Disentangling the interactions between microbial loop and grazing food web at the southern ECS

Bacteria and bacterivorous nanoflagellates are two critical trophic levels in a marine food web. In a marine food web, energy and nutrients in the bacteria trophic level are typically transferred to bacterivorous nanoflagellates, which are typically heterotrophic nanoflagellages (HNF), and then cycled back to bacteria. This loop formed by bacteria and heterotrophic nanoflagellages (HNF) are called the microbial loop in marine food webs. The energy and nutrients in the microbial loop can be transferred to the classic grazing food webs when phytoplankton production is low in an oligotrophic environment. When phytoplankton production is low, small size copepods (e.g. nauplii and copepodites) consume more HNFs and thus link the microbial loop to the classic grazing food web. Consequently, the microbial loop plays an important role in biochemical cycling and energy transfer in the marine food web. However, most of the studies only investigate the trophic interactions either in the microbial loop or in the classic grazing food chain. To investigate the trophic interactions among bacteria, heterotrophic nanoflagellates (HNF), phytoplankton and microzooplankton, we design a modified dilution experiment (Exp. I) consisting of two series of dilutions. The first dilution series is designed to estimate the trophic interaction between bacteria and HNF, i.e. the microbial loop. The second dilution series is designed to estimate the trophic interactions between HNF/phytoplankton and microzooplankton (mainly nauplii and copepodites), i.e. the classic grazing food chain. To further estimate the trophic transfer efficiency between HNF/phytoplankton and microzooplankton, we design artificial cohort incubation for microzooplankton growth rate and sample the microzooplankton community biomass to calculate the secondary production of microzooplankton (Exp II).

**Exp I**

The modified dilution experiment (Exp. I) will have 11 treatments, each with two replicates. The first 6 treatments are the first dilution series, which represent 0%, 20%, 40%, 60%, 80% and 100% of the nanoflagellate density in the untreated seawater. In these treatments, zooplankton that are larger than 20 μm will be removed. The 6th to the 11th treatments are the second dilution series, which represent 0%, 20%, 40%, 60%, 80% and 100% of microzooplankton density in the seawater. In these treatments, zooplankton that are larger than 200 μm will be removed. According to the rationale of dilution experiment, the first series of dilutions (treatment 1-6) allows to measure the consumption of bacteria by HNF and the second series of dilutions (treatment 6-11) allows us to measure the consumption of HNF/phytoplankton by microzooplankton.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| % Microzooplankton | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 40 | 60 | 80 | 100 |
| % HNF | 0 | 20 | 40 | 60 | 80 | 100 | 80 | 60 | 40 | 20 | 0 |
| % Particle free seawater | 100 | 80 | 60 | 40 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |

To prepare the 1st to the 6th treatments, we will use a CTD-General Oceanic Rosette assembled with Go-Flo bottles to collect 30 liters of seawater from 5-m depth. 15 L of which will be filtered through a 0.2 μm pore size polycarbonate filter using a peristaltic pump to obtain particle free seawater. The other 15 L will be filtered through a 20 μm screen mesh to retain HNF that are smaller than 20 μm. The 1st to the 6th treatments will finally be created by gently mixing 0, 0.5, 1, 1.5, 2, 2.5L of particle free seawater with 2.5, 2, 1.5, 1, 0.5, 0L of the 20 μm-filtered seawater in 2L-carboys bottles.

To prepare the rest of the 5 treatments, we will first prepare seawater with only HNF as well as with HNF and microzooplankton that are smaller than 200 μm. To prepare seawater with only HNF, we use a CTD-General Oceanic Rosette assembled with Go-Flo bottles to collect 30 liters of seawater from 5-m depth. 10 L of which will be filtered through a 20 μm screen mesh to retain only HNF that are smaller than 20 μm. The rest 20 L will be filtered through a 20 μm screen mesh to prepare the seawater that will be used to temporarily keep microzooplankton. We will then vertically tow a 50 μm Norpac net with a flowmeter at 5 m depth for 10 minutes to collect microzooplankton. The water volume flowing through the net by flowmeter is recorded for back-calculating the *in situ* density of microzooplankton. The net-concentrated microzooplankton will be resuspended in the 20 L 20 μm-filtered seawater. Finally, we will gently mix 2, 1.5, 1, 0.5, 0 L seawater with only HNF with 0.5, 1.0, 1.5, 2, 2.5 L seawater with microzooplankton through 200 μm-mesh reverse filtration to create the 7th to the 11th treatment. All 22 bottles will then be placed in a 100L dark incubation tank for 12-hour incubation.

