[working title]: **AN EXPERIMENTAL TEST OF BIOTIC AND ABIOTIC DRIVERS OF SPATIAL VARIATION IN ABUNDANCE OF EPIPHYTE *SMITHORA NAIADUM* ON SEAGRASS *ZOSTERA MARINA***

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[ABSTRACT, < 250 words] Ecological communities vary in space at a variety of scales. In marine communities, dramatic changes in species composition can occur across a small spatial distance, resulting in communities that vary across seascapes. Through an experimental manipulation of seagrass (*Zostera marina*), we examined the processes that drive the abundance and distribution of *Smithora naiadum*, an epiphytic red alga, in *Zostera marina* meadows on the central coast of British Columbia. At, the shoot level, we hypothesized that *Smithora* could be controlled by invertebrate grazing or bacterial facilitation. Whereas, at the site level, we hypothesized that Smithora could be controlled by environmental factors leading to the recruitment and persistence of the epiphyte on seagrass shoots. We used a reciprocal transplant to investigate whether *Smithora* load was affected by shoot level characteristic, location (and associated environmental characteristics) or an interaction between them. We found that uncolonized blades were colonized when placed in a high *Smithora* environment, while colonized blades did not lose their existing *Smithora* when moved to a *Smithora* free environment. Using illumina sequencing of bacterial DNA isolated from surface swabs of seagrass transplants, we found a significant difference between bacterial communities on shoots with and without *Smithora*. Further, we found that shoots with *Smithora* had a higher invertebrate abundance, indicating that *Smithora* is likely not grazer controlled. Our results suggest that seagrass associated bacterial and invertebrate communities could be altered by the colonization of epiphytic *Smithora,* or could be subject to the same spatial processes as *Smithora*. This research suggests potential drivers of epiphytic community composition in seagrass meadows.

**Highlights**

**Graphical Abstract (anyone?)**

1. **Introduction**

[***spatial variation in host - epiphyte relative abundance***]

What drives community variation across spatial scales continues to puzzle ecologists in a variety of ecosystems (Boström et al., 2006, A. Driscoll, 2008; Levins, 1969). In communities strongly influenced by foundation species, variation the presence and abundance of resident species may reflect spatial patterns in the host species or in the environment (Wahl 2008).

[***seagrass host - epiphyte system***]

Seagrasses are a foundation species that hosts richly diverse animal and algal communities. Algal epiphytes living on the seagrass blades support secondary productivity (Fry 1984) (Figure 1), yet also compete with seagrasses for light and nutrients (Mcroy and Goering 1974), (Sand-Jensen 1977), (Penhale 1977), (Harlin 1973a) (Coleman and Burkholder 1994) (Lin et al. 1996) (Morgan and Kitting 1984). Thus the foundation species and the biodiversity it hosts exist in a complex network of positive and negative interactions (Connolly 1994) (Boström and Bonsdorff 1997) (Sheridan 1997) (Webster, Rowden, and Attrill 1998) (Heck and Orth 1980; Heck Jr and Orth 1980) (Attrill, Strong, and Rowden 2000) (Tolan, Holt, and Onuf 1997) (Harlin 1975) (Fong, Lee, and Wu 2000). When the interaction network is perturbed, the system may change dramatically and produce extreme or undesirable states including seagrass die-offs (Burkholder, Tomasko, and Touchette 2007)(Best and Stachowicz 2012).

[***shoot-level or abiotic drivers: possibilities and examples. (shoot-level: microbes, other?; location: abiotic, or grazers…)***]

Spatial variation in the types and abundance of epiphytes on a single foundation species such as Zostera can reflect local environmental conditions, such as nutrient concentration or flow rates (Lavery and Vanderklift 2002) (Milchakova 2000) (Alcoverro, Duarte, and Romero 1997) (Kendrick and Burt 1997; Cebrian et al. 1999) (Reyes and Sansón 1997; Johnson et al. 2005). For example, large changes in epifaunal communities can be associated seagrass meadow edges (Bowden, Rowden, and Attrill 2001; Bell et al. 2001)[*find a better example*]. In general, edge effects affect communities due to changes in physical structure, abiotic conditions or grazing pressure (Soule 1986) (Renhorn et al. 1996; Fagan, Cantrell, and Cosner 1999). Abundances of invertebrates can vary significantly both between and within meadows due to predation by fish (Amundrud, Srivastava, and O’Connor 2015) (Boström and Mattila 1999) (Tanner 2005). Understanding the drivers of this spatial variation helps to understand the dynamics of the eelgrass-based community, supporting better understanding of temporal variation and events such as algal blooms. Epiphytes may also be used as indicators of nutrient pollution, if their abundance is known to reflect water column nutrients (ref).

