Isolating and Imaging Horseshoe Crab Symbionts

Miniproject for the MBL Microbial Diversity Course 2017

Mette Busck
Aarhus University
mette.busck@bios.au.dk

Table of Contents

1 Summary

Because of a personal interest in bacterial symbionts of arthropods I set out to identify microbial symbionts in the digestive system and on the surface of juvenile Atlantic horseshoe crabs (*Limulus Polyphemus*), collected at Sippewissett salt marsh. 16 bacterial strains were isolated, and 12 of these identified. Fluorescent in situ hybridization imaging showed bacteria colonizing the gut wall and surface of a 5mm juvenile horseshoe crab.

2 Introduction

All animals live in close association with bacteria (Gilbert et al. 2012) and marine invertebrates are no exception (Dubilier et al. 2008). The Atlantic horseshoe crab is well studied for a marine invertebrate, but not much is known about horseshoe crab associated bacteria (Ismail et al. 2015; Nolan & Smith 2009). The two main reasons I find the horseshoe crabs and their possible microbiota particularly interesting are the crabs' very peculiar immune response and their status as "living fossils". Limulus amebocytes react to even the slightest contact with gram negative bacteria by inducing a very fast and extensive vascular clotting (Shirodkar et al. 1960). This is taken advantage of in the limulus amebocyte lysate test for gram negative bacteria, and raises the bar for any true endosymbiotic relationship which would either have to be very specifically adapted, or only involve gram positive bacteria. Horseshoe crabs are seen as "living fossils" because their basic body plan has remained largely unchanged for 450 million years. Assuming that this is not only a superficial conservation, this opens the possibility of very long evolutionary history with possible symbionts.

3 Methods

3.1 Animals

Juvenile horseshoe crabs (*Limulus Polyphemus*), were caught in Sippewissett salt marsh (41°34'31.8"N 70°38'17.0"W) on July 28th (crab fuzz) and August 9th (guts and poop) 2017. Crabs were kept in filtered sea water at room temperature, and were not fed before samples were taken. Several lab-hatched juvenile crabs were given to me by Dr. Dan Gibson who also helped me catch the wild crabs. All larger crabs (above 8mm) were set free again at Sippewissett after sampling.

3.2 Enrichments and isolation

Four different inocula were used: 1) A Poop sample taken from a 27mm crab shortly after capture. 2 & 3) diverticula and hindgut dissected from the same 5mm crab on the day of capture. 4) Bacterial growth ("crab fuzz") which grew on the surface of a 35mm crab after 10 days in the lab. Inoculum 1-3 were washed in several baths of filtered seawater. 11 different enrichments were set up in dilution series. See *Table 1* Anoxic conditions were obtained by collecting and homogenizing tissues in a medium with added cysteine (100mM), followed by enrichment in sealed Balch tubes flushed under a stream of N_2/CO_2 (80%/20%) gas. Pasteurization was done by placing samples at 65 °C for an hour.

Enrichment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Inoculum	Poop				Hindgut				Diverticula		Crab Fuzz
O2	yes	no	yes	no	yes	no	yes	no	yes	no	yes
Pasteurized	yes	yes	no	no	yes	yes	no	no	no	no	no

Table 1 Enrichment specifications

The SWC (see appendix 1A) medium was used for oxic enrichment and culture, and a specially made "SAS" medium (*Table 2*) was used for the anoxic enrichment and culture.

Salty Anaerobic Symbiont medium (SAS)		
Concentration	**Ingredient**	**Role**
1mM	MOPS (ph7)	buffer
250uM	KPHOS	Phosphate
1mM	NH_4Cl	Nitrogen
1g/L	Tryptone	Amino acids
1g/L	Yeast extract	Trace minerals and metals
The rest	1x SW base	Sea water salinity
Add filtered after autoclave		
5mM	$NaHCO_3$	Carbon for autotrophs
1mM	Sodium Sulfide	Sulfur and possible e-donor
10mM	Maltose solution	Carbon and e- donor

Table 2 Medium for anoxic enrichment

Each enrichment was diluted in series to 10^{-5}. The oxic dilutions were spreadplated right away, anoxic dilutions were left until obvious turbidity and single dilutions were picked for new dilution series in shaketubes with 1% agar. All incubations were at room temperature. Single colonies were picked and restreaked (or re-suspended in shaketubes) and colonies from pure cultures were picked for 16S sequencing.

3.3 16S colony PCR and sequencing

PCR amplification of 16S variable regions V1-V8 was done according to lab manual – see appendix 1B.

PCR products were run on agarose gels to check for DNA of the proper size. Then products were cleaned and sent to sequencing.

A quick maximum likelihood tree was made in MEGA 7 (Kumar et al. 2016) using the Muscle aligner.

3.4 MiPACT

A 5mm lab-hatched crab was killed by drowning in ethanol and washed in several baths of filtered sea water. The dorsal carapace of the prosoma (front of the body) was removed to expose the tissues underneath. Microbial identification after passive clarity technique passive clarity technique (MiPACT) was performed as presented in (DePas et al. 2016) with the following modifications: worms were fixed using 4% paraformaldehyde overnight at 4C, washed 3x in PBS, and then embedded in polyacrylamide gel using 1.5mL eppendorf tubes as a mold (fixed and washed worms were placed in 4% 29:1 acrylamide:bis-acrylamide and 0.25% VA_044 hardener in 1xPBS overnight at 4C, then incubated at room temperature in an anaerobic hood for ~5 m with the tube cap open to remove oxygen, and finally hardened by incubate in a 37C water bath for 12 hours). Embedded worms were carefully removed from eppendorf tubes and placed in 50mL Falcon tubes with 10mL of 8% SDS in 1X PBS for 5 days to clear lipids (only partial clearing was seen).

3.5 Cryosections

A 5mm Juvenile horseshoe crab was killed by drowning in ethanol. The crab was washed in filtered PBS, holes were cut in the carapace to let in the fixative, and the crab was fixed in 4% paraformaldehyde overnight at 4°C. The crab was washed in filtered PBS and frozen in Tissue Tek by dipping in liquid nitrogen. It was left at -20°C for a day before cryosectioning. 20μm sections were cut at -20°C on a Microm HM 505N Cryostat at the MBL Central Microscopy Facility. Sections were mounted on a poly lysine covered glass slide by simply touching the side to the frozen section. After air drying, slides were washed in milliQ to get rid of the Tissue Tek and dehydrated for 1-3 minutes in each of 50-75-96% ethanol.