To preserve bacterial community before incubation, we will preserve 3 replicates of 2mL of the 20 μm-filtered seawater in 0.2 % paraformaldehyde. These samples will be frozen in liquid nitrogen on-board. For the initial HNF community and density, we will also preserve 3 replicates of 50mL of the 20 μm-filtered seawater in 1% glutaraldehyde. These samples will be frozen in -20 °C refrigerator on-board. The initial microzooplankton (mainly nauplii and copepodites) community will be preserved in 5% formalin. We finally will use the following to estimate the initial natural microzooplankton density.

 Ratio of Norpac net-concentration: *I*net = *F* /*V*cod (*F* is the water volume (liter) recorded by flowmeter; *V*cod is the volume of codend).

 Ratio of concentration after resuspension: *I*res = *I*net/(20+ *V*cod) (20 L is the volume of 50 μm-filtered seawater for resuspension).

 Real microzooplankton density: *N*meso = *N*0, meso/*I*res. (*N*0,meso is the density of microzooplankton in the beginning of the incubation).

After incubation, we will count bacteria density in the 1st to the 6th treatments to estimate the consumption of bacteria by HNF. We will preserve 3 replicates of 2mL of the seawater by 0.2 % paraformaldehyde and freeze the sample in liquid nitrogen on-board. We will also count the HNF abundance in the 6th to 11th treatments to estimate the consumption of HNF by microzooplankton. To do so, we will preserve 3 replicates of 50mL of the seawater by 1% glutaraldehyde and freeze the sample in -20 °C refrigerator on-board. To estimate the biomass change of autotrophic nanoflagellate, we will filter respectively 150 mL of fresh sample on GF/F (0.75 μm), 3 μm filter and GF/A (20 μm) from the 6th to 11th treatments before and after incubation. After back to the lab, the paraformaldehyde-fixed samples will be used to count bacteria abundance with flow cytometry and the glutaraldehyde-fixed samples and size-fractionated chl-a will be used to count HNF abundance with epifluorescence microscope and estimate the biomass of autotrophic nanoflagellates by spectrophotometry.  
Microzooplankton at T0 and in treatment 11 at T12 will be stained by neutral red for 15 minutes before fixation to estimate the mortality. At T0, 5mL of neutral red will be added to microzooplankton sample filled in a 500 mL bottle. At T12, 20 mL neutral red will be added to the two replicates of treatment 11.

[Equipment]

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Quantity  for 1 set of exp | Quantity  for all XX sets | Extras |
| 20L carboys |  | 4 |  | 1 |
| 20L bucket |  | 1 |  | 0 |
| 2L carboys | Exact volume is 2.41 L | 22 |  | 3 |
| 20 μm nylon filters | 2m x 2m | 2 |  | 0 |
| Silicone tubes | No filter, >100 cm | 2 |  |  |
| Silicone tubes covered with 20 μm mesh |  | 4 |  | 0 |
| Large 200μm nylon sieve for reverse filtration | Diameter 30 cm | 1 |  | 0 |
| 50μm stainless sieve | Collecting microzooplankton at T12 |  |  |  |
| 50μm Norpac net |  | 5 |  | 0 |
| Flowmeter |  | 2 |  | 0 |
| Cod end |  | 2 |  |  |
| Peristaltic pump |  | 1 |  | 0 |
| Silicone tubes 2 #1 | For pump rotate | 4 |  | 0 |
| Silicone tubes 3 #2 | For pump to filter holder | 4 |  | 0 |
| Filter holder |  | 2 |  | 0 |
| Plastic joint 1 | For tube 2 to tube 3 | 1 for 1 pump set |  | 0 |
| Plastic joint 2 | For filter holder to tube 3 | 1 for 1 pump set |  | 0 |
| Wash bottle | 500mL | 2 |  | 0 |
| 500 mL Plastic bottle | For neutral red staining | 2 |  | 0 |
| Plastic syringe and dropper | For liquid chemicals | 2/5 |  | 0 |
| 1.2 μm filter #3 |  | 2 |  | 0 |
| 0.2 μm filter #3 |  | 2 |  | 0 |
| Kimwipes Kimtech | 11 x 21cm | 1 |  | 0 |
| Tweezer | For arranging filters | 1 |  | 0 |
|  |  |  |  |  |
| 100L dark incubation tank |  | 2 |  | 0 |

#1 Silicone tube 2 needs to match the peristaltic pump

#2 Silicone tube 3 needs to match the filter holder

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

[Chemicals]

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Amount  for 1 set of exp | Amount  for all XX sets | Extras |
| 10% paraformaldehyde |  | 1.68 mL |  | 0 |
| 50% glutaraldehyde |  | 12 mL |  | 0 |
| 40 % formalin  10% formalin |  | 35 mL  140 mL |  | 0 |
| 1:2000 (w/v) neutral red |  | 45 mL |  | 0 |
| 75% Ethanol | For sterilization | 0.5L |  | 0 |

[Consumables]

