Gracilaria vermiculophylla in the Virginia coastal bays: Documenting the distribution and effects of a non-native species

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Abstract

Non-native species are of worldwide concern in both terrestrial and aquatic systems. Macroalgal introductions in coastal environments can have varied and often harmful effects, especially when surrounding habitats are altered by the invasion. *Gracilaria vermiculophylla* is a red macroalga that is native to East Asia and has been introduced to temperate estuaries around the world. It cannot be easily identified based on morphology alone, and is frequently mistaken for native congeners if genetic testing is not used. Mats of the macroalga can accumulate on subtidal and intertidal substrate within the Virginia coastal bays, USA and are held in place by tube decorating polychaetes on the order of months to years.

The broad goals of this dissertation were to determine how widespread the *G. vermiculophylla* invasion was in the Virginia coastal bays and to document potential effects of *G. vermiculophylla* mats on biogeochemistry, trophic cascades, and on public health in the region. I found that the introduction was widespread in both subtidal and intertidal habitats, with higher intraspecific genetic richness and diversity than currently documented in other invasions. In addition, I found that intertidal sediment, marsh cordgrass, and mudflat invertebrates all incorporated nitrogen of *G. vermiculophylla* origin, which indicates that the macroalga is an important mediator of nutrient transfers in the system. Work on intertidal mudflats showed that the presence of *G. vermiculophylla* in this system, at moderate densities, could increase oxic-anoxic heterogeneity in the sediment and thus increase coupled nitrification-denitrification. In addition, although *G. vermiculophylla* was associated with an overall increase in invertebrate biomass,

shorebirds chose to forage on bare mudflats. Lastly, I found that *G. vermiculophylla* was a reservoir for the pathogenic bacteria, *Vibrio parahaemolyticus* and *V. vulnificus* which can cause gastroenteritis, severe wound infections, septicemia, and death in humans. In addition, oysters, sediment, and water collected in close proximity to mats of *G. vermiculophylla* had higher concentrations of both bacterial species when compared to samples collected on bare mudflats. Taken together, data collected within the Virginia coastal bays indicate that this widespread habitat modifier can have important effects on nitrogen availability, food web interactions, and shellfish sanitation.

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Chapter 1: Introduction to the Dissertation

Background

Species introductions are of worldwide concern because they can have deleterious economic and ecological consequences (Sakai et al. 2001). Ecosystems that are affected by either natural or anthropogenic stresses, such as global warming and pollution, are often more susceptible to species introductions (Occhipinti-Ambrogi & Savini 2003; Occhipinti-Ambrogi 2007; Piola & Johnston 2008). Of particular concern are invasive species that modify habitat or resources in the invaded ecosystem (Ruiz et al. 1997; Ruesink et al. 2006; Grosholz & Ruiz 2009). Marine macroalgae often fit into this paradigm of habitat modifiers in estuarine waters where vectors of introduction like shipping, aquarium/food trade, and aquaculture are common (Ruiz et al. 1997, 1999; Williams & Grosholz 2008).

Gracilaria vermiculophylla is a red macroalga that has proliferated in temperate estuaries in the Western and Eastern Atlantic, North and Baltic Seas, and Eastern Pacific. This macroalga is native to the Western Pacific, where it can be a dominant member of the macroalgal assemblage (Yamamoto 1978). *G. vermiculophylla* is a cryptic invader, meaning that it cannot be distinguished from native species of *Gracilaria* in invaded regions using morphology alone. Rather, morphology coupled with hybridization testing (Ohmi 1956; Yamamoto 1978; Yamamoto & Sasaki 1988; Terada & Yamamoto 2002) or genetic analyses are necessary to accurately identify *G. vermiculophylla* (Thomsen et al.

2006a; Gulbransen et al. 2012). Because hybridization testing is time consuming and often complicated, most researchers now prefer to use genetics for identification (Bellorin et al. 2002; Gurgel & Fredericq 2004).

As is true of most invasive species, *G. vermiculophylla* is tolerant of many environmental stresses, including fluctuations in salinity, temperature, sedimentation, light intensity, and nutrient availability (eg. Yokoya et al. 1999; Thomsen & McGlathery 2007; Thomsen et al. 2007; Nejrup & Pedersen 2010, 2012; Nejrup et al. 2012; Sfriso et al. 2012). It is typically unpalatable to herbivores when compared to native species of macroalgae in invaded locations (Thomsen & McGlathery 2007; Nejrup & Pedersen 2010; Jensen et al. 2011; Nejrup et al. 2012). Because of its tolerance to both biotic and abiotic stress, *G. vermiculophylla* often becomes a dominant macroalgal species in invaded ecosystems (Thomsen 2004a; Freshwater et al. 2006; Gulbransen & McGlathery 2013).

Multiple modes of introduction have been proposed for *G. vermiculophylla,* including shellfish aquaculture (Mollet et al. 1998; Rueness 2005; Thomsen et al. 2006a, 2007; Thomsen & McGlathery 2007; Nyberg et al. 2009; Sfriso et al. 2010, 2012; Jensen et al. 2011; Gulbransen et al. 2012), transport via ballast water, fishing gear, or boat propellers (Thomsen et al. 2007; Weinberger et al. 2008; Nyberg & Wallentinus 2009), and fouling of ship hulls (Weinberger et al. 2008; Nyberg et al. 2009). In addition, asexual reproduction via fragmenation can be common, however this more likely accounts for dispersal within an invaded location (Thomsen 2004a, 2004b; Thomsen & McGlathery 2005; Thomsen et al. 2007, 2009; Weinberger et al. 2008). Once introduced

to a system, *G. vermiculophylla* attaches to shells of bivalves and other mollusks, floats around as drifting mats, or becomes incorporated into tube caps of decorating worms (Thomsen 2004b; Thomsen & McGlathery 2005; Thomsen et al. 2007; Abreu et al. 2011; Berke 2012).

Once *G. vermiculophylla* becomes established in a region it can have significant effects on biogeochemistry, macrophytes, and higher trophic levels on intertidal marshes and mudflats, subtidal flats, seagrass beds, and oyster reefs. Biogeochemical effects are often complex, with researchers finding the macroalga can be a potential source of nitrogen (Gulbransen & McGlathery 2013), but also can compete for nutrients (Hammann et al. 2013) and increase losses of reactive nitrogen from the system (Gulbransen et al. in review L & O). Several studies have found that seagrass beds can be negatively affected when *G. vermiculophylla* biomass is high (Martínez-Lüscher & Holmer 2010; Höffle et al. 2011), but moderate levels of the macroalga can enhance densities of native macroalgae and invertebrates (Thomsen et al. 2006b; Thomsen 2010; Byers et al. 2012). Mats of the macroalgae have been shown to both inhibit settlement of oysters (Thomsen & McGlathery 2006), and enhance habitat for juvenile blue crabs (Falls 2008; Mahalak 2008; Johnston & Lipcius 2012) and scallops (Hernández Cordero et al. 2012). .

Site Description

G. vermiculophylla studies were conducted in the Virginia coastal bays at the Virginia Coastal Reserve Long Term Ecological Research (VCR LTER) site. This region comprises 110 km of the southern part of the Delmarva Peninsula and is bounded to the

east by a series of barrier islands. The coastal bays are shallow with half of the area < 1 m at mean low water, a tidal range of 1.2-1.5 m, and 37% of the benthic surface area covered by marsh and intertidal flats (Oertel 2001). Land use in this region of the Delmarva Peninsula is primarily agriculture and forest. There are no major riverine inputs into the coastal bays; rainfall and groundwater are the primary sources of freshwater in the region.

G. vermiculophylla can be found in the Virginia coastal bays floating as drifting mats, attached to tube caps of the polychaete *Diopatra cuprea* on subtidal bare flats or among seagrass, wound around stems of the cordgrass *Spartina alterniflora* or lying unattached on the sediment on marshes, attached to live and dead bivalve shells, or attached to *D. cuprea* tube caps on intertidal mudflats. Rarely, it can also be seen washed up as wrack on top of marsh cordgrass. Although routine monitoring of *Gracilaria* biomass in this region began in 1998, it was not until 3 samples of *Gracilaria* were sequenced in 2004, that researchers realized they had likely been collecting the non-native *G. vermiculophylla* rather than its native congener *G. tikvahiae* (Thomsen et al. 2006a). Work conducted shortly after this discovery in Virginia focused on potential reasons for the successful *G. vermiculophylla* invasion including resistance to burial, grazing, desiccation, changes in light and nutrient levels, and an association with *D. cuprea* that enhanced asexual reproduction and available surface for attachment (Thomsen & McGlathery 2005, 2006, 2007). Building on these findings, I will address biogeochemical, trophic and public health consequences of this invasive species.

Approach

In this dissertation, I will address five main questions:

(a) How widespread is the *G. vermiculophylla* introduction and how genetically rich is the non-native population in Virginia compared to other areas in the world?

(b) Can *G. vermiculophylla* mediate nitrogen transfers to sediment, macrophytes, and invertebrates on marshes and mudflats?

(c) How does the presence of *G. vermiculophylla* on intertidal mudflats affect net denitrification rates?

(d) How do *G. vermiculophylla* mats affect invertebrates and the foraging behavior of migratory shorebirds during their spring migration stopover in the Virginia coastal bays?

(e) Is *G. vermiculophylla* a reservoir for pathogenic species of *Vibrio* bacteria? Is the presence of a *G. vermiculophylla* mat correlated with concomitant increases in the densities of these pathogens in water, sediment, and oyster tissue?

Each of these questions has been written up as a separate chapter and formatted for publication. Chapter 2, "*Gracilaria vermiculophylla* (Rhodophyta, Gracilariales) in the Virginia costal bays, USA: cox1 analysis reveals high genetic richness of an introduced macroalga" was published in the Journal of Phycology in 2012. Chapter 3, "Nitrogen transfers mediated by a perennial, non-native macroalga: A ^{15}N tracer study" has been accepted for publication in Marine Ecology Progress Series. Chapter 4, "Mats of the non-native macroalga, *Gracilaria vermiculophylla*, alter net denitrification rates and

nutrient fluxes on intertidal mudflats" has been submitted for publication in Limnology and Oceanography. Chapter 5, "A non-native intertidal macroalga influences invertebrate densities and shorebird foraging" has been submitted for publication in Biological Invasions. Lastly, Chapter 6, "Association of *Gracilaria vermiculophylla*, a non-native, mat forming macroalga, with increased concentrations of *Vibrio* bacteria in sediment, water, and oysters on intertidal mudflats" has been submitted to Applied and Environmental Microbiology.

The concluding remarks of this dissertation have been structured as a comprehensive review of what is currently known about *G. vermiculophylla* both in its native and invaded ranges. As part of this review, publications were split into groups based on the topic they addressed (genetic confirmation of species, environmental tolerances, vectors of dispersal and colonization, effects on intertidal, subtidal, and seagrass communities, effects on commercially important seafood, and industry applications). This review will be submitted to Annual Reviews of Ecology, Evolution, and Systematics.

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Chapter 2: Gracilaria vermiculophylla (Rhodophyta, Gracilariales) in the Virginia costal bays, USA: cox1 analysis reveals high genetic richness of an introduced macroalga

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Abstract

Gracilaria vermiculophylla (Ohmi) Papenfuss is an invasive alga that is native to Southeast Asia and has invaded many estuaries in North America and Europe. It is difficult to differentiate *G. vermiculophylla* from native forms using morphology and therefore molecular techniques are needed. In this study we used three molecular markers (*rbc*L, *cox*2-*cox*3 spacer, *cox*1) to identify *G. vermiculophylla* at several locations in the western Atlantic. *Rbc*L and *cox*2-*cox*3 spacer markers confirmed the presence of *G. vermiculophylla* on the east coast of the USA from Massachusetts to South Carolina. We used a 507 base pair region of *cox*1 mtDNA in order to (i) verify the widespread distribution of *G. vermiculophylla* in the Virginia (VA) coastal bays, and (ii) determine the intraspecific diversity of these algae. *Cox*1 haplotype richness in the VA coastal bays was much higher than that previously found in other invaded locations, as well as some native locations. This difference is likely attributed to the more intensive sampling design used in this study, which was able to detect richness created by multiple, diverse introductions. On the basis of our results, we recommend that future studies take differences in sampling design into account when comparing haplotype richness and diversity between native and non-native studies in the literature.

Key index words: *cox*1, *cox*2-*cox*3 spacer, DNA barcode, *Gracilaria vermiculophylla*, marine algae, rbcL, species introductions, Virginia

Abbreviations: BC, British Columbia, Canada; bp, base pair; CA, California; MA, Massachusetts; mtDNA, mitochondrial DNA; NJ, New Jersey; NC, North Carolina; RI, Rhode Island; SC, South Carolina; USA, United States of America; VA, Virginia

Introduction

Biological invasions are occurring at an increasing rate globally, and are known to impact native habitats by altering physical structure, species composition and ecosystem functions (Ruiz et al. 1997, 1999). Often invasive macroalgae are resilient to control by native herbivores, alter competition in invaded systems, cause native species declines, increase toxicity, and change community structure and habitat availability (Schaffelke and Hewitt 2007, Williams and Smith 2007, Thomsen et al. 2009). It is important that scientists have early information concerning the presence and potential sources of an invasion in order to have the best chance of managing introductions and preventing negative impacts. For red macroalgae, identification of invasive species from morphological characteristics is usually not conclusive (Gurgel and Fredericq 2004, Thomsen et al. 2006), and hybridization testing is often slow and provides less information than molecular techniques (Bellorin et al. 2002). Therefore, researchers advocate the inclusion of genetic testing to identify cryptic macroalgal invasions and to determine potential vector-pathways and source regions (Miura 2007).

Gracilaria vermiculophylla is a macroalga that is native to Japan (Ohmi 1956), widespread in Southeast Asia, and has invaded several parts of the temperate northern hemisphere (Kim et al. 2010). There are around 110 different species of *Gracilaria* worldwide and species identification is often difficult when only morphological data are

used (Gurgel and Fredericq 2004). Molecular techniques have therefore been used to detect *G. vermiculophylla* in the eastern Atlantic (Rueness 2005, Guillemin et al. 2008, Saunders 2009, Kim et al. 2010), western Atlantic (Thomsen et al. 2006, Freshwater et al. 2006, Saunders 2009, Kim et al. 2010), eastern Pacific (Bellorin et al. 2004, Saunders 2009, Norris and Gurgel in press), and in its native range in the western Pacific (Yang et al. 2008, Skriptsova and Choi 2009, Kim et al. 2010).

Kim et al. (2010) included samples from 3 continents and found that the haplotype (intraspecific) richness and diversity of *G. vermiculophylla* was substantially higher in its native range (17 haplotypes, diversity (Hd) 0 to 0.933) than in its invaded range (3 haplotypes, diversity (Hd) 0 to 0.327). Low haplotype richness in invaded areas was also found in the United States, Canada and Europe (2 haplotypes, Saunders 2009). However, in these studies, more sites were sampled in the species native range which could have artificially increased measured diversity in these areas when compared to invaded areas. More intensive sampling in invaded regions is needed before comparisons between native and invaded regions can be accurately made.

G. vermiculophylla was first identified in the VA coastal bays, USA in 2004 by analyzing the *cox*2-*cox*3 mitochondrial spacer region of three samples (*cox*2-3 spacer in remaining of paper, Thomsen et al. 2006). Prior to this study, *Gracilaria* samples in the region were morphologically misidentified as the native *G. tikvahiae* (Thomsen et al. 2006). A larger survey was needed to confirm whether *G. vermiculophylla* is widespread in the VA coastal bays.

To date, several different molecular markers have been used to identify *G. vermiculophylla.* In this study, we used *cox*2-3 spacer (Rueness 2005, Thomsen et al.

2006), *rbc*L (Rueness 2005, Yang et al. 2008, Skriptsova and Choi 2009, Hommersand and Freshwater 2009, Sfriso 2010), and *cox*1 (Yang et al. 2008, Saunders 2009, Skriptsova and Choi 2009, Kim et al. 2010) markers to identify *G. vermiculophylla* samples in the western Atlantic.

The goals of this study were to: (i) document the current distribution of *G. vermiculophylla* in the western Atlantic using three molecular markers and (ii) compare *cox*1 haplotype richness and diversity in the VA coastal bays to other reported invaded and native populations.

Materials and Methods

Sample collection

Samples were collected from eastern USA sites (South Carolina (SC), VA, Massachusetts (MA), and Rhode Island (RI)) for *cox*2-3 spacer and *rbc*L identification between 1999 and 2000. *Cox*1 analysis was used for samples collected at 39 marsh, mudflat, and seagrass sites within the VA coastal bays (37° to 38° N, 75°17' to 75°56' W) between June 3rd and July 8th, 2009, and then on September 25th 2009. One to nine algal samples were haphazardly collected at each site. All specimens were preserved with silica gel. Further collection details including vouchers and GenBank accession numbers can be found in Table 2.S1 and 2.S2.

Molecular analyses

For *cox*1 analyses, DNA was extracted from tissue samples using NucleoSpin® 96 Plant II kits (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's

protocol. We targeted the mtDNA *cox*1 region with primers GazF1 and GazR1 from Saunders (2005)**.** Gene amplification followed protocols outlined in Lin et al. (2001). PCR products were cleaned with ExoSAP-IT (GE Healthcare, Picataway, New Jersey, USA) following manufacturer's instructions. Sequencing reactions were conducted in both forward and reverse directions using the Big Dye Terminator chemistry (Applied Biosystems, Carlsbad, CA, USA). Sequences were cleaned with Millipore Multiscreen Sequencing plates (Millipore, Darmstadt, Germany) and capillary separation was outsourced to the Australian Genome Research Facility (AGRF), Adelaide node, South Australia. Generated sequence data were compiled with Sequencher v. 4.9 (Gene Codes Corp., Ann Arbor, MI), aligned for phylogenetic analysis in ClustalX 2 (http://www.clustal.org), proof read for misaligned sections, gaps, and stop codons, and further edited by hand in MacClade 4.08 (Maddison and Maddison 2005). North Carolina (NC) *rbc*L DNA sequences were provided by Dr. Wilson Freshwater (UNCW) and other *rbc*L sequences newly generated in this study used protocols and primers described in Gurgel and Fredericq (2004). For *cox*2-3 spacer, we used protocols described in Zuccarello et al. (1999).

Haplotype parsimony networks

Published *cox*1 sequences (394 samples) were obtained from GenBank and aligned with VA sequences. Aligned sequences were clipped to a 507 bp overlap region and a parsimony network was constructed in TCS v.1.2.1 (Clement et al. 2000) using a 95% connection limit. In order to prevent confusion with haplotype labeling, we conformed to the labeling system of Kim et al. (2010). Haplotypes that corresponded to

one of the 19 haplotypes reported in Kim et al. (2010) were assigned the appropriate number, and those that had not been reported or numbered in the literature were given new values (starting at 20) and new GenBank accession numbers.

*Rbc*L and *cox*2-3 spacer sequences from sites other than VA were aligned with respective GenBank sequences. The *rbc*L alignment of 51 specimens encompassed the entire 1467 nucleotide gene. *Cox*2-3 spacer alignment of 28 specimens encompassed 345 nucleotides as used in Rueness (2005). A parsimony network was generated for the *rbc*L data using TCS as per above. *Rbc*L haplotypes that corresponded to one of the 3 haplotypes reported in Yang et al. (2008) were assigned the same name (R1-R3) whereas new haplotypes were given new names starting at R4. It is important to note that the naming of the R2 and R3 haplotypes in Fig. 2.1 from Yang et al. (2008) was inverted, so we followed the naming as per their Supplementary 1. Due to the paucity of *cox*2-3 spacer sequences, only pair-wise distances between our data and published sequences using p-distances in PAUP* (Swofford 2002) were used to confirm sample identity.

Cox1 haplotype richness and diversity

Haplotype richness and diversity (Nei and Tajima 1981) were calculated based on the 507 bp *cox*1 region (Table 2.S3). Preliminary examination of the dataset showed that higher haplotype richness could be related to either the number of sites sampled or the total number of samples collected. To test this, Spearman's rank correlation analyses were conducted in SAS (SAS 9.2; SAS Institute, Carey, NC, USA) to compare the number of haplotypes detected with the number of sites sampled within a region and the total number of samples collected in each study.

Since we collected considerably more samples than previous studies, bootstrap resampling was used to determine how VA *cox*1 haplotype richness would have changed if fewer sampling sites had been used (SAS 9.2; SAS Institute, Carey, NC, USA). Each analysis used 1000 replicates and was run to simulate sampling anywhere from one to 39 sites. The mean number of haplotypes found at each site sampling replicate were calculated and plotted. The maximum and minimum number of haplotypes found in each replicate were also plotted in order to show the range of values possible when a specific number of sites were re-sampled. Literature values of site quantity versus haplotype detection were plotted on the same figure.

Results

Sequencing and parsimony networks

*Rbc*L, *cox*2-3 spacer, and *cox*1 sequencing confirmed *G. vermiculophylla* presence in the western Atlantic as far north as MA and as far south as SC. Intra-specific genetic variation of *rbc*L ranged between 0.0 and 1.73%, with 12 haplotypes named R1 to R12 (Fig. 2.S2) detected. The central haplotype in the network, R1 was also the most common haplotype found in the native range in Japan and Korea, and in introduced locations such as Norway, the Netherlands, Italy, NC, MA and CA. New Jersey (NJ; R9), NC (R5, R7, R8) and RI (R10) have new *rbc*L haplotypes not yet found in other studies (Fig. 2.S2). Only 2 haplotypes separated by one single mutation were found when *cox*2-3 spacer obtained from 4 SC, VA, MA, and RI samples were compared to 19 sequences from Rueness (2005) collected in Japan (AY725145), Korea (AY725144) and introduced populations in Europe (i.e. France: AY725152-60, Sweden: AY725147, Portugal:

AY725146, Netherlands: AY725142, and Spain: AY725143) (data not shown). Eighteen of the 19 *cox*2-3 spacer samples were the same haplotype, the exception being a specimen from SC. *Cox*2-3 spacer pair wise uncorrected p-distance genetic divergence ranged between 0.0-0.3%. *Cox*2-3 spacer haplotypes were not named but showed that SC, MA and RI samples were indeed *G. vermiculophylla* with little to no genetic variation with their European, Japanese and Korean counterparts.

*Cox*1 sequences were obtained for 174 samples with seven different haplotypes from 39 sites within the coastal bays. At 30 of these sites we found only haplotype number 6, and at the remaining 9 sites we found 2 to 3 haplotypes (Fig. 2.1). Five of the haplotypes reported in this study have not been documented previously and were assigned haplotype numbers 20-24 (Table 2.S1).

The Spearman's rank correlation analysis comparing the number of sites sampled within a region and the number of haplotypes found in each study showed a significant correlation of $r_s = 0.54$ ($p = 0.03$). The Spearman's rank correlation analysis comparing the total number of samples collected within a region with the number of haplotypes detected did not show a significant correlation ($p = 0.14$) but still a small positive correlation coefficient, $r_s = 0.38$.

Bootstrap resampling returned mean haplotype numbers ranging from 1.21 ± 0.48 (mean ± SD) when only one site was resampled, to 5.26 ± 1.12 when 39 sites were resampled. Once the program had reached 14 sites being resampled it was possible for all 7 haplotypes to be detected in some trials, as indicated by the maximum line on Fig. 2.2. Data collected in Korea by Kim et al. (2010) and Yang et al. (2008) as well as the data

presented in our study, show that when a large number of sites are sampled, haplotype richness is high.

Discussion

Our multi-marker dataset showed that the current distribution of *G. vermiculophylla* in the western Atlantic extends from MA to SC, USA. *Rbc*L was used in CA, NC, VA, MA, RI and NJ (Table 2.S2), *cox*2-3 spacer in SC, and *cox*1 in VA (Table 2.S1, 2.S3). The newly generated *rbc*L sequences from our USA samples, together with GenBank sequences, revealed the existence of at least 12 distinct *rbc*L haplotypes worldwide (R1-R12, Table 2.S2, Fig. 2.S2).

*Cox*1 haplotype richness in the VA coastal bays (7 haplotypes) was much higher than that described previously, not only in other invaded regions (1-3 haplotypes, Saunders 2009, Kim et al. 2010), but also in several native regions (Japan 3 haplotypes, China 2 haplotypes; Yang et al. 2008, Kim et al. 2010). The only location where more haplotypes have been recorded than VA is Korea, which had 10 different *cox*1 haplotypes (Fig. 2.2, Kim et al. 2010). While haplotype diversity in VA is still lower than that found at these native sites, it is the most diverse when compared to other invaded sites (Table 2.S3).

There are three potential reasons why high haplotype richness was detected in this study: (i) a large single introduction of *G. vermiculophylla* into the region from a diverse donor population; (ii) several introductions; and (iii) more intensive sampling discovered more haplotypes missed in previous poorly sampled studies. *G. vermiculophylla* in the VA coastal bays likely came from the western Pacific because the two areas share several

haplotypes (Fig. 2.S1). In addition, our study found unique haplotypes in VA. It is unlikely that the unique haplotypes can be attributed to recent mutations in the mitochondrial DNA after an initial introduction and are likely due to a lack of data in the native range.

While there are many modes of *G. vermiculophylla* transport such as entanglement in boat propellers, anchors, and fishing gear, hull fouling and ballast water (Rueness 2005, Weinberger et al. 2008), attachment to oyster shells is the most likely mode of long distance dispersal (Thomsen et al. 2006). Multiple introductions of the Japanese oyster, *Crassostrea gigas,* have been well documented in the Chesapeake Bay from 1988 to 1990 and NJ in the 1930s (Carlton 1992, Nelson 1946). It is possible that *G. vermiculophylla* could have attached to these imported oysters and been introduced to the western Atlantic. Subsequent oyster trades among the states on the east coast of the USA, which have been common since at least 1935 (Armstrong et al. 1935), could have provided a mechanism for multiple introductions in coastal waters throughout the region.

This study documented high haplotype richness in the VA coastal bays because of the intensive sampling, as seen in other studies where sampling has been increased (Zuccarello et al. 2006). We conducted correlation analyses to compare the number of haplotypes found in our study and in the literature to the number of samples collected and the number of sites visited in each study. There was no significant correlation between haplotype richness and the number of samples collected, but there was a significant correlation between haplotype richness and the number of sites visited.

