CHALMERS

Presence of phenolic compounds in acetone extracts from *Saccharina latissima* and their antibacterial and ferrous ion-chelating activities

Förekomst av fenoliska föreningar i acetonextrakt från *Saccharina latissima* och deras antibakteriella och järnjonskelaterande aktiviteter

Degree project

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ABSTRACT

An increased interest to use biomass for alternative fuels has led to a competition between crops for energy production and food. The larger demand for food crops is causing a growing concern about food scarcity, food price as well as pollution of agricultural land. This has led to researchers looking at algae for a solution. Algae provides a significantly higher production yield per unit area compared to terrestrial biomass and contains polysaccharides, proteins, lipids as well as other high value products. They can also be cultivated in most forms of water, sea water and municipal waste water being the most interesting. These qualities combined with the lack of competition with food production make algae an interesting area to explore.

The aim of this project was to investigate acetone extracts from the seaweed *Saccharina latissima* for the presence of phenolic compounds, which have potential future uses as food preservation additives and in the medicinal sector. Apart from investigating the presence of phenolic compounds, the aim was also to investigate the extracts' ferrous ion-chelating- and anti-bacterial activities as well as the difference between three seasonal variations (June, August and October) and three locations (Ulvillarna, Ursholmen and Yttre Vattenholmen). There were, however, storms during October resulting in Ulvillarna and Ursholmen having no seaweeds left. Extraction was done with 70 % aqueous acetone using freeze dried algae biomass. Quantification of total phenolic content was done with the folin-ciocalteu reagent. Ferrous ion-chelating activity was done using $FeCl_2$ and ferrozine, and the anti-bacterial activity was done on two bacterial strains, *Escherichia coli* and *Klebsiella oxytoca*, using filter discs dotted with algae extract and put on bacterial lawns in petri dishes.

The generally largest concentration of phenolic content was found in June and in samples from the collection site Yttre Vattenholmen. Yttre Vattenholmen extracts from October had close to the same average amount of phenolic content to those of August. The ferrous ion-chelating activity of the extracts were the strongest during October but showed no clear linear correlation with total phenolic content. The anti-bacterial activity did not show any clear growth inhibiting effect on either bacterial strain.

Sammanfattning

Ett ökat intresse för användandet av biomassa till alternativa bränslen har lett till en ökad konkurrens om livsmedelsgrödor mellan energiproduktion och mat. Den större efterfrågan på livsmedelsgrödor skapar en växande oro för matbrist, höjda matpriser samt förorening av jordbruksmarker. Detta har lett till att forskare tittar på alger som en alternativ biomassa. Alger ger en betydligt högre produktionsavkastning per ytenhet jämfört med markbunden biomassa och innehåller polysackarider, proteiner, lipider samtidigt som de innehåller andra högvärdiga produkter. De kan också odlas i de flesta former av vatten där havsvatten och kommunalt avloppsvatten är de mest intressanta. Dessa egenskaper i kombination med en obefintlig konkurrens med livsmedelsproduktion gör alger till ett intressant område att utforska.

Projektets mål var att undersöka acetonextrakt från tången *Saccharina latissima* med avseende på förekomsten av fenoliska ämnen, vilka har potentiella framtida användningsområden som konserveringsmedel och för medicinska syften. Utöver förekomsten av fenoliska ämnen undersöktes också extraktens järnjonskelaterande förmåga, antibakteriella egenskaper och hur extraktens egenskaper varierade med tre olika säsonger (juni, juli och augusti) och skördeplatser (Ulvillarna, Ursholmen och Yttre Vattenholmen). Under oktober var det höststormar som resulterade i att Ulvillarna och Ursholmen inte hade någon tång kvar. Extraktionen gjordes med 70 % aceton och totalfenolhalten mättes med folin-ciocalteu reagens. Järnjonskelaterande förmåga mättes med $FeCl_2$ och ferrozin, och den antibakteriella aktiviteten gjordes på två olika bakteriestammar, *Escherichia coli* och *Klebsiella oxytoca* med hjälp av filterdiskar impregnerade med algextrakt som sedan placerades på bakterieodlingar i petriskålar.

Den generellt högsta koncentrationen av fenoliska föreningar i extrakten fanns i juni och i prover från Yttre Vattenholmen. Yttre Vattenholmens extraktkoncentrationer under oktober var ungefärligt lika höga som de under augusti. Järnjonskelaterande aktiviteten hos extrakten var högst under oktober men de visade inte på någon tydlig linjär korrelation med totalfenolhalten. Det antibakteriella testet visade inte på någon tydlig växtinhiberande aktivitet för varken *E. coli* eller *K. oxytoca*.

Contents

1. Introduction

There is an increased interest to use terrestrial biomass for production of alternative fuels and other products. To meet this end, biorefineries producing a wide range of products from biomass, are developed which are utilizing mainly agricultural crops. This development has led to intensified competition for resources between food and energy production. Since the fuel applications create a larger demand for food crops there is a growing concern about food scarcity, higher food price as well as pollution of agricultural land (Rojan JP *et al*., 2011), contributing to the unstable global economies. However, not only the economy has been affected but the environment as well. The direct or indirect use of land for energy crop cultivation leads to both a larger carbon debt and water consumption (Fargione J *et al.,* 2008; Dominguez-Faus *et al.,* 2009). In addition, the increased demand for arable land may lead to exploitation of valuable ecosystems such as the already threatened rainforests. With a large and growing population, this is not sustainable, which has led researchers to look at alternatives to the use of terrestrial biomass. Biomass provided from algae is therefore an interesting alternative.

Algae are, just like plants, photosynthetic organisms. It is a very large and diverse group ranging from simple unicellular to multicellular organisms able to grow in most forms of water, such as fresh, sea and municipal waste water with the two latter being an especially important quality (Rojan JP *et al*., 2011). The largest and most complex forms of algae are called seaweeds. Seaweeds have a significantly higher production yield per unit area compared to terrestrial biomass (Brinkhuis BH *et al*., 1987; Gao K *et al*., 1994) because of their rapid synthesis of biomass through conversion of sunlight, carbon dioxide and inorganic nutrients (Subhadra B *et* al., 2010). Furthermore, seaweeds do not require irrigation, fertilization or pesticide applications in contrast to terrestrial plants. These abilities make marine autotrophs very attractive since there will not be a competition with food crops requiring fresh water nor any potential environmental hazards from pesticides while providing a more substantial amount of biomass.

There are three different types of seaweeds that can be grouped in red, green and brown seaweeds. Archeological finds in Chile show that the use of seaweeds by humans date as far back as 14000 years ago (Dillehay TD *et al*., 2008) although the cultivation of macroalgae in the sea did not start until 1600-1800 AD in Japan (Tamura T, 1966). During the last 50 years the increasing demand of seaweed has exceeded the ability to supply it from natural stocks which has led to a dramatic growth from aquaculture sources. Traditionally, seaweeds have been used as a direct source of food but are now also an important source of food additives, hydrocolloids and for other applications.

Brown seaweeds (kelps), such as *Saccharina latissima,* are as of now harvested in large volumes every year for the production of alginate but there are many other potential future uses. These include production of high-value biologically active agents, such as phlorotannins, that have been linked to several disease inhibiting activities. Altogether, this has led to a higher demand of kelp.