Another possible driver of variation in algal epiphytes is variation in the host plant’s structure or chemistry. *Z. marina* plants could be changing their blade surface chemistry to discourage epiphyte colonization (Bell, Lang, and Mitchell 1974) (Mejia et al. 2016). Colonization of *Zostera marina* by spores of epifaunal organisms is accomplished through the establishment of a suitable bacterial community before the spores attach (Sieburth and Thomas 1973). Unique bacterial groups are associated with a specific blade surface chemistry (Bagwell et al. 2002) (Crump and Koch 2008) (Hamisi et al. 2009) (Weidner et al. 2000) (Duarte, Holmer, and Marba 2005), and the growth of unique bacterial communities can discourage the attachment of spores on marine macrophytes.

*can we comment on whether we are aware of any other reciprocal transplant experiments on epiphytes? that makes our study novel.*

In the northeast pacific, the red alga *Smithora naaidum* is one of the more common macroalgal epiphytes on eelgrass. *Smithora* abundance varies substantially among meadows, present on eelgrass primarily in marine environments (not brackish). Smithora is one of the highest quality algae for grazers, extremely high in fatty acid content, suggesting an important role in the seagrass-based food chain. Smithora also changes the physical structure of the seagrass microenvironment, potentially enhancing protection. Due to *Smithora*’s large variation over a small distance, *Smithora* on *Zostera marina* is an interesting system in which to investigate the drivers of changes in epiphyte abundance in *Zostera marina* meadows (Kitting, Fry, and Morgan 1984).

[***our objectives***]

The main objective of this work was to investigate potential drivers of changes in *Smithora* abundance within a single *Zostera marina* eelgrass meadow. We performed a reciprocal transplant experiment of seagrass shoots between zones of high and low *Smithora*. We tested the hypotheses that *Smithora* abundance on eelgrass is dictated by a shoot level characteristic rather than by the local environment. If shoot-level attributes dominate, we expected that abundance of *Smithora* would not change with a change in shoot location.

2. Materials and Methods

2.1 Study System

*Zostera marina* is a meadow forming eelgrass common along coastlines in the northern hemisphere (Olsen et al XXX). *Z. marina* provides habitat for hundreds of invertebrate and fish species that in turn provide food for fish and other large consumers. Thus, eelgrass meadows are highly productive environments, and much of this secondary productivity is derived from epiphytic algae growing on the seagrass blades rather than the seagrass itself (Valentine and Heck, Edgar and Shaw 1995, Taylor 1998).

*Smithora* *naiadum* is a red alga thought to specialize on *Z. marina. Smithora* abundance and distribution varies widely along the Pacific Northwest coast (Harlin 1975). After colonizing as a microscopic spore, it forms tough basal cushions and then grows into lobed blades (Hansen 1986, Harlin 1973b, Hawkes 1988). *Smithora*’s successful colonization depends on the survival of spores as well as the microenvironment of the *Z. marina* blade.

2.2 Spatial variation in *Smithora*, *Zostera,* and associated organisms

We studied the interaction between *Smithora* and *Z. marina* on the central coast of British Columbia, Canada, in June-August 2015 ([Figure 2](https://docs.google.com/document/d/1fnmB9FjXoJj9Wm9wLuwfoJh-fRqCLzDUWRTvhR-4bhM/edit?usp=sharing)) in Choked Pass, Calvert Island. In a large continuous eelgrass meadow approximately 367,000 square meters in area (Hakai geospatial team), *Smithora* is prevalent along the edges of the meadow, but not in the meadow interior. At the wolf beach study site temperatures range from 6-10 degrees, light, etc.