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Quantity  for 1 set of exp | Amount  for all XX sets | Extras |
| 2 mL cryogenic tubes  (bacteria) |  | 3 (T0) +  36 (T12; trmt1-6 \* 2rep \* 3subsample) |  | 11 |
| 50 mL centrifuge tubes  (HNF) |  | 3 (T0) +  36 (T12; trmt6-11 \* 2rep \* 3subsample) |  | 11 |
| GF/F |  | 1 (T0)+12 (T12; trmt6-11\*2rep) |  |  |
| GF/A |  | 1 (T0)+12 (T12; trmt6-11\*2rep) |  |  |
| 3 μm filters |  | 1 (T0)+12 (T12; trmt6-11\*2rep) |  |  |
| Embedding cassettes for storing GF/A and GF/F |  | 3 (T0)+ 36 (T12; trmt6-11\*2rep\*3size) |  |  |
| 100 mL plastic bottle  (mzp) |  | 1 (T0) +  2 (T12; trmt11 \* 2rep) |  | 3 |

[Seawater volume]

65 L (at least) =

15 L 0.2μm-filtered water (treatment 1-6) +

25 L 20μm-filtered water (treatment 2-10) +

20 L 20μm-filtered water (temporarily keep microzooplankton)+

5 L seawater (for rinsing all the carboys)

**(Exp III) Microzooplankton (mainly nauplii and copepodites) growth and production**

Secondary production of microplankton group *i* (*SPi*) is defined as

,

where *GRi* is the mass-specific growth rate and *Bi* is the biomass of group *i*. We will incubate two microzooplankton size classes: 50-80 μm (nauplii) and 100-150 μm (copepodites).

20 L cubitainers will be filled with 18 L 50 μm-filtered incubation water (at 5 m depth) with plankton < 50 μm. Nauplii and copepodites will be collected by 10-min vertical tow of 50 and100 μm plankton nets at 5 m depth and gently resuspended in a bucket of 20 L 50 μm-filtered incubation water. Fill the remaining space of cubitainers with nauplii/copepodites grazer by reverse filtering the seawater with nauplii/copepodites through 80/150 μm sieves. Nauplii and copepodites will be incubated for 24 and 48 hrs, respectively. Microzooplankton community before and after incubation will be preserved in 5% formalin for analysis.

[Equipment]

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Quantity  for 1 set of exp | Quantity  for all XX sets | Extras |
| 20 L cubitainers |  | 6  (2trmt\*3reps) |  | 6  (1 set) |
| dark incubation tank |  | 2 |  | 0 |
| 50 μm Norpac net |  | > 15  (in case it breaks) |  | 0 |
| 100 μm Norpac net |  | > 5  (in case it breaks) |  | 0 |
| Flowmeter for Norpac |  | 2 |  | 0 |
| Cod End for Norpac |  | 5 |  | 0 |
| 80 μm sieves for reverse filtration |  | 2 |  | 0 |
| 150 μm sieves for reverse filtration |  | 2 |  | 0 |
| 50 μm nylon filter |  | 2 |  | 0 |
| 50 um hand-made sieve |  | 2 |  | 0 |
| 20 L plastic buckets |  | 2 |  | 0 |
| 100 mL plastic bottles |  | 8 |  | 16 |
| Cleaning Sponge with Scouring Pad |  | 2 |  | 0 |
| Pressure sprayer  (https://tg.pe/rJu) |  | 2 |  | 0 |
| Alcohol thermometer |  | 2 |  | 0 |
| Wash bottle |  | 3 |  | 0 |

[Chemicals]

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Item | Type | Amount  for 1 set of exp | Amount  for all XX sets | Extras |
| formalin | 40 % or  10% | 40 mL  or  160 mL |  | 0 |

[Seawater volume]

160 L (at least) =

120L 50μm-filtered water (cubitainers) +

40L 50μm-filtered water (buckets)

**[I haven’t modified any of the following]**

**\* Time for net tows per station**

|  |  |  |  |
| --- | --- | --- | --- |
| Sampling depth | Mesh-size | Spend-time (minutes) | Experiment |
| 5-m vertical tow | 50 μm Norpac with a flowmeter | 15 (The net will be set to 10 m depth and allowed to drift with the ship for 10 minutes) | I |
| 5-m vertical tow | 50 μm Norpac\*2 and 100 μm Norpac attached on ring | 15 (The net will be set to 10 m depth and allowed to drift with the ship for 10 minutes) | II |
| From bottom to surface oblique tow | 50 μm Norpac with Flowmeter | 15-50 min, depending in the bottom depth | II |

**\* Go-Flo bottle water collection per station**

|  |  |  |
| --- | --- | --- |
| Sampling depth | # of Go-Flo (20L) | Experiment |
| 5-m | 4 | I |
| 5-m | 8 | II |