# The effect of diet on the gut microbiota in *Strongylocentrotus droebachiensis*

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# Abstract

The sea urchin, *Strongylocentrotus droebachiensis*, is a prominent grazer in coastal ecosystems with the ability to graze down large areas of kelp forests and turn them into marine deserts, or so called barren grounds. Kelp are usually avoided by most grazers due to low protein content, thick leaf structure and grazing deterrents. The ability to consume kelp might be caused by a bacterial symbiosis in the sea urchin gut, where the bacteria contribute to the degradation of tough structural components and provide nutrients through nitrogen fixation. To investigate this, the aim of this thesis was to study how the bacterial diversity and composition of bacteria responded to various diets, and if bacteria related to the degradation of structural components and nitrogen fixation could be identified in the sea urchin gut. In order to investigate this, a no-choice feeding experiment was conducted, and the diets were: *Saccharina latissima* (kelp), *Fucus serratus* (wrack) and *Palmaria palmata* (dulse, red algae), each representing a uniform diet. Starved urchins served as control, and sea urchins collected prior to the experiment served as reference for natural microbiomes. The bacterial communities were analyzed from sequenced 16S rRNA gene fragments, and 614 amplicon sequence variants (ASVs) were identified, and one ASV (related to *Psychromonas marina* sp. nov.) accounted for 44 % of the total sequence reads and was present in all samples, suggesting that this is an important symbiont in the sea urchins. The sea urchins given uniform diets, had a significantly lower ASV diversity compared to the control and reference samples. This indicates that uniform diets promote dominant bacterial groups and a lower richness compared to the reference samples, and thus the sea urchins in nature, were probably not restricted to only one type of food source. Several types of ASVs were found related to bacteria that can degrade structural components like alginate, and possible nitrogen fixing representatives, although the latter was likely a bit restrained due to the low C:N ratio of the kelp which indicated a high nitrogen content. These findings can provide a better understanding of how sea urchins in nature are able to survive on high-carbon food sources like kelp.

# Acknowledgements

I have learned a lot about marine biology and a lot about myself, in the past two years at the University of Oslo (UiO). Ever since I was a child going along the shore looking for small creatures in the water and being enthusiastic owning an aquarium, I knew that I had to learn more about the life below the surface.

I would like to thank my supervisor Kjell Magnus Norderhaug for introducing me to the world of sea urchins and kelp forests, and co-supervisor Mia Bengtsson for introducing me to the invisible but very important microbial world. The symbiotic interactions between higher organisms and microbes are found to be very important in the acquisition of nutrients, and there is still a lot more to uncover. I think my master's project was very exciting, because I got to do my own experiment and got a better understanding of the whole process. I would like to thank Kjell Magnus, Stein Fredriksen and the people at the Biological station in Drøbak (Hans Erik, Grete, Jens Ådne and Rune), who helped me get the material to set up the experiment, and with questions along the way.

I enjoyed working at the laboratory, and would like to thank Mia Bengtsson and Sissel I. Brubak for giving me valuable practice in lab techniques and assistance during the lab work at Greifswald and UiO. Thank to Mia for inviting me to her lab at the University of Greifswald (Germany) and the people I met there, for making my stay enjoyable, even though my samples were unfortunately damaged due to shipping. Doing statistics with R (software), can sometimes give "red error signs", making life a bit harder… Therefore I am very thankful for Mia and Jan D. Heuschele for helping me resolve the errors. Despite some obstacles, statistics is actually quite interesting and a valuable tool for analyzing data. Kjell Magnus, Mia and Stein, I am grateful for your advices during the analysis of the results and the writing process.

The past two years at UiO would not have been as good without the other students at my study room (4405), where I got support, and where we had lots of great laughs and many interesting talks and discussions. Finally, I would like to thank my family, especially Nora for reading through my thesis and Daniel for helping me make a map, and friends and boyfriend for supporting me along the way!

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# 1 Introduction

## 1.1 The ecological function of sea urchins

Sea urchins belong to the phylum Echinodermata and have a rounded symmetrical body covered in spines and tube feet, which facilitate locomotion along the substrate and can function as a photosensory organ (Florey & Cahill 1980, Lesser et al. 2011). Sea urchins are found on hard substrata and feed on a variety of food items, like macroalga, diatoms, dead fish, and other dead material (Himmelman & Steele 1971). Sea urchins can induce a substantial grazing pressure on kelp forests, and several events of destructive grazing have been reported around the world (Bernstein et al. 1981, Scheibling et al. 1999). From Carmel Bay in California (Watanabe & Harrold 1991) to the Gulf of Maine on the east coast of the US (Kirby et al. 2001), and to northern Chilean waters (Contreras & Castilla 1987), are just some examples of places that have experienced kelp deforestations due to sea urchin grazing. The urchins have also been responsible for transforming large stretches of kelp forests along the Norwegian coast into marine deserts, or barren grounds since the 1970s (Norderhaug & Christie 2009). Even if each case is unique, the mass grazing events are often associated with an imbalance in the ecosystem (Ling et al. 2009). The removal of sea urchin predators caused by e.g. overfishing, disrupts the fine balance between the predator and its prey (sea urchins), and the reduced predation pressure offer the sea urchins an opportunity to increase their numbers (Steneck et al. 2013). Sea urchins can only affect the local ecosystem in a destructive way once they form dense aggregations, or so called "wavefronts" (Mann 1977), and become the ecologically dominant species. Aggregations of more than 100 individuals per $m^2$ are common in these "wavefronts", in contrast, no more than 5 to 10 individuals per $m^2$ are typically found in healthy kelp forests (Bernstein et al. 1981). A low and scattered population of sea urchins can perform a valuable function in a kelp forest, like preventing overgrowth by epiphytes (Tomas et al. 2005). But when they form dense aggregations, they graze down everything including the kelp itself, and continue to reside on the barrens, thus preventing regrowth of new kelp (Chapman 1981). Going from intensively grazing to barely feeding on the barrens is made possible by the sea urchins great plasticity in adapting to various conditions. The sea urchins can survive through periods with low food availability, and they are able to maximize the utilization of the food particles that come by (Russell

1998). The jaw size can be adjusted according to the food available, and when starved, the urchin jaw will increase its size to scrape a larger area of sediment in the search for food particles (Ebert et al. 2014). A study conducted by Russell (1998) found that the sea urchin gonad plays an essential role in adapting to various types of diets and storing nutrients, and this may be an important reason why sea urchins can survive without regular access to food.

In the North Atlantic, the green sea urchin, *Strongylocentrotus droebachiensis* O.F. Müller 1776, is the only grazer with the greatest impact on controlling large areas of algal abundance (Witman 1985). Macroalgae (kelps, seaweeds and seagrasses) are estimated to cover about 8000 $km^2$ along the Norwegian coast, of which 97 % is comprised of kelp forests (Gundersen et al. 2011), and these numbers illustrate that kelp forests are the dominant macroalgal habitat (in Norway). The two common kelp species in Norway occupy different types of environments: *Laminaria hyperborea* is found on wave exposed sites, while *Saccharina latissima* is found on sheltered rocky shores (Christie et al. 2009, Andersen et al. 2011). Grazing by sea urchins has been the most prominent threat factor in restricting the extensiveness of these kelp communities (*L. hyperborea* and *S. latissima*), and climate change might become an emerging threat (Norderhaug & Christie 2009, Andersen et al. 2011, Gundersen et al. 2011). When kelp forests are grazed down to local extinction, the community shifts into barren grounds with low productivity, low complexity and low diversity, where only few species of crust forming red algae are able to persist (Norderhaug & Christie 2009).

Kelp forests are among the most productive habitats on earth, and support great numbers of species (Christie et al. 2003). The three-dimensional structure of kelp forests creates complex habitats in an otherwise empty body of water (Tegner & Dayton 2000). The macrofauna found in Norwegian kelp forests are represented within several phyla: gastropods, bivalves, echinoderms, crustaceans, fish and mammals (Norderhaug et al. 2012), and this includes several commercially fished species (Norderhaug et al. 2005). Kelp canopies along the coast can dampen waves, which influence water flow, coastal erosion, sedimentation, benthic productivity and recruitment (Duggins et al. 1990, Alonso et al. 2012), and it is suggested that kelp forests could be important contributors to the carbon sink (Nunes et al. 2016). Primary production by kelp forests is an important food source, and enters the ecosystem through direct consumption by grazers, or as particulate organic matter that is consumed by

filter feeding organisms (e.g. mussels), or further processed through bacterial degradation (Christie at al. 2009).

Thus, from an ecological perspective, habitat destruction by sea urchins is highly relevant, and it is important to understand the underlying mechanisms of these occurrences.

## 1.2 Macroalgae as a food source

There are several factors that influence the likelihood that an herbivore will consume a macroalga. These factors include morphology (size, shape, toughness), chemical composition (grazing deterrents, digestibility reducers) and nutritional qualities (e.g. protein, carbohydrate content) (Lubchenco & Gaines 1981). The nutritional quality of the alga is essential for the organism that is going to consume it, because if the alga is of poor quality (e.g. nitrogen limited), the organism has to exhibit compensatory feeding to meet the nutrient demand (Liess 2014). The elemental properties of macroalgae differ among species and with seasonal conditions, and generally red and green algae are known to contain high amounts of the desired proteins (Morgan et al. 1980). In contrast, brown algae like kelp contain high amounts of carbohydrates (Schiener et al. 2015), and the lack of protein which is an important source of nitrogen, results in a C:N ratio (Carbon and Nitrogen ratio) that exceeds what is found in most marine organisms, making the kelp considered to be of poor nutritional quality (Sterner & Hessen 1994, Christie et al. 2009). The ability to live on fresh kelp tissue is rare, and few organisms other than sea urchins can live directly from fresh kelp (Mann 1977). However, as kelp have a perennial lifestyle, the carbon and nitrogen content changes through the year (Broch et al. 2013). In northern temperate seas, the C:N ratio increases during summer, and decreases in the winter season (Nielsen et al. 2014). However, even if the nitrogen content in kelp reach maximum levels during the winter, it is unlikely that it will reach the high levels of red and green algae (Schiener et al. 2015).

Macroalga with a tough thalli structure and secondary metabolites can deter organisms from ingesting it (Daggett et al. 2005, Iken et al. 2009, McDonald & Bingham 2010). A thick and leather-like leaf structure prevents grazing, as most organisms will prefer food were the cost of handling is low (Lemire & Himmelman 1996). This is also true for *S. droebachiensis*, as Daggett et al. (2005) observed in a study that the urchins ingested and absorbed the

formulated feed (a feed developed to enhance the urchin gonad quality for human consumption) faster than though macroalgae like *Palmaria palmata*. However, feeding on structurally complex algae does not deter sea urchins from consuming it, due to a specialized feeding apparatus (Aristotle's lantern) that can chew through tough structures (Wang et al. 1997).

Even if a macroalga can be readily consumed, it may contain defensive toxins to deter hungry grazers, and various types of algae have developed different types of toxins. The green alga *Ulvaria obscura* protects itself against grazing with dopamine, which is a common neurotransmitter in animals. When ingested, the dopamine transforms into a reactive substance that can harm the consumer, and this was found to be effective in deterring *S. droebachiensis* (Van Alstyne et al. 2006). Brown algal phlorotannins are secondary metabolites, and are related to several functions, they protect against UV-radiation, anti-microbial, antifoulant, and anti-herbivory properties (Iken et al. 2009). These substances are commonly found in species of fucoids and Laminariales (kelp), and are proposed to serve an important role in deterring grazing (Geiselman & McConnell 1981, Estes & Steinberg 1988, Levinton et al. 2002). The concentration of phenols varies with grazing pressure and within the different parts of the algae, often concentrated in the meristematic parts (Estes & Steinberg 1988), which is likely due to a trade-off between defensive toxins and growth (Johnson & Mann 1986, Iken et al. 2009). However, in recent years, the anti-herbivore properties of phlorotannins have been debated (Norderhaug et al. 2006, Schuster & Konar 2014), instead Deal et al. (2003) proposed a non-phenolic metabolite as the actual grazing deterrent in brown algae.

In addition to ingesting the algal material, the following degradation is important for the absorption of the nutrients. This requires an intestinal apparatus adapted to handle such material. Enzymes derived from the host and symbiotic bacteria are essential, otherwise the feed might pass through the digestive system and the organism could starve.

## 1.3 The role of bacteria in digestion

To fully understand the biology of the sea urchin, one should also understand the role of its associated microbes, the microbiome. The genes of the microbiome are often much more numerous than the genome of the host organism (Dillon & Dillon 2004, Turnbaugh et al. 2007), and they serve many important functions to the host. The microbial community in the intestine is of particular interest, as it has been recognized as one of the most important host-microbial interactions (Van Horn et al. 2012). The surrounding environment and the resources ingested, have been found to have key roles in modifying the bacterial composition in intestinal flora, as laboratory reared individuals, which are not exposed to the same factors, have different bacterial assemblages (Dillon & Dillon 2004, Ringo et al. 2006, Zhang et al. 2014). Harris (1993) made a summary of the various associations between invertebrates and gut microbes, and only the host-favorable interactions are mentioned here. By ingesting and lysing the bacterial cells, the bacterial enzymes can contribute to the digestion of tough plant material. Some ingested microbes may survive the passage through the gut, and the release of nutrients from the bacterial production, can be absorbed by the host organism (Harris 1993). The symbiotic relationship between the host and the microbes living in the gut is very important, and determines what types of resources are required for the organism to survive. Organisms living on food with poor quality are dependent on microbes for the extraction of vital nutrients, e.g. termites have a strong connection with gut bacteria that play an important role in the degradation of cellulose components (Breznak & Brune 1994).

Sea urchins are omnivores as they consume a broad diversity of organisms from several trophic levels, however, the primary food source is macroalgae like kelp (Himmelman & Steele 1971). As described earlier, kelp is difficult to break down, due to structural components of cellulose and alginate. Breaking down tough algal components can be performed by specialized enzymes from the host, bacteria or protozoans (Lasker & Giese 1954). A study investigating the digestibility of algal tissue in *Strongylocentrotus purpuratus*, observed that the intact alga was not digested by the urchin enzymes, instead they found that the bacteria were able to digest it (Lasker & Giese 1954). Based on these findings, a bacterial symbiosis in the gut was suggested, where the bacteria could digest the structural components of the algae and subsequently release some of the nutrients that could be absorbed by the urchin host. A bacterial symbiosis was also suggested for *S. intermedius* based on bacterial

analyses from gut and stool samples, and the presence of the bacteria *Psychromonas* sp. and *Saccharophagus degradans* could indicate that they contributed to the degradation of alginate and several other structural components (Zhang et al. 2014). When the food source has a low nitrogen content, a symbiotic relationship with nitrogen fixing bacteria in the gut can convert gaseous atmospheric dinitrogen ($N_2$) into the biologically available ammonia ($NH_3$), and thus provide a source of nitrogen (Guerinot & Patriquin 1981). In an experiment by Guerinot and Patriquin (1981), a correlation was found between the nitrogen content in kelp and the nitrogenase activity (enzymes that fix nitrogen) in the sea urchin *S. droebachiensis*, and the nitrogenase activity was activated by a low nitrogen content. However, a more complete characterization of the bacteria found in the gut lacked in this study, as it was based on cultured bacteria. Today, there are more advanced methods to study the bacterial assemblages and their functional properties, which do not require bacterial cultures. Sequencing the 16S rRNA gene amplicons is a culture-independent method, and identifies the various bacteria present. Compared to traditional culturing methods, sequencing the 16S rRNA gene amplicons yields a much higher diversity of microbial populations, and has given researchers a better tool to study the diversity of bacteria living in the gut (Yun et al. 2014). To study the functional properties of the bacteria, metagenomic and metatranscriptomic analyses look directly into the gene and protein expression (Madigan 2015), however these methods were not implemented for this thesis.

