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RESEARCH ARTICLE

Soil microbial communities in restored and unrestored coastal dune ecosystems in California

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Most restoration projects involving invasive plant eradication tend to focus on plant removal with little consideration given to how these invasives change soil microbial communities. However, soil microorganisms can determine invasibility of habitats and, in turn, be altered by invasives once established, potentially inhibiting native plant establishment. We studied soil microbial communities in coastal dunes with varying invasion intensity and different restoration approaches (herbicide, mechanical excavation) at Point Reyes National Seashore. Overall, we found evidence of a strong link between bacterial and fungal soil communities and the presence of invasives and restoration approach. Heavily invaded sites were characterized by a lower abundance of putatively identified nitrifiers, fermentative bacteria, fungal parasites, and fungal dung saprotrophs and a higher abundance of cellulolytic bacteria and a class of arbuscular mycorrhizal fungi (Archaeosporomycetes). Changes in soil microbiota did not fully dissipate following removal of invasives using herbicide, with exception of reductions in cellulolytic bacteria and Archaeosporomycetes abundance. Mechanical restoration effectively removed both invasives and soil legacy effects by inverting or "flipping" rhizome-contaminated surface soils with soils from below and may have inadvertently induced other adverse effects on soils that impeded reestablishment of native dune plants. Land managers should consider additional measures to counteract lingering legacy effects and/or focus restoration efforts in areas where legacy effects are less pronounced.

Key words: dune, herbicide, invasive plant, microbial, restoration, soil

Implications for Practice

- Even when invasive plant species are successfully removed, soil microbial legacy effects may persist that could derail efforts to reestablish functioning, intact, and resilient native dune ecosystems.
- Legacy effects may impede efforts to reestablish native dune plant species and/or promote either re-invasion by primary target species or invasion by new weeds or "secondary" invaders better adapted to changed soil conditions.
- Project managers may need to consider additional restoration measures (e.g. iceplant duff removal, soil amelioration) to ensure the success of restoration efforts or focus restoration efforts on low-risk, high-yield activities such as invasives removal in sparsely to moderately invaded areas or in foredune areas, where sand overwash can counteract beachgrass legacy effects.

Introduction

Invasive plants pose one of the largest threats to coastal ecosystems, along with development and climate change (Pickart & Sawyer 1998). Two of the most problematic invasive species within coastal dunes are European beachgrass (*Ammophila arenaria*) and iceplant (*Carpobrotus* spp.), which are native to northern Europe and South Africa, respectively, but have been introduced to southern Europe, South Africa, Australia/New Zealand, and North America (Weber 2003; van der Putten et al. 2005). While the spread of invasive plants in some coastal ecosystems often builds slowly over time due to accidental introductions, these two species were in many instances deliberately planted to stabilize coastal areas and protect roads, railroads, and other infrastructure or land uses. This gives these invasives a tremendous jump-start in wholescale conversion of previously diverse native ecosystems into monotypic, dense stands of European beachgrass and iceplant (Wiedemann & Pickart 1996, 2008). These invasive plants not only displace, outcompete, and directly reduce native plant species diversity, but can induce indirect effects on native plant communities by altering soils and insect-plant relationships and providing habitat for predators of both plants and animals (Barbour et al. 1976; Vilà et al. 2006; Conser & Connor 2009). Through these

direct and indirect impacts, they also threaten the wildlife and rare plants that depend on intact ecosystems for persistence (Dangremond et al. 2010; Muir & Colwell 2010).

Plant–soil feedbacks are important for aboveground community composition (Wardle et al. 2004; van der Heijden et al. 2008). In particular, soil microbial communities might facilitate or inhibit the establishment and success of invasive plant species, as well as native plant ones (Wolfe & Klironomos 2005; Inderjit & van der Putten 2010; Kardol & Wardle 2010). Among the ecosystems where plant–soil feedbacks have been of interest are coastal dunes. Many studies have focused on understanding factors enabling certain dune species such as European beachgrass and iceplant to be more invasive in terms of prevalence and persistence in their introduced ranges than in their native ranges. In general, results from these studies suggest that factors potentially differ depending on geographic region. For example, in some areas, European beachgrass and iceplant appear to flourish when they are either completely or partially released from their natural enemies that inhibit expansion and persistence of these species in their "home" range (Beckstead & Parker 2003; Knevel et al. 2004; Van Grunsven et al. 2009). Another California study argued that, rather than being wholly or partially released from its enemies, European beachgrass accumulates local pathogens and that it can tolerate higher pathogen levels than native plants, giving beachgrass a competitive advantage (Eppinga et al. 2006). Invasives may also be able to spread because they disrupt native species' relationships with symbiotic mutualists or because they are able to form stronger symbiotic relationships than native species (Pringle et al. 2009; Inderjit & van der Putten 2010). For example, germination of European beachgrass in iceplant-invaded soils resulted in lower aboveground biomass of beachgrass than in non-iceplant-invaded soils, which may relate to suppression of beneficial mycorrhizal interactions by iceplant (de la Peña et al. 2010).

However, it has been argued that introduced species are not always successful in invading native communities, because strong interaction between soil biota and native species can hinder their establishment and spread (Knevel et al. 2004; Suding et al. 2013). Along these lines, a study on South African dunes found that one species, *Sporobolus virginicus*, might actually contribute to soil feedback resistance against European beachgrass and concluded that not only plant species diversity, but also plant community composition, might be important in determining the potential for biotic resistance (Knevel et al. 2004). In a meta-analysis of native–exotic feedback comparisons, researchers concluded that soils conditioned by native species can resist invaders when invader frequency is low, even if the invader exerts a positive plant–soil feedback, but that this resistance can be negated by disturbance or other events that enable invaders to spread quickly or establish in high numbers, particularly when non-native species have dispersal or competitive advantages over native ones (Suding et al. 2013). Experiments suggested that the soil community in some Mediterranean dune systems may initially resist iceplant, but the resistance breaks down over time, with an increase in abundance of a symbiotic

fungal species (chytrids) eventually being detrimental to native plant species (Vilà et al. 2006; de la Peña et al. 2010).

