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DEPARTAMENTO DE CIÊNCIAS DA VIDA

Carrageenan yield and quality of *Chondrus crispus* Stackhouse (Rhodophyta) cultivated in an Integrated Multi-Trophic Aquaculture (IMTA) system

Filipa Antónia Mota Ferreira da Costa Meireles

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Rendimento e qualidade das carragenanas da macroalga *Chondrus crispus* Stackhouse (Rhodophyta) cultivada em sistema de Aquacultura Multi-Trófica Integrada (AMTI)

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal

Orientador: Prof. Dr. Leonel Carlos dos Reis Tomás Pereira, Professor Auxiliar da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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The work presented in this dissertation results from the collaboration between the Department of Life Sciences of the University of Coimbra, the research centre IMAR-CMA in Coimbra and the company Algaplus Lda., located in Ílhavo, in Vouga estuary (Ria de Aveiro).

All IMTA-cultivated algal material and all wild harvested algal material from the North Portuguese coast was supplied by Algaplus Lda.

The laboratory work was conducted at the Department of Life Sciences of the University of Coimbra.

FTIR-ATR spectroscopy was conducted at the Department of Chemistry of the University of Aveiro.

Filipa Antónia Mota Ferreira da Costa Meireles

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SEA CHANGES

Each rock a pool garden
Of colour, bronze and
Blue gleam of Irish moss,
Rose of coral algae,
Ochre of sponge where
Whelk and starfish turn
In an odour of low tide;
Faint odour of stillness.

John Montague, New Collected Poems, 2012

Abstract

Chondrus crispus Stackhouse, Irish moss, is a traditionally harvested seaweed of the Portuguese coast. The tetrasporophyte life phases produce lambda-type carrageenan, which has a wide application in the food industry and is increasingly promising in the pharmaceutical and cosmetic industries. Combining a carrageenophyte with promising new market niches, such as cold-water *C. crispus* lambda-carrageenan, may contribute to boost the economic viability of Integrated Multi-Trophic Aquaculture (IMTA) in Portugal, while converting land-based intensive fish farms into an ecological and more sustainable aquaculture. In the present study, IMTA-cultivated *C. crispus* had good carrageenan yields with alkaline extraction process when compared to wild harvested *C. crispus* from the Centre and North Portuguese coast. FTIR-ATR spectroscopy analysis of the carrageenans produced by IMTA-cultivated specimens, both in their native state and the alkaline extracted, was in conformity with results from previous studies on wild *C. crispus* from the Portuguese coast.

Keywords: *Chondrus crispus*, Irish moss, carrageenan, alkaline extraction, Integrated Multi-Trophic Aquaculture (IMTA), FTIR-ATR spectroscopy.

Resumo

Chondrus crispus Stackhouse, musgo-irlandês (‘Irish moss’), é uma macroalga colhida tradicionalmente na costa portuguesa. A geração tetrasporófita produz carragenana do tipo lambda, que tem uma aplicação vasta na indústria alimentar e é cada vez mais promissora nas indústrias farmacêutica e cosmética. Associar uma carragenófita a novos e promissores nichos de mercado, como é o caso da carragenana-lambda de *C. crispus* de águas frias, poderá contribuir para a viabilidade económica da Aquacultura Multi-Trófica Integrada (AMTI) em Portugal, ajudando converter as pisciculturas de regime intensivo, de tanques situados em terra, numa aquacultura mais ecológica e sustentável. No presente estudo, espécimes de *C. crispus* de cultivo em sistema AMTI apresentaram bons rendimentos na extracção alcalina de carragenanas comparativamente a espécimes de *C. crispus* colhidos nas regiões centro e norte da costa portuguesa. As análises de espectroscopia FTIR-ATR das carragenanas produzidas por espécimes de *C. crispus* de AMTI, tanto no estado nativo como as obtidas por extracção alcalina, apresentaram-se em conformidade com resultados obtidos em estudos prévios de *C. crispus* selvagem da costa portuguesa.

Palavras-chave: *Chondrus crispus*, musgo-irlandês, carragenana, extracção alcalina, Aquacultura Multi-Trófica Integrada (AMTI), espectroscopia FTIR-ATR.

Table of Contents

List of Figures

Appendix B

List of Tables

Appendix A

1. Introduction

1. 1 Marine algal resources of Portugal – an overview

The coast of mainland Portugal occupies most of the South-West part of the Iberian Peninsula, between latitudes 37º N and 42º N, and it is approximately 830 km long. Biogeographically, Portugal is situated in the warm temperate Mediterranean-Atlantic region and the Portuguese coast is under unique circumstances, receiving climatic influences from the North Atlantic Ocean and the Mediterranean Sea, in this way generating a sharp latitudinal gradient in the macroalgal flora. In the coastline, rocky shores are separated by extended areas of sandy beaches. Most of the beaches are very exposed and algae in the intertidal zone are mainly found closest to the low tide level. In the West littoral, algae fall into two groups: the North zone algae (between Minho river mouth and Tagus river mouth) and the South zone algae (between Tagus river mouth and Algarve province). The intertidal algal flora in the northern zone is similar to that of the coast of Central Europe (Brittany and South of British Isles), while the intertidal algal flora of the southern zone is very different, suffering a marked influence from the Mediterranean and the North-West African coast species. Temperate species gradually decline in number southward along the Portuguese coast, where some *taxa* have their southern limits (**Pereira, 2004; Sousa-Pinto and Araújo, 2006**).

The intertidal flora of the North zone is dominated by *Himanthalia elongata*, *Gelidium corneum*, *Bifurcaria bifurcata*, *Chondrus crispus*, *Mastocarpus stellatus*, *Calliblepharis jubata*, *Gigartina pistillata*, *Chondracanthus acicularis*, *Osmundea pinnatifida*, *Gelidium pulchellum*, *Pterosiphonia complanata*, *Saccorhiza polyschides*, *Corallina elongata* and *Gelidium pusillum*. The South zone is dominated by *Caulacanthus ustulatus*, *Corallina elongata*, *Chondracanthus acicularis*, *Gelidium pusillum*, *Osmundea pinnatifida* and *Chondria coerulescens*, where *Codium adhaerens* may have a significant presence in the rocky shores (**Sousa-Pinto and Araújo, 2006**). In the southward direction of the Portuguese coast, the number of species of red algae (Rhodophyta) increases in the presence of warmer waters and in systems subject to less anthropogenic pressure, where they naturally dominate in numbers over brown algae (Phaeophyceae, Ochrophyta) and green algae (Chlorophyta). With increasing disturbances, the number of Rhodophyta *taxa* declines, although their proportions may

not decline as clearly as in numbers, and ultimately affects diversity and species richness (**Ardré, 1970** *apud* **Pereira, 2004**; **Gaspar *et al*., 2012**; **Mesquita Rodrigues, 1963**; **Palminha, 1951**).

The main references on Portuguese marine macroalgae (i.e., seaweeds) are the works of Palminha (**1951**, **1971**) and Ardré (**1970**, **1971**), and Araújo *et al*. (**2009**), who updated the benthic marine macroalgal checklist of the North coast of Portugal.

Until the mid-20th century, seaweed harvest was a relevant activity in the Portuguese economy, supplying European carrageenan production (*Chondrus crispus*, *Mastocarpus stellatus* and *Gigartina pistillata*) and agar production (*Gelidium corneum*, *Gracilaria* spp. and *Pterocladiella capillacea*). During World War II, when agar from Asia became scarce, the phycocolloid industry emerged in Portugal and we became one of the biggest agar producers in the world. "Sargaço" (the Portuguese name given to the accumulation of seaweed thrown on to the beaches by the tide) in the North of the country from Matosinhos to Viana do Castelo, and "moliço" (a mixture of seaweeds and seagrasses) in Vouga estuary (Ria de Aveiro), were both used as fertilisers in the agricultural fields close to the sea. These traditional activities are almost extinct today, except for *Pterocladiella capillacea* in Azores and *Gelidium corneum* in the South-West of the mainland coast, which supply the raw material for the only Portuguese land-based agar extraction company (Iberagar S.A.). Socioeconomic factors, lack of innovation in the sector and direct competition with Morocco were the reasons for the decline in the activity.

In the past few years, research scientists have been trying to resurrect the exploitation of this natural resource. There are several research centres in Portugal (CIIMAR, CCMAR, IMAR-CMA, Centro de Oceanografia-FCUL, 3B's, FEUP, among others) studying these organisms at different levels (taxonomy, physiology, ecology, biochemistry, resource management), screening for new applications: developing materials for biomedical and industrial applications (biofilms, bioplastics), pharmaceutical and cosmetic formulations, animal nutrition additives, and the improvement of the extraction procedures for agar and carrageenans (**Abreu *et al*., 2011b**).

1.2 Rhodophyta, the red algae

1.2.1 Systematics of Rhodophyta

The term *algae* includes a large and diverse group of aquatic organisms that comprehends unicellular organisms, the microalgae, and more complex, large, multicellular organisms with differentiated tissues, commonly known as 'seaweeds'. Red algae, or Rhodophyta, are a widespread group of uni- to multicellular aquatic photoautotrophic plants.

Phylogenetically, Rhodophyta are true plants as they share a single common ancestor with the green lineage – green algae and higher plants (**Adl *et al.* 2005** *apud* **Gurgel and Lopez, 2007**), although algae do not possess true stems or leaves. They exhibit a broad range of morphologies, simple anatomy and display a wide array of life cycles. About 98% of the species are marine, 2% freshwater and a few rare terrestrial/sub-aerial representatives (**Gurgel and Lopez, 2007**).

The phylum Rhodophyta is distinguished from other groups of eukaryotic algae due to: total absence of centrioles and any flagellate phase; presence of chlorophylls *a* and *d*, and accessory pigments (light-absorbing) called phycobilins (phycoerythrin and phycocyanin); plastids with unstacked thylakoids, and no external endoplasmic reticulum; absence of parenchyma and presence of pit-connections between cells (i.e. incomplete cytokinesis); and floridean starch as storage product (**Pereira, 2012**).

In regard to carotenoids, Rhodophyta can be divided into two groups based on carotenoid composition; the unicellular type contains only β-carotene and zeaxanthin, and the macrophytic type contains additional α-carotene and lutein (**Takaichi, 2011**).

Traditionally, red algae can be morphologically separated in three major groups:

(1) a unicellular group with reproduction by binary cell division only;

(2) a multicellular group where a carpogonial branch is absent or incipient – Bangiophyceae *sensu lato*;

(3) and a multicellular group with well-developed carpogonial branches – Florideophyceae.

Algal cell walls are composed of cellulose fibrils (rarely xylan fibrils) and a matrix of hydrocolloids. Many algae produce hydrocolloids, associated with the cell wall and intercellular spaces.

1.2.2 Florideophyceae

Reproduction by fragmentation of the thallus in red algae is rare and all of them produce one or more types of non-flagellated spores.

Most Florideophyceae have a triphasic life cycle, indicating three distinct phases: the gametophyte, the carposporophyte and the tetrasporophyte. The male and female gamete-producing reproductive structures are the spermatangium and carpogonium, respectively, and both develop from the gametophyte.

Florideophyceae sexual reproduction includes the development of a specialised female filament called the carpogonial branch. Originally formed from inner cortical cells, it arises from a supporting cell, which is very large and also serves as the auxiliary cell once the cell is fertilised. At the end of the carpogonial branch is the female reproductive structure, the carpogonium, which consists of an elongated, gelatinous tip, called the trichogyne, and an inflated basal region. The trichogyne is responsible for receiving the male gametes (spermatium). The spermatia are produced on spermatangial mother cells, which branch off from the male gametophyte. When the spermatia are released, they take with them a mucilage sheath which will aid them later in adhering to the female trichogyne. As these cells are non-flagellated, they must rely on water currents to passively carry them to the female egg. Fertilisation begins with the fusion of the spermatium to the trichogyne. The cell walls of each dissolve, and the male nucleus moves down into the basal portion of the carpogonium where it fuses with the female nucleus.

After fertilisation, the zygotic nucleus develops, directly or indirectly, into a diploid phase, the carposporophyte, which grows parasitically on the female gametophyte. The carposporophyte is usually composed of gonimoblastic filaments (vegetative diploid cells) and carposporangia. Mature carposporangia release diploid carpospores (reproductive diploid cells) into the water.

The cystocarp is composed of the carposporophyte plus all protective sterile haploid tissue of the female gametophyte encircling and interacting with it, the pericarp. It is an interesting structure as the envelope is haploid and the internal carposporophyte is diploid. These cystocarps are pushed out from the thallus and housed in the wart-like papillae. Carpospores are released, settle, and then develop into a second free-living phase called tetrasporophyte. Tetrasporophytic thalli produce tetrasporangia by meiosis, which release tetraspores. This pattern of meiotic cell division in the tetrasporangium is

stable in red algae and can be one of three types: cruciate (including decussate), tetrahedral and zonate. When released, each tetraspore will give rise to either a male or a female haploid gametophyte (**Gurgel and Lopez, 2007**).

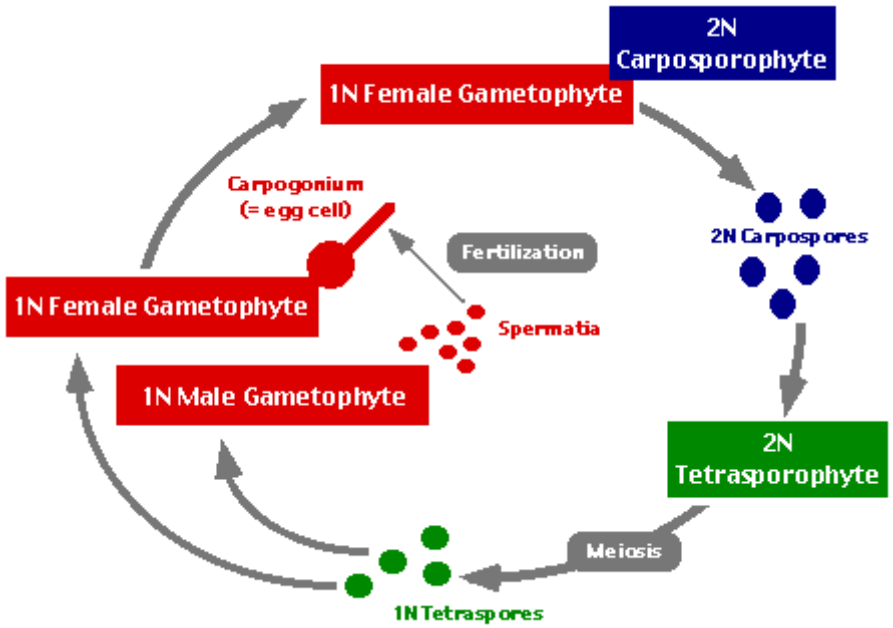

Figure 1.1 – Diagram of triphasic life history in the class Florideophyceae. Haploid (1N) male gametophytes produce spermatia that are released, while haploid female gametophytes produce carpogonia (=egg cells) that are retained on the female gametophyte. After fertilisation, the diploid (2N) zygote is still retained on the female gametophyte and develops into the diploid carposporophyte. The carposporophyte produces diploid carpospores that are released and develop into the diploid tetrasporophyte. The tetrasporophyte produces tetrasporangia where meiotic divisions result in haploid tetraspores. These tetraspores then develop into haploid gametophytes completing the life cycle. (Adapted from '**Tree of Life**': http://tolweb.org/Florideophyceae.)

Florideophyceae includes 17 orders and it was found to be monophyletic by Freshwater *et al*. (**1994**) by *rbc*L analysis. The large order Gigartinales was revealed polyphyletic. A large number of genera of high economic interest, the carrageenophytes, are members of this order and most of them are phylogenetically related. All Gigartinales species have an auxiliary cell, which originated from a cortical cell. This auxiliary cell normally produces one or more gonimoblast initials, with connecting sterile filaments present or absent, and tetrasporangia can be either cruciate or zonate. In the Gigartinaceae, each auxiliary cell cuts off several gonimoblast initials, the

connecting filaments are absent, and the tetrasporangia are cruciately divided (**Freshwater *et al*., 1994**).

1.2.3 *Chondrus crispus* Stackhouse

Classification: domain Eukaryota (Eukarya), kingdom Plantae, phylum Rhodophyta, class Florideophyceae, order Gigartinales, family Gigartinaceae, genus *Chondrus*, species *Chondrus crispus* Stackhouse 1797.

Chondrus crispus is the type species (lectotype) for the genus *Chondrus*.

Common names in English are Irish moss, Carragheen, Carragheen moss, Dorset weed, Pearl moss, Sea moss, Sea Pearl moss, Jelly moss, Rock moss, Gristle moss, Curly moss, Curly gristle moss, Carrageen, Carraghean, Carrageenin. In Irish (Gaelic): "Cruibín chait", "Carraigín", "Cosáinín carraige". In Portuguese: "Musgo gordo", "Botelho", "Botelha", "Cuspelho", "Musgo", "Limo-folha", "Folha-de-alface". Common names used in commerce, often for edible algae: Irish moss, pearl moss, carrageen moss, carrageen, jelly moss.

Description, according to 'MACOI – Portuguese Seaweeds Website': cartilaginous, dark purplish-red, red, yellowish or greenish fronds to 150mm high, gametophyte plants are often iridescent under water when in good condition. Stipe compressed, narrow, expanding gradually to a flat, repeatedly dichotomously branched frond, in tufts from a discoid holdfast. Axils rounded, apices blunt or subacute, frond thicker in centre than margins. Very variable in breadth of segments. Very variable in branching, colour and thickness. Highly variable (polymorphous) thalli may reach 15 cm long, cartilaginous consistency and reddish-pink or brown colour and iridescent in water. These algae are fixed by a disk whose start unbranched stipe gradually expanding into fan-like blade, repeatedly dichotomously divided, with ends rounded or truncated. On the surface of the blades may appear small dilations (2-3mm in diameter) which are the reproductive structures.

Habitat: on rocks, lower intertidal and shallow subtidal, in pools in the mid-intertidal in some locations (Fig. 1.2).

Distribution: widely distributed in the North-eastern and North-western Atlantic, as well as in the Bering Sea from Russia to Alaska. It can be found to a limited extent in the Western Baltic Sea. In the NE Atlantic, is common in Great Britain, Ireland, Iceland

and between Norway and the Iberian Atlantic coast. In the NW Atlantic, it can be found from Newfoundland, Canada, to Delaware, U.S.A. In Portugal, the species has its South limit in Vila Nova de Milfontes (**Ardré, 1970**). According to 'MACOI – Portuguese Seaweeds Website', *C. crispus* can be found in: Praia do Norte (Viana do Castelo), Aguçadoura, Apúlia, A-Ver-o-Mar, Lavadores, Aguda, Buarcos, Nazaré, S. Martinho do Porto and Peniche.

Life cycle: *Chondrus crispus*, like other Florideophyceae, has a triphasic life cycle comprising free living isomorphic gametophytes and tetrasporophytes.

Figure 1.2 *Chondrus crispus* Stackhouse in an intertidal pool at Buarcos bay ('**MACOI – Portuguese Seaweeds Website**': http://macoi.ci.uc.pt).

1.3 Phycocolloids

Colloids are compounds that form colloidal solutions, an intermediate state between a solution and a suspension, and are used as thickeners, gelling agents, and stabilisers for suspensions and emulsions. Hydrocolloids are carbohydrates that when dissolved in water form viscous solutions. Cell wall hydrocolloid matrixes in red algae are formed by sulfated polysaccharides which are classified in two main groups: agars and carrageenans. Brown algae (Ochrophyta, Phaeophyceae) produce uronates, e. g. alginates.

The properties of phycocolloids are generating an increasing commercial and scientific interest, having already a wide range of industrial, medical, pharmaceutical and food applications, with more than one million tonnes of algae extracted annually for hydrocolloid production. The processed food industry is still the primary market for the algal hydrocolloids (**Bixler and Porse, 2011**; **Ioannou and Roussis, 2009**; **Jiao *et al.*, 2011**).

