
# Antibiotic treatment of the breadcrumb sponge Halichondria panicea and subsequent recolonization V.2

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Symbiosis Model Systems
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## ABSTRACT

This protocol generates sponges (*Halichondria panicea*) with a disturbed microbiome under controlled experimental conditions, in order to study bacterial recolonization dynamics. Bacteria-bacteria interactions can be analysed with this set-up within the host environment aiming at a better understanding of sponge-microbe symbiosis *in vivo*.

It is divided into the sections 1) preparation, 2) antibiotic treatment and recovery phase, 3) recolonization with the natural microbiome and 4) sampling.

## GUIDELINES

**Keywords:** host-microbe interactions, sponge model, symbioses, microbiome, metaorganism, holobiont, antibiotic treatment

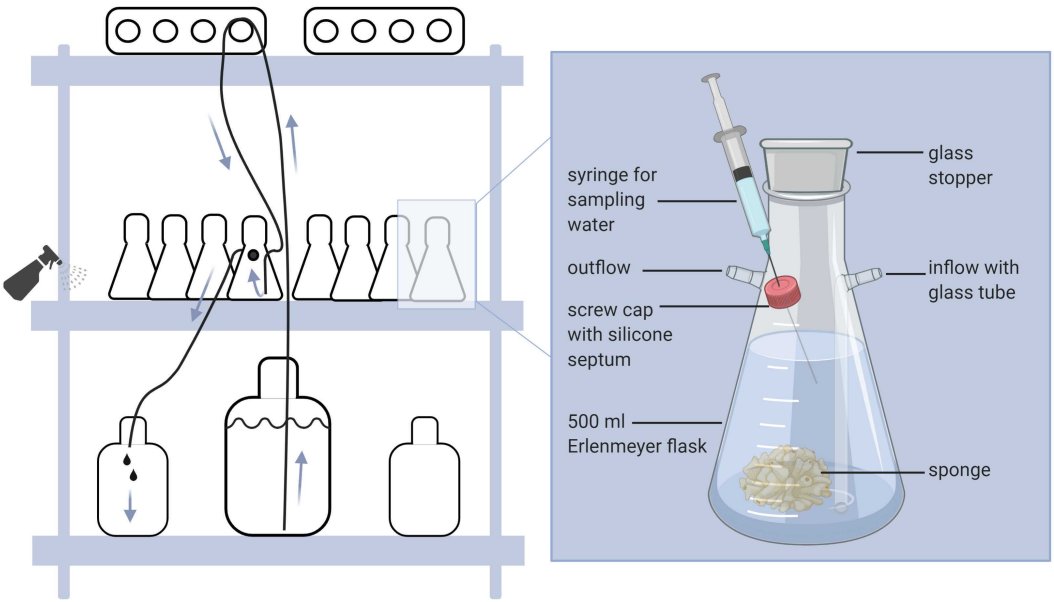



Figure: Experimental set-up in the gnotobiotic chambers in GEOMAR Helmholtz Centre for Ocean Research, Kiel.
Four shelfs like the one depicted here are available, each holding 8 x 500 mL modified Erlenmeyer flasks, in total 32 flasks.

**The flow-through set-up** (Figure) consists of 32 x 500 mL modified Erlenmeyer flasks distributed on 4 shelfs. Each set of 8 flasks is supplied with fresh, sterile filtered artificial seawater from one 20 L carboy.

**The culture flasks** are customized flow-through Erlenmeyer flasks (500 mL) that are fully autoclavable and re-usable (Figure). The flasks are closed with a hollow glass stopper. Water is exchanged via a hose barb and attached glass tube reaching near the bottom of the Erlenmeyer flask for water inflow, and a hose barb on the opposite side for water outflow. A thread with a screw cap and silicone septum allows for sampling culture water with a sterile syringe and needle without opening the flasks.

