A pilot study of lumpfish (*Cyclopterus lumpus*) skin health, reared with three different treatments in land-based facilities and commercial net-pens

by

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Abstract

Biological delousing by the means of deploying cleanerfish is one of the methods applied in Atlantic salmon aquaculture to combat the sea lice (*lepeophtheirus salmonis*) infestation problem. The method is based on the natural behaviour of certain fish species exhibiting mutualistic cleaning behaviour, to then graze on the parasite on the salmon. The lumpfish (*Cyclopterus lumpus*) is one such cleanerfish species. Lumpfish have been used commercially as a cleanerfish since mid-2010s, and the hatchery production of eggs from wild caught broodstock has become an industry of its own. The peak in production volume was in 2019 when over 42 million lumpfish were hatchery produced and deployed in Norwegian Salmon cages.

Extensive research has however shown that the lumpfish health and welfare are often poorly maintained during their time at sea, and modest estimations of mortality is 46 %, and observations of 100 % have also been made. The industry has grown tremendously in just a few years, and thus the research regarding how to maintain the species health and welfare have not caught up to speed. As long as there is a lack of knowledge regarding basic lumpfish physiology, the welfare of the cleanerfish will not improve.

To get a better understanding of the factors influencing lumpfish health, two preliminary research projects consisting of two land-based (Agder and Austevoll) and one sea-based (Fitjar), experiments were conducted during 2020. In these experiments the lumpfish were reared with the different treatments natural kelp, plastic kelp, and no kelp, to investigate if the treatments had an influence on the skin health of the lumpfish. Lumpfish from all three experiments (n=140) were sampled, and skin analyses were applied through mucosal mapping. This is an objective and quantitative measure of skin health, applied to a range of fish species. Mucous cell mean area (MA), volumetric density (VD), and defence activity (DA) exhibited by the mucous cells in the lumpfish skin were then calculated, and found to be within the range of other species. No significant differences between the different treatments in the experiments were found, but significant differences within experiments between sampling dates were observed. The results also show an increase in the mean area of the mucous cells and increased defence activity with increasing exposure to particles and pathogens in the water.

Additionally, microbiological analyses were obtained from researchers at BIO (UiB), and the relative abundance (RA) of different bacterial taxa were measured again MA, VD and DA, of the lumpfish skin, however few significant correlations were found. The present study is the first of its kind to scientifically use natural kelp as a substrate for lumpfish in commercial salmon production and several commercial like systems. This research is an important element in establishing a baseline for lumpfish health, which is currently missing.

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1 Introduction

1.1 Basic biology of *Cyclopterus lumpus*

The lumpfish is a marine semi-pelagic, semi-demersal cold water teleost and the only extant species in the genus *Cyclopterus* in the family *Cyclopteridae* (Blacker, 1983; Davenport, 1985; Kennedy et al., 2016; Nelson et al., 2016). The distribution of the species ranges over large parts of the North Atlantic Ocean and the Barents sea (Blacker, 1983; Moring, 2001; Powell et al., 2018a), dividing them into three genetic clusters, the western Atlantic (Maine, Canada, Greenland), the eastern Atlantic (Iceland, Norway) and the Baltic sea (Pampoulie et al., 2014).

The anatomy consists of one caudal and anal fin, two dorsal fins, two pectoral fins and the pelvic fins are modified, forming a suction disc (Davenport, 1985). The first dorsal fin is not visible but covered by a high crest made of tough skin containing compressed ossicles (Davenport, 1985; Powell et al., 2018a). Below the crest, laterally, three parallel rows of ossicles are found. The first one starts above the eye and runs mid laterally on the body, reaching the middle base of the caudal fin. The second row is most protruding, giving the lumpfish a spherical shape. The third row runs from the edge of the pectoral fins at the ventral side of the body and runs along the ventral edge to the anal fin. Located all over the ventral surfaces between the larger ossicles, numerous smaller ossicles lye unorganised. The abdomen may bulge or be smooth, and the suction disk is located cranially below the pectoral fins (Budney & Hall, 2010; Davenport, 1985). Lumpfish also lack a swim bladder. However, the skeleton has a density close to seawater (1.04 g ml^{-1}), and the crest and abdomen consist of subcutaneous jelly-like tissue. These adaptations have reduced the body's density, making the lumpfish fit the semi-pelagic lifestyle (Davenport & Kjørsvik, 1986).

Little is known about the pelagic migration of the Lumpfish (Blacker, 1983; Davenport, 1985; Powell et al., 2018a). Mature lumpfish enters shallow water during spring and spawn during early summer (April – July) (Davenport, 1985; Mitamura et al., 2007). The males arrive first and find a nest sub-tidally between rocks and kelps, favourably *Laminaria*, in shallow water (Davenport, 1985). During the spawning season, the female lays 100 – 400 thousand demersal eggs in two to three batches in different males nests, waiting 8 - 14 days between each batch (Davenport, 1985). After fertilisation, the ovarian fluid coating the eggs makes the eggs cluster and sticks to the substrate. After spawning, the female shortly returns to the pelagic zone (Mitamura et al., 2007), while the male stays, guarding and ventilating the eggs for 6 – 10 weeks until hatching (approximately 300 day-degrees) (Davenport, 1985). Nor male or female eat during the spawning season, and the males are aggressive towards anyone approaching the nest (Davenport, 1985).

After hatching, the juveniles (app. 5.5 mm long) are dispersed in the water column. They are then equipped with a small yolk sack that is spent in a few days, together with a functional digestive system and suction disk to latch on to seaweed and rocks (Davenport, 1985; Ingólfsson & Kristjánsson, 2002; Moring, 2001). Some have been found to recruit to the neustonic community short after hatching, often in connection with floating seaweed (Davenport, 1985; Ingólfsson & Kristjánsson, 2002), but most stay in the intertidal zone during the first year. They live in intertidal pools and the kelp forest for 1 -2 years until they out-grow these protective habitats and join the pelagic life stage (Davenport, 1985; Moring, 2001).

The females can reach a size of 60 centimetres in length and weigh 10 kilograms. Thus, they can be more than twice as big as their counterpart, which may only reach 30 centimetres in length (Davenport, 1985). There are few morphological differences between male and female lumpfish outside of the breeding season, although males' dorsal crest tends to be less protruding. However, mature males tend to get a red colouration in the abdomen during the breeding season, while females tend to stay blue-green (Davenport, 1985). Males also sexually mature earlier, at four years, while females usually mature between 5 and 6 and spawn between the age of 5 and 8. The oldest females found have been 12 - 14 years old, but most specimens have been 5 -8 years old (Davenport, 1985).

The lumpsucker is omnivorous, and the diet mainly consists of large planktonic organisms, often comprising crustaceans (Davenport, 1985). They may also eat ctenophores when available (Davenport, 1985; Ingólfsson & Kristjánsson, 2002). Juvenile lumpfish are selective feeders and feed on planktonic crustaceans while still having a yolk sack and may also exhibit cannibalistic behaviour (Ingólfsson & Kristjánsson, 2002). Still, the primary source of food for juvenile lumpfish is harpacticoid copepods, often associated with seaweed (Davenport, 1985; Ingólfsson & Kristjánsson, 2002).

1.2 Kelp ecology and cultivation

In the wild, the lumpfish is a seaweed specialist, and lives its first years in the kelp forest and intertidal pools (Blacker, 1983; Davenport, 1985; Powell et al., 2018a). As for the lumpfish, the kelp forests are a nursing ground for a myriad of other species.

Kelps are large macroalgae in the order Laminariales (Steneck et al., 2002). They make extensive underwater forests along the arctic and temperate regions of shallow rocky shores all over the globe (Filbee-Dexter et al., 2019; Steneck et al., 2002). Kelp forests are some of the most vital ecosystems on earth, much due to the ecosystem services they account for (Gundersen et al., 2017; Vondolia et al., 2020).

They are nursing ground, feeding ground and habitats for several ecological and economically important fish species, such as costal Cod (*Gadus Morhua*), Pollock (*Pollachius virens*) and Ballan wrasse (*Labrus bergylta*) (Gundersen et al., 2017; Norderhaug et al., 2005; Vondolia et al., 2020). Additionally, the kelp forests provide food, shelter and habitats for various terrestrial and marine organisms alike (Fredriksen, 2003; Gundersen et al., 2017; Steneck et al., 2002).

The production of macroalgae for harvest is a global industry in growth (FAO, 2020). The production volume was more than tripled from 2000 to 2018, and the production of temperate to cold water species are rapidly increasing (FAO, 2020; Olafsen et al., 2012). In 2012 it was predicted that the seaweed production in Norway would reach 50 million tonnes harvest by 2050 (Olafsen et al., 2012). The kelps found in Nordic waters comprise five species, *Laminaria hyperborea, Laminaria digitata, Saccharina latissima, Alaria esculenta,* and *Saccorhiza polyschides* (Gundersen et al., 2017). Today only the kelp *L. hyperborea* and the brown seaweed *Ascophyllum nodosum* are harvested commercially from the wild in Norway (Directorate of fisheries, 2020). Still, aquaculture cultivation of kelp has become an increasingly popular industry. In 2015 there were 54 commercial licences for aquaculture cultivation of seaweeds in Norway. Fast forward to 2020, and the number of licenses was 511, divided by 93 site permits (Directorate of Fisheries, 2021). The cultivated species are *Saccharina latissima, Laminaria digitata, Alaria esculenta, Palmaria palmata* and what is defined as "other species".

1.3 Biological delousing – the implementation of cleanerfish

Rearing kelp is still just a small production of the Norwegian aquaculture, which the Atlantic salmon (*Salmo salar)* clearly dominates, with over 300 million salmon put at sea in sea cages each year (Norwegian Directorate of Fisheries, 2021). However, the sector is troubled by the ectoparasite *Lepeophtheirus salmonis*, commonly known as the sea lice (Costello, 2009; Overton et al., 2020; Torrissen et al., 2013). Numerous methods have been applied to combat this problem, such as chemical (Aaen et al., 2015; Denholm et al., 2002) and non-chemical delousing treatments (Gismervik et al., 2017; Overton et al., 2019; Poppe et al., 2018; Sommerset et al., 2021) (Appendix A). However, the effectiveness of these methods has stated to wear off (Aaen et al., 2015) or maintain the welfare of the salmon poorly (Gismervik et al., 2017; Poppe et al., 2018). A third delousing method that is effective and neither harm the environment nor the salmon thus had to be conjured, leading to the implementation of cleanerfish (Powell et al., 2018b). Cleanerfish are different species of fish deployed into the same net-pen as the salmon, with the only task to graze on the ectoparasite from the skin of the salmon and keep the reproduction number of sea lice down (Imsland et al., 2018a; Skiftesvik et al., 2014). In doing this, the need for chemical and mechanical treatments are reduced, lowering the economic expense the sea lice treatments cause and increasing the

welfare of the salmon (Brooker et al., 2018; Costello, 2009; Torrissen et al., 2013). It is thus regarded as a sustainable and environmentally friendly delousing method (Overton et al., 2019).

The practice first started in the late 1980s using wrasses and their natural symbiotic activity of eating ectoparasitic copepods off the salmon (Bjordal, 1988, 1992). Wild-caught wrasses were the only used cleanerfish for a long time (Brooker et al., 2018). However, a negative side effect was that they go into winter torpor and are not effective lice eaters when the sea temperature drops below 6 - 10 degrees Celsius (Kelly et al., 2014; Yuen et al., 2019). A new species that are tolerant to colder temperatures, therefore, had to be implemented. In a study regarding wrasses as cleanerfish in northern Norway in late 1990/ early 2000, they at random caught a lumpfish in the net-pen. This lumpfish had ingested 160 sea lice, and there were more lumpfish in the net-pen, most of them also having consumed sea lice (Willumsen, 2001). Although cleaning behaviour has not been observed in lumpfish in the wild such as with other cleanerfish species (Family: *Labridae*), especially on coral reefs (Clague et al., 2011; Grutter, 1995), it was clear that if mature sea lice were presented to the lumpfish on the salmon, the lumpfish would eat it (Imsland, 2019; Willumsen, 2001). Thus, early experiments were conducted in the 2000s, and lumpfish was first mentioned as a cleanerfish in the annual Norwegian fish health report in 2011 (Olsen & Hellberg, 2011).

1.4 The status of Lumpfish in Norwegian Aquaculture

The preliminary research project NOLICE regarding lumpfish in aquaculture started simultaneously as the commercial production of lumpfish as cleanerfish started (Imsland, 2019; Olsen & Hellberg, 2011). It is thus apparent that the species was used without substantial knowledge regarding its physiology or limitations. Due to the industrial expanse co-occurring as the first scientific projects, the annual fish health report and other reports are the primary written sources of information on how the status of the lumpfish in salmon aquaculture was in the beginning. The Veterinary Institutes annual report series regarding fish health in Norwegian aquaculture was first published in 2005 and have ever since grown to be an essential collaboration and symposium of information regarding the status and health of farmed fish in Norway (Bornø et al., 2005; Sommerset et al., 2021).

1.4.1 Early reports on cleanerfish health

In the beginning, the mortality in the net-pens was high, and no shelter or resting places for the cleanerfish were present. Thus, the cleanerfish used the dead-fish net as shelter, causing even more mortality when it was emptied (Johansen, 2013). High mortality came to be a problem. An early statement was that the basic

knowledge regarding cleanerfish welfare and their limitations were lacking and that it was needed to map out potential diseases and causes of death (Olsen & Hellberg, 2011). By implementing the new lice limit in 2013, making the allowed number of lice per salmon to be half a mature female lice per salmonid (Forskrift om lakselusbekjempelse, 2013), the demand for cleanerfish increased to a greater extent (Hjeltnes, 2014; Nilsen et al., 2014). From 2013 to 2020, considerations regarding cleanerfish health and welfare were increasingly implemented. To compensate for rapid currents and fast swimming salmon, plastic installations mimicking natural kelp were put in the net-pen to give the lumpfish a substrate to rest on. Still, a recurring statement was that although the knowledge and attention towards the welfare and limitations of the cleanerfish had increased, the knowledge base was too small, and the mortality too high (Bornø & Linaker, 2015; Hjeltnes et al., 2016, 2017, 2018, 2019; Hjeltnes, 2014; Sommerset et al., 2020, 2021). During this time, the hatchery production of lumpfish became an industry of its own, the second-largest sector in Norwegian aquaculture. It has gone from just a few wild-caught lumpfish to over forty-two million hatchery-reared lumpfish in 2019, sold to the salmon aquaculture to a value of 935 020 000 NOK (Sommerset et al., 2021).

To assess the status of the cleanerfish in the net-pens several campaigns regarding cleanerfish health and welfare have been conducted (Nilsen et al., 2014; Sommerset et al., 2021; Stien et al., 2020). The first was the report mapping mortality and causes of death regarding cleanerfish health from the veterinary Institute (Nilsen et al., 2014). Based on daily dead fish registrations during six months from June to November 2013, looking at five sea farms holding lumpfish during this time, the mortality ranged from 39 to 100 per cent, with a mean mortality rate of 48 % (Nilsen et al., 2014). This is the most accurate public dataset regarding mortality on lumpfish in Norwegian aquaculture. The most apparent cause of death was categorised as bacterial infections (Nilsen et al., 2014). Of primary pathogens, *Aeromonas salmonicid*a, *Pasteurella* sp., *Vibrio anguillarum* and *Tenacibaculum* sp. were found in histological samples (Nilsen et al., 2014).

The report by Nilsen et al., together with several scientific publications by Imsland et al. regarding the use of lumpfish (2014a), its efficacy (2014b) and behaviour (2014c), shed some needed light on the situation. In 2014 the welfare of the cleanerfish was thus more implemented in the sea-based farms (Bornø & Linaker, 2015), incorporating shelters and feeding as part of holding cleanerfish, especially lumpfish. The welfare was thus seemingly increased, increasing the survival rate and the efficacy of the cleanerfish. However, the mortality reported was approximately 40 % overall, and during freshwater treatments, it was expected that all the cleanerfish would die since they were not sorted out before the treatment (Bornø & Linaker, 2015).

In 2018, cleanerfish were implemented in the akvakulturdriftsforskriften, and the law enforced demand regarding sorting out cleanerfish before delousing events was implemented (§28, Akvakulturdriftsforskriften, 2008). After implementing cleanerfish in several paragraphs in the legislation (§28, 44), the food safety authority issued a new national supervisory campaign directed at cleanerfish welfare (Akvakulturdriftsforskriften, 2008; Mattilsynet, 2020). From the questionnaire, the median mortality of lumpfish was estimated to be 46%. The geographical representation of the dataset shows clear division with higher mortality in the regions south-Norway (57%) and mid-Norway (48%), contrary to northern Norway (21 %) (Stien et al., 2020).

As seen in the previous fish health reports and other reports regarding cleanerfish mortality, it is apparent that a lot of cleanerfish disappear during the production cycle at sea (Mattilsynet, 2020; Sommerset et al., 2021). Eaten, escaped or lost cleanerfish are not registered as dead, which is a strong indication that the number of cleanerfish dying, is actually much higher (Nilsen et al., 2014; Sommerset et al., 2021; Stien et al., 2020). In addition, some farmers do not register the surviving cleanerfish, and mortality or survival analyses are thus not possible to conduct (Stien et al., 2020). In the annual risk assessment from the Institute of marine research, the risk of lumpfish experiencing poor welfare in salmon sea cages is considered to be high (Grefsrud et al., 2021). The lumpfish cannot fulfil its mission as a lice eater when dead or sick. Consequently, other parameters than mortality to evaluate welfare must be pursued.

1.5 Welfare indicators

One way to measure or obtain information on a livestock species health and welfare are by developing species-specific welfare indicators (WI) (Espmark et al., 2019; Kolarevic et al., 2017; Toni et al., 2019). WIs can be used in operations (OWIs) or labs (LABWIs) and are either environmental or animal-based, and thus refer to either environmental, individual or group explained parameters (Brooker et al., 2018; Martins et al., 2012; Noble et al., 2019). The first scientific symposium of welfare indicators for lumpfish in aquaculture was "An introduction to operational and laboratory-based welfare indicators (OWI) for lumpfish (Cyclopterus Lumpus L.)" published in 2019 (Noble et al., 2019). Although this is a fact sheet series and not standardised OWIs as for salmon (Noble et al., 2018), much is accounted for.

1.5.1 Environment, individual and lab-based WIs for lumpfish

Some welfare requirements have been well studied, such as optimal dissolved oxygen during different situations (Jørgensen et al., 2017; Remen & Jonassen, 2017; Treasurer, 2018), optimum temperatures during different ontogenetic stages (Hvas et al., 2018; Imsland et al., 2014c; Powell et al., 2018a; Skår et al.,

2017), and visual cues regarding light colour, intensity and wavelength (Espmark et al., 2019; Skiftesvik et al., 2017). Lumpfish have also been found to have a limited aerobic scope (Hvas et al., 2018), making them vulnerable to environmental fluctuations and rapid currents (Killen et al., 2007). Still, there are many parameters with little knowledge about it, such as density, turbidity, CO_2 and pH tolerance ranges (Noble et al., 2019)

Individual-based OWIs often describe the external appearance of the organism, and sores and epidermal damage are especially well-used WI in aquaculture (Martins et al., 2012; Noble et al., 2012). Epidermal injuries in lumpfish are often caused by bacterial infections (Noble et al., 2019), and the evidence base regarding bacterial diseases in lumpfish is substantial (Hjeltnes et al., 2017; Nylund et al., 2020; Rimstad et al., 2017; Sandlund et al., 2021; Sommerset et al., 2021). The OWI fin damage, often caused by tail nipping, disease or other factors, is also one of the main OWIs for lumpfish (Ellul et al., 2019; Espmark et al., 2019; Rimstad et al., 2017).

Lab-based WIs regarding physiological parameters such as plasma cortisol, glucose, osmolality, magnesium, and chloride are well-established welfare indicators, but few standardised ranges have been developed regarding lumpfish (Espmark et al., 2019; Jørgensen et al., 2017; Remen & Jonassen, 2017). Changes in plasma cortisol levels (corticosteroids) in the bloodstream are a widely used measure of the initial stress response (Barton & Iwama, 1991; Mommsen et al., 1999; Wendelaar Bonga, 1997). Unstressed lumpfish have been found to have a plasma cortisol level below 10 ng mL^{-1}, and stresses lumpfish have been found to have a plasma cortisol level below 60 ng mL^{-1} (Hvas et al., 2018; Jørgensen et al., 2017). Barton and Iwama reported that Atlantic salmon had a plasma cortisol level of 40 ng mL^{-1} before five minutes chasing event and 190 ng mL^{-1} afterwards (1991). Rather than being frenetic, the lumpfish adhere to the present substrate and sit still (Remen & Jonassen, 2017). They also lack mauthner neurons (Hale, 2000), and the flight response expressed by lumpfish is thus quite different from other fish. Hence, it is difficult to distinguish a resting lumpfish from a stressed lumpfish without conducting physiological analyses (Remen & Jonassen, 2017).

1.5.2 WIs - measuring sickness or health?

The lumpfish is a new aquaculture species, and currently, there are few standardised classification scales regarding WIs in Lumpfish aquaculture, and benchmarking health is therefore tricky. Two studies have recently been done to the author's knowledge, creating applicable, non-lethal Lumpfish scoring schemes or indexes (LOWSI) developed for assessing the health status of lumpfish in commercial operations (Gutierrez Rabadan et al., 2021; Imsland et al., 2020). Futierres Rabadan et al.'s scoring scheme consists of the

parameters skin and fin damage, weight, sucker deformities, and eye condition. They found that fin damage and sucker deformities were most prevalent in the hatcheries and that body damage and poor eye condition were the most dominant welfare issues in sea cages (Gutierrez Rabadan et al., 2021). Additionally, a lab-WI liver score for lumpfish based on different welfare/ feed parameters have recently been developed (Eliasen et al., 2020).

Another way of measuring health and welfare that looks at the current status of the fish, and can say something about the robustness of the fish in confrontation with stressors or disease in the future, is mucosal mapping (Myre & Pittman, 2019). Numerous studies have investigated the morphology, structure and distribution of mucous cells in the fish skin, and it is agreed upon in the literature that abiotic and biotic factors play a prominent role in mucous cell dynamics, and studying mucous cells as an index for tertiary stress response, is therefore of high relevance, and a significant way of characterising mucosal health (Dang et al., 2019; Jonassen et al., 2019a; Pittman et al., 2011, 2013; Vatsos et al., 2010). As a measure of skin health, mucosal mapping would then be a novel lab-based welfare indicator for lumpfish.

Lumpfish has thus been seen to exhibit different physiological limitations and ranges than other species, especially salmon (Noble et al., 2019). Without the knowledge of lumpfish physiology and how it deals with different stressors and changes in the environment, knowing that it is healthy is impossible. Thus, current welfare indicators focus on diseases rather than health, and new methods that focus on health and robustness should be implemented. In establishing a range of both invasive and non-invasive WIs, making room for standardisation, the overall health and welfare of the species can be secured in a much broader way than the current status. For the WIs to be applicable, the range or the optimums of the parameters must be documented and established. In this study, the ranges of size, density and defence activity exhibited by clinically not ill lumpfish reared in three different environments have been investigated, with three different treatments. The lumpfish were thus investigated while reared with the treatments natural kelp, plastic kelp and without kelp.

1.6 Use of kelp

The interest in utilising natural kelp and seaweeds as biological resources such as biofuel, medicine, cosmetics, food and feed in different industries is increasing (Adams et al., 2011; Ferreira et al., 2020; Gundersen et al., 2017; Olafsen et al., 2012). Alginates, glucose, pigments and other biochemical compounds of the kelp are being extracted, processed and used in different industries. In addition, several secondary metabolites and other compounds exhibited by seaweed have been found to be antimicrobial,

antiseptic, stress-reducing, and a range of other desirable properties (Adams et al., 2011; Bengtsson et al., 2012; Ferreira et al., 2020; Reichelt & Borowitzka, 1984; Wiese et al., 2008). Increasing interest has led to macroalgae becoming a more frequent feed additive in aquaculture due to many health benefits (Thépot et al., 2021). However, seaweeds do not need to be processed to be an aquaculture resource.

1.6.1 Microbial community

As well as providing a habitat for a range of species due to their three-dimensional structure, seaweeds also provide habitats for epibionts, their associated microbiota (Egan et al., 2013; Malik et al., 2020). Marine eukaryotes can depend on epiphytic bacteria for survival since they may play a significant role in maintaining the kelps health, performance and resilience. (Egan et al., 2013; Malik et al., 2020; Wahl et al., 2012). These bacteria are present because of the kelp surface's local hydrodynamics and physiochemical properties and its mucosal layer. Since all marine surfaces possess a biofilm (Geesey, 2001), the bacteria found on the kelp surface lives in and on the biofilm. The biofilm produced by the bacteria is mainly a matrix made of eDNA, polysaccharides and protein (Geesey, 2001). In this biofilm, some bacteria also release secondary metabolites and compounds with antimicrobial effects (Holmström et al., 2006).

The kelp itself also exhibit secondary metabolites, inhibiting fouling by the microbiota (Holmström et al., 2006; Reichelt & Borowitzka, 1984). Kelps of the order Laminariales comprises laminarin, a water-soluble polysaccharide found to inhibit virus proliferation (Wang et al., 2012). Thus, kelps can promote the growth of beneficial bacteria that defend their surface and inhibit the growth of pathogenic and fouling bacteria (Goecke et al., 2010; Wahl et al., 2012). The biofilm of some kelps is thus a cocktail of host-specific bacteria exhibiting antimicrobial compounds, functioning as a second skin displaying a borrowed immunity (Malik et al., 2020; Steinberg et al., 1997; Wiese et al., 2008).

Bacteria exhibiting antimicrobial activity mainly belong to the gram-positive phyla *Firmicutes* and *Actinobacteria*, including antibiotic strains, and the gram-negative phyla *Proteobacteria* and *Bacteroidete*s, who have shown to contain bacteria that exhibit secondary metabolites showing antimicrobial activity (Wiese et al., 2008). Within the phylum *Proteobacteria* and the class *Alphaproteobacteria,* species within the *sulfitobacte*r genus have exhibited antimicrobial activity. In the class *Gammaproteobacteria*, the genera *seudomonas*, *pseudoalteromonas*, *vibrio* and *Aeromonas* are some of the bacteria exhibiting antimicrobial substances (Bérdy, 2005; Wiese et al., 2008). Bacteria in the genus *Bacillus* from the class *Bacilli* have also shown to produce antibiotics (Bérdy, 2005). *Flavobacteria* in the phylum *Bacterioidetes* were found to degrade algal compounds (Bérdy, 2005).

In a study done on the bacterial community in association with the kelp *S. latissima*, they found that the community consisted of bacteria exhibiting antimicrobial activity (Wiese et al., 2008). Some of the antibiotic substances produced by the bacteria had the potential to inhibit growth of pathogenic bacteria. (Wiese et al., 2008), and several papers have studies the bacterial community and the antimicrobial activity exhibited by these bacteria on the kelp, such as towards gram-positive test strains (Staufenberger et al., 2008; Wiese et al., 2008).

1.7 Fish physiology – mucosal barriers

The mucosal barrier of the fish skin is the first part of the body in touch with the environment and the first line of defence against pathogens (Ángeles Esteban, 2012; Kryvi & Poppe, 2016). Contrary to terrestrial animals, where the skin is keratinised, all layers of the fish (teleost) skin consists of living cells (Castro & Tafalla, 2015; Kryvi & Poppe, 2016). The outermost layer, the epidermis, consists of stratified squamous epithelia, where cell division occurs in all depths of the layer (Kryvi & Poppe, 2016). The thickness varies from species to species and regarding the location on the body, averaging at 5 – 10 cells in thickness (Peterson, 2015). The skin of all fishes produce mucous, and the epithelial layer facing the surface consists of numerous microridges on the surface, making a better grip for the mucosal layer (Kryvi & Poppe, 2016). Among the epithelial cells, there are several other types of cells such as pigment cells, mucous cells (also called goblet cells), sensory neurons, and some fish groups also have saccular cells and club cells (Kryvi & Poppe, 2016). Some fish have scales embedded in the dermis, thus always remaining under the epidermis and the mucosal layer, and others do not.

The mucosal layer is secreted from different types of cells in the epidermis, primary from mucous cells. Exactly how the mucous cells arise is still not scientifically proven, but they arise in the basal part of the epidermis, migrating towards the apically external surface (Kryvi & Poppe, 2016). As the mucous cells migrate, they may increase in size. The mucous cells and their content also vary between species, location on the body, and local environmental factors. (Gona, 1979; Kryvi & Poppe, 2016; Peterson, 2015). These cells are larger than squamous epithelial cells and contain a basal nucleus and apically large round secretion vesicles (Kryvi & Poppe, 2016). When the mucous cells reach the surface, they burst and empty the content on the surface. The content then reacts with the water and swells to a viscoelastic gel, making the mucosal layer adhere evenly to the fish skin. The biochemical composition of fish mucous mainly consist of large macromolecules such as glycoproteins (mucins), and to a lesser extent, humoral immune components such as immunoglobulins (Igs), enzymes, lectins, antimicrobial peptides and lysozymes, varying with species, environment and life stage (Ángeles Esteban, 2012; Kryvi & Poppe, 2016). The migration of mucous cells is

continuous, and the mucous has a high turnover rate when healthy. The fish is then constantly equipped with a fresh layer of mucous (Merrifield & Rodiles, 2015).

As the first line of defence, the mucosal barrier is a part of the innate immune system (Castro & Tafalla, 2015). The mucosal barrier has an immunological aspect, to protect the fish against microbes such as parasites and bacteria, as well as physiological and mechanical elements to preserve the fish again physical damage, environmental stressors, drag reduction, and acid/base and osmoregulation (Castro & Tafalla, 2015; Kryvi & Poppe, 2016). The skin is often the main entry point for pathogens. However, entering the fish via a healthy mucosal layer is close to impossible due to its physical, chemical and biological properties (Ángeles Esteban, 2012). In a bath challenge where Atlantic salmon were infected with *Vibrio anguillarum* and *Aeromonas salmonicida*, the results showed that the fish with a removed mucosal layer (wiping the fish dry) had a higher mortality rate than the fish that had been externally wounded (Svendsen & Bøgwald, 1997). The protective mucosal barrier is vital for all marine aquatic life and hence the most effective defence against pathogens in fishes (Ángeles Esteban, 2012).

1.7.2 Lumpfish skin

The lumpfish is a scaleless fish, but the skin is tough. It consists of numerous ossicles protruding the epidermis and the spongious and compact layers of the dermis, which on average accounts for 72 % of the skin (Klingenberg, 2019). The lumpfish skin is a novel site of research, which is reflected in the ambiguous remarks regarding anatomical structures, especially suited in the epidermis.

In 2019 the normal histology of lumpfish (<5 g and up to 140 g) regarding the main organs, including skin, was mapped out (Klingenberg, 2019). Transverse histological sections were made, and the epidermis was observed to contain stratified squamous epithelia, melanophores, mucous cells and structures classified as large vacuoles (Klingenberg, 2019). In another study regarding histologically transverse sectioned lumpfish skin, these structures, the vacuoles, were classified as saccular cells (Patel et al., 2019). In a third study regarding lumpfish skin (tangentially sectioned for stereology based investigations), these structures were thought to be novel structures and gave them the name Q-cells (Jonassen & Pittman, 2019).

In the last article, the structure of the epithelial cells was also addressed. Usually, the most used tissue sectioning method for histology has been transverse, vertical sectioning (Ross & Pawlina, 2006). Regarding sectioning for stereological analyses, the tissue is sectioned tangentially, namely as a cheese slicer. With

tangential sectioning, the area of interest is thus much larger, and the cells of interest greater in number (Dang et al., 2020). This way of sectioning the epidermis thus led to the findings of a new epithelial structure, named rose petal cells (Jonassen & Pittman, 2019). These cells are arranged peripherally, and with their large surface area, are similar to wild rose petals (Figure 1) (Jonassen & Pittman, 2019).

A possible link between the lumpfish habitat, the kelp forest, and this novel biological structure of the lumpfish skin have been hypothesised. When the lumpfish adhere to or swim by the kelp, the rose petal cells flutter with the waves, grabbing onto the mucous of the kelp and thus coats themselves in the microbiota of the kelp (Jonassen & Pittman, 2019).

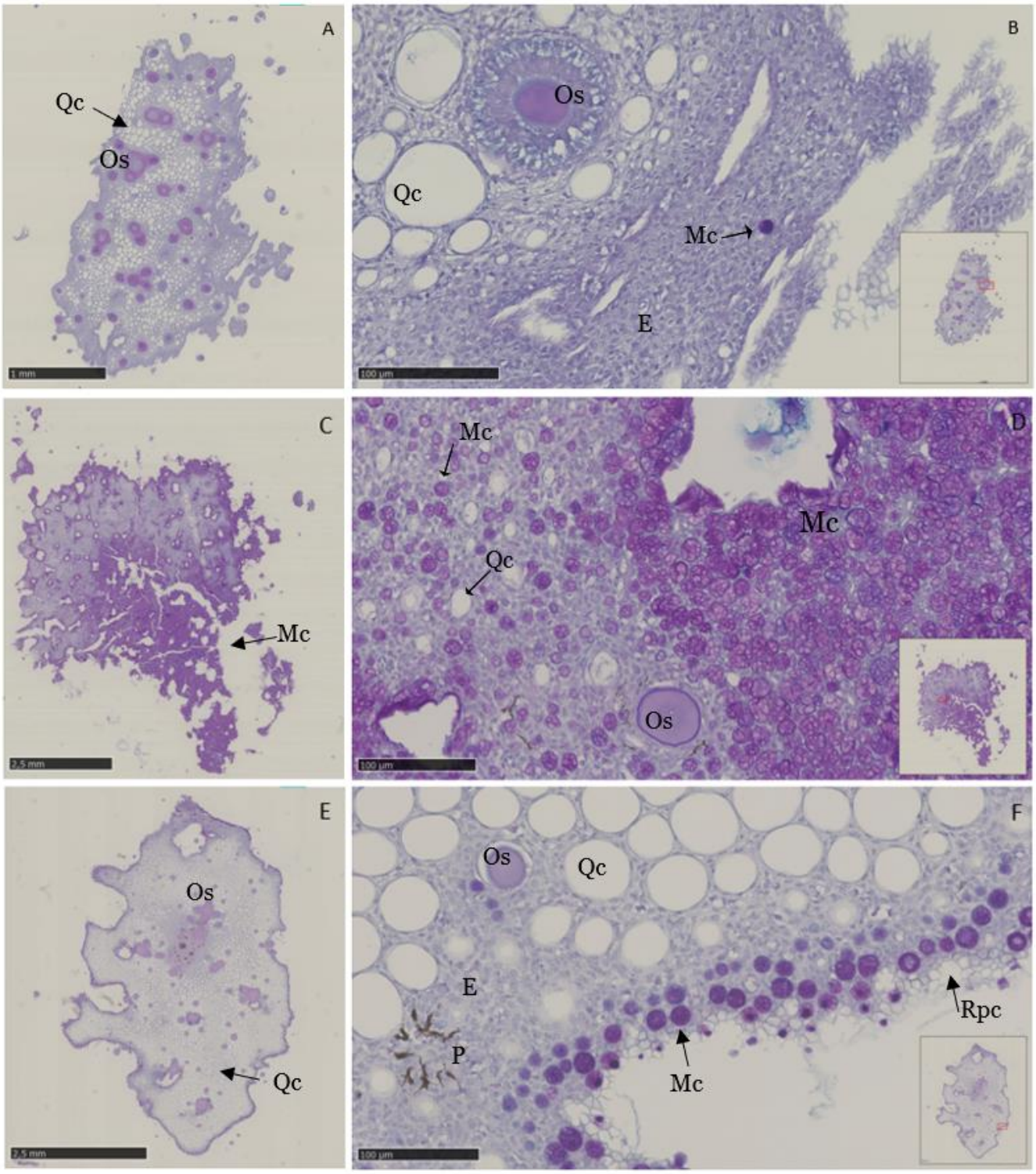

Figure 1: Tangentially sectioned epidermis of Lumpfish (*C. lumpus*) skin (mid laterally, PAS-AB stain). A) Lumpfish from the treatment with natural kelp in Agder (scalebar:1 mm). B) Closeup of A, scalebar 100 µm. C) Lumpfish from the 2. Sampling at Austevoll from the treatment with plastic kelp, scalebar 2.5 mm. D) Close-up of C, scalebar 100 µm. E) Lumpfish from Fitjar from the sea cage with no kelp, scalebar 2.5 mm. F) Close-up of E, scalebar 100 µm. In reading order: Qc: Q-Cells, Os: Ossicle, Mc: Mucous cell, E: Epithelia, P: Pigment cell and Rpc: Rose petal cells. Qs and Rpc are novel structures discovered by K. Pittman and T. Jonassen (2019).

1.8 Hypothesis and research aims

The hypothesis is thus that the lumpfish borrows external immunity from the kelp.

The main objective of this master thesis was to investigate how the mucous cells in the lumpfish skin react to being reared with the different treatments natural kelp, plastic kelp and no kelp in different environments (landbased facilities and a commercial salmon farm), and whether the treatments affect the mucous cells systematically. By applying the standardised, quantifiable method of mucosal mapping on the lumpfish skin, the following research aims were asked:

1) What are the ranges of the mucous cells mean area, volumetric density and defence activity?

2) Is there a difference in mucous cell mean area, volumetric density or defence activity based on the different methods of rearing the lumpfish with the treatments natural kelp, plastic kelp or no kelp?

3) Is there a difference in the mucous cells based on location?

4) Can the bacterial communities established on the natural kelp, artificial kelp or tank walls be transferred to the lumpfish skin, and will it have an impact on the fish's skin health?

To answer these research questions, three different experiments rearing lumpfish in different environments and with the treatments natural kelp, plastic kelp and no kelp were conducted. Secondly, skin samples were obtained from all experiments, and mucosal mapping by the semi-automated technology of Veribarr was applied. Lastly, nested one-way analysis of variance (ANOVA) to check for statistical significance was conducted.

2 Materials and methods

Ethical statement

This thesis comprises two different research projects, divided into three experiments. All experiments conducted in connection to this thesis were performed in accordance with legislations (Norwegian animal welfare act, akvakulturforskriften and 2010/63/EU) concerning animal welfare and experimentation. The legislations are based on the principle of humane experimental techniques (Russell & Burch, 1959) and the five freedoms (Webster, 2008).

2.1 Agder

The experiment was financed by Agder county municipality through "Blått kompetansesenter sør" with the RFF Agder project title "May kelp facilitate better fish health for lumpfish in aquaculture?" ("Kan tare gi bedre fiskehelse for rognkjeks i oppdrett?"). The owner of the project was Landbasert Akvakultur Norge AS. The experiment took place in an industrial area in Hausvik, Lyngdad, Norway (58°02'59.3"N 6°58'55.9"E) close to their hatchery, from the 1st of July with sampling on the 14th of July (Figure 2). Two treatments were tested: rearing lumpfish in tanks containing live kelp and rearing lumpfish in tanks with no kelp.

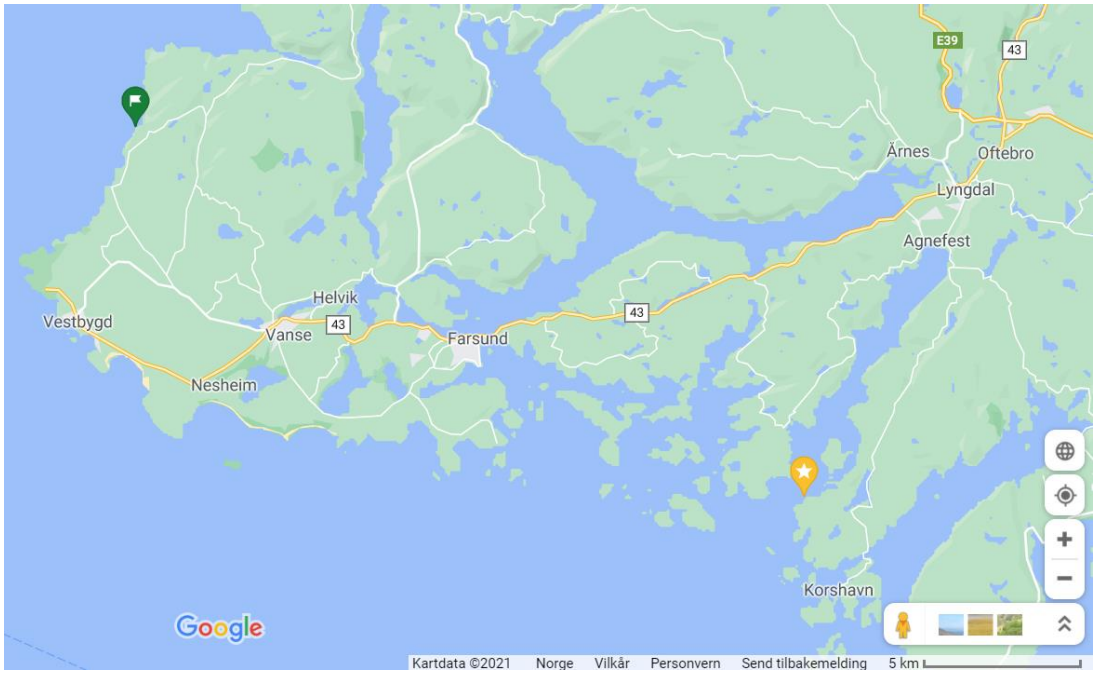

Figure 2: The location of kelp (*Saccharina latissima*) sampling point (green flag pin) and research facility/hatchery (yellow star pin) in Agder, Norway (01.07.2020 – 14. 07.2020).

2.1.1 Experimental design and fish husbandry

The experimental setup consisted of a seawater flow-through system including four green fibreglass tanks placed inside a container (Figure 3 and 4). The tanks were square (1.1x1.1x0.6m), holding a volume of approximately 500 L. The outlet was centred, and the water exchange rate was 1000 L/h. The seawater was pumped up from 100 m depth from Rosfjorden outside the facility. The inlet water was treated through a sand filter and UV filter and was maintained at 8 - 9.5 ° C, with 100 % dissolved oxygen (DO) saturation. The light regime was 24 h light, and the fish were fed Atlantic gold 1.0 mm (Pacific Trading Aquaculture Ltd, Dublin, Ireland) daily according to satiation using a belt feeder.

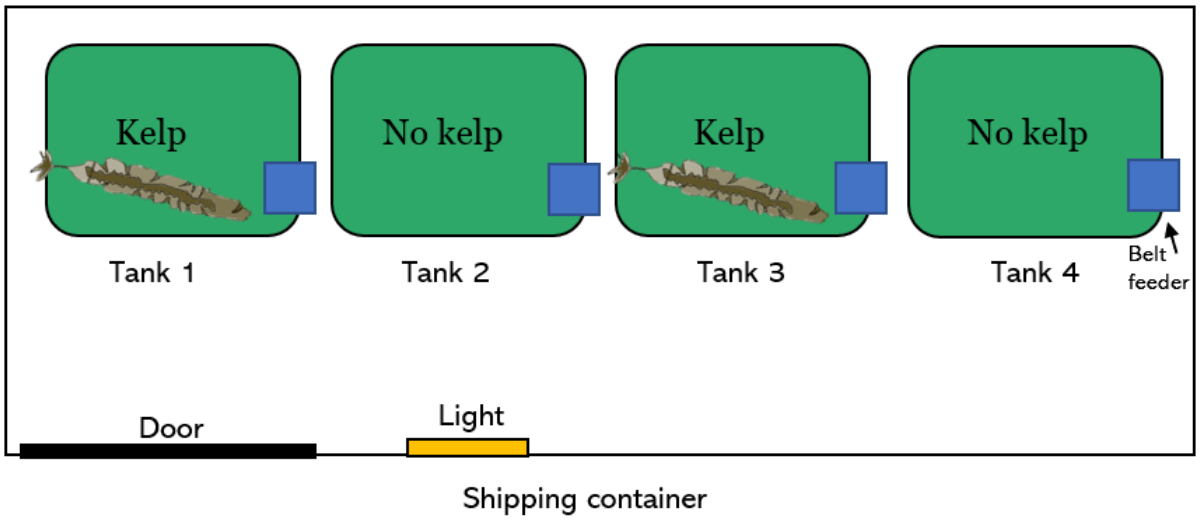

Figure 3: Experimental design and facility in Agder. Four green square tanks (1.1x1.1x0.6m) located inside a shipping container. Tank 1 and 3 were equipped with the treatment natural kelp (*Saccharina latissima*), and tank 2 and 4 had no kelp (control). One hundred lumpfish (*Cyclopterus lumpus*) were allocated to each tank, and they were fed daily by a belt feeder. The experiment lasted from 01.07.2020 – 14.07.2020 and had one sampling point (lethal, n = 5 per tank, N=20). The results from the experiments Agder, Austevoll and Fitjar are compiled in Figure 15.

The lumpfish were sourced from Landbasert Akvakultur Norge's own hatchery and were vaccinated with Amarine Micro 3-1 (Pharmaq Analytic, Bergen, Norway). A small quantity of kelp was harvested (no permits required) from the public access point Snekkestø (58°09'21.8"N, 6°37'05.0"E) prior to the experiment and was not changed. At day zero, 100 lumpfish were placed in each of the four tanks (n= 400). The experiment consisted of two treatment replicates (Figure 3). Tank number one and three were equipped with the treatment natural kelp (*Saccharina latissima*), and tank two and four contained no kelp (control). The kelps (6-8 individuals per tank) were tied together at their stripe and attached with a string to the edge of the tanks, making the thallus float in the water column (Figure 4).

Figure 4: The experimental setup in Agder. A) Experimental facility B) Tank setup C) Tank with the treatment real kelp (*Saccharina latissima*) D) Tank with the treatment no kelp (Experiment Agder, sampling 14. 07.2020, n=20). Photos: Karin Pittman, UiB/QuantiDoc.

2.1.2 Sampling

The sampling was conducted on the 14th of July. An appropriate working station was set up, and a lethal dose of anaesthetics was mixed out in a bucket containing seawater (Finquel, MS222). The sampling order of the tanks was 4, 3, 2 and lastly, tank 1. Five random fish from each tank were fished out using a small net and placed in the bucket and euthanised. The fish were then measured in length to nearest mm (14 of 20 samples), and skin sampled. The sampling consisted of cutting out one 1-2 cm^2 piece of skin on the lateral side of the body using scalpel and forceps, and carefully not touching the skin, placing it in a histocassette (Sigma-Aldrich, Darmstadt, Germany). The histocassette was then placed in a small retainer containing 10 % buffered formalin, fixing the samples. The samples, 10 per treatment (n=20), were then sent to staining, slicing and digitalisation. This is the standardised sampling method and processing, following the QuantiDoc manual regarding preparations for mucosal mapping (Pittman et al., 2011, 2013).

2.2 Austevoll

This experiment was conducted by researchers and master students from the University of Bergen, project leader Akvahub AS, and project owner Engesund oppdrett AS as part of the preliminary project Seaweed symbiosis (project number 317935) funded by regional research funds (RFF).

2.2.1 Experimental species and facilities

The experiment took place at the institute of marine research (IMR) research station Sauvaneset II (location number 16195, 60,087259° N, 5,265122° Ø) Austevoll, Norway (Figure 5). The experimental species was lumpfish *(C. lumpus*), and 600 lumpfish from the same batch were sourced from Vest Aqua Base, where Engesund oppdrett AS is a co-owner. The lumpfish were randomly distributed between the tanks, each tank having 100 lumpfish. The experiment lasted for six weeks and consisted of three sampling days: Day 2 (08.09.2020), day 23 (29.09.2020) and day 40 (16.09.20). Ten fish from each treatment (five fish from each

tank) were collected at each sampling day. 90 fish were sampled in total. During the experiment, there were no mortalities except the sampled fish. One additional fish was used as a test on day 23. During the whole experiment, 91 out of 600 fish were sampled.

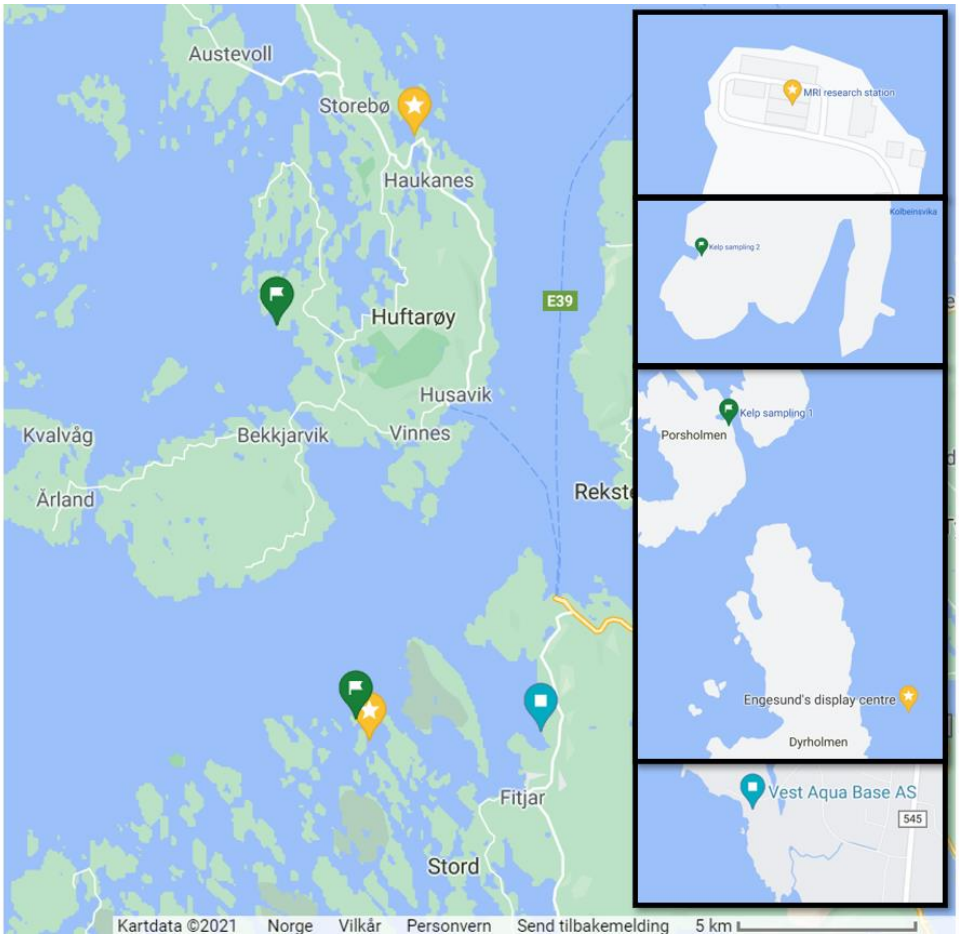

Figure 5: Research facility and sampling points for lumpfish and kelp in in Austevoll and Fitjar. Austevoll: Research facility and sampling of lumpfish (upper yellow star pin). First and second kelp (*Laminaria digitata*) sampling point (green flag pins. Fitjar: Engesund's fish farm and sampling point for lumpfish in Fitjar (lower yellow star pin) and the hatchery when the lumpfish were sourced from (turquoise square pin). (Map sourced from google.maps.no)

2.2.2 Experimental design and fish husbandry

At the IMR's research station, the experimental design consisted of a seawater flow-through system involving six circular plastic tanks located in an indoor facility (Figure 6). The seawater was pumped from 160 m outside the facility, mechanically filtered in a sand filter (filter size 50 µm), and then brought straight to the tanks. The tanks were circular, 1 m in diameter, holding a volume of approximately 400 L. The tanks contained an inlet pipe with a single outlet, and the outlet pipe was centred. The water flow in the tanks was 800L/min throughout the experiment in each tank. Water temperature and oxygen saturation were monitored daily in the outlet drain of each tank. Water temperature was maintained at 8.4 ± 0,01 °C (Mean ± SEM), and the percentage oxygen saturation was 92 ± 0.6 (Mean ± SEM). The feed regime consisted of

hand-feeding the first and second day of the experiment. After that, the fish were increasingly fed, 50 -100 g feed a day, regulated after satiation, the commercial dry feed Lumpfish Grower (Biomar, Haugesund, Norway). The feed was supplied using a belt feeder (Figure 6). There was no light regime during the experiment except light from the ceiling during the working hours of the personnel. Faeces and feed residue in the tanks were cleaned daily by the personnel.

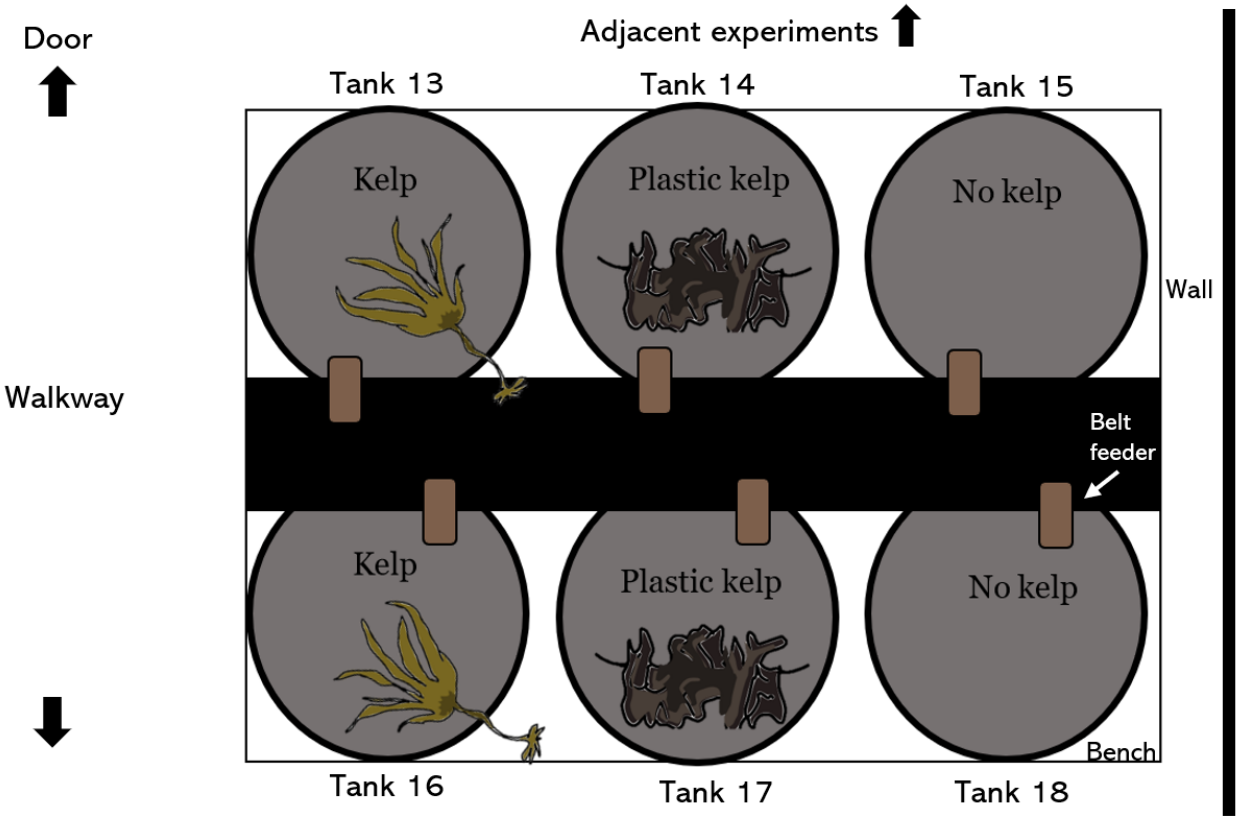

Figure 6: Experimental design and facility in Austevoll. Six round (1x0.6m) black tanks located inside a research hall. Tank number 13 and 16 were equipped with the treatment real kelp (*Laminaria digitata*), tank number 14 and 17 were equipped with plastic kelp, and tank number 15 and 18 had no kelp (control). One hundred lumpfish were allocated to each tank, and they were fed daily by a belt feeder. The experiment lasted from 08.09.2020 – 16.10.2020 and had three sampling points, on day 2, 23 and 40. Sampling was lethal, n= 5 per tank per sampling, N=90. The results from the experiments Agder, Austevoll and Fitjar are compiled in Figure 15.

The experimental design consisted of three groups of treatments. Each group had two tank replicates (Figure 6). The first treatment consisted of tank 13 and 16 equipped with real kelp (*Laminaria digitata*) (Figure 8). The first batch of kelp was harvested on the south side of Porsholmen, just north of Dyrholmen Øst (Figure 5). The second batch of kelp was harvested in an inlet inshore of Boene, which is located near Kolbeinsvik (Figure 5). Both harvests were done in small quantities, at locations open for public access. The harvested kelp was acclimated to a colder water temperature by being placed in tanks with a water temperature of 12 degrees a few days before being placed in the experimental tanks containing 8 degrees

seawater. Eight individual kelps were then placed in each tank. Initially, the kelp already attached to rocks were placed in one tank, and in the other tank, the individuals were sewn onto a rope and hung down into the tank (Figure 7). This method led to excessive faeces on the bottom of the tanks, and the kelp was disintegrating in both tanks. The kelps were thus changed on day 22 with the second batch of kelp, and this time the holdfast of the kelps was tied to the side of the tanks, and the thallus of the kelps was tied to pieces of buoyant Styrofoam, making the thallus drift at different levels in the water column in both tanks (Figure 7). One additional tank was used to contain the extra live kelp.

Figure 7: Left: The first batch of kelps attached to rocks at the bottom of the tank. The tank contains natural kelp and lumpfish (*Cyclopterus lumpus*). Right: Second batch of kelp attached to the edge of the tank and Styrofoam. The tank contained lumpfish and kelp. Photos: Frida Sol M Svendsen.

The second treatment consisted of tank 14 and 17 equipped with approximately 1x 0.5 m "plastic kelp". The material used was thick PE plastic sheets commonly used in the industry as hides and resting place for cleanerfish (OKmarine, Kristiansand, Norway). The plastic kelp was tied with strings across the tank (Figure 8). The third treatment, the control group, consisted of tank 15 and 18 with no content in the tank except the body of water containing fish and the tank walls themselves (Figure 8).

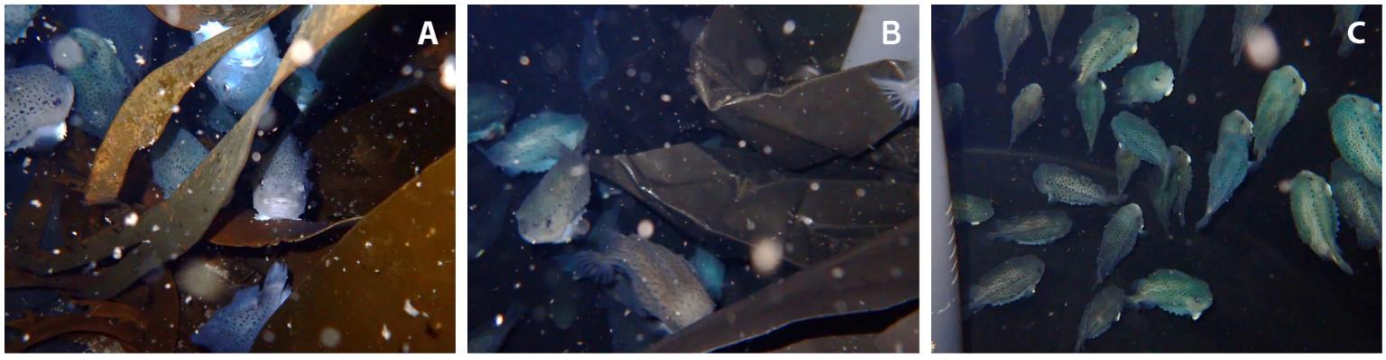

Figure 8: The three different treatments used in the experiment at Austevoll (07.09.2020 – 16.10.2020) A) Lumpfish (*C.lumpus)* attached on the natural kelp *(L.digitata*). B) Lumpfish in a tank containing artificial PE-plastic-kelp. C) Lumpfish resting on the tank walls in a tank with no kelp. Photos: Bettina Wickman Kvamme, Akvahub AS.

2.2.3 Sampling

Sampling was conducted on day 2 (8. September 2020), day 23 (29. September 2020) and day 40 (16. October 2020).

Austevoll 1. sampling - day 2 (8. September 2020)

On the first day of sampling, a suitable working station was set up and the equipment used was prelabelled. The histocassettes (Sigma-Aldrich, Darmstadt, Germany) were numbered 1 to 30 in accordance with the fish-ID of sampling. The fish were sampled from the tank order of 13 (Real kelp), 14 (plastic kelp), 15 (no kelp), 16 (real kelp), 17 (plastic kelp) and lastly, tank 18 (no kelp). For each tank, five fish were caught by a small net (15x15 cm) at random and placed in a bucket containing a lethal dose (≥1,6 gL^{-1}) of Finquel (MS222). The fish were thus moribund and humanely euthanised. The ratio between the anaesthetic compound and the medium was unknown, resulting in what was believed to be a solid euthanising dosage. When the fish stopped moving around and had lost equilibrium for about 30 seconds, they were gently pushed around, and if no reaction or gill flare was observed, the sampling proceeded. One by one, the fish were then picked up from the bucket, weighed in grams to one decimal point precision on a scale, and measured to the nearest mm on a measuring table before transferred to another bench for skin sampling. All the fish were laid down on their left (lateral) side and sampled on their right side (Figure 12).

The skin sampling was conducted according to Quantidoc's protocol using protective medical gloves, a sterile scalpel, forceps, plastic histocassettes and biopsafes. The first procedure of the skin sampling was to puncture the heart before making a dorsolateral excision of 1-2 cm^2 skin area adjacent to the first row of protruding ossicles (Figure 12). The biopsy was then placed facing up, carefully not touching the skin surface, in a pre-labelled histocassette (Sigma-Aldrich, Darmstadt, Germany) which was placed in a 20 mL BiopSafe® (Biopsafe, Vedbaek, Denmark) and the lid was screwed on. The button on the top of the biopsafe was pushed down, and the sample was immersed in 10 % phosphate-buffered formalin solution (4% formaldehyde). Three histocassettes were placed in each biopsafe. This procedure was conducted for all six tank replicates (n=30). The biopsafes were handled and stored according to the manufacturer's instructions.

Austevoll 2. sampling - day 23 (16. September 2020)

On day 23, the skin sampling was conducted as previously explained for day 1. The sampling order was made based on treatments and was thus in the order of tank 13 (real kelp), 16 (real kelp), 14 (plastic kelp),

17 (plastic kelp), 15 (no kelp) and last tank 18 (no kelp). The external appearance of the fish was also noted (Table 4). Additionally, microbiological sampling was conducted. The sampling consisted of collecting pieces of the live kelp, swabbing the sampled fish, the treatments (e.g., natural kelp and plastic kelp) and the tank walls, including the outlet pipe.

The kelp was sampled using a pair of scissors and forceps, cutting out a 2x7 cm random piece of healthy kelp and placing it in a petri dish. The mucosal layer on the kelp was then scraped off using a sterile scalpel blade into a container (CryoTube™, Thermo scientific) and then placed in a barrel containing liquid nitrogen for flash-freezing. All samples were stored at -80°C until DNA extraction.

During this sampling, one fish was used as a test fish to combine skin and microbiological sampling. Each fish was swabbed three times, and the swabbing occurred before the skin was sampled. Thus, the microbial sampling was conducted on the skin and around the ossicles, avoiding the area where skin sampling would occur. The tank walls and the kelp (natural and plastic) were swabbed twice each. The swabbing was done using sterile cotton swabs (Applimed SA, Châtel St. Denis, Switzerland), and all the swabs were put in individual vials (CryoTube™, Thermo scientific), in DNA later. All the samples were placed in liquid nitrogen for flash freezing and were stored at -80°C until DNA extraction.

Austevoll 3. sampling - day 40 (16. October 2020)

The sampling on day 40 was conducted like day 22, with skin and microbiological sampling. As a result of this experiment, 90 skin samples were obtained for further analyses.

2.3 Fitjar

This experiment was also a part of the RFF project (nr 317935) Seaweed symbiosis. The experiment took place at Engesund's fish farm and display-centre Dyrholmen Øst in Fitjar, Norway (location number 32117, position 59,9332° N 5,239467° E) (Figure 5). The farm is located on the east side of the islet Dyrholmen, inside the Selbjørnsfjord and consists of a display fleet and six sea cages (Figure 9). Two sea cages were used in this experiment, which lasted for approximately six weeks (5.10.2020 - 10.11.2020). Two skin samplings were conducted and one microbiological sampling. Due to external limitations regarding the first sampling at Fitjar, only the second sampling containing both skin and bacteria samples were chosen to be further analysed. Fitjar is thus regarded as an experiment with one sampling date.

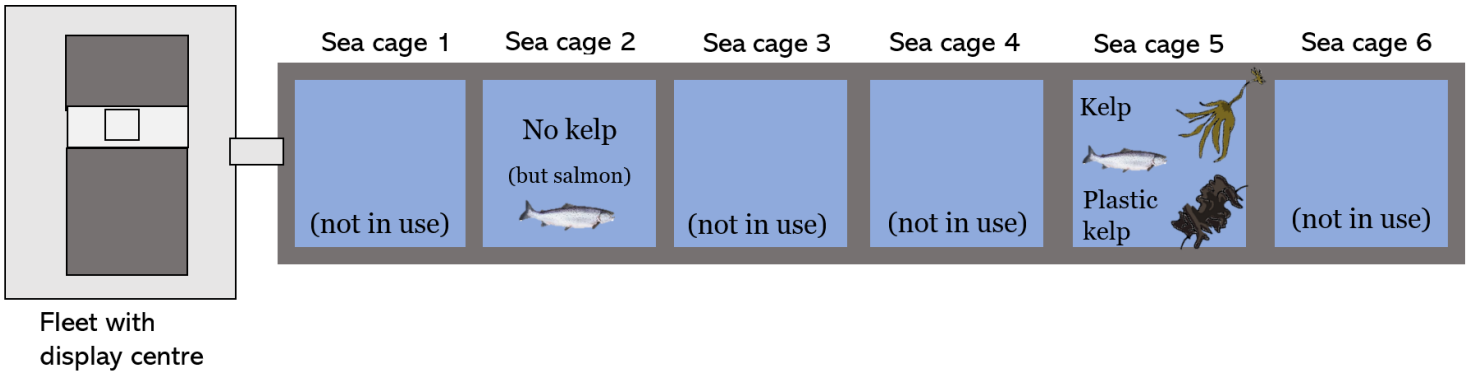

Figure 9: Experimental design and facility in Fitjar. Engesund's sea farm at Dyrholmen Øst (59,9332° N 5,239467° E) including a fleet with a display centre and six square sea cages (24x24x27m). The experimental setup consisted of the treatment "no kelp" in sea cage 2, and both treatments natural kelp (*Laminaria digitata)* and plastic kelp in adjacent corners in sea cage 5. The experiment lasted from 05.10.2020 – 10.11.2020 with one endpoint of sampling (n= 10 per treatment, N=30). The results from the experiments Agder, Austevoll and Fitjar are compiled in Figure 15.

2.3.1 Experimental design and husbandry

The experimental design consisted of two square ned pens (24 x 24 x 27 m) with a con at the bottom amounting to 17000 m^3 holding 144 000 salmon each at the time (Figure 9). The fish were exposed to natural daylight. The lumpfish was fed with Atlantic Gold SEA 2.0 (Pacific Trading Aquaculture Ltd, Dublin, Ireland), and the Atlantic salmon were fed a combination of the prescription feed Performance (Havbrukspartner, Norway) and the health feed Control Gill Q (Biomar, Norway). Throughout the experiment, the sea temperature decreased from 14.4 °C to 11.5 °C (Barentswatch, 2020). The lumpfish were deployed in the sea cages in January and September 2020. The first batch consisted of 32 000 lumpfish distributed between the cages, and the second batch (one batch younger) consisted of 15 000 lumpfish. The September lumpfish came from the same batch as those used in the experiment at Austevoll. All the lumpfish were vaccinated against *furunculosis* and *vibrio anguillarum.*

In the two net-pens, the three treatments were installed (Figure 9). The first treatment consisted of real kelp (*laminaria digitata*). The kelp used in this experiment was also harvested on the south side of Porsholmen, just north of Dyrholmen Øst near Fitjar (Figure 5 and 10). The kelp was deployed in the sea cages on the 5th of October, 2020. Twenty-eight kelp individuals were threaded onto a leaded rope and locked in place at their holdfast using cable ties (Figure 10). The individuals were placed with approximately 40 cm spacing between each other in a curtain pattern (Figure 10). The kelp-rope was then cut into nine pieces, one 18 m long rope and eight 12 m long ropes, and each piece were attached with 20-25 cm spacing onto a long rope. The rope system was then installed in the bottom right corner of net-pen number 5.

Figure 10: Harvesting and installation of the treatment real kelp (*L. digitata*). A) harvest of the kelp. B) mounting of the kelp onto the rope using cable ties. C) finished kelp-rope ready for deployment. D) Lumpfish (*C. lumpus*) attached on the natural kelp in the net-pen. Photos: Kjetil Kolbeinsvik, former employee of Engesund Oppdrett AS.

The second treatment consisted of a commercial frame cover (CleanGOOD, OKmarine, Kristiansand, Norway). This "artificial kelp" was made of black PE plastic and were 11 m deep and had a circumference of 12 m (2 x 4m + 2 x 2m). The plastic kelp was placed in the adjacent corner of the live kelp (Figure 9). The third treatment, the control, consisted of a net-pen only containing salmon and lumpfish.

