The effects of sediment origin, microbiome and mesograzer presence on *Zostera marina* seedling settlement

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Abstract

Seagrasses form the foundation of valuable coastal ecosystems. They provide numerous ecosystem services to people living in coastal areas but are currently rapidly declining on a global scale. To preserve and re-establish seagrass populations, many attempts are being undertaken to restore valuable seagrass meadows, with limited successes. To improve the success rates of restoration practices, more knowledge on the ecology of seagrasses is needed. I thus looked into factors affecting successful seedling establishment for seed-based restoration. Specifically, I aimed to unravel the effects of 1) sediment origin (donor vs. restoration site), 2) sediment microbiome presence (sterilized vs. non-sterilized sediment) and 3) mesograzer presence (control, mudsnails, periwinkles) on eelgrass (*Zostera marina*) seedling establishment and development. I tested this in a mesocosm experiment comparing growing seedlings from seeds within the aforementioned treatments and looking at plant biomass, morphology and the sediment microbiome.

Sediment origin (donor vs restoration site) did not affect seedling count at harvest, biomass and morphology, despite differences in sediment characteristics (OM/grain size/nutrients) and microbiome. However, seedling settlement over time was twice as high in the donor site. Sediment sterilization (sterilized vs. non-sterilized) positively affected seedlings settlement, and seedling biomass/sizes. This might be due to higher nutrient availability in the sterilized sediment, as well as decreased microbial diversity, which is potentially linked to lower pathogen presence. Mesograzer presence negatively affected seedling settlement but did not affect plant morphology, potentially as a result of consumption of sprouted seeds, however biomass did increase with mudsnail presence. At the same time, either mesograzers presence negatively affected archaea diversity, but not bacterial diversity.

Based on these results it seems that 1) sediment of the restoration site is suitable for establishment and developing of eelgrass seedlings, 2) sterilization promotes seedling settlement and development, possibly as a result of enhanced nutrient availability and/or reduced amount of detrimental bacteria, 3) mesograzers negatively affect seedling settlement, most likely due to consumption of the seeds, however, mudsnails do promote seedling development. Altogether this study suggests that the presence of a microbiome can promote seedling settlement and development after re-colonisation, possibly due to unknown pathogens in the sediment (also known to be detrimental in terrestrial plants). This indicates that a site-specific microbiome at a site without seagrass presence can become suitable as a restoration site. Further research linking the functional and ecological interaction between the microbiome and seedling settlement and growth has to be done to be able to conclude the influence of potentially healthy microbiomes for restoration.

Keywords: ***Seagrass*, *eelgrass (*Zostera marina*)*, *seagrass restoration, restoration framework, microbiome, sediment sterilization, (meso)-grazers, mudsnails (*Peringia ulvae*)*, *periwinkles (*Littorina littorea*)***.

Introduction

Seagrass beds are one of the most productive ecosystems on earth (van Katwijk et al. 2016). They are highly valued due to the ecosystem services they provide, for example, they form the foundation of many coastal communities worldwide, regulate nutrient cycling, provide productive fisheries, contribute to erosion control and attenuate wave strength (Orth et al. 2006a, van Katwijk et al. 2016, Maxwell et al. 2016, Folmer et al. 2016, Ettinger et al. 2017, Sullivan et al. 2017). Seagrasses are the only marine angiosperms that evolved from terrestrial plants (Fahimipour et al. 2017, Martin et al. 2018), making them unique in morphology and physiology (Orth et al. 2006a). For example, having roots within the sediment ensures trapping and storing of sediments, leading to sediment stabilization and consequently lower water turbidity (Orth et al. 2006a, van Katwijk et al. 2016). In general seagrass beds increase 1) biodiversity of animal life (van der Heide et al. 2009, van der Zee et al. 2016, Govers et al. 2017) by modifying the habitat through creating shelter for a diverse range of species (van Katwijk et al. 2016), especially in their juvenile stages (Beck et al. 2001, Orth et al. 2006a), and 2) the number of interactions species have, which in turn contributes to link density of the food web (van der Zee et al. 2016).

Despite the high ecological value of seagrass beds they are currently declining all across the world with an acceleration in losses to almost 7% per year, which also strongly affects associated species (Orth et al. 2006a, Waycott et al. 2009). To counteract losses, restoration efforts worldwide have been made (Paling et al. 2009, van Katwijk et al. 2009, 2016, Unsworth et al. 2019), but there are only a handful of documented large-scale, long-term restoration successes (Orth et al. 2017a, Lefcheck et al. 2018, Rezek et al. 2019). However, in the past decades, seagrass restoration has become a common management tool for recovering coastal ecosystem services and ecological functions (Greiner et al. 2013, van Katwijk et al. 2016). A literature review from 2016 reported that seagrass survival rates are overall only 37% among restoration trials (van Katwijk et al. 2016). In Florida (USA) a more recent study stated that the low initial survival rate there (50%) is in contrast with the long-term restoration persistence rates, as these approach 90% (Rezek et al. 2019). The aim of nature restoration in general is to transition a particular natural environment towards an envisioned restored state (Paling et al. 2009, Floor et al. 2018), which is relatively hard with seagrass due to their self-regulatory tendencies (Nyström et al. 2012, Maxwell et al. 2016, Moksnes et al. 2018). As each restoration attempt varies per seagrass species, ranging from planting

specific plant fragments to distribution of seeds or seedlings, and per temperate zone and water depth, (van Katwijk et al. 2016, Eriander et al. 2016, Lefcheck et al. 2018, Paulo et al. 2019), it is difficult to create one clear step-by-step plan for restoration. Even though there have been workshops and guidelines for improving restoration (van Katwijk et al. 2009, 2016, Cunha et al. 2012), there are still many gaps in knowledge. It has become clear that their self-regulatory propensity created by feedback mechanisms are crucial for each seagrass species, wherever they are (Moksnes et al. 2018).

Seagrass feedback mechanisms can positively influence seagrass survival as well as negatively (van Katwijk and Hermus 2000, Maxwell et al. 2016, Moksnes et al. 2018). For example, high seagrass densities increase sulfide in the sediment leading to seagrass reduction, whereas a high abundance of seagrasses reduces water turbidity and therewith positively enhance their own photosynthesis (Longstaff et al. 1999, Burkholder et al. 2007, Maxwell et al. 2016). Disruption of feedback mechanisms contribute to a switch between different stable states, which convert abruptly when specific tipping points are met (Maxwell et al. 2016, Moksnes et al. 2018). If this happens, an algae dominated state can take over, resulting in seagrass degradation (Burkholder et al. 2007, Duffy et al. 2015, Maxwell et al. 2016, Campbell et al. 2018). Mesograzers (crustaceans and gastropods) consume epiphytic algae and therefore (partially) diminish the competition over light between algae and seagrasses, leading to a mutualistic relationship between the two (Hootsmans and Vermaat 1985b, Valentine and Duffy 2006, Burkholder et al. 2007, Campbell et al. 2018). Before a tipping point is met, the cropping of algae by mesograzers can even lead to promoting seagrass dominance (Baden et al. 2010, Whalen et al. 2013, Reynolds et al. 2014). Exclusion of grazers in experimental settings has led to a reduction of seagrass biomass, where in some instances more than half of total seagrass cover was lost (Valentine and Duffy 2006, Duffy et al. 2015, Campbell et al. 2018). Even though there is proof presence of mesograzers contribute as a positive feedback mechanism on seagrass abundance, it's only recently been taken into account as an important contributing factor for seagrass restoration (Reynolds et al. 2014, Duffy et al. 2015, Scott et al. 2018). Another feedback mechanism which has not been considered often is between the sediment and seagrass roots, which most likely creates an extra factor influencing seagrass survival (Crump et al. 2018, Martin et al. 2018), like it does in terrestrial plants, where sediment-specific compounds influence plant growth and health (Maxwell et al. 2016,

Moksnes et al. 2018). So far there are only a couple of instances where possible effects of sediment-specific compounds and (micro)organisms are considered in light of seagrass-restoration (Maxwell et al. 2016, Crump et al. 2018).

The collection of all microorganisms in a specific niche is called a microbiome (Figure 1). A lot of research is done on the niche in the soil around (terrestial) plant roots, including the rhizosphere, which is the narrow zone of soil directly surrounding the roots (Walker et al. 2003, Bais et al. 2006, Lugtenberg and Kamilova 2009, Philippot et al. 2013). Terrestrial plants directly interact with the tens of thousands of microorganisms species in their rhizosphere via exchange of either inhibiting or stimulating species-specific compounds, creating the possibility to shape their rhizosphere microbiome (Bais et al. 2006, Berendsen et al. 2012, Mendes et al. 2013, Philippot et al. 2013, Martin et al. 2018). Similarly, microorganisms can regulate plant health and/or performances (Berendsen et al. 2012, Martin et al. 2018) by providing plant nutritions and hormones that improve plant growth in exchange for food (Lugtenberg and Kamilova 2009, Philippot et al. 2013), fixating otherwise toxic compounds (Mendes et al. 2013, Fahimipour et al. 2017), while some induce herbivore resistance (Bais et al. 2006) or control soilborne plant diseases (Lugtenberg and Kamilova 2009). Even though for terrestrial plants most types of microbiomes and their effects are well studied, there haven't been many studies focussed on marine plant-microbiome interactions (but see: Cúcio et al. 2016, Fahimipour et al. 2017). The studies so far have shown a high abundance in certain microbal orders (Table 1) and that a seagrass microbiome 1) contains many microbes associated with reducing toxic compounds like sulfides (Nielsen et al. 1999, Fahimipour et al. 2017), 2) is the same for different seagrass species in the same location (Cúcio et al. 2016), 3) is selected for by individual plants (Cúcio et al. 2016), 4) differs significantly from the surrounding sediment microbiome (Ettinger et al. 2017), and 5) gets altered by low light availability (Martin et al. 2018). In general, the link between microbiome and seagrass functioning has not

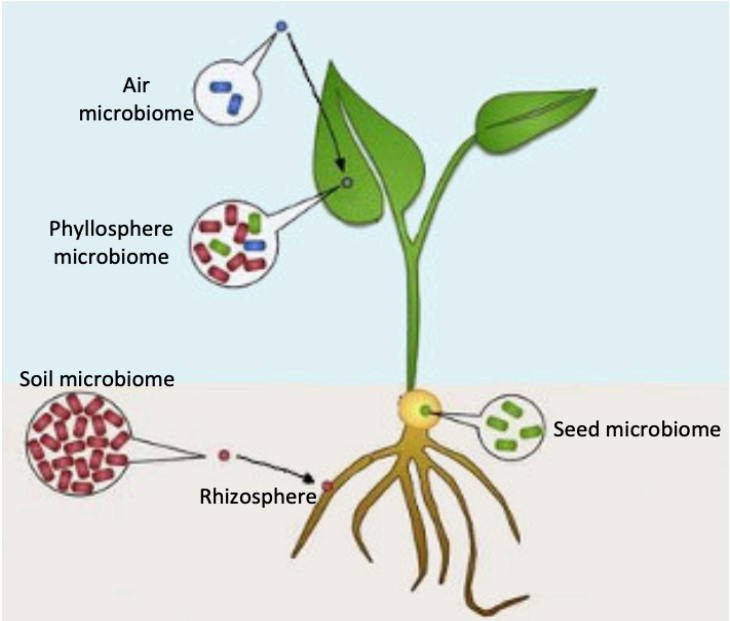


Figure 1. A simplified illustration of the different microbiomes associated with plants

been thouroughly researched yet, which means the possible influence on restoration efforts is lacking as well.

Table 1. Known orders that are in relative high abundance around the roots of seagrass species. This table is based on data gathered in Martin et al. (2018) and Ettinger et al. (2017)

Order	Seagrass species	Associated function
Alteromonadales	*Zostera marina*	
Bacteroidales	*Zostera marina, Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Campylobacterales	*Zostera marina, Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Cerasicoccales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Clostridiales	*Zostera marina*	
Desulfobacterales	*Zostera marina, Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	Anaerobic, reduces sulphates to sulphides to obtain energy
Desulfovibrionales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	Obligatory anaerobic, sulphate-reducing bacteria
Desulfuromonadales	*Zostera marina*	
Flavobacteriales	*Zostera marina*	
Kiloniellales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Leptospirales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Methylophilales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Rhodocyclales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
TG3-1	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	
Thiotrichales	*Zostera marina*	
Vibrionales	*Halophila ovalis, Halodule uninervis, Cymodocea serrulata*	

Altogether, there is a gap in knowledge on the effects of both grazer presence and different microbiomes on seagrass restoration success, which might conctribute to low restoration success rates. For example, hystorically natural occuring *Zostera marina* (*Z. marina*, eelgrass) fields have disappeared over time in the Waddensea (Floor et al. 2018). Improving water quality has lead to natural recovery in the German parts, but in the Dutch parts eelgrass has not come back naturally yet (van Katwijk et al. 2009, Floor et al. 2018). In the past 50 years many attempts to restore the fields have been made and only in the past three years these active restoration efforts have prompted a growth from 30 hectares to 170 hectares of eelgrass in Griend (Wadden island near the Dutch coast), leading to ten times more plants and an increase in density from 0.01 plants per m^2 to 50 plants per m^2. Unfortunately, this succes is not (yet) visible in other parts of the Dutch Waddensea. *Z. marina* is used because it is a model seagrass species that is the most widespread and geographically distributed, most frequently used in restoration attempts (van Katwijk et al. 2016) and a foundation species in shallow coastal areas (Moksnes et al. 2018). Methods of

restoration vary with each location, ranging from shoot-based transplants to seed plantations (van Katwijk et al. 2016). The use of seeds has certain advantages (Unsworth et al. 2019): there is a high genetic diversity in the restored population (Reynolds et al. 2012, Ort et al. 2014), it's more cost-effective than other approaches (Busch et al. 2008), pathogens carried by the seeds can be reduced via copper treatment (Govers et al. 2017), and there is a reduction of seed loss compared to natural conditions, as seed dormancy in the sediment naturally leads to significant losses (Marion and Orth 2010). However, the effects of the microbiome and sediment types on the survival of seeds and therewith the establishment of seedlings is still lacking. To improve restoration efforts, more information is needed.

I therefore aimed to gain more knowledge on the influence on seedling settlement and seedling characteristics of 1) sediment origin (donor population vs. restoration site), 2) sediment microbiome presence (sediment with neutralized microbiome vs. normal sediment) and 3) mesograzer presence (control, mudsnails, periwinkles). I hypothesized that sediment from a natural seagrass location, as well as the presence of mesograzers and an intact sediment microbiome, will lead to better seedling settlement and development (Figure 2). To test these hypotheses, I conducted a mesocosm experiment.

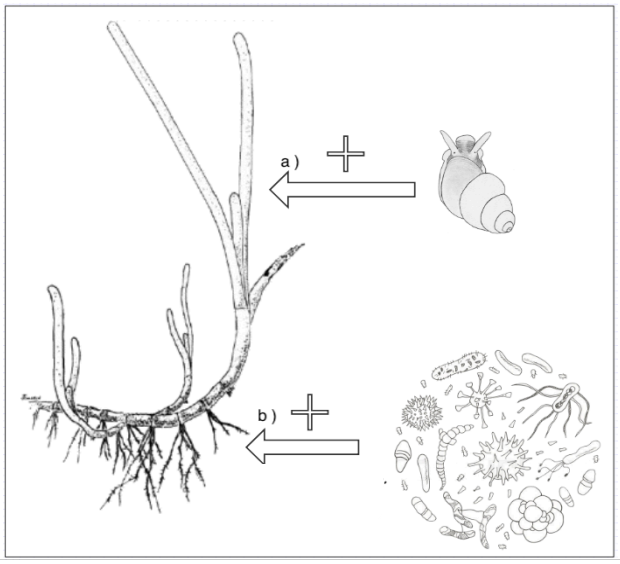


Figure 2. A schematic visualisation of the hypothesis made. (a) Grazers will have a positive influence on seedling settlement and development. (b) The microbiome has a positive influence on seedling settlement and development.

Material and methods

Treatments

The mesocosm experiment had three different factors: sediment origin (donor population vs. restoration location), sterilization (natural microbiome vs. neutralized microbiome) and grazer presence. Sterilization leads to destruction of microorganisms and macromolecules, enabling the possibility to differentiate between effects on seeds from the microbiome and abiotic sediment characteristics. Both sediment origin and sterilization had two levels, donor versus restoration respectively natural versus sterilized (Figure 3). Grazer presence had three levels, control and two different grazer species: *Peringia*

ulvae (*P. ulvae,* mudsnail) and *Littorina littorea* (*L. littorea*, periwinkle). The sediment in the sterilization factor came from the restoration site, while sediment in the grazer factor was unsterilized and from the donor site. There were five replicas for each treatment.

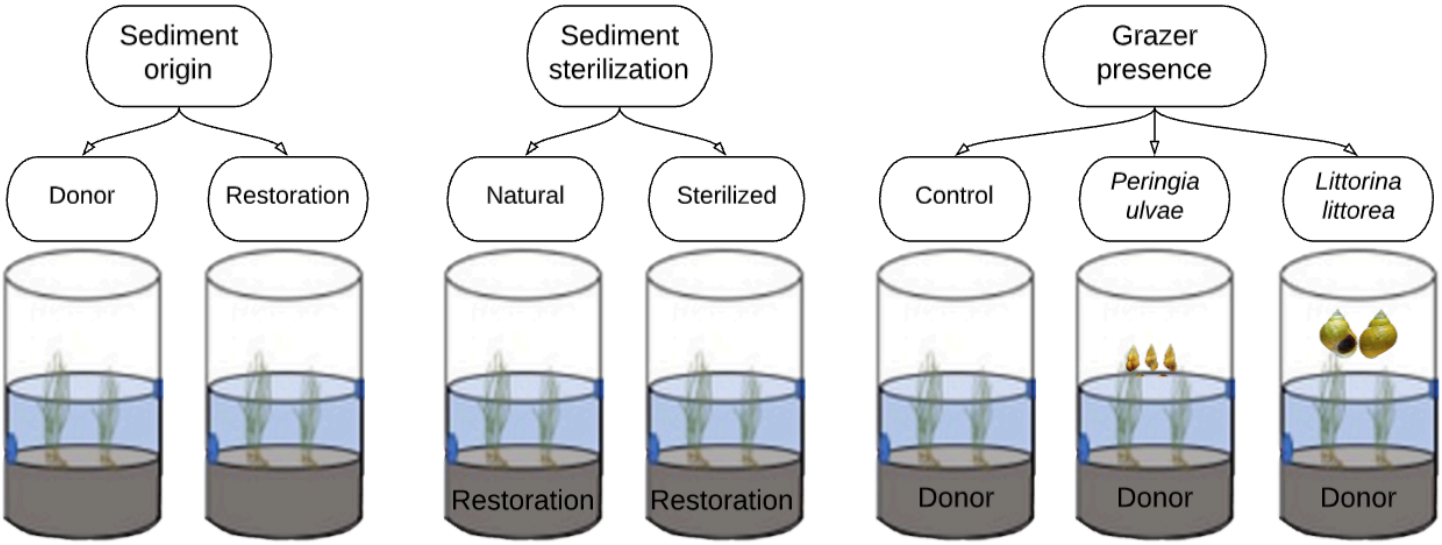


Figure 3. The different treatments. An illustration of all the different treatments within this experiment. The sterilization treatment was done with soil from the restoration site and the grazer treatments on sediment from the donor site.

Experimental set-up

The experiment was conducted in 30 cm-high, plastic cylinders with a 12 cm diameter, filled with a ± 10 cm-high sediment layer and on top ± 10 cm-high synthetic seawater (30‰) (Figure 4), from now on called experimental units (EU). Each EU had a porous membrane at sediment level, penetrated by two needles through which a constant air and seawater (30 ppt) supply ensured full aeration of the water column. The needle inserting seawater was attached to one of three peristaltic pumps (Masterflex® 7568–10 Peristaltic Tubing Pumps, Cole-Parmer, USA) that continuously inserted water from one of two basins at approximately 40 ml/h, refreshing all water in one EU twice a week. The excess water left each EU through an overflow hole at ±20 cm height and was then disregarded, to ensure no cross contamination of microbes between EUs. The synthetic seawater was prepared with deionized water combined with Tropic Marin

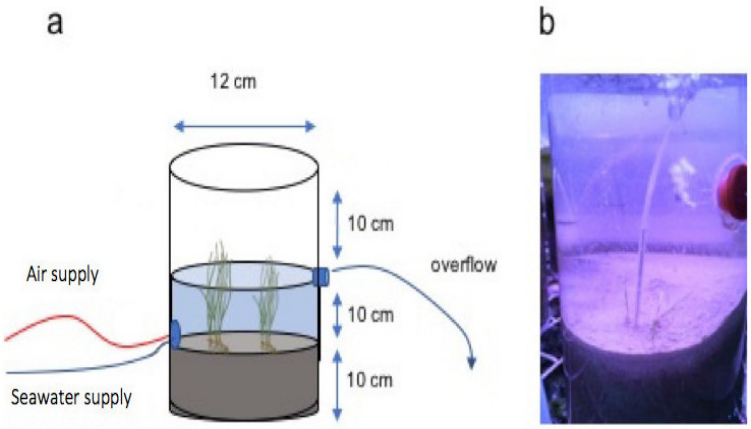


Figure 4. Experimental set-up. (a) Schematic representation of the set-up with all measurements, seawater supply, air-supply and overflow mechanism. (b) Picture of the experimental set-up.

synthetic sea salt and checked every other day for its salinity level using a multimeter salinity probe. The EUs were located in a controlled climate chamber at the University of Groningen, with a constant temperature of 20 ºC, LED-lights of 240 µmol fotons/m^2/s and a natural day/night rhythm (12 h/12 h) (conditions slightly adapted from Govers et al. 2014). Per EU one rhizon soil moisture samplers (Eijkelkamp agrisearch Equipment, giesbeek, the Netherlands) pushed to 5 cm depth was added. Treatments were assigned randomly. Twenty seeds were planted in each EU at ±0.5 cm depth using two ethanol-disinfected tweezers. After three days grazers were added.

Origin of the sediment

The donor location was Sylt (around 54.7998º N, 8.2968º E), a barrier island in the Wadden Sea off the German coast. There is a high abundance of *Z. marina* and *Zostera noltii,* as well as the two gastropod species *L. littorea* and *P. ulvae* (visual observations). The restoration site was Uithuizen (53.452º N, 6.639º E), an area in the Dutch Wadden Sea that used to have high abundance of *Z. marina* (van Katwijk et al. 2000, van der Heide et al. 2007, Paling et al. 2009), but the meadows never recovered, despite a positive assessment of habitat suitability (van Katwijk et al. 2000, Folmer et al. 2016) and several restoration attempts (van Katwijk and Hermus 2000, Floor et al. 2018). Besides origin, mesograzer, and seagrass presence, the two sediment types differ in sediment characteristics as well. Uithuizen has a higher organic matter content (15% vs 0.64% in Sylt) and a smaller grain size (muddier, 24 µm vs 334 µm) than Sylt (Table 2).

Table 2. Known sediment characteristics of Sylt and Uithuizen sediment. Shows soil characteristics of both sediment origins. Based on data from Govers et al. (2016) and Govers et al. (in review)

Sediment	Median grain size (µm)	% Organic matter
Sylt (DE)	334	0.64%
Uithuizen (NL)	24	15.01%

Sediment was sieved (5 mm) right after collection (April 2018) and homogenized, after which it was stored at 5 ºC until start of the experiment. For the sterilization treatment half of the sediment from

Uithuizen was sterilized through autoclaving in plastic heat-proof bags with airholes, at 120 ºC for two consecutive hours. Autoclaving leads to enhancement of dissolved organic matter content (%OM), increase in organic carbon concentrations, changes in soil organic matter structure by carbon mobilization, releases of nutrients and substrates, and greater soil surface area of the grains (Berns et al. 2008, Otte et al. 2018).

Seeds

The seeds used were collected on Sylt in August 2017, at the same site as sediment collection. Govers *et al.* (2016) showed that up to 99% of seeds produced by the *Z. marina* populations in Sylt are infected by *Halophytophthora* sp. Zostera and *Phytophthora gemini*, which leads to a six time reduction of germination when compared to non-infected seeds (Govers et al. 2016). To reduce these infections, all seeds underwent copper treatment after collection (Govers et al. 2017). First the seeds were put in a basin with running synthetic seawater, located in a controlled dark room with a constant temperature of 5 ºC. Subsequently, water with 0.2 ppm copper sulphate and 30 ppt salinity washed over the seeds in a flow-through system. Copper concentrations were checked on a weekly basis, and manually corrected if concentrations dropped below 0.1 ppm. The seeds were collected in the beginning of May 2018 and individually rinsed with deionized fresh water for 24 hours to induce faster germination (Liu et al. 2016) prior to the experiments.

Grazers

L. littorea and *P. ulvae* were both collected in the beginning of May 2018 in Sylt. After collection, they were transferred back to Groningen in a box with sediment, seawater, and a mesh cover to keep them aerated. The box was put inside a cooler with a temperature of approximately 6 ºC, to resemble the winter period in Sylt, during which the snails reduce their metabolic rates to stay alive due to reduced food availability. In Groningen the box was placed inside a water tank with a constant synthetic seawater-flow (same as during the experiment), ambient temperatures, a mesh cover and an aeration tube. The number of grazers added at the start of the experiment was based on calculations concerning abundance data from Sylt and surface area of each EU, which was 10400 cm^2, to create a realistic abundance. I added approximately 20,000 *P. ulvae* per square meter (Govers et al. 2014), resulting in ±200 individuals per EU. For *L. littorea*

up to 917 individuals per square meter (Eschweiler et al. 2009) can be found, leading to approximately 10 individuals per EU. The grazing rate of *L. littorea* individuals is dependent on body size. In Sylt the average snail size is approximately 1 cm (Eschweiler et al. 2009), therefore only individuals bigger than 0.5 cm and smaller than 1.5 cm were used.

Sample collection during the experiment

Every weekday, seedling numbers and grazer presence were determined by visual observations. During the first two weeks *P. ulvae* individuals buried themselves into the sediment, which made them impossible to take into account during daily counts. When *L. littorea* grazers escaped their EUs, they were put back manually with disinfected tweezers. During the first three weeks approximately 15% all *L. littorea* died due to accumulation of sulphide within the sediment, leading to a necessary addition of new *L. littorea* at the end of week three. New individuals were added so the total would be at ten.

