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Preliminary Microbiome Analysis of Freshwater Bivalves from Turnbull National Wildlife Refuge

Dechen Edwards

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INTRODUCTION

Eukaryotic hosts and their associated microbes exhibit variable relationships.(1) Some are driven by well-documented benefits including microbial contributions to host digestion; others are less understood.(1, 2) In this project, we seek to understand how a host's microbiome is differentiated from the surrounding, free-living microbial community and whether this differentiation is altered by the presence of pollution. As a first step, we present a protocol developed for the extraction, isolation, and identification of the microbial population found in freshwater "fingernail" clams (*Sphaeriidae*).

Our methodology is based on methods from the Earth Microbiome Project (EMP) and studies of both freshwater amphibian and marine bivalve microbiomes. (3, 4, 5) This protocol is appropriate for downstream microbial DNA extraction and analysis via next-generation sequencing and bioinformatics platforms.

OBJECTIVES

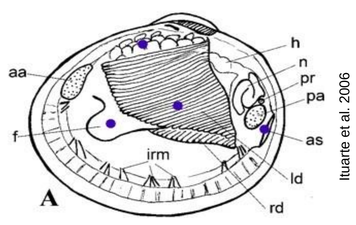
- Develop methodology for collection and dissection of individual clams
- Develop methodology for bacterial DNA extraction from whole clam tissue facilitating downstream analysis of clam microbiome

FINGERNAIL CLAMS

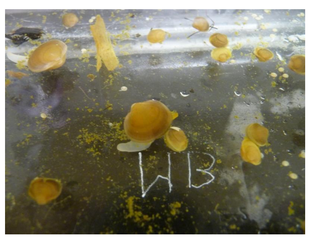
- Cosmopolitan, bioindicator species
- Filter-feed at sediment-water interfaces
- Hermaphroditic, brood internally
- In constant contact with environment via gill filtration
- May contain resident microbes in foot (f), gill (ld/rd), siphon (as), and digestive gland (unlabeled) (Fig 1A) based on marine bacterial isolate studies
- Using whole-clam tissue for this project as clams are small

DISSECTION METHODS AND RESULTS

- Sampled fingernail clams from TLES pond
- Gathered by sweep netting near emergent plant matter; clams are patchily abundant
- Retained individuals over 5mm in shell length

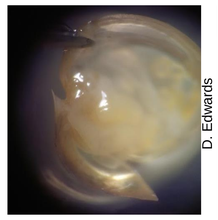


Ituarte et al. 2006



J. Matos lab

Figure 1. (L-R) Fingernail clam internal anatomy, purple dot indicates likely microbial habitat based on marine bivalve microbiome studies; live fingernail clams (larger) and extruded juvenile clams



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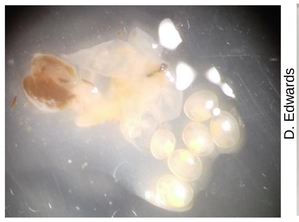

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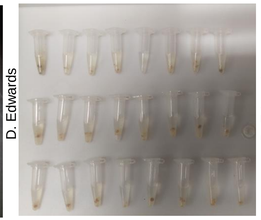

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Figure 2: (L-R) partially dissected clam, dissected clam with shell removed showing brood, individual clam tubes for post-dissection processing

- Shell removal, brood separation and count (Fig 2)
- Dissected based on methods from 2011 paper (6) altered to reduce shell shattering
- Tested dissection in multiple fluids:
- deionized water; made clam tissue sticky and tough
- lysis buffer used for initial DNA extraction step; evaporated quickly under the dissecting scope light
- TLES pond water; pond water from the clam sampling location
- Pond water dissection yielded best results; minimal evaporation and tissues remain flexible
- After dissection, whole clam tissue in tube for freezing or immediate DNA extraction
- First DNA extraction and PCR showed bacterial DNA but increasing contamination in dissection controls
- Adjusted dissection procedures, used autoclaved liquid components and new tool sterilization procedure
- Subsequent controls did not have contamination

MOLECULAR METHODS AND RESULTS

- Extracted DNA from whole clam tissue (DNeasy Blood and Tissue Kit)
- Quantified DNA using Qubit 2.0 Fluorometer (Table 1)
- PCR using universal 16 rRNA primers to amplify only bacterial DNA

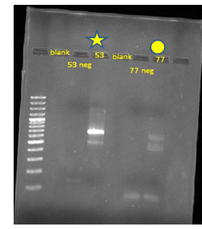
Sample	Type	Sample DNA	Estimated whole DNA
T01	Fresh, H20	4.02 ug/mL	80.4 ug/mL
T02	Control	<0.010	
T03	Fresh, H20	2.98	59.7
T04	Fresh, lysis buffer	2.39	47.8
T05	Control, lysis buffer	<0.010	
T06	Fresh, lysis buffer	4.40	88
T07	Frozen, H20	7.8	160
T08	Control, freeze	<0.010	
T09 ★	Frozen, H20	4.54	90.8
T10 ●	Fresh, H20	0.743	14.9 *(small clam)*
T11	Control, fresh	<0.010	
T12	Fresh, H20	1.41	28.2 *(small clam)*

Table 1: Qubit 2.0 results for first 12 samples pre microbial processing. Markers indicate samples used for genomic sequencing

- Modified version of EMP protocol
- Visualized sample bacterial DNA via gel electrophoresis
- Initial result showed contamination in dissection control but not PCR control, modified dissection methods
- Frozen clams yielded enough DNA for proper sequencing

PRELIMINARY MICROBIOME

- Selected one fresh and one frozen clam for bacterial genomic sequencing (Illumina MiSeq) (Table 1, Fig 3)
- Samples prepared using 16s Illumina MiSeq Sequencing Protocol from Walke Lab: 3x PCR using V4-5 primer and a negative control for each sample
- Both samples assigned to unused barcodes in shared Illumina sequencing run, samples pooled after PCR by Walke Lab
- Initial QIIME2 and BLAST analysis indicated over 1700 alignments; ~700 have 100% sequence identity



Class	Species	Associated habitats
Gammaproteobacteria	*Halomonas* sp. (multiple)	Variety of pH and temperature
Gammaproteobacteria	*Serratia* sp. (multiple)	Environmental
Gammaproteobacteria	Rahnella aquatilis	Freshwater, soil, snails, beetle gut
Gammaproteobacteria	*Vibrio metschnikovii* *V. cincinnatiensis*	Aquatic environments, seafood, human (pathogen)
Gammaproteobacteria	*Alishewanella* sp.	Metal resistant bacteria, Baltic Sea
Betaproteobacteria	*Dechloromonas* sp. A34	Mining overburden, ID

(L-R) Fig 4: Gel electrophoresis of the PCR controls and 2 clam samples (fresh, frozen) sent for sequencing, see Table 1; Table 2: some frequently-occurring 100% sequence matches from initial BLAST analysis of the two samples

NEXT STEPS

This protocol was developed with clams that live in a pollution-free environment; it now will be used to ask whether trace metal pollution alters the relationship between a clam microbiome and the aquatic microbial community.

ACKNOWLEDGMENTS

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