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Masters in Aquaculture and Fisheries

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"Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída."

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Resumo

As macroalgas do género *Ulva* crescem em locais abrigados, águas litorais eutrofizadas e por vezes são consideradas uma praga. No entanto, a sua capacidade biorremediadora, associada ao seu crescimento rápido, fornece um serviço ecossistémico importante principalmente no que diz respeito à mitigação de excesso de nutrientes. Para além desta tem outras propriedades que podem vir a ser valiosas, tal como a sua composição bioquímica que é caracterizada pela existência de vários compostos que podem ser usados para fins medicinais (e.g. antioxidante, anti-inflamatória, anti cancro), nutraceuticos, para alimentação animal e até mesmo como biomaterial (e.g. bioplástico). O seu cultivo em sistemas fechados e a sua capacidade de captação de nutrientes, particularmente amónia, conferem às *Ulva* spp. características valiosas para a sua integração em sistemas de produção multitróficos. No entanto, a comunidade científica necessita de compreender como os parâmetros ambientais influenciam o crescimento da macroalga e seus compostos bioativos.

Este trabalho pretende contribuir para um melhor conhecimento da ecologia de Ulva e da forma como a concentração de nutrientes e o sistema de produção (semi-intensivo e extensivo) afetam o seu crescimento, e as suas propriedades antioxidantes e anti-inflamatórias. De modo a estudar a influência dos nutrientes uma quantidade conhecida de biomassa de Ulva foi cultivada em caixas flutuantes de 0.7 m^3 perfuradas, instaladas dentro de um reservatório de água para aquacultura com valores comparativamente baixos de nutrientes e de um tanque de decantação/sedimentação de uma piscicultura com valores mais elevados. No estudo dos sistemas de produção a eficiência destas caixas foi comparada com a de "*raceways*" tendo-se usadas a água do tanque de sedimentação. Os parâmetros ambientais (temperatura, turbidez da água e radiação), crescimento, utilização de nutrientes, composição proximal (humidade, matéria seca, cinzas e matéria orgânica), conteúdo total de polifenóis, atividades antioxidantes (DPPH, FRAP, ABTS) e anti-inflamatória foram estimadas a partir da biomassa cultivada. Os períodos de cultivo analisados correspondem a amostras de Inverno e de Primavera. O efeito da concentração de nutrientes e o efeito dos tipos de sistema de produção foram inferidos através de análises de componentes principais (PCA).

O efeito dos nutrientes e sistema de produção influenciaram significativamente o crescimento, matéria orgânica, conteúdo de proteína e lípidos, polifenóis e atividades

antioxidantes e anti-inflamatórias. As caixas flutuantes e o tanque de decantação forneceram condições ótimas para um crescimento mais estável, maior conteúdo proteico e polifenólico e elevadas atividades antioxidantes e anti-inflamatórias, comparativamente com os "*raceways*" e o reservatório. Adicionalmente, foi constatado que o caudal nos "*raceway*" foi um fator limitante que comprometeu a utilização de nutrientes pelas algas, o seu crescimento, o conteúdo proteico e polifenólico e respetivas atividades. A grande parte da atividade antioxidante e anti-inflamatória foi maior durante o inverno do que na primavera. No entanto, o aumento de certas atividades antioxidantes ao longo do tempo, poderá ser justificado pelo incremento do metabolismo antioxidante em resposta ao aumento da temperatura e da radiação. Deste modo, o presente estudo concluiu que o conteúdo proteico, antioxidante e anti-inflamatório foi significantemente limitado pela disponibilidade de nutrientes face ao sistema de produção e local.

Palavras-chave: *Ulva* spp.*;* Aquacultura Integrada Multitrófica; Compostos bioativos; Antioxidante; Anti-inflamatório.

Abstract

Ulva spp. grow in sheltered, eutrophic coastal waters, covering large areas and being sometimes considered a nuisance. However, they have valuable bioremediation capabilities that, associated with the fast growth rate, provide important ecosystem services mainly in respect to waste nutrient impact mitigation. Other very important *Ulva* properties are associated to its biochemical composition characterized by several compounds useful in fields like medicine, nutraceuticals, animal feed, and bio-material construction. Their cultivation efficiency in closed systems and efficient nutrient uptake makes them valuable macroalgae for integrated multitrophic aquaculture (IMTA). Nevertheless, the scientific community still needs to understand how environmental parameters influence the macroalgae growth and their biological active compounds.

The present work used two different water sources with different nutrient concentration (settling pond (*SP*) and reservoir (*R*) from an aquaculture research centre) and two different production systems (raceway system (*Rw*) and floating cages (*Fc*)) to study the temporal and spatial effect of nutrient concentration and production system on growth, nutrient uptake, and antioxidant and anti-inflammatory properties of *Ulva* spp. biomass. *Ulva* biomass samples were analysed in periods representative of Winter and early Spring conditions. Additionally, the nutrient effect (*SP vs R*) and production system effect (*Rw* vs *Fc*) were analysed through two separate principle component analysis.

The nutrient levels and different production systems influenced growth, organic matter, protein and lipid contents, antioxidant and anti-inflammatory activities of *Ulva* spp. *Fc* and *SP* provided an optimal environment for growth, and higher protein, polyphenol content and anti-activities. Contrarily, restrained water flow in *Rw*, compromised the nutrient uptake, growth, protein and polyphenol yields and anti-activities. Also, most anti-activities were higher during winter, and lower in early spring. However, some antioxidant activities were found to increase over season due to the rise of antioxidant metabolism in response to temperature and radiation. Therefore, the present study concludes that protein, antioxidant and anti-inflammatory activities were significantly lower where the nutrient availability and uptake were limited.

Keywords: *Ulva* spp.; Integrated Multitrophic Aquaculture (IMTA); Bioactive compounds; Antioxidant; Anti-inflammatory.

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1. Introduction

For millennia, seaweed have been used as a food source, but the earliest written record of their human usage originates from China, about 1700 years ago (Yang et al.,2017). Coastal populations harvested a wide variety of seaweeds from all algal groups. Initially, seaweeds were most often used for domestic purposes as food and feed, whereas later, industrial uses (gels, fertilizers) emerged (Delaney et al., 2016). Seaweed farming at sea is becoming an increasingly competitive biomass production candidate for food and related uses. In 2012, just eight Asian nations produced 99% of 24 million tons harvested, while most of the world's 150 countries and territories with coasts were yet to begin seaweed farming. The main producing countries were China, Indonesia and the Philippines, which were also those that cultivated the greatest diversity of seaweed species (FAO, 2014, 2016). By 2016, the world seaweed production reached over 30 million tonnes and *Undaria pinnatifida*, *Porphyra spp*. (nori), and *Caulerpa spp.*, were almost produced exclusively for direct human consumption, although scraps from processing factories are used for other purposes, including feed for abalone culture (FAO, 2018).

Using current technology, extensively available sea areas may be cultivated to produce crops that require no freshwater or fertilizers, while providing a variety of valuable ecosystem services. Seaweed farming is an extractive industry whose very process of production of valuable biomass renders the sea's various ecosystem services with ecological and economic values (Chopin et al., 2008, 2010; Neori et al., 2009; Radulovich et al., 2015). The farming of seaweed in combination with the production of fish (nutrient source) and organic extractive feeders (e.g., abalone, bivalves, sea urchin, and sea cucumbers) is used to uptake the excess of inorganic nutrients from the production system. This integrated multitrophic aquaculture (IMTA) concept is becoming more and more important as a sustainable food production system (Ben-Ari et al.,2014; Shpigel et al., 2018; Cunha et al., 2019). Kelps (e.g. *Laminaria digitata*), *Ulva* spp., *Gracilaria* and other seaweeds are being farmed in integrated modules (Broch et al. 2013; Macchiavello et al., 2014). For example, in Portugal (see www.algaplus.pt), farmers and researchers used the IMTA concept to sustainably produce macroalgae either by cultivating them together with fish (Glauco et al., 2018; Cunha et al., 2019) or using the effluent water from fish that is rich in nutrients (Cohen et al., 1991, Diamahesa, et al., 2017).

Seaweed can be produced for more valuable reasons than commodity purposes. Their biomass is raw material for the nutraceutical, cosmeceutical, and pharmaceutical industries, and recent studies point towards biofuel and bioplastic as other possible applications (Holdt et al., 2011; Helmes et al., 2018). A holistic "biorefinery approach" would minimize waste and environmental impacts of the biomass processing, while sequentially extract the most valuable components from the seaweeds, leaving the remainder untouched for commodity uses such as feed (Buschmann et al., 2017). Although several techniques for seaweed transformation are known, the impacts of seasonal and geographical variation on the composition and nutritional value of seaweed biomass remain poorly investigated (Wells et al., 2016). This information could bring great benefits to management procedures like culturing, harvest planning, de-watering methods, drying, storing and processing (Buschmann et al., 2017).

Marine seaweeds are believed to be important reservoirs of several bioactive compounds, such as sulphated polysaccharides, peptides, and phlorotannins. These are relevant for the improvement of human and animal welfare, as these compounds display antioxidant, anti-inflammatory, and antitumoral properties (Andrade et al., 2013). Generally, seaweeds contain all the essential amino acids and are rich in acidic amino acids, aspartic and glutamic acids (Holdt et al., 2011). Some peptides, such as carnosine and glutathione, recognized for their strong antioxidant properties and previously identified in animal tissue, have been detected in seaweed (Shiu and Lee, 2005). It is worthy to report that certain algal peptides can support the prevention and treatment of diseases such as high cholesterol, high blood pressure, AIDS, and even some types of cancer (Sato et al. 2002, Suetsuna et al. 2004; Holdt & Kraan, 2011).

Even within a small geographic area, growth rate and chemical composition might vary depending on harvest season (Schiener et al., 2016), light exposure (Boderskov et al., 2016), salinity (Nielsen et al., 2016), depth (Sharma et al., 2018), local water currents, or closeness to high nutrient source, like aquaculture effluent (Marinho et al., 2015; Je et al., 2015).

The seaweed industry is mainly focused on food supply, feed, fertilizers, pharmaceuticals, cosmeceuticals, and nutraceuticals (Holdt et al., 2011; Buschmann et al., 2017; Lerat et al., 2018). Interestingly, products based on seaweeds are present in everyday life items. Red and brown seaweeds are used to produce hydrocolloids, which are non-crystalline substances with very large molecules that can be dissolved into water or solubilized to

give a thickened (viscous) mixture. Hydrocolloids such as agar, alginates and carrageenans are water-soluble carbohydrates that are used to thicken aqueous solutions, to form gels and water-soluble films and/or to stabilize products such as ice cream or toothpaste.

The object of this study, the green seaweed *Ulva* spp., presents several bioactive compounds with commercial interest, such as polyphenols (Jahan et al., 2017), salts (Magnusson et al., 2016), essential amino acid (Robertson-Andersson et al., 2008), cellulose, dietary fibres, lipids and ulvan, a polysaccharide with a high capacity of increasing viscosity and antioxidant power (Lahaye et al., 2007; Chiellini et al., 2011; Gajaria et al., 2017), have been reported.

Recently literature refers to the utilization of *Ulva* for the production biofuel, methanol, bioplastics (Helmes et al., 2018; Sivaprakash et al., 2019), and bioactive compounds. *Ulva* spp. has been proven to be a rich source of bioactive compounds with commercial interest, such as polyphenols (Jahan et al., 2017), minerals (Magnusson et al., 2016), proteins rich in essential amino acids (Bolton et al., 2008), cellulose, dietary fibres, lipids and ulvan, a polysaccharide with a high capacity of increasing viscosity and antioxidant activity (Lahaye et al., 2007; Chiellini et al., 2011; Gajaria et al., 2017). Among bioactive compounds, ulvans have special importance, being highly branched polymers of soluble dietary fibres, which have structural similarity to mammalian glycosaminoglycans (present in species such as *Ulva lactuca* and *Ulva rigida*). This polymer can be extracted and applied to modified adhesion and proliferation of normal and tumoral human colonic cells, presenting cytotoxic and cytostatic effects. Furthermore, it can be used for the treatment of gastric ulcers and demonstrates anti-influenza capacity (Holdt et al., 2011). Studies about bioactivate compounds in *Ulva spp.* revealed that polymers of two different units of uronic acids (*D*-mannoronic acid and *L*-glucoronic acid) present in this green seaweed promote regeneration of the mucous membranes and suppress inflammation in the stomach. Moreover, these polymers also exhibited anti-diabetic activity, aided the prevention of postprandial increase of glucose and insulin, thus promoting maintenance of human health (Holdt et al., 2011).

However, the scientific community still need to understand how environmental parameters influence the macroalgae growth and their biological active compounds (Critchley et al., 2019). In the present work, we will try to understand the influence of the

nutrients levels using two different water sources and the effect of two production systems on growth, composition and bioactive compounds of *Ulva* spp.

1.1. *Ulva* spp.

The genus *Ulva*, also known as "sea lettuce", is present in marine, freshwater and brackish environments found in the intertidal and shallow subtidal areas of rocky shores all over the globe (Ismail, 2017) and it was first identified by Linnaeus in 1753 (Kong et al., 2011). *Ulva* species are notoriously difficult to classify due to the morphological plasticity expressed by several representatives and lack of reliable characters available for taxon differentiation (Heesch et al., 2009; Wolf, 2012). The morphology of the genus is highly influenced by environmental conditions, thallus age, and life-stage, which hinders the description of the species by morphological features alone (Wolf et al., 2012). Leaf-like forms and tubular shapes were generally used to distinguish the genera *Ulva* and *Enteromorpha*, respectively, until 2003. However, intermediate states have been observed, and evidence that species could switch from a morphotype to another was found (Ismail, 2017). Thus, identification assessment turned from morphological characteristics to genetics, the latter by means of DNA extraction and amplification techniques such as Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) (Ismail, 2017). In 2017, a study performed at IPMA's Aquaculture Research Station, (EPPO-IPMA, Estação de Piscicultura Piloto de Olhão - Instituto Português do Mar e da Atmosfera) by Favot et al. (2017) identified six species of the genus *Ulva* in the earthen ponds by internal transcribed spacer (ITS)-based DNA barcoding, namely *Ulva flexuosa* (Wulfen, 1803), *U. clathrata* ((Roth) C. Agardh, 1811), *U. intestinalis* (Linnaeus, 1753), *U. sapora1* (Phillips et al., 2016), *U. torta* ((Mertens) Trevisan, 1842) and *U. prolifera* (O.F.Müller, 1778). The cultivated species in the earthen ponds was *U. flexuosa* (Favot et al., 2019). The study presented here was conducted in the same facilities, but, since no genetic determination was performed during this trial, the specimen will be designated as *Ulva spp.*

1.2. *Ulva* spp. aquaculture

Ulva spp. grows in sheltered, eutrophic coastal waters, covering large areas and being sometimes considered a nuisance. However, they have valuable bioremediation capabilities that, associated with the fast growth rate, provide important ecosystem services mainly in respect to waste nutrient impact mitigation (Buschmann et al., 2017). Other very important *Ulva* properties are associated to its biochemical composition characterized by several compounds that can be used in fields like medicine (antioxidant, anti-inflammatory, anti-fungal, and anti-cancer), nutraceuticals, animal feed, and bio-material construction (e.g. bioplastic) (Holdt et al., 2011; Helmes et al., 2018). Their added value compounds combined with their cultivation efficiency in closed systems and efficient nutrient uptake (particularly ammonium) makes them valuable macroalgae for integrated multitrophic aquaculture (IMTA) (Lamprianidou et al. 2015, Neveux et al. 2017). Nonetheless, *Ulva* spp. is produced all over the world, ranging from Portugal, to Israel, Canada to South Africa, to the inlands of Germany, Australia and India (Bushmaan et al., 2017; FAO 2018).

Farming requirements are necessary to produce *Ulva*. Usually water nutrients need to be in the range of 10 to 48 µM of ammonia (Neori et al., 1991), and 1 to 750 µM phosphorus (Floreto et al., 1996). Growth is usually measured in fresh weight (FW) or dry weight (DW) grams per each square meter of farming area per week ($g.m^{-2}\ week^{-1}$) and can reach up to 2,250 $g.m^{-2}week^{-1}$, over 20% daily growth, depending on temperature, nutrient concentration, light and water flow (Neori et al., 1991, 1998). *Ulva* and many other seaweeds can be cultivated in tanks. Thus, water flow and aeration are necessary to promote the renewal of the boundary layer between algae and water surfaces in order to promote the diffusion of the nutrient into the biomass (Mann et al., 1996; Diamahesa et al., 2017). Relying on nutrient concentration and the water flow, that can vary between 1 to 16 water exchanges (per day), it is possible to increase nutrient uptake and growth (Neori et al., 1991, 2003). For example, Diamahesa et al. (2017) stated that in tank culture waterflow is one important aspect to manage nutrient availability. By increasing the water flow, nutrient flux increases, and thus prevent the algae from becoming nutrient-limited, allowing higher biomass production. On the other hand, if the water flow is low, nutrients will become limiting, biomass production will decrease, but the nutrient uptake efficiency will increase (Diamahesa et al., 2017). Some recent studies are now trying to control algal physiology in order improve their biochemical composition. For example, a study

conducted to provide further insight into the environmental impacts of producing *Ulva* spp. to manufacture bioplastic, through a nitrogen deprived media, increased the starch yield that at a later stage could be used as feedstock (Helmes et al., 2018).