In week five vacuumed syringes were placed on the rhizons of each EU to extract ±20 ml of porewater, which was one week after the rhizons were individually cleaned with deionized water. The porewater samples were frozen and stored in a -20 ºC freezer until the end of the experiment, after which a subset was used to determine ammonium, phosphate, nitrate and iron levels in an analytical lab at the University of Groningen. All analyses were done according to the Manual for Soil and Water Analysis edited by P. Buurman, B. van Lagen and E. J. Velthorst (Wageningen, June 1996 Backhuys Publishers Leiden) on porewater from all samples without grazers present, based on the assumption that grazers do not influence nutrient levels. Nitrate, ammonium, and phosphate were analysed with a Skalar spectrophotometer. Nitrate levels were determined by sulfanilamide, after reduction of nitrate to nitrate in a cadmium-column. Ammonium and phosphate concentrations were measured using a salicylate-solution and respectively ammonium molybdate combined with ascorbic acid. Iron samples were first acidified with 4% HCl, after which they were measured in an Atom Absorption Spectrophotometer from Varian.

When an EU had a thick biofilm that completely covered the sediment surface, the biofilm was removed carefully by hand, using latex gloves and ethanol-disinfected tweezers, making sure there was no cross contamination and the seedlings were unharmed and left in place.

Sample collection at harvest

Seedlings

The experiment was terminated after 6 weeks, when the first seedlings started to develop into adult plants. At termination, every seedling was manually removed from the EUs. Manually wet weight (aboveground and belowground), with a 0.1 mg accuracy, and plant morphology, to the nearest mm (leaf length, width, longest root length), were measured. Subsequently, for every shoot, maximum leaf length was determined, specific leaf area was calculated by multiplying leaf lengths with leaf widths and aboveground/belowground weight ratio was noted. After these measurements, aboveground fragments were disregarded, while the belowground fragments were kept in a -80ºC freezer till further analysis.

Sediment

Upon harvest, sediment samples were collected from each EU in sterile 15 mL tubes, which subsequently were stored in a -80 ºC freezer. These sediment samples were used for DNA extraction to determine species composition of both archaea and bacteria within the sediment. DNA extraction was done with RNA Powersoil Total RNA Isolation Kit (MO BIO Laboratories, INC., Carlsbad, CA, USA) and the Powersoil DNA Elution Accessory Kit (MO BIO Laboratories, INC., Carlsbad, CA, USA), according to the manufacturer's protocol (MO BIO Laboratories, Inc. 1993a, 1993b). Before placing the sediment in the PowerBead tubes, it was thawed and homogenized via stirring. Sediment from the same treatment but different EUs were pooled together. The microbial 16S rRNA genes were amplified using a protocol targeting almost the entire genome using universal 17F and 1692R primers for bacteria and A519F and arch1017R primers for archaea. The Thermo Scientific 2X Phire Tissue Direct PCR Master mix was used for amplifications. The thermocycling conditions applied are represented in Table 3. The PCR products were purified, and libraries were sequenced according to the Ligation Sequencing Kit 1D (SQK-LSK108) and the PCR Barcoding Kit (EXP-PBC001) (Oxford Nanopore Technologies, Oxford, OX4, UK) protocols.

Table 3. The PCR protocol used for the microbial 16S rRNA amplifications. These are the thermocycling conditions used to amplify the 16S rRNA of bacteria and archaea species

Cycle step	Temp.	Time	Cycles
Initial denaturation	98 °C	5min	1
Denaturation	98 °C	7sec	
Annealing	57 °C	7sec	25
Extension	72 °C	40sec	
Final extension	72 °C	2min	1
Hold	12 °C	2min	1

Data visualization and statistical analysis

All data visualizations and statistical analyses were done exclusively in R version 3.4.2 (2017) and R-studio version 1.1.383 (2017). All analyses with a p-value < 0.05 were considered significant.

Seedlings

All results were visualised as mean ± SE with packages: ggplot2, plyr and dplyr. Data for morphology, biomass, average final count, and nutrient levels of both sediment origin and microbiome treatments, was analysed using a two-sample student's t-test, whilst for the same data from the grazer treatment, a one-way ANOVA was used. Preceding student's t-test, the data was checked for homogeneity of variances via an f-test and a Shapiro-Wilk tested for normal distribution. If variances were not homogenous, a Welch t-test, instead of a student's t-test ensued. If the data was not normally distributed, it was log-transformed. Prior to the ANOVA, normality of data was determined via visual observations of dotcharts and a statistical evaluation via a Shapiro-Wilk test on residuals of the model. A log-transformation followed detection of non-normally distributed data. If a result was significant, a post-hoc Tukey test followed. Statistical analysis for average seedling counts over time was a one-way ANOVA (Type II, Wald chisquare test) done on a fit generalized linear mixed-effects model with poisson distribution, where time in weeks functioned as random factor and treatment (origin of sediment, microbiome, or grazer presence) as a fixed factor. Statistical analysis was done using the following functions: lme (nlme package), lmer (lmerTest package), glht (multcomp package), glmer (lme4 package), Anova (car package), and anova.

Sediment

Based on the nanopore tech libraries, 16S classifications generated a list of all identifiable archaea (61% was successfully classified) and bacteria (90% was successfully classified). From this list, an OTU-table was generated, which consists out of a list with all found organisms, named as 'OTU#', and their corresponding count in different samples, which were numbered from one to twelve. Using https://www.ezbiocloud.net/ a taxonomy table was created, where the 'OTU#'s were linked to the corresponding domain, phylum, class, order, family, genus, and species. To connect each sample number mentioned in the OTU-table with the

correct treatment, a metadata file was produced, containing corresponding sediment type, sterilization treatment and grazer presence. All three files were combined to create a phyloseq file in R using the following packages: ggplot2, vegan and phyloseq. Visualisation of the data was done using the ggplot package.

Diversity

To calculate intra-sample (alpha) diversity Chao1 and Shannon diversity metrics were used, which check if there are differences in richness and evenness of the microbial communities of each treatment (Ettinger et al. 2017). To conclude if these differences were significant, a two-sample student t-test (for sediment origin and microbiome), and a one-way ANOVA (for grazers) was done. After significant values for the ANOVA's, a post-hoc Tukey test ensued. To be sure that all assumptions for a student t-test were met, the data was checked for homogeneity of variances via an f-test. All treatments were homogenous and therefore no Welch t-test was needed.

Beta diversity indicates differentiation among habitats (Martin et al. 2018) and was determined via Unifrac dissimilarities using unconstrained principal coordinate analyses (PCoA). Due to the wide range of abundances weighted Unifrac dissimilarities were analysed. To test for significant differences between different EUs within treatments, a PERMANOVA test was performed using the adonis function from the vegan package with 999 permutations, but because there were only two replicas for each treatment, the tests did not have a high statistical power. Therefore, the analysis was solely done based on the figures.

Abundance

The count data was transformed to relative abundance using the transform_sample_counts function within phyloseq. Subsequently, this newly generated data was plotted on class-level using a stacked barplot from the ggplot package in combination with coloration from the RColorBrewer package. An abundance heatmap based on order-level was created using the tax_glom function of the phyloseq package. No statistical analysis of the abundance data was done.

Results

Sediment origin

Seedling numbers, morphology, biomass and nutrient levels

Seedling numbers

Over time, significantly more seedlings establised on Sylt sediment than on Uithuizen (Figure 5.b, Linear Mixed model, Chisq = 10.471, p = 0.001). The difference, twice as many seedlings in Sylt than Uithuizen, initiated itself after week two, when seedling count in Sylt doubled, whilst in Uithuizen it stayed relatively constant. However, the difference between the two sediment origins was not significant when only looking at total seedling numbers at harvest (Figure 5.a, t-test, df = 8, p = 0.108).

Morphology and biomass

Morphological traits (specific leaf area, mean leaf length, mean leaf width and maximum root length) did not differ between sediment origins (donor vs restoration) (Figure 6). Maximum leaf length of seedlings grown on Sylt was 1.3 times longer than seedlings grown on Uithuizen, however these results were not significantly different (t-test, df = 14, p = 0.8705) (Figure 6.b).

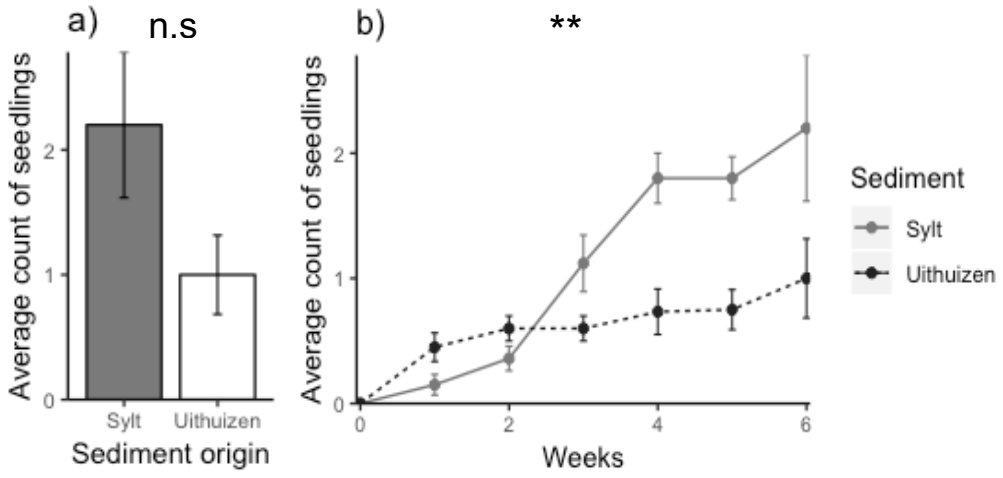


Figure 5. Results of the average count of seedlings grown on Sylt (grey) vs Uithuizen (white) sediment. (a) This barplot represents the total average amount of counted seedlings on the last count on the y-axis, compared with the sediment origin on the x-axis. The counts do not differ significantly from one another (t-test, df = 8, p = 0.108). (b) This is the sprouting of new seedlings over time, where the y-axis shows the average observed count and the x-axis the time in weeks. The average seedling counts from both sediment origins over time differ significantly from each other (Linear Mixed model,

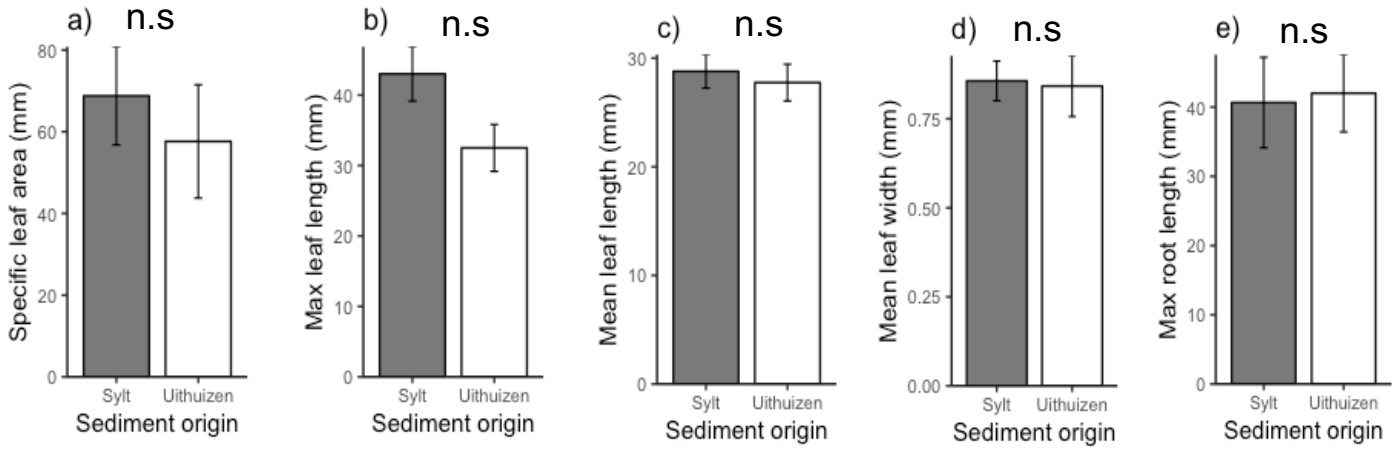


Figure 6. Results of the morphology of seedlings grown on Sylt (grey) vs Uithuizen (white) sediment. To compare the seedlings from Uithuizen and Sylt, the different morphology variables taken into account are portraited on the y-axis and the two sediment origins are shown on the x-axis. The results show the following: (a) the specific leaf areas do not differ significantly (t-test, df = 14, p = 0.8591); (b) the maximum leaf lengths do not differ significantly (t-test, df = 14, p = 0.8705); (c) the mean leaf lengths do not differ significantly (t-test, df = 14, p = 0.7216); (d) the mean leaf widths do not differ significantly (t-test, df = 14, p = 0.6214); (e) the maximum root lengths do not differ significantly (t-test, df = 14, p = 0.917).

Although seedling biomass seemed higher on donor sediment (Figure 7) for almost all biomass traits (mean plant biomass, total plant biomass, aboveground biomass, and belowground biomass), these results were not significant (t-tests, p >0.05). The aboveground/belowground ratio seemed higher for seedlings grown on Uithuizen, however, this was also statistically not significant (Welch t-test, df = 4.878, p = 0.1941).

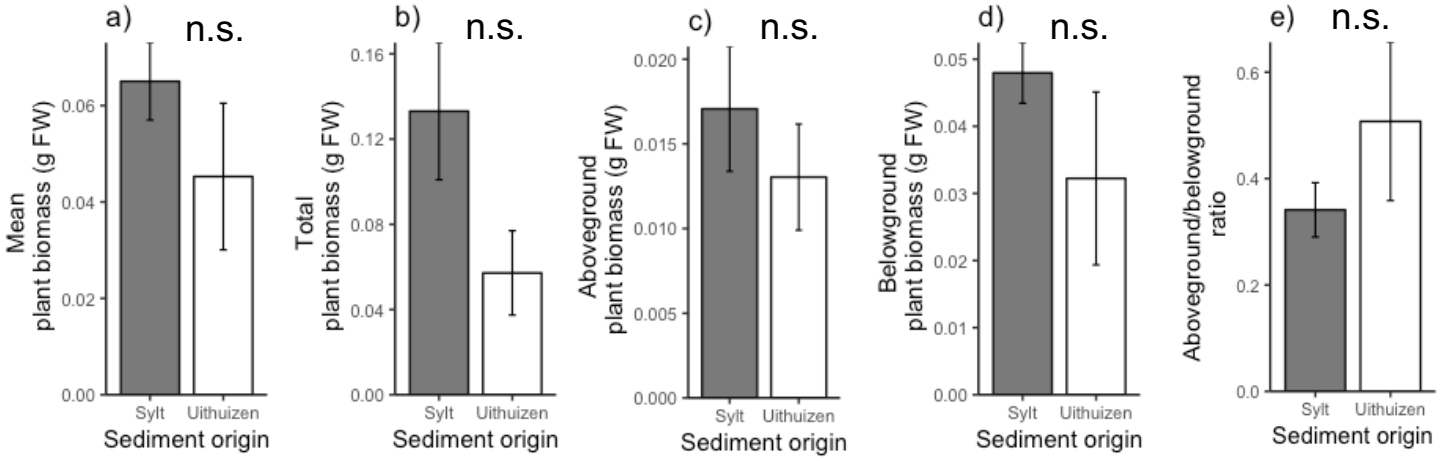


Figure 7. Results of the biomass data of seedlings grown on Sylt (grey) and Uithuizen (white) sediment. Diifferent biomass variables are shown on the y-axis, while the x-axis show the different sediment origins. The graphs show the following: (a) the mean plant biomasses do not differ significantly (t-test, df = 14, p = 0.543); (b) the total plant biomasses of the do not differ significantly (t-test, df = 7, p = 0.1025); (c) the aboveground biomasses do not differ significantly (t-test, p = 0.6473); (d) the belowground biomasses do not differ significantly (t-test, df = 14, p = 0.3377); (e) a Welch t-test was done and the results show that the aboveground/belowground biomass ratios do not differ significantly (Welch t-test, df = 4.8785, p = 0.1941).

Nutrients

Nutrient composition differed between donor and restoration sediment (Figure 8). Ammonium and phosphate levels were, respectively, four times and 2.3 times higher in Sylt (donor) (Figure 8.b & d). However, only ammonium levels were significantly so (t-test, df = 8, p = 0.005). The iron levels in Uithuizen were about 80 times higher compared to Sylt (Figure 8.c, t-test, df = 7, p = 0.003). The nitrate levels of both sediment origins were relatively low, showing a slightly higher level (0.1322 mg/l more) in Uithuizen, but this was not significant (Figure 8.a, t-test, df = 8, p = 0.1204).

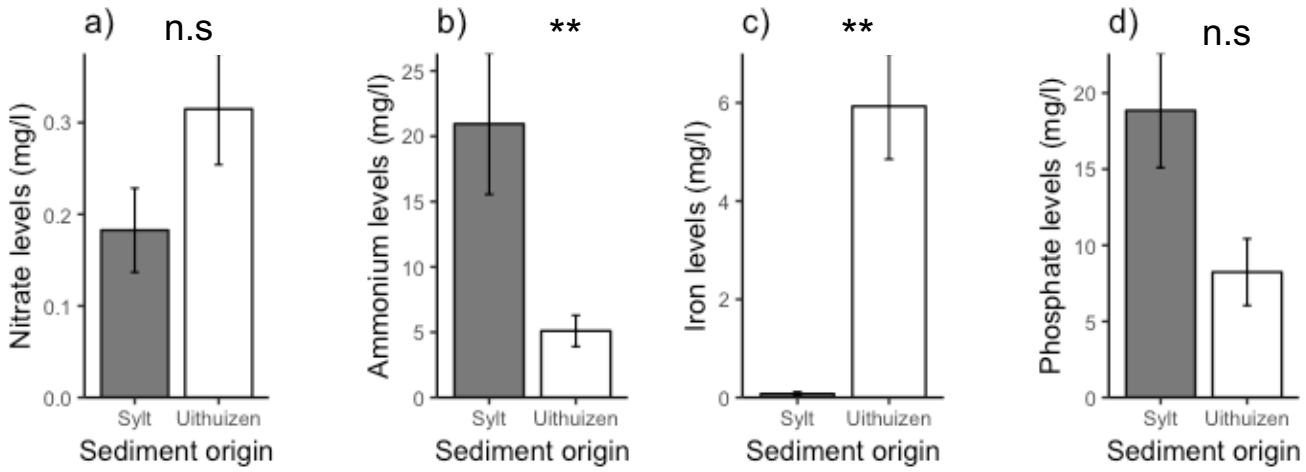


Figure 8. Results of the nutrient levels measured in the sediment origin treatments. In figures a to d different graphs with four different nutrient levels on the y-axis are portrayed. The x-axes all represent sediment origins: Sylt and Uithuizen. The graphs show the following: (a) nitrate levels do not differ significantly (t-test, df = 8, p = 0.1204); (b) ammonium levels within Sylt sediment are significantly higher than in Uithuizen (t-test, df = 8, p = 0.005); (c) the iron levels in Uithuizen sediment are significantly higher than in Sylt (t-test, df = 7, p = 0.003); (d) the phosphate levels between both sediment types do not differ (t-test, df = 7, p = 0.057).

Diversity of the sediment microbiome

In both sediment origins combined, 56.146 reads of bacterial DNA were found of which 92% were classified with an accuracy of 83%. Of this total 83% (45.952 reads) was found in the donor site (94% of which were classified) and 18% (10.194 reads) in the restoration site (84% classified). For the archaea 310.420 reads labelled as archaeal DNA were identified with an accuracy of 82%, however, this was somewhat diluted with DNA from cyanobacteria. The donor site contributed 92.996 reads (30% of total reads where 70% were classified with an accuracy of 84%), while the restoration site represented 70%, of which only 44% were classified (accuracy of 81%).

Archaea

Alpha diversity of archaea species was three times higher in Uithuizen according to the Shanon index, while only 1.4 times higher according to Chao1 (Figure 9.a). The difference in diversity according to the Shannon index measure was significant (t-test, df = 2, p = 0.0018), while the Chao1 metric was borderline significant (t-test, df = 2, p = 0.0556).

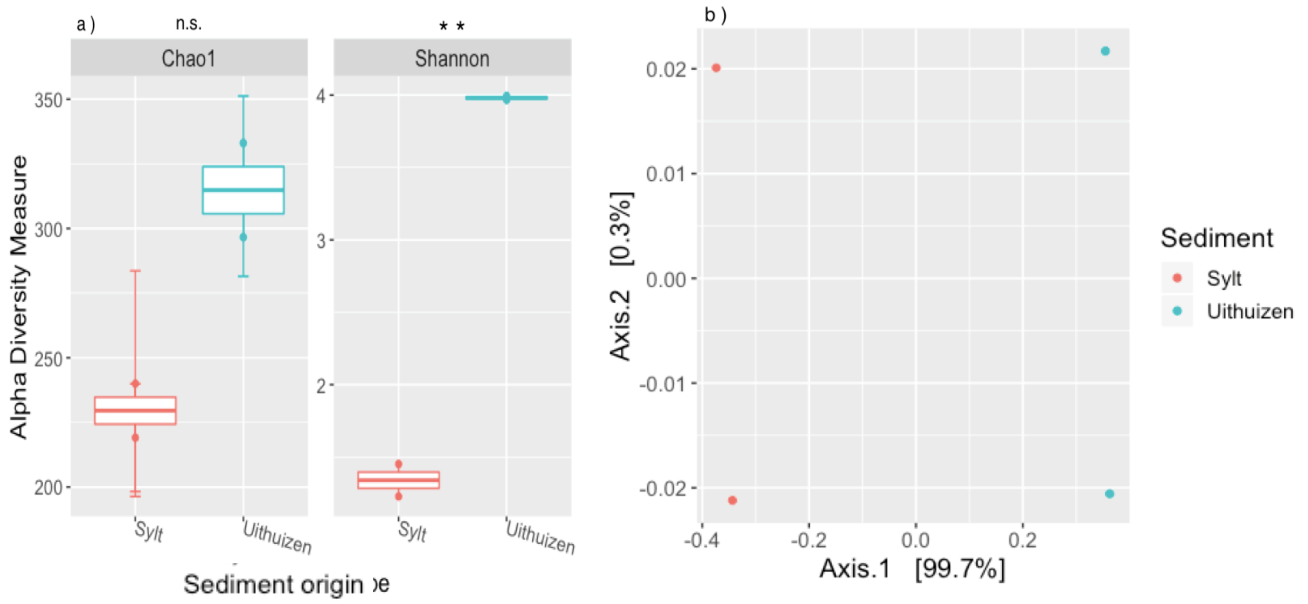


Figure 9. The diversities for the archaea species found in Sylt and Uithuizen sediment. (a) Two alpha-diversity metrics, Chao1 and Shannon, shown as boxplots. Both the x-axes show the sediment origin treatments, while the y-axis show the specific alpha-diversity measure per metric. The Chao1 metric is not significant (t-test, df = 2, p = 0.0556), while the Shannon metric is (t-test, df = 2, p = 0.0018). (b) A principal coordinates analysis of microbial communities based on weighted Unifrac distances. Both axis portray a possible way to explain the in-sample variation, where axis.2 is responsible for 0.3% and axis.1 99.7%. Samples are coloured by treatment.

Axis.1 from the PCoA (Figure 9.b), which explained 99.7% of total variation, showed inter-sample variation between both sediment origins. Axis.2 showed that one sample per treatment had a similar archaeal composition to the other treatment, yet a contrasting composition to the other sample within treatment. However, as this axis only explains 0.3%, it will not be taken into further consideration. Consequently, the microbial communities detected in each sediment origin were distinctly different from one-another, while constant per sediment.

Bacteria

The alpha-diversity metrics for the bacterial composition had contrasting results (Figure 10.a). The Chao1-metric indicated that Sylt had a 1.5 times higher alpha-diversity, whereas the Shannon-index suggested that Uithuizen had a 1.4 times higher alpha-diversity. As the Shannon index lays more weight on richness and Chao1 index more on low abundance species (Kim et al. 2017), Chao1 is more suitable for the current situation. However, neither differences were significant (Chao1, t-test, df = 2, p = 0.3509; Shannon, t-test, df = 2, p = 0.1443).

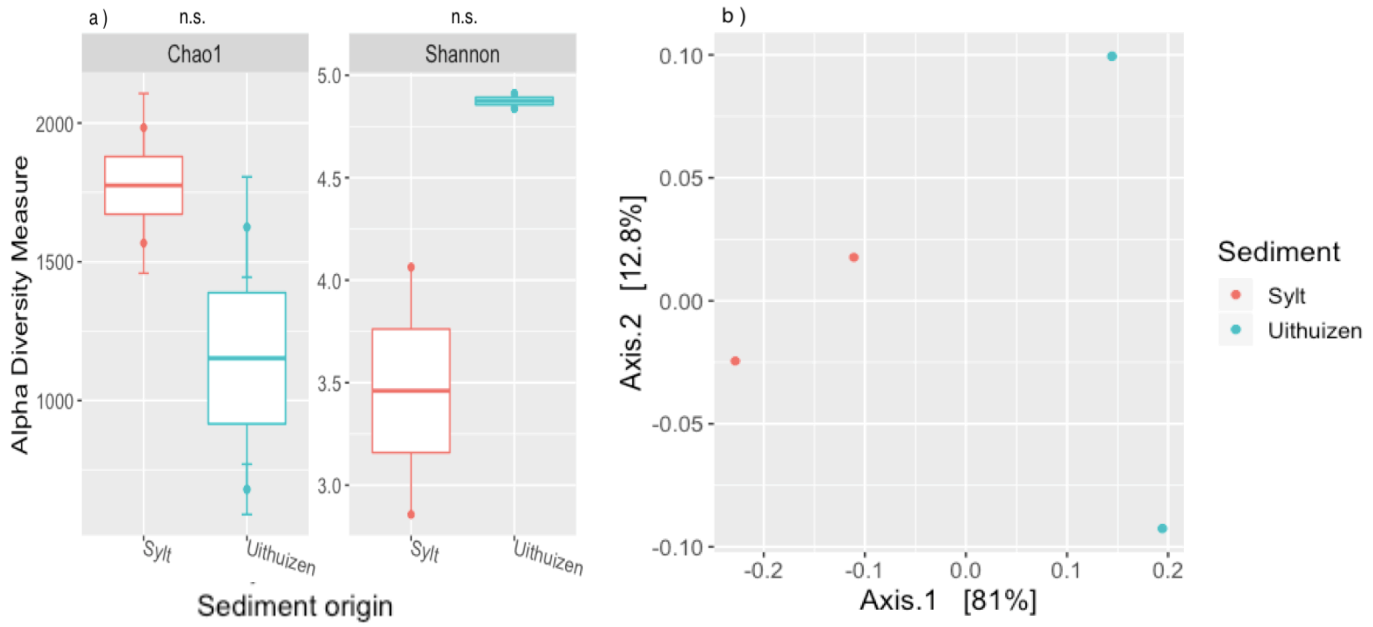


Figure 10. The diversities for the bacteria species found in Sylt and Uithuizen sediment. (a) Two alpha-diversity metrics, Chao1 and Shannon, shown as boxplots. Both the x-axes show the sediment origin treatments, while the y-axis show the specific alpha-diversity measure per metric. Both metrics are not significant (Chao1, t-test, df = 2, p = 0.3509; Shannon, t-test, df = 2, p = 0.1443). (b) A principal coordinates analysis of microbial communities based on weighted Unifrac distances. Both axes portray a possible way to explain the in-sample variation, where axis.1 explains 81% of the total variation and axis.2 12.8%. Samples are coloured by treatment.