The amount of high-value compounds in seaweeds varies depending on environmental stress factors and seasons, which has yet to be fully understood, requiring more research to be done in this field.

In this project samples of *Saccharina latissima* from the western shore of Sweden were collected at three different dates and locations. The aim of the project was to study the amount of phenolic compounds extracted by the acetone, how the concentration varies depending on seasons and locations and the ferrous ion-chelating- and anti-bacterial activity of the extracts. Since the western shore of Sweden offer a unique marine environment, the composition of the kelps may vary and could provide either more or less of high-value products which is important to know for seaweed farming.

2. Background

2.1 Seaweeds

Seaweeds have been used by man for a long time. Archeological finds in Chile suggest that it dates at least as far back as 14000 years ago (Dillehay TD *et al*., 2008) but the earliest written record tell that they were consumed approximately 1500 years ago in Japan during the Asukaand Nara Era. When the fishermen started to use artificial substrates for fish farming in Japan, 1600-1800 AD, those substrates inadvertently allowed for various seaweeds to grow on them. Since then seaweed have been cultivated in sea (Tamura T, 1966) but during the last 50 years, the increasing demand of seaweed has exceeded the ability to supply it from natural stocks which has led to a dramatic production growth from aquaculture sources.

As of now, farmed seaweeds have many applications. They are used for food, pharmaceuticals, textiles, cosmetics and in the biotechnological industry (Bartsch I *et al*., 2008). Their present most important use, as it has been for thousands of years, is the food sector. In the Asia-Pacific region direct consumption is the most common, but worldwide the algal hydrocolloids are used as emulsifying, gelling or water retention agents in a large variety of food items (Dillehay TD *et al*., 2008; Indergard M *et al*., 1991; Murata M and Nakazoe J, 2001). Hydrocolloids comprise carrageen, alginates and agar, and in 2009 a total amount of 86100 tons algal hydrocolloids were traded globally (Baxter HJ and Porse H, 2011). Although they are used in great deal as food thickener (among other), agar is indispensable in the medical bacteriology and microbial research as a solid culture medium (Figure 1). This is due to the inability of most microorganisms to break down the polysaccharide.

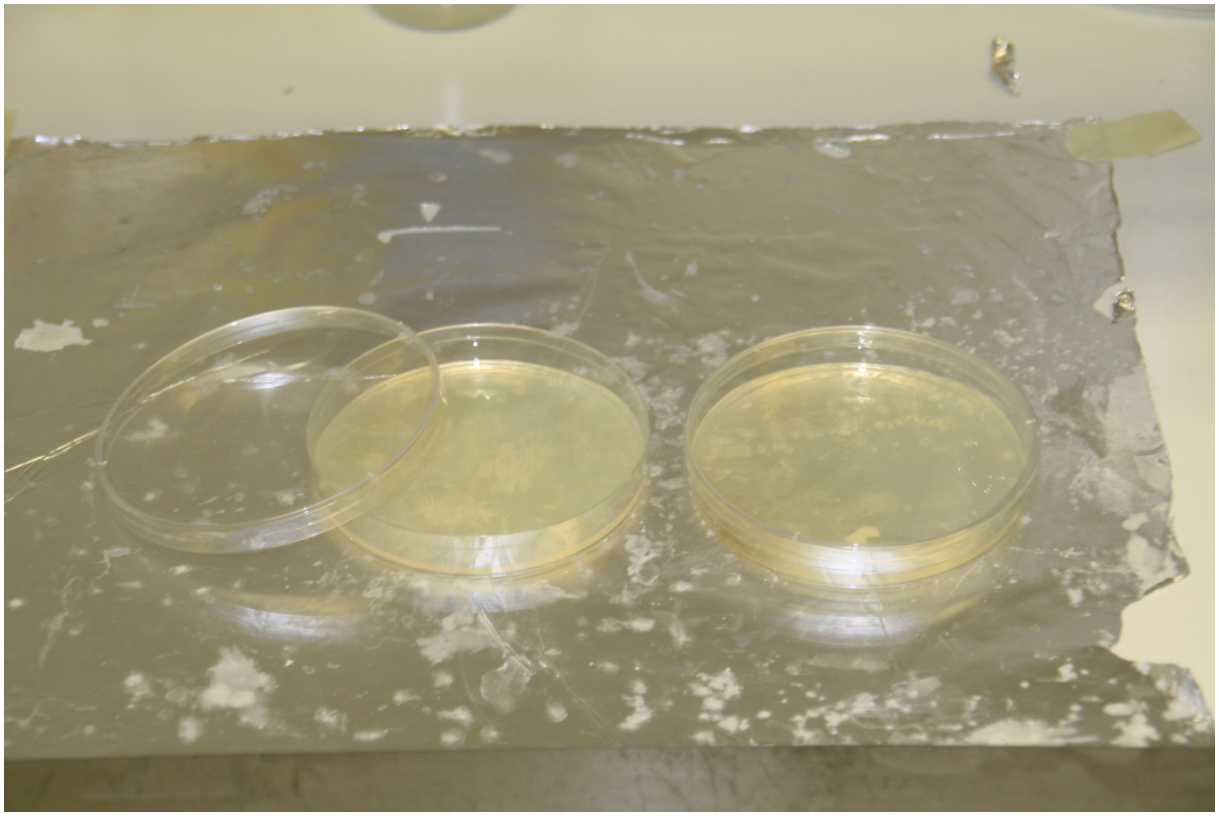

Figure 1 Agar dishes used to grow bacterial colonies on.

2.2 *Saccharina latissima*

Saccharina latissima is a kelp species that iscommon around Scandinavia. Kelp species are the only algae containing phlorotannins, which are phenolic compounds of interest given their biological activities (See further section 2.3).

Figure 2 Western Shore of Sweden. *Google maps.*

There can be significant variations in TPC content in kelp according to several studies. These variations seems to correlate to environmental factors such as salinity, water depth, light and nutrient availability, as well as plant factors such as age, tissue type and plant size (Pavia H and Toth GB, 2000). Variations also seem to depend on herbivore grazing intensity and mechanical wounding (Hemmi A *et al.*, 2005).

Studies on seasonal variations report that the TPC levels are generally higher in the summer and early fall compared to the amounts during winter (Connan S *et al.*, 2004). Sun-exposed kelps, compared to their shaded counterparts, have also been found to have substantially higher TPC which suggests that phlorotannins have important photoprotective roles (Pavia H and Toth GB, 2000).

This project focuses on *Saccharina latissima* collected at Tjärnö, Stömstad (Figure 2), since it is one of the most common kelp species around Sweden and because the western shores of Sweden offer a unique marine environment. This is due to the waters from the Baltic Sea flowing through the southern shore of Sweden and along the western coast mixing with the waters coming in from the northern sea. The average salinity level in the North Sea is 35 ppm while the Baltic Sea's salinity level averages at around 6 to 8 ppm. The mixture of these two waters creates different conditions for the species living there, forcing them to adapt and it is thus an interesting area to explore how they might differ from other *S. latissima* specimens found elsewhere, such as in Iceland or Spain, in terms of composition.

