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Abstract

During the recent years, environmentally friendly packaging has become increasingly important for the consumer. However, this shift towards a greener alternative should not come as a compromise on the safety of the product being packaged. The main aim of this study was to investigate two different biopolymers and their usage as packaging material for food packaging applications. Films made from natural biopolymers such as chitosan and alginate were made, and their functional properties were studied. However, in order to achieve films with enhanced functional properties (mechanical, barrier, etc.) suitable for food packaging applications, seaweed was added to the film forming solution followed by HPP treatment.

Chitosan is typically dissolved in acetic acid, which has a quite pungent smell which might lead to rejection by the consumer. Thus, it was investigated if citric acid as a solvent could compare to the properties of chitosan-acetic acid films. The films made with the two different acids showed similar water vapor transmission rates. However, chitosan-acetic acid films were tougher, more flexible and had a higher concentration of free amino groups. Nevertheless, films made with both citric and acetic acid were observed to have relatively good antioxidant properties. HPP treatment was performed on the film-forming solution prior to casting the films. Generally, HPP treatment at pressures of 200 and 600 MPa either decreased the properties in the chitosan films, or the properties remained unchanged. In addition, seaweed was added in different concentrations to investigate if the properties of the chitosan films could improve. However, no such improvement was seen except for increased antioxidant activity at high seaweed concentrations.

Alginate is dissolved in water, so only the effect of HPP treatment and seaweed addition was studied for this polymer. HPP treatment showed some promising effects on the tensile strength of the alginate films at 200 MPa pressure. However, the barrier properties in the films remained unchanged by HPP treatment. Some decrease in antioxidant activity was observed with HPP treatment at 600 MPa pressure. Addition of seaweed showed more promising results. The antioxidant activity of the alginate films increased with added

seaweed and some improvement was seen in the WVTR. Nevertheless, the tensile strength of the films was not improved by addition of seaweed, and the elasticity decreased.

Both chitosan and seaweed has been reported to have good antimicrobial properties. These properties were investigated by two types of assays: disc diffusion and liquid-based assays. The disc diffusion assay was not successful due to the films curling in on themselves upon contact with the slight wet media. However, two liquid-based assays were performed at both ideal growth temperate of 37 °C, and 10 °C to simulate more realistic storage temperatures. Chitosan made with citric acid or acetic acid showed higher antimicrobial effect against *E. coli* than *S. aureus* at both 24 h incubation at 37 °C and 5 and 10 days incubation at 10 °C. The log concentration in the chitosan samples was below the detection limit for *E. coli* after 5 and 10 days incubation at 10 °C. Seaweed had no antimicrobial effect at 37 °C, however, some effect was seen for *S. aureus* at 10 °C. The combination of chitosan and seaweed was also studied at 10 °C. Reduction in CFU/mL were observed for both *E. coli* and *S. aureus* after 5 and 10 days incubation at 10 °C, however, the effect was biggest for *S. aureus* with a complete inhibition after 10 days incubation at 10 °C.

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Table of Contents

List of Tables

List of Figures

Abbreviations

DPPH - 2,2-Diphenyl-1-picrohydrazyl
DA – Degree of acetylation
HPP – High pressure processing
MHA – Mueller-Hinton agar
MHB – Mueller-Hinton broth
TSA – Tryptic Soya agar
TSB – Tryptic Soya broth
PCA – Plate Count agar
CFU – Colony forming units

1. Introduction

In today's society, foods are transported over great distances before reaching the customers. They go through periods of cooling and possibly rough handling. Nutritional value and physical appearance may decrease, and microbial activity might increase. Ultimately, this leads to rejection by the consumer and to increased food waste [1]. Almost one third of all food produced worldwide gets thrown away, especially fruits and vegetables [2]. A large portion of the plastic produced is used as packaging material [3], and only around 68% of the packaging bought by consumers is recycled [4], resulting in several metric tons of plastic ending up in the oceans each year [3]. Because of these problems, the use of biopolymers as packaging material has become increasingly popular over the last few decades [5], [6]. Although biopolymers could be attractive alternatives to conventional plastic, generally, films made from biomaterials have low mechanical strength and poor barrier properties [7]. However, these properties could be improved by for example the addition of fillers [8]–[10] or by crosslinking [11]–[13].

The main aim of this study was to investigate the functional properties of the two polysaccharides, chitosan and alginate. Chitosan is produced from chitin, which is found naturally in crustaceans [14]. The polymer has several beneficial properties, including being non-toxic, effective against bacteria and fungi [15]–[18], as well as having film-forming abilities [19]. They are also reported to be tough, clear and flexible [20]. In the last decades, the use of chitosan has been extensively researched, and the results are positive [16], [17], [19], [21]. However, most studies use acetic acid to dissolve the chitosan. The acetic acid has a quite pungent smell, which is unwanted by potential customers. Thus, this study looked at potentially using citric acid as a solvent instead, and how these films compare to those made with the previously preferred acetic acid.

The second biopolymer under investigation was alginate, which is readily available by extraction from algae. It is a cheap biopolymer, which has low toxicity and a high biocompatibility, making it a candidate for use in food and medical packaging. However, studies with alginate typically consist of reacting alginate with metal ions, usually calcium.

This is mostly due to the poor water resistance of alginate on its own. Nevertheless, alginate films are reported to be strong [22], and it has been found that addition of seaweed extract reduces the water solubility of alginate films [10].

For both polysaccharides, the weight and thickness, mechanical and barrier properties, as well as the DPPH radical scavenging activity was studied. In addition, the free amino group concentration in the chitosan was determined using the ninhydrin method. The effect of addition of seaweed in different concentrations as well as treatment with high pressure processing (HPP) at two different pressures on the properties of the films were also studied. Though research on application of HPP treatment and addition of fillers such as seaweed has previously been conducted on other biopolymers, limited literature is available on the combination of the two.

For chitosan, the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* was studied at both a comfortable temperature of 37 °C, and a stressful temperature of 10 °C for the bacteria over time. This was also performed on seaweed to check for potential antimicrobial activity, as well as on the combination of seaweed and chitosan.

2. Theory

In 2017, the average plastic waste generated in the EU per capita was 173.8 kg. Only 117.3 kg were recycled [4]. About 36% of the plastic produced is used as packaging (2015). 9 metric tons of plastic ended up in the ocean in 2010 [3]. Films produced from biological materials that would otherwise have been thrown away may be a solution to these challenges.

Biopolymers create a barrier between food and the environment, protecting against oxygen, moisture and solute movement. Some of these films are also edible. Films made from biopolymers are used around individual products such as fruits and nuts, applied between layers in foods consisting of different components [14], or used as antimicrobial [11], [15]–[17] and antioxidant barriers [23]–[25]. Sometimes, they are used in combination with traditional plastic packaging [26]. Typically, biopolymer films are made from hydrocolloids

(polysaccharides and alginates), lipids, and proteins. Hydrocolloids usually have better mechanical properties than films made from lipids and hydrophobic components [27].

2.1 Chitosan

Chitosan is a biopolymer which is non-toxic, has a low cost and is tasteless [28]. The use of chitosan has been widely studied, and it has been found to positively impact the consumable product by acting as an antimicrobial factor [15], [18], [29], extending the shelf life, reducing the respiration rate and conserve tissue firmness [30]. Recently, it has become increasingly popular to study the effect of fillers on the properties of chitosan films. Several studies report increasing properties for chitosan by addition of for example bone collagen [31], quercetin [32], fruit extract [33] and α-tocopherol [34].

Chitosan is produced by deacetylation of chitin, which is found naturally in the exoskeleton of crustaceans such as shrimp and crab [35]. It is soluble in dilute acids and has many applications, for instance, within food, pharmaceutical and biomedical fields. It is capable of forming films, gels and sponges [19]. Chitosan films has been reported to be clear, tough and flexible, and quite stable during storage. These films are used as gas barrier on fruits, and as antimicrobial coatings on fruit and vegetables [20]. No et al. (2002) reported that even low concentrations of chitosan was effective against *Bacillus sp*, and a significant log reduction was observed for *Bacillus megaterium* and *Bacillus cereus* [36].

The structure of chitosan is made up of randomly repeating segments of β-(1->4)-linked D-glucosamine and N-acetyl-D-glucosamine (Figure 1), where the two units are acetylated and de-acetylated, respectively [35]. An important factor when it comes to the solubility of chitosan, is its molecular weight. Typically, chitosan is commercially available with low, medium and high molecular weight. The lower the molecular weight, the easier it is to dissolve [19].

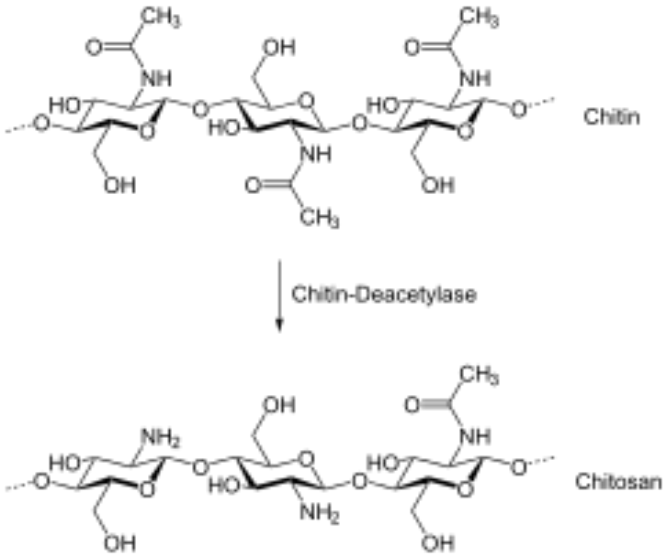

Figure 1. Production of chitosan from chitin [35]

The degree of acetylation (DA) of chitosan influences the biological properties of the films, including molecular weight and mechanical properties [37], [38]. Films made from high molecular weight chitosan is reported to have higher tensile strength than those of lower molecular weight [39]–[41]. In addition, the antioxidant activity of the chitosan films are also dependent on the molecular weight and degree of acetylation, with a higher radical scavenging activity in chitosan of low molecular weight [39].

Like previously mentioned, chitosan can be dissolved in dilute acid solutions. Typically, acetic acid is used due to having the best properties. Chitosan films made with acetic acid as the solvent has been reported to have high tensile strength, however, typically, chitosan films made with citric acid has a higher elongation at break than those made with acetic acid [13], [41], [42]. Addition of citric acid to a chitosan-acetic acid film forming solution has been reported to result in films with better barrier properties [13], whereas others report a decrease in barrier properties in films made with citric acid [41]. These observations are mainly due to the binding of citric acid to the chitosan molecule, forming C-O and amide bonds [43], resulting in higher scavenging activity [44].

2.2 Alginate

Alginate is a readily available and cheap biopolymer which has a low toxicity and a high biocompatibility. The polymer is usually extracted from brown algae. It is filtered, in this case with sodium, to produce alginic acid sodium salt [45]. Alginate films and coatings act by reducing the dehydration, controlling respiration and improving mechanical properties, among others, of fruits, meats and seafoods, thereby extending the shelf life. Several research teams have worked on improving alginate films by addition of antimicrobial, antioxidant and anti-browning agents [10], [46], [47]. Flavors, colors and nutritional ingredients has also been added to said films. Especially fresh fruits and vegetables that are processed (peeling, cutting, slicing etc.) before reaching the consumer, benefit from alginate films [48]. Recently, films made from sodium alginate with additives has been used for preservation of cheese [49] and for water cleanup [50].

Research on alginate films with no additives are sparse, possibly because of its poor water resistance. However, Augusto et al. (2018) has showed that addition of *Codium tomentosum* extract reduces this solubility [10]. Alginate films with added salt has been proved to increase the warming efficiency in microwaveable foods [51], and alginate coatings incorporated with antimicrobial additives such as sodium sorbate and potassium sorbate delayed the growth of *Listeria monocytogenes*, a highly dangerous pathogen [52]

Alginate is a linear polymer consisting of two residues. These are (1,4)-linked β-D-mannuronic acid and α-L-guluronic acid (Figure 2). These are abbreviated M and G residues, respectively. Depending on the alginate´s origin, these residues make up the blocks in the polymer. Either there are only one type of residue, or the two residues alternate [53]. The strength of alginate gels is based on ion binding, and this is linked to the G residues. Thus, the mechanical strength of the gels is higher in alginate with larger G content [54].

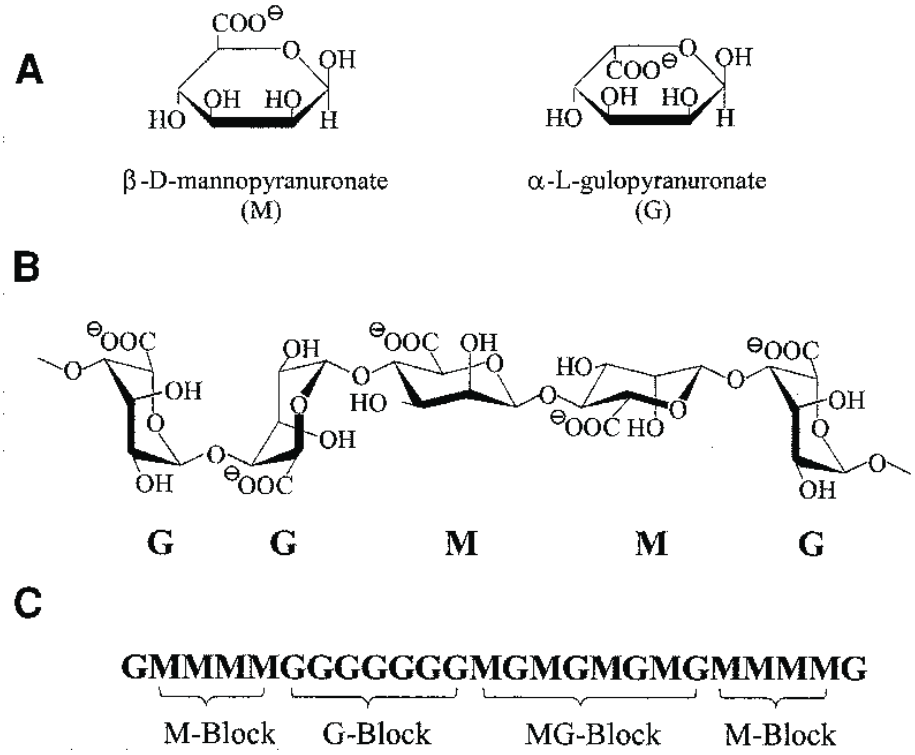

Figure 2. Alginate structures. A) the two monomers that makes up alginate. B) polymer conformation. C) G, M and GM blocks in the polymer [55].

Currently, alginate films are made by reacting with polyvalent metal ions to produce strong films that are insoluble. Typically, calcium ions are used. In foods that are restructured before selling, such as onion rings and crabsticks, this technology is used [22]. Within the field of biotechnology, alginates with calcium has been used to immobilize living cells, as well as in the controlled release of drugs such as vaccines [56]. Because alginate is so readily available and reproductible, it is interesting to look at as a biodegradable edible film. However, gels or films made of alginate has poor water resistance despite being strong. Addition of calcium is thus preferrable, due to their insolubility. The disadvantage in this method is that the gel formation is so instantaneous, that casting films are is prevented in some cases [22].

2.3 HPP treatment

High pressure processing (HPP) has gained popularity based on its potential to inactive microorganisms without significantly compromising on the quality of the product. It has

been used extensively on juices, tomato salsa and ready-to-eat foods [57]. High pressure processing is the act of treating materials with high pressure, which might result in structural changes within the materials such as alteration of hydrogen, ionic and hydrophobic bonds [28]. Pressures ranging from 100 to 1000 MPa are typically applied to the product [57]. The use of HPP treatment on the two biopolymers chitosan and alginate is scarce. However, Niu et al. (2002) studied the effect HPP treatment ranging in pressures from 100 to 500 MPa on chitosan. They reported that the tensile strength increased up to 400 MPa pressure. In addition, Niu et al. (2002) reported that the water vapor permeability of the films made from HPP treated chitosan significantly decreased at all pressures [28].

Research on the effect of HPP treatment on other biopolymers is quite extensive. For example, Montero et al. (2002) reported an increase in the strength of gelatin gels after HPP treatment, explained by the stabilization of intermolecular hydrogen bonds in the gelatin structure [58]. Molinaro et al. (2015) also studied the effect of HPP treatment on gelatin films, and reported an increase in tensile strength and barrier properties after HPP treatment at 600 MPa pressure for 30 min. They also reported decreased oxygen transmission rate through the films [59]. Similar research has also been conducted on protein-based films. HPP treatment has been reported to improve the mechanical properties of the protein films, and in addition decreasing the water solubility and water vapor permeability of the films due to increased crosslinking and unfolded proteins as a consequence of HPP treatment [60]. Starch based films made from both buckwheat and tapioca also exhibited improved tensile strength and elongation at break with HPP treatment, and improvements in water vapor permeability has been reported [61].

HPP treatment has also been reported to improve the dispersion and interaction of fillers in different biofilms. Lian et al. (2016) reported that HPP treatment improved the properties of polyvinyl alcohol (PVA)-chitosan films with added nano-TiO_2 particles. The water vapor permeability and oxygen permeability decreased in the films with TiO_2 particles and HPP treatment, in comparison to non-treated films with TiO_2. In addition, they reported a significantly improved tensile strength with HPP treatment at 200, 400 and 600 MPa pressure [62]. Similarly, Chi et al. (2018) reported improved effect between Poly(lactic acid) (PLA) and Ag nanoparticles after HPP treatment, resulting in a more compact

network structure of the PLA/Ag nanocomposite film. HPP treatment at 200, 400 and 600 MPa pressure decreased the water vapor permeability in the PLA films with Ag nanoparticles, in addition to increased tensile strength caused by stiffness in the structure through development of hydrogen bonds [26].

2.4 Seaweed

Seaweed can be used as a filler in edible films. It is used to produce alginate, but the seaweed itself has beneficial factors. It has a relatively high protein content, with green or red seaweeds having a higher content. In brown seaweed, about 3-15% of the dry weight is proteins, whereas in green or red seaweed, this varies between 10 and 47%. This protein content depends on the seasonal period. Typically, the higher content can be found in late winter and spring, and low contents during the summer. For both humans and animals, mainly fish, seaweed protein can act as a nutritional source [63]. Incorporation of seaweed or other fillers can improve the properties of biofilms. Seaweed was selected for the present study for its reported antimicrobial [24], [64], [65] and antioxidant activity [24], [64], [66], as well as being readily available in Norway.

Seaweed is also used to create edible films. Cian et al. (2014) used extracts from red seaweed combined with phycocolloids or phycobiliproteins, which are mainly used as thickening agents to produce films [67]. Whereas Albertos et al. (2019) looked at two different seaweed (*Himanthalia elongata* and *Palmaria palmata*) and seaweed extracts and how they extended the shelf-life of fresh fish burgers. They found that *H. elongata* showed a significantly higher antioxidant activity than *P. palamate*. In addition, they showed that films made with seaweed extracts, regardless of species, had a lower antioxidant capacity. The water activity in the fish burgers also was controlled during storage using these films, and the microbial growth was reduced [68].

Addition of seaweed to chitosan or alginate has not been studied much previously. However, Augusto et al. (2018) reported that addition of *Codium tomentosum* extract to chitosan films decreased the tensile strength of the films, while increased elongation at break was

observed. They also looked at addition of seaweed to alginate films. The strength of the films was unchanged after addition of seaweed, however, the water vapor permeability in the alginate films decreased [10]. This decreased water vapor permeability was also observed after addition of oregano oil to alginate films [11], and seaweed to fish skin gelatin films [11], [69], explained by enhanced crosslinking and longer route of travel for the water through the film [11], [69].

Scarce literature is available on the effect of seaweed addition to chitosan or alginate on the antioxidant capability of the biopolymers. However, addition of plant extracts such as ginseng extract [70] and protein hydrolysates [71] has been reported to generally increase the scavenging activity of alginate films. Similarly, addition of tea extracts [72], [73], α-tocopherol [74] and *Pistacia terebinthus* [75] has been reported to increase the antioxidant activity of chitosan films.

2.3 Antimicrobial effect of Chitosan and seaweed

Extensive research has been done on the antimicrobial potential of chitosan [15]–[17]. Several mechanisms have been reported as potential modes of action. Firstly, chitosan affects the permeability of the cell membrane by interacting with the negatively charged phospholipids, since it is positively charged. This leads to disruption of the cell membrane, leaking of the intercellular content, and ultimately to cell death. Another potential mechanism is attributed to the metal ion chelating properties of chitosan. Lastly, some reports claim that chitosan is able to penetrate the cell wall and inhibit mRNA synthesis by binding to DNA [76].

Several factors also affect the antimicrobial effect of chitosan. For example, chitosan with a lower molecular weight has a higher antimicrobial effect than chitosan in its native form. In addition, the degree of polymerization impacts the effect, high molecular fractions exhibit higher antimicrobial efficiency. Due to higher solubility and charge density, chitosan which is highly deacetylated also has a higher antimicrobial activity compared to ones with more amino groups [18]. Chitosan has also been reported to have synergistic antimicrobial effect

when combined with other agents. Addition of nano-particles such as TiO_2 [77] and Ag-Nanoparticles [8] has been shown to increase the antimicrobial effect of chitosan films, which on their own has been reported to not be satisfactory for food packaging applications [78]. Incorporation of gelatin into chitosan films improved the preservative activity on pork [79], and addition of grapefruit seed extract enhanced the antimicrobial activity of chitosan on cherry tomatoes [80] and shrimp [81].

Table 1 presents a study overview on the antimicrobial activity of chitosan at different incubation times, temperatures and assays.

Table 1. Overview of antimicrobial studies conducted on chitosan.

Biopolymer (MW)	**Additives**	**Solvent / extraction**	**Method**	**Test microorganisms**	**Incubation temperature and time**	**Findings: Log reduction/zone of inhibition**	**Culture media**	**Reference**
Chitosan (MW not reported)		Acetic acid	Disc diffusion	*E. coli* and *Bacillus cereus*	37 °C for 24 h	*E. coli*: 17 mm *B. cereus*: 19 mm	MHA	Nataraj et al. (2018) [13]
Chitosan (MW not reported)		Acetic acid + citric acid	Disc diffusion	*E. coli* and *B. cereus*	37 °C for 24 h	*E. coli*: 23 mm *B. cereus*: 26 mm	MHA	Nataraj et al. (2018) [13]
Chitosan (MW not reported)		Acetic acid	Disc diffusion	*S. aureus*, *Staphylococcus epidermidis* and *E. coli*	37 °C for 24 h	No antimicrobial effect	Nutrient agar	Foster and Butt (2010) [82]
Chitosan (medium MW)		Acetic acid	Filter disc assay	*E. coli* and *S. aureus*	37 °C for 24 h	*E. coli*: 22 mm *S. aureus*: 23 mm	MHA	Koc et al. (2020) [83]
Chitosan (224 kDa)		Acetic acid	Liquid assay (0.1% w/v chitosan)	*E. coli* and *S. aureus*	37 °C for 24 h	*E. coli*: 3.11 log reduction *S. aureus*: 6.64 log reduction	MHB	No et al. (2002) [36]
Chitosan (MW not reported)			Liquid assay (150 ppm chitosan powder) – growth curve	*E. coli*	4 – 37 °C for 48 h	4 °C: Rapid reduction before stabilization at log 3 – 4 CFU/mL 37 °C: None detectable	Nutrient broth	Tsai and Su (1999) [84]
Chitosan (190-310 kDa)		Acetic acid or lactic acid	Film applied to black radish	*Listeria monocytogenes*	4 °C for 7 days	Complete inhibition after 4 days		Jovanovic et al. (2016) [85]

			(1% w/v chitosan)					
Chitosan (2025-1110 kDa)		Acetic acid or lactic acid	Liquid assay (0.05% w/v chitosan)	*L. monocytogenes, S. enteritidis, E. coli* and *S. aureus*	4 °C for 15 weeks	Log reduction (2025 and 1110 kDa): *L. monocytogenes*: 5.72-8.45 *S. aureus*: 5.65-6.18 *S. enteritidis*: 4.59-6.37 *E. coli*: 2.51-2.67	MHB	No et al. (2006) [86]
Chitosan (900-1000 kDa)	Garlic oil	Acetic acid	Disc diffusion (100 - 400 μL oil added)	*E. coli, S. aureus, Salmonella* Typhimurium*, L. monocytogenes* and *B. cereus*	37 °C for 24 h	No effect on *E. coli* or *S. typhimurium.* *S. aureus*: 20.39 – 34.46 mm. *L. monocytogenes*: 26.47 – 40.83 mm *B. cereus*: 21.56 – 34.83 mm	MHA	Pranoto et al (2004) [87]
Chitosan (450 kDa)	Essential oils	Acetic acid	Disc diffusion (3.1 – 12.3 μL oil per disc)	*L. monocytogenes* and *E. coli*	35 °C for 48 h	Oregano oil had the strongest antimicrobial activity. Zone of inhibition at 4% oil: *E. coli*: 6 mm *L. monocytogenes:* 32 mm	MHA	Zivanovic et al. (2005) [14]

The antimicrobial activity of seaweed depends on several factors, such as type of seaweed and method in which the seaweed is extracted [88], [89], in addition to the season and growth stage of the seaweed [90]. The highest activity has been found for seaweed collected in the spring, followed by winter, summer and autumn, respectively [91]. Though the antimicrobial activity of seaweed has been widely studied (Table 2), the exact mechanism behind the action is not known [90]. Nevertheless, *Laminaria hyperborea* has a high phenolic content [89], which has been reported to increase the antimicrobial activity [24], [89], [64].

Table 2. Overview of antimicrobial studies conducted on seaweed

Biopolymer (Mw)	**Solvent / extraction**	**Species (seaweed)**	**Method**	**Test microorganisms**	**Incubation temperature and time**	**Findings: Log reduction/zone of inhibition**	**Culture media**	**Reference**
Seaweed	Soxhlet extraction	*Sargassum polycycstum*	Agar cup plate diffusion	*Bacillus subtilis, S. aureus, E. coli, Erwinia caratovora* etc.	37 °C for 24 h	Zone of inhibition for all tested extraction methods and microorganisms	Nutrient agar	Kausalya and Narasimha (2015) [88]
Seaweed	Soxhlet extraction	*Sargassum tenerrimum*	Agar cup plate diffusion	*B. subtilis, S. aureus, E. coli, E. caratovora* etc.	37 °C for 24 h	Zone of inhibition for all tested extraction methods and microorganisms	Nutrient agar	Kausalya and Narasimha (2015) [88]
Seaweed	Extraction	*Lamibaria hyperborea*	Disc diffusion	*E. coli* and *S. aureus*	37 °C for 24 h	MIC: *E. coli*: 21.0 – 45.6 mg/mL *S. aureus*: 5.3 – 11.4 mg/mL	Not reported	Kadam et al. (2015) [89]
Seaweed	Extraction	*Laminara digitata, Laminara saccharina, Himanthalia elongata, Palmaria palmata, Chondrus crispus* and *Enteromorpha spirulina*	Liquid assay (8 mg/mL seaweed)	*L. monocytogenes, Salmonella* Abony, *E. faecalis* and *P. aeruginosa*	Not reported. 24 h	Inhibition depended upon type of seaweed. Largest inhibition for *L. digitata*: 100 – 72% inhibition	TSB	Cox et al. (2010) [24]

3. Materials and method

The methodology for this study is divided into three parts. The first part consists of chitosan´s abilities and properties on its own, dissolved in either acetic acid or citric acid. Then, the chitosan film-forming solution was HPP treated, and seaweed was added as a filler. The next section is alginate, which was dissolved in water. For this polymer as well, the film-forming solution was HPP treated, and seaweed was added. Finally, the antimicrobial properties of chitosan and seaweed, as well as the two combined, at different incubation temperatures and times were studied.

3.1 Materials

Chitosan of medium molecular weight (75-85% deacetylated, molecular weight: 190-310 kDa), citric acid 99% (molecular weight: 192.12 g/mol), sodium alginate (alginic acid sodium salt from brown algae) with low viscosity, L-alanine (non-animal source), ninhydrin reagent 2% solution and 96% ethanol were purchased from Sigma-Aldrich, St. Louis. Acetic acid (glacial) 100% was purchased from Merck, Germany. DPPH (2,2-Diphenyl-1-picrohydrazyl free radical, 95%) was procured from Alfa Aesar, Germany. Tryptone soya broth and peptone water was purchased from Oxoid, Germany. Sodium chloride (analysis grade), plate count agar, tryptic soya agar and Mueller-Hinton agar were purchased from Merck, Germany.

The seaweed (*Laminaria hyperborean)* powder with a water content of 6.3% were supplied by Dolmøy House of Seafoods (Frøya, Norway). The plants were harvested in May 2020, the stipe and holdfast were removed, and the square-shaped samples were stored at 4 °C. Milling of the seaweed was performed by Nofima. Briefly, the procedure was to take frozen seaweed, thaw it and then remove the stems. Next, the leaves were finely milled using a Comitrol 1700 (Urschel laboratories, Chersterton, IN) with cutting heads 3K-025040U which had an opening of 1.016 mm, and with 3K-010010 which had an opening of 0.354 mm. The leaves were then frozen over night at -20°C, followed by lyophilization for 48 h in a GAMMA 1-16 LSC dryer (MARTIN CHRIST GmbH, Osterode, Germany). For the next two days, the dried leaves were conditioned against air at ambient temperature, before freezing again at -80 °C. Finally, the frozen leaves were ground on a Retch ZM-1 Centrifugal mill (Retsch GmbH), using a ring sieve with aperture of 0.5 mm.

3.2 Chitosan

3.2.1.1 Film preparation

In order to create a film forming solution, 2 g of chitosan was added in 100 mL of 1% acetic acid or 1% citric acid solution. The solution was then heated to 70°C and stirred at 500 rpm for 40 minutes, followed by centrifugation at 14500 rpm for 20 minutes to get rid of non-dissolved particles. The pH was measured with a FiveEasy Plus pH meter (Mettler Toledo, US) equipped with a LE410 electrode, and noted. Carefully, 30 mL of the solution was

poured into petri dishes with a diameter of 90 mm. The plates were left to dry in a fume hood for around 48 h. The films were also weighed. Figure 3 represents the films made from citric acid (A) and acetic acid (B) as a solvent to prepare the chitosan film forming solution.

A second bottle of chitosan had to be acquired halfway into the research work. This bottle was from another batch and although the second bottle was also medium-molecular weight, it proved more difficult to dissolve. Thus, the method to dissolve chitosan powder was slightly modified. After heating and stirring for 40 minutes, the sample was centrifuged, and the undissolved chitosan particles were discarded. Then, the process was repeated. This resulted in a homogenous film forming solution. No significant difference was observed between the weight and thickness of the films made using both methods.

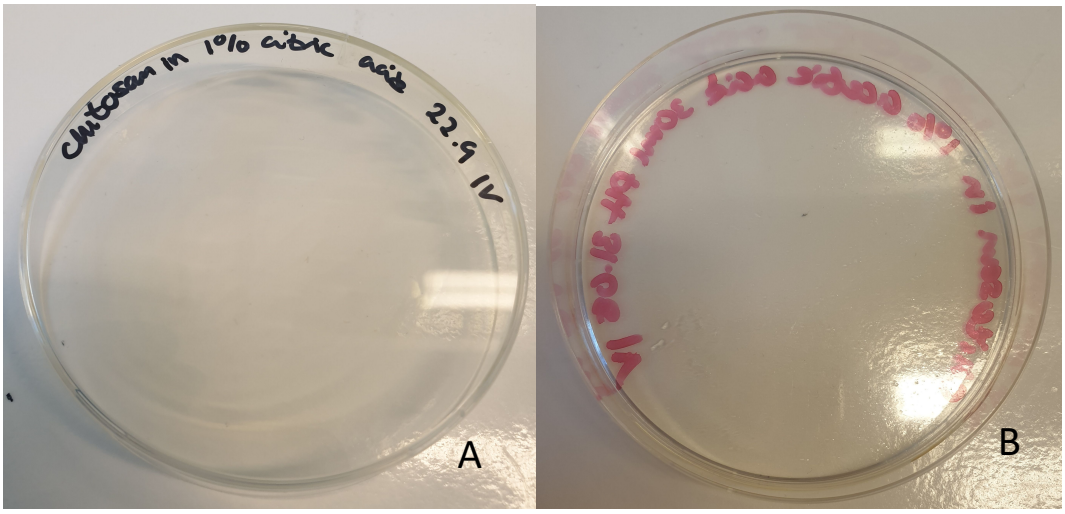

Figure 3. Film made from chitosan dissolved in citric acid (A), and acetic acid (B)

3.2.1.2 High pressure processing

High pressure processing (HPP) was performed on the film forming solutions to explore the potential effects it had on the mechanical, barrier and antioxidant properties of the films. The solutions were put into sous-vide bags and vacuum packaged at 20% vacuum (Figure 4). Two different pressures (200 MPa and 600 MPa) were used in the HPP machine for 15 min.

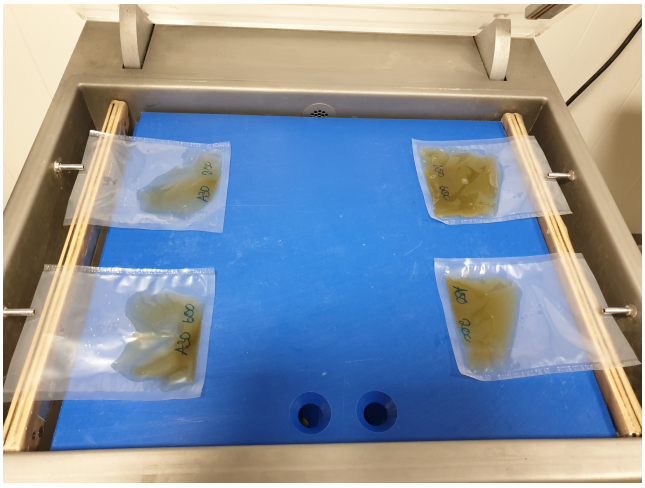

Figure 4. Vacuum packaging of alginate samples with seaweed to prepare for HPP treatment

3.2.1.3 Seaweed addition

In order to evaluate the effect of seaweed addition on the functional properties of chitosan films, only chitosan prepared from acetic acid was considered due to the films properties which will be discussed later. The chitosan solution in acetic acid was prepared as described above, and seaweed was added in concentrations of 10, 30 and 50% (of dry weight of polymer) to the film forming solution. Before addition to the chitosan solution, the seaweed powder was sieved through a 200 μm sieve to get rid of bigger particles in order to achieve a better dispersion of the seaweed powder into the solution. The films were prepared using the solvent casting method as mentioned before.

3.2.2 Dissolving films

For some of the characterization processes the films needed to be dissolved and one whole film was dissolved in 100 mL distilled water. Because the initial concentration of both chitosan and alginate is 2% and films prepared via drying 30 mL solution, the final concentration was 0.6 g/100 mL or 6 mg/mL. To dissolve chitosan films with no HPP treatment, around 2 hours at 70°C was sufficient. The chitosan films with HPP treatment were stirred for 1 hour at 70°C to dissolve, regardless of pressure and acid used.

3.2.3 Mechanical properties

Tensile strength and elongation at break of the films were measured using a TA.XT Plus texture analyzer (STable Micro Systems Lts, Godalming, UK), equipped with tensile grips (Figure 7) and a 50 kg loading cell, a span distance of 25 mm and speed of 0.85 mm/s , following a modified version of the procedure by Han et al. (2011) [92]. The dimensions of the films tested were 40 mm x 10 mm (length x width), as presented in Figure 5. The thickness of the films varied between each sample. Film thickness was measured with a Limit Digital Caliper (150 mm, 0.01 mm resolution). For each film, 4 strips were cut and least three replicates of each sample were performed and analyzed using the software Exponent (version 6.1.16.0). Some of the films were very brittle, making it close to impossible to get rectangles (Figure 6).