Comparable to the archaea data, beta diversity of the bacteria showed inter-sample variation. Axis.1 explained 81% of total variation and showed that both EUs per sediment origin were clustered together, while the treatments were widespread. Axis.2, which explained 12.8%, suggested a similar trend, with one exception: bacterial compositions of the Uithuizen samples were more widespread over the axis. Altogether, there was inter-sample variation between sediment origins.

Composition of the sediment microbiome

In Uithuizen sediment 93% of the total micro-organisms detected were classified as archaea species (82% accuracy), while in Sylt the composition seemed to be more dominated by bacteria (68%). In Uithuizen 1.5

times more archaea taxas were classified and 3.4 times more archaea sequences detected. Sylt had almost twice the number of bacteria taxa compared to Uithuizen and five times higher amount of sequences classified.

Archaea

Sylt was more homogenous than Uithuizen, as 78.73% of total relative abundance was contributed to one species in the ten most abundant archaea species (Table 4), namely *Methanobacterium alcaliphilum*, while in Uithuizen 53.46% was explained by the entire top ten. All species in Table 4 are methanogens: methanogenic microorganisms that gain energy by reducing carbon dioxide into methane in anaerobic circumstances (Garcia et al. 2000, Sparks 2007).

Table 4. The top 10 most abundant archaea species in both sediment origins. The data showed is the mean of the two samples per treatment. Therefore, the total count in both treatments was two times as high. The order of the species shown is based on the abundance in the Sylt treatment. The % columns show the relative species abundance.

	Sylt		Uithuizen	
	Count	%	Count	%
Methanobacterium alcaliphilum	4768	78.73%	821	3.98%
Methanobacterium beijingense	123.5	2.04%	1319.5	6.40%
Methanocaldococcus jannaschii	56	0.92%	708	3.44%
Methanosarcina horonobensis	29	0.48%	792.5	3.85%
Methanocaldococcus vulcanius	25.5	0.42%	632.5	3.07%
Methanothermobacter thermophilus	12.5	0.21%	1212.5	5.88%
Methanobrevibacter thaueri	4.5	0.07%	2326	11.29%
Methanoculleus horonobensis	3.5	0.06%	1562.5	7.58%
Methanocaldococcus infernus	2.5	0.04%	578.5	2.81%
Methanobrevibacter woesei	2	0.03%	1062.5	5.16%
Total	**6,056**	**83.01%**	**20,607**	**53.46%**

Total count of archaea species in Uithuizen was three times higher than total count in Sylt, leading to an overall higher abundance for all orders (Figure 11.a). The large manifestation of methanogens was also visible in the relative abundance stacked barplot (Figure 11.b) as 85% of species within Sylt sediment and 45% in Uithuizen were from *Methanobacteria* class, known methanogens. In Uithuizen *Methanomicrobia* and *Methanococci* had a higher, respectively 20% and 8% more, relative abundance when compared with Sylt, leading to a different composition between the two. All three classes, however,

are part of a diverse group called methanogenic bacteria which have three uniting factors: they are all 1) archaea, 2) methanogens and 3) strictly anaerobe (Sparks 2007).

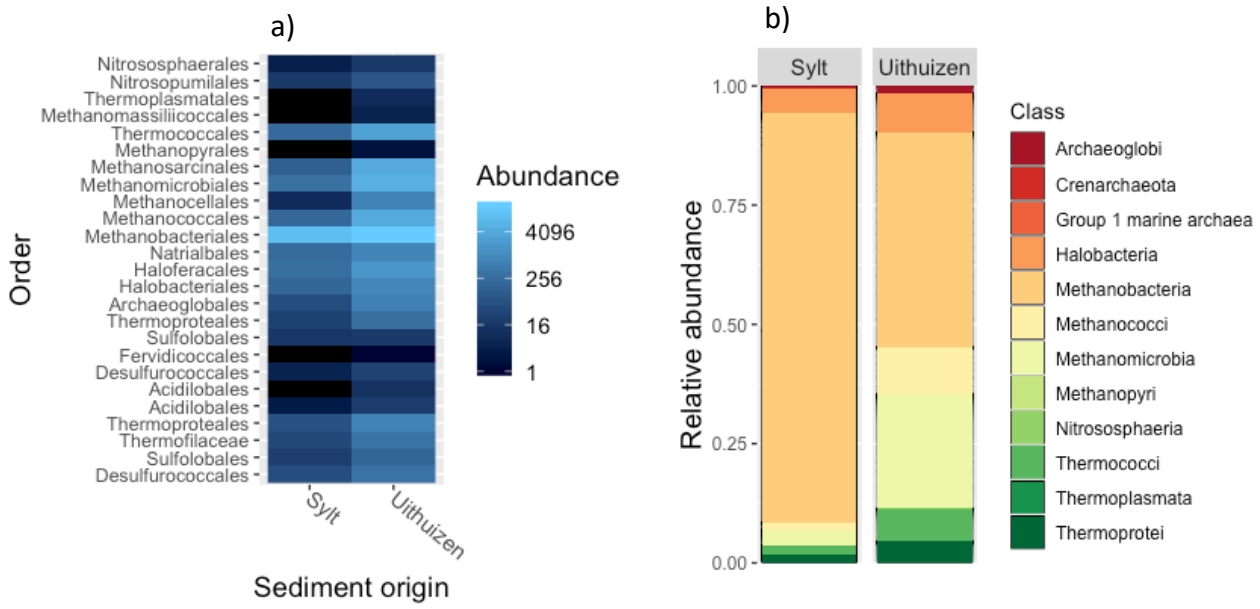


Figure 11. Two different abundance plots for the sediment origin treatment. (a) This is a heatmap showing the abundances of all the found orders on the left. The lighter the color, the more counts the order has. A black bar resembles no count data. (b) This stacked barplot shows the composition of the classes of the archaea species via a relative abundance. The y-axis shows the relative abundance.

Bacteria

The composition of Sylt sediment was more homogenous than Uithuizen sediment, as 66,44% of total counts were composed of the top 10 most abundant species, while in Uithuizen this was only 32.45% (Table 5). In total 40.60% of Sylt's relative abundance constituted out of one species, *Nodularia spumigena*, which is a toxic, hazardous, planktonic cyanobacteria, known to cause algal blooms during an increase in phosphorus and nitrogen (Krüger et al. 2009). Within Uithuizen the abundances were more spread out, with the highest abundance (14.72%) for a sulphur-, nitrate-, and thiosulfate reducing bacteria called *Sulfurovum aggregans* (Mino et al. 2014).

Table 5. The top 10 most abundant bacteria species in both sediment origins. The data shown is the mean of the two samples per treatment. Therefore, the total count in both treatments was two times as high. The order of the species shown is based on the abundance in the Sylt treatment. The % columns show the relative species abundance.

	Sylt		Uithuizen	
	Count	%	Count	%
Nodularia spumigena	5139.5	40.60%	41	2.66%
Ilumatobacter nonamiensis	1095.5	8.65%	75	4.86%
Ilumatobacter fluminis	891.5	7.04%	81.5	5.28%
Fulvivirga lutimaris	266	2.10%	15.5	1.00%
Sulfurovum aggregans	262.5	2.07%	227	14.72%
Loktanella rosea	177.5	1.40%	26	1.69%
Lewinella cohaerens	177	1.40%	3.5	0.23%
Cylindrospermum stagnale	138.5	1.09%	0.5	0.03%
Marivita cryptomonadis	133.5	1.05%	20	1.30%
Loktanella maricola	128.5	1.02%	10.5	0.68%
Total	**12,658**	**66.44%**	**1,543**	**32.45%**

Sylt sediment had an eight times higher total count of identified species, which lead to an overall higher counted abundance (Figure 12.a), yet the same orders were present in both sediment origins. Nonetheless, there were certain variations on class level (Figure 12.b), e.g. *Cyanophyceae* had a 44% relative abundance in Sylt, while in Uithuizen this was only 4% (caused by the earlier mentioned *N. spumigena* species). Other differences in composition were smaller, like *Deltaproteo-* and *Epsilonproteobacteria* having a six, respectively four, times higher abundance in Uithuizen than in Sylt. *Epsilonproteobacteria* are mostly known for their pathogenic genera, however some members of this class also play a ecologically important role due to their capability to combine sulphur oxidation with other chemical processes (Waite et al. 2017). Most species from *Epsilonproteobacteria* found here are known for their sulphur oxidizing traits, not unlike the species found from the *Deltaproteobacteria*, which are also well-known for their sulphate-reducing capacities (Mussmann et al. 2005).

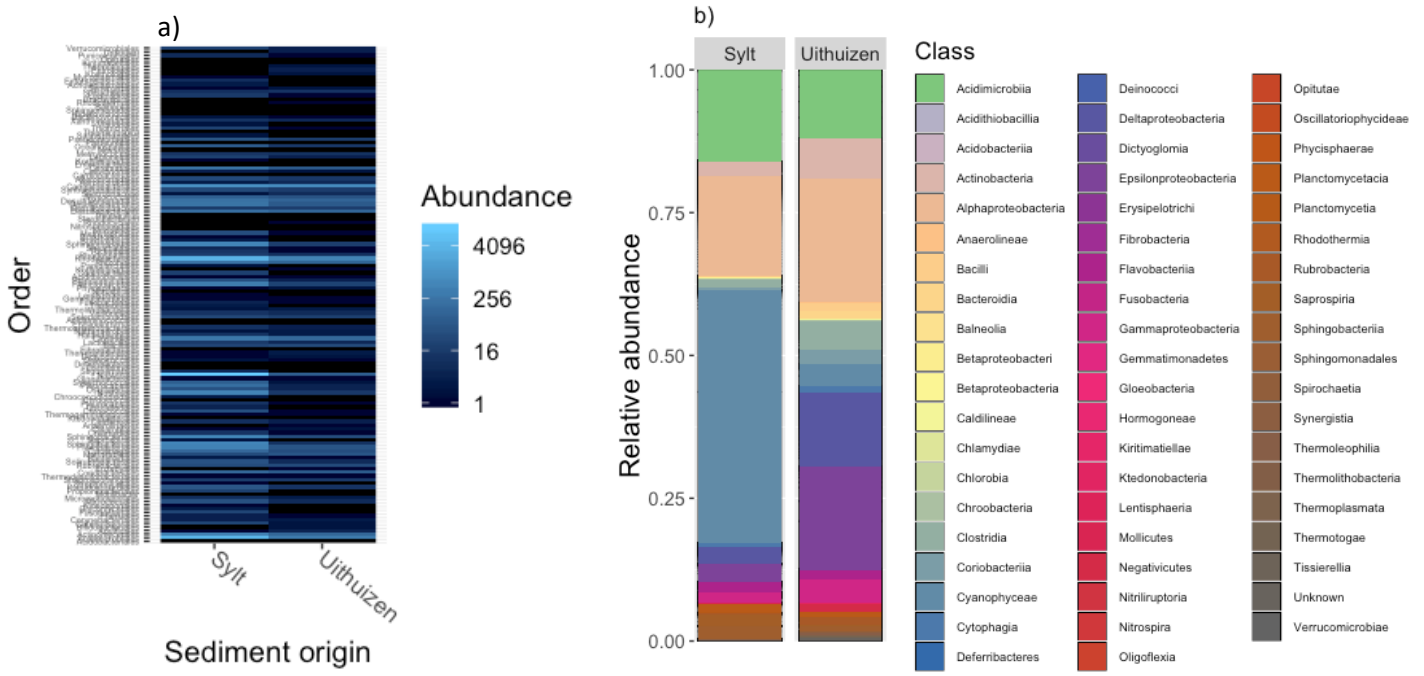


Figure 12. Two different abundance plots representing the sediment origin treatment. (a) This is a heatmap showing the abundances of all the found orders on the left. The lighter the color, the more counts the order has. A black bar resembles no count data. (b) This stacked barplot shows the composition of the classes of the bacteria species via a relative abundance. The y-axis shows the relative abundance.

Sediment sterilization

Seedling numbers, morphology, biomass and nutrient levels

Seedling numbers

A 2.2 times higher count of sprouted seedlings was found on the day of harvest within sterilized sediment (Figure 13.a), however, this difference was not significant (t-test, df = 8, p = 0.2005). Germination rates were significantly different between sterilized and not-sterilized sediment (Figure 13.b, Linear Mixed model, Chisq = 17.194, $p < 0.001$). During week three, more seedlings began sprouting within sterilized sediment, while there was little change within normal Uithuizen sediment. In week four the average of sterilized sediment was a little over two seedlings, while non-sterilized sediment only had an average of one seedling after six weeks.

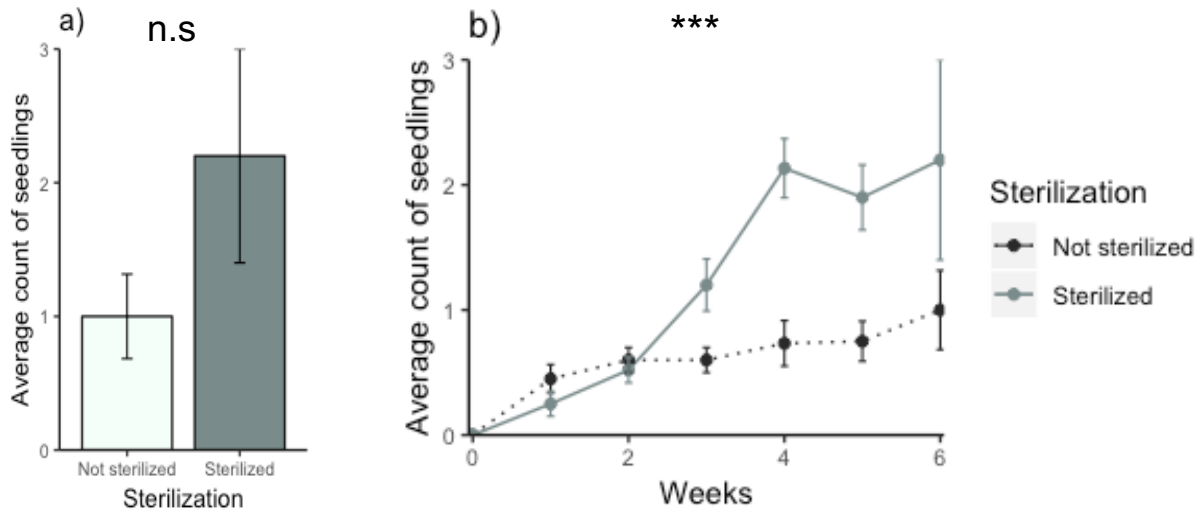


Figure 13. Results of the average count of seedlings grown on sterilized (dark blue) and not-sterilized (light blue) sediment. (a) This barplot represents the total average amount of counted seedlings on the last count on the y-axis, compared with the sterilization treatment on the x-axis. The counts do not differ significantly from one another (t-test, df = 8, p = 0.2005). (b) This is the sprouting of new seedlings over time, where the y-axis shows the average observed count and the x-axis the time in weeks. The average seedling counts from both sediment origins over time differ significantly from each other (Linear Mixed model, Chisq = 17.194, p <0.001).

Morphology and biomass

Seedlings grown on sterilized sediment had longer (mean leaf length 1.6 times and max leaf length 2.2 times) and wider leaves (1.2 times) with bigger (almost three times) specific leaf areas than ones grown on normal Uithuizen sediment (Figure 14). Differences in maximum leaf length and mean leaf length were both significant (Figure 14.b, Welch t-test, df = 13.783, p = 0.01067; Figure 14.c, Welch t-test, df = 13.969, p = 0.02191), while specific leaf area and mean leaf width were marginally insignificant (Figure 14.a, t-test, df = 14, p = 0.06875; Figure 14.d, t-test, df = 14, p = 0.0606). Maximum root length of seedlings was 1.5 times longer within not-sterilized sediment, but this difference was not significant (Figure 14.d, t-test, df = 14, p = 0.1136).

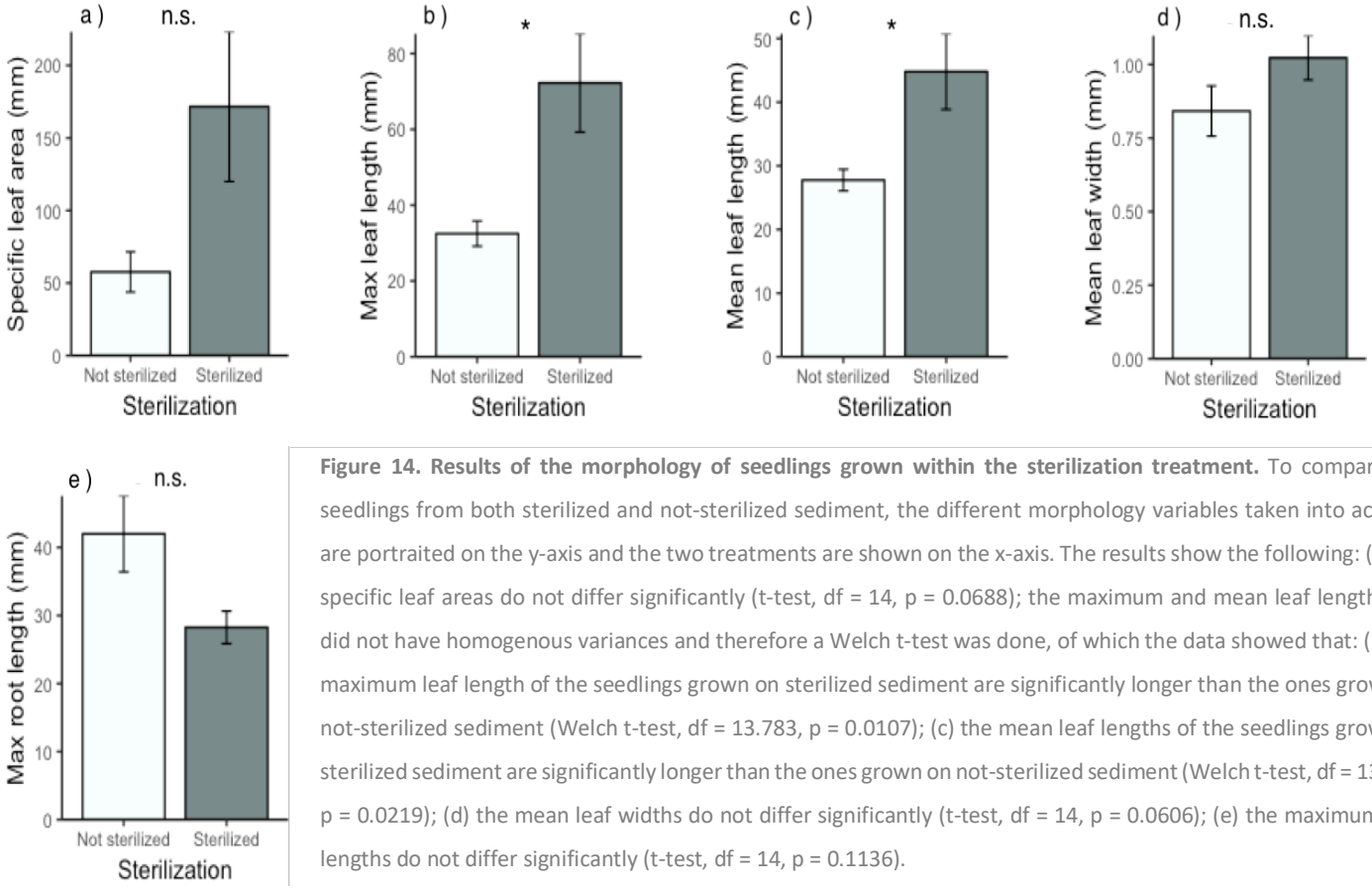


Figure 14. Results of the morphology of seedlings grown within the sterilization treatment. To compare the seedlings from both sterilized and not-sterilized sediment, the different morphology variables taken into account are portraited on the y-axis and the two treatments are shown on the x-axis. The results show the following: (a) the specific leaf areas do not differ significantly (t-test, df = 14, p = 0.0688); the maximum and mean leaf length data did not have homogenous variances and therefore a Welch t-test was done, of which the data showed that: (b) the maximum leaf length of the seedlings grown on sterilized sediment are significantly longer than the ones grown on not-sterilized sediment (Welch t-test, df = 13.783, p = 0.0107); (c) the mean leaf lengths of the seedlings grown on sterilized sediment are significantly longer than the ones grown on not-sterilized sediment (Welch t-test, df = 13.969, p = 0.0219); (d) the mean leaf widths do not differ significantly (t-test, df = 14, p = 0.0606); (e) the maximum root lengths do not differ significantly (t-test, df = 14, p = 0.1136).

Seedlings grown on sterilized sediment were overall (mean 1.7 times, total 4.2 times, aboveground 2.5 times, belowground 1.3 times and aboveground/belowground ratio approximately three times) heavier than seedlings from normal sediment (Figure 15). However, this trend was marginally insignificant for mean, total and aboveground plant biomass (Figure 15.a-c, t-test, $0.10 > p > 0.05$) and insignificant for belowground biomass and aboveground/belowground ratio (Figure 15.d, t-test, df = 14, p = 0.3499; Figure 15.e, Welch t-test, df = 13.449, p = 0.2946).

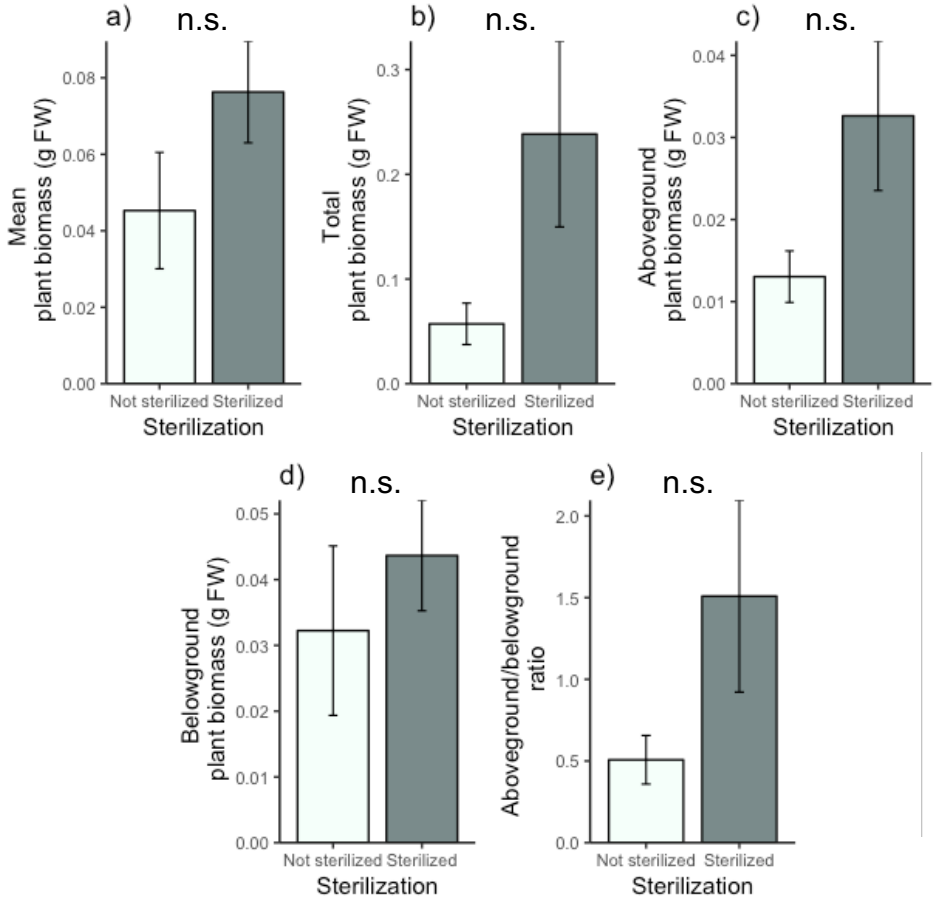


Figure 15. Results of the biomass data of seedlings grown on not-sterilized (light blue) vs sterilized (dark blue) sediment. In all cases, the different biomass variables are shown on the y-axis, while the x-axis show if the sediment has been sterilized or not. The different variables give the following results: (a) the mean plant biomasses do not differ significantly (t-test, df = 14, p = 0.0596); (b) the total plant biomasses do not differ significantly (t-test, df = 6, p = 0.0967); (c) the aboveground biomasses do not differ significantly (t-test, df = 14, p = 0.0977); (d) the belowground biomasses do not differ significantly (t-test, df = 14, p = 0.3499). The variances of the ratio data were not homogenous and therefore a Welch t-test was used, which showed: (e) the aboveground/belowground biomass ratios do not differ significantly (Welch t-test, df = 13.449, p = 0.2946).

Nutrient levels

With a difference of only 0.0248mg/l, nitrate levels were the same between treatments (Figure 16.a, t-test, df = 8, p = 0.778). However, the levels of ammonium, iron, and phosphate were all higher (respectively, 11 times, two times and two times) within sterilized sediment (Figure 16.b-d). All three differences were significant (ammonium, t-test, df = 8, p <0.001; iron, t-test, df = 7, p = 0.007; phosphate, t-test, df = 7, p = 0.0144).