The different phycocolloids used in food industry as natural additives are: alginic acid – E400, sodium alginate – E401, potassium alginate – E402, ammonium alginate – E403, calcium alginate – E404, propylene glycol alginate – E405, agar – E406, carrageenan – E407, semi-refined carrageenan or processed *Eucheuma* algae – E407A (European Union additive codes).

Sulphated polysaccharides are recognised to possess several biological activities including anticoagulant, antiviral, antitumor, anti-inflammatory, and immunostimulating activities that might find further relevance in nutraceutical/functional food, cosmetic, and pharmaceutical applications. In particular, carotenoids from *Chondrus crispus* have been reported to dietary antioxidant activity with benefit in vascular health and to have anti-coagulant activity (**Cornish and Garbary, 2010**).

Agar was the first colloid to be developed and it has applications as a gelling agent for food and also as an inert support medium for microbial culture and many other biotechnological applications, such as electrophoresis, chromatography and DNA sequencing. It is a powerful gel-forming gum because of the unusual length of its carbohydrate molecules. Most agars are extracted from species of *Gelidium* and *Gracilaria*.

Agars and carrageenans, each bear a basic sugar skeleton consisting of 1,3-linked ß-D-galactopyranose plus either 1,4-linked 3,6-anhydro-α-L-galactopyranose, for agars, or 1,4-linked 3,6-anhydro-α-D-galactopyranose units, for carrageenans. These polysaccharides are galactans, i.e. galactose polymers. Some groups of red algae exhibit higher concentrations of one particular class and thus are known as either agarophytes (agar-producers), e. g. Gracilariales, Gelidiales and Ceramiales, or carrageenophytes (carrageenan-producers), with most families belonging to the Gigartinales, e. g. Caulacanthaceae, Cystocloniaceae, Dumontiaceae, Furcellariaceae, Gigartinaceae, Hypneaceae, Kallymeniaceae, Polyideaceae, Rhizophyllidaceae, Solieriaceae, Sphaerococcaceae, Tichocarpaceae (**Gurgel and Lopez, 2007**; **Pereira *et al*., 2003**).

Cell wall polysaccharides in macroalgae are of chemotaxonomic significance. The pioneer in marine phycocolloids investigation was Tseng, who coined the terms 'agarophyte' and 'carrageenophyte' in 1944. In 1957, using 66 species from 22 different genera, Stoloff and Silva investigated the properties of water extractable polysaccharides as potential chemotaxonomic tools in red algae. Many types of phycocolloid biochemical analyses have been developed and among which must be referred Chopin and Wallen (**1993**), who developed a method for the identification of phycocolloids using FTIR (Fourier transform infrared spectroscopy and, more recently, van de Velde *et al.* (**2002**) using NMR (nuclear magnetic resonance**)** spectroscopy, specifically, ^{1}H-RMN, allowed precise determination (quantitative and qualitative) of the composition of the main phycocolloids, especially carrageenans (**Pereira, 2004**).

The cell walls of most algae and higher plants are composed of cellulose microfibrils embedded in a matrix consisting of proteins and polysaccharides. In marine macroalgae, the composition and organisation of the extracellular matrix is different from that of terrestrial plants. As the latter need a structural rigidity to resist gravity, algae need flexibility to resist alternating turbulent and calm water conditions. Nevertheless, the physiological role of algal cell walls in what concerns to mechanical regulation, hydration and osmotic regulation is still a matter of debate (**Pereira, 2004**).

1.4 Carrageenophytes and carrageenan industry

In 1862, British pharmacist Stanford coined the term ‘carrageenin’ for the gelatinous material extracted by water from Irish moss, *Chondrus crispus*. The term is derived from the colloquial Irish name for this alga, ‘carrageen’ or ‘carraigín’, meaning “little rock” (from the Irish place name, probably Carrigeen Head in County Donegal). The name was later changed to ‘carrageenan’ to comply with the ‘-an’ suffix for the names of polysaccharides. Carrageenan has a 600 year-long folk history in Ireland that includes milk puddings thickened by boiling sweetened milk with dried *Chondrus*. *C. crispus* continues to be used world-wide, but in limited quantities. The most commonly used, commercial carrageenans are extracted from *Kappaphycus alvarezii* and *Eucheuma denticulatum* (**Pereira, 2011**).

Carrageenans represent one of the major texturising ingredients used by the food industry. They are natural ingredients, which have been used for decades in food applications and are generally regarded as safe, ‘GRAS’, by the U. S. Food and Drug Administration. They are the third most important hydrocolloid in the food industry, after gelatine (animal origin) and starch (plant origin). Modern carrageenan industry dates from the 1940s, receiving its impetus from the dairy industry where carrageenan was found to be the ideal stabiliser for the suspension of cocoa in chocolate milk. Next to dairy products, carrageenans are widely used in ice cream, paints, water gels, pharmaceuticals, and even oil well drilling (**McHugh, 2003; Pereira *et al*., 2011**).

These are some applications of carrageenan in food, according to FAO (Food and Agriculture Organization of the United Nations): milkshake and instant breakfast powder, cooked flans and custards, cooked pudding and pie fillings, cold prepared flans and custards, chocolate milk, chocolate syrup, ice cream and sherbet, filled and skim milk, cottage and cream cheese products, evaporated milk (canned), infant formulations, ready-to-eat milk puddings (canned), whipping cream, aerosol spray cream topping, yogurt, frozen whipped toppings, imitation milk, dessert gels, fruit drinks, low-calorie jellies, pet foods, fish gels, frozen fish coating, and relishes, pizza and barbecue sauces.

Large carrageenan processors have fuelled the development of *K. alvarezii* (‘cottonii’ to the trade) and *E. denticulatum* (‘spinosum’ to the trade) farming in several countries including the Philippines, Indonesia, Malaysia, Tanzania, Kiribati, Fiji, Kenya, and Madagascar. Indonesia has recently overtaken the Philippines as the world’s largest producer of dried carrageenophyte biomass. Primarily, wild-harvested genera

such as *Chondrus*, *Furcellaria*, *Gigartina*, *Chondracanthus*, *Sarcothalia*, *Mazzaella*, *Iridaea*, *Mastocarpus*, and *Tichocarpus* are also mainly cultivated as carrageenan raw materials and producing countries include Canada, Mexico, Argentina, Chile, Japan, North Korea, South Korea, Russia, Denmark, France, Spain, Portugal, Morocco and the U.S.A. Carrageenan production exceeded 50,000 tonnes in 2007/2008 with a value of over US$600 million (not including China) (**Bixler and Porse, 2011**; **Pereira *et al.*, 2009a**).

As for the Iberian Atlantic coast, natural populations of *C. crispus* on the coast of Galicia, NW Spain, have long been harvested for industrial purposes. Yet, carrageenan obtained from natural beds are limited and unable to supply all industrial requirements, and the raw material is currently supplied by importing algae (≈2500 tonnes a year) and by harvesting natural beds of *C. crispus* and *Mastocarpus stellatus* (≈250 tonnes a year) (**Tasende *et al.*, 2012**). In Portugal, *C. crispus* and *M. stellatus* are mainly harvested in Viana do Castelo, but not at significant industrial level.

It must be referred that *Mastocarpus stellatus* is frequently harvested with *Chondrus crispus* and sold as a mixture under the name Carrageen or Irish moss. One of carrageenan's major attributes is its reactivity with proteins, particularly those in milk. Concentrations as low as 0.01% carrageenan can form a network with milk proteins to suspend cocoa particles in chocolate milk; whereas ten times this concentration of carrageenan alone would be needed to form such a network. Therefore, it is not surprising that dairy applications are still growing in use. However, the dairy market has not grown as rapidly as the processed meat market, particularly ham and other deli meats such as turkey breast. In processed meats, carrageenan serves as a water binding agent that prevents loss of moisture during cooking that improves cooked yields and prevents an undesirable dry texture or bite. Carrageenan has long had attributes as a toothpaste binder (texture, cellulase resistance, etc.), but it is losing ground to cheaper CMC (carboxymethyl cellulose) (**Bixler and Porse, 2011**).

1.5 Chemistry of carrageenans

Carrageenans are built on a disaccharide backbone of alternating 3-linked β-D galactopyranose (**G**) and 4-linked α-D-galactopyranose (**D**). Several types of carrageenans are recognised according to the position of sulphate/s (**S**) in the disaccharide repeating unit, cyclization of the **D** units forming an anhydro ring (**DA**), and presence of pyruvate (**P**) on **G** units. These letter codes refer to the nomenclature developed by Knutsen *et al*. (**1994**). The most common types of carrageenan are traditionally identified by a Greek prefix. The three commercially most important carrageenans are the kappa (κ), iota (ι) and lambda (λ) carrageenans, and their corresponding IUPAC-inspired names and letter codes are carrageenose 2,4'-disulfate (G4S-DA2S), carrageenose 4'-sulfate (G4S-DA) and carrageenan 2,6,2'-trisulfate (G2SD2S,6S) (**van de Velde *et al*., 2004**). The idealised disaccharide repeating units of these carrageenans are given in Fig. 1.3.

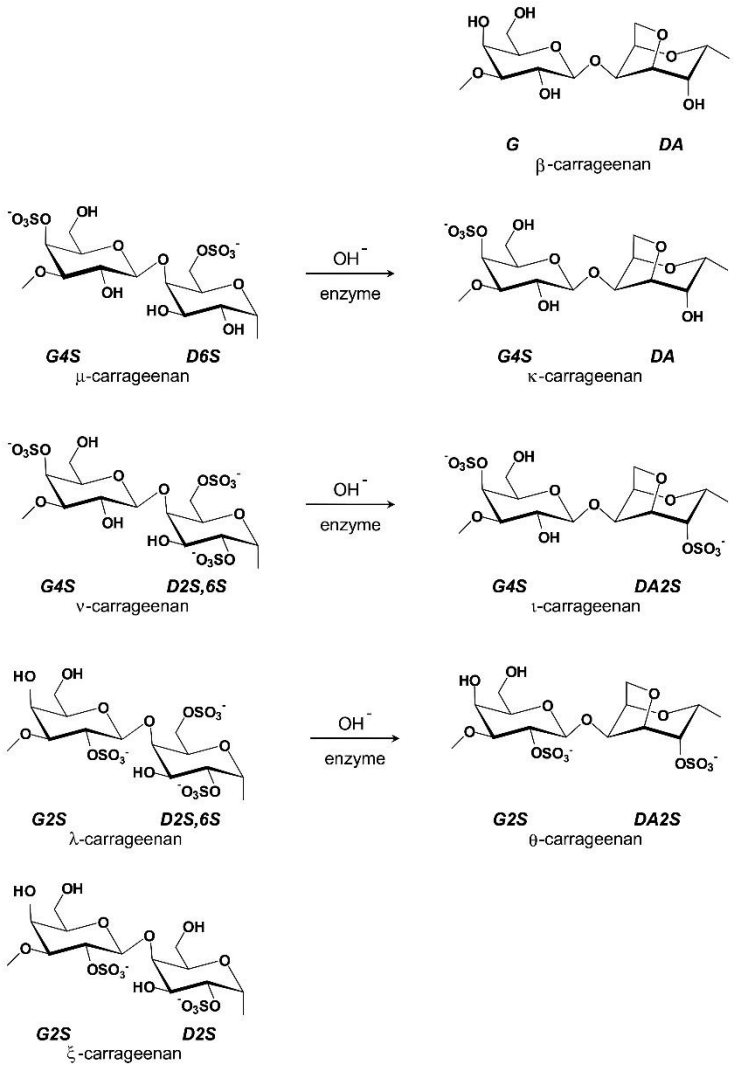

Figure 1.3 Schematic representation of the different structures of the repeating units of carrageenans (adapted from **van de Velde *et al*., 2004**).

Generally, algae do not produce these idealised and pure carrageenans, but rather a range of hybrid structures. Several other carrageenan repeating units exist, e.g. xi (ξ), theta θ, beta (β), mu (μ) and nu (ν). The precursors (mu and nu), when exposed to alkaline conditions, are modified into kappa and iota, respectively, through formation of the 3,6 anhydro-galactose bridge. Different types of carrageenan are obtained from different species of the Gigartinales.

Kappa-carrageenan is predominantly obtained by extraction from the cultivated, tropical alga *K. alvarezii* (‘cottonii’). *Eucheuma denticulatum* (trade name ‘spinosum’) is the main species for the production of iota-carrageenan.

Lambda-carrageenan is obtained from different species from the genera *Gigartina* and *Chondrus*. *Chondrus crispus* tetrasporophyte stages produce lambda-type

carrageenan, which has a wide application in the food industry and is increasingly promising in the pharmaceutical and cosmetic industries.

Other related lambda-type carrageenans include xi- and theta-carrageenan. Small amounts of theta-carrageenan have been found in extracts of tetrasporic *Gigartina* and *Chondracanthus* species. Theta-carrageenan is also formed by alkaline treatment of lambda-carrageenan.

Kappa and iota hybrid carrageenans and their biochemical precursors are found in the gametophytic life phase of various species in the family Gigartinaceae. Co-polymers of kappa and iota carrageenan are referred to as "k-2" carrageenan, "kappa/iota-hybrids" or "weak-gelling" kappa by the algae processing industry and all produce gels under certain conditions.

The rheological properties of the gelling carrageenans (e.g. kappa and iota) are quite distinct: the kappa-type forms gels that are hard, strong and brittle, whereas iota carrageenan forms soft and weak gels. The common feature of these carrageenans is the anhydro-galactose bridge of the 4-linked galactose residue, respectively DA and DA2S, which adopts the $^{1}C_{4}$-chair conformation. This conformation is crucial for the formation of the helical structure and, thereby, for the ability to form a gel. Lambda-carrageenan and the precursors mu- and nu-carrageenan lack the 3,6-anhydro bridge and, therefore, the 4-linked residue adopts the $^{4}C_{1}$-chair conformation, which disturbs the helical conformation. Thus, lambda-carrageenan forms viscous solutions and acts simply as a thickening agent (**Pereira and van de Velde, 2010**).

The κ-carrageenan and the hybrid forms (κ-ι) occur normally in Gigartinaceae and Petrocelidaceae gametophytes; the λ-family carrageenans appear habitually in the tetrasporophytic stages. Finally, the ι-carrageenan (and ι-κ hybrids) is produced mainly by species of the genus *Eucheuma* (*Eucheuma denticulatum*) and also by some other species of the Cystocloniaceae and Phyllophoraceae families (**Pereira and Mesquita, 2003**).

1.6 Extraction of carrageenans

Industrial extraction is performed under extreme alkaline conditions (usually 0.1 M $Ca(OH)_2$) and high temperatures (70-90 ^oC) as at industrial level extraction processes are intended to enhance profitability and increase the gelling power of the carrageenans obtained. Other procedures of carrageenan alkaline extraction, under less aggressive conditions, have been developed in order to obtain specific fractions or carrageenans with high level of precursors. This way, a lower percentage of 6-sulphated precursor units are converted into the corresponding 3,6-anhydrous form in shorter duration processes (2–4 hours) and with a gentle alkaline (0.02–0.1 M NaOH). The process of moderate alkaline extraction is also used at industrial level when cold soluble carrageenans and/or with the ability to increase viscosity are required.

Extraction performed in hot water is perhaps the oldest procedure and, this way, water-extraction methods (which normally use distilled or bi-distilled water) produce "native carrageenans" because they reflect the "native composition" of the phycocolloids present in the alga.

Undesirable compounds, especially water-soluble organic compounds (e.g. pigments), are usually removed with mixtures of acetone and methanol, or diethyl ether. By coupling different times and extraction temperatures it is possible to obtain different types of carrageenan. For a more fine extraction, salts can be added, sodium chloride (NaCl) or sodium bicarbonate ($NaHCO_3$). Amyloglucosidase can be added to digest floridean starch. Several impurities of low molecular weight can be removed by dialysis. Finally, cell wall components such as cellulose microfibrils can be removed by centrifugation and/or successive filtrations. Carrageenan precipitates in ethanol or isopropanolol and then it is dried to constant weight, using a ventilated oven or by lyophilisation. The latter is a fine method but rather expensive (**Pereira, 2004**).

1.7 Chemical analysis of carrageenans: FTIR-ATR, FT-Raman and Nuclear magnetic resonance spectroscopy (NMR)

Vibrational spectroscopy (infrared and Raman) can reveal detailed information concerning the properties and structure of materials at a molecular level.

Vibrational spectrometers are now standard equipment in many laboratories and the techniques described below are useful for studying carrageenophyte populations, in substitution of the traditional tests of iridescence and resorcinol, used to distinguish tetrasporophytes from female gametophytes (**Brown, Neish and Harwood, 2004**; **Pereira, 2004**). Spectroscopy techniques are also useful for the development and the implementation of strategies of sustainable algae harvest, the evaluation of the natural algae composition with industrial potential and the evaluation and control of the quality of the different batches of algal material harvested and/or cultivated. The increments of the knowledge of the polysaccharide composition of the different species contribute also to the development of the phycocolloid chemotaxonomy. Spectroscopy techniques can be especially useful to analyse the composition of food ingredients, and pharmaceutical and cosmetic excipients (**Pereira *et al.*, 2009b**).

Since the 1960's, Infrared (IR) spectroscopy has been the most frequently used vibrational technique for the study of the chemical composition of phycocolloids. This technique presents two main advantages: it requires minute amounts of sample (milligrams), and it is a non-aggressive method with reliable accuracy. However, conventional IR spectroscopy requires laborious procedures to obtain spectra with a good signal/noise ratio (**Chopin and Whalen, 1993**). This limitation was overcome with the development of interferometric IR techniques (associated with the Fourier transform algorithm), known as FTIR spectroscopy (Fourier transform IR). More recently, FTIR-ATR (from attenuated total reflectance) spectroscopy allowed the determination of the composition of the different phycocolloids from dried ground algae (**Pereira *et al.*, 2009b**).

The Raman spectroscopy is considered a complementary technique of the IR spectroscopy by some authors, presenting similar spectra to the ones of infrared. However, certain bands of weak intensity in FTIR appear clear in FT-Raman, which increases the information and allows the correct interpretation of the vibrational spectra. In comparative studies of carrageenan types, the FTIR spectra provide enough

information, however, variants of the lambda-family carrageenans can be more clearly identified by FT-Raman (**Pereira *et al*., 2003**).

While FTIR analysis requires no further treatment of the samples, FT-Raman requires depigmentation, which can be achieved by sun drying (a similar process used by harvesters/producers of commercial algae) or by pigment removal in the laboratory by the addition of a calcium hypochlorite solution (4%, 30 to 60 s, 4 °C), according to Pereira (**2006**) and Pereira *et al*. (**2009b**).

Combining FTIR-ATR with FT-Raman results in accurate identification of the natural composition of the phycocolloids presents in the alga. ^{1}H-NMR spectroscopy, more sensible than ^{13}C-NMR, not only provides identification but also quantification of the different carrageenan types, based on the intensity and chemical shift of the resonances of the anomeric protons. It controls sample purity and it is especially useful for quantifications in hybrid carrageenans. Quantitative analysis of natural carrageenan batches is of greatest importance for both ingredient suppliers and food industries to ensure ingredient quality (**van de Velde *et al*., 2004**).

1.8 Aquaculture

1.8.1 Aquaculture – environments and systems, an overview

Worldwide, aquaculture continues to be the fastest growing animal food producing sector. In 2006, it had already equalled wild fisheries in the world's fish supply (**FAO, 2009a**). Aquaculture is the breeding, rearing and harvesting of fish, crustaceans, molluscs, aquatic plants, algae and other organisms in freshwater, brackish water or saltwater under controlled conditions. Different aquaculture systems can be categorised into three major groups: extensive, semi-intensive and intensive systems.

Extensive farming is usually conducted in medium-size to large-size ponds or water bodies in a 'natural' habitat, relying entirely on the food web, thus, with no supplementary food added and with minimum impact on the environment, although fertilisation may be done. In intensive farming, the cultivated species are kept at high stocking densities and they are totally dependent on the feed provided, fertilisers (chemical and organic) and pesticides. Water must be replenished at a high rate to maintain oxygen levels and remove waste. Semi-intensive systems use stocking densities higher than extensive systems and use supplementary feeding. Intensive and semi intensive systems use small pond compartments, of up to one hectare in size, for ease of management. (**Bostock *et al,* 2010**; **Baluyut, 1989**).