## 1.4 Aims and hypotheses

The main aim of this study was to find out if the bacterial communities in the sea urchin gut were sensitive to dietary changes, and if there were differences in bacterial diversity between different food sources. To test this, a no-choice feeding experiment was conducted, with three different algae as treatments and starved urchins as control. The microbial 16S rRNA gene amplicons were analyzed from fecal pellet samples that were collected directly from the gut. Fecal pellet samples were also collected from urchins before the experiment, to serve as reference data for the bacterial diversity found in sea urchins feeding in their natural habitat.

To gain knowledge about the ecological function of the sea urchin microbiome, and how it may contribute to the urchin's diverse diet and its ability to consume low nutritious algae, the following hypotheses were tested:

1. Sea urchins collected in their natural environments are not restricted to a particular food source, as they were in the experiment, and the bacterial community will likely reflect the diverse food alternatives found in the natural environment. Consequently, the bacterial diversity will be different between urchins sampled before the experiment, and the algal treatments.

   **$H0_1$: The bacterial diversity will not differ between the urchins sampled before the experiment and the algal treatments.**

   **$HA_1$: The bacterial diversity will be different in the urchins sampled before the experiment compared to the algal treatments.**

2. Macroalgae that differ in structural and chemical compositions may favor a composition of bacteria that are adapted to process these molecules (Zhang et al. 2014). Living on a monotonous diet may have an effect on the microbial community, as the specific diet may support certain bacterial groups, that will result in a different bacterial composition between the different treatments.

   **$H0_2$: The bacterial composition will be the same for all the algal treatments.**

   **$HA_2$: The bacterial composition will be different among the various algal treatments.**

3. The ability to live on a carbohydrate rich diet, indicates specialized bacteria in the gut that can digest the structural components, and nitrogen fixing bacteria that provide a source of nitrogen, which increases the nutritional value of the diet.

   **$H0_3$: Bacteria involved in degrading algal structural components and fixing nitrogen will not be present in the urchin gut.**

   **$HA_3$: Bacteria involved in degrading algal structural components and fixing nitrogen will be present in the sea urchin gut.**

# 2 Materials and methods

## 2.1 Sea urchin sampling

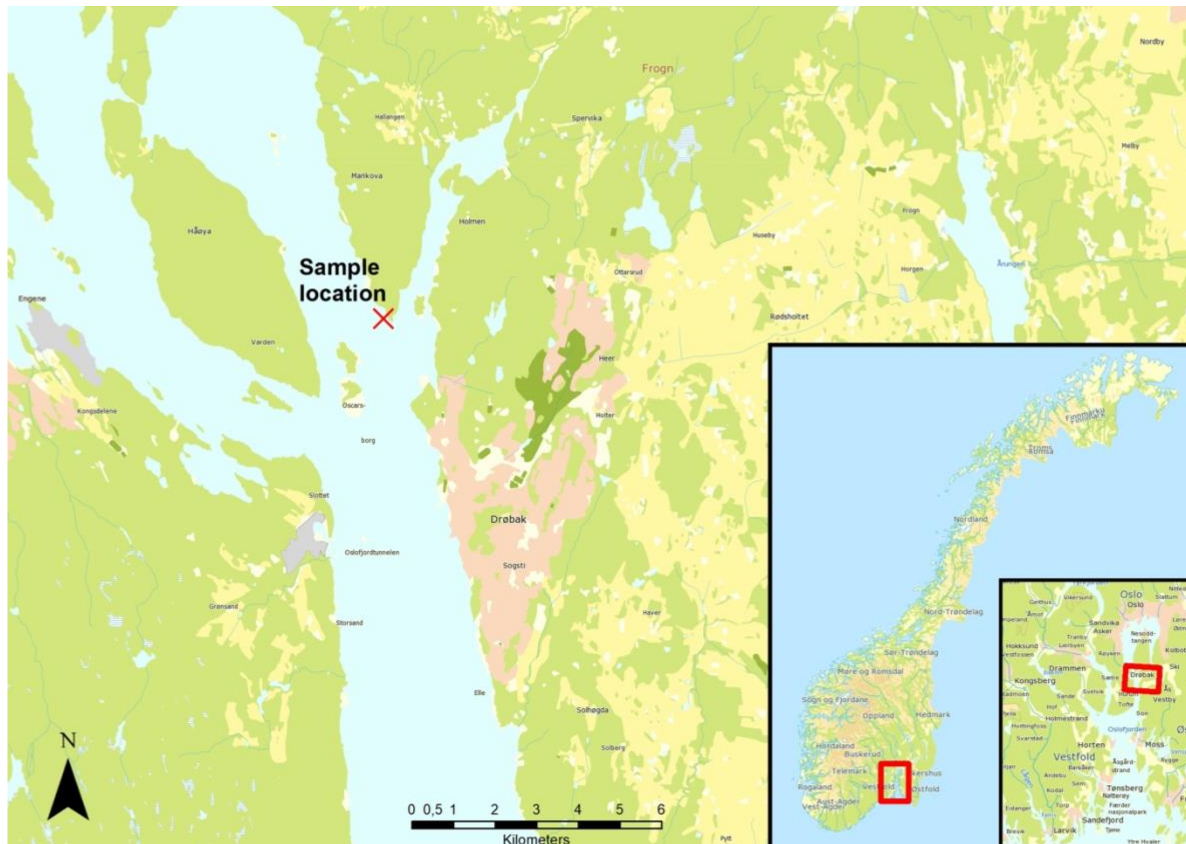


**Figure 1.** The sea urchin sample location was next to Hallangstangen, approximately 2.5 km from the Biological station in Drøbak. Map made with ArcGIS by ESRI.

Wild sea urchins of the species *S. droebachiensis*, approximately 40-60 mm in diameter were collected next to Hallangstangen (59°40'58.8"N, 10°36'49.0"E, Figure 1), 2.5 km north of the main harbor in Drøbak on January 4th, 2017. The seabed topography was a mosaic composed of both soft sediment and hard bottom. A triangular dredge was used to collect the sea urchins from the rocky shores at 6-15 m depth. The collected sea urchins were kept in plastic containers with ambient seawater on the vessel, and transported to the Biological station in Drøbak ("Biologen").

## 2.2 The study area

The Oslofjord is separated into an inner fjord and an outer fjord by a shallow sill at 19.5 m depth near Drøbak (Webb et al. 2009). The outer fjord has free connection to the open sea, while the inner fjord is restricted in circulation due to the sill in Drøbak (Gade 1968). Oslofjord has a history of strong pollution due to sewage discharge and agricultural discharge through rivers and streams running into the fjord, which have restricted the growth of macroalgae at greater depths. In recent years there has been less pollution, but the registration of the lower most growth limit in the fjord has been difficult due to extensive grazing by sea urchins (Thaulow & Faafeng 2014). Sea urchins are mostly found on hard substrata, where they can get a firm attachment to the surface. The depth distribution of the sea urchins depends on temperature and salinity (IMR 2009), and as *S. droebachiensis* is a cold water species, they will retreat to greater depths in the summer season (in northern temperate seas). Urchins living on depths where there is no photosynthetic activity rely on drift algae and other dead material to survive, and have been observed to accumulate on algal pieces of *Fucus* (Kjell Magnus Norderhaug, personal observation). A study by Nyhagen (2015) investigated the occurrence of sea urchins (*S. droebachiensis*) on three sites in the inner Oslofjord, and despite some reduction in size and a change of depth distribution, the sea urchin populations were still regarded as healthy.

## 2.3 Study design

### 2.3.1 Experimental design

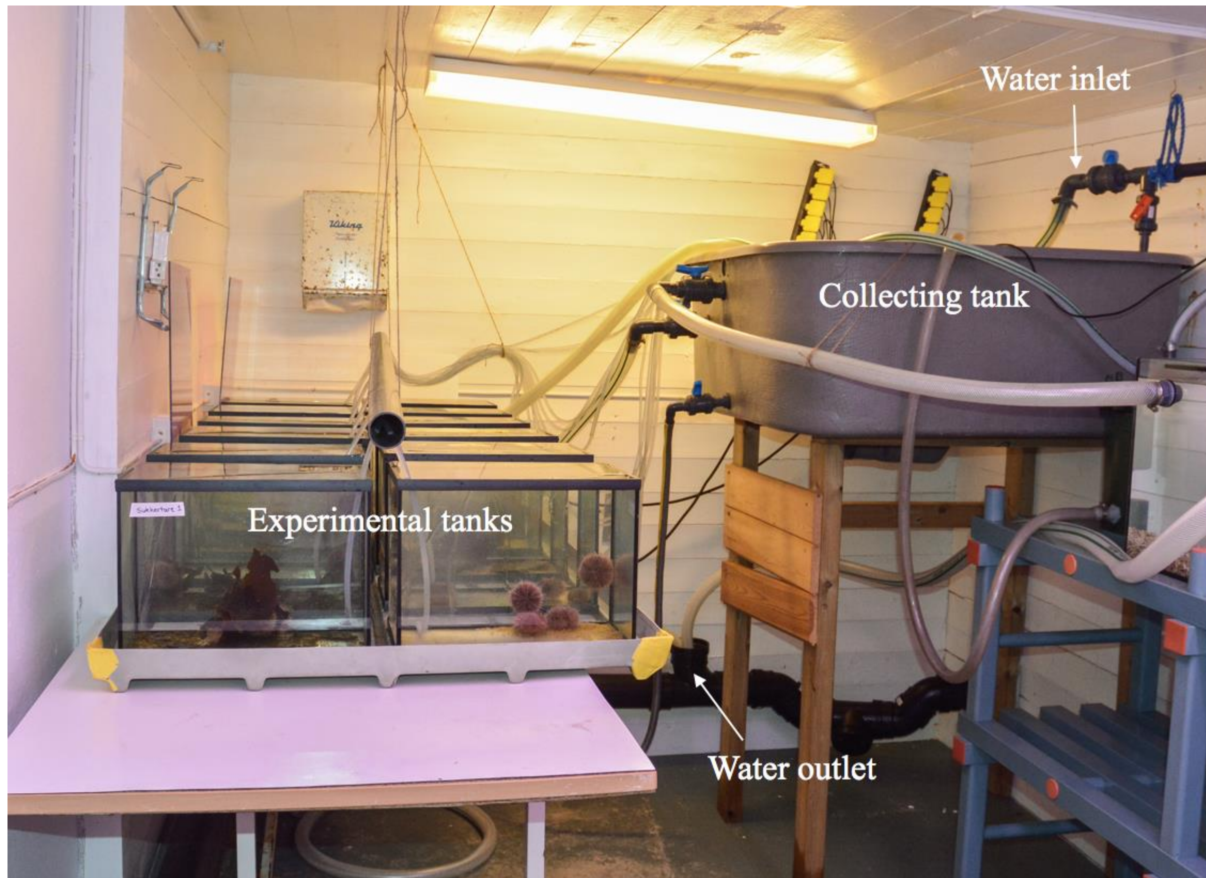


**Figure 2.** Picture of the experimental design. The collecting tank gathers water from the inlet outside the building. There is one pump for each experimental tank which provide fresh water to the tanks continuously (Image by the author).

| 1<br>*S. latissima* | 2<br>*F. serratus* | 3<br>Control | 4<br>*F. serratus* | 5<br>*P. palmata* | 6<br>Control |
|---|---|---|---|---|---|
| 7<br>*P. palmata* | 8<br>Control | 9<br>*S. latissima* | 10<br>*P. palmata* | 11<br>*S. latissima* | 12<br>*F. serratus* |

**Figure 3.** The arrangement of the experimental tanks and the different treatments allocated to each tank.

The sampled sea urchins were randomly distributed among the tanks (Figure 3), and there were five individuals in each tank (5 urchins* 12 fish tanks = 60 urchins). There were twelve glass tanks (L x W x H: 35 x 20 x 25 cm = 17.5 L) aligned into two rows of six. The set-up was designed as a flow-through system, where each tank had its own filter transporting new seawater from a large container (Figure 2), that was continuously re-filled from an inlet next to the Biological station in Drøbak. This design was selected to prevent mixing of water among tanks, and to provide natural water conditions for the sea urchins. The tanks were placed on a table which was raised on one side creating an angle to let water discharge on the other side. The rate of water flow was between 33 to 67 L/h. The urchins were starved for ten days prior to the experiment, to prevent previous feeding influence the experiment and to let them acclimatize to the new conditions. Each tank represented a treatment, and all treatments had three replicate tanks. The water quality (temperature and PSU) was monitored on a regular basis to assure good conditions for the sea urchins (Appendix 1, Table 5). The lights were turned off during the experiment (except when handling), to reduce the impact of undesirable growth by algae in the tanks.

### 2.3.2 Treatments

To find out if the type of diet alters the bacterial composition in the gut, a no-choice feeding experiment was conducted. The diets consisted of three macroalgae species: *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, *Fucus serratus* Linnaeus and *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr. These algae were selected as they could be sampled close to Oslo. *S. latissima* was chosen as it has common features with *L. hyperborea* (both are kelp with similar structural components, and a preferred food source for *S. droebachiensis*), and the other two algae were selected to have some variation in food alternatives for the sea urchin treatments.

*Saccharina latissima* belongs to the order Laminariales (kelps). This is a large macroalga (1–3m) and has thalli composed of holdfast (rhizoid), stipe (cauloid) and lamina (phylloid) (Sjøtun 1993, Wilkinson 1995, Christie et al. 2003, Andersen et al. 2011). It grows on rocky shores from the sublittoral fringe and down to >30 meters, if there are sufficient light conditions. In Europe, its distribution ranges from Portugal to Spitsbergen (Van den Hoek & Donze 1967). It is a perennial species with a seasonal growth pattern. Through the spring and

summer (March to July) kelp performs photosynthesis and the photosynthetic product, carbohydrate, is stored in the kelp. As kelp do not spend resources on growth in the summer season, the carbohydrate content accumulates, and thus the C:N ratio is the highest during this period (Nielsen et al. 2014, Schiener et al. 2015). In northern temperate seas, the nutrient concentration is higher during the winter before the phytoplankton bloom has started (Wroblewski 1989), due to upwelling of nutrient rich water in the fall. During the winter season (from November to January), kelp absorb nutrients from the ambient seawater and with the stored carbon from summer months, the kelp will finally start to grow (Broch et al. 2013, Nielsen et al. 2014). The kelp will now spend the stored carbohydrate on growth and the C:N ratio will decrease. The growth is maximized during spring (March and April), and decreases through summer and autumn due to depletion of its reserves and available nutrients in the water (Sjøtun 1993, Nielsen et al. 2014). The total carbohydrate content (cellulose, laminarin, alginate and mannitol) is on average high throughout the year, and it is estimated to be 63.1 ± 11.4 % of the total biomass (Schiener et al. 2015).