Therefore, the present work used two different water sources with different nutrient concentration and two different IMTA production systems (floating cages and raceways) to study the temporal and spatial effect of nutrient concentration and production system on the productivity, nutrient uptake, and bioactive properties of *Ulva* spp. biomass.

2. *Material and Methods*

2.1. *Facilities*

The experiment was carried at the Aquaculture Research Station of the Portuguese Institute of Sea and Atmosphere, (EPPO from the Portuguese Estação Piloto de Piscicultura de Olhão) located in the south of Portugal (37º 02´ N; 07º 49´W). EPPO has an area of 7 hectares inside the Natural Park of the Ria Formosa coastal lagoon and comprises one hatchery/nursery building (Fig. 1a), 17 semi-intensive earthen ponds used for farming trials (1 to 17 in Fig. 1), one water reservoir (Fig. 1b) and one settling pond (Fig. 1c). The earthen ponds are used for the growth of different fish species (mainly gilthead seabream *Sparus aurata*, sea bass *Dicentrarchus labrax*, and meagre *Argyrosomus regius*) and bivalves (mainly oyster *Crassostrea gigas*). The reservoir pond is the saltwater source for the entire station, and it is filled during high tide with water from the coastal lagoon. The water is held in the pond by a sluice gate until the next high tide. The settling or sedimentation pond, receives the water effluents from the entire research station, being rich in dissolved nutrients like ammonia and phosphate The effluents from the aquaculture tanks are treated to remove solid wastes in the settling lagoon and the resulting clarified water is released back to the Ria Formosa lagoon. In the present experiment the reservoir and settling pond were used as the source of water with contrasting nutrient levels.

Fig. 1 - Aerial view of the Aquaculture Research Centre (EPPO, IPMA) facilities localization and spatial layout: a – Hatchery; b – Reservoir; c – Settling pond; 1-17 experimental earthen ponds

2.2. *Experimental design*

The effect of two distinct nutrient levels and two different production systems in the levels of bioactive compounds and respective bioactivities (total phenolic antioxidant activity, anti-inflammatory activity) in *Ulva* biomass was evaluated for 3 months. The effect of nutrient concentration was determined using the same production system inside two water sources with different nutrient concentrations, the aquaculture settling pond (Fig. 1c) and the water reservoir pond (Fig. 1b). The production system were floating cages (Fig. 2A) consisting of 1-m^2 perforated plastic boxes to enable the water to circulate freely. Three were put inside the settling pond and three in the reservoir and they were attached to a longline to prevent dispersion. The net water volume of the floating cages was 0.7 m^3 (1.2 m^2 x 0.6 m depth) and no aeration was used. Two production systems were used to determine which would give better results in the *Ulva* production and levels of bioactive compounds: floating cages (1.2 m^2 x 0.6 m depth; Fig. 2A) and raceways (12 m^2 x 0.5 m depth; Fig. 2B). A diagram of how the experiment was set up is given in Fig. 3. The water origin was the same, the nutrient rich water from the settling pond. The raceways were two 6.0-m^3 fibre glass tanks. The water from the settling pond was pumped into them and the flow controlled at the inlet. The water outlet was at the opposite side and there was central aeration to promote water homogenization and biomass resuspension in the water

column. The information from the three floating cages was the same than in the previous experiment. In total, data was collected from 6 floating cages and 2 raceways.

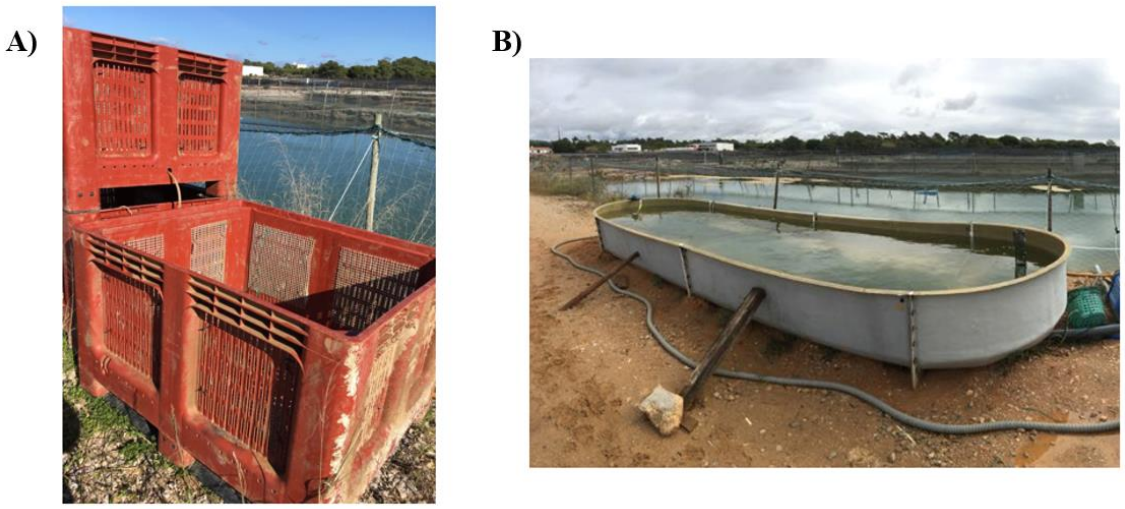

Fig. 2 - Experimental units. A) floating cages; B) fiber glass raceway tank.

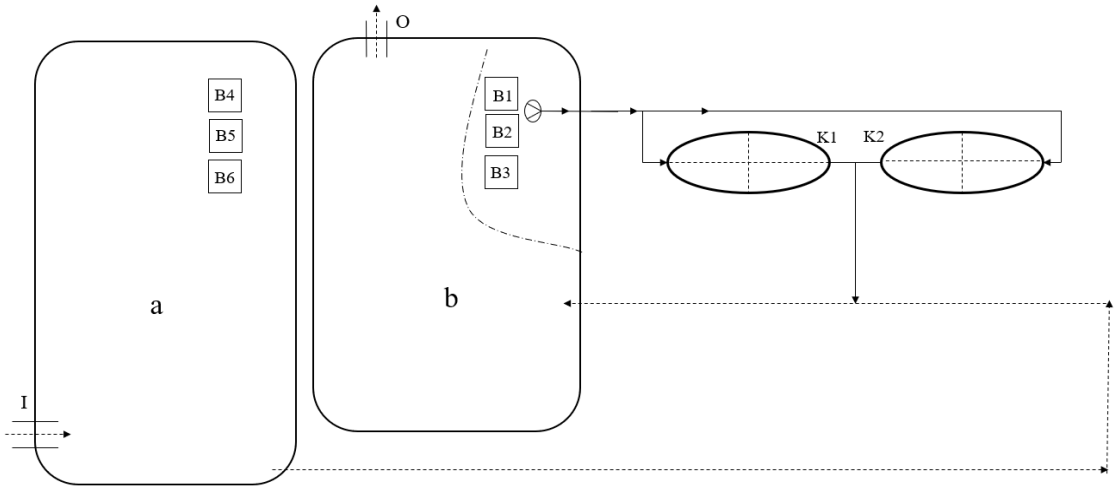

Fig. 3 - Diagram representative of the experimental system, where: a – reservoir pond; b – settling pond; I – reservoir water inlet; O – settling pond water outlet; B – floating cages; K - raceways; arrows indicate water flow; Triangle within circle– water pump.

2.3. *Sample collection and preparation*

Water and environmental parameters (temperature, dissolved oxygen, turbidity, salinity and irradiation) were daily recorded. *Ulva* grown was determined from 23/01/2019 to 30/01/2019, 30/01/2019 to 06/02/2019, 06/02 to 13/02/2019, 27/03 to 03/04//2019, and 03/04 to 11/04 /2019 being considered as representative of Winter and early Spring growth conditions. Initial stocking densities were 1 kg per m^3. At the end of each grown

period, the entire *Ulva* biomass were harvested, hand-drained and weighted. At each collection (6 from the floating cages and 2 from the raceways) 50g of fresh biomass were sampled, cleaned and frozen at -20 ºC for analysis of antioxidant and anti-inflammatory activity, and proximal composition.

Water samples for dissolved nutrient (ammonia, nitrates, nitrites, phosphates) were collected previously of collection, at the inlet and outlet of the raceways and inside each floating cage and frozen at -20 ºC for later determination. The levels of nitrite nitrate, ammonia and total phosphate were determined in a Segmented Flux Sanplus Skalar Colorimetric Autoanalyzer. The results are expressed in µM.

Biomass growth (g m^2 $week^{-1}$) was expressed in fresh weight (FW) and estimated using the following formula:

$$\frac{(Wf - Wi \times 1000)/A}{t} \times 7$$

Where:

Wf – Final weight (g)
Wi – Initial weight (g)
A – Surface of production system (m^2)
t – Culture time (days)

The fresh biomass samples were kept frozen at -20 ºC, then at -80ºC, freeze-dried and thoroughly cleaned of epiphytes. Biomass pools were made to ensure enough biomass, where each replica for a given sampling and system were ground using a Grindomix GM200 (Retsch, Haan, Germany): 2 cycles of 8 seconds at 3 000 rpm followed by 1 cycle of 8 seconds at 5 000 rpm. The biomass pools were stored at -80ºC until usage.

2.4. *Proximal composition*

2.4.1.1. *Moisture and Ash*

Moisture and ash content were determined according to AOAC methods (AOAC, 1995). These methods enable the determination of the water and inorganic matter contents in the samples. From the biomass pool, 2.5 g of each sample was weighed in duplicate to crucibles that were previously heated in the oven (100ºC for 30 minutes) and weighed

after cooling. The samples were then placed in the oven overnight (100 ºC) and weighed the next day, once cooled. The moisture content was calculated according to:

$$\%\ Moisture\ (\%\ m/m)\ =\ \frac{m1\ -\ m2}{m1\ -\ m3} \times 100$$

Where:

m1 = Crucible weight with moist sample (g)
m2 = Crucible weight with dry sample (g)
m3 = Crucible weight (g)

The crucibles with the respective dried samples were then placed in a furnace at 500 ºC overnight. Afterwards, the crucibles were removed and allowed to cool for 45 minutes, weighed, and placed again in the furnace for 30 minutes at the same temperature. After this period, the crucibles were weighed again once cooled. If the weight stabilized, up to 1 mg threshold, the process would terminate. The ash content, on dry matter terms, was then calculated using the following formula:

$$\%\ Ash\ (\%\ m/m) = \frac{m1\ -\ m2}{m3\ -\ m2} \times 100$$

Where:

m1 = Crucible weight with ash (g)
m2 = Crucible weight (g)
m3 = Crucible weight with dry sample (g)

The dry weight and organic matter were estimated using the following formulas, respectively:

$$Dry\ weight\ (\%\ FW)\ =\ 100\ \% - \%\ Moisture$$

$$\%\ Organic\ matter\ (\%\ DW)\ =\ \%\ DW\ \% - Ash\ \%$$

DW % = sample dry weight (% DW)
Ash % = sample ash content (% DW)

Moisture, dry weight and ash content results were expressed as percentage of fresh weight (% FW) and organic matter as percentage of dry weight (% DW)

2.4.1.2. *Total protein*

The protein content was quantified according to Dumas method (Saint-Denis and Goupy, 2004) using a nitrogen conversion factor to protein of 5.5, which is specific to seaweed (Angell et al., 2016).

For the quantification, 100 mg of each sample were weighed in duplicate and place in a LECO FP-528 analyzer which through a combustion process (900ºC) volatilized the nitrogen content and calculated the nitrogen percentage and the respective protein content of each sample. The ethylenediamine tetraacetic acid (EDTA) was used to calibrate the standards. Total protein values were expressed as percentage of dry weight (% DW).

2.4.1.3. *Total lipids*

The total lipid content of the samples was attained by a modified Folch extraction method (Folch et al., 1957, Campos et al., 2017). Therefore, 200 mg of each sample were weighed in triplicate and mixed with 3 ml of chloroform:methanol (2:1) solution. The tubes containing the mixture were placed in a shaking water bath (room temperature, 400 rpm) for 10 min, and then 3 ml of hydrochloric acid 0.1 N and 300 µl of magnesium chloride 0.5% were added. The tubes were later centrifuged (2,000 × g, 4ºC, 10 minutes) and the organic phase (supernatant) was collected and filtered through a column filled with cotton and anhydrous sodium sulphate (to retain any aqueous phase or suspended particle) to a previously weighed tube. This process was repeated twice to ensure maximum lipid extraction. At a later stage, 3 ml of chloroform:methanol (2:1) solution were added to the initial tubes containing the biomass sample, placed in the shaking bath again (room temperature, 400 rpm) and centrifuged (5 minutes, 4ºC, 2,000 × g). The new organic phase was collected and filtered through the previous column to the respective sample. The column was then washed with 3 ml of pure chloroform to wash out any retained lipids. The solvent was later evaporated using a nitrogen continuous stream. Once the weight of the sample stabilized (full evaporation of the chloroform), the total lipid content was calculated using the following formula:

$$Total\ lipids\ (\%) = \frac{Mf - Mi}{Ms} \times 100$$

Where:

Mf = Tube weight with lipids (final) (g)
Mi = Tube weight (initial) (g)

Ms = Sample weight (g)

Total lipid content results were expressed as percentage of dry weight (% DW).

2.4.2. *Antioxidant activity*

2.4.2.1. *Extract preparation*

Aqueous (*AQ*) and ethanolic (*ETA*) 96 %, w/w, were prepared according to Campos et al. (2017).

To prepare the extracts, 1.25 g of freeze-dried biomass was weighed, homogenized in 25 ml of mili-Q water or ethanol 96 % w/w, using a model Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 30,000 rpm during 1 minute, and later agitated for 18 hours on an orbital shaker at 100 rpm. Subsequently, the mixtures were centrifuged (5,000 × *g* at room temperature for 20 minutes), and the supernatant was transferred into a tube. The volume of the extracted supernatants was then made up to 25 ml with the respective solvent. When calculating the activities, the dilution factor was considered.

2.4.2.2. *Total polyphenol content*

Total polyphenol content was estimated in accordance to an adapted version of the Singleton and Rossi method using the Folin-Ciocalteu reagent (Singleton and Rossi, 1965; Ripol et al., 2018; Campos et al., 2018).

A volume of 100 µl of each sample extract was added to a thread tube in triplicate. To each tube, 600 µl of mili-Q water and 150 µl of twice-diluted Folin-CioCalteau reagent were added and allowed to react for 5 minutes at room temperature. Next, 750 µl of a 2% w/v sodium carbonate solution was added. The mixture was left to react for 1 hour in the dark at room temperature and after this period samples were measured in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. The phenolic content was expressed as gallic acid equivalents (mg GAE·100 g^{-1}) and then transformed into milligrams of polyphenols per gram of dried weight (mg.g^{-1}DW).

2.4.2.3. *DPPH method*

The antioxidant activity was measured through the determination of the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Miliauskas et al., 2004; Campos et al., 2018).

A volume of 1 ml of each extract (prepared in 2.4.2.1.) was added to 2 ml DPPH (Sigma, Steinheim, Germany) 0.15 mM methanolic solution and thoroughly mixed and left to react for 30 minutes at room temperature in the dark. After, the reaction time, the absorbance of the samples and blanks (either mili-Q water or ethanol 96, w/w) was measured at 517 nm in a Helios Alpha (Unicam, Leeds, UK) UV-Vis light spectrophotometer. Results were expressed in mg of ascorbic acid equivalents (AA Eq.) per litre. Radical scavenging activity was estimated by the following formula:

$$\%\, Inhibition\ = \frac{A0\ -\ Asample}{A0} \times 100$$

Where:

A0 – Absorbance of the blank
Asample – Absorbance of the sample

2.4.2.4. *FRAP*

The Ferric Ion Reducing Antioxidant Power (FRAP) method was applied based on a modified Benzie and Strain (1996) technique as performed by Campos et al. (2018) and Ripol et al. (2018). The used techniques are based on the previous work done by Campos et al. (2018), where a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the Fe^{2+} form, it will exhibit an intense blue colour. This means that in FRAP method Fe^{3+} is used in excess and what limits the formation of Fe^{2+} is the reducing ability of the sample under study.

The FRAP solution is composed of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution diluted in 40 mM HCl, and 20 mM of $FeCl_3 \bullet 6H_2O$ solution. The fresh solution was prepared by mixing 100 ml of acetate buffer, 10 ml TPTZ, and 10 ml of $FeCl_3 \bullet 6H_2O$.

A volume of 100 µl of each extract (see 2.4.2.1.), in triplicate, was transferred into thread tubes, and was allowed to react with 3 ml of the FRAP solution for 30 minutes in the dark at 37 ºC. The absorbance of the triplicates was read at 595 nm using in a Helios Alpha (Unicam, Leeds, UK) UV-Vis light spectrophotometer. The % inhibition results were then expressed in mM Fe^{2+} and ascorbic acid (AA) was used as a standard for the calibration curve.

2.4.2.5. *ABTS*

The 2,2'-azino-bis(3-ethylenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was determined using the method described in Campos et al. (2018).