Freshwater environments were the source for 60 per cent of the world aquaculture production in 2008 (**FAO, 2010** *apud* **Bostock *et al,* 2010**). Freshwater aquaculture is mostly conducted in earth ponds, open-air concrete tanks and raceways; they can have flow-through systems (river water enters tanks upstream and leaves downstream) or recirculating systems, where water remains in a closed circuit and is recycled so it can re-enter the tanks. The latter are more energetically costly, but offer better control of breeding conditions, such as temperature and oxygen, and better water quality. Mariculture is the aquaculture of marine organisms and it can be done in coastal cage farms, coastal ponds and lagoons, or in onshore tanks, where most use pumped water that passes through the tanks once before being discharged to the environment. Onshore recirculation systems can also be used for the farming of marine species. On a large scale, marine farming may pose several threats to marine and coastal environments, such as degradation of natural habitats, nutrients and waste discharge,

transmission of diseases to wild stocks, and displacement of local and indigenous communities (**Phillips, 2009**). In fact, in water downstream from aquaculture farms, changes in the levels of oxygen, suspended organic matter, inorganic nutrient, heavy metals and even drugs can be measured and may negatively impact the downstream biological communities (**Abreu, 2011c**).

The EU-funded project SEACASE (2007-2010) – "Sustainable extensive and semi-intensive coastal aquaculture in Southern Europe"– had as a purpose the development of effective tools for the sustainability of extensive and semi-intensive aquaculture production in Southern Europe, while minimising its environmental impacts, improving the quality and public image of its products. Extensive and semi-intensive aquaculture systems in Southern Europe are responsible for a significant production, at least 100,000 tonnes/year, and for using a large area, at least 92,000 ha, along the southern European coastal zone (e.g., confined areas of coastal lagoons, natural and managed deltas, and semi-closed bays and estuaries, encompassing polders with earthen ponds) (**Dinis, 2010**).

In regard to seaweed aquaculture, at the present time, the commercial carrageenan industry is largely based on cultivated sub-tropical species (approximately 88.3%) from the major world producers, in Indonesia and the Philippines (**Pereira 2009b**). Commercial demand for carrageenan has renewed the interest of collectors of raw materials and major companies of food additives for cold water carrageenophyte species. However, non-sustainable procedures can have severe economic and environmental impacts, including total loss of valuable biomass (**Pereira, 2004**; **Pereira and Mesquita, 2004** *apud* **Pereira, 2009b**). The integrated culture of algae is an alternative option to traditional carrageenophyte harvest, especially when there is a renewed interest in the cultivation (both intensive tank and extensive open-water operations) of cold water carrageenophytes, e.g., *C. crispus* (**Pereira 2009b**).

1.8.2 Integrated Multi-Trophic Aquaculture (IMTA)

IMTA is a practice in which the by-products (wastes) from one species are recycled to become inputs (fertilisers, food and energy) for another. Fed aquaculture species (e.g. finfish/shrimps) are combined, in the appropriate proportions, with organic extractive aquaculture species (e.g. suspension feeders/deposit feeders/herbivorous fish) and inorganic extractive aquaculture species (e.g. seaweeds). Multi-trophic refers to the incorporation of species from different trophic or nutritional levels in the same system (**Chopin, 2006** *apud* **FAO, 2009b**; **Chopin and Robinson, 2004** *apud* **FAO 2009b**). Ideally, the biological and chemical processes in an IMTA system should balance. This is achieved through the appropriate selection and proportions of different species providing different ecosystem functions. The co-cultured species should be more than just biofilters; they should also be harvestable crops of commercial value (**Chopin, 2006** *apud* **FAO, 2009b**). A working IMTA system should result in greater production for the overall system, based on mutual benefits to the co-cultured species and improved ecosystem health, even if the individual production of some of the species is lower compared to what could be reached in monoculture practices over a short term period (**Neori *et al*., 2004** *apud* **FAO, 2009b**).

The IMTA concept is very flexible. IMTA systems can be land-based or open-water systems, marine or freshwater systems, and may comprise several species combinations (**Neori *et al*., 2004** *apud* **FAO, 2009b**). Some IMTA systems have included such combinations as shellfish/ shrimp, fish/seaweed/shellfish, fish/shrimp and seaweed/shrimp (**Troell *et al*., 2003** *apud* **FAO, 2009b**).

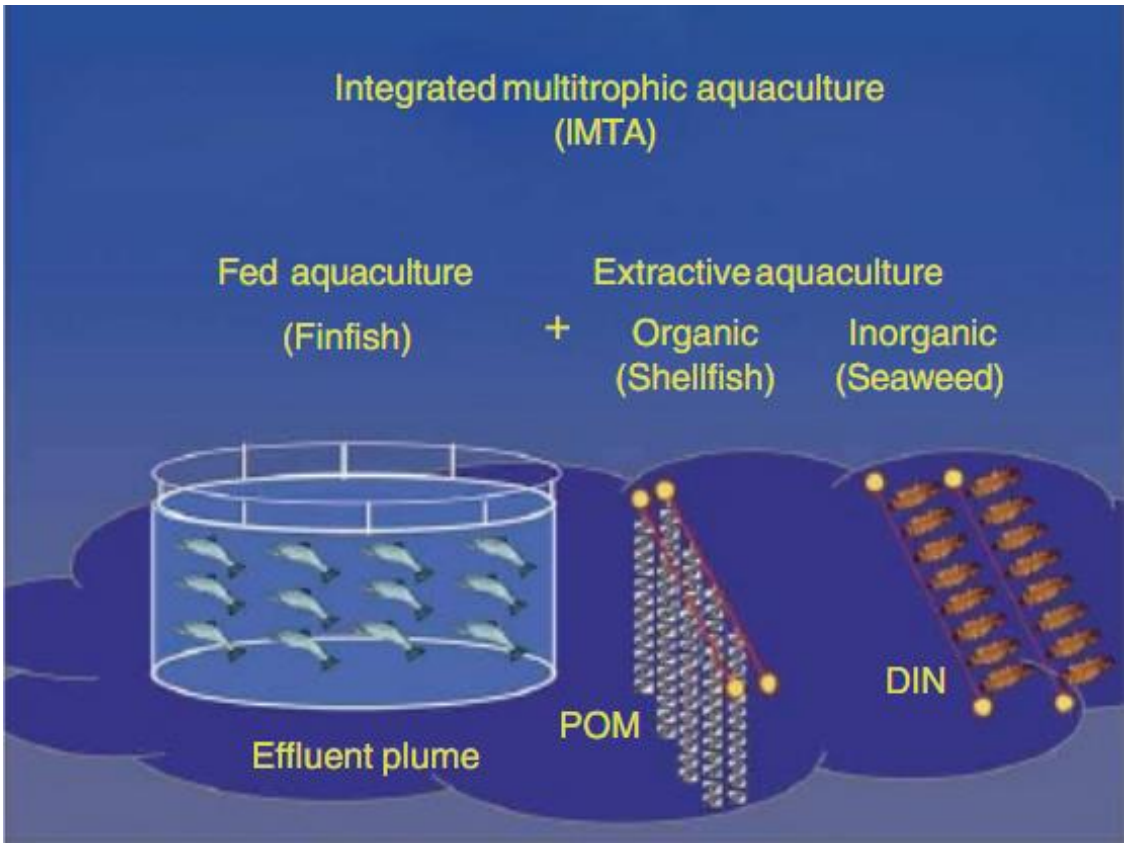

Figure 1.4 Conceptual diagram of an integrated multi-trophic aquaculture (IMTA) operation including the combination of fed aquaculture (e.g., finfish) with organic extractive aquaculture (e.g., shellfish), taking advantage of the enrichment in particulate organic matter (POM), and inorganic extractive aquaculture (e.g., seaweeds), taking advantage of the enrichment in dissolved inorganic nutrients (DIN) (adapted from **Chopin *et al.*, 2008**, 'Multitrophic Integration for Sustainable Marine Aquaculture', in *Encyclopedia of Ecology*: Elsevier, p. 2466).

Another EU-funded project, SEAPURA (2001-2003) – Species diversification and improvement of aquatic production of seaweeds purifying effluents from fish and other waste sources – was the first project to implement IMTA in Portugal. Portuguese researchers from the University of Porto and the University of Algarve (CIIMAR and CCMAR) and partners from Germany, Spain, United Kingdom and France studied seaweeds in IMTA systems, monitoring productivity, biofiltration, physiological responses, uses of produced biomass, and developed guidelines on the viability of these systems in Europe. With this project the concept of IMTA was widely disseminated through workshops and scientific papers (**Abreu *et al.*, 2011b**).

While non-sustainable harvesting can have severe economic and environmental impacts including total loss of valuable biomass (**Pereira, 2004**; **Pereira and Mesquita, 2004**), the IMTA concept can also provide for simultaneous and natural

bioremediation of eutrophication resulting from intensive fish aquaculture. It may be necessary to educate end-users on their potential negative attitudes towards growing food grade products in “fish waste” (**Pereira, 2004; Chopin and Robinson, 2006** *apud* **Pereira 2009b**).

Note: The culture of seaweeds is generally referred to as 'seaweed farming', while the term 'algaculture' refers specifically to the culture of microalgae, prokaryotic or eukaryotic photosynthetic microorganisms, in bioreactors.

1.8.3 Aquaculture in Portugal

The total length of the coastline of Portugal, including Archipelago of Madeira and Archipelago of Azores, is 2830 km. Portugal has the biggest Exclusive Economic Zone in Europe with 1,727,408 km^2, which includes an area of territorial waters with 64,145 km^2 and a continental shelf area of 20,141 km^2. The mainland coast is in a transition zone to warmer ecosystems, with high biodiversity, but with relatively low values of capture fisheries per species. The majority of the Portuguese population (76%) lives along the coastal area. Portugal holds the 4^{th} biggest European fishing fleet, after Greece, Italy and Spain. The Portuguese eat 56.9 kg/person/year of fish, molluscs and crustaceans, numbers surpassed only by Japan and Iceland. However, the national production of seafood, ca. 217,000 tonnes/year, is insufficient to assure that consumption. Portuguese aquaculture produces only 7,000 tonnes of that volume, which makes only 0,3% of the total European production (2,400,000 tonnes/year). That way, aquaculture is seen as an activity in need of rapid growth as the Portuguese Government wants to expand offshore aquaculture in order to reach the goal of 21,000 tonnes/year by 2015 (**Abreu *et al*., 2011b**; **DGPA, 2007**).

Most of the Portuguese aquaculture takes hold in extensive and semi-intensive culture systems in estuarine zones, using the intertidal zone and land-based tanks. In extensive aquaculture systems, Ria Formosa in Algarve has the highest production, followed by Vouga estuary (Ria de Aveiro), Tagus, Sado, Mira and Mondego estuaries. Mollusc production is the most significant (with *Crassostrea gigas* and *Ruditapes decussatus*). Production of rainbow trout (*Oncorhynchus mykiss*) has decreased, while saltwater species with higher commercial value are currently dominating national fisheries production, such as gilt-head bream (*Sparus aurata*), European seabass

(*Dicentrarchus labrax*), brill (*Scophthalmus rhombus*), turbot (*Scophthalmus maximus*) e Senegalese sole (*Solea senegalensis*) (**Abreu *et al*., 2011b**).

Extensive and semi-intensive aquaculture systems are highly dependent on natural processes and they have low production levels (500 Kg/ha/year to 20 tonnes/ha/year) when compared to intensive systems reaching hundreds of tonnes a year. This type of culture system is mainly established in salt marsh zones with special conservation status (Natura 2000), thus facing several environmental challenges, followed by enormous bureaucratic obstacles, all of this obviously not very appealing to potential investors, especially when direct competition with Spain, Turkey, Tunisia and Greece is reducing market price and minimising profit (**Abreu *et al*., 2011b**).

Seaweed production systems of several Portuguese native species with economic value have been studied. In the South of Portugal, the most successfully cultivated species belong to genera *Asparagopsis* and *Ulva*. In the North, testing was done with *Chondrus crispus*, *Gracilaria vermiculophylla*, *Palmaria palmata*, *Ulva rigida* and *Mastocarpus stellatus*. The seaweed cultivation was mainly performed in white cylindrical polyethylene tanks (Allibert-Buckhorn) outside the aquaculture area (**Abreu *et al*., 2011b**).

The priorities in the investigation of these systems were the optimisation of seaweed productivity and its efficiency in nutrient removal. Up to 2011, the most complete studies of IMTA in Portugal were performed with *Asparagopsis armata* and *Gracilaria vermiculophylla*, as they have the best balance between productivity and commercial use (**Abreu *et al*., 2011b**).

Algal protein content can be raised when seaweeds are cultivated in IMTA systems, by the accumulation of nitrogen (N) in the algal issue, which was proved for *Gracilaria vermiculophylla* (**Pereira *et al*., 2010**, **Abreu *et al*. 2011c** *apud* **Abreu *et al*. 2011b**). The quality and quantity of phycocolloid can vary according to N content in the biomass **(Buschmann *et al*., 1994**; **Troell *et al*., 1997** *apud* **Abreu *et al*., 2011b**). The influence of IMTA cultivation in the characteristics of polysaccharides was also studied in Portugal. Sousa *et al*. (**2010**) concluded that the agar extraction process in *Gracilaria vermiculophylla* can be affected: cultivation density and nutrient flux affect significantly the yield and strength of the agar. The effect of IMTA cultivation in the extracted carrageenan from *Mastocarpus stellatus* and *Chondrus crispus* has been monitored by the ongoing project 'CARRAGEENAN' (**Abreu *et al*., 2011b**).

In Portugal, seaweeds are beginning to be integrated in aquaculture farming by research scientists who work with these organisms and took interest in studying the IMTA concept. At the present time, Algaplus Lda., is the only Portuguese company cultivating seaweeds in an IMTA system, in partnership with Materaqua Lda., in Ílhavo, in Ria de Aveiro.

Abreu *et al*. (**2011c**) outlined criteria useful in identifying suitable seaweed species for IMTA: wide tolerance of environmental conditions, similar growth characteristics between life phases, high rates of nutrient uptake with preference for ammonium-nitrogen, and commercial value for extractive and/or consumption purposes. This way, it is important to select local and representative species. Good examples for the fish aquaculture when adopting IMTA already exist with *Gracilaria*, *Ulva* and kelp species, with sufficient biomass for effective bioremediation.

1.9 Aims of the present study

The purpose of this collaboration between the Department of Life Sciences of the University of Coimbra, the research centre IMAR-CMA and Algaplus Lda. was to understand if the quantity of polysaccharides – carrageenans – in IMTA-cultivated *C. crispus* specimens were higher than in *C. crispus* wild specimens and if its morphological and histological and chemical properties were maintained.

The aims of the present study were:

– to undertake a comparative morphological and histological analysis of IMTA-cultivated *C. crispus* and wild *C. crispus* harvested in the Centre and North coast of Portugal;

– to analyse and compare carrageenan yields, after alkaline extraction, in IMTA-cultivated *C. crispus* and in wild *C. crispus* harvested in the Centre and North coast of Portugal;

– to analyse the chemical characteristics of the carrageenans, both in their native state and the alkaline extracted, produced by IMTA-cultivated *C. crispus* by FTIR-ATR spectroscopy analysis and compare them with previous studies of wild *C. crispus* from the Portuguese coast.

2. Methodologies

2.1 Algal material

All specimens of IMTA-cultivated *C. crispus* and all specimens of wild harvested *C. crispus*, except the ones from Buarcos bay, were provided by Algaplus Lda.

Voucher specimens of cultivated *C. crispus* were deposited in the Algae Herbarium Collection of the University of Coimbra (COI).

The sets of samples of harvested *C. crispus* from Algaplus were all received as dehydrated specimens, as they were harvested in the summer of 2012. These samples were harvested in Mindelo (41º18' N, 8º44' W) and Labruge (41º16' N, 8º43' W) in Vila do Conde, and Aguda (41º03' N, 8º39' W) in Gaia.

Individual fronds of all three life cycle phases of *C. crispus* were sampled from an intertidal population at Buarcos (40º10' N, 8º53' W) in the spring of 2013.

IMTA-cultivated samples of *C. crispus* were received as fresh specimens. Their original provenance was Mindelo, Vila do Conde. They were harvested in the spring of 2013 (Figs. 2.2 to 2.4) and cultivated for 5 weeks (May-June) in tanks, in a land-based Integrated Multi-Trophic Aquaculture (IMTA) system, at Algaplus.

Algaplus Lda. detains the seaweed aquaculture sector of a land-based IMTA system, in partnership with finfish aquaculture farm Materaqua Lda., at Ria de Aveiro, combining a semi-intensive finfish earth-pond system with a continuous-flow system, in an overall open system. Main gates regulate the exchange of water between the finfish ponds and the estuarine tidal stream, without any energy consumption as opposed to a pumping system, which is high energy consuming. A schematic representation of this IMTA system is given in Fig. 2.1.

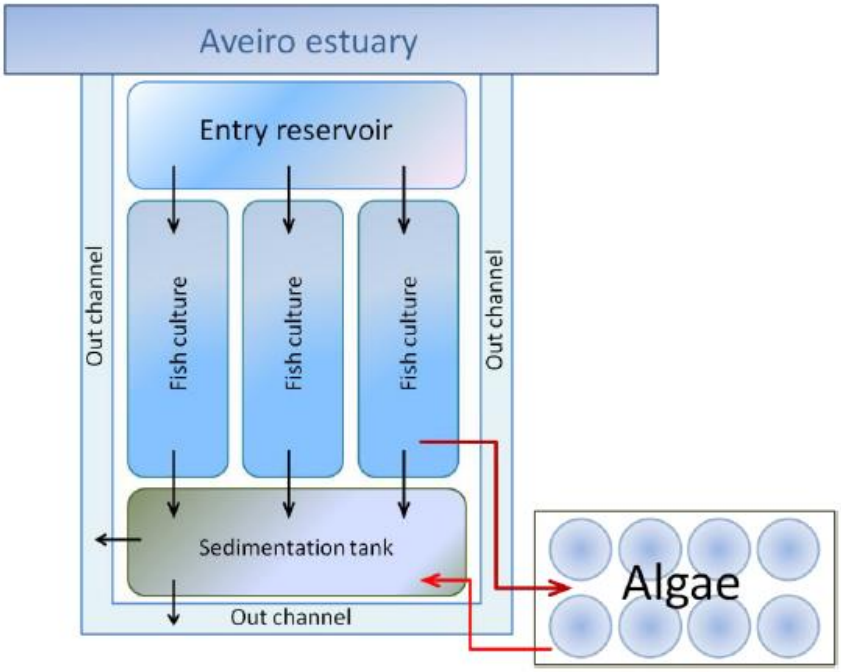

Figure 2.1 Schematic representation of the IMTA system at Algaplus Lda. in cooperation with Materaqua Lda. at Ria de Aveiro.

The water comes from the aquaculture fish tanks, in continuous flow, and feeds the seaweed tanks. In the tanks, the controlled variables are the water renewal (water inflow and water outflow for nutrient removal), water aeration and the cultivation density. *C. crispus* tanks had a cultivation density of 5 Kg/m^2. The seaweeds are under natural temperature and luminosity variations.

For Algaplus Lda. confidentiality reasons, permission to take pictures or give away any specifications of this IMTA system was not granted.

Figure 2.2 Sampling individual fronds of *C. crispus* from an intertidal population at Buarcos: non-fructified fronds.

Figure 2.3 Sampling individual fronds of *C. crispus* from an intertidal population at Buarcos: female gametophytes.

Figure 2.4 Sampling individual fronds of *C. crispus* from an intertidal population at Buarcos: tetrasporophytes.

Except for the spectroscopy analysis, all the laboratory work took place at the Department of Life Sciences of the University of Coimbra.

At the laboratory, all samples were separated according to their life cycle phases: female gametophytes, tetrasporophytes and non-fructified thalli.

Fresh specimens (IMTA-cultivated specimens and the ones sampled in Buarcos) were preserved at ca. -10 ºC and only unfrozen for life cycle phase identification immediately before histological studies were conducted and cleaned from epiphytic organisms, especially parasitic Crustaceans. Despite the presence of these grazers, the sampled specimens did not have damaged thalli. (Thalli length measurements were not conducted in this study.)