Moreover, the collection has been done at 3 different harvest sites (Figure 3) offering different environmental stress factors. The currents at these sites may vary, causing different levels of mechanical wounding, as well as a varying depth, sun exposure and herbivore grazing intensity which may result in varying concentrations of TPC in the samples.

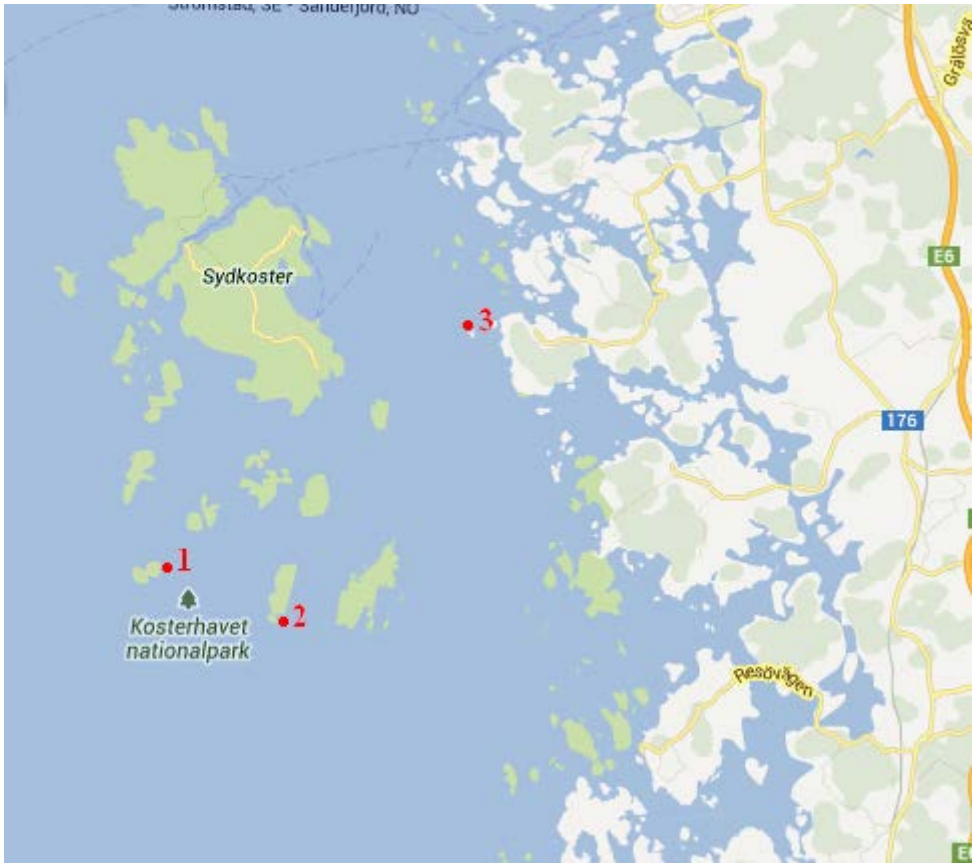

Figure 3 Sample harvest site. 1; Ursholmen, 2; Ulvillarna, 3; Yttre Vattenholmen. *Google maps*.

2.3 Phlorotannins in seaweeds

Naturally occurring phlorotannins (Figure 4) are oligomers and polymers of phloroglucinol only found in brown algae. The structure and molecular weight of these can differ greatly, ranging from 126 Da to 650 Da. The structural difference may also depend on different developmental stages in the kelp. They can occur in algal cells of young thallus in the form of short oligomers that may later, during development, convert into more complex and longer polymeric phlorotannins (Kovikko R *et al*., 2005).

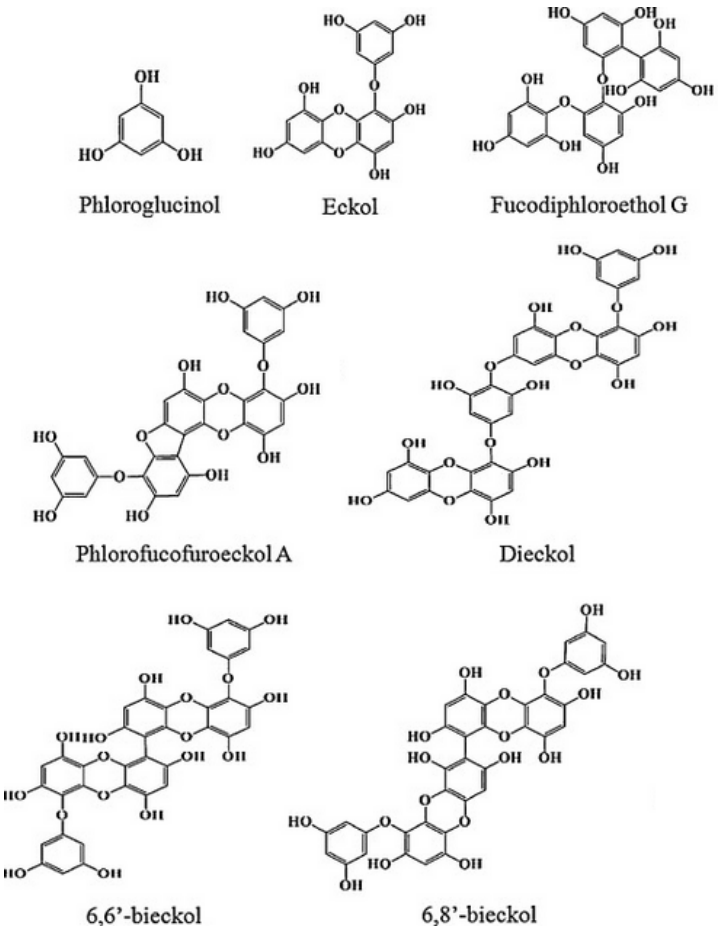

Figure 4 Examples of different phlorotannins.

The potential biological benefits of these phenols are many. Studies show that they can be anti-diabetic, anti-cancer, angiotensin-converting-enzyme (ACE) inhibitory, photo ageing preventive, matrix metalloproteinase inhibitory, reactive oxygen species scavenging, anti-HIV, anti-pruritic inflammatory etc. (Thomas NV and Se-Kwon K, 2011).

The radical scavenging activity of several phlorotannin derivatives, including eckol, phlorofucofuroeckol A, dieckol and 8,8'-bieckol, was evaluated in several antioxidant assays (Shibata T *et al*., 2008). The phlorotannin scavenging activities of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical anion were 2 to 10 times higher than that of the commercially available antioxidants catechin, α-tocopherol and ascorbic acid. The phlorotannins were also shown to have significant protective effects against peroxidation of phospholipid liposomes.

Phenolic compounds have generally been shown to be the main contributors to the antioxidant activity of various seaweed extracts since there is a positive correlation between the antioxidant activity of different seaweed extracts and their total phenolic content (TPC) (Nagai T and Yukimoto T, 2003). In brown algae, the only phenolic compounds detected have been shown to be phlorotannins according to many studies (Jormalainen V and Honkanen T, 2004; Koivikko R *et al.*, 2007).