Figure 5. Alginate film with 50% seaweed cut into rectangles ready for the texture analyzer

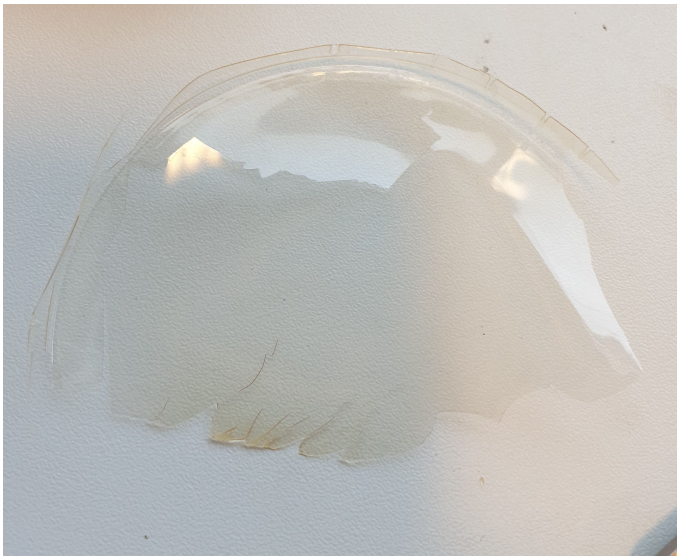

Figure 6. Example of how brittle the citric acid films treated with HPP were. The films fractured while cutting.

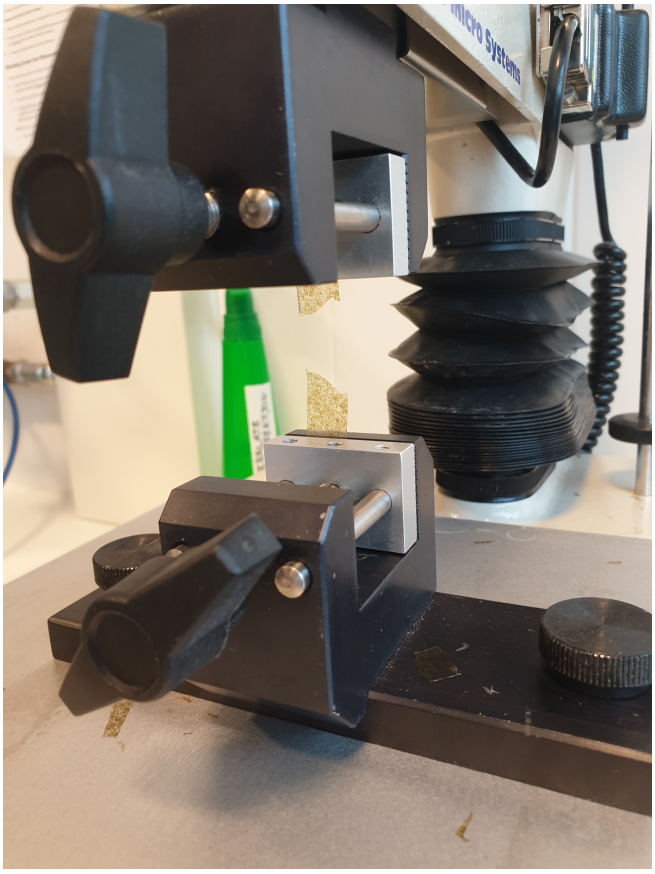

Figure 7. Film with seaweed after stretching on the texture analyzer.

The texture analyzer puts out data including max force (N) and distance at max force (mm). These two values were used to calculate the elongation at break (%) and tensile strength (MPa). The tensile strength is the most important factor for strength analysis of the different films, but elongation at break was also interesting in some cases. Elongation at break was calculated using Equation 1. Tensile strength was calculated using Equation 2.

$$Equation\ 1:\ Elongation\ at\ break\ \% = \frac{Distance\ at \max force}{Distance\ between\ grips} * 100$$

$$Equation\ 2:\ Tensile\ strength\ (MPa) = \frac{Max\ force}{Length\ x\ thickness\ of\ film}$$

3.2.4 Barrier properties

The water vapor transmission rate (WVTR) of the films was measured according to the method by Sarwar et al. (2018) [93]. 10 mL of distilled water was added to test tubes with a

diameter of 13 mm. The films were cut into appropriate sizes and fitted to the top of the tubes and secured with Parafilm (Figure 8). The weight of the tube was measured and then placed in an oven at 45 °C for 24 h. After 24 h, the tubes were weighed again and the WVTR was measured using Equation 3. Two measurements for each film were taken.

$$Equation\ 3: WVTR = [W_i - W_t/A]\ x\ T\ (g/m^2h)$$

Where; W_i = initial weight of the tube, W_t = final weight of the tube, A = area of the tube, T = 24 h.

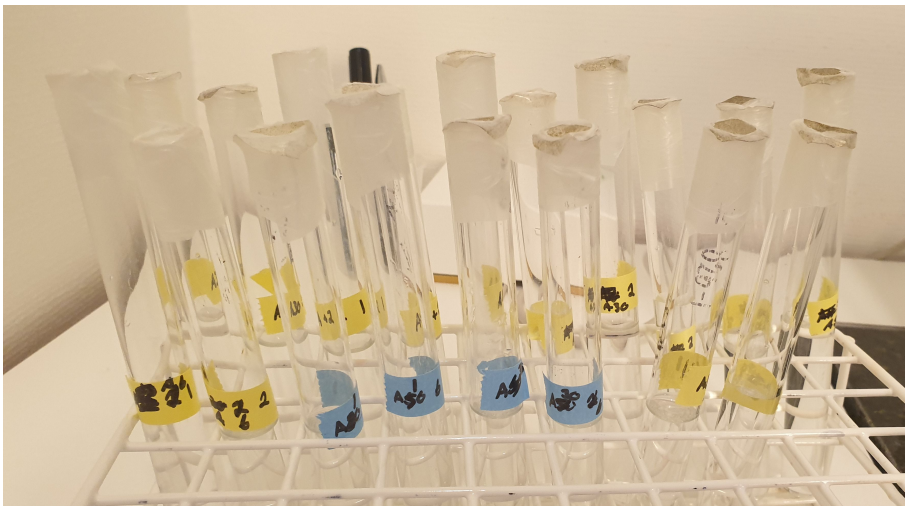

Figure 8. Barrier testing. Pieces of the films are secured to the top of the tube with parafilm.

3.2.5 Ninhydrin assay

The crosslinking of the chitosan films was analyzed with a ninhydrin test, using a slightly modified version of that by Sun et al. (2008) [94]. Because this test detects free amino groups, samples with more crosslinking will have less free amino groups. A calibration curve was prepared by dissolving an amino acid, in this case Alanine, in distilled water, following a modified version of the protocol by Li et al. (2011) [95] . The alanine solution was further diluted to concentrations of 0.05, 0.10, 0.15, 0.20 and 0.25 mg/mL. 0.25 mL of each dilution was added to an Eppendorf tube (2 mL), followed by 0.25 mL premade ninhydrin reagent. The bottles were capped and briefly shaken by hand (Figure 9 A) before heating in a water

bath at 90°C for 30 minutes (Figure 9 B). Next, the tubes were cooled to below 30°C before addition of 1.25 mL 50% (v/v) ethanol/water. To oxidize the excess hydrindantin, the tubes were vortexed for 15 seconds.

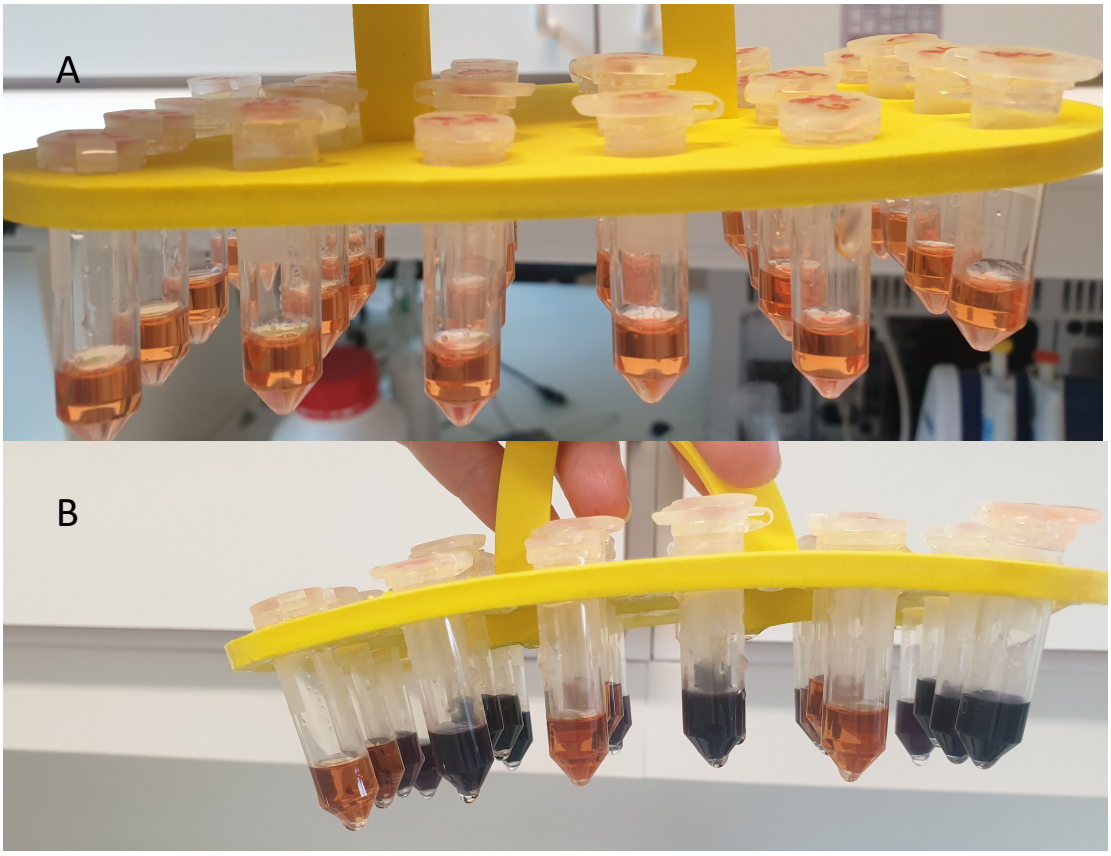

Figure 9. The ninhydrin samples before (A) and after (B) heating. The purple color indicates presence of free aminos.

Absorbance of the chitosan samples after reaction with ninhydrin was read at 570 nm on a microplate reader (SpectraMax Paradigm Multi-Mode Microplate Reader at CORE Stavanger). From the read values, a calibration curve was made and a linear trendline (Equation 4) was made.

$$Equation\ 4: y = ax + b$$

All films containing only chitosan, both with and without HPP treatment, were dissolved in 100 mL water to create a concentration of about 6.0 mg/mL based off of weight of the films. Further dilutions were made to final concentrations of 0.5 and 1.0 mg/mL. The same

procedure as described for the calibration curve was used to measure the absorbance of the samples at 570 nm. Three parallels of each sample were measured, in addition to a blank consisting of the ninhydrin reagent with no sample added. The equation from the linear trendline found previously (Equation 4) were used to calculate the amount of free amino groups.

3.2.6 Antioxidant properties

The DPPH scavenging activity of the samples was measured using a modified version of the method by Chen et al. (2015) [23]. The DPPH in ethanol reagent was made by dissolving 5.92 mg DPPH in 100 mL 96% ethanol to get a concentration of 0.15 mMol DPPH. The solution was light sensitive, so the beaker needs to be covered in foil before adding the DPPH. After around 15 minutes of stirring, the bottle was placed at 4°C for at least an hour before use. It can be used for up to 2 days if kept at 4°C.

Diluted samples were prepared by first dissolving a film in 100 mL water, resulting in a concentration of 6 mg/mL chitosan. These were further diluted to 0.5, 1.0, 2.0 and 3.0 mg/mL chitosan. 1 mL of each sample was mixed with 1 mL DPPH in ethanol in an Eppendorf tube (2 mL) on a vortex for 10 seconds and placed in the dark for 60 minutes at room temperature. A control consisting of just DPPH reagent was also prepared, along with a blank of only water. Using a microwell plate, 250µl sample was added to the wells in three parallels (Figure 10), and the absorbance was read at 517 nm (SpectraMax Paradigm Multi-Mode Microplate Reader at CORE Stavanger). The scavenging effect was expressed as:

$$Equation\ 5: Scavenging\ activity(\%) = \frac{A_{control} - A_{Sample}}{A_{control}} * 100$$

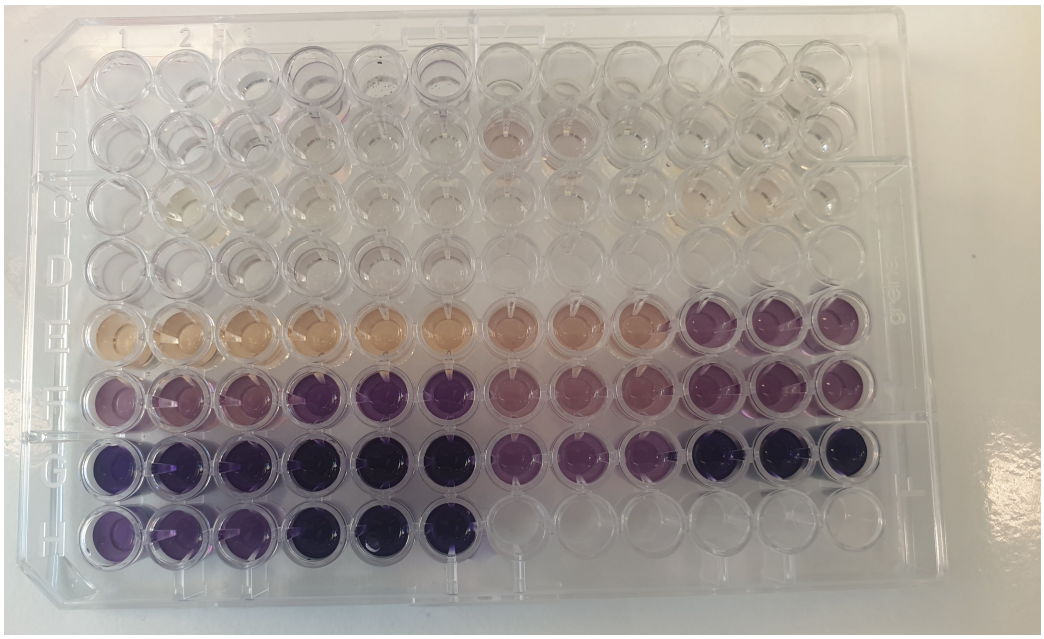

Figure 10. DPPH assay. Microwell plate filled with samples to be read.

3.3 Alginate

3.3.1.1 Film preparation

Different samples were prepared with alginate. 2 g alginate were dissolved over-night on a stirrer at 550 rpm in 100 mL water at room temperature, to create a 2% biopolymer solution. The films were prepared using solvent casting method as described before.

3.3.1.2 High pressure processing (HPP)

The alginate films were treated with HPP as described previously in section 3.2.1.2.

3.3.1.3 Seaweed addition

Samples with alginate in water with added seaweed was prepared as described in section 3.2.1.3. Figure 11 represents the alginate films with no fillers (A), followed by addition of 10 (B), 30 (C), and 50% (D) seaweed.

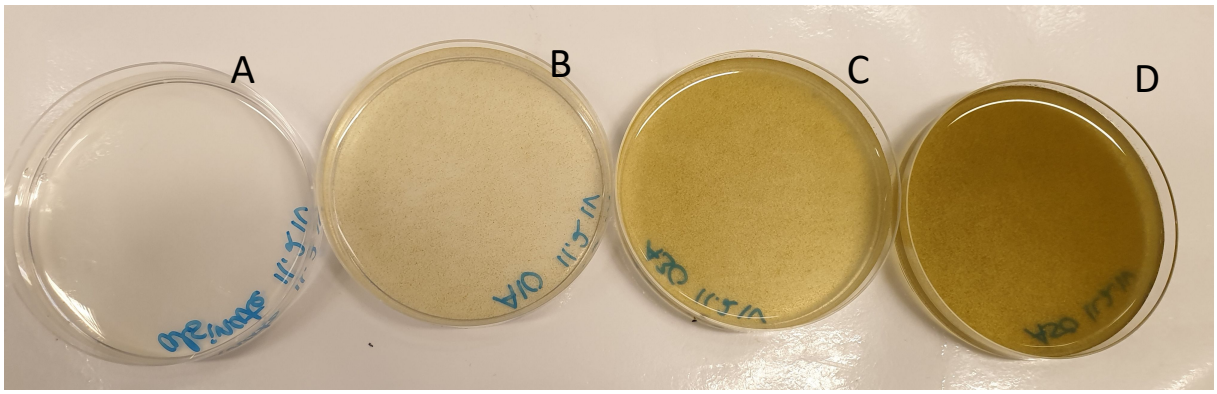

Figure 11. Alginate films before drying. Alginate with no fillers on the (A), followed by 10 (B), 30 (C) and 50% (D) seaweed addition.

3.3.2 Dissolving films

All alginate films were dissolved in room temperature within 15 minutes.

3.3.3 Mechanical, barrier and antioxidant properties.

The method described in section 3.2.3 was followed to assess the tensile strength and elongation at break of the alginate films. The barrier properties of the alginate films were studied according to the protocol in section 3.2.4. Finally, the same procedure described in section 3.2.6 was followed to assess the antioxidant properties of the alginate films.

3.7 Antimicrobial studies

In order to study the antimicrobial properties of the developed films, the disc diffusion assay [25] was initially followed for chitosan films. However, due to the limited reproducibility, a liquid-based assay [96] was then set up for chitosan, seaweed and their combination. Assays with alginate films were not conducted since antimicrobial properties have not been reported for this biopolymer [47]. The antimicrobial assays were conducted at 37 and 10 °C to simulate temperature abuse (optimal for the growth of the tested bacterial strains) and

refrigeration conditions. The bacterial strains used for the trials, the Gram-negative *Escherichia coli* (CCUG 10979) and the Gram-positive *Staphylococcus aureus* (CCUG 1828), were acquired from the Culture collection at the University of Gothenburg (Sweden). Method for preparation of the media can be found in appendix A2.1.

3.7.2 Disc diffusion assay

A Microbank™ (Microbank, Pro-lab Diagnostics, Canada) bead stored at -80 °C was placed in a 15 mL Falcon tube containing 8 mL TSB broth, respectively for *E. coli* and *S. aureus*. After 24 h at 37 °C, a loop of the stationary phase bacterial cultures was spread onto TSA plates, which were also incubated at 37 °C for 24 h. One singe well-isolated colony from the TSA plate was transferred with a sterile loop into 5 mL TSB broth and incubated at 37 °C for 24 h.

After 24 hours, the exact bacterial concentration was determined using a Thoma cell counting chamber [97] (Celeromics Technologies, Cambridge, UK), and the samples further diluted to a level of 10^4-10^5 CFU/mL using Peptone water. Onto each plate (PCA, MHA and TSA), 100 μl of suspension was spread evenly using a sterile spreader [98]. The chitosan films prepared in citric and acetic acid were cut into 1-cm-side squares using sterile scissors and two of them were placed onto each plate. Squares of similar size cut from sterile stomacher bags were used as a control. For each chitosan sample and each bacterium, three replicates for each plate type were made. Thus, 18 plates in total for *E. coli* and 18 plates for *S. aureus* were prepared. After incubation at 37 °C for 24 h, the potential inhibition halo around the samples was measured.

3.7.3 Liquid assay

3.7.3.1 Chitosan films (37 °C)

Microbank™ beads of *E. coli* and *S. aureus* stored at -80 °C (Figure 12 A) were spread onto PCA plates and incubated at 37 °C overnight. Then, a single colony (Figure 12 B) was

transferred to a Falcon tube containing 5 mL of TSB (3 replicates per bacterial strain) (Figure 12 C) and incubated overnight at 37 °C.

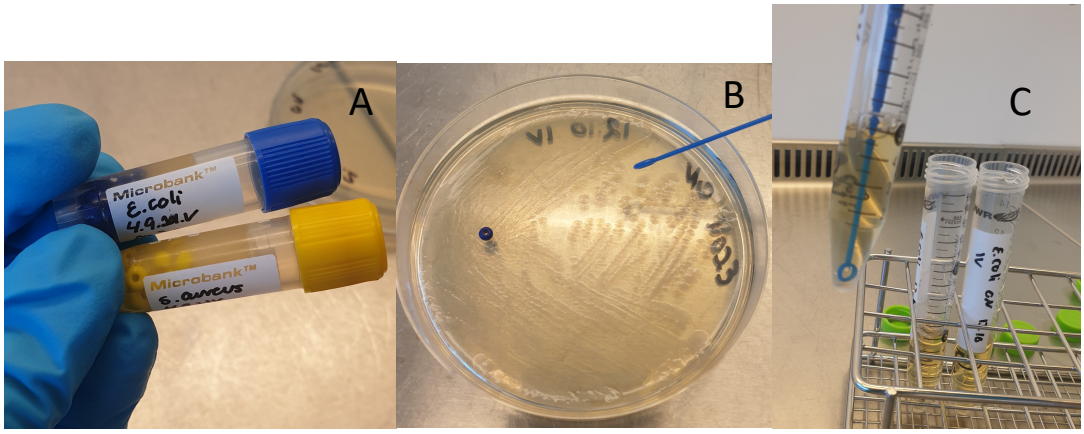

Figure 12. Preparation of cell suspension. A Microbank™ bead (A) was taken and spread onto PCA agar and incubated overnight. A single well-isolated colony was taken from the plate (B) and placed into Falcon tubes containing TSB (C).

For each bacterial strain, the 3 independent stationary phase cultures were combined, and appropriate dilutions were prepared in salt water in order to achieve cell suspensions of about 10^7 CFU/mL, further used for the antimicrobial assays. To confirm the exact concentration in the bacterial suspensions, appropriate decimal serial dilutions of the already diluted stationary phase cultures were prepared in salt water, plated onto MHA in triplicate and incubated at 37 °C for 24 h, prior to enumeration.

Chitosan films prepared in either citric or acetic acid were dissolved in water, resulting in solutions with pH 4.0 ± 0.0 and 5.4 ± 0.2, respectively. In order to avoid the effect of the acidic pH when conducting the antimicrobial assays, the pH of both solutions was adjusted to 5.2 ± 0.1 using 0.1 M HCl for the citric acid solution and 0.1 M NaOH for the acetic acid solution. The pH was measured using a FiveEasy plus pH meter (Mettler Toledo, US) equipped with a LE427 electrode.

100 µL of the dissolved samples or TSB (controls) was added to Eppendorf tubes (1.5 mL), followed by 200 µL of the diluted cell suspension. The Eppendorf tubes were incubated in a VorTemp 56 Shaking incubator (Labnet, US) at 37 °C and 300 rpm for 24 h. Appropriate decimal serial dilutions prepared in salt water were then plated onto MHA plates in triplicate

and incubated at 37 °C for 24 h, prior to enumeration. Moreover, the pH of the different samples was measured before and after incubation.

3.7.3.2 Seaweed (37 °C)

To determine the antimicrobial properties of the *Laminaria hyperborea* extract, the protocol for the liquid-assay was applied with slight modifications. Moreover, different samples were tested: (A) alginate films containing 50% (w/w) seaweed (concentration expressed as the ratio seaweed:alginate levels), dissolved in 100 mL water under magnetic stirring for 15 min at room temperature; (B) The film-forming solution of alginate containing 50% seaweed, i.e. the solution before casting the films; and (C) TSB with 10 mg/mL seaweed, concentration calculated in relation to the seaweed:alginate ratio in the films. In this case, the pH of the samples was not adjusted as it was close to neutral. 100 μL of the corresponding sample or TSB (control) was added to an Eppendorf tube, followed by 200 μL of diluted stationary-phase cell suspensions prepared as previously described (see 3.7.3.1). Three replicates were prepared per sample and bacterial strain. Colony counts and initial and final pH were determined as above mentioned (see 3.7.3.1).

3.7.3.3 Antimicrobial assays at 10°C

To determine the antimicrobial activity under more realistic conditions for food storage, the liquid-based assay was also conducted at 10 °C with samples of chitosan (film prepared with acetic acid, dissolved in water and adjusted to pH 5.2 ± 0.1), seaweeds (TSB with 10 mg/mL seaweed) and their combination (chitosan film prepared with acetic acid, dissolved in water at pH 5.2 ± 0.1 and supplemented with 10 mg/mL seaweed).

In this case, the samples were incubated at 10 °C and 70 rpm in an Innova 40 (New Brunswick Scientific, US) orbital shaker. pH and colony counts were determined after 5 and 10 days incubation, as described in previous sections.

3.8 Optimization

For several of the experiments, the methods described above were optimized.

- Two different volumes of film-forming solution (30 and 40 mL) were used to prepare the films based on previous studies conducted in the group on different polymers. Films made from 40 mL film-forming solution did not dry to a homogenous film. Several bubbles were present, and they were more difficult to peel off the petri dish compared to the 30 mL films. Films made from 20 mL solution would give films which would have been too thin and thus difficult to handle.
- The protocol for making the films were optimized for chitosan. Stirring over-night or over several days at room temperature was tried, but the chitosan particles did not dissolve into the acidic solution. In addition, the temperature at which the chitosan was dissolved was investigated. Elevating the temperature to 90 °C did not aid the dissolving and 40 °C was too low to dissolve the chitosan, so it was decided to use 70 °C to avoid excessive heating of the solution.
- The films were also dried in different ways in an attempt to shorten the drying time. Drying the films in an oven set at either 40 or 70 °C resulted in films which had several bubbles, and some of them had browning sections. Though drying the films in a fume hood took longer, it did not cause changes in the films.
- To begin with, the film-forming solution instead of the dissolved films were used in both the DPPH and ninhydrin assay. However, because the product of interest was the finished film, the first method was rejected. In addition, in the beginning of these two trials there were large standard deviations in the results. This was solved by switching to another spectrometer, namely the one at CORE Stavanger.

3.9 Statistics

Each value is reported as a mean of at least three parallels (if not stated otherwise) with standard deviation. Using SPSS statistics version 26 by IBM, general linear model two-way ANOVA tests were performed on the different sets of data with t-tests (Tukey´s) to detect variance between the means of each group. Statistics were performed on alginate and the

potential effect of seaweed and HPP, and chitosan with both the potential effect of seaweed and HPP as well as solvent (acetic and citric acid) and HPP. A significance level of $p \leq 0.05$ was used. For the antimicrobial studies, individual t-tests (Tukey B´s) were performed with a significance level of $p \leq 0.05$. Equal variance was not assumed.

4. Results

4.1 Chitosan

4.1.1 Film characteristics

4.1.1.1 Chitosan

Table 3 represents the average weight and thickness of the chitosan films prepared via dissolving in acetic acid and citric acid.

Table 3. The average weight in grams and thickness in millimeters of all films of chitosan dissolved in acetic acid and in citric acid, with standard deviation.

Sample	Weight (g)	Thickness (mm)
Chitosan in citric acid	0.7755 ± 0.0897	0.10 ± 0.03
Chitosan in acetic acid	0.6548 ± 0.0698	0.10 ± 0.02

As seen from the table, the average weight of the chitosan films prepared in citric acid (0.7755 ± 0.0897) was significantly ($p \leq 0.05$) higher than the average weight of the chitosan films prepared in acetic acid (0.6548 ± 0.0698). However, use of different acidic conditions (citric acid and acetic acid) did no impart any significant effect ($p > 0.05$) on the thickness of the films. As mentioned in the methodology section, the films were prepared via drying 30 mL of 2% chitosan solution, so each film should contain approximately 0.6 g of chitosan.

The pH of the film-forming solution of chitosan in citric acid and acetic acid was 3.95 ± 0.06 and 4.15 ± 0.15, respectively. However, the pH of the solution prepared via resolving the dried film into 100 mL water was 4.00 ± 0.01 and 5.44 ± 0.23, for citric acid films and acetic acid films, respectively.

4.1.1.2 Chitosan + HPP treatment

The average weight and thickness of the chitosan films prepared using HPP treated solution are presented in Table 4.

Table 4. The average weight in grams and thickness in millimeters of all films of chitosan dissolved in either acetic acid or citric acid treated with HPP at 200 and 600 MPa pressures, with standard deviation. *For chitosan in citric acid and 200 MPa treatment, only one film was weighed due to the brittle nature of the film, thus no standard deviation.

Sample	Weight (g)	Thickness (mm)
Chitosan in citric acid 200 MPa	0.7056*	0.09 ± 0.01
Chitosan in citric acid 600 MPa	0.6731 ± 0.0146	0.08 ± 0.02
Chitosan in acetic acid 200 MPa	0.6093 ± 0.0659	0.10 ± 0.02
Chitosan in acetic acid 600 MPa	0.5970 ± 0.0417	0.09 ± 0.02

HPP treatment did not cause any significant ($p > 0.05$) change in the weight of the films compared to the non HPP treated ones. Similarly, no significant effect ($p > 0.05$) of the different pressure used during the HPP treatment was observed on the thickness of the films. The films prepared via drying HPP treated chitosan solution in citric acid was very brittle and difficult to handle and characterize, especially at 200 MPa. However, the films prepared from a HPP treated solution at 600 MPa were less brittle and more films were able to be weighed. The films with acetic acid were easier to handle and characterize. These were also significantly lighter ($p \leq 0.05$) than those made with citric acid.

Moreover, for the HPP treated films, no significant effect ($p > 0.05$) of solvent, HPP or the two combined was seen on the thickness of the films. The citric acid films had a thickness of 0.09 ± 0.01 mm when treated at 200 MPa pressure, and 0.08 ± 0.02 mm for the 600 MPa pressure ones. For acetic acid films, the thickness was 0.10 ± 0.02 mm and 0.09 ± 0.02 mm after treatment at pressures of 200 and 600 MPa, respectively.

Interestingly, the films prepared from HPP treated solution, regardless of pressure and solvent used, took much less time to dissolve compared to the non-treated films. Whereas the non-treated films took around 2 hours at 70°C, the HPP treated films only took 1 hour to

completely dissolve at the same temperature, regardless of the solvent used. The pH was similar to that of the non-treated films.

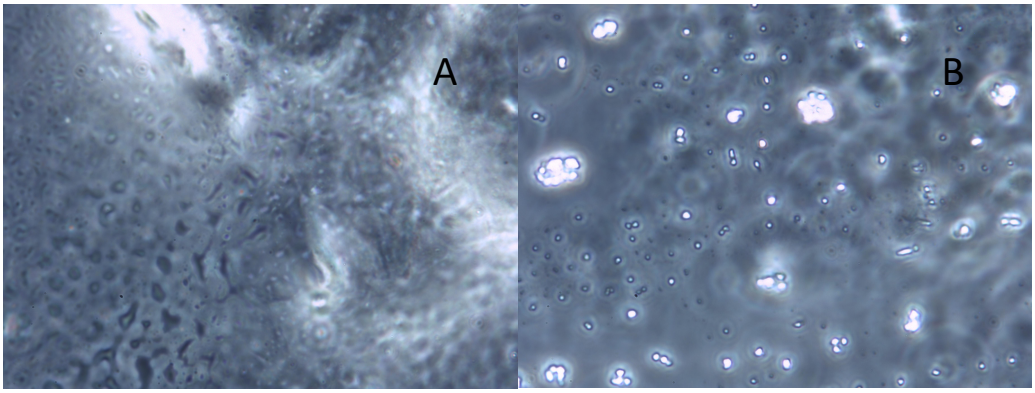

Figure 13. Microscopic images of the chitosan films with acetic acid as the solvent (magnification 40x). Non-treated film (A) and the HPP treated one at 200 MPa pressure (B).

The images in Figure 13 show the chitosan films with acetic acid as the solvent on a microscopic level (40x magnification). The non-treated film (A) is more homogenous than the HPP treated at 200 MPa pressure one, where bubbles can be seen. The presence of these bubbles might decrease the mechanical and barrier properties of the HPP treated films.

4.1.1.3 Chitosan + Seaweed

The weight and thickness of chitosan films with added seaweed are presented in Table 5. Since the films with citric acid as a solvent and HPP treatment were much more brittle compared to the acetic acid ones, only the chitosan solution prepared with acetic acid was used to study the effect of seaweed on the functional properties of the films.

Table 5. The average weight in grams and thickness in millimeters of all films of chitosan dissolved in acetic acid with added seaweed in concentrations of 10, 30 and 50%, with standard deviation.

Sample	Weight (g)	Thickness (mm)
Chitosan + 10% SW	0.7371 ± 0.0229	0.18 ± 0.03
Chitosan + 30% SW	0.8504 ± 0.0348	0.19 ± 0.02
Chitosan + 50% SW	0.9809 ± 0.0315	0.22 ± 0.02

With addition of a filler such as seaweed, the films were expected to be heavier and thicker. This is also the case for our seaweed containing samples. With increasing seaweed addition, the weight and also the thickness increased significantly ($p \leq 0.05$). The weight of the chitosan film without seaweed was 0.6548 ± 0.0698 g, which increased to 0.7371 ± 0.0229, 0.8504 ± 0.0348 and 0.9809 ± 0.0315 g, with added seaweed in concentrations of 10, 30 and 50%, respectively, when the same 30 mL solution was dried to prepare the films. Similarly, the thickness increased from 0.10 ± 0.02 mm for the chitosan film with no seaweed, to 0.18 ± 0.03, 0.19 ± 0.02 and 0.22 ± 0.02 mm with added seaweed in concentrations of 10, 30 and 50%, respectively.

The chitosan films with seaweed had a similar time to being completely dissolved as the ones with acetic acid as a solvent and no-treatment, thus, around 2 hours at 70°C heat was sufficient.

4.1.1.4 Chitosan + HPP treatment + Seaweed

The final group of films with chitosan as the polymer was prepared via dissolving chitosan in acetic acid with added seaweed in combination with HPP treatment at 200 and 600 MPa. The weights and thicknesses of the dried films are presented in Table 6.

Table 6. The average weight in grams and thickness in millimeters of all films of chitosan dissolved in acetic acid and with added seaweed in concentrations of 10, 30 and 50%, in addition to HPP treatment at pressures of 200 and 600 MPa. With standard deviation.

Sample	Weight (g)	Thickness (mm)
Chitosan + 10% SW 200 MPa	0.6881 ± 0.0603	0.17 ± 0.02
Chitosan + 10% SW 600 MPa	0.6766 ± 0.0347	0.18 ± 0.02
Chitosan + 30% SW 200 MPa	0.7813 ± 0.0294	0.18 ± 0.02
Chitosan + 30% SW 600 MPa	0.7698 ± 0.0319	0.19 ± 0.02
Chitosan + 50% SW 200 MPa	0.8773 ± 0.0344	0.21 ± 0.02
Chitosan + 50% SW 600 MPa	0.9054 ± 0.0317	0.22 ± 0.02

It has already been mentioned in the previous section that addition of seaweed has a significant effect ($p \leq 0.05$) on the weight and thickness of the films. HPP treatment also significantly affected the weight of the chitosan films, however the two did not have a

significant interaction effect ($p > 0.05$). For the films with 10% seaweed, the weight of the ones treated with a pressure of 200 MPa was 0.6881 ± 0.0603 g, whereas it was 0.6766 ± 0.0347 g for the 600 MPa one. For 30% seaweed containing films, it was 0.7813 ± 0.0294 g and 0.7698 ± 0.0319 g for the ones treated at 200 and 600 MPa pressure, respectively. Finally, for the films with 50% seaweed, the weight of the film with 200 MPa pressure treatment was 0.8773 ± 0.0344 g, and for the 600 MPa pressure treated ones it was 0.9054 ± 0.0317 g. Moreover, although HPP treatment showed a significant effect on the weight of the films, the seaweed addition had a much larger effect on the weight as expected.

HPP treatment showed no significant effect ($p > 0.05$) on the thickness of the films. However, like already stated, seaweed did. The films with 10% seaweed in combination with 200 MPa was 0.17 ± 0.02 mm, and with 600 MPa it was 0.18 ± 0.02 mm. For the 30% seaweed addition, the thickness was 0.18 ± 0.02 mm and 0.19 ± 0.02 mm for the two different pressures, respectively. Whereas for the films with 50% seaweed, the 200 MPa treated films were 0.21 ± 0.02 mm, and the 600 MPa treated ones were 0.22 ± 0.02 mm. Therefore, there was no significant interaction effect ($p > 0.05$) between seaweed addition and HPP treatment.

Seaweed had no effect on the time it took to dissolve the films, so these films also took 1 hour at 70°C to be completely dissolved.

4.1.2 Mechanical properties

4.1.2.1 Chitosan

The code names used throughout this study along with corresponding solvent, HPP treatment and concentration of seaweed are given in Table 7.

Table 7. Code names of the different chitosan samples and their conditions.