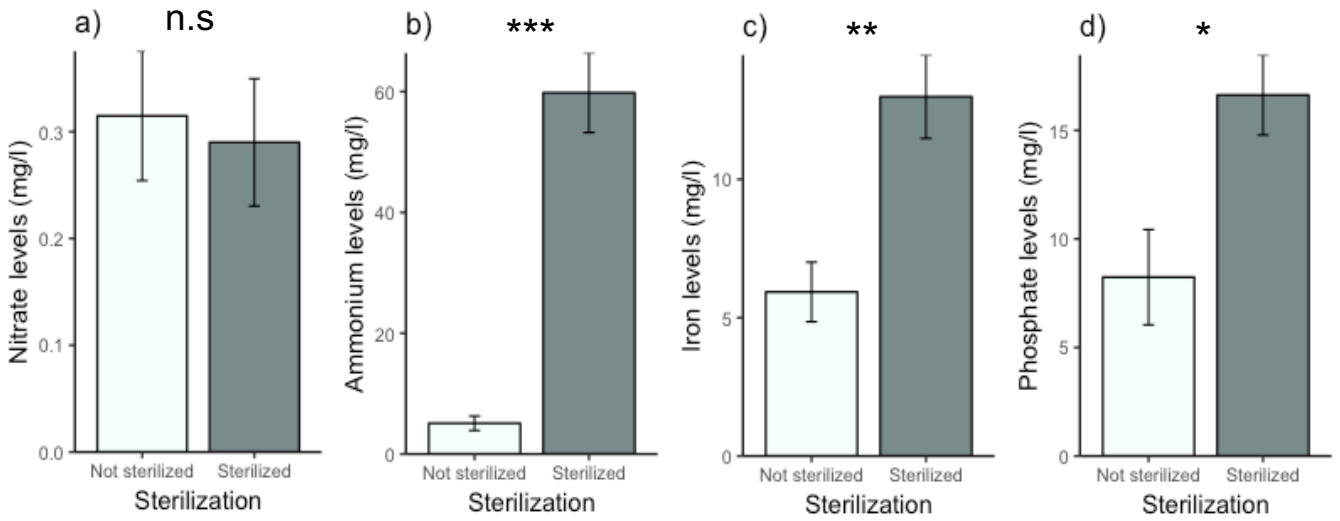


Figure 16. Results of the nutrient levels measured in the sediment origin treatments. In figures a to d different graphs with four different nutrient levels on the y-axis are portrayed. The x-axis represent sediment origins: Sylt and Uithuizen. The graphs show the following: (a) nitrate levels do not differ significantly (t-test, df = 8, p = 0.778); (b) ammonium levels within Sylt sediment are significantly higher than in Uithuizen (t-test, df = 8, p <0.001); (c) iron levels in Uithuizen sediment are significantly higher than in Sylt (t-test, df = 7, p = 0.007); (d) the phosphate levels between both sediment types differ significantly (t-test, df = 7, p = 0.0144).

Diversity of the sediment microbiome

In total 971 unique species were identified (average accuracy of the original reads was 81.5%) within the sediment microbiome of normal Uithuizen sediment, while in sterilized sediment this total was 892 (same average accuracy). In sterilized sediment 92% of these species were bacteria, in not-sterilized sediment this was 69%.

Archaea

Chao1 indicated a 4.6 times higher alpha diversity measure for not sterilized sediment when comparing to sterilized sediment, and Shannon index a 1.5 times higher alpha diversity measure (Figure 17a). The differences between treatments were significant for both diversity metrics (Chao1, t-test, df = 2, p = 0.0238; Shannon, t-test, df = 2, p = 0.0216) indicating not sterilized sediment had an overall higher diversity in archaea species.

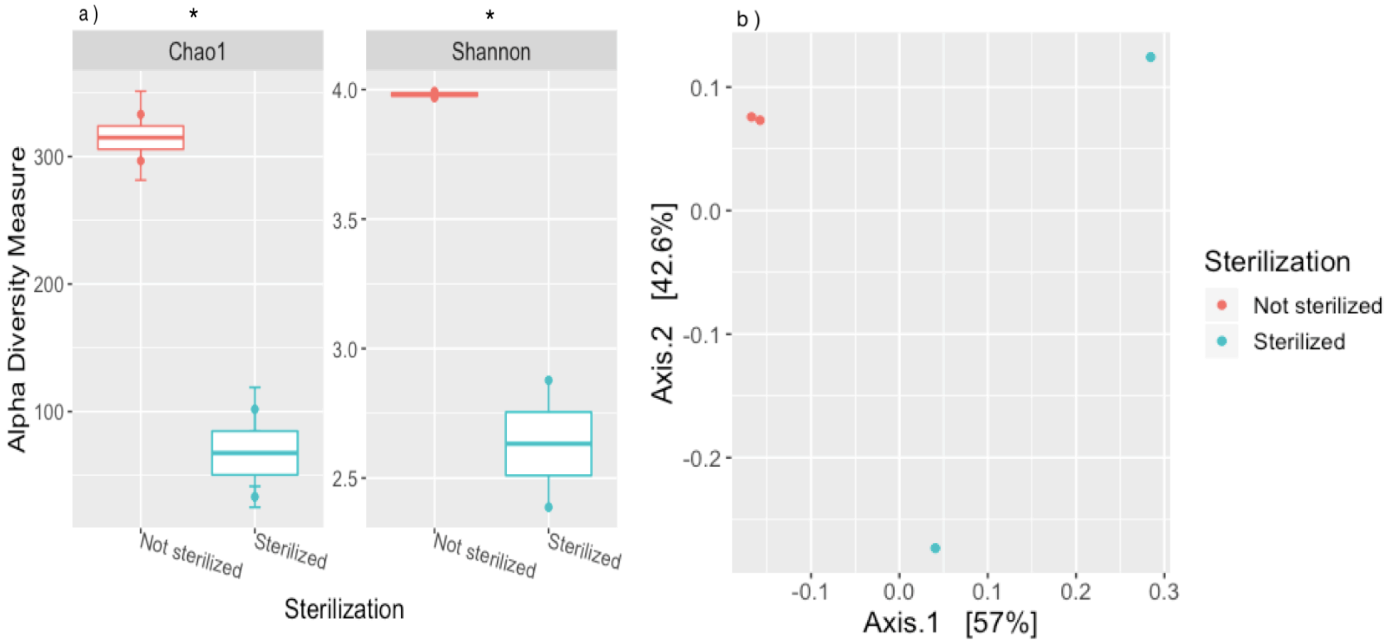


Figure 17. The diversities for the archaea species found in sterilized and not-sterilized sediment. (a) Two alpha-diversity metrics, Chao1 and Shannon, shown as boxplots. On the x-axes the treatments are portrayed. The y-axes show the two alpha-diversity measures. Both boxplots show a significant higher alpha-diversity for not-sterilized sediment (Chao1, t-test, df = 2, p = 0.0238; Shannon, t-test, df = 2, p = 0.0216). (b) A principal coordinates analysis of microbial communities based on weighted Unifrac distances. Both axes portray a possible way to explain the in-sample variation, where axis.1 explains 57% and axis.2 42.6%. Samples are coloured by treatment.

The beta diversity revealed an overall clustering of not-sterilized samples, while sterilized samples were more widespread (Figure 17.b). On Axis.2, which explained 42.6% of total variation, one of the sterilized samples seemed to have an archaeal composition that resembled not-sterilized samples more than it did the other sterilized sample. This pattern was, however, not visible within axis.1, which explained 57%. Axis.1 insinuated that the treatments had inter-sample variation, as the treatments were spread out.

<u>Bacteria</u>

The Chao1 index indicated that alpha-diversity in both treatments were similar for bacteria species, which is confirmed by an insignificant result of the t-test (Figure 18.a, t-test, df = 2, p = 0.8639). In the Shannon index, the average alpha-diversity measure of normal sediment was 0.6 times higher than the average of sterilized sediment due to the high richness and evenness within the not sterilized samples, which was not paralleled in one of the sterilized samples. However, this difference was not significant (Figure 18.a, t-test, df = 2, p = 0.4311).

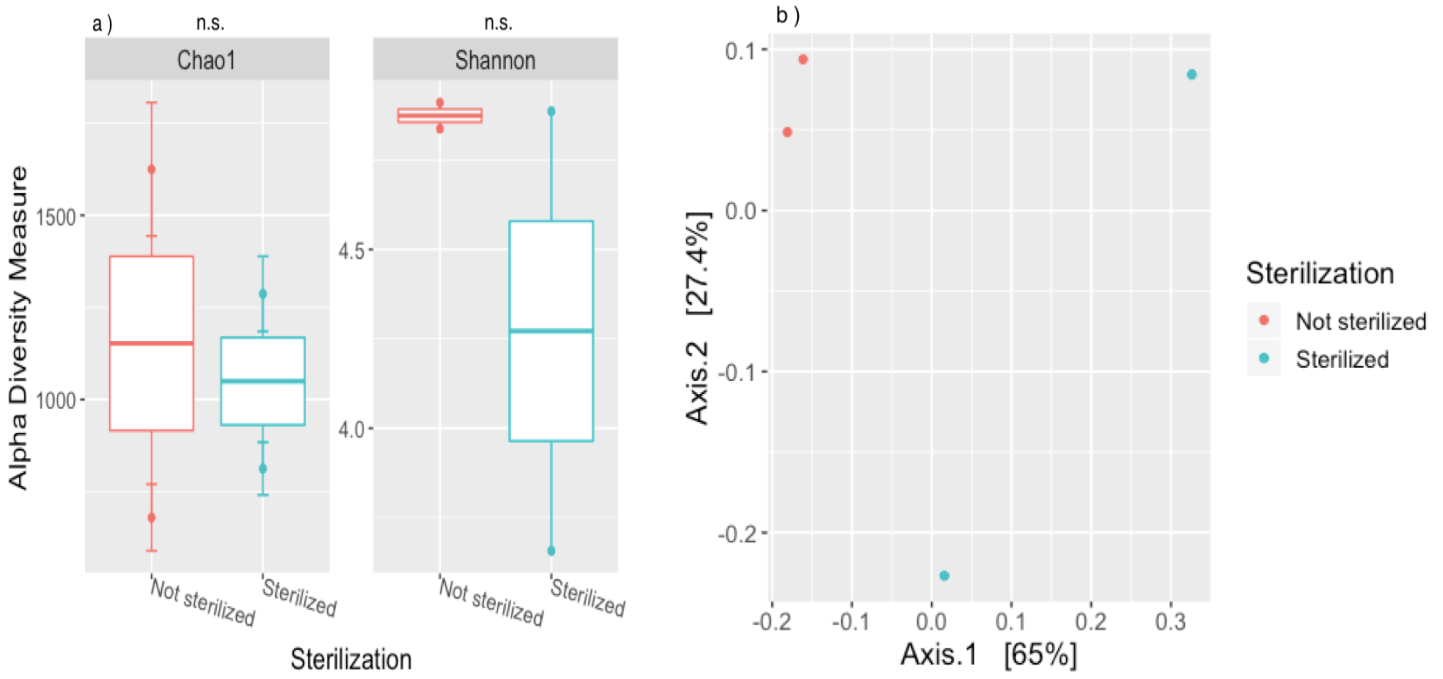


Figure 18. The diversities for the bacteria species found in sterilized and not-sterilized sediment. (a) Two alpha-diversity metrics, Chao1 and Shannon, shown as boxplots. Both the x-axes show the sterilization treatments, while the y-axis show the specific alpha-diversity measure per metric. Both metrics are not significant (Chao1 metric, t-test, df = 2, p = 0.8639; Shannon metric, t-test, df = 2, p = 0.4311). (b) A principal coordinates analysis of microbial communities based on weighted Unifrac distances. Both axes portray a possible way to explain the in-sample variation, where axis.2 is responsible for 27.4% and axis.1 65%. Samples are coloured by treatment.

Weighted Unifrac dissimilarities (Figure 18.b) displayed the same as the archaea PCoA: a clustering of not sterilized sediment on both Axis.1 and Axis.2, which respectively explained 65% and 27.4% of total variation. Sterilized samples, based on Axis.2, had more variation in their bacterial composition as the samples were very widespread and one of the samples resembled not-sterilized sediment. However, most of the variation can be explained by Axis.1, which showed that there was inter-sample variation between sterilized and not-sterilized Uithuizen sediment.

Composition of the sediment microbiome

The total count of identified bacteria and archaea microorganisms within not-sterilized sediment was 44.299, which was almost four times higher than total count in sterilized sediment. In not sterilized sediment 93% were archaea species, while in sterilized sediment 93% were bacteria.

<u>Archaea</u>

Not sterilized sediment had a heterogenous distribution, as the entire top 10 only contributed to 53.46% of total abundance, with ranging percentages from 2.81% to 11.29% (Table 6). In sterilized sediment the top 10 most abundant species constituted 66,36% of total abundance and the distribution was more homogenous than in not-sterilized sediment, as *Methanothermobacter thermophilus* contributed to 39.35% of total abundance. The *Methanothermobacter* genus consists out of thermophilic methanogenic microorganisms, who are strictly anaerobic and use ammonia as their sole nitrogen source (Wasserfallen et al. 2000, Boone 2015). All species within the top 10 are methanogens.

Table 6. The top 10 most abundant archaea species in both sterilization treatments. The data shown is the mean of the two samples per treatment. Therefore, the total count in both treatments was two times as high. The order of the species shown is based on the abundance in the not-sterilized treatment. The % columns show the relative species abundance.

	Not sterilized		**Sterilized**	
	Count	%	Count	%
Methanobrevibacter thaueri	2326	11.29%	1.5	0.39%
Methanoculleus horonobensis	1562.5	7.58%	0	0.00%
Methanobacterium beijingense	1319.5	6.40%	3.5	0.91%
Methanothermobacter thermophilus	1212.5	5.88%	151.5	39.35%
Methanobrevibacter woesei	1062.5	5.16%	0.5	0.13%
Methanobacterium alcaliphilum	821	3.98%	45	11.69%
Methanosarcina horonobensis	792.5	3.85%	7.5	1.95%
Methanocaldococcus jannaschii	708	3.44%	0.5	0.13%
Methanocaldococcus vulcanius	632.5	3.07%	9.5	2.47%
Methanocaldococcus infernus	578.5	2.81%	36	9.35%
Total	**20,607**	**53.46%**	**385**	**66.36%**

Total count of archaea microorganisms was approximately 54 times higher within not sterilized sediment, which lead to an overall higher recorded abundance (Table 6, Figure 19.a). The heatmap indicated that all orders present within sterilized sediment were also present within not-sterilized sediment, but with a lower recorded abundance and slightly different composition. The relative abundance barplot showed a similar pattern on class level (Figure 19.b). The most prominent classes were *Methanobacteria*, *Methanococci* and *Methanomicrobia*, which were the three classes the top 10 most abundant species belong to.

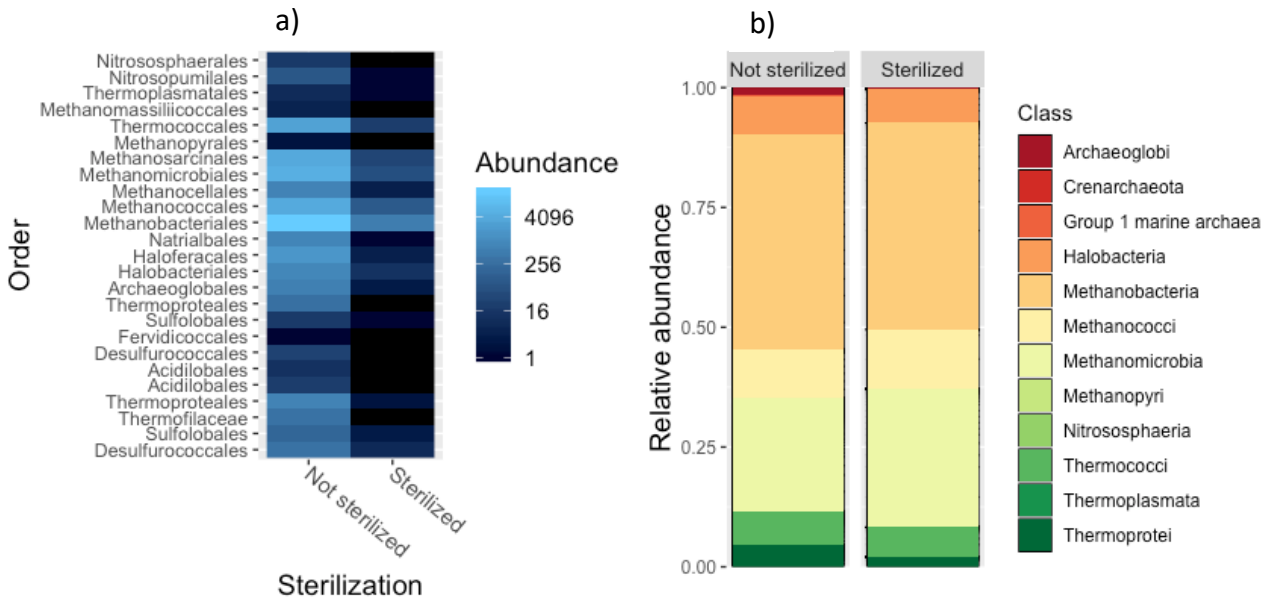


Figure 19. Two different abundance plots for the archaea in both sterilization treatments. (a) This is a heatmap showing the abundances of all the found orders on the left. The lighter the color, the more counts the order has. A black bar resembles no count data. (b) This stacked barplot shows the composition of the classes of the archaea species via the relative abundance. The y-axis shows the relative abundance.

Bacteria

Within not-sterilized sediment, the top 10 most abundant bacteria species comprised 17.5% of total abundance of which 14.72% is contributed to *S. aggregans* (Table 7). In sterilized sediment this contribution was a little higher, namely 46.8%, but percentages per species did not surpass 9.74%, indicating both treatments had heterogenous distribution. Three different species of the *Arcobacter* genus were detected within sterilized sediment (Table 7), while these were not found within not-sterilized sediment. Arcobacters grow optimally under micro-aerobic conditions and some are found in marine environments, although most species are identified as pathogenetic to humans and animals (Ho et al. 2006, Pati et al. 2010, Collado and Figueras 2011). However, as the arcobacters associated with marine environments are involved in nutrient cycling, the detrimental effects found in warm-blooded animals will most likely not be consequential for plant species (Pati et al. 2010). For example, all three *Arcobacter* species found in sterilized sediment – *A. bivalviorum, A. suis* and *A. nitrofigilis* – are known to produce sulphur from sulphides (Campbell et al. 2006) and reduce nitrate to nitrite (Pati et al. 2010, Collado and Figueras 2011, Levican et al. 2013, Pérez-Cataluña et al. 2018). Not unlike the second most abundant species in sterilized sediment: *Sulfurimonas gotlandica* (Han and Perner 2015). In general, *Sulfurimonas* is a group of sulphur-oxidizing bacteria able to utilize many kinds of reduced sulphur compounds (Han and

Perner 2015), not unlike the *Sulfurovum* genus, which contributed the most abundant species within not-sterilized sediment: *S. aggregans*. Relative abundance of *S. aggregans* was 14.72% in not sterilized sediment, while only 0.51% in sterilized.

Table 7. The top 10 most abundant bacteria species in both sterilization treatments. The data shown is the mean of the two samples per treatment. Therefore, the total count in both treatments was two times as high. The order of the species shown is based on the abundance in the sterilized treatment. The % columns show the relative species abundance.

	Not sterilized		Sterilized	
	Count	%	Count	%
Arcobacter nitrofigilis	0	0.00%	527.5	9.74%
Sulfurimonas gotlandica	6	0.39%	510	9.42%
Arcobacter bivalviorum	0	0.00%	322.5	5.96%
Sulfurimonas autotrophica	12	0.78%	308	5.69%
Terasakiella brassicae	0	0.00%	230.5	4.26%
Fusibacter fontis	1.5	0.10%	181	3.34%
Arcobacter suis	0	0.00%	146.5	2.71%
Calothrix desertica	3.5	0.23%	141	2.60%
Marivita cryptomonadis	20	1.30%	139	2.57%
Sulfurovum aggregans	227	14.72%	27.5	0.51%
Total	**1,543**	**17.50%**	**5,414**	**46.80%**

The orders and classes with the highest abundance in not sterilized sediment were also high in abundance in sterilized sediment (Figure 20), namely *Alphaproteobacteria* (20% in not-sterilized and 26% in sterilized sediment) and *Epsilonproteobacteria* (22% in not-sterilized and 39% in sterilized sediment). *Alphaproteobacteria* include the most abundant of marine cellular organisms, with a variety of metabolic strategies, including photosynthesis, nitrogen fixation, ammonia oxidation, and methylotrophy (Williams et al. 2007) and *Epsilonproteobacteria* are known for their sulphur-reducing species (Mussmann et al. 2005, Campbell et al. 2006). Many of the orders (and therefore classes) detected in lower abundance were only so within one of two treatments. An example: the relative abundances of classes *Acidimicrobiia* and *Deltaproteobacteria* had respectively 11% and 13% in not sterilized sediment, while only 0.7% and 3% in sterilized sediment (Figure 20.b). *Acidimicrobiia* are mostly species isolated from extremely acidic environments, except for the one genus found here, *Ilumatobacter*, which is found in estuary sediments or seashore sand (Hu et al. 2018) and *Deltaproteabacteria* are known for sulphur-reducing species (Mussmann et al. 2005).

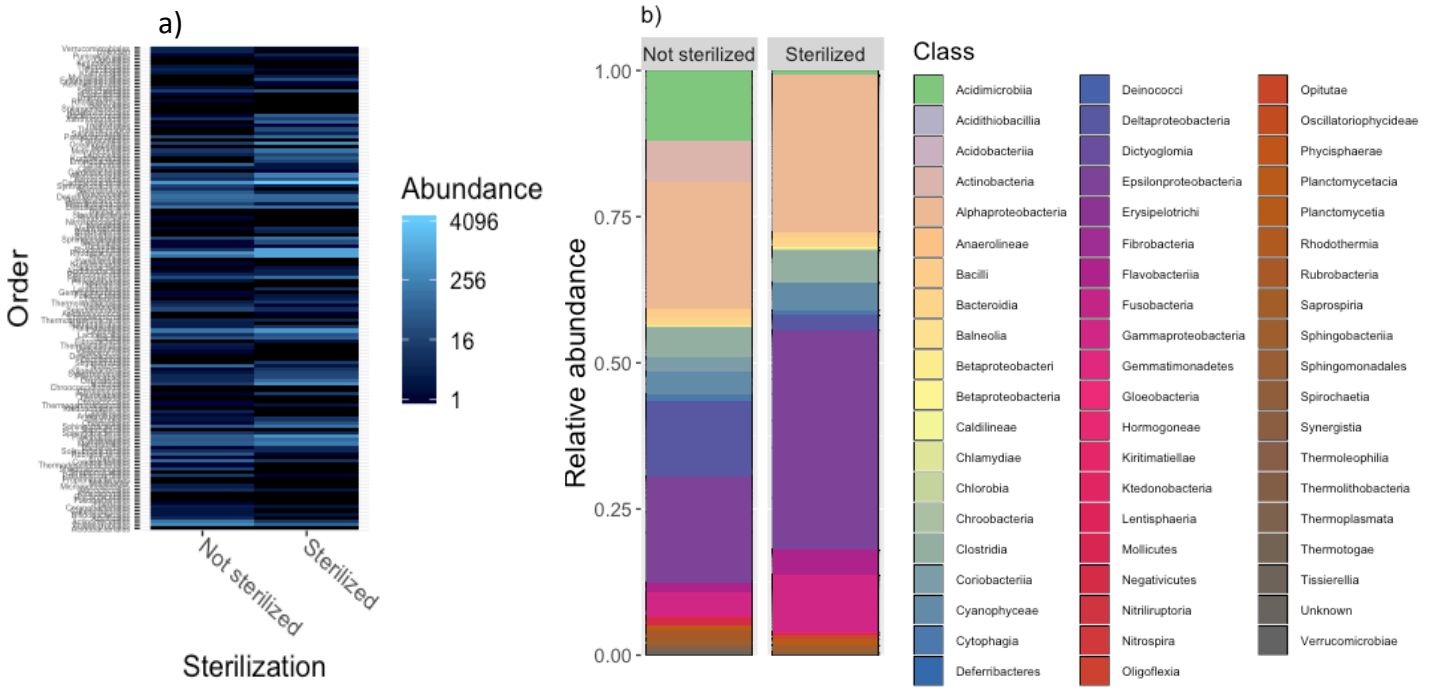


Figure 20. Two different abundance plots for the bacteria in both sterilization treatments. (a) This is a heatmap showing the abundances of all the found orders on the left. The lighter the color, the more counts the order has. A black bar resembles no count data. (b) This stacked barplot shows the composition of the classes of the bacteria species via a relative abundance. The y-axis shows the relative abundance.

Grazers

Grazer abundance

For both species there was a drop in percentage of visual observations over time (Figure 21). Due to the burrowing behaviour of *P. ulvae* this drop went very rapid, resulting in a percentage of less than 5% at week three. Noteworthy, this did not indicate the mudsnails were deceased, just invisible for observation. After week three some resurfaced, which resulted in an observation increase. The decline of *L. littorea* was due to sulphide poisoning and therefore more gradual over time, eventually dropping to 75%.

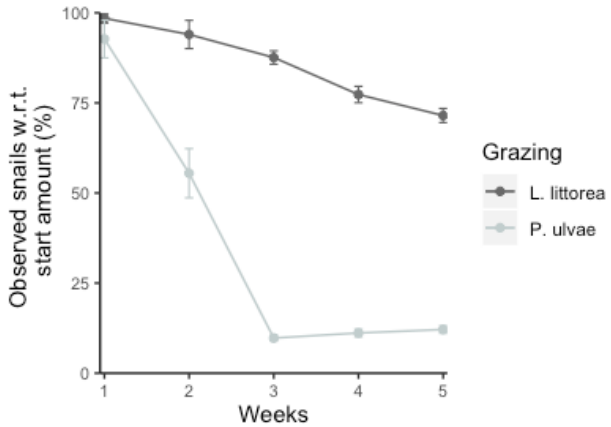


Figure 21. Graphs showing the percentage of observed snails over time. The percentage of visible grazers is shown on the y-axis and the time in weeks on the x-axis. Dark grey color represents the *L. littorea*, and light grey represents the *P. ulvae*.

Seedling numbers, morphology and biomass

Seedling numbers

More seedlings sprouted in control treatment compared to both grazer treatments (1.4 times more than *L. littorea* and 1.6 times more than *P. ulvae*, Figure 22.a). However, the differences were not significant (anova, F = 0.2897, p = 0.7536). Germination rate was significantly higher in control treatment (Figure 22.b, Linear Mixed model, Chisq = 12.49, p = 0.0019), which established itself after week two, when seedling count in control EU's became gradually higher than the other two (Tukey t-test, *L. littorea*, Z = -3.311, p = 0.0027, *P. ulvae*, Tukey t-test, Z = -2.442, p = 0.0385). Germination rate for both grazer treatments was the same, with a gradual rise over time (Tukey t-test, Z = 0.905, p = 0.6361).