Even in the dried algal samples, identifying female fruiting bodies was rather easy at macroscopic scale, as cystocarps protrude strongly as concave-convex swellings of ca. 2 mm in diameter. As for tetrasporophytes, tetrasporangial sori are also macroscopically identifiable against backlight. Specimens not fully grown and/or with few fruiting bodies were identified with the help of a stereomicroscope: Figs. 2.5 to 2.10.

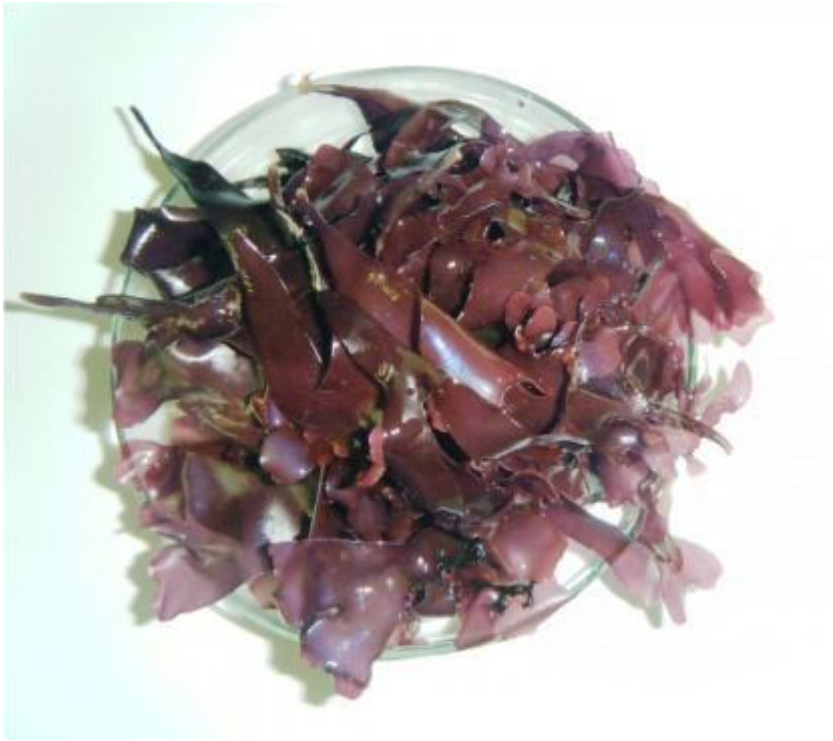

Figure 2.5 Life cycle phase identification of *C. crispus* specimens: non-fructified thalli.

Figure 2.6 Life cycle phase identification of *C. crispus* specimens: female gametophytes.

Figure 2.7 Life cycle phase identification of *C. crispus* specimens: tetrasporophytes.

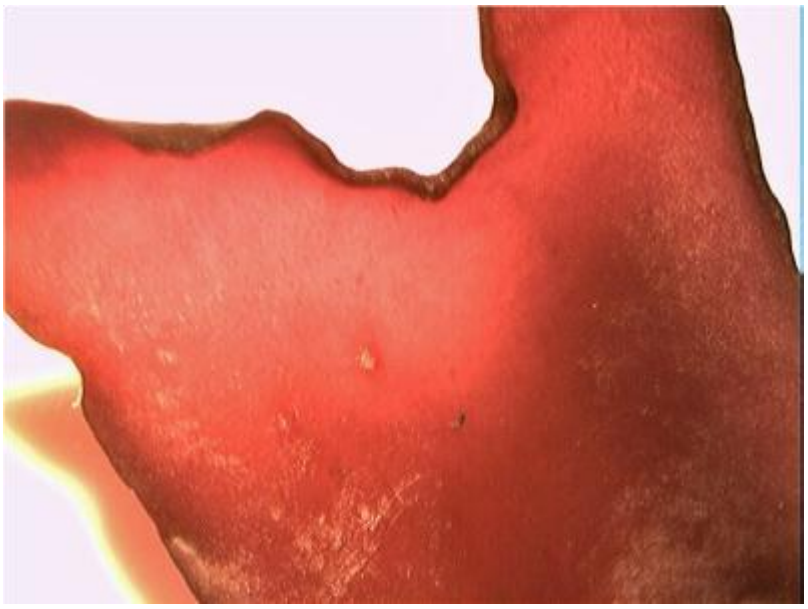

Figure 2.8 Details of the fronds of the different life cycle phases of *C. crispus* at stereomicroscope: non-fructified thalli (7X).

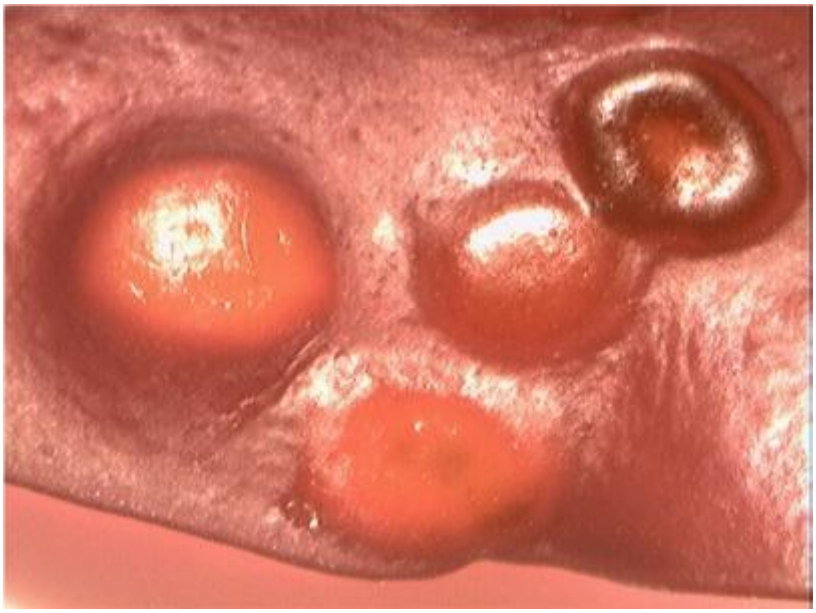

Figure 2.9 Details of the fronds of the different life cycle phases of *C. crispus* at stereomicroscope: female gametophytes (7X).

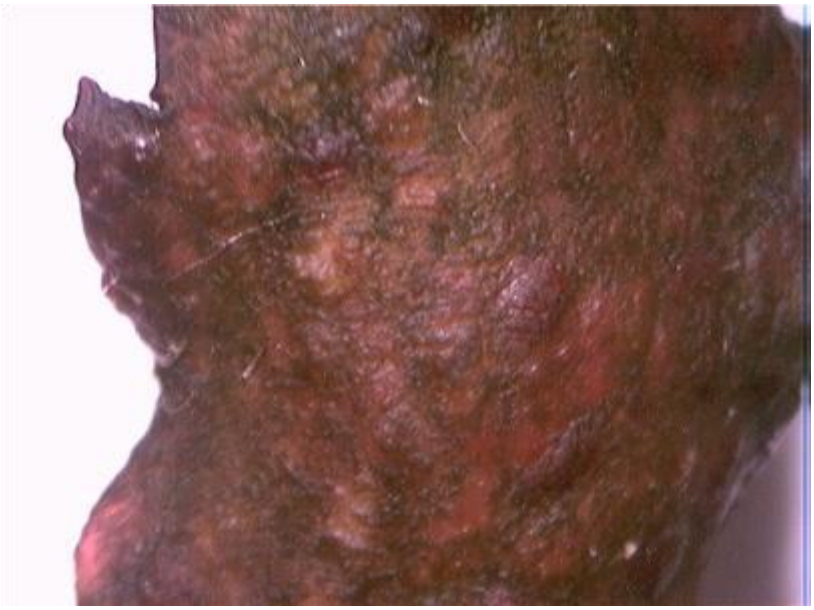

Figure 2.10 Details of the fronds of the different life cycle phases of *C. crispus* at stereomicroscope: tetrasporophytes (8X).

2.2 Morphological and histological studies

For morphological and histological studies, the cultivated specimens and the ones sampled in Buarcos were unfrozen at room temperature (21 ^{o}C) for approximately 1 hour. Hydration was maintained with filtered seawater (non-sterilised).

After life phase identification and removal of epiphytic organisms, cross-sections with ca. 30 μm in thickness of were made using a manual microtome. They were mounted in young stems of *Sambucus nigra* and cut to obtain undisrupted serial sections. Temporary preparations were sealed with clear nail polish all around the cover slip. This is a common laboratory procedure to maintain hydration of the biological material and to prevent the room air from entering the preparation and forming air bubbles. The preparations were kept for 24 hours at 4 ^{o}C.

Photographs of cross-sections were digitally obtained at the optical microscope.

2.3 Alkaline extraction of carrageenan

The fresh specimens destined to alkaline extraction, the IMTA-cultivated and the ones harvested in Buarcos, were rinsed in distilled water, to eliminate debris and salt, and then dried in a ventilated oven, to a constant weight, at 60 ^{o}C (**Pereira *et al.*, 2003**; **Pereira and Mesquita, 2004**).

Harvested *C. crispus* samples from Algaplus, as mentioned above, were already dehydrated and ready for grounding.

Carrageenan extraction was performed as similarly as possible to industrial extraction.

1.00 g of dried, finely ground samples, with three replicates for each sample, were rehydrated and then immersed in a methanol-acetone (1:1) mixture to eliminate the organ-soluble fraction, e. g. pigments, for 24 hours, at room temperature below 25 ^{o}C, as the extraction process begins at this temperature (**Pereira *et al.*, 2003**; **Zinoun and Cosson, 1996**).

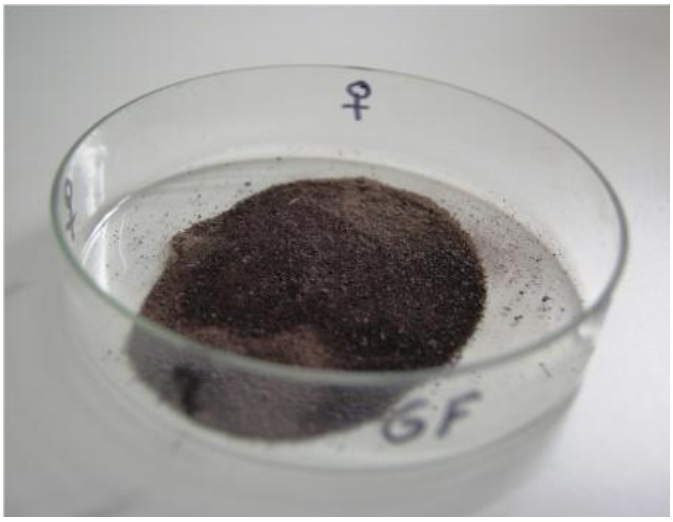

Figure 2.11 Dried, finely ground *C. crispus* sample.

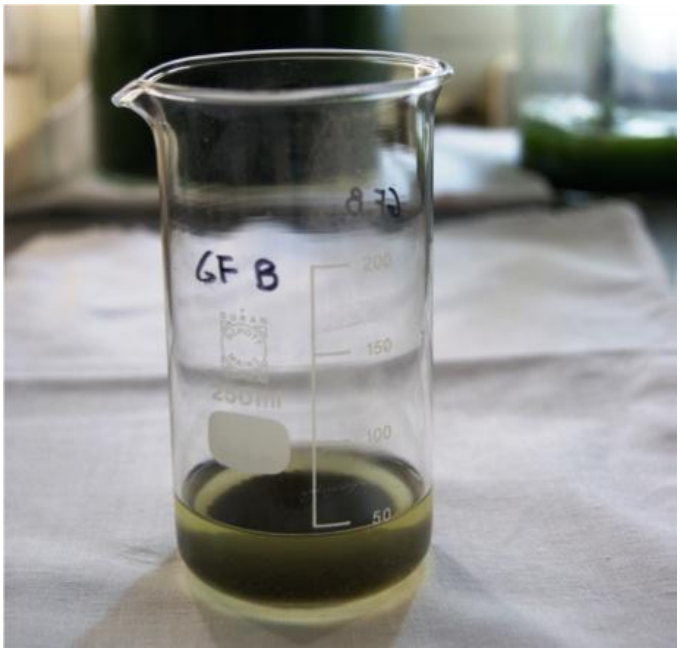

Figure 2.12 Ground *C. crispus* sample immersed in a methanol-acetone mixture.

After 24 hours, the methanol-acetone mixture was carefully decanted and rejected, and the samples returned to the ventilated oven, at 60 ºC, for a few minutes. The samples were then placed in 150 mL NaOH (1 M), at 80 ºC, for at least 3 hours for alkaline treatment (Fig. 2.13). It is important to agitate the solution during alkaline treatment, so that the carrageenan stays in constant suspension for optimal extraction.

Hot filtration was performed, under suction: cotton filtration, followed by filtration in a silica filter (Fig. 2.14). The carrageenan precipitated adding 300 mL of ethanol (96%) – twice the initial volume of the solution (Fig. 2.15). The carrageenan was then removed with the help of a glass rod and placed in ethanol (96%) for 24 hours at 4 ºC (**Pereira and Mesquita, 2004**; **Pereira and van de Velde, 2011**).

Figure 2.13 Alkaline treatment of a group of samples at 80 ^{o}C.

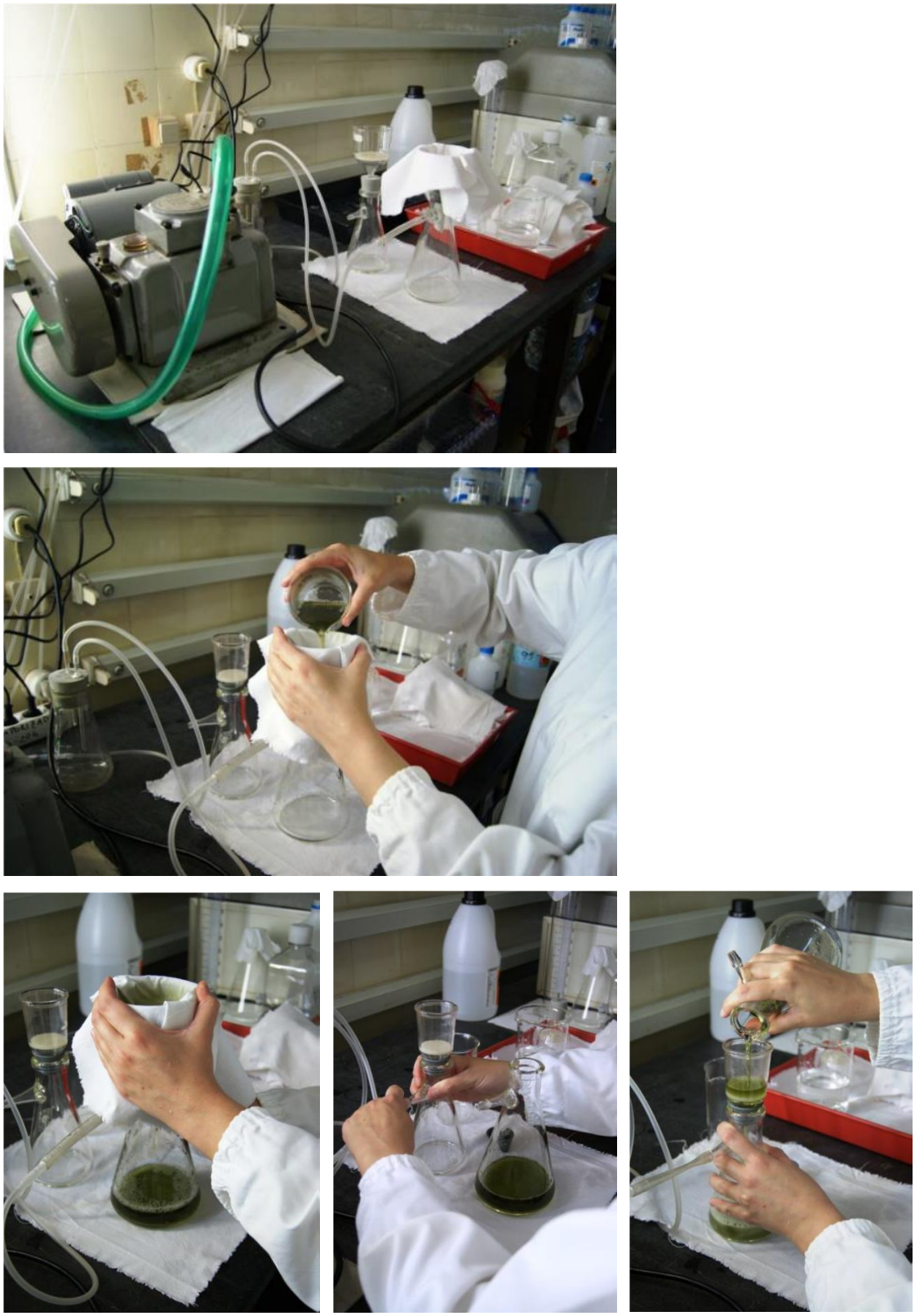

Figure 2.14 Hot filtration of one of the samples performed under suction: cotton filtration followed by filtration in a silica filter.

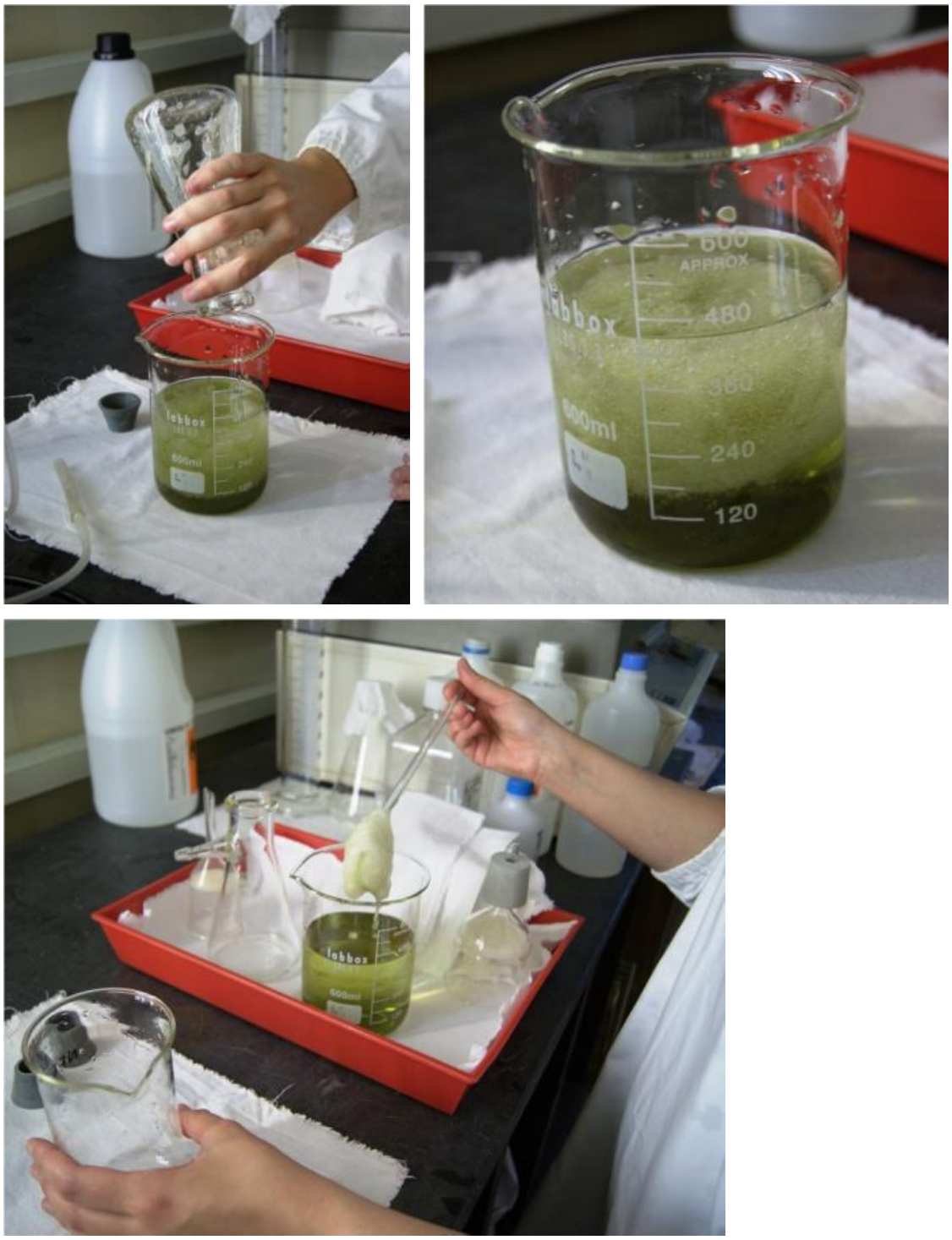

Figure 2.15 Carrageenan of one of the samples precipitating in ethanol (96%).