Once established within dune systems, invasive non-native species have developed a number of mechanisms that enable them to rapidly spread and displace native counterparts. These mechanisms include physical, chemical, and biological "legacy" effects. European beachgrass leaves a lasting physical legacy on foredune systems by capturing sands and creating larger, taller foredune systems than would have existed naturally (Wiedemann & Pickart 1996; Corbin & D'Antonio 2012). Iceplant exerts a very well-documented physiochemical influence on invaded dunes by reducing soil pH and available water and increasing organic matter, nitrogen, carbon, and sodium levels (D'Antonio & Mahall 1991; Conser & Connor 2009; Novoa et al. 2014). Iceplant leaves have high levels of tannin and antibacterial compounds (van der Watt & Pretorius 2001) that may reduce litter decomposition rates (Vilà et al. 2006) and acidify soils, thereby reducing availability of nitrate, calcium, and magnesium (Conser & Connor 2009). Physiochemical legacy effects can later lead to further changes in the soil microbial communities that may reinforce the competitive advantage for invasive species. A study in Spain documented that soil microbial communities changed substantially with invasion, which they attributed to iceplant creating higher levels of microbial carbon sources that resulted in an increase in total microbial biomass, a decrease in the bacteria/fungi ratio, and a shift in microbial species composition (Badalamenti et al. 2016). Once invaded, these persistent legacy effects can have tremendous ramifications on dunes and the potential for restoring them in the future.

In this study, we assessed soil microbial communities by sequencing environmental DNA (Breed et al. 2019) in a large-scale coastal dune restoration program that Point Reyes National Seashore (PRNS; California, U.S.A.) has been conducting since 2001 to eliminate European beachgrass and iceplant. These species were believed to have been planted in PRNS in the early to mid-1900s by ranchers and other residents trying to prevent coastal dune sands from migrating into adjacent pastures and ranch facilities and on to roads. By 2009, more than 60% of the park's roughly 890 ha of coastal dune, bluff, and scrub habitat was estimated to be dominated by European beachgrass and iceplant, with beachgrass being more prevalent in areas where most of the restoration has been conducted to date (National Park Service 2009).

We hypothesize that differences in soil microbiota exist between areas invaded by European beachgrass and uninvaded areas, and these changes may promote persistence and expansion of invaders while suppressing that of native plant species. We also hypothesize that the approach used for removing European beachgrass (mechanical removal, herbicide treatment) may have differential effects on soil microbial communities, which may thereby affect the potential for re-establishing a native flora, as well as the potential for being re-invaded by either beachgrass or other weeds. There has been little study about the effect of mechanical removal on soil microbial communities, but research has suggested that herbicides might alter soil biota

due to persistence of chemicals in soils and the fact that herbicides such as glyphosate can affect microbial, as well as plant, metabolic pathways (Issa 1999; Durkin 2011).

The availability in PRNS of areas with varying invasion intensity, as well as of restored and unrestored invaded habitats, offers a valuable opportunity to evaluate impacts of invasion and restoration approach on soil microbial communities in coastal dune ecosystems. Understanding these mechanisms would enable land managers to optimize site selection and approaches for habitat restoration.

Methods

Study Area

PRNS is a unit of the National Park Service and is located approximately 48 km north of San Francisco, California, U.S.A. (Fig. 1A). The climate is Mediterranean, with hot/dry summers and cool/wet winters. Coastal areas in California are generally cooler and have less intra-annual variability in temperature than inland regions, with fog and winds being important climatic influences. Annual temperature is estimated to average 12.1°C, and precipitation is estimated to average 45–52 cm per year. Soil types in dune areas consist of hummocks/mounds and hills of loose sand blown from nearby beaches with no profile development (Soil Conservation Service 1985). Coastal dune sands tend to be nutrient limited, especially for the essential nutrients nitrogen, phosphorus, and potassium (Barbour et al. 1985). PRNS's dunes provide habitat for up to 11 species listed by the U.S. Fish and Wildlife Service as endangered or threatened.

Between 2001 and 2014, approximately 200 gross hectares of coastal dunes have been restored using a combination of manual removal (e.g. hand pulling or small hand tools), mechanical removal, and chemical treatment. Mechanical removal involves use of excavators and bulldozers to invert or "flip" the surface 1–1.5 m of rhizome-contaminated soils with less-contaminated soils 2–3 m below the soil surface. Initial chemical treatment and subsequent re-treatment are performed annually in the late summer with herbicide backpacks and directed spraying using a single nozzle wand of a mixture of 1.5% imazapyr, 2% glyphosate, a modified vegetable oil surfactant, and blue dye onto target invasive plants.

The park's restoration efforts have focused on invaded areas that would have the most benefit for critical listed species. While invasive plants often account for more than 90% of the vegetation in these targeted restoration areas, some retain vestiges of native dune mat or dune scrub habitat. These "islands" of native dune mat habitat have been surrounded by dense invasive stands for decades, yet retain the ability to resist encroachment despite patchy colonization by iceplant. Native dune refugia often support sizeable rare plant populations, as well as a diverse native plant community, and range in size from 1 to 20.8 ha.

Selection of Sites for Soil Sampling

The sampled areas included Abbotts Lagoon (122°57′33.46″W, 38°6′36.272″N), AT&T/North Beach (122°57′51.737″W, 38°5′47.147″N), and B Ranch North (123°0′0.289″W, 38°1′12.915″N; Fig. 1A). Soil sampling in native dune and unrestored invaded areas occurred along a continuum of invasion, from lightly invaded (native dune "islands" with <5% cover of invasives, primarily iceplant) to moderately invaded (approximately 25–50% cover of European beachgrass, iceplant, or secondary invaders such as European searocket/*Cakile maritima*) to heavily invaded (>75% cover of invasives, primarily beachgrass) (Fig. 1B–D).