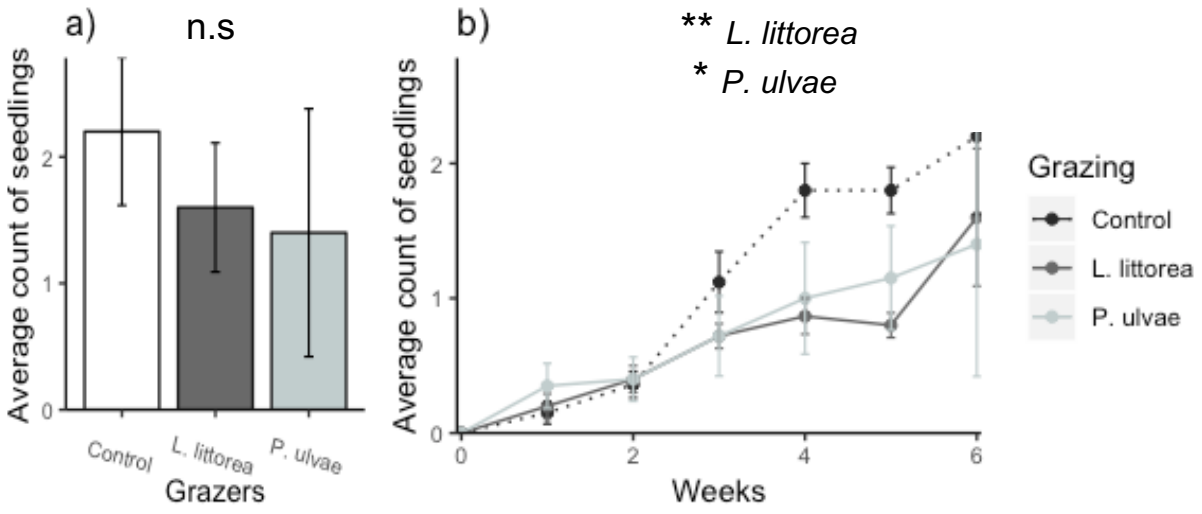


Figure 22. Results of the average count of seedlings grown without any grazers presence (white), with *L. littorea* present (dark grey) and with *P. ulvae* present (light blue). (a) This barplot represents the total average count of seedlings on the last count, which shows on the y-axis, compared with the different grazer treatments on the x-axis. The counts do not differ significantly from one another (anova, F = 0.2897, p = 0.7536). (b) This is the sprouting of new seedlings over time, where the y-axis shows the average observed count and the x-axis the time in weeks. The average seedling counts from the three grazer treatments over time differ significantly from each other (Linear Mixed model, Chisq = 12.49, p = 0.0019) The average seedling counts from the seedlings grown without grazers, over time, differ significantly from both grown with *L. littorea* (Tukey t-test, Z = -3.311, p = 0.0027) and those grown with *P. ulvae* (Tukey t-test, Z = -2.442, p = 0.03852). Both grazer treatments do not differ from one another (Tukey t-test, Z = 0.905, p = 0.63615)

Morphology and biomass

Although all leaf morphology traits seemed bigger in *P. ulvae* treatment, only leaf width was significantly bigger (anova, F = 4.8161, p = 0.0424) than both treatments and maximum leaf length of *P. ulvae* treatment was significantly longer than control treatment (Tukey t-test, Z = 2.461, p = 0.0366), not *L. Littorina* treatment (Tukey t-test, Z = 2.008, p = 0.9439). The leaves were 1.6 times wider than both treatments, and

maximum leaf length was approximately 1.8 times longer than control and 1.6 times than *L. littorea* (Figure 23). Maximum root length was significantly shorter (anova, F = 7.3781, p = 0.0153) in seedlings grown with *L. Littorina* compared to both other treatments (5 times shorter than control, 4 times shorter than to *P. ulvae*).

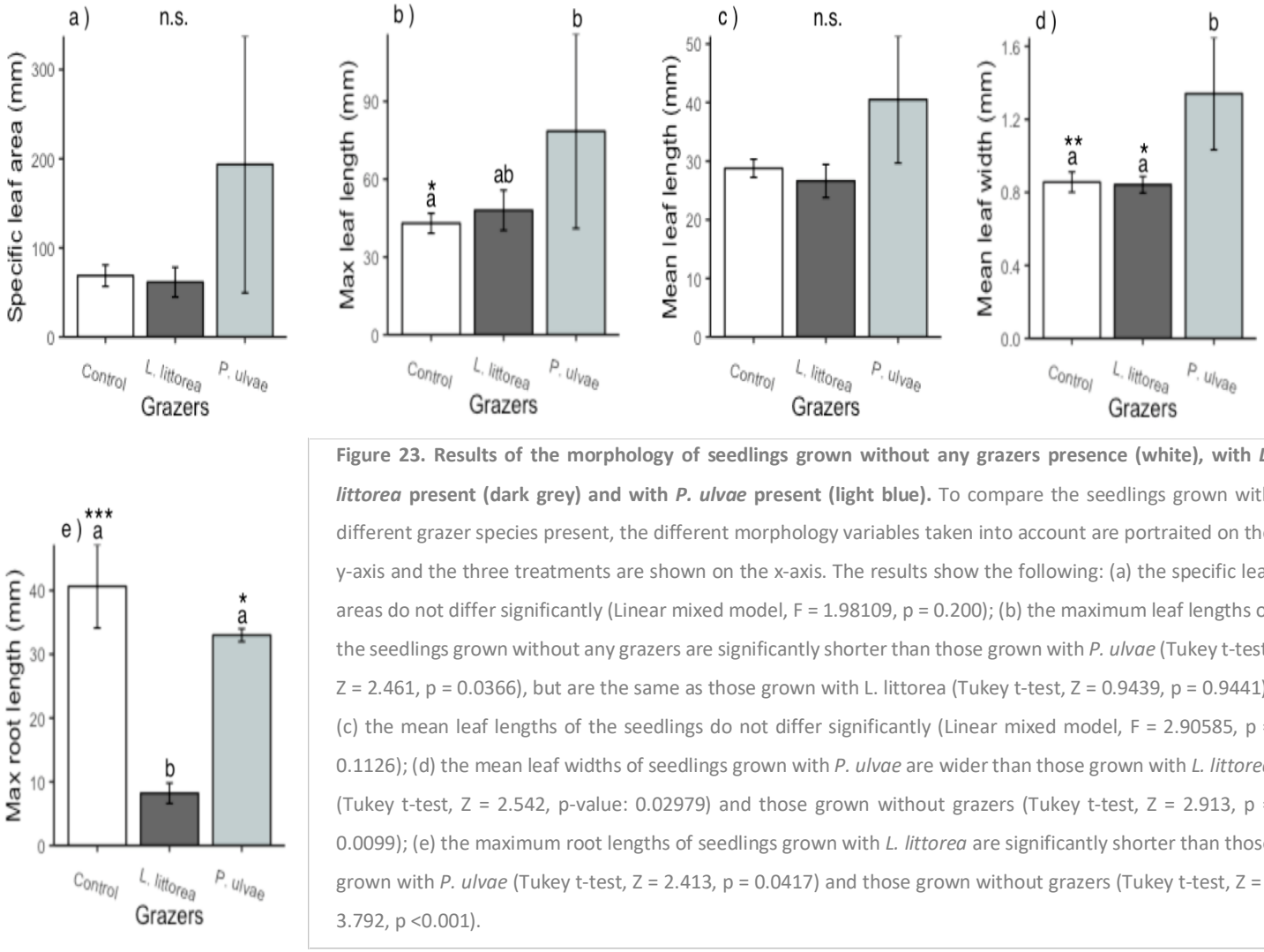


Figure 23. Results of the morphology of seedlings grown without any grazers presence (white), with *L. littorea* present (dark grey) and with *P. ulvae* present (light blue). To compare the seedlings grown with different grazer species present, the different morphology variables taken into account are portraited on the y-axis and the three treatments are shown on the x-axis. The results show the following: (a) the specific leaf areas do not differ significantly (Linear mixed model, F = 1.98109, p = 0.200); (b) the maximum leaf lengths of the seedlings grown without any grazers are significantly shorter than those grown with *P. ulvae* (Tukey t-test, Z = 2.461, p = 0.0366), but are the same as those grown with L. littorea (Tukey t-test, Z = 0.9439, p = 0.9441); (c) the mean leaf lengths of the seedlings do not differ significantly (Linear mixed model, F = 2.90585, p = 0.1126); (d) the mean leaf widths of seedlings grown with *P. ulvae* are wider than those grown with *L. littorea* (Tukey t-test, Z = 2.542, p-value: 0.02979) and those grown without grazers (Tukey t-test, Z = 2.913, p = 0.0099); (e) the maximum root lengths of seedlings grown with *L. littorea* are significantly shorter than those grown with *P. ulvae* (Tukey t-test, Z = 2.413, p = 0.0417) and those grown without grazers (Tukey t-test, Z = -3.792, p <0.001).

Seedlings grown in *P. ulvae* presence were the heaviest of all treatments for all biomass traits: mean (3.5 times heavier than control, 5.5 times heavier than *L. littorea*), total (7 times heavier than control, 11 times heavier than *L. littorea*), aboveground (almost 7 times heavier than control, 6 times heavier than *L. littorea*) and belowground (almost 2.5 times heavier than control, 5 times heavier than *L. littorea*) (Figure 24). This was significant for total plant biomass (anova, F = 6.6921, p = 0.0196), while insignificant for aboveground plant biomass (anova, F = 2.1039, p = 0.1844). The difference in mean and belowground plant biomass were only significant when comparing seedlings in *P. ulvae* treatment with *L. littorea* treatment (mean plant biomass, Tukey t-test, Z = 2.548, p = 0.0292; belowground plant biomass, Tukey t-test, Z =

2.827, p = 0.0131), but not significant when comparing to control treatment (Tukey t-test, p > 0.05). The average aboveground/belowground ratio for seedlings grown in control treatment was 2.3 times lower than *P. ulvae* and 2.7 times lower than *L. littorea*, however, this was not significant (anova, F = 1.5719, p = 0.2656).

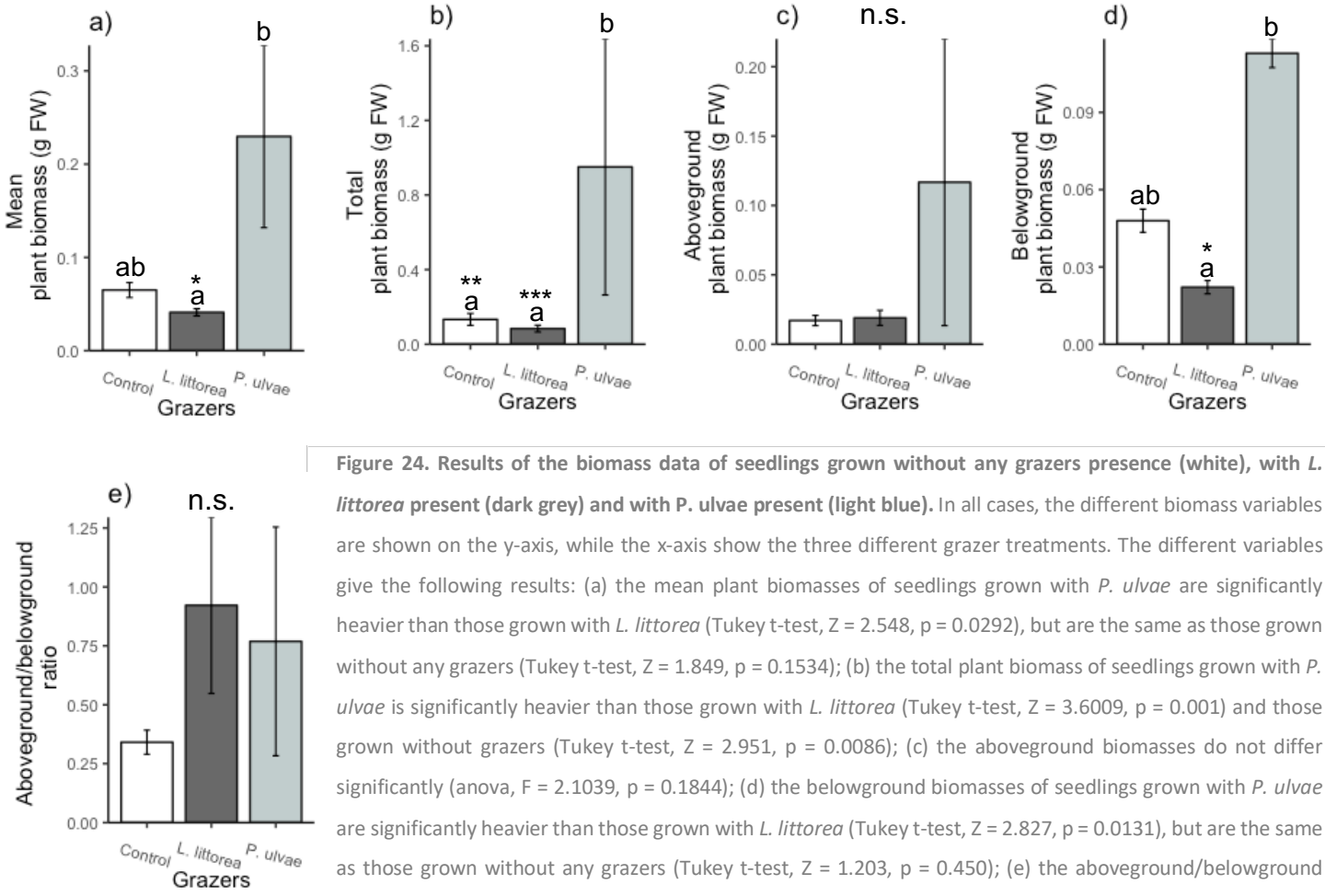


Figure 24. Results of the biomass data of seedlings grown without any grazers presence (white), with *L. littorea* present (dark grey) and with P. ulvae present (light blue). In all cases, the different biomass variables are shown on the y-axis, while the x-axis show the three different grazer treatments. The different variables give the following results: (a) the mean plant biomasses of seedlings grown with *P. ulvae* are significantly heavier than those grown with *L. littorea* (Tukey t-test, Z = 2.548, p = 0.0292), but are the same as those grown without any grazers (Tukey t-test, Z = 1.849, p = 0.1534); (b) the total plant biomass of seedlings grown with *P. ulvae* is significantly heavier than those grown with *L. littorea* (Tukey t-test, Z = 3.6009, p = 0.001) and those grown without grazers (Tukey t-test, Z = 2.951, p = 0.0086); (c) the aboveground biomasses do not differ significantly (anova, F = 2.1039, p = 0.1844); (d) the belowground biomasses of seedlings grown with *P. ulvae* are significantly heavier than those grown with *L. littorea* (Tukey t-test, Z = 2.827, p = 0.0131), but are the same as those grown without any grazers (Tukey t-test, Z = 1.203, p = 0.450); (e) the aboveground/belowground biomass ratios do not differ significantly (anova, F = 1.5719, p = 0.2656).

Diversity of the sediment microbiome

Within the sediment microbiome of the three grazer treatments – control, with *P. ulvae*, and with *L. littorea* – respectively, 88%, 90% and 90% of total unique species count were bacteria species. In control treatment this meant 1.310 bacteria species and the 186 archaea were distinguished, while for *P. ulvae* treatment it added up to 1.273 bacteria species and 135 unique archaea. Within *L. littorea* treatment 1.338 different bacteria and 128 archaea species were detected.

Archaea

The Chao1 index distinguished a significant difference between the control group and both grazer treatments, as the control group had a ±1.7 times higher average alpha diversity measure (*P. ulvae* Tukey t-test, t = -6.818, p = 0.013, *L. littorea* Tukey t-test, t = -7.507, p = 0.009, Figure 25.a). The Shannon index indicated that the control group had the lowest alpha diversity, being 1.6 times lower than *P. ulvae* and 1.4 times lower than *L. littorea*, but this was not significant (anova, F = 0.9072, p = 0.4919). Altogether the Chao1 and Shannon had contrasting results, but due to the high quantity of species with low abundance, Chao1 is better applicable for these samples.

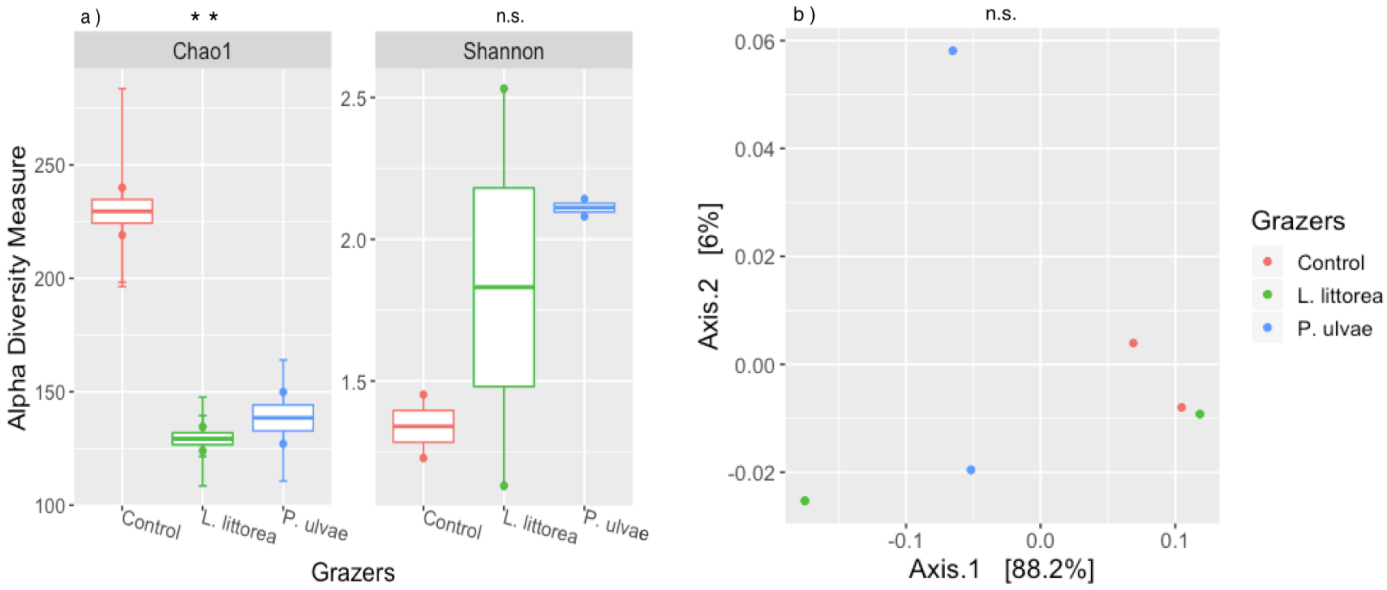


Figure 25. The diversities for the archaea species found in the sediment of the grazer treatments. (a) Two alpha-diversity metrics, Chao1 and Shannon, shown as boxplots. Both the x-axes show the grazer treatments, while the y-axis show the specific alpha-diversity measure per metric. The Chao1 metric shows a significantly higher alpha-diversity for the control group over both grazer treatments (*L. littorea*, Tukey-test, t = -7.507, p = 0.0104; *P. ulvae*, Tukey-test, t = -6.818, p = 0.0131). The Shannon metric has no significant results (anova, F = 1.7372, p = 0.3154) (b) A principal coordinates analysis of microbial communities based on weighted Unifrac distances. Both axes portray a possible way to explain the in-sample variation, where axis.2 is responsible for 6% and axis.1 88.2%. Samples are coloured by treatment.

Axis.2 of the PCoA (Figure 25.b) explained 6% of total variation and revealed a strong resemblance in beta diversity between the *L. littorea* samples, the control samples and one *P. ulvae* sample, while the other *P. ulvae* sample seemed to be distinguished from the rest. On Axis.1, which explained 88.2% of total variation, the archaeal composition of the two *P. ulvae* samples was very similar, while the samples of the *L. littorea* treatment were spread out, one of which strongly resembled the composition of the control treatment. As Axis.1 explained most of the variation, it can be concluded there was inter-sample variation

between *P. ulvae* and the control treatment, but not between *L. littorea* and the other treatments, due to the wide variation of the *L. littorea* samples.

Bacteria

The bacterial alpha diversity measure calculated with the Chao1 index was the same for all three treatments (Figure 26.a), which was substantiated by the statistical analysis (anova, F = 0.1364, p = 0.8776). The average alpha diversity measure of the Shannon index for *L. littorea* was almost 1.2 times higher than *P. ulvae* and 1.4 times higher than control treatment, while the average of *P. ulvae* was 1.3 times higher than the control treatment, but none of these differences were significant (anova, F = 1.7372, p = 0.3154).

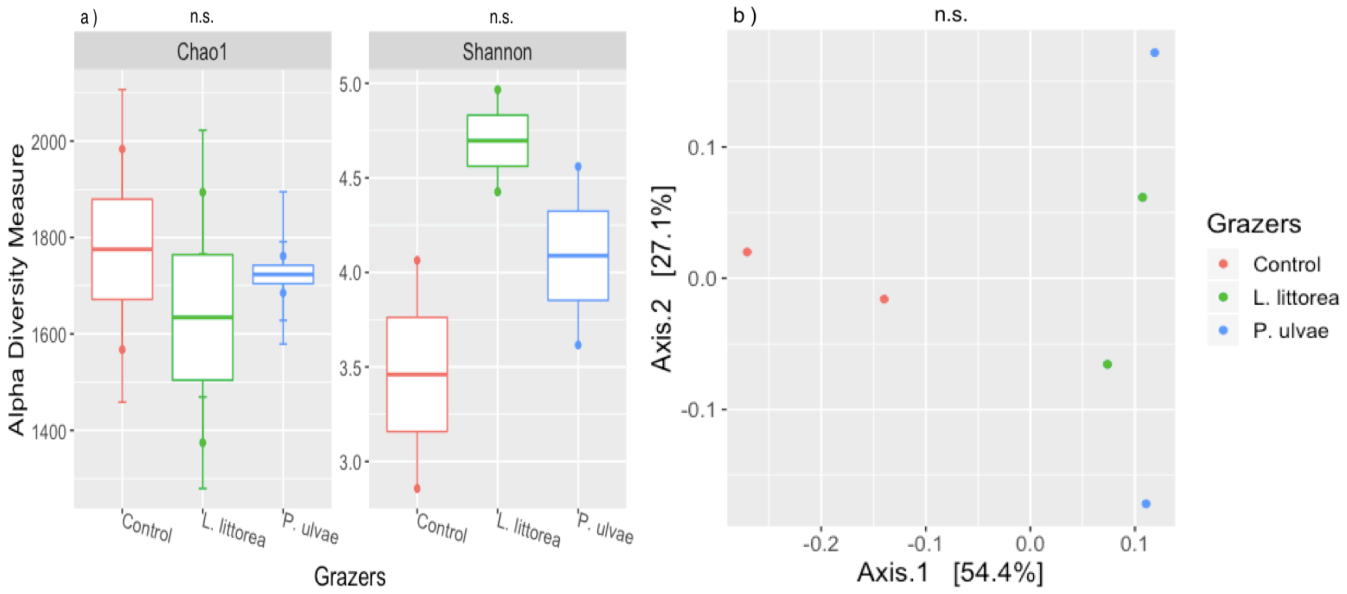


Figure 26. The diversities for the bacteria found in sterilized and not-sterilized sediment. (a) Two alpha-diversity metrics, Chao1 and Shannon, shown as boxplots. Both the x-axes show the grazer treatments, while the y-axis show the specific alpha-diversity measure per metric. The Chao1 metric and the Shannon metric had no significant results (Chao1, anova, F = 0.1364, p = 0.8776; Shannon, anova, F = 1.7372, p = 0.3154) (b) A principal coordinates analysis of microbial communities based on weighted Unifrac distances. Both axes portray a possible way to explain the in-sample variation, where axis.2 is responsible for 27.1% and axis.1 54.4%. Samples are coloured by treatment.

Beta diversity (Figure 26.b) based on Axis.2 (27.1% of total variation) depicted a wide variation in the bacterial compositions for both grazer treatments, while the control samples were similar. Axis.1 (54.4% of the variation) displayed a difference in bacterial composition between control treatment and either grazer treatments, but similarities between the grazer treatments. On both axes was a difference

between the control treatment and the grazer treatments, therefore it can be concluded that there was inter-sample variation.

Composition of the sediment microbiome

In all three treatments combined a total of 128.707 bacteria reads were detected of which 95% were classified with an accuracy of 84%. For archaea the total was 231.491 reads of which 76% were classified with an accuracy of 84%. After identification of the bacteria and archaea total count was 25.315 respectively 12.112 in control treatment, 18.261 respectively 2.733 in *P. ulvae* treatment, and 12.042 respectively 3.261 for *L. littorea*. The total amount of identified archaea and bacteria species within grazer treatments were only a fraction of the amount found in control treatment, namely 23% respectively 72% for *P. ulvae* and 27% respectively 48% for *L. littorea.*

Archaea

There was a homogenous distribution in archaeal composition for all three treatments (Table 8), based on the fact that in all three sediments at least 63.41% of total count was contributed to one species: *Methanobacterium alcaliphilum*. This is an alkaliphic methanogonic species that grows optimally within a pH-range of 8.1 and 9.1 (Worakit et al. 1986). However, their niche does not have to be alkaline in nature, as long as the transient alkalinity arises through biological activity, such as ammonification, sulphate reduction or photosynthesis (Grant et al. 1990). Six out of the ten most abundant species are methanogens.

Table 8. The top 10 most abundant archaea species in all grazer treatments. The order is based on the abundance in the first EU. The % EUs show the relative species abundance.

	Control		***L. littorea***		***P. ulvae***	
	Count	%	Count	%	Count	%
Methanobacterium alcaliphilum	4768	78.73%	1179	72.31%	866.5	63.41%
Methanobacterium congolense	131	2.16%	36.5	2.24%	23	1.68%
Methanobacterium beijingense	123.5	2.04%	43.5	2.67%	32.5	2.38%
Methanospirillum hungatei	62	1.02%	10	0.61%	20.5	1.50%
Halovivax ruber	61	1.01%	30	1.84%	23.5	1.72%
Methanocaldococcus jannaschii	56	0.92%	23	1.41%	27.5	2.01%
Methanobacterium aggregans	44	0.73%	11.5	0.71%	9	0.66%
Natronoarchaeum mannanilyticum	39	0.64%	19.5	1.20%	19	1.39%
Halorubrum terrestre	33.5	0.55%	17	1.04%	12.5	0.91%
Natrinema altunense	32	0.53%	11.5	0.71%	15.5	1.13%
Total	**6,056**	**88.34%**	**1,631**	**84.73%**	**1,367**	**76.80%**

The total count of detected archaea species was highest in control group, as it was 3.7 times more than *L. littorea* treatment and 4.4 times more than *P. ulvae* treatment (Table 8, Figure 27.a). Composition of the archaeal sediment microbiome was similar in all treatments, as there were only a few orders not represented in all of them (Figure 27.a). *Methanobateria* had the highest relative abundance of all classes (85% in control, 80% in *L. littorea* and 73% in *P. ulvae*, Figure 27.b). *Halobacteria* class was the second highest contributor, but only with 6% in control, 8% in *L. littorea* and 10% in *P. ulvae* and *Methanomicrobia* represented 4% in control, 4% in *L. littorea* and 7% in *P. ulvae. Halobacteria* is a class known to contain only halophilic species (Gupta et al. 2016), while *Methanomicrobia* and *Methanobateria* only have methanogens (Sparks 2007).