3.6 General Fluorescence in situ hybridization (FISH)

I had enough slides with horseshoe crab cryosections to test 3 types of FISH: monoFISH CARD-FISH and HCR-FISH. Each method was done on independent glass slides with sections separated by XXX pen. monoFISH was done with Cy3 labeled EUB probe, CARD-FISH with horseradish peroxidase labeled EUB I-III probe and Alexa594 labeled tyramide, and HCR-FISH with EUB338 probe and Alexa594 labeled hairpins. Control sections were made on each slide, including empty (no stain at all), NON338 (fluorophore on nonsense probe), only DAPI, and, for the CARD-FISH slide, non-permeablized and only tyramide-treated(no probe).

Permeabilization, hybridization, and amplification were all done with drops of buffer on each section and the whole slide incubated in 50mL falcon tube "chambers" with buffer soaked paper towels. Washes and inactivation were done by submersion of whole slides in the liquids.

3.7 Cryosection CARD FISH

Because it worked the best, here is a detailed description of the CARD-FISH method (slightly modified from course manual)

Permeabilization

Lysozyme solution: 10mg/mL lysozyme in MilliQ with 0.05M EDTA and 0.1 M Tris-HCl.
Incubation: 20µL on each tissue section, 30 minutes at 35°C
Wash: 1 minute each in MilliQ and 96% EtOH

Inactivation of endogenous peroxidases

Buffer: 0.15% H_2O_2 in Methanol
Incubation: slide fully immersed 30 minutes at RT
Wash: 1 minute each in MilliQ and 96% EtOH
Let dry

CARD hybridization buffer (final volume 20mL)

- 3.6 mL 5 M NaCl
- 0.4 mL 1 M Tris HCl (pH 8.0)
- 20 µL SDS (20% w/v)
- 2.0 mL blocking solution (10% blocking reagent (Roche) in buffer of 100mM maleic acid, 150mM NaCl, pH 7.5)
- 7 mL formamide and 7mL milliQ (->35% formamide for EUB probe)
- 2.0 g of dextran sulfate
- Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. Small portions of the buffer can then be stored at –20°C for several months.

Buffer and HPR-labeled probe working solution (50 ng DNA/µl) is mixed 300:1 (be careful and do not pipet up and down too much, or it will break)
Incubation: 60µL drop on each tissue section, at least 2 hours at 46°C

CARD washing buffer (final volume 50mL)

- 700µL 5M NaCl
- 1mL 1M Tris HCl
- 500µL 0.5M EDTA
- 25µL 20% SDS (add last to avoid precipitation)
- milliQ to 50 mL

Incubation: slide fully immersed, 10 minutes at 48°C
Wash in filtered 1xPBS for 10 minutes at 48°C
Do not let it dry completely!

CARD amplification buffer (final volume 40 mL)

- 4 mL of 10 × PBS, pH 7.4 (important for proper enzyme function)

- 0.4 mL blocking solution
- 16 mL 5 M NaCl
- milliQ to 50 mL
- Add 4 g of dextran sulfate
- Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. The amplification buffer can be stored in the refrigerator for several weeks.

Mix amplification buffer with freshly made 0.15% H_2O_2 100:1. Add 1 µL of fluorescently labeled tyramide (1 mg/mL), volume of tyramide can be adjusted if too low.
Incubation: drops on each tissue section, 40 minutes at 46°C.
Wash in filtered 1xPBS 10 minutes, milliQ, 96% EtOH, let dry.
Remember to keep slides in dark after fluorophore labeling!

DAPI staining

DAPI with Citifluor/Vectashield mounting medium (final DAPI concentration: 1µg/mL) was spotted on to each tissue section and immediately coverslipped and sealed at the edges with clear nail polish.

3.8 MiPACT CARD-FISH

I also did CARD FISH on the partially cleared MiPACT sample, skipping the ethanol washes and keeping the sample in its hydrogel, submerging the whole thing in buffers in eppendorph tubes. A note on inactivation: DO NOT USE METHANOL ON MiPACT SAMPLES – dehydration will result in gel opacity and tissue shriveling. This can be at least partially reversed by submersion in PBS, but may have damaged the sample.

3.9 Imaging

All microscopy images were acquired with ZEN and processed with Fiji (Schindelin et al. 2012).

Fluorescence microscopy was done on a Zeiss Axioplan A.2, and by Laser Scanning Confocal Microscopy on a Zeiss LSM 880 Confocal with FAST Airyscan.

4 Results and Discussion

4.1 Enrichments and isolates

I successfully isolated 16 microbial strains. For 12/16 if the strains partial 16S gene was successfully sequenced. The surface growth and the poop proved to be the best source of isolates. These were also the inocula that looked most densely colonized from the start. Nothing was isolated from the diverticula. Nothing ever grew in the dilution series or on any of the plates. Only a single isolate was obtained from the gut, there was a lot of growth in the gut enrichments, but it was very uniform in colony morphology, and tended to overgrow every spread plate before single colonies could be picked.

Table 3 lists colony and cell morphology of the isolates, along with the best hit from a blast of 16S sequences and *Figure 1* shows a phylogenetic tree of the isolates which worked for sequencing.