We also sampled areas restored by either herbicide treatment or mechanical removal, ensuring equal sampling effort between foredunes and backdunes, as backdunes represent older, more stabilized dunes with a different vegetation community (Miller 2015) that could, therefore, have different soil chemistry and belowground biota. Chemical treatment of European beachgrass and, to a lesser degree, iceplant has been performed at all three areas, but mechanical removal was only conducted at Abbotts Lagoon in 2011 due to high cost per acre and indirect impacts from sand remobilization on adjacent native habitats and ranchlands. Due to these issues, PRNS switched restoration approach primarily to herbicide treatment following that project, with initial herbicide treatment occurring in 2011–2014 (Abbotts), 2014 (B Ranch North), and 2016 (AT&T/North Beach). Soil sampling represented a range in terms of restoration age: mechanical removal (6 years) and herbicide treatment (1–6 years).

Soil Sampling Methodology

In general, two to three subsampling areas were selected within each of these sampling categories (invasion intensity/restoration approach) at all areas (Table 1). At each soil sampling location, four surface soil cores (approximately 7 cm depth) were collected in the peak growing season (26 April, 2017) that varied in proximity to plants, although areas directly beneath plants with dense roots were avoided. Composite soil samples were homogenized, sieved to 4 mm to remove roots and stones, placed in plastic bags, and frozen in the field until samples could be transported on ice at −20°C to the laboratory for storage at −80°C until analysis.

Molecular Analyses

We used DNA metabarcoding coupled with high-throughput sequencing to describe the soil microbial communities based on taxonomic markers. Genomic DNA was extracted using the QIAGEN DNeasy PowerLyzer PowerSoil kit. For bacteria and archaea, we sequenced the V4 hypervariable region of the 16S rRNA, using the 515-F (GTGCCAGCMGCCGCG GTAA) and 806-R (GGACTACHVGGGTWTCTAAT) primer pair, and for fungi, we sequenced the first internal transcribed spacer (ITS1) region of the rRNA operon, using the ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCT-GCGTTCTTCATCGATGC) primer pair (Barberán et al. 2015). Primers included Illumina adapters and unique error-correcting 12-bp barcodes. PCR products were quantified and pooled in equimolar concentrations for sequencing on an Illumina MiSeq at the University of Arizona (Tucson, AZ, U.S.A.).
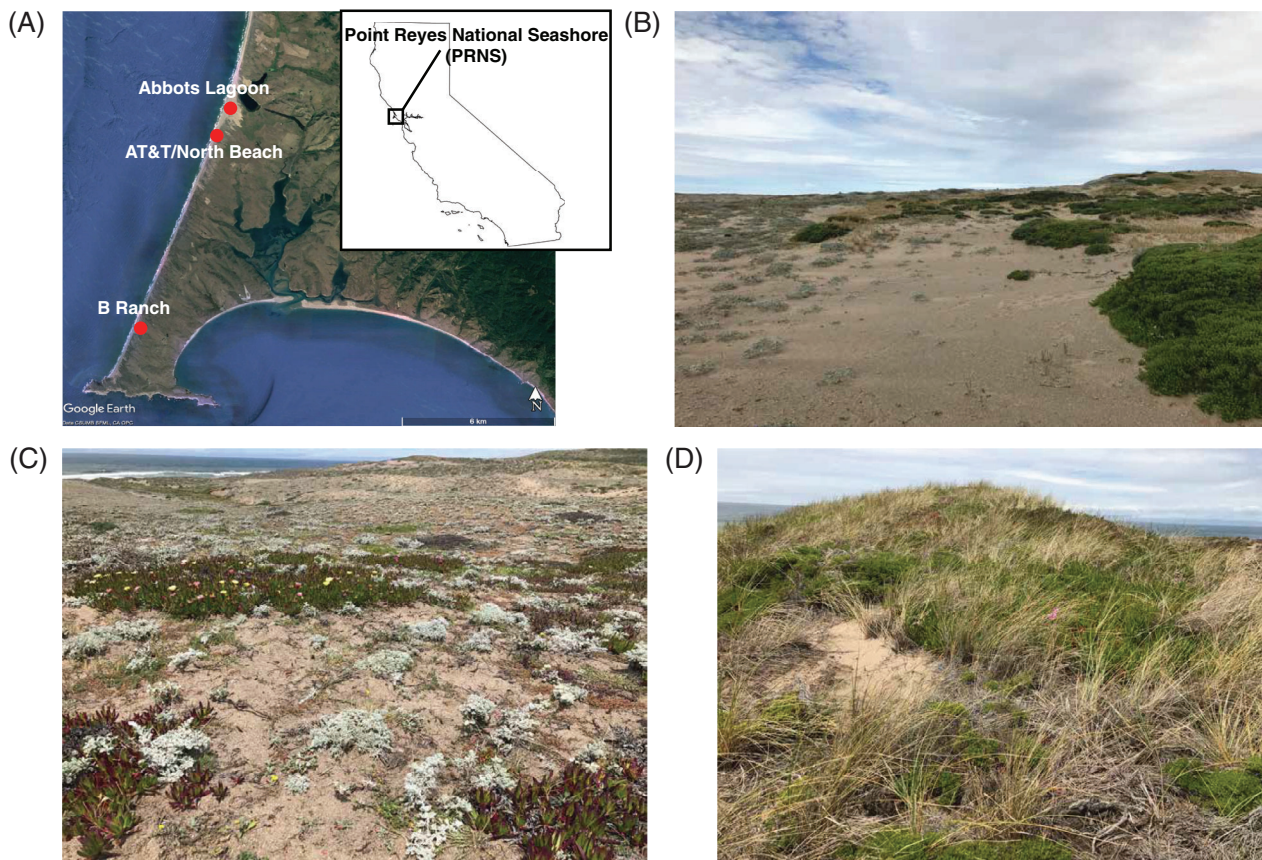
Figure 1. Map of the study site in Point Reyes National Seashore (PRNS), California (A). Pictures showing examples of lightly invaded dunes (B), moderately invaded dunes (C), and heavily invaded dunes (D).

Table 1. Number of soil samples analyzed from restored and unrestored dunes in Point Reyes National Seashore. NA = not available (i.e. mechanical restoration not performed in all areas). NS = present, but not sampled.