The coagula were dried in the ventilated oven, at 60 ºC, for 24 hours, and then weighed to determine the carrageenan content or yield (% of dry weight) (**Pereira & Mesquita, 2004**; **Pereira and van de Velde, 2011**).

2.4 Carrageenan yield determination

The formula used to determine the carrageenan yield of each sample was:

$$Yield = \frac{We}{Wds}\ x\ 100$$

where W_e is the extracted carrageenan weight (g) and W_{ds} is the dried seaweed weight (g) used for alkaline extraction.

One-way ANOVA was used to test statistical significance.

Data analysis was performed on Windows Excel 2010 (Microsoft) and Origin 8.6 (OriginLab).

2.5 Analysis by FTIR-ATR spectroscopy

2.5.1 Preparation of ground algal samples for FTIR-ATR analysis

The IMTA-cultivated algal specimens for FTIR-ATR sampling were rinsed in distilled freshwater to eliminate salt and debris from the thallus surface and dried to a constant weight, at 60 ^{o}C. The dried algae were finely ground in order to render the samples uniform. For FTIR-ATR analysis the samples do not need additional treatment and, this way, the determined composition represents, as accurately as possible, the natural colloid composition of each alga (**Chopin and Whalen, 1993**; **Pereira *et al*. 2013**).

2.5.2 Interpretation of FTIR-ATR analysis

The chemical characteristics of the carrageenans, both in their native state and the alkaline extracted, produced by IMTA-cultivated *C. crispus* were analysed by FTIR-ATR spectroscopy and compared with previous studies of wild *C. crispus* from the Portuguese coast

The FTIR-ATR analysis was carried out at the Department of Chemistry of the University of Aveiro.

The FTIR spectra of sample materials – ground-dried seaweed and alkaline modified carrageenans – were recorded on an IFS 55 spectrometer, using a Golden Gate single reflection diamond ATR system, with no need for sample preparation. All spectra are the average of two independent measurements with 128 scans, each at a resolution of 2 cm^{-1} (**Pereira *et al*., 2003**; **Pereira e Mesquita, 2004**).

Figure 2.16 FTIR-ATR equipment at the Department of Chemistry of the University of Aveiro.

3. Results and Discussion

3.1 Morphological and histological studies

Cultivation conditions such as cultivation density, water renewal and water aeration in the tanks, among others, could have affected morphological and histological aspects of the alga, which were not found to be significant in the IMTA-cultivated specimens when compared to the specimens harvested at Buarcos bay (although thalli length measurements were not conducted in this study).

As in wild specimens, IMTA-cultivated tetrasporophytes exhibited tetrasporangial sori with an appearance of dark red spots, prominent in the thallus, on the main axis and lateral branches. In the female gametophytes, the cystocarps, usually in large numbers on the pinnules, margins of the branches and thalli surface in wild specimens, had similar density and distribution as in the IMTA-cultivated individual fronds. Histologically, cortical and medullary zones of wild and IMTA-cultivated algae in all three life cycle phases appeared undistinguishable, and the tetrasporophytes in the medullary zone exhibited the same elongated cells, with apparent star shapes (not shown in the figure). The cystocarps in cross-section appear with the same density and form with regard to the carpospores. The tetrasporangial sori, with the typical cruciately divided tetrasporangia, also appear with same density and as in the Buarcos thalli.

Cross-sections of both wild and IMTA-cultivated *C. crispus* specimens' thalli at optical microscope (OM) are shown in Figs 3.1 to 3.4.

Although this is a preliminary study, morphological differences between the IMTA-cultivated and the wild seaweed were not found.

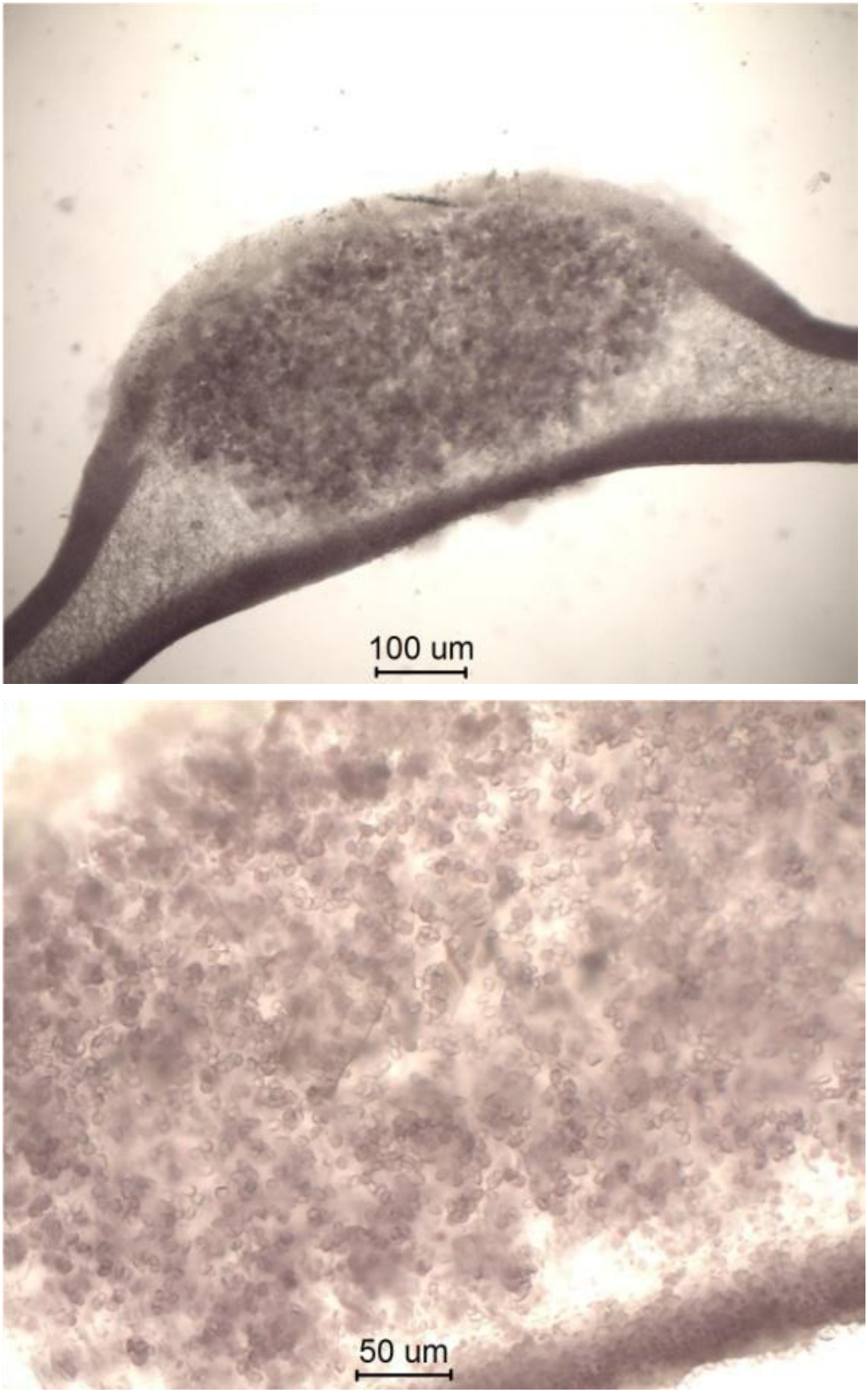

Figure 3.1 Thallus cross-section of a female gametophyte of *C. crispus* wild specimen harvested in Buarcos (OM). Cystocarp cross-section with carpospores within.

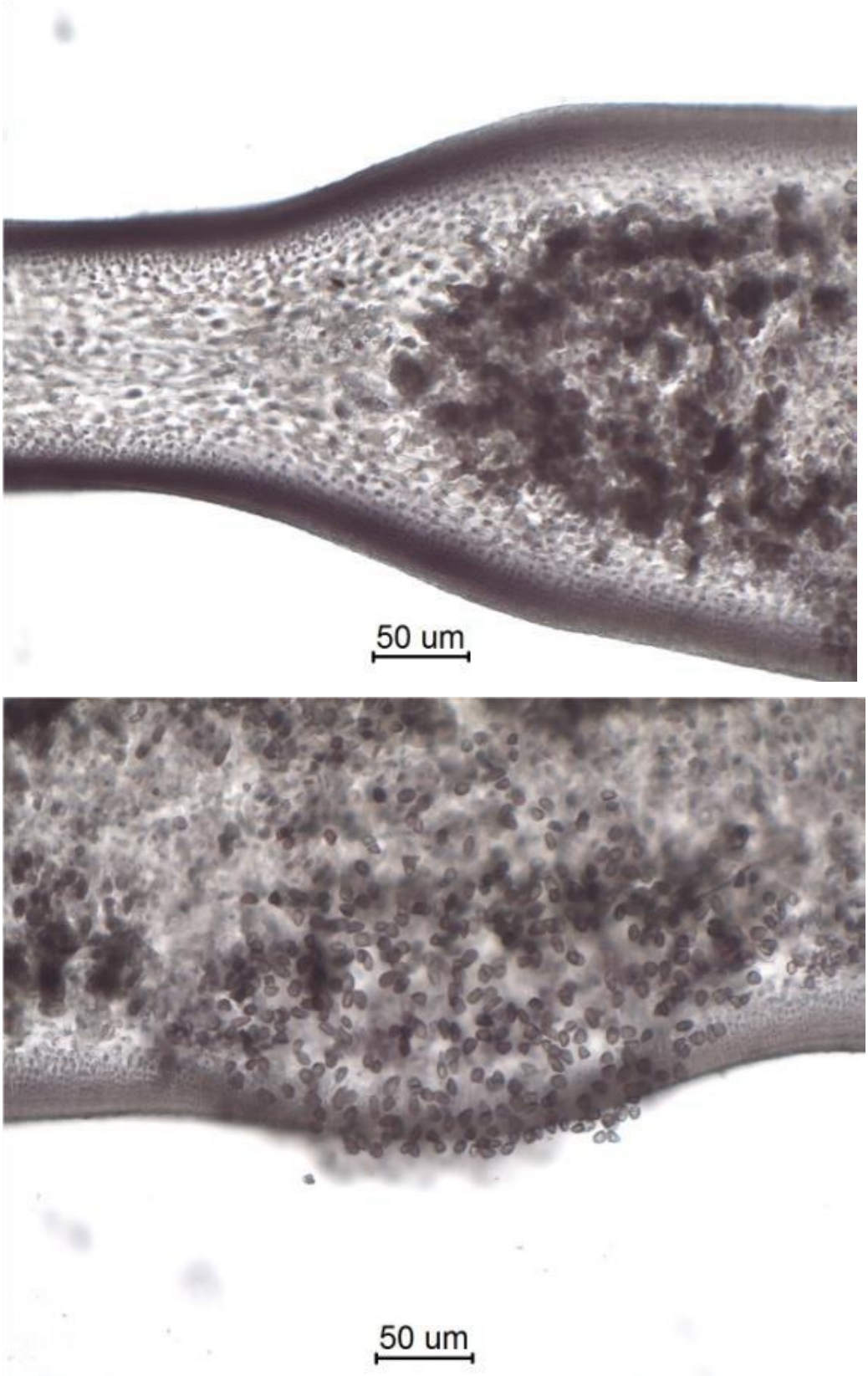

Figure 3.2 Thallus cross-section of a female gametophyte of *C. crispus* IMTA-cultivated specimen (OM). Cystocarp cross-section with carpospores within.

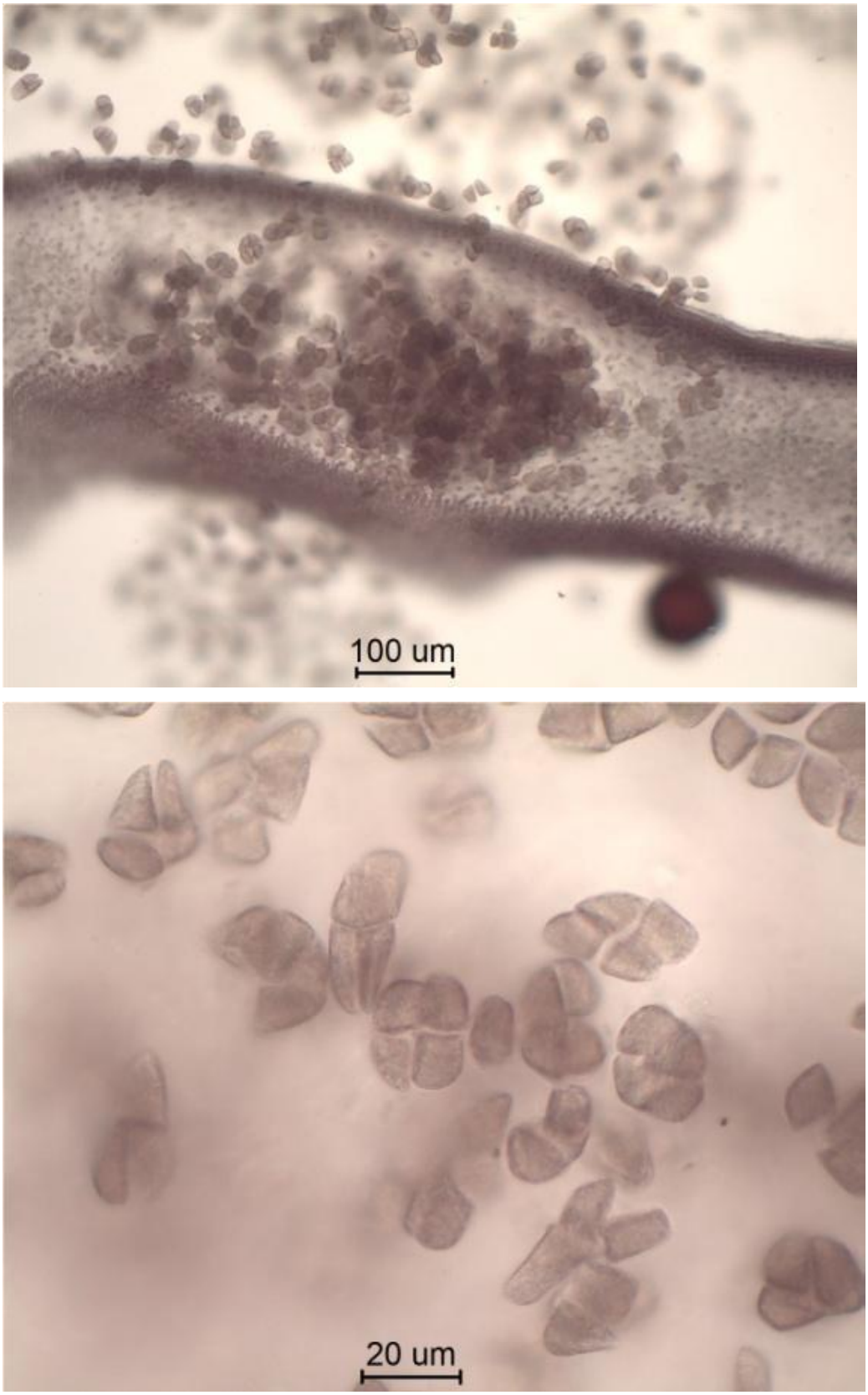

Figure 3.3 Thallus cross-section of a tetrasporophyte of *C. crispus* wild specimen harvested in Buarcos (OM). Tetrasporangial sori cross-section and detail of tetraspores.

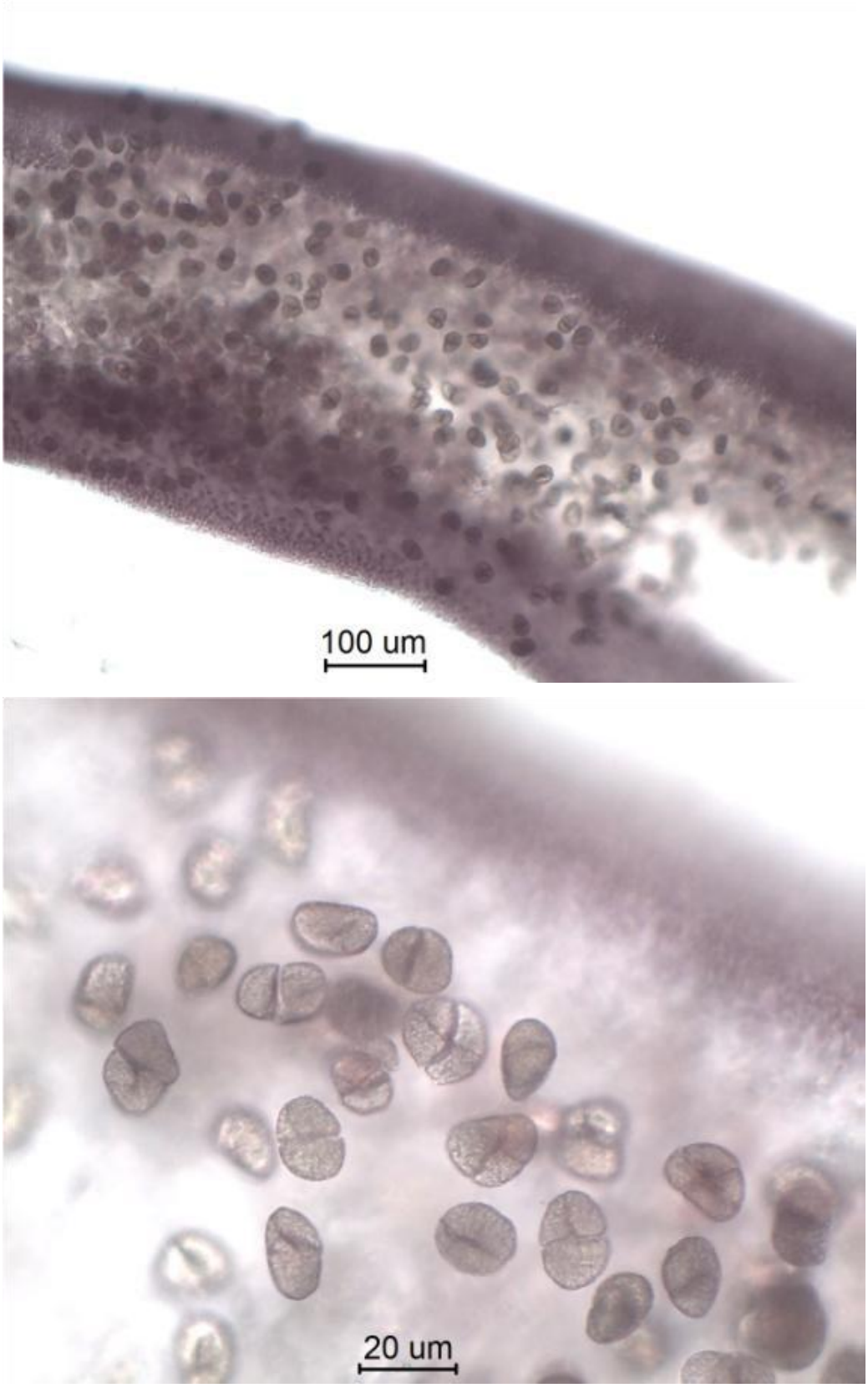

Figure 3.4 Thallus cross-section of a tetrasporophyte of *C. crispus* IMTA-cultivated specimen (OM). Tetrasporangial sori cross-section and detail of tetraspores.