Sample code	Solvent	HPP treatment (MPa)	Seaweed addition (%)
CH-CS	Citric acid	0	0
CH-CA	Acetic acid	0	0
CH-CA-200	Acetic acid	200	0
CH-CA-600	Acetic acid	600	0
CH-CA-10SW	Acetic acid	0	10
CH-CA-10SW-200	Acetic acid	200	10
CH-CA-10SW-600	Acetic acid	600	10
CH-CA-30SW	Acetic acid	0	30
CH-CA-30SW-200	Acetic acid	200	30
CH-CA-30SW-600	Acetic acid	600	30
CH-CA-50SW	Acetic acid	0	50
CH-CA-50SW-200	Acetic acid	200	50
CH-CA-50SW-600	Acetic acid	600	50

The tensile strength of the chitosan films with no treatment and no fillers are presented in Figure 14 below.

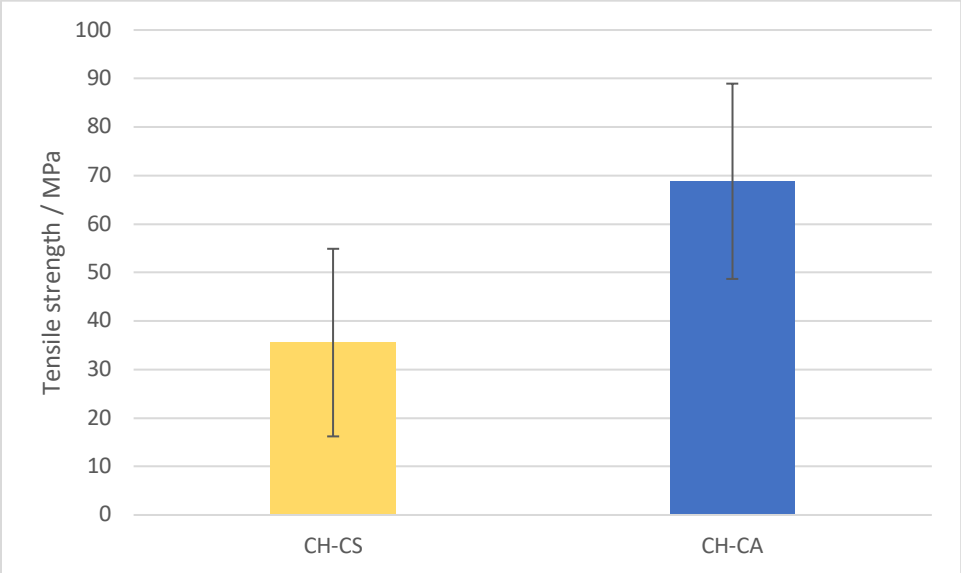

Figure 14. Tensile strength (MPa) of films of chitosan dissolved in either citric or acetic acid. Averages of all films measured with standard deviation as error bares.

The chitosan films with citric acid as a solvent showed a significant lower ($p \leq 0.05$) tensile strength compared to those with acetic acid as the solvent. For the citric acid films, the

tensile strength was 35.55 ± 19.34 MPa, whereas for the acetic acid ones the tensile strength increased to 68.81 ± 20.13 MPa.

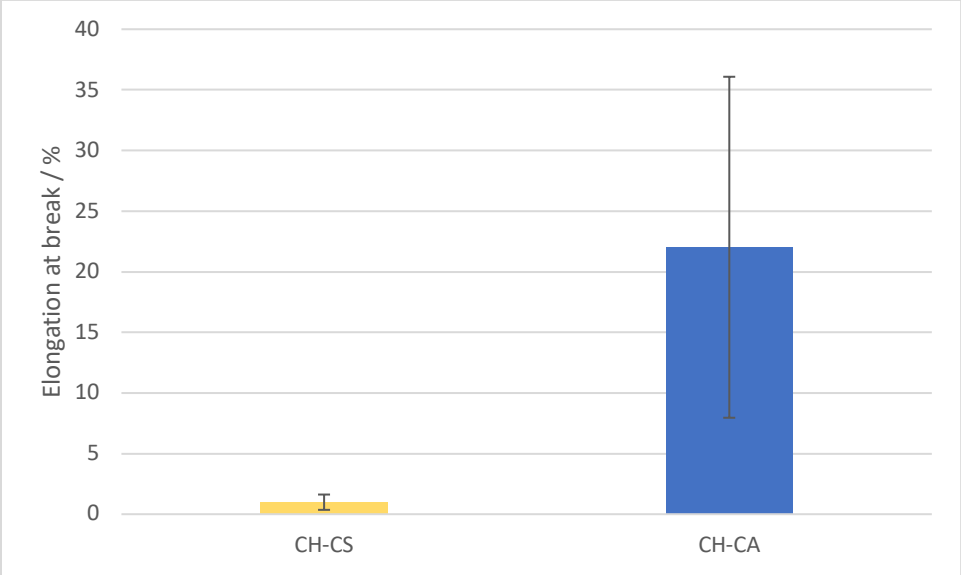

Figure 15. Elongation at break (%) of films of chitosan dissolved in either citric or acetic acid. Averages of all films measured with standard deviation as error bars.

The elongation at break of chitosan films (Figure 15) made with acetic acid was significantly higher ($p \leq 0.05$) than the films made with citric acid. The citric acid films had an elongation at break of 0.99 ± 0.63%, while the acetic acid films had one at 22.02 ± 14.06%. A large error bar was observed for the acetic acid films which could be due to variations within the films and uneven thickness across the films.

4.1.2.2 Chitosan + HPP treatment

Figure 16 and 17 presents the effect of HPP on the tensile strength of chitosan samples made with acetic acid. For the films with citric acid as a solvent, the films with HPP treatment were too brittle to cut into the shapes necessary for the texture analyzer. Thus, no statistical analysis was performed on citric acid and only films with acetic acid as a solvent is presented.

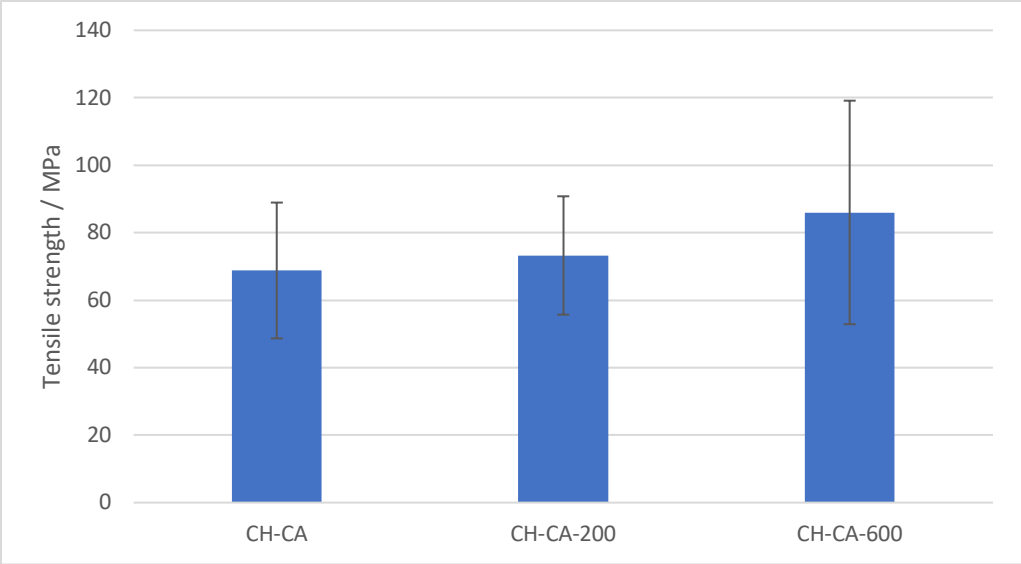

Figure 16. Tensile strength (MPa) of films made of chitosan dissolved in acetic acid, HPP treated with 200 MPa and 600 MPa pressures. Averages of all films measured with standard deviation as error bars.

No significant effect ($p > 0.05$) was seen on the tensile strength for films treated with 200 MPa pressure. However, a significant effect ($p \leq 0.05$) was seen for the samples where the film-forming solution was treated at 600 MPa pressure. As mentioned before, the tensile strength for the chitosan sample made with acetic acid and with no treatment was 68.81 ± 20,13 MPa. For the films with 200 MPa treatment, the tensile strength was 73.26 ± 17.54 MPa, and for the ones with 600 MPa treatment, it was 86.02 ± 33.13 MPa.

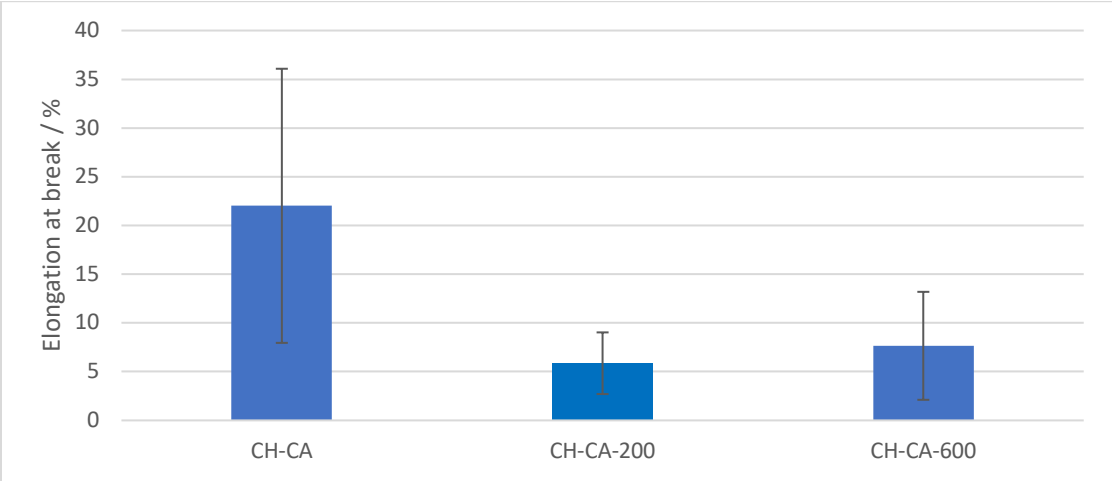

Figure 17. Elongation at break (%) of films made of chitosan dissolved in acetic acid, HPP treated with 200 MPa and 600 MPa pressures. Averages of all films measured with standard deviation as error bars.

For elongation at break, there was a significant decrease ($p \leq 0.05$) after HPP treatment for both the 200 MPa and 600 MPa samples. The elongation at break for the non-treated

chitosan sample was 22.02 ± 14.06%, which reduced to 5.87 ± 3.16% and 7.65 ± 5.54% for HPP treatment at 200 MPa and 600 MPa HPP treatment, respectively.

4.1.2.3 Chitosan + Seaweed

Chitosan samples made with acetic acid was used to explore the effect of seaweed addition on the functional properties of the films. The effect of seaweed addition on the tensile strength and elongation at break of chitosan samples made with acetic acid are presented in Figure 18 and 19 below.

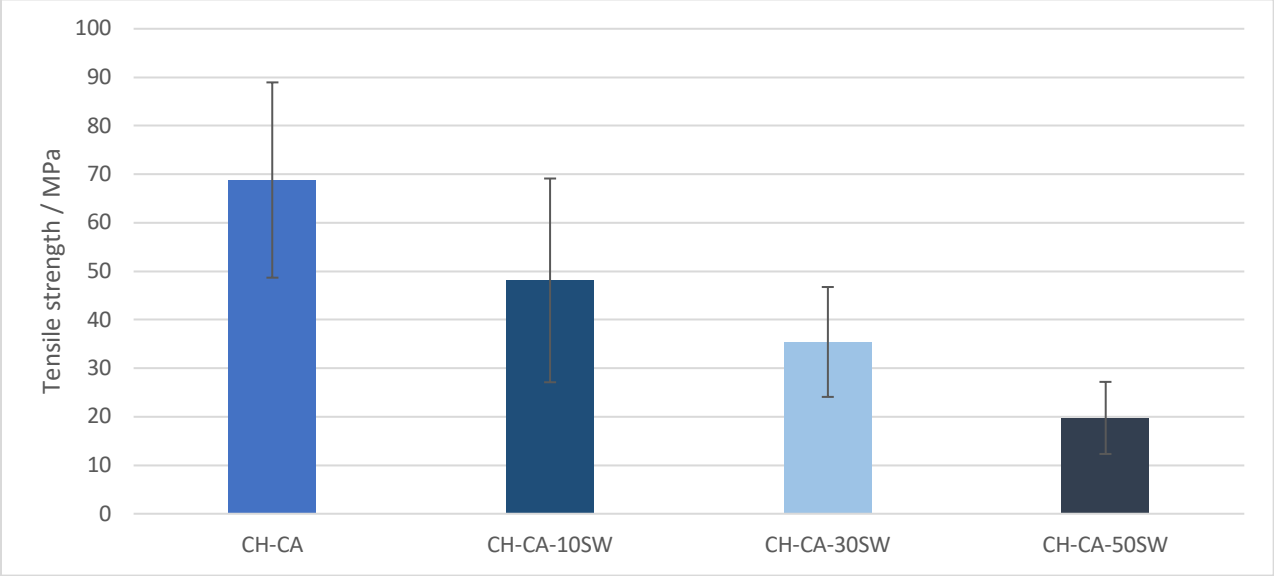

Figure 18. Tensile strength (MPa) of films made of chitosan dissolved in acetic acid, with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. Averages of all films measured with standard deviation as error bars.

Seaweed addition had a significant effect ($p \leq 0.05$) on the tensile strength of the chitosan samples. Like what is clear in Figure 18, the tensile strength decreased after addition of seaweed. The tensile strength of the chitosan in acetic acid film with no filler was 68.81 ± 20.13 MPa. After addition of seaweed in concentrations of 10, 30 and 50%, the tensile strength decreased to 48.12 ± 21.02, 35.42 ± 11.33 and 19.76 ± 7.43 MPa, respectively. A significant decrease ($p \leq 0.05$) with increasing seaweed concentration was seen.

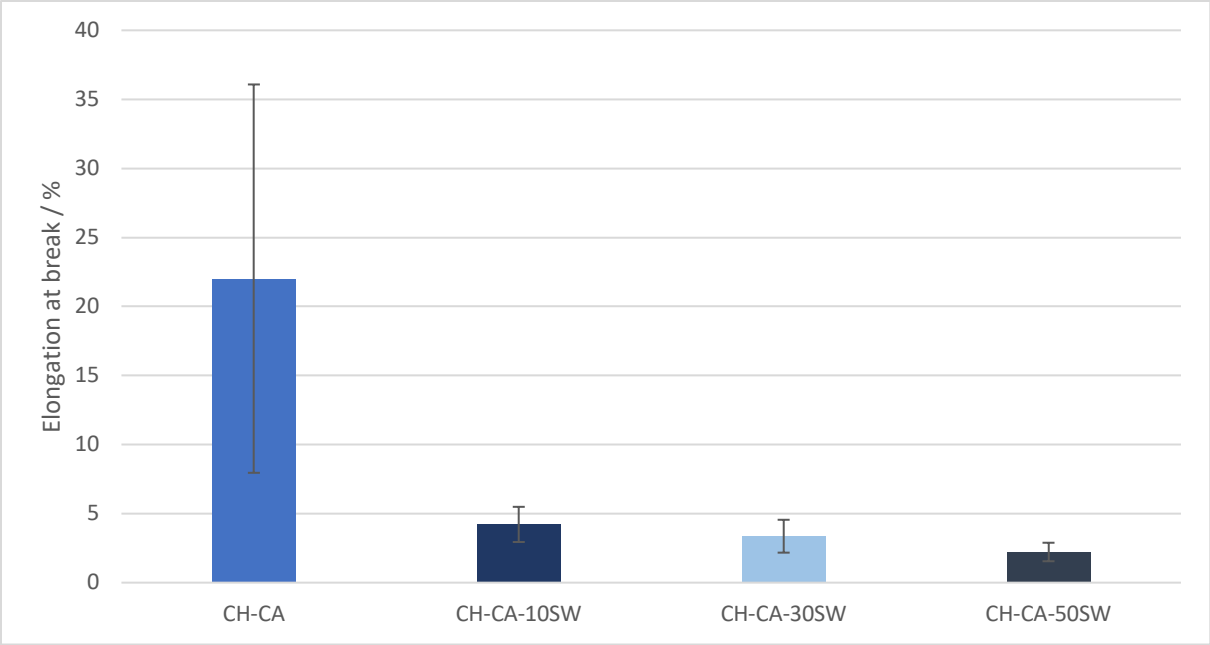

Figure 19. Elongation at break (%) of films made of chitosan dissolved in acetic acid, with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. Averages of all films measured with standard deviation as error bars.

Similar decreasing trend with seaweed addition was also observed for elongation at break values. This effect was significant ($p \leq 0.05$). For chitosan in acetic acid without any seaweed, the elongation at break was 22.02 ± 14.06%, whereas for 10% seaweed addition, the elongation at break was reduced to 4.22 ± 1.27%. For 30% seaweed addition, the films had an elongation at break of 3.36 ± 1.19%, and for 50% seaweed it was 2.22 ± 0.67%.

4.1.2.4 Chitosan + HPP treatment + Seaweed

Figure 20 and 21 illustrates the effect of combination of seaweed addition and HPP treatment on the chitosan in acetic acid samples.

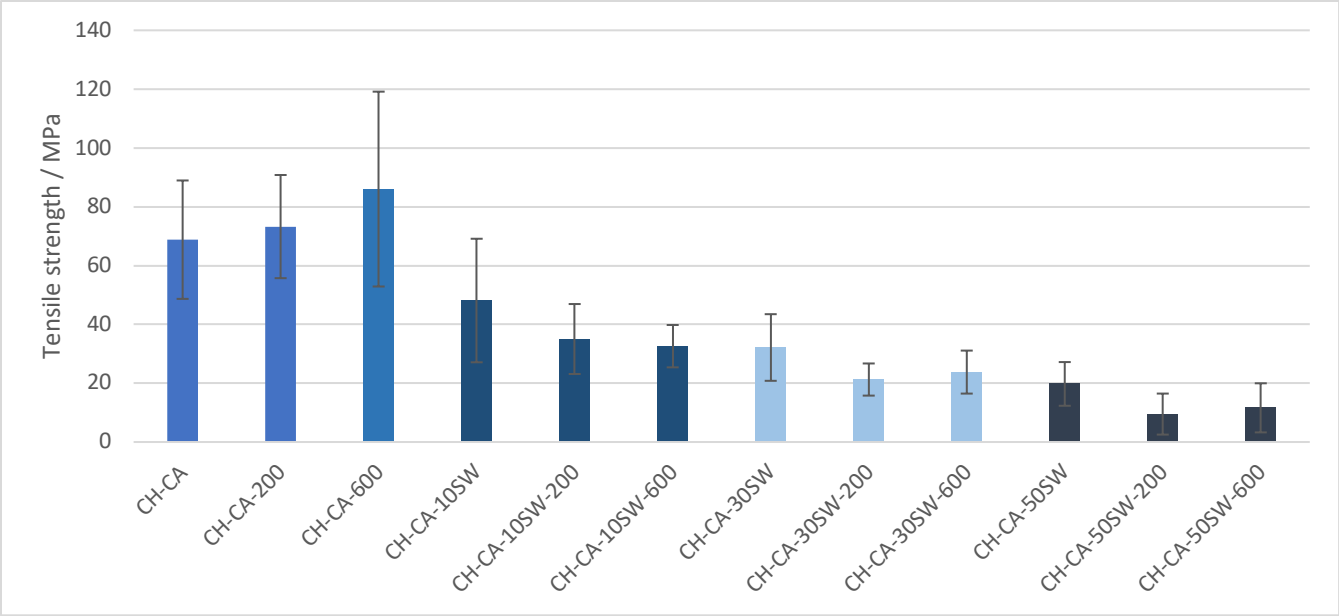

Figure 20. Tensile strength (MPa) of films made of chitosan dissolved in acetic acid, with seaweed added as a filler in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. In addition, samples with HPP treatment at pressures of 200 MPa and 600 MPa. Averages of all films measured with standard deviation as error bars.

It has already been presented that addition of seaweed had a significant effect ($p \leq 0.05$) on the tensile strength of chitosan films. Overall, treatment with HPP also impacts the tensile strength, except for the samples with no seaweed.

The tensile strength of the chitosan samples with 10% seaweed was 48.12 ± 21.02 MPa, which reduced to 35.03 ± 11.90 and 32.59 ± 7.20 MPa for treatment at 200 and 600 MPa pressures, respectively. For the films with 30% seaweed without any HPP treatment, the tensile strength was 32.14 ± 11.33 MPa. After HPP treatment of the film-forming solution at 200 MPa and at 600 MPa pressure, the tensile strength reduced to 21.25 ± 5.46 MPa and 23.78 ± 7.31 MPa, respectively. A similar effect was seen for the films with 50% seaweed as well, with an initial tensile strength of 19.76 ± 7.43 MPa, which reduced to 9.50 ± 6.97 and 11.63 ± 8.35 MPa with HPP treatment at 200 and 600 MPa, respectively.

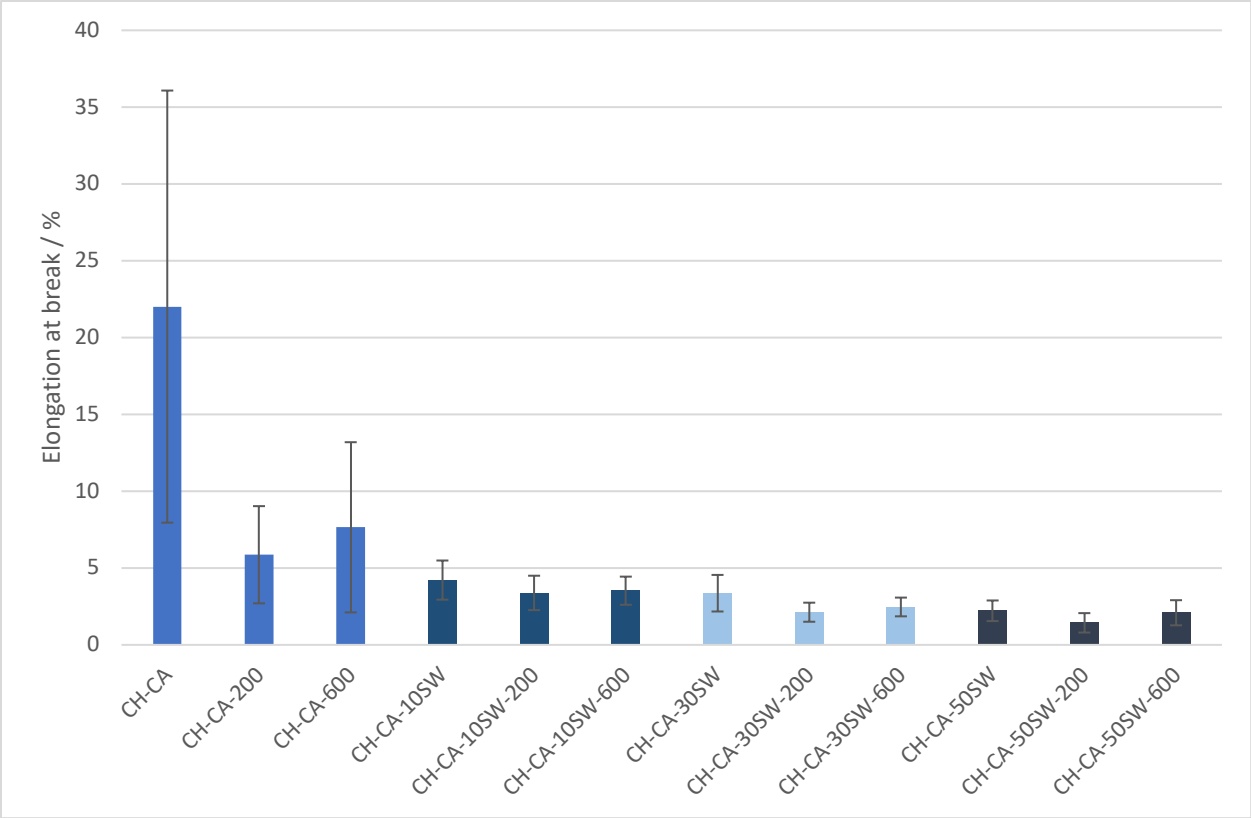

Figure 21. Elongation at break (%) of films made of chitosan dissolved in acetic acid, with seaweed added as a filler in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. In addition, samples with HPP treatment at pressures of 200 MPa and 600 MPa. Averages of all films measured with standard deviation as error bars.

For elongation properties, both seaweed addition and HPP treatment had a significant effect ($p \leq 0.05$) on the chitosan films. In addition, there was a significant interaction effect ($p \leq 0.05$) between the two. For the samples with 10% seaweed, the initial elongation at break was 4.22 ± 1.27 % which decreased to 3.38 ± 1.12% for treatment with 200 MPa pressure, and to 3.53 ± 0.92% for treatment with 600 MPa pressure. For the films with 30% seaweed, the elongation at break decreased from an initial value of 3.36 ± 1.19%, to 2.13 ± 0.62 and 2.47 ± 0.61% for HPP treatment at 200 MPa and 600 MPa pressures, respectively. Similarly, for films with 50% seaweed, the elongation at break reduced from 2.22 ± 0.67% to 1.43 ± 0.63 and 2.09 ± 0.82%.

Therefore, it could be concluded that in general the HPP treatment at 200 MPa significantly reduced ($p \leq 0.05$) the elongation at break as compared to the non-treated samples, whereas a slight though non-significant ($p > 0.05$) improvement could be observed for treatment at 600 MPa. However, although the chitosan samples treated at 600 MPa

pressure showed higher elongation at break than the samples treated at 200 MPa, it was still lower than the non-treated samples.

4.1.3 Barrier properties

4.1.3.1 Chitosan

The water vapor transmission rate of the films made of chitosan dissolved in citric or acetic acid is presented below in Figure 22.

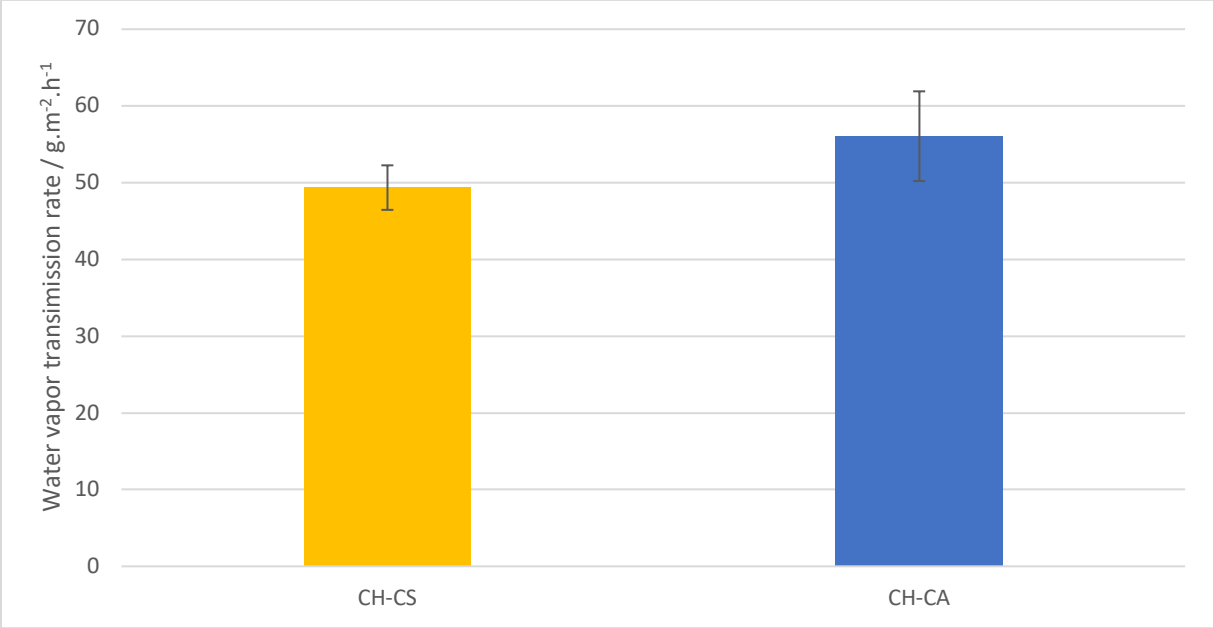

Figure 22. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of chitosan dissolved in either citric or acetic acid. Averages of two measurements with standard deviation as error bars.

The barrier properties of the films are important factors for maintaining the quality of the packaged product [99]. The water vapor transmission rate for the chitosan films made with citric acid was 49.36 ± 2.90 $g.m^{-2}.h^{-1}$. For the films made with acetic acid as the solvent, the WVTR was 56.06 ± 5.85 $g.m^{-2}.h^{-1}$. There was no significant variance ($p > 0.05$) between the two, so it could be concluded that the water vapor transmission rate is not impacted by the type of solvent used to create the chitosan films.

4.1.3.2 Chitosan + HPP treatment

The impact of HPP treatment on the barrier properties of the chitosan films with no fillers is presented in Figure 23 below. Like mentioned previously, the citric acid samples with HPP treatment were very brittle and thus impossible to fit on top of the glass tubes for the barrier test. Thus, only the chitosan films prepared using acetic acid were considered for further experiment and are presented in the next sub-chapters.

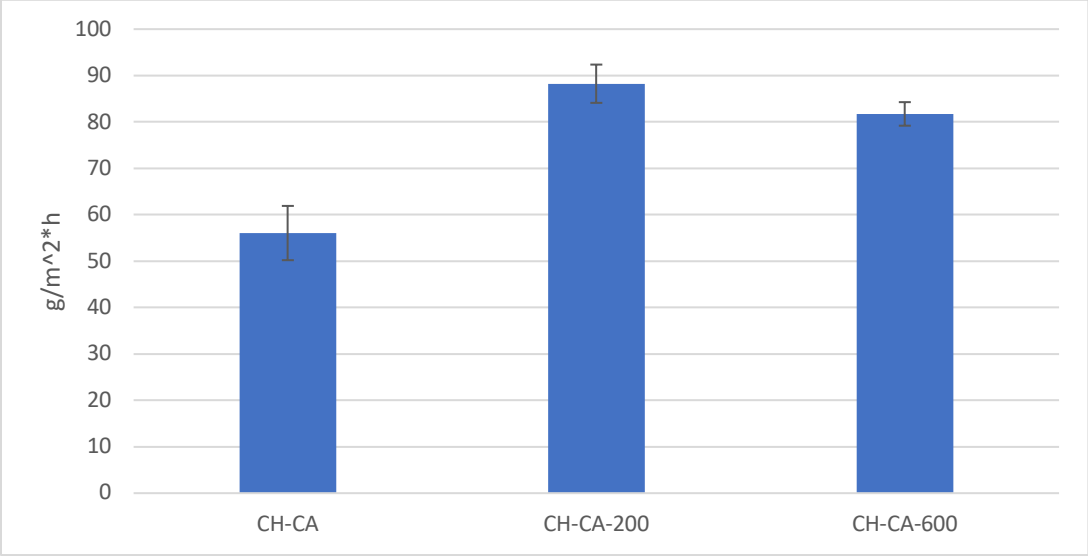

Figure 23. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of chitosan dissolved in acetic acid, HPP treated at pressures of 200 and 600 MPa. Averages of two measurements with standard deviation as error bars.

Decreased barrier properties were observed in the chitosan films where the film-forming solution was HPP treated. As mentioned before, the WVTR of the films made from chitosan dissolved in acetic acid was 56.06 ± 5.85 $g.m^{-2}.h^{-1}$, which increased to 88.24 ± 4.12 $g.m^{-2}.h^{-1}$ and 81.73 ± 2.54 $g.m^{-2}.h^{-1}$ with HPP treatment at 200 and 600 MPa pressure, respectively. However, no significant difference ($p > 0.05$) between the two pressures were found.

4.1.3.3 Chitosan + Seaweed

The WVTR of chitosan films with added seaweed were also evaluated, and the results are presented in Figure 24.

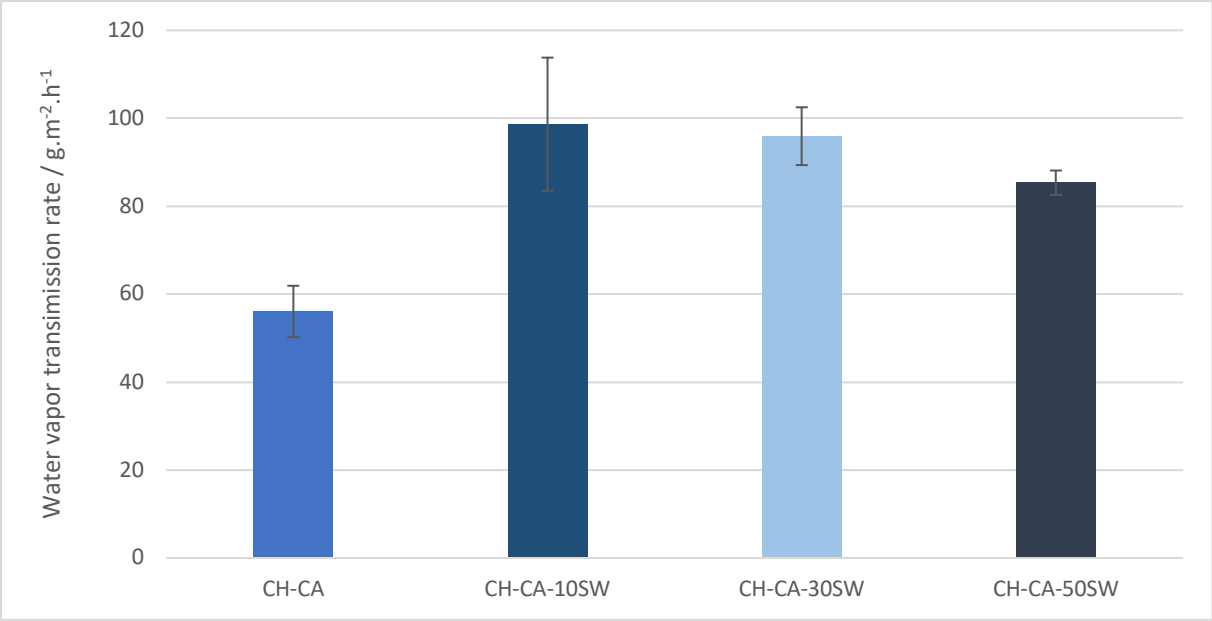

Figure 24. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of chitosan dissolved in acetic acid with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. Averages of two measurements with standard deviation as error bars.

An increase in WVTR was seen for all concentrations of seaweed added to the chitosan samples. The water vapor transmission rate of the chitosan film with no fillers was 56.06 ± 5.85 $g.m^{-2}.h^{-1}$, which increased to 98.65 ± 15.14, 95.93 ± 6.57 and 85.35 ± 2.76 $g.m^{-2}.h^{-1}$ for 10, 30 and 50% seaweed addition, respectively. No significant ($p > 0.05$) difference in WVTR between the different seaweed concentrations was found.

4.1.3.4 Chitosan + HPP treatment + Seaweed

The effect of HPP treatment in combination with seaweed addition on the WVTR of chitosan samples are presented below in Figure 25.

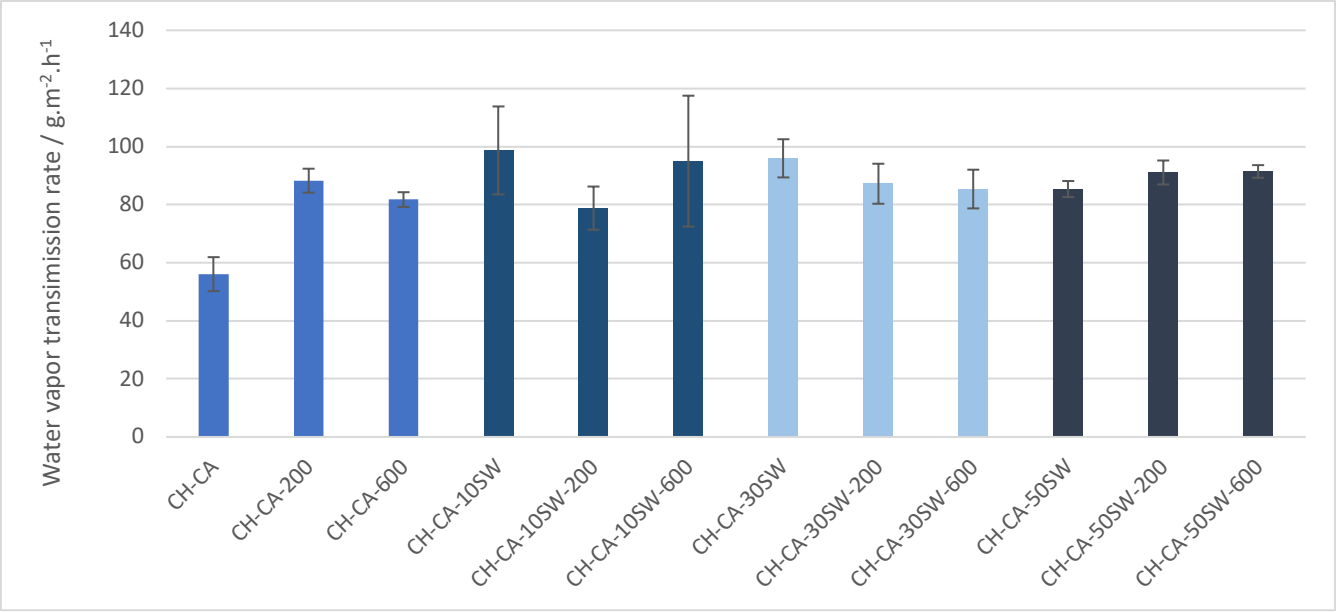

Figure 25. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of chitosan dissolved in acetic acid with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. In addition, HPP treatment at pressures of 200 and 600 MPa. Averages of two measurements with standard deviation as error bars.