**Water flow-through** is realized by GHL2 pumps that pump water from a 20 L source tank into the Erlenmeyer flasks; the replaced water is collected in 10 L canisters.
Important! Calibrate each pump head prior to an experiment via the GHL software.

**The gnotobiotic chambers** are equipped with an air filtration system (Expansion

Electronic CP500/SNATURE SYSTEM) constantly removing microorganisms from the air. The temperature and light regime can be chosen according to the season. Surface area is minimized and regularly surface sterilized (Curacid Medical wipes). All material is either autoclavable, sterile packed or sterilizable by surface sterilization prior to entering the room. Access is restricted and only allowed with lab coat, gloves and shoe covers. A transitioning zone in front of the gnotobiotic chambers provides space for changing, storage of additional material, cleaning of material etc..

**The experiment can be set up one day prior to the start (start = placing sponges in the flasks), but some things have to be prepared 1-2 weeks in advance (start with section "preparation").**

## MATERIALS

**Culture:**

- Breadcrum sponge *Halichondria panicea* (Pallas) collected from Schilksee, Kiel, Germany 54.424792, 10.175010
- Aqua Medic Seasalt EAN Code: 4025901100075
- *Nannochloropsis salina* freeze dried powder - algova UG
- Stainless steel filter holder, diameter 142 mm - Sartorius SM 16275
- 0.22 µm PVDF filter, diameter 142 mm
- Silicone tubing - inner diameter 4 mm, outer diameter 6 mm
- Silicone tubing - inner diameter 8 mm, outer diameter 12 mm
- Erlenmeyer - custom built by Eydam, Kiel, Germany
- Waste canisters for chemicals 10 l
- 20 l Nalgene® carboy (PP, for autoclaving)
- 100 l barrel
- Eheim® 1250 universal aquarium pump
- GHL® Doser 2.1
- Curacid® medical soaked whipes for surface sterilization

**Antibiotics:**

- Rifampicin - CAS :13292-46-1; stock solution as 50 mg/ml in 100 % DMSO
- Ampicillin - CAS :69-52-3; stock solution as 50 mg/ml in MilliQ water
- Nalidixic acid - CAS :389-08-2; stock solution as 50 mg/ml in 0.3 M NaOH
- Neomycin - CAS: 1405-10-3; stock solution as 50 mg/ml in MilliQ water
- Polymyxin B - CAS :1405-20-5; stock solution as 2 mg/ml in MilliQ water

**Other chemicals:**

- Difco™ Marine Agar
- Difco™ MarineBroth 2216
- RNase AWAY®
- RNAlater

- Ethanol
- Glutaraldehyde 2.5 %, 6.25 % in sterile filtered 1xPBS
- Paraformaldehyde 4 % in sterile filtered 1xPBS
- Glutaraldehyde 0.1 % + Paraformaldehyde 10 % in sterile filtered 1xPBS
- For calcium-magnesium-free artificial seawater (CMF-ASW): NaCl, $Na_2SO_4$, KCl, $NaHCO_3$

**Consumables and other material:**

- Forceps
- Scalpel
- Sterile plastic petri dishes
- 2 ml polypropylene tubes
- 50 ml polypropylene tubes
- 1.8 ml Cryovials DNase/RNase free
- Corning® cell strainer 40 µm
- Sterile syringes 5 ml
- Sterile needles diameter 0.6 mm, length 80 mm
- 200 ml autoclavable bottles (for food stock)
- 1 l autoclavable bottles
- Bottle top vacuum filter holder, diameter 47 mm
- Re-usable syringe filter holder, diameter 25 mm
- 0.22 µm PVDF filter, diameter 25 mm
- 0.22 and 5 µm PVDF filter, diameter 47 mm

**Equipment:**

- Autoclave
- Fume hood
- Biosafety cabinet
- Orbital shaker
- Climate chamber with controlled light and temperature
- Salinometer
- Water bath
- Centrifuge - for 2 and 50 ml polypropylene tubes
- Vacuum pump