3.2 Variation in carrageenan yield

Carrageenan yield values from wild harvested *C. crispus* samples are shown in Table 1. Carrageenan yield values from IMTA-cultivated *C. crispus* samples are shown in Table 2. Yield is expressed as percentage of dry weight (average ± standard error).

The maximum carrageenan content was found in a non-fructified thalli sample from Mindelo, in early August, with 33.2 ± 16.2 % (n = 3) of dry weight. The minimum carrageenan content was found in a female gametophyte sample also from Mindelo, in early August, with 8.7 ± 3.5 % (n = 3) of dry weight (Table 1).

The overall average content in carrageenan was 18.3 ± 8.4 % (n = 45), with an average of 14.9 ± 4.0 % (n = 12) in the tetrasporophytes, 18.0 ± 9.3 % (n = 18) in the female gametophytes and 21.5 ± 9.2 (n = 15) in non-fructified thalli (Table 1).

Table 1 Wild harvested *C. crispus* carrageenan yield**.**

Harvesting sites	Harvesting dates	Life cycle phase	Yield (% dry weight)
Mindelo	04-07-2012	T	9.8 (n=3)
		NF	16.1±1.1 (n=3)
	18-07-2012	FG	26.0±7.3 (n=3)
		NF	17.7±1.9 (n=3)
	01-08-2012	FG	8.7±3.5 (n=3)
		NF	33.2±16.2 (n=3)
Labruge	18-07-2012	T	15.8±0.2 (n=3)
		FG	16.4±1.9 (n=3)
Aguda	17-09-2012	T	14.0±2.1 (n=3)
		FG	14.3±1.3 (n=3)
	03-10-2012	FG	25.1±17.9 (n=3)
		NF	16.8±3.3 (n=3)
Buarcos	06-06-2013	T	20.1±1.5 (n=3)
		FG	17.3±3.6 (n=3)
		NF	23.6±3.3 (n=3)

T: tetrasporophytes; FG: female gametophytes; NF: non-fructified thalli; yield is expressed as percentage of dry weight (average ± standard error).

The data on geographical variation (harvesting sites) of carrageenan content are shown in Fig. 3.5. An average of 18.6 ± 10.9 % (n = 18) was found in the samples from Mindelo, 16.1 ± 1.2 % (n = 6) in Labruge, 17.6 ± 9.2 % (n = 12) in Aguda and 20.3 ± 3.8 % (n = 9) in Buarcos. The sampling has statistical significance (one-way ANOVA, p <0.05).

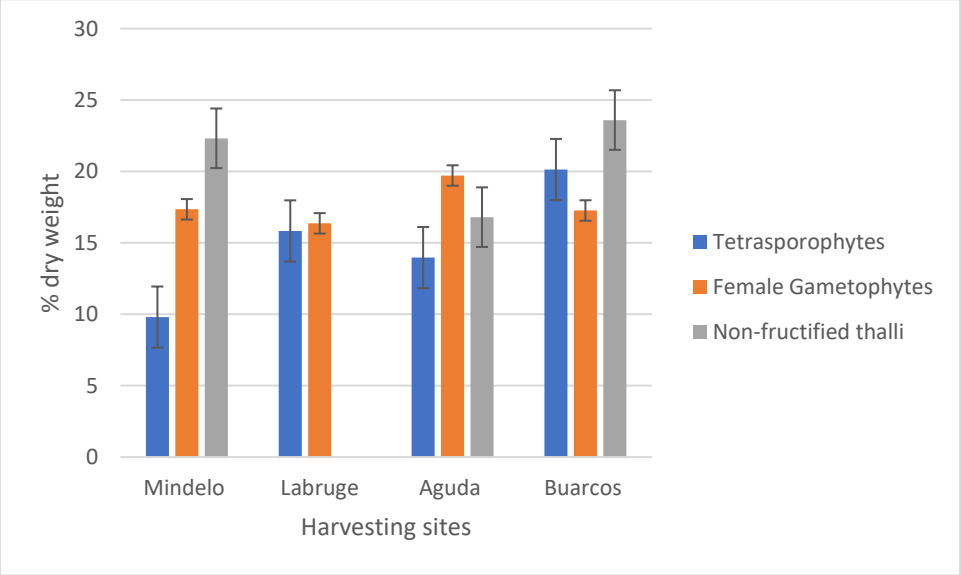

Figure 3.5 Geographical variation in carrageenan yield of wild harvested *C. crispus* (average ± standard error, n=3). The sampling has statistical significance (one-way ANOVA, p <0.05).

The data on temporal variation (harvesting time of the year) of carrageenan content are shown in Fig. 3.6. The highest values were found in samples collected in early August, with 21.0 ± 17.0 % (n = 6) of dry weight and in early October, with 21.0 ± 12.4 % (n = 6). However, tetrasporophytes were absent in those samples. The highest carrageenan content in tetrasporophytes was found in samples from early July, with 20.1 ± 1.5 % (n = 3) of dry weight and mid-July, with 15.8 ± 0.2 % (n = 3) of dry weight. The sampling has statistical significance (one-way ANOVA, p <0.05).

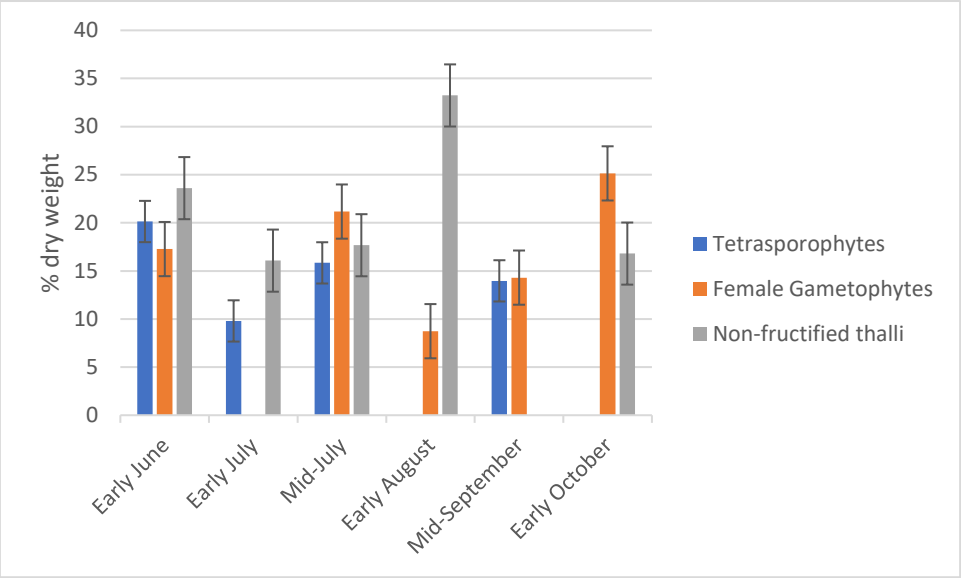

Figure 3.6 Temporal variation in carrageenan yield of wild harvested *C. crispus* (average ± standard error, n=3). The sampling has statistical significance (one-way ANOVA, $p < 0.05$).

The interest in cultivating *C. crispus* for commercial purposes is focused on the production of lambda-carrageenan, which is produced by *C. crispus* tetrasporophyte stages (**McCandless and Craigie, 1974** *apud* **Pereira, 2004**). The highest carrageenan content in tetrasporophytes was found in early June in Buarcos. However, while all samples were collected randomly in regard to life phase, exception was made for Buarcos because of the need to use fresh specimens from this location, only about 40 km close to Coimbra, for comparative morphological and histological studies on IMTA-cultivated specimens. Thus, it can be said that the highest result in carrageenan content from tetrasporophyte stages, while more representative of the local cover within the sampling, was found in mid-July in Labruge. This result is in conformity with the studies of Pereira (**2004**), who investigated extensively the carrageenophytes of the Portuguese coast, and found the highest carrageenan contents in the summer (with a small increment recorded in early spring) with the highest value in July (interestingly in tetrasporic thalli).

Table 2 IMTA-cultivated *C. crispus* carrageenan yield.

Cultivation provenance	Cultivation period	Life cycle phase	Carrageenan yield (% dry weight)
Mindelo	5 weeks May-June	T	17.5 (n=3)
		FG	22.1±2.4 (n=3)
		NF	24.6±4.5 (n=3)

T: tetrasporophytes; FG: female gametophytes; NF: non-fructified thalli; yield is expressed as percentage of dry weight (average ± standard error).

IMTA-cultivated samples of *C. crispus* original provenance was Mindelo, Vila do Conde. They were harvested in the spring of 2013 and cultivated for 5 weeks (May-June). They were received as fresh specimens and a minority of tetrasporophyte fronds in the fresh weight of these samples was recorded (data not shown).

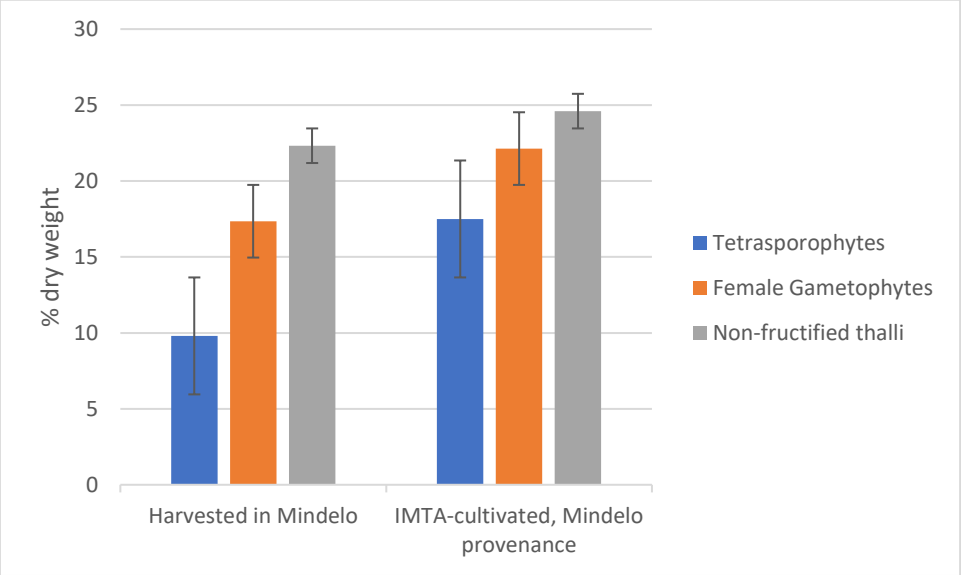

Figure 3.7 Carrageenan yield variation in *C. crispus* wild harvested in Mindelo *versus* IMTA-cultivated *C. crispus* of Mindelo provenance (average ± standard error, n=3). The sampling has statistical significance (one-way ANOVA, $p < 0.01$).

A comparative analysis of carrageenan yield variation in *C. crispus* wild harvested in Mindelo with IMTA-cultivated *C. crispus* of Mindelo provenance is shown in Fig. 3.7. Overall average carrageenan content in IMTA-cultivated *C. crispus* of

Mindelo provenance was 21.4 ± 4.0 % (n = 9), with an average of 17.5 ± 0.0 % (n = 3) in the tetrasporophytes, 22.1 ± 2.4 % (n = 3) in the female gametophytes and 24.6 ± 4.5 % (n = 3) in non-fructified thalli. The sampling has statistical significance (one-way ANOVA, $p < 0.01$).

All IMTA-cultivated *C. crispus* results were higher than the ones from wild harvested *C. crispus* in Mindelo, which is in accordance with previous citations of Abreu *et al*. (**2011b**) (see p. 23 of the Introduction).

Factors responsible for season patterns in phycocolloid content include photoperiod, life cycle phase, growth level, air temperature, seawater temperature, pH, salinity and nutrients, e. g., nitrogen (N) and phosphorous (P) (**Pereira, 2004**). It has been known since the 1950s that the manipulation of the N nutrition of algae in culture could be used to control the composition of the seaweed, including the percentage of carbohydrates. Seasonal variations in carrageenan contents in the Gigartinales, and in particular *C. crispus*, besides being a reflection of the degree of heterogeneity of the samples, i.e., the number of dichotomies and frond length (**Chopin, 1999**), depend on complex interactions of several factors in which the nutrients play a key controlling role. Chopin *et al*. (**1995**) refer that seasonal variations in carrageenan content of *C. crispus* have an increase during the summer and a decrease during the fall and that a second increase occurs through the winter, followed by a sharp decrease in the spring. The same study refers that when other parameters are constant, carrageenan content is inversely related to the ambient seawater N concentration – the 'Neish effect' – and the same is valid for the ambient P concentration – the 'Chopin effect' (**Chopin *et al*. 1995**; **Chopin, 1999**). As mentioned above, the same results were obtained by Pereira (**2004**) with tetrasporic thalli of carrageenophytes of the Portuguese coast.

Additionally, it should be referred that when analysing alkaline extraction yields, Chopin and collaborators warn that these data should be considered with caution, as alcoholic precipitation for the extraction of carrageenan often coprecipitates floridean starch, resulting in an overestimation of carrageenan content (**Chopin, 1991** *apud* **Chopin, 1995**), especially because floridean starch varies seasonally in inverse proportion to the carrageenan content (**Zinoun, 1993**). A smaller quantity of N and a bigger sunlight exposure in the spring/summer are, possibly, the responsible factors for redirecting cellular metabolism, with a preferential synthesis of carrageenan in detriment of floridean starch (**Pereira, 2004**).

3.3 FTIR-ATR spectroscopy

Carrageenan analysis by FTIR-ATR spectroscopy usually reveals the presence of very strong absorption bands in the 1210-1260 cm^{-1} region (S=O of sulfate esters) and in 1010-1080 cm^{-1} region (glycosidic linkage) (**Pereira *et al.*, 2003**), which our results also show, in all the six spectra obtained.

The biochemical alternation of generations in Gigartinaceae is well established: gametophytes produce hybrid polysaccharides belonging to the kappa-carrageenan family (hybrid kappa/iota/mu/nu carrageenan), while the tetrasporophyte stages produce carrageenans of the lambda family (hybrid xi/theta- or xi/lambda-carrageenan) (**Pereira and Mesquita, 2003**; **Pereira *et al.*, 2009a**; **Pereira *et al.*, 2009b**).

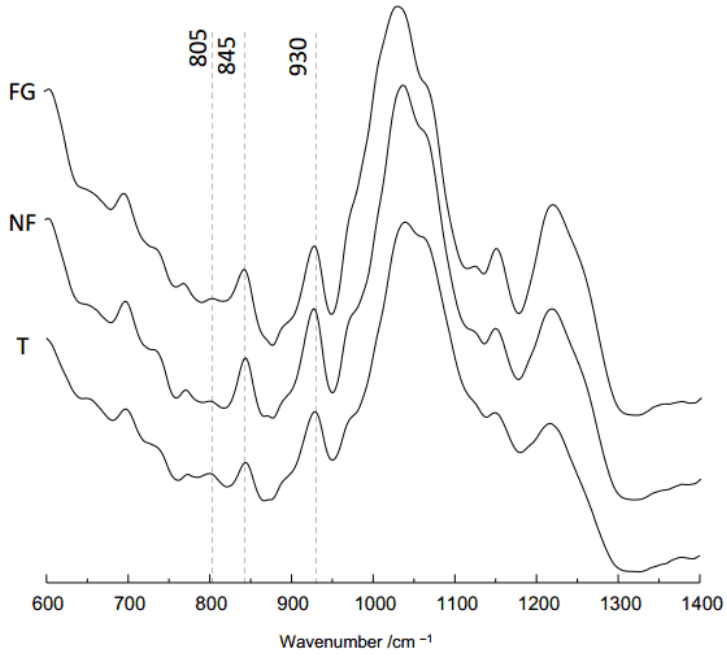

Figure 3.8 FTIR-ATR spectra of IMTA-cultivated *C. crispus* – native carrageenan from ground seaweed samples from all three life phases. (NF: non-fructified thallus; FG: female gametophyte; T: tetrasporophyte.)

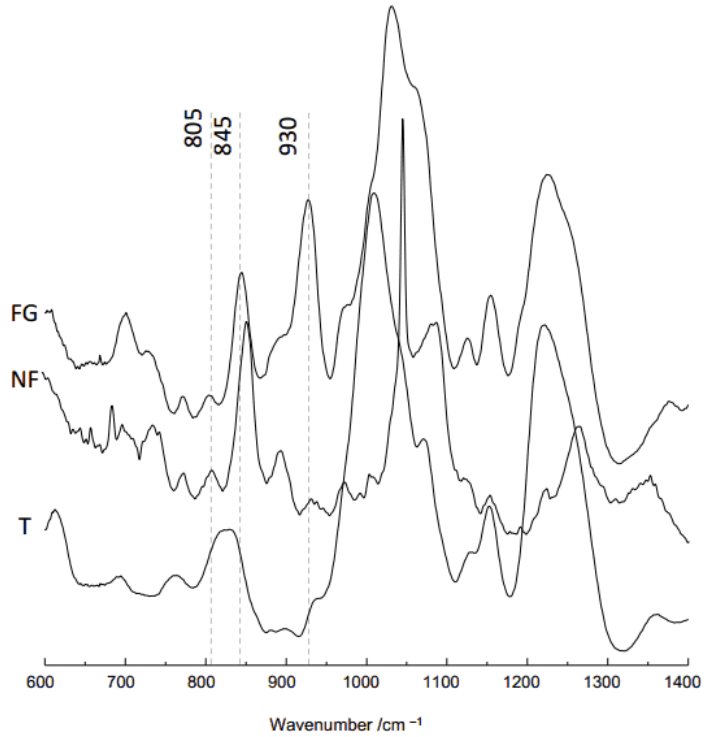

Figure 3.9 FTIR-ATR spectra of IMTA-cultivated *C. crispus* – alkaline extracted carrageenan from all three life phases. (NF: non-fructified thallus; FG: female gametophyte; T: tetrasporophyte.)

Non-fructified thalli carrageenan spectra in our results appear very similar to the ones presented by the female gametophytes, which show a strong absorbance at 1218 cm^{-1} for sulphate esters and strong absorption bands in the region of 845 cm^{-1} (G4S, or D-galactose-4-sulphate) typical of the presence of kappa-carrageenan, and in the 930 cm^{-1} region (DA, or 3,6-anhydro-D-galactose). They present lower absorbance in the 805 cm^{-1} (DA2S, or 3,6-anhydro-D-galactose-2-sulphate), which indicates a presence of low quantities of iota-carrageenan (**Pereira and Mesquita, 2003**; **Pereira and Mesquita, 2004**). The presence of these three bands is typical of hybrid kappa-iota carrageenan. Alkaline treatment of carrageenan from non-fructified thalli and female gametophytes produced a strong narrow peak at 845 cm^{-1}, which corresponds to the expected conversion of the precursor mu- into kappa-carrageenan.

The ratio between 805 and 845 cm^{-1} absorption peaks was calculated and used as a parameter to determine the degree of the iota/kappa hybridisation. The ratio 805/845 in the alkaline treated carrageenan as compared to ground whole algal samples, decreased, especially in the female gametophytes, which corresponds to an increment of the kappa fraction relatively to the iota fraction: 0.81 to 0.73 in the non-fructified and 0.88 to 0.59 in the female gametophytes.

In *C. crispus* tetrasporophytes, a shoulder peak appears at 1062 cm^{-1} in the ground sample and a narrow peak at 1070 cm^{-1} (DA) in the alkaline extracted carrageenan, which is related to the presence of theta-carrageenan, formed by alkaline treatment of lambda-carrageenan.

Alkaline extracted carrageenan from *C. crispus* tetrasporophytes shows a broad peak at approximately 820-830 cm^{-1} (G/D2S), characteristic of lambda-carrageenan, which is absent in the carrageenan spectrum from tetrasporophytes grounded samples. This may be due to an incorrect separation of life phases of non-fully grown fronds.