Because we believe that the high haplotype richness detected in this study could be related to the relatively high number of distinct sites sampled, we wanted to model

how our results would have looked if we had collected samples from fewer sites. Bootstrap re-sampling demonstrated that with a lower number of sampling sites, fewer haplotypes would have been detected.

When studying macroalgal introductions, it is important to maintain constant collections and sequencing standards across distinct institutions. The use of multiple markers by different research groups is a hindrance to the study of *G. vermiculophylla* worldwide (Saunders 2009). Molecular data in this study suggest that: (1) the current range of *G. vermiculophylla* along the Eastern North American coast is from MA to SC; and (2) a more extensive sampling design in VA documented higher *cox*1 haplotype diversity which is indicative of multiple introductions from multiple geographic source locations. We believe that earlier studies investigating *G. vermiculophylla* haplotype richness and diversity in invaded regions may have collected too few samples which resulted in reporting lower haplotype richness. Future studies should take note of differences in sampling methods when comparing haplotype richness and diversity in the literature. In addition, further collection and sequencing of *G. vermiculophylla* samples in invaded and native ranges will develop a more complete *cox*1 library and would allow future studies to better analyze introduction transport pathways.

Acknowledgments

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Coastal Research Center in VA and the Smithsonian Marine Station at Fort Pierce, Florida for field and lab support; Dr. Loren Coen (Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston), for providing time aboard the R/V *Silver Cresent* to dredge on the Charleston shrimp bars and Shaun Norris for collection assistance; The Nature Conservancy in the VA coastal reserve for research permits. This is Smithsonian Marine Station at Fort Pierce contribution. Funding for part of this work was provided by the Virginia Coastal Reserve Long Term Ecological Research Grant DEB-0621014.

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Figures

Fig. 2.1. World map showing *cox*1 haplotype distributions. For more detailed information see Table 2.S1. BC = British Columbia, Canada, CA = California, USA, NC = North Carolina, USA, and RI = Rhode Island, USA.

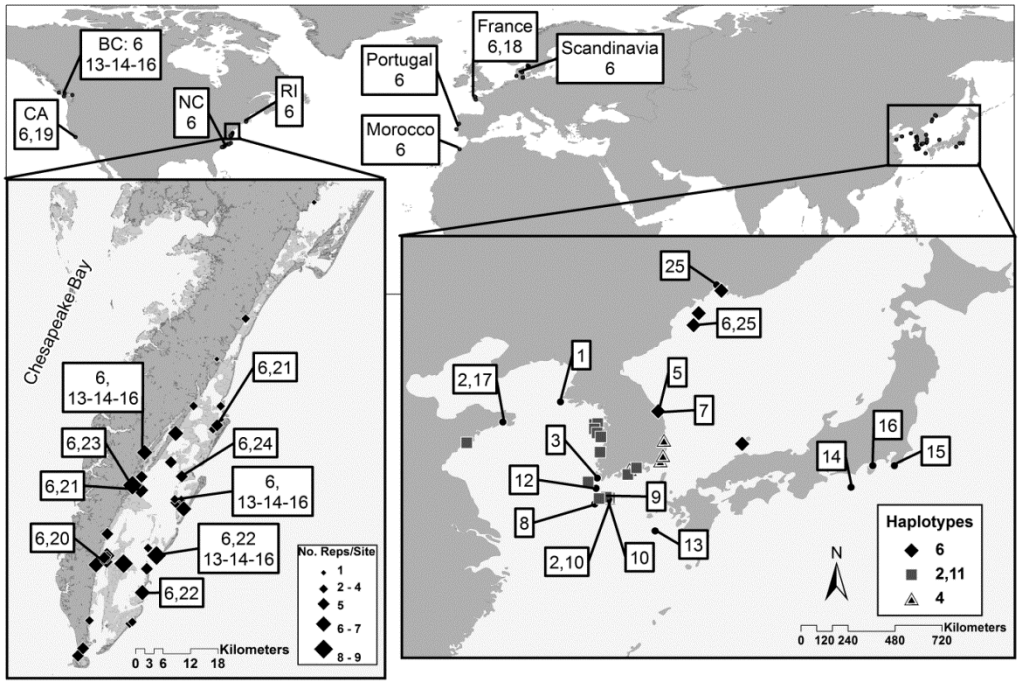


Fig. 2.2. Graph showing the number of *cox*1 haplotypes found (y-axis) as a function of the number of sites sampled (x-axis). Crosses and diamonds show literature results from native and non-native regions, respectively. Each bootstrap analysis re-sampled the haplotype results in this study (n = 1000) for 1 to 39 sites. Mean (triangles), minimum (gray line), and maximum (black line) number of haplotypes found within each analysis are displayed on the graph.

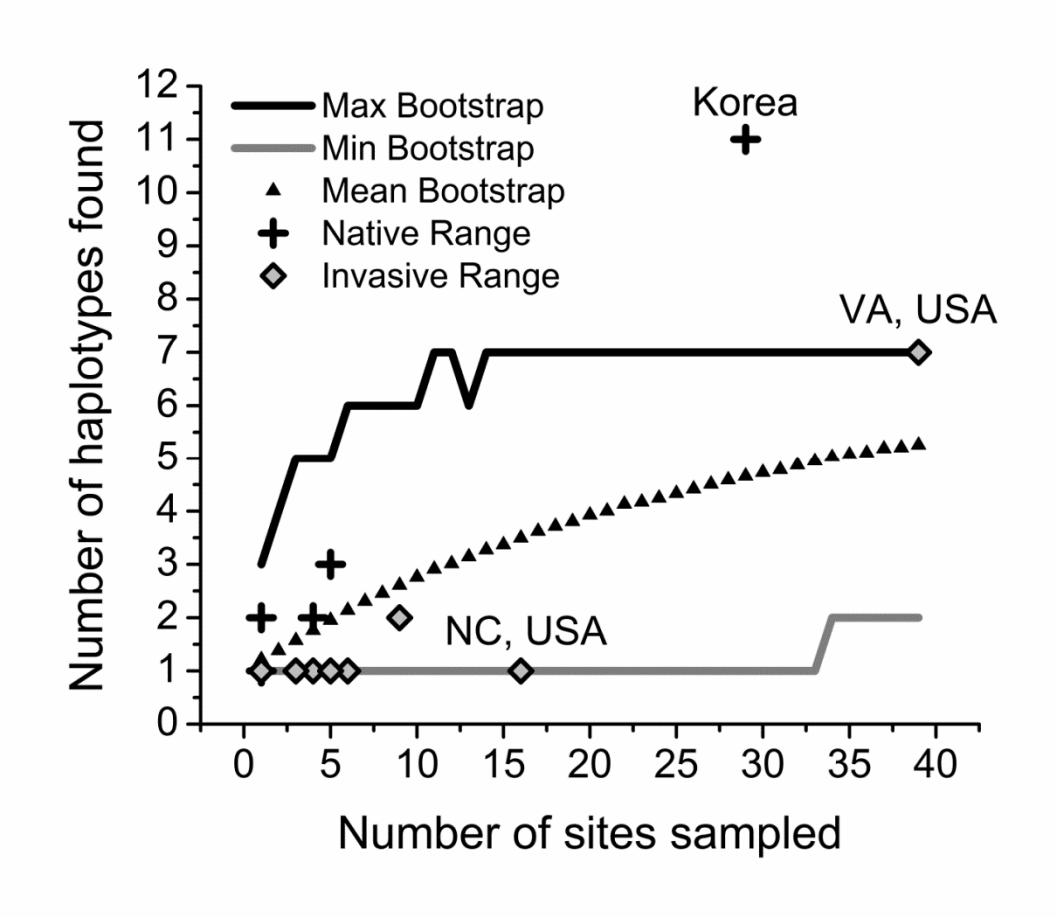


Fig. 2.S1. Parsimony network from a 507 base pair overlap of *cox*1 DNA sequences from 568 *G. vermiculophylla* samples, 174 from VA (this study) and 394 literature samples (GenBank). For more detailed information on the sequences see Table 2.S1.

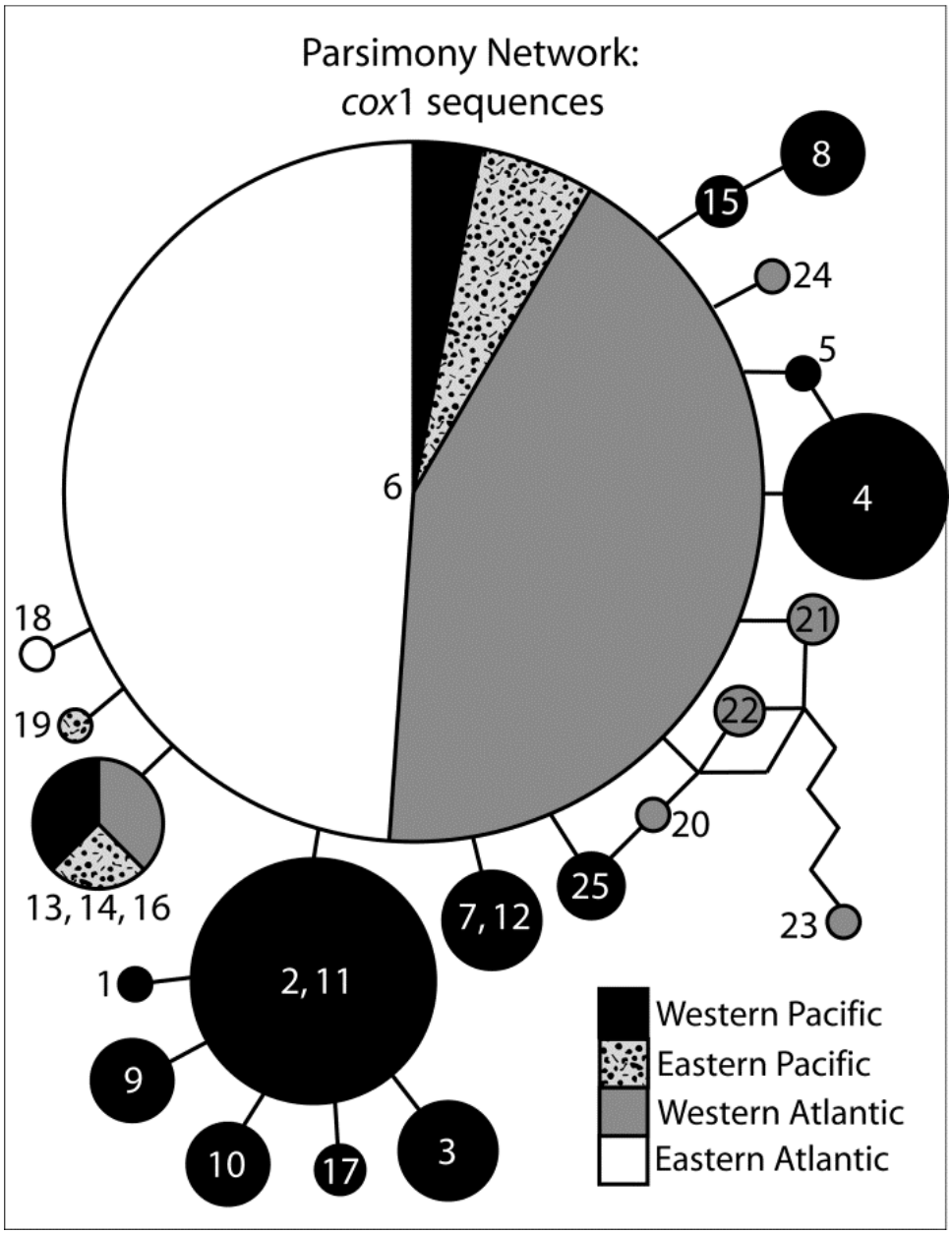
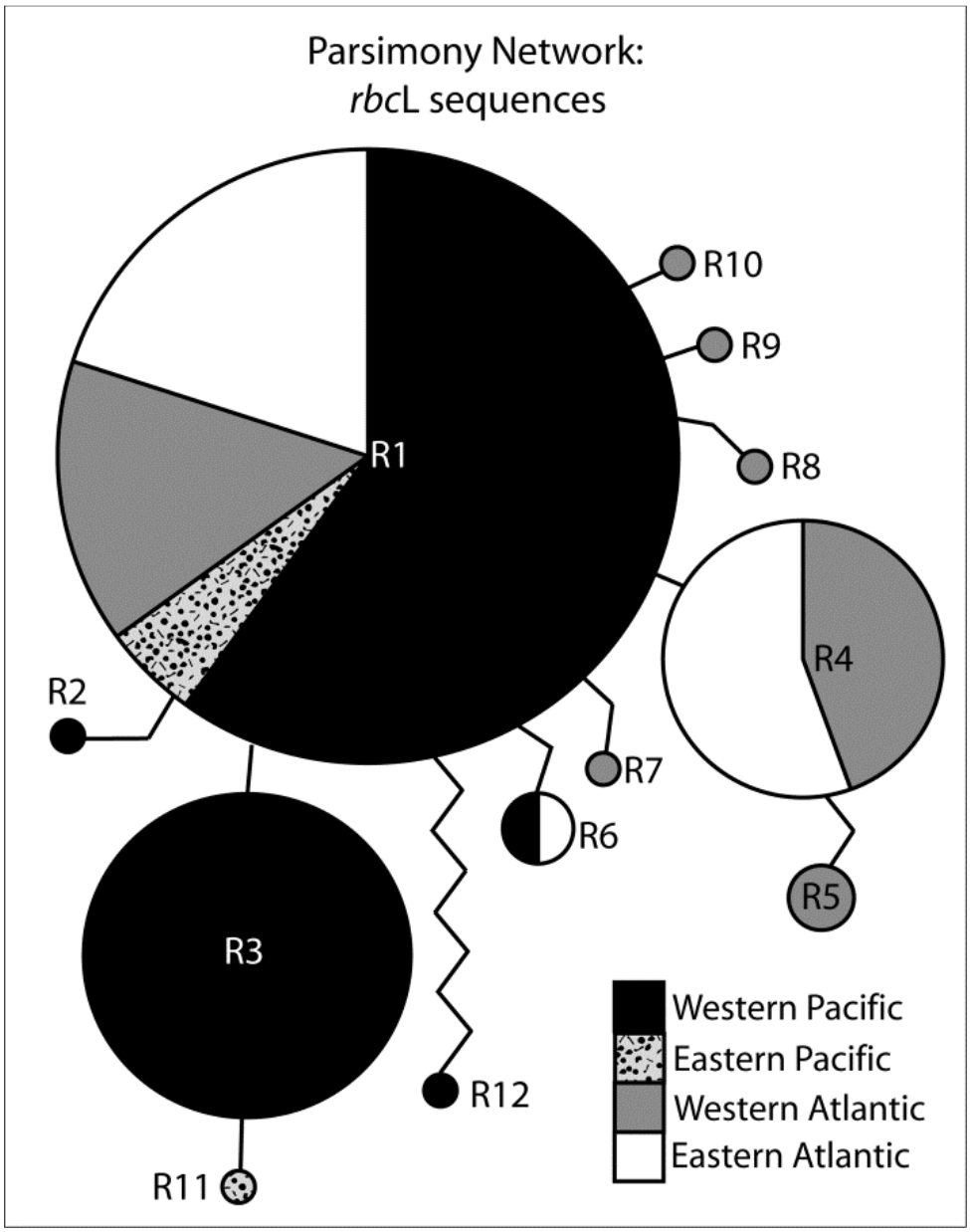


Fig. 2.S2. Parsimony network for *G. vermiculophylla rbc*L sequences from the literature and this study. For more detailed information on the sequences see Table 2.S2.



Tables

Table 2.S1. Additional information for *G. vermiculophylla cox*1 haplotypes included in 507 bp parsimony network (Fig. 2.S1). For more details on sampling dates and locations see cited references.

Haplotype	Location	Total no. of samples	Reference sequence, GenBank accession number(s) and remarks	Reference
1	Korea	1	GU907110	Kim et al. (2010)
2	Korea	5	EF434928, EF434932-4, EF434936	Yang et al. (2008)
2	Korea	30	30 out of 78 individuals, all with same sequence as EF434936	Kim et al. (2010)
2	China	17	EF434936	Kim et al. (2010)
3	Korea	5	GU907108	Kim et al. (2010)
4	Korea	12	GU907109	Kim et al. (2010)
5	Korea	1	EF434926	Yang et al. (2008)
6	Korea	1	EF434927	Yang et al. (2008)

6	Japan	1	EF434939	Yang et al. (2008)
6	Morocco	1	All same as EF434927	Kim et al. (2010)
6	Europe: Denmark, France, Germany, Sweden	175	All same as EF434927	Kim et al. (2010)
6	Korea	2	All same as EF434927	Kim et al. (2010)
6	Russia	3	All same as EF434927	Kim et al. (2010)
6	USA: CA, NC, VA	27	All same as EF434927	Kim et al. (2010)
6	British Columbia, Canada	15	FJ499551-FJ499556, FJ499567-FJ499571, FJ499580, FJ499616, FJ499619, FJ499622	Saunders (2009)
6	Portugal	16	FJ499557-FJ499566, FJ499572-FJ499577	Saunders (2009)
6	USA: RI	40	FJ499578, FJ499579, FJ499582-FJ499615, FJ499617, FJ499618, FJ499620, FJ499621	Saunders (2009)

6	Russia	6	GQ292864, GQ292867, GQ292869-GQ292872	Skriptsova and Choi (2009)
6	USA: VA Coastal Bays	164	JQ794754	This study
7 & 12	Korea	6	GU907106	Kim et al. (2010)
8	Korea	4	EF434929	Yang et al. (2008)
9	Korea	4	GU907111	Kim et al. (2010)
10	Korea	4	GU907112	Kim et al. (2010)
11	Korea	3	EF434930, EF434931, EF424935	Yang et al. (2008)
13	Japan	1	GU907105	Kim et al. (2010)
14 & 16	Japan	2	EF434937, EF434938	Yang et al. (2008)
15	Japan	2	GU907104	Kim et al. (2010)
13, 14, & 16	British Columbia, Canada	2	FJ499550, FJ499581	Saunders (2009)
13, 14, & 16	USA: VA Coastal Bays	3	JQ794753	This study
17	China	2	GU907103	Kim et al. (2010)

18	Europe: France	1	GU907102	Kim et al. (2010)
19	USA: CA	2	GU907113	Kim et al. (2010)
20	USA: VA Coastal Bays	1	JQ794759	This study
21	USA: VA Coastal Bays	2	JQ794755	This study
22	USA: VA Coastal Bays	2	JQ794756	This study
23	USA: VA Coastal Bays	1	JQ794757	This study
24	USA: VA Coastal Bays	1	JQ794752	This study
25	Russia	3	GQ292865, GQ292866, GQ292868	Skriptsova and Choi (2009)

Table 2.S2. Additional information for *G. vermiculophylla* rbcL sequences included in Fig. 2.S2 parsimony network. For more details on sampling dates and locations see references.

Haplotype	Location	Total no. of samples	GenBank	Citation
R1	USA: CA, NC, MA	4	JQ768762, JQ768768, JQ768771, JQ768772	This study
R1	Europe: Norway, Netherlands	2	AY725171, AY725174	Rueness (2005)
R1	Europe: Italy	1	FN400862	Sfriso (2010)
R1	USA: NC	1	EU600293	Hommersand and Freshwater (2009)
R1	Korea	10	DQ095815-DQ095821, EF434909-EF434911	Yang et al. (2008)
R1	Japan	2	EF434912-EF434913	Yang et al. (2008)
R2	Japan	1	DQ095822	Yang et al. (2008)
R3	Russia	9	GQ292855-GQ292863	Skriptsova and Choi (2009)

R3	Korea	2	EF434907-EF434908	Yang et al. (2008)
R4	USA: NC, VA	4	JQ768761 (2 samples), JQ768763, JQ768766	This study
R4	Europe: France	1	AY725172	Rueness (2005)
R4	Europe: France	4	DQ241572, DQ241574, DQ241583, DQ241586	Weinberger et al. (2010)
R5	USA: NC	2	JQ768764, JQ768765	This study
R6	Europe: Sweden	1	AY725173	Rueness (2005)
R6	Korea	1	AY725175	Rueness (2005)
R7	USA: NC	1	EU605702	Hommersand and Freshwater (2009)
R8	USA: NC	1	JQ768767	This study
R9	USA: NJ	1	JQ768774	This study
R10	USA: RI	1	JQ768773	This study
R11	USA: CA	1	JQ768769	This study
R12	Japan	1	JQ768770	This study

Table 2.S3. Summary table comparing sampling methods, haplotype richness, and diversity. Diversity calculated per the methods of Nei and Tajima (1981).

Location	Citation	Total no. of samples	Total no. of sites	Haplotype richness	Haplotype diversity (Hd)
Korea	Kim et al. (2010)	78	29	10	0.729
VA	This study	172	39	7	0.112
Japan	Kim et al. (2010)	6	5	3	0.733
France	Kim et al. (2010)	56	4	2	0.036
BC	Saunders (2009)	17	4	2	0.221
CA	Kim et al. (2010)	11	1	2	0.327
China	Kim et al. (2010)	11	2	2	0.389
Russia	Skriptsova and Choi (2009)	9	4	2	0.500
Denmark	Kim et al. (2010)	4	1	1	0
Germany	Kim et al. (2010)	43	3	1	0
Morocco	Kim et al. (2010)	1	1	1	0
NC	Kim et al. (2010)	16	16	1	0
Portugal	Saunders (2009)	16	3	1	0
RI	Saunders (2009)	40	3	1	0
Russia	Kim et al. (2010)	1	1	1	0
Sweden	Kim et al. (2010)	73	5	1	0
VA	Kim et al. (2010)	2	1	1	0

Chapter 3: Nitrogen transfers mediated by a perennial, non-native macroalga: A ^{15}N tracer study

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Abstract

Mats of macroalgae can alter nutrient regimes in intertidal communities, such as mudflats, marshes and beaches, by transferring nutrients to the surrounding habitat. Previous work has focused on ephemeral species of macroalgae that decompose in these intertidal environments. However, unlike ephemeral macroalgae, perennial species can be long-lived, resident members in intertidal systems and their role in mediating nutrient transfers may therefore be different. In this study, we used a ^{15}N isotope tracer to determine if nitrogen from a perennial, non-native macroalga (*Gracilaria vermiculophylla*) could be found in other macrophytes and in higher trophic groups on salt marshes and mudflats in shallow coastal bays of Virginia. We found that sediment on marshes and mudflats, the marsh cordgrass, *Spartina alterniflora*, and mudflat invertebrates all incorporated nitrogen from *G. vermiculophylla,* indicating that this perennial alga is important in the transfer of nutrients within, and between, trophic levels.

Keywords: nitrogen transfer, isotope, non-native, *Gracilaria vermiculophylla*, perennial, marsh, mudflat

Introduction

Prior work on intertidal macroalgal mats has found that intertidal macroalgal communities can mediate nutrient transfers to higher trophic levels as well as other macrophytes. However, until now, a distinction has not been made between ephemeral and perennial macroalgae. Ephemeral algae typically bloom in areas with high nutrient inputs and then collapse when they are limited by oxygen and/or light availability (Sfriso et al. 1992, Valiela et al. 1997). Perennial fucoids (brown macroalgae) and rhodophytes (red macroalgae), in contrast, form mats that persist on longer time scales (Gerard 1999, Thomsen et al. 2006, Dijkstra et al. 2011). Because their growth cycles are different, information on both ephemeral and perennial macroalgae is needed to understand the potential role of macroalgae in nutrient transfers on marshes and mudflats.

Gerard (1999) hypothesized that mats of the perennial brown macroalga *Ascophyllum nodosum* enhanced the survival of marsh cordgrass (*Spartina alterniflora*) by releasing nutrients during senescence; however, this hypothesis was never tested experimentally. All prior studies that have experimentally documented intertidal macroalgal nutrient transfers have focused on ephemeral species (Boyer and Fong 2005, Rossi 2007). However, these results cannot be directly applied to ecosystems with perennial macroalgae that persist in intertidal environments.

Gracilaria vermiculophylla is a perennial rhodophyte that is native to Southeast Asia and has invaded temperate estuaries across North America and Europe (Kim et al. 2010, Gulbransen et al. 2012). It is a successful invader in the mid-Atlantic region due to its resistance to desiccation, sedimentation, and grazing relative to native species, and to

its association with a common mudflat polychaete, *Diopatra cuprea*, that stabilizes populations and facilitates asexual reproduction (Thomsen and McGlathery 2005, 2007). *G. vermiculophylla* has been the dominant macroalgal species in the Virginia coastal bays since at least 1998 (Thomsen et al. 2006), and high densities on marshes (mats up to 3 cm deep, maximum biomass 88 gdw m^{-2}) and mudflats (mats up to 15 cm deep, maximum biomass 800 gdw m^{-2}) can persist on the order of months to years (pers. obs.).

We hypothesized that *G. vermiculophylla* was present year-round on marshes and mudflats and would transfer nitrogen to the sediments, the marsh cordgrass *Spartina alterniflora*, and invertebrates, including the marsh snail *Littorina irrorata*, the mud snail *Ilyanassa obsoleta*, gammarid amphipods, and the tube-building polychaete *D. cuprea* that are all common in these systems. Using a natural abundance mixing model that incorporated ^{13}C, ^{15}N, and ^{2}H on a Virginia coastal bay marsh, we were unable to resolve trophic interactions because the isotopic composition of end-members in the community were not sufficiently different (unpub. data). Therefore, we enriched *G. vermiculophylla* with a ^{15}N stable isotope tracer and recorded the changes in ^{15}N levels in sediments, macrophytes and invertebrates to determine if the macroalga mediated nitrogen transfers on marshes and mudflats.

Methods

This study was conducted in the Virginia coastal bays at the Virginia Coastal Reserve Long Term Ecological Research (VCR LTER) site. The Virginia coastal bays extend 110 km along the mid-Atlantic coast and are bounded to the west by the Delmarva Peninsula and to the east by a series of barrier islands. They are shallow with 50% < 1 m

at mean low water, a tidal range of 1.2-1.5 m, and 37% of the benthic surface area covered by marsh and intertidal flats (Oertel 2001).

Seasonal macroalgal biomass was determined along transects at 5 marshes from June 2009 to January 2012 and 3 mudflats from December 2010 to January 2012 (Figure 3.1). At each marsh site a 100 m transect was run perpendicular to the edge of the marsh-mudflat interface and five haphazard 0.25 m^2 samples were collected in each 20 m segment, for a total of 25 samples. Mudflat transects were conducted the same way but were 30 to 50 m in length, with samples collected in 10 m sections.