It has already been mentioned that the seaweed addition and HPP treatment separately increased the WVTR of the chitosan films. However, HPP treatment showed no significant effect ($p > 0.05$) on the chitosan samples with added seaweed. Only two measurements were taken, which could cause large error bars and thus this non-significant effect.

For the films with 10% seaweed, the non-treated films had a WVTR of 98.65 ± 15.14 $g.m^{-2}.h^{-1}$ which were 78.78 ± 7.42 $g.m^{-2}.h^{-1}$ and 94.97 ± 22.53 $g.m^{-2}.h^{-1}$ with HPP treatment at 200 and 600 MPa, respectively. The 30% seaweed containing films had a WVTR of 95.93 ± 6.57 $g.m^{-2}.h^{-1}$, which after HPP treatment at pressures of 200 and 600 MPa were 87.18 ± 6.89 and 85.35 ± 6.66 $g.m^{-2}.h^{-1}$, respectively. The non-treated film with 50% seaweed had a WVTR of 85.35 ± 2.76 $g.m^{-2}.h^{-1}$. After HPP treatment at 200 and 600 MPa, the WVTR was 91.06 ± 4.12 and 91.41 ± 2.18 $g.m^{-2}.h^{-1}$, respectively. The variations within each group with the same seaweed concentration were not significant ($p > 0.05$).

4.1.4 Ninhydrin assay

4.1.4.1 Calibration curve

The calibration curve was made using an amino acid in concentrations of 0.05, 0.10, 0.15, 0.20 and 0.25 mg/mL in water, as well as a blank. The resulting equation which can be seen in Figure 26 below was only valid for samples with a free amino concentration within the range of 0.05 to 0.25 mg/mL. For concentrations below, the curve was not linear meaning the method was not sensitive enough for very low amino concentrations. In addition, at concentrations above 0.25 mg/mL, the curve plateaued. However, using the amino acid in concentrations between 0.05 to 0.25 mg/mL, a relatively linear line was created, with an R^2 value of 0.9626.

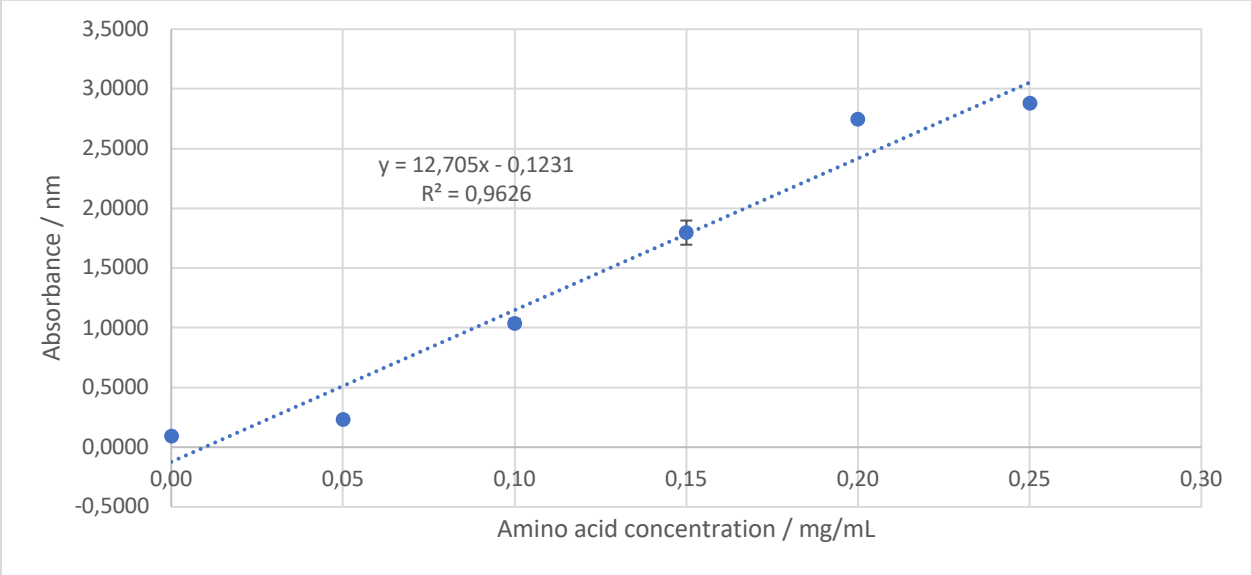

Figure 26. Calibration curve for ninhydrin assay, using Alanine as the amino acid. Only valid between concentrations of 0.05 and 0.25 mg/mL. Blank consists of ninhydrin reagent with water

4.1.4.2 Chitosan

The free amino concentration in the chitosan samples with no treatment is presented in Figure 27 below. Chitosan has a free amino group in its molecular structure, which is quantified by the ninhydrin method. The presence of amino groups on the surface of chitosan helps it interact both chemically and physically with other substances such as cells, polymers and nanoparticles [100]. The effect of the different solvent used, either acetic acid or citric acid, was studied.

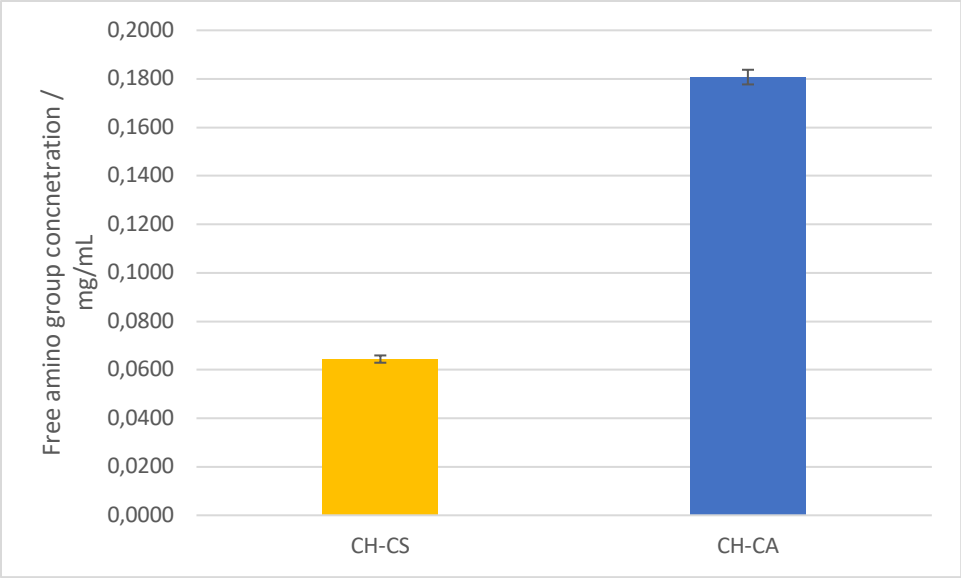

Figure 27. Free amino group concentration in films of chitosan dissolved in either citric or acetic acid. Averages of three measurements with standard deviation as error bars.

Using the equation obtained from the calibration curve in Figure 26, the concentrations of free amino groups present in chitosan samples were calculated. As seen from the figure above, the concentration of free amino groups in the chitosan samples prepared using citric acid was significantly lower ($p \leq 0.05$) than in the samples prepared with acetic acid. The samples with chitosan dissolved in acetic acid had the highest concentration of amino groups which was 0.1807 ± 0.0030 mg/mL. Whereas, for the sample with chitosan dissolved in citric acid, this concentration of amino groups was reduced to 0.0644 ± 0.0015 mg/mL.

4.1.4.3 Chitosan + HPP treatment

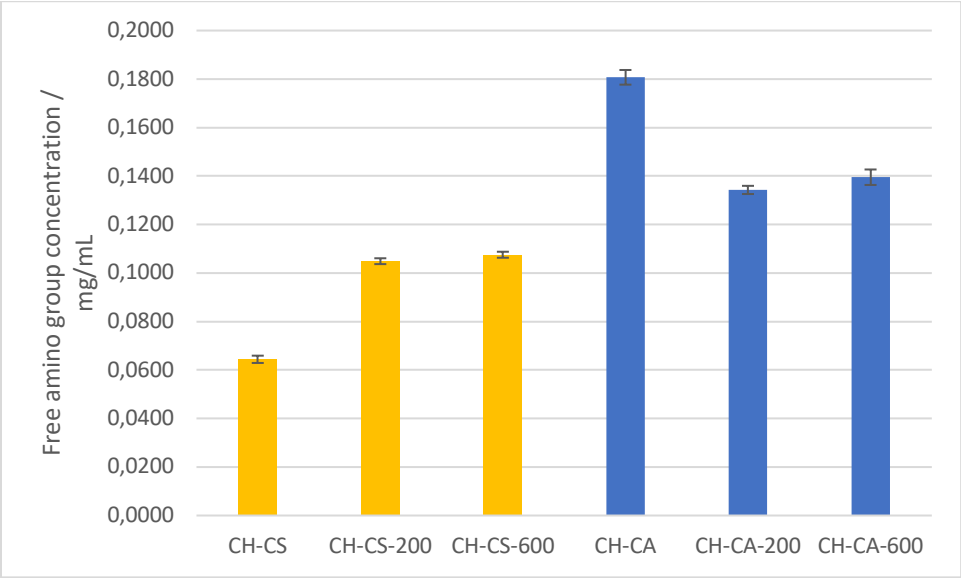

Figure 28. Free amino group concentration in films of chitosan dissolved in either citric or acetic acid, treated with HPP at pressures of 200 and 600 MPa. Averages of three measurements with standard deviation as error bars.

HPP treatment caused the opposite effect for chitosan with the two different solvents (Figure 28). Whereas HPP treatment had a significant increasing ($p \leq 0.05$) effect on the free amino group concentration in the chitosan with citric acid as the solvent, HPP treatment had a significant decreasing ($p \leq 0.05$) effect on the free amino group concentration in the chitosan with acetic acid as the solvent. For chitosan in citric acid, the sample with no treatment had a concentration of free amino groups of 0.0.0644 ± 0.0015 mg/mL, which after HPP treatment at 200 MPa and 600 MPa increased to 0.1049 ± 0.0012 and 0.1075 ± 0.0012 mg/mL, respectively. The chitosan in acetic acid sample with no treatment had a free amino group concentration of 0.1807 ± 0.0030 mg/mL. After HPP treatment at 200 and 600 MPa, this concentration decreased to 0.1343 ± 0.0017 and 0.1395 ± 0.0032 mg/mL, respectively. No significant ($p > 0.05$) difference in free amino group concentrations was seen between the two different HPP treatment pressures.

4.1.5 DPPH assay

4.1.5.1 Chitosan

The DPPH radical scavenging activity assay gives a measure of the polymer's antioxidant activity and its ability to protect against free radicals which might be harmful to the packaged material. Free radicals can damage cells due to their unstable state where an electron is unpaired. Antioxidants can prevent and stabilize the damage done by free radicals by supplying electrons to the damaged cells [101]. The scavenging activity of the chitosan samples with no fillers or treatment and with citric or acetic acid as a solvent is presented in Figure 29 below. Concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL chitosan was evaluated.

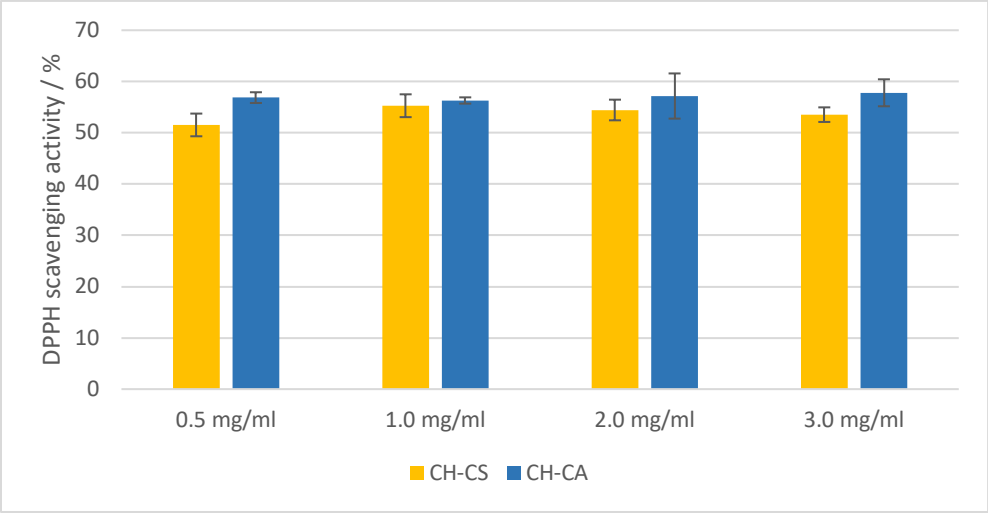

Figure 29. Scavenging activity (%) of films made of chitosan dissolved in either citric or acetic acid. Averages of three measurements with standard deviation as error bars.

For chitosan concentrations at 0.5 and 1.0 mg/mL, there was no significant effect ($p > 0.05$) of solvent on the scavenging activity. Thus, no significant difference ($p > 0.05$) between the chitosan samples made with citric acid and acetic acid were found. For the samples with citric acid as the solvent, the scavenging activity at 0.5 and 1.0 mg/mL was 51.53 ± 2.21 and 55.27 ± 2.23%, respectively, and for the acetic acid samples the scavenging activity was 56.86 ± 1.05 and 56.30 ± 0.61%, respectively. However, at chitosan concentrations of 2.0 and 3.0 mg/mL, a significant effect ($p \leq 0.05$) on the type of solvent used was observed on the scavenging activity. Generally, the films with acetic acid as the solvent had a higher scavenging activity than the citric acid ones. For the acetic acid samples, the scavenging

activity was 57.17 ± 4.41% at 2.0 mg/mL and 57.80 ± 2.63% at 3.0 mg/mL chitosan, the scavenging activity of the citric acid samples were 54.45 ± 2.02 and 53.54 ± 1.42%, respectively

4.1.5.2 Chitosan + HPP treatment

The effect of HPP treatment at pressures of 200 and 600 MPa on the scavenging activity of chitosan samples with citric acid and acetic acid as solvents are presented in Figure 30.

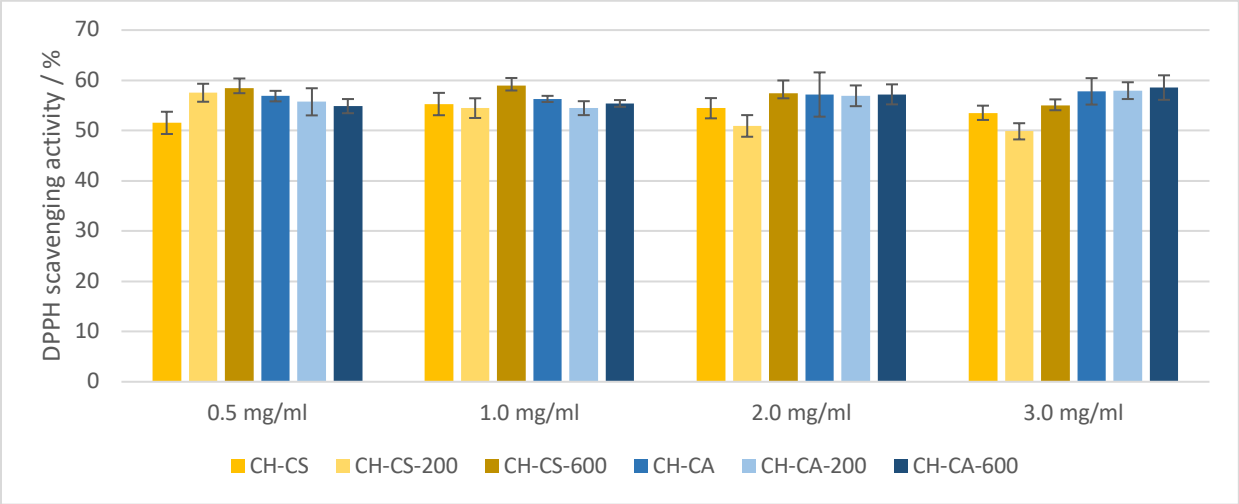

Figure 30. Scavenging activity (%) of films made of chitosan dissolved in either citric or acetic acid, HPP treated at 200 and 600 MPa pressures. Averages of three measurements with standard deviation as error bars.

HPP treatment showed a significant effect ($p \leq 0.05$) on the scavenging activity at chitosan concentrations of 0.5 and 1.0 mg/mL, considering all the samples with both solvents. At 0.5 mg/mL, the samples treated at 600 MPa pressure had a significantly higher ($p \leq 0.05$) scavenging activity than the non-treated samples. At 1.0 mg/mL, the samples treated at 600 MPa pressure had a significantly higher ($p \leq 0.05$) scavenging activity than both the non-treated samples and those treated with HPP at 200 MPa. No significant effect ($p > 0.05$) of HPP at 200 MPa pressure was found. HPP treatment did not create any significant effect ($p > 0.05$) on the scavenging activity of the films at higher concentration levels (2.0 and 3.0 mg/mL).

A significant interaction effect ($p \leq 0.05$) of solvent type and HPP treatment was only found at a chitosan concentration of 0.5 mg/mL. At this concentration, the samples with citric acid

and HPP treatment had on average a significantly higher ($p \leq 0.05$) scavenging activity than the chitosan samples with acetic acid and HPP treatment.

The chitosan sample with citric acid as the solvent and no treatment had a scavenging activity of 51.53 ± 2.21%, which after HPP treatment at 200 and 600 MPa pressure, significantly increased to 57.53 ± 1.79 and 58.44 ± 1.93%, respectively. No significant ($p > 0.05$) difference between the two pressures were found. At chitosan concentrations of both 1.0 and 2.0 mg/mL, there were no significant effect ($p > 0.05$) of HPP treatment on the citric acid samples. At 1.0 mg/mL, the non-treated sample had a scavenging activity of 55.28 ± 2.23%. After HPP treatment at 200 and 600 MPa pressure, the scavenging activity was 54.47 ± 1.96 and 58.98 ± 1.49%, respectively. At 2.0 mg/mL, the scavenging activity of the non-treated sample was 54.45 ± 2.02%, and after HPP treatment at 200 and 600 MPa pressure, it was 50.92 ± 2.15 and 57.42 ± 2.55%, respectively.

For the citric acid samples with a chitosan concentration of 3.0 mg/mL, a significant decrease ($p \leq 0.05$) in scavenging activity was seen after HPP treatment at 200 MPa. There was a significant increase ($p \leq 0.05$) in the scavenging activity between the samples where the film-forming solution was treated with HPP at 200 MPa and those treated at 600 MPa pressure, however, there were no significant increase ($p > 0.05$) from the non-treated sample to the 600 MPa treated sample. The scavenging activity of the non-treated sample at a chitosan concentration of 3.0 mg/mL was 53.54 ± 1.42%. After HPP treatment at 200 and 600 MPa, the activity was 49.85 ± 1.61 and 55.05 ± 1.16%, respectively.

For chitosan films with acetic acid as the solvent, no significant effect ($p > 0.05$) of HPP treatment at 200 MPa was found at all chitosan concentrations. The non-treated films had a scavenging activity of 56.86 ± 1.05, 56.30 ± 0.61, 57.17 ± 4.41 and 57.80 ± 2.63% at chitosan concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL, respectively, which after HPP treatment at 200 MPa were 55.72 ± 2.70, 54.45 ± 1.39, 56.91 ± 2.07 and 57.95 ± 1.67%, respectively. After HPP treatment at 600 MPa, a significant increase ($p \leq 0.05$) in scavenging activity was seen at chitosan concentration of 1.0 mg/mL. Whereas the non-treated sample had a scavenging activity of 56.30 ± 0.61%, it increased to 55.40 ± 0.67 after HPP treatment at 600 MPa. A significant increase ($p \leq 0.05$) was also seen between the sample treated with HPP at

200 MPa and the one treated with HPP at 600 MPa pressure at this chitosan concentration. At all other chitosan concentrations, no significant effect ($p > 0.05$) was seen. The scavenging activities of chitosan films with acetic acid as the solvent and HPP treatment at 600 MPa pressure at chitosan concentrations of 0.5, 2.0 and 3.0 mg/mL were 54.86 ± 1.41, 57.21 ± 1.99 and 58.56 ± 2.44%, respectively.

No significant ($p > 0.05$) increase or decrease was seen with increasing concentration of chitosan for the acetic acid samples. For the citric acid samples, a significant decrease ($p \leq 0.05$) was seen for the samples with HPP treatment at 200 MPa with increasing chitosan concentration.

4.1.5.3 Chitosan + Seaweed

Figure 31 presents the effect of addition of seaweed in concentrations of 10, 30 and 50% on the scavenging activity of chitosan samples with acetic acid as the solvent in concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL.

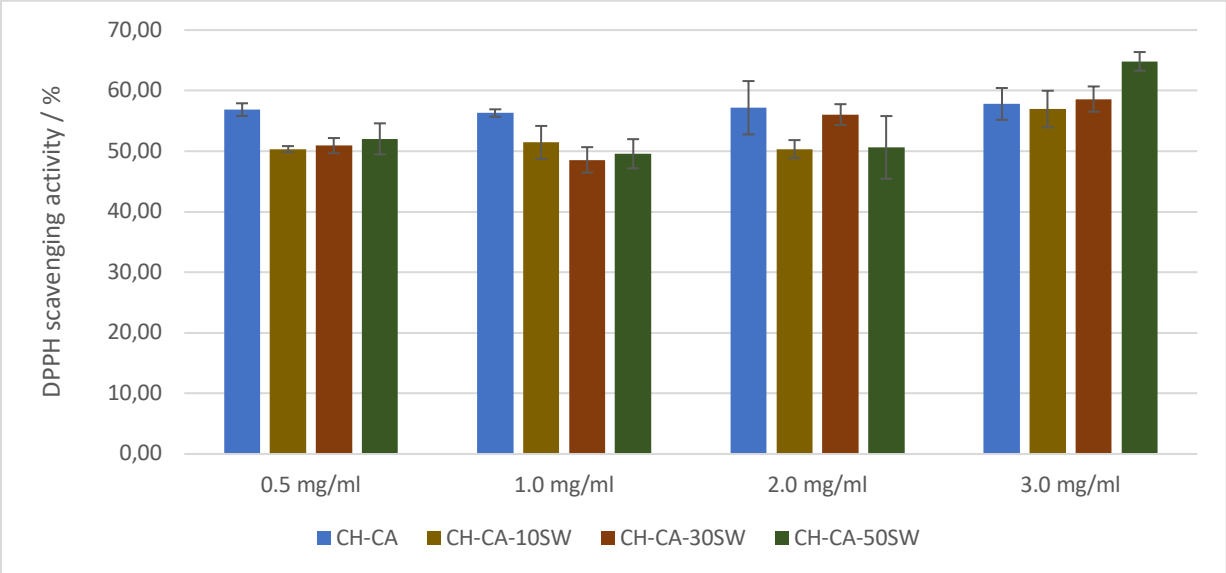

Figure 31. Scavenging activity (%) of films made of chitosan dissolved in acetic acid, with seaweed added in concentrations of 10, 30 and 50% compared to the chitosan concentration. Averages of three measurements with standard deviation as error bars.

Addition of seaweed in all concentrations to the chitosan films had a significant effect ($p \leq 0.05$) on the scavenging activity at some of the chitosan concentrations. At a chitosan

concentration of 0.5 mg/mL, the scavenging activity significantly decreased ($p \leq 0.05$) with addition of seaweed in all concentrations. The sample with no fillers had a scavenging activity of 56.86 ± 1.05%. After seaweed addition of 10, 30 and 50%, the scavenging activity decreased to 50.34 ± 0.50, 50.91 ± 1.25 and 52.04 ± 2.56%, respectively. No significant variance ($p > 0.05$) within the samples with different seaweed concentrations at chitosan concentration of 0.5 mg/mL were found.

The chitosan sample with no filler at a concentration of 1.0 mg/mL, had a scavenging activity of 56.30 ± 0.61%. A significant decrease ($p \leq 0.05$) was seen after addition of seaweed at all concentrations. The scavenging activities of 1.0 mg/mL chitosan after addition of seaweed were 51.44 ± 2.73, 48.57 ± 1.10 and 49.58 ± 2.41% at seaweed concentrations of 10, 30 and 50%, respectively. However, no significant effect ($p > 0.05$) of seaweed addition was seen on the chitosan samples at a concentration of 2.0 mg/mL. The scavenging activity of the sample with no filler was 57.17 ± 4.41%, and after seaweed addition at 10, 30 and 50%, it was 50.34 ± 1.49, 56.04 ± 1.72 and 50.62 ± 5.17%, respectively.

At chitosan concentration of 3.0 mg/mL, only the sample with 50% seaweed showed a significant difference ($p \leq 0.05$) in the scavenging activity. However, unlike previously discovered in this study, this effect is increasing. The chitosan sample with no seaweed had a scavenging activity of 57.80 ± 2.63%, which after seaweed addition of 10 and 30% were 56.96 ± 3.02 and 58.60 ± 2.09%, respectively. Addition of 50% seaweed increased the scavenging activity to 64.83 ± 1.54%.

4.1.5.4 Chitosan + HPP treatment + Seaweed

The effect of both HPP treatment at 200 and 600 MPa pressure and seaweed addition in concentrations of 10, 30 and 50% on the scavenging activity of chitosan samples are presented in Figure 32.

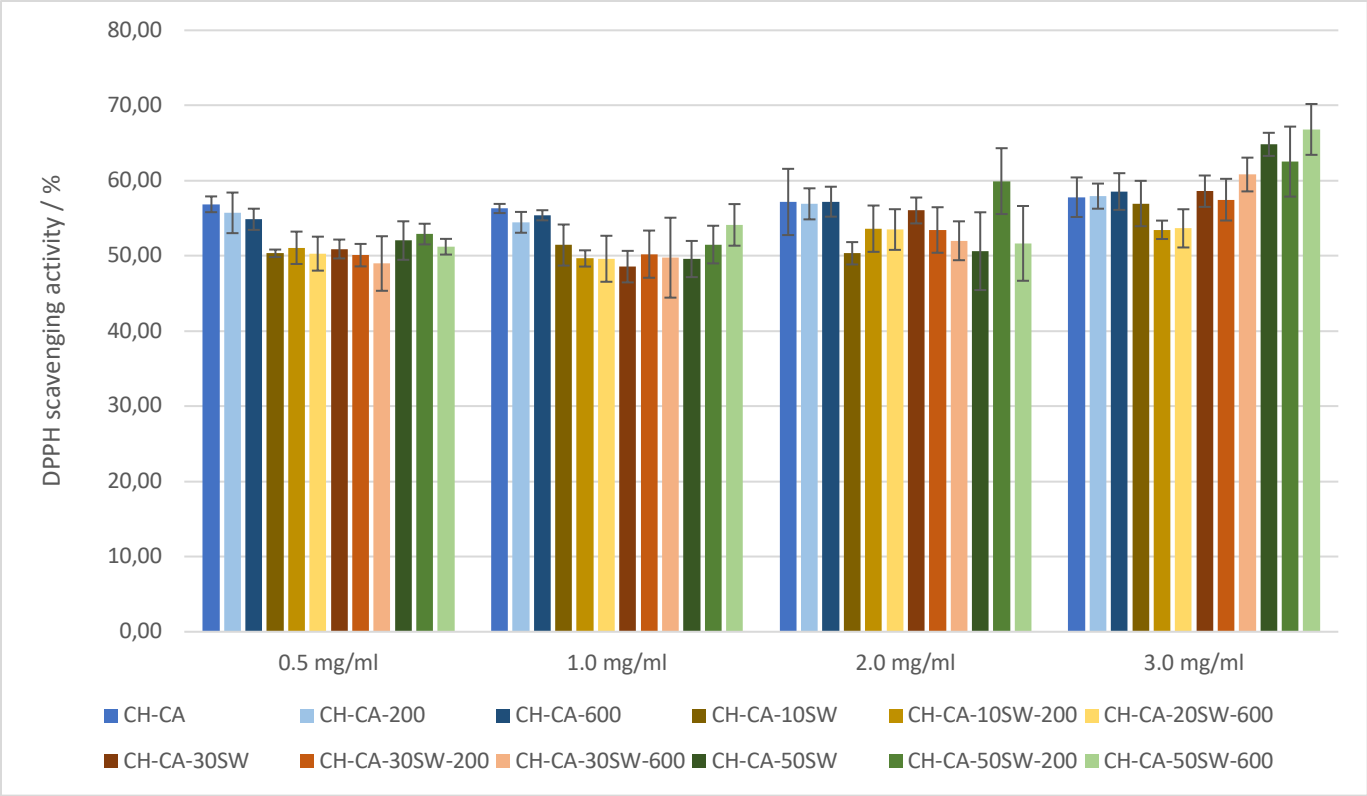

Figure 32. Scavenging activity (%) of films made of chitosan dissolved in acetic acid, HPP treated at 200 and 600 MPa pressure and with added seaweed in concentrations of 10, 30 and 50% compared to the chitosan concentration. Averages of three measurements with standard deviation as error bars.

It has already been presented that HPP treatment had a significant effect ($p \leq 0.05$) on the scavenging activity of the chitosan samples. In addition, addition of seaweed had a significant effect ($p \leq 0.05$) at all concentrations. However, a significant combination effect between HPP treatment and seaweed addition was not found for any of the concentrations tested. Thus, no significant effect ($p > 0.05$) of HPP treatment on the samples with seaweed was found.

The scavenging activity of the sample with 10% seaweed at chitosan concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL were 50.34 ± 0.50, 51.44 ± 2.73, 50.34 ± 1.49 and 56.96 ± 3.02%, respectively. The scavenging activity was not significantly increased or decreased ($p > 0.05$) with HPP treatment. In the samples with no treatment and 10% seaweed addition, a significant increase ($p \leq 0.05$) in scavenging activity was seen between the sample at 0.5 mg/mL chitosan and the one at 3.0 mg/mL. However, no significant difference ($p > 0.05$) was seen with increasing chitosan concentrations in the samples with HPP treatment.

The scavenging activity of the samples with 30% seaweed was 50.91 ± 1.25, 48.47 ± 2.10, 56.04 ± 1.72 and 58.60 ± 2.09% at chitosan concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL, respectively. Although HPP treatment had no significant effect ($p > 0.05$) on the scavenging activity of the samples with seaweed, a significant increase ($p \leq 0.05$) was seen with increasing chitosan concentration from 0.5 mg/mL to 3.0 mg/mL with HPP treatment at pressures of 200 and 600 MPa. Similarly, a significant increase ($p \leq 0.05$) in scavenging activity with increasing chitosan concentrations was seen in the samples with 30% seaweed and no HPP treatment.

Like previously presented, no significant effect ($p > 0.05$) of HPP treatment was found on the scavenging activity of samples with 50% added seaweed. However, a significant increase ($p \leq 0.05$) was seen with increasing chitosan concentrations from 0.5 to 3.0 mg/mL for both the HPP treated and non-treated samples. The non-treated samples with 50% seaweed added had scavenging activities of 52.04 ± 2.56, 49.58 ± 2.41, 50.62 ± 5.17 and 64.83 ± 1.54% at chitosan concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL, respectively.

Overview of all the properties of the chitosan films can be found in appendix A1.

4.2 Alginate

4.2.1 Film characteristics

4.2.1.1 Alginate + HPP treatment

Alginate can be completely dissolved in water and as such, no added solvent was used to aid the dissolution process. The effect of HPP treatment on the weight and thickness of alginate films with no filler addition is presented in Table 8 below.

Table 8. The average weight in grams and thickness in millimeters of all films of alginate dissolved in water, treated with HPP at pressures of 200 and 600 MPa. With standard deviation.

Sample	Weight (g)	Thickness (mm)
Alginate	0.6574 ± 0.0089	0.09 ± 0.01
Alginate 200 MPa	0.6048 ± 0.0266	0.09 ± 0.03
Alginate 600 MPa	0.6157 ± 0.0182	0.09 ± 0.02

The weight of the alginate films with no treatment and no filler was 0.6574 ± 0.0089 g. Since the films were made from drying 30 mL (2% w/v) film-forming solution, it was expected that each film should weigh approximately 0.6 g. HPP treatment significantly decreased ($p \leq 0.05$) the weight of the dried alginate films to 0.6048 ± 0.0266 g for the solution treated at 200 MPa, and for the films from the solution treated at 600 MPa, the weight was 0.6157 ± 0.0182 g. No significant effect ($p > 0.05$) of HPP treatment on the thickness of the films were observed. HPP treatment did not have any effect on the dissolution rate of the films and both treated and non-treated films dissolved in water at room temperature after 15 minutes.

4.2.1.2 Alginte + Seaweed

The effect of 10, 30 and 50% seaweed addition on the weight and thickness of the alginate films are presented in Table 9.

Table 9. The average weight in grams and thickness in millimeters of all films of alginate dissolved in water, with added seaweed in concentrations of 10, 30 and 50%. With standard deviation.

Sample	Weight (g)	Thickness (mm)
Alginate + 10% SW	0.7261 ± 0.0242	0.11 ± 0.01
Alginate + 30% SW	0.7877 ± 0.0197	0.12 ± 0.01
Alginate + 50% SW	0.9406 ± 0.0479	0.12 ± 0.01

The addition of seaweed significantly increased ($p \leq 0.05$) the weight of alginate films. The alginate film with no filler had a weight of 0.6574 ± 0.0089 g. After addition of seaweed in

concentrations of 10, 30 and 50%, the weight increased to 0.7261 ± 0.0242, 0.7877 ± 0.0197 and 0.9406 ± 0.0497 g, respectively. The addition of seaweed also had a significant effect ($p \leq 0.05$) on the thickness of the alginate films. While the alginate film with no seaweed had a thickness of 0.09 ± 0.01 mm, seaweed addition increased the thickness to 0.11 ± 0.01, 0.12 ± 0.01 and 0.12 ± 0.01 mm for concentrations of 10, 30 and 50% seaweed addition, respectively.

4.2.1.3 Alginate + HPP treatment + Seaweed

The thickness and weight of the seaweed containing alginate films in combination with HPP treatment are shown in Table 10.

Table 10. The average weight in grams and thickness in millimeters of all films of alginate dissolved in water with added seaweed in concentrations of 10, 30 and 50%, in addition to HPP treatment at pressures of 200 and 600 MPa. With standard deviation.

Sample	Weight (g)	Thickness (mm)
Alginate + 10% SW 200 MPa	0.6742 ± 0.0123	0.10 ± 0.01
Alginate + 10% SW 600 MPa	0.6692 ± 0.0186	0.10 ± 0.01
Alginate + 30% SW 200 MPa	0.7742 ± 0.0185	0.11 ± 0.01
Alginate + 30% SW 600 MPa	0.7450 ± 0.0165	0.11 ± 0.01
Alginate + 50% SW 200 MPa	0.8569 ± 0.0337	0.12 ± 0.01
Alginate + 50% SW 600 MPa	0.8438 ± 0.0219	0.11 ± 0.01

Treatment with HPP on the film forming solution had a significant effect ($p \leq 0.05$) on the weight of the alginate films. For the films with 10% seaweed in combination with HPP treatment at 200 MPa pressure, the weight was 0.6742 ± 0.0123 g, whereas for HPP treatment at a pressure of 600 MPa, the weight was reduced to 0.6692 ± 0.0186 g. A similar trend was also observed for 30% and 50% seaweed addition. For 30% seaweed containing films, the weight was 0.7742 ± 0.0185 g and 0.7450 ± 0.0165 g for HPP treatment at

200 MPa and 600 MPa pressure, respectively. Therefore, the combination of seaweed addition and HPP treatment had a significant interacting effect ($p \leq 0.05$) on the weight.