The results of the FTIR-ATR spectroscopy analysis of the chemical characteristics of the carrageenans, both in their native state and the alkaline extracted, produced by IMTA-cultivated *C. crispus* are in conformity with results from previous studies on wild *C. crispus* from the Portuguese coast by Pereira and collaborators (**Pereira, 2004**; **Pereira *et al.*, 2013**; **Pereira and Mesquita, 2003**; **Pereira and Mesquita, 2004**). Satisfactory results were obtained, especially with the alkaline extracted carrageenan from *C. crispus* tetrasporophytes, which is the focus in *C. crispus* IMTA-cultivation.

4. Conclusions and perspectives

The opportunity of this collaboration between the Department of Life Sciences of the University of Coimbra, the research centre IMAR-CMA in Coimbra and the company Algaplus Lda., located in Ria de Aveiro, is a contribution to preliminary studies in understanding if *C. crispus* cultivated in an IMTA system maintains its morphological and histological characteristics, as well as the chemical properties of its polysaccharides, when compared to wild *C. crispus*.

Although this is a small, preliminary study, morphological and histological differences between the IMTA-cultivated and the wild seaweed specimens were not found.

Using an alkaline extraction procedure that resembles the industrial one, the IMTA-cultivated *C. crispus* specimens, all from Mindelo provenance, had an overall good carrageenan yield when compared with wild harvested *C. crispus* from the Centre and North Portuguese coast, particularly when compared to wild harvested *C. crispus* from Mindelo. The interest in cultivating *C. crispus* for commercial purposes is focused on the production of lambda-carrageenan, which is produced by *C. crispus* tetrasporophyte stages. The highest result in carrageenan content from tetrasporophyte stages, while more representative of the local cover within the sampling, was found in specimens collected in mid-July in Labruge. This result is in conformity with previous studies on carrageenophytes of the Portuguese coast, that found the highest carrageenan contents in the summer (with a small increment recorded in early spring) with the highest value in July.

The FTIR-ATR analysis of carrageenans from IMTA-cultivated *C. crispus*, both in their native state and the alkaline extracted, was in conformity with results from previous studies on wild *C. crispus* from the Portuguese coast.

Lambda-carrageenan is very promising at the moment for pharmaceutical and cosmetic industries, making it a potential niche market product. In the carrageenan food industry sector, cold-water species are unable to compete with the sub-tropical Asian carrageenophyte species. Nevertheless, some authors predict that, in medium- to long-term horizons, Asian carrageenophyte and agarophyte industries may succumb due to climate change and consequent dramatic changes in algal flora. Thus, combining a cold-water carrageenophyte with promising new market niches, such as cold-water *C. crispus* lambda-carrageenan, may contribute to boost the economic viability of Integrated

Multi-Trophic Aquaculture (IMTA) in Portugal, while converting land-based intensive fish farms into an ecological and more sustainable aquaculture.

Bibliographical references

Abreu, M. H., Pereira, R., Buschmann, A. H., Sousa-Pinto, I. and Yarish, C. (2011a) 'Nitrogen uptake responses of *Gracilaria vermiculophylla* (Ohmi) Papenfuss under combined and single addition of nitrate and ammonium', *Journal of Experimental Marine Biology and Ecology*, 407(2): 190-199.

Abreu, M. H., Pereira, R., Mata, L. and Sousa-Pinto, I. (2011b) *Aquacultura Multi-Trófica Integrada em Portugal. Macroalgas en la Acuicultura Multitrófica Integrada Peninsular: Valorización de su Biomasa* Vigo, Spain: Centro Tecnológico del Mar – Fundación CETMAR, p. 54-77.

Abreu, M. H., Pereira, R., Yarish, C., Buschmann, A. H. and Sousa-Pinto, I. (2011c) 'IMTA with *Gracilaria vermiculophylla*: Productivity and nutrient removal performance of the seaweed in a land-based pilot-scale system', *Aquaculture*, 312: 77-87.

Abreu, M. H., Varela, D. A., Henriquez, L., Yarish, C., Sousa-Pinto, I. and Buschmann, A. H. (2009) 'Traditional vs. integrated aquaculture of *Gracilaria chilensis* C.J. Bird, McLachlan *et* E.C. Oliveira: Productivity and physiological performance', *Aquaculture*, 293: 211-220.

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R. and others (2005) 'The new higher level classification of eukaryotes with emphasis on the taxonomy of protists', *Journal of Eukaryotic Microbiology*, 52: 399-451.

Araújo, R., Bárbara, I., Tibaldo, M., Berecibar., E., Tapia, P. D., Pereira, R. and others (2009) 'Checklist of benthic marine algae and cyanobacteria of northern Portugal', *Botanica Marina*, 52(1): 24-46.

Ardré, F. (1970) 'Contribution a l'étude des algues marines du Portugal. I – La Flore', *Portugaliae Acta Biologica*, 10(1-4): 1-423.

Ardré, F. (1971) 'Contribuition à l'étude des algues marines du Portugal, II. Ecologie et chorologie', *Bulletin du Centre d'Études de la Recherche Scientifique de Biarritz*, 8(3): 359-574.

Azevedo, G., Hilliou, L., Bernardo, G., Sousa-Pinto, I., Adams, R. W., Nilsson, M. and Villanueva, R. D. (2013) 'Tailoring kappa/iota-hybrid carrageenan from *Mastocarpus stellatus* with desired gel quality through pre-extraction alkali treatment', *Food Hydrocolloids*, 31: 94-102.

Baluyut, E. A. (1989) *Aquaculture Systems and Practices: A Selected Review. FAO* Rome, Italy: Food and Agriculture Organization of the United Nations.

Barrington, K., Chopin, T. and Robinson, S. (2009) *Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. Integrated mariculture: A global review. FAO Fisheries and Aquaculture Technical Paper.* Rome: Food and Agriculture Organization of the United Nations.

Barrington, K., Ridler, N., Chopin, T., Robinson, S. and Robinson, B. (2010) 'Social aspects of the sustainability of integrated multi-trophic aquaculture', *Aquaculture International,* 18: 201-221.

Bixler, H. J. and Porse, H. (2011) 'A decade of change in the seaweed hydrocolloids industry', *Journal of Applied Phycology,* 23(3): 321-335.

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I. and Corner, R. (2010) 'Aquaculture: global status and trends', *Philosophical Transactions of the Royal Society B: Biological Sciences,* 365: 2897–2912.

Brown, M. T., Neish, A. and Harwood, D. (2004) 'Comparison of three techniques for identifying isomorphic phases of *Chondrus crispus* (Gigartinaceae)', *Journal of Applied Phycology,* 16: 447-450.

Buschmann, A. H., Mora, O. A., Gómez, P., Buitano, S., Retamales, C., Vergara, P. A. and Gutierrez, A. (1994) '*Gracilaria chilensis* outdoor tank cultivation in Chile: use of land-based salmon culture effluents', *Aquaculture Engineering,* 13: 283-300.

Chopin, T. (2006) 'Integrated multi-trophic aquaculture. What it is, and why you should care… and don't confuse it with polyculture', *Northern Aquaculture,* 12, pp. 4.

Chopin, T., Gallant, T. and Davison, I. (1995). Phosphorus and nitrogen nutrition in *Chondrus crispus* (Rhodophyta): Effects on total phosphorus and nitrogen content, carrageenan production, and photosynthetic pigments and metabolism. *Journal of Phycology*. 31(2): 283-293.

Chopin, T., Hanisak, M. D., and Koehn, F. E. (1991) Effects of seawater phosphorous concentration on floridean starch content in *Agardhiella subulata* (C. Agardh) Kraft *et* Wynne (Rhodophyceae), *Botanica Marina*, 34: 369-373.

Chopin, T., MacDonald, B., Robinson, S., Cross, S., Pearce, C., Knowler, D., Noce, A., Reid, G.K., Cooper, J., Speare, D., Burridge, L., Crawford, C., Sawhney, M., Pee Ang, K., Backman, C. and Hutchinson, M. (2013). 'The Canadian Integrated Multi-

Trophic Aquaculture Network (CIMTAN) – A Network for a New Era of Ecosystem Responsible Aquaculture', *Fisheries*, 38: 297-308.

Chopin, T. and Robinson, S. (2006) 'Rationale for developing integrated multi-trophic aquaculture (IMTA): an example from Canada'. *Fish Farmer Magazine*, 1: 20-21.

Chopin, T., Robinson, S. M. C., Troell, M., Neori, A., Buschman, A. H. and Fang, J. (2008) 'Multitrophic Integration for Sustainable Marine Aquaculture', in Jørgensen, S.E. and Fath, B.D. (eds.) *Encyclopedia of Ecology*: Elsevier, pp. 2463-2475.

Chopin, T., Sharp, G., Belyea, E., Semple, R. and Jones, D. (1999) 'Open-water aquaculture of the red alga *Chondrus crispus* in Prince Edward Island, Canada', *Hydrobiologia*, 398: 417-425.

Chopin, T. and Whalen, E. (1993) 'A New and Rapid Method for Carrageenan Identification by FT IR Diffuse-Reflectance Spectroscopy Directly on Dried, Ground Algal Material', *Carbohydrate Research*, 246: 51-59.

Cornish, M. L. and Garbary, D. J. (2010) 'Antioxidants from macroalgae: potential applications in human health and nutrition', *Algae*, 25: 155-171.

Correa-Díaz, F., Aguilar-Rosas, R. and Aguilar-Rosas, L. E. (1990) 'Infrared analysis of eleven carrageenophytes from Baja California, Mexico', *Hydrobiologia*, 204-205: 609-614.

Craigie, J. S. and Correa, J. A. (1996) 'Etiology of infectious diseases in cultivated *Chondrus crispus* (Gigartinales, Rhodophyta)', *Hydrobiologia*, 326: 97-104.

Delattre, C., Fenoradosoa, T. A. and Michaud, P. (2011) 'Galactans: An Overview of their Most Important Sourcing and Applications as Natural Polysaccharides', *Brazilian archives of Biology and Technology*, 54(6): 1075-1092.

DGPA (2007) *'Programa Operacional Pescas 2007-2013'*. Direcção-Geral das Pescas e Aquicultura, Portugal.

Dinis, T. (2010) *SEACASE – Sustainable extensive and semi-intensive coastal aquaculture in Southern Europe – First Publishable Activity Report*, Brussels, Belgium. Available at: http://www.seacase.org/PDF/Publications/Final%20Publishable%20Activity%20Report_FINAL.pdf.

EU (2009) 'Regulamento (CE) N. 710/2009 da Comissão de 5 de Agosto de 2009', *Jornal Oficial da União Europeia*, pp. 15-33.

FAO (2009a) *Integrated mariculture: A global review. FAO Fisheries and*

Aquaculture Department Rome, Italy: Food and Agriculture Organization of the United Nations.

FAO (2009b) *The State of the World Fisheries and Aquaculture 2008. FAO Fisheries and Aquaculture Department* Rome, Italy: Food and Agricultural Organization of the United Nations.

FAO (2010) *FishStat fishery statistical collections: aquaculture production (1950–2008). FAO* Rome, Italy: Food and Agriculture Organization of the United Nations.

Gaspar, R., Pereira, L. and Neto, J. M. (2012) 'Ecological reference conditions and quality states of marine macroalgae sensu Water Framework Directive: An example from the intertidal rocky shores of the Portuguese coastal waters', *Ecological Indicators,* 19: 24-38.

Gayral, P. and Cosson, J. (1986) *Connaître et reconnaître les algues marines.*

Gurgel, C. F. D. and Lopez-Bautista, J. (2007) 'Red Algae', *Encyclopedia of Life Sciences, Wiley Online Library*, Available: John Wiley & Sons, Ltd., Chichester.

Hilliou, L., Larotonda, F. D. S., Abreu, P., Abreu, M. H., Sereno, A. M. and Gonçalves, M. P. (2012) 'The impact of seaweed life phase and postharvest storage duration on the chemical and rheological properties of hybrid carrageenans isolated from Portuguese *Mastocarpus stellatus*', *Carbohydrate Polymers,* 87(4): 2655-2663.

Ioannou, E. and Roussis, V. (2009) 'Natural Products from Seaweeds', in Osbourn, A.E. and Lanzotti, V. (eds.) *Plant-derived Natural Products: Synthesis, Function, and Application*. New York, NY, USA: Springer US, pp. 51-81.

Jiao, G., Yu, G., Zhang, J. and Ewart, H. S. (2011) 'Chemical structures and bioactivities of sulfated polysaccharides from marine algae', *Marine Drugs,* 9: 196-223.

Knutsen, S. H., Myslabodski, D. E., Larsen, B. and Usov, A. I. (1994) 'A modified system of nomenclature for red algal galactans', *Botanica Marina,* 37: 163-169.

Lee, R. E. (2008) *Phycology*. 4th edn. Cambridge: Cambridge University Press, pp. 560.

MACOI, Portuguese Seaweeds Website. World-wide Electronic Publication. IMAR, Department of Life Sciences, University of Coimbra, http://macoi.ci.uc.pt (Accessed: 2013-06-03).

Matos, J., Costa, S., Rodrigues, A., Pereira, R. and Sousa-Pinto, I. (2006) 'Experimental integrated aquaculture of fish and red seaweeds in Northern Portugal',

Aquaculture, 252: 31-42.

Mccandless, E. L. and Craigie, J. S. (1974) Re-Evaluation of Seasonal Factors Involved in Carrageenan Production by *Chondrus crispus* - Carrageenans of Carposporic Plants, *Botanica Marina*, 17(3): 125-129.

McHugh, D. J. (2003) *A guide to the seaweed industry. FAO Fisheries Technical Paper* Rome, Italy: Food and Agriculture Organization of the United Nations.

Melo, R. A. (1998) '*Gelidium* commercial exploitation: Natural resources and cultivation', *Journal of Applied Phycology,* 10(3): 303-314.

Mesquita Rodrigues, J. E. (1963) 'Contribuição para o conhecimento das *Phaeophyceae* da costa portuguesa', *Boletim da Sociedade Broteriana,* 16: 1-124.

Moseley, C. M. (Ed.) (1990) 'The effect of cultivation conditions on the yield and quality of carrageenans in *Chondrus crispus*' in *Introduction to Applied Phycology*. The Hague, Netherlands: SPB Academic Publishing.

Neish, A. C., Shacklock, P. F., Fox, C. H. and Simpson, F. J. (1977) 'The cultivation of *Chondrus crispus*. Factors affecting growth under greenhouse conditions', *Canadian Journal of Botany*, 55(16): 2263-2271.

Neori, A. (2008) 'Essential role of seaweed cultivation in integrated multi-trophic aquaculture farms for global expansion of mariculture: an analysis', *Journal of Applied Phycology,* 20(20): 567-570.

Palminha, F. P. (1951) 'Contribuição para o estudo das algas marinhas portuguesas', *Boletim da Sociedade Portuguesa de Ciências Naturais,* 2(3): 226-250.

Palminha, F. P. (1971) *Exploração e utilização de algas marinhas na plataforma portuguesa e nas ilhas do Arquipélago dos Açores*. Junta Nacional do Fomento das Pescas.

Pereira, L. (2004) *Estudos em macroalgas carragenófitas (Gigartinales, Rhodophyceae) da costa portuguesa - aspectos ecológicos, bioquímicos e citológicos. Ph.D. Thesis*, Coimbra, Universidade de Coimbra, Portugal, pp. 293.

Pereira, L. (2006) 'Identification of phycocolloids by vibrational spectroscopy', in Critchley, A.T., Ohno, M. and Largo, D.B. (eds.) *World Seaweed Resources – An authoritative reference system*.

Pereira, L. (2011) 'A Review of the Nutrient Composition of Selected Edible Seaweeds', in Pomin, V.H.E. (ed.) *Seaweed: Ecology, Nutrient Composition and Medicinal Uses*. New York, U.S.A.: Nova Science Publishers Inc., pp. 15-47.

Pereira, L. (2012) 'Cytological and Cytochemical Aspects in selected

Carrageenophytes (Gigartinales, Rhodophyta)', in Heimann, K. and Katsaros, C. (eds.) *Advances in Algal Cell Biology*: De Gruyter, pp. 81-104.

Pereira, L., Amado, A. M., Critchley, A. T., van de Velde, F. and Ribeiro-Claro, P. J. A. (2009a) 'Identification of selected seaweed polysaccharides (phycocolloids) by vibrational spectroscopy (FTIR-ATR and FT-Raman)', *Food Hydrocolloids*, 23, pp. 1903- 1909.

Pereira, L., Critchley, A. T., Amado, A. M. and Ribeiro-Claro, P. J. A. (2009b) 'A comparative analysis of phycocolloids produced by underutilized *versus* industrially utilized carrageenophytes (Gigartinales, Rhodophyta)', *Journal of Applied Phycology*, 21: 599-605.

Pereira, L., Gheda, S. F. and Ribeiro-Claro, P. J. A. (2013) 'Analysis by Vibrational Spectroscopy of Seaweed Polysaccharides with Potential Use in Food, Pharmaceutical and Cosmetic Industries', *International Journal of Carbohydrate Chemistry*, Vol. 2013.

Pereira, L. and Mesquita, J. F. (2003) 'Carrageenophytes of occidental Portuguese coast: 1-spectroscopic analysis in eight carrageenophytes from Buarcos bay', *Biomolecular Engineering*, 20: 217-222.

Pereira, L. and Mesquita, J. F. (2004) 'Population studies and carrageenan properties of *Chondracanthus teedei* var. *lusitanicus* (Gigartinaceae, Rhodophyta)', *Journal of Applied Phycology*, 16: 369-383.

Pereira, L., Sousa, A., Coelho, H., Amado, A. M. and Ribeiro-Claro, P. J. A. (2003) 'Use of FTIR, FT-Raman and 13 C-NMR spectroscopy for identification of some seaweed phycocolloids', *Biomolecular Engineering*, 20(4-6): 223-228.

Pereira, L. and van de Velde, F. (2011) 'Portuguese carrageenophytes: Carrageenan composition and geographic distribution of eight species (Gigartinales, Rhodophyta)', *Carbohydrate Polymers*, 84: 614-623.

Pereira, R., Valente, L., Sousa-Pinto, I. and Rema P. (2010) Evaluation of IMTA produced Seaweeds *Gracilaria*, *Porphyra* and *Ulva* as dietary ingredients in nile tilapia (*Oreochromis niloticus*). *Aquaculture Europe 2010 – Seafarming tomorrow* – Porto, Portugal, October 5-8, 2010.

Phillips, M. (2009) 'Mariculture Overview', in Steele, J.H. (ed.) *Encyclopedia of Ocean Sciences.* Second ed: Academic Press, pp. 537-544.

Rhodophyta (Red algae). Available at: http://www.mbari.org/staff/conn/botany/flora/reds.htm (Accessed: 2013-07-17).

Santos, R. and Duarte, P. (1991) 'Marine plant harvest in Portugal', *Journal of Applied Phycology,* 3(1): 11-18.

Sousa, A. M. M., Alves, V. D., Morais, S., Delerue-Matos, C. and Gonçalves, M. P. (2010) 'Agar extraction from integrated multitrophic aquacultured *Gracilaria vermiculophylla*: Evaluation of a microwave-assisted process using response surface methodology', *Bioresource Technology,* 101: 3258-3267.

Sousa-Pinto, I., Abreu, M. H. and Pereira, R. (2012) 'The Integrated Multi-Trophic Aquaculture in Portugal: history, major results and futures perspectives', in Chopin, T., Neori, A., Robinson, S. and Troell, M. (eds.) *Integrated Multi-Trophic Aquaculture (IMTA) or the Turquoise Revolution. A greener ecosystem approach to the Blue Revolution*. Berlin: Springer.

Sousa-Pinto, I. and Araújo, R. (2006) 'Seaweed Resources of Portugal', in Critchley, O. (ed.) *Seaweed Resources of the World*. Yokosuka, Japan: JICA.

Stoloff, L. and Silva, P. C. (1957) 'An attempt to determine possible taxonomic significance of the properties of water extractable polysaccharides in red algae', *Economic Botany,* 11: 327-330.

Takaichi, S. (2011) 'Carotenoids in Algae: Distributions, Biosynthesis and Functions', *Marine Drugs,* 9(6): 1101-1118.

