

Benthic Macrofauna Abundance Along a Transect from False Bay, San Juan Island

Michael G. Tassia¹

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¹Department of Biology, University of Washington, Seattle, WA, 98195

Contact Information:
Michael Tassia
Biology Department
University of Washington
Seattle, WA 98195
mtassia@uw.edu

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ABSTRACT:

Benthic macrofauna are a diverse group of organisms found in both deep-sea and shallow-water systems. Understanding the influence of biotic and abiotic factors in shallow-water systems can translate to further understanding inaccessible deep-sea community structure. This study observes the change in community structure as a function of 'Altitude Above Mean Low-tide Sea-level' (AAML) and distance from shore through push-core sediment sampling. In addition, this study identifies several specimens through genetic barcoding methods. The results show closer relationship among 90cm AAML sites than between any 90cm and 60cm site. Finally, specimens identified through barcoding could not be identified down to a species level. Future population genetic analyses will benefit from increasing accessibility to metagenetics and high-throughput sequencing.

BACKGROUND AND INTRODUCTION:

Infaunal macrofauna form a substantial portion of the biosphere **REFERENCE**. Macrofaunal organisms are those retained by a 500 μ m sieve from shallow water samples, or 300 μ m sieve from deep-sea samples, yet are too small to be identified by the naked eye. In both shallow and deep-sea systems, the sediment-water interface provides a rich environment that supports a diverse assemblage of organisms (Gust & Harrison 1981, Smith & Hinga 1983).

In the deep-sea, where the system is often food/nutrient poor (Smith et al. 2008), benthic macrofauna are highly diverse and often dominated by polychaetes (Smith & Demopoulos 2003). Although reasons behind the high diversity may not be well

understood (Gage 2004), there is importance in understanding benthic macrofaunal community structure as we continue to assess diversity and animal interactions in the deep sea. In addition to the vast abyss, benthic macrofauna assemblages are also present at ephemeral nutrient input sites such as whale falls, wood falls, kelp falls, vents, and seeps (Bernardino et al. 2010, Bernardino et al. 2012, Smith & Baco 2003). Despite their small size, they should not be overlooked as they form a significant portion of the deep-sea ecosystem (Bernardino et al 2012).

Unfortunately, studying deep-sea systems proves difficult. Instead, understanding shallow-water benthic macrofauna communities can provide us insight into the questions to be asked for deep-sea systems. Previous studies have shown physical and biotic factors, such as slope, tide distance, detrital input, and vegetation, impact community structure (Netto & Lana 1997, Ysaebaert & Herman 2002, Beukema 1976). However, studies of community structure can require accurate identification of species. Thus, results may change depending on the researchers ability to correctly identify one small animal from another of similar form (Leasi & Norenburg 2014).

One method of circumnavigating this issue is through utilization of genetic barcoding. This method provides a reliable method to place morphospecies into their corresponding taxa. Various genes can be used to determine different levels of relationships among collected individuals (Peterson et al. 2013, Cannon et al 2013, Cameron et al 2000) making this a powerful tool when looking at both higher and lower taxa. Barcoding also lends great capability to population connectivity through phylogeography and haplotype networks (Wilson et al 2008, Miyamoto et al 2012, Allcock & Strugnell 2012).

False Bay provides a large intertidal area of mixed sand and mud for benthic animals to thrive. Common organisms found here include polychaetes (including spionids, nereids, and chaetopterids), nemertean, mud shrimp, holothuroids, bivalves, and more. Using a mixture of traditional microscopy and genetic sorting for species identification, sample community structure will be evaluated as a function of distance from shore and as average altitude above mean low-tide sea-level.

METHODS AND MATERIALS:

Collection and Sorting:

Five cylindrical push-core samples (diameter = 6.5cm) were collected along a single transect at False Bay, WA on August 9, 2014 at 11:00pm. False Bay is a shallow, sandy environment with very shallow tidal elevation change (60-90cm across the 1km intertidal system). The first core was collected just beyond the tide-line. Following this, each core was collected every 200 paces (approximately 100m) along a transect perpendicular to the tide-line towards the inner bay (**Figure 1**). Each site's altitude above mean low-tide sea level (AAML) was determined through Google Earth®. Cores were brought back to the University of Washington Friday Harbor Laboratories and placed in a water table at 11°C for 24 hours to maintain animal integrity.