Invasion Intensity/Treatment Approach	Foredune vs. Backdune (Where Applicable)	Abbotts Lagoon	AT&T/ North Beach	B Ranch North
Lightly invaded native dune mat	Foredune	3	2	3
	Backdune			
Moderately invaded native dune mat	Foredune	3	3	3
	Backdune			
Heavily invaded dunes—untreated	Foredune	NA	1	NS
	Backdune		2	3
Heavily invaded dunes—restored using herbicide	Foredune	3	3	3
	Backdune	3	3	3
Heavily invaded dunes—restored mechanically	Foredune	2	NA	NA
	Backdune	2		

Sequence Processing

After demultiplexing, raw reads were subject to quality filtering, dereplication, chimera detection, and merging of paired-end reads using dada2 (Callahan et al. 2016). The dada2 algorithm uses a parametric error model to correct and group sequences into unique phylotypes (Callahan et al. 2016). The ITS region is highly variable in length, and thus the first step prior to sequence processing was the removal of primers using cutadapt (Martin 2011). Unique sequence phylotypes were assigned taxonomic affiliation using the Ribosomal Database Project naïve Bayesian classifier (Wang et al. 2007) trained on the 16S rRNA SILVA database (Quast et al. 2012) or the ITS UNITE database (Abarenkov et al. 2010) for bacteria and fungi, respectively. Sequences unclassified at the domain level or classified as mitochondria, chloroplasts, or Eukaryota were removed. We removed potential contaminants by discarding phylotypes present in the control blanks. We normalized phylotype counts using a cumulative-sum scaling approach (Paulson et al. 2013).

Finally, we inferred putative functions using FAPROTAX (Louca et al. 2016) and FUNGuild (Nguyen et al. 2016) for bacteria and fungi, respectively. Although each DNA metabar-coding step can potentially introduce artifacts and biases, we sequenced gene regions shown to provide accurate taxonomic information; we employed broadly used primers with few biases against specific taxa; we filtered out sequencing errors and chimeras using astringent thresholds; we used up-to-date taxonomic reference databases, and we removed potential contaminants through sequencing negative controls (Zinger et al. 2019). Sample information, phylotype abundance tables, and representative sequences are publicly available in FigShare (https://doi.org/10.6084/m9.figshare.9036740.v1).

Statistical Analyses

We used analysis of variance (invasion levels nested within sites) to assess the effect of the different treatments on micro-bial richness and taxonomic or inferred functional differences. We represented microbial community similarity patterns by non-metric multidimensional scaling using Bray-Curtis dis-tance metric. We used permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) to assess the effect of the different treatments on microbial community similarity patterns. We corrected for multiple testing using false discovery rate (FDR) correction (Benjamini & Hochberg 1995). All statistical analyses were implemented in the R environment (www.r-project.org) using the *vegan* package (vegan.r-forge.r-project.org).

Results

The total number of unique phylotypes was 28,295 for bacteria across 41 soil samples and 8,249 for fungi across 39 soil samples. The average number of unique phylotypes per soil sample was 1,791 and 969 for bacteria and fungi, respectively. Overall, bacterial community composition in PRNS dune soils was dominated by the taxonomic classes Actinobacte-ria (19.5%), Alphaproteobacteria (19.1%), Thermoleophilia (10.3%), Bacteroidia (9.2%), Gammaproteobacteria (8.6%), Verrucomicrobiae (4.4%), Acidobacteria (4.3%), Deltapro-teobacteria (4.1%), and Bacilli (3.5%). Fungal communities were dominated by Dothideomycetes (29.2%), Sordari-omycetes (25.9%), Eurotiomycetes (17.9%), Agaricomycetes (9.2%), Leotiomycetes (6.9%), Tremellomycetes (4.3%), and Mortierellomycetes (1.5%).

Bacterial richness showed no significant differences among sites (Abbotts, AT&T/North Beach, B Ranch; analysis of vari-ance [ANOVA], $df = 2$, $F = 0.06$, $p = 0.94$) or among inva-sion levels nested within sites (ANOVA, $df = 5$, $F = 0.54$, $p = 0.74$; Fig. 2A). Similar results were observed using Shan-non's diversity index (ANOVA, $df = 2$, $F = 0.78$, $p = 0.48$ for sites; $df = 5$, $F = 0.83$, $p = 0.55$ for invasion nested within sites). Bacterial community similarity patterns did vary among sites (PERMANOVA, $df = 2$, $r^2 = 0.29$, $p < 0.001$) and by invasion levels nested within sites (PERMANOVA, $df = 5$, $r^2 = 0.38$, $p < 0.001$; Fig. 2B). Inferred methylotrophy, nitrifi-cation, denitrification, nitrate reduction, nitrogen respiration, chitinolysis, cellulolysis, dark oxidation of sulfur compounds,
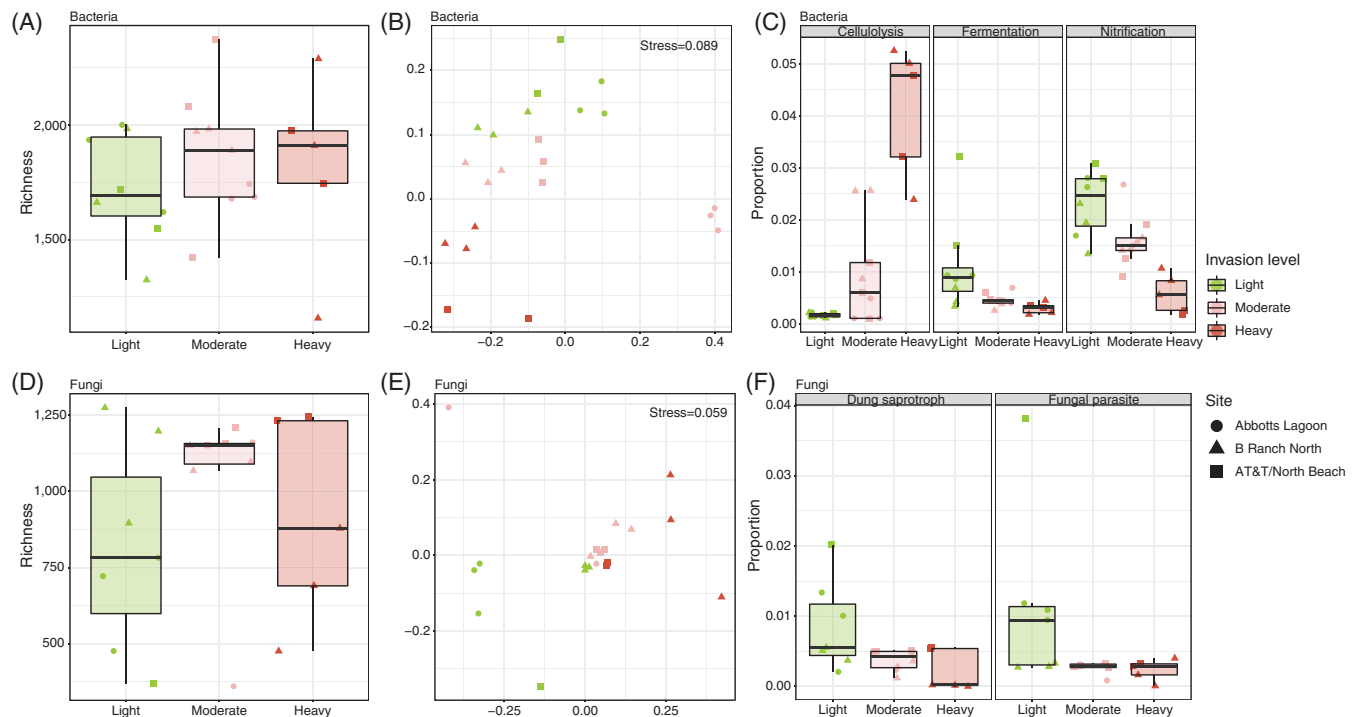