The 7 mM $ABTS^{+\bullet}$ solution comprises 10 mg of ABTS dissolved in 2.6 ml of 2.45 mM potassium persulfate solution. The mixture was placed in the dark for 16 hours at room temperature before use. After this period, the $ABTS^{+\bullet}$ was diluted in 5 mM sodium phosphate buffer (pH 7.4) until reaching an absorbance value of 0.70 ± 0.02 at 734 nm.

Furthermore, 20 µl of each extract prepared (see 2.4.2.1) was added to the $ABTS^{+\bullet}$ solution, the mixture homogenized and incubated in the dark at 30 ºC for 6 minutes. The sample activity was expressed as percentage of inhibition and in µmol of Trolox equivalents (Trolox Eq.) per g freeze-dried biomass. The absorbance of the samples was measured at 734 nm in a Helios Alpha model (Unicam, Leeds, UK). The following formula was used to estimate the percentage of inhibition:

$$\%\, Inhibition = \frac{A0 - Asample}{A0} \times 100$$

Where:

A0 – Absorbance of the blank
Asample – Absorbance of the sample

2.4.3. *Anti-inflammatory activity*

2.4.3.1. *Extract preparation*

The anti-inflammatory activity of the collected *Ulva* spp. biomass was determined in alcoholic extracts, because during the preparation of the aqueous extract the extraction of the supernatant was affected by a very high viscosity.

Approximately 200 mg of freeze-dried biomass was weighed and homogenized with 2 ml of ethanol 96 % w/w, using a Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 25,000 rpm during 1 min. The homogenized extracting mixture was then placed in a bath (80ºC for 1 hour), and later centrifuged (10 minutes, 4ºC,5,000 × *g*). The supernatant was then collected, and the solvent evaporated using a vacuum rotary evaporator with the water bath at 65ºC, 120 rpm and 300 mbar. The residue

was then dissolved in 100% dimethyl sulfoxide (DMSO) to prepare a stock solution at 10 mg/ml.

2.4.3.2. *Cyclooxygenase (COX-2) inhibition method*

The anti-inflammatory activity was determined according to the cyclooxygenase (COX-2) inhibition method (Campos et al. 2018; Ripol et al. 2018) through an *in vitro* technique using a specific kit.

The extract previously prepared (see 2.4.3.1) was tested at 10 mg/ml using a commercial kit cyclooxygenase (COX) inhibitory screening assay kit, Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, MI, USA). To 10 µl of each extract or DMSO, 10 µl 10 mM AA was added to initiate the reaction. Then each reaction tube was incubated at 37ºC for 2 minutes. The reaction was terminated by addition of 50 µl 1 N HCL and saturated stannous chloride.

The assay was executed using 100 units of human recombinant COX-2. The prostaglandin produced by the reaction was then quantified at 412 nm by spectrophotometry via enzyme immunoassay (ELISA) after an 18-hour period of incubation. Then, the microplate was washed and Ellman's reagent was added, followed by 90-minute incubation. The results were then expressed as a percentage of inhibition of COX-2.

2.5. *Statistical analysis*

Normality and homogeneity of variance was determined by the Kolmogorov-Smirnov's test and Levene's test. Data that did not follow parametric assumptions was transformed by being multiplied by the natural logarithm of base e (ln(x)). Figures which corroborated the later, were analysed by a factorial ANOVA distribution using the Tukey HSD to determine the differences between, winter and early spring samples, nutrient effect (*SP* vs *R*), production system (*Fc* vs *Rw*), and aqueous (*AQ*) and ethanolic (*ETA*) extracts. Statistical tests were performed using a significance level (α) of 0.05.

The effect of nutrient level (settling pond *vs* reservoir) and production system (floating cages *vs* raceways) was evaluated through two separate Principle Component Analysis (PCA). The variables were the environmental parameters (temperature, radiation, turbidity, ammonia, nitrite, nitrate, and phosphate), and *Ulva* parameters (growth, protein content, lipid content, dry weight, moisture, ash, organic matter, anti-inflammatory

activity and the antioxidant variables for ethanolic and aqueous extracts of DPPH, ABTS, FRAP and polyphenol content) totalizing 23 variables. Environmental parameters were averaged for each production period. The PCA's were based on the correlation matrix of variables obtained in each production period. To decrease the number of eigenvectors in PCA plots and the loads of just few highly correlated variables, paired correlation was inspected. When paired correlations were higher than 0.90 only one of the variables was chosen as representative. Before the PCA the data was ln (x+1) transformed and centred. All data analysis was performed using STATISICA 6 (Stat-sof, Inc. USA, 2003).

3. *Results*

3.1. *Effect of nutrient level (settling pond vs reservoir)*

3.1.1. *Environmental parameters*

Temperature (Fig. 4a) and radiation (Fig. 4b) increased from winter to early spring. Temperature was significantly higher on the settling pond (*SP*) than in the reservoir (*R*), with average values of 17.79±1.32 ºC and 16.43±1.75ºC respectively, varying between 15 to 18 ºC, and from 14 to 18 ºC. However, no significant differences were found between winter and early spring and between *SP* and *R*.

Turbidity (Fig. 4c) was higher in *SP* than the *R* averaging 3.37±0.38 FNU and 1.43±0.38 FNU respectively. No statistical difference was observed between turbidity values in *SP*, whereas in *R*, significant differences were detected over the seasonality effect.

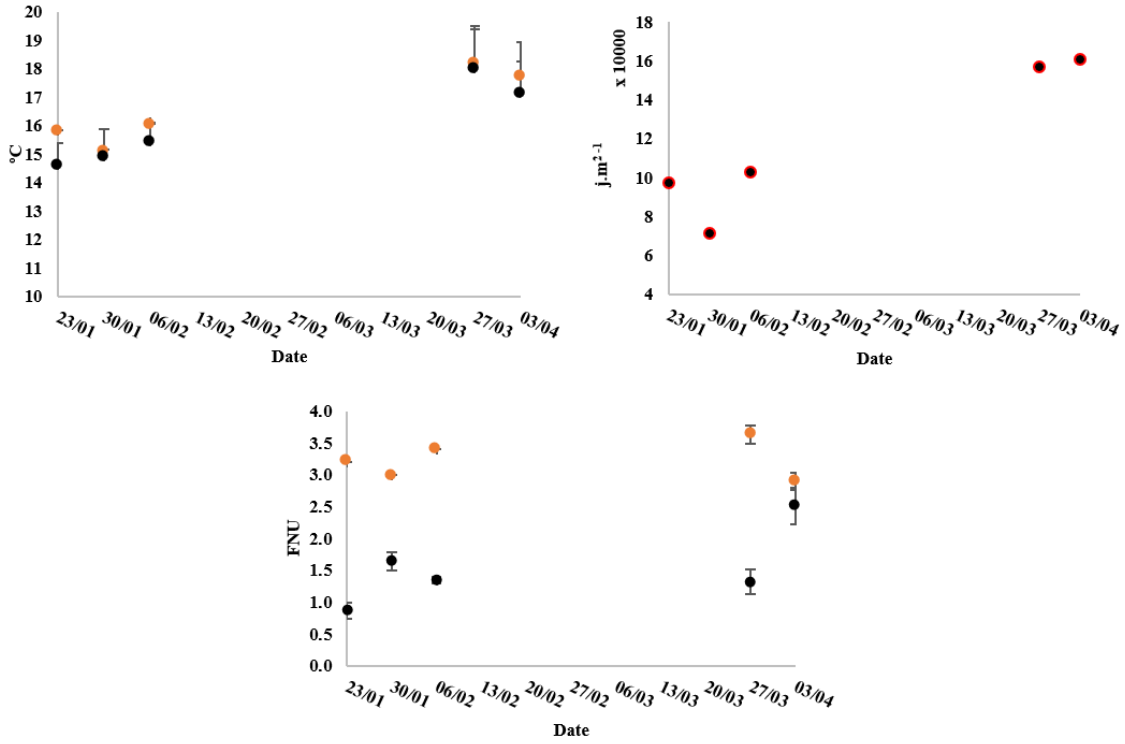

Fig. 4 - Environmental parameters: a) temperature (ºC); b) radiation ($j \cdot m^{-2}$); c) turbidity (Formazin Nephelometric Units (FNU)). Orange dots represent *SP* values and black dots stand for *R* values. Values respective to 13/02 until 20/03 were not analyzed.

Ammonia concentration in *SP* was significantly higher than *R* (Fig. 5a), averaging 40.06±8.30 µM and 30.56±12.42 µM, respectively. Significant differences were observed between *SP* and *R* samples, and between winter and early spring samples.

Nitrite (Fig. 5b) concentration varied between 3.58 and 5.38 µM in *SP*, and 2.90 and 3.55 µM in *R*. Significantly higher concentration of nitrites were observed in *SP* than *R*, with mean values averaging 4.55±0.84 µM, and 3.18± 0.47 µM. No significant difference was observed between and within winter and early spring with exception in *SP* at 27/03.

Nitrate (Fig. 5c) ranged 6.84 to 30.24 µM and 19.29 to 37.85 µM in *SP* and *R* with averages of 23.49±4.47 µM and 26.77±9.62 µM, correspondingly. No significant differences were observed between and within *SP* and *R* values, suggesting similar concentrations in winter and early spring, except for one sample at *R* (27/03).

Phosphate in *SP* was significantly higher than *R*, with mean values 2.21±0.28 µM and 1.87±1.24 µM, respectively (Fig. 5d). Phosphate concentration was significantly different between winter and early spring samples.

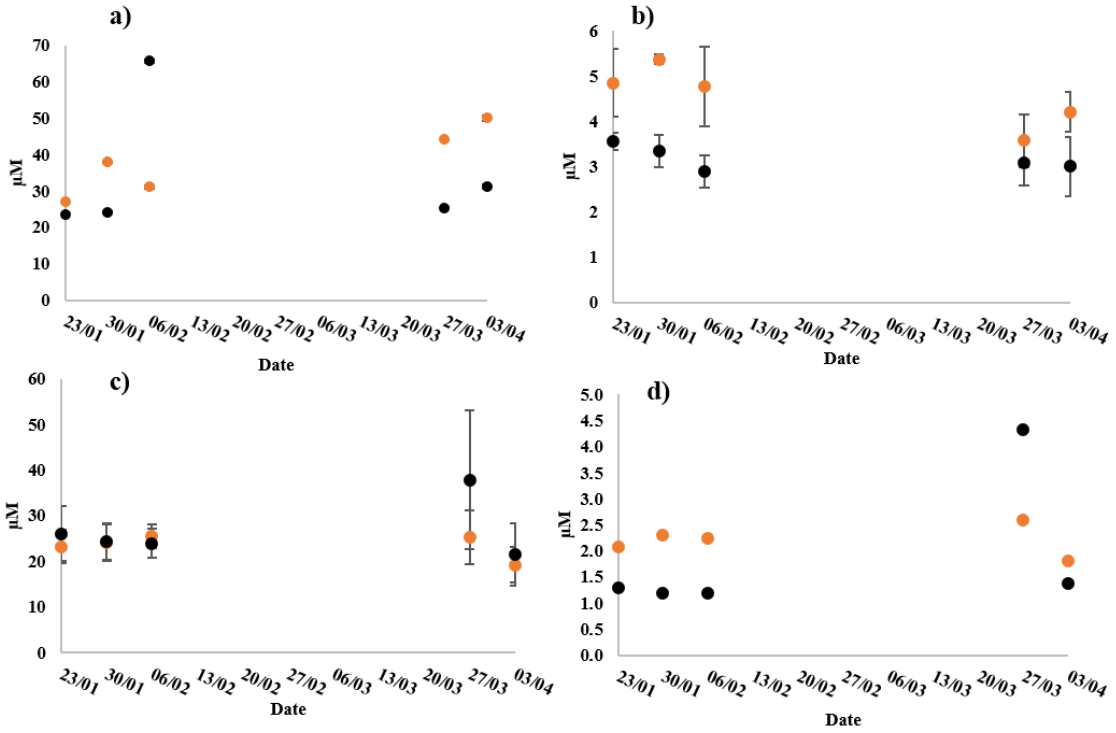

Fig. 5 - Nutrient related results: a) Ammonia; b) Nitrite; c) Nitrate; d) Phosphate. Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.2. *Growth*

The settling pond registered higher growth then the reservoir during winter being similar in spring (Fig. 6). The average growth was 28.56±136.58 FW $g \cdot m^{-2}$ $week^{-1}$ in the settling pond and significantly higher than in the reservoir with a mean of -113.123±174.34 FW $g \cdot m^{2}$ $week^{-1}$. Moreover, growth during winter was significantly lower than early spring. During winter, growth averaged -5.30±174.90 FW $g \cdot m^{-2}$ $week^{-1}$ in *SP*, and FW -230.91±126.46 $g \cdot m^{-2}$ $week^{-1}$ in *R*. On the other hand, during early spring, mean values reached 79.38± 14.63 FW $g \cdot m^{-2}$ $week^{-1}$, and 63.55±15.64 FW $g \cdot m^{-2}$ $week^{-1}$, in *SP* and *R*, respectively.

With exception for January 23, where negative growth was recorded, the *SP* growth ranged between 57 to 130 fresh weight FW $g \cdot m^{-2}$ $week^{-1}$ with no significant differences among periods of collection. The biomass grown in *R*, was only positive on the early spring (27/03 and 03/04) with values between 52.50 and 74.56 FW $g \cdot m^{-2}$ $week^{-1}$ and not statistically different from the values observed in the settling pond.

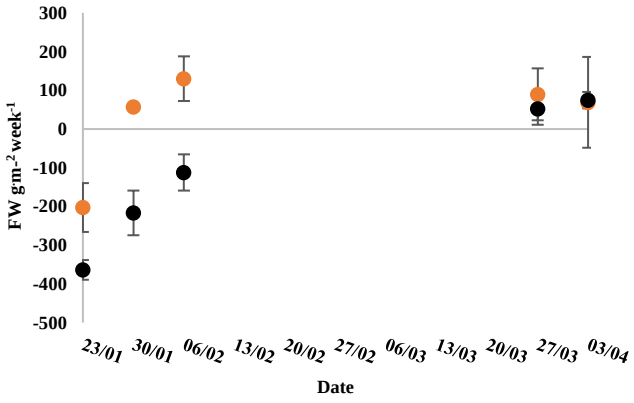

Fig. 6 - Graphic depicting the results regarding growth. Y axis – growth (FW $g.m^{-2}$ $week^{-1}$); X axis – date (days). The orange dots represent the *SP* and the black dots the *R*. Values respective to 13/02 until 20/03 were not analyzed.

3.1.3. *Proximal composition*

No significant differences were found between moisture content in *Ulva* from *SP* and *R*, neither between and within samples regarding winter and early spring (Fig. 8a). Values fluctuated between 80 and 83 % FW (83.7±2.4 % FW) and 80 and 85 % FW (83.5±2.4 FW%) for *SP* and *R* . The dry weight was always higher in *SP* except at 23/01 and 03/04 when the values were similar to *R*.

Ulva biomass dry weight decreased along time (Fig. 7b) with significant differences only observed between winter and early spring on the settling pond. The dry weight on *SP* ranged from 16 to 20 % FW (16.3±2.4 % FW) whereas in *R* varied between 16 and 19 % FW (16.5±2.4 % FW).

The ash content presented a similar trend than dry weight (Fig. 7c). During winter, *SP* in *Ulva* ash content was higher than in *R*, but in early spring the values were similar. No differences were observed between and within *SP* and *R*, and no difference was recorded between winter and early spring samples. Ash content in *Ulva* varied from 5.9 to 6.0 % FW with an average of 6.1±2.4% FW in *SP*, while in *R* they varied between 4.8 and 6.4 % FW with an average of 5.9±0.8 % FW.

The organic matter (OM) varied between 58 and 70% DW (61.7±4.3 % DW) in the settling pond (*SP*), whereas in the reservoir (*R*) ranged from 60 to 67 % DW (64.5±3.1 % DW) being, with exception of 23/01, always higher in *R* (Fig. 7d). Significant differences were found between *SP* and *R* values, and values did not differ between winter and early spring samples, apart from 23/01.