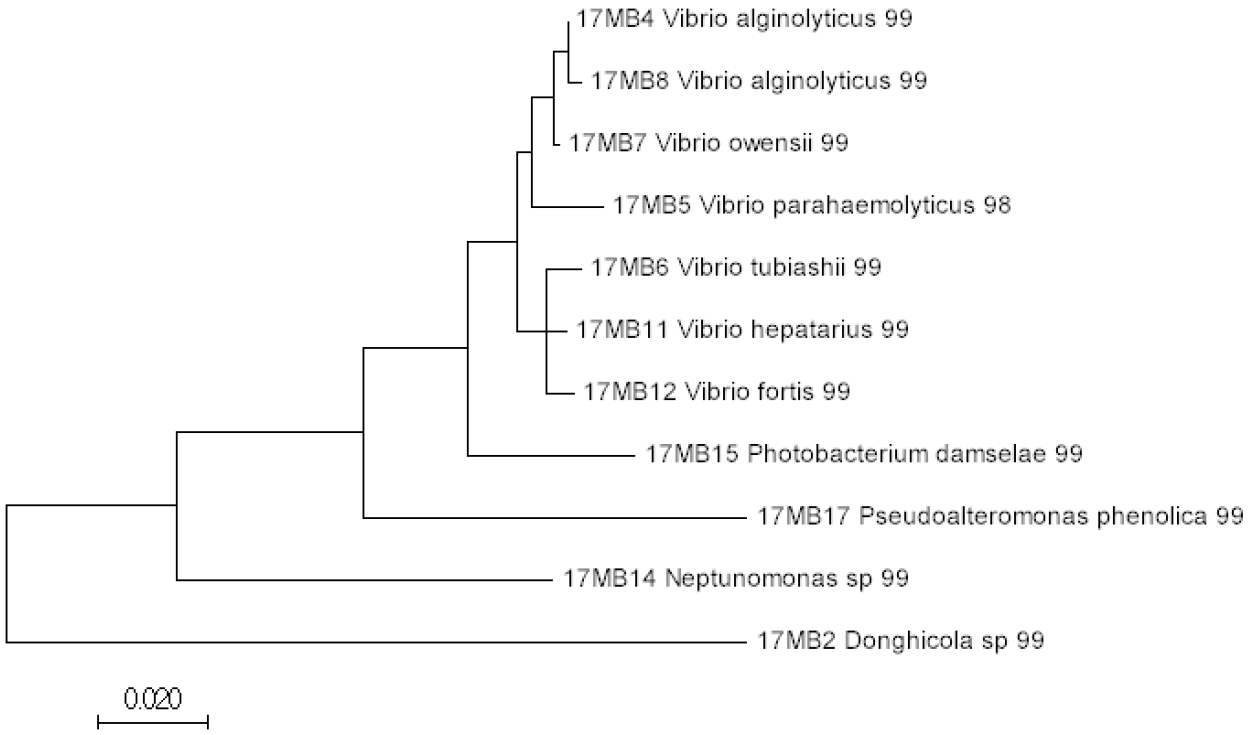
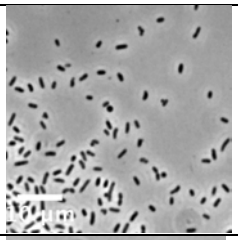
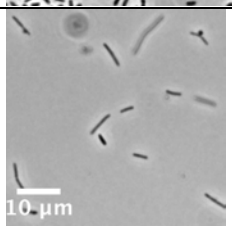
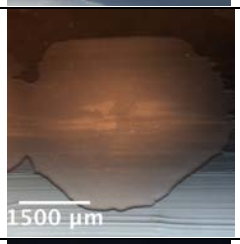
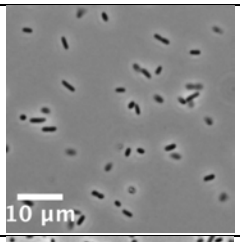
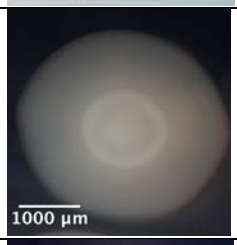
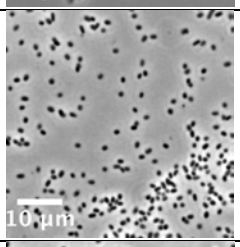
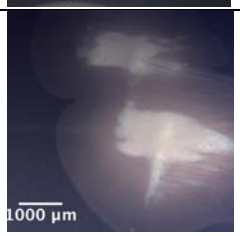
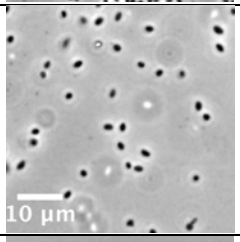
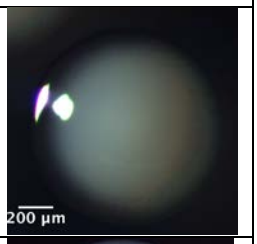
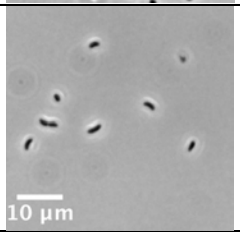
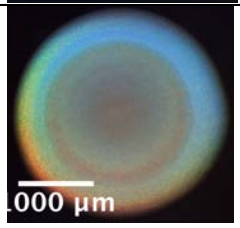
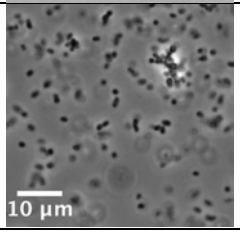
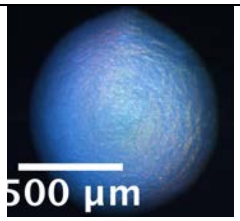


Figure 1 Molecular Phylogenetic analysis by Maximum Likelihood method Caption curtesy of MEGA 7: *The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993).The tree with the highest log likelihood (-2539.9820) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 754 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)*. Names show Isolate ID, name of closest match according to blast and percent match.

Isolate ID	Isolation source	Enrichment	Best match 16S	Cell morphology	Colony morphology
17MB4	Crab Fuzz	oxic	*Vibrio alginolyticus* 99%		
17MB8	Poop	oxic	*Vibrio alginolyticus* 99%		
17MB7	Poop	ocix	*Vibrio owensii* 99%		
17MB5	Crab Fuzz	oxic	*Vibrio parahaemolyticus* 98%		
17MB6	Poop	oxic	*Vibrio tubiashii* 99%		
17MB11	Poop	oxic	*Vibrio hepatarius* 99%		
17MB12	Poop	oxic	*Vibrio fortis* 99%		