Figure 2. Observed richness (number of different phylotypes per sample) for bacterial and fungal soil communities (A, D). Similarity patterns for bacterial and fungal soil communities represented using non-metric multidimensional scaling (B, E). Significant differences ($p < 0.05$, nested ANOVA after false discovery rate correction) were found in the inferred abundance of bacterial metabolic functions and fungal functional guilds (C, F).
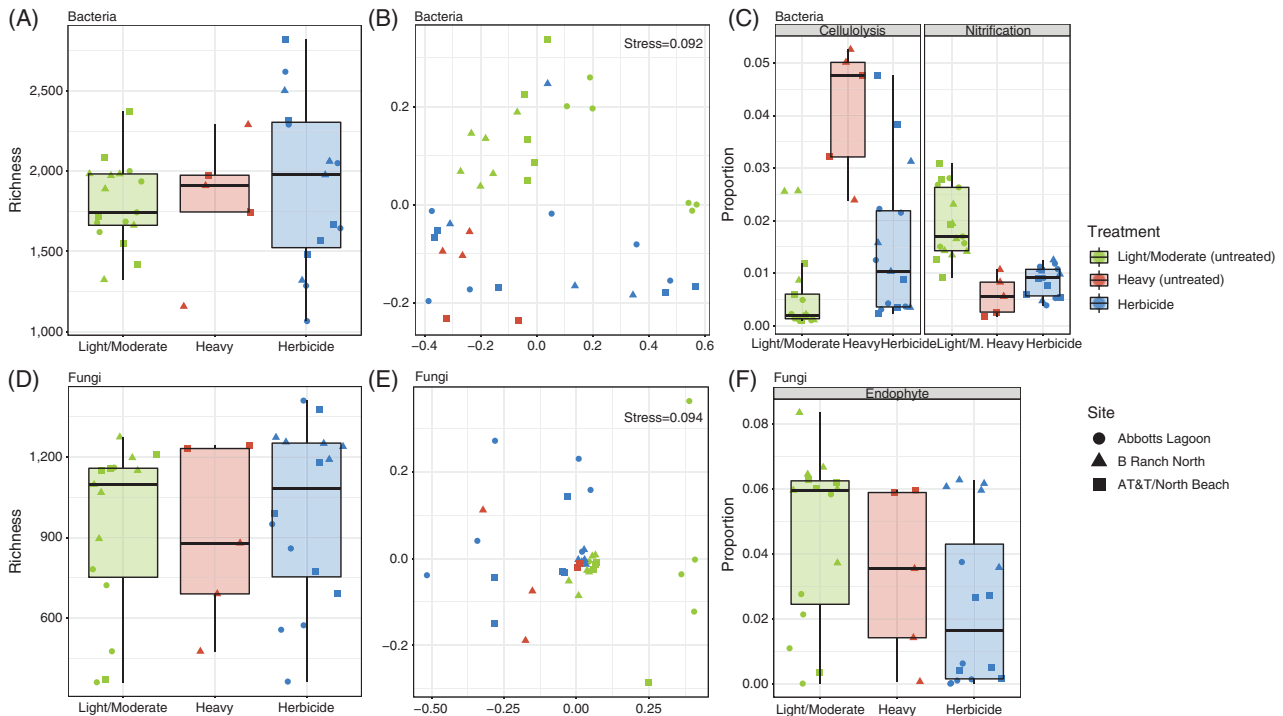
Figure 3. Observed richness (number of different phylotypes per sample) for bacterial and fungal soil communities (A, D). Similarity patterns for bacterial and fungal soil communities represented using non-metric multidimensional scaling (B, E). Significant differences ($p < 0.05$, nested ANOVA after false discovery rate correction) were found in the inferred abundance of bacterial metabolic functions and fungal functional guilds (C, F).

manganese oxidation, fermentation, and oxygenic photoautotrophy exhibited significant differences among invasion levels ($p < 0.05$ after FDR correction). The metabolic functions differing the most were cellulolysis, nitrification, and fermentation (Fig. 2C). Inferred cellulolysis showed increasing levels with increasing invasion intensity (in parallel to an increasing proportion of Alphaproteobacteria with increasing invasion; $p < 0.05$ after FDR correction), while nitrification and fermentation showed the opposite pattern (as did classes Nitrososphaeria, Nitrospira, Chloroflexia, Chthonomonadetes, and Gemmatimonadetes; $p < 0.05$ after FDR correction).