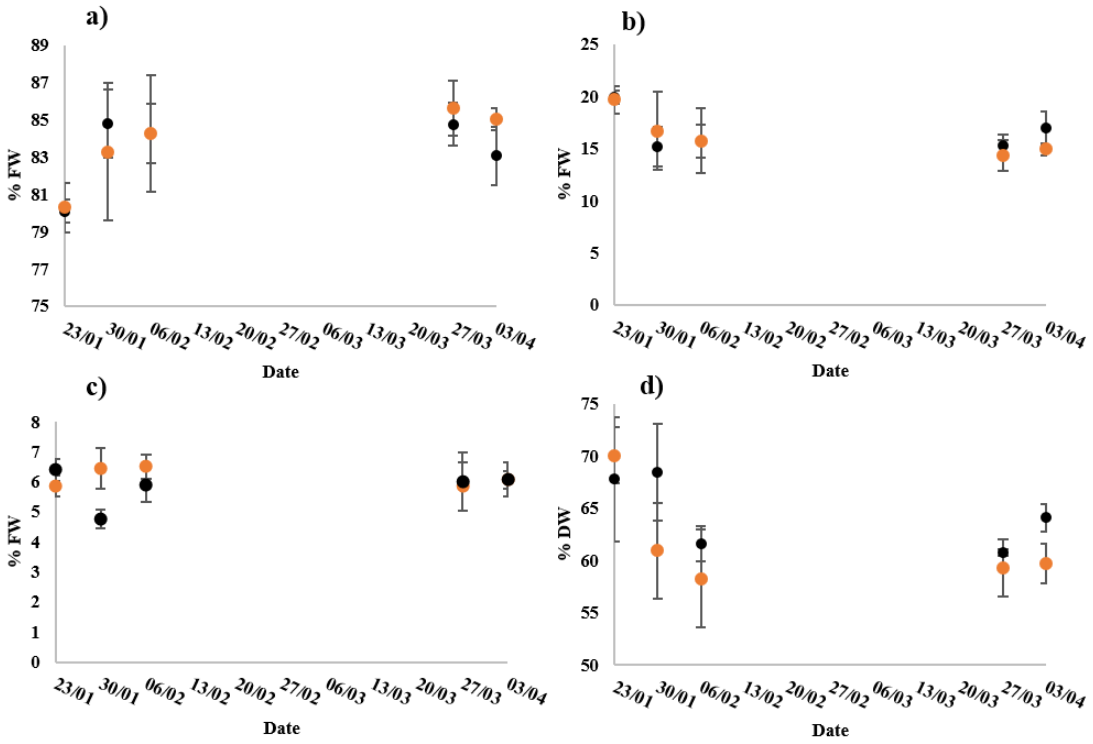

Fig. 7 - Seasonal variation in moisture (a), dry weight (b) ash content (c) and organic matter (d). Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

Total protein content in *Ulva* was significantly higher in *SP* than *R*. With time, *Ulva* protein content in *R* approached *SP* (Fig. 8). During winter, the *Ulva* protein content in *SP* was higher than in early spring. *SP* protein content ranged between 16.30 and 12.3 % DW with an average of 14.8±1.3 % DW, whereas in *R* they varied between 11.1 and 14.9 % DW with a mean of 13.3±1.4 % DW.

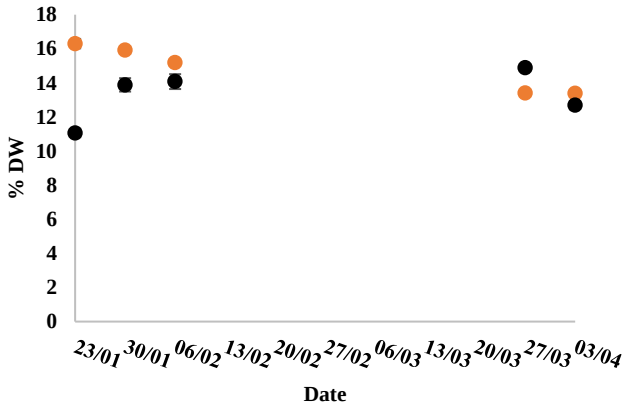

Fig. 8 - Results regarding total protein content. X axis – percentage (% DW); Y axis – date (days). Black dots refer to R related samples; Orange dots refer to SP related samples. Values respective to 13/02 until 20/03 were not analyzed.

The SP total lipidic content (Fig. 9), exhibited no significant differences between samples. Thus, no difference was observed between winter and early spring samples in *SP*. However, regarding *R* biomass results showed significant differences within samples, meaning that the lipid content varied between winter and early spring samples. Nevertheless, no significant difference was found between *SP* and *R* lipid content. Levels varied between 1.26 and 1.66 % DW on *SP*, and 0.41and 2.80 % DW in *R* and averaged 1.46±0.20 % DW and 1.37±1.05 % DW, correspondingly.

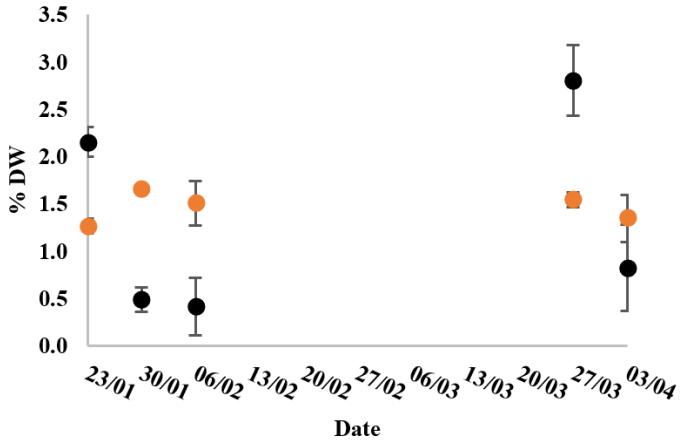

Fig. 9 - Total lipid content. Y axis – percentage in biomass dry weight (% DW); X axis – date (days). Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.4. *Antioxidant properties*

3.1.4.1. *Total polyphenol content*

Regarding the aqueous extracts (*AQ*) (Fig. 10a), the polyphenol content was significantly higher in *SP* biomass than in the *R* , except at 23/01 when it was lower, and at 30/01 where both values were similar. Significant differences were found between and within winter an early spring samples; more specifically, during winter, polyphenol content increased, later decreasing in early spring samplings. Therefore, maximum values recorded were 0.69 and 0.59 mg GAE.g^{-1} DW, for *SP* and *R* respectively. On the other hand, registered minimum values reached 0.23 and 0.43 mg GAE.g^{-1} DW, correspondingly. Polyphenol content averaged 0.54±0.17 mg GAE.g^{-1} DW on *SP* biomass, and 0.25±0.07 mg GAE. g^{-1} DW on *R*.

With respect to the ethanolic extracts (*ETA*) (Fig. 10b), polyphenol content at *SP* biomass was significantly higher than *R* , and values averaged 0.33±0.06 mg GAE.g^{-1} DW on *SP*,

and 0.17±0.11 mg GAE.g^{-1} DW on *R*. The *SP* polyphenol content ranged between 0.23 and 0.43 mg GAE.g^{-1} DW and *R* between 0.02 to 0.29 mg GAE.g^{-1} DW. The values followed a trend similar to the *AQ* extracts, where the content increased over winter, and decreased during early spring samples .

In summary, the *AQ* extract had significantly higher polyphenol content than *ETA*. Average documented values were 0.40±0.19 mg GAE.g^{-1} DW for *AQ*, whilst *ETA* recorded 0.25±0.12 mg.g^{-1} DW. The richest biomass was found to be *SP* cultivated, which produced levels up to 0.44±0.16 mg.g^{-1} DW, whereas *R* presented 0.21±0.19 mg GAE.g^{-1} DW.

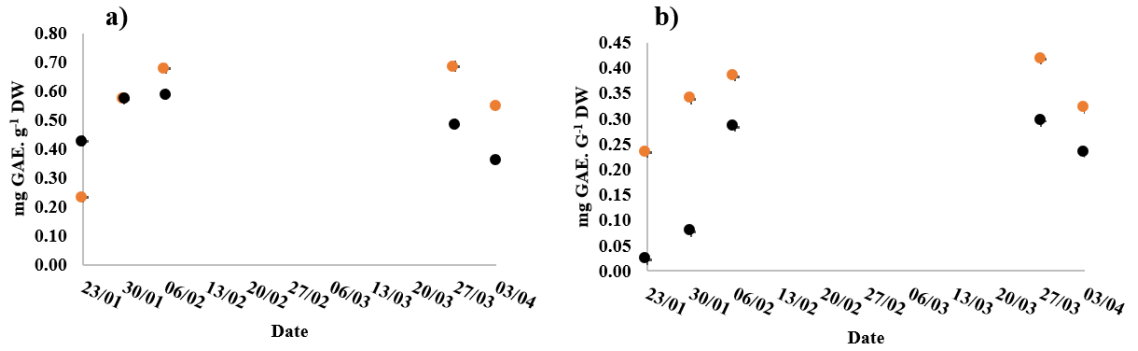

Fig. 10 - Total polyphenol content: a) aqueous extract results; b) ethanolic extract results; X axis – date (days); Y axis – polyphenol content mg GAE.g^{-1} DW); Black dots refer to R related samples; Orange dots refer to SP related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.4.2. *DPPH*

Through the determination of the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) the antioxidant activity of *AQ* and *ETA* extracts was measured.

Regarding the *AQ* extract (Fig. 11a), *R* exhibited significantly higher activity than *SP*. The results show that values increased during winter and decreased in early spring . Consequently, significant differences were observed between and within winter and early spring samples. Maximum values were 445.72 and 41.32 mg AA Eq.L^{-1} for *SP* and *R*, respectively. Minimum values were noted during early spring, being 25.4 and 31.55 mg AA Eq.L^{-1} for *SP* and *R*, respectively. Moreover, *SP* averaged 34.13±7.14 mg^{-1}AA Eq^{-1}L, while *R* 36.18±3.50 mg AA Eq.L^{-1} . Concerning the *ETA* extract, values from *R* were significantly higher than *SP*.

The values observed on *ETA* extract (Fig. 11b) increased from winter to early spring . Minimum values were recorded on 23/01, with 3.95 and 5.94 mg AA Eq.L^{-1} for *SP* and *R* biomass, respectively. On the other hand, maximum values were noted in early spring reaching 8.66 mg AA Eq.L^{-1}, and 10.13 mg AA Eq.L^{-1} for *SP* and *R*, respectively.

The *AQ* extract displayed a higher antioxidant activity, averaging 35.19± 5.62 mg AA Eq.L^{-1}, whereas *ETA* registered only 7.38±1.62 mg AA Eq.L^{-1}. Furthermore, *R* biomass generated the strongest antioxidant activity, with a mean value of 21.93± 14.73 mg AA Eq.L^{-1}, while *SP* averaged 20.15±14.65 mg AA Eq.L^{-1}.

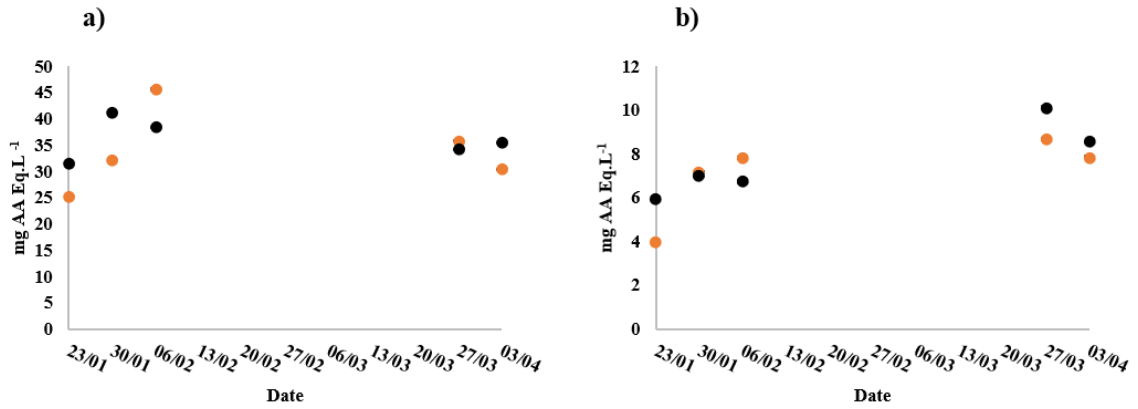

Fig. 11 - DPPH antioxidant results: a) aqueous extract results; b) ethanolic extract results; X axis – date (days); Y axis – DPPH activity (mg AA Eq.L^{-1}); Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.4.3. *ABTS*

The 2,2'-azino-bis(3-ethylenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity is used to assay lipophilic and hydrophilic antioxidants.

Regarding the *AQ* extract (Fig. 12a), during winter the biomass cultured in *R* presented higher activity, whereas during early spring *SP* surpassed it. The results show that the activity increases significantly from winter to spring. The maximum value for *R* biomass was recorded during winter on 06/02, with 36.89 µmol Trolox Eq.g DW^{-1} and the minimum at 23/01, with 23.61 µmol Trolox Eq.g DW^{-1}. Although the minimum value for *SP* was similar to *R*, the peak was observed at 03/04 reaching at 46.13 µmol Trolox Eq.g DW^{-1} . Nevertheless, *SP* presented more activity than *R*, with mean values 32.22±9.01 µmol Trolox Eq.g DW^{-1} and 29.98±5.59 µmol Trolox Eq.g DW^{-1} , respectively.

Concerning the *ETA* extract (Fig. 12b), *SP* activity was significantly higher than *R*, with mean values of 31.80±10.76 µmol Trolox Eq.g DW^{-1} , and 12.90±4.17 µmol Trolox Eq.g DW^{-1} . The activity of *SP* samples increased continually from 16.62 µmol Trolox Eq.g DW^{-1} noted at 23/01, peaking at 27/03 with 42.47 µmol Trolox Eq.g DW^{-1} , when values decreased. Concerning *R*, results seem to follow the same pattern as *SP*, with a minimum value recorded at 23/01, 8.85 µmol Trolox Eq.g DW^{-1} , and the maximum at 27/03, reaching 19.19 µmol Trolox Eq.g DW^{-1} .

Briefly, the *AQ* extracts and *SP* biomass (encompassing both extracts) presented the most powerful antioxidant activity, averaging 31.14±7.54 µmol Trolox Eq.g DW^{-1} , and 32.01±9.75 µmol Trolox Eq.g DW^{-1} , whilst *ETA* and *R*, averaged 22.35±12.35 µmol Trolox Eq.g DW^{-1} , and 21.15± 9.95 µmol Trolox Eq.g DW^{-1} .

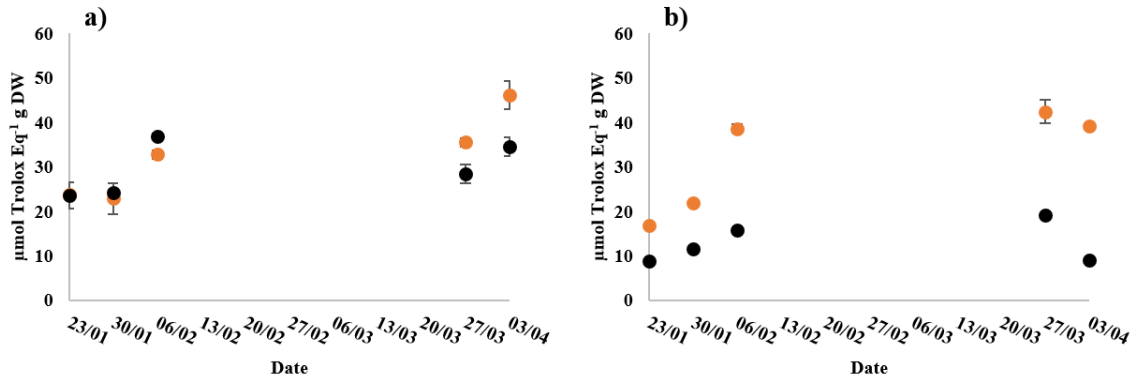

Fig. 12 -ABTS antioxidant results: a) aqueous extract; b) ethanolic extract; X axis – date (days); Y axis – ABTS antioxidant activity (mmol Trolox Eq.g DW^{-1}); Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.4.4. *FRAP*

This method is based on redox reactions where the oxidizing species reacts with the antioxidant instead of the substrate.

Regarding the *AQ* extracts (Fig. 13a), the activity of *R* biomass was higher during winter, whereas at early spring the *SP* values were significantly higher. The activity on *SP* samples increased over time, whereas *R* samples decreased from winter to early spring. Minimum values were recorded during winter with 37.39 mM and 92.01 mM for *R* and *SP*, respectively. On the other hand, *SP* reached its peak at 03/04 with 1215.98 mM, and

R at 30/01 with 454.78 mM. Therefore, *SP* and *R* were significantly different, averaging 515±538.86 mM and 222.97±121.82 mM, respectively.

Concerning the *ETA* extracts (Fig. 13b), *SP* had significantly higher activity than *R*. Values gradually increased during winter, until they reached the maximum at 27/03, except for 06/02 where *SP* peaks at 1,312.76 mM. Thus, values were significantly different between winter and early spring samples. Biomass cultured at R peaked at 889.57 mM on the 27/03 and the minimum value was recorded on the 23/01 at 92.01 mM. On the other hand, SP minimum was recorded also on the 23/01 at 429.41 mM, and not considering 27/03, the maximum was also recorded on the 27/03. Hence, the average values for SP and R ethanolic extract were 766.3±330.52 mM and 469.66±277.79 mM.

In sum, both extracts had similar activity and followed a similar trend. Whenever *SP* activity increased, *R* activity also increased. *SP* produced a stronger antioxidant biomass than *R*, since mean values reached 640.93±457.36 mM and 350.52±247.44 mM, respectively. In addition, the *ETA* extracts had a stronger antioxidant activity, averaging 617.98±335.78 mM, whereas *AQ* extract had only 374.26±417.41 mM.

