfully grew up and reproduced (gametogenesis). This culture method of G. bulloides would be better for growing their shells.

The feedings of G. quinqueloba are enigmatic. In spite of spinose species, G. quinqueloba never caught the motile zooplankton and algae in the laboratory. The rhizopodia of G. quinqueloba looks like less sticky than that of G. bulloides. Almost G. quinqueloba in the 0.2 μm-filtered seawater showed shorter lifetime. However in no-treated natural seawater, their cytoplasm increased and survival time extended clearly. At present, we suppose that G. quinqueloba would be particulate organic materials and/or bacterial feeder. However, little is known about the possible role of bacteria in the diets of planktonic foraminifera.

Survival time of each species was shown in Fig. 5. N. pachyderma fed diatoms every day and almost N. pachyderma finished their life time within 2 months. However, several N. pachyderma lived more than 3 months in culture vessel without gametogenesis. As mentioned above, G. quinqueloba did not capture algae and zooplankton and they almost starved. It should be important to establish the method supplying prey for G. quinqueloba. G. bulloides fed Artemia nauplii in 3 – 5 days and lived more than 1 month.

4.3. Chamber Formation

Chamber formations were frequently observed in the culture vessels, especially in N. pachyderma and G. bulloides. The process of chamber formation is obvious under the microscope (Fig. 6) and the process is basically same with that of deeper living non-spinose species (e.g. Bé et al., 1979; Hemleben, et al., 1989). Chamber

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**Table 1** Variations of temperature, salinity, and foraminiferal assemblages in the Tsugaru Strait from March to June, 2003.

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<tr>
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<th>March</th>
<th>April</th>
<th>May</th>
<th>June</th>
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<tbody>
<tr>
<td>temperature (°C)</td>
<td>4.70</td>
<td>9.09</td>
<td>9.96</td>
<td>12.8</td>
</tr>
<tr>
<td>salinity (psu)</td>
<td>32.32</td>
<td>33.70</td>
<td>33.03</td>
<td>33.72</td>
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</table>

<table>
<thead>
<tr>
<th>Foraminifera</th>
<th>%</th>
<th>%</th>
<th>%</th>
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<tbody>
<tr>
<td>N. pachyderma</td>
<td>66.7</td>
<td>26.7</td>
<td>22.4</td>
</tr>
<tr>
<td>G. quinqueloba</td>
<td>19.4</td>
<td>71.1</td>
<td>72.4</td>
</tr>
<tr>
<td>G. bulloides</td>
<td>13.9</td>
<td>2.2</td>
<td>5.2</td>
</tr>
</tbody>
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![Diagram of survival time in each species from March to May, 2003. (cultivated at 4.7–10.0°C condition).](chart.png)
The relationships with survival time and shell length of *N. pachyderma* and *G. bulloides* were shown in Fig. 8. Shell growth (chamber formation) rate in *N. pachyderma* is gentle (1.25 ~ 3.75 μm par day in average) and shows like "plateau" that keeps shell length constantly. Moreover, growth rates and intervals of shell forming are different with each specimen. On the other hand, growth rate of *G. bulloides* shows linear (8.75 μm par day in average) and is almost same in each cultivated temperatures. These differences in each species would be related with the difference of metabolism, feeding interval, and kind of food.

formation of *N. pachyderma* generally completes within a half day. But some *N. pachyderma* often created aberrant (abnormal) chamber (Fig. 7). Such abnormal shells are seldom occurred in the natural condition. The cause of this phenomenon is not understood yet, but some stressful environment (e.g. water condition, nutrition, light intensity etc.) would be affected shell formation. Hemleben (personal comm.) reported that *Orbulina universa* formed abnormal shell in culture condition when feeding (*Artemia*) continued every day. In our experiment, *N. pachyderma* which attached a lot of diatoms around the shell created abnormal chambers. It is suggestive that creating of irregular chamber has any relationships with nutrition.
4.4. Gametogenesis

Some *G. bulloides* cultured in our laboratory successfully released gametes (swarmers). Just prior to reproduction, *G. bulloides* shed own spines. After that, several thousand of gametes are released with cytoplasmic fragments (Fig. 9). Gametes had a flagella on their cell and were spinning very quickly. In several hours, gametes dispersed in culture vessel and some of them began to die at the same time. The host (parent) almost die when they released swarmers, but some host lived continually in several days. Approximately 1 ~ 2 weeks later, all gametes died. We have not ever observed syngamy (fusion of swarmers) in this experiment. Ketten and Edmond (1979)⁸ have reported the zygote (proloculi) of *Hastigerina pelagica* in the culture bath, but it
has never replicated in the laboratory experiment. At this time, it is difficult to maintain the life of swarmers. We could never observed gametogenesis for *N. pachyderma* and *G. quinqueloba*.

### 4.5. Coccolithophorids

Coccolithophorids we found from surface seawater in the Tsugaru Strait are: (Fig. 4)

- *Emiliania huxleyi* (Lohman)
- *Gephyrocapsa oceanica* Kamptner
- *Coccolithus pelagicus* (Wallich)
- *Braarudosphaera bigelowii* (Gram and Braarud)
- *Syracosphaera* spp.

Isolation of single cell of coccolithophorid failed several times in this experiment because of the rare coccolithophorids in spring season in the Tsugaru Strait. At present, We only succeeded in isolating *Gephyrocapsa oceanica* in our laboratory.

### 5. Conclusions and future works

In this study, we created cultivation methods for observing ecology of calcareous plankton and growing. On this procedure, creating chambers and growing of planktonic foraminifera succeeded in the laboratory. *G. bulloides*, a spinose and zooplankton feeder, are grow up quickly and its lifetime is approximately one month. Moreover gametogenesis are observed in all cultured *G. bulloides*. *N. pachyderma*, a non-spinose and phytoplankton feeder, grow slowly and has relatively longer lifetime (approximately two months). Occasionally created aberrant chambers. The ecology for *G. quinqueloba*, a spinose species are still unclear. If more stable continuous culturing is possible, the studies of morphology, shell chemistry of foraminifera and relationships with environmental factor will be advanced. But continuous cultivation of plankton are still difficult and repeating trial and error. Our culturing protocols also under construction and are frequently revised. Now we are trying to monitor of other environmental factors in the culture vessels (e.g. pH, dissolved oxygen, nutrients, etc.). Further trial are needed for the establish the cultivation protocols of plankton.

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### Appendix: Observation system for living plankton of MIO (Fig. 10).

![Fig. 10 Observation and non-linear editing system of digital images for living plankton in MIO.](image)

1) Inverted microscope (IX70, Olympus), 2) binocular microscope (SZX12, Olympus), 3) HR 15 inch analog monitor (Sony), 4) Color video recording system (CS900, Olympus), 5) 2.1 Mega-Pixel digital microscope (VH8000, Keyence), 6) A/D converter (ADVC-10, Canopus), 7) Digital image editing system (PowerMac G4,1.25GHz dual processor; Final Cut Pro 3, Apple).

### References

1. Hemleben, C., Spindler, M. and Anderson, O. R.
8. Ketten, D. R. and Edmond, J. M.

(Manuscript received 15 August 2003)