All macroalgal samples were rinsed with distilled (DI) water, identified, dried in a 60 °C oven for at least 48 hours, and weighed. A subset of samples were saved for C:N analysis, which was conducted on a Carlo Erba elemental analyzer. Biomass data from each sampling period were pooled seasonally and plotted relative to *Ulva* spp., another prominent macroalgal species in the region.

The ^{15}N enrichment studies were conducted on the Ramshorn channel marsh and mudflat (Figure 3.1; 37° 18.133' N, 75° 54.036 W). Prior to the start of the experiment, *G. vermiculophylla* was labeled with 98% + $^{15}NO_3NH_4$ for 1 week in the lab. Each day, enough 98% + $^{15}NO_3NH_4$ was added to fuel 0.05 gdw growth per day and a cumulative 1% tissue enrichment (i.e. 0.0312 mg N day^{-1} for 100 g algae).

For the marsh experiment, 20 paired control (no *G. vermiculophylla* added) and experimental (with added live, ^{15}N labeled *G. vermiculophylla*) cages (circular, 1/16 m^2) were anchored on the marsh on May 17th, 2010 using PVC stakes. At each of 5 sampling

periods, 38, 71, 93, 138, and 249 days after initial launch, 4 replicate cages were collected. Within each cage, any remaining *G. vermiculophylla*, one sediment plug (30 cc syringe, 5 cm depth), *S. alterniflora*, and all macrofauna were collected. We replaced enriched algae within experimental cages that were not collected at each sampling period at biomass amounts that reflected seasonal variations. At 38 and 71 days into the experiment, the equivalent of 110 gdw m^{-2} of labeled *G. vermiculophylla* was added to experimental cages, followed by 45 gdw m^{-2} at 93 and 138 days, and 28 gdw m^{-2} at 249 days.

For the mudflat experiment, 30 cages (circular, 1/8 m^2), 10 control without *G. vermiculophylla,* and 20 experimental with live, ^{15}N labeled *G. vermiculophylla,* were anchored to the mudflat on June 9th, 2010 using PVC stakes. All cages were collected after 1 month because maintaining accurate algal biomasses within cages on the mudflats for a longer period of time was not possible due to tidal effects. Algae were placed into experimental cages on the mudflat at densities between 90 to 500 gdw m^{-2} to represent estimates of patchy *in situ* conditions, which could be as low as 60 gdw m^{-2} and as high as 800 gdw m^{-2} (unpub. data). At the completion of the study, all remaining algae, one sediment plug (30 cc syringe, 5 cm depth), and all macrofauna were collected as described above.

Macroalgal samples were rinsed with DI water, dried in a 60 °C oven, and weighed. Sediment samples were picked free of roots and invertebrates were placed into separate containers and allowed to expel their guts for 24 hours before drying. All samples were ground, packaged and shipped to the University of California Davis Stable

Isotope Facility (UCD SIF) for ^{15}N tissue content analysis using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Marsh data were analyzed using three mixed model ANOVAs in SAS (SAS 9.2, SAS Institute, Carey, NC, USA) to test the effects of sampling time since launch, the presence or absence of ^{15}N enriched *G. vermiculophylla*, and the interaction of these two factors on the ^{15}N levels detected in the sediment, *S. alterniflora*, and *L. irrorata*. Data for sediment and *L. irrorata* ^{15}N levels satisfied ANOVA assumptions, but data for *S. alterniflora* ^{15}N levels had to be natural log transformed in order to satisfy ANOVA assumptions. Data for sediment ^{15}N levels on the mudflat were log transformed and analyzed using a one-way ANOVA. Amphipod, *D. cuprea*, and *I. obsoleta* ^{15}N data on the mudflat were all analyzed using non-parametric Wilcoxon tests.

Results

Seasonal transects showed that *G. vermiculophylla* was a dominant member of the macroalgal community and was present year-round (Figure 3.2, Table 3.S1). Average *G. vermiculophylla* tissue nitrogen values at the marsh and mudflat sites varied seasonally, with highest values documented in fall (Table 3.S2). In addition, tissue nitrogen values were higher, and C:N levels were lower on mudflats when compared to marshes year-round.

Over the course of the ^{15}N experiment, *G. vermiculophylla* on the marsh lost biomass (Table 3.S3). In addition, $\delta^{15}N$ tissue levels were always lower at the end of the experiment than at the beginning, but this difference was only significant in the third

sampling period (July 27th to August 18th, 2010, $p = 0.0396$, Table 3.S3). Cages with labeled *G. vermiculophylla* had significantly higher $\delta^{15}N$ levels in marsh sediment ($p < 0.0001$) and *S. alterniflora* ($p < 0.0001$), but there were no significant differences in *L. irrorata* tissue ($p = 0.2127$, Figure 3.3). Differences in $\delta^{15}N$ levels in sediment, *S. alterniflora*, and *L. irrorata* were not significantly affected by the number of days after launch before the samples were collected ($p = 0.2369$, 0.0780, and 0.0651, respectively). In addition, the interaction between enriched treatment and sampling time was not significant for sediment ($p = 0.2735$) or *L. irrorata* ($p = 0.2246$), indicating that their $\delta^{15}N$ levels changed at the same rate in each enrichment treatment. The enrichment treatment and sampling time interaction was significant for *S. alterniflora* measurements ($p = 0.0492$).

On the mudflat, $\delta^{15}N$ levels in *G. vermiculophylla* collected at the completion of the experiment were significantly lower than initial levels (Table 3.S4, $p = 0.0013$). Sediments underlying cages with labeled *G. vermiculophylla* added had significantly higher $\delta^{15}N$ levels ($p < 0.0001$), as well as significantly higher amphipod ($p < 0.0001$), *I. obsoleta* ($p = 0.0007$), and *D. cuprea* ($p = 0.0002$) tissue levels when compared to cages without labeled *G. vermiculophylla* (Figure 3.4).

Discussion

We present evidence of nitrogen transfers from the perennial macroalga, *G. vermiculophylla,* to sediment on the marsh and mudflat, to the marsh cordgrass *S. alterniflora*, and to mudflat invertebrates. *G. vermiculophylla* nitrogen could be entering the marsh and mudflat systems either via leakage of nitrogen during active growth or by

release during decomposition of the algae (Tyler and McGlathery 2006). This released nitrogen may have been subsequently incorporated into cordgrass on the marsh (Gerard 1999) or benthic microalgae (BMA) and bacteria and then further transferred through the trophic food web via consumption by macrofauna. Alternately, direct consumption of *G. vermiculophylla* by macrofauna could result in the incorporation of the tracer to the system.

On the mudflat, the mechanisms of trophic transfer of ^{15}N tissue from *G. vermiculophylla* to invertebrates were likely direct consumption of the labeled *G. vermiculophylla* or BMA, and/or other invertebrates that were enriched in ^{15}N from *G. vermiculophylla*. For amphipods, many species, including gammarids, have been shown to consume *Gracilaria* spp. tissue in both laboratory and *in situ* studies (e.g. Mancinelli and Rossi 2001). Other studies have found that amphipods prefer to eat diatoms (e.g. Kanaya et al. 2007). Thus, it is likely that the amphipods in our experiments had elevated ^{15}N levels from eating labeled *G. vermiculophylla* and/or BMA. The mud snail, *I. obsoleta,* is a non-selective omnivore (Feller 1984). Therefore, it is probable that mud snails assimilated ^{15}N either directly by consuming labeled *G. vermiculophylla* (Giannotti and McGlathery 2001) or indirectly by grazing on labeled BMA (Connor and Edgar 1982). Finally, our data indicate that the polychaete worm, *D. cuprea,* consumed labeled *G. vermiculophylla*, BMA, and/or invertebrate tissue. This is supported by prior work that examined gut contents of *D. cuprea* and found that it is omnivorous and will consume animal tissue, microalgae, and macroalgae (Mangum 1968).

In contrast to the mudflat, on the marsh we found that the dominant periwinkle snail, *Littorina irrorata*, did not incorporate labeled nitrogen from *G. vermiculophylla*. Previous studies have shown that periwinkle snails consume macroalgae (Norton et al. 1990), BMA (Sommer 1999), live *S. alterniflora* tissue (Bertness 1984, Silliman and Zieman 2001), detritus (Newell and Bärlocher 1993, Currin et al. 1995), and fungi growing on standing dead *S. alterniflora* stems (Newell and Bärlocher 1993, Silliman and Newell 2003). We often collected *L. irrorata* on *S. alterniflora* shoots, but since the snails did not incorporate the ^{15}N signal, we conclude that these snails were likely consuming fungi on *S. alterniflora* stems rather than the enriched cordgrass tissue directly, which resulted in a ^{15}N signal that was not significantly different from controls. Unfortunately, the inefficiency of collecting all fungi mycelia, as documented in Newell et al. (1986), prevented us from measuring fungal isotopic signatures.

Variations in *G. vermiculophylla* total nitrogen content followed the trend previously documented for algal species in the coastal bays, with highest nitrogen availability in late summer and early fall (Anderson et al. 2003, Tyler et al. 2003). The tissue nitrogen and C:N levels in *G. vermiculophylla* also indicated nitrogen was likely more limiting to macroalgae on the marsh compared to the mudflat.

This study demonstrates that a perennial, non-native macroalga is important in the transfer of nitrogen to sediments, *S. alterniflora,* and invertebrate consumers. It differs from prior studies which used ephemeral macroalgae in microcosms or buried as detritus in intertidal sediments. Our study was done under more realistic environmental

conditions for perennial macroalgae and confirmed that the non-native macroalga can transfer nitrogen to marshes and mudflats during both active growth and decomposition.

In order to determine if macroalgal-mediated nutrient transfers can result in nutrient subsidies to a system, researchers need to know how macroalgae move in space and time and if the addition of macroalgae results in increased growth and production of flora and fauna in recipient communities. Before the ultimate effects of *G. vermiculophylla* nutrient mediation on marshes and mudflats can be determined, more information is needed on these dynamics.

Acknowledgements

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Figures

Figure 3.1. Map of seasonal transect locations and ^{15}N study in the Virginia (VA) coastal bays, USA.

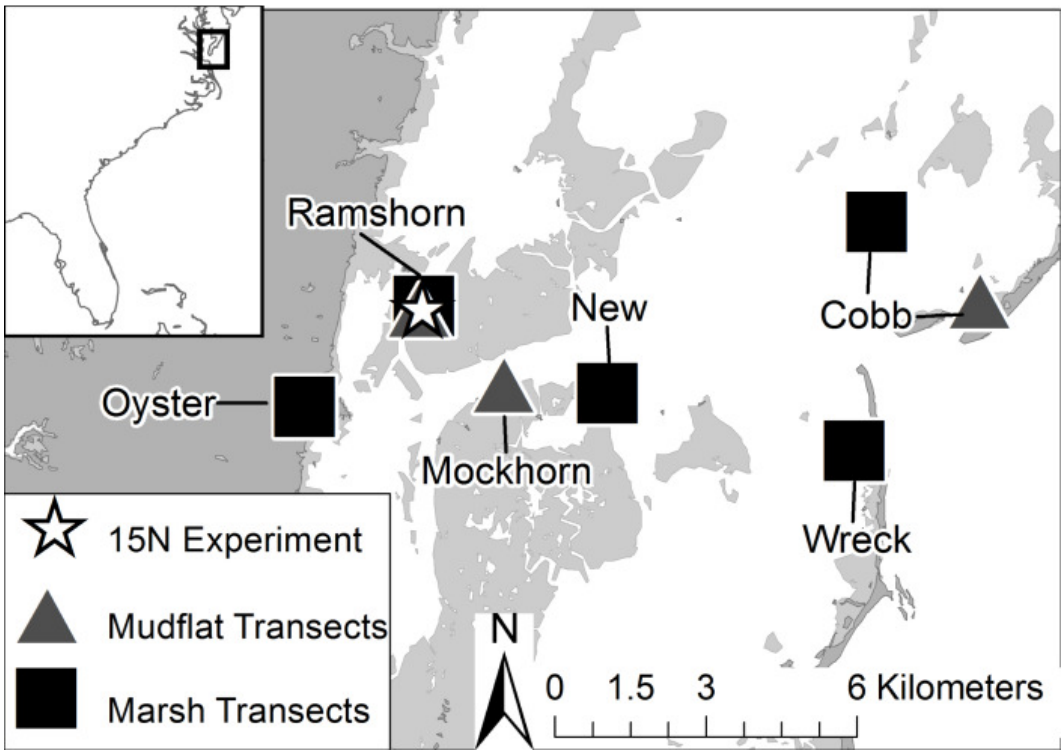


Figure 3.2. Average macroalgal biomass collected on (a) marshes and (b) mudflats seasonally from June 2009 to January 2012 (± SE). For more detailed information, see Table 3.S1.

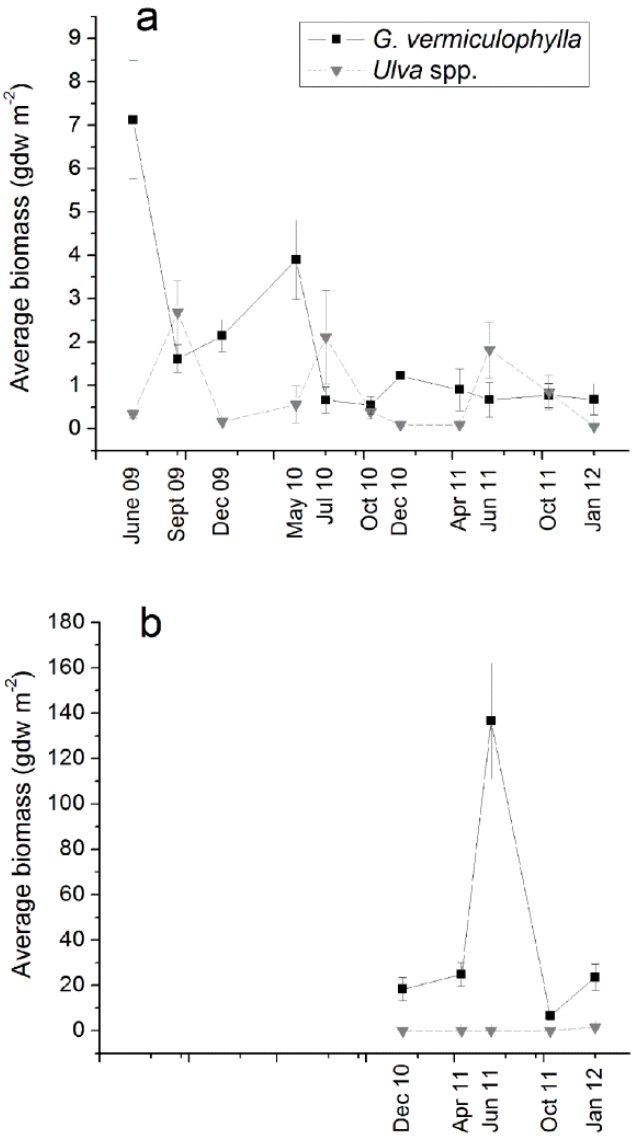
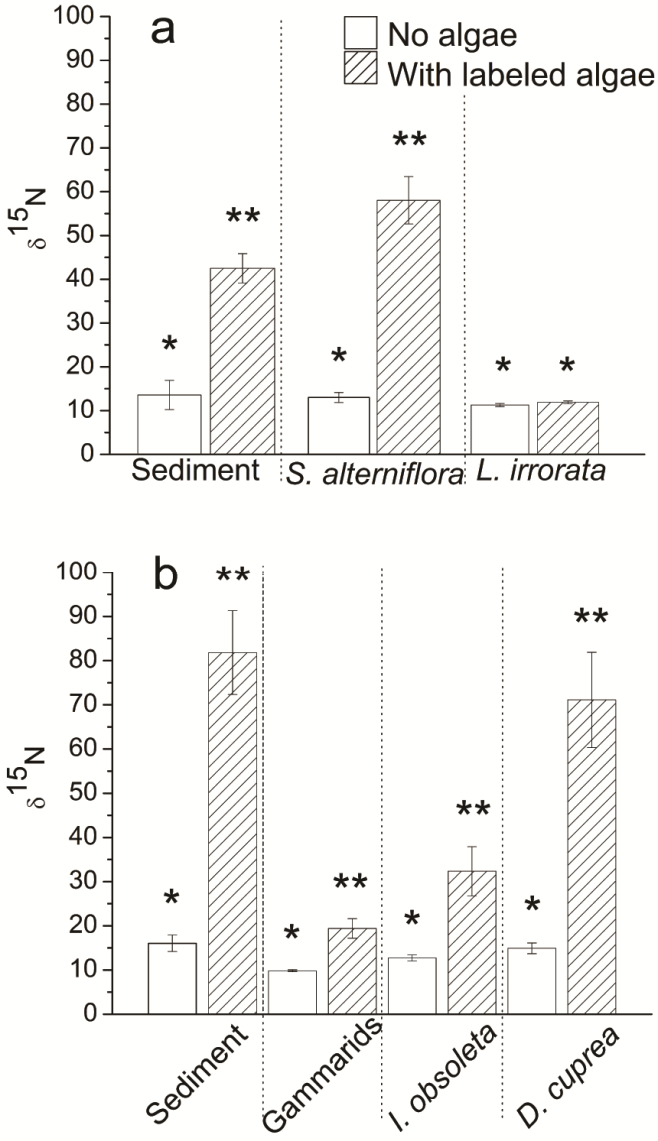


Figure 3.3. Compiled ^{15}N results for (a) marsh sediment, *Spartina alterniflora*, and *Littorina irrorata* and (b) mudflat sediment, gammarids, *Ilyanassa obsoleta*, and *Diopatra cuprea* in cages with and without labeled *Gracilaria vermiculophylla* (all ± SE). Significant differences within each category are denoted by asterisks (i.e. * is significantly different from **).



Tables

Table 3.S1. Average *Gracilaria vermiculophylla* biomass (gdw m^{-2} ± SE) on marshes sampled from June 2009 to January 2012 and mudflats sampled December 2010 to January 2012.

Date	Cobb Marsh	New Marsh	Oyster Marsh	Ramshorn Marsh	Wreck Marsh	Cobb Mudflat	Ramshorn Mudflat	Mockhorn Mudflat
Jun 2009	6.72 ± 2.06	1.02 ± 0.35	16.04 ± 5.44	11.29 ± 3.70	2.33 ± 1.02	NA	NA	NA
Sep 2009	4.60 ± 1.27	0.91 ± 0.46	1.00 ± 0.45	1.11 ± 0.30	0.40 ± 0.14	NA	NA	NA
Dec 2009	1.89 ± 0.69	0.80 ± 0.42	5.09 ± 1.48	2.26 ± 0.57	0.66 ± 0.20	NA	NA	NA
May 2010	3.12± 0.90	7.94 ± 3.80	6.01 ± 2.06	0.90 ± 0.43	1.52 ± 0.39	NA	NA	NA
Jul 2010	0	0.32 ± 0.25	2.86 ± 1.43	0.11 ± 0.10	0.02 ±0.01	NA	NA	NA
Oct 2010	0.26 ± 0.21	1.26 ± 0.46	1.29 ± 0.86	0	0.01 ± 0.01	NA	NA	NA
Dec 2010	0.16 ± 0.07	2.80 ± 2.10	2.61 ± 1.88	0.03 ± 0.02	0.82 ± 0.69	18.78 ± 6.96	17.98 ± 7.38	NA
Apr	0.10 ±	0.83 ±	2.44 ±	0	1.24 ±	10.46 ±	13.86 ±	58.90 ±

2011	0.05	0.32	2.06		1.24	3.35	5.07	14.24
Jun 2011	0.14 ± 0.13	0.22 ± 0.16	2.71 ± 1.87	0.14 ± 0.10	0.04 ± 0.03	1.11 ± 0.53	57.77 ± 11.96	336.36 ± 51.08
Oct 2011	0.57 ± 0.24	0.03 ± 0.03	0.71 ± 0.31	2.41 ± 1.22	0	0.12 ± 0.09	2.84 ± 0.60	16.04 ± 5.44
Jan 2012	0.52 ± 0.30	2.12 ± 1.99	0.73 ± 0.67	0.03 ± 0.02	0.27 ± 0.17	9.12 ± 5.87	52.51 ± 14.66	12.76 ± 5.39

Table 3.S2. Average seasonal nitrogen (N) and carbon (C) ± SE in *Gracilaria vermiculophylla* collected on marshes and mudflats.

Season	Marsh N	Marsh C	Marsh C:N	Mudflat N	Mudflat C	Mudflat C:N
Spring	1.69 ± 0.09	34.78 ± 0.81	20.58	2.29 ± 0.08	33.76 ± 0.22	14.74
Summer	1.95 ± 0.05	34.31 ± 0.14	17.59	2.93 ± 0.11	33.72 ± 0.24	11.51
Fall	2.51 ± 0.10	34.13 ± 0.21	13.60	3.16 ± 0.09	35.08 ± 0.50	11.10
Winter	2.33 ± 0.10	34.80 ± 0.19	14.94	2.60 ± 0.16	34.03 ± 0.35	13.09

Table 3.S3. Percent dry weight lost day^{-1}, initial and final $\delta^{15}N$, atom% ^{15}N, and %N tissue levels (all ± SE) of *Gracilaria vermiculophylla* in each sampling period of the marsh study. P values reported for t-test between initial and final $\delta^{15}N$ values in each sampling period.

Sample Date	Percent dry weight lost day^{-1}	Initial $\delta^{15}N$ of caged algae	Final $\delta^{15}N$ of caged algae	p for $\delta^{15}N$	Initial atom% ^{15}N	Final atom% ^{15}N	Initial %N	Final %N
6.24.2010	1.90 ± 0.29	2402.11 ± 798.60	599.27 ± 51.52	0.0871	1.55 ± 0.09	1.60 ± 0.09	1.23 ± 0.28	0.58 ± 0.02
7.27.2010	3.02 ± 0.01	1289.05 ± 226.31	156.46 ± 0	0.1106	2.48 ± 0.06	2.21 ± 0	0.83 ± 0.08	0.42 ± 0
8.18.2010	3.44 ± 0.46	506.45 ± 56.82	279.86 ± 71.24	0.0396	3.12 ± 0.23	2.66 ± 0.15	0.55 ± 0.02	0.47 ± 0.03
10.12.2010	1.33 ± 0.31	693.06 ± 244.90	346.10 ± 160.89	0.3560	3.07 ± 0.30	3.09 ± 0.08	0.62 ± 0.09	0.49 ± 0.06
1.31.2011	0.19 ± 0.17	431.58 ± 103.67	256.20 ± 167.22	0.3822	3.23 ± 0.24	2.38 ± 0.09	0.52 ± 0.04	0.46 ± 0.06

Table 3.S4. Initial and final $\delta^{15}N$, atom% ^{15}N, and %N tissue levels (all ± SE) of *Gracilaria vermiculophylla* in mudflat study.

Sample Time	$\delta^{15}N$	At%	%N
Initial	2060.54 ± 454.70	1.11 ± 0.16	2.33 ± 0.05
Final	233.49 ± 25.47	0.45 ± 0.009	2.47 ± 0.08

Chapter 4: Mats of the non-native macroalga, *Gracilaria vermiculophylla*, alter net denitrification rates and nutrient fluxes on intertidal mudflats

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Abstract

Managers often advocate for coastal restoration in areas that can enhance removal of reactive nitrogen from these systems, namely denitrification, because excessive nutrient loading to coastal zones can have many deleterious consequences. Prior work investigating effects of macroalgal mats on denitrification rates have produced conflicting results, indicating that mats can have positive, negative, or no effects on denitrification rates. In this study, we hypothesized that mats of a non-native macroalga, *Gracilaria vermiculophylla*, which is often found incorporated several cm into intertidal mudflat sediments, would increase net denitrification rates relative to bare sediments. We found that at moderate densities (~40 gdw m^{-2}), net denitrification rates in June (182.37 ± 16.87 μmol N-N_2 m^{-2} h^{-1}), July (213.19 ± 16.30 μmol N-N_2 m^{-2} h^{-1}), and September (124.82 ± 11.17 μmol N-N_2 m^{-2} h^{-1}) were higher than rates previously documented with macroalgal mats in the literature. Compared to rates from bare sediment in June (25.48 ± 15.09 μmol N-N_2 m^{-2} h^{-1}) and September (46.47 ± 15.79 μmol N-N_2 m^{-2} h^{-1}), net denitrification was significantly higher when *G. vermiculophylla* was present. Rates measured on bare sediment in July (254.81 ± 19.86 μmol N-N_2 m^{-2} h^{-1}) were not significantly different from *G. vermiculophylla* counterparts, most likely due to highly reduced conditions in *G. vermiculophylla* cores, which could have limited nitrification. July incubations also demonstrate that at higher densities (~120 gdw m^{-2} *G. vermiculophylla*), denitrification rates can drop, suggesting a potential biomass threshold for macroalgal enhancement of denitrification.

Introduction

Human interactions with the environment, including the introduction of non-native species and alterations to nutrient regimes, have led to many changes in ecosystem functioning. Biological invasions can change species composition and interactions, and also the habitat structure in a system (Ruiz et al. 1997, 1999). Increased fluxes of reactive nitrogen from nitrogen fixing crops, fossil fuel combustion, and the Haber-Bosch process have led to increased anoxic and eutrophic conditions around the world (Vitousek et al. 1997; Galloway et al. 2003). Estuaries and coasts are hotspots for both species introductions and alterations to nutrient regimes. Non-native species dispersal mechanisms such as ballast water exchange, ship fouling, aquaculture, and aquarium and food trade are all common in these systems (Ruiz et al. 1997, 1999; Williams and Grosholz 2008). Species introductions can change food web interactions, biodiversity, and nutrient dynamics (Ruiz et al. 1997; Ruesink et al. 2006; Grosholz and Ruiz 2009). Anthropogenic nutrient enrichment can also lead to shifts in primary producer communities including dominance by phytoplankton or blooming ephemeral macroalgae (Valiela et al. 1992; McGlathery et al. 2007), reductions in seagrass coverage (Short et al. 1995; Hauxwell et al. 2001; McGlathery 2001), increased anoxia, and reductions in benthic fauna (Karlson et al. 2002). In order to remediate these negative effects, managers often focus on increasing the nitrogen removal capacity of systems (Galloway et al. 2003; Brush 2009).