The top 10cm of each core was extruded from their respective samples and filtered through a 500µm sieve. Sediment and animals retained were washed into a 500mL bottle and kept in a flow-through tank to maintain constant temperature. Animals in each sample were sorted to morphospecies and counted using a dissecting microscope. At least one member from each morphospecies was preserved in 4% formalin for future

taxonomic identification (Hobson & Banse 1981, Banse & Hobson 1974, and Kozloff, 1987). In addition, individuals belonging to the Lophotrochozoa were preserved in 95-100% ethanol for further genetic barcoding (see below). If there was only one individual for any given lophotrochozoan morphospecies it was preserved only in ethanol.

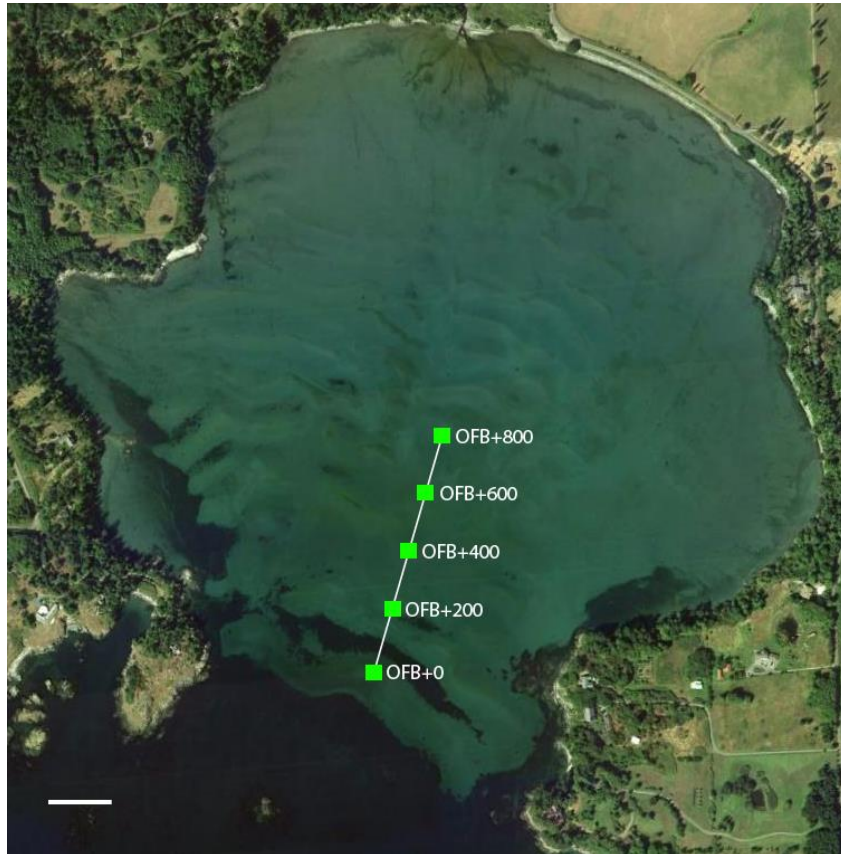


Figure 1: Displays the five sample sites along a transect from False Bay, San Juan Island. Samples were taken at 11:00am on August 9th, 2014. Samples were taken at 100m intervals and are indicated by yellow boxes on the figure. Scalebar = 100m.

Genetics and bioinformatics:

To determine relationships among select lophotrochozoan specimens, 16S mtDNA trees were constructed. DNA was extracted from 14 individuals using the Qiagen DNeasy© Blood & Tissue Kit (Cat. No. 69504). PCR was performed on each sample using universal 16S primers (16Sa & 16Sb). The following reagents volumes were used

per 50µL reaction: 10µL 5x Colorless Promega GoTaq® Flexi Buffer, 5µL 25mM MgCl₂, 1µL 10mM PCR Nucleotide Mix, 0.5µL 10µM 16Sa Primer, 0.5µL 10µM 16Sb Primer, 0.25µL 5 µ/µL GoTaq® G2 Flexi DNA Polymerase, 1µL Template DNA, and 31.75µL ddH₂O. Thermocycler steps are as follows: (1) 2min 94°C, (2) 30sec 94°C, (3) 1min 42°C, (4) 1min 72°C, (5) Repeat 2-4 34x, (6) 5min 72°C, and (7) 12hr 6°C.

PCR Products were purified using Qiagen QIAquick® PCR Purification Kit (Cat. No. 28104) ; DNA was eluted into 30µL ddH₂O. All DNA extraction, PCR, and PCR purification products were visualized by gel electrophoresis (1% agarose/1X TBE). Sequencing was performed by GENEWIZ®, Inc. (Seattle, WA) using their pre-defined sequencing service.