2.3.2 Collection and sampling

Sampling was conducted using a stationary workspace on the fleet, scalpels, forceps and a 10 L bucket filled with seawater mixed with Benzoat Vet 200 mg/mL, according to the sampling protocol. To optimise the sampling of the different treatments and prevent cross-contamination, the rope containing kelp was first loosened and brought onto the railway (Figure 11). The ten first caught lumpfish still sucked onto the kelp or just falling off the kelp were then collected using a net and placed in the bucket containing the anaesthetics. The ten fish were then skin and microbial sampled as previously explained (Figure 12). For the second treatment, the rope containing the plastic kelp commercial frame cover (OKmarine, Kristiansand, Norway) was loosened and brought onto the railway (Figure 11). The ten first caught fish falling off or still sucked onto the plastic were sampled and put in the bucket containing anaesthetics. The fish were then sampled as previously explained (Figure 12). For the third treatment, the control, the lumpfish in a separate net-pen containing only lumpfish and Atlantic salmon, ten random lumpfish were caught by a net from the railway and placed in a bucket and anesthetised as explained.

Figure 11: The collection of lumpfish (*C. lumpus*) for sampling at Engesund's facility in Fitjar (10.11.2020). A) Collecting lumpfish from the natural kelp (*L. digitata*). B) Collecting lumpfish from the artificial kelp. C) Overview of the fish farm. Photos: Frida Sol M Svendsen.

The lumpfish caught on/from the live kelp were sampled first, then from the plastic kelp and last from the net-pen with only salmon and lumpfish. The sampling was conducted as previously explained (Figure 12).

Figure 12: Sampling procedure in the project Seaweed symbiosis, following the Quantidoc protocol. A: Lumpfish skin microbiological sampling with a cotton swab. B: Sample location on the lumpfish skin. C: Sampled lumpfish, skin piece placed in a histocassette. Photos: Frida Sol M Svendsen.

2.4 Processing / Histological preparations

After the sampling was done and the skin samples embedded in 10 % buffered formalin, they were then sent to Albert Girons at Ictiovet in Barcelona, Spain, for processing. The skin samples were embedded in paraffin and tangentially sliced into 3-µm-thick sections and then stained according to Quantidoc's mucosal mapping protocol with Periodic Acid Schiff-Alcian Blue (PAS-AB) pH 2.5 (Pittman et al., 2013, 2011). The prepared slides were then scanned by the digital slide scanner NanoZoomer 2.0-RS (Hamamatsu phototonic K.K, Hamamatsu, Japan) in order to obtain high-resolution digital images in NDPI format.

2.4.1 Gram staining

In addition to the standardised PAS-AB staining, a few selected samples were also gram stained. The samples were first sliced tangentially into 3-µm-thick sections, and the section immediately after that used for PAS-AB staining was used for Gram staining. The fish chosen for gram staining were adjusted for weight and treatment. Thus two fish per treatment from each trial (Austevoll 2 and Fitjar) were Gram-stained (n=12). This was done as a test to see if it was possible to make objective, unbiased measures of the gram-positive bacterial amount in the upper epithelial layers of the skin, using a modified mucosal mapping technique.

2.5 Mucosal mapping

2.5.1 Unbiased stereology

Mucosal mapping is a design-based, unbiased, and quantitative method of measuring and analysing stereological images of mucosal barriers (Brown, 2017; Dang et al., 2020). From all skin samples, 152 high-resolution digital images were analysed, namely 140 images from the PAS-AB-stained skin samples and 12

images from the gram-stained samples (Appendix B). Thus, 12 skin samples were used twice. Once the NDPI images were obtained, the analyses could begin. To analyse these images, Quantidoc's stereology based, semi-automatic software technology Veribarr™ and Mucomaster™ (Quantidoc, Bergen, Norway) were used for the mucosal mapping of the lumpfish skin (Pittman et al., 2013, 2011). These tools were used to estimate the mean area of mucous cells and calculate the volumetric density of the mucous cells (Equation I) in the mucosal epithelia of the skin. The defence activity, formerly described as barrier status (Dang et al., 2020), was further calculated (Equation II). A modified mucosal mapping technique was used for the gram-stained samples.

Equation I:

$$Mucous\ cell\ volumetric\ density = \frac{Mucous\ cell\ area\ x\ mucous\ cell\ number}{Epithelial\ area}$$

Equation II:

$$Defence\ activity = \frac{1}{\left(\frac{Mucous\ cell\ area}{Volumetric\ density}\right)}\ x\ 1000$$

2.5.2 Veribarr - Mucomaster

For each slide, the first thing that was done was establishing a region of interest (ROI), marking the sample area. Several randomised counting frames, dividing the sample further into 1-80% of the total ROI, were added. The number of counting frames ranged between 10 and 234 as a prediction of the number of mucous cells in the samples. The next step was marking all the individual MCs in the counting frames and marking the epithelia (figure 13).

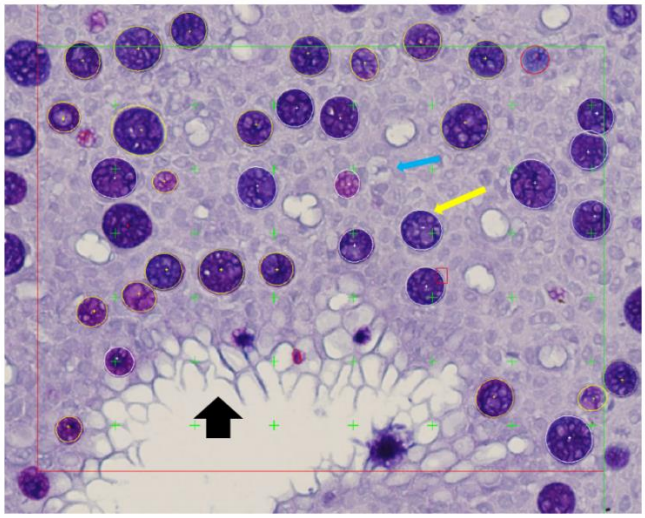

Figure 13: A mucosal mapping counting frame. Mucous cell (yellow arrow), stratified squamous epithelia (blue arrow), and the newly discovered rose petal cells (black arrow) from a digitalised skin sample displaying one counting frame (red and green lines) and counting cells (white circles) and size cells (yellow circles). PAS-AB stain, experiment Seaweed symbiosis at Austevoll (8. Sept. – 16. Oct. 2020).

Q-cells, pigment cells, ossicles, muscles, and other non-epithelial structures were not considered epithelia (Figure 14). The number of marked MCs ranged between 210- 841 with a mean of 415 counting cells (included in the mean - three samples from Agder containing merely 8, 13 and 19 cells). The average number of size cells was 96, ranging between 50 and 211 (included in the mean – the three samples containing merely 1, 3 and 4 size cells). The next step was to estimate the cells and then verify them. In this step, the AI's best-predicted circumference was chosen by the AI, then manually (Figure 13). Most of the cells were considered counting cells, and some, if they were touching a probe in the counting frame, they were considered size cells (Figure 13). This procedure was done for all 140 samples. When the analyses using Mucomaster™ were finished, the data were imported and stored in Microsoft Excel (2016).

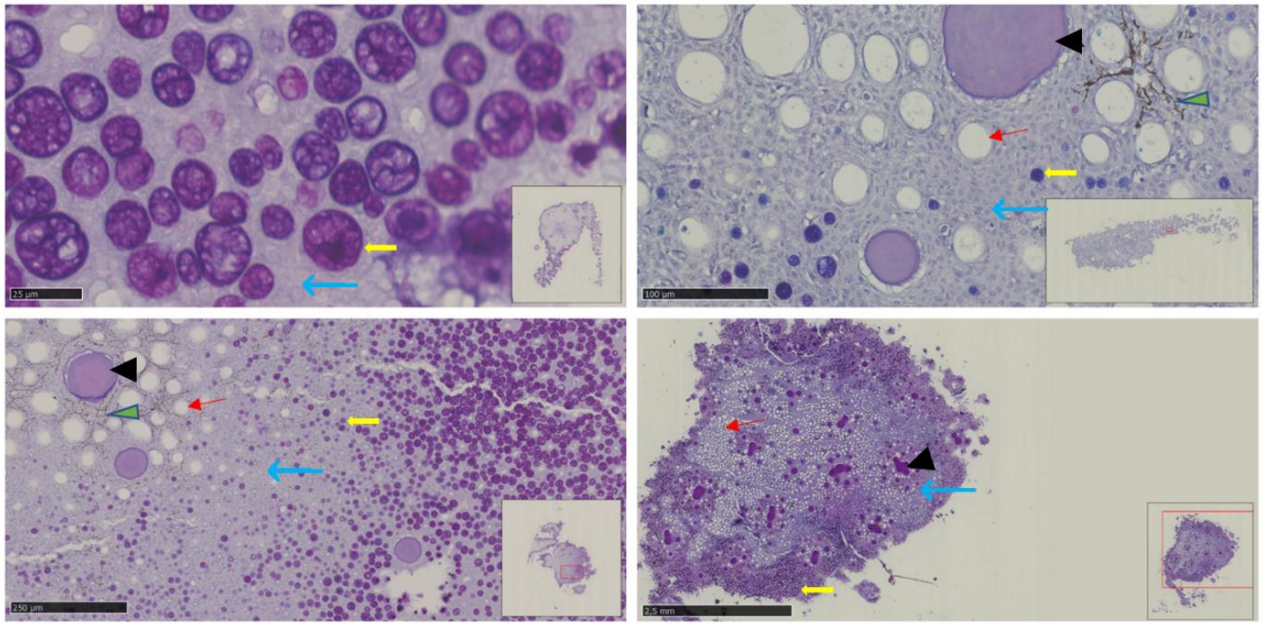

Figure 14: Different samples of tangentially sectioned lumpfish skin *(C. lumpus*) Top left: close-up of densely arranged mucous cells (scale bar: 25 µm). Top right: Example of the complexity of the lumpfish skin (scale bar:100 µm). Lower left: Dense mucous cell aggregation towards the edges of the skin (scale bar: 250 µm). Bottom right: Whole skin section (scale bar: 2,5 mm). Yellow arrows: Mucous cells, Blue arrows: Squamous epithelia, Red arrows: Q-cells, Black arrowhead: Ossicles, Blue and green arrowhead: Pigment cells (All pictures were taken with the digital application NDP.view 2, Hamamatsu phototonic K.K, Hamamatsu, Japan).

2.5.3 Modified mucosal mapping for gram-stained samples

The novel modified mucosal mapping technique for gram-stained samples was applied to twelve samples, six from Austevoll and six from Fitjar. In this method, the ROI was first marked and counting frames ranging from 29 to 50 were randomly applied, covering 2.75 – 12.61 % of the total ROI. For each counting frame, the epithelia were marked as for the normal mucosal mapping and excluded mucous cells as part of the epithelia. Thus, only what was observed as epithelia were measured. To distinguish between normal

epithelia and gram-positive affected epithelia, the probes touching these areas were marked. Each counting frame included 12 probes, amounting to twelve equally sized squares per counting frame. Hence the counting frames exclusively consisted of probes either containing normal epithelia, gram-positive epithelia or not epithelia. The epithelial area could then be divided into gram-positive or normal epithelia (Equation III-V, Figure 14b).

III) $Area\ per\ epithelial\ group = \frac{epithelial\ areal}{epithelial\ groups}$

IV) $Gram\ area = area\ per\ epithelial\ group\ x\ marked\ probes$

V) $Gram\ density\ (\%)\ of\ the\ epithelia\ = \frac{Gram\ area}{Epithelial\ area} x100$

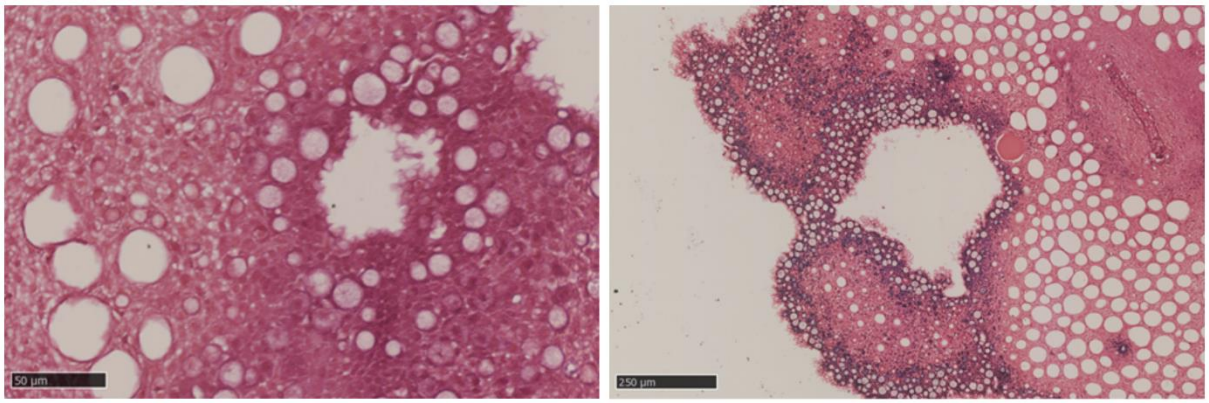

Figure 14b: Left: Normal gram-stained tangentially sliced slide. Squamous epithelia coloured red. Right: Gram-positive affected skin. Gram-positive detected bacteria coloured black, and normal epithelia coloured red, mucous cells and Q-cells have no colour (white). Digitally photographed with Hamamatsu phototonic K.K, Hamamatsu, Japan).

2.6 Microbiological processing and analysis

Microbiological samples were obtained from Austevoll (3. sampling) and Fitjar. Several research groups were involved in the project "seaweed symbiosis". Thus, the microbial part of the project was compiled and analysed by a microbiological team from the department of biological sciences at the University of Bergen. The group consisted of David John Rees (chief engineer), Stefan Thiele (post-doc) and Lotte Svengård Dahlmo (master student), led by prof. Lise Øverås. The statistical analyses regarding bacterial data in this thesis are based on Lotte Dahlmo's BIO299 project from this experiment.

Although first, gene sequences of the archaea and bacterial community from the samples were constructed using the Ion torrent technology (Thermo Fischer) for DNA extraction, amplification, and 16S rRNA sequencing. By analysing the assembled gene sequences in the R package DADA2 (DADA2, version 1.18, R3.6.1), the number of amplicon sequence variants (ASVs) were mapped out, and the Relative abundance (RA) calculated. In the dataset, some ASVs were mapped down to genus level and some only to class level. The degree of accuracy in covering relative abundance is therefore not 100 % but varies. In addition to the analyses of the total bacterial community, a selection of ASVs representing known pathogenic or pathogen-including species and genera were extracted from the dataset and analysed to more extent. Some ASVs coded for a single species, and some species had multiple ASVs, and some ASVs only led to pathogenic or pathogen-including genera.

The samples analysed were:
Austevoll: Ten skin samples from lumpfish from the treatment with natural kelp, nine skin samples from the treatment with plastic kelp and ten skin samples from the control. Additionally, seven samples from the treatment natural kelp, nine samples from the treatment plastic kelp and two samples from the treatment no kelp (tank walls). In Fitjar, ten skin samples from lumpfish found on the natural kelp, eight skin samples from lumpfish found on the plastic kelp, and ten skin samples from lumpfish with the treatment no kelp were analysed. In addition, five microbiological samples from the real kelp and four microbiological samples from the plastic kelp were analysed.

After obtaining the microbial raw data, the relative abundance of the bacteria was tested against the mucous cell mean area, mucous cell volumetric density, and defence activity in all the samples analysed from both Austevoll and Fitjar was calculated. Additionally, the relative abundance of the most prominent classes was calculated. In Austevoll, eight classes, nine families, 14 genera and four species were tested (Table 1). In Fitjar, seven classes, six families, 12 genera and eight species were tested (Table 1).

Table 1: The Bacterial classes, families, genera and species analysed in this thesis. The relative abundance of each taxonomic unit was tested against mucous cell mean area, volumetric density and defence activity for correlation in Austevoll (3. sampling) and Fitjar. The raw data was received from Lotte Dahlmo.

Taxonomic unit	**Austevoll (16.10.2020)**	**taxonomic unit**	**Fitjar (10.11.2020)**
class	Alphaproteobacteria	class	Alphaproteobacteria
class	Bacilli	class	Bacilli
class	Bacteroidia	class	Bacteroidia
class	Campylobacteria	class	Campylobacteria
class	Gammaproteobacteria	class	Gammaproteobacteria
class	Planctomycetes	class	Planctomycetes
5class	Polyangia	class	Verrucomicrobiae
class	Verrucomicrobiae		
family	Alteromonadaceae	family	Arcobacteraceae
family	Cellvibrionaceae	family	Burkholderiales_Incertae_Sedis
family	Flavobacteriaceae	family	Cryomorphaceae
family	Methylophagaceae	family	Flavobacteriaceae
family	Moraxellaceae	family	Saprospiraceae
family	Pseudoalteromonadaceae	family	Vibrionaceae
family	Rhodobacteraceae		
family	Saprospiraceae		
family	Vibrionaceae		
genus	Aliivibrio	genus	Aliiroseovarius
genus	Aureispira	genus	Aliivibrio
genus	Dokdonia	genus	Candidatus_Branchiomonas
genus	Litoreibacter	genus	Colwellia
genus	Maribacter	genus	Dokdonia
genus	Micrococcus	genus	Francisella
genus	Moritella	genus	Halarcobacter
genus	Paraglaciecola	genus	Moritella
genus	Paraperlucidibaca	genus	Pseudoalteromonas
genus	Polaribacter	genus	Psychrobacter
genus	Sulfitobacter	genus	Sulfitobacter
genus	Tenacibaculum	genus	Tenacibaculum
genus	Ulvibacter	genus	Vibrio
genus	Vibrio		
species	A.logei	species	A.wodanis
species	P.chitinilyticum	species	P.salmonis
species	T.maritimum	species	T.finnmarkense
species	T.soleae	species	T.maritimum
		species	V.kanaloae
		species	V.metschnikovii
		species	V.tapetis

2.7 Statistical analyses

The compilation of datasets, analyses, tables and graphs were made in Microsoft Excel (2016) and R version 4.0.4 (R core team 2021, Vienna, Austria) using RStudio version 1.4.1106 (RStudio Team, 2021, Boston, MA, USA). The package used was Tidyverse (Wickham et al., 2019).

A one-way analysis of variance (ANOVA) using a nested linear mixed-effects model (lme) with a single categorical predictor was applied to compare the differences between the means in the different experiments and between the different treatment groups regarding lumpfish size, mucous cell mean area, volumetric density and defence activity. Statistical significance was set to $p < 0.05$. Q-Q plots were applied to test for normality and homogeneity. Q-Q plots were applied to the variables mean area, volumetric density, defence activity, weight and length (Appendix F).

Correlation between the bacterial relative abundance and the mucous cell measurements was tested using a linear regression model (lm). The coefficient of determination (R^2) was then applied to see how well the model fit the data. A statistically significant correlation was set to $R^2 > 0.6$.

3 Results

The aim of this study was to see the range of and how the mucous cells in the epidermis of the skin of *Cyclopterus lumpus* act in land-based rearing tanks and open sea cages, with three different treatments: real kelp, plastic kelp and without kelp.

3.1 Growth

The total length (cm) was measured for all treatment groups and experiments, and the weight (g) was measured for all treatment groups in experiment Austevoll and Fitjar, excluding Agder.

3.1.1 Agder

In this experiment, two treatments were used, real kelp and no kelp. 20 fish were sampled. There were no mortalities beyond sampling. From the measured *C. lumpus,* the final length was 8.9 ± 0.6 cm and 9.2 ± 0.4 cm (Mean ± SEM) for the treatments with kelp and without kelp, respectively (Table 2). No weight measurements were conducted but comparing the length measures with the first sampling in Austevoll, the lumpfish was estimated to weigh approximately 30 grams.

3.1.2 Austevoll

In Austevoll, three treatments were applied, and the experiment lasted for six weeks. 90 of 600 lumpfish distributed in six tanks were sampled, and there were no mortalities beyond sampling. At the first sampling, the initial measurements were 8.6 ± 0.6 cm and 28.7 ± 5.2 g (Mean ± SEM) for length and weight, respectively (Table 2). There was no significant difference ($p>0.05$) between the initial measurements between the treatments (8.5 ± 0.6 cm and 27.1 ± 5.2 g, 8.9 ± 0.6 cm, 31.6 ± 5.3g, 8.5 ± 0.4cm, 27.4 ± 4.1g for the treatments with kelp, without kelp and plastic kelp, respectively, Table 2). The means at the second sampling was 10.4 ± 0.6 cm, 10.2 ± 0.6cm and 10.1 ± 1.2cm for length, and 59.1 ± 9.7g, 59.2 ± 12.4 g, and 55.2 ± 1.,5g for weight, for the treatments real kelp, no kelp and plastic kelp, respectively (Table 2). The final length and weight measurements for the treatment groups were 11.8 ± 0.7 cm and 82.1 ± 13.5 g for the treatment real kelp, 11.2 ± 1.0 cm and 73.7 ± 14.4 g for the treatment no kelp, and 11.6 ± 0.8 cm and 81.0 ± 12.6 g for the treatment plastic kelp (Table 2). The was no significant difference in length or weight between the treatments in the final sampling ($p > 0.05$).

3.1.3 Fitjar

The third experiment constituted of 30 sampled lumpfish in three treatments over six weeks. Due to the fact this is a commercial farm, it was difficult to make assumptions about the total amount of lumpfish and, consequently, the mortality. In Fitjar, the mean weight and length for the lumpfish found on/near the real kelp, *L. digitata* was 12.5 ± 1.4 cm and 76.0 ± 18.0 g (Mean ± SEM) (Table 2). The fish located on or near the plastic kelp were 12.2 ± 1.9 cm long and weighed 74.0 ± 29.4 g (Table 2). The lumpfish in the net-pen with no kelp were 11.3 ± 0.6 cm long and weighed 64.5 ± 8.0g (Table 2). The difference in length between the sea cages were approaching significance ($p = 0.0675$), but there was no significant difference in weight ($p > 0.05$).

Table 2: Length and weight measurements of the lumpfish (c. lumpus) from Agder, Austevoll and Fitjar. The mean per treatment and sampling was calculated (Mean ± SEM).

Location (date)	Mean per location		Mean per Treatment	Length (cm)	Weight (g)
	Length (cm)	Weight (g)			
Agder (14.07.2020)	9.0	Ca. 30 g	Natural kelp	8.9 ± 0.6	Ca. 30
			Without kelp	9.2 ± 0.4	Ca. 30
Austevoll 1. sampling (08.09.2020)	8.6 ± 0.6	28.7 ± 5.2	Natural kelp	8.5 ± 0.6	27.1 ± 5.2
			Without kelp	8.9 ± 0.6	31.6 ± 5.3
			Plastic kelp	8.5 ± 0.4	27.4 ± 4.1
Austevoll 2. sampling (29.09.2020)	10.2 ± 0.8	57.8 ± 11.4	Natural kelp	1.4 ± 0.6	59.1 ± 9.7
			Without kelp	10.2 ± 0.6	59.2 ± 12.4
			Plastic kelp	10.1 ± 1.2	55.2 ± 12.5
Austevoll 3. sampling (16.10.2020)	11.5 ± 0.9	78.9 ± 13.6	Natural kelp	11.8 ± 0.7	82.1 ± 13.5
			Without kelp	11.2 ± 1.0	73.7 ± 14.4
			Plastic kelp	11.6 ± 0.8	81.0 ± 12.6
Fitjar (10.11.2020)	12.0 ± 1.5	71.0 ± 20.3	Natural kelp	12.5 ± 1.4	76.0 ± 18.0
			Without kelp	11.3 ± 0.6	64.5 ± 8.0
			Plastic kelp	12.2 ± 1.9	74.0 ± 29.4

3.2 Mucous cell mean area, volumetric density and defence activity

Subsequential to the sampling and processing of the skin, the mean area (MA), volumetric density (VD) and defence activity (DA) of the mucous cells were computed. The units were calculated for each sample (n=140), and the mean per treatment per sampling per experiment is further the applied entity of comparison.

3.2.1 Mucous cells mean area

Agder: When looking at all experiments, Agder had the lowest mucous cells mean area (MA) with a mean of 110.006 ± 21.897 μm^2 for the treatment real kelp, and 114.540 ± 38.746 μm^2 for the treatment with no kelp (Mean ± SEM) (Figure 15A). There was no significant difference regarding mucous cell MA between the treatments in this experiment (p=0.8797).

Austevoll 1. sampling: The treatment real kelp had the smallest MA (122.114 ± 27.114 μm^2), and the mucous cells from the plastic kelp treatment had a mean of 128.438 ± 15.538 μm^2 (Figure 15B). The mucous cells with the largest MA in this sampling were from the treatment with no kelp (133.372 ± 21.569 μm^2, Mean ± SEM). There were no significant differences between the treatment real kelp and the treatment plastic kelp (p=0.739), the treatment real kelp and no kelp (p=0.589), or the treatment no kelp and plastic kelp (p=0.6167).

Austevoll 2. sampling: In the second sampling at Austevoll the treatment without kelp had the smallest MA, and the plastic kelp had the largest (Figure 15B). In the order of real kelp, no kelp and plastic kelp, the MA from the second sampling were 169.421 ± 23.820 μm^2, 153.627 ± 24.169 μm^2 and 170.431 ± 23.666 μm^2 (Mean ± SEM) (Figure 15B). There were no significant differences between the treatments real kelp and plastic kelp (p=0.9329), real kelp and no kelp (p=0.2789) or the treatments plastic kelp and no kelp (p=0.2568).

Austevoll 3. sampling: The largest MA when comparing all experiments was found in the treatment without kelp with a mean of 185.286 ± 33.286 μm^2 (Figure 15B). The second-largest MA was also from the third sampling at Austevoll and from the treatment real kelp, with a mean area of 176.962 ± 32.616 μm^2 (Figure 15B). The lowest mean area in this sampling was from the treatment plastic kelp (169.469 ± 21.380 μm^2, mean ± SEM) (Figure B). No significant differences were found between the treatments real kelp and plastic kelp (p=0.6423), real kelp and no kelp (p=0.7137) or plastic kelp and no kelp (p=0.385).

Fitjar: The mucous cell mean area was 139.422 ± 31.700 μm^2 for the treatment of real kelp, 138.176 ± 25.534 μm^2 for the treatment of plastic kelp, and 167.911 ± 28.412 μm^2 for the treatment of no kelp (mean

± SEM) (Figure 15C). When comparing the sea cages, there was a significant difference in mucous cell MA between the sea cage with no kelp and the sea cage with natural and plastic kelp (p= 0.0126) (Figure 16C).

3.2.2 Volumetric density

The volumetric density (VD) is the percentage of the mucosal epithelia filled with mucous cells (Equation I).

Agder: The volumetric density was 7.17 % for the treatment with kelp and 4.98 % for the treatment with no kelp (Figure 15D). There was no significant difference between the treatments (p=0.6002).

Austevoll 1. sampling: In the first sampling at Austevoll, the volumetric density per treatment was 6.32 % (real kelp), 5.25% (no kelp) and 6.33 % (plastic kelp) (Figure 15E). There was no significant difference between the treatment real kelp and plastic kelp (p=0.9984), real kelp and no kelp (p=0.7263) or plastic kelp and no kelp (p=0.6344).

Austevoll 2. sampling: In the second sampling, the mucous cell volumetric density increased for the treatment plastic kelp (10.98%) but decreased for the treatment real kelp (5.07%) and no kelp (4.60%) (Figure 15E). The difference between the treatments was not significant (p=0.4091 between the treatment real kelp and plastic kelp, p=0.8699 between the treatment real kelp and no kelp, and p=0.3513 between the treatment plastic kelp and no kelp).

Austevoll 3. sampling: In the third sampling at Austevoll, the volumetric density increased to 12.47% in the treatment plastic kelp and increased to 11.76% for the lumpfish in the treatment with real kelp, and to 11.14 % for the lumpfish without kelp (Figure 15E). No significant differences between real kelp and plastic kelp (p=0.8842), real kelp and no kelp (p=0.9226), or plastic kelp and no kelp (p=0.7585) were found.

Fitjar: the highest volumetric density was measured in Fitjar in the treatment real kelp with 17.38% (Figure 15F). In the treatment with plastic kelp, the density was 13.49 %, and in the treatment with no kelp, 10.27% (Figure 15E). When comparing the cages, there was a significant difference (p=0.0331) in volumetric density between the lumpfish in cage 2 (no kelp) and cage 5 (real kelp and plastic kelp) (Figure 16F).

3.2.3 Defence activity

Defence activity (DA) is an objective, quantitative measure of barriers. It is based on the relationship between the mean area and volumetric density of the mucous cells in the mucosal epithelia (Equation II).

Agder: The defence activity was higher (0.600) for the treatment real kelp and lower without kelp (0.361) (Figure 15G), but the difference between the treatments was not significant (p=0.4358).

Austevoll 1. sampling: In the first sampling at Austevoll, the mean defence activities were 0.459 (real kelp), 0.373 (no kelp) and 0.466 (plastic kelp) (Figure 15H). There was no significant difference between the treatments real kelp and plastic kelp (p=0.9655), real kelp and no kelp (p=0.6033), or plastic kelp and no kelp (p=0.5034).

Austevoll 2. sampling: In the second sampling, the defence activity increased for the treatment plastic kelp (0.595), but as for the volumetric density, it decreased for the treatment real kelp (0.277) and no kelp (0.282) (Figure 15H). There was no significant difference in defence activity between the treatment real kelp and plastic kelp (p=0.3955), real kelp and no kelp (p=0.9659), or plastic kelp and no kelp (p=0.3769).

Austevoll 3. sampling: In the third sampling, the defence activity increased for the treatment with plastic kelp (0.710) and also increased for the treatment real kelp (0.627) and the treatment no kelp (0.562) (Figure 15H). Nor here was there any significant difference between the treatments. p=0.7283 between the treatment real kelp and plastic kelp, p=0. 0.8251 between the treatment real kelp and no kelp, and p=0.4737 between the treatment plastic kelp and no kelp.

Fitjar: The highest mean defence activity throughout the experiments was found in the treatment with real kelp in Fitjar (1.225) (Figure 15I). The second-highest defence activity was also found in Fitjar in the treatment with plastic kelp (0.980) and the defence activity in the no kelp treatment at Fitjar was 0.603 (Figure 15I). When comparing sea-cages there was a significant difference in defence activity between the sea cage with no kelp, and the sea cage with both real and plastic kelp ($p<0.005$) (Figure 16I).

3.2.4 MA, VD and DA per treatment per sampling at Austevoll

Austevoll - Treatment: Real kelp

Mean area: In the treatment real kelp, a significant difference between the mucous cell mean area from day 2 to day 23 ($p<0.005$) and day 2 and day 40 ($p<0.005$) was found, but there was no significance between day 23 and day 40 (p=0.5621).
Volumetric density: The difference in volumetric density from day 2 to day 23 was not significant (p=0.6717). However, the difference was approaching significance between day 2 and day 40 (p=0.0796). The difference in VD from day 23 to day 40 was significant (p=0.0344).

Defence activity: The difference in Defence activity between day 2 and day 23 was not significant (p=0.2330), nor between day 2 and day 40 (p=0.2709). However, the difference in DA from day 23 to day 40 was significant (p=0.0154).

Austevoll - Treatment: No kelp

Mean area: In the treatment with no kelp, the difference in MA from day 2 to day 23 was approaching significance (p= 0.0592), and a significant difference was observed between day 2 and day 40 ($p<0.005$) and between day 23 and day 40 (p=0.02).

Volumetric density: There was no significant difference between day 2 and day 23 (p=0.7065). however, there was a significant difference in VD between day 2 and day 40 (p=0.0319), and between day 23 and day 40 (p=0.0190).

Defence activity: There was no significant difference in defence activity in the treatment no kelp between day 2 and day 23 (p=0.3435), or between day 2 and day 40 (p=0.1167), but a significant difference was found between day 23 and day 40 (p=0.0215).

Austevoll - Treatment: Plastic kelp

Mean area: In the treatment with plastic kelp, a significant difference in mucous cell mean area between day 2 and 23 ($p<0.005$) and day 2 and day 40 ($p<0.005$) was found, but there was no significant difference between day 23 and 40 (p=0.9251).

Volumetric density: There was no significant difference in volumetric density between day 2 and day 23 (p=0.3095). The difference between day 2 and day 40 however was significant (p = 0.0498), but the difference between day 23 and day 40 was not significant (p=0.7613).

Defence activity: There was no significant difference in defence activity between the samplings ((p=0.5476 between day 2 and day 23, p =0.1379 between day 2 and day 40 and p=0.6177 between day 23 and day 40).

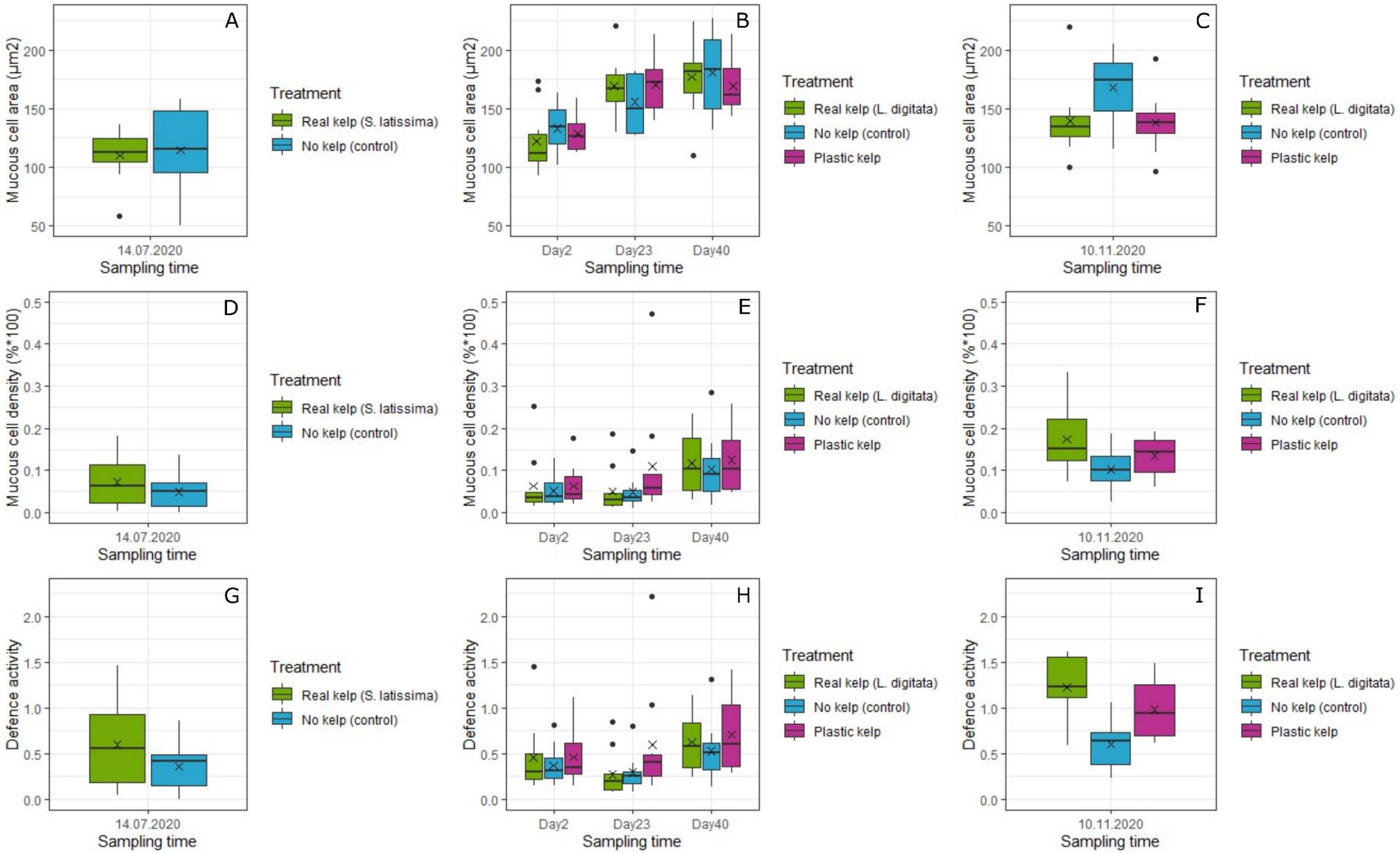

Figure 15: Mean mucous cell mean area (A-C), Mean mucous cell volumetric density (D-F) and mean defence activity (G-H) per treatment per sampling of *Cyclopterus lumpus* skin from the experiments Agder, Austevoll and Fitjar. All samples were lethal (n per treatment = 10, total N=140). The cross represents the mean, the bar represents the median, the upper and lower rang of the boxes represents the 1st and 3rd Quartile, the vertical bars represent the upper and lower whiskers, and the dots represents outliers. The colour green represents the treatment with real kelp (*Saccharina latissima* or *Laminaria digitata*), the blue represents the treatment with no kelp and the magenta represents the treatment with plastic kelp (PE plastic, commercial hides).
15A, 15D and 15G: Agder (14.07.202). 15B, 15D and 15H: Austevoll (day2: 08.09.2020, day23: 29.09.2020 and day 40: 16.10.2020). 15C, 15F and 15I: Fitjar (10.11.2020).

3.2.5 Ranges of mean area, volumetric density, and defence activity

Because of differences in experimental design and setup, it was not possible to directly compare the results from the different experiments. Still, it was possible to look at the ranges exhibited by the lumpfish reared in the different environments, from the experiments Agder, Austevoll and Fitjar, and within each experiment (Table 3).

Table 3: The ranges of mucous cell mean area, volumetric density and defence activity found in experiment Agder, Austevoll and Fitjar.

	Limits	Mean area (μm^2)	Volumetric density (%)	Defence activity
· Total range	· Low	· 50,266	· 0,0237	· 0,004
	· Mean	· 148,513	· 9,1	· 0,580
	· High	· 226,741	· 47,1	· 2,212
Agder	Low	50,265	0,0237	0,004
	mean	112,273	6,1	0,48
	High	158,197	18,2	1,456
Austevoll 1	Low	92,643	1,6	0,147
	Mean	127,975	6,0	0,433
	High	173,464	25,3	1,456
Austevoll 2	Low	126,782	1,1	0,081
	Mean	164,493	6,9	0,385
	High	220,912	47,1	2,212
Austevoll 3	Low	109,925	1,9	0,141
	Mean	177,239	11,8	0,633
	High	226,741	28,6	1,417
Fitjar	Low	96,708	2,7	0,23
	Mean	148,503	13,7	0,936
	High	219,951	33,2	1,611

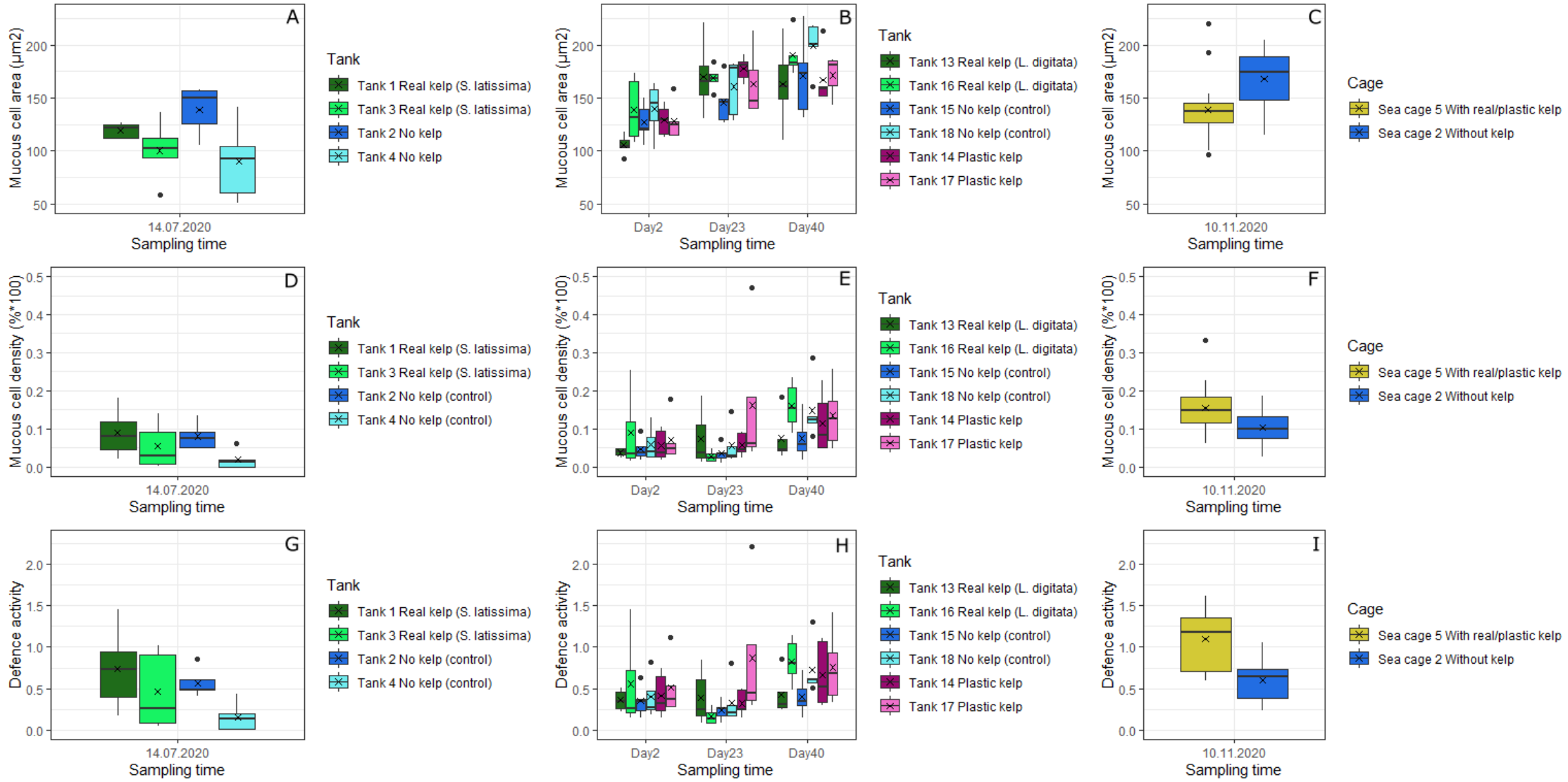

Figure 16: Mean mucous cell mean area (A-C), Mean mucous cell volumetric density (D-F) and mean defence activity (G-H) per tank/ sea cage treatment per sampling of *Cyclopterus lumpus* skin epithelia from the experiments Agder, Austevoll and Fitjar. All samples were lethal (n per tank = 5, n per treatment = 10, total N=140). The light and dark greens represents the treatment with real kelp (*Saccharina latissima* or *Laminaria digitata*), the light and dark blues represents the treatment with no kelp, the purple and magenta represents the treatment with plastic kelp (PE plastic, commercial hides), and the yellow represents the combination of real and plastic kelp.
16A, 16D and 16G: Agder (14.07.202). 16B, 16D and 16H: Austevoll (day2: 08.09.2020, day23: 29.09.2020 and day 40: 16.10.2020). 16C, 16F and 16I: Fitjar (10.11.2020).

There were no correlations between length and mucous cell mean area, volumetric density or defence activity when looking at Agder, Austevoll and Fitjar combined (Figure 17-19). Additional calculations where no trend was found can be found in appendix C.

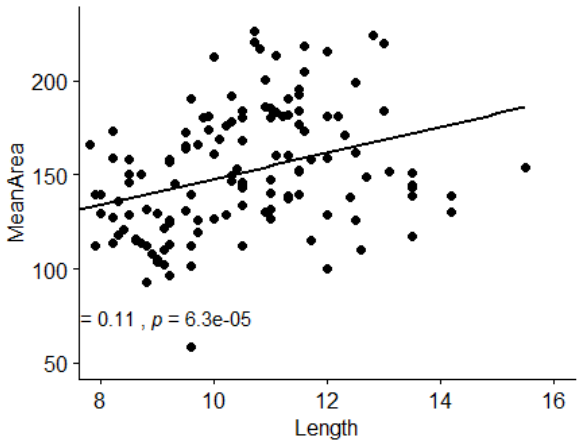

Figure 17: Mucous cells mean area versus length combining experiment Agder, Austevoll and Fitjar (Six lumpfish from Agder were not measured, n=134).

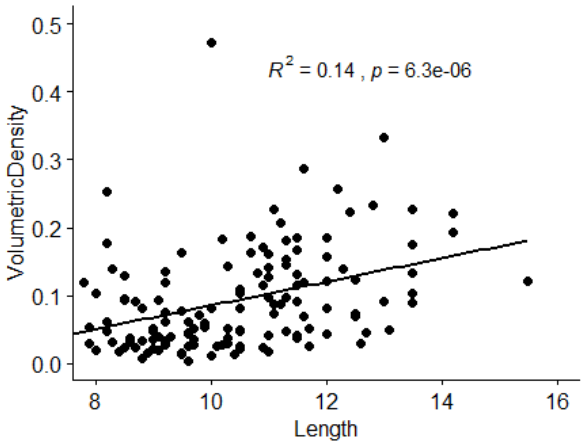

Figure 18: Mucous cells volumetric density versus length from experiment Agder, Austevoll and Fitjar (Six lumpfish from Agder were not measured, n=134).

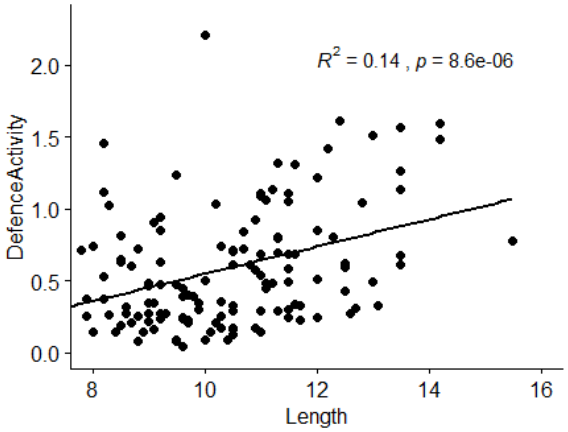

Figure 19: Defence activity versus length from experiment Agder, Austevoll and Fitjar (Six lumpfish from Agder were not measured, n=134).

3.3 Novel mucosal mapping of Gram-stained samples

The novel method of mucosal mapping of Gram-stained lumpfish skin was applied to six samples from the experiment at Austevoll and six samples from the experiment in Fitjar (Figure 20). The gram-positive epithelia were not size-dependent (Figure 21).

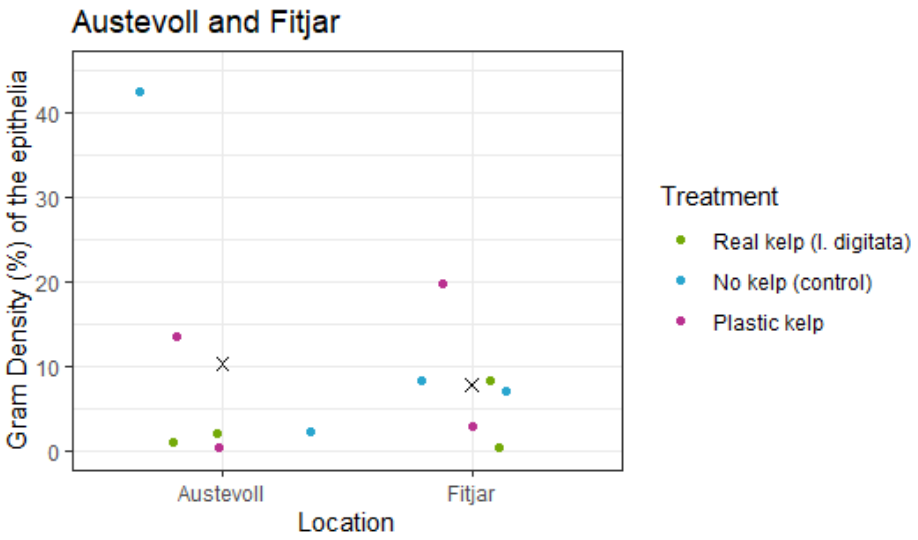

Figure 20: Gram density (%) of the epithelia that were gram positive (gram-stain), in experiment 2, 3. sampling (Austevoll) and experiment 3 (Fitjar).

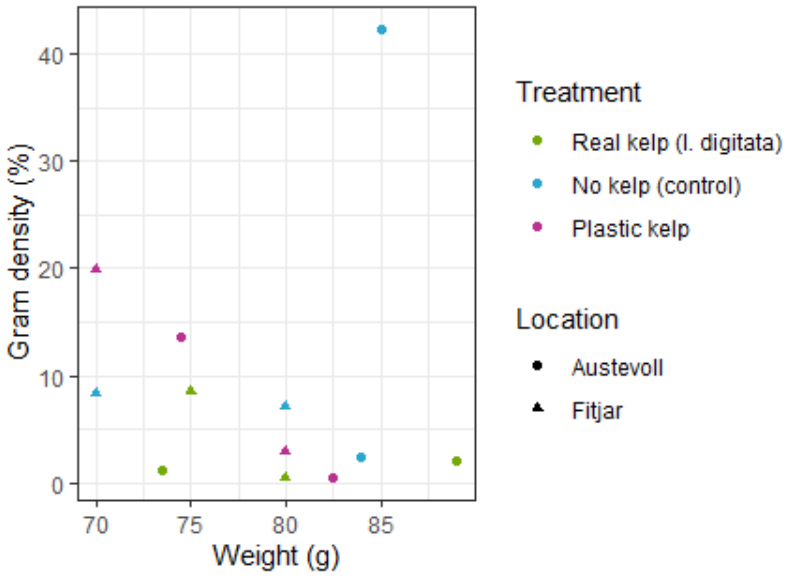

Figure 21: Gram-density (%) of the epithelia that were gram-positive (gram-stain) versus weight (g), in Austevoll 3. Sampling and Fitjar.

3.4 Microbiological results

Microbiological samples were obtained from Austevoll (3. Sampling) and Fitjar. The microbiological team obtained these data. Each sample was analysed, and the amount of amplicon sequence variants (ASV) in the samples were found. For each processed sample, the taxa's relative abundance (RA) comprising the bacterial community was calculated.

3.4.1 Disease-inducing/ pathogenic bacteria in Austevoll

From the analysis of a selection of known pathogenic species or pathogen-including genera, the relative abundance was calculated for 12 species and 11 genera (Figure 22). The most abundant pathogenic bacteria on the fish skin was *Aliviibrio logei*. in one specimen, there was a higher abundance of the bacteria *Photobacter chitinilyticum* (Figure 22).

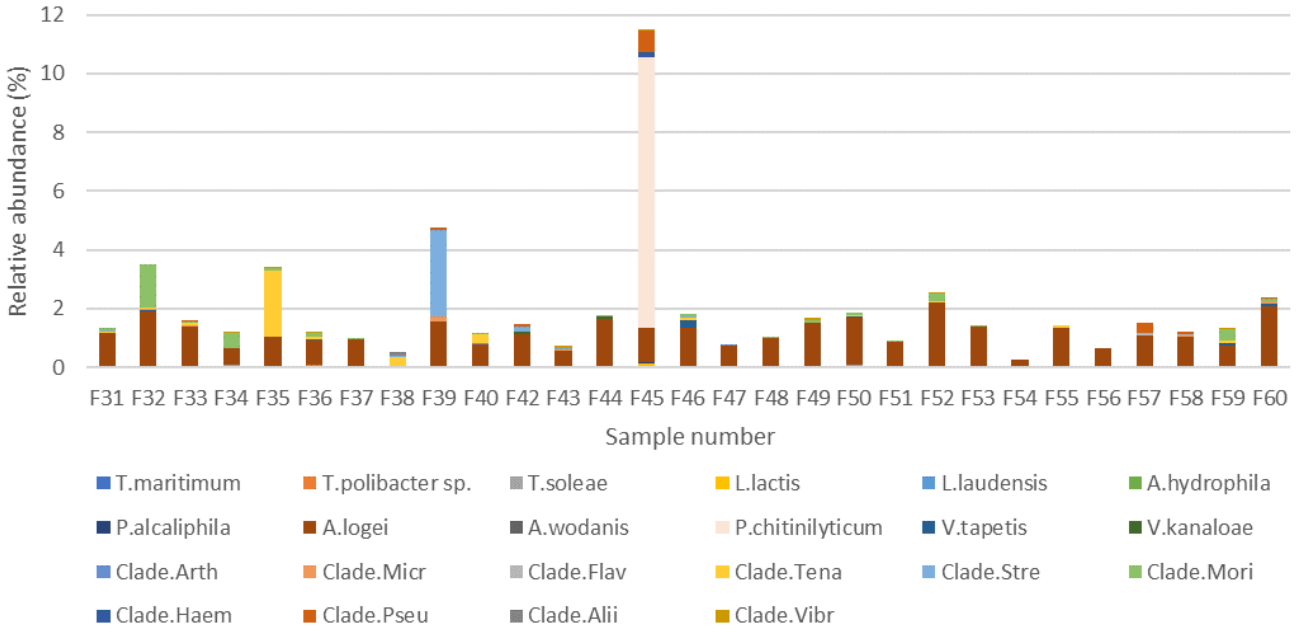

Figure 22: Accumulated relative abundance of a selection of pathogenic species and genera on the lumpfish skin, from the different treatment real kelp, plastic kelp, and tank walls in Austevoll (08. Sept. - 16. Oct. 2020). Sample F31 – F40 are from fish reared with real kelp (*Laminaria digitata*), F42 – F50 are from lumpfish reared with plastic kelp (PE plastic sheet) and F51 – F60 are from the lumpfish with no kelp.

In Austevoll, two pathogenic bacteria and six genera were found on the treatments real kelp (tank 13 and 16), plastic kelp (tank 14 and 17), and tank walls from the treatment no kelp (Tank 15 and 18). The number of pathogenic species or genera on the treatments was very low and did not exceed 1.4 % of total relative

abundance (Figure 23). The most abundant pathogenic genus was *Moritella* (highest RA in one sample was in T54 with 0.58 %).

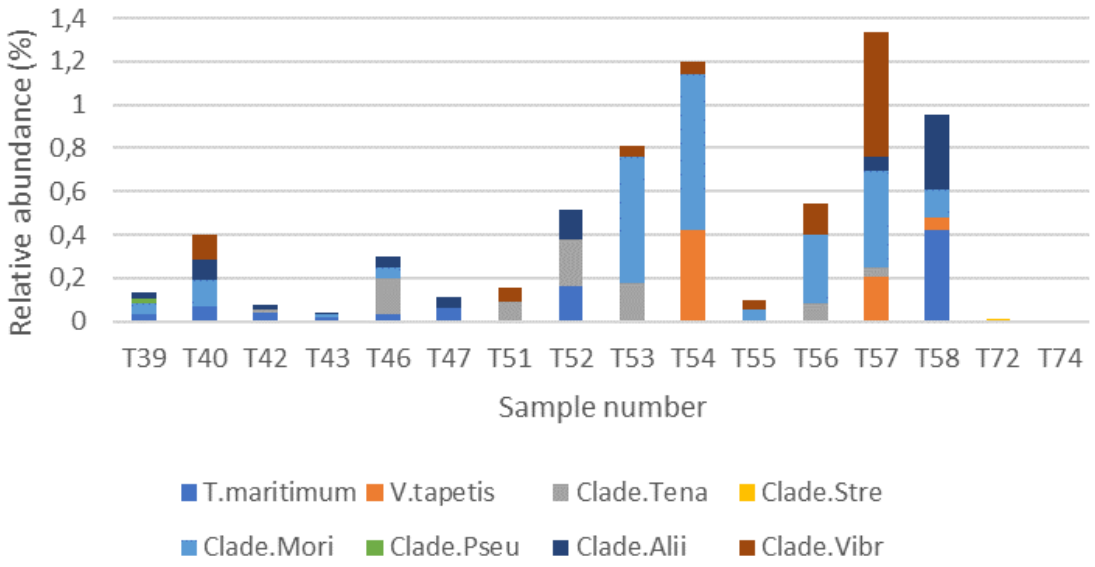

Figure 23: Accumulated relative abundance of a selection of pathogenic species and genera on the different treatment real kelp, plastic kelp and tank walls in Austevoll. Sample T39 – T47 are from the treatment real kelp (*laminaria digitata*), T51 – T58 are from the plastic kelp (PE plastic sheet), and T72 and T74 are from the tank walls with no kelp.

3.4.2 Total bacterial community in Austevoll

In Austevoll, the relative abundance of seven classes, nine families, fourteen genera and four species were analysed against mucous cell area, volumetric density and defence activity to check for correlation (Appendix D).

The bacterial community consisted of *Alphaproteobacteria, Bacilli, Bacteroidia, Gammaproteobacteria, Planctomycetes, Campylobacteria* and *Verrucomicrobiae* (86.8 – 99.0 %) (Figure 24). In both treatment samples and skin samples, *Alphaproteobacteria, Gammaproteobacteria and Bacteroidia* represent most of the community. In the skin samples, the relative abundance of *Alphaproteobacteria* was 15.8±3.9 %, *Gammaproteobacteria* 61.6±10.7 % and *Bacteroidia* 17.8 ±6.3 % (Mean ± SEM). In the treatment samples, the relative abundance of *Alphaproteobacteria* was 24.1±5.2 %, *Gammaproteobacteria* 34.8±6.6 % and *Bacteroidia* 30.9 ±10.3 % (Mean ± SEM). The class *Planctomycetes* had a mean RA of 10±5.6 % from the real kelp treatment samples, while in the treatments no kelp and plastic kelp the RA was <1 % (Figure 24). The skin samples from the treatment with real kelp had a mean of 0.75%. There were no treatment samples with a RA >1% of the class *Bacillus*, but the class was represented in the skin samples from the treatments real kelp and plastic kelp (Figure 24).

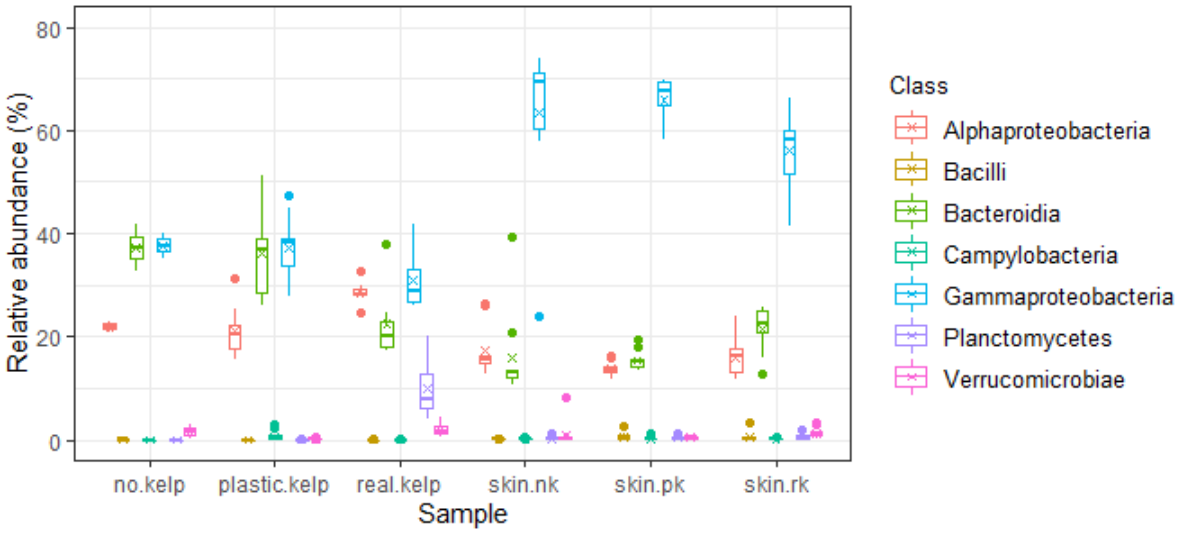

Figure 24: Relative abundance (%) of the bacterial classes *Alphaproteobacteria* (red), *Bacilli* (orange), *Bacteroidia* (lime), *Campylobacteria* (turquoise), *Gammaproteobacteria* (Blue), *Planctomycetes* (purple) and *Verrucomicrobiae* (pink), per sample. The different samples were, from left to right, tank walls (n=2), plastic kelp (n= 9), real kelp (*L. digitata*) (n=7), lumpfish skin from the treatment without kelp (n=10), skin from the treatment with plastic kelp (n=9) and skin from the treatment with real kelp (10). All classes include at least one sample where RA >1%.

The most abundant genus on the lumpfish skin in Austevoll was *Paraperlucidibaca* (family *Moraxellaceae,* class *Gammaproteobacteria*, phylum *Proteobacteria (Appendix D).* Mean RA was 31.4 % in the treatment real kelp, 35.0 % in the treatment plastic kelp and 39.2 % in the treatment no kelp. The families analysed represents 61.7 – 84.2 % RA of the bacterial community and the genera analysed represent 28.5 – 71.6 % RA, and the species analysed represents 0 – 10.4 % of the bacterial community. The low coverage of species is due to the fact that the level of pathogenic species was low. No other ASVs were analysed to species level.

3.4.3 Disease-inducing/ pathogenic bacteria in Fitjar

In Fitjar, thirteen species and nine genera were pathogenic or pathogen-including (Figure 25). Here the species that dominated the bacterial community on the skin, even across the treatments, was the pathogen *Tenacibaculum maritimum* (Figure 25). When combining all ASVs representing the genus Tenacibaculum, the mean relative abundance was 34%, 38 % and 46 % for the treatments plastic kelp, real kelp, and no kelp, respectively. The second most abundant group of bacteria was the genus *Aliivibrio*, also a pathogen including genus (Figure 25).

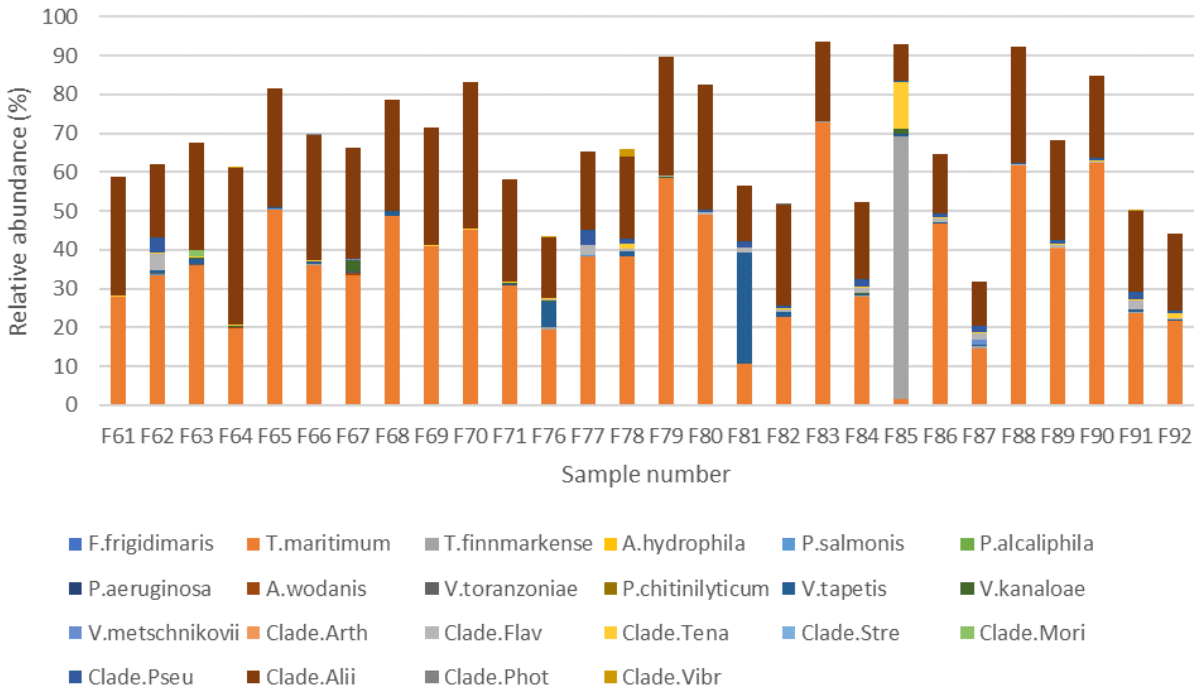

Figure 25: Accumulated relative abundance of a selection of pathogenic species and genera on the fish skin from the different treatments real kelp, plastic kelp and no kelp in Fitjar. Sample F61 – 70 are from the real kelp (*Laminaria digitata*), sample F71 – F82 are from the treatment with plastic kelp (PE plastic sheet) and sample F83 – 92 are from the treatment without kelp.

The amount of pathogenic or pathogen-containing species and genera on the treatments in Fitjar (Real kelp and plastic kelp) did not exceed 3 % combined relative abundance (Figure 26). The most abundant was the genus *Tenacibaculum* (the highest RA in one sample was in T79 with 1.33 %).

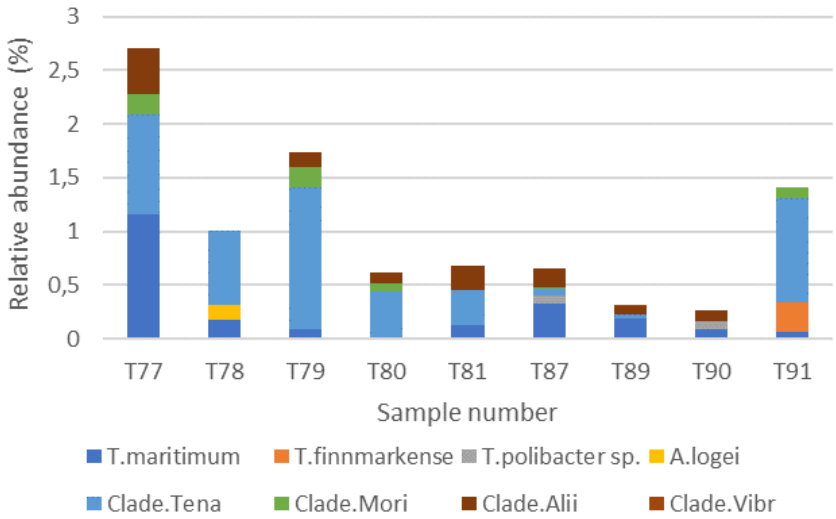

Figure 26: Accumulated relative abundance of a selection of pathogenic species and genera on the different treatment real kelp and plastic kelp in Fitjar. Sample T77 – T81 are from the real kelp (*Laminaria digitata*), and sample T87 – T91 are from the plastic kelp (PE plastic sheet).

3.4.4 Total bacterial community in Fitjar

In Fitjar, seven classes, six families, fourteen genera and seven species were analysed against mucous cell area, volumetric density and defence activity to check for correlation (Appendix E).

The bacterial community on the kelp (real and plastic) and on the lumpfish skin consisted mainly of the classes *Alphaproteobacteria, Bacilli, Bacteroidia, Campylobacteria, Gammaproteobacteria, Planctomycetes* and *Verrucomicrobiae* (81.6 – 100 %) (Figure 27). In both treatment samples and skin samples, *Gammaproteobacteria and Bacteroidia* represent most of the community. In the skin samples, the relative abundance of *Alphaproteobacteria* was 2.6±2.4 %, *Gammaproteobacteria* 47.5±14.5 % and *Bacteroidia* 41.6 ±12.2 % (Mean ± SEM). In the treatment samples, the relative abundance of *Alphaproteobacteria* was 22.5±4.5 %, *Gammaproteobacteria* 36.4±7.6 % and *Bacteroidia* 21.4 ±4.9 % (Mean ± SEM) (Figure 27). The class *Planctomycetes* had a mean RA of 9.7±8.6 % from the plastic kelp treatment samples, while in the treatments real kelp and no kelp, the RA was <1 % (Figure 27). The highest RA of Planctomycetes found in a skin sample was 0.27 %.

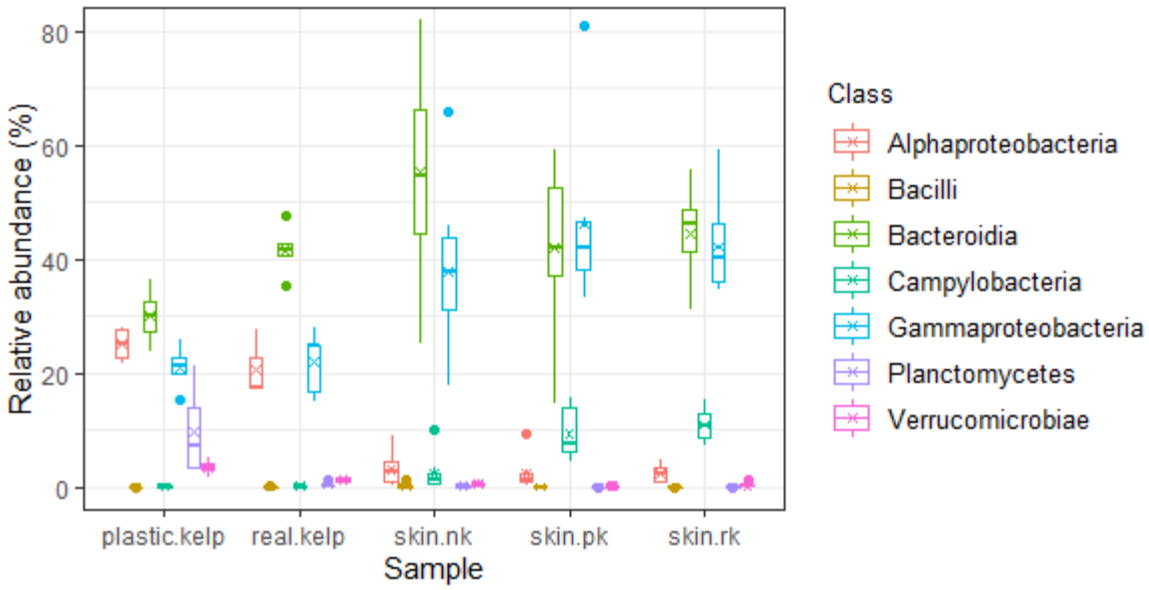

Figure 27: Relative abundance (%) of the bacterial classes *Alphaproteobacteria* (red), *Bacilli* (orange), *Bacteroidia* (lime), *Campylobacteria* (turquoise), *Gammaproteobacteria* (Blue), *Planctomycetes* (purple) and *Verrucomicrobiae* (pink), per sample. The different samples were, from left to right, plastic kelp (n= 4), real kelp (*L. digitata*) (n=5), lumpfish skin from the treatment without kelp (n=10), skin from the treatment with plastic kelp (n=8) and skin from the treatment with real kelp (10). All classes include at least one sample where RA >1%.