In coastal systems, many processes interact to affect retention and removal of nitrogen. Nitrogen can be lost from estuaries three ways: burial, physical transport, and denitrification (Nixon et al. 1996, Vitousek et al. 1997, Seitzinger et al. 2006). Anaerobic

ammonium oxidation (anammox) can also represent a net loss of reactive nitrogen to the system (Burgin and Hamilton 2007), though it is often considered less prevalent than denitrification in coastal systems (Dalsgaard et al. 2005; Koop-Jackobsen and Giblin 2009). Nitrate for denitrification can either come directly from the surrounding environment (direct denitrification), or from coupling with nitrification (coupled nitrification-denitrification). Coupling, rather than direct denitrification, is more common in estuaries with low dissolved nutrients and good water quality (Seitzinger et al. 2006). While denitrification requires anoxic conditions, nitrification requires aerobic conditions. Estuaries are dynamic environments where tidal fluctuations can create oxygenated conditions for nitrification at low tide, facilitating nitrate loss through denitrification after the sediments are inundated and reduced at high tide (Ensign et al. 2008, 2012). However, estuarine rates of denitrification can be increased if oxygen conditions in the sediments are more heterogeneous, with many oxic-anoxic "hotspots" for coupled denitrification (McClain et al. 2003; Eyre and Ferguson 2009; Santos et al. 2012). Systems where anoxic conditions dominate will meet carbon and oxygen state requirements for denitrification, but can be nitrate limited because of inhibition of nitrification (Kemp et al. 1990; Joye and Hollibaugh 1995; Childs et al. 2002; Webster and Harris 2004; Conley et al. 2009; Eyre and Ferguson 2009). Conversely, entirely oxic conditions will have nitrate available from nitrification, but will lack the anoxic conditions necessary for denitrification to proceed (Webster and Harris 2004; Eyre and Ferguson 2009).

To date, data on how macroalgal mats can affect denitrification rates remain equivocal. Macroalgal mats can be associated with decreases in denitrification rates due

to macroalgal competition for nitrate (Dalsgaard 2003). Published data have also shown that denitrification rates on macroalgal vegetated sediments can be no different from those on bare sediments, due either to a shift in the oxic-anoxic boundary for coupled nitrification-denitrification into the macroalgal mat or enough DIN being available to satisfy both macroalgal growth and denitrification requirements (Krause-Jensen et al. 1999; Bartoli et al. 2012). Alternatively, recent work by Eyre et al. (2011b) has indicated that biomass of the invasive macroalga, *Caulerpa taxifolia*, can be associated with increased rates of denitrification, most likely because the macroalgae oxygenates sediments around its rhizoids and thus increases oxic-anoxic hotspots for coupled nitrification-denitrification within the sediments. This relationship between root oxygenation and increased denitrification rates has been well documented for many marine macrophytes (see Risgaard-Petersen and Jensen 1997 and references therein).

The goal of this study was to determine how the introduction of a non-native macroalga, *Gracilaria vermiculophylla,* affects net denitrification on mid-Atlantic, USA intertidal mudflats. This macroalga is native to Southeast Asia and has been introduced to temperate estuaries around the world. It has been hypothesized that this introduction unintentionally occurred in the 1970s in the mid-Atlantic region via attachment to traded oysters (Thomsen et al. 2006; Gulbransen et al. 2012). It has been the dominant macroalgal species in the region since routine monitoring began in 1998 and recent seasonal surveys have documented biomasses on mudflats as high as 800 gdw m^{-2} (Gulbransen and McGlathery 2013). Rather than forming mats that only lie on the surface of the sediment, *G. vermiculophylla* thalli are often found incorporated several cm into the sediment (pers. obs.). While prior work has shown that this macroalga can increase

epifaunal densities on mudflats (Byers et al. 2012) and mediate transfers of nitrogen to higher trophic levels (Gulbransen and McGlathery 2013), little is known about how this introduction could be affecting sediment nitrogen dynamics on intertidal mudflats.

We hypothesized that *G. vermiculophylla* presence on intertidal mudflats would enhance rates of net denitrification compared to bare substrate. We also hypothesized that at high densities, *G. vermiculophylla* coverage would be associated with highly reduced conditions that would inhibit nitrification and reduce overall coupled denitrification. In order to test these hypotheses, we collected microcosms with (vegetated) and without (bare) *G. vermiculophylla* biomass for continuous-flow, incubations twice in the summer and once in the fall of 2012.

Methods

Study Site

Samples were collected from an intertidal mudflat within the Virginia Coast Reserve Long Term Ecological Research (VCR LTER) site (37°18’20” N, 75°53’59” W). The coastal bays that make up the VCR LTER site span 110 km of coastline on the eastern shore of the Delmarva Peninsula and are enclosed by barrier islands to the east. The site has been minimally affected by humans and water quality, as assessed using dissolved nutrient concentrations and chlorophyll a content, has remained stable for the last 20 years (McGlathery et al. 2012).

Sample Collection

Microcosm cores (6.4 cm diameter x ~ 17 cm sediment depth, ~400 mL overlying water) were collected within 2 hours of low tide on three dates. Two of these sample

dates were in the summer, once when macroalgal coverage was moderate (11 June 2012) and once when coverage was much higher (23 July 2012). One additional fall sampling was conducted after much of the summer biomass had been removed by storm activity (28 September 2012).

At each collection time, four bare microcosms, with only mudflat sediment, and four vegetated microcosms, with *G. vermiculophylla* densities approximately equivalent to 40 gdw m^2, were collected (Table 4.1). In July, an additional four cores were collected with over twice as much *G. vermiculophylla* biomass as in the other vegetated cores. Because of problems with bubble formation, which could have affected dissolved gas concentrations, in three of these cores, only gas fluxes from one core with the equivalent of 122 gdw m^{-2} of *G. vermiculophylla* were used, however all four cores were used for nutrient fluxes and sediment carbon and nitrogen content.

Preliminary experiments showed that adding *G. vermiculophylla* onto collected bare sediments underestimated denitrification rates, compared to sediments collected with *G. vermiculophylla* intact. Thus, vegetated cores were collected intact, with care taken to not disturb the algae-sediment interface. In addition to sediment microcosms, 190 L of water were collected from the channel adjacent to the mudflats for the continuous flow incubations. Water column temperature, dissolved oxygen, and salinity were measured using a handheld YSI Model 556.

Continuous-Flow Incubation

Upon collection, water and microcosms were transported in the dark, on ice, with water overlying the headspace to the University of North Carolina Institute of Marine

Sciences in Morehead City, North Carolina. Microcosms were submerged in an aerated water bath in an environmental chamber (Bally Inc.) at *in situ* temperatures in the dark for 12-16 hours (Fulweiler and Nixon 2011). Each microcosm was capped with an air-tight plexiglass top that was equipped with an inflow and outflow sampling port, and incubated in a continuous-flow system. To reduce the effects of benthic microalgae, dark conditions were maintained throughout the incubations. Aerated and unfiltered water was passed over each microcosm at a flow rate of 1.5 mL min^{-1}, which created a well-mixed water column within the chamber (Lavrentyev et al. 2000).

Microcosms were acclimated in the system for 24 hours prior to sampling to allow the system to reach steady state (Eyre et al. 2002; McCarthy and Gardner 2003). Five mL water samples were collected at 0, 8, and 24 hours after the 24-hour acclimation period to ensure steady-state conditions were reached with respect to dissolved gasses. Water entering (measured from a bypass line that flowed directly into sample vials) and leaving the microcosms were analyzed for N_2, O_2 and Ar dissolved gases in water using a Balzers Prisma QME 200 quadropole mass spectrometer (MIMS; Pfeiffer Vacuum, Nashua, NH, USA; Kana et al. 1994). Concentrations of O_2 and N_2 were determined using the ratio with Ar (Kana et al. 1994; Ensign et al. 2008).

The MIMS technique has a rapid analysis time, requires a small sample volume and little sample preparation, and has a high precision (CV of N2/Ar <0.05%). This method determines net N_2 fluxes such that a positive N_2 flux is attributed to net denitrification, while a negative N_2 flux is attributed to net nitrogen fixation. This method does not discern between the sources of N_2, therefore net denitrification refers to N_2 production from heterotrophic denitrification, anammox, and any other N_2 producing

process. Fluxes of oxygen directed into the sediment were considered to represent rates of biological oxygen demand (BOD; Kana et al. 1994, Piehler and Smyth 2011). In cases when it was evident that an invertebrate in the core had died, or bubble formation was an issue, we did not use that replicate in the analysis. Therefore, for some sampling periods we had 3 rather than 4 replicate cores within each bare and vegetated treatment.

Nutrients

Water samples (50ml) were collected for nutrient analysis from the bypass line and the outflow port of each core once during the incubation after steady state conditions were reached. Water was filtered through Whatman GF/F filters (25 mm diameter, 0.7 µm nominal pore size), and the filtrate was analyzed with a Lachat Quick-Chem 8500 (Lachat Instruments, Milwaukee, WI, USA) automated ion analyzer for nitrate (NO_3^- and NO_2^-, NO_x in remainder of paper), ammonium (NH_4^+), phosphate (PO_4^{3+}), and total organic nitrogen (TON). Lower detection limits for ammonium, nitrate, and TON were 0.36 µM, while the detection limit for phosphate was 0.16 µM. Dissolved organic nitrogen (DON) was calculated by subtracting NH_4^+ and NO_x from TON.

At the end of each experiment sediment samples from the upper 2 cm of sediment within each microcosm were collected for C:N analysis using a Carlo Erba elemental analyzer. *G. vermiculophylla* within each vegetated core were rinsed with distilled (DI) water, dried in a 60 °C oven, and weighed.

Flux Calculations

Flux calculations determined in the dark incubations were based on the assumption of steady-state conditions and a homogenous water column (Miller-Way and

Twilley 1996). Fluxes of dissolved nutrients and gasses were calculated as the difference between the concentration leaving and entering the microcosm, the flow rate of water entering the microcosm and expressed relative to the area of the microcosm (Laventyev et al. 2000). A positive flux indicated production within the microcosm and a negative flux indicated uptake within the microcosm. Mass balance equations were used to determine the percent of denitrification that occurred coupled to nitrification (Groffman et al. 2006; Fennel et al. 2009). Coupled nitrification-denitrification was calculated by subtracting the absolute value of the nitrate flux from denitrification. Denitrification from the water column (direct denitrification) was calculated based on the nitrate flux.

Data Analysis

Net fluxes of N_2, O_2, ammonium, nitrate, phosphate, and DON for each sample period on bare and vegetated areas were compared using analysis of variance (ANOVA) with Tukey-Kramer post-hoc tests in SAS (SAS 9.2, SAS Institute, Carey, North Carolina, USA). All data met ANOVA assumptions and were not transformed. Significant Pearson correlations (r) between all fluxes, *G. vermiculophylla* biomass, and sediment nitrogen and carbon within each core were calculated in SAS.

Results

Water chemistry and algal biomass

Water temperature was highest in July (27 °C) and salinity ranged from 31 to 33 ppt (Table 4.2). Dissolved oxygen (DO) in the reservoir water ranged from 2.8 mg L^{-1} in July to 6.2 mg L^{-1} in September. Nitrate, ammonium, and phosphate were below detection in June and July, and only slightly above detection limits in September (Table

4.2). *G. vermiculophylla* biomass within microcosms was near 40 gdw m^{-2} in all incubations (Table 4.1).

N_2 Fluxes

Net N_2 fluxes were significantly different between bare and *G. vermiculophylla* covered areas in June ($p = 0.0023$) and September ($p = 0.0133$), but not in July ($p = 0.1806$, Fig. 4.1). Lowest net denitrification was recorded on bare sediments in June (25.48 μmol m^{-2} hr^{-1}, Table 4.1). Highest N_2 production occurred in July on both bare (254.81 μmol m^{-2} hr^{-1}) and vegetated substrates (213.19 μmol m^{-2} hr^{-1}, Table 4.1). N_2 fluxes had strong positive correlations to *G. vermiculophylla* biomass in June ($r = 0.98$, $p = 0.0007$) and September ($r = 0.79$, $p = 0.0359$). The July high biomass microcosm (122 gdw m^{-2} of *G. vermiculophylla*) had a net N_2 flux of 70.76 μmol m^{-2} hr^{-1}.

Biological Oxygen Demand (BOD)

BOD was significantly higher in *G. vermiculophylla* microcosms compared to bare mudflat sediments in June ($p = 0.0019$) and September ($p = 0.0062$), but not significantly different in July ($p = 0.3265$, Fig. 4.1). The lowest measured BOD was found in bare microcosms in June (522.50 μmol m^{-2} hr^{-1}), while the highest was measured in the same month in microcosms with *G. vermiculophylla* biomass (3460.50 μmol m^{-2} hr^{-1}, Table 4.1). BOD had a strong, significant positive correlation to N_2 fluxes in June ($r = 0.98$, $p = 0.0007$), July ($r = 0.86$, $p = 0.0297$), and September ($r = 0.95$, $p = 0.0009$). The July high biomass microcosm (122 gdw m^{-2} of *G. vermiculophylla*) had a BOD of 1214.24 μmol m^{-2} hr^{-1}.

Nutrient Fluxes

Nitrate fluxes were always the same in bare and vegetated microcosms and were either undetectable in June or negative in July and September (Fig. 4.2, Table 4.3). Ammonium fluxes were undetectable in July and negative but not significantly different between bare and vegetated microcosms in September ($p = 0.7175$). In June, there was production of ammonium from vegetated cores when compared to the negligible flux in bare microcosms, but this difference was not significant ($p = 0.0715$, Table 4.3). While phosphate fluxes where higher in vegetated cores in both June and July, these differences were only significant in July ($p = 0.1501$ and 0.0033, respectively, Table 4.3). Phosphate fluxes in September were always negative and were not significantly different from one another ($p = 0.3524$). Dissolved organic nitrogen (DON) fluxes were positive in all incubations (Fig. 4.2, Table 4.3). However, only in July were there significantly higher DON fluxes in vegetated cores ($p = 0.0138$). The July high biomass microcosms had no nitrate flux, but high ammonium (814.91 ± 280.41), DON (1758.36 ± 1046.56), and phosphate (47.28 ± 32.52) fluxes.

Based on mass balance calculations, all denitrification in bare and vegetated microcosms in June was coupled to nitrification. In July, 95% to 100% of denitrification was coupled to nitrification in all microcosms. In contrast, in September, coupled nitrification-denitrification accounted for 0% to 77% of denitrification in bare microcosms and 82% to 86% of denitrification in vegetated microcosms.

Sediment carbon and nitrogen

Both carbon and nitrogen content in sediments were highest in July microcosms when bare cores and vegetated cores were compared among dates (Table 4.4). Within

each sample date, sediment percent carbon was significantly higher in *G. vermiculophylla* vegetated microcosms in June ($p = 0.0059$), July ($p = 0.0007$), and September ($p = 0.0030$). In addition, sediment percent nitrogen content was significantly higher when *G. vermiculophylla* was present in June ($p = 0.0073$), July ($p = 0.0007$), and September ($p = 0.0021$). While there was a positive correlation between *G. vermiculophylla* biomass and sediment percent carbon and nitrogen content during all sample periods, the correlations were only significant in June (carbon: $r = 0.90$, $p = 0.0141$; nitrogen: $r = 0.90$, $p = 0.0159$) and July (carbon: $r = 0.87$, $p = 0.0213$; nitrogen: $r = 0.87$, $p = 0.0242$). Sediment percent carbon (2.36 %) and percent nitrogen (0.25 %) were highest in the July high biomass microcosms.

Discussion

N_2 Fluxes

In June and September higher rates of net denitrification were measured when *G. vermiculophylla* was present. This increased production was likely attributable to increased carbon availability and increased habitat heterogeneity associated with the algal biomass (Table 4.4, Eyre and Ferguson 2009; Eyre et al. 2011b). Under those conditions, *G. vermiculophylla* likely increased oxic-anoxic "hotspots" in the sediments and thus increased the potential for coupling of nitrification and denitrification compared to bare areas. This interpretation is supported by our mass balance calculations that estimate that 80% of denitrification was coupled to nitrification in vegetated cores during all sampling periods.

In July there were no significant differences in net denitrification from bare and vegetated cores. During this time the sediments also had the highest carbon content measured in all of the incubations. Therefore, it is possible that increased metabolism in the peak of summer led to increased production of high quality organic matter on both bare and vegetated substrates, which in turn led to high rates of denitrification everywhere. The slight drop in net denitrification in vegetated microcosms compared to bare microcosms could be attributed to reduced conditions, as supported by the phosphate efflux. These reduced conditions could limit nitrification and thus reduce net denitrification (Joye and Hollibaugh 1995; Childs et al. 2002; Webster and Harris 2004; Conley et al. 2009; Eyre and Ferguson 2009). Macroalgal effects on net denitrification are likely biomass-dependent, with biomass beyond a certain threshold inhibiting denitrification due to homogeneous, anoxic conditions that reduce nitrification. Lower rates of net denitrification within the high biomass microcosm in July (122 gdw m^{-2} *G. vermiculophylla*) and increased phosphate and ammonium fluxes from all high biomass microcosms, provide limited evidence to support this hypothesis.

Net denitrification rates from vegetated microcosms in this experiment were on the upper end of rates seen in other studies with other macrophytes, in particular macroalgae. When compared to fluxes measured in seagrass beds, our rates were much higher than those seen annually in sediments vegetated with *Z. marina* in Virginia (6-98 μmol m^{-2} hr^{-1}, Cole 2011), *Halophilia ovalis and H. spinulosa* in Australia (77-109 μmol m^{-2} hr^{-1}, Eyre et al. 2011a), and *Z. capricorni* in Australia in summer (average under 50 μmol m^{-2} hr^{-1}, Eyre et al. 2011b), but were similar to fluxes seen in mixed beds of *Halodule wrightii* and *Z. marina* in North Carolina (under 200 μmol m^{-2} hr^{-1} in each

season, Piehler and Smyth 2011; Smyth et al. 2013) and lower than winter fluxes seen in winter *Z. capricorni* beds in Australia (412 μmol m^{-2} hr^{-1}, Eyre et al. 2011a). All prior studies on denitrification fluxes with macroalgal presence have found lower rates of denitrification that range from almost zero up to 55 μmol m^{-2} hr^{-1} (Krause-Jensen et al. 1999; Dalsgaard 2003; Eyre et al. 2011b; Bartoli et al. 2012). It is likely that because our incubations were done in the dark, competition for available nitrogen between macroalgae and denitrifying bacteria was reduced and therefore net denitrification rates were higher. Of previous macroalgal studies, only one specifically used macroalgae that protruded into the sediments (Eyre et al. 2011b) and all others used macroalgae lying on top of sediments (Krause-Jensen et al. 1999; Dalsgaard 2003; Bartoli et al. 2012). It is possible that by protruding into the sediments, *G. vermiculophylla* may have increased oxygen heterogeneity in the sediments and led to more oxic-anoxic microzones for coupled nitrification-denitrification.

Biological Oxygen Demand (BOD)

There was a strong positive relationship between BOD and net N_2 flux in all of the incubations, further supporting prior assertions that oxygen demand can be used to predict denitrification in systems where coupled nitrification-denitrification is common (Fennel et al. 2009; Piehler and Smyth 2011). In June and September BOD was positively correlated to *G. vermiculophylla* biomass under dark incubation conditions, which indicated that, at higher *G. vermiculophylla* biomasses, there was an active microbial community breaking down organic matter and more reduced conditions that enhanced net denitrification rates (Piehler and Smyth 2011). The relationship between BOD and *G.*

vermiculophylla biomass was negative, but not significant in July, which might indicate that all microcosms had highly active microbial communities and similarly favorable conditions for denitrification, as suggested by the lack of differences between N_2 fluxes. This conclusion is also supported by high C and N levels found in the sediments in both bare and vegetated microcosms. The negative relationship between BOD and macroalgal biomass in July might also indicate that when *G. vermiculophylla* was present at that time, more homogeneous, anoxic conditions were favored.

Future scenarios

Current densities of *G. vermiculophylla* on Virginia coastal bay mudflats vary greatly in space and time from negligible amounts to biomasses as high as 800 gdw m^{-2} at some sites in warmer months (Gulbransen and McGlathery 2013). In this study we found that at moderate densities (~40 gdw m^{-2}), *G. vermiculophylla* biomass enhanced net denitrification from mudflat communities. However, preliminary data from one microcosm incubation suggests a threshold density, above which, *G. vermiculophylla* inhibited net denitrification. Therefore, it is important to note that under a higher nutrient loading regime in the Virginia coastal bays, variable outcomes are possible. We would likely see increased *G. vermiculophylla* biomass on mudflats, which would lead to a more anoxic, homogeneous environment not conducive to coupled nitrification-denitrification. In addition, the reduced conditions created by the dense mats of macroalgae would likely result in high sulfide levels that could cause a shift from nitrification-denitrification to an increased retention of nitrogen (An and Gardner 2002; Childs et al. 2002; Banks et al. 2012). It is likely that, under this regime, increased flushing of nitrate into the water

column would be converted to ammonium rather than N_2 gas, thus keeping reactive nitrogen in the system. Therefore, while our results indicate that at moderate densities, *G. vermiculophylla* can enhance net denitrification, care should be taken when trying to extrapolate these results to different water quality and macroalgal biomass regimes.

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Figures

Fig. 4.1. Cumulative N_2 - N flux and biological oxygen demand (BOD) for June (a), July (b), and September (c) incubations. An asterisk indicates a significant difference between N_2 – N fluxes or BOD individually.

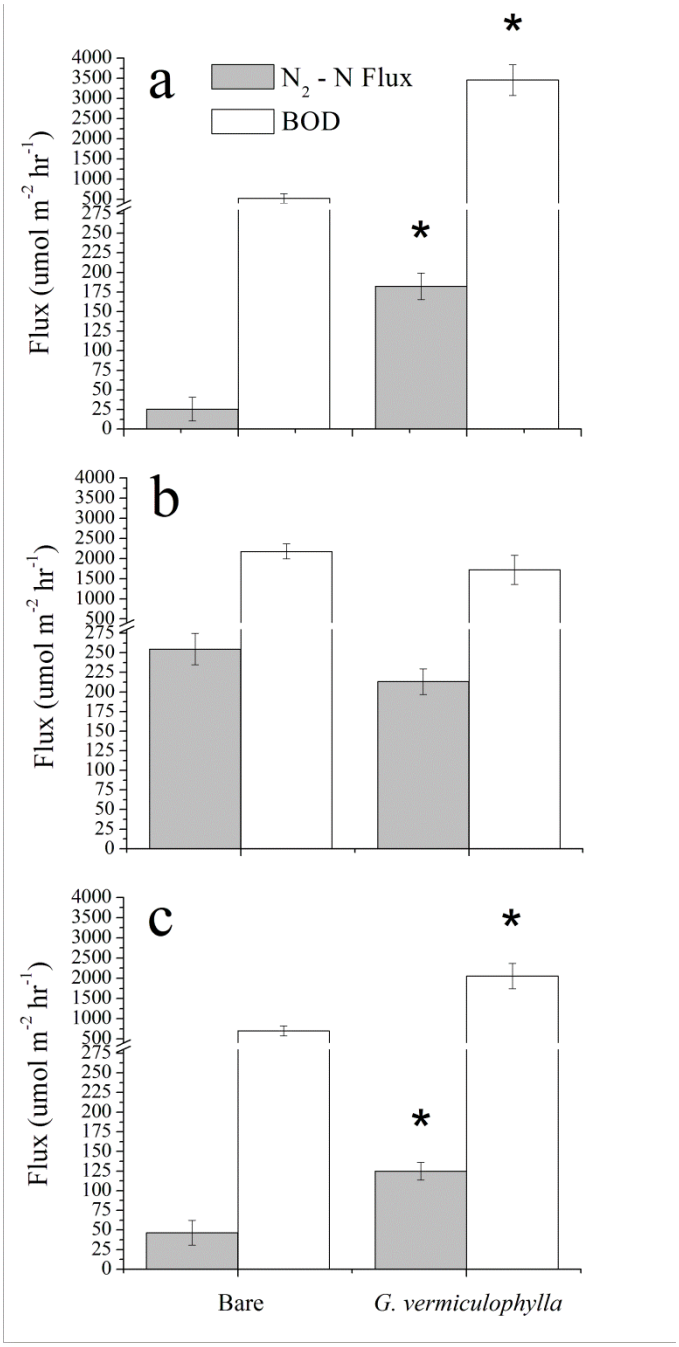
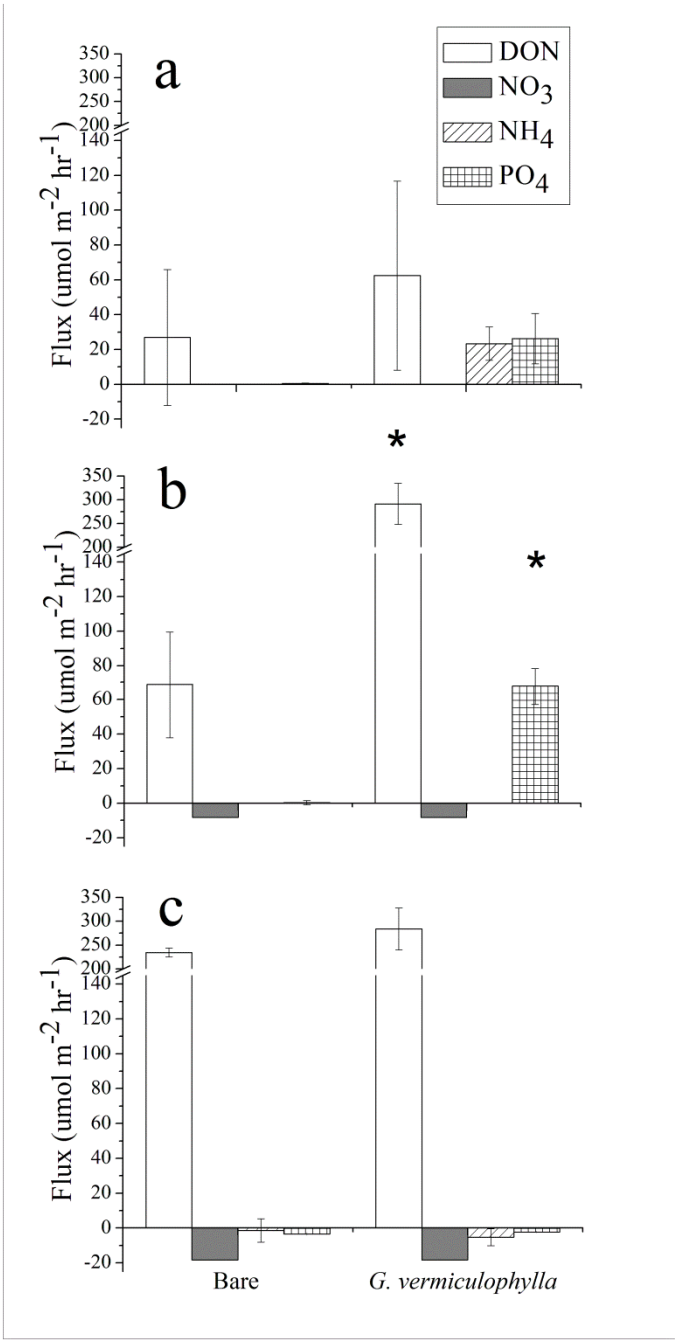


Fig. 4.2. Dissolved organic nitrogen (DON), nitrate (NO_3^-), ammonium (NH_4^+), and phosphate (PO_4^{3+}) fluxes for June (a), July (b), and September (c) incubations. An asterisk indicates a significant differences between fluxes from bare and *G. vermiculophylla* vegetated microcosms.