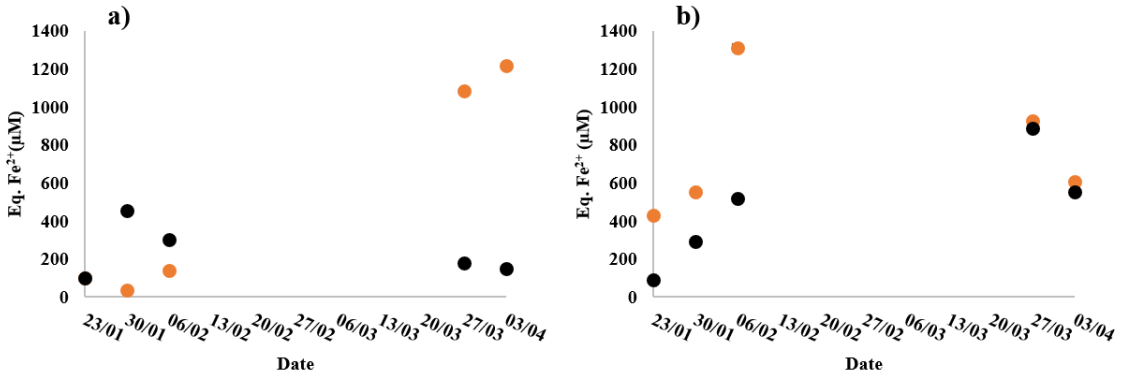

Fig. 13 - FRAP antioxidant results. a) aqueous extract; b) ethanolic extract; X axis – date (days); Y axis – FRAP antioxidant activity (Eq. Fe^{2+} (mM)); Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.5. *Anti-inflammatory activity*

During winter, *SP* anti-inflammatory activity was significantly higher than R, whilst during early spring, R activity was higher, except on the 27/03 when values were similar. The biomass produced at the SP had maximum inhibition on the 23/01 with 100 %, decreasing with time, reaching the minimum value on the 03/04, with 77.17%. Regarding the biomass cultivated at *R*, highest value was recorded on the 30/01 with 90.53% inhibition, and the minimum was recorded on the 06/02, presenting 68.25%. In summary, the results showed that *SP* anti-inflammatory activity was higher than *R*, with mean values 88.66±7.81 % and 85.03±9.01 %, respectively (Fig. 14).

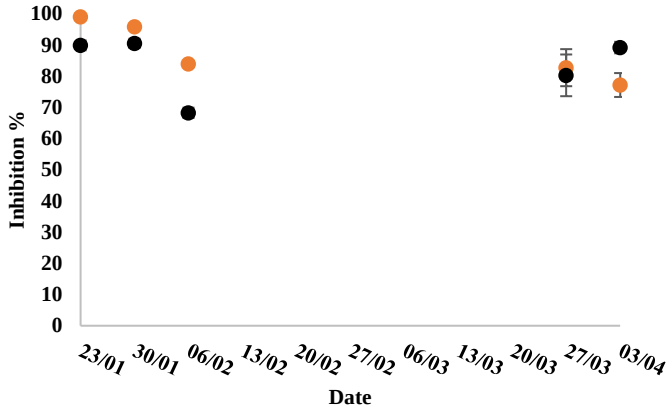

Fig. 14 - Anti-inflammatory activity of the ethanolic extract. X axis – date (days); Y axis – anti-inflammatory inhibition (%); Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.1.6. Principle component analysis for nutrient source

The explanatory variables used for PCA were dry weight (*DW*), growth (*Growth*), total lipid content (*Fat*), total protein (*Prot.*), radiation (*Rad.*), turbidity (*Turb.*), phosphate (*PO4*), ammonia (*NH3*), nitrite (*NO2*), nitrate (*NO3*), polyphenol content aqueous and ethanolic extracts (*Poly. AQ* or *ETA*), DPPH aqueous and ethanolic extracts (*DPPH AQ* or *ETA*) activity, FRAP aqueous and ethanolic extract activity (*FRAP AQ* or *ETA*) and ABTS aqueous and ethanolic extract activity (*ABTS AQ* or *ETA*).

The PCA correlation biplot for explanatory variables and nutrient sources were plotted in two biplots where the first and second principle components explained 55.84% of the variability (Fig. 15, A and B). PC1 that explains 36.13% of the variation, separates the low nutrient source (*R*) and winter samples (*W1* and *W2*) clustering to left upper corner and the high nutrient source (*SP*) and late winter and early spring samples (*W3*,*Sp1*, and *Sp2*) to the right. Within late winter and early spring cluster, there is a separation between reservoir and settling pond. *SP* formed a homogeneous group to the middle right, mainly explained by growth, polyphenol *ETA* content and DPPH *ETA* antioxidant activity. *R* samples clustered to the left upper corner, were mostly explained by organic matter content and the anti-inflammatory activity. One early spring *R* value (*R/Sp1*), strays further from the main cluster, explained mostly by phosphate, nitrate levels and lipid content, suggesting a possible water sample contamination by organic matter. The antioxidant activities were all positively correlated with ammonia (NH3). Turbidity (Turb) present a high correlation with polyphenol content. Growth is positively correlated with radiation and nitrite, whilst negative correlation is found for organic matter (OM) and anti-inflammatory activity (Inflam).

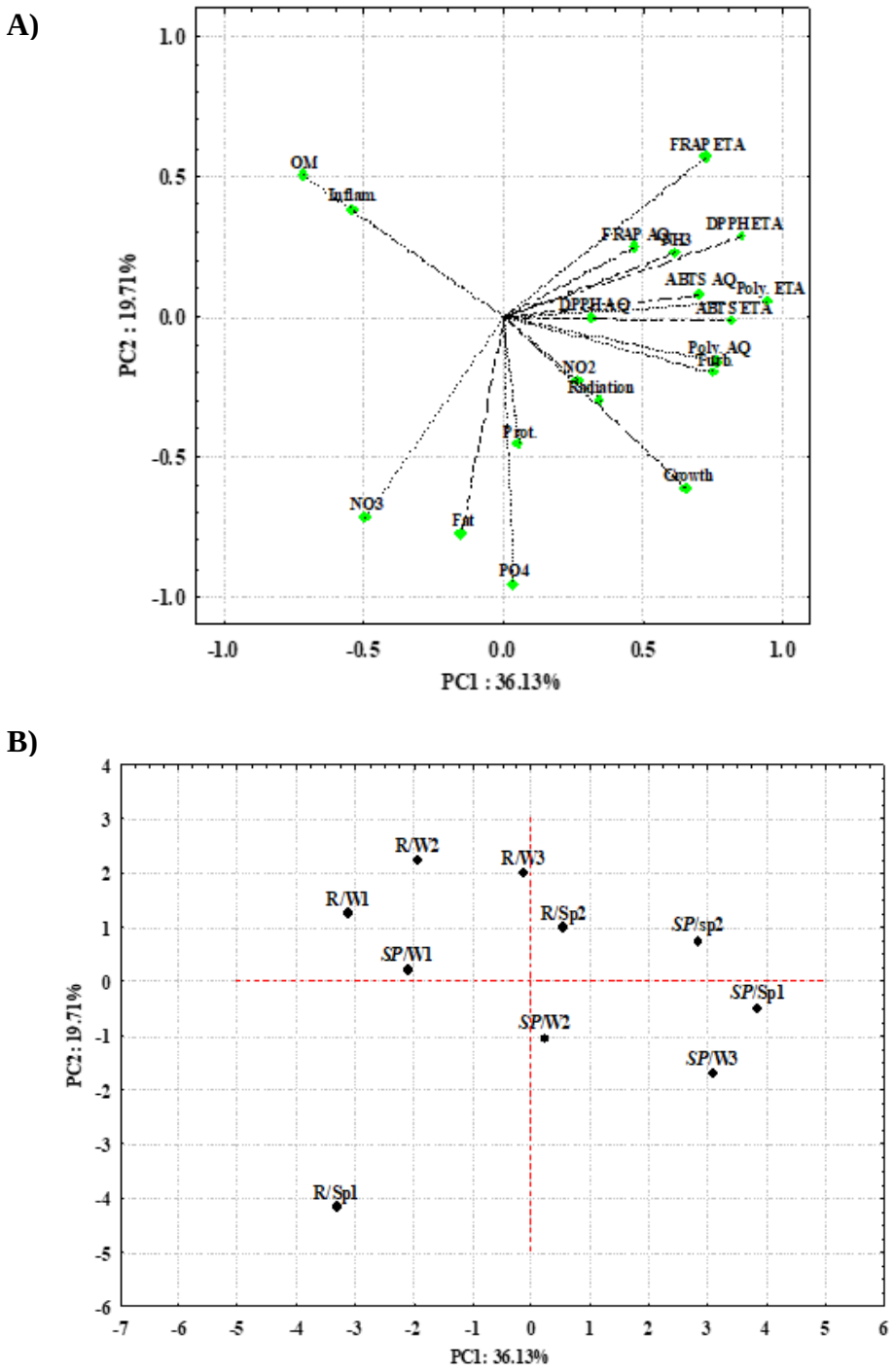

Fig. 15 - PCA correlation biplot for Nutrient Source data: A) PCA correlation biplot on explanatory variables (PC1 x PC2). Legend: organic matter (*OM*), growth (*Growth*), total lipid content (*Fat*), total protein (*Prot.*), radiation (*Rad.*), turbidity (*Turb.*), phosphate (*PO4*), ammonia (*NH3*), nitrite (NO2), nitrate (*NO3*), polyphenol content aqueous and ethanolic extracts (*Poli. AQ* or *ETA*), DPPH aqueous and ethanolic extracts (*DPPH AQ* or *ETA*) activity, FRAP aqueous extract activity (F*RAP AQ*), and anti-inflammatory activity (*Inflam.*); B) PCA correlation biplot applied on nutrient source data. Legend: R (Reservoir), SP (settling pond), W*1* (winter, 23/01), *W2* (winter, 30/01), *W3* (winter 06/02), *Sp1* (spring, 27/03), and *Sp2* (spring 3/04).

3.2. *Effect of production system*

3.2.1. *Environmental parameters in the raceway and floating cages*

Temperature on the floating cages (*Fc*) ranged 15.16 and 18.26 ºC, whereas in the raceways (*Rw)* fluctuated between 14.01 to 17.39 ºC. Temperature increased from winter to early spring (Fig. 16a). As temperature increased, so did radiation, varying from 71,351 to 161,063 $j \cdot m^{-2}$. The temperature in *Fc* averaged 17.79 ± 1.33 ºC, while in the *Rw* averaged 16.55±2.05 ºC. No significant difference was found between Fc and Rw regarding temperature.

Turbidity was higher during early spring on the *Fc* (Fig.16b). During winter, values were very similar, although the *Fc* were more turbid. Turbidity ranged 1.14-3.70 (3.36±1.97) FNU in the *Rw* and 3.08-5.82 (4.52±1.13) FNU in *Fc*, but differences were not significant.

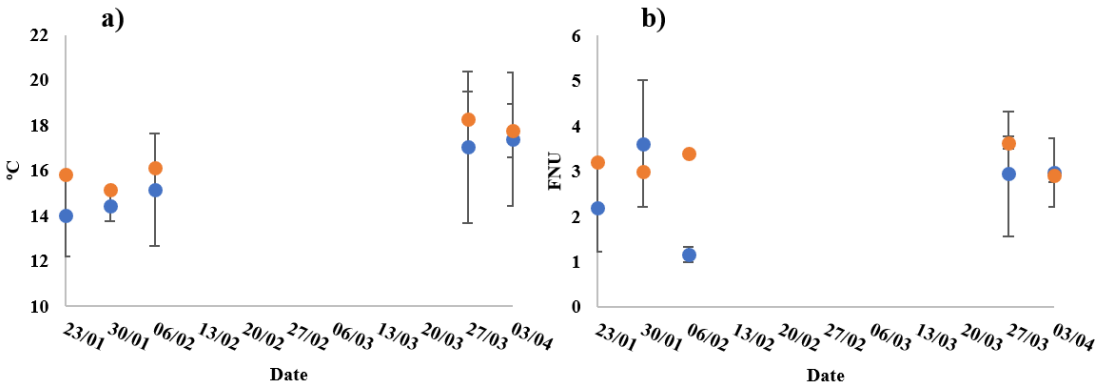

Fig. 16 - Seasonal variation of temperature (ºC) (a) and turbidity (FNU) (b) in the *Rw* (blue dots) and *Fc* orange dots). Values respective to 13/02 until 20/03 were not analyzed.

Ammonia was significantly higher in the *Rw* in comparison to *Fc*, averaging 59.60 ± 23.53 µM, and 40.07 ± 8.30 µM, respectively. NH_3 ranged from 20.14 to 89.01 µM in the *Rw*, and 23.89 and 56.92 µM in *Fc* (Fig. 17a). Winter values were significantly different from early spring.

Nitrite concentration was significantly higher on the *Fc* (4.55 ± 0.88 µM) than in the *Rw* (1.19 ± 0.22 µM), and values differed significantly over time, ranging from 3.58 to 5.38 µM in the *Fc*, whereas in the *Rw* they varied between 0.98 and 1.52 µM (Fig. 17b).

Nitrates, averaged 21.10 ± 6.59 µM in *Rw*, and 23.49 ± 4.47 µM in *Fc*. NO_3 varied between 19.29 to 25.44 µM in the *Fc*, and from 20.32 to 22.46 µM in the *Rw*. No statistical difference was observed over time and between groups (Fig. 17c).

About phosphate levels averaged 2.21 ± 0.21 µM and 2.57 ± 0.49 µM, and differences were observed between groups and between winter and early spring samples. PO_4^{3-} values varied from 1.82 µM to 2.60 µM in the *Fc*, and from 2.07 to 3.29 µM in *Rw* (Fig. 17d).

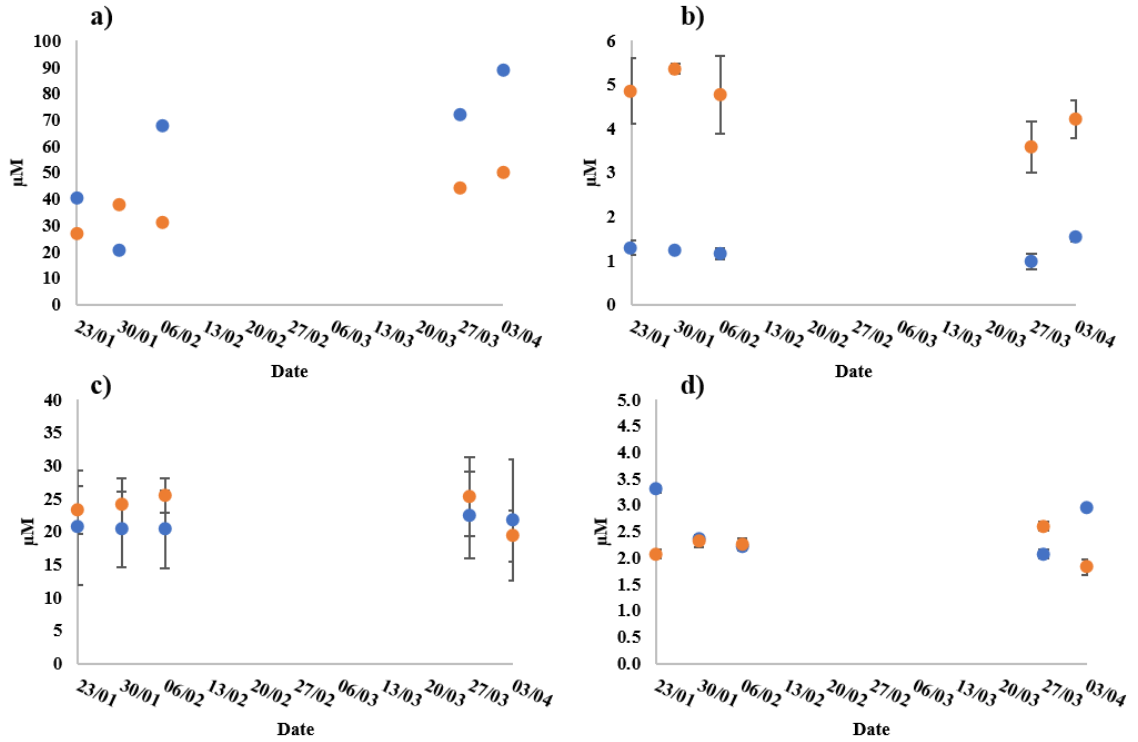

Fig. 17 - Nutrient related results: a) ammonia; b) nitrite ; c) nitrate; d) phosphate . Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.2. *Growth*

Growth averaged 92.87 and 28.56 FW $g \cdot m^{-2}$ $week^{-1}$ in raceways (*Rw*) and floating cages (*Fc*). Over time, productivity was always higher on the *Fc*, except on 23/01 and 03/04 where the productivity in *Rw* surpassed (Fig. 18). Growth ranged between 56.72 to 130 FW $g \cdot m^{-2}$ $week^{-1}$, and 13.62 and 233.33 FW $g \cdot m^{-2}$ $week^{-1}$, for the floating cages and raceways, respectively. The results show that the productivity in the *Fc* was more constant in comparison to the *Rw*, and that growth increased over time. Significant differences were observed between production systems, and between winter and early spring samples.