The families analysed from the lumpfish skin represents 39.3 – 97.3 % RA, the genera analysed represents 43.8 – 93.0 % RA and the species analysed represents 16.7 – 73.0 % of the bacterial community. In Fitjar,

there were few correlations between the taxonomic units of class, family, or genus and the lumpfish skin regarding mucous cell area, volumetric density, and defence activity (Appendix E).

3.4.5 Bacterial relative abundance vs mucous cell MA, VD and DA

There was no difference between the classes regarding the mucous cells and the relative abundance, i.e., the classes were found in all ranges of mucous cell mean area, volumetric density or defence activity (Figure 28-30).

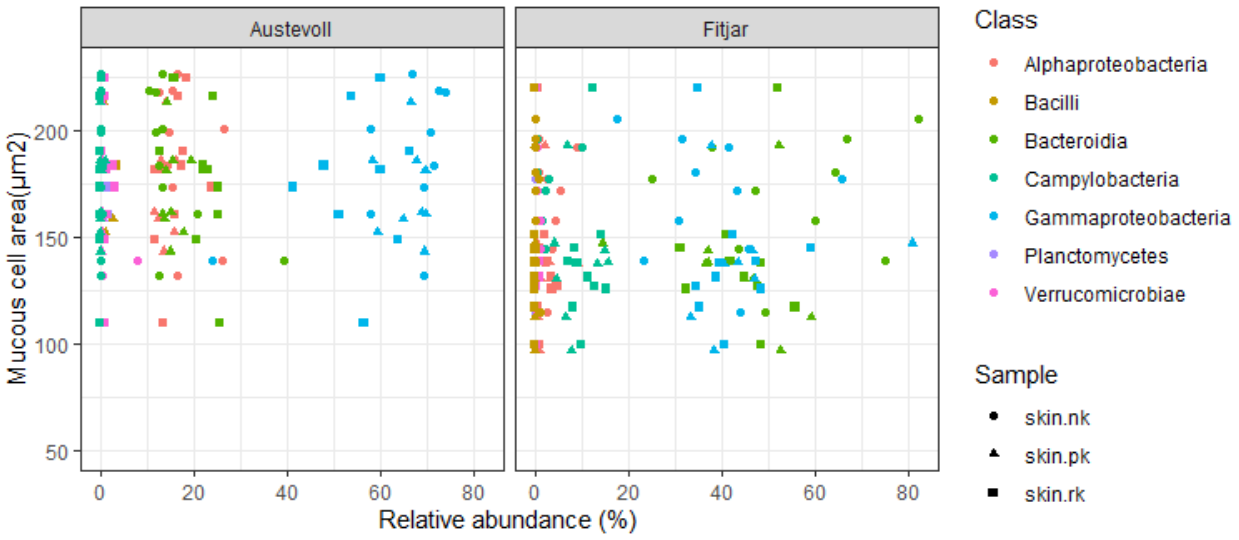

Figure 28: The mean area of the mucous cells in the epithelia in the epidermis of *Cyclopterus lumpus* vs relative abundance (%) of the bacterial classes *Alphaproteobacteria* (red), *Bacilli* (orange), *Bacteroidia* (lime), *Campylobacteria* (turquoise), *Gammaproteobacteria* (Blue), *Planctomycetes* (purple) and *Verrucomicrobiae* (pink) from experiment Austevoll (08.09.2020 – 16.10.2020) and Fitjar (05.10.2020 – 10.11.2020).

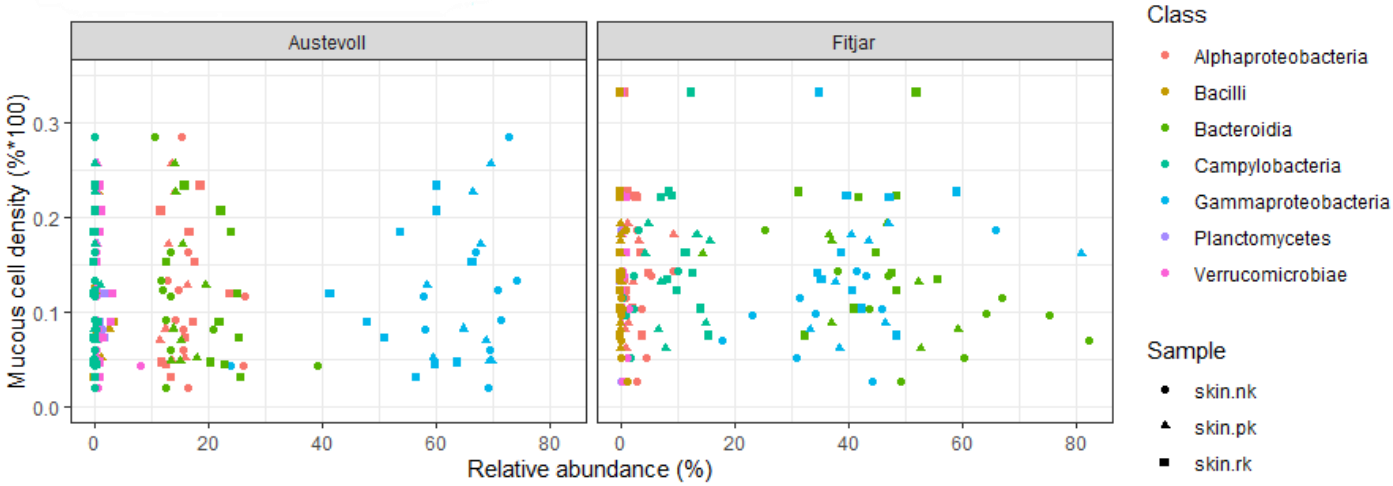

Figure 29: The volumetric density of the mucous cells in the epithelia in the epidermis of *Cyclopterus lumpus* vs relative abundance (%) of the bacterial classes *Alphaproteobacteria* (red), *Bacilli* (orange), *Bacteroidia* (lime), *Campylobacteria* (turquoise), *Gammaproteobacteria* (Blue), *Planctomycetes* (purple) and *Verrucomicrobiae* (pink) from experiment Austevoll (08.09.2020 – 16.10.2020) and Fitjar (05.10.2020 – 10.11.2020).

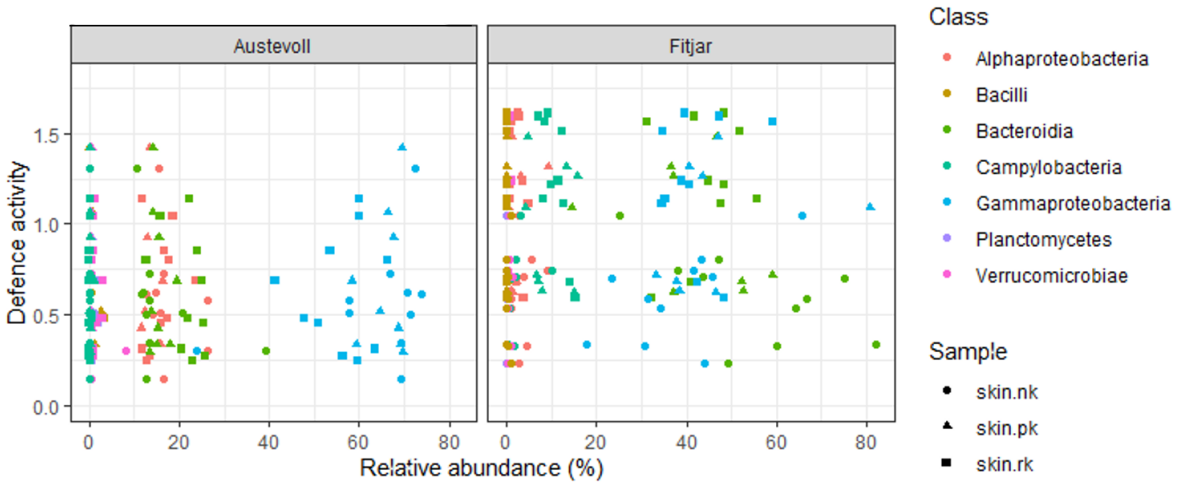

Figure 30: Defence activity of the mucous cells in the epithelia in the epidermis of *Cyclopterus lumpus* vs relative abundance (%) of the bacterial classes *Alphaproteobacteria* (red), *Bacilli* (orange), *Bacteroidia* (lime), *Campylobacteria* (turquoise), *Gammaproteobacteria* (Blue), *Planctomycetes* (purple) and *Verrucomicrobiae* (pink) from experiment Austevoll (08.09.2020 – 16.10.2020) and Fitjar (05.10.2020 – 10.11.2020).

In Austevoll, no correlation between the taxonomic units of class, family, genus or species and the lumpfish skin regarding mucous cell area, volumetric density, or defence activity was found (appendix D). However, the relationship between mucous cells mean area in the treatment real kelp and the relative abundance of the genus *Tenacibaculum* did approach significant correlation, $R^2 = 0.51$, and in the genus *Aureispira* ($R^2 = 0.51$), also in the treatment real kelp (Appendix D).

In Fitjar, a significant correlation between mucous cell area and relative abundance (<1%) was found in the class *Planctomycetes* (treatment plastic kelp, $R^2=0.63$) and *Verrucomicrobiae* (treatment plastic kelp, $R^2=0.77$), and in the family *Francisella* (treatment plastic kelp, $R^2=0.72$), *Moritella* (treatment plastic kelp, $R^2=0.62$), and in the species *Vibrio metschnikovii* (treatment plastic kelp, $R^2=0.64$) (Appendix E). A significant correlation was also found between mucous cell area in the treatment real kelp and RA (>3.5%) in the genus *Halarcobacter* ($R^2=0.77$) (Figure 31).

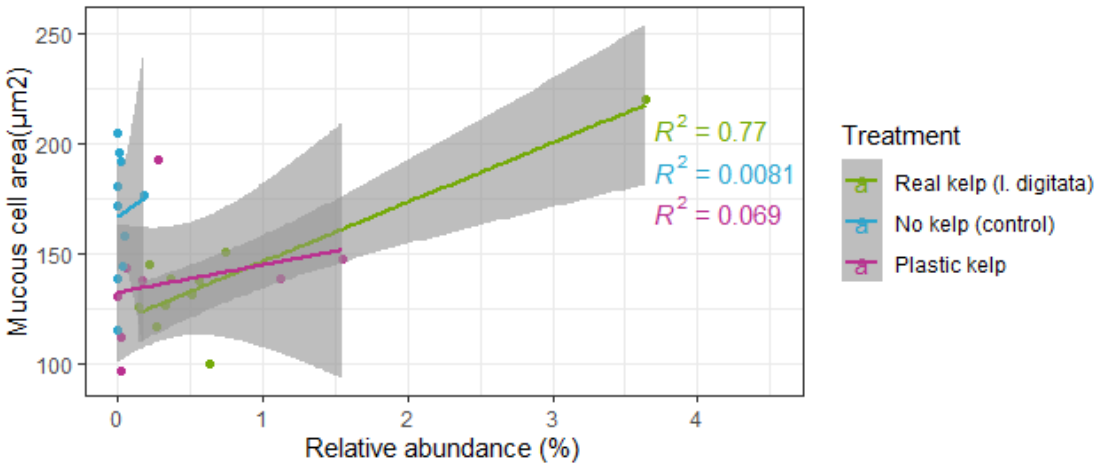

Figure 31: Relative abundance (%) of the genus *Halarcobacter* measured against mucous cell mean area (µm^2). Lethal sampling (n=10, N=30) from the third sampling of *C. lumpus* skin at Fitjar (10.11.2020).

There was no correlation between volumetric density and relative abundance on any of the taxonomic levels of class, family or species in Fitjar. However, approaching significant correlation between VD and RA was found in the genera *Aliivibrio* (treatment plastic kelp, R^2=0.58) and *Halarcobacter* (treatment real kelp, R^2=0.50) (Appendix E).

Regarding defence activity, a significant correlation was found in the family *Arcobacteraceae* (treatment real kelp, R^2=0.61) *(*Figure 32*).* This was the only family analysed in the class *Campylobacteria (Appendix E)*. The DA vs RA was approaching significance in the genus *Colwellia* (treatment real kelp, R^2=0.56).

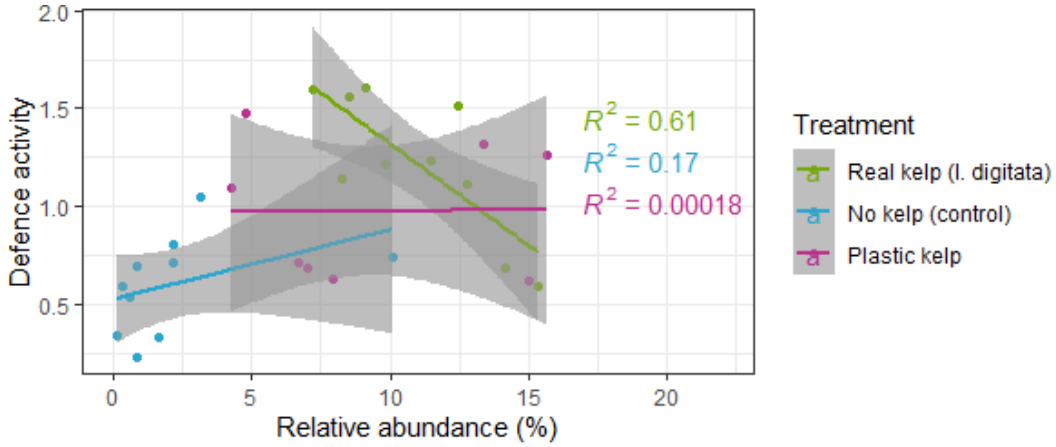

Figure 32: Relative abundance (%) of the family *Arcobacteraceae* from the class *Campylobacteria* measured against defence activity. Lethal sampling (n=10, N=30) from the third sampling of *C. lumpus* skin at Fitjar (10.11.2020).

3.5 Observations on the appearance of the lumpfish in Austevoll and Fitjar

There were no comments regarding observations on the lumpfish in Agder. In Austevoll, in the first sampling, the caudal fin was observed to be short in some individuals, and the gills were observed to be pale in the treatment with real kelp and some pale in the treatment with plastic kelp (Table 4). In the second and third sampling at Austevoll, different degrees of tail damage was most commented upon, but other comments regarded thin fish, flared anal fin, or abnormally round shape (Table 4). The lumpfish reared with the treatment plastic kelp had the most comments (Table 4).

In Fitjar, the comments mostly regarded observations concerning presumed bacterial infections, sores and discolouration (Table 4). Lumpfish from the treatment with plastic kelp were also here commented the most.

3.5.1 Visualisation of the mucous cells in the epithelia

The mean defence activity per treatment in Agder Austevoll and Fitjar were visually represented by the Quantidoc's Dicer App v2 (Figure 33). By feeding the app the mean area and density of the mucous cells, the program calculated the defence activity, displaying it as mucous cells (blue circles) in the epithelia (white square). Here the size and number of the circles represent the size and density of the mucous cells per 10000 μm^2 epithelia. However, the defence activity calculated by the app deviated slightly from the values given in this thesis due to different ways of calculating the defence activity. The app uses the already calculated treatment means for MA and VD but calculates the DA directly from these means, thus missing the range of the samples and the grand mean (used in the thesis, Figure 15).

Table 4: Comments on the external appearance of the lumpfish (*Cyclopterus lumpus*) in Austevoll (08.09.2020 – 16.10.2020) and Fitjar (sampling 10.11.2020).

Location	Tank /cage	Treatment	Fish	Length	Weight	Comment
Austevoll 1	13	Real kelp	-	-	-	All: Pale/very pale gills
Austevoll 1	16	Real kelp	-	-	-	All: more normal gills
Austevoll 1	14	Plastic kelp	-	-	-	All: Pale gills
Austevoll 1	17	Plastic kelp	-	-	-	All: Some pale and some normal gills
Austevoll 1	15	No kelp	-	-	-	All: Not so pale gills, more normal
Austevoll 2	16	Real kelp	7	10,5	69	Some tail damage
Austevoll 2	16	Real kelp	8	9,7	61	Some tail damage
Austevoll 2	16	Real kelp	10	9,5	45	Some tail damage
Austevoll 2	14	Plastic kelp	12	7,1	28,5	Much tail damage
Austevoll 2	14	Plastic kelp	14	9,6	54,5	Some tail damage
Austevoll 2	17	Plastic kelp	19	11	60,5	Narrow tail
Austevoll 2	17	Plastic kelp	20	10,2	54	Some tail damage
Austevoll 2	15	No kelp	21	9,8	49,5	Flared anal fin
Austevoll 2	15	No kelp	23	10	59,5	Some tail damage, extremely round
Austevoll 2	15	No kelp	24	9	32	Very thin
Austevoll 2	15	No kelp	25	10,5	58	Very round/deformed
Austevoll 2	18	No kelp	26	9,9	62,5	Much tail damage
Austevoll 3	13	Real kelp	63	12	89	Tail damage
Austevoll 3	16	Real kelp	67	11,3	83,1	Some tail damage
Austevoll 3	14	Plastic kelp	72	11,1	76,5	Some tail damage
Austevoll 3	14	Plastic kelp	74	13,1	104	Much tail damage
Austevoll 3	17	Plastic kelp	77	10,5	70,5	Tail damage
Austevoll 3	17	Plastic kelp	78	12,2	74,5	Much tail damage
Austevoll 3	17	Plastic kelp	80	10,9	67,5	Some tail damage
Austevoll 3	18	No kelp	88	11,6	76,5	Some tail damage
Fitjar	5	Real kelp	95	13,5	90	Yellow on one ossicle
Fitjar	5	Real kelp	96	9,5	45	White in the face - bacteria? + yellow on the ossicles
Fitjar	5	Real kelp	97	13,5	85	sore? + white lumps in the face, nice fins
Fitjar	5	Real kelp	98	13	75	some yellow on one ossicle
Fitjar	5	Real kelp	100	12	60	thin
Fitjar	5	Plastic kelp	101	13,5	80	Banana shape + yellow ossicles
Fitjar	5	Plastic kelp	102	15,5	130	white spots on the head, otherwise fine
Fitjar	5	Plastic kelp	104	13,5	85	discolouration, sore?
Fitjar	5	Plastic kelp	105	14,2	115	bacteria (?) / pale ossicles laterally and dorsally
Fitjar	5	Plastic kelp	106	11,5	70	discolouration - mid row of ossicles - bacteria?
Fitjar	5	Plastic kelp	107	10,5	45	discolouration around the ossicles
Fitjar	5	Plastic kelp	108	9,2	40	white ossicle (artefact?)
Fitjar	5	Plastic kelp	109	11	55	brown ossicles
Fitjar	5	Plastic kelp	110	11,3	65	starting to be pale around the ossicles + short tail
Fitjar	2	No kelp	111	11,3	60	bacterial accumulation (?) - ossicles near the tail
Fitjar	2	No kelp	113	11,6	70	thin
Fitjar	2	No kelp	114	11,7	70	white
Fitjar	2	No kelp	115	11,5	65	some white (artefact?)
Fitjar	2	No kelp	116	11	55	white spots near the tail, head and ossicles
Fitjar	2	No kelp	117	11,7	70	sore around the anterior ossicles,
Fitjar	2	No kelp	119	10,5	60	fringing tail

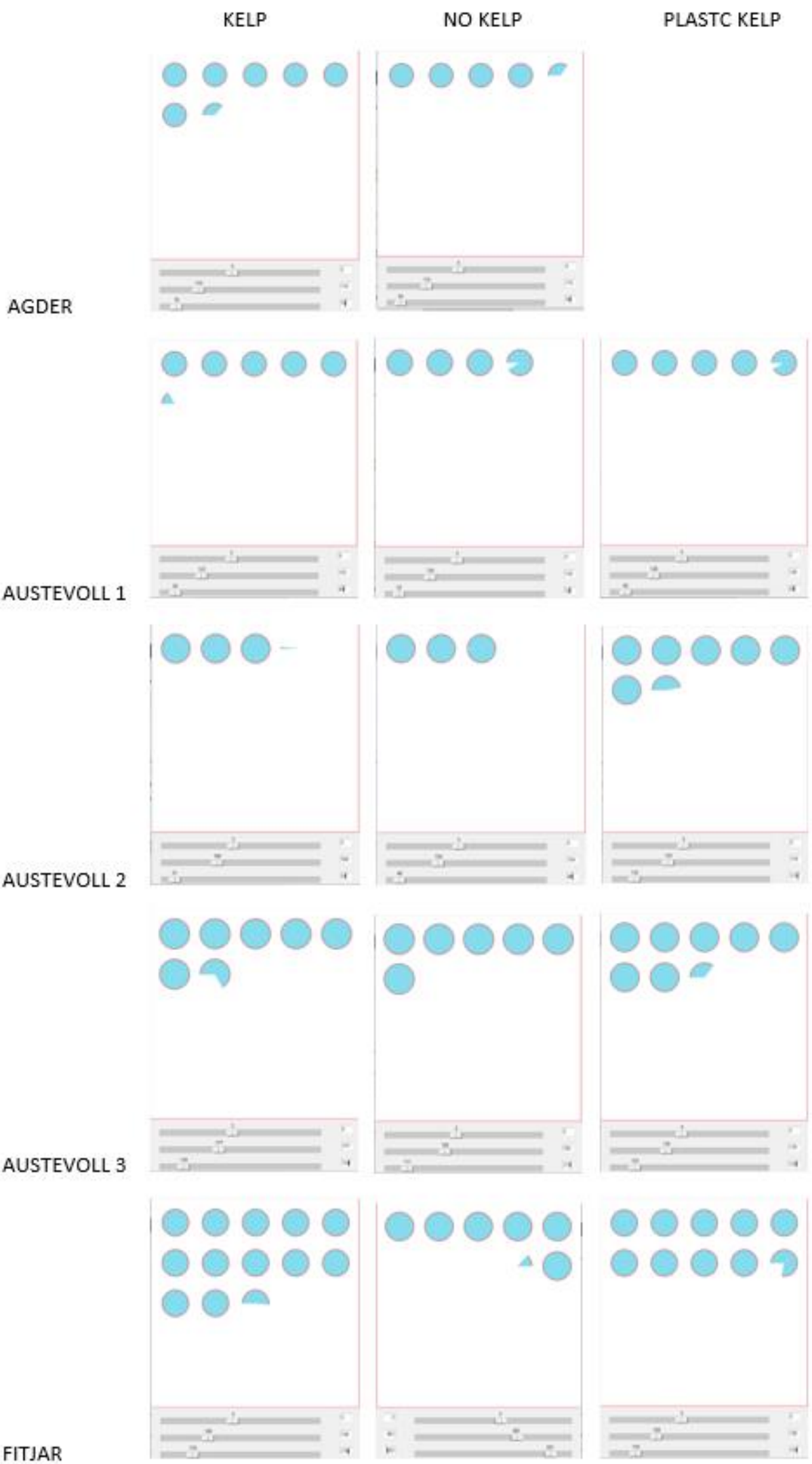

Figure 33: Visual representation of the defence activity from Agder, Austevoll and Fitjar through Quantidoc's Dicer App v2. The blue circles represent the mucous cells size and density in the epithelia (white square) in the Lumpfish (*C. lumpus*) skin. Reference area: 10000 μm^2.

4 Discussion

The results accomplished in this master thesis are based on two different RFF preliminary projects, plus supplementary microbiological data comprised from Lotte Dahlmo, Stefan Thiele, David Rees and Lise Øverås. This study is the first to show the ranges of the mucosal barrier of the skin, of clinically not ill lumpfish across different treatments, and physical conditions regarding location.

4.1 The skin defence of lumpfish

Individual differences within the same groups were grand regarding MA, VD, and DA in Agder, Austevoll and Fitjar (Figure 17 – 19, Table 5). This observation concurs with the variation between individuals from the same group regarding mucous cell concentration in the epidermis found in brown trout (*Salmo trutta*) and char (*Salvelinus alpinus)* (Pickering, 1974). Individual variations in mucous cell dynamics between salmon have also been suggested to follow a repeatable pattern, making unbiased comparisons between individuals from different treatments possible (Pittman et al., 2013). The mucous cells in the lumpfish skin did not vary significantly regarding mean area, volumetric density, or defence activity between the different treatments in the three experiments (Figure 15). However, significant differences were found between some sampling points within treatments in Austevoll and between sea cages in Fitjar (Figure 15 and 16). Statistical testing across experiments was not possible to conduct due to differences in experimental design and setup, but the ranges were measured (Table 5).

Currently, the database and studies regarding lumpfish skin are few, but by comparing the present results to the literature that exists, it is apparent that the mucous cells in the lumpfish skin, sampled mid laterally, aligns with previous studies done on different species, using the same methods of investigating the morphometrics of the mucous cells in the epidermis (Dang et al., 2020; Lazado et al., 2020; Pittman et al., 2013; Torrecillas et al., 2015) (Tabel 5).

Table 5: A comparison of skin mucous cell mean area, volumetric density and defence activity across different studies and species. All studies applied mucosal mapping. Some numbers are approximated.

Study	Species	Sampling	Range Mucous cell MA	Mean MA	Mean Volumetric density	Defence activity	Skin sectioning location	Source
Agder	Lumpfish (*C. lumpus*)		50 – 158 µm^2	112 µm^2	6 %	0.48	Mid lateral	Present study
Austevoll	Lumpfish (*C. lumpus*)	1. sampling	93 – 173 µm^2	128 µm^2	6 %	0.43		
		2. sampling	127 – 221 µm^2	165 µm^2	7 %	0.39		
		3. sampling	110 – 227 µm^2	177 µm^2	12 %	0.63		
Fitjar	Lumpfish (*C. lumpus*)		97 - 220 µm^2	149 µm^2	14 %	0.94		
Stress and Transportation T1 &T2 same facility same batch T3 & T4 Same facility same batch	Lumpfish (*C. lumpus*)	T1	Before transport	68 µm^2	1 %	0.09	Mid lateral	(Jonassen et al., 2019b)
			1 week at sea	71 µm^2	1 %	0.10		
		T2	Before transport	77 µm^2	0.2 %	0.001		
			1 week at sea	75 µm^2	0.1 %	0.002		
		T3	Before transport	119 µm^2	3 %	0.31		
			1 week at sea	103 µm^2	5 %	0.50		
		T4	Before transport	110 µm^2	4 %	0.35		
			1 week at sea	95 µm^2	3 %	0.27		
Field study - Parasitic infections / Pb pollution	Shorthorn sculpin (*M. Scorpius*)	Station 1	-	167 µm^2	4 %	0.24	Tail region	(Dang et al., 2019)
		Station 2	-	187 µm^2	5%	0.27		
		Station 3	-	113 µm^2	4 %	0.30		
Feed trial	European sea bass (*D. labrax*)		150 – 200 µm^2	170 µm^2	< 5 %	-	Dorsolateral	(Torrecillas et al., 2015)
peracetic acid-based disinfectant	Atlantic Salmon *(S. salar)*		150 – 250 µm^2	188 µm^2	-	0.80 – 1.00	Dorsal region	(Lazado et al., 2020)
Feed trial	Atlantic Salmon *(S. salar)*		10 – 666 µm^2	160 µm^2	8 %	-	Dorsolateral and lateral	(Pittman et al., 2013)

Jonassen et al. Investigated the mucous cell mean area, volumetric density, and defence activity regarding stress in relation to transportation (2019a). Four different transportations methods were applied, with a sampling point before the transport and after one week at sea (n = 20). The lumpfish had a mean weight between 25 – 40 g (personal communication, T. Jonassen, 13.04.2021). The mucous cell mean area from all four transportations were smaller than that of Austevoll and Fitjar, and only the mean area in T3 (before transportation) was larger than mean area found in Agder, despite having similar sizes as the lumpfish from

both Agder and Austevoll 1. sampling (Table 2 and 5). Thus, the mean area in T1 is the smallest mucous cell mean area observed in any experiment regarding lumpfish skin, across sizes and location, although the range went lower in Agder (Jonassen et al., 2019a) (Table 5).

The largest lumpfish (130 g, from sea cage 5, Fitjar) had a mean mucous cell area of 154 μm^2, while a smaller individual (64,5 g, treatment with no kelp in Austevoll 3. Sampling) had the largest mucous cell area of 227 μm^2. Thus, the present study shows that the mucous cell area in the skin varies with other environmental factors (Esteban & Cerezuela, 2015; Vatsos et al., 2010) rather than size. The size of the fish is thus not included in the comparison of the mucous cell morphometrics between the species (Table 5).

Comparing the volumetric density of the first samples in all the studies of Lumpfish, it is apparent that Location plays a clustering role (Figure 15, Table 5). The density was relatively low in the study of Jonassen et al., with the lowest mean being <1 % and the highest mean being approximately 5 %, and only a few percentages change between the sampling points (Jonassen et al., 2019a). In Austevoll, on the other hand, the density was doubled from 6 to 12 % from the first to last sampling (Table 5). However, comparing the volumetric density across all studies compiled in Table 5, the range exhibited within the lumpfish is extraordinary. The range goes from a mean density close to 0 in T2 (Jonassen et al., 2019a), up to a mean density of 14 % in Fitjar (Table 5). This is a volumetric density far above that reported in the other species (Dang et al., 2019; Pittman et al., 2013; Torrecillas et al., 2015)(Table 5). Even stranger perhaps is the volumetric density exhibited by the lumpfish in Austevoll, ranging from 1.1 – 47.1 % (Table 2). The lumpfish having 47.1 % of the epithelia covered by mucous cells were nonetheless an obvious outlier, and no other observation like this has been done (Pers. Comm. K. Pittman, 1.12.2020) (Fish ID 17 from the treatment plastic kelp, Appendix B). However, the mucous cell in the epidermis of lumpfish has been found to be individually and sparsely distributed, indicating a low density (Jonassen et al., 2019b; Klingenberg, 2019). The elevated densities found in this thesis might thus partly be caused by the rejection of Q-cells as epithelial tissue, which in fact dominates the epidermis (Appendix B). The area occupied by the Q-cells was thus not included in calculating volumetric density, which would have substantially lowered the density of the mucous cells by increasing the epithelial area.

The lumpfish's defence activity in this thesis aligns with that of previous research (Dang et al., 2019; Jonassen et al., 2019b; Lazado et al., 2020). The number of parasites on the shorthorn sculpin was positively correlated to the mucous cell's defence activity and volumetric density (Dang et al., 2019). In this thesis, the relative abundance of bacteria on the lumpfish skin versus mean area, volumetric density or

defence activity were tested for the lumpfish in Austevoll 3 sampling and Fitjar, but only a few sporicidal significant correlations were found in Fitjar (Figure 31 and 32) (Appendix D and E). As to why these specific bacteria and the mucous cell morphometrics were significant (although a significant R^2 was regarded as 0.6), is not known, and is a field for further research (Appendix D and E).

4.1.1 Novel structures in the lumpfish skin

When studying the lumpfish skin, it is not possible to miss the characteristic Q-cells. The number, distribution and size of the Q-cells vary with ontogenetic stage and environment (Klingenberg, 2019). In this study, specific measurements or statistical testing regarding the Q-cells were not made. Still, the Q-cells were observed to be dominating the epidermis of the lumpfish skin samples from all experiments, with few exceptions (Appendix B). However, a significant ($p<0.05$) systematic increase in Q-cell density was found after sea transfer in the stress and transportation study of Jonassen et al. (2019a). They found that these cells may be up to 200 times larger than mucous cells (> 2200 µm2) and have a density ten folds that of mucous cells. Thus, it is hypothesised that the Q-cells are the main driver regarding immunological responses in the lumpfish skin rather than the mucous cells (Jonassen et al., 2019b).

4.2 Factors influencing the mucous cells morphometrics

In nature, the lumpfish spends its first years in close proximity to seaweeds and kelp, and current rearing facilities are far from their natural habitats (Davenport, 1985; Imsland et al., 2018b; Ingòlfsson, 2000). The natural habitat of salmon is also quite far from that of aquaculture facilities, but the salmon have been bred through several decades, hence being finetuned for a life in the net-pen, and there are clear differences between wild Atlantic salmon and aquaculture Atlantic salmon (Glover et al., 2017; Houston & Macqueen, 2019). The production of functional spawning broodstock of lumpfish, on the other hand, is still in its infancy (Pountney et al., 2020). Since the lumpfish industry thus still rely on wild-caught broodstock, there have been no breeding to make it more robust and suitable for life as a cleanerfish (Imsland et al., 2019a; Pountney et al., 2020). Since the hatchery-reared lumpfish are always generation f1, and the natural and tank environments are very different, it would be interesting to investigate the epidermis of wild-caught lumpfish to see how exactly the rearing facilities affects the skin health of hatchery-reared lumpfish.

4.2.1 Treatments, water quality and location

In all three experiments, one tank or cage consisted of the treatment natural kelp, however, the species used in Agder and Austevoll (and Fitjar) were different. Therefore, it is uncertain whether the different species of kelp had a different effect on the mucous cells in the lumpfish skin in the different experiments. Yet, the microbiome associated with these species consists of mainly the same bacterial taxa (Ihua et al.,

2020; Schiener et al., 2015; Staufenberger et al., 2008). Nonetheless, since no microbiological analyses have been conducted regarding the lumpfish in Agder, it is impossible to make assumptions about the kelps influence on the bacterial load on the lumpfish skin in Agder.

In the two weeks long experiment in Agder, there were no reported observations of the kelp (*S. latissima*) disintegrating, and the water flow and quality were monitored as good throughout the experiment. Although mucous cell changes in the epidermis have been discovered after only 48 hours (Vatsos et al., 2010), a more extended experiment should in the future be conducted with multiple sampling points with the kelp species *Saccharina latissima* to be able to detect systematic changes in the mucous cells in the epidermis, as seen in Austevoll (Figure 15) (Vatsos et al., 2010).

In Austevoll, the water flow was not optimal, and the current in the tanks with the treatments natural kelp and plastic kelp were almost non-existing. However, the slow water current may still be an advantage (Roalkvam et al., 2019). To achieve a stable microbial community over time and favours slow growing bacteria, the water flow must be slow enough for the bacteria to establish in the tanks and not flush away (De Schryver & Vadstein, 2014; Roalkvam et al., 2019). However, the kelp *(L. digitata*) disintegrated midway, and all kelp was changed on day 22. The likely reason was that the kelp did not get enough light to sustain photosynthesis and thus deteriorated. *L. digitata* is defined as a "sun kelp", and the rate of photosynthesis have been shown to be 50 – 100 % higher in blue light compared to red light (Dring, 1989). This suits well with the light preferences found for lumpfish (Espmark et al., 2019; Skiftesvik et al., 2017) and rearing lumpfish with natural kelp under blue light should therefore be considered in future research.

The disintegration of the kelp in combination with many faecal particles in the tanks, may have lowered the water quality and consequently the welfare of the lumpfish in Austevoll. After the kelp had been changed and a new method of deploying the kelp was in place, the particle load in the water was reduced but not gone. Slow water in combination with a lot of organic matter is not a favourable situation, potentially leading to an increase in opportunistic pathogenic bacteria that may cause harm (De Schryver & Vadstein, 2014; Roalkvam et al., 2019). Since the mucosal layer is the first barrier between the fish and the environment (Salinas, 2015), the living cells creating this barrier will be affected by the environment (Cabillon & Lazado, 2019). This also applies to the newly discovered rose petal cells in the epidermis of lumpfish (Jonassen & Pittman, 2019). These cells have an increased surface area that unintentionally may trap particles and organic matter floating in the water column, potentially causing harm and disease. Hence, it is vital when using live kelp as shelter in land-based facilities to have the tanks big enough so that

the kelp does not break up the water flow and give the kelp enough light to sustain photosynthesis, preventing disintegration.

In an experiment on carp where they elevated the bacterial load (*Aeromonas hydrophila*) in the water for twenty days, the fish produced more mucous, and the number of mucous cells increased (van der Marel et al., 2010). Thus, the bacterial load in the water affects the mucous cells in the fish skin. Contrary to treated seawater used in aquaculture, the microbial load in the ocean is estimated to be 10^3 microalgae, 10^3 fungi, 10^5 bacteria and 10^7 viruses per mL, in addition to planktonic larvae and spores (Giovannoni & Stingl, 2005; Goecke et al., 2010). The gradient from optimal water quality, treater through a mechanical and UV filter in Agder, though suboptimal tank conditions in Austevoll, to full-on microbial soup in the ocean in Fitjar, can thus be observed in the visualisation of the defence activity displayed by the lumpfish skin, as mucous cells size and number per reference area increasing with the different conditions (van der Marel et al., 2010)(Figure 33). Here, the differences in treatments, although not significant (despite cage differences in Fitjar), can be seen (Figure 33).

4.2.3 Bacteria influencing the mucous cells morphometrics

The pathogenic load in Austevoll was very low compared to Fitjar, although the pathogenic load on the treatments in both locations was low (Figure 22, 23, 25 and 26). The most abundant pathogenic bacteria on the lumpfish skin in Austevoll was *Aliviibrio logei* (Figure 22), a species that has been found in samples from deceased lumpfish, although the pathology of the species is unknown (Hjeltnes et al., 2019). The bacterial community comprising of gram-positive bacteria on the fish skin and the treatments, represented only by the gram-positive class *Bacilli*, was very low, and did not follow any pattern (Figure 20 and 21, Table 4).

The bacterial community on the lumpfish skin in Fitjar, on the other hand, mainly consisted of the pathogenic genus *Tenacibaculum* (mean 34 – 46 %) (Figure 25). Pathogenic bacteria in this genus cause tenacibaculosis, an ulcerative disease, which is a significant problem in aquaculture worldwide, affecting numerous marine and anadromous species such as Atlantic salmon (*Salmo salar*), Dover sole (*Solea solea)* and striped trumpeter (*Latris lineata)* (Fernández-Álvarez & Santos, 2018; López et al., 2009; Wakabayashi et al., 1986). The dominant species found on the lumpfish skin in Fitjar was *T. maritimum,* and the first isolation of this bacteria in Norway was from deceased Lumpfish in 2016 (Småge et al., 2016). *T. maritimum* has been found to be the causative agent of mortality in lumpfish and have also been found to facilitate other bacteria, creating a gateway for secondary pathogens (Fernández-Álvarez & Santos, 2018; Småge et al., 2016; Van Gelderen et al., 2009).

Experimentally, the Norwegian isolates of *T. maritimum* have been transmitted horizontally from lumpfish to salmon and henceforth caused disease in salmon (Nylund et al., 2020). In lumpfish, *T. maritimum* is known to cause "doughnut-disease" (kratersyke), skin lesions, white spots and fin rot (Småge et al., 2016), and a lot of these characteristics were observed in the lumpfish sampled from Fitjar (Table 4). In a study done by Roalkvam et al., they found that in a natural, healthy production cycle, a large portion of the bacteria present was from the genus *Tenacibaculum* and that the *Tenacibaculum* could be connected to a low hatching rate (2019). Investigating the possibility that lumpfish may work as a pathogen vector, bringing pathogenic bacteria from the hatchery to the sea cages, should therefore be of interest.

The bacterial communities established on the natural kelp, artificial kelp and tank walls was not mirrored in the lumpfish skin, and thus did not have an observable impact on the fish's skin health regarding correlation between bacterial relative abundance and mucous cell mean area, volumetric density or defence activity (Appendix D and E).

4.2.4 bacteria on the kelp

The bacterial community represented on the natural kelp (*L. digitata)* in both Austevoll and Fitjar is in coherence with previous research (Bengtsson et al., 2010; Ihua et al., 2019, 2020). Interestingly, the relative abundance of *Planctomycetes* in the present study was much lower (highest reported was 19 % RA in one kelp sample) compared to Ihua et al., where the mean relative abundance of *Planctomycetes* on the kelp *L. digitiata* was 36 % (2020). Additionally, *Planctomycetes* was found by Bengtsson and Øverås to be the most dominant bacterial phylum in the biofilm on surfaces of the kelp *L. hyperborea*, accounting for 53 % of the RA during August - September (2010). Even stranger perhaps is that in Fitjar, the mean RA of *Planctomycetes* was higher on the treatment plastic kelp, while on the treatment real kelp, the mean RA was <1 % (Figure 27). A feasible reason for this might be the fact that the treatment real kelp and plastic kelp were in the same net-pen, and lumpfish can thus have freely moved between the two, and in this way brought planctomycetes from the kelp to the plastic kelp. Ihua et al., found that the bacterial communities are highly distinctive between the different thallus regions of the kelp *Laminaria digitata,* and that only 1.1 % of the surface-attached bacteria were present at all regions of the kelp (2020). Sampling location and area on the kelp is therefore important and might be one of the reasons for the low RA of planctomycetes found on the kelp in the present study (Ihua et al., 2020).

4.3 Welfare indicators

Evaluation of morphology, structure and distribution of mucous cells in the fish skin can be an important way of characterising mucosal health in fish (Dang et al., 2019; Jonassen et al., 2019a; Pittman et al., 2011, 2013; Vatsos et al., 2010). Current welfare indicators tend to focus on disease and already visible signs of poor welfare, such as emaciation, sores and fin rot, rather than factors that may prevent these things from happening and make the fish more robust (Gutierrez Rabadan et al., 2021; Imsland et al., 2020; Noble et al., 2012, 2019). A new study has shown that lumpfish from commercial farms may be chronically stressed, revealing a cortisol level of 85 ng mL^{-1} (Gutierrez Rabadan et al., 2021). Including mucosal mapping as a tool to assess lumpfish health should therefore be implemented as a standardised LAB-WI to prevent lumpfish skin health from deteriorating beyond repair from chronic stress.

4.4 The way forward

In the fish health report of 2020, anecdotal reports of sound effects of the cleanerfish were presented, but the scientific documentation is still missing (Sommerset et al., 2021). It is thus apparent that even though much effort has been put into bettering the welfare of the cleanerfish, there is a mismatch between the problems and the solutions (Imsland et al., 2019b, 2020; Powell et al., 2018b; Sommerset et al., 2021). Despite the high focus on health and welfare over the past years, the situation has not changed much, which causes alarm (Sommerset et al., 2020). Many farmers probably do their best to facilitate good welfare such as applying adequate hides, feed regime and gentle handling (Grefsrud et al., 2021). But still, routines regarding sorting and delousing events are lacking or maintain the welfare of the cleanerfish poorly, reflecting that the focus lies on the practicalities and technology rather than the biology (Sommerset et al., 2021).

There is a growing concern about the potential for disease transfer from the cleanerfish to the salmon. Lumpfish are susceptible to multiple pathogenic and disease-causing agents simultaneously, and the primary cause of death is often difficult to establish (Nylund et al., 2020; Sommerset et al., 2021). *Atypical furunculosis, Vibrio anguillarum, pseudomonas anguilliseptica, Pasteurella* sp., and *Tenacibaculum* spp. are the most prominent bacterial pathogens causing infections in lumpfish in aquaculture, and some of the diseases caused may transfer between species (Hjeltnes et al., 2017, 2018, 2019; Sommerset et al., 2020, 2021). it is all the more reason to strengthen the health of the lumpfish, making it as robust as possible before being deployed at sea.

Excluding handling and delousing events, fin rot, sores and bacterial diseases are the most prominent issues experienced by cleanerfish (Sommerset et al., 2020). Additionally, a considerable cause of concern is the lack of an overview of the status and mortality of the cleanerfish being deployed at sea. Whether the sufficient knowledge and technology regarding keeping cleanerfish as a sustainable lice strategy and at the same time maintain the welfare and health of the cleanerfish are also raised by the governing powers and the public (Sommerset et al., 2020).

In Norwegian aquaculture, lumpfish have equal rights and are equally protected as salmon by law and legislations (Akvakulturdriftsforskriften, 2008; Dyrevelferdsloven, 2010). Regardless, the mortality of food-fish Atlantic salmon was 14.8 % in 2020, whereas there are no official numbers regarding lumpfish (Sommerset et al., 2021). Previous reports have however mentioned mortalities up to 100 % (Nilsen et al., 2014; Sommerset et al., 2020). A thought experiment is that if the mortalities were reversed, there would surely be a forced immediate cease in salmon production. The objectification of the lumpfish as a replaceable tool is hence in a way, collectively agreed upon.

The number of hatchery-raised lumpfish put out in the net-pens were lower in 2020 than in 2019. One possible explanation is the lack of progress and increasing awareness of the welfare issues (Sommerset et al., 2021). Lumpfish was supposed to be the green and sustainable solution to the sea lice problem, but instead, the problems are heaping up for the lumpfish itself (Brooker et al., 2018; Powell et al., 2018b). Consumer awareness regarding the sustainable production of salmon have also increased during recent years, and the problems have been made accessible for the public through numerous news articles (Kyst.no, 2020; Stranden, 2020). In the documentary "public enlightenment" (Folkeopplysningen) regarding salmon, available from the Norwegian broadcasting corporation (Wahl, 2020), the deep dismay regarding the lumpfish industry is expressed, and the conclusion of the national supervisory campaign is brough up (Mattilsynet, 2020;Wahl, 2020). The food health authority are currently debating the possibility of prohibiting the of using cleanerfish if the mortality cannot be documented to go down (Mattilsynet, 2020). For the whole industry to not fall under, it is thus not a question of willingness, but a demand of change.

In 2015 the united nations came together to form 17 sustainable development goals (SDG) to make the world a better place by 2030 (*THE 17 GOALS | Sustainable Development*, n.d.). As the human population is predicted to reach 9 billion people by 2050, the demand for food production is estimated to increase by 50 %, and aquaculture is thus becoming a source of food for an increasing number of people (FAO, 2020; FAO et al., 2020). These goals, primarily SDG 12 responsible consumption and production and SDG 14 life below

water, are thus crucial pieces in the management puzzle regarding sustainable fisheries and aquaculture (FAO, 2020; *THE 17 GOALS | Sustainable Development*, n.d.). Many Norwegian aquaculture companies have adopted these goals, and the word sustainable are frequently used by the industry for itself, and to achieve the perception of the public, certifications of sustainability are increasingly popular (Bailey & Eggereide, 2020). However, to achieve a genuinely sustainable aquaculture industry of Atlantic salmon that still use cleanerfish, the current challenges must be dealt with (Brooker et al., 2018; Gutierrez Rabadan et al., 2021).

4.5 IMTA

Saccharina latissima is one of the most prominent species of cultivated kelps in the Nordic countries (Bak et al., 2018; Broch et al., 2019; Marinho et al., 2015). The implementation of seaweeds around salmon farms as integrated multitrophic aquaculture (IMTA) systems, as well as seaweed as feed additives, are gaining more interest (Bak et al., 2018; Broch et al., 2019; Ferreira et al., 2020; Mols-Mortensen et al., 2017; Thépot et al., 2021). In a recent harvest from the commercial project "tarelaks", small juvenile and larger lumpfish were found in between or sucked onto the harvested kelp, which they were clearly bathed in the mucous and pigments from (B. W. Kvamme, personal communication, May 06, 2021). This is an example from nature where the lumpfish unoubtedly interact with the mucosal layer of the kelp.

Saccharina latissima was the kelp species used in Agder, and positive effects of this can be seen (Figure 15 and 33). The bacterial community in association with the kelp *S. latissima*, also consist of bacteria exhibiting antimicrobial activity (Wiese et al., 2008). this show that the microbial community on the kelp *S. latissima* may exhibit favourable compounds that might interact in a potential symbiotic relationship with the lumpfish and other species inhabiting the kelp.

The lumpfish found in the harvested kelp were bathed in pigments from the kelp. Fucoxanthin is a pigment exhibited by many brown algae, and it will easily leak out of the cells and colour to the surrounding water, skin and supposedly even eyes. As the more familiar carotenoid astaxanthin known for its supplementation in salmon feed, fucoxanthin has also shown to exhibit a range of health benefits (Bae et al., 2020; Thépot et al., 2021). Fucoxanthin has also been found to be one of the factors driving the regulation of the bacterial colonisation of algae (Malik et al., 2020). Both project owners of these preliminary projects produce kelp themselves. The project owner of the Seaweed symbiosis experiments, Engesund Oppdrett AS, found that rearing the kelp (*Saccharina latissima*) inside the net-pen with the salmon had a higher growth rate than outside (Engesund Fiskeoppdrett, n.d.). Implementing the live kelp as a resting and hiding place for the

lumpfish may increase the lumpfish's health and be a valuable resource for harvest (Bae et al., 2020; Ferreira et al., 2020; Mols-Mortensen et al., 2017).

Unpublished data from the commercial company Hiddenfjord in the Faroe Islands, clearly show a connection between the biofouling on the net-pens i.e. the amount of macroalgae growing on the net, and the mortality rate of lumpfish, where the mortality was highest when the nets were clean (Esbern Patursson, Pers. Comm. 26.05.2021). This also led to fewer empty stomachs when assessing the lumpfish, and a better welfare score. In the present thesis, the kelp was applied as a substrate inside the net for the lumpfish to rest on, but applying this method in locations with stronger currents could possibly have great effect, as lumpfish are not strong swimmers, and have been observed to be pushed against the net in such locations (Davenport & Kjørsvik, 1986; Hvas et al., 2018; Noble et al., 2019).

4.6 Further research

The interest in mucosal mapping as a quantitative measure of health is increasing. Skin samples for mucosal mapping in regard to a study investigating lumpfish exposed to different salinities (freshwater test), as well as broodstock have been obtained, but these data have not yet been analysed due to economic considerations (Personal communication, T. Jonassen,13.04.2021). As the method is applied in an increasing number of studies, the empirical dataset increases, and it will be possible to benchmark what is a healthy and robust lumpfish and determine what is not, and thus make action.

4.6.1 Mounting the kelp rope and scientific error

The seaweed symbiosis experiment was a pilot-project, and the resources and manpower at hand were simply not adequate. It took a technician's full day of work to mount the kelp-rope and put it into the net-pen. The experimental error with placing both kelp types in the same net-pen can in the future be avoided with a better scientific communication between the parts, since this is after all a commercial farm, and the personnel is not expected to have a scientific education and knowledge about experimental design. It is also worth mentioning that due to the low scale of this project, and the risks taken with this novel method, of potentially harm or destroy the existing fish-farm, was a risk they were not willing to take, namely, to use one whole net-pen for each treatment. Each net-pen contain approximately 150 000 Atlantic salmon, and if something were to go wrong with the experiment, the economic losses would be tremendous. Thus, the economical and labour-demanding aspect of the project led to a shortage in experimental design. However, placing two treatments in the same net-pen is a severe error and should be avoided in the future.

5 Conclusion

This research is an essential element in establishing a baseline for lumpfish health that are currently missing. Lumpfish are susceptible to a high range of novel and well-known pathogens (Erkinharju et al., 2021). The health and welfare of the lumpfish are also compromised in farm operations, where the focus often is on practicality and technology rather than biology and the needs of the different species in the net-pen (Brooker et al., 2018; Powell et al., 2018b).

Adequate resting and hiding space are vital for lumpfish to thrive (Imsland et al., 2015, 2014c; Ingòlfsson, 2000), which is also shown in this thesis by the lower defence activity and volumetric density but large mucous cells exhibited by the lumpfish reared with no kelp (Natural or artificial) (Figure 15). The difference between the treatments in Austevoll was not significant, but the lumpfish still had the tank walls to adhere to, and the water was treated. In Fitjar, on the other hand, since it is not possible to attach onto the net-pen, the lumpfish had nowhere to rest, and the microbial and particle load in the ocean is substantially higher than in land-based facilities (Giovannoni & Stingl, 2005). The skin defence expressed by lumpfish is thus increasingly activated in coherence with the microbial and particle load in the surrounding water (Figure 33). This observation concur with previous research (van der Marel et al., 2010).

Studies regarding adequate substrate for the lumpfish have previously been investigated, but only regarding materials mimicking the lumpfishes natural habitat, often made of plastic (Imsland et al., 2015, 2018b; Imsland & Conlon, 2019). It is thus clear that no studies have been done to compare or use natural kelp on its own as a suitable substrate for the lumpfish in the net-pens. This experimental study is the first of its kind to scientifically use and measure the effect of natural kelp as a substrate for lumpfish in commercial salmon production and several commercial like systems. This was a pilot project, and there were no significant differences between the treatments, but the results show that natural kelp is just as suited as substrate in the net-pens as the commercially available plastic hides.

By applying the stereology based, objective and quantitative measure of mucosal mapping, it was possible to investigate the mucous cells in the lumpfish skin and find differences within treatments over time and between sea cages in a commercial farm setting. The goal would then be to implement mucosal mapping in the industry as a laboratory-based welfare indicator, as a standardised, objective way of measuring health in lumpfish.

6 References

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Appendix A – Introduction elements

History of the industry – the salmon lice

Aquaculture production of Atlantic salmon started commercially in Norway in the 1970s. since then, the industry has grown from just a few commercial farms to over one thousand permits, and an annual production of 1,4 million metric tonnes to a value of 12 billion USD in 2018 (FAO, 2020). Despite the rapid growth, the problems that shorty followed in the beginning is still present today (Overton et al., 2019). The ectoparasite *Lepeophtheirus salmonis*, commonly known as the sea lice (salmon lice), is native to Norwegian waters, but started to become a serious problem for Atlantic salmon (hereafter referred to as salmon) aquaculture farmers as early as in the 1970s (Brandal & Egidius, 1979). Fast forward forty years and the parasite is now the most prominent issue in salmonid aquaculture (Costello, 2006), and the sea lice infestation pressure on wild stocks of Salmonids in Norway is the main factor regulating prospects of expansion of the industry (Costello, 2009; Johansen et al., 2011; Overton et al., 2019; Torrissen et al., 2013) (Figure 1).

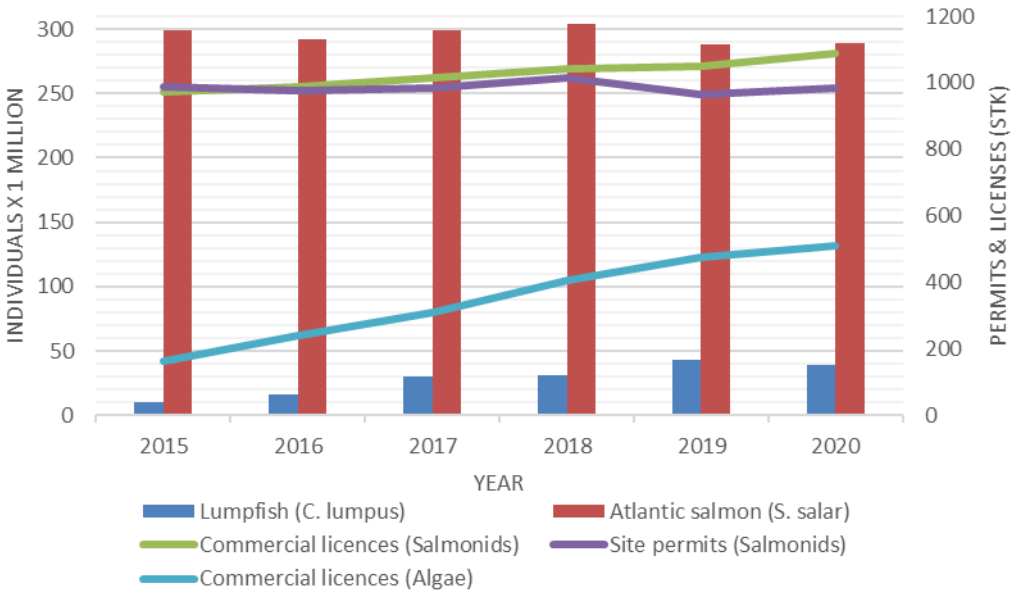


Figure 1: The number of individuals times 1 million of Lumpfish (*C. lumpus) (*blue bar) and Atlantic salmon *(S. salar)* smolt (<250 g) (red bar) put out in food-fish production of Atlantic salmon aquaculture per year. Green line represents the number of commercial licences regarding salmonid grow out production per year and the purple line represents the number of site-permits in sea water regarding salmonid grow out production per year. Pale blue line represents the number of algae licences per year. (Data sourced from: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier)

Chemical sea lice treatments

To combat the sea lice problem, numerous pest control strategies have been tried out, and the first solution was to use chemotherapeutic substances. The chemicals were administer as bath treatments, such as organophosphates, pyrethroids, and hydrogen peroxide (Aaen et al., 2015), or as feed additives such as emamectin benzoate and benzoyl ureas (Aaen et al., 2015; Haya et al., 2005). The chemical bath treatments were effective, until reduced sensitivity and resistance became a problem (Denholm et al., 2002; Helgensen et al., 2017; Helgesen et al., 2015; Sevatdal et al., 2005; Treasurer et al., 2000). Resistance towards the feed additives have also been observed along the Norwegian coast (Helgensen et al., 2017; Jones et al., 2013; Lees et al., 2008; NIVA, 2011). The mode of action of the active substance of organophosphates it that they block the cleavage of acetyl-choline, which is vital in arthropod nerve system, and benzourone-ureas block ecdysis by inhibiting chitin synthesis. The chemotherapeutants involved in chemical delousing will evidently come in contact with the surrounding environment, and may thus inflict substantial harm or even death to arthropods in near vicinity to the farm (Fjørtoft et al., 2017; Macken et al., 2015; Overton et al., 2019). The use of these chemicals have thus halted, much due to the negative effects the chemicals may have on the non-target species (Macken et al., 2015; NIVA, 2011). Through the extensive use, sea lice now exhibit a reduced sensitivity to chemotherapeutants in Norwegian salmon aquaculture. Resistance have even been found in sea lice from wild salmonids (Fjørtoft et al., 2017). New methods to combat the sea lice infestation thus had to be implemented (Helgesen et al., 2015).

Non-chemical sea lice treatments

The non-medical treatments consist of mechanical, thermic and freshwater treatments (Svåsand et al., 2017). The freshwater delousing treatment consists of holding the salmon in freshwater to make the lice detach from the salmon (Powell et al., 2015; Reynolds, 2015). Mechanical delousing methods consist of flushing the Salmon using seawater, alone or in combination with brushes to make the lice fall off or dragged off the salmon (Gismervik et al., 2017). The thermic method consists of the salmon begin exposed to heated seawater, at a temperature of 28 – 34 degrees Celsius, for a short period of time (30 sec), which immobilizes the sea lice (Poppe et al., 2018; Sommerset et al., 2020).

These treatments do not expose the environment to potentially unwanted and harmful chemicals, but the fish welfare is compromised (Cerbule & Godfroid, 2020; Overton et al., 2019), and the use of these non-medical treatments have increased tremendously. From 2015 to 2020 the amount of time spent on thermic delousing went from merely 36 to 1736 weeks (Sommerset et al., 2021). The number for mechanical delousing was 34 and 816 for 2015 and 2020, respectively. The freshwater treatment as a means to combat

sea lice, have not been not as popular as the two previous, but the number of weeks using this treatment in 2015 was 28, and between 2019 and 2020 the use of the freshwater treatment increased by 53 % to 238 weeks in total (Sommerset et al., 2021).

Since these treatments are based on the biology of the ectoparasite, it is a growing worry that, as seen with the chemotherapeutants, the lice will evolve tolerance to a higher temperature and lower salinity, e.g. the thermic and freshwater treatments that are currently being used (Ljungfeldt et al., 2017). Another concern is the welfare issues these treatments give rise to, and the lack of unbiased scientific information describing these unwanted side effects (Cerbule & Godfroid, 2020).

It is stated in the fish health report (2020) that mechanical damages caused by delousing is the main welfare issue in Norwegian salmon farming (Sommerset et al., 2021). The use of thermic and mechanical delousing caused a higher mortality post treatment than chemical and freshwater treatments, and for both treatments loss of scales and haemorrhage in the gills increased significantly after the treatments (Gismervik et al., 2017; Sommerset et al., 2020). Serious damages such as haemorrhage in the brain and loss of epidermis have also been found in thermically treated salmon, and seem to be a recurring problem (Grøntvedt et al., 2015; Poppe et al., 2018). However, a third, preventive delousing method that neither harm the environment nor the salmon exists, namely biological delousing.