Fungal richness differed among sites (ANOVA, $df = 2$, $F = 4.22$, $p = 0.04$) and invasion levels nested within sites (ANOVA, $df = 5$, $F = 4.29$, $p = 0.02$; Fig. 2D). Similar results were observed using Shannon's diversity index (ANOVA, $df = 2$, $F = 5.72$, $p = 0.02$ for sites; $df = 5$, $F = 3.14$, $p = 0.04$ for invasion nested within sites). AT&T/North Beach samples and moderate/heavily invaded samples tended to have higher fungal diversity. Fungal community similarity patterns also varied among sites (PERMANOVA, $df = 2$, $r^2 = 0.23$, $p < 0.001$) and invasion levels nested within sites (PERMANOVA, $df = 5$, $r^2 = 0.41$, $p < 0.001$; Fig. 2E). Focusing on inferred fungal functional guilds, lightly invaded areas had significantly higher abundance of putative dung saprotrophs and fungal parasites than moderate and heavily invaded areas ($p < 0.05$ after FDR correction; Fig. 2F). This pattern was opposite to the one observed for Archaeosporomycetes ($p < 0.05$ after FDR correction).

Herbicide treatment did not affect bacterial richness, with no significant differences between formerly heavily invaded areas that had been herbicide-treated, heavily invaded untreated areas, and lightly to moderately invaded untreated areas (ANOVA, $df = 5$, $F = 0.14$, $p = 0.98$; Fig. 3A). Shannon's diversity index was also not significant (ANOVA, $df = 5$, $F = 0.43$, $p = 0.82$). Bacterial community patterns varied, though, between treatments nested within sites (PERMANOVA, $df = 5$, $r^2 = 0.24$, $p < 0.001$; Fig. 3B). Bacterial community composition of lightly to moderately invaded areas remained distinct from those of heavily invaded untreated and herbicide-treated sites, which were strongly overlapping (Fig. 3B). Inferred cellulolysis levels were the highest in heavily invaded untreated areas, while nitrification (as taxonomic classes Nitrososphaeria, Chloroflexia, and Entotheonellia; $p < 0.05$ after FDR correction) was highest in lightly to moderately invaded untreated samples ($p < 0.05$ after FDR correction; Fig. 3C). Halobacteria abundance was present and significantly highest in herbicide-treated samples and absent from all the untreated samples whether lightly, moderately, or heavily invaded ($p < 0.05$ after FDR correction).

Similarly, fungal species richness showed no significant differences among herbicide-treated areas, heavily invaded untreated areas, and lightly to moderately invaded untreated areas (ANOVA, $df = 5$, $F = 2.00$, $p = 0.11$; Fig. 3D). Shannon's diversity index was also not significant (ANOVA; $df = 5$, $F = 2.29$, $p = 0.07$). Fungal community composition varied significantly between treatments nested within sites (PERMANOVA, $df = 5$, $r^2 = 0.25$, $p < 0.001$). As with

bacterial communities, fungal composition showed separation between lightly/moderately invaded areas and herbicide-treated and heavily invaded untreated areas, which overlapped considerably (Fig. 3E). Fungal endophytes were significantly different across treatments with herbicide-treated samples showing the lowest relative abundance ($p < 0.05$ after FDR correction; Fig. 3F). At the taxonomic level, Archaeosporomycetes were significantly more abundant in heavily invaded untreated samples compared to both lightly to moderately invaded untreated and herbicide-treated samples ($p < 0.05$ after FDR correction).

Similar results were found when comparing heavily invaded areas that had been treated either by herbicide or through mechanical removal. Bacterial phylotype richness or diversity showed no significant differences between mechanically treated areas and herbicide-treated ones (ANOVA, $df = 2$, $F = 1.37$, $p = 0.32$ for richness; $df = 2$, $F = 2.69$, $p = 0.15$ for Shannon's diversity). Similarly, as with all of the other analyses, there were no observed significant differences based on dune location (foredune/backdune; ANOVA, $df = 1$, $F = 0.35$, $p = 0.58$ for richness; $df = 1$, $F = 0.63$, $p = 0.46$ for Shannon's diversity). Bacterial community composition, however, varied significantly among treatment approach nested within dune position (PERMANOVA, $df = 2$, $r^2 = 0.36$, $p = 0.013$) and dune position (PERMANOVA, $df = 1$, $r^2 = 0.19$, $p = 0.013$). Also, in contrast to bacterial results, there was a weakly significant difference in soil fungal phylotype richness/Shannon's diversity for treatment approach nested within dune position (ANOVA, $df = 2$, $F = 4.25$, $p = 0.08$ for richness; $df = 2$, $F = 6.92$, $p = 0.04$ for Shannon's diversity). There was also a stronger relationship between richness and dune position (foredune/backdune) for fungi (ANOVA, $df = 1$, $F = 9.23$, $p = 0.03$), with fungal richness generally higher for mechanical treatment and foredune areas, although dune position did not influence Shannon's diversity (ANOVA, $df = 1$, $F = 2.49$, $p = 0.18$). No significant differences were observed for fungal community composition as a function of restoration treatment or dune position (PERMANOVA, $df = 1$, $r^2 = 0.14$, $p = 0.127$ for dune position; $df = 2$, $r^2 = 0.34$, $p = 0.127$, for restoration treatment nested within dune position). In this case, we did not evaluate specific differences in taxonomic classes or inferred functions due to low sample size.

Discussion

Although plant–soil feedbacks might facilitate or inhibit the initial establishment and later success of invasive plant species (Wardle et al. 2004; Kardol & Wardle 2010), above-belowground interaction mechanisms and outcomes are complex, highly variable, and ecosystem-specific (Shannon et al. 2012; Carey et al. 2015; Bansal & Sheley 2016; Gornish et al. 2016). In this study, we assessed soil microbial communities using DNA metabarcoding in the presence of plant invaders and approaches for removing these invaders in coastal dunes. Overall, we found evidence of a strong link between soil microbial communities and the presence of invasives, invasion intensity, and restoration approach.