Obtained sequences were assembled using Geneious® 7.1.7 'De Novo Assembly'. Sequences were then compared to known sequences on the NCBI GenBank® using BLAST®. Datasets of similar sequences were compiled from GenBank® and used to build phylogenetic trees to identify unknown species further. Multiple sequence alignments were performed using MUSCLE and maximum likelihood trees were constructed using the RAxML Blackbox with *Mytilus edulis* 16S as an outgroup for each tree. Bootstraps were run at 1000 replicates followed by a maximum likelihood best-tree search.

Table 1: 16mtDNA sequences collected from GenBank. Individuals in blue were collected during the course of this study.

| Species | GenBank Accession # | Species | GenBank Accession # |
|-------------------------------|------------------------|----------------------------------|------------------------|
| Annelids (Spionidae): | | Nemerteans: | |
| <i>Aonidella cf. dayi</i> | KF434510.1 | <i>Amphiporus formidabilis</i> | AJ436787.1 |
| <i>Aurospio dibranchiata</i> | EU340083.1 | <i>Amphiporus imparispinosus</i> | AJ436788.1 |
| <i>Aurospio foodbanesia</i> | EU340078.1 | <i>Carinoma mutabilis</i> | AJ436832.1 |
| <i>Boccardia proboscidea</i> | FJ972547.1 | <i>Cerebratulus marginatus</i> | AJ436821.1 |
| <i>Dipolydora bidentata</i> | JX228112.1 | <i>Emplectonema buergeri</i> | AJ436792.1 |
| <i>Dipolydora cardalia</i> | JX228123.1 | <i>Gurjanovella littoralis</i> | AJ436794.1 |
| <i>Dipolydora carunculata</i> | JN048703.1 | <i>Micrura alaskensis</i> | AJ436827.1 |
| <i>Laonice norgensis</i> | KF434512.1 | Nemertean sp. 1 | - |
| <i>Marenzelleria viridis</i> | EF431973.1 | Nemertean sp. 2 | - |
| <i>Marenzelleria neglecta</i> | EF431979.1 | <i>Nipponemertes bimaculatus</i> | AJ436799.1 |
| <i>Marenzelleria wireni</i> | EF431980.1 | <i>Paranemertes peregrina</i> | AJ436805.1 |
| <i>Polydora triglanda</i> | JN048709.1 | <i>Paranemertes sanjuanensis</i> | AJ436807.1 |
| <i>Prionospio ehlersi</i> | EU340081.1 | <i>Parborlasia corrugatus</i> | EU194801.1 |
| <i>Spio filicornis</i> | FR823435.1 | <i>Procephalotrix spiralis</i> | AJ436837.1 |
| Spio sp | FR823439.1 | <i>Riserius pugetensis</i> | AJ436831.1 |
| Spionid sp. 2 | - | <i>Tubulanus sexlineatus</i> | AJ436840.1 |
| Spionid sp. 3 | - | Molluscs (Outgroup): | |
| Spionid sp. 4 | - | <i>Mytilus edulis</i> | AF023550.1 |
| <i>Spiophanes kroeyeri</i> | EU340082.1 | | |

Abundance Analysis:

Abundance analyses were performed using Primer© 6.1.15. Fourth-root whole dataset transformations were used to equally reduce variability across all data for further use. Bray-Curtis Similarity tests were performed followed by clustering by average distance and NMDS. Percent abundance charts were produced through Microsoft® Excel® 2010.

RESULTS:

Collecting and Sorting:

Three physical parameters were recorded at each site: ‘Distance from Shoreline’, ‘Altitude Above Mean Low-tide Sea Level’ (AAML), and ‘Water Column Height’. The values recorded have been summarized in (**Table 2**). At the time of collection, tides were at -1.0’ below mean low-water height (NOAA Tide Charts). Three of the five sites were found to be 90cm AAML, the other two were 60cm AAML (**Table 2**).

Table 2: Physical parameters measured at each collection site. Altitude from Sea Level was determined after collection through Google Earth.

| Site | Distance from Shore (m) | Altitude from Sea Level (cm) | Water Coverage (cm) |
|---------|-------------------------|------------------------------|---------------------|
| OFB+0 | 0 | 90 | 360 |
| OFB+200 | 100 | 90 | 150 |
| OFB+400 | 200 | 90 | <30 |
| OFB+600 | 300 | 60 | 30 |
| OFB+800 | 400 | 60 | <30 |

A total of 22 morphospecies were identified across all five samples (**Table 3**). In two of the five sample sites (OFB+200 and OFB+400), a single morphospecies of amphipod heavily dominated. In the other three samples, a spionid polychaete (identified as a member of *Spiophanes*) dominated. Of the 22 morphospecies identified, 13 were fixed in 4% formalin and identified further (**Figure 2**).