Appendix B – Digital sections of the lumpfish skin from Agder, Austevoll and Fitjar

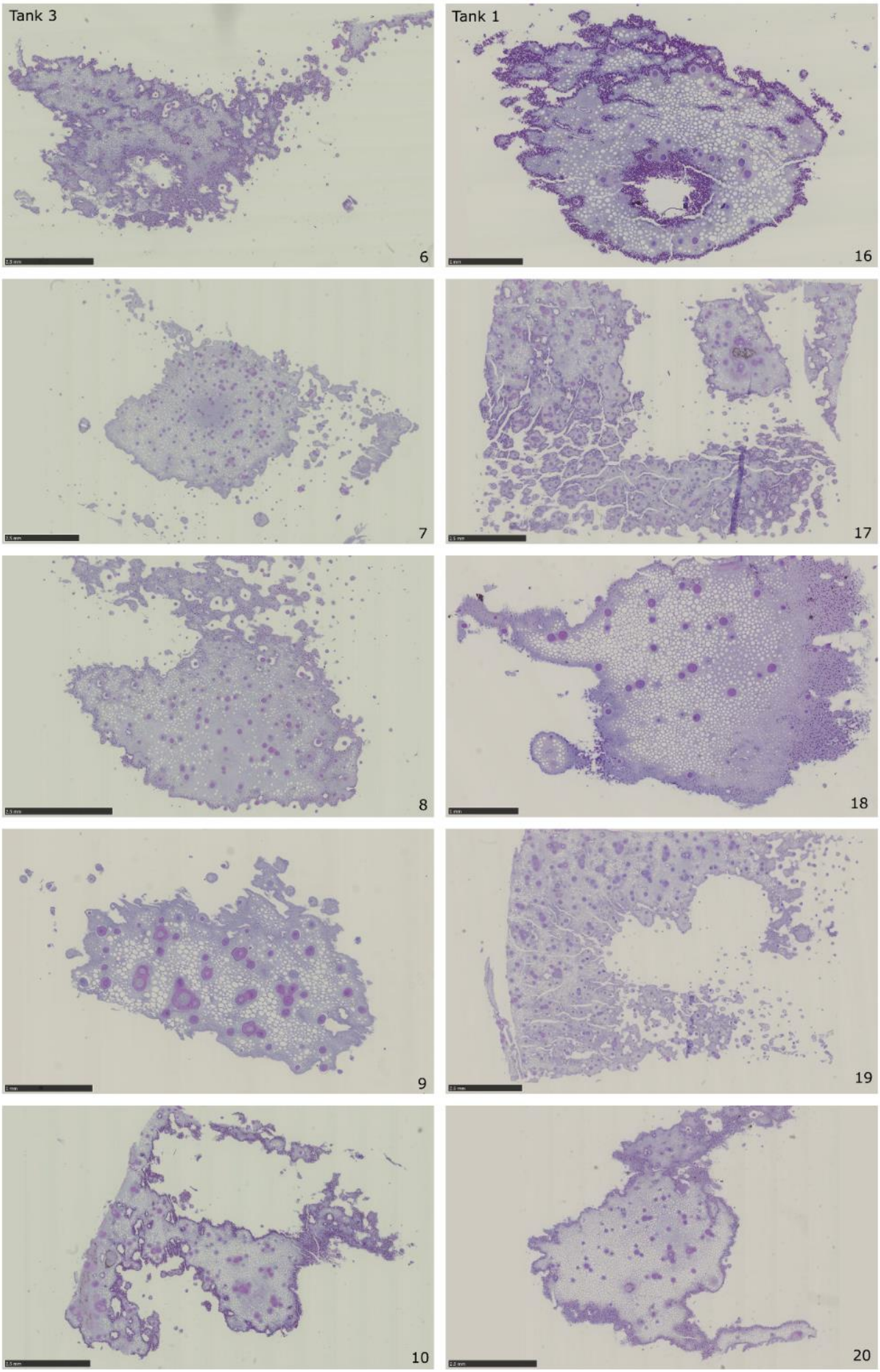


Figure 1: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 1 - Agder: Tank 1 and 3: treatment with natural kelp (*Saccharina latissima*)

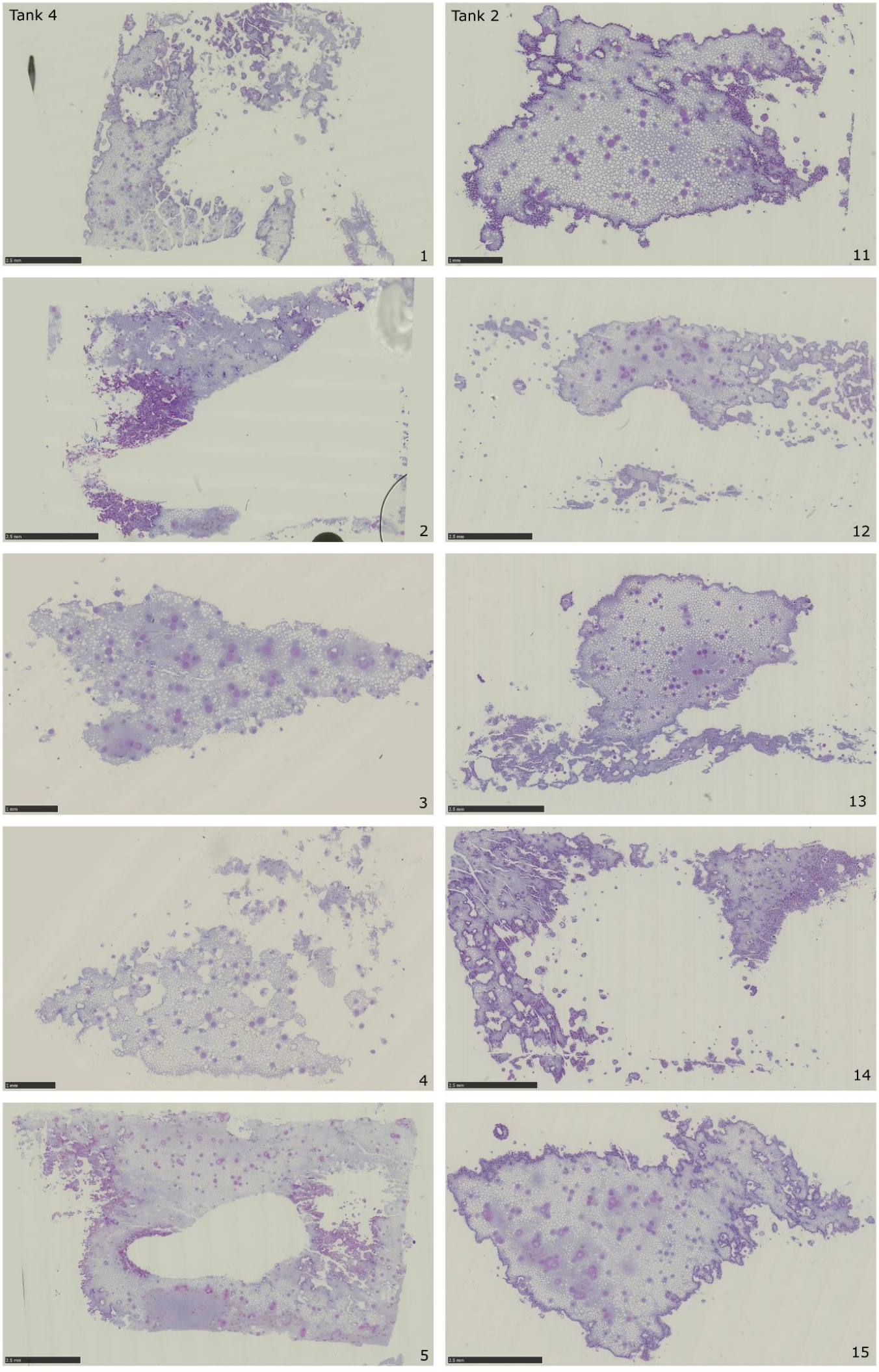


Figure 2: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 1 - Agder: Tank 2 and 4: treatment with no kelp

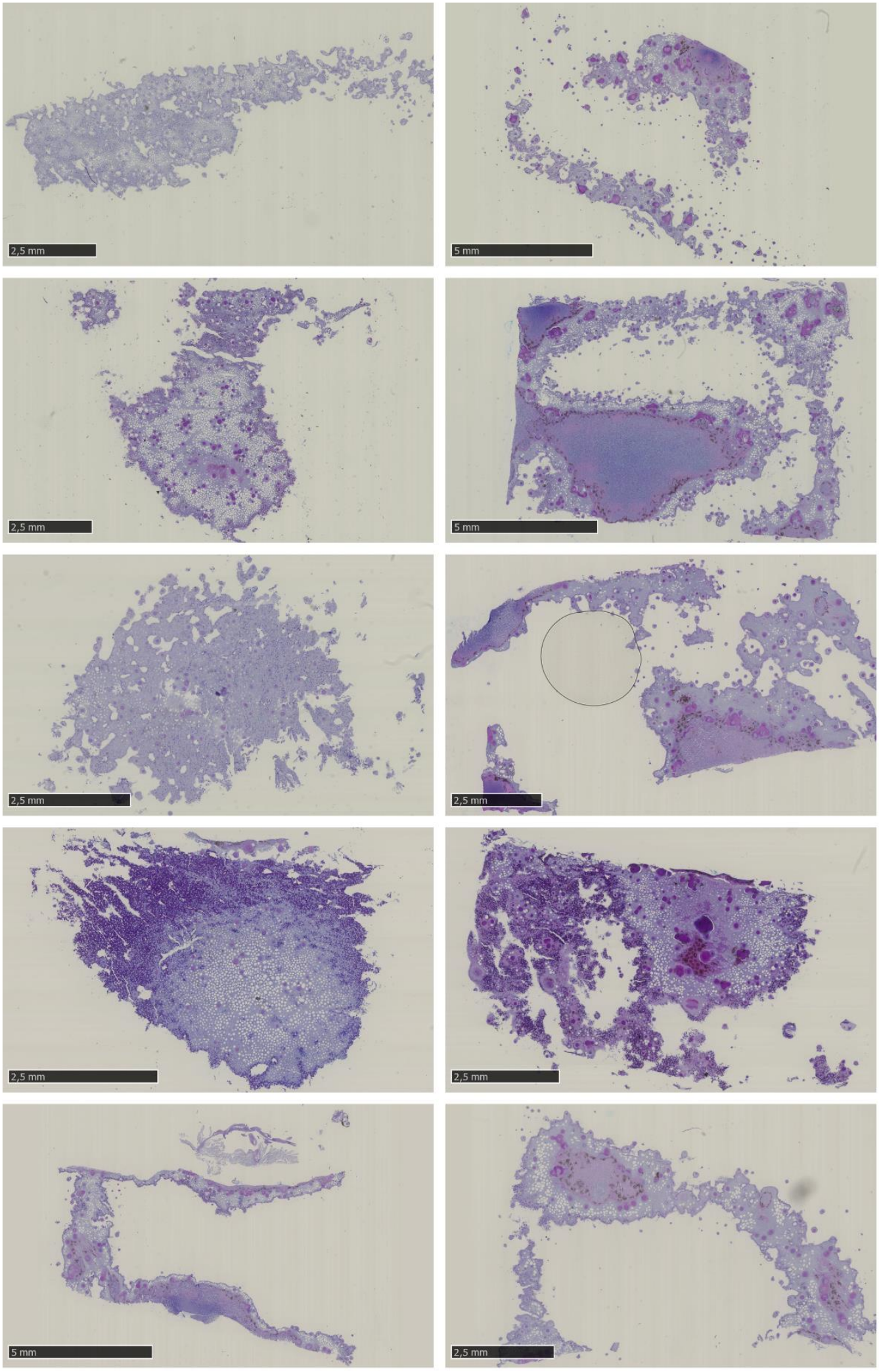


Figure 3: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (Sampling 1, 08.09.2020): Tank 13 and 16: treatment with natural kelp (*Laminaria digitata*)

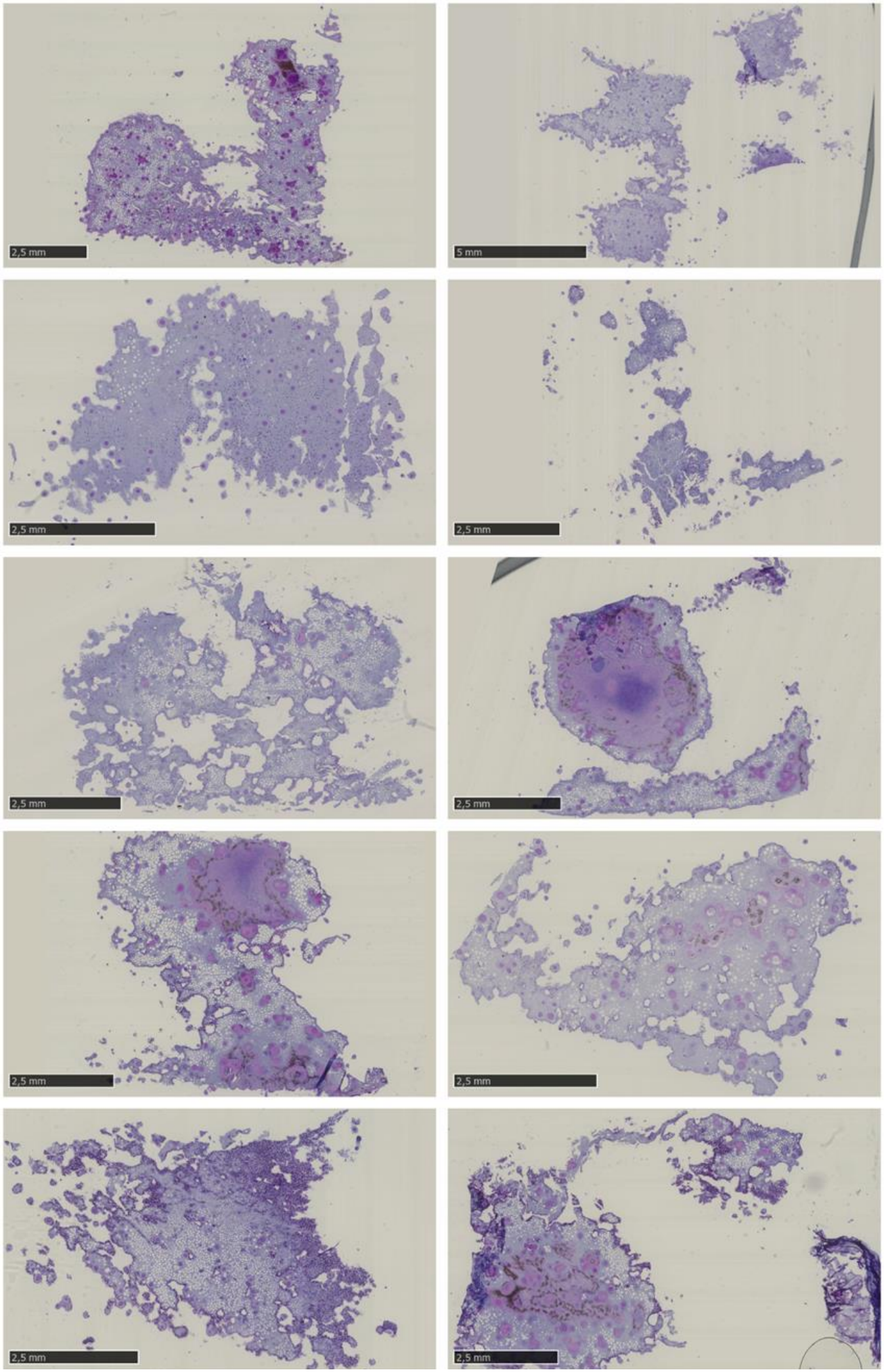


Figure 4: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (Sampling 1, 08.09.2020): Tank 14 and 17: treatment with plastic kelp

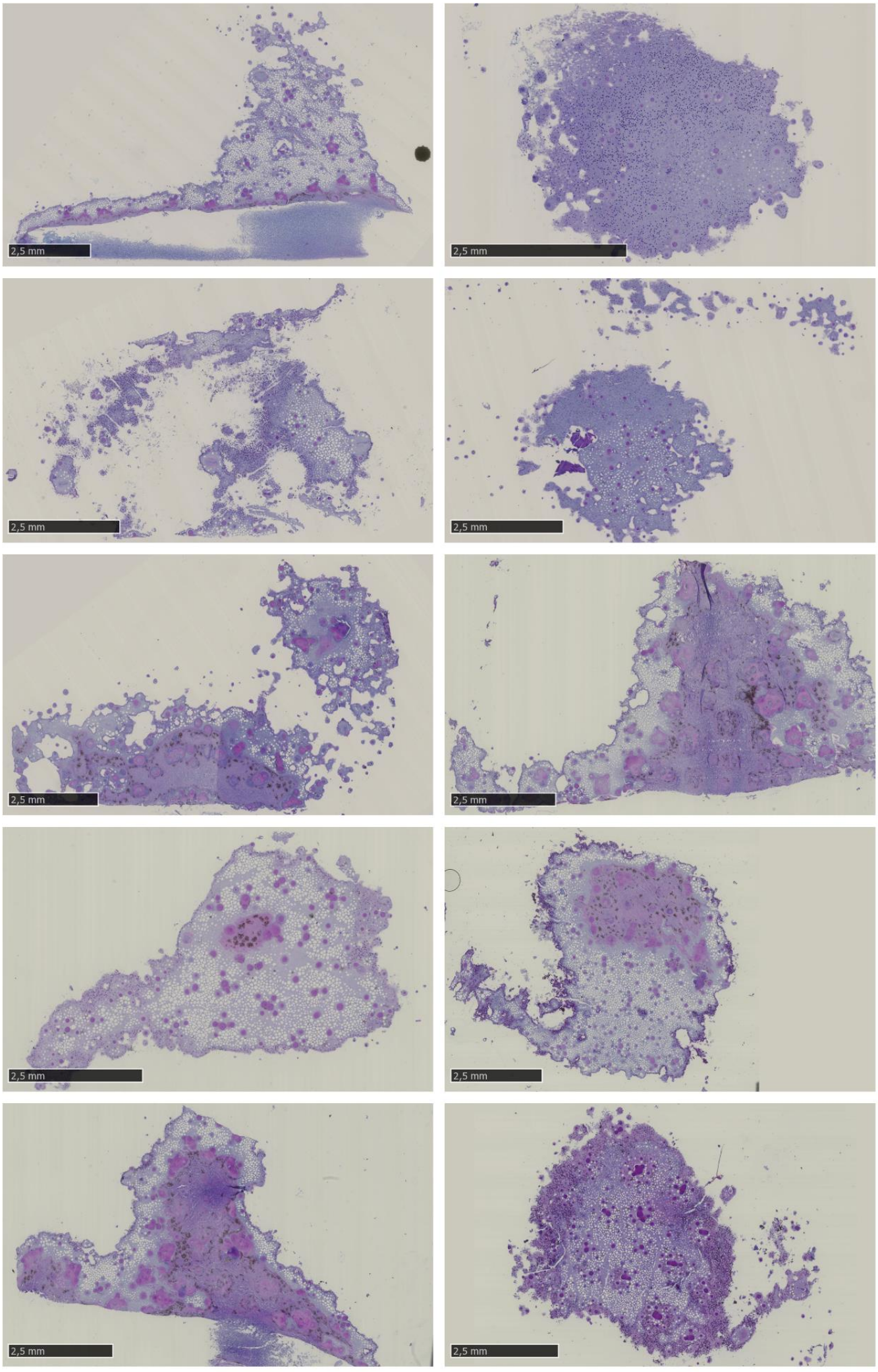


Figure 5: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (Sampling 1, 08.09.2020): Tank 15 and 18: treatment with no kelp (control)

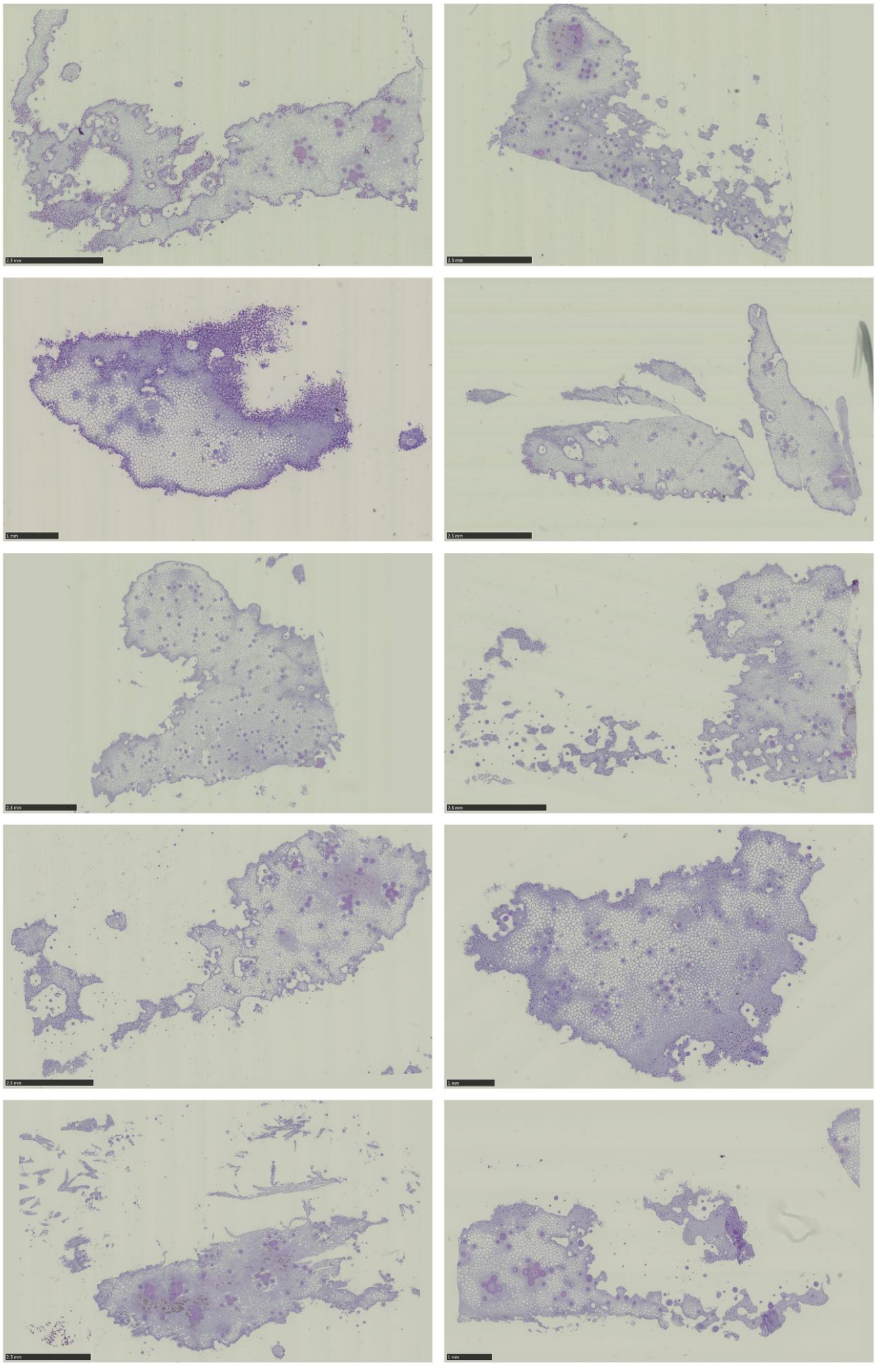


Figure 6: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (2. sampling 29.09.2020): Tank 13 and 16: treatment with natural kelp (*Laminaria digitata*)

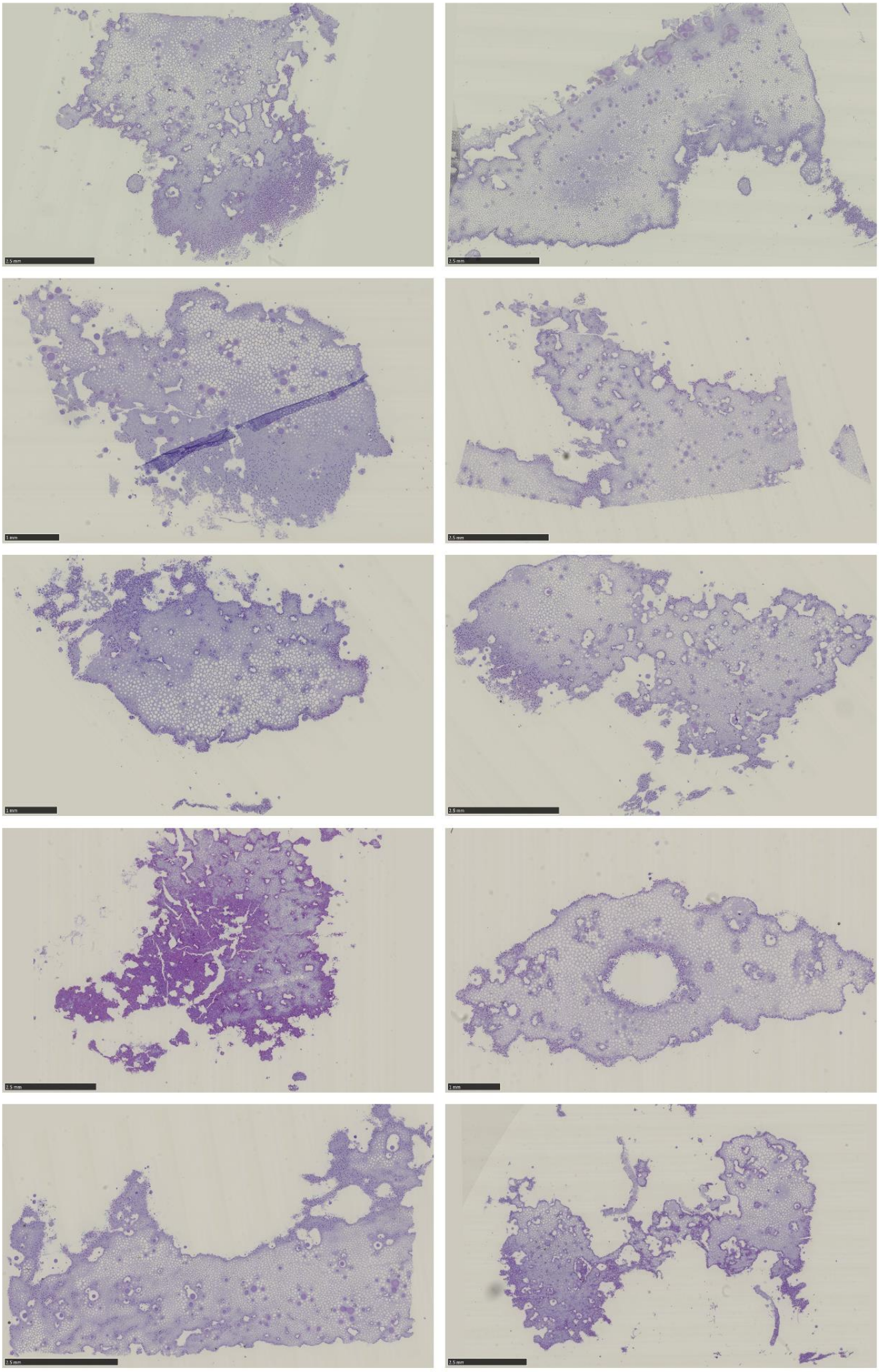


Figure 7: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (2. sampling 29.09.2020): Tank 14 and 17: treatment with plastic kelp. (Lightning mark - Fish nr 17: VD of 47.1 %)

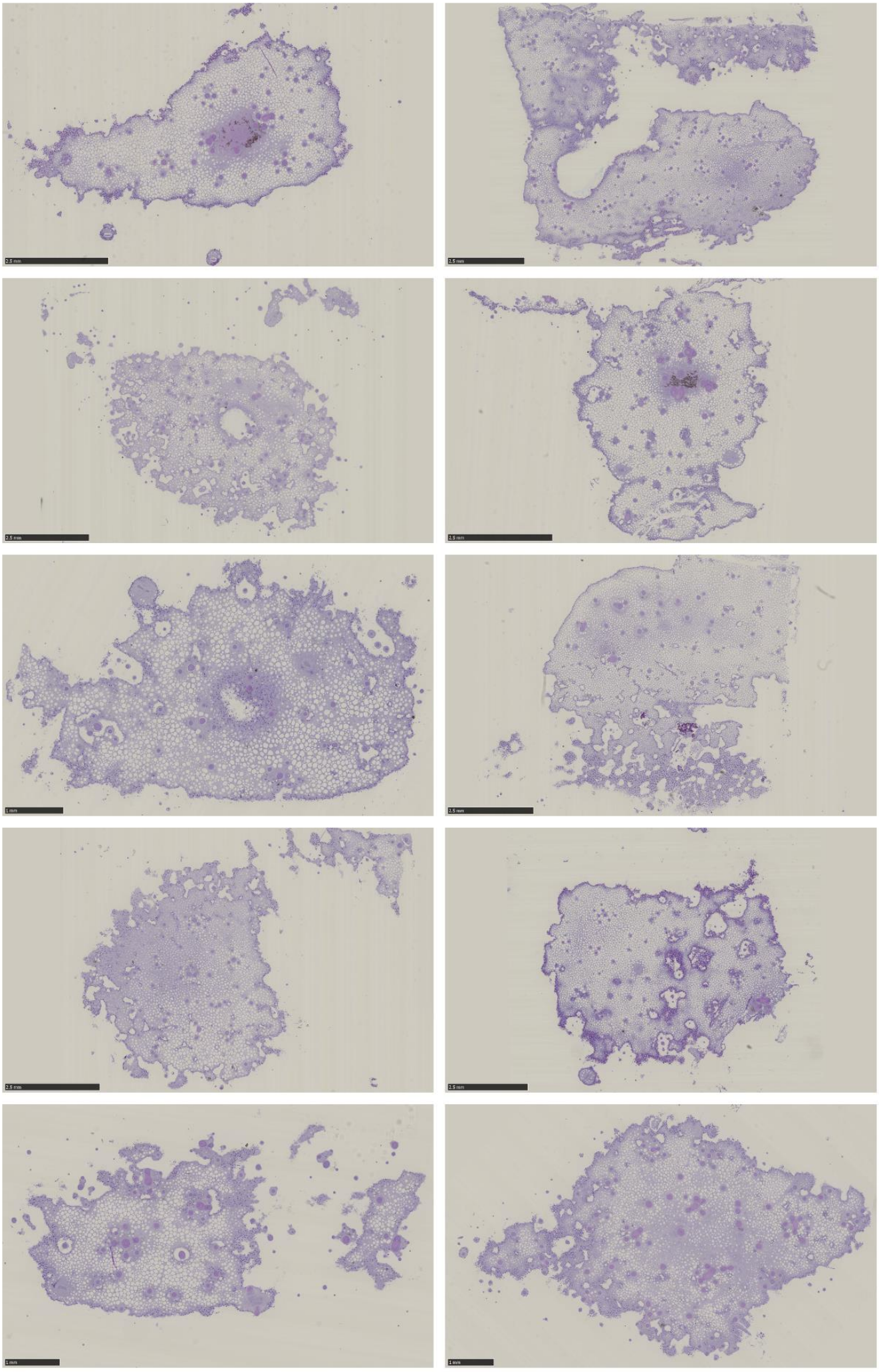


Figure 8: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (2. sampling 29.09.2020): Tank 15 and 18: treatment with no kelp (control).

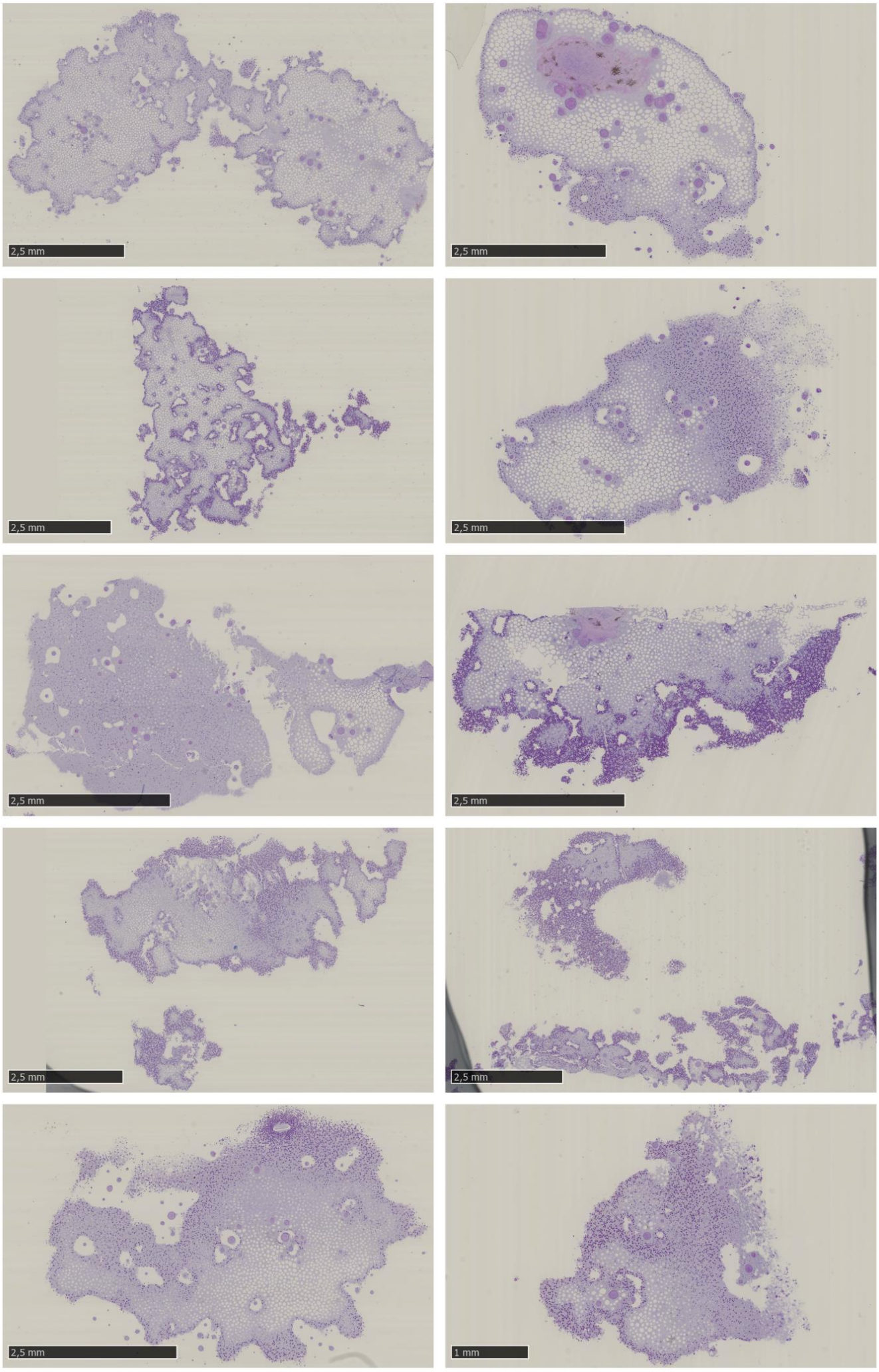


Figure 9: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (3. sampling 16.10.2020): Tank 13 and 16: treatment with natural kelp (*Laminaria digitata).*

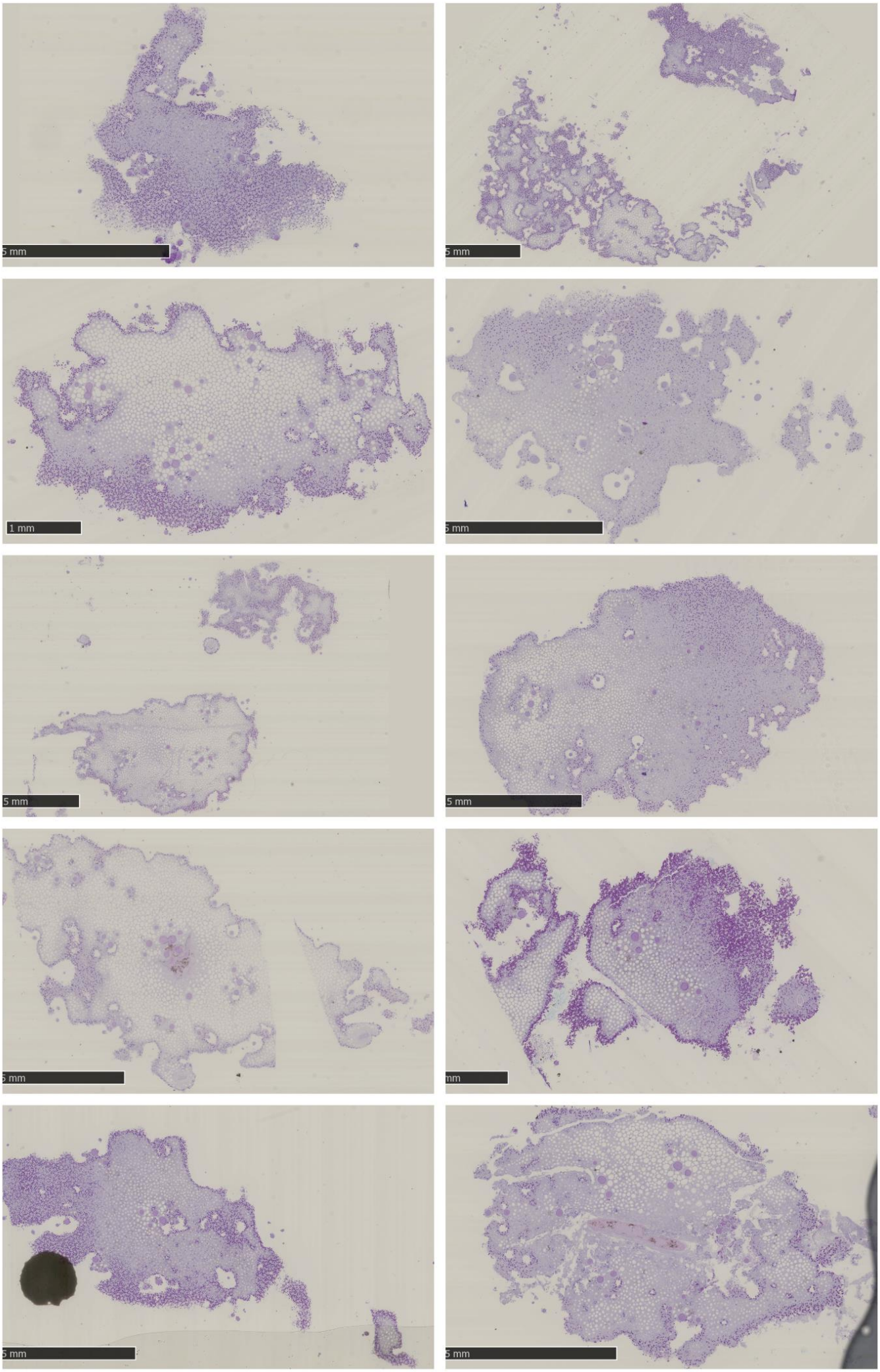


Figure 10: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (3. sampling 16.10.2020): Tank 14 and 17: treatment with plastic kelp.

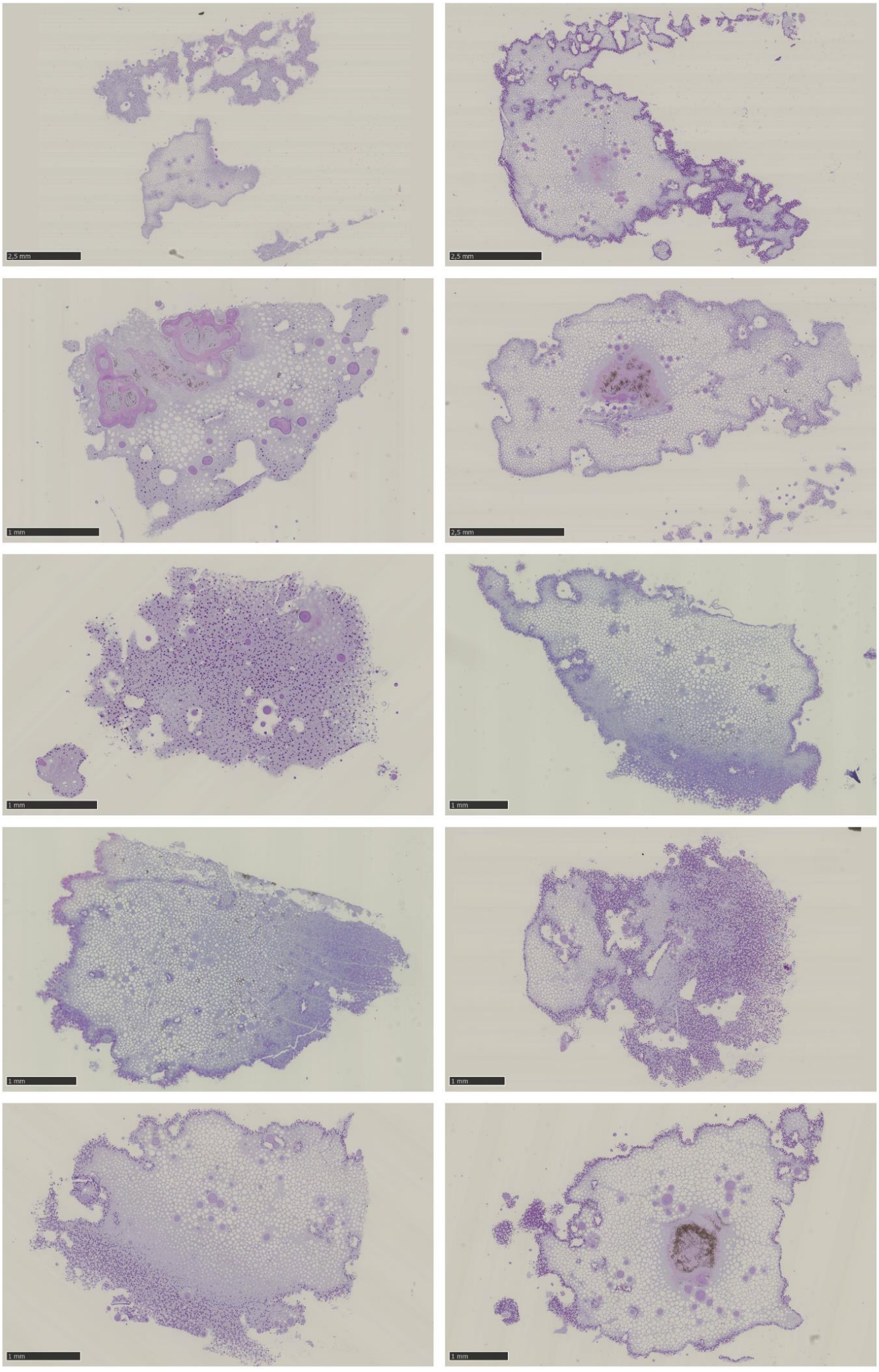


Figure 11: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 2 – Austevoll (3. sampling 16.10.2020): Tank 15 and 18: treatment with no kelp (control).

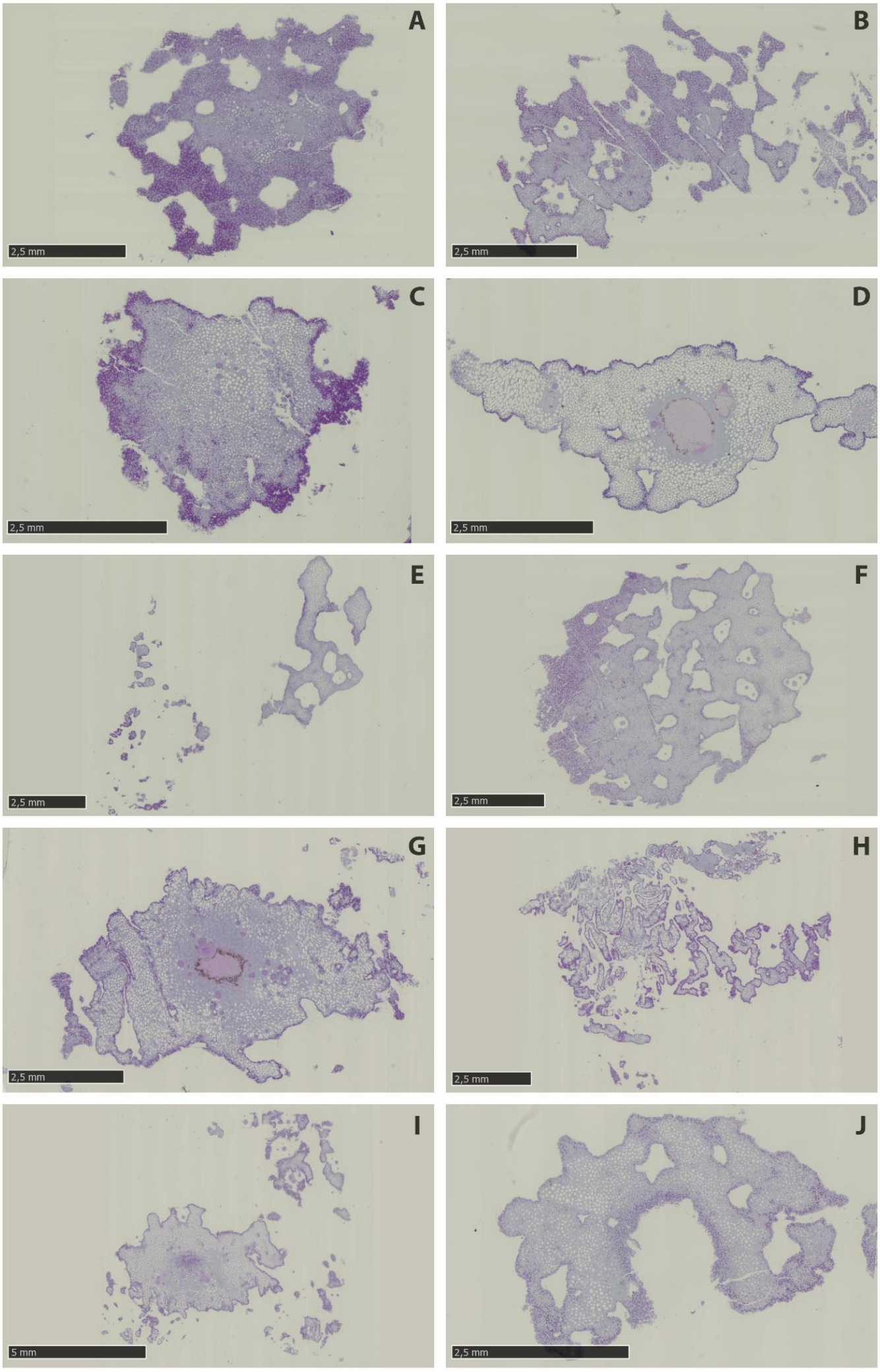


Figure 12: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 3 – Fitjar (10.11.2020): Sea cage 5: treatment with natural kelp (*Laminaria digitata*).

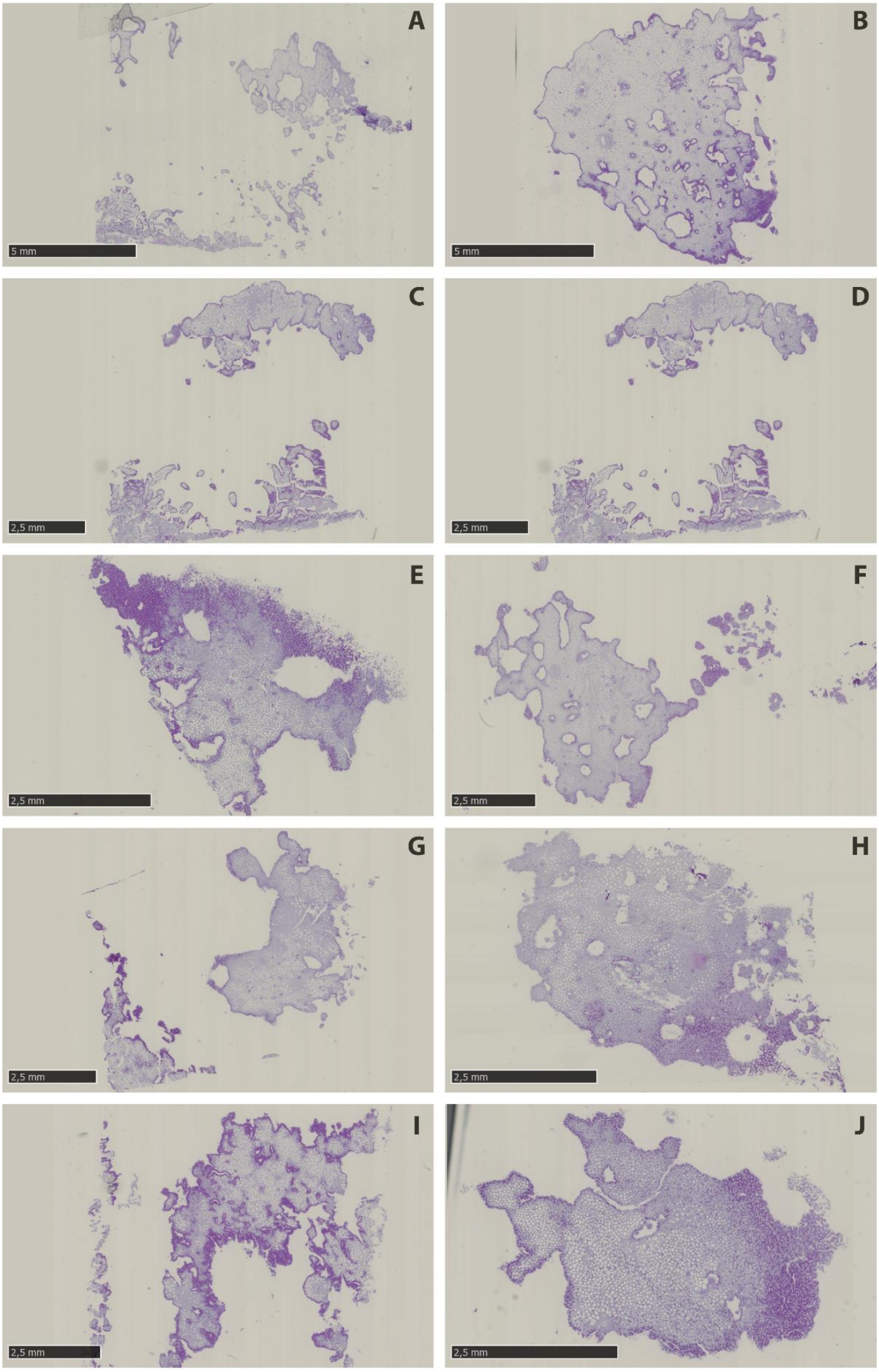


Figure 13: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 3 – Fitjar (10.11.2020): Sea cage 5: treatment with plastic kelp.

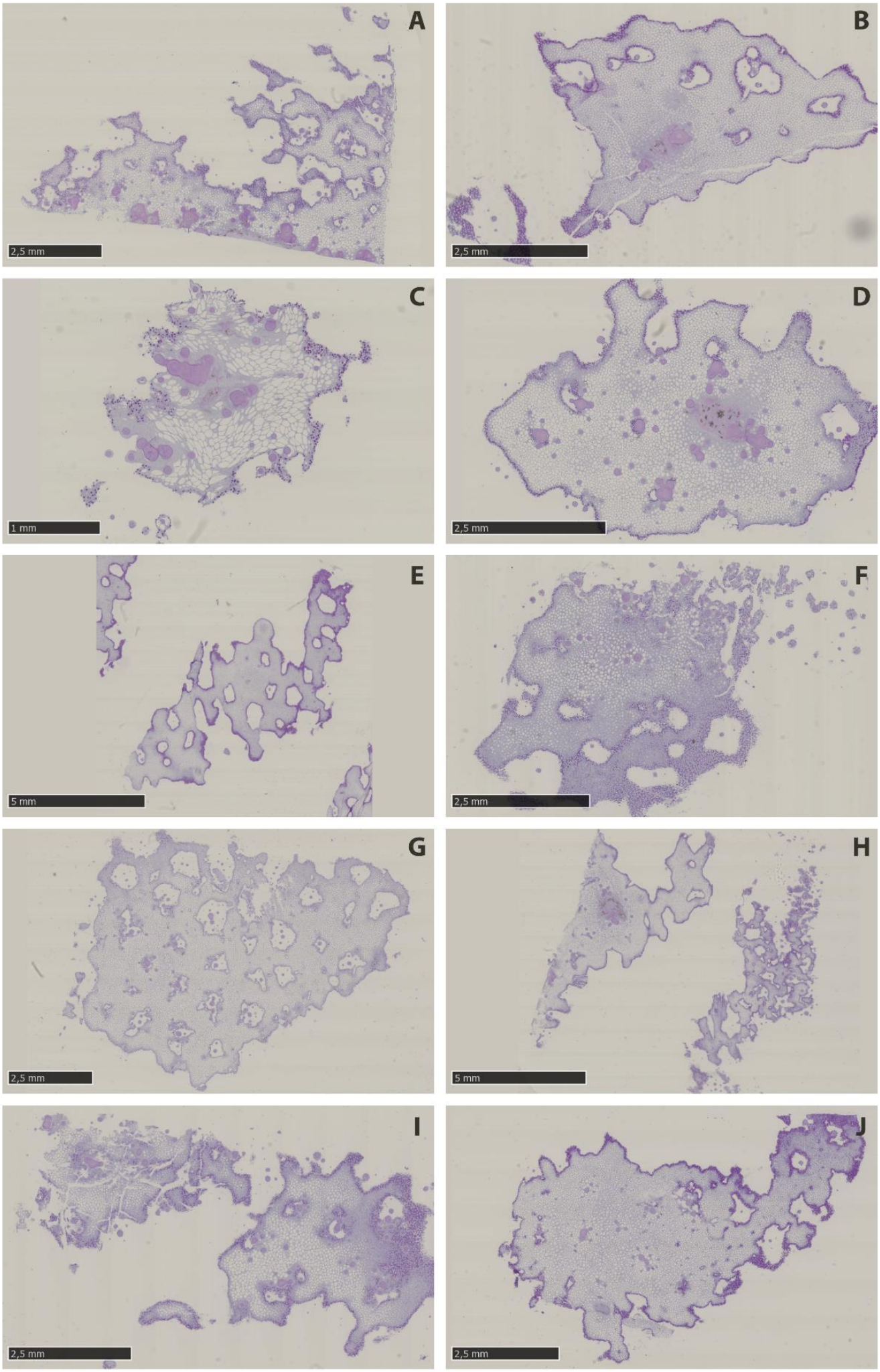


Figure 14: Tangentially sectioned lumpfish (Cyclopterus lumpus) skin slides, from experiment 3 – Fitjar (10.11.2020): Sea cage 5: treatment with no kelp (control).

Appendix C – Mucous cell morphometrics

Table 1: Means and standard error per treatment for the measurements mucous cell mean area, volumetric density and defence activity per location.

Agder 14.07.2020						
Treatment	*Mean area*	*SD Mean area*	*Density*	*SD Density*	*Defence activity*	*SD Defence activity*
Real kelp	*110,006048*	*21,8965088*	*0,07174288*	*0,0610247*	*0,59975845*	*0,47904025*
Without kelp	*114,540127*	*38,7456078*	*0,04975344*	*0,04338662*	*0,36071703*	*0,27320657*
Austevoll 1 - 08.09.2020						
Treatment	*Mean area*	*SD Mean area*	*Density*	*SD Density*	*Defence activity*	*SD Defence activity*
Real kelp	*122,113618*	*27,1135447*	*0,06319018*	*0,07254293*	*0,45863869*	*0,39032101*
Without kelp	*133,372287*	*21,5687891*	*0,05247595*	*0,03665305*	*0,37278625*	*0,21217261*
Plastic kelp	*128,438053*	*15,5383235*	*0,06325404*	*0,049212*	*0,46620782*	*0,29712152*
Austevoll 2 - 29.09.2020						
Treatment	*Mean area*	*SD Mean area*	*Density*	*SD Density*	*Defence activity*	*SD Defence activity*
Real kelp	*169,420847*	*23,8195359*	*0,05071247*	*0,0557466*	*0,27650072*	*0,25406727*
Without kelp	*153,626758*	*24,1687942*	*0,04598687*	*0,03909867*	*0,28249025*	*0,20206758*
Plastic kelp	*170,431266*	*23,6657223*	*0,1097974*	*0,13474683*	*0,59483083*	*0,6197563*
Austevoll 3- 16.10.2020						
Treatment	*Mean area*	*SD Mean area*	*Density*	*SD Density*	*Defence activity*	*SD Defence activity*
Real kelp	*176,962469*	*32,6161198*	*0,11756827*	*0,07311242*	*0,62720371*	*0,32397574*
Without kelp	*185,28589*	*33,2860674*	*0,11143336*	*0,07544852*	*0,56225114*	*0,31410117*
Plastic kelp	*169,469482*	*21,3797048*	*0,12472664*	*0,07781065*	*0,70987956*	*0,39583358*
Fitjar - 10.11.2020						
Treatment	*Mean area*	*SD Mean area*	*Density*	*SD Density*	*Defence activity*	*SD Defence activity*
Real kelp	*139,421643*	*31,7953346*	*0,17380066*	*0,07669988*	*1,22504083*	*0,36505484*
Without kelp	*167,911322*	*28,4122728*	*0,10270059*	*0,04662839*	*0,60318079*	*0,2521922*
Plastic kelp	*138,176045*	*25,5353151*	*0,1349319*	*0,04602314*	*0,98020399*	*0,32688746*

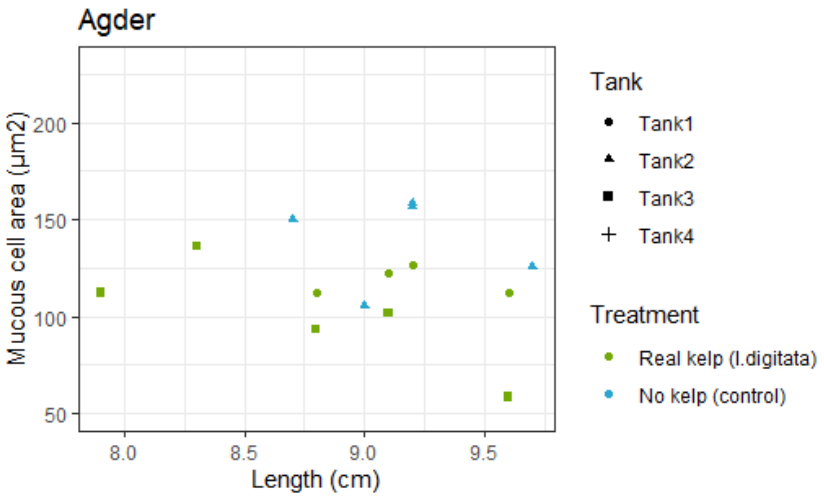


Figure 15: Length vs. mucous cell mean area (Experiment 1, 14.07.2020, n= 20)

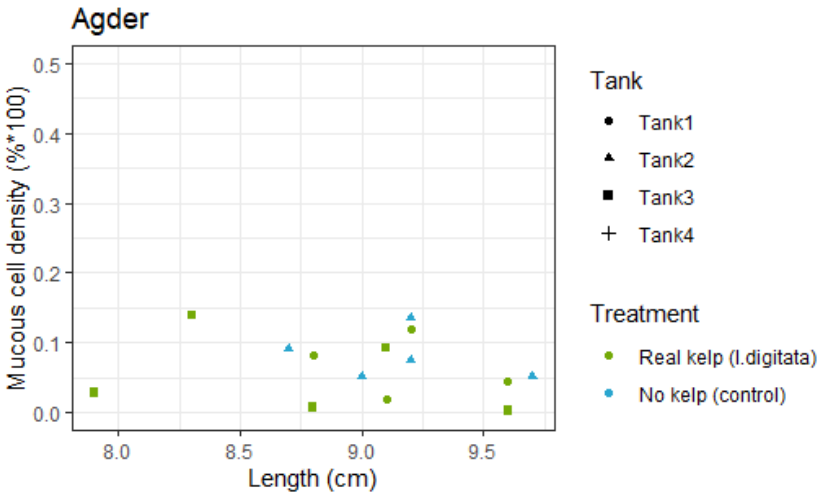


Figure 16: Length vs. mucous cell density (Experiment 1, 14.07.2020, n= 20)

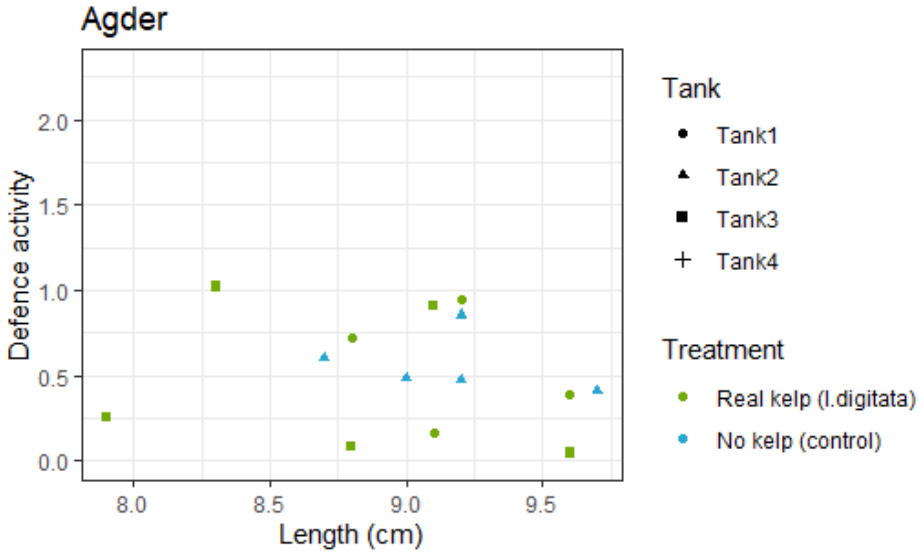


Figure 17: Length vs. defence activity (Experiment 1, 14.07.2020, n= 20)

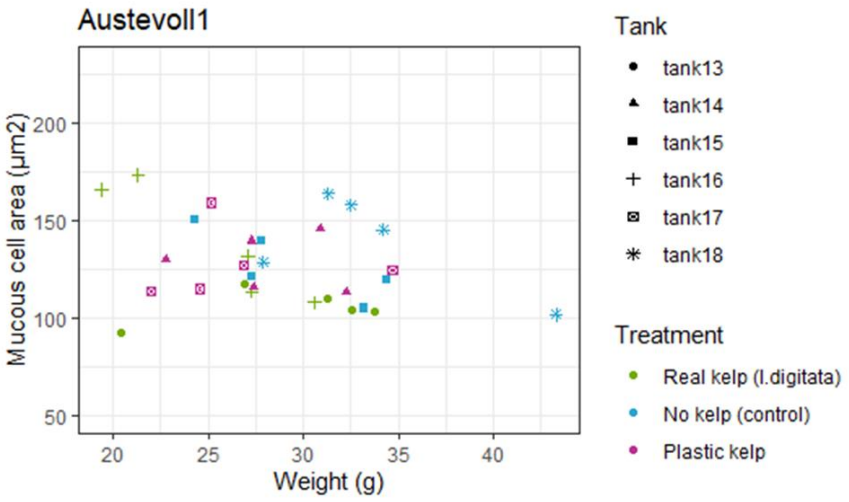


Figure 18: Weight vs. mucous cell mean area (Experiment 2, 1. Sampling, 08.09.2020, n= 30)

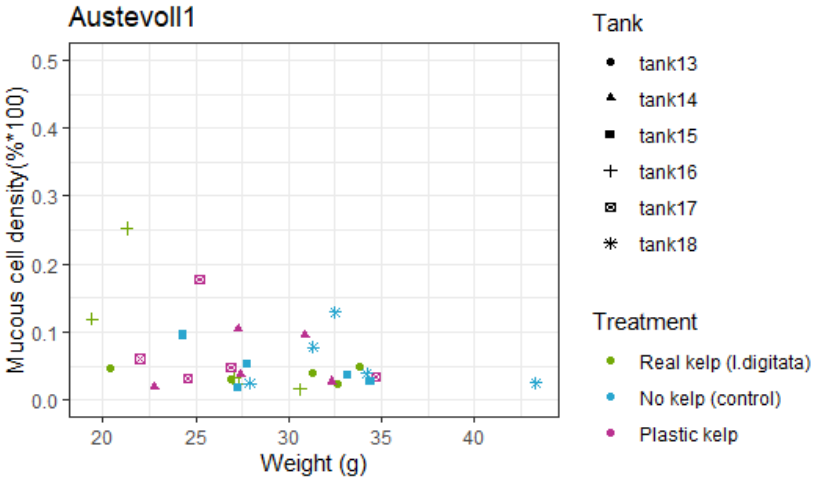


Figure 19: Weight vs. mucous cell density (Experiment 2, 1. Sampling, 08.09.2020, n= 30)

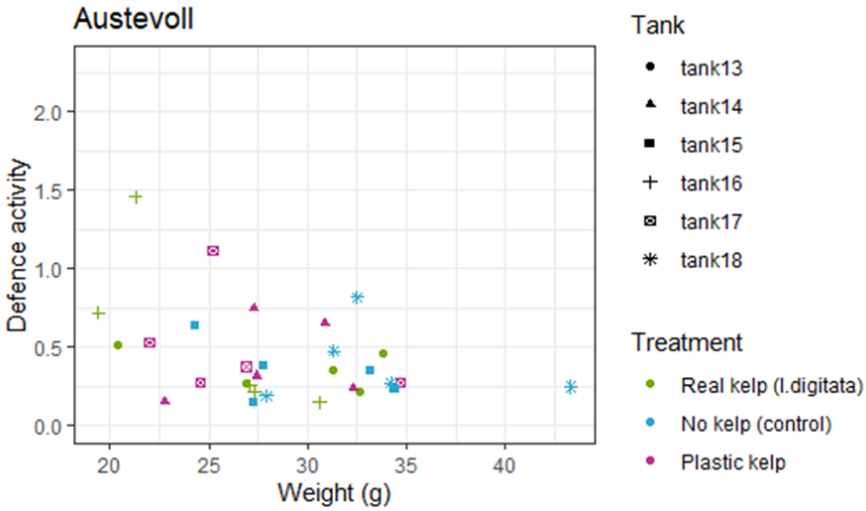


Figure 20: Weight vs. defence activity (Experiment 2, 1. Sampling, 08.09.2020, n= 30)

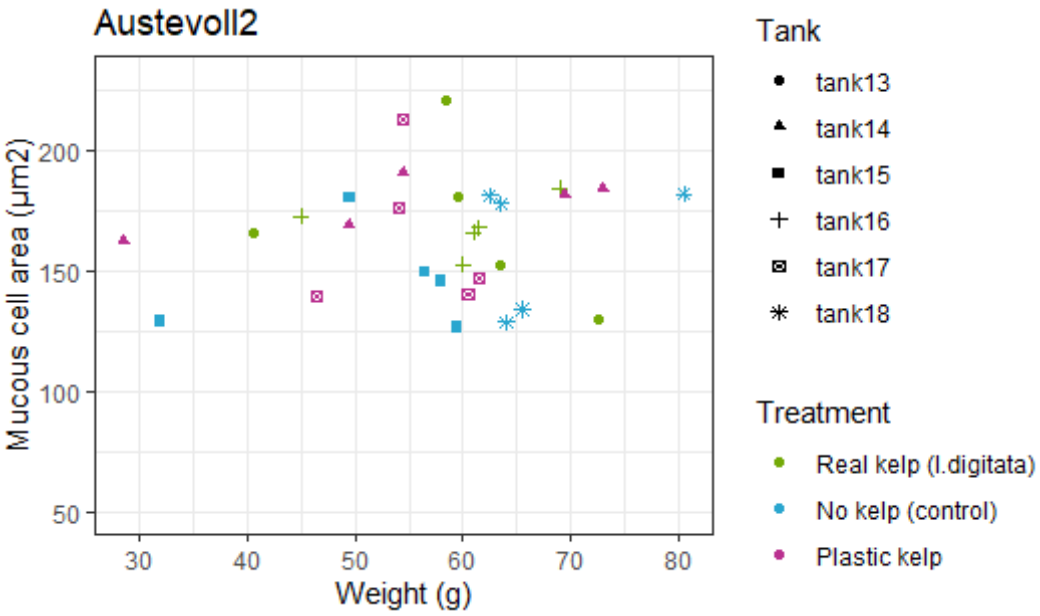


Figure 21: Weight vs. mucous cell mean area (Experiment 2, 2. Sampling, 29.09.2020, n= 30)

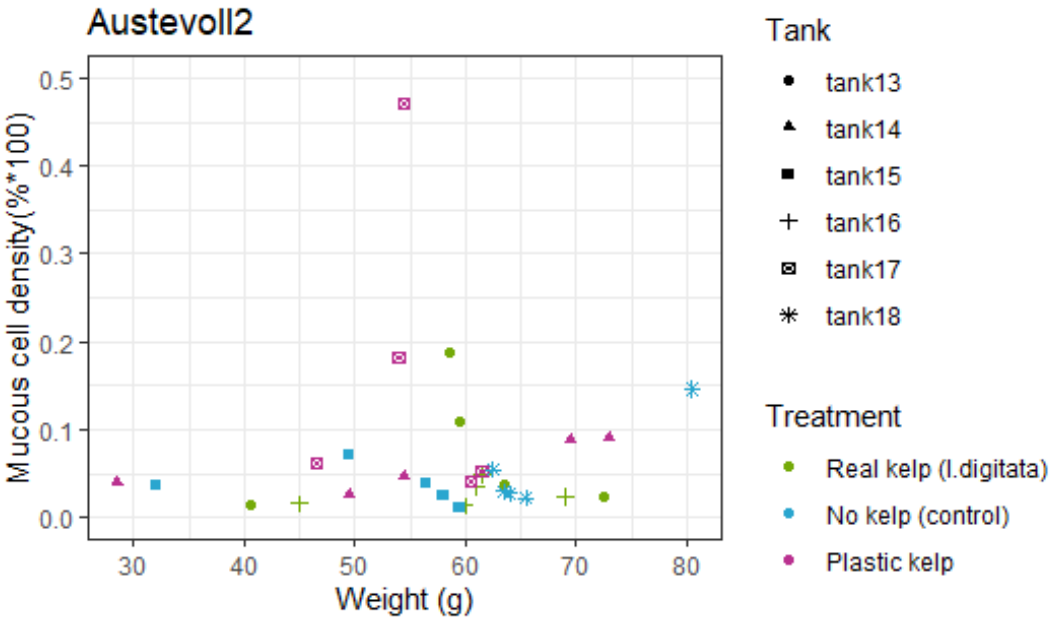


Figure 22: Weight vs. mucous cell density (Experiment 2, 2. Sampling, 29.09.2020, n= 30)

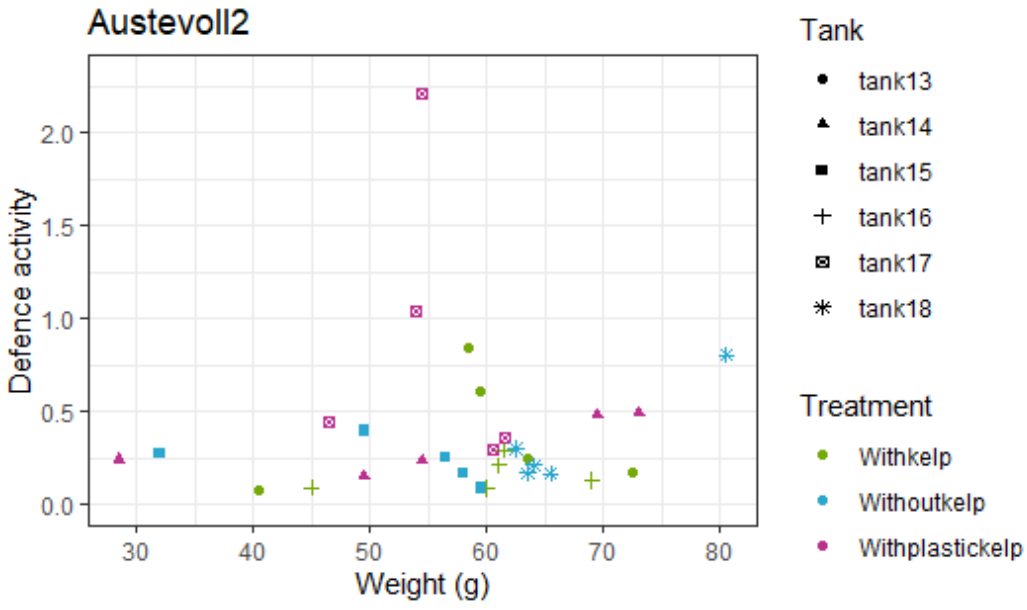


Figure 23: Weight vs. defence activity (Experiment 2, 2. Sampling, 29.09.2020, n= 30)

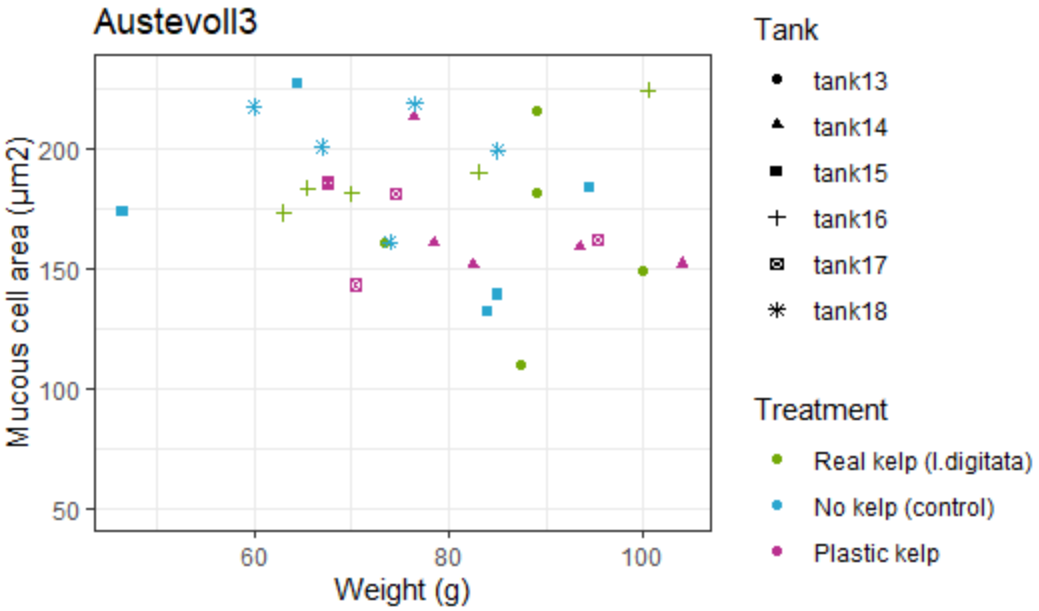


Figure 24: Weight vs. mucous cell area (Experiment 2, 3. Sampling, 16.10.2020, n= 30)

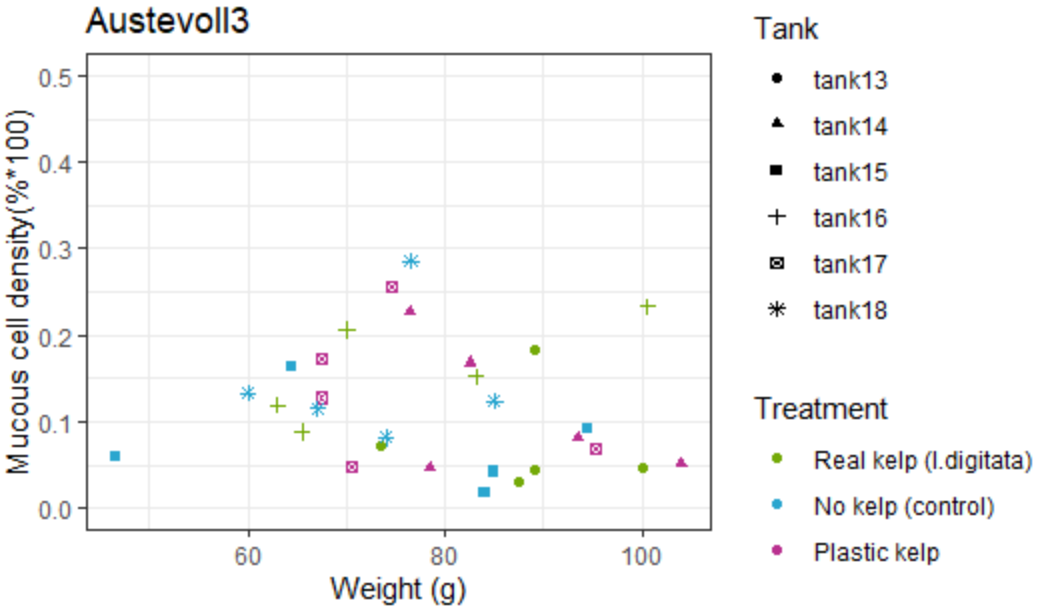


Figure 25: Weight vs. mucous cell density (Experiment 2, 3. Sampling, 16.10.2020, n= 30)

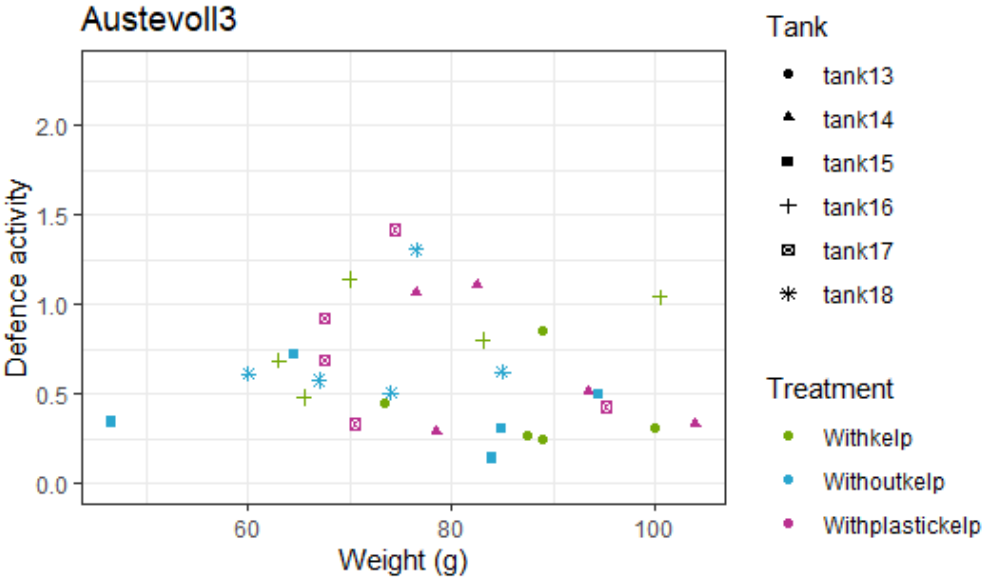


Figure 26: Weight vs. defence activity (Experiment 2, 3. Sampling, 16.10.2020, n= 30)