Our results show that soil microbial community composition of coastal dunes at PRNS varies dramatically with invasion intensity. Whether soils in currently invaded areas supported different microbial communities prior to invasion than current "native dune refugia" that increased their vulnerability to invasion or whether active planting and spread of invasives broke down initial biotic resistance and subsequently changed soil biota is unknown. The fact that islands of remnant native dune have persisted at PRNS for decades despite being surrounded by a sea of beachgrass points to some possible role of soil biota in resisting invasives when native communities are intact and not overwhelmed by anthropogenic or natural disturbance events as postulated by Suding et al. (2013). There are little data available showing how quickly biotic and abiotic conditions in native dune soils change once invaded, but, in Mediterranean island ecosystems, differences in soil chemistry and vegetation communities became apparent only 8 years after iceplant invasion (Badalamenti et al. 2016).

Heavily invaded sites were characterized by a lower abundance of nitrifiers, fermentative bacteria, fungal parasites, and fungal dung saprotrophs and a higher abundance of cellulolytic bacteria. All these soil microbial changes imply shifts in carbon and nitrogen cycling with invasion success. While the observed increase in cellulolytic bacteria is expected due to the higher plant biomass in highly invaded soils and increased volume of plant material to be broken down through cellulolysis (Kourtev et al. 2002; Badalamenti et al. 2016), reduced nitrification might be due to bacteria competing with invasive plant roots for available ammonium (Kaye & Hart 1997) and/or due to acidification (Nicol et al. 2008). The decrease in taxa associated with fermentative processes together with the increase in taxa with cellulolytic potential might indicate that carbon is being processed differently in heavily invaded sites. The increase in taxa with non-fermentative characteristics suggests an increased turnover of carbon and other nutrients to support invasive species growth.

Fungal guilds were also influenced by invasion levels. Although we did not observe significant changes in overall fungal plant pathogen or arbuscular mycorrhizal fungi (AMF) abundance, Archaeosporomycetes, a class of AMF, showed a significant increase with invasion levels. Several studies have explored the importance of AMF for native dune plants, with mycorrhizal associations enhancing phosphorus uptake, promoting sand aggradation, improving salt tolerance, and increasing nitrogen fixation in legumes (Koske & Polson 1984; Tsang & Maun 1999). In Dutch dune systems, AMF infection rates were positively associated with vigor of beachgrass stands (Kowalchuk et al. 2002), but not necessarily in areas outside its native range, with AMF infection rates showing no relationship with beachgrass biomass in Oregon dunes (David et al. 2016). Invasive plants can either arrive with AMF already present in intact root systems, or they may be mycorrhizal generalists that can take advantage of AMF species already present in their new home (Pringle et al. 2009; Vogelsang & Bever 2009; Lekberg et al. 2013; Gornish et al. 2016). Several studies have shown that invaders, once established, can increase abundance of AMF or influence abundance of particular AMF species that facilitates growth of the invader, but suppresses

that of native plant species (Pringle et al. 2009; Zhang et al. 2010). It is possible that widespread establishment of European beachgrass in Point Reyes selectively promoted expansion of Archaeosporomycetes, which may have been naturally present, but at low levels, and that higher abundance of this particular AMF had a positive feedback on beachgrass while having a negative feedback on native dune plant species.

As other studies have shown, simply removing invasive plants does not necessarily eliminate the legacy effects that these species have had on native ecosystems. Coastal scrub sites in southern California that had been restored 11 years previously with herbicide treatment of invasive Harding grass (*Phalaris aquatica*) still suffered from microbial legacy effects, with symbiotic nitrogen fixers found in plant root nodules, nitrate-reducing bacteria, and rhizophilic AMF being more abundant in post-invasive soil, while the nitrite-oxidizing bacteria, *Nitrospira*, was more abundant in uninvaded soil (Pickett et al. 2019). In our study, soil prokaryotic and fungal communities from heavily invaded untreated sites were almost indistinguishable from formerly heavily invaded sites that had been herbicide-treated anywhere from 1 to 6 years previously. With the exception of nearshore areas affected by beach sand overwash, herbicide treatment areas would be expected to have similar soil microbiota to heavily invaded untreated areas, as herbicide treatment is intended to only eradicate aboveground biomass and associated roots of target invasives. However, herbicide treatment has the potential to affect soil biota through translocation by plants of herbicides into the soil through roots and rhizomes, infiltration of herbicides into the soil due to overspray, or run-off of chemicals from sprayed plants into the soils. Some laboratory studies have shown impacts of glyphosate on soil microorganisms (Irvine et al. 2013), but field studies on glyphosate and imazapyr have been more equivocal, showing either no effect or even an enhancement of microorganisms (Busse et al. 2004; Lupwayi et al. 2004; Sederholm et al. 2018).

The strong similarity between soils from heavily invaded untreated and herbicide-treated sites would suggest that herbicide treatment did not in and of itself shift overall bacterial and fungal composition or cause any change in bacterial and fungal richness. The one exception might be that Halobacteria, a class of halophilic archaea that are extremely salt tolerant, showed the highest abundance in herbicide-treated sites while literally being absent from the rest of them, pointing to herbicide changing salinity or some degradation by-product enhancing halophilic microbe activity (Le Borgne et al. 2008). Along these lines, glyphosate and imazapyr are often used in their isopropylamine salt rather than acid forms, and it has been shown that halophilic bacteria can break down glyphosate by utilizing carbon released after the organism breaks down the carbon-phosphorus bond (Sharifi et al. 2015).

However, while herbicide may not have had a large overall direct or indirect impact on the soil community, it also did not release restored systems from some of the soil legacies left behind by beachgrass. For example, nitrifiers were as scarce in herbicide-treated sites as in heavily invaded untreated sites, suggesting legacy effects in nitrogen cycling. The decrease in nitrifiers with invasion disputes previous findings that showed how invasive plants can increase nitrification by over 50% (Hawkes et al. 2005; Liao et al. 2008). This persistent legacy may have implications for the success of restoration efforts in that, given the continued absence of nitrifiers in herbicide-treated areas, species that are capable of fixing nitrogen such as bush lupine may have a competitive advantage in colonizing these areas, which could thwart the native vegetation recovery process.