*Fucus serratus* belongs to the order Fucales and is a major component in the lower part of the rocky intertidal communities in the North Atlantic (Coyer et al. 2006). Living in the intertidal zone it must be able to cope with stressors like wave exposure, exposure to air, fluctuating temperature, salinity and irradiation levels (Harley & Helmuth 2003). The adult individuals have receptacles, while the juveniles lack these structures (Malm et al. 2001). Alginate is the main carbohydrate component, and the content of mannitol and laminarin is maximized in the growing tips. *F. serratus* has a dioecious life strategy with separate male and female plants (Black 1949). Fucoids are known to contain high concentrations of phenols, and will be avoided or least preferred by most grazers (Johnson & Mann 1986). Its distribution is restricted to the coasts of the North Atlantic with a southern boundary of 40°N (northern Portugal) (Jueterbock et al. 2013) and has been discovered on the coasts of Svalbard (Spitsbergen) >74°N (Gulliksen et al. 1999).

*Palmaria palmata* is a red alga and belongs to the order Palmariales. It is found in the intertidal and the subtidal zone down to 20 meters in both sheltered and exposed regions. Its lifestyle can be epiphytic and it is often observed growing on the stipes of *L. hyperborea*, or epilithic growing on rock substrata in the intertidal (Whittick 1983). Its distribution is confined to oceans in the North Atlantic with a southern boundary on the shores of Portugal

(Faes & Viejo 2003). The nutritional value of *P. palmata* is considered to be of good quality, because of the high nitrogen content and the presence of vitamins commonly found in fruits and vegetables (Morgan et al. 1980).

The macroalgae were collected in January 2017 from Flødevigen in Arendal (*P. palmata* and *S. latissima*) and from Drøbak (*F. serratus*). The various urchin tanks were presented one species of algae, and were fed three times a week for ten days, to stabilize the microbial community in the gut. Only the algae that appeared free from epiphytes and in good condition were selected for the feeding experiment. The wet weight of the algae was measured and cut into smaller pieces and placed into their respective tanks.

Control tanks with sea urchins, not given any food, were also set up to evaluate if the experimental conditions impacted the results. In addition, samples were taken from sea urchins not included in the experiment, to represent a natural microbiome (referred as "before").

The urchin tanks were cleaned frequently by removing the fecal pellets with a siphon before new feed was given. The urchin behavior appeared to be normal, they usually responded when new feed was dropped into the tanks. There were no mortalities during the experiment.

### 2.3.3 Elemental analysis

To estimate the elemental composition of the algae used in the experiment, samples of algae were dried at 60°C, and when completely dry, they were crushed into fine powder using a mortar. 5 replicate samples of each algal species were analyzed for the carbon and nitrogen content using an elemental analyzer from Thermo Finnigan. As the sea urchins were presented with various parts of the algae during the experiment, different parts of the algae were sampled for the elemental analysis to give a good representation of the food alternatives: the base close to the stem and the middle part from *S. latissima*, the receptacle and thalli from *F. serratus* and new and old growth from *P. palmata*.

### 2.3.4 Sea urchin dissection

Even if the sea urchins produced high amounts of fecal pellets in the tanks, these samples were likely contaminated by the surrounding water, and it would be difficult to know the time of discharge. Thus, fecal pellet samples were dissected directly from the sea urchin gut. Fecal pellet samples were taken from 3 urchins from each tank (3 urchins * 12 tanks = 36 samples), in addition, samples were taken from urchins before the experiment (6 before samples + 36 experiment samples = 42 samples in total). The sea urchins were dissected as described in the sea urchin dissection protocol by Whalen (2008). The width of each sea urchin was measured with a caliper, and a garden shear was used to cut open the sea urchin though the circumference (Figure 4). By cutting through the circumference of the urchin body, the aboral side was separated from the oral side, and the gut content could be sampled carefully by tweezers (see illustration from Figure 5). The large intestine and fecal pellets were identified and placed into separate cryovials and then frozen directly with liquid nitrogen to prevent DNA from degrading. Between each dissection, the equipment was sterilized with decanox, sterilized water and ethanol (70 %). The samples were stored at -80°C until DNA isolation. Fecal pellets were not found in all sea urchins, especially the control urchins, thus the sample size in all groups were not the same. Sample sizes: before = 6, control = 5, all three algal treatments (*Fucus*, *Palmaria*, *Saccharina*) = 9 samples each.

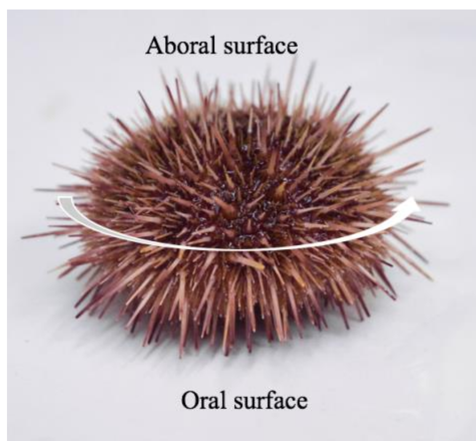


**Figure 4.** The sea urchin was cut in half through the circumference (white arrow) of its body (Image by the author).

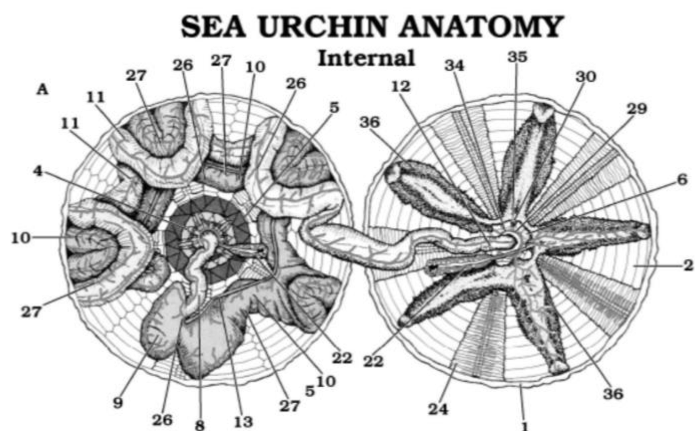


**Figure 5.** Sea urchin internal anatomy. Oral side (left) with intestines (no. 10&11), and aboral side (right) with gonads (no. 36) Whalen (2008).

## 2.4 DNA analysis

A couple of pilot isolations and PCRs were performed to evaluate if there were any adjustments required on the isolation process. Based on the pilot assays (see Appendix 4 Figure 14), the fecal pellets were selected for DNA isolation, as they gave the highest yield in DNA extract compared to isolates from intestine samples (DNA quantification from the Invitrogen™ Qubit™ 3.0 fluorometer).

### 2.4.1 Isolation of microbial DNA

DNA isolation was performed with the commercial DNeasyPowerSoil® (Qiagen, formerly MoBio Laboratories). The fecal pellet samples were thawed and kept cooled on an ice tray, and the DNA isolation was carried out according to the protocol provided from the manufacturer, Qiagen (Appendix 2), with some minor changes: Solution C1 was added before the sample, the amount of fecal pellet samples added was between 0.02 to 0.08 g, and half the amount of solution C6 was used to elute the isolated DNA in the final step. The amount of DNA in isolates from each sample were measured with a Qubit 3.0 fluorometer. The DNA isolates were stored at -20°C until they were shipped for sequencing.

### 2.4.2 Polymerase Chain Reaction (PCR)

Prior to the PCR, the isolated DNA was diluted to about 5 ng/µl in the C6 solution (Appendix 3, Table 9). PCR and sequencing were carried out by LGC Genomics in Germany, and the procedure was as follows: 1 µl of isolated DNA was combined with 15 pmol of the forward and reverse primer, and 20 µl 1 x MyTaq buffer that was made of 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl BioStabll PCR Enhancer (Sigma). The primers used were: forward: 515F-mod (5'-GTGYCAGCMGCCGCGGTAA-3') and reverse: 806R-mod (5'-GGACTACNVGGGTWTCTAAT-3') (Walters et al. 2015). These primers target the V4 region of the prokaryotic 16S rRNA gene. The PCR was set to run for 30 cycles, and the parameters were as follows: 2 min 96°C pre-denaturation, 15 s denaturation at 96°C, 30 s annealing at 50°C, 90 s elongation at 70°C. The DNA concentration was determined by gel electrophoresis.

### 2.4.3 DNA sequencing

Illumina MiSeq paired-end sequencing was used to sequence the PCR products of 16S rRNA gene of bacteria and archaea in the sample. About 20 ng amplicon DNA of each sample was pooled for up to 48 samples carrying different barcodes. One volume AMPure XP beads (Agencourt) was used for the purification of the amplicon pools, which binds PCR amplicons from 100bp and larger and leaves out primer dimers and other mispriming products that can be removed by a simple washing procedure. MinElute columns (Qiagen) were used to remove the non-PCR products. 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 Chemistry (Illumina).

### 2.4.4 Quality-filtering the Illumina amplicon data

To perform statistical analysis from the sequence data, it had to be refined to correct defect sequences. Quality-filtering of the Illumina amplicon data was carried out by my co-supervisor Mia Bengtsson. The DADA2 package (Callahan et al. 2016) was used to purify the Illumina amplicon data. DADA2 involves the implementation of a novel algorithm that corrects the errors introduced during amplicon sequencing, and generates the true sample composition based on the error models. The traditional processing involves the generation of operational taxonomic units (OTUs), which are sequences that are grouped together based on a dissimilarity threshold of normally 3 %. By merging these sequences together, one neglects the fine variation that can distinguish commensal strains from pathogenic strains. The DADA2 provides a better sensitivity and creates a table of sequences with a higher resolution, termed “amplicon sequence variants” (ASV) (Callahan et al. 2017). The package filters low quality sequences and trims the ends of the reads, as the ends often contain errors generated in the sequencing process. It combines the forward and reverse reads, and removes the reads that do not match. Chimeras, which are formed during the PCR, consists of a part of one sample and a part of another, and are identified by the DADA2 package and removed.

## 2.5 Data analysis

### 2.5.1 Statistical analysis of bacterial diversity and community composition

All statistical analyses were carried out using the R software (version 3.4.1) for macOS (R Development Core Team 2010). Prior to the statistical analyses, the dataset had to be processed, and data containing chloroplast sequences, sequences with low or zero abundance (less than 10 000), and samples dominated by plastid sequences were removed. If these sequences were not cut out, then samples with similar diets would generate false relationships based on common chloroplast sequences (derived from the algal food source). The concept of diversity can be described by richness and evenness (Wilsey & Potvin 2000), and to examine the differences in diversity between treatment groups, univariate tests for richness (S) and evenness (J) were performed. ASV richness (S) was calculated with the rarefy function from the vegan package, and rarefied at the lowest recorded number of read counts (min = 17 650). The ASV richness was visualized by boxplots. The "rarefy" function returns the expected ASV richness by subsampling the community data on a ASV level, and calculates the total number of amplicon sequence variants found in the urchin gut. A rarefaction curve was also made to visualize if the sea urchin samples represent the majority of the sequences available. Evenness describes the relative abundance of the different species within the groups. This method can be used to find out if there are dominating bacterial groups, or if the bacterial groups are evenly distributed in the samples.

Pielou's evenness (J) was calculated using this equation: $J = \frac{H}{\log(S)}$

From the equation: Shannon diversity (H) and richness (S).

One-way analysis of variance (ANOVA) was performed to compare the means for each diversity test with the Linear Mixed-Effects Models (lme) function from the nlme package (Pinheiro et al. 2017). As the urchin samples were nested in 3 replicate fish tanks for each treatment, a random nested effect (of tank) were added to the R code to account for this variation. If ANOVA detected significant differences in one or more group means, a Tukey post hoc test was performed to test the difference between treatment pairs. The Tukey test was performed with the General Linear Hypotheses (glht) function from the multcomp package (Hothorn et al. 2008). Prior to the statistical tests, the diversity estimates had to be normally distributed, as this is assumed in the tests. The normality of the models was

evaluated by plotting histograms, and the data were transformed accordingly to achieve normality.

To analyze the bacterial composition within the groups, multivariate statistics were implemented. The response variable for the multivariate statistics consisted of a dataset with all the amplicon sequence variants and the amount of each sequence in each urchin sample. The dataset was Hellinger-transformed with the decostand function from the vegan package (Oksanen et al. 2017). The transformed values were used as basis for Non-metric multidimensional scaling (NMDS) plot and permutation analyses of variance (PERMANOVA), that were generated from the metaMDS and adonis functions respectively. The NMDS plot is based on a Bray-Curtis dissimilarity, and creates a two-dimensional visualization of a multidimensional dataset, and was generated to display the bacterial communities in the different treatments. The distance between the points are based on the degree of similarity of the bacterial communities, where adjacent points have more similar bacterial profiles than non-adjacent points. The stress value indicates how well the plot present the relationship between the bacterial compositions, and a value below 0.2 is considered acceptable. Permutation analyses of variance (PERMANOVA) compares bacterial communities between treatment groups, and identifies if the variation in the community composition is explained by treatments. As there is a risk of sea urchins within the same tank having similar community compositions, and that there might be an interaction between treatment and tank variables, an interaction segment (treatment*tank) was added to the formula. One can interpret the degree of the interaction between treatment and tank by evaluating the $R^2$ value from the PERMANOVA table. An $R^2$ value close to 1, indicates that the specific explanatory variable describes most of the variation in the response variable. The Euler diagram compares the amplicon bacterial sequences (ASVs) among groups and returns a diagram with separate and overlapping regions (ASVs they have in common). The Euler diagram can give a general impression of how the bacteria within the different treatment groups are distributed. It is important to notice that this method combines all samples within a treatment, and thus the number of ASVs will be higher than the richness estimated for each sample in the richness boxplots.

In addition to looking for differences in treatment groups, the bacteria driving these differences were also of interest. A similarity percentages analysis (SIMPER) was conducted to examine the species that differentiate between treatments. This method performs a pair-wise comparison of the treatments using Bray-Curtis dissimilarities with 1000 permutations, and lists the ASVs according to their average contribution to the overall dissimilarity (Oksanen et al. 2017). As there can be numerous ASVs that differentiate between two treatments, only a subset of the data was analyzed. The subset was selected based on the top 10 ASVs with the highest average contribution and a significant p-value $< 0.05$.

### 2.5.2 Taxonomic analysis of bacterial phyla

The taxonomic relationship used for the stacked bar plot was determined using the SILVA Incremental Aligner (SINA), version 1.2.11 (Pruesse et al. 2012). The settings were set to "search and classify", and the gene as "SSU" for small subunit rRNA, otherwise default settings were applied. This method will align rRNA gene sequences and taxonomically classify them from an rRNA database accepted by researchers worldwide. The phylogeny was presented by class-level, and classes with an abundance less than 2 000 sequence variants were assembled together in one group called "Others". The stacked bar plot displays an overall view of the most abundant class-level bacteria within the treatment groups. However, as some taxonomic groups consist of uncultured sequences, they are named after the clone sequence submitted earliest (Pruesse et al. 2012).
Analysis of specific sequence variants of interest were identified to closest relatives with the BLAST function (Altschul et al. 1990), and the assumptions made of their functionality, are based on literature studies and different indicators.