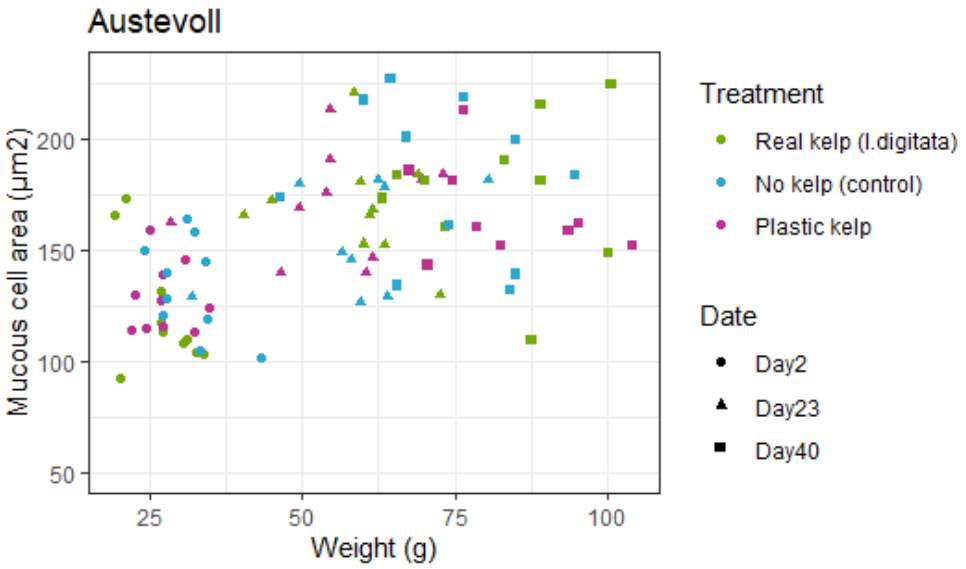


Figure 27: Weight vs. mucous cell area (Experiment 2, n= 90)

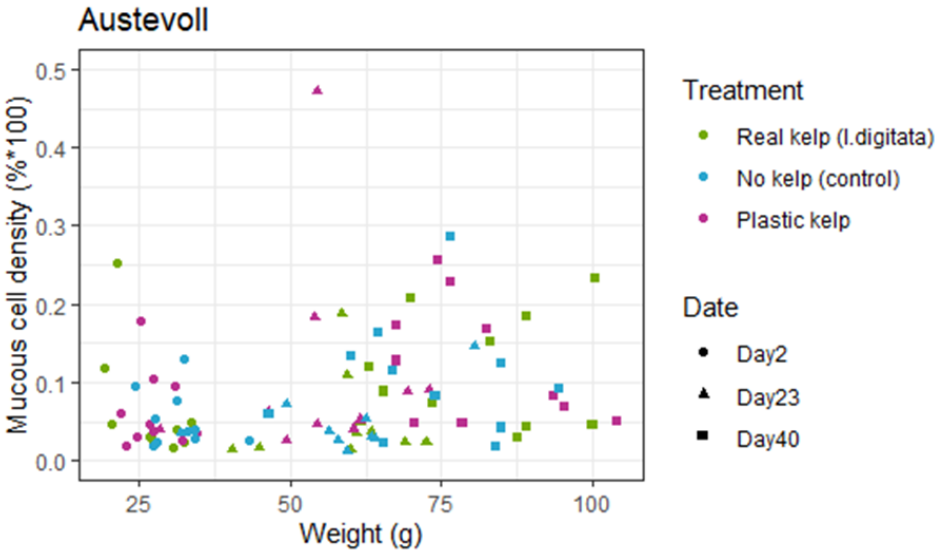


Figure 28: Weight vs. mucous cell density (Experiment 2, n=90)

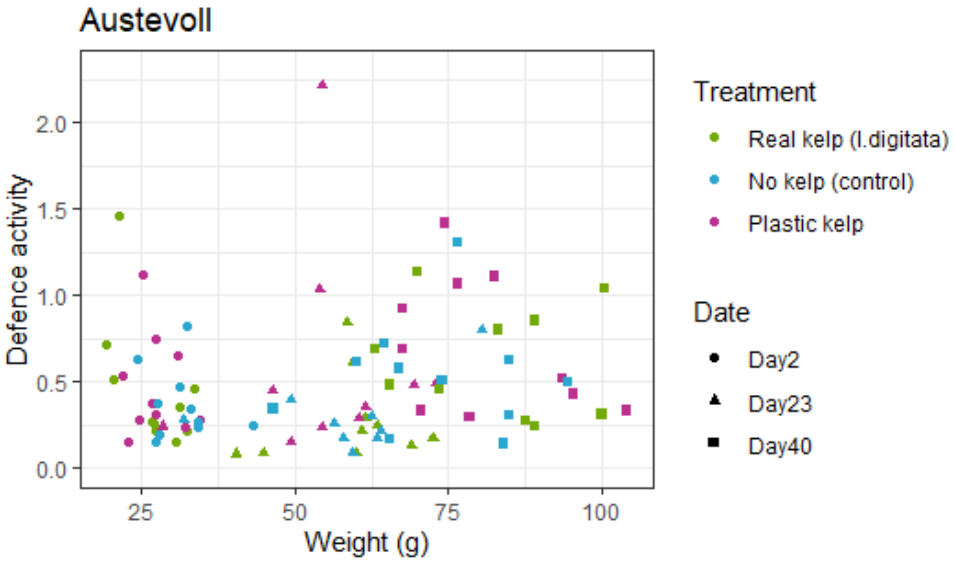


Figure 29: Weight vs. defence activity (Experiment 2, n=90)

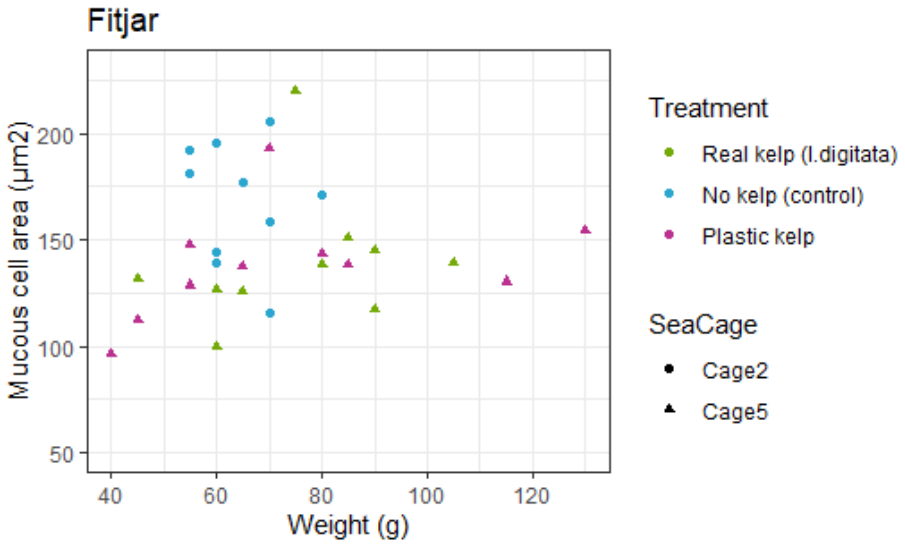


Figure 30: Weight vs. mucous cell area (Experiment 3, n= 30)

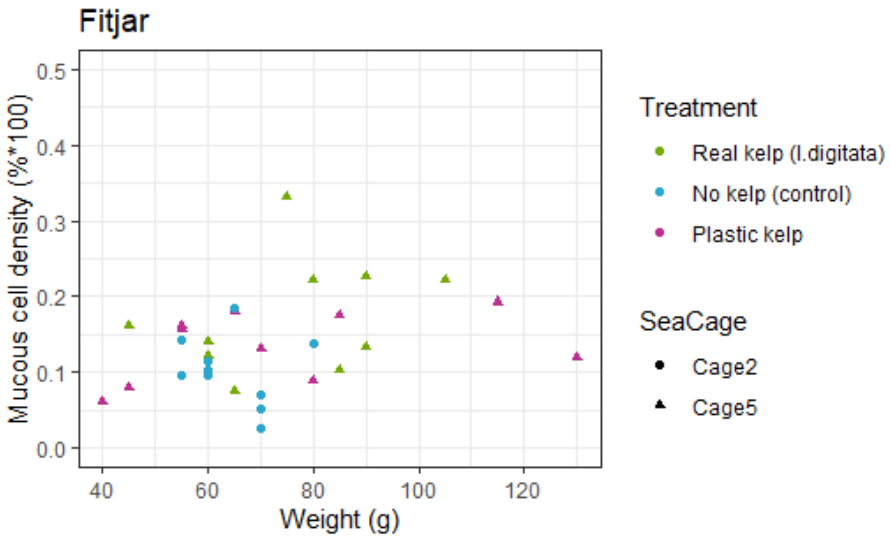


Figure 31: Weight vs. mucous cell density (Experiment 3, n=30)

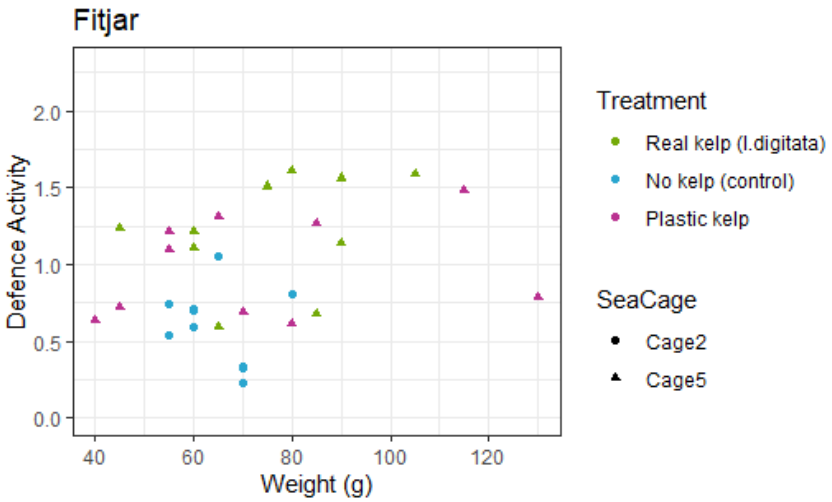


Figure 32: Weight vs. defence activity (Experiment 3, n=30)

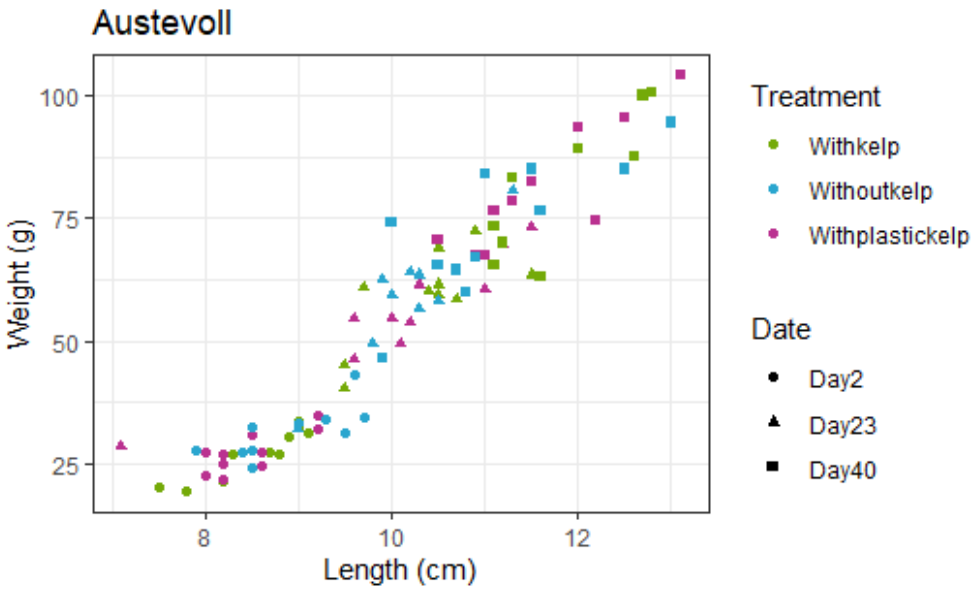


Figure 33: Weight vs. length (Experiment 2, n=90)

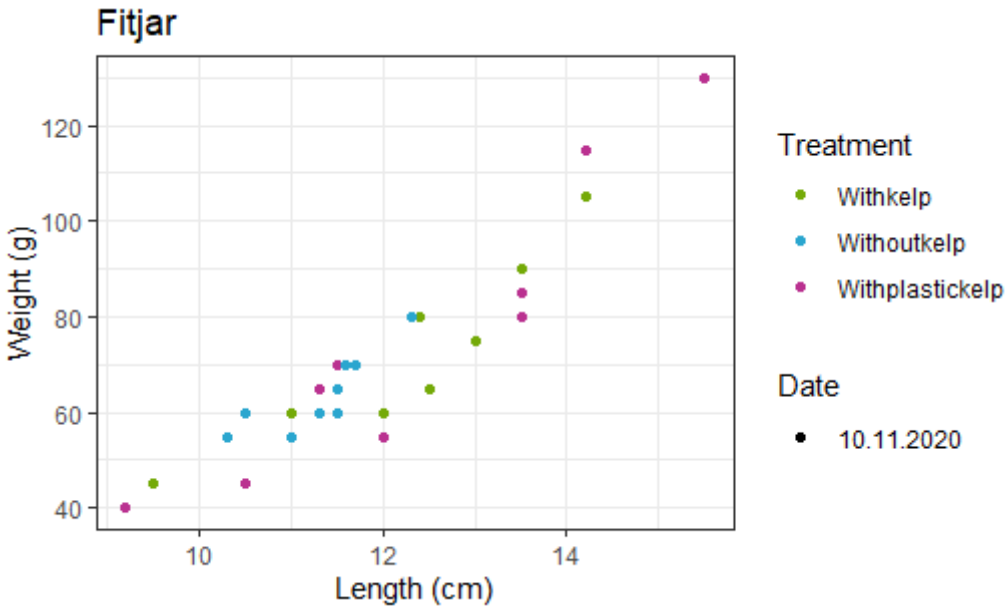


Figure 34: Weight vs length (Experiment 3, n=30)

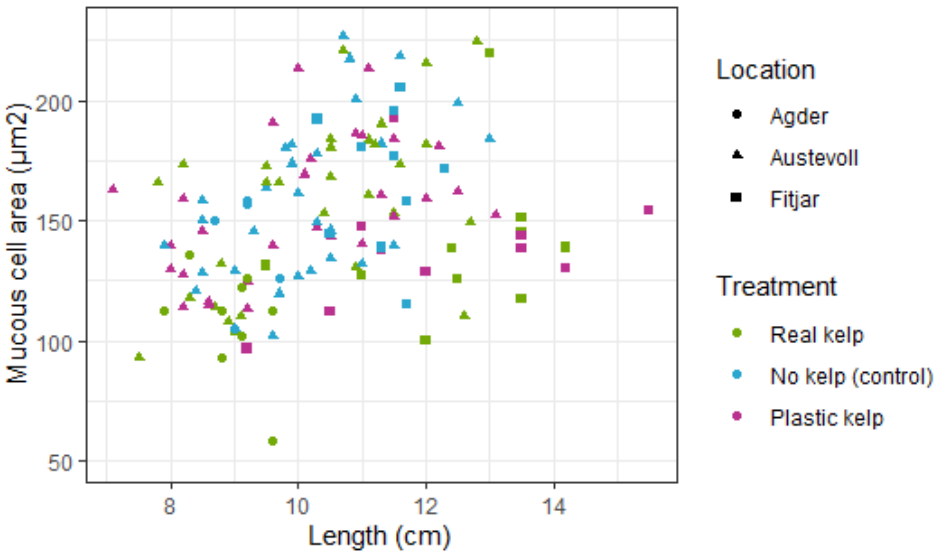


Figure 35: Length vs mucous cell area (Experiment 1, 2, and 3, n=134)

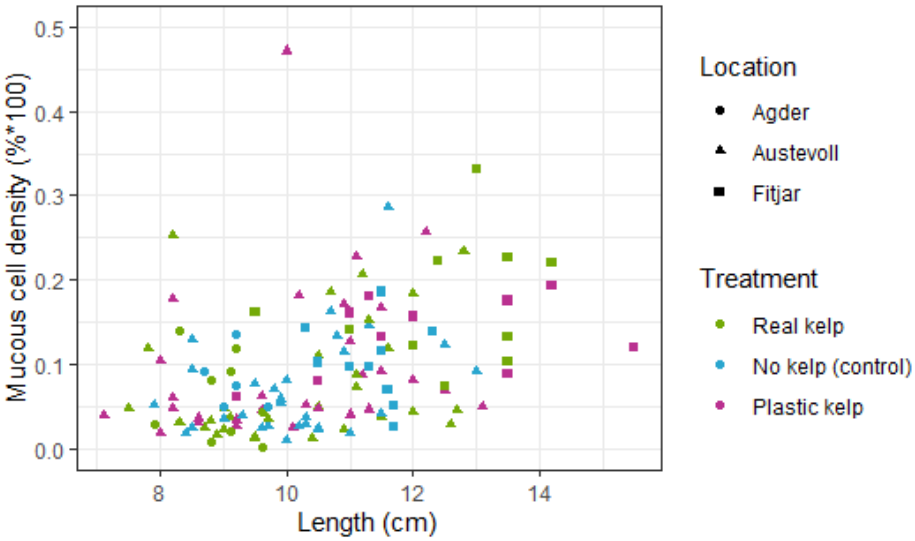


Figure 36: Length vs mucous cell density (Experiment 1, 2, and 3, n=134)

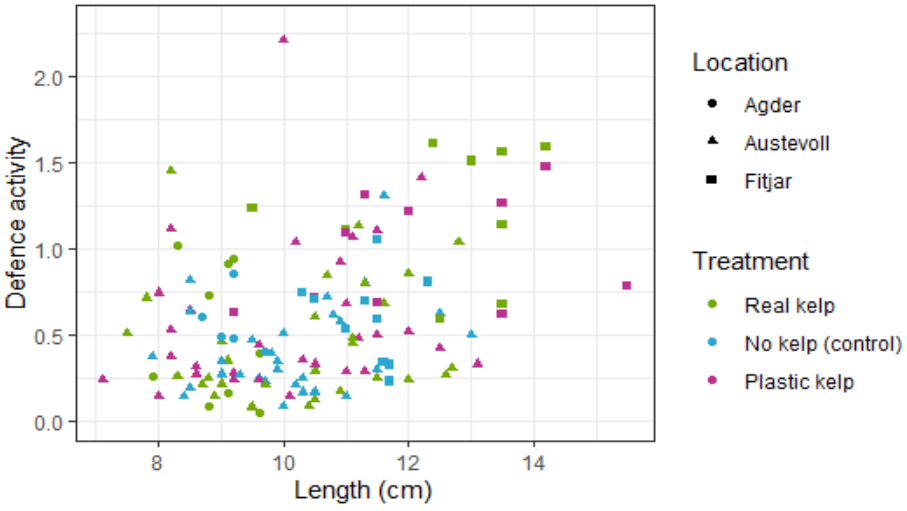


Figure 37: Length vs defence activity (experiment 1,2 and 3, n=134)

Table 3: Gram measurements from Austevoll and Fitjar

Location	FishID	Marked probes	Counting Frames	Volumetric Density	%Gram	Gram Area	Epithel-Groups	Area per group	Epithel Area
Fitjar	2,0	1,0	30,0	0,0	0,5	2741,3	207,0	2741,3	567459,0
Fitjar	8,0	20,0	48,0	0,1	8,4	54827,0	237,0	2741,3	649699,5
Fitjar	11,0	7,0	50,0	0,0	2,9	19189,4	239,0	2741,3	655182,2
Fitjar	16,0	54,0	42,0	0,2	19,9	148032,8	272,0	2741,3	745646,7
Fitjar	22,0	19,0	31,0	0,1	7,1	52085,6	267,0	2741,3	731939,9
Fitjar	27,0	16,0	39,0	0,1	8,3	43861,6	193,0	2741,3	529080,2
Austevoll	3,0	4,0	41,0	0,0	2,0	10965,4	198,0	2741,3	542786,9
Austevoll	4,0	2,0	30,0	0,0	1,1	5482,7	181,0	2741,3	496184,0
Austevoll	11,0	1,0	31,0	0,0	0,4	2741,3	227,0	2741,3	622286,0
Austevoll	18,0	27,0	29,0	0,1	13,6	74016,4	198,0	2741,3	542786,9
Austevoll	23,0	4,0	31,0	0,0	2,4	10965,4	169,0	2741,3	463287,8
Austevoll	30,0	67,0	32,0	0,4	42,4	183670,3	158,0	2741,3	433133,0

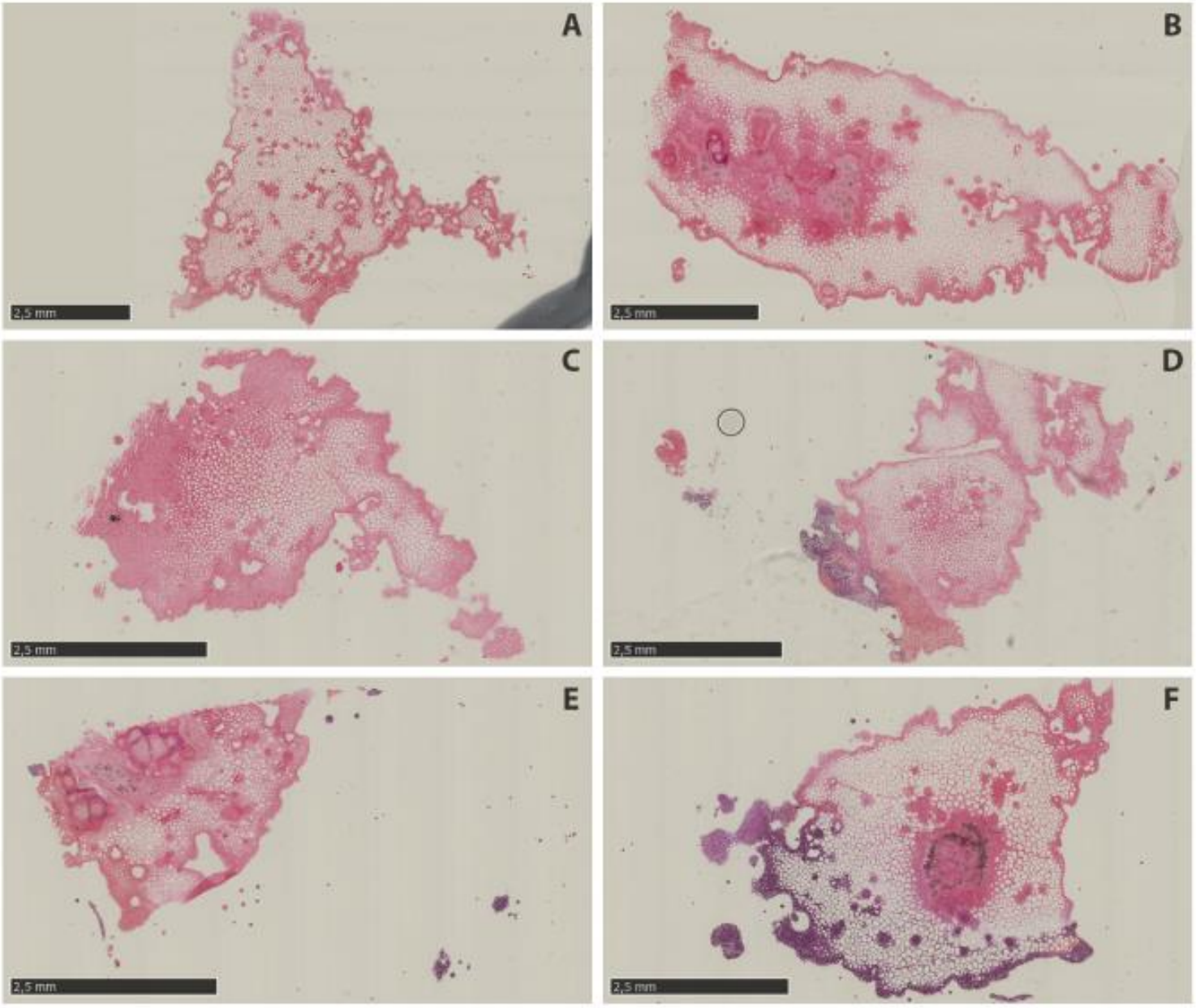


Figure 38: Gram-stained samples from Austevoll. A+B from the treatment with natural kelp, C+D from the treatment with plastic kelp and E+F from the treatment with no kelp.

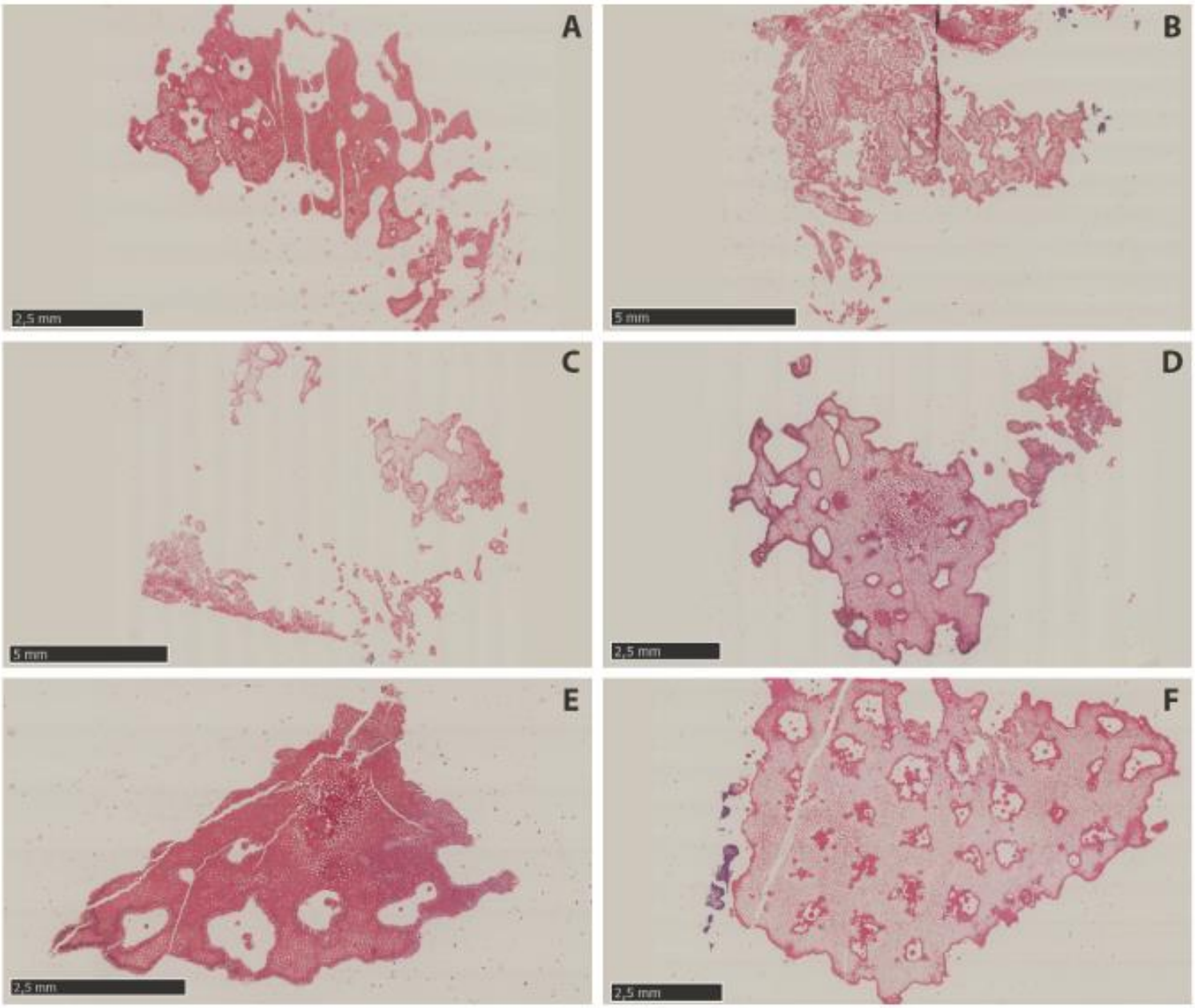


Figure 39: Gram-stained samples from Fitjar. A+B from the treatment with natural kelp, C+D from the treatment with plastic kelp and E+F from the treatment with no kelp.

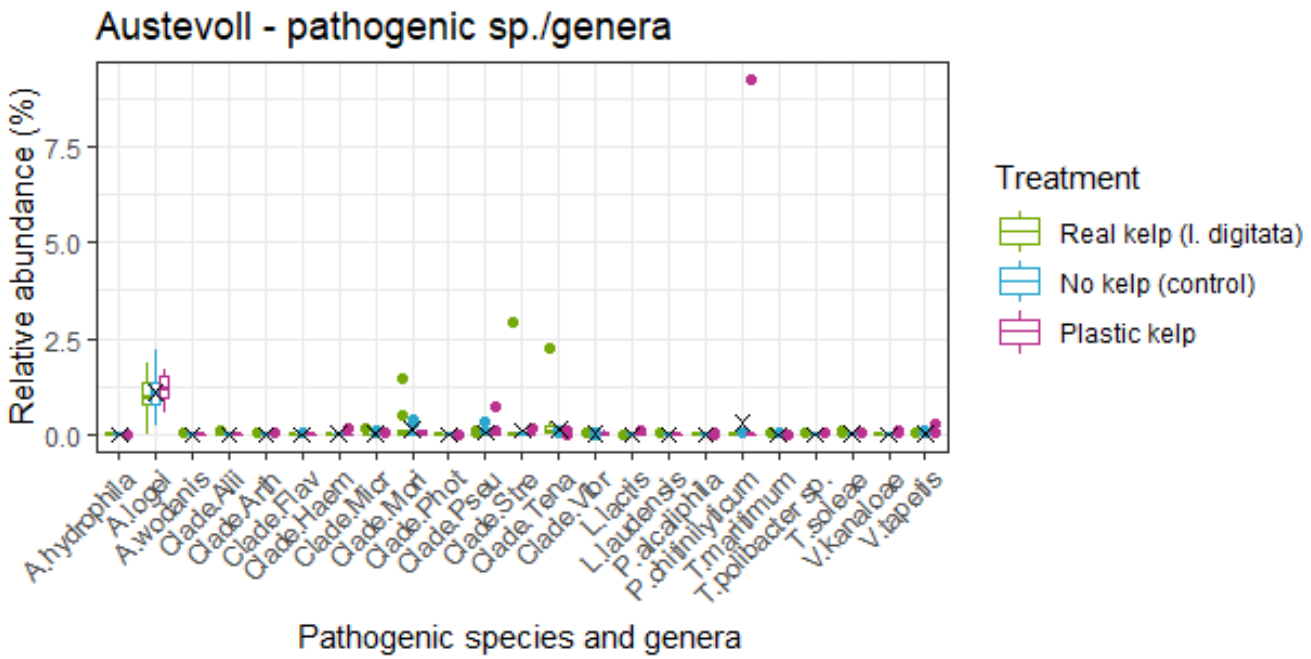


Figure 40: Pathogenic species and genera (clade) on the lumpfish skin in experiment 2 (3. Sampling, n= 30).

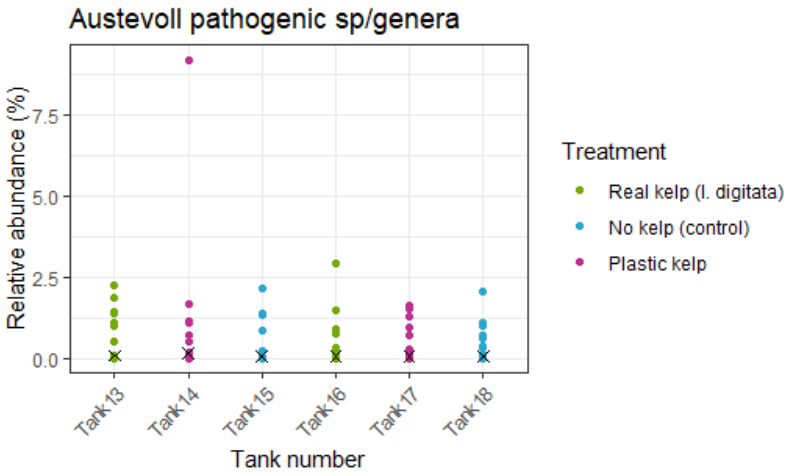


Figure 41: Relative abundance of the pathogenic species and genera on the lumpfish skin in experiment 2 per tank (3. Sampling, n= 30).

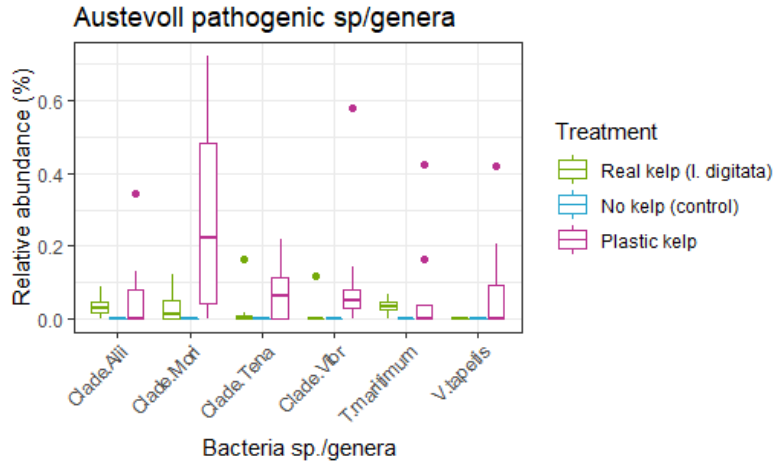


Figure 42: Pathogenic species and genera on the treatments in experiment 2 (3. Sampling, n= 11).

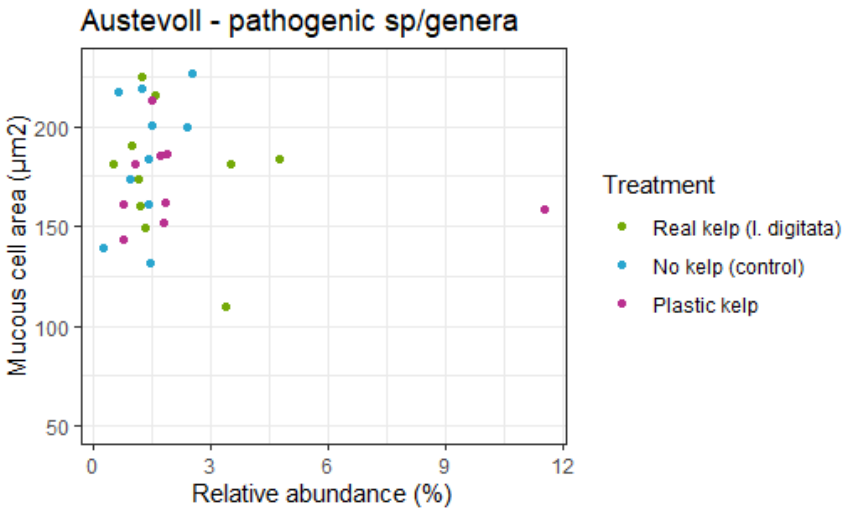


Figure 43: Relative abundance of pathogenic species and genera on the fish skin versus mucous cell area in experiment 2 (3. Sampling, n= 30).

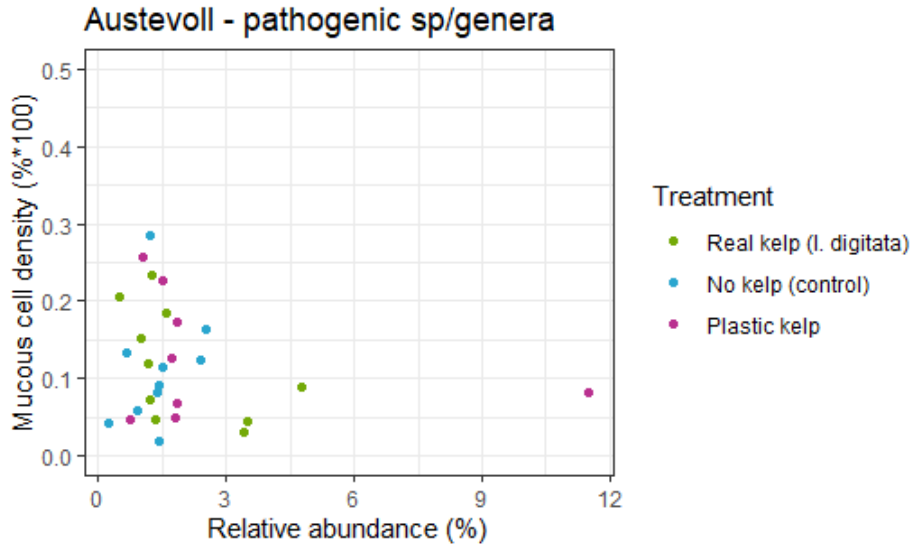


Figure 44: Relative abundance of pathogenic species and genera on the fish skin versus mucous cell density in experiment 2 (3. Sampling, n= 30).

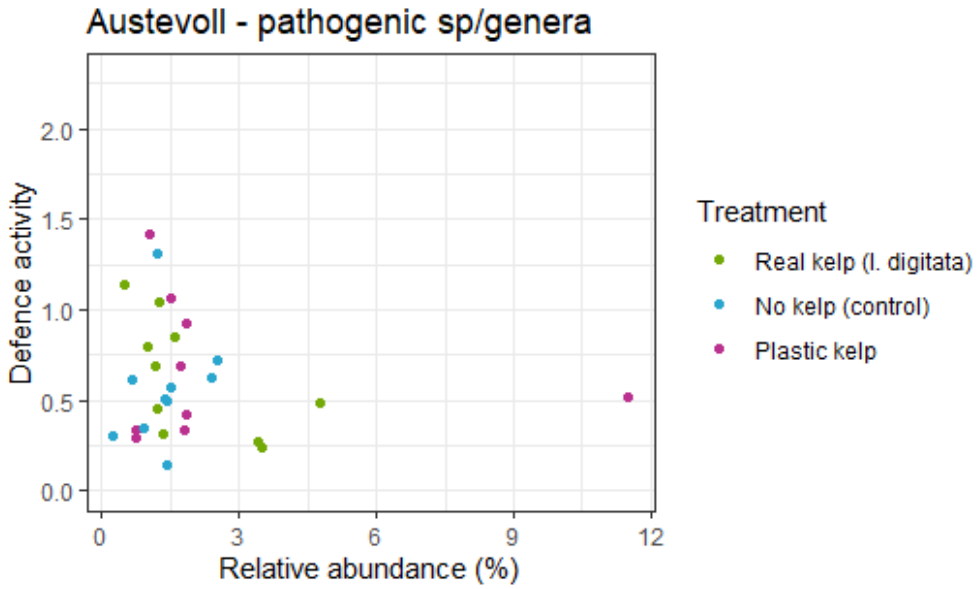


Figure 45: Relative abundance of pathogenic species and genera on the fish skin versus defence activity in experiment 2 (3. Sampling, n= 30).

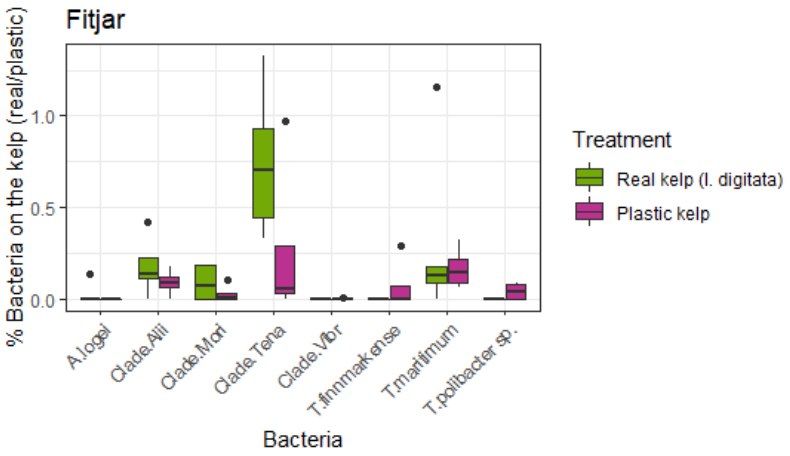


Figure 46: Pathogenic bacteria or genera (clade) on the treatment real kelp and plastic kelp in Fitjar (experiment 3)

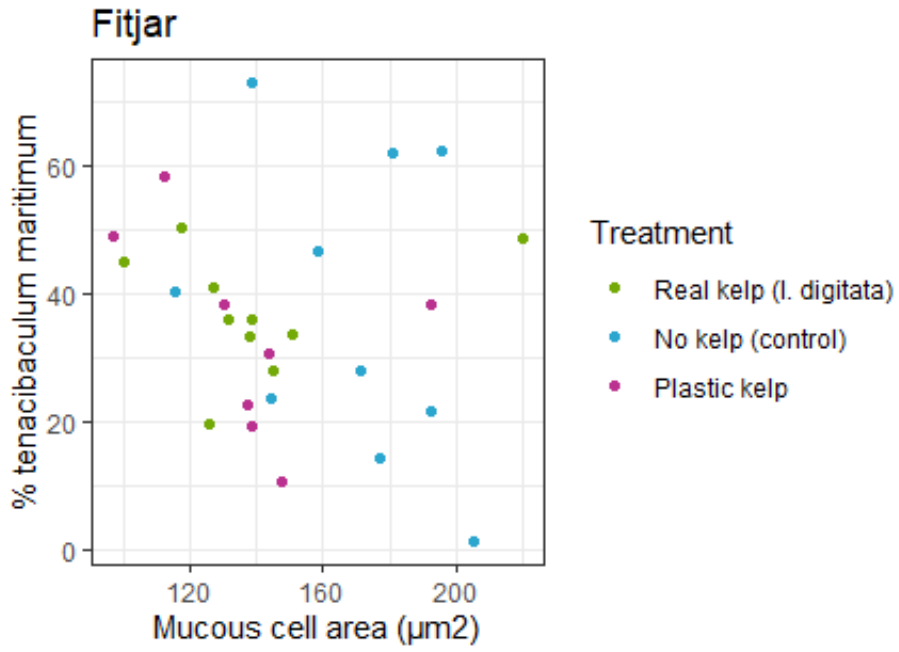


Figure 47: *Tenacibaculum Maritimum* vs. Mucous cell mean area (Fitjar, 11-01-2020)

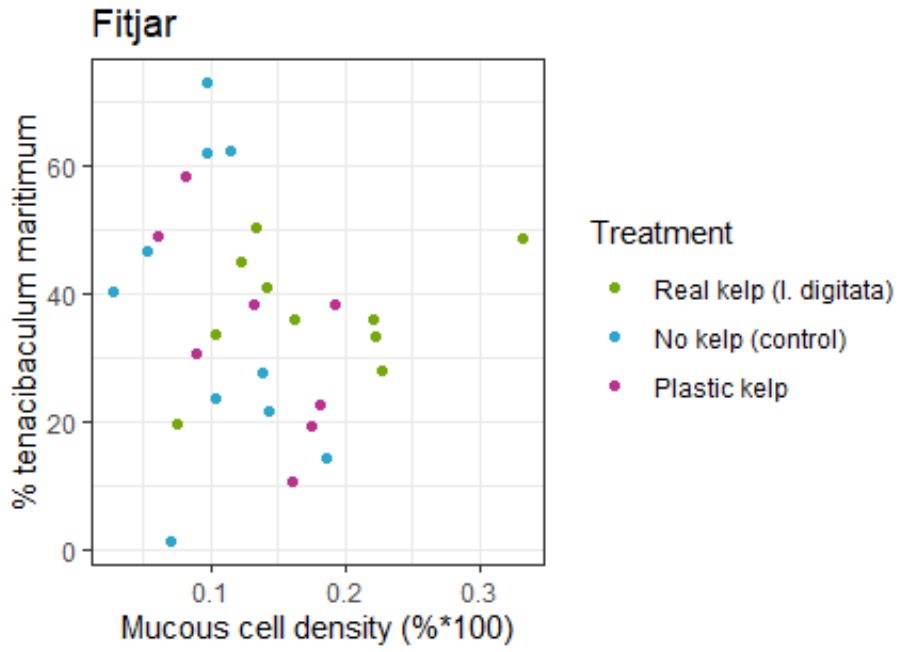


Figure 47: *Tenacibaculum Maritimum* vs. Mucous cell volumetric density (Fitjar, 11-01-2020)

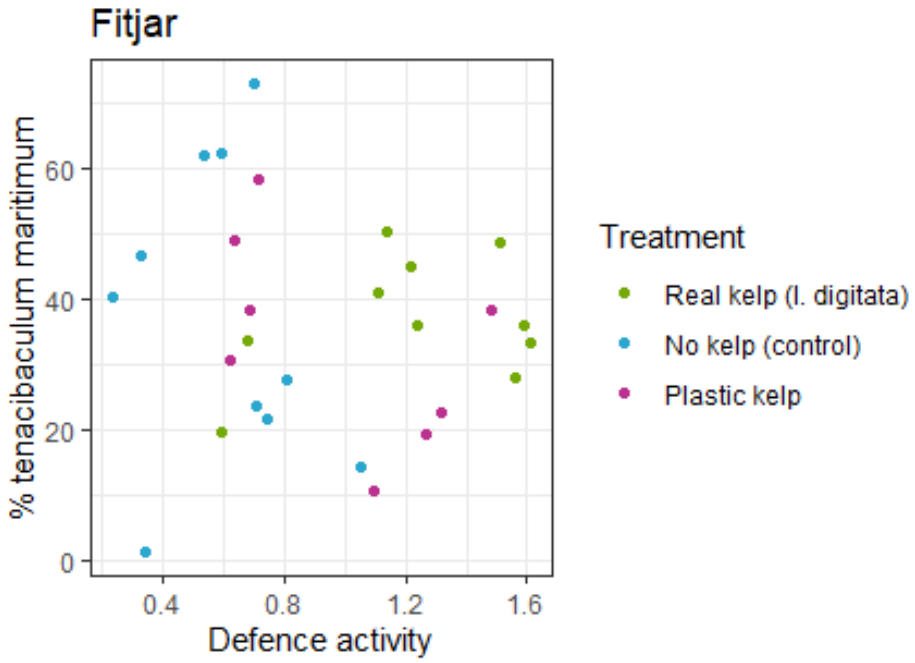


Figure 48: *Tenacibaculum Maritimum* vs. Defence activity (Fitjar, 11-01-2020)

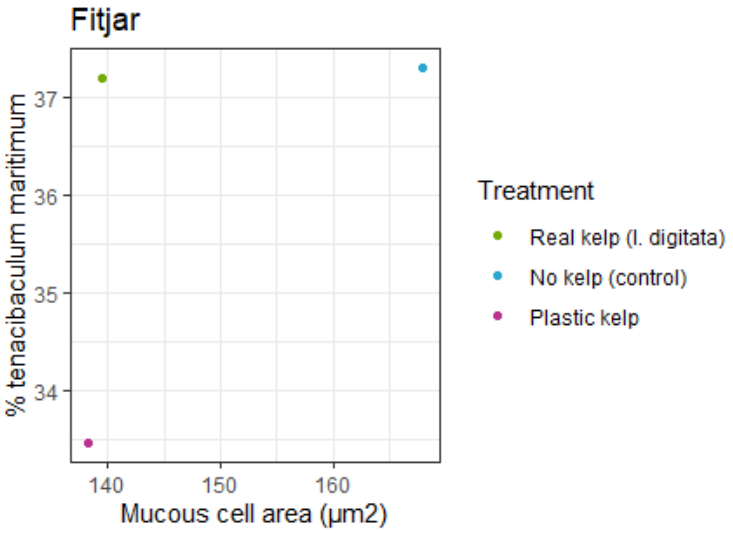


Figure 49: *Tenacibaculum Maritimum* vs. Mean MA (Fitjar, 11-01-2020)

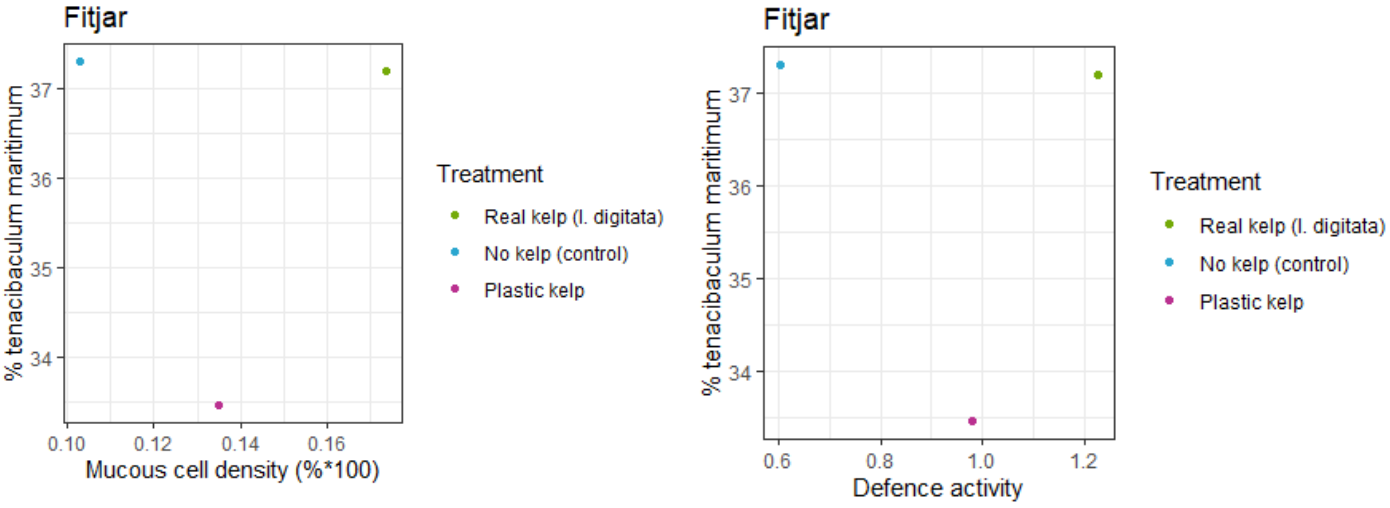


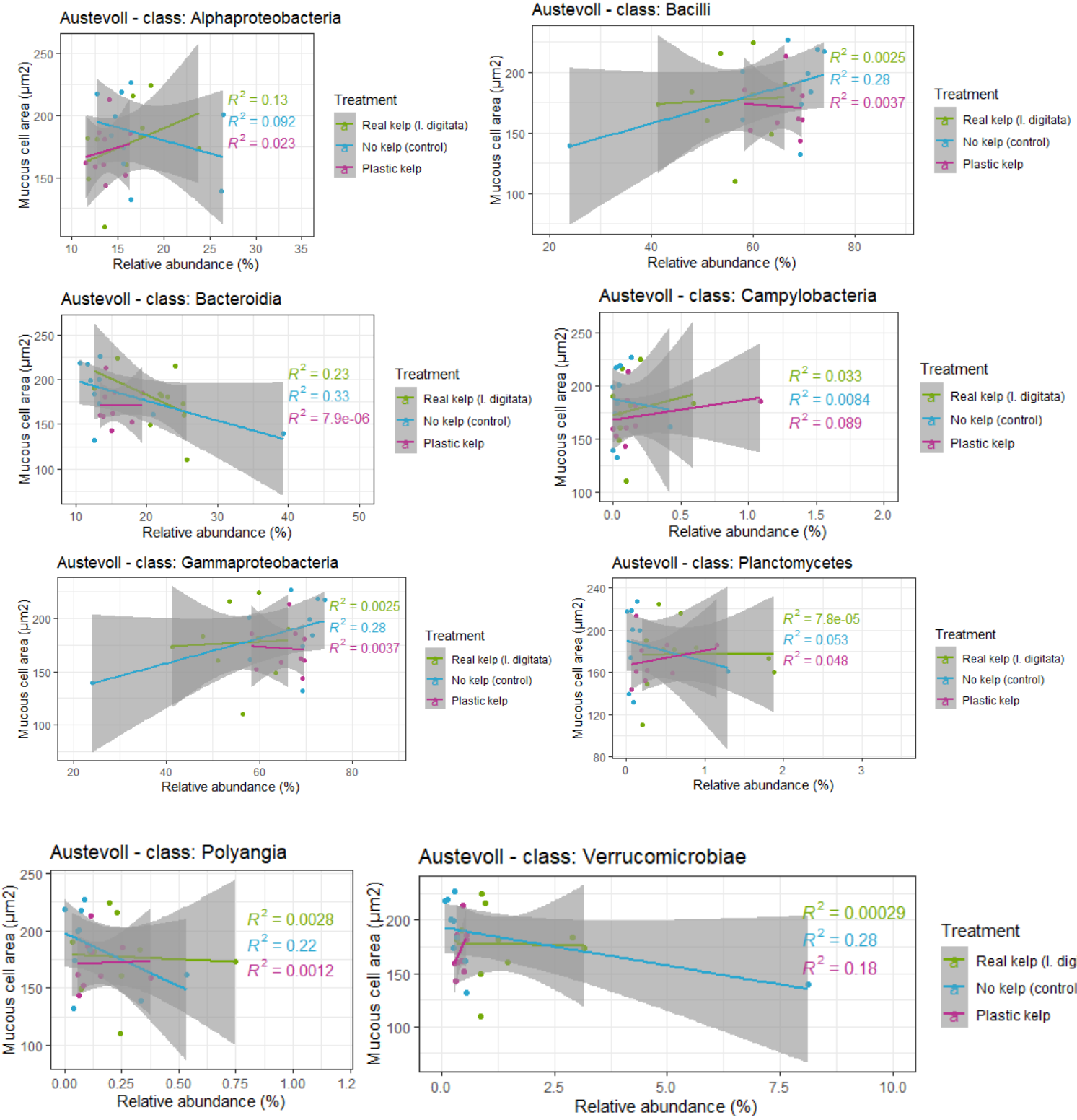
Figure 50: *Tenacibaculum Maritimum* vs. Mean VD and DA (Fitjar, 11-01-2020

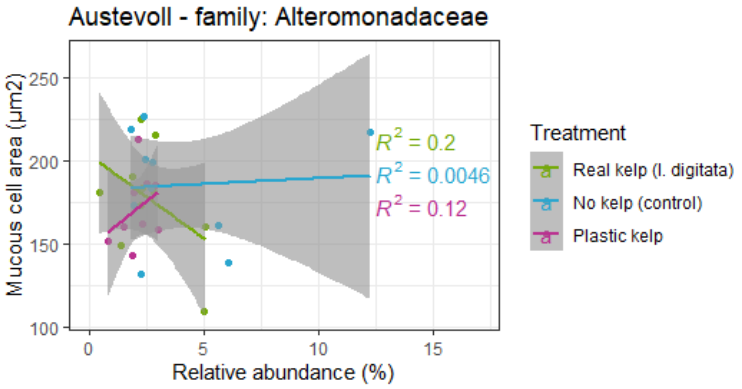
Appendix D – Bacterial RA vs MA, VD and DA in Austevoll

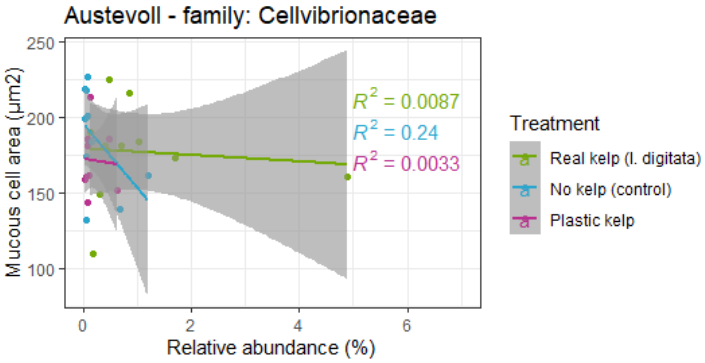
Austevoll (16.10.2020) *Cyclopterus lumpus*

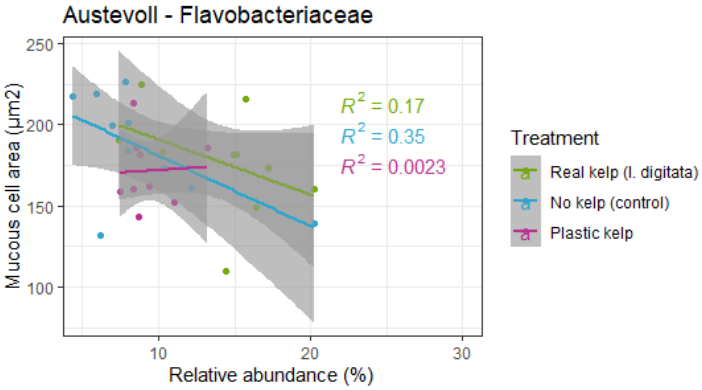
Mucous cell mean area, volumetric density and defence activity, versus relative abundance of class, family, genus and species – of bacteria found on the lumpfish skin. R^2 = correlation coefficient, line= linear regression, grey area= 95% confidence interval. (All of the figures should have a figure legend under the figure, but due to the time consuming event that would be, the figures are left as is, with taxonomic unit and name on top)

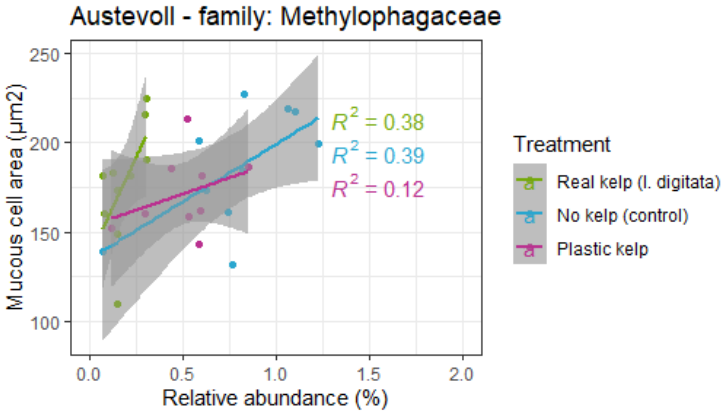
Mucous cell mean area vs. relative abundance

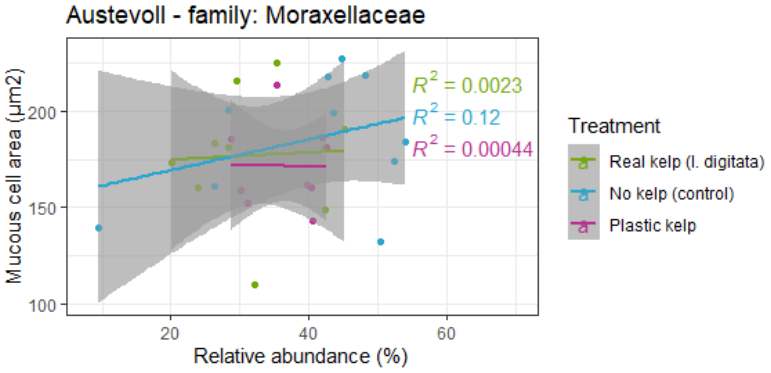


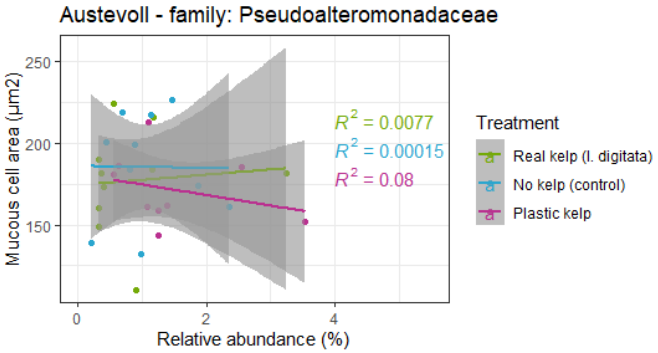

Austevoll - family: Alteromonadaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.2
R^2 = 0.0046
R^2 = 0.12
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

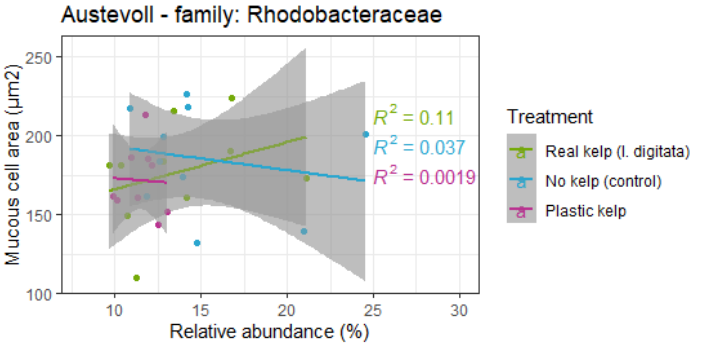

Austevoll - family: Cellvibrionaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.0087
R^2 = 0.24
R^2 = 0.0033
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

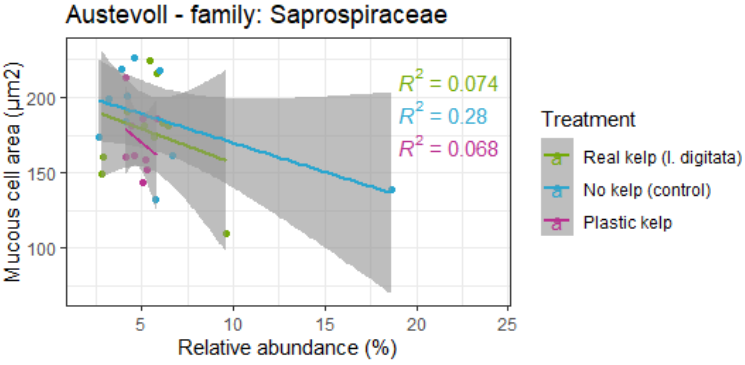

Austevoll - Flavobacteriaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.17
R^2 = 0.35
R^2 = 0.0023
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

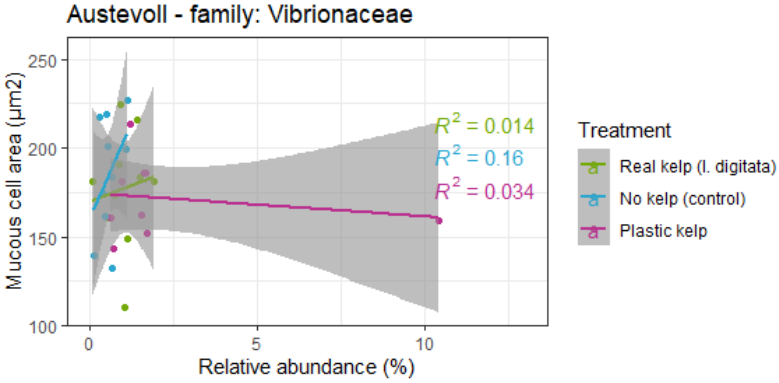

Austevoll - family: Methylophagaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.38
R^2 = 0.39
R^2 = 0.12
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp


Austevoll - family: Moraxellaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.0023
R^2 = 0.12
R^2 = 0.00044
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

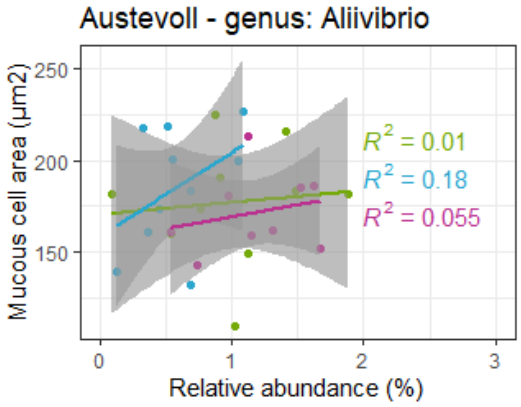

Austevoll - family: Pseudoalteromonadaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.0077
R^2 = 0.00015
R^2 = 0.08
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

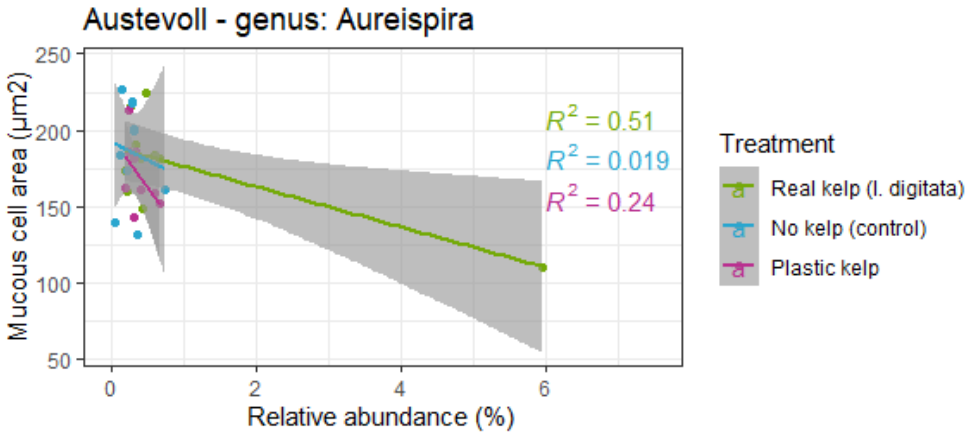

Austevoll - family: Rhodobacteraceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.11
R^2 = 0.037
R^2 = 0.0019
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

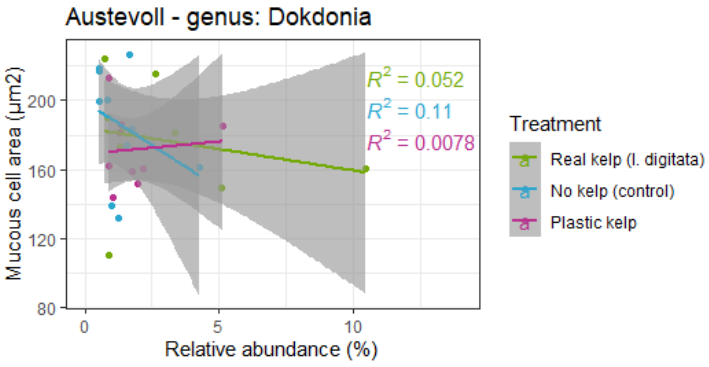

Austevoll - family: Saprospiraceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.074
R^2 = 0.28
R^2 = 0.068
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

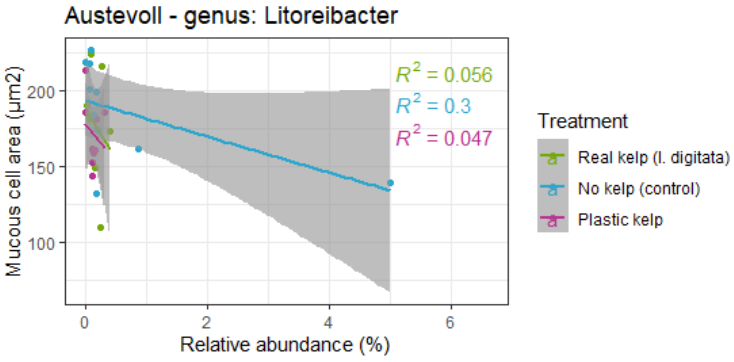

Austevoll - family: Vibrionaceae
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.014
R^2 = 0.16
R^2 = 0.034
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