Table 3: Number of individuals for each species at each site. Individuals were identified to lowest taxonomic unit.

| Species | OFB+0 | OFB+200 | OFB+400 | OFB+600 | OFB+800 |
|-----------------------------|-------|---------|---------|---------|---------|
| Arthropods: | | | | | |
| Amphipod sp.1 | 6 | 204 | 126 | 0 | 1 |
| Amphipod sp.2 | 0 | 0 | 0 | 4 | 0 |
| Amphipod sp.3 | 0 | 0 | 0 | 0 | 1 |
| Cumella sp. | 5 | 23 | 17 | 0 | 2 |
| <i>Leptochelia</i> sp. | 3 | 2 | 0 | 0 | 0 |
| <i>Nebalia pugettensis</i> | 3 | 0 | 0 | 0 | 0 |
| Annelids: | | | | | |
| Eusyllis sp. | 0 | 2 | 13 | 0 | 0 |
| Lumbrineris sp.1 | 3 | 2 | 0 | 1 | 0 |
| Nereid sp. | 0 | 0 | 1 | 0 | 0 |
| Oligochaete sp.1 | 0 | 0 | 0 | 0 | 1 |
| Polychaete sp.1 | 0 | 0 | 0 | 1 | 1 |
| Spionid sp.1 | 1 | 0 | 0 | 0 | 0 |
| Spionid sp.2 | 0 | 0 | 0 | 2 | 0 |
| Spionid sp.3 | 0 | 0 | 0 | 0 | 2 |
| Spiophanes sp.1 | 28 | 2 | 1 | 9 | 86 |
| Unknown Nectichaete | 0 | 0 | 1 | 0 | 0 |
| Other Phyla: | | | | | |
| Bivalve sp.1 | 1 | 7 | 1 | 0 | 0 |
| <i>Leptosynapta clarki</i> | 2 | 23 | 3 | 0 | 0 |
| Nemertean sp.1 | 0 | 1 | 0 | 0 | 0 |
| Nemertean sp.2 | 0 | 0 | 1 | 0 | 0 |
| <i>Phoronis cf. harmeri</i> | 1 | 5 | 0 | 0 | 0 |
| Platyhelminth sp.1 | 0 | 1 | 0 | 0 | 0 |

Genetics and Bioinformatics:

Of the thirteen DNA extractions performed, only five sequences were of sufficient quality to assemble into consensus sequences. These consensus sequences were aligned and trimmed against the sequences available at the time of this experiment via MUSCLE (**Table 1**). Alignments were run through a maximum likelihood analysis using RAxML Blackbox and processed into two trees (**Figures 3**).

