Reagents and Procedures for Culturing Marine Microbes

A. Stock solutions (See "Seawater Plate Recipe")

- Asparagine (100 mM)
- Adenosine monophosphate (0.33 M)
- $NaH_2PO_4 \cdot H_20$ (25 mM)
- $NaNO_3$ (75 g/L)
- $Na_2EDTA \cdot 2H_20$ (1 g/L)

1. Make 100 mL of each reagent in an autoclavable bottle by weighing out the appropriate amount into the bottle and then adding 100 mL DI water. You will need to calculate the amount of each substance to weigh out based on the concentration, volume, and formula weight.

***For adenosine monophosphate, only make 10 mL and sterilize by sterile filtering.

Seawater Plate Recipe (with 1.5% agar)

		Stock solution				
Quantity per 500 mL media	**Compound**	**Concentration**	**Per 1 L**	**Per 100 mL**	**Per 50 mL**	**Sterilize**
75% (375 mL)	Seawater	No stock solutions				Autoclave
1.5% (7.5 g)	Agar					Autoclave
1.0 mL	$NaH_2PO_4 \cdot H_20$	25 mM in dH_2O	3.45 g	345 mg	172.5 mg	Autoclave
0.5 mL	$NaNO_3$	0.882 M in dH_2O	75 g	750 mg	375 mg	Filter
2.5 mL	$Na_2EDTA \cdot 2H_20$	2.686 mM in dH_2O	1 g	100 mg	50 mg	Autoclave
0.5 mL	SN Trace metals	Borrow from Martiny Lab				Filter
1.5 mL (1 mM final conc.)	Adenosine 5' monophosphate	0.33 M ***	114.6 g	11.5 g	5.75 g	Filter
5.0 mL (1 mM final conc.)	Asparagine	100 mM in dH_2O	15 g	1.5 g	750 mg	Filter

*** Dissolve 347 mg of AMP in 1.5 mL 1 M NaOH; Add 1.5 mL dH_2O for final concentration of 0.33 M in 3 mL total volume

(OR Dissolve 1.15 g of AMP in 5 mL 1 M NaOH; Add 5 mL dH_2O for final concentration of 0.33 M in 10 mL volume)

B. Growth media for agar plates

- You will need a separate flask for each different substrate type and concentration you plan to test
- Substrates: Asparagine (1 mM final conc.), Adenosine monophosphate (1 mM final conc.)

1. Weigh out agar at a rate of 15 g/L (1.5% final volume)
2. Add magnetic stir bar to container
3. Wash agar 3 times with DI water
   a. Stir briefly for 1-2 minutes
   b. Let agar settle to the bottom of the container
   c. Siphon clean water from surface with vacuum trap
4. Add 75% seawater
5. Bring to volume with DI water if necessary
6. Autoclave 15 min at 121˚C

7. Let cool to ~50ºC
8. While stirring gently on magnetic stirrer, add appropriate amounts of sterile amendments (phosphate, nitrate, and trace element solutions)
9. Add appropriate amount of sterile carbon amendment to obtain correct final concentration
10. Add 200 µL sterile 1 M NaOH for every 500 mL asparagine media and 350 µL sterile 1 M NaOH for every 500 mL adenosine monophosphate media (to correct pH)
11. Pour plates and let cool; Store upside-down in humid environment

C. Seawater sampling and filtration

1. Autoclave all components of the 47 mm seawater filtration apparatus and the 47 mm GF/D filters, or autoclave with filter already in place. Use sterile tweezers if you move the filter.
2. Obtain 2 sterile sampling vessels (50 mL centrifuge tubes) and bring to the field
3. Fill each vessel with fresh seawater from the bucket (pour, do not scoop)
4. Filter each sample of seawater through a separate GF/D filter.
5. Place the filter in a sterile 50 mL centrifuge tube. Resuspend the trapped particles from the filter in 25 mL filtered, sterilized seawater by vortexing for 2 min.
6. Pour ~25 mL filtrate into separate 50 mL centrifuge tube. Discard the remaining filtrate.

D. Plate inoculation

- The 25 mL filtrate is the inoculum of "free" cells, while the suspended filter material is the inoculum of "attached" cells
- Use sterile technique

1. Add 50 µL free cell inoculum to each type of agar plate. Use undiluted, 10-fold diluted, and 100-fold diluted inoculum. Dilute in sterilized, filtered seawater.
2. Add 50 µL attached cell inoculum to each type of agar plate. Use undiluted, 10-fold diluted, and 100-fold diluted inoculum. Dilute in sterilized, filtered seawater.
3. Incubate plates for >1 week at room temperature
4. Streak plates on the same type of agar plate to obtain single colonies

E. Liquid cultures (*Not yet revised*)

- Pipet 230 µl liquid seawater medium into 96 wells of 4 sterile, clear microplates
- Pick up to 90 colonies with sterile toothpicks and transfer to microplate wells. Transfer one colony to duplicate microplates. Leave 6 wells uninoculated as controls on each plate.
- Incubate plates at room temperature on microplate shaker at 100 rpm for up to 5 days, reading optical density at 600 nm every 24 h

F. Enzyme screening (*Not yet revised*)

- On the last day of incubation, read optical density and transfer 5 µl of each well into a new plate with identical seawater medium
- Transfer another 5 µl into liquid seawater medium with 5 mM complex substrate
- Add enzyme substrate solutions for alkaline phosphatase to one set of plates and substrate for leucine aminopeptidase to the other set. Follow protocol for seawater enzyme assays.