Tables

Table 4.1. Average *G. vermiculophylla* biomass, cumulative N_2 – N fluxes and biological oxygen demand (BOD), mean ± SE, for each sample date. Biomass is in gdw m^{-2} while N_2 – N flux and BOD are in μmol m^{-2} hr^{-1}. Only one core from the high biomass set in July is reported here because all other cores had bubbles.

Date and Coverage	*G. vermiculophylla* biomass	N_2 – N flux	BOD
June Bare	0	25.48 ± 15.09	522.50 ± 122.30
June Vegetated	39.48 ± 2.48	182.37 ± 16.87	3460.50 ± 382.17
July Bare	0	254.81 ± 19.86	2176.62 ± 186.92
July Vegetated	42.13 ± 9.79	213.19 ± 16.30	1718.77 ± 364.72
July High Biomass (core 1)	122.41	70.76	1214.24
September Bare	0	46.47 ± 15.79	697.34 ± 122.37
September Vegetated	44.60 ± 16.39	124.82 ± 11.17	2053.40 ± 312.11

Table 4.2. In situ water properties at each sampling date.

Date	Temperature (°C)	Salinity	DO (mg L^{-1})	DO (MIMS)	NO_x (μM)	NH_4^+ (μM)	PO_4^{3+} (μM)
11 June	25	32	8.9	5.3	0	0	0
23 July	27	31	NA	2.8	0.24	0	0.05
28 September	22	33	7.16	6.2	0.70	0.48	0.34

Table 4.3. Dissolved organic nitrogen (DON), NO_3^-, NH_4^+, and PO_4^{3+} fluxes at each sample date (all ± SE). All fluxes are in μmol m^{-2} hr^{-1}. All four July high biomass cores were included here because bubble formation should not have altered nutrient fluxes.

Date and Coverage	DON	NO_x	NH_4^+	PO_4^{3+}
June Bare	26.87 ± 39.09	0	0	0.41 ± 0.36
June Vegetated	62.41 ± 54.34	0	23.33 ± 9.58	26.23 ± 14.52
July Bare	68.70 ± 30.78	-8.24 ± 0	0	0.34 ± 1.17
July Vegetated	291.24 ± 43.27	-8.24 ± 0	0	67.77 ± 10.70
July High Biomass	1758.36 ± 1046.56	0	814.91 ± 280.41	47.28 ± 32.52
September Bare	234.42 ± 9.35	-18.30 ± 0	-1.36 ± 6.69	-3.35 ± 0.79
September Vegetated	383.88 ± 44.28	-18.30 ± 0	-5.21 ± 4.90	-2.26 ± 0.60

Table 4.4. Sediment percent nitrogen and percent carbon content in bare and vegetated microcosms for all sampling dates (mean ± SE). Sample size (*n*) indicates the number of microcosms.

Date and Coverage	Sample Size (*n*)	Sediment carbon (%)	Sediment nitrogen (%)
June Bare	3	0.92 ± 0.04	0.07 ± 0.004
June Vegetated	3	1.55 ± 0.11	0.13 ± 0.01
July Bare	3	1.14 ± 0.06	0.09 ± 0.01
July Vegetated	3	1.68 ± 0.01	0.16 ± 0.002
July High Biomass	4	2.36 ± 0.12	0.25 ± 0.02
September Bare	4	1.04 ± 0.01	0.08 ± 0.001
September Vegetated	3	1.30 ± 0.05	0.11 ± 0.005

Chapter 5: A non-native intertidal macroalga influences invertebrate densities and shorebird foraging

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Abstract

Non-native species can have multi-trophic effects that 'cascade up' the food webs in the communities where they are introduced. In this study, we determined how the introduction of a mat-forming macroalga from Southeast Asia, *Gracilaria vermiculophylla*, affects invertebrate prey availability as well as shorebird abundance and foraging behavior at an important migratory stopover site in the mid-Atlantic region, USA. Prior work on the consequences of introductions of mat-forming macroalgae has produced conflicting results. Often, shorebirds with flexible modes of foraging are more resilient to intertidal macroalgal mat formation, while those with more stringent prey or foraging substrate needs can be negatively affected by macroalgae. Our results indicated that although *G. vermiculophylla* mats often contained more invertebrate prey, black bellied plovers, semipalmated sandpipers, and dowitchers all chose to forage on mudflats without coverage. Conversely, dunlin densities and foraging effort on mudflats with and without *G. vermiculophylla* coverage were not significantly different, indicating that they may be more resilient to macroalgal mat introduction. Correlation analyses and multiple linear regression (MLR) models were used to compare variables that have been shown to be associated with shorebird foraging abundance (i.e. invertebrate, meteorological and sediment properties). Results from these analyses indicated that dunlin abundance was associated most with maximum daily temperature, above ground invertebrates, and number of roosting birds, while their foraging attempts were most associated with below-ground invertebrate richness, *G. vermiculophylla* biomass, and sediment grain size. Wind direction and a metric of invertebrate abundance were all associated with black bellied plover, semipalmated sandpiper, and dowitcher abundance.

Key words: non-native, macroalgae, shorebirds, foraging, invertebrates, *Gracilaria vermiculophylla*

Introduction

Non-native species introductions can affect the overall functioning of the ecosystems they invade by changing the structure of the system and thus changing which species are able to live there (Ruiz et al. 1997, 1999). Estuaries and coasts are particularly susceptible to introductions of non-native species partly because vectors of dispersal such as ballast water, ship fouling, aquaculture, and aquarium/food trade are common (Ruiz et al. 1997, 1999, Williams and Grosholz 2008). Invasive species that modify the habitats they invade can alter biodiversity and food-web structure, trophic flow of energy and materials, and nutrient dynamics (Ruiz et al. 1997; Ruesink et al. 2006; Grosholz and Ruiz 2009). While there has been much recent interest in marine macroalgal invasions, our understanding of the impacts on native organisms, community structure, and ecosystem services, and of the linkages to management are limited (Williams and Smith 2007; Williams 2007; Williams and Grosholz 2008).

This study addressed the effects of a non-native macroalga, *Gracilaria vermiculophylla,* on shorebird behavior on mid-Atlantic intertidal mudflats in the United States. *G. vermiculophylla* is from Southeast Asia and has been introduced to many temperate estuaries worldwide (Kim et al. 2010; Gulbransen et al. 2012). In the mid-Atlantic region it forms mats on intertidal mudflats that remain stable for months to years (Gulbransen and McGlathery 2013) due to an association with a tube-building polychaete, *Diopatra cuprea* (Thomsen and McGlathery 2005). *G. vermiculophylla* has

been the dominant macroalga in the Virginia coastal bays since at least 1998, largely due to its resistance to desiccation, sedimentation, and grazing (Thomsen et al. 2006; Thomsen and McGlathery 2007).

In addition to being the location of a widespread macroalgal introduction (Gulbransen et al. 2012), the Virginia coastal bay region is an important stopover site for migratory shorebirds (Watts and Truitt 2000). As such, it is important that managers in the area understand how mats of introduced *G. vermiculophylla* affect shorebird foraging habitat and behavior.

Studies investigating the effects of intertidal macroalgal mats on shorebird densities and foraging behavior often focus on native species, and have found diverse and sometimes conflicting results that are typically species dependent. Work done in Portugal by Cabral et al. (1999) and Lopes (2006) found that shorebird abundance on mudflats was negatively correlated with native macroalgal mat presence. However, a different study in the same location found no net effect of native macroalgal mats on shorebirds, and hypothesized that this was most likely because tactile feeders such as dunlins were able to adapt their foraging strategies to the presence of macroalgal mats (Murias et al. 1996). Similarly, several studies have found that shorebirds that are able to adjust to the presence of macroalgal mats by changing their mode of foraging were more successful than those with specific substrate or dietary needs (Garcia et al. 2010; Green 2011). Other studies have found that intertidal macroalgal mats led to increases in invertebrate densities and thus increases in foraging shorebirds (Dugan et al. 2003; Martinetto et al. 2010).

In addition to often being species dependent, shorebird responses to macroalgal mat formation are likely correlated to changes in invertebrate prey availability and/or changes to the foraging surface. Work by Byers et al. (2012) has shown that *G. vermiculophylla* acts as an ecosystem engineer and enhances epifaunal invertebrate densities by providing habitat and resources. Additional studies of native and non-native macroalgal effects have found that invertebrates can be positively (Cabral et al. 1999; Rossi 2007; Martinetto et al. 2010; Thomsen 2010; Piova-Scott et al. 2011) or negatively (Lopes et al. 2006) associated with the mats. Regardless of the overall effects on epifaunal densities, it should be noted that macroalgal mats can inhibit visual shorebird foragers from seeing where they are hunting (Green 2011). Sediment grain size and below-ground penetrability under macroalgal mats can change and cause alterations to the below ground invertebrate community and the ability of shorebirds to forage in those habitats, respectively (Yates et al. 1993; Green 2011). However, much like shorebird studies, macroalgal effects on invertebrate densities and foraging surface characteristics can be highly variable and therefore should be determined in each location where shorebird foraging behavior is observed.

We combined invertebrate enumeration, total avian species abundance and individual feeding focal observations of dunlin (*Calidris alpina*) to determine how the introduced macroalga, *G. vermiculophylla,* has affected shorebirds in the region. We chose dunlin as our focal species because they were common in the area and we were able to find individuals on mudflats with and without *G. vermiculophylla* coverage, allowing us to evaluate their behavior in both habitats. Because ecological systems are

complex and bird foraging choices may encompass more than only macroalgal and invertebrate densities, correlation analyses and multiple linear regression (MLR) models were employed to compare a variety of biotic and abiotic variables to shorebird abundance and dunlin foraging behavior, a technique that has been applied in other shorebird studies (e.g. Cabral et al. 1999).

Methods

This study was conducted on mudflats within the Virginia coastal bays at the Virginia Coast Reserve Long Term Ecological Research (VCR LTER) site. The bays comprise 110 km of coastline and are bounded to the west by the Delmarva Peninsula and to the east by barrier islands. This region has high conservation value and was established as a marine reserve by the Nature Conservancy in 1974. Data were collected during the shorebird spring migration of 2012 on 5 mudflats, 2 unvegetated, without any macroalgal coverage, and 3 vegetated, with non-native *G. vermiculophylla* mats present. All sites were sheltered, lagoonal mudflats within a 3 km boating distance to one another. This close proximity facilitated frequent trips between sites within tidal cycles.

Invertebrate biomass was determined on May 1, 2012 at all 5 mudflats by haphazardly collecting 8 replicates cores (10 cm diameter X 5 cm depth) within a 100 m^2 area at each site. At vegetated sites, all cores were collected with the overlying *G. vermiculophylla* layer intact, and all cores at unvegetated sites were bare. Cores were immediately separated into above-ground (from vegetated sites) and below-ground (sediment to 5 cm depth) sections and sieved through 500 μm mesh. All invertebrates were identified, weighed, dried and ashed in a muffle furnace at 500 °C to determine ash

free dry weight (AFDW, mg AFDW). *G. vermiculophylla* from within each sample core was rinsed, dried at 60 °C, and weighed. Average *G. vermiculophylla* and invertebrate densities, species richness counts, and above and below AFDW were calculated for unvegetated and vegetated mudflats. In this study, above ground AFDW will refer to combined gammarid and small snail AFDW, while below ground AFDW will refer to combined polychaete and total worm AFDW. T-tests or nonparametric Wilcoxon tests were used to statistically compare these averages (see table 5.2 for details). At each site, three sediment samples were collected for grain-size analysis using an LS 13 320 Laser Diffraction Particle Size Analyzer (Beckman Coulter, Brea, CA) and analyzed for differences between site types using a non-parametric Wilcoxon test.

Bird abundances were recorded between April 30, 2012 and May 17, 2012 when migratory shorebird densities were highest. During each observation period, counts of foraging shorebird species, roosting birds, and number of gulls were all recorded every 10 min. Differences in counts of the four most common species, dunlin (*Calidris alpina*), black-bellied plover (*Pluvialis squatarola*), semipalmated sandpiper (*Calidris pusilla*), and dowitcher (*Limnodromus* spp.), on unvegetated and vegetated mudflats were calculated using t-tests. Gull densities on the two types of mudflats were compared using a non-parametric Wilcoxon test because they did not meet parametric assumptions.

From May 3 to 17, 2012 individual focal observations of dunlin were recorded using a Sony Handycam HDR-CX260V (30X optical zoom) on both unvegetated and vegetated mudflats. A total of 123, 2-min observations were tabulated for pecks and probes on bare and *G. vermiculophylla* covered substrate by slowing down the recorded

video. Differences in average total feeding attempts on unvegetated and vegetated mudflats were compared using a t-test.

Pearson's correlations (r) were calculated using the PROC CORR function in SAS (SAS 9.2, Raleigh, NC). All significant and nearly significant correlations between variables listed in Table 5.1 were noted. A stepwise multiple linear regression (MLR) model that maximized R^2 was used to determine which independent variables (Table 5.1) were most highly associated with each species density and with dunlin foraging attempts. The PROC GLMSELECT function in SAS was used for this analysis with a maximum of 3 explanatory parameters allowed in the output. The amount of variation described by the model equations were evaluated using model R^2 values. Variance inflation factors (VIF) were calculated using the PROC REG function to test for collinearity issues. All VIF values were below 10, indicating that there were no significant issues with collinearity between predictor variables. A total of 72 observations were used for each bird density model and a total of 123 observations were used for the dunlin feeding attempt model.

Results

Data collected from invertebrate samples are displayed in Table 5.2. There were significantly more gammarid amphipods collected on vegetated mudflats when compared to unvegetated mudflats ($p < 0.0001$). However, there were also significantly fewer small snails on vegetated mudflats, though this percent difference was much smaller than that between gammarids (snails, $p = 0.0443$). No significant differences between polychaete and total worms were found between the two mudflat types ($p = 0.3905$ and 0.7178, respectively). Overall, there was significantly more invertebrate AFDW on vegetated

mudflats when compared to unvegetated mudflats ($p = 0.0002$). There were no significant correlations between any meteorological variables and invertebrate parameters. Grain size on unvegetated mudflats (56.55 ± 0.78 μm) was smaller than on vegetated mudflats (72.68 ± 3.58 μm), but this difference was not significant ($p = 0.0737$).

There was an overall trend of fewer shorebirds counted on vegetated mudflats (Fig 5.1), with fewer black-bellied plovers, semipalmated sandpipers, and dowitchers on vegetated mudflats. However, bird species richness was significantly higher on unvegetated mudflats (3.04 ± 0.35) when compared to vegetated mudflats (1.85 ± 0.14, $p = 0.0013$). Gull densities were significantly lower on unvegetated (0.79 ± 0.38) versus vegetated (3.79 ± 0.77) mudflats ($p = 0.0048$). Dunlins were the most common shorebird observed on both types of mudflats, but differences between counts on unvegetated and vegetated mudflats were not significantly different ($p = 0.1160$). In addition, dunlin feeding attempts on unvegetated mudflats (558.94 ± 38.61) did not significantly differ from the number of attempts made on vegetated mudflats (581.24 ± 23.28, $p = 0.6000$).

All significant and nearly significant Pearson correlations are displayed in Table 5.3. *G. vermiculophylla* was negatively correlated with all bird abundance counts and positively correlated with gull abundance, though this correlation was not significant to the 0.05 level for dunlins ($p = 0.0962$). In addition, *G. vermiculophylla* was positively correlated with sediment grain size, but had no significant correlations to meteorological parameters. *G. vermiculophylla* was significantly correlated with all invertebrate parameters; however these relationships were both positive and negative.

Dunlin abundance was not correlated with other shorebird densities, however all non-dunlin densities were positively correlated with one another. All bird species abundance counts were correlated with temperature, and all but semipalmated sandpipers were correlated with wind and level of sunlight. While black-bellied plovers, semipalmated sandpipers, and dowitchers were correlated with virtually all invertebrate parameters, dunlins were only correlated to above-ground invertebrate species richness and above-ground invertebrate AFDW. Dunlin feeding attempts were significantly correlated to below ground invertebrate richness and AFDW, small snail densities, and total worm density.

Estimated effect size and variation described for each of the explanatory parameters in the five MLR models are displayed in Table 5.4. Dunlin densities were highly associated with the maximum daily temperature (72% of variation), above-ground invertebrate richness (17% of variation), and the number of birds roosting (11% of variation), while their feeding attempts were associated with below-ground invertebrate species richness (63% of variation), *G. vermiculophylla* biomass (23% of variation), and sediment grain size (14% of variation). Black bellied plover densities were associated with below ground invertebrate richness (66% of variation), wind direction (22% of variation), and sediment grain size (12% of variation). Semipalmated sandpiper densities were associated with total above and below-ground AFDW (72% of variation), minimum daily temperature (22% of variation), and wind direction (6% of variation). Dowitcher densities were correlated with total above and below-ground invertebrate AFDW (43% of

variation), wind direction (39% of variation), and total worm densities (18% of variation).

Discussion

In this study we found that non-native macroalgal mat presence was associated with both increases and decreases in availability of specific invertebrate prey. *G. vermiculophylla* presence was directly correlated to increases in above-ground gammarids and also indirectly related to increases in below-ground invertebrate metrics, most likely through changes in sediment grain size. *G. vermiculophylla* associated increases in gammarid amphipods, a common prey item for shorebirds, could have increased the overall food supply on vegetated mudflats (see also Cabral et al. 1999; Dugan et al. 2003; Byers et al. 2012). However, there was also a less dramatic, but still significant decrease in small snails on vegetated mudflats which should also be taken into consideration when assessing overall food availability for shorebirds at each mudflat type.

It is possible that *G. vermiculophylla* presence may have caused changes to the surrounding sediments which resulted in changes to the invertebrate community (Yates et al. 1993). Although unvegetated and vegetated mudflats did not significantly differ in mean sediment grain size, there was a significant positive correlation between grain size and *G. vermiculophylla* biomass. In addition, sediment grain size had a significant positive correlation to below-ground invertebrate metrics such as species richness, AFDW, polychaete densities, and worm densities.

Although we observed a large increase in above-ground invertebrate food sources on vegetated mudflats, these increases were not reflected in the shorebird assemblage. There were always fewer shorebirds on vegetated mudflats when compared to unvegetated mudflats and all shorebird species abundances were negatively correlated with *G. vermiculophylla* biomass, though these differences were not significant for dunlins. Gulls are kleptoparasites, often stealing food from other shorebirds, and have been found to be negatively associated with shorebird densities and foraging behavior in other studies (Cabral et al. 1999). It is therefore possible that the positive association between gull densities and *G. vermiculophylla* biomass could have led to reductions in other species of shorebirds on vegetated mudflats.

In our study, dunlins, which are tactile foragers, were able to exploit mudflats with non-native macroalgal coverage, while other species were not. Dunlin densities as well as their foraging intensity were not significantly different on unvegetated and vegetated mudflats, though there was a trend for fewer dunlins on mudflats with *G. vermiculophylla* coverage. Significant reductions in other shorebird species on vegetated mudflats as well as their significantly negative relationship with *G. vermiculophylla* biomass could be attributed to changes in foraging substrate and decreased visibility of prey for visual hunters like plovers (Green 2011). However, shorebirds with more flexible foraging strategies, like dunlins, can often adapt to changes in prey visibility (Murias et al. 1996). This difference associated with foraging strategy was also seen in previous work in Ireland where black-tailed godwits avoided macroalgal mats and redshanks were undeterred, often preferring macroalgal covered areas (Lewis and Kelly

2001). Similarly, Green (2011) found that macroalgal mats negatively affected only shorebirds with specific prey preferences and/or substrate needs.

In addition to relationships with sediment properties and invertebrate metrics, all shorebird abundances were significantly correlated to different meteorological parameters. It is possible that differences in temperature, wind, and sunlight affected shorebird energy budgeting and thus their overall activity rate and foraging behavior (Evans 1976). While other studies have found that cooler temperatures are associated with reductions in invertebrate activity and increases in invertebrate depths (Zwarts and Wanink 1993), none of the correlations between temperature and the different invertebrate parameters were significant in this study, although it should be noted that the temperature range in this study was limited; under more variable temperature conditions we may have seen significant relationships.

Explanatory parameters that were most highly associated with individual species densities and dunlin feeding attempts help to elucidate how the species differ from one another. Maximum daily temperature describes the most variation in the dunlin abundance model, most likely due to temperature-driven effects on overall activity levels (Evans 1976). In addition, dunlins were the only species that showed a positive correlation to other roosting birds. This result is not surprising as dunlins often flock together on mudflats and other studies have shown that their foraging behavior can be positively correlated to total bird abundance (Cabral et al. 1999). It should, however, be noted that the overall R^2 of this model is 0.17 which indicates that there are other parameters that are associated with dunlin abundance and have not been considered in

this study. Dunlin feeding attempts are most highly associated with below-ground invertebrate richness. This follows the classic finding that shorebird densities should mirror availability of prey items (Goss-Custard et al. 1991). Although there was no significant reduction in dunlin foraging attempts on unvegetated and vegetated mudflats, there was a negative relationship between non-native *G. vermiculophylla* biomass and dunlin feeding attempts in the MLR model. This result indicates that there may be a threshold level of *G. vermiculophylla* biomass, which was not seen during the current study period, and above which dunlin densities would be significantly lower.

The dominant explanatory parameters in the MLR models for black bellied plovers, semipalmated sandpipers, and dowitchers all relate to overall invertebrate prey availability for the shorebirds. Prior studies have also found that shorebird distribution is highly correlated to the location of prey items (Goss-Custard et al. 1991). Dowitcher abundance was also highly associated with wind, which could affect energy budgeting and overall activity rate (Evans 1976).

The data presented here indicate that non-native *G. vermiculophylla* mat formation on mudflats likely reduces suitable foraging substrate for several migratory shorebirds in the Virginia coastal bays. However, as has been shown in prior work (Lewis and Kelly 2001; Green 2011), shorebirds with flexible foraging strategies, in this case dunlins, are still able to exploit these vegetated mudflats. It should be noted that there is likely a threshold density of *G. vermiculophylla* above which all shorebird foraging, including dunlin, will be significantly reduced. This hypothesis is supported in part by the negative correlation between dunlin abundance and *G. vermiculophylla* biomass. In

addition, observations in May 2011 on a mudflat with very dense *G. vermiculophylla* coverage indicated that only gulls chose to roost in very dense *G. vermiculophylla* mats. Future work should quantify this threshold and determine how very dense mats of *G. vermiculophylla* might affect invertebrate densities and shorebird foraging behavior.

Acknowledgments

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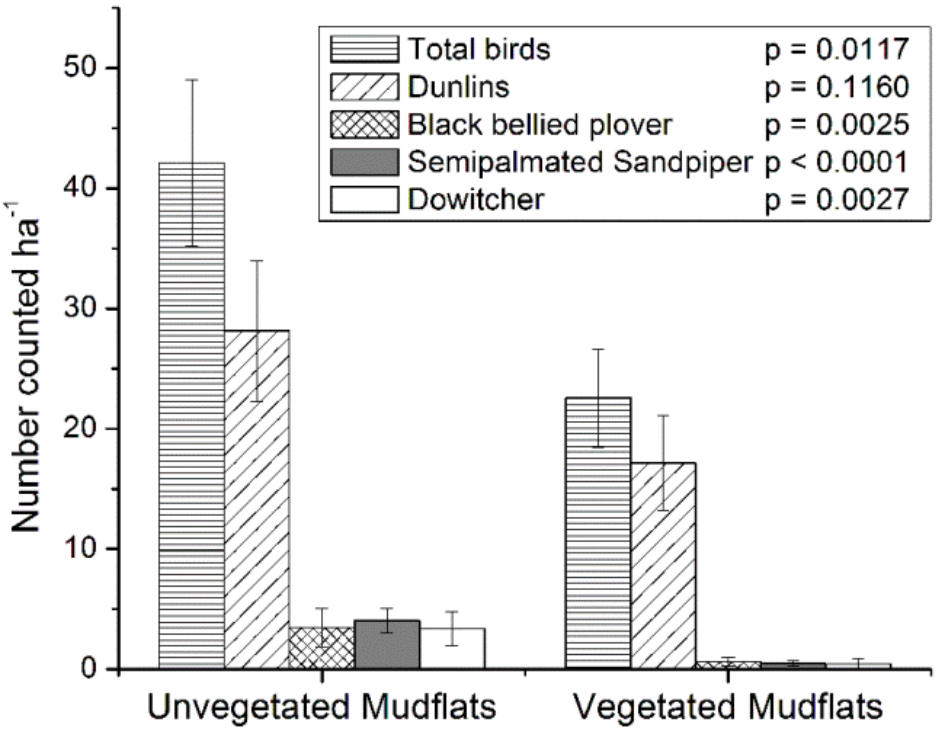
Figures

Figure 5.1 Graph showing number of total birds, dunlins, black bellied plovers, semipalmated sandpipers, and dowitchers counted per hectare on unvegetated and vegetated mudflats (all ± SE). P values displayed indicate significant differences.