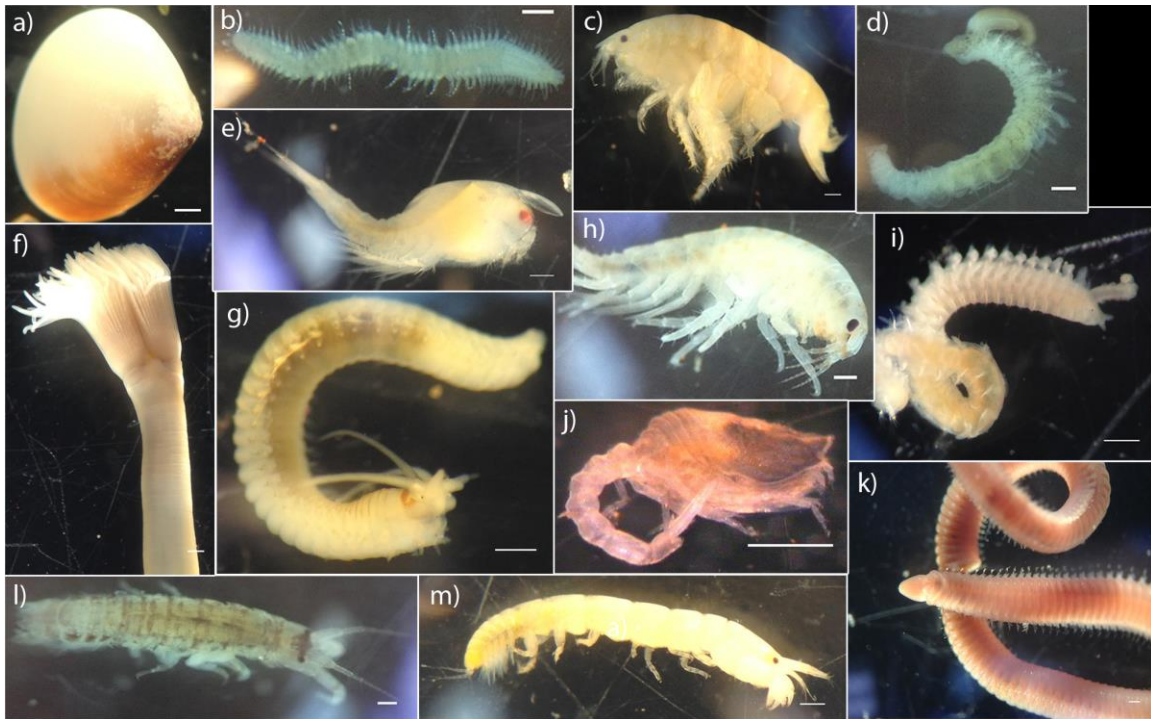


Figure 2: a) Bivalve sp. , b) Eusyllis sp. , c) Amphipod sp. 1, d) Spionid sp. 3, e) *Nebalia pugettensis*, f) *Phoronopsis cf. harmeri*, g) Nereid sp. , h) Amphipod sp. 3, i) Spionid sp. 1, j) *Cumella* sp. , k) *Lumbrinereis* sp. , l) Amphipod sp. 4, m) *Leptocheila* sp. Scale bars = 250 μ m

Tree and alignment data show high support for Spionid sp. 3 and Spionid sp. 4 as one species. However, given the sequences available on GenBank, the genus containing these specimens could not be identified using molecular data. Spionid sp. 2 aligned with members of the *Spio* genus with high support (**Figure 3a**). Nemeritean sp. 1 allied closely with the genus *Micrura*, confidently placing it within the Heteronemertea (Thollessen & Norenburg 2003). Furthermore, Nemeritean sp. 2 placed sister to *Gurjanovella* with high support lending to its position within the Hoplonemertea (Thollessen & Norenburg 2003).