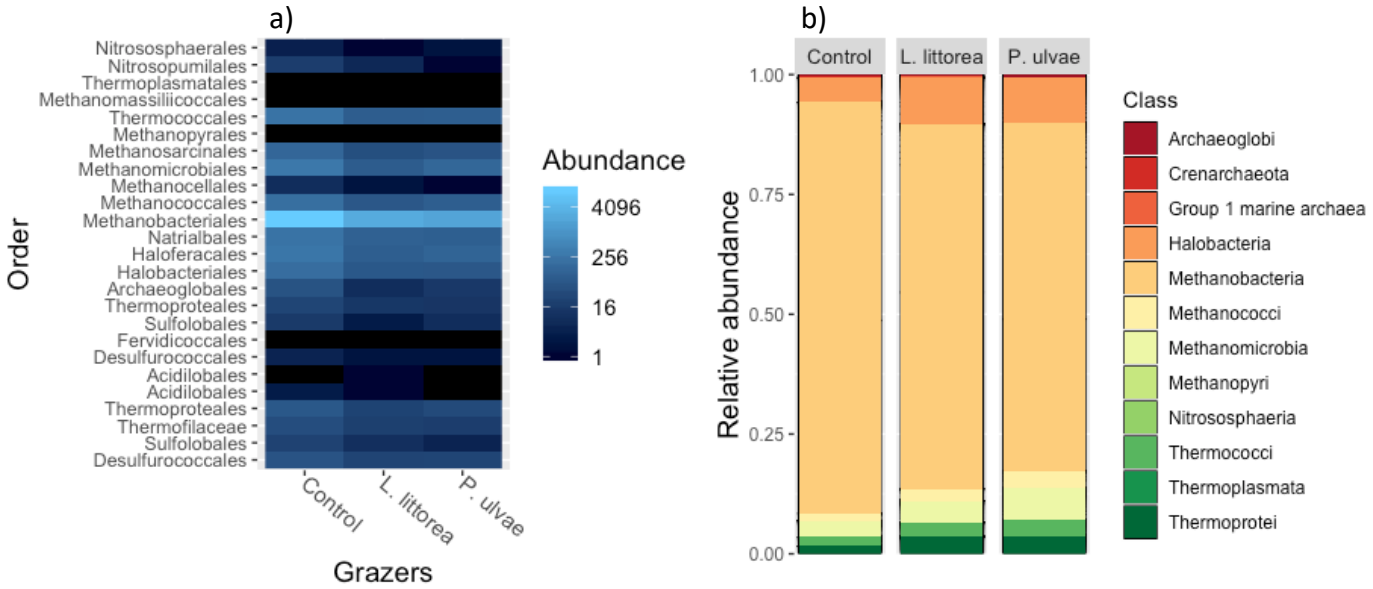


Figure 27. Two different abundance plots for the archaea in both microbiome treatments. (a) This is a heatmap showing the abundances of all the found orders on the y-axis and the treatments on the x-axis. The lighter the color, the more counts the order has. A black bar resembles no count data. (b) This stacked barplot shows the composition of the classes of the bacteria species via a relative abundance. The y-axis shows the relative abundance.

Bacteria

The bacteria species with the highest abundances were similar in the grazer treatments, while the control treatment differed from either (Table 9). The biggest difference was the fact that *N. spumigena* accounted for 40.60% of total count in control treatment, while for both grazer treatments this was < 1%. In *L. littorea* and *P. ulvae* treatments the highest relative abundances were two species from *Ilumatobacter* genus: *I. nonamiensis,* respectively 12.36% and 15.56%, and *I. fluminis,* 10.09% and 17.92%. These two species are

1) two out of three species within this genus, 2) very similar to one other (95% gene sequence similarities), 3) aerobic and non-motile and they grow in a pH range of 6-10 (Matsumoto et al. 2009, 2013). Besides a difference in most abundant species, the control treatment was also more homogenous than the grazer treatments, as 64.4% of all detected bacteria were within the top 10 (of which 40.60% was by one species), while in the *L. littorea* and *P. ulvae* treatments total relative abundances of the top 10 added up to, respectively, 41.06% and 49.63%.

Table 9. The top 10 most abundant bacteria species in all grazer treatments. The order is based on the abundance in the first EU. The % EUs show the relative species abundance.

	Control		***L. littorea***		***P. ulvae***	
	Count	%	Count	%	Count	%
Nodularia spumigena	5139.5	40.60%	8	0.13%	2	0.02%
Ilumatobacter nonamiensis	1095.5	8.65%	744	12.36%	1421	15.56%
Ilumatobacter fluminis	891.5	7.04%	607.5	10.09%	1636.5	17.92%
Fulvivirga lutimaris	266	2.10%	98	1.63%	16.5	0.18%
Sulfurovum aggregans	262.5	2.07%	162	2.69%	6	0.07%
Lewinella cohaerens	177	1.40%	66	1.10%	33	0.36%
Marivita cryptomonadis	133.5	1.05%	77.5	1.29%	826	9.05%
Erythrobacter longus	114.5	0.90%	310.5	5.16%	237	2.60%
Sulfurovum lithotrophicum	58	0.46%	38	0.63%	322	3.53%
Erythrobacter seohaensis	17	0.13%	361	6.00%	31.5	0.34%
Total	**12,658**	**64.43%**	**6,021**	**41.06%**	**9,130**	**49.63%**

Total count of control treatment was 2.1 times higher than *L. littorea* and 1.4 times higher than *P. ulvae*. This difference was mostly due to *Nostocales*, which is the order of *N. spumigena* (Figure 28.a). Their class is *Cyanophyceae* (nitrogen fixating cyanobacteria) which accounted for the biggest difference between control compared to *L. littorea* and *P. ulvae* treatments, as the relative abundance is 15, respectively 18 times higher in the control group (Figure 28.b). Other differences were smaller, for example: 1) *Alphaproteobacteria* was 37% in *L. littorea,* while 27% in *P. ulvae* and 18% in control and 2) *Acidimicrobiia* was 34% in *P. ulvae,* but 16% in control and 23% in *L. littorea*.

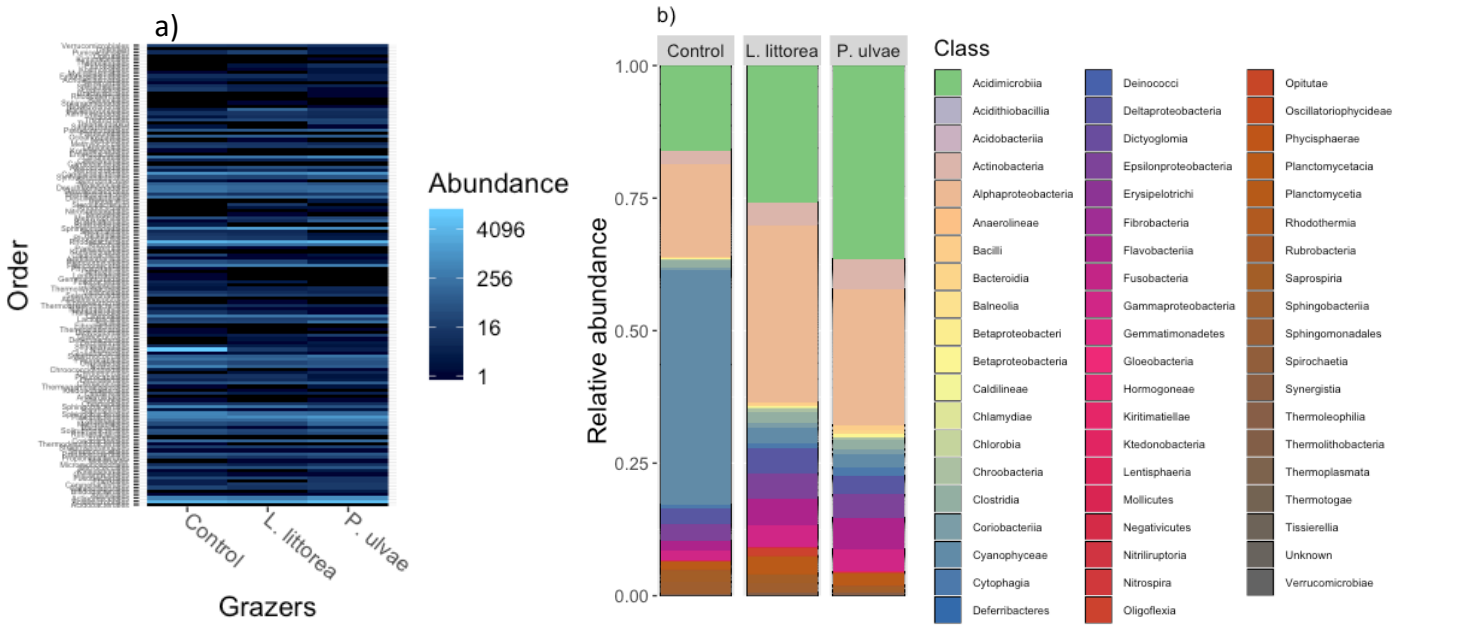


Figure 28. Two different abundance plots for the bacteria in all three grazer treatments. (a) This is a heatmap showing the abundances of all the found orders on the y-axis and the treatments on the x-axis. The lighter the color, the more counts the order has. A black bar resembles no count data. (b) This stacked barplot shows the composition of the classes of the archaea species via a relative abundance. The y-axis shows the relative abundance.

Discussion

Seagrass meadows are declining on a global scale and numerous restoration attempts are currently undertaken with mixed successes (Orth et al. 2017b, Lefcheck et al. 2018, Unsworth et al. 2019, Rezek et al. 2019). To improve seed-based seagrass restoration efforts, more knowledge about processes affecting successful seedling development is necessary. I therefore looked into the importance of 1) sediment origin (donor vs. restoration site), 2) microbiome presence (sterilized vs. unsterilized sediment) and 3) mesograzers presence (control, mudsnails, periwinkles) on eelgrass (*Z. marina*) seedling settlement and development. Sediment from the seagrass seed donor site (Sylt) differed substantially from the restoration site (Uithuizen) in terms of sediment grain size (coarser sediment on Sylt), %OM (more %OM in Uithuizen) and porewater nutrients (more NH_4, PO_4 and Fe in Uithuizen). I found that twice as many seedlings settled on donor site sediment compared to restoration site sediment, although seedling development was similar on both sediment types. Seedling settlement might have been impaired by dense microfilm development on the restoration sediment. Very likely as a result of different sediment properties, the sediment microbiome also differed substantially among sediment origins: the fine-grained restoration site sediment

had higher archaea diversity with a community dominated by methanogens, whereas the donor site sediment had a bacterial community with more nitrogen fixing cyanobacteria. Secondly, sediment sterilization resulted in rapid recolonization by bacteria (incl. sulphur oxidizing bacteria) and a slower recolonization by archaea. Seedling recruitment in sterilized soils was two times higher than in unsterilized soil and seedlings growing in sterilized sediments were up to three times bigger than seedlings growing in unsterile soil. Seed germination in unsterilized sediment may have been hampered by presence of harmful microbes or microfilm development, whereas increased seedling development in sterilized soils might be due to the higher nutrient availability (six times more ammonium, 2.5 times more iron and twice as much phosphate) in this sediment. Finally, grazer presence slightly limited seedling recruitment, but seedling development was positively affected by *P. ulvae* (mudsnail) presence, leading to wider and longer leaves and higher seedling biomass in this treatment. Grazer presence changed the community composition of the bacterial microbiome; especially less nitrogen fixing cyanobacteria were present possibly as a result of mesograzer grazing activity on topsoil bacterial communities. Altogether, these results show that linking the microbiome community composition (archaea and bacteria) to seedling recruitment and development is challenging. However, sediment characteristics such as organic matter content and grain size, that are linked to the sediment microbiome, strongly seem to affect seedling recruitment: lower recruitment on sediments with microbial biofilms. In addition, seedling development is positively affected by high porewater nutrient concentrations. Mesograzers, especially mudsnails, may in turn limit seedling recruitment, but may be positive for seedling development due to the removal of microbial films on seedling leaves and topsoil microphytobenthic mats. I conclude that taking sediment properties (incl. the microbial community composition, sediment grain size, %OM and porewater nutrients) into account may be key to seagrass seed-based restoration success, although the functional role of the sediment microbiome still needs to be linked to seed and seedling performance. Additionally, presence of sufficient mudsnails may enhance seedling development in seed-based eelgrass restoration.

Sediment origin

Over time seagrass restoration has been used worldwide to recover ecosystem services and ecological functions in coastal ecosystems (van Katwijk et al. 2016). Although great successes in eelgrass restoration

have been made in specific areas of the Dutch wadden sea (Griend), restoration attempts in other restoration sites (Uithuizen) has not yet established eelgrass beds (Floor et al. 2018), despite positive assessments of seagrass suitability (van Katwijk et al. 2000). Therefore, I compared Uithuizen sediment with sediment from a donor site (Sylt) and evaluated their differences and consecutively seedling establishment and development. Not only did the restoration site differ in terms of sediment characteristics (coarser particles, less %OM and less NH_4, PO_4 and Fe in donor sediment), the microbiomes differed substantially as well. The donor site soil had a bacterial community with more nitrogen fixing cyanobacteria, while the fine-grained restoration sediment had higher archaea diversity with a community dominated by methanogens. Over time, twice as many seedlings established on donor soil, while the development of seedlings was similar on both sediment types. On restoration sediment a thick microfilm developed, which might have impaired seedling settlement. Reduced settlement could also be a result of the sulphide concentrations in the restoration soil, as hydrogen sulphide is toxic for plant cells (Koch 2001). Fine-grained particles with a high organic matter content (like Uithuizen) are known to have high levels of sulphides (Koch 2001, Govers et al. 2016). In aerobic circumstances, fully grown eelgrasses have physical structures to diminish negative effects of hydrogen sulphide (Pedersen et al. 2004), however, during the sensitive time as seedlings these structures are underdeveloped and a relative low sulphide concentration would increase mortality significantly (Lamers et al. 2013, Dooley et al. 2013).

Within Sylt sediment the microbiome was highly dominated by a nitrogen fixating cyanobacteria. This increased abundance was most likely due to the nitrate and phosphate levels in donor soil (nitrates were similar, but phosphates were almost 2.5 times higher in donor soil), because cyanobacteria increase in abundance with low N:P ratios (de Tezanos Pinto and Litchman 2010). However, the ratios were so uncommonly low (well under 0) for marine sediments (de Tezanos Pinto and Litchman 2010) that most likely something went wrong during measurements. This suspicion is supported by the uncharacteristically low nitrate levels (Govers et al. 2014). Therefore, it is difficult to be certain if the high abundance of nitrogen fixating cyanobacteria in Sylt was a consequence of the N:P ratios. In Uithuizen the microbiome was dominated by methanogens, which are generally abundant in habitats where common electron acceptors (e.g. O_2, NO_3^-, Fe^{3+}, and SO_4^{2-}) are limiting (Liu and Whitman 2008). When electron

acceptors other than CO_2 are present sulphate-reducing bacteria outcompete methanogens (Whitman et al. 2006, Ferry and Lessner 2008). This is relatively uncommon in marine sediments due to high levels of SO_4^{2-} in seawater, however, the fine-grained nature of Uithuizen sediment limits the exchange of nutrients between the soil and the surrounding water below the top layer (Cúcio et al. 2016), creating an anaerobic environment with limited sulphate sources. Combine this with the high %OM in Uithuizen, which reduces sulphate levels under aerobic circumstances (Ferry and Lessner 2008), and the possibility for methanogens to outcompete sulphate-reducing bacteria ensues.

Even though methanogens were dominating Uithuizen sediment, there was a high relative abundances of sulphur oxidising and sulphate reducing bacteria in the top layer. Normally these bacteria positively influence nutrient cycling and are therefore considered beneficial for seagrasses (Hansen et al. 2000). Nonetheless, the oxidation of sulphur into sulphate creates the opportunity for sulphate reduction into sulphides, which are known to be toxic for eelgrass (Koch 2001, Lamers et al. 2013, Dooley et al. 2013). The combination of these bacteria and the already higher sulphide concentrations in Uithuizen, could explain the lower seedling settlement compared to donor soil (Ugarelli et al. 2017). However, the fact that development of seedlings in both sediment types were similar contradicts this, as the negative effects of sulphide would also stunt development in established seedlings (Mascaró et al. 2009). The lower seedling settlement in restoration soil is therefore most likely a result of the anaerobic circumstances below the top layer created by the fine-grained nature (Plus et al. 2003), harmful microbes or microbial biofilms.

As donor soil was dominated by nitrogen fixating bacteria, and fixation of atmospheric nitrogen into ammonia is known to be an additional source of nitrogen in the nutrient requirements of seagrasses (Hansen et al. 2000, Welsh 2000, Garcias-Bonet et al. 2016), it would be expected that seedlings settled on donor soil would have an enhanced development compared to the restoration soil. However, this was not the case, presumably due to the specific species of nitrogen fixating bacteria found: *N. spumigena*, which is known to cause algae mats (Krüger et al. 2009). Algae mats have several negative effects on seagrass functioning (Han and Liu 2014). Even though all observed mats were removed, this could still lead to a detrimental effect on development.

Altogether the results demonstrate that sediment characteristics, such as grain size, organic matter content and nutrient levels, have a strong influence on the soil microbial community and on seedling

settlement and development. Therefore, it is important to consider these elements during assessment if a restoration site is suitable for seed-based seagrass restoration.

Sediment sterilization

In terrestrial plants the positive influence of microbes has been known and used for years (Walker et al. 2003, Lugtenberg and Kamilova 2009, Berendsen et al. 2012, Philippot et al. 2013). For marine environments the effects are less well studied (Crump and Koch 2008, Cúcio et al. 2016, Ettinger et al. 2017, Wainwright et al. 2019), nevertheless suggestions of positive influence on plant health and growth by microbial communities in the soil have already been made (Crump et al. 2018). Even restoration studies have suggested that the microbiome of donor sites may play a larger role than previously thought and may be responsible for the success or failure of transplanted seagrasses (Milbrandt et al. 2008, Christiaen et al. 2013). To discern the effects a microbiome could have on seed-based restoration, I extinguished all microbes in one treatment and compared seedling settlement and development of seeds with those grown on intact soil. The sterilization was done via autoclaving, a form of thermal neutralization, which is known to lysis cells, change mineral phases and affect geochemistry, leading to (among other things) 1) a release of nutrients and substrates, and 2) an increase in organic carbon concentrations (Berns et al. 2008, Andersson et al. 2018, Otte et al. 2018). Sediment sterilization lead to rapid recolonisation by bacteria and slow recolonisation by archaea. Seedling settlement was positively influenced, as twice as many seedlings settled in sterilized soil. The development was also increased, as the seedlings were three times bigger than the ones from normal soil.

Lower seedling germination and reduced development in unsterilized sediment compared to sterilized sediment could be due to presence of harmful microbes or microfilm development (Han and Liu 2014). However, side effects of sterilization could also have increased seedling settlement and development in sterilized soils, as autoclaving heightens nutrient levels (six times more ammonium, 2.5 times more iron and twice as much phosphate) significantly and nutrient availability is commonly a limiting factor for seagrass growth (Short 1987). As sulphides can be sequestered in the soil by metals such as iron (Lamers

et al. 2013, Ugarelli et al. 2017), the rise in nutrients also reduces sulphides and therewith their toxic effects on seagrasses (Koch 2001, Dooley et al. 2013).

It should be mentioned that even though autoclaving inactivates most fungi and protozoans, there are strains of bacteria and archaea with sterilization survival strategies, including spore formation, repair and resistance mechanisms, and dormant states (Otte et al. 2018). The only species known for these strategies within the donor soil belong to a class with a relative abundance of 5% in both treatments: *Clostridia*. If survival of micro-organisms after sterilization was the case in this study, a higher relative abundance of *Clostridia* compared to species who wouldn't have survived in sterilized sediment was to be expected. Therefore, rapid re-colonization is a more likely conclusion.

The most abundant microbe species in both sterilized and unsterilized soil were sulphur oxidizing species. In sterilized soil the abundance was about 3.5 times higher compared to unsterilized soil. This rapid re-colonization was most likely a result of autoclaving. Studies have shown that an increase in bacterial growth rates following autoclaving is common, and it is hypothesized to be a result of the increase in carbon concentrations, as that is most commonly the limiting factor in bacterial growth (Jannasch 1969, Ammerman et al. 1984, Postma et al. 1990, Andersson et al. 2018). A noteworthy difference between the sterilized and not sterilized microbiome was the high abundance of the *Arcobacter* genus within sterilized sediment, whereas they were unaccounted for in normal restoration soil. Although the metabolic capabilities of most arcobacters have not been studied in detail, it is known that most of them produce filamentous sulphur with sulphide and oxygen (Campbell et al. 2006). Uithuizen sediment naturally has high sulphides (Koch 2001, Govers et al. 2016), therefore it is to be expected both treatments would have sulphide reducing species. Nevertheless, only sterilized had any *Arcobacters*, which suggests they could only establish after sterilization. This could be a result of other micro-organisms within donor soil that outcompete them or suppress their occurrence. Either way, this presumable lead to the improved seedling germination and development in sterilized sediment when comparing with normal soil (Borum et al. 2005).

All things considered, I would conclude that it is challenging to link the microbial community composition (archaea and bacteria) to seedling establishment and development, as the improvements in sterilized sediment could also be from side effects of autoclaving. To be sure, further research on the microbiome has to be done, preferably with different types of sterilization. However, if it was not due to side effects, the results suggest there are interactions within the donor sediment between micro-organisms

that negatively impact seagrass establishment. This could be the presence of harmful microbes or the repression of beneficial microbes, both of which could have dampening effects on restoration efforts in Uithuizen. An assessment of the microbiome is therefore recommended before restoration attempts, as is it possible to determine if there are beneficial micro-organisms that could promote development and settlement (e.g. nitrogen-fixating and sulphide-reducing (Crump et al. 2018)), or it could indicate if a sediment is unsuitable. Based on these indicators, restoration efforts could be adjusted accordingly by, for example, introducing certain microbes to the sediment surrounding the seeds (Milbrandt et al. 2008). In the instance of Uithuizen, addition of sulphide-oxidising bacteria (e.g. cable bacteria from *Desulfobulbaceae* genus (Martin 2019)) could help improve restoration attempts.

Grazers

The addition of algae consumers, e.g. *L. littorea* and *P. ulvae*, was expected to increase seedling settlement and development, as they reduce algae in the EU's. *L. littorea* (periwinkles) is a common omnivorous, mobile gastropod known to be a key species in many European coastal habitats (Seuront et al. 2007, Perez et al. 2009, Chang et al. 2011) and *P. ulvae* (mudsnails) is one of the most common hydrobiid, deposit feeding invertebrates in the intertidal zones of Europe, most commonly found on the sediment surface or within the top two cm layer (Bolam 2011, Campana et al. 2013). For seeds the germinate the influence of salinity, temperature, oxygen levels, seed size, and light (Orth et al. 2006a, Jørgensen et al. 2019) are consequential. Algae are the only source of possible disturbance of one of these elements within the EU's: light. Algae live throughout the entire water column, and can therefore block light reaching seagrasses (Ralph et al. 2007). Seagrass light requirements are approximately four to twelve times higher (depending on location, water circumstances and specific species) than algae's (Dennison et al. 1993, Lee et al. 2007, Ralph et al. 2007) and will consequently easily be outcompeted.

Contrary to expectation, grazer presence resulted in a slightly lowered seedling settlement. Presumably, this is a result of predation by the grazers. Studies have shown that predation on seagrass seeds is not uncommon and leads to reduced germination (Orth et al. 2006b, 2007). Normally this is done via boring through the seed coat with force (Nakaoka 2002), which is unlikely for *P. ulvae* and *L. littorea* as

they lack the body parts to do so. Nevertheless, when seedlings start to sprout, the coat is opened, making the seeds more vulnerable for predation. Hence it is probable that both grazers consumed the newly sprouted seedlings and therewith reduced seedling settlement.

During the first three weeks some of the seeds in *P. ulvae* units resurfaced repeatedly after burial, leading to believe that the snails moved them up through the sediment. As mudsnails are commonly found within the first two centimetres of the soil, it could be a side effect of their burying movements relocating the sediment (and therewith the seeds), especially because resurfacing was not found in EUs with periwinkles nor control. The precise reason for this phenomenon remains unclear, but it could have inhibited seedling settlement. Nevertheless, seedling development was positively affected by mudsnail presence, as the leaves were wider and longer with higher biomass compared to either other treatment. This is most likely due to the removal of microbial films on seedling leaves (Hootsmans and Vermaat 1985a, Jaschinski et al. 2009). The same cannot be said for the perwinkles, presumably due to their larger size. The consumption of epiphytic algae by snails significantly larger than the seedlings could result in uprooting, which would disturb seedling development.

The bacterial microbiome of the grazer treatments differed from control: there was a lower microbiome diversity and a reduced abundance of nitrogen fixing cyanobacteria. Both phenomena were most likely a result of consumption. Mudsnails and periwinkles are both consumers of microphytobenthos (MPB), which is an assemblage of green algae, photosynthetic diatoms, flagellates, and cyanobacteria that live in coastal environments (MacIntyre et al. 1996, Pivato et al. 2019, Xie et al. 2019). MPB and seagrasses have light and nutrients as limiting factors (MacIntyre et al. 1996, Pivato et al. 2019, Xie et al. 2019), making them natural competitors for resources. *P. ulvae* snails are especially known for their efficiency in consumption of MPB (Jaschinski et al. 2009, Araújo et al. 2015, Lange et al. 2019), whereas the preference of periwinkles lie in consumption of (macro)algae (Jaschinski et al. 2009, Peckol and Putnam 2017, Putnam and Peckol 2018). The higher efficiency in removal of topsoil microphytobenthic mats by mudsnails in combination with snail size probably explains why mudsnails did improve seedling development, while periwinkles did not.

Altogether it seems that inclusion of mudsnails in seed-based restoration attempts would improve the outcome, whereas periwinkles are unfavourable at this stage of development. When mudsnails are to

be included it should be taken into consideration that more than an average amount of seeds is used, to counteract the dampening of seedling establishment via consumption.