We surveyed the Choked Pass eelgrass meadow to quantify spatial variation in *Smithora* abundance on eelgrass. We surveyed 8 40-m transects, four in the meadow interior (>200m from the closest edge) and four at the meadow edge (2m from bordering sand habitats) throughout the primary growing season, May to August (Olson 2017; Fig. 2). All transects were in permanently subtidal seagrass, and were separated by at least 100 m. Using SCUBA, we collected one shoot every 10m along each transect (n = 5 shoots per site visit) by covering shoots with a Ziplock bag and detaching at the rhizome. From each shoot, we measured *Z. marina* shoot dry weight and *Smithora* dry weight.

In a separate survey, we quantified ambient grazer abundance and diversity at the meadow edge and interior at two adjacent cites: WF and IA (Figure 1). *Z. marina* shoots were collected from 0.25m X 0.25m plots (n = 6) from the interior and edge at the Wolf Beach study site in early June, 2015. We used a standardized processing protocol similar to Whippo (2013), the only difference being that we separated (using a Whatman GCF X filter) and weighed periphyton biomass. All invertebrates were removed and preserved with 95% ethanol for diversity analysis. Invertebrates > 500 um in diameter were classified to the closest possible taxonomic grouping using a stereo microscope, and invertebrates known to associate with *Zostera* and graze epiphytic algae were enumerated. Gammaridian amphipods were identified to Order. Otherwise, every invertebrate in each quadrat was classified to as specific a grouping as possible, see Appendix 1 for exact groupings.

2.3 Reciprocal Transplant Experiment

We conducted a reciprocal transplant experiment to test whether *Smithora* abundance on an eelgrass shoot reflects the shoot’s location (environmental conditions) or the shoot itself (defenses, microbiota, age, etc). We identified two adjacent source sites within the Choked Pass meadow typical of the high *Smithora* zone (WF) at the meadow edge and the low *Smithora* zone (IA) at the meadow interior. These zones differed in *Smithora* abundance on shoots, from 0.37 + 0.39 Smithora / Z. marina (g/g dry wt) in the high *Smithora* zone at WF to 0.02 + 0.06 Smithora / Z. marina (g/g dry wt) in the low *Smithora* zone at IA. Depth and substrate (sandy) were consistent, and the two sites were 5 meters apart and connected by continuous eelgrass habitat.

From each zone, we collected twelve shoots and exposed them to one of two treatments (n = 6): transplant and control. Transplanted shoots were collected and moved to the other zone (WF shoots moved to IA zone, and IA shoots moved to WF zone). Control shoots were collected and replaced in their zone of origin to control for the effect of uprooting on *Smithora* abundance and bacterial community. Collection, initial sampling, and replanting procedures consisted of the following steps: Shoots were collected on SCUBA on July 9th. They were clipped in the field at the sediment surface, leaving the rhizomes. Shoots were placed in a ziploc bag in the field underwater, and transported to the lab immediately. In the lab, shoots were photographed for morphometric analysis and swabbed for initial bacterial community analysis. When not being processed they remained submerged in seawater. They were given an ID using flagging tape so that each shoot could be re-sampled at the end of the experiment. Shoots were replaced in the field on July 10th by attaching them by the sheath with zipties to PVC submersible platforms. In the field, platforms were secured to the sediment surface to keep shoots on the sandy substrate and floating upright. On August 10th, all 24 treatment and control shoots were collected and processed and photographed in the lab. We also collected 2 ambient shoots next to each transplant platforms to compare transplanted shoots to unmanipulated shoots. We removed invertebrate grazers were removed from the shoots upon collection.

2.4 Shoot characteristics: morphometrics and microbiota

For all shoots collected for the experiment (N = 12) and environmental surveys (N = 120), we measured the following shoot characteristics: length, width, biomass (dry weight, after 48 hours at 60°C), and microbiota. For shoots collected as part of environmental surveys, we also counted the number of blades per shoot. Shoots were brought to the lab, where epiphytes were gently scraped off *Z. marina* shoots with a microscope slide and grouped taxonomically (*Smithora*, porphyra, and periphyton). Dried biomass of *Z. marina* shoots and their associated *Smithora* epiphytes was obtained by drying samples at 60°C for 48hrs.