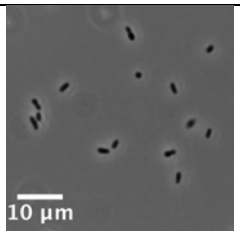
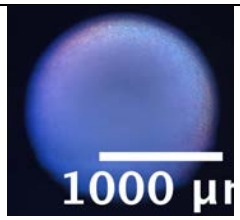
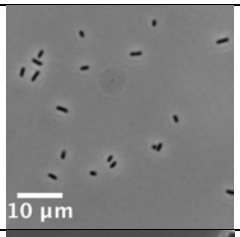
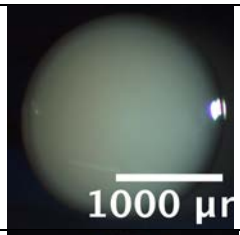
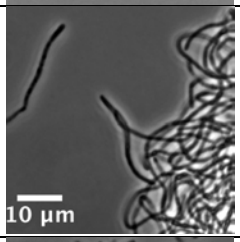
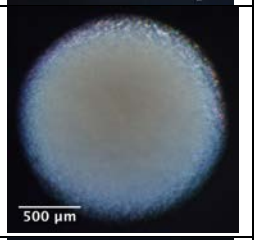
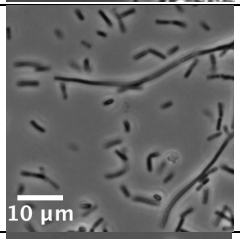
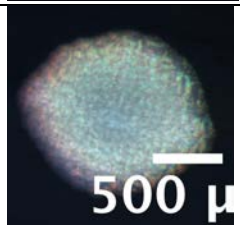
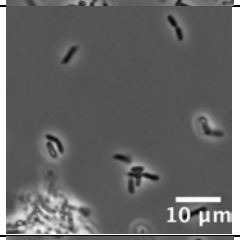
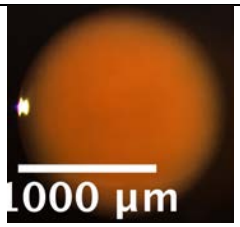
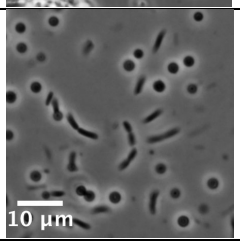
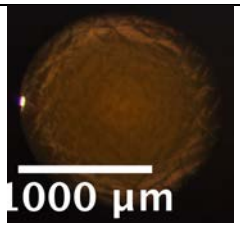
Isolate ID	Isolation source	Enrichment	Best match 16S	Cell morphology	Colony morphology
17MB15	Poop	anoxic	*Photobacterium damselae* 99%	NA	NA
17MB17	Gut	oxic	*Pseudoalteromonas phenolica* 99%		
17MB14	Crab Fuzz	oxic	*Neptunomonas* sp. 99%		
17MB2	Crab Fuzz	oxic	*Donghicola* sp. 99%		
17MB13	Poop	oxic	Sequencing reaction failed		
17MB9	Poop	oxic, pasteurized	Sequencing reaction failed		
17MB10	Poop	oxic, pasteurized	Sequencing reaction failed		
17MB16	Poop	anoxic, pasteurized	Sequencing reaction failed	NA	NA

Table 3 Isolate Morphology and identity

It is notable that out of the 4 isolates which failed in sequencing, 3 were selected for by pasteurization. If these isolates were indeed sporeformers, this may have interfered with sequencing in some way.
The fact that fewer isolates were obtained from the anoxic enrichments, may be more due to the fact that this was my first time working with anoxic conditions, and not because less anaerobic bacteria are associated with the horseshoe crab. In light of my FISH findings, if I were to redo these experiments, I would focus more on the anoxic and maybe a microaerophilic enrichment too.

The identities of my isolates make a lot of sense, as most of them are from genera previously found to be associated with limulus (Nolan & Smith 2009), or other species of horseshoe crab (Ismail et al. 2015). Two of the isolates (*Vibrio fortis* and *Neptunomonas* sp.) are most closely related to bacteria found freely in the watercolumn. The rest are most closely related to bacteria isolated from marine invertebrates. Many of them are pathogens. It is possible that both of these facts reflect a sequencing bias.

4.2 MiPACT – a promising beginning

The tissue clearing worked at least in part (see *Figure 2*), but the combination of a lacking depth of field in the confocal microscope, and problems with the CARD-FISH staining (see section 3.8)

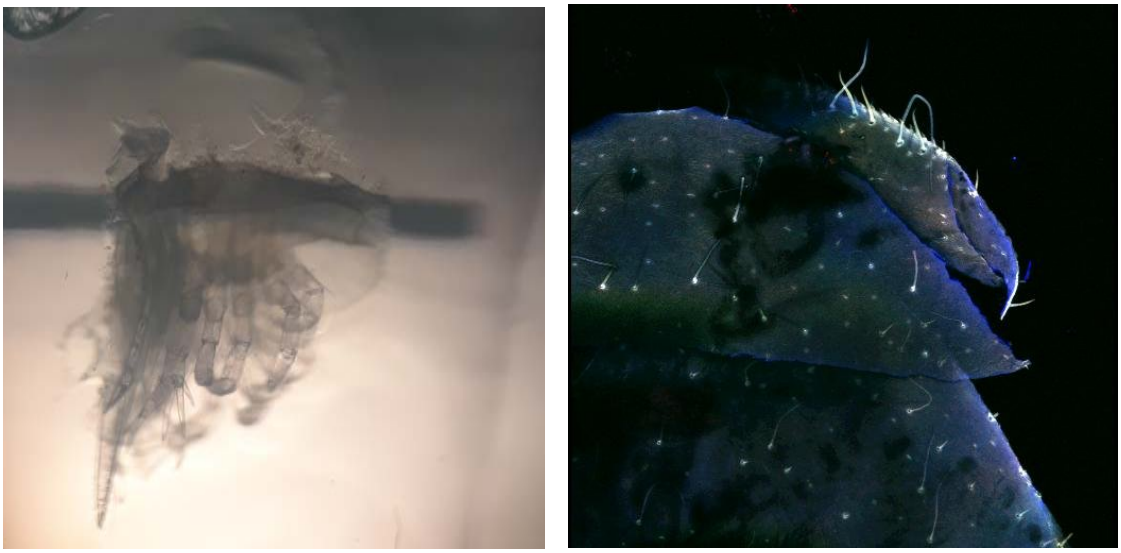


Figure 2 (left): Partially cleared 5mm horseshoe crab (you can see the line through it) (right): Confocal 3D projection of crab shell and claw

Figure 2 shows the only image of the cleared sample obtained at the confocal: A 3D projection of a z-stack with the 10x objective. Only the autofluorescent chitin was clearly visible. The image does show a beautiful resolution, and with access to special objectives and optimization of the MiPACT protocol for the sample type, this method could be extremely valuable in understanding spatial relationships of symbionts on hosts.