2.4 Antioxidants

Since aerobic organisms use oxygen to support their energy supply, destructive oxygen radicals are continuously formed. It is a result of the respiratory chain in the mitochondria leaking reactive superoxide radicals (Petersson G, 2012) or of ionizing radiation (UV light from the sun) interacting with water molecules, creating hydroxyl radicals (Figure 5) (Birgeson B *et al.*, 2010).

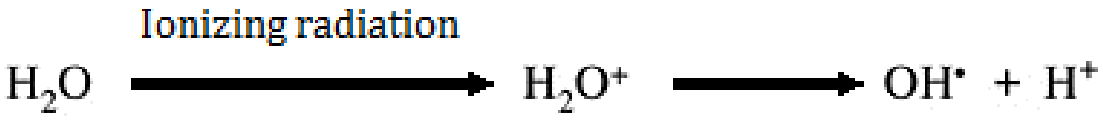

Figure 5 Ionizing radiation interacting with a water molecule creating a hydroxyl radical.

The body has developed a defense against these radicals in the form of effective enzymes (e.g. superoxide dismutase and catalase) but they must also be complemented by non-enzymatic antixoidants. Examples of well-known antioxidants are L-ascorbic acid (vitamin C), α-tocopherol (Vitamin E), flavonoids and carotenoids (Figure 6). These vary in structure and hydro- and lipophilic solubility.

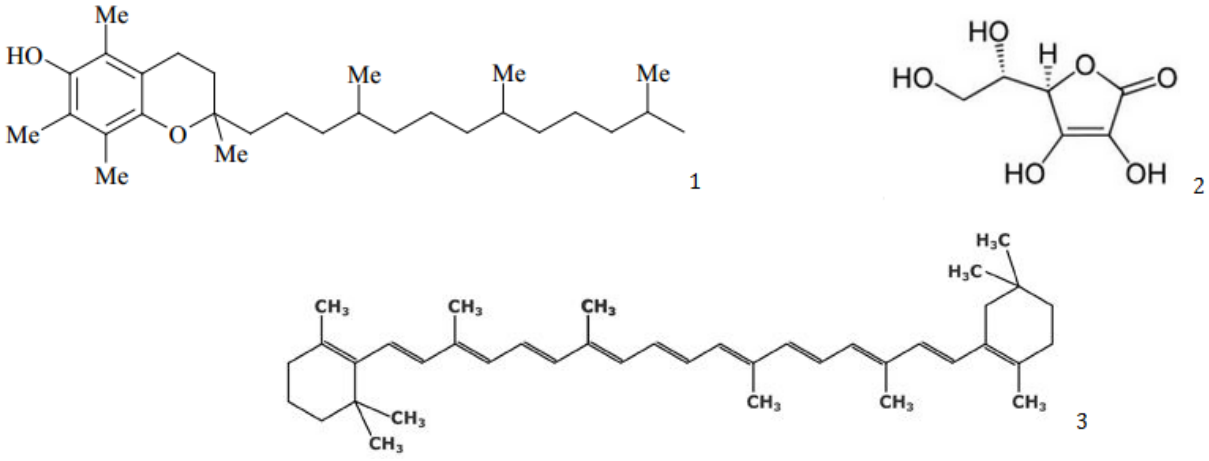

Figure 6 Antioxidants; 1: α-tocopherol, 2: L-ascorbic acid, 3: β-carotene.

The difference in hydro- and lipophilic solubility gives the antioxidants different functions and areas of effect. Since α-tocopherol has one nonpolar and one polar end, the long carbon chain penetrates into the biological lipophilic membranes with the polar end pointed outwards toward the aqueous solution of the cytoplasm or the blood plasma. The antioxidant stops lipid peroxidation since polar peroxyl radicals tend to move towards the membrane surface, where the phenolic group is located, and cause the OH-group to react with the radical. The newly created phenoxyl radical is then stabilized by the oxygen ring and methyl groups (Petersson G, 2012). L-ascorbic acid is water soluble and is thus able to stop radicals in the cytoplasm or blood plasma. β-carotene, also found in cellular membranes, has several double bonds in the structure with which it can bind peroxyl radicals and is then stabilized by the adjacent double bond.

Ion-chelating compounds may indirectly be categorized as antioxidants since, for example, ferrous iron(II) can become oxidized by hydrogen peroxide to ferric iron(III) creating a hydroxyl radical and hydroxyl anion. Iron(III) is then, by hydrogen peroxide, again reduced back to iron(II) creating another peroxide radical and an additional proton.

$$(1)\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-}$$
$$(2)\mathbf{Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+}$$

Several methods measuring antioxidant activities have been developed targeting different mechanisms.

Oxygen radical absorbance capacity (ORAC) is a method that relies on the degradation of a fluorescent molecule (beta-phycoerythrin or fluorescein) mixed with free radical generators. Antioxidants protect the fluorescent molecule from the formed free radicals that would otherwise damage it, causing it to loose fluorescence.

DPPH (diphenylpicrylhydrazyl) is a method named after the molecule. DPPH is a radical with an absorbance at around 520 nm and has a deep violet color in solution. When DPPH reacts with antioxidants the solution becomes colorless or pale yellow.

The assay for ferrous ion-chelating capacity is described in section 2.7.

2.5 Extraction of polyphenols

While phenolic groups have different affinity toward different solvents (ranging from very polar such as water to nonpolar such as hexane) resulting in not all phenolic groups being extracted, acetone has been proven to be the most efficient extraction medium for polyphenols in several kelp species (Koivikko R *et al.,* 2005). This is due to acetone's presumable ability to inhibit protein-polyphenol complexes during extraction (Hagerman AE, 1988) or its ability to break down hydrogen bonds that are formed between protein carboxyl groups and phenolic groups (Kallithraka S *et al.,* 1995).

2.6 Total phenolic content

In order to analyze the total amount of phenolic groups in an extract, the Total Phenolic content (TPC) method can be applied. In this method the folin-fiocalteu (FC) reagent is used. Under alkaline conditions, electron-transfer reactions occur between phenolic compounds and molybdenum causing formations of blue complexes that can be spectrophotometrically detected in the 725-765 nm range (Magalhães LM, 2008). Advantages of this assay are that it is easy to perform, inexpensive and it is reproducible. It is, however, not suitable for lipophilic antioxidants given that the reactions take place in aqueous media. Another disadvantage with the method is that a range of non-phenolic compounds may interfere with the assay resulting in an overestimation of TPC.

2.7 Ferrous ion-chelating activity

The absorbance of the stable complex created by sulfonated ferroin (ferrozine) combined with ferrous iron is directly proportional to the concentration of iron in a sample. The assay has the advantages of being simple to perform; It is inexpensive and has satisfactory reproducibility. The disadvantage with this method is that it does not evaluate chemicals affinity towards iron ions, availability of coordination sites, rigidity of ligand conformations and stoichiometry of complex formation (Moore J and Yu L, 2008). The formation of the ferrous-ferrozine complex is also a time-dependent reaction and thus requires consistent timing between sample addition, mixing and absorbance measurement for optimal reproducibility.

2.8 Anti-bacterial activity

Bacterial growth is linear and easily detected by cultivation on petri plates. Halo assays, in which bacterial growth inhibiting effects can be noticed by a visible halo around the tested chemicals, are therefore easily reproducible and they are inexpensive. The major disadvantage with the method is that readily available bacterial strains may be resistant against the chemicals, demanding a more hazardous strain to be used that may also be expensive and demand harsher regulations which in turn consumes more time.