# 3 Results

## 3.1 16S rRNA gene amplicon sequencing results

The Illumina amplicon sequencing resulted in a total of 1 275 250 amplicon sequences across all treatments, and after quality filtering, the number was reduced to 1 097 148 sequences. The total number of filtered sequence reads present in each sample were between 17 650 to 43 870, with a mean of 28 870 reads. A total of 614 prokaryotic amplicon sequence variants, ASVs (a high-resolution analogue to the OTUs), were identified. The majority of the sequences belong to the domain Bacteria, some are unclassified and only one sequence has been identified as Archaea. Thus, 16S rRNA gene sequences will be referred to as bacteria onwards.

ASV no. 1 accounted for 44 % of all sequence reads, while the next most abundant (no. 2) accounted for only 12 %, demonstrating that ASV no.1 is exceptionally dominant. A BLAST search found that ASV no. 1 had a 100 % sequence identity with the species *Psychromonas marina* sp. nov.

How well the DNA analysis is able to detect the most abundant ASVs in the sea urchin gut can be viewed from rarefaction curve (Appendix 4, Figure 15), where the curves level in most samples, indicating a good representation of the abundant ASVs.

## 3.2 Bacterial diversity

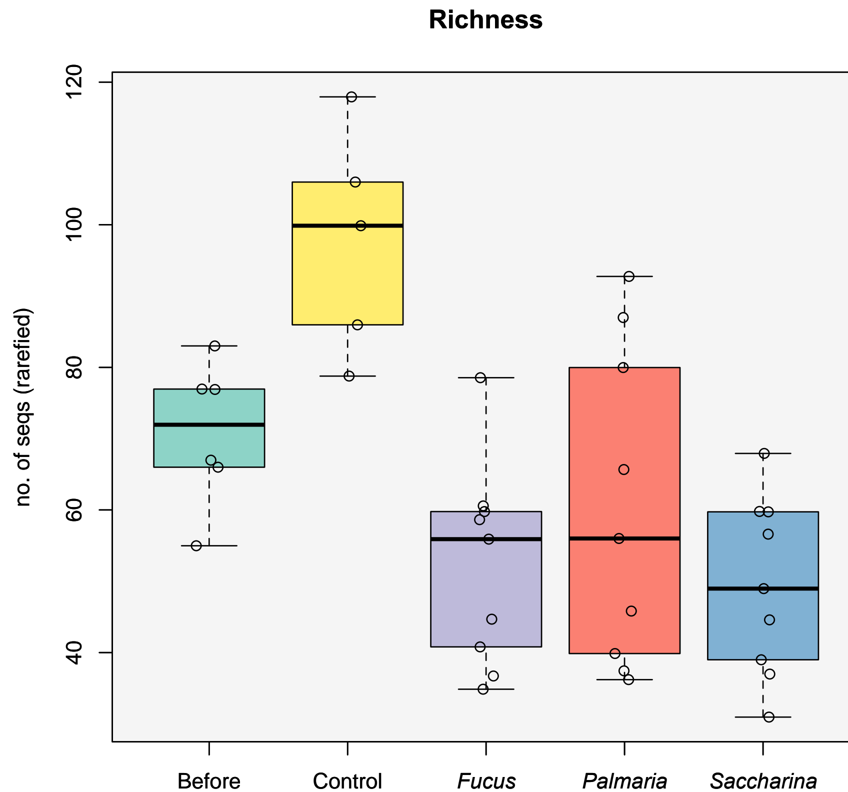


**Figure 6.** Richness boxplots of amplicon sequence variants (total number of ASVs) of the different treatments are listed on the x-axis. The circles in the plot represent each data point. The group labelled "before" represents the urchin samples taken before the experiment, i.e. with a bacterial richness reflecting the natural environment.

The before group consists of the sea urchins sampled before the experiment, and represents the natural microbiome of the urchin intestine, this group had a median value higher than the algal treatments, suggesting that the ASV richness in on average higher (Figure 6). The *Palmaria* group overlaps the boxplot of the before group and the other algal groups, which suggests a spread of the data points. One can observe that the ASV richness of the control group, which was urchins not fed during the experiment, was considerably higher than for all the other groups. The sea urchins fed a uniform diet experienced a decrease in the ASV numbers, as the median in all the algae treatments (*Fucus*, *Palmaria* and *Saccharina*) had similar values just below 60 ASVs, compared to medians of 70 and 100 ASVs in the before and control groups.

Performing an ANOVA analysis on the effect of treatment on ASV richness found that treatment is significant (p = 0.0107) in determining the ASV richness in the samples (Table 1).

**Table 1.** One-way ANOVA table of differences in square root transformed richness among treatments. Formula used: richness ~ treatment with tank as a random nested effect.

| | numDF | denDF | F-value | p-value |
|---|---|---|---|---|
| (Intercept) | 1 | 26 | 2116.845 | <0.001 |
| treatment | 4 | 7 | 7.665 | 0.0107 |

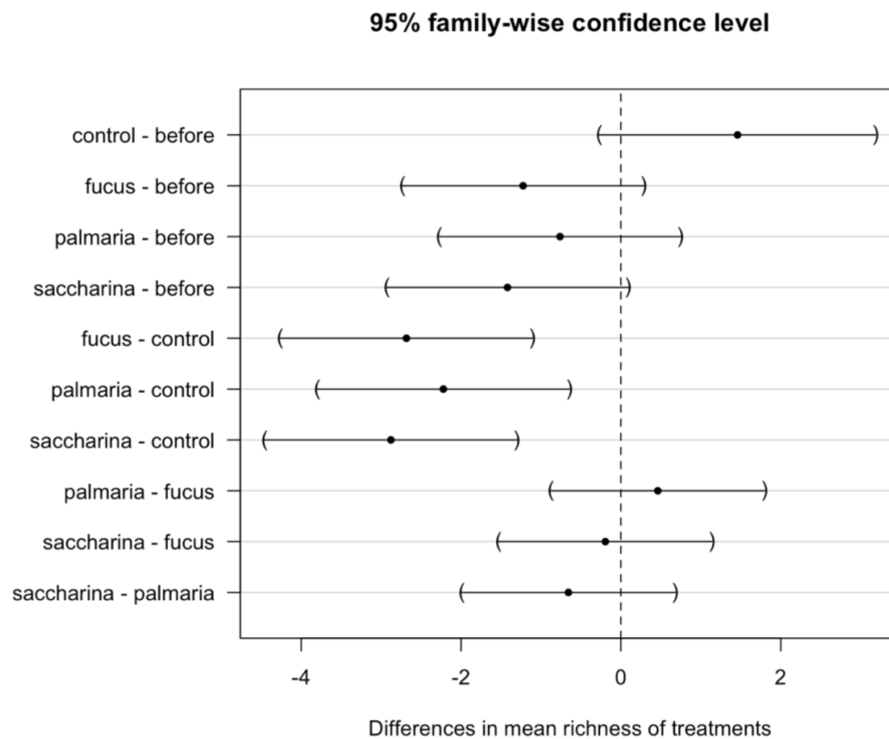


**Figure 7.** Pair-wise comparisons of a Tukey test of (square root transformed) mean richness for the treatments. Confidence intervals that do not overlap 0 on the x-axis provide evidence for significant differences between groups compared. Table of the Tukey test with p-values can be viewed from Appendix 5, Table 10.

As the ANOVA detected a significant effect of treatment on the sequence richness, a Tukey post hoc test was performed to find out which treatments were different from each other (Figure 7). The Tukey test confirmed several of the observations made from the box plots in Figure 6. None of the algal treatments were significantly different from each other, as all the confidence intervals overlap the dashed line. This confirms the observation that the treatments had very comparable results, due to overlapping box plots and similar median

values (Figure 6). The only detectable significant difference between the groups compared, was found between control samples and the all the algal treatments. Even if the algal treatments were not proven to be significantly different from the before samples, one should not assume that they were the same. Because the dashed line overlaps the confidence intervals on the outer ends, one can believe that the algal treatments and the before samples are more different than alike.

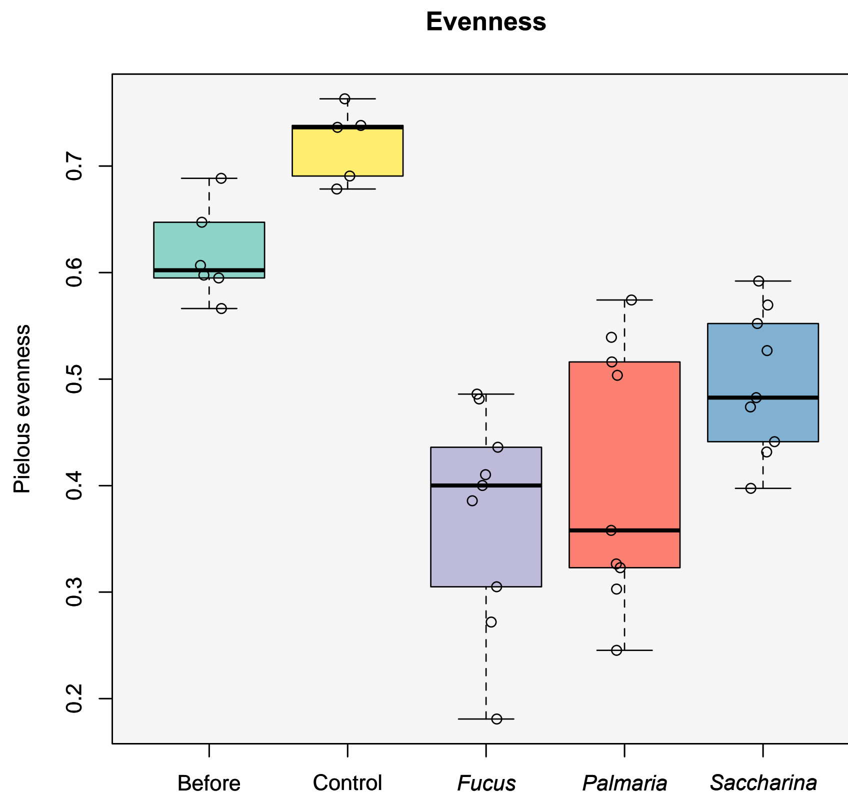


**Figure 8.** Evenness. The distribution of the relative ASV abundances in each treatment on the x-axis (before, control, *Fucus*, *Palmaria* and *Saccharina*). The circles represent each data point that produced this plot. A high evenness score (close to 1) indicates that the abundant ASVs are equally distributed, and that there are few or no dominant ASVs. A low evenness (closer to 0) indicates the presence of dominant ASVs.

The ASV evenness was greatest in the groups before and control (Figure 8). The control group had the highest score in evenness. Evenness decreased in the urchins fed a uniform diet, which indicates the presence of dominant bacterial sequences. The algal box plots are

more displaced from each other and the median values are more distant, compared to the richness results in Figure 6, which had a more similar distribution.

The ANOVA table shows a strong and significant ($p < 0.001$) effect of treatment on the evenness distribution (Table 2).

**Table 2.** One-way ANOVA table of differences in evenness among treatments. Formula used: evenness ~ treatment with tank as random nested effect.

| | numDF | denDF | F-value | p-value |
|---|---|---|---|---|
| (Intercept) | 1 | 26 | 1216.077 | <0.001 |
| treatment | 4 | 7 | 17.760 | 0.000905 |

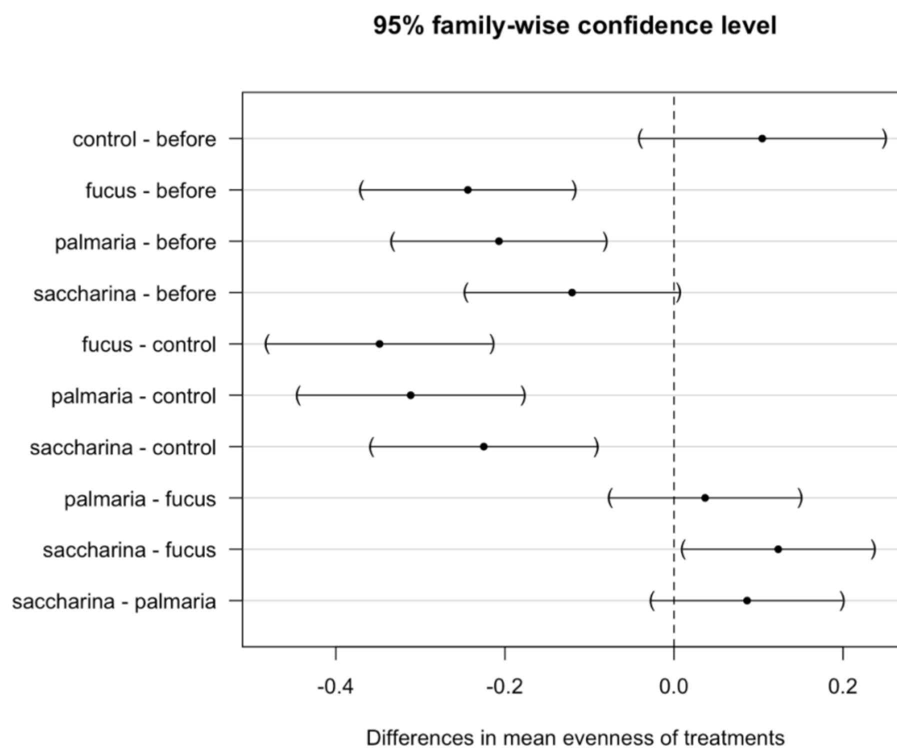


**Figure 9.** Pair-wise comparisons of a Tukey test of mean evenness for the different treatments (control, before, *Fucus*, *Palmaria*, *Saccharina*). Confidence intervals that do not overlap 0 on the x-axis provide evidence for significant difference between groups compared. Table of the Tukey test with p-values can be viewed from Appendix 5, Table 11.

The Tukey test showed that several groups were significantly different from each other (Figure 9). Almost all algal treatments are significantly different from the before sample (except *Saccharina*), which suggests that a uniform diet promotes dominant bacterial sequences. All algal treatments are significantly different from the control. The Tukey test detects a slight significant difference between the mean values of *Saccharina* and *Fucus*, an

indication of this can be observed in the evenness box plots (Figure 8), as they are located further apart from each other.

The first null hypothesis stated that there were no differences in diversity between samples before the experiment and the algal treatments in the experiment. As significant differences between the groups were detected, both in the one-way ANOVA (Table 1 and 2) and the Tukey tests (Figure 7 and 9), the null hypothesis can be rejected.

