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

*Chien-Yun Yang, Chih-hao Hsieh  
Institute of Oceanography, National Taiwan University*

In the marine food web, bacteria and bacterivorous nanoflagellates (NF) are the two critical trophic levels of the microbial loop. Within the microbial loop, the energy and nutrients are transferred from bacteria to nanoflagellate and cycle back to bacteria. When phytoplankton production is low in oligotrophic environment, nanoflagellates are alternative prey to small size copepods (e.g. nauplii and copepodites) and the energy and nutrients can be transferred from microbial to the classic food chain. Consequently, the microbial loop plays an important role for biochemical cycling and energy transfer in the marine food web. However, most of the studies only focus on the individual controlling factors of community structures of either the bacteria or the nanoflagellate. Few studies have investigated the trophic interactions between the two trophic levels and how trophic interactions influence the community structures of bacteria and nanoflagellates. In the cruise, we aim to achieve two objectives:

1. First, we will investigate the relationships between bacteria and nanoflagellate (NF) community structures, in terms of their abundance and diversity.
2. Second, we design experiments to understand not only the trophic interactions between the bacteria and nanoflagellate trophic levels, but also the energy transfer from nanoflagellate (microbial loop) to small size copepods (classic food chain).

To achieve the first objective, we will use a CTD-General Oceanic Rosette assembled with X-Niskin bottles to collect 40 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 150mL (50mL for bacteria abundance and 100mL for NF abundance) out of the 40L seawater through a 20 μm mesh. For bacteria abundance, duplicates of 2mL subsamples will then be taken and fixed with paraformaldehyde solution (final concentration of 0.2%). Samples fixed in 0.2 % paraformaldehyde will be frozen in liquid nitrogen (if not, preserved in -20 °C refrigerator on-board). For NF abundance, duplicates of 50mL filtered seawater will be fixed with glutaraldehyde (final concentration of 1%). Samples preserved in 1% glutaraldehyde will be frozen in -20 °C refrigerator on-board. Back to the lab, we will use a flow cytometry to count bacteria abundance in the paraformaldehyde-fixed samples, and use an epifluorescence microscope to count nanoflagellate abundance in the glutaraldehyde-fixed samples.

To estimate the diversity of bacteria and nanoflagellate, the rest of the seawater (~40L) will be first filtered through a 20 μm mesh. The 20 μm mesh-filtered seawater will then be filtered first through a 1.2 μm, then followed by a 0.2 μm pore size polycarbonate filter sequentially by a peristaltic pump. The 0.2 μm and 1.2 μm pore size polycarbonate filters will then be frozen in liquid nitrogen (if not, preserved in -20 °C refrigerator on-board). Finally, from the 1.2 μm and 0.2 μm pore size polycarbonate filters, we will extract 18S and 16S rDNA with illumina Miseq to estimate the nanoflagellates and bacterial biodiversity, respectively.

[Equipment, consumables and chemicals]

Bacteria abundance (estimated with 40 stations+10 extra stations)

|  |  |  |
| --- | --- | --- |
| 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 |

NF abundance (estimated with 40 stations+10 extra stations)

|  |  |  |
| --- | --- | --- |
| 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 |

Diversity (DNA) (estimated with 40 stations+10 extra stations)

|  |  |  |
| --- | --- | --- |
| 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°C samples on board? (storage size for NF samples): ≈ 140 of 50mL centrifuge Tubes

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

=> See telegram group for Mini’s experience

To achieve the second objective, we will design a modified dilution experiment (Exp. 1) to first estimate the trophic interaction between bacteria and nanoflagellates (NF) and an artificial cohort incubation (Exp. 2) to estimate the energy transfer from nanoflagellates to small size copepods in 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 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. 1 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 with two replicates. The first treatment represents 100% of the nanoflagellate density with zooplankton 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 in which zooplankton larger than 20um than are removed. To prepare these treatments, we will use a CTD-General Oceanic Rosette assembled with X-Niskin bottles to collect 28 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 (24L) will all be filtered through a 20 μm screen mesh to retain bacterivorous nanoflagellates that are smaller than 20 μm. 12L of these 20 μm-filtered seawater will be filtered again 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 refrigerator 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 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 each that represent 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 160 liters of seawater from 5-m depth. The seawater will be filtered through a 50 μm mesh filter 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 for the two copepod size classes. We will then add the copepods in 50-80 and 100-150 μm size ranges 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 in two plastic buckets. The seawater 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).