To quantify the diversity and composition of the shoot’s external microbiota, bacterial samples were taken from each experimental shoot (both before and after the transplant), as well as from ambient shoots collected at the time of transplant retrieval. An area halfway up the shoot that was free of *Smithora* was chosen bacterial sampling. This area was rinsed with filtered sterilized seawater for 10 seconds, and then a Puritan® sterile swab was used to swab the area for ten seconds, avoiding any *Smithora* basal thallus cushions. Swabs of *Smithora* blades were taken as above for comparison. The swab was stored in an individual sterile cryovial (VWR) and placed on ice for transport back to the lab, and were transferred to -80˚C for storage within 8 hours.

DNA was extracted from swabs and water filters using the MoBio PowerSoil®-htp 96 well DNA extraction kit (Carlsbad, CA) following the manufacturer’s recommended protocol. The V4 region of 16S rRNA in Bacteria and Archaea was targeted for amplification using redesigned versions of the primers 515f/806r (Caporaso et al. 2012): 515f: 5’–GTGYCAGCMGCCGCGGTAA–3’, 806r: 5’–GGACTACNVGGGTWTCTAAT–3’. Forward primers were tagged with a 12bp Golay barcode to facilitate sample pooling. Each PCR contained 10µl of 5-Prime Master Mix, 1µl of each primer (final concentration = 0.2µM each), 0.5µl of peptide nucleic acid (PNA) chloroplast blocking primer (Lundberg et al. 2013; 0.2µM final concentration, purchased from PNA Bio Inc., Thousand Oaks CA), 2µl of DNA, and PCR grade water to a final volume of 25µl. PCR was carried out with an initial denaturation step at 94˚C for 3 minutes, followed by 25 cycles of denaturation at 94˚C for 45 seconds, PNA clamping at 75˚C for 60 seconds, primer annealing at 50˚C for 60 seconds, and extension at 72˚C for 90 seconds, with a final extension step of 72˚C for 10 minutes. PCR products were quantified using Quant-IT Pico Green® ds DNA Assay Kit (Life Technologies). Equal amounts (25ng) of each sample were pooled and then purified using the MoBio UltaClean® PCR clean-up kit. Pooled library quantitation and paired-end Illumina MiSeq sequencing (2 x 300bp) was carried out at the Integrated Microbiome Resource facility in the Centre for Genomics and Evolutionary Bioinformatics at Dalhousie University (Halifax, Canada).

Raw sequencing reads were demultiplexed using split libraries within the Quantitative Insights into Microbial Ecology (QIIME v.1.9) analysis pipeline (Caporaso et al. 2010b), and then then trimmed to 250 base pairs using FastX Toolkit (<http://hannonlab.cshl.edu/fastx_toolkit/>). Reads were then clustered into “species” level operational taxonomic units (OTUs) using Minimum Entropy Decomposition (MEDs; Eren et al. 2015), with the minimum number of reads per MED node set to 90 (-M parameter). All other parameters were run with default settings; the maximum variation allowed per node (-V) was automatically set at three nucleotides.

Taxonomy was assigned to MED-nodes (hereafter referred to as operational taxonomic units; OTUs) using uclust (Edgar 2010) as implemented in the Assign Taxonomy function of QIIME v.1.9 retrained on the GreenGenes (gg\_13\_8) database (DeSantis et al. 2006). OTUs annotated to either chloroplast or mitochondrial sequences were removed as putative host contamination. Additional OTUs were removed if they occurred in only a single sample. Representative sequences for the remaining OTUs (n = 1984) were aligned with PyNAST v.1.2.2 (Caporaso et al. 2010a) using the GreenGenes 13\_8 alignment as a template, and a tree was constructed using FastTree (Price et al. 2010) as implemented in QIIME v.1.9. Samples with fewer than 1000 reads were removed from the analysis. Sequence data and MiMARKs compliant metadata are deposited at the European Bioinformatics Institute, accession number (XXXXXXXX).