## 3.3 Bacterial composition

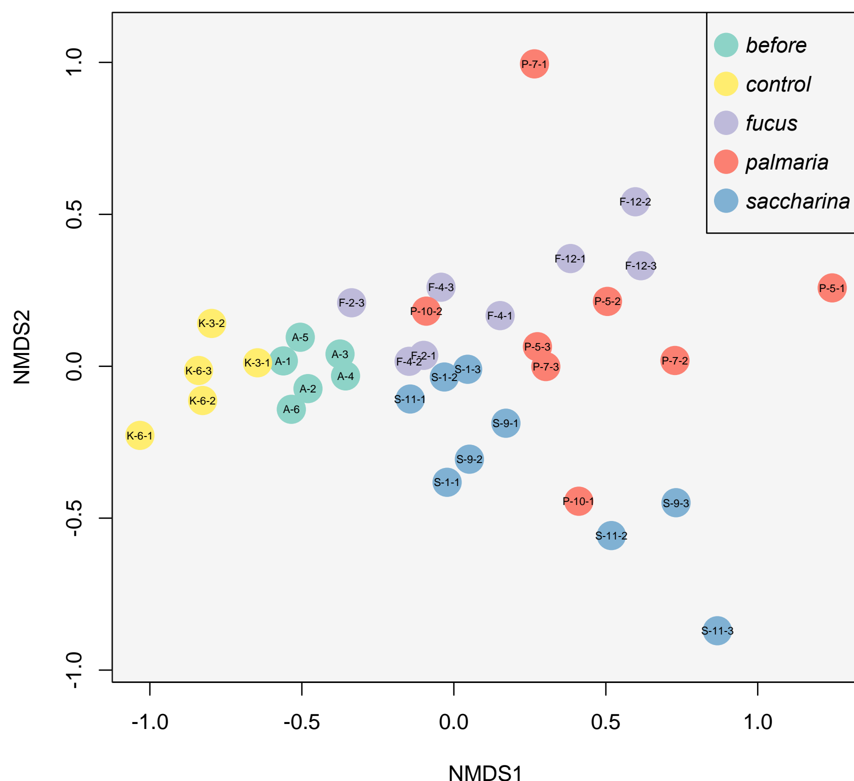


**Figure 10.** Non-metric multidimensional scaling (NMDS) ordination plot of community dissimilarities based on hellinger transformed data obtained from amplicon sequence variants from the various treatments (control, before, *Fucus*, *Palmaria*, *Saccharina*). Data points next to each other, represents similar bacterial assemblages in the different treatments (presented as different colors). The data points represent the urchin identity (ID), and the first letter in the ID represents the treatment. Chloroplast sequences are removed to avoid false treatment relationship. Stress value = 0.12.

The stress value was 0.12, which indicates a good representation (Figure 10). A gradient can be observed along the first axis (NMDS1) where the bacterial composition changes in relation to the different groups. The gradient starts with the control samples (left side), and

next is the before samples, which then transitions into the algal treatments. The end points of the gradient represent distant bacterial communities, which suggest that the control samples have a different bacterial composition than the algal treatments. There seems to be a strong relationship between the samples within the before group and within the control group, as the data points were located close together. However, the two groups did not overlap, which suggests that a different bacterial profile develops when the sea urchins are starved. There seems to be an association between samples in the algal treatment groups, except *Palmaria*, due to scattered data points in the plot.

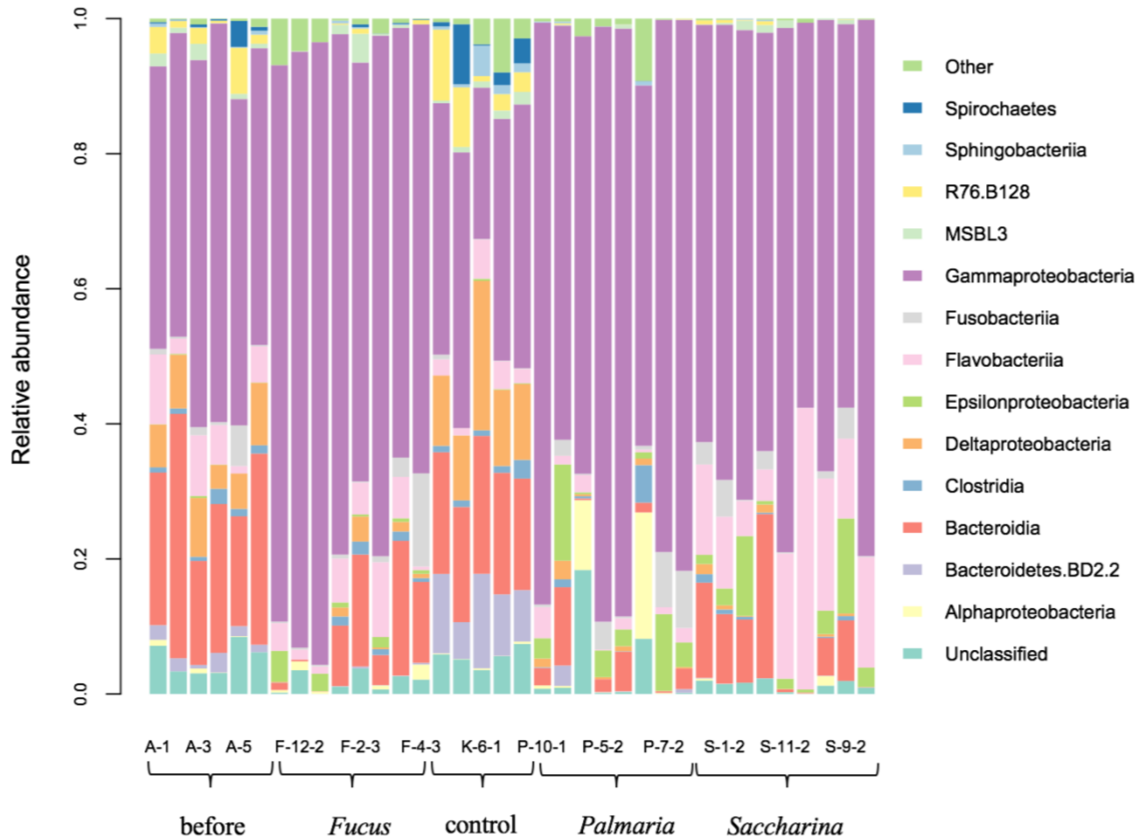


**Figure 11.** Stacked bar graphs showing relative abundance (y-axis) of class-level bacteria (listed to the right) occurring in the various treatments on the x-axis: control, before, *Fucus*, *Palmaria*, *Saccharina* (A-1, A-3 and so on represents the individual samples). “Other” represents low abundance classes with less than 2000 sequence reads, and “Unclassified” are sequences that have not been cultured yet.

The most abundant class-level bacteria found across all the samples are listed in the sidebar of Figure 11. Gammaproteobacteria (dark purple color) were the dominating class, and abundant across all samples. The before group seemed to have a similar bacterial composition to the control group, however, two bacterial groups appeared to have increased in the control group, which is Bacteroidetes.BD2.2 and Spirochaetes. Flavobacteriia was more common in the urchins fed with *Saccharina*, and it seems that there was a low representation of minor groups labelled “Other”. Some of the urchins fed *Palmaria* have a high abundance of Alphaproteobacteria, while this strain was virtually absent both in the before and control group. The Spirochaetes class can be seen in the control and before groups, but was virtually absent in all the algal treatments.

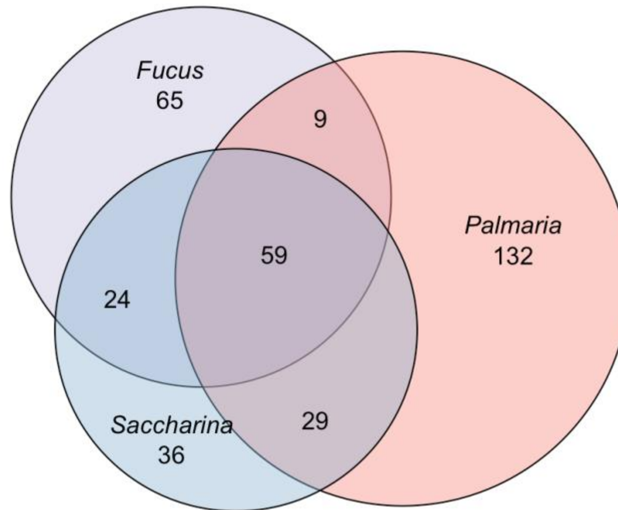


**Figure 12.** Euler diagram showing overlaps of amplicon sequence variants (ASV) in the algal treatments (*Palmaria*, *Saccharina* and *Fucus*). The Euler diagram is scaled to the size of the bacterial composition of the different treatments, and the numbers in each overlapping region show how great the overlap is. It shows the shared sequences, and the ones who are unique to each algal treatment.

The algal treatments (*Palmaria*, *Saccharina* and *Fucus*) shared 59 amplicon sequence variants (Figure 12). The *Saccharina* group shared almost equal amounts of ASVs with *Palmaria* and *Fucus* (29 and 24 ASV). Only 9 ASVs were shared between the *Palmaria* and *Fucus* group alone. Looking at the size of the circles, one can observe that *Palmaria* have the largest sample size of 132 separate ASVs, followed by *Fucus* and *Saccharina*.

111 ASVs were shared between control and before samples, and control had 76 separate ASVs and before had 34 separate ASVs.

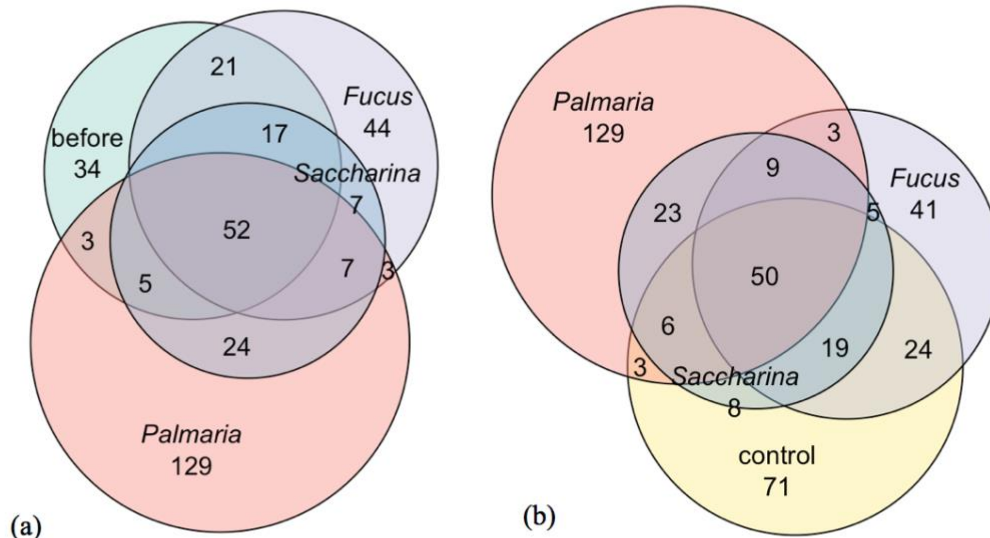


**Figure 13.** Euler diagrams showing overlaps of amplicon sequence variants (ASV) in the treatments. The numbers in each overlapping region show how great the overlap is. It displays the shared sequences, and the ones who are unique to each treatment. **a.** This Euler diagram show the relationship between treatments: before, *Fucus*, *Saccharina* and *Palmaria*. **b.** This Euler diagram show the relationship between treatments: control, *Fucus*, *Saccharina* and *Palmaria*.

The *Saccharina* treatment in both Euler diagrams (Figure 13) do not have any separate ASVs. The *Palmaria* treatment is still the largest group with 129 separate ASVs in both comparisons (Figure 13). Compared to *Fucus* in Figure 12, there is less ASVs that are separate when before and control samples are included (44 ASVs in a. and 41 ASVs in b., Figure 13). 52 ASVs were shared between all treatments in Figure 13a, and 50 ASVs in Figure 13b.

PERMANOVA compares the bacterial community dataset with treatment and tank as explanatory variables (Table 3). The p-value for the treatment factor is low ($p = 0.001$), which means that PERMANOVA detects significant differences in bacterial communities between treatments. There is also a significant effect of tank ($p = 0.003$) and the interaction of treatment and tank on the bacterial community. However, the $R^2$ value states which variables explains most of the variation in the response, and treatment is the variable that explains most of the variation in the bacterial composition, and that a minor effect is provided by the other two variables (tank and treatment: tank).

**Table 3.** Results of the multivariate permutational analysis (PERMANOVA) of differences in (hellinger transformed) bacterial communities between treatments (Interaction between the variables treatment and tank are inspected. Treatments: control, before, *Fucus*, *Palmaria*, *Saccharina*. Formula used: table of amplicon sequence variants (ASV) ~ treatment*tank. Significance level is indicated by the significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

| | Df | Sums of Sqs | Mean Sqs | F. Model | $R^2$ | Pr(>F) | Sign. c. |
|---|---|---|---|---|---|---|---|
| treatment | 4 | 2.7252 | 0.68131 | 6.9766 | 0.43443 | 0.001 | *** |
| tank | 1 | 0.3647 | 0.36472 | 3.7347 | 0.05814 | 0.003 | ** |
| treatment: tank | 3 | 0.5464 | 0.18214 | 1.8651 | 0.08711 | 0.015 | * |
| Residuals | 27 | 2.6367 | 0.09766 | | 0.42032 | | |
| Total | 35 | 6.2731 | | | 1.00000 | | |

The second null hypothesis stated that the composition of the bacteria in the gut would not be different for the various treatment groups including the before samples. By analyzing the NMDS plot (Figure 10), one can observe clear distinctions between the bacterial communities of the various samples, and that samples belonging to the same treatment tended to assemble together. A PERMANOVA test found a significant effect of the treatment in structuring the bacterial communities. Based on these findings, the second null hypothesis can be rejected.

The third hypothesis stated that no bacteria involved in degrading structural components and fixing nitrogen would be found in the sea urchin gut. Spirochaetes and Clostridia found in the bar plot (Figure 11), contain bacteria known to fix nitrogen and degrade carbohydrates. The third null hypothesis is difficult to reject, because even if those bacteria are present, one cannot know if the bacteria perform these functions based on sequenced 16S rRNA gene analyses.

## 3.4 Similarity percentage (SIMPER) analysis

The results from the SIMPER analysis can be viewed from Appendix 7, Table 12. The subset of the SIMPER analysis resulted in 46 different amplicon sequence variants (ASV), and there was also a consistency regarding which treatment that had a higher contribution of the specific ASV, e.g. ASV no. 15 were found in higher amounts in the *Palmaria* treatments (seen row-wise in Appendix 7, Table 12). Most of the ASVs were related to marine origin,

and several were also found in the sea urchin *Strongylocentrotus intermedius*. ASV no. 1 was found to differentiate between several treatments, and although it was present in all samples, the "difference" was likely derived from the variable amounts of read counts in each sample. Some ASVs were related to epiphytic bacteria on brown algae, suggesting that these may have been introduced through the food source. ASV no. 17 was related to Colwellia sp. which was known to hydrolyze agars and carrageenans. Bacteria associated with sulfur-metabolism were found in elevated amounts (sequence reads) in the controls and the *Palmaria* treatments (e.g. ASV no. 20, 43, 49, 62). There is also an indication of similarity between the before and control samples, as some ASVs are prominent in both groups and the same ASV do not differentiate between before and control, e.g. ASV no. 9, 10, 19.

## 3.5 Carbon and nitrogen content in the food

A high C:N ratio indicates that there is a high portion of carbon compared to the nitrogen content in the algae. The C:N ratio was lowest in *S. latissima* and highest in *P. palmata* (Tabl).