3 Material and methods

3.4 Kelp biomass sample collection and preparation

Six specimens of the brown algae *Saccharina latissima* were collected at each sampling time at three different sites close to Tjärnö, Strömstad, Sweden in collaboration with scientists at the Marine Station, Tjärnö. At two locations, Ulvillarna (UL) and Ursholmen (UR), samples were collected at two different dates (5/6-2012 and 8/8-2012) and at one location, Yttre Vattenholmen (VH), samples were collected at three different dates (5/6-2012, 8/8-2012 and 23/10-2012). Unfortunately, due to storms during fall, there were no kelp samples to collect at UL and UR in October. After collection of kelp samples the whole blades were put in a -20 °C freezer within 8 h and temporarily stored at -20 °C. After the last collection date, the blades (stipes were removed) were crushed and homogenized with a mortal in liquid nitrogen and stored at -80 °C. The algae were freeze-dried and ground into a fine powder with a mortal before going ahead with further extraction and analyses.

3.5 Acetone extraction of kelp biomass

For extraction of phlorotannins, acetone was used. 0.25 g of freeze dried kelp biomass in 0.75 ml of Milli-Q water was homogenized using a FastPrep-24 (MP) with 0.2 g glass beads at speed 6.5 m/s, for 40 s and repeated 8 times. Subsequently 0.75 ml Milli-Q water and 3.5 ml acetone were added to create a 70:30 acetone:water ratio for extraction. The samples were incubated in a rotating wheel overnight at room temperature. After the incubation, the samples were centrifuged at 3000 g for 5 minutes and the supernatants were transferred to glass vials and stored at -80 °C until analysis.

3.6 Analysis of total phenolic content

A standard curve was done using phloroglucinol at concentrations 20, 50, 80 and 100 µg/ml. The analysis were done by mixing 100 µl of sample/standard with 50 µl folin-ciocalteu reagent (10% in MilliQ water, Sigma-Aldrich) and 50 µl $NaHCO_3$ (7.5% in MilliQ water) (Sigma-Aldrich) directly in a microplate. The microplate was then read at 765 nm in a plate reader (Safire2, Tecan) 3 times with a shaking step of 5 s before each measuring cycle. The results were expressed as phloroglucinol equivalents/dry algae powder.

3.7 Anti-bacterial activity of extracts

3.7.1 Bacteria, media and cultivation conditions

The bacteria *Escherichia coli* (Escherichia coli DH5α, Department of Chemical and Biological Engineering, Chalmers) and *Klebsiella oxytoca* (Klebsiella oxytoca SIK307, Department of Chemical and Biological Engineering, Chalmers) were used to test for anti-bacterial activity. Both species were prior to use cultivated in shake flasks containing growth media overnight. For *E. coli*, luria broth (LB) medium and for *K. oxytoca*, nutrient broth (NB) medium was used. The LB or NB agar was prepared and autoclaved (see appendix). After autoclaving, 20 ml of agar medium were added to petri plates in a LAF bench and left to solidify after which they were stored at room temperature.

3.7.2 Halo assay and anti-bacterial activity of extracts

Media agar plates (LB or NB) were placed at 37 °C an hour before the experiment. A sterile shake flask was filled with 30 ml of medium and 0.6 ml of the overnight bacteria culture was added. A solution with 1% agar in distilled water was melted using a microwave (450 W, 1 min). 30 ml of the melted agar was added to the shake flask containing medium and bacteria during swirling of the flask. Immediately afterwards, 4 ml of the agar mix was pipetted onto the pre-heated petri plates and spread evenly. Filter discs (Whatman 3MM filter paper, made by a perforator and sterilized by autoclavation) were spotted with 5 µl algae extract of each sample and left to let the acetone and water evaporate. For tests using *K. oxytoca*, additional 5 µl of algae extracts were added after evaporation. Two filter discs were spotted with a 200 µg/ml Na-ampicillin in distilled water for *E. coli* and 34 mg/ml chloramphenicol in 99% ethanol for *K. oxytoca* as a positive control and two filter discs with 70:30 acetone:water as a negative control. After evaporation, four filter discs were put on each petri plate. *E. coli* was incubated for 2 days and *K. oxytoca* was incubated for 36 hours.

3.8 Ferrous ion-chelating assay

100µl of the extracts were mixed with 50 µl of 0.2 mM $FeCl_2$ and 100 µl of 0.5 mM ferrozine in a microplate. Blanks were prepared by mixing 100 µl of each sample with 50 µl of 0.2 mM $FeCl_2$ and 100 µl of milliQ water and controls were prepared by mixing 100 µl 70 % aqueous acetone with 50 µl of 0.2 mM $FeCl_2$ and 100 µl of 0.5 mM ferrozine. The microplates were then incubated in a shaker (SW22, Julabo) at 80 RPM at room temperature for 30 min. After incubation the microplate was read at 560 nm in a plate reader (Safire2, Tecan) 3 times with a 5 s shaking step in between each measuring cycle.

The ion-chelating activity was calculated using the following formula:

$$\textbf{Chelating activity (\%)} = (Abs_{control}-(Abs_{sample}-Abs_{control})/Abs_{blank})*100$$

3.9 Dry weight measurement

The moisture content of the freeze-dried algae powder was measured in a dry weight scale (HA 300, Precisa). Approximately 0.11 g of algae powder was heated gradually to 80 °C with the decrease in weight measured.

4 Results

4.1 Quantification of total phenolic content

S. latissima samples were collected from 3 different locations at 3 different time points in order to compare the changes in phenolic contents depending on harvesting time and location, as described above. Extraction using 70 % acetone was performed on the samples and the extracts were then analyzed using the folin-ciocalteu reagent to form complexes between phenolic compounds and molybdenum that may be quantified with a spectrophotometer. The results indicated that there was a generally higher content of phenolics in the samples collected in early June (Figure 7, see appendix for a full table of data). Yttre Vattenholmen (VH) sample extracts had an average amount of 0.35 mg phlorglucinol equivalents (PGE) per g of dry algae more during June than August, while the difference between August and October extracts only averaged around 4.7 µg. Ulvillarna (UL) had an average of 0.37 mg PGE per dry g of algae more in June compared to August while Ursholmen (UR) had an average of 0.57 mg PGE per dry g of algae more. In addition, it can clearly be seen that the extracts from VH had a higher content of phenolic content than the extracts from UL or UR. Extracts from VH had an average of 1.12 mg PGE per dry g of algae in June and 0.77 mg PGE in August. Extracts from UL had an average of 0.83 mg PGE in June and 0.46 mg PGE in August while extracts from samples collected at UR had an average of 0.97 mg PGE and 0.41 mg PGE respectively. Overall, the content of PGE in of the sample extracts from VH did not change much from August to October.