4.3 FISH on cryosections – anatomy and autofluorescence

Of the three FISH methods, monoFISH and CARD-FISH worked best. HCR-FISH was only attempted on one slide, and many tissue sections were lost during the protocol, making it difficult to evaluate how well it worked.

The anatomy of the sections was reasonably well preserved (*Figure 3*), and even with quite a lot of autofluorescent tissue, the probes and DAPI stain were clearly identifiable.

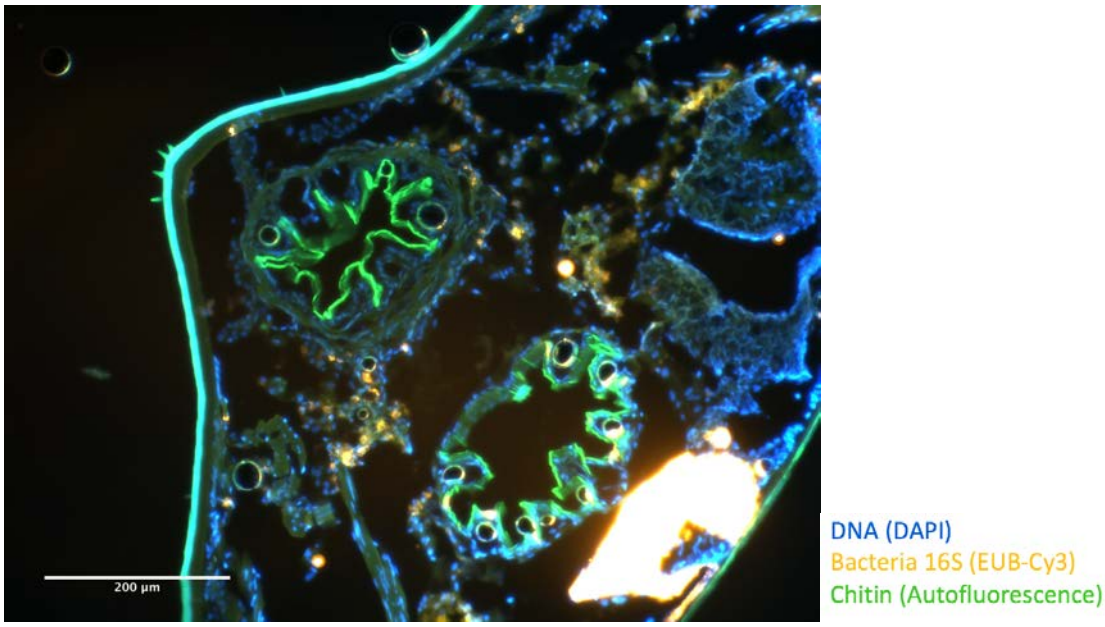


Figure 3 monoFISH with EUB probe on a anterior crossectional section of horseshoecrab, showing how the ailmentary canal turns back on itself (=2xgut lumen), and a lot of autofluorescence.

4.4 FISH on cryosections - Non specific binding in intracellular granules

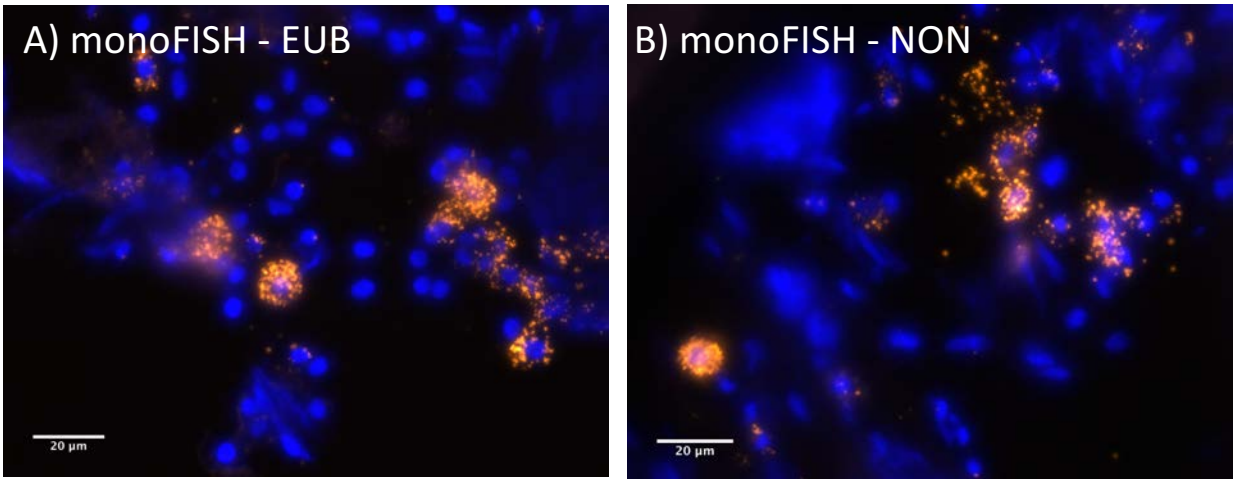


Figure 4 monoFISH with EUB probe and with NON probe show nonspecific binding of intracellular granules

An interesting cell type was observed in all of the tissue sections. (*Figure 4*). Host cells with clearly DAPI stained nuclei contain granules of an unknown nature. First thought to be bacteriocytes, the granules in these cells had a high degree of unspecific binding of the probe in monoFISH (*Figure 5*)

meaning that they are probably not bacterial. The same pattern was found in CARD-FISH (not shown).

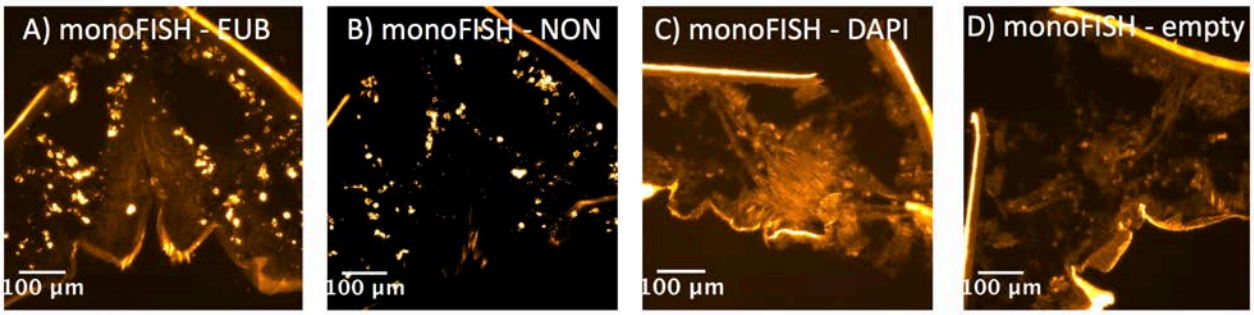


Figure 5 monoFISH with EUB probe and three controls show nonspecific binding of probe in certain host cells.