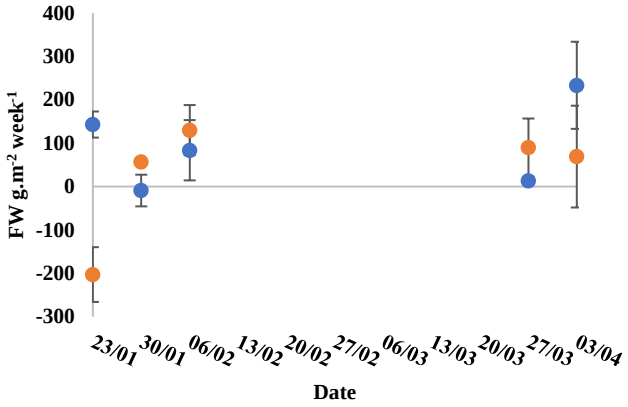

Fig. 18 - Seasonal variation of growth (FW $g \cdot m^{-2}$ $week^{-1}$) in *Fc* (orange dots) and in the *Rw* (blue dots). Values respective to 13/02 until 20/03 were not analyzed.

3.2.3. *Proximal composition*

Moisture was always higher in the *Fc Ulva* biomass than in the *Rw* biomass, averaging 83.72 ± 2.41 % FW and 81.76 ± 2.38 % FW, correspondingly. The water content varied between 79.34 and 83.68 % FW, and 80.30 and 85.64 % FW on the raceways and floating cages. No significant difference was observed within *Rw* samples from winter to early spring, whereas the *Fc* winter samples were statistically different from early spring (Fig. 19a)

Concerning the dry matter of the samples, the *Ulva* cultured on the raceways presented higher values in comparison to the floating cages, with means of 18.23 ± 2.38 % FW and 16.27± 2.41 % FW, respectively. Dry matter varied between 14.36 and 19.70 % FW, in *Fc* and 16.87 and 20.66 % FW, and *Rw* . Significant differences were observed between

Fc and *Rw* . Dry matter of the *Ulva* samples differed significantly from winter to early spring on *Fc*, whereas no differences were observed within *Rw* samples (Fig. 19b).

The ash content presents no statistical difference between *Fc* and *Rw*, and over time. However, the biomass produced in *Rw* had higher ash content than the biomass produced in the *Fc*, averaging 6.44 ± 0.53 % FW and 6.12 ± 0.49 % FW, correspondingly. Values ranged between 6.12 and 6.19 % FW in *Rw* and 5.86 and 6.51 % FW in *Fc*. (Figure 19c).

The organic matter (OM) was always higher in *Ulva* cultivated in the raceways, with exception from 23/01 and 03/04. OM ranged 58.3 to 70.18 % DW in the *Ulva* from *Fc*, and 59.7 and 68.6 % DW in the *Rw*. The organic matter content was higher during winter, decreasing until early spring. There was no significant difference in OM content on samples from different dates between *Rw* and *Fc*. OM in the *Rw* averaged 65.0±5.0 % DW and was significantly higher than *Fc*, that averaged 61.8±5.3 % DW (Fig. 19d).

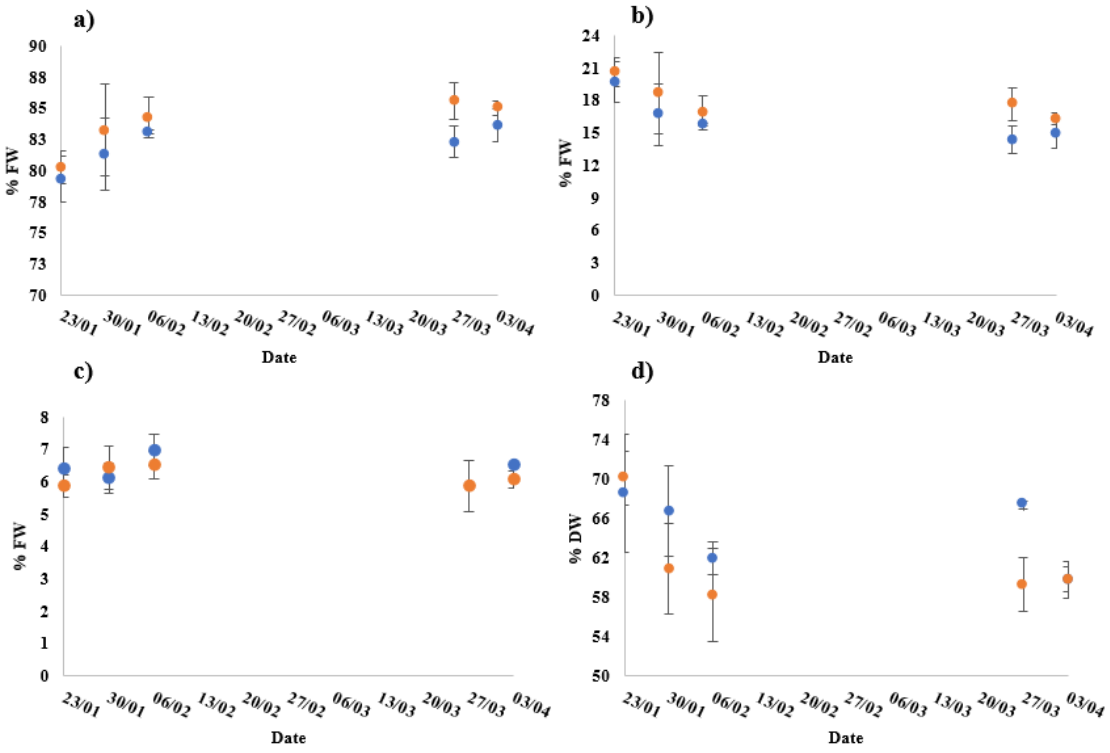

Fig. 19 - Seasonal variation: moisture (a), dry weight (b) ash content (c) and organic matter (d). Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

Total protein content was significantly higher in the *Fc Ulva* produced biomass than in that obtained from *Rw*, averaging 14.84±1.82 % DW and 10.60±1.82 % DW,

respectively. The protein levels were significantly higher during winter than in early spring. The content varied between 13.38 and 16.30 % DW, and 8.30 and 12.85 % DW in the *Fc* and *Rw*, respectively. According to the results, *Fc* and *Rw* appear to follow the same trend (Fig. 20)

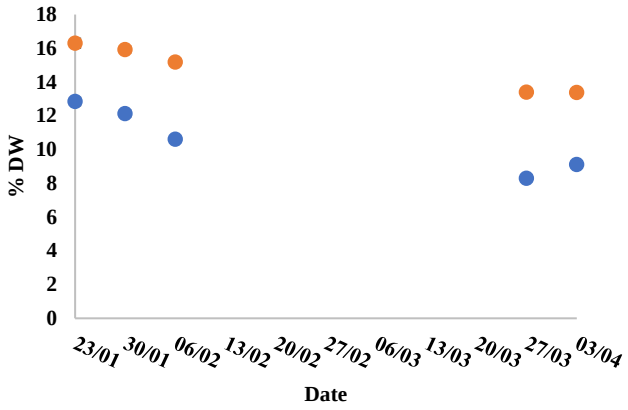

Fig. 20 -- Results regarding total protein content. X – percentage (% DW); Y axis – date (days). Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

Total lipidic content was significantly higher on *Fc* than *Rw*. However, no significant difference was observed between *Fc* and *Rw* regarding early spring samples. *Rw* lipids averaged 1.35±0.57 % DW while *Fc* mean percentage was 1.46±0.20 % DW. No significant difference was observed within *Fc* samples, whereas lipid content differed within *Rw* winter samples. Briefly, values ranged between 0.71 and 1.90 % DW on *Rw* , whist *Fc* varied from 1.26 to 1.66 % DW. Results also show that the lipid content decreased from winter to early spring (Fig. 21).

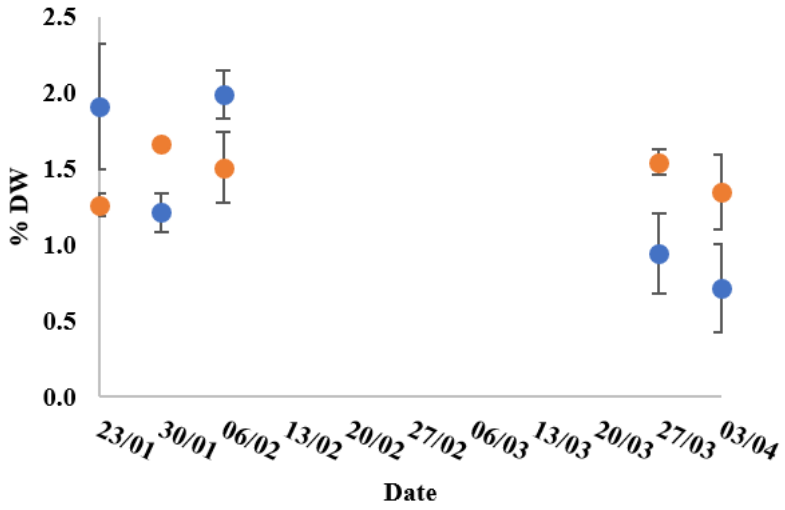

Fig. 21 - Results related to the total lipid content. Y axis – percentage (% DW); X axis – date (days). Black dots refer to *R* related samples; Orange dots refer to *SP* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.4. *Antioxidant properties*

3.2.4.1. *Total polyphenol content*

Regarding the *AQ* extracts (Fig. 22a), the polyphenol content was significantly higher on the biomass produced in *Fc*, except for the sample 23/01 where the level was higher in *Rw* biomass. Levels ranged from 0.05 and 0.12 mg GAE.g^{-1} DW, and 0.23 and 0.43 mg GAE.g^{-1} DW in the *Fc* and *Rw* biomass and averaged 0.54±0.18 mg GAE.g^{-1} DW, and 0.37±0.08 mg GAE.g^{-1} DW, respectively. Significant differences were found between *Fc* and *Rw*, and between winter and early spring samples.

Concerning the *ETA* extracts (Fig. 22b), the *Fc* produced the biomass with the higher polyphenol content, and differences were observed between the later and *Rw*. The polyphenol levels increased during winter, peaked on 27/03 and then decreased on the last sampling. In fact, during early spring the polyphenols decreased on *Fc* and increased on *Rw* biomass. Significant differences were found between groups and winter and spring samples. In the *ETA* extract, values varied between 0.23 and 0.69 mg GAE.g^{-1} DW, and 0.25 and 0.48 mg GAE.g^{-1} DW in the *Fc* and *Rw* samples and averaged 0.09±0.02 mg GAE.g^{-1} DW and 0.33±0.06 mg GAE.g^{-1} DW, respectively.

Water was the best solvent to extract the polyphenols. Hence, the *AQ* had significantly higher activity, averaging 0.45±0.16 mg GAE.g^{-1} DW and *ETA* 0.22±0.13 mg GAE.g^{-1}

DW. Furthermore, *Fc* biomass produced the extracts with higher polyphenol content, averaging 0.43±0.16 mg GAE.g^{-1} DW, whereas *Rw* averaged 0.24±0.15 mg GAE.g^{-1} DW.

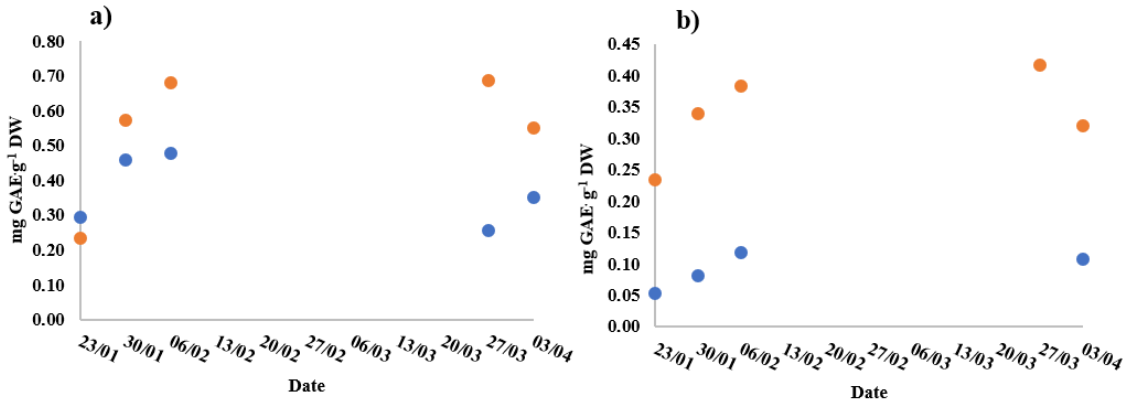

Fig. 22 - Total polyphenol content results: a) aqueous extract results; b) ethanolic extract results; X axis – date (days); Y axis – polyphenol content (mg GAE.g^{-1} DW); Blue dots refer to Rw related samples; Orange dots refer to Fc related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.4.2. *DPPH*

Regarding the *AQ* extracts (Fig. 23a), *Fc* was significantly more active than the *Rw*. The activity increased from winter until early spring in *Rw* biomass, while the *Fc* biomass only increased during winter, and then levels seem to have stabilized after peaking at 06/02. Hence, significant differences were observed between and within winter and early spring samples. Concerning the *Fc*, values averaged 34.13±7.24 mg AA Eq.L^{-1} and ranged from 25.36 and 45.72 mg AA Eq.L^{-1}, whilst in the *Rw* biomass ranged between 17.02 and 30.36 mg AA Eq.L^{-1} with a mean value of 22.21±6.03 mg AA Eq.L^{-1}.

Focusing on the activity of the *ETA* extracts (Fig. 23b), *Fc* biomass produced higher activity than *Rw*, except on the 23/01 and 30/01, and levels increased from winter to early spring. Afterwards, in *Rw*, although the values during winter were very close to *Fc*, no increase was noted in early spring, showing a more constant activity. Values averaged 5.49±1.47 mg AA Eq.L^{-1}, and 7.10±1.69 mg AA Eq.L^{-1} on the *Rw* and *Fc* and ranged between 5.16 and 7.90 mg AA Eq.L^{-1} ,and 3.95 and 8.66 mg AA Eq.L^{-1}, respectively. Nevertheless, significant differences were observed between and within winter and early spring samples.

Briefly, the *AQ* extracts presented higher activity than the *ETA* extracts, with average values of 28.17±17.53 mg AA Eq.L^{-1} and 6.29±1.76 mg AA Eq.L^{-1}. Values differed

statistically between winter and early spring samples, and *Fc* showed significantly stronger antioxidant power than *Rw*, with mean values of 20.15±14.65 mg AA Eq.L^{-1} and 13.56±9.50 mg AA Eq.L^{-1}.

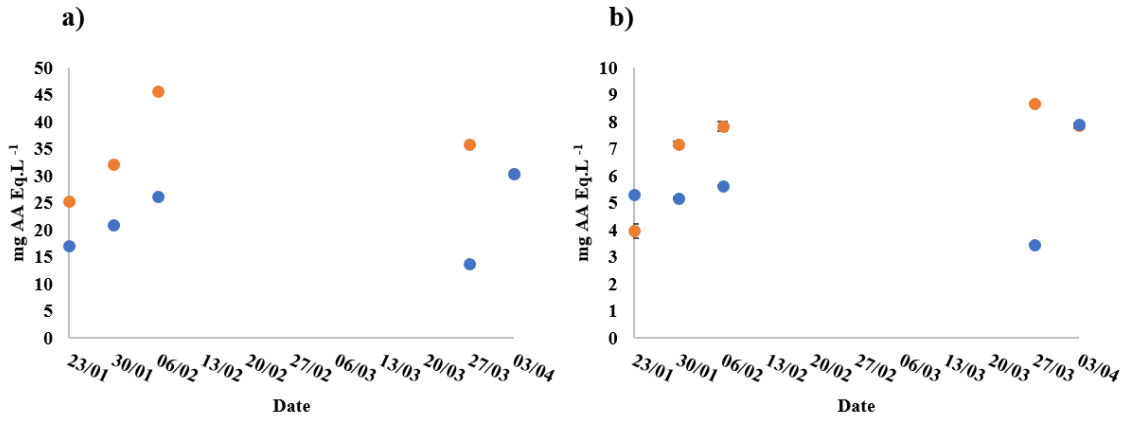

Fig. 23 - DPPH antioxidant results: a) aqueous extract results; b) ethanolic extract results; X axis – date (days); Y axis – DPPH activity (mg AA Eq.L^{-1}); Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.4.3. *ABTS*

The *AQ* extract produced from *Fc* presented significantly higher activity than *Rw* produced biomass, averaging 32.22 ± 9.01, and 19.64 ± 1.54 µmol Trolox Eq.g^{-1} DW, respectively (Fig. 24a). On *Fc*, values increased from winter until early spring. However, no statistical difference was observed between winter and early spring samples, although *Fc* and *Rw* were significantly different. *AQ* extract readings ranged from 22.90 to 46.13 µmol Trolox Eq.g^{-1} DW on *Fc*, and 17.38 and 22.44 µmol Trolox Eq.g^{-1} DW in *Rw* biomass.

Concerning the *ETA* extracts (Fig. 24b), *Fc* biomass had significantly higher activity than *Rw*. Values increased during winter and peaked at early spring, averaging 31.80 ± 10.76 µmol Trolox Eq.g^{-1} DW for *Fc*, and 8.72 ± 1.5 µmol Trolox Eq.g^{-1} DW for *Rw*. The results varied between 16.92 and 42.47 µmol Trolox Eq.g^{-1} DW, and 6.30 and 10.80 µmol Trolox Eq.g^{-1} DW, in *Fc* and *Rw*, respectively. In *Fc* and *Rw* biomass, winter and early spring readings were significantly different.