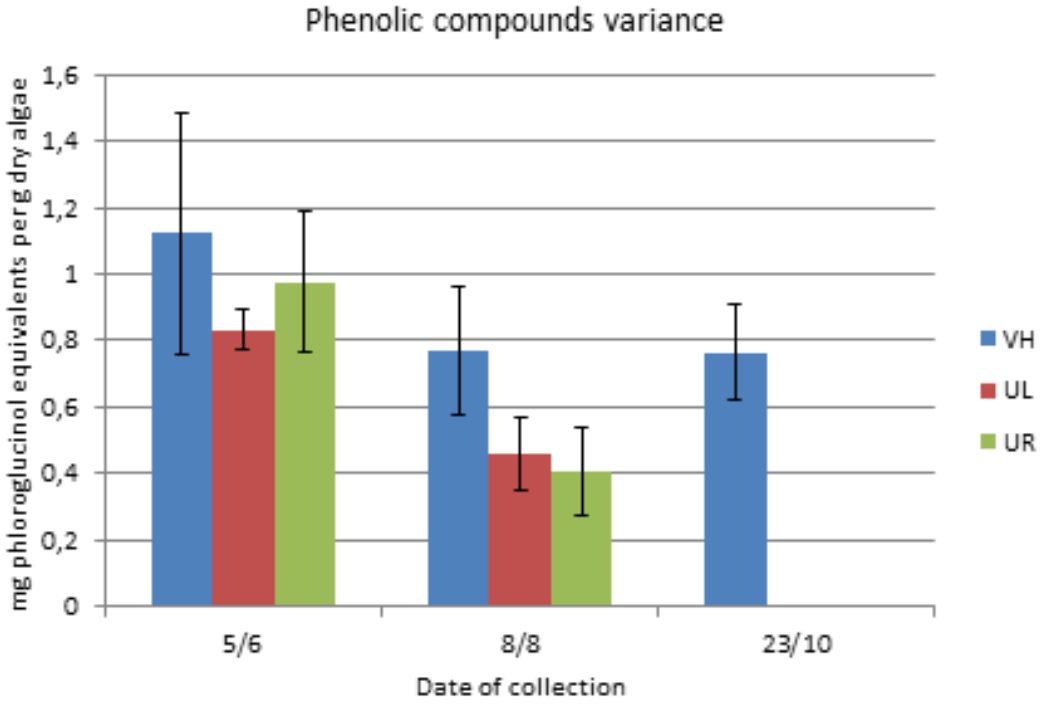

Figure 7 TPC measurement in acetone extracts of six specimens of *Saccharina latissima* at each sampling occasion. VH represents Yttre Vattenholmen, UL represents Ulvillarna and UR represents Ursholmen. The averages are shown with standard deviations as error bars.

4.2 Ferrous ion-chelating activity

The acetone extracts of phenolic contents described above were also tested for ion-chelating activities (Figure 8). The metal ion-chelating ability in all samples ranged between 1.64% to 81.98% with no clear linear correlation between ferrous ion-chelating ability and TPC. There was neither a clear correlation of the ion-chelating activity to date of harvest nor location (Figure 9). There was, however, a tendency that the June samples had the lowest ion-chelating activity.

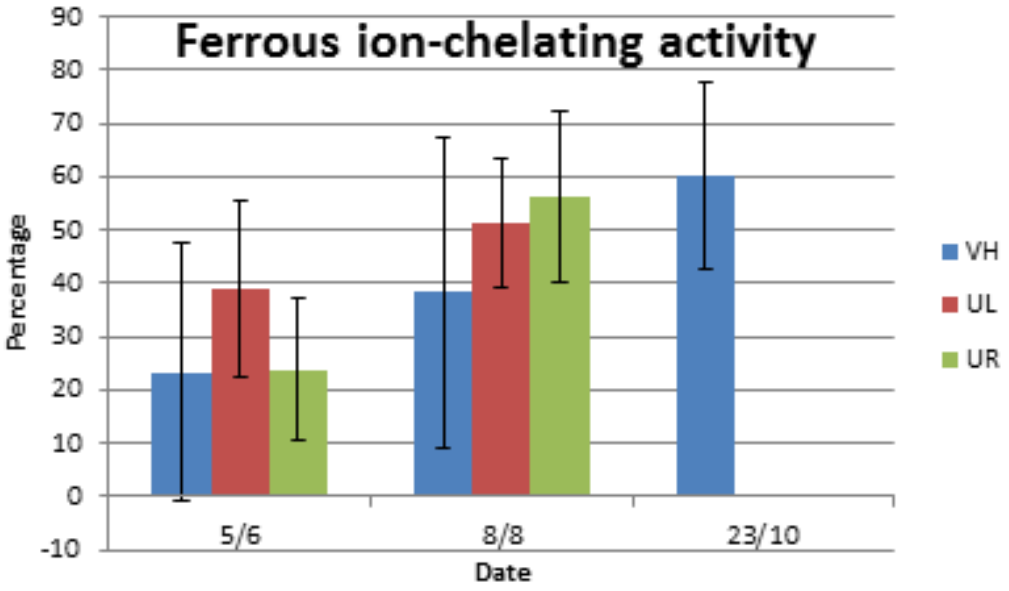

Figure 8 Ferrous ion-chelating measurement in acetone extracts of six specimens of *Saccharina latissima* at each sampling occasion. VH represents Yttre Vattenholmen, UL represents Ulvillarna and UR represents Ursholmen. The averages are shown with standard deviations as error bars.

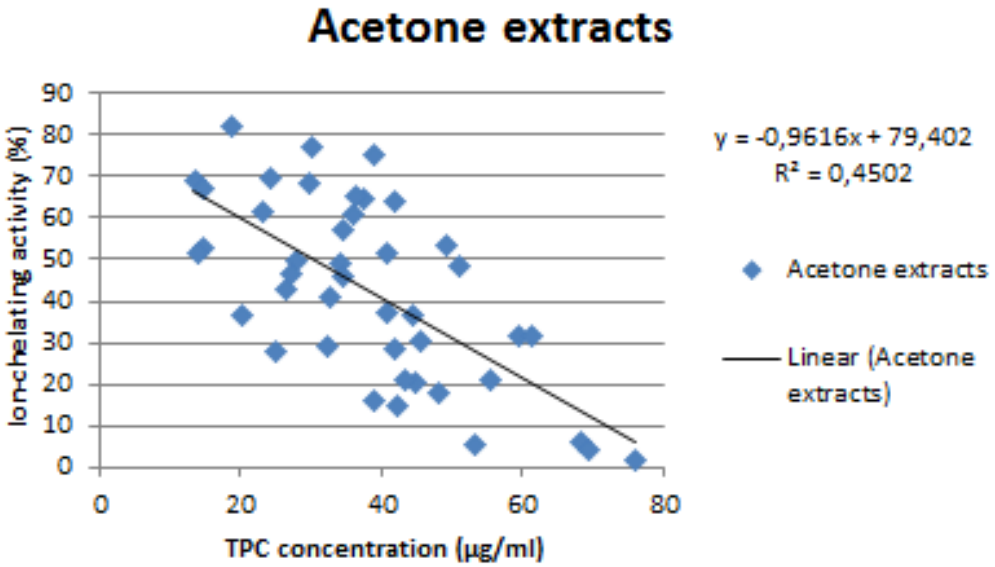

Figure 9 Ferrous ion-chelating activity against TPC concentration trend of all the extracts. The blue dots represent different acetone extracts.