The cells are found throughout the tissue, but never in the lumen of gut or diverticula. A close up (*Figure 6*) was taken to attempt comparison with TEM images from Microscopic Anatomy of Invertebrates volume 8A (Fahrenbach 1999) – but no conclusion about cell type could be made.

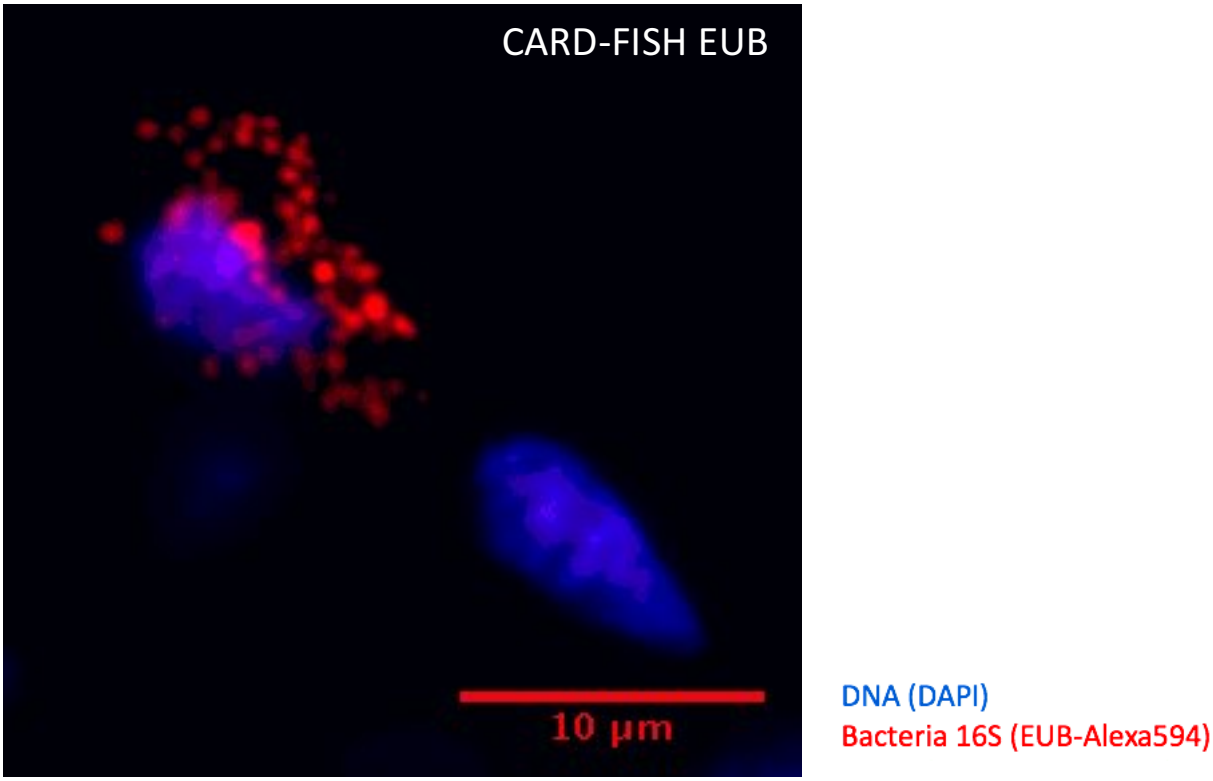


Figure 6 Z-stack average of cell with granules

4.5 FISH on cryosections - Bacteria on the outside

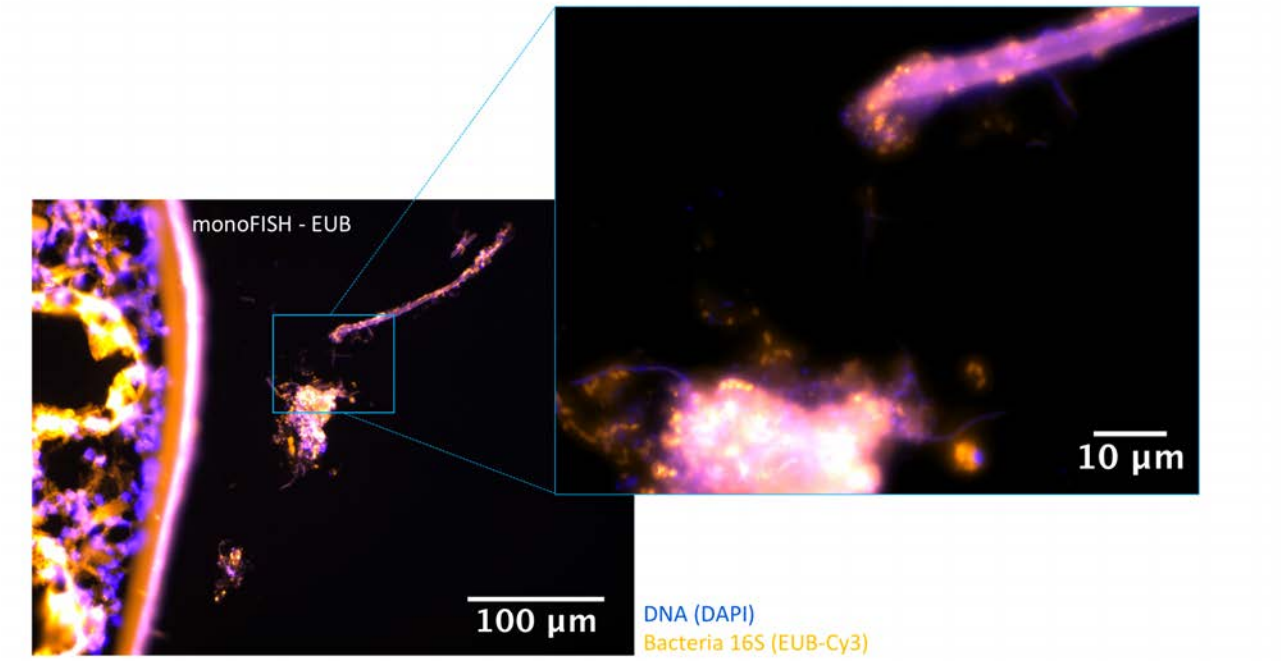


Figure 7 bacteria outside the horseshoe crab tissue and on a hair from the horseshoe crab

Figure 7 shows the combination of DAPI and Cy3 on the same cells indication eubacteria on the outside of the horseshoe crab. In the NON control, aggregates like this were only stained in DAPI (*Figure 8*). Images are from monoFISH, but a similar pattern was seen in the CARD FISH tissue sections.