**Table 4.** Nitrogen and carbon content (%) and the C:N ratio in the various species of algae after 4 to 9 days of storage in seawater.

| Alga | Mean N % | Mean C % | C:N ratio | Days of storage |
|---|---|---|---|---|
| *F. serratus* | 2.45 | 35.72 | 14.60 | 4 |
| *P. palmata* | 2.46 | 37.37 | 15.21 | 9 |
| *S. latissima* | 2.62 | 28.80 | 11.00 | 9 |

# 4 Discussion

The results obtained in this thesis, indicate that there were clear distinctions of bacterial diversity (measured as ASV richness and evenness) and community compositions (viewed from NMDS plot, stacked bar plot and Euler diagrams) between the different feeding regimes. This suggests that the bacterial community shift occurs as a response to the food ingested, and that it may be an adaptive response to ensure a better extraction of the current resources.

## 4.1 Diet was found to modify the bacterial diversity

The various algae fed the sea urchins during the experiment, represented uniform diets, and these sea urchins had a significantly lower bacterial diversity compared to the control (starved sea urchins) and reference samples (natural bacterial communities) (Figure 7 and 9). Studies that have investigated the microbiota of insects with different feeding regimes, found that the bacterial diversity (number of OTUs) were greatest in the insects with an omnivorous diet. Insects specialized to consume one type of material, e.g. wood-feeding termites, had a lower diversity (Sabree & Moran 2014), which corresponds to the results in this thesis. The bacterial diversity in the gut is affected by the microbes associated with the food ingested and with the nutrients provided to the gut, as diverse nutrients can support diverse microbiota (Heiman & Greenway 2016). This indicates that the urchins representing the before samples had a diverse diet, but there can be a certain degree of doubt as we cannot know for sure what they were feeding on before they were collected. However, as all the sea urchins were collected as the same site, it is likely that they were exposed to similar food sources, and that the bacterial diversity in the algal treatments resembled the before samples initially. The sea urchins fed only one type of algae, had a low bacterial richness (Figure 6), suggesting that they lacked some of the bacteria that are common in urchins with a diverse diet (as seen in the before samples, Figure 6). This could be due to different sources of nutrition which facilitate different bacteria. New types of bacteria could be introduced through epiphytic bacteria, which are bacteria living on the surface of macroalgae (Lachnit et al. 2011). Thus, when the sea urchins were fed single algal diets during the experiment, the food could function as a source of new and more competitive bacteria. The epiphytic bacteria may vary through the season and on young vs. old tissue, and a study by Bengtsson et al. (2010) found

that the bacterial profile on the kelp tissue (*L. hyperborea*) exhibited a seasonal cycle, with February having the highest diversity of OTUs.

The bacterial evenness was significantly lower in urchins presented only one type of algae compared to the before samples (Figure 9), indicating dominant bacterial groups. Feeding on a uniform diet could reduce the bacterial evenness due to the lack of common vitamins from a diverse diet, and consequently some bacterial groups may have lost their competitive advantage and become less abundant. In some cases, the reduction in abundance can lead to the elimination of the bacterial group, as observed in the corresponding low sequence richness in the algal treatments (Figure 6). In other cases, the algae may contain defense toxins, like brown algal phlorotannins, that could eliminate some bacteria in the gut. A change in ASV richness and evenness can affect the functionality of the community in different ways. If the dominating bacteria can perform a function better than several different bacteria combined, a positive effect could be the outcome of a reduction in diversity. If the functionality is enhanced by the synergistic interactions, a reduced diversity can have a negative effect (Hillebrand et al. 2008). The results of this study indicate that a low diversity may be beneficial when the urchin is presented one type of diet, as some bacteria are better adapted to process the consumed algae. However, a low diversity could be problematic when the urchin is re-introduced to diverse food alternatives, because the appropriate functional bacteria may no longer be present to process the diverse food sources.

A low ASV richness and evenness from a uniform diet, may not always be true for all organisms, as the opposite outcome was observed from a feeding experiment on threespine stickleback fish, *Gasterosteus aculeatus* (Bolnick et al. 2014). The single-diet fish had a higher microbial diversity than fish fed a mixed diet (Bolnick et al. 2014). A review combining various studies on biodiversity, found that invertebrate animals tended to have a strong positive correlation between richness and evenness, less positive for vertebrates and a negative correlation for plants (Hillebrand et al. 2008), and a positive correlation was also found in this thesis (Appendix 6, Figure 16). This suggests that communities of species belonging to different phyla may differ in their relationship between richness and evenness, and their microbiome might respond differently to mixed or single-diets. Even if contrasting results were observed for the fish from the study by Bolnick et al. (2014) and the sea urchins from this study, both of these findings support diet as an important factor in structuring bacterial communities.

The diversity was also high in the control group, which was starved urchins (Figure 6 and 8), and this observation challenges the assumption that a high bacterial diversity derives only from a varied diet. The control and before groups had several ASVs in common and this could indicate that the starved sea urchins retained a lot of the original bacteria, however, the ASV richness in the control was higher than the before group which needs to be explained by something else. When urchins are starved, the digestion rate slows down, and enables the urchin to hold the remains of food in the gut for a longer time (Lasker & Giese 1954). The study by Lasker and Giese (1954) observed that the feces of the starved sea urchins (*S. purpuratus*) were almost completely decomposed and contained high amounts of bacteria, suggesting that bacteria accumulates on the food available. This could give an explanation to the high ASV richness found in the starved urchins from this thesis. A study investigating the effects of starvation on the diversity of the gut microbiota in locusts, found that the starvation led to an increase of the microbial diversity in the gut, and it was proposed that the microbial diversity could act as a barrier and prevent the settlement of pathogenic bacteria, known as the colonization resistance (Dillon & Charnley 2002). Although sea urchins are very robust and adapted to periods of low food availability (Russell 1998), starvation is stressful and can interrupt the development of gonads in order to preserve energy (Minor & Scheibling 1997). The starved urchins from the experiment in this thesis, were constantly foraging for food in the tank. Although they were not fed anything, and the tanks were frequently cleaned with a siphon, a biofilm were likely formed from small particles in the incoming seawater. Previous studies of food sources in urchins residing on barren grounds, where there is virtually no algal growth, found that urchins frequently ingest small particles of diatoms, cyanobacteria and filamentous red and brown algae (Chapman 1981). This indicates that urchins will begin to ingest alternative food sources when the preferred food (kelp) is absent. This may also influence the microbial diversity to some extent, as new bacteria can be introduced by the small food particles found in the tanks.

In addition to starving, the study by Dillon & Charnley (2009) found that the age of the organism was also influencing the bacterial diversity, and that older specimens had a more diverse microbiome. The diameter of the sea urchins from the experiment was measured (Appendix 1, Table 8), and the mean diameter was 46.2±0.8(SE) mm, which is regarded as an adult sized urchin. In a study by Fagerli et al. (2015) green sea urchins have been aged by use of growth rings and an age up to 14 years have been identified. Based on Fagerli et al. (2015) urchins with a diameter of 46 mm would be around 4 to 6 years old. However, a more

precise determination of age was not estimated in this study. As only the largest urchin specimen where selected for the experiment, and thus large specimens were present in all tanks (Table 8), one can assume that age was not the driving factor to the species diversity in the starved urchins, but for future studies this factor could be investigated further.

## 4.2 Different bacterial compositions and the identification of functional bacteria

The PERMANOVA analysis (Table 3) found that the various treatments were significantly different ($p < 0.002$) in modifying the bacterial communities, and this could be viewed from the NMDS plot as all treatments were clustered at different places in the plot (Figure 10). As discussed earlier, even starved urchins (referred as controls) exhibited discrete bacterial communities, indicating that the bacterial diversity and composition were affected by the environmental conditions, or a so-called laboratory effect. Thus, the results from the PERMANOVA analysis (Table 3) indicated that tank had a significant effect of the bacterial composition. Given that experimental conditions among tanks were kept as similar as possible, some circumstances were difficult to prevent. Environmental factors could be slightly different from tank to tank (e.g. water flow), or that the small size of the tanks (17.5 L) limited the space between each specimen, and that the chance of encounter was high. During feeding, several urchins could feed on the same piece of alga, which could facilitate a pathway for the transfer of microbiota between urchin specimens. This effect is common in social organisms, as the transfer of microbiota can occur between organisms that encounter each other (Martinson et al. 2012). Although sea urchins are not considered to be social organisms, they can encounter each other due to grazing activities, e.g. when the sea urchins aggregate into feeding fronts, and could possibly transfer beneficial bacteria to the neighboring individuals. However, tank displayed a low $R^2$ value, 0.058 compared to 0.434 for treatment (Table 3), which indicates that that tank had a minor effect, and that type of treatment was the driving factor to modify the bacterial compositions.

Even if the bacterial compositions were different from each other in relation to treatment, there was also some ASVs present in all samples, as seen in the overlapping regions on the Euler diagrams (Figure 12 and 13). Another interesting feature was that the algal treatments were located closer to the before samples than the controls in the NMDS plot (Figure 10), and

shared 52 ASVs (Figure 13a) compared to 50 ASVs (Figure 13b). This could suggest that sea urchins in nature ate a combination of the algae used in the experiment, as similar species of algae have similar epiphytic bacteria (Lachnit et al. 2011). Some epiphytic bacteria were found to differentiate between the treatments in the SIMPER analysis (Appendix 7, Table 12), which suggests that these bacteria were acquired through the food ingested, and were not initially present. An example is ASV no. 15 which is related to *Shewanella* sp., and the *Palmaria* treatment had the highest sequence reads of this ASV. However, this do not eliminate the possibility that the sea urchins in nature were not feeding on this alga, but as the alga were collected from another location (Flødevigen) to where the sea urchins were collected (Drøbak), new epiphytic bacteria could have been introduced. Sometimes the ingested epiphytic bacteria can form a symbiotic relationship with the marine host, and alginate lyase-excreting bacteria is an example of this (Wong et al. 2000). These bacteria have the ability to degrade alginate, which is a major constituent of the cell-wall in brown algae (Kovalenko et al. 2011). Several marine alginate lyase-excreting bacteria have been identified in literature (Wong et al. 2000, Li et al. 2011, Dong et al. 2012), and some of these were related to ASVs found in various samples from this experiment: *Photobacterium*, *Pseudoalteromonas* (present in treatments *Palmaria*, *Saccharina* and before) and *Psychromonas* (present in all treatments). A symbiotic relationship of these bacteria and the sea urchins, may facilitate the digestion of structural components in brown algae, like *Saccharina* and *Fucus*. As new bacteria can be introduced through the food, bacterial samples from algal tissue should also be exanimated for future studies, in order to get a better perception of the origin of the bacteria in the sea urchin gut. In addition, an analysis of the structural components in the different algae could be implemented.

The SIMPER analysis revealed that several ASVs were also found in a related sea urchin, *Strongylocentrotus intermedius* (e.g. ASV no. 4, 5, 6 and 7, Appendix 7, Table 12). However, as most of the amplicon sequence variants (ASVs) did not have a cultured relative, it was difficult to predict their functionality. One ASV was identified as *Lutibacter holmesii* which belongs to the class Flavobacteriia, and was found in elevated amounts in the *Saccharina* treatment both in the SIMPER analysis (ASV no. 6 in Appendix 7, Table 12) and is likely the distinct pink-colored bands seen from *Saccharina* in the stacked bar plot (Figure 11). *Lutibacter holmesii* were also found in the sea urchin *Strongylocentrotus nudus*, and including *S. droebachiensis* from this thesis, there has been three species of the genus *Strongylocentrotus* that retain relatives of this bacterium. *Lutibacter holmesii* is described as

a strictly aerobic bacterium, meaning that it requires oxygen to grow. In addition, it can perform several hydrolytic activities and could be an important factor in the metabolic processes of the host. Based on these findings, Nedashkovskaya et al. (2015) proposed that *Lutibacter* could be a resident microflora in the genus *Strongylocentrotus*.

There were not many abundant ASVs, but one that accounted for 44 % of all sequence reads, and was present in all samples regardless of dietary treatment. This ASV was related to the species *Psychromonas marina* sp. nov., a bacterium described by Kawasaki et al. (2002). This bacterium belongs to the class Gammaproteobacteria and is likely the dominating bacteria observed in the stacked bar plot (Figure 11). It is considered a facultative psychrophilic bacterium, meaning that it is cold water adapted, and can tolerate temperatures down to 0°C and no growth on temperatures above 26°C, which is comparable to the preferences to the cold water adapted sea urchin *S. droebachiensis*. *Psychromonas marina* can produce polyunsaturated fatty acids (PUFA) which is an important material in cell and tissue membranes, and an important factor in tolerating cold environments (Miquel et al. 1993). A review article concerning fatty acids in marine organisms, listed several studies of PUFA-producing bacteria that were isolated from the intestines of marine invertebrates, and suggested that these bacteria could transfer PUFA to the host (Bergé & Barnathan 2005). The fact that this bacterium did not lose its abundance from the experimental conditions in this thesis, suggests that it can be recognized as an important symbiotic bacterium providing PUFA to *S. droebachiensis*. Another aspect of abundant bacteria is their influence on the sampling method, as the relative abundance of ASVs rather than the absolute abundance, are measured. When a bacterium is this dominant, the method for obtaining the relative bacterial abundances can be biased, and the abundant bacteria may conceal other bacteria present in the samples (Bolnick et al. 2014). This is a common issue and because of this, some of the bacterial diversity in the sea urchin gut may not have been recorded.

It was also hypothesized that nitrogen fixing representatives would be present in the sea urchin gut because of low nitrogen values in kelp. However, the kelp treatment (*Saccharina*) used in this study was probably not nutrient poor after all, as the C:N ratio did not differ notably to the other algal treatments (Table 4). The C:N ratio obtained for *Saccharina* may occur as a response of several factors, like storage during the experiment or the method used for extraction of total carbon and nitrogen. But most likely the time of year was crucial, as the

chemical composition in kelp is strongly influenced by season. The kelp fronds were collected in January, which is the when the nitrogen content in the kelp peaks (Schiener et al. 2015), and consequently the C:N ratio is low. Similar results on carbon and nitrogen content were obtained in another study done in Scotland (Schiener et al. 2015), which can validate the results observed in this thesis. Because of this, nitrogen fixing representatives may not be very prominent, and for future studies, it would be more interesting to examine the effect of kelp diet when the nutrient level is at its lowest during the summer months (high levels of carbon), and one could also hypothesize that the presence of nitrogen fixing bacteria would be more prominent.

Still, this does not exclude the possibility of finding bacteria related to nitrogen fixation. One group of bacteria known from the termites are the class Spirochaetes, which is known to have nitrogen fixing representatives, and recognized as an important nutrient contributor in termites (Lilburn et al. 2001). The Spirochaetes class were also present in the sea urchins from this study, and they may function as nitrogen fixing units. Nitrogen fixing can only occur in anaerobic environments, as the activity of the enzyme nitrogenase is inhibited by the presence of oxygen (Madigan 2015). There was some evidence of anaerobic conditions in the sea urchin gut. The presence of the class Clostridia gives a good indication, as it represents an obligate anaerobic class of bacteria, involved in the degradation of complex carbohydrates in termites and cockroaches (Tracy et al. 2012). The presence of these microbes in the sea urchin intestine indicates that there were conditions appropriate for nitrogen fixing. However, this does not necessarily mean that the sea urchin gut is completely anaerobic, as the previously mentioned bacterium *Lutibacter holmesii* (from the SIMPER analysis) require oxygen to survive. This could suggest that the sea urchin gut is divided into compartments, with oxygenic sections and non-oxygenic sections that reside different types of bacteria. The study by Zhang et al. (2014) found that the diversity of microbial species was different between the large and the small intestine in *Strongylocentrotus intermedius*, which could indicate a compartmentalization in the sea urchin gut. As the DNA isolations were based on fecal pellet samples from the gut, some of the bacterial DNA could be remains of dead bacteria from another compartment, which could explain the presence of both aerobic and anaerobic bacteria in the same samples.