**2.5 Statistical analyses**

To quantify variation in *Smithora* among edge and interior sites in Choked Pass, multiple generalized linear models were fit using an inverse Gaussian distribution, with LAI and site as explanatory variables for *Smithora* biomass. Models were compared using Akaike Information Criterion (AIC). To compare seagrass density and *Smithora* biomass at the edge vs. interior, one-way ANOVA’s were used with R. 325 statistical software. Both *Smithora* biomass and shoot density fit a normal distribution and so a linear model was used to fit the data.

We constructed a dissimilarity matrix on rarefied data (5000 sequences/sample) using the UniFrac metric, which takes phylogenetic distance into account (Lozupone & Knight 2005), to compare microbiota composition among sites and before and after transplanting of shoots. The matrix was constructed in Phyloseq (McMurdie and Holmes 2013) within R. Beta-diversity patterns were visualized with non-metric Multi Dimensional Scaling (NMDS) plots created in Phyloseq. A two-way PERMANOVA (Permutational Analysis of Variance) was used to compare the effect of *Smithora* presence, transplant, and their interaction on bacterial community. We did not include bacterial families with relative abundances lower than 0.02.

We compared epifaunal abundance and diversity between edge and interior plots using ANOVA. We used NMDS plots to visualize invertebrate community dissimilarity using a bray-curtis distance metric. Invertebrate community data was analyzed in the Vegan 2.3-4 package in R (Okansen et al. 2016). A PERMANOVA was used to test the effect of location (edge vs. interior) on invertebrate communities. A one-way ANOVA compared amphipod abundance at the edge vs. interior locations. All R analyses used R 3.2.4.

**3. Results**

3.1 Spatial variation in *Smithora* and *Zostera* and associated organisms

Across eight sites at the landward side of the Choked Pass eelgrass meadow, *Smithora* presence and abundance on eelgrass shoots varied strongly from site to site (two-way ANOVA: site type (interior vs edge): F = 63.46, df = 1, p = < 0.001; Site: F = 8.06, df = 6, p = < 0.001, residuals: df = 108). There was a significant difference in *Smithora* abundance on *Z. marina* between meadow edge and interior sites (Figure 1C).

Additional, plot-scale sampling the Wolf Beach site, where the experiment was conducted, revealed similar patterns in *Smithora* abundance in June 2017 (Figure 2). Shoot density was higher at the edge vs the interior (one-way anova: F = 15.29, df = 1, 10, p = 0.003; Appendix A), and so was *Smithora* load (one-way anova: F = 6.57, df = 1, 10, p = 0.028).

Grazer abundance and diversity also varied substantially among the inner and outer seagrass samples at Wolf Beach, such that interior seagrass shoots with less *Smithora* also had fewer grazing epifaunal invertebrates (one-way anova: F = XX, df = 1, X, p = XX; Figure 2C). *Species composition differences?*

*Initial microbial assemblages.* And mention in the second paragraph that *Smithora* abundance patterns at the local site were like the general patterns.

3.3 Reciprocal transplant

At the end of the experiment, shoots at the edge of the meadow had high *Smithora* load regardless of their source location (Figure 3; two-way anova with interaction term: Source (interior vs edge): F = 32.04, df = 1, p = < 0.001; Treatment (control vs unmanipulated: F = 0.28, df = 1, p = 0.61, Source X Treatment: F = 4.67, df = 1, p = 0.05; residuals: df = 11). Shoots that were transplanted from the edge to the interior site retained *Smithora,* while interior shoots that stayed in the interior were not colonized. Controls (uprooted but locally planted) and unmanipulated shoots did not differ in *Smithora* load at the time of the end of the experiment (two-way anova: Source (interior vs edge): F = 26.34, df = 1, p = < 0.001; Treatment (control vs unmanpulated: F = 1.59, df = 1, p = 0.27, residuals: df = 10).

*Bacterial results on transplanted shoots.* A two-way PERMANOVA showed that there was a significant effect of transplant and *Smithora* presence on shoot level bacterial community.

July edge: Sapro,Methy only consistent ones that edge share

July interior: Flavo, Sapro,Rhodo,Thio,unknown, Rickett

August experiments: colonized by everything

**4. Discussion**