Conclusion

Since the start of seagrass restoration in 1939 a lot of progress has been made. Several well-established frameworks and considerations for restoration have been published (Addy 1947, Campbell 2002, van Katwijk et al. 2016, Tan et al. 2020) and large-scale restoration successes have occurred, e.g. in Whangarei Harbor, New Zealand 600 ha of *Zostera muelleri* have been restored (Matheson et al. 2016), a recovery of 1700 ha of *Z. marina* in the Virginia Coast reserve (Orth and McGlathery 2012), and even in Griend within the Dutch Wadden Sea a growth from 30 hectares to 170 hectares of *Z. marina* has taken place. However, there have also been plenty instances with lower rates of success (van Katwijk et al. 2016). While most seagrass restoration efforts have relied on adult plants (Paling et al. 2009), seed-based restoration has been acknowledged as one of the most cost-effective methods of restoration (Unsworth et al. 2019). Nevertheless, there are three main limitations to this method: acquisition and processing of seeds, maintenance of viable seed supplies and low initial seedling establishment rates (Marion and Orth 2010). Improvements in harvesting resulted in twice the amount of seeds collected per labour-hour, with minimal damage to donor meadows (Marion and Orth 2010) and research in maintenance of viable seeds has led to a decrease in disease contamination and therefore a inhibition in reduction of germination rates (Govers et al. 2016). Despite improvements, even successful seed-based restoration efforts had low (8%-28%) seedling establishment (Orth et al. 2009, Marion and Orth 2010, Zhang et al. 2015, Infantes et al. 2016, Unsworth et al. 2019). Local environmental conditions, health of the seeds, and/or disease contamination are speculated to be the source of the low germination rates, but nothing has been proven yet. This current study shows that seedling establishment is influenced by a multitude of aspects, e.g. mesograzers limit seedling recruitment and even though it has been difficult to link the microbiome community composition (archaea and bacteria) directly to seedling recruitment, sediment characteristics (organic matter content (%OM) and grain size), which are linked to the sediment microbiome, strongly seem to affect recruitment as well. Even though there is a growing understanding of the seagrass

microbiome (Fahimipour et al. 2017), and it's potential mutualistic role in seagrass growth and production (Crump et al. 2018) for further research the link to seedling recruitment and performance still needs to be determined. Nevertheless, I conclude that to asses if the conditions of a restoration site are suitable, not only the environmental conditions (van Katwijk et al. 2016) but also the sediment properties, incl. the microbial community composition, sediment grain size, %OM and porewater nutrients, have to be taken into account for seed-based restoration success.

References

Addy, C. E. 1947. Eel grass planting guide. Maryland Conservationist 24:16–17.

Ammerman, J., J. Fuhrman, Å. Hagström, and F. Azam. 1984. Bacterioplankton growth in seawater: I. Growth kinetics and cellular characteristics in seawater cultures. Marine Ecology Progress Series 18:31–39.

Andersson, M., N. Catalán, Z. Rahman, L. Tranvik, and E. Lindström. 2018. Effects of sterilization on dissolved organic carbon (DOC) composition and bacterial utilization of DOC from lakes. Aquatic Microbial Ecology 82:199–208.

Araújo, C. V. M., M. Moreira-Santos, J. Patrício, I. Martins, I. Moreno-Garrido, J. Blasco, J. C. Marques, and R. Ribeiro. 2015. Feeding niche preference of the mudsnail *Peringia ulvae*. Marine and Freshwater Research 66:573.

Baden, S., C. Boström, S. Tobiasson, H. Arponen, and P.-O. Moksnes. 2010. Relative importance of trophic interactions and nutrient enrichment in seagrass ecosystems: A broad-scale field experiment in the Baltic–Skagerrak area. Limnology and Oceanography 55:1435–1448.

Bais, H. P., T. L. Weir, L. G. Perry, S. Gilroy, and J. M. Vivanco. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology 57:233–266.

Beck, M. W., K. L. Heck, K. W. Able, D. L. Childers, D. B. Eggleston, B. M. Gillanders, B. Halpern, C. G. Hays, K. Hoshino, T. J. Minello, R. J. Orth, P. F. Sheridan, and M. P. Weinstein. 2001. The Identification,

Conservation, and Management of Estuarine and Marine Nurseries for Fish and Invertebrates. BioScience 51:633.

Berendsen, R. L., C. M. J. Pieterse, and P. A. H. M. Bakker. 2012. The rhizosphere microbiome and plant health. Trends in Plant Science 17:478–486.

Berns, A. E., H. Philipp, H.-D. Narres, P. Burauel, H. Vereecken, and W. Tappe. 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. European Journal of Soil Science 59:540–550.

Bolam, S. G. 2011. Burial survival of benthic macrofauna following deposition of simulated dredged material. Environmental Monitoring and Assessment 181:13–27.

Boone, D. R. 2015. Methanothermobacter. Pages 1–8 Bergey's Manual of Systematics of Archaea and Bacteria. American Cancer Society.

Borum, J., O. Pedersen, T. M. Greve, T. A. Frankovich, J. C. Zieman, J. W. Fourqurean, and C. J. Madden. 2005. The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, *Thalassia testudinum*. Journal of Ecology 93:148–158.

Burkholder, J. M., D. A. Tomasko, and B. W. Touchette. 2007. Seagrasses and eutrophication. Journal of Experimental Marine Biology and Ecology 350:46–72.

Busch, K. E., R. R. Golden, T. A. Parham, L. P. Karrh, M. J. Lewandowski, and M. D. Naylor. 2008. Large-Scale *Zostera marina* (eelgrass) Restoration in Chesapeake Bay, Maryland, USA. Part I: A Comparison of Techniques and Associated Costs. Restoration Ecology 18:490–500.

Campana, O., A. Rodríguez, and J. Blasco. 2013. Evaluating the suitability of *Hydrobia ulvae* as a test species for sediment metal toxicity testing applying a tissue residue approach to metal mixtures in laboratory and field exposures. Chemosphere 91:1136–1145.

Campbell, B. J., A. S. Engel, M. L. Porter, and K. Takai. 2006. The versatile ε-proteobacteria: key players in sulphidic habitats. Nature Reviews Microbiology 4:458–468.

Campbell, J. E., A. H. Altieri, L. N. Johnston, C. D. Kuempel, R. Paperno, V. J. Paul, and J. E. Duffy. 2018. Herbivore community determines the magnitude and mechanism of nutrient effects on subtropical and tropical seagrasses. Journal of Ecology 106:401–412.

Campbell, M. 2002. Getting the foundation right: A scientifically based management framework to aid in the planning and implementation of seagrass transplant efforts. Bulletin of Marine Science 71:1405–1414.

Chang, A. L., A. M. H. Blakeslee, A. W. Miller, and G. M. Ruiz. 2011. Establishment Failure in Biological Invasions: A Case History of *Littorina littorea* in California, USA. PLOS ONE 6:e16035.

Christiaen, B., A. McDonald, J. Cebrian, and A. C. Ortmann. 2013. Response of the microbial community to environmental change during seagrass transplantation. Aquatic Botany 109:31–38.

Collado, L., and M. J. Figueras. 2011. Taxonomy, Epidemiology, and Clinical Relevance of the Genus *Arcobacter*. Clinical Microbiology Reviews 24:174–192.

Crump, B. C., and E. W. Koch. 2008. Attached Bacterial Populations Shared by Four Species of Aquatic Angiosperms. Applied and Environmental Microbiology 74:5948–5957.

Crump, B. C., J. M. Wojahn, F. Tomas, and R. S. Mueller. 2018. Metatranscriptomics and Amplicon Sequencing Reveal Mutualisms in Seagrass Microbiomes. Frontiers in Microbiology 9.

Cúcio, C., A. H. Engelen, R. Costa, and G. Muyzer. 2016. Rhizosphere Microbiomes of European + Seagrasses Are Selected by the Plant, But Are Not Species Specific. Frontiers in Microbiology 7.

Cunha, A. H., N. N. Marbá, M. M. van Katwijk, C. Pickerell, M. Henriques, G. Bernard, M. A. Ferreira, S. Garcia, J. M. Garmendia, and P. Manent. 2012. Changing Paradigms in Seagrass Restoration. Restoration Ecology 20:427–430.

Dennison, W. C., R. J. Orth, K. A. Moore, J. C. Stevenson, V. Carter, S. Kollar, P. W. Bergstrom, and R. A. Batiuk. 1993. Assessing Water Quality with Submersed Aquatic Vegetation. BioScience 43:86–94.

Dooley, F. D., S. Wyllie-Echeverria, M. B. Roth, and P. D. Ward. 2013. Tolerance and response of *Zostera marina* seedlings to hydrogen sulfide. Aquatic Botany 105:7–10.

Duffy, J. E., P. L. Reynolds, C. Boström, J. A. Coyer, M. Cusson, S. Donadi, J. G. Douglass, J. S. Eklöf, A. H. Engelen, B. K. Eriksson, S. Fredriksen, L. Gamfeldt, C. Gustafsson, G. Hoarau, M. Hori, K. Hovel, K. Iken, J. S. Lefcheck, P.-O. Moksnes, M. Nakaoka, M. I. O'Connor, J. L. Olsen, J. P. Richardson, J. L. Ruesink, E. E. Sotka, J. Thormar, M. A. Whalen, and J. J. Stachowicz. 2015. Biodiversity mediates top-down control in eelgrass ecosystems: a global comparative-experimental approach. Ecology Letters 18:696–705.

Eriander, L., E. Infantes, M. Olofsson, J. L. Olsen, and P.-O. Moksnes. 2016. Assessing methods for restoration of eelgrass (*Zostera marina* L.) in a cold temperate region. Journal of Experimental Marine Biology and Ecology 479:76–88.

Eschweiler, N., M. Molis, and C. Buschbaum. 2009. Habitat-specific size structure variations in periwinkle populations (*Littorina littorea*) caused by biotic factors. Helgoland Marine Research 63:119–127.

Ettinger, C. L., S. E. Voerman, J. M. Lang, J. J. Stachowicz, and J. A. Eisen. 2017. Microbial communities in sediment from *Zostera marina* patches, but not the *Z. marina* leaf or root microbiomes, vary in relation to distance from patch edge. PeerJ 5:e3246.

Fahimipour, A. K., M. R. Kardish, J. M. Lang, J. L. Green, J. A. Eisen, and J. J. Stachowicz. 2017. Global-Scale Structure of the Eelgrass Microbiome. Applied and Environmental Microbiology 83:e03391-16.

Ferry, J. G., and D. J. Lessner. 2008. Methanogenesis in Marine Sediments. Annals of the New York Academy of Sciences 1125:147–157.

Floor, J. R., C. S. A. (Kris) van Koppen, and J. P. M. van Tatenhove. 2018. Science, uncertainty and changing storylines in nature restoration: The case of seagrass restoration in the Dutch Wadden Sea. Ocean & Coastal Management 157:227–236.

Folmer, E. O., J. E. E. van Beusekom, T. Dolch, U. Gräwe, M. M. van Katwijk, K. Kolbe, and C. J. M. Philippart. 2016. Consensus forecasting of intertidal seagrass habitat in the Wadden Sea. Journal of Applied Ecology 53:1800–1813.

Garcia, J.-L., B. K. C. Patel, and B. Ollivier. 2000. Taxonomic, Phylogenetic, and Ecological Diversity of Methanogenic Archaea. Anaerobe 6:205–226.

Garcias-Bonet, N., J. M. Arrieta, C. M. Duarte, and N. Marbà. 2016. Nitrogen-fixing bacteria in Mediterranean seagrass (*Posidonia oceanica*) roots. Aquatic Botany 131:57–60.

Govers, L. L., W. A. Man in 't Veld, J. P. Meffert, T. J. Bouma, P. C. J. van Rijswick, J. H. T. Heusinkveld, R. J. Orth, M. M. van Katwijk, and T. van der Heide. 2016. Marine *Phytophthora* species can hamper conservation and restoration of vegetated coastal ecosystems. Proceedings of the Royal Society B: Biological Sciences 283:20160812.

Govers, L. L., T. Pieck, T. J. Bouma, W. Suykerbuyk, A. J. P. Smolders, and M. M. van Katwijk. 2014. Seagrasses are negatively affected by organic matter loading and *Arenicola marina* activity in a laboratory experiment. Oecologia 175:677–685.

Govers, L. L., E. M. van der Zee, J. P. Meffert, P. C. J. van Rijswick, W. A. Man in 't Veld, J. H. T. Heusinkveld, and T. van der Heide. 2017. Copper treatment during storage reduces *Phytophthora* and *Halophytophthora* infection of *Zostera marina* seeds used for restoration. Scientific Reports 7:43172.

Grant, W. D., W. E. Mwatha, and B. E. Jones. 1990. Alkaliphiles: Ecology, diversity and applications. FEMS Microbiology Letters 75:255–269.

Greiner, J. T., K. J. McGlathery, J. Gunnell, and B. A. McKee. 2013. Seagrass Restoration Enhances "Blue Carbon" Sequestration in Coastal Waters. PLoS ONE 8:e72469.

Gupta, R. S., S. Naushad, R. Fabros, and M. Adeolu. 2016. A phylogenomic reappraisal of family-level divisions within the class *Halobacteria*: proposal to divide the order *Halobacteriales* into the families *Halobacteriaceae*, *Haloarculaceae* fam. nov., and *Halococcaceae* fam. nov., and the order *Haloferacales* into the families, *Haloferacaceae* and *Halorubraceae* fam nov. Antonie van Leeuwenhoek 109:565–587.

Han, Q., and D. Liu. 2014. Macroalgae blooms and their effects on seagrass ecosystems. Journal of Ocean University of China 13:791–798.

Han, Y., and M. Perner. 2015. The globally widespread genus *Sulfurimonas*: versatile energy metabolisms and adaptations to redox clines. Frontiers in Microbiology 6.

Hansen, J., J. Udy, C. Perry, W. Dennison, and Ba. Lomstein. 2000. Effect of the seagrass *Zostera capricorni* on sediment microbial processes. Marine Ecology Progress Series 199:83–96.

van der Heide, T., E. H. van Nes, G. W. Geerling, A. J. P. Smolders, T. J. Bouma, and M. M. van Katwijk. 2007. Positive Feedbacks in Seagrass Ecosystems: Implications for Success in Conservation and Restoration. Ecosystems 10:1311–1322.

van der Heide, T., E. Peeters, D. C. R. Hermus, M. M. van Katwijk, J. G. M. Roelofs, and A. J. P. Smolders. 2009. Predicting habitat suitability in temperate seagrass ecosystems. Limnology and Oceanography 54:2018–2024.

Ho, H. T. K., L. J. A. Lipman, and W. Gaastra. 2006. Arcobacter, what is known and unknown about a potential foodborne zoonotic agent! Veterinary Microbiology 115:1–13.

Hootsmans, M. J. M., and J. E. Vermaat. 1985a. The effect of periphyton-grazing by three epifaunal species on the growth of *Zostera marina* L. under experimental conditions. Aquatic Botany 22:83–88.

Hootsmans, M. J. M., and J. E. Vermaat. 1985b. The effect of periphyton-grazing by three epifaunal species on the growth of Zostera marina L. under experimental conditions. Aquatic Botany 22:83–88.

Hu, D., G. Cha, and B. Gao. 2018. A Phylogenomic and Molecular Markers Based Analysis of the Class *Acidimicrobiia*. Frontiers in Microbiology 9:987.

Infantes, E., L. Eriander, and P. Moksnes. 2016. Eelgrass (*Zostera marina*) restoration on the west coast of Sweden using seeds. Marine Ecology Progress Series 546:31–45.

Jannasch, H. W. 1969. Estimations of Bacterial Growth Rates in Natural Waters. Journal of Bacteriology 99:156–160.

Jaschinski, S., N. Aberle, S. Gohse-Reimann, H. Brendelberger, K. H. Wiltshire, and U. Sommer. 2009. Grazer diversity effects in an eelgrass–epiphyte–microphytobenthos system. Oecologia 159:607–615.

Jørgensen, M. S., R. Labouriau, and B. Olesen. 2019. Seed size and burial depth influence *Zostera marina* L. (eelgrass) seed survival, seedling emergence and initial seedling biomass development. PLOS ONE 14:e0215157.

van Katwijk, M. M., A. R. Bos, V. N. de Jonge, L. S. A. M. Hanssen, D. C. R. Hermus, and D. J. de Jong. 2009. Guidelines for seagrass restoration: Importance of habitat selection and donor population, spreading of risks, and ecosystem engineering effects. Marine Pollution Bulletin 58:179–188.

van Katwijk, M. M., D. C. R. Hermus, D. J. de Jong, R. M. Asmus, and V. N. de Jonge. 2000. Habitat suitability of the Wadden Sea for restoration of *Zostera marina* beds. Helgoland Marine Research 54:117–128.

van Katwijk, M. M., A. Thorhaug, N. Marbà, R. J. Orth, C. M. Duarte, G. A. Kendrick, I. H. J. Althuizen, E. Balestri, G. Bernard, M. L. Cambridge, A. Cunha, C. Durance, W. Giesen, Q. Han, S. Hosokawa, W. Kiswara, T. Komatsu, C. Lardicci, K.-S. Lee, A. Meinesz, M. Nakaoka, K. R. O'Brien, E. I. Paling, C. Pickerell, A. M. A. Ransijn, and J. J. Verduin. 2016. Global analysis of seagrass restoration: the importance of large-scale planting. Journal of Applied Ecology 53:567–578.

van Katwijk, M., and D. Hermus. 2000. Effects of water dynamics on *Zostera marina*: transplantation experiments in the intertidal Dutch Wadden Sea. Marine Ecology Progress Series 208:107–118.

Kim, B.-R., J. Shin, R. B. Guevarra, J. H. Lee, D. W. Kim, K.-H. Seol, J.-H. Lee, H. B. Kim, and R. E. Isaacson. 2017. Deciphering Diversity Indices for a Better Understanding of Microbial Communities. Journal of Microbiology and Biotechnology 27:2089–2093.

Koch, E. W. 2001. Beyond Light: Physical, Geological, and Geochemical Parameters as Possible Submersed Aquatic Vegetation Habitat Requirements. Estuaries 24:1.

Krüger, T., R. Oelmüller, and B. Luckas. 2009. Comparative PCR analysis of toxic *Nodularia spumigena* and non-toxic *Nodularia harveyana* (Nostocales, Cyanobacteria) with respect to the nodularin synthetase gene cluster. European Journal of Phycology 44:291–295.

Lamers, L. P. M., L. L. Govers, I. C. Janssen, J. J. Geurts, M. E. Van der Welle, M. M. Van Katwijk, T. Van der Heide, J. G. Roelofs, and A. J. Smolders. 2013. Sulfide as a soil phytotoxin—a review. Frontiers in Plant Science 4.

Lange, G., J. A. Schmitt, I. Kröncke, S. D. Moorthi, S. Rohde, S. Scheu, and P. J. Schupp. 2019. The role of invasive marine plants for macrofauna nutrition in the Wadden Sea. Journal of Experimental Marine Biology and Ecology 512:1–11.

Lee, K.-S., S. R. Park, and Y. K. Kim. 2007. Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review. Journal of Experimental Marine Biology and Ecology 350:144–175.

Lefcheck, J. S., R. J. Orth, W. C. Dennison, D. J. Wilcox, R. R. Murphy, J. Keisman, C. Gurbisz, M. Hannam, J. B. Landry, K. A. Moore, C. J. Patrick, J. Testa, D. E. Weller, and R. A. Batiuk. 2018. Long-term nutrient reductions lead to the unprecedented recovery of a temperate coastal region. Proceedings of the National Academy of Sciences 115:3658–3662.

Levican, A., L. Collado, and M. J. Figueras. 2013. *Arcobacter cloacae* sp. nov. and *Arcobacter suis* sp. nov., two new species isolated from food and sewage. Systematic and Applied Microbiology 36:22–27.

Liu, Y., and W. B. Whitman. 2008. Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic Archaea. Annals of the New York Academy of Sciences 1:171–189.

Liu, Y., X. Zhang, W. Song, and Z. Wang. 2016. Artificial seed germination and seedling production of *Zostera marina L.* by salinity manipulation. Acta Oceanologica Sinica 35:99–105.

Longstaff, B. J., N. R. Loneragan, M. J. O'Donohue, and W. C. Dennison. 1999. Effects of light deprivation on the survival and recovery of the seagrass *Halophila ovalis* (R.Br.) Hook. Journal of Experimental Marine Biology and Ecology 234:1–27.

Lugtenberg, B., and F. Kamilova. 2009. Plant-Growth-Promoting Rhizobacteria. Annual Review of Microbiology 63:541–556.

MacIntyre, H. L., R. J. Geider, and D. C. Miller. 1996. Microphytobenthos: The Ecological Role of the "Secret Garden" of Unvegetated, Shallow-Water Marine Habitats. I. Distribution, Abundance and Primary Production. Estuaries 19:186.

Marion, S. R., and R. J. Orth. 2010. Innovative Techniques for Large-scale Seagrass Restoration Using *Zostera marina* (eelgrass) Seeds. Restoration Ecology 18:514–526.

Martin, B. C. 2019. Oxygen loss from seagrass roots coincides with colonisation of sulphide-oxidising cable bacteria and reduces sulphide stress. The ISME journal 13:707–719.

Martin, B. C., D. Gleeson, J. Statton, A. R. Siebers, P. Grierson, M. H. Ryan, and G. A. Kendrick. 2018. Low Light Availability Alters Root Exudation and Reduces Putative Beneficial Microorganisms in Seagrass Roots. Frontiers in Microbiology 8.

Mascaró, O., T. Valdemarsen, M. Holmer, M. Pérez, and J. Romero. 2009. Experimental manipulation of sediment organic content and water column aeration reduces *Zostera marina* (eelgrass) growth and survival. Journal of Experimental Marine Biology and Ecology:9.

Matheson, F., J. Reed, V. Dos Santos, G.Mackay, and V. Cummings. 2016. Seagrass rehabilitation: successful transplants and evaluation of methods at different spatial scales. New Zealand Journal of Marine and Freshwater Research:1–14.

Matsumoto, A., H. Kasai, Y. Matsuo, S. Ōmura, Y. Shizuri, and Y. Takahashi. 2009. *Ilumatobacter fluminis* gen. nov., sp. nov., a novel actinobacterium isolated from the sediment of an estuary. The Journal of General and Applied Microbiology 55:201–205.

Matsumoto, A., H. Kasai, Y. Matsuo, Y. Shizuri, N. Ichikawa, N. Fujita, S. Omura, and Y. Takahashi. 2013. *Ilumatobacter nonamiense* sp. nov. and *Ilumatobacter coccineum* sp. nov., isolated from seashore sand. International Journal of Systematic and Evolutionary Microbiology 63:3404–3408.

Maxwell, P. S., J. S. Eklöf, M. M. van Katwijk, K. R. O'Brien, M. de la Torre-Castro, C. Boström, T. J. Bouma, D. Krause-Jensen, R. K. F. Unsworth, B. I. van Tussenbroek, and T. van der Heide. 2016. The fundamental

role of ecological feedback mechanisms for the adaptive management of seagrass ecosystems - a review: Review of feedbacks in seagrass. Biological Reviews 92:1521–1538.

Mendes, R., P. Garbeva, and J. M. Raaijmakers. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiology Reviews 37:634–663.

Milbrandt, E. C., J. Greenawalt-Boswell, and P. D. Sokoloff. 2008. Short-term indicators of seagrass transplant stress in response to sediment bacterial community disruption. Botanica Marina 51.

Mino, S., H. Kudo, T. Arai, T. Sawabe, K. Takai, and S. Nakagawa. 2014. *Sulfurovum aggregans* sp. nov., a hydrogen-oxidizing, thiosulfate-reducing chemolithoautotroph within the *Epsilonproteobacteria* isolated from a deep-sea hydrothermal vent chimney, and an emended description of the genus Sulfurovum. INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY 64:3195–3201.

MO BIO Laboratories, Inc. 1993a. RNA PowerSoil Total RNA isolation kit. Pages 1–15. Instruction manual, QIAGEN, Carlsbad, CA USA.

MO BIO Laboratories, Inc. 1993b. RNA PowerSoil DNA Elution Accessory Kit. Pages 1–14. Instruction manual, QIAGEN, Carlsbad, CA USA.

Moksnes, P.-O., L. Eriander, E. Infantes, and M. Holmer. 2018. Local Regime Shifts Prevent Natural Recovery and Restoration of Lost Eelgrass Beds Along the Swedish West Coast. Estuaries and Coasts 41:1712–1731.

Mussmann, M., K. Ishii, R. Rabus, and R. Amann. 2005. Diversity and vertical distribution of cultured and uncultured *Deltaproteobacteria* in an intertidal mud flat of the Wadden Sea. Environmental Microbiology 7:405–418.

Nakaoka, M. 2002. Predation on seeds of seagrasses *Zostera marina* and *Zostera caulescens* by a tanaid crustacean *Zeuxo* sp. Aquatic Botany 72:99–106.

Nielsen, J. T., W. Liesack, and K. Finster. 1999. *Desulfovibrio zosterae* sp. nov., a new sulfate reducer isolated from surface-sterilized roots of the seagrass *Zostera marina*. International Journal of Systematic and Evolutionary Microbiology 49:859–865.

Nyström, M., A. V. Norström, T. Blenckner, M. de la Torre-Castro, J. S. Eklöf, C. Folke, H. Österblom, R. S. Steneck, M. Thyresson, and M. Troell. 2012. Confronting Feedbacks of Degraded Marine Ecosystems. Ecosystems 15:695–710.

Ort, B. S., C. S. Cohen, K. E. Boyer, L. K. Reynolds, S. M. Tam, and S. Wyllie-Echeverria. 2014. Conservation of Eelgrass (Zostera marina) Genetic Diversity in a Mesocosm-Based Restoration Experiment. PLoS ONE 9:e89316.

Orth, R. J., T. J. Carruthers, W. C. Dennison, C. M. Duarte, J. W. Fourqurean, K. L. Heck, A. R. Hughes, G. A. Kendrick, W. J. Kenworthy, and S. Olyarnik. 2006a. A global crisis for seagrass ecosystems. Bioscience 56:987–996.

Orth, R. J., W. C. Dennison, J. S. Lefcheck, C. Gurbisz, M. Hannam, J. Keisman, J. B. Landry, K. A. Moore, R. R. Murphy, C. J. Patrick, J. Testa, D. E. Weller, and D. J. Wilcox. 2017a. Submersed Aquatic Vegetation in Chesapeake Bay: Sentinel Species in a Changing World. BioScience 67:698–712.

Orth, R. J., W. C. Dennison, J. S. Lefcheck, C. Gurbisz, M. Hannam, J. Keisman, J. B. Landry, K. A. Moore, R. R. Murphy, C. J. Patrick, J. Testa, D. E. Weller, and D. J. Wilcox. 2017b. Submersed Aquatic Vegetation in Chesapeake Bay: Sentinel Species in a Changing World. BioScience 67:698–712.

Orth, R. J., M. C. Harwell, and G. J. Inglis. 2006b. Ecology of Seagrass Seeds and Seagrass Dispersal Processes. Pages 111–133 Seagrasses: Biology, Ecology and Conservation. Springer-Verlag, Berlin/Heidelberg.