4.3 Anti-bacterial activity

All the algae acetone extracts described in 3.2 were also tested for antibacterial activity. The growth inhibiting effect on two different bacterial species, *Escheria coli* and *Klebsiella oxytoca*, was investigated using a halo assay where filter discs containing algae extract were put on top of bacterial lawns. Positive controls of commercial antibiotics were used; ampicillin for *E. coli* and chloramphenicol for *K. oxytoca.* A negative control consisting of 70 % aqueous acetone solution was used. There was a clear growth of both bacterial species on the plates after two days, with the discs containing antibiotics having caused an impaired growth resulting in a halo around the discs on the plates (Figure 10). However, for the algae extracts there were no clear halos around any of the discs (Figure 11), but there were slight indications of halos around some of the extract discs in the *K. oxytoca* assay, although not clear enough to determine if the extracts had a true growth-inhibiting effect.

Figure 10 Positive and negative control for *K. oxytoca.*

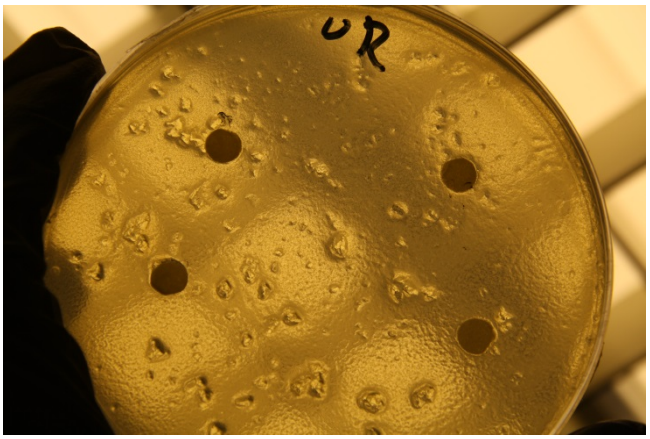

Figure 11 Four filter discs dotted with different phlorotannin extracts.

4.4 Moisture content of freeze-dried algae

The freeze-dried algae powder had slightly adsorbed moist and showed an average dry weight of 95 %. Their moisture absorption was accounted for in the calculations of TPC and ion-chelating activity.

5 Discussion

The aim of this project was to determine the total phenolic content, ion-chelating activity and anti-bacterial activity of acetone extracts from the kelp *Saccharina latissima* from the west coast of Sweden. Since the only phenolic groups in kelps are phlorotannins, according to earlier studies (Jormalainen V and Honkanen T, 2004; Koivikko R *et al.,* 2007), the total phenolic content measurement gave an indication of the phlorotannin content.

Variations in TPC were observed in *Saccharina latissima* in June, August and October. Generally, a higher content of phenolic compounds was found in the June samples, while the August samples had less. October, which was only measured in one sample group, had similar concentrations to that of August, which contradict studies showing that summer months and early fall tend to yield the highest content of phenolic compounds (Pavia H and Toth GB, 2000; Wang T, 2009)]. The amount of phenolic compounds in the algae depends on the growth conditions. A higher exposure to sunlight and other environmental stress factors could result in a higher concentration of antioxidants, as mentioned in 2.4. This study did not take this into account, but it could be the reason for the high quantity in late fall. Yttre Vattenholmen is a location found closer to the shore of Sweden, and leaked nitrogen compounds from fertilized ground, or other products, may be the reason for a higher yield of high-value products in the sample extracts.

Phlorotannins have also been shown to have an anti-bacterial activity (Shanmughapriya S *et al.,* 2008; Nagayama K *et al.,* 2002). However, *E. coli* is a strain that has been observed to have a high resistance towards some algae extracts (Shanmughapriya S *et al.,* 2008; Nagayama K *et al.,* 2002), which could be the reason for the lack of effect in the halo assay. *Klebsiella pneumoniae* has been shown to have a low resistance to phlorotannins, but was unavailable for this study. For that reason, the related species *Klebsiella oxytoca* was used for the assays instead. However, *K. oxytoca* did not display any clear sensitivity in the assays either which could be because it is more resistant, or that the concentrations of the extracts were too low. To investigate this further, the concentration of sample extracts could be increased by evaporation.

The ferrous ion-chelating activity did not show any linear correlation with total phenolics which is in agreement with previous studies (Wang T *et al.,* 2010; Wang T *et al,* 2009). A reason for the lack of linear correlation could be because the algal dietary fibers have a high metal binding capacity (Wang T *et al,* 2009). Proteins are other compounds that also have been reported to chelate metal ions. In future studies, both fibers and proteins should be analyzed.

Furthermore, during this project some extracts were noticed to have changed colors from green-brownish towards more brown-reddish while stored in the glass vials. The reason for this could be the acetone dissolving the plastic in the caps, which in turn could have distorted some test results, both in activity and spectrophotometry readings. The total phenolic content measurement during the earlier stage showed no trend of distortion. However, the ion-chelating capacity assay showed a variation from around -10 % to 21 % in the triplicate analysis of the same sample (VH-8/8-6). The ion-chelating capacity assay was performed as the last part of this project while the total phenolic content measurement was performed soon after extraction. Possibly, for the ion-chelating test, the acetone had had enough time to dissolve the plastic for distortion.

6 Conclusion

The results of this study shows that acetone extracts from *S. latissima* harvested in June had higher concentrations of phenolic contents than the other harvest dates. Furthermore, the extracts collected at Yttre Vattenholmen had a generally higher content of phenolic compounds compared to sample extracts from Ulvillarna and Ursholmen. The bacteria tested for phlorotannin anti-bacterial activity had no clear sensitivity, suggesting that either the concentration of the extracts were too low or the bacterial strains were resistant against the extracts. Ferrous ion-chelating activity had no clear linear correlation with the total amount of phenolic groups, suggesting that the phlorotannins are not very good metal chelators. Slightly higher activities were seen in the samples from the later harvest dates.

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9 Appendix

9.1 Media preparations

9.1.1 LB and agar recipe

For 1 liter of lysogeny broth (LB) media;

Measure 900 ml of distilled water and add 16 g Tryptone, 10 g yeast extract and 5 g of NaCl. Adjust the pH to 7 with NaOH and dilute the solution to 1 l with distilled water. Sterilize by autoclaving.

For 1 liter of LB agar;

Follow the same recipe as for the media but add 20 g agar before adjusting pH.

9.1.2 NB media and agar recipe

For 1 liter of nutrient broth (NB) media;

Measure 900 ml of distilled water and add 5 g peptone and 3 g meat extract. Adjust the pH to 7 with HCl and dilute the solution to 1 l with distilled water. Sterilize by autoclaving.

For 1 liter of NB agar;

Follow the same recipe as for the NB media, but add 20 g agar powder before adjusting pH.