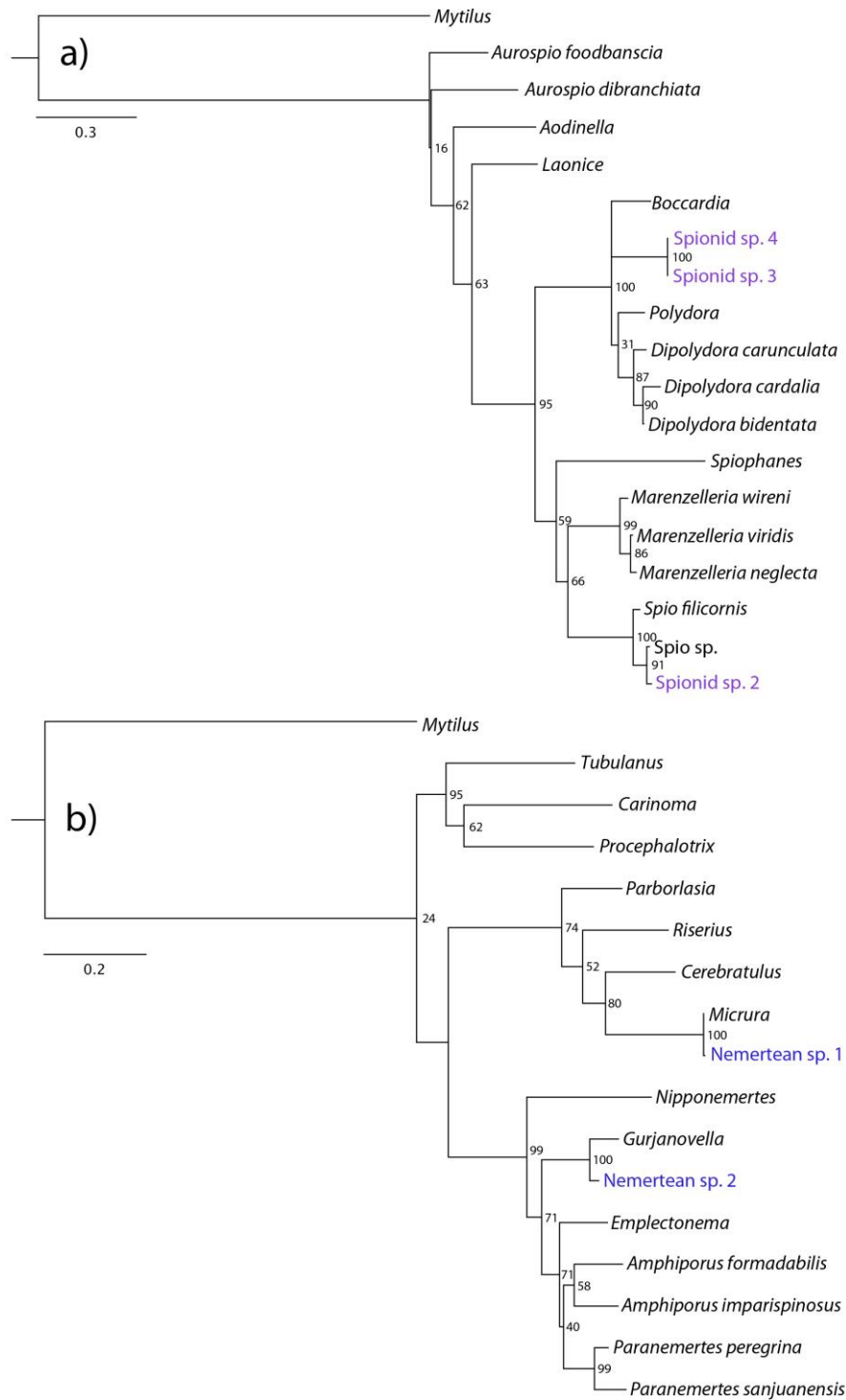


Figure 3: Best trees produced by RAxML Blackbox. Leaves in blue are sequences collected during this study. Bootstrap values are indicated at nodes. a) Best scoring spionid phylogeny – Spionid sp. 3 and sp. 4 were united with high confidence. b) Best scoring nemertean phylogeny – Nemertean sp. 1 was placed sister to *Micrura*, placing it within the Heteronemerteia. Nemertean sp. 2 was placed sister to *Gurjanovella*, placing it within the Hoplonemerteia.

Abundance Analyses:

Samples were not taken in replicate when conducting the initial sample collection due to time constraint. However, given the physical parameters at each site, replicates of ‘Altitude Above Mean Low-tide Sea Level’ were compiled with the data. Percent abundance of each morphospecies were calculated to reflect abundance against both site and altitude (**Figure 4**). Sites OFB+0, +600, and +800 show dominance by Spiophanes sp. 1, whereas sites OFB+200 and +400 show dominance by Amphipod sp. 1 (**Figure 4a**). However, dominance by Amphipod sp. 1 was much stronger (**Table 3**). When comparing species abundance to AAML, 60cm sites showed high dominance by Spiophanes sp. 1 and 90cm sites showed high dominance by Amphipod sp. 1 (**Figure 4b**).

Given the developed replicates of AAML, fourth-root transformed NMDS and Cluster diagrams were produced showing a close similarity for samples at 90cm AAML. A summary of abundance data is provided in **Figure 5**. Resemblance data indicates a closer relationship among 90cm AAML than any of their similarity to the 60cm sites. However, similarity between OFB+200 and OFB+400 does not appear to be strongly influenced by the high proportion of Amphipod sp. 1. **Figure 5a** shows OFB+200 and OFB+400 have a near identical Bray-Curtis score as OFB+200 and OFB+0.