On the contrary, only seaweed addition and HPP treatment separately have a significant effect ($p \leq 0.05$) on the thickness of the films. No significant interaction effect ($p > 0.05$) was seen. Nevertheless, the alginate films with 10% seaweed were 0.10 ± 0.01 mm for HPP treatment at both conditions. The films with 30% seaweed also had the same thickness at both HPP treatments, which was 0.11 ± 0.01 mm. For alginate films with 50% seaweed, the thickness for those treated with a pressure of 200 MPa was 0.12 ± 0.01 mm, whereas for those treated with a pressure of 600 MPa, the thickness reduced to 0.11 ± 0.01 mm.

4.2.2 Mechanical properties

4.2.2.1 Alginate + HPP treatment

The code names used throughout this study along with corresponding solvent, HPP treatment and concentration of seaweed are given in Table 11.

Table 11. Code names for the different alginate samples at different seaweed concentrations and HPP treatment.

Sample code	Solvent	HPP treatment (MPa)	Seaweed addition (%)
A	Water	0	0
A-200	Water	200	0
A-600	Water	600	0
A-10SW	Water	0	10
A-10SW-200	Water	200	10
A-10SW-600	Water	600	10
A-30SW	Water	0	30
A-30SW-200	Water	200	30
A-30SW-600	Water	600	30
A-50SW	Water	0	50
A-50SW-200	Water	200	50
A-50SW-600	Water	600	50

The tensile strength of the alginate films which are created from film-forming solution treated with HPP at pressures of 200 and 600 MPa are presented in Figure 33.

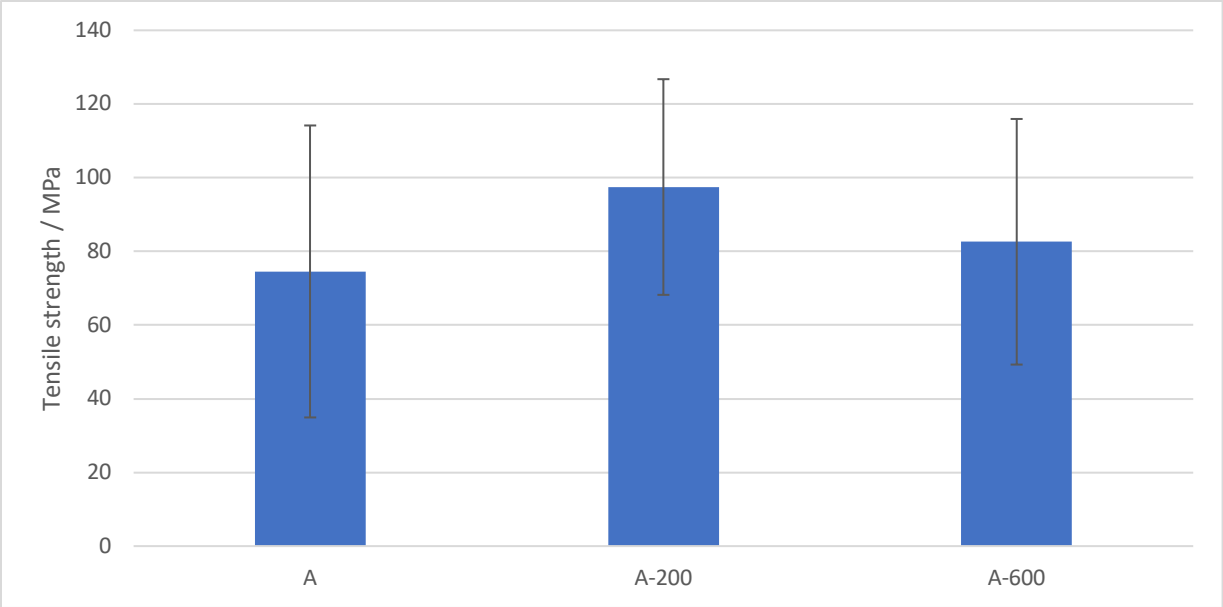

Figure 33. Tensile strength (MPa) of films made of alginate dissolved in water, treated with HPP at pressures of 200 and 600 MPa. Averages of all films measured with standard deviation as error bars.

Alginate on its own had a tensile strength of 74.55 ± 39.63 MPa. After HPP treatment at 200 MPa and 600 MPa pressures of the film-forming solution, the tensile strength changed to 97.47 ± 29.27 and 82.61 ± 33.33 MPa, respectively. Thus, a significant effect ($p \leq 0.05$) was seen for the samples which had HPP treatment at 200 MPa, whereas no such significant effect was seen at pressure of 600 MPa.

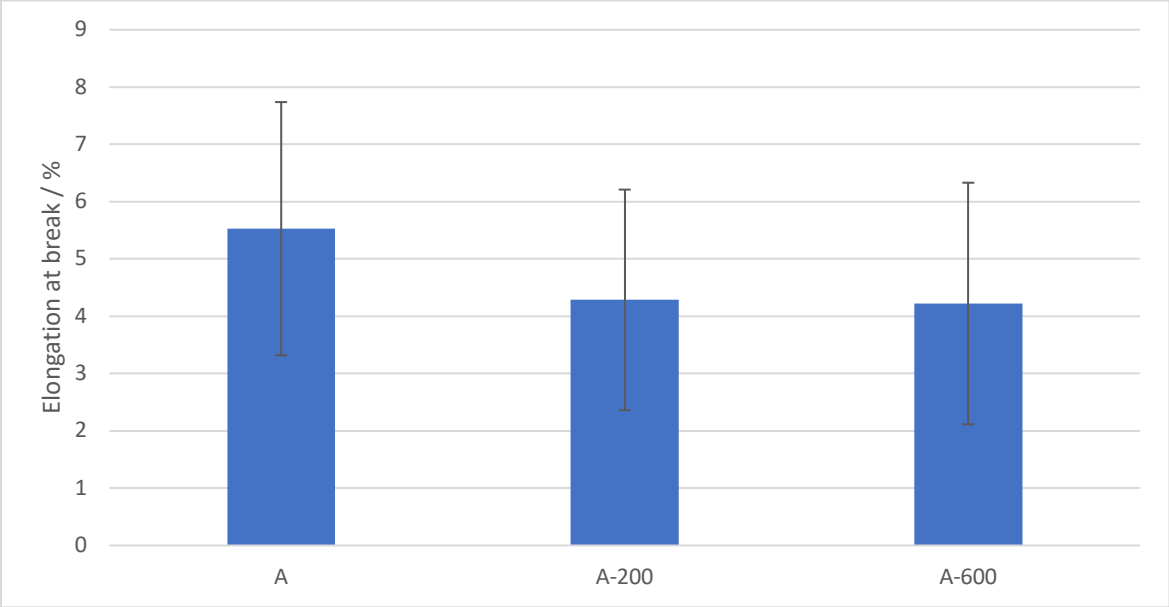

Figure 34. Elongation at break (%) of films made of alginate dissolved in water, treated with HPP at pressures of 200 and 600 MPa. Averages of all films measured with standard deviation as error bars.

For elongation at break (Figure 34), HPP treatment at both 200 MPa and 600 MPa pressure had a significant effect ($p \leq 0.05$) on the alginate films. While the alginate films with no treatment had an elongation at break of 5.52 ± 2.21%, after HPP treatment at 200 MPa and 600 MPa pressures, the elongation at beak reduced to 4.29 ± 1.92 and 4.22 ± 2.11%, respectively.

4.2.2.2 Alginate + Seaweed

The effect of seaweed addition on the tensile strength and elongation at break of the alginate films are presented in Figure 35 and 36 below.

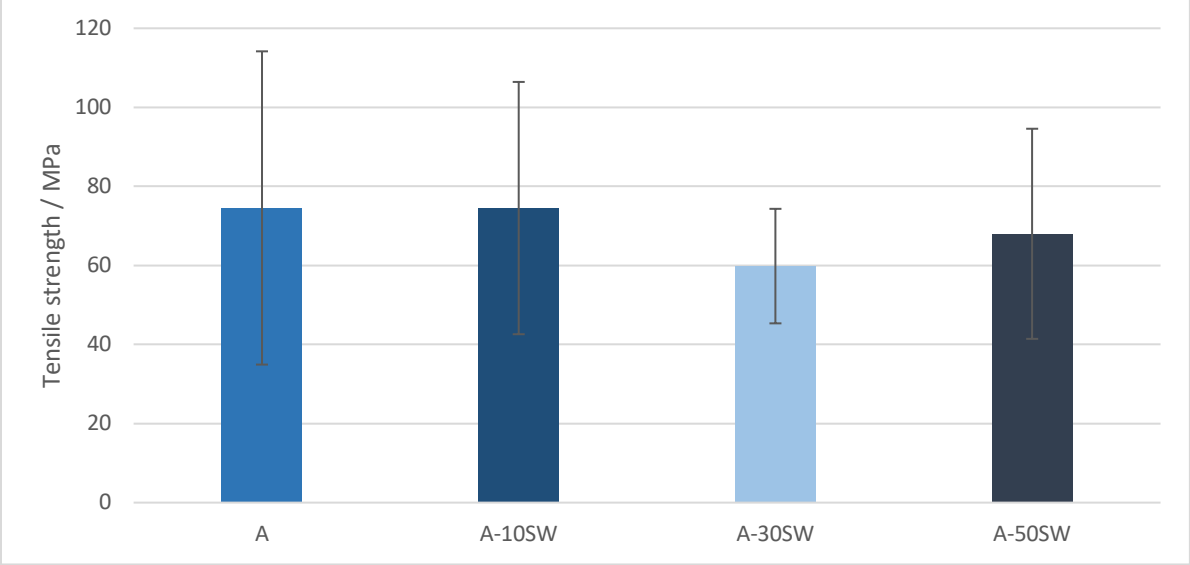

Figure 35. Tensile strength (MPa) of films made of alginate dissolved in water, with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. Averages of all films measured with standard deviation as error bars.

Addition of seaweed to the alginate films had no significant effect ($p > 0.05$) on the tensile strength of the alginate films at all seaweed concentrations. It has already been presented that alginate with no seaweed had a tensile strength of 74.55 ± 39.36 MPa. The tensile strength of the alginate films containing 10, 30 and 50% of seaweed was 74.52 ± 31.92, 59.84 ± 14.49 and 68.01 ± 26.84 MPa, respectively.

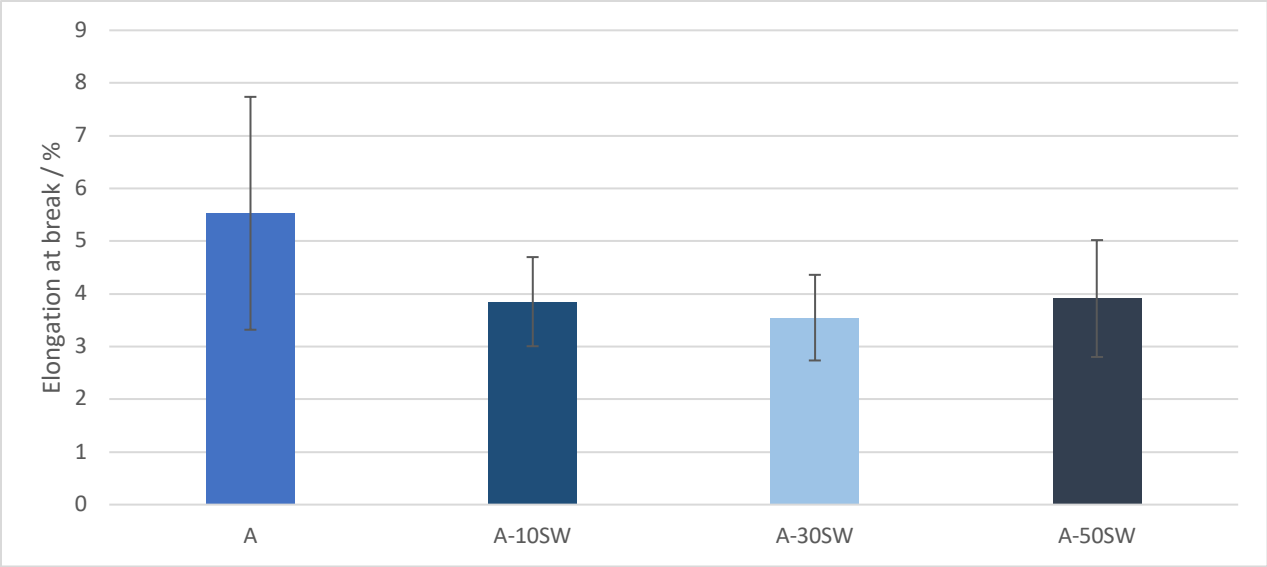

Figure 36. Elongation at break (%) of films made of alginate dissolved in water, with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. Averages of all films measured with standard deviation as error bars.

However, addition of seaweed had a significant effect ($p \leq 0.05$) on the elongation at break for the alginate films. Whereas the elongation at break was 5.53 ± 2.21% for the alginate films with no seaweed, it decreased to 3.85 ± 0.85% after addition of 10% seaweed. The elongation at break for the films with 30 and 50% seaweed was similar at 3.55 ± 0.82 and 3.91 ± 1.11%, respectively.

4.2.2.3 Alginate + HPP treatment + Seaweed

Figure 37 and 28 presents the effect of combination of HPP treatment and seaweed addition on the tensile strength and elongation at break of alginate films.

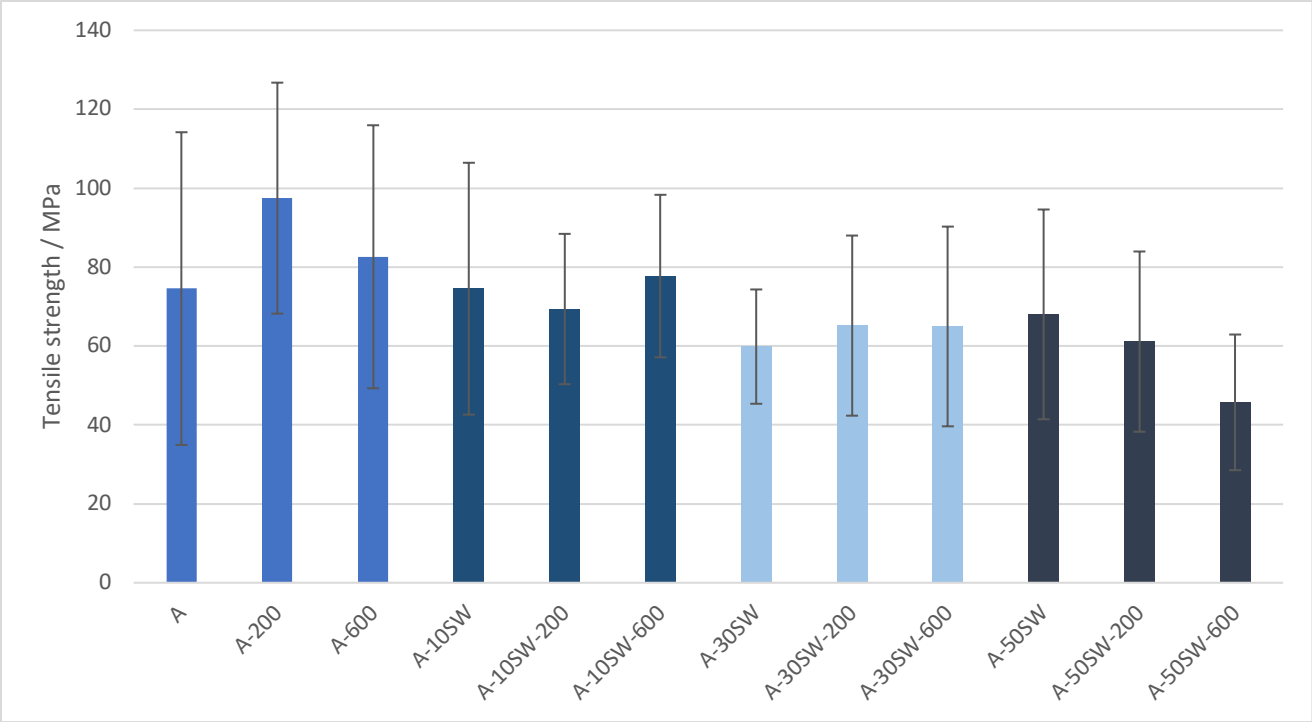

Figure 37. Tensile strength (MPa) of films made of alginate dissolved in water, with seaweed added as a filler in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. In addition, samples with HPP treatment at pressures of 200 MPa and 600 MPa. Averages of all films measured with standard deviation as error bars.

The combination of HPP treatment and seaweed addition on the alginate films showed an overall significant interaction effect ($p \leq 0.05$). However, between the non-treated films with seaweed and those with seaweed and treatment, a significant effect ($p \leq 0.05$) of HPP was only seen between A-50SW and A-50SW-600. The large error bars as a result of differences between each film could explain why this effect is not as clear in the figure.

For the films with 10% seaweed, the tensile strength was 74.52 ± 31.92 MPa. After HPP treatment at 200 and 600 MPa pressure, the tensile strength was 69.37 ± 19.05 and 77.73 ± 20.59 MPA, respectively. The films with 30% seaweed and no treatment had a tensile strength of 59.84 ± 14.49 MPa, which was 65.16 ± 22.83 and 64.95 ± 25.31 MPa after the film-forming solution was treated with HPP at pressures of 200MPa and 600 MPa, respectively. For films with 50% seaweed, the initial tensile strength was 68.01 ± 26.58 MPa. After HPP treatment at pressures of 200 MPa and 600 MPa, the tensile strength of the 50% seaweed films were 61.11 ± 22.84 and 45.72 ± 17.19 MPa, respectively.

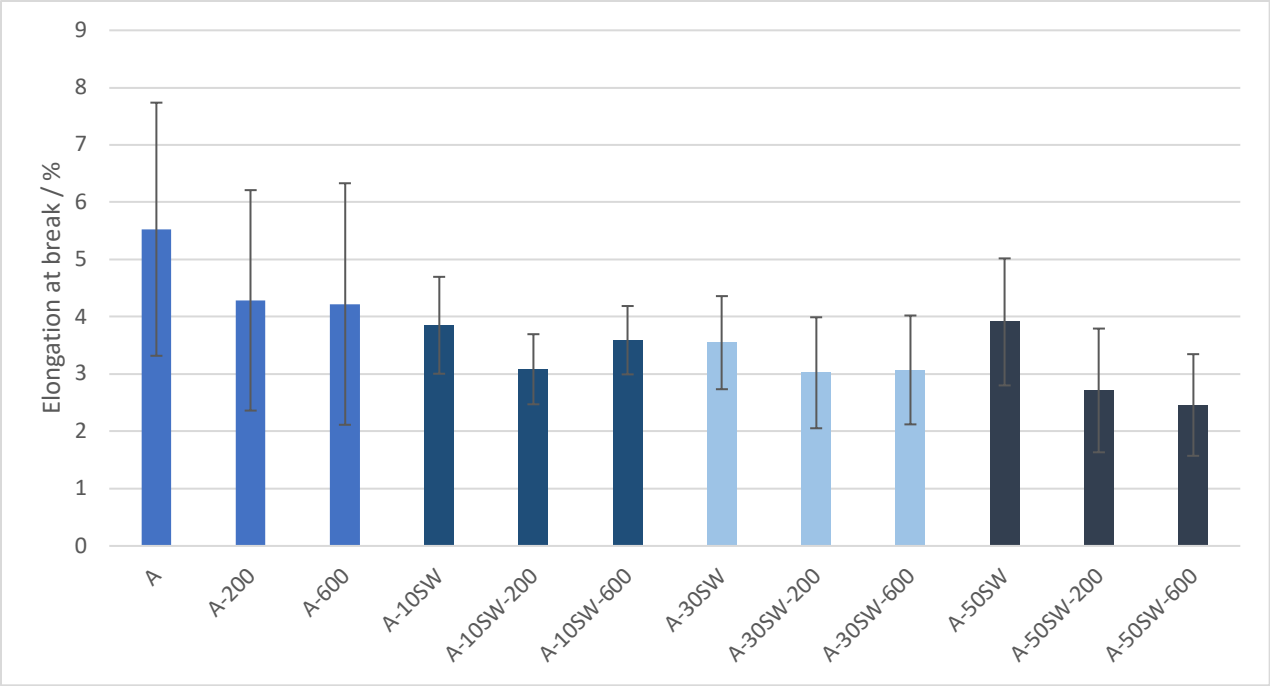

Figure 38. Elongation at break (%)) of films made of alginate dissolved in water, with seaweed added as a filler in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. In addition, samples with HPP treatment at pressures of 200 MPa and 600 MPa. Averages of all films measured with standard deviation as error bars.

It has previously been presented that addition of seaweed caused the elongation at break of the alginate films to decrease. HPP treatment also showed a significant effect on the films. However, there was no significant ($p > 0.05$) combined effect the two variables. The films with 10, 30 and 50% added seaweed had elongation at breaks of 3.85 ± 0.85, 3.56 ± 0.81 and 3.91 ± 1.11%, respectively.

4.2.3 Barrier properties

4.2.3.1 Alginate + HPP treatment

The water vapor transmission rate of the alginate films is presented below in Figure 39.

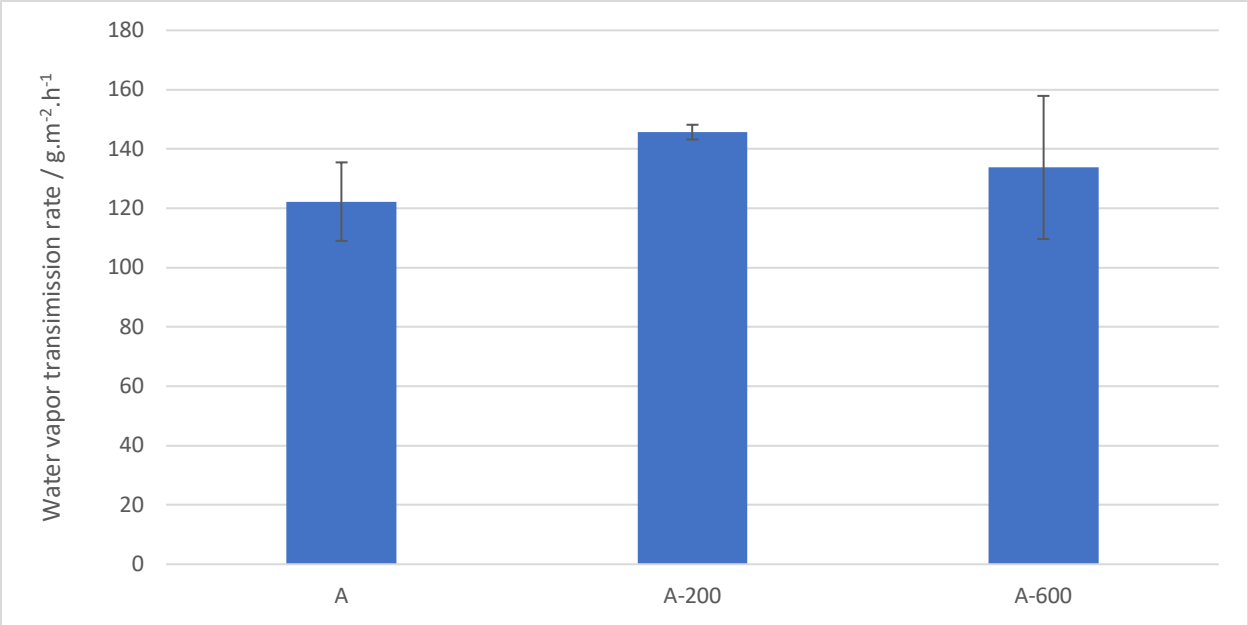

Figure 39. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of alginate dissolved in water, HPP treated at pressures of 200 and 600 MPa. Averages of two measurements with standard deviation as error bars.

No significant effect ($p > 0.05$) of HPP treatment on the WVTR of alginate films were found. The non-treated alginate film had a WVTR of 122.24 ± 13.24 $g.m^{-2}.h^{-1}$, which did not significantly change after HPP treatment at 200 and 600 MPa pressures.

4.2.3.2 Alginate + Seaweed

The effect of seaweed addition in different concentrations on the water vapor transmission rate of alginate films were also evaluated. The results are presented in Figure 40.

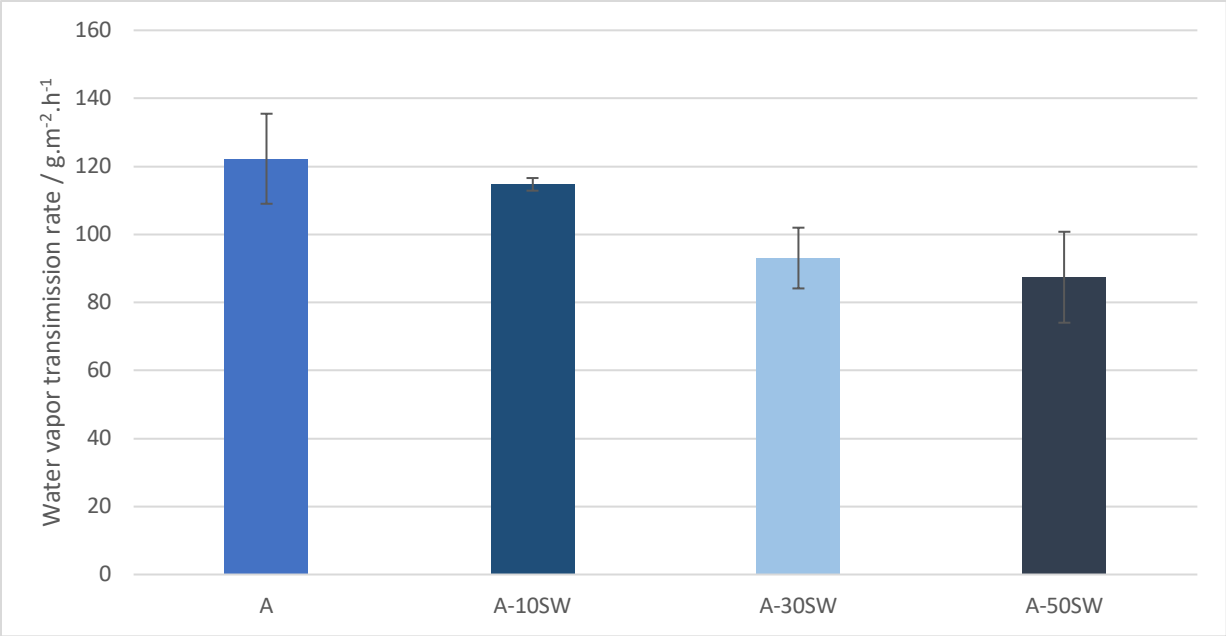

Figure 40. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of alginate dissolved in water with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. Averages of two measurements with standard deviation as error bars.

Addition of 30 and 50% seaweed decreased the WVTR of the alginate films. The WVTR of the alginate film with no fillers were 122.24 ± 13.24 $g.m^{-2}.h^{-1}$, which after addition of 10% was 114.71 ± 1.86%. After addition of 30 and 50% seaweed, the WVTR decreased to 93.04 ± 8.93 and 87.40 ± 13.37 $g.m^{-2}.h^{-1}$, respectively.

4.2.3.3. Alginate + HPP treatment + Seaweed

The effect of HPP treatment in combination with seaweed addition on the WVTR of alginate films are presented below in Figure 41.

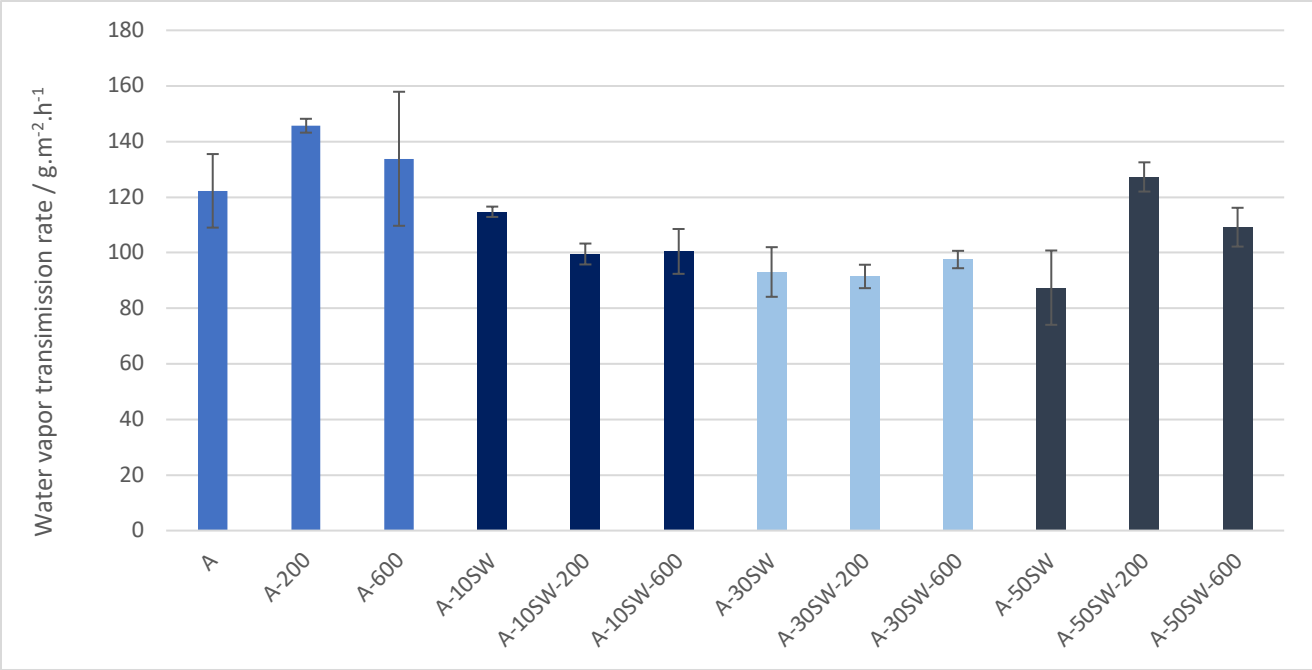

Figure 41. Water vapor transmission rate ($g.m^{-2}.h^{-1}$) of films made of alginate dissolved in water with added seaweed in concentrations of 10, 30 and 50% in comparison to the chitosan concentration. In addition, HPP treatment at pressures of 200 and 600 MPa. Averages of two measurements with standard deviation as error bars.

It has already been mentioned that HPP treatment had no significant effect ($p > 0.05$) on the WVTR of the alginate films, whereas seaweed addition did. HPP treatment on the alginate films with seaweed had varying results. At 10% seaweed, the WVTR decreased with HPP treatment, whereas at 50% seaweed, the WVTR increased. For the alginate films with 30% seaweed, no effect of HPP treatment was observed.

4.2.4 DPPH assay

4.2.4.1 Alginate + HPP treatment

The scavenging activity of the alginate samples in concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL alginate with HPP treatment at 200 and 600 MPa pressures are presented in Figure 42 below.

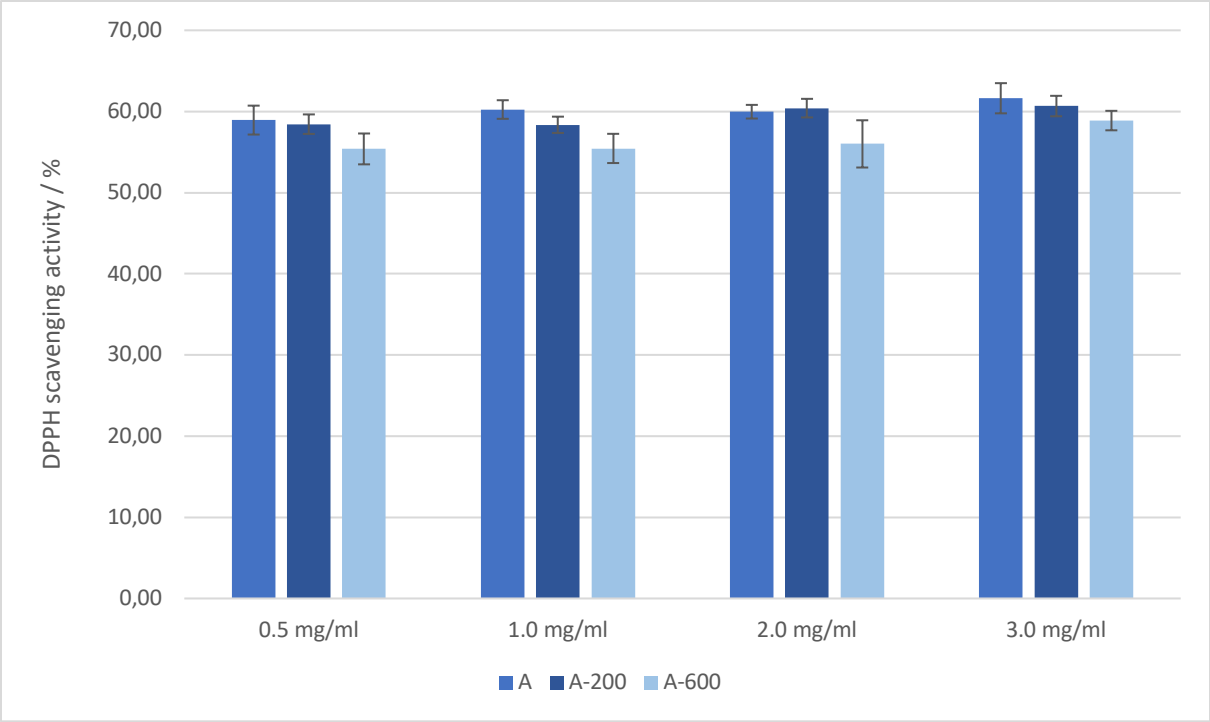

Figure 42. Scavenging activity (%) of films made of alginate dissolved in water, HPP treated at pressures of 200 and 600 MPa. Averages of three measurements with standard deviation as error bars.

No significant ($p > 0.05$) difference on the scavenging activity was found between the non-treated sample and those treated with HPP at 200 MPa pressure for any of the concentrations. However, HPP treatment at 600 MPa had a significant effect ($p \leq 0.05$) on the scavenging activity of the samples with an alginate concentration of 1.0 mg/mL. Whereas the non-treated alginate sample at 1.0 mg/mL had a scavenging activity of 60.24 ± 1.15%, it decreased to 55.45 ± 1.80% after HPP treatment at 600 MPa pressure.

At alginate concentrations of 0.5, 2.0 and 3.0 mg/mL, the scavenging activity was 58.94 ± 1.78, 59.97 ± 0.84 and 61.63 ± 1.86%, respectively. HPP treatment had no significant effect ($p > 0.05$) on the scavenging activity at these alginate concentrations. No significant ($p > 0.05$) increase or decrease in scavenging activity was seen for the non-treated samples with increasing alginate concentration. The same observation was seen for the samples with HPP treatment at 200 and 600 MPa pressures.

4.2.4.2 Alginate + Seaweed

Figure 43 presents the effect of seaweed addition in concentrations of 10, 30 and 50% on the scavenging activity of alginate samples in concentrations of 0.5, 1.0, 2.0 and 3.0 mg/mL.

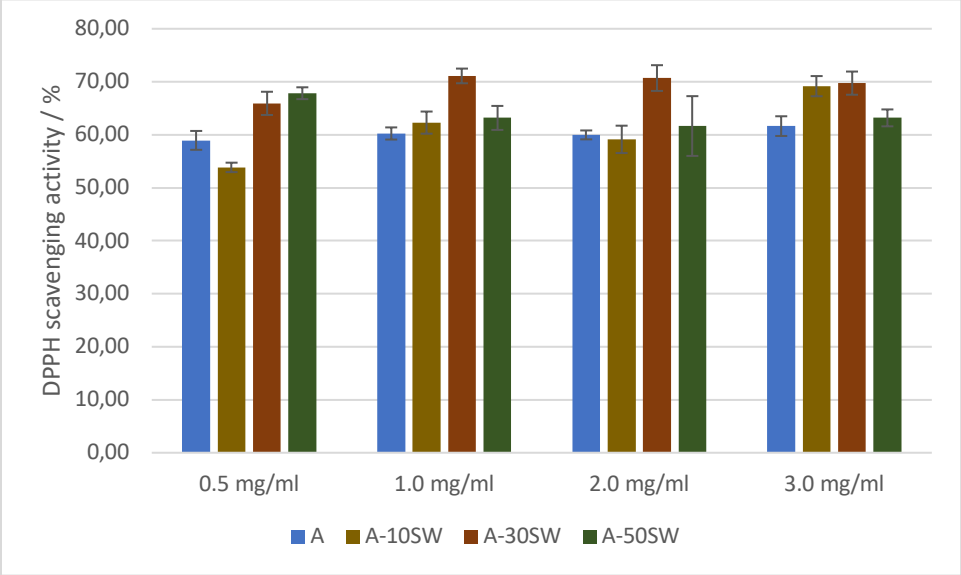

Figure 43. Scavenging activity (%) of films made of alginate dissolved in water, with seaweed added in concentrations of 10, 30 and 50% compared to the alginate concentration in the film. Averages of three measurements with standard deviation as error bars.