,

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*. We will collect the copepod community using 50μm mesh Norpac net with flowmeter by oblique tow at 200 m depth and the copepods will be preserved in 10% formalin solution. The abundance and biomass of copepods will be estimated using anatomical microscopes in lab.

**[Equipment, consumables and chemicals]**

**We plan to conduct both experiments at 3 stations (the most offshore and inshore and the middle one in between) in each of the 6 transect line**

Modified dilution experiment (Exp. 1)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Quantity  for 1 set of exp | Quantity  for all 18 sets of exp | Extras |
| Peristaltic pump | with LS25 tube | 2 | 2 (reusable) | 2 |
| Silicone tubes 2 #1 | For pump rotate | 2 | 2 (reusable) | 2 |
| Silicone tubes 3 #2 | For pump to filter holder | 2 | 2 (reusable) | 2 |
| Filter holder |  | 2 | 2 (reusable) | 2 |
| 20L carboys |  | 3 | 3 (reusable) | 2 |
| 2L carboys |  | 14 | 42  (14\*3 sets) | 6 |
| dark incubation tank #3 | 100L | 2 | 2 (reusable) |  |
| 20 μm filters | 2m x 2m | 2 | 2 (reusable) | 1 |
| 1.2 μm filter #4 | 1.2 μm polycarbonate filter  (Diameter = 142 mm) | 2 | 1 box (50 / box) |  |
| 0.2 μm filter #4 | 0.2 μm polycarbonate filter  (Diameter = 142 mm) | 1 | 1 box (50 / box) |  |
| Kimwipes Kimtech | 11 x 21cm | 1 | 1 box  (280 papers/box) |  |
| 75% Ethanol | For sterilization | 0.5L | 0.5 L |  |
| Tweezer | For arranging filters | 1 | 1 |  |
| Liquid nitrogen |  |  |  |  |
| 50 mL centrifuge tube | 50 mL | 16  (2 trmt\*2 reps\*2 subsamples\*2 Times) | 288 (16\*18) | 12 |
| 2 mL cryogenic tubes | 2 mL | 56  (7 trmt\*2 reps\*2 subsamples\*2 Times) | 1008 (56\*18) | 32 |
| Paraformaldehyde (10%) | Final 0.2% | 1.12 mL | 20.16 mL (1.12\*18) |  |
| Glutaradehyde (50%) | Final 1% | 8 mL | 144 mL (8\*18) |  |

#1 Silicone tube 2 needs to match the peristaltic pump

#2 Silicone tube 3 needs to match the filter holder

#3 Both Exp. 1 and 2 need this

#4 The 1.2 and 0.2 μm filters need to match the filter holder

Artificial cohort incubation (Exp. 2)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Quantity  for 1 set of exp | Quantity  for all 18 sets of exp | Extras |
| 20 L cubitainers | 20 L | 6  (2trmt\*3reps) | 18  (6\*3 sets) | 6  (1 set) |
| dark incubation tank | 100L | 2 | 6  (2\*3 sets) |  |
| 50 μm Norpac net | with Flowmeter | > 15  (in case it breaks) | > 15 |  |
| 100 μm Norpac net |  | > 5  (in case it breaks) | > 5 |  |
| Flowmeter for Norpac |  | 2 | 2 (reusable) |  |
| Cod End for Norpac |  | 5 | 5 (reusable) |  |
| 80 μm sieves for reverse filtration | diameter 50 cm | 2 | 2 (reusable) |  |
| 150 μm sieves for reverse filtration | diameter 50 cm | 2 | 2 (reusable) |  |
| 50 μm nylon filter | 2m x 2m | 2 | 2 (reusable) |  |
| 50 um hand-made sieve |  | 2 | 2 (reusable) |  |
| 20 L plastic buckets | 20 L | 2 | 2 (reusable) |  |
| 100 mL plastic bottles | 100 mL | 8 | 144 (8\*18) | 16 |
| Formalin (40% or 10%) | Final 5% (incubation)  Final 10% (community) | 140 mL (40%)  560 mL (10%) | 3 L (40%)  12 L (10%) |  |
| Cleaning Sponge with Scouring Pad |  | 2 | 2 (reusable) |  |
| Pressure sprayer  (https://tg.pe/rJu) |  | 2 | 2 (reusable) |  |
| Alcohol thermometer |  | 2 | 2 (reusable) |  |
| Wash bottle |  | 3 | 3 (reusable) |  |