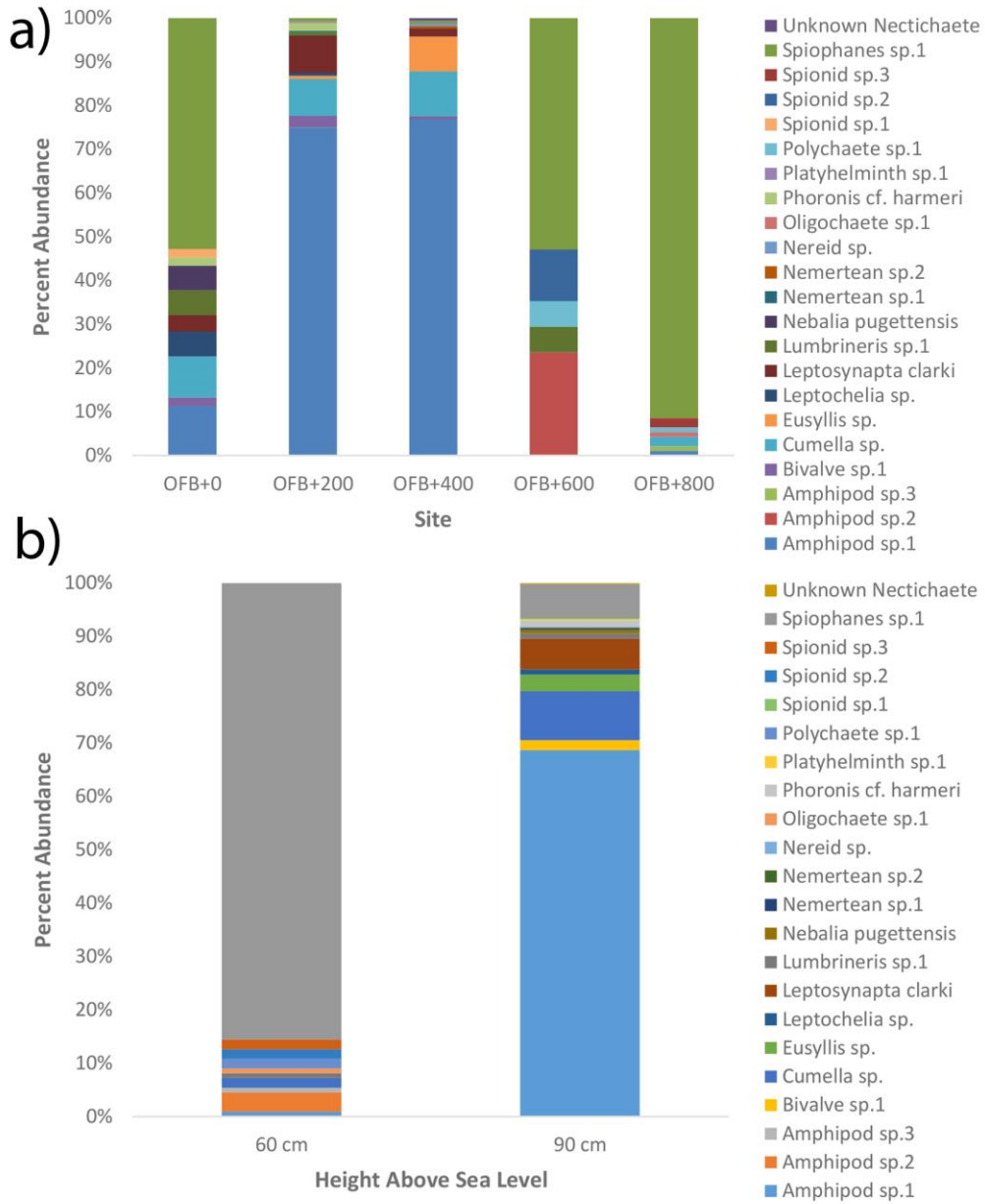


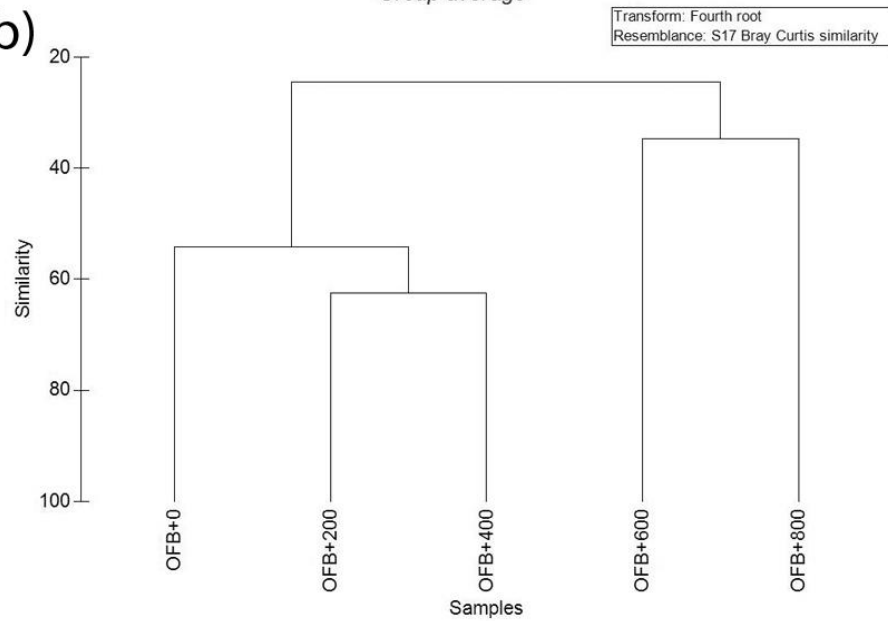
Figure 4: Percent abundance of morphospecies for each dataset: a) Percent abundance against site, b) Percent abundance at each altitude. Spiophanes sp. 1 shows high abundance in 60cm sites, whereas Amphipod sp. 1 shows high abundance at 90cm sites.