Tables

Table 5.1. Dependent and independent variables used in correlations and multiple linear regression (MLR) models with units.

Variable	Units
Dependent Variables	
Dunlins abundance	Number counted ha^{-1}
Dunlin feeding attempts	Number pecks and probes $2min^{-1}$
Black bellied plovers abundance	Number counted ha^{-1}
Semipalmated sandpipers abundance	Number counted ha^{-1}
Dowitchers abundance	Number counted ha^{-1}
Independent Variables	
G. vermiculophylla biomass	gdw m^{-2}
Mean sediment grain size	µm
Above ground invertebrate richness	Number counted 0.0314 m^2
Below ground invertebrate richness	Number counted 0.0314 m^2
Above ground invertebrate AFDW	mg AFDW m^{-2}
Below ground invertebrate AFDW	mg AFDW m^{-2}
Total invertebrate AFDW	mg AFDW m^{-2}
Gammarids	Number counted m^{-2}
Small Snails	Number counted m^{-2}
Polychaetes	Number counted m^{-2}
Total worms	Number counted m^{-2}
Number roosting birds	Number counted ha^{-1}
Number gulls	Number counted ha^{-1}
Precipitation	mm day^{-1}
Maximum temperature	°C
Minimum temperature	°C
Mean temperature	°C
Wind speed	m s^{-1}
Wind direction	degrees
Vapor pressure	mb
Solar radiation	kJ m^{-2}
PAR	µE m^{-2}

Table 5.2. *G. vermiculophylla* density, invertebrate densities, and AFDW from cores collected on unvegetated and vegetated mudflats (all ± SE) in May 2012. P values and type of test reported in final row.

Site Type	*G. vermiculophylla* (gdw m^{-2})	Above ground Invertebrate Species Richness (number per 10 cm core)	Gammarids (number m^{-2})	Total Small Snails (number m-2)	Below ground Invertebrate Species Richness (number per 10 cm core)	Total Polychaetes (number m-2)	Total Worms (number m-2)	Invertebrate above ground AFDW (mg AFDW m-2)	Invertebrate below ground AFDW (mg AFDW m-2)	Total Invertebrate AFDW (mg AFDW m-2)
Unvegetated	0	0	17.91 ±7.67	67.68 ± 13.28	3.75 ± 0.21	9.95 ± 4.79	270.70 ± 41.83	0	14.30 ± 1.18	14.30 ± 1.18
Vegetated	54.49 ± 4.11	2.08 ± 0.22	322.45 ± 69.60	30.52 ± 11.58	3.38 ± 0.29	17.25 ± 6.06	290.61 ±34.84	22.32 ± 2.91	30.22 ± 7.19	49.75 ± 6.98
p value and statistical test	< 0.0001 Wilcoxon	< 0.0001 Wilcoxon	< 0.0001 Wilcoxon	0.0443 t-test	0.3472 t-test	0.3905 t-test	0.7178 t-test	< 0.0001 Wilcoxon	0.0814 t-test	0.0002 t-test

Table 5.3. Pearson correlation statistics. Only Pearson correlations (r) with $p < 0.1$ are listed. ^ indicates a significance level between 0.05 and 0.10 while a * indicates that data were not available for the correlation.

	G. vermiculophylla biomass	Sediment grain size	Dunlin abundance	Dunlin feeding attempts	Black bellied plover abundance	Semipalmated sandpiper abundance	Dowitcher abundance
G. vermiculophylla biomass		0.500	-0.198^		-0.237	-0.494	-0.299
Dunlin abundance	-0.198^			*			
Black bellied plover abundance	-0.237			*		0.576	0.744
Semipalmated sandpiper abundance	-0.494	-0.282		*	0.576		0.640
Dowitcher abundance	-0.299			*	0.744	0.640	
Gull abundance	0.235			*			
Sediment grain size	0.500					-0.282	
Above ground invert richness	0.933	0.761	-0.221^			-0.469	-0.272
Below ground invert richness	-0.467	0.297		-0.377	0.419	0.351	0.288
Above ground invert AFDW	1.000	0.516	-0.200^		-0.237	-0.495	-0.298
Below ground invert AFDW	0.679	0.382		0.239	-0.372	-0.446	-0.296
Total invert	0.978	0.531			-0.300	-0.527	-0.337

AFDW							
Gammarid density	0.711					-0.310	-0.212^
Small snail density	-0.607			-0.306	0.415	0.415	0.293
Polychaete density	0.639	0.327				-0.324	-0.250
Total worm density	0.255	0.429		0.272	-0.202^	-0.293	-0.304
Number roosting birds				*			
Precipitation							
Maximum temperature			-0.353				
Minimum temperature			-0.326		0.236	0.275	0.278
Mean temperature			-0.343			0.254	0.201^
Wind speed			0.273				
Wind direction					0.269		0.294
Vapor pressure			-0.301		0.201^	0.267	0.250
Solar radiation			0.231^		-0.285		-0.239
PAR			0.229^		-0.265		-0.203^

Table 5.4. Explanatory parameters for shorebird densities and dunlin feeding attempts from stepwise MLR model output.

Parameter	Estimate	% Variation Described
DUNLIN ABUNDANCE n = 72		Model R^2 = 0.17
Intercept	86.272	
Maximum daily temperature	-2.553	72.02
Above ground invertebrate richness	-5.654	17.05
Number of birds roosting	2.941	10.92
DUNLIN FEEDING ATTEMPTS n = 123		Model R^2 = 0.23
Intercept	1780.474	
Below ground invertebrate richness	-396.367	62.64
***G. vermiculophylla* biomass**	-3.999	23.42
Sediment grain size	4.450	13.94
BLACK BELLIED PLOVER ABUNDANCE n = 72		Model R^2 = 0.27
Intercept	-15.786	
Below ground invertebrate richness	5.467	65.94
Wind direction	0.021	22.09
Sediment grain size	-0.063	11.97
SEMIPALMATED SANDPIPER ABUNDANCE n = 72		Model R^2 = 0.38
Intercept	-0.185	
Total invertebrate AFDW	-0.137	72.40
Minimum daily temperature	0.326	22.08
Wind direction	0.009	5.52
DOWITCHER ABUNDANCE n = 72		Model R^2 = 0.27
Intercept	5.039	
Total invertebrate	-0.100	42.73

AFDW		
Wind direction	0.026	38.75
Number worms m^{-2}	-0.012	18.51

Chapter 6: Association of *Gracilaria vermiculophylla*, a non-native, mat forming macroalga, with increased concentrations of *Vibrio* bacteria in sediment, water, and oysters on intertidal mudflats

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Abstract

Vibrio spp. are bacteria that are naturally found in a range of aquatic environments. In estuaries, they are recognized as being biogeochemically and ecologically important. While most species are harmless, some pathogenic species can cause symptoms of disease in humans that range from gastrointestinal and wound infections to septicemia and death. *V. parahaemolyticus* and *V. vulnificus* are two important human pathogens. Recent research efforts have focused on potential reservoirs and environmental conditions that can increase human exposure to and infection with these species of bacteria. In this study we investigated how the proliferation of a non-native macroalga, *Gracilaria vermiculophylla*, within the mid-Atlantic region, USA, could be related to concentrations of total *Vibrio, V. parahaemolyticus,* and *V. vulnificus* in water, sediment, and oysters on intertidal mudflats where the macroalga is found. Our data indicated that total *Vibrio, V. parahaemolyticus,* and *V. vulnificus* were commonly found on the macroalga in both summer and early fall. Summer and fall seasonal samplings indicated that mudflats with mats of *G. vermiculophylla* were associated with higher total *Vibrio, V. parahaemolyticus,* and *V. vulnificus* concentrations of proximal water, sediment, and oysters when compared to mudflats without macroalgal coverage. In addition, of all isolates confirmed to be *V. vulnificus*, regardless of source, 68% were confirmed as a highly virulent genotype.

Introduction

Vibrio bacteria are ubiquitous in coastal and estuarine environments, and comprise as much as 40% of the culturable bacterial population, with coastal abundance as high as 10^7 cells/ 100 ml (1, 2, 3). They are recognized for their importance in nutrient

cycling, including N_2 fixation, carbon cycling, nitrate reduction, and phosphorus recycling (1, 3, 4, 5, 6). These bacteria are important degraders of chitin (1) and polycyclic aromatic hydrocarbons (7). One species, *V. tubiashii,* is a lethal pathogen for oyster larva, and the presence of this organism can have devastating effects (8). Other examples of marine vertebrate and invertebrate pathogens include, *V. alginolyticus* and *V. splendidus*, which harm clam larvae (9) and *V. harveyi* which can negatively affect marine fish and invertebrates (10).

While most members of this genus are harmless to humans, some pathogenic strains, such as *V. parahaemolyticus* (Vp), *V. vulnificus* (Vv)*, V. cholerae*, and *V. alginolyticus*, can cause gastrointestinal illnesses, wound infections, or septicemia. Infection can occur via consumption of raw or undercooked seafood or via exposure of wounds to seawater (11). Typically, infections occur in warmer months, when *Vibrio* spp. densities are highest (11). In susceptible individuals, like those with diabetes, liver disease, or the elderly, septicemia can result in death about 44% of the time with *Vibrio* spp. infections (12).

Over the last several decades, reports of *Vibrio* ssp. infections have been increasing, most likely due to climate change, a shift to more elderly people in the population, and increased human exposure to coastal waters via recreation and consumption of shellfish (13, 14, 15, 16). Global climate change will increase sea level height, overall aerial extent of estuaries, and year-round sea surface temperatures, which could increase overall concentrations of warm-water loving *Vibrio* spp. (15, 16). These increases in overall *Vibrio* concentrations, combined with increased storm frequency, as

predicted by many climate change scenarios, will likely result in greater human exposure and infection from both Vp and Vv (16). Because of these increases in exposure and potential infection, it is important that researchers and managers understand the ecology of, and possible reservoirs for, Vp and Vv.

In estuarine waters, macrophytes, microalgae, invertebrates, and sediment can act as *Vibrio* reservoirs. While initial studies of this bacterial genus focused primarily on reservoirs of *V. cholerae* (17, 18, 19, 20), increasing emphasis has been placed on expanding this knowledge to reservoirs for Vp and Vv. Benthic diatoms (21, 22), zooplankton, copepods, sediments (4, 23), estuarine snails (22, 24), freshwater fish (25), and seaweed (26, 27) can all be associated with Vp in coastal ecosystems. In addition, currently documented reservoirs of Vv include several size classes of zooplankton (28), shellfish, crab, finfish intestines (29), and algae (30). Often, when looking at Vv in estuarine systems, researchers classify molecularly confirmed Vv species into one of two genotypes, the avirulent E-genotype, or the more virulent C-genotype, that is commonly associated with human infection (31). To date, most of these studies have reported Vv isolates collected from environmental samples to be primarily of the avirulent E-genotype (31, 32, 33).

Gracilaria vermiculophylla is a non-native, red macroalga from East Asia that has been introduced to temperate estuaries around the world (34, 35). It often accumulates on intertidal mudflats to form dense mats (up to 15 cm deep in Virginia) that can remain on the order of months to years due to attachment to the tube building polychaete, *Diopatra cuprea* (36, 37). Preliminary testing showed that Vp and Vv could be recovered from *G.*

vermiculophylla thalli, a finding which led us to question whether this macroalga could act as a reservoir for *Vibrio* spp. bacteria on the intertidal mudflats where it persists.

Virginia epidemiological datasets support the paradigm previously described in Baker-Austin et al. (16) of increasing *Vibrio* infections over time with reported infections more than doubling in the past 20 years (38). It is important that managers and watermen in the area understand how the habitat surrounding oyster reefs might affect *Vibrio* levels in harvested oysters. We hypothesized that if *G. vermiculophylla* was acting as a *Vibrio* reservoir, this could have important consequences for concentrations of the bacteria in sediment, water, and oyster tissue on mudflats where the macroalga is found.

The goals of this study were to: (i) quantify concentrations of total *Vibrio*, Vp, and Vv associated with *G. vermiculophylla* thalli, (ii) compare differences in total *Vibrio*, Vp, and Vv concentrations in sediment, water, and oysters on mudflats proximal to areas either with (vegetated) or without (bare) *G. vermiculophylla* mats, and (iii) investigate the relative public health concern associated with the presence of Vv, by determining which genotype of the species was present in the samples (C-genotype or E-genotype). We collected samples a total of seven times, once in July 2012, three times in August 2012, and three times in September 2012. Even though the sampling window was temporally narrow, we captured conditions during summer, when recreational water quality is of high importance, and during early fall, when shellfish harvesting commences in the Virginia coastal bays.

Methods

Study Site

Sampling was conducted on mudflats with and without *G. vermiculophylla* coverage within the Virginia Coastal Reserve Long Term Ecological Research (VCR LTER) site (Figure 6.1). The Virginia coastal bays extend from the tip of the Delmarva Peninsula, 110 km north to the Maryland border, and are enclosed to the east by several barrier islands. All sample sites were within 6 km of one another, facilitating rapid sample collection.

Sample Collection

G. vermiculophylla samples were collected on each of the seven sampling days to determine concentrations of total *Vibrio,* Vp, and Vv associated with the macroalgal thalli. On each sampling date, *G. vermiculophylla* samples were collected within a 100 m^2 section of the mudflat, and stored in sterile plastic bags until analysis.

Sampling done on July 2, 2012 was conducted at a larger spatial scale and covered six bare and six vegetated sites, with one replicate sample of water, sediment and *G. vermiculophylla* processed per each study site. This sampling was focused on quantification of total *Vibrio,* Vp, and Vv concentrations in water and sediments in areas either associated with or not associated with *G. vermiculophylla*; oysters were not sampled. Sampling in August and September covered three bare and three vegetated sites, with three replicate samples each of water, sediment, oysters, and *G. vermiculophylla* processed at each study site. Because temperature and salinity have been shown to affect *Vibrio* concentrations, we measured both variables at all sample sites, on each sampling date.

Water samples were collected in autoclaved, 1 L bottles and sediment samples were collected using a modified, sterile 60 cc syringe. At each site four, 1 cm deep sediment cores were combined in a sterile Whirl-pak® bag. Five to ten oysters were collected from each sample site in August and September, placed into a sterile plastic bag, and transported to the laboratory. All samples were stored in a cooler after collection and processed within 6 hours of collection in the field.

Average *G. vermiculophylla* biomass was determined once in July, August, and September through biomass surveys. Briefly, for each site that was determined to be *G. vermiculophylla* covered, all visible algae found within ten, 0.25 m^2 randomly thrown quadrats were collected. Algae were rinsed with distilled water, dried in a 60 °C oven, and weighed in order to determine average dry weight of *G. vermiculophylla* m^{-2} at each site.

Laboratory Processing

All samples were plated on thiosulfate-citrate-bile salts-sucrose medium (TCBS, Oxoid, Hampshire, England) for total *Vibrio* enumeration and CHROMagar™ Vibrio media (CHROMagar, Paris, France) to determine presumptive concentrations of Vp and Vv. Water samples were filtered onto 0.45 µm sterile, gridded filters (Pall Corporation, Ann Arbor, Michigan), which were placed onto each of these. Sediment samples were combined with equal parts phosphate buffered saline (i.e. 10 wet g of sediment in 10 mL PBS; PBS, Amresco, Solon, Ohio), vortexed for 5 minutes, and shaken for 1 minute. This slurry was then immediately serially diluted in PBS and spread on TCBS and CHROMagar Vibrio plates. In order to control for variations in the initial water content

of sediment samples, 2 mL of each sediment slurry were filtered onto duplicate, pre-dried and weighed glass fiber (GF) filters, dried in a 60°C oven for 48 hours, and reweighed. This average dry weight of sediment was later used in calculations to determine total *Vibrio*, Vv, and Vp concentrations per dry g of sediment. Each replicate of 10 g of *G. vermiculophylla* from vegetated sites was combined with 100 mL of PBS, vortexed for 5 minutes, and shaken for 1 minute. Immediately after vortexing and shaking, subsamples of the resulting liquid were removed for serial dilutions and spread plating.

Oysters were rinsed first with distilled water to remove any excess sediment and then with ethanol and patted dry. All shucking of oysters was done with an ethanol and flame sterilized knife. Once opened, the meat was rinsed with PBS, aseptically separated from the shell, and placed into sterile containers. Tissues from 5 oysters was combined and homogenized in a blender (Waring Commercial, Torrington, Connecticut) with a 1 to 1 w:v ratio of grams of oyster meat to PBS (minimum of 25 mL PBS) using three 15 sec long blending cycles separated by a 5 sec pause. Three replicate, homogenized samples from each site were then serially diluted in PBS and spread on TCBS and CHROMagar Vibrio media.

All plated samples were incubated for 24 hours per manufacturer instructions (35 °C for TCBS and 37 °C for CHROMagar Vibrio). Colony forming units (CFUs) were counted on each plate after the incubation period in order to determine the presumptive CFUs per g or mL of sample. Isolated colonies (300-400 per each sampling period) were picked from CHROMagar Vibrio using sterile loops into nuclease-free water and boiled for 10 minutes to release DNA for molecular typing. Presumptive Vp and Vv

concentrations within each sampling period were multiplied by the proportion of isolates that were molecularly confirmed to be Vp and Vv to yield an estimate of confirmed Vp and Vv concentrations. These values were used for statistical analyses.

Molecular Typing

Tubes containing released DNA were centrifuged at 10,000 x g for 10 minutes and supernatant was then transferred to a fresh tube to be used as template DNA for polymerase chain reaction (PCR) confirmation of species identification. Multiplex PCR reactions were used to confirm either Vp or Vv species identity by detecting amplification of species-specific DNA fragments. Vp isolates were confirmed using primers specific for *flaE* (39). Vv confirmation targeted a sequence located in the *vvhA* gene which encodes for Vv specific hemolysin (32). The genotypes of confirmed Vv isolates were determined via multiplex PCR, examining for *vcgC* or *vcgE* alleles (31).

Statistical Analysis

All statistical analyses to determine differences in total *Vibrio,* Vp, and Vv concentrations were performed on data separated by sample period (July, August, September) and sample type (water, sediment, oysters) in SAS (SAS 9.2, Cary, NC). In July, t-tests were used to compare average total *Vibrio,* Vp, and Vv concentrations at bare sites and vegetated sites. Significance required a p-value ≤ 0.05 (alpha = 0.05). While all data collected from sediments did not need any transformations, Vv values from water had to be log transformed to satisfy ANOVA assumptions. Because transformations did not resolve homogeneity of variance issues with data for Vp levels in water, we used a nonparametric Wilcoxon test to analyze the data.

Total *Vibrio,* Vp, and Vv concentration data for August and September were analyzed using mixed model ANOVAs to determine differences between *G. vermiculophylla* coverage type (vegetated or bare), each of the three sample dates within each sample period, and the interaction of these two variables. All data satisfied ANOVA assumptions and were therefore analyzed without transformation. Significance required a p-value ≤ 0.05 (alpha = 0.05), however differences with p-values ≤ 0.10 (alpha = 0.10) were also noted.

Results

Site Conditions

Salinity and temperature were not significantly different between sites at each sampling period (Table 6.1). Average *G. vermiculophylla* biomass at vegetated sites was highest in July and tapered off in August and September (Table 6.1). Total *Vibrio,* Vp, and Vv were found in relatively high abundance on *G. vermiculophylla* biomass in July, August, and September 2012 (Figure 6.2, Table 6.S1).

July 2, 2012 Survey

There was an overall trend for higher total *Vibrio*, Vp, and Vv levels in water and sediment samples collected on vegetated, rather than bare mudflats (Figure 6.3). These differences were significant for total *Vibrio* concentrations in water samples collected on bare (2.0e2 ± 3.3e1 CFU mL^{-1}) and vegetated (1.3e3 ± 2.7e2 CFU mL^{-1}) mudflats ($p = 0.0021$). Differences were significant for total *Vibrio* levels in sediments with $p < 0.10$ ($p = 0.0592$) when mean densities on bare (6.3e4 ± 1.5e4 CFU g^{-1}) and vegetated (1.5e5 ± 3.6e4 CFU g^{-1}) mudflats were compared. Vp levels in sediments ($p = 0.0349$), as well as

Vv in water samples ($p = 0.0020$) were significantly different as well (Figure 6.3). No Vv was found in the sediment on either vegetated or bare mudflats.

August 27-29 and September 19-21, 2012 Surveys

There was a trend in both August and September for higher levels of total *Vibrio*, Vp and Vv in water, sediments, and oyster tissue collected proximal to mats of *G. vermiculophylla* when compared to concentrations from samples collected from bare mudflats (Figure 6.4). Total *Vibrio* in August water samples were significantly different ($p = 0.0877$), with $p < 0.10$, when bare (1.5e2 ± 2.8e1 CFU mL^{-1}) and vegetated (2.3e2 ± 4.0e1 CFU mL^{-1}) mudflat means were compared. Sediment total *Vibrio* levels were significantly higher ($p = 0.0325$) at vegetated sites (2.7e5 ± 9.9e4 CFU g^{-1}) when compared to bare sites (8.7e4 ± 2.1e4 CFU g^{-1}). Total *Vibrio* levels in oyster tissue were significantly different ($p = 0.0875$), with $p < 0.10$, between bare (5.7e3 ± 2.1e3 CFU g^{-1}) and vegetated (1.8e4 ± 8.4e3 CFU g^{-1}) sites.

August water Vp ($p = 0.0980$) and Vv ($p = 0.0887$) levels were significantly higher at *G. vermiculophylla* covered sites with $p < 0.10$ (Figure 6.4). Sediment ($p = 0.0422$) and oyster tissue ($p = 0.0382$) Vp levels were significantly higher when *G. vermiculophylla* was present (Figure 6.4). Vv levels were also higher in oyster meat when *G. vermiculophylla* was present nearby and were significant at the $p < 0.10$ level ($p = 0.0589$, Figure 6.4). The interaction between *G. vermiculophylla* coverage state and sample date were not significant for total *Vibrio*, Vp, or Vv measurements in water or sediment during the August sampling period.

In September, total *Vibrio* levels were not significantly different for water ($p = 0.1345$) on bare (6.2e1 ± 8 CFU mL^{-1}) or vegetated (1.2e2 ± 3.4e1 CFU mL^{-1}) mudflats. Sediment ($p = 0.2478$) total *Vibrio* levels were also no different on bare (1.3e5 ± 3.4e4 CFU g^{-1}) or vegetated (2.6e5 ± 9.0e4 CFU g^{-1}) mudflats. Differences in total *Vibrio* concentrations in oysters were not significant between bare (1.1e3 ± 3.1e2 CFU g^{-1}) and vegetated (2.0e3 ± 5.9e2 CFU g^{-1}) mudflats ($p = 0.1686$). Only Vp levels measured in sediments were significantly higher when *G. vermiculophylla* was present ($p = 0.0363$); all other densities of Vp and Vv were not significantly different between bare and vegetated mudflats (Figure 6.4). Sample date and the interaction between sampling date and coverage type was never significant for Vp, Vv, or total *Vibrio* measurements.

Molecular Typing

Vp molecular analysis determined that, overall, CHROMagar Vibrio medium correctly identified Vp colonies 81% of the time (total 846 isolates) from all three sampling periods. Specifically, 81% of the 348 samples collected in July, 76% of the 271 samples collected in August, and 89% of the 227 samples collected in September were molecularly confirmed via PCR as Vp.

PCR confirmation of Vv on 163 isolates over the course of all three study periods, demonstrated that 35% of isolates were confirmed as being this species. Specifically, 15% of 41 isolates in July, 36% of 36 isolates in August, and 44% of 86 isolates in September were confirmed as Vv. Of isolates confirmed to be Vv, regardless of source, 68% were C-genotype (the more virulent genotype, associated with human infections) and 32% were E-genotype (relatively avirulent genotype, not typically associated with

human infections). Isolates collected from water and sediment were C-genotype in 64% and 56% of confirmed Vv species, respectively. In addition, 80% of confirmed isolates collected from *G. vermiculophylla* and 75% of confirmed isolates from oyster tissue were confirmed as being the C-genotype.

Discussion

G. vermiculophylla as a Vibrio Reservoir

While other studies have looked at seaweeds, in general, being a reservoir for Vp (26, 27) and Vv (30), no studies have looked at the invasive macroalga, *G. vermiculophylla,* as a potential reservoir for *Vibrio* bacteria. Results from all sampling dates confirmed that *G. vermiculophylla* biomass was associated with measureable concentrations of total *Vibrio*, Vp and Vv in July, August and September (Figure 6.2, Table 6.S1), thus confirming that this macroalga is a reservoir of *Vibrio* bacteria, including the pathogens Vp and Vv.

G. vermiculophylla Effects on Water, Sediments, and Oyster Tissue

Data from all sampling periods support the hypothesis that *G. vermiculophylla* presence can be associated with an increase in total *Vibrio*, Vp, and Vv densities in water, sediment, and oyster tissue. These differences were significant for total *Vibrio* in sediments in August, Vp in sediments during all three sampling periods, Vp in oysters in August, and total *Vibrio* and Vv in water in July. Although not all differences were significant, it is likely that if more samples were collected at more study sites, many of the differences would have been detected at the 0.05 level, since at a 0.10 level there were more significant differences.

These trends have important management implications for the Virginia coastal bays for both fisheries and human health risks. Total *Vibrio* measurements encompass both human pathogens and other species like *V. tubiashii*, *V. alginolyticus*, *V. splendidus,* and *V. harveyi* which can negatively affect marine vertebrates and invertebrates (8, 9, 10). Reductions in oyster, clam, shrimp, or finfish yields due to exposure to these bacteria could have drastic effects on fisheries. In addition, our species-specific measurements that indicated higher levels of the human pathogens Vp and Vv in water, sediments, and oysters nearby mats of *G. vermiculophylla,* have important public health implications that managers and watermen should be aware of. In the context of increasing concentrations of *Vibrio* bacteria in the coming years (15, 16), these associations could result in higher incidence of infection in the public.

Molecular Typing

In addition to overall *Vibrio* concentration trends, 68% of all Vv isolates collected, regardless of source were C- rather than E-genotype strains. While most studies have reported a majority of environmentally collected Vv isolates to be of the E-genotype (31, 32, 33), Yokochi et al. (40) found as many as 91% of the Vv isolates from bay waters in Japan to be C-genotypes. Since the sampling period for this study was relatively short, it would be interesting to understand the prevalence of C- versus E-genotypes of Vv over a range of seasons and matrices. In particular, additional work is warranted to investigate potential causes for higher C-genotype Vv isolates in Virginia.