Some changes in soil microbiota did occur, however, after restoration. The abundance of cellulolytic bacteria in herbicide-treated sites dropped compared to heavily invaded untreated sites even though large volumes of undecomposed beachgrass material remained. Also, Archaeosporomycetes were more abundant in heavily invaded untreated sites than in herbicide-treated ones, reinforcing the idea that invasive plants such as European beachgrass may establish symbiotic relationships to enhance its success. By removing beachgrass, restoration may be able to reverse at least some of the changes in soil biota associated with invasion, which may have been precluding re-establishment by native species. In addition, loss of this AMF from herbicide-treated areas may shift the competitive advantage away from beachgrass in terms of re-invasion, making it easier to preclude re-establishment of this particular primary invasive.

In addition to microbial legacy effects, it is likely that restored areas must also contend with other types of legacy effects, as well. At PRNS, most of the herbicide-treatment areas (particularly in the backdunes where sand overwash from adjacent beaches is unlikely) may have also been impacted by the physical legacy effects associated with the long-lingering stands of dead European beachgrass (Parsons et al. 2019), with standing dead material decreasing space available for colonization and the slowly decomposing thick layer of "thatch" or litter material potentially suppressing plant germination and/or establishment (Xiong & Nilsson 1999). Recruitment rates of native dune plant species remained lower than those of weedy species or so-called secondary invaders, which include ripgut grass (*Bromus diandrus*), brome fescue (*Festuca bromoides*), common groundsel (*Senecio sylvestris*), and sow thistle (*Sonchus* spp.; Parsons et al. 2019). Only herbicide treatment in foredune areas has resulted in almost immediate colonization by native dune plants, with sand overwash burying the dense beachgrass stands (and soils) and providing a new substrate for colonization (Parsons et al. 2019).

In the restored backdune areas, these non-native plant species may benefit from the lingering microbial and physical legacy effects of beachgrass, as well as any potential changes that beachgrass may have had on soil organic matter, nutrient pools, and chemistry. It stands to reason that, given the very large volume of plant matter produced by dense beachgrass monocultures, this species could have a dramatic impact on organic matter content of dune soils. However, unlike iceplant, there appears to have been no studies published on how beachgrass changes soil chemistry and the persistence of these changes once beachgrass is removed. Studies on chemical legacy effects of other species suggest that changes can be persistent. After removal of iceplant from coastal dunes in Spain, soil pH, organic matter, ammonium, and nitrate/nitrite levels were still altered 12 months

after removal of iceplant from coastal dunes in Spain (Novoa et al. 2014). Similarly, areas mowed to remove bush lupine (*Lupinus arboreus*) showed no difference from still invaded ones in soil nitrogen levels 5 years after eradication (Maron & Jefferies 2001).

At Abbotts Lagoon, mechanical treatment conducted by "horizon flipping" almost instantaneously eliminated both beachgrass and seemingly any of its potential microbial, physical, or chemical legacy effects. As discussed earlier, this resulted in different soil microbial composition than herbicide-treated areas. However, while releasing restored areas from the legacy effects of beachgrass, this approach may have had its own repercussions. A soil inoculum study conducted 2 years after the mechanical restoration project was completed found that aboveground biomass of several native dune plant species grown in "flipped" soils was considerably reduced relative to that of plants grown in native dune soils and concluded that microorganisms in flipped soils could be detrimental to growth of germinated individuals (Winsemius et al. 2015). Vegetation monitoring results seemed to support this conclusion. Mechanical removal resulted in almost immediate eradication of primary invasives, but also left vast expanses of barren sand and little to no colonization by native dune plants, with cover ranging from 0.5 to 2.3% up through Year 5 post-restoration (Parsons et al. 2019). There was one notable exception: the federally endangered plant species Tidestrom's lupine (*Lupinus tidestromii*), a leguminous species capable of nitrogen fixation, quickly expanded throughout mechanical removal areas immediately after restoration (Parsons et al. 2019).

On the basis of some of these dune restoration project results, many researchers have argued that ecosystem restoration cannot be accomplished without taking additional steps to remediate some of the physical, chemical, and biological legacy effects. Habitat restoration cannot just rely on removing invasives, but may need to include "a perturbation that mitigates biogeochemical and physical effects" caused by invasives (Suding et al. 2004). These perturbations could include soil amelioration, topsoil removal, iceplant duff/litter removal, inoculation with fungal or bacterial taxa, mowing/removal of biomass, overseeding by native plant species, carbon addition, or selective removal of non-native species or species with strong biogeochemical feedbacks (Suding et al. 2004; Corbin & D'Antonio 2012; Novoa et al. 2014).

It remains to be seen whether intact, resilient native dune plant and soil microbial communities will eventually re-establish in these restored areas, with or without additional restoration measures. Not only has re-colonization by natives been very slow regardless of restoration approach, but both herbicide-treated and mechanically restored areas have been invaded by new weeds or secondary invaders that are taking advantage of the decrease in primary invader abundance and disturbance associated with restoration to gain a foothold in restored systems (Parsons et al. 2019). Ultimately, more information is needed on the persistence of legacy effects of invaders on coastal dune soils and how these effects might be most effectively countered to jumpstart dune restoration efforts and improve their success. Both aboveground and belowground

interventions may be necessary to restore functional plant–soil interactions characteristic of a native ecosystem (Kardol & Wardle 2010). In all cases, vigilant, long-term re-retreatment will be critical to success of restoration efforts, as coastal ecosystems are disturbance-prone and therefore vulnerable to invasion and re-invasion events (Carboni et al. 2010). Until effective measures to remediate legacy effects are developed, land managers might best be served to focus restoration efforts on low-risk, high-yield restoration efforts such as removal of iceplant in lightly or very recently invaded areas or herbicide treatment of European beachgrass or iceplant in foredune areas where sand overwash creates a new substrate for native dune plant establishment unimpacted by lingering legacy effects. Future studies should delve more deeply into changes in soil chemistry, carbon-nitrogen cycling, and biota associated with beachgrass invasion and restoration of invaded areas, as well as evaluate potential remediation measures that might mitigate persistent legacy effects that could ultimately preclude native plant reestablishment.

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