The *AQ* extracts had higher activity than the *ETA* extracts, and mean values were 25.93 ± 9.17 µmol Trolox Eq.g^{-1} DW and 20.26 ± 13.96 µmol Trolox Eq.g^{-1} DW. *Fc* averaged

32.01±9.75 µmol Trolox Eq.g^{-1} DW, thus being the biomass with stronger antioxidant activity, and *Rw* averaged 14.18±5.99 µmol Trolox Eq.g^{-1} DW.

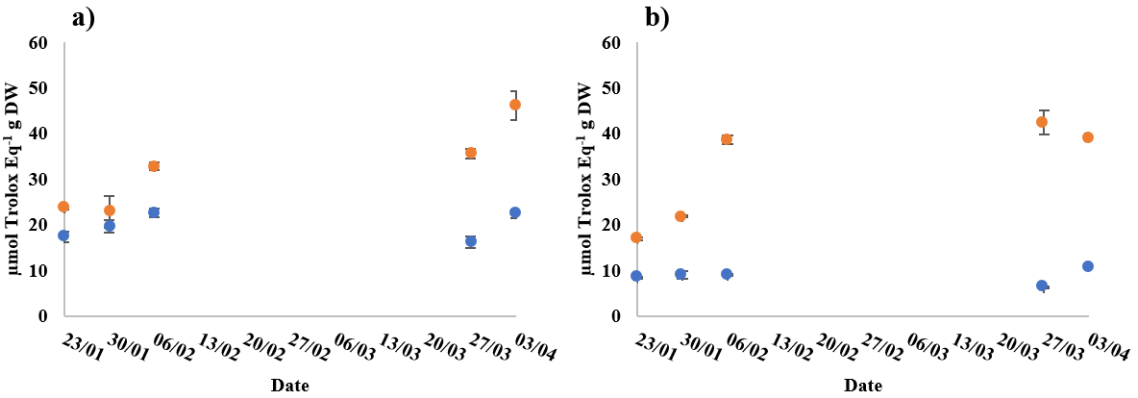

Fig. 24 - ABTS antioxidant results: a) aqueous extract results; b) ethanolic extract results; X axis – date (days); Y axis – FRAP antioxidant activity (µmol Trolox Eq.g $^{-1}$ DW); Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.4.4. *FRAP*

With respect to *AQ* extracts (Fig. 25a), during winter, both *Fc* and *Rw* showed similar activity. On the other hand, during early spring, levels on *Fc* increased significantly, whereas *Rw* increased slightly. Readings ranged from 37.39 and 1,215.98 mM and between 92.16 and 198.84 mM, on *Fc* and *Rw*, respectively. The *Fc* biomass showed more activity than *Rw*, averaging 515.55±538.86 mM and 152.03±48.8 mM, respectively. Furthermore, results significantly differed between and within winter and early spring samples.

Concerning the *ETA* extracts (Fig. 25b), the *Fc* biomass activity was significantly higher than the *Rw*. *Fc* values increased from winter to early spring, whilst the *Rw* values remained low and constant, similar to what the *AQ* extracts exhibited. Nevertheless, significant differences were observed within winter and early spring values, and between *Fc* and *Rw*. Maximum values were recorded during early spring in the floating cages, although a peak reaching 1,312 mM was noted amongst winter samples (06/02). Values ranged between 429 and 929 mM, and from 139 and 257 mM in *Fc* and *Rw*, averaging 766.30±330.51 mM and 179.65±49.12 mM, respectively.

The *Fc* biomass showed to have significantly more antioxidant activity than *Rw*, with respective mean values of 640.93±457.36 mM, and 165.84±457.36 mM. Additionally,

the *ETA* extract FRAP levels were significantly higher than *AQ*, with mean values 505.56±384.12 mM and 354.991±437 mM, respectively.

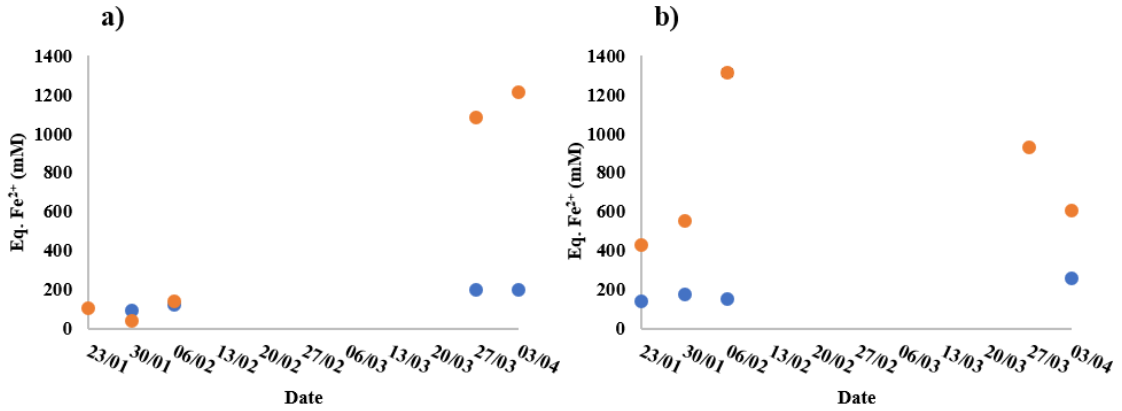

Fig. 25 - FRAP antioxidant results: a) aqueous extract results; b) represents ethanolic extract results; X axis – date (days); Y axis – FRAP antioxidant activity (Eq. Fe^{2+} (mM)); Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.5. *Anti-inflammatory activity*

The anti-inflammatory activity (Fig. 26) was significantly higher in *Fc* biomass than in *Rw*. Inhibition averaged 88.7±7.8%, and 77.7±7.2%, for *Fc* and *Rw*, respectively. Levels ranged from 77.2 to 99.0 %, and 70.3 and 88.2 %, in *Fc* and *Rw* samples, correspondingly. The results also show that the activity decreased significantly from winter to early spring samples and differences were found between *Fc* and *Rw* systems.

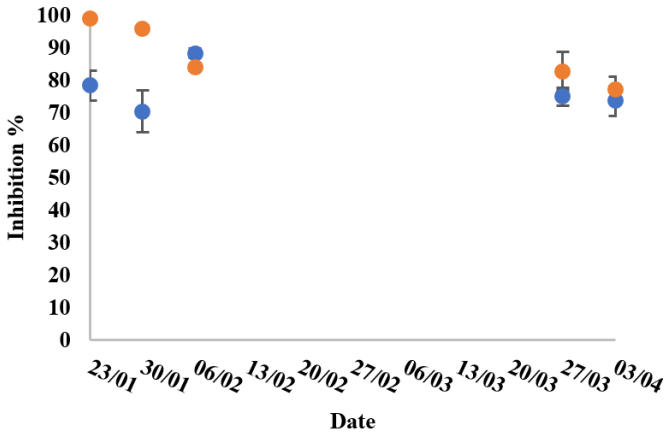

Fig. 26 - Anti-inflammatory activity of the ethanolic extract. X axis – date (days); Y axis – anti-inflammatory inhibition (%); Blue dots refer to *Rw* related samples; Orange dots refer to *Fc* related samples. Values respective to 13/02 until 20/03 were not analyzed.

3.2.6. *Principle component analysis for production systems*

The explanatory variables used for PCA were organic matter (*OM*), growth (*Growth*), total lipid content (*Fat*), total protein (*Prot.*), radiation (*Rad*.), turbidity (*Turb.*), phosphate (*PO4*), ammonia (*NH3*), nitrite (*NO2*), nitrate (*NO3*), polyphenol content aqueous and ethanolic extracts (*Poly. AQ* or *ETA*), DPPH aqueous and ethanolic extracts (*DPPH AQ* or *ETA*) activity, FRAP aqueous and ethanolic extract activity (*FRAP AQ* or *ETA*) and ABTS aqueous and ethanolic extract activity (*ABTS AQ* or *ETA*).

The PCA correlation biplot for explanatory variable (Fig. 27 A) and production system (Fig. 27 B) were plotted with the first and second principal components explaining 60.24% of the total variations. There is a clear separation between production systems along the PC1 that explains 34.58% of the variability, with the raceway (*Rw*) cluster on the right and the floating cages (*Fc*) on the left (Fig. 27 B). On the other hand, PC2 that explains 22.97%, separates production periods in two distinct groups, winter (W1 and W2) and late winter and early spring samples (W3, Sp1, and Sp2). Late winter and early spring clusters are mainly explained by FRAP AQ, DPPH ETA, growth and radiation, while winter is mainly associated with organic matter content and anti-inflammatory. Within late winter and early spring cluster, there is a separation between raceways and floating cages. Late winter and early spring raceways formed a homogeneous cluster to the left upper corner and are explained by the ethanolic extracts antioxidant activity. The cluster of late winter and early spring *Fc* to the right upper right are mainly explained by the aqueous extract antioxidant activity.

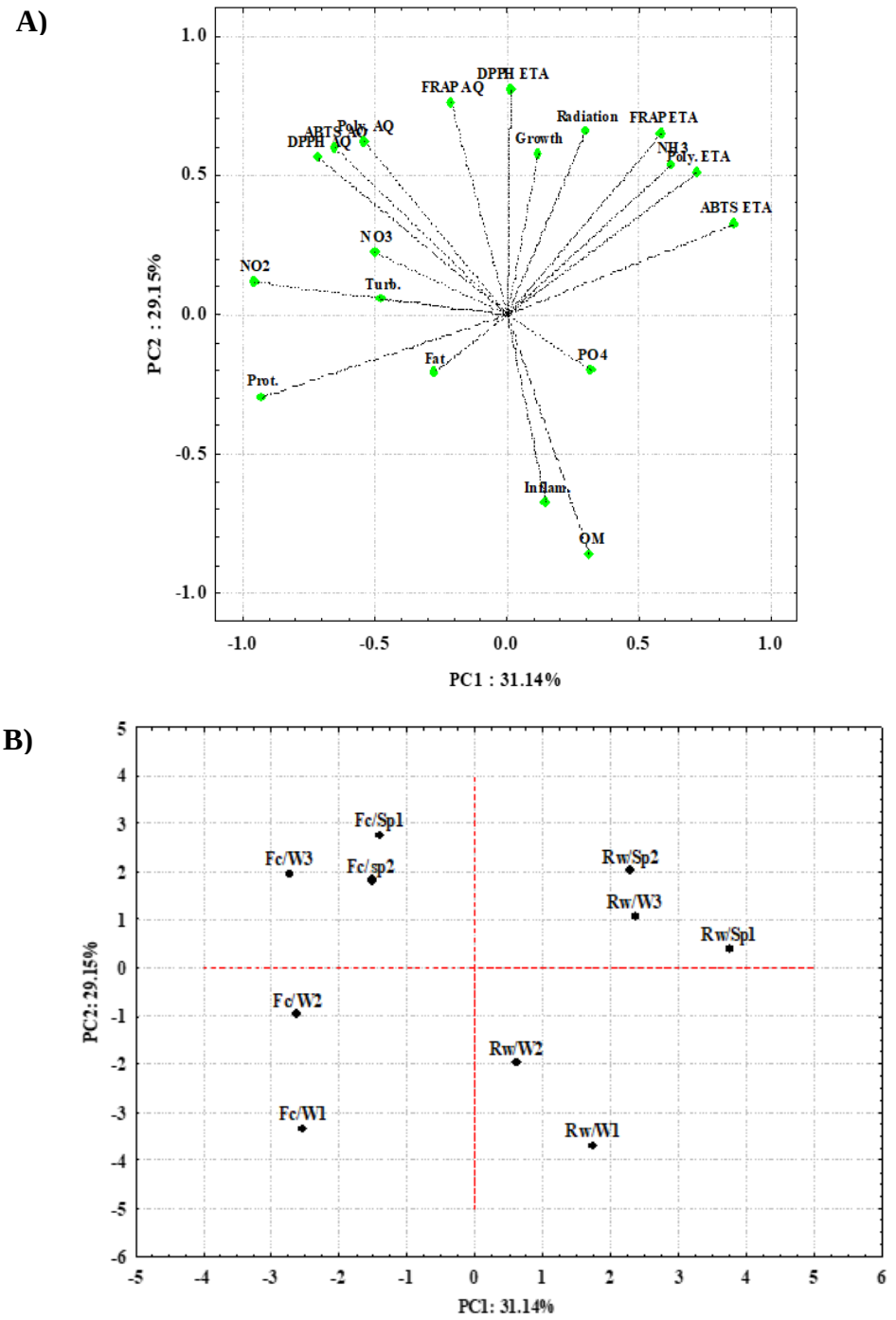

Fig. 27 - PCA correlation biplot for the effect of production system (raceways (*Rw*) vs floating cages (*Fc*)): A) PCA correlation biplot of explanatory variables: organic matter (*OM*), growth (*Growth*), total lipid content (*Fat*), total protein (*Prot.*), radiation (*Rad.*), turbidity (*Turb.*), phosphate (*PO4*), ammonia (*NH3*), nitrite (*NO2*), nitrate (*NO3*), polyphenol content aqueous and ethanolic extracts (*Poly*. *AQ* or *ETA*), DPPH aqueous and ethanolic extracts (*DPPH AQ* or *ETA*) activity, FRAP aqueous and ethanolic extract activity (F*RAP AQ*) and ABTS aqueous and ethanolic extract activity (*ABTS AQ* or *ETA*); B) PCA correlation biplot of production systems data. Legend: floating cages (*Fc*) and raceways (*Rw*), *W1* (winter, 23/01), *W2* (winter, 30/01), *W3* (winter 06/02), *Sp1* (spring, 27/03), and *Sp2* (spring 3/04).

4. *Discussion*

4.1. *The effect of nutrient concentration*

The present work used two different water sources with different nutrient concentrations to study the temporal and spatial effect of nutrient levels on the productivity, nutrient uptake, and bioactive compounds of *Ulva* spp. using a system of floating cages inside a pond reservoir (*R*) and in a settling pond (*SP*) of an aquaculture research centre.

Nutrient sources were indeed distinct. Statistical differences were observed in ammonia, nitrites and phosphates. The *SP* had more ammonia, nitrites, and phosphate than *R*. Temperature and radiation were similar between locals, but significant differences were detected in turbidity. Turbidity is mostly comprised by particulate organic matter and phytoplankton. Once temperature rose, so did fish metabolism and feed consumption, resulting in a rise turbidity and ammonia levels on the *SP*, which are consequence of the increase of uneaten feed and fish excretory metabolites. So, higher turbidity and nutrient levels detected were linked to the water source. Because *R* is nourished by a coastal lagoon, the water does not have direct influence from the fish production, hence turbidity and nutrient concentration were lower. The differences registered in the nitrites are linked to the ammonia decomposition by phytoplankton and therefore, higher ammonia and nitrate levels were recorded on the *SP*.

Although this experiment was performed from winter (January) until early spring (April), results showed that even at low temperatures (16.90±1.74 ºC) growth could be attained at 1.0 $kg \cdot m^{-3}$, which is considered an optimal stocking density (Neori et al., 1991). Nevertheless, while biomass loss was recorded at 23/01 in *SP*, all winter samplings in *R* lost biomass compared to early spring. Still, *SP* exhibited significantly higher growth than *R* cultured biomass. Neori et al. (1991) achieved 678-2,025 $g \cdot m^{-2}$ $week^{-1}$ with ammonia inflows between 10-32 µM and similar temperature, and light exposure, which implies that environmental parameters of the present study were not a limiting factor in either *SP* or *R* (Neori et al., 1991; Floreto et al., 1996). However, environmental stress due to lack of acclimatization may explain the biomass loss recorded.

Between *SP* and *R* samples, no significant differences were observed regarding moisture, dry weight and ash content, and values decreased from winter to early spring. Moisture and dry weights were higher than those reported for green seaweeds by prior findings (Satpati et al., 2011). In addition, the ash content obtained was significantly higher than

those reported for *Ulva rigida* and *Ulva prolifera* (> 1 % FW; Ripol et al., 2017). The organic matter varied significantly, and *R* values were always higher than *SP*.

Subsequently, as the DW and OM decreased from winter to spring so did protein content, since it can comprise up to 16 % of the Ulva dry weight, and values were similar to *Ulva lactuca*, *Ulva prolifera*, and *Ulva rigida* (Setthamongkol et al., 2015; Ripol et al., 2017). The settling pond samples contained more protein than those from the reservoir, which suggests a positive correlation between nutrients and protein content, as shown by Msuya et al. (2008). The lipid content in *SP* did not vary over time, and fluctuations were recorded in *R*. No significant differences were found between *SP* and *R* lipid contents, and values followed the standards for *Ulva* spp. (Gosch et al., 2012; Ripol et al., 2017), except at 23/01 and 27/03. The samples collected in *R* on 23/01 and 27/03 might possibly be an overestimation due to contamination, as it was noted before regarding the nitrates and phosphates readings.