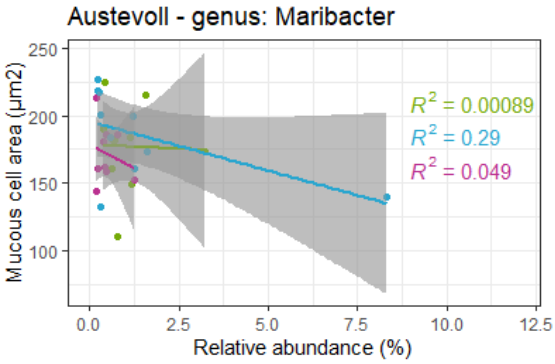
Taxonomic level: Genus

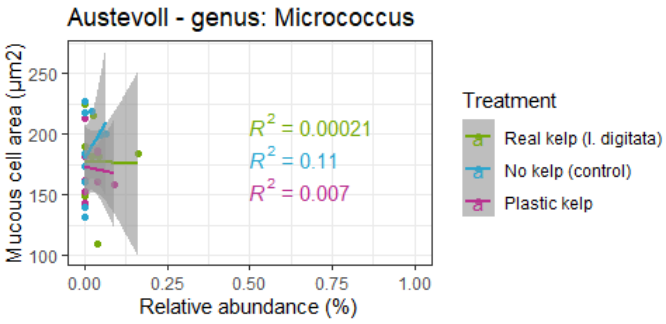


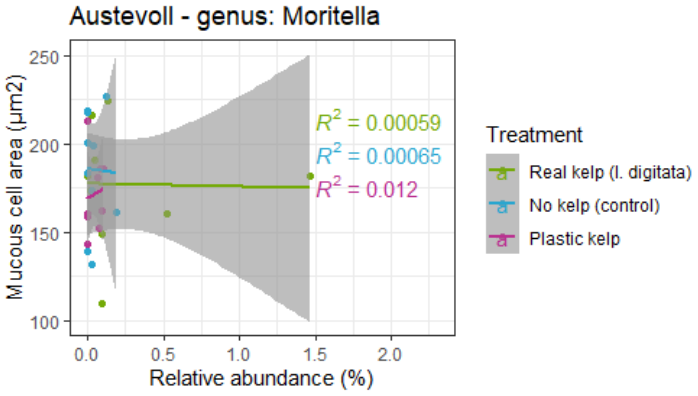


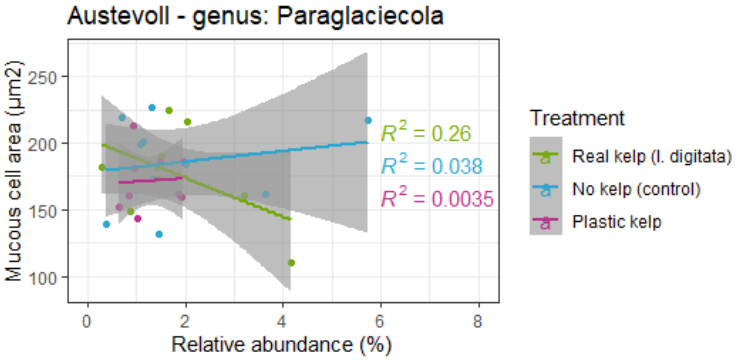


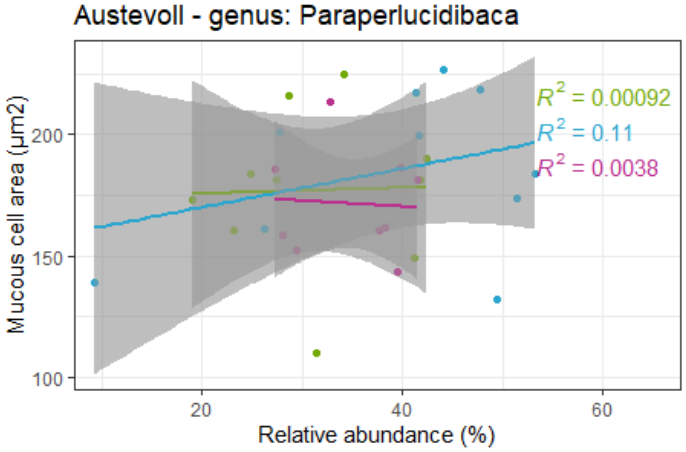


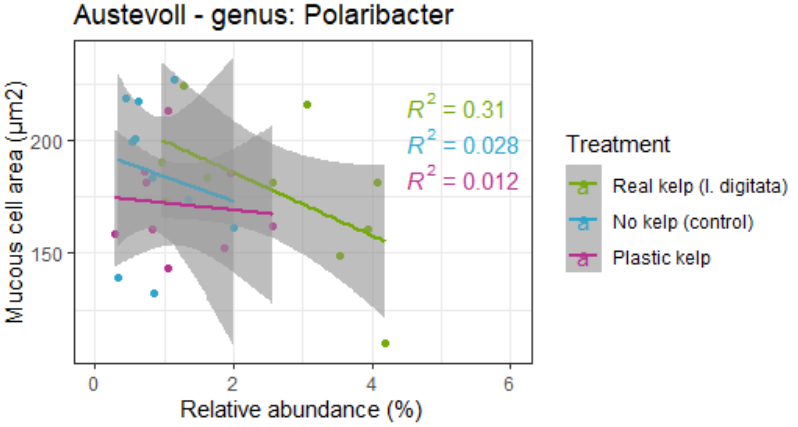


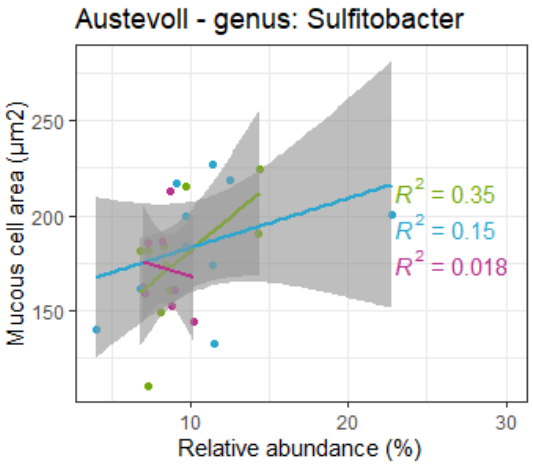


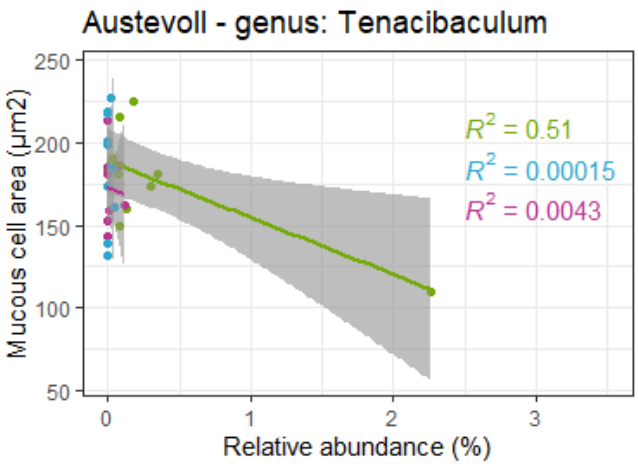


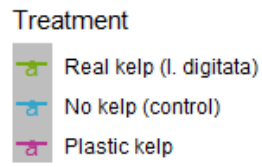


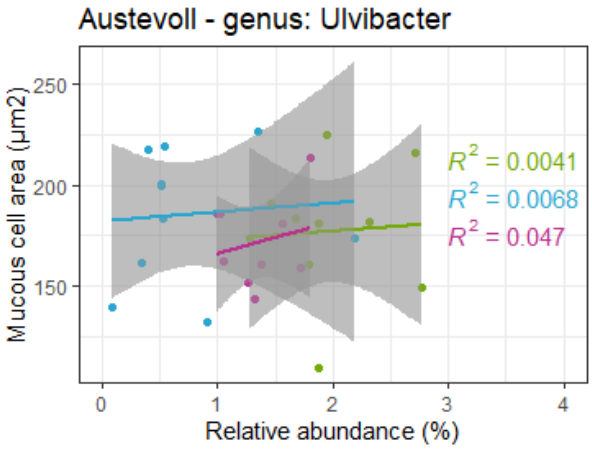


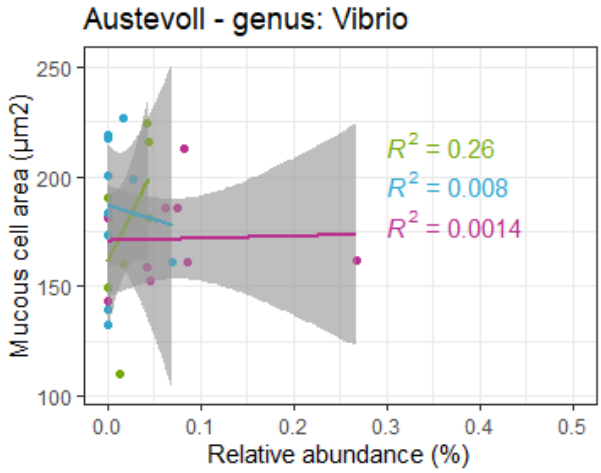


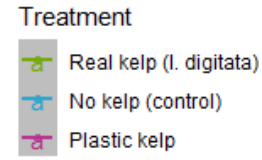




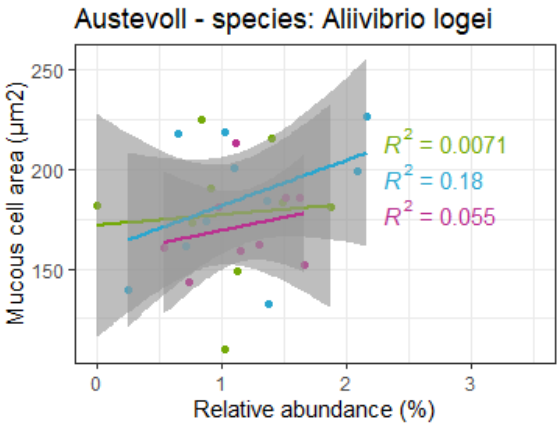


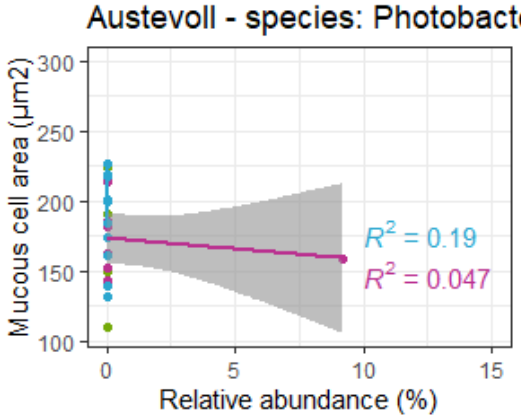


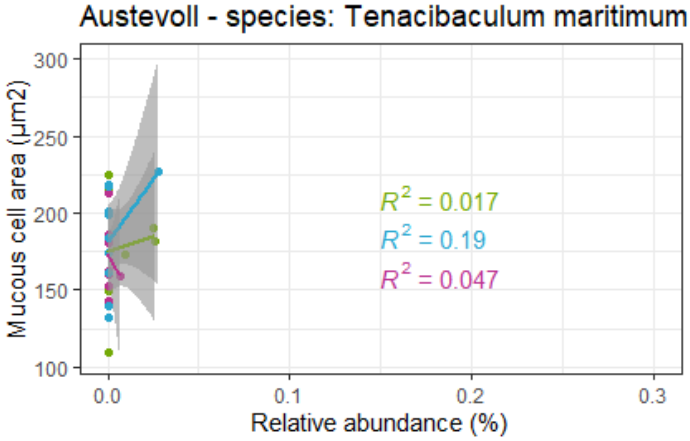


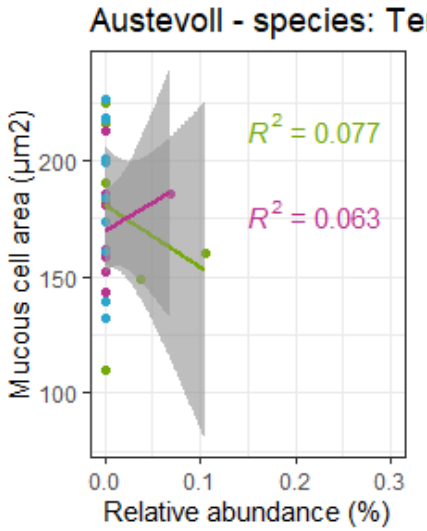


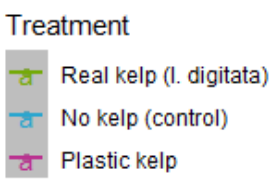
Taxonomic level: Species





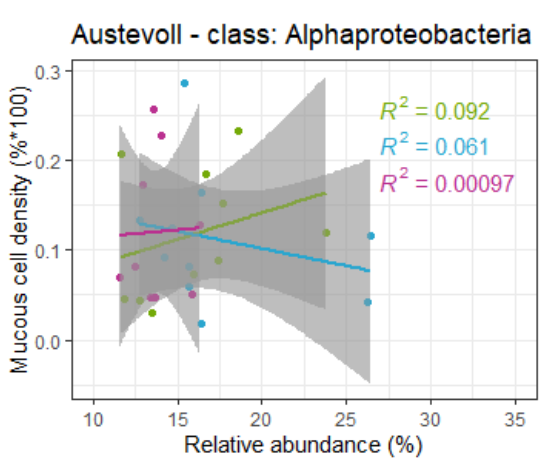


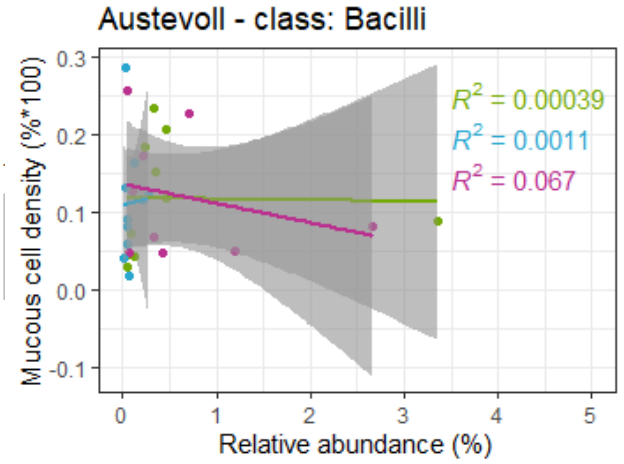


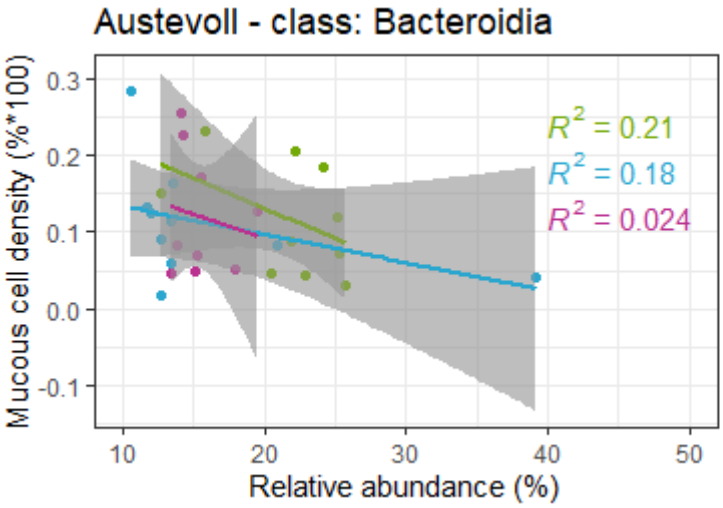


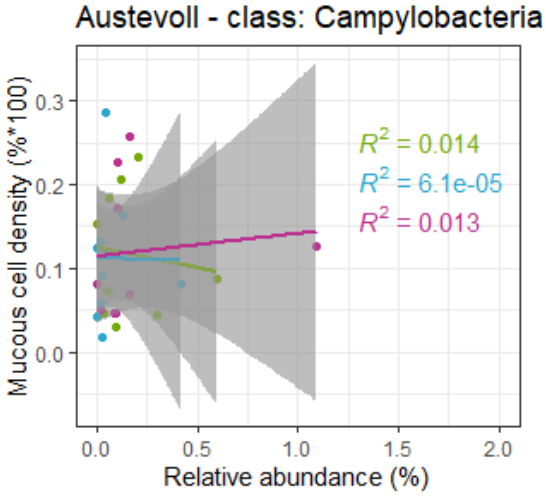
Mucous cell volumetric density vs. relative abundance

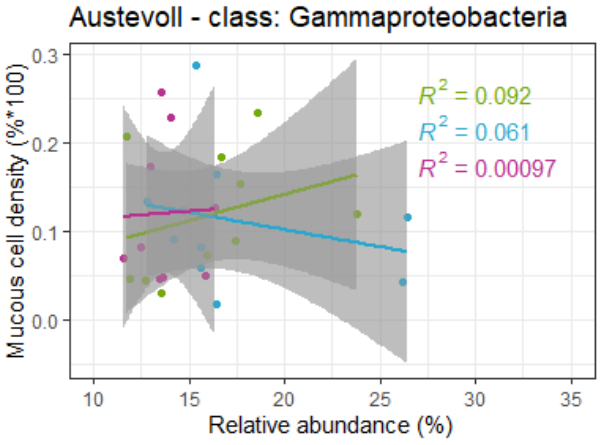
Taxonomic level: Class

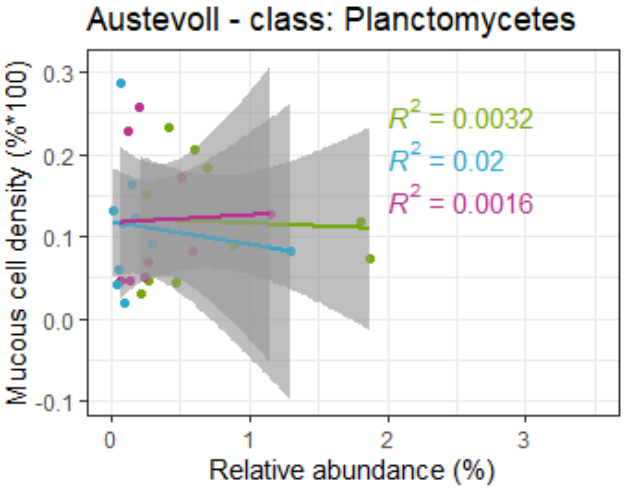


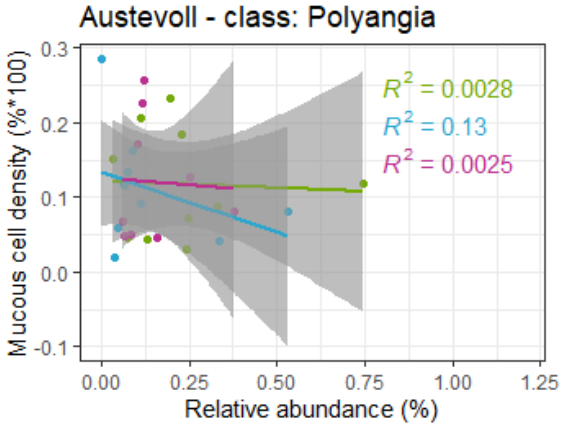


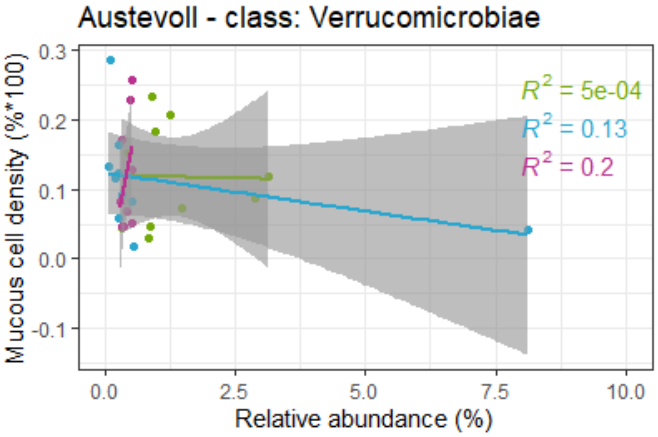




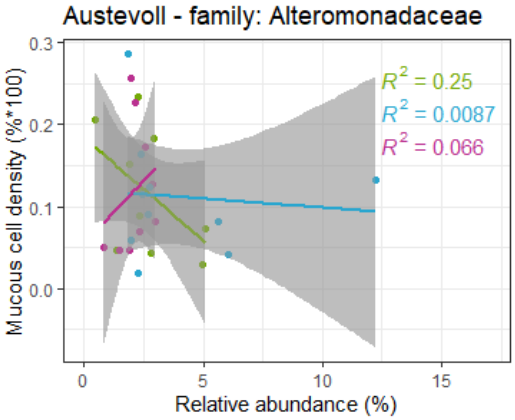


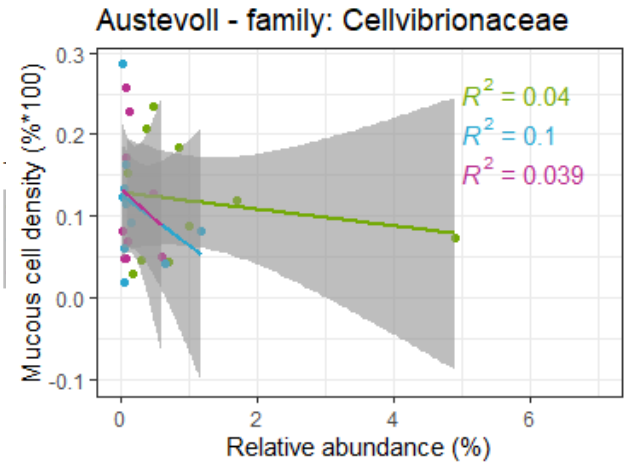


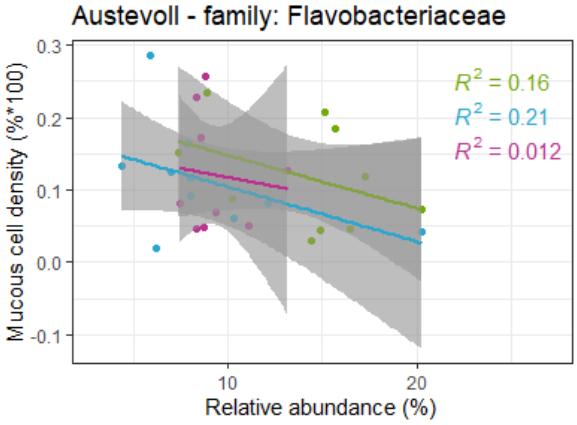


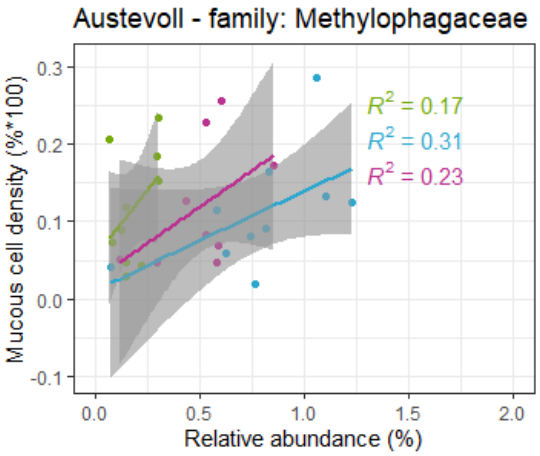


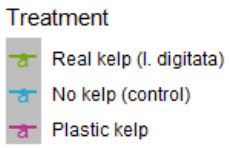
Taxonomic level: Family

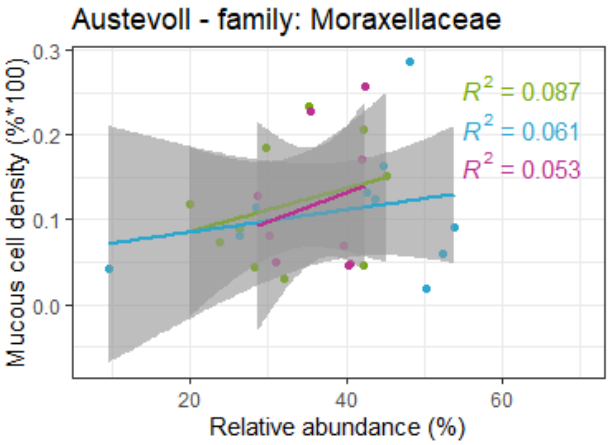


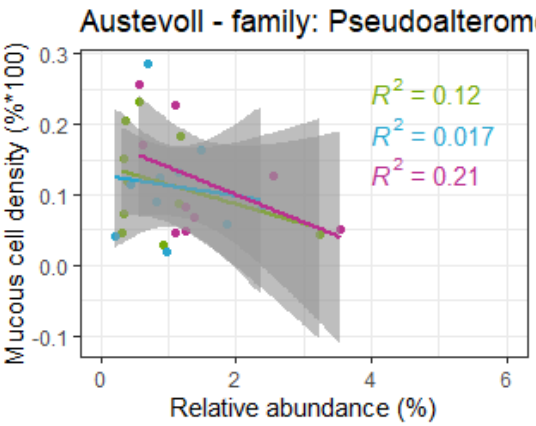


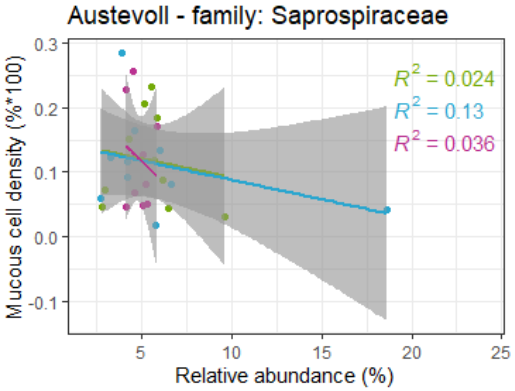


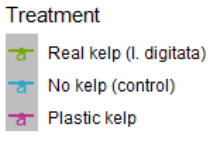


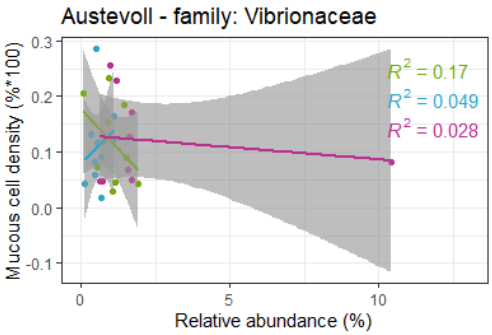


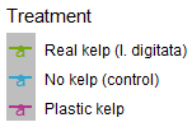


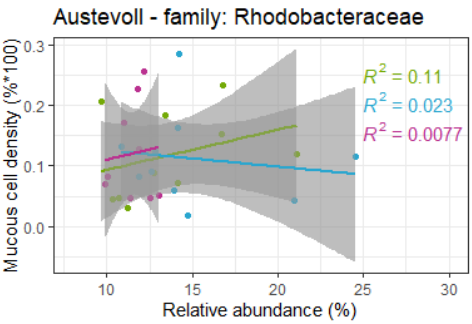




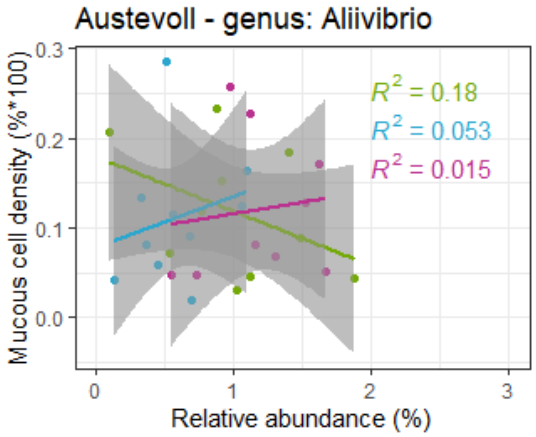


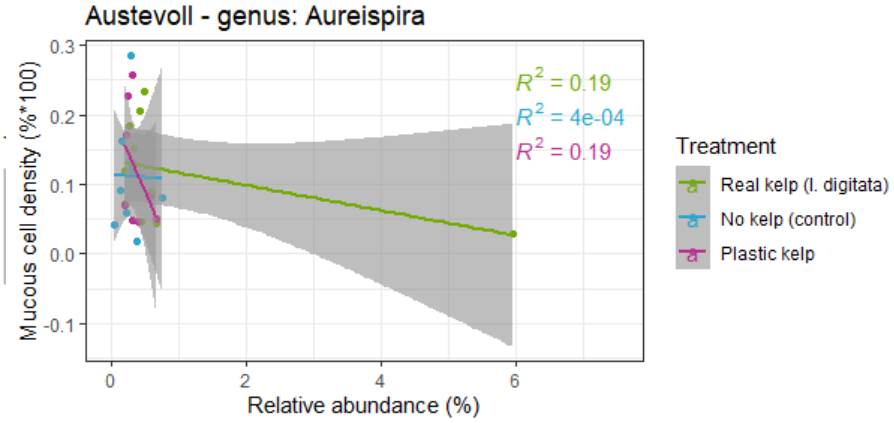


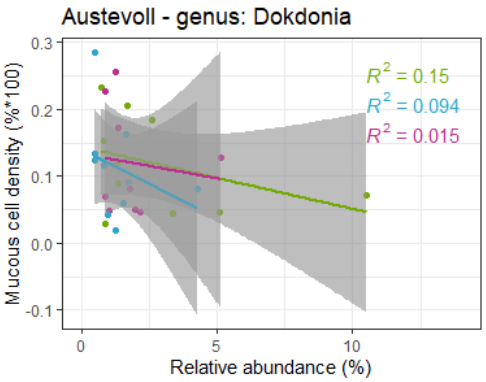


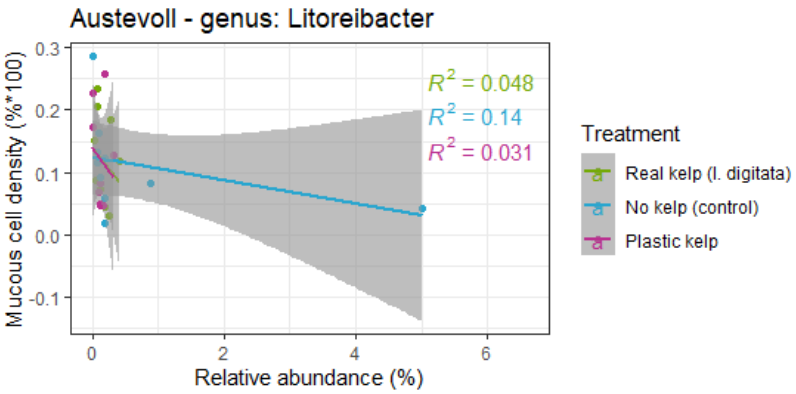


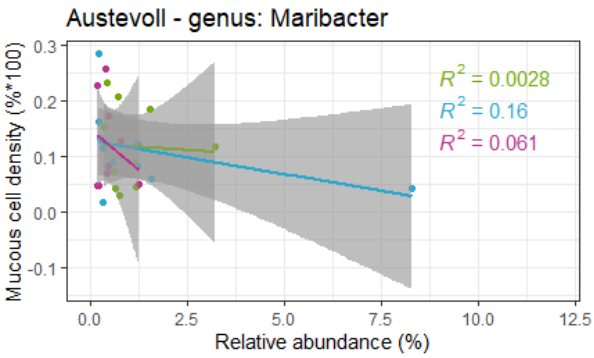
Taxonomic level: Genus

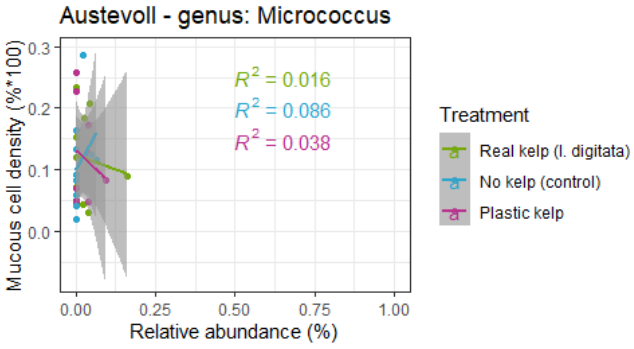


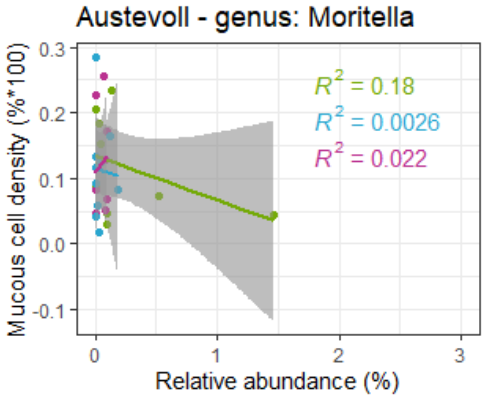


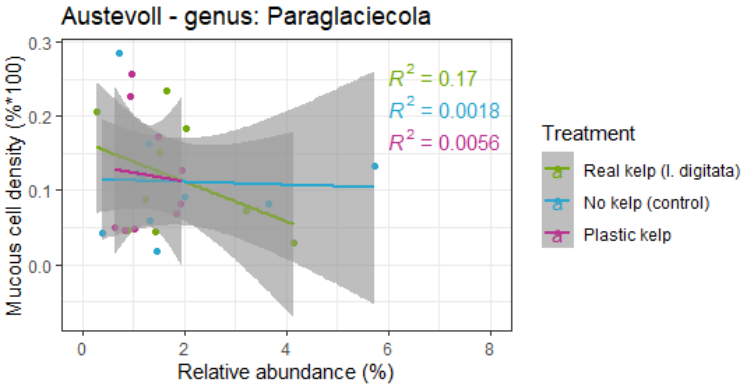


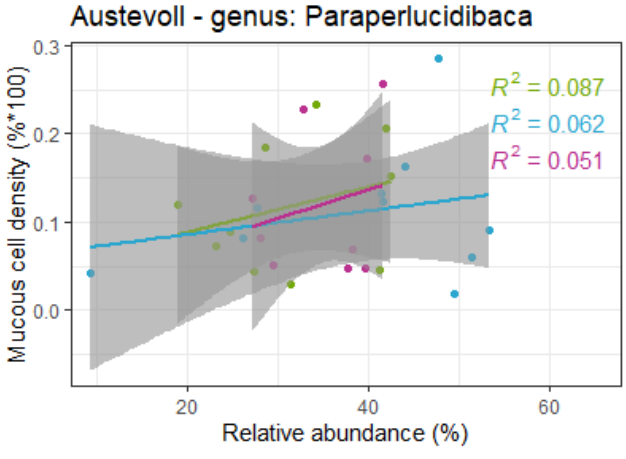


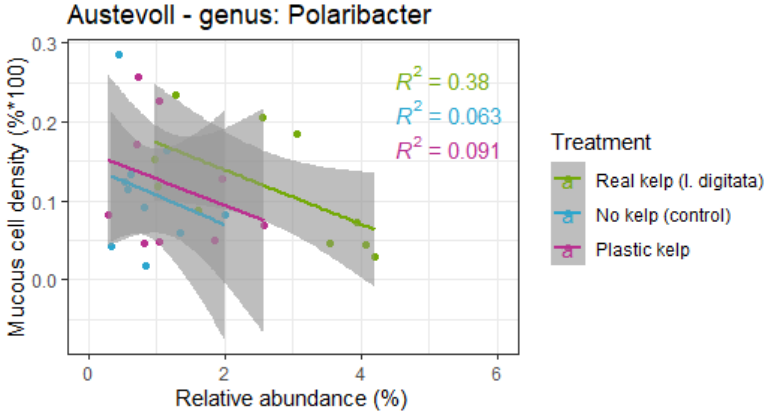


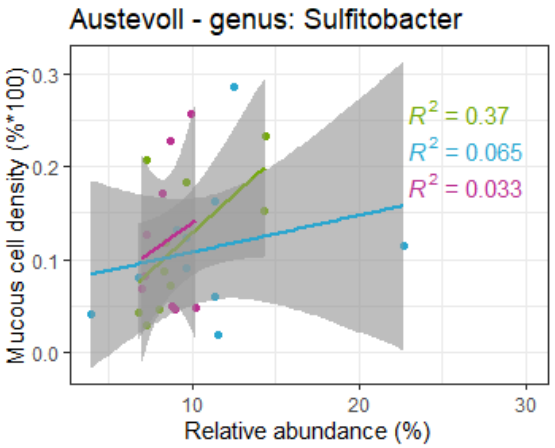


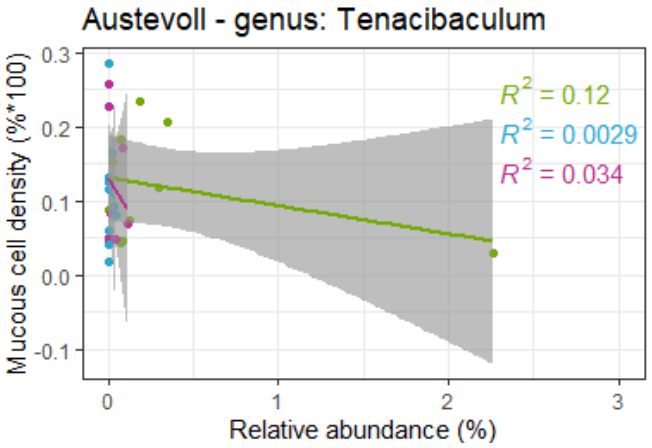


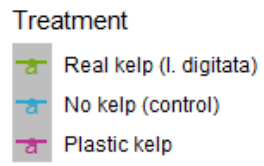


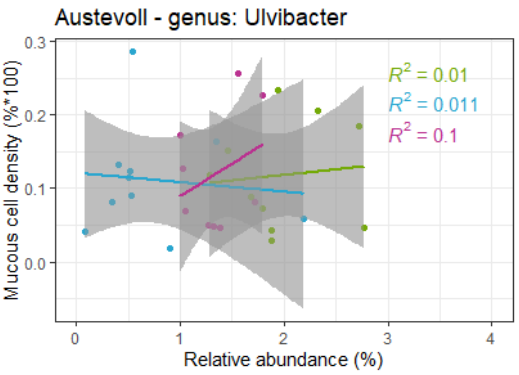


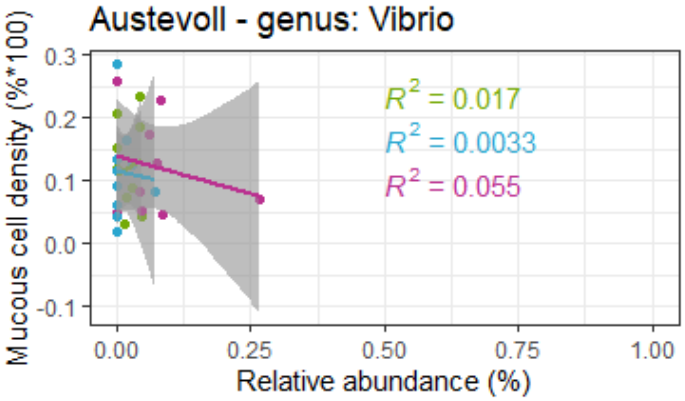


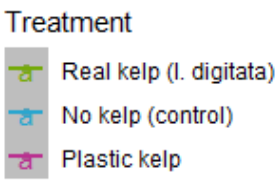




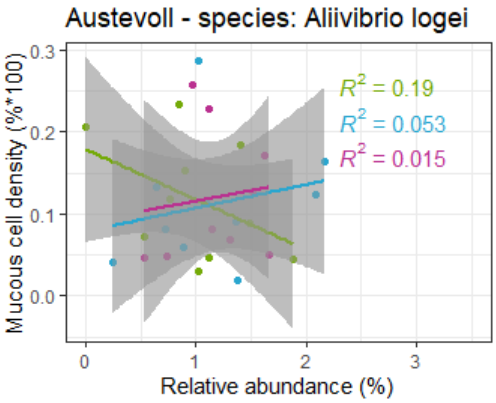


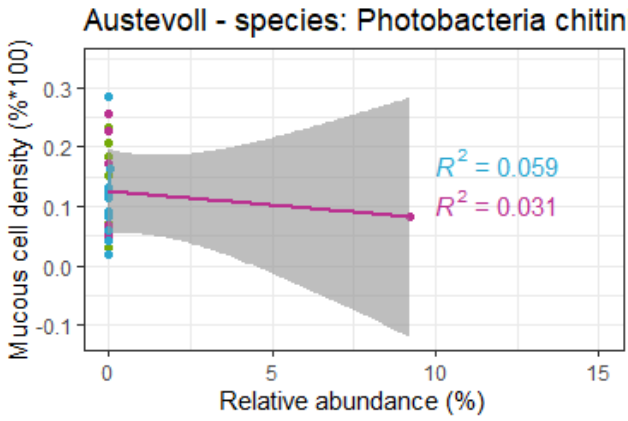


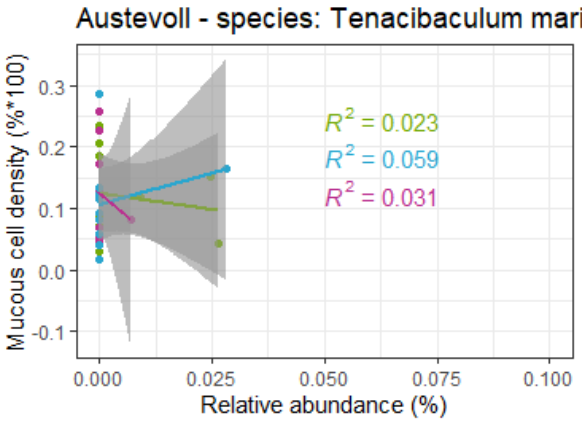


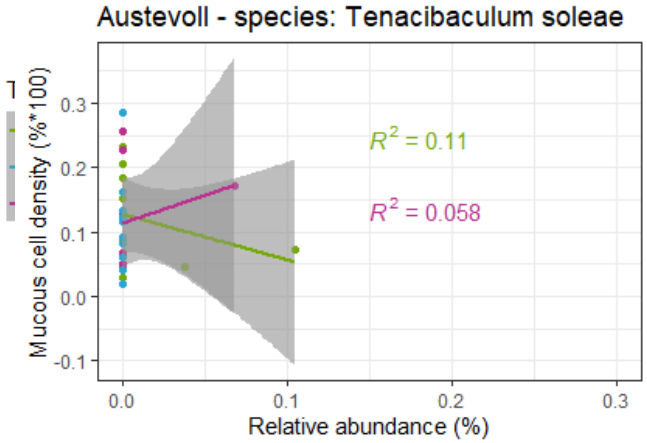


Taxonomic level: Species



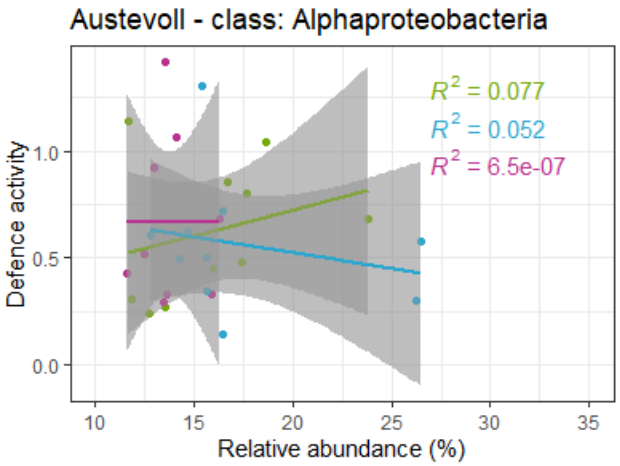


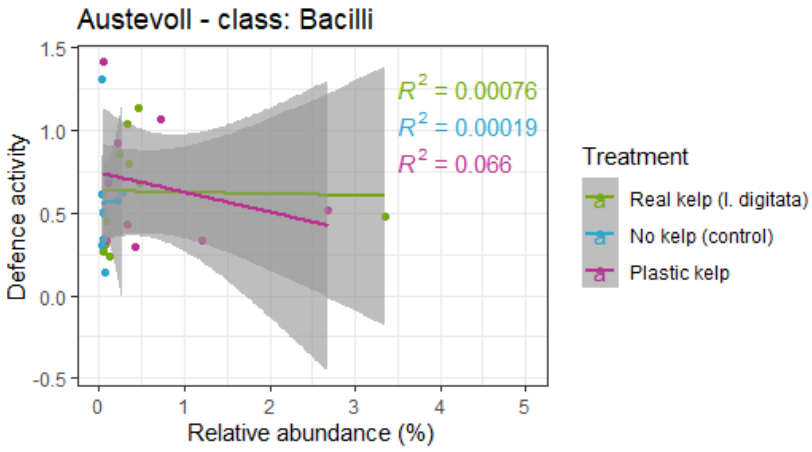


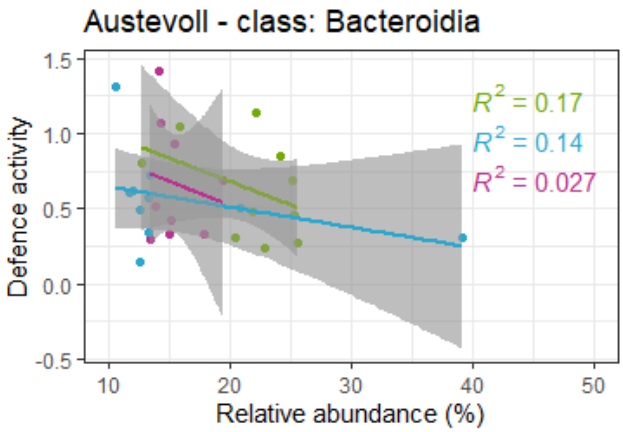


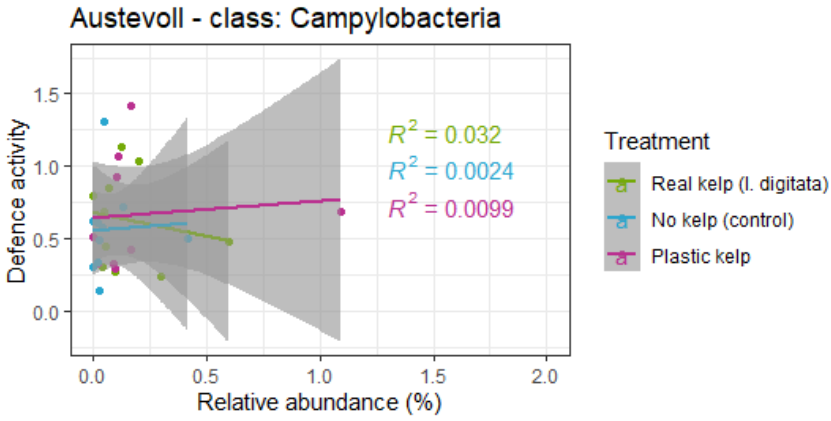
Defence activity vs. relative abundance

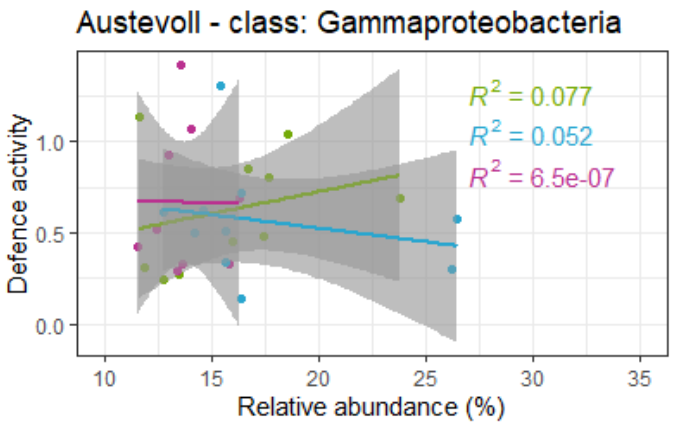
Taxonomic level: Class

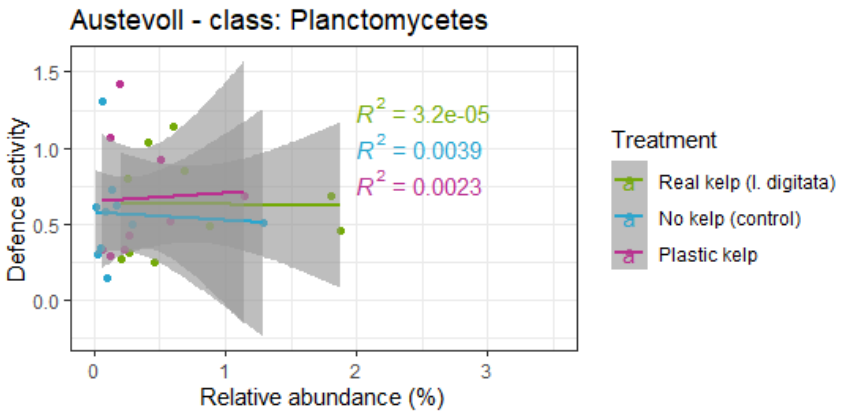


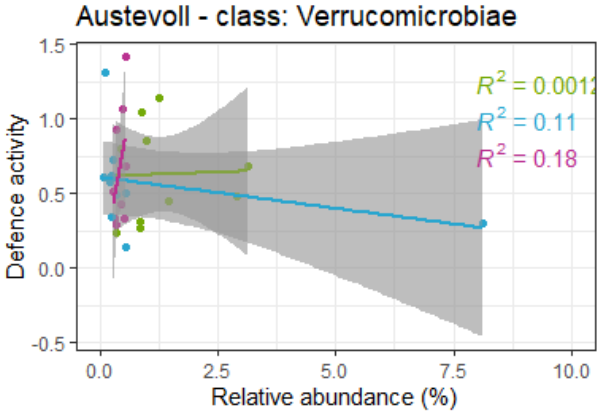


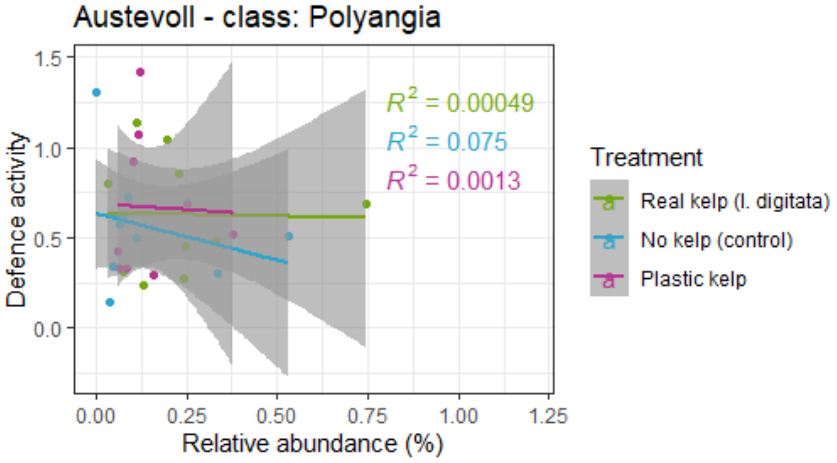




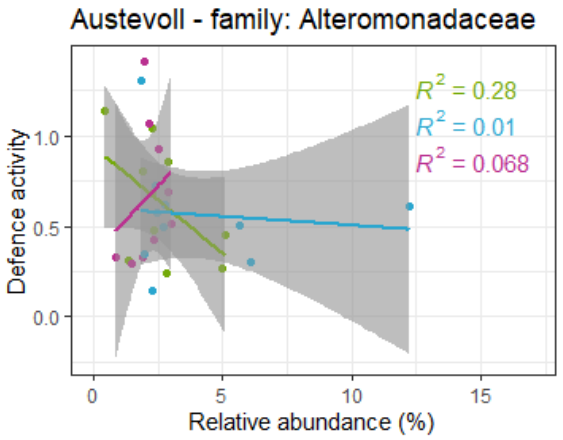


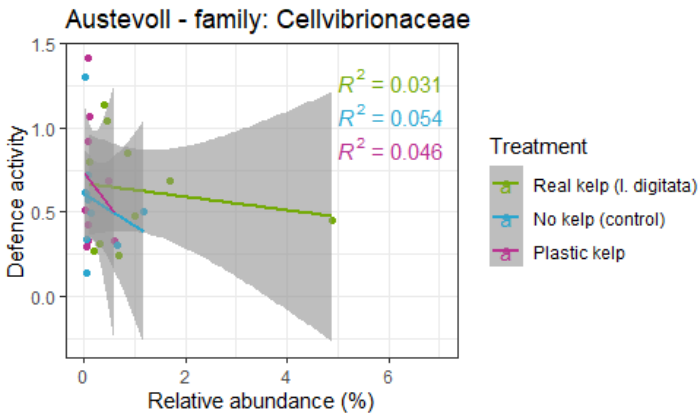


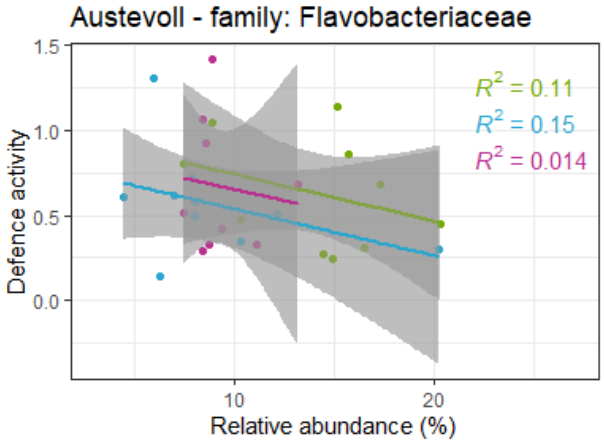


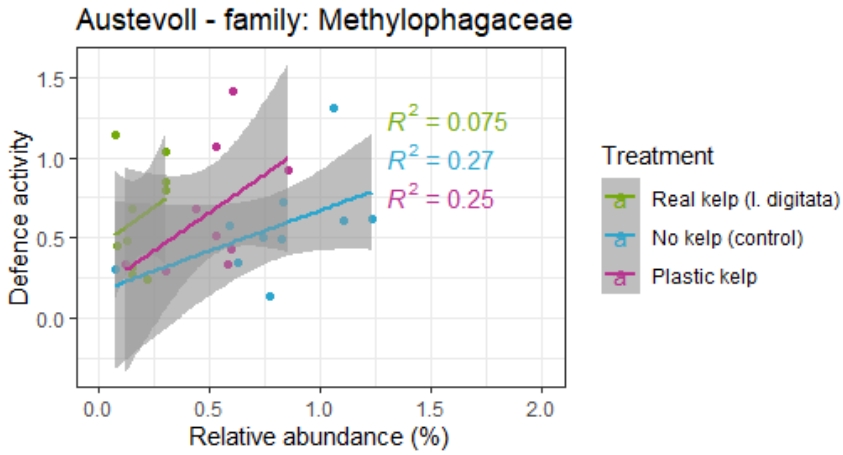


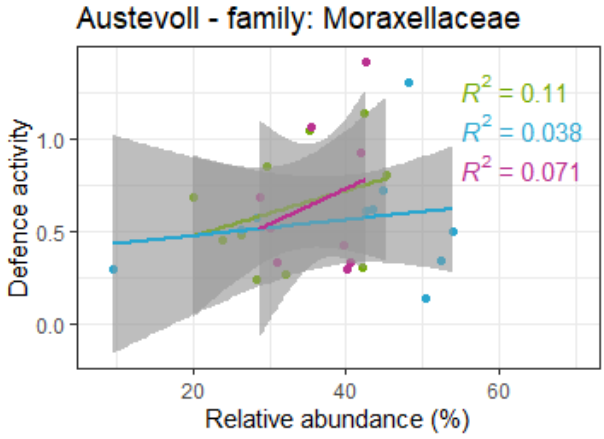
Taxonomic level: Family

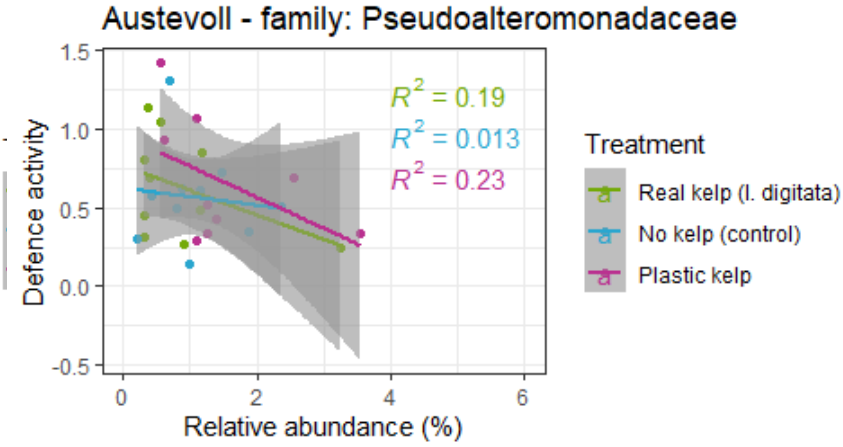


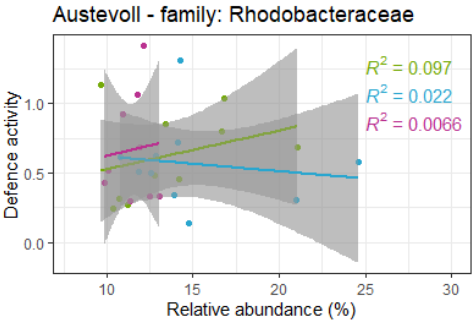


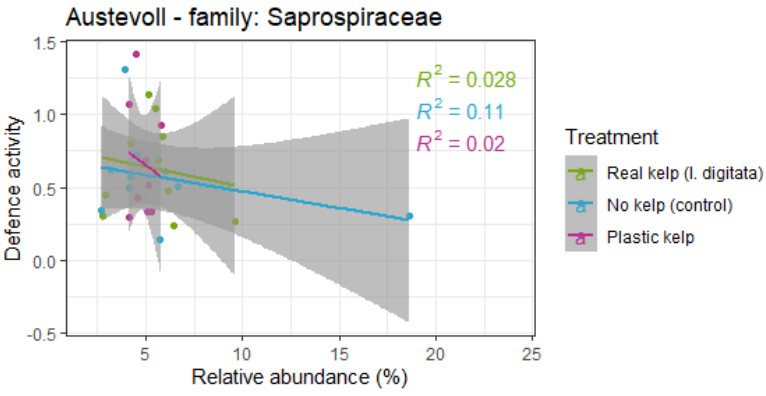


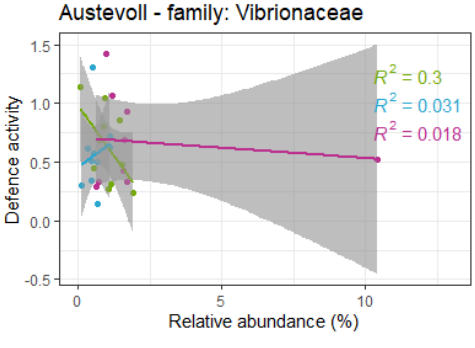




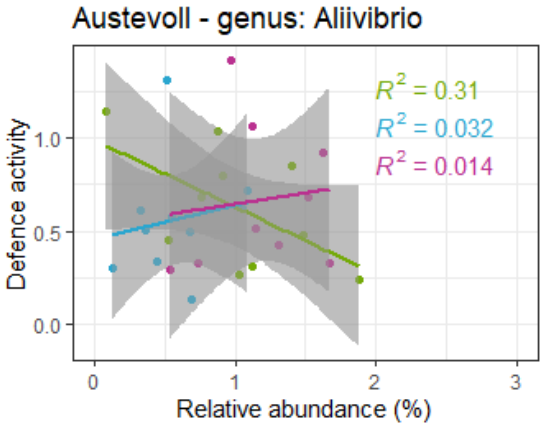


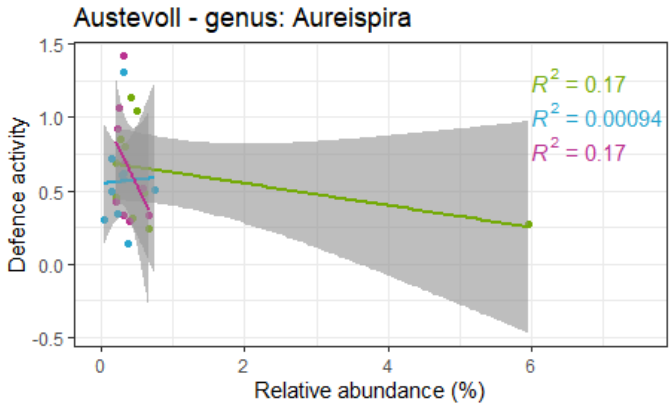


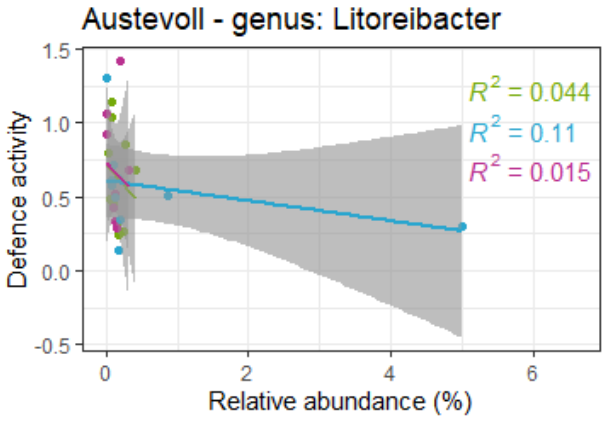


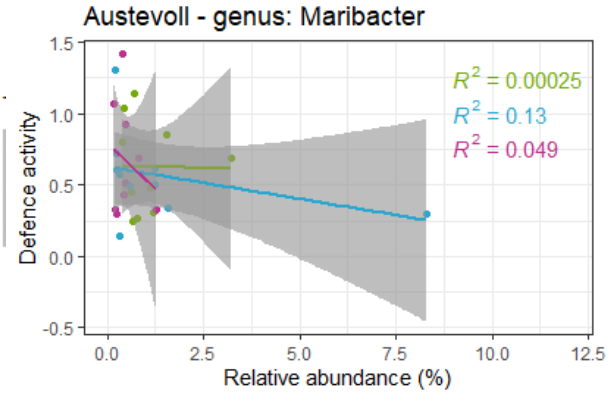


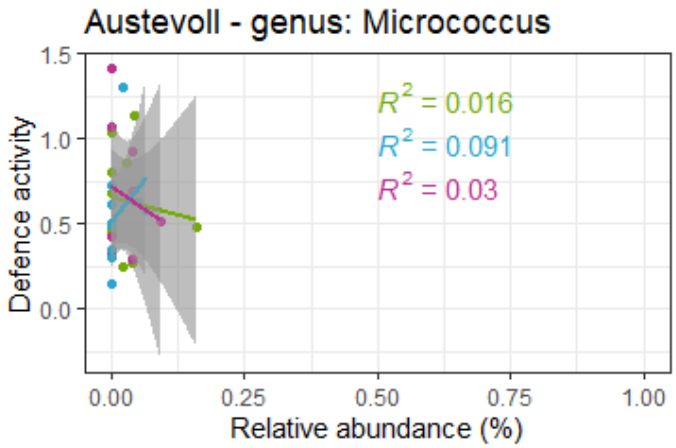
Taxonomic level: Genus

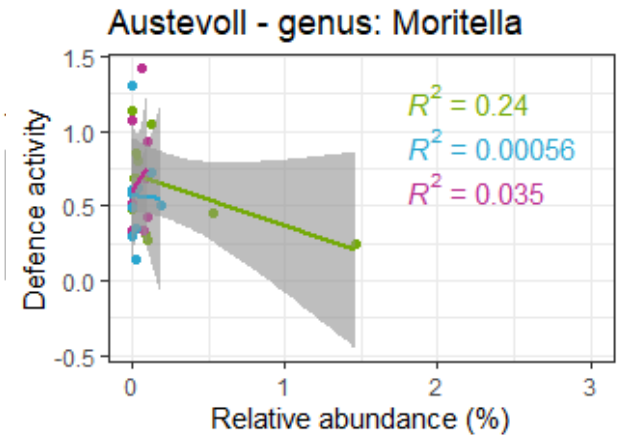


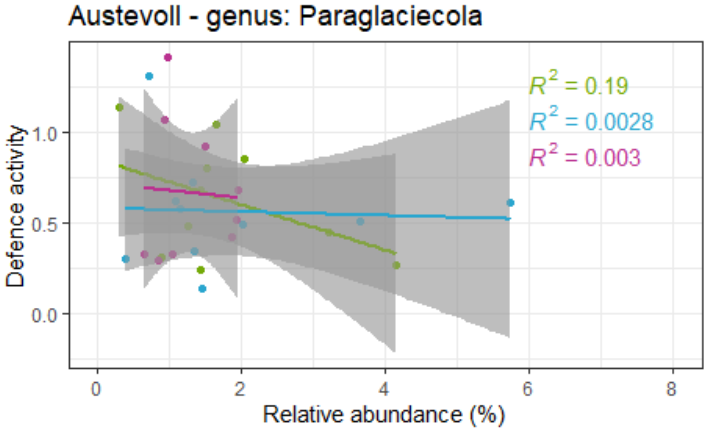


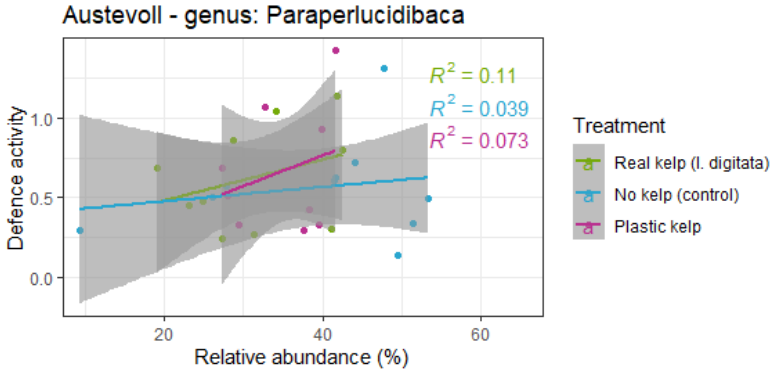


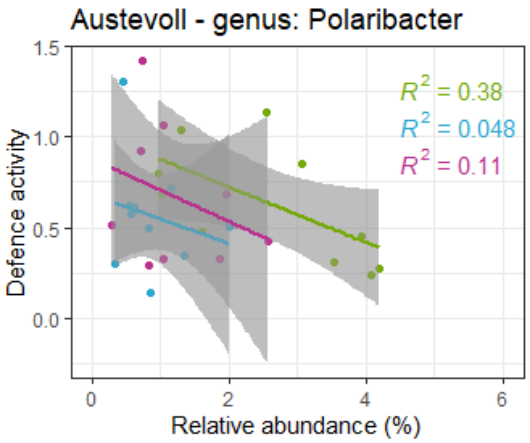


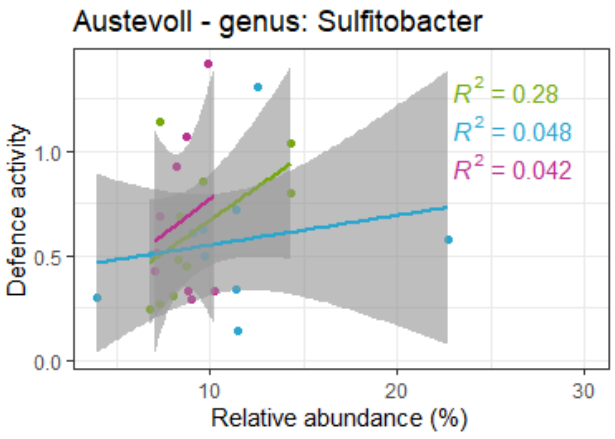


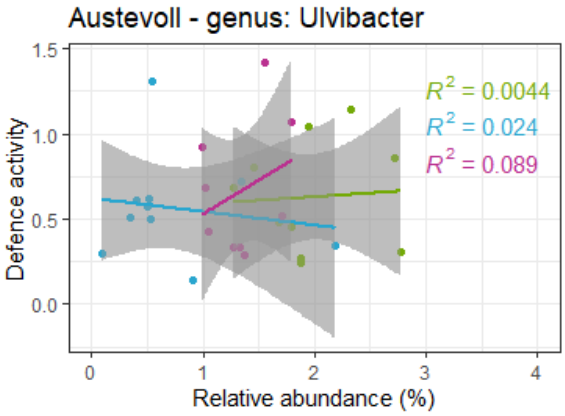


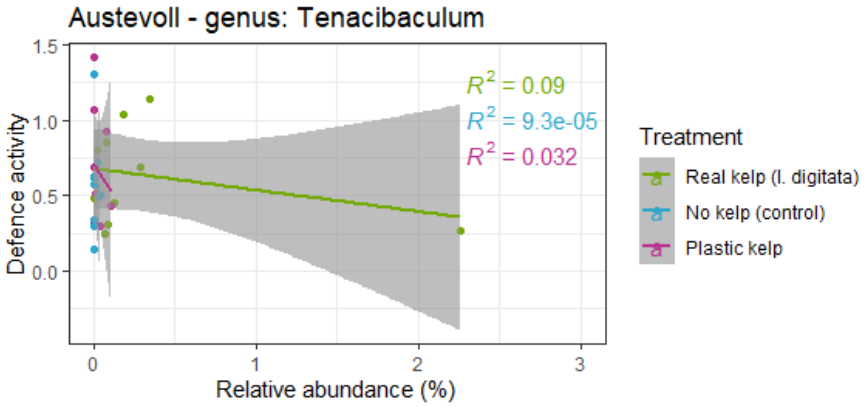


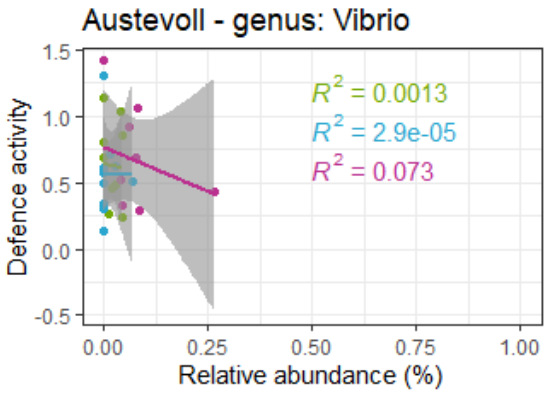



Austevoll - genus: Polaribacter
Defence activity
Relative abundance (%)
R^2 = 0.38
R^2 = 0.048
R^2 = 0.11

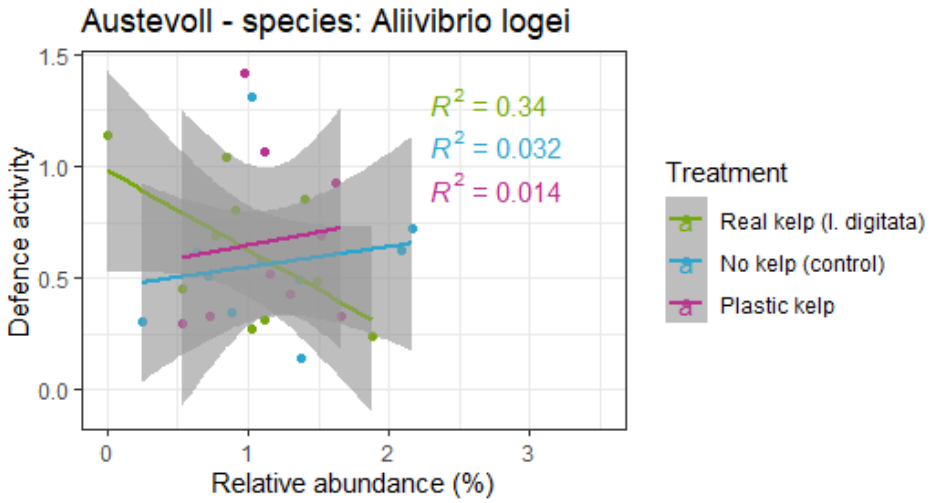

Austevoll - genus: Sulfitobacter
Defence activity
Relative abundance (%)
R^2 = 0.28
R^2 = 0.048
R^2 = 0.042
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

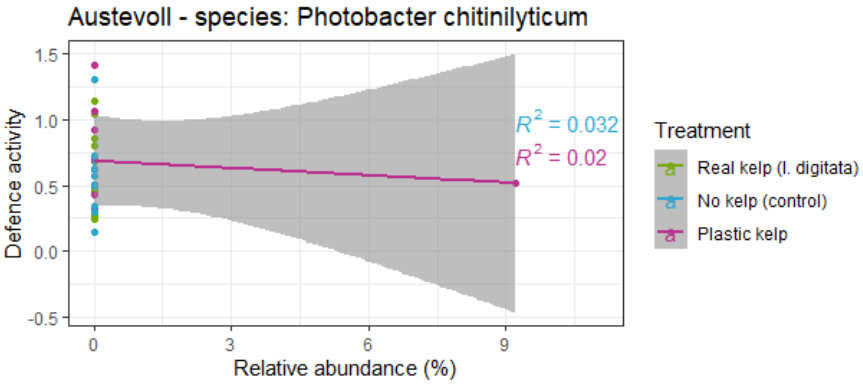

Austevoll - genus: Ulvibacter
Defence activity
Relative abundance (%)
R^2 = 0.0044
R^2 = 0.024
R^2 = 0.089

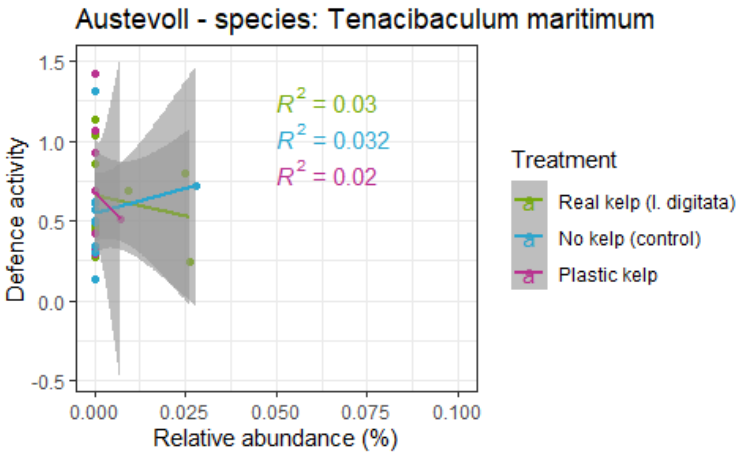

Austevoll - genus: Tenacibaculum
Defence activity
Relative abundance (%)
R^2 = 0.09
R^2 = 9.3e-05
R^2 = 0.032
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

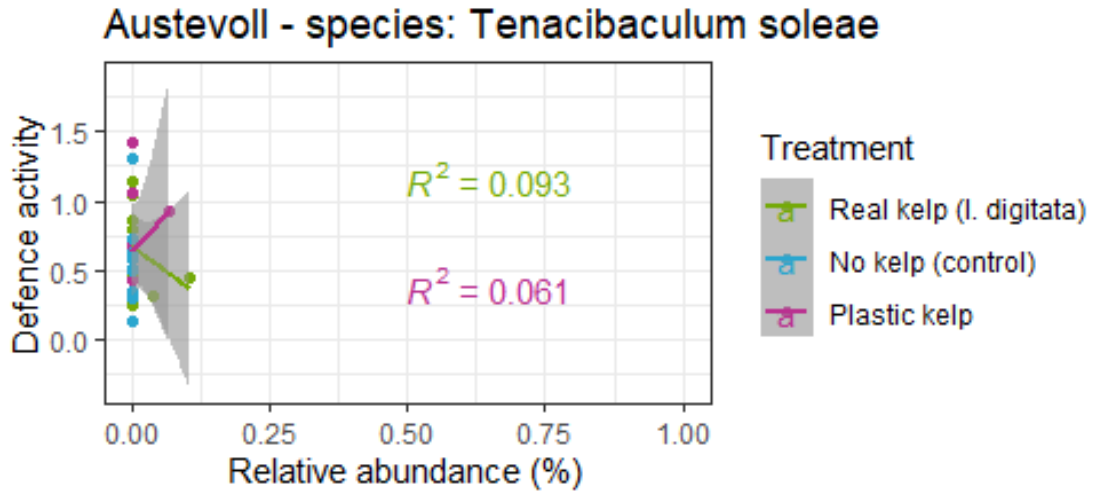

Austevoll - genus: Vibrio
Defence activity
Relative abundance (%)
R^2 = 0.0013
R^2 = 2.9e-05
R^2 = 0.073
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

Taxonomic level: Species









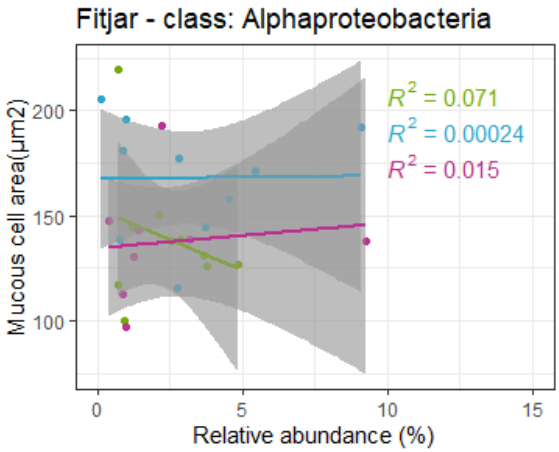
Appendix E – Bacterial RA vs Ma, VD and DA in Fitjar

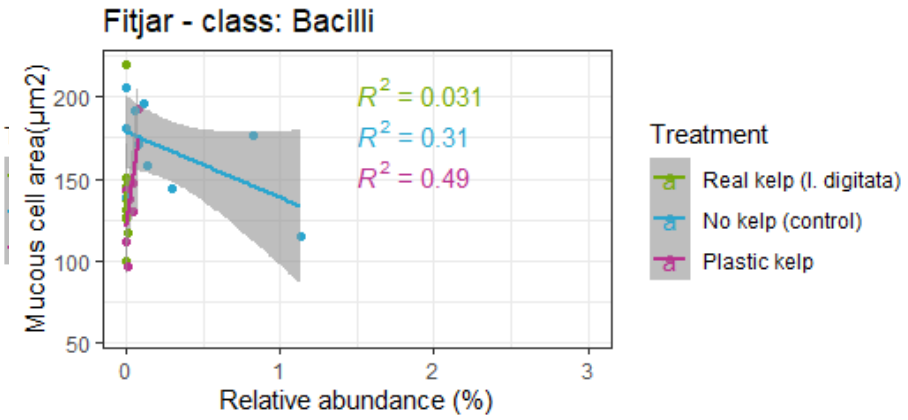
Fitjar (1011.2020) *Cyclopterus lumpus*

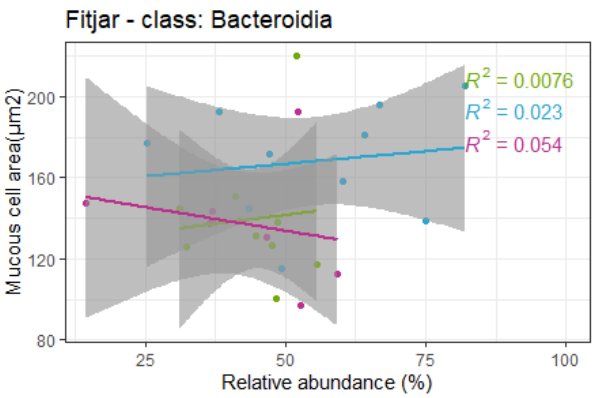
Mucous cell mean area, volumetric density and defence activity, versus relative abundance of class, family, genus and species – of bacteria found on the lumpfish skin. R^2 = correlation coefficient, line= linear regression, grey area= 95% confidence interval.