9.2 Data tables

9.2.1 Total phenolic content

Sample	Well 1	Well 2	mg PGE/g dry algae powder
SL-VH-5/6-1	0,5529	0,5485	0,681335
SL-VH-5/6-2	1,2291	1,1827	1,516519
SL-VH-5/6-3	0,5248	0,5882	0,688553
SL-VH-5/6-4	1,0807	1,0893	1,355044
SL-VH-5/6-5	0,8343	0,9358	1,104919
SL-VH-5/6-6	1,0554	1,1529	1,391617
SL-VH-5/6 average			1,12299768996862
SL-VH-5/6 standard deviation			± 0,364690428382643
SL-UL-5/6-1	0,6067	0,5951	0,741954
SL-UL-5/6-2	0,609	0,6979	0,81859
SL-UL-5/6-3	0,6119	0,6917	0,804546
SL-UL-5/6-4	0,5529	0,7839	0,834955
SL-UL-5/6-5	0,6946	0,758	0,913494
SL-UL-5/6-6	0,7103	0,7182	0,885678
SL-UL-5/6 average			0,833202726601712
SL-UL-5/6 standard deviation			± 0,0609332904521272
SL-UR-5/6-1	0,4835	0,5805	0,664389
SL-UR-5/6-2	0,727	0,8058	0,956863
SL-UR-5/6-3	0,8984	0,9912	1,18769
SL-UR-5/6-4	1,0394	0,9166	1,220207
SL-UR-5/6-5	0,7688	0,9307	1,058466
SL-UR-5/6-6	0,63	0,729	0,851604
SL-UR-5/6 average			0,97498533996483
SL-UR-5/6 standard Deviation			± 0,211205254614748
SL-VH-8/8-1	0,298	0,3188	0,373197
SL-VH-8/8-2	0,7873	0,8422	1,028691
SL-VH-8/8-3	0,5506	0,6172	0,726899
SL-VH-8/8-4	0,5662	0,5479	0,687199
SL-VH-8/8-5	0,6722	0,7503	0,885848
SL-VH-8/8-6 A	0,6048	0,6445	0,773097
SL-VH-8/8-6 B	0,6333	0,6815	0,811626
SL-VH-8/8-6 C	0,6728	0,7113	0,859177
SL-VH-8/8 average			0,768216800195426
SL-VH-8/8 standard deviation			± 0,191302917479661
SL-UL-8/8-1	0,4411	0,4656	0,560092
SL-UL-8/8-2	0,4464	0,4334	0,542237
SL-UL-8/8-3	0,2252	0,2277	0,270251
SL-UL-8/8-4	0,4033	0,4522	0,527116
SL-UL-8/8-5	0,3256	0,3414	0,40621
SL-UL-8/8-6	0,3719	0,3833	0,460694
SL-UL-8/8 average			0,461099942913914
SL-UL-8/8 standard deviation			± 0,109706154107705

Sample	Well 1	Well 2	mg PGE/g dry algae powder
SL-UR-8/8-1	0,458	0,5021	0,59133
SL-UR-8/8-2	0,2439	0,2451	0,291117
SL-UR-8/8-3	0,3816	0,4101	0,484006
SL-UR-8/8-4	0,2424	0,2527	0,295782
SL-UR-8/8-5	0,4111	0,4028	0,500142
SL-UR-8/8-6	0,2252	0,2435	0,276751
SL-UR-8/8 average			0,406521601728402
SL-UR-8/8 standard deviation			± 0,135162679721126
SL-VH-23/10-1	0,7936	0,783	0,990857
SL-VH-23/10-2	0,5918	0,6571	0,78267
SL-VH-23/10-3	0,473	0,4966	0,599263
SL-VH-23/10-4	0,5407	0,6161	0,721455
SL-VH-23/10-5	0,498	0,5397	0,643098
SL-VH-23/10-6	0,6803	0,6653	0,843791
SL-VH-23/10 average			0,763522285542999
SL-VH-23/10 standard deviation			± 0,142648779105639

PGE: Phloroglucinol equivalents

9.2.2 Ferrous ion-chelating activity

Sample	TPC Concentration (µg/ml)	Ion-Chelating (%)
SL-VH-5/6-1	34,18801	49,03515
SL-VH-5/6-2	76,03826	1,636392
SL-VH-5/6-3	34,55848	57,06273
SL-VH-5/6-4	68,31589	6,226522
SL-VH-5/6-5	55,54428	20,99007
SL-VH-5/6-6	69,53908	4,456337
SL-VH-5/6 average	56,3640000333333	23,2345323247397
SL-VH-5/6 standard deviation	± 18,2835103316845	± 24,1802765151298
SL-UL-5/6-1	37,39449	64,89477
SL-UL-5/6-2	40,75107	51,3405
SL-UL-5/6-3	40,64567	37,21505
SL-UL-5/6-4	41,70598	28,29723
SL-UL-5/6-5	45,40429	30,46879
SL-UL-5/6-6	44,6346	20,55781
SL-UL-5/6 average	41,7560162333333	38,7956911782363
SL-UL-5/6 standard deviation	± 2,92794728080032	± 16,4511865443763
SL-UR-5/6-1	32,68218	41,14136
SL-UR-5/6-2	47,96563	17,87681
SL-UR-5/6-3	59,36076	31,77585
SL-UR-5/6-4	61,48137	31,77585
SL-UR-5/6-5	53,28953	5,742809
SL-UR-5/6-6	42,41498	14,7018
SL-UR-5/6 average	49,5843072166667	23,8357433232131
SL-UR-5/6 standard deviation	± 10,771441076995	± 13,2050514200084

Sample	TPC Concentration (µg/ml)	Ion-Chelating (%)
SL-VH-8/8-1	34,59361	46,10199
SL-VH-8/8-2	44,44298	36,60783
SL-VH-8/8-3	38,91149	16,05002
SL-VH-8/8-4	41,00337	-10,1477
SL-VH-8/8-5	43,2166	20,99521
SL-VH-8/8-6	18,71134	81,98425
SL-VH-8/8-6	51,05394	48,45366
SL-VH-8/8-6	36,30863	65,51742
SL-VH-8/8 average	38,5302461625	38,195337827407
SL-VH-8/8 standard deviation	± 9,51126142531255	± 29,1732296087658
SL-UL-8/8-1	27,96988	49,76586
SL-UL-8/8-2	27,11077	46,63716
SL-UL-8/8-3	13,47687	68,93943
SL-UL-8/8-4	26,3347	43,12767
SL-UL-8/8-5	20,31458	36,60783
SL-UL-8/8-6	23,13142	61,63227
SL-UL-8/8 average	23,05637045	51,1183725278307
SL-UL-8/8 standard deviation	± 5,48849949609553	± 12,0370260266307
SL-UR-8/8-1	29,67531	68,39911
SL-UR-8/8-2	14,62979	52,99748
SL-UR-8/8-3	24,29712	69,47461
SL-UR-8/8-4	14,82461	67,2876
SL-UR-8/8-5	25,00612	27,99876
SL-UR-8/8-6	13,98147	51,32507
SL-UR-8/8 average	20,40240575	56,2471054392014
SL-UR-8/8 standard deviation	± 6,75297175195956	± 15,9830283015373
SL-VH-23/10-1	49,36447	53,36798
SL-VH-23/10-2	38,89872	75,37694
SL-VH-23/10-3	29,97872	77,02877
SL-VH-23/10-4	35,95732	60,88098
SL-VH-23/10-5	32,15362	29,05882
SL-VH-23/10-6	41,98703	64,25668
SL-VH-23/10 average	38,0566470666667	59,9950256436645
SL-VH-23/10 standard deviation	± 7,05024466913968	± 17,5878635501545