Tasende, M. G., Cid, M. and Fraga, M. I. (2012) 'Spatial and temporal variations of *Chondrus crispus* (Gigartinaceae, Rhodophyta) carrageenan content in natural populations from Galicia (NW Spain)', *Journal of Applied Phycology,* 24: 941-951.

Troell, M., Halling, C., Nilsson, A., Buschmann, A. H., Kautsky, N. and Kautsky, L. (1997) 'Integrated open sea cultivation of *Gracilaria chilensis* (Gracilariales, Rhodophyta) and salmons for reduced environmental impact and increased economic output', *Aquaculture,* 156: 45-62.

Tseng, C. K. (1945) 'The Terminology of Seaweed Colloids', *Science,* 101(2633): 597-602.

van de Velde, F. (2008) 'Structure and function of hybrid carrageenans', *Food Hydrocolloids,* 22: 727-734.

van de Velde, F. and de Ruiter, G. A. (2002) 'Carrageenan', in Vandamme, E.J., Baets, S.D. and Steinbèuchel, A. (eds.) *Biopolymers*: Weinheim: Wiley, pp. 245-274.

van de Velde, F., Knutsen, S. H., Usov, A. I., Rollema, H. S. and Cerezo, A. S. (2002) 'H-1 and C-13 high resolution NMR spectroscopy of carrageenans: application in research and industry', *Trends in Food Science & Technology,* 13(3): 73-92.

van de Velde, F., Peppelman, H. A., Rollema, H. S. and Tromp, R. H. (2001) 'On the structure of k/i-hybrid carrageenans', *Carbohydrate Research*, 331: 271-283.

van de Velde, F., Pereira, L. and Rollema, H. S. (2004) 'The revised NMR chemical shift data of carrageenans', *Carbohydrate Research*, 339: 2309-2313.

Zhanjiang Fisheries College – People's Republic of China (1990) 'Training Manual on *Gracilaria* Culture and Seaweed Processing in China', *FAO Corporate Document Repository*, Available: Food and Agriculture Organization of the United Nations (Accessed 2013-07-16).

Zinoun, M. (1993). Études biochimiques des polysaccharides parietaux produits par deux Gigartinales (Rhodophycées) des côtes de la Manche, *in situ* et en cultures controlées: *Calliblepharis jubata* et *Gigartina teedii*. *Ph.D. Thesis*, Université de Technologie de Compiegne, 219 pp.

Zinoun, M. and Cosson, J. (1996) 'Seasonal variation in growth and carrageenan content of *Calliblepharis jubata* (Rhodophyceae, Gigartinales) from the Normandy coast, France', *Journal of Applied Phycology*, 8(1): 29-34.

Appendix A – *C. crispus* carrageenan yield calculations in alkaline extractions

Table A-1 Samples weights of IMTA-cultivated *C. crispus* for 5 weeks, May-June 2013.

	Wds (g)	We (g)
NF (1)	1.32	0.30
NF (2)	1.01	0.30
NF (3)	1.03	0.22
FG (1)	1.07	0.25
FG (2)	1.02	0.24
FG (3)	1.08	0.21
T (1)	1.03	0.18
T (2)	1.03	0.18
T (3)	1.03	0.18

Table A-2 Samples weights of *C. crispus* harvested in Mindelo in 04/07/2012.

	Wds (g)	Wfe(g)
NF (1)	1.04	0.16
NF (2)	1.04	0.18
NF (3)	1.03	0.16
T (1)	1.02	0.10
T (2)	1.02	0.10
T (3)	1.02	0.10

Table A-3 Samples weights of *C. crispus* harvested in Mindelo in 18/07/2012.

	Wds (g)	We (g)
NF (1)	1.02	0.17
NF (2)	1.01	0.20
NF (3)	1.09	0.18
FG (1)	0.53	0.11
FG (2)	1.02	0.35
FG (3)	1.01	0.23

Table A-4 Samples weights of *C. crispus* harvested in Mindelo in 01/08/2012.

	Wds (g)	We (g)
NF (1)	1.00	0.24
NF (2)	1.01	0.24
NF (3)	0.54	0.28
FG (1)	1.03	0.12
FG (2)	1.04	0.10
FG (3)	1.03	0.05

Table A-5 Samples weights of *C. crispus* harvested in Labruge in 18/07/2012.

	Wds (g)	We (g)
FG (1)	1.01	0.17
FG (2)	1.00	0.18
FG (3)	1.05	0.15
T (1)	1.00	0.16
T (2)	1.01	0.16
T (3)	1.02	0.16

Table A-6 Samples weights of *C. crispus* harvested in Aguda in 17/09/2012.

	Wds (g)	We (g)
FG (1)	1.04	0.15
FG (2)	1.03	0.16
FG (3)	1.08	0.14
T (1)	1.00	0.16
T (2)	0.67	0.08
T (3)	1.00	0.14

Table A-7 Samples weights of *C. crispus* harvested in Aguda in 03/10/2012.

	Wds (g)	We (g)
NF (1)	1.00	0.13
NF (2)	1.04	0.19
NF (3)	1.05	0.20
FG (1)	1.01	0.45
FG (2)	1.07	0.23
FG (3)	0.43	0.04

Table A-8 Samples weights of *C. crispus* harvested in Buarcos in 06/06/2013.

	Wds (g)	We (g)
NF (1)	1.06	0.29
NF (2)	1.01	0.21
NF (3)	1.10	0.25
FG (1)	1.06	0.22
FG (2)	1.04	0.18
FG (3)	1.02	1.14
T (1)	1.02	0.19
T (2)	1.04	0.21
T (3)	1.02	0.22

Table A-9 Samples yields of IMTA-cultivated *C. crispus* for 5 weeks May-June 2013.

	NF	FG	T
Yield (1)	0.227	0.234	0.175
Yield (2)	0.297	0.236	0.175
Yield (3)	0.214	0.194	0.175
Average	0.246	0.22133	0.175
SE	0.04464	0.02369	0

Table A-10 Samples yields of *C. crispus* harvested in Mindelo in 04/07/2012.

	NF	FG	T
Yield (1)	0.154	−	0.098
Yield (2)	0.173	−	0.098
Yield (3)	0.155	−	0.098
Average	0.16067	−	0.098
SE	0.01069	−	0

Table A-11 Samples yields of *C. crispus* harvested in Mindelo in 18/07/2012.

	NF	FG	T
Yield (1)	0.167	0.208	−
Yield (2)	0.198	0.343	−
Yield (3)	0.165	0.228	−
Average	0.17667	0.25967	−
SE	0.0185	0.07286	−

Table A-12 Samples yields of *C. crispus* harvested in Mindelo in 01/08/2012.

	NF	FG	T
Yield (1)	0.24	0.117	−
Yield (2)	0.238	0.096	−
Yield (3)	0.519	0.049	−
Average	0.33233	0.08733	−
SE	0.16166	0.03482	−

Table A-13 Samples yields of *C. crispus* harvested in Labruge in 18/07/2012.

	NF	FG	T
Yield (1)	−	0.168	0.16
Yield (2)	−	0.18	0.158
Yield (3)	−	0.143	0.157
Average	−	0.16367	0.15833
SE	−	0.01888	0.00153

Table A-14 Samples yields – *C. crispus* harvested in Aguda in 17/09/2012.

	NF	FG	T
Yield (1)	−	0.144	0.16
Yield (2)	−	0.155	0.119
Yield (3)	−	0.13	0.14
Average	−	0.143	0.13967
SE	−	0.01253	0.0205

Table A-15 Samples yields of *C. crispus* harvested in Aguda in 03/10/2012.

	NF	FG	T
Yield (1)	0.13	0.446	−
Yield (2)	0.183	0.215	−
Yield (3)	0.191	0.093	−
Average	0.168	0.25133	−
SE	0.03315	0.17928	−

Table A-16 Samples yields of *C. crispus* harvested in Buarcos in 06/06/2013.

	NF	FG	T
Yield (1)	0.273	0.208	0.186
Yield (2)	0.208	0.173	0.202
Yield (3)	0.227	0.137	0.216
Average	0.236	0.17267	0.20133
SE	0.03342	0.0355	0.01501

*NF: non-fructified thalli; FG: female gametophytes; T: tetrasporophytes.

**Yield is expressed as % dry weight calculated with the formula: Yield = $(W_e/W_{ds}) \times 100$, where W_e is the extracted carrageenan weight (g) and W_{ds} is the dried seaweed weight (g) used for alkaline extraction.

***SE: standard error.

Appendix B – FTIR-ATR spectra

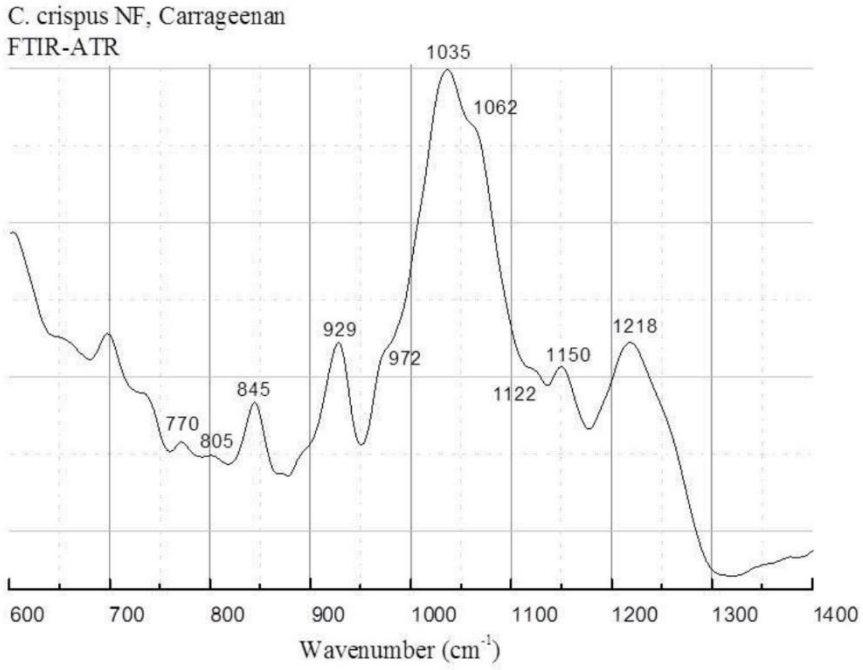

Figure B-1 FTIR-ATR spectrum of IMTA-cultivated *C. crispus* – non-fructified thallus ground sample.

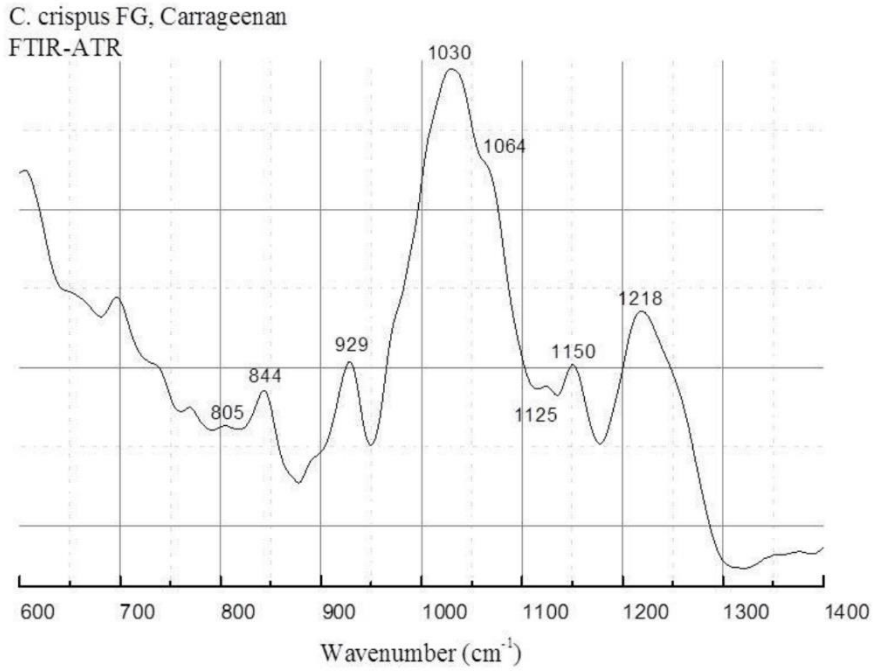

Figure B-2 FTIR-ATR spectrum of IMTA-cultivated *C. crispus* – female gametophyte ground sample.

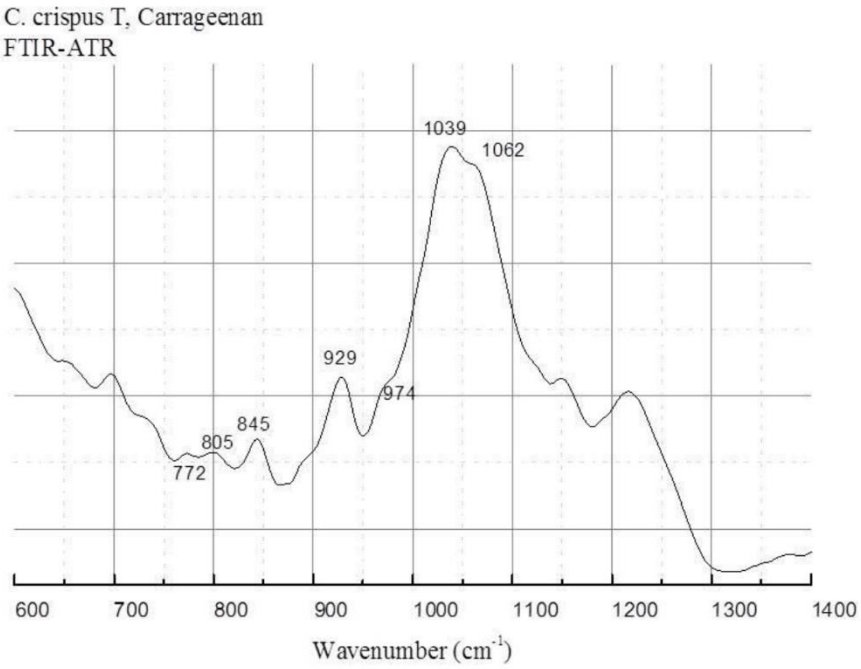

Figure B-3 FTIR-ATR spectrum of IMTA-cultivated *C. crispus* – tetrasporophyte ground sample.

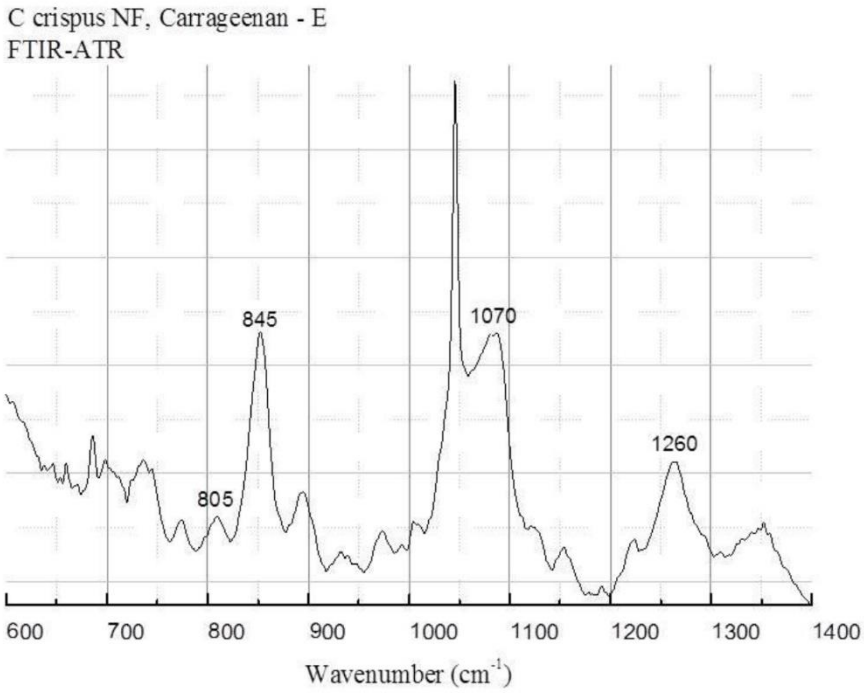

Figure B-4 FTIR-ATR spectrum of IMTA-cultivated *C. crispus* – alkaline extracted carrageenan from non-fructified thallus sample.

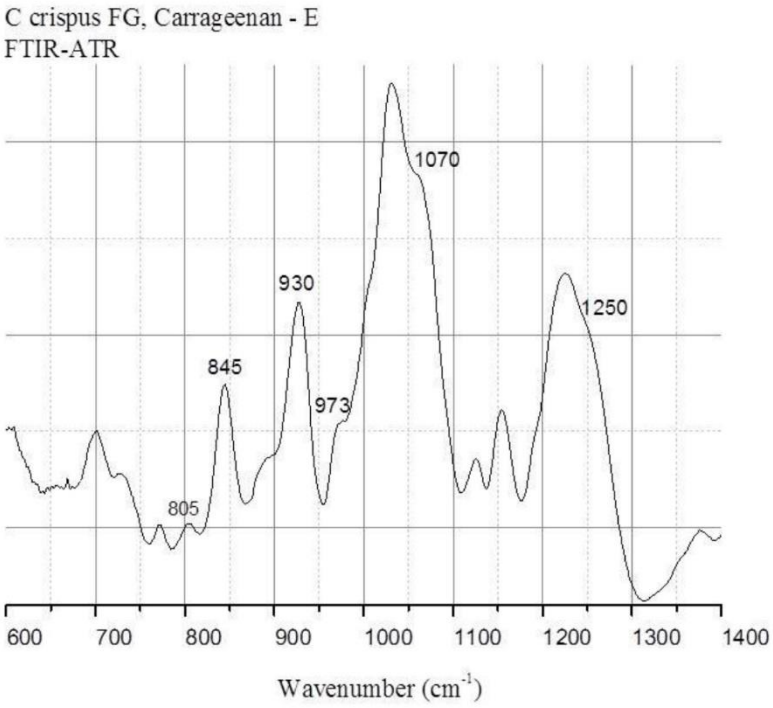

Figure B-5 FTIR-ATR spectrum of IMTA-cultivated *C. crispus* – alkaline extracted carrageenan from female gametophyte sample.

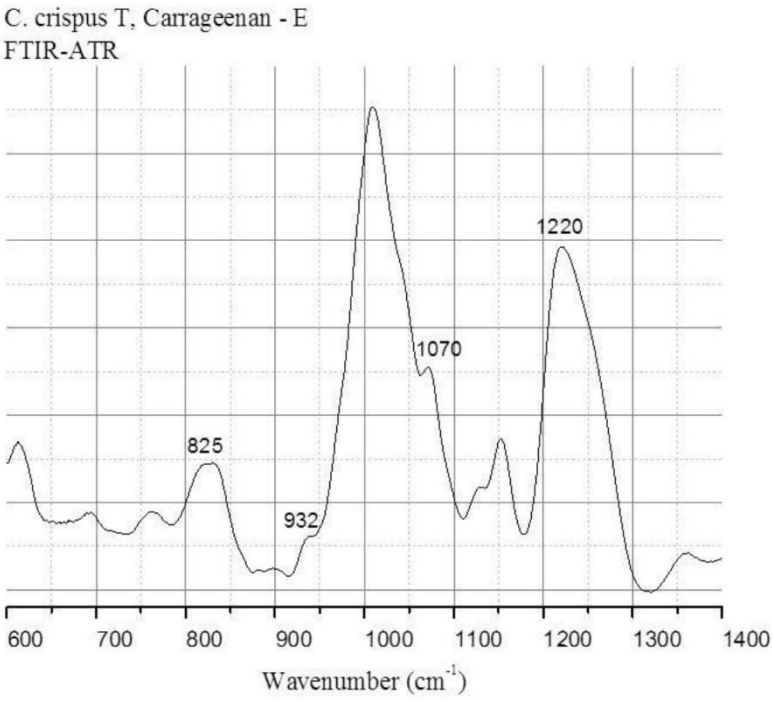

Figure B-6 FTIR-ATR spectrum of IMTA-cultivated *C. crispus* – alkaline extracted carrageenan from tetrasporophyte sample.