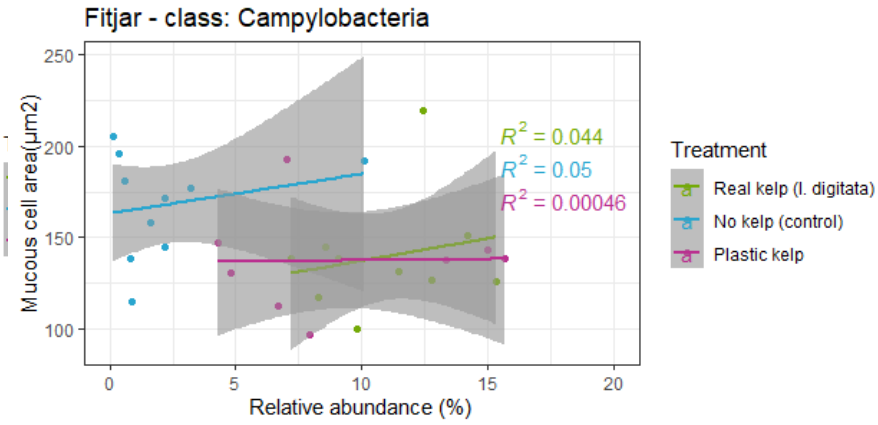
Mucous cell mean area vs. relative abundance

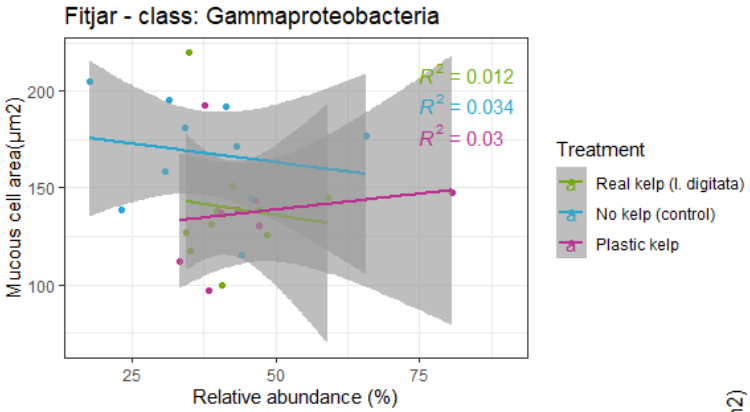
Taxonomic level: Class

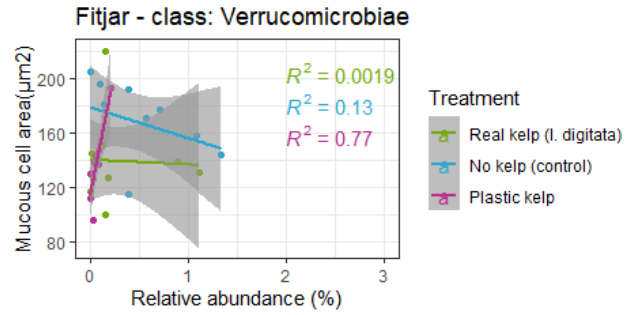


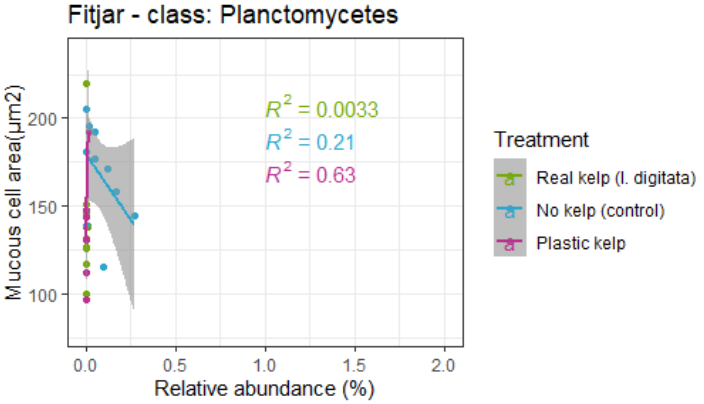




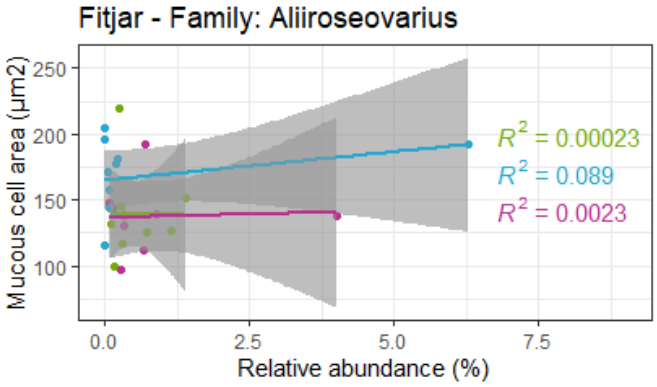


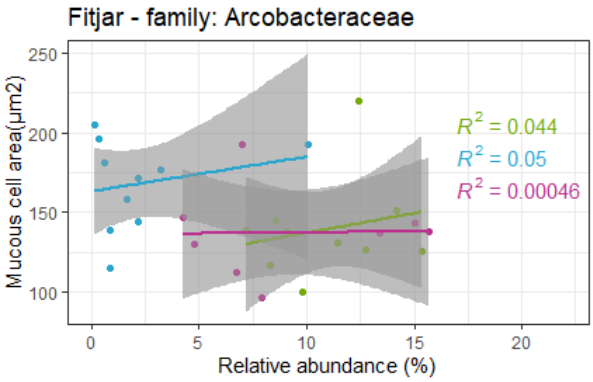


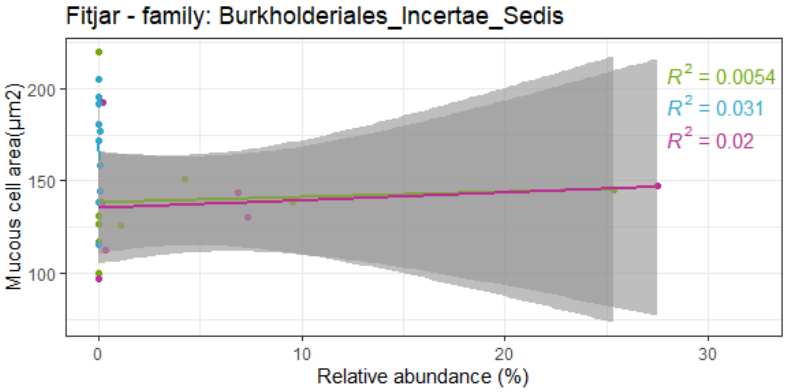


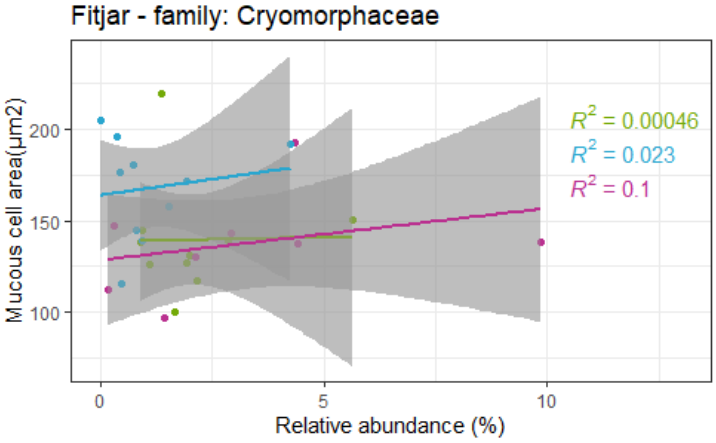


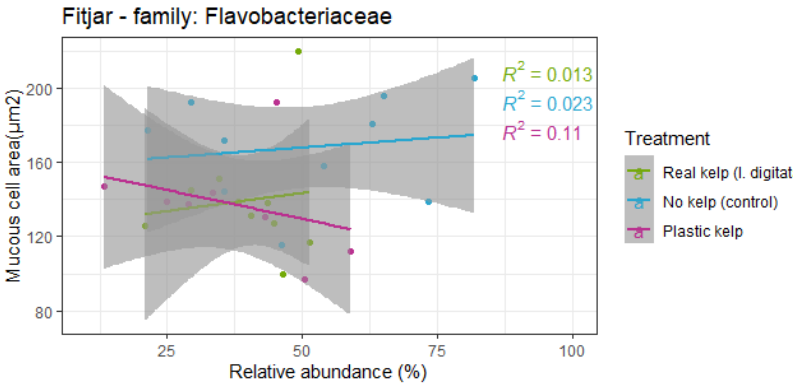
Taxonomic level: Family

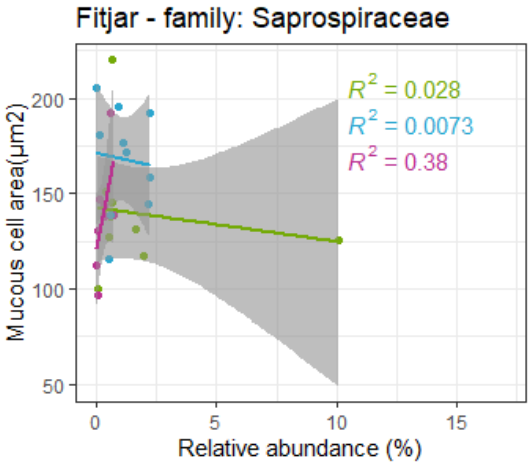


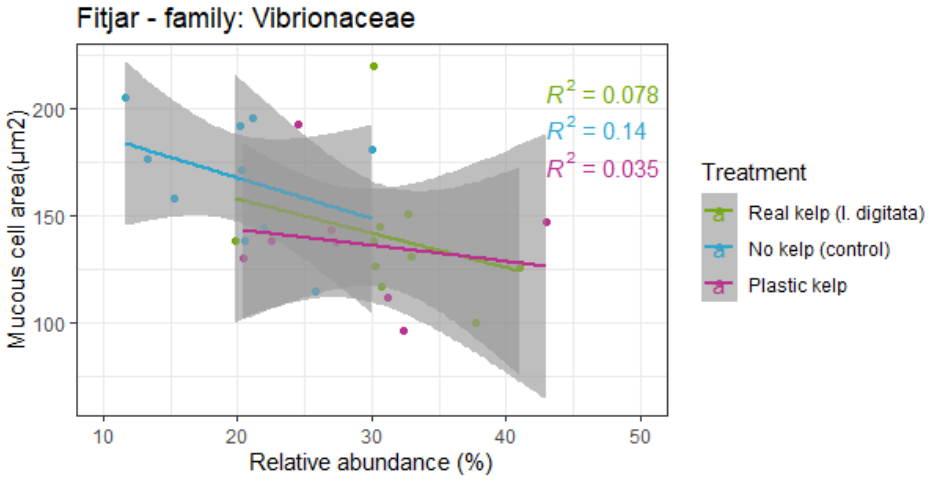




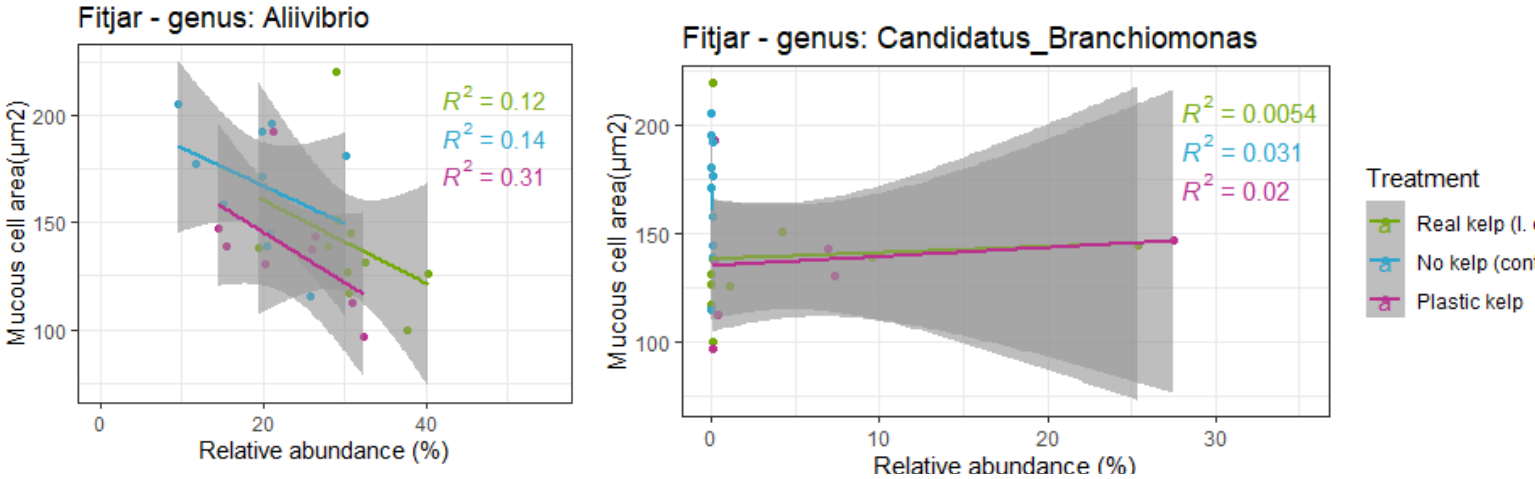


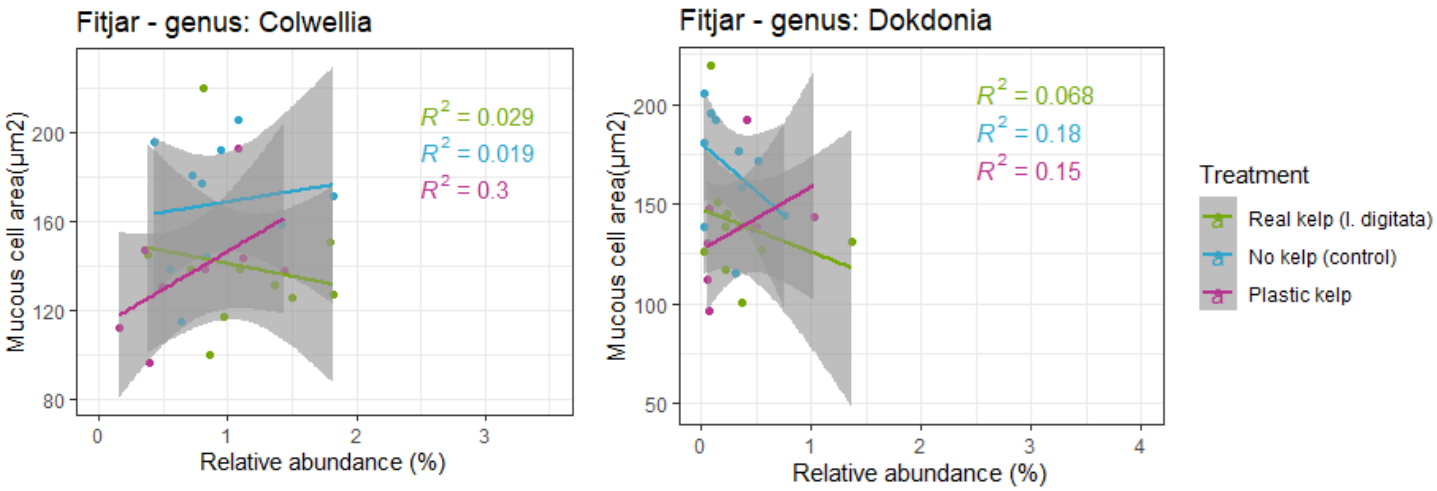


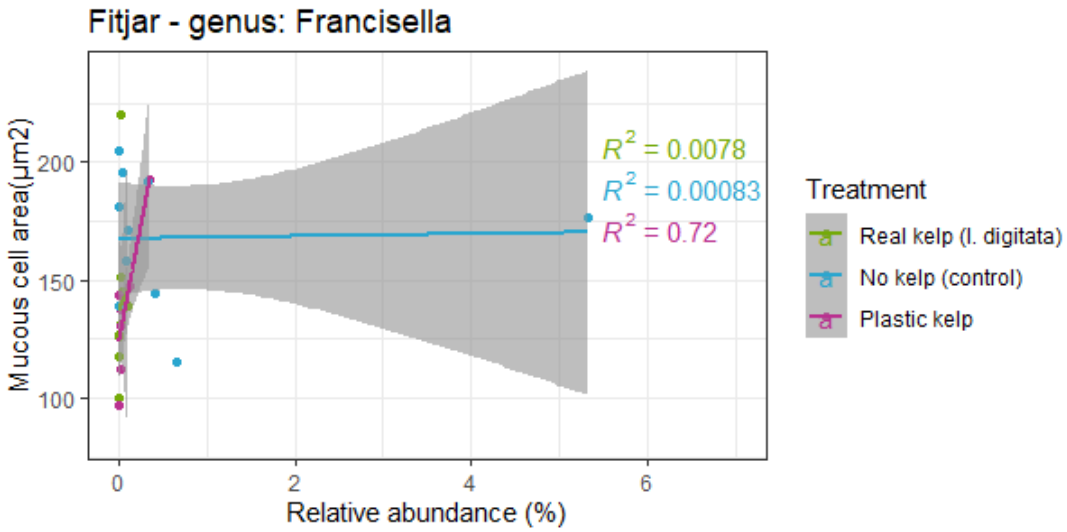


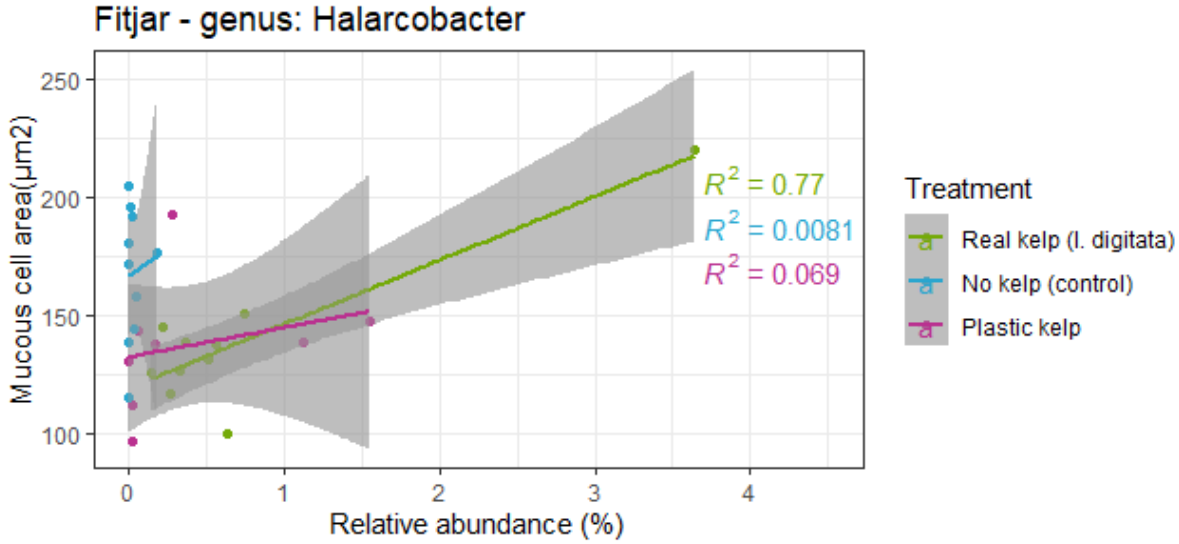


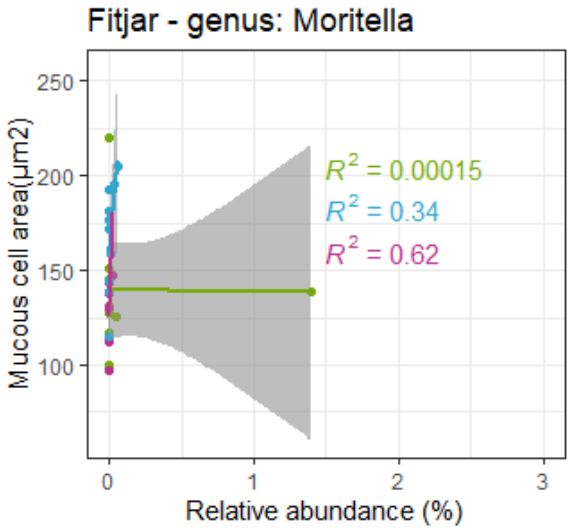
Taxonomic level: Genus

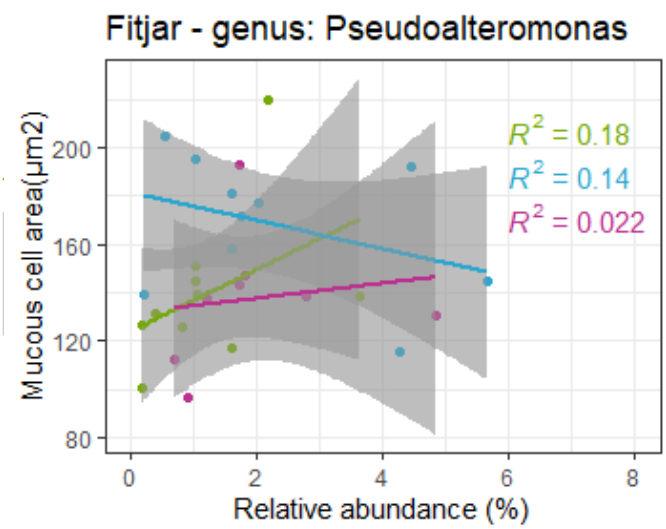


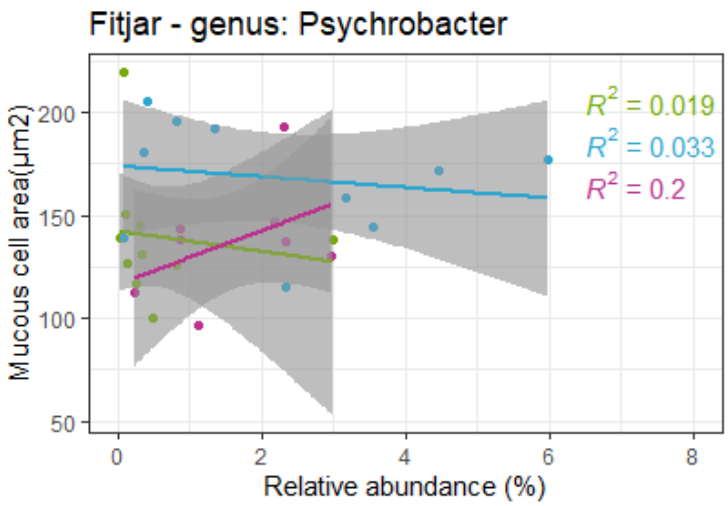


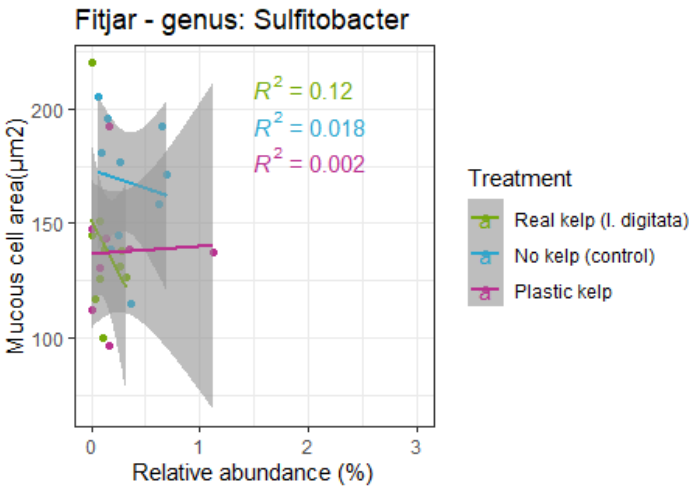


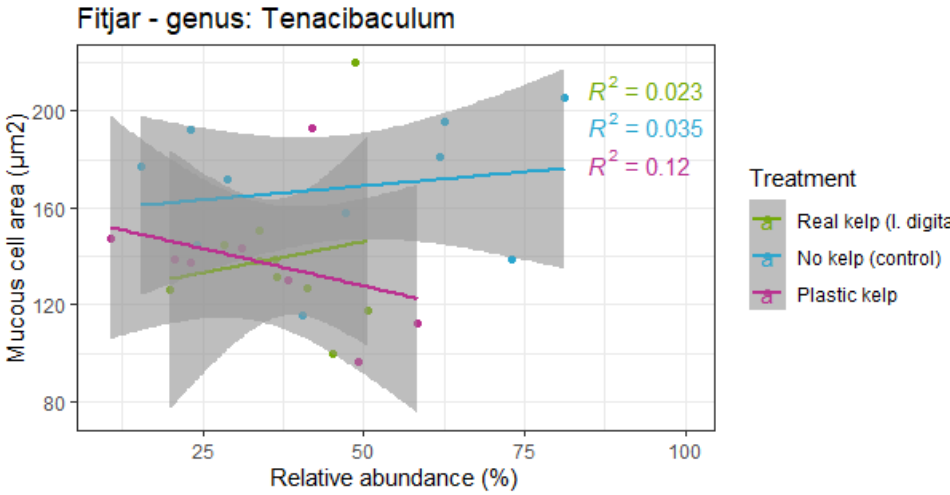


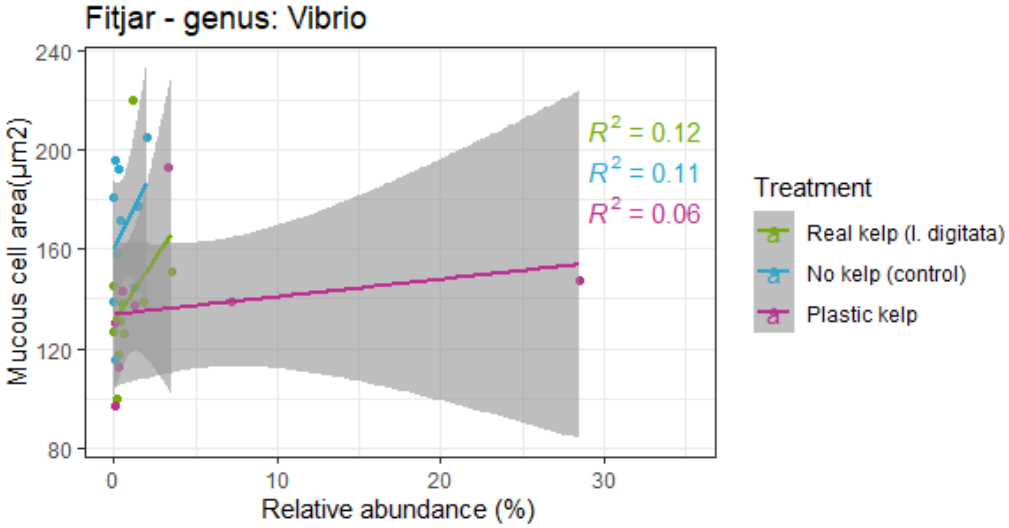

Fitjar - genus: Moritella
Mucous cell area(µm2)
Relative abundance (%)
R^2 = 0.00015
R^2 = 0.34
R^2 = 0.62


Fitjar - genus: Pseudoalteromonas
Mucous cell area(µm2)
Relative abundance (%)
R^2 = 0.18
R^2 = 0.14
R^2 = 0.022
Treatment
Real kelp (I. digit
No kelp (control)
Plastic kelp

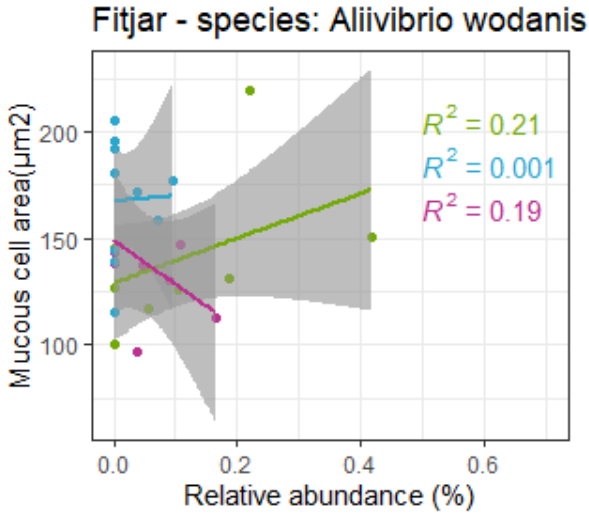

Fitjar - genus: Psychrobacter
Mucous cell area(µm2)
Relative abundance (%)
R^2 = 0.019
R^2 = 0.033
R^2 = 0.2

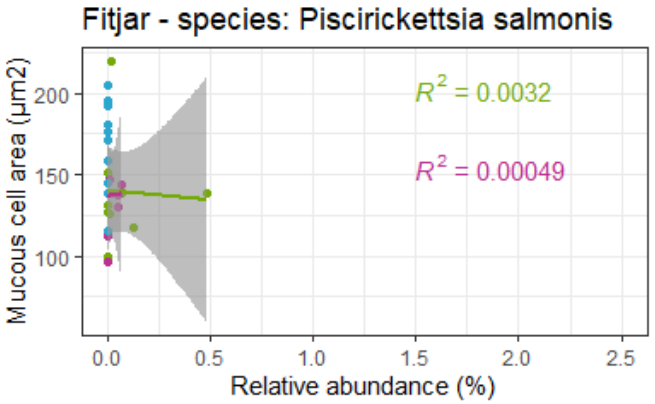

Fitjar - genus: Sulfitobacter
Mucous cell area(µm2)
Relative abundance (%)
R^2 = 0.12
R^2 = 0.018
R^2 = 0.002
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

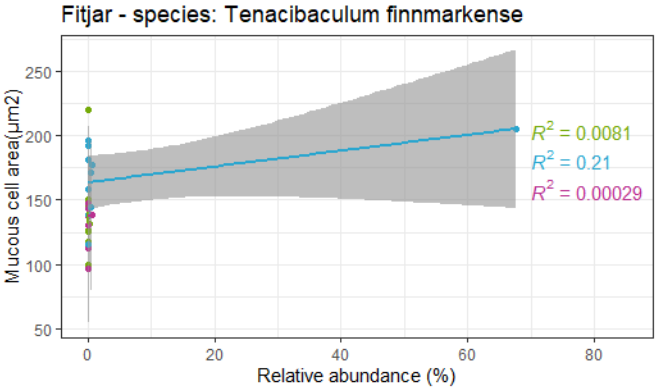

Fitjar - genus: Tenacibaculum
Mucous cell area (µm2)
Relative abundance (%)
R^2 = 0.023
R^2 = 0.035
R^2 = 0.12
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

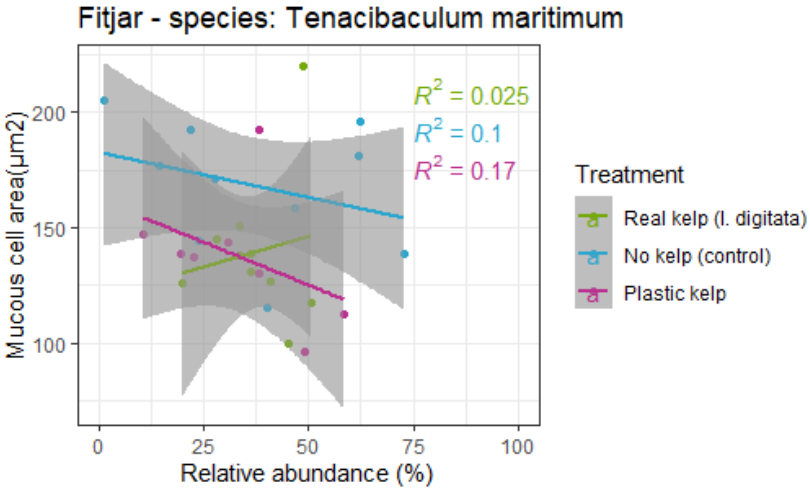

Fitjar - genus: Vibrio
Mucous cell area(µm2)
Relative abundance (%)
R^2 = 0.12
R^2 = 0.11
R^2 = 0.06
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

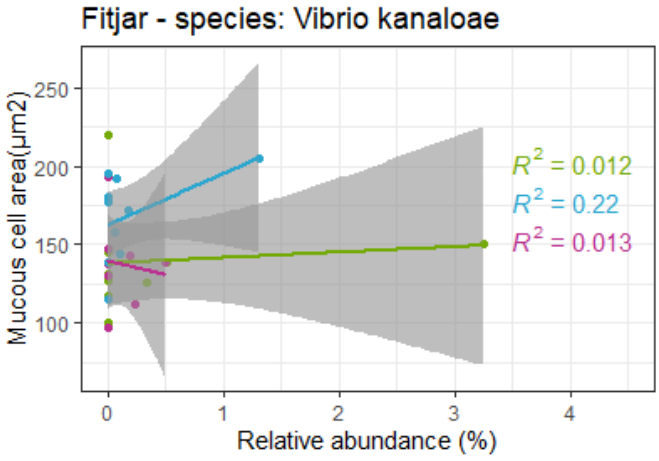
Taxonomic level: Species

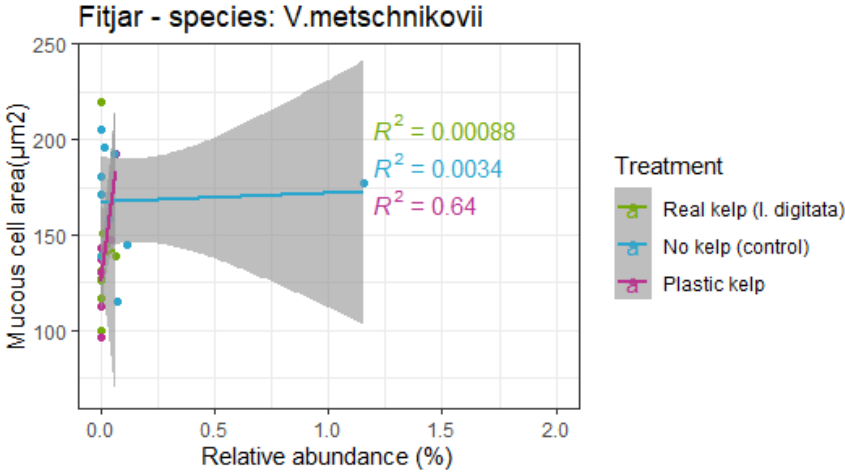


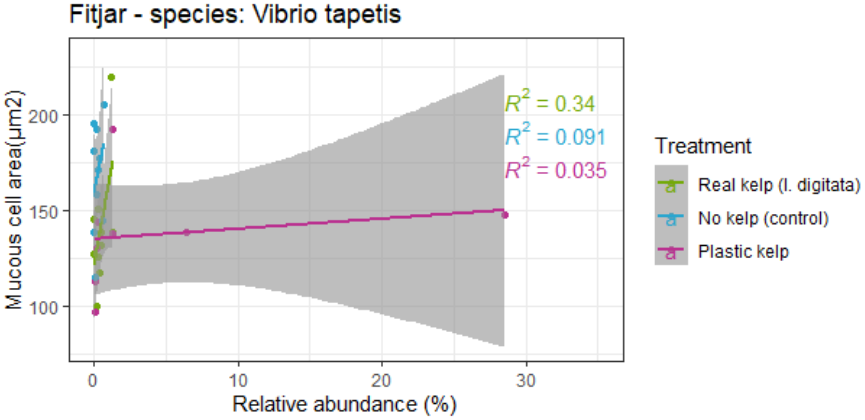






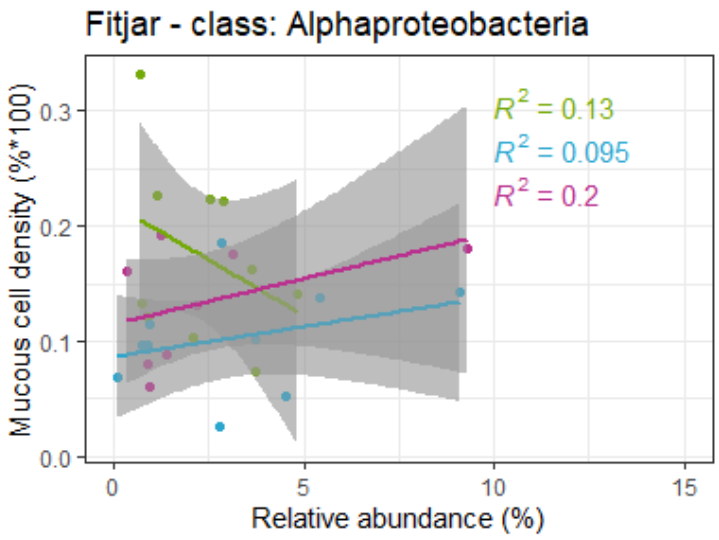


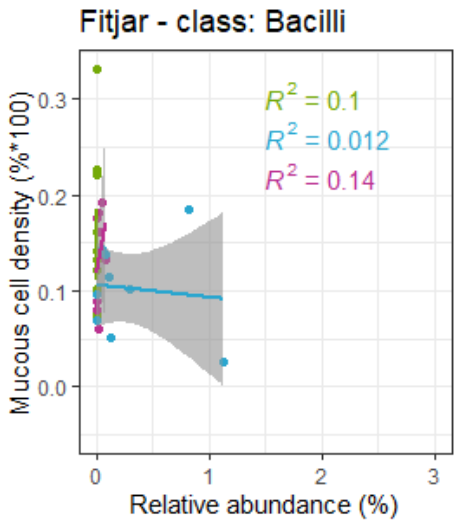


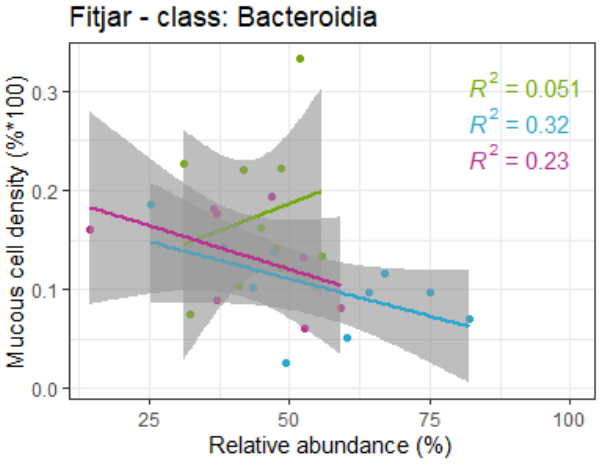


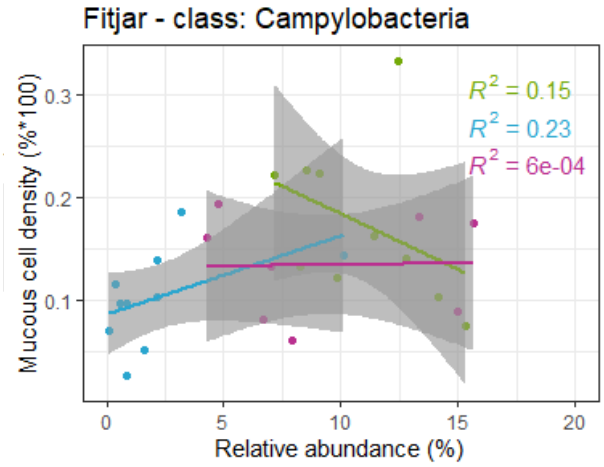
Mucous cell volumetric density vs. relative abundance

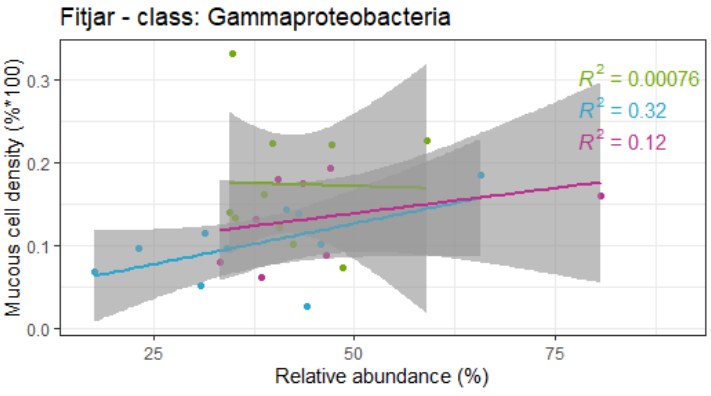
Taxonomic level: Class

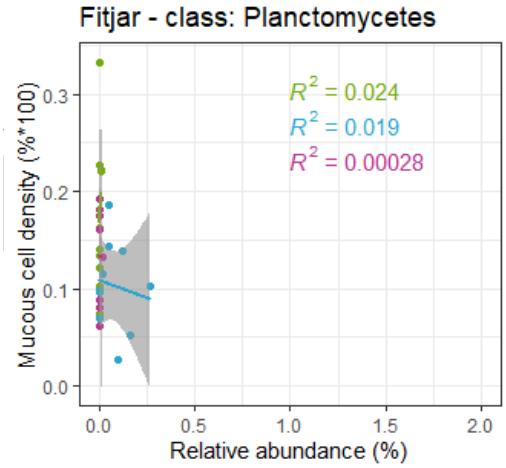


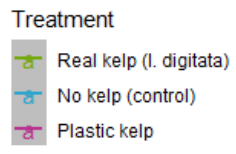


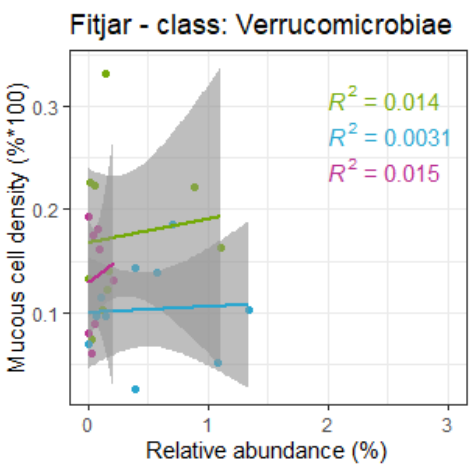


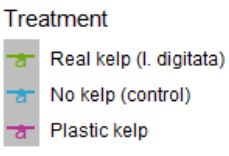




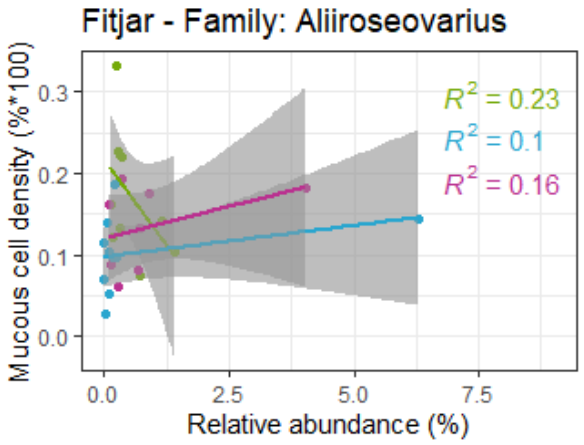


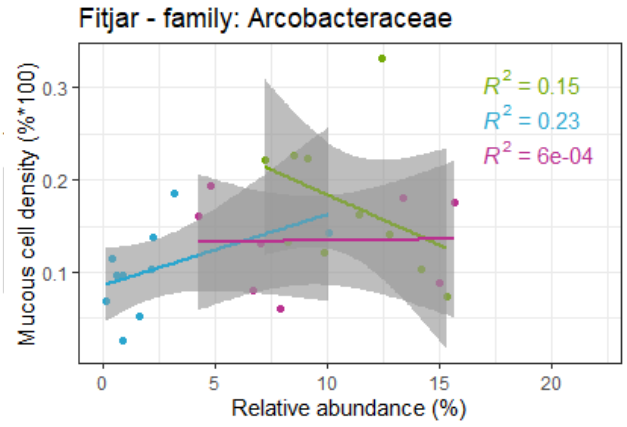


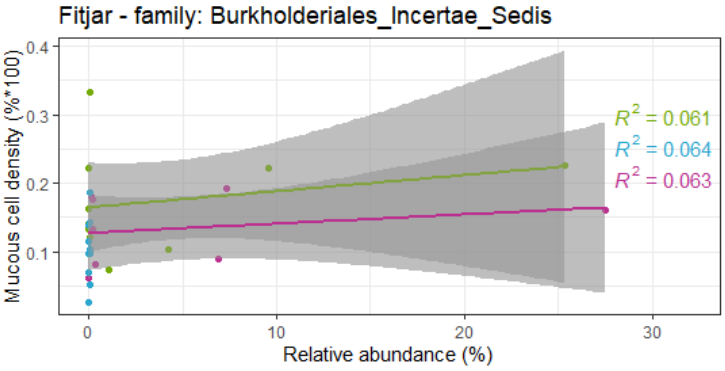


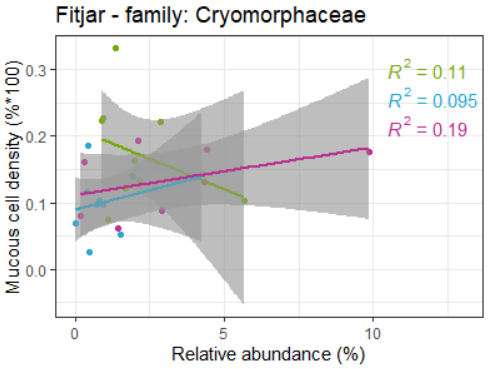


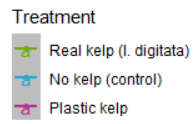
Taxonomic level: Family

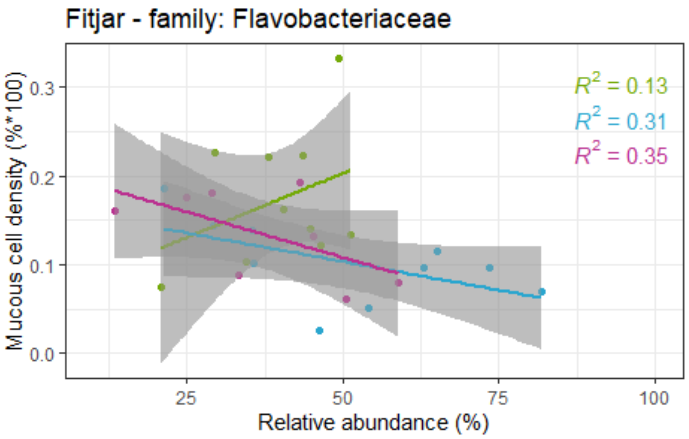


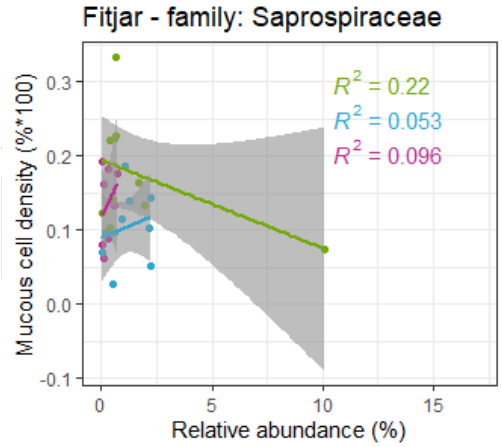


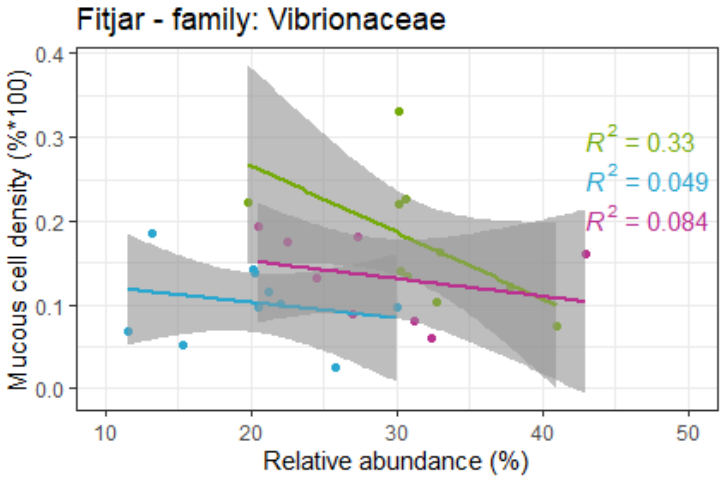




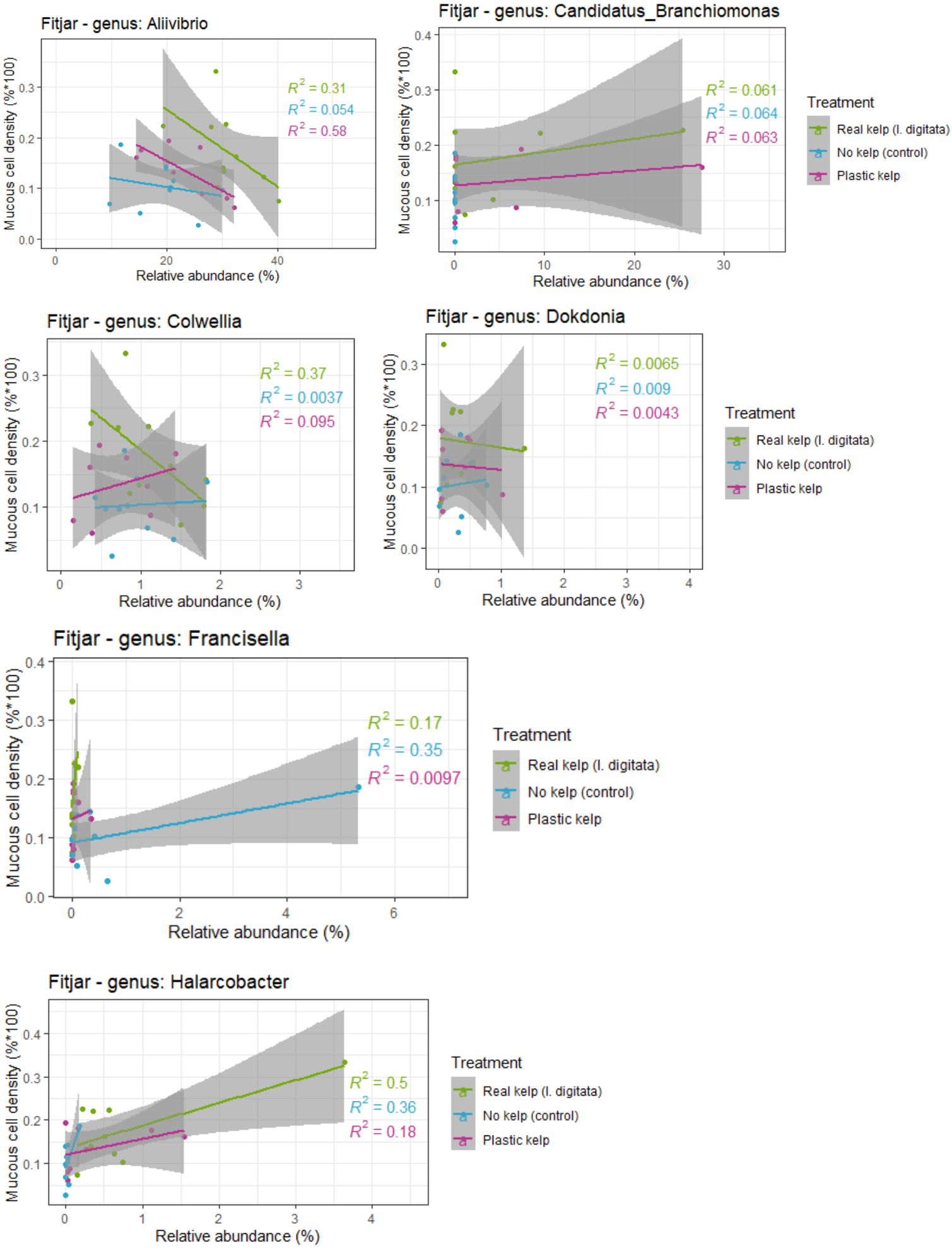


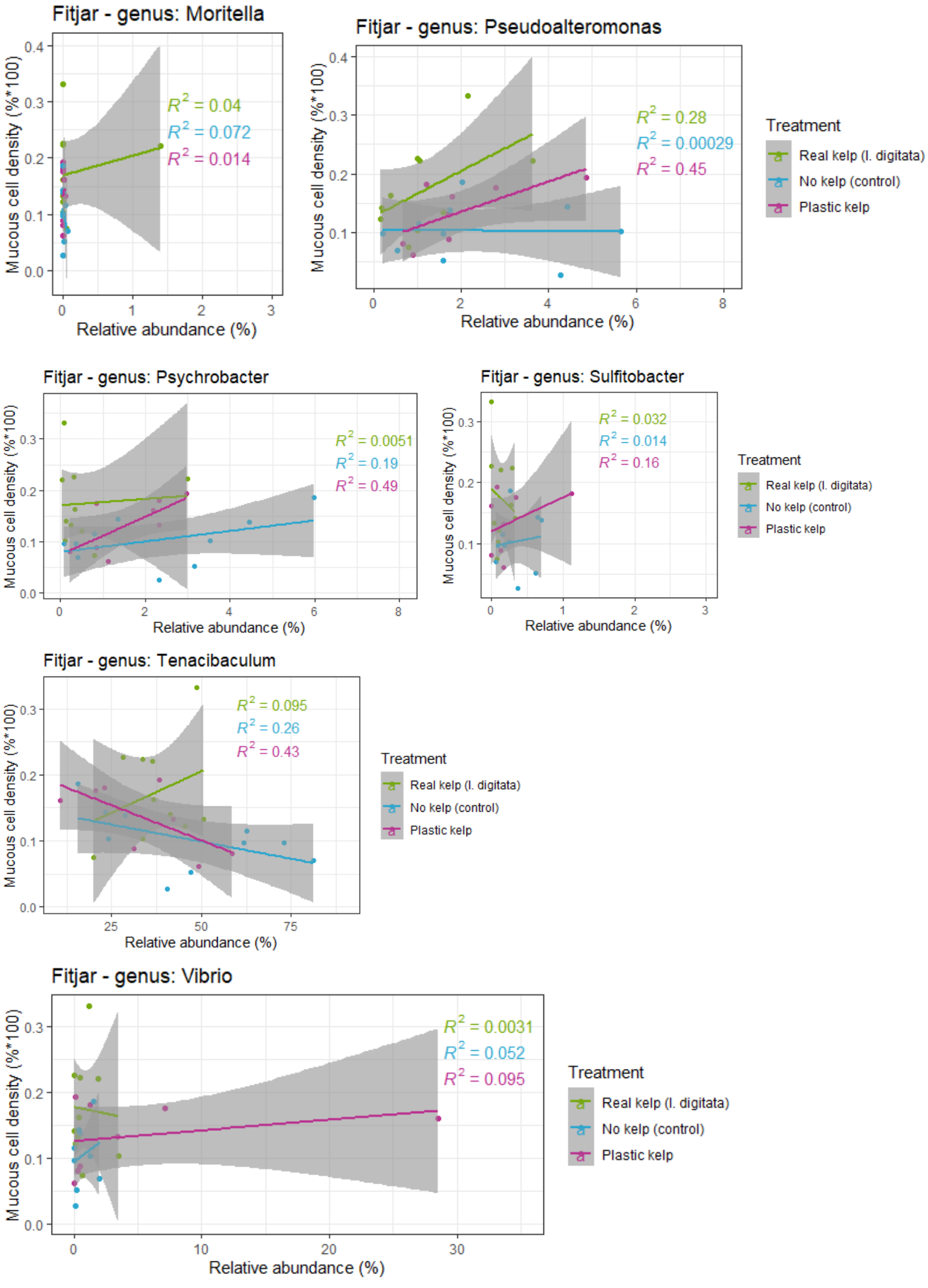




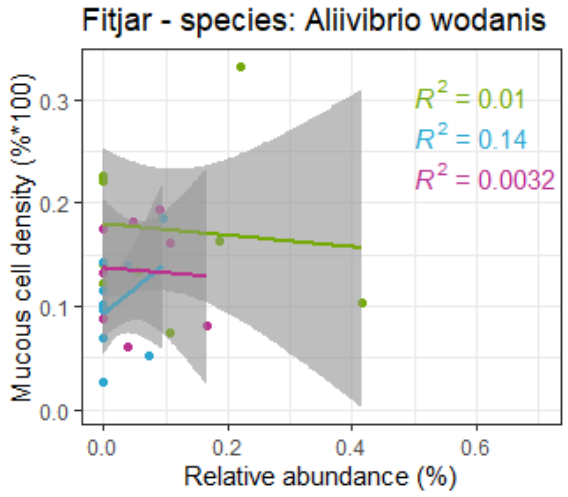


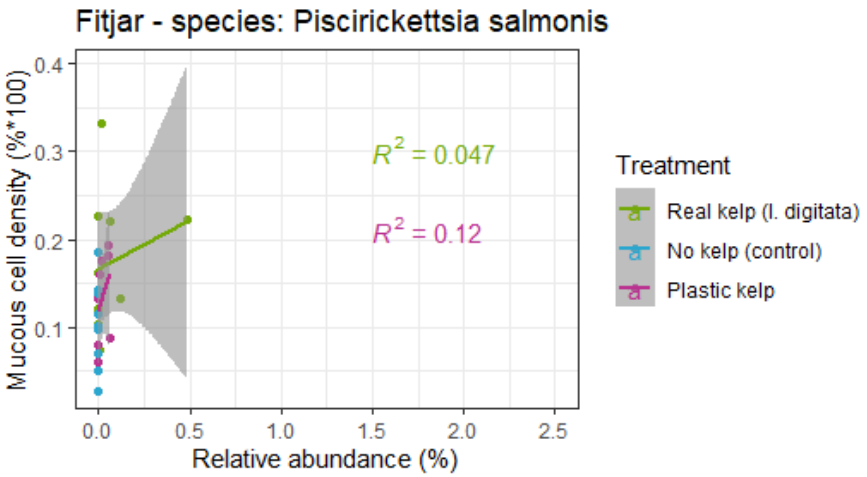
Taxonomic level: genus

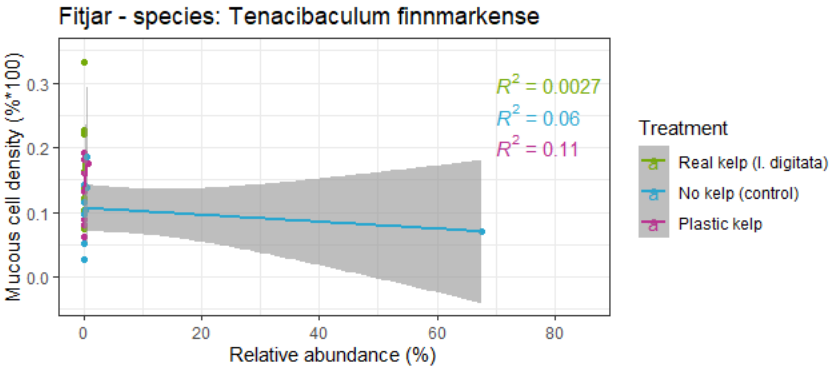


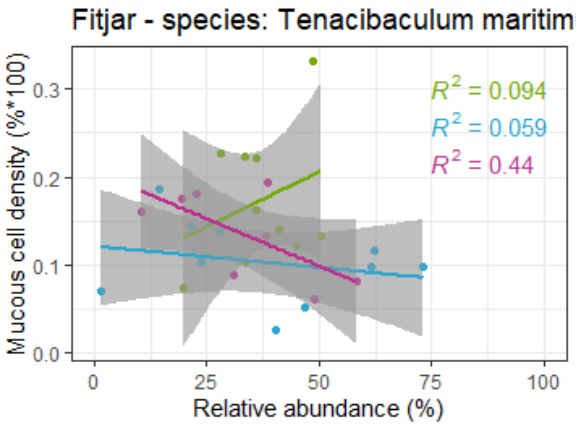

Fitjar - genus: Moritella
Mucous cell density (%*100)
Relative abundance (%)
R^2 = 0.04
R^2 = 0.072
R^2 = 0.014
Fitjar - genus: Pseudoalteromonas
R^2 = 0.28
R^2 = 0.00029
R^2 = 0.45
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp
Fitjar - genus: Psychrobacter
R^2 = 0.0051
R^2 = 0.19
R^2 = 0.49
Fitjar - genus: Sulfitobacter
R^2 = 0.032
R^2 = 0.014
R^2 = 0.16
Fitjar - genus: Tenacibaculum
R^2 = 0.095
R^2 = 0.26
R^2 = 0.43
Fitjar - genus: Vibrio
R^2 = 0.0031
R^2 = 0.052
R^2 = 0.095

