**Biodiversity and abundance of bacteria and nanoflagellates in the Kuroshio Region**

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In the marine food web, bacteria and their main consumers, nanoflagellates (NF), are the two critical trophic levels of the microbial loop, which is called a loop because the energy and nutrients transferred from bacteria to nanoflagellate might cycle back to bacteria and not being transferred to small size copepods (e.g. nauplii and copepodites) at higher trophic levels, i.e. the classic food chain. Consequently, the microbial loop plays an important role for energy transfer and biochemical cycling in the marine food web. However, most of the studies only focus on the community structure and the controlling factors of either the bacteria or the nanoflagellate trophic level. Relative few studies have investigate the relationships as well as trophic interactions between the two trophic levels. In the cruise, we propose to achieve two objectives:

1. The first is to investigate the relationships between bacteria and nanoflagellate (NF) trophic levels in terms of their community structures, including the abundance and diversity.
2. We also design experiments to understand not only the trophic interactions between the bacteria and nanoflagellate trophic levels but also the energy transfer from nanoflagellate to small size copepods (nauplii and copepodites), i.e. the classic food chain.

To achieve the first objective, we will use a CTD-General Oceanic Rosette assembled with X-Niskin bottles to collect 9 liters of seawater from 5-m depth and subsurface chlorophyll maximum (SCM) layers at every station. To estimate the abundance of bacteria and nanoflagellate (NF), we will first filter 50mL out of the 9L seawater through a 20 μm mesh. Duplicates of 2mL subsamples will then be taken and fixed with paraformaldehyde solution to the final concentration of 0.2%. The rest of the 50mL filtered seawater will be fixed with glutaraldehyde to the final concentration of 1%. All these samples will be preserved in -20 °C liquid nitrogen on-board. Finally, the paraformaldehyde-fixed samples will be used to count bacteria abundance with flow cytometry and the glutaraldehyde-fixed samples will be used to count nanoflagellate abundance with epifluorescence microscope. To estimate the diversity of bacteria and nanoflagellate, the rest of the 9L seawater samples will be filtered sequentially through a 20 μm mesh, and a 1.2 μm as well as a 0.2 μm pore size polycarbonate filter using a peristaltic pump. The 0.2 μm and 1.2 μm pore size polycarbonate filters will then be frozen in -20 °C liquid nitrogen on-board. Finally, from the 0.2 μm pore size polycarbonate filters, we will extract 18S and 16S rDNA from the with illumina Miseq to estimate the nanoflagellates and bacterial biodiversity respectively.

[Equipment]

NF abundance

|  |  |  |
| --- | --- | --- |
| Item | Type | Quantity |
| Silicone tubes 1 (mesh) | Short (CTD-50mL) | 2 |
| Plastic dropper | 3mL | 5~10 |
| 50mL Centrifuge Tubes | 50mL | 200 |
| Labels | Print Out | 200 |
| Basket | Market type | 1 |
| Metal rank | For 50mL x 9 tubes | 1 |
| Latex gloves | M | 2 boxes |
| Neoprene rubber Glove | M (for preventing gluta) | 1 pair |
| Glutaraldehyde (50%) | 1mL each sample (final 1%) | >200 mL |

Bacteria abundance

|  |  |  |
| --- | --- | --- |
| Item | Type | Quantity |
| Soft Tubes with mesh | Short (CTD-50mL) | 2 |
| 2 mL Cryogenic tubes | 2mL | 200 |
| Spray bottle (for Ethanol) | 200mL-400mL | 1 |
| Ethanol | 75% for sterilizing | 1L-2L |
| Pipette | 1000uL | 3 |
| Pipette | 20uL | 3 |
| Tip | 1000uL | 6 boxes |
| Tip | 20uL | 6 boxes |
| Liquid Nitrogen?? | ??? | for a month? Dip & freeze? |
| Paraformaldehyde (10%) | 2uL each sample (final 0.2%) | 15mLx3 tubes |

Diversity (DNA)

|  |  |  |
| --- | --- | --- |
| Item | Type | Quantity |
| Carboys | 10-20L | 6 |
| Polycarbonate filter # | 1.2μm (D = 142 mm) | 3 boxes (300) |
| Polycarbonate filter # | 0.2μm (D = 142 mm) | 3 boxes (300) |
| 5mL cryogenic tube | 5mL | 200 |
| Tweezer | For arranging filter | 4 |
| Silicone tubes 1 (mesh) | Long (CTD- 20L Bottle) | 6 |
| peristaltic pump | with LS25 tube X 4  (2 for backup) | 2 set with 4 tubes∆ |
| Filter holder # | hold the 142 mm filter | 3 sets |
| Silicone tubes 2 | For pump rotate | 4 tubes∆ |
| Silicone tubes 3 | For pump to filter holder | 4 tubes∆ |
| Silicone gel | For pump-tube | 1 |

＊How to preserve -20 samples on board? (storage size for NF samples)

＊How to deliver -20 samples from cruise to NTU laboratory? (shipping method and fee for all samples)

To achieve the second objective, we will design a modified dilution experiment (Exp. 1) to first estimate the trophic interaction between bacteria and nanoflagellate (NF) and an artificial cohort incubation (Exp. 2) to estimate the energy transfer from nanoflagellate to small size copepods, i.e. classic food chain. First, to estimate the trophic interaction between bacteria and nanoflagellate, we plan to conduct the classic dilution experiment, which allows us to estimate the consumption of bacteria by nanoflagellate. In addition, we plan to modify the dilution experiment by coupling it with a predator removal experiment. This modification allows us to estimate not only the growth rate and thus the secondary production of nanoflagellates. Finally, we will also conduct artificial cohort incubations to estimate the secondary production of small size copepods (nauplii and copepodites; Exp. 2). From Exp. 1b and Exp. 2, we can estimate the energy transfer efficiency from nanoflagellates to small size copepods.