a)

| | OFB+0 | OFB+200 | OFB+400 | OFB+600 | OFB+800 |
|---------|------------|------------|------------|------------|---------|
| OFB+0 | | | | | |
| OFB+200 | 62.2604941 | | | | |
| OFB+400 | 46.1330147 | 62.5041599 | | | |
| OFB+600 | 27.5496375 | 17.9639268 | 10.0344752 | | |
| OFB+800 | 39.1724997 | 24.6046658 | 27.7087545 | 34.6725714 | |

False Bay Macrofauna
Group average

b)



c)

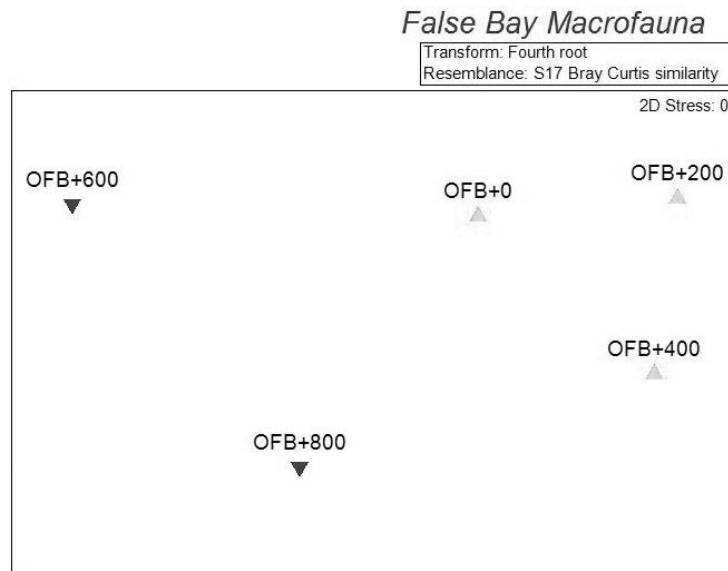


Figure 5: Summary of abundance statistics. a) Table of Bray-Curtis similarity used to produce b) Average distance tree, showing close alliance of OFB+0, +200, and +400 which are the three samples at 90cm above AAML, and c) NMDS showing data consistent with b)

DISCUSSION & CONCLUSIONS:

During collection of samples, express replicates were not obtained at each site. Thus, analyzing trends in diversity or abundance as a function of distance from the shore is difficult to make. Netto & Lana (1997) showed macrofauna abundance and diversity could vary with elevation and other biotic factors in salt-marsh systems. Furthermore, Ysaebaert & Herman (2002) showed macrofauna abundance varies significantly given many physical and biological parameters. Finally, Beukema (1976) showed benthic macrofauna diversity in mudflats is drastically reduced by sediment type and by proximity to the extremities of the system.

Given these previous studies, we might predict that community structure will change given different parameters. Although the present study does not provide replicates for distance from the shoreline, future studies in community structure along a gradient will prove interesting.

Alternatively, replicates of AAML were taken along this study's transect. This dataset showed zones 90cm AAML were more similar than they were to 60cm sites. False Bay varies between 30-90cm in AAML (Google Earth). Thus, 90cm sites would be 'peaks' among 'valleys' of sand and stagnate water. These physical effects alone may provide evidence for the similarity seen in animal abundance. Further study into the relationship between AAML and community structure will be important in evaluating the relationship between the two variables.

Five species were identified through genetic barcoding and maximum likelihood analyses. In the case of the nemerteans, identification on morphology alone can be quite difficult and may require internal anatomy studies (Stricker 1987). However, through

barcoding we can accurately identify individuals to lower levels of taxonomy (given identified sequences within a database of known species). This study uses the 16S mtDNA to assess the relationships among collected individuals to sequences collected from GenBank. Although the specimens collected for this study are identifiable with respect to their closely related taxa, the addition of other genes or faster evolving genes will prove useful for lower taxonomic identification (Hajibabaei et al. 2007).

An emerging method for assisting in community structure studies for benthic macrofaunal communities is metagenetics via high-throughput sequencing. Other studies have shown high-throughput sequencing of environmentally extracted DNA can be extremely affective at determining sediment community structure (Bik 2012, Creer 2010, Lindeque et al. 2013). As sequencing becomes more accessible, the ability to identify study organisms using a worldwide database of sequences becomes a very strong tool for identifying micro-, meio-, and macrofauna in any given sample.

For this study, several individuals were identified using traditional Sanger sequencing methods (**Figure 3**). Although Sanger sequencing provides long, high-quality reads, high-throughput sequencing provides rapid evaluation of massive datasets (Metzker 2010). In the case of this study, and future genetic diversity and connectivity studies, high-throughput sequencing will prove to be an invaluable asset for providing accurate estimates to community structure.

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