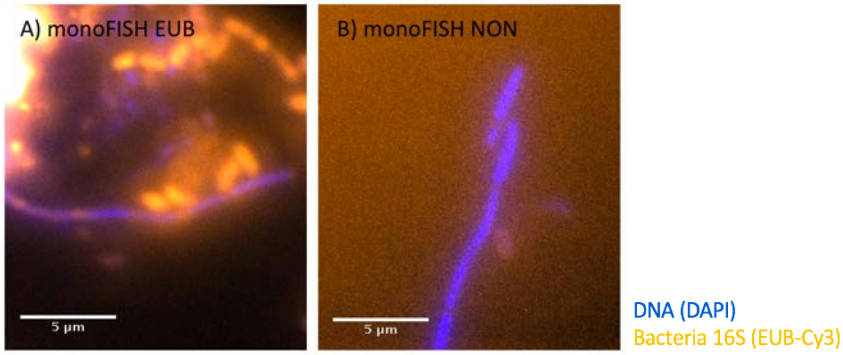


Figure 8 bacteria in EUB tissue section (A) are fluorescent in both DAPI and Cy3, in the NON tissue section (B) they are only fluorescent in DAPI (notice the orange background as the Cy3 intensity is set to max, and still no spots of staining is seen).

4.6 CARD-FISH on cryosections - Gut wall bacteria

The final and most interesting finding in this project, is evidence of bacteria colonizing a patch of the horseshoe crab gut wall. Figure 9 shows increasing magnifications of a patch on the lumen side of the chitinous gut wall which was brightly fluorescent in red from the alexa594 EUB probe. Several of these patches were seen in the EUB stained tissue sections, but never in the NON, DAPI-only or Tyramide-only sections. A further line of evidence that this is indeed bacteria, is the co-localization with DAPI staining. This was only possible to see at high magnification (63x objective) on the confocal microscope, as autofluorescence and host cell DAPI staining otherwise tended to drown out the signal.

With only one isolate from the gut, I don't have much to compare the observed bacteria to. The cells from the gut isolate have the right shape, but seem to be a bit larger than the bacteria observed by FISH (compare *Table 3*: 17MB17 and *Figure 9*).

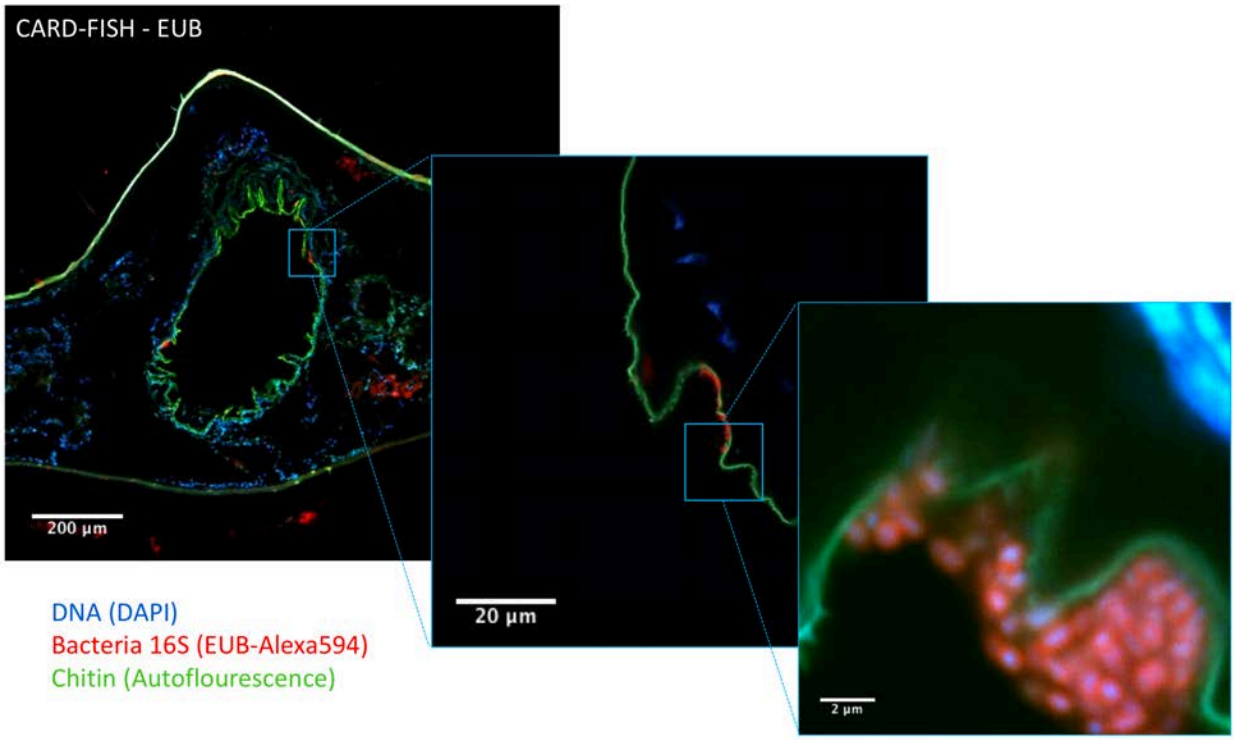


Figure 9 The money shot.