The effect of seaweed addition on the scavenging activity of the alginate samples varied across seaweed and alginate concentrations. At an alginate concentration of 0.5 mg/mL, the scavenging activity of the sample with no seaweed was 58.94 ± 1.78%. After addition of 10% seaweed, it significantly decreased ($p \leq 0.05$) to 53.84 ± 0.89%, and with 30% and 50% seaweed it significantly increased ($p \leq 0.05$) to 65.93 ± 2.19 and 67.84 ± 1.12%, respectively. No significant difference ($p > 0.05$) between the samples with 30% seaweed and those with 50% seaweed was observed.

The scavenging activity of the alginate sample at 1.0 mg/mL was 60.24 ± 1.15%, which after 30% seaweed addition significantly increased ($p \leq 0.05$) to 71.10 ± 1.38%. Addition of 10 and 50% seaweed had no significant effect ($p > 0.05$) on the scavenging activity. The same observation was seen at alginate concentration of 2.0 mg/mL. Whereas the sample with no filler had a scavenging activity of 59.97 ± 0.84%, addition of 30% seaweed significantly increased ($p \leq 0.05$) the activity to 70.70 ± 2.42%. Addition of 10 and 50% seaweed had no significant effect ($p > 0.05$) on the scavenging activity.

At an alginate concentration of 3.0 mg/mL, addition of 10 and 30% seaweed had a significant effect ($p \leq 0.05$) on the scavenging activity. Whereas the sample with no filler had a scavenging activity of 61.63 ± 1.86%, it increased to 69.17 ± 1.91 and 69.74 ± 2.19% after addition of 10 and 30% seaweed, respectively. Addition of 50% seaweed did not have a significant effect ($p > 0.05$) on the scavenging activity of the alginate films.

In the samples with 10% seaweed, higher scavenging activities were seen for alginate concentrations of 1.0, 2.0 and 3.0 mg/mL compared to that of 0.5 mg/mL. However, there were no significant ($p > 0.05$) difference in the scavenging activity between the samples at 1.0 and 2.0 mg/mL, so a linear increasing trend in scavenging activity with increasing alginate concentration was not observed. For the samples with 30% seaweed, there was a significant increase ($p \leq 0.05$) in scavenging activity between the samples at 0.5 and 1.0 mg/mL, but not at the higher concentrations. Both the samples with 50% seaweed at 1.0 and 3.0 mg/mL alginate were significantly lower ($p \leq 0.05$) than the ones at alginate concentration of 0.5 mg/mL. However, no significant increase ($p > 0.05$) was observed at alginate concentration of 2.0 mg/mL.

4.2.4.3 Alginate + HPP treatment + Seaweed

The effect of both HPP treatment at 200 and 600 MPa pressure and seaweed addition in concentrations of 10, 30 and 50% on the scavenging activity of alginate samples are presented in Figure 44.

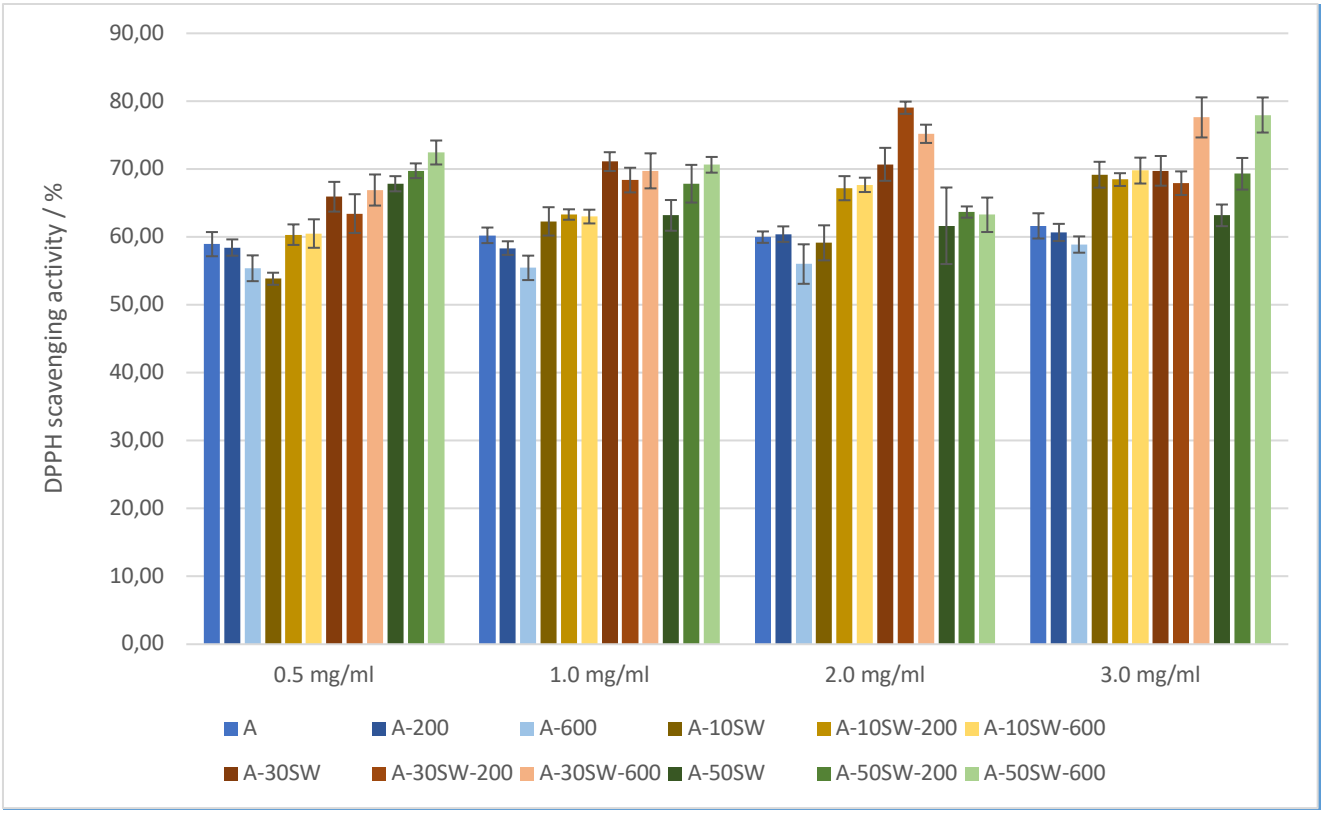

Figure 44. Scavenging activity (%) of films made of alginate dissolved in water, HPP treated at pressures 200 and 600 MPa and with added seaweed in concentrations of 10, 30 and 50% compared to the alginate concentration. Averages of three measurements with standard deviation as error bars.

Like previously described, there was some significant effect ($p \leq 0.05$) of HPP treatment and seaweed addition on the scavenging activity of the alginate samples. In addition, there were some significant ($p \leq 0.05$) interaction effect of seaweed and HPP treatment combined. The samples with 10% seaweed and 0.5 mg/mL alginate had an initial scavenging activity of 53.84 ± 0.89%, which significantly increased ($p \leq 0.05$) to 60.34 ± 1.51 and 60.50 ± 2.10% after HPP treatment at 200 and 600 MPa pressures, respectively. At 1.0 mg/mL alginate, no significant effect ($p > 0.05$) on the scavenging activity after HPP treatment was seen. The same observation was seen for an alginate concentration of 3.0 mg/mL. The scavenging activity of the non-treated sample with 10% seaweed was 62.29 ± 2.09% at a alginate concentration of 1.0 mg/ml, whereas at 3.0 mg/mL alginate it was 69.17 ± 1.91%. At alginate concentration of 2.0 mg/mL, a significant increase ($p \leq 0.05$) was seen from the non-treated sample at 59.13 ± 2.58%, to those treated with HPP at 200 and 600 MPa pressures, where the scavenging activity was 67.18 ± 1.78 and 67.68 ± 1.06%, respectively.

For the samples with 10% seaweed added, the scavenging activity significantly increased ($p \leq 0.05$) with increasing alginate concentration of 0.5 mg/mL to 3.0 mg/mL in the non-treated samples. However, no increase was seen between 1.0 and 2.0 mg/mL alginate. A significant increase ($p \leq 0.05$) in scavenging activity was seen with increasing alginate concentration in the HPP treated samples with 10% seaweed added.

No significant effect ($p > 0.05$) of HPP treatment on the alginate samples at concentrations of 0.5 and 1.0 mg/mL with 30% seaweed added was observed. The non-treated samples had scavenging activities of 65.93 ± 2.19 and 71.10 ± 1.39% for the alginate concentrations of 0.5 and 1.0 mg/mL, respectively. However, a significant increase ($p \leq 0.05$) was seen at alginate concentration of 2.0 mg/mL. Whereas the non-treated sample had a scavenging activity of 70.70 ± 2.43%, after HPP treatment at 200 and 600 MPa pressures it increased to 79.04 ± 0.90 and 75.20 ± 1.34%, respectively. Nevertheless, HPP treatment at 200 MPa caused a significantly higher ($p \leq 0.05$) scavenging activity than did treatment at 600 MPa pressure. At alginate concentration of 3.0 mg/mL, the non-treated sample with 30% seaweed had a scavenging activity of 69.74 ± 2.19%. No significant effect ($p > 0.05$) was seen after HPP treatment at 200 MPa pressure, where the scavenging activity was 67.92 ± 1.74%. However, with HPP treatment at 600 MPa, a significant increase ($p \leq 0.05$) to 77.61 ± 2.95% was observed.

No linear increase in scavenging activity was observed in the alginate samples with 30% seaweed. Only the samples with 30% seaweed at 1.0 mg/mL alginate concentration was significantly higher ($p \leq 0.05$) than at 0.5 mg/mL for the non-treated samples. In the samples with HPP treatment at 200 MPa pressure, a significant increase ($p \leq 0.05$) in scavenging activity was seen from 0.5 mg/mL alginate to 2.0 mg/mL. However, no significant increase ($p > 0.05$) was seen on the other alginate concentrations. The samples with HPP treatment at 600 MPa pressure showed a significant increase ($p \leq 0.05$) in scavenging activity with increased alginate concentration.

There was no significant effect ($p > 0.05$) of HPP treatment at 200 MPa pressure on the scavenging activity of the 0.5 mg/mL alginate samples with 50% seaweed added. The scavenging activity of the non-treated sample was 67.84 ± 1.12%, and after HPP treatment

at 200 MPa pressure it was 69.76 ± 1.08%. However, a significant increased ($p \leq 0.05$) to a scavenging activity of 72.44 ± 1.77% was seen after HPP treatment at 600 MPa pressue. The same trend was seen at alginate concentration of 1.0 mg/mL. The non-treated sample with 50% seaweed had a scavenging activity of 63.84 ± 2.26%. After HPP treatment at 200 MPa pressure it was 67.84 ± 2.78%, whereas at 600 MPa pressure it significantly increased ($p \leq 0.05$) to 70.63 ± 1.16%. No significant effect ($p > 0.05$) effect of HPP treatment on the scavenging activity of 2.0 mg/mL alginate with 50% added seaweed was seen. The non-treated sample had a scavenging activity of 61.65 ± 5.64%. Finally, at alginate concentration of 3.0 mg/mL, HPP treatment at both pressures had a significant increasing ($p \leq 0.05$) effect on the scavenging activity of the samples with 50% seaweed. Whereas the non-treated sample had a scavenging activity of 63.19 ± 1.60%, after HPP treatment at 200 and 600 MPa pressure, it significantly increased ($p \leq 0.05$) to 69.31 ± 2.33 and 77.97 ± 2.58%, respectively. The scavenging activity of the sample after HPP treatment at 600 MPa were significantly higher ($p \leq 0.05$) than after HPP treatment at 200 MPa pressure.

A significant decrease ($p \leq 0.05$) in scavenging activity of the non-treated alginate samples with 50% seaweed added was observed with an increase in alginate concentration. For the samples with HPP treatment at 200 MPa, a significant decrease ($p \leq 0.05$) was found from 0.5 mg/mL alginate to 2.0 mg/mL. However, at the other alginate concentrations, there were no significant increase or decrease ($p > 0.05$) with increased concentration. Finally, the scavenging activity of the alginate samples with 50% seaweed added and HPP treatment at 600 MPa pressure showed a significant decrease ($p \leq 0.05$) from 0.5 mg/mL alginate to 2.0 mg/mL alginate. However, at alginate concentration of 3.0 mg/mL, an increase in scavenging activity compared to the lowest concentration (0.5 mg/mL) of alginate was found.

Overview of all the properties of the alginate films can be found in appendix A1.

4.3 Antimicrobial properties

4.3.1 Disc diffusion assay – Chitosan

Using the disc diffusion assay, inhibition of bacterial growth was not visually detected for any of the conditions tested (chitosan films in acetic or citric acid). Although underneath the films there was limited bacterial proliferation, as compared to the rest of the plate, this could be attributed to the anaerobic conditions and the absence of head-space for colony formation. Moreover, practical limitations were faced when following this method, e.g. the films curled upon contact with the slightly wet media in the Petri dishes (Figure 45). Attempts were made to gently push the film down onto the agar using sterile swabs, but irregularities in the film surface and thus, the attachment to the agar medium were also observed during storage. Therefore, a new liquid-based assay was set up to determine the antimicrobial properties of the developed films.

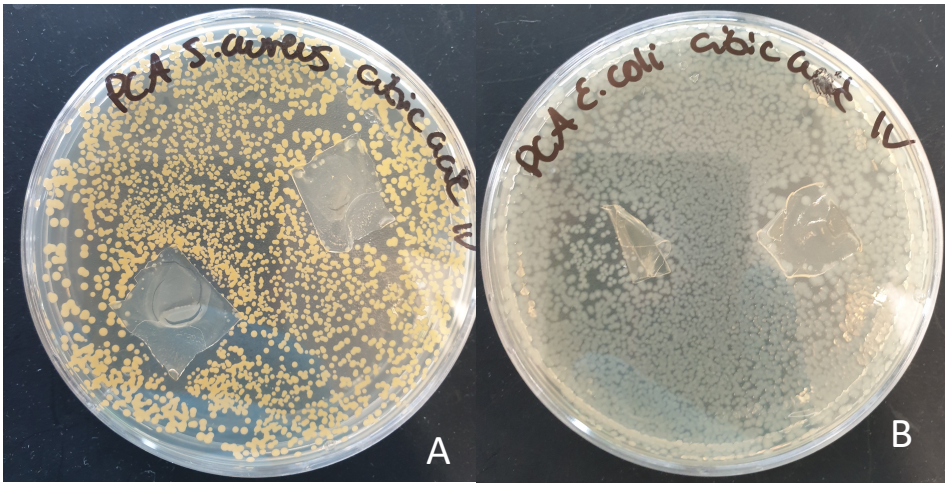

Figure 45. Example of the disc diffusion assay for the chitosan films prepared with citric acid; A: *S. aureus*, B: *E. coli*.

4.3.2 Liquid assay 37 °C – Chitosan and Seaweed

Figures 46 and 47 show the concentration (average and standard deviation) of *E. coli* and *S. aureus*, respectively, in the chitosan samples (film prepared with either citric or acetic acid and dissolved in water at pH 5.2 ± 0.1) and seaweed samples (alginate film with 50% seaweed and TSB with 10 mg/mL seaweed) after incubation at 37 °C. This temperature was selected as optimal for microbial growth, while representing temperature-abuse conditions. The average initial log concentration for all the conditions tested was 6.71 ± 0.10 for *E. coli* and 6.90 ± 0.13 for *S. aureus*. After 24 h incubation at 37 °C, the log concentration in the control samples (TSB), calculated as an average of the levels achieved in all the different trials, was 9.06 ± 0.09 and 8.60 ± 0.29, respectively, for *E. coli* and *S. aureus*.

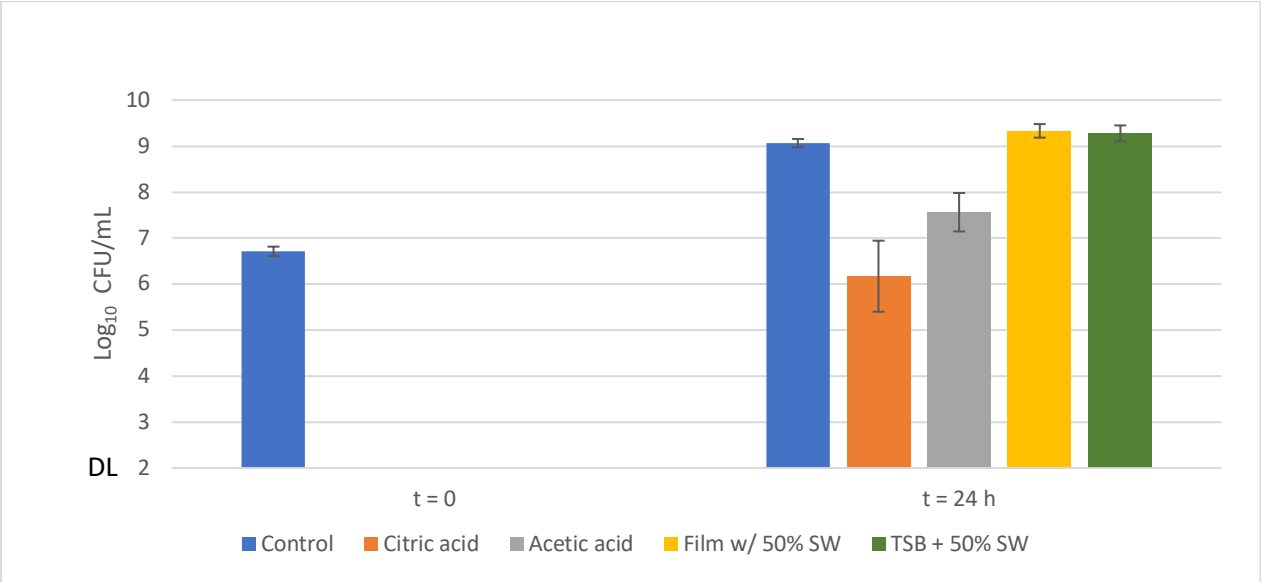

Figure 46. Concentration (CFU/mL) of *E. coli* at t = 0 (initial concentration for all the assayed conditions) and after 24 h incubation at 37 °C (blue: control, orange: dissolved chitosan-citric acid film, grey: dissolved chitosan-acetic acid film, yellow: dissolved alginate film with 50% seaweed, green: TSB with 10 mg/mL seaweed corresponding to the proportion of seaweed:alginate in the films). DL: detection limit.

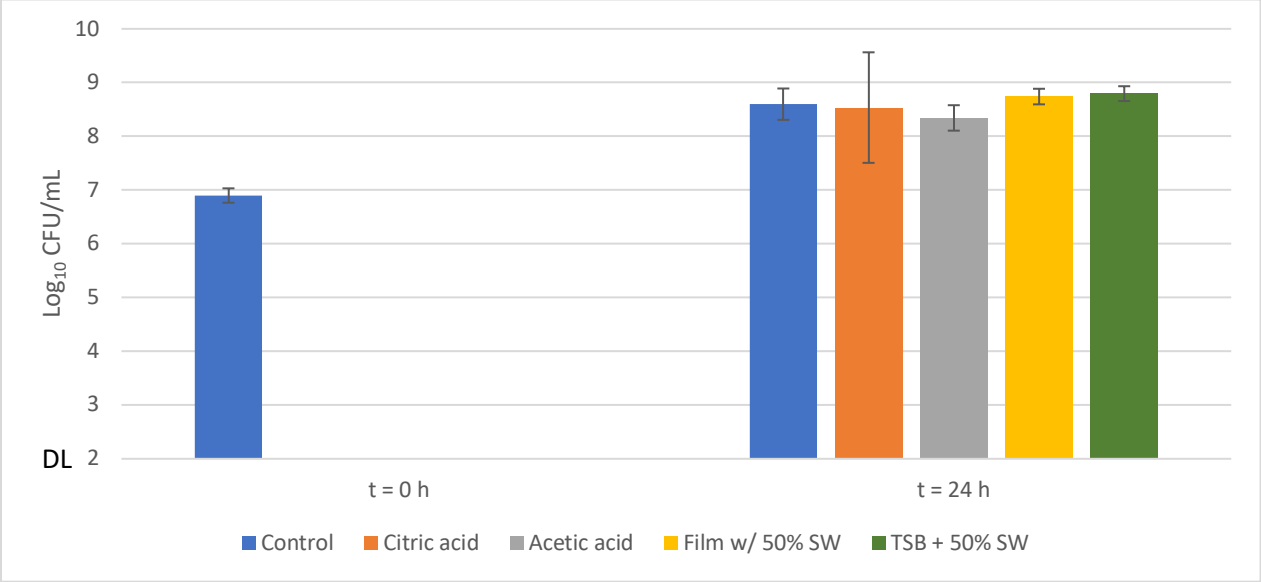

Figure 47. Concentration (CFU/mL) of *S. aureus* at t = 0 (initial concentration for all the assayed conditions) and after 24 h incubation at 37 °C (blue: control, orange: dissolved chitosan-citric acid film, grey: dissolved chitosan-acetic acid film, yellow: dissolved alginate film with 50% seaweed, green: TSB with 10 mg/mL seaweed corresponding to the proportion of seaweed:alginate in the films). DL: detection limit

In the samples prepared from chitosan films containing citric acid (orange bar in Figures 46 and 47), the log concentrations for *E. coli* and *S. aureus* after 24 h incubation at 37 °C were 6.17 ± 0.77 and 8.54 ± 1.03, respectively. Thus, no significant differences ($p > 0.05$) in the levels of *S. aureus* were observed after 24 h incubation at 37 °C, as compared to the respective concentration of this bacterium in the control samples. However, for *E. coli*, a significantly lower concentration ($p \leq 0.05$) was observed in the chitosan-citric acid samples, in relation to the control samples.

For chitosan films containing acetic acid (grey bar in Figures 46 and 47), the log concentrations after 24 h at 37 °C were 7.56 ± 0.42 for *E. coli*, and 8.34 ± 0.24 for *S. aureus*. For *E. coli*, the concentration in the chitosan-acetic acid films after 24 h incubation at 37 °C was significantly lower ($p \leq 0.05$) than the corresponding one in the control samples. Despite the large standard deviation, statistical t-tests showed a significant variance ($p \leq 0.05$) as well between the control after 24 h incubation at 37 °C and the concentration of *S. aureus* in the acetic acid-based chitosan films.

To determine the antimicrobial properties of seaweeds, two different samples were tested using the liquid-based assay: alginate film-forming solution (prior to casting) with 50% seaweed (yellow bar in Figures 46 and 47), and TSB with 10 mg/mL (green bar in Figures 52 and 53) at 37 °C. For both experiments, no significant differences ($p > 0.05$) in the log concentration of *E. coli* (9.33 ± 0.15 and 9.28 ± 0.17) and *S. aureus* (8.74 ± 0.15 and 8.79 ± 0.14) were observed after 24 h incubation at 37 °C, as compared to the control samples (9.06 ± 0.09 and 8.60 ± 0.29).

4.3.3 Liquid-based assay at 10 °C – Chitosan and Seaweed

Figures 48 and 49 show the concentration (average and standard deviation) of *E. coli* and *S. aureus*, respectively, in the chitosan samples (film prepared with either citric or acetic acid and dissolved in water at pH 5.2 ± 0.1), seaweed sample (TSB with 10 mg/mL seaweed) and chitosan combined with seaweed sample (film prepared with acetic acid dissolved in water at pH 5.2 ± 0.1 and supplemented with 10 mg/mL added seaweed), after 5 and 10 days

incubation at 10 °C. As mentioned in materials and method, this temperature was selected to mimic more realistic food storage conditions. The average initial log concentration for all conditions tested was 6.74 ± 0.16 for *E. coli* and 7.00 ± 0.08 for *S. aureus*. After 5 days incubation at 10 °C, the log concentration in the control samples (TSB), calculated as an average of the levels achieved in the different trials, was 8.01 ± 0.11 for *E. coli* and 6.84 ± 0.28 for *S. aureus*. After 10 days incubation at 10 °C, the log concentration in the control samples (TSB) was 8.35 ± 0.19 and 6.78 ± 0.29, respectively, for *E. coli* and *S. aureus*. For *E. coli,* the log concentration in the control tube increased after 5 days incubation at 10 °C and remained stable after 10 days incubation. However, for *S. aureus*, the log concentration in the control did not increase after 5 or 10 days incubation.

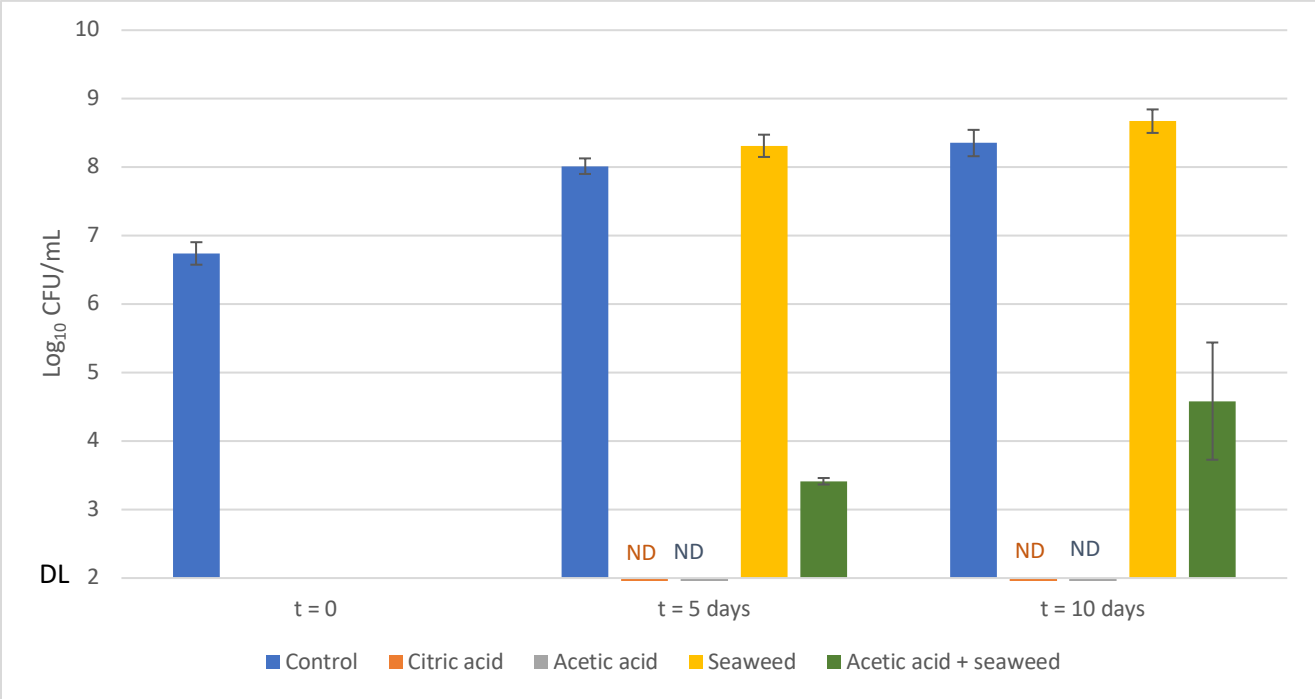

Figure 48. Concentration (CFU/mL) of *E. coli* at t = 0 (initial concentration for all the assayed conditions) and after 5 and 10 days incubation at 10 °C (blue: control, orange: dissolved chitosan-citric acid film, grey: dissolved chitosan-acetic acid film, yellow: TSB with 10 mg/mL seaweed corresponding to the proportion of seaweed:alginate in the films, green: dissolved chitosan acetic-acid film with 10 mg/mL seaweed addition). ND: not detected DL: detection limit

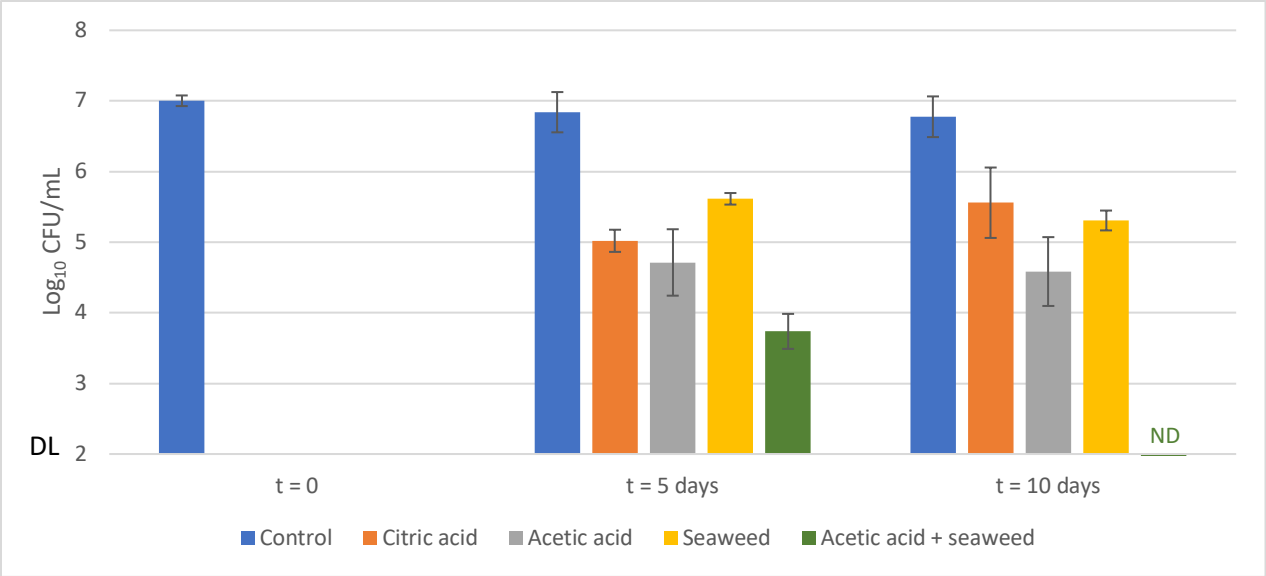

Figure 49. Concentration (CFU/mL) of *S. aureus* at t = 0 (initial concentration for all the assayed conditions) and after 5 and 10 days incubation at 10 °C (blue: control, orange: dissolved chitosan-citric acid film, grey: dissolved chitosan-acetic acid film, yellow: TSB with 10 mg/mL seaweed corresponding to the proportion of seaweed:alginate in the films, green: dissolved chitosan acetic-acid film with 10 mg/mL seaweed addition). ND: not detected DL: detection limit

For the chitosan films containing citric acid (orange bar in Figures 48 and 49), the log concentrations after 5 days at 10 °C were 5.01 ± 0.16 for *S. aureus* and below the detection limit (log 2) of the colony-counting technique for *E. coli*. After 10 days incubation at 10 °C, the log concentration in the chitosan-citric acid samples was 5.56 ± 0.50 for *S. aureus* and again below the detection limit for *E. coli*. For both *E. coli* and *S. aureus*, the concentration in the chitosan-citric acid films after both 5 and 10 days were significantly lower ($p \leq 0.05$) than the corresponding ones in the control samples. Despite relatively larger standard deviation bar for *S. aureus*, there was no significant variability ($p > 0.05$) in the viable counts between 5 and 10 days incubation.

In the samples prepared from chitosan films containing acetic acid (grey bars in Figures 48 and 49), the log concentrations for *E. coli* and *S. aureus* after 5 days incubation at 10 °C were 4.71 ± 0.47 for *S. aureus* and below the detection limit for *E. coli*. After 10 days incubation, the log concentration was 4.48 ± 0.49 for *S. aureus* and non-detectable for *E. coli*. Thus, a significant reduction ($p \leq 0.05$) was observed in the concentration of both microorganisms when comparing the acetic acid-based chitosan films with the control samples (TSB), regardless of the incubation time at 10 °C.

TSB containing 10 mg/mL seaweed (yellow bars in Figures 48 and 49) was also tested for antimicrobial properties at 10 °C. For *E. coli*, significant cell proliferation ($p \leq 0.05$) was observed in the seaweed samples after 5 days incubation at 10 °C, as compared to the initial bacterial levels. However, no significant difference ($p > 0.05$) in the log concentration of *E. coli* was observed between the 5-day and 10-day seaweed samples. The control samples (TSB) for *E. coli* presented a log concentration of 8.01 ± 0.11 after 5 days incubation at 10 °C and 8.35 ± 0.19 after 10 days. The log concentrations of *E. coli* in the seaweed samples after 5 and 10 days incubation at 10 °C were 8.31 ± 0.16 and 8.67 ± 0.17, respectively. Thus, no significant differences ($p > 0.05$) were found in the concentration of *E. coli* after 5 and 10 days incubation at 10 °C in the seaweed samples, as compared to the respective concentration in the control samples. For *S. aureus*, a significant reduction ($p \leq 0.05$) in the bacterial concentration was observed in the seaweed samples after 5 days incubation at 10 °C, as compared to the initial levels. However, no significant difference ($p > 0.05$) in the log concentrations of *S. aureus* was observed between the 5-day and 10-day seaweed samples. The control samples (TSB) for *S. aureus* presented a log concentration of 6.84 ± 0.28 and 6.78 ± 0.29, respectively, after 5 and 10 days incubation at 10 °C. The *S. aureus* log concentrations in the seaweed samples after 5 and 10 days incubation at 10 °C were 5.61 ± 0.08 and 5.31 ± 0.50, respectively. Thus, the concentration in the seaweed samples after both 5 and 10 days incubation were significantly lower ($p \leq 0.05$) than that in the corresponding control samples.

In the samples prepared from chitosan films containing acetic acid with added seaweed (green bars in Figures 48 and 49), the log concentrations after 5 days were 3.41 ± 0.05 for *E. coli* and 3.74 ± 0.25 for *S. aureus*. After 10 days incubation at 10 °C, the log concentrations in the chitosan-seaweed samples were 4.58 ± 0.86 for *E. coli* and non-detectable for *S. aureus*. Thus, a significant reduction ($p \leq 0.05$) in the levels of both *E. coli* and *S. aureus* were observed after 5 and 10 days incubation at 10 °C, as compared to the respective concentrations in the control samples. On the other hand, no significant variance ($p > 0.05$) in the log concentration of *E. coli* in the chitosan-seaweed samples was observed between day 5 and day 10 incubation at 10 °C, although a significant reduction ($p \leq 0.05$) was observed, as compared to the initial levels. Interestingly, a significant ($p \leq 0.05$) reduction in

the concentration of *S. aureus* in the chitosan-seaweed samples was observed between 0, 5 and 10 days incubation at 10 °C.

Overview of all the log concentrations and pH values of all the tested samples at both incubation temperatures can be found in appendix A2.2.

5. Discussion

5.1 Chitosan

5.1.1 Film characteristics

5.1.1.1 Chitosan

No significant difference ($p > 0.05$) in the thickness was observed between the films made with citric or acetic acid as the solvent. Bégin and Van Calsteren (1999), studied the effect of five different acids (hydrochloric, formic, acetic, lactic and citric acid solution) on the functional properties of the chitosan films and reported that the films made with citric acid was thinner than films made with acetic acid because of the acid´s properties and structure [16]. It is worth to mention here that Bégin and Van Calsteren (1999) used 1% (w/v) chitosan solution which was lower than the concentration used in our current study. They reported that during the drying process, the solution with citric acid starts to form a gel before the molecules can pack and align with each other resulting in thinner films. It was also mentioned that citric acid has the ability to form salt bridges with aminos, which not only promotes this gel formation, but also introduce porosity in films [16].