The polyphenol content was higher during winter and decreased in early spring samples which indicate a correlation with the environmental parameters. Literature reveals that temperature and radiation can influence negatively the polyphenol content (Roleda et al., 2019). Therefore, since *SP* generated always the richest biomass, our results imply that at higher nutrient levels the polyphenol content is also higher. Although literature reports greater polyphenol content for *U. clathrata, U. linza*, *U. instestinalis, U. flexuosa*, and *U. intestinalis* (Farasat et al., 2014)., the extraction methods used vary significantly from the present study, meaning that the content might be solvent-dependent.

Even though *R* biomass generated higher DPPH inhibition, *SP* readings were similar. Moreover, the same seasonal variation was observed in the *AQ* extracts for the DPPH radical scavenging activity. Values were significantly higher during winter, and lower in early spring. Contrary to the *AQ* extracts values, the *ETA* extracts inhibitions increased over time, and a possible reason is the fact that different solvents extract different compounds. Trigui et al. (2013) studied the seasonal variation of polyphenol composition and antioxidant activity on *Ulva rigida*, and determine higher polyphenol content in late winter and early spring compared to late summer and early autumn. Hence, the increase in polyphenol content and antioxidant activity were related to the decrease in temperature and radiation. Regardless of the increase in DPPH inhibition observed in our results, the samplings only covered a small scale of time (January-April). If the experiment continued, the decrease described by Trigui et al. (2013) may have been visible. Still,

DPPH results were significantly higher than the readings attain by Trigui et al (2013), meaning that factors beyond temperature influenced the quality and or quantity of polyphenols and antioxidant compounds.

ABTS inhibition also demonstrated that *SP* biomass comprised the most powerful antioxidant activity. Despite the methodology, our results were significantly higher than those found in Srikong et al. (2017). Prior studies have proved that the antioxidant activity is concentration-dependent (Tariq et al., 2015). Therefore, the ABTS antioxidant activity also suggests that some compounds displaying antioxidant activity are related to environmental stress, such as temperature, sun exposure and nutrients limitation.

Again, samples screen for FRAP antioxidant activity revealed that *SP* provided the higher inhibition, therefore corroborating the results seen in ABTS and total polyphenol content. FRAP antioxidant activity was significantly higher than those reported by (Kazir et al., 2018). The results also show that values increase during winter and decrease over early spring. However, *SP AQ* extract ABTS values increased significantly over season, suggesting the presence of a different kind of compounds with high affinity to redox reactions. Sampath-Wiley et al. (2008) suggested that once macroalgae are exposed to environmental stress, e.g., sun radiation, increase their antioxidant metabolism for protection, and revealed that the greatest antioxidant increases observed were recorded during summer months when irradiance levels and temperatures usually peak. Therefore, the variation observed within FRAP results might have had been related to the increase in temperature and radiation throughout the experiment, and the nutrient levels might explain the significant difference observed between *SP* and *R.*

The anti-inflammatory activity can be expressed through several pathways, so some compounds can trigger different anti-inflammatory activities. Literature suggests that compounds like polyphenols can have more than antioxidant activities. In Margret et al. (2009), *Ulva* methanolic extracts anti-inflammatory activity were able to inhibit up to 80% of carrageenan-induced paw oedema in rats. Kim et al. (2018) confirmed the presence of anti-inflammatory, and antioxidant activity in ethanolic extracts produced from *U. linza*, and stated both anti-activities were dose-dependent, which implies the presence some specific constituents with both anti-activities. Kim et al. (2018) also stated that the ethanolic extract was able to decrease the inflammatory expression of nitric oxide (NO), which is a free radical linked to immunoregulation in humans, that when produced in excess promotes inflammatory reactions and may cause bronchitis. Therefore, the

existence of polyphenols in the alcoholic extracts suggests the possibility of a double anti-activity, and that the antioxidant and anti-inflammatory activities might be corelated. Furthermore, Joseph et al. (2015) provided an overview of human clinical trials investigating the acute and chronic (feeding) effect of polyphenols from commonly consumed fruits and berries and derived products on inflammation and showed that dietary polyphenols are effective in enhancing inflammatory stress. Consequently, the same holds for the polyphenol content comprised in algae.

Proteins, and peptides may also play an important role on the antioxidant and anti-inflammatory activities. The protein content was significantly higher in *SP* samples, which had access to higher amount of nutrients (mainly ammonia), two factors that are directly corelated (Msuya et al., 2008). According to Shiu et al. (2005) and Kazir et al. (2018), certain peptides have antioxidant activity. Moreover, Ma et al. (2016) isolated and characterized anti-inflammatory peptides from whey protein hydrolysates (soluble milk proteins) and found that peptide DQWL (Aspartic acid–Glutamine-Tryptophan-Leucine) showed the strongest inhibitory ability against cyclooxygenase-2. So, the decrease of protein content over season might also explain the decreasing trend observed in the anti-inflammatory activity. Consequently, the higher anti-inflammatory activity found in the *SP*, can be explained by the protein content. Therefore, the nutrient availability can be directly related to the antioxidant and anti-inflammatory activity through the polyphenol content and total protein content.

In fact, the PCA brings to light the correlations observed before. The *SP* and late winter and early spring samples were mainly explained by growth, turbidity, ammonia and most antioxidant activities, showing that the biomass produced with the higher nutrient source presented greater protein content, growth, and antioxidant activity. The high correlation between turbidity and ammonia to polyphenol content and DPPH, FRAP, and ABTS activities explains the influence of the nutrients present in *SP* on the *Ulva* quality.

Moreover, the PCA eigenvectors show a high correlation between the anti-inflammatory activity and the organic matter, which may reflect the high protein content observed during winter. Moreover, the negative correlation between the anti-inflammatory activity and growth mirrors the increase of growth from winter to early spring with the decrease of the anti-inflammatory activity and protein content, possibly explained by the presence of anti-inflammatory peptides as described before. Therefore, the present work suggests

that the protein content may be a reference for both antioxidant and ant-inflammatory activities.

4.2. *The effect of production system*

On this experiment, the *SP* results are from now on referred to those obtained with floating cages. Therefore, with the same water source, two different production systems, raceways (*Rw*) and floating cages (*Fc*) were compared in other to evaluate the influence of the production system on algal growth, composition and respective bioactive compounds

Temperature, radiation and turbidity were similar between *Rw* and *Fc*. However, the *Rw* fiberglass composition may have increased its thermal amplitude. Significant differences were only observed with respect to turbidity during early spring, and *Fc* were significantly more turbid. Differences in turbidity levels can be explained by the settling of sediments, while water was diverted to the raceways.

Despite of the same water source, greater nutrient content was observed in *Fc*, where nitrite and phosphate levels were significantly higher. However, ammonia was superior on the *Rw*. In fact, the average ammonia levels in the *Rw* were 3 times higher than what was recorded on the *Fc*, which imply that either the nutrients were not being used by the macroalgae or the uptake was too low to be reflected on the ammonia levels. Previous studies conducted with *Ulva lactuca*, tested several ammonia and phosphate influxes and demonstrated that growth was attained with 10-48 μM of ammonia and 1-750 μM of phosphorus using high water flows rates (4-16 daily water exchanges) (Neori et al., 1991; Floreto et al., 1996). Because the daily water renewal in *Rw* (1 daily water exchange) was fairly low compared to what literature suggests, low biomass yields were obtained even though the nutrient levels were over any limiting factor. The bottom centered aeration was set to increase turbulence in order to thin the diffusive boundary layers (DBL's) around frond surfaces, to increase nutrient uptake (Gao et al. 1992). However, only a part of the biomass comprised within *Rw* kept floating, whilst the majority sank. Therefore, the aeration used was not enough to transport the fronds through the water column, limiting the renewal of the DBL's and nutrient uptake. Msuya et al. (2008) stated that with proper nutrient concentration (above 4 μM of total ammonia nitrogen (TAN)), aeration is not essential for effective growth and biofiltration. (Neori et al. 1991; Diamahesa et al., 2017). According to Menéndez et al. (2001), *Ulva* sp.

photosynthetically activity decreased when grown at pH levels above 8.5 and below 6.5. Although pH results are not presented, values up to 9.0 pH were recorded in the *Rw*, but not on *Fc*. Therefore, our result might imply that the water flow was extremely decisive for the nutrient uptake efficiency, and accumulation of inhibitory compounds. Although growth was significantly higher on the *Rw*, proper water flow would have promoted greater nutrient uptake and greater growth Nevertheless, *Rw* results show that high thermal amplitude and the lack of nutrient biofiltration generated greater fluctuations in growth. In a farming point of view, inconsistent growth yields do not ease the predictability of the production, making harvesting planning difficult. Although growth was lower on the *Fc*, the stability of the settling pond provided conditions for a steadier growth.

Despite no differences were observed in moisture and ash contents between production systems, dry and organic matter were significantly higher on *Ulva* cultivated on the *Rw*. Furthermore, from winter to early spring, dry weights and organic matter decreased. Hence, the dry and organic matter noted in *Rw* may be related to the nutrient availability. For example, through water flow and N (nitrogen) deprivation, researchers managed to increase starch yields in *Ulva* spp. (Helmes et al., 2018). Although the present study did not estimate the starch, they average 14-40% of *Ulva* dry matter (Kazir et al., 2018). Therefore, the N and water flow deprivation observed in *Rw* may explain the high dry and organic matter contents compared to *Fc* biomass. Nevertheless, moisture, dry weight, ash and organic matter of *Rw* and *Fc* followed the normal standards for *Ulva* spp. (Abirami et al., 2011; Satpati et al., 2011; Ripol et al., 2017). The compounds that comprise most of the dry weight and organic matter, such as proteins and carbohydrates, possibly explain the decrease observed from winter to early spring.

Recognizing the effect of low water flow on nutrient availability, *Rw* negatively affected the total protein content and *Fc* generated biomass with greater protein content. As stated before, the nutrient availability is directly related to the protein content. Msuya et al. (2008) revealed that the difference in protein content in *Ulva lactuca* cultivated at high and low nutrient treatments depended on water velocity, and higher protein yields were obtained with high flow rates., Protein levels in *Rw* decrease 35% from winter to early spring, whereas in *Fc* decreased 17%. However, the protein content estimated on the *Fc* biomass was under normal standards for *Ulva* spp. (Setthamongkol et al., 2015; Gosch et al., 2012). Thus, the *Fc* was exposed to enough hydrodynamic action to promote elevated

nutrient availability and uptake, allowing higher protein biosynthesis. Diamahesa et al. (2017) stated that water flow is important to manage the nutrient availability. By increasing the water flow, nutrient flux rises, and the biomass will not be nutrient limited, allowing greater biomass production, but lower nutrient uptake efficiency. On the other hand, if the flow rate is low, nutrients will become limiting, restricting the biomass production, and increasing the nutrient efficiency. Therefore, our results suggest that the low *Rw* protein yields were due to low nutrient availability caused by the low water flow. Hence, the *Fc* provided the best conditions to obtain higher protein on *Ulva* biomass.

Regarding to lipid content, a tendential decrease from winter to spring was noted, and figures were normal for *Ulva* spp. (Gosch et al., 2012, Ripol et al., 2017). Literature states that temperature is conversely related to the lipid content (Sánchez-Machado et al., 2004), and that temperature, light, and nutrients are intimately associated to the algal lipid and fatty acid content. Tatsuzawa et al. (1996) reported significant changes in lipid and fatty acid composition on *Pavlova lutheri* (microalgae) cultivated at 15ºC and 25ºC. Moreover, *Ulva fenestrate* glycoproteins MGDG (monogalactosyldiacylglycerol) and DGDG (digalactosyldiacylglycerol) that represent 40-55 and 15-35% of thylakoid lipids, increased 2-3.5 times when the biomass was grown at lower radiation compared to high (Khotimchenko et al., 2004). Hence, the decrease observed from winter to early spring may be explained by the increase of temperature and radiation. Thus, the difference observed between production systems cannot be explained by the nutrient limitation but is possibly related to *Rw* high thermal amplitude.

Furthermore, *Fc* biomass presented greater polyphenol content than *Rw*. Figures were higher in winter, and lower during early spring. According to Roleda et al. (2019), the polyphenol content decreases from winter to early spring, and such effect was observed in both production systems. However, *Fc* and *Rw* total polyphenol contents were lower than the figures estimated by previous studies (Ripol et al., 2017). Nevertheless, the difference between production systems must be explained by the inefficient nutrient uptake observed in the *Rw*. Goiris et al. (2015) studied the impact of nutrient stress in three species of microalgae and revealed that the phenolic content was significantly reduced by nitrogen and phosphate limited treatments. Hence, the present work suggests that the high nutrient availability in *Fc* is positively corelated to the polyphenol content.

As discussed above, the nutrient limitation caused by low water flow may have compromised the production of polyphenol and protein levels. Therefore, the antioxidant

activities were also affected by the production systems. Briefly, the *Fc* antioxidant readings were mostly greater than *Rw*. Roleda et al. (2019) suggested that environmental parameters, such as temperature and radiation (sun exposure), are corelated conversely to the polyphenol content. Observing DPPH radical scavenging activity, values were higher in *Fc* and increased in winter, whereas *Rw* was lower and figures decreased over early spring. According to Sampath-Wiley et al. (2008), once macroalgae are exposed to environmental stress, such as sun exposure (radiation), their antioxidant metabolism increases for protection. So, in *Fc AQ* extracts high readings observed during early spring (ABTS and FRAP activity) can be possibly be explained by the increase in radiation and temperature. The absence of certain antioxidant compounds might justify the low ABTS and FRAP antioxidant activity observed in *Rw ETA* and *AQ* extracts (Fig. 24b and Fig.25. Therefore, our study proposes that antioxidant compounds, such as peptides (Shiu et al., 2005; Kazir et al., 2018) and polyphenols (Trigui et al., 2013; Roleda et al., 2019), were influenced by the *Rw* limiting conditions.

Overall the anti-inflammatory inhibition decreased over time. Anti-inflammatory polyphenols (Joseph et al., 2015; Kim et al., 2018) and peptides (proteins) (Ma et al., 2016) might clarify the difference between *Fc* and *Rw*. The *Fc* offered greater nutrient availability, so higher polyphenol and protein yields were obtained. On the other hand, *Rw* conditions provided low nutrient availability to the biomass, restraining the polyphenol and protein biosynthesis. Therefore, the anti-inflammatory activity was higher on *Fc* biomass. Concerning the seasonal effect on the anti-inflammatory activity, values decreased from winter to early spring. Thus, the decreasing activity may possibly have been connected to the decrease of total protein and polyphenol content conversely to the increase of temperature. Since the total polyphenols content and protein levels were measured through quantitative methods, it was not possible to release which compounds were responsible for the decrease of the anti-inflammatory activity.

The PCA exhibited a clear separation between production systems and separated production periods in two distinct groups, winter (W1 and W2) and late winter and early spring (W3, Sp1, and Sp2). Late winter and early spring clusters were mainly explained by *AQ* extracts FRAP activity , *ETA* extracts DPPH activity , growth and radiation, while winter production periods were mainly associated to the organic matter and anti-inflammatory activity.

The separation of production systems occurred within winter and early spring production periods where *Fc* and *Rw* antioxidant activities diverged the most. Interestingly, the *Fc* antioxidant activity was mostly associated to *AQ* extracts, while *Rw* was to ETA ones. Moreover, the positive correlation between growth, radiation, and ammonia with DPPH, FRAP, ABTS and polyphenol content may justify the low values attained at *Rw* during early spring. Greater growth implies proper radiation and nutrient availability. High nutrient uptake leads to higher production of polyphenol and other antioxidant compounds. However, the lack of nutrient availability on *Rw* produced the contrary effect even though those explanatory variables (polyphenol content, ABTS, FRAP, and ammonia) were positively corelated. Moreover, winter production periods were intimately corelated with protein, anti-inflammatory activity and the organic matter. Hence, the converse correlation between temperature and protein, may reflect the positive correlation between organic matter and anti-inflammatory activity observed in winter. Thus, the present work suggests that the organic matter and protein are positively corelated to anti-inflammatory activities. Further research is needed to clarify the seasonal variation in the anti-inflammatory activity of *Ulva* spp. protein and polyphenolic compounds.

5. *Conclusions*

The different nutrient concentration and different production systems influenced significantly the growth, composition and bioactive compounds of *Ulva* spp. In the case of the *Rw,* the high ammonia levels reflected the low nutrient uptake caused by the low water flow and inefficient aeration. Therefore, *Rw* conditions restrained the nutrient availability, compromising protein and polyphenol yields. Conversely, *Fc* provided enough hydrodynamic action for efficient algal nutrient uptake, which resulted in a biomass with higher protein and polyphenol content. Consequently, the antioxidant and anti-inflammatory activities were affected, and readings were significantly lower where the nutrient uptake was limited. However, some antioxidant activities were found to increase over season, due to the rise of antioxidant metabolism in response to temperature and radiation. Therefore, the present study concludes that protein, antioxidant and anti-inflammatory activities were significantly lower where the nutrient availability and uptake were limited.

Further research should focus in understanding if tank (raceway) cultivation coupled with proper water flow rates and aeration leads to an increase of the bioactive compounds and respective bioactivities, as suggested by the *Fc* results. Additionally, more study is needed to clarify the seasonal variation in the anti-inflammatory activity of *Ulva* spp. protein and polyphenolic compounds.

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