Orth, R. J., G. A. Kendrick, and S. R. Marion. 2007. *Posidonia australis* seed predation in seagrass habitats of Two Peoples Bay, Western Australia. Aquatic Botany 86:83–85.

Orth, R. J., S. R. Marion, S. Granger, and M. Traber. 2009. Evaluation of a mechanical seed planter for transplanting *Zostera marina* (eelgrass) seeds. Aquatic Botany 90:204–208.

Orth, R., and K. McGlathery. 2012. Eelgrass recovery in the coastal bays of the Virginia Coast Reserve, USA. Marine Ecology Progress Series 448:173–176.

Otte, J. M., N. Blackwell, V. Soos, S. Rughöft, M. Maisch, A. Kappler, S. Kleindienst, and C. Schmidt. 2018. Sterilization impacts on marine sediment---Are we able to inactivate microorganisms in environmental samples? FEMS Microbiology Ecology 94.

Paling, E. I., M. Fonseca, M. M. van Katwijk, and M. van Keulen. 2009. Seagrass restoration. Coastal wetlands: an integrated ecosystem approach:687–713.

Pati, A., S. Gronow, A. Lapidus, A. Copeland, T. Glavina Del Rio, M. Nolan, S. Lucas, H. Tice, J.-F. Cheng, C. Han, O. Chertkov, D. Bruce, R. Tapia, L. Goodwin, S. Pitluck, K. Liolios, N. Ivanova, K. Mavromatis, A. Chen, K. Palaniappan, M. Land, L. Hauser, Y.-J. Chang, C. D. Jeffries, J. C. Detter, M. Rohde, M. Göker, J. Bristow, J. A. Eisen, V. Markowitz, P. Hugenholtz, H.-P. Klenk, and N. C. Kyrpides. 2010. Complete genome sequence of *Arcobacter nitrofigilis* type strain (CIT). Standards in Genomic Sciences 2:300–308.

Paulo, D., A. H. Cunha, J. Boavida, E. A. Serrão, E. J. Gonçalves, and M. Fonseca. 2019. Open Coast Seagrass Restoration. Can We Do It? Large Scale Seagrass Transplants. Frontiers in Marine Science 6:52.

Peckol, P., and A. B. Putnam. 2017. Differential toxic effects of *Ulva lactuca* (Chlorophyta) on the herbivorous gastropods, *Littorina littorea* and *L. obtusata* (Mollusca). Journal of Phycology 53:361–367.

Pedersen, O., T. Binzer, and J. Borum. 2004. Sulphide intrusion in eelgrass (*Zostera marina* L.). Plant, Cell and Environment 27:595–602.

Perez, K. O., R. L. Carlson, M. J. Shulman, and J. C. Ellis. 2009. Why are intertidal snails rare in the subtidal? Predation, growth and the vertical distribution of *Littorina littorea* (L.) in the Gulf of Maine. Journal of Experimental Marine Biology and Ecology 369:79–86.

Pérez-Cataluña, A., N. Salas-Massó, A. L. Diéguez, S. Balboa, A. Lema, J. L. Romalde, and M. J. Figueras. 2018. Revisiting the Taxonomy of the Genus *Arcobacter*: Getting Order From the Chaos. Frontiers in Microbiology 9:2077.

Philippot, L., J. M. Raaijmakers, P. Lemanceau, and W. H. van der Putten. 2013. Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology 11:789–799.

Pivato, M., L. Carniello, I. Moro, and P. D'Odorico. 2019. On the feedback between water turbidity and microphytobenthos growth in shallow tidal environments. Earth Surface Processes and Landforms 44:1192–1206.

Plus, M., J.-M. Deslous-Paoli, and F. Dagault. 2003. Seagrass (*Zostera marina*L.) bed recolonisation after anoxia-induced full mortality. Aquatic Botany:14.

Postma, J., C. H. Hok-A-Hin, and J. H. O. Voshaar. 1990. Influence of the inoculum density on the growth and survival of Rhizobium leguminosarum biovar trifolii introduced into sterile and non-sterile loamy sand and silt loam. FEMS Microbiology Ecology 6:49–57.

Putnam, A., and P. Peckol. 2018. Asymmetric interference competition between herbivorous gastropods, introduced *Littorina littorea* and indigenous *L. obtusata*. Marine Ecology Progress Series 594:135–147.

Ralph, P. J., M. J. Durako, S. Enríquez, C. J. Collier, and M. A. Doblin. 2007. Impact of light limitation on seagrasses. Journal of Experimental Marine Biology and Ecology 350:176–193.

Reynolds, L., M. Waycott, K. McGlathery, R. Orth, and J. Zieman. 2012. Eelgrass restoration by seed maintains genetic diversity: case study from a coastal bay system. Marine Ecology Progress Series 448:223–233.

Reynolds, P. L., J. P. Richardson, and J. E. Duffy. 2014. Field experimental evidence that grazers mediate transition between microalgal and seagrass dominance. Limnology and Oceanography 59:1053–1064.

Rezek, R. J., B. T. Furman, R. P. Jung, M. O. Hall, and S. S. Bell. 2019. Long-term performance of seagrass restoration projects in Florida, USA. Scientific Reports 9:15514.

Scott, A. L., P. H. York, C. Duncan, P. I. Macreadie, R. M. Connolly, M. T. Ellis, J. C. Jarvis, K. I. Jinks, H. Marsh, and M. A. Rasheed. 2018. The Role of Herbivory in Structuring Tropical Seagrass Ecosystem Service Delivery. Frontiers in Plant Science 9.

Seuront, L., A.-C. Duponchel, and C. Chapperon. 2007. Heavy-tailed distributions in the intermittent motion behaviour of the intertidal gastropod *Littorina littorea*. Physica A: Statistical Mechanics and its Applications 385:573–582.

Short, F. T. 1987. Effects of sediment nutrients on seagrasses: Literature review and mesocosm experiment. Aquatic Botany 27:41–57.

Sparks, D. L. 2007. Microbial Ecology of Methanogens, and Methanotrophs. Page Advances in agronomy. Academic, London.

Sullivan, B. K., S. M. Trevathan-Tackett, S. Neuhauser, and L. L. Govers. 2017. Review: Host-pathogen dynamics of seagrass diseases under future global change. Marine Pollution Bulletin.

Tan, Y. M., O. Dalby, G. A. Kendrick, J. Statton, E. A. Sinclair, M. W. Fraser, P. I. Macreadie, C. L. Gillies, R. A. Coleman, M. Waycott, K. van Dijk, A. Vergés, J. D. Ross, M. L. Campbell, F. E. Matheson, E. L. Jackson, A. D. Irving, L. L. Govers, R. M. Connolly, I. M. McLeod, M. A. Rasheed, H. Kirkman, M. R. Flindt, T. Lange, A. D. Miller, and C. D. H. Sherman. 2020. Seagrass Restoration Is Possible: Insights and Lessons From Australia and New Zealand. Frontiers in Marine Science 7:617.

de Tezanos Pinto, P., and E. Litchman. 2010. Interactive effects of N:P ratios and light on nitrogen-fixer abundance. Oikos 119:567–575.

Ugarelli, K., S. Chakrabarti, P. Laas, and U. Stingl. 2017. The Seagrass Holobiont and Its Microbiome. Microorganisms 5:81.

Unsworth, R. K. F., C. M. Bertelli, L. C. Cullen-Unsworth, N. Esteban, B. L. Jones, R. Lilley, C. Lowe, H. K. Nuuttila, and S. C. Rees. 2019. Sowing the Seeds of Seagrass Recovery Using Hessian Bags. Frontiers in Ecology and Evolution 7:311.

Valentine, J. F., and J. E. Duffy. 2006. The central role of grazing in seagrass ecology. Pages 463–501 Seagrasses: Biology, Ecologyand Conservation. Springer Netherlands, Dordrecht.

Wainwright, B. J., G. L. Zahn, J. Zushi, N. L. Y. Lee, J. L. S. Ooi, J. N. Lee, and D. Huang. 2019. Seagrass-associated fungal communities show distance decay of similarity that has implications for seagrass management and restoration. Ecology and Evolution 9:11288–11297.

Waite, D. W., I. Vanwonterghem, C. Rinke, D. H. Parks, Y. Zhang, K. Takai, S. M. Sievert, J. Simon, B. J. Campbell, T. E. Hanson, T. Woyke, M. G. Klotz, and P. Hugenholtz. 2017. Comparative Genomic Analysis of the Class *Epsilonproteobacteria* and Proposed Reclassification to *Epsilonbacteraeota* (phyl. nov.). Frontiers in Microbiology 8.

Walker, T. S., H. P. Bais, E. Grotewold, and J. M. Vivanco. 2003. Root Exudation and Rhizosphere Biology. Plant physiology 132:44–51.

Wasserfallen, A., J. Nölling, P. Pfister, J. Reeve, and E. Conway de Macario. 2000. Phylogenetic analysis of 18 thermophilic Methanobacterium isolates supports the proposals to create a new genus, Methanothermobacter gen. nov., and to reclassify several isolates in three species, Methanothermobacter thermautotrophicus comb. nov., Methanothermobacter wolfeii comb. nov., and Methanothermobacter marburgensis sp. nov. International Journal of Systematic and Evolutionary Microbiology 50 Pt 1:43–53.

Waycott, M., C. M. Duarte, T. J. B. Carruthers, R. J. Orth, W. C. Dennison, S. Olyarnik, A. Calladine, J. W. Fourqurean, K. L. Heck, A. R. Hughes, G. A. Kendrick, W. J. Kenworthy, F. T. Short, and S. L. Williams. 2009. Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proceedings of the National Academy of Sciences 106:12377–12381.

Welsh, D. T. 2000. Nitrogen fixation in seagrass meadows: Regulation, plant–bacteria interactions and significance to primary productivity. Ecology Letters 3:58–71.

Whalen, M. A., J. E. Duffy, and J. B. Grace. 2013. Temporal shifts in top-down vs. bottom-up control of epiphytic algae in a seagrass ecosystem. Ecology 94:510–520.

Whitman, W. B., T. L. Bowen, and D. R. Boone. 2006. The Methanogenic Bacteria. Pages 165–207 *in* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, editors. The Prokaryotes: Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes. Springer New York, New York, NY.

Williams, K. P., B. W. Sobral, and A. W. Dickerman. 2007. A Robust Species Tree for the Alphaproteobacteria. Journal of Bacteriology 189:4578–4586.

Worakit, S., D. R. Boone, R. A. Mah, M.-E. Abdel-Samie, and M. M. El-Halwagi. 1986. *Methanobacterium alcaliphilum* sp. nov., an H2-Utilizing Methanogen That Grows at High pH Values. International Journal of Systematic Bacteriology 36:380–382.

Xie, Y., L. Wang, X. Liu, X. Li, Y. Wang, and B. Huang. 2019. Contrasting responses of intertidal microphytobenthos and phytoplankton biomass and taxonomic composition to the nutrient loads in the Jiulong River Estuary. Phycological Research 67:152–163.

van der Zee, E. M., C. Angelini, L. L. Govers, M. J. A. Christianen, A. H. Altieri, K. J. van der Reijden, B. R. Silliman, J. van de Koppel, M. van der Geest, J. A. van Gils, H. W. van der Veer, T. Piersma, P. C. de Ruiter, H. Olff, and T. van der Heide. 2016. How habitat-modifying organisms structure the food web of two coastal ecosystems. Proceedings of the Royal Society B: Biological Sciences 283:20152326.

Zhang, P.-D., C. Fang, J. Liu, Q. Xu, W.-T. Li, and Y.-S. Liu. 2015. An effective seed protection method for planting *Zostera marina* (eelgrass) seeds: Implications for their large-scale restoration. Marine pollution bulletin v. 95:89–99.

Appendix

P-values Unifrac dissimilarities

Archaea

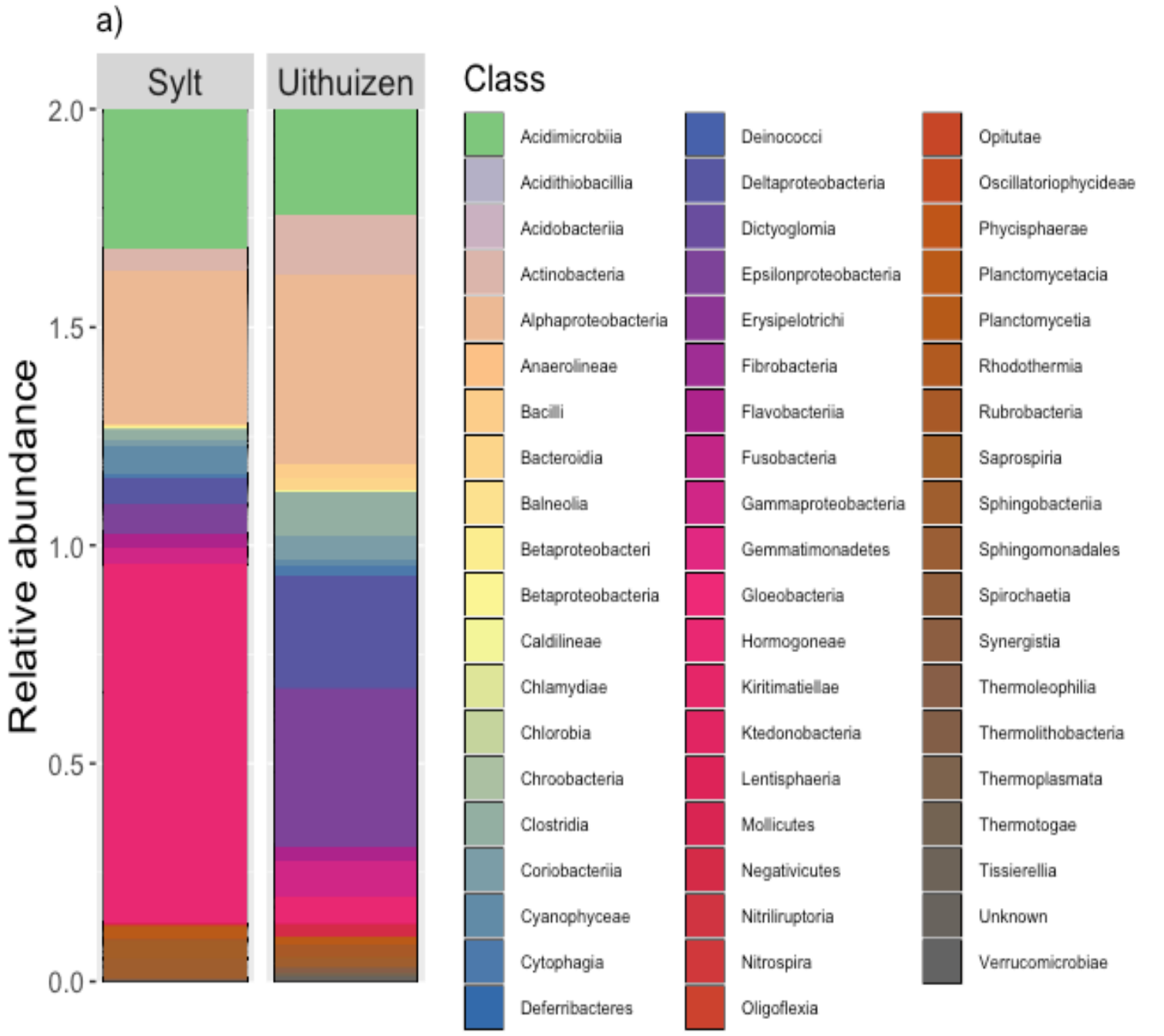
Treatment	Diversity metric	Pseudo-F	R2	P (perm)
Sediment origin	Weighted Unifrac	250.47	0.99208	0.3333
	Unweighted Unifrac	3.4885	0.6356	0.3333
Sterilization	Weighted Unifrac	1.1993	0.37487	0.3333
	Unweighted Unifrac	1.6264	0.4485	0.3333
Grazers	Weighted Unifrac	1.2885	0.46207	0.6
	Unweighted Unifrac	1.3186	0.46782	0.2667

Bacteria

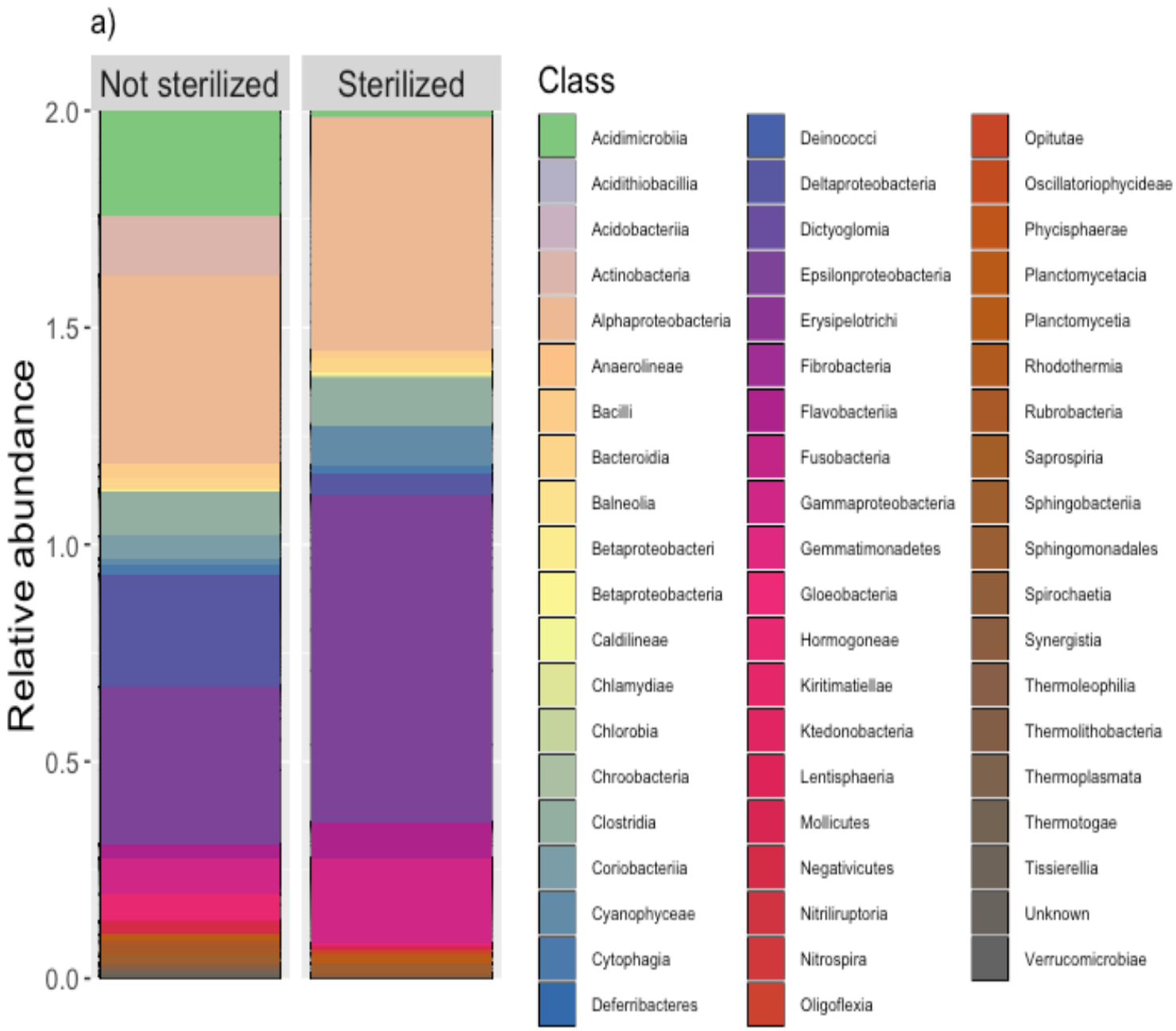
Treatment	Diversity metric	Pseudo-F	R2	P (perm)
Sediment origin	Weighted Unifrac	1.1469	0.36445	0.6667
	Unweighted Unifrac	1.321	0.39777	0.3333
Sterilization	Weighted Unifrac	5.0604	0.71673	0.3333
	Unweighted Unifrac	2.1137	0.51382	0.3333
Grazers	Weighted Unifrac	0.92664	0.38186	0.5333
	Unweighted Unifrac	0.98371	0.39607	0.5333

Enlarged relative abundance plots bacteria

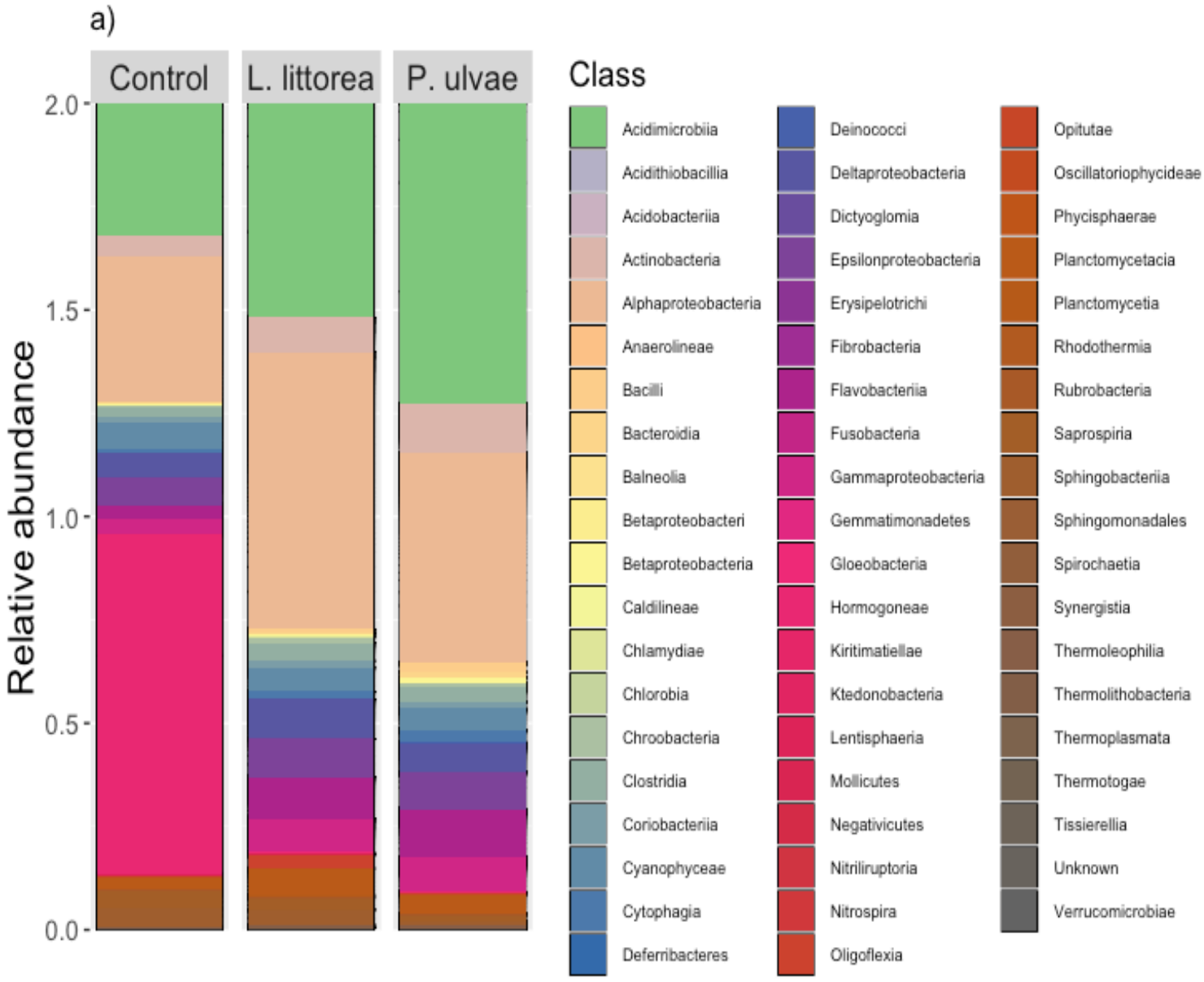
Sediment origin



Sterilization


a)
Not sterilized
Sterilized
Relative abundance
2.0
1.5
1.0
0.5
0.0
Class
Acidimicrobiia
Acidithiobacillia
Acidobacteriia
Actinobacteria
Alphaproteobacteria
Anaerolineae
Bacilli
Bacteroidia
Balneolia
Betaproteobacteri
Betaproteobacteria
Caldilineae
Chlamydiae
Chlorobia
Chroobacteria
Clostridia
Coriobacteriia
Cyanophyceae
Cytophagia
Deferribacteres
Deinococci
Deltaproteobacteria
Dictyoglomia
Epsilonproteobacteria
Erysipelotrichi
Fibrobacteria
Flavobacteriia
Fusobacteria
Gammaproteobacteria
Gemmatimonadetes
Gloeobacteria
Hormogoneae
Kiritimatiellae
Ktedonobacteria
Lentisphaeria
Mollicutes
Negativicutes
Nitriliruptoria
Nitrospira
Oligoflexia
Opitutae
Oscillatoriophycideae
Phycisphaerae
Planctomycetacia
Planctomycetia
Rhodothermia
Rubrobacteria
Saprospiria
Sphingobacteriia
Sphingomonadales
Spirochaetia
Synergistia
Thermoleophilia
Thermolithobacteria
Thermoplasmata
Thermotogae
Tissierellia
Unknown
Verrucomicrobiae

Grazers


a)
Control
L. littorea
P. ulvae
Relative abundance
2.0
1.5
1.0
0.5
0.0
Class
Acidimicrobiia
Acidithiobacillia
Acidobacteriia
Actinobacteria
Alphaproteobacteria
Anaerolineae
Bacilli
Bacteroidia
Balneolia
Betaproteobacteri
Betaproteobacteria
Caldilineae
Chlamydiae
Chlorobia
Chroobacteria
Clostridia
Coriobacteriia
Cyanophyceae
Cytophagia
Deferribacteres
Deinococci
Deltaproteobacteria
Dictyoglomia
Epsilonproteobacteria
Erysipelotrichi
Fibrobacteria
Flavobacteriia
Fusobacteria
Gammaproteobacteria
Gemmatimonadetes
Gloeobacteria
Hormogoneae
Kiritimatiellae
Ktedonobacteria
Lentisphaeria
Mollicutes
Negativicutes
Nitriliruptoria
Nitrospira
Oligoflexia
Opitutae
Oscillatoriophycideae
Phycisphaerae
Planctomycetacia
Planctomycetia
Rhodothermia
Rubrobacteria
Saprospiria
Sphingobacteriia
Sphingomonadales
Spirochaetia
Synergistia
Thermoleophilia
Thermolithobacteria
Thermoplasmata
Thermotogae
Tissierellia
Unknown
Verrucomicrobiae