There were also found bacteria related to sulfur-metabolism, due to the presence of both sulfate-reducing and sulfur-oxidizing representatives (e.g. ASV no. 43 and 20, Appendix 7,

Table 12). Sulfate is a common electron donor which allow chemoautotrophic bacteria to fix carbon dioxide, and thereby convert inorganic carbon to organic carbon that can be taken up by the host organism (Dubilier et al. 2008). Chemoautotrophic bacteria are important primary producers in hydrothermal vents, but symbioses with chemoautotrophic bacteria can occur in a wide range of habitats, not only near hydrothermal vents in the deep sea (Dubilier et al. 2008). Meziti et al. (2007) also found several sulfur metabolizing bacteria in the gut of the sea urchin *Paracentrotus lividus*, and suggested that they facilitated sulfur cycling, and consequently, sulfate reducing bacteria (SRB) utilized the fermentation products, and the sulfur oxidizing bacteria detoxified the $H_2S$ produced by SRB. A similar process could occur in *S. droebachiensis*. Much focus have been on the organic carbon production by the chemoautotrophic bacteria, but a study by Welsh et al. (1996) recorded nitrogen fixation (acetylene reduction method) by SRB associated with seagrasses, and Petersen et al. (2016) found evidence of genes encoding nitrogen fixation in sulfur-oxidizing bacteria associated with a bivalve (*Loripes lucinalis*) and a nematode (*Laxus oneistus*), suggesting that chemoautotrophic bacteria could be an important contributor of fixed nitrogen. Related bacteria were also found in the sea urchins from this thesis, suggesting that they could form a symbiotic relationship and provide a source for nitrogen as well.

## 4.3 Summary and conclusion

During the 10 days of the feeding experiment, the diversity and composition of the microbiome was found to be significantly different from the control and before samples. The bacterial diversity and composition were also different in the starved urchins, indicating that the bacterial community does not reflect the diet alone (Bolnick et al. 2014). There are possibly several other factors involved in modifying the microbial community in addition to nutrition, and these could include age, genetics and abiotic factors (Wu et al. 2011). Several ASVs related to bacteria with different functions were proposed (like alginate degradation and nitrogen fixation), and there are likely even more interesting bacteria to analyze, but that was beyond the scope of this thesis. The results indicate that the sea urchin microbiome could be a dynamic community, and respond to different feeding regimes. The response could possibly occur at the same moment the urchin feeds on a new type of diet, as previous studies on the human microbiome found detectable changes in microbiome composition after only 24 hours of treatment (Wu et al. 2011). Understanding the functioning of the gut microbiota

could provide important information about the feeding habits of the sea urchins. The ability to exhibit such plasticity in terms of modifying the bacterial community in the gut, could be an important strategy, enabling the degradation of the diverse food items ingested by the sea urchins. Thus, urchins grazing on kelp forests and urchins residing on barrens could have completely different bacterial communities, each with a microbiome adapted to maximize the food available. The results in thesis suggests that the ecological functioning of the sea urchins could be strongly connected to the microbial associations in the gut. Thus, if there were no symbiotic relationship with microbes in the gut, the sea urchins could probably have a completely different feeding behavior, and the effect of grazing would likely not be as prominent as we have experienced until now.

## 4.4 Methodological aspects and future work

In addition to the proposed improvements stated in the previous chapters, there are also opportunities of improvements focusing on the DNA analysis, which will be stated here. The sea urchin experiment was done twice, once in late summer, and the second in mid-winter. In the first attempt the sea urchin samples were thawed due to shipping to Germany, which made it extremely difficult to isolate DNA, as the DNA may have been destroyed by active enzymes. The next time, the samples were isolated at a lab at the University of Oslo. However, it was still difficult to isolate DNA from intestine samples with the isolation kit provided. For this reason, only the fecal pellet samples were analyzed as they yielded more DNA (Appendix 4, Figure 14). As it was difficult to find fecal pellet samples in the control urchins, the sample size in all groups were not the same, and the experiment was not fully balanced, which could affect the outcome of the post hoc test. For future feeding-studies with sea urchins, one could choose another method for the control, e.g. feed them a formulated feed that contain a variety of nutrients to resemble a diverse diet, or find another DNA isolation kit, that gives better results using tissue samples from the intestine. Another suggestion could be to include a stabilizing substance that inactivates the enzymes from degrading the DNA.

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# Appendix 1

**Table 5.** Measured water parameters during the experiment.

| Date | Temperature (°C) | Salinity (ppt) |
|---|---|---|
| 05.01.17 | 4.5 | 29.2 |
| 11.01.17 | 5.5 | 29.0 |
| 12.01.17 | 4.5 | NA |
| 13.01.17 | 4.0 | NA |
| 16.01.17 | 4.6 | 29.6 |
| 18.01.17 | 5.4 | 30.3 |
| 20.01.17 | 4.3 | 28.2 |
| 23.01.17 | 3.5 | 26.2 |
| 25.01.17 | 4.0 | 27.1 |

**Table 6.** The quantity of algae fed the different tanks. All replicate tanks were given the same amount of algae.

| Date | Treatment (species of algae) | Wet weight algae (g) |
|---|---|---|
| 16.01.17 | *P. palmata* | 15 |
| | *F. serratus* | 15 |
| | *S. latissima* | 15 |
| 18.01.17 | *P. palmata* | 15 |
| | *F. serratus* | 15 |
| | *S. latissima* | 15 |
| 20.01.17 | *P. palmata* | 10 |
| | *F. serratus* | 10 |
| | *S. latissima* | 20 |
| 23.01.17 | *P. palmata* | 0 |
| | *F. serratus* | 0 |
| | *S. latissima* | 11 |

**Table 7.** The remains of algae in the tanks at end of the experiment.

| Treatment | Aquarium No. | Wet weight remains (g) |
|---|---|---|
| *P. palmata* | 5 | 6 |
| | 7 | 4 |
| | 10 | 4 |
| *F. serratus* | 2 | 8 |
| | 4 | 4 |
| | 12 | 4 |
| *S. latissima* | 1 | 6 |
| | 9 | 3 |
| | 11 | 0 |

**Table 8.** Size measured by the diameter of the urchin body at the end of the experiment. Mean and standard deviation values were calculated for each treatment.

| Treatment | Diameter (mm) | Mean | SD |
|---|---|---|---|
| before | 40 | 40.8 | 2.0 |
| before | 38 | | |
| before | 42 | | |
| before | 44 | | |
| before | 41 | | |
| before | 40 | | |
| control | 50 | 46.3 | 5.4 |
| control | 48 | | |
| control | 43 | | |
| control | 51 | | |
| control | 49 | | |
| control | 37 | | |
| *Fucus* | 52 | 47.4 | 3.7 |
| *Fucus* | 54 | | |
| *Fucus* | 46 | | |
| *Fucus* | 44 | | |
| *Fucus* | 42 | | |
| *Fucus* | 47 | | |
| *Fucus* | 49 | | |
| *Fucus* | 46 | | |
| *Fucus* | 47 | | |
| *Palmaria* | 45 | 48 | 5.0 |
| *Palmaria* | 47 | | |
| *Palmaria* | 38 | | |
| *Palmaria* | 47 | | |
| *Palmaria* | 47 | | |
| *Palmaria* | 51 | | |
| *Palmaria* | 52 | | |
| *Palmaria* | 49 | | |
| *Palmaria* | 56 | | |
| *Saccharina* | 44 | 46.6 | 5.7 |
| *Saccharina* | 46 | | |
| *Saccharina* | 46 | | |
| *Saccharina* | 47 | | |
| *Saccharina* | 43 | | |
| *Saccharina* | 52 | | |
| *Saccharina* | 58 | | |
| *Saccharina* | 45 | | |
| *Saccharina* | 38 | | |

# Appendix 2

## EXPERIENCED USER PROTOCOL

**PowerSoil® DNA Isolation Kit** Catalog No. 12888-50 & 12888-100

**Please wear gloves at all times**

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

    **Note** If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

    **Note** Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

    **Note** A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x g.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

# Appendix 3

**Table 9.** Summary of the isolated DNA, and values used for the dilution prior to the PCR.

Abbreviations: S = *S. latissima*, F= *F. serratus*, P = *P. palmata*, C = control, A = before.

| Diet-tank-ID | DNA (ng/mL) | DNA (ng/µL) | Dilution factor | New cons. (ng/µl) | Volume of extract (µl) | C6 buffer (µl) | Final volume (µl) |
|---:|---:|---:|---:|---:|---:|---:|---:|
| S-1-1 | 32800 | 32.8 | 6 | 5.5 | 4 | 20 | 24 |
| S-1-2 | 52400 | 52.4 | 10 | 5.2 | 2 | 18 | 20 |
| S-1-3 | 32000 | 32 | 6 | 5.3 | 4 | 20 | 24 |
| S-9-1 | 16860 | 16.9 | 3 | 5.6 | 8 | 16 | 24 |
| S-9-2 | 13720 | 13.7 | 3 | 4.6 | 8 | 16 | 24 |
| S-9-3 | 84000 | 84 | 16 | 5.3 | 1.5 | 22.5 | 24 |
| S-11-1 | 26200 | 26.2 | 5 | 5.2 | 4 | 16 | 20 |
| S-11-2 | 77800 | 77.8 | 15 | 5.2 | 1.5 | 21 | 22.5 |
| S-11-3 | 14740 | 14.7 | 3 | 4.9 | 7 | 14 | 21 |
| F-2-1 | 90400 | 90.4 | 17 | 5.3 | 1.4 | 22.4 | 23.8 |
| F-2-2 | 64800 | 64.8 | 12 | 5.4 | 2 | 22 | 24 |
| F-2-3 | 78200 | 78.2 | 15 | 5.2 | 1.5 | 21 | 22.5 |
| F-4-1 | 12680 | 12.7 | 3 | 4.2 | 7 | 14 | 21 |
| F-4-2 | 83600 | 83.6 | 16 | 5.2 | 1.5 | 22.5 | 24 |
| F-4-3 | 97200 | 97.2 | 18 | 5.4 | 1.3 | 22.1 | 23.4 |
| F-12-1 | 30800 | 30.8 | 6 | 5.1 | 4 | 20 | 24 |
| F-12-2 | 96800 | 96.8 | 18 | 5.4 | 1.3 | 22.1 | 23.4 |
| F-12-3 | 98600 | 98.6 | 19 | 5.2 | 1.3 | 23.4 | 24.7 |
| P-5-1 | 2740 | 2.74 | 1 | 2.7 | 20 | 0 | 20 |
| P-5-2 | 82400 | 82.4 | 15 | 5.5 | 1.5 | 21 | 22.5 |
| P-5-3 | 62800 | 62.8 | 12 | 5.2 | 2 | 22 | 24 |
| P-7-1 | 3480 | 3.5 | 1 | 3.5 | 20 | 0 | 20 |
| P-7-2 | 46000 | 46 | 9 | 5.1 | 2.5 | 20 | 22.5 |
| P-7-3 | 49600 | 49.6 | 10 | 5.0 | 2 | 18 | 20 |
| P-10-1 | 1702 | 1.7 | 1 | 1.7 | 20 | 0 | 20 |
| P-10-2 | 26000 | 26 | 5 | 5.2 | 4 | 16 | 20 |
| P-10-3 | 16800 | 16.8 | 3 | 5.6 | 7 | 14 | 21 |
| C-3-1 | 87000 | 87 | 16 | 5.4 | 1.5 | 22.5 | 24 |
| C-3-2 | 34400 | 34.4 | 7 | 4.9 | 3 | 18 | 21 |
| C-3-3 | 998 | 1.0 | 1 | 1.0 | 20 | 0 | 20 |
| C-6-1 | 25600 | 25.6 | 5 | 5.1 | 4 | 16 | 20 |
| C-6-2 | 33000 | 33 | 6 | 5.5 | 4 | 20 | 24 |
| C-6-3 | 9220 | 9.2 | 2 | 4.6 | 10 | 10 | 20 |
| A-0-1 | 54600 | 54.6 | 11 | 5.0 | 2 | 20 | 22 |
| A-0-2 | 104000 | 104 | 20 | 5.2 | 1 | 19 | 20 |
| A-0-3 | 34000 | 34 | 7 | 4.9 | 3 | 18 | 21 |
| A-0-4 | 25000 | 25 | 5 | 5 | 4 | 16 | 20 |
| A-0-5 | 48600 | 48.6 | 9 | 5.4 | 2.5 | 20 | 22.5 |
| A-0-6 | 86400 | 86.4 | 16 | 5.4 | 1.5 | 22.5 | 24 |

# Appendix 4

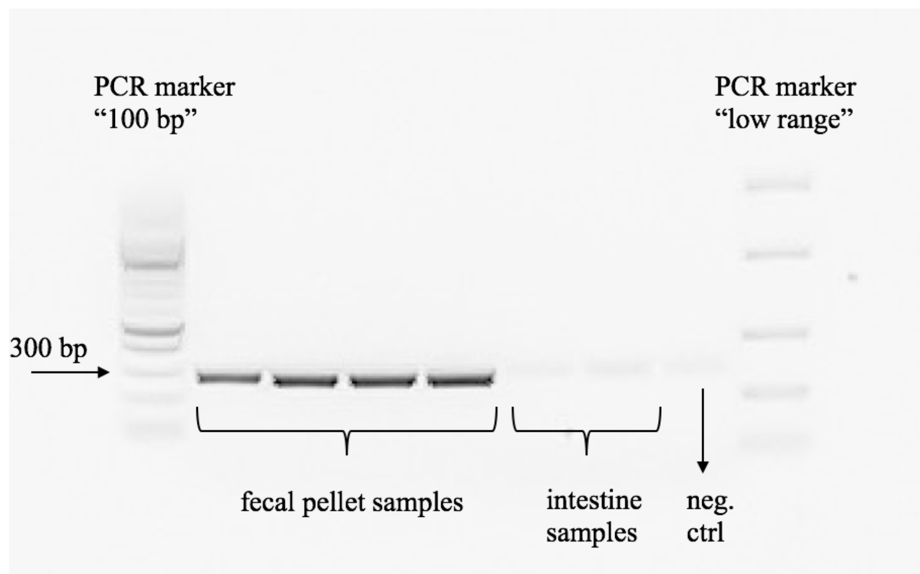


**Figure 14.** Image of a PCR result showing that fecal pellet samples gave a better result compared to samples from the intestine.