5 Conclusions and Outlook

The 16 bacterial strains isolated from horseshoe crab are similar to bacteria associated with other marine invertebrates, but whether the association is more than transient or the bacteria are parasitic, commensal or beneficial would need much more investigation to answer. The FISH imaging of bacteria colonizing a pocket on the gut wall of the horseshoe crab show that bacteria are not just passing through, but actually colonizing the internal surface of the crab.

There are interesting bacterial species associated with the Atlantic horseshoe crab, and I believe that my findings warrant further investigation. A first step could be designing FISH probes to specifically locate bacteria from the isolated species.

6 Acknowledgements

I would like to thank everyone involved in the Microbial Diversity course at MBL especially Dianne, Jared and Viola. A huge thank you to Dr. Dan Gibson for help with everything horseshoe crab and for being so welcoming and motivation to talk to. I am deeply grateful to the Simons foundation for making my participation in this course a possibility, and to all the companies who have donated equipment to the course – especially Zeiss for their awesome microscopes. My supervisor Andreas Schramm for recommending the course to me, for help with funding and for encouraging me to take the time to participate. Lastly, I would like to thank my fellow students of the course for making these 6 weeks so memorable.

7 References

DePas, W.H. et al., 2016. Exposing the Three-Dimensional Biogeography and Metabolic States of Pathogens in Cystic Fibrosis Sputum via Hydrogel Embedding , Clearing , and rRNA Labeling. , 7(5), pp.1–11.

Dubilier, N., Bergin, C. & Lott, C., 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology*, 6(10), pp.725–740. Available at: http://www.nature.com/doifinder/10.1038/nrmicro1992.

Fahrenbach, W.H., 1999. Merostomata. In F. W. Harrison & R. F. Foelix, eds. *Microscopic Anatomy of Invertebrates, volume 8A*. Wiley-Liss, pp. 21–117.

Gilbert, S.F., Sapp, J. & Tauber, A.I., 2012. A Symbiotic View of Life : We Have Never Been Individuals. *The Quarterly Review of Biology*, 87(4), pp.325–341.

Ismail, N. et al., 2015. Marine Bacteria Associated with Horseshoe Crabs, Tachypleus gigas and Carcinoscorpius rotundicauda. In *Changing Global Perspectives on Horseshoe Crab Biology, Conservation and Management*. Springer International Publishing, pp. 313–320.

Kumar, S., Stecher, G. & Tamura, K., 2016. MEGA7 : Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets Brief communication. *Molecular Biology and Evolution*, 33(7), pp.1870–1874.

Nolan, M.W. & Smith, S.A., 2009. Clinical Evaluation , Common Diseases , and Veterinary Care of the Horseshoe Crab , Limulus polyphemus. In J. Tanacredi, M. Botton, & D. Smith, eds. *Biology and Conservation of Horseshoe Crabs*. New York: Springer Publishing, pp. 479–499.

Schindelin, J. et al., 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7).

Shirodkar, M. V, Warwick, A. & Bang, F.B., 1960. The in Vitro Reaction of Limulus amebocytes to Bacteria. *Biological Bulletin*, 118(2), pp.324–337.

Tamura, K. & Nei, M., 1993. Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees. *Molecular Biology and Evolution*, 10(3).

Wetter, M. et al., 2008. Osmoregulation and acid base regulation of the Asian Horseshoe crab Carcinoscorpius rotundicauda. *Annual Main Meeting of the Society of Experimental Biology, Abstracts / Comparative Biochemistry and Physiology*, Part A 150, pp.S97–S114.

Appendix 1

APPENDIX 1A – Seawater base and Seawater Complete
Excerpt from 2017 MBL MicDiv Lab Manual

1X Seawater ("SW") Base (per 20 liters)

Component	Amount	FW	Final Conc.
NaCl	400 g	58.44	342.2 mM
$MgCl2 \cdot 6H2O$	60 g	203.30	14.8 mM
$CaCl2 \cdot 2H2O$	3 g	147.02	1.0 mM
KCl	10 g	74.56	6.71 mM

CULTIVATION MEDIUM - "SWC" Sea Water Complete
1X SW (seawater base) 1000 ml
Bacto tryptone 5 g
yeast extract 1 g
glycerol 3 ml
1 M MOPS, pH 7.2 5 mL
agar (for plates) 15 g

APPENDIX 1B
Slightly modified excerpt from 2017 MBL MicDiv Lab Manual

Preparation of genomic DNA from bacterial colonies
1. Touch a colony with a 10-µl tip or sterile toothpick and suspend the material in 20µl of ALP reagent (alkaline PEG200)
2. Boil the ALP cell suspensions to lyse the cells and inactivate bacterial enzymes. The PCR machines are set-up for this step with a program entitled "BOIL".
3. Vortex briefly and pellet the cellular debris by a quick spin in a microcentrifuge. The supernatant contains your bacterial DNA in solution. Use 2 µl of this supernatant for small subunit (ssu) rDNA PCR (below).
NOTE: Cell material does not have to be visible on the pipette tip or toothpick (due to the sensitivity of PCR). In fact, too much cell material can be inhibitory to the PCR reaction. Resist the urge to pick too much cell material.

PCR Amplification
The components below should be combined into one master solution. For multiple reactions combine the appropriate factor of the volumes below and aliquot to individual tubes accordingly.

Master Mix recipe for one reaction:
25.0 µl Promega GoTaq® G2 Hot Start Green Master Mix
1.0 µl 16S_8F (15 pmol) – for Bacteria
1.0 µl 16S_1391R (15 pmol) – for Bacteria
20.0 µl Nuclease-free water
---

47 µl Total Volume

Prepare PCR reactions:
1. Immediately before running PCR reaction, aliquot 47 µl of the above master solution into individual 0.2 mL PCR reaction tubes. Label each tube and record in your notebook the template for each PCR reaction.
2. To each tube add 3 µl from one of your DNA samples. (Note: you may want to do a 1:10 dilution of your DNA supernatant if the reaction fails)
3. For the positive control add 3 µl control DNA.
4. Add 3 µl of nuclease free water to your negative control tube.
5. Place your PCR tubes within the PCR thermocycler.

The PCR thermocycler program should be set up as follows (for 8F/1492R):

Step	Temperature	Time
Initial Denaturation	95 °C	2 min.
Denaturation	95 °C	30 sec.
Annealing	55 °C	30 sec.
Extension	72 °C	1.5 min.
Final Extension	72 °C	10 min.
Hold	12°C	∞

30 cycles.