The modified dilution experiment (Exp. 1) will have 7 treatments each has two replicates. The first treatment represents 100% of the nanoflagellate density and the presence of zooplankton that are larger than 20um in untreated seawater. The other 6 treatments represent 100, 80%, 60%, 40%, 20% and 0% of the nanoflagellate density in untreated seawater but with the absence of zooplankton that are larger than 20um. To prepare the these treatments, we will use a CTD-General Oceanic Rosette assembled with X-Niskin bottles to collect 40 liters of seawater from 5-m depth. 4L of the untreated seawater will first be taken to prepare the first treatment by filling two 2L-carboys bottles. The rest of the seawater will all be filtered through a 20 μm screen mesh. 20L of the 20 μm-filtered seawater will then be filtered through a 0.2 μm pore size polycarbonate filter using a peristaltic pump to obtain particle free seawater. To prepare the rest of the treatment (treatment 2 to 7), we will gently mixed 2, 1.6, 1.2, 0.8, 0.4, 0L of 20 μm-filtered seawater with 0, 0.4, 0.8, 1.2, 1.6, 2L of particle free seawater in 2L-carboys bottles. All 14 bottles will then be placed in a 100L dark incubation tank for 12-hour incubation.

To count the nanoflagellate abundance in all treatments, we will preserve 50mL of the seawater before and after incubation by 1% glutaldehyde and freeze the sample in -20 °C liquid nitrogen on-board. To count bacteria abundance in the 2nd to the 7th treatments, we will preserve 2mL of the seawater before and after incubation by 0.2 % paraformaldehyde and freeze the sample in -20 °C liquid nitrogen on-board. Finally, the paraformaldehyde-fixed samples will be used to count bacteria abundance with flow cytometry and the glutaraldehyde-fixed samples will be used to count nanoflagellate abundance with epifluorescence microscope.

To conduct the artificial cohort incubation (Exp. 2), we will have two treatments with three replicates representing two copepod size class, i.e. 50-80 and 100-150 μm, which are corresponding to the nauplii and copepodites stage of copepods. In each treatment (size class), we incubate the copepod with their food consisting mostly of plankton < 50 μm. To prepare each treatment, we will use a CTD-General Oceanic Rosette assembled with X-Niskin bottles to collect 120 liters of seawater from 5-m depth. The seawater will be filtered through a 50 μm to retain only plankton that are smaller than 50 μm. The filtered seawater is the incubation water. We will then fill three replicates of 20 L cubitainers with 18 L incubation water. We will then add the copepods in 50-80 and 100-150 μm size range, i.e. nuaplii and copepodites, to the cubitainers. To capture the nauplii and copepodites, we will vertically tow the 50 and 100 μm plankton nets respectively at 5-m depth for 20 minutes. The nauplii and copepodites will then be gently suspended in 20 L incubation sea water. The incubation with nauplii and copepodites will then be reversely filtered through 80 and 150 μm sieves respectively to be added into the 20 L cubitainers. The nauplii and copepodites treatment (50-80 and 100-150μm) will be incubated for 24 and 48 hours respectively. The nauplii and copepodites before and after incubation will be preserved in 5% formalin and bring back to Taiwan for analysis. With the following equation, we can calculate the secondary production and growth rate of small size copepods (nauplii and copepodites).

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where *SPi* is the secondary production of nauplii and copepodites group *i*, *GRi* is the mass-specific growth rate and *Bi* is the biomass of group *i*.

[Equipment]

Exp. 1

- 100L dark incubation tanks or 100L portable coolers with lid, 1 per set of experiment (2 for the cruise)

- Peristaltic pump

- Filter holders for 1.2 and 0.2 μm filter

- 20L carboys, 2 per set of experiment

- 2L carboys, 14 per set of experiment (3 sets for the cruise)

- Silicone tubes covered by 20 μm nylon filter.

- Liquid nitrogen (1-2 containers)

- 1.2 and 0.2 μm filters (3+3 per set of experiment)

- 2 mL cryogenic tubes, 48 per set of experiment

- 50 mL centrifuge tube, 8 per set of experiment

Exp. 2

- 20L cubitainers, 6 per set of experiment (3+1 sets for the cruise)

- 100L dark incubation tanks or 100L portable coolers with lid, 2 per set of experiment (3 sets for the cruise)

- 50 μm and 100 μm Norpac net (10/3 for the cruise)

- codends (5 for the cruise)

- Flowmeter

- 80 and 150 μm sieves (diameter 50 cm) for reverse filtration

- Silicone tubes covered by 50 μm nylon filter

- Long (~1.5 m) silicone tube

- 20 L plastic buckets, 2 for the cruise

- 50 μm nylon hand-made sieves for collecting incubated copepods (20cm height cylinder sieve; 2 for the cruise)

- Pressure sprayer

- Wash bottle

- 100mL plastic bottles, 8 per set of experiment

- 1000L plastic bottles, 1 per set of experiment.

[Chemicals]

Exp. 1

- 50% Glutaraldehyde 8mL per set of experiment (final conc. is 1%)

- 10% Paraformaldehyde 960 μL per set of experiment (final conc. is 0.2%)

Exp. 2

- ~140mL 40% formalin or ~560mL 10% formalin per set of experiment

[Seawater volume]

Exp. 1

* 2L\*2 (1st trmt) + (2 + 1.6 + 1.2 + 0.8 + 0.4L)\*2 (20 μm-filtered surface seawater) + (0.4 + 0.8 + 1.2 + 1.6 + 2L)\*2 (0.2 μm-filtered surface seawater) = 28 L

Exp. 2

* 20L\*6 for cubitainers + 20L\*2 for buckets =160 L