Acknowledgements

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Figures

Figure 6.1. Map of study sites visited during July, August, and September 2012 surveys.

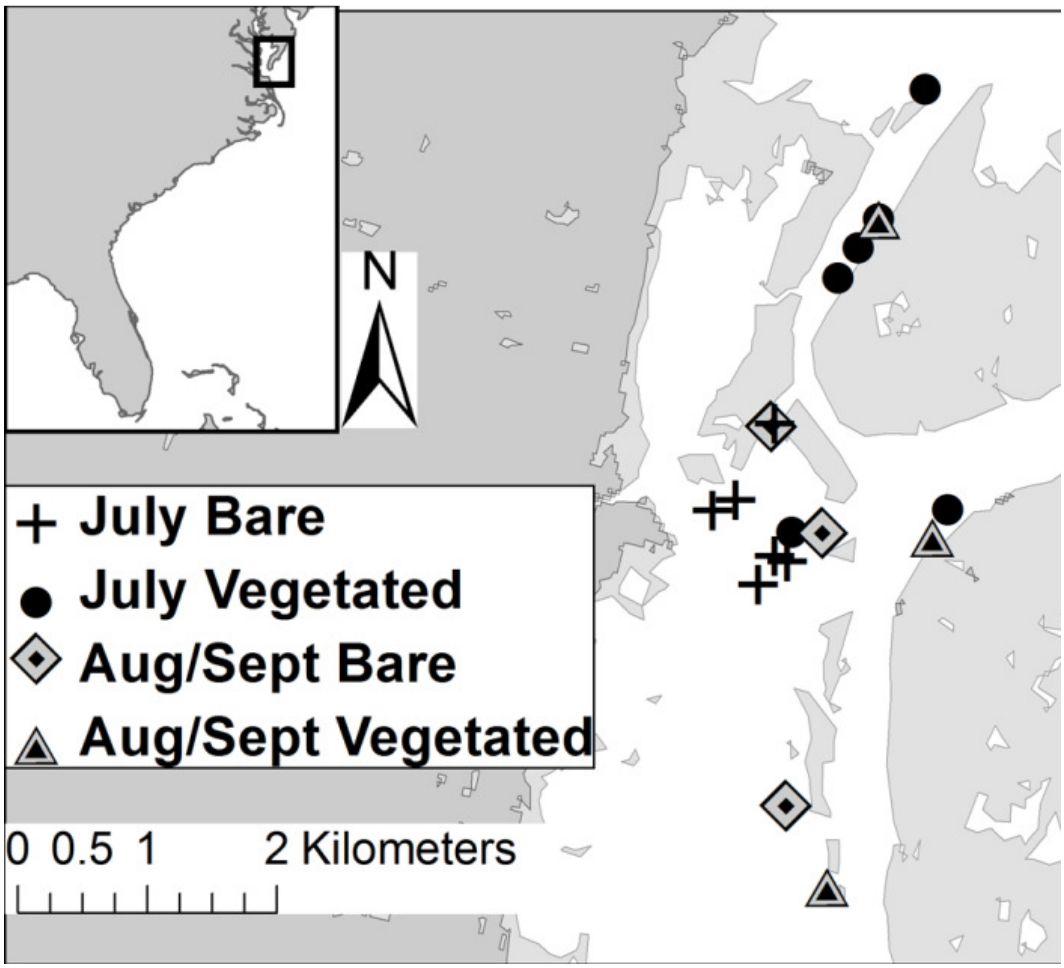


Figure 6.2. Average total *Vibrio*, *V. parahaemolyticus* (Vp), and *V. vulnificus* (Vv) concentrations documented on *G. vermiculophylla* tissue (mean ± SE) in July, August, and September 2012. For specific numbers, see table 6.S1.

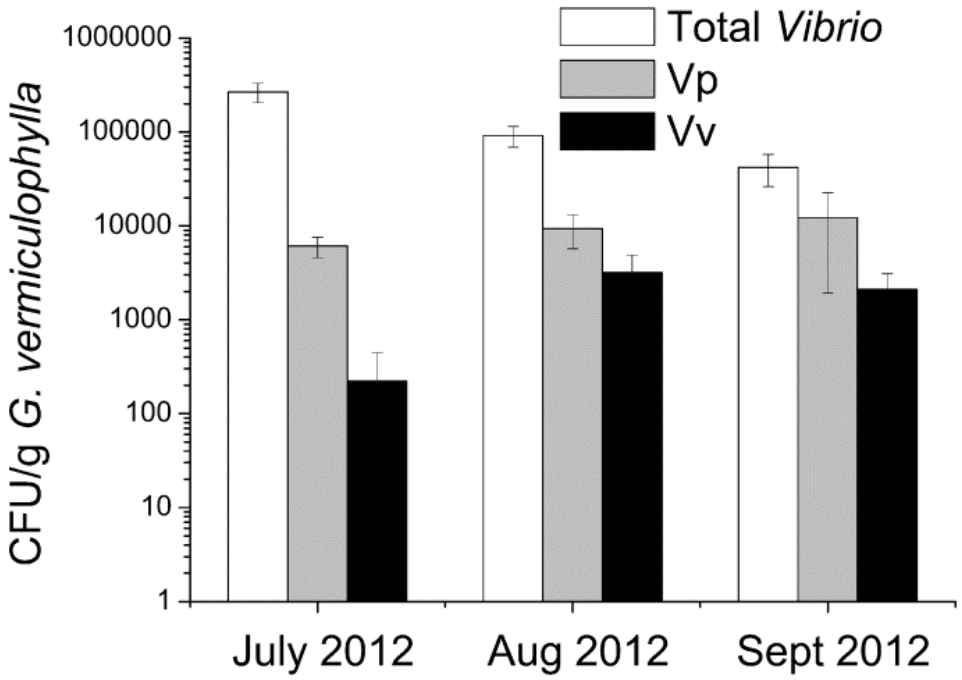


Figure 6.3. CFUs of *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) from water and sediment on mudflats with and without *G. vermiculophylla* coverage from the widespread survey at 6 vegetated and 6 bare mudflats in July 2012. Significant differences between concentrations on bare and vegetated mudflats indicated by an asterisk between hatched and solid bars for each bacterial species. P-values for statistics between coverage types within each sample type displayed on x-axis.

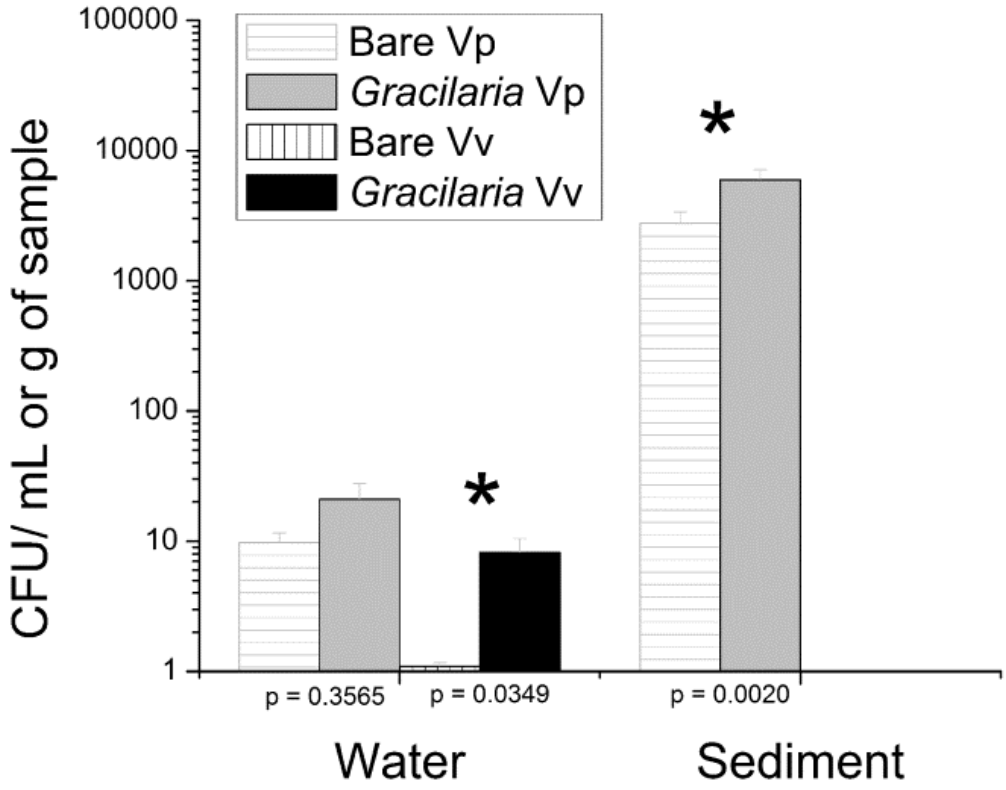
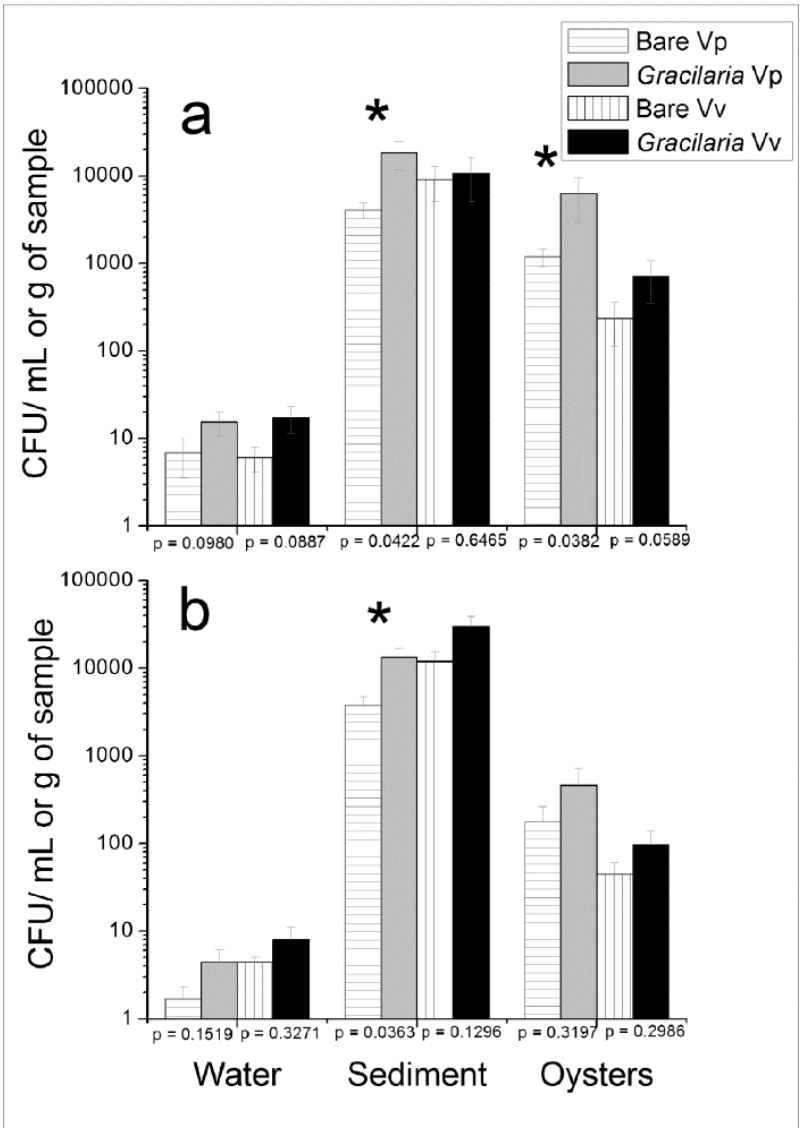


Figure 6.4. CFUs of *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) from water, sediment, and oysters with and without *G. vermiculophylla* coverage nearby on three sample days in (a) August and (b) September 2012. Significant differences between concentrations on bare and vegetated mudflats indicated by an asterisk between hatched and solid bars for each bacterial species. P-values for statistics between coverage types within each sample type displayed on x-axis.



Tables

Table 6.1. Average salinity, temperature, and *G. vermiculophylla* biomass at each sample period (mean ± SE).

Date	Salinity (ppt)	Temperature (°C)	*G. vermiculophylla* biomass (dry g m^{-2})
02 July 2012	31.86 ± 0.07	30.62 ± 0.34	112.02 ± 13.28
27, 28, 29 August 2012	29.95 ± 0.16	27.66 ± 0.19	26.55 ± 3.46
19, 20, 21 September 2012	31.36 ± 0.04	22.09 ± 0.27	15.27 ± 2.29

Table 6.S1. Average total *Vibrio, V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) concentrations (CFUs g^{-1}) found on *G. vermiculophylla* (± SE) in July, August, and September 2012.

Date	Sample Size (n)	Total *Vibrio* (CFUs g^{-1})	Vp (CFUs g^{-1})	Vv (CFUs g^{-1})
02 July 2012	6	2.7e5 ± 6.2e4	6.1e3 ± 1.5e3	2.3e2 ± 2.2e2
27, 28, 29 August 2012	9	9.1e4 ± 2.3e4	9.4e3 ± 3.7e3	3.2e3 ± 1.7e3
19, 20, 21 September 2012	9	4.2e4 ± 1.6e4	1.2e4 ± 1.0e4	2.1e3 ± 9.7e2

Chapter 7: A global perspective on the *Gracilaria vermiculophylla* invasion: What is currently known and what is still needed

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Abstract

Consequences of species introductions are often complex, especially when the introduced species is able to modify the habitat it invades. Such is the case with the macroalgal invader, *G. vermiculophylla*, which often becomes established in a system before it is recognized as an invader due to its cryptic nature. Here we discuss the currently known distribution of this macroalga, confirmed using genetic barcoding, as it cannot be distinguished from native congeners based on morphology alone. In addition, we explain why the macroalga is a successful invader in diverse habitats. Ecological effects in intertidal, subtidal, and seagrass communities, as well as consequences for commercially important seafood and industry are also discussed.

Introduction

Species invasions occur in both aquatic and terrestrial ecosystems worldwide and can have dramatic ecological and financial consequences (Sakai et al. 2001). Often, invasions occur more readily in systems that are already stressed by some type of disturbance (Occhipinti-Ambrogi & Savini 2003). For example, increased temperatures as a result of global warming can allow non-native species that may have been limited by cold stress, to invade an area (Occhipinti-Ambrogi 2007; Hellmann et al. 2008; Rahel & Olden 2008; Walther et al. 2009; Sorte et al. 2010b). Often these increased temperature effects are seen most dramatically during the winter, when cold temperatures and winter hypoxia, which can act as natural inhibitors of invasions, are minimized by global warming (Rahel & Olden 2008). In addition, increased pollution can make a system more susceptible to invasions (Piola & Johnston 2008). Because they have a higher level of

connectivity, marine systems are often more readily affected by the interaction of climate change and species invasions (Pyšek & Richardson 2010; Sorte et al. 2010a).

Of particular concern are macroalgal invaders, which can have significant effects on the ecosystems they invade because of their ability to change habitat structure and function (Schaffelke et al. 2006). Often, macroalgal invaders act as habitat engineers which can have cascading effects, both positive and negative, on system function, food web structure, water movement, biogeochemistry, and sediment suspension (Schaffelke et al. 2006; Wallentinus & Nyberg 2007; Byers et al. 2012). Common vectors of macroalgal introduction include recreational boating, shipping, aquaculture, and aquarium trade (Johnson et al. 2001; Padilla & Williams 2004; Chapman et al. 2006; Minchin et al. 2009; Clarke Murray et al. 2011). Coastal systems often become susceptible to macroalgal invasions when unused nutrients are prevalent and space is available for establishment (Bax et al. 2003; Chapman et al. 2006). In addition, successful invaders are typically resistant to fluctuations in salinity and temperature, wave stress, herbivore pressure, and have efficient reproductive strategies (Chapman et al. 2006).

Gracilaria vermiculophylla is a red macroalga from East Asia that has invaded many temperate estuaries around the world (Figure 1). It is a cryptic invader, meaning that it cannot be easily distinguished from native *Gracilaria* species and is therefore difficult to classify as native or introduced based on morphology alone (see inset; Saltonstall 2002). Therefore, researchers must rely on hybridization studies coupled with morphological analyses (Ohmi 1956; Yamamoto 1978; Yamamoto & Sasaki 1988;

Terada & Yamamoto 2002) or DNA sequencing (Bellorin et al. 2002; Gurgel & Fredericq 2004) to identify *G. vermiculophylla*. Improved genetic methods are now being used to document the expansion and invasion history of *G. vermiculophylla* (Yang et al. 2007; Saunders 2009; Skriptsova & Choi 2009; Kim et al. 2010a, 2010b; Rueness 2010; Gulbransen et al. 2012; Nettleton et al. 2013). As is seen with many invasive species, *G. vermiculophylla* is highly resilient to environmental changes and has many potential vectors of dispersal and colonization (eg. Thomsen & McGlathery 2005, 2007; Thomsen et al. 2007; Nyberg & Wallentinus 2009; Abreu et al. 2011b).

G. vermiculophylla thrives in shallow, low-energy environments and can often be found living in three main habitat types in coastal ecosystems: intertidal marshes and mudflats, shallow subtidal systems, and seagrass-dominated systems. On marshes, *G. vermiculophylla* often winds around cordgrass stems, which help to keep it in place at the marsh surface (Thomsen et al. 2009; Gulbransen & McGlathery 2013). On mudflats, tube building worms and shellfish provide substrates for attachment (Thomsen 2004a; Thomsen & McGlathery 2005; Thomsen et al. 2007; Abreu et al. 2011b; Berke 2012). Subtidal populations are found either attached to tube worms or as drifting mats (Thomsen 2004b; Thomsen & McGlathery 2006; Cacabelos et al. 2012; Lawson et al. 2012). In both native (Western Pacific) and introduced (North Sea/Baltic Sea, Eastern Atlantic/Mediterranean, Western Atlantic, Eastern Pacific) locations, *G. vermiculophylla* can affect biogeochemical cycles, macrophytes, higher trophic levels, and commercially important seafood and industry.

Until the mid 2000's, minimal research was conducted on *G. vermiculophylla*, with most published studies coming from its native range in the Western Pacific, and focusing primarily on species identification based on morphology and hybridization testing, environmental effects on growth, and agar production using the macroalga (Figure 2). Since the mid 2000's publications from around the world have dramatically increased, with the highest number of peer-reviewed publications in 2010 (15 studies) and 2012 (18 studies). This lag in research may be attributed to the cryptic nature of *G. vermiculophylla*. For example, within the Virginia coastal bays, routine monitoring of *Gracilaria* biomass commenced in 1998, however, it was not until 3 specimens were genetically analyzed in 2004 that researchers in the region realized they were dealing with the non-native *G. vermiculophylla* rather than its native congener *G. tikvahiae* (Thomsen et al. 2006a).

Overview

We review a total of 93 *G. vermiculophylla* studies, based on 8 main topics discussed in the literature: genetic confirmation of species, environmental tolerance, vectors of dispersal and colonization, ecological effects on marshes and mudflats, ecological consequences on shallow subtidal systems, ecological effects on seagrass communities, effects on commercially important seafood, and industrial applications. We will explain what is currently known about where invasions have occurred, why *G. vermiculophylla* is such a successful invader, and its potential ecological and economic consequences.

Distribution and Spread

Genetic Confirmation of Species

Historically, researchers would use a combination of morphological analyses and hybridization testing to identify *G. vermiculophylla* (Ohmi 1956; Yamamoto 1978; Yamamoto & Sasaki 1988; Terada & Yamamoto 2002), however these techniques are more cumbersome, time consuming, and difficult than genetic analyses (Bellorin et al. 2002; Gurgel & Fredericq 2004). Therefore, most researchers have transitioned to using molecular techniques for *G. vermiculophylla* identification.

Several different genetic markers have been used for *G. vermiculophylla* identification including: SSU rDNA, ITS regions, *rbc*L, *cox*2-*cox*3, Rubisco spacer, and *cox*1. Genetic analyses using these markers have been applied in the Western Pacific (Rueness 2005a, 2005b, 2010; Yang et al. 2007; Skriptsova & Choi 2009; Kim et al. 2010a, 2010b) to confirm the presence of *G. vermiculophylla* in its native range. In addition, introductions have been genetically confirmed in the Eastern Pacific (Bellorin et al. 2004; Saunders 2009; Kim et al. 2010b; Gulbransen et al. 2012), Western Atlantic (Freshwater et al. 2006b; Thomsen et al. 2006a; Hommersand & Freshwater 2009; Saunders 2009; Kim et al. 2010b; Gulbransen et al. 2012; Nettleton et al. 2013), Eastern Atlantic/ Mediterranean Sea (Rueness 2005a, 2005b, 2010; Guillemin et al. 2008; Saunders 2009; Kim et al. 2010b; Sfriso et al. 2010; Weinberger et al. 2010), and the North Sea/ Baltic Sea (Rueness 2005a, 2005b; Kim et al. 2010b).

It is difficult to make direct comparisons across all genetic studies because of the use of multiple markers to identify *G. vermiculophylla* (Saunders 2009). However, recent work is placing greater emphasis on using the *cox*1 gene from mtDNA for both species

identification and analysis of within species, or haplotype, richness and diversity (see inset; Saunders 2005; Robba et al. 2006). The great advantage of the *cox*1 DNA barcode approach is marker standardization, which circumvents the limitations of research compatibility (Hebert et al. 2003). In addition, only a small section of the *cox*1 gene needs to be sequenced (usually the first 650 nucleotides of the 5' end) with one set of primers, and alignment of multiple sequences is easier (Hebert et al. 2003; Robba et al. 2006). The c*ox*1 marker is ideal for determining both inter- and intra-specific diversity in red algae; when sequences of different red algal species are compared there are generally over 30 base pair differences, whereas within species there are often fewer than 11 base pair differences (Saunders 2005; Robba et al. 2006). Base pair differences between samples that are identified as the same species can be used to assess haplotype richness and diversity (Gulbransen et al. 2012).

Presumably, one could use *cox*1 haplotype richness and diversity to assess potential founder effects in introduced locations. However, this approach is currently limited by the lack of *cox*1 data in invaded locations, in particular. It has been proposed that when an introduction occurs, there are likely specific non-native haplotypes that will dominate because of their increased tolerance to environmental stress (Saltonstall 2002). In addition, if multiple introductions occur in one region, founder effects should be reduced and be reflected in the haplotype richness and diversity of the region (Roman 2006). Work published in 2010 comparing worldwide *cox*1 haplotype distribution and diversity has indicated that one haplotype (haplotype 6) dominates the introduced assemblages, with haplotype richness and diversity significantly lower in introduced

populations when compared to native populations (Kim et al. 2010b). However, the sampling design employed in this study was not balanced, with more samples collected in native regions than invaded regions, which could have artificially reduced the haplotype richness and diversity documented in invaded locations (Gulbransen et al. 2012). Work in Virginia has shown that when sampling intensity was increased in an invaded region, detection of less common haplotypes was possible, and documented haplotype richness was therefore higher (Gulbransen et al. 2012). It is possible that if more samples are collected and sequenced in invaded regions, more haplotypes will be discovered (Gulbransen et al. 2012). However, Gulbransen et al. (2012) still saw a predominance of haplotype 6 throughout the region, indicating that this haplotype may have a competitive advantage over other haplotypes.

Limited evidence for this hypothesis can be found in a recent publication, which proposes that *G. vermiculophylla* collected in invaded locations is less palatable to herbivorous snails, from native and invaded locations, than samples collected in the native range (Hammann et al. 2013b). Although the study generally alluded to the importance of genetics and haplotype identity, analysis of the genetic sequences submitted by the authors to GenBank presents some interesting preliminary data. As has been found in prior genetic work, *G. vermiculophylla* collected in invaded locations, that were less palatable for both snail species, can be assigned to haplotype 6. Many of the more palatable samples collected in the native range were assigned to haplotype 2 or 3, which, to date have only been documented in the native range of the alga (Kim et al. 2010b; Gulbransen et al. 2012). In addition, samples from the native range in Donghae,

Korea, that were also unpalatable for both snail species, can be assigned to haplotype 6. It is possible that this haplotype dominates invaded regions because it is resistant to control by herbivores. More work investigating *cox*1 haplotype richness and diversity of *G. vermiculophylla* in both native and invaded locations is warranted to determine if haplotype 6 is the most dominant and invasive form of *G. vermiculophylla*. In addition, future research should determine if haplotype 6 is resistant to a range of herbivores when compared to other haplotypes that are currently only documented in the native range (eg. haplotypes 1-5, 7-12 in Gulbransen et al. 2012).

Environmental Tolerance

G. vermiculophylla, like many invasive species, is highly resistant to both abiotic and biotic stresses. It has been suggested that *G. vermiculophylla* outcompetes native macroalgae under persistent eutrophic conditions, tolerates changes and reductions in nutrient availability (Thomsen & McGlathery 2007; Nejrup & Pedersen 2010; Jensen et al. 2011; Sfriso et al. 2012) and can take up multiple forms of nitrogen, including urea, amino acids, ammonium, and nitrate (Tyler et al. 2005; Tyler & McGlathery 2006; Abreu et al. 2011a).

In addition to nutrient tolerance, *G. vermiculophylla* can withstand changes in salinity and temperature, desiccation, light levels, sedimentation, and burial when compared to native species (Thomsen & McGlathery 2007; Kim et al. 2012b). Studies in native and invaded locations have found that *G. vermiculophylla* has a high tolerance for salinities ranging from 5 to 60 ppt, with the optimal salinity for maximum growth between 15 and 30 ppt (Yokoya et al. 1999; Raikar et al. 2001; Rueness 2005b; Thomsen

et al. 2007; Jensen et al. 2011; Kim et al. 2012b; Nejrup & Pedersen 2012). Salinities below 5 ppt can reduce photosynthesis rates that do not recover to normal rates even after the macroalga is returned to a favorable 15 ppt salinity (Nejrup & Pedersen 2012). *G. vermiculophylla* can also grow at a range of temperatures between 5 and 30 °C, with maximum growth generally occurring between 20 and 25 °C (Yokoya et al. 1999; Raikar et al. 2001; Phooprong et al. 2008; Kim et al. 2012b; Nejrup et al. 2013). Spore germination occurs most readily at 20 °C and is limited at 5 °C, with no spore survival at the low temperature (Abreu et al. 2011b). Temperatures above 30 °C are associated with reductions in growth and photosynthesis, with thalli damage at 35 °C and death at 37 °C (Raikar et al. 2001; Phooprong et al. 2008).