Despite the thickness not varying significantly between the films made from the two different solvents in this study, a significant difference ($p \leq 0.05$) was observed between the weight of the two films. This could be explained by the cross-linking abilities of citric acid. Because of the multiple functional groups present in citric acid, including carboxyl and hydroxyl groups, barrier and mechanical properties can be improved by crosslinking. In addition, citric acid has been reported to increase sorption of chitosan by several folds in certain applications [13][102]. Between the carboxyl groups present in citric acid and the

hydroxyl groups on the polysaccharide [103], covalent intermolecular di-ester linkages form resulting in film which are stronger, have higher water resistance and better antimicrobial properties [104][105].

Though the films in this study were not crosslinked, Nataraj et al. (2018) also reported that non-crosslinked chitosan films with citric acid had a higher water sorption capacity than did those with acetic acid [13]. However, Nataraj et al. (2018) used chitosan films made by creating an acetic acid film-forming solution, and then added citric acid. Thus, the chitosan film with citric acid also contained acetic acid. Nevertheless, it is possible that due to citric acid´s water sorption abilities [13], that there was more water present in the finished film compared to that with acetic acid as a solvent after drying.

5.1.1.2 Chitosan + HPP treatment

There were no significant effect ($p > 0.05$) effect of HPP treatment on the weight or thickness of the films. The HPP treated films with citric acid as a solvent were very fragile and near impossible to handle. However, no such issues appeared with the acetic acid films which were very easy to peel off from the petri dish and prepare for characterization.

It has been reported by Molinaro et al. (2015) that HPP treatment have an effect on the bonds in the structure of gelatin films, and thus the thickness of the films. The FTIR spectra of the HPP treated films showed slightly different absorbances at the characteristic wavenumbers as compared to the non-treated films meaning that the HPP treatment altered some of the bonds such as C = O and amide bonds in the gelatin structure. They also observed that HPP treated films were thicker than the non-treated counterparts [59]. However, a study on protein films showed no such increase in thickness after HPP treatment [60].

5.1.1.3 Chitosan + Seaweed

There was scarce literature available on the effect of seaweed addition on the weight and thickness of chitosan films. However, Gomaa et al. (2018) reported that for films consisting

of alginate and chitosan incorporated with fucoidan, which is found in seaweed, the thickness of the films significantly increased with addition of this filler [106]. A study conducted using a different species of seaweed (*Codium tomentosum*) also found the thickness of films to increase with added seaweed [10]. This increase in thickness of films with seaweed addition, and presumably also weight, which was also found in the present study, is attributed to the increase of the total solid mass of the films.

5.1.1.4 Chitosan + HPP treatment + Seaweed

For the films that were both treated with HPP and had seaweed added in different concentrations, there were a significant effect ($p \leq 0.05$) of both seaweed addition and HPP treatment on the weight of the films. In general, addition of seaweed increased the weight of the films, whereas the HPP treatment decreased the weight. According to Le Chatelier´s principle, increased pressure will shift the equilibria to a state with reduced volume. Thus, the HPP treatment may create a denser polymer structure which will eventually result in improved mechanical and barrier properties. [57]. A denser structure could also mean a heavier film; however, this is not the case here.

It is interesting that HPP treatment had no effect on the weight of the films with no seaweed, whereas for the films with seaweed, it showed significant effect ($p \leq 0.05$). Possibly, the high pressure alters some of the bonds within the solution containing seaweed. Nevertheless, a further study is required for more clarification. HPP treatment had no effect on the thickness of the films, however seaweed addition did. Like previously mentioned, this is probably due to added solid mass of the films.

5.1.2 Mechanical properties

5.1.2.1 Chitosan

Bégin and Van Calsteren (1999) reported that chitosan films made with citric acid as a solvent, had a higher elongation at break than did films made with acetic acid as a solvent [16]. Qiau et al. (2021) also observed the same trend [42]. However, the exact opposite was found in this study, where acetic acid films had a significantly higher ($p \leq 0.05$)

elongation at break. Park et al. (2002) also observed similar effect as Bégin and Van Calsteren (1999), with films made from citric acid having a higher elongation at break in comparison to chitosan films with acetic acid as the solvent, regardless of chitosan concentration. They found no significant difference ($p > 0.05$) in elongation at break for chitosan films with acetic acid at different concentrations of chitosan, whereas the films with citric acid as a solvent, showed a decreasing trend in elongation at break with increasing chitosan concentration. They explain this observation could be due to the high acid concentration of citric acid (4%) compared to the other acids tested (2%) [41].

However, Park et al. (2002) observed that the tensile strength of the citric acid films was lower than the acetic acid films [41], which correlated well with the current study. However, Park et al. (2002) used 4% citric acid in the film-forming solution, which they explain might result in weaker films. In addition, the molecular weight of chitosan dissolved in acetic acid was found to be higher than in the other acids, due to formation of dimers which result in strong intermolecular interactions. Park et al. (2002) explains that because of this, chitosan films prepared with acetic acid has a tighter structure than those prepared with other acids. An increase in tensile strength was observed with increasing chitosan concentration for both acids [41]. Qiau et al. (2021) offer another explanation, which is that the citrate ions possibly interact more strongly with chitosan, which destroys the interchain hydrogen bonds in chitosan, resulting in lower mechanical strength [42]. Nataraj et al. (2018) also found that for films with both acetic acid and citric acid, addition of citric acid generally decreased the tensile strength [13], which is consistent to the findings in this thesis for citric acid as a solvent.

According to Park. et al. (2002), the tensile strength of chitosan films should increase with increasing molecular weight of chitosan. This was explained by the amino groups in chitosan forming hydrogen bonds with the hydroxyl groups, which they found to be increasing with increasing chitosan concentration. It was also reported that films with lower elongation at break generally had a higher tensile strength [41]. However, this was not the case in the present study. The tensile strength of the lowest molecular weight of the chitosan films Park et al. (2002) studied, is quite similar to the results of this thesis. They do not report the molecular weight but rather the viscosity and degree of acetylation (95%) [41], which is

higher than the degree of acetylation used in this study (75-85%). The results to do not match with those of citric acid. It is important to mention, that the concentration of acetic (2%) and citric acid (4%) used by Park et al. (2002) was higher than the concentration used in this current study. The elongation at break values observed in the present study did not correspond at all with the values observed by Park et al. (2002) They reported elongation at break values 4.1 ± 1.2% for acetic acid, and 117 ± 19.5% for citric acid, for the lowest molecular weight chitosan [41].

Suyatma et al. (2004) reported the tensile strength of 1% chitosan solution in 1% acetic acid to be 82.4 ± 8.5 MPa [107], whereas in this study it was 68.81 ± 20.13 MPa. The difference in the two values could be due to higher chitosan concentration used in the present study (2%), as well as variance in molecular weight. Suyatma et al. (2004) had a chitosan molecular weight of 49,000 [107], which is much lower than what was used in the present study (190,000 – 310,000). The elongation at break of the 1% chitosan films was reported to be 5.2 ± 0.9% [107], whereas it was 22.02 ± 14.06% in the present study. It is difficult to draw conclusions based on the massive standard deviation, however the elongation at break were still larger than that of Suyatma et al. (2004) The difference could be explained by the much higher molecular weight chitosan that was used in the present study, in addition to the higher chitosan concentration.

5.1.2.2 Chitosan + HPP treatment

As mentioned before, HPP treatment could lead to enhances barrier properties and mechanical strength, explained by a denser structure as a result of the HPP [57].

Niu et al. (2012) observed that HPP enhanced the tensile strength of films made of chitosan and also lowered the elongation at break up to 400 mpa. They explain this observation by the high pressure breaking the structure of chitosan, breaking the hydrogen and hydrophobic bonds and exposing the polar groups. As a result, the films were more compact but with lower elasticity. At HPP treatment at 500 MPa pressure, they saw a decrease in tensile strength explained by saturation of large molecules in the solution [28].

In addition, Montero et al. (2002) looked at the strength of gelatin gels treated with high pressure. For one of the gelatins used, HPP treatment gels had a better strength than the non-treated gels, whereas the opposite was true for another gelatin type. However, they also found that pressurizing at low temperatures (7°C) had a higher effect than did those at 20°C, and a higher pressure resulted in higher strength. Montero et al. (2002) explain this observation by stability of hydrogen bonds resulting in a well-structured network in the films after HPP treatment [58].

Another study conducted using gelatin-based films showed a higher tensile strength for HPP treated films, whereas the differences in elongation at break was not significant. The explanation behind the increased tensile strength is that the mechanical forces which are applied during HPP treatment, results in a stiffer film through formation of hydrogen bonds [59]. The same result was found for protein films, attributed to increased amount of crosslinking [60]. For poly (lactic acid)/Ag nanocomposite films, the tensile strength increased with HPP treatment, and the elongation at break decreased. There was also a decrease in elongation at break with higher HPP pressures, explained by improvement in the crystallinity of the films by high pressure. The internal structure is more compact and compressed after HPP treatment [26].

The results from this thesis confirm most of the results from the literature mentioned. Generally, HPP treatment significantly increases ($p \leq 0.05$) the tensile strength, whereas the elongation at break is lower for the treated films compared to the non-treated ones. This observation is possibly due to formation of hydrogen bonds and a more compact and compressed structures following HPP treatment. Another explanation could be that the presence of bubbles in the HPP treated film (Figure 13) can result in films with decreased tensile strength, which is possibly why others report increased tensile strength with HPP treatment, while no effect was after treatment at 200 MPa pressure in the present study.

5.1.2.3 Chitosan + Seaweed

Augusto et al. (2018) used Tween 80 as a plasticizer in their chitosan films, and the seaweed concentration was 0.5% (w/v). With a chitosan concentration of only 1% (w/v) in 1% (w/v) citric acid, this is a 50% seaweed concentration. However, seaweed extract instead of powder was used. Augusto et al. (2018) saw an increase in elongation at break, but a significant lower ($p \leq 0.05$) puncture strength for the samples containing seaweed than those without. They explain this observation by the possibility of the film structure to soften and become more flexible, and that seaweed can act as a plasticizer to increase the mobility of the polymers, resulting in increase in elongation at break and decrease of puncture strength [10].

The worse strength in samples with the filler is consistent to the findings in this study. Addition of seaweed in all concentrations significantly decreased ($p \leq 0.05$) the tensile strength of the chitosan films. However, Augusto et al. (2018) saw an increase in elongation at break with added seaweed [10], which is the opposite to the findings in this thesis. They also used citric acid, which Bégin and Van Calsteren (1999) found that had significantly longer elongation at break compared to films made with acetic acid [16], like mentioned previously. However, Augusto et al. (2018) used seaweed extracted in water or ethanol in the chitosan films [10], whereas in the present study, seaweed was added directly in powder form. So, it is possible that instead of improving the flexibility of the films, the seaweed particles disrupt the structure within the film, resulting in a more brittle product. Finding literature where the particle size is similar to that used in this study (≤200 μm) was unsuccessful, possibly because fillers of this size does not improve the mechanical strength of biopolymers.

5.1.2.4 Chitosan + HPP treatment + Seaweed

Lian et al. (2016) reported that the addition of nano-TiO_2 particles to PVA + chitosan films increased the tensile strength of the films. In addition, a significant improvement ($p \leq 0.05$) in the tensile strength was also found with increasing HPP treatment from 200 MPa to 600 MPa. They reported that the elongation at break of the non-treated films were much

higher than those HPP treated. However, no significant difference ($p > 0.05$) in the elongation at break between the HPP treated samples at the different pressures were reported. Addition of TiO_2 also had a significant ($p \leq 0.05$) and positive effect on the elongation at break. They explain this observation by reinforced microstructure of the films with addition of nano-TiO_2 particles. A high concentration of particles however, could lead to agglomeration and thus reduction of mechanical properties [62]. In the present study, addition of a filler in the form of seaweed significantly decreased ($p \leq 0.05$) the tensile strength of the chitosan films. Moreover, a decreasing trend of tensile strength was also seen with increasing seaweed concentration from 10 to 50%, in addition to HPP treatment at both 200 and 600 MPa.

The findings of Lian et al. (2016) in terms of elongation at break was also the opposite of the findings in this study. While addition of TiO_2 particles had a positive effect on the elongation at break for chitosan + PVA films, a negative effect was seen in this study. Also, treatment with HPP in combination with seaweed negatively impacted the elongation at break. Nevertheless, Lian et al. (2016) used citric acid to dissolve the chitosan, which previously presented has been found to have better elongation at break than chitosan dissolved in acetic acid [16][41]. As mentioned earlier, Lian et al. (2016) explained that a high concentration of filler could cause agglomeration and decreased mechanical strength [62]. Li et al. (2009) also use this reasoning for ZnO nanoparticles in polyurethane coatings [9]. Thus, the use of seaweed in powder form is most likely the reason the results from this present study vary from the literature available where either extracts or nanoparticles were used.

5.1.3 Barrier properties

5.1.3.1 Chitosan

No significant effect ($p > 0.05$) of solvent type was found on the water vapor transmission rate of the chitosan films. Nataraj et al. (2018) made films by first dissolving chitosan in acetic acid and then adding citric acid to the solution prior to casting the film. They found that the chitosan films which had both acids had significantly better ($p \leq 0.05$) barrier

properties than the ones with just acetic acid. They reported that this observation could be due to citric acid´s ability to absorb more water. It has also been reported that the presence of COOH groups found in citric acid makes it difficult for water vapor to transmission through the film [13].

Park et al. (2002) studied the effect of different acids used as solvents for chitosan films and their water vapor permeability. They reported that chitosan films made with 4% citric acid did not have a significantly higher water vapor permeability compared to 2% acetic acid chitosan films at low molecular weight, both containing 2% chitosan. However, at higher molecular weights of chitosan, films made with citric acid had significantly lower barrier properties explained by acetic acid´s ability to form a tighter structure with chitosan than other acids and thus making it more difficult for the water molecules to pass through the film [41]. While a medium molecular weight chitosan was used to create the films for barrier testing in the present study, the findings were consistent with those Park et al. (2002) found for low molecular weight chitosan. They tested only low and high molecular weight, so it is possible that medium molecular weight chitosan films follow the trend of those at low molecular weight.

5.1.3.2 Chitosan + HPP treatment

Niu et al. (2012) reported that the water vapor permeability of chitosan films made from HPP treated solution was lower than for the non-treated ones, explained by the breaking of hydrogen and hydrophobic bonds and exposing polar groups, resulting in a more compact film [28]. Molinaro et al. (2015) found that HPP treated gelatin films had significantly better ($p \leq 0.05$) barrier properties than non-treated films explained by the possible formation of more hydrogen bonds, resulting in a more compact film which is more resistant to diffusion of water molecules [59]. A study using protein films also found that HPP treatment improved the barrier properties of films explained by higher crosslinking of films or unfolding of proteins by HPP which retard the passage of water through the film [60]. Despite these reports, films made of chitosan in this present case had poorer barrier properties after HPP treatment. This is possibly also linked to the alteration in structure of the chitosan during treatment.

5.1.3.3 Chitosan + Seaweed

In a study with PVA + chitosan films incorporated with nano-TiO_2 particles, the water vapor permeability of the films with nano-TiO_2 were better than the films with no TiO_2, as the route of water vapor transmission were prolonged by the particles in the film [62]. Another explanation could be the poor solubility of the TiO_2 particles in water compared to the chitosan + PVA. However, at higher concentrations of TiO_2, a decrease in the water vapor permeability was observed which was probably due to the agglomeration of the TiO_2 particles [62]. Chi et al. (2018) also explained the better barrier properties in poly (lactic acid)/Ag nanocomposite films with higher nanoparticles with the water having a longer route around the particles [26]. However, Augusto et al. (2018) did no observe any significant increase of decrease in barrier properties of chitosan films with added seaweed where citric acid was used as a solvent [10]. In a study with chitosan-starch films with added red cabbage extract, the incorporation of extract did not significantly change ($p > 0.05$) the water vapor permeability [108]. Whereas for chitosan-starch films with added PLA, the barrier properties were increased with increasing poly (lactic acid) concentration according to Suyatma et al. (2004). They explain that the smaller concentrations had the most impact due to the poor miscibility with chitosan, whereas the increase in water vapor permeability is also attributed to the hydrophobicity of PLA [107].

The common factor in all these studies, is that addition of fillers either improved the barrier properties of the different films, or there was no significant effect. Despite this, the exact opposite was found in this case. Addition of seaweed significantly increased ($p \leq 0.05$) the water vapor transmission rate, and thus lowered the barrier properties of the films. Though the films with the lowest seaweed concentration had poorer barrier properties than the ones with the highest concentration, they were all significantly lower ($p \leq 0.05$) than the films with no fillers. A possible explanation could be that seaweed has a higher solubility than chitosan, however because the films with seaweed took around the same time to dissolve as the ones without seaweed, this is most likely not the case.

5.1.3.4 Chitosan + HPP treatment + Seaweed

Although HPP treatment significantly decreased ($p \leq 0.05$) the barrier properties of the chitosan films with no fillers, there were no effect of HPP treatment on the films with seaweed. Lian et al. (2016) observed that the water vapor permeability of chitosan-PVA films previously mentioned decreased with added TiO_2 nanoparticles and with HPP treatment, thus resulting in films with better barrier properties explained by increased interaction between PVA and chitosan molecules in the film matrix and a more homogenous incorporation of the TiO_2 particles after HPP treatment [62]. The same phenomenon was seen for the study using poly (lactic acid)/Ag nanocomposite films, with added nanoparticles the barrier properties increased explained by better interaction between the nanoparticles and the PLA. In addition, HPP treatment improved crystallinity which makes the structure more compact and thus makes it more difficult for water molecules to transmission though the film [26]. A common factor in both the studies however, were that HPP treatment at 200 mpa resulted in films with better barrier properties compared to those treated at 600 mpa [62][26]. Because there were no definite trends in the barrier properties of the chitosan films, it is difficult to see whether the same conclusion could be drawn for this study.

5.1.4 Ninhydrin assay

5.1.4.1 Chitosan

The Ninhydrin assay quantifies the free amino group concentration in the chitosan films and how the type of solvent used affects this concentration. It is the β-(1->4)-linked D-glucosamine units of chitosan that form colored products in the Ninhydrin assay [109]. Cui et al. (2011) studied the effect of addition of citric acid in different concentrations to chitosan already dissolved in acetic acid on the water resistance of chitosan through IR spectroscopy. They observed a broader peak at ranges corresponding to increased OH groups with addition of citric acid, as well as a broader peak for the chitosan C-O with added citric acid C-O bonds. The remaining changes in the spectra were mainly attributed to the

formation of amide bonds between citric acid and the chitosan amide group not found in the chitosan with no citric acid (Figure 50 and 51) [43].

Based on the reports by Cui et al. (2011), theoretically, the chitosan samples with citric acid as a solvent could have lower free amino group concentration than the chitosan samples with acetic acid as a solvent because of the formation of amide bonds between citric acid and chitosan (Figure 50). This is exactly what was found in this present study as well from the ninhydrin assay, where the type of solvent used had a significant effect ($p \leq 0.05$) on the free amino concentration in the samples.

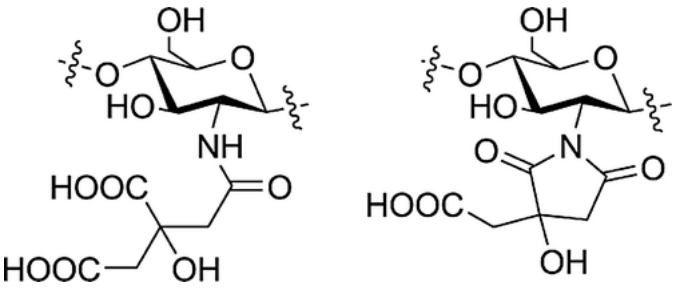

Figure 50. The possible amide bonds between citric acid and chitosan [43]

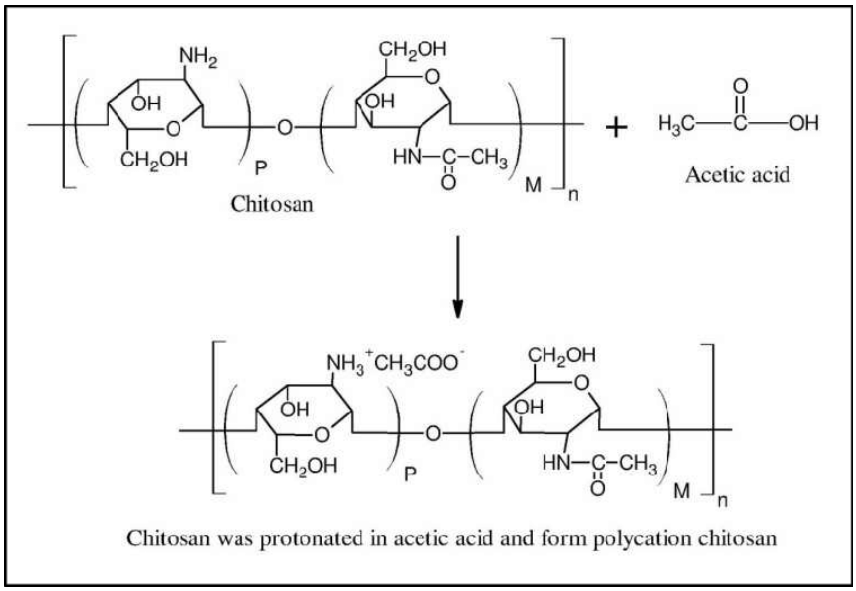

Figure 51. Chitosan reacting with acetic acid [110]

However, it is important to note that the expected yield of free amino groups from the ninhydrin assay is found to theoretically be lower in acidic solutions due to the shift in the equilibrium of the reaction [111]. Moreover, after dissolving the chitosan films in water, the ones with citric acid solvent had a lower pH of 4.00 ± 0.01, whereas the acetic acid samples

had a pH of 5.44 ± 0.23. This lower pH value of citric acid could contribute to the fact that there was almost a three-fold higher free amino group concentration in the acetic acid samples compared to the citric acid samples. According to Bottom et al. (1978), there is insignificant hydrolysis of the amine for pH 5-7 which leads to free ammonia, whereas at pH 1-2, no purple color is because of production and protonation of ammonia which reacts much slower with ninhydrin [112]. No report was given of what happens between pH of 2 and 5, but it could be expected that some protonation took place and thus less Ruhemann´s purple is formed, and the free amino group yield is less.

5.1.4.2 Chitosan + HPP treatment

Though the ninhydrin assay is widely used to quantify the protein content in foods both before and after high pressure processing, it is very rarely used on biopolymers. A study conducted on PVA + chitosan films incorporated with TiO_2 particles showed that HPP treatment did not alter the chemical bonds in the chitosan molecule. Acetic acid was used as a solvent in this case [62]. However, the free amino group concentration in the citric acid samples increased after HPP treatment, whereas for the acetic acid samples, it decreased. Therefore, it could be expected that HPP treatment induced some structural changes within the chitosan structure. Like previously mentioned, Cui et al. (2011) reported that citric acid forms amide bonds with chitosan [43]. Because the free amino group concentration in the chitosan samples with citric acid increased after HPP treatment, it is possible that these bonds are disrupted by the high pressure. However, the opposite was observed in the chitosan in acetic acid samples after HPP treatment. The pH was similar to the non-treated films, so the acidity of the solution was not responsible for the change.

5.1.5 DPPH assay

5.1.5.1 Chitosan

Ai et al. (2008) reported that chitosan isolated from larvae of houseflies with oxalic acid as a solvent had a scavenging activity of 57.1% at 0.5 mg/mL [113], which is very similar to the values from this present study. However, they saw a much higher scavenging activity at

concentrations of 1.0 and 2.0 mg/mL, whereas only little differences was found in the present study. Other studies report scavenging activities of 16.5% at 5.0 mg/mL for 150,000 Da chitosan [114] and 58.3-70.2% at 1.0 mg/mL (MW not reported) [115].

The molecular weight and degree of acetylation of the chitosan is important in regard to the antioxidant activity reported. Generally, low molecular weight chitosan has the highest radical scavenging activity [39]. The molecular weight of the chitosan used in this study had a range of 190,000-310,000 Da, with a deacetylation degree of 75-85%. Because this range is quite large, it is difficult to compare directly to other studies as long as the molecular weight and degree of acetylation apparently has such a large impact on the scavenging activity.

Besada et al. (2016) reported that chitosan dissolved in citric acid had the highest scavenging activity of different acids tested at all concentrations (1.0, 2.0, 3.0 and 5.0 mg/mL). They also reported that the scavenging activity of both chitosan with acetic acid and chitosan with citric acid increased with increasing chitosan concentration [44]. Nevertheless, there were no significant effect of solvent type on the scavenging activity at concentrations of 0.5 and 1.0 mg/mL in the present study, whereas at 2.0 and 3.0 mg/mL, the acetic acid films actually had a significantly higher scavenging activity compared to the citric acid ones. The scavenging activity of chitosan is based on the free amino group reacting with free radicals to form stable molecules, followed by formation of ammonium groups by absorbing hydrogen ions from the solution [116]. It has already been described how citric acid binds to the chitosan molecule at the free amino group, which is a possible explanation why the scavenging activity of chitosan dissolved in acetic acid was higher than chitosan dissolved in citric acid at chitosan concentrations of 2.0 and 3.0 mg/mL.

5.1.5.2 Chitosan + HPP treatment

No literature was available on the potential effect of HPP treatment on the antioxidant activity of chitosan films. However, on food extracts, HPP treatment generally either increased the scavenging activity, or it was unchanged. On grapefruit juice, scavenging activity increased with increasing HPP pressure and also with temperature explained by cell interruption and distraction with HPP treatment [117], whereas HPP treatment did not

dramatically increase the DPPH scavenging activity of beet extracts [118]. Sun et al. (2019) found the HPP treated samples to be similar to the non-treated samples of Djulis grains [119]. However, studies suggest that above a certain threshold, the effect of HPP on the scavenging activity might be negative [119].

All these studies are with food extracts and not polymers, but the general trend is similar to that found in the present study. HPP treatment had no significant effect ($p > 0.05$) on the DPPH scavenging activity at chitosan concentrations of 2.0 and 3.0 mg/mL, whereas at the two lower concentrations it did. The scavenging activity of chitosan with citric acid as the solvent in concentrations of 0.5 and 1.0 mg/mL increased with HPP treatment at 600 MPa pressure, possibly because of interruption of cells like for grapefruit juice. Like previously mentioned, the scavenging activity is based on the free amino group reacting with a free radical [101]. It has also been presented that HPP treatment of chitosan samples with citric acid as the solvent caused an increase in free amino groups determined by the ninhydrin assay. Therefore, this increase in scavenging activity with HPP treatment in samples with citric acid could be attributed to the increase in free amino groups. Nevertheless, if this was the case then theoretically HPP treatment should decrease the scavenging activity in the samples with acetic acid as the solvent, which it did not. In addition, the same observation should be seen at higher chitosan concentrations of 2.0 and 3.0 mg/mL.

5.1.5.3 Chitosan + Seaweed

Peng et al. (2013) studied the effect of adding tea extract on the scavenging activity of chitosan films. Interestingly, they observed close to no scavenging activity in the samples with no extracts, whereas addition of tea extracts significantly increased ($p \leq 0.05$) the activity in all concentrations. They explain this observation by intermolecular interactions between tea extracts and chitosan [72]. The same observation was reported by Siripatrawan and Harte (2010), who also investigated the effect of addition of tea to chitosan [73]. Incorporation of α-tocopherol enhanced the antioxidant capacity of chitosan films as well, according to Martins et al. (2012), though lactic acid was used as a solvent and the chitosan had a slightly higher degree of acetylation at 95% and the fact that tocopherol has a reported antioxidant activity of its own. However, the films with tocopherol had a

scavenging activity of around 97%, whereas for the control films with just chitosan, the scavenging acidity was just 10.69% [74]. According to Martins et al. (2012), this observed scavenging activity in the chitosan controls is mainly due to the residual free amino groups in the sample, which reacts with free radicals forming stable ammonium groups and macromolecular radicals. The increase in scavenging activity with added α-tocopherol was due to the interaction between the filler and chitosan, possibly due to hydrogen and covalent binding [74].

On chitosan films with or without added polyvinyl alcohol, the potential effect of addition lignin nanoparticles was studied by Yang et al. (2016). They reported that the high molecular weight chitosan films with no fillers had a scavenging activity of 20.3% [116]. Like Martins et al. (2012) stated, they explain this as a result of the amino group reacting with free radicals. After addition of lignin nanoparticles, the antioxidant activity increased drastically [116]. This general observation of increased scavenging activity with added filler is also seen in chitosan films with *Pistacia terebinthus* due to the high antioxidant activity of the filler [75], and for mango leaf extracts [120]. However, in the present study, an increase in scavenging activity in the films with 50% seaweed as a filler was only seen at a chitosan concentration of 3.0 mg/mL. There was a decrease in scavenging activity for the other concentrations tested after addition of seaweed, and thus in general, the results obtained from this study contradicts those of others. An explanation might be that the seaweed particles somehow disrupt the absorbance reading due to the sheer size of the particles at ≤ 200 μm, as well as the viscosity of the chitosan samples causing uneven distribution of seaweed.

5.1.5.4 Chitosan + HPP treatment + Seaweed

There was no significant effect ($p > 0.05$) of HPP treatment on the scavenging activity of the chitosan samples with added seaweed. Thus, only seaweed and HPP treatment separately had an effect. Because the lower concentrations of seaweed did not increase the scavenging activity, it is possible that this effect is only seen in large concentrations of both chitosan and seaweed. Alternatively, the seaweed interferes with the reading due to the size, like previously mentioned. Nevertheless, all the samples showed a relatively good scavenging activity above or close to 50%.

5.2 Alginate

5.2.1 Film characteristics

5.2.1.1 Alginate + HPP treatment

HPP treatment had a significant effect ($p \leq 0.05$) on the weight of the alginate films. The non-treated films were significantly lighter ($p \leq 0.05$) than the treated films. This decrease in weight is most likely a result of HPP treatment changing the bonds in the alginate molecules. In gelatin films, it has been reported that HPP treatement altered amide and C=O bonds [59], which might result in films with different thickness and weights due to altered structure. However, no effect of HPP treatment was seen on the thickness of the films in the current study.

5.2.1.2 Alginate + Seaweed

Addition of seaweed significantly increased ($p \leq 0.05$) both the weight and thickness of the alginate films. This increase is attributed to the increase of the total mass of the films. Gomaa et al. (2018) reported that addition of fucoidan, which is found in seaweed, to films made of both chitosan and alginate significantly increased the thickness of the films [106]. The same conclusion of increased thickness of alginate films has also been concluded for addition of another seaweed species (*Codium tomentosum*) [10]. It was easy to disperse the seaweed into the alginate film-forming solution (Figure 52) because the viscosity was low compared to other biopolymers such as chitosan. This could explain why no dramatic increase in thickness was seen with added seaweed.

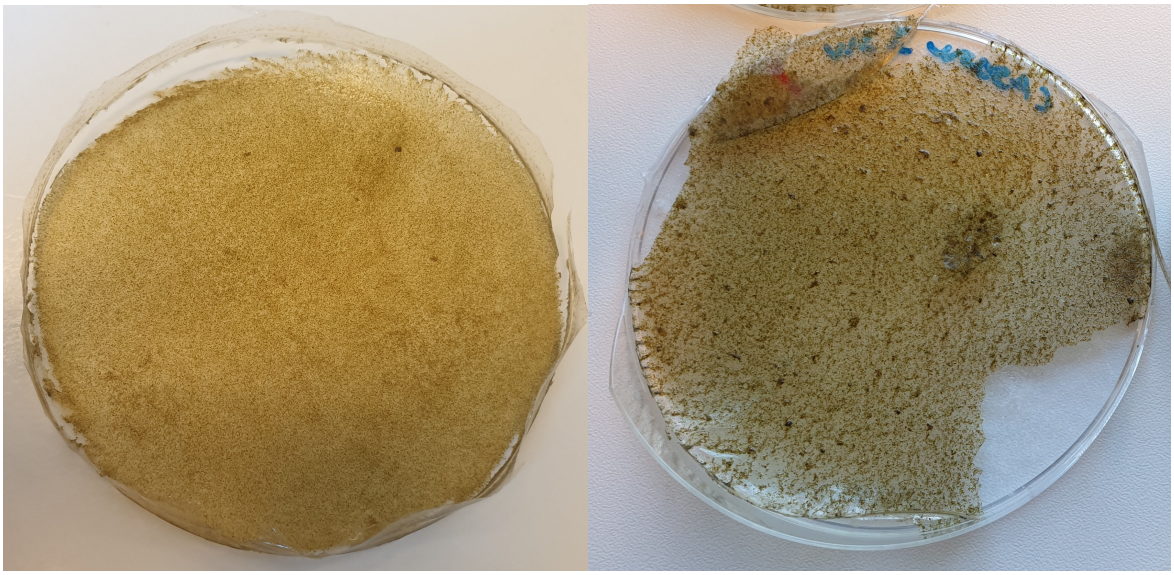

Figure 52. Alginate film with 50% seaweed vs chitosan film with 50% seaweed.

5.2.1.3 Alginate + HPP treatment + Seaweed

HPP treatment significantly decreased ($p \leq 0.05$) the weight of the films containing seaweed as compared to the non-treated alginate films with seaweed, most likely as a result of altering of the bonds in the alginate structure. However, HPP treatment in combination with added seaweed did not affect the thickness of the films.

5.2.2 Mechanical properties

5.2.2.1 Alginate + HPP treatment

HPP treatment significantly increased ($p \leq 0.05$) the tensile strength of the alginate films at 200 MPa pressure, whereas at 600 MPa pressure, no significant effect ($p > 0.05$) was observed. For gelatin [58], gelatin-based [59] and poly (lactic acid)/Ag nanocomposite films [26], an increase in tensile strength was seen after HPP treatment explained by stability in the structure due to an increase in hydrogen bonds [58], stiffer and more compact structure [59], increased crosslinking and improved crystallinity [26]. In the present study, it was found that HPP treatment at 200 MPa pressure significantly increased ($p \leq 0.05$) the tensile strength similarly to other reports, whereas at HPP treatment at 600 MPa pressure a decrease in tensile strength was observed. A study on chitosan films treated with HPP

reported that with HPP treatment up to 400 MPa pressure, the tensile strength increased and then decreased at higher pressures due to the saturation of large molecules [28], which could be the case in the present study.

Generally, it has been found that films treated with HPP had lower elongation at break than their non-treated counterparts [26], [28], which is consistent with the observations in the present study.

5.2.2.2 Alginate + Seaweed

Augusto et al. (2018) reported a decrease in elongation at break after addition of seaweed to alginate films [10]. However, the parameters were slightly different as they used a different seaweed extract (*C. tomemtosum*), as well as added glycerol in the samples and a lower alginate concentration at 1% (w/v). Instead of testing tensile strength, they tested the puncture strength, which was not significantly higher in any of the samples [10]. However, the puncture strength still gives a picture of the strength of the films. Similarly, the tensile strength of the alginate films in the present study was not significantly affected ($p > 0.05$) by seaweed addition. In addition, the elongation at break significantly decreased ($p \leq 0.05$). Nevertheless, like previously mentioned, the alginate concentration was different, and the seaweed extract was of a different strain. In addition, they added glycerol to the films which acts as a plasticizer and might impact the mechanical properties of the films.

Like already mentioned, the tensile strength of the alginate films was not affected by addition of seaweed. This is not consistent with other studies. Chi et al. (2018) reported that for Poly (lactic acid)/Ag nanocomposite films, the tensile strength increased with addition of nanoparticles. They also found that the elongation at break decreased, which is similar to the results of the present study with alginate and seaweed [26]. For gelatin films with added seaweed, the tensile strength did not significantly increase ($p > 0.05$) with added seaweed, whereas the elongation at break increased explained by compounds in the seaweed linking to the gelatin to form longer chains which are more elastic [69].

5.2.2.3 Alginate + HPP treatment + Seaweed

The combination of HPP treatment and seaweed showed some varying results. HPP treatment had a significant decreasing effect ($p \leq 0.05$) on the tensile strength of the alginate films with 50% seaweed and pressure of 600 MPa. No literature with previous studies were found that could explain this observation. There was no effect of HPP treatment on elongation at break of the samples with added seaweed at all concentrations.