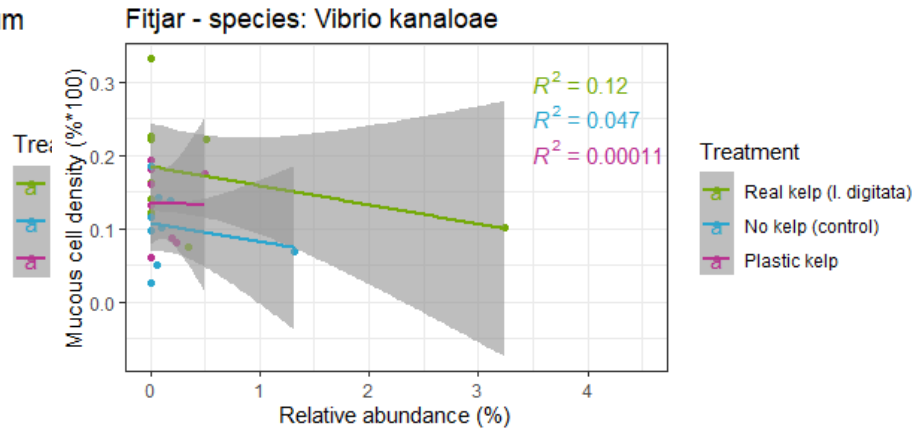
Taxonomic level: Species

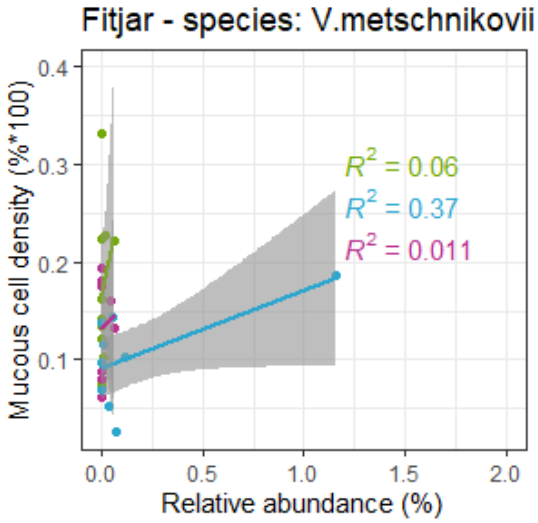


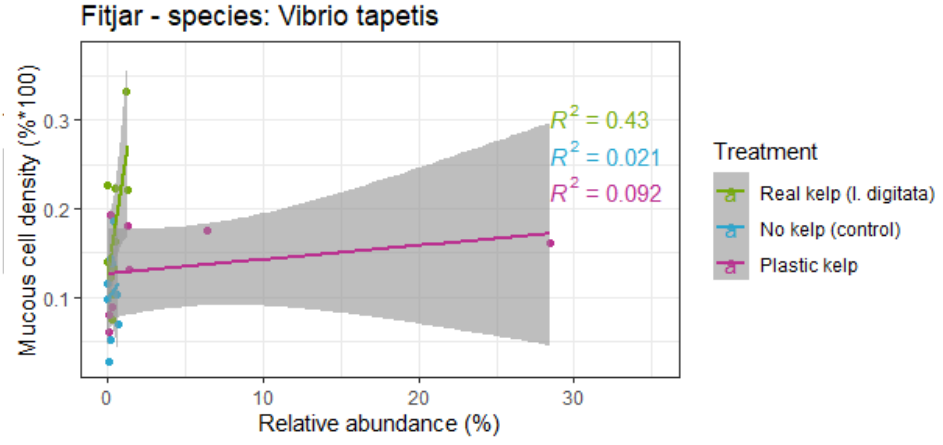






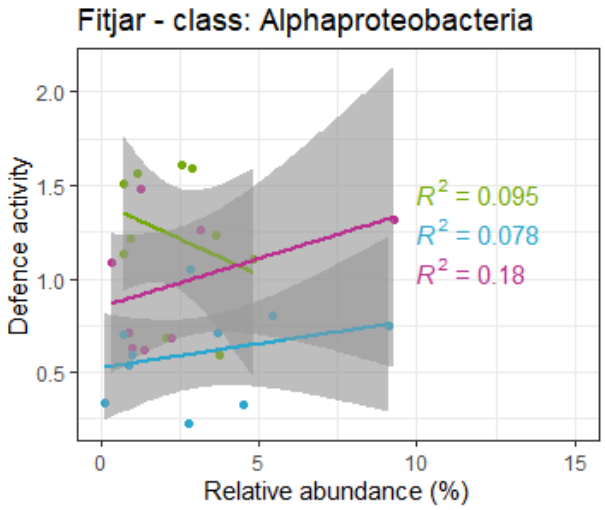


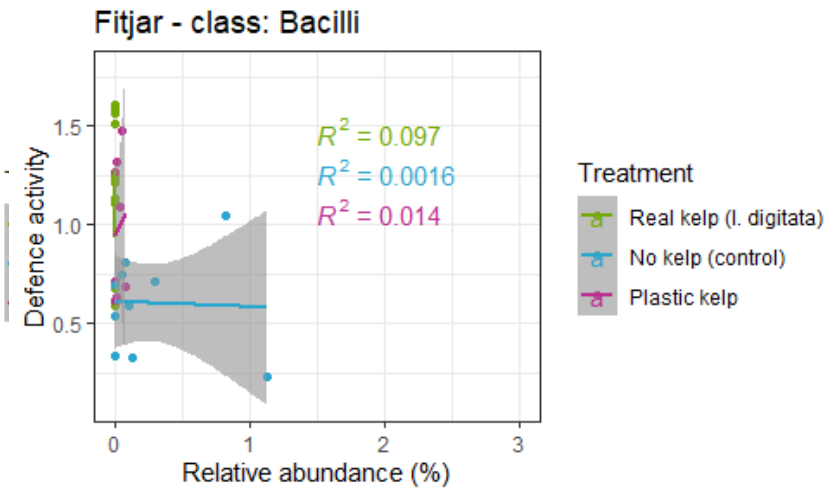


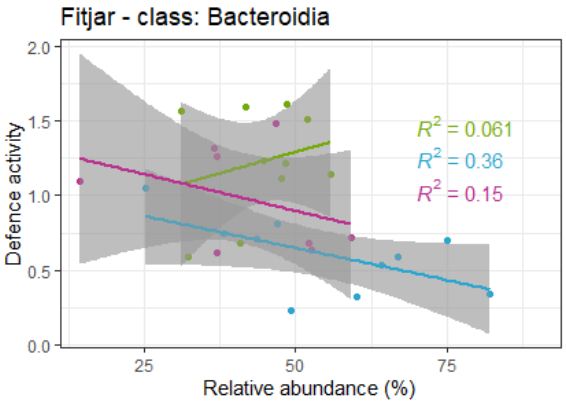


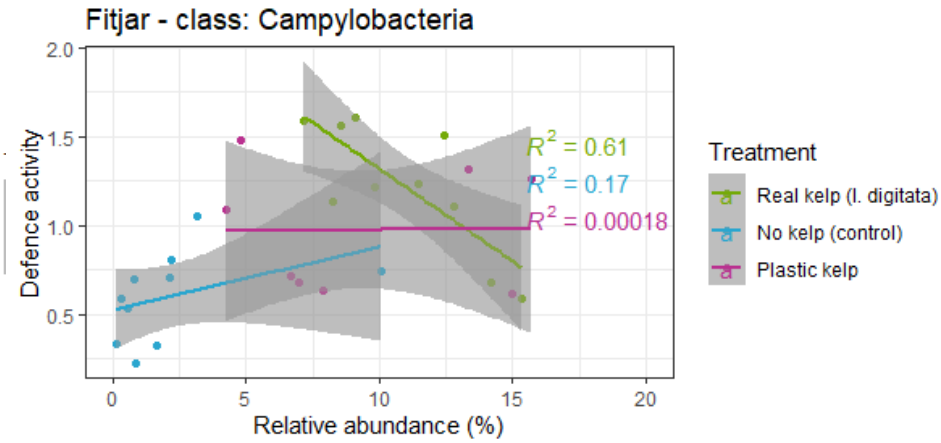
Defence activity vs. relative abundance

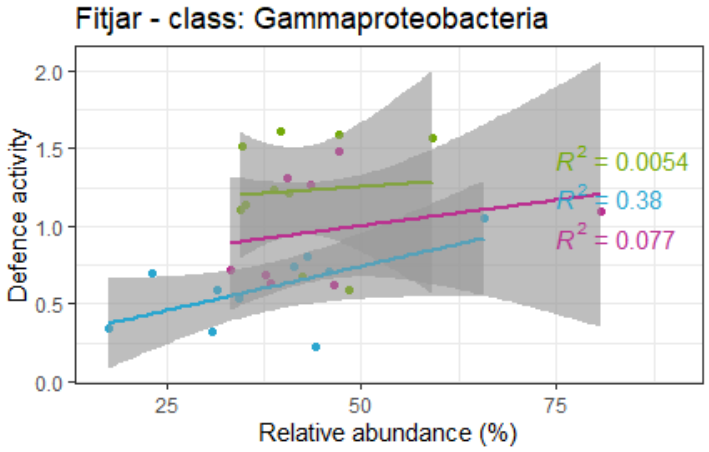
Taxonomic level: Class

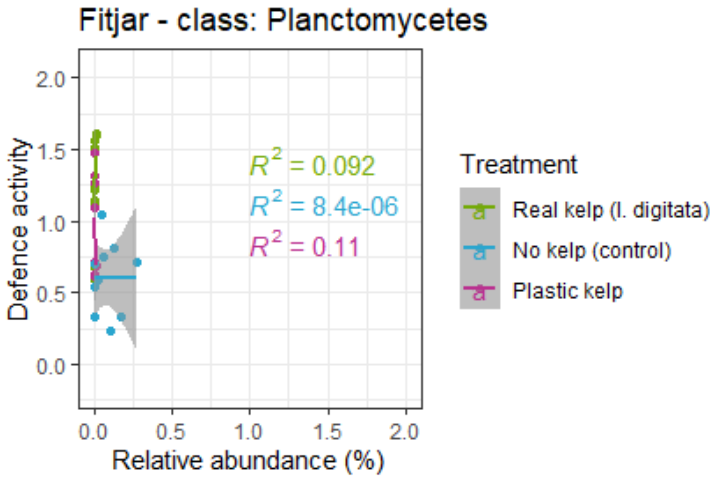


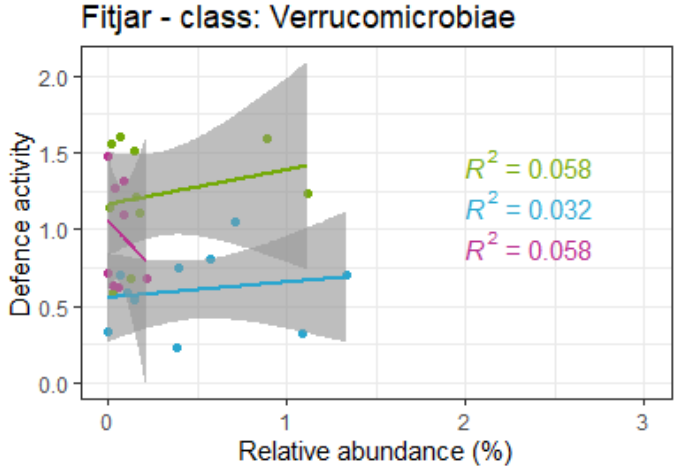




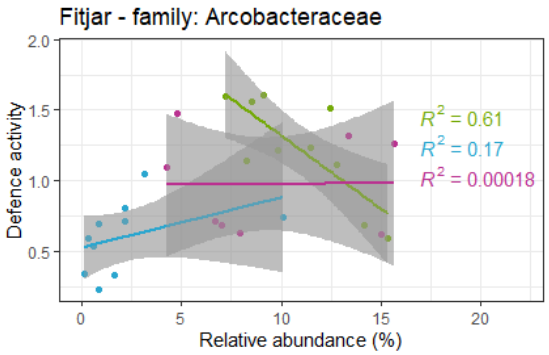


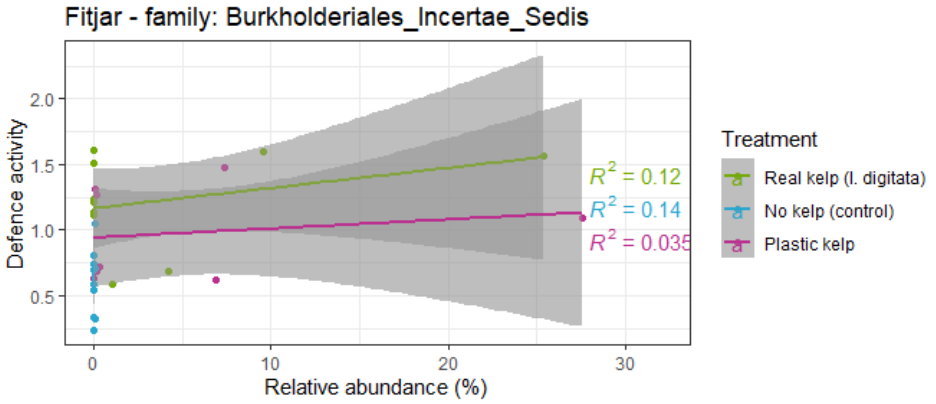


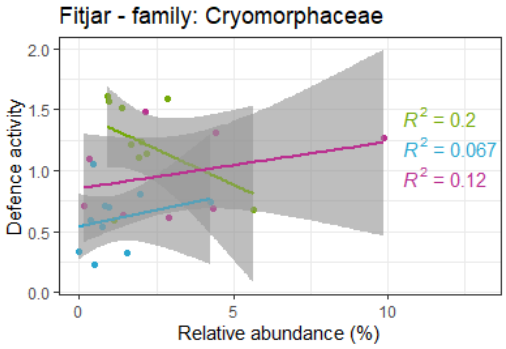


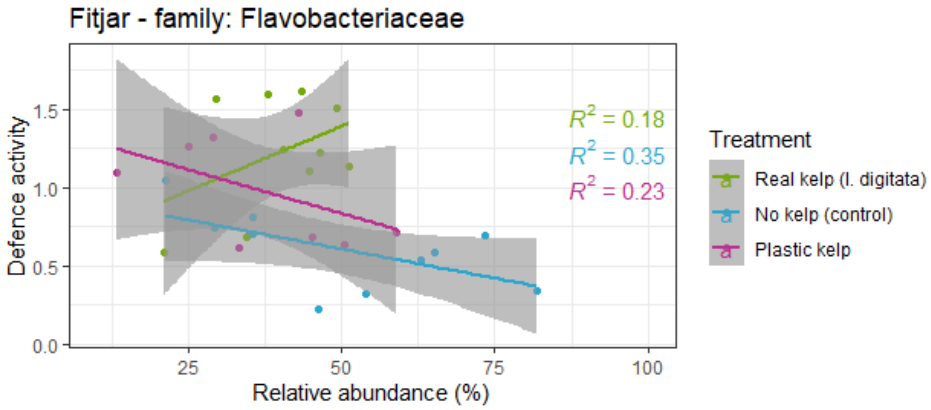


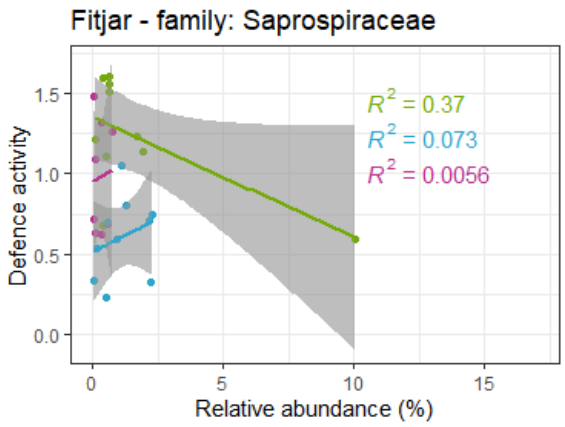
Taxonomic level: Family

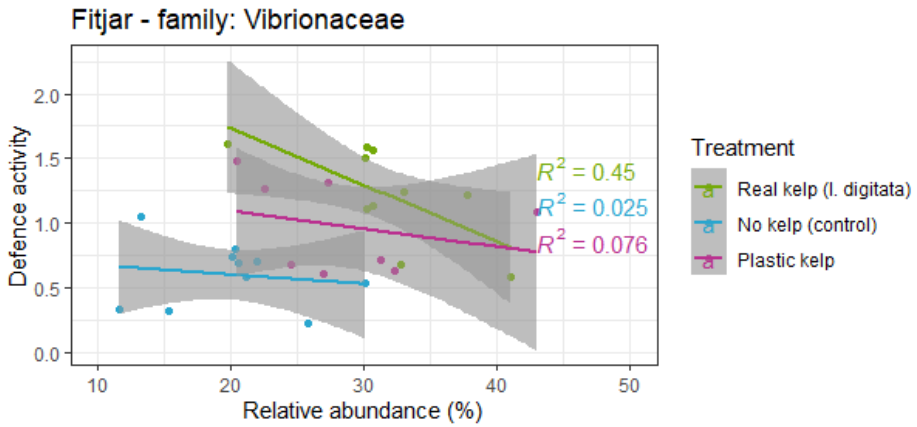




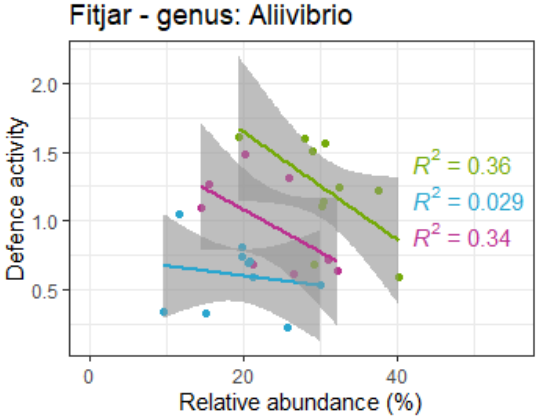


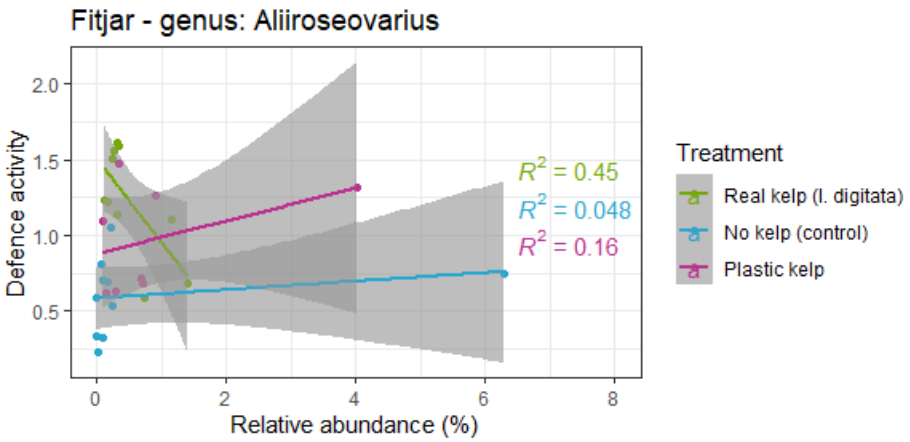


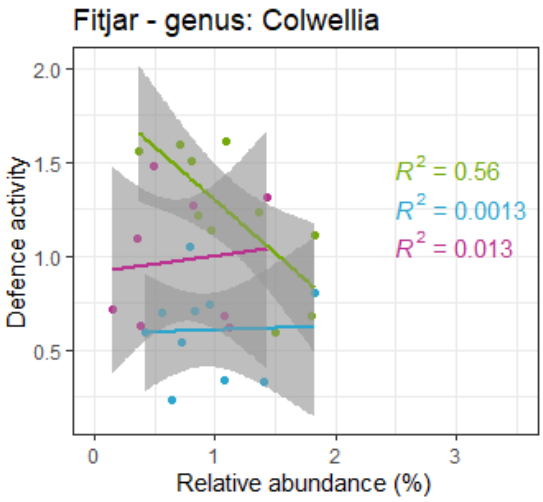


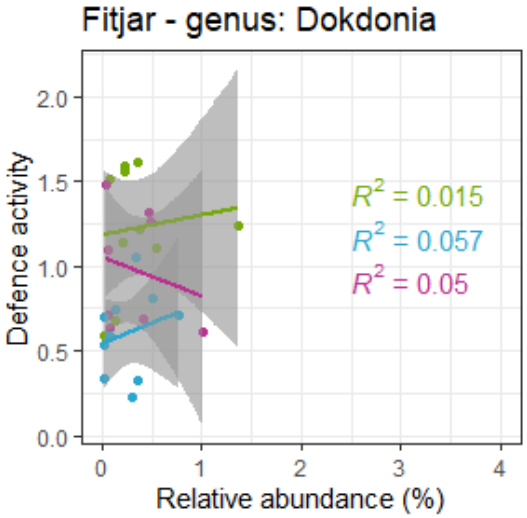


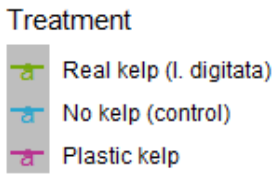
Taxonomic level: Genus

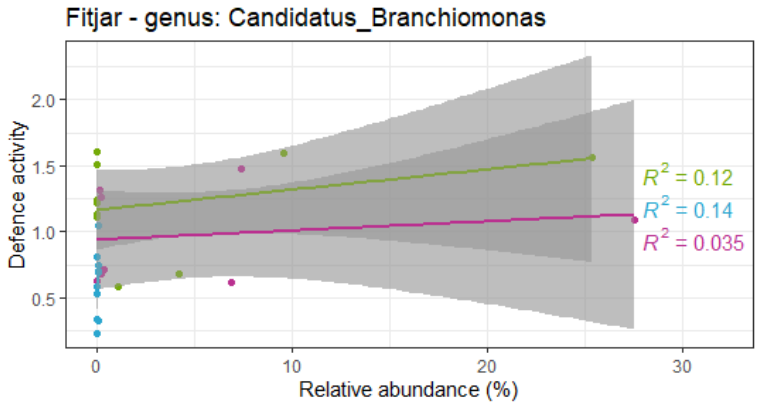


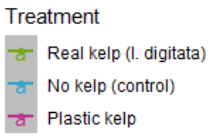


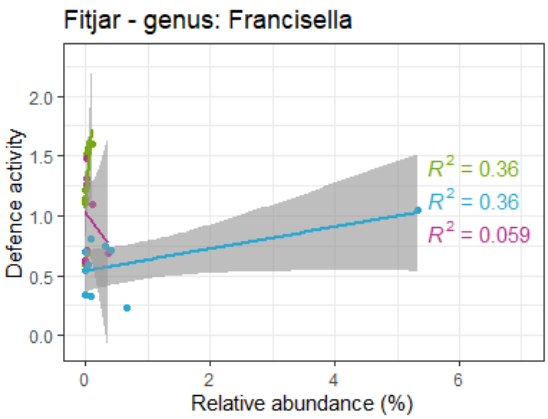

Fitjar - genus: Colwellia
Defence activity
Relative abundance (%)
R^2 = 0.56
R^2 = 0.0013
R^2 = 0.013

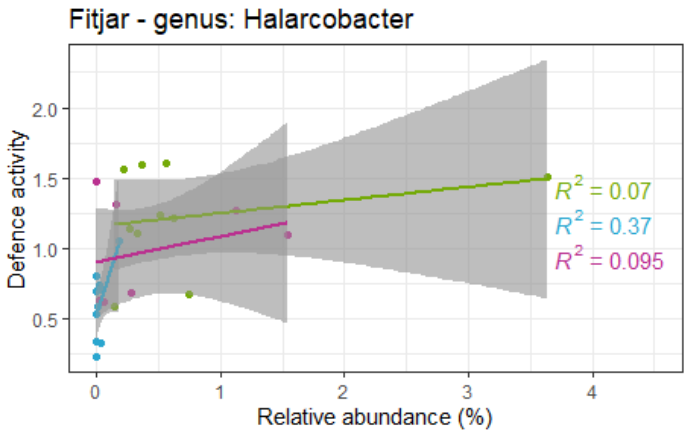

Fitjar - genus: Dokdonia
Defence activity
Relative abundance (%)
R^2 = 0.015
R^2 = 0.057
R^2 = 0.05

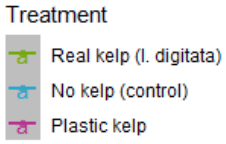

Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

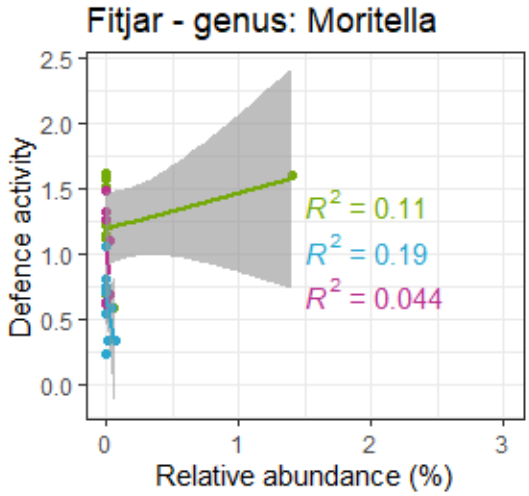

Fitjar - genus: Candidatus_Branchiomonas
Defence activity
Relative abundance (%)
R^2 = 0.12
R^2 = 0.14
R^2 = 0.035

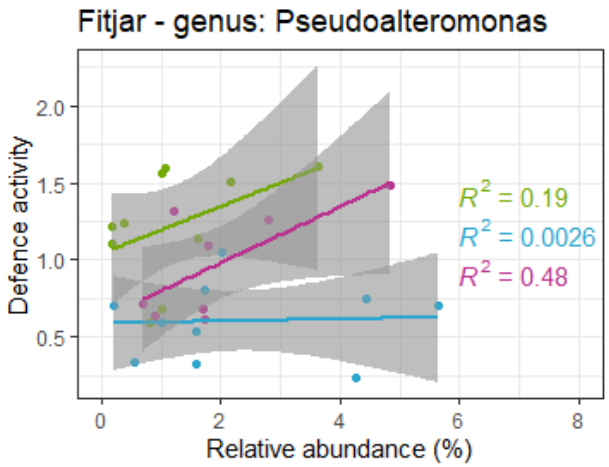

Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp


Fitjar - genus: Francisella
Defence activity
Relative abundance (%)
R^2 = 0.36
R^2 = 0.36
R^2 = 0.059

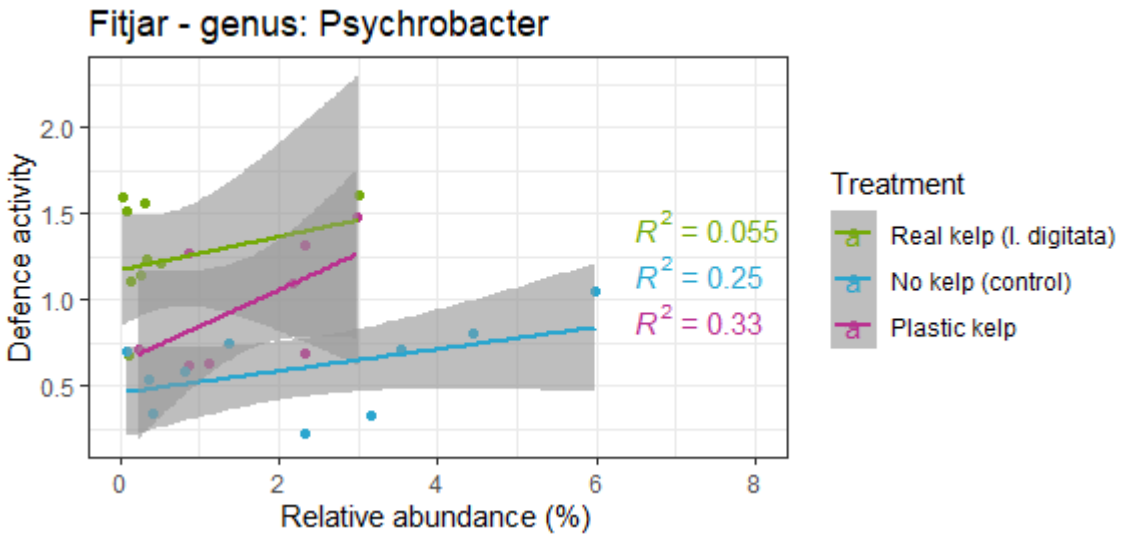

Fitjar - genus: Halarcobacter
Defence activity
Relative abundance (%)
R^2 = 0.07
R^2 = 0.37
R^2 = 0.095

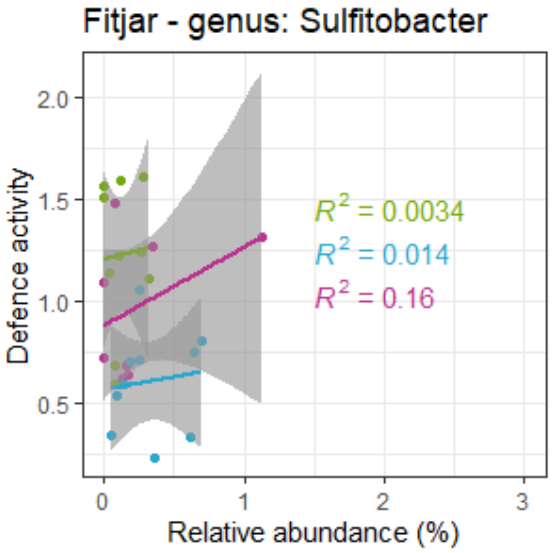

Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

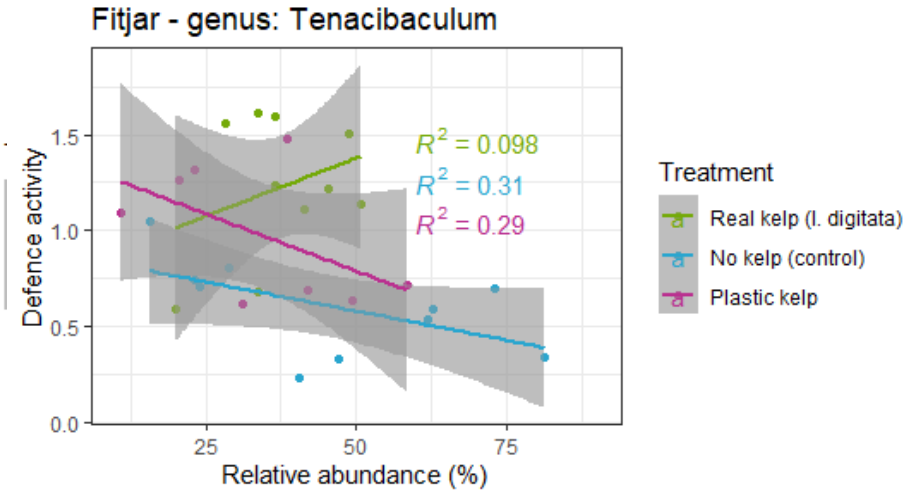

Fitjar - genus: Moritella
Defence activity
Relative abundance (%)
R^2 = 0.11
R^2 = 0.19
R^2 = 0.044

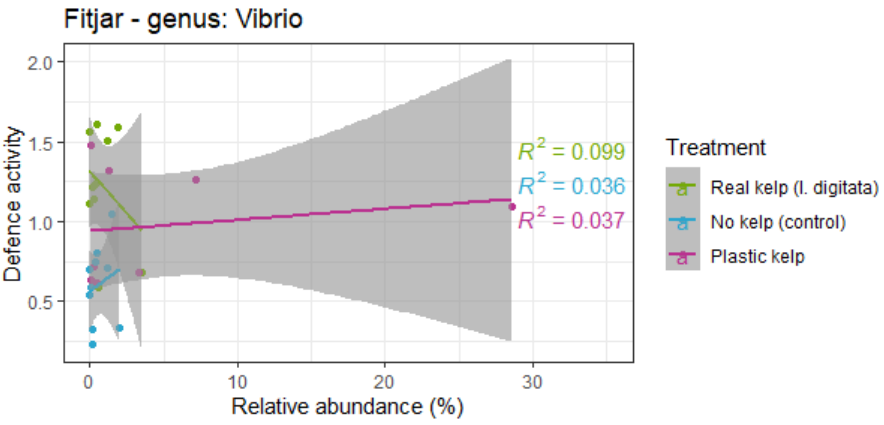

Fitjar - genus: Pseudoalteromonas
Defence activity
Relative abundance (%)
R^2 = 0.19
R^2 = 0.0026
R^2 = 0.48


Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

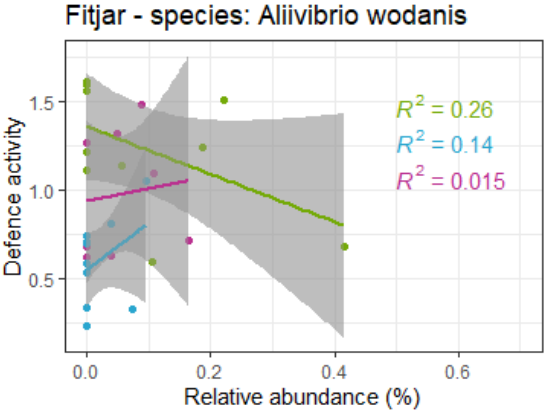


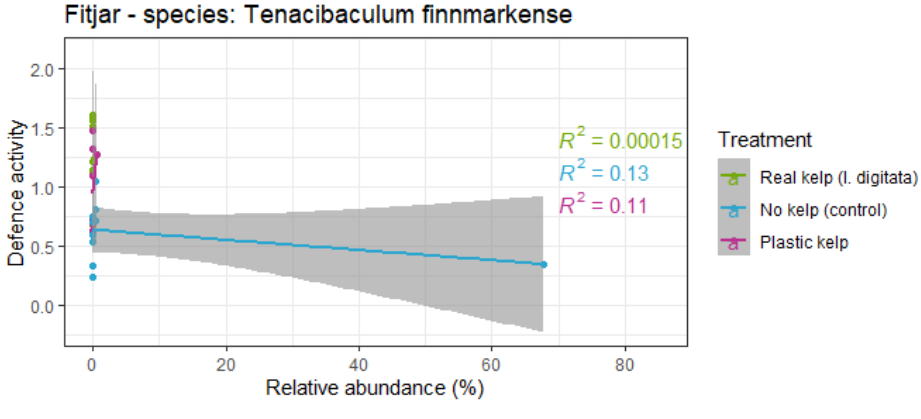


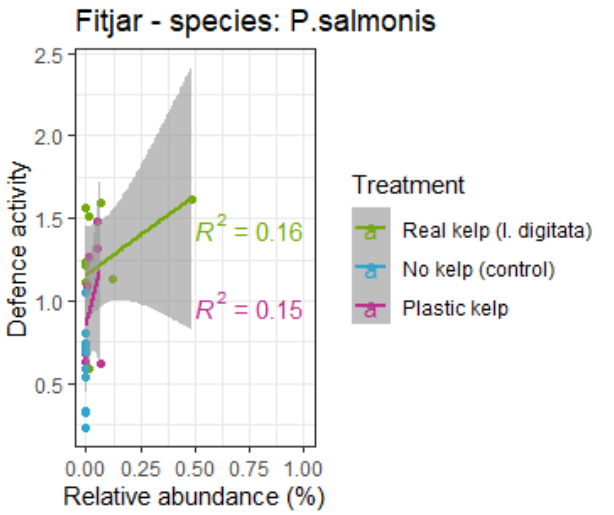


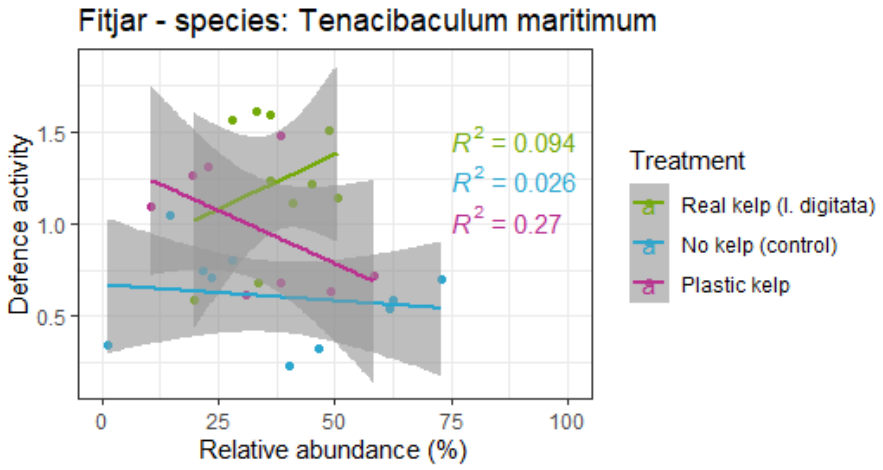


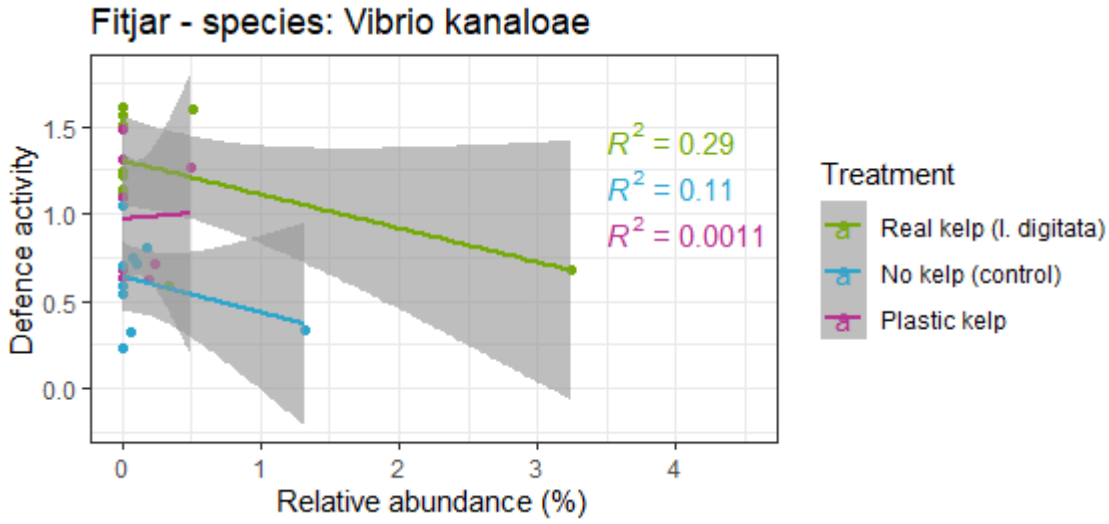
Taxonomic level: Species

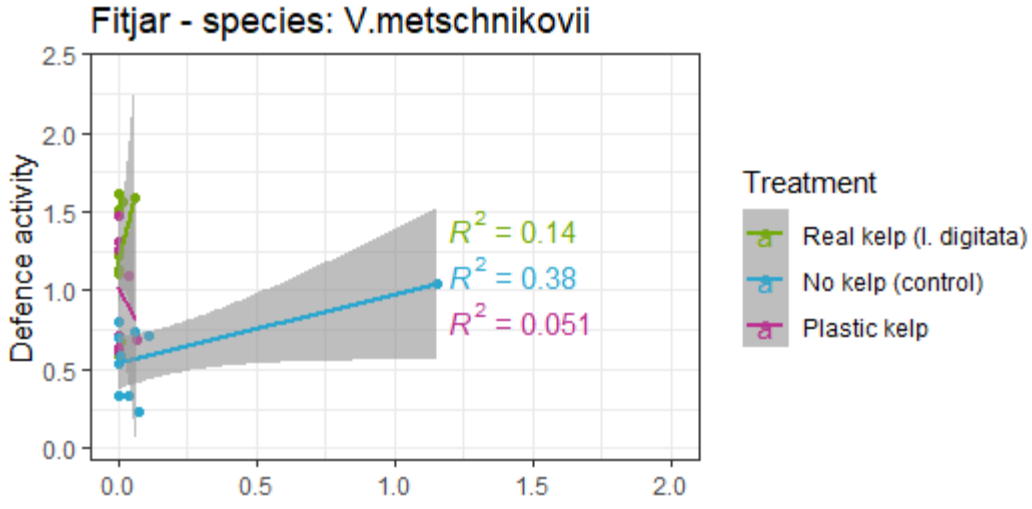


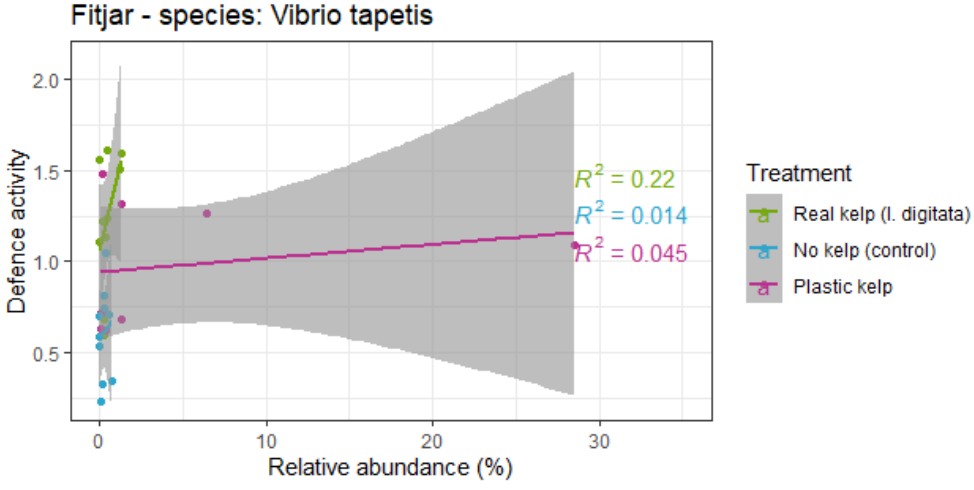



Fitjar - species: P.salmonis
Defence activity
Relative abundance (%)
R^2 = 0.16
R^2 = 0.15
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp


Fitjar - species: Tenacibaculum maritimum
Defence activity
Relative abundance (%)
R^2 = 0.094
R^2 = 0.026
R^2 = 0.27
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp


Fitjar - species: Vibrio kanaloae
Defence activity
Relative abundance (%)
R^2 = 0.29
R^2 = 0.11
R^2 = 0.0011
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp


Fitjar - species: V.metschnikovii
Defence activity
R^2 = 0.14
R^2 = 0.38
R^2 = 0.051
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp


Fitjar - species: Vibrio tapetis
Defence activity
Relative abundance (%)
R^2 = 0.22
R^2 = 0.014
R^2 = 0.045
Treatment
Real kelp (I. digitata)
No kelp (control)
Plastic kelp

Appendix F – Q-Q plots

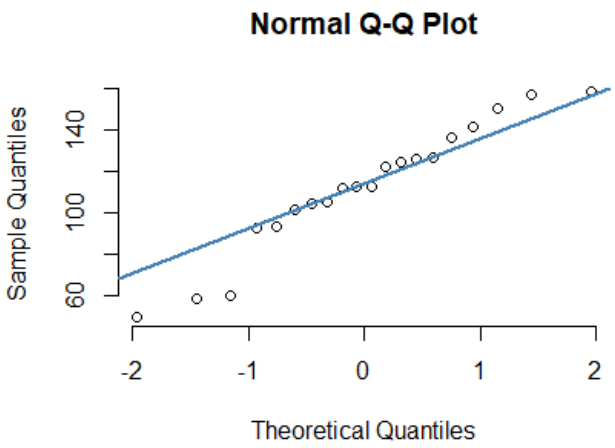


Figure 1: Q-Q plot Agder - Mean area

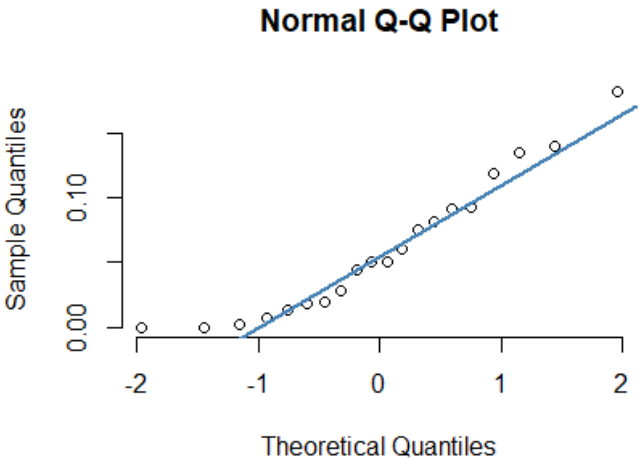


Figure 2: Q-Q plot Agder- Volumetric density

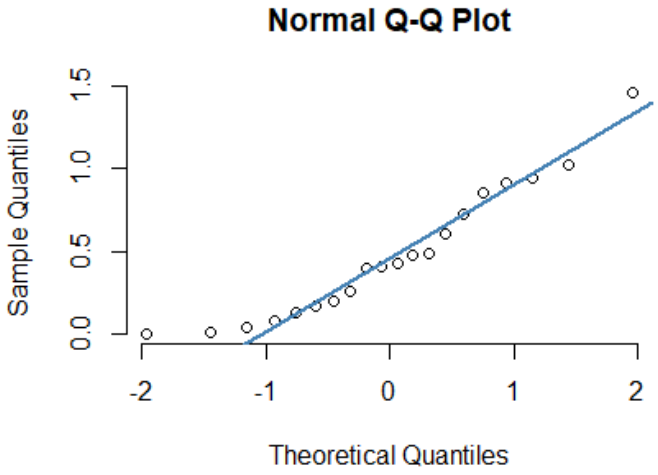


Figure 3: Q-Q plot Agder - Defence activity

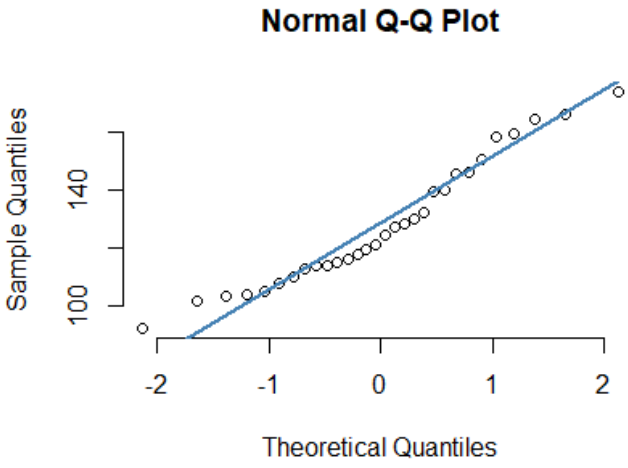


Figure 4: Q-Q plot Austevoll 1 - Mean area

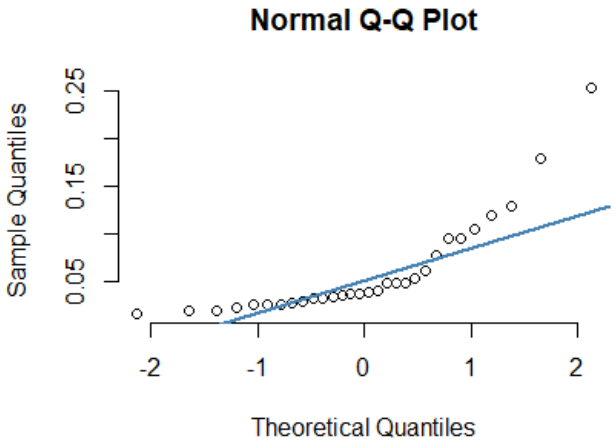


Figure 5: Q-Q plot Austevoll 1 - Volumetric densit

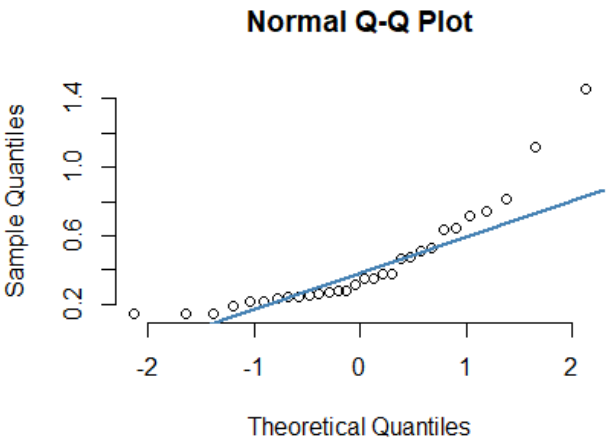


Figure 6: Q-Q plot Austevoll 1 - Defence activity

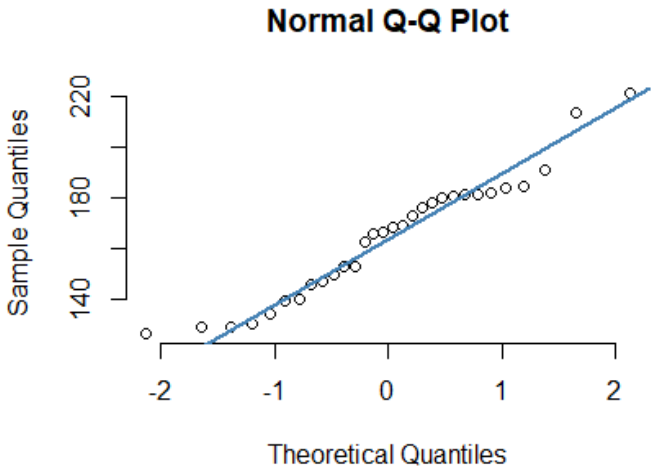


Figure 7: Q-Q plot Austevoll 2 - Mean area

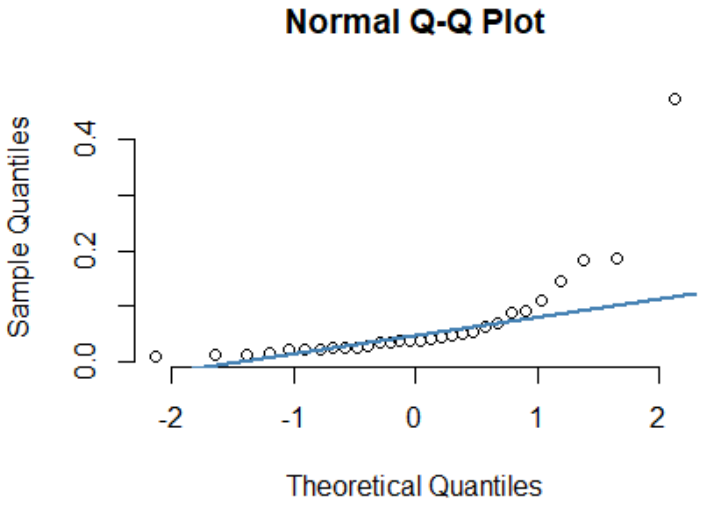


Figure 8: Q-Q plot Austevoll 2 - Volumetric density

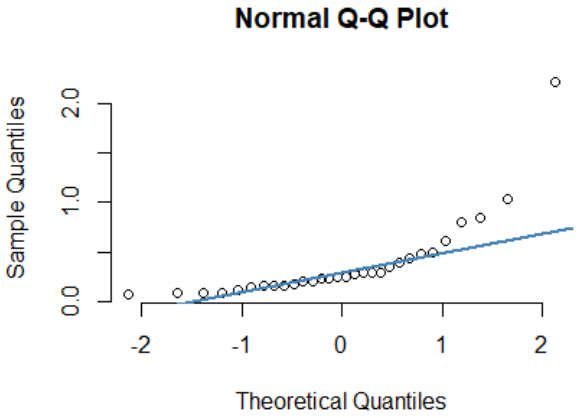


Figure 9: Q-Q plot Austevoll 2 - Defence activity

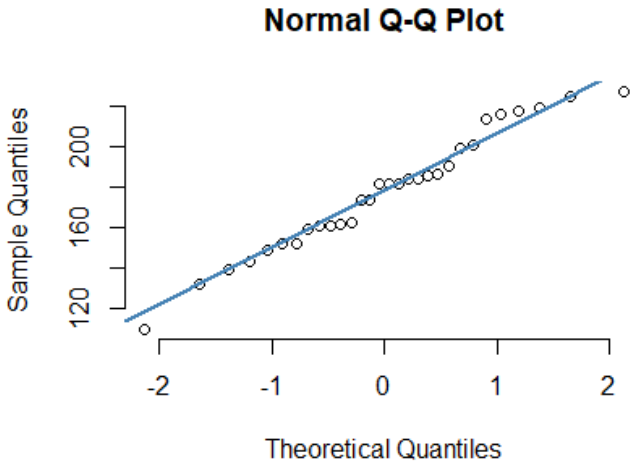


Figure 10: Q-Q plot Austevoll 3 - Mean area

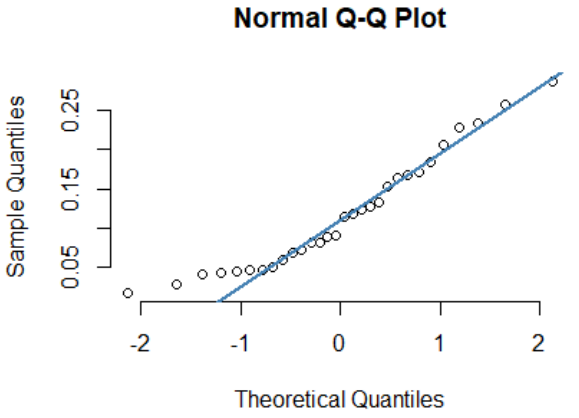


Figure 11: Q-Q plot Austevoll 3 - Volumetric density

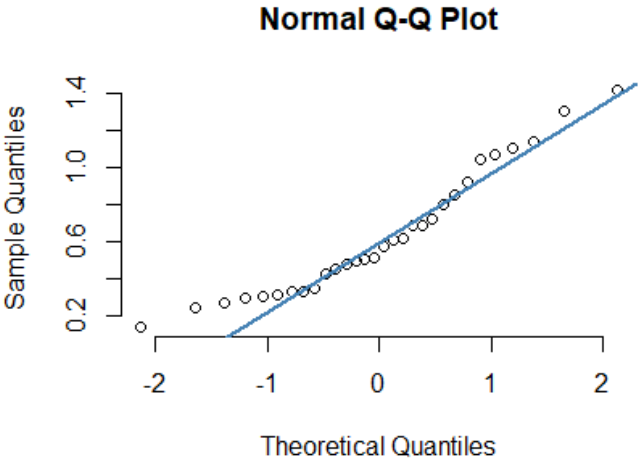


Figure 12: Q-Q plot Austevoll 3 - Defence activity

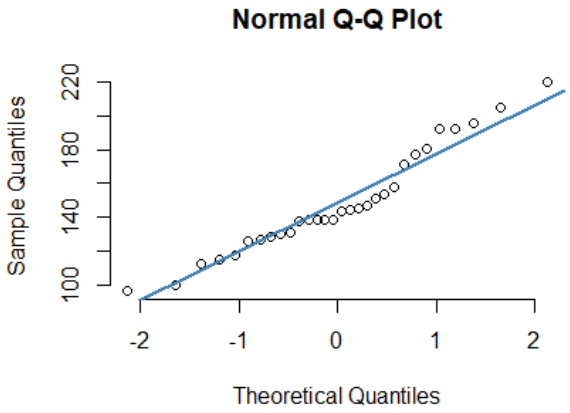


Figure 13: Q-Q plot Fitjar - Mean area

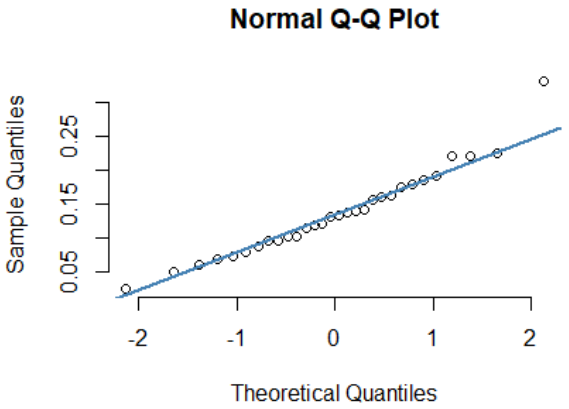


Figure 14: Q-Q plot Fitjar Volumetric density

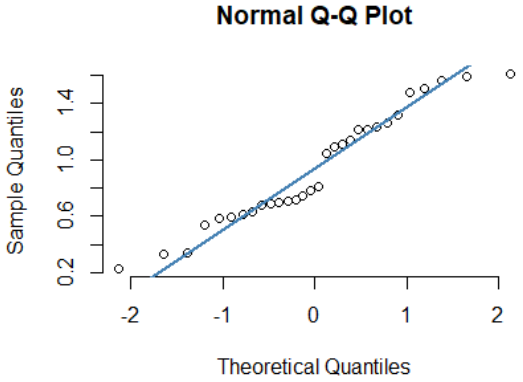


Figure 18: Q-Q plot Fitjar - Defence activity

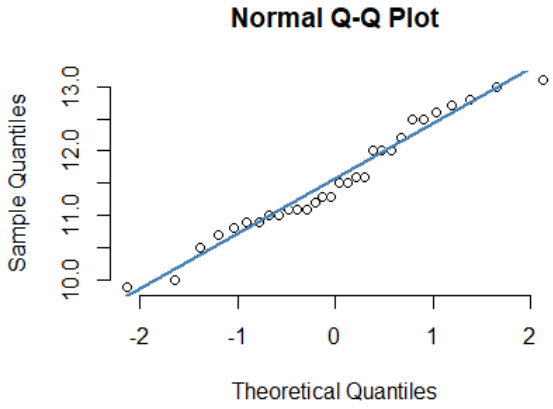


Figure 15: Q-Q plot Austevoll 3. sampling - length

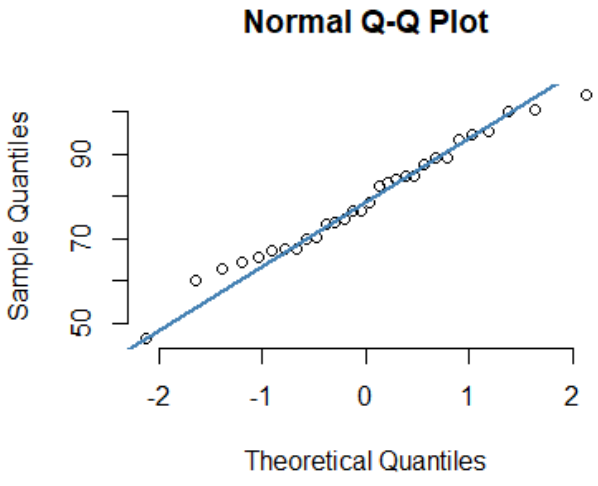


Figure 16: Q-Q plot - Austevoll 3. sampling - weight

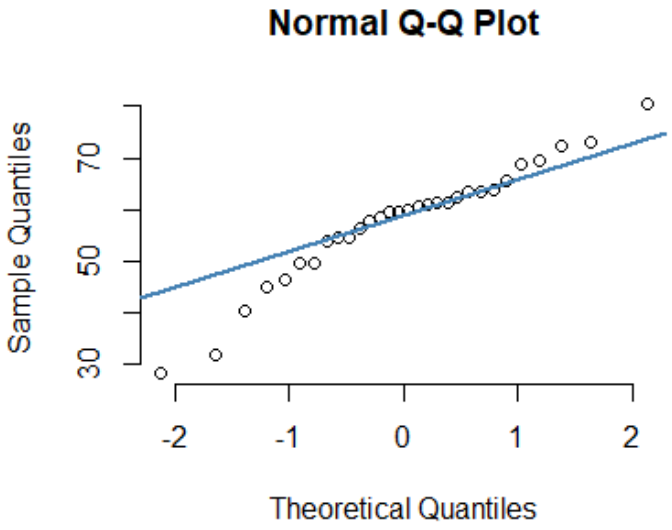


Figure 17: Q-Q plot - Austevoll 2. sampling - weight

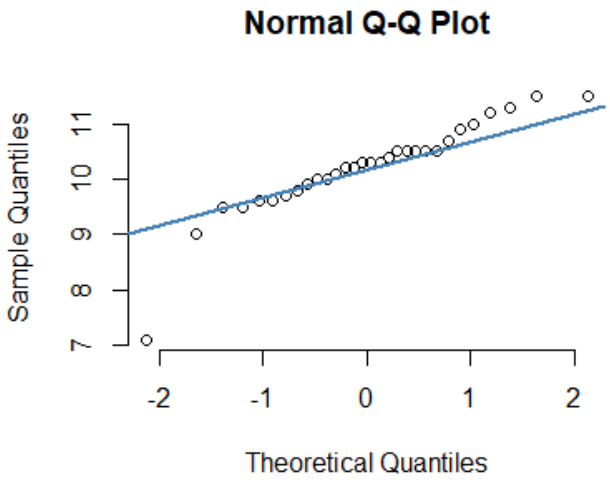


Figure 19: Q-Q plot - Austevoll 2. sampling - length

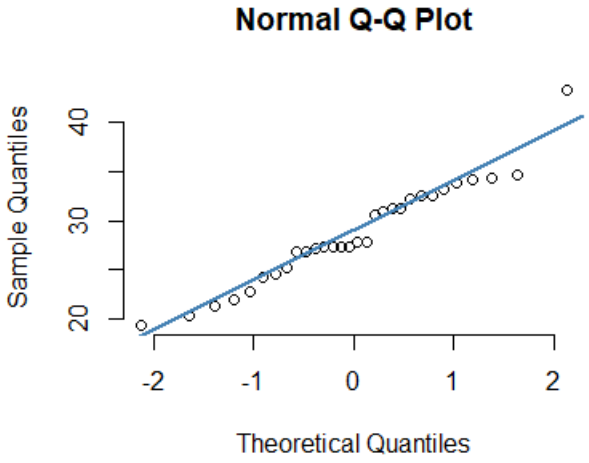


Figure 20: Q-Q plot - Austevoll 1. sampling - weight

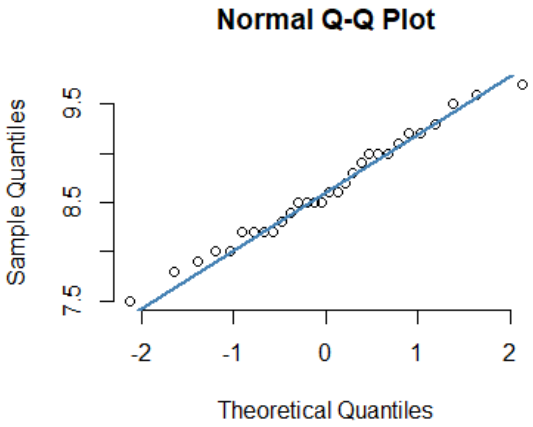


Figure 23: Q-Q plot Austevoll 1 sampling - length

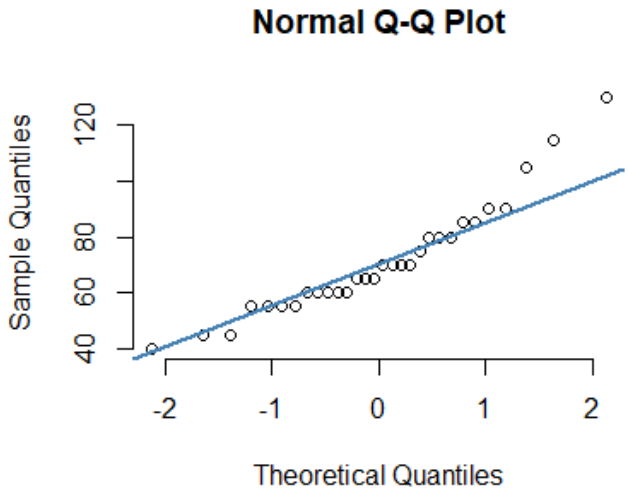


Figure 22: Q-Q plot - Fitjar - Weight

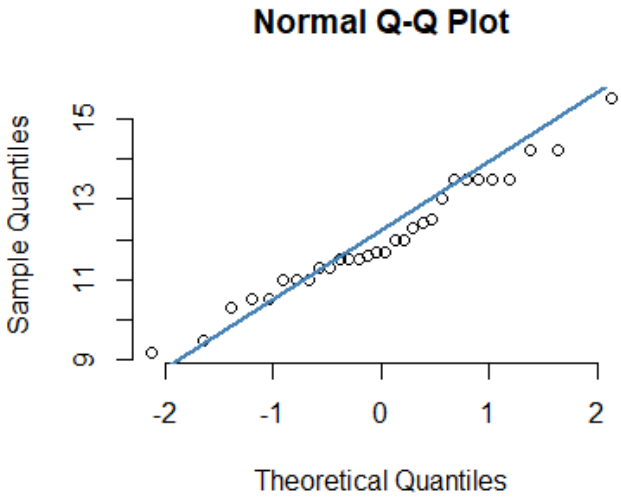


Figure 21: Q-Q plot - Fitjar - length

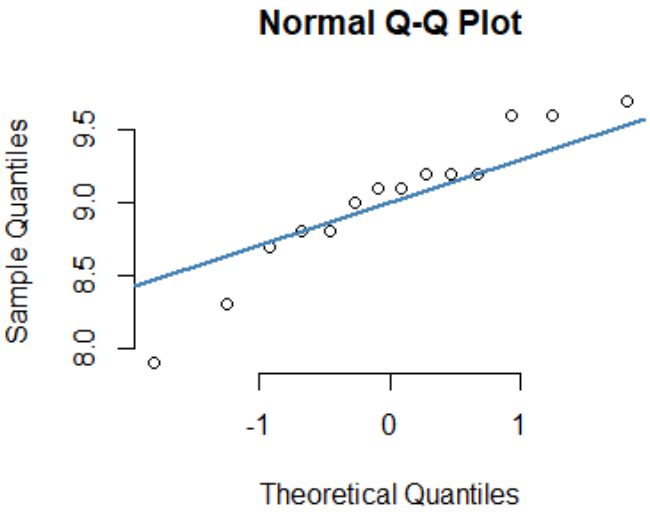


Figure 24: Q-Q plot - Agder length

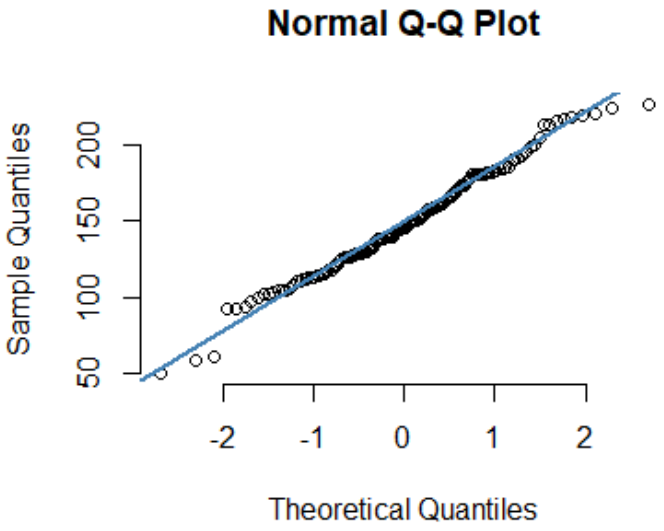


Figure 26: Q-Q plot Agder, Austevoll and Fitjar - MA

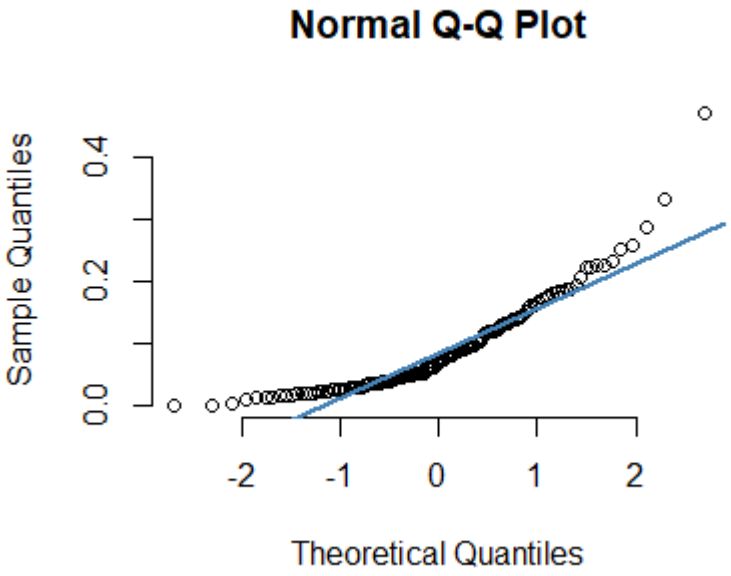


Figure 25: Q-Q plot Agder, Austevoll and Fitjar - VD

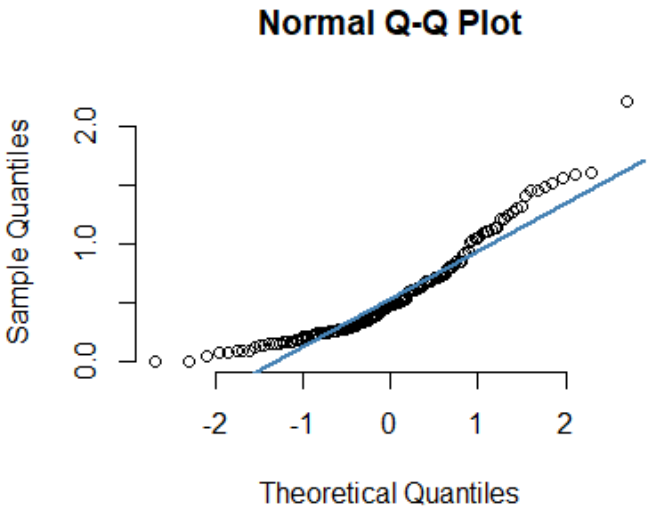


Figure 27: Q-Q plot Agder, Austevoll and Fitjar - DA