G. vermiculophylla is well adapted to both low and high light availability. Tolerance to reductions in both light and temperature were demonstrated in one study that found that the macroalga could be kept in the dark at 8 °C for 175 days and still grow when placed back in seawater at 11.5 °C (Nyberg & Wallentinus 2009). Other studies have shown that the macroalga can survive with light intensities as low as 1 µmol photons m^{-2} s^{-1} (Nejrup et al. 2013) and can reach maximum growth rates at 40 µmol photons m^{-2} s^{-1} when levels between 0 and 163 µmol photons m^{-2} s^{-1} were tested (Jensen et al. 2011). Similar laboratory studies found maximum growth rates at the highest irradiances tested, 80-100 µmol photons m^{-2} s^{-1} (Yokoya et al. 1999; Raikar et al. 2001). *In situ* light intensities that the macroalga tolerates can be as high as 1600 µmol photons m^{-2} s^{-1} and compounds such as mycosporine-like amino acids and antioxidants can protect *G. vermiculophylla* from exposure to high light levels and ultraviolet wavelengths

(Yakovleva 2008; Roleda et al. 2012). In addition, it has been proposed that burial under sediment could be an adaptation to ultraviolet light exposure (Roleda et al. 2012), as the macroalga can be buried for over a week while still maintaining healthy tissue capable of photosynthesis (Thomsen & McGlathery 2007). This resistance to burial stress is advantageous for invasions in lagoons and estuaries where sediment redistribution can easily occur (Thomsen & McGlathery 2007).

While most studies report that herbivores avoid eating *G. vermiculophylla*, there is some evidence to indicate that the gastropod *Littorina littorea* may be able to consume the macroalgae under ideal laboratory conditions (Thomsen et al. 2007). However, it is possible that *in situ* these invertebrates would choose to eat more palatable algae (Thomsen et al. 2007). Other studies have shown that secondary metabolites within *G. vermiculophylla* tissue (Nylund et al. 2011; Rempt et al. 2012) make the macroalga unplatable to herbivores who often choose to consume native macroalgae (Thomsen & McGlathery 2007; Nejrup & Pedersen 2010; Jensen et al. 2011; Nejrup et al. 2012). Based on these studies it is unlikely that herbivores would be able to control the spread of *G. vermiculophylla* in the regions it invades. In fact, one study found that the mudsnail *Ilyanassa obsoleta* can facilitate *G. vermiculophylla* growth by providing nitrogen to fuel algal growth (Thomsen & McGlathery 2007).

Because of its adaptability to numerous environmental conditions and stresses, *G. vermiculophylla* is often the dominant macroalga in both native (Yamamoto 1978) and invaded (Thomsen 2004b; Freshwater et al. 2006a) locations. However, *G. vermiculophylla* is not successful under all conditions; highlighting its vulnerabilities to

environmental stresses may be advantageous for both researchers and managers in invaded locations. *G. vermiculophylla* tends to dominate protected estuarine environments, where tidal currents and wave energy are minimal; it does not appear to do well on rocky coasts (Rueness 2005b; Thomsen et al. 2007). Temperatures below 5 °C and salinities below 5 ppt can reduce *G. vermiculophylla* biomass and limit sexual reproduction (Abreu et al. 2011b; Nejrup & Pedersen 2012). *G. vermiculophylla* is typically found at shallow depths and rarely forms dense mats below 2 to 3 m depth because of light limitation (Thomsen et al. 2007; Weinberger et al. 2008). In fact, *G. vermiculophylla* biomass placed deeper than 3 m depth typically loses biomass, with samples placed at 5 m depth loosing 84% of their biomass within one year (Weinberger et al. 2008). Anchoring to the substrate is important for both sexually produced spores that attach to hard substrate and for asexual fragments that can be partially buried in the sediment or attached to tube worms (Thomsen et al. 2007). In Virginia, where attachment to tube worms is more common than attachment to shellfish or drifting mats of algae (Thomsen & McGlathery 2005), mats of *G. vermiculophylla* are not found on mudflats that do not have tube worms (pers. obs.).

Vectors of Dispersal and Colonization

Vectors of dispersal can be separated into long-distance vectors, which are likely to introduce *G. vermiculophylla* into a new region, and short-distance vectors, which will transport the algae within an invaded system. Most studies have suggested that the predominant long distance vector for *G. vermiculophylla* introductions to new regions is through shellfish importation and farming (Mollet et al. 1998; Rueness 2005a; Thomsen

et al. 2006a, 2007; Thomsen & McGlathery 2007; Nyberg et al. 2009; Sfriso et al. 2010, 2012; Jensen et al. 2011; Gulbransen et al. 2012). When spores are produced by the macroalga (Weinberger et al. 2008; Xie et al. 2010), attachment to the surface of live and dead bivalve shells, cockles and snails is possible (Thomsen 2004a; Thomsen et al. 2007; Abreu et al. 2011b). Once attached, *G. vermiculophylla* can be transported with introduced and traded shellfish. For example, researchers in the mid-Atlantic hypothesize that *G. vermiculophylla* was introduced to the region attached to the non-native Pacific oyster, *Crassostrea gigas* (Thomsen et al. 2006a; Gulbransen et al. 2012). Oysters have been introduced to virtually all regions where *G. vermiculophylla* has been found and it is possible that there are cryptic populations of the macroalga in regions where oyster introductions have occurred, but genetic testing of *Gracilaria* spp. has not been conducted. Additional barcoding in regions where oyster trade is common, especially if the oysters come from areas where *G. vermiculophylla* presence has been confirmed, is needed in order to test this theory.

Because *G. vermiculophylla* can survive for long periods of time in dark, dry, and low-temperature conditions, long-distance transport via ballast water is possible (Nyberg & Wallentinus 2009). Ballast water transport has been proposed as the most likely mode of long distance introduction in the Baltic region, where shellfish aquaculture is uncommon (Weinberger et al. 2008). Long distance transport on boat hulls would rely on *G. vermiculophylla* forming spores, a process that doesn't necessarily occur in all systems (Weinberger et al. 2008; Xie et al. 2010). We are not aware of any studies that have documented *G. vermiculophylla* being attached to ship hulls or found in ballast water

(Thomsen et al. 2007), and more work on this topic is warranted, especially in the Baltic region.

Asexual reproduction via fragmentation can be a common vector of short-distance dispersal within an invaded location (Thomsen 2004a, 2004b; Thomsen & McGlathery 2005; Thomsen et al. 2007, 2009; Weinberger et al. 2008). Fragments can come from boat propellers and fishing gear or from breakage during attachment to tube worms (Thomsen & McGlathery 2005; Thomsen et al. 2007). Sexual reproduction and spore formation are required for holdfast generation and attachment to hard substrate; therefore, two methods of attachment for asexual fragments are partial burial in the sediment (which can be facilitated by lugworms) or attachment to tube worms on mudflats (Thomsen et al. 2007). Tube decorating worms such as *Diopatra cuprea* and *D. neopolitana* often attach floating fragments of *G. vermiculophylla* to their mucus-based tube caps which protrude out of the sediment (Thomsen 2004a; Thomsen & McGlathery 2005; Thomsen et al. 2007; Abreu et al. 2011b; Berke 2012; Byers et al. 2012). This association with worm tube caps provides a stable attachment to intertidal flats that can remain on the order of months (Gulbransen & McGlathery 2013). In some regions, this type of attachment is more common than attachment to oyster shells or drifting mats of algae (Thomsen & McGlathery 2005).

Ecological Effects

Because *G. vermiculophylla* is a cryptic invader, it typically becomes established in a region before it is genetically identified, at which time it is difficult to eradicate.

Therefore, it is important that researchers and managers understand how this invasion can affect intertidal, subtidal, and seagrass dominated systems.

Intertidal Marshes and Mudflats

Mudflat populations of *G. vermiculophylla* can affect biogeochemical reactions, invertebrate densities and the behavior of higher trophic groups. Enrichment of *G. vermiculophylla* mats with a ^{15}N tracer has shown that nitrogen from the macroalgae can be transferred to both sediments and invertebrates in the mudflat community (Gulbransen & McGlathery 2013). The mechanism for this transfer is likely the release of nitrogen during both active growth and decomposition (Tyler & McGlathery 2006), which can then be incorporated into benthic microalgae on the sediment surface (Gulbransen & McGlathery 2013). Consumption of labeled *G. vermiculophylla* or benthic microalgae by invertebrates on the mudflat demonstrates that the macroalga plays an integral role in nutrient transfers among trophic groups in the system (Gulbransen & McGlathery 2013). In addition, recent work on intertidal mudflats has shown that, at moderate densities, *G. vermiculophylla* can increase oxic-anoxic hotspots of coupled nitrification-denitrification and potentially aid in the removal of reactive nitrogen from the system (Gulbransen et al. in review L&O). Mudflat populations of *G. vermiculophylla* can be extremely productive and may also affect detrital food when this biomass decomposes (Byers et al. 2012).

Invertebrate densities on mudflats are typically enhanced by *G. vermiculophylla* presence, as the macroalga provides a novel habitat for invertebrates to live in compared to bare substrate (Byers et al. 2012; Johnston & Lipcius 2012; Gulbransen et al. in review Biological Invasions). In addition, mats of the algae in its native range are often

associated with increased amphipod abundance that can in turn affect benthic microalgae densities on which amphipods graze (Aikins & Kikuchi 2002). Although the mats are associated with high food availability for migratory shorebirds (amphipods, small snails, worms), the shorebirds tend to avoid mudflats with mats of *G. vermiculophylla*, and tend to forage at bare locations (Gulbransen et al. in review Biological Invasions).

Mats of *G. vermiculophylla* from subtidal communities and intertidal mudflats can be important source populations of algae for nearby marshes (Thomsen et al. 2009). These marsh populations of *G. vermiculophylla* can mediate nitrogen transfers to marsh sediments and cordgrass (Gulbransen & McGlathery 2013). While not yet experimentally tested, it is possible that this transfer of nitrogen could fuel nitrogen-limited cordgrass growth on the marsh. More work is needed to investigate potential distribution and effects of *G. vermiculophylla* on both marsh and mudflat communities.

Shallow Subtidal Communities

G. vermiculophylla in subtidal communities can be attached to hard substrate or tube worms and remain in place, travel around in small clumps as bedload, or drift as large floating mats. Regardless of its mode of attachment or travel, *G. vermiculophylla* can release up to 67% of the its gross daily nitrogen uptake back into the water column (Tyler & McGlathery 2006). Clumps of *G. vermiculophylla* that drift along the benthic surface as bedload, due to its negative buoyancy, can increase benthic sediment suspension (Lawson et al. 2012). Desorption of nutrients from this resuspended sediment can elevate nutrient levels in the water column (Lawson et al. 2012). In addition, although *G. vermiculophylla* can quickly absorb available carbon and nitrogen from the

water column, only 6 to 50% of the nitrogen and 2 to 9% of the carbon are incorporated into the sediments for long term storage when the macroalga decomposes; the remainder of the nutrients are released to the water column (Hardison et al. 2010). Bacteria and benthic microalgae in the system are important for the retention of the macroalgal nutrients that are incorporated into the sediments (Hardison et al. 2010). Dissolved organic matter released by decomposing *G. vermiculophylla* is absorbed by heterotrophic bacteria and benthic microalgae (Hardison et al. 2010). In turn, mineralized carbon and nitrogen that are released by the heterotrophic bacteria can be retained in the system via benthic microalgal or bacterial absorption (Hardison et al. 2010).

As is true on intertidal mudflats, *G. vermiculophylla* increases habitat availability for both algae and invertebrates when attached or drifting (Thomsen et al. 2006b, 2010; Weinberger et al. 2008; Nyberg et al. 2009). While populations in the Western Atlantic have been shown to increase native filamentous macroalgae by providing additional habitat (Thomsen et al. 2006b), the relationship between *G. vermiculophylla* and native *Fucus vesiculosus* in the Baltic region is less positive. There, *G. vermiculophylla* competes with *F. vesiculosus* for limiting nutrients and enhances grazer densities that prefer to eat *F. vesiculosus* rather than *G. vermiculophylla* (Weinberger et al. 2008; Hammann et al. 2013a).

Seagrass Communities

Studies looking at the interaction of macroalgal mats and seagrass beds typically report reductions in seagrass beds due to increases in toxic compounds and reductions in light levels as a result of macroalgal presence (Hauxwell et al. 2001). Recent studies

investigating *G. vermiculophylla* and seagrass interactions have addressed the combined impact of rising sea surface temperatures and increases in macroalgal biomass (Martínez-Lüscher & Holmer 2010; Höffle et al. 2011). These studies have found that declines in seagrass beds that are already stressed by higher temperatures (27-30 °C), can be increased when *G. vermiculophylla* is present in large quantities (~2-4 kg WW m^{-2}; Martínez-Lüscher & Holmer 2010; Höffle et al. 2011). Lower oxygen availability and increased levels of sulfide are likely the primary causes of the reduced seagrass metabolism and survival (Martínez-Lüscher & Holmer 2010; Höffle et al. 2011).

Stress imposed by *G. vermiculophylla* mats and rising water temperatures on seagrass beds can cause systems to transition from seagrass-dominated states to macroalgal-dominated states, which can have noteworthy effects on system productivity. Cacabelos et al. (2012) used CO_2 and O_2 fluxes to estimate metabolism in seagrass-dominated, mixed-seagrass and *G. vermiculophylla*, and *G. vermiculophylla* dominated systems. They found higher overall production in the *G. vermiculophylla* dominated system when compared to the other two states (Cacabelos et al. 2012). However, when production measurements were normalized in order to get a measure of ecosystem efficiency relative to overall macrophytes biomass, the seagrass-dominated system was the most efficient (Cacabelos et al. 2012).

Although dense macroalgal mats can be associated with reductions in seagrass biomass and ecosystem efficiency, moderate densities (0.1-0.4 kg WW m^{-2}) of *G. vermiculophylla* can increase habitat availability for bivalves, gastropods, and crustaceans and thus increase their densities (Thomsen 2010). In addition, green turtles

have been shown to consume more *G. vermiculophylla* than any other macrophyte, which may indicate that the alga plays an important role in sea turtle ecology (Talavera-Saenz et al. 2007).

Effects on Commercially Important Seafood and Industry

In addition to understanding the ecological effects of *G. vermiculophylla* introductions, it is important that research provide a metric for economic consequences. Potential costs and benefits to both commercially important seafood and industry applications can be used as metrics for these economic consequences.

Seafood

G. vermiculophylla has a tendency to foul fishing nets and at high biomasses (2.7 kg WW m^{-2}) it can result in reduced recruitment of the commercially important oyster *Crassostrea virginica,* when compared to areas without the macroalga (Freshwater et al. 2006a, 2006b; Thomsen & McGlathery 2006). However, researchers in the mid-Atlantic have also found that mats of *G. vermiculophylla* could be alternative habitats for juvenile blue crabs (Falls 2008; Mahalak 2008; Johnston & Lipcius 2012) and scallops (Hernández Cordero et al. 2012), both of which are commercially important in the region. Using *G. vermiculophylla* as an alternative habitat for commercially harvested seafood may not always be advisable. The macroalga is a reservoir for pathogenic species of *Vibrio* bacteria (*V. parahaemolyticus* and *V. vulnificus*) that can cause gasteroentritis, necrotizing wound infections, septicemia (blood poisoning), and death in at-risk individuals (Gulbransen et al. in review AEM). In addition, *G. vermiculophylla* presence near oyster reefs is associated with a concomitant increase in the two human pathogens in

oyster tissue (Gulbransen et al. in review AEM). More research is needed to determine if scallops and blue crabs found within *G. vermiculophylla* mats are also associated with higher pathogen concentrations.

In the Western Pacific (native) range of *G. vermiculophylla* it has been shown that the macroalga can be used to increase prawn growth (Tahara & Yano 2001). In addition, the direct consumption of *G. vermiculophylla* as a prepared dish called 'ogonori' is also common in Asia and can be a good source of dietary antioxidants (Terasaki et al. 2012). However, the alga should be eaten with caution as prostaglandins within the tissue have been associated with ogonori poisoning in humans (see inset; Fusetani & Hashimoto 1984). Prostaglandins are important messenger molecules, and when purified in the laboratory, they can be used to produce pharmaceuticals (Illijas et al. 2008; Kanamoto et al. 2011; Imbs et al. 2012; Varvas et al. 2013). *G. vermiculophylla* tissue can also be used to produce anti-obesity medication (Kim et al. 2012a).

Industrial Applications

G. vermiculophylla can be a good source of high quality food agar, a substance that is used to make many products in the scientific, food science, and cosmetic industries. In addition, agar from *G. vermiculophylla* can be used to make a fruit and vegetable coating, that has been shown to prolong shelf-life of produce (Sousa et al. 2010b). Therefore, many studies have looked at the best methods for extraction of agar from the macroalga as well as the properties of the resulting agar (Mollet et al. 1998; Arvizu-Higuera et al. 2008; Orduña-Rojas et al. 2008a, 2008b; Vergara-Rodarte et al. 2010; Villanueva et al. 2010; Souza et al. 2012). *G. vermiculophylla* grown in culture

produces a stronger, higher quality agar when compared to agar produced from field-collected samples (Sousa et al. 2010a; Abreu et al. 2011c). Because *G. vermiculophylla* is also efficient at absorbing excess nutrients from the water column, it is a good candidate for coupling nutrient removal in Integrated Multi-trophic Aquaculture (IMTA) systems with harvest of excess macroalgae for agar production (Sousa et al. 2010a; Abreu et al. 2011a, 2011c; Skriptsova & Miroshnikova 2011). *G. vermiculophylla* has also been proposed as a macroalga whose natural stocks could be removed from estuaries as a method to mitigate eutrophication (Abreu et al. 2011a). Excess *G. vermiculophylla* biomass could be used as a substrate for biochemical methane production to produce energy, although *Ulva* spp. may be a more efficient substrate (Costa et al. 2012).

Future Research Needs

Current knowledge of the distribution of *G. vermiculophylla* worldwide is still limited by lack of genetic barcoding data. Therefore, additional genetic testing of *Gracilaria* spp. in locations were shellfish introductions and trade are common will likely find new *G. vermiculophylla* introductions, as these are primary vectors of invasion. In addition, future barcoding studies would benefit from using the *cox*1 marker to enable comparisons to the currently documented haplotype distribution. More interdisciplinary studies combining genetic haplotype analysis with environmental tolerance traits would help to determine if haplotype 6 can be considered a super-invasive strain. Knowledge of ecological effects in both intertidal and subtidal systems is still rather limited across a range of geographical regions. More information is still needed to determine how nitrogen transferred from *G. vermiculophylla* to intertidal communities directly affects

other primary producers and consumers. In addition, a better understanding of how *G. vermiculophylla* presence affects carbon sequestration on marshes, mudflats, oyster reefs, and seagrass beds is still needed. While work in Virginia has shown potential trophic effects on migratory shorebirds (Gulbransen and McGlathery in review, Biol. Inv.), there are still many consumers within invaded ecosystems that have not yet been addressed. While it is interesting that *G. vermiculophylla* may present a novel habitat for commercially important seafood, more research on how the macroalga may be affecting concentrations of pathogenic bacteria is still needed.

G. vermiculophylla is a cryptic invader with high tolerance to environmental stresses and is therefore able to invade temperate estuaries and become established before local researchers and managers notice its presence. Because of this, it is imperative that we understand potential ecological consequences of these introductions and find novel ways of using excess algal biomass as some have for agar creation, energy production, and/or nutrient removal (*in situ* and in IMTA systems).

Figures

Figure 7.1. Locations where *G. vermiculophylla* has been genetically confirmed (hollow triangles) and where non-genetic studies have been conducted (solid squares) in the 5 geographic regions discussed in this review (Eastern Pacific, Western Atlantic, North Sea/Baltic Sea, Eastern Atlantic/Mediterranean, Western Pacific).

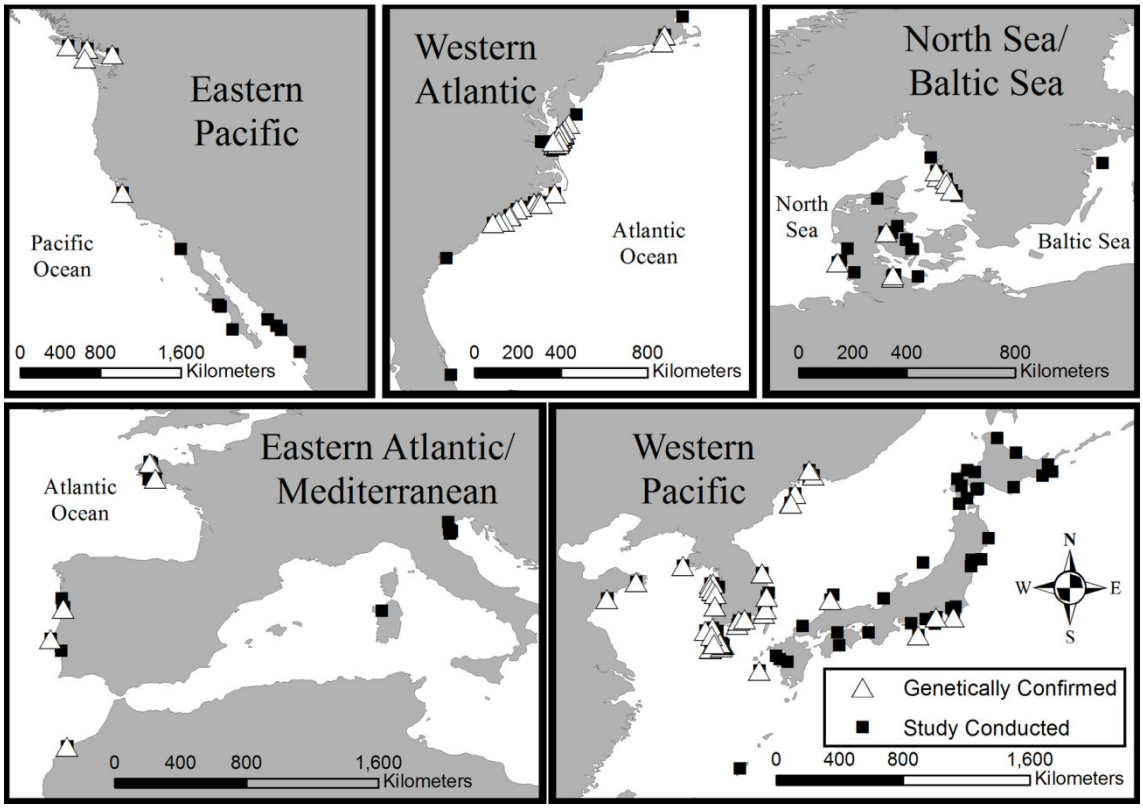
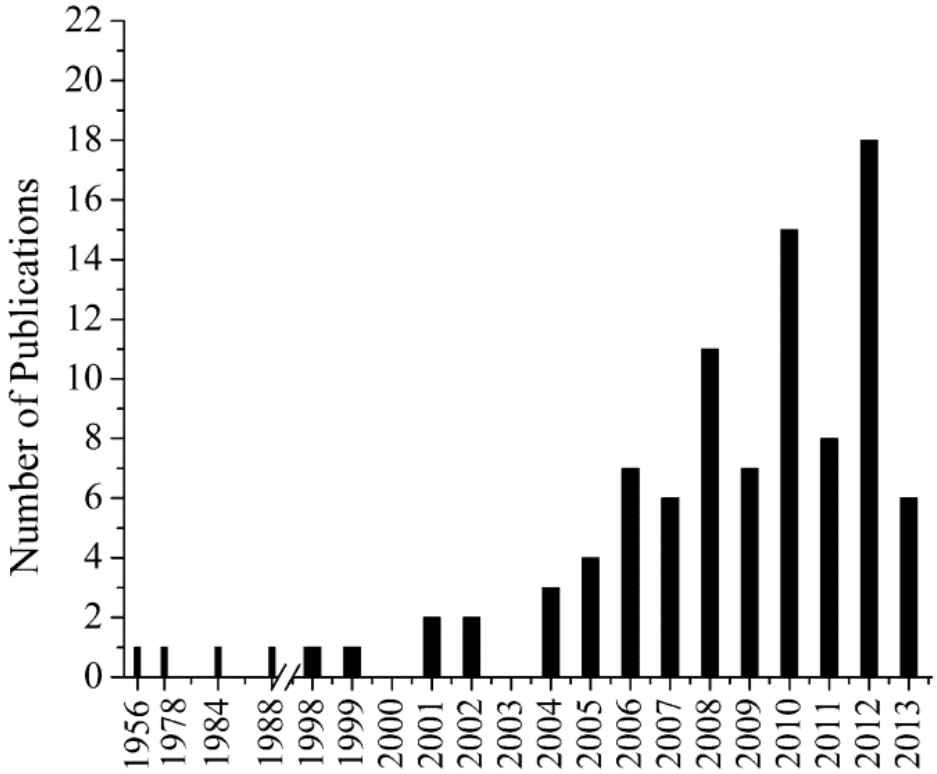


Figure 7.2. Number of worldwide publications from 1978 to March 2013.



Definition List (inset)

Invasive Species

A non-native species that proliferates in an introduced region and maintains a self-sustaining population there.

Cryptic Invader

A non-native species that cannot be distinguished from native congeners based on morphology alone. Typically hybridization testing and/or genetic analyses are necessary to identify cryptic species. Therefore, cryptic invaders often establish self-sustaining populations in invaded ecosystems before researchers and managers identify their presence.

Haplotype Diversity

Measure of within species diversity base on variation in sequences of barcoding DNA used for species identification (for example the *cox*1 region on mtDNA).

Ogonori poisoning

Food poisoning that can occur after consuming *Gracilaria* spp. in a dish called ‘ogonori’ which often involves pickling the algae in lime or vinegar. Initial symptoms include nausea, vomiting, and diarrhea and can progress to low blood pressure, unconsciousness, and death in serious cases.

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