In general, like previously mentioned, for other bio films like those made of gelatin, protein and poly(lactic acid), HPP treatment increases the tensile strength [26], [59], [60], and reduces the elongation at break [26], [28]. On its own, HPP treatment at 200 MPa pressure significantly increased ($p \leq 0.05$) the tensile strength, whereas for elongation at break, HPP treatment at both pressures had a decreasing effect. Addition of seaweed had no significant effect ($p > 0.05$) on the tensile strength, whereas the elongation at break was decreased. Though no studies were found where HPP treatment was performed on alginate samples with seaweed, however for PVA+Chitosan films with TiO_2 nanoparticles, the combination of filler and HPP treatment increased the tensile strength explained by a reinforced microstructure after HPP treatment [62]. Similarly, HPP treatment of Poly(lactic acid)/Ag nanocomposite films increased the tensile strength due to stiffness in the films through development of hydrogen bonds, as well as a more homogenous films as a result of HPP treatment. A reduction in elongation at break was found as a consequence of higher tensile strength and higher crystallinity of the films [26].

This is contradictory to the findings in the present study. Except for the alginate sample with 50% seaweed and HPP treatment at 600 MPa pressure, there were no effect of combination of seaweed addition and HPP treatment on the tensile strength of the films. Although separately both HPP treatment and seaweed had significant effects ($p \leq 0.05$) on the elongation at break, the combination of the two showed no interaction effect, contrary to other reports. It is important to note, however, that in most reports, either extracts or nanoparticles are used, whereas in this present study seaweed is used in powder form with a size of ≤ 200 μm. It has been explained that large amounts of fillers can result in agglomeration in the films [62], which is possibly the case in this study.

5.2.3 Barrier properties

5.2.3.1 Alginate + HPP treatment

No reports on the barrier properties of alginate after HPP treatment were found. However, studies have been conducted on several other biofilms like those made of protein and chitosan. Condés et al. (2015) reported that for protein films, the water vapor permeability decreased after HPP treatment as a consequence of higher degree of crosslinking in addition to unfolding of proteins which retard the passing of water molecules through the film [60]. Similarly, HPP treatment resulted in lower water vapor permeability of chitosan films due to breaking of hydrogen and hydrophobic bindings, as well as formation of a more compact film [28].

Chi et al. (2018) studied the effect of high-pressure treatment on Poly (lactic acid)/Ag nanocomposite films and reported that application of pressure of 200 and 400 MPa significantly improved the barrier properties of the films. They also reported that films treated with a lower pressure showed a significantly lower ($p \leq 0.05$) water vapor permeability compared to those treated with a pressure of 400 MPa, resulting in better barrier properties at lower HPP treatment. However, the barrier properties of the films treated with 400 MPa were still significantly better than the non-treated films [26]. Despite numerous reports on increased barrier properties after HPP treatment, no significant effect ($p > 0.05$) was seen in the case of alginate in the present study.

5.2.3.2 Alginate + Seaweed

Augusto et al. (2018) reported a significant decrease ($p \leq 0.05$) of water vapor permeability in alginate films with added seaweed. They explained this observation by the changes in the crosslinking in the polymer chain, caused by seaweed. The seaweed interfere with the hydrophilic portion of the film, which affects the water vapor transfer through the film at the hydrophilic zone [10]. In alginate films with added calcium, this decrease in water vapor permeability was also observed as a result of increased crosslinking which reduced the mobility of the polymeric chains [11], and in gelatin films with seaweed added which was also explained by enhancement of crosslinking resulting in a decrease of free volume in the

matrix and thus a more torturous pathway for the water through the film [69]. Although addition of 10% seaweed had no impact on the WVTR of the alginate samples in the present study, addition of 30 and 50% seaweed decreased the WVTR. The reported decrease in WVTR with increased seaweed concentration in other studies, could be due to accumulation of seaweed particles in the film, longer route for the water vapor to travel around the particles, or seaweed´s poor solubility in water compared to alginate [62].

5.2.3.3 Alginate + HPP treatment + Seaweed

There was no effect of combination of HPP treatment and seaweed addition on the water vapor transmission rate of the alginate films. However, improvement in the water vapor transmission rate was seen in the films with 30% seaweed and HPP treatment. However, the reason for the reduction in WVTR for only seaweed concentrations of 30% seaweed is unknown. A large amount of seaweed in the film could cause agglomeration of particles which should reduce the WVTR, however if this was the case, the films with 50% seaweed should have even better WVTR.

Chi et al. (2018) observed that for poly (lactic acid)/Ag nanocomposite films, the water vapor permeability decreased with addition of nanocomposites and with HPP treatment, explained by increasing interaction between the nanoparticles and PLA resulting in a more homogenous film by improved crystallinity [26]. Similarly, Lian et al. (2016) reported that addition of Nano-TiO_2 particles in combination with HPP treatment on polyvinyl alcohol/chitosan films decreased the water vapor permeability. They explain this observation could be the result of low water solubility of the particles compared to the film forming materials, prolonged pathway of water molecules through the film due to the presence of TiO_2 particles and increased interaction between PVA and chitosan as a result of HPP treatment [62].

5.2.4 DPPH assay

5.2.4.1 Alginate + HPP treatment

The molecular weight and ratio of G and M blocks in the alginate are important factors impacting the scavenging activity. A larger proportion of G blocks are described as having higher antioxidant activity due to the hindered rotation around the glycosidic linkage. This causes the flexibility to decrease and thus the hydroxyl groups lose some of their ability to donate H-atoms [121]. Khajouei et al. (2018) reported a scavenging activity of 66.4% for alginate from *Nizimuddinia zanardini* at a concentration of 2.0 mg/mL. Alginate from *Cystoseira barbata*, which had a higher G/M ratio, had a scavenging activity of 74% at 0.5 mg/mL, whereas alginate from *Turbinaria conoides* had a scavenging activity of 62% at 5.0 mg/mL, due to a smaller G/M ratio [121]. The scavenging activity of alginate films with calcium chloride and glycerol added, showed almost no scavenging activity according to Norajit et al. (2010). However, the films were not dissolved in water, but frozen and then grounded, before adding methanol. Prior to this, $CaCl_2$ solution was poured onto the films [70].

The molecular weight or G/M ratio in the sodium alginate used in the present study is unknown, but the scavenging activity is around the same value as Khajouei et al. (2018) reported. The activity was around 60%, regardless of concentration used. No significant effect ($p > 0.05$) was seen after HPP treatment for all samples except at 1.0 mg/mL and treatment at 600 MPa pressure. No literature was found where the effect of HPP treatment on the scavenging activity of alginate was tested. However, the general trend on food extracts is that HPP treatment either increases the scavenging activity or it stays relatively unchanged. Aadil et al. (2017) reported that the scavenging activity of grapefruit juice increased with increasing HPP pressure explained by cell disruption and interruption [117]. Selig et al. (2018) observed no dramatic increase in scavenging activity of beet extract as a consequence of HPP treatment [118], and Sun et al. (2019) observed similar scavenging activity in HPP treated and non-treated samples of Djulis grains. It has however been suggested that above a certain threshold, the effect of HPP treatment on the scavenging activity of samples is negative [119]. In the present study, a significant decrease ($p \leq 0.05$) was seen after HPP treatment at 600 MPa pressure on the alginate sample at 1.0 mg/mL.

However, because there were no significant changes ($p > 0.05$) at all the other concentrations, it is unlikely that there is a threshold at 600 MPa HPP treatment where the effect becomes negative. Especially since there was no positive effect of HPP treatment at lower pressures like other research suggests.

5.2.4.2 Alginate + Seaweed

In general, addition of plant extracts to alginate films increases the scavenging activity. Norajit et al. (2010) saw almost no scavenging activity in the alginate control with no fillers, whereas after adding ginseng extract, the activity was dramatically higher at all concentrations explained by the antioxidant activity of the ginseng itself [70]. Addition of protein hydrolysates to alginate films had similar results [71]. Though the alginate sample with no fillers in this present study had a scavenging activity of around 60% and not close to nothing like the literature suggests, the addition of seaweed generally increased the scavenging activity. Seaweed is also reported to have a scavenging activity on its own [66], which explains this increase.

The effect of seaweed addition on the scavenging activity of the alginate films varied between different seaweed and alginate concentrations. Only addition of 30% seaweed resulted in an increased scavenging activity at all alginate concentrations. The variation could be explained by the sheer amount of seaweed particles in the diluted samples when the absorbance was read which could cause interference. In addition, in the present study, seaweed in powder form was used. However, in most research that has been previously performed, the seaweed was extracted in either ethanol [10] or methanol [66], [69] before adding it to the film-forming solutions. The use of alcoholic extraction might result in other properties of the seaweed.

5.2.4.3 Alginate + HPP treatment + Seaweed

The combination of seaweed addition and HPP treatment had a significant effect ($p \leq 0.05$) on the scavenging activity of some of the alginate samples, generally an increase in the scavenging activity. No literature is available explaining the reasoning behind this. However,

HPP treatment has been found to decrease the antioxidant capacity in some seaweeds but causing small changes in others, mostly due to structural changes caused by HPP treatment [122].

However, separately, it has been found that both HPP treatment and addition of fillers increased the DPPH scavenging activity like previously mentioned. Thus, it is possible that combined they also have a positive effect on the antioxidant activity. Especially at high seaweed and alginate concentrations, HPP treatment resulted in scavenging activities of 70-80%, which is relatively high. So overall, alginate films with or without fillers and HPP treatment, are effective antioxidants and can protect against free radicals.

5.3 Antimicrobial properties

5.3.1 Liquid assay 37°C - Chitosan and Seaweed

Though the disc diffusion assay was unsuccessful to characterize the antimicrobial activity of the films developed in the present study, works in the literature have reported antimicrobial properties of chitosan films. For instance, Nataraj et al. (2018) reported that chitosan films prepared with acetic acid resulted in inhibition zones of 17 and 19 mm, when applied to *E. coli* and *Bacillus cereus*, respectively, on MHA. After crosslinking the film with citric acid (1%), the inhibition zones increased up to 23 and 26 mm for *E. coli* and *Bacillus cereus*, respectively [13]. Another study using filter discs embedded with the chitosan-in-acetic-acid sample, reported an inhibition zone of around 22 mm for *E. coli* and 23 mm for *S. aureus* on MHA [83]. On the other hand, another literature study reported no antimicrobial effect of chitosan films on *E. coli*, *S. aureus* or *Staphylococcus epidermidis* using the disc diffusion assay on nutrient agar [82]. In conclusion, the suitability of the antimicrobial activity of chitosan-based-systems seems to depend on the properties of the test item and the agar support, and the experimental set up (e.g., films vs. soaked discs).

No significant difference ($p > 0.05$) in the levels of *S. aureus* were observed after 24 h incubation at 37 °C as compared to the respective concentration of this bacterium in the control samples for the chitosan-citric acid sample, as well as for seaweed. For chitosan-

acetic acid samples, there was, however, a significant reduction ($p \leq 0.05$) in log concentration of *S. aureus* after 24 h incubation at 37 °C. On *E. coli*, the log concentration was significantly reduced ($p \leq 0.05$) for both chitosan samples (citric and acetic acid) after 24 h incubation at 37 °C. However, seaweed had no significant ($p > 0.05$) effect on the log concentration of *E. coli* after 24 h incubation at 37 °C.

Chitosan has been reported to be more effective on Gram-positive rather than Gram-negative bacteria due to the presence of lipid bilayers [13]. However, it is also reported the interaction of chitosan with bacteria is mainly based on the positively charged chitosan molecules reaction with the negatively charged cell membranes on the microbes. The outer membrane of Gram-negative bacteria consists mostly of lipopolysaccharides which contains phosphate and pyrophosphates, generating a negative charge which is higher than it is in Gram-negative bacteria due to the presence of peptidoglycan. Nevertheless, the effectiveness is controversial. Another theory is that the hydrophilicity in Gram-negative bacteria is higher than in Gram-positive bacteria, which makes the Gram-negative bacteria more sensitive to chitosan [123]. Other factors such as pH, temperature and culture medium also impact the potential antimicrobial effect of chitosan. *E. coli* grows in a temperature range of 4-45 °C, however, the optimum temperature of growth is 37 °C. Though *E. coli* can grow within the temperature range mentioned, it might be slowed by the stressful conditions. In addition, it can grow in acidic environments down to a pH of 3.6 [124]. Similarly, *S. aureus* can grow between 4 and 46 °C, although the optimum temperature is 37 °C. *S. aureus* is a bit more pH sensitive than *E. coli*, with a range of pH 4.8 to 8.0 [125].

This effect is also directly related to the concentration of chitosan [15], as well as the molecular weight. No et al. (2002) studied the effect of chitosan of different molecular weight (from 28 kDa to 1671 kDa) and prepared in acetic acid on several Gram-negative and Gram-positive bacteria. A log reduction of 3.11 CFU/mL was reported for *E. coli* after 24 h incubation at 37 °C in Mueller Hinton broth containing 0.1% (w/v) chitosan (224 kDa) [126]. The results obtained in the present work are consistent with the literature, although less inhibition was observed in the present study with a log reduction of 1.50 CFU/mL for chitosan with acetic acid as the solvent, and 2.89 CFU/mL for chitosan-citric acid.
No et al. (2002) also studied the effect of chitosan on *S. aureus*, reporting a log reduction of

6.64 CFU/mL (molecular weight 224 kDa) after 24 h incubation at 37 °C [126]. In the antimicrobial studies performed in the present work, only a small reduction of 0.26 CFU/mL was observed in the concentration of *S. aureus* for the chitosan films prepared with acetic acid, and no reduction for those with citric acid. However, it is noteworthy that, for the chitosan of medium molecular weight, No et al. (2002) reported a minimum inhibitory concentration of 0.1% (w/v) for *E. coli*, and 0.08% for *S. aureus* [126], values much higher than the final chitosan concentration (0.004% w/v) used in the present study.

Interestingly, almost complete inhibition of both *E. coli* and *S. aureus* at chitosan concentrations identical to that used in the present study were reported by Foster and Butt (2010), although a longer incubation period (36 h) at 37 °C was assayed [82].

The higher inhibitory effect on *E. coli* of chitosan films prepared with citric acid (2.89 log reduction), as compared to acetic acid (1.50 log reduction), could be attributed to the chitosan structure when dissolved in the acid, like described in the previous chapters, and thus its ability to bind to the cell exterior of the bacteria. The low pH of citric acid helps reduce the internal pH of the bacteria because of ionization of undissociated acid molecules, which in turn alters the permeability of the membrane [13]. However, because the pH of the chitosan-citric acid sample is adjusted to 5.2 ± 0.1 before adding the cell suspension and was thus the same as the chitosan-acetic acid sample, this is unlikely the cause.

No antimicrobial effect of seaweed on *E. coli* or *S. aureus* after incubation at 24 h at 37 °C was observed in the present study. However, antimicrobial activity of several species of seaweed at various concentrations has been shown against relevant foodborne pathogens, including *Listeria monocytogenes* and *Enterococcus faecalis* [24]. In addition, studies of different species of seaweed (not including *Laminaria hyperborea*) on *E. coli* and *S. aureus* showed promising results after 24 h incubation at 37 °C [65], and *Sargassum polycustum* and *Sargassum tenerrimum* after 24 incubation at 37 °C [88]. It has been reported that the antimicrobial effect of seaweed is highest when the leaves are harvested in the spring [91]. However, different species of seaweed was studied, in addition to it taking place in Egypt which has a widely different climate to where the seaweed was sampled in the present case, Norway.

Despite numerous studies reporting the antimicrobial properties of seaweed, no reduction in viable counts has been observed in this present case for both bacteria. Although different seaweed species could have different antimicrobial activities, a study conducted by Kadam et al. (2015) with an extract of *Laminaria hyperborea* concluded a minimum inhibitory concentration (MIC) of 21.0 mg/mL (solid-liquid extraction) after 24 h incubation at 37 °C, 45.6 mg/mL (ultrasound extraction) for *E. coli*, and 5.3 mg/mL (solid-liquid extraction) and 11.4 mg/mL (ultrasound extraction) for *S. aureus* [89]. It is noteworthy that the seaweed powder used in the present study was not subjected to the extraction processes reported by Kadam et al. (2015). Therefore, it is possible that higher concentrations of non-extracted seaweed, as compared to that used in the present work (10 mg/mL), would be necessary to cause significant inhibition, especially for *E. coli*.

5.3.2 Liquid assay 10 °C – Chitosan and Seaweed

In the present work, more pronounced log reduction for both *E. coli* and *S. aureus* were observed at 10 °C rather than 37 °C after incubation of chitosan samples, regardless of the solvent. Although No et al. (2006) reported that there was more reduction for *S. aureus* with chitosan than with *E. coli* [86], the opposite was seen in the present study. In the present work, the concentration of *E. coli* was below the detection limit (log 2) after 5 and 10 days incubation at 10 °C in both chitosan solutions. For *S. aureus*, acetic acid as chitosan solvent caused a more pronounced inhibitory effect than citric acid, especially after 10 days. However, the opposite trend, i.e., higher antimicrobial efficacy of citric acid as chitosan solvent, was observed at 37 °C. To the knowledge of the authors, there is not available scientific literature reporting this effect.

Tsai and Su (1999) reported that chitosan (150 ppm) actually had a more pronounced inhibitory effect on *E. coli* at higher temperatures than at lower ones. At both 4 and 15 °C, the levels of *E. coli* declined quickly within the first 5 hours ,and then stabilized, whereas at 37 °C, the negative effect was more significant. It was reported that *E. coli* cells may change the structure of the cell surface under cold temperatures, resulting in fewer available binding sites or electronegativity on the cell surface. However, they stress that there needs

to be more research done on the effect of temperature on the cell surface to draw any conclusion [84]. Studies on another bacteria, *L. monocytogenes*, concluded promising results at 4 °C for chitosan dissolved in acetic acid. Only 4 days were necessary to completely inhibit *L. monocytogenes* [85]. In addition, No et al. (2006) reported that chitosan prepared in acetic acid (final concentration of 0.05%) had inhibitory effects on both *E. coli* and *S. aureus* after storage at 4 °C for 15 weeks, although a much more pronounced effect was observed for *S. aureus* [86].

Although no inhibitory effect of seaweed on *E. coli* or *S. aureus* was seen at incubation temperature of 37 °C, some reduction in log concentration was observed after 5 and 10 days incubation at 10 °C for *S. aureus*. Scarce research has been conducted on how low temperature impacts the antimicrobial activity of seaweed. However, it is logical that the antimicrobial effect could be amplified by additional temperature stress. Despite this, since bacterial growth was observed in the control samples (TSB) incubated at 10 °C, the observed inhibitory effect on *S. aureus* is attributed to the addition of seaweed and not the temperature.

Interestingly, the inhibitory effect of chitosan combined with seaweed was lower for *E. coli* as compared to chitosan alone. On the other hand, the combination of chitosan and seaweed had a synergistic inhibitory effect on *S. aureus*, as compared to the separate effects of the individual agents. Thus, the concentration of *S. aureus* was not detected after 10 day incubation at 10 °C in the mixture. Other studies have also reported enhanced antimicrobial effect of chitosan when other antimicrobial substances were added to the films. For example, Pranoto et al. (2004) reported that incorporation of garlic oil to chitosan edible films enhanced the antimicrobial activity against *S. aureus*, *L. monocytogenes* and *B. cereus*. However, Pranoto et al. (2004) observed no inhibitory effect of garlic oil infused chitosan films on *E. coli* or *Salmonella typhimurium* [87]. Similarly, Zivanovic et al. (2005) observed that chitosan films infused with essential oils exhibited antimicrobial activity against *L. monocytogenes* and *E. coli*, with oregano oil having the highest inhibitory activity. Zivanovic et al. (2005) also reported that there were less effect on *E. coli* than the other pathogen, in this case *L. monocytogenes* [14]. They explain that this larger effect on Gram-

positive bacteria as compared with Gram-negative bacteria is because of the protective lipopolysaccharides or outer wall proteins [14], like presented previously.

6. Conclusion

Two natural biopolymers, namely chitosan and alginate, were studied for potential food packaging application in the current study. Both biopolymers showed promising properties in terms of potential usage within food packaging or the biomedical field. Though the chitosan films made by dissolving the polymer in acetic acid has a quite pungent and unpleasant smell, these films were shown to have better properties, which is what others have found as well. The chitosan films with acetic acid as the solvent were stronger and had longer elongation at breaks than did those with citric acid as a solvent. The barrier properties of the two films were similar, whereas the free amino group concentration was much greater in the acetic acid films which helps chitosan interact with nanoparticles, cells and polymers. In addition, the free radical scavenging activity was slightly higher in the chitosan in acetic acid films at higher chitosan concentrations. Nonetheless, both solvents proved to create films with relatively good antioxidant abilities.

The alginate films had a similar weight to the chitosan films, whereas they were thinner. However, the alginate films proved to be stronger, though with a smaller elongation at break. Like previously mentioned, alginate films are reported to have poor barrier properties, which was confirmed in the present study. The scavenging activity of the alginate films were relatively good at around 55-60%.

HPP treatment generally resulted in inferior or unchanged properties of the films, including strength, elongation at break, barrier properties and scavenging activity for both chitosan and alginate. However, HPP treatment significantly increased the free amino acid concentration in the chitosan films with citric acid, whereas for the ones with acetic acid as a solvent, the free amino acid concentration decreased. Seaweed addition resulted in weaker films, with better barrier properties of the alginate films and worse for the chitosan films. An improvement in scavenging activity was only seen at high concentrations of chitosan and seaweed, whereas for alginate the effect was generally positive. Others generally report

improvement in most properties with both added fillers and HPP treatment, which was not the case here, especially for chitosan. Because the result of the present study contradicts that of others, further research on the topic should be conducted.

The effect of seaweed addition in combination with HPP treatment had varying results. For chitosan, the effect was negative on both strength and barrier properties, with no effect on the scavenging activity. For alginate however, the scavenging activity increased. Nevertheless, no effect was seen on the barrier properties, and generally, weaker films was the result. Thus, overall, the chitosan films with no treatment and no fillers had better qualities. For alginate, positive results were seen for the barrier properties and scavenging activity for addition of seaweed in combination with HPP treatment.

Liquid-based antimicrobial studies showed that at 37 °C, chitosan samples prepared in both acetic and citric acid had a significant inhibitory effect on *E. coli*, although the growth of *S. aureus* remained unaffected. At 10 °C, both chitosan samples completely inhibited the growth of *E. coli* already after 5 days incubation, and a significant decrease was also observed in the *S. aureus* levels. In general, the inhibitory effect was more pronounced when acetic acid was used as a solvent in the preparation of chitosan films*. L. hyperborea* did not have an inhibitory effect on *E. coli*, regardless of the incubation temperature, although a significant reduction was observed in *S. aureus* levels at 10 °C. Overall, the combination of chitosan and seaweed had lower antimicrobial activity than chitosan on its own, but more pronounced than seaweed. Interestingly, a synergistic effect was observed for *S. aureus*, with complete inhibition after 10 day incubation at 10 °C in the mixture. Although liquid-based antimicrobial studies showed promising antimicrobial effects of chitosan and seaweed at a temperature a refrigerator might be at (10 °C), the main aim of this study was to look at films made from biopolymers within packaging. Thus, the results from the liquid-based assay cannot be directly related to the antimicrobial properties of films which are not dissolved.

7. Future recommendations

To further explore the properties of chitosan and alginate and their usage as packaging material, several steps can be taken. Mainly, the structural changes caused by HPP treatment for both polymers and all solvents should be studied by for example FTIR. This could help explain the observations in free amino group concentration and antioxidant properties in the samples after HPP treatment. In addition, the polymers could be further characterized. The molecular weight and degree of acetylation of the chitosan could be determined to further help explain the observed antimicrobial studies and antioxidant capability. In addition, the G/M ratio in the alginate could be determined, which also further explain the biopolymer´s antioxidant capabilities. Finally, most studies use seaweed extract as fillers in their biopolymers. Thus, it would be interesting to examine how seaweed with smaller particle size affects the properties of the biopolymers.

Liquid-based antimicrobial studies at 10 °C, which might resemble the consumer´s refrigerator temperature, showed promising results. However, if chitosan and seaweed is to be used as films in food packaging, an antimicrobial study using films instead of dissolved ones should be performed. Although the disc-diffusion assay was unsuccessful in this instance, this method could be developed further by for example placing something on top of the films to avoid lifting from the plate, in addition to applying the films to perishable foods such as fish and fruits. In vivo studies of the films made from both chitosan and alginate should be performed.

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Appendix

A1 Chitosan and alginate - Properties

Table 12. Overview of all chitosan and alginate films and their tensile strength, elongation at break, water vapor transmission rate, free amino group concentration (chitosan only) and scavenging activity.

Sample	TS (MPa)	EB (TS)	WVTR (g.m^{-2}.h^{-1})	Free amino group conc. (mg/mL)	Scavenging activity (%) 0.5, 1.0, 2.0 and 3.0 mg/mL			
CH-CS	35.55 ± 19.34	0.99 ± 0.63	49.36 ± 2.90	0.0644 ± 0.0015	51.53 ± 2.21	55.28 ± 2.23	54.45 ± 2.02	53.54 ± 1.42
CH-CS-200				0.1049 ± 0.0012	57.53 ± 1.79	54.47 ± 1.96	50.92 ± 2.15	49.85 ± 1.61
CH-CS-600				0.1075 ± 0.0012	58.44 ± 1.93	58.98 ± 1.49	57.42 ± 2.55	55.05 ± 1.16
CH-CA	68.81 ± 20.13	22.02 ± 14.06	56.06 ± 5.85	0.1807 ± 0.0030	56.86 ± 1.05	56.30 ± 0.61	57.17 ± 4.41	57.80 ± 2.63
CH-CA-200	73.26 ± 17.54	5.87 ± 3.16	88.24 ± 4.12	0.1343 ± 0.0017	55.72 ± 2.70	54.46 ± 1.39	56.92 ± 2.07	57.95 ± 1.67
CH-CA-600	86.02 ± 33.13	7.65 ± 5.54	81.73 ± 2.54	0.1395 ± 0.0032	54.86 ± 1.41	55.40 ± 0.67	57.21 ± 1.99	58.56 ± 2.44
CH-CA-10SW	48.12 ± 21.01	4.22 ± 1.27	98.65 ± 15.14		50.34 ± 0.50	51.44 ± 2.73	50.34 ± 1.49	56.96 ± 3.02
CH-CA-10SW-200	35.03 ± 11.90	3.38 ± 1.12	78.78 ± 7.43		51.07 ± 2.15	49.66 ± 1.08	53.62 ± 3.08	53.47 ± 1.22
CH-CA-10SW-600	32.59 ± 7.20	3.53 ± 0.92	94.97 ± 22.53		50.30 ± 2.26	49.61 ± 3.06	53.50 ± 2.70	53.66 ± 2.54
CH-CA-30SW	35.42 ± 11.33	3.36 ± 1.19	95.93 ± 6.57		50.91 ± 1.25	48.57 ± 2.10	56.04 ± 1.72	58.60 ± 2.09
CH-CA-30SW-200	21.25 ± 5.46	2.13 ± 0.62	87.18 ± 6.89		50.09 ± 1.49	50.23 ± 3.14	53.44 ± 3.03	57.47 ± 2.77
CH-CA-30SW-600	23.78 ± 7.31	2.47 ± 0.61	83.35 ± 6.66		48.98 ± 3.63	49.76 ± 5.32	52.01 ± 2.59	60.82 ± 2.25

CH-CA-50SW	19.76 ± 7.43	2.22 ± 0.67	85.35 ± 2.76		52.04 ± 2.56	49.58 ± 2.41	50.62 ± 5.17	64.83 ± 1.54
CH-CA-50SW-200	9.50 ± 6.97	1.43 ± 0.63	91.06 ± 4.12		52.90 ± 1.37	51.50 ± 2.50	59.94 ± 4.38	62.54 ± 4.66
CH-CA-50SW-600	11.63 ± 8.35	2.09 ± 0.82	91.41 ± 2.18		51.22 ± 1.04	54.12 ± 2.77	51.66 ± 4.97	66.82 ± 3.38
A	74.55 ± 39.63	5.53 ± 2.21	122.24 ± 13.24		58.94 ± 1.78	60.24 ± 1.15	59.97 ± 0.84	61.63 ± 1.86
A-200	97.47 ± 29.27	4.29 ± 1.92	145.67 ± 2.49		58.44 ± 1.20	58.36 ± 1.00	60.41 ± 1.14	60.67 ± 1.26
A-600	82.61 ± 33.33	4.22 ± 2.11	133.78 ± 24.11		55.39 ± 1.90	55.45 ± 1.80	56.01 ± 2.91	58.88 ± 1.20
A-10SW	74.52 ± 31.92	3.85 ± 0.85	114.71 ± 1.86		53.84 ± 0.89	62.29 ± 2.09	59.13 ± 2.58	68.17 ± 1.91
A-10SW-200	69.37 ± 19.05	3.08 ± 0.61	99.52 ± 3.76		60.34 ± 1.51	63.31 ± 0.77	67.18 ± 1.78	68.45 ± 0.94
A-10SW-600	77.72 ± 20.59	3.59 ± 0.60	100.45 ± 8.07		60.50 ± 2.10	63.00 ± 1.01	67.68 ± 1.06	69.78 ± 1.91
A-30SW	59.84 ± 14.49	3.55 ± 0.81	93.04 ± 8.93		65.93 ± 2.19	71.10 ± 1.38	70.70 ± 2.43	69.74 ± 2.19
A-30SW-200	65.16 ± 22.83	3.02 ± 0.97	91.44 ± 4.22		63.44 ± 2.85	68.38 ± 1.82	79.04 ± 0.90	67.92 ± 1.74
A-30SW-600	64.95 ± 25.31	3.07 ± 0.95	97.53 ± 3.13		66.92 ± 2.29	69.74 ± 2.58	75.20 ± 1.34	77.61 ± 2.95
A-50SW	68.01 ± 26.58	3.91 ± 1.11	87.40 ± 13.37		67.84 ± 1.12	63.18 ± 2.26	61.65 ± 5.64	63.19 ± 1.60
A-50SW-200	61.11 ± 22.84	2.71 ± 1.08	127.24 ± 5.26		69.76 ± 1.08	67.84 ± 2.78	63.67 ± 0.83	69.31 ± 2.33
A-50SW-600	45.72 ± 17.19	2.46 ± 0.89	109.17 ± 6.98		72.44 ± 1.77	70.63 ± 1.16	63.27 ± 2.54	77.97 ± 2.58

A2 Antimicrobial properties

A2.1 Preparation of media

Tryptic soya broth (TSB)

15 g of TSB was dissolved in 500 mL distilled water. The medium was heated until boiling while subjected to magnetic stirring. Using a digital pH meter, the pH was adjusted to 7.1 ± 0.1 using NaOH. Finally, the medium was sterilized by autoclaving at 121°C and 15 psi pressure for 15 min and cooled down before storage at 4°C.

Peptone water

0.5 g of peptone water was dissolved in 500 mL distilled water using magnetic stirring. Using a digital pH meter, the pH was adjusted to 7.1 ± 0.1 using NaOH. Finally, the medium was sterilized by autoclaving at 121°C for 15 minutes and cooled down before storage at 4°C.

Saline solution, 0.9% (w/v)

4.5 g of laboratory grade NaCl was placed in a 500 mL volumetric flask and dissolved in distilled water. The solution was transferred to a Scotch bottle and sterilized by autoclaving at 121 °C for 15 min. For long time storage, the medium was best kept cool at 4 °C.

Plate count agar (PCA)

12.5 g of PCA was dissolved in 500 mL distilled water. The medium was heated until boiling while subjected to magnetic stirring and the pH adjusted to 7.1 ± 0.1 with HCl. The medium was sterilized by autoclaving at 121°C for 15 minutes, before cooling down to 45°C. About 15 mL of the agar was poured in 90 mm diameter sterile Petri dishes and allowed to dry in the laminar flow cabinet. The plates were bagged and stored at 4 °C for up to 30 days.

Tryptic soya agar (TSA)

20 g of TSA was dissolved in 500 mL distilled water. The medium was heated until boiling while subjected to magnetic stirring and the pH adjusted to 7.1 ± 0.1 with HCl. The medium was sterilized by autoclaving at 121°C for 15 minutes, before cooling down to 45°C.

About 15 mL of the agar was poured in 90 mm diameter sterile Petri dishes and allowed to dry in the laminar flow cabinet. The plates were bagged and stored at 4°C for up to 30 days.

Mueller-Hinton agar (MHA)

17 g of MHA was dissolved in 500 mL distilled water. The medium was heated until boiling while subjected to magnetic stirring and the pH adjusted to 7.1 ± 0.1 with HCl. The medium was sterilized by autoclaving at 121°C for 15 minutes, before cooling down to 45°C. About 15 mL of the agar was poured in 90 mm diameter sterile Petri dishes and allowed to dry in the laminar flow cabinet. The plates were bagged and stored at 4°C for up to 30 days.

A2.2 Liquid-based assays

Table 13. Log concentration of *E. coli* in the chitosan and seaweed samples before and after 24 h incubation at 37 °C, with pH values. pH value of the controls is an average of all assays performed at 37 °C. NM – not measured

Sample with *E. coli*	**t = 0**		**t = 24 h (37 °C)**	
	Log CFU/mL	**pH**	**Log CFU/mL**	**pH**
Control (TSB)	6.71 ± 0.10	7.06	9.06 ± 0.09	6.59
Chitosan – citric acid	6.71 ± 0.10	6.83	6.17 ± 0.77	6.65
Chitosan – acetic acid	6.71 ± 0.10	7.15	7.56 ± 0.42	6.88
Alginate film w/ 50% seaweed	6.71 ± 0.10	7.37	9.33 ± 0.15	7.34
TSB + 10 mg/mL seaweed	6.71 ± 0.10	NM	9.28 ± 0.17	NM

Table 14. Log concentration of *S. aureus* in the chitosan and seaweed samples before and after 24 h incubation at 37 °C, with pH values. pH of the controls is an average of all assays performed at 37 °C. NM – not measured

Sample with *S. aureus*	**t = 0**		**t = 24 h (37 °C)**	
	Log CFU/mL	**pH**	**Log CFU/mL**	**pH**
Control (TSB)	6.90 ± 0.13	7.03	8.60 ± 0.29	6.62
Chitosan – citric acid	6.90 ± 0.13	6.95	8.54 ± 1.03	6.31
Chitosan – acetic acid	6.90 ± 0.13	7.13	8.34 ± 0.24	6.72
Alginate film w/ 50% seaweed	6.90 ± 0.13	7.34	8.34 ± 0.15	7.35
TSB + 10 mg/mL seaweed	6.90 ± 0.13	NM	8.79 ± 0.14	NM

Table 15. Log concentrations of *E. coli* in the chitosan and seaweed samples before and after 5 and 10 days incubation at 10 °C, with corresponding pH values. pH value of controls is an average of all assays performed at 10 °C.

Sample with *E. coli*	**t = 0**		**t = 5 days (10 °C)**		**t = 10 days (10 °C)**	
	Log CFU/mL	**pH**	**Log CFU/mL**	**pH**	**Log CFU/mL**	**pH**
Control (TSB)	6.74 ± 0.16	7.20	8.01 ± 0.11	6.44	8.35 ± 0.19	6.86
Chitosan – citric acid	6.74 ± 0.16	6.90	0	6.66	0	7.06
Chitosan – acetic acid	6.74 ± 0.16	7.04	0	6.84	0	6.95
TSB + 10 mg/mL seaweed	6.74 ± 0.16	7.82	8.31 ± 0.16	6.69	8.67 ± 0.17	6.96
Chitosan – acetic acid + 10 mg/mL seaweed	6.74 ± 0.16	7.35	3.41 ± 0.05	7.05	4.58 ± 0.86	6.94

Table 16. Log concentrations of *S. aureus* in the chitosan and seaweed samples before and after 5 and 10 days incubatoin at 10 °C, with corresponding pH values. pH value of the controls is an average of all assays performed at 10 °C.

Sample with *S. aureus*	**t = 0**		**t = 5 days (10 °C)**		**t = 10 days (10 °C)**	
	Log CFU/mL	**pH**	**Log CFU/mL**	**pH**	**Log CFU/mL**	**pH**
Control (TSB)	7.00 ± 0.08	7.17	6.84 ± 0.28	7.12	6.78 ± 0.29	6.85
Chitosan – citric acid	7.00 ± 0.08	6.92	5.02 ± 0.16	6.77	5.56 ± 0.50	7.01
Chitosan – acetic acid	7.00 ± 0.08	7.08	4.71 ± 0.47	6.81	4.58 ±0.49	7.01
TSB + 10 mg/mL seaweed	7.00 ± 0.08	7.68	5.61 ± 0.08	7.76	5.31 ± 0.14	6.91
Chitosan – acetic acid + 10 mg/mL seaweed	7.00 ± 0.08	7.10	3.74 ± 0.25	7.00	0	6.94