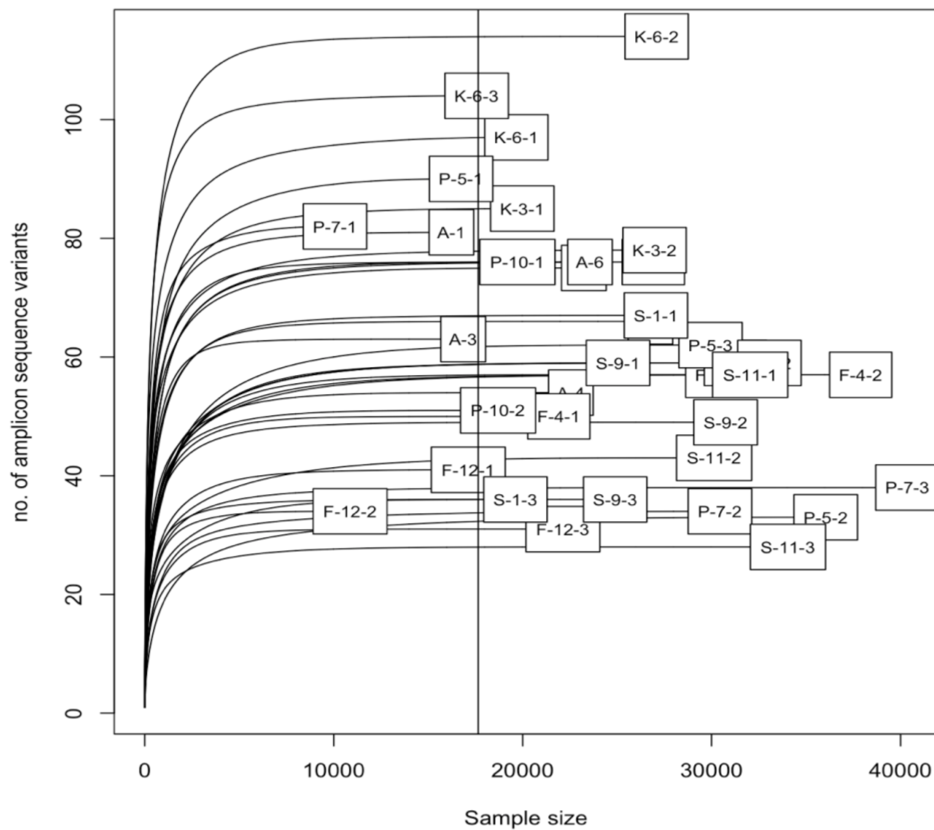


**Figure 15.** Rarefaction curves of the samples, with the lowest sequence read by a vertical line. The curves have reached the plateau phase, which means that the majority of abundant species are sampled, and thus the data represents a good sampling depth.

# Appendix 5

**Table 10.** Square root transformed Tukey test of richness with samples nested in tank. The significance level is indicated by the significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

| Linear Hypotheses: | Estimate | Std. Error | z value | Pr(>\|z\|) | Sign. c. |
|---:|---:|---:|---:|---:|---:|
| control - before == 0 | 1.4591 | 0.6340 | 2.301 | 0.14289 | . |
| *Fucus* - before == 0 | -1.2237 | 0.5540 | -2.209 | 0.17455 | |
| *Palmaria* - before == 0 | -0.7620 | 0.5540 | -1.375 | 0.64128 | |
| *Saccharina* - before == 0 | -1.4187 | 0.5540 | -2.561 | 0.07689 | |
| *Fucus* - control == 0 | -2.6828 | 0.5790 | -4.633 | < 0.001 | *** |
| *Palmaria* - control == 0 | -2.2211 | 0.5790 | -3.836 | 0.00115 | *** |
| *Saccharina* - control == 0 | -2.8777 | 0.5790 | -4.970 | < 0.001 | *** |
| *Palmaria* - *Fucus* == 0 | 0.4617 | 0.4901 | 0.942 | 0.8794 | |
| *Saccharina* - *Fucus* == 0 | -0.1949 | 0.4901 | -0.398 | 0.99467 | |
| *Saccharina* - *Palmaria* == 0 | -0.6567 | 0.4901 | -1.340 | 0.66404 | |

**Table 11.** Tukey test of evenness with samples nested in tank. The significance level is indicated by the significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

| Linear Hypotheses: | Estimate | Std. Error | z value | Pr(>\|z\|) | Signif. c. |
|---:|---:|---:|---:|---:|---:|
| control - before == 0 | 0.10445 | 0.05301 | 1.970 | 0.2785 | |
| *Fucus* - before == 0 | -0.24388 | 0.04614 | -5.285 | <0.001 | *** |
| *Palmaria* - before == 0 | -0.20705 | 0.04614 | -4.487 | <0.001 | *** |
| *Saccharina* - before == 0 | -0.12054 | 0.04614 | -2.612 | 0.0674 | . |
| *Fucus* - control == 0 | -0.34833 | 0.04883 | -7.133 | <0.001 | *** |
| *Palmaria* - control == 0 | -0.31150 | 0.04883 | -6.379 | <0.001 | *** |
| *Saccharina* - control == 0 | -0.22499 | 0.04883 | -4.607 | <0.001 | *** |
| *Palmaria* - *Fucus* == 0 | 0.03682 | 0.04127 | 0.892 | 0.8990 | |
| *Saccharina* - *Fucus* == 0 | 0.12334 | 0.04127 | 2.989 | 0.0232 | * |
| *Saccharina* - *Palmaria* == 0 | 0.08651 | 0.04127 | 2.096 | 0.2197 | |

# Appendix 6

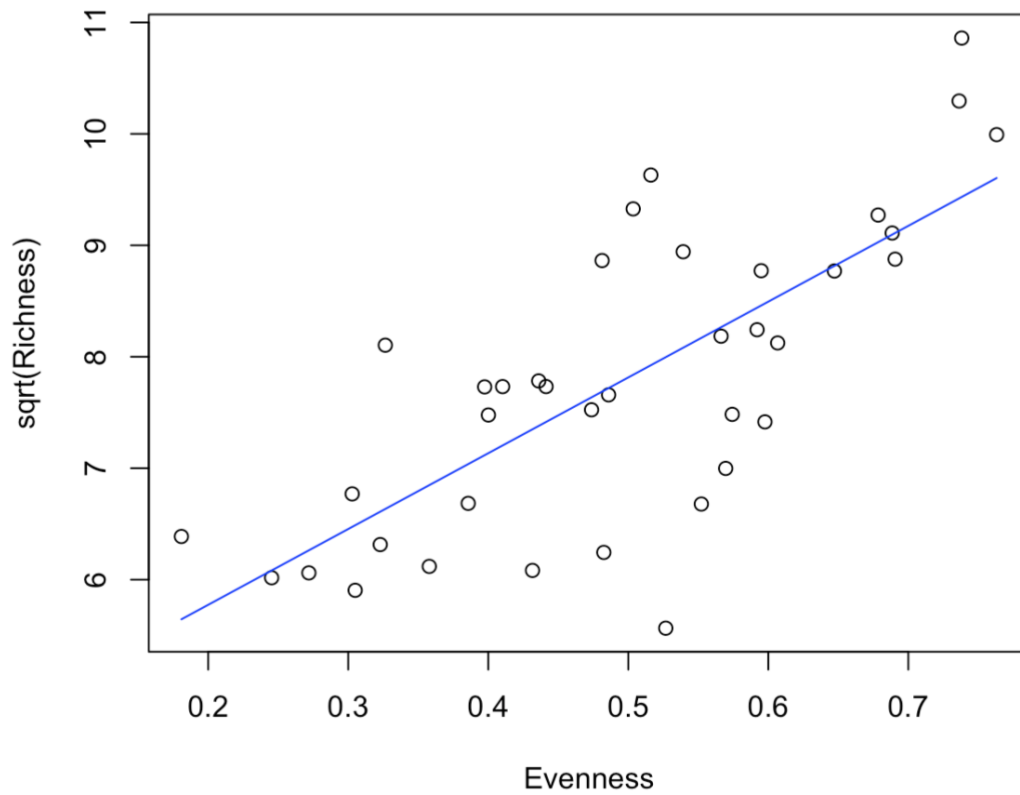


**Figure 16.** A plot of richness and evenness with a regression line through the data points.

# Appendix 7

**Table 12.** Similarity percentage (SIMPER) abundance analysis. Sequences were explored in the BLAST database and assigned to the closest relative. Abbreviations: C= control, P=*Palmaria* treatment, F=*Fucus* treatment, B=before, S=*Saccharina* treatment. “C-P” means that the SIMPER analysis compares ASVs between controls and the *Palmaria* treatment. Symbols in the table indicates which treatment had the highest sequence counts of the specific number of amplicon sequence variant (ASV no.).

| ASV no. | C-P | F-C | C-S | B-F | B-P | F-P | P-S | B-C | F-S | B-S | Closets relative (BLAST) | Similarity (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | P | F | | F | | | | | | | *Psychromonas marina* sp. nov. | 100 |
| 4 | | | | | | F | S | | | | *Lutibacter holmesii* | 99 |
| 5 | | | | B | B | | | | | B | Uncultured Bacteroidetes | 99 |
| 6 | | | | | | | S | | S | S | *Lutibacter holmesii* | 100 |
| 7 | | | | | | | S | | S | | Uncultured Bacteroidetes | 100 |
| 8 | | | | | | P | | | | | Uncultured bacterium | 100 |
| 9 | C | | | B | B | | | | | B | *Colwellia* & *Shewanella* | 100 |
| 10 | C | C | C | B | B | | | | | B | Uncultured Deltaproteobacterium | 99 |
| 11 | | | | B | B | | | | | B | Uncultured Bacteroidetes | 100 |
| 12 | | | | | | | S | | S | S | *Psychromonas marina* | 99 |
| 13 | | | | | | | S | | S | S | Uncultured Bacteroidetes | 96 |
| 14 | | | S | | | | S | | S | S | *Psychromonas profunda* | 100 |
| 15 | P | | | | P | P | P | | | | *Shewanella* sp. | 100 |
| 16 | | | | | | | S | | S | S | *Psychromonas* sp. | 100 |
| 17 | | | | | B | F | | | | | *Colwellia* sp. | 96 |
| 18 | | | | B | B | | | | | B | Uncultured Bacteroidetes | 99 |
| 19 | C | C | C | B | B | | | | | | Uncultured bacterium/*Verrucomicrobia* | 99 |
| 20 | P | | | | | P | P | | | | *Sulfurimonas* sp. | 99 |
| 21 | C | C | C | | | | | C | | | Uncultured bacterium | 96 |
| 23 | | | | | B | F | | | | | Uncultured Bacteroidetes | 99 |
| 25 | | | | | | | S | | S | | *Psychromonas* sp. | 100 |
| 26 | | | | | | | | | S | | Uncultured bacterium | 99 |
| 31 | C | C | C | B | | | | | | | Uncultured bacterium | 97 |
| 32 | | C | | B | B | | | | | | *Psychromonas arctica* | 100 |
| 34 | | | | B | | | | | | | Uncultured bacterium | 98 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 35 | C | C | C | | C | | Uncultured bacterium | 99 |
| 36 | C | C | C | | C | | Uncultured Spirochaetes | 99 |
| 38 | | | C | | | | Uncultured bacterium | 99 |
| 42 | C | C | C | | C | | Uncultured Deltaproteobacterium | 98 |
| 43 | | | C | | | | Deltaproteobacterium | 97 |
| 47 | | | | P | | | *Shewanella* sp. | 100 |
| 49 | | | | P | | | Uncultured bacterium | 100 |
| 50 | | C | | | | | Uncultured bacterium | 97 |
| 57 | | | | | C | | Uncultured bacterium | 98 |
| 58 | | | | | | F | Uncultured bacterium | 100 |
| | | | | | | | Alphaproteobacterium | |
| 62 | | | | | C | | Uncultured bacterium | 98 |
| 66 | | | | P | | | Uncultured bacterium | 100 |
| 69 | | | | | C | | Uncultured bacterium | 99 |
| 73 | | | | | C | | Uncultured bacterium | 97 |
| 74 | | | | P | | | *Vibrio* sp. (several species) | 100 |
| 75 | | | | | C | | Uncultured/*Cytophaga* sp. | 100 |
| 78 | | | | | | F | Uncultured planctomycete | 100 |
| 95 | | | | | C | | Uncultured bacterium | 98 |

**Table 13.** The corresponding taxonomic relationship of the SIMPER analysis (Table 12) based on the SILVA aligner.

| ASV no. | Domain | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|
| 1 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Psychromonadaceae | Psychromonas |
| 4 | Bacteria | Bacteroidetes | Flavobacteriia | Flavobacteriales | Flavobacteriaceae | Lutibacter |
| 5 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Bncultured |
| 6 | Bacteria | Bacteroidetes | Flavobacteriia | Flavobacteriales | Flavobacteriaceae | Lutibacter |
| 7 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Proliibacteraceae | Proliibacter |
| 8 | Bacteria | Fusobacteria | Fusobacteriia | Fusobacteriales | Fusobacteriaceae | |
| 9 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Colwelliaceae | Colwellia |
| 10 | Bacteria | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobulbaceae | Desulfotalea |
| 11 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Uncultured |
| 12 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Psychromonadaceae | Psychromonas |
| 13 | Bacteria | Proteobacteria | Epsilonproteobacteria | Campylobacterales | Campylobacteraceae | Arcobacter |

| | | | | | | |
|---|---|---|---|---|---|---|
| 14 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Psychromonadaceae | Psychromonas |
| 15 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Shewanellaceae | Shewanella |
| 16 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Psychromonadaceae | Psychromonas |
| 17 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Colwelliaceae | Colwellia |
| 18 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | uncultured |
| 19 | Bacteria | Verrucomicrobia | R76-B128 | | | |
| 20 | Bacteria | Proteobacteria | Epsilonproteobacteria | Campylobacterales | Helicobacteraceae | Sulfurimonas |
| 21 | Bacteria | Bacteroidetes | Bacteroidetes BD2-2 | | | |
| 23 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Uncultured |
| 25 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Psychromonadaceae | Psychromonas |
| 26 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Uncultured |
| 31 | Bacteria | Bacteroidetes | Bacteroidetes BD2-2 | | | |
| 32 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Psychromonadaceae | Psychromonas |
| 34 | Unclassified | | | | | |
| 35 | Bacteria | Verrucomicrobia | R76-B128 | | | |
| 36 | Bacteria | Spirochaetae | Spirochaetes | Spirochaetales | Spirochaetaceae | Spirochaeta 2 |
| 38 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Uncultured |
| 42 | Bacteria | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobulbaceae | |
| 43 | Bacteria | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobacteraceae | |
| 47 | Bacteria | Proteobacteria | Gammaproteobacteria | Alteromonadales | Shewanellaceae | Shewanella |
| 49 | Bacteria | Proteobacteria | Epsilonproteobacteria | Campylobacterales | Campylobacteraceae | Arcobacter |
| 50 | Bacteria | Bacteroidetes | Bacteroidetes BD2-2 | | | |
| 57 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Uncultured |
| 58 | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | Uncultured |
| 62 | Bacteria | Bacteroidetes | Bacteroidetes BD2-2 | | | |
| 66 | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | Sedimentitalea |
| 69 | Bacteria | Proteobacteria | Deltaproteobacteria | Desulfobacterales | Desulfobulbaceae | Desulfotalea |
| 73 | Bacteria | Bacteroidetes | Bacteroidetes BD2-2 | | | |
| 74 | Bacteria | Proteobacteria | Gammaproteobacteria | Vibrionales | Vibrionaceae | Vibrio |
| 75 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Marinilabiaceae | Saccharicrinis |
| 78 | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Blastopirellula |
| 95 | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Uncultured |