## SAFETY WARNINGS

Waste water from the experiment has to be disposed as chemical waste:
- during antibiotic treatment as AVV 20 01 19 toxic pesticides
- after antibiotic treatment as AVV 18 01 03 medical waste for infection prevention (potentially antibiotic resistant bacteria selected by antibiotic treatment)

Work under the fume hood when handling toxic fixatives (Paraformaldehyde, Glutaraldehyde)

## BEFORE START INSTRUCTIONS

Start with section "preparation" and take ~1-2 weeks to prepare

# Preparation

1w 0d 9h

### 1 Autoclaving material prior to use for 15 min at 121°C

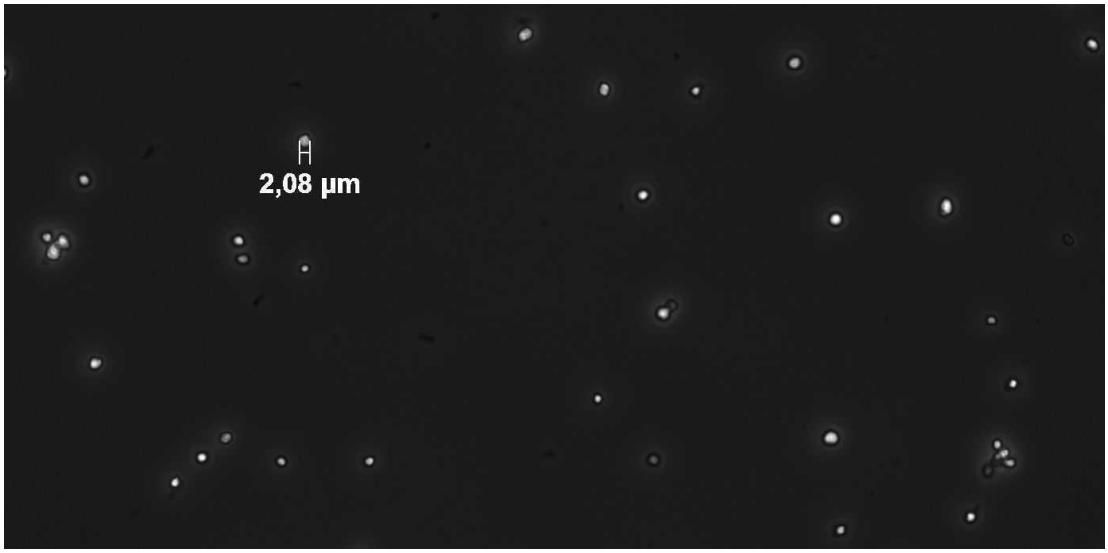
1w

- culture bottles
- silicone tubing (in- and outflow tubes can be connected to culture bottles before autoclaving)
- 20 L carboys with tin foil wrapped around lid but don't close lid! otherwise carboys dent when they cool down
- filtration unit with inserted filter paper 0.22 µm, diameter 142 mm
- saline (1.5 % NaCl in MilliQ water)
- food stock
- CMF-ASW

### 2 Food stock

3h

Autoclaved *Nannochloropsis salina* unicellular algae solution




These small (~2 µm) unicellular algae stay intact after autoclaving and retain their chlorophyll fluorescent signal. Also, Dan et al. 2018 showed that the nutritional value of closely related *Nannochloropsis* is not affected by autoclaving

Dan2018.pdf

**2.1** Weigh 16 g *Nannochloropsis salina* powder dried algae and dissolve in 2 L MilliQ water with 30 g artificial seasalt

Shake rigourously for several minutes or use magnetic stirrer

**2.2** Filter through 40 µm cell strainer to remove algae clumps

**2.3** Determine concentration in stock (flow cytometer or counting under the microscope)

adjust to a stock solution of 5 x $10^7$ cells/ml (final concentration is $10^5$ cells/ml)

**2.4** Autoclave in 160 mL portions

Store at RT

## 3 Antibiotic stocks

3h

Per experiment, prepare 350 ml of each of the following antibiotic stocks:

- 50 mg/ml Rifampicin in 100 % DMSO (working conc. 50 mg/l)
- 50 mg/ml Ampicillin in MilliQ water (working conc. 50 mg/l)
- 50 mg/ml Nalidixic acid in 0.3 M NaOH (4.2 g NaOH in 350 ml MilliQ water) (working conc. 50 mg/l)
- 50 mg/ml Neomycin in MilliQ water (working conc. 50 mg/l)
- 2 mg/ml Polymixin B in MilliQ water (working conc. 2 mg/l)

Note

Antibiotics were chosen based on their different mechanisms of actions, compound classes and based on previous studies (Franzenburg et al. 2012 PNAS, Domin et al. 2018 Frontiers in Microbiology, Weiland-Bräuer et al. 2015 AEM).
rifampicin: ansamycine (RNA synthesis inhibition of most Gram +, many Gram-)
nalidixic acid: quinolone (blocks DNA replication of most Gram-, some Gram+)
ampicillin: beta-lactame (inhibits cell wall formation during cell division of Gram+)
neomycin: aminoglycoside (blocks ribosomal subunit of Gram-, some Gram+)
polymixin B: polypeptide (disrupts cell membrane of Gram-)

**3.1** Freeze in portions of 20 ml and store at -20°C

**3.2** Use freshly thawed solutions, do not re-freeze

## 4 Sterile filtered artificial seawater (F-ASW)

6h

F-ASW should be prepared every day during the antibiotic treatment and every second day during the recovery phase and recolonization

**4.1** Fill a 100 l barrel with destilled water while continuously adding 1.5 kg artificial seasalt
Dissolution can be sped up by adding a water pump into the barrel to mix the water

4.2 Mix well! Check salinity at bottom and top of the barrel and prevent foramtion of a salinity gradient. Slowly add missing salt until the desired salinity is reached

Since the salinity constantly fluctuates in the Baltic Sea, we choose the ambient salinity on the starting day of each experiment (between 14 and 18 PSU)

4.3 Connect autoclaved stainless steel filter holder (142 mm) unit to pump and fill 4 autoclaved 20 l carboys with F-ASW

4.4 When all carboys are filled, stop water pump and rinse it in fresh water

Rinse filter unit and change filter paper; autoclave including silicone tube for the next filtration

4.5 Add 40 ml *Nannochloropsis salina* stock solution to each 20 l carboy

During antibiotic treatment, add 20 ml per antibiotic to each 20 l carboy. The final working concentrations will be:

- 50 mg/l Rifampicin
- 50 mg/l Ampicillin
- 50 mg/l Nalidixic acid
- 50 mg/l Neomycin
- 2 mg/l Polymixin B

Mix well (by rolling closed carboys)

4.6 After use, rinse carboys twice with fresh water and autoclave for the next use

## 5 MB medium

1d

Prepare 15 g/l Difco agar and 37.5 g MarineBroth in 1 l destilled water

Prepare ~350 plates per experiment (= 10 bottles of MB medium) and store at 4°C

5.1 Cook in water bath for 10-15 min to dissolve medium

5.2 Autoclave and store at 60°C until pouring plates

5.3 Pour plates under biosafety cabinet and store upside down at 4°C

## 6 CMF-ASW (Calcium-Magnesium-free artificial seawater) after Rottmann et al. 1987

3h

Prepare e.g. 10 l as a stock and autoclave in portions of 500 ml

**Safety information**

attention! The recipe for CMF-ASW is thought for marine organisms. Since we are working on sponges from brackish water here, dilute the stock 1:1 before autoclaving

6.1 For 1 l stock solution:

27 g NaCl
1 g $Na_2SO_4$
0.8 g KCl
0.18 g $NaHCO_3$

add 1 l MilliQ water

6.2 Dilute stock 1:1 with MilliQ water and autoclave in portions of 500 ml

Can be stored at RT, but it is used ice-cold. Put bottles in freezer 1 h before they are needed

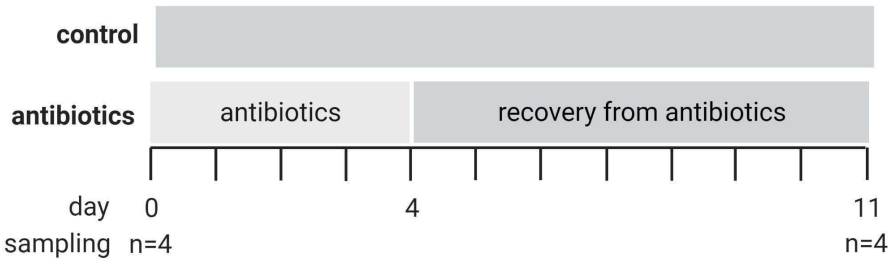
# Antibiotic treatment and recovery phase

5d

## 7 Antibiotic treatment

5d

During the first four days of the experiment prepare ASW with antibiotics and change water source daily




Timeline of the antibiotic treatment. T0 = start of the experiment; T4 = end of the antibiotic treatment; T11 = end of the recovery phase and start of the recoloniziation. Figure created with Biorender.com

**7.1** Start pumps on the first day prior to placing sponges in the Erlenmeyer flasks
Set pumps to **150 doses/day of 12 ml** to exchange the volume of each bottle 3.5 times per day

**7.2** When Erlenmeyer flasks are filled, add one sponge in each Erlenmeyer flask (about 3x3x3 cm)
Start the experiment early in the morning

**7.3** Prepare fresh antibiotic water and change carboys daily to prevent degradation and loss of function of antibiotics

## 8 Antibiotic wash-out

4h

In the afternoon of T4 start wash-out of antibiotics by exchanging the carboys with ASW+antibiotics to carboys with ASW only

**8.1** Flush the Erlenmeyer flasks several times with 250 ml fresh ASW each (by programming the pumps to dose 250 ml. Attention! The GHL2 pumps are not designed to continuously run; after each flush they need to stop for at least 15 min)

8.2 Since Rifampicin is coloured bright orange, you clearly see the reduction of antibiotics in the water by flushing

Aim at completely clear culture water until the next morning to prevent remaining antibiotics at low concentrations
It helps to manually pour out water (without opening the Erlenmeyer; simply tilt the bottle and empty it via the outflow barb) and reduce the water level to about 200 ml before flushing

9 **Recovery phase**

During the recovery phase, exchange the water source every second day with freshly filtered ASW

Set the pumps to **100 doses/day of 10 ml** to exchange to volume of each bottle twice per day

## Recolonization with the natural microbiome

2d

10 **Differential centrifugation**

3h

To prepare a bacterial inoculum for recolonization with the natural microbiome dissociate fresh, healthy sponges

The volume of sponge tissue should match the volume of sponge that you want to recolonize

10.1 Remove large pieces of algae and place sponges in a beaker with sterile, ice-cold CMF-ASW for 5 min to remove loosely attached bacteria

10.2 Replace CMF-ASW

10.3 Cut sponge tissue with a razor and forceps in a petri dish with sterile, ice-cold CMF-ASW
Only process a small piece at a time and try to keep tissue submerged
Remove algae or other non-sponge material

10.4 Transfer the pieces into 50 ml polypropylene tubes filled with ~30 ml sterile, ice-cold CMF-ASW and keep them on ice during the process
Add no more than 10 ml sponge tissue

Top up tubes until 50 ml with sterile, ice-cold CMF-ASW

**10.5** Incubate tubes on ice on an orbital shaker at 200 rpm for 20 min

**10.6** Filter through 40 µm cell strainer in fresh 50 ml polypropylene tubes, squeeze remaining tissue with forceps
Top up until 50 ml with sterile, ice-cold CMF-ASW

**10.7** Centrifuge for 20 min at 700 g and 4°C to remove sponge cells

**10.8** Transfer supernatant to a fresh 50 ml polypropylene tube and discard the sponge cell pellet

**10.9** Centrifuge supernatant for 15 min at 4000 g and 4°C

**10.10** Resuspend the pellet in ~5 ml CMF-ASW and pool the bacteria fraction from several tubes
This is the recolonization inoculum. Keep on ice!

**10.11** Proceed as quickly as possible to prevent dying of bacteria. Always keep on ice!

First recolonize, but keep a few milliliters to sample (step 12)

## 11 Recolonization

30m

Recolonize sponges by injection of 2 ml recolonization mix with sterile syringes and needles
Inject gradually and change location 5 times per sponge to distribute the inoculum throughout the whole animal

11.1 For the control treatment, use a sham control with 2 ml sterile CMF-ASW per sponge

12 **Viability and composition of inoculum** 30m

Take different samples to ensure viability of inoculum and analyse bacterial composition later with qPCR and amplicon sequencing

12.1 Plate 100 µl pure inoculum, 1:10, 1:100 and 1:1000 dilution on MB agar plates at incubate at 25°C for 1 day
Check lower dilutions after 7 days

12.2 Freeze several aliquots of 1 ml pure inoculation mix in cryovials and flash freeze in liquid nitrogen
Store at -80°C

12.3 Filter 2 x 2 ml inoculum mix onto 0.22 µm filter with a reusable syringe filter holder (diameter 25 mm), store filter in cryovials and flash freeze in liquid nitrogen
Store at -80°C

12.4 Fix 2 x 1.8 ml inoculum mix with 0.2 ml Glutaraldehyde+Paraformaldehyde (final concentration 0.01 % and 1 %)

Fix for 10 min at RT

Freeze at -80°C

13 **Repeat recolonization three times within 48 h to ensure transfer of bacteria** 2d

## Sampling

14 **Plating of culture water**

To monitor bacterial growth in the Erlenmeyer flasks, culture water is plated every 2-3 days. The

sterile filtered ASW and the food stock solutions should be plated before the start and at least once during the experiment to ensure sterility

**14.1** Sample ~1 ml culture water from 4 replicate tanks per treatment via the silicone septum with a sterile needle and syringe. Rinse syringe once with culture water before taking the sample and take care to do it very slowly, so the sponge does not get kicked around by the water current

Store samples in fridge no longer than 3 h

**14.2** Under the clean bench: prepare dilution series by pipetting 100 µL culture water and 900 µL saline (1:10) and repeat twice until a dilution of 1:1000 that is used for plating

Mix well!

**14.3** Plate 100 µL of the 1:1000 dilution in triplicates on MB agar plates; use glass spatula (sterilize by dipping in ethanol and burning, cool down)

**14.4** Wrap plates with parafilm and incubate upside down at 25°C for 7 days

**14.5** Count colony forming units (CFUs)

## 15 Sponge tissue samples

At T0, T11 and after the recolonization take sponge tissue samples and preserve for different analyses

Take a picture of each sponge before sampling

Clean dissection place with ethanol and RNAseaway

Work quickly

**15.1** **Cut sponge in 6 pieces with sterile forceps and scalpel on a sterile petri dish.**

Clean utensils after each sponge with ethanol and RNAseaway and discard petri dish

## 15.2 DNA/RNA extraction

Preserve two pieces in 2 x cryovials with 1.6 ml RNAlater

Incubate at 4°C overnight and transfer to -80°C the following day

For DNA/RNA extractions and qPCR see protocol:
dx.doi.org/10.17504/protocols.io.bxwwppfe

## 15.3 Backup

Flash freeze one piece (or all leftover sponge tissue) in cryovial